Integration of Stem Cell-Derived Neurons with Multi-Electrode Array

Master's thesis in Biotechnology

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DEPARTMENT OF LIFE SCIENCES

CHALMERS UNIVERSITY OF TECHNOLOGY
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Abstract

The prevalence of serious mental illnesses (SMI) in the US adult population, including bipolar disorder (BP), major depressive disorder (MDD), and schizophrenia (SCZ), is estimated at 4-6%. While the creation of *in vitro* models from human induced pluripotent stem cells (hiPSCs) has transformed disease modeling in such illnesses, further research is needed to optimize these models.

This project utilizes the human embryonic stem cell (hESC) line H1 to investigate the electrophysiological activity of stem cell-derived cortical neurons using Multi-Electrode Arrays (MEA). By integrating micro-electrodes into cell well-plates, neural activity can be non-destructively measured over several weeks with MEA. The objective is to optimize the integration of stem cell-derived neurons with MEA for monitoring electrophysiological activity, evaluating different cell densities, coatings, and culturing mediums.

Comprising a pilot study followed by a more extensive investigation, this project compares metrics such as activity and electrode coverage across various culturing conditions to identify optimal parameters. The most promising condition entails a cell density of 50k cells per well, coated with both Poly-L-Ornithine (PLO) and Biolaminin 521 (LN521), and cultured in either Neural Maintenance Medium (NMM) or BrainPhys (BP).

Acknowledgements

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Isabella Simonsson, Gothenburg, June 2024

Contents

1
Introduction

This chapter begins with a brief background on serious mental illnesses and how induced pluripotent stem cells has enabled *in vitro* tissue models of such illnesses. It also introduces the cells as well as the analysis tool that will be used in the project. Subsequently, the aim and the limits of the project are described and specified. Finally, societal and ethical aspects are addressed.

1.1 Background

Serious mental illness (SMI) commonly refers to a diagnosis of psychotic disorders, including bipolar disorders (BP), major depression disorder (MDD) and schizophrenia (SCZ) [2]. In the US, the prevalence of SMIs is estimated to 4-6% for adults.

Bipolar disorders (BD) are chronic mental disorders, with an estimated prevalence ranging from 1-4% [3]. There are several types of BD, including bipolar I disorder (BD-1) and bipolar II disorder (BD-2) [4]. While both types have episodes of depression for at least two weeks, the episodes typically last longer for people with BD-2 [5]. Another difference is that people with BD-1 experience episodes of mania which lasts at least one week and typically require hospitalization, while people with BD-2 experience episodes of hypomania (a less severe form of mania) that lasts a few days and does not require hospitalization.

In recent years, genome-wide association studies (GWAS) have been used to detect several genetic markers associated with BD, which have overlaps with other severe mental disorders such as SCZ and MDD [3]. With GWAS, BD-1 has been found to be highly genetically correlated with SCZ, while BD-2 is highly genetically correlated with MDD [4][6].

Several recent studies suggest that the classical psychedelic psilocybin can be used as a clinical treatment for MDD [7][8]. The active compund in psilocybin is psilocin [9]. It has been shown to significantly reduce the depressive symptoms and functional dysfunction, with few side effects. Another study found that a single dose of psilocybin lead to a 10% increase of the dendritic spine size and density in mice, as well as elevated excitatory neurotransmission [10].

The new generation of induced pluripotent stem cells (iPSCs) enables *in vitro* modeling of human tissues [11]. iPSCs are pluripotent stem cells derived by reprogram-

ming adult somatic cells, such as skin and blood cells. Thus, they have the ability to differentiate into multiple cell types. One major advantage with tissues derived from iPSCs is their nearly identical match to the cell donor, which is an important factor in disease modeling [12]. By using such models, phenotypes such as morphology, gene expression and cellular functions can be analyzed [3] as well as population neural activity and electrophysiology [11].

One way of measuring the electrical activity exhibited by neural populations is by using multi-electrode arrays (MEAs) [13]. MEA mesurements are non-destructive to the neural integrity, which enables monitoring over several weeks or months, providing insight to the long-term neurodevelopmental processes [14]. In MEA systems, the cell culture dishes are provided with a grid of small electrodes embedded in the bottom of each well [14]. The neuronal cells are cultured directly on top of the electrodes, which record extracellular action potentials exhibited by the cells. Thus, with simultaneous recording of spiking activity from multiple spatial locations, the firing activity of the whole network is measured. This can show how network dynamics can differ between patient groups or culturing conditions.

To perform these MEA analyses, a robust protocol must be established in the lab. For this, the human embryonic stem cell (hESC) line H1 will be used, which is one of the oldest and most commonly used hESC lines [15]. It is well-characterized and established, and is therefore a suitable line to use in protocol development, which is partly what will be done in this project.

1.2 Aim

The overall aim is to establish a protocol for integrating stem cell-derived neurons with MEA. This enables analyzation and comparison of electrophysiological behaviour between different culturing conditions. The specific aims are:

- Design and optimize the integration of stem cell-derived neurons with MEA for monitoring of electrophysiological activity.
- Analyze and compare the electrophysiological behavior of stem cell-derived neurons between different culturing conditions, including different cell densities, coatings, and mediums.

1.3 Scope/limitations

The brain consists of two main cell types, neurons and glia. A major distinction between the two is that glial cells do not participate directly in synaptic interactions and electrical signaling [16], while neurons are electrically excitable cells that transmit signals throughout the body [17]. Because of this, only neurons will be cultured and evaluated with MEA in this project. Although both spikes and bursts are detected in the MEA recordings, the bursts are not analyzed in this project due

to the time limit. Moreover, only the hESC line H1 will be used.

1.4 Societal and ethical aspects

A major controversy in the biotechnology field is the use of human embryonic stem cells (hESCs) which have ethical challenges [18]. To produce hESCs, human embryos need to be destroyed, leading to the debate about when life begins. Its use is consequently restricted or prohibited in several countries. Thus, the stem cell research field was revolutionized with the derivation of human iPSCs [19]. The iPSCs have the same differentiation abilities as hESCs, but are derived from the patients own somatic cells, overcoming the ethical challenges [18]. However, hESC lines such as H1 are commonly used, well-studied, and relatively easily available. This can be beneficial to hiPSC, making them difficult to replace despite ethical controversies. Moreover, the use of iPSCs creates new ethical and societal issues regarding the collection of genetic material and confidential personal information, making informed consent crucial.

Mortality studies have indicated that SMI are associated with a loss of approximately 10-20 years of life [20][4]. Moreover, suicide is more common with people with BD than with any other mental illness, and compared with the general population, people with BD are 20-30% more likely to die by suicide. Besides these individual burdens, there are economic burdens both for the individual and the society [21]. The per-patient lifetime burden of SMI has been estimated to $1.85 million dollars [22]. Moreover, the health care costs for BD are high, although not well studied [21]. In a study from 1991 the total annual costs for BD in the US was estimated to $45 billion: $7 billion were direct costs from inpatient and outpatient care, and $38 billion were indirect costs. The indirect costs included lost productivity of wage earners, caregivers, homemakers, persons in institutions, and persons who committed suicide.

It is thus of great importance to find correct treatments for people with SMI in order to reduce both individual and societal burdens. This can be facilitated using disease models with iPSCs. Although it requires extensive further research, this project can be a stepping stone towards the understanding of the neuronal electrophysiological behaviour of SMI patients.

2
Methods

In this chapter, the method used for differentiation of the stem cells to cortical neurons is briefly explained. Thereafter, the MEA system and its setup are described, followed by the experimental setup and data analysation. All reagents, chemicals used and media compositions as well as specified protocols mentioned in this chapter are found in Appendix A.

The project mainly consists of two parts: a pilot to test different cell densities and coatings when doing MEA recordings, followed by a more extensive experiment, based on the pilot, where different cell densities, coatings and medias were tested. Both experiments started with thawing neural progenitor cells (NPCs) that has previously been differentiated from the human embryonic stem cell (hESC) line H1. The NPCs where then further differentiated into cortical neurons.

