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PhD in Biomedical Science

RESEARCH PROJECT:

“A Bottom-up Approach for the Generation of Neural Circuits”

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RESEARCH PROJECT:

“A Bottom-up Approach for the Generation of Neural Circuits”

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“A Bottom-up Approach for the Generation of Neural Circuits”

I. PROJECT SUMMARY

Neural circuits consist of ordered arrays of neurons connected by synapses that process information within the nervous system, from the reception of an input signal to the generation of a neural response. The complexity of neural circuits makes the mapping, manipulation, and replication of biological neural networks challenging for experimental studies. Current state-of-the-art techniques are time- and resource-intensive and require large amounts of data to produce reliable and reproducible results. Novel high-throughput methodologies that enable the precise and accessible in vitro fabrication of neural circuits could significantly enhance our understanding of information processing within neural circuits.

This Thesis presents a bottom-up workflow to generate in vitro arrays with a defined structure from scratch, through controlled manipulation and positioning of individual neurons from primary cultures. Combined with the adaptation of an open-source automated workstation, FrescoM, to improve precision, efficiency and reproducibility, the developed process provides a consistent approach for reproducing specific architectures of simple neural networks.

The Thesis describes the design and incorporation of additional components to enable the manipulation of individual neurons as a new workstation function, the optimisation of primary culture conditions using zebrafish neurons as cell sources for the experiments, and the testing of controlled neuronal positioning. Using the automated workstation, it was possible to define circuit architecture, induce axonal growth, and control synaptic connection formation.

The discussion explores the possibility of increasing the complexity of these newly generated circuits by progressively adding neurons to the network, as well as the potential application of the developed process to provide neural circuits as biological samples for high-throughput experiments.

II. INTRODUCTION

PART 1:

A Bottom-Up approach for the generation of neural circuits

1.1 Overview of Neural Circuits

A **neural circuit** is defined as an organised array of neurons with a defined structure comprising its constituent elements (neurons) and their interactions (chemical and electrical synaptic connections). Upon activation, this system functions as a computing unit that processes received information in a recurring pattern to produce and transmit a response related to cell stimulation, neural firing, and nerve function (**Figure 1.1**), reflected in holistic functions such as learning, memory, emotion, and sensory and motor functions. In this process, neurons act as nodes, generating action potentials in response to node activation, with depolarisation travelling down the axon as the means of information transmission, and the activation of synapses as the response (Purves et al., 2001).

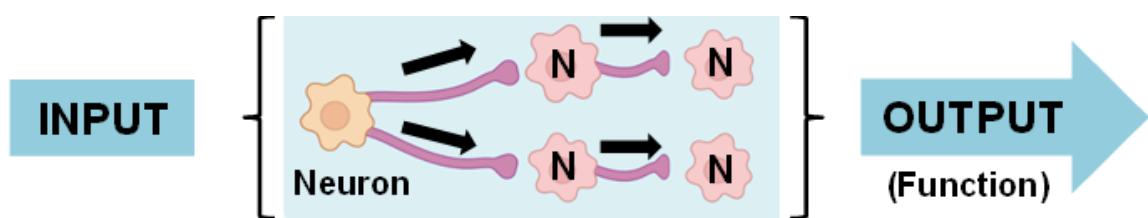


Figure 1.1: Overview of a neural circuit

Representation on the structure of a typical neural circuit as an information processing system. The information follows a defined route from initial entry neurons that trigger the signal to effector neurons, where the nerve function is produced.

The effect of circuit activation can be local, when the system is restricted to groups of adjacent neurons, or remote, when distant neurons participate, such as interactions among different nerve structures or brain areas. Also, one circuit output can serve as the input to another, creating sequential processing pathways. Besides, neural signal processing can take diverse routes through the involved neurons, and the sequence of information processing is not always linear. Ramified paths can also occur, including diverging, converging, reverberating, and parallel-after-discharge neural circuits (**Figure 1.2**). The diverse circuit architecture enables the propagation of signals, providing dissemination, integration, and feedback capabilities.

From a broader perspective, the nervous system comprises multiple excitable cells, known as neurons, that coexist with other cell types, including glial cells and astrocytes. Its ultimate function is to coordinate physiological processes by receiving information from both the internal and external environments, processing it through intricate networks, and generating a response to preserve homeostasis and maintain bodily functions. However, neurons do not work independently. Rather, their function depends on assembling circuits or networks with other neurons.

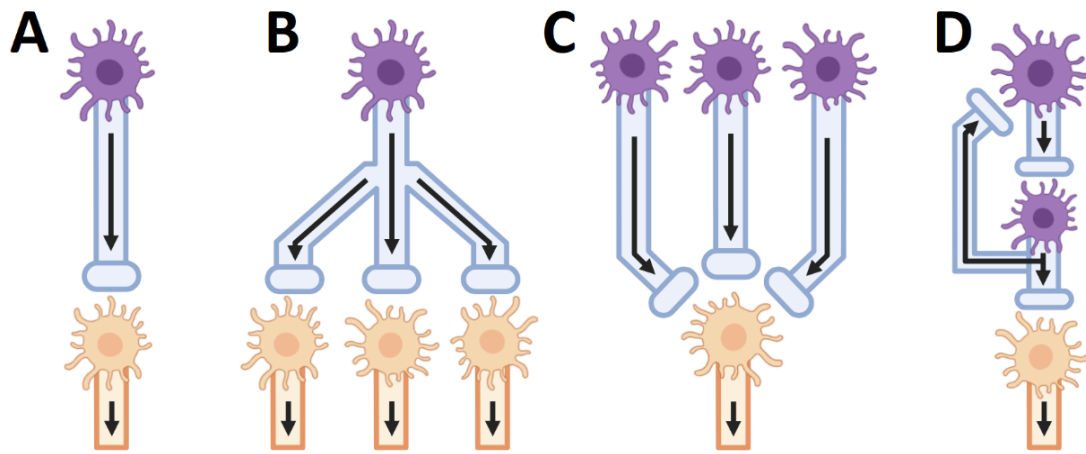


Figure 1.2: Types of neural circuit based on their structure

Depending on the direction of the information flow and the number of neurons present in the input and output layers, circuits can be classified as follows:

A. Linear Circuit: Information is transmitted in a straightforward, uninterrupted flow. One presynaptic neuron is connected to a postsynaptic neuron, uninfluenced by external neurons. All the input information from the presynaptic level is received by the postsynaptic level.

B. Diverging Circuit: Information from a single source is divided and transmitted as a ramified flow. The activation of one presynaptic neuron affects several postsynaptic neurons, and individual outputs may vary between these targets.

C. Converging Circuit: Information from multiple sources is integrated and transmitted as a single flow. The activation of several presynaptic neurons affects a unique postsynaptic neuron, and individual inputs may vary to this target.

D. Reverberating Circuit: Information is transmitted as a non-linear flow. One neuron can be connected to an upstream neuron, regulating its transmission properties on the system.

Since the beginning of the study of the nervous system, it has become clear that its functional design depends on the components and interactions within neural circuits. The anatomical role of neurons as the building units of circuits has been observed since the classical works of Santiago Ramón y Cajal in the late 19th century (Cajal, 1906, 1911). The first attempt to develop an explanatory model of how neurons are organised and work together to process information and produce a response was postulated in 1943 by Warren S. McCulloch and Walter Pitts in their published work “A Logical Calculus of the Ideas Immanent in Nervous Activity”. They define neurons as “the logic units of a computing system that can be individually activated or remain inactivated” (McCulloch & Pitts, 1943). Thus, from this two-state perspective, connectivity can be presented as a coordinated network whose structural links between paired elements are represented as binary values indicating whether information flow (or neural activation) is present (‘1’) or absent (‘0’), in an “all-or-not” manner. These processing nodes are connected in a defined configuration, commonly arranged in layers or levels, with variable activation properties. The information this system receives (Input) follows a specific pathway and exits the system as a response (Output), in type and intensity, depending on the activation patterns through the cell layers. This work also highlighted the importance of defining neural constituents to understand brain architecture and function. In biological organisms, the input to the nervous system can be sensory information or homeostatic signals, and the response is mediated by neural function, passing through a series of connected neurons along consistent pathways (White et al., 1986). This idea of information processing in the nervous system was later extended to other computing systems, including computational systems, to create the broader concept of “neural networks” for modelling information processing. The present project is only focused on biological neural circuits.

The previous description of neural circuits applies to most of the structures involved in nerve function, with some variants found later. All share the neuron as the elementary component. A representative example of visualising the canonical structure and operation of a biological neural circuit is the retina (**Figure 1.3**), where cells of six main subtypes are organised into three defined nuclear layers (described in detail by Masland et al., 2012). Each discrete region contains neurons with similar properties and information is transmitted from one layer to the next through chemical synaptic connections. Lateral information flow also occurs between cells in the same layer that may include other types of interactions, such as gap junctions. Photoreceptors in the outer nuclear layer receive and process the sensory information from the eye (light). The data is converted into a chemical signal, passing from the neural soma of the first-layer cells to axons, and then to the following cells in the inner layers via synaptic connections. The signal flows through parallel streams and objects to their processing properties. Finally, the signal is integrated within the cells at the third layer, and an output response is generated in the optic nerve, conveying information about light and colour perception. In turn, the optical nerve output is transported to new circuits. It serves as a starting point for further sensory information processing in specialised regions of the brain, converting this information into three-dimensional images and objects, and supporting other neural functions such as memory.

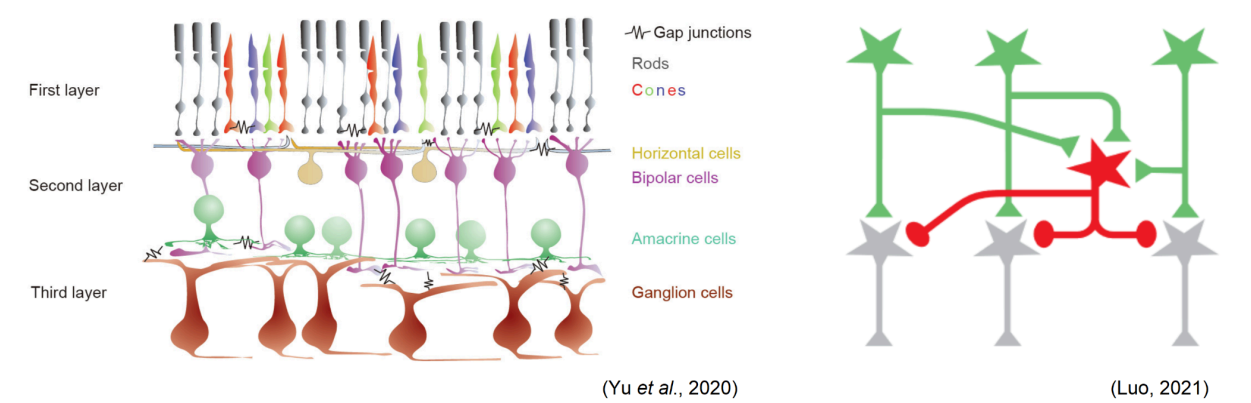


Figure 1.3: Retinal neural layers as an example of neural circuit modelling

Left: Sensory information from the retina passes through several layers, each with a distinct cell composition, shown in colours. The input sensory information related to light perception is received in the outer layers and processed through inner layers composed of different neural types. A resulting output signal is transmitted to the brain via the optic nerve.

Right: Representation of an information processing route. Information does not always follow a straightforward direction. Individual neurons can form multiple connections and core circuit motifs, transmitting, disseminating and integrating processed information.

Subsequent studies on neural circuits expanded the investigation of local circuits to larger scales, such as distinct brain regions. For instance, Zeki and Shipp investigated the visual regions in the primate cortex, leading to one of the first descriptions of brain networks linking distant areas. They identified the neural circuit responsible for visual information processing in the cortex. Their findings showed that functional organisation and segregation reflected the specialisation of brain regions and recurrent patterns in anatomical connections that served as essential blueprints for neural circuits (Zeki & Shipp, 1988). Later studies confirmed that the identified neural areas are connected by interregional pathways of serial synaptic connections (Felleman & Van Essen, 1991).

A comprehensive understanding of how neural circuits function requires integrating findings across multiple levels. Based on the initial findings on intact nerve structures, it is necessary to reproduce the circuit in the laboratory to elucidate the mechanisms involved in the elemental processing events at the individual cell and synaptic levels. Only with this resolution is it possible to confirm the initial inferences about the role of specific neurons, the regulatory mechanisms, alternative processing routes and the total contribution of the nerve function. Researchers have focused their efforts on imaging and mapping the circuits at the ultrastructural level of individual neurons using microscopy (Denk et al., 2012). However, obtaining suitable and accessible in vitro neural circuits is one of the main obstacles to high-throughput experiments. **This thesis proposes a methodology to address current limitations in the precise in vitro construction of neural circuits, with the potential for scalable applications in neural circuit research.**

Recent progress in high-resolution neural fluorescent labelling and imaging techniques has initiated the “connectomic maps” field, which is described in the following section. This discipline can be described as the generation of comprehensive descriptions of networks that account for information processing at specific anatomical and functional stages in an organism, ideally encompassing the entirety of the involved neurons and synapses (Livet et al., 2007).

1.2 The Human Connectome as Ultimate Goal in Circuit Mapping

The connectome is one of the ultimate goals of investigating neural circuits. The term refers to the entire map of neural elements and connections in a given organism, which can precisely explain the mechanisms of functional, regulatory, developmental, and pathological events at the cellular and synaptic levels (Sporns, 2013). As other terms that employ the suffix “ome”, a connectome must comply with specific features:

Totality: A connectome must be a complete and finite description of the nerve network in an organism, representing entirely the neurons participating in all brain functions and the rest of the nerve structures, as well as all nerve functions.

Universality: A connectome must apply to all individuals of the same species. When performing the same task in similar conditions, all the neural circuits must be shared between individuals. The same set of neurons must be part of the same networks regarding identity and location. The information must flow through similar paths and nerve structures.

One of the main obstacles tracking neural circuits as part of the search for connectomes is the intricate architecture of the brain and the constituent neural networks. To give a better perspective, the human brain comprises an estimated 86 billion neurons, each forming, on average, about 7,000 synaptic connections with other neurons. This number corresponds to approximately 600 trillion synaptic connections in the human brain (Herculano-Houzel, 2009). Moreover, neural distribution is not uniform throughout the brain mass, with regions where the neurons are more abundant or tightly packed. When focusing on neural circuit mapping, new complications arise. Frequently, axons form bundles, thus requiring high-resolution tracking techniques to elucidate individual cell interactions. At the regional scale, neural circuits involving different brain areas tend to include axons that cross long distances, traverse various structures, and follow non-linear paths, such as those found in diverging, converging, and reverberating circuits. Because neurons form complex but precise networks, the continuous tracking of neural circuits at single-cell resolution in the human brain and other organisms represents a current challenge to understanding brain function and structure. Thus, ongoing efforts include searching for more effective strategies and experimental tools to enhance progress in the field of neural circuits, aiming for highly targeted, efficient, accurate, and affordable options for conducting in vitro and in vivo studies (Lyu, 2025).

Researchers have recently sought to gather information on identified neural circuits in model organisms to generate the first full connectomes. This assembly process results from integrative

approaches that complement data from anatomical and functional sources. The outcome finally culminated in 1986 with the generation of the first connectome from *Caenorhabditis elegans*, which was derived from reconstructions of electron micrographs of ultra-thin serial sections. This full brain map identified 302 neurons of several subtypes, approximately 5,000 chemical synapses, and 2,000 neuromuscular junctions, all arranged in an invariant structure in the organism (White et al., 1986). This initial map was later refined using physical connectivity matrices and fluorescent labelling of synapses to include all synaptic connections from sensory input to end-organ output across the whole animal body and including both sexes, as depicted in **Figure 1.4**. The study revealed sexual dimorphism in structure and connectivity that can be reflected in adaptive behaviour differences (Cook et al., 2019).

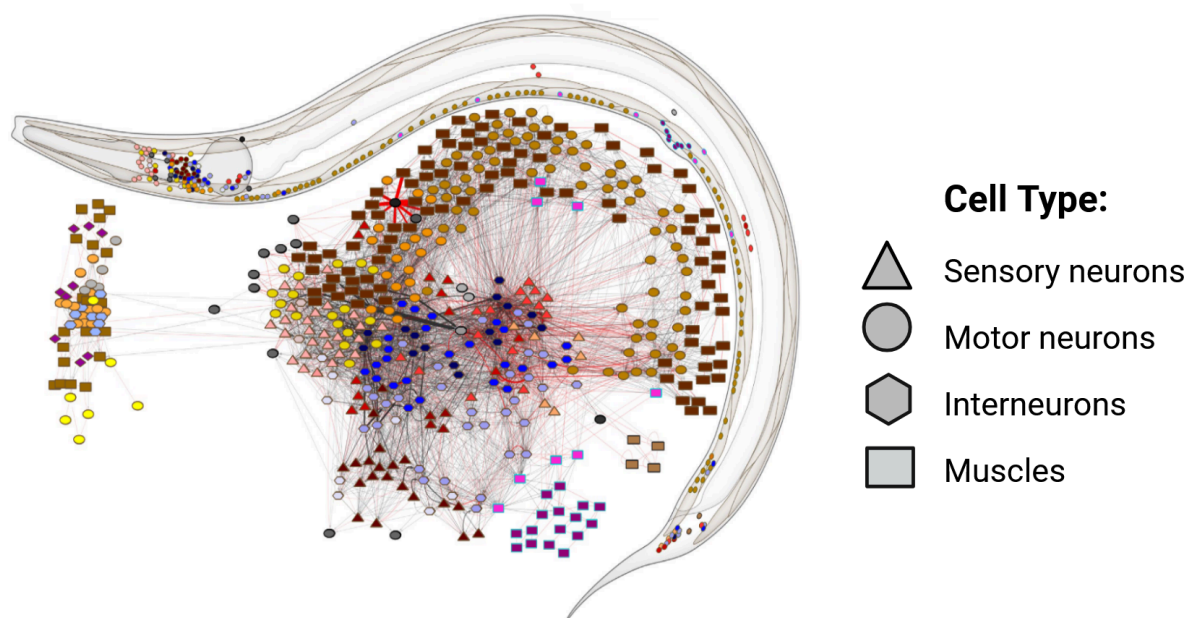


Figure 1.4: Visual representation of the neural connectome in *Caenorhabditis elegans*

After integrating tracing data, a map of the whole nervous system in the adult organism for both sexes was generated. Cell components and interactions are represented as connected symbols. Using the developed AllegroViva clustering algorithm, the vertex layout indicated heavily connected cell pairs. Directed edge colours indicate if the interactions are chemical synapses or gap junctional connections. Symbol shapes indicate cell type, and symbol colours, similar cell categories and properties, as in the case of neurons forming layers (modified from Cook et al., 2019).

As the number of neurons increases from one organism to another, so do synaptic connections and the intricacy of the network. From the few hundred neurons that form the *C. elegans* connectome (Cook et al., 2019), the mouse brain contains approximately 100 million neurons (Oh et al., 2019), and the human brain contains around 86 billion neurons (Herculano-Houzel, 2009). To address the challenge of exponential complexity, novel tools are needed to process and integrate large amounts of data from diverse sources. For this purpose, computational analysis has proven to be an invaluable tool, as it can generate structure-function models and predict interactions based on previously processed data. Generated maps also must be tested and validated before producing a consistent brain model that can fit and explain the experimental results, which can be done simultaneously with new data acquisition.

The most remarkable example of this coordinated analysis is the *Drosophila melanogaster* connectome, presented in 2024 by the Neurobiology Division of the UKRI MRC Laboratory of Molecular Biology and collaborators at Cambridge University, Princeton and the University of

Vermont. This series of works, constituting the FlyWire Consortium, produced an entire map of the adult fly brain, comprising approximately 140,000 neurons of 8,000 different subtypes and around 5×10^7 synapses, making it the first complete synaptic resolution wiring map of the brain of an adult animal capable of performing complex behaviours (Dorkenwald et al., 2024). The technical work involved slicing the brain into several thousand sections, followed by high-resolution electron microscopy to resolve local circuits in each sample. Then, novel AI deep neural networks were employed to segment the images and identify individual neurons and synapses. However, manually correcting every segmented image was required, greatly increasing the project duration. It is estimated that the generation of the *D. melanogaster* connectome took around 33 years of the combined work of more than 50 labs worldwide to proofread and correct the segmentation (Schlegel et al., 2024).

The technical and data processing improvements have led to new endeavours targeting more complex brains and, inevitably, the human brain. The combined scientific efforts to unify the generated data in this regard have led to the foundation of the Human Connectome Project (HCP, www.humanconnectome.org), which aims to create an ultimate database for acquiring and sharing information on neural connectivity (Van Dijk et al., 2010). Local governments have firmly supported this initiative. For example, in 2010, the NIH awarded \$40 million to characterise the healthy human brain connectome and its variability (News, 2010). This study in progress has involved a collaboration between Washington University, the University of Minnesota, and Oxford University, forming the “WU–Minn–Ox” consortium, aiming to map the structural and functional brain connectivity in 300 healthy adult families to determine alterations related to genetic and environmental contributions and optimise MRI technologies (Van Essen et al., 2013). In addition, efforts in the UK have led to the establishment of the UK Biobank, which utilizes brain imaging to create the first comprehensive database on human structure and function, initially gathering information from 5,000 participants. **Figure 1.5** shows that ongoing global large-scale initiatives aim to map the human brain connectome in detail, including its age- and disease-related changes (Elam et al., 2021). This includes identifying changes in connectivity throughout the lifespan, understanding the causes of brain diseases, developing more effective markers for therapeutic interventions, and advancing non-invasive imaging techniques (Miller et al., 2016).

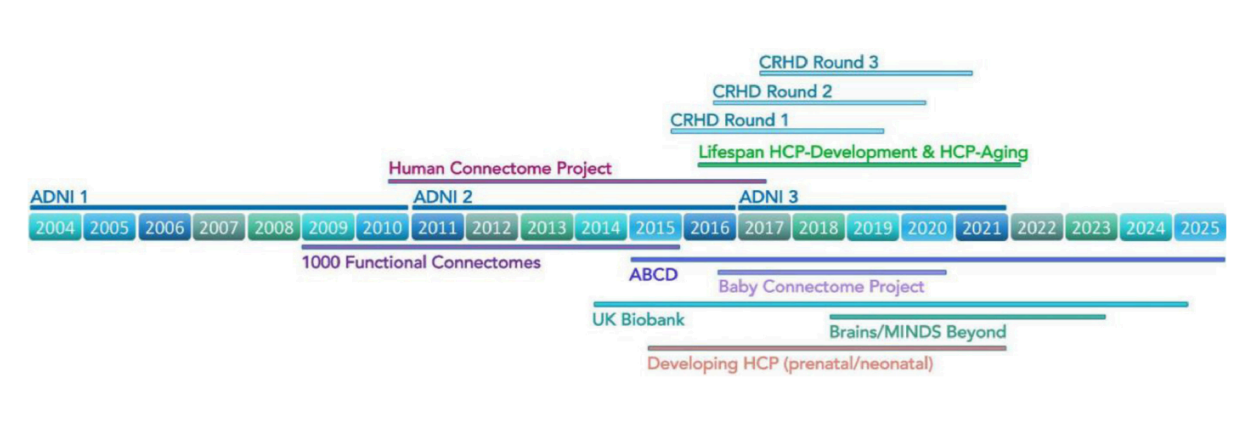


Figure 1.5: Timeline of large-scale imaging projects on the human brain connectome

Recent initiatives that have contributed to the human brain connectome by scanning, processing and integrating local neural circuits. Progressive assembly of regional maps has extended the coverage to wider regions of the brain. Alongside the Human Connectome Project, centred on the healthy adult brain, other projects have aimed to cover different developmental stages and relevant pathological conditions. Abbreviations: ADNI, Alzheimer’s Disease Neuroimaging Initiative; ABCD, Adolescent Brain and Cognitive Development Study; CRHD, Connectomes Related to Human Disease; HCP, Human Connectome Project (from Elam et al., 2021).

The immense amount of data generated from large-scale imaging projects necessitates efficient access, management, and sharing. Dedicated databases have been developed to meet this purpose. For instance, data from the HCP is stored in the ConnectomeDB database, which has

led to tremendous success in reaching the scientific community. Elam et al. (2021) followed the data sharing derived from the initial HCP data release over a 10-year time period from 2010 to 2021, covering the subsequent data release stages. They found an exponential increase in the usage of HCP outputs, reflected in the high volume of data shared, the constant development of software tools, and the growing number of publications derived from connectivity data sharing (**Figure 1.6**). This trend is expected to persist in the upcoming data releases from the HCP project and related initiatives.

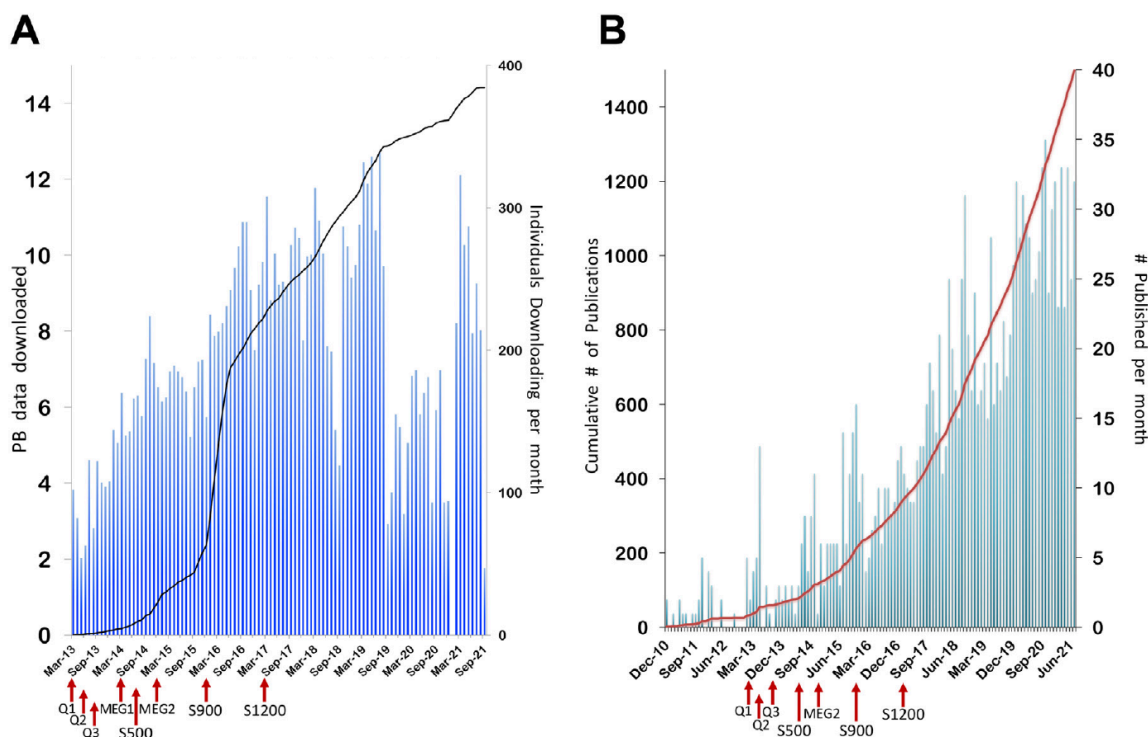


Figure 1.6: Usage of data from the Human Connectome Project

Cumulative downloaded data amount (in Petabytes of information) from PubMed database related to the Human Connectome Project. Data release dates of each project stage are indicated in the X-axis with red arrows. Usage has exponentially increased with each data release during recent years.

A. Downloaded data from the ConnectomeDB database, related to mapping of local circuits of the human brain and integration into the Human Connectome Project.

B. Number of publications available in PubMed database using Human Connectome Project as their core topic, using related imaging or resulting circuit mapping methods. (from Elam et al., 2021)

All mapping data requires validation in a biological environment, and experimental procedures must be performed to confirm that the generated products accurately reflect the structure and function of the nervous system. Here, an upcoming challenge related to the experimental biological models used for this purpose is presented. Several *in vivo*, *ex vivo*, and *in vitro* models have been developed to identify and test nerve connectivity, which will be discussed in the following section. However, as described, all of them have specific limitations related to the fidelity in reproducing natural-occurring circuits, accessibility in manipulating individual network elements, ease of obtaining biological materials, and consistency in the circuits. The increasing reach within the scientific community and the growing use of data from imaging initiatives necessitate an urgent escalation in generating suitable biological samples to support experimental work for connectivity validation. **This thesis aims to provide technical tools to meet the growing scientific demand by developing processes that improve access to *in vitro* biological models for studying neural circuits.**

1.3 Experimental Strategies for Studying Neural Circuits

The study of neural circuits, with analysis spanning various levels of complexity, can be viewed as assembling a puzzle, as illustrated in **Figure 1.7**. The big picture, the entire circuit or a connectome, can be inspected from its elemental parts, namely individual neurons and synapses. Understanding the big picture (the structure and function of the circuit) can be explored in opposite directions. Depending on the chosen strategy, the required biological samples will vary, ranging from fully formed circuits to individual neurons. Aside from the specific benefits and drawbacks of each circuit fabrication technique, as described in the next sections, certain strengths and weaknesses are shared based on the employed approach, which comprises the current scenario and challenges in the field of neural circuits research.

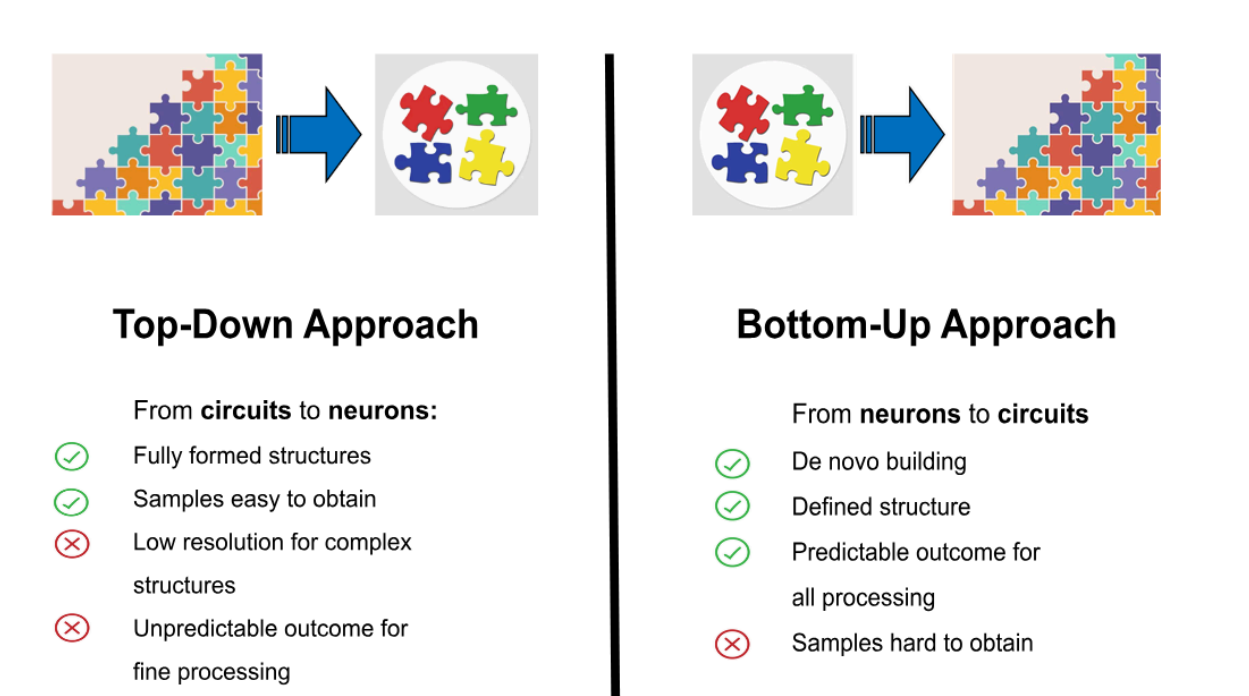


Figure 1.7: Top-Down and Bottom-Up approaches for studying neural circuits

Comparison of the advantages and disadvantages in each case. Due to the costs and accessibility of manipulating individual circuit elements, Top-Down approaches have been primarily used.

On one hand, the straightforward and reductionist study of natural circuits from nerve tissues, represented by the Top-Down Approach, represents an integral network analysis that encompasses all neurons and synapses as a unity, irrespective of their previous identification as isolated units or discrete circuits (Jeon & Kim, 2023). The existence of non-linear information processing is acknowledged, but the examination is focused solely on input and output information and confined to the neurons involved at those levels. The hidden layer remains an unmapped entity, similar to a black box, where diverse inner motifs interact through regulatory mechanisms, horizontal processing, and inter-circuit interactions (Budinger & Heneka, 2025). Following a trial-and-error experimental validation, the mapping and uncovering of the circuit details rely on the repeated acquisition of samples from the same (or similar) type of biological specimens to confirm initial assumptions.

The structural confirmation is based on comparisons with previously known neural circuits, leaving little room for novel structural and functional features. Consequently, a rudimentary description of the information processing mechanisms and the pathways through nerve structures can be derived from molecular, cellular, and behavioural studies. Gradually, research

refinement may lead to identifying most elements and interactions present and how they contribute to anatomical structures. However, some participants whose contributions require clarification (such as glial cells or poorly understood neural types) fall outside the scope of any validation process. Moreover, their involvement is regarded as irrelevant to the circuit map. When the Top-Down strategy is translated from experimental results in natural circuits to generate a model, the incomplete map encounters precision issues regarding information processing. The analysis relies on inference, and achieving manageable uncertainty levels is sufficient to meet mapping resolution requirements (Jonas & Kording, 2017).

In contrast, a Bottom-Up approach to mapping and studying neural circuits centres on precision rather than extension. The spatial and functional properties at each level require a full definition before increasing complexity, ranging from elementary units, such as single cells and synapses, to the connectome level while maintaining mapping resolution. At every stage, the complete circuit map is achieved, and information processing is validated to ensure the inclusion of all elements and synapses, attributing equal relevance to each. Experimental manipulations encompass all input, output, and hidden layers, as accessibility allows for every neuron to be evaluated for its function and participation. Anatomical and functional confirmations define the specific contributions of each network component, adjusting parameters until the partially generated model behaves as expected. Considering elements that were poorly understood from early mapping efforts allows for a reevaluation of their roles, leading to the discovery of novel functions and regulatory mechanisms. Circuit modelling also benefits from this approach, as translating biological events into mathematical terms requires not only defining each element and its interactions within the system but also providing a complete description of the parameters involved in each processing event. Both requirements resemble logical data assignments in informatics.

Consequently, an accurate description of a biological circuit can lead to appropriate mathematical and computational modelling. Model calculations often align with observable experimental results from other *in vitro* circuits or *in vivo* structures that display predictable behaviour. Understanding basic information processing rules can facilitate complex analysis, requiring only minor corrective measures when dealing with complex systems. Discrepancies can be solved by analysing individual constituent elements with minimal levels of uncertainty. Thus, solid anatomical and functional evidence can support causal relations in information processing.

1.4 Top-Down Approach to Studying Neural Circuits

Mapping complex circuits is challenging, often requiring the system to be divided into segments to dissect the structure and function of smaller circuits based on simple roles. This can be achieved by stimulating individual neurons or regions, and monitoring the effects on other neurons and the overall area. To exemplify this, we can compare the nervous system to a mechanical machine, such as a car engine (**Figure 1.8**). Explaining its function requires a complete structural and functional description of the components, represented by the individual parts, and their interactions when performing a specific task, such as traction generation. To understand the working mechanisms without prior structural information from the machine blueprints, the function of each part is often examined in isolation, with an impact on the overall system. Removing a vital component will halt machine operation, and altering one component affects other components directly related to it.

The nervous system, as in this example, is spatially constrained and composed of parts (i.e., organs, ganglia, nuclei and layers) with various properties (i.e., neuron subtypes) and specific interactions (i.e., synapses) to perform their designated functions (i.e., nerve function). These relationships can affect the function of the same part in different ways, influencing both adjacent and distant elements as well as the overall performance of the machine.

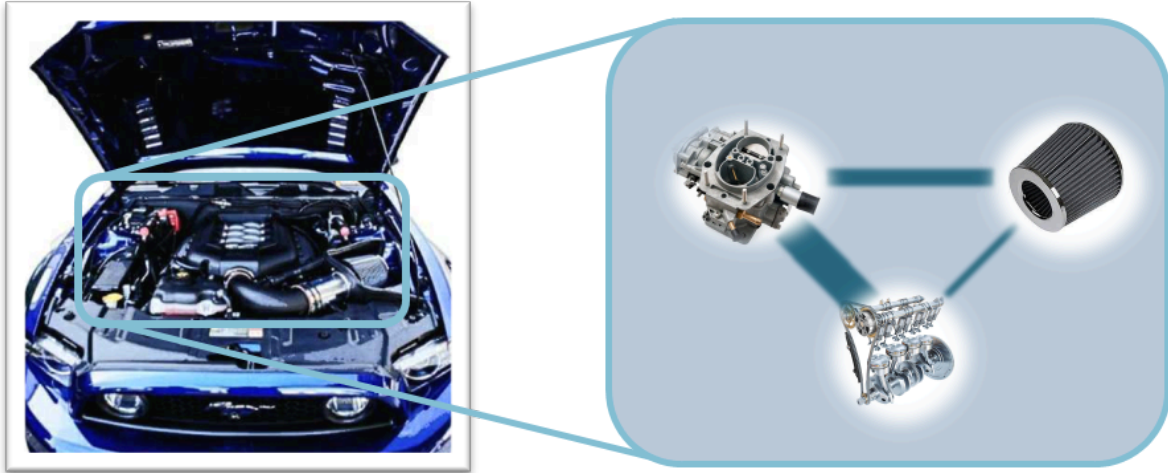


Figure 1.8: Analogy to represent the Top-Down approach for the study of systems

Traditional reductionist mapping approaches follow a Top-Down direction. This could be viewed as disassembling a car engine to inspect individual parts and elucidate their contribution and interactions during traction generation.

With the previous information, a preliminary explanation of these interactions can be formulated, assuming the occurrence of the simplest events, following the principle of parsimony, which can serve as a starting point for testing other assumptions. A similar situation occurs if we focus on a specific subfunction of the machine and interfere with one or more of its components. The examination will proceed in a trial-and-error manner to formulate assumptions about the components and their interactions and contributions to local and systemic functions. Collecting sufficient information can generate local network maps, which can progressively be assembled to reconstruct the blueprints of the entire machine. Subsequently, new tests may be necessary to confirm the validity of these assumptions and to make appropriate adjustments to better understand the functions of the entire system under varying conditions. Moreover, this validation process can uncover new interactions that a straightforward manipulation of the elements may not detect. These interactions may include parallel, redundant, or branched information exchange pathways. Hence, the system blueprint will be consistently updated and enhanced as new information is acquired.

The same strategy and concepts, translated into biological terms, can be applied to studying the Nervous System and neural circuits. Understanding nerve functions and mapping neural arrays by examining isolated parts of a complex network, followed by an assembly and testing phase, is referred to as a Top-Down approach to studying neural circuits. By employing this strategy, the intricate architecture of the Nervous System has been dissected, leading to the partial mapping of the brain and the identification of primary neural circuits that control remarkable functions (Kveraga et al., 2007). Despite the technical diversity and the biological samples used in each study, Top-Down approaches share some characteristics, represented in (**Figure 1.9**):

1. Initially, intact nerve structures are divided into discrete areas, such as anatomical brain regions, containing arrays of neurons.
2. Subsequently, synaptic connections are identified through cell visualisation or activity recordings. In the case of cell staining, single synapses are labelled. In contrast, recordings follow outputs that can be produced after one or more neurons process the stimulus. Only portions of the circuit, consisting of single information processing paths or even single synapses between pairs of neurons, can be identified in this step.

3. The next step involves an anatomical reconstruction to integrate these sections and build a unique network map that covers all the identified neurons and synaptic connections. The cell location in the intact structure is also identified to relate the observed results to the physiological properties known for each nerve structure. Three main groups of neurons or layers are established: the input layer, where the stimulus is directly received and processed; the output layer, where the nerve response is generated; and the hidden layer, which includes all the neurons and intermediate stages of information processing. A model of the neural circuit is generated.
4. Some adjustments to the model may be necessary to align with the experimental results. Inferences about cell interactions are tested. This can be achieved through techniques such as stimulating specific cells and recording the response or by monitoring whole nerve activity, as in the case of fMRI. New neural interactions, such as activity-regulatory mechanisms or interactions with other circuits, may emerge at this point. After a mapping refinement process, the obtained circuit model should explain the mechanisms of information processing that produce nerve function based on the empirically observed structure, interactions, and neural activity.

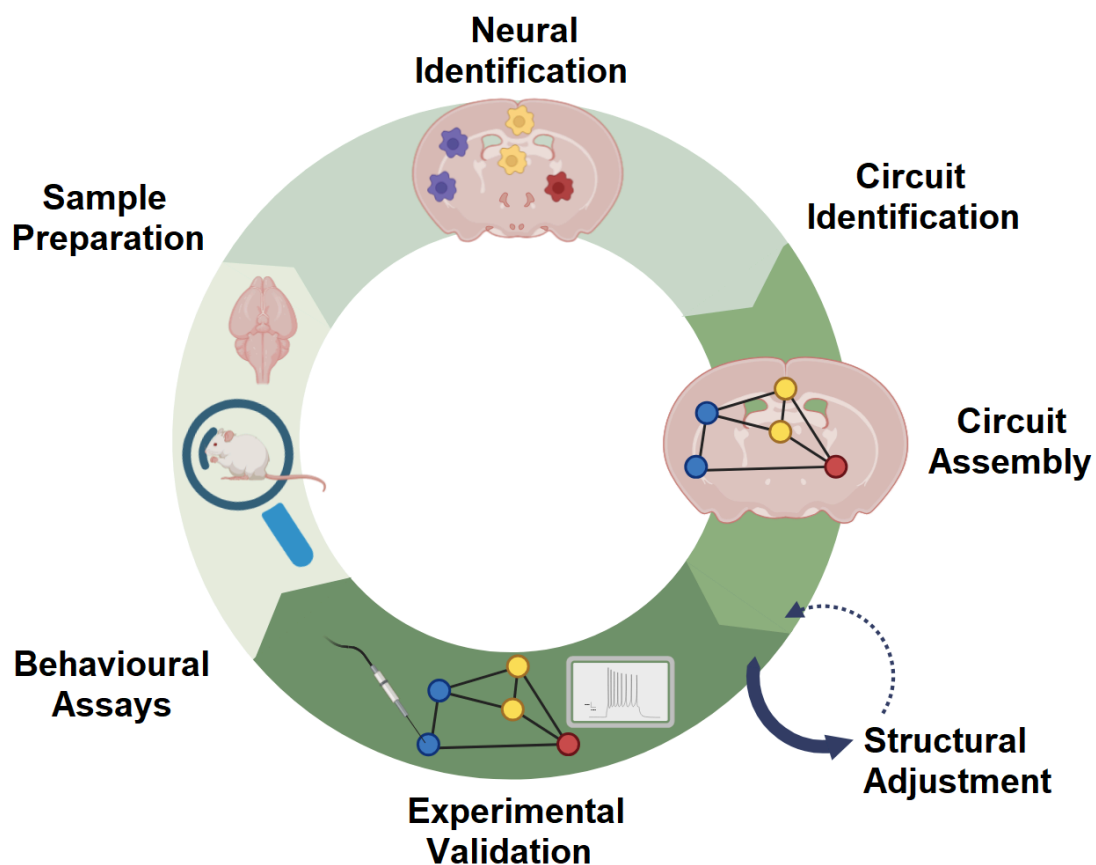


Figure 1.9: Experimental strategy for the Top-Down mapping of neural circuits

Mapping neural circuits is a process of continuous improvement. Nerve function is associated with the activation of specific neurons and their synaptic connections following a processing route. Anatomical and structural data is integrated into models that require further experimental validation to confirm, refine or modify initial assumptions about circuit structure and function.

1.5 Building Neural Circuits Using the Top-Down Approach

To determine the role of individual neurons and simple neural connections within the intricate architecture of the nervous system, which consists of millions of neurons and a complex network of synaptic connections (approximately 86 billion neurons and 600 trillion synapses), it has been essential to develop experimental strategies to dissect these highly complex networks into suitable samples for practical examination. Several factors have shaped the strategy for analysing neuronal circuit architecture. Ideal biological samples for circuit research must capture snapshots of the intact circuit under physiological conditions, preserving the complete shape and spatial distribution of the constituent neurons and their processes (i.e., dendrites and axons), as well as the synaptic connections and their mechanisms for information processing (Heckman & Doe, 2021). The following paragraphs describe the main techniques for studying and mapping neural circuits using a Top-Down approach, including their current limitations and how to overcome them.

Direct Neural Staining and Markers

The discovery of neurons as elemental constituents of the Nervous System immediately raised questions about their structure and function. Histological descriptions in *ex vivo* brain sections by Camillo Golgi identified the main features of neural structure, including their entire shape with axons and dendrites, as well as physical contact between these cells (Golgi, 1873). Golgi stain, based on potassium dichromate and silver nitrate, was used in these initial descriptions to stain entire neurons in a contrasting dark colour, following local groups of neurons (**Figure 1.10**). The concept of neurons forming networks as the basis for information processing in the brain sparked interest in studying neural interactions. Decades later, Ramón y Cajal's studies utilized the Golgi stain to observe neural morphology and demonstrated that neurons and neural connections are the fundamental components of the nervous system (Ramón y Cajal, 1911).

Despite their success in pioneering studies, the technical capabilities of staining techniques soon faced challenges when applied to complex circuits, such as the brain. The colour mark of this stain is very limited by the diffusion of the compound, and only a small group of neurons close to the application site can be stained. In addition, all cells are uniformly stained in the same colour, making it impossible to distinguish between axons and dendrites from different cells. For many decades, the Golgi stain became useful only for the study of isolated neurons and more sensitive and selective methods were required to be developed. Still, after 150 years of its invention, Golgi stain continues to be refined with new technologies for high-resolution 3-D imaging of individual neurons (Vints, 2019).

From the initial descriptions of neural structure, physical contact in specialised extensions between pairs of neurons was suspected to be the site of information transmission in the nervous system. Light microscopy studies assumed the existence of a synaptic connection when the axons of one stained neuron overlapped with the dendrites of another (Cajal, 1911). Due to the inability to stain all neurons simultaneously in the samples, the first attempts to trace and map neural circuits involved repeatedly labelling neurons in a limited area, aiming to identify as many connections as possible. Subsequently, single connections were organised into libraries and manually assembled to reconstruct neural circuits section by section. In addition to Golgi staining, other methods, such as intracellular dye filling (Gilbert & Wiesel, 1979) and, more recently, genetically encoded markers, were developed. The latter first enabled the marking of specific neural subtypes (Feng et al., 2000). However, advances in the mechanisms of synapse formation have raised issues regarding the criteria for classifying simple physical proximity as indicative of a functional synaptic connection.

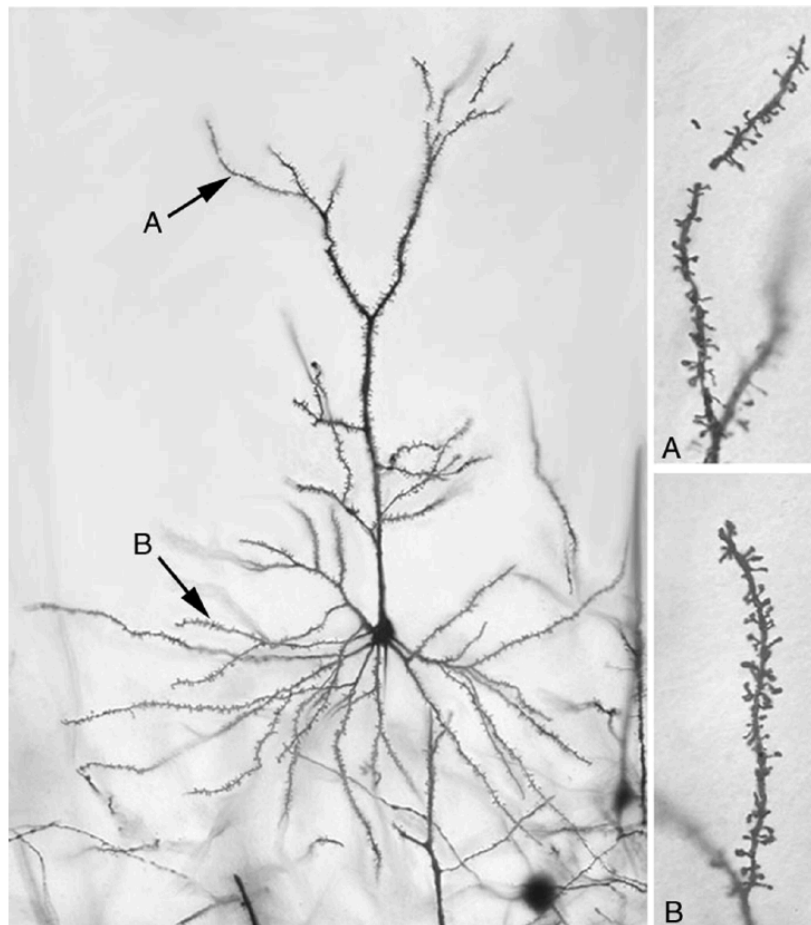


Figure 1.10: Golgi staining for morphology visualisation in cortical pyramidal neurons

Golgi staining is a standard technique to study neural morphology. After its invention by Ramón y Cajal in the early XX century, the method has been refined in recent years to enhance its resolution and enable the identification of subcellular features in neurons, such as dendrite spines, with high resolution. **A, B.** Amplification of the regions indicated with arrows (from Kolb & Gibb, 2015)

Trans-synaptic Tracing

Several tracking molecules were developed in subsequent years to reveal the complete neuronal morphology with enhanced resolution, including their axonal projections, compared to direct neural staining. These molecules, termed tracers, are applied directly inside specific neurons by injection or internalised by endocytosis. In these techniques, after being internalised by a cell, the posterior distribution of the tracking molecule is identified, and its propagation to adjacent cells indicates physical contact between them. The spread of the molecule depends on intracellular transport systems, resulting in a more intense signal at the application site, with progressive signal intensity decreasing proportionally to the distance. It is also possible to distinguish when molecules have been transported anterogradely and retrogradely through the neural axon. This group of molecules includes the anterograde tracers agglutinin and the retrograde tracers fluorogold, cholera toxin subunit B and carbocyanine. The use of tracers as a standard for studying neural connections over the next few decades confirmed the existence of conserved circuits between different brain structures and peripheral nervous system projections. Additionally, the input-output identities of neurons were established based on the direction of tracer displacement (Saleeba, 2019).

One concern in using these tracers is the limited cell marking, which produces only local maps of the circuits. The extent covered by this tracking method depends on the diffusion properties of the tracer molecule and the functionality of transport mechanisms, typically spreading within a single neuron, including somatodendritic compartments. A maximum of a few connected cells

per sample can be monitored through repeated tracer application to adjacent neurons. Furthermore, diffusible tracers cannot differentiate between various cell types, and additional markers cannot be incorporated into the same molecule. However, antibodies can be developed to target the molecule, as seen in the case of fluorogold processed with biotinylated secondary antibodies. Toxicity presents another significant concern when using certain neuronal tracers, as they disrupt normal axonal transport mechanisms, potentially leading to neural death due to a diminished supply of vital molecules to the soma (Hu et al., 2013).

Recently, viral vector technology has significantly enhanced tracing capacity by linking fluorescent markers to non-pathogenic viruses that naturally infect and migrate within neurons, allowing them to spread across synaptic connections (Aschauer et al., 2013). Adeno-associated virus (AAV) has been a widely used viral vehicle for tracing neurons and for anterograde and retrograde labelling of synaptic connections (**Figure 1.11**). In the last decade, AAV cocktails have been developed that express more than one fluorescent protein (i.e. red, blue, green) in the same sample. These AAV-expressing neurons fluoresce in different colours, facilitating the differential labelling of individual neurons, including all their synaptic processes, over long distances (Han et al., 2023). Also, the inclusion of specific promoter sequences in these DNA constructs can restrict the expression of the fluorescent marker to specific neural populations. These tracers have also been combined with advanced 3-D imaging tools to reconstruct large brain sections at single-cell and single-synapse resolution. The most remarkable applications of this technical combination are ongoing initiatives to generate connectomes (Oh et al., 2014). Despite these advantages and the significant amounts of data these tools can generate, an identified issue with tracers is the frequent incomplete characterisation of synaptic partners (false negatives) and the labelling of non-synaptic patterns (false positives).

Consequently, anatomical results still require experimental validation, such as functional studies, to refine the generated models and ensure accurate reconstruction of neural circuits. **Therefore, developing methods to reconstruct neural circuits in vitro with specific structures for validation will enhance the potential of viral vectors for circuit mapping.**

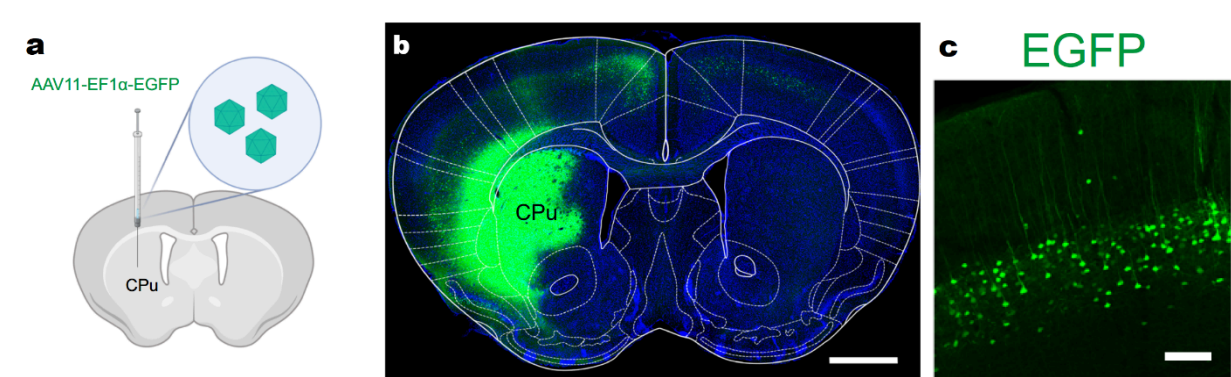


Figure 1.11: Neural tracing using AAV vehicles conjugated with fluorescent proteins

Cell labelling using viral constructs has enabled the visualisation of specific neural populations in discrete brain areas. Adenoviruses containing gene constructs can be injected locally into the brain to integrate exogenous genes into the neuronal genome and enable the production of fluorescent reporters. Here, mouse brain slices were injected with an EGFP-based fluorescent probe, which was subsequently expressed in the caudate-putamen (CPu) neurons to visualise their distribution and anatomical features. Scale bars: b = 1 mm, c = 100 μ m (from Han et al., 2023).

Serial Electron Microscopic Reconstruction

Neural and axonal tracing facilitated the identification of neural circuits composed of a limited number of cells. Libraries of local circuits were created to map distinct nerve structures. However, integrative methods to analyse high-throughput data were essential for mapping the brain at single-cell and single-synapse resolution. In contrast to previous techniques, which generated data from numerous samples and individuals while aiming for consistent connectivity patterns, serial electron microscopic (EM) reconstruction emerged as an innovative method for elucidating complete maps of complex neural networks from individual subjects. Enhanced by the development of computational imaging analysis, this method involves labelling neurons and synapses using earlier techniques on ultra-thin tissue sections (measuring a few micrometres), encompassing the entire brain or nerve structure into a collection of electron microscopy images. Individual images are then annotated to identify the presence and location of distinct neurons and synapses, forming local maps within each image. Based on spatial position, the information is organised sequentially, followed by confirmation of neural locations and interactions through comparison with preceding and subsequent tissue sections. This process is repeated for each image, consolidating the neural positions and the existence of synapses. Gradually, a complete 3-D brain map is constructed by integrating information from all images (Chklovskii et al., 2010).

It is necessary to emphasise that the generated map corresponds only to individual cases. To produce results that adhere to the principles of totality and universality, which are crucial for a reliable connectome, the process must be repeated, generating a connectome for each case. This demanding task has been successfully carried out to create the first precise connectomes using EM reconstruction, among other species, in *C. elegans* and, more recently, in *D. melanogaster*. Aside from the substantial time and information processing power required for high-throughput data analysis, which needs dedicated hardware and software, data generated by EM reconstructions still require in vitro experimental validation to ensure accuracy (Briggman & Denk, 2006).

Brain Lesions

After confirming the existence of conserved synaptic connections between different brain structures, researchers progressed to link structured neural arrays with observable nerve functions. This was achieved by interfering with the neurons responsible for initiating, propagating, or integrating information into a nerve response. However, a significant challenge emerged due to the lack of technical resolution to selectively ablate or interfere solely with the neurons forming the circuit of interest without affecting nearby cells. Consequently, researchers were required to induce permanent lesions in the entire brain area where the neuron of interest was presumed to be located to assume its removal from the neural network. Following this, researchers examined the observable behaviour related to the affected brain circuit to determine whether the neural removal impacted nerve function (Lavond & Steinmetz, 2003).

The first example confirming the existence of a brain circuit that governs complex nerve functions was the identification of the medial temporal lobe, specifically the hippocampus, in the process of memory formation. In the 1950s, the case of patient H.M. underscored the link between hippocampal damage and memory impairment following surgery. This issue was observed over subsequent years and attributed to focal damage in the hippocampus (Corkin, 1984). Subsequent studies involving bilateral local ablation as a treatment for psychosis, which damaged the anterior hippocampus and the hippocampal gyrus, resulted in a severe loss of recent memory in the patients examined. In contrast, lesions in adjacent brain areas did not lead to similar impairments. These findings indicated that only specific regions of the hippocampus are critical for memory formation, and disrupting these structures can compromise nerve function (Scoville & Milner, 1957), establishing the significance of hippocampal neural circuits in memory formation.

The development of animal models of brain lesions has enhanced research by allowing for precise control of the compromised area rather than relying on previous patient conditions. Injections of chemical compounds, such as kainic acid, are the most common method used to induce local neural death (Foster et al., 2003). Genetically-targeted ablation using the diphtheria toxin (DT) gene under a promoter of interest is also possible (Saito, 2001). In addition, localised electrodes can be placed in the brain areas to induce electrical injuries (Reisner, 2013). Moreover, tissue cooling using specialised probes has been tested to produce reversible lesions (Brooks, 1983). From classical studies to recent research, brain lesion models have been used in human and animal models, similar to hippocampus studies, to relate localised brain areas to complex nerve functions and pathologies, including language comprehension in the cortical superior temporal gyrus (Wernicke, 1874), visual control upon stimulus (Hausen & Wehrhahn, 1990) and the generation of epileptic seizures (Ben-Ari et al., 1981). Finally, axotomy, the physical interruption of axon integrity, has been extensively used to disrupt neural circuits at specific sites.

Despite its historical significance and the various experimental models based on brain lesions, this technique remains subject to criticism. Firstly, there is the paradoxical nature of the method, as it aims to study “nerve function” by creating a state of “dysfunction.” In other words, ensuring that the nerve circuits can still be regarded as normal after an irreversible alteration is not always possible. Compensatory mechanisms may be activated to mitigate the impact of this injury, concealing the true effect of the experimental manipulation on the system. Secondly, techniques used to induce lesions rarely affect individual neurons. This lack of selectivity suggests that the environment has been altered rather than just affecting a single circuit element, which includes other neurons and non-neural supporting elements, such as glial cells (Lomber, 1999). Chemical and physical stimuli, such as inflammatory processes, can also elicit tissue responses that extend beyond the nervous system, generating signalling molecules that indirectly influence nerve function. Furthermore, procedures to induce the lesions are associated with precision issues. Chemical compounds often employ diffusion to reach cells from the application site, making it challenging to affect only specific neurons or nerve processes. In conclusion, the main issues associated with using lesions to interfere with neural circuits are their irreversibility, lack of precision, and indirect effects on the tissue.

Electrophysiological Recordings

Information about neural circuits obtained during the anatomical analysis of nerve structures inherently necessitated a method to validate the suspected relationships concerning the functional properties of the neurons within those areas. Neurons, being excitable cells, communicate by altering their electrical properties, resulting in rapid membrane voltage changes due to ion exchange across the membrane. This electric potential is transmitted along the axon to the terminal ends, where it generates an action potential. As a result, neurotransmitters are released at the synapse to affect adjacent neurons. Electrophysiology encompasses all techniques for measuring and controlling electrical activity in neurons, as well as monitoring changes in voltage and electric currents in cells and tissues in response to a stimulus. These electrical changes can be produced and recorded using electrodes in individual cells and even at sub-cellular levels, such as the single ion channel (Sanziani & Häusser, 2009). Direct stimulation of individual neurons and recording of the response in the same neurons and innervated targets have represented the gold standard for testing interactions in a circuit. If Neuron A is connected to Neuron B, stimulation of Neuron A via electrical or chemical means will generate an action potential, which, in turn, will elicit a response in Neuron B. This response can be recorded as a voltage change.

An electrode can be placed on one of the involved neurons to map and examine the neural circuit. Local stimulation generates an input signal, which can be recorded as an output response in a second neuron. Although this principle does not imply a straightforward connection between two distinct neurons, varying the electrode position to target different neurons can provide a comprehensive understanding of the cellular interactions in the studied area. Furthermore, many of the mechanisms involved during signal propagation can be

identified by combining the recordings with the application of molecules that alter neural activity, such as ion channel agonists and blockers.

After several decades, electrodes have significantly improved their stimulation and recording capabilities. Initially intended for voltage and current applications in individual neurons, it is now feasible to manipulate single molecules within membranes and, with the development of mini electrode arrays, to precisely stimulate and record from individual neurons within groups. By integrating the spatial and temporal responses, a local map of the circuit can be generated. In this technique, several microelectrodes are grouped or contained in fabricated chips (Pelkonen et al., 2021). Then, the array is placed on a neural sample (as a culture) to stimulate and record neural responses in the contact sites between electrodes and neurons, and the recorded responses are integrated into a local circuit map. (**Figure 1.12**).

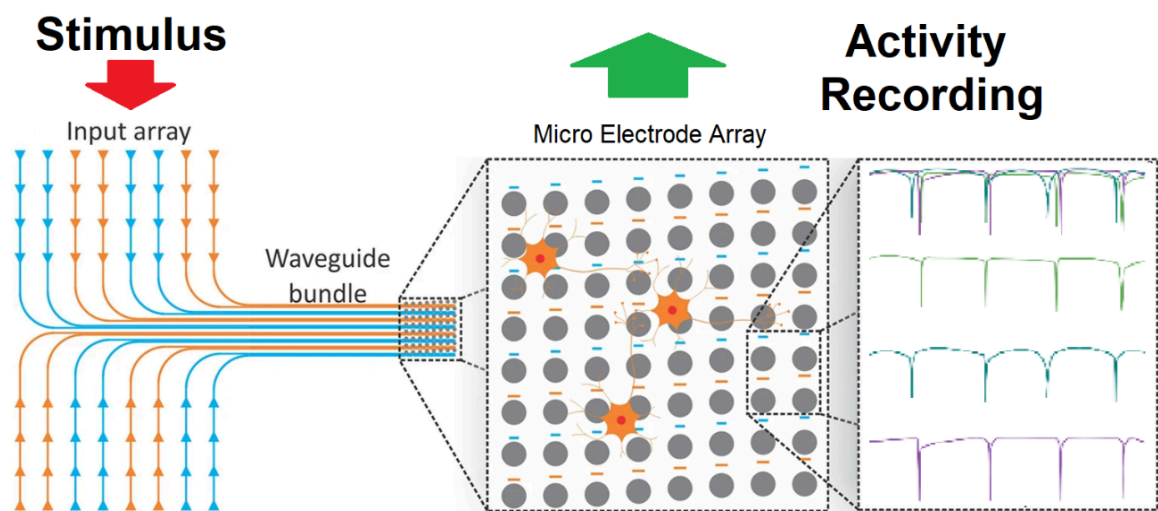


Figure 1.12: Microelectrode arrays for mapping neural circuits

Devices formed by numerous electrodes at regular intervals, capable of stimulating and recording neural activity, can be placed over a biological sample with neurons forming circuits. The activation of a single electrode stimulates a neuron in proximity and records this activity. The stimulus can be transmitted to connected neurons, with a delayed recording of their own activity patterns. The subsequent identification of the spatial and temporal distribution of the activation patterns can be used to map the activated neural circuit. (from Pelkonen et al., 2021)

Functional magnetic resonance imaging (fMRI)

Functional magnetic resonance imaging (fMRI) is a technique for non-invasively visualising brain activity, developed in the early 1990s. It relies on exposing the brain to magnetic fields and detecting electromagnetic response signals with a scanner. Protons in the nuclei of certain atoms react to strong magnetic fields, and applying a radiofrequency pulse increases the magnetisation levels of these nuclei. When the radiofrequency pulse is removed, they return to their original states, producing an energy emission that the scanner can track and integrate into 2-D and 3-D spatial brain maps based on differences in local magnetic properties (Huettel et al., 2009).

