



Pázmány Péter Catholic University
Faculty of Information Technology
and Bionics



Institute of Cognitive
Neuroscience and Psychology

37. OTDK Biológia Szekció

Exploring Human Organotypic Neuronal Tissue Cultures for Investigating Central Nervous System Changes

Rebeka Stelcz
Medical biotechnology MSc
2023

Supervisor: Estilla Zsófia Tóth
Faculty mentor: Lucia Wittner D.Sc.

1. Table of Contents

1. Table of Contents	2
2. Introduction	3
3. Literature	4
3.1. Importance of investigating human tissue	4
3.2. The organisation of the neocortex	5
3.3. The characteristics of the neurons in the neocortex	6
3.4. The application of organotypic tissue cultures	8
3.5. Viral vector application on human tissue	10
3.6. Human cerebrospinal fluid as a culturing medium	11
3.7. Neuronal nuclear protein antibody	12
3.8. Fluoro-Jade C	13
4. Planning, materials, and methods	14
4.1. Origin of the human neocortical brain slice cultures	14
4.2. Preparation of human brain slices	15
4.3. Human brain slice cultures	15
4.4. In vitro electrophysiological recordings	17
4.5. Immunohistochemistry	17
4.5.1. Immunostaining protocol for light microscopy	18
4.5.2. Fluorescent staining protocol for the visualisation of degenerating neurons	19
4.6. Microscopic investigations	19
4.7. Data analysis and statistical testing	20
5. Results	20
5.1. Qualitative analyses	20
5.1.1. Patient data and tissue origin	20
5.1.2. Culturing medium composition	21
5.1.3. Structure and preservation of the cultured tissue	22
5.1.4. Intracellular recordings	23
5.1.5. Extracellular recordings	23
5.1.6. NeuN staining	24
5.1.7. FJC staining	25
5.1.8. Transgenic expression through viral vector infection	27
5.2. Statistical evaluation of the results	28
5.2.1. Cell density based on the NeuN-positive (NeuN+) cells	29
5.2.2. Differences caused by the culturing medium	30
5.2.3. Characteristics of the cell number based on the length of the culturing period	30
5.2.4. Tendency in the reduction of cell number	31
5.2.5. Investigation of damaged cells by FJC staining	32
5.2.6. FJ staining protocols	32
5.2.7. Comparing the samples based on the culturing medium	33
5.2.8. Pixel intensity histogram among cultures	34
5.2.9. Histogram of given days	34
5.3. Correlation between FJC and NeuN staining	35
6. Discussion	35
6.1. Conservation of the cultured tissue	35
6.2. Change in the viability of the neurons	36
6.3. Influencing factors	37
6.3.1. Viral vector infection	37

7. Conclusions	38
7.1. Future perspectives	38
8. Acknowledgement	39
9. References	39

2. Introduction

The microstructure of the brain was investigated as early as the 19th century by Ramon y Cajal and other researchers. For the study of the healthy and diseased central nervous system, several animal models exist. These can be utilized for the examination of cellular and populational level neuronal activity and for getting a deeper understanding of CNS related diseases, like epilepsy and neurodegenerative disorders. However, research over the past few decades has demonstrated that examination of animals in vivo, ex vivo, or in vitro falls short in capturing the full spectrum of relevant insights, primarily due to disparities in anatomical and electrophysiological characteristics as compared to the human brain. Though there are certain species-to-species comparisons and analogies that can be made, it is difficult to determine how well the results obtained from these models can be applied to the human brain. Truly reliable and complex information can only be obtained if we are able to study the functions of the human CNS tissue directly. The technological advancements in recent decades have allowed researchers to carry out ex vivo or in vitro experiments on surgically removed specimens in laboratories in accordance with ethical standards-[1], [2], [3], [4].

Nonetheless, numerous experiments need prolonged observations and therefore are not feasible with acute brain slices. To address this limitation, in vitro brain tissue cultures can be established. Employing this methodology, tissue slices can be sustained in an artificial milieu for several weeks, facilitating the examination of cellular and network-level alterations over an extended duration. Furthermore, this approach enables the execution of a broader range of experiments, including the introduction of exogenous proteins via viral vector application [5], [6], [7].

I was able to join the Integrative Neuroscience Research Group at the Research Centre for Natural Science, where one of the main research areas are electrophysiological, and anatomical investigations combined with two-photon microscopy on human brain tissues. In the last two years, one of the main projects has been to create a well-working protocol for culturing human organotypic neuronal tissues, measuring their intra-, and extracellular properties and analysing the anatomy of the fixed tissue samples. In addition to assisting in sample preparation and measurements, my primary responsibilities regarding to this work are doing immunostaining on the fixed tissue samples and assessing the changes in the

cytoarchitecture.

In this work, I start by explaining the core ideas and difficulties associated with human organotypic cultures. Afterwards, I detail the step-by-step protocol we followed during the experiments. Finally, I discuss the outcomes we achieved and lay out our plans for the future. The aim of this study is to utilize analysis to assess the viability of pyramidal cells in organotypic tissue culture and to identify the structural alterations on the cellular level that may occur during this process.

3. Literature

3.1. Importance of investigating human tissue

Researchers have been studying the mechanism of the information flow in the brain and the elements that control it for many years using electrophysiological and anatomical investigations on rodent brain slices. Although there are many similarities between the anatomy and function of rodents and human brains, the human brain is capable of higher order thinking not only because its volume but also because the cells of the human brain are capable of more efficient, diverse information processing at both the network and subcellular levels. It is crucial to investigate human samples since there are many differences between the structure of the human brain and other mammals (such as cell types, signal integration, receptor localization and anatomy). [1], [4], [8]

From the operation, through the transport of the brain slices to the culturing and the measurement, care must be taken to ensure that as many of the artificially preserved brain slices as possible remain in high quality. Particular attention must be paid to ischemia, which can lead to cell damage. It is important to consider the patient's age, health, anaesthesia, and medications before and during surgery because they all have an impact on the condition of the tissues. A greater focus should be given to this because there is not much proof in human samples yet of how diseases and medical conditions affect the fundamental physiological characteristics of neuronal and glial cells [9].

In the last few decades, the use of tools like the Cre-lox system, optogenetics, and virus-mediated gene targeting, knockout and delivery has allowed for a better understanding of how various cell types work together to create functional circuitry in the brain of rodents [7], [10], [11]. These methods might open an entirely novel field of investigating how the healthy or pathological human central nervous system functions once they are adapted for human brain slice cultures that closely mimic the environment of the human brain. Through these techniques, we would be able to answer several questions which have been raised during the

establishment of the diagnosis or the treatment. How is the function of complex CNS networks influenced? What is the role of the morphological and functional integrity of neurons? How does the tissue condition influence the integrity and viability of the network activity [12]? To answer these questions, organotypic culturing seems to be an optimal method to be able to perform long-lasting experiments that ensure deep insight into the processes of the CNS. However, in this case, the optimization of the culturing conditions is an additional challenge due to the alteration in the specimen quality gained from patients under antiepileptic drug treatment or living with a brain tumour. In our project, we have had to adapt to several different conditions and modify our protocols based on the gained empirical knowledge.

3.2. The organisation of the neocortex

The appearance of the neocortex is one of the fundamental events in evolution. The complexity of this region of the brain refers to how complex cognitive functions the given species has [13].

The largest part of the mammalian cerebral tissue is the neocortex, which is composed of grey matter. It is the outermost layer of the mammalian brain with a thickness of circa 2-4 mm depending on the species. The neocortical structure varies significantly across different mammalian species and some brain regions. While smaller animals typically exhibit a more limited number of functional divisions, approximately 20, humans, on the other hand, possess nearly 200 distinct cortical areas within their neocortex [14].

The human cerebral cortex can be divided into 4 functional parts based on the arrangement of striations and the gyri, allowing the frontal, parietal, occipital and temporal lobes to be distinguished. In smaller animals, such as rodents, the brain surface is smooth, and the neocortex is proportionally smaller in volume compared to the brain as a whole. The neocortex is responsible for complex information processing (e.g. higher-order thinking), execution of associative tasks, motor movement and perception. The dysfunction of neurons found in the neocortex can lead to diseases such as epilepsy, Parkinson's disease and Alzheimer's disease.

The neocortex can be divided into 6 continuous layers based on the cytoarchitecture, helping to increase the efficiency of complex computations at the network level. The numbering of layers increases from the pia mater towards the white matter.

Several kinds of histological neurochemical techniques can be applied to study the density and organization of neurons, or their size and shape. The integration of modern (e.g.

electrophysiological techniques, swift ion beam-based and synchrotron radiation techniques, scanning transmission ion microscopy) and traditional histological tissue staining techniques (e.g. immunostaining, hematoxylin and eosin staining) presents a compelling approach to the exploration of neocortical cell circuitry and spatial distribution in normal and pathological tissue [15].

While each cortical layer possesses its distinct cytoarchitectural structure, they do exhibit certain shared characteristics. Firstly, all cortical layers have a principal source of input and a primary output destination. Secondly, cells within a specific region form lateral and columnar connections with neighbouring cells. Additionally, cells with analogous functions tend to organize into radial clusters spanning all cortical layers, often with radial or columnar inputs. Finally, interneuron axons frequently extend horizontally, fostering connections between cell groups with similar functions [16]. The six layers within the cortex do not exhibit uniform prominence in the different brain regions [17]. (Figure1).

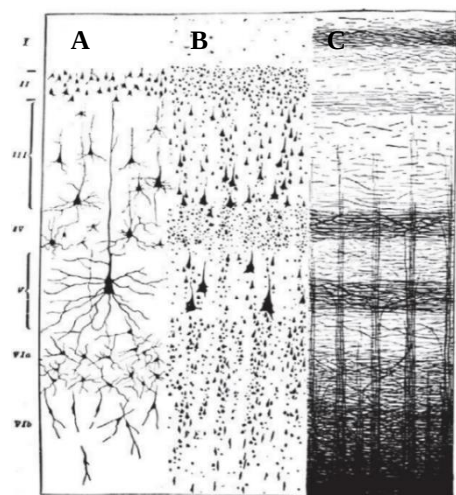


Figure 1: Laminar structure of the adult human neocortex. They display distinct connections, various cell densities, and diverse cell types. These layers, when counted from the dura mater, include the molecular layer (L1), external granular cell layer (L2), external pyramidal cell layer (L3), internal granular cell layer (L4), internal pyramidal cell layer (L5), polymorphic layer (L6). Different staining techniques reveal variations in pyramidal cell size and fiber across different layers of the neocortex. A: Golgi staining showing the neuronal cell body with the dendrites and the axon mainly in L2/3 and L5, B: Nissle staining showing the soma of the neuronal cells (L2/3, L5, L6) and granular cells across the neocortex (L4, L6) C: Myelin staining providing insights into the organization and structure of neural pathways [18].

3.3. The characteristics of the neurons in the neocortex

A significant part of the neocortex is composed of pyramidal cells. These neurons with triangular-shaped soma and other non-pyramidal, heterogeneous neurons and glial cells participate in the hierarchical and parallel circuits, that enable the most complex signal processing and communication among distinct cortical areas and targeted areas of the spinal cord [9], [12], [15]. Pyramidal neurons send excitatory outputs to various brain regions. The apical dendrites of these cells extend towards the cortical surface, while the basal dendrites spread within the layer. Pyramidal cells in L2/3 communicate horizontally with neighbouring regions, contributing to local processing. Additionally, pyramidal cells in L5 form long-range connections, projecting to subcortical structures like the thalamus and brainstem, also L6 pyramidal neurons are involved in corticocortical and corticothalamic projections. The region-

specific connectivity of pyramidal cells is essential for information processing and integration across different cortical layers and brain regions. The pyramidal cells receive excitatory input from intrinsic afferent pathways (other pyramidal cells and excitatory interneurons) and extrinsic afferent pathways (thalamus and cortical areas). Inhibitory input comes from interneurons presented in the cerebral cortex and in subcortical structures [19].

The neurons are connected to each other through synapses where neurotransmitters are released at the axon terminal of the presynaptic neurons and taken up by the postsynaptic densities located at the dendrite spine, shaft or cell body of the postsynaptic neurons. Pyramidal cells and spiny stellate cells, which are excitatory interneurons, release molecules with excitatory effects (glutamate, aspartate) into asymmetric synapses [20]. Inhibitory interneurons, like basket, chandelier and double bouquet cells release the primary inhibitory neurotransmitter, gamma-aminobutyric acid (GABA) into the synaptic cleft.

Pyramidal cells receive inhibitory and excitatory signals through symmetrical and asymmetrical synapses from different sources [20]. In addition to their unique shape, pyramidal cells can be distinguished from other neurons by their high variety of firing patterns. This feature is due to the diverse density of ion channels presented on the cell membrane. The electrophysiological properties of the cells are type specific. Based on the firing pattern of cells, it is possible to determine their ability to process information and their role in the circuit [18]. The human brain's capacity for signal processing is enhanced by the distinct membrane properties and firing patterns of neocortical neurons, which are achieved through the specific density of various ion channels, for instance A type K^+ channels and hyperpolarisation-activated cation channels (HCN II) [4], [21], [22], [23].

Specific morphological types, with particular molecular attributes and connections, have been shown to present distinct physiological characteristics [24]. The pyramidal cells in the L2/3 and L5 have more extensive dendritic trees and their morphology is more unique than the pyramidal cells in L6. [17], [19], [25]. A human pyramidal cell has approximately 30,000 synapses, twice the number of synapses in a rodent. Furthermore, L2/3 pyramidal cells' resting potential is characterized by stronger hyperpolarization and their input resistance is lower than that found in other layers [26].

Human pyramidal cells differ from that of rodents at the subcellular level, with more branching in the dendritic tree and a proportionally greater overall length. These characteristics are not associated with the age of the patient or the thickness of the neocortex. In addition, in both mouse and human pyramidal cells, the length of dendrite segments increases between two branches as the distance from the soma increases, but in second- and

third-order branches, human segments are significantly longer. This type of cell architecture influences the rate of change in the signals propagating on the dendrite, with attenuation on human dendrites being on average 1.5-2.5 times more significant than in mice [26]. The human neocortex is much larger and more diverse than the mouse neocortex, especially in the supragranular layers that connect with other brain regions. Based on patch clamp recording combined with RNA sequencing there are different type of glutamatergic neuron types in the human and mouse neocortex. In the human supragranular neocortex, five types of glutamatergic neurons were identified, whereas only three in the mouse, with characteristic features in their shape, size, dendritic branching, membrane properties, action potential shape and firing patterns. These features reflect on their transcriptomic and functional specialization too [27]. Furthermore, another investigation has explored the transcriptomic diversity of L1 interneurons in both humans and mice. This exploration suggests that L1 has undergone evolutionary divergence, adapting to accommodate the development of an expanded neural circuitry in the human cortex [28].