2.1 Experiment 1

In this section, the methods and reagents used in experiment 1 are presented. It begins with an introduction of the conditions that were tested, and the experimental setup that was used. Thereafter, the differentiation of ESCs to cortical neurons is briefly explained, as well as the final seeding onto the MEA plate.

2.1.1 Conditions

Eight conditions with no replicates were studied in experiment 1. Two different coatings were used in the wells: Poly-L-Ornithine (PLO) together with Biolaminin 521 (LN521); and only LN521. See Appendix A.5.3 and A.5.2 for more detailed information. Furthermore, four different cell densities were used: 25k, 50k, 100k, and 150k cells per well. The media that was used in all wells was Neural Maintenance Media (NMM) (see Appendix A.2). Thus, the eight conditions are:

- 25k, PLO + LN521
- 25k, LN521
- 50k, PLO + LN521
- 50k, LN521
- 100k, PLO + LN521
- 100k, LN521
- 150k, PLO + LN521
- 150k, LN521

This setup is illustrated in Figure 2.1.

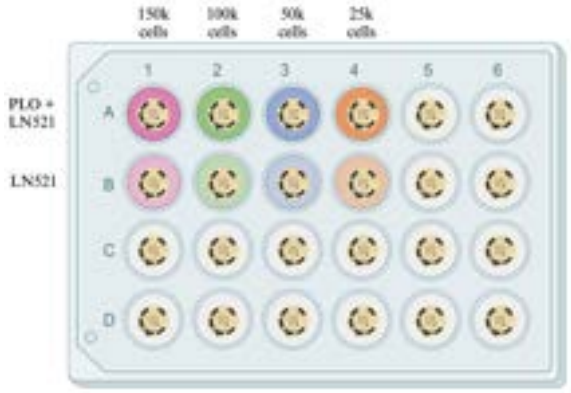


Figure 2.1: Experimental setup in experiment 1. Two different coatings: PLO + LN521 and LN521; and four different cell densities: 25k, 50k, 100k, and 150k cells per well were used. This resulted in eight different culturing conditions. The remaining wells were left empty. The figure is created using Biorender [23].

2.1.2 Differentiation of ESCs to cortical neurons

There is already an established protocol in the laboratory for differentiation of iPSCs to cortical neurons, based on Shi et al [24] that were used for the H1 cells in this project as well. The protocol is based on dual-SMAD inhibition. SMAD proteins maintain pluripotency and induce ectoderm and mesoderm germ layer specifications and their inhibition induce in vitro neuroectoderm formation [25]. The SMAD signaling inhibitors that were used were LDN193189 and SB431542. The protocol is found in Appendix A.8.

The cells that were used in this experiment were NPCs, from celline H1, that had been frozen on day 23 according to the protocol in Appendix A.4. One vial of H1 NPCs were thawed according to the protocol in Appendix A.3. The cells were seeded onto two wells in a 6-well plate coated with Laminin-521, and were resuspended in 2 ml Neural Maintenance Media (NMM) supplemented with 20 µl/ml FGF2. On day 28 after start of differentiation, the cells were passaged with accutase according to the protocol in Appendix A.6.

2.1.3 Final seeding on MEA plate

The method for the final seeding onto the MEA plate was based on a protocol from Axion Biosystems, which is found in Appendix A.9. The preparation of the MEA plate started two days before the final seeding of the cells. 50 µl PLO was added directly onto the electrodes on four of the wells (A1-A4), and the plate was incubated at 37°C, 5% CO2 overnight. The next day, the PLO was removed, and 50 µl PBS+/+ supplemented with 2,5 µl LN521 were added onto all eight wells (A1-A4, B1-B4).

On day 35 after start of differentiation, the cells were dissociated with Accutase according to the protocol in Appendix A.6, and finally seeded onto the MEA plate.

Before the last washing step, the cells were counted and distributed in four eppendorph tubes to obtain the wanted cell densities. After removing the supernatant, the cells were resuspended in 20 µl NMM combined with 20 µl/ml LN521. The laminin was removed from the MEA wells, after which 10 µl cell suspension (1/2 of the volume in each eppendorph tube) was added onto the electrodes in the coated wells.

The MEA plate was put in an incubator at 37°C, 5% CO2 for 1 h before 250 µl NMM was carefully added to the wells. After another hour in the incubator, additional 250 µl NMM was added to the wells, resulting in a final volume of 500 µl media. On day 1 and 2 post plating, 50% of the media was exchanged with new NMM, then for every two days after that. The first MEA recording was made on day 39, and the last on day 71. The recordings lasted approximately 15 minutes and were made about once a week.

2.2 Experiment 2

In this section, the methods and reagents used in experiment 2 are presented. It also begins with an introduction of the conditions that were tested, and the experimental setup that was used. Thereafter, the differentiation of ESCs to cortical neurons is briefly explained, as well as the final seeding onto the MEA plate. Finally, the timeline of how the different conditions were added is explained.

2.2.1 Conditions

The second experiment investigated twelve different conditions, each with at least three technical replicates. Two different coatings were used: PLO + LN521 and LN521 (see Appendix A.5.3 and A.5.2); two different cell densities were used: 50k and 90k cells per well; and three different medias were used: NMM (See Appendix A.2), Brainphys (BP) (see Appendix A.3) and NMM + Psilocin. Thus, the final twelve conditions are:

- 50k, PLO + LN521, NMM
- 50k, LN521, NMM
- 50k, PLO + LN521, BP
- 50k, LN521, BP
- 50k, PLO + LN521, NMM + Psilocin
- 50k, LN521, NMM + Psilocin
- 90k, PLO + LN521, NMM
- 90k, LN521, NMM
- 90k, PLO + LN521, BP
- 90k, LN521, BP
- 90k, PLO + LN521, NMM + Psilocin
- 90k, LN521, NMM + Psilocin

This resulted in two full 24-well plates, and the final setup is illustrated in Figure 2.2. However, initially, all wells contained only NMM as the culturing medium. Later in the experiment, on day 52 and 73 respectively, the media was replaced with BP or NMM supplemented with psilocin. This process is described in more detail in Section 2.2.4.

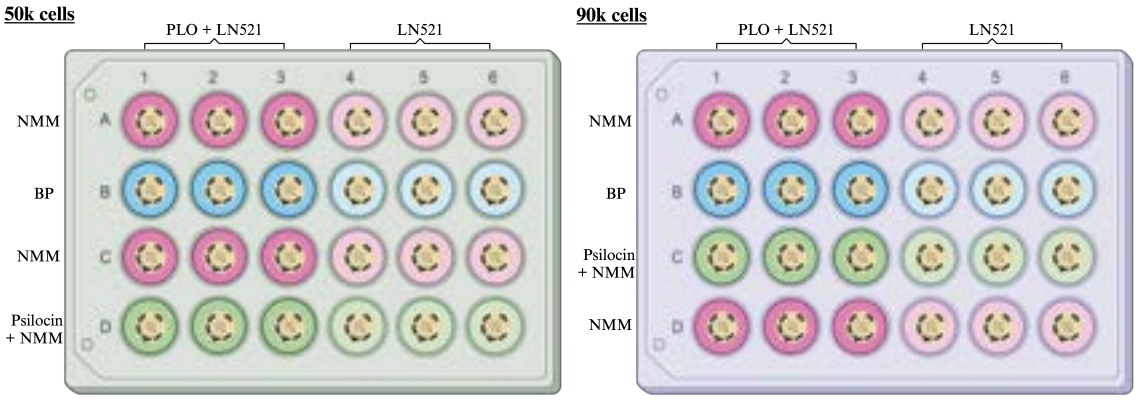


Figure 2.2: Experimental setup of experiment 2. Two different coatings were used: PLO + LN521 and LN521; two different cell densities were used: 50k and 90k cells per well; and three different medias were used: NMM, BP and NMM + Psilocin. The figure is created using Biorender [23].

