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Master of Bioengineering

**Spectral dynamics of extracellular
neuronal signaling in developing 2D
hiPSC-derived cortical networks on
MEAs**

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Abstract

Functional maturation of human induced pluripotent stem cell (hiPSC)-derived neuronal networks on microelectrode arrays (MEAs) is predominantly characterized using spike-based metrics, such as firing, bursting, and network bursting. Conversely, the low-frequency local field potential (LFP) is typically filtered out and discarded. However, the LFP reflects subthreshold population activity and shares a biophysical origin with clinical signals like the electroencephalogram, serving as a vital bridge between *in-vitro* models and clinical recordings. Whether LFP maturation signatures, such as spectral power redistribution and rhythm coupling, emerge in two-dimensional (2D) cortical cultures remains largely unexplored.

This thesis investigated the longitudinal evolution of the LFP in 2D cortical networks derived from the TUBA1B hiPSC line, recorded on MEAs at four timepoints between DIV 47 and 59. Two independent batches were acquired on systems with different low-frequency recording limits and were first compared to exclude technical bias. Because the older system attenuated the slow component via hardware filtering, spectral analyses focused on the batch-preserving broadband LFP, while spike metrics were reported for both. Three signal properties were evaluated: the distribution and ratios of power across canonical frequency bands, the aperiodic spectral component, and delta-broadband phase-amplitude coupling (PAC). These metrics were condensed via principal component analysis into a unified maturation index: the LFP-based neural activity score (LFP-NAS).

Spike-based metrics reproduced expected developmental trajectories. Spectral power progressively shifted from low to high frequencies with a stable aperiodic component, indicating band-specific changes rather than global reorganisation. Delta-broadband PAC emerged and strengthened over time, demonstrating increasing population-level rhythm locking. The LFP-NAS successfully tracked single-well maturation, rising significantly with DIV from negative to positive values. Its trajectory was driven primarily by gamma power, the two PAC measures, relative delta power, and the delta-to-gamma ratio.

Overall, this study shows that the LFP in 2D hiPSC-derived networks is an informative substrate of functional maturation. LFP-based descriptors and their multivariate integration into the LFP-NAS offer a powerful complement to spike-based characterisation, with promising applications in developmental neuroscience, disease modelling, and drug screening.

Chapter 1

Introduction

The human brain is one of the most complex biological systems; it is made up of billions of cells, the neurons, which are interconnected via synapses and organised into circuits within the brain. These internal brain circuits enable information to be processed and generate a coordinated activity that is equally complex, as it is organised in space and time and evolves as the brain matures. (Kandel et al., 2021) Studying such a complex organ directly in humans is complicated both for practical reasons and, above all, for ethical reasons (Farahany et al., 2018). Consequently, *in-vitro* systems have been developed in order to approximate the organisation and dynamics of the human brain, but under controlled conditions (Gordon et al., 2013). Animal *in-vivo* and *in-vitro* models, particularly those derived from rats, have contributed to this endeavour for years, but they have several inherent limitations. First and foremost, the species-specific differences between mice and humans limit the translational relevance of animal models, and this becomes even more significant when studying human-specific diseases (Hodge et al., 2019). Furthermore, ethical concerns have been raised, leading to efforts to reduce the use of animals (Carter & Shieh, 2015).

These limitations were overcome with the advent of the human induced pluripotent stem cell (hiPSC) technique. With two groundbreaking papers, Yamanaka and Takahashi demonstrated that hiPSCs could be obtained by reprogramming adult somatic cells (Takahashi et al., 2007; Takahashi & Yamanaka, 2006). When subsequently differentiated, these cells yielded neurons with the same genetic background as the donor. This discovery has opened up major opportunities in the field of personalised medicine, where neurons derived directly from patients with genetic diseases can be studied *in-vitro*, enabling the modelling of patient-specific neurological diseases and the testing of drugs on the corresponding *in-vitro* model.

These hiPSC-derived neuronal models are often characterised electrophysiologically using micro-electrode arrays (MEAs) (Pelkonen et al., 2022), devices consisting of electrode arrays that record activity simultaneously, thereby enabling the recording of the

extracellular activity of the whole neuronal network. In particular, the MEA records broadband extracellular activity, sampling at a few kHz. Based on the frequency content, this signal can be divided into two components distinguished by the frequency range they occupy: Multi-Unit Activity (MUA), which represents the high-frequency component ($\sim 300\text{--}3000$ Hz) and describes the supra-threshold activity of a few neurons, and the low-frequency component (< 300 Hz) known as the Local Field Potential (LFP), which instead represents the collective, mainly subthreshold, activity of a local population of neurons in the network. (Einevoll et al., 2013; Obien et al., 2015) The scientific community has primarily focused on characterising *in-vitro* neuronal networks solely on the basis of high-frequency MUA, describing the network's electrophysiological activity through spikes, bursts, network bursts and the metrics derived from them (Mossink et al., 2021). In this context, LFPs have often been discarded and filtered out, creating a marked imbalance between the two components and a clear gap in the literature. LFPs are derived from the spatio-temporal sum of the transmembrane currents of various neurons in the vicinity of the electrode, thus reflecting the activity, primarily synaptic, and the processing of a local population of neurons. Although the contribution of action potentials (APs) to the LFP is not clear yet, the synchronous firing of many neurons can also contribute to its generation. (Buzsáki et al., 2012) Despite the plausible influence of suprathreshold processes, the LFP signal is mainly characterised by subthreshold synaptic activation of neurons (Buzsáki et al., 2012; Einevoll et al., 2013). The information contained in this component is therefore complementary to that included in the MUA. However, a particularly relevant feature of LFPs is that they share the same biophysical origin as Electroencephalography (EEG), which is a purely non-invasive clinical signal. Therefore, LFPs can be compared with EEG across different spatial scales (Buzsáki et al., 2012).

This LFP component, characterised by oscillations and rhythms, has mainly been studied *in-vivo*, using electrodes implanted within the brain, where cortical networks in particular have been shown to exhibit oscillations of up to 500 Hz (Buzsáki & Draguhn, 2004). The main characteristic of these oscillations is that power is inversely proportional to frequency, following a $1/f$ scaling; this means that slow oscillations predominate over fast oscillations. The $1/f$ trend in power was initially described as pure noise, but has recently been investigated in greater depth as it is believed to contain information regarding the excitation-inhibition (E/I) balance of the network (Donoghue et al., 2020; Gao et al., 2017).

The contribution of each rhythm to the overall LFP spectrum is assessed in terms of frequency bands, each of which represents a rhythm at a different frequency and is associated with a specific state of the network. *In vitro*, the LFP's analysis of dissociated rodent networks has a longer history than that of human cultures. Leondopoulos et al. (2012) were among the first to describe spontaneous oscillations from the delta to the slow gamma range in dissociated hippocampal networks on MEAs, together with spikes phase-locked to the slow gamma, and noted that earlier MEA studies of dissociated neurons had largely ignored frequencies between 1 and 140 Hz. The same LFP analysis was later applied to cortical cultures to study sleep-like slow oscillations and their modulation by carbachol (Colombi et al., 2016, 2021).

In the developing brain, the frequency component evolves as the brain matures. Some studies on preterm EEG have shown that the signal spectrum reorganises in a characteristic manner, indicating a redistribution of power and, consequently, the emergence and modulation of brain rhythms, probably due to synaptic maturation (Niemarkt et al., 2011). This opens the possibility that a hiPSC-derived network could exhibit a similar spectral shift in its LFP during maturation.

In addition to power distribution, the activity of the neural network can be studied by analysing the relationships between oscillations at different frequencies, known as cross-frequency coupling (CFC). In particular, the phase-amplitude coupling (PAC) has been the subject of in-depth study in *in-vivo* cortical networks. It represents the dependence of the amplitude of a fast oscillation on the instantaneous phase of a slow oscillation, and has been linked to the temporal organisation of the network and to mechanisms to transfer information between neuronal assemblies. An organised and functionally mature network exhibits, in fact, a well-defined coupling between rhythms of different frequencies (Canolty & Knight, 2010). In 2024, Salimpour and colleagues demonstrated that the PAC is also present in simple two-dimensional (2D) *in-vitro* cultures and that it strengthens as the network matures. This effectively presents the PAC as an intrinsic characteristic of the neuronal networks that is maintained even outside the brain, and consequently, that can therefore be studied under experimental control using *in-vitro* preparations. (Salimpour et al., 2024)

This type of characterisation based on oscillations, rhythms and frequencies mainly performed *in-vivo*, has recently also been applied *in-vitro*, primarily using hiPSC-derived

three-dimensional (3D) networks which, by better replicating the spatial structure of the human brain compared to 2D cultures, allow for the observation of brain-like activity (Pelkonen et al., 2022; Trujillo et al., 2019). In contrast, oscillation- and LFP-based analyses of human 2D cultures on MEAs remain barely explored (Pelkonen et al., 2022). Nevertheless, using 2D cultures to examine LFPs and the frequency component offers a complementary strategy that is well justified when dealing with the early stages of network characterisation or the analysis of a specific spectral feature of neuronal activity via MEAs. This argument is supported by the fact that, compared to 3D preparations, planar preparations mature more quickly and become electrophysiologically active sooner; furthermore, there are numerous differentiation protocols that have demonstrated their suitability for use in combination with MEAs (Frega et al., 2017), not to mention the economic and reproducibility benefits (Pelkonen et al., 2022). Despite this, as they do not replicate the complex 3D geometry of the human brain (Cerina et al., 2023), the interpretation of LFP may differ and requires caution in the case of 2D cultures, and its analysis remains an open question in the literature.

The LFP analysis, like the MUA analysis, generates an enormous number of parameters such as band powers, parameters of the aperiodic component, and descriptors of the coupling between oscillations, each of which can partially describe the state of functional network maturation (Trujillo et al., 2019; Passaro et al., 2021). However, this multitude of parameters can be summarised by a single multivariate parameter. In the spike-based context, this has already been presented by Passaro and colleagues, who, by combining multiple spiking and bursting metrics, defined a single index called the Neural Activity Score (NAS) that describes the functional activity of the network recorded by the MEA (Passaro et al., 2021). A similar descriptor that also utilises information from the slow component of the extracellular signal, and thus allows for an even more refined description of the activity and maturation of the network, does not yet exist.

The aim of this work is to investigate how spectral properties of 2D hiPSC-derived neuronal cortical neurons evolve during development *in-vitro*. By analysing band-power and inter-band ratios, and performing aperiodic decomposition of the LFP Power Spectrum, this thesis aims to verify and investigate the underlying cause of an expected spectral shift in the LFP Power. In addition, phase-amplitude coupling (PAC) analysis is also performed. This type of cross-frequency coupling is related to network activity

organisation (Canolty & Knight, 2010), and its emergence and development are examined in this work. A multivariate analysis is then applied, adapting the NAS framework, to construct a single representative index. This index considers all extracted LFP-based metrics to assess the functional maturation of the network. By characterising the maturation of hiPSC-derived networks using LFP-based descriptors, this thesis addresses a gap in the literature, where characterisation is often based on spikes and largely neglects the low-frequency component of the extracellular signal recorded by the MEA. LFP-based metrics are, in principle, directly comparable to *in-vivo* markers and allow for the construction of a multivariate index of functional maturation for the study of human neuronal models.

The remainder of the thesis is organised in five chapters. Chapter 2 reviews the background: *in-vitro* models of neuronal networks, from rodent-derived to hiPSC-derived cultures, the MEA technique and the spike and field-potential information it provides, and the analytical concepts used later, namely the Neural Activity Score, the aperiodic component of the spectrum, phase-amplitude coupling and electrophysiological markers of maturation. Chapter 3 describes the cell line, the differentiation protocol and the recording setup with the two MEA systems, followed by the analysis pipeline, from spike and network burst detection to LFP extraction, spectral parameterisation, PAC, the construction of the LFP-NAS and the statistical tests. Chapter 4 reports the results in the order of the analyses: spike-based characterisation of both batches, comparison of the two acquisition systems, band power, the aperiodic component, PAC, the multivariate index and the univariate follow-up of its main drivers. Chapter 5 discusses these findings against the literature on developing networks, from preterm EEG to cortical organoids, and considers what the LFP-NAS adds to spike-based description and where its interpretation is limited. Chapter 6 concludes the thesis with a summary of the main findings, the limitations of the study, the experiments that would follow from it and its possible impact on the characterisation of hiPSC-derived models.

Chapter 2

State of the Art

2.1 *In-vitro* Models of Neuronal Networks

2.1.1 The rationale for *in-vitro* systems

Brain development involves overlapping and highly regulated processes, including neuronal proliferation and migration, synapse formation and network maturation, which take place over a long period and depend strongly on continuous changes at the molecular level (Budday et al., 2015; Li et al., 2020). This makes understanding of the human brain development and information processing a difficult and extensive scientific challenge. When faced with such a complex system, an effective approach is to study the individual processes in isolation and under controlled conditions. *In-vitro* neuronal cultures are among the most accessible and widely used tools for studying neuronal processes in a controlled manner. The first *in-vitro* approach dates back to 1909, when Ross Granville Harrison, an American biologist and anatomist, demonstrated that embryonic nerve fibres could survive and develop outside their original host organism. This discovery laid the foundations, at least in conceptual terms, for neuronal cell culture (Harrison, 1959). From the 20th century to the present day, methods for isolating, culturing and functionally characterising neuronal populations *in-vitro* have been progressively improved and refined, providing the basis for an experimental toolkit for classical and translational neuroscience (Millet & Gillette, 2012). The real strength of *in-vitro* cultures lies in the level of experimental control they offer. By isolating neurons from the complexity of the brain, researchers can precisely regulate the cellular composition, the culture medium, the pharmacological environment and the physical parameters of the culture (Carter & Shieh, 2015). Furthermore, *in-vitro*

platforms provide access to individual neurons and network-level dynamics through high-resolution imaging and electrophysiological techniques that are impossible *in-vivo* (Grienberger & Konnerth, 2012; Obien et al., 2015).

2.1.2 Rodent-derived models and their structures

The gradual development of cell culture protocols has been driven predominantly by animal-derived models, and particularly rodent models (Banker & Goslin, 1988; Nicholls, 1992), because rodents provide a good animal source of primary neurons. This is mainly due to the fact that mice are of a controlled and manageable size, reproduce rapidly, are inexpensive to maintain and share a notable genomic similarity with humans (Carter & Shieh, 2015). As regards rodent models, neurons are extracted from the brains of embryonic or early postnatal rodents and subsequently isolated and maintained in various configurations, which differ in the degree to which they retain the structure of the tissue of origin. *In-vitro* models derived from rodents can be divided into 2D neuronal cultures and 3D neuronal cultures derived from dissociated cells, as well as organotypic brain slice cultures derived from sections of brain regions. (Cerina et al., 2023) (Figure 2.1)

In dissociated cultures, the tissue is enzymatically and mechanically disaggregated into isolated neurons, which are then plated and maintained in culture media for survival and maturation. These cultures retain the molecular, morphological and electrophysiological properties of the region of the brain from which they are derived and can be grown either as a 2D monolayer or, more recently, within 3D scaffolds that restore part of the volumetric organisation (Carter & Shieh, 2015). Brain slices are another possibility besides dissociated cultures. They are defined as thin sections of tissue that are kept in culture while preserving the native three-dimensional cytoarchitecture and local circuitry of the source region. In fact, brain slices preserve much of the cellular and synaptic organisation of the original tissue and provide reproducible models for studying synaptic plasticity, network formation, and pharmacological responses (Carter & Shieh, 2015; Millet & Gillette, 2012). For their three-dimensional structure, organotypic slices are described as a more *in-vivo*-like environment than dissociated cells (Gähwiler et al., 1997; Hutter-Schmid et al., 2015).

Among these formats, dissociated 2D cultures are widely used for the functional study of network activity, and they are considered in more detail in the following section.

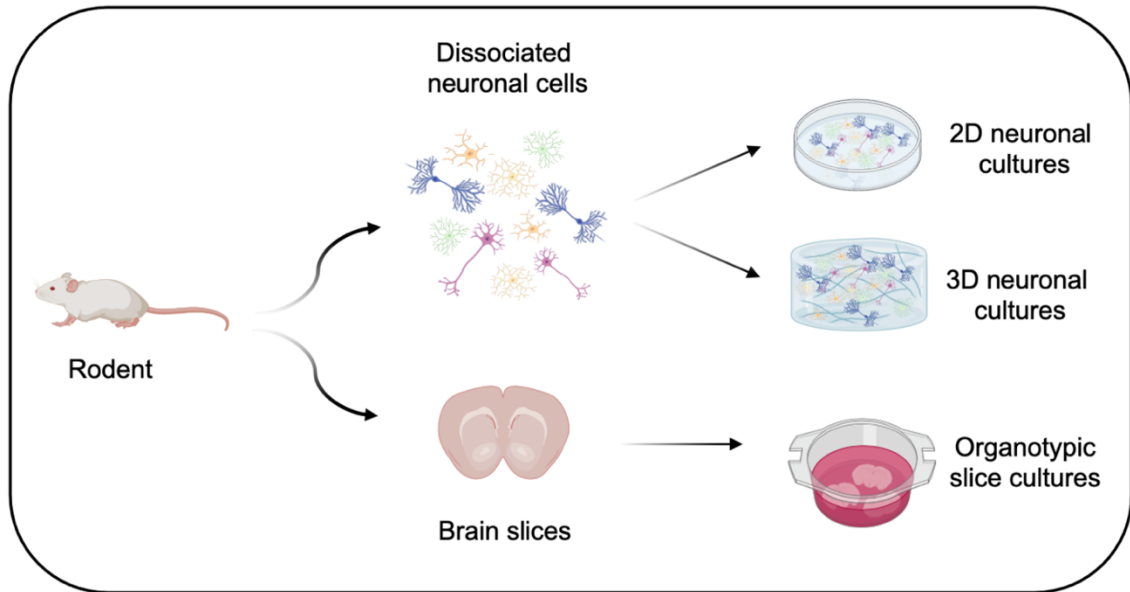


Figure 2.1. Sketch illustrating *in-vitro* neural models derived from rodents. These include 2D and 3D neuronal cultures derived from dissociated cells and organotypic brain slice cultures. Created using BioRender, with elements inspired by Cerina et al. (2023).

2.1.3 Dissociated Two-Dimensional Cultures

Among the rodent models described in the previous section, 2D dissociated cultures have made a significant contribution to our understanding of the functional behaviour of neuronal networks *in-vitro*. In the brain, neurons grow in a 3D environment, comprising axonal projections, dendritic arborisation, glial scaffolding and the extracellular matrix. Consequently, when neurons grow as a 2D monolayer, the architecture of intact brain tissue cannot be recreated. Confining growth to a single plane thus eliminates volumetric connectivity, cell-type heterogeneity and the layered organisation of the tissue, limiting the ability of 2D cultures to model circuit-level phenomena that depend on spatial structure. (Centeno et al., 2018)

This structural simplicity, however, is also accompanied by a practical advantage when combined with MEA technology. Network activity can be recorded non-invasively across the whole culture using planar electrodes, and since each electrode is directly in contact with the culture, the recorded signal reflects the activity of neurons in its vicinity without having to travel across layers of tissue before arriving at the sensor. For this reason, the

electrophysiological readout of 2D culture on an MEA can be related more straightforwardly to cellular and synaptic mechanisms than those from 3D cultures. (Obien et al., 2015; Cerina et al., 2023)

Before the advent of human cultures, 2D rodent-derived cultures had been extensively studied and thus characterised from an electrophysiological perspective. In particular, dissociated cortical cultures derived from rodents develop, over the course of weeks *in-vitro*, characteristic patterns of synchronised discharges. This distinctive feature of *in-vitro* dynamics has been extensively described in longitudinal studies (Wagenaar et al., 2006; Chiappalone et al., 2006), and this type of culture has thus become an established system for the longitudinal characterisation of neuronal network development (van Pelt et al., 2004).

2.1.4 From Rodent to Human-Derived Models

Dissociated cultures are traditionally prepared from embryonic or neonatal rodent tissue and the resulting neuronal networks retain many of the molecular, morphological and electrophysiological properties of the region from which they originate (Carter & Shieh, 2015; Millet & Gillette, 2012). However, when the aim is to replicate the physiology or diseases of the human brain, these rodent models have mainly two limitations. The first limitation comes from the fact that neurons are post-mitotic and therefore cannot proliferate (Gordon et al., 2013). For this reason, every experiment requires a fresh animal dissection, which raises ethical and practical concerns. Second, and more fundamentally, humans and rodents are different biological systems. Interspecies differences are present in gene expression, astrocyte morphology and the pace of neuronal development and reduce the fidelity with which findings in rodent neurons generalise to humans (Hodge et al., 2019; Oberheim et al., 2009; Napoli & Obeid, 2016). A direct example was given by Napoli and Obeid (2016); they showed that rodent cultures developed strong spontaneous network activity in about ten days. In contrast, human neurons needed nearly forty days to reach a significantly weaker level of activity under the same conditions. This difference in developmental kinetics is something that rodent models cannot capture.

These limitations have motivated a complementary class of models, human-derived neuronal cultures. By generating neurons directly from human cells, these systems bypass

the interspecies barrier and allow the study of human-specific neurophysiology, developmental trajectories, and disease mechanisms, including patient-specific genetic variants, in a fully controlled setting (Hyvärinen et al., 2019; Mossink et al., 2021).

2.2 Human-Derived Neuronal Cultures

2.2.1 hiPSC technology and translational relevance

hiPSC technology represents one of the most influential innovations in biomedical research over the last two decades. In 2006, Takahashi and Yamanaka demonstrated that mouse somatic cells could be reprogrammed to a pluripotent state by inducing the expression of four transcription factors, OCT3/4, SOX2, KLF4 and c-MYC, subsequently known as the ‘Yamanaka factors’ (Takahashi & Yamanaka, 2006). The following year, the same four factors were found to be capable of reprogramming adult human fibroblasts, giving rise to human iPSCs comparable to human embryonic stem cells in terms of morphology, proliferation and pluripotency (Takahashi et al., 2007).

The main strength of hiPSCs is that they retain the donor’s genetic background: this makes them a platform for studying human-specific cellular mechanisms and for directly modelling, *in-vitro*, diseases with a defined genetic aetiology. A further advantage offered by this technology is that it also provides an ethically acceptable alternative to human embryonic and foetal stem cells, which raise ethical concerns and are in limited supply (Cerina et al., 2023).

2.2.2 Formats of hiPSC-derived networks

Following reprogramming, hiPSCs are differentiated into neurons and organised into different culture formats, which vary both in terms of how faithfully they replicate the architecture of brain tissue and in terms of how easily their activity can be measured (Figure 2.2). The literature distinguishes between three types: 2D cultures, 3D cultures and brain organoids. In 2D cultures, neurons grow as a single layer on a flat surface: this configuration provides direct optical and electrical access to the network and allows for non-invasive recordings from the same culture over time (Obien et al., 2015; Cerina et al.,

2023). In the case of engineered 3D cultures, the neuronal cells are embedded into a biocompatible scaffold along with glial cells, imitating partially the 3D cytoarchitecture of brain tissue; nevertheless, recording their activity is more demanding since require using dedicated techniques rather than a planar electrode array (Cerina et al., 2023). In brain organoids, by contrast, neurons derived from hiPSCs organise themselves into three-dimensional structures that more accurately replicate certain aspects of cortical development and cell type diversity; however, accessing their internal activity and recording it in a controlled and reproducible manner is more complex (Centeno et al., 2018).

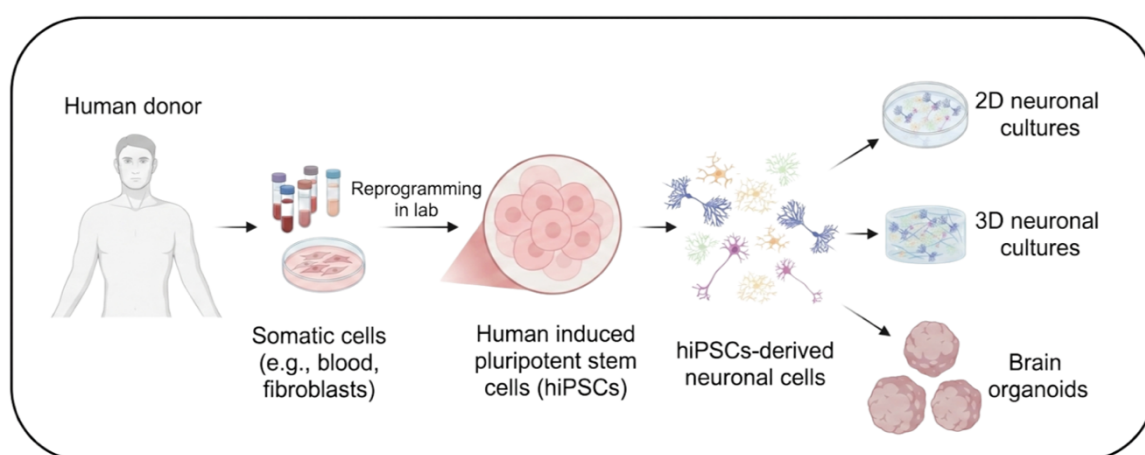


Figure 2.2. Sketch showing *in-vitro* neural models derived from hiPSCs. Somatic cells obtained from a human donor are reprogrammed into hiPSCs and subsequently differentiated into neuronal cells, which can be cultured as 2D neuronal cultures, 3D neuronal cultures, or brain organoids that recapitulate aspects of the 3D architecture of the brain tissue. The figure was created using BioRender and is inspired by Cerina et al. (2023).

2.2.3 Differentiation toward neuronal subtypes

For hiPSCs to serve as neuronal models, the reprogrammed cells must be directed towards specific neuronal identities. This requires reliable and reproducible differentiation protocols. A breakthrough in this regard came in 2009 with the dual SMAD inhibition protocol proposed by Chambers and colleagues, which utilises two SMAD signalling inhibitors to achieve rapid and efficient neural conversion from both human embryonic stem cells and iPSCs. Much of the subsequent work on differentiating hiPSCs into neuroectoderm and various neuronal subtypes was based on this method, and it remains widely used today (Chambers et al., 2009; Engle et al., 2018). In general, the choice

between one differentiation protocol and another is driven by scientific question, as each offers optimal culture characteristics for a different type of study.

Specifically, by replicating the stages of embryonic neuroinduction, dual-SMAD cultures pass through all the intermediate stages —from neural progenitor cells (NPCs) to neural rosettes, and on to mature neurons — and so the development of the network follows a physiologically realistic trajectory, allowing us to observe processes that would otherwise be invisible using other differentiation techniques. These intermediate stages make the dual-SMAD-based protocols suitable for studying human neuronal development and modelling neurodevelopmental disorders and neurodegenerative diseases, where the maturation trajectory itself is under investigation. (Chambers et al., 2009; Puspita et al., 2025)

On the other hand, it is precisely these intermediate steps that make dual-SMAD protocols comparatively long and labour-intensive. Added to this is the fact that the resulting cell populations are heterogeneous, as the proportions of the different cell types are not directly controlled, and that achieving sufficiently mature neurons capable of functional network activity can take months of culture (Zhang et al., 2013; Yang et al., 2017).