Unlike structural MRI, which focuses exclusively on brain shape and integrity, fMRI can track changes in blood flow in specific regions according to energy demands, referred to as the hemodynamic response. This change results from the magnetic properties of oxygen-rich blood and is facilitated by a specific contrast scanning technique known as blood-oxygen-level-dependent (BOLD) imaging. Thus, fMRI can detect variations in signal

based on blood oxygen levels (Ogawa & Lee, 1990). By linking blood flow to brain activity, an increase in neural activity within a particular brain region is expected to manifest as an increased energy demand and enhanced blood flow. The resulting brain activity maps are produced using a colour code, highlighting areas with elevated brain activity. Consequently, fMRI has been employed to investigate brain regions associated with specific functions or experimental tasks. The primary benefit of fMRI is that it does not necessitate invasive manipulation of the area under investigation or the use of injected contrast agents.

The EM-fMRI variant of the technique has been developed through electrical microstimulation (EM) of individual neurons in specific brain regions using implanted electrodes, primarily focused on the cerebral cortex or subcortical nuclei (**Figure 1.13**) due to electrode invasiveness. Following this local stimulation, whole-brain activity is monitored. Consequently, causal links between the activated foci and the areas exhibiting modified activity can be identified as excitatory or inhibitory effects of neural activation. These are signs of interconnected neurons in the corresponding brain regions, forming a circuit (Zhang et al., 2023). Due to the direct structural approach and the capacity to precisely stimulate individual neurons in specific areas, EM-fMRI has quickly become one of the primary strategies for dissecting the structure of neural circuits involving neurons from various brain areas. Despite certain resolution limitations, such as the inability to microstimulate particular types of neurons and the identification of functional local changes rather than individual neural activation in target regions, the extensive application of EM-fMRI has facilitated the generation of the first local cortical connectomes in primates (Xu et al., 2022).

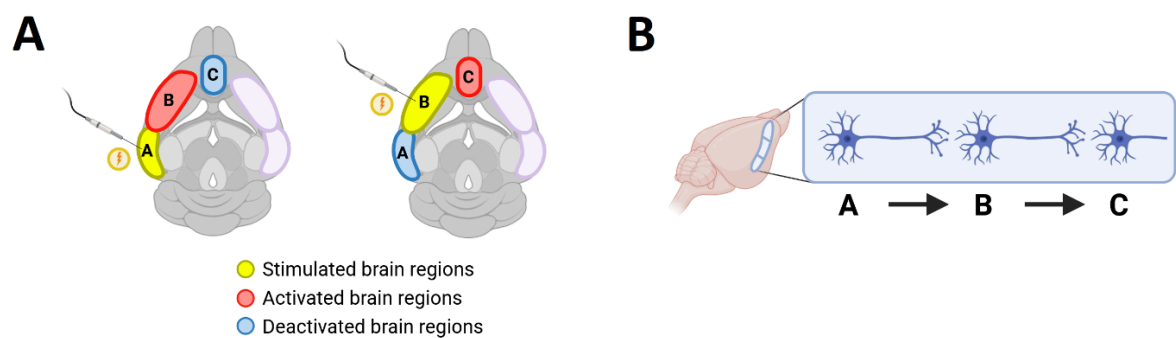


Figure 1.13: EM-fMRI to map neural circuits in the brain

Electrical microstimulation-related imaging techniques have been successfully applied to map superficial areas of the brain. Monitoring cell responses by changes in electromagnetic response signals can be used to infer immediate neural interactions, such as the case of a synapse between a pair of neurons, and to assemble sequential maps of neural information transference. The resulting map constitutes a variable fraction of a neural circuit, which can be fully obtained by the integration of partial maps and may include different nerve structures.

A: Local areas of the brain can be electrically stimulated using electrodes positioned on specific neurons, while fMRI monitors whole-brain activity by detecting electromagnetic response signals. The brain areas responding to the stimulus are recorded and the presence of neural synapses between those areas are assumed. In contrast, neural interactions related to the stimulus are discarded in non-responding areas.

B: The identification of active synaptic connections in each area results in the assembling of a sequential map for the neural information flow that derives in the progressive assembling of local and general neural circuits.

Transgenic Targeting (Chemogenetics / Optogenetics)

Molecular tools based on the expression of exogenous genes in neurons have introduced highly specific mechanisms to control the activation and inactivation of neurons, allowing the study of their participation, contribution, and behavioural effects on neural circuits while facilitating the mapping of neural projections. These techniques rely on the expression of a transgenic receptor in neurons that responds selectively to a specific activator administered by the researcher to either activate or inactivate that neuron, similarly to a switch. The activator affects only the transgenic receptor, leaving neurotransmitter release and neural firing mechanisms undisturbed. Due to the nature of the expression system, the expression of the transgenic receptor can be restricted to a specific population of neurons, such as a particular neural subtype. These molecular tools are named according to the primary mechanisms that promote receptor activation, which include the administration of a selective molecule (chemogenetics) or exposure to light (optogenetics). These are illustrated in **Figure 1.14**.

The transgenic targeting system that allows the expression of the transgenic receptor consists of a viral vector (primarily AAV-derived vehicles), a cell-specific promoter or enhancer sequence (often including a fluorescent reporter tag), and a recombinase inducible transcription system. This combination packages and delivers the transgenic gene into host cells, and the fluorescent indicator enables confirmation of transfection. To target specific cell types, a distinct promoter is employed. For instance, the human synapsin (hSyn) 1 promoter is used for pan-neural expression, whereas the CaM-KII promoter specifically targets excitatory and GAD67-inhibitory GABAergic neurons. Neurotransmitter identity can also serve as targeting criteria, such as the DRD1a promoter for dopaminergic neurons (Dimidschstein et al., 2016).

Chemogenetic tools utilise a natural or engineered macromolecule to agonise the inserted transgenic receptor, primarily G-protein-coupled receptors and ligand-gated ion channels. The agonist is chosen for its potent and selective effect on the transgenic receptor while lacking other effects within the organism. Additionally, the receptor is designed to be unaffected by endogenous neurotransmitters. When the agonist is systemically applied to the organism or biological sample, it triggers receptor activation, either promoting or inhibiting neural activity, depending on the receptor type, in a dose-response manner (Armbruster et al., 2007). Due to the molecular nature of the activator, the subject does not require any further treatment or manipulation beyond the traditional administration of the agonist. Furthermore, the behavioural effects of neural activation can be monitored in freely moving individuals (Conklin et al., 2008). However, the primary limitation of chemogenomic tools is the lack of temporal resolution in monitoring neural activation. The expression of the receptor and neural response events can also be assessed at discrete time intervals. Only posterior reporter expression analysis can confirm the presence and spatial distribution of the transgenic receptor, as well as the titration of the agonist. Consequently, the real coupling of the agonist and behavioural effects throughout the progression of the experiment is only assumed. Additionally, the high doses of ligands required to achieve effective receptor activation are frequently accompanied by non-specific effects. Developing improved engineered ligands is desirable (Boehm et al., 2021).

Optogenetic tools depend on the expression of a family of light-sensitive ion channels, known as retinylidene proteins or rhodopsins, within neurons. These G-protein-coupled receptors transform the presence of a light photon into an electrochemical signal through a conformational change that opens the channel, allowing a selective influx of ions (Boyden et al., 2005). The channelrhodopsin subfamily facilitates the entry of sodium and calcium ions into neurons, while halorhodopsins enable the entry of chloride ions. As a result, neural excitation is produced in the first group, and inhibition is produced in the second. Therefore, when expressed in neurons, rhodopsins can mediate the activation and inactivation of specific neurons upon exposure to light of the appropriate wavelength. Several rhodopsins have been developed to vary in their ion permeability, activation kinetics, and light sensitivity to specific wavelengths (Duebel et al., 2015). The primary advantage of optogenetic tools lies in their ability to reliably activate or inactivate individual neurons with precise temporal control when a light stimulus is applied.

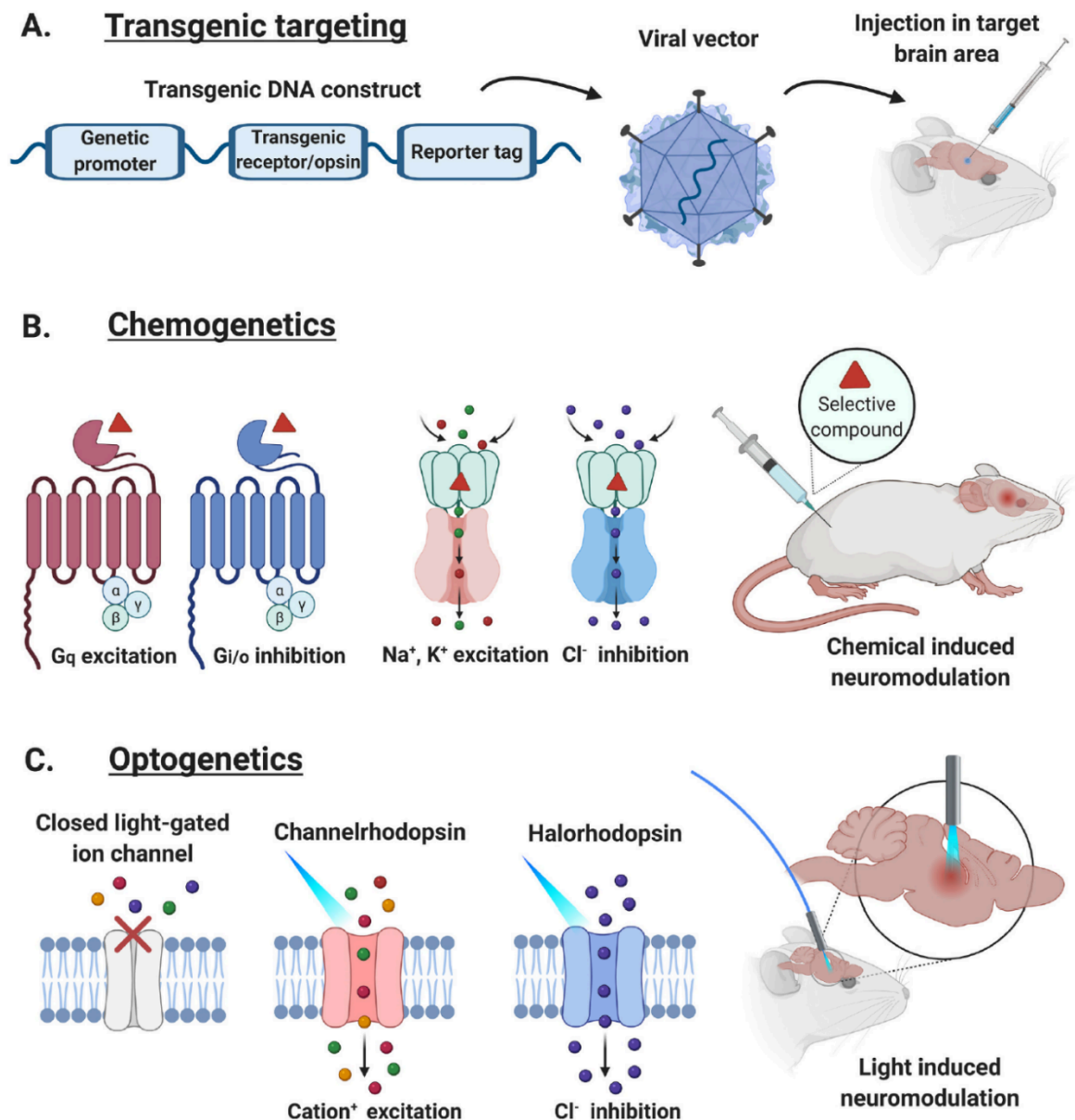


Figure 1.14: Transgenic targeting tools to study neural circuits

Several techniques based on the introduction of genetically engineered DNA constructs have been developed to express inducible receptors in specific neural populations and precisely control the activation of individual neurons forming circuits and facilitate the identification of neural connections (modified from Boehm et al., 2021).

A. Structure of the genetic constructs to express exogenous activable receptors in neurons. The viral construct is injected into specific regions of the brain, where local neurons are infected and express the engineered channel in addition to a reporter molecule.

B. Overall of chemogenetic tools. A selective ligand can activate an introduced exogenous receptor to excite or inhibit specific populations of neurons selectively.

C. Overall of optogenetic tools. Light-sensitive receptors of the channelrhodopsin family are introduced in neurons, which result in the excitation or inhibition of the expressing neurons upon local exposure of the tissue to a light beam.

Furthermore, their application in studying neural activation and circuits can be extended to various in vitro and in vivo experimental models. However, implementing optogenetic tools requires substantial hardware implementation. It is important to consider that light penetration in living tissue confines activation to superficial tissue layers. For that, tethered animals are essential for experiments, which limits their movement and may result in altered behaviours that

mask the true effects of neural manipulation. Also, implants and prolonged exposure to light can harm neurons and disrupt their normal firing properties in long-term manipulations. Finally, invasive methods pose a significant barrier to translating research to human applications. It is essential to develop effective routes for administering DNA and activators (Boehm et al., 2021).

In conclusion, the primary advantage of transgenic targeting tools is their ability to overcome the precision issues associated with other techniques by selectively acting within specific neural populations, such as neural subtypes. This capability enables the precise activation or inactivation of specific neurons within a circuit, allowing the observable effects of circuit manipulation to be attributed solely to certain network elements while leaving the remainder intact. Concurrently, the behavioural effects of neural manipulation in the organism can be monitored, which is beneficial for elucidating the role of neural populations in information processing. Selectivity is desirable not only for in vitro models but also from the perspective of pre-clinical studies. However, a significant challenge of these techniques lies in the inability to track the location and function of neurons expressing the transgenic receptors in vivo.

Ongoing research into human circuit mapping aims to integrate transgenic targeting tools in patients with minimally invasive imaging methods, and positron emission tomography (PET) imaging presents a promising solution. PET imaging entails injecting a radiotracer, a radiolabelled compound, into the patient to track biological functions in vivo. Within the nervous system, these functions may include monitoring brain activity by measuring glucose metabolism or blood flow in specific brain regions (Saha, 2005). Initially, a radiolabeled analogue of glucose is used to track glucose metabolism as an indicator of energy usage following neural activation. In the second instance, radiolabelled H₂O is used to observe changes in cerebral blood flow related to cellular activity. Despite recent advancements, the limitations of PET for widespread use in brain imaging stem from issues concerning adequate radiotracer uptake by the patient and the necessity for dedicated facilities to produce radioactive isotopes in cyclotrons for immediate application due to the rapid radioactive decay of the radiotracers, which have half-lives of about a few minutes (Zimmer & Luxen, 2012). This results in elevated costs that must be considered for implementation as part of routine studies or experimental work involving neural circuits.

This thesis seeks to establish an experimental model that can be utilised to harness transgenic targeting tools, such as chemogenetics and optogenetics. This model facilitates real-time manipulation and visualisation of neurons forming circuits.

1.6 Limitations of the Top-Down Approach Study of Neural Circuits

Despite the extensive implementation of Top-Down approaches to studying neural circuits, the endeavour to describe complex nerve circuitry has encountered significant challenges. Processing pathways are more than continuous and unidirectional highways for information flow. Instead, multiple nonlinearities exist in nerve structures, including intricate axonal and dendritic geometries, loops, redundant paths, lateral connections, regulatory mechanisms, and diverse neural subtypes. The interconnected networks of neurons and synapses tightly regulate information processing, even at the most fundamental level. Hence, the nervous system is regarded as one of the most intricate systems studied by science. Observable nerve functions, such as memory, planning, and task execution, result from orchestrated activity at different levels of complexity. Therefore, findings from circuit mapping using a Top-Down approach must be experimentally validated to confirm their accuracy in truly representing the original circuit. However, even if the complete neural composition of neurons and the existing synaptic connections are known, the circuits rarely behave exactly as anticipated.

To better understand this scenario, it is helpful to describe the characters involved. **Figure 1.15** presents a graphic visualisation of the components of a neural circuit derived from mapping and experimental testing. In addition to the cellular constituents (neurons, synaptic connections), information processing is affected by the intrinsic properties of the pathway, determined by factors such as the environment at the synaptic buttons (the presence of specific neurotransmitters, channels, and receptors), the number and type of nerve fibres, the myelin sheath of the axon, and the existence of parallel pathways serving the same function. This results in additional properties within the system.

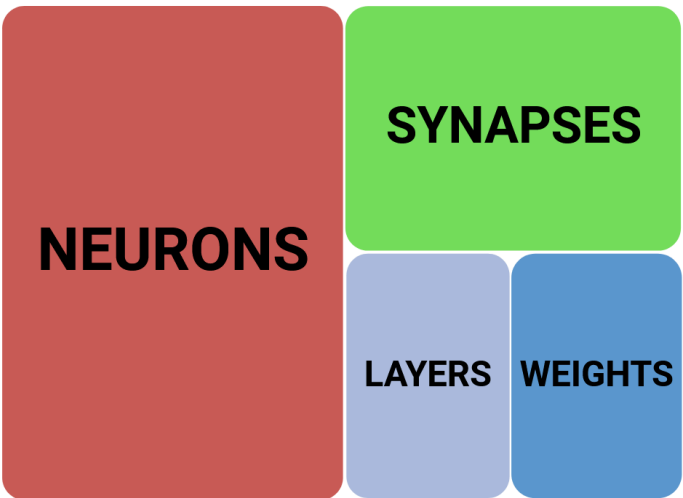


Figure 1.15: Relative contribution of neural circuit components to nerve function

Information processing in neural circuits is influenced by several factors. The main component is constituted by the number and identity of the neurons forming the network. However, the functional properties of the synaptic connections and particular features in nerve structures can influence the manner the information is processed and, therefore, the generated output of the circuit activation.

Neurons form the fundamental building blocks of circuits, determining the information processing characteristics through their individual activation patterns and the type of response generated at each network stage. Synapses comprise various neurotransmitters and receptors with distinct effects, which determine how information is transmitted, propagated, integrated, or regulated at each step. This can lead to alterations in the course of information and the destination, ultimately shaping the final effect on nerve function. The layers component divides

the entire circuit into groups of neurons representing the main features of information input, output, and the hidden steps corresponding to all intermediate information processing. This definition is pertinent to understanding circuit behaviour, including the events that trigger circuit activation and the effects anticipated following information processing through the network. The weights or synaptic strengths of the elements within the circuits describe the influence of an individual neuron activation on the next neuron. This can be affected by the type of neuron, the number and types of interactions with adjacent neurons, and the cellular environment, which encompasses the presence of signalling molecules and the largely unknown contribution of a diverse population of glial cells and neural progenitors to neural function (Yuste, 2015).

In this complex scenario, refining neural circuits at single-synapse resolution was only possible with the development of sensitive labelling techniques (fMRI), transgenic targeting tools (optogenetics, chemogenetics), circuit mapping tools (transsynaptic viral mapping), and imaging techniques (2-photon imaging, high-throughput imaging). This technical progress has begun to fill the gaps in circuit maps that had existed since pioneering studies. However, despite these advancements, some issues remained in generating complete and universal maps that comply with the principles of connectome totality and universality (Van Dijk et al., 2010). These unresolved issues are detailed in the following paragraphs and illustrated in **Figure 1.16**.

Firstly, individual variability represents a prominent challenge. A connectome is not a unique dataset applicable to all members of a species. The universality principle in circuit mapping stems from extensive statistical analyses and comparisons among populations, which eventually have provided representative and consistent results during empirical measurements. For example, the WU–Minn–Ox consortium, part of the Human Connectome Project, has aimed to compare identical twin pairs to minimise this issue when generating brain connectivity maps (Van Essen et al., 2013). However, this approach is not feasible for individual cases, and results must be compared with the most commonly available circuitry data in the databases. Since the available data was gathered from a limited number of individuals with similar genetic and environmental backgrounds, precision issues arise the further an individual is from the original profile. Additional adjustments are required for individuals from underrepresented populations. This can be addressed by increasing the number of individuals and the diversity of their backgrounds when assembling the neural circuit libraries, which are currently constrained by time and cost restraints. **A method for the long-scale building of neural circuits with a defined structure and the ability to assemble multiple variations of the same circuit is part of the proposal in the current thesis.**

Another aspect related to interindividual variability has been the association of certain anatomical features among individuals and the presence of invariant neural connections. The structural inference process has often linked the presence of certain neural components in the visible layer to specific contributions in nerve processing. Only cell-specific recordings can analyse these structure-function links (Friston, 2011). Moreover, fine-tuning mechanisms are often part of the hidden layer of the circuit, taking the form of inaccessible synapses by traditional mapping techniques, such as the case of lateral synapses on cell layers, neurotransmitter segregation from multiple axon fibres within the same neuron, silent synapses, or multiple axon fibres that cannot be separated due to the resolution limitations of the techniques. Recent single-cell-resolution techniques have helped to address this issue. Still, the lack of high-volume sample processing has constrained the application to resolve partial structures of complex circuits or multiple variants of the same circuit.

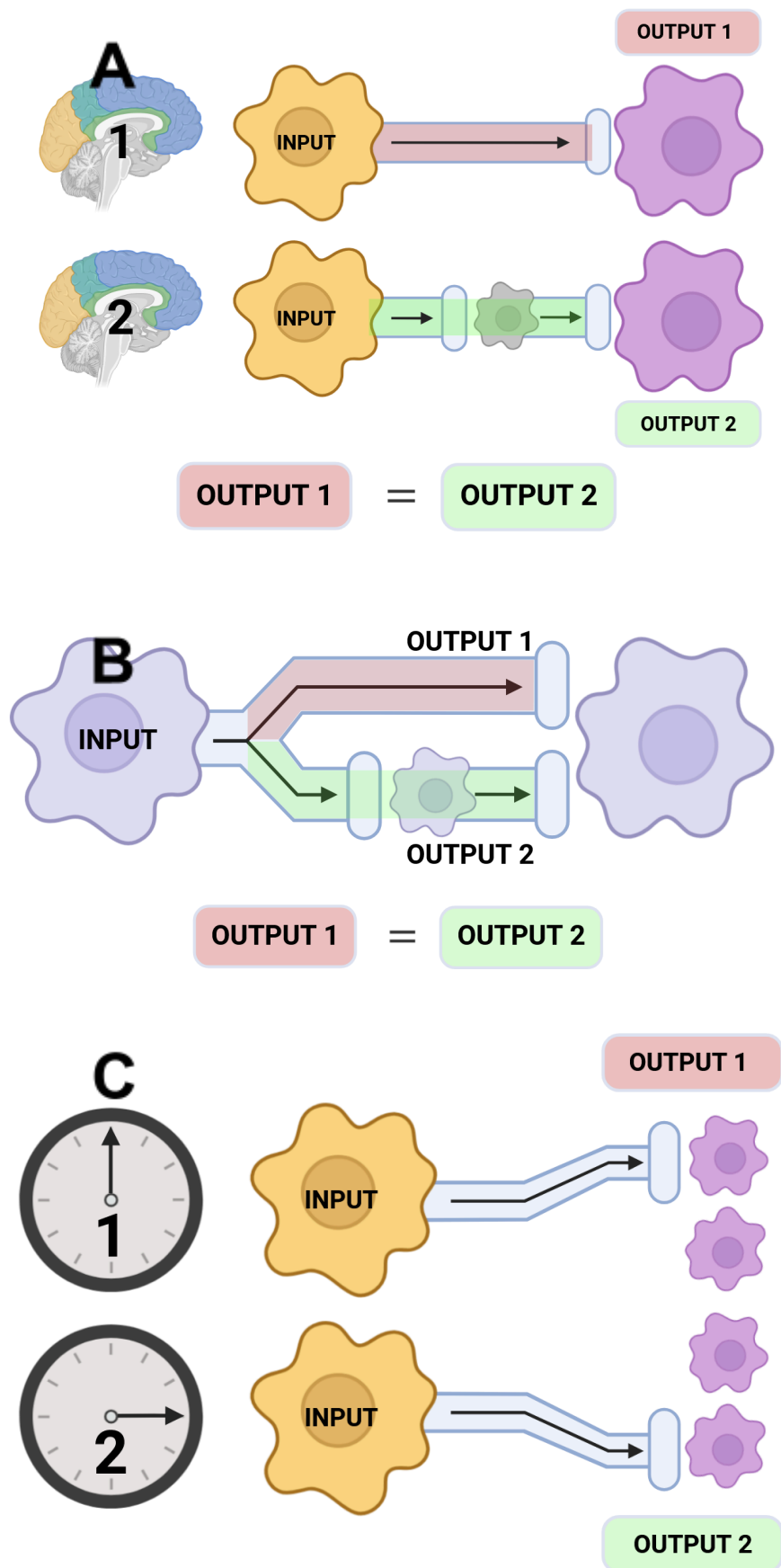


Figure 1.16: Anatomical features that difficult neural circuit mapping

Circuits with diverse structures can generate similar outputs after information processing.

A. Individual Variability: The same pathway is conserved, but the number of neurons involved in the intermediate steps varies.

B. Nerve Degeneracy: A single stimulus is processed through more than one pathway, involving structural and functional differences, but the final output is similar.

C. Dynamic Changes Over Time: A neuron forming a pathway can change its connections with other neurons over time, and the outputs of its activation depend on the involved circuit.

However, the technical limitations of current circuit-mapping methods in biological samples rarely yield complete network maps. Instead, local maps are obtained from discrete areas and then assembled manually or with the aid of computer-assisted tools. Due to the nature of these mapping techniques, some neurons and intercellular interactions are excluded from their local maps and, consequently, are not incorporated in the final version of the model. Experimental validation does not detect their presence to correct the model, as it only focuses on previously identified neurons when refining the initial results (Budinger & Heneka, 2025). Consequently, the resulting description of the hidden layer (i.e., the intermediate processing steps) tends to be incomplete, with missing components affecting the model fit with the observable results, as described in the next section.

Another second main consideration is termed nerve degeneracy. This refers to the ability of structurally different elements to perform the same function. In other words, degeneracy refers to the fact that there is often no single path to process information and generate an output, as is the case with redundant nerve circuits. That is, the same response can be the effect of the activation of several neural circuits running in parallel. Some neurons can even be shared between these parallel circuits, converging and diverging their paths at some points of the information processing. In contrast, these paths may not be identical in terms of the sequence of neural synapse steps involved, but they can generate the same output. Degeneracy makes it difficult to establish consistent structure-function relationships in neural circuits and decode their function due to the multiple yet independent effects that an alteration in a single circuit node can trigger. Recovering the function of one path does not mean the same for the rest. On the other hand, compensation can occur, and when the function of one neural circuit is interfered with, the other parallel paths can compensate for the effect in the studied circuit. To prevent this, it is necessary to identify the entire repertory of neural circuits controlling the nerve function under study and isolate it from parallel circuits before applying any experimental manipulation. Additionally, data integration from populations comprising many individuals can help minimise this degeneracy effect (Tononi et al., 1999). **The outputs of the present project can be applied to reduce the time requirements to build circuits in vitro and perform experiments to study repertories of circuit variants.**

Dynamic changes in nerve circuitry over time have also been emphasised (Lewis et al., 2009). Neurons can modify their synaptic connections as part of developmental processes in response to activity patterns following synaptic contact (such as short-term adaptations to activity levels during acute neural stimulation) and during long-term plasticity and circuit consolidation events. A connectome must be viewed as a snapshot of nerve connectivity at a specific moment. Therefore, techniques for dynamic tracking are necessary to account for these temporal changes in the validity of the theoretical framework. Furthermore, to bridge the gap between single-neuron studies and complex network analysis, neural ensembles have recently been proposed as an intermediate method to generate accessible, high-throughput data on the contributions of cell-to-cell events to complex circuit mechanisms through a hierarchically modular organisation (Yuste et al., 2024). Finally, it is essential to emphasize the use of artificial circuits as a means to replicate in vitro the various forms of a neural circuit that encompass all temporal stages, thereby enabling structural and functional tests to better understand these dynamics. **The present project has the potential to produce these circuit variants.**

Other considerations concern analysis methods. For instance, one strategy in dissecting complex neural networks is to divide them into segments by defining a minimal unit or node for study. However, since the cells in the brain are not uniformly distributed, there are variations in cell and synapse densities. Based on the anatomical and functional properties of specific brain regions correlated with those densities, such as fMRI profiles, gene expression profiles, or myelination, a particular node can be defined and maintained for other areas with comparable parameters to yield consistent results (Lashkari et al., 2010). This division of the brain into analogous regions is referred to as parcellation. The minimal node definition can later be adjusted to suit different cell and synaptic density environments, allowing for the dissection of

node connectivity within the node and achieving a detailed mapping of a complex network following a subsequent assembly process (Bryce et al., 2021).

When describing and modelling circuits, a final consideration is that biological neurons should not be treated as electronics that perform a single function with anatomically invariable wiring in contrast with synthetic neural networks. In fact, growing evidence supports the idea that neurons act as multifunctional entities, assuming different roles at various times, even when the network structure remains constant (Katz, 2024). The first example originated from the central pattern generators (CPGs) of molluscs, simple circuits that control reflexes related to fundamental animal functions. CPGs provided the first examples supporting the neural multi-function concept in living organisms. For instance, the dorsal swim interneurons, a set of serotonergic neurons that control swim episodes, exhibit different firing properties, ranging from rhythmic bursts of action potentials to sustained elevated firing rates, depending on the time elapsed since swim initiation. Some other neurons were identified as capable of orchestrating swimming regulation, but their circuits are mostly inactive (Hill et al., 2024). Current questions include elucidating the conditions that swimming control can switch to these parallel circuits. In consequence, consistent behaviours can be controlled by different neural mechanisms at different times (Hennig, 2023). This neural multifunctionality has been recently explored in other organisms, including humans (Li et al., 2025a).

Additional limitations arise when addressing the computational modelling of neural circuits. To represent information flow as a logical system, biological events occurring at each implicated neuron must be numerically defined, employing abstract language to describe the rules that an input signal follows to generate the output neural function. The first step in this translation involves a precise inventory of all the participating elements in the circuit, followed by a comprehensive account of all the interactions within the circuit and their numerical contributions to both the local and general streams. The relative weights of these descriptions were previously illustrated in **Figure 1.15**.

However, the technical limitations of current circuit mapping methods in biological samples rarely yield complete network maps. Instead, local maps are obtained from discrete areas and then assembled manually or with the aid of computer-assisted tools. Due to the nature of these mapping techniques, some neurons and intercellular interactions are excluded from their local maps and, consequently, are not incorporated in the final version of the model. Experimental validation does not detect their presence, as it only focuses on previously identified neurons (Budinger & Heneka, 2025). Consequently, the resulting description of the hidden layer (i.e., the intermediate processing steps) tends to be incomplete, with unaccounted components impacting the model ability to fit the observable results. Additionally, further non-monitored neural stimuli and responses from external networks interacting with the circuit of interest are ignored, even if their contribution is unknown (**Figure 1.17**).

Due to the assembled and inferential nature of the Top-Down approach to studying neural circuits, the issues remain unresolved even if a hypothetical method is developed to map all neurons. For example, the construction process from components primarily considers the anticipated interactions of simple circuits when integrating the local elements. However, complex neural circuits may entail entirely different types of interactions. In this context, gap junctions are attracting attention as important regulators of neural circuits, particularly in controlling neurogenesis and synaptogenesis, as well as neuron-to-neuron communication and synchronous activation, including transient electrical coupling events (Cao et al., 2023). Moreover, the role of glial cells, which make up a substantial proportion of the cell population in nerve structures, has only recently started to be clarified. Their function encompasses circuit formation and contributes to higher-order nerve functions, such as cognition and behaviour, which has been evidenced in recent years (Rangel-Gomez et al., 2024) and is beginning to be incorporated into some mathematical and computational models (Linne, 2024).

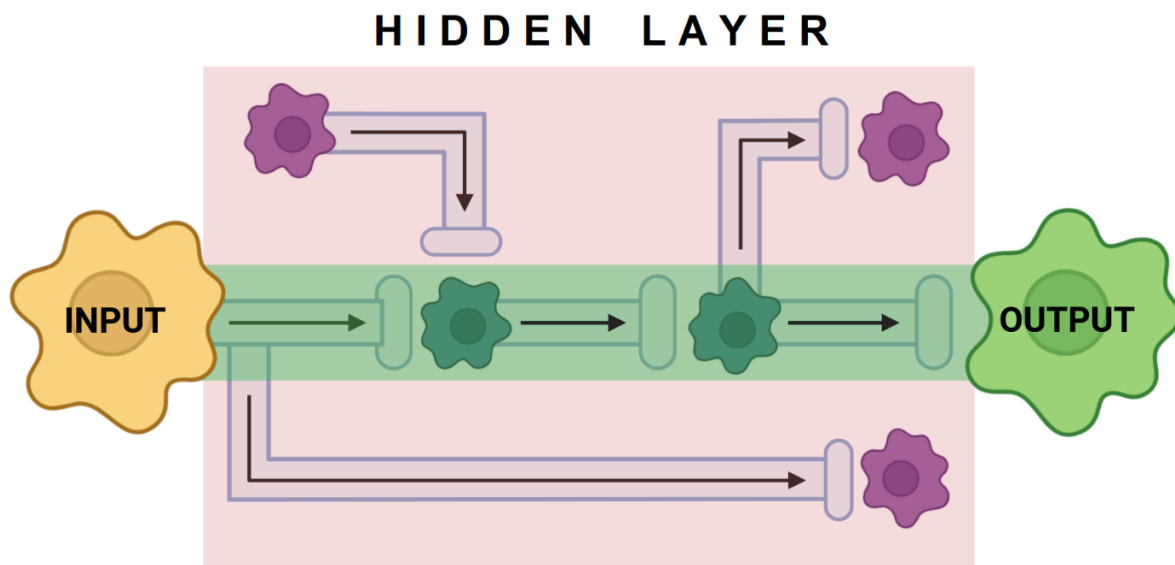


Figure 1.17: Hidden layer affects information processing during circuit modelling

Representation of elements in the Hidden Layer that can affect information processing in circuit modelling. Despite information processing running through the main neural pathways forming a circuit, additional neural connections may be present, producing detectable effects on processing outcomes, resulting in deviations from modelled circuits.

Additionally, relative synaptic weights vary in different situations, including developmental processes and plasticity events. Rather than being a fixed process, neural circuit formation and consolidation should be viewed as a temporal integration process in which transient activity changes become persistent (Bashin et al., 2024). The presence of silent synapses and parallel processing pathways, which are typically inactivated but can assume a significant role when activated. Primarily driven by circuit activity, the recruitment of these components has been noted during specific developmental stages as well as in events of short-term facilitation and depression (Atwood & Wojtowicz, 1999). Also, the effect of neuromodulators other than neurotransmitters can modulate neural activity. These molecules can alter the processing mechanisms even when the circuit structure remains intact. This family includes hormones, produced in distant tissues and causing variable effects on the nervous system depending on the target neurons. Other regulatory effects of neuromodulators include ion channel opening, neural excitability, synaptic formation, and plasticity events (Marder, 2012). Lastly, decades of research have demonstrated that other unknown regulatory mechanisms may still be discovered. A remarkable example emerged in the last decade, when the detailed mapping of brain cortex circuits in primates were completed, including the relative synaptic weights of individual neurons. Without a reasonable explanation, researchers found that by forcibly increasing one synaptic weight parameter (the scaling factor for connections from excitatory to inhibitory neurons) to double the values reported in the literature, the resulting computational model adapted significantly better to the realistic conditions observed during experimental validation (Rasch et al., 2011).

Given the aforementioned limitations in neural circuit mapping and model generation, particularly in terms of completeness, accuracy, invasiveness, and applicability in high-throughput studies, alternative approaches must be explored to overcome the shortcomings of each discussed methodology. In line with current trends in neural circuit research encapsulated by the phrase “What we cannot model, we do not understand” (Anderson & Kreiman, 2011), this thesis proposes adopting the Bottom-Up approach for studying neural circuits and addressing the previously outlined limitations of the Top-Down strategy.

1.6 Bottom-Up Approach for the Study of Neural Circuits

A reductionist strategy for studying neural circuits, such as the Top-Down Approach, relies on the idea that complex neural functions, including behaviour and emotions, can be explained by isolating parts of a network, dissecting information from these isolated components, and then reassembling the system to elucidate the entire nerve function (Marei, 2025).. Due to the limited elements that can be tested simultaneously, often constrained to readily accessible neurons and nerve terminal endings, certain inferences must be made to bridge the information gaps regarding the non-monitored cells (Budinger & Heneka, 2025). This leads to a “black box effect”, where the input and output of the network are analysed, attempting to deduce the causal mechanisms behind the observed measurements without understanding what is occurring within the hidden layer (**Figure 1. 18**).

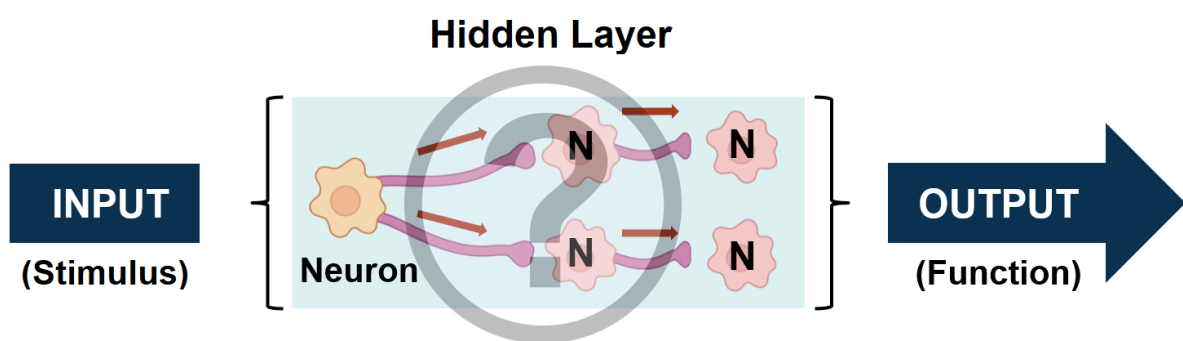


Figure 1.18: “Black Box” effect studying neural circuits

Modelled circuit maps using a Top-Down approach often result in missing components in their structure, preventing a full explanation of the events happening in the hidden layer. A complete description of a neural circuit requires the identification of all neurons and interactions in all layers.

Inference relies on evidence; consequently, the reconstruction process adheres to established rules, predominantly from simpler networks, seeking to identify recurring patterns (Berg, 2017). Nevertheless, as network complexity grows, novel types of interactions emerge. Furthermore, unique roles are assigned to neurons despite their simultaneous involvement in multiple networks. Conversely, the labelling techniques for neurons outlined in the previous section are not wholly effective in identifying all cells in a biological sample, resulting in some unidentified neurons being excluded from the calculations. Both situations frequently arise in the study and mapping of neural circuits, leading to precision issues derived from these unaccounted elements and interactions, which are reflected in the generation of circuit models and the experimental validation phase. Additional issues include fine-tuning mechanisms regulating nerve function, nonlinear information pathways, dynamic changes over time, and uncharacterised features of neural circuits. As a result, discrepancies persist, and the experimental outcomes do not always align with the expected values for the modelled circuits.

To address these cumulative issues, alternative procedures for generating and integrating research data are necessary. The Bottom-Up approach for studying neural circuits addresses the previously outlined limitations of obtaining information on nerve structure and interactions that run in the opposite direction to the traditional Top-Down approach. This strategy involves examining the structure and function of a neural circuit, starting with its most basic elements, individual neurons, confirming their identities and properties as isolated entities and forming single interactions. Once the properties of these simple systems are confirmed, the following steps aim to progressively increase the complexity of the studied system, considering more neurons involved, until a comprehensive understanding of intricate circuits is achieved. To

broaden this perspective, the following paragraphs outline the general principles of in vitro experimental models for building neural circuits.

Firstly, in vitro biological models are essential in research efforts as they simplify complex systems by focusing solely on the elements of interest, thereby creating an isolated environment for those elements while minimising interference from other sources. In other words, they provide controlled conditions where manipulations are precise and easy to follow, allowing for a clearer understanding of the basis of biological functions. This environment is also suitable for observing the effects of specific manipulations and alterations from physiological conditions. Regarding the study of nerve function, in vitro models offer access to a potentially greater number of biological samples than animal models, due to the reduced sample processing required compared to ex vivo tissue. This can accelerate the acquisition of samples for experiments and lessen ethical concerns associated with using live animals. Furthermore, they eliminate the need for animal maintenance during husbandry, growth, and the duration of experiments, which can also lead to cost reductions. Individual variability issues are also eliminated, as all in vitro samples can be identical. Before an in vitro model can be deemed suitable for reliable data outcomes, a validation process must ensure that it accurately represents the intended natural environment while maintaining the expected composition and functions of the natural tissue (Chow et al., 2022).

Several techniques have been developed intended to build neural circuits in vitro, each relying on different principles but sharing the following parameters:

1. The process must be standardised and reproducible.
2. Neurons must be placed in fixed positions.
3. Axonal growth is promoted until axon-dendrite contacts with adjacent neurons are achieved.
4. The growth must be restricted to specific areas or paths.
5. Neurons must survive long enough to form and consolidate synaptic connections.
6. When synapses are formed, a proper stimulus in the pre-synaptic neuron must trigger a response in the post-synaptic neuron.
7. Monitoring the process by microscopy observation of the cultured neurons is highly desirable.

The listed strategy can establish a neural circuit with a fully understood structure of the participating neurons and their interactions. To achieve this, the elements and interactions must be continuously regulated. Thus, the circuit is constructed from the ground up to prevent any external components from influencing it, with the building blocks being added one at a time. Unfortunately, this strategy is not applicable when working with natural circuits due to the complex environment encompassing various cell types, spatiotemporal distributions and the reduced accessibility to cells forming a tissue. The Bottom-Up approach requires in vitro work to create a contained environment ideal for de novo assembly. Consequently, the in vitro construction of the circuit can commence using live neurons, followed by the controlled manipulation of the cells throughout the building process.

Considering that the basic element of a neural circuit is the neuron, the starting point of the building process comprises individual neurons with single synapses between two cells, which equals a 2-node, 1-link system, as the simplest structure regarding circuit occurrence and information processing where information goes from point A to point B following a single path. Then, more neurons and synaptic connections can be considered to model more complex systems with multiple nodes and links until a complex system is represented that resembles the intricate interactions present in the natural circuits of the nervous system (**Figure 1.19**).

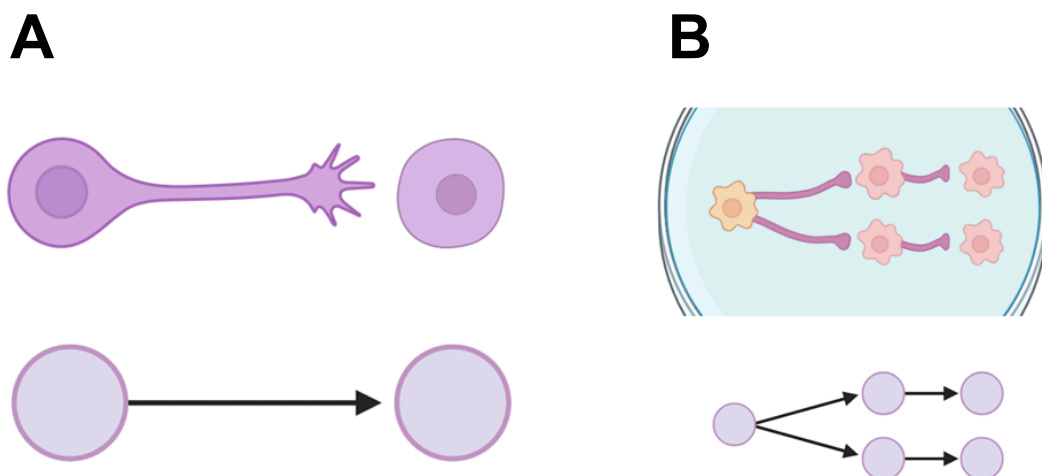


Figure 1.19: Schematic representation of neural circuits.

Biological neural circuits can be modelled as abstract elements to facilitate the representation of complex systems and establish rules for logical and mathematical interpretation of physiological events. Neurons, as building entities of neural circuits, adopt the identity of nodes, while the synaptic connections work as the link to share and transmit information through the system.

A: A basic neural circuit composed of two neurons and one synapse can be represented as a 2-node, 1-link system. The arrow direction represents the information flow, based on the presence of presynaptic and postsynaptic structures.

B: Representation of complex circuits requires multiple nodes and links. The identification of all the constitutive elements and interactions can lead to the modelling of the circuit.

In vitro cultured neurons retain key neural features crucial for shaping a neural circuit, including their survival as individual units, the growth of axons (both spontaneously and in response to external stimuli), the formation of synaptic connections with other cells, and the generation of action potentials (Zhang et al., 2022). Maintaining these functions is essential for replicating cellular interactions during the development of the neural circuit and its response to stimulation, which is its ultimate function, even at a fundamental level.

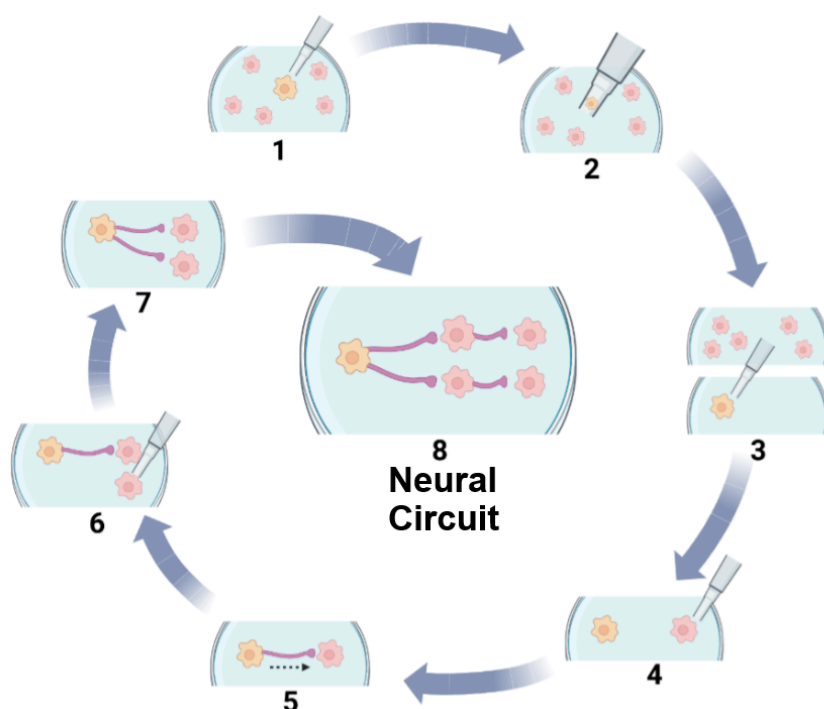
Several considerations must be taken into account when constructing a circuit from isolated cultured neurons. Firstly, a suitable source of cells is essential. In vitro neural cultures have been shown to fulfil this requirement and can be obtained either from primary cultures of nerve tissues (Banker & Cowan, 1977) or artificially generated neurons, such as differentiated neurons derived from stem cells and reprogrammed iPSCs (Thao & Zhang, 2016). Animal-isolated neurons are preferred because they can be easily obtained, monitored, and precisely manipulated. While using stem cell-derived neurons is possible, it is not recommended due to the current low efficiency of the in vitro neural differentiation process, which results in only a fraction of the cells used becoming functional neurons. Nevertheless, recent advances in stem cell technologies have enhanced the repertoire of generated cells and improved differentiation yields for experimental applications (Song et al., 2018). Non-isolated neurons in a 3-D array, such as groups forming clusters or brain organoids, can also serve as cell sources. These grouped cells retain all key neural functions. Moreover, they exhibit improved survival over prolonged cultures, acquire structural features resembling the developing brain (such as forming neuron-subtype layers), and establish limited connections with other clusters. However, the reduced control over their functions at the single-cell level does not prevent the formation of cell-specific synaptic connections, as internal tangling occurs within each cluster (Shariati et al.,

2021). Consequently, clusters and brain organoids represent systems with a low inner resolution regarding information processing mechanisms.

Building a neural circuit *de novo*, from a cell culture of isolated neurons to a finished network with a defined structure, can be represented as a linear manufacturing process, depicted in **Figure 1.20**. With some minor variations, depending on the assembling technique, the main steps can be described as follows:

1. The first step involves obtaining dissociated neurons from cultures. Although biological samples containing neurons can also be acquired from slice and explant cultures, dissociated cells represent the most suitable source for the building process. They enable the manipulation and visualisation of isolated neurons with distinctly identifiable outlines from the nerve tissue without requiring simultaneous procedures, typically mechanical or enzymatic treatments, to completely separate them from other cells. Instead, the dissociating treatment is applied before the procedure, allowing the isolated cells to be maintained *in vitro* for several days (Carter & Shieh, 2015). Once the neural source has been selected, whether dissociated cells from tissue, differentiated stem cells, or cell lines, neurons in dissociated cultures are plated and maintained on two-dimensional coated surfaces or three-dimensional substrates with culture media suitable for attachment and growth. However, to transfer the neurons from these cultures, cell attachment must be prevented through immediate handling after cell plating or by prior treatment similar to that initially applied to the tissue.
2. Individual neurons are typically selected using pipettes mounted on micromanipulators or supports for precision. After positioning the pipette over the designated neuron, negative pressure is applied using pumps or other devices to draw a controlled volume of media into the pipette along with the individual neuron. The diameter and pressure of the pipette are critical for achieving precise manipulation. Ideally, the parameters should be adjusted to pick only one cell without disturbing the adjacent ones. Employing low-density cultures can also facilitate this task. Furthermore, the manoeuvre should be gentle enough to prevent any forces compromising the manipulated cell survival and integrity. Additionally, selective markers are recommended if the procedure aims to select a specific cell type among a heterogeneous cell population.
3. Resembling a blank canvas for painting, constructing a neural circuit from scratch requires an empty culture area for placing the selected neurons. Depending on the experimental requirements, this second culture may feature similar or varied growth conditions. Nevertheless, it should be capable of promoting cell survival, attachment, and growth of neural processes to some extent. The previously selected neurons are transferred and released into this culture in a controlled manner, applying negative pressure to reverse the internal media handling and release the cell from the previous step. The circuit planning includes a prior design of the intended circuit architecture. Therefore, the release of neurons must follow this blueprint to achieve the desired circuit architecture.
4. Repeating Steps 2 and 3, successive picking and releasing of individual neurons from the same cell source progressively completes the circuit structure. Moving the pipette (or the culture surface) can modify the pipette position to select other neurons and release them in new positions. Selecting only individual neurons in this process prevents the inclusion of external elements (neurons) in the assembly. Since neurons only attach loosely to the culture surface, cell release and pipette displacement should not disturb the working area. Still, some minor manipulations can be made to cells to correct their position and preserve the intended circuit structure. Neurons are then placed in culture conditions to recover. Cells progressively attach to the culture surface after some stabilisation period, which can last minutes to hours, depending on the neural type.

5. The next step in building a neural circuit is to form connections in the attached neurons. Axonal growth can be induced in several ways, as described in Section X. The presence of permissive molecules in restricted areas of the growth surface or the application of inducible molecules in the culture medium can stimulate an unprocessed neuron to grow one or more axons toward adjacent cells. Several experimental procedures can also direct the growth direction. The axon will extend upon making contact with another neuron. However, physical contact does not necessarily mean successful synaptic formation and additional confirmation tests are required to verify functional neural connections. Besides, in synapses, defining pre-synaptic and post-synaptic neural identity is crucial to establishing the information flow direction (Colón-Ramos, 2009).
6. A neural circuit can be formed by more than one pair of connected neurons. Consequently, additional neurons can be integrated into the network at various positions. Ideally, neurons that comprise the input and output layers should be situated on opposite sides of the circuit structure to facilitate visualisation and manipulation, while neurons in the hidden layer remain in the central area. The positioning process must adhere to the circuit blueprints to ensure that the in vitro construction aligns with the intended circuit. Continuous or frequent microscopic visualisation of the process is beneficial for this purpose. Additionally, it is beneficial that neurons corresponding to the same level of information processing (i.e., input and output neurons) are aligned at consistent distances, as axonal growth is promoted under similar conditions for multiple neurons.
7. Axonal growth can be promoted in newly positioned neurons. This may occur in sequential steps or simultaneously across multiple neurons. Similar to the formation of a single synapse, physical contact between neurons does not guarantee the existence of a functional synapse, and further tests must be conducted to confirm synaptic consolidation. Besides, the direction of information flow is critical for facilitating the experimental procedures. For instance, having all input neurons in the same region enables the application of local stimulation techniques to all neurons in that layer. Successful synaptic formation in the positioned neurons completes the node-connection structure of a typical circuit while enabling mathematical or computational modelling for calculations.
8. The constructed circuits can encompass more than just input and output layers and straightforwardly connected synapses. By varying the positioning of cells and strategies for axonal growth, the circuit can comprise neurons at multiple levels and connections. The resultant building process can be visualised using microscopy to confirm the intended structure. Additionally, neural identity can be established with markers to enhance the structural and functional information. Typically, markers that differentiate subtypes of neurons are employed to ascertain the potential effects of activating specific neurons.



GENERAL STEPS:

1. NEURAL CULTURE
2. Picking
3. Transferring
4. Positioning
5. Induction of axonal growth
6. Number of neurons increase
7. Number of synapses increase
8. DEFINED NEURAL CIRCUIT

Figure 1.20: Bottom-Up building of a neural circuit

The in vitro formation of neural arrays with a defined structure can be experimentally achieved by sequentially manipulating individual neurons from a cell source to an isolated system. Starting from samples of neurons, such as primary cultures or neural-derived cell lines, individual neurons with desired features (1) can be selected using dedicated devices as manipulators (2) and transferred to an empty culture system (3). The progressive incorporation of additional individual neurons at specific positions defines, one by one, the number of components, their identity, and the spatial distribution of the neural system under assembly (4). The transition from a group of isolated units to a coordinated array capable of processing information occurs when neurons are connected through synaptic communication, which can be promoted by growth-promoting factors such as permissive molecules in the environment (5). Precision in circuit structure results from a controlled positioning (6) and formation of specific connections (7) during the subsequent incorporation of more neurons into the system, while preserving the circuit architecture. Communication between neurons from different layers can generate an in vitro system for complex information processing, with a known structure and predictable behaviour (8).

The Bottom-Up construction of neural circuits by direct manipulation of cells operates within a two-dimensional coordinate plane. This means that the position of the cells is defined according to an X-Y Cartesian plane on a permissive growth surface. To achieve this, neurons require a uniform matrix similar to their natural environment for survival and growth. This matrix is commonly created in vitro using proteins that form a layer resembling the natural extracellular matrix, such as laminin, fibronectin, poly-lysine, or poly-ornithine, to cover the bottoms of Petri dishes or well plates (Dertinger et al., 2002). The simplicity of creating this matrix as a layer facilitates treatments that remove protein from specific areas at the microscopic scale, a process termed microlithography. Thereby, restricting cell location and growth of neural processes, including axons and dendrites, a neural growth system can be generated that restraint the location of neurons at specific positions and favour the formation of a limited number of connections. For instance, **Figure 1.21** illustrates a culture system where the presence of the growth permissive protein, laminin, has been restricted to stripes in the culture surface. In the case of neurons, these stripes can serve as lanes for neural attachment and the growth of axons along these paths. The protein removal can be performed using application devices for protein solutions, microseals containing the protein or manual printing (Kam et al., 2001). The treatment may also be assisted by dedicated hardware and software design tools, such as the UV-micropatterning system PRIMO and the Leonardo software (© Alvéole), to generate precise protein patterns that can serve as a neural growth surface.

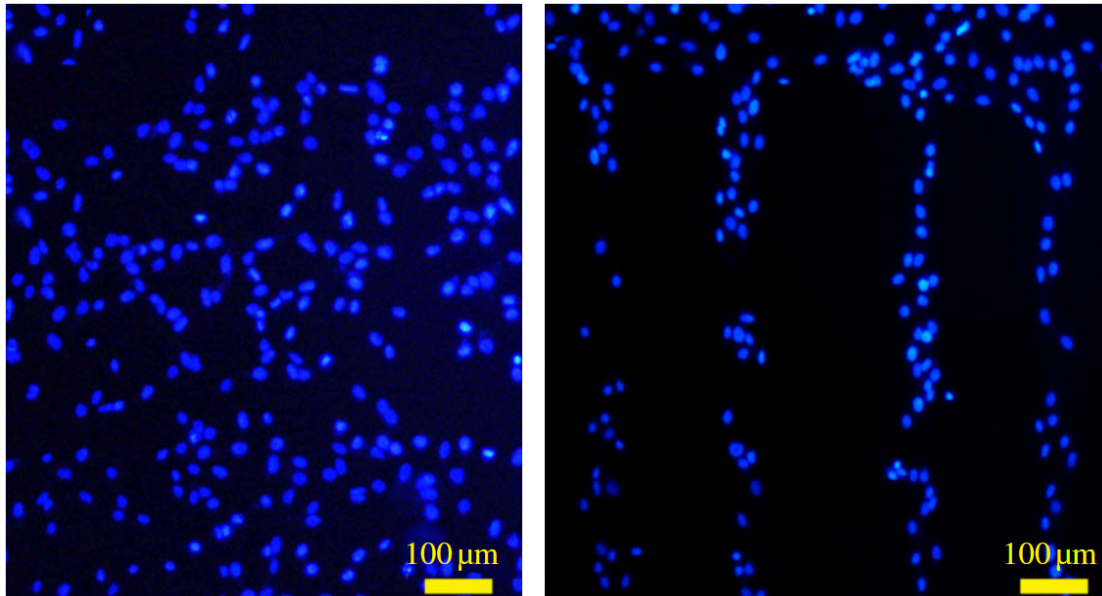


Figure 1.21: Effect of laminin patterns on cell distribution in cultures

Protein patterns can be generated in growth surfaces to restrict cell growth. Here, laminin patterns were created in polystyrene Petri dishes following parallel lines. Subsequently, 1321N1 astrocytoma cells were cultured and visualised with DAPI. Cell distribution was confined to areas with laminin. (Modified from Chelli et al., 2014)

Left: Unpatterned Petri dish, homogeneous distribution

Right: Laminin-patterned Petri dish, restricted distribution

The micropatterning effect is not only restricted to cell attachment but also limits the area of axonal growth. This effect is crucial for the building of circuits in vitro. In contrast to the natural neural environment, where growth control is orchestrated by chemical cues (such as molecular gradients, trophic factors, and paracrine cell communication), in vitro cell cultures are maintained in a uniform media composition. It is essential to locally alter this environment to trigger axonal growth processes, as the neuron interprets these signals to promote axonal extension, determine the growth direction, and facilitate the formation of synaptic connections. While other techniques, such as physical barriers and the establishment of local molecular gradients, can be employed to consistently promote and regulate growth, creating protein patterns on the growth surface remains the most prevalent method for restricting cell placement and axonal growth in laboratory-made neural circuits (James et al., 2000).

Microimprint lithography, or microsealing, was the first technique developed for this purpose (**Figure 1.22**). The process involves a silicon-patterned device, fabricated using an electron beam, that acts as a mold or seal. The mold surface is mechanically pressed against a double layer consisting of a protein matrix or substrate for cell growth (e.g., laminin) at the bottom and a thermoplastic resist material (polymethyl methacrylate) at the top. The mechanical force removes the resist material in the contact areas in a process termed etching. After the mold separation, the protein substrate layer is exposed in restricted areas following the mold design, achieving traces even at the nanoscale (Chou et al., 1996). By regulating the groove size and shape, limited areas can be printed to contain cells in chambers or as paths for crossing axons.

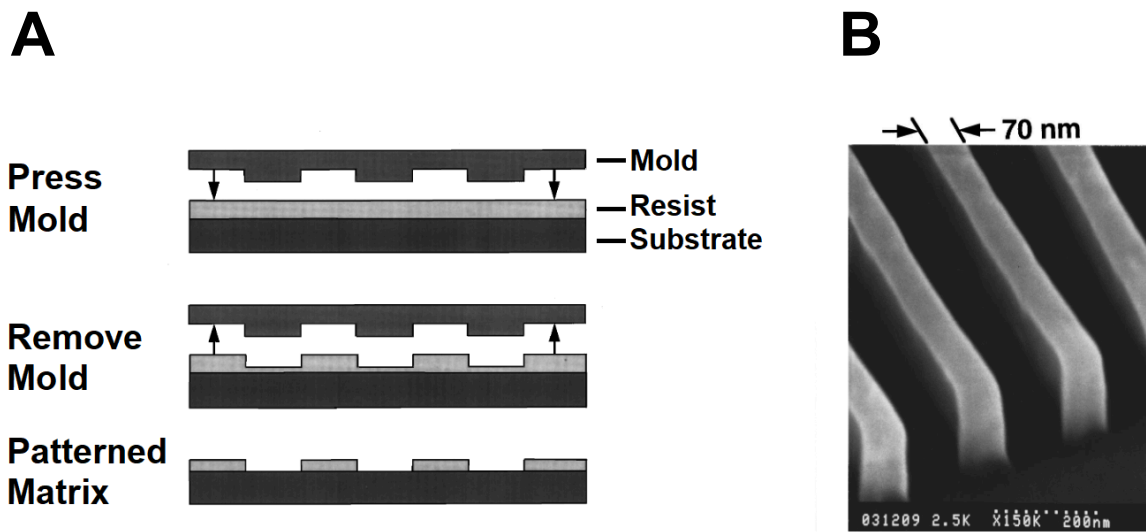


Figure 1.22: Microimprint lithography

A microscopic seal can be used to apply mechanical force against a protein matrix in a culture surface to selectively expose the protein in the contact zones (Modified from Chou et al., 1996)

A: Steps to generate a patterned protein matrix or substrate using a mould or seal, requiring a fabricated rigid mold with the intended pattern, an intermediate etching material layer and a surface coated with a protein matrix.

B: SEM micrograph visualising the generated line pattern with 200 nm depth grooves and 70 nm interspaces. Engraved areas represent the zones where laminin has been exposed and, thus, after the incorporation of a cell sample, those permissive to cell attachment and growth.

As printing technologies progressed, so did the resolution of the fabricated patterns. Although moulds and seals effectively generate patterns at micro and nanoscales, it has been necessary to minimise the effects of topographic alterations to the culture surface during the imprinting process to allow access for experimental manipulations in cultured cells and prevent other cell adhesion alterations (Roach et al., 2010). Additionally, biocompatibility presents another significant concern during imprinting, as mould and debris can affect cell cultures (Zhai, 2024). Consequently, alternative lithography techniques have been developed that directly apply or remove the protein matrix from specific areas. The following paragraphs describe other common micropatterning techniques based on these deposition and etching principles, and are illustrated in **Figure 1.23**.

Extended protein droplets

This technique directly applies protein solution droplets (e.g., laminin) to the culture surface, then displaces the solution in a specific direction by microcontact to draw lines and, progressively, patterns. The first devices to perform this technique consisted of a thin, rigid device made of an absorbent material or a flexible filament, resembling a paintbrush used for “painting”. However, novel brushes made of polymer filaments have been developed to increase the precision and consistency of the trace drawing (Pardo-Figuerez et al., 2018). The thickness of the filaments in these devices determines the line size, which in turn defines the area intended for cell attachment and axonal growth.

Microinject molding in capillaries

Also known as micro sealing, dedicated moulds have been created to evenly distribute a protein solution in contact with the cell culture surface. Firstly, the mold is fabricated by microprinting as a “negative” version of the intended pattern. Subsequently, the mould is placed in contact with the growth surface, sealing the contact area and creating internal capillaries, and the protein solution is applied nearby. The solution enters through the mold grooves and is spread across

the empty spaces, forming the “positive” version of the pattern once the protein forms a matrix in the culture surface. Finally, the mold is removed, leaving the pattern formed. Leak prevention during capillary formation is essential for accurately creating patterns (Cavallini et al., 2019).

Protein removal using UV light

When exposed to intense radiation, proteins are vulnerable to alterations compromising their native structure and functional properties (Lenci et al., 2011). UV light beams are an appropriate radiation source to induce such changes at manageable safety levels, using equipment commonly found in research laboratories. Regarding UV-based micropatterning techniques, the culture surface is initially covered with a uniform protein layer. Subsequently, a patterned reflective mask made from an inert material is placed on top of the surface, which possesses the desired pattern structure. Then, the entire surface is exposed to a high-intensity UV beam (with a wavelength of around 240 nm), compromising the protein function in the UV-exposed areas while leaving the rest unaffected. The removal of functional permissive proteins prevents cell attachment and axonal growth in the exposed areas, limiting growth to specific paths (Lan & Liu, 2013). Considering the risks associated with UV radiation is relevant when implementing this technique.

Protein removal using lasers

The negative impact of high-energy radiation exposure on protein structure and function extends to other sources, such as lasers. These tools have the advantage over UV-beams of not requiring a mask to cover the initially formed protein layer. Instead, the laser is directly applied to the culture surface to remove or inactivate the exposed protein (Kaehr et al., 2006). Due to the focused nature of lasers, exposure is restricted to defined limits, allowing the creation of patterns with nanometric-scale resolution (Costantino et al., 2005). In addition, exposure can be controlled by using specific software to regulate the beam activation in restricted areas and adjust the beam intensity, exposure time, and line thickness, allowing for the fast and precise generation of complex patterns. However, a 2-photon microscope laser or similar is required for this technique, creating a cost barrier for extensive application.

2-D Ink-jet Printing

A solution consisting of a co-polymer and an extracellular matrix protein mixture can supply an ink-jet printer dispenser to create line patterns directly on the growth surface, similar to domestic ink printing (Lindner et al., 2019). The primary advantage of this method is its simplicity, as it does not require masks or highly specialised equipment to achieve reliable lithography designs. Materials such as X-poly(dimethylsiloxane) have been used for this purpose. The drawn lines dictate the presence of protein, without requiring additional surface treatment, and two-layer stamps can be achieved. Moreover, the thickness of the lines can be adjusted by controlling the volume of the drop shots. The minimum drop volume contains at least 30 pL of mixture, resulting in a line width of approximately 30 µm. Considering the average size of a neuron is between 10 and 25 µm, this method enables a surface patterning with sufficient size resolution to handle individual neurons (Turku et al., 2003).