2.2.2 Differentiation of ESCs to cortical neurons

The same protocol for differentiation was used in experiment 2 as in experiment 1, i.e. the protocol that is found in Appendix A.8. The cells that were used in this experiment were also NPCs, from celline H1, that had been frozen on day 23 according to the protocol in Appendix A.4.

Four vials of H1 NPCs were thawed according to the protocol in Appendix A.3 and seeded onto eight LN521-coated 6-wells in 2 ml NMM supplemented with 20 µl/ml FGF2. On day 24, two of the wells were confluent enough for a 1:3 accutase passage according to the protocol in Appendix A.6. On day 25, another four wells were passaged 1:1,5 with accutase. On day 26, the remaining two wells were also passaged 1:1,5 with accutase.

2.2.3 Final seeding on MEA plate

The method for the final seeding onto the MEA plate was, again, based on a protocol from Axion Biosystems, which is found in Appendix A.9. The preparation of the MEA plate started two days before the final seeding of the cells. 50 µl PLO was added directly onto the electrodes on half of the wells (A1-A3, B1-B3, C1-C3, D1-D3) on both plates, and the plates were incubated at 37°C, 5% CO2 overnight. The next day, the PLO was removed, and 50 µl PBS+/+ supplemented with 2,5 µl LN521 were added onto all 48 wells.

On day 32 for the 90k well-plate and on day 33 for the 50k well-plate, the cells were dissociated with accutase according to the protocol in Appendix A.6, and seeded onto their respective MEA plates. After the first washing step the cells were resuspended in NMM instead of wash media. They were counted and distributed in two falcon tubes to obtain the wanted cell densities, and were not washed a second time. After removing the laminin from the MEA wells, a 25 µl droplet of the cell

suspension was added onto the electrodes in each well.

The MEA plates were put in an incubator at 37°C, 5% CO2 for 1 h before 250 µl NMM was carefully added to the wells. After another hour in the incubator, additional 250 µl NMM was added to the wells, resulting in a final volume of 500 µl media. On day 1 and 2 post plating, 50% of the media was exchanged with new NMM, then every Monday, Wednesday and Friday after that. Additional LN521 (20 µl/ml medium) was added to all wells every Friday to increase the adherence. The first MEA recordings were made on day 43 and the last on day 89. The recordings were made for 30 minutes every Monday, Wednesday and Friday before the medium change. However, an exception from this was made on day 47, where the recordings only lasted for 15 minutes.

2.2.4 Addition of conditions

Until day 52 after start of differentiation, all wells contained NMM as their culturing medium, resulting in only four different condition between day 43 and day 52:

- 50k, PLO + LN521, NMM
- 50k, LN521, NMM
- 90k, PLO + LN521, NMM
- 90k, LN521, NMM

After the measurement made on day 52, half of the medium in twelve of the wells (see Figure 2.2) was replaced with 250 µl BP instead of NMM. BP has been found to accelerate neuronal and synaptic maturation compared to NMM in iPSC-derived cortical neurons[26], which is why it is assessed in this experiment. Thus, the measurements made from day 54 and forth contained eight different conditions:

- 50k, PLO + LN521, NMM
- 50k, LN521, NMM
- 50k, PLO + LN521, BP
- 50k, LN521, BP
- 90k, PLO + LN521, NMM
- 90k, LN521, NMM
- 90k, PLO + LN521, BP
- 90k, LN521, BP

The last four conditions were added on day 73, after the measurement was made. In twelve of the wells (see Figure 2.2), the NMM was supplemented with psilocin with a final concentration of 1 µM per well. Thus, the 250 µl NMM that was used in the medium change in each well contained 2 µM psilocin, since only half of the medium was replaced. The NMM supplemented with psilocin was added to the well rows with the highest resistance per electrode. The following measurements hence contain all twelve conditions:

- 50k, PLO + LN521, NMM
- 50k, LN521, NMM
- 50k, PLO + LN521, BP
- 50k, LN521, BP
- 50k, PLO + LN521, NMM + Psilocin
- 50k, LN521, NMM + Psilocin
- 90k, PLO + LN521, NMM
- 90k, LN521, NMM
- 90k, PLO + LN521, BP
- 90k, LN521, BP
- 90k, PLO + LN521, NMM + Psilocin
- 90k, LN521, NMM + Psilocin

2.3 MEA

This section describes the multi-electrode array (MEA) system used in the project. It begins with an introduction to the hardware and software used for recordings. Next, it explains the metrics measured during the recordings and provides definitions. Finally, the data analysis process is described.

The MEA system measures neurophysiological properties of neuronal cultures *in vitro* and provide recordings of their spontaneous electrical activity [27]. Thus, proper development of the neuronal activity of a culture can be investigated by this method. The MEA system that was used was Maestro Edge from Axion Biosystems [28], and the plates that were used were CytoView MEA 24 from Axion Biosystems [29].

2.3.1 MEA system

The MEA measurements were performed on assay days using Maestro Edge, and Axion Integrated Studio (AxIS) Navigator software was used to record the measurements, according to the user guide [30]. The spontaneous neural real-time configuration was used to acquire spontaneous spikes and bursts, i.e. extracellular action potentials exhibited by the cells attached to the electrodes.

The Environment Control panel was used to configure the Maestro Edge prior to the recordings. The temperature was set to 37°C and the CO2 to 5%, which is the same condition as in the cell incubator. Moreover, all recordings were made prior to medium changes.

2.3.2 MEA plates

The plates CytoView MEA 24 contain 24 wells, each containing 16 electrodes in a 4 x 4 grid [31]. 15 of the electrodes have a diameter of 50 µm, while the last electrode has a 300 µm diameter and is used for stimulation. The electrode spacing is 350 µm, and the recording area is 1.1 mm x 1.1 mm. The microelectrodes are made of PEDOT, and SU-8 is the insulation/surface material.

The electrodes allows for detection of local field potentials displayed as spikes (arising from single action potentials) and burst activity (arising from groups of action

potentials) at different positions and times within a neuronal network [27].

2.3.3 Cell viability acquisition

Cell coverage and viability were measured with the MEA Viability Software Module, using the Maestro's impedance technology to assess the cells over each microelectrode [32]. An electrical signal of 41.5 kHz was delivered to the electrodes, and the recorded impedance was used to measure cell density, coverage and viability. The number of cells attached to the electrodes, as well as their membrane integrity, determines how easily the signal passes through the cell-electrode interface to the media. Thus, a higher impedance was measured when viable cells cover the electrode. Similarly, when the electrode was uncovered or when the cell membrane integrity was disrupted, the electrical signal passed through the cell-electrode interface more easily, and impedance was decreased. The resistance at 41.5 kHz is highly correlated with cell density and coverage, which is why MEA viability is reported as the resistance. For an electrode to be considered covered, the resistance had to be greater than the Covered Electrode Threshold, which was set to 18 kΩ by default.

2.3.4 Neuronal activity

Using the AxIS Navigator software, the spontaneous neuronal activity was recorded [30]. Mammalian neurons typically produce action potential widths of approximately 1-2 ms, with peak amplitudes ranging from 20 μV and 150 μV [32].

The measured 'spikes' are defined as threshold crossings in the continuous data stream. They are detected by the Spike Detector, which identifies the spike timing and location. The threshold detection method that is used is called Adaptive Threshold, and is set on a per electrode basis as a multiple of the noise of the continuous data, each electrode threshold is specific to that electrode. The adaptive threshold is set to 6 x standard deviations, which minimize both false-positives and missed detections.

Furthermore, the firing rate is defined as the total number of spikes divided by the duration of the analysis, in Hz. For an electrode to be defined as 'active', more than 5 spikes per minute must be detected. This means that an electrode has to have a mean firing rate greater than 0.08333 Hz to be considered active.

2.4 Data Analyzation

The data from the recordings were first exported as .csv files into Excel, where the data from each individual electrode within each well was extracted and imported into R studio. In addition, a metadata file was created for each measurements, containing information about the cell density, coating and culturing medium for each well. All code used in the data analyzation are available through GitHub.