A complementary strategy focuses on the forced overexpression of neurogenic transcription factors, which yields more homogeneous populations in a shorter time. Overexpression of Neurogenin-2 (Ngn2) transforms hiPSCs into functional glutamatergic neurons with high yield in less than two weeks, and into mature neurons within about three weeks. This is precisely what is required for electrophysiological studies that demand a controlled cellular composition and reproducible network formation (Zhang et al., 2013; Frega et al., 2017). For inhibitory neurons, however, the transient co-expression of Achaete-scute homolog 1 (Ascl1) and Distal-less homeobox 2 (Dlx2) generates exclusively GABAergic populations, with a good degree of synaptic maturation in around four weeks (Yang et al., 2017). By combining the two strategies, the composition of the network can be determined experimentally: By co-culturing excitatory neurons induced with Ngn2 and inhibitory neurons induced with Ascl1/Dlx2 in defined ratios, hiPSC-derived networks with a controlled excitation–inhibition balance are obtained, which reproduce *in-vitro* a fundamental property of cortical microcircuitry (Heider et al., 2024).

These two differentiation protocols are presented in Figure 2.3, with the aim of highlighting their complementarity. The dual-SMAD protocol is represented as biologically realistic in reproducing neuronal developmental stages, but at the same time it generates heterogeneous networks, and this complicates the reproducibility of experiments (Chambers et al., 2009; Puspita et al., 2025). On the other hand, protocols based on overexpression of neurogenic transcription factors are faster and more reproducible, but by skipping the stages of neural development, they do not reflect what actually happens in the early stages of network maturation (Zhang et al., 2013; Frega et al., 2017).

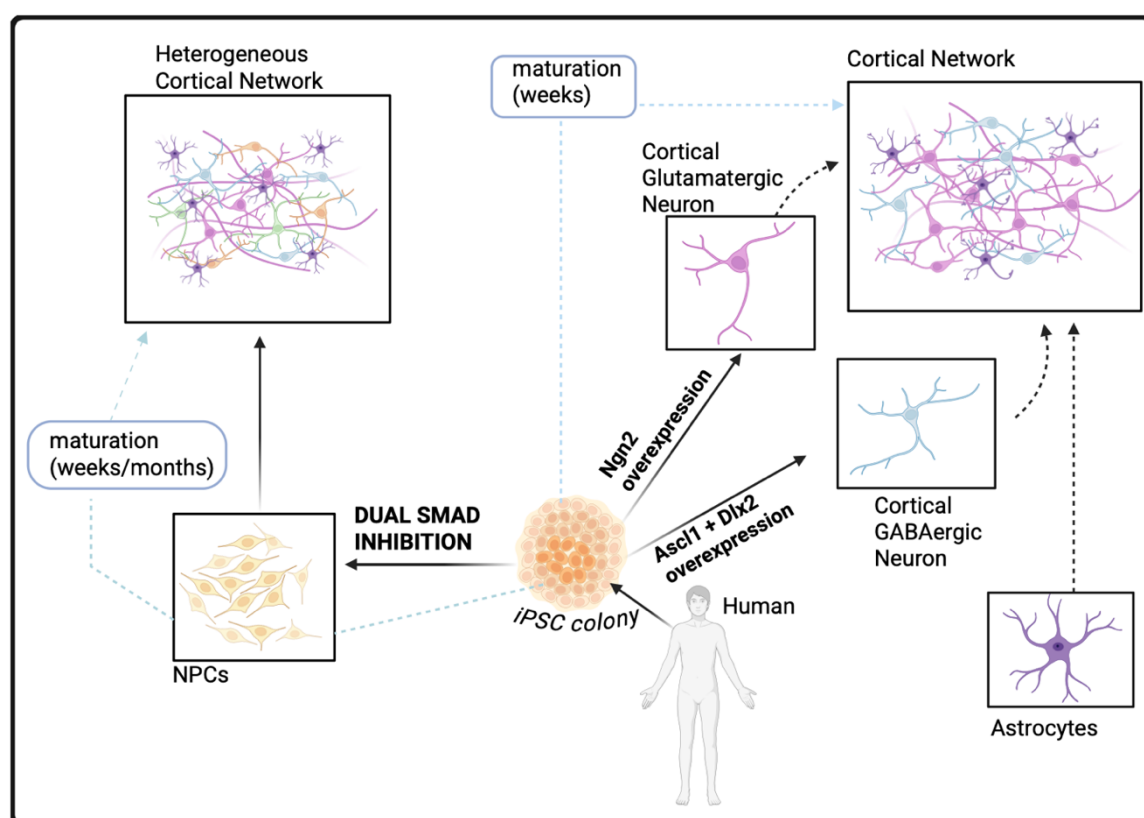


Figure 2.3 Strategies for generating hiPSC-derived cortical neuronal networks. In directed differentiation by dual SMAD inhibition, iPSCs are first converted into neural progenitor cells (NPCs) and then mature, over weeks to months, into a heterogeneous cortical network (Chambers et al., 2009). Forced overexpression of neurogenic transcription factors converts iPSCs into defined neuronal subtypes on a shorter timescale of weeks: Ngn2 yields cortical glutamatergic neurons (Zhang et al., 2013), whereas Ascl1 and Dlx2 yield cortical GABAergic neurons (Yang et al., 2017). Co-culturing the two populations at a defined ratio, together with astrocytes, reconstitutes a cortical network with a controlled excitation/inhibition balance (Heider et al., 2024). Created with BioRender.com.

2.2.4 Maturation on Micro-Electrode Arrays, substrate and glial support

Using the differentiation protocols described in the previous section, neural networks are obtained which must further be characterised at an electrophysiological level. In recent years, there has been growing interest in the functional characterisation of the electrical activity of neural networks derived from hiPSCs using MEAs. (Pelkonen et al., 2022) In addition to the differentiation protocol, which has a significant influence, the functional development and maturation of the network depend on the conditions under which the neurons are cultured. In this context, it is important to have a substrate that can guarantee the development and recording of the network on MEAs (Hyvärinen et al., 2019), as well as adequate support from the glial cells that accompany the neurons (Frega et al., 2017).

In 2017, Hyysalo and colleagues compared various human laminin isoforms, discovering that if the substrate contained an $\alpha 5$ chain, such as laminin-521, benefits were observed. In particular, this substrate promoted neuronal attachment, network formation and viability, and yielded a greater number of electrophysiologically active electrodes in the MEA. (Hyysalo et al., 2017) This highlights the importance of the substrate in neuronal cultures: maximising the number of electrophysiologically active electrodes expands the spatial sampling of the network and enables the calculation of metrics related to synchrony and functional connectivity, which can be reliably estimated only when the network activity is recorded simultaneously from multiple points. (Cerina et al., 2023; Chiappalone et al., 2006)

For protocols based on the forced overexpression of neurogenic factors, glial support is equally important. Co-culture with astrocytes has shown several benefits in terms of neuronal survival, synaptic maturation and the regularity of network activity. In various protocols, this support is provided by primary rodent astrocytes (Frega et al., 2017). On the other hand, human glial models derived from stem cells are becoming increasingly available. This is because protocols for deriving astrocytes, oligodendrocytes and microglia from human pluripotent stem cells are being refined and are becoming increasingly widely used (Lanjewar & Sloan, 2021). In dual-SMAD inhibition protocols, the culture is instead

heterogeneous and already includes a fraction of endogenously generated astrocytes, so that exogenous co-culture is not strictly necessary.

Under conditions conducive to growth and maturation, hiPSC-derived neuronal networks develop spontaneous electrophysiological activity that can be observed while the culture is maintained on the MEA for several weeks. This electrophysiological activity includes action potentials, synaptic transmission and, once the culture is mature, synchronised activity within the network. In 2019, Hyvärinen and colleagues compared hiPSC-derived networks with rat cultures during MEA characterisation, noting that, whilst undergoing similar stages of development, the human networks exhibited different patterns of activity. Specifically, human networks and rat cultures differed in certain characteristics of bursting activity (Hyvärinen et al., 2019). This shows that, when maintained under favourable conditions, human networks mature and develop properties that differ from those of animals. This diversity is attributed to species-specific features.

2.2.5 Application to genetic disorders

The fact that hiPSC-derived neurons retain the donor's complete genetic background makes this type of model particularly suitable for studying neurological diseases with a genetic cause. In these models, in fact, neurons derived directly from the patient can be studied at the preclinical stage to see how the mutation alters neuronal functions and the behaviour of the network, without having to use animal models. An example is given by drug-resistant epilepsies such as Dravet syndrome, a form of epilepsy often caused by loss-of-function mutations in SCN1A, the gene encoding the α -subunit of the voltage-gated sodium channel Nav1.1 (van Hugte et al., 2023).

This type of epilepsy has been analysed in studies that, through the use of hiPSC-derived neurons, have allowed us to delve deeper into the causes of the observed phenotype. In 2013, Higurashi and colleagues (Higurashi et al., 2013) showed that, using patient-derived neurons, a characteristic decrease in firing rate was observed during sustained stimulation. This study was based predominantly on GABAergic neurons, and therefore the observed phenotype was attributed to a deficit in inhibition. Subsequent studies have scaled back this interpretation. van Hugte and colleagues (van Hugte et al., 2023) analysed excitatory neuronal networks derived from patients with different SCN1A mutations and, using

MEAs, identified mutation-specific phenotypes. Furthermore, they showed that all networks had aggravated network activity at febrile temperature and that antiepileptic drugs acted on networks of patients with milder epilepsy but not on Dravet. Another study worth mentioning in this field is that of Mzezewa and colleagues (Mzezewa et al., 2025). The researchers analysed both GABAergic-enriched cultures and heterogeneous cultures on MEAs, showing that functional alterations were more prevalent in cultures composed of both glutamatergic, e.g., excitatory and GABAergic neurons. Further, they showed that the extent of functional impairment reflected the clinical severity of the disease and, through a principal components analysis (PCA), were able to clearly separate patient networks from those of controls. The studies by van Hugte and Mzezewa, evaluated overall, update the initial idea that attributed the phenotype solely to inhibition, demonstrating that excitatory neurons also contribute to the network phenotype.

Beyond single-gene mechanisms, hiPSC-derived networks recorded on MEAs have been proposed as a robust platform for relating genotype to network-level phenotype. Mossink et al. (2021) showed that MEA recordings of hiPSC-derived neuronal networks yield reproducible measures of network activity and can distinguish disease-specific phenotypes from healthy controls, supporting the use of these networks both for disease phenotyping and for preclinical drug screening on human-relevant systems.

2.2.6 Limitations of hiPSC-derived models

As described in the previous sections, hiPSC-derived neuronal models offer numerous advantages, particularly in translational neuroscience. Despite this, they have certain limitations that should be highlighted. The main limitation is their intrinsic variability, which limits and complicates comparisons between experiments (Ortmann & Vallier, 2017; Mossink et al., 2021). Indeed, hiPSC lines exhibit variability between donors, between clones from the same donor, and even between differentiation batches. Another limitation is related to limited maturation. Even after an extended culture period and under optimal conditions, hiPSC-derived neurons often exhibit immature-like activity rather than the characteristics of adult, fully mature neurons (Mateos-Aparicio et al., 2020). This immaturity reduces the extent to which their activity can be compared to that of the mature human brain. Furthermore, co-culture with rodent glial support introduces, in addition to the benefits discussed in the previous sections, limitations intrinsic to all cross-species

systems (Frega et al., 2017). In the case of disease modelling, it is important to bear in mind that there is a mismatch between human-derived neurons and rodent astrocytes, in particular when interpreting recorded network dynamics. Despite the limitations described, neural models derived from hiPSCs are considered one of the most promising *in-vitro* models in the modelling of human neurological genetic disorders and drug testing (Engle et al., 2018; Centeno et al., 2018). However, the fact remains that *in-vitro* models, and therefore also those derived from hiPSCs, do not replicate the systemic and behavioural complexity of an entire organism. *In-vivo* animal models allow the study of physiological and behavioural mechanisms that cannot be reproduced *in-vitro* and, despite translational limitations related to differences between species, still constitute the gold standard in preclinical research. (Loewa et al., 2023) In neuroscience as in other fields, hiPSC models are therefore complementary, and not alternative, to *in-vivo* studies (Centeno et al., 2018; Engle et al., 2018).

2.3 Micro-Electrode Arrays (MEAs)

2.3.1 The MEA technique: rationale and working principle

MEAs are widely used to characterise network electrophysiological activity *in-vitro* (Cerina et al., 2023; Vinogradov et al., 2025). This is because the investigation of neuronal network activity requires tools capable of resolving the collective behaviour of large populations of cells simultaneously. For decades, electrophysiology relied almost exclusively on patch-clamp, a technique introduced by Neher and Sakmann in 1976 that enables highly precise intracellular recordings of ionic currents and membrane potentials at the level of individual cells, and even of single ion channels (Neher & Sakmann, 1976). For their work on the function of single ion channels, Neher and Sakmann were awarded the Nobel Prize in 1991, and patch-clamp has since remained the gold standard for studying ion channel activity (Conforti, 2012).

Patch-clamping provides an unparalleled level of insight into electrophysiology on a single-neuron basis. In the whole-cell configuration, a high-resistance seal is formed between the pipette tip and the cell membrane, which is then ruptured to give direct access to the cytoplasm and allow high-precision recording of the intracellular potential (Gao et al., 2021). This makes it the method of choice for resolving membrane dynamics and ion-channel behaviour at the level of the individual neuron. It is, by its nature, a single-cell technique: neurons are patched individually through manual positioning of the pipette, and dialysis of the cytoplasm progressively degrades the signal, so that the same cell cannot be recorded for long periods. (Conforti, 2012) The dynamics of a neuronal network, by contrast, are defined at a different level. Properties such as synchronised firing and network-wide bursting are collective since they exist only across many neurons active at the same time, and they emerge gradually as the network matures (van Pelt et al., 2004; Wagenaar et al., 2006). Capturing them therefore requires a recording method that samples many neurons simultaneously, rather than a single neuron in a single session.

MEAs are devices in which a grid of extracellular micro-electrodes, integrated into the culture substrate, record the electrical activity of an entire neural network. They allow extracellular recordings without damaging the cells, and for long durations (Gross et al., 1977; Cerina et al., 2023). With MEA, the electrodes never come into contact with the interior of the cell, leaving the viability and function of the neuron intact, and for this reason, the same culture can be recorded multiple times: days, weeks or even months apart. This characteristic makes MEAs particularly well-suited to longitudinal studies of the maturation and development of neural networks. The network signal recorded by the MEA describes the collective state of the network, and varies depending on the balance between excitatory and inhibitory synaptic transmission, the degree of functional connectivity, and the maturity of the culture. (Cerina et al., 2023)

2.3.2 Historical development and technological evolution

The use of MEA technology in *in-vitro* electrophysiology dates back to the landmark research conducted by Thomas, Gross, and Pine (Thomas et al., 1972; Gross et al., 1977; Pine, 1980; Cerina et al., 2023). The first-ever MEA device was described in 1972, in which Thomas recorded biological electric activity from cultured chick cardiomyocytes by employing about thirty platinised gold microelectrodes that were arrayed in two rows, each consisting of fifteen electrodes spaced at 100 μm distance on a glass substrate (Thomas et al., 1972). After five years, the approach was extended to neuronal cultures (Gross et al., 1977). From that time until now, the MEA technology has made many breakthroughs, such as an increase in the number of electrodes, greater recording resolution, varied forms of devices and related software, whilst maintaining the essential extracellular recording concept. The following section discusses the different categories of devices currently being used and distinguishes them in terms of electrode density, configuration, and well design.

2.3.3 Different MEA platforms

The most widely used format is the standard low-density single-well MEA (swMEA; Figure 2.4a), which is made on a biocompatible electrically insulating substrate like

polyimide or silicon nitride. This ensures that electrode traces do not come into direct contact with the electrolyte solution, thus preventing a short circuit. (Cerina et al., 2023) The material used to make the electrodes is chosen on the basis of its cytocompatibility, electrical conductivity and low impedance properties. The materials used include gold, titanium nitride (TiN), poly(3,4-ethylenedioxythiophene) (PEDOT)-coated gold and black platinum (Obien et al., 2015) that have an impedance lower than 500 k Ω at 1 kHz, which minimises thermal noise and ensures a high signal-to-noise ratio (Cerina et al., 2023). There are standardised geometrical arrangements for electrodes on the swMEA, with 8 $\times$ 8 grids often being used for a single-quadrant swMEA. The number of electrodes varies from 60 to 256, with distances between the electrodes of 100–500 μ m and electrode diameter ranging between 10–30 μ m (Cerina et al., 2023). These geometrical features determine the resolution of the recorded data. These spacings result in each electrode capturing extracellular electrical potentials of the local neuronal network rather than single neurons. Although this is a limitation compared to the recording resolution of fine metal electrodes and tetrodes as well as high-density MEAs (HD-MEAs), the setup is sufficient for measuring network activity and synchronisation.

The growing need for single-cell spatial resolution has driven the development of HD-MEAs (Figure 2.4b), in which the number and density of recording sites have increased significantly thanks to the integration of active electronic components. These devices are typically based on CMOS (complementary metal-oxide-semiconductor) technology, integrated onto the same substrate as the electrodes (Berdondini et al., 2009; Cerina et al., 2023). Conventional passive thin-film MEAs require each electrode to be connected individually to the chip's periphery and to an external amplifier. This severely limits the number and density of electrodes and is overcome by CMOS-based active MEAs, which integrate amplification, multiplexing and analogue-to-digital conversion directly onto the chip. (Obien et al., 2015) In 2009, Berdondini and colleagues developed such a device: a CMOS MEA with an active-pixel sensor comprising 4,096 electrodes (Berdondini et al., 2009). Subsequent systems have since reached from thousands to tens of thousands of electrodes, with pitches down to 17.5 μ m (Cerina et al., 2023). The reduced distance between the electrodes of HD-MEAs provides access to the microcircuit dynamics of local neuronal populations. Combined with spike-sorting algorithms, this allows the propagation of electrical activity to be tracked with sub-cellular resolution. However, this higher

resolution comes at a high cost. Indeed, a ten-minute recording from a 4096-electrode array sampled at 20 kHz produces approximately 90 GB of raw data (Cerina et al., 2023), and the proximity of the electrodes necessitates careful assessment of cross-talk between adjacent sites (Naughton et al., 2022). For these reasons, HD-MEAs are preferred in studies requiring high spatial resolution, such as the measurement of axonal conduction velocity or synaptic transmission at the level of individual connections, whilst standard low-density MEAs remain widely used for high-throughput functional characterisation of neural networks.

A development that considerably extended the use of MEAs in biomedical research was the introduction of multi-well MEA (mwMEA; Figure 2.4c) platforms, in which several independent wells, each with its own electrode array, are fabricated on a single plate and recorded at the same time. Commercial mwMEA systems are available in a range of formats, commonly spanning from 24 to 96 wells, with 3 to 64 electrodes per well depending on the plate format, and inter-electrode spacings of 150 to 700 μm (Cerina et al., 2023). Their key advantage is throughput. By simultaneously acquiring electrophysiological data from many networks under potentially different experimental conditions, mwMEAs offer a powerful platform. They are used for dose-response studies of pharmacological compounds, neurotoxicity screening and, particularly relevant for hiPSC-based research, for comparing multiple patient-derived and control cell lines within a single MEA device (Mossink et al., 2021). Because all wells on a plate are seeded and maintained under the same conditions, this format also improves experimental control and helps to limit variability between parallel experimental conditions. The multi-well format is what makes the benchmarking study of Mossink et al. (2021) possible. Using 278 wells from ten healthy hiPSC lines, cultured across several batches, they measured the normal range of network-activity parameters and identified the metrics that are most robust across lines.

The MEA formats described so far are based on planar electrodes fabricated on a flat substrate. These devices are perfectly suited to 2D cultures, but are poorly suited to 3D preparations such as organoids and tissue slices. To work with 3D networks, MEAs with non-planar microelectrodes have therefore been developed, in which the recording sites take the form of spikes, pillars, mushrooms, volcanoes or needles (Cerina et al., 2023, Figure 2.4d). These devices offer two main advantages for recording from 3D networks.

The first benefit relates to the recording process and, in particular, to the distance between the electrode and the active neurons, which is reduced by the penetration of the electrodes into the tissue. Indeed, by reaching the inner layers of the 3D *in-vitro* model, the surface layer – which is often made up of damaged cells – is bypassed (Spanu et al., 2020; Cerina et al., 2023). In practice, reported electrode heights range from around 110 μm for electroplated gold pillars (Spanu et al., 2020) to roughly 1 mm for free-standing actuated probes (Soscia et al., 2020). This is enough to clear the superficial layer of damaged cells and reach the first active layers, but well below the several-millimetre size of larger organoids, whose core is therefore not reached. Secondly, their increased geometric surface area lowers the electrode impedance, improving the signal-to-noise ratio (Obien et al., 2015; Cerina et al., 2023). The limitation is that all the microelectrodes are at the same height: as a result, the recording takes place on a single horizontal plane, which is simply raised within the tissue. The depth of the network along the vertical axis therefore remains inaccessible, and in effect the recording takes place in two dimensions.

A different approach is offered by 3D MEAs (Figure 2.4e), which record simultaneously from multiple 2D planes thanks to microelectrodes distributed throughout the volume of the neural tissue (Cerina et al., 2023). Several prototypes have been proposed, based on the insertion of micro-electrodes at different heights along free-standing probes. Soscia and colleagues, for example, have developed a flexible 3D array of 80 thin-film electrodes distributed across 10 actuated polymer probes, designed to record from within a 3D *in-vitro* brain model (Soscia et al., 2020; Cerina et al., 2023). Overall, these developments extend MEA recording from a single plane to the entire volume of the culture, progressively aligning the geometry of the recording array with that of 3D networks.

With the recent development of organ-on-chips (OoCs), microfluidic devices designed to *in-vitro* reproduce the functions of an organ, the recording of these systems through in-chip sensors is of particular interest (Ferrari et al., 2020). The idea is to have a more realistic device that can be measured in real time and non-invasively. The same idea is applied for brain-on-chips, in which brain tissue and its modularity are reproduced in a more controlled way than conventional 2D and 3D cultures, thanks to microfluidic channels that allow the network architecture to be controlled (Xu et al., 2023). With these devices, it is possible to guide neuronal growth pathways and create compartmentalised networks, in which two or more populations are in separate chambers that can communicate through microfluidic

channels. At this point, the possibility of using MEA devices in combination with these engineered circuits becomes clear. Specifically, the electrodes can be in custom experimental positions based on the required readout: for example, they could be placed inside microchannels to detect axonal signals that would otherwise be difficult to record due to the small size and complexity of the connections (Xu et al., 2023). In general, the integration of MEAs allows recording and stimulating at specific points and monitoring functional connectivity and information transmission between two or more regions (Xu et al., 2023).

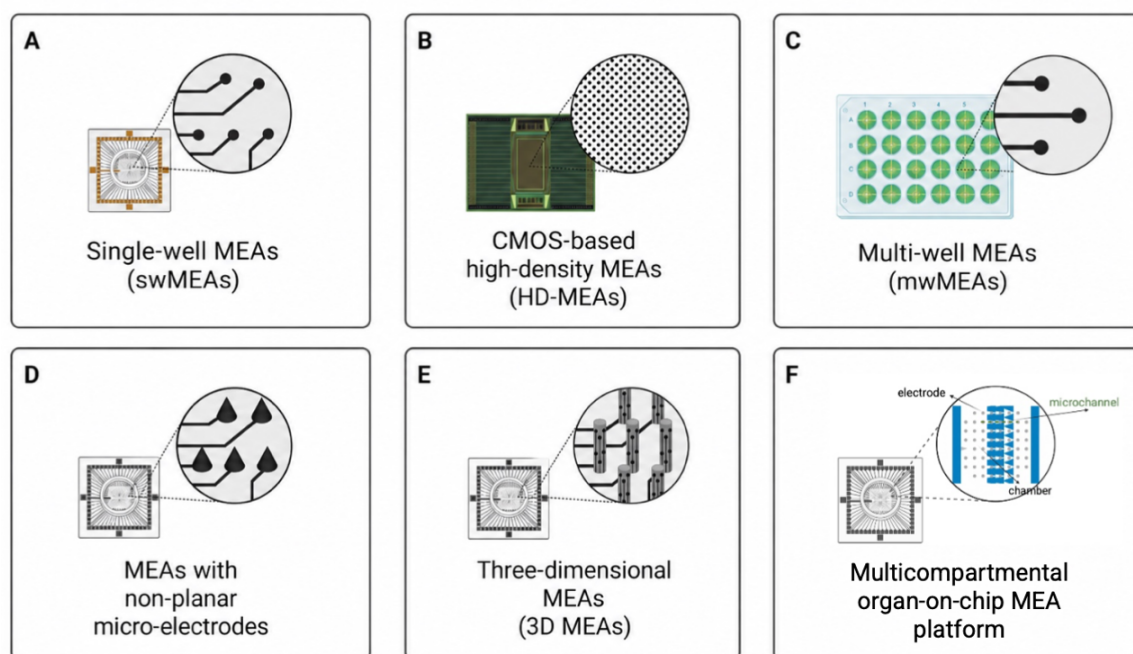


Figure 2.4. The main MEA platforms. (A) Single-well MEAs (swMEAs), low-density planar arrays for network-level recording. (B) CMOS-based high-density MEAs (HD-MEAs), with thousands of electrodes and (sub)cellular resolution. (C) Multi-well MEAs (mwMEAs), parallel arrays on one plate for high-throughput screening. (D) MEAs with non-planar micro-electrodes for recording from 3D preparations. (E) Three-dimensional MEAs (3D MEAs), with electrodes spanning the tissue volume. (F) Multicompartmental organ-on-chip MEA platform, where microchannels guide axons between compartments to impose a defined network architecture. Created with BioRender.com and inspired by Cerina et al. (2023) and Xu et al. (2023).

2.3.4 MEA signal acquisition and frequency content

An MEA records an extracellular voltage signal whose information spans a spectrum of frequencies from slow components to fast components. The specific part of this spectrum captured depends on the acquisition system, so the recording configuration dictates what can later be analysed. As good practice, the signal is acquired with a wide-band filter, for example from about 1 to 7000 Hz, so that the full frequency content is preserved, and narrower filters are applied only at the analysis stage to isolate the components of interest (Obien et al., 2015; Cerina et al., 2023).

During signal acquisition, each electrode records a signal which is then amplified by a headstage connected to the MEA plate. This amplified signal passes through filtering stages. It is first digitised by an analogue-to-digital converter and then transferred to recording software for visualisation and storage. (Vinogradov et al., 2025; Cerina et al., 2023). The frequency band of this chain determines which signals are retained: components that fall outside the recorded band are lost and cannot be recovered afterwards. For instance, a pipeline tuned to high frequencies uses a high-pass filter to eliminate the slower signal components and suppress the substantial low-frequency noise from the electrode-electrolyte interface (Obien et al., 2015; Mossink et al., 2021). For this reason, the acquisition system and its filters must be matched to the frequency range of the activity to be studied rather than adopted by default.