A recent study has delved into the behaviour of fast-spiking interneurons, crucial for neocortical synchronization and cognitive functions, and has identified conserved mechanisms across species. Notably, the activation/inactivation properties of sodium currents are similar, while morphological and other electrophysiological properties exhibit notable differences, including increased strength and speed [29]. It is important to consider the differences between species [30].

3.4. The application of organotypic tissue cultures

The first research paper about organotypic cultures was published in the 1950s on the differentiation of the chick embryo eye [31]. The first, detailed descriptions of brain tissue cultures are from the 70s with pioneering technical approaches [32]. Brain organoids, which enable the in vitro development of "humanoid" neurons, and embryonic stem (ES) or induced pluripotent stem (iPS) cell technologies are two alternative methods for studying the CNS network. Although they are major improvements, they are still highly limited and cannot ensure the reconstruction of the complex tissue that the human central nervous system provides [33]. Organotypic brain tissue cultures are a bridge between cell cultures, and acute in vitro and in vivo experiments providing a unique tool to study physiological and pathological mechanisms in mature human neurons at a more complex, organized level than neuronal cultures provide.

Over the last decades, organotypic cultures have been found to be an important step forward

in simulating more in vivo-like situations compared to cell cultures since they allow several aspects of the structural and synaptic organization of the original tissue to be preserved and experiments that require longer periods of time can be performed [34]. They may serve as a platform enabling investigation of certain mechanisms and components of neuronal network plasticity, and long-term effects of novel chemical compounds relevant to drug development, or studies involving viral-mediated overexpression of proteins of interest. Even the effect of neuroprotective (growth factors, neuroactive drugs) or neurogenerative molecules on the network and cellular activity can be investigated [33].

Several modern experimental procedures can be performed on these samples in the same way as in the case of acute tissue slices, such as repeated multi-electrophysiological recordings and stimulations, retrograde tracing using fluorescent dyes, long-term live imaging, and exclusively gene transfer techniques are also possible [35]. The fixed tissues can be immunohistochemically stained (chromogenic or fluorescent) and transferred to glass slides for analysis under the microscope [34].

There are several ways for culturing brain tissue slices. In the case of the most advanced technique, the slices are placed on a semipermeable membrane covered with a medium, that allows the culturing of samples originating from a mature brain. These samples tend to be more sensitive to environmental changes compared to cultures derived from embryonic tissue. The components of the medium can vary based on the purpose of the experiment. Artificial culture medium or human cerebrospinal fluid is added to the tissues with or without growth factors, serum and antibiotics to optimize or adapt the environment for specific conditions [34]. The applied medium can influence the length of the culturing time; the results of different research groups are contradictory in this case [9], [12], [33], [35], [36]. We have to consider the fact that not only the origin and the quality of the obtained tissues can influence the success of the culturing but also the culture medium.

Establishing long-term survival of human brain slices opens new possibilities for exploring relatively long-term treatment outcomes by gaining more information on cellular viability or getting a better insight into neurodegenerative diseases. Another advantage of the organotypic culturing techniques is that in this case not only the cells but even the extracellular matrix is preserved and the unique integration of the cells or the biomolecular processes between the neurons and glial cells can be observed [37].

Several questions that arise during the testing of new chemicals, can be answered more precisely. Such as the degree of survival of the excitatory neuronal population in human brain slice cultures following injection of reagents over time or the effectiveness of viral vector

application on cells for optogenetics and morphology analysis [12]. A number of studies using human tissue have already advanced the field of study by exposing novel insights into the pathological mechanisms underlying temporal lobe epilepsy, assisting in the creation of new antiepileptic medications while also broadening our understanding of the physiological mechanisms of cortical activity both on the cellular and network-level [35].

3.5. *Viral vector application on human tissue*

The use of viral vectors in human organotypic cultures has become increasingly popular in recent years, due to their unique ability to efficiently and deliver genetic material into targeted cells. The viral tools cannot be applied to acute tissue, because the expression of exogenous transgenes is slower than the time range in which the neurons keep their viability, and the membrane properties remain intact in acute tissue [9]. With the help of this powerful method, detailed cellular analyses of the neuronal compartments or the electrophysiological properties can be performed throughout the time of culturing which is not possible on acute brain tissue [35].

On a long scale, one aim is to be able to deliver therapeutic genes to the CNS in order to treat neurological disorders without pathogenic effects. In vector design, the objective is to minimize potential risks by knocking out its pathogenic genes and incorporating only essential viral components for the transduction of the target cell and transgene expression, all while maintaining production efficiency [5].

Nowadays there is a wide range of different types of vectors to choose from when planning an experiment. The decision-making, of which vector is the best, relies on several factors. Based on the characteristics of the applied viral vector, different features of the tissue and the neuronal network are revealed. If fluorescent proteins will be expressed, attention must be paid to the emitted wavelength of the expressed protein and whether it will interfere with the wavelength of the dyes applied for the anatomical analyses. The work of C. Humpel et. al. (2015) illustrates briefly, how pyramidal neurons of human brain slice cultures show robust structural and electrophysiological stability after the transduction of green fluorescent protein (GFP)-encoding adeno-associated viral (AAV) vectors [12]. In another study, the application of viral vector-based optogenetic tools was used to express opsins, such as channel rhodopsin 2 (ChR2), in human neurons up to a predefined level that enables functional manipulation of these neurons by creating action potential, that shows great potential for finding new therapeutic strategies. [6]. One also must decide what are the target cells and based on that cell-specific vectors have to be designed and injected into the culture. The specificity depends

on the targeted promoter of the host cell. The capacity to target specific cell types in adult human brain tissue with viral vectors and a wide range of contemporary molecular genetic tools may speed up the study of the human brain, with a focus on cellular resolution functional studies. Fine-tuning the initial viral vector concentration in the cultures is essential because if it is too high, the expression of the desired protein may cause the early apoptosis of the cultured cells and the swelling of the tissue. While lower virus titre may cause a slow transgene expression, thus, the low concentration of the protein in the cells leads to weak staining. In both cases, it can be difficult to understand the measurements' records [9]. Based on the comparative study of Duigou et al (2018) the time point of the injection does not impact the transduction level of different vectors or the survival rates of the neurons, since after 7-28 days of the injection the value of these properties did not change significantly, the transgene was stably expressed.

In addition to assessing the tool's effectiveness and potency, it is crucial to consider the inherent biological properties of the vector. Organotypic cultures serve as an ideal platform for evaluating potential tissue toxicity. Even though these injected viruses carry modified genetic material, they may still trigger immune responses, mutagenesis, and changes in the physiological characteristics of the infected cells. In the previously mentioned study different vectors, primarily AAV and lentivirus, and different kinds of promoters (hSyn, e/Syn, CMV, CaMKII) were combined to provide cell-type specific transgene expression. Researchers investigated the crucial role of finding the optimal combination as it can influence both the selectivity of transduction and the efficiency of transcription [35].

The combination of the novel method of genetic labelling in adult human brain slices may be appropriate for addressing a variety of experimental questions that were unfeasible before about the activities of human brain cell types and circuits. Until now characteristics of the AAV and lentiviral vectors show great potential for treating diseases in the nervous system ex vivo or in vivo [38]. There is only a limited amount of data about these techniques in human brain tissue cultures. So further experiments are required to improve and broaden the genetic expression approaches and the scale of accessible viruses. Such as using HSV-1 vectors for cell-type specific transduction and transgene expression, that can be used with the human in vitro slices [9], [39].

3.6. Human cerebrospinal fluid as a culturing medium

Human cerebrospinal fluid (hCSF) is a colourless fluid found in the subarachnoid cisterns, between the brain surface and the skull, and in all the ventricles as well as in and around the

spinal cord. It is characterized by a low percentage of protein and a high percentage of salts. It is in constant contact with the interstitial fluid of the parenchyma and contains a variety of neuromodulators, including neurotransmitters, neuropeptides, neurosteroids, purines, and endocannabinoids. Additionally, it functions as a mediator in neuromodulatory signalling [36]. The composition of hCSF can change upon ion channel activation, hormonal changes and due to diseases and medications. Analysing the composition of hCSF might be a solution for the identification of important neuroprotective molecules which help the survival of the neurons in the human CNS and might open new therapeutic routes [33]. Moreover, biomarkers associated with neurodegenerative diseases, such as amyloid-beta oligomers in Alzheimer's disease, can be detected in cerebrospinal fluid even during preclinical stages, possibly enabling the early identification of these health conditions [40].

Determining the ideal culture medium composition is a complex and non-trivial task. The origin and quality of the culturing medium greatly influence neuron viability and network activity. In one study it was described that the electrophysiological activity of human tissues cultured in hCSF is closer to the state recorded in vivo if during electrophysiological measurement the tissues are perfused with hCSF and not with artificial cerebrospinal fluid (aCSF). Moreover, perfusion of hCSF led to increased activity and single-cell firing compared to the activity recorded during aCSF perfusion [36].

The viability of human neocortical organotypic brain slice cultures kept in an artificial culture medium or hCSF was compared in an article. The result of the study shows that the hCSF significantly improved the long-term viability and network function of neurons in the brain slice cultures due to the presence of growth factors and nutrients. The spontaneous activity of the neurons was more typical and not only single but repetitive trains of action potential appeared. On the other hand, increased network activity and tonic activity in aCSF with higher potassium concentration (8mM) was typical. Moreover, the resting membrane potential was more depolarized than in the case of the hCSF [33]. In contrast to this another report has revealed that human neocortical brain slice cultures optimized for rapid-genetic manipulation can exhibit exceptional viability. The researchers applied another type of artificial culturing medium for their cultures which was developed at the Allen Institute for Brain Science (Seattle, WA, USA) [9]. It is advantageous that it can be prepared in advance and the quality can be controlled.

3.7. Neuronal nuclear protein antibody

Neuronal nuclear protein (NeuN) was discovered in the 1990s, and in the last decade, it has

been used as a universal neuro-specific marker[41]. It is a protein that is localized in the majority of the neurons in the central nervous system of mammals, either in their nuclei (46 kDa isoform) or perinuclear cytoplasm (48 kDa isoform). It has not yet been detected in glial cells, Cajal-Retzius cells or Purkinje cells [42]. It is frequently utilized for immunohistochemical analysis in scientific and clinical research because monoclonal antibodies can bind to it. The intensity of the immunocytochemical reactions is influenced by its concentration, which varies between different cell types. For instance, compared to the neocortex, neurons in the substantia nigra of both rats and humans are less intensely stained. In fact, there is no clear correlation relating the degree of NeuN immunoreactivity to specific types of neurons. However, damage to the neurological system (such as axon damage, ischemia, or hypoxia) can result in lower expression of the protein and, as a result, lower staining intensity [41]. Its staining can be helpful for quantitative and semi-quantitative statistical analysis of neuronal cell loss in epilepsy, or neurodegenerative diseases [42].

3.8. *Fluoro-Jade C*

It has been reported in several articles that the classic methods (suppressed silver techniques and hematoxylin and eosin staining), which aim to detect degenerating neurons, are not always able to detect degenerating neurons reliably due to the presence of artefacts and changes in staining intensity, furthermore, they are incompatible with other histochemical methods and consist of many steps that are difficult to implement [43], [44], [45].

An advance was the development of Fluoro-Jade dyes (FJ) as an alternative method from the traditional labelling of degenerating neurons in the year 1997. It can be used with fluorescent Nissl staining or UV nuclear stains such as DAPI or fluorescent immunohistochemistry, performed last to help visualize the cells and cell nuclei with the best results. Because some steps in the FJS staining process are harsh to fluorophores (particularly the KMnO_4 treatment), double staining with FJ and fluorescence immunostaining is not always sufficient. However, utilizing cell-type-specific antibodies is important to provide more precise information on the cell type. The fluorescence intensity will only be strong enough for successful double staining if the quantity and quality of primary antibodies are optimal [46]. The staining mechanism of FJ remains unclear. It is excited with blue light (485-506 nm), while DAPI counter-stained cell nuclei are visualized with ultra-violet illumination. Its emission peak is at 525-527 nm based on the kit protocol.

This staining technique has several advantages against the classical methods. This is a flexible technique, not requiring precise reagent concentration and long incubation time. Secondly, it

is compatible both with fixed and not fixed tissue [43].

There are 3 different types of these anionic, fluorescent derivatives in the market: the simple Fluoro-Jade, the B, and the C variants. Their structure is related but differs in their fluorochrome content. FJ-C exhibits the greatest affinity for degenerating neurons and has the highest resolution and contrast. It seems to be the most optimal dye for histological application. Even the finest neuronal processes, such as distal dendrites, axons, and terminals, can be precisely localized. This variant has the highest affinity for endogenous neurodegeneration and requires the shortest staining time. Additionally, it is highly photoactive, therefore quick fluorochrome fading is not a problem. Lastly, it is less acidic because of one additional sulphate ester group on the fluorescent molecule [43]. However, it has been noted that under some special experimental conditions, the FJC labelling is not suitable to distinguish between degenerating and non-degenerating glial cells. The double staining has to be optimized [46].

4. Planning, materials, and methods

Different examination methods were applied to investigate the integrity, viability of the neurons and the condition of the brain tissue. The first level was the electrophysiological analysis of the electrical properties of a single neuron or a population by intracellular and extracellular recordings in vitro. Following this, the fixed tissue was prepared for immunohistological investigation. In this section, I describe the basic methods and procedures for preparing, maintaining organotypic brain slice.

4.1. Origin of the human neocortical brain slice cultures

Human neocortical brain slice cultures were prepared from tissue obtained from patients undergoing resected surgery due to epilepsy or tumour formation in the National Institute of Clinical Neuroscience (NICN). The resected specimen may contain neocortical tissue from the epileptic focus, and in the case of tumour patients, it usually includes the tissue surrounding the tumour. Furthermore, if the tumour is in the deep brain structures, neocortical tissue has to be resected for technical reasons. In cases of sample infiltration, we can identify it only later through staining techniques like NeuN, parvalbumin (PV), and glial fibrillary acidic protein (GFAP) staining. Typically, we obtain access tissues, which are resected to reach the diseased areas of the brain.

All patients gave informed written consent prior to surgery and in compliance with the General Data Protection Regulation. The procedures and the use of the resected human brain tissue were approved by the Regional and Institutional Committee of Science and Research

Ethics of the Scientific Council of Health (ETT TUKEB IV/8358-3/2021/EKU) and performed in accordance with the Declaration of Helsinki.