In R studio, the data was first processed and prepared for downstream analysis. The data was filtered by removing data from uncovered electrodes, i.e. electrodes with a resistance < 18 kΩ. Next, the electrode data was combined within each well, and means and standard deviations were calculated for all relevant parameters, to get the average well data. After that, the data was prepared for plotting by grouping the well data within each condition, to get the means and standard deviation for each condition.

The p-values were calculated with Anova and Tukey's HSD test. An adjusted p-value was considered significant if it was 0.05 or lower. The significance is indicated in the plots with asterisks: * if $P \leq 0.05$; ** if $P \leq 0.01$; and *** if $P \leq 0.001$.

3
Results

This chapter contains all results from the data analysis, where the data from the MEA recordings were analysed and different conditions were compared. First, the results from experiment 1 are presented, followed by the results from experiment 2.

3.1 Experiment 1

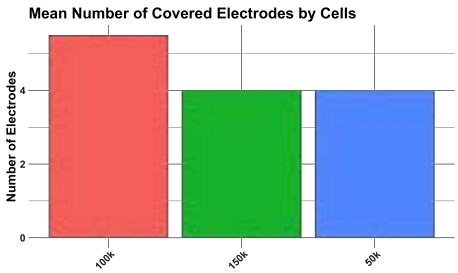
In this section, the results from Experiment 1 are presented and compared. First, bar charts compare the cell densities and coatings respectively, where the significant differences are indicated with asterisks. Images of the wells at different time points are then presented, providing a visual insight of how the networks develop over time.

The results from the measurement made on day 47 are shown in Figure 3.1. For the mean number of covered electrodes there is a significant difference between the coatings, where LN521 has a higher value (see Figure 3.1 (b)). The only other parameter showing any significant differences is mean firing rate (Figure 3.1 (e)), where the cell densities have a significant effect. The wells with 50k cells have the highest firing rate, and is significantly higher than both 100k and 150k. Moreover, 150k has a significantly higher firing rate than 100k.

When comparing Figures 3.1 (a) and (c), it can be seen that all electrodes that are covered are also active for 150k and 50k, but none of the covered electrodes for 100k are considered active. Furthermore, there are no bars showing the results from the wells with 25k cells, meaning that their coverage was too low to be included in the statistical analysis.

Figure 3.2 shows the results from the measurement made on day 59. As in the previous measurement, the wells with 50k cells have the highest mean firing rate (see Figure 3.2 (e)), which is significantly higher than for the other cell densities. Apart from that, there are no statistical differences between the cell densities or coatings. However, the number of covered electrodes has increased for most cell densities compared to the previous measurement, while the activity has decreased for the 150k wells.

The last measurement of Experiment 1 was made on day 71, and the results are found in Figure 3.3. The mean firing rate has decreased for the 50k wells, and is now significantly lower than for the 100k wells, but still higher than for the 150k



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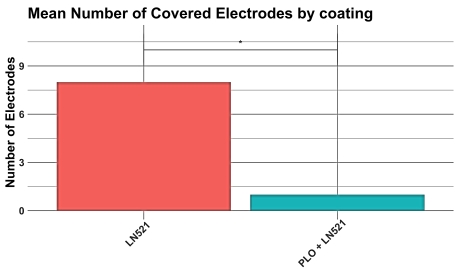

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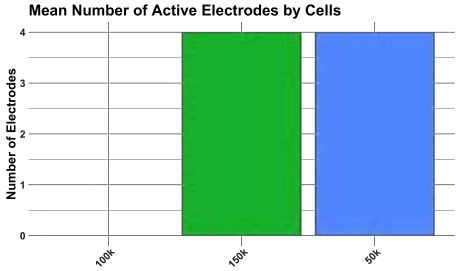

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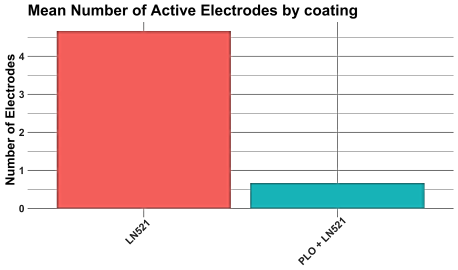

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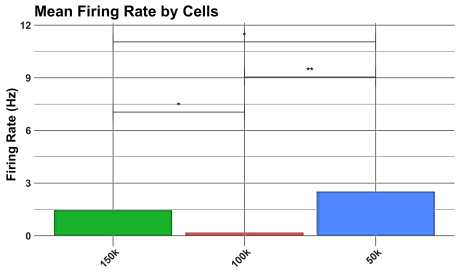

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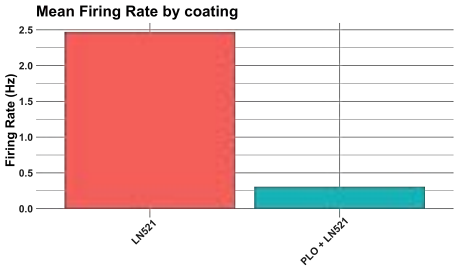

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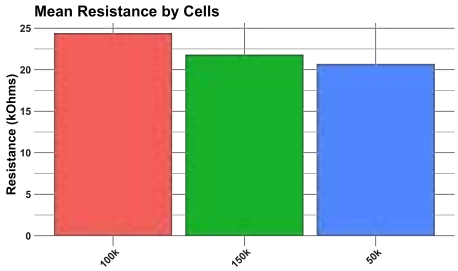
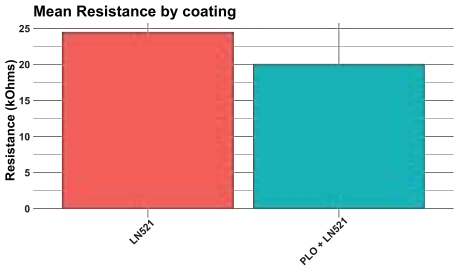
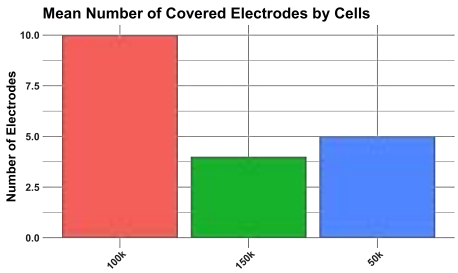


(g)

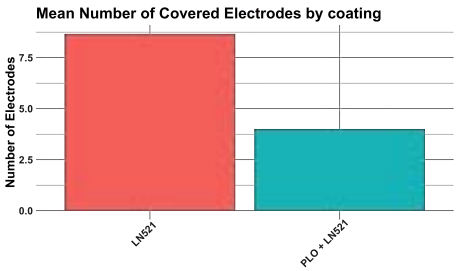


(h)

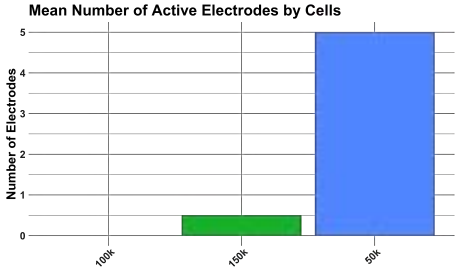
Figure 3.1: Results from MEA recording Day 47. Sub-figures (a) and (b) depict the mean number of covered electrodes per well, with each well containing 16 electrodes. The results in the plots are grouped by cell density or coating respectively. Similarly, sub-figures (c) and (d) show the mean number of active electrodes per well. Sub-figures (e) and (f) show the mean firing rate per well, i.e. spikes per second. Finally, sub-figures (g) and (h) depict the mean resistance per covered electrode within each group. Significance levels are indicated in the plots with asterisks: * if $P \leq 0.05$; ** if $P \leq 0.01$; and *** if $P \leq 0.001$.