2.4 Neuronal Dynamics and Electrophysiological Signals

Neurons are defined by electrical excitability, which implies their capability of encoding and transmitting information via transient changes in the transmembrane voltage. Under resting conditions, the voltage across the neuronal membrane remains constant at about -70 mV due to the differential distribution of ions and their selective permeability through ion channels. Major ions involved include Na^+ , K^+ , Ca^{2+} and Cl^- , the concentration gradients of which are maintained by the activity of ion transporters such as the Na^+/K^+ -ATPase pump described by Skou in 1957. Subsequently, the principles of membrane excitability were formulated by Hodgkin and Huxley in 1952 (Hodgkin & Huxley, 1952). Their model describes the specific trajectory of the membrane potential during an AP, which can be described in distinct phases (Figure 2.5). A depolarising input results in an increase in the membrane voltage to a value exceeding the firing threshold (≈ -55 mV). In response, rapid opening of voltage-gated Na^+ channels produces a fast inward current driving membrane voltage up. This marks the depolarisation phase of the AP. Next comes repolarisation when Na^+ channels deactivate, and K^+ channels activate to restore the resting potential. Finally, there is hyperpolarisation as a result of the increased K^+ permeability and subsequent membrane potential decrease below the resting value until baseline is restored (Kandel et al., 2021). This period is divided into two phases: the absolute and relative refractory periods. In the first phase, the inactivation of the Na^+ channels makes it impossible to generate a new AP, regardless of the intensity of the stimulus applied to the neuron. In the second phase, however, it is possible to generate an AP, but the threshold is temporarily higher and therefore requires a more intense stimulus to elicit a response. This time constraint imposed by the refractory period sets an upper limit on the firing rate and helps to define the temporal pattern of the spikes (Bean, 2007; Hodgkin & Huxley, 1952). Critically, stereotyped in amplitude and duration, AP carry information only in terms of the rate and pattern of spikes. Upon propagation through the axon, the AP reaches presynaptic terminals where voltage-gated Ca^{2+} channels open, allowing Ca^{2+} entry and causing the fusion of vesicles and release of neurotransmitters to the synaptic cleft and formation of postsynaptic currents (Kandel et al., 2021). When many neurons are active at

the same time, these currents add up over time and space, giving rise to the extracellular field potentials that MEA electrodes are able to measure (Buzsáki et al., 2012).

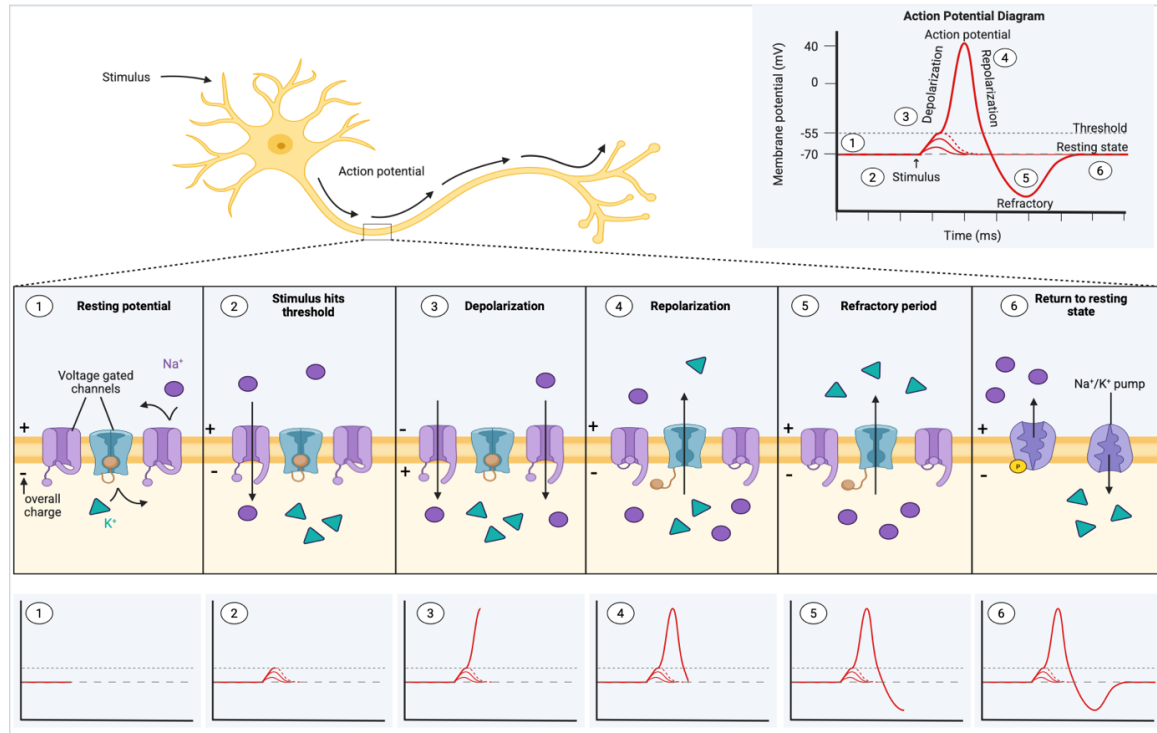


Figure 2.5. Phases of the action potential. Schematic of the membrane events underlying a single AP (Bean, 2007; Kandel et al., 2021): a suprathreshold stimulus opens voltage-gated Na⁺ channels (depolarisation), which then inactivate as voltage-gated K⁺ channels open (repolarisation), followed by a brief K⁺-mediated hyperpolarisation before the resting state is restored. The top panels show the propagating AP and its membrane-potential trajectory over time; the six central panels show the corresponding Na⁺ and K⁺ channel states, numbered (1) to (6); the lower row shows the progressive emergence of the spike waveform. The Na⁺/K⁺-ATPase shown in panel (6) re-establishes the ionic gradients over slower timescales and does not itself restore the membrane potential after a single spike. Created with Biorender.com

2.4.1 Spike-Based Activity in MEA signal

MEA electrodes detect individual APs extracellularly as brief voltage deflections, lasting 1 or 2 milliseconds, that represent the transmembrane currents related to the depolarisation and repolarisation of the cell membrane. These detected APs are the most basic readout of neuronal activity; they are often called spikes, and the distance between two consecutive spikes is called inter-spike interval (ISI). (Cerina et al., 2023; Bean, 2007) As described previously, the information carried by APs lies in the frequency and temporal pattern of

discharge; this leads to the definition of a spike train, in which each spike is represented uniquely by the instant it was emitted (Figure 2.6 A).

At the level of individual electrodes, neuronal activity can be classified according to its temporal organisation, described by the spike train, into distinct firing regimes (Cerina et al., 2023). The simplest pattern consists of isolated, temporally sparse APs, referred to as random or isolated spiking, which reflect the baseline activity of the network in the absence of stimulation (Chiappalone et al., 2006). The most common descriptor of the spiking activity is the mean firing rate (MFR), defined as the number of spikes per unit time recorded at a given electrode (Cerina et al., 2023). A more complex and functionally significant pattern is bursting, in which neurons emit clusters of APs over short periods of time. Each cluster is separated from adjacent clusters by intervals of relative silence known as inter-burst intervals (IBIs) (Wagenaar et al., 2006; Chiappalone et al., 2006). In dissociated cultures, bursting has been extensively characterised and constitutes a dominant feature of spontaneous network dynamics (van Pelt et al., 2004; Chiappalone et al., 2006). Standard metrics used to characterise bursting at the electrode level include the mean bursting rate (MBR), burst duration (BD) and the percentage of spikes occurring outside burst events, the latter offers an index of how network activity is organised into structured episodes rather than being randomly distributed over time. (Chiappalone et al., 2006; Bologna et al., 2010).

As neuronal cultures mature and recurrent synaptic connectivity strengthens, bursting activity at individual electrodes becomes progressively coordinated across the network, creating network bursts. A network burst is defined as a near-simultaneous activation of a large fraction of active electrodes, each generating a burst within a short temporal window, resulting in a population-level synchronisation event (van Pelt et al., 2004; Bologna et al., 2010, Figure 2.6. B). This behaviour is commonly observed in mature *in-vitro* cultures and is thought to emerge from the intrinsic connectivity of the network, involving both excitatory and inhibitory neurons (Bologna et al., 2010; van Pelt et al., 2004).

Functionally, network bursts are analogous to the 'up' states observed in the cortex during slow-wave sleep *in-vivo* and are believed to play a key role in synaptic plasticity, learning processes and the maintenance of rhythmic oscillatory behaviour (Timofeev et al., 2000; Kamioka et al., 1996). Network bursts can be identified through several detection methods, including approaches based on the statistical distribution of inter-spike intervals and

adaptive-threshold algorithms that account for the variability of spontaneous activity across different culture conditions and developmental stages (Pasquale et al., 2010). The resulting events are commonly characterised through parameters such as the network burst rate (NBR), the network burst duration (NBD), and the spatial activation across the electrode array (Chiappalone et al., 2006; Bologna et al., 2010).

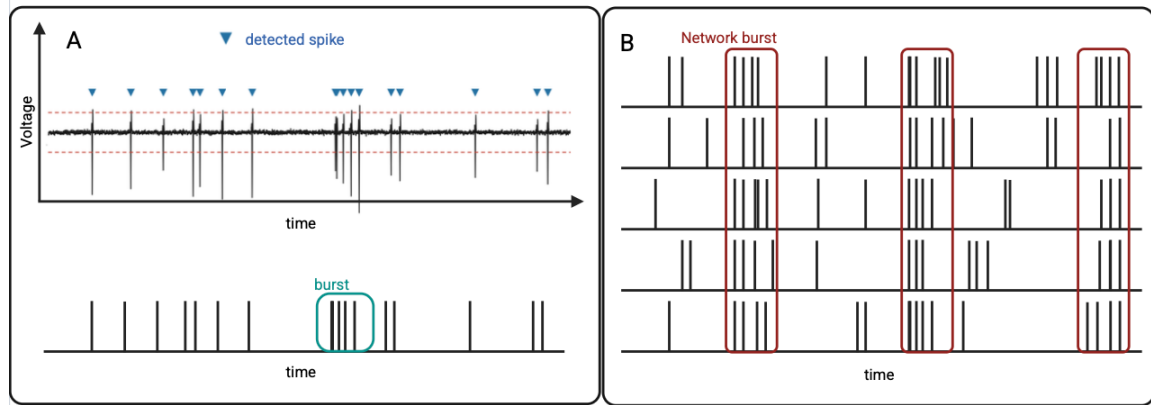


Figure 2.6. Sketch describing the spike-based readouts of MEA activity. **(A)** *Top*: simulated extracellular voltage trace, with detected spikes (blue triangles) identified by a positive and negative threshold (dashed red lines). *Bottom*: the same signal shown as a spike train, displaying isolated spikes and a burst. **(B)** Raster plot of spike trains from multiple electrodes, with network bursts highlighted in red. Created with BioRender.com

2.4.2 The Neural Activity Score (NAS)

The multiplicity of descriptors available from MEA recordings, spanning firing rate, burst characteristics, network synchrony, and connectivity metrics, poses a significant analytical challenge. In practice, researchers most commonly rely only on the mean firing rate and on metrics for overall activity. This underutilises the full informational content of the recordings and, in screening applications where changes in network connectivity may occur independently of overall activity levels, increases the risk of false-negative results (Passaro et al., 2021). To address this limitation, Passaro et al. (2021) developed the NAS, a composite index designed to capture the multidimensional nature of MEA data in a single, objectively derived scalar value.

The NAS is obtained through PCA applied to a comprehensive set of MEA parameters spanning spiking, bursting, network bursting, and synchrony metrics. Prior to decomposition, each parameter is standardised to a z-score:

$$X_i = \frac{x_i - \mu_i}{\sigma_i} \quad (2.1)$$

where x_i is the raw value of the i -th parameter and μ_i and σ_i are its mean and standard deviation across the dataset. This normalisation process was done to define a common dimensionless scale in which all metrics, which can differ by several orders of magnitude, can be represented.

Principal component analysis (PCA) is then applied to the standardised parameters. The first principal component (PC1) was found to be strongly correlated with time in culture and was identified as the axis that captures the progressive functional maturation of the neuronal network. Its factor loadings β_i were therefore retained as the weights of the index, and the NAS for a given well at a given time point is defined as the linear combination of all standardised parameters, as described by formula 2.2.

$$\text{NAS} = \sum_{i=1}^n \beta_i X_i \quad (2.2)$$

In formula 2.2, β_i is the PC1 factor loading of the i -th parameter, X_i its z-normalised value, and n the number of parameters considered. Because the loadings act as relative weights, each metric contributes to the score in proportion to its association with network maturation.

In the work of Passaro and colleagues, the MFR was ranked only as the 11th strongest contributor, while the strongest contribution was found from network-level parameters such as burst percentage and network burst percentage. This result shows the information loss incurred when considering only simple measures of overall activity rather than also including informative network-level metrics. (Passaro et al., 2021). NAS proved to be a valid measure in different conditions, showing responsiveness in both the enhancement and disruption of network activity and in toxicity tests, where it showed to be more sensitive compared to single MEA parameters. This supports its use as a single, sensitive readout of network maturation (Passaro et al., 2021). The NAS framework therefore can represent an important methodological reference for longitudinal MEA studies, providing a standardised and quantitative basis for comparing network maturation trajectories across experimental conditions, cell lines, and laboratories.

2.4.3 Field potentials and the frequency domain

The extracellular signal recorded by a microelectrode is a wide-band voltage trace that contains, superimposed, two functionally distinct components that are conventionally separated by frequency filtering. The high-frequency band (typically 300–3000 Hz) isolates the extracellular action potentials (EAPs), the brief deflections produced by the firing of individual neurons close to the electrode. The low-frequency band (typically 1–200 Hz), instead, isolates slow voltage fluctuations also described as local field potentials (LFPs) (Pelkonen et al., 2022; Einevoll et al., 2013). The distinction is not only spectral but conceptual. Whereas spikes report the output of single cells, the LFP reflects the summed transmembrane currents of many neurons around the electrode and is therefore read as a mesoscopic signal. LFP reflects the integrative, mainly synaptic, activity arriving at and processed by a local population (Buzsáki et al., 2012; Einevoll et al., 2013). The previous sections characterised network activity through spiking, bursting and network bursting; the LFP provides a complementary, lower-frequency view of the same network, giving access to population dynamics that spike trains alone do not capture.

The LFP is the extracellular potential generated by the superposition of all transmembrane currents flowing in the tissue volume around the electrode. Its dominant source is synaptic activity. Excitatory and inhibitory postsynaptic currents create local current sinks and sources that, together with their return currents along the neuronal membrane, form electric dipoles (Buzsáki et al., 2012; Einevoll et al., 2013). Although with a minor contribution, non-synaptic processes such as Ca^{2+} spikes, voltage-dependent intrinsic currents, spike afterhyperpolarisations, and the spikes themselves at higher frequencies also contribute to the signal (Buzsáki et al., 2012).

Because the contribution of each current scales with the inverse of its distance from the electrode, and because a dipole field decays faster than a monopole, the LFP is shaped not only by how much current flows but also by the spatial arrangement of its sources and by the position of the electrode relative to them (Buzsáki et al., 2012; Einevoll et al., 2013). For this reason, the geometry of the contributing neurons is a primary determinant of the LFP. For instance, pyramidal cells, which are characterised by their long apical dendrites aligned in parallel, generate "open-field" dipoles along their somatodendritic axis. When many such cells are activated, their dipoles sum synchronously, producing large field

potentials. Spherically symmetric neurons, by contrast, tend to generate "closed fields" whose currents largely cancel and contribute little to the LFP (Buzsáki et al., 2012). The consequences of this geometric dependence are that the amplitude of the LFP is governed more by source geometry than by the degree of synchronisation or by the properties of the medium (Herreras, 2016). In fact, much of the recorded signal can originate from distant rather than nearby sources, and the polarity of a deflection does not by itself indicate whether the underlying activity is excitatory or inhibitory (Herreras, 2016). These consequences are important when interpreting the LFP as a proxy for local network activity.

Much of the interest in the LFP lies in its frequency content. Cortical networks oscillate over a broad range of frequencies, spanning roughly 0.05–500 Hz, which are conventionally organised into bands (delta, theta, alpha, beta, gamma and faster gamma), each associated with distinct network states (Buzsáki and Draguhn, 2004). A robust feature of these oscillations is that their power is inversely related to frequency, following a $1/f^n$ scaling, so that slow components carry most of the power and fast components progressively less (Buzsáki and Draguhn, 2004; Buzsáki et al., 2012). The standard tool for examining this frequency structure is the power spectral density (PSD) (Figure 2.6 B), where genuine oscillatory activity appears as a peak, or "bump", rising above the aperiodic $1/f$ background of the spectrum. This provides an objective criterion for identifying and quantifying rhythms in the recorded signal (Trujillo et al., 2019).

The relevance of the frequency-based approach for stem-cell-derived models has been demonstrated most clearly in 3D systems. Trujillo et al. (2019) placed cortical organoids on planar MEAs and tracked their electrical activity over time. As the organoids matured, periodic oscillatory events emerged spontaneously, visible as distinct peaks in the delta band (1–4 Hz) rising above the aperiodic $1/f$ background of the power spectrum. These oscillations were also coupled to broadband gamma activity through phase–amplitude coupling, and both phenomena required balanced glutamatergic and GABAergic signalling. Although organoids offer a more brain-like microenvironment and tissue architecture, 2D hiPSC-derived cultures continue to serve as valid functional models and, on MEA, display network activity comparable to that of the human brain (Pelkonen et al., 2022). Examining the LFP and its spectral content in 2D cultures is thus a justified and complementary strategy, well suited to the early stages of characterising these networks,

where the simpler and more accessible 2D geometry allows the frequency-domain properties of the activity to be studied under more controlled and reproducible conditions before moving to more complex 3D systems. Given that, the LFP recorded from 2D cultures should be interpreted with some caution. The large, well-organised cortical LFP described above arises from the laminar architecture and the parallel alignment of pyramidal dendrites, a geometry that dissociated 2D cultures might not reproduce (Buzsáki et al., 2012; Pelkonen et al., 2022; Figure 2.7C). In addition, since the amplitude and polarity of the signal also depend on the position of the electrode relative to the sources (Herreras, 2016), in 2D networks the temporal and spectral features of the LFP are more informative than its absolute amplitude or polarity.

Importantly, because *in-vitro* and *in-vivo* field potentials share the same biophysical origin, the LFP recorded in culture is in principle comparable to the field signals recorded clinically in humans, such as the electroencephalogram (EEG) and electrocorticogram (ECoG). These two signals are recorded respectively from the scalp and from the cortical surface, and are captured and analysed in the corresponding frequency bands (Buzsáki et al., 2012; Pelkonen et al., 2022). This shared structure is what gives the spectral analysis of *in-vitro* LFPs its translational value: oscillatory and frequency-domain features measured in hiPSC-derived networks can be related to the rhythms that characterise human brain activity in physiological and pathological conditions, making the frequency-domain characterisation of 2D cultures a meaningful step toward bridging cellular models and clinical electrophysiology.

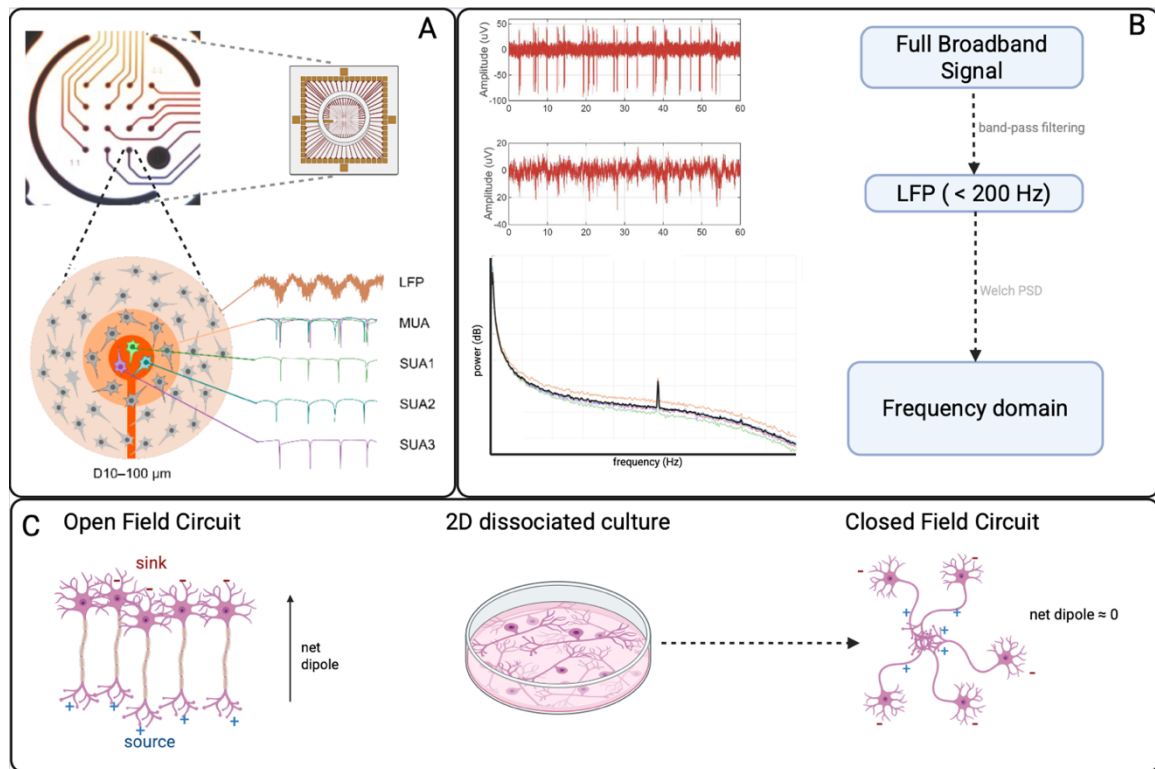


Figure 2.7: Sketch representing LFP in 2D hiPSC-derived cultures on MEA. (A) Single-well MEA device composed of 16 electrodes: each records the full broadband signal containing single-unit activity of individual neurons (SUA1-3), multi-unit activity (MUA), and local field potential (LFP). (B) Pipeline for LFP analysis. From the full broadband signal acquired by the electrodes, the LFP is obtained by band-pass filtering between 0.5 and 200 Hz. The representation in the frequency domain is obtained through a Welch PSD. (C) Influence of network geometry on LFP. Two ideal source geometries are defined: open-field circuit (e.g., aligned cortical pyramidal neurons) and closed-field circuit (random orientation of sources; 2D cultures resemble this circuit). Sink (-) and source (+) indicate current sinks and sources; the arrow denotes the resulting net dipole, which cancels out (≈ 0) in the closed-field case. Created with BioRender.com, with panel A adapted from Pelkonen et al., 2022.

2.4.4 The Aperiodic Component of the LFP: Spectral Parameterisation and Excitation/Inhibition Balance

Apart from the description of oscillatory bands, there is another important element of the LFP power spectrum that can be relevant in the analysis. This component is the aperiodic part of the LFP power spectrum. It typically decreases monotonically with frequency following the inverse power law, usually described as a $1/f$ trend. The aperiodic component is not related to any oscillatory activity; instead, for many years it was treated as background noise or as an unstructured baseline over which the oscillatory peaks of interest were superimposed. Accumulating evidence has since established that it carries physiological information and that its parameters vary systematically with brain state, age, and cognitive demands. (Donoghue et al., 2020)

The aperiodic component is described by two parameters extracted from the log-log representation of the PSD. The offset b reflects the overall level of broadband power and correlates with neuronal population spiking, and therefore with the MFR of the local population. The exponent χ corresponds to the negative slope of the spectrum in log-log space and reflects the integration of the underlying synaptic currents. (Donoghue et al., 2020) The slope has additionally been proposed as a proxy for the balance between excitatory and inhibitory synaptic activity. A flatter spectrum, described by a smaller exponent, is associated with relatively stronger excitation and a steeper spectrum, modelled by a larger exponent, with stronger inhibition. (Gao et al., 2017) The decomposition of neural power spectra into periodic and aperiodic components has been formalised in the FOOOF algorithm, later renamed specparam (Donoghue et al., 2020). The model represents the log-power spectrum as the sum of an aperiodic component and a set of periodic peaks. In the algorithm, the aperiodic component is modelled as

$$L(F) = b - \log_{10}(k + F^\chi) \quad (2.3)$$

where F is the vector of frequencies, b is the offset, χ is the exponent, and k is a "knee" parameter that accounts for a possible bend in the spectrum. When the spectrum is fitted

over a restricted frequency range with no bend, k is set to zero, and the expression reduces to a straight line in log-log space. (Donoghue et al., 2020)

$$L(F) = b - \chi \cdot \log_{10}(F) \quad (2.4)$$

In this case, the slope is described by $-\chi$.

Equation 2.4 therefore represents the $1/f$ background of the power spectrum, and it is obtained by a fitting procedure which consists of estimating the parameters b and χ that best describe this aperiodic component of the data. The periodic part of the spectrum is then modelled as the sum of a small number of peaks, each representing a region of power rising above the aperiodic component. Each peak is characterised by a Gaussian, and its mathematical model is described by equation (2.5) where c_n is the centre frequency, a_n the power above the aperiodic component, and w_n the width of the peak. (Donoghue et al., 2020)

$$G_n(F) = a_n \cdot \exp\left(-\frac{(F - c_n)^2}{(2w_n^2)}\right) \quad (2.5)$$

The algorithm estimates these parameters iteratively, and it is described in Figure 2.8. An initial aperiodic component is fitted to the log-power spectrum (Figure 2.8A) and subtracted from it, yielding a flattened residual spectrum (Figure 2.8B). The largest residual peak is then identified and, if it exceeds a noise threshold defined from the standard deviation of the residuals, a Gaussian is fitted and subtracted. This step is repeated, and the iteration stops when no further peak exceeds the threshold (Figure 2.8C). A multi-Gaussian model is then fitted to account jointly for all the detected peaks (Figure 2.8D) and subtracted from the original spectrum (Figure 2.8E). After these steps the aperiodic component is re-estimated on this peak-removed spectrum, so that the final aperiodic fit is less distorted by the oscillations (Figure 2.8F). The re-fitted aperiodic component and the multi-Gaussian model are finally recombined into the full model (Figure 2.8G), whose agreement with the original spectrum is evaluated to assess the goodness of fit (Figure 2.8H). (Donoghue et al., 2020)

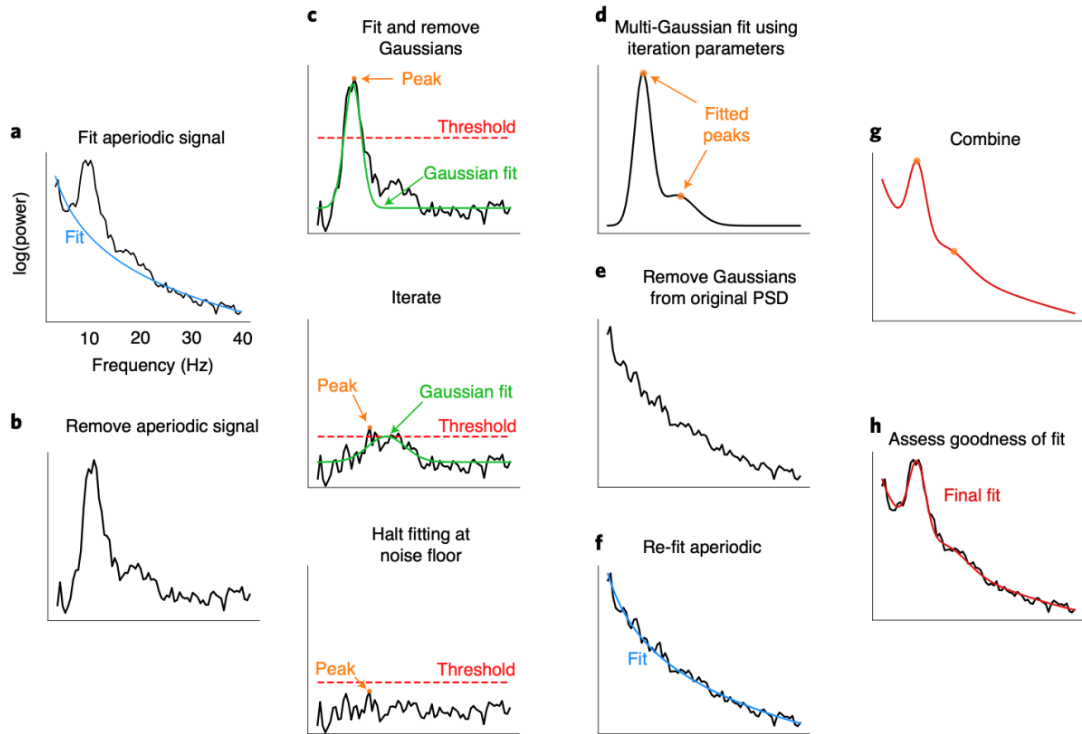


Figure 2.8. The PSD is decomposed into an aperiodic component (blue) and a set of Gaussian-modelled periodic peaks (green) through an iterative procedure. The aperiodic component is fitted and removed, oscillatory peaks are detected above a noise threshold and fitted iteratively, and the aperiodic component is then re-fitted on the peak-removed spectrum. Finally, the two components are recombined into the final model (red). Reproduced from Donoghue et al. (2020).