From the listed options, the best micropatterning technique would depend on several factors, including the required resolution, the number of processed cell samples, accessibility to specialised equipment (such as lasers and electron microscopes), the side effects of used reagents, and the particular properties of cell samples that respond effectively to changes in protein matrix concentration at different zones. Regardless of the technique used, culture surface preparation is followed by incorporating cell samples under optimal growth conditions. The purpose of the patterned growth surface is to limit or, ideally, avoid the presence of cells in the area treated to remove the protein matrix.

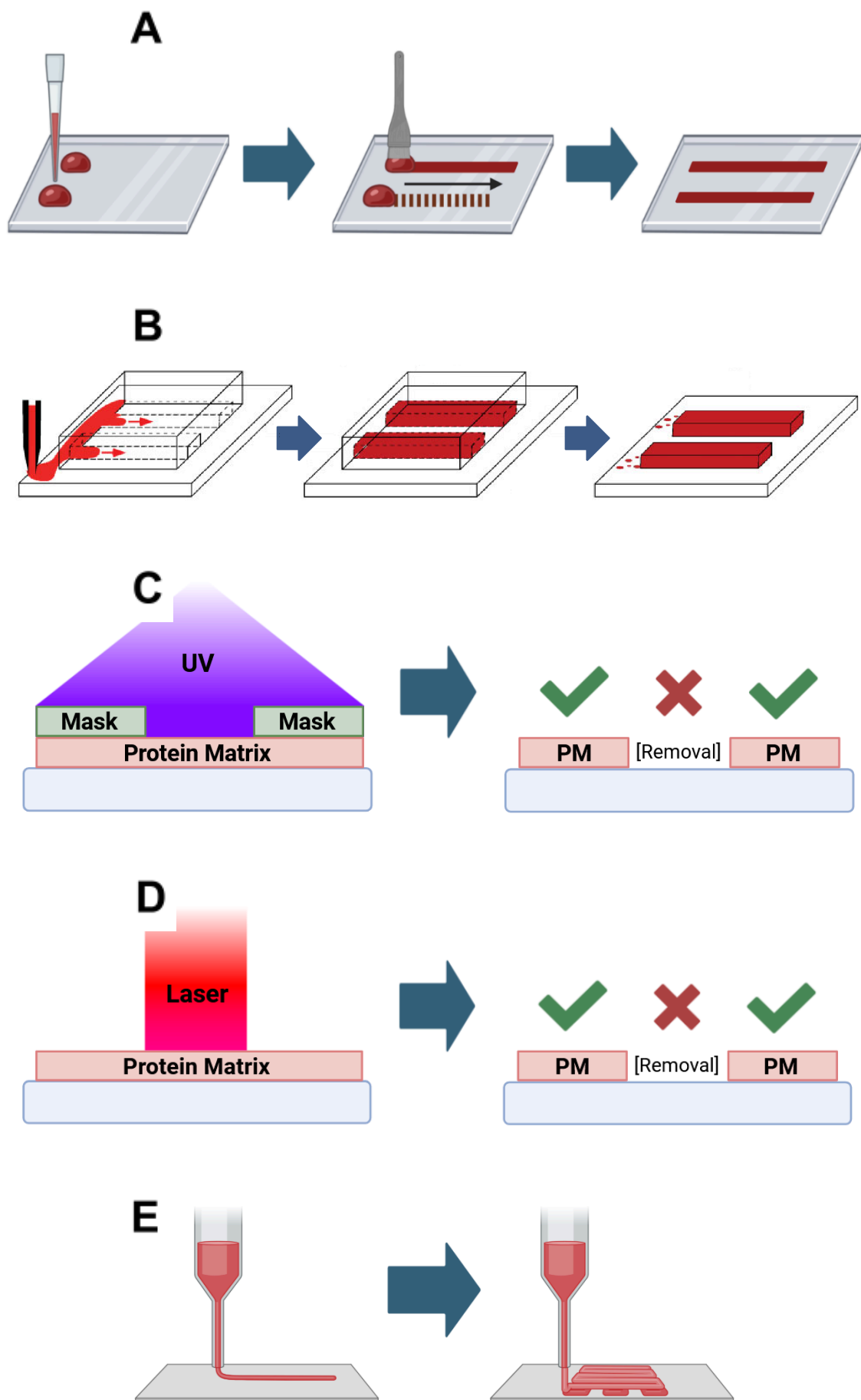


Figure 1.23: Protein micropatterning techniques on a culture surface

A: Extended protein droplets (“Painting”)

B: Microinject moulding in capillaries (from Cavallini et al., 2019)

C: Protein removal using UV light

D: Protein removal using lasers

E: 2-D Ink-jet printing

1.7 Building Neural Circuits Using the Bottom-Up Approach

Researchers have long sought to obtain biological samples in the laboratory that accurately replicate naturally occurring circuits to understand the basis of fundamental nerve functions. A suitable in vitro neural circuit model must reproduce the minimal elements and interactions required to perform a specific nerve function and, when manipulated, exhibit responses similar to those expected in other biological samples. Constructing artificial circuits has proven challenging because the method must integrate conditions that facilitate sustained neural survival, directed growth of processes in confined areas, structural precision of the generated circuit, straightforward microscopic observation of the sample, and accessible manipulation for experimental purposes.

Under appropriate conditions, neurons tend to grow axons randomly. This process is naturally triggered by an external chemical stimulus that activates intracellular signalling cascades, inducing cytoskeletal changes that lead to the sprouting of a growth cone from the neural soma, thereby elongating the axon. The growth direction is determined by molecular cues, such as neurotrophins, which are typically produced by the target cells and guided by additional molecules during a process known as axonal pathfinding (Zang et al., 2021). However, reproducing this process in vitro presents technical challenges in controlling each step, from positioning isolated neurons to promoting axonal growth and forming specific synaptic connections. Research efforts have identified solutions and developed standardised techniques to construct and examine neural circuits with precise structure from isolated neurons (Bang et al., 2021). In contrast to the Top-Down approach for studying circuits, which frequently utilises naturally formed circuits from nerve tissues, the Bottom-Up approach relies on techniques to maintain and manipulate neurons to create circuits in vitro. Each option employs one or more principles to assemble neural arrays by regulating the formation of specific neural contacts in cultured cells, aligning with the intended circuit architecture. Among these are mechanical manipulations, physical barriers, regulating protein distribution, utilising scaffolds, and adapting technological devices. Some of the main strategies developed are listed in **Figure 1.24**.

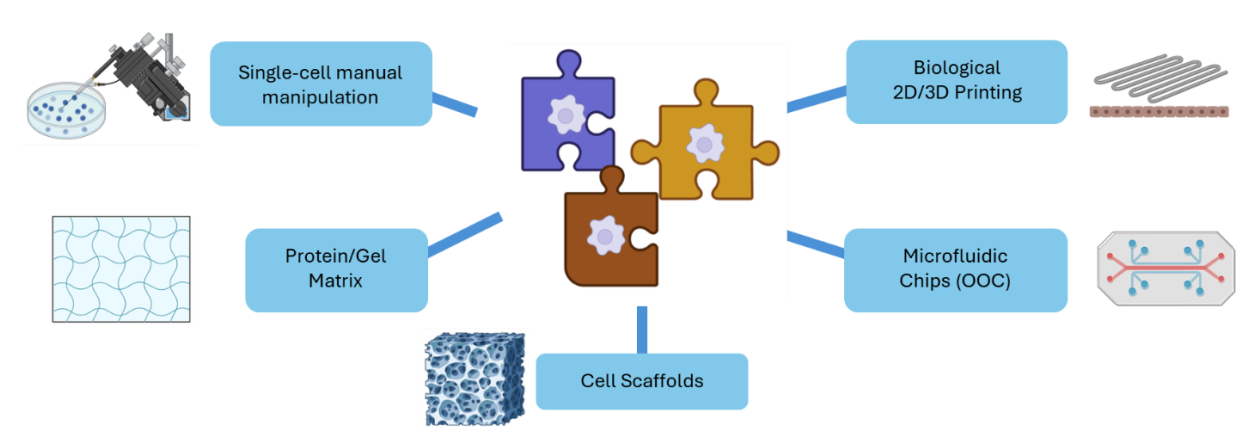


Figure 1.24: Bottom-Up strategies for building neural circuits

Several experimental strategies, based on mechanical manipulation, physical barriers and chemical cues, have been developed to handle individual cells and form circuits controlling the number and position of the constituting elements.

As mentioned in the previous section, those techniques require obtaining a suitable neural source. The optimal source depends on the experimental objectives, but the requirements for neural in vitro maintenance and growth must be taken into account. Additionally, each method employs different equipment, reagents and procedures, which affect the process cost in terms of time and resources. Also, the resolution of the obtained circuit depends on the manipulation capabilities of isolated or grouped cells. The key characteristics of each instance are outlined in the following paragraphs, including their strengths and limitations.

Cultures of Isolated Neurons

The endeavour to produce the first functional in vitro biological models of neural circuits was initially supported by the understanding of nerve function control through simple circuits composed of neurons confined to specific nerve areas and, in theory, involving a limited number of neurons. Neurons can be isolated from nerve structures and cultured, maintaining most of their morphological and functional properties in vitro. Primary cultures, consisting of neurons directly dissociated from tissues and placed in culture media, were developed more than 50 years ago from rodent brains and other nerve structures (Banker & Cowan, 1977), refining since then the study of isolated neurons over several days by artificially reproducing the growth conditions that enable survival and growth in the organism, including culture media composition, nutrient and oxygen requirements as well as the supply of other crucial molecules such as vitamins, hormones and growth factors. Culture media optimisation, the process of refining the media composition to achieve the desired culture outcomes, has been a persistent issue in research and cell processes in the field of Cell Biology (Zhou et al., 2023).

The search for artificially reproducing neural circuits was based on the principle of recreating the minimal constitution in terms of elements and interactions necessary to perform a nerve function using cultured cells. For this purpose, similar to the development of mapping techniques, central pattern generators (CPGs) in molluscs provided a suitable biological model to test this principle. In certain organisms from the *Aplysia* and *Limnaea* genera, CPGs govern reflexes related to fundamental functions such as breathing reflexes. In contrast to the intricately structured brains of mammals, the entire nervous system in these animals comprises a series of interconnected ganglia that form a ring, with a fully identified profile of their constituent neurons, including their subtype, location, position, primary neurotransmitter, and direction of information flow. Furthermore, several of these neurons are large, facilitating their visualization, manipulation, and activity recording. Foundational research using neurons from *Aplysia californica* first demonstrated the potential for forming individual synapses in neural cultures with high specificity (Camardo et al., 1983). A later discovery in the development of in vitro neural circuits was made in the pond snail *Lymnaea stagnalis*, where a relevant central pattern generator (CPG) function related to the breathing reflex was shown to be regulated by the coordinated activity of only three types of neurons (**Figure 1.25**). This CPG system was minimised to its essential scale, comprising three interconnected individual neurons in culture, which were sufficient to replicate the specific synapse formation observed in the whole organism and the rhythm generation apparent in the entire nerve ganglia (Syed et al., 1990). The isolated cultured neurons grew axons and formed chemical synapses, but only with certain types of neurons, mirroring the synaptic targets seen in the live animal. Subsequent recordings from these in vitro connected neurons also matched the in vivo firing behaviour of similar synapses.

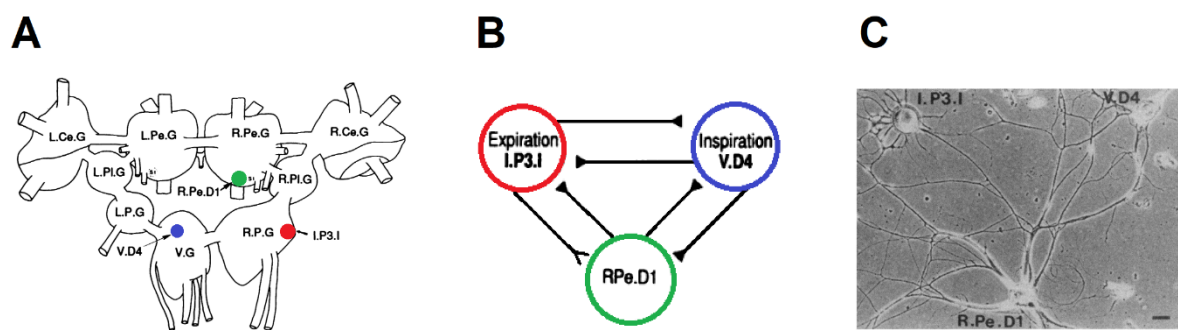


Figure 1.25: Generation of the first in vitro neural circuit from an *Aplysia californica* CPG (modified from Syed et al., 1990)

- A:** Anatomical representation of the CPG in *A. californica*. Coloured dots indicate the structures for the obtention of neurons
- B:** The CPG was reduced in vitro to its essential components, comprising three neurons
- C:** Isolated cultured neurons formed synaptic connections similar to the natural CPG

Further research with cultured neurons and other cell types has successfully reproduced additional natural circuits, comparing in vitro recordings with the nerve activity observed in live organisms. It is important to note that these simple neural cultures do not hinder axonal growth, and synaptic connections form due to the intrinsic properties of each neural subtype. However, the cellular mechanisms involved in axonal growth and synaptic connections may differ among organisms such as mammals. Thus, additional strategies are required to limit and guide growth and establish specific connections, particularly when managing a large number of neurons and synapses, to maintain control over the formed synaptic connections. This is necessary to provide an accurate explanation of the experimental results based on the circuit architecture.

Manual Single-Cell Manipulation:

The diverse cell composition in the nervous system requires single-cell resolution techniques to accurately track neural circuit components. The precision and accuracy during circuit mapping and element identification mean that any measurement or modification is limited to one element at a time. Therefore, spatially isolating cells from groups and setting them as an independent system has been a common technique for studying cell function. Neurons are small, with an average soma size of 15 μm . Hence, they require suitable tools to precisely move them without compromising their integrity. Micromanipulators are tools specifically designed for this purpose, consisting of a pipette mounted on a mobile frame that can be manually displaced at a micron scale through screws or using motors (**Figure 1.25**), while the cells and their movements can be observed through an inverted microscope.

The pipette incorporated in the manipulator is frequently filled with a standard physiological or modified solution to maintain a suitable environment, depending on the experimental aims. The pipette is then connected to a vacuum pump, which applies positive or negative pressure inside the pipette to release or introduce the inner solution, respectively. This device may also include electrodes or probes for recording neural activity, such as changes in electric potential, thereby expanding the technical capabilities of the device. By combining other devices that control the application and recording of electrical parameters, including current and voltage, several standard techniques for measuring neural activity have been developed. Currently, there are three main types of micromanipulators, based on their operational characteristics: mechanical, pneumatic/hydraulic and piezoelectric, based on direct displacement, limited by the presence of an air or water sample and electronically assisted, respectively (Molecular Devices, 2012). With this, micromanipulators are frequently used to perform various experimental tasks, encompassing, but not limited to, cell manipulations, microinjections, patch clamp experiments, activity recordings, and in vitro fertilisation (Perkins, 2006). With numerous commercial suppliers and models available, micromanipulators are the most common strategy for performing cell positioning and interaction in experimental procedures involving neurons.

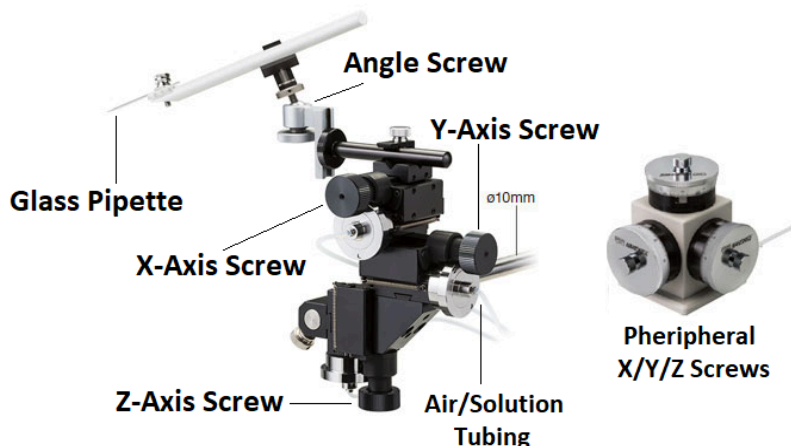


Figure 1. 26: Schematic of a typical micromanipulator with its main parts
(© NARISHIGE Group, <https://products.narishige-group.com/group1/wr-6/electro/english.html>)

Protein / Gel Matrix

In addition to direct physical manipulation, neural position in a culture can also be controlled using chemical cues. Cells in culture require the presence of an extracellular matrix composed of proteins to allow them to adhere, grow, and support their development (Zhao et al., 2025). In the case of neurons, these protein networks are also crucial to provide mechanical stiffness, establish cell polarity and promote and sustain the differentiation and growth of axons and dendrites (Letourneau, 2017). In contrast to the extracellular matrix, which naturally contains proteins such as collagen, elastin, and fibronectin, an in vitro system requires a protein matrix to replace it. These requirements can be met by incubating the glass or plastic culture surfaces with a protein solution, commonly laminin, polylysine, or fibronectin, or a gel-forming polymer before adding the cell suspension. In the resulting uniform layer, isolated neurons can survive and randomly grow processes with no limitation other than the presence of other surrounding cells. To in vitro assemble a neural circuit in these conditions, it is necessary to confine the migration and growth of neural processes. Otherwise, neurons will grow and form synapses with other neurons without a pre-existing assembly plan. For this purpose, micropatterning techniques, previously described, have been developed to remove or inactivate the protein substrate in specific areas, thereby creating morphologies on the surface at the micro- and nanoscale. The possibilities include drawing patterns such as chambers and groves to contain the presence of neural bodies and processes, respectively (**Figure 1.27**). As described later, a micropatterning method based on sustained exposure to UV light was employed in this project.

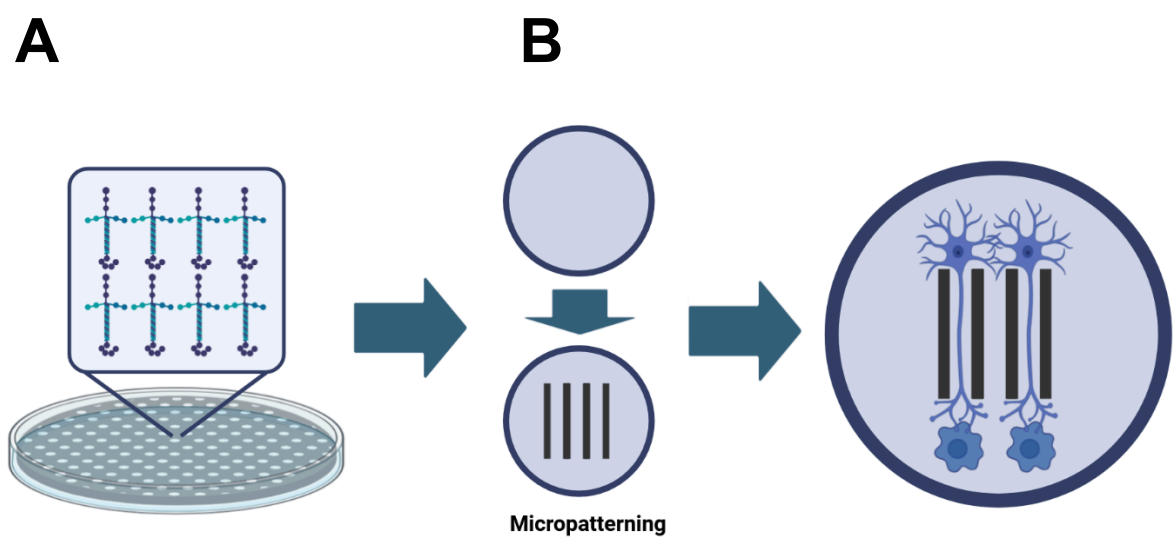


Figure 1.27: Formation of a protein matrix to control neural growth

- A.** Extracellular matrix proteins, such as laminin, are added as a solution to polymerise and form a uniform layer on the culture surface, allowing cell attachment and growth.
- B.** By using micropatterning techniques, the protein can be selectively removed or inactivated from specific areas, thereby limiting neural growth and development.

In addition to forming a uniform layer, proteins can be mixed with gelatin to create other coating structures, such as fibers. Fibronectin matrices have been widely used in cell cultures as they form bundles of filaments and 3D arrangements resembling those present during developmental processes, which can be visualised using antibodies as shown in **Figure 1.28** (from Sharma & Schwarzbauer, 2022). However, micropatterning of these structures is challenging due to the variable exposure of the matrix surface to treatments, with no alternative options available to restrict the growth of neurons and nerve processes during the in vitro growth of neural processes. Thus, gelatin-based matrices are more intended for neural maintenance with unrestricted growth procedures.

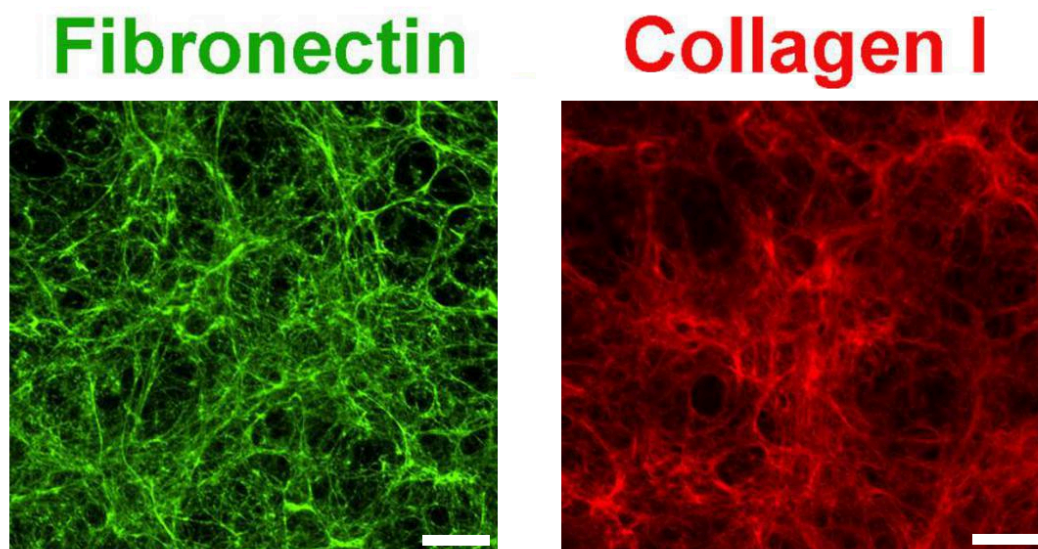


Figure 1.28: Visualisation of different types of gel matrix with fluorescent antibodies

Matrix proteins can be mixed with gelatin to form a fibre-based matrix on the culture surface, serving a scaffold to allow cell attachment and growth. Protein matrix can be labelled with fluorescent antibodies for visualisation to adjust suitable parameters including morphology, porosity and pore size (modified from Sharma et al., 2023).

Cell Scaffolds

The development of cell culture systems has also led to the creation of scaffolds that produce 3D cell structures mimicking the structure of intact tissue while simultaneously creating local environments that reproduce close interactions between neural and non-neural cells (Madhusudanan et al., 2020). These scaffolds have been constructed using hydrophobic materials derived from natural polymers, such as gelatin, fibrin, hyaluronic acid, type I collagen, and Matrigel, as well as synthetic polymers, including polyethylene glycol (PEG), polyacrylamide, and poly(2-hydroxyethyl methacrylate) (p-HEMA). These three-dimensional networks feature an inert, highly porous structure that facilitates the placement and support of cells in empty spaces (**Figure 1.29**). Neurons and other cell types can form 3D structures when they grow attached to a permissive surface, as in the case of polymer scaffolds serving as support structures. The polymerisation conditions can be adjusted to regulate pore size and accommodate other types of cell structures, such as clusters and brain organoids, in addition to individual neurons. Besides, the cellular composition of these culture systems is not limited to neurons alone. Glial cells are commonly included as supporting cells for the proper development of neurons due to their known involvement in the production of extracellular matrix proteins, growth factors, and neurotransmitter management.

The scaffold surface can contain other molecules, such as adhesion proteins or growth factors, which serve as chemical cues to direct cell growth. In this regard, molecular and drug delivery can be achieved by incorporating them into the material composition during the polymerization process. In contrast, multiphoton lithography techniques have recently been developed, using a laser to remove proteins from discrete areas of the 3D scaffold and spatially restrict the presence of cells to certain areas (Sivun et al., 2024).

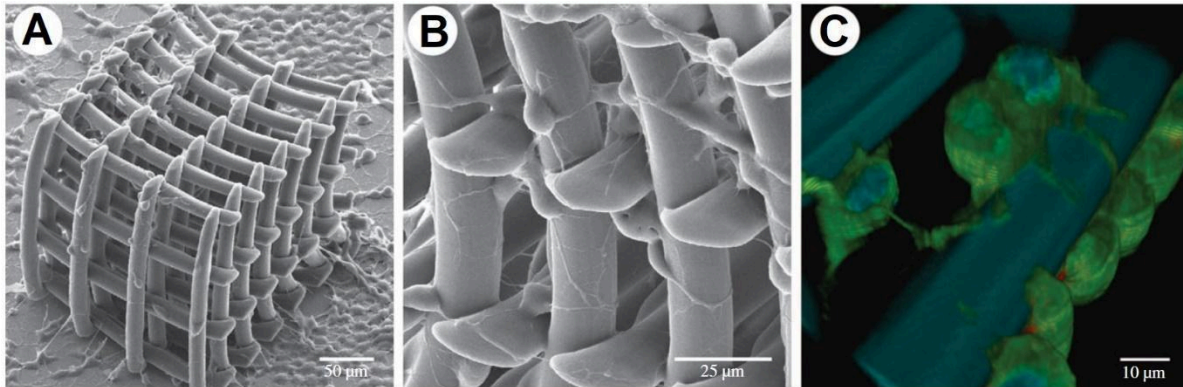


Figure 1.29: Cell Scaffolds for Artificial Nerve Tissue Generation

A , B: Scanning electron microscopy images of a 3D-PEGDA-hydrogel scaffold cultured with neuro2A cells. Initially, neurons are homogeneously distributed in the cultures. Most of the neurons attach to the flat culture surface. However, a fraction can be found attached to the scaffold material. In these neurons, axonal growth is physically supported by the scaffold filaments. When cultured neurons form connections, neural circuits can be generated with structure following the scaffold design.

C: The attached growing cells are shown coloured in green. Note that the number of cells attached to each scaffold segment is variable (from Madhusudanan et al., 2020).

The artificial microenvironments created using scaffolds are capable of accommodating large populations of neurons and non-neural cells. Some innovative models for studying neural events during development and the early formation of neural circuits have been brain organoids. In this case, stem cells are placed in specific places on scaffolds to promote their proliferation and differentiation into neurons and other cell types (Choi et al., 2025). The scaffold structure is designed to facilitate the formation of interactions between specific cell groups through interconnected pores by inner channels .

The application of scaffold technologies has not been restricted to the field of neural circuit research. Some promising applications include the development of tissue engineering techniques for therapies involving the implantation of laboratory-generated nerve tissue to replace damaged structures (Loh & Choong, 2013). However, despite the increasing yields in generating suitable biological samples, some technical limitations persist. The resulting 3D cultures using scaffolds present reproducibility issues associated with the unrestricted growth of cell groups, making circuit mapping at single-cell resolution challenging. In addition, a considerable proportion of the cells are not directly attached to the scaffold surface but to other cells, making it difficult to deliver molecular cues, such as during axonal growth events, which affects the formation of precise synapses

Biological 2D/3D Printing

Cell therapies, such as tissue regeneration procedures, require the development of cell culture systems that generate structures resembling natural tissues to increase compatibility with the host during transplantation. For this purpose, several techniques have aimed to produce laminar structures by using printing devices. The processes rely on using cell suspensions, either neurons or progenitor cells, which are injected into a dispenser system and then released onto the culture surface as droplets using a dedicated printer device and progressively arranged into layers. The cells are subsequently maintained until the neurons attach in the intended positions and start to grow. This process, known as bioprinting, enables the precise definition of cell distribution in a 2D array and is followed by the facilitation of the growth and synapse formation in these neurons by controlling media composition (Knowlton et al., 2018). Although this system

has enabled the formation of layer-based circuits between specific neural populations, the growth process still has operated as a random process, and controlling synapse formation at the individual level is not possible. In addition, only a fraction of the neurons forming the layers survive after the manipulations of the printing process. For this reason, the resulting circuits have limited mapping resolution. Despite this, the bioprinting fabrication process has been useful for generating synthetic cell culture arrays that resemble tissues, as in the case of transplantation therapies, where neurons are intended to continue growing into the host, and a precise initial number of neurons is not critical for allowing tissue implantation.

The refinement of bioprinting techniques has enabled the creation of complex cell arrangements, including those with multiple layers. These 3D culture systems have further expanded similarities with the natural environment and recapitulate developmental processes. These devices handle and position cells that form an initial layer, followed by the controlled addition of more cells on top as subsequent layers. However, cell manipulation comprising several layers has faced challenges in maintaining circuit structure due to the limited capacity of micromanipulators to work with samples containing high cell densities. The use of free-floating cell aggregates forming coated droplets, termed neurospheres (**Figure 1.30**), has facilitated manipulation by allowing the delivery of groups of progenitor cells and neurons to specific sites during the building process (Reynolds & Weiss, 1992).

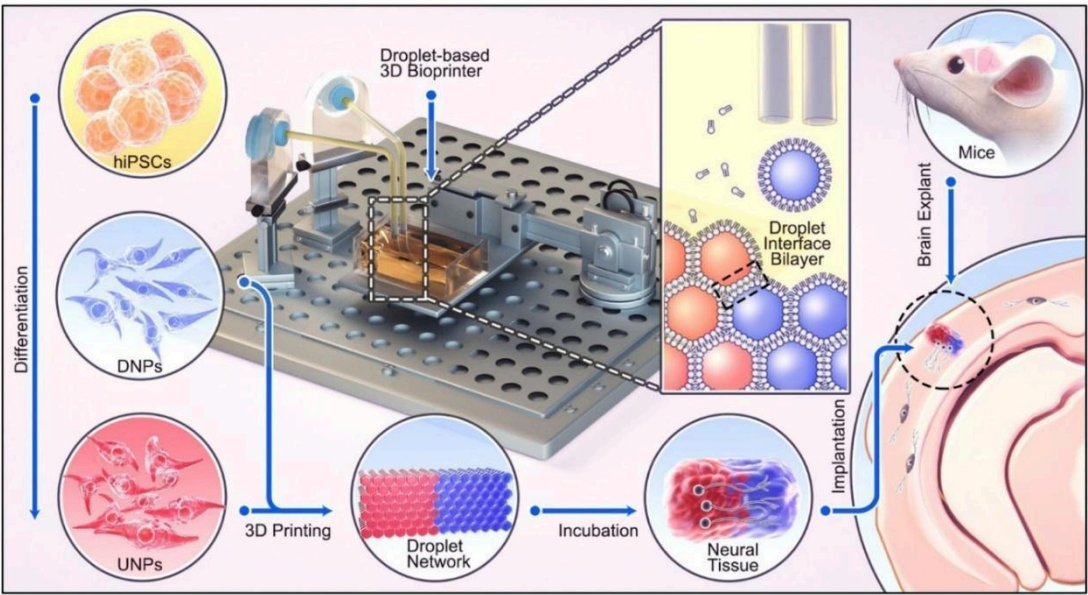


Figure 1.30: Droplet-based 3D bioprinting

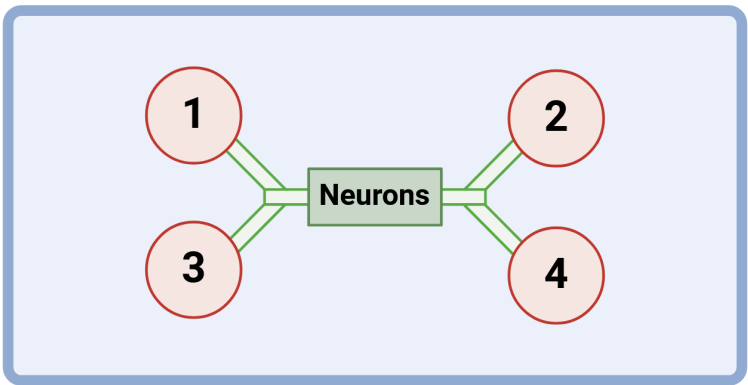
Functional 3D structures resembling tissues can be fabricated in printed droplets droplets using live neurons from various sources, such as pluripotent stem cells and progenitor-derived neurons, maintained for cell replication, growth, and subsequently used for cell implantation in living organisms to integrate into the in vivo nerve tissue (from Jin et al., 2023)

However, droplet delivery systems have limited internal control in cell numbers, as they only restrict access to small groups without limiting the pass of individuals. Eventually, this unpredictable amount of neurons forms interactions with their surroundings. Consequently, the final number, positions and interactions between them are variable, and the building process is far from being reproducible at the elementary level of individual synapses, making circuit modelling a difficult task.

Microfluidic Chips (Organ-On-a-Chip)

Microfluidic devices have emerged due to advancements in microengineering and 3D printing technologies. These devices consist of interconnected chambers that hold isolated cells or cell arrays within, restricting their movement by a reduced size in the channel between the chambers, similar to a sieve effect (**Figure 1.31**). The media in the chambers mimics specific environments with a composition that preserves their natural cell functions (Servais et al., 2024). By using physical barriers, it is possible to vary the media composition between chambers to reproduce different cell conditions, thereby simulating the environmental variations observed in organs and tissues. Microfluidic devices are intended to contain all the single-cell elements and direct interactions that represent the structure and function of an organ. As a result, the system is also referred to as an “organ-on-a-chip (Cao et al., 2023)”. Therefore, microfluidic chips can be viewed as miniature organs for experimental purposes. In the case of nerve cells, the communication channels between the chambers can play an additional role, allowing the crossing of neural processes but impeding those of neural somas due to their reduced size (approximately a few micrometres in width). The resulting chips, termed Brain-On-a-Chip, contain interconnected neurons or groups of neurons whose disposition can be used to model neural circuits and investigate their behaviour (Amirifar et al., 2022).

A



B

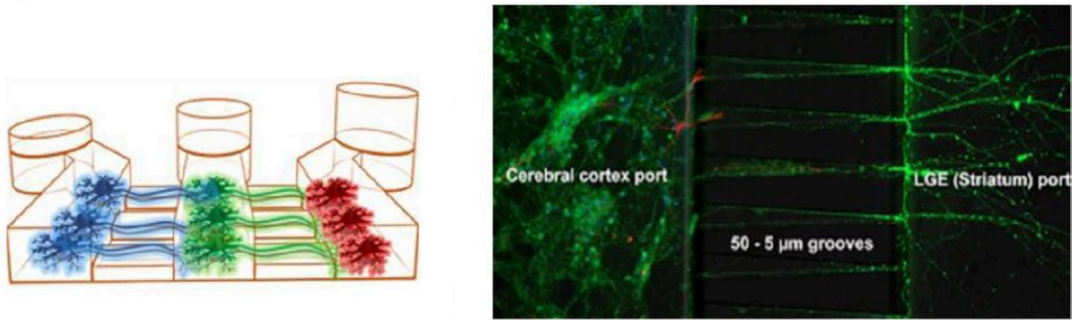


Figure 1.31: Microfluidic devices for studying neural circuits.

A. Diagram of a printed microfluidic device. Separated compartments enable the maintenance of cultured neurons in different microenvironments and facilitate the formation of synapses through connecting channels.

B. Compartmentation in engineered devices can mimic the occurrence of natural circuits composed of different kinds of neurons. Neurons can be held in discrete areas, with spatially restricted growth of axons (from Bang et al, 2021; and Kamudzandu et al., 2019)

The search for in vitro models that accurately represent neurological disorders has led to the development of chips with cell compositions and interactions that reproduce specific pathologies. Despite the entire mechanism of nerve diseases not being fully understood, some characteristic traits can be produced at the most elementary level. For instance, Park and his colleagues developed a microfluidic chip composed of clustered neurons, termed neurospheres, to mimic the brain microenvironment in healthy individuals and those with Alzheimer's Disease (AD), using previously identified cell population profiles to study the effects of the A β precursor protein on cell viability, neural loss, and synaptic dysfunction. These systems enabled the following of the neural alterations related to the progression of the disease as an in vitro model (Park et al., 2015). Similar chips have been developed based on identified cellular profiles for other nerve pathologies, including Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and other conditions, for a closer investigation of the involved cellular, molecular, and functional changes (Cerutti & Brofiga, 2024).

The possibility in these devices to precisely administer molecules to individual neurons and record the functional effects has increased the resolution of the information obtained by other experimental approaches. For example, chips resembling the brain-blood barrier for studying drug delivery in the brain. Although microfluidic devices represent notable progress in the study of neural circuits using a Bottom-Up approach, they still present critical limitations for their extensive use in research. Among them are the continuous monitoring and manipulation of the cultured cells required to ensure their correct positioning and growing, design rigidity that restricts the testing to a small predefined type of circuits, shear stress caused by the manufacturing materials, frequent impairment in nutrient delivery and the complicated fabrication process of the devices that limits their application for high-throughput experiments.

To date, the entire process of building a neural circuit in vitro using previous techniques is time-consuming due to the low yields obtained when collecting the required biological samples. The successful formation and consolidation of synaptic connections in vitro, a crucial step in developing any circuit, is a process that generally yields a low success rate using current methodologies. In addition, neural growth requires an optimal physical and chemical environment, which varies according to the specific cell requirements and does not always yield the same results. A higher number of repetitions is required to achieve sufficient neuron numbers with properly formed synaptic connections. Additionally, the sequential execution of these steps as a routine procedure allocates fixed time intervals at specific steps, such as cell growth periods. The overall consequence is a low-efficiency process. This is especially relevant considering the high number of experiments required to produce reliable results and reach conclusions during neural circuit study.

At this point, the proposal for the current project involves improving the efficiency of this process through optimization and automation, a strategy aimed at enhancing the efficiency of each individual step in building neural circuits using a Bottom-Up approach.

1.8 Limitations of the Bottom-Up Approach

The Bottom-Up approach to studying neural circuits is far from an extensive and swift process. Considerable data regarding the primary neural subtypes, anatomical interactions, and functional behaviour must be obtained beforehand. Firstly, this information dictates the construction plan for creating the intended circuit. Secondly, this is necessary to determine the experimental requirements, such as culture conditions, growth times, and monitoring equipment. Lastly, other similar circuits can serve as a reference to compare the structure and information processing in the obtained circuit (Hennig, 2023).

Furthermore, comprehensive information on developmental mechanisms is essential. Numerous neural interactions, including axonal growth and synapse formation, are restricted to specific temporal and spatial events, such as the establishment of molecular gradients, intercellular communication, and protein expression profiles. To achieve the desired circuit, it may be necessary to accurately replicate these dynamic conditions in vitro. Only validated platforms can ensure the technical accuracy and consistency needed for sample manipulations to reach reliable conclusions. Optimising each assembly step is also advantageous for ensuring the best product with efficient use of time and resources.

Another obstacle in the large-scale fabrication of neural circuits is consistency. The building process must replicate identical circuits even during large-scale operations. This can ensure experimental reproducibility, comparable results, and conclusions based on reliable statistical analysis (Striebel et al., 2025). Paradoxically, the process parameters must be flexible to address frequent challenges in circuit mapping, such as individual variability and nerve degeneration (previously illustrated in **Figure 1.16**). In this context, evaluating multiple variations on the same circuit is necessary to achieve robust conclusions. Therefore, the circuit fabrication technique must be versatile enough to incorporate specific modifications into the structure as part of the process. Commercial platforms offer limited possibilities for parameter alterations during the assembly process, so the ability to generate libraries of circuit variations by other means would be beneficial for experimental evaluation.

Considering the previous limitations, process automation implementation offers a solution to the current obstacles of building large-scale and precise neural circuits for experimental purposes.

PART 2:

Automated Platforms for the Study of Neural Circuits

2.1 Automation of Biological Processes

The term automation refers to the replication of a process using mechanical or electronic devices to reproduce the physical manipulations that would normally be performed manually by a staff member or a tool operator (Pavan, 2001). Originally, automated devices consisted only of mechanical devices that made physical contact with the raw materials and processed samples to reproduce standard staff procedures, offering several advantages, including precision improvement and associated risk reduction. With the introduction of electronics and computational technologies, automated processes later incorporated self-operating and monitoring devices into the systems to further reduce the required interactions between the process and staff members (Bissell et al., 2023).

Automated processes typically run in controlled environments, starting from a specific point, and repeat predefined or programmed tasks on raw materials or samples to achieve a desired delivery state. The process runs in a single direction, following sequential steps at demand with the possibility of monitoring the process progression. Automation has been applied to various disciplines, including life sciences, to perform routine tasks and techniques (Holland & Davies, 2020). Application fields have included hematology, chemistry, microbiology and molecular biology, from basic research to clinical testing (Choi et al., 2018; Croxatto et al., 2016; Stephenson et al., 2023). The ultimate aim of automation is an increase in research efficiency, which can be achieved by combining several shared features when applying automated devices (**Figure 30**), listed in the following paragraphs:

- **Fidelity**

The process must strictly reproduce the original manual process, following similar principles to convert the initial materials into the same products. The outputs have to be equivalent in terms of properties and quality. Any additional material requirements or outputs must not be related to the process itself but only to the device. Additionally, the process must be performed identically by similar equipment. A validation stage is required to ensure the accuracy of each step and the overall performance of the automated process (Hawker & Schlank, 2000).

- **Reproducibility**

The process must remain identical after multiple repetitions, including the overall process and the involved partial steps. The required materials and generated products have also to remain constant in terms of identity and amounts at all steps. This excludes the use of consumables and replacement parts for the operation and periodical maintenance of the equipment. The constancy must be extensible to processes running at different times and places and using other equipment from the same model (Goodman, 2016).

- **Time Reduction**

As the process does not require direct human manipulation or supervision, it can proceed uninterrupted over extended periods of time. Depending on the device capabilities, it can even be continuously performed as long as the necessary materials are properly supplied and the delivered products are properly managed. In contrast to the limited staff working hours in human-supervised processes, uninterrupted automated processes result in a reduced time frame required to generate the same amount of products (Burger et al., 2020).

- **Staff Dependency Reduction**

Automated tasks do not require constant interaction between staff and materials, products, and intermediates for direct processing. Instead, the main duties are related to the equipment maintenance to keep it running. Reproducibility in processing steps by automated means also diminishes dependency on the skills of particular staff members, as all step repetitions are identical and executed independently by the involved operators. Moreover, as dedicated equipment can also perform several steps of the process and cover multiple staff roles, implementing an automated device can result in a reduction in staff requirements.

- **Search for Optimisation**

The possibility of automated devices to test an elevated number of conditions within a reduced timeframe, allowing for comparison of outputs and identification of the best parameters, can result in determining the most suitable conditions for each step to maximise product outputs while minimising material usage. This strategy, termed optimisation, can also be used to improve the quality and desired properties of the product (Check Hayden, 2014; Bédard et al., 2018).

- **Resources Savings**

Optimisation is leveraged to achieve a more efficient use of materials, time, and working hours, generating equivalent amounts of product. This is translated into a reduction in the economic and human resources required to run the process, yielding the expected outputs. This serves as the foundation for enhancing research efficiency.

- **Safety**

Scientific research often requires the employment of chemical compounds and other sources of hazard, such as radiation, for routine procedures. Reducing the exposure of research staff to these agents, which can potentially cause harm, is a desirable measure mentioned in health and safety regulations (Movsisyan et al., 2016). When procedures are performed by automated devices instead of staff members, potential risks associated with research activities are reduced.

- **Trustability**

In the current scientific research system, projects are planned and executed to deliver results within specific timeframes, complying with the requirements of commercial, institutional, and funding agencies. A reduced risk of human errors, which may delay progress through automation of processes, can be reflected in better chances of delivering results on time (Hua et al., 2018).

In life sciences, some remarkable accomplishments have been achieved in developing automated processes for experimental applications. After a progressive technological transition from the chemical manufacturing industry, it was demonstrated that automated devices can be used to generate and maintain cell cultures (Kempner, 2002). From the 1990s, commercial manufacturers developed automated devices consisting of closed environments with integrated systems that replicated typical manipulations of cell culture protocols, such as cell passaging, cell counting, and culture media changes. The first standardised processes achieved by commercial initiatives were intended to utilize bacterial organisms as a live factory for the large-scale production of molecules of interest, resulting in a dramatic yield increase in product obtention, along with a cost reduction compared to animal and vegetal sources of the molecules

(Doulgkeroglou et al., 2020). These cell culture systems have generated interest in their application for maintaining eukaryotic cell lines, with potential applications including the preservation of stem cells, cell lines and human-derived cell cultures for clinical use (Ochs et al., 2017; Moutsatsou et al., 2019). The incorporation of additional tools, such as imaging processing algorithms, micromanipulators, and microinjectors, was later introduced to extend technical capabilities, as exemplified by automated cell transfection methods.

The previous automated systems for cell culture have been constantly refined to adapt to more specialised tasks. A keystone in biological research was the discovery of iPSCs, which enabled in vitro stem cell differentiation into somatic cells, including neurons, by applying specific molecules during targeted temporal windows and spatial distributions that triggered differentiation mechanisms (Bassil et al., 2021). The efforts to precisely reproduce the related molecular dynamics and obtain specific phenotypic outputs consistently led to the development of automated cell culture platforms with tight control over culture media composition and rapid changes via dedicated rapid perfusion systems. The discovery of iPSCs for the production of diverse neural types later enhanced the development of automated protocols for the large-scale generation of specific neural types from these reprogrammed stem cells, also extending their application to the culture of non-neural populations present in the nervous system. However, limited consistency in generating specific cell types has been a current challenge in the field (Dolmetsch & Geschwind, 2011).

The development of automated platforms for maintaining and experimenting with neural populations has not been limited to generating structures that resemble physiological conditions. There is a growing interest in developing in vitro models that represent pathological conditions to explore the cellular mechanisms involved in disease onset and progression, as well as to study therapeutic approaches. Biological samples featuring the temporal changes related to developmental processes is another interesting challenge addressed by ongoing initiatives. For instance, novel in vitro systems of cultured neurons and stem cells are intended not only to constitute the cell population at a specific developmental stage but also to autonomously trigger the formation of layers similar to the developing neural plate. (Meinhardt, 2014) These novel biological models for research can be leveraged not only at the basic research level but also for therapeutic development by spreading to clinical field research.

The interest in automation in biological sciences extends beyond devices performing single tasks and isolated functions to the development of full manufacturing processes that benefit from efficiency and consistency. This search has been reflected in the production of workstations by commercial initiatives. The incorporated functions in these devices include automated pipettes for liquid handling, biosafety cabinets for maintaining sterility, incubators for controlling temperature and atmosphere, and robotic arms for manipulating labware. In addition, they can work coordinately with other specialised equipment, such as imaging systems, plate readers, and PCR thermocyclers (Greub et al., 2016). The technical applications are diverse, and there is a growing interest in applying novel experimental procedures. For instance, in the field of stem cell biology, cell reprogramming techniques using iPSCs, the generation of stem cell banks, and screening assays require large amounts of biological samples for testing. Novel discoveries have revealed promising applications in regenerative and personalised medicine, leading to the development of dedicated workstations to optimise and increase yields in these areas. Currently, the field requires improved efficiency and reproducibility in generating large amounts of cell process outputs, such as controlling differentiation from progenitor cells, creating in vitro disease models, and maintaining cell profiles in prolonged cultures (Truong et al., 2025; Yang et al., 2025). This is relevant for prospective applications of research outputs due to the limited ability to translate experimental findings from basic research to clinical applications (Mobley et al., 2013).

Some automated devices extensively used in industrial cell processes, such as cell culture sample management systems, are listed in **Table 1**, along with their primary applications. Most of them are involved in routine tasks during cell culture, such as solution dispensing, programmed media changes, cell counting, and handling of consumables. More recently developed functions may also include cell passaging and advanced imaging processing.

Model	Manufacturer	Main Purpose
Freedom EVO™	Tecan Integration Group ©	Liquid handling in cell cultures
Biomek Cell Workstation™	Beckman Coulter ©	Liquid handling in cell cultures
Cellhost™	Hamilton Robotics ©	Cell culture maintenance
SelecT™	TAP Biosystems ©	Cell culture maintenance
C.Station™	Cytana ©	Cell culture maintenance
Petaka bioreactors™	Celartia ©	Cell culture maintenance
CELLSTAR Autoflasks™	Greiner ©	Tissue culture
BioCel™	Agilent ©	Labware management

Table 1: Examples of commercial automated platforms

2.2 Open-Source Platforms for Automation

Despite the advantages of automated processes, the adoption of automated platforms has been limited by several factors. Firstly, their high acquisition cost makes them unaffordable for most laboratories (Storrs, 2013). In 2025, commercial devices with capabilities similar to those described above typically cost between £60,000 and £100,000, depending on the manufacturer, model, and additional features. Ongoing expenses must also be considered, including specialised consumables, periodic maintenance, calibration, and staff training provided by the manufacturer (Thomson & McElvania, 2019). Similar limitations apply to confocal and imaging systems designed for high sample volumes. Typical microscopes equipped with integrated X-Y stages used in biomedical research laboratories for multi-well plates, such as the Olympus BX61, Nikon Ti widefield, or Nikon A1 confocal systems, range in price from £15,000 to £150,000. Even so, the range of tasks that can be automated with these devices remains limited. This is particularly significant in academic settings, where research budgets depend on funding bodies such as government agencies (e.g., UKRI in the United Kingdom), charities, and grants. Because project requirements vary widely across different funding periods, the affordability of laboratory equipment is a major concern. As a result, manual experimental work has been often preferred due to its lower operational cost and its role in developing human expertise in line with academic missions, leaving most automation-related work to large commercial and industrial laboratories.

Another important concern is space requirements. Because automated processes require controlled environments, most of these devices incorporate incubators and isolated chambers as workspaces. When combined with the other necessary components, they tend to be bulky and heavy (**Figure 1.32**), requiring dedicated laboratory space and facilities and making them difficult to move once installed. Academic spaces are often shared among research groups, so allocating substantial space to specialised equipment can be difficult to justify. In addition, the required consumables are frequently sold by the same manufacturer and are incompatible with standard labware. As a result, storing these additional materials increases not only operational costs but also space requirements, due to the need to accommodate extra routine consumables.



Figure 1.32: Demanding space requirements of commercial automated cell culture systems

Size reference of the CompactT™ SelecT™ workstation (TAP Biosystems ©). Commercial automated culture systems often require large spaces to install and operate. Besides, they work using specialised consumables from the same manufacturer that are difficult to interchange, increasing further space requirements due to consumable storage.

Consequently, the elevated implementation costs of automation have generated a considerable gap between academic and industry research activities, with the former being almost absent in including automation as part of routine processes. This difference has been continuously widened by the progressive cost escalation in laboratory equipment, provided by a reduced group of commercial partners, and the lack of affordable alternatives to implement automated devices in lower-income academic facilities, which are more intended in knowledge generation and human resources formation and do not prioritise the generation of economical benefits upon processes. To exacerbate this scenario, industrial manufacturing processes are often tied to standard operational procedures, leaving any potential improvements on the processes to a dedicated Research and Development area, which incurs additional company expenses and impacts the product acquisition price. Consequently, due to the implementation and operational cost restraints faced by commercial devices, the application of automated processes has been limited in biological research, particularly in academic settings. The development of automated devices at lower costs could be beneficial as the scientific community continually strives for research efficiency and technological improvement. **The present project proposes a technological proposal to automate a specific process in Neuroscience research, specifically the in vitro generation of neural circuits.**

The growing demand to test and analyse extensive amounts of samples in the field of Cell Biology, such as in the case of drug screening experiments to test molecular effects or the optimisation of processes, has entailed the development of tools that ensure reliable and reproducible procedures to performing experimental tasks as visualising the generated experimental data. Moreover, when deep analysis processes are incorporated into this process, as in the case of using algorithms for data analysis, the amount of required data increases exponentially, and the experimental variability inherent to experimental procedures is a major concern. The previously mentioned experimental strategies for building neural circuits in vitro can generate consistent biological samples for experimental purposes with more predictable behaviour. However, due to the implementation and operational cost restraints faced by commercial devices, their application as an experimental standard is limited. It comprises only their application to a few experimental models, a discrete number of testing conditions per experiment and delegated to serve as a low-scale validation tool for other biological models.

In recent years, open-source devices have gained a presence in the biological sciences as a solution to address cost-related constraints in laboratory equipment requirements (Saggiomo,

2023). The term "open-source device" refers to any piece of equipment that provides intentional, unrestricted access to its full documentation, encompassing both hardware and software designs, component lists and assembly instructions (Niezen et al., 2016). Open-source initiatives often operate as non-profit organisations or provide accessible alternatives to commercial devices. The information sharing is intended to maximise their contribution to addressing current technological needs by creating a collaborative community to improve the original designs continually. Instead of relying on large-scale manufacturing processes, as in the case of commercial platforms, open-source technologies are intended for low-scale applications, often by assembling homemade or lab-made parts controlled by simple electronic components. The development of 3-D printing technologies in a variety of materials, such as plastic polymers, resins and metals, in combination with the flexibility in computational tools to create and manipulate 3-D object models, the tendency to create user-friendly interfaces in design software packages and the STEM (science, technology engineering and mathematics) orientated interest in early educational programs have been reflected in a growing public community interested in open-source technological development at a professional and amateur level (Baldwin et al., 2025). The creation of online repositories has also been a crucial enhancer of this progress, generating databases and discussion forums where contributors can share, test, and update their ideas. This overall process yields the creation of low-cost devices that can rival commercial counterparts in terms of technical capabilities and performance. Moreover, collaborative improvement can also lead to rapid product refinement and evolution, ultimately driving innovation.

Academic initiatives have played a major role in the development of open-source devices. Due to their close involvement in frontier research projects, awareness of specific technical demands in their field, and a public-oriented vision based on their human resource formation mission, academics have developed open-source devices aimed at combining efficiency, simplicity, and accessibility to perform routine experimental procedures. This repertoire of applications has also expanded into the fields of Cell Biology and Neurosciences. More specifically, some open-source platforms have been developed to maintain, monitor, and manipulate cell cultures, including those of neurons. Additionally, some simple imaging acquisition tasks have recently begun to be automated using customisable protocols that perform simultaneous image capturing and analysis (Casas-Moreno et al., 2023) and long-term microscopy recordings (Chiron et al., 2022). **This possibility to automate tasks as part of standardised laboratory processes is helpful in the search for suitable tools to apply the bottom-up approach for generating neural circuits in vitro, as seen in the present project.**

In this scenario, the Dr. Anton Nikolaev group at the University of Sheffield has developed the Fresco Project initiative, a series of open-source automated workstations designed for maintaining cultured cells, performing real-time imaging analysis, and manipulating culture media during extended experimental procedures. These devices possess the technical capabilities to handle and monitor multiple experimental conditions and analyse data in real-time, serving as a suitable platform for high-throughput experiments. **To develop an effective system for building and studying neural circuits in vitro, the FrescoM workstation was selected as the starting point for this project.** The following paragraphs describe the development and key features of this workstation.

Studying individual cells as the anatomical and functional units of biological systems requires the development of tools for proper handling. Generating reliable data from single cells relies on repeatable manipulations at a microscopic scale while minimising any harm that could compromise normal cellular functions. The proposed Bottom-Up approach for building neural circuits will require the precise manipulation and positioning of individual cells in cultures. Traditionally, single-cell manipulations have been performed manually using fine pipettes.

2.3 Overview of the FrescoM Workstation

The FrescoM workstation was introduced in 2021 in the original paper “An Open-Source Framework for Automated High-Throughput Cell Biology Experiments” (Katunin et al., 2021), resulting from the combined efforts of Dr Anton Nikolaev research team and his collaborators at the School of Biosciences in the University of Sheffield, United Kingdom. Their main motivation stemmed from the frequent need during biological experimental procedures to generate large and reproducible datasets, which could be extensively analyzed using modern data analysis methods, such as optimization algorithms and deep learning. The developed framework consisted of an epifluorescent microscope mounted in a mobile metal frame designed to hold cell cultures in standard plastic multi-well plates, as well as a perfusion manifold for the controlled application of solutions to the culture media. This system was designed with a modular structure, where the constituent parts function as separate modules that combine their roles, orchestrated by a microcontroller, to perform specific experimental tasks such as maintaining cultures, real-time cell imaging, and rapid manipulations of the media composition. **Figure 1.33** depicts the overall design and functional modules of the FrescoM automated workstation.

As an open-source initiative, the full list of components, software files and detailed assembly instructions has been publicly shared. They can be accessed and downloaded from the referenced paper, the FrescoLabs GitHub repository (URL: github.com/frescolabs/FrescoM), and **Appendix 1** at the end of this thesis. Depending on their function, the components fall into four main categories:

- Structure: Metal (aluminum) extrusion bars and rails.
- Assembly: PLA plastic 3-D printed parts and adapters
- Diverse Functions: Specialised parts (for instance, microscope parts)
- Electronics: For hardware control

Parts from each group were assembled into blocks to perform independently one of the workstation functions. For instance, several microscopy components, including a lens, a camera, 3D-printed plastic supports, and cabling, were used to construct a lab-made epifluorescence microscope. This example illustrates the functional module principle used in the overall design. The main functional module constitution of the original design is described in the following paragraphs:

The first module that constitutes the workstation is an aluminum mobile frame. This frame serves as a scaffold for mechanical, electronic and 3D-printed plastic components that can be fixed using standard steel M3 screws and nuts. The frame was built using aluminium MakerBeam-XL™ extrusion bars (15mm x 15mm size) of different lengths (5, 10 and 30 cm). The bars were connected using I, L and T-shaped metal brackets. The resulting frame for the workstation, originally designed and assembled for this project, was designed to be compact in size, addressing space-related issues commonly associated with workstations. The final size was 33 cm in length, 23 cm in width, and 23 cm in height, making it small enough to place on a small laboratory bench or inside an average-size incubator to better control the environment. However, this size is not fixed and can be modified depending on space requirements and availability. The stability and vibration resistance during prolonged work must be ensured when choosing the standing place for the workstation.

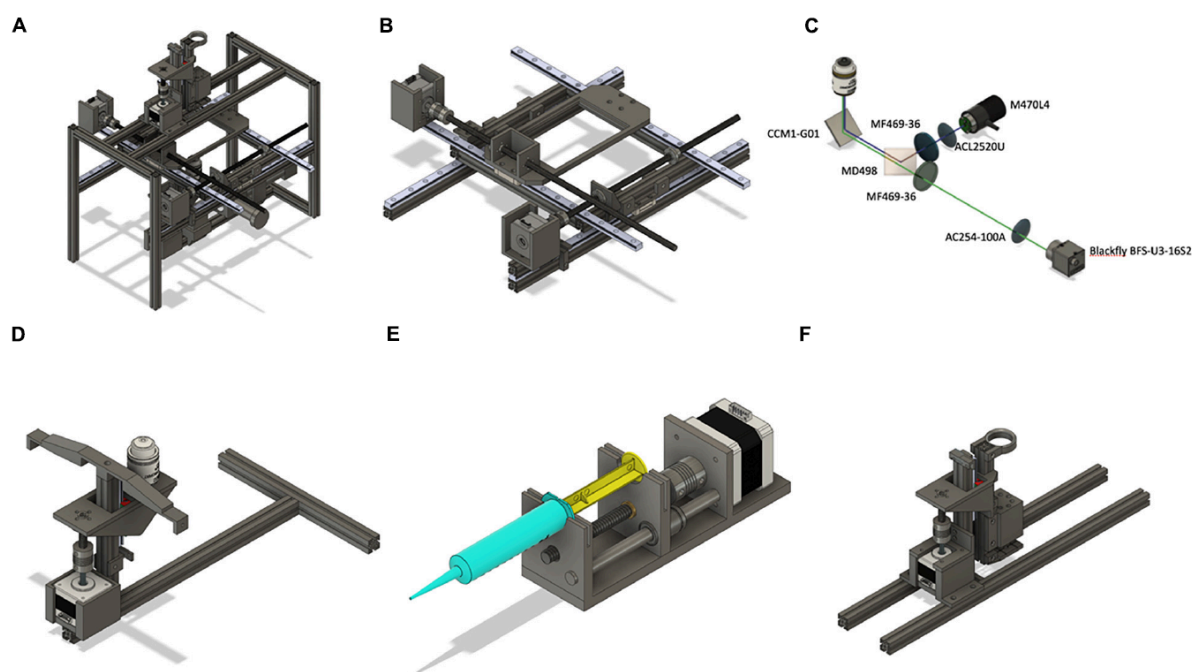


Figure 1.33: General structure of the FrescoM automated workstation (from Katunin et al., 2021)

This automated workstation comprised components arranged in functional modules and mounted on an aluminium MakerBeam-XL™ frame. Some electronic components are connected to a central PCB and controlled by an Arduino™ UNO™ microcontroller.

A. Diagram of the assembled workstation with the three original functional modules to maintain cell cultures and perform high-throughput imaging procedures. A perfusion manifold was placed on the top, aligned with an epifluorescence microscope and a culture plate to administer solutions on cultured cells at variable positions of a well plate, while capturing images in real time.

B. The workstation included a X-Y stage that was used to hold and move a well plate via stepper motors and rails. A plastic 3D-printed adapter was designed to fit the shape and size of commercial culture well plates. The adapter ran through metal rails and was moved by the action of a pair of NEMA™ stepper motors, whose rotation was converted to displacement in the X and Y axes, respectively.

C. Optical design of the incorporated epifluorescence microscope. Thorlabs™ part codes and camera models are included.

D. Assembled microscope stage. Focusing is controlled by a single stepper motor, which enables the Z-displacement of the objective.

E. Syringe holder for solution administration. Syringe content release is controlled by a stepper motor that moves the plunger in forward and backward directions.

F. The solution dispenser stage consists of a manifold that holds the plastic tubing required for solution exchange in the sample, connected to the syringe.

The second module was a mobile X-Y stage (Figure 5B), built from extrusion bars arranged in a square shape. This inner frame was designed as a platform to hold a standard culture plate and to move along the X and Y axes using two pairs of MGH12 metal rails (one pair per axis) and stepper motors. Some PLA plastic adapters were required to connect the parts together. The current plate holder was designed for a standard disposable 96-well plate, but similar parts can be printed and incorporated for other types of plates. For instance, an additional holder for 35mm Petri dishes and microscope slides was used during some experiments.

The third module is an epifluorescence microscope. In contrast to commercial microscopes with a fixed design, this component was assembled using ThorLabs™ aluminium parts, mirrors, and lenses to allow for optical magnification. The image was recorded using a BlackFly microscope camera (Teledyne Vision Solutions) mounted at one end of the light beam. The camera parameters were adjusted by the manufacturer using the Spinnaker SDK Spinview™ through a computer or laptop connected via a USB cable. The resulting microscope was capable of capturing and transferring images for storage and analysis. The addition of a blue LED and filters enabled the conversion of the microscope into a fluorescence microscopy system. Finally, the microscope objective was mounted on a 3D-printed plastic adaptor, which could be vertically moved for focusing using a stepper motor. The real-time image of the microscope, along with relevant parameters, was monitored and controlled through the workstation user interface.

The fourth module was a system that controlled the application of solutions to the sample through a pipette, which was operated by the movement of an incorporated syringe. The plunger displacement was regulated by a designed 3-D printer support for the syringe. This semi-mobile system consists of a static case for the syringe body and a mobile unit that displaces the syringe forward and backwards via an additional stepper motor. The original design for this part was intended to hold a standard 50 mL plastic disposable syringe. Although it did not have a fixed position on the main frame, this module was commonly held close to the manifold during experimental procedures.

The original fifth module was a manifold designed to hold the plastic tubing system during experimental manipulations. It consists of a 3D-printed plastic nozzle that keeps all tubing in position at all times. A commercial multi-channel nozzle could also be adapted. Using a fifth stepper motor, the manifold was moved along the Z-axis to bring the plastic tubing closer to the sample, thereby performing a liquid exchange on the culture well content. The manifold also contained a series of white LEDs to illuminate the sample within the visual frame of the microscope, enabling the capture of bright-field microscopy images.

As mentioned, some of the components required the design of dedicated adapters for assembly and fixation with other parts and into the frame. For this purpose, several 3-D printed parts were used. It is worth highlighting the contribution of 3D printing technologies to the enhancement of open-source device development. Hardware manufacturers commonly fabricate components by design using industrial processes such as mould injection. This method is effective for large-volume production but far from being profitable for limited numbers, as in the case of customised lab equipment. Accessible 3D printers have enabled end-users to design individual components from scratch, with endless possibilities, assemble unique products, and conduct trial-and-error testing for validation and design improvement at a low cost. The same process was applied to the Fresco-M platform, and these 3D-printed components were intended to be effective, focusing on functionality, durable by evaluating and reinforcing resistance over extended use, accessible, employing PLA plastic as material, and compact, leveraging spatial distribution with multiple fixation points.

The mentioned modules were connected and controlled by an Arduino MEGA™ microcontroller mounted on a dedicated printed circuit board (PCB), specially developed for this platform, which also contained the required motor and LED drivers. The microcontroller was plugged into a personal computer or laptop via a USB cable. This system was chosen due to its customisation capabilities to connect and control diverse electronic components through the common programming language C++ and a user-friendly interface.