4.2. *Preparation of human brain slices*

The tissue was transported from the operating room (NICN) to the laboratory (RCNS), which takes approximately 30-minutes. During the transportation, the tissue was in an ice-cold, oxygenated solution (NMDG). During the subsequent cutting phase, the brain block is submerged in the same solution, which does not contain sucrose but contains HEPES, to help prevent excessive oedema, because it has cell-protective properties. The composition of the NMDG is the following: 93mM N-Methyl-D-glucamine, 2.5mM KCl, 1.2mM NaH₂PO₄, 30mM NaHCO₃, 20mM HEPES, 25mM glucose, 5mM ascorbic acid, 3mM Na-pyruvate. The solution's pH is adjusted with HCl in the range of 7.3-7.4, the end osmolarity is 300-305 mOsm. This version of NMDG can be stored at 4 °C for one week; only before usage should it be supplemented with 10mM (MgSO₄)₇H₂O and 0.5mM (CaCl₂)₂H₂O.

The slices of 300-350 µm and 500 µm thickness were cut with a vibratome (LEICA VT 1200S, Germany). The typical size of the excisions from the surgeries is less than 1 cm³, and after the trimming steps (removing the pia mater and the blood vessels), it is sectioned perpendicular to the cortical layers, and each slice contains all the layers. The thinner slices were sorted alternately for culturing and the thicker ones for acute electrophysiological measurement (more conserved neuronal network-optimal for populational activities) to determine the initial state of the acute tissue, and whether any activity may be expected later in the cultured tissue and for various ongoing projects not mentioned. After sorting we can start the culturing with an averaged 9 slice (SD=3). After that, the ones chosen for measurement were incubated at 35–37 °C (for extracellular measurement) and at 29 °C (for intracellular measurement) in an interface chamber perfused with artificial cerebral spinal fluid (ACSF) containing (in mM): 124 NaCl, 26 NaHCO₃, 3.5 KCl, 1 MgCl₂, 1 CaCl₂ and 10 D-glucose, equilibrated with 5% CO₂ and 95% O₂. Additionally, the pH was calibrated for 7.3–7.4 and the osmolarity for 305–310 mOsm. We use the same techniques and solutions when it comes to tissue cultures.

4.3. *Human brain slice cultures*

After the slices are cut, the culturing can start in the prepared culturing medium under a laminar airflow cabin in sterile conditions. The slices are placed in a sterile 6-well culture plate with 3 cm PTFE (polytetrafluor-ethylene) inserts, in a way that the slices are located on top of the insert with 0.4 µm pore size and the culture medium (750 µl) is underneath it, so it

only contacts the slices from the bottom through the membrane not causing any mechanical damage. In our case, only during the first 13 cultures, the culturing medium was human cerebrospinal fluid (hCSF), which has several advantages based on the reports. (Table 1) [36] We were able to gain hCSF from NICN and store it at -80°C.

We also considered applying an artificial culturing medium based on the protocol developed at the Allen Institute for Brain Science (Seattle, WA, USA), because they reported that in this medium the cells were not dispersed even after 18 days in vitro, and the neuronal density was well-preserved. [9] Until now we applied this medium on 5 cultures. (Table 1)

Sample	HOC	Date of operation	Age	Gender	Disease	Region
HP67	1	2021.11.	70	female	tumour	frontal
HP68	2	2021.11.	65	female	tumour	parietal
HP70	3	2022.01.	66	female	tumour	frontal
HP72	4	2022.02.	68	male	tumour	temporal
HP73	5	2022.03.	82	female	tumour	temporal
HP74	6	2022.03.	43	male	tumour	temporal
HP75	7	2022.04.	61	male	tumour	temporal
HP77	8	2022.07.	42	male	epilepsy	temporal
HP78	9	2022.09.	27	male	epilepsy	temporal
HP79	10	2023.02.	24	male	tumour	temporal
HP80	11	2023.03.	62	female	tumour	frontal
HP82	12	2023.03.	76	male	tumour	temporal
HP84	13	2023.04.	58	female	tumour	temporal
HP88	14	2023.05.	62	male	tumour	n.a.
HP89	15	2023.06.	n.a.	n.a.	tumour	n.a.
HP90	16	2023.06.	71	male	tumour	temporal
HP93	17	2023.07.	74	male	tumour	temporal

Table 1: Patients included in this work (HP: Human Patient, HOC: Human Organotypic Culture)

The composition of this artificial culturing medium is the following: 8.4 g/L MEM Eagle medium, 20% heat-inactivated horse serum, 30 mM HEPES, 13 mM D-glucose, 15 mM NaHCO₃, 1 mM ascorbic acid, 2 mM MgSO₄, 1 mM CaCl₂, 0.5 mM GlutaMAX-I, and 1 mg/L insulin, carefully adjusted to pH 7.2–7.3, also the osmolarity is set to 300–310 mOsm by adding distilled water, following this the medium is sterile-filtered and can be stored at 4°C for up to two weeks. Although the plates are in a sterile environment, antibiotics and fungicides are added to the culturing medium during the first week. Since on the first 3 cultures fungi appeared, the composition of the antibiotics was modified, penicillin (100unit/ml) and streptomycin were extended with amphotericinB (100unit/ml).

The culturing plates were placed in the laminar airflow cabin at 35°C and while the hCSF

medium was changed every day, the artificial culturing medium was replaced only every 2-3 days.

4.4. *In vitro electrophysiological recordings*

Since the electrophysiological properties of a single neuron and population activity in the tissue can change over time, we perform electrophysiological measurements on the cultured tissue. In this way, we can observe how the preservation of the tissue, or the intrinsic and extrinsic properties of the neurons vary in relation to the electrophysiological properties during culturing. Based on several studies, the cells become more depolarized after 2-3 days in vitro (DIV), but after that, they stay stable over the remaining time in vitro [9][12]. Another group examined epileptic activity after electrical stimulation in epileptic tissues at DIV 30. [47]

For analysing the intrinsic characteristics of the pyramidal cells, we do whole-cell patch clamp recordings with glass pipettes combined with two-photon imaging, and the extracellular activity recording is carried out with a multi-channel silicon laminar microelectrode. Later during analysis, the recordings are filtered based on the frequency and based on it the population and the single-cell activity can be distinguished. Two types of recording systems are setup for in vitro measurements. (Figure 2)

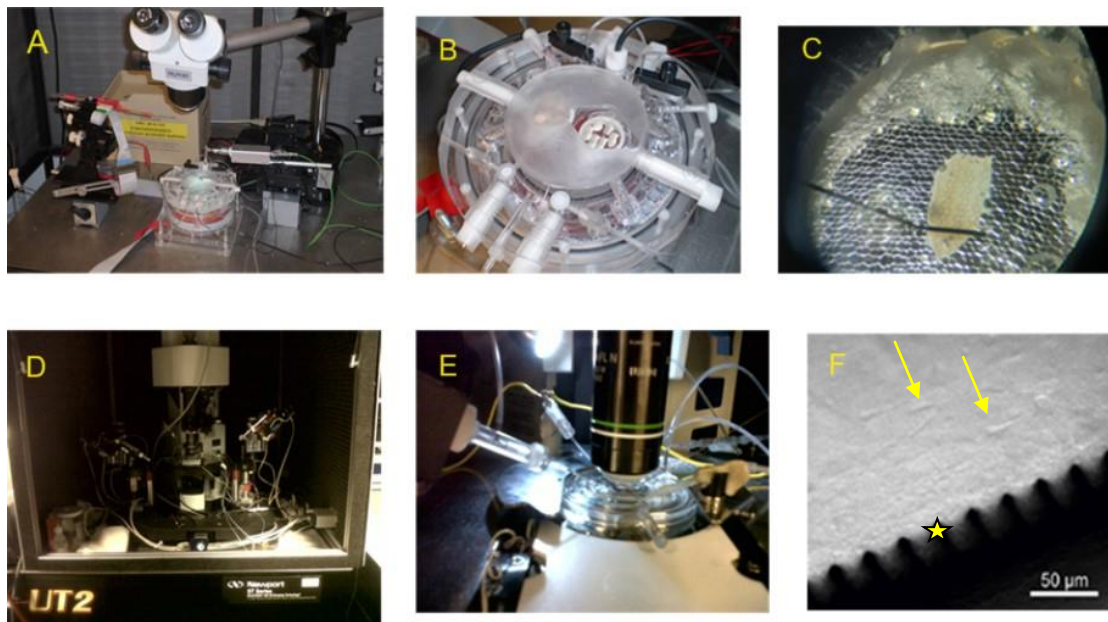


Figure 2: Setups for electrophysiological recordings. A: Setup for extracellular recording equipped with stereomicroscope and (B) an interface chamber. C: The slices are placed on a mesh, and the laminar microelectrode is placed above the slice. D: Setup for intra-, and extracellular measurements equipped with a two-photon microscope. On the right handside is the holder for the extracellular electrode, on the left handside the patch pipett holder (left), E: the slice is placed in a dual perfusion chamber under the microscope. F: 25 μm Spiky probe above rat brain tissue under bright field camera mode, where the spikes are visible. Pyramidal cells are signed with arrows and the end contacts with yellow stars. (Modified [48])

4.5. *Immunohistochemistry*

Immunostaining was performed on the fixed tissue to visualize the viable cells.

At predefined intervals during culturing or after the electrophysiological recordings the slices were preserved with a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB).

The tissue cultures become typically flattened because of the specific properties of the culturing. During immunohistochemistry, they must be treated cautiously since the cultured tissue gets more and more fragile and delicate the longer the culturing period is because of the changes in the content of the extracellular matrix or the cell integrity. Before immunostaining the 300-350 µm thick cultured tissue was resected into 60 µm thick slices.

4.5.1. Immunostaining protocol for light microscopy

The samples were prepared for immunostaining using the neuronal cell body marker NeuN antibody (1:2000, EMD Millipore, Billerica, MA, USA, RRID: AB_2298772). As an antibody mouse monoclonal antibody was used. The protocol we use for human tissue's immunostaining is the following.

After fixation, the slides were washed with 0.1 M phosphate buffer (PB) and stored in a 30% D-saccharose diluted in 0.1 M PB at 4°C for a night. It was followed by a freezing above liquid N₂ repeated three times, after this, the slices could be stored at -80°C in the refrigerator. The next step was the blocking of the endogenous peroxidase activity by 1% H₂O₂ in PB for 10 min after a washing step (3×10 minutes). 1% H₂O₂ (diluted with PB) helps to prevent the non-specific binding of the peroxide enzymes found naturally in the tissue. Next, non-specific immunostaining was blocked by 2% normal goat serum and 2% normal horse serum for one hour and then washed with PB for 5 minutes. After that, the primary antibody was added and applied for two days at 4 °C. A biotinylated anti-mouse IgG (1:250, Vector, Burlingame, CA, USA) was used as a secondary antibody to visualize the immunopositive components for two hours and washed again with PB 3x10 minutes long. This was followed by an avidin-biotinylated horseradish peroxidase complex (ABC, 1:250, Vector, Burlingame, CA, USA) for 1.5 hours. 3,3'-diaminobenzidine tetrahydrochloride (DAB) used as a chromogen and dissolved in 0.1 M PB (1:9), was used for 20 minutes to create the immunoperoxidase reaction. As a result, by adding 10 µl of 1% H₂O₂ (diluted in 0.1 M PB) to the 1 ml DAB-PB solution a colour change occurred at the location of the antigen which can be visualized under a light microscope. After that, the slices could be placed in chrome-gelatine and right after mounted on slides in order. They dried for 24 hours, then they were dehydrated and immersed in Xylol for 2x10 minutes and lastly covered with Depex.

4.5.2. *Fluorescent staining protocol for the visualisation of degenerating neurons*

The protocol of the double-labelling with FJC and an antibody is usually the following.

Prepared tissue sections were mounted on chrome-gelatine coated slides and dried at 50°C for at least 30 minutes to one hour for good adhesion and drying. The staining steps were carried out in cuvettes. The applied solutions are from a kit containing 4 types of solutions (TR-100-FJ, BioSensis, Thebarton, Australia):

- SolutionA: sodium hydroxide (prior use 1:10 diluted with 80% ethanol)
- SolutionB: potassium permanganate (prior use 1:10 diluted with distilled water)
- SolutionC: FJC (prior use 1:10 diluted with distilled water)
- SolutionD: DAPI

First, the slides were incubated in SolutionA. They were transferred into a solution containing 70% ethanol then into distilled water for 2-2 minutes. The second step was the fluorescent background blocking and contrast optimization by SolutionB for 10 minutes and then the slides were rising for 2 minutes in fresh distilled water. The fluorescence quencher potassium permanganate is rather potent, therefore also able to affect the FJ staining. Timing can be changed to improve the fluorescent signal-to-background ratio. The FJC staining is performed in the dark; the slides are incubated for 10 minutes in SolutionC. After that, the slides are rinsed for 3x1 minutes in distilled water and dried on a slide warmer. Lastly, they are covered with Depex, which is a non-aqueous, low fluorescent, styrene-based mounting media. Later, FJC-labelled degenerating neurons are visualized with blue light, excitation peak is at 485-506 nm and the emission peak is at 525-527 nm.

To gain a better contrast and less noisy background we changed the protocol multiple times and tried out different intervals for staining. The length of the double staining namely the time spent in KMnO_4 and DAPI solutions varied between 10 and 15 minutes and between 20 and 30 minutes. (Table 2)

Protocol	1	2	3	4
KMnO_4	10	15	15	10
FJC	20	20	30	30

Table 2: Protocols for the FJC staining. The time length spent in the KMnO_4 and FJC solution was varied in minutes.

4.6. *Microscopic investigations*

After the tissues were fixed and immunostaining with the NeuN antibody was performed, images of the stained slices were made under a light microscope for the histological analysis (Leica DM 2500 microscope with an Olympus DP73 camera). Magnifications of 2.5x, 5x, 10x, and 20x are generally used. Images of the tissue are made not only in low magnifications

where the changes in the laminar architecture of the neocortex can be seen but also in higher magnifications of smaller parts of the tissue where neuronal integrity is in the focus. Subsequently using these images, cell counting can be done.

Since FJC is a fluorochrome, for the evaluation of the staining of the degenerating neurons we use confocal fluorescence (Zeiss LSM 710, Germany) microscope or epifluorescence microscopy (Leica DM2500, Germany), which is easier to handle, more cost effective but the resolution and the sign-noise ratio is not as great as in confocal microscopes. The previous is a powerful tool for visualising fluorescently labelled cells with high resolution while the latter is perfect for quick mainly preliminary investigations [49].