To conclude, offset and exponent of the aperiodic fit are also informative in a developmental context. The offset was linked to population firing rate and is expected to track the overall increase in network activity during maturation, as the number of active neurons and functional synapses increase. On the other hand, as described before, the exponent provides a complementary index of the changing balance between excitatory and inhibitory drives (Donoghue et al., 2020; Gao et al., 2017) that can be used to observe and analyse changes during maturation. This makes the aperiodic component a compact and biologically meaningful readout in longitudinal MEA studies of hiPSC-derived neuronal networks.

2.4.5 Cross-Frequency Coupling: Phase-Amplitude Coupling and the Modulation Index

Neural oscillations across different frequency bands do not operate independently. Rather, they interact through a class of phenomena collectively referred to as cross-frequency coupling (CFC), in which the phase or amplitude of oscillations at one frequency modulates the dynamics of oscillations at another. Among the various forms of CFC, phase-amplitude coupling (PAC) has received considerable theoretical and experimental attention. PAC describes the statistical dependence between the instantaneous phase of a low-frequency oscillation and the amplitude envelope of a higher-frequency oscillation, such that the power of fast rhythms is rhythmically modulated by the phase of a slower carrier wave. (Canolty & Knight, 2010)

The neurophysiological significance of PAC has been demonstrated across various brain regions and species. For example, in the rodent hippocampus and human neocortex, coupling between theta phase and gamma amplitude has been proposed as a mechanism for organising neuronal assemblies. This coupling allows for the coordination of local computations within individual gamma cycles and their sequential ordering across theta cycles. This nested oscillatory structure has been associated with learning, memory and decision-making, supporting the view that PAC is a fundamental organising principle of cortical information processing. More broadly, PAC has been interpreted as a reflection of the hierarchical organisation of neural dynamics, in which slow rhythms, entrained over large spatial and temporal scales, modulate the excitability windows within which faster and spatially local computations occur. (Canolty & Knight, 2010)

The quantification of PAC requires a measure that captures the statistical dependence between the phase time series of a low-frequency band and the amplitude envelope of a high-frequency band. Several metrics have been proposed for this purpose, including the mean vector length (MVL), the phase-locking value (PLV), and the modulation index (MI). The MVL, calculated as the magnitude of the average complex-valued amplitude-phase signal, is mathematically simple but is sensitive to non-stationarity and outliers in the amplitude envelope. This sensitivity can inflate the coupling estimate when there are transient high-amplitude bursts (Tort et al., 2010). The PLV, instead, while useful for

detecting the existence of coupling, is not suitable for quantifying its intensity and shows low tolerance to noise (Tort et al., 2010). The MI introduced by Tort et al. (2010) addresses these limitations and has been proven to be a particularly attractive PAC measure due to its robustness and interpretability.

The MI is computed by first extracting the instantaneous phase of the low-frequency signal and the amplitude envelope of the high-frequency signal through the Hilbert transform. The amplitude values are then averaged within bins defined by the phase of the low-frequency oscillation, and the resulting phase-amplitude distribution is compared to a uniform distribution using an adaptation of the Kullback-Leibler divergence. An MI value of zero indicates that the high-frequency amplitude is uniformly distributed across all phases of the low-frequency oscillation, reflecting the absence of coupling, while higher values indicate a non-uniform, phase-locked distribution consistent with genuine PAC. (Tort et al., 2010)

To assess which frequency band combinations contribute most strongly to PAC, the comodulogram computes the MI across a systematically varied grid of phase-frequency and amplitude-frequency pairs, producing a two-dimensional map that identifies the dominant coupling interactions within a given recording. This approach enables an unbiased exploration of cross-frequency interactions without requiring a priori assumptions about the relevant frequency bands. (Tort et al., 2010)

The application of PAC analysis to *in-vitro* neuronal cultures is a relatively recent development. In 3D systems, Trujillo et al. (2019) reported phase-amplitude coupling between delta-frequency phase (1-4 Hz) and broadband gamma amplitude (100-400 Hz) in cortical organoids, with stronger coupling during network events than during quiescent periods. The extension of this analysis to 2D cultures was provided in 2024 by Salimpour and colleagues, who reported the first demonstration of PAC in human 2D hiPSC-derived cortical neurons co-cultured with hiPSC-derived astrocytes on mwMEA plates. Using the MI, with the phase extracted from a low-frequency band (1-10 Hz) and the amplitude from a high-frequency band (20-200 Hz), they showed that PAC is also present in bi-dimensional cultures. Furthermore, they showed that the PAC is comparable across independent wells at the same time point but increases over development in culture. Importantly, pharmacological disinhibition with bicuculline modulated the coupling, and this was driven

by network-level interactions rather than individual neurons, suggesting PAC is an emergent property of the network.

Taken together, these observations suggest that PAC is not a property of individual neurons but is best understood as a network-level signature. It only emerges as the network matures, is largely absent in immature cultures, and its modulation reflects changes in neuron interactions rather than individual firing rates (Canolty & Knight, 2010; Salimpour et al., 2024). In this sense, the presence and strength of PAC can be interpreted as an index of the degree of functional organisation and coordination reached by the network, which makes it a particularly informative readout for tracking the maturation of hiPSC-derived neuronal networks over time.

2.4.6 Electrophysiological Markers of Network Maturation: From Neonatal EEG to *In-vitro* Models

Electrophysiological signals have been, and continue to be, used in clinical neuroscience to characterise the maturation of neuronal networks in the human brain, as well as to detect epileptic seizures. Among the various techniques available, EEG is a primary tool for monitoring brain development in preterm infants and neonates (Niemarkt et al., 2011; Pavlidis et al., 2017). A series of changes in the neonatal EEG signal has been documented during the perinatal period. These changes reflect the progressive maturation of synaptic connections and the development of inhibitory circuits (Vanhatalo & Kaila, 2006). One of the most consistent of these transformations is a systematic redistribution of spectral power from lower to higher frequencies (Niemarkt et al., 2011). In a quantitative analysis of preterm infants with normal neurological outcome, Niemarkt et al. (2011) showed that, with advancing postmenstrual age, the absolute power of the delta and theta bands decreases, while in relative terms the weight of the lowest delta band declines and that of the alpha and beta bands increases, suggesting an overall shift towards higher frequencies. Both absolute band power and relative, inter-band measures of power therefore provide a sensitive and clinically informative index of brain maturation, capable of detecting

deviations from normal development in conditions such as preterm birth and hypoxic-ischaemic encephalopathy (Niemarkt et al., 2011).

However, simply transferring the power from low to high frequencies is not always a sign of changing the oscillatory activity. As pointed out in Section 2.4.4, the aperiodic part spreads the power over the whole spectrum. Therefore, a change in its slope over development can independently alter the relative weight of low and high frequency bands, effectively mimicking a band-power shift even without any genuine changes in oscillations (Donoghue et al., 2020). For this reason, the periodic and aperiodic contributions to the spectrum are increasingly regarded as complementary, and the spectral signatures of network maturation are most reliably characterised when band-power is interpreted together with the aperiodic component of the PSD. (Donoghue et al., 2020)

The translation of these frequency-domain markers to *in-vitro* MEA models has gained considerable momentum in recent years. Evidence, so far primarily from 3D hiPSC-derived systems, indicates that the extracellular activity recorded on MEA undergoes developmental changes that parallel, at least in part, those observed *in-vivo* (Trujillo et al., 2019). At early time points, recordings are typically dominated by broadband aperiodic activity with limited oscillatory structure, which is indicative of an immature state of synaptic connectivity, whereas progressively more structured spectral patterns emerge as the network matures.

In this context, Trujillo et al. (2019) showed that the spontaneous activity of human cortical organoids evolves progressively over months in culture, with the emergence of oscillatory dynamics that resemble the discontinuous patterns characteristic of preterm neonatal EEG (Trujillo et al., 2019). It is important to remember that in 2D hiPSC-derived cultures, LFP is often filtered out and thus not analysed, nor even recordable with all commercial MEA systems (Obien et al., 2015; Mahadevan et al., 2022); consequently, it remains largely unexplored whether a spectral trajectory analogous to that observed *in-vivo* and in 3D models also occurs in these 2D models.

The application of such frequency-domain maturation markers to hiPSC-derived neuronal cultures holds particular translational relevance because variation from common maturation trajectories in patient-derived lines may reflect pathological mechanisms related to neurodevelopmental disorders. MEA recordings of hiPSC-derived networks are

already an established and robust tool for this purpose, where network function is typically characterised through spike, burst and network-burst metrics. (Frega et al., 2017; Mossink et al., 2021) This spike-based approach has been used to identify disease-specific network phenotypes in patient-derived neurons and to reverse them pharmacologically, as demonstrated in a model of Kleefstra syndrome (Frega et al., 2019). Quantitatively, LFP-based indices would complement this spike-based toolkit, extending the analytical repertoire available for the characterisation of human neuronal networks on MEA systems.

Chapter 3

Materials and Methods

3.1 Cells and Data Acquisition

3.1.1 Cell Line and Cortical Neuron

Differentiation

The human induced pluripotent stem cell (hiPSC) line used in this study was TUBA1B (Coriell Institute, USA), a commercially available hiPSC line in which one allele of the TUBA1B gene has been monoallelically tagged with monomeric enhanced green fluorescent protein (mEGFP). The donor was a healthy male aged 30 years. The TUBA1B line has been previously employed in MEA-based studies of cortical network development within the NeuroGroup (Mzezewa et al., 2025). All experimental procedures were conducted under the supportive statement of the Ethics Committee of the Expert Responsibility Area of Tampere University Hospital for the use of named hiPSC lines in neuronal research (R20159).

Cortical neurons were differentiated from hiPSCs using a previously published dual-SMAD inhibition protocol (Hyvärinen et al., 2019), applied consistently across both culture batches analysed in this study. Following 32 days of neural induction and maturation, cells were dissociated and seeded onto CytoView MEA 48-well plates (Axion Biosystems, USA) pre-coated with poly-ethylene-imine (PEI) and human recombinant laminin (LN521; Biolamina, Sweden) at a density of 80,000 cells per well. Cultures were maintained in BrainPhys™ maturation medium (BMM; STEMCELL Technologies) at 37°C in a humidified incubator with 5% CO₂. Each well contains a 4×4 array of 16 electrodes, yielding a maximum of 16 recording channels per biological replicate

3.1.2 MEA Recording Setup and Experimental Design

Spontaneous neuronal network activity was recorded using Axion Biosystems MEA systems (Axion Biosystems, USA) operated with AxIS software at a sampling frequency of 12,500 Hz under controlled temperature conditions (37°C). Before each recording session, MEA plates were equilibrated outside the incubator for approximately 5 minutes, and the culture medium was refreshed the day before each recording to ensure stable spontaneous activity. Each recording session lasted 600 seconds. Two independent culture batches of the TUBA1B line-derived neurons were analysed in this study, recorded on two different Axion Maestro models, the Maestro Pro and the earlier Maestro, and referred to throughout as TUBA-new and TUBA-old, respectively. Both culture batches were produced and recorded by the NeuroGroup at Tampere University as part of ongoing research activities. The raw MEA recordings were made available for the analysis presented in this work. An overview of the two datasets, the recording systems used, and their respective analytical applications is provided in Table 3.1.

For the system comparison, all 24 wells of both datasets were included, and LFP power spectral density (PSD) was computed for both systems. Visual inspection of the mean PSD revealed that TUBA-old recordings exhibit a marked attenuation of spectral power below 200 Hz, consistent with the hardware high-pass filter applied by the AxIS v2.5.2.1 system, formally confirming the unsuitability of this dataset for LFP-based spectral analysis (see Section 4.2). Consequently, all LFP spectral analyses were restricted to the TUBA-new dataset. For longitudinal LFP analysis, eight wells in row A were retained. This was because the TUBA-new plate contained 24 wells distributed across three rows cultured under different conditions. Therefore, analysing a single row ensured consistent experimental conditions across the *days in-vitro* (DIV) timepoints. Spike-based network activity metrics were extracted from both datasets across all available wells and are reported in parallel for comparative purposes.

Note. As observable by the table 3.1, the two datasets were recorded one day apart. The four longitudinal recording sessions correspond to DIV 47/52/54/59 in TUBA-new and

DIV 48/53/55/60 in TUBA-old, and, as a consequence, the paired notation “DIV 47/48”, “52/53”, “54/55”, “59/60” is used in between-system comparisons.

Table 3.1. Overview of the two TUBA1B datasets, recording systems, and their use in the analyses presented in this thesis.

Dataset	System	AxIS version	Wells (<i>n</i>)	DIV	Used for
TUBA-new	Axion Maestro Pro	v3.12.8. 6	24 (rows A–C, 8 per row)	47, 52, 54, 59	System comparison (LFP & spike, <i>n</i> =24); longitudinal analysis (LFP, row A, <i>n</i> =8)
TUBA-old	Axion Maestro	v2.5.2.1	24 (plates N2894, N2905)	48, 53, 55, 60	System comparison (LFP & spike, <i>n</i> =24)

3.2 Data Analysis

The analytical pipeline developed for this work is organised into seven sequential stages. Spike and NB detection was performed following the NeuroGroup MEA analysis pipeline, and the resulting spiking activity was subsequently used to identify electrophysiologically active electrodes (Section 3.2.1), which were then retained for LFP extraction and analysis (Section 3.2.2). Mean firing rate and network burst metrics derived from the spike data were additionally extracted as reference indices of overall network function. The spectral content of the LFP is then characterised through two complementary approaches: classical narrowband power analysis across canonical frequency bands (Section 3.2.3), and parametric decomposition into an aperiodic component via the specparam algorithm (Section 3.2.4). Cross-frequency interactions are subsequently quantified using the Modulation Index (Section 3.2.5). The resulting set of fourteen LFP-derived metrics is then condensed into a single multivariate index of network maturation through principal component analysis, designated as LFP-NAS (Section 3.2.6), and the six metrics contributing most strongly to the maturation axis are examined individually through a confirmatory univariate follow-up (Section 3.2.7). All statistical procedures, including outlier removal, group-level comparisons, and longitudinal trend analysis, are described separately in 3.2.8. Software dependencies and code availability are described in 3.2.9.

3.2.1 Spike Detection and Network Activity

Metrics

Spike detection was performed using the previously described MEA analysis pipeline of the Tampere NeuroGroup (Hyvärinen et al., 2019), which combines two independent detection methods to reduce false positives. A first detection was performed with a simple amplitude-based detector (ABS), which reported a spike when the amplitude of the filtered signal exceeded $4.5 \times$ the estimated standard deviation of the noise (Quiñero et al., 2004). Subsequently, a detector based on the stationary wavelet transform–Teager energy operator algorithm (SWTTEO; Lieb et al., 2017; Mayer et al., 2018) was applied; one spike was confirmed only if detected by both detectors. Based on spike detection, an electrode

was defined as active if its MFR (calculated over the full recording session) was equal to or greater than 0.1 Hz. Single-channel burst detection was performed starting from the spikes thus identified, using an algorithm adapted from LogISI (Pasquale et al., 2010): the minimum number of spikes to define a burst was 5, and both the ISI cutoff and the maxISI were set at 100 ms. All spike and burst metrics were finally calculated with the R package *meaRtools* (Gelfman et al., 2018) and averaged for well.

Network-burst detection was performed using a MATLAB pipeline developed at NeuroGroup, Tampere University. This detection was based on the ISI-N logarithmic histogram introduced by Pasquale et al. (2010). In particular, a cumulative spike train was created by pooling the spikes in all electrodes with a firing rate greater than 10 spikes/min and the distribution of ISIs in $N=20$ consecutive spikes was evaluated. In case of network bursting activity, the ISI histogram has a double peak, one indicating bursting periods and one indicating silent ones, and the minimum between the two peaks defines the threshold for detecting an NB. Once the algorithm has identified potential NBs, it may happen that two consecutive NBs are separated by a very short time interval; a merging process was used to distinguish whether the two NB candidates are a single event or not. In particular, the merging of the two candidates into a single NB occurred if the IBI was less than the duration of the longest candidate multiplied by a merging fraction = 0.5. A significant participation of at least three electrodes is further required to detect an NB: an electrode was considered a significant contributor when its contribution reached at least 30% (contribution fraction = 0.3) of the average contribution per channel within the event. The parameters described in the NB detection algorithm coincide with those presented by Mzezewa in 2025. (Mzezewa et al., 2025) The following network burst metrics were extracted for each well: the network burst rate (NBR, bursts per minute) and the network burst duration (NBD, seconds). Because the network burst duration is an unstable estimator when computed from a small number of events, each well was required to display at least three detected network bursts over the recording to contribute to the NBD analysis at a given DIV. For the same reason, NBD was summarised across wells by the median rather than the mean. This approach is justified by the fact that the burst-duration distribution is right-skewed, and each per-well estimate rests on a small and variable number of events; therefore, the median is markedly more robust to occasional long-duration bursts and to the residual instability of estimates from sparsely bursting wells. The rate-based metrics

(MFR, BR, NBR), computed over the full recording duration and consequently better behaved, were summarised by the mean.

3.2.2 Preprocessing and LFP Extraction

Raw extracellular voltage signals were pre-processed in MATLAB (R2025b) to isolate the LFP component before spectral analysis. LFP signals were extracted by applying two cascaded zero-phase Butterworth filters sequentially using the `filtfilt` function, which eliminates phase distortion by performing forward and reverse filtering passes (Gustafsson, 1996). A second-order high-pass filter with a cutoff frequency of 0.5 Hz was first applied to remove the DC offset and slow down recording drift. Then a third-order low-pass filter with a cutoff frequency of 200 Hz was used to attenuate high-frequency spiking activity and broadband noise. A 0.5 Hz high-pass cutoff is compatible with preserving slow LFP components, including the delta range, while attenuating very slow drift. This filter choice is broadly consistent with the low-pass nature and slow dynamics of LFP signals described in the literature (Buzsáki et al., 2012). No power-line notch filter was applied, as spectral inspection confirmed negligible 50 Hz contamination in the recordings.

The filter was applied in a forward-reverse manner to obtain a zero-phase response, thereby avoiding phase distortion in the signal. The choice of a cascaded two-stage design also tackles the numerical instability that can affect direct-form bandpass filters at high sampling rates. This instability arises from the wide separation between the lower and upper cutoff frequencies (0.5 Hz and 200 Hz spanning over two decades), which can lead to ill-conditioned filter coefficients (Widmann et al., 2015).

LFP traces were extracted for each electrode in every well and organised into a matrix of dimensions $N \times E$. Here N is the total number of samples and E is the number of electrodes per well. All subsequent spectral analyses focused on electrodes classified as electrophysiologically active based on spiking activity (see Section 3.2.1).

3.2.3 LFP Spectral Analysis

PSD estimation was performed on the pre-processed LFP signals of all active electrodes using Welch's method (Welch, 1967), implemented via the `pwelch` function in MATLAB.

Welch's method improves upon the classical periodogram by dividing the signal into overlapping segments. It then computes a modified periodogram for each segment and averages the results.

Each LFP trace was segmented using a Hann window of 2 seconds duration (25,000 samples at a sampling frequency of 12,500 Hz) with 50% overlap between consecutive segments. The Hann window was chosen for its lower sidelobes compared to a rectangular window, which helps minimise spectral leakage from strong low-frequency components into adjacent frequency bins (Harris, 1978). Consecutive Hann-windowed segments were used with 50% overlap to ensure uniform signal coverage and enhance spectral estimation by reducing leakage from these low-frequency components. Each segment's discrete Fourier transform was computed using a zero-padded FFT of 32,768 points (2^{15}), exceeding the window length and providing interpolation in the frequency domain. This results in a smoother spectral representation. The true spectral resolution, however, remains determined by the window duration at $1/T = 0.5$ Hz, independently of the degree of zero-padding. PSD values were expressed in units of $\mu\text{V}^2/\text{Hz}$.

Figure 4.2A presents a qualitative time-domain comparison of the two systems using representative single-electrode recordings displayed in four complementary formats. The first column displays the unfiltered extracellular signal for both datasets, while the second column shows the same signal after applying a third-order zero-phase Butterworth low-pass filter via the `filtfilt` function. This filter has a cutoff of 250 Hz, slightly above the 200 Hz cutoff used for quantitative spectral analysis. This choice ensures the upper transition band is retained for visualisation. The third column further emphasises the slow envelope of the LFP waveform by applying additional Gaussian-weighted moving-average smoothing (a window length of 500 samples equivalent to 40 ms at a 12.5 kHz sampling frequency). The fourth column shows the time-frequency representation of the low-pass LFP, computed by the short-time Fourier transform (MATLAB spectrogram function) using a Hamming window of 2,048 samples (approximately 164 ms) with 50% overlap (1,024 samples) and a 2,048-point FFT. Spectrogram power was expressed in decibels as $10 \cdot \log_{10}$ of the squared magnitude, with the colour scale bounded by the 5th and 95th percentiles of the displayed power distribution. These time-domain representations are illustrative and were not used for any quantitative metric.

PSD estimates were computed independently for each active electrode within a well. The resulting per-electrode spectra were subsequently averaged arithmetically across all active electrodes, producing a single well-level mean PSD estimate per recording session. This well-level average constitutes the primary unit of analysis for all spectral comparisons across DIV timepoints, consistent with the multi-well experimental design in which each well represents one biological replicate.

For the group-level spectral comparison between the two recording systems (Section 4.2, Figure 4.2B), a further criterion was applied at the well level. Only wells containing at least two active electrodes were retained. This ensured that each well-level mean PSD relied on a minimum of two independent electrode estimates rather than relying on a single channel. The criterion was applied identically to both systems, and wells failing it at a given timepoint were excluded from the corresponding group-level PSD. The per-timepoint sample sizes reported in Section 4.2 reflect the number of wells satisfying it. The retained well-level PSDs were summarised across wells, separately for each system and timepoint, as the across-well arithmetic mean with a 95% confidence interval computed as $\text{mean} \pm 1.96 \times \text{SEM}$, where SEM denotes the standard error of the mean, under a normal approximation. The mean and standard error were evaluated on the linear power spectral density in $\mu\text{V}^2/\text{Hz}$. For display on a logarithmic ordinate, the resulting mean and confidence bounds were expressed in decibels as $10 \cdot \log_{10}$ of the power spectral density.

Absolute band power was extracted from the well-level mean PSD for each of five canonical frequency bands by numerical integration of the PSD using the trapezoidal rule over the following ranges: delta (δ , 1–4 Hz), theta (θ , 4–8 Hz), alpha (α , 8–12 Hz), beta (β , 12–30 Hz), and gamma (γ , 30–100 Hz). The gamma band was limited to 100 Hz, and a separate high/fast gamma band (>100 Hz) was not defined. LFP power above 100 Hz is dominated by spectral dispersion of spiking activity rather than true subthreshold oscillations (Manning et al., 2009; Ray & Maunsell, 2011) and therefore, extending the analysis to this range would reintroduce multiunit spiking energy into the LFP metrics and blur the separation between the MUA and LFP components on which this work relies. Furthermore, the >100 Hz range overlaps with the 100 and 150 Hz harmonics of 50 Hz power line interference, further limiting its reliability. It is important to note that band boundaries are not absolute physical quantities, but rather slightly different conventions used across different studies. Hence, all that matters is that the bands are used consistently

throughout the analysis and that the band powers are computed in the frequency bins of interest for each electrode. This way, any longitudinal changes in the derived metrics reflect a redistribution of spectral powers across the analysed ranges. Because adjacent bands share boundary frequencies, the trapezoidal integration assigns half-weight to boundary samples on each side, ensuring that the five bands partition the 1–100 Hz range without double-counting.

Relative band power for each band was defined as its absolute power divided by the sum of absolute powers across all five bands. This construction ensures that relative powers sum to unity. By restricting the total power reference to the 1–100 Hz range rather than integrating the full spectrum, sub-delta drift below 1 Hz and high-frequency content above 100 Hz are excluded from the denominator. This ensures that relative powers accurately reflect only the contribution of physiologically meaningful oscillatory components.

Two inter-band power ratios were additionally computed from the relative power estimates as candidate indices of neuronal network maturation: the delta-to-gamma ratio (δ/γ) and the delta-to-beta ratio (δ/β). They are defined as the ratio between the absolute power in one band and the absolute power in another band and were motivated by the maturational redistribution of EEG spectral power observed in preterm infants, in which power shifts from lower to higher frequencies over postmenstrual age (Niemarkt et al., 2011). All spectral metrics were computed per well at each longitudinal timepoint. Outlier detection and removal procedures, as well as group-level and longitudinal statistical comparisons, are described in Section 3.2.8.

3.2.4 Aperiodic Component Decomposition (specparam / FOOOF)

The aperiodic component of the LFP power spectrum was parameterised using the specparam algorithm (Donoghue et al., 2020), implemented in Python via the SpectralGroupModel class from the specparam library (v2.0.0rc6), which fits the model to a group of spectra in batch mode. Before fitting, PSD estimates were recomputed from the pre-processed LFP signals using Welch's method with a 4-second Hann window and 50% overlap. This window length was chosen to be longer than the 2-second window adopted for band power estimation, yielding a finer frequency resolution of 0.25 Hz that improves

the accuracy of aperiodic slope fitting over the 2–40 Hz range. The aperiodic fitting was performed in fixed mode. This assumes a purely linear spectral slope in log-log space without a low-frequency knee (see Section 2.4.4, equation 2.4). This is suitable given the absence of a discernible low-frequency knee within the fitting range of these recordings. In addition, the aperiodic component is well approximated by a single linear log-log relationship as shown in Donoghue et al. (2020).

The fitting range was restricted to 2–40 Hz based on two considerations. The lower bound of 2 Hz avoids the influence of the high-pass filter roll-off at frequencies near 0.5–1 Hz, where the aperiodic fit would otherwise be biased by attenuation artefacts not related to the underlying physiological signal. The upper bound of 40 Hz balances the inclusion of low-gamma oscillatory content with the exclusion of higher gamma and high-frequency broadband activity, where putative narrowband peaks may interfere with the linear aperiodic fit and where signal-to-noise ratio declines progressively.