In terms of software, the FrescoM platform was developed using a Python-based interface and scripts. The user interface, constructed using the Python 3 Tkinter package, served as the microscope display and included a series of buttons to control each function. The aforementioned FrescoM GitHub repository (URL: github.com/frescolabs/FrescoM) contains all the software and hardware documentation necessary to run the user interface, control the components, and perform predefined functions and simple experimental tasks. One of the main advantages is the ability to develop custom scripts using the Python programming language to execute specific experimental protocols, thereby performing particular experiments.

Building on the previous advantages, one of the main objectives during the design of the FrescoM workstation was to provide an affordable solution to expand automation implementation in laboratories, which would otherwise be limited by acquisition and maintenance costs. In contrast to manufactured parts with dedicated designs, the use of low-cost assembly kits, complemented by plastic 3D-printed parts and open-source electronic components, drastically reduces assembly costs compared to commercial automated platforms. Summing all the conforming elements, the total cost of the FrescoM workstation ranged from £400 to £ 600 in the most basic version, which included only a bright-field microscope, and around £2,500 with a fluorescent microscope incorporated, representing a fraction of at least a tenth compared with the most basic models of equivalent commercial platforms for cell culture and visualisation. Thus, this workstation is more affordable for underrepresented groups to consider it for task automation of their particular processes involving cell cultures.

Finally, a notable characteristic of the FrescoM workstation is its organisation into functional modules. Each individual component can perform a specific function in the system, such as the microscope or the solution application manifold. Instead of a fixed allocation of the components in specific places with invariable connections with other parts, their position can be adjusted depending on specific designs. However, the final position shown in the figure was chosen to minimise the required cabling and displacements. It is worth noting that this conformation allows for the easy insertion of additional components into the empty spaces of the metal frame. The incorporated parts can be directly plugged into the rest of the components or via the PCB to the customisable microcontroller, which also holds extra pins for this purpose. In terms of software, the Python elements loaded into the microcontroller have been designed to add new parts simply and intuitively. For instance, incorporating a new motor requires including an additional index number in the respective script, with the rest of the motors and functions already loaded, and relating the forthcoming actions of this new motor to that index number. However, different components not included in the original design may require adjustments and calibration to ensure proper performance. As a result, the FrescoM workstation is an adaptable and versatile workstation that can incorporate diverse components, enabling it to perform coordinated tasks and fulfil additional experimental procedures. This can expand technical capabilities and open up the possibility of increasing technical applications by incorporating new functions. **This advantage was leveraged in the present thesis, serving as the starting point for the development of an automated device capable of performing the required steps to build in vitro neural circuits.**

The first stage of this project aimed to design new components for the FrescoM workstation, adding an original function for the controlled manipulation of single cells in cultures. After this, the next aim was to convert this workstation into a device capable of producing neural circuits in vitro. For this purpose, the component performance was tested to handle, select, and release neurons from primary culture samples, in which optimal growth conditions were also tested to maximise yields, followed by confirmation of the neural integrity after these manipulations. The next stage of the project aimed to develop a method to promote and direct axonal growth in positioned neurons, to form synaptic connections and generate simple neural circuits in vitro. Finally, the project also implemented automated tasks for individual steps of the process, thereby constituting an automated manufacturing process for in vitro neural circuit fabrication.

III. AIMS AND OBJECTIVES

MAIN OBJECTIVE:

This project aimed to develop an experimental paradigm for the in vitro construction of neural circuits de novo. This involves establishing a procedure to manipulate individual neurons from a selected cell source sequentially, generating in vitro arrays of neurons with a defined structure as a constant output, using a Bottom-Up approach-based procedure. The project involved an initial refinement of a current technological tool, an improvement of neural culture protocols, and the development of an assembly process using cultured neurons, followed by the automation of tasks to increase precision and reduce task times, in search of improved output yields.

The process development firstly required adapting the automated workstation FrescoM (Katunin et al., 2021) by designing and installing additional components and modifications to allow the precise manipulation of individual cells in the managed cell samples. Primary cultured neurons from embryonic zebrafish were proposed to serve as a cell source for in vitro circuit building due to the convenient expression of a genetically encoded neural marker and an activity reporter in a specific strain (HuC-GCaMP6). Thus, a protocol was implemented to consistently generate neurons from zebrafish primary cultures, with enhanced in vitro survival and the ability to grow processes, thereby producing a suitable source of neurons for procedures involving cell handling. The subsequent stage established procedures to handle individual cultured cells using the developed workstation, involving the identification, selection and positioning of neurons by the coordinated work of the workstation components. The project next investigated manipulations in positioned neurons which promoted neurite growth, directed axonal extension and consolidated synaptic connections formation between these neurons to form stable neural arrays. Subsequently, the structure and cellular properties of the produced circuits were evaluated to compare the outputs of the circuit formation process to resemble a functional neural circuit. The project was complemented by the inclusion of programming tools to allow the automation of the involved tasks for sample manipulations in cell cultures.

The proposed process of in vitro building of neural circuits is aimed to provide an accessible solution to be implemented as a routine procedure to generate suitable biological samples, in a reduced frame of time and using minimal resources, to model neural interactions and information processing in neural circuits at the most elementary level, for experimental purposes.

Specific objectives of the study can be divided as follows:

1. To develop an automated workstation, based on the FrescoM workstation design, to manipulate individual cells from biological cultures as part of controlled procedures, combining with the maintenance and imaging by microscopy of cultured neurons.

Leveraging the modular structure of the FrescoM workstation, the first aim was to enhance the technical capabilities of the FrescoM platform to function not only as an imaging system for maintaining cell cultures, but also to physically interact with individual cells in the samples as part of experimental procedures. The initial task towards the in vitro generation of circuits using cultured neurons was the development of a cell micromanipulator, based on a custom micropipette, to be installed in the FrescoM workstation. The inclusion of the new functional module required the design of the involved components and software modifications to perform precise solution dispensing on the handled cell samples, identifying the necessary technical parameters to serve as a suitable tool for selecting individual neurons from managed cultures and releasing them in specific positions into new cultures, forming arrays.

2. To implement a protocol to consistently produce samples of cultured neurons from zebrafish embryos, capable of surviving for extended periods and growing neural processes in the presence of promoting molecules, to serve as a suitable cell source for the in vitro building of neural circuits.

The HuC-GCaMP6 zebrafish strain offered convenient features for use as a cell source during in vitro circuit building, allowing for the rapid formation of neural arrays, a highly specific neural identification and real-time activity monitoring. However, protocols for the obtention and extended maintenance of primary cultures of zebrafish neurons were limited, with little information regarding the induction and guidance of process growth. A repertoire of conditions was tested to identify those which favoured neural survival and axonal growth of cultured zebrafish neurons, thereby finding optimal parameters for their use in cell manipulation procedures and consolidating them as a valuable tool for experimental purposes.

3. To develop a method to use cultured zebrafish neurons to form in vitro circuits with defined structure by using the developed micromanipulator to identify and select individual cultured neurons, and positioning them forming arrays, to subsequently direct axonal growth and the formation of specific synaptic connections.

A main challenge in the project was to achieve a controlled manipulation of the cultured neurons, preserving their integrity and functions, to build specific cell arrays. Once a tool capable of handling individual cells was acquired, the next stage focused on transferring neurons from a sample of dissociated neurons into new cultures of isolated neurons at specific positions. This involved adjusting the parameters to selectively identify neurons from samples using microscopy, followed by a “pick and release” procedure with the micromanipulator, while minimising harm to the cells during the task.

4. To induce controlled axonal growth in positioned neurons to produce physical contact and the formation of functional synaptic connections.

To achieve the successful building of a neural circuit, the next aim was to replicate growth-stimulating conditions in the previously positioned neurons, promoting axonal growth and the formation of specific physical contacts between these neurons. This involved the application of strategies to continuously direct the growing axons to defined targets and match the intended structure of the neural circuit. The maintenance of these physical interactions was designed to consolidate into the formation of functional synapses on the contacting neurons.

IV. MATERIALS AND METHODS

Assembly of the Cell Manipulator Functional Module for the FrescoM Workstation

The complete 3D design of the modified workstation, component list, assembly instructions, and software files for operation were uploaded to a dedicated folder in the GitHub repository for this project (URL: <https://github.com/FrescoM/CellManipulator>). The modified workstation was based on the FrescoM framework design described by Katunin et al. (2021). Similar to the original version, the structural frame was assembled using aluminium 15 mm Makerbeam™ extrusion bars of several lengths, aluminium L-shaped and T-shaped brackets, and M3 screws. A second, smaller XY stage was mounted on the top of the workstation, replacing the upper manifold. A 100 mm one-push-pull solenoid, a 2-port pipette holder (MEH1RF, World Precision Instruments; or ESW-MxxP, Warner Instruments), and a fabricated glass pipette were mounted on this mini-stage, using 3D-printed plastic adapters to secure them in place. The pipette for cell manipulation consisted of a 10 cm borosilicate glass pipette (1.5 O.D., 0.8 I.D.), heat-pulled to a diameter of 50 µm in one tip using an automated two-step puller (Narishige © model PC-10; step 1 = 79.8°C; step 2 = 47.2°C). Upon activation of the push-pull solenoid, the pipette extended and retracted on the Z-axis. The pipette holder was connected to a precision syringe (Hamilton ©, Microliter Series™ 500 µL, CAL80865) via a flexible polyvinyl chloride tube, allowing the application or retrieval of liquid solutions inside the pipette when the syringe plunger was displaced. When placed on liquid culture media containing cells, the system worked as a cell manipulator, picking up, moving and releasing cells with the pipette. Procedure positions were defined by controlling the main XY-stage movement. The hardware operation was controlled by the native FrescoM software, previously developed in a Python v3.8 environment (Python Software Foundation, Wilmington, DE, USA) and run via an Anaconda3 prompt, with some modifications to operate the new components, via a dedicated PCB connected to a 12V power supply and a laptop.

Precision Assessments on the X, Y and Z Displacement of the Implemented Mini-Stage

A Python protocol was developed to measure deviations during positioning of the cell manipulator pipette, consisting of automated movements of the pipette along each axis to assess movement consistency and the precision of returning to the same point after repeatedly performing a displacement similar to routine cell manipulation procedures. A heat-pulled pipette with a 50 µm diameter tip was mounted in the pipette holder and placed in a culture well of a 96-well plate filled with Phosphate Buffer Saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄). Then, the pipette tip was focused by the workstation microscope. Then, a short protocol was run, which travelled the pipette a distance of 300µm along the X, Y or Z axis in a series of 20 partial equidistant steps of 15µm, by activating the corresponding stepper motors (X and Y axis) or the push-pull solenoid (Z axis) and returning to the initial position at the end. An image was captured at each step of the individual sequence using the “Capture Image” internal function of the FrescoM software.

After completing one pipette displacement sequence, the main X-Y stage moved the plate to a different position, and the sequence was repeated until 50 repetitions were completed. A minimum of 6 experimental rounds (300 individual protocols in total) were performed for each axis testing. To analyse precision during pipette displacement, the initial and final captured images were compared, with the pipette tip visible in both. A similar protocol was used to assess precision in the Z-axis, in which the push-pull solenoid was enlarged and retracted to perform the pipette vertical movement. All image measurements were made using the FIJI software (Schindelin et al., 2012). The centre of the pipette tip was manually annotated in the first and the last pictures of each movement cycle, and the pipette tip distance from both images was measured by drawing a straight line between the two annotated points. Median values were calculated to obtain typical values expected for repeated displacement.

Primary Culture of Embryonic Zebrafish Neurons

Zebrafish (*Danio rerio*) HuC-GCaMP6 transgenic embryos were provided by the Bateson Centre Aquarium of the University of Sheffield. Embryos were manually selected at 2-3 days post-fertilisation (dpf) based on the expression of the GCaMP6 fluorescent protein in neurons, visible in the brain and spinal cord, under widefield fluorescence microscopy (excitation λ = 488nm). Animals were used under the Procedure Project Licence PB8AF89D6, issued by the Home Office of the United Kingdom. Due to the developmental stage of the fish embryos (under 5 dpf), no Personal Project Licence for protected species was required to perform experimental work, including approved Schedule 1 procedures. Competence in all techniques was confirmed by the PPL holder (Dr Anton Nikolaev) before unsupervised work.

To obtain a primary culture of neurons, 12 to 15 embryos from 1 to 3 dpf were used per experiment, yielding approximately 12 high-density neural cultures in 7.0-mm culture wells. Selected embryos were placed in E3 Medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl_2 , 0.33 mM MgSO_4 , 0.00001% Methylene Blue) and anaesthetised with MS-222 solution (tricaine 168 mg/L). Embryos were euthanised by decapitation, followed by the immediate dissection of the brains, which were partially fragmented using a steel scalpel blade, and transferred to cold Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific Inc. 12082739) in a 15mL conical tube.

Tissue fragments were collected by centrifugation (200 RCF, 5 min). HBSS was almost completely removed from the conical tube using a micropipette, leaving just enough to cover the tissue pellet and brain fragments, and 400 μ L of TrypLE™ reagent (ThermoFischer Scientific Inc., Cat. 10043382) was added to the tube to dissociate cells from the tissue. The tube with the enzymatic solution was placed in an incubator (37°C, 10 min) with periodic gentle mixing to prevent tissue fragment aggregation. Then, the enzymatic reaction was stopped by the addition of 400 μ L HBSS. Brief centrifugation collected non-dissociated tissue fragments, and the supernatant containing dissociated cells was transferred to a new conical tube. Cells in suspension were centrifuged (200 RCF, 5 min) to create a pellet. The medium was almost completely removed from the tube, leaving just enough to cover the tissue pellet, which was subsequently resuspended in 150 μ L pre-heated (37°C) Neurobasal™ culture medium (ThermoFischer Scientific Inc., Cat. 21103049). Culture wells in a polystyrene 96-well plate (0.7mm well diameter, 400 μ L capacity) were used to maintain the cells. Each well was previously incubated overnight with 80 μ L of a protein solution, either poly-L-lysine 10 mg/mL or laminin 10 μ g/mL, as indicated in each case, to create a uniform coating on the well bottom. From the obtained cell suspension, 10 μ L was taken per experimental condition, mixed with 300 μ L of supplemented Neurobasal medium (see **Table 2** for medium composition), and transferred into a culture well.

Following the previous protocol, multiple high-density cultures of dissociated cells were obtained, mainly neurons ($\sim 1\text{--}3 \times 10^5$ cells per cm^2). Cell fluorescence confirmed the neural identity of HuC-GCaMP6 neurons due to the constitutive expression of the huC promoter as a neural marker in neurons, when observed using the incorporated epifluorescence microscope (LED excitation λ = 488nm). The 96-well plate containing the cell samples was maintained in a cell culture incubator dedicated to cell growth, at 37°C $\pm$ 0.5°C, 5% CO_2 and 95% humidity. Every 24 hours, 150 μ L of the culture medium was replaced with fresh medium of similar composition. For experiments using the modified FrescoM automated workstation, the device was placed inside the incubator, except for the electronic components (the PCB and the control computer). Additionally, to study the effect of media composition on neuronal survival and growth, the media composition was modified as indicated for each case. Cell count values were obtained using the native “Cell counter” plugin in FIJI for the captured images.

Component	Final Concentration
Neurobasal Medium (NB)	-
Fetal Bovine Serum (FBS)	10 %
B-27 Supplement	2 %
GlutaMAX™ Supplement	1 %
Penicillin / Streptomycin mix solution	100 µg/ml

Table 2: Composition of Supplemented Neurobasal Medium

Optimisation of Survival and Growth Conditions in Cultured Zebrafish Neurons

Primary cultures of brain neurons from 1 to 3 dpf Huc-GCaMP6 zebrafish embryos were prepared using a previously described method to obtain a cell suspension consisting mainly of dissociated neurons, which was mixed with 300 µL of culture medium of the specific composition for each test and placed in a single well of a 96-well plate.

To determine the effect of serum on neural survival, media without FBS (Fetal Bovine Serum (Sigma Aldrich, Co. F0392) was used to reconstitute cells in the final step of the protocol. The rest of the media composition remained unaltered. One additional cell sample was reconstituted in supplemented Neurobasal media containing 10% FBS. Cultures were maintained in a cell culture incubator, at 37°C ± 0.5°C, 5% CO2 and 95% humidity. For extended cultures (more than 24 hours), culture manipulations were performed inside a cell incubator. The FrescoM workstation size was small enough to fit inside a standard 170L incubator for cell cultures. Although cell manipulations were performed with the culture plate lid removed, adding antibiotics to the culture media was sufficient to prevent culture contamination.

To study the effect of protein coating on neural survival and growth of neural processes, cells were placed in culture wells incubated overnight with poly-L-lisine (10 mg/mL, Sigma Aldrich Co. P4832) or laminin (10 mg/mL, mouse laminin from Engelbreth-Holm-Swarm murine sarcoma, Sigma-Aldrich Co. L2020) coating solution before adding the dissociated cells. The protein solution in the wells was washed twice with 200 µL PBS before placing the cell suspension. The effect of neurotrophins on cell growth was investigated using neurobasal medium supplemented with NGF (100 ng/mL; human β-nerve growth factor, Sigma-Aldrich Co., N1408), BDNF (human brain-derived growth factor, Sigma-Aldrich Co., L2020 SRP 3014), or a combination of both neurotrophins.

To monitor cell survival and axonal growth, images were captured every 24 hours using brightfield and fluorescence microscopy, with the epifluorescence microscope incorporated in the workstation and the “Capture Image” internal function of the FrescoM software. Cultures were maintained in a cell culture incubator, at 37°C ± 0.5°C, 5% CO2 and 95% humidity for a maximum of three days. After the imaging session, 150 µL of the culture medium was replaced with fresh medium with identical composition. A minimum of four pictures were captured from each culture well from random positions per session, moving the XY-stage of the plate holder to a different location within the well between frames and comparing with previously captured images to avoid capturing the same area. Unless indicated otherwise, pictures were captured using a 20X objective and manually adjusting the "Exposure time" and "Gain" from camera parameters to facilitate visual identification of cell features, focusing on soma shape and integrity, and the presence of neural processes.

Survival and axonal growth were calculated by capturing images of each culture well from randomly selected areas containing a homogeneous number of dissociated cells, in both

brightfield and fluorescent channels (LED excitation at $\lambda = 488$ nm). Captured images at each time point were stored in JPG format and analysed using the software FIJI and the Plugins “Cell-counter” and “NeuronJ” to count neurons in the captured area and, when present, measure process length. Measurements were calibrated using a 0.01mm micrometre slide. A surviving HuCGCaMP6 neuron was defined as a round-shaped cell in the culture, with a soma diameter around 15-20 μm , no visible disruption of membrane integrity and expressing fluorescence when the microscope illumination system was switched to a blue LED (LED excitation $\lambda = 488\text{nm}$), corresponding to epifluorescent microscopy function. Cells forming clusters were only counted when their individual limits were clearly defined. The presence of axonal growth in neurons was established by the presence of at least one neural process of 5 μm length. Axonal length measurements were taken from the longest neural process when present. In some images, contrast was linearly enhanced via pixel-intensity histogram stretching using Brightness and/or Contrast adjustments in FIJI, without altering the intrinsic pixel-intensity values.

Manipulation of Cultured Cells Using the Developed Cell Manipulator

Primary cultures of brain neurons from 2dpf Huc-GCaMP6 zebrafish embryos were obtained using the previously described method, yielding cultures consisting mainly of dissociated neurons. They were initially placed in wells of 96-well plates without any protein coating to reduce cell attachment and to serve as a cell source, each well containing 300 μL of Neurobasal culture medium. The culture plate was left in the incubator ($37^\circ\text{C} \pm 0.5^\circ\text{C}$, 5% CO_2 , and 95% humidity) for 15 minutes to recover before subsequent manipulations.

A heat-pulled borosilicate glass pipette (1.5 O.D., 0.8 I.D) was mounted in a 2-port pipette holder (MEH1RF, World Precision Instruments ©) to work as a cell manipulator for cultured cells when connected to a 500 μL precision syringe, controlled by the FrescoM software via a stepper motor pushing or pulling the syringe plunger. This cell manipulator was used to transfer ~100 neurons per experiment from the wells serving as the cell source to new culture wells, coated with poly-L-Lysine (0.1 mg/mL). Transferred neurons were individually released and positioned in regular square-shaped arrays separated by a 50 or 100 μm gap. The destination culture wells were filled with 300 μL of the corresponding culture media, in the absence or presence of one of the following neurotrophins in the culture media: Nerve growth factor (β -NGF, 100 ng/mL), Brain-derived growth factor (Recombinant human BDNF, 150 ng/mL) Fibroblast growth factor-2 (Recombinant mouse FGF2/FGFB, 100 ng/mL) or a combination of both, as specified in each experiment. Culture plates were maintained in an incubator for the full duration of the experiment, at $37^\circ\text{C} \pm 0.5^\circ\text{C}$, 5% CO_2 and 95% humidity. To allow access to the culture well during cell manipulations, the plate lid was removed for the duration of the procedure and was returned to its position thereafter. To monitor cell survival and axonal growth, images were captured every 24 hours by brightfield and fluorescence microscopy, using the epifluorescence microscope incorporated in the workstation and the “Capture Image” internal function of the FrescoM software. The criteria to define cell survival and presence of a neural process were the same as the previous step.

Precision of the cell release was evaluated using a Python protocol that filled a pipette with media containing ~50 neurons and then, using short motor steps moving the syringe plunger, releasing the media and the individual cells in a new culture dish during 15 minutes, capturing images every five seconds. Individual neurons were eventually released and left for free attachment in the culture surface. After an additional 15 minutes, neurons were partially attached to the bottom. Then, the position of the centre of the manipulator pipette was annotated in the captured images using FIJI, as well as the final position of the released neurons. Distance was measured from a total of 100 individual repetitions of the task.

The method for detecting neuronal picking and release events by the pipette consisted of positioning the pipette in proximity to fluorescent dissociated neurons from the cell source samples, and in the centre of the camera visual display. Then, a FIJI dynamic analysis was initiated, which scanned a region of interest (ROI) measuring 840x840 pixels, corresponding to

the central area of the visual display. The analysis generated a Z-profile of total pixel intensities in the area, producing a numerical intensity value every second. Then, a sliding data frame was created, and the average intensity was calculated from the last 10 recorded values. A conditional instruction was included in the script to continue media retrieval (activation of the syringe plunger) until the last measured pixel intensity was 0.5 times the average intensity, following a logical comparison. This marked decrease in intensity coincided with the pipette retrieval of the neuron in the scanned area, triggering progression to the next step of the protocol. The release event identification used the same script, but with the logical comparison direction inverted to detect an increase of 0.5 in total pixel intensity in the area. This increase occurred when a fluorescent neuron entered the central region from inside the pipette, triggered by pushing the syringe plunger. The method for the formation of discrete cell layers consisted of the variation of the final position of the pipette before cell release in the empty culture well, to generate a 50 or 100 μm gap between neurons by adding a constant increment in the distance travelled in the X or Y axis at the end of each release cycle. At the end of each protocol run, cultures were grown in a supplemented Neurobasal medium in an incubator at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$, 5% CO_2 , and 95% humidity. After 24 hours, imaging was performed to assess neural survival and axonal process growth.

Stimulation of Positioned Huc-GCaMP6 Neurons

Neural arrays, typically consisting of 2-3 neurons, were generated using the previously described procedure. After 24 hours of free growth in a supplemented Neurobasal medium with at NGF 100 ng/mL to allow neural processes to grow, the well plate with the culture was placed in the FrescoM workstation. The micromanipulator pipette was replaced with another pipette measuring 10 μm in diameter, which was filled with 50 μL of 20 mM KCl. The new pipette was placed in proximity to one neuron composing a neural array. A protocol was developed to capture an image every second to detect GCaMP6 fluorescence using the fluorescence microscope. After a 30-second baseline period, the syringe was activated, releasing the pipette content next to the centred neuron. Image capturing continued for a total of 3 minutes. Fluorescence values were normalised against the average value from the baseline period. To analyse fluorescence in individual neurons, an ROI was selected using FIJI to limit the measurement to a discrete area around each neuron.

Laminin Strip Application from Droplets

A 20 μL laminin droplet was applied in the centre of a standard culture well and left undisturbed for 2 hours to allow laminin to form a uniform layer in the covered area. A solution applicator was fabricated, consisting of a ~ 30 μm gauge heat-pulled PLA filament placed inside the micromanipulator pipette with a segment of $\sim 20\mu\text{m}$ coming out from the pipette tip. The repeated and manually assisted movement of the micromanipulator stage enabled the spreading of the laminin droplet over short distances, generating stripes of ~ 100 μm with 50 μm gaps between each other, after several repetitions.

Formation of an NGF Gradient

A 10 μm -diameter heat-pulled glass pipette was placed in the manipulator pipette holder and filled with a 0.1% fluorescein solution (Sigma-Aldrich Co., Cat. 46955). The pipette filled with the dye was then placed in an empty culture well. A protocol was developed to release a small amount of laminin every 15 seconds and capture images every second using the fluorescence microscope in the workstation. Gradient formation was evaluated by annotating the pipette centre position and the closest point where fluorescence was sufficiently intense to be distinguishable from the background. ($R/R_0 > 1$). The distance between the two points was measured using tracing a straight line and the native FIJI Measure function, and the average value was obtained from four independent protocol runs to calculate the gradient size.

Effect of an NGF Gradient Application on Axonal Growth

A pipette filled with NFG-containing NB media (150 nM) was positioned in the proximity of cultured HuC-GCaMP6 zebrafish neurons after 2 hours of growth in NB media without NGF. The NGF solution was released at a constant rate of ~5 $\mu\text{L/h}$ and culture images were captured every 10 min with the brightfield microscope of the FrescoM workstation. The centre of the pipette and visible axonal cones were manually annotated in the captured images using ImageJ. The net displacement of the axonal cones was calculated as $\Delta x = x_2 - x_1$, where x_1 and x_2 were the initial and final distances from the axonal cone to the pipette centre in each frame.

Laminin Removal Using UV Lithography

A custom UV lamp was fabricated, composed of a commercial LED-UV emitter module (1.05 W, 365 nm), an 8 mm Plano-convex lens, 150 mm diameter (Thorlabs), a 1.2A LED driver (T-Cube™ LED Driver, Thorlabs) and a 60 mm metal frame for microscope slides, working as support (Figure 5.35). The LED beam intensity was controlled using a knob in the driver. Laminin-coated culture wells from a 96-well plate were incubated overnight with a laminin solution, as previously described, to create a uniform laminin layer on the well surface. After this, a 3mm nickel TEM mesh disc was placed in the centre of the culture well using fine forceps. The discs contained a pattern of either repeated parallel lines (Bar width = 35 μm ; Hole width = 48 μm , Athene™, Agar Scientific Ltd., Cat. AGG2015) or a mesh (Bar width = 10 μm ; Hole Size = 50 μm , Athene™, Agar Scientific Ltd., Cat. AGG2004). After disc adhesion to the well bottom surface, a 100 mW UV beam was applied using the fabricated UV lamp. Beam intensity was measured using an optical power meter. The beam distance to the convex lens was adjusted to equal the TEM disc diameter (~3mm).

Once the exposure time had elapsed, the disc was carefully removed from the well surface to avoid disturbing the protein pattern, and the well was washed with sterile PBS, followed by the addition of a freshly dissociated neuron sample obtained after following the primary culture protocol for HuC-GCaMP6 zebrafish neurons. Cells were cultured in supplemented Neurobasal culture medium in optimal environmental conditions. The neural cultures were maintained in an incubator at $37^\circ\text{C} \pm 0.5^\circ\text{C}$, 5% CO_2 , and 95% humidity. After 24 hours, imaging was performed to assess neural survival and axonal process growth. Culture images were captured using the FrescoM imaging system using brightfield and fluorescent microscopy. Image analysis was performed using the FIJI CellCounter plugin to evaluate the presence of neurons with growing axons in the captured frames. A neuron presenting at least one neural process of 5 μm length was considered positive for axonal growth. At least five frames were counted from four independent cultures per condition, as indicated in each experiment.

Laminin Removal Using Laser Lithography

The laser-based lithography system, working as a galvo switcher UV, consisted of a confocal fluorescence microscope (Nikon A1 Confocal System™, Nikon©) with an incorporated laser (LU-N3 3-laser unit, Nikon©), operated at 1KHz to perform engraving procedures, and a FRAP/photobleaching device (Galvo Miniscanner™, Nikon©). The hardware was controlled by the NIS-Elements Advanced Research™ software (Nikon©). All used devices were part of the Wolfson Light Microscopy Facility of the University of Sheffield and operated by authorised staff from the facility.

Laminin coated culture wells from a 96-well plate were incubated overnight with a laminin solution, as previously described, to create an uniform laminin layer on the well surface. Two linear patterns were designed for engraving the protein coating: a pattern of repeated parallel lines (Bar width = 35 μm ; Hole width = 48 μm) and an enclosed chamber. The second consisted of a square area (100 x 50 μm), with sides referred to as “long side” and “short side” for explanatory purposes. The two short sides were continuous lines, and the two long sides

contained apertures of different sizes, one a completely open side and the other containing a 20 μm aperture in the centre. This smaller aperture was the entry to a 40 μm -long corridor with the same width as the aperture (see **Figure 5.28** for display).

Once the engraving was completed, the well was washed with sterile PBS, followed by the addition of a freshly dissociated sample of neurons, obtained after following the protocol of primary culture of HuC-GCamp6 zebrafish neurons. Cells were cultured in supplemented Neurobasal culture media, in optimal environmental cond. The neural cultures were maintained in an incubator at a temperature of $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$, 5% CO_2 and 95% humidity. After 24 hours, image capturing was performed to assess neural survival and growth of axonal processes. Culture images were captured using the FrescoM imaging system using brightfield and fluorescent microscopy. Image analysis was performed to evaluate the presence of neurons with growing axons using the FIJI CellCounter plugin to count the presence of neurons growing axons in the captured frames. A neuron presenting at least one neural process of 5 μm length was considered as positive for axonal growth. At least five frames were counted from a total of four independent cultures per condition, as indicated in each experiment.

Count of Neural Processes in Generated Neural Arrays

After cell positioning in protein coated dishes, UV, or laser preretreated dishes, generated neural arrays were grown in standard conditions for primary cultures and images were captured every 24 hours by both brightfield and fluorescence microscopy. Cells were classified as neurons depending on fluorescence expression in the soma and the presence of neural processes. Neural processes were counted using the FIJI CellCounter and measured using the NeuronJ plugin. A neuron was considered to have a neural process when it showed at least one neurite longer than 10 μm . A synaptic contact was considered positive if the distance of the observable end of a neural process was closer than 10 μm to any adjacent neuron.

Data Management

All FrescoM project files, including 3-D models on the structure of the FrescoM workstation, assembling structure, microcontroller firmware, software files and Python protocol scripts, were uploaded to an open Github repository (URL: github.com/frescolabs/FrescoM), created by Katunin et al. (2021). 3-D models files were created employing Autodesk Fusion™ (v.2604.1.48) and stored as .f3d and .stl files. A dedicated section was created in the repository to include the new components, updated software files and protocol scripts for the experimental procedures described in this project.

According to University of Sheffield policies, all images files, annotated pictures and raw data files from experiments have been uploaded to a dedicated data storage drive. All image analysis was performed using the ImageJ v.1.53t-based software FIJI (Schindelin et al., 2012). Culture captured images were stored as .png files. Numerical data on measurements were stored and used for further analysis as .csv files. In some cases, previous data management was performed using Microsoft Excel. All figures were generated in .png format.

Data analysis was performed in R (v4.1.1), using the RStudio integrated development environment (v.2021.09.0, Build 351), generating new data frames for statistical analysis values. For single comparison of two unique experimental conditions, a 2-tail Welch's unpaired T-test was performed. For multiple comparisons of more than 2 independent experimental conditions, a two-way ANOVA test was performed. In both cases, a $P < 0.05$ was considered a statistically significant difference between compared groups. Figures were generated using the ggplot2 package of R and RStudio, or using SigmaPlot (v15.0, © Inpixon).

Several figures and diagrams of this thesis were created using icons and templates from BioRender© software. A free-user account was created for this purpose.

V. RESULTS

Section 1:

Development and implementation of a functional module for single-cell manipulation procedures

Obstacles for the development of an efficient in vitro generation of neural circuits have been related to the lack of affordable technological tools that combine the execution of precise cell manipulations in cultured neurons, the constant monitoring of cells by microscopy and the versatility to adapt to particular experimental procedures. The FrescoM automated workstation, composed of a customizable modular structure, could solve these challenges. However, technical capabilities were limited to the maintenance and imaging of cultured cells, requiring additional components to also function as a cell manipulator, enabling physical interaction with individual cells present in the managed samples.

Section 1 of this thesis describes the development and testing of an automated workstation, based on the FrescoM workstation design, capable of combining the in vitro maintenance of cultured neurons, the visualisation in real time of cell samples by microscopy and the ability to manipulate cells in the culture as part of controlled procedures.

Particular objectives during this project stage were:

- To include a new functional module composed of a customised pipette capable of moving in any direction and handling small volumes of culture media at demand, to be installed as part of a modified design of the FrescoM automated workstation intended to function as a cell manipulation for cultured neurons.
- To test the performance of the functions of this pipette over extended operation periods and execute unsupervised experimental protocols, including precise positioning and image capturing by microscopy.

5.1 Overview of the Modified Design of the Workstation and the Developed Cell Manipulator

The present project introduced an example of functional module development for customisable workstations to solve current technical challenges required for a specific technical process involving cultured cells. As previously described, the *in vitro* generation of neural circuits has been possible by manipulating neurons from culture samples by physical means to form arrays with defined structures, followed by the induction of axonal growth and formation of synaptic connections between these neurons. The FrescoM workstation is a device capable of maintaining cell samples, performing live imaging, and applying solutions to cultured cell samples. However, functions related to physical cell manipulations have not been considered. For this reason, if the required components are developed, the FrescoM platform could surpass this unique step limitation and be converted into a suitable tool to handle individual cells, while monitoring progression by microscopy in real time. Besides, the tasks listed in the project aims could be automated to increase the performance and yields of the process while reducing time requirements and experimental variability. Altogether, the orchestrated events could be applied to generate a process for the *in vitro* production of neural circuits, thereby achieving the specific objectives of this project.

The proposed component arrangement for this modified version is displayed in **Figure 5.1**. The dimensions of the workstation, including all the components, were: 42 cm x 35 cm x 23 cm. Same as the original design of the FrescoM workstation (Katunin et al., 2021), the adapted workstation was composed of a metal frame assembled with aluminium Makerbeam™ extrusion bars, fixed together with aluminium L-shaped and T-shaped brackets and M3 screws. The frame served as a scaffold to place the rest of the components and as a stable support for the mobile parts. To minimise vibration, rubber pads were added to each of the four support legs and to the camera support. The central area of the frame was dedicated to holding a culture plate with the biological samples, requiring a dedicated holder fabricated in plastic (PLA) using 3D printing, to ensure its proper fixing. Fitting other components, such as motors and microscope parts, also required 3D-printed adapters. The displacement of mobile elements, including the culture plate, was achieved by custom linear actuators consisting of a NEMA™-17 stepper motor connected to a metal screw rod. The motor rotation was converted into linear displacement of the components requiring movement by running along steel MGH12 linear rails and using plastic 3D-printed adapters in forward or reverse direction, depending on motor rotation. One linear actuator was installed to control the horizontal and vertical displacements of the culture plate holder, through the X and Y axes, respectively, forming a main X-Y stage. A second mini X-Y stage, with two more linear actuators, was installed on top of the workstation, as described in detail later. The only exception was the movement on the Y-axis, initially used for moving a manifold. In the modified design used in this project, the manifold was replaced by a cell manipulator. Instead of a linear actuator, a push-pull solenoid was installed to improve displacement precision over short distances. Additionally, rounded plastic caps were incorporated at the pointy ends of metal parts (extrusion bars and screw rods) to increase user safety.

Next, an inverted epifluorescence microscope was installed in the bottom area of the workstation. The structural parts were assembled with Thorlabs™ aluminium tubes, while the optical components were: a non-infinity-corrected objective (Nikon Plan 20/0.4), a GFP cube consisting of blue and green Thorlabs filters (MF469-35 and MF525-39) and a dichroic mirror (MD498, Thorlabs). A FLIR Blackfly S® USB camera (BFS-U3-120S4C-CS, Teledyne™) placed at the end of the microscope tube was used to capture images at 1440 x 1080 resolution. Apart from the objective, the microscope components were fixed in the aluminium tubular assembly. To allow microscope focusing, the objective was moved along the Z-axis using a stepper motor as a linear actuator, controlling the focal distance until a clear image was achieved. The system was able to perform two types of microscopy using two different installed LED systems. On the one hand, a set of white LEDs, serially connected to a MOSFET (IRF520) and a 12V power supply, enabled bright-field microscopy. On the other hand, a blue LED, driven by a binary

ON/OFF pulse sent via a Thorlabs LED driver, produced an excitation beam at 488 nm, enabling fluorescence microscopy in this wavelength range. Imaging was performed by connecting the camera to a computer via a dedicated area in the FrescoM software to display the camera signal in real time, and by using previously developed functions in a Python 3 environment to adjust capture parameters, such as exposure time and gain.

Lastly, one more stepper motor and linear actuator were included in the cell manipulator workstation to control the plunger movement in the contained syringe. As mentioned, a push-pull solenoid controlled the Z-axis displacement of a mounted pipette in this module. The detailed structure and function of the incorporated cell manipulator functional module are described in the next section.

Summarising, to control component movement of the workstation components, six motors in total and one push-pull solenoid were installed for the following functions:

- Horizontal displacement of the well plate holder (X-axis)
- Vertical displacement of the well plate holder (Y- axis)
- Microscope focusing (Z- axis)
- Syringe plunger displacement (Y-axis)
- Horizontal displacement of the pipette (X-axis)
- Vertical displacement of the pipette (Y-axis)
- Push-pull displacement of the pipette (Z-axis)

Motor rotation was controlled by the FrescoM software platform using previously developed Python functions. Thus, a unique software for all workstation components, including those designed for this project, used the same electronic setup: CNC shields and a tailored PCB with input and output pins, an Arduino MEGA microcontroller for command processing and a user interface running Python functions. The software components, in turn, were unified within a Python environment that included all the required libraries and functions to execute commands, access variables, and customise protocols to perform tailored experimental procedures and even allow further modifications on the workstation. The constitution as an environment also allowed loading the software on any computer properly configured to run identical protocols once the pertinent files were installed.

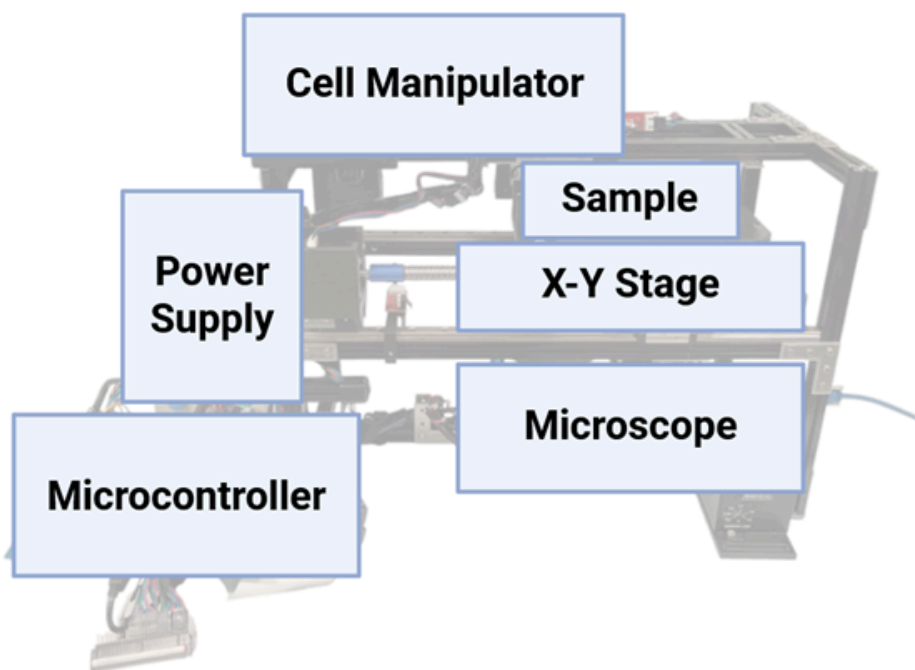
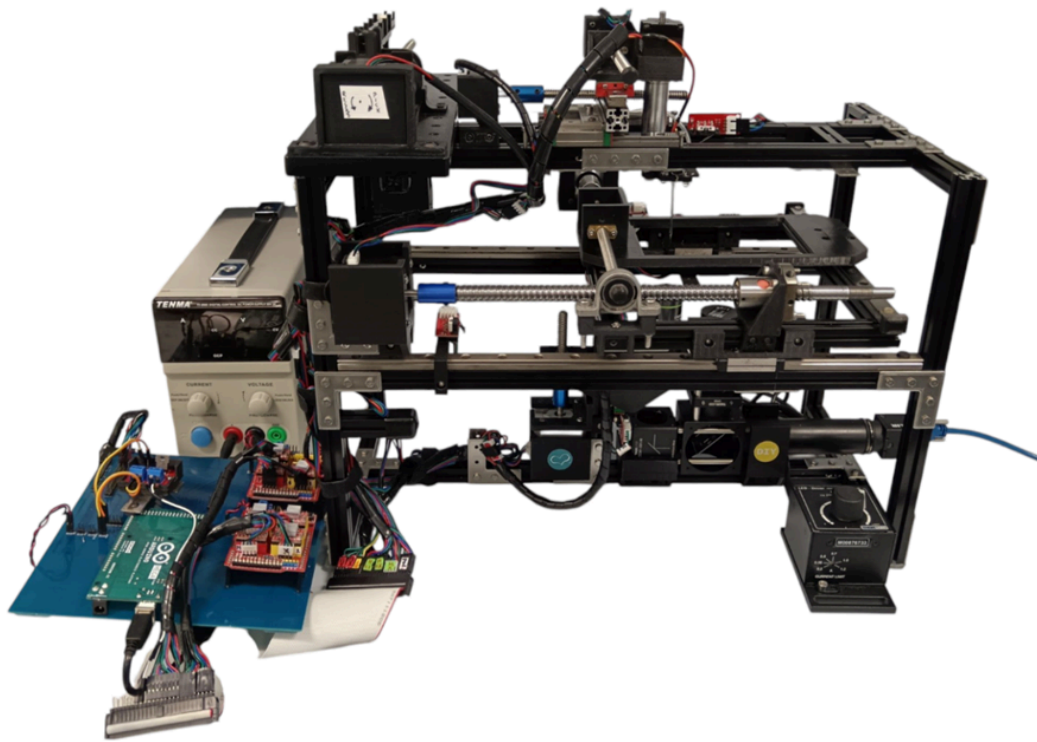


Figure 5.1 : Overview of the assembled FrescoM workstation, including the additional parts required for cell manipulations on cultured cells

The platform was mounted on a metal frame constructed using aluminium extrusion bars, including space in the centre for a stage to hold a culture plate with the cell samples. The stage was fixed through adapters to blocks running along metal linear guide rails. The stage moved in the X and Y axes by stepper motors. The developed cell manipulator was mounted on the top of the metal frame. This module was formed by a pipette placed in a holder and moved using an independent X-Y stage, also by motors. A push-pull linear solenoid controlled the Z-displacement of the pipette. The holder contained a glass pipette connected to a precision syringe, which applied pressure through a flexible plastic tubing system. An additional motor moved the syringe plunger and controlled the entry and release of cell-containing culture media inside the pipette. At the bottom of the metal frame, an epifluorescence microscope with a camera was installed to perform live imaging of the cultured cells. The components are operated by a microcontroller using scripts programmed in a Python-based environment.

5.2 Hardware Adjustments to the FrescoM Workstation

The initial stage of the project adapted the FrescoM workstation, developed by Dr Anton Nikolaev group at the School of Biosciences in the University of Sheffield, to extend the technical capacity of the hardware and software components to serve as an automated platform that combines the performance of cell culture maintenance, live imaging and individual cell manipulation on cultured cells, for the in vitro formation of neural circuits. This intended modification required incorporating additional components to perform the new function of manipulating individual cells from the maintained cultures. Hence, a micromanipulator was developed. The main components of the developed micromanipulator are depicted in **Figure 5.2**. In general terms, the developed micromanipulator consisted of a pulled glass pipette mounted vertically on a commercial pipette holder using a 3D-printed adapter. This adapter was designed to secure both parts in place during operation, while being detachable for pipette mounting and replacement. The pipette was connected to a precision syringe via a tubing system to control the entry and release of cell-containing media into the pipette. A set of fixed motors on the workstation scaffold enabled the movement of the pipette in the X, Y and Z directions. Finally, the pipette was aligned with the workstation microscope to visualise its function and displacement. In the following paragraphs, the rationale for choosing each part of the module is explained.

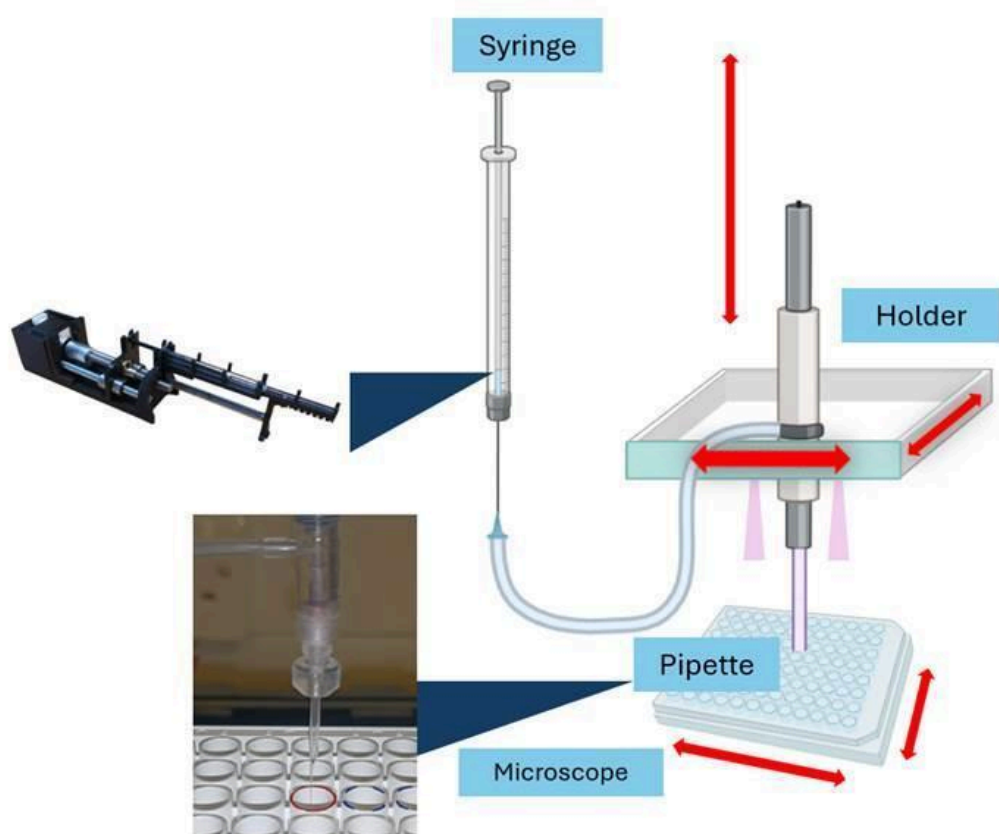


Figure 5.2: Schematics of the developed cell manipulator for cultured cell handling

A glass pipette was vertically mounted on a pipette holder and fixed in a mobile frame via a custom plastic adapter. This mini frame, placed at the top of the FrescoM workstation, was capable of moving through the X and Y axes along metal rails by the action of stepper motors. The Z-axis displacement of the pipette was achieved using an extensible push-pull solenoid. The content of the pipette was controlled by triggering a syringe connected to the pipette holder via a flexible tubing system. The pipette was illuminated by installing a set of LED lights in the plastic adapter. The culture plate containing the culture samples was moved by the previously installed main X-Y stage of the framework. The pipette was aligned with the workstation microscope to visualise its function and displacement.

The core part of the manipulator consists of a pulled glass capillary tube placed in a pipette holder. The selected capillary tube was a commercial borosilicate tube (1.5 O.D. x 0.86 I.D. x 10 cm) due to the easy availability to most research laboratories, reduced cost in case of replacement and the possibility to reduce the diameter of its cylindrical shape at one tip using commercial pipette pullers by means of heat application, as frequently done for Cell Biology applications such as microelectrodes fabrication. A Narishige PP-830 2-step pipette puller was used for this purpose (First pull = 79.8°C; Second pull = 24.8°C) to obtain a pipette diameter of approximately 50 μm (**Figure 5.3**). Size and tip integrity were confirmed by direct measurement using a microscope reticle. The end of the pipette was fire-polished with a microforge to eliminate sharp edges that could disrupt cells or the liquid flow during sample handling. Given that the average soma diameter of a brain neuron is around 15 μm , the selected size enabled this pipette to handle most neural types found in the brain. Because cultured cells were involved in subsequent procedures, these pipettes were sterilised by autoclaving before use to prevent contamination of the culture.

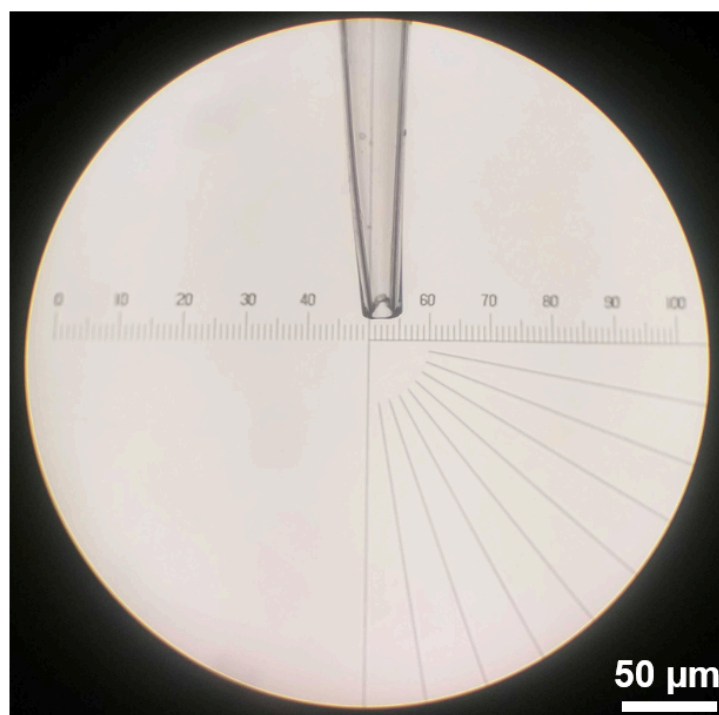


Figure 5.3: Size reference of the fabricated pipette for use in the cell manipulator

To fabricate a suitable pipette for the custom cell manipulator, a commercial borosilicate glass capillary tube (1.5 mm O.D.) was pulled using a 2-step pipette puller to reduce the tip diameter to 50 μm , followed by a fire polishing. After visualisation in a microscope reticle for size and integrity confirmation, the resulting pipette was then mounted in the pipette holder, firmly fixed to the micromanipulator arm. One division = 10 μm , Bar = 100 μm

Instead of direct pumping of the solution through the pipette, the solution handling was controlled by an air pressure control system. After the fabrication of a suitable pipette, this was mounted on an commercial electrophysiology pipette holder to obtain a larger air chamber to better control the pressure application parameters. Among the different types of electrode holders, the conceived cell manipulator required the controlled entry and exit of air in one port connected to the pipette, and the controlling of the air pressure, on the other by using a second port. After consulting the product catalogues of the main manufacturers, two models of pipette holders (MEH1RF from World Precision Instruments © and ESW-MxxP from Warner Instruments ©) were selected, based on the technical requirements compliance and price (**Figure 5.4**). Both pipette holders presented two ports in their main structure: one in the end for placing the glass pipette (Diameter = 1.5 mm), which also had a rubber o-ring for sealing, and another laterally placed for connecting a flexible plastic tubing (Diameter = 2.0 mm).

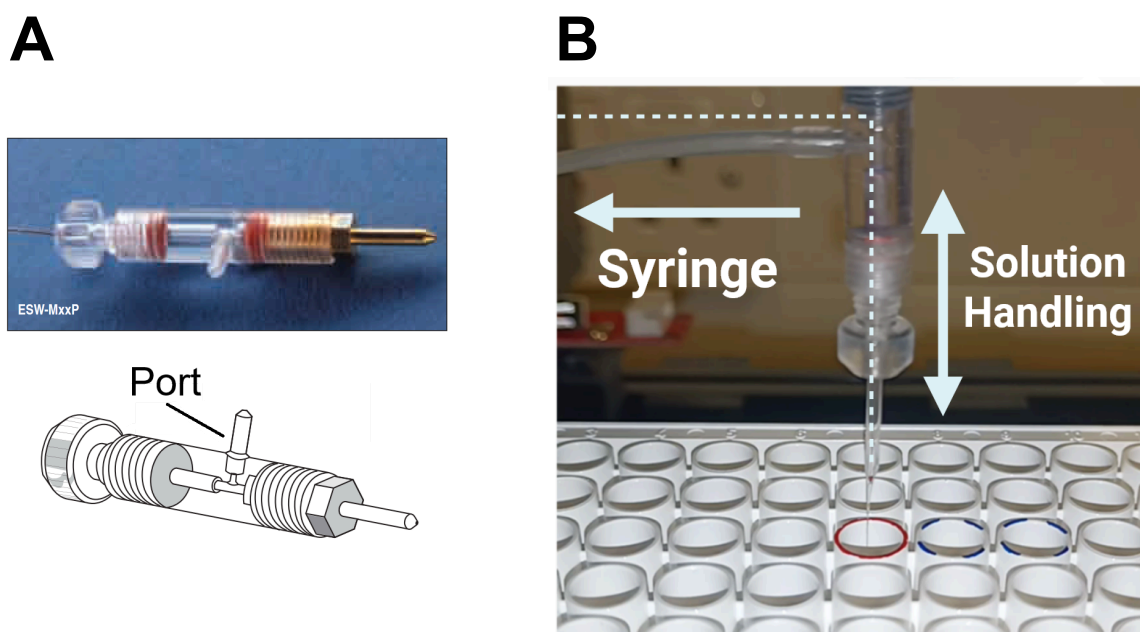


Figure 5.4: Pipette mounting for micromanipulator assembling

A. A commercial 1-port microelectrode holder was used to mount the fabricated glass pipette and to connect to the syringe and control the air pressure. MEH1RF (World Precision Instruments ©) and ESW-MxxP (Warner Instruments ©).

B. A syringe regulated the entry and removal of air to the inner chamber of the pipette holder via a tubing system. This air pressure change was transferred to the inner side of the pipette and then to the pipette tip. When the pipette tip was placed inside a liquid culture media sample, these changes resulted in the taking or removal of cell-containing media into the pipette. The syringe plunger movement was controlled using a stepper motor (shown in **Figure 5.5**).

The chosen manipulator type was based on standard cell manipulators, which use pressure-control systems to handle small volumes of culture media containing isolated cells in suspension. In the selected device, the pipette holder was directly connected to the glass pipette, and an installed tubing system led to a precision syringe, forming a hermetically closed system with variable internal air pressure. The inner pressure was controlled by a syringe, which produced positive or negative pressure. When the syringe plunger was moved backwards using a stepper motor, the resulting air cushion created a pressure drop within the pipette, drawing liquid culture media from the sample inside. To avoid the influence of liquid on the air system caused by filling the pipette or pipette holder with media, which would reduce the linear relationship between volume and pressure, the maximum volume of culture media handled was limited to 50 μL . On the other hand, when the media was released by moving the syringe plunger forward, it occurred before the pipette was empty, preventing bubbles in the pipette and the sample. As a result, the change in pressure by moving the syringe plunger can be used to fill or empty the pipette with cell-containing solutions.

The change in the air system volume, determined by the movement of the syringe plunger, was crucial to determine the applied pressure on the pipette and, consequently, the correct functioning of the cell manipulator. In the original design of the FrescoM platform, the movement could be refined by mounting a stepper motor on the syringe to move the plunger forward and backwards when the motor is activated, depending on the direction of rotation. The same principle was applied to the present cell manipulator. In contrast to the liquid volumes used in other experimental applications, such as cultures, which ranged from hundreds of microlitres to millilitres of media, the manipulations required for the present project were smaller. Thus, a refined version to control the syringe plunger displacement was required.

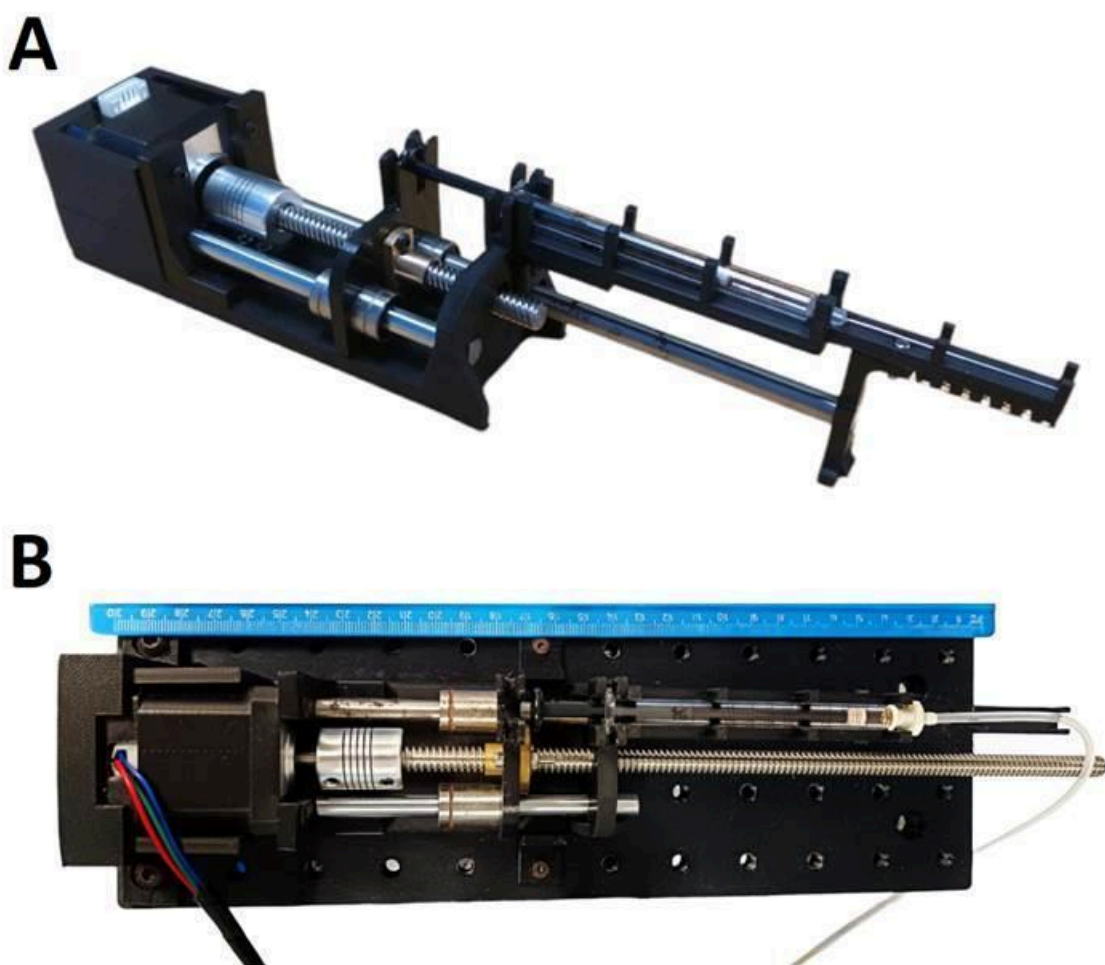


Figure 5.5: Developed syringe to control liquid handling in the cell manipulator

A. A 1000 μL glass precision syringe was horizontally mounted into a 3-D printed case. An incorporated stepper motor controlled the plunger displacement via a 3-D printer mobile adapter. This resulted in a pulling movement of the plunger when the motor was rotated clockwise and a pushing movement when rotated counterclockwise. The end of the syringe had a metal capillary with a Luer adapter, hermetically sealed and connected to a flexible plastic tubing to the input port of the pipette holder.

B. Top view, including size reference in cm. Rotation of the stepper motor was linked to a screw rod. An adaptor transformed this rotation into a linear displacement of the syringe plunger.

The resulting device is shown in **Figure 5.5**. It comprised a 500 μL Hamilton™ glass precision syringe (Hamilton 700 Microliter, 80600) with a metal plunger and glass inner barrel. This syringe type offered increased control to dispense specific volumes in a 0.5 μL to 500 μL range, as less friction exists when moving the plunger compared with its plastic counterparts. Also, the Microliter series was designed to translate the displacement distance of the plunger to the application of a specific volume, as a linear function. For these properties, precision syringes are frequently used for precision applications such as HPLC.

The syringe was placed in a specially designed case made using 3D printed plastic parts with some metal components to ensure fixing to the platform metal frame and proper control of its function. An horizontal position of the syringe was chosen to avoid any gravity effect during the plunger displacement and maintain a compact design of the entire workstation. In this matter, to ensure a constant holding of the pipette body in a horizontal position, a plastic enclosure case was tailored to the selected syringe size, including the needle, which, in addition, protected this delicate device from accidental shocks. The case rigidity was preserved by a fixed metal bar

running along the syringe axis as a backbone. As described earlier, the plunger movement was controlled by a NEMA™-17 motor placed at one end of the enclosure, in which the rotation was linked to the movement of a metal screw rod and a copper adapter by sliding the backbone metal bar. With this system, each motor step was reflected in a small and constant displacement of the syringe plunger, making it possible to define the distance and direction of pushing or pulling. Additionally, a plastic case was made for the motor to maintain a fixed position. The syringe components, including the motor and the plastic cases, were later fixed to an aluminium breadboard (Thorlabs Mb1030) to serve as a stable platform to avoid any movement of the parts and minimise vibrations during motor operation. This base, in turn, was fixed to the upper part of the workstation aluminum frame.

Finally, a mobile stage was installed to position the pipette freely during the intended cell manipulation procedures and to vertically align the pipette with the framework microscope to follow the course of the performed actions (**Figure 5.6**). This component substituted the manifold for solution dispensing in the original design of the workstation due to space constraints and the redundancy in function with the new manipulator, which can also be used to administer small volumes of solutions into the culture media when used as an injection system. Leading micromanipulator manufacturers agree that an ideal micromanipulator must prioritise stability through a solid construction to prevent any vibration or inaccurate movement of the device that can cause instrumental interference and, consequently, restrict any recorded measurement to the pure activity in the cell. Also, an ideal device must be compact to access the sample and the other experimental instruments, such as a microscope or electrodes (Molecular Devices, 2012).

Thus, an X-Y mini stage was mounted on the top of the aluminium scaffold of the workstation, which resembled the main stage to move the culture well-plate but at a smaller scale by installing shorter rails and using two motors (one for each axis) with reduced capacity according to the load size. This lower power was compensated with more precision for movements and liquid handling, while adapting the electronic components consumption to the original PCB power supply, preventing overcharging. The movement on the Z-axis was critical because an inexact movement of the pipette tip could harm the cultured cells, and also produce flow variations during the media handling due to an inaccurate distance to the dish surface. Hence, to achieve the movement of the pipette in the Z-axis, instead of a rail-based system, a push-pull solenoid was installed to increase the positioning precision further. This change in the displacement principle also prevented backlash when changing the direction of the movement, a concerning issue with motor-driven devices. Upon current application, the vertical movement device extended or retracted a 3D printed plastic adapter carrying the previously described pipette holder. This adapter also included a set of white LEDs to illuminate the pipette and the working area during the module work, as well as the microscope objective for imaging procedures. The frame also served to align the pipette with the microscope objective for imaging capturing during the cell manipulation procedure described in the next sections.

The mobile frame designed to displace the pipette was complemented by incorporating new functions in the Python-based interface (“tkinter” package) of the FrescoM software. An extended control panel, with additional buttons, was incorporated to control the syringe plunger displacement by the syringe motor activation and to direct the pipette displacement using the additional mobile frame described in the previous paragraph. Consequently, the operation of the described components using the Python user interface enabled a delicate and smooth movement of the installed micropipette in any direction when performing experimental procedures towards the cell samples as well as providing a simple but precise system to control the filling and release of the pipette content to work as a precise cell manipulator. The mobile frame also aligned the pipette with the microscope objective for imaging during these manipulation events.