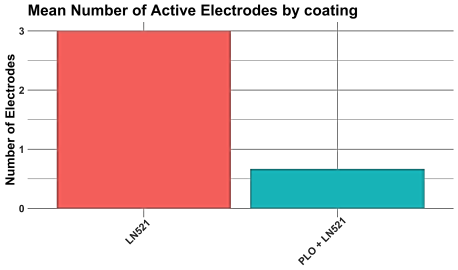
(a)



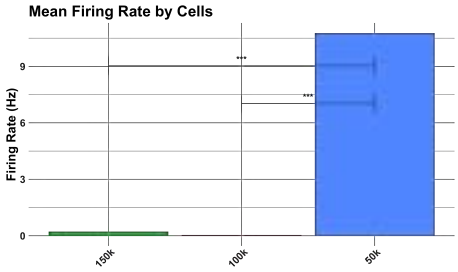
(b)



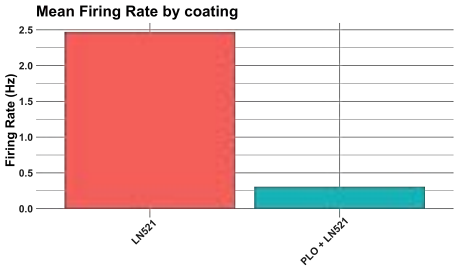
(c)



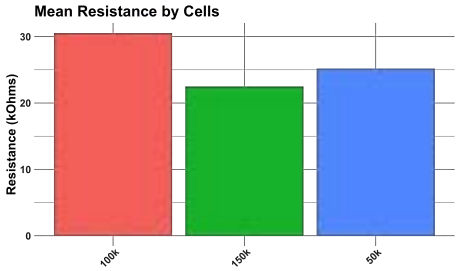
(d)



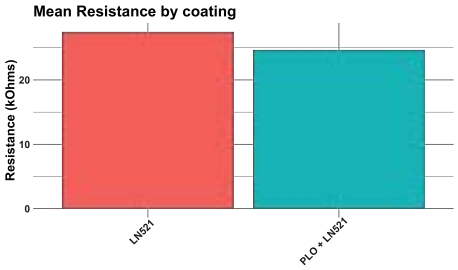
(e)



(f)



(g)



(h)

Figure 3.2: Results from MEA recording on Day 59. Sub-figures (a) and (b) depict the mean number of covered electrodes per well, with each well containing 16 electrodes. The results in the plots are grouped by cell density or coating respectively. Similarly, sub-figures (c) and (d) show the mean number of active electrodes per well. Sub-figures (e) and (f) show the mean firing rate per well, i.e. spikes per second. Finally, sub-figures (g) and (h) depict the mean resistance per covered electrode within each group. Significance levels are indicated in the plots with asterisks: * if $P \leq 0.05$; ** if $P \leq 0.01$; and *** if $P \leq 0.001$.

wells. This can be seen in Figure 3.3 (c), as well as that the 100k wells also have significantly higher firing rate than the 150k wells.

There are no figures showing the mean number of active electrodes because there were no active electrodes during this measurement. Moreover, Figure 3.3 (e) shows that the 100k wells have significantly higher mean resistance than the other cell densities. But there are no significant differences between the coatings.

Figures 3.4 - 3.11 show how the cell networks for all conditions develop over time between day 42 and day 67. It can be seen in Figures 3.4 and 3.5 that the cells in the 25k wells do not change much over time, and that the cells do not cover the electrodes. This is consistent with the results in the plots. Moreover, complex networks have formed in the 100k and 150k wells, as can be seen in Figures 3.8, 3.9, 3.10 and 3.11. This is in line with the increasing number of covered electrodes found in Figures 3.1, 3.2 and 3.3. In Figure 3.6, the cells have formed clumps, but not as intricate networks as in the wells with 100k and 150k cells.

To summarize experiment 1, no data from the 25k wells were included in the results due to the electrode coverage not being sufficient. Moreover, some significant differences were found, mainly between the cell densities. In the first two measurements, the 50k wells had the highest firing rates, but this had changed in the last measurement where the 100k wells had the highest firing rate.

The coatings had a significant difference in the first measurement, where LN521 had a significantly higher number of covered electrodes than LN521 + PLO. Although LN521 had higher values in all measurements, that was the only significant difference. However, no big differences between the coatings were observed from the images.

3.2 Experiment 2

This section contains the results from experiment 2, and is divided into three parts based on the number of conditions at different times. The bar chart compare each condition, and significant differences are indicated with asterisks. Finally, brightfield images of the four most active conditions are presented in a timeline.

3.2.1 Day 43 - Day 52: 4 Conditions

The first MEA recording was made on day 43, i.e. 10 and 11 days after the final seeding on the MEA plates for the 50k plate and 90k plate respectively. The results from that recording is found in Figure 3.12. It can be seen in Figure 3.12 (a) that almost all wells had covered electrodes, however in Figure 3.12 (b) it can be seen that both [90k, PLO + LN521, NMM] and [90k, LN521, NMM] have significantly higher numbers of covered electrodes in total. Moreover, [50k, PLO + LN521, NMM] has