Additional model hyperparameters were configured following Donoghue et al.’s (2020) recommendations with minor adjustments personalised to the spectral characteristics of the dataset of *in-vitro* recordings. Peak width limits were set between 0.5 and 12 Hz to constrain peaks within physiologically plausible bandwidths and exclude both spurious narrow peaks caused by residual line noise and wide peaks indistinguishable from the aperiodic background. The maximum number of peaks was set to four, and the minimum peak height was set to 0.05 log units above the aperiodic fit to suppress fluctuations within the noise envelope of the spectral estimate.

The quality of model fits was assessed per well using the coefficient of determination R^2 . This was calculated between the original log-transformed PSD and the full model fit over the fitting range. Wells with an R^2 of 0.9 or higher were deemed to have acceptable fit quality. This threshold was then used to determine the proportion of well-level fits meeting quality criteria across different DIV timepoints. The aperiodic exponent and offset were extracted for each well and DIV and subjected to IQR-based outlier removal ($k = 1.5$) applied independently per DIV and per parameter before statistical analysis. Statistical comparisons of aperiodic parameters across timepoints followed the same procedure described in Section 3.2.8.

For group-level visualisation (Figure 4.5A), well-level log-transformed PSDs were averaged across all wells with a valid aperiodic fit at each DIV; a 1–80 Hz display range was used to show the spectral context beyond the 2–40 Hz fitting range. The group-level aperiodic fit was obtained by averaging the per-well aperiodic components (in $\log_{10}$ power) over the fitting range. Under the fixed mode adopted here, this averaging is equivalent to the line defined by the mean exponent and mean offset. For the exponent and offset trajectories (Figure 4.5B, C), SEM was preferred over a bootstrap confidence interval because the spaghetti-plus-mean format already displays the full within-DIV distribution, so a parametric one-standard-error band is a more concise summary of central-tendency uncertainty.

3.2.5 Phase-Amplitude Coupling (PAC)

Phase-amplitude coupling was quantified using the MI method (Tort et al., 2010), applied to the pre-processed LFP signals of all active electrodes. The instantaneous phase of the low-frequency signal and the amplitude envelope of the high-frequency signal were extracted via the Hilbert transform applied to the respective LFP traces. After comodulogram inspection, the delta band (1–4 Hz) was selected as the phase-providing frequency. This choice is consistent with the dominant low-frequency oscillatory component observed in immature hiPSC-derived cortical cultures and with the peak coupling frequencies (1–2 Hz) reported in analogous *in-vitro* recordings by Salimpour et al. (2024). The amplitude-providing band was defined as the broadband high-frequency range (20–200 Hz). The low-frequency component was isolated from the pre-processed LFP (see Section 3.2.2) using a second-order zero-phase Butterworth bandpass filter and the high-frequency component using a third-order zero-phase Butterworth bandpass filter, both applied via forward-reverse filtering. The selection of the 20–200 Hz amplitude band is consistent with the bandwidth criterion discussed by Aru et al. (2015), according to which the amplitude filter should be wide enough to capture the sidebands generated by modulation from the phase frequency. With a maximum phase frequency of 4 Hz, the minimum amplitude bandwidth needed is 8 Hz. This is comfortably exceeded by the 180 Hz bandwidth used here. The phase time series was discretised into 18 bins of 20° each covering the full range from $-\pi$ to π . Within each bin, the mean amplitude envelope was calculated. The choice of 18 phase bins follows the original specification of Tort et al.

(2010) and represents a balance between phase resolution and bin occupancy because a broader binning would obscure the shape of the phase-amplitude distribution, while a finer binning would reduce the number of amplitude samples per bin and inflate the variance of the empirical distribution. The MI was computed as the Kullback-Leibler divergence between the resulting amplitude-phase distribution and the uniform distribution, normalised by $\log(N)$, where $N = 18$.

MI was computed at the electrode level for each active electrode within each well. Well-level MI was obtained by averaging per-electrode MI values arithmetically across all active electrodes. Statistical significance at each electrode was assessed against a surrogate null distribution of 100 MI values. Surrogates were generated by applying independent circular time shifts to the amplitude envelope prior to phase-binning, with each shift drawn randomly from the interval $[1 \text{ s}, T - 1 \text{ s}]$, where T is the total signal duration. The surrogate distribution provides a null reference for the MI value expected in the absence of genuine phase-amplitude coupling by applying circular time shifts to the amplitude envelope while leaving the phase time series intact. In this way, the temporal relationship between phase and amplitude is destroyed while their individual spectral properties and autocorrelation structures are preserved.

An electrode was classified as exhibiting significant PAC if its observed MI exceeded the 95th percentile of the surrogate distribution. The fraction of electrodes with significant PAC per well was computed as the number of significant electrodes divided by a fixed denominator of 16, corresponding to the total number of recording electrodes per well on the Axion CytoView MEA 48-well plate. This convention, rather than normalisation by the number of active electrodes, was adopted to ensure that across-well comparisons of the fraction of electrodes with significant PAC per well reflect a uniform baseline regardless of the variability in active electrode counts between wells.

A group-level comodulogram was computed at the early (DIV47) and late (DIV59) timepoints by evaluating the MI across a two-dimensional grid of phase frequencies (1–15 Hz in 1 Hz steps) and amplitude frequencies (20–200 Hz in 10 Hz steps), with half-bandwidths of ± 2 Hz and ± 10 Hz, respectively. MI values were averaged across all active electrodes pooled over wells at each timepoint to produce a mean comodulogram for each DIV. All PAC metrics were subjected to the same IQR-based outlier removal and statistical comparisons described in Section 3.2.8.

For the single-electrode illustration in Figure 4.6, a comodulogram was also calculated for the representative electrode on a finer grid than the group-level one. This grid spanned phase frequencies from 1 to 10 Hz in 0.5 Hz increments (half-bandwidth ± 1 Hz) and amplitude frequencies from 20 to 200 Hz in 5 Hz increments (half-bandwidth ± 10 Hz). In the same figure, the temporal zoom of the highlighted burst (Figure 4.6B and C) is centred on the instant of the highest positive peak of the broadband high-frequency filtered trace within the displayed window. This peak is identified using local-maximum detection. The low-frequency and high-frequency traces overlaid on the broadband signal in Figure 4.6A and B are amplitude-scaled for visualisation only and do not affect the values used in the MI computation.

3.2.6 Multivariate LFP Maturation Index (LFP-NAS)

A multivariate maturation index analogous to the NAS framework introduced by Passaro et al. (2021) was derived from the full set of LFP-based metrics via PCA. The metric pool comprised 14 features: five absolute band powers, five relative band powers, two inter-band power ratios (δ/β , δ/γ), mean well-level MI, and the fraction of electrodes with significant PAC. All metrics were computed at the well level as described in Sections 3.2.3 and 3.2.5.

As done for the original NAS (see Section 2.4.2), before PCA, each metric was standardised independently across all wells and timepoints by subtracting the mean and dividing by the standard deviation, yielding a z-scored matrix of dimensions $n \times 14$, where n denotes the number of wells with complete observations across all metrics. Wells with missing values in any of the 14 metrics were excluded from the PCA to ensure that the decomposition was performed on a fully observed data matrix. PCA was applied to the z-scored matrix with MATLAB's `pca` function (which calculates singular value decomposition of its input), and the sign of PC1 is flipped if the Spearman rank correlation between PC1 scores and DIV is negative to make sure PC1 increases with DIV. The resulting PC1 score was designated as the LFP-NAS.

Validation of the LFP-NAS was performed by assessing the association between PC1 scores and DIV across all wells. The Spearman rank correlation (ρ) was preferred over

Pearson correlation because PC1 scores are not normally distributed and ρ is more robust to outliers and small sample sizes. The coefficient of determination (R^2) was reported alongside ρ as a complementary descriptive measure of the linear component of the association. PC1 loadings were inspected to characterise the relative contribution of each metric to the maturation axis and to identify the dominant spectral and PAC features driving longitudinal change. The proportion of variance explained by PC1 was reported relative to the total variance. Considering the exploratory nature of the analysis and the sample size limitations of the design, the PCA was assessed against the widely accepted heuristic of at least five observations per variable ($n \geq 5p$, Hair et al., 2010).

3.2.7 Univariate Follow-up on Top PC1 Drivers

Once LFP-NAS had been defined, a univariate follow-up of its main constituents was carried out for the purpose of: i) verifying if the longitudinal trend reflected by the multivariate index can be extracted also using the individual metrics alone; ii) as a way to validate the results obtained with the spectral and PAC methods, using a totally independent methodology. As described in Section 3.2.6, PC1 was sign-aligned with DIV, so that metrics with a positive loading are those that increase along the maturation axis and metrics with a negative loading are those that decrease. The three metrics with the highest (most positive) and the three with the lowest (most negative) PC1 loadings were therefore examined individually, yielding six drivers, three of each sign. Because metric selection was data-driven from the same dataset used for the PCA, this analysis is explicitly exploratory and confirmatory in nature rather than an independent statistical test. The resulting p-values quantify the strength of longitudinal trends in the chosen metrics but are unrelated to the multivariate maturation hypothesis already tested in Section 3.2.6.

For each of the six selected metrics, the Spearman rank correlation coefficient (ρ) between the metric values and DIV was computed across all wells with valid observations, providing a non-parametric measure of the monotonic association between the metric and time in culture. The linear coefficient of determination (R^2) was additionally computed via ordinary least squares regression of the metric on DIV, quantifying the proportion of variance explained by a linear longitudinal trend. To account for the multiplicity of tests across the three metrics, p-values were corrected using the Benjamini-Hochberg false discovery rate (FDR) procedure (Benjamini & Hochberg, 1995), with corrected p-values

denoted as q-values. All computations were performed in MATLAB using custom functions.

3.2.8 Statistical Analysis

All statistical analyses were performed in MATLAB (R2025b) unless otherwise specified. A significance threshold of $\alpha = 0.05$ was applied throughout.

Outlier removal. Before statistical testing, outlier detection was performed independently for each metric and each DIV timepoint using Tukey’s IQR fence method, with a fence multiplier of $k = 1.5$. This criterion corresponds to the standard inner-fence rule for identifying outliers and is a conservative, nonparametric approach suitable for modest sample sizes. For absolute band power, the IQR criterion was applied to $\log_{10}$ -transformed values rather than linear values, as LFP power estimates follow an approximately log-normal distribution and applying the IQR to linear power would bias the fence asymmetrically toward the upper tail. For relative band power and inter-band power ratios, the IQR criterion was applied to the untransformed linear values, which are bounded on $[0, 1]$ and exhibit less severe skewness. For the aperiodic exponent and offset, IQR removal was applied independently to each parameter on linear values, given that these quantities are already extracted from the log-transformed PSD and are approximately normally distributed. For PAC metrics, IQR removal was applied to linear MI values independently per DIV. For band power metrics, a unified exclusion mask was additionally constructed per band such that a well was excluded at a given DIV if identified as an outlier in either the absolute or the relative power distribution, ensuring consistency between the two representations of the same underlying signal.

Group-level comparisons. Differences across DIV timepoints were assessed using the Kruskal-Wallis test, a non-parametric rank-based alternative to one-way ANOVA, applied independently to each metric with DIV as the grouping variable. The Kruskal-Wallis test was preferred over parametric alternatives given the small and unequal sample sizes per group, the absence of normality guarantees for the well-level distributions, and the ordinal nature of the DIV grouping variable. When the Kruskal-Wallis test yielded a statistically significant result ($p < 0.05$), pairwise post-hoc comparisons between all DIV pairs were performed using the Dunn-Šídák test, which applies a Šídák correction to the Dunn rank-

sum statistic to control the family-wise error rate across multiple simultaneous comparisons. Post-hoc tests were implemented using MATLAB's `multcompare` function with the 'dunn-sidak' option.

Longitudinal trend analysis. To characterise the directional trajectory of each metric over the DIV range studied, a linear mixed-effects model (LMM) was fitted for each metric using MATLAB's `fitlme` function; the modelling framework follows Pinheiro & Bates (2000). The model was specified as $Y \sim \text{DIV} + (1 \mid \text{Well})$, where DIV was included as a continuous fixed effect to capture the linear change over time, and Well was modelled as a random intercept to account for repeated measurements from the same well across different DIV timepoints. For absolute band power metrics, Y was log10-transformed before fitting, in line with the skewed, approximately log-normal distribution of power values. Relative band power, inter-band ratios, and PAC metrics were analysed on their original scale. For each model, the DIV slope and its p-value were extracted as the main measures of longitudinal change.

Between-system comparisons. Differences in spike-based metrics between the TUBA-new and TUBA-old datasets (Section 4.1, Figure 4.1, Table 4.1A) were assessed at each DIV using the two-sided Mann–Whitney U test (Mann & Whitney, 1947), implemented in MATLAB via the `ranksum` function. The non-parametric test was chosen for the same reasons as the within-system Kruskal–Wallis analysis (small, unequal samples and non-normal, right-skewed distributions). Comparisons with fewer than three valid wells in either group were not performed. To control the family-wise error rate across the four DIV timepoints, p-values were corrected with the Šidák procedure (Šidák, 1967).

Confidence intervals via bootstrap. The across-well summary statistics shown in the figures of Sections 4.1, 4.3, and 4.4 are reported with 95% non-parametric bootstrap confidence intervals (Efron & Tibshirani, 1993), computed from 2000 resamples drawn with replacement from the well-level distribution. The bootstrap was preferred over parametric alternatives because the well-level distributions of LFP metrics cannot be assumed to be normally distributed, particularly at early DIV timepoints where sample sizes are small and between-well variability is high. The bias-corrected and accelerated (BCa) interval (Efron, 1987) was used when at least ten observations were available. For smaller samples down to three observations, the simpler percentile interval was used instead, as the BCa correction becomes unreliable with very few data points. Wells with

fewer than three observations were reported without a confidence interval. All intervals were computed in MATLAB using the `bootci` function with a fixed random seed for reproducibility.

3.2.9 Software and Code Availability

All signal-processing, spectral analysis, statistical, and visualisation routines were implemented in MATLAB (R2025b; MathWorks Inc., Natick MA), making use of the Signal Processing Toolbox (`filtfilt`, `butter`, `hilbert`, `pwelch`), the Statistics and Machine Learning Toolbox (`kruskalwallis`, `multcompare`, `fitlme`, `pca`, `corr`), and custom-developed analysis functions. The parametric decomposition of power spectra into aperiodic and periodic components was performed in Python (v3.11) using the `specparam` library v2.0.0rc6 (<https://github.com/fooof-tools/fooof>). PAC analysis was implemented entirely in MATLAB using custom functions based on the algorithmic specification of Tort et al. (2010).

Chapter 4

Results

4.1 Spike-Based Characterisation

The present chapter opens with a spike-based characterisation of network development in both available datasets (TUBA-new and TUBA-old; see Section 3.1.3), serving two complementary purposes. First, the analysis aims to confirm that the hiPSC-derived cortical cultures examined in this study display the canonical hallmarks of progressive spontaneous network activity over the DIV range examined, providing the biological baseline against which the LFP-based analyses presented in the rest of the chapter are interpreted. Second, by establishing that spike-detected activity is captured by both acquisition systems regardless of their differing LFP suitability (which is examined in Section 4.2), it justifies the retention of both datasets for spike-based metrics throughout the chapter. Four canonical spike-based network indices were extracted per well at each DIV using the metric definitions and detection parameters specified in Methods 3.2.1: the mean firing rate (MFR), the single-electrode burst rate (BR), the network burst rate (NBR), and the median network burst duration (NBD). At each DIV, the two systems were compared using the Mann-Whitney U test with Šídák correction across the four timepoints. The longitudinal trajectory of each metric within each system was characterised using a linear mixed-effects model $\text{metric} \sim \text{DIV} + (1|\text{Well})$ (see Methods 3.2.8). Results are reported in Figure 4.1 and Table 4.1A and 4.1B.

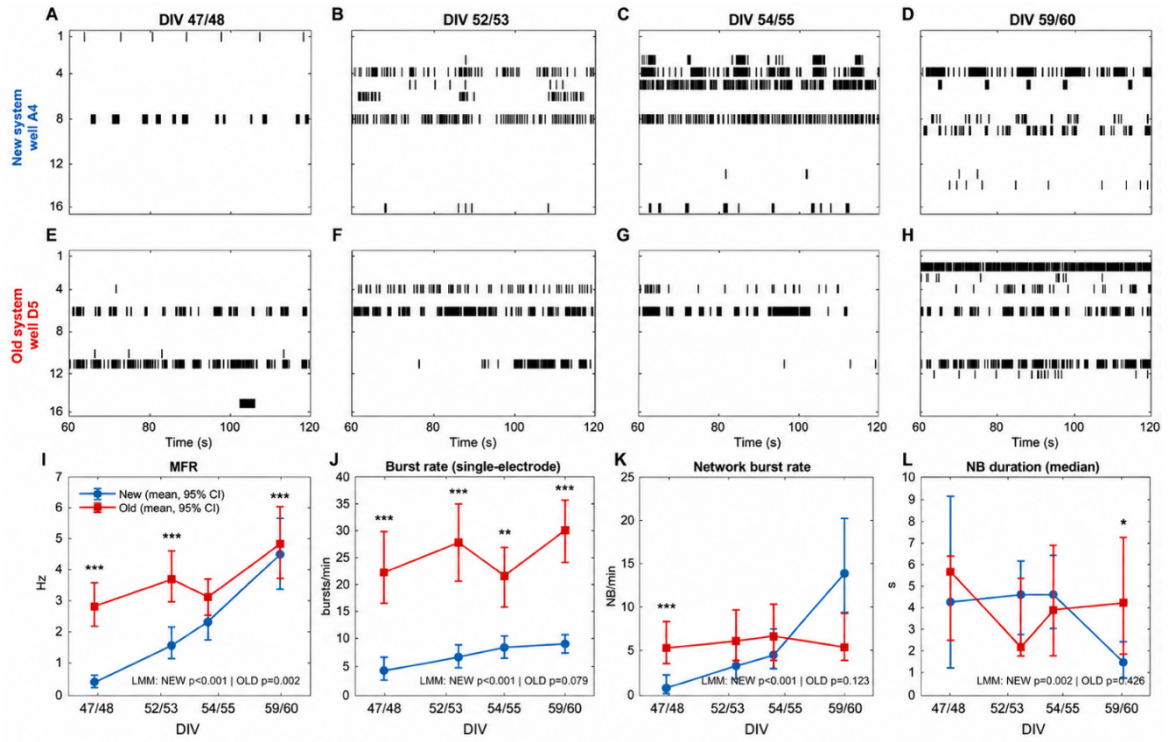


Figure 4.1. Spike-based characterisation of spontaneous network development in TUBA cultures recorded on the new and old Axion Maestro systems. (A–D) Single-well raster plots from one representative well of the new system (well A4), shown over a 60-second window (60–120 s) at DIV 47, 52, 54, and 59. Each row corresponds to one of the 16 electrodes of the well; black ticks indicate detected spikes. **(E–H)** Analogous raster plots from one representative well of the old system (well D5), shown at the matched timepoints DIV 48, 53, 55, and 60. **(I)** Mean firing rate (MFR, Hz) across wells for the new (blue) and old (red) systems at each DIV. **(J)** Single-electrode burst rate (BR, bursts/min). **(K)** Network burst rate (NBR, NB/min). **(L)** Median network burst duration (NBD, s). In panels I to K, markers represent the across-well mean and error bars the bootstrap 95% confidence interval (BCa method for $n \geq 10$, percentile method otherwise; see Methods 3.2.8); in panel L, markers represent the median and error bars the corresponding bootstrap 95% confidence interval. Between-system Mann-Whitney significance after Šídák correction is indicated above each DIV pair (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The LMM p-values for the longitudinal trend within each system are reported in the bottom-right of each panel. For panel L, only wells with at least 3 detected network bursts contributed to the NBD estimate (see Methods 3.2.1). Complete statistical reporting (means, CIs, Mann-Whitney p-values, and LMM parameters) is provided in Table 4.1A and 4.1B.

Table 4.1A. Between-system comparison per DIV for spike-based network metrics (Mann-Whitney U test with Šídák correction).

Metric	DIV	New: n, mean (95% CI)	Old: n, mean (95% CI)	p (Šídák)
MFR (Hz)	47/48	24, 0.37 (0.25, 0.58)	19, 2.81 (2.17, 3.51)	4.8×10^{-7} ***
	52/53	24, 1.58 (1.16, 2.18)	17, 3.66 (2.96, 4.56)	8.9×10^{-4} ***
	54/55	24, 2.34 (1.73, 3.17)	17, 3.13 (2.56, 3.65)	0.111
	59/60	24, 4.50 (3.38, 5.71)	19, 4.84 (3.81, 6.40)	0.912
BR (bursts/min)	47/48	20, 3.89 (2.40, 6.45)	19, 21.75 (16.16, 29.41)	2.7×10^{-4} ***
	52/53	24, 6.36 (4.69, 8.39)	17, 27.53 (19.91, 35.79)	3.8×10^{-4} ***
	54/55	24, 8.10 (6.18, 9.89)	17, 21.31 (15.40, 26.19)	0.002 **
	59/60	24, 8.86 (7.34, 10.23)	19, 30.26 (24.44, 36.28)	2.9×10^{-6} ***
NBR (NB/min)	47/48	24, 0.60 (0.15, 2.12)	11, 4.93 (3.37, 7.80)	1.7×10^{-5} ***
	52/53	24, 3.30 (1.57, 5.91)	11, 5.79 (3.59, 9.35)	0.121
	54/55	24, 4.56 (2.66, 7.19)	11, 6.44 (4.01, 9.95)	0.534
	59/60	24, 14.01 (9.66, 20.05)	11, 5.11 (3.71, 8.98)	0.360
NBD (s)	47/48	4, 4.17 (1.15, 9.17)	11, 5.59 (2.37, 6.27)	1.000
	52/53	8, 4.51 (2.68, 6.07)	11, 2.11 (1.70, 5.29)	0.542

Metric	DIV	New: n, mean (95% CI)	Old: n, mean (95% CI)	p (Šídák)
	54/55	12, 4.49 (2.93, 6.32)	11, 3.79 (1.65, 6.80)	0.999
	59/60	19, 1.39 (0.64, 2.32)	11, 4.09 (1.76, 7.27)	0.027 *

Notes. Per-DIV summary values for the TUBA-new and TUBA-old datasets are reported as n, mean (95% bootstrap CI) for MFR, BR, and NBR, and as n, median (95% bootstrap CI) for NBD. Bootstrap confidence intervals were computed using the BCa method when $n \geq 10$ and the percentile method otherwise (Methods 3.2.8). Between-system p-values were obtained from the Mann-Whitney U test and corrected for multiple comparisons across the four DIV timepoints using the Šídák correction. † NBD is summarised by the across-well median, and only wells with at least 3 detected network bursts contributed to the NBD estimate (Methods 3.2.1). Significance codes: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 4.1B. Longitudinal linear mixed-effects model (LMM) analysis per system for spike-based network metrics. Model: metric ~ DIV + (1|Well).

Metric	System	n_obs	n_wells	Slope (per DIV)	SE	p
MFR (Hz)	New	96	24	+0.345	0.032	5.3×10^{-18} ***
MFR (Hz)	Old	72	20	+0.159	0.050	0.002 **
BR (bursts/min)	New	92	24	+0.442	0.077	1.4×10^{-7} ***
BR (bursts/min)	Old	72	20	+0.622	0.349	0.079
NBR (NB/min)	New	96	24	+1.104	0.182	2.7×10^{-8} ***
NBR (NB/min)	Old	44	14	+0.045	0.029	0.123
NBD (s)	New	43	22	-0.292	0.090	0.002 **
NBD (s)	Old	44	14	+0.078	0.097	0.426

Notes. The LMM was fitted independently for each system, with DIV as a continuous fixed effect and a random intercept per well to account for the repeated-measures structure of the data (Methods 3.2.8). The reported slope is expressed in the native units of each metric per unit DIV and SE is the standard error of the estimated slope. NBD is summarised by the across-well median. Significance codes: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Qualitative inspection of the raster plots (Figure 4.1A–H) shows that both systems captured progressively richer spontaneous activity patterns across the DIV range studied. For the TUBA-new dataset (panels A–D, well A4), the earliest timepoint (DIV 47) displayed sparse activity restricted to one or two electrodes per well, progressively giving way to denser firing distributed across an increasing number of electrodes by DIV 59, with visually identifiable burst structures emerging from DIV 52 onwards. For the TUBA-old (panels E–H, well D5), single-electrode firing was already substantial at DIV 48 and remained dense throughout the four timepoints, with comparatively smaller visual changes over time.

Mean firing rate (Figure 4.1I) increased temporally in both systems, but with different starting baselines and trajectory magnitudes. For TUBA-new, MFR rise from approximately 0.37 Hz at DIV 47 to approximately 4.50 Hz at DIV 59, an order-of-magnitude increase that the LMM captured as a highly significant linear trend (slope = +0.35 Hz/DIV, $p = 5.3 \times 10^{-18}$). For TUBA-old, MFR also increased over the same interval, but from a higher initial baseline of approximately 2.81 Hz at DIV 48 to a comparable endpoint of approximately 4.84 Hz at DIV 60, with a more modest but still significant LMM trend (slope = +0.16 Hz/DIV, $p = 0.002$). The comparison between the TUBA-old and TUBA-new datasets revealed that the TUBA-old dataset had a higher MFR at the two earliest recording sessions. However, by the two latest timepoints, the two datasets converged to statistically indistinguishable values. The single-electrode burst rate (Figure 4.1J) followed a qualitatively similar pattern, with the TUBA-new displaying a highly significant LMM increase from approximately 3.89 to 8.86 bursts/min across the DIV range studied (slope = +0.44/DIV, $p = 1.4 \times 10^{-7}$), and the TUBA-old maintaining systematically higher values throughout the four timepoints (approximately 21.75 to 30.26 bursts/min) with only a marginal LMM trend (slope = +0.62/DIV, $p = 0.079$) and significant between-system differences at every DIV (all Šidák-corrected $p < 0.01$). These two metrics jointly indicate that both batches displayed progressive maturation of single-

electrode spontaneous activity. The TUBA-new cultures showed an increase in the single-electrode activity in the DIV window that was examined. On the other hand, the TUBA-old cultures had greater activity than TUBA-new at the very beginning, but also had sustained (and detectable) increases in activity over the DIVs. This indicates that while the TUBA-old were already well-developed, they were not quite at developmental plateau.