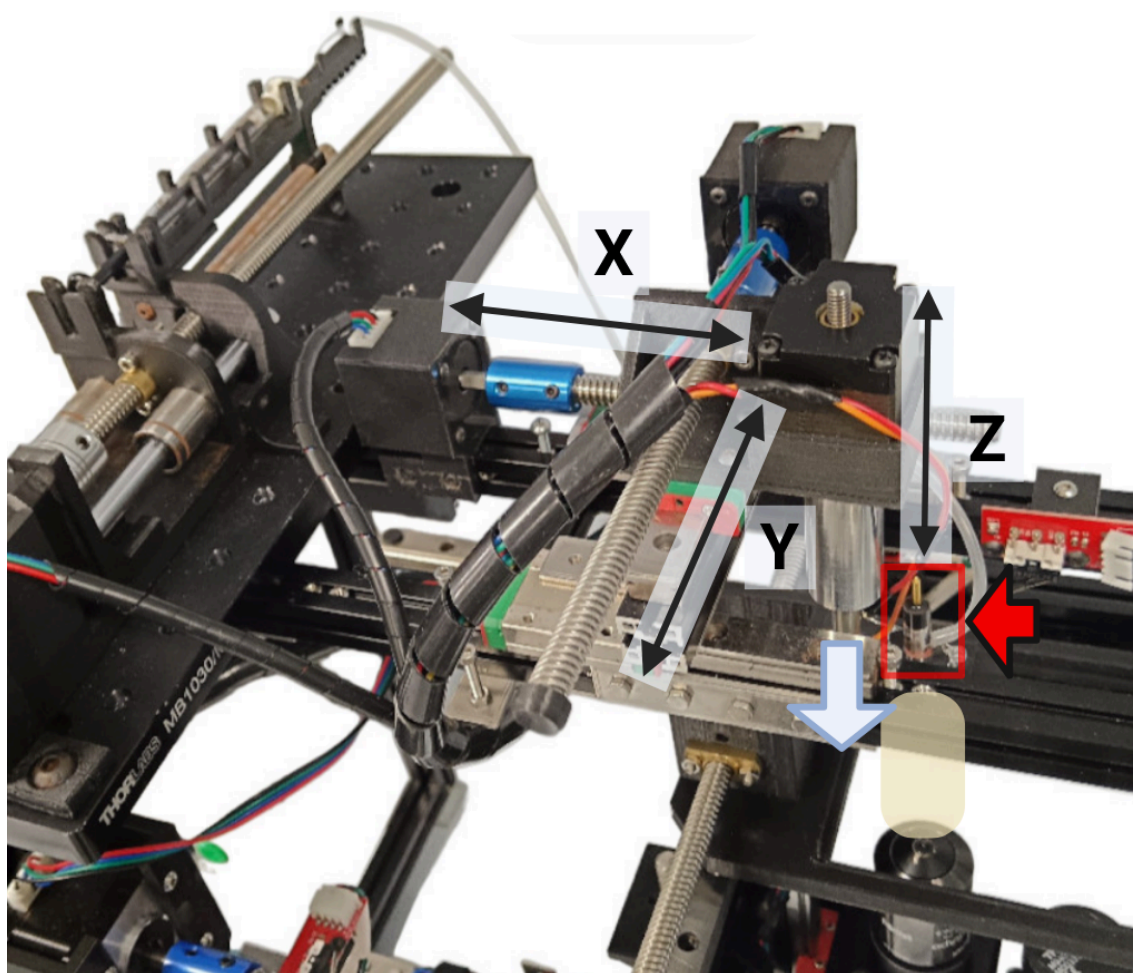


Figure 5.6: Overview of the developed mobile stage to control pipette positioning in the cell manipulator

A X-Y-Z mini-frame, replacing the solution dispensing manifold, was installed to move the micromanipulator. The frame consisted in an adapter for the pipette holder (red arrow) moved along rails for the X and Y axis, moved by individual stepper motors for each axis. The Z movement was controlled by the contraction of a push-pull solenoid to increase precision. The resulting mobile frame enabled a movement of the installed micropipette in any direction when performing experimental procedures towards the cell samples (blue arrow).

Besides, an optional function was included in the module to lean the pipette adopting a 45°-angle conformation, commonly used for cell injection. This manoeuvre also required horizontal adjustment with the microscope, which has now been added as a single-step function. However, apart from a few experiments related to the application of molecules in cell proximity to form gradients of molecules, described in later chapters, this pipette position was not widely used for this project, and the completely vertical position of the pipette was used for most procedures. It is worth noting that the 45°-angle conformation was not feasible to be used in 96-well plates due to the narrow space. Testing different culture plate options, convenient X-Y displacement of the pipette using this conformation was only achieved using 6-well plates and Petri dishes.

The whole design of the adapted automated workstation to perform cell manipulations in cultured cells, including mechanical and electrical components and the possibility to visualise isolated parts and interactions, has been uploaded as part of the FrescoM GitHub public repository in a subsection called “FrescoM Cell Manipulator” (URL: github.com/FrescoM/CellManipulator), as a series of Autodesk Fusion™ files. This shared drive complements the original FrescoM database. It includes free access to the STL files for 3D printing of the required plastic adapters, a full list of the components, as well as a descriptive tutorial to assemble the cell manipulator platform from scratch.

5.3 Software Adjustments to the FrescoM Workstation

The incorporation of additional parts required the corresponding modifications in the software for operating and coordinating with other functions during procedures. The open-source software enabled these changes by accessing the FrescoM code master, composed of Python (.py) files. The original version of the software included the required functions to control the displacement of the stepper motors of the X-Y stage, the microscope objective for image focusing and the retrieval of the manifold for solution dispensing. However, several software adjustments were required to enable a stable operation of the new components:

Firstly, from functions related to experimental protocols of cell maintenance and imaging, some parameters related to image capturing, camera settings, positioning of the culture plate, LED switching on/off and solution dispensing were adjusted to image quality in the cell samples (primary cultures of neurons) used in this project. The access to all these functions were maintained through the original FrescoM user interface, in the form of a window with buttons. The central area, displaying a real-time image from the camera, was kept.

Secondly, the X-Y-Z definition of the new cell manipulator module, which included the X-Y mini stage and the pipette push-pull solenoid, was included in the hardware definition of the workstation, in a new file named “manipulator.py”. Dedicated functions and classes were generated to control their functions and call them during protocol execution, based on the same commands previously used for the original manifold motors. As additional motors were connected to the same PCB, they also required their inclusion in the pin definition of the Arduino microcontroller. The values of some variables, such as the step size of the motors, were adjusted to operate within the working micrometre range, ensuring proper displacement according to the procedure scale. The step size of the push-pull solenoid was also adjusted to ensure a slow and smooth displacement when moving the pipette next to the cell samples, reducing the risk of accidentally breaking the pipette.

Thirdly, new software files and some predefined scripts required to perform automated and semi-automated experimental procedures, described in the following sections, were developed and incorporated in the Software section of the GitHub repository. The scripts were annotated in most of the individual steps to facilitate their understanding by other users, highlighting variable names to adapt the protocols to specific experimental conditions.

Finally, some modifications were made to the FrescoM user interface, running with the previously employed “tkinter” package. These additions included new buttons for controlling the new X-Y-Z stage and syringe motors, placed in a dedicated area of the interface window in a separated area (**Figure 5.7**). Also, some marks were affixed to the camera display frame to facilitate the positioning of the pipette in the centre of the working area. Finally, some minor changes, such as button colours and visual references, were added to the original version of the software to obtain an overall more friendly and intuitive interface for the user.

During the project, the potential use of an external hardware device, such as a buttoned joystick, to facilitate motor and functional operations was explored. However, this idea was discarded due to hardware recognition conflicts with the Python environment, the limited use on the Windows® operating system, and the lack of accessible commercial options with a suitable button layout for an intuitive operation during routine protocol runs.

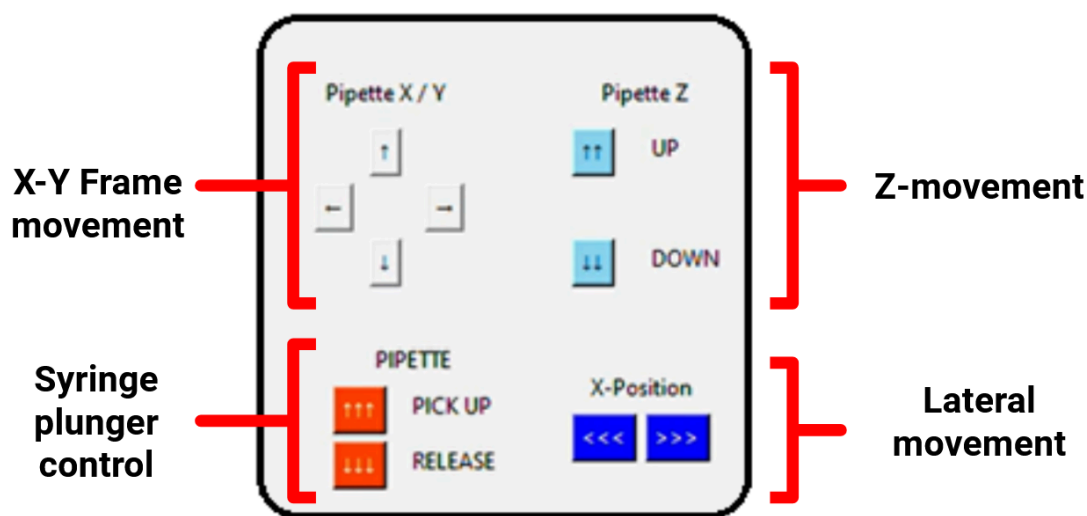


Figure 5.7: Incorporated UI in the FrescoM interface to control the cell manipulator

The user interface, based on the Python “tkinter” package, was updated to include new buttons that activate functions related to the cell manipulator, specifically controlling the additional motors to move the pipette and activate the syringe for solution handling.

5.4 Precision Tests on Pipette Displacement

Once the functional module was assembled and incorporated into the FrescoM workstation, relevant testing was performed to assess its functionality. The first assessments involved the capacity to perform repeated movements of the pipette with precision. That is, to ensure the pipette could move in the desired direction with the correct distance by activating the corresponding motor, while this output movement remained constant. For this purpose, an experimental protocol was developed to move the pipette to fixed distances and measure the tip position after displacement to evaluate whether the pipette returned to its original position or any deviation occurred. Due to the intended application of the device to handle individual neurons, whose soma sizes range from 10 to 20 μm , a variation of only a few micrometres when moving the tip of the pipette could result in an inability to reach the intended target. When translating these situations to cell manipulation procedures, a lack of precision would mean that the process could not start or proceed. Even more, an extended repetition of the same protocol would result in a progressive increase in the deviation from the target. In other words, an error accumulation would occur. A deviation caused by an imprecise mobile stage, which may include a flawed motor operation or an uneven displacement on the rails, should be reflected in a misposition over the same axis. This could be later improved by improving the moving mechanisms. However, a deviation in any other direction might be caused by a structural failure requiring a scaffold or adapter fixing.

The displacement of the mobile frame along the X and Y axes, caused by the operation of the corresponding stepper motors, was assessed first. For this purpose, a pipette of 50 μm inner diameter was mounted in the pipette holder as previously described and placed in a standard culture well filled with PBS to mimic operational conditions. A testing protocol was established, starting with positioning and focusing the pipette tip in the centre of the display area of the microscope. The initial position was annotated and then the pipette was moved along each axis by activating the corresponding stepper motor (X and Y axes) or the push-pull solenoid (Z axis) in the mini frame, and then returned to its initial position to recreate typical stage operation. Once this displacement was completed 50 times, the final position of the pipette was annotated again and measured to the initial position (**Figure 5.8**). Since this function was optimised initially to recognise large objects across the display area, such as cells in samples, the manual focusing of the small centred area corresponding to the pipette tip was preferred. In this position, when focused, the pipette tip was visible as a dark ring with a shadow produced by the illuminated pipette body. Any movement could be followed in real time in the camera display.

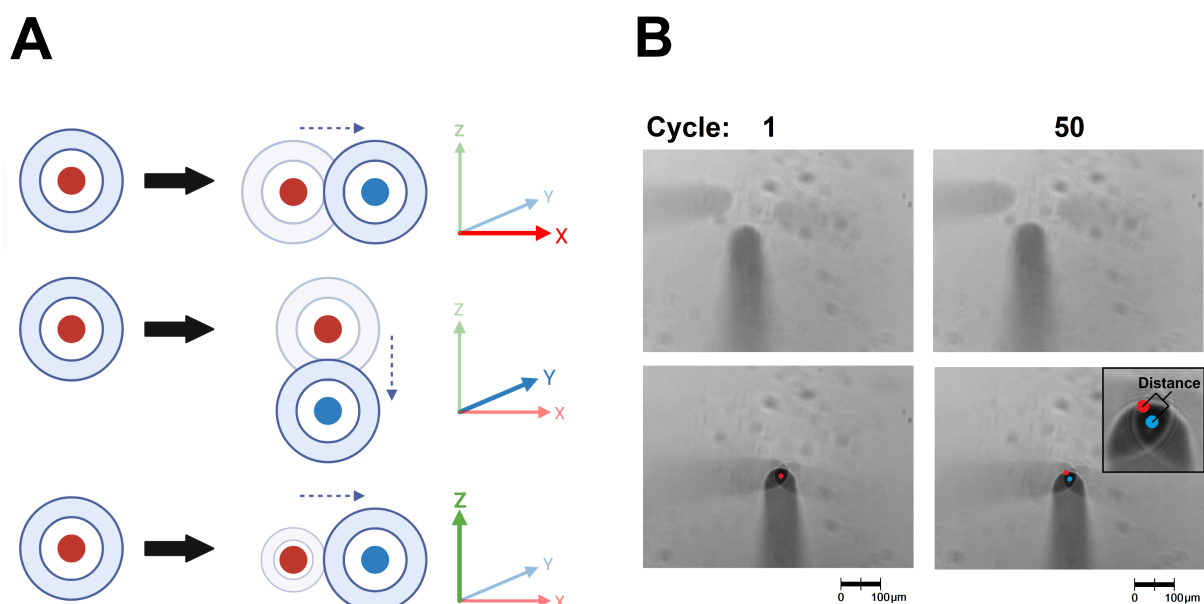


Figure 5.8: Precision assessment on the X, Y and Z axis pipette displacement over extended operation of the mobile stage

A. Pipette displacement testing consisted in a repeated movement of the pipette along each axis (X, Y and Z axis) to recreate typical stage operation. The initial and final position of the pipette were annotated, measuring the displacement as the distance between these points.

B. Images of the pipette moving along the Z-axis, from the first (left) and the last (right) displacement cycles. The initial (red) and final (blue) annotated positions of the pipette tip are indicated.

After positioning the pipette tip in the centre of the visual display, the protocol consisted of moving the pipette tip along a fixed distance of 300 μm in the current direction (left to right or up to down) in a total of 20 steps, each 15 μm in length. This distance is similar to the space between cells in dissociated cultures, described in the next section. After completing the path, the pipette was returned to the initial position in a single step, completing a cycle. A picture of the current display area was captured during each partial step to follow the pipette movement. Also, to recreate the movements of other components in the workstation that may affect pipette stability, such as an eventual structural malfunction, the position of the pipette was changed between sequences by moving the main XY frame to a random different position. The protocol completed a total of 50 repetitions per run. To test precision during the movement of the pipette, the captured picture at the beginning of the cycle was annotated using the software FIJI (v.1.53t, ImageJ) to indicate the initial position of the pipette tip. This position was then compared with the pipette tip centre at the end of the sequence, measuring the distance between these two points. The measurement was performed with all protocol repetitions and from six different experimental runs to account for possible variations among experiments, resulting in a total of 300 measurements per assessment.

The results obtained from the precision analysis, which determined the occurrence of a specific distance measurement within discrete intervals, during repeated movement of the pipette by operating the X, Y, and Z axes, are presented in the frequency density histograms shown in **Figure 5.9** for the Z axis and in **Figure 5.10** for the X and Y axes. A frequency density histogram was generated with the pipette displacement measurements to visualise the distribution of the observed variation (red line) arranged in discrete intervals. Median value was calculated (blue dashed line) in each case. It is pertinent to mention again that the mechanism for moving the pipette along the X and Y axes differs from that for the Z axis, employing stepper motors or a push-pull solenoid, respectively. Thus, a different behaviour on each displacement mechanism was expected.

From the measurements on each protocol, the median (Med) values were calculated as an indicator of non-symmetrical central tendency or typical values observed over extended repetition of the pipette displacement, as well as standard deviation (SD) to identify the variation on the expected position of the pipette. The calculated values for each axis were: Med=2.32 μ m, SD=1.041 for the X-axis; Med=4.08 μ m, SD=1.328 for the Y-axis; and Med=2.93 μ m, SD=2.105 for the Z-axis. All these observed values indicated a low variation among the measurements, and as a result, a pipette displacement that maintained a close distance to the expected position when operating the stage. Median values were chosen over Mean values because outlier measurements were a rare event that was mainly caused by the malfunctioning of one component of the workstation, which markedly differed from observed fluctuation through normal experimental runs. These isolated events occurred during pipette positioning in all three axes. However, the occurrence of these events was no more than 2% of the total number of recordings and, in all cases, the measured deviation values were less than 10 μ m. This distance was less than the average neural soma size or the intended distances between cells during manipulation procedures. Thus, even in the occurrence of these events, a manipulated cell would remain within reach of the pipette. Consequently, both systems for moving the mobile frame have been demonstrated to be precise enough to displace the pipette in the micrometre range and maintain this optimal performance during experimental protocols involving extended operation of the frame.

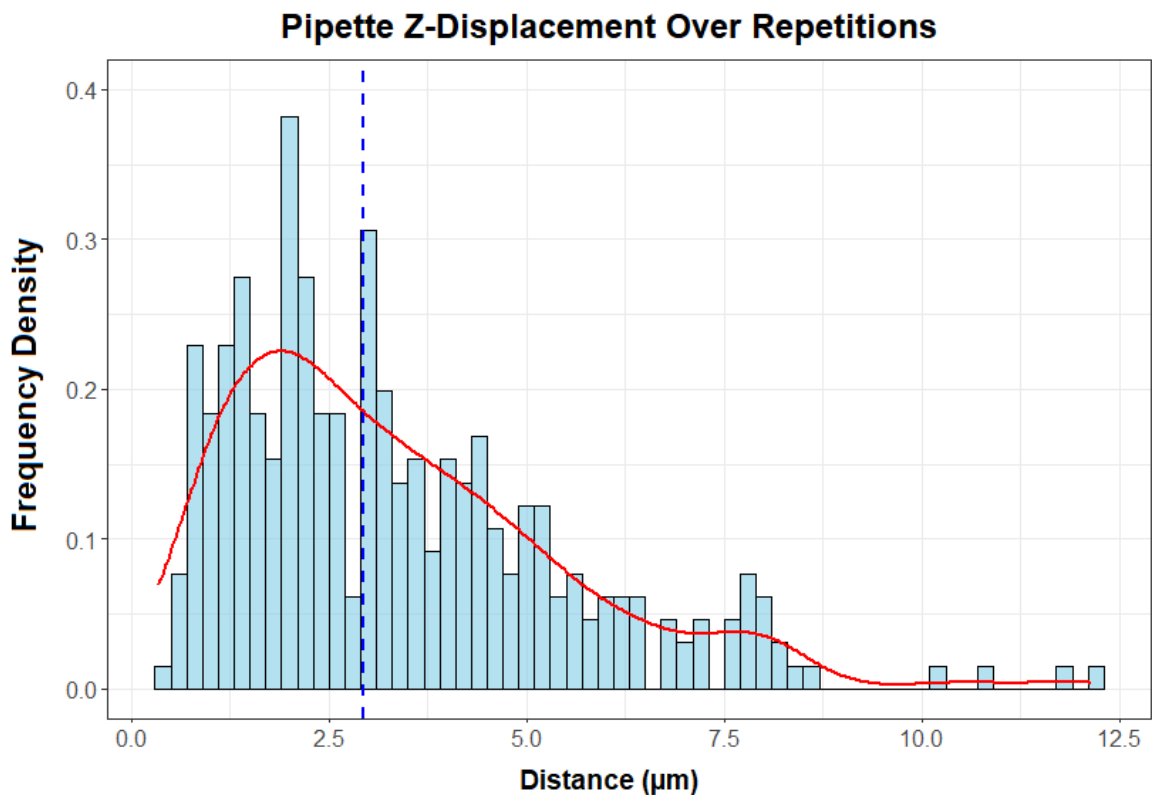


Figure 5.9: Precision of Z-displacement over repeated operation of the mobile stage

A protocol was developed to move the cell manipulator pipette in cycles to determine positioning deviations during operation of the push-pull solenoid for pipette displacement along the Z axis. The initial and final positions of the pipette tip were annotated in captured images. Then, the distance between these points were measured along repetitions, over extended experimental runs (n= 6 experimental rounds, 50 repetitions each). A density histogram was generated with measurements to visualise the distribution of the observed variation (red line). Median value was calculated (blue dashed line) to 2.93 μ m, which was minimal compared to the average neural soma size or the intended distances between cells during manipulation procedures.

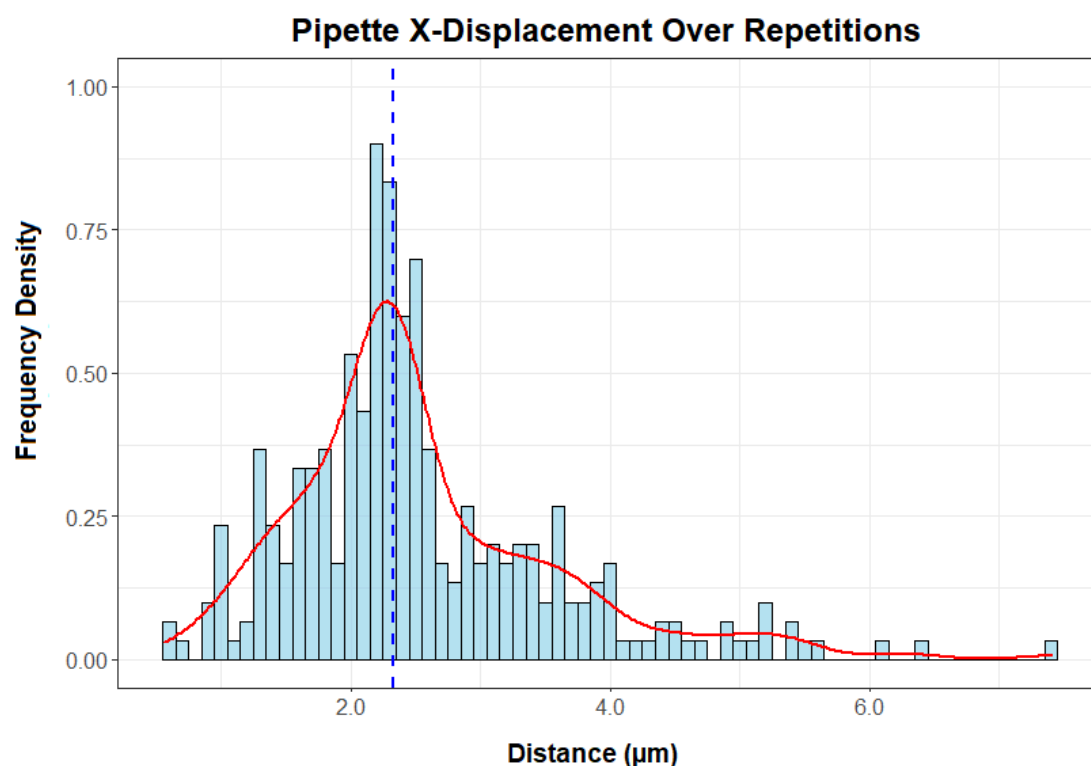
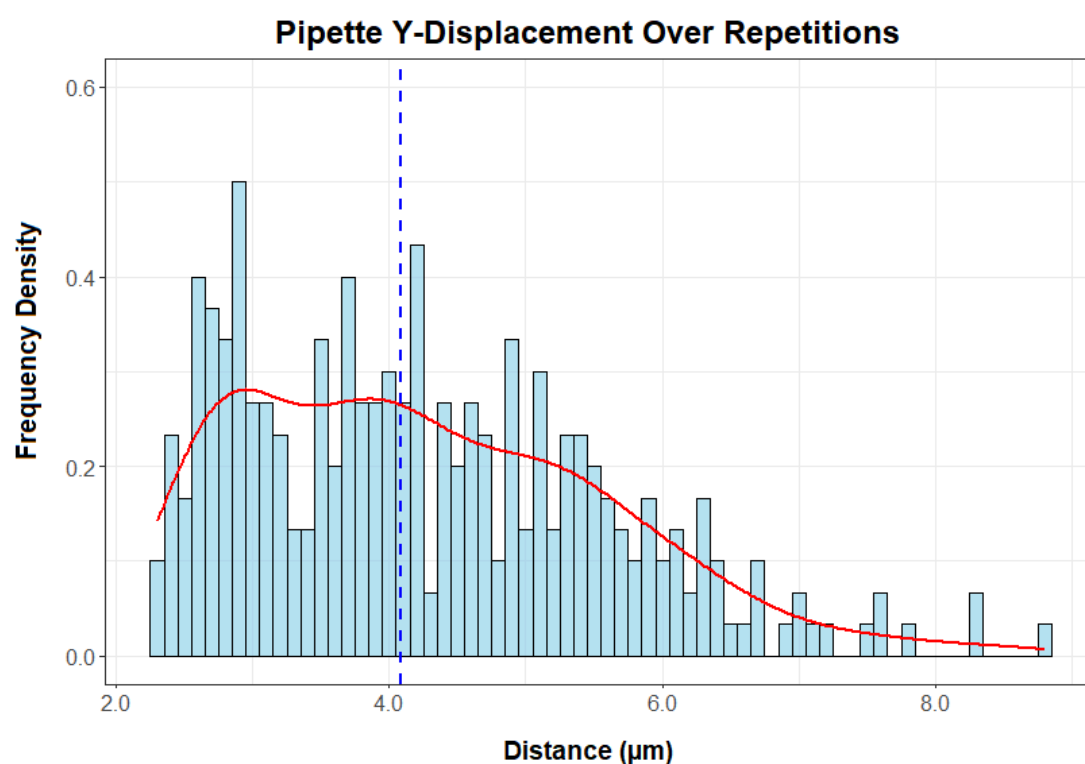
A**B**

Figure 5.10: Precision of the pipette on the X and Y axis over repeated operation of the mobile stage

A protocol was developed to move the cell manipulator pipette in cycles to determine positioning deviations during operation of the XY stage. The initial and final positions of the pipette tip were annotated in captured images. Then, the distance between these points were measured along repetitions, over extended experimental runs ($n=6$ experimental rounds, 50 repetitions each). Density histogram of the measurements on pipette displacement along the X (**A**) and Y (**B**) axis. Distribution (red line) is displayed after several repetitions and median value (blue dashed line) was calculated as $2.32\ \mu\text{m}$ for X, and $4.08\ \mu\text{m}$ for Y axis, with was both less than the average neural soma size or the intended distances between cells during manipulation procedures ($n=6$ experimental rounds per axis, 50 repetitions each).

The obtained values from displacement measurements revealed that both, the Z-axis push-pull solenoid and the XY-axes motorised stage, were capable of positioning the pipette with precision (**Figure 5.11**). The movement precision of the installed X-Y frame to control the micromanipulator movement was tested under variable operational parameters. Two different step sizes, representing long movements and fine positioning adjustment respectively, were independently compared during extended displacement of the frame. Also, two vertical positions of the pipette, with a vertical leaning of 0° (completely vertical) to 45° (used in future procedures of local administration of solutions) were compared to assess structural stability. In all cases, pipette positioning deviation after repeated operation was under 10 µm, which was considered acceptable compared to the average neural soma size of 15µm, or the intended 50 µm distance between cells during manipulation procedures in the next steps. Even the less frequent variations in the upper limits were within this size, which means that in a worst-case scenario, the pipette displacement was precise enough to be in the proximity of the target cell for procedures using the cell manipulator pipette. The median displacement was similar despite the different mechanisms to move the pipette in each axis, providing evidence that motors and pull-pull solenoids are reliable systems to move a pipette-based cell manipulator in the micrometre range. Besides, the displacement consistency was observed to be independent of the position on the culture plate, with similar behaviour when varying the well position used for the testing.

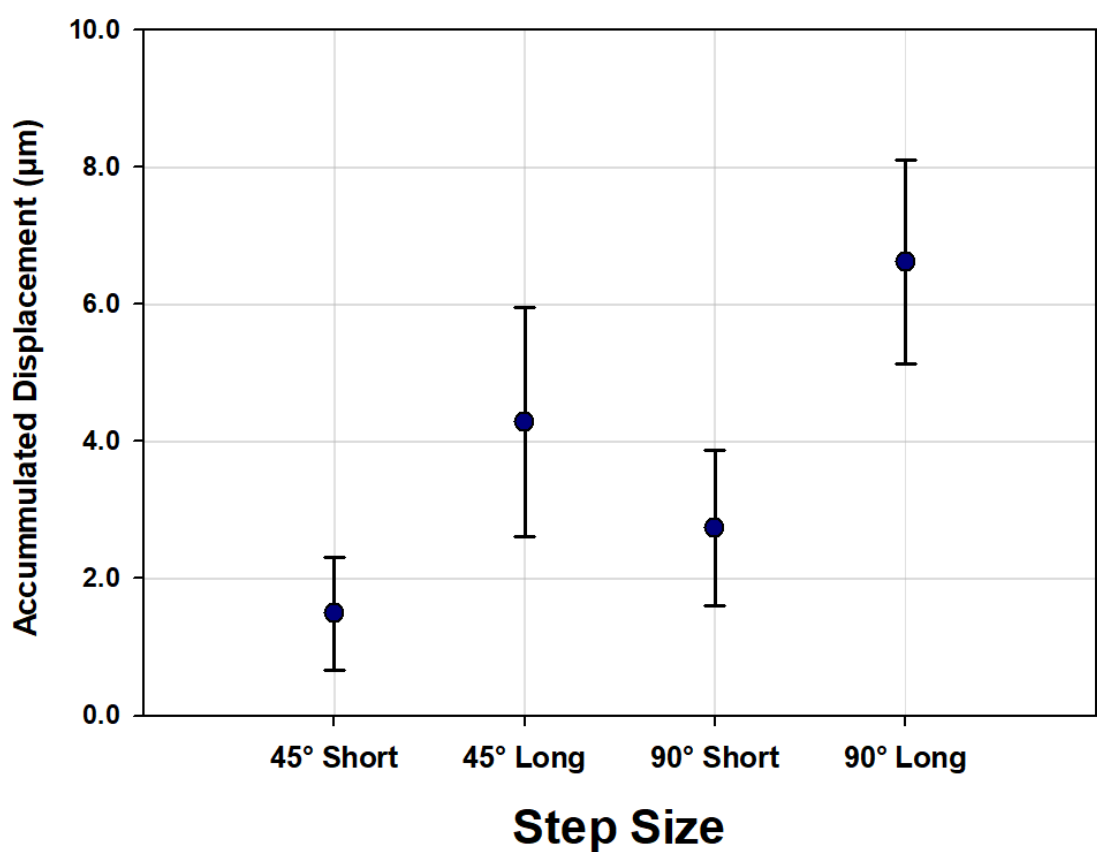


Figure 5.11: Effect of step size and pipette orientation on displacement deviation over extended operation of the XY mini-stage

Pipette displacement was measured after repeated movement of the pipette mounted in the XY-ministage in two different angles, to perform either cell manipulations or application of substances. Deviation was found within an acceptable range in all cases. (n=100 displacement repetitions)

On a next step, outputs regarding the improvements on the FrescoM workstation, which led to technical enhancement by the inclusion of a new function with all the involved components and operational software, maintained the compromise of data sharing with the scientific community. With this in mind, all the contributions from this stage of the project, including all the information on the components and assembly instructions of the incorporated mini stage, as well as the Python protocols to perform testing of the pipette displacement in all the X,Y and Z axes, were included in a dedicated folder for additional testing (URL: github.com/FrescoM/CellManipulator).

To conclude with Section 1 of this thesis, the main outputs can be listed as follows:

- A new functional module was developed for the FrescoM workstation, intended to manipulate cultured cells. This system was composed of an additional XYZ-stage, an injection system and a mounted pipette, capable of applying and retrieving small volumes of solutions in cell samples. Some modifications were made to the original design to install the required components in the workstation structure and control their operation.
- The precision of the developed cell manipulator was confirmed by testing variation degree during pipette positioning. The performance of the components during prolonged operation generated trustable outputs with minimal variations, to be applicable to a controlled positioning and navigation across cell samples with visual monitoring of the cell culture status in real time.

This section of the project provided a technical solution for a current restraint for the in vitro generation of neural circuits using an open-source platform with a customisable design. However, this progress only represented one of several missing aspects for an applicable process development. The possibility of the developed automated workstation to impact the ongoing efforts in the field, to create a consistent method for manipulation of cultured neurons, was not limited to technological obstacles related to the piece of equipment. Instead, the current landscape required solving other issues, such as the availability of suitable biological samples and the prosecution of known cell functions to desirable outputs, such as in this case, the controlled in vitro formation of neural arrays. This thesis also aimed to contribute to another crucial component of the building process, related to the generation of a consistent method to generate cultured neurons from zebrafish, which was the core part of Section 2.

Section 2:

Optimisation of Neural Primary Cultures from Zebrafish Brain

Once the hardware and software components required to perform cell manipulations were successfully developed, the next step towards the standardisation of an automated process to generate neural circuits in the laboratory was to obtain a suitable neural source for the experimental procedures, building neural arrays. As previously mentioned, artificial neural circuits can be generated from a variety of cell sources, including but not restricted to primary cultures and artificially differentiated neurons. For this project, primary cultures of neurons from zebrafish (*Danio rerio*) were used as the biological source of neurons due to the convenient advantages of this organism to obtain samples of cultured neurons with convenient features and scalable potential, including sample obtention in a reduced frame of time, a robust genetic repertory with powerful molecular markers and expression-based reporters and a simple visualisation of neural developmental processes.

Section 2 of this thesis describes some features of the organism and the advantages of using it as a convenient source of isolated neurons for in vitro maintenance, cell-based selection, and induction of process growth, leading to the formation of controlled synaptic connections and circuits with defined structure. Aiming at the consolidation of the neural circuit building in this project as a consistent process, an optimisation of the culture conditions of zebrafish neurons was required, searching for experimental conditions that favour the neural survival and neurite growth to be used in subsequent applications.

Particular objectives during this project stage were:

- To design a standardised protocol to consistently produce samples of high-density isolated neurons, from primary cultures of zebrafish embryos, capable of surviving for extended periods and growing neural processes in the presence of promoting molecules, to serve as a suitable cell source for the process of in vitro building of neural circuits.
- To select the most convenient conditions for cultures, in terms of cell number yields and process growth features, based on a repertoire of promoting molecules, including protein coating of the culture surface, culture media composition and the administration of different trophic factors.
- To compare the effect of embryonic age on the ability to generate samples of neurons with the aforementioned desired features.

5.5 Zebrafish As Model Organism for Experiments

Animal models have represented invaluable tools for scientific progress. Extensive observation and testing of biological events are required before formulating theoretical explanations of the constituting elements, interactions, and alterations under specific conditions. Even this information is continuously challenged with new advances as part of scientific progress. The use of animals in experimentation has filled a crucial gap, from laboratory bench work to solving scientific questions and generating tangible applications, such as developing treatments for diseases.

Due to the ethical issues and practical constraints of experimental testing in humans, many model organisms have been implemented, with their own advantages and limitations. Rodents, such as mice and rats, have been the most extensively used in Physiology and Medicine due to the close resemblance of their bodily functions to those of humans. However, depending on the research purpose, other organisms may be more suitable options. Among these, the zebrafish (*Danio rerio*) has emerged in recent decades as an appropriate animal model, particularly for studying body development, nerve function, behaviour, and the effects of genetic alterations (Teame et al., 2019). Some of the main advantages are displayed in **Figure 5.12**.

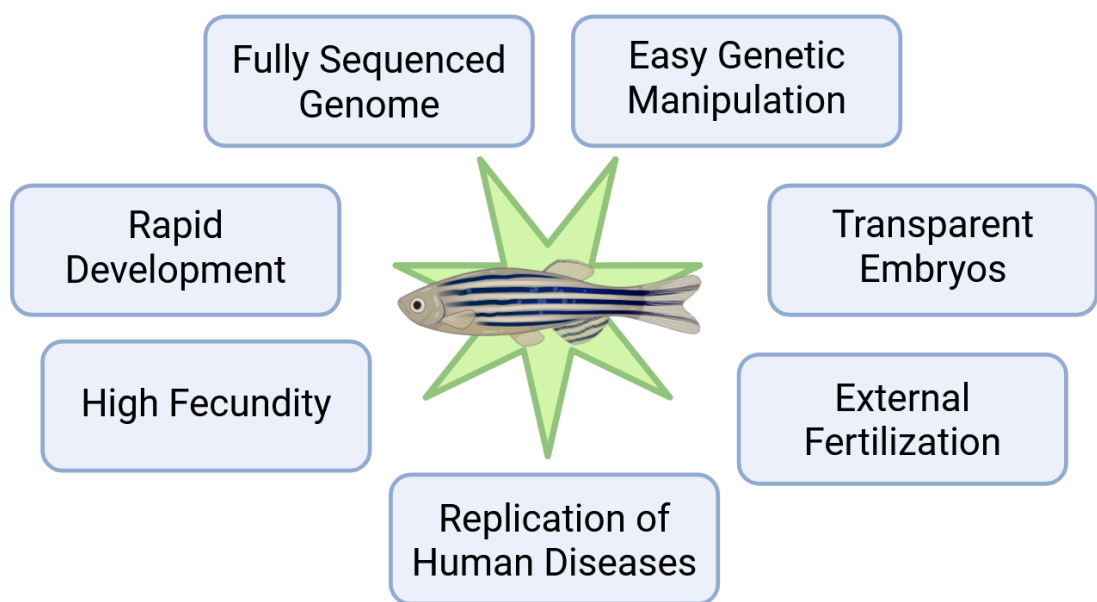


Figure 5.12: Advantages of using zebrafish (*D. rerio*) as an experimental animal model

Besides innate features in the organism that facilitate its handling, breeding and maintenance in research facilities, the deep understanding of the genetic background of the zebrafish and the directed generation of genetically modified individuals has positioned it as one of the main experimental organisms for studying body development, gene function and modeling of human diseases.

One of the more powerful tools extensively explored in zebrafish research is the generation of targeted genetic modifications, resulting from its fully sequenced genome, compatibility with recent gene editing tools, such as CRISPR/Cas9 (Hwang et al., 2013); and the possibility of selecting organisms with specific genetic profiles through generations by selective breeding in a reduced time frame. This combination has led to the generation of extensive libraries of genetically altered strains for numerous experimental applications (Choi et al., 2021). These include the replication of several human nerve diseases with most of the involved events during their onset and progression (Veldman & Lin, 2008). Additionally, the easier breeding and maintenance of zebrafish in dedicated facilities, compared to rodents and other animal models, is beneficial for procedures that require the use of large numbers of individuals, such as genetic screenings on large populations (Paige et al., 2014). Besides, embryonic samples require minimal tissue treatment for visualisation because of their external embryonic development and

the optical clarity of the embryos, which is almost translucent, making them an advantageous model for monitoring labelled molecules in lines expressing fluorescent reporters, even in live organisms (Holder & Xu, 1999). In this regard, a nomenclature to refer to the developmental stage of the fish, based on the post-fertilisation age (in days) of the embryo, has been established (i.e. 0 dpf, 1 dpf, 2 dpf, etc), which would be used for the rest of this project.

For these main reasons, genetically modified zebrafish with molecular markers, which allowed for the precise labelling of neurons and neural processes, were used as the selected animal model during this project and as the source of biological samples for the development of the intended process of neural culturing and the in vitro construction of neural circuits.

5.6 HuC-GCaMP6 Zebrafish Line

A zebrafish strain of special interest was HuC-GCaMP6, developed at the Bateson Centre for Disease Mechanisms at the University of Sheffield. The genetic modification in this strain comprises the incorporation of a two-component genetic construct into the genome, which enables the expression of a fluorescent protein in neural cells.

Firstly, the construct includes the promoter of the HuC gene, also known as ELAV-like RNA-binding protein 3 (HGNC:3314), which encodes an RNA-binding protein expressed during neurogenesis in early embryonic stages. Its expression is ubiquitous to neural progenitors and is maintained during differentiation processes in all neural subtypes. This converted HuC promoter into a trustworthy neural marker gene in embryonic and subsequent developmental stages when combined with fluorescent protein expression (Park et al., 2000). In other words, the use of the HuC promoter can be used to enable specific fluorescent protein expression in neurons (**Figure 5.13**). As part of this confirmation of identity, embryonic development of the local HuC-GCaMP6 zebrafish strain was observed from egg fertilisation to 5dpf to confirm previous observations on fluorescent protein distribution along the embryo bodies. As expected, the expression of the GFP protein was faintly visible within the first 24 hours from fertilisation, with no distinguishable structures in the beginning. The anatomical features of the brain and spinal cord appeared around 36 hours from fertilisation and were clearly defined from 2 dpf. At the same time, GFP fluorescence intensity reached its maximum in these structures, which was not uniform across the population. Thus, a quick visual inspection was performed to manually select the fish showing the most intense fluorescence for their use in experiments.

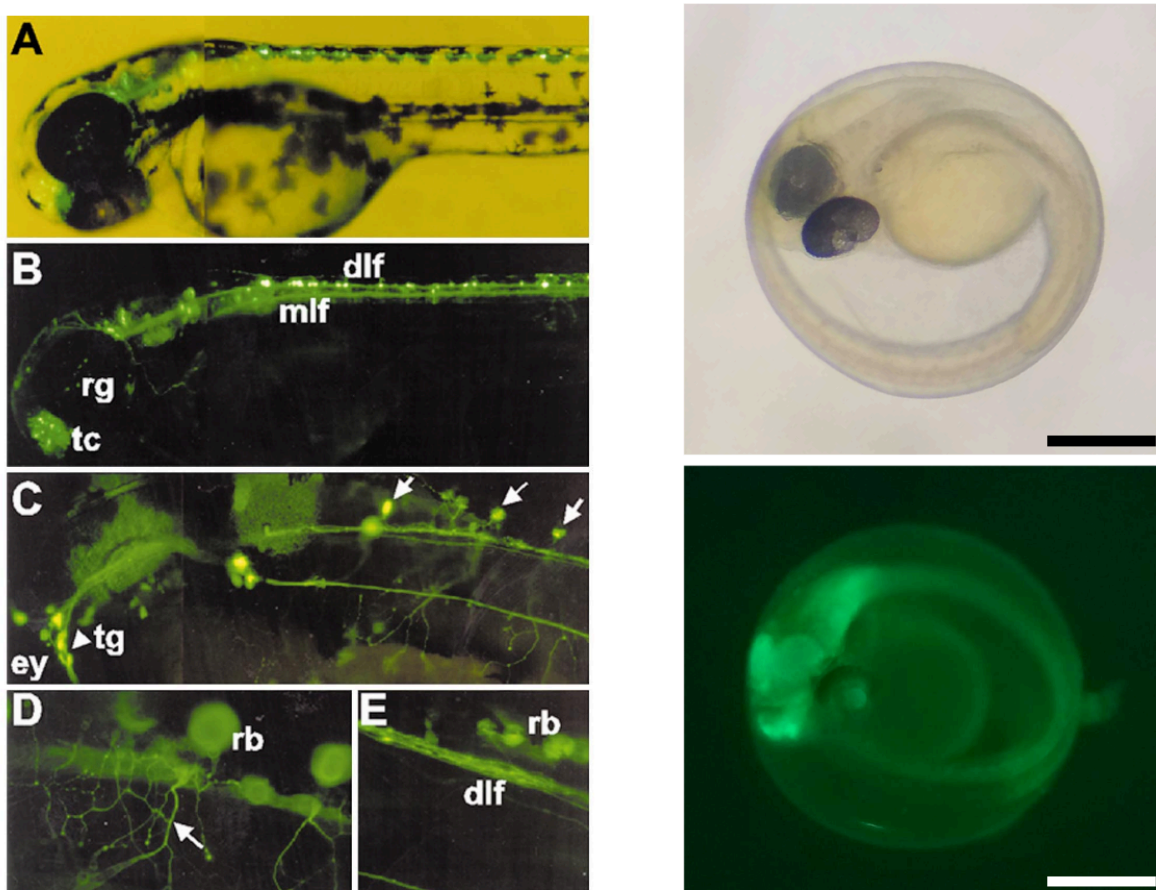


Figure 5.13: Neural specificity of the HuC promoter expression

HuC is a promoter ubiquitously expressed in neurons. In zebrafish, when combined with GFP expression, the HuC promoter can be used as a neural marker from early developmental stages to adult organisms.

Left: HuC-GFP distribution in zebrafish embryos from early developmental stages. Promoter expression is extended but limited to neurons, including soma, dendrites and axons (arrows) in all nerve structures (modified from Park et al., 2000). Abbreviations: dlf, dorsal longitudinal fasciculus; mlf, medial longitudinal fasciculus; rg, retinal ganglion; tc, telencephalic cluster; rb, Rohon-Beard neurons; tg, trigeminal ganglion; ey, eye; rb: Rohon-Beard neurons.

Right: Superimposition of a bright-field image on a fluorescence image of a 2 dpf embryo strain from the local HuC-GCaMP6 zebrafish strain used in this project. The same promoter expression distribution in nerve structures was conserved when using more recently developed fluorescent indicators, such as the GCaMP6 sensor. This strain is part of the Bateson Centre zebrafish collection, in the University of Sheffield (Bar = 0.2 mm).

Secondly, the HuC-GCaMP6 construct incorporates the GCaMP6 gene sequence. The product of this gene is a member of a novel family of highly sensitive calcium fluorophores, developed through directed structure-based mutagenesis for enhanced performance, and designed for use in calcium-mediated excitable cells, such as neurons (Chen et al., 2013, **Figure 5.14**). The non-invasive intracellular calcium monitoring achieved using the GCaMP6 sensor has allowed the detection of rapid calcium changes in neural somas and intracellular compartments, including dendritic spines, axons, and nerve endings, in response to action potentials and neural stimulation. As a result, the GCaM family of genetically encoded calcium indicators has become a valuable tool for studying neural activity in live cells. For this research project, the HuC-GCaMP6 strain of zebrafish was used to leverage the combination of the tools mentioned above in the employed cultured cells: a neural marker and a calcium sensor. Thus, the identity of cultured neurons could be confirmed to be selected by the cell manipulator and used to build in vitro neural circuits. In addition, the chosen neurons also possessed an internal tool to study their activity, as part of information processing events.

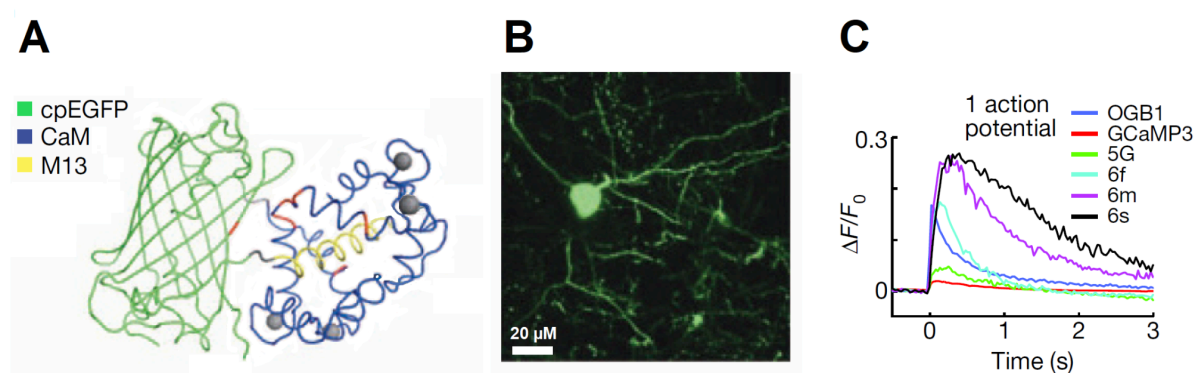


Figure 5.14: Use of GCaMP6 as calcium indicator for neurons

The genetically-encoded calcium indicator GCaMP6 represents a valuable tool for labelling neurons and monitoring rapid changes in intracellular calcium upon generation of action potentials. Calcium binding induces a conformational change in the protein that is reflected in an increased intensity of emitted fluorescence (Modified from Chen et al., 2013).

A. Molecular structure of GCaMP6, composed of a circularly permuted green fluorescent protein (cpEGFP), a calcium-binding protein calmodulin (CaM) and an M-13 peptide.

B. In vitro expression of GCaMP6 in transfected neurons, showing the indicator presence both in neural soma and dendritic processes.

C. Comparison of fluorescence intensity changes of GCaMP6 family with other related calcium indicators after the generation of one action potential in the monitored neurons.

5.7 Considerations for Zebrafish Primary Cultures of Neurons

Most previous experimental work on neural circuits has utilised primary cultures of mammalian cells. However, as explained earlier, other animal models have arisen with interesting advantages to facilitate and extend the scope of the experimental work. In this respect, zebrafish offer the possibility of studying nerve structures at the in vitro level through the production of primary cultures. This is achieved by a similar procedure of dissection, nerve tissue dissociation, and cell dissociation using mechanical or chemical agents (Russo et al., 2018). Along the main steps required for primary culture generation, to routinely obtain dissociated neurons with acceptable yields and cell integrity, suitable for representing a cell source for cultures, some adjustments were necessary to address the different features and cell composition of zebrafish tissues (Taylor & Houart, 2024). In contrast to mammalian primary cultures, which have been extensively used and refined to obtain and maintain isolated neurons, protocols related to zebrafish are limited. Previous protocols have included homogenisation of the whole animal to obtain extracts of a mix of diverse cell types (Kinikoglu et al., 2013) or the use of enzymatic dissociation reagents on the dissected brain (Patel et al., 2019). Those related to culture maintenance in automated workstations are currently nonexistent. Protocol optimisation is primarily achieved through algorithms that test a repertoire of parameters known to have a positive effect on cell growth, such as environmental factors or culture media composition (Zhou et al., 2023).

Therefore, some culture parameters were tested in the culture system used in this project, aiming to promote zebrafish as a valuable organism for obtaining neural primary cultures, especially in research involving gene targeting. Exploring the conditions that lead to better culture yields can position this model organism in current in vitro research trends, with a special interest in cell culture processes that require consistency and reproducibility during cell culture procedures.

5.8 Effect of Serum in the Culture Media

Firstly, the effect of serum on the culture media composition was tested. In the intact nerve tissue, an optimal environment for the growth and survival of neurons is achieved by molecules secreted by all the cell types present. Glial cells play a prominent role in producing molecules to control the spatial and temporal proliferation and differentiation events in neurons. Despite lacking a complete understanding of the molecular mechanisms involved in cell culture sources, the administration of external blood serum in the culture medium has been a standard method to cover any requirement that cultured cells may have. However, differences in serum requirements have been observed when culturing some cell lines, capable of growth and initiating morphological differentiation when culturing in media without serum (Qui et al., 1997). To determine the behaviour of primary cultures of neurons from HuC-GCaMP6 zebrafish, a comparison of cultures grown in the presence and absence of serum was performed. Based on standard protocols used for primary cultures in other organisms, dissociated neurons from 2 dpf embryonic brains were cultured in Neurobasal media, a standard for neural cultures, and the effect on neural survival was followed during several days. Serum concentration in the media was 10% of fetal bovine serum (FBS). Culture images were obtained by placing the culture dish in the modified FrescoM plate holder and using the incorporated microscope to capture images of the cells. Random frames were obtained from wells containing dissociated neurons grown in each condition, and the number of surviving neurons in the picture was counted using the “Cell Counter” plugin, part of the default plugin collection of FIJI software, from a total of six independent primary cultures.

Despite starting with a similar number of cells in both conditions, after 1 day of growth, a decrease of 40% in the number of surviving cells was observed in the culture, from an average of 40 cells/frame to a final count of 24 cells/frame when the serum was present or absent, respectively (**Figure 5.15**). This cell number decrease persisted during the experiment, reaching a 47% decrease in counted cells at day 2, and 49% at day 3.

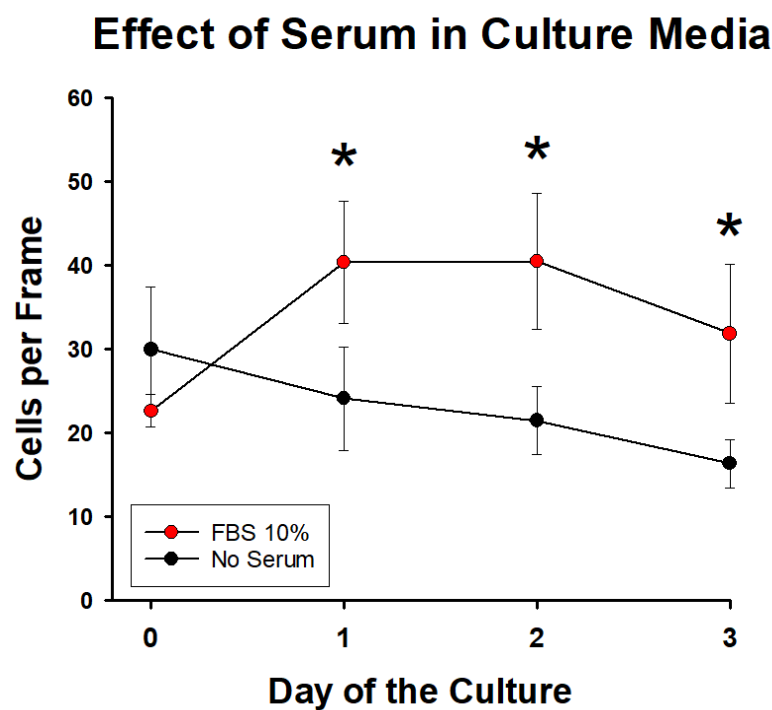


Figure 5.15: Effect of serum in culture media on zebrafish primary cultures of neurons

Dissociated neurons from embryonic brains were dissociated and cultured over several days in neurobasal media with or without FBS 10%. Culture pictures were obtained every 24 hrs and surviving cells were counted. The average number of cells per day was calculated and compared between both conditions. A positive effect of FBS on cell survival was observed, as the number of neurons was maintained until the end of the experiment (n= 6 cultures; * , Statistical difference of FBS use compared with media with no serum after performing a two-tailed unpaired T-test in each group and $p<0.05$)

5.9 Effect of Neurotrophins and Poly-Lysine Matrix on Neural Survival

Secondly, the effects of combining other molecules relevant to neural survival with observed effects in different culture systems were studied, aiming to increase further neural maintenance and growth in the present culture system. From special interest was the impact of a family of growth factors known as neurotrophins, known for orchestrating developmental processes, survival, and signalling events in the nervous system (reviewed by McPhee et al., 2020) by regulating axonal and dendritic growth, formation and maintenance of synaptic connections, neurotransmitter release, and events of synaptic plasticity (Gómez-Palacio-Schjetnan & Escobar, 2013). Neurotrophins act mainly through the activation of tyrosine kinase-type receptors, with different specificity and affinity for each neurotrophin, with variable effects depending on the receptor activation patterns. With this in mind, the following experimental culture conditions tested the effect of two remarkable members of the neurotrophin family: nerve growth factor (NGF) and brain-derived growth factor (BDNF), administered separately and together in 2 dpf zebrafish neurons cultured in Neurobasal media. Neurotrophins were used at concentrations at which promotion of neural survival and growth of processes has been observed in primary cultures of neurons from other organisms (100 ng/mL and 150 ng/mL, respectively). The culture media included FBS 10% in all conditions. Also, culture plates were previously coated with poly-L-lysine to reproduce the extracellular matrix, a critical requirement for the in vitro growth of cells. Culture images were obtained by placing the culture dish in the workstation plate holder and using the incorporated microscope to capture images of the cells at random positions of the culture well. Four frames were taken in each condition, and the number of surviving neurons in the picture was counted using the “Cell Counter” FIJI plugin. A total of five independent primary cultures were included, and values were evaluated using a one-way ANOVA analysis for differences in the effect of each growth condition.

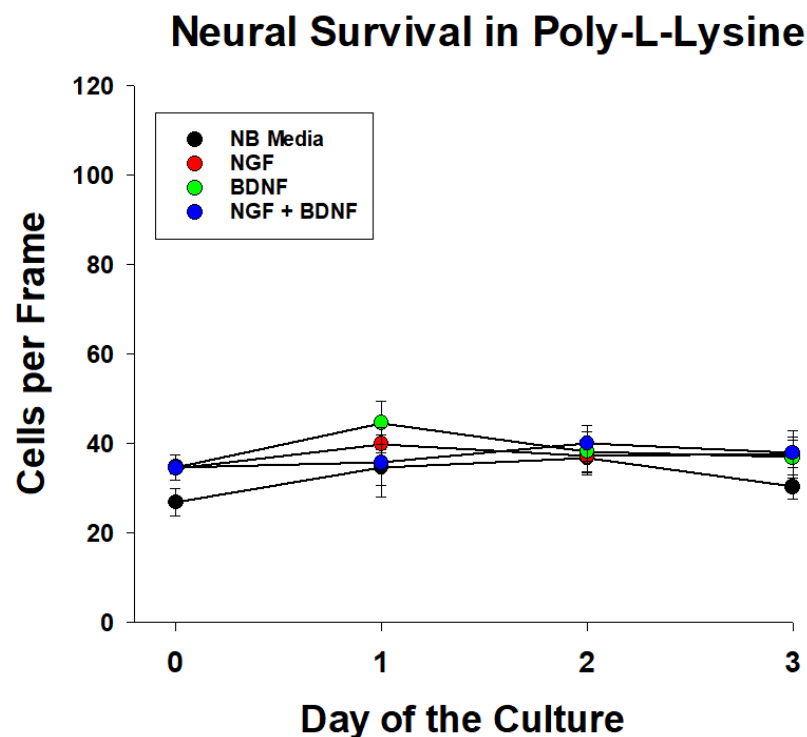


Figure 5.16: Effect of neurotrophins on neural survival in zebrafish primary cultures of neurons using poly-L-lysine coated culture dishes

Primary cultures of 2dpf embryonic zebrafish neurons were grown in the presence of NGF (100ng/mL), BDNF (150ng/mL) or a combination of both neurotrophins in supplemented neurobasal media, in poly-L-lysine-coated culture dishes. Cultures were maintained in an incubator for 3 days, and surviving neurons were counted each day. No effect of neurotrophins on neural survival, expressed in surviving cells per imaged captured frame, was observed after 3 days of culture (n = 5 independent cultures, 4 frames of each culture; * : Difference vs. NB media, after a one-way ANOVA test and $p < 0.05$)

Starting with a similar number of cells in all conditions, no effect on cell survival was found on zebrafish neurons cultured in wells coated with poly-L-lysine and exposed to NGF and BDNF (**Figure 5.16**). After 3 days of culture, the cell number was maintained in a similar cell number range in all conditions (mean cell count per day: Day 0 = 32.6 cells/frame; Day 1 = 38.7 cells/frame; Day 2 = 38.1 cells/frame; Day 3 = 35.7 cells/frame). After performing a one-way ANOVA test to compare the cell count values between the media without and containing neurotrophins, no significant change was observed. Neither any tendency to increase nor decrease in the number of neurons was detected in any condition. Therefore, no isolated or combined effect of neurotrophins on neural survival was observed in this culture system.

As mentioned earlier, in vitro cultured neurons require the presence of a protein matrix in the growth surface to recreate extracellular matrix, including the natural electrostatic interactions of cytoskeleton-related molecules to promote cell adhesion, maintain cell shape and control growth processes (Widge et al., 2007). In the case of neurons, the function of this external matrix also includes the guidance of neural extensions, such as axons, and participates in long-distance processes, as in the case of axonal pathfinding to reach distant targets (Koh et al., 2008). Aiming to find growth condition variants that improve culture yields, the protein coating of the dishes was modified.

5.10 Effect of Neurotrophins and Laminin Matrix on Neural Survival

The neurotrophin effect testing was repeated, replacing the poly-L-lysine in the culture dishes with laminin. Instead of a synthetic polymer, laminin is a glycoprotein naturally found in tissues that play mechanical support roles by interacting with the cell surface and other extracellular matrix proteins (Lander, 1987). Laminin-coated dishes have been preferred in neural cultures that required a sustained growth of axons and dendrites, such as nerve regeneration studies (Manthorpe et al., 1982), since laminin alone is sufficient to promote axonal growth and even potentiate the action of trophic factors such as NGF (Collins & Lee, 1984). Therefore, the next experimental primary cultures tested the effect of NGF (100 ng/mL) and BDNF (150 ng/mL), administered separately and in combination, in 2 dpf zebrafish neurons cultured in Neurobasal media with FBS 10% in all conditions. Again, culture images were obtained by positioning the culture dish in the workstation plate holder and employing the incorporated microscope to capture images of the cells at random positions of the culture well. Four frames were taken in each condition, where the number of surviving neurons in the picture was similarly determined using the “Cell Counter” FIJI plugin. A total of five independent primary cultures were included.

Some differences in neural survival resulted from this protein coating change in the culture surface coating, shown in **Figure 5.17**. After an initial cell attachment period, an early increase in the number of counted cells was found in all conditions, compared with the cultures using poly-L-lysine, rising from an average of 32.6 cells/frame to 84.8 cells/frame attached per captured frame. A different effect of neurotrophins was observed in each condition as the culture progressed. Neuron numbers in cultures grown in Neurobasal media without neurotrophins, as well as containing BDNF alone, progressively decreased over the 3-day testing period. However, cell count in neural cultures containing NGF and both tested neurotrophins was effectively maintained until the last recorded day, and significantly greater than the less effective conditions, as determined by a one-way ANOVA test comparing cell count values from each at each day when neurotrophins were present or absent in the used culture media.

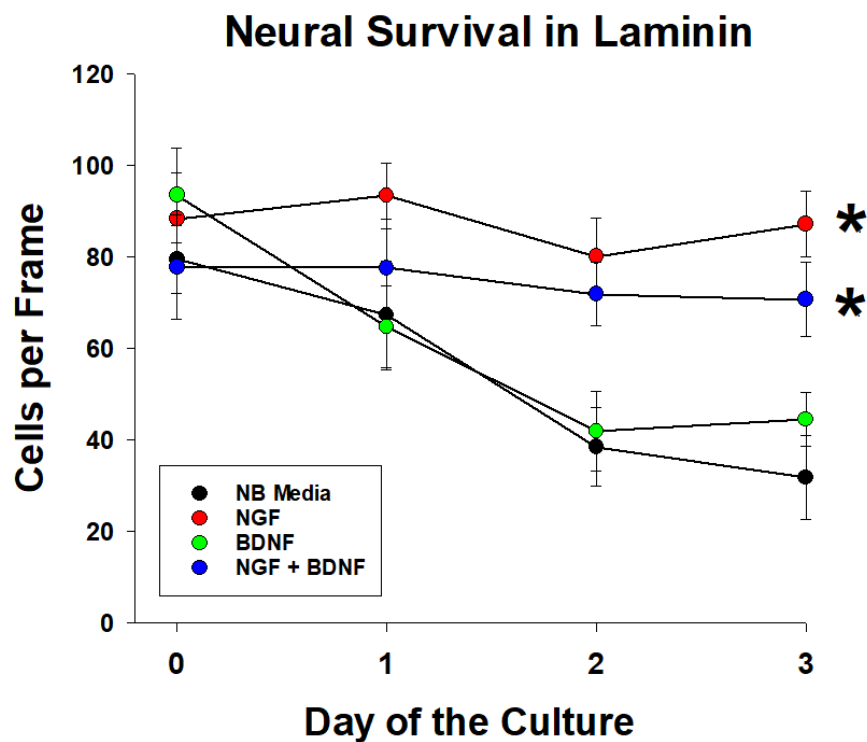


Figure 5.17: Effect of neurotrophins on neural survival in zebrafish primary cultures of neurons using laminin-coated culture dishes

Primary cultures of 2dpf embryonic zebrafish neurons were grown in the presence of NGF (100ng/mL), BDNF (150ng/mL) or a combination of both neurotrophins in supplemented neurobasal media, in laminin-coated culture dishes. Cultures were maintained in an incubator for 3 days, and surviving neurons were counted each day.

An increased number of initially attached neurons in the dishes was observed, compared with poly-L-lysine. After 3 days, the number of surviving neurons decreased when no neurotrophins or BDNF alone was present in the culture media. When NGF was present alone or in combination with BDNF, cultures maintained neural numbers during the experiment duration (n = 5 independent cultures, 4 frames of each culture;* : Difference vs. NB media, after a one-way ANOVA test and $p < 0.05$)

5.11 Effect of Protein Coating on Processes Growth

Subsequent testing aimed to expand the obtained information about the positive effect of poly-lysine and laminin on increasing the yields of cultured zebrafish neurons over extended periods by analysing the growth of neural processes, another critical feature relevant to cultured neurons. Since the ultimate objective of the project was the development of a reproducible method to in vitro build neural circuits, obtaining parameters which could be used to induce axonal growth to connect cultured neurons effectively was critical. In the initial culture system, adapted from a literature protocol, neural processes in cultured rodent neurons were visible in some neurons from as early as 3 hours after placing the cells in poly-L-lysine-coated dishes. During the following hours, after cell attachment and growth in optimal environmental conditions, axonal growth could be observed. The number and length of processes reached a maximum in the first, which progressively decreased as neural integrity decayed with time.