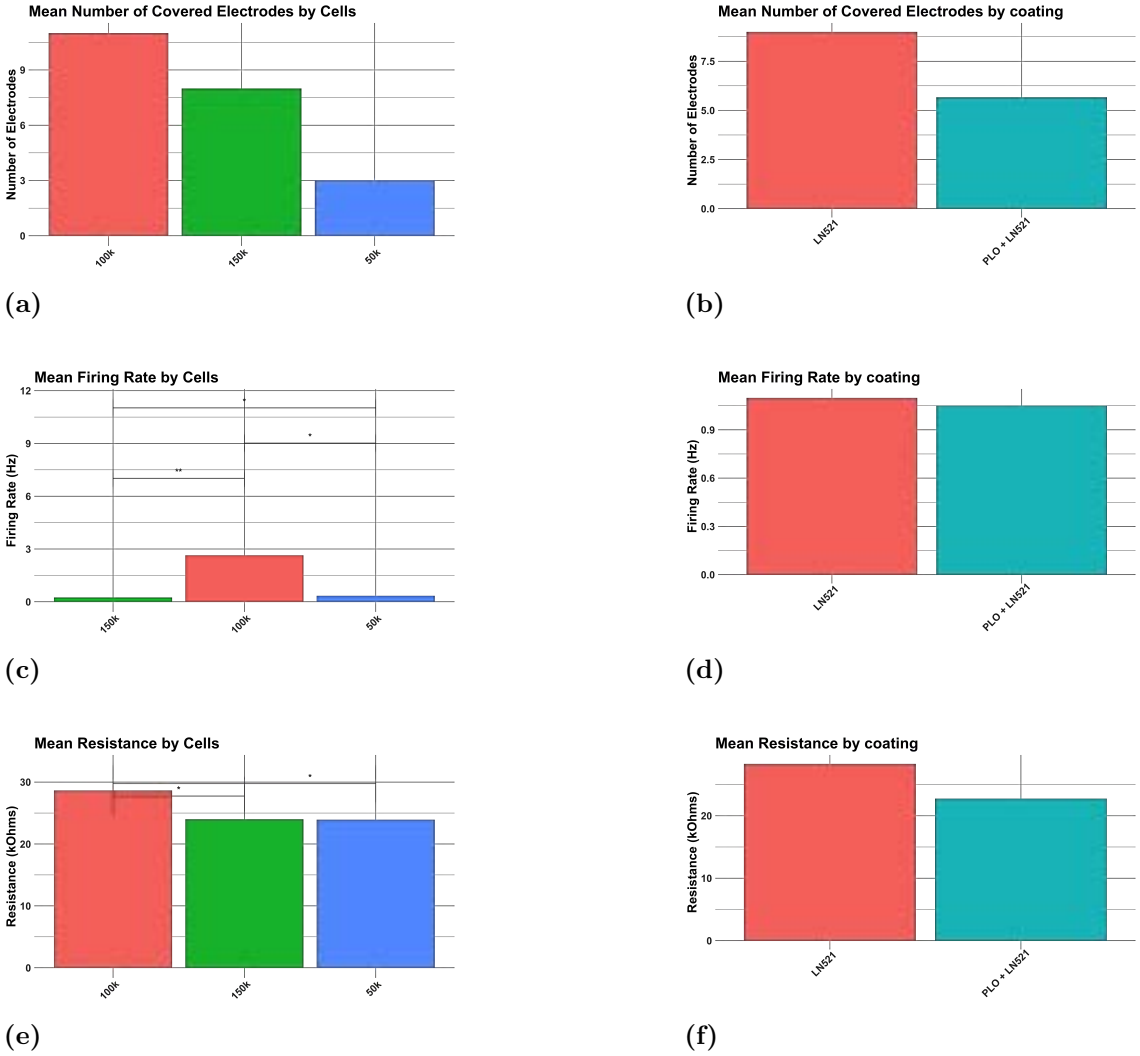
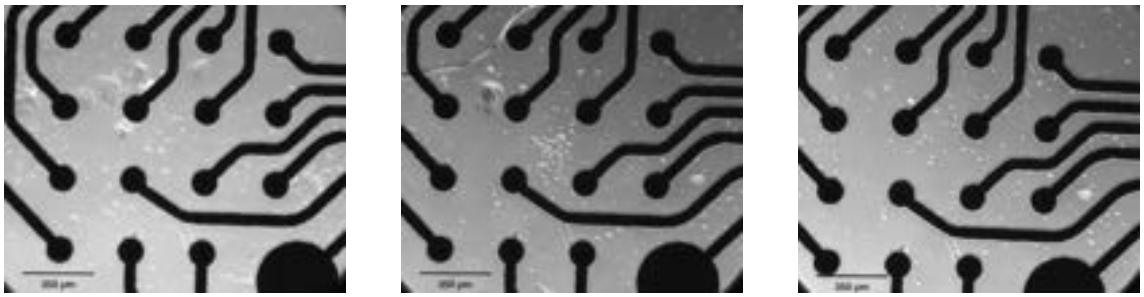
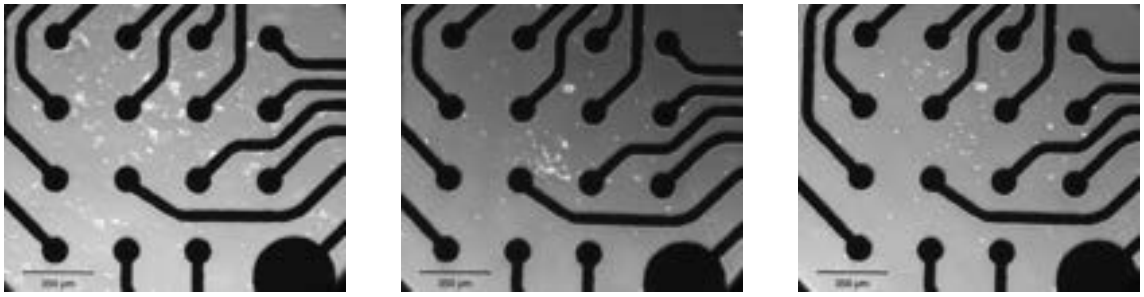


Figure 3.3: Results from MEA recording on Day 71. Sub-figures (a) and (b) depict the mean number of covered electrodes per well, with each well containing 16 electrodes. The results in the plots are grouped by cell density or coating respectively. Sub-figures (c) and (d) show the mean firing rate per well, i.e. spikes per second. Finally, sub-figures (e) and (f) depict the mean resistance per covered electrode within each group. Significance levels are indicated in the plots with asterisks: * if $P \leq 0.05$; ** if $P \leq 0.01$; and *** if $P \leq 0.001$.



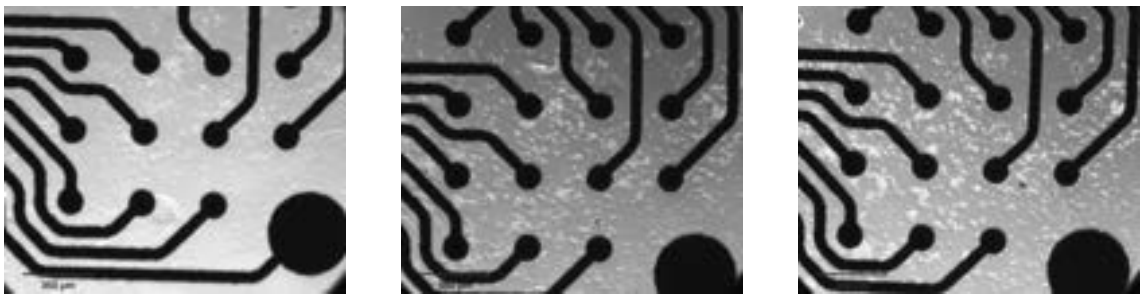
(a) Day 42 (b) Day 59 (c) Day 67

Figure 3.4: Brightfield images of the well with condition [25k, PLO + LN521] at three time points (Day 42, Day 59, and Day 67). Images are captured at 10x magnification.



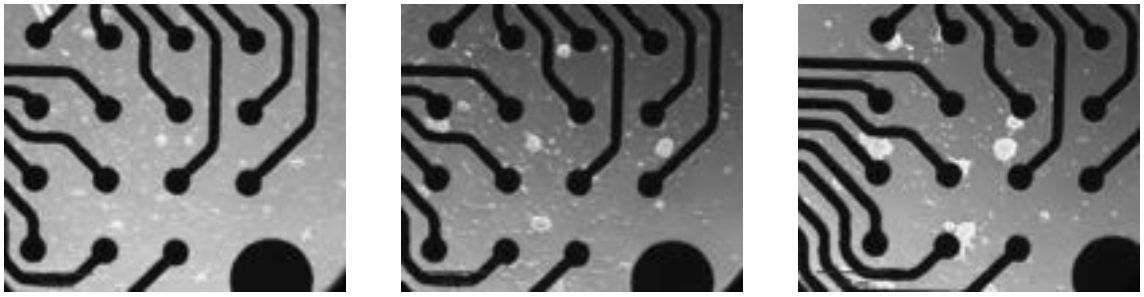
(a) Day 42 (b) Day 59 (c) Day 67

Figure 3.5: Brightfield images of the well with condition [25k, LN521] at three time points (Day 42, Day 59, and Day 67). Images are captured at 10x magnification.



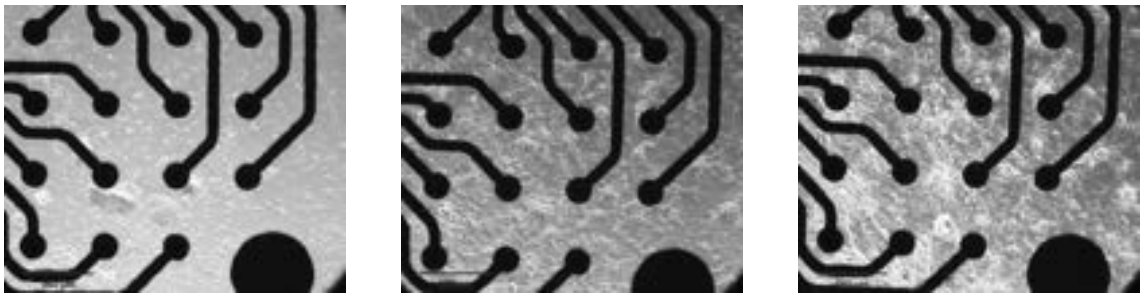
(a) Day 42 (b) Day 59 (c) Day 67

Figure 3.6: Brightfield images of the well with condition [50k, PLO + LN521] at three time points (Day 42, Day 59, and Day 67). Images are captured at 10x magnification.