The clearest longitudinal effect was observed for the network burst rate (Figure 4.1K). In the TUBA-new dataset, NBR underwent a substantial increase from approximately 0.60 NB/min at DIV 47 to approximately 14.01 NB/min at DIV 59. The LMM's capture of this more than 20-fold gain is the strongest longitudinal effect in this chapter (slope = +1.10 NB/min per DIV, $p = 2.7 \times 10^{-8}$). The TUBA-old dataset, by contrast, displayed an essentially stationary NBR profile of approximately 4.93 to 6.44 NB/min across the four timepoints, with no significant LMM trend (slope = +0.05 NB/min per DIV, $p = 0.123$). The two datasets differed significantly only at the earliest timepoint, where the TUBA-old showed higher NBR than the TUBA-new (DIV 47/48: Šídák-corrected $p = 1.7 \times 10^{-5}$), and the trajectories crossed between the third and fourth recording sessions (DIV 54/55 and DIV 59/60). For NBR, the TUBA-new value reached about three times the TUBA-old value during the fourth recording session. However, the between-system contrast at DIV 59/60 didn't reach Šídák-corrected significance. Figure 4.1L illustrates a complementary pattern in the median NBD. Within the TUBA-new dataset, the NBD decreased significantly from around 4.17 seconds at DIV 47 to approximately 1.39 seconds at DIV 59. This trend, described by an LMM slope of -0.29 seconds per DIV, reflects the network's progressive maturation and the emergence of shorter and more focused bursts. The TUBA-old, however, didn't show a significant change in NBD across different divisions (slope = +0.08 s/DIV, $p = 0.426$). The between-dataset comparison reached significance only at the fourth recording session (DIV 59/60), where the TUBA-new NBD was significantly lower than the TUBA-old NBD (Šídák-corrected $p = 0.027$). The number of TUBA-new wells contributing valid NBD estimates increased markedly across DIVs ($n = 4$ at DIV 47; $n = 19$ at DIV 59), reflecting the parallel increase in NBR and the consequent fraction of wells satisfying the ≥ 3 NB inclusion criterion (Methods 3.2.1).

Collectively, these spike-based analyses support progressive spontaneous network maturation in these hiPSC-derived cortical cultures within the range of DIVs investigated. The TUBA-new dataset demonstrates all four canonical maturation hallmarks: significant

increases in mean firing rate (MFR), burst rate (BR) and network burst rate (NBR) and a significant decrease in network burst duration (NBD). The TUBA-old dataset, however, shows only a significant longitudinal effect for MFR, suggesting that cultures start with a higher baseline of network activity during the earliest recording session. Interpreting the spike-based metrics result, the two datasets differ in the timing and magnitude of these trajectories. Notably, the TUBA-new cultures begin with lower baseline spiking but achieve stronger network synchronisation and shorter, more focused bursts by the final recording session (DIV 59). The validity of both datasets for spike-based characterisation is therefore established, while the suitability of each system for LFP-based analyses requires separate verification, which is the subject of the following section.

4.2 LFP Acquisition System

Comparison

As detailed in Methods 3.1.3, spontaneous activity was recorded across two independent TUBA1B culture batches using two different Axion Maestro systems, TUBA-new and TUBA-old. While spike-based metrics (Section 4.1) were analysed from both datasets, the longitudinal LFP analyses presented in Chapter 4.3 rely on the TUBA-new dataset alone. This section explains the asymmetry by demonstrating that the LFP component extracted from TUBA-old recordings is incompatible with quantitative spectral analysis. This is despite the underlying spiking activity being intact. Representative recordings from a matched late timepoint were examined in both the time and frequency domains (Figure 4.2).

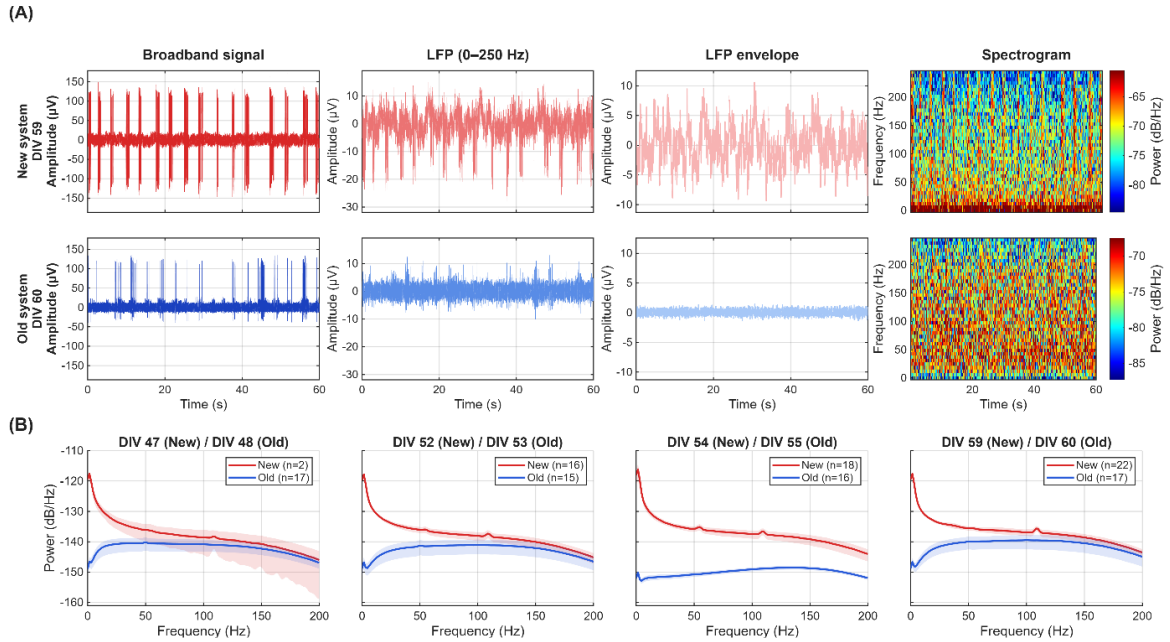


Figure 4.2. Comparison of LFP signal content between the new and old Axion Maestro recording systems. (A) Representative 60-second segments from single active electrodes recorded with the new (red, top row) and old (blue, bottom row) TUBA datasets at the latest available timepoint (DIV 59 for TUBA-new, DIV 60 for TUBA-old), shown across four signal representations: broadband signal, low-pass filtered LFP (0–250 Hz), Gaussian-smoothed LFP envelope, and time–frequency spectrogram. Colour scale in spectrograms represents power in dB. (B) Well-level mean power spectral density (PSD) estimated by Welch's method (Methods 3.2.3) for the TUBA-new (red) and TUBA-old (blue) at DIV 47, 52, 54, and 59 (TUBA-new) and at the corresponding timepoints DIV 48, 53, 55, and 60 (TUBA-old) (see Section 3.1.3 for Dataset description). Shaded bands represent 95% confidence intervals across wells; sample sizes reflect the number of wells meeting the minimum active-electrode criterion (≥ 2 active electrodes) at each timepoint. PSDs are expressed in dB/Hz ($10 \cdot \log_{10}(\mu V^2/Hz)$). DIV, days *in-vitro*; LFP, local field potential; PSD, power spectral density.

Figure 4.2A shows the time domain, where a similar amplitude of the broadband signal was observed from the two systems (approximately 100–150 V at peak deflection). Bursting and spiking were observed in both systems but qualitatively resembled each other rather than being exactly identical. This is consistent with the assumption that the two batches are independent biological replicates. After low-pass filtering at 250 Hz to isolate the LFP component, however, a marked divergence emerged. While slow fluctuations were still clearly observable in TUBA-new's LFP signal, the LFP trace of the old system was substantially attenuated across the same recording epoch. The divergence was most noticeable in the Gaussian-smoothed LFP, where the TUBA-new signal exhibited

significant low-frequency fluctuations while the TUBA-old envelope remained remarkably flat throughout the recording.

The time-frequency representation offered further support. The TUBA-new spectrogram clearly showed a dominant low-frequency band throughout the recording while the TUBA-old spectrogram appeared relatively uniform across the 0–250 Hz range. This qualitative finding was supported at the group level as shown in Figure 4.2B where the group mean PSDs for each system are plotted at the four DIV-matched time points. At every time point, the new system PSD displayed the canonical $1/f$ -like profile expected of extracellular field recordings (Buzsáki et al., 2012), with peak power on the order of -115 to -120 dB/Hz at low frequencies and a progressive decline toward higher frequencies. In contrast, the TUBA-old PSDs were nearly flat across the full 1–200 Hz range. The separation between the two systems was most pronounced at the lowest frequencies, with a power deficit on the order of 25–30 dB in the delta range, and the two profiles converged to comparable power levels only above approximately 150 Hz. This pattern was largely consistent across timepoints, apart from DIV 55, in which the old-system PSD showed a partially distinct profile. Nevertheless, the fundamental characteristics of the TUBA-old dataset remained unchanged across all timepoints. This dataset is characterised by the absence of low-frequency emphasis and the systematic power deficit in the low-frequency range relative to the TUBA-new dataset.

Taken together, these observations indicate a substantial low-frequency signal component present in the TUBA-new recordings that is absent from the TUBA-old recordings. While differences in spontaneous network activity between the two datasets cannot be entirely excluded, as suggested by the spike-based metrics reported in Section 4.1. Such differences alone cannot account for the pronounced frequency-dependent attenuation observed, because a genuine divergence in network dynamics would be expected to produce a broadband power difference rather than the selective low-frequency deficit seen here. Rather, the spectral signature is consistent with the hardware high-pass filter implemented in AxIS v2.5.2.1 before digitisation, which suppresses precisely the low-frequency range that supports the low-frequency band power, aperiodic, and phase-amplitude coupling analyses constituting the primary LFP-derived metrics of interest in this study (Methods 3.1.3). For all these reasons, the TUBA-old dataset cannot be considered for LFP-based studies, and therefore, TUBA-old dataset cannot be used anymore. Since the spike-based

metrics do not rely on the low-frequency filter applied to either dataset, they remain valid in both datasets and continue to be reported in parallel where relevant.

4.3 LFP Spectral Analysis

Having restricted LFP analyses to the TUBA-new dataset as outlined in the previous section, the longitudinal evolution of spectral content across four DIV timepoints (47, 52, 54 and 59) on the eight wells of row A was characterised. Absolute and relative band power were extracted from the well-level mean PSD for each of the five canonical frequency bands, and two inter-band power ratios (δ/β and δ/γ) were derived from the relative power estimates (Methods 3.2.3). For all measures, within-group change across DIV were analysed using a Kruskal-Wallis omnibus test with post-hoc Dunn-Šídák comparisons as well as a linear mixed-effects model to model the longitudinal change and incorporate the repeated-measures structure of the data (Methods 3.2.8). Results are presented in Figure 4.3 for the band powers, in Figure 4.4 for the inter-band ratios, and summarised in Table 4.2.

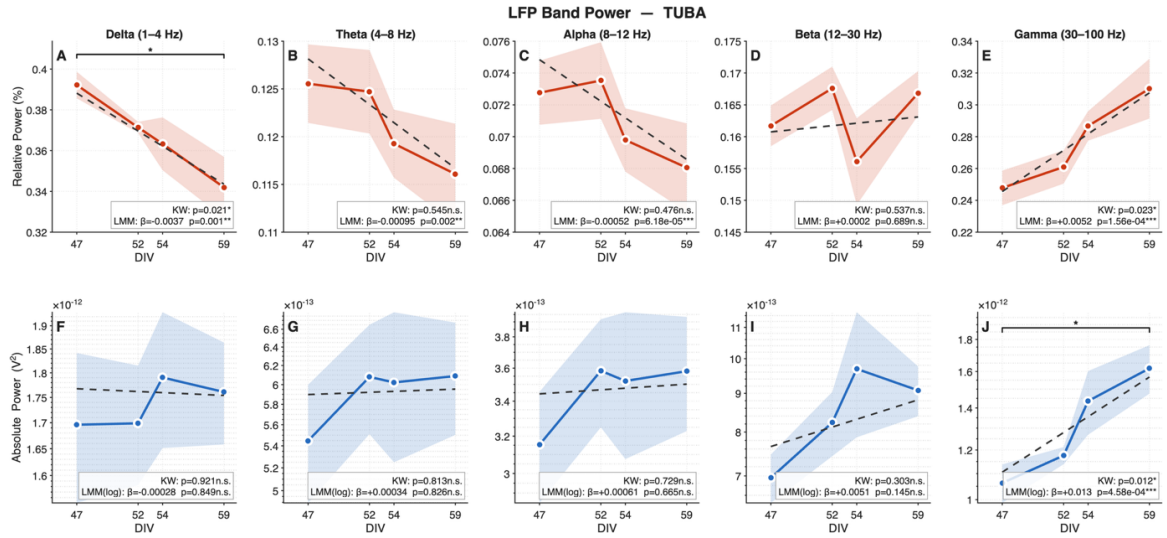


Figure 4.3. Longitudinal evolution of LFP band power in TUBA-new cultures across DIV 47–59. Top row (A–E): relative band power expressed as a fraction of the total 1–100 Hz power, for delta (1–4 Hz), theta (4–8 Hz), alpha (8–12 Hz), beta (12–30 Hz), and gamma (30–100 Hz) bands. Bottom row (F–J): absolute band power for the same five bands, plotted on a logarithmic ordinate. Solid markers represent the across-well mean and shaded bands the bootstrap 95% confidence interval (Methods 3.2.8) at each DIV; dashed grey lines indicate the LMM regression fit. Inset boxes report the Kruskal–Wallis omnibus p-value, the LMM slope (β/DIV), and the LMM p-value for each metric. For absolute power, the LMM was fitted on $\log_{10}$ -transformed values and is denoted as LMM(log). Significant pairwise Dunn–Šídák comparisons are indicated by brackets above the data. Significance codes: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n.s., not significant. IQR-based outlier removal ($k = 1.5$) was applied independently per band and per DIV before statistical testing (Methods 3.2.8). Complete statistical reporting is provided in Table 4.2.

The relative power distribution across the five canonical bands exhibited a coherent longitudinal redistribution from low to high frequencies (Figure 4.3A–E; Table 4.2). Delta power decreased monotonically from approximately 0.39 at DIV47 to 0.34 at DIV59, reaching significance under both the Kruskal–Wallis omnibus test and the LMM linear-trend test, with the DIV47–DIV59 pairwise contrast surviving Dunn–Šídák correction (Figure 4.3A). On the other hand, gamma power increased symmetrically from around 0.25 to approximately 0.31 over the same period. This trend was confirmed by convergent evidence from both the omnibus and linear-trend tests as shown in Figure 4.3E. The DIV47–DIV59 pairwise contrast for relative gamma did not reach significance under Dunn–Šídák correction ($p = 0.052$). The missing significance most likely stems from the small sample size ($n \leq 8$ wells per DIV) and the Šídák correction applied across all six pairwise comparisons. This limitation doesn't reflect a weakness in the underlying trend, which is clearly shown by both the omnibus and LMM tests. To summarise relative band-power analysis, from this data, delta and gamma are hence the top metrics capturing the spectral signature of a maturing network, and will be explored further with inter-band power ratios.

The intermediate bands provided supportive but weaker evidence of the same redistribution. Both theta and alpha showed a significant linear decrease under the LMM (Table 4.2) without reaching significance on the omnibus Kruskal–Wallis test (Figure 4.3B,C). This is consistent with a gradual, monotonic decrease in low-to-mid frequency content that becomes detectable once within-well correlations are accounted for. In contrast, beta power was largely stable across both tests with a non-monotonic trend and wide confidence intervals suggesting significant session-to-session variability (Figure 4.3D; Table 4.2). The relative-power profile thus describes a spectral shift in which the dominant change is the redistribution of power away from the delta band and into the gamma band, with the intermediate bands following the same low-to-high direction more subtly.

Absolute band powers were quite stable across DIVs for each band, except for the gamma band (Fig. 4.3F–J; Table 4.2). In the delta, theta, alpha, and beta frequency bands, absolute power was relatively consistent across time points and did not reach significance under either statistical framework. Only gamma absolute power showed a clear longitudinal increase. The pairwise difference between DIV47 and DIV59 reached significance under

Dunn–Šídák correction (Figure 4.3J). The apparent drop of delta in relative terms indicates a redistribution rather than an absolute loss of low-frequency power, but the convergence of relative and absolute increases in the gamma band indicates that gamma is the only spectral component exhibiting a strong longitudinal trend. This finding led to the addition of a gamma-based inter-band ratio to the traditional delta-to-beta ratio in the analysis that follows. The gamma band is expected to be the most sensitive spectral marker of network maturation in the present dataset.

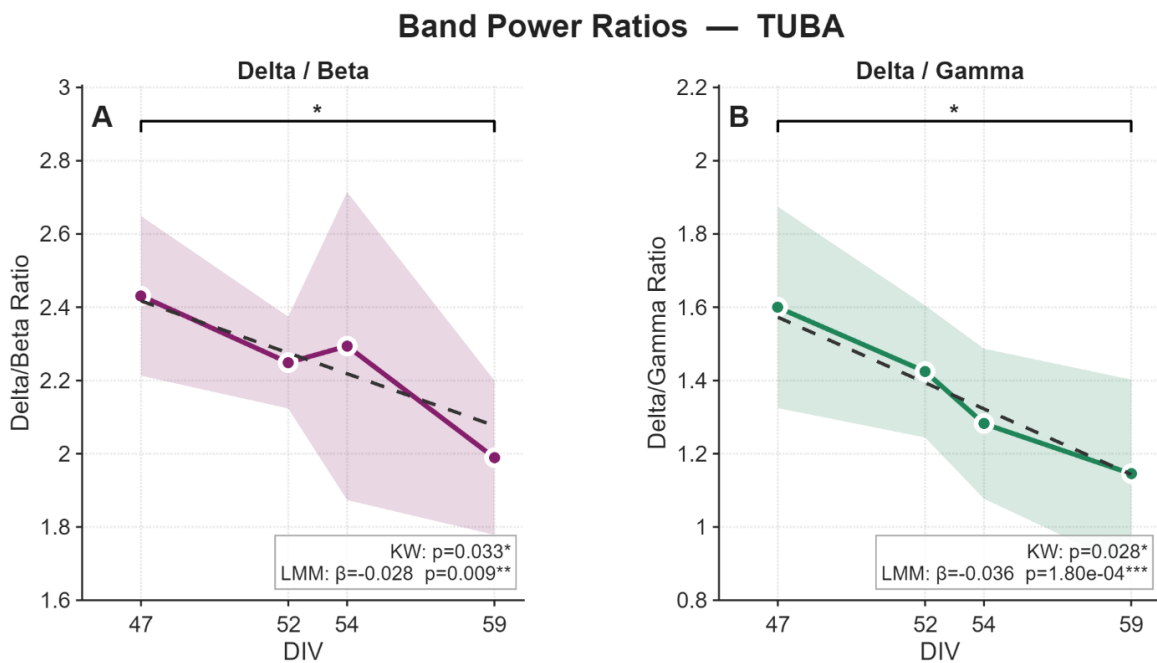


Figure 4.4. Longitudinal evolution of LFP band power ratios in TUBA-new cultures. (A) Delta-to-beta power ratio (δ/β). (B) Delta-to-gamma power ratio (δ/γ). Solid markers represent the across-well mean and shaded bands the bootstrap 95% confidence interval (BCa method for $n \geq 10$, percentile method otherwise; Methods 3.2.8) at each DIV; dashed grey lines indicate the LMM regression fit. Inset boxes report the Kruskal–Wallis p-value, LMM slope, and LMM p-value. Significant Dunn–Šídák pairwise comparisons are indicated by brackets. Significance codes as in Figure 4.3. IQR-based outlier removal was applied independently per ratio and per DIV (Methods 3.2.8). Complete statistical reporting is provided in Table 4.2.

The two inter-band power ratios, described as candidate maturation indices, both showed significant decline over the DIV range observed in this study (Figure 4.4; Table 4.2). The δ/β ratio decreased from around 2.43 at DIV47 to 2.07 at DIV59. This difference between DIV47 and DIV59 was statistically significant (Figure 4.4A). The δ/γ ratio displayed a more pronounced and more monotonic decline, from approximately 1.60 to 1.15 across the same interval, with a markedly stronger LMM trend (Table 4.2) and a smoother trajectory

than the δ/β ratio (Figure 4.4B). The greater regularity of the δ/γ trajectory is consistent with the greater stability of the gamma band noted above. In fact, beta was the only band to present non-monotonic variation with large uncertainty intervals, carrying this variation to the corresponding ratio. In contrast, gamma was nearly monotonously increasing on both the relative and absolute scales. Given that, gamma was the most consistent spectral marker of network development in the present dataset, a gamma-based ratio may offer a more sensitive readout of the underlying maturational trend. However, both ratios are retained as candidate indices in the analyses that follow. Taken jointly, the relative-power redistribution, the gamma-driven absolute power increase, and the two converging ratio trajectories provide three methodologically independent and biologically convergent indices of a low-to-high-frequency spectral shift over time in culture, consistent with the maturational redistribution previously reported in neonatal EEG (Niemarkt et al., 2011).

A possible explanation for this low-to-high-frequency shift is a gradual and simultaneous shift in the developing network's excitation-inhibition balance, which should modify the broadband spectral slope. The aperiodic exponent, proposed as a non-invasive proxy for excitation-inhibition balance (Gao et al., 2017), is therefore explored in the following section as a complementary measure of network maturation.

Table 4.2. Statistical summary of LFP band power and inter-band ratio analyses across DIV 47–59 in TUBA-new cultures (n = 8 wells of row A).

Metric	n	KW $\chi^2(3)$	KW p	LMM β/DIV	LMM p	Significant Šidák pairs	Dunn–
Relative δ	26	9.75	0.021*	-3.7×10^{-3}	0.001**	DIV47–DIV59 (p = 0.013)	(p = 0.013)
Relative θ	28	2.13	0.545	-9.5×10^{-4}	0.002**	—	—
Relative α	28	2.50	0.476	-5.2×10^{-4}	< 0.001***	—	—
Relative β	27	2.18	0.537	$+2.0 \times 10^{-4}$	0.689	—	—
Relative γ	27	9.55	0.023*	$+5.2 \times 10^{-3}$	< 0.001***	— (DIV47–DIV59 p = 0.052)	—

Metric	n	KW $\chi^2(3)$	KW p	LMM β/DIV	LMM p	Significant Šídák pairs	Dunn–
Absolute δ	26	0.49	0.920	-2.8×10^{-4}	0.849	—	
Absolute θ	28	0.95	0.813	$+3.4 \times 10^{-4}$	0.826	—	
Absolute α	28	1.30	0.729	$+6.1 \times 10^{-4}$	0.665	—	
Absolute β	27	3.64	0.303	$+5.1 \times 10^{-3}$	0.145	—	
Absolute γ	27	10.97	0.012*	$+1.3 \times 10^{-2}$	$< 0.001^{***}$	DIV47–DIV59 (p = 0.017)	
δ / β ratio	25	8.74	0.033*	-2.8×10^{-2}	0.009**	DIV47–DIV59 (p = 0.024)	
δ / γ ratio	26	9.07	0.028*	-3.6×10^{-2}	$< 0.001^{***}$	DIV47–DIV59 (p = 0.034)	

Notes. n denotes the total number of well-by-DIV observations retained after IQR-based outlier removal ($k = 1.5$; Methods 3.2.8) from a maximum of $n = 32$ (8 wells $\times$ 4 DIVs). Per-DIV counts ranged between 5 and 8, with DIV47 consistently exhibiting the lowest count (5 wells across all metrics) due to the limited oscillatory activity at the earliest timepoint. † LMM fitted on $\log_{10}$ -transformed values, consistent with the IQR cleaning convention for absolute power (Methods 3.2.8). Significance codes: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Pairwise Dunn–Šídák comparisons are reported only when significant ($p < 0.05$); marginal trends ($0.05 \leq p < 0.10$) are reported in parentheses.

4.4 Aperiodic Component

The longitudinal redistribution of LFP power from low to high frequencies established in Section 4.3 raises the question of its underlying spectral origin. As predicted in Methods 3.2.3, two mechanisms can cause this shift. One possibility is a steepening of the broadband 1/f-like aperiodic slope that would proportionally redistribute power from low to high frequencies without involving band-specific oscillatory dynamics (Gao et al., 2017; Donoghue et al., 2020). Alternatively, another hypothesis suggests band-specific changes in oscillatory or broadband content that selectively impact specific frequency ranges. In order to address these two cases, LFP power spectra were parameterised using the specparam algorithm (Methods 3.2.4), and the aperiodic exponent (χ) and offset were extracted per well and DIV. Their longitudinal trajectories are reported in Figure 4.5.

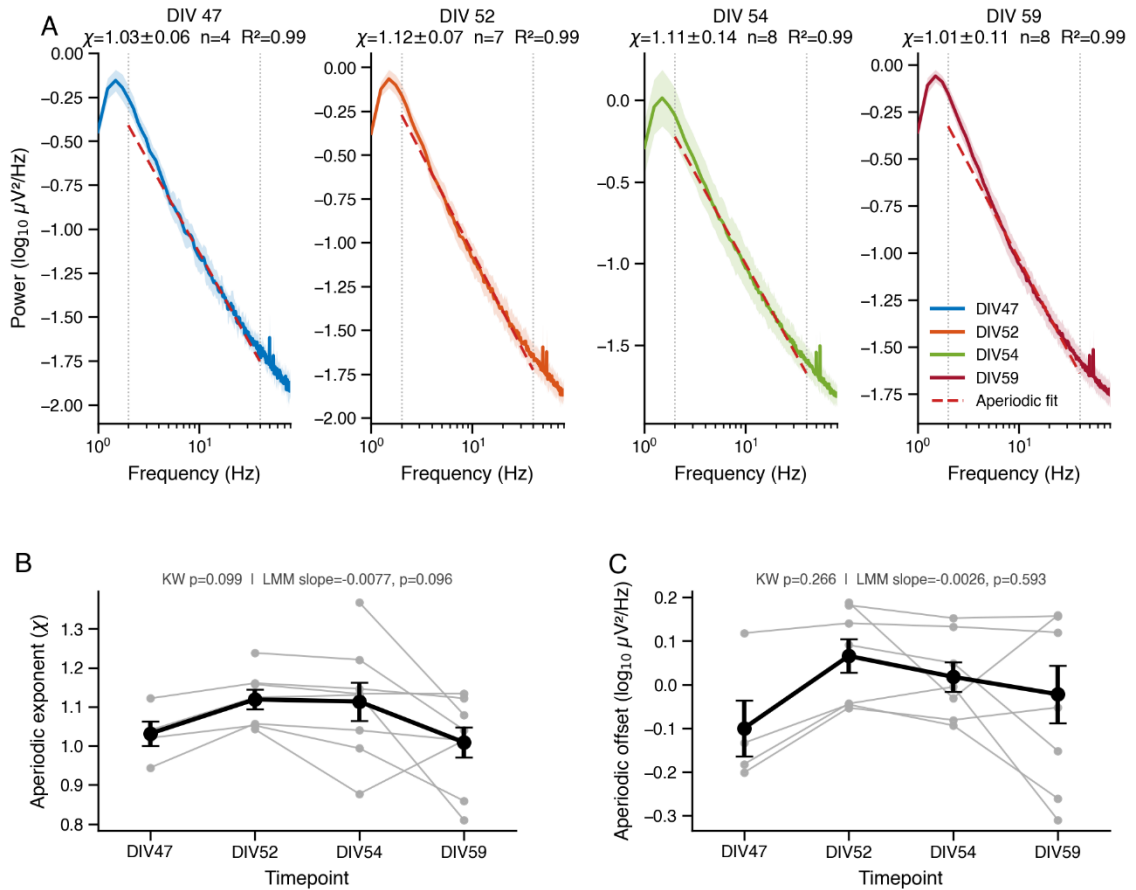


Figure 4.5. Longitudinal aperiodic component of LFP power spectra in TUBA-new cultures. (A) Mean log-transformed PSD $\pm$ 95% confidence interval across active wells at each recording timepoint (DIV 47, 52, 54, 59), displayed on a log–log scale (1–80 Hz). Dashed red lines indicate the mean aperiodic fit estimated by specparam in fixed mode over the 2–40 Hz range (Donoghue et al., 2020); vertical dotted grey lines mark the fitting-range boundaries. Panel titles report the mean aperiodic exponent $\chi \pm$ the standard deviation (SD), the number of wells contributing valid fits (n), and the mean coefficient of determination (R^2) for each DIV. (B) Longitudinal trajectory of the aperiodic exponent χ . (C) Longitudinal trajectory of the aperiodic offset. In B and C, grey lines represent individual well trajectories and the black line with error bars indicates the group mean $\pm$ SEM (Methods 3.2.8). Panel subtitles report the Kruskal–Wallis omnibus p-value and the LMM linear-trend slope and p-value (Methods 3.2.8). IQR-based outlier removal ($k = 1.5$) was applied independently per parameter and per DIV.