4.7. Data analysis and statistical testing

Electrophysiological data were analysed using custom analysis software. Based on our protocol three minutes long continuous recordings are saved for later analysis. After the pre-processing, the files are visualized by the NeuroScan 4.5 Software package, and the neuronal activity is extracted with automatic Neuroscan Edit4.5 program (Compumedics Neuroscan, Charlotte, NC, USA) and home written routines or MATLAB (The MathWorks, Natick, MA, USA, RRID:SCR_001622).

The images being made under the microscopes can be investigated with several programs. In this case, I used the ImageJ program (National Institute of Health, Bethesda, MA, USA) for semi-automated cell counting for validation and a program code written in Python language, which enables a faster way to count the cells on the stained tissues. In this program, basic image processing steps are applied to create a better contrast between the background and the cells. It allows the marking of the region of interest (ROI) and calculates the cell density. I chose the ROIs based on the tissue structure, so I counted the cells systematically on each layer, where it was possible to distinguish the layers of the neocortex. The cell density was normalised for 1 mm².

The images were edited subsequently with Adobe Photoshop (Adobe System, San Jose, USA). For data analysis, I applied GraphPad Prism (Dotmatics, Boston, Massachusetts, USA).

5. Results

5.1. Qualitative analyses

5.1.1. Patient data and tissue origin

Organotypic tissue cultures were established using samples from 17 patients who underwent surgeries between November 2021, and September 2023, at the National Institute of Clinical

Neuroscience (NICN). Out of these patients, 15 were treated for tumours, while 2 underwent surgery due to epilepsy (see Table 1). Based on the size and quality of the resected brain block the number of slices initially cultured varied between 3 and 20 (with an average of 10/culture), determining the maximum length of the culturing period, ranging from 6 to 35 days. (Table 3)

HOC	Number of slices	Culturing medium	Antibiotics (7days)	Applied Treatment	Length of the culturing
1	7	hCSF	P/S		27
2	17	hCSF	P/S		16
3	19	hCSF	P/S		12
4	10	hCSF	P/S		23
5	9	hCSF	P/S		16
6	19	hCSF	P/S		20
7	9	hCSF	P/S		22
8		hCSF	Anti/Anti		14
9	20	hCSF	Anti/Anti		19
10	11	hCSF	Anti/Anti		21
11	7	hCSF	Anti/Anti		21
12	6	hCSF	Anti/Anti		6
13	8	hCSF/AM	Anit/Anti	Virus1	28
14	3	AM	Anti/Anti	Virus 2	14
15	5	AM	Anti/Anti	Virus1	35
16	4	AM	Anti/Anti	Virus 2	14
17	12	AM	Anti/Anti	HCPC	10

Table 3: Cultures included in this work (hCSF: human cerebral-spinal fluid, AM: artificial medium, P: penicillin, S: streptomycin, Anti/Anti: penicillin, streptomycin, amphotericinB) Virus1- Virus expressing GCaMP-(neurons, glial cells). Virus 2-virus with an interneuron specific promoter expressing CYP. HCPC-human hippocampal progenitor cells expressing GCaMP, transduces neurons.

5.1.2. Culturing medium composition

We encountered several challenges related to the quality of the culturing medium after a few culturing sessions. The first 12 tissue cultures were maintained in hCSF and after HOC7 the supplemented with streptomycin and penicillin for the first week of culturing. The quality of the hCSF depends on several factors which we cannot influence. It was transported to our lab only after a larger amount (125-150 ml) had been collected during the various health screenings, the time varied between a few days and four weeks. The quality of the liquid was therefore not perfect by the time we received it. In addition, it should be centrifuged immediately after the procedure to remove cellular particles, but in our case, this was only possible later. Moreover, we would need to add hCSF from multiple patients with different clinical statuses during a single culturing session as we required 750 μ l of it daily, which is impossible to collect from a single patient. If the quality was good, it wouldn't necessarily be a problem if it came from different patients.

Considering the issues mentioned above, we made the decision to transition to the artificial culturing medium (AM) developed by the Allen Brain Institute. The shift was motivated by the demonstrated high preservation and viability of tissue slices in AM, as reported in published papers. Notably, the surface of the slices and the laminar architecture of the neocortex more closely resembled those in acute tissue when compared to hCSF. The activity of the cells and the quality of the tissue was also better. (Figure 3) Additionally, in the article, they are cultured in AM for only 18 days, but in our case, the activity persisted for up to 28 days. This is also a noteworthy result because one of the advantages of hCSF was expected to be its ability to support longer-term culture. In future experiments, we intend to use the artificial culturing medium extensively due to its superior ability to retain tissue structure and its established reliability [9].

Beside the hCSF, antibiotics (a combination of penicillin and streptomycin) were added to the tissue cultures during the first 7 days to prevent the development of any. In the first three cultures fungi appeared on the tissues, so the composition of the antibiotics was supplemented with an additional fungicide. Later, no fungi, bacteria or mycoplasma developed in the cultures. These concentrated antibiotics (100-100 unit/ml penicillin, streptomycin, and amphotericin) luckily did not affect the viability of the tissue cultures based on the day-to-day photos and the electrophysiological measurement.

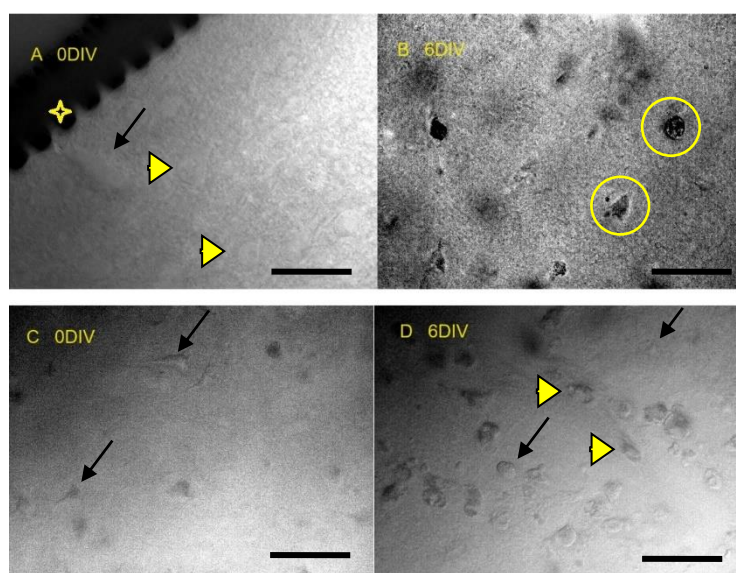


Figure 3: Light microscopic images made before extracellular measurements with 10x magnification. A: Acute slice (HOC6) with the Spiky (yellow star). The cells look healthy (arrow) or their soma is little ischemic (arrowhead). B: Tissue after 6 DIV (HOC6) in hCSF, the cells are apoptotic (circle), and vesicles are visible. C: Acute slice with healthy looking cells. D: Tissue after 6 DIV (HOC17) in AM, the cells are swollen and the soma is contrasted in some cases, but the tissue is more homogeneous, and the cells are preserved better. (Scalebar: 50 μ m)

5.1.3. Structure and preservation of the cultured tissue

The transformation of the tissue was noticeable even without magnification. The quality of the culture can be inferred from the colour of the slices, but the good appearance does not mean its good health state. Furthermore, AM is pH-sensitive, if it turns yellow it means a lower pH, which could indicate bacterial contamination. (Figure 4)

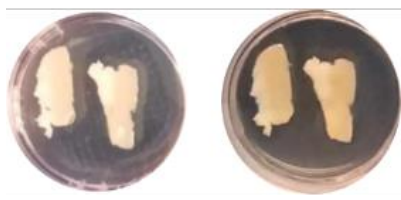


Figure 4: Transformation of AM-cultured tissue slices over time. A: Organotypic tissue culture at 0 DIV closely resembles the initial acute slices, displaying a white colour, and adhering firmly to the bottom of the insert. B: 5 DIV, the colour of the slices has changed, become thinner.

Although in early cases we have not managed to make interpretive electrophysiological recordings, we performed immunohistochemical staining on the fixed tissues as described in the section Material and Methods. The resulting images offer valuable insights into the alterations occurring within the tissue. These images were captured using a light microscope to observe the NeuN staining, while the samples stained with the fluorescent FJC were examined under an epifluorescent microscope.

5.1.4. Intracellular recordings

Despite our multiple attempts to carry out patch clamp experiments and record intracellular neuronal activity, we encountered persistent challenges in rupturing the cell membrane using the glass pipette. In experiments, when the tissue culture was from hCSF, the real-time image displayed different characteristics compared to those observed in acute tissue samples on several occasions. (Figure 3 A, B) Additionally, in some cases when tried to approach the cell with the pipette, the cell suddenly vanished before or after we touched it with the pipette, probably due to the pressure. Although the pipette functions effectively with acute slices, its parameters are not well-suited for use with cultured tissue, leading us to develop a new protocol. Notably, the soma of the cells often appeared overly contrasted, while the membrane indicated decreased cell viability. Additionally, manipulating the pipette became more challenging, as it frequently adhered to the tissue and was difficult to keep open. This suggests that the tissue's integrity was compromised. Importantly, we observed that the preservation of the tissue was better when AM was applied, because it was easier to approach the cells, although in this case the cells also changed. (Figure 3 C, D)

5.1.5. Extracellular recordings

Electrophysiological measurement was not performed on each culture, but we managed to record extracellular activity from one sample (HOC7) at 7 DIV from hCSF and from another sample (HOC15) at 28 DIV. The extracellular activity measurement was carried out with the laminar electrode containing 24 independent channels with 150 μ l inter-contact distance (Figure 5). Furthermore, 2 cultures were injected with non-selective (expressing in both neurons and glial cells) virus expressing GCaMP and change in calcium level was observed. These phenomena draw attention to two important aspects. Firstly, it is worth trying out other

protocols for glass pipette pulling because there are active cells. Secondly, it suggests the potential for introducing transgene-containing viral vectors or pharmacological compounds into the tissue cultures.

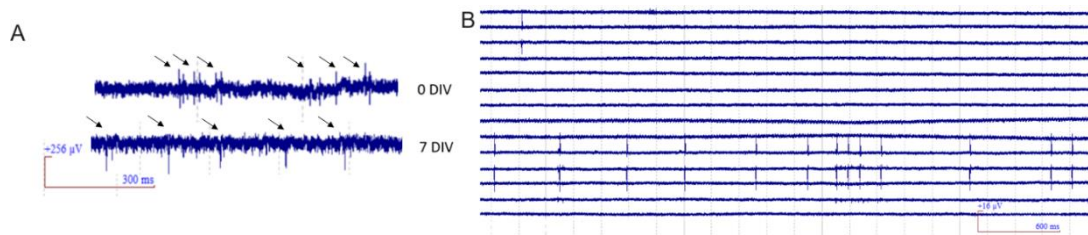


Figure 5: Spikes represent the activity of single cells. A: Recordings of extracellular activity on one channel out of 24 measured an acute tissue (top) and on a tissue after 7 DIV in hCSF (bottom). The probe was placed above the neocortex in both cases. Action potentials of a single cell were detected on both specimen (marked with black arrows) with Spiky. B: Row recording of extracellular activity with the same electrode. The tissue was maintained in AM for 27 DIV and action potential of single cells can be seen on 5 channels. The electrode was placed above the L2/3. This suggest that viable cells are in the tissue even at almost 4 weeks of culturing.

5.1.6. NeuN staining

During the histological analysis, we compared 14 cultures originating from 14 different surgical specimens. These cultures were maintained under slightly different conditions, as outlined in Table 3. Notably, it became apparent, both through visual observation and during the staining process, that the slices fixed at later time points were thinner and less structured. Consequently, they could be harmed easily with the brush and the pipettes during washing. Additionally, the extracellular matrix within these slices became more transparent.

In the early cases, the laminar architecture of the neocortex did not remain intact. The neuronal density decreased over time based on the comparison of the images of the acute and cultured slices. While each layer of the neocortex is visible on the acute tissue, after 7 DIV the pyramidal neurons are not as contrasted as in the acute slices, the proximal dendrites and the axon are less visible, the extracellular matrix is less dense, the tissue laminar architecture changed, and the neuronal integrity decreased, but the tissue still can be applied for experiments. (Figure 6)

In several samples pyramidal cells remained in distinct groups. This could be attributed to several factors. (Figure 7) On one hand, these subpopulations might have more synaptic connections with each other within the resected tissue, potentially supporting their mutual viability. On the other hand, even though the resected specimen were categorized as originating from "healthy" brain regions, the presence of nearby tumours could alter the local environment, affecting factors such as nutrient supply and pressure, which might contribute to changes in the cell density pattern. (Figure 7) NeuN stains every neuron in the brain. Although most neurons in the neocortex are excitatory pyramidal cells and spiny stellate cells, inhibitory interneurons are also important in the generation of neocortical activity. (Figure 8

first column) Furthermore, the loss of inhibitory cells is a common phenomenon in the epileptic brain. To gain information about the fate of these cells, we applied specific interneuron markers.

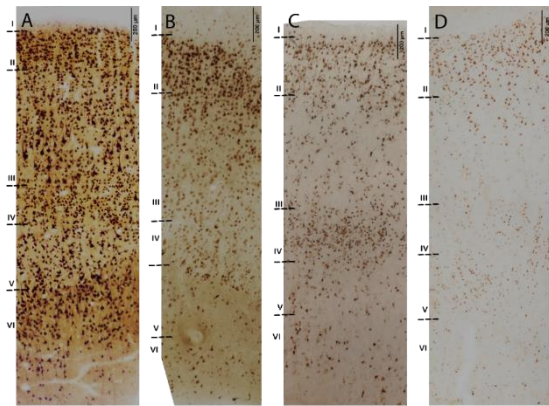


Figure 6: Changes during the culturing of a brain slice with NeuN. The first layer is on the top, the images are made under light microscope. A: On the acute tissue the 6 layers are well detectable; B-C: The neocortical brain slice can maintain its laminar architecture and integrity at 7 DIV and 14 DIV; D: In the late culturing period (DIV23) the layers are not as visible as in the early stages, mainly the degrading cell bodies are stained in L2/3. (Examples are not necessary from the same culture.)