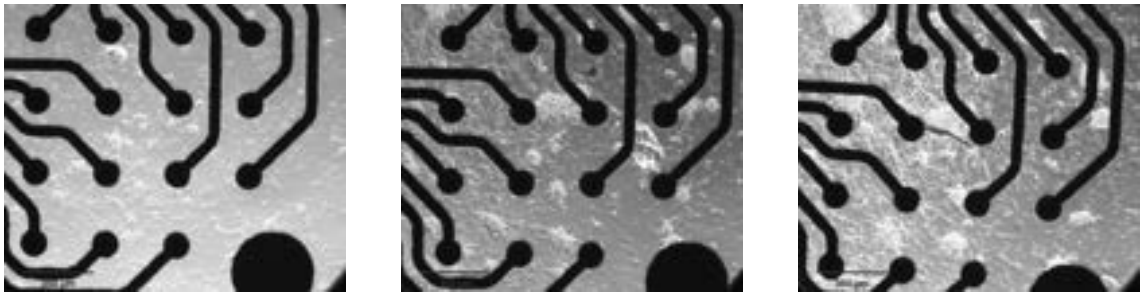
(a) Day 42 **(b)** Day 59 **(c)** Day 67

Figure 3.7: Brightfield images of the well with condition [50k, LN521] at three time points (Day 42, Day 59, and Day 67). Images are captured at 10x magnification.



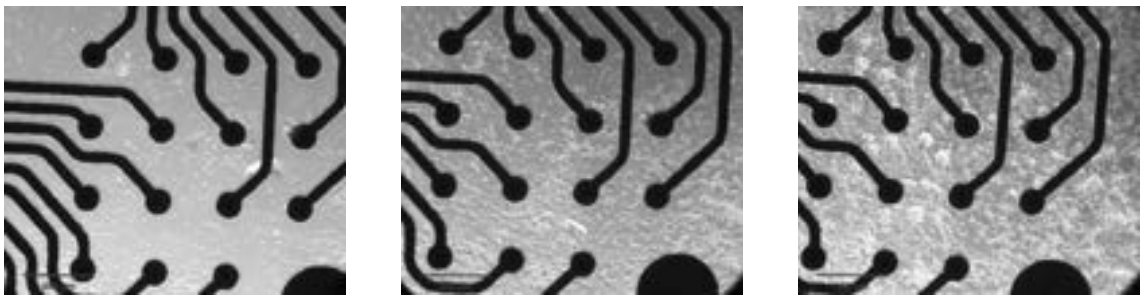
(a) Day 42 **(b)** Day 59 **(c)** Day 67

Figure 3.8: Brightfield images of the well with condition [100k, PLO + LN521] at three time points (Day 42, Day 59, and Day 67). Images are captured at 10x magnification.



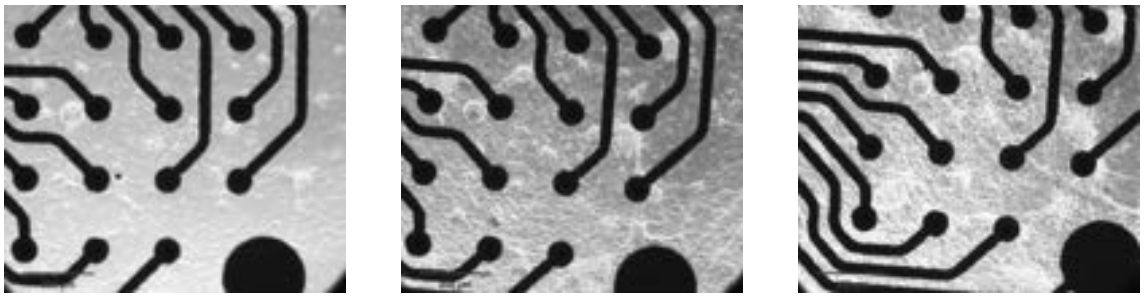
(a) Day 42 **(b)** Day 59 **(c)** Day 67

Figure 3.9: Brightfield images of the well with condition [100k, LN521] at three time points (Day 42, Day 59, and Day 67). Images are captured at 10x magnification.



(a) Day 42 **(b)** Day 59 **(c)** Day 67

Figure 3.10: Brightfield images of the well with condition [150k, PLO + LN521] at three time points (Day 42, Day 59, and Day 67). Images are captured at 10x magnification.



(a) Day 42 **(b)** Day 59 **(c)** Day 67

Figure 3.11: Brightfield images of the well with condition [150k, LN521] at three time points (Day 42, Day 59, and Day 67). Images are captured at 10x magnification.

a significantly higher number of covered electrodes than [50k, LN521, NMM].

Figures 3.12 (c), (d) and (e) show that [90k, PLO + LN521, NMM] has a significantly higher number of active electrodes, as well as mean number of spikes and mean firing rate compared to the other conditions. Moreover, it has a significantly higher mean resistance than the other conditions, and Figure 3.12 (f) also shows that [90k, LN521, NMM] has a significantly higher mean resistance than [50k, LN521, NMM] as well.

The results from the recording made on day 47 are found in Figure 3.13. The number of wells per condition, number of covered electrodes and mean resistance have remained approximately the same since the measurement made on day 43, as can be seen in Figures 3.13 (a), (b) and (f) respectively. However, in Figures 3.13 (c), (d) and (e) a decrease in the activity of [90k, PLO + LN521, NMM] can be seen. This has lead to [50k, PLO + LN521, NMM] having the highest activity, and it has a significantly higher mean number of spikes and mean firing rate than both [50k, LN521, NMM] and [90k, LN521, NMM].

Figure 3.14 shows the results from the measurement made on day 52. A loss of coverage for [50k, LN521, NMM] can be seen in Figure 3.14 (a), as the number of wells has decreased. Out of twelve wells, only seven have any covered electrodes. Besides that, the number of wells per condition, number of covered electrodes and mean resistance show similar results as previous measurements, as can be seen in Figures 3.14 (a), (b) and (f). [90k, PLO + LN521, NMM] and [90k, LN521, NMM] continue to have significantly higher number of covered electrodes and mean resistance than [50k, PLO + LN521, NMM] and [50k, LN521, NMM].

Furthermore, all conditions have an increased number of active electrodes compared to the last measurement, as well as increased mean firing rate. This can be seen in Figures 3.14 (c) and (e). Additionally, the mean number of spikes, in Figure 3.14 (d) have increased compared to the last measurement. However, this is at least partly due to the different lengths of the measurements.

To summarize, [50k, PLO + LN521, NMM] and [90k, PLO + LN521, NMM] consistently showed higher numbers of active electrodes, mean number of spikes and mean firing rates than [50k, LN521, NMM] and [90k, LN521, NMM], although not always significantly higher. However, there are big differences between the number of covered electrodes and the number of active electrodes. Moreover, [50k, LN521, NMM] had significantly lower number of covered electrodes than all other conditions throughout all recordings. Furthermore, [90k, PLO + LN521, NMM] had the highest mean resistance per covered electrode, which was significantly higher than for the other conditions.