For this project, the previous observations in rodent primary neural cultures were tested in zebrafish neurons, focusing on the presence of neural processes and their length over an experimental time frame of 3 days. For this, primary cultures of 2-dpf zebrafish neurons were prepared. Dissociated neurons were placed in culture dishes coated with poly-L-lysine, laminin and without any coating as a reference. Every 24 hours after placing the cells, the culture plate was situated in the workstation holder, and four pictures were captured at random positions, using the brightfield microscope incorporated in the automated workstation. The epifluorescence microscope could also be considered to capture HuC-GCaMP6 neurons, leveraging the

expressed fluorescent marker distributed through neural soma and processes. However, lighting issues in the culture room, unrelated to the culture, made the imaging sessions and proper cell identification difficult. All neurons were counted for the first time using the “Cell Counter” FIJI plugin to obtain the total number of neurons in the image frame. Then, to determine the presence of neural processes, all extrusions coming from cultured cells were measured using the Neuron J (v.1.53t) plugin of FIJI software (Meijering et al., 2004), a free tool which performs a semiautomated delineation tracing in cell images with soma extensions, such as neurons, and transforms the trajectory of the processes into a linear object to measure in standard units (μm). A thin soma extension with a length of 5 μm , corresponding to half of the average size of a neural soma, was the established criterion to serve as a threshold to include a given neuron into the count of neurons with processes. This type of extension was distinguishable when observed with the brightfield microscope and was considered less likely to be an extension of the neural soma. Neurons that met the selection criteria were counted a second time using the “Cell Counter” FIJI plugin. It is worth noting that neural processes were present in the form of axons and dendrites. Still, identifying a given neural process within any of these groups in isolated neurons was complicated without using cell or molecular markers, which were not considered at this moment. For this reason, the counting procedure indistinguishably enclosed both types of neural processes in the cultured neurons. Besides, neurons showing more than one process were counted only once, even if more than one of them met the length threshold.

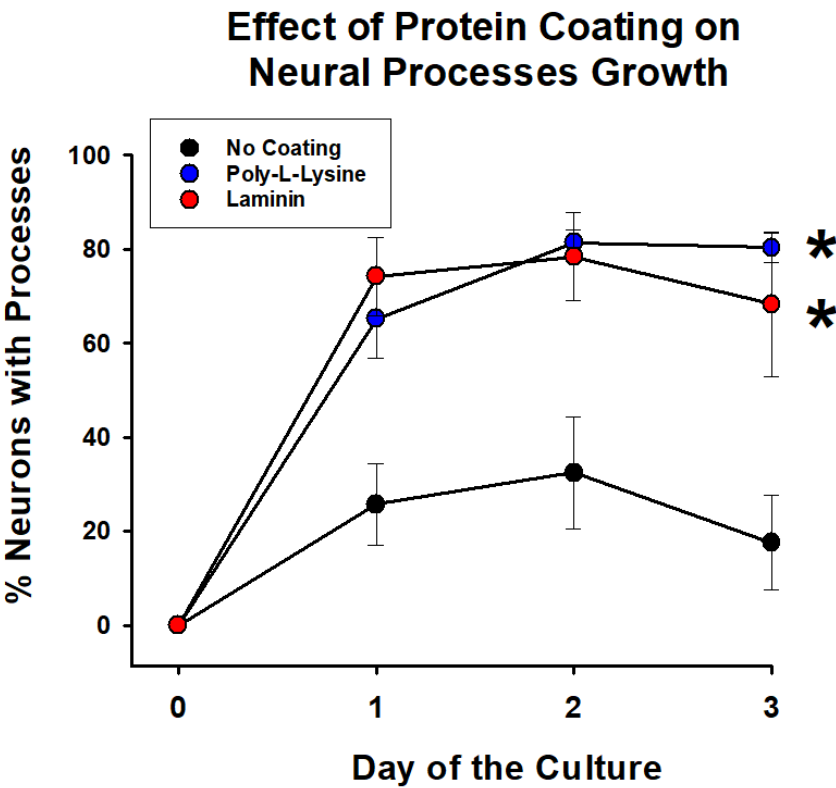


Figure 5.18: Effect of protein coating on processes growth in cultured neurons

Primary cultures of 2 dpf zebrafish neurons were grown using two different protein coatings to find optimal parameters favouring axonal growth. Dissociated neurons were cultured in poly-L-Lysine and laminin-coated wells. Every 24 hrs, the neurons with processes were counted and the percentage of the total number of cells was calculated for each group.

Protein coating was necessary to allow optimal process growth in cultured neurons. After 3 days of culture, when the protein coating was absent, the proportion of neurons with growing processes was markedly reduced (17%). Most neurons grown in poly-L-lysine or laminin developed neural processes (80.3% and 68.3%, respectively), with no difference in the proportion between these two groups (n = 4 independent cultures, 4 frames of each culture; Error bars, SEM; *, Statistical difference comparing each protein coating to control [no coating] after performing a two-tailed unpaired T-test and $p < 0.05$).

The growth proportion, expressed as the percentage of neurons with at least one neural process of 5 μm length, from the total number of neurons in the image frame, was followed during 3 days. Results are displayed in **Figure 5.18**, following in parallel the effect of each protein coating variant. Four frames were taken in each condition, from four independent cultures. In all conditions, growth of processes was detected after 24 hours of culture, even in neurons growing without a protein matrix (25.6% of total neurons) and with a greater proportion when a poly-L-lysine or laminin coating was used (65.2% and 74.2% of total neurons, respectively). The tendency was maintained during the experimental period, with process growth observed in most neurons growing in poly-L-lysine or laminin-coated dishes, with average growth proportions of 80.3% and 68.3%, respectively, from the total of neurons in the culture. These values were found to be statistically significant compared with the absence of a protein coating, in both conditions. These observations confirmed the *in vitro* requirement in zebrafish neurons of a protein coating on the culture surface to provide mechanical stiffness and permit cell attachment and growth, extensively described in rodent primary cultures of neurons. The proportion values in both types of protein coating remained similar during the 3-day duration of the cultures.

Intending to achieve an effective method to *in vitro* connect neurons in circuits, culture conditions that permit the growth of long axons were of particular interest. The ability to connect neurons positioned at longer distances could enhance neural array designs. With this objective, a deeper analysis focused on the measurement of process length was performed in the previous image frames. As mentioned, process length was measured using the Neuron J (v.1.53t) plugin of FIJI software, which performs a semiautomated delineation tracing followed by a linear measurement of the neural processes. The same exclusion criteria were used, considering only processes longer than 5 μm coming from a neural soma into the analysis. No upper limit was established, although some processes that extended beyond the boundaries of the image frame were cut. In the case of neurons growing multiple processes, only the longest one was considered. Again, for this testing, the classification of processes as axons or dendrites was omitted due to the lack of a reliable cell marker. Mean length values were obtained from the cells present in four captured image frames from each culture at a given experimental day. The comparison was repeated in four independent cultures.

Measurements on the process length are displayed in **Figure 5.19**. In contrast with the similar proportion of neurons growing axons, marked differences were found in process length between conditions, depending on the protein used as coating. After 24 hours of culture, the absence of protein coating on the culture dish surface produced only very short neural processes in the cells (mean = 14.6 μm), which decreased in length during the following days until being barely above the minimum length threshold at day 3 (6.37 μm). This supported the general idea of a neural dependence on the extracellular matrix to allow axonal extension. Conversely, zebrafish neurons grown in protein-coated dishes extended long processes from the first hours. A maximum length was observed around 24 hours, when axons grew a length equivalent to several times the soma diameter. Some neurons presented more than one growing process, but this event was not analysed at the moment. Process length was greater in laminin cultures than in poly-L-lysine cultures in all the analysed days, as a statistically significant difference was found after comparing measured length values from both groups every day. The mean length of processes was 91.1 μm and 161.1 μm for poly-L-lysine and laminin, respectively. Despite the spontaneous removal of some axons during the following days, which resulted in a decrease in average length, the observed neural processes mainly remained unaltered during the experiment. The initial difference between conditions was also maintained through this period. Average process length at day 3 was 68.8 μm and 147.9 μm for poly-L-lysine and laminin, respectively. It is relevant to highlight that this growth was enough for some neurons to make physical contact with adjacent neurons and randomly form synapses and circuits. Because protein was forming a uniform layer in the well bottom, no preference in growth direction in neurons was observed.

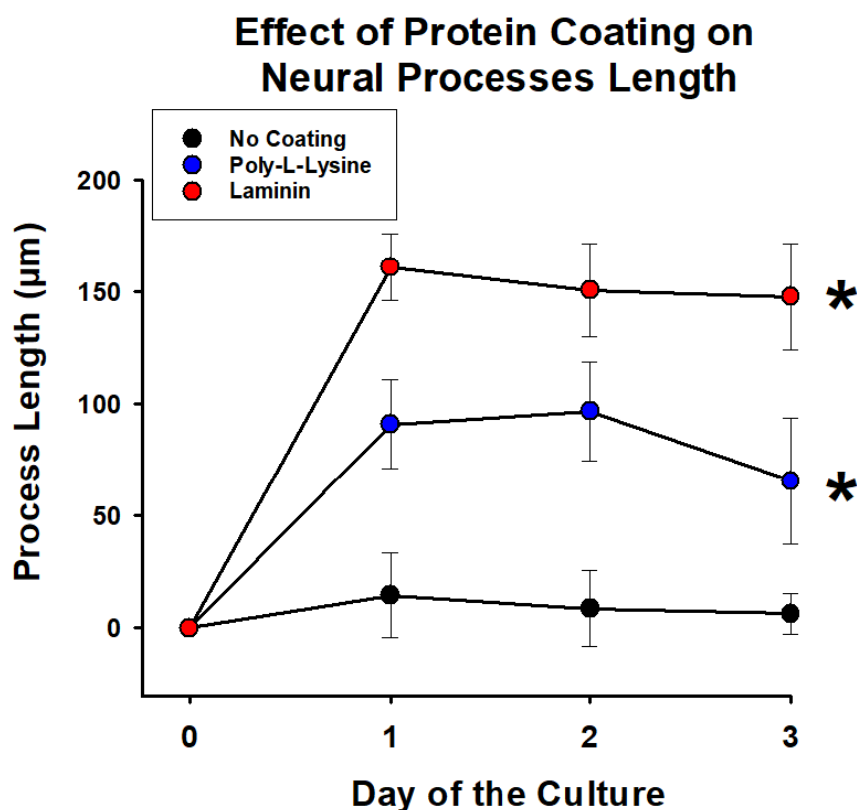


Figure 5.19: Effect of protein coating on processes length in cultured neurons

Primary cultures of 2 dpf zebrafish neurons were grown using two different protein coatings to find optimal parameters favouring axonal extension. Dissociated neurons were cultured in poly-L-Lysine and laminin-coated wells. Every 24 hrs, the process length in cells showing growing processes was measured, and means were calculated for each group.

A protein coating was necessary to allow process growth in cultured zebrafish neurons. The process extension reached a maximum within the first 24 hrs, with a minor decrease during the experimental period. After 3 days, when the protein coating was absent, the axonal length was markedly reduced (mean = 4.3 µm). The process extension of neurons grown in laminin was longer (mean = 147.9 µm) than that of neurons grown in poly-L-lysine (mean = 65.7 µm). The effect of both protein coatings was significantly greater compared with dishes without coating (n = 4 independent cultures, 4 frames of each culture; Error bars, SEM; *, Statistical difference comparing each protein coating to control [no coating] after performing a two-tailed unpaired T-test and $p < 0.05$).

5.12 Effect of Zebrafish Embryonic Stage on Neural Survival

The following stage exploring culture parameters for zebrafish neurons investigated the differences related to the developmental stage of the animals used for brain obtention. Zebrafish is an organism among vertebrates with a rapid embryonic development of the nervous system, with observable changes regarding the formation and maturation of the neural tube, and neural structures. Cell differentiation into neural-line progenitors and cell migration occur immediately after egg fertilisation. Main nerve structures appear in the next hours, followed by brain and spinal cord maturation in the subsequent days (Kimmel et al., 1995). When using zebrafish embryos as a source for primary neural cultures, age selection was crucial because anatomic and metabolic properties of neurons, as well as cell composition, may vary as a consequence of a highly dynamic neural environment and interactions. For this reason, a better understanding of specific cell responses during each developmental stage was essential aiming to produce higher culture yields with desirable features for in vitro neural circuit building, which include better cell survival and an increased ability to grow neural processes. To integrate these experiments with previous results, an exploration of the effect of neurotrophins in the aforementioned features was included, aiming to obtain the ultimate conditions that led to optimal culture parameters.

A pilot test revealed that samples of cultured neurons obtained by the use of the developed protocol responded to neurotrophins by growing axons in the first hours after delivery in the culture wells and maintained in optimal environmental conditions (**Figure 5.20**). However, a deeper analysis was required to establish the trophic effect of neurotrophin administration to select the media composition producing the better yields in terms of neural survival and growth.

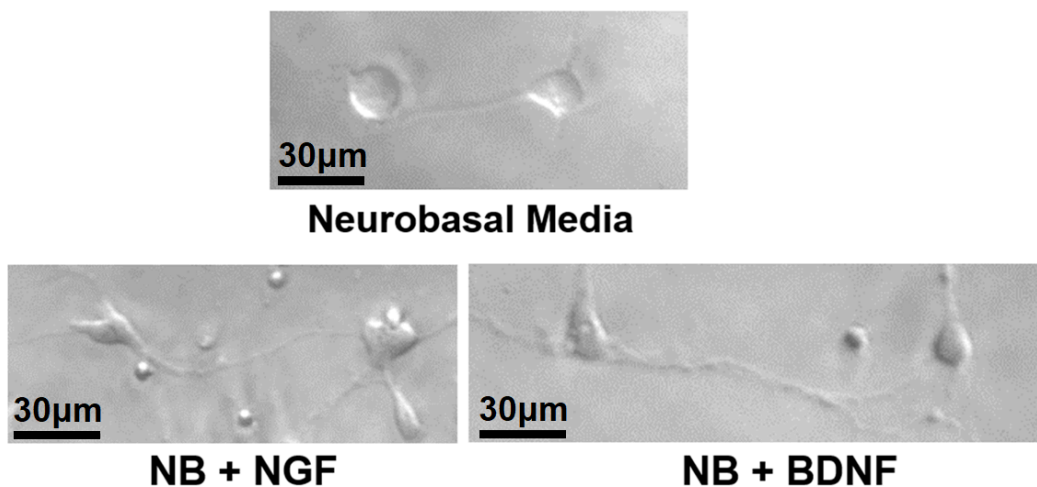


Figure 5.20: Growth of neural processes in the presence of neurotrophins

Dissociated neurons were grown in supplemented Neurobasal media and, after a few hours, axonal growth was observed. The addition of neurotrophins, NGF 100 ng/mL and BDNF 150 ng/mL, in the media resulted in a noticeable increase in axonal growth length.

With this in mind, the effect of neurotrophins on cell survival was tested in three different developmental stages of the zebrafish embryo. Zebrafish developmental times use the elapsed hours after egg fertilisation as the initial time reference and, for longer monitoring, days after fertilisation (equivalent to 24, 48, 72, etc. hours after fertilisation). An immature brain and spinal cord are visible from 1dpf, which continue their maturation process in the following days. Embryos spontaneously hatch from eggs at 2 dpf and show adult traits and completely formed nerve structures from 5dpf (Lowery et al., 2009). During this time, the brain and nervous system increase in size, form defined anatomical structures and express genetically encoded markers. In the case of the HuC-GCaMP6 line, the fluorescence in nerve structures is barely visible from 1dpf and becomes clearly identifiable from 2 dpf (Chen et al., 2013).

HuC-GCaMP6 zebrafish embryos from 1, 2 and 3 dpf were employed as a source to generate primary cultures of neurons. Cultures were grown for 3 days in supplemented Neurobasal media with NGF (100 ng/mL), BDNF (150 ng/mL) or a combination of both neurotrophins. One additional culture using media without neurotrophins was included as a control. All cultures were grown in dishes coated with laminin due to the increased neural survival and process growth observed in previous testing. Culture images were obtained by positioning the culture dish in the workstation plate holder and employing the incorporated microscope to capture images of the cells at random positions of the culture well. Four frames were taken in each condition, using the “Cell Counter” FIJI plugin to count the surviving cells and the mean number of cells was calculated. A total of four independent primary cultures were included per group.

The effects of developmental stage and neurotrophins on neural survival in zebrafish primary cultures of neurons are displayed in **Figure 5.21**. Survival was expressed in cells per captured image frame. Several differences were observed in each embryonic stage. Firstly, yields after the cell dissociation protocol were higher when younger embryos were used. The mean number of initially attached cells at day 0 in cultures was 86.4 cells/frame in 1dpf, 79.5 cells/frame in 2dpf and 53.7 cells/frame in 3dpf zebrafish embryos grown in reference conditions (Neurobasal media without any neurotrophin), revealing a progressive decrease in dissociation efficiency of the procedure as the brain grew and matured.

Regarding the neurotrophin effect on neural survival, neurons from 1dpf embryos were less sensitive to the positive impact of these trophic molecules. Mean values on surviving cell counts progressively decreased in all conditions, lowering from 86.4 to 42.1 cells/frame (52% decrease) in the reference culture at day 3. The use of a single neurotrophin did not modify this observed decrease, with similar values of NFG and BDNF compared to the reference culture. Only the combination of both neurotrophins partially ameliorated the cell number decrease, counting 70.4 cells/frame (32% decrease) using this culture media composition.

The survival of neurons from 2dpf embryos followed two markedly different paths. A progressive decrease in the number of surviving neurons was similarly observed in the reference culture, from 79.5 to 31.8 cells/frame (60% decrease), and in the presence of BDNF. In contrast, use of NFG alone and combined with BDNF minimised the decline in the number of neurons after 3 days, counting 87.2 cells/frame in NFG and 70.8 cells/frame in NGF+BDNF cultures (only a 2% and 9% decrease, respectively).

Cultures from 3dpf embryos showed a similar effect of neurotrophins on neural survival. The progressive reduction in the number of surviving neurons was found again in the reference culture, from 53.7 to 21.8 cells/frame (59% decrease), and in the presence of BDNF. Conversely, including NFG alone and combined with BDNF in the media diminished the decline in the number of neurons after 3 days, counting 67.4 cells/frame in NFG and 68.8 cells/frame in NGF+BDNF cultures (22% and 7% decrease, respectively).

The previous findings suggested an association of the presence of neurotrophins in the culture media and an increased neural survival in cultured zebrafish neurons. This effect was more evident in 2dpf and 3dpf embryos, with NFG playing a leading role during prolonged in vitro neural maintenance by preserving the number of surviving neurons over several days.

In addition to the results on cell count, it is relevant to mention some observed differences during the cell culture process, which may account for differences in the cell counts. Dissected brains from 1dpf embryos were more easily dissociated into isolated cells when incubating with TrypLE enzymatic solution. After ~8 minutes of incubation at 37°C, all the brain fragments have completely dissociated into isolated cells, visible in a brightfield microscope. Brains from 2dpf embryos required periodic gentle shaking of the solution, but at the end of the 10-minute incubation with TrypLE solution, most of the large tissue fragments had dissociated. A few cell clusters were visible when the cell suspension was observed in the microscope, but most of the cells appeared as isolated units. Brains from 3dpf embryos required more shaking to break the tissue fragments. After the 10-minute incubation with TrypLE solution, some undissociated tissue pieces and cell clusters persisted. A quick test showed that even a 15-minute incubation with the enzymatic solution was not enough to completely dissociate the remaining fragments, compounded by the known cell harm caused by a more prolonged incubation with dissociating enzymes.

On the other hand, even with the prompt enzymatic dissociation of tissues observed in 1dpf zebrafish embryos, brain dissection was complicated using these embryos because of the early development of nerve structures. Brain and spinal cord sizes are considerably smaller, and their limits are barely visible even when observing with a microscope. Thus, removal of other tissues during dissection, such as muscle and connective tissue, was imprecise, and the obtained brain was often accompanied by fragments of other tissues, increasing the number of non-neural cells in the generated cultures. The presence of different cell types in the samples could mean additional cell interactions within the cultures, such as paracrine signalling events, which might alter responses expected to be due to internal cell mechanisms in the neurons.

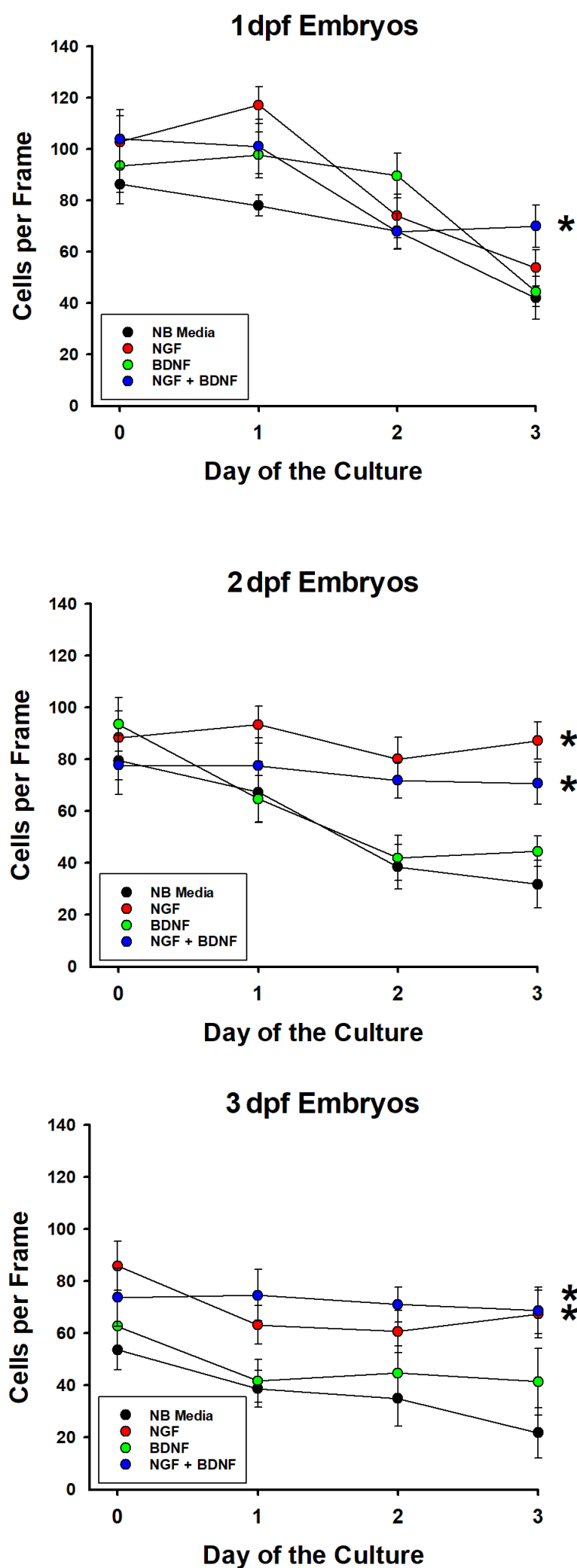


Figure 5.21: Effects of developmental stage and neurotrophins on neural survival in zebrafish primary cultures of neurons (continues in next page)

(from previous page)

Primary cultures of embryonic zebrafish neurons from different developmental stages (1, 2 and 3 dpf) were grown in the presence of NGF (100ng/mL), BDNF (150ng/mL) or a combination of both neurotrophins, in supplemented neurobasal media and culture plates coated with laminin. Cultures were maintained in an incubator for 3 days, and surviving neurons were counted each day.

Adding neurotrophins to the culture media enhanced neural survival. Greater survival was observed when combining both neurotrophins, followed by NGF and BDNF alone. The difference was more marked in later days of the culture. A greater number of initially attached neurons and overall neurotrophin-dependent survival were observed in primary cultures from an early developmental stage (1dpf), which was reduced in later developmental stages, 2dpf and 3dpf (* : Significant difference vs. NB media, after a One-way ANOVA test and $p < 0.05$)

The considerations above led to the conclusion that the optimal procedure for the obtention of isolated neurons in culture should combine enhanced neural survival over extended periods, facilitation of the growth of neural processes, such as axons, easy-to-follow steps, and minimal presence of non-neural cells in the samples. Based on the obtained results and the tested parameters, the selected cell source culture media for subsequent procedures in this project involved the use of 2dpf zebrafish embryos. This developmental stage had a convenient balance between an easy method to identify and dissect brains from the anatomical observation of the animal, combined with sufficient tissue dissociation by enzymatic action, and a favourable yield in terms of the initial number of attached cells in the cultures. Regarding culture media composition, the obtained values on neural survival and neural process growth led to the decision to base the composition on the supplemented Neurobasal media, including 10% FBS and NGF 100 ng/mL. Despite the combination with BDNF giving similar effects in the mentioned parameters, results suggested that NGF alone was sufficient to promote in vitro survival and process growth in zebrafish neurons during prolonged procedures. Besides, laminin was chosen as coating for culture dishes due to the enhanced neural survival and process growth when neurotrophins were present in the culture media. These conclusions dictated the methods to follow to obtain dissociated neurons for in vitro neural circuit building, developed in the following stages of this project.

To conclude with Section 2 of this thesis, the main outputs can be listed as follows:

- A protocol for the obtaining of primary cultures of embryonic zebrafish neurons from the HuC-GCaMP6 strain was implemented. This procedure resulted from testing several developmental stages of the source organism, protein coatings on the culture dishes and culture media composition. In this latter parameter, the effect of providing the neurotrophins NGF and BDNF to cultured zebrafish neurons was compared to select an adequate supply which favoured neural survival and growth of processes over prolonged cultures.
- By applying the previous protocol, cultured neurons from 2dpf HuC-GCaMP6 zebrafish were selected as the most suitable cell source for the subsequent use in the modified FrescoM workstation, pointing towards the in vitro construction of neural circuits. Also, neurons cultured in laminin-coated dishes, and using Neurobasal culture media containing FBS 10% and NGF 100 ng/mL, were found to be the most convenient conditions for sufficient culture yields, extended survival of neurons and the consistent growth of processes with the required length for the in vitro formation of neural arrays.

This section of the project improved the limited availability of technical protocols regarding primary cultures of neurons in zebrafish. Several parameters were translated from other organisms and were first explored in the zebrafish research field. The involved mechanisms were known to participate in the in vitro survival of neurons and the developmental processes during the formation of synaptic connections with other neurons. These parameters were tested due to their critical roles as part of an intended process for the in vitro generation of neural circuits. The conditions that produced improved yields and desirable effects on neural processes growth were selected to implement them as part of routine procedures to obtain isolated neurons in culture, which were used later for the in vitro circuit building. The intended automated process incorporated the utilisation of the previously described FrescoM automated workstation to perform the selection, manipulation and positioning of neurons from these biological samples, which was the subject of Section 3.

Section 3:

In vitro Building of Neural Circuits

Standard techniques for in vitro modelling of the pathways of information processing through neurons have shared a lack of sufficient resolution to shape fabricated circuits at a single-cell resolution. The availability of adequate biological material has been a notable restraint. Besides, access to suitable tools for cell maintenance, precise sample manipulation and continuous monitoring are also among the most remarkable issues faced by researchers in bridging the gap from laboratory bench testing to the large-scale production of neural circuits as an applicable manufacturing output. In the first two sections of this thesis, the development of an accessible and customizable automated platform for controlled manipulation of individual cells, summed with the standardization of a protocol to produce high yields of fluorescence-expressing neurons in cultures that maintain their survival and trophic properties over extended periods, provided a solution to account for limitations to generate circuits in the laboratory as a continuous process.

Section 3 describes the development of a procedure to use the modified FrescoM workstation to use dissociated neurons from primary cultures as building material for the building of neural circuits, by the identification and selection of individual cultured neurons followed by the positioning into arrays and the promotion of axonal growth to form and consolidate specific connections with other neurons. This project stage encompassed the testing of the micromanipulator capability to retrieve and release individual cultured neurons, the application of molecular cues to induce and direct axonal growth in these neurons and a functional evaluation upon the formation of synaptic connections. The potential to automate the overall building process was also explored.

Particular objectives during this project stage were:

- To achieve a precise manipulation of the cultured neurons, using the developed cell manipulator in the FrescoM Workstation, to transfer individual neurons from dissociated samples to new cultures in specific positions.
- To induce and direct the axonal growth in positioned neurons towards the formation of specific neural contacts and synaptic connections with neighbouring neurons in the fabricated arrays.

5.13 Criteria for Neural Selection

The application of the protocol for obtaining high-density cell samples from dissociated zebrafish brains provided a suitable source of neurons for subsequent manipulations and in vitro circuit formation. The procedure was designed, from tissue obtention to cell dissociation conditions and the selection of culture media composition, to favour the obtention of a majority of neurons in the resulting cell samples. However, the obtained cultures were not composed of a completely homogeneous neural population, but of a mix of cells, as illustrated in **Figure 5.22**.

The expression of the GFP molecule in the GCaMP6 indicator, expressed under the neural-specific promoter huC, was the main criteria to define neural identity. Besides, uniform soma size around 15 μm and spontaneous process growth in these cells during extended monitoring confirmed this assumption. To facilitate single-cell manipulations, individual cells were preferred for further steps.

With respect to other cell types present in the samples, a population of non-fluorescent small cells (with a soma diameter of approximately 5 μm) was commonly observed in the cultures. During the first hours of culture, these small cells aggregated, forming small clusters with other similar cells and around neurons without the growth of any visible neurites. Based on their features, they appeared to be microglial cells, as observed by Patel et al. (2019), and were not targeted for the manipulations intended for this project. Additionally, undissociated small neural clusters and tissue debris were not uncommon, but they were not selected due to pipette dimensions and the inability to ensure cell identity during cell selection.

Additionally, not all the cells that covered all the features of neurons expressed the neural marker with the same intensity. In some cases, intensity of the fluorescent signal was dim, absent or variable during prolonged cultures. Considering that the ultimate goal of the project was the eventual automation of the tasks involved in building the neural circuit, a criterion based on fixed parameters was implemented for the reliable identification and subsequent selection of solely HuC-GCaMP6-positive neurons in the cultures. An average signal/background value on fluorescence intensity was obtained from a population of neurons ($n = 50$ neurons) during the implementation of culture conditions, using the normal camera set up for image capturing.

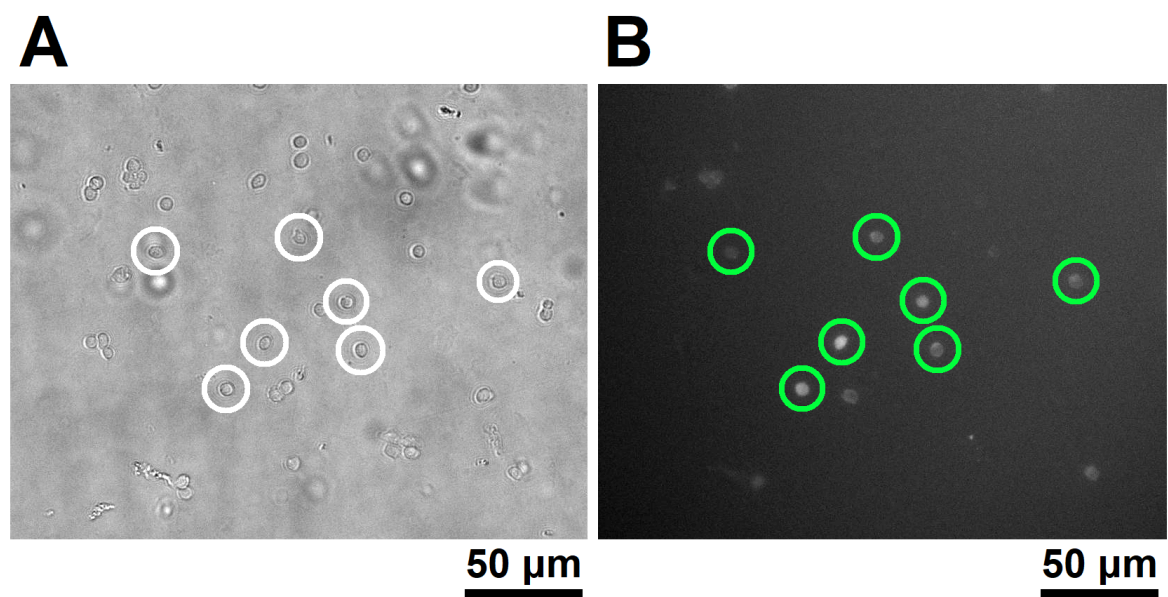


Figure 5.22: Fluorescence expression in primary cultures of neurons from HuC-GCaMP6 zebrafish

Circles in the brightfield (**A**) and epifluorescence (**B**) images on the same cell sample indicate typical neurons chosen for further manipulations. Neural identity was confirmed as neurons by GFP expression, part of the GCaMP6 indicator. Occurrence as individual cells, size and uniform fluorescence intensity were also selection criteria for neurons.

Next, the manipulation of individual neurons to build arrays in this project followed the Bottom-Up principle. **Figure 5.23** illustrates this process as an assembling process from basic building blocks, which comprised cycles of a coordinated sequence of four main events:

1. **Neural collection or picking** from the original primary culture, by entering culture media containing the neuron into the micromanipulator pipette. This demanded neuron targeting by the pipette and activation of the micromanipulator syringe to pull the plunger and retrieve the neuron.
2. **Transferral** of the neuron to a new culture dish or well with media, serving as place for the circuit in production. This involved synchronized function of motors in the mobile frames to move the culture plate and situate the pipette containing the cell in the final position into the array.
3. **Release** or delivery of the neuron into the new culture, by retrieving the culture media from the micromanipulator pipette, on the target position. This required activation of the micromanipulator syringe to push the plunger until the neuron is released.
4. **Return** the empty pipette to the original primary culture, once the neuron release is complete, and select another neuron, starting a new cycle.

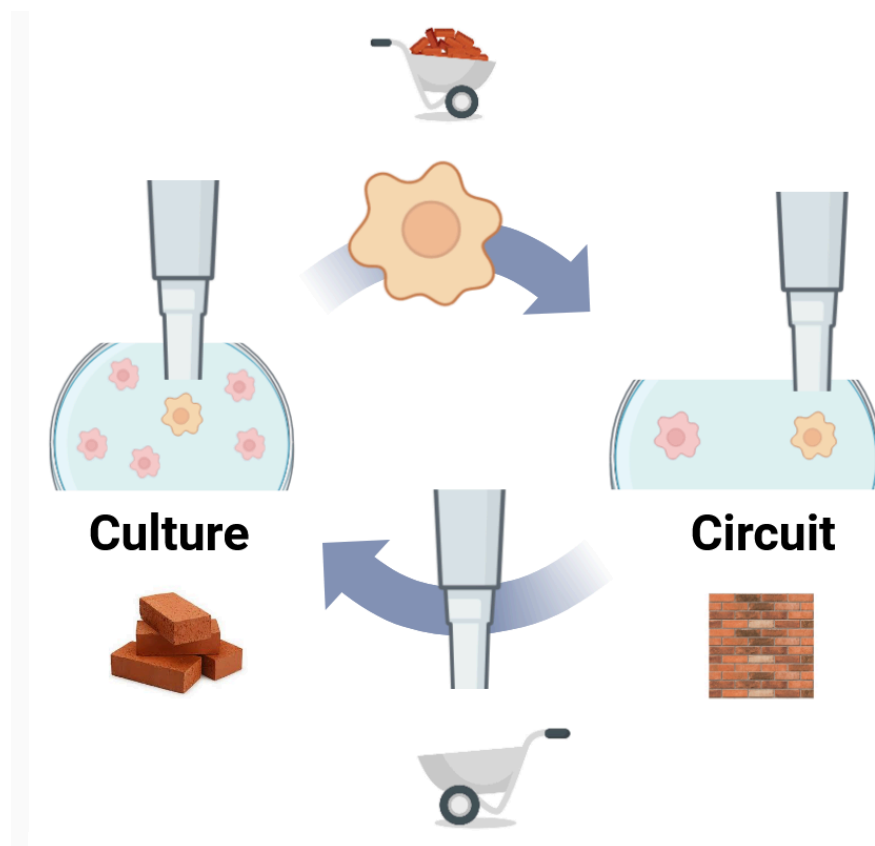


Figure 5.23: Cycle for the Bottom-Up construction of neural circuits

Individual neurons were identified in the primary cultures, retrieved into the manipulator pipette and released in a new culture at a specific position. The cycle was finalised by moving the pipette back to the initial culture to transfer additional neurons. This procedure of sequential steps can be exemplified as a building process in building construction, using elementary construction blocks, requiring a transport device to move the parts to their final positions.

5.14 Building of Neural Arrays Using the Cell Manipulator

The cell manipulator incorporated in the FrescoM workstation was designed to retrieve and release small volumes of liquid culture media from biological samples using a pipette. When used on neural cultures, this function can be used to pick, transfer, and release floating or poorly attached cells as a standard cell manipulator.

In the next stage of the project, the technical capabilities of the cell manipulations were tested to build arrays of neurons with regular shapes, using primary cultures of neurons from HuC-GCaMP6 neurons as a cell source. The Bottom-Up approach to building neural circuits offered the possibility of establishing specific circuit architectures from the most elementary scale, which represents individual neurons. Although complex circuits could eventually be achieved by simple escalation of this process, the present project only studied simple circuits, grouping neurons to serve only for two primary purposes: presynaptic and postsynaptic neurons. For this purpose, the chosen architectures were regular shapes with a two-column structure. The subtype neural identity was not considered at this time, as the initial appearance and fluorescence expression by all neurons in primary cultures were similar, and the identification of subpopulations would have required the use of additional cell markers.

To select individual neurons from the HuC-GCaMP6 primary culture samples and transfer them into new cultures at specific positions, the coordinated functioning of diverse components of the workstations was essential. Among these parts were:

- Nema motors, controlling the movement of the pipette and the well plate
- Micromanipulator syringe motor, dispensing the media
- Microscope camera, monitoring the cell status and progress of the manipulation
- LED illumination system, allowing cell identification by brightfield and fluorescence microscopy

Only the activation of these functions at specific times could achieve success in this task and, after gathering enough information on operational parameters, an automated script to automate the involved steps could be considered.

To direct the cell manipulation function in building neural arrays, an assisted protocol was initially developed. Controlled by an operator and using the modified FrescoM workstation software and hardware, the manipulator was employed to pick individual cells for freshly prepared primary cultures of HuC-GCaMP6 zebrafish neurons. After a short recovery period, the culture plates were placed in the dedicated area to handle the well plates, and the cultured cells were observed under the microscope. At this point, neurons were loosely attached to the culture well surface. Thus, a close positioning of the micromanipulator pipette, followed by the retrieval of media, could cause the cell to detach from the surface and enter the pipette. After confirmation of neural identity, by fluorescence expression in the cells due to GCaMP6 protein expression, individual cells were picked into the pipette. The pipette was then moved to an empty culture well containing fresh media. The pipette was slowly placed in proximity to the bottom of the culture well, making barely any contact, and the micromanipulator syringe was operated in the opposite direction in small steps to release the media containing the isolated neuron into the new culture. The coordinates of the release defined the architecture of the intended neural array, which was built from scratch in this manner.

Following the previous assisted steps, the FrescoM workstation was capable of performing neural selection, transfer and positioning in new cultures at defined positions, as shown in **Figure 5.24**. The target structures for this testing were variants of square-shaped arrays, consisting of two columns separated by a gap of approximately 150 μm , to reduce the risk of cells making physical contacts after a short displacement, a spontaneous phenomenon often observed in cultured cells in proximity. The array consisted of at least one neuron on each

column, being more frequent the positioning of a total of four neurons. Apparently, random displacement was observed in the hours following cell positioning, which slightly altered the array structure. This variation in position was assessed, and the results are displayed in the next section. It is also relevant to mention that, although the initial cell-picking step was intended to select only one neuron, sometimes the pipette collected more than an individual neuron, which could be liberated from the pipette during the release step without affecting the general pipette operation.

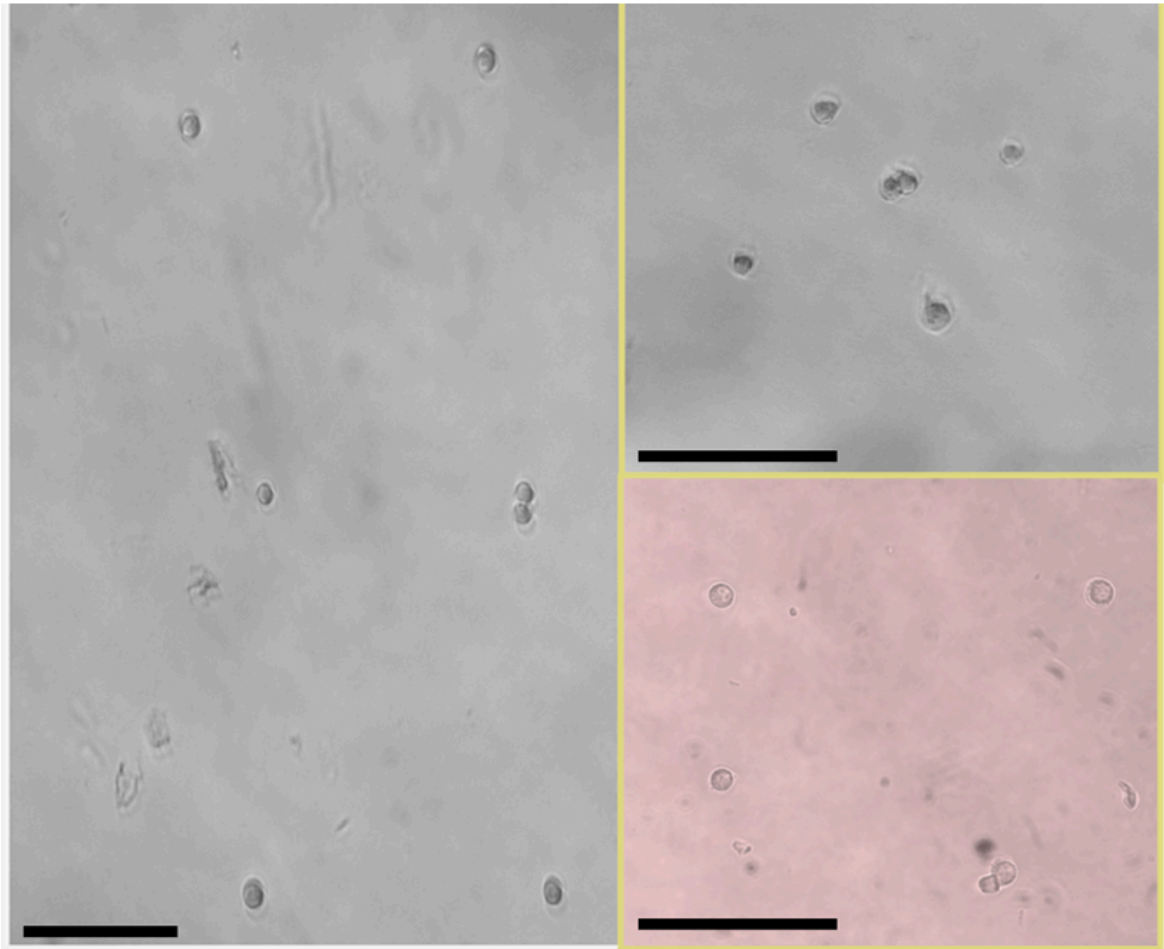


Figure 5.24: Generation of neural arrays after repeated positioning of neurons

Representative examples of output cell arrays after an assisted protocol run for fluorescent-based neural selection, single-cell pick, transferral and controlled release in specific coordinates into the new cultures. The FrescoM cell manipulator was used to transfer individual Huc-GCamp6 zebrafish neurons to new cultures forming square-shaped arrays. Several minutes after release, neurons attached to the dish surface in the new culture and continued growing while maintaining the array design. (Bar = 100 μ m)

Considering that the observed cell displacement could affect the structure of the generated circuit in both the short and long term, the next aim was to define the precision of the device in releasing neurons by measuring the distance a positioned cell travelled after being delivered from the pipette. This movement resulted from either the immediate flow of media out from the pipette or a posterior cell displacement before attachment to the bottom of the culture well. For this purpose, an assisted protocol was developed involving the repeated selection of individual zebrafish neurons from primary culture samples and the supervised release into empty cultures. Several neurons were simultaneously loaded into the pipette from the first culture, and after transferring to the second culture, the neurons were released using short steps of the syringe motor. Progressively, the neurons were delivered from the pipette at a fixed position, one unit at a time. After a 30-minute recovery period, the neurons attached to the bottom of the culture

sufficiently to maintain their position. At this point, an image of the final state of the culture, constituting a snapshot of the intended circuit, was captured with the microscope camera. Using linear measurement functions incorporated in the FIJI software, the centre of the pipette was annotated and defined as the initial delivery point. Then, the final position of released cells, enclosing the displaced distance during the procedure before cell attachment, was marked. The distance to the pipette was measured for all released cells, and distance values were grouped in discrete intervals. Obtained values on cell release precision are depicted in **Figure 5.25**. Distance measurements showed a calculated average travelled distance of the released neurons of 50.4 μm . A frequency density analysis was performed on the obtained distance intervals, revealing that most neurons were displaced by less than 100 μm . These results indicated that the developed cell manipulator was capable of transferring individual neurons from cultures of dissociated neurons, performing transfers using the pipette and positioning the neurons in specific positions, forming arrays with precision and minimal cell displacement.

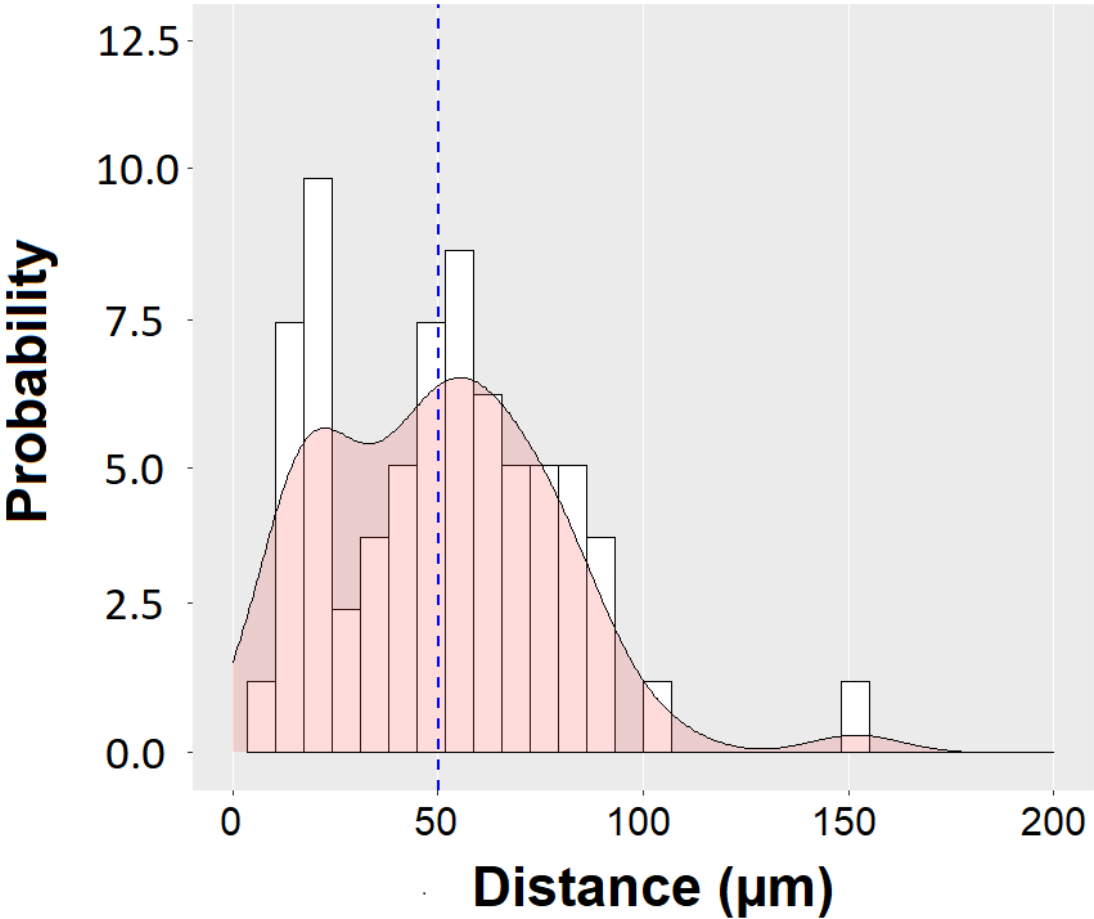


Figure 5.25: Precision of the cell release step using the developed cell manipulator.

The pipette loaded with neurons from primary cultures was programmed to release the cells into new culture wells. After cell attachment to the culture surface, the distance from the pipette tip to the final position of the released neurons was measured. Bars show the histogram of the measured distances, arranged in discrete intervals, from the initial position to the final position of the neural soma. Values represent the probability of finding a neuron at a specific distance from the pipette tip centre. The average distance to the released cell in the task task was 50.4 μm , indicated by a blue dashed line (n=100 individual repetitions of the task).

5.15 Fluoresce-Based Selection of HuC-GCaMP6 Neurons

The previously mentioned cycle for neural selection and positioning could be performed manually to progressively generate a neural array with a desired architecture, just as any other manual micromanipulator. However, the intended outputs of this project were centred on the eventual implementation of task automation to increase the building process efficiency. In this regard, the neural expression of fluorescence in the HuC-GCaMP6 zebrafish strain and the ability of the FrescoM workstation to perform real-time imaging in cell samples represented advantageous features of the primary circuits by providing a quantifiable parameter in the cells which could be measured to trigger the sequential activation of the workstation components involved in the retrieval, transfer and positioning of neurons.

A method of automated neural identification and selection was developed, based on the real-time measurement of neural fluorescence. This method required the visualisation and constant monitoring of the primary culture of HuC-GCaMP6 neurons using the workstation epifluorescent microscope. As previously mentioned, some (but not all) neurons in the culture expressed a considerable amount of fluorescence above the background signal, making them distinguishable from other cells present in the culture. The identification method consisted in the following rules:

1. The first step of the selection method consisted of positioning the tip of the micromanipulator pipette at the centre of the pipette tip, which was slightly ($\sim 10\ \mu\text{m}$) above the culture dish surface. At this distance, the pipette was brought into focus by the microscope. Also, it was possible to move the pipette in proximity to the cultured cells without making physical contact. The target neuron, which expressed fluorescence above the background level when illuminated with the blue LED, was centred in the visual display of the microscope, with the pipette tip positioned over it.
2. The monitoring of cell fluorescence was possible by running a Python script within the framework software, which performed continuous image capturing on the microscope display (1 captured image every second), followed by analysis of the saved frames. From the entire image in the camera display, the analysis software cropped the original image (from 840×840 pixels in size) to a smaller square-shaped area in the centre (180×180 pixels in size, equivalent to $\sim 50 \times 50\ \mu\text{m}$ using the $20\times$ microscope objective) for image analysis, corresponding to the central area containing the target fluorescent neuron and the pipette tip, as illustrated in **Figure 5.26**. Due to the cell density in the cultures, only the targeted neuron was present in this scanned area. The fluorescence intensity, defined as total pixel brightness in the analysed region of the frame, was measured. The obtained value was included in a generated data frame, managed by the “numpy” Python library.
3. The constant monitoring of the cell fluorescence was achieved by analysing the generated data frame on fluorescence values over time. When the first ten values were obtained after ten seconds had elapsed, mean fluorescence and standard deviation values in the data frame within this range were calculated. These values were used to represent the cell fluorescence status at that particular moment. Afterwards, the data frame consisted of sliding computed values. Thus, for any additional measurements, only the last ten captured frames were considered to obtain new mean and standard deviation values, updating the cell fluorescence status as the protocol progressed over time. The target neuron, the pipette and the analysed region remained at the same position, maintaining the analysis focused on the same neuron over time.

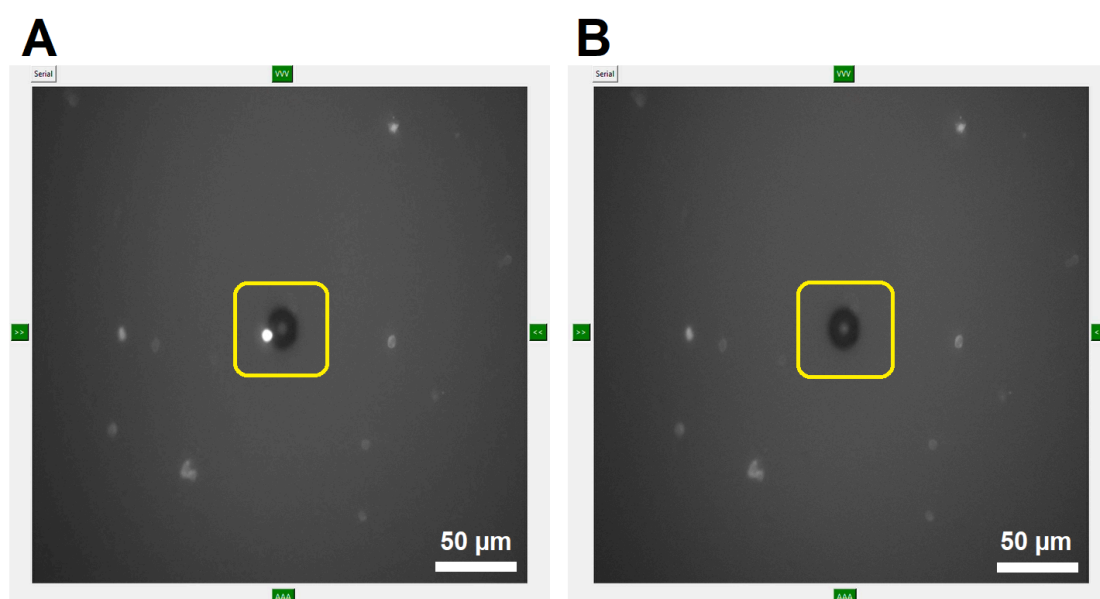


Figure 5.26: Detection of neural picking events by fluorescence monitoring in discrete areas of the camera display

A protocol based on continuous fluorescence quantification was developed to automatically identify neural picking events during the collection of individual neurons from cell samples. The recognition of release events followed the same principle, but running in the opposite direction.

A. The workstation software continuously captured images from the micromanipulator pipette positioned over a fluorescent neuron. The resulting pictures were continuously analysed in a discrete area in the centre of the camera display (yellow square) to quantify fluorescence intensity.

B. The micromanipulator syringe activation resulted in the entering of the neuron into the pipette and a decrease in the measured fluorescence over the monitored area. This event triggered the progression of the cycle and subsequent transfer events in the selected neuron.

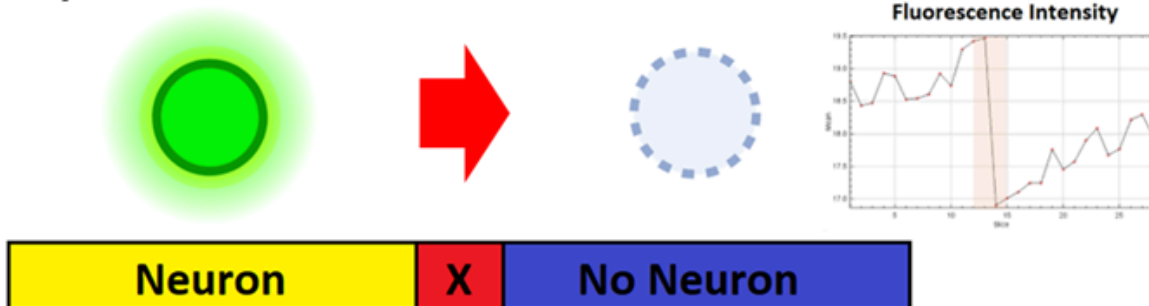
4. Regarding the protocol for cell picking using the micromanipulator pipette, once the first mean fluorescence value was calculated, an additional step involving pulling the syringe was included as part of the cycle. This was accomplished by activating the respective Nema motor, which controlled the plunger displacement. As a consequence, at the end of each cycle, the pipette extracted a small amount of culture media from the culture. As the neurons in culture were not firmly attached to the dish bottom at this point, there was a possibility that this action would dislodge the target neuron and collect it into the pipette.
5. At the end of each cycle, mean fluorescence values were evaluated. As long as the fluorescent neuron remained in the analysed area, the centre of the visual display, similar mean fluorescence values were maintained. In operational progress, this meant that the neuron had not been picked yet, and, therefore, the same cycle was repeated. However, if a relevant decrease in mean fluorescence values was detected, this incident was identified as a “Pick Event”, indicating that the last cycle had dislodged the neuron from the culture surface and placed it inside the pipette. A decrease of 20% in the fluorescence intensity was established as the threshold to define the occurrence of this event. The observed reduction in calculated mean fluorescence values is represented in **Figure 5.27, panel A**. It is worth highlighting that comparing mean fluorescence values from the last ten cycles, rather than the single latest value, prevented the false positive occurrence of a Pick event caused by unexpected sources, such as cell debris or floating particles, which could alter measurements.

6. The occurrence of a “Pick Event” triggered the repositioning of the pipette in the new culture in construction, at predefined coordinates, by activating the respective XYZ motors in the frame, moving the culture plate holder. As the working position in the cultures was controlled by moving the plate instead of moving the pipette, the pipette tip remained in the centre of the visual display at all times. However, the pipette was required to move only in the vertical direction.
7. The spatial repositioning was followed by a transition to a new type of cycle, which delivered the neuron held inside the pipette to a defined position in the new culture. The process followed a similar principle to the “Picking Event” identification, involving the constant monitoring of a small square-shaped area in the centre of the visual display to calculate a mean fluorescence value from a sliding range of a generated data frame. However, in this case, the evaluation ran in the reverse direction. A pushing displacement replaced the pulling of the syringe plunger in the micromanipulator. Consequently, the liquid inside the pipette was progressively released, and eventually, the contained fluorescent neuron was released. The fluorescence monitoring system also operated as an opposite evaluation, as illustrated in **Figure 5.27, panel B**. The fluorescence intensity in the same centred area was similarly measured in continuous intervals, which were added and updated in a data frame. However, instead of identifying a decrease in the mean fluorescence value, the script recognised the occurrence of an increase in this value, as a consequence of the neuron entering the scanned area of the microscope display. An increase of 20% in the fluorescence intensity was established as the threshold to define the occurrence of “Release Event”.
8. The incidence of a “Release Event” triggered the progression of the cycle into its last stage, where the pipette was repositioned again in the initial cell culture. The cycle was finalised by the identification of a new fluorescent neuron in the sample of dissociated cells and the centring in the microscope display, inside the scanned area. The reposition was achieved by activating the XYZ motors in the frame, initially moving the culture plate holder with long steps and later with short ones for fine positioning. All the data frames were then reset, and the target coordinates for the next neuron were updated. The framework was then ready to start another cycle.

This fluorescence-based recognition of target sites was implemented to measure pixel intensity within a discrete square region to identify localised areas of increased fluorescence intensity. This approach provided a computationally simple and robust method while adequately approximating the generally circular morphology of cells. In contrast, direct recognition of the pipette tip was also considered, but it would require a more complex image-processing algorithm. The glass pipette appeared as a dark structure in brightfield images, making it susceptible to false identification caused by non-fluorescent cells, debris, or other image artefacts. Furthermore, accurate detection of the pipette tip would require scanning a ring-shaped region corresponding to the pipette geometry, increasing both the computational complexity and the likelihood of recognition errors.

The proposed cycle for neural selection represented a reproducible protocol to pick and release individual neurons from the cultures of dissociated HuC-GCaMP6 zebrafish neurons. Progression through the steps depended on meeting specific criteria, which could be adjusted to particular conditions, such as experimental variation in fluorescence intensity or an increase in the background signal, making the parameters adjustable. The method was also designed to be robust, capable of handling unexpected fluorescence variations by utilising mean values contained in a constantly updated data frame, as described earlier. Most importantly, running the procedure as an operational script in the framework opened the possibility of implementing this method as an automated task, for continuous and extended activity with a large number of neurons.

A) Neuron Pick:



B) Neuron Release:

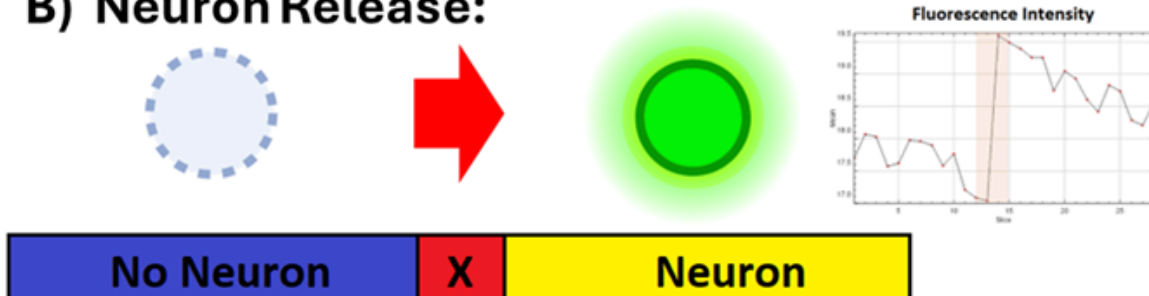


Figure 5.27: Automated identification of neural pick and release events by fluorescence changes to target HuC-CaMP6 cultured neurons

To increase experimental efficiency, a procedure to automate the picking and releasing of neurons was developed based on the fluorescence expressed from HuC-GCaMP6 neurons in primary cultures. Using the epifluorescent microscope of the workstation, these fluorescent neurons were identified and placed in the centre of the camera display of the microscope. The software was programmed to monitor a square-shaped area around this central point, measure the light signal intensity and obtain an average from the last recorded values.

A. Picking a neuron required the entry of cell-containing media into the pipette. Hence, the manipulator syringe was activated as part of the protocol, moving the precision syringe to apply negative pressure inside the pipette, aiming to pick the centred neuron. When the monitoring system detected a decrease in the average fluorescence, the event was identified as a “Picking event”, which stopped the plunger movement and the entry of media, to proceed with subsequent steps of neural transferal.

B. Releasing a neuron involved the delivery of the cell contained inside the pipette into a new culture well. Once the pipette was placed in the final position, the precision syringe was activated to apply positive pressure and release the pipette content. When the monitoring system detected an increase in the average fluorescence, the event was identified as a “Release Event”, which stopped the plunger movement and media delivery, to proceed with the remaining steps of the protocol.

5.16 Axonal Growth in Positioned HuC-GCaMP6 Neurons

Delivery of neurons to specific positions, forming layers, was achieved by following initial circuit blueprints that defined the number and arrangement of neurons in the circuit under construction. After a recovery period, positioned neurons attached to the culture dish surface. As previously explained, neural circuits are composed of neurons, functioning as nodes, and specific connections, both of which allow information processing along the system. The next aim was to convert these arranged neurons into a functional circuit by forming the proper connections, according to the intended structure of the circuit.

The first approach to fulfil these requirements was to provide the conditions that proved to favour the spontaneous growth of neural processes in zebrafish neurons. For this, fabricated neural arrays with 50 μm separation between cells were grown in supplemented Neurobasal media containing NGF (100 ng/mL), which showed the best performance in terms of process growth and neural survival in primary culture samples. After a few hours, some axons, initially visible as short processes, became apparent in the positioned neurons. Cultures were maintained in optimal environmental conditions, inside an incubator. Consistent with the original primary cultures, a maximal length in axonal growth was observed after 24 hours, when a picture of the built circuit was captured. A representative image of the obtained neural behaviour is displayed in **Figure 5.28**. The proposed structure of the circuit under construction consisted of separated neurons serving as layers. To achieve this structure, selected neurons from cultures were individually released in specific positions intended to restrict physical contact with other neurons, allowing synaptic contacts to form through axonal growth between different layers.

Some relevant events were detected. Firstly, not all the neurons grew axons. When it was the case, the axonal length was variable and pointed in random directions, with no apparent tendency to follow a specific pattern. Secondly, even the presence of other adjacent neurons was not observed as a sufficient feature to alter the trajectory of a growing process. Even though some neurons were connected with other adjacent neurons, as aimed, at variable times. Additionally, some neurons exhibited the previously observed spontaneous displacement over the culture surface, which affected their positioning with respect to the original circuit design. Measures to reduce this cell displacement were considered later.

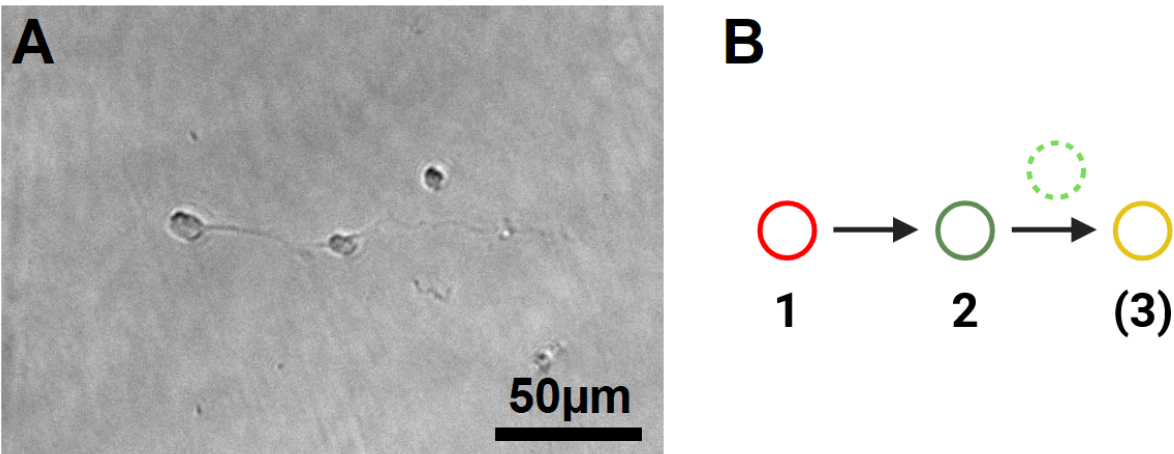


Figure 5.28: Formation of defined cell layers during in vitro circuit building

A: Image of released neurons positioned in three different layers. After 24 hours of growth, neurons grew axons in apparently random directions, which could produce physical contact with neurons in the next layer if a neuron was present in the axonal extension trajectory.