The quality of the aperiodic fits was uniformly excellent across all four timepoints, with the mean R^2 exceeding 0.99 in every condition (Figure 4.5A). This confirms that, over the 2-40 Hz fitting range, the decomposition successfully represents the spectral shape. This is important because in the case of poor conditioning, any longitudinal behaviour of the parameters could be explained by adaptation failures instead of spectral changes. Due to the low activity available at the initial timepoint, DIV47 retained the fewest fits. The number of wells generating valid fits varied across DIV ($n = 4$ at DIV47; $n = 7–8$ at later timepoints).

The aperiodic exponent χ did not differ significantly across DIVs under either the Kruskal–Wallis omnibus test ($p = 0.099$) or the LMM linear-trend test ($\beta = -7.7 \times 10^{-3}/\text{DIV}$, $p = 0.096$; Figure 4.5B). The group-level trajectory was non-monotonic, with a modest mean increase from $\chi \approx 1.03$ at DIV47 to ≈ 1.12 at DIV52, followed by stabilisation at DIV54 ($\chi \approx 1.11$) and a return to baseline at DIV59 ($\chi \approx 1.01$). Therefore, the tiny linear trend suggested by LMM was the opposing direction to the increasing slope expected by a maturation-dependent aperiodic redistribution (Gao et al., 2017; Donoghue et al., 2020), although neither the direction nor the magnitude of this trend reached statistical significance. Per-well trajectories were substantially heterogeneous, with individual wells showing increases, decreases, or no clear trend across DIV, reflecting a high inter-well variability in the aperiodic component over the studied range.

The aperiodic offset displayed a similar pattern of non-significant change (KW $p = 0.266$; LMM $\beta = -2.6 \times 10^{-3}/\text{DIV}$, $p = 0.593$; Figure 4.5C). The group-level trajectory was even more variable than that of the exponent, with no discernible monotonic component. The absence of a systematic offset change is consistent with the absence of a systematic exponent change. In fact, under a strict aperiodic-redistribution scenario, an increase in

slope steepness would typically be accompanied by a parallel adjustment of the offset and the joint stationarity of both parameters argues against such a redistribution within the DIV range examined.

Considered jointly with the band-power findings of Section 4.3, the absence of a significant longitudinal change in either aperiodic parameter has an important interpretive consequence: the redistribution of relative power from delta to gamma, and the corresponding decline of the δ/β and δ/γ ratios, cannot be explained by a progressive steepening of the aperiodic component within the DIV range examined. The spectral shift quantified by the band-power ratios could therefore reflect band-specific changes in oscillatory or broadband content rather than a global $1/f$ redistribution. In the following section, the nature of these band-specific effects and specifically how the increased power in gamma relates to ongoing delta-range oscillatory activity is assessed by studying the cross-frequency phase-amplitude coupling.

4.5 Phase-Amplitude Coupling (PAC)

A natural mechanistic candidate for such a band-specific change is the emergence of cross-frequency coupling between the slow oscillatory dynamics that persist in the delta range and the higher-frequency content that accumulates with culture age (Section 4.3, Figure 4.3E, J). To evaluate this possibility, the phase-amplitude coupling was measured using the MI introduced by Tort et al. (2010). The frequency bands selected for the measurement were justified by the previous comodulogram as described in the Methods section (Methods 3.2.5). The PAC is first illustrated at the single-electrode level in Figure 4.6, then quantified at the network level across the longitudinal DIV range in Figure 4.7.

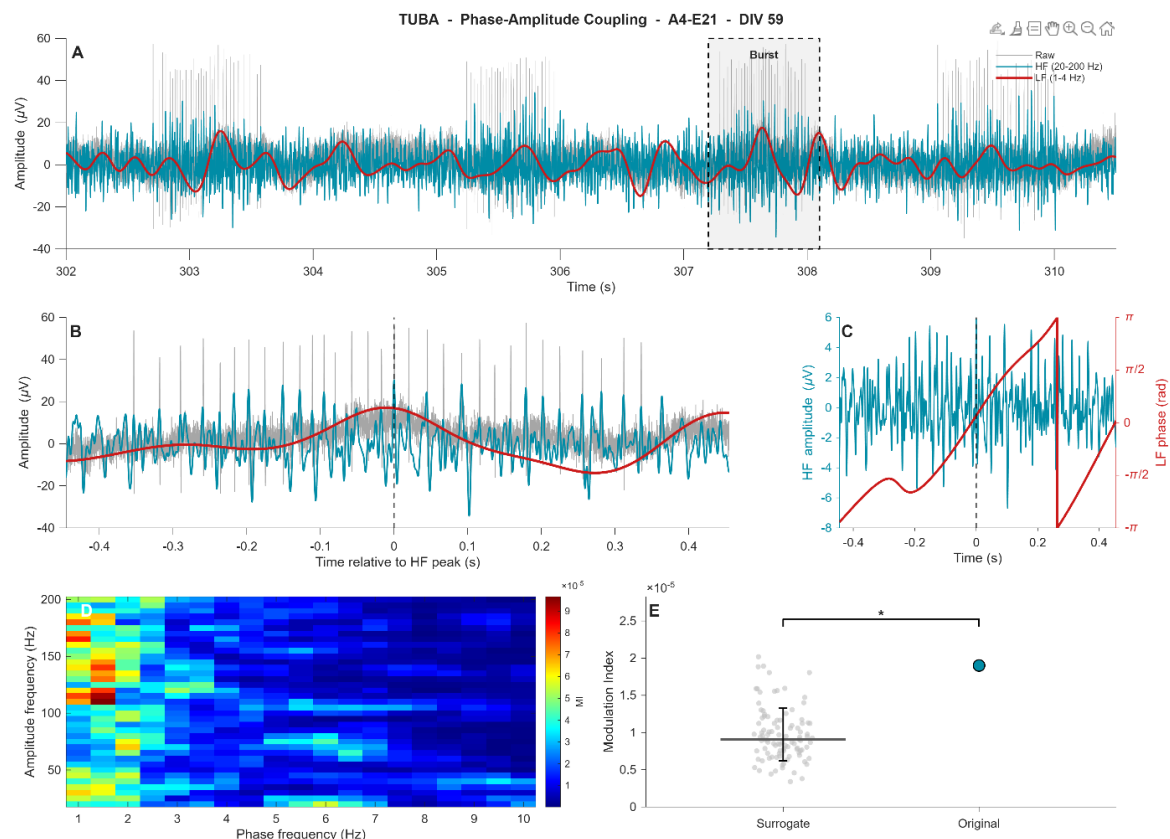


Figure 4.6. Representative single-electrode illustration of delta-broadband phase-amplitude coupling at DIV59 (well A4, electrode E21). (A) Full LFP trace over a representative 8.5-second window showing the raw signal (grey), the broadband HF component (20–200 Hz, teal), and the delta LF component (1–4 Hz, red). HF and LF traces are shown rescaled vertically to be visible on the same amplitude axis as the broadband signal (Methods §3.2.5). The dashed box marks the burst region used for the temporal zoom of panels B and C. (B) Temporal zoom on the highlighted burst, with time expressed relative to the highest-amplitude HF peak ($t = 0$). (C) Instantaneous delta-phase trace (right axis, red) overlaid on the broadband HF amplitude (left axis, teal) over the same zoom window as in B. (D) Single-electrode comodulogram (phase frequencies 1–10 Hz, amplitude frequencies 20–200 Hz) (E) MI of the original signal (teal point) compared against the distribution of 100 circular-shift surrogates (grey points; horizontal bar = median, vertical bar = mean $\pm$ SD). The asterisk indicates statistical significance ($p < 0.05$) under the non-parametric surrogate test.

A representative recording is shown in Fig 4.6, showing the type of phenomenon being investigated. This particular recording provides a good demonstration of the population of phenomena the MI tries to capture. Panel A displays an 8.5-second LFP segment characterised by a noticeable delta-range oscillation (red, 1–4 Hz) alongside high-frequency broadband activity (teal, 20–200 Hz). This broadband activity’s envelope seems to closely follow the delta cycle especially within the highlighted burst region. The temporal zoom of Panel B, centred on the highest-amplitude broadband peak in the burst,

shows the delta cycle reaching its positive excursion approximately at the same time as the broadband amplitude envelope reaches its maximum. The instantaneous delta-phase trace of Panel C suggests a tendency for the broadband amplitude to peak around a preferred phase of the delta cycle. This illustration demonstrates phase-amplitude coupling at the single-electrode level for illustrative purposes. Quantitative assessment is provided by the group-level analyses in Figure 4.7. The single-electrode comodulogram in Panel D shows that the highest MI values for this electrode are localised at low-delta phase frequencies across the broadband amplitude range, and the MI of the original signal in Panel E lies substantially above the surrogate distribution, with a non-parametric p-value below the significance threshold.

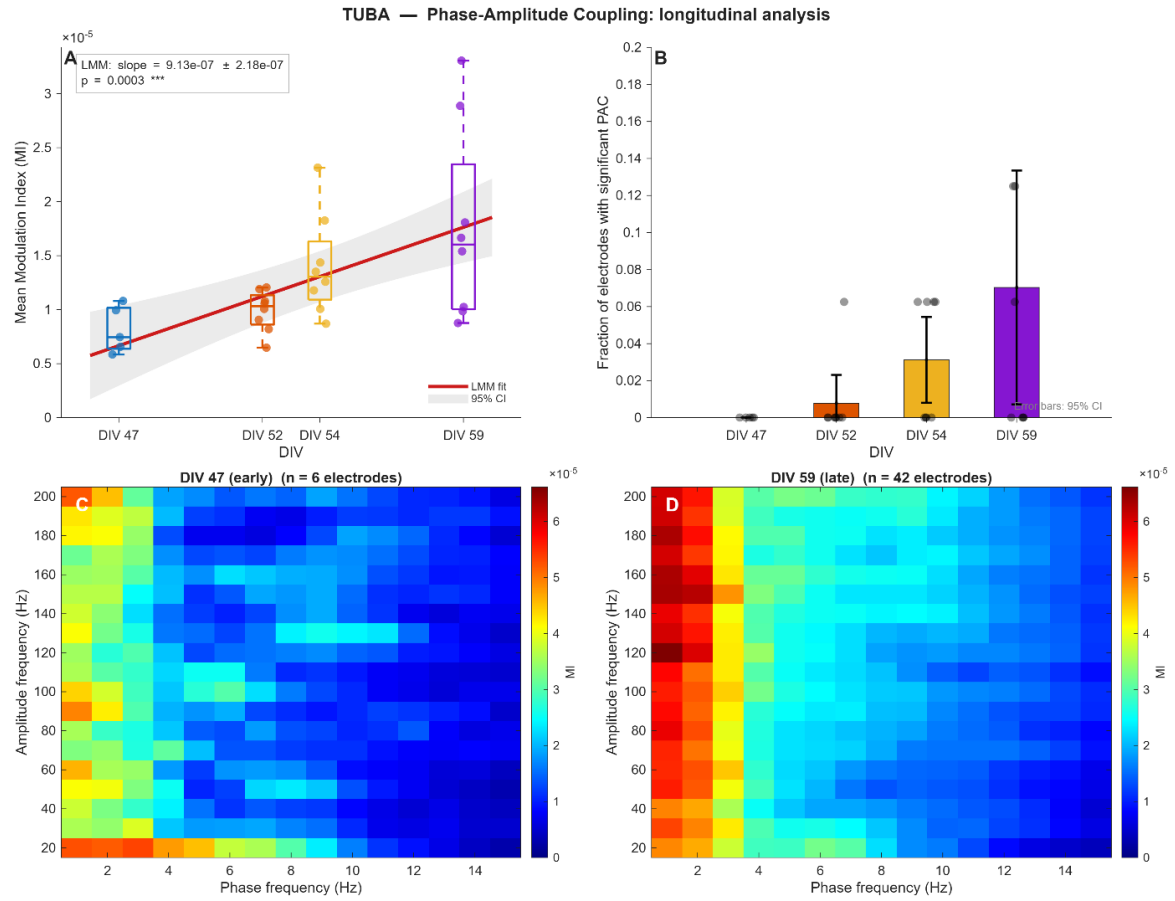


Figure 4.7. Longitudinal evolution of phase-amplitude coupling in TUBA-new cultures. (A) Mean MI per well across DIV 47, 52, 54, 59. Boxplots show the median and interquartile range across wells, individual-coloured points represent single-well MI averaged across all active electrodes per well, and the whiskers extend to $1.5 \times \text{IQR}$. The red line indicates the LMM regression fit (Methods §3.2.8); the grey shaded band represents the 95% confidence interval around the fit. Inset: LMM slope and p-value. **(B)** Fraction of active electrodes per well exhibiting statistically significant PAC against the circular-shift surrogate distribution (Methods §3.2.5). Bars represent the mean across wells with error bars indicating non-parametric bootstrap 95% confidence intervals (Methods 3.2.8); individual-coloured points represent single-well values. **(C, D)** Mean group-level comodulograms across active electrodes at DIV47 (early, panel C; n = 6 electrodes) and DIV59 (late, panel D; n = 42 electrodes). Each pixel reports the mean MI obtained for the corresponding combination of phase frequency (1–15 Hz, 1 Hz steps, ± 2 Hz bandwidth) and amplitude frequency (20–200 Hz, 10 Hz steps, ± 10 Hz bandwidth). Colour scale is matched across the two panels.

MI increased monotonically from a group-level mean of approximately 0.8×10^{-5} at DIV47 to approximately 1.8×10^{-5} at DIV59, with intermediate timepoint values of approximately 1.0×10^{-5} at DIV52 and 1.4×10^{-5} at DIV54 falling consistently along the trajectory (Figure 4.7A). The linear mixed-effects model detected a highly significant positive slope across DIV ($\beta = 9.13 \times 10^{-7}/\text{DIV}$, $\text{SE} = 2.18 \times 10^{-7}$, $p = 3 \times 10^{-4}$), and the omnibus Kruskal–Wallis

test confirmed an overall longitudinal effect ($p = 0.0092$). Pairwise Dunn–Šídák comparisons between individual DIVs did not survive correction for multiple testing, with the strongest pairwise contrast (DIV47 vs DIV59) approaching but not reaching significance ($p = 0.079$). Inter-well variability increased visibly with DIV, most pronouncedly at DIV59, where the well-level distribution included two outliers reaching $MI \approx 3 \times 10^{-5}$, approximately twice the median of that group. This enlargement of the distribution is consistent with the heterogeneous timing of network maturation across replicate wells within the same batch and contributes to the relatively broad CI envelope of the LMM fit at the upper end of the DIV range.

The fraction of active electrodes per well showing statistically significant PAC against the circular-shift surrogate distribution (Figure 4.7B) offered a methodologically different measure of the same phenomenon. The mean fraction increased from essentially zero at DIV47 to approximately 0.008 at DIV52, 0.03 at DIV54, and 0.07 at DIV59, indicating a first phase of a progressive enrichment of the population of electrodes capable of supporting significant phase-locking between delta phase and broadband amplitude. As described in 3.2.5, the number of electrodes showing a significant PAC is then normalized used a fixed denominator of 16. This choice ensures that this metric is directly comparable across wells regardless of inter-well differences in the absolute number of active electrodes. The omnibus Kruskal–Wallis test on this metric failed to reach significance ($p = 0.094$). This rising group-level trend closely mirrors the longitudinal pattern seen in the well-averaged MI of Panel A. Although this fraction metric did not reach significance under the omnibus test ($p = 0.094$), it provides qualitative convergent support, consistent with the significant increase in the well-averaged MI (Figure 4.7A), for the interpretation that maturation strengthens PAC at the individual coupling level.

A group-level comodulogram analysis (Figure 4.7C, D) was applied to discover, empirically, the phase-frequency and amplitude-frequency bands exhibiting the strongest coupling in this culture model. This allows localisation of the coupling before any longitudinal analysis. At the latest timepoint analysed (DIV59), the comodulogram revealed a clearly localised hot region. This region showed the highest MI values at low phase-frequencies between 1 and 3 Hz and across a wide amplitude-frequency range from about 20 Hz to the grid’s upper limit of 200 Hz. A weak signature of the same coupling region was already visible at DIV47, where only a few electrodes were active (Methods

3.2.1) and available for PAC computation ($n = 6$). Overall, the absolute MI values in the delta-broadband region increased substantially between DIV47 ($n = 6$) and DIV59 ($n = 42$ electrodes). The pattern shown in the group-level comodulogram (Figure 4.7 C, D) agrees with the single-electrode comodulogram of Figure 4.6D, in which the same delta-broadband region emerges as the dominant pair of bands at the individual-channel level. On this empirical basis, the delta band (1–4 Hz) was retained as the phase carrier and the broadband HF range (20–200 Hz) as the amplitude envelope, for all longitudinal PAC analyses presented in the same figure in panels A and B.

In general, longitudinal analysis of PAC shows that the coupling between the delta phase and the broadband high-frequency amplitude frequencies strengthens monotonically within the considered DIV range. Strengthening is robustly accompanied by the average MI (Figure 4.7A) and qualitatively by the parallel increase in the fraction of individually significant electrodes (Figure 4.7B). Longitudinal strengthening of phase-amplitude coupling, together with the results of band power measurements and ratios presented in sections 4.3 and 4.4, constitutes a multivariate input for the LFP-derived NAS introduced in the next section.

4.6 Multivariate LFP Maturation

Index

The analyses in Sections 4.3, 4.4 and 4.5 collectively identified several LFP-derived indices of network maturation. These indices capture partially overlapping aspects of spectral and cross-frequency dynamics that develop across different DIVs. To combine the above diverse indices into a single compound summary that would serve as a proxy descriptor of LFP-based maturation for use as a downstream descriptor, PCA was applied to the ensemble of LFP metrics, following the NAS framework applied to spike-based MEA recordings by Passaro et al. (2021) (see methods 3.2.6). The resulting PC1 as a function of DIV is shown in Figure 4.8A with corresponding metric loadings in Figure 4.8B.

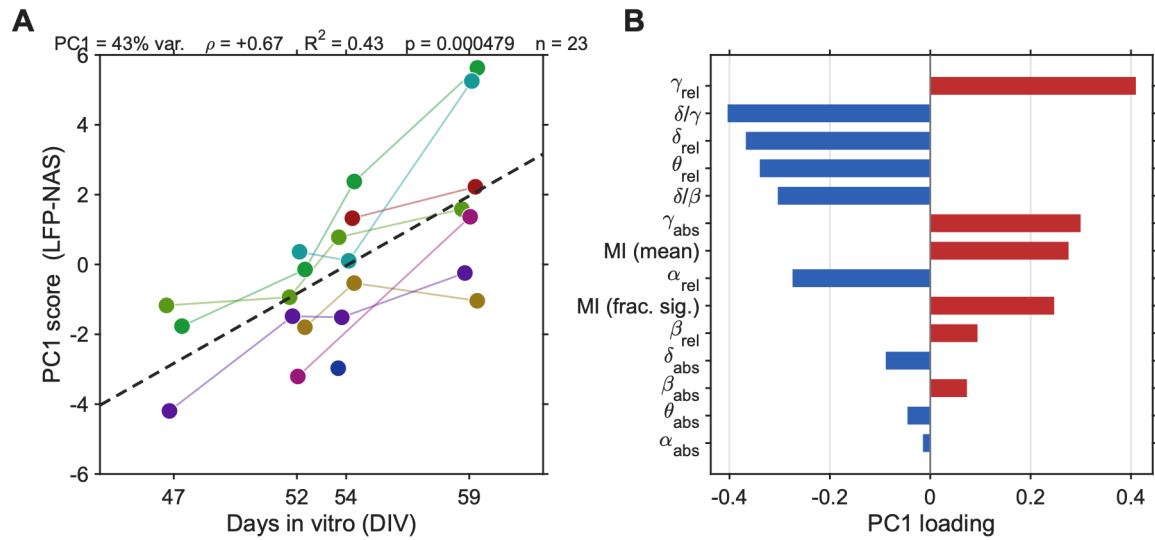


Figure 4.8. Multivariate LFP maturation index (LFP-NAS) in TUBA-new cultures. (A) First principal component score (PC1, "LFP-NAS") computed on the standardised LFP metric pool, plotted against DIV for each well-by-DIV observation. Markers are colour-coded by individual well, with longitudinal measurements from the same well across time points (DIV) linked by matching coloured lines; the dashed grey line indicates the linear regression of PC1 on DIV. Panel title reports the fraction of variance explained by PC1, the Spearman rank correlation between PC1 and DIV (ρ), the linear coefficient of determination (R^2), the Spearman p-value, and the number of well-by-DIV observations retained after listwise deletion of incomplete cases. **(B)** PC1 loadings of the 14 LFP metrics included in the PCA, sorted by decreasing absolute magnitude. Negative loadings (blue) and positive loadings (red) indicate metrics that decrease and increase, respectively, along the PC1 maturation axis. Subscripts: rel = relative power, abs = absolute power; MI (mean) = mean Modulation Index per well; MI (frac. sig.) = fraction of electrodes with significant PAC per well.

The PCA pool comprised fourteen LFP metrics: the absolute and relative power of the five canonical bands (δ , θ , α , β , γ), the two inter-band relative power ratios (δ/β and δ/γ), and the two phase-amplitude coupling metrics introduced in Section 4.5 (mean MI per well and fraction of electrodes with significant PAC; Methods 3.2.6).

The PC1 captured 42.9% of the total variance across the standardised metric pool, with the second component accounting for an additional 36.2%, so that the two together explained 79.1% of the total variance. The PC1 score correlated strongly and positively with DIV (Spearman $\rho = +0.67$, linear $R^2 = 0.43$), with the correlation reaching high statistical significance ($p = 4.8 \times 10^{-4}$; Figure 4.8A). The PC1 versus DIV scatter shows a clear group-level positive trend across all four timepoints, with the centre of mass of the PC1 distribution shifting from negative values at DIV47 to positive values at DIV59. As already observed in Sections 4.3 and 4.5, within-DIV dispersion is most pronounced at the latest timepoint. In particular, two wells showed PC1 scores above +5 while the remaining DIV59 observations clustered between approximately 0 and +2. This widening of the late-DIV distribution, which might be seen as a negative outcome, aligns with the previously observed heterogeneous timing of network maturation across replicate wells. This is reflected in the LFP-NAS without losing the overall strong trend.

The PC1 loading structure (Figure 4.8B) provides an interpretable view of the multivariate axis. The strongest positive loading on PC1 corresponds to relative gamma (γ_{rel} , loading = +0.41), while the strongest negative loadings correspond to the δ/γ ratio (loading = -0.40), relative delta (δ_{rel} , loading = -0.37), and relative theta (θ_{rel} , loading = -0.34). The next-strongest negative driver is the δ/β ratio (loading = -0.30), and the next-strongest positive driver is absolute gamma (γ_{abs} , loading = +0.30). The two phase-amplitude coupling metrics introduced in Section 4.5 both show moderate positive loadings (MI mean of +0.28 and fraction of significant electrodes of +0.25). This aligns with the maturation axis direction of relative and absolute gamma and supports the longitudinal strengthening of PAC reported in Section 4.5. The β -band powers (β_{rel} , +0.09; β_{abs} , +0.07) and the absolute powers of the low- and mid-frequency bands (δ_{abs} , -0.09; θ_{abs} , -0.05; α_{abs} , -0.02) contribute negligibly to PC1, this aligns with the absence of a longitudinal effect for these metrics as observed in Section 4.3. The loading pattern thus internally reproduces, on a single multivariate axis, the same low-to-high frequency redistribution and concurrent PAC strengthening that the previous analyses had identified piecewise.

Collectively, the LFP-NAS model summarizes these LFP maturation measures into a single, informative and quantitatively significant index that can be evaluated over time within the individual well. The strong correlation of PC1 with DIV, obtained despite the insufficient sample size relative to the dimensionality of the metric pool, supports the interpretation of PC1 as an authentic signature of the underlying biological process rather than as a noise-driven projection. The results of LFP-NAS confirm that the approach presented by Passaro and colleagues (Passaro et al., 2021) can be applied to metrics derived from LFP frequency analysis with convergent results.

4.7 Univariate Follow-up on Top PC1 Drivers

The LFP-NAS presented in the previous section allowed us to condense 14 LFP-based metrics into a single maturation axis. This section further seeks to distinguish whether its trend derives from only a few metrics that contribute strongly or is spread across multiple metrics. To resolve this and to make the biological substrate of PC1 explicit, the metrics with the largest PC1 loadings were followed up univariately against DIV (Methods 3.2.7). PC1 was sign-aligned with DIV (Section 4.6, Methods 3.2.6), which means that metrics with positive loading are those that increase along the maturation axis, while metrics with negative loading are those that decrease. In this section, a univariate analysis of the top three drivers was performed for each sign, then the three up-drivers (relative gamma, absolute gamma, mean MI) and three down-drivers (δ/γ ratio, relative delta, relative theta) were analysed. It is important to remember that since the choice of drivers is data-driven, it depends on the previous multivariate analysis; this analysis is solely confirmatory rather than an independent statistical test (Methods 3.2.7). In particular, for each metric, the Spearman rank correlation against DIV was computed for all valid well-by-DIV observations, with p-values corrected through the Benjamini–Hochberg correction and presented as q-values. Results are shown in Figure 4.9.