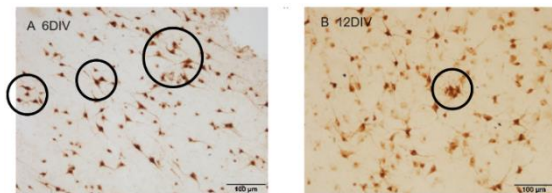


Figure 7: In some sample a denser cluster of the neurons (marked with circles) is visible. This is typical in epileptic tissue A: The sample is from epileptic patient (6 DIV), B: The sample is from tumour patients in this case our sample is near to the tumour, infiltration or changed pressure and supplement of nutrition can cause this phenomenon (12 DIV). (Examples are not from the same culture.)

5.1.7. FJC staining

The result from the FJ staining visualising degenerating cells (n=15) suggest similar changes to the ones that we observed with the NeuN staining. In the acute tissue, neuronal cells were mainly unstained, however in some cases activated glial cells and blood vessels were visible. (Figure 8 second column A) However, as time progressed, the tissue displayed hollow spaces, most likely representing the locations of former neural cells. The FJC staining revealed two types of FJC+ pyramidal cells, those with strongly stained proximal dendrites and the ones where the staining was mainly visible in the cell body (Figure 8 second column B, C, D), the latter were probably more viable, since the dye labelled only the soma is.

In addition to pyramidal cells, other cell types and parts of the extracellular matrix were also stained. Some cultures exhibited the presence of glial cells. Notably, the appearance of activated glial cells did not show a specific pattern. They were predominantly observed in seven cultures, mainly on acute slices (HOC1,4,8,9), and even after the first week of culturing in some cases (HOC14,16,17). These glial cells were often located at the edge of the slice, near blood vessels, or in proximity to pyramidal cells. Based on the studies, these appear to be activated microglia. Immunostaining with GFAP staining astrocyte which reveals the glial scar was performed on some cultures, and gliosis was revealed in HOC14, 16. However, the literature is contradictory regarding which type of glia can be stained with FJC. Further investigations are needed to clarify this topic. [46], [50] (Figure 9)

NeuN

FJC

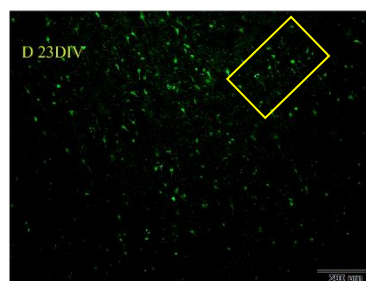
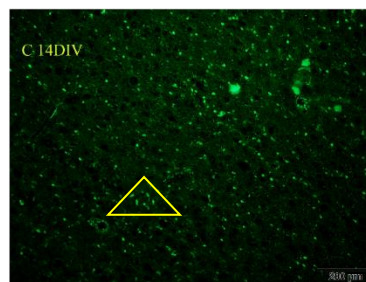
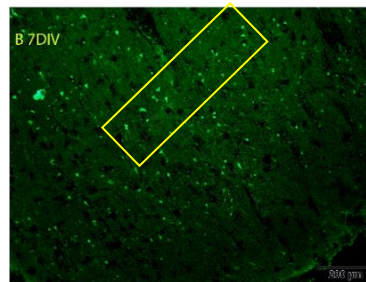
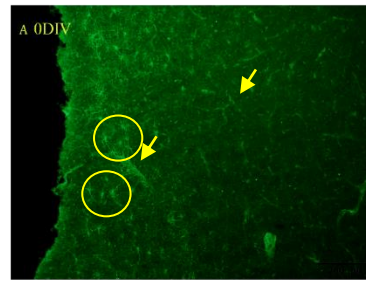
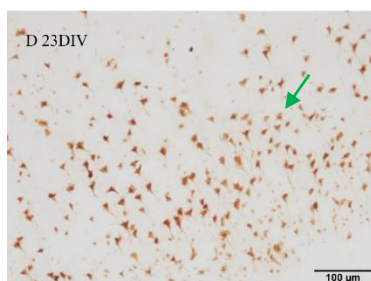
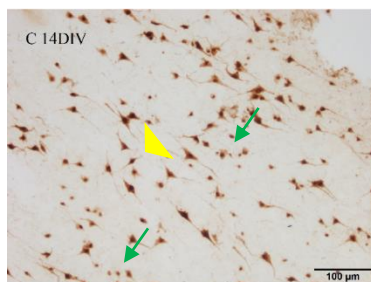
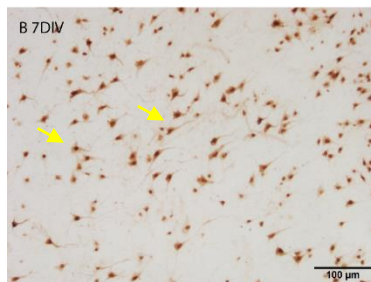
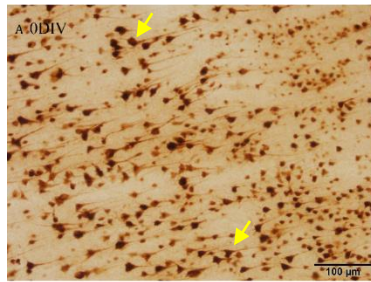


Figure 8: Changes in the neuronal density and viability between 0 DIV and 23 DIV. First column: Staining of NeuN positive neurons. The images show the change of the neural viability over time (A: 0 DIV, B: 7 DIV, C: 14 DIV, 23 DIV). The number of stained neurons decreases over time. A: On the acute tissue not only the cell bodies can be distinguished but the proximal dendrites too. On day 7 (B) and 14 (C) both healthy-looking and degrading (smaller round cellbodies) neurons are visible, and the tissue is more transparent. Beside the pyramidal cells the interneurons (yellow arrowhead) can be stained too. D: 23 DIV we can see less neurons with soma. (The examples are from different cultures. Examples of viable pyramidal cells are marked with yellow arrows, degrading with green arrows.)

Second column: FJC staining shows changes in the preservation of the tissue and the viability of the neuronal cells. A: On the acute sample slice autofluorescent vessels (marked with arrow) are visible beside the activated glia (circle) supporting the vessel. B: During culturing the neurons lose their viability, the stained cells are stressing or are apoptotic. The dead cells were engulfed by glial cells visibly showing as hollow space in the tissue, the stained part is mainly the soma, but in some cases the axon and dendrites are also well contrasted (rectangle). (7 DIV) C: The tissue's tension changed; the

viable cells' density is lower. The parts of a single cell are not as contrasted anymore, and the soma is swollen (triangle). (14 DIV) D: After 3 weeks of culturing the autofluorescence of the tissue decreases, it is cleaned out, more hollow spaces appeared, and most of the proximal processes are stained too (23 DIV) (The examples are not necessary from one culture.)

Blue light excitation can induce autofluorescence in organotypic tissues, and lipofuscin, NADH, collagen, and elastin are among the common contributors to this autofluorescence. Fibrillar autofluorescence was most prominent at the edge of the slices, close to the culturing medium, or in larger groups on the slice. (Figure 10)

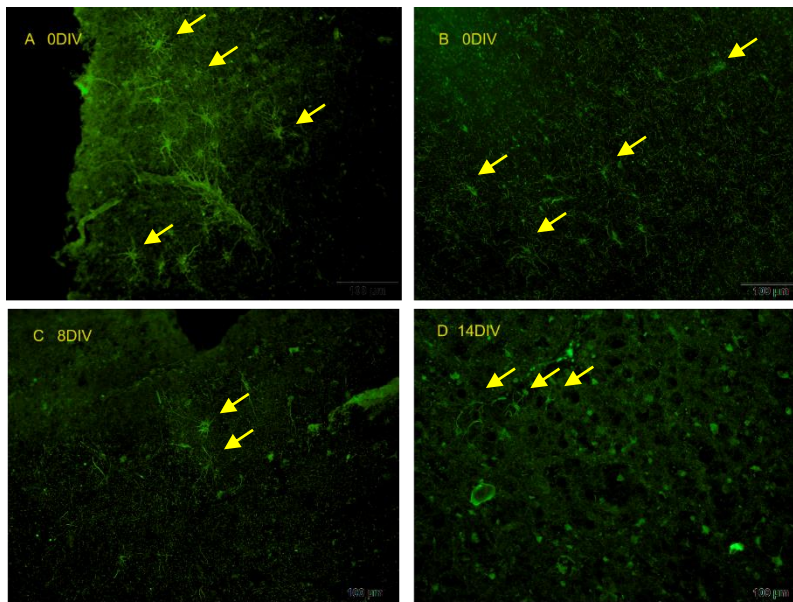


Figure 9: Activated glial cells (marked with arrows) were observed not only on acute tissue after FJC staining. A, B: The activated glial cells are stained mainly around vessels and inside of the acute tissue. C, D: After several DIV on some sample glial cells appear at the edge of the slice, and their processes extended towards vessels or neurons. (The examples are not from the same culture.)

The autofluorescence of the red blood cell-containing blood vessels was the most intensive during the early culturing period since the blood that may have remained in the tissue had been cleaned up, and the changes in the glial cells may potentially affect the autofluorescence of the blood-brain barrier (BBB). In the beginning, the blood vessel wall and the inner content were autofluorescent, as time went on after circa 7 DIV small particles appeared on the blood vessels' wall. The presence of macroglia/macrophages might be a potential explanation for these observations [51].

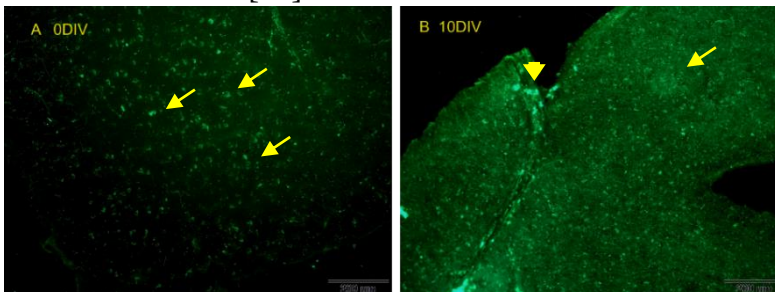


Figure 10: After excitation not only the stained cells are visible. A: On the acute tissue the number of lipofuscins is high. In the human tissue appearance of lipofuscins is typical (arrow). B: The number of lipofuscins increases during culturing. The collagen content of the extracellular matrix possesses autofluorescent properties. The non-homogeneous distribution of the

appearance may be linked to variations in the tissue's structural integrity and cellular composition. On the blood vessel activated microglia/macrophage are visible (arrowhead).

5.1.8. Transgenic expression through viral vector infection

Viral vector was injected into 5 tissue cultures, totalling 14 slices. The expression of 3 different viruses was tested (see Table 3). The injection occurred on the 0 DIV in all three cases. Based on widefield and two-photon imaging, the expression of the first virus, which transduces all cell types with a generic promoter, was successful. The change in calcium ion concentration of the cells led to the change in light intensity under both microscopes due to the GCaMP content, even after one day of the injection, and we managed to capture the activity until 28 DIV. expression The expression was more effective on the slices cultured in AM, suggesting that this medium preserves the viability of the cells better, ensuring a better

environment for transgene expression. (Figure 11) The effectiveness of the transgene expression was high, leading to the problem that the activity of the cells could not be observed precisely due to the excessively intense baseline activity of the virus.

The second virus targeting inhibitory cells was not calcium-sensitive; the activity of the cells could not be seen during real-time imaging. The result must be evaluated on the fixed tissues. The third virus targeting neurons was ATP-sensitive, and the cells should express mCherry after infection. Based on immunostaining, the transduction of this virus was unsuccessful.

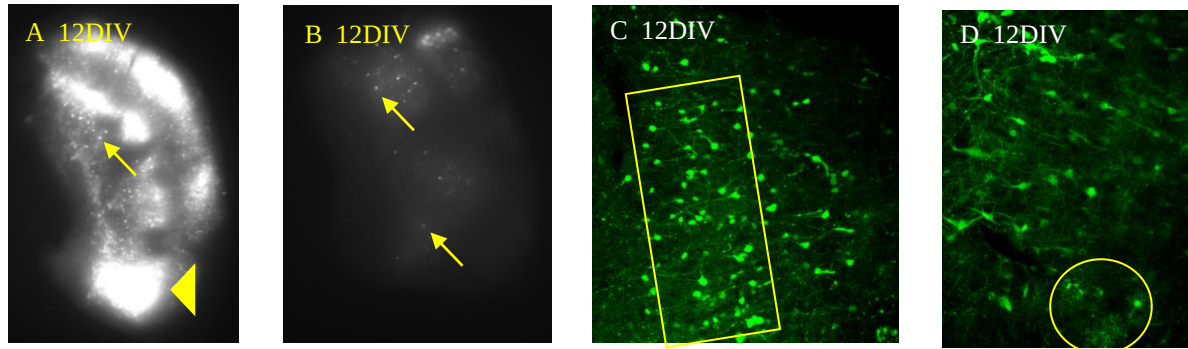


Figure 11: Widefield imaging (A, B) on cultured tissue and confocal imaging (C, D) on fixed cultured tissue shows the successful expression of virus 1 on HOC13 DIV12. A: The virus injected to the slice cultured in AM express the transgene strongly, and overexpression masks the true activity of the cells (arrowhead). White dots indicate strong expression (arrow). B: The slice was cultured in hCSF, the transgene expression and the viral activity is lower. C-D: Without any additional staining the pyramidal cells (rectangle) and astroglial cells are visible (circle).

5.2. Statistical evaluation of the results

Analysing the data in this project presents unique challenges due to various factors beyond our control, such as the origin of the specimens and their initial quality. After the fixation the cultured slices were recut to 60 μm thin slices for anatomical investigation (anatomical slices), introducing four crucial variables into the analysis.

Firstly, even when focusing on a single culture, the samples originate from different depths of the cultured slice resulting in anatomical slices of varying quality (e.g., size, structure). (Figure 12) Anatomical slices from the middle of the cultured slice tend to retain a more defined structure, while those from the top exhibit less conservation due to other biological processes that may disrupt the integrity of the tissue. Various areas of the tissue may suffer damage for different reasons. The upper and lower layers may experience harm during the cutting process, as cells are damaged, or due to glial scar formation at the top and mechanical damage at the bottom. The inner part is susceptible to ischemic damage.

Secondly, the survival time of the cultured slices are not consistent and vary across cultures. This variability makes it challenging to establish correlations, as it is not always the case that only one variable differs in the comparisons. Consequently, the initial amount of data suitable for specific analyses had to be restricted and as a result, the amount of data available for

various comparisons has significantly decreased.