B. Schematic of the cell culture in (A), displaying three neural layers. Despite the initial positioning of neurons (solid circles), cells presented displacement from their original position, which in some cases prevented contact with growing axons.

5.17 Calcium Response in Positioned HuC-GCaMP6 Neurons

After observing physical contact between positioned neurons, the next question that arose was whether this physical approach was reflected in the formation of a synaptic connection involving the growing axon and the target neural soma. Currently identified neural mechanisms indicate that mere axon-dendrite physical contact is not sufficient for the formation of a functional synaptic connection. Rather, regional remodelling of the pre- and postsynaptic neurons is required to convert a growing synaptic ending and the corresponding somatodendritic region at the particular environment present in the synaptic cleft, through a process known as synaptogenesis (reviewed by Südhof, 2018). The formation of a synapse can only proceed when specific conditions are met, which include activation of receptors in both membranes, production of signalling molecules and trophic factors, cytoskeleton remodelling, expression of neurotransmitters, changes in vesicle trafficking, generation of membrane potentials due to ion fluxes, and generation of action potentials. This last outcome is a crucial event that strengthens a newly formed connection between two neurons by serving as a trigger to discern those connections properly formed during developmental processes that sculpt the structure of neural circuitry and participate in remodelling events of circuits throughout life (Mittermaier, 2024). A wide repertoire of neurotransmitters can mediate neural function, but they share the initiation of their action in response to changes in membrane potential, with intracellular calcium playing a prominent role. Typically, an action potential transiently increases the intracellular calcium concentration in the presynaptic terminal, which triggers neurotransmitter release via synaptotagmins (Südhof, 2012). For this reason, monitoring intracellular calcium was selected as a valuable method to determine the functional features of synaptic connections that may form during contact between positioned neurons in the implemented process.

The next aim in the project was to determine the effect of local stimulation of one neuron forming a neural array on the calcium responsiveness of contacting neurons. The experimental samples consisted of neural arrays formed by the previously described circuit-building method after 24 hours, when some of the positioned neurons formed physical contact with other adjacent neurons. If these neural junctions were reflected in the formation of functional synapses, the stimulation of the first neuron would generate an action potential that could be transmitted to chemical synapses, which in turn would produce changes in intracellular calcium concentration in the postsynaptic neurons. The occurrence of a synaptic transmission event was monitored by detecting changes in expressed fluorescence in the system using the workstation epifluorescence microscope. The developed protocol, performed in the FrescoM workstation, linked the local administration of a depolarising agent to the continuous fluorescence imaging on the target neural array. To achieve the selective depolarisation of one neuron in the neural array, a KCl solution was applied using the manipulator components, but replacing the pipette used for cell manipulation with a smaller one, designed to handle reduced volumes and to dispense them in a narrow area adjacent to the target cell, by means of the syringe activation. The administration of a high concentration of potassium has been a standard method to induce neural depolarisation in vitro (Rienecker, 2020).

The developed protocol involved a rapid application of 50 μ L KCl 20 mM after a baseline period of 30 seconds, using the modified pipette over one neuron previously centered in the microscope display. Next, pictures of the neural array were captured at one-second intervals. The observed results are illustrated in **Figure 5.29**. As expected, local application of KCl produced depolarisation of the targeted neuron, as indicated by an immediate GCaMP6 fluorescence increase. Only the stimulated neuron showed a prominent and transitory increase in fluorescence intensity at this point. However, after a few seconds, connected neurons also presented a similar increase in fluorescence intensity, indicating a rise in intracellular calcium concentration. In physiological terms, results suggested that neural depolarisation in the first neuron was propagated to other contacted neurons by generating and spreading action potentials, a reflection of the formation of functional synapses in the positioned neurons after 24 hours. It is relevant to mention that this stimulation was not restricted to neurons adjacent to the stimulation site. Some neurons extended longer contacting axons, which also propagated the stimulation signal to more distal sites as long as a physical contact was present with the first

neuron, directly or via neural intermediates. Moreover, in the case of non-contacting neurons in the array, an increase in fluorescence was observed in some but not all cases. Also, this rise occurred in posterior times compared with contacting neurons. These observations suggested the presence of functional synapses that rapidly propagated the initial depolarising stimulus, compared with the slower effect of the eventual diffusion of the KCl solution in the media.

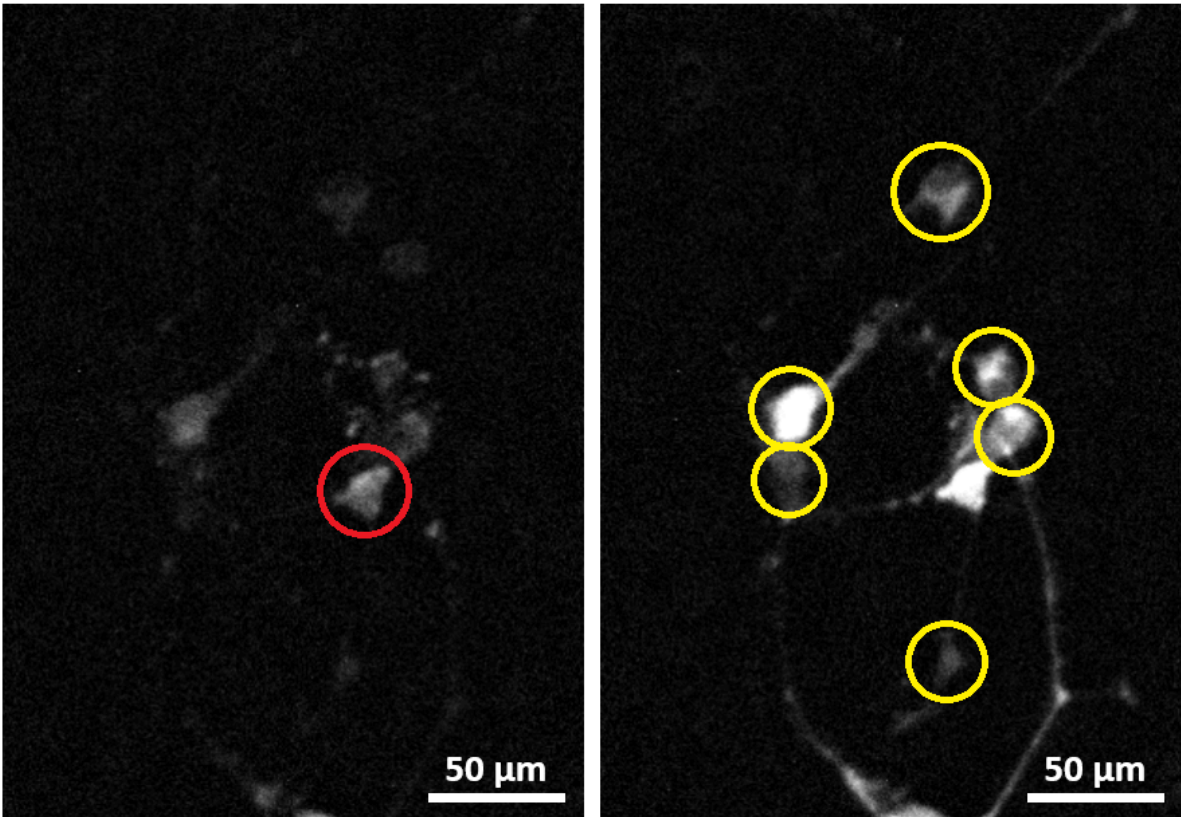


Figure 5.29: Intracellular calcium fluorescence response in locally stimulated HuC-GCamP6 neurons and contacting neurons forming arrays

Effect of neural stimulation on cultured HuC-GCamP6 zebrafish neurons forming circuits. Positioned neurons were cultured for 24 hours to randomly grow axons, create physical contacts, and form neural arrays. A pipette filled with a KCl 20 mM solution was positioned over one of the neurons forming the built neural array using the FrescoM workstation. After 30 seconds, the pipette content was released, followed by image capturing using the epifluorescent microscope. Panels show captured images from a representative experiment. The targeted neuron showed an immediate and transitory increase in intracellular calcium fluorescence (left panel, red circle), which was propagated to other contacting neurons in the array (right panel, yellow circles).

An analysis of calcium responses in individual neurons was performed to determine the temporal progression of intracellular calcium changes during the previously observed stimulation. For this, sequences of captured images were analysed with the FIJI software. Square-shaped areas containing individual neurons were selected to obtain the Z-axis profile function, corresponding to total pixel intensity over the selected area, representing the total fluorescence intensity changes over time. Normalised fluorescence values were calculated and are displayed in **Figure 5.30**.

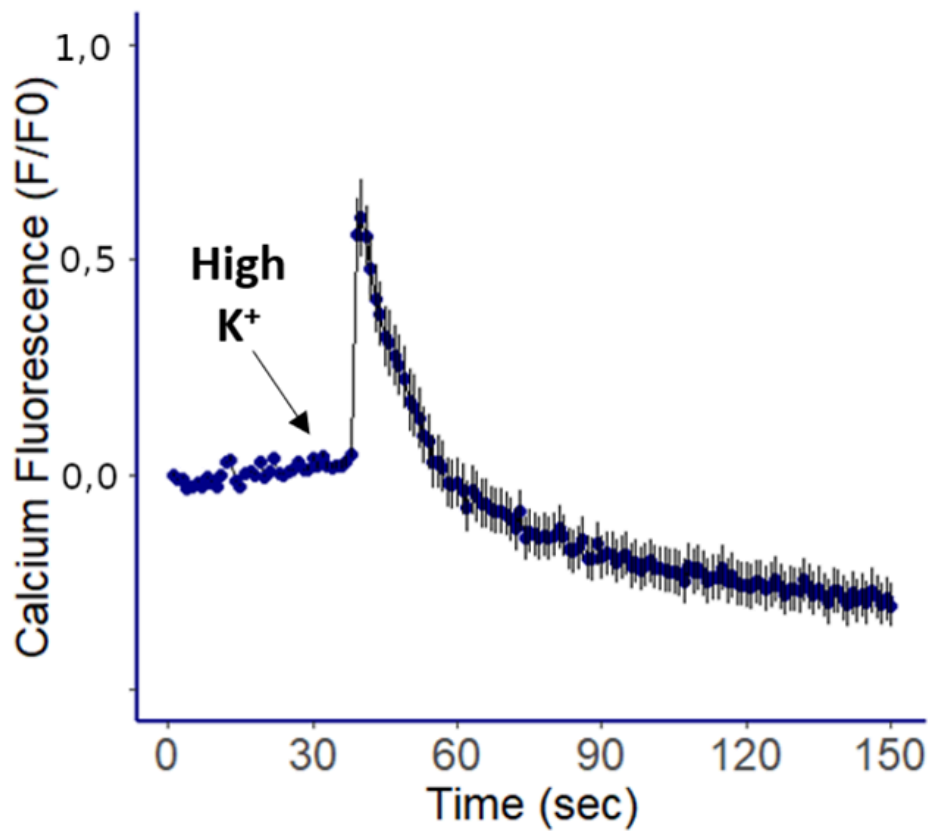


Figure 5.30 : Calcium fluorescence increase in positioned neurons upon stimulation.

Post-manipulated Huc-GCaMP6 zebrafish neurons retained their ability to respond to depolarising agents by an increase of intracellular calcium concentration. An automated protocol was developed in the workstation to apply solutions from a loaded pipette close isolated cultured neurons (24 hrs after positioning) and capture images each second for the next 2 min, using the fluorescence microscope. A single local application of 20mM KCl induced a rapid increase of intracellular calcium in adjacent neurons, monitored as changes in GCaMP6 fluorescence in the neurons. Values were normalised by subtracting each obtained value to the average basal fluorescence, during a 30 seconds interval prior to KCl application (n= 220 stimulated neurons).

Calcium imaging results showed that connected neurons in the generated arrays presented the typical response of stimulated neurons when a chemical synapse is present. After the baseline period, the application of KCl 20 mM in only one neuron produced a fast and transient increase in the intracellular calcium concentration in all the neurons forming the array. Therefore, these observed calcium dynamics resulted from the propagation of an initial action potential that subsequently triggered calcium entry in the postsynaptic neurons. The consequence, in physiological terms, was the activation of synapses present in the cultured neurons. This finding supported the presence of functional synaptic connections in the in vitro-generated arrays from the implemented process, which fulfilled the expected properties for its use as a biological model resembling a natural neural circuit. It is worth mentioning that the formation of these connections resulted from the free extension of axons after 24 hours, without any control system applied to limit their formation, including their number, structure, or involvement with specific neurons. Besides, displacement of some neurons from their original positions was observed again, producing connecting axons of variable length. However, this change was not long enough to modify the layer, presynaptic or postsynaptic, to which each neuron belonged.

5.18 Axonal Growth Direction in Positioned Neurons

At this stage, the generated process using the modified FrescoM workstation was capable of positioning neurons from primary cultures as a cell source using cell manipulation procedures. Besides, maintaining the cells in growth-permissive conditions induced growth of axons in the neurons, physical interaction with proximal neurons, and the later formation of synaptic connections. However, this formation of connections lacked the specific formation of synaptic connections to generate neural circuits matching a predefined structure. When cultured in vitro, zebrafish neurons were capable of forming synaptic connections that varied in number and length. Ramifications of a single axon were also a common feature in the cultures, either in the high-density samples used as the initial cell source or in the built arrays of neurons after the manipulation process. To prevent the growth of axons in random directions and the formation of connections other than those included in the original plan for the circuit under construction, a growth control system in neurons, other than fully permissive conditions, was required.

As previously described, several methods have been developed to restrict and direct axonal growth in cultured neurons, including the use of physical barriers, the formation of chemical cues, and the interaction with the neurons and processes. The next step in the process implementation was to find a suitable method to control the formation of precise neural connections in the positioned neurons, aiming to produce in the generated circuits only the intended number of synaptic connections. Aiming to generate an effective, simple and reproducible method to limit growth of axons, the explored options centered around the presence of chemical cues in the culture environment. Besides, considering that the culture dishes were coated with laminin and, as shown in the previous section, its presence dictated the number and length of axonal growth in cultured zebrafish neurons, several options to limit laminin to specific areas of the culture surface were investigated.

5.19 Laminin Strip Application from Droplets

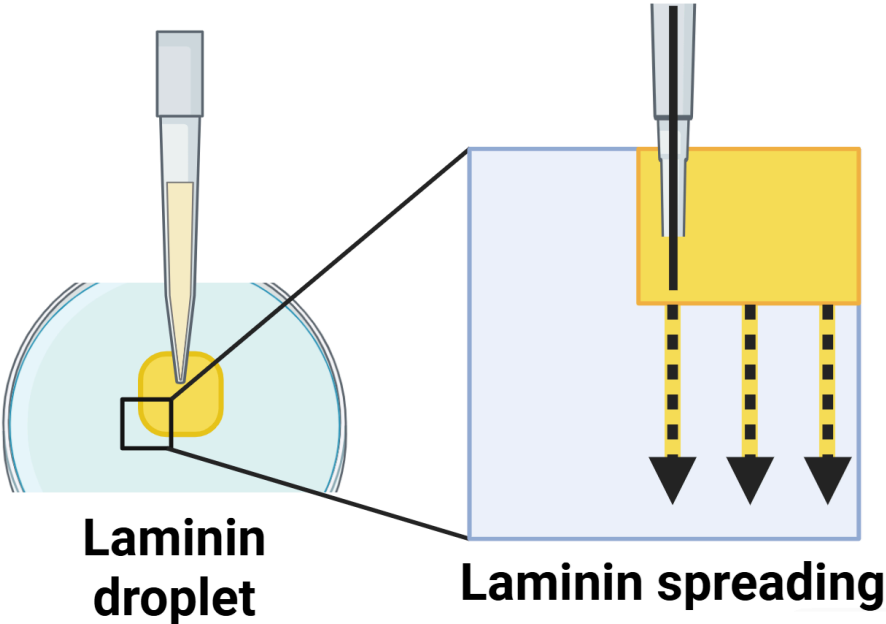
The first attempt to limit axonal growth involved the direct application of laminin to cover only specific areas of the culture dishes before the incorporation of cultured cells. These areas, forming strips, were intended to serve as lanes to restrict axonal growth to those regions to favour the connection of neurons positioned along the paths, substituting the reliance on random growth and eventual cell contact just by chance. Instead of applying a sufficient amount of laminin solution to cover the entire bottom dish and form a uniform layer, a small droplet of solution was applied in a limited area in the centre of the culture well. In parallel, a small device was fabricated by modifying the glass pipette of the FrescoM cell manipulator. A 50 μm gauge filament of polylactic acid (PLA) was made using a hot nozzle of a commercial 3D printer, and carefully placed inside the glass pipette with a short segment of the filament, around 50 μm , coming out from the pipette tip. This device was installed in the pipette holder from the cell manipulator and was intended to work as a brush to spread the droplet of laminin solution along discrete areas of the culture surface, similar to a painting procedure. The movement of this device was manually controlled using the same mechanisms as the cell manipulator and was restricted to the drawing of linear parallel patterns. **Figure 5.31** illustrates the procedure, including a schematic of the device and the output of extending a laminin droplet by performing the linear displacement of the filament along the well bottom.

Using the developed filament-based device, centrifugal laminin spreading was achieved, drawing parallel lines of around 35 μm thickness and 100 to 150 μm length when the filament was moved following a straight-line direction. Multiple repetitions of the procedure were required to create a uniform laminin layer along the lanes. Replication of the task in different x-axis coordinates allowed the progressive drawing of laminin strips that could be used to position neurons in defined layers. However, extension beyond length limits was difficult because the loaded solution amount in the pipette depended only on capillarity. Modifying the pipette diameter or filament length or gauge did not produce any improvement. Besides, the required amount of laminin in the droplets was elevated compared to the generated workable space on

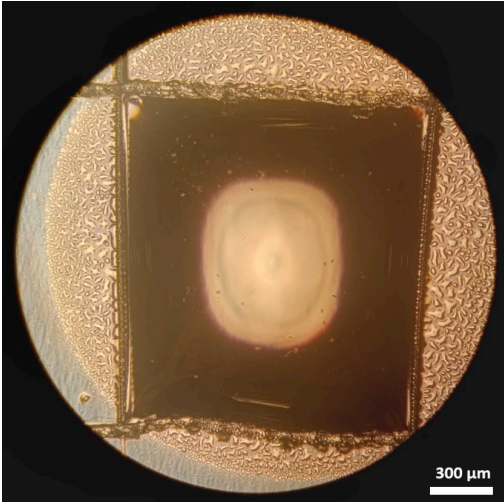
the strips. Moreover, the process was found to be time-consuming and inconsistent across multiple samples, requiring an approximate of 15-20 minutes per drawn lane by a trained user. Constant adjustment of the filament movement was needed, with unpredictable behaviour of the laminin distribution and, therefore, little space for automation of the task.

For these limitations, it was concluded that this process was not suitable as a reproducible and applicable large-scale procedure for the intended sample management of this project and, thus, was discarded for future application.

A



B



C

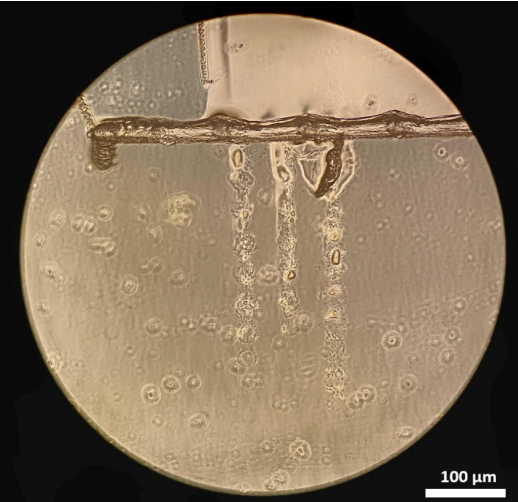


Figure 5.31: Laminin spreading from droplets

- A.** Aiming to restrict axonal growth on positioned neurons, laminin lanes were generated by applying a laminin solution droplet in the culture surface and manually extending the drop using a thin PLA filament mounted on the FrescoM micromanipulator pipette.
- B.** Application of laminin solution in the centre of the culture surface.
- C.** After multiple repetitions of spreading the laminin from the laminin droplet to the outer areas using the filament, short parallel laminin lanes were produced.

5.20 Formation of an NGF Gradient in Cultured Neuron Proximity

The second attempt to control axonal growth was based on a different principle. The method was intended to leverage neural trophic responses to growth factors. As previously mentioned, axonal growth direction is determined by molecular cues, such as neurotrophins, which are produced by the target cells during a process known as axonal pathfinding (Zang et al., 2021). These signals are present, forming concentration gradients that are sensed by the growing axonal cone (Paves & Saarma, 1997). Therefore, the next tested method aimed to artificially reproduce the formation of a neurotrophin gradient in the culture environment to direct axonal growth towards specific targets. If the neurotrophin concentration in one area of the culture was higher, i.e., around one layer of positioned neurons, the axonal growth of the cultured neurons should point towards this area. Moreover, the growth-stimulating signal could progressively move to direct the axon to a specific direction as the neurotrophin concentration remains constant.

The first test on this method analysed the gradient formation. To create a neurotrophin gradient employing the FrescoM components, the micromanipulator pipette was again used as a solution dispenser, as the original manifold of the workstation, but taking advantage of the now improved precision systems for positioning and solution application, as shown in **Figure 5.32**. A pipette of reduced diameter, around 8 μm , replaced the one used for cell manipulations to reduce the handled volume during the functioning of the connected syringe. The pipette was then filled with a solution of NGF 200ng/mL, which was chosen according to the increased trophic effects in cultured zebrafish neurons, in a concentration that doubled those used in culture media. This modification considered the fast protein diffusion with the distance when a soluble gradient is formed in a liquid, and the minimal amount of NGF required by cells for sensing and triggering of trophic responses. A Python protocol for the workstation was developed to progressively release the NGF from the pipette in pulses with constant intervals, positioned at a 45° angle, at a rate of ~5 μL NGF solution per minute. The total length of the protocol was 5 minutes of constant NGF solution release. Initially, the NGF solution was mixed with fluorescein to visualise the gradient formation with the epifluorescence microscope of the workstation and capture images during short and extended procedures.

As depicted in **Figure 5.33**, the protocol run resulted in the formation of a constant and uniform NGF gradient. As expected, fluorescein extended from the release site in the pipette tip to the surrounding area, reaching an area of ~50 μm diameter with marked fluorescence. The gradient was immediately observed a few seconds after the syringe activation, remaining constant in size and intensity throughout the entire testing period. **Panel A** displays the size of the generated gradient and the relative fluorescence intensity of fluorescein over distance. Values were calculated from the grey intensity value of pixels conforming to the gradient area in the captured images, throughout the protocol run period. These results confirmed the consistent gradient formation starting from the pipette tip, releasing the fluorescein solution to the surrounding areas, linearly decreasing the fluorescence intensity as the distance from the centre increased (Slope = -0.1437; R^2 = 0.942). The observed shape of the gradient, although mainly circular, was not always identical for all experiments. In an attempt to solve this, the gradient was applied in a single test, positioning the release pipette at a 45-degree angle. However, this resulted in no effect on gradient shape.

Once the size of the gradient was measured, the next step was to follow the consistency of these applications over extended periods, as in the case of cell growth experiments. **Panel B** displays measurements of gradient intensity over time, represented as grey values in the analysed area of the captured images, over several hours. Calculated intensity values found that the fluorescein intensity remained constant during the duration of the experiment (Mean Relative Intensity = 0.731). Despite maximal intensity being reached after 30 min of protocol run, these intensity values persisted throughout the entire experiment. No influence of fluorescence diffusing into the media was detected.

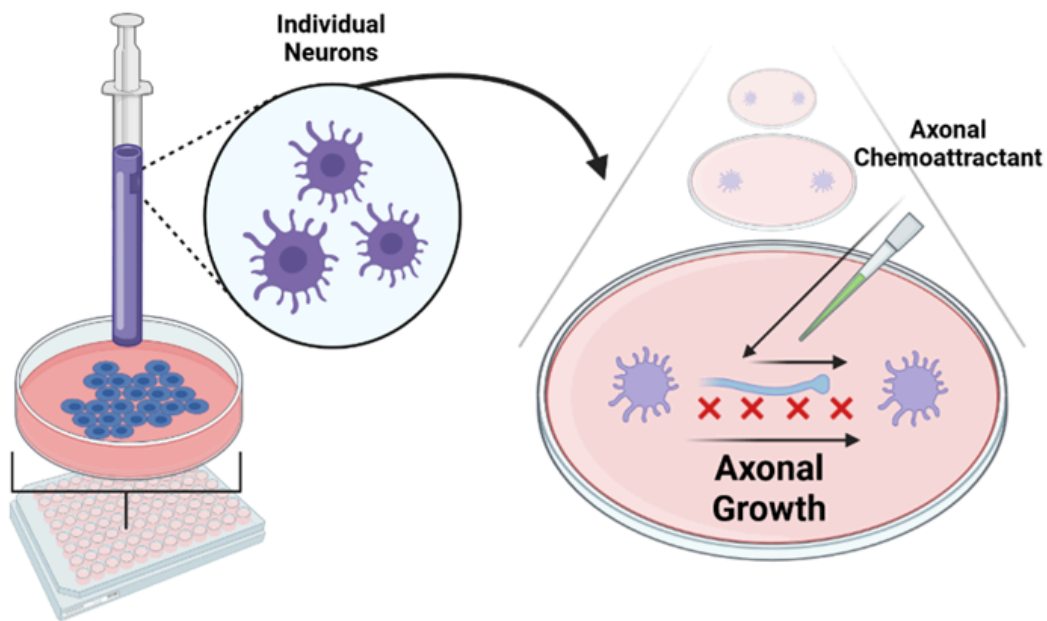
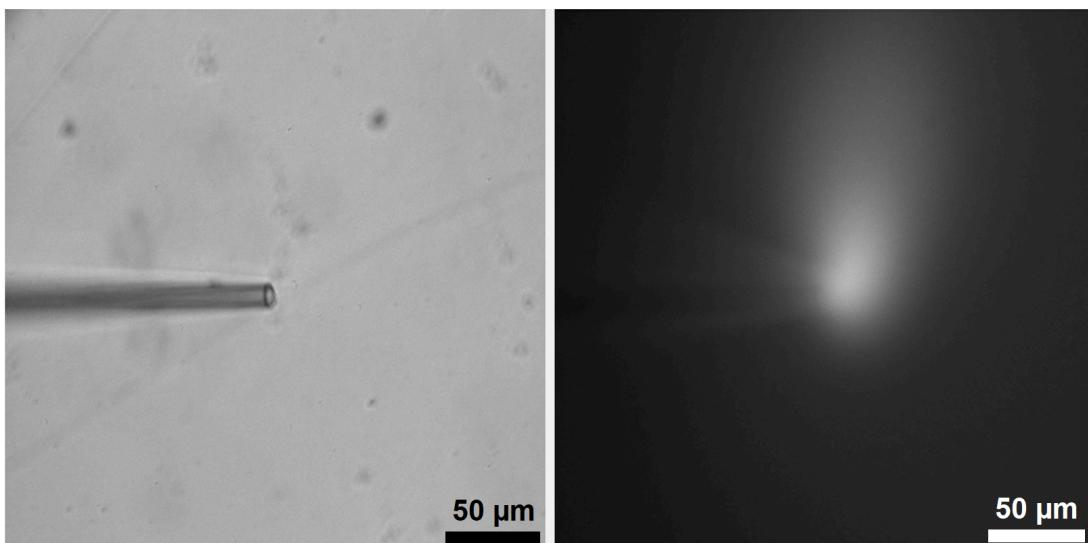
A**B**

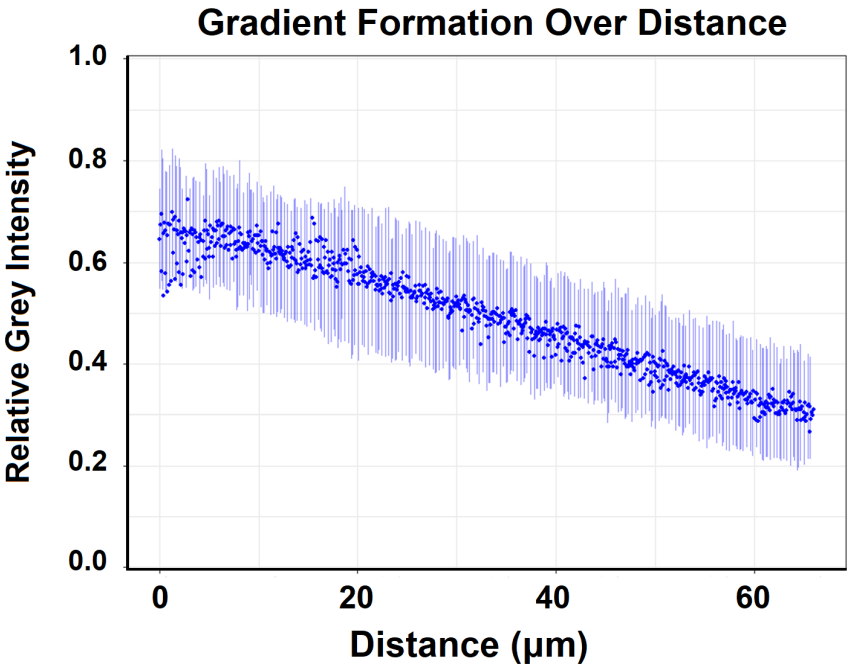
Figure 5.32: Neurotrophin gradient formation to direct axonal growth in cultured neurons

A. A method for the local application and formation of gradients of molecules intended to control neurite growth direction in the cell cultures was implemented. The procedure consisted of a modified pipette filled with an NGF-containing solution (200 ng/mL) mounted on the cell manipulator in a leaned position. The activation of the syringe in pulses controlled the release of the NGF solution adjacent to the neurons in culture. A gradient was formed by the free diffusion of the molecule of interest into the culture media, which could be moved as the axon grew to maintain the trophic stimulus constant.

B. Brightfield microscopy image displaying the size and position of the pipette used for gradient formation. The used pipette had a reduced diameter (around 8 μm), compared with the one used for cell manipulation, and was held at a 45° angle from the vertical to favour the release of the NGF-containing media in a specific direction.

C. The release and the gradient formation by free diffusion were visualised by mixing with the NGF solution with fluorescein dye before a sustained application of the solution into the culture media.

A



B

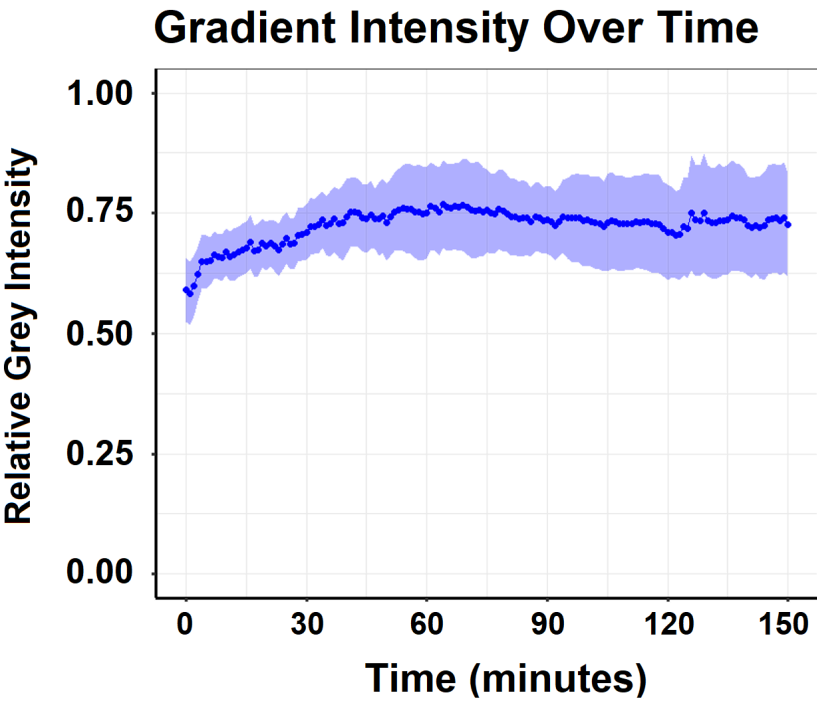


Figure 5.33: Features of the generated gradient using the FrescoM manipulator

The radiant formation was followed by filling the FrescoM micromanipulator pipette with a fluorescein dye and running a protocol for continuous solution delivery and imaging.

A. The amount of dye, expressed as relative grey intensity in the captured images, decreased with respect to the distance from the release site, confirming the presence of a gradient coming from the pipette release in the loaded solution (Mean $\pm$ SE, n = 4).

B. The intensity of the area covered by the gradient remained constant after prolonged application of the dye, reflecting the gradient consistency over extended protocols of cell growth (Mean $\pm$ SE, n = 4).

Once the sustained formation of a gradient was confirmed, the next step was aimed to apply a NGF gradient in the proximity of cultured HuC-GCaMP6 zebrafish neurons and to determine the effect on directing the growth of axons. This procedure applied the gradient formation parameters with the dispensing pipette, but reducing the released volume to a minimum (around 5 $\mu\text{L/h}$) by narrowing the pipette size, to prevent NGF diffusion through the totality of media volume in the culture well and avoid reaching a concentration that may influence neural growth. Also, the NGF was removed from the culture media composition, restricting the supply only to the NGF released by the pipette, through the formed gradient. The pipette was filled with NFG-containing NB media (150 nM) and positioned in the proximity of positioned neurons after 2 hours, when some neural processes became visible in the neurons. Then, the pipette started forming a radial NGF gradient over 10 hours and culture images were captured every 10 min.

The centre of the NGF-releasing pipette as well as the position of visible axonal cones, identified as dark spots at the distal end of processes, were annotated in the captured images. The net displacement of the axonal cones was calculated as $\Delta x = x_2 - x_1$, where x_1 and x_2 were the initial and final distances from the annotated axonal cone to the pipette centre in each frame (**Figure 5.34**). As shown, the tested method did not produce a marked attraction to the NGF release site. Although a minimal approach to the gradient occurred (Mean Distance = 10.3 μm), it was insufficient to bridge the minimal 50 μm distance separating individual neurons in the hypothetical arrays intended during further steps of circuit building. Thus, an NGF gradient was not an effective method for directing axonal growth over long distances in cultured zebrafish neurons and was not considered for subsequent use in the neural circuit assembling process of this project. Consequently, alternative methods were explored in the following sections.

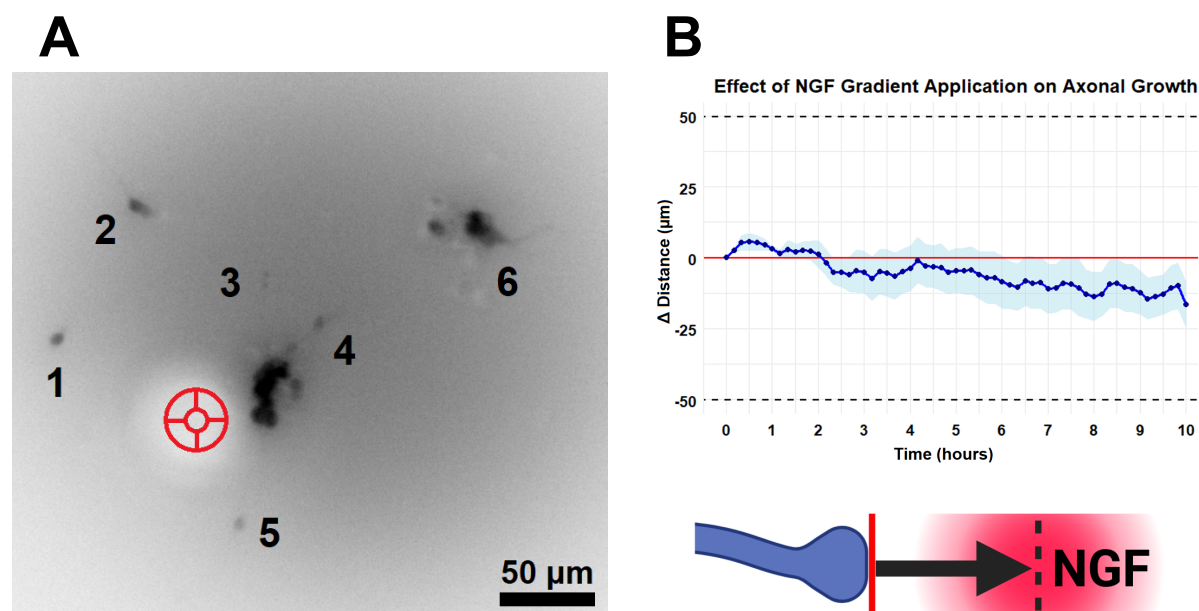


Figure 5.34: Effect of NGF gradient application on axonal growth

A. Annotation of the centre of the application pipette, and axonal cones from surrounding neurons with visible development of short neural processes, for monitoring axonal displacement.

B. NGF gradient effect on axons was estimated by measuring the displacement (Δd) from the pipette to each axonal cone caused by the local application of an NGF gradient over 10 hours. Horizontal lines indicate the initial distance (red solid line) and the minimal distance (50 μm , black dashed lines) required for axons to travel and connect positioned neurons in hypothetical neural circuits built, as shown in the lower panel (Mean $\pm$ SE, $n = 12$ samples, from 4 independent cultures).

5.21 Laminin Removal Using UV Lithography

The third attempt to control axonal growth tested an alternative technique to limit the presence of growth-permissive molecules on the culture surface, such as laminin. In contrast to the direct application of the protein in discrete areas of a clean surface, other methods can remove the protein after the formation of a uniform layer. In other words, the protein coating of the culture surface could be prepared as usual, followed by an additional treatment of the dishes or wells to remove the laminin from specific areas, leaving the growth-permissive properties in the untreated areas. These techniques include several types of microlithography, which rely on the exposure of the culture surface to a protein removal or inactivating agent, such as a UV-light or a laser beam, and the use of a physical device to limit this exposure to specific areas, as illustrated in **Figure 5.35**. This procedure can be performed using commercially manufactured discs of reflective materials, such as metals, containing predefined patterns at a microscopic level. When culturing neurons in microlithography pretreated surfaces, the laminin lines forming patterns serve as paths to restrict the growth of axons and dendrites to the areas where the protein matrix has been maintained and thus, guide the growing to reach specific targets. As neurons can form synapses after cell contact by proximity, this procedure can be applied to produce particular synaptic contacts in the cultured neurons, as cells and processes are confined to the laminin-containing areas.

A UV-based lithography method was implemented to apply this principle to the positioned zebrafish neurons in cultures, aiming to direct the growth of axons and create growth patterns in the culture well surface after subsequent culture maintenance. For this, laminin-coated culture wells were exposed to a UV beam after partial covering with a mask consisting of 3 mm transmission electron microscopy (TEM) nickel disks. These disks presented precise patterns at the microscopic level that reflected the incident UV light while allowing it to pass through the holes. Grid and parallel patterns were selected as initial patterns in the TEM discs, with a chosen line thickness of 48 μm and gaps between lines of 35 μm . To limit the permissive effects of laminin presence to the treated area, laminin pretreatment was only applied to the area covered by the disc. After laminin formed a layer on the culture surface, the solution was partially removed from the well, and one TEM disc was placed in this area. Then, the culture well was exposed to a lab-made UV lamp constantly generating a uniform UV beam for 1 hour, until the exposed protein was expected to be inactivated. Special care was taken to remove most of the well content, but to prevent complete drying of the well bottom, which was observed to affect the protein surface.

The custom UV lamp was composed of an LED-UV emitter module, a convex 8 mm lens, an LED driver and a metal support (**Figure 5.36**). Beam intensity was controlled using a knob in the driver. This lab-made device was employed instead of a commercial UV lamp due to the greater maximum intensity of the light, which was also focused into a beam instead of diffuse illumination. The beam size was similar to the TEM disc diameter. Once the exposure time had elapsed, the disc was carefully removed from the well surface to prevent disturbing the protein pattern, and the well was filled with a freshly dissociated cell suspension in supplemented culture media. The neural cultures were maintained in optimal conditions inside an incubator, and the growth was analysed after 24 hours. Culture images were captured using the FrescoM imaging system using brightfield and fluorescent microscopy. Image analysis was performed to evaluate the presence of neurons with growing axons.

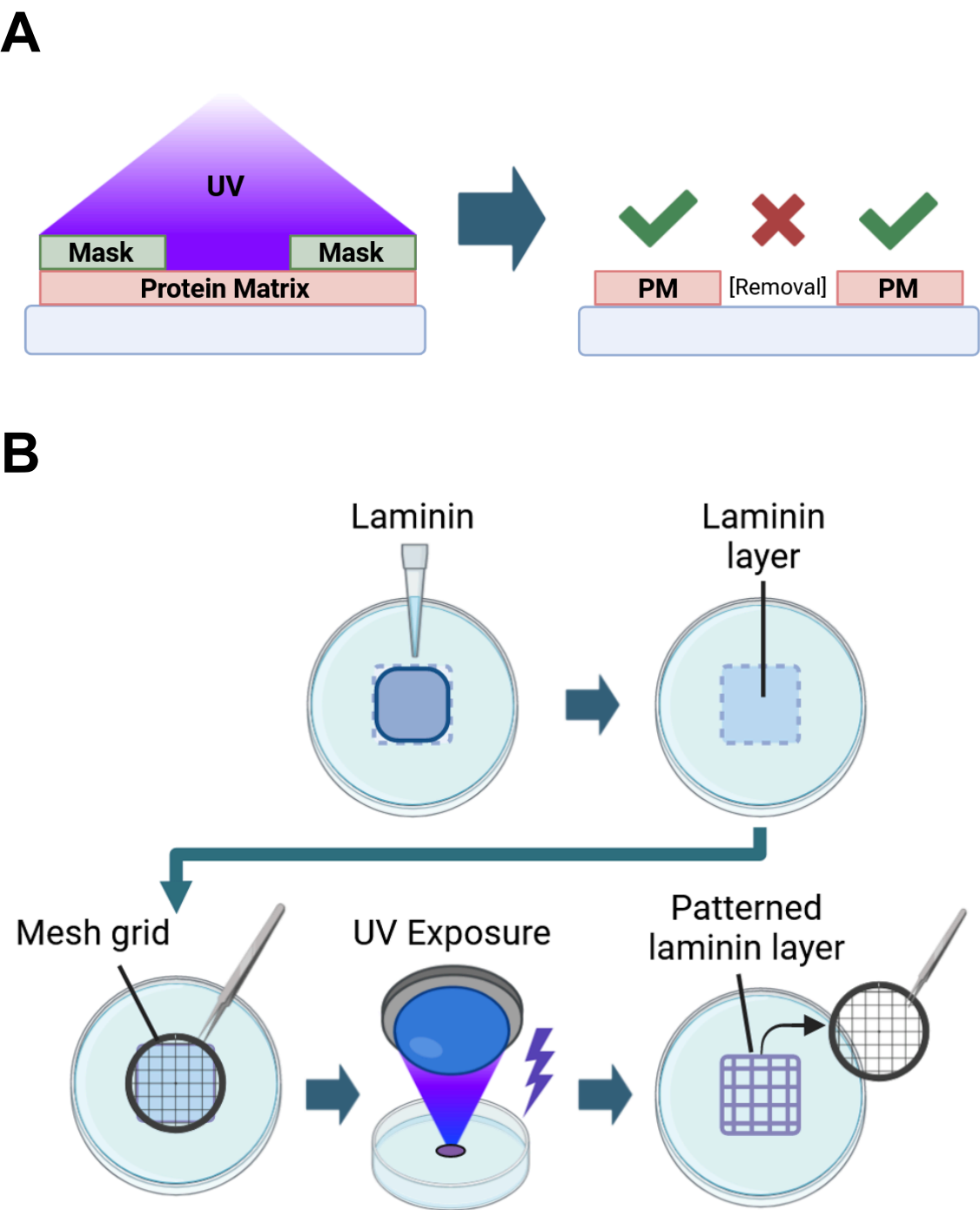


Figure 5.35: UV-based lithography to generate laminin patterns on the culture surface

A. The exposure of a uniform protein layer to a UV-light beam results in protein inactivation. By using a reflecting material as a mask, such as a TEM mesh grid, the exposure to a UV beam can be restricted to discrete areas, resulting in the generation of laminin patterns in the culture well surface that can be used to restrict axonal growth to the laminin-containing areas.

B. Laminin was applied in the culture dish surfaces to form a protein layer. Then, a TEM mesh grid disc was placed on the coated area and the well was exposed to a focalised UV beam to remove laminin from the exposed areas, resulting in a patterned lamin layer to hold cultured neurons and restrict growth to laminin-containing areas.

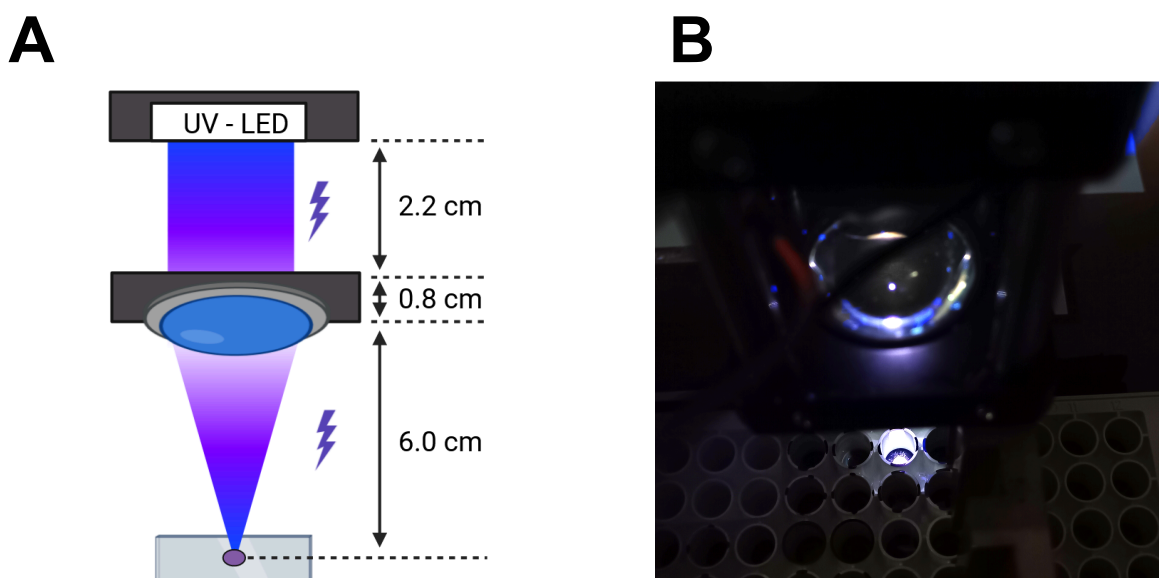


Figure 5.36: Custom UV Lamp used for UV-based microlithography procedures

A lab made UV-beam, composed of a LED-UV emitter module, a 150mm convex lens, an LED driver and a metal support was fabricated to fulfil the technical requirements for UV lithography in a 96-well system.

A. Schematic representation of the UV beam device components, including technical specifications for its use in a commercial 96-well plate (0.7mm well diameter). The dashed line indicates the focalised UV beam, corresponding to the illuminated grid disc location.

B. Picture of the UV beam device performing an lithography procedure in a 96-well plate. Note the UV-light beam focused on the mesh grid disc surface, placed on the well bottom.

The implemented microlithography method generated visible modifications on the culture well surface when observed with the brightfield microscope. After the TEM disc with parallel lines was removed from the culture well, some equidistant parallel lines were visible, matching the size and position of the original disc grid. These lines might have been due to the different presence or absence of laminin on the UV-exposed and covered areas, respectively. However, no additional analysis on protein identity was made to confirm this idea. After the UV exposure treatment, primary neural cultures of HuC-GCaMP6 neurons were placed in the wells, and they were maintained in optimal growth conditions in an incubator. However, in contrast to the expected outcome, no neurons were observed in any of the stripes after 24 hours of growth, as illustrated in **Figure 5.37**. Only a homogeneous population of small non-neural cells was observed in the laminin-containing bands, lacking the typical fluorescence expression of HuC-GCaMP6 neurons. Considering size, lack of the neural marker expression and the ability to survive in the absence of laminin, these cells were probably microglial, suggesting that the irradiated surface was not suitable for neural growth. The cause of this unexpected growth inhibition was not further explored. Still, it may include the total inactivation of laminin due to reflective beams that ended up irradiating the entire culture surface or the cytotoxic effect of nickel from the mesh grid discs.

Despite the potential of UV-based lithography to generate precise growth patterns after proper refinement, subsequent efforts shifted to alternative lithography types to maintain an intact laminin layer. Also, despite this method was no further explored culturing isolated neurons, it remained as an option to explore other possibilities in cell composition that could be suitable with this lithography-generated culture system.

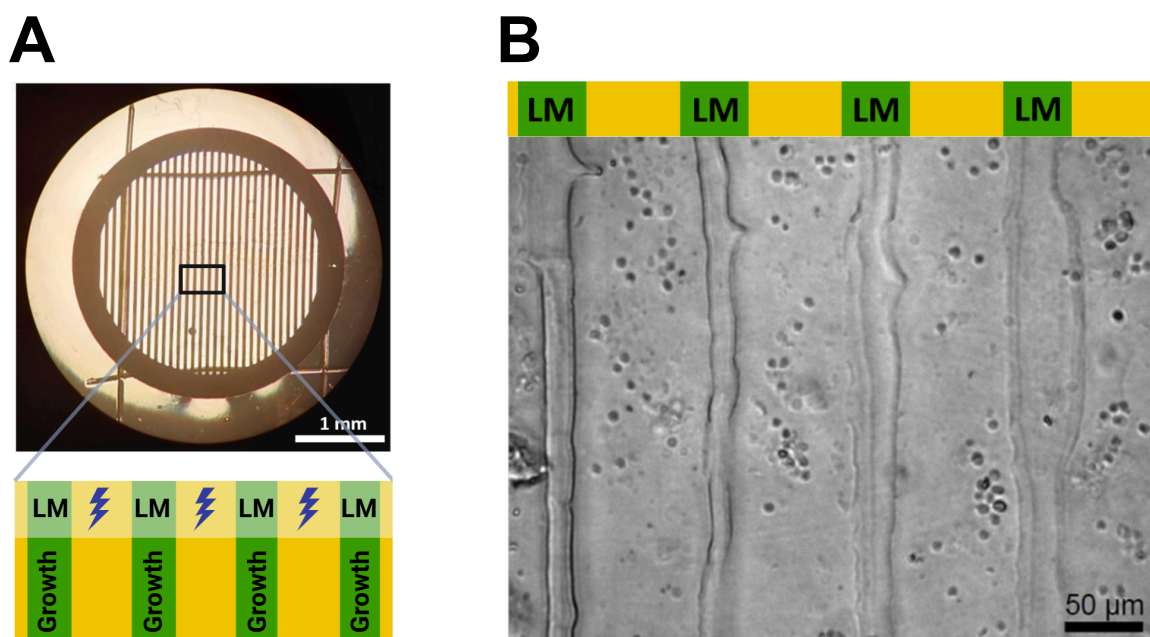


Figure 5.37: Cell growth on UV-generated patterns by microlithography

- A.** Resulting patterned laminin layer after UV irradiation. An altered pattern of stripes was observed, where laminin was either present or absent. Neural growth was expected in non-exposed bands.
- B.** After 24 hours of adding dissociated cells, only small non-neural cells, but no fluorescent neurons, were observed in the laminin-containing lanes, suggesting that the irradiated surface was not suitable for neural growth. The top banner indicates the expected laminin presence.

5.22 Laminin Removal Using Laser Lithography

Similar to UV light, other sources of high-energy beams can be used to remove or inactivate proteins forming a matrix locally. Lasers emit light that possesses spatial coherence, which means that the radiation beam travels in a completely straight trajectory from the source to the target. The incident beam interacts only with a focused area in the target material, without disturbing the surrounding external areas and, therefore, does not require the use of a reflective covering as a mask (**Figure 5.38**). Several commercial and custom laser lithography systems have been developed, linking a laser activation to a microscope system to control laser exposure on discrete areas at a small scale. The system can move the incident site of the beam, resulting in the generation of linear patterns. This has been refined with the development of dedicated photopatterning software that incorporates pattern drawing tools into a microscope user interface. Aiming to increase effectiveness and precision in laminin removal, a laser lithography method was tested to generate patterns on the laminin coating of culture dishes and restrict axonal growth in cultured neurons.

A laser-based lithography system, adapted from a FRAP device, was employed to work as a galvo switcher UV to perform the laser-based lithography. The system consisted of a confocal fluorescence microscope with an incorporated laser and a FRAP/photobleaching device, all controlled by the microscope software. This adapted system was capable of applying a 1KHz laser stimulus to a reduced area to engrave surfaces of organic films and plastic polymers. The laser power was in the range used for FRAP procedures and calibrated to produce an immediate and drastic reduction in rhodamine fluorescence on the culture dish surface when applied, without burning the material.

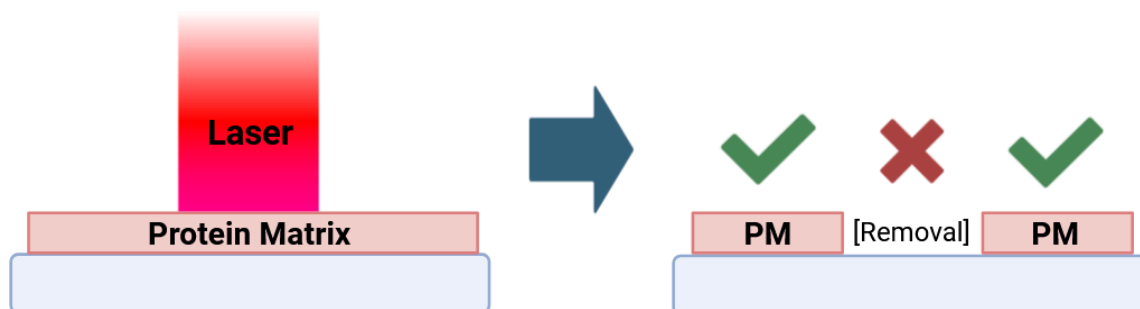


Figure 5.38: Laser-Based Lithography

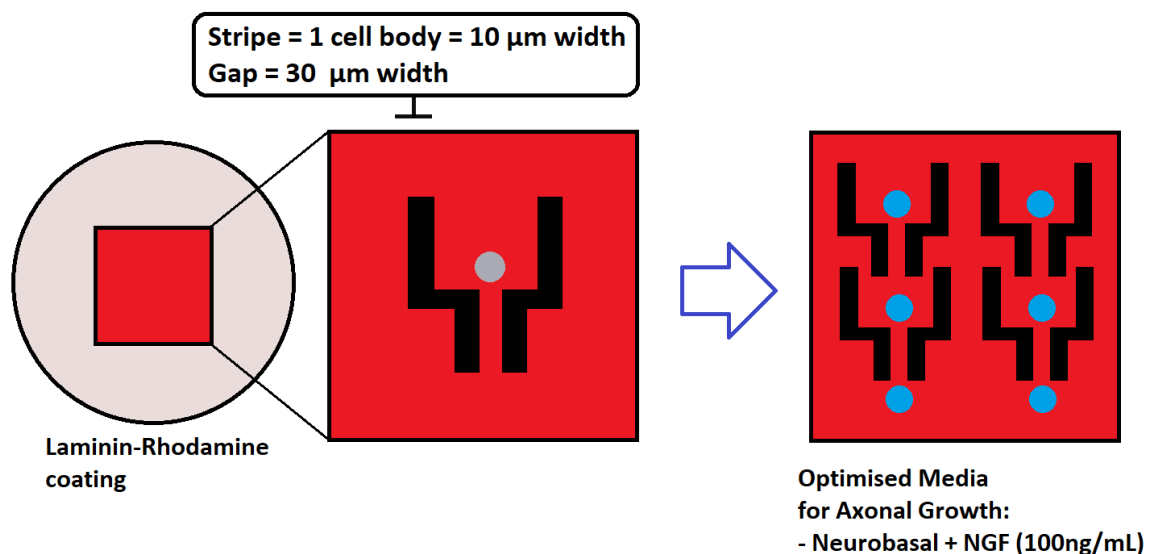
The laminin coating in culture dishes was exposed to a high-energy laser beam from a microscopy system, assisted by a software control to expose the culture surface to the beam and move the plate through the X and Y axis. This procedure produced specific linear patterns where the laminin was either removed or inactivated, without requiring a covering mask.

Culture dishes were coated with laminin-rhodamine, incubated similarly to a standard laminin solution to create a uniform protein matrix on the bottom of the culture wells. The conjugated rhodamine molecule allowed the visualisation of laminin by fluorescence microscopy. The culture dishes coated with laminin-rhodamine were subsequently exposed to the microscope laser beam (1KHz power) to remove protein from discrete areas, following the predefined pattern. The software-assisted procedure formed linear patterns on the culture surface, which prevented later rhodamine detection in these areas, identified as dark spots, when observed with the fluorescence microscope using the absorbance maximum for rhodamine at 565 nm. This lack of fluorescent signal in the surface was inferred to be caused by the removal or inactivation of laminin-rhodamine molecules in these areas. However, no extensive procedures, such as antigen identification, were performed to confirm these assumptions.

The microscopic resolution obtained with this lithography method was sufficient to sequentially create designs composed of horizontal and vertical lines of 35 μm thickness and variable length. Leveraging this engraving resolution, a couple of patterns were designed to restrain axonal growth in positioned zebrafish neurons. The first pattern consisted of a parallel straight lines pattern, same in size to the parallel pattern tested with the UV lithography method. The second pattern created an enclosed central area, similar to a chamber, with two apertures, with the upper one being wider (**Figure 5.39**). Lanes (35 μm width) were intended to enclose cells and neural processes, while the drawn lines (10 μm width), where laminin was removed, were used to prevent their positioning or crossing. Repeated production of these engraved chambers was intended to position a group of neurons inside, to produce an array forming linear cell layers, with the number of neurons established by the number of chambers in each row and column, and the separation distance defined by the chamber size.

Before running the lithography protocol, culture wells from a 96-well plate were prepared by incubating the wells with a laminin-rhodamine solution, similar to standard laminin. These laminin-covered surfaces were subsequently exposed to the microscope laser beam, following the predefined pattern and assisted by the photolithography software. Laminin removal or inactivation was confirmed by visualisation of rhodamine in the culture surface with the fluorescence microscope. Subsequently, neural samples from primary cultures of zebrafish neurons were added to the laser-exposed culture wells. After 24 hours of growth in optimal conditions, with supplemented Neurobasal media containing NGF, neural growth was analysed using the FrescoM workstation to capture images from the cultures. The performed analysis and results are described in the next section.

A



B

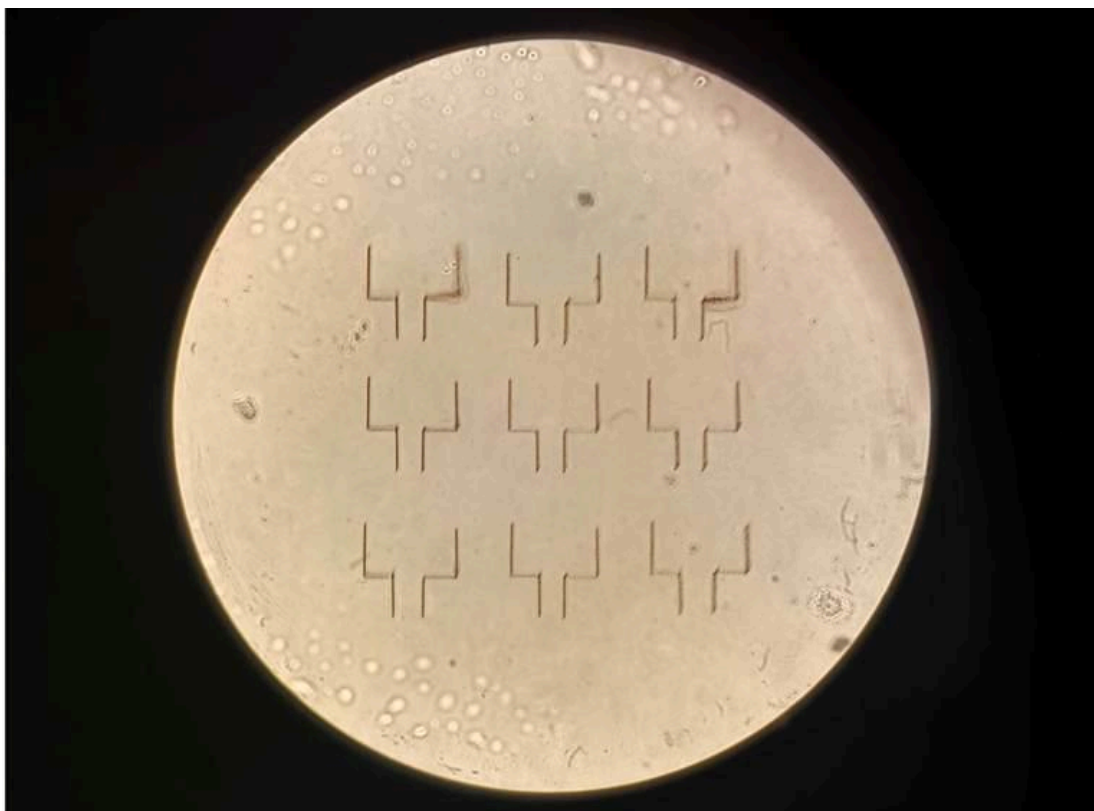


Figure 5.39 : Generation of patterns of laminin removal in the culture surface to restrict axonal growth using laser lithography

The linear removal of laminin by the X-Y displacement of the culture dish during the lithography process can be adjusted to produce more complex patterns designed to enclose cells in laminin-containing areas and limit the extension of axons, while promoting the growth to specific directions.

A. Proposed design of a chamber to promote the formation of simple neural circuits in cultured neurons. The dimensions of the central area were intended to contain individual neurons. Absence of laminin in the surrounding area, except for short segments, was aimed to serve as gates to allow maximal axonal extension only through these gaps. Reaching neurons positioned on the other side of the gates could promote physical contact between these neurons and eventual formation of a synaptic connection.

B. Example of the proposed lithography-designed culture system. This example was aimed at generating a 4x3 neural circuit after cell positioning on each side of the chamber gates.

5.23 Comparison of UV and Laser Lithography to Guide Axonal Growth in Cultured Neurons

The culture dishes with the engraved patterns, created using the laser, were then used to test axonal growth restriction on cultured neurons. To compare the performance of both lithography-based methods, similar patterns were generated either using UV or laser irradiation of the laminin coating, and cell samples from primary cultures were grown in parallel. With this purpose, HuC-GCamP6 neurons were transferred from 2dpf primary cultures to one dish treated with each lithography method, and maintained in growth-permissive conditions, Neurobasal media containing 100ng/mL NGF, to induce the appearance and elongation of growing axons. A generated pattern consisting of parallel lines of 35 μm thickness, corresponding to the laminin lanes, and 50 μm spacing was used for the lithography comparison. After 24 hours of cell growth in optimal conditions, axonal growth was analysed using the FrescoM workstation by capturing images from the cultures and analysing the number of neurons presenting axons using the CellCounter FIJI plugin, with the inclusion criteria previously described. Apart from isolated neurons, some cell aggregation was observed in the cultured cells, involving neurons and other non-neural cells, presumably neuroglia. Thus, counted cells were also grouped into one of these categories.

The observed axonal growth in culture dishes after each lithography treatment to remove laminin from discrete areas is illustrated in **Figure 5.40**. Some regions, indicated as bands in the figure, were expected to allow the growth and development of axons in the cultured neurons, corresponding to those stripes where laminin was not removed by each lithography method. In contrast, a reduced occurrence of them was anticipated for the bands without laminin. After 24 hours of culture growth, despite the occurrence of cells in both areas, axon development in neurons was only observed on the laminin stripe, where extending axons were found to exceed the established target length of 50 μm , connecting positioned neurons in the generated neural arrays. Axons were found growing along laminin lanes, with reduced or no presence outside of these boundaries in the analysed culture images. When present, axons running along other directions were shorter. The separation between the laminin lanes matched the size and position of the generated pattern, supporting the idea that the restricted growth was due to the alternating presence of laminin on the culture surface. It is noteworthy to mention that many long axons were observed in clustered neurons. However, the potential effect of this cell association on favouring neural growth was not further explored in this project. The generated axons and physical contacts observed after 24 hours of growth were followed in the subsequent hours, finding that they were maintained up to 72 hrs in some cases, and progressively disappeared as neural integrity in the cultures decayed over time. Finally, some migration of the cell somas along the culture surface was observed, either in the period between release from the pipette and attachment or during the first hours of the culture.

Considering the previous detection of cell movement, a quantification of the neural survival or axonal growth in each area would be influenced by the displacement of neurons from their original position. Therefore, a general counting of neurons was performed instead, independently of the cell location in the laminin stripes. Four groups were included in this analysis, depending on the presence or absence of growing axons, and the occurrence as individual neurons or forming clusters with other neurons and non-neural cells. After 24 hours of neural growth in Neurobasal media containing NFG 100 ng/mL, cell counting in these groups revealed a different occurrence of axonal growth depending on the neural association with other cells, as shown in **Figure 5.41** and **Figure 5.42**. Axonal growth, expressed in the percentage of the total number of neurons in the culture presenting at least one axon of 10 μm length or longer, was observed in 48% of clustered neurons and 28% of isolated neurons in the UV-pretreated cultures.