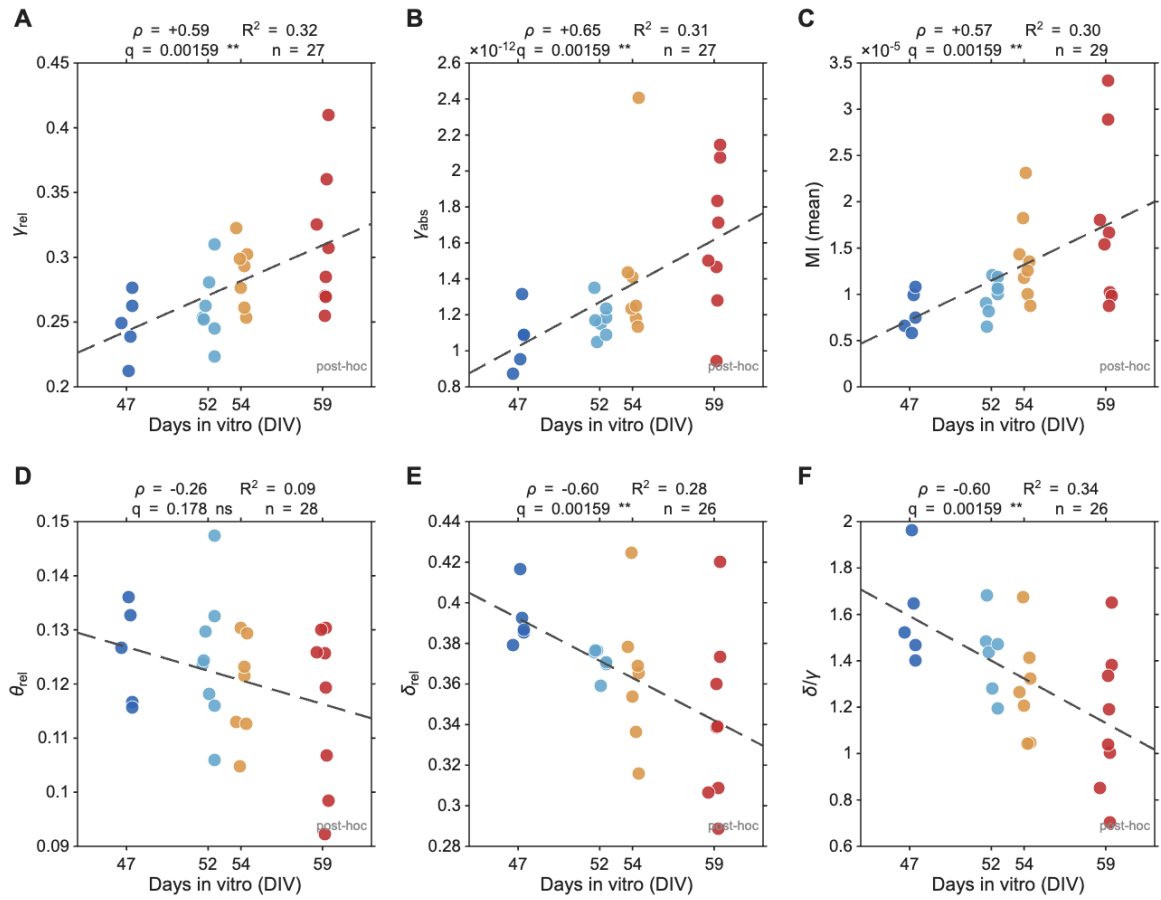


Figure 4.9. Univariate post-hoc follow-up on the top PC1 drivers of the LFP-NAS. Each panel plots one LFP metric against DIV for every well-by-DIV observation, with markers colour-coded by DIV and the dashed grey line indicating the ordinary least-squares linear fit (Methods 3.2.7). Top row (A–C): the three metrics with the strongest positive PC1 loadings with (A) relative gamma power (γ_{rel}), (B) absolute gamma power (γ_{abs}), (C) mean modulation index, MI (mean). Bottom row (D–F): the three metrics with the strongest negative PC1 loadings with (D) relative theta power (θ_{rel}), (E) relative delta power (δ_{rel}), (F) delta-to-gamma power ratio (δ/γ). Panel titles report the Spearman rank correlation between the metric and DIV (ρ), the linear coefficient of determination (R^2), the Benjamini–Hochberg FDR-corrected p-value (q) with significance code, and the number of well-by-DIV observations with valid data for that metric (n). The analysis is exploratory/confirmatory rather than an independent statistical test, as marked by the "post-hoc" annotation in each panel. Axis scale factors: γ_{abs} , $\times 10^{-12}$; MI (mean), $\times 10^{-5}$. Significance codes: * $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$; n.s., not significant. Subscripts: rel = relative power, abs = absolute power.

The three positive drivers each showed a significant monotonic increase with DIV (Figure 4.9A–C). Relative gamma power, the single strongest PC1 contributor (loading +0.41), rose across the DIV range with a Spearman $\rho = +0.59$ and a linear $R^2 = 0.32$ ($n = 27$). Absolute gamma power (loading +0.30) displayed the strongest univariate association of all six drivers ($\rho = +0.65$, $R^2 = 0.31$, $n = 27$), confirming that the gamma absolute power increase characterised in Section 4.3 re-emerges as a leading component of the multivariate axis on both the relative and the absolute scale. The mean modulation index (loading +0.28)

increased in parallel ($\rho = +0.57$, $R^2 = 0.30$, $n = 29$), recovering at the single-metric level the longitudinal strengthening of phase-amplitude coupling established in Section 4.5. The three up-drivers thus jointly reproduce the gamma-and-PAC signature that the multivariate model had compressed into the positive loadings.

Among the negative drivers, the two power-redistribution metrics behaved as expected while the theta relative power contribution was markedly weaker (Figure 4.9D–F). The δ/γ ratio, the strongest negative loader (-0.40), declined with the steepest linear fit of any of the six drivers ($\rho = -0.60$, $R^2 = 0.34$, $n = 26$), and relative delta (loading -0.37) decreased to a comparable degree ($\rho = -0.60$, $R^2 = 0.28$, $n = 26$). Both confirm, as single metrics, the low frequency reduction and the δ/γ decline characterised in Sections 4.3 and 4.4. Relative theta (loading -0.34) was the only one of the six drivers not to reach significance, showing a weak, non-significant negative trend ($\rho = -0.26$, $R^2 = 0.09$, $q = 0.18$, $n = 28$). This is consistent with Section 4.3, where theta relative power reached significance only under the LMM and not under the omnibus test, indicating that its contribution to PC1 is carried largely by its covariance with the other low-frequency metrics rather than by a strong longitudinal trend.

Chapter 5

Discussion

This thesis aimed to investigate how spectral properties of 2D hiPSC-derived cortical networks (TUBA1B cell line) evolve during *in-vitro* development, focusing on LFP-based descriptors to characterise network maturation. This analysis addresses a gap in the MEA literature on hiPSC-derived neuronal networks, where functional characterisation has relied predominantly on spike-based metrics such as firing, bursting, and network burst descriptors (Mossink et al., 2021), while the low-frequency component of the extracellular signal has often been excluded, with only few reports on LFPs or neural oscillations in human 2D cultures (Pelkonen et al., 2022).

Two independent batches of the same cell line (TUBA-old and TUBA-new) were analysed at four timepoints from DIV 47 to DIV 60. The extracellular recordings for the two batches were acquired using two different MEA systems: the Axion Maestro for TUBA-old and the Axion Maestro Pro for TUBA-new. Importantly, the two systems differ in their low-frequency limits: the Maestro Pro preserves the broadband signal, including the low-frequency content of the LFP, whereas the earlier Maestro applies a hardware high-pass filter at the acquisition level (AxIS v2.5.2.1), attenuating spectral power below approximately 200 Hz (Methods 3.1.2).

This thesis focused on three properties of the signal: the distribution of power across canonical frequency bands and their ratios, the aperiodic component of the spectrum, and the phase-amplitude coupling (PAC) between the phase of the slow delta rhythm and the amplitude of high-frequency broadband activity. Ultimately, these complementary metrics were integrated via principal component analysis into a unified maturation index, the LFP-based neural activity score (LFP-NAS), providing a robust quantitative framework to track network progression.

5.1 Spike-based metrics confirmed the expected maturation trajectory in both batches

Despite differences between TUBA-old and TUBA-new, the analysis of spike-based metrics confirmed a maturation trajectory coherent with what is expected from hiPSC-derived networks on MEAs (Frega et al., 2017; Mossink et al., 2021). In particular, on TUBA-new networks, four canonical hallmarks were present: an increase of MFR, BR, and NBR and a decrease of NBD are coherent with networks becoming more active and synchronous while becoming mature. TUBA-old networks were characterised by a higher baseline activity compared with TUBA-new, and only the MFR increase showed a statistically significant longitudinal trend. The main difference between the two batches concerned NBD, which decreased considerably in TUBA-new while staying stable in TUBA-old. This divergence is consistent with the intrinsic batch-to-batch variability of hiPSC-derived cultures, in which network burst parameters, including NBD, are among the metrics most affected by the experimental batch (Mossink et al., 2021), and it did not compromise the reliability of spike-based metrics in either dataset.

Before the spectral analysis, the two batches were compared at the spectral level to exclude technical bias. TUBA-old showed a clear deficit at low frequency, described by a flat and compressed PSD. Since this deficit is frequency-selective, meaning that it concerns only low frequencies while spiking and high frequencies are preserved, it can be attributed to hardware high-pass filtering applied at the acquisition level rather than a real biological difference that would produce broadband deficits. As a consequence, the low-frequency component is most likely present in both batches, but it is reliably captured only in the TUBA-new recordings. Spectral analysis was therefore restricted to this dataset and carried out on eight wells of TUBA-new networks. While not the sole reason, such hardware constraints may partially explain why earlier literature predominantly focused on spike-based metrics, highlighting how the availability of modern broadband systems like the Maestro Pro can facilitate the exploration of these slow-wave dynamics.

5.2 LFP power redistributed from low to high frequencies during maturation

The distribution of relative power among canonical bands showed a low-to-high frequency redistribution: delta relative power decreased significantly across DIVs, while gamma relative power increased over the same period. As for absolute power, only gamma power increased significantly over DIVs, while other bands stayed more stable, showing broad inter-well variability. Given that only gamma varied in absolute terms, it is important to note that the decrease in delta power is relative and represents a redistribution of the power instead of a loss at low frequencies, which is in line with the maturational redistribution described in developing neural systems, where the relative weight of slow activity decreases in favour of faster components (Niemark et al., 2011; Trujillo et al., 2019). As a direct numerical consequence of these opposite band trends, both inter-band ratios decreased significantly, with the delta-to-gamma ratio showing a stronger monotonic trend. The lower significance of the delta-to-beta ratio is expected to derive from the higher variability of beta band power, which is transferred to the ratio, making the delta-to-beta ratio less precise than the delta-to-gamma counterpart as a network maturation descriptor.

The first interpretation attributes this frequency redistribution to synaptic and network maturation. During development, the frequency content emerges at the same time as network maturation, and in particular the fast activity accelerates to the gamma-range in parallel with the maturation of fast-spiking interneurons (Bitzenhofer et al., 2020; Le Magueresse & Monyer, 2013). However, this increase in power in the high-frequency range of the LFP must be interpreted with caution. Since the firing rate of the network increased with DIV and the high-frequency broadband power of the LFP covaries with, and is partly generated by spiking activity (Manning et al., 2009; Ray & Maunsell, 2011; Herreras, 2016), the increase in the gamma band is more properly read as an index of increasing activity and synchrony rather than as evidence of the emergence of a gamma oscillation. On the other hand, it should be observed that spike contribution to the LFP power spectrum becomes relevant above 50 Hz (Ray & Maunsell, 2011). Since the observed redistribution also involved lower frequencies, the phenomenon cannot be uniquely attributed to spike contamination.

These two readings are not necessarily in contrast: developing cortical cultures display a well-described transition in which long network bursts are progressively replaced by shorter and more frequent ones (Chiappalone et al., 2006), a reorganisation linked to the maturation of synaptic connectivity and of the excitation/inhibition balance (Chiappalone et al., 2006; Le Magueresse & Monyer, 2013), and the resulting denser and more precise firing inside NBs can be reflected in high-frequency broadband power (Manning et al., 2009; Ray & Maunsell, 2011). In this perspective, while NBs became shorter and more frequent, the intra-network burst firing rate (INBFR) could increase, producing an increase in the high-frequency bands' power. Although this interpretation would unify the reorganisation of spiking and spectral shift into a single mechanism, the INBFR was not quantified in this thesis, and consequently its verification remains among the future perspectives. This reading is in line with rodent MEA studies in which LFP power in dissociated networks was tied to burst activity and changed when bursting was altered (Leondopulos et al., 2012; Colombi et al., 2016), although in those studies the spectrum was altered experimentally, whereas here the shift emerged spontaneously with culture age and concerned relative rather than absolute delta power.

Overall, this low-to-high spectral shift recalls the maturational power redistribution of the preterm EEG (Niemarkt et al., 2011), linked to the maturation of GABAergic interneuronal signalling (Vanhatalo & Kaila, 2006), and parallels the developmental evolution of network oscillations reported in human cortical organoids on MEA (Trujillo et al., 2019). Band power analysis, however, quantifies the shift without clarifying its origin: it remains to be determined whether it reflects band-specific dynamics or a global change in the aperiodic structure of the spectrum, a question addressed by the aperiodic analysis.

The low-to-high redistribution presented above can arise from two origins: a global steepening of the aperiodic $1/f$ component, which would shift power from low to high frequencies without band-specific dynamics, or band-specific changes (Donoghue et al., 2020). To distinguish them, the spectrum was parameterised with `specparam`, extracting the aperiodic exponent (χ) and the offset. Neither χ nor the offset varied significantly along the DIVs, and the very weak trend of χ , besides not being significant, went in the opposite direction to that expected for maturational steepening (Gao et al., 2017; Donoghue et al., 2020). Consequently, the delta-to-gamma redistribution cannot be explained by a global steepening of $1/f$ in the examined window and reflects band-specific changes. On the E/I

level, this means that the hypothesis of a shift in the excitation/inhibition balance readable in the spectral slope (Gao et al., 2017) is not confirmed in this dataset, consistent with the band-specific/spiking-related interpretation. Overall, the aperiodic decomposition excludes the simplest explanation, a global 1/f redistribution, and strengthens the idea that the shift reflects band-specific dynamics, preparing the PAC analysis.

5.3 PAC emerged and strengthened with network maturation

PAC describes the temporal organisation in which the phase of a slow rhythm modulates the amplitude of faster activity, and its presence is considered a signature of hierarchical temporal structure in cortical networks (Canolty & Knight, 2010). In this thesis, PAC was quantified through the Modulation Index (MI), which measures how non-uniformly the amplitude of the fast component is distributed across the phase of the slow rhythm (Tort et al., 2010). In developing networks, the slow delta component reflects the alternation of network-wide excitability associated with NB events, while the broadband amplitude is a surrogate of population spiking (Manning et al., 2009), so the emergence of coupling indicates that population firing becomes progressively confined to a preferred phase of the slow excitability cycle, as described for the nested oscillations of cortical organoids (Trujillo et al., 2019).

To verify whether the growing high-frequency content was temporally organised by the slow delta rhythm, the MI was computed for each electrode across DIVs. The mean MI increased monotonically and significantly with DIV, with the coupling empirically localised at low-delta phase frequencies (1–3 Hz) across the broadband amplitude range (20–200 Hz). In parallel, the fraction of electrodes with significant PAC increased across DIVs, confirming the trend through a measure independent of the mean MI and indicating that the coupled regime progressively recruited a larger portion of the network, consistent with the network-level nature of the phenomenon (Salimpour et al., 2024), albeit on a limited number of active electrodes, especially at the earliest timepoints.

This result is particularly relevant considering the present scientific literature. PAC is widely characterised *in vivo* (Canolty & Knight, 2010), and it has been found in cortical

organoids (Trujillo et al., 2019), but in 2D cultures of human hiPSC-derived neurons it has been reported, to date, solely by Salimpour et al. (2024). The study of Salimpour and colleagues is described as the first to demonstrate PAC in 2D cultures, at around DIV 200, but it did not address how the phenomenon evolves over time. This thesis attempted to add this evolutionary dimension, showing the progressive emergence and strengthening of PAC at early DIV (47–59). However, since this is an early stage characterised by a small number of active electrodes, the PAC analysis in this thesis must be interpreted as explorative, although the trend is consistent across two independent metrics and reproduces the same low-frequency-phase/broadband-amplitude coupling reported in 2D hiPSC-derived neuronal cultures (Salimpour et al., 2024), and the delta/broadband localisation described in 3D organoids (Trujillo et al., 2019).

Consistently, Salimpour et al. (2024) showed that PAC is a network-level phenomenon rather than a single-neuron phenomenon, which supports interpreting the coupling observed here as genuine network organisation rather than a single-channel artefact. The coupling measured here differs from the spike-phase locking described in rodent hippocampal cultures (Leondopulos et al., 2012), which relates spike timing to the phase of a band-limited rhythm, whereas the PAC quantified in this thesis relates broadband amplitude to delta phase. Overall, the emergence of PAC constitutes an early maturation signature, complementary to the spectral shift, and represents one of the first pieces of evidence of the phenomenon in 2D hiPSC-derived cultures.

5.4 The multivariate LFP-NAS index tracked network maturation across wells

Individual spectral metrics describe only partial aspects of maturation, and combining multiple electrophysiological features into a single composite descriptor is an established strategy across scales. In the clinical domain, the maturation of preterm cortical activity has been summarised into an EEG maturational age, obtained by combining many computational EEG features into a single estimate that tracks functional development

longitudinally (Stevenson et al., 2017). *In-vitro*, Passaro et al. (2021) combined multiple spiking and bursting metrics through PCA into the NAS, PCA-based approaches have been applied to characterise hiPSC-derived network activity on MEAs (Hyvärinen et al., 2019; Mossink et al., 2021), multivariate network-level pipelines have been developed to track the development of 2D cultures and organoids (Sit et al., 2024), and a model trained on preterm EEG features has been used to map organoid development onto a single developmental trajectory (Trujillo et al., 2019). All these approaches, however, are built either on spike-derived metrics or on clinical EEG: a composite index based on the LFP of 2D hiPSC-derived cultures was missing. Constructing one is motivated by the complementarity of the two signal components, since the LFP reflects mainly subthreshold synaptic and population-level processes that spike-based metrics do not capture (Buzsáki et al., 2012; Einevoll et al., 2013). On this basis, the LFP-NAS was constructed by applying PCA to the set of 14 LFP metrics, adapting the NAS method introduced for MUA (Passaro et al., 2021).

PC1, indicated as LFP-NAS, captured the dominant maturation axis of the dataset and correlated strongly and significantly with DIV, moving from negative values at DIV 47 to positive values at DIV 59. The structure of the loadings is biologically coherent with the processes discussed in the previous sections. The index was driven up by gamma power (relative and absolute) and by the two PAC metrics, that is, by the features indexing the increasing activity, synchrony, and temporal organisation that characterise network maturation (Bitzenhofer et al., 2020; Canolty & Knight, 2010; Salimpour et al., 2024). It was driven down by the relative power of delta, theta and alpha and by the δ/γ and δ/β ratios, the features that decrease as power redistributes toward higher frequencies (Niemarkt et al., 2011). Finally, the beta band and absolute powers at low and medium frequencies weighed little, contributing negligibly to the index as they did not display a clear longitudinal trend in the analysed window. In this reading, the LFP-NAS did not introduce new information: it condensed the spectral redistribution and the PAC strengthening, previously shown separately, into a single well-level descriptor of functional maturation, complementary to spike-based measures.

To explore what drove the LFP-NAS, the metrics with the highest loadings were analysed one by one against DIV. The three positive drivers (relative gamma, absolute gamma, and mean MI) all increased significantly with DIV, and the two negative drivers related to

power redistribution (the delta-to-gamma ratio and relative delta) decreased equally significantly. The only exception was relative theta, whose decrease did not reach significance, in line with the band power analysis. Overall, the LFP-NAS maturation axis was supported by a few biologically consistent metrics, related to gamma and PAC and contrasted by the decline in low-frequency relative power, confirming at the single-metric level the trends identified by the multivariate analysis.

The behaviour of the LFP-NAS parallels that of the spike-based NAS, whose first principal component was likewise strongly correlated with culture age and was designated as an index of neural maturation on that basis (Passaro et al., 2021). Obtaining the same result from the low-frequency component shows that network maturation remains legible in a portion of the extracellular signal that spike-based indices discard. Part of what carries the LFP-NAS, namely the coupling metrics and the redistribution among the slow bands, has no counterpart in spike-based descriptions, so the index adds information rather than restating it in a different form.

Nevertheless, the index must be interpreted within clear limits. As a PCA-derived descriptor, its weights are dataset-dependent and cannot be assumed to generalise to other cell lines, protocols, or recording systems. PC1 captured slightly less than half of the total variance, summarising the dominant maturation axis but not the full structure of the data. Moreover, part of the input metrics are direct derivatives of each other (absolute and relative powers, and their ratios), and the built-in redundancy can inflate the shared variance captured by the first component. For these reasons, and given the limited sample size, the LFP-NAS should be regarded as an exploratory descriptor rather than a validated biomarker, pending confirmation on independent datasets.

Ultimately, this thesis shows that the LFP of hiPSC-derived 2D networks is not noise to be discarded, but an informative maturation substrate: spectral shift, PAC, and LFP-NAS trace their longitudinal evolution, opening the way to LFP-based characterisation as a complement to spike analysis. Since band power, band ratios and PAC are defined in the same way for EEG and for in-vivo LFP, these descriptors make it possible to relate 2D human networks to clinical recordings. The LFP recorded on MEAs and the EEG recorded in patients share the same origin, the synaptic currents of groups of neurons (Buzsáki et al., 2012). The spatial scales differ by orders of magnitude, so raw amplitudes cannot be compared, but the same features, and the way they change over time, can. In this thesis,

relative band power and PAC changed with maturation in a simple 2D culture, and the descriptors are in principle comparable with the metrics used to follow maturation in the human brain (Niemarkt et al., 2011; Stevenson et al., 2017). Spike-based metrics do not offer this link, since they are not available in routine clinical recordings. The comparison holds at the level of the features and does not imply that a culture is equivalent to the developing cortex. It also opens a possible workflow for disease modelling: an altered EEG rhythm in a patient could be searched for in networks derived from the patient's cells, compounds could be screened in vitro on that feature, and the effect of a candidate could then be checked on the EEG. The present work is a first step, based on one healthy cell line, and shows that such a comparison is feasible.

Chapter 6

Conclusions

6.1 Summary of the findings

This thesis followed the electrophysiological maturation of 2D hiPSC-derived cortical networks (TUBA1B line) between DIV 47 and DIV 59, using the low-frequency component of the MEA signal that spike-based characterisation normally discards. Spike metrics reproduced the expected developmental trajectory in both batches. Because the older system attenuated the slow component through hardware filtering, the spectral analyses were restricted to eight wells of the TUBA-new batch.

In these wells, relative delta power decreased and gamma power, both relative and absolute, increased with DIV, so that the delta-to-gamma and delta-to-beta ratios fell. The aperiodic exponent and offset did not change significantly, which points to band-specific changes rather than a global steepening of the spectrum, although the sample size does not allow the aperiodic component to be declared stable. Delta-broadband PAC appeared and strengthened over the same window. Combining 14 LFP metrics by PCA produced the LFP-NAS, whose first component increased with DIV from negative to positive values and was driven mainly by gamma power, the two PAC measures, relative delta power and the delta-to-gamma ratio. These results answer the question posed in Chapter 1: the LFP of 2D hiPSC-derived networks changes systematically during maturation, and the changes can be summarised in a single well-level descriptor. Since the data come from one cell line and one batch, the descriptor remains exploratory.

6.2 Limitations

Some limitations must be kept in mind when interpreting the results. The first concerns sample size: spectral analyses were based on eight wells and a small number of active electrodes, especially at the first timepoints. This condition inevitably limited statistical

power, as shown by some pairwise comparisons that did not pass the correction for multiple comparisons and as suggested by the absence of a significant change in the aperiodic component, which in such a small sample ($n \leq 8$; $n = 4$ at DIV 47) cannot be taken as evidence that the aperiodic component was genuinely stable, since the analysis was underpowered to detect a small effect. A second limitation arises from the use of a single cell line and, for the LFP part, of only one suitable batch, since the other presented a deficit at low frequencies of technical origin. The variability observed between the two batches at the spiking level also reminds us how reproducibility remains an open question in hiPSC-derived models, where individual control lines can differ in specific network parameters, and the use of multiple lines and batches is recommended (Mossink et al., 2021) and calls for caution in generalising the conclusions. The time window considered is also a limiting factor. The four time points between DIV 47 and DIV 59 represent only a narrow window of development, within which the networks had already matured considerably. Hence, it can be expected that the data represent only a specific stage of development, and that neither the onset nor the cessation of the developmental process could be identified based on this dataset. (Trujillo et al., 2019; Salimpour et al., 2024) Finally, the multivariate index was constructed with a number of observations per variable below the minimum commonly recommended for PCA (Hair et al., 2010), so that its structure has exploratory value and the individual loadings should not be overestimated.

6.3 Future perspectives

These limitations suggest natural future working directions. The most immediate regards the origin of the spectral shift. Since the high-frequency power mostly reflects spiking activity in the neuronal population, it would be useful to check whether it is influenced by a higher firing rate inside NBs, which in the meantime became shorter and more frequent. Calculating the INBFR over DIVs and correlating it with gamma power would allow one to determine whether the spiking reorganisation inside the NB events is the cause of the spectral shift. Further, extending the DIV range would be appropriate in order to follow the whole maturation trajectory. Other future perspectives are based on repeating the analysis on multiple cell lines and a larger number of wells: it serves both to understand whether the observed trajectories hold in general and to validate the LFP-NAS on independent data.

In fact, with more samples one could also confirm the weight structure of the index, which here remains only descriptive due to the small sample ($n = 23$, below the recommended threshold). It would also be useful to record over a wider time window, both at earlier and more mature stages, to follow the entire course of the spectral shift and PAC, with the latter observed here only at the beginning (DIV 47–59) and described at full maturity by Salimpour et al. (2024) around DIV 200.

On the mechanistic level, in addition to the direct measurement of INBFR already mentioned, pharmacology could be used to causally verify the role of excitation and inhibition. For example, reducing inhibition with GABA-A receptor antagonists, such as bicuculline or gabazine, which Salimpour et al. (2024) have already shown to modulate PAC in 2D cultures, or, conversely, enhancing it and, in parallel, block excitatory (glutamatergic) transmission (Trujillo et al., 2019; Chiappalone et al., 2006), would allow researchers to separate the contribution of the two systems to spectral shift and coupling, completing the mechanistic framework proposed here.

6.4 Impact and outlook

The first practical consequence concerns data acquisition. The comparison of the two recording systems showed that a hardware high-pass filter can remove the slow component of the extracellular signal without affecting spike-based metrics, so the loss goes unnoticed when only spikes are analysed. A laboratory using MEAs can check the low-frequency limit of its recording settings and, where a broadband system was used, analyse the LFP of recordings that were already acquired for spike analysis.

The second consequence concerns comparability across scales. Relative band power, band ratios and delta-broadband PAC are defined in the same way for EEG and for invasive in-vivo LFP, so a culture can be described with the vocabulary used for the developing brain. The spectral shift observed here recalls the redistribution reported in preterm EEG (Niemarkt et al., 2011), and in the clinical domain a maturational age has already been derived from EEG features (Stevenson et al., 2017). The LFP-NAS is an in-vitro counterpart of such a score, but the two are not interchangeable: a culture lacks the thalamocortical input, the laminar architecture and the cell-type diversity of the developing

cortex, and any cross-scale validity would have to be tested by applying the same features to both kinds of recordings.

A third direction is the combination with spike-based descriptors. The NAS of Passaro et al. (2021) and the LFP-NAS are built from different components of the same recording, and a joint index could be tested for whether it tracks maturation better than either one alone. The same features could also be computed in cortical organoids on MEA, where oscillatory activity has already been reported (Trujillo et al., 2019), to see whether 2D and 3D preparations follow comparable LFP trajectories.

Rodent MEA studies indicate that LFP spectra respond to interventions on the network. Chronic stimulation raised theta and slow gamma power (Leondopulos et al., 2012), carbachol fragmented network bursts (Colombi et al., 2016) and reduced LFP power, most strongly in delta and theta (Colombi et al., 2016, 2021), and GABA-A antagonists modulated PAC in human 2D cultures (Salimpour et al., 2024). If LFP-based descriptors respond in a similar way in hiPSC-derived networks, they could serve as readouts of compound effects that complement firing and bursting rates. The response of the LFP-NAS to such treatments was not tested here.

Finally, applying LFP-NAS to patient-derived neurons would offer a way to identify maturation alterations in neurodevelopmental disease models, extending the use of the index from studying normal development to screening phenotypes and drugs.

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