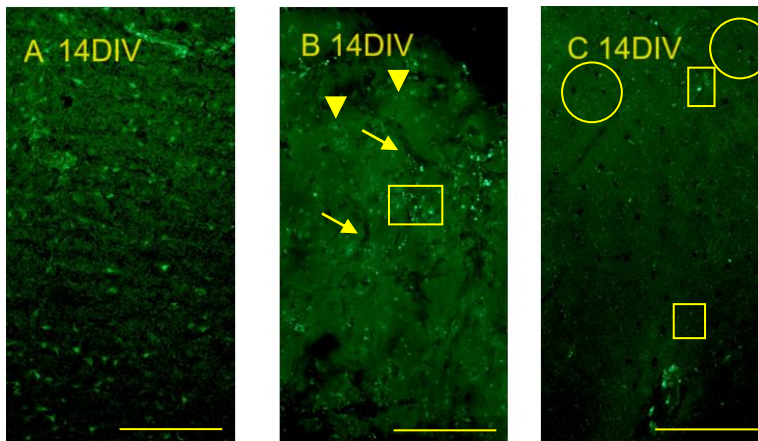


Figure 12 Changes in the quality of the tissues revealed with FJC staining. These images are taken of one tissue culture after 14 DIV in AM. A: The sample is rich in stained degrading cells, suggesting that it originates from the inner part of the cultured tissue. B: The tissue contains a lot of blood vessels (arrow), a few contains autofluorescent particles, the smaller might be lipofuscins (rectangle), the bigger apoptotic cells (arrowhead). C: This part contains hollow spaces (circle), stained cells and lipofuscins (rectangle). (Scalebar: 200 μ m)

Thirdly, the quality of staining significantly influenced the effectiveness and reliability of the output data generated by the custom-coded algorithms. To address this issue, cell counting was semi-automated, and I manually adjusted the real-time filtering or altered the count of detected cells when necessary. However, this approach introduces a degree of subjectivity.

Lastly, it is worth noting that the number of neuronal cells in the neocortex exhibits a strong negative correlation with age, which further complicates the analysis [52].

5.2.1. Cell density based on the NeuN-positive (NeuN+) cells

The staining intensity is proportional to the NeuN content of the given cells. During culturing the cells lose their viability leading, for instance, to changes in the concentration of proteins found in the nuclei. Based on the images I marked that the later the slice was fixed, the contrast of the stained cells decreased, furthermore the tissue quality also changed, I identified 3 groups based on the staining: (1) the tissue is not transparent and the cells are well contrasted, (2) the tissue is transparent and the cells are well contrasted, (3) the tissue is transparent and the cells are not well contrasted.

I identified three factors that may affect the counting of NeuN-positive cells, as determined by Spearman's nonparametric correlation, which align with my initial assumptions (Figure 13). Firstly, the later the tissue was fixed, the more challenging it became to reconstruct the slice due to increased fragility, resulting in more frequent tearing of the samples. Additionally, the intensity of staining decreased as the NeuN content of the cells decreased. Also, according to multiple linear regression analysis, patient age, gender, and the culture medium had a significant impact on the outcome of cell counting. To assess the normality of the data sets, a Shapiro-Wilk test was conducted.

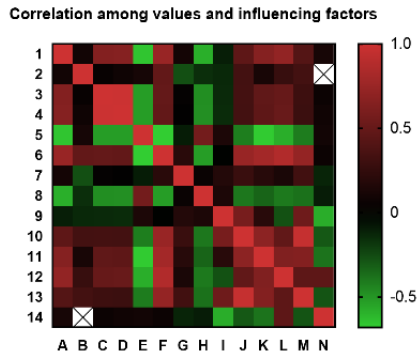


Figure 13: Spearman correlation analysis for NeuN and FJC staining data. Red squares indicate strong positive correlations, while green squares indicate strong negative correlations. Black squares denote no significant correlations. Factors (indicated with numbers and letters) included in the analysis are the following: 1A: black point ratio on fluorescent images, 2B: cell number based on NeuN staining, 3C: Kurtoses of histogram, 4D: Skewness of histogram, 5E: Mean pixel intensity on fluorescent images, 6F: HOC, 7G: DIV, 8H: Gender, 9I: Age, 10J: culturing medium, 11K: fluorescent protocol, 12L: antibacterial treatment, 13M: viral treatment, 14N: Disease of the patient. Markable is that the black point ratio has negative correlation with the mean pixel intensity, so as the number of stained neurons increases the level of the tissue autofluorescence also increases. Number of NeuN⁺ cells negatively correlate with DIV, which means the decrease of cells

over time, but there is weak positive correlation with culturing medium and viral treatment, meaning that in AM was the viability slightly higher, the same with the virus containing samples, also these were maintained in AM as well. The mean pixel intensity negatively correlates with almost everything, but with the HOC and the fluorescent protocol is this connection the most significant, suggesting that the cultures exhibit unique fluorescence intensity.

5.2.2. Differences caused by the culturing medium

Two different culturing media, hCSF and AM, were used on the slices, each with its unique composition and properties. While previous research from other groups has promising results for both media, our qualitative analysis indicated that the cultured tissues showed greater stability in AM. This led to my investigation of whether the choice of medium made a difference in cell preservation in our specific case.

Concerning the cell density across all six layers, there is no statistically significant difference between the hCSF and AM protocols (Paired t-test, $p=0.7699$, $n_1=19$ $n_2=10$ different slices for 6, 7, 8, 10, 12, 14, 21, 24 DIV) (Figure 14). Although it is important to note that NeuN staining identifies not only pyramidal cells but also interneurons. Additionally, differences in tissue quality were observed under the light microscope during electrophysiological measurements. Changes in the composition of the antibacterial (penicillin/streptomycin) and fungi-preventing (penicillin/streptomycin/amphotericinB) solution were made; however, the number of detected cells did not exhibit significant differences after treatment with penicillin or antibiotics (Paired t-test, $p=0.1368$, $N=13$).

5.2.3. Characteristics of the cell number based on the length of the culturing period

First, I investigated the number of detected cells on each slice, grouped by the length of the culturing period. It showed significant differences in the cell number (One-way ANOVA $p=0.0023$, number of different DIV=24, number of values=59), indicating a substantial change in cell density over time. Based on the mean of the cell numbers to each day there was an almost 50% drop in the cell number after the first week. (Figure 14 A) A research group reviewed 5-22% decrease in the cell number, although other type of culturing medium was applied [9].

To analyse changes in neuronal cell density in the neocortex, cells were counted in L2/3 and L5 using higher magnification images. In these layers, larger pyramidal cells with

homogenous morphological characteristics are presented, are well distinguishable from the other layers containing granules, and smaller pyramidal cells. The initial sample size in this analysis was smaller compared to the analysis of whole slices, because distinguishing between layers was not always possible, because of the quality or size of the tissue or the decreased number of stained cells.

As a result, a nonparametric statistical method was applied to assess significance among different cultures. First, I tested whether the distribution of the cell numbers is the same among cultures within the layer between 0-17 DIV. There were no significant differences neither within L2/3 or L5 (Kruskal-Wallis test, $p_1=0.75332$ and $p_2=0.2248$, $n_1=18$ and $n_2=16$). Furthermore, no significant differences were found between the two layers (Paired t-test, $p=0.0068$, $n_1=18$ and $n_2=16$ for 10 different days). Therefore, the distance from the white matter to the given layer or the projection characteristics of neuronal cells in different layers does not impact cell density. Visual inspection also revealed distinct cell populations in both layer groups in pictures taken during the late culturing period, suggesting that they have more contact with each other (Figure 14 B, C).

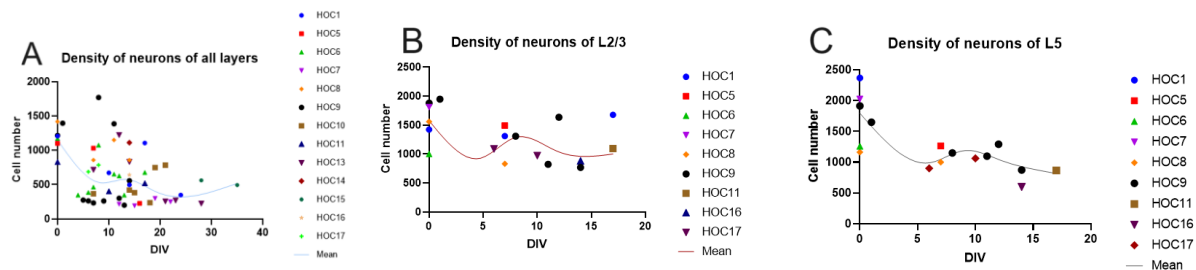


Figure 14: Change in neuronal cell density during culturing based on NeuN staining. Cell counting was performed at varying magnifications, leading to initial number shifts. Each point represents the number of cells on one tissue on the given day. Spline curves are fitted using four knots based on the mean values of each day. A: Cellular density in the whole slice based on 5x magnifications; B: Cellular density in L2/3 and L5 based on 10x magnifications. The cell number decreases during the first week significantly, this is the critical timespan for adaptation to the new environment. This is followed by a stabilisation on each area. (Due to the length limitation of the competition essay the tables containing the exact numbers of the diagrams are available at the following link: https://drive.google.com/file/d/1b1Q5x1H_QOxduqXdDsEqdklXJq2acWyg/view?usp=drive_link)

5.2.4. Tendency in the reduction of cell number

Based on the previous studies the change of the cell density in the organotypic tissue cultures during the first seven days, a significant reduction in cell numbers was observed [53].

To validate this trend in our data, a spline with four knots was fitted to the mean values of cell numbers from whole slices, L2/3, and L5 results. In all three cases, the results were consistent with previous findings. Between 0- and 5-7-days DIV, the number of detected cells decreased rapidly (-50%, -31%, -40%), followed by a period of stabilization until the 13-14th day (Figure 14). The first days are the most critical because, despite the cultured slices being 300-350 μm thick, they have been removed from their natural environment. In this new

environment, the cells need to adapt, and their intercellular connections may be disrupted, causing stress and apoptosis. The decrease in L2/3 is more prominent, which might be addressed to the differences in the pattern of the afferent and efferent pathways in the given layer and the flattening in the cultured tissue was not considered, hence corrected.

5.2.5. *Investigation of damaged cells by FJC staining*

In the case of FJC staining, fluorescence after excitation can originate from two potential sources: FJC-positive (FJC+) cells and autofluorescent intracellular structures and biomolecules within the tissue. This autofluorescence can be attributed to red blood cells, to lipofuscins, which accumulate within lysosomes, and can be excluded during analysis but it can also lead to false positive signals from larger cells like macroglia/macrophages. [51] Additionally, the level of autofluorescence tends to increase with the age of the patient.

To analyse changes in FJC+ cells and autofluorescence among cultures, a set of images was captured with the same exposure time and light intensity. This analysis aimed to determine whether there were significant correlations with factors such as the ratio of black pixels or the mean pixel intensity across entire slices. With these properties I wanted to present, how the level of fluorescent and autofluorescent signals change, and finding what can influence the intensity of the staining, how does it change based on different factors. Additionally, a region of interest (250x500 pixels) was marked on each image where cells were visible, and a histogram was created to assess how the fluorescent profile of the images correlated with different factors. These factors included the type of culture medium, the type of antibiotic treatment, patient gender, age, disease, DIV, and the applied protocol. Four staining protocols were tested with different staining length: (1) 10 minutes KMnO₄, 20 minutes FJC; (2) 15 minutes KMnO₄, 20 minutes FJC; (3) 15 minutes KMnO₄, 30 minutes FJC; (4) 10 minutes KMnO₄, 30 minutes FJC, to find the most optimal sign and noise ratio.

Interestingly, both the mean pixel intensity and the ratio of black pixels were significantly higher in the first four cultures, not only visually, which is intriguing since no aspect was found through multiple linear regression to influence these cultures more than the others. Additionally, based on Spearman correlation, both parameters were highly correlated with the applied staining protocol (black pixel ratio: $t=0.649$, mean: $t=-0.674$, see previous chapter for details, protocols: Table 1) and moderately correlated with the culturing medium.

5.2.6. *FJ staining protocols*

Four different staining protocols were applied, with variations in the time spent in a cuvette containing KMnO₄ or FJC to increase the signal to noise ratio (see Table 2). Although there

were visual differences in the contrast of FJC+ cells in the images, in the staining of the agar and the autofluorescence of the tissue, statistically, no differences were observed based on the mean pixel intensity of entire slices (One-way ANOVA, $p=0.6963$, $n=18$). It was hypothesized that staining intensity would increase with time spent in the solutions and the black ratio should increase. Furthermore, ANOVA was carried out on the skewness and kurtosis of the histograms and in both cases based on the p-values ($p_1=0.42$, $p_2=0.513$) the protocols are not significantly different. So, the staining protocol do not affect the quality of the staining (Figure 15).

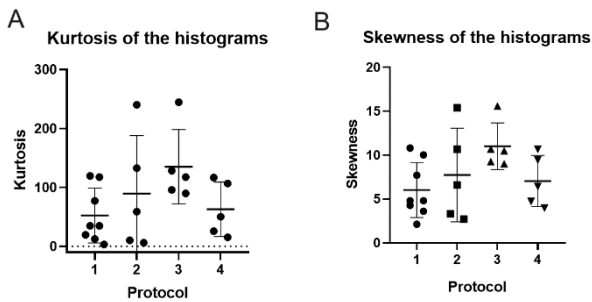


Figure 15: Columned scatter plot of the different FJC staining protocols represented with kurtosis (A) and skewness (B). This scatter plot displays different FJC staining protocols with means and standard deviations. KMnO_4 treatment durations vary, with 10 minutes in the 1st and 4th protocols and 15 minutes in the 2nd and 3rd protocols. The 2nd and 3rd protocols exhibit higher mean kurtosis and skewness values, suggesting sharper and more asymmetric histograms, meaning that the pixel intensity is far from normal distribution.