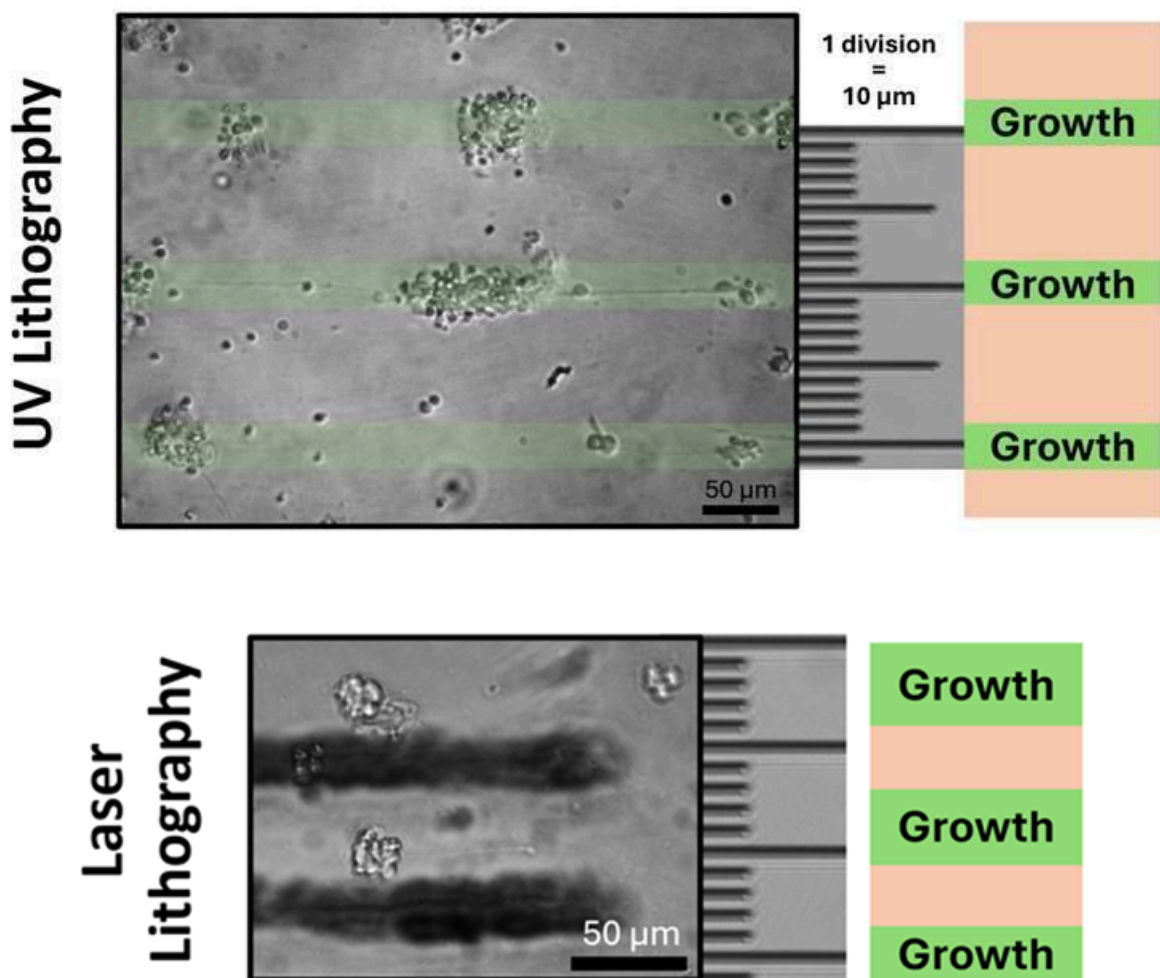


Figure 5.40: Comparison of the effects of UV vs. laser lithography to restrict axonal growth in cultured clustered neurons.

Selected neurons transferred from primary cultures were grown for 24 hours in culture dishes with patterns generated by either UV or laser lithography. The colours in the right indicate the areas permissive to axonal growth, depending on the maintenance or removal of laminin from the culture surface. In both cases, the presence of laminin restricted the axonal growth to the areas corresponding to the lithography-generated patterns. Cell clusters, formed by neurons and non-neural cells, were also observed in these regions.

Similarly, axonal growth was found in 41% of clustered neurons and 21% of isolated neurons employing laser lithography. Raw values on cell counts were compared, finding a statistically significant difference in axonal growth between the cell arrangements after performing a paired T-test comparing both groups (P-value = 0.028 and P-value = 0.030 for UV and laser lithography groups, respectively). Therefore, observed differences in axonal growth between clustered and individual neurons appeared to be independent of the method used for laminin removal.

The obtained results from this section confirmed the potential of microlithography techniques to restrict the axonal growth in cultured zebrafish neurons. Despite noticeable changes in the growth control that were observed, depending on the cell arrangement, the culture system was able to generate parallel growth of axons in both treatments. Although the presence of non-neural cells in the cell clusters could be a relevant event triggering axonal growth in cultured neurons, this role was not further explored during this project. Nevertheless, the potential contribution of these cells should be considered and further investigated, not only during the final maintenance of cultures but also from the design of cell purification methods and selection of maintenance conditions for the samples.

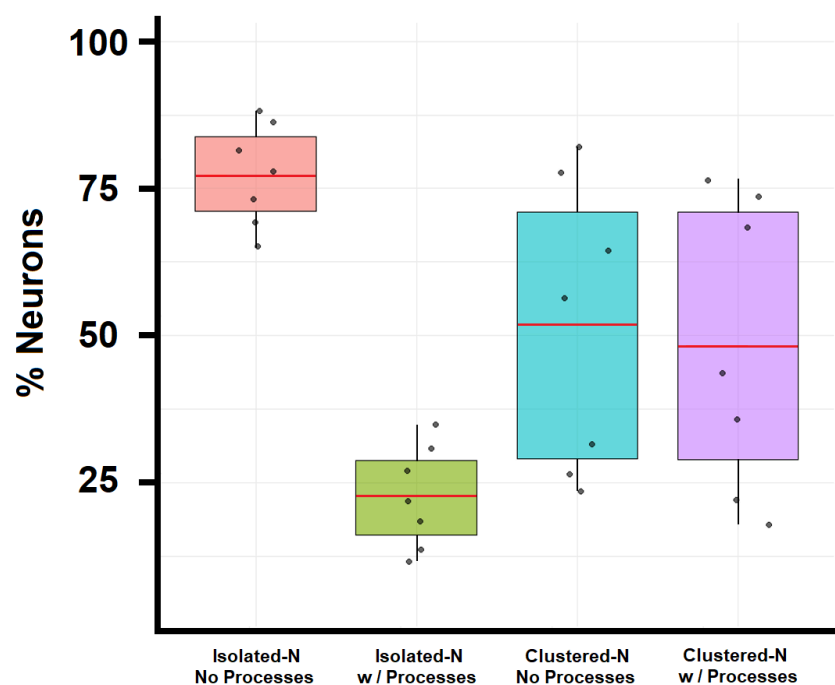


Figure 5.41: Development of growing axons on different cell arrangements in UV-treated culture dishes with laminin

After positioning in culture dishes with UV lithography-generated patterns, axonal growth was observed in neurons either individually and forming clusters with other neurons and microglial cells. Counting of neurons revealed that neurons forming clusters showed an increased prevalence of axonal growth compared to isolated neurons (n=6 UV-treated wells from 4 independent cultures).

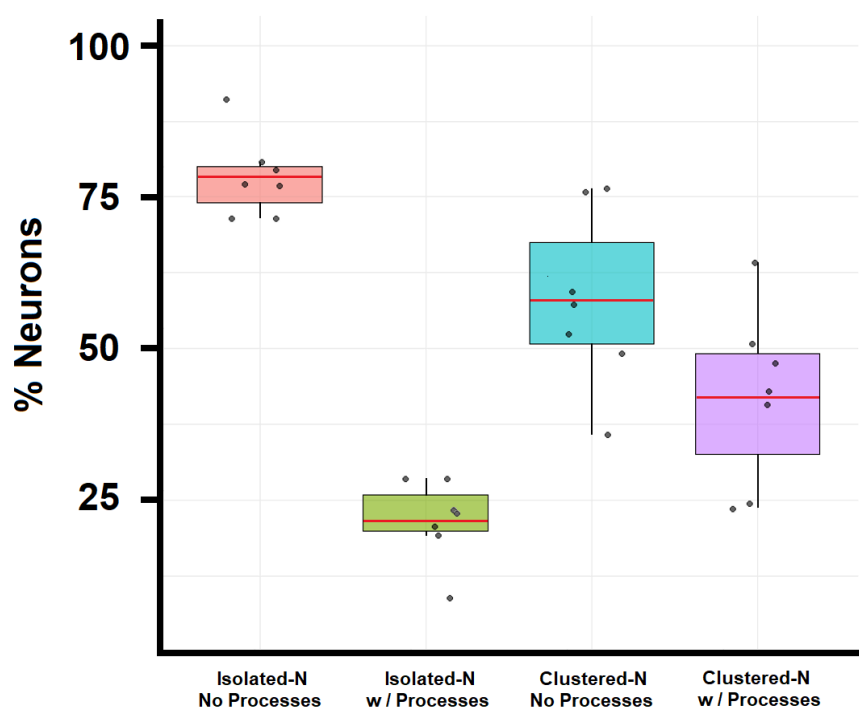


Figure 5.42: Development of growing axons on different cell arrangements in laser-treated culture dishes with laminin

After positioning in culture dishes with laser lithography-generated patterns, axonal growth was observed in neurons either individually and forming clusters with other neurons and microglial cells. Consistently with the previous method, counting of neurons found that neurons forming clusters showed an increased prevalence of axonal growth compared to isolated neurons (n=7 laser-treated wells from 3 independent cultures).

Finally, UV treatment of the dishes was selected to track the prolonged growth of axonal processes over the 3 days following neuron positioning. Results are shown in **Figure 5.43**, counting neurons with visible presence of neural processes and contacts with other positioned cells. It is worth noting that the number of contacts was highest in the first 24 hrs and then progressively decreased. A closer inspection by brightfield microscopy revealed cell death as a major cause of this reduced count.

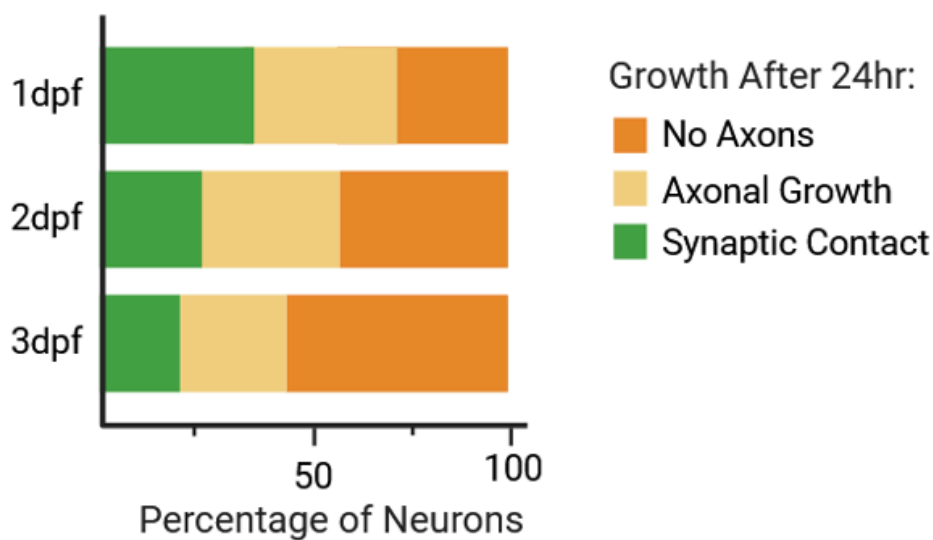


Figure 5.43: Proportion of positioned neurons in a UV-treated dish with observable growth of neural processes over time

Percentage calculated from counts on images captured every 24 hrs after positioning the neurons. A neuron was considered to have a neural process when it showed at least one neurite longer than 10µm. A synaptic contact was included if the distance of the observable end of a neural process was closer than 10µm to an adjacent neuron (n=26 cells).

5.23 Protocol Automation to Select, Transfer and Position Individual Neurons from Cell Samples

The full automation of the building steps described in the previous sections was required to integrate the individual project outputs into a methodology applicable to large-scale procedures. That is, the ability to perform cell selection and manipulation, and to build neural arrays, unsupervised and continuously. Besides, automation enabled adjusting the manipulation parameters, such as the movements of the workstation components, the pressure applied by the pipette, or the required times to complete each intermediate step, aiming to improve the performance of individual steps and, ultimately, the whole process. This optimisation aimed to generate consistent results that match the outputs with the original structural plan of the circuits after completing a routine protocol run.

The manual and semi-automated operation of the cell manipulator consisted of filling the pipette with one or more cells from a culture well containing dissociated neurons, transferring to the new culture and operating the syringe until one cell was released. The process was repeated with subsequent cells, varying the position of the cell release at demand. In contrast, the development of a fully automated protocol involved defining a fixed distance “d”, at which the final position of the pipette was displaced at the end of each round in either direction on an X-Y Cartesian plane, following the circuit structural plan. Initially, automation was tested in the simplest circuit structures, such as 1x2 and 1x3 arrays, consisting of 2 or 3 cells aligned in a

row, respectively. In this case, the pipette was displaced only through the X axis (**Figure 5.44**). It is worth noting that once the protocol run started, no further interaction with the software was required for the remainder of the protocol. Also, images were captured at the start and end of each intermediate step (cell picking or release) to track protocol progression.

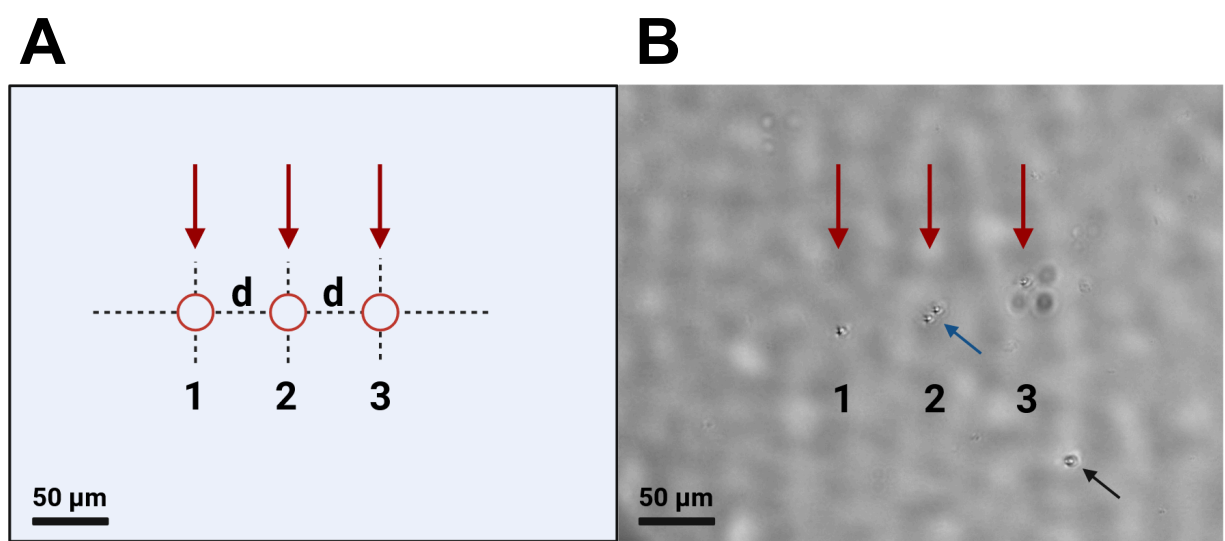


Figure 5.44: Automated positioning of neurons in predefined positions

A customisable automated protocol was developed to perform the complete building of simple neural circuits from samples of dissociated neurons. The protocol consisted in cycles including the selection of individual neurons from the cell samples (primary cultures of HuC-GCaMP6 zebrafish neurons) and the transferring of the picked neurons into new cultures at specific positions.

A. The tested protocol was based in an X-Y cartesian plane. It consisted on a progressive transferral of individual neurons to target sites (circles) at a given distance d , forming arrays of three aligned neurons. After releasing a neuron in the first position, the protocol adjusted the position for the next cell release (red arrows) by moving the culture well plate and, therefore, the relative position of the pipette releasing neurons.

B. Example of an obtained neural circuit after completing a run of the automated protocol. Resulting arrays consisted mainly in single neurons positioned in the proximity of the target sites. However, in some situations the cell manipulation placed more than one neuron at a given site (blue arrow), no neurons at all, or out of the target position (black arrow).

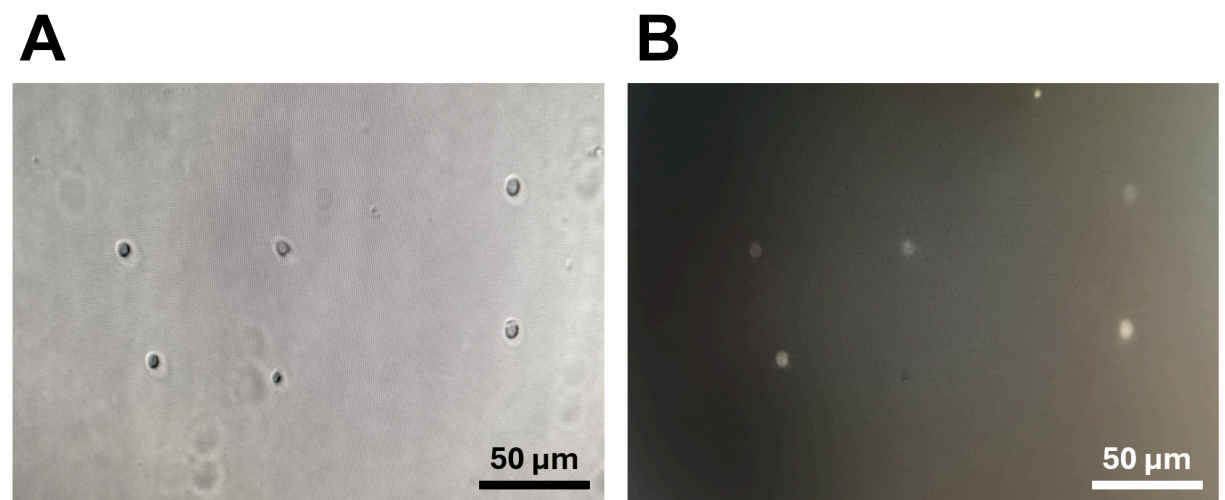


Figure 5.45: Automated generation of neural circuits with multiple layers

The capability to generate more complex circuits as a fully automated procedure was confirmed by producing 2x3 neural arrays. The number of cells along the X and Y axes was customisable. Neural identity of the selected cells was confirmed by GCaMP6 fluorescence. Tracking of the building process was performed by brightfield (**A**) and fluorescence (**B**) imaging on the sample.

Displacement along the Y-axis was subsequently incorporated into the automated protocols to produce multilayered circuits. Generation of 2x3 arrays was selected as the structural plan for this test, yielding the desired structure as output (**Figure 5.45**). The same distance “d” was maintained during displacements through either the X or the Y axes. Some movement of the positioned cells was observed after release, to the same extent as previously observed during manual operation of the manipulator (see Figure 5.24). Due to time limitations, more complex arrays were not included in this project. However, the corresponding variable defining the number of cells along the X and Y axes was included in the Python protocol files for easy identification and customisation. Finally, the placement position can be adjusted by modifying the “d” parameter or by including multiple distances to meet specific structural specifications. Although culture wells were completely coated with laminin at this stage, the distance can match, for instance, the laminin lines drawn by any of the lithography methods implemented in this project, thereby positioning the neurons in areas where their growth can be controlled.

To conclude with Section 3 of this thesis, the main outputs can be listed as follows:

- A procedure was implemented to select and release individual neurons from primary cultures of neurons to empty cultures using the installed cell manipulator in the FrescoM workstation. The task showed high precision when applied to the repeated transfer of cultured neurons from samples of dissociated cells.
- An automated method, based on fluorescence expressed by HuC-GCaMP6 neurons and continuous image scanning, was developed to identify the selection and release of individual neurons from the micromanipulator pipette.
- Three methods to promote the growth and direction of axons in the cultured neurons were adapted to the culture system, including the formation of a neurotrophin gradient and microlithography of the culture surface using UV light and a laser. Although variable outputs were obtained, the microlithography methods generated growth of axons in positioned neurons following specific pathways.
- A customisable automated protocol was developed to constantly perform the complete process of identification, manipulation and positioning of fluorescent neurons from cultures into simple neural arrays of defined structure.

This section provided experimental evidence supporting the use of the FrescoM workstation to handle primary cultures of neurons to select and transfer individual neurons at specific positions, to generate simple linear arrays. Although this progress only represented a first step in the in vitro replication of complex neural networks, this contribution laid the foundations towards the fabrication of neural circuits at the single-neuron resolution.

To further improve the efficiency and reproducibility of the generated circuits, it was still necessary to refine certain tasks, such as long-term monitoring of axonal growth in positioned neurons and the formation of synaptic connections. This could enable performing the circuit-building protocol as a completely unsupervised, long-term process and exploring parameters to find the optimal conditions for neural culture, growth control, and consolidation of the circuit structure. Still, this section, combined with the rest of the project, has provided the technological and methodological bases to serve as a starting point for process optimisation and for generating other output circuits with specific architectures and cell compositions.

VI. DISCUSSION

6.1 A Bottom-Up Approach for the Study of Neural Circuits

Neural circuits are anatomical entities that translate the unique structural and functional properties of individual neurons into complex networks capable of performing specialised neural functions, such as sensory perception, motor tasks, learning, or memory. An accurate elucidation of the structural and functional properties of neural circuits is essential for describing normal neural function and the alterations associated with pathological states, such as the onset and progression of neurological and neuropsychiatric diseases (Marei et al., 2025). Experimental models that replicate naturally occurring neural circuits *in vitro* have aided this endeavour by integrating recent discoveries in cell biology and technological devices. The result has been the production and maintenance of *in vitro* samples that enable interaction with individual circuit components and allow measurement of information processing in controlled environments (Bang et al., 2021; Chow et al., 2022). In this project, the main objective was to establish a procedure to consistently generate small *in vitro* arrays that resemble elementary neural processing systems composed of a few neurons.

The Bottom-Up construction of neural circuits involves adding individual elements to a system built from scratch, providing an environment in which the number and positions of constituent neurons are tightly controlled (Mofazzal Jahromi et al., 2019). The ideal progression of this building process yields an *in vitro* system composed of specific neural elements and connections, serving as an experimental model to follow neural processing from the entry of a neural stimulus to the generation of a response. An accurate description of the information processing, as the transition through a limited number of intermediate steps, requires the identification of all neurons involved at each stage, their interactions with other neurons, firing patterns, plasticity capacity, spatial orientation, distribution molecules involved in synaptic communication and other features, which can integrate a profile of what we can expect as an output from a given input. This principle, the basis for circuit modelling, explains deviations in the circuit behaviour due to structural inaccuracies (Koren et al., 2023). Besides, a stable circuit structure over time and the prevention of interference from external elements are desirable features for identifying internal connectivity patterns or network motifs. This internal processing, involving a limited number of neurons within the circuit, is often masked by its low signal-to-noise ratio and represents the core of activity-regulatory mechanisms (Braganza & Beck, 2018).

Techniques for generating *in vitro* circuits from scratch require the direct physical manipulation of neurons and the progressive performance of a series of steps on neural samples from the most elementary scale, starting by selecting a neuron, positioning in a specific arrangement with others, defining the circuit architecture, and providing all the necessary conditions that allow these units to maintain and grow into ensembles with stable synaptic connections (Hasan & Berdichevsky, 2016). However, structural inaccuracies have been a common problem in their construction (Swanson et al., 2022). In many fabricated systems, such as organoids, 2D and 3D cultures, and transplanted cells, groups of neurons are grown unrestrained in permissive environments, with minimal control over the number and identity of existing neural connections (Li et al., 2025b; Paşca, 2024). This lack of structural restraint produces different structural outputs from the same process. An explanation lies in the developmental mechanisms that shape natural nerve circuitry over the lifetime, including intrinsic plasticity, compensatory effects, and chronic adaptations that modify the occurrence of synaptic connections and tune nerve function (Südhof, 2015). Methods for generating invariant artificial neural circuits enable experimental reproducibility and lead to reliable, robust conclusions (Park & Carmel, 2016).

A proposed solution to increase building precision has been the incorporation of one cell element at a time into the system, in the correct spatial and temporal order, and the restriction of neural process growth by mechanical confinement or by using a pretreated surface to discrete areas (Liu et al., 2022). Although this is a laborious task, this method can limit the number of cells and synaptic connections in the system, ensure circuit mapping (Goshi et al., 2020), and facilitate tracing neural processing events at single-neuron resolution (Rabadan et al., 2022).

The current project applied the combined strategy of manipulating cultured cells from primary cultures and employing a lithography-generated culture surface. To ensure reproducibility, the automated FrescoM workstation (Katunin et al., 2021) was adapted to produce a first-of-its-kind device specifically designed for the in vitro construction of neural circuits.

Automated workstations have become increasingly relevant in biological research in recent years. Their applications have included handling, maintaining, and processing biological samples; large-scale drug assays; and generating high-throughput data across task repetitions. Also, their use has extended to clinical environments to obtain fast, accurate, replicable and accessible results for diagnosis (Antonios et al., 2021). These favourable features have been made possible by consistent control of testing conditions and the large number of samples processed simultaneously. In the field of Neuroscience, automated workstations have also gained relevance by assisting some routine procedures, such as in vitro cell detection and patch-clamp recordings (Wu et al., 2016; Holst et al., 2019).

The original design of the FrescoM workstation leveraged automated manipulations to develop a standardised, reproducible circuit-assembly process from cultured neural samples, capable of generating simple neural arrays with defined structure and incorporating monitoring tools to track neural function, with the potential for process automation to enable continuous task execution and, therefore, completion within a shorter timeframe compared to manual manipulations. A few examples of the use of automated workstations have recently emerged to study neural circuits in cultured cells, such as the placement of neurons in microstructures to limit the total number of processes forming connections during electrophysiological recordings (Girardin et al., 2022) or the transfer of individual cultured neurons to microscaffolds (Striebel et al., 2025). However, these examples require manual operation of the workstation for a considerable part of their processes. Also, these workstations were less suitable for routine implementation because they relied on expensive equipment and materials.

6.2 Development and Implementation of a Functional Module for Single-Cell Manipulation

The FrescoM workstation represented a notable option for experimental automation, designed for technical procedures involving cell samples that require continuous imaging and manipulation of media composition (Katunin et al., 2021). Effective operation relied on customisable parts running in a Python-based environment, enabling unsupervised execution of specific tasks. Among its main advantages over similar commercial platforms, this workstation features a compact structure, low acquisition and operational costs, easy assembly, and an open-source design. Other similar open-source systems have recently been developed to automate single-laboratory tasks involving the handling of cell samples, mainly cell culture pipetting systems, cell incubators, and microscopy systems (Rothmiller et al., 2020; Dettinger et al., 2022). Functional enhancement has also been achieved by incorporating several manipulation and monitoring devices into a single workstation, as in the OpenWorkstation (Eggert et al., 2020). However, its design, inspired by a linear assembly line, required continuous repositioning of the sample container, resulting in substantial increases in space and mechanical requirements. In contrast, versatility in the FrescoM workstation was achieved by fixing functional modules in an enclosed metal frame and displacing the components around the sample at the centre, thereby minimising space requirements while increasing the repertoire of functions performed on the processed sample.

This project demonstrated the adaptability of the FrescoM workstation by proposing a novel technical procedure for the controlled assembly of neural circuits from cultured neurons. To achieve this main objective, the framework incorporated the appropriate equipment into the design to replicate the necessary manipulations of neural samples in the FrescoM workstation. However, the potential of the FrescoM workstation can be extended to more procedures involving the handling and imaging of cultured cells. For instance, ongoing work in Dr Anton Nikolaev research group has modified the workstation with distinct components and functional modules to extend the applications, such as extensive calcium imaging in cultured cells by repetitive cycles of image capturing in cells upon stimulation, differentiation of cultured stem

cells by modification of culture media composition at specific growth stages, and the generation of processing algorithms for imaging analysis by the generation of large-scale databases of cell cultures. The outputs of these research projects can be found in Zhang et al. (2025) and in the University of Sheffield 2025 and 2026 thesis databases.

Maintaining a new design of workstation in the form of functional modules enclosed the necessary components and cabling to perform specific functions in discrete areas, with simple installation requirements for fixing to the structural extrusion bars and the cabling network of the electronic components, allowing access to a power supply and sharing information with the rest of the platform. Also, the distinguishable component grouping was easy to install and to repair in the event of a malfunction. This advantage was reinforced by the preference for simple mechanisms over complex ones to perform functional tasks at the workstation without sacrificing precision, which also helped keep assembly costs (£400–600 without and £2,500 with a 1-channel fluorescent microscope) and maintenance needs within an affordable range. The addition of the new components did not represent a considerable increase in cost (around £50), since some parts, such as the motors and extrusion bars, were repurposed. Connecting components was also easy, thanks to additional plug pins on the main PCB, which expanded the number of components the microcontroller could manage. Also, a new PCB can be designed thanks to the accessible PCB blueprints.

The openness philosophy of the developed platform was maintained in the incorporated Python-based software by allowing the straightforward inclusion of additional elements into the system to receive, process, and utilise data generated by the components. Python constitutes one of the most extended and accessible programming languages, known for its simple syntax for new users, as well as a wide and enthusiastic community. A growing collection of Python libraries have provided a repertory of tools serving as an efficient interface for user-machine communication (Perez et al., 2011). A few applications of Python developments applied for this project comprised the establishment of functions and operative protocols, extensive data management, coordination of hardware operation and generation of user interfaces.

These advantages of a design based on functional modules also contrast with the conventional designs of commercial workstations, which often feature inflexible designs and reduced customisation options. High acquisition and maintenance costs are also major concerns, with prices starting from £10,000 for basic automated culture system devices. Task programming, maintenance and repair were also significant issues faced by users due to the required assistance of staff from the manufacturer, which often resulted in stopping the process performed by the workstation until the problem was solved.

For this project, the new functional module, which performed cell manipulations, replaced the original top manifold on the previous version of the workstation. Instead of eliminating a pre-established function, the new module provided increased performance and precision not only for applying and retrieving liquid samples, as demonstrated by the adapted pipettes used for local solution application, but also for applying this refined media handling to manipulate cells at the bottom of a culture surface. Reliable control of flow-rate generation has been a key step in the development of useful automated dispensing systems for handling liquids and, especially, solutions containing dissociated cells (Lee et al., 2019). The ability of the developed micromanipulator, consisting of a mounted pipette connected to a precision syringe, to operate with precision was also confirmed during this project. However, it is worth noting that the increased control in the modified system for handling small volumes also reduced the former ability to perform a rapid media washout in the culture wells prior to the modifications. The resulting device complied with the requirements to serve as a cell manipulator, but operating parameters may vary in the event of future technical needs.

Component customisation was maintained in the workstation design and extended to individual parts. A notable example was the set of variants of the fabricated pipettes mounted in the cell manipulator. Depending on the pipette diameter, the same device was used for diverse functions, including cell handling, local solution application, and a painting device for lithography when installing an internal filament. The ability of a given component to perform multiple tasks has been explored by other authors, who coined the term “Swiss-army-knife component” for

such multifunctional parts (Rupp et al., 2025). The chosen manipulator parts also considered other potential applications not explored in this project, such as incorporating an electrode into the pipette holder to record cell activity. Other modifications were intended to increase precision on previously established functions. For instance, steel ball screws replaced standard screw rods in the main X-Y mobile stage to reduce step size and backlash during well plate movement. Finally, other changes only facilitated the user experience, such as adjustments to the software interface or aesthetic enhancements, including a 3-D-printed rigid console to enclose the PBC and some electronic components.

6.3 Implementation of Optimal Conditions for the Obtention of Primary Cultures of Neurons from Embryonic Zebrafish

Accurate models for investigating human physiology are crucial for understanding biological processes and diseases, including research on neural circuits. The ethical and physical constraints on performing experiments in human patients have led to the use of animal models to study conserved structures and processes. Small mammals, such as mice and rats, have commonly served for this purpose with a wide repertoire of cellular and molecular techniques applied to study circuit function and structure in individuals. Their contribution to research has extended to in vitro studies, establishing refined protocols for maintaining and monitoring isolated neurons and neural arrays (Neziri et al., 2024). However, the advent of large-scale studies as a prominent strategy to yield robust conclusions on circuit mapping and descriptions of information-processing pathways has exacerbated the flaws of rodent models in producing the large numbers of biological samples required for these studies (Vandamme, 2014). Rodents are expensive to maintain in dedicated facilities, and reproduction rate is dictated by a prolonged gestation period of several weeks, a small litter size and the occurrence of oestrous cycles (Parra-Vargas et al., 2023). Besides, rodents are difficult to manipulate at embryonic stages, when obtaining nerve tissue for dissociated cells or primary cultures is more feasible. Also, this developmental stage is employed to produce genetically modified strains to investigate the function of specific genes and proteins.

The inherent limitations of rodent models have been addressed by exploring alternative species, such as zebrafish. The advantages of this organism for obtaining large numbers of nerve tissue samples for extensive studies stem from its ability to maintain and breed large numbers of individuals in limited space with reduced care compared with rodents. This is complemented by a wide availability of organisms for experimental purposes, due to a rapid early development, capable of generating usable animals in a period as fast as two days after egg fertilisation, combined with a frequent breeding availability of the adults, able to repeat mating cycles in a few days, and the abundant offspring with each breeding, typically producing hundreds of eggs. The external fertilisation of zebrafish and the transparency of their eggs have also been leveraged to facilitate embryonic manipulation, in contrast to the physical obstacles faced in rodents, enabling genetic editing techniques such as directed mutagenesis, gene overexpression, and knockouts. The advent of the complete sequencing of the *D. rerio* genome over the last decade has enhanced the repertoire of available genetic libraries in zebrafish. Also, the detailed inspection of genetic expression profiles and their effect on nerve function and neural circuit conformation in zebrafish, has confirmed their close similarity to in vitro mammalian neurons, including human neurons (Barbazuk et al., 2000), which poses a major drawback when using other sources, such as laboratory-stable cell lines. In conclusion, the particular advantages of zebrafish have established it as a convenient animal model for procedures requiring sustained, extensive access to nerve tissue and the incorporation of genetically encoded tools into these biological samples, as in the present project. The requirement of an accessible source of nerve tissue and the inclusion of a double genetic tool, a molecular marker to identify neurons and a highly sensitive intracellular calcium indicator, were conveniently covered by the use of the HuC-GCaMP6 zebrafish.

Despite the previously mentioned advantages of employing zebrafish to obtain primary cultures of neurons, little information was available on the procedures to efficiently obtain isolated neurons from the intact nerve tissue. Moreover, its high fecundity and accessibility to embryos

and mature organisms within a reduced time frame make the zebrafish an ideal candidate to serve as a cell source for demanding procedures requiring cultured cells. Despite all the advantages of zebrafish as an animal model in research, a relevant consideration for implementation in projects is the requirement of specialised facilities to breed and maintain fish colonies, which differ from other organisms, such as rodents. Dedicated rooms, trained staff, certifications and Home Office licences are mandatory to include zebrafish in operative animal units in the UK. Also, the cellular and molecular techniques during the generation of genetic libraries, such as genotyping testing, are similar. Nevertheless, the time frame to cross and breed transgenic lines is reduced compared to some animal models.

Currently, most available culture protocols for zebrafish neurons process whole embryo extracts using enzymatic reagents to obtain a highly heterogeneous mix of cells (Patel et al., 2019; Acosta et al., 2018), followed by the use of neural-favouring culture media. A method that processes only nerve structures, such as the dissected brain, could facilitate the obtaining of pure neural cultures. This targeting could increase the culture yields by selecting the best parameters for them during the entire process and maximising the use of reagents and culture media. Purified zebrafish neural cultures have only recently been considered a suitable in vitro model for studying nerve diseases (Odierna et al., 2025). However, optimal conditions for increased yield obtention still need to be explored.

This project investigated the effect of relevant culture parameters on neural survival and growth in cultured zebrafish neurons, to determine their potential as a cell source for in vitro building of neural circuits. These parameters included the culture media composition, the presence of a protein layer resembling the extracellular matrix and the administration of neurotrophins that promote survival and extension of neural processes. Additionally, other factors related to the physiological state of the organism, such as the embryonic developmental stage, were also taken into consideration. As a result of this project, the permissive effects of laminin on the growth of zebrafish cultured neurons were confirmed (Chen et al., 2014). The presence of laminin positively influenced in vitro neural survival and axonal development, as observed in dissociated neurons and later in the implementation of lithography techniques. Thus, controlling laminin coating in the culture surface was confirmed to be a potent and versatile method to control neural attachment and direct axonal pathfinding (Calvo-Garrido et al., 2021). Still, large-scale testing of the circuit building method is required to ensure the capacity to generate reproducible outputs, and automation of the task using the FrescoM workstation can be used for this purpose.

Regarding optimal culture media composition, listed in **Table 2**, monitoring of cell survival and process growth in the cultures found that supplemented neurobasal media containing NGF was sufficient to sustain neurons in vitro up to three days, thereby preserving critical neural functions. The long-known positive effects of NGF in neural survival and growth were leveraged in the zebrafish model, with no observed enhanced effects when combined with other neurotrophins, as tested in combination with BDNF. This can be explained by typical NFG action in different organisms, through canonical NGF effector responses mediated by TrkA receptors found in neurons, and involving protein kinase C and phosphatidylinositol 3-kinase pathways (Pierchala et al., 2004). In other words, trophic responses triggered by NGF are enough to enhance neural survival and axonal growth in primary cultures of zebrafish neurons. In the developed methodology, neural survival in the cultures was extended up to three days under optimal conditions, with minimal cell loss and the maintenance of main neural features, such as axonal growth, formation of synaptic connections with neighbouring neurons and the generation of calcium responses upon stimulation. The survival time frame was enough to perform experimental procedures related to axonal growth, which was observed after 24 hours of neural maintenance. However, for procedures requiring more prolonged neural survival, further refinement of growth conditions is required. Recently developed cultures for zebrafish neurons have been able to maintain neurons alive up to several weeks (Odierna et al., 2025). However, there are current discrepancies in the best media composition and neurotrophin requirements to allow extended survival of zebrafish cultured neurons. In this matter, the FrescoM workstation can be a valuable tool for optimisation as it is capable of testing numerous growth conditions, for instance, those related to testing different media composition, in cultured samples while performing simultaneous imaging.

The HuC-GCaMP6 zebrafish strain chosen for this project has been demonstrated to be a convenient choice to exploit the available genetic tools in this organism to follow the process of building neural circuits. Due to the prominent feature of containing a genetically engineered construct that expressed a highly sensitive calcium sensor, cultured neurons possessed a selective marker to distinguish their neural lineage. Simultaneously, calcium responses could be followed by changes in internal fluorescence intensity, which is directly related to the monitoring of neural activity in isolated neurons and groups forming arrays. No invasive treatments related to neural labelling, such as the induced entry of dye molecules into cells, were required that may affect neural integrity, survival, or growth processes. In contrast to previous technologies using permeable calcium dyes, the fluorescent proteins of the GCaMP6 family have proven increased selectivity and performance, enabling continuous response to rapid intracellular calcium changes with a low signal-to-noise ratio (Chen et al., 2014). Moreover, the expressed fluorescence was sufficient to visualise not only the neural soma but also the growing processes, thereby consolidating the strain as suitable for investigating axonal growth events. However, the development of better calcium sensors in the future is not discarded, including members of the GCaMP family, as the case of the later developed GCaMP7 and GCaMP8 (Zhang et al., 2023).

Despite the general expression of fluorescence in all types of neurons resulting from control by the HuC gene promoter, other promoters can be used to express the GCaMP6 molecule in specific neural subtypes by utilising targeted promoters. This could result in the identification and selection of neurons with a specific profile for generating arrays with particular neural components in their structure. For instance, circuits entirely composed of neurons producing one specific neurotransmitter, or with different properties in each circuit layer, can be made. The generation of this kind of genetic library in zebrafish has been considered a prospective future direction in genetic design, providing a repertoire of building blocks for neural circuit construction to allow following a circuit building plan at the individual neuron level.

6.4 Controlled Positioning of Neurons from Dissociated Cultures

Common techniques to obtain and manipulate dissociated neurons involve the mechanical or chemical isolation of neurons from nerve tissue, such as the brain or peripheral ganglia, and the physical manipulation of the neurons with dedicated instruments (Powel et al., 2014). In this project, a method for selecting, transferring, and releasing individual neurons from primary cultures was established. The method demonstrated coordinated operation between the workstation components, including mobile parts, microscope components and a solution handling device, within the developed Python-based software. All scripts were uploaded as freely accessible through an online repository. Code lines were annotated to facilitate change of relevant parameters and customisation by new users. The developed protocol files were intended to work with cultures using commercial 96-well plates. However, variable values can be adjusted in the commands to accommodate specific conditions, such as culture dishes of different sizes and varying volumes of the handled solution in each step. Consequently, the protocol is not restricted to manipulating zebrafish neuron samples but any other cell culture.

Fluorescence-based cell detection was implemented for genetically modified fish neurons expressing GCaMP6, but the same detection principle can be extended to other fluorescent cells, including non-neural laboratory cell lines. To enable monitoring of intracellular calcium at a later stage, dyes such as Fura-2 and Fluo-4 can be loaded into cells before transferring them to the culture well, where they can be used as a cell source for manipulations. Moreover, because the camera used for real-time image analysis captures only black-and-white images and treats fluorescence as pixel intensity, alternative fluorescent molecules in the cells may not be detected. However, parameter calibration may require testing. The same adaptability applies to the fluorescence-based method for cell recognition during manipulation procedures, whose sensitivity can be adjusted to accommodate different ranges of the fluorescent intensity, molecule excitation and emission wavelengths, or microscope detection capabilities.

One major pending issue during the manipulation procedure was the complete automation of the task. As previously mentioned, the unsupervised cell picking, transfer, and release required the initial positioning of the pipette tip in proximity to the target fluorescent neuron. The protocol ended with the manual return of the pipette to the well position containing the primary culture of neurons. However, a second method would be required in the future to achieve the initial positioning of the pipette without supervision and to translate to the next target neuron at the end, thereby completing the transition between cycles and allowing the concatenation of several rounds of neural transfer. The FrescoM software included a previously developed autofocus function for the microscope that was optimised to adjust the microscope objective to obtain defined pictures of the cell samples. This function might also need improvements since a new object, the micromanipulator pipette, was now present in the analysed area by the microscope camera, which may affect the focus function performance on the surrounding cells.

Another aspect to be addressed during posterior procedure refinement is the restraining of positioning neurons to minimise spontaneous displacement after release in the new cell cultures. Although the observed movement was commonly within the range of a few micrometres, repeated accumulation of deviations in positioning might result in a decrease in the precision of the generated array structure, which is a fundamental priority when applying a bottom-up approach to building a network. The restraining effect must be strong enough to be maintained during the several days comprising the events of circuit formation. Laminin removal or inactivation from the culture surface, such as in the case of laser-based lithography, can be an applicable strategy to restrict neural movement. However, its effect must be sufficiently strong to prevent the need for an additional method for displacement control.

One additional challenge in using the automated workstation was to ensure the sterile conditions required for cell culture handling. Some commercial platforms include their own enclosed sterile environments (Storrs, 2013), reflected in increased size and the device operating costs. Although media contamination was not a common issue in the long-term procedures tested, potential contamination can easily arise when handling high volumes of cell samples, and adherence to aseptic techniques is crucial to ensure the quality of the generated cell samples (Dan et al., 2024).

6.5 Induction and Direction of Axonal Growth in Positioned Neurons

Experimental control of axonal growth can be achieved by reproducing molecular cues triggering the formation of a growing axon from the neural soma, followed by the guidance of the neural cone to follow a specific trajectory. Several mechanisms have been described that direct wiring formation in the developing nervous system (Dorskind & Kolodkin, 2020), including the formation of molecular gradients, competition for limited trophic factors, mechanical cell contacts and neural firing. However, studies of the individual neuron responses in live organisms have been restrained by accessibility issues due to the intricate structure of synaptic connections in the intact nerve tissue. However, the identification of the molecular function of some molecules have been exploited to reproduce developmental events in engineered neural circuits, following the entire process of forming neural connection and functional consolidation (Bang et al., 2021). The presented project provides an experimental example of fabricated biological circuits using live cells to reproduce the information processing events. Aside from the in vitro maintenance of neurons over prolonged periods, a crucial component of the project was to develop a method to connect a selection of neurons following a specific circuit plan.

Axonal growth guidance by laminin extension on the culture surface yielded a potent method for achieving neural growth. However, the tested methodologies to cover discrete areas of the culture still have a long way to go to be fully optimised. For instance, laminin application using a filament faced severe limitations regarding the applicability over long areas, compounded by a complicated and time-consuming process. The formation of an NGF gradient, although expected to attract growing axons supported by previous NGF induction of neural growth, did not result in an effective attractant in positioned neurons. Additionally, the tested UV lithography method to treat and inactivate laminin from limited regions generated variable effects from the

expected one when the neurons grew along laminin stripes, resulting in neither neural attachment nor growth of individually positioned neurons on untreated areas. A poor coverage of the UV beam by the disk mask, or even the toxic effects of nickel, the material used in the employed mesh grid discs, could explain the lack of extensive neural growth. Thus, this technique required competent skills to manipulate grid discs using forceps to prevent breaking the protein patterns when removing the disc from the dish bottom. Despite pending improvements in the lithography process, the performance of the fabricated UV lamp was considered positive, being capable of generating a UV beam with sufficient power to remove the laminin layer from the culture surface, coupled with easy assembly and low cost. This device is proposed to be used as an alternative device for commercial UV lamps for controlled and focused irradiation of culture plates.

Similar variable effects were observed in laser lithography, where only a fraction of neurons managed to grow axons, either growing as isolated units or forming clusters with other cells. Additionally, the increased effectiveness in removing or inactivating laminin in narrow areas must be weighed against the equipment requirements for performing this task, primarily the microscopy-class laser, as well as trained staff for operation and safety concerns related to the use of lasers, which elevated the cost of producing laser-based engraving culture systems.

Considering the intricate cell mechanisms involved in axonal pathfinding, the experimental experience gathered through this project, in the form of variable results along repetitions, indicates that an effective and reproducible method to induce and direct axonal growth is likely to depend on the combination of more than one method. For instance, in the localised presence of laminin plus the administration of a chemical attractant, as NGF, in the form of a persistent gradient. Therefore, a prospective next step following this project would be to apply together the engraved dishes with patterns, produced by either UV or laser microlithography, with the local administration of an NGF gradient, as described in the Results section. A combined effect of these techniques could result in a better performance in promoting axonal guidance, surpassing the observed effects during isolated testing and being sufficient to cover the gap between positioned neurons and allow an effective connection and formation of synaptic connections.

6.6 Automation of the Circuit Building Process

The project aimed not only to reproduce the experimental manipulations related to in vitro neural circuit building, but also to develop a process that could be scalable and applicable as a biological sample for experimental purposes. Current scientific research requires the generation of substantial amounts of high-quality data to yield robust and trustworthy conclusions (O'Mahony et al., 2019). Task automation is a crucial step in converting manual management of samples into a continuous and invariable process (Bultelle et al., 2024). One of the primary challenges of fabricated biological neural circuits is the time and resources required to obtain sufficient biological material for generating experimental data. Here, a process based on automation is proposed to replace typical manipulations by the researcher with machine-operated tasks. It is worth noting that the presented outputs only represent a portion of the entire process. Efforts were mainly focused on the development of the operative components, namely the modifications of the FrescoM workstation to deal with this process, the obtention of a suitable source of biological material, represented by the optimisation of culture conditions for zebrafish neurons, and the development of a consistent method of cell recognition and monitoring, based on a genetically expressed neural sensor. Subsequent experimental work should focus on task refinement, aiming to achieve better performance in long-term procedures, as well as intermediate steps, such as the previously mentioned cell positioning concatenation. Despite some relevant progress achieved by using an automated workstation in terms of time savings and increased precision to perform the described experimental tasks, the full benefits of process automation can only be achieved if all steps can proceed consistently.

One main difference found during the transition from assisted cell manipulations to running fully automated processes was an increase in the time required to perform each step. Manual operation allowed constant evaluation of the handling progression, with immediate parameter

rectification towards the intended outputs based on experience. In contrast, the automated tasks tested in this project relied on real-time imaging and proceeded through each step following fixed parameters. Increasing the time was found to be the best strategy to refine cell handling, favouring a small amount of handled media and reducing liquid disturbance during picking until a change in fluorescence was detected, thereby reducing the risk of capturing more than one fluorescent cell. This also resulted in a captured cell maintained next to the pipette tip, facilitating the release in the next step. However, this often resulted in more attempts until the contained cell was picked or released. Nevertheless, automating the task can enable constant repetition to counter this initial time investment. More sophisticated methods for enhancing performance based on real-time analysis have been applied to automated workstations performing cell cultures. These include AI-driven quality monitoring to modify parameters according to real-time evaluations (Drobnjakovic et al., 2023; Singh et al., 2025), but these require generating an initial collection of generated data after altering individual parameters before obtaining any robust conclusion on outcome prediction. Recent innovations on task automation in biomedical research and clinics have introduced additional tools to further increase reproducibility, precision and capacity on sample handling and processing, such as auto-verification, AI-guided machine learning, quality control, quality assurance systems (Brown & Badrick, 2022). Combined with the development of more accessible, customisable and specific user-oriented devices, automation is currently transforming the routine hands-on laboratory work toward a progressive implementation of total laboratory automation in scientific research (Yun et al, 2019).

Finally, of special relevance was the increased technical capabilities of the FrescoM workstation. In a research environment that demands the optimal generation of reliable data, this project opened up novel options for implementing automation in laboratories, particularly those limited by resources, space, and the rigidity of commercial platforms. Additionally, the development of this automated system as a shared data project fostered the growth of an enthusiastic scientific community, sharing information, component development, and continuous improvements to address upcoming technical challenges through the use of accessible and trustworthy technological tools.

VIII. FUTURE PERSPECTIVES

The limited methodological tools for the study of neural circuits using a bottom-up approach are, to a great extent, a consequence of the lack of efficient processes for producing samples with precision and reproducibility. Here, a process for producing simple neural arrays using an automated platform to perform repetitive tasks was developed. Although substantial progress has been made, especially in testing of the platform performance, establishment of HuC-GCaMP6 zebrafish as a convenient cell source and exploration of strategies to control axonal growth in cultured neurons, some relevant issues faced during the project remain to be refined before obtaining a fully automated, reproducible and scalable process for the large-scale production of artificial neural arrays.

Firstly, sources other than primary cultures of embryonic HuC-CaMP6 zebrafish neurons can be used to obtain neurons. One potential direction is the generation of circuits composed of specific neural subtypes. Neural identity and its functional properties, as part of circuits, are defined by the set of neurotransmitters produced and released at nerve terminals. Response depends on the number and type of receptors activated in the target cells (Purves et al., 2001). Similar to the use of the huC-gene promoter as a pan-neural marker, the sequence of other promoters can be used to restrict expression to specific neural subtypes. These may include genes involved in the production and transport of neurotransmitters in neurons (Lake et al., 2016). Some proposed examples for this purpose are listed in **Table 3**. With a confirmed expression pattern of homologous genes to all of these markers in zebrafish (Zhang et al., 2021), the sequenced genome provides the exact sequences needed to generate DNA constructs for gene editing. Then, current molecular tools in DNA designing, cloning, and embryonic transfection can produce zebrafish lines with genetically incorporated neural markers. As with the HuC-GCaMP6 line tested here, neural marker expression can be linked to a calcium indicator to enable identification during cell selection based on fluorescence and monitoring of neural responses in these neurons as changes in fluorescence intensity.

Neurotransmitter	Marker DNA Sequence
Glutamate	• Vesicular glutamate transporter 1 & 2 (VGLUT1, VGLUT2) • Special AT-rich sequence-binding protein 2 (STAB2)
GABA	• Vesicular GABA transporter (VGAT) • Glutamic acid decarboxylase 65 & 67 (GAD65, GAD67)
Acetylcholine	• Choline acetyltransferase (ChAT) • Vesicular acetylcholine transporter (VACHT)
Dopamine	• Tyrosine hydroxylase-1 (TH1) • Dopamine transporter (SLC6A3)
Serotonine	• Tyrosine hydroxylase-2 (TH2) • Serotonin transporter (Slc6a4a, Slc6a4b)

Table 3: Subtype-specific neural markers in zebrafish based on neurotransmitter expression

The selective choice of the elements used to assemble the circuits could increase the building process reproducibility by dealing with neurons with identical functions and trophic responses that can be more easily adjusted to result in a uniform effect of variable parameters on all the circuit elements, such as applying a trophic factor to the culture media or growth ability on a pretreated culture surface. On the other hand, controlling the neural identity of specific elements

can determine the type of synaptic connections that a selected neuron is capable of forming, such as excitatory or inhibitory synapses, depending on the nature of the expressed neurotransmitter, and consequently, the properties of that node with respect to the overall information processing through the circuit.

Other options for controlling neural identity include using neurons derived from stem cells, such as embryonic stem cells or reprogrammed iPSCs. However, an efficient neural differentiation method for specific subtypes is still a major obstacle. Current efforts in our research group focus on optimising the differentiation yields of the human-derived cell line SH-SY5YH into several neural subtypes by adding retinoic acid and neurotrophic factors in a precise temporal window (McCourt H, University of Sheffield Thesis Database, 2026). The inclusion of the FrescoM workstation in this ongoing project has produced neural samples of specific subtypes within the same environment used in this project. An ideal scenario may link the generation of a pool of neurons of different subtypes to an internally uniform population, as a versatile repertoire of building blocks for assembling neural circuits on demand at single-cell resolution.

The potential to generate a repertoire of structures with defined structural patterns, by controlling the identity of individual elements, could enable accurate representation of modelled circuits using live, interconnected neurons. In other words, the assembled circuits should integrate specific inputs, in the form of individual stimulation of presynaptic neurons, to produce an expected response by the activation of a postsynaptic neuron. Considering the currently limited number of neurons formed into arrays by the developed methodology, within the range of 2 and 3 cells, an initial proposal for circuit function testing consists of pairs of cells, in which the presynaptic neuron is activated to expect a response in a unique postsynaptic neuron. Transgenic targeting molecular tools, such as chemogenics or optogenetics, might be a suitable strategy to achieve precise neural activation within an experimental timeframe, and their expression in zebrafish can be linked to specific neural markers (Dimidschstein et al., 2016). For instance, channelrhodopsin-expressing neurons could be used to form pairs of neurons, as shown in **Figure 7.1**. Next, a localised beam of blue (480 nm) light could be used to activate one of the two neurons by limiting the area of incidence. When recording the activity on the postsynaptic neurons, the expected consequence would be a detectable response resembling long-term potentiation (LTP) or long-term depression (LTD), depending on whether activation of both neurons was correlated or uncorrelated. Both events represent biological mechanisms to control the strength of synaptic connections (Xie et al., 2013).

The previous assembly principle could be extended by adding one more neuron into the system to build elementary logical gates. These systems represent the most basic examples of information processing, performing system activation as a Boolean function (McCulloch & Pitts, 1943). Considering the possibility of assembling circuits with neurons of specific subtypes, the composing elements to recreate the gate could be provided by using neural populations expressing specific neurotransmitters. For instance, excitatory glutamatergic neurons may serve as information-transmitting units, since inhibitory GABAergic neurons could play the opposite role. Two simple types of logical gates are proposed to test this idea: logical AND and logical XOR AND gates (**Figure 7.2**). In the former case, the circuit consists of two excitatory glutamatergic neurons connected to a single target neuron, which would exhibit a detectable activation only when both presynaptic neurons fire. In the latter case, the gate consists of one presynaptic glutamatergic neuron and one GABAergic neuron that innervate the same target neuron, which only exhibits a postsynaptic response when the glutamatergic neuron is activated alone. It is worth mentioning that this conceptual building of circuits considers neurons as entities following Dale's principle of one neurotransmitter for each neuron (Haber & Schneidman, 2022). On the other hand, the consideration of additional gate types would depend on the consistency and reproducibility of the circuit assembly process when dealing with cells from distinct sources. It is also relevant to note that any procedure involving cell activity recording in the automated workstation environment would require incorporating the corresponding components to perform such techniques.

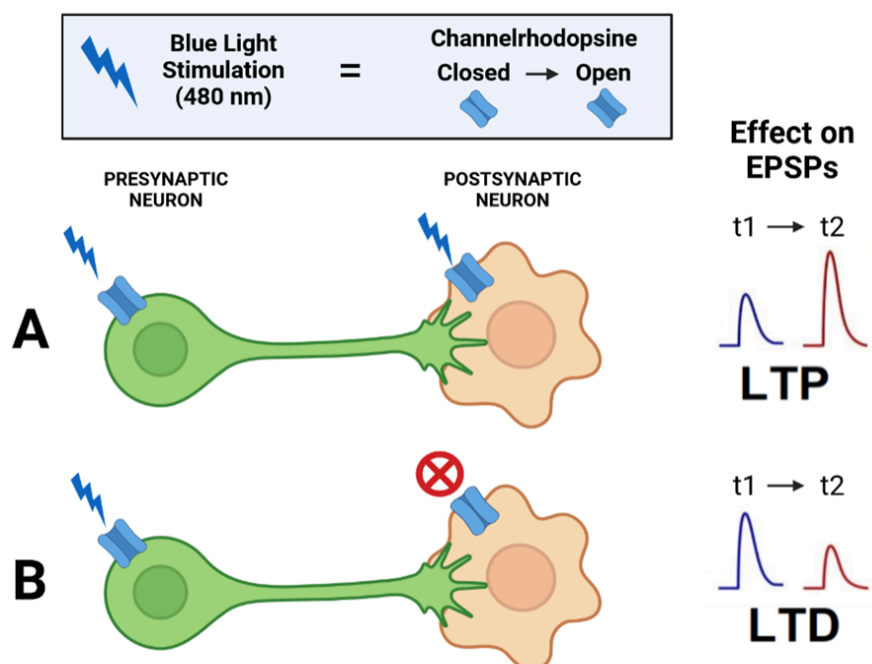


Figure 7.1: Generation of LTP and LTD responses in activable pairs of neurons

Prospective activable circuits can be generated from pairs of neurons expressing channelrhodopsin. Limiting the incidence of the light beam, either (A) or only one (B) neuron could be activated. Simultaneous neural activation may produce a detectable LTP response in the postsynaptic neuron activity, or an LTD response otherwise.

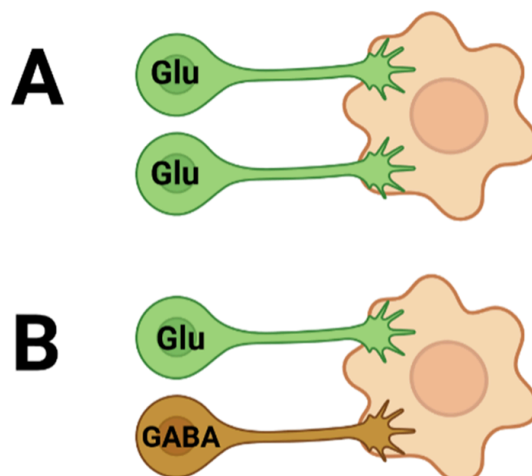


Figure 7.2: Prospective building of neural circuits using specific neural subtypes

Logical gates represent simple information processing systems that can be assembled using specific neural subtypes to test the functional properties of the generated neural circuits.

A. Logical AND gate consists of two presynaptic glutamatergic neurons connected with the same postsynaptic neuron. The postsynaptic neuron will only fire when both presynaptic neurons are depolarized simultaneously.

B. Logical XOR AND gate will consist of one glutamatergic and one GABAergic presynaptic neuron connected to the same postsynaptic neuron. The postsynaptic neuron will only fire when the glutamatergic but not the GABAergic neurons fire.

Following current project challenges, the induction and direction of axonal growth were among the most demanding steps in implementing this project. Three different strategies were tested to restrict neural processes to discrete areas and to favour their growth in specific directions, without achieving definitive control over the growth events. The best strategy was a combination of techniques: microlithography and an NGF gradient. This, combined with an optimal growth environment provided by the culture media composition. Therefore, until consistent results are obtained when processing a high number of neural samples, none of the tested methods alone would be sufficient to generate suitable biological samples for further applications. Special attention is required for UV-based and laser-based lithography techniques. Although they were shown to be efficient methods for directing axonal growth, culture plates were treated individually during experiments. To achieve process escalation, a procedure for the rapid, efficient treatment of large numbers of culture plates may be required.

Next, the fluorescence-based method for neural selection and release could be refined. The real-time image analysis considered only single numerical values, derived from the pixel intensity over a defined area in a captured image. However, this analysis is prone to estimation errors due to many factors that may affect image quality. For instance, light beam incidence over the curved surface of the culture well produced variable brightness across the captured image. Therefore, an ideal analysis method should analyse multiple image parameters to identify cells. A convenient approach could be to implement cell shape identification, as in cell segmentation algorithms, to confirm the presence or absence of cells in the analysed field of view of the microscope. Methods for identification of cells of irregular shapes, such as the Morphoseg algorithm developed by our research group (Zhang et al., 2025), have just been developed, based on high-contrast light microscopy. However, enhanced image processing may be required to efficiently identify neurons from fluorescence microscopy images, as well as increased processing capabilities on the workstation computer.

Ensuring reproducibility in the circuit-assembling process was the first step toward increasing the complexity of the generated samples in the future. Although this project focused on creating elementary neural arrays composed of only a few cells, the cell-manipulation platform could potentially be adapted to form more complex architectures. Since one of the main challenges in producing lab-grown biological substitutes is achieving structural precision, an effective, reproducible, and affordable automated method for generating neural samples with specific structures could become a valuable tool for producing suitable samples for experimental studies (Healy & Deans, 2019). On-demand structural design also makes it possible to model both physiological and pathological states of neural circuitry (Bang et al., 2021). As Alan Turing envisioned in his report *Intelligent Machinery*, building artificial systems capable of exhibiting information-processing behavior resembling that of a living entity is an extensive endeavor requiring multiple conceptual and experimental approaches, but based on the effective and adaptable performance of the simplest tasks.

Finally, this project emphasizes the value of open access in research. The project outputs and future improvements, whether as part of the FrescoM workstation or through new initiatives, have been developed with accessibility, equity, and collaboration in scientific research in mind. This project aims to foster interest in open-source initiatives in the life sciences, broaden the range of potential applications, and provide a biological and technological tool capable of competing with commercial devices. It also welcomes ongoing improvement through collaboration with the scientific community and broader audience, and is intended to serve as an adaptable solution for addressing new scientific questions.

VIII. CONCLUSIONS

The present project aimed to implement a procedure assisted by an open-source automated platform to in vitro generate neural circuits from zebrafish cultured neurons. This thesis summarises the current paradigm in the field, provides evidence of the experimental barriers faced during this process, and proposes strategies using the workstation to overcome obstacles at each stage of the circuit assembly process. The output samples were intended to serve as experimental models for in vitro experiments requiring biological modelling of simple neural circuits with high resolution and reproducibility.

The FrescoM automated workstation was successfully modified to increase its functionality, serving as a cell manipulator to handle cultured cells from primary cultures by incorporating a custom cell manipulator into the framework. Technical specifications on displacement precision of an adapted mobile frame, and on the positioning of a pipette used for manipulations were tested, demonstrating sufficient precision to perform procedures at single-cell scale.

To obtain a suitable source of neurons for use in the circuit assembly process, culture conditions for primary cultures of zebrafish neurons were investigated to determine the optimal conditions that favoured neural survival and axon growth for extended periods. This project also explored the effect of relevant culture parameters on neural survival and growth in cultured zebrafish neurons, including culture media composition, the presence of a protein layer resembling the extracellular matrix and the administration of neurotrophins that promote survival and extension of neural processes. Additionally, other factors related to the physiological state of the model organism were taken into account, including the embryonic developmental stage. Culturing neurons on laminin-coated dishes and in serum and NGF-containing media provided the best results among the tested neurotrophins.

Subsequently, the developed device was used to identify individual neurons from the cell samples by expression of a genetically encoded marker. Next, the device was capable of repeatedly positioning the manipulator, picking, transferring, and releasing individual cells at specific positions. Starting from dissociated neurons obtained from primary cultures of HuC-CaMP6 zebrafish, the neural selection task was assisted by a fluorescence-based method developed to confirm neural identity and monitor cell picking and releasing events in the handled samples.

Neurons were then transferred to new cultures to define the circuit architecture, composed of arrays of a few neurons. These neural samples were able to survive up to three days in supplemented culture media and to grow axons spontaneously after 24 hours. This axonal growth led to the formation of neural arrays that maintained their responses to depolarisation as determined by monitoring intracellular calcium dynamics using the GCaMP6 calcium sensor. Then, several methods were implemented and tested to control axonal growth in these arrays, including local administration of neurotrophins and restricting the presence of laminin on the culture surface by UV and laser-induced microlithography. Isolated neurons failed to present consistent growth of axons, but neurons forming clusters managed to form connections following a predefined growth pattern.

Despite pending technical challenges, the project developed a partially automated procedure to generate simple neural arrays from cultured zebrafish neurons, which included selective manipulation of neurons and can be used for the reproducible and extensive production of in vitro systems representing neural circuits of defined structure. Further improvements can be directed to the complete automation of the process.

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This project was developed in the Development, Regeneration and Neurophysiology cluster of the School of Biosciences, in the University of Sheffield