5.2.7. Comparing the samples based on the culturing medium

While the type of culturing medium did not impact the number of the detected cells at the NeuN staining, the mean pixel intensity and black pixel ratio exhibited significant differences (t-test with Welch's correction, $p_1=0.0003$, $p_2<0.0001$), meaning that the quality of the medium influences the sign-to-noise ratio. Based on a spline with four points, the mean pixel intensity of hCSF samples was nearly double compared to AM. This may be due to the different composition of the two solutions. However, interestingly, the mean intensity in both cases increased until the 7th DIV, followed by a slight drop, and then a slow increase after the 19th and 22nd DIV. As mentioned previously, the initial week of culturing is a critical phase during which neurons undergo adaptation. Some neurons may have compromised dendritic trees, cut axons, hence begin to experience stress and start apoptosis, which leads to a significant portion of them being stained. However, it's important to note that FJC specifically stains neurons that are under stress, and these neurons can still remain active in the tissue. The decrease in intensity observed after 7 DIV may indicate the appearance of hollow spaces at the sites of apoptotic cells and the clearance of additional blood and autofluorescent particles from the tissue. The subsequent increase in staining intensity around 20 DIV could be explained by the stress experienced by stable neurons up to that point. (Figure 16) Previous literature has suggested that the longest time for which cell activity was measured in AM was 18 DIV, so at this point stress and apoptosis is expected from the most cells. However, we were able to measure activity at 28 DIV.

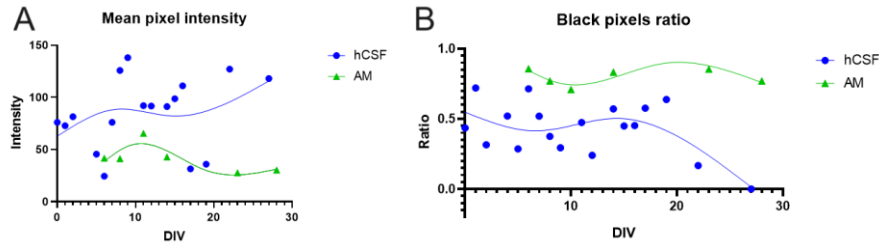


Figure 16: Mean intensity of the coloured pixels (A) and the black pixels ratio representing non-fluorescent pixels (B) on each fluorescent images. In this case the coloured pixels represent fluorescent cells and the autofluorescent parts of the tissue. The number of stained cells cannot be determined, the differences in the tendency of the changes and values among mediums are markable. Green triangles represent tissues cultured in AM, while blue circles represent tissues cultured in hCSF. Notably, the two features are inversely proportional on both cases, indicating that as autofluorescence of the tissue decreases, the number of stained pyramidal cells increases. The mean pixel intensity of hCSF samples is nearly twice that of AM, while the black pixel ratio is half, likely due to solution compositions. On picture A the slope of the mean pixel intensity in hCSF is steeper after DIV20 meaning intensively increasing number of apoptotic and stressing cells. Based on picture B the background fluorescence of the tissue cultured in hCSF is more prominent due to its quality, and the weaker capability of tissue preserving.

5.2.8. Pixel intensity histogram among cultures

To see the pixel intensity profile of the images more accurately, I investigated the histograms of given regions (ROIs) of each sample. The ROI was chosen based on the appearance of stained cells. Based on the histogram the brightness or darkness of the image can be investigated, or the artefacts with different pixel intensity as the cells could be filtered out based on peaks.

The values of histograms for each culture were averaged and compared with each other, revealing significant differences among all 12 cultures (ANOVA, $p < 0.0001$). Post hoc testing (Friedman test) indicated that the 1st and 2nd cultures differed the most from the others, particularly from the latest cultures. These differences suggest that the staining is highly patient specific, although there are other factors that cannot be excluded, like the type of the culturing medium. The observed differences between the two mentioned cultures may stem from two factors. Firstly, in these two cultures, fungi appeared, unlike in any other cultures. Secondly, these cultures were maintained in hCSF, which, as previously observed, is significantly different compared to AM, in which the most recent cultures were kept. Additionally, the composition of AM is known and standardized, unlike hCSF, which is sourced from different patients and may vary in composition slightly. (Figure 17)

5.2.9. Histogram of given days

Based on changes in cell numbers in NeuN staining and mean pixel intensity, three milestone days (0, 7, 14 DIV) during culturing were selected for histogram comparison. Four cultures (HOC3,4,8,9) met these criteria, and statistically significant differences were observed (ANOVA, $p < 0.0001$). Post hoc comparisons (Friedman test) revealed that only in one culture (HOC9) were the histograms significantly different between 0-7 DIV, 0-14 DIV. The

distinguishing factor in this case was that the culture was from an epileptic patient, while the others were from tumour patients. Furthermore, in HOC4 between 0-7 DIV was significant difference. These data suggest and confirm that the most noticeable change in neuronal viability occurs during the first week.

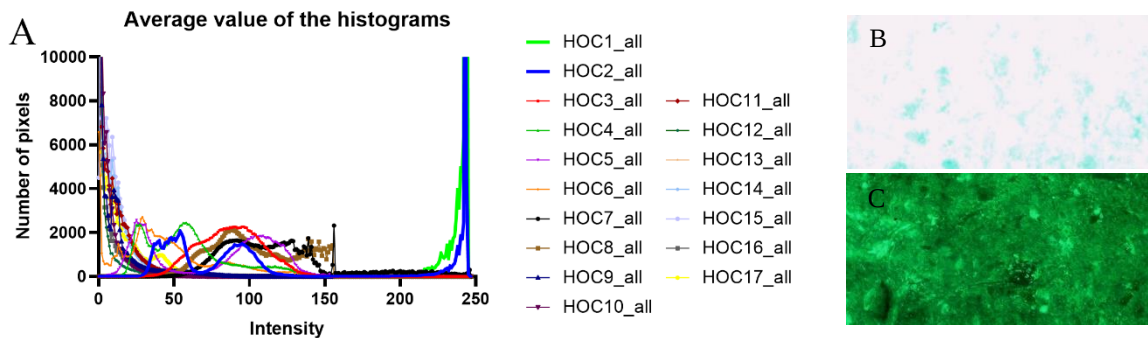


Figure 17: Histogram comparison of each culture. A: HOC1 and HOC2 differ from the other cultures the most significantly based on post hoc tests. HOC4 and HOC5 have two peaks suggesting appearance of strong noise, there is a strong shift in the black direction in the later cultures. B: ROI of HOC1. The strong right shift in the intensity can be observed, the tissue is bleached out suggesting wrong staining. C: ROI of HOC5, the intensity of the pixels is not consistent, the colour of the tissue and the cells is not well distinguishable.

5.3. Correlation between FJC and Neun staining

Pearson correlation analysis was conducted in order to assess the relationship between the two different staining. Based on the characteristics of the fluorescent staining (kurtosis, skewness of the histograms which refer to the symmetry and sharpness of it), mean pixel intensity, black pixel ratio) and the number of detected cells on the tissues treated with immunostaining strong correlation was not detected. Although based on the properties of these staining techniques, one might expect negative correlation, since as the number of the NeuN+ decrease, the number of FJC+ cells should increase due to the growing number of damaged cells. (Figure 13)

6. Discussion

In this work I aimed to gain information on the maintenance and application of human neuronal tissue cultures, which provide promising interface for establishing long-term experiments and testing of new therapeutic approaches against neuronal diseases.

6.1. Conservation of the cultured tissue

The use of organotypic brain tissue cultures from 17 patients, consisting of tumour and epilepsy cases, provided a diverse set of samples. For anatomical investigation we performed immunostaining and fluorescence staining on the cultured tissues.

Throughout the culturing process, in some cases we observed visible changes in the tissue's appearance. but appearance alone does not necessarily reflect tissue quality, since occasionally we were able to record extracellular cell activity even in these slices. A crucial

aspect of our experimental protocol was the transition from using human cerebrospinal fluid (hCSF) to an artificial culturing medium (AM). This transition significantly contributed to the preservation of tissue integrity over extended periods. While one previous study suggested the advantage of hCSF for culturing purposes, our experience demonstrated that the maintenance in AM is possible over 4 weeks. [33]. This observation was influenced by the varying quality of hCSF we received, compared to the fixed composition of AM exhibiting tissue-preserving properties. [9] In response to fungal contamination appearing in the initial three cultures, we supplemented the culturing medium with amphotericin B, in addition to penicillin and streptomycin.

The structure of the neocortex and the extracellular matrix of the tissue changed after approximately 7 DIV as revealed by NeuN staining. In a few specific cases, the layers remained distinguishable even after 28 DIV. However, it is important to note that tissue quality exhibited a rapid decline beyond 28 DIV in our experimental setup, on one hand suggesting that after 4 weeks of culturing even the most viable neurons start to stress, but on the other hand the freshly made AM was applied after 28 DIV, which could influence the viability of the cells. Based on the publication it is rare to maintain a culture more than 5 weeks, and this time is enough for performing other long-lasting experiments [9], [33], [35].

6.2. *Change in the viability of the neurons*

To see the viability of the neurons electrophysiological experiments were carried out on the acute and cultured tissues before fixation. Performing intracellular recording was challenging because the cells were showing a change in their membrane properties after 7 DIV, so modification on the pulling protocol for our glass pipette is an important next step. But successful extracellular recordings ensured that there are viable cells in the tissue even at 28 DIV, this is indeed an achievement because recording was made from slices cultured in hCSF up to 28 DIV [33], [35], but in the case of AM 18 DIV was reported. [9]. Based on the recordings there is a potential to introduce transgene-containing viral vectors or pharmacological compounds into the tissue cultures.

NeuN staining indicated changes in neuronal density over time. The observation of a substantial reduction in cell numbers (-50% all layers, -31% L2/3, -40%, L5), during the first week and subsequent stabilization highlights a critical period for neuronal adaptation and viability. These results align with previous findings regarding the initial challenges of culturing brain tissue [53]. We did not perform analysis based on the depth although it is known that the neurons near to the edge of the cultured slices tend to die due to the loss in

their processes after cutting, and later on the cells with decreased connections are dying. Although in one study 88% of the cells were preserved, they applied growth factors and other type on culturing medium [46][35]. The analysis of specific layers within the neocortex demonstrated the consistency in cell density and provides insights into the potential interaction and adaptation of neuronal populations within these layers.

FJC staining of stressing and apoptotic neurons resulted in changes mirroring the ones observed with NeuN staining, pointing to the viability and integrity of the tissue. In the slices fixed later the number of the stained neurons increased and after one week of culturing hollow spaces appeared at the place of the dead cells engulfed by glia. Changes in the conservation of the tissue could be observed, based on the change of the black ratio on the images and pixel intensity. On some sample activated glia appeared, mainly on the first day, but even after one week of culturing could be observed at the edge of the slices reaching in direction of vessels pyramidal cells. Analysis of histograms at milestone days emphasizes the dynamic nature of the culturing process. The presence of activated microglia in some cultures underlined the need for further investigation into staining specificity. And the importance of the quality of the received tissue and the differences that can arouse from this [43], [46].

6.3. *Influencing factors*

FJC staining results reveal both the effects of tissue conditions and staining protocols on the quality of the data. The patient-specific differences in staining intensity underscore the complexity of optimizing staining conditions and interpreting results.

Statistical challenges were faced due to variations in patient data and tissue quality, making it important to acknowledge the impact of these variables on results.

Variations in histograms among cultures suggest that multiple factors, including the patients' health conditions (resulting in differing tissue quality) and the choice of culturing medium, influence staining quality. The presence of fungi and differences in the composition of hCSF and AM adds complexity to the staining outcomes.

6.3.1. *Viral vector infection*

The effectiveness of transgene expression was tested using various viral vectors targeting specific cells and expressing unique transgenes. Out of the three attempts, one resulted in clear and successful expression, one requires further analysis, and one proved unsuccessful. The optimization of titer emerges as a critical factor to attain the ideal level of gene expression, ensuring visibility without inducing premature cell death in transfected cells. Subsequent investigations are important to refine this experimental method [35].

7. Conclusions

My research on this topic showed me the importance of investigating human organotypic brain slice cultures since this is the only in vitro method that mimics the in vivo conditions for studying the cellular integrity in the human brain. Although this model has its limitations, in the sense of getting access to such tissue, and protocols have to be standardised and optimized for maintaining reliable, stable long-term cultures, our protocol modified after more than 10 individual cultures should be more and more optimal for performing electrophysiological measurements and further histological analysis.

Overall, this study offers a detailed understanding of how various factors, including culturing time, staining protocols, and patient attributes, influence cell density and staining quality in organotypic tissue cultures. While our results contribute to the knowledge of in vitro neural cultures, it's important to acknowledge the need for a larger sample size to strengthen the statistical evidence. The multitude of comparisons made in this study suggests that other unaccounted factors may have influenced the results. Furthermore, variations between the visible properties of the tissue and the data extracted from images underscore the complexity of this research field. These findings help the way for future investigations and potential refinements in experimental methodologies.

The key points of my analyses are that the NeuN staining revealed a decrease in cell density during the first seven days of culturing, followed by a period of stabilization until the 13-14th day. There were no significant differences in cell density between different layers (L2/3 and L5) or between cultures with varying lengths of time in vitro. Age was found to have a strong negative correlation with the number of neuronal cells in the neocortex. Autofluorescence levels increased with the age of the patient. The mean pixel intensity and the ratio of black pixels were significantly higher in the initial cultures, with notable differences based on the applied protocol and culturing medium. Differences in autofluorescence were observed among cultures from epileptic patients, which correlated with the culturing time.

Additionally, antibiotics and the culturing medium did not significantly impact cell density based on the NeuN staining, although on the images there was a visible difference in the preservation of the tissue. The staining quality on the fluorescence samples varied among different staining protocols. Histogram analyses revealed significant differences in fluorescent profiles among different cultures and time points.

7.1. *Future perspectives*

Our study has opened up numerous questions that require further investigation. To gain a

more comprehensive understanding of tissue characteristics, it is important to assess immunostaining with antibodies targeting different cell types, such as glial cells, activated macroglia or macrophages and interneurons. Additionally, exploring the use of molecular markers like lactate dehydrogenase for the detection of apoptotic neuronal cells can offer valuable insights. The search for an optimal viral vector that can effectively penetrate the cytosol without compromising cell viability is also a crucial next step. And last but not least the glass pipette pulling protocol has to be modified to the special properties of neuronal tissue cultures. This modification is crucial for ensuring the success of future experiments and optimizing the precision of intracellular recordings.

8. Acknowledgement

I would like to thank the supervision and care of my supervisor Estilla Tóth, and faculty mentor Lucia Wittner. Furthermore, I would like to thank all the members of the research group who helped me in my work. I also like to thank the physicians and patients who contributed to the success of this project.

9. References

- [1] L. Beaulieu-Laroche *et al.*, “Enhanced Dendritic Compartmentalization in Human Cortical Neurons,” *Cell*, vol. 175, no. 3, pp. 643–651.e14, Oct. 2018, doi: 10.1016/J.CELL.2018.08.045.
- [2] T. E. Bakken *et al.*, “Comparative cellular analysis of motor cortex in human, marmoset and mouse,” *Nature* 2021 598:7879, vol. 598, no. 7879, pp. 111–119, Oct. 2021, doi: 10.1038/s41586-021-03465-8.
- [3] A. Napoli and I. Obeid, “Comparative Analysis of Human and Rodent Brain Primary Neuronal Culture Spontaneous Activity Using Micro-Electrode Array Technology,” *J Cell Biochem*, vol. 117, no. 3, pp. 559–565, Mar. 2016, doi: 10.1002/JCB.25312.
- [4] B. E. Kalmbach *et al.*, “h-Channels Contribute to Divergent Intrinsic Membrane Properties of Supragranular Pyramidal Neurons in Human versus Mouse Cerebral Cortex,” *Neuron*, vol. 100, no. 5, pp. 1194–1208.e5, Dec. 2018, doi: 10.1016/J.NEURON.2018.10.012.
- [5] T. B. Lentz, S. J. Gray, and R. J. Samulski, “Viral Vectors for Gene Delivery to the Central Nervous System,” *Neurobiol Dis*, vol. 48, no. 2, p. 179, Nov. 2012, doi: 10.1016/J.NBD.2011.09.014.
- [6] M. Andersson *et al.*, “Optogenetic control of human neurons in organotypic brain cultures,” *Scientific Reports* 2016 6:1, vol. 6, no. 1, pp. 1–5, Apr. 2016, doi: 10.1038/srep24818.
- [7] K. Deisseroth, “Optogenetics,” *Nat Methods*, vol. 8, no. 1, pp. 26–29, Jan. 2011, doi: 10.1038/NMETH.F.324.
- [8] A. Beauchamp, Y. Yee, B. C. Darwin, A. Raznahan, R. B. Mars, and J. P. Lerch, “Whole-brain comparison of rodent and human brains using spatial transcriptomics,” *Elife*, vol. 11, Nov. 2022, doi: 10.7554/ELIFE.79418.
- [9] J. T. Ting *et al.*, “A robust ex vivo experimental platform for molecular-genetic dissection of adult human neocortical cell types and circuits,” *Scientific Reports* 2018 8:1, vol. 8, no. 1, pp. 1–13, May 2018, doi: 10.1038/s41598-018-26803-9.
- [10] S. M. Sternson and B. L. Roth, “Chemogenetic tools to interrogate brain functions,” *Annu Rev Neurosci*, vol. 37, pp. 387–407, 2014, doi: 10.1146/ANNUREV-NEURO-071013-014048.
- [11] D. Atasoy and S. M. Sternson, “Chemogenetic Tools for Causal Cellular and Neuronal Biology,” *Physiol Rev*, vol. 98, no. 1, pp. 391–418, Jan. 2018, doi: 10.1152/PHYSREV.00009.2017.
- [12] N. Schwarz *et al.*, “Long-term adult human brain slice cultures as a model system to study human CNS circuitry and disease,” *Elife*, vol. 8, Sep. 2019, doi: 10.7554/ELIFE.48417.
- [13] J. DeFelipe, “The evolution of the brain, the human nature of cortical circuits, and intellectual creativity,” *Front Neuroanat*, vol. 5, no. MAY, May 2011, doi: 10.3389/FNANA.2011.00029.
- [14] J. H. Kaas, “Neocortex in early mammals and its subsequent variations,” *Ann N Y Acad Sci*, vol. 1225, no. 1, pp. 28–36, 2011, doi: 10.1111/J.1749-6632.2011.05981.X.
- [15] P. F. C. Jobim *et al.*, “Human Neocortex Layer Features Evaluated by PIXE, STIM, and STXM Techniques,” *Biol Trace Elem Res*, vol. 201, no. 2, pp. 592–602, Feb. 2023, doi: 10.1007/S12011-022-03182-X/FIGURES/5.
- [16] D. Purves *et al.*, “An Overview of Cortical Structure,” 2001, Accessed: Nov. 07, 2023. [Online]. Available: <https://www.ncbi.nlm.nih.gov/books/NBK10870/>
- [17] J. Schwartz, T. Jessell, and E. Kandel, “Principles Of Neural Science”.
- [18] S. Jacobson and E. M. Marcus, “Cerebral Cortex Functional Localization,” *Neuroanatomy for the Neuroscientist*, pp. 169–186, 2011, doi: 10.1007/978-1-4419-9653-4_10.
- [19] L. R. Squire *et al.*, “Fundamental neuroscience,” 2012, Accessed: Nov. 10, 2023. [Online]. Available: https://books.google.com/books?hl=hu&lr=&id=QGzJFu_NyzcC&oi=fnd&pg=PP1&dq=fundamental+of+neurosciences&ots=Hx9uPXsb82&sig=w7uMhbmGWJCyBfDsjOvyHj0SkPQ
- [20] J. DeFelipe, L. Alonso-Nanclares, and J. I. Arellano, “Microstructure of the neocortex: comparative aspects,” *J Neurocytol*, vol. 31, no. 3–5, pp. 299–316, Mar. 2002, doi: 10.1023/A:1024130211265.

- [21] H. Inibhunu, H. M. Chameh, F. Skinner, S. Rich, and T. A. Valiante, "Hyperpolarization-Activated Cation Channels Shape the Spiking Frequency Preference of Human Cortical Layer 5 Pyramidal Neurons," *eNeuro*, vol. 10, no. 8, pp. 215–238, Aug. 2023, doi: 10.1523/ENEURO.0215-23.2023.
- [22] S. Rich, H. Moradi Chameh, V. Sekulic, T. A. Valiante, and F. K. Skinner, "Modeling Reveals Human-Rodent Differences in H-Current Kinetics Influencing Resonance in Cortical Layer 5 Neurons," *Cerebral Cortex*, vol. 31, no. 2, pp. 845–872, Feb. 2021, doi: 10.1093/CERCOR/BHAA261.
- [23] Q. Zhang, Y. Zeng, T. Zhang, and T. Yang, "Comparison Between Human and Rodent Neurons for Persistent Activity Performance: A Biologically Plausible Computational Investigation," *Front Syst Neurosci*, vol. 15, Sep. 2021, doi: 10.3389/FNSYS.2021.628839/PDF.
- [24] G. Silberberg, A. Gupta, and H. Markram, "Stereotypy in neocortical microcircuits," *Trends Neurosci*, vol. 25, no. 5, pp. 227–230, May 2002, doi: 10.1016/S0166-2236(02)02151-3.
- [25] "Az orvosi élettan tankönyve Fonyó, Attila," 2011.
- [26] H. Moradi Chameh *et al.*, "Diversity amongst human cortical pyramidal neurons revealed via their sag currents and frequency preferences," *Nat Commun*, vol. 12, no. 1, Dec. 2021, doi: 10.1038/S41467-021-22741-9.
- [27] J. Berg *et al.*, "Human neocortical expansion involves glutamatergic neuron diversification," *Nature* 2021 598:7879, vol. 598, no. 7879, pp. 151–158, Oct. 2021, doi: 10.1038/s41586-021-03813-8.
- [28] T. Chartrand *et al.*, "Morphoelectric and transcriptomic divergence of the layer 1 interneuron repertoire in human versus mouse neocortex," *Science* (1979), vol. 382, no. 6667, Oct. 2023, doi: 10.1126/SCIENCE.ADF0805.
- [29] B. R. Lee *et al.*, "Signature morphoelectric properties of diverse GABAergic interneurons in the human neocortex," *Science* (1979), vol. 382, no. 6667, Oct. 2023, doi: 10.1126/SCIENCE.ADF6484.
- [30] B. W. Connors and M. J. Gutnick, "Intrinsic firing patterns of diverse neocortical neurons," *Trends Neurosci*, vol. 13, no. 3, pp. 99–104, 1990, doi: 10.1016/0166-2236(90)90185-D.
- [31] E. F. Moreira and R. Adler, "Effects of follistatin overexpression on cell differentiation in the chick embryo retina," *Dev Biol*, vol. 298, no. 1, pp. 272–284, Oct. 2006, doi: 10.1016/j.ydbio.2006.06.035.
- [32] R. Azzarelli, R. Oleari, A. Lettieri, V. Andre', and A. Cariboni, "In Vitro, Ex Vivo and In Vivo Techniques to Study Neuronal Migration in the Developing Cerebral Cortex," *Brain Sci*, vol. 7, no. 5, May 2017, doi: 10.3390/BRAINSKI7050048.
- [33] N. Schwarz *et al.*, "Human Cerebrospinal fluid promotes long-term neuronal viability and network function in human neocortical organotypic brain slice cultures," *Scientific Reports* 2017 7:1, vol. 7, no. 1, pp. 1–12, Sep. 2017, doi: 10.1038/s41598-017-12527-9.
- [34] C. Humpel, "Organotypic brain slice cultures: A review," *Neuroscience*, vol. 305, pp. 86–98, Oct. 2015, doi: 10.1016/J.NEUROSCIENCE.2015.07.086.
- [35] C. Le Duigou *et al.*, "Imaging pathological activities of human brain tissue in organotypic culture," *J Neurosci Methods*, vol. 298, p. 33, Mar. 2018, doi: 10.1016/J.JNEUMETH.2018.02.001.
- [36] J. Wickham *et al.*, "Human Cerebrospinal Fluid Induces Neuronal Excitability Changes in Resected Human Neocortical and Hippocampal Brain Slices," *Front Neurosci*, vol. 14, p. 283, Apr. 2020, doi: 10.3389/FNINS.2020.00283/BIBTEX.
- [37] V. M. Ravi *et al.*, "Human organotypic brain slice culture: a novel framework for environmental research in neuro-oncology," *Life Sci Alliance*, vol. 2, no. 4, p. e201900305, Aug. 2019, doi: 10.26508/lsa.201900305.
- [38] J. N. Warnock, C. Daigre, and M. Al-Rubeai, "Introduction to viral vectors," *Methods Mol Biol*, vol. 737, pp. 1–25, 2011, doi: 10.1007/978-1-61779-095-9_1.
- [39] E. Braun *et al.*, "Neurotropism of herpes simplex virus type 1 in brain organ cultures," *J Gen Virol*, vol. 87, no. Pt 10, pp. 2827–2837, Oct. 2006, doi: 10.1099/VIR.0.81850-0.
- [40] S. K. Maji, A. Anoop, P. K. Singh, and R. S. Jacob, "CSF Biomarkers for Alzheimer's Disease Diagnosis," *Int J Alzheimers Dis*, vol. 2010, 2010, doi: 10.4061/2010/606802.
- [41] V. V. Gusel'nikova and D. E. Korzhevskiy, "NeuN As a Neuronal Nuclear Antigen and Neuron Differentiation Marker," *Acta Naturae*, vol. 7, no. 2, p. 42, 2015, doi: 10.32607/20758251-2015-7-2-42-47.
- [42] H. K. Wolf *et al.*, "NeuN: a useful neuronal marker for diagnostic histopathology," *J Histochem Cytochem*, vol. 44, no. 10, pp. 1167–1171, 1996, doi: 10.1177/44.10.8813082.
- [43] L. C. Schmued, C. C. Stowers, A. C. Scallet, and L. Xu, "Fluoro-Jade C results in ultra high resolution and contrast labeling of degenerating neurons," *Brain Res*, vol. 1035, no. 1, pp. 24–31, Feb. 2005, doi: 10.1016/J.BRAINRES.2004.11.054.
- [44] A. Rosario, A. Howell, and S. K. Bhattacharya, "A revisit to staining reagents for neuronal tissues," *Ann Eye Sci*, vol. 7, Mar. 2022, doi: 10.21037/AES-21-31/COIF.
- [45] T. Ikenari, H. Kurata, T. Satoh, Y. Hata, and T. Mori, "Evaluation of Fluoro-Jade C Staining: Specificity and Application to Damaged Immature Neuronal Cells in the Normal and Injured Mouse Brain," *Neuroscience*, vol. 425, pp. 146–156, Jan. 2020, doi: 10.1016/J.NEUROSCIENCE.2019.11.029.
- [46] T. Ikenari, T. Kawaguchi, R. Ota, M. Matsui, R. Yoshida, and T. Mori, "Improvement in Double Staining With Fluoro-Jade C and Fluorescent Immunostaining: FJC Staining Is Not Specific to Degenerating Mature Neurons," *J Histochem Cytochem*, vol. 69, no. 9, pp. 597–610, Sep. 2021, doi: 10.1369/00221554211043340.
- [47] E. Eugène *et al.*, "An organotypic brain slice preparation from adult patients with temporal lobe epilepsy," *J Neurosci Methods*, vol. 235, pp. 234–244, Sep. 2014, doi: 10.1016/J.JNEUMETH.2014.07.009.
- [48] D. Meszéna *et al.*, "A silicon-based spiky probe providing improved cell accessibility during in vitro slice recordings," *Sens Actuators B Chem*, vol. 297, p. 126649, Oct. 2019, doi: 10.1016/J.SNB.2019.126649.
- [49] A. D. Elliott, "Confocal Microscopy: Principles and Modern Practices," *Curr Protoc Cytom*, vol. 92, no. 1, p. e68, Mar. 2020, doi: 10.1002/CPCY.68.
- [50] T. Ikenari, H. Kurata, T. Satoh, Y. Hata, and T. Mori, "Evaluation of Fluoro-Jade C Staining: Specificity and Application to Damaged Immature Neuronal Cells in the Normal and Injured Mouse Brain," *Neuroscience*, vol. 425, pp. 146–156, Jan. 2020, doi: 10.1016/J.NEUROSCIENCE.2019.11.029.
- [51] T. R. Riew, H. L. Kim, J. H. Choi, X. Jin, Y. J. Shin, and M. Y. Lee, "Progressive accumulation of autofluorescent granules in macrophages in rat striatum after systemic 3-nitropropionic acid: a correlative light- and electron-microscopic study," *Histochem Cell Biol*, vol. 148, no. 5, pp. 517–528, Nov. 2017, doi: 10.1007/S00418-017-1589-X/FIGURES/6.
- [52] R. D. Terry, R. DeTeresa, and L. A. Hansen, "Neocortical cell counts in normal human adult aging," *Ann Neurol*, vol. 21, no. 6, pp. 530–539, 1987, doi: 10.1002/ANA.410210603.
- [53] J. Dyhrfeld-Johnsen, Y. Berdichevsky, W. Swiercz, H. Sabolek, and K. J. Staley, "Interictal spikes precede ictal discharges in an organotypic hippocampal slice culture model of epileptogenesis," *J Clin Neurophysiol*, vol. 27, no. 6, p. 418, Dec. 2010, doi: 10.1097/WNP.0B013E3181FE0709.