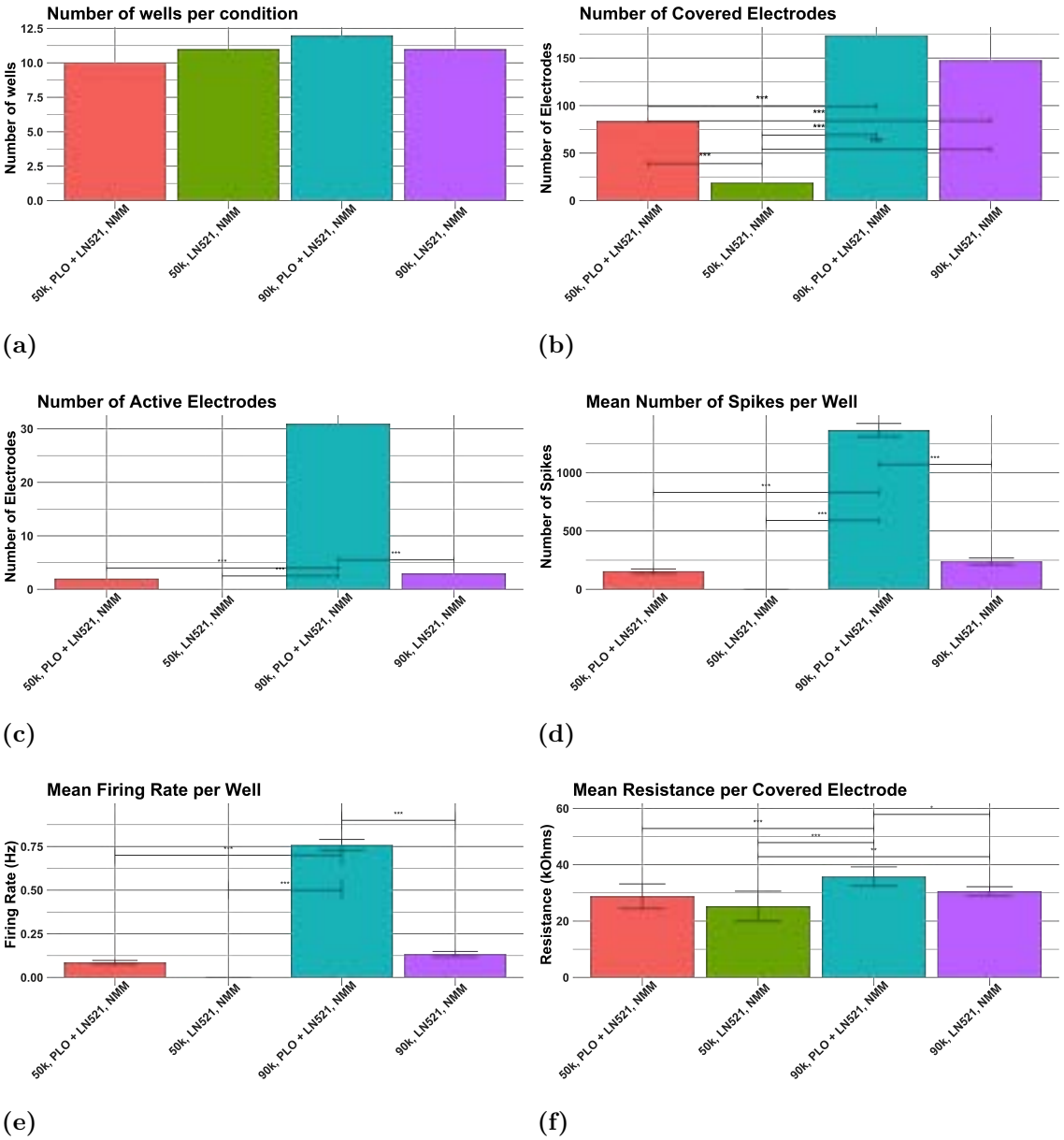


Figure 3.12: Results from MEA recording on day 43. Sub-figure (a) depict the number of wells with at least one covered electrode for each condition. Each condition has 12 wells. Sub-figure (b) depict the total number of covered electrodes per condition, with each well containing 16 electrodes. Similarly, sub-figure (c) shows the total number of active electrodes per condition. Sub-figure (d) shows the mean number of spikes per well that were measured during the entire length of the recording, while sub-figure (e) shows the mean firing rate per well, i.e. spikes per second. Finally, sub-figure (f) depict the mean resistance per covered electrode within each group. Significance levels are indicated in the plots with asterisks: * if $P \leq 0.05$; ** if $P \leq 0.01$; and *** if $P \leq 0.001$.

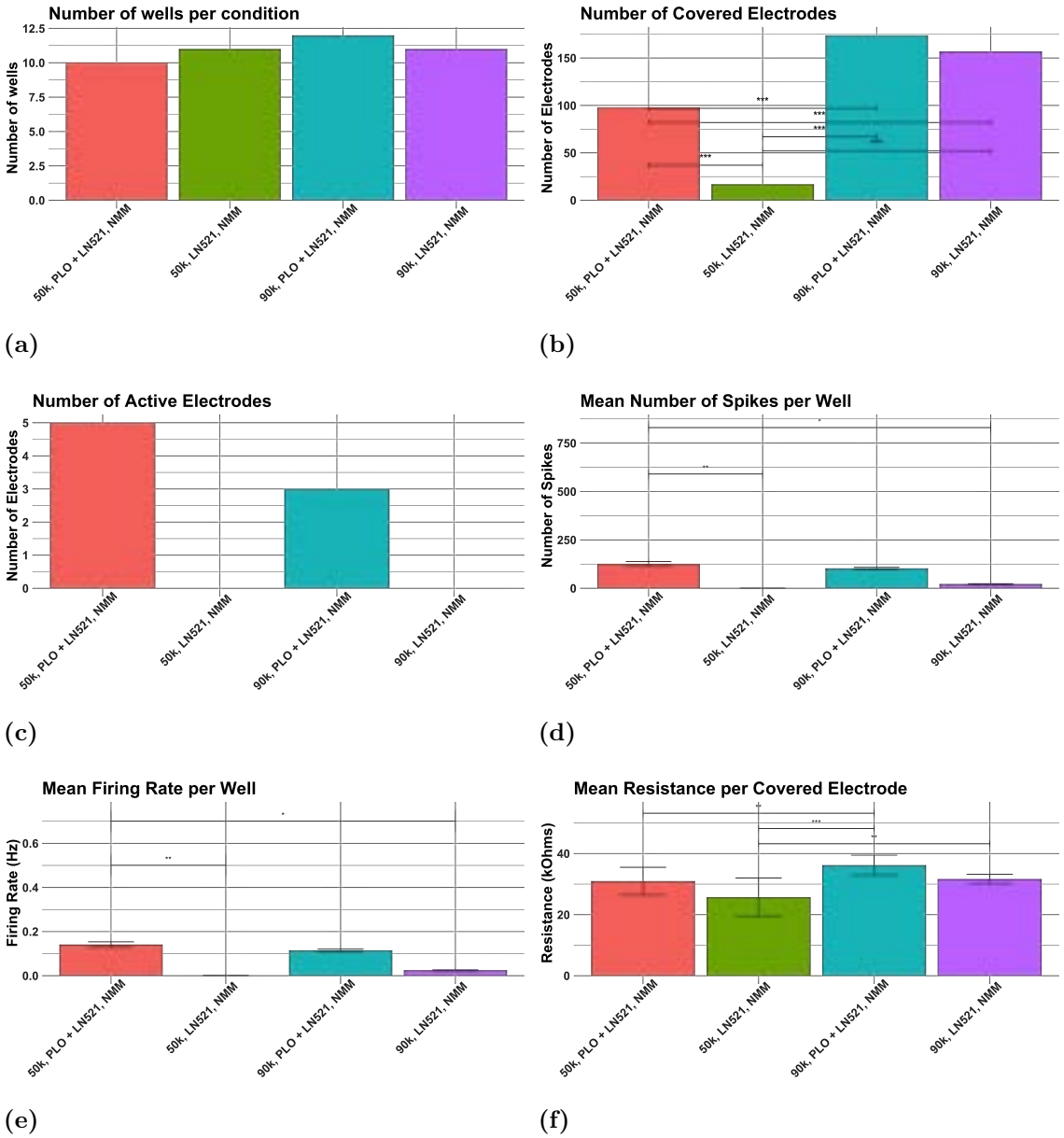


Figure 3.13: Results from MEA recording on day 47. Sub-figure (a) depict the number of wells with at least one covered electrode for each condition. Each condition has 12 wells. Sub-figure (b) depict the total number of covered electrodes per condition, with each well containing 16 electrodes. Similarly, sub-figure (c) shows the total number of active electrodes per condition. Sub-figure (d) shows the mean number of spikes per well that were measured during the entire length of the recording, while sub-figure (e) shows the mean firing rate per well, i.e. spikes per second. Finally, sub-figure (f) depict the mean resistance per covered electrode within each group. Significance levels are indicated in the plots with asterisks: * if $P \leq 0.05$; ** if $P \leq 0.01$; and *** if $P \leq 0.001$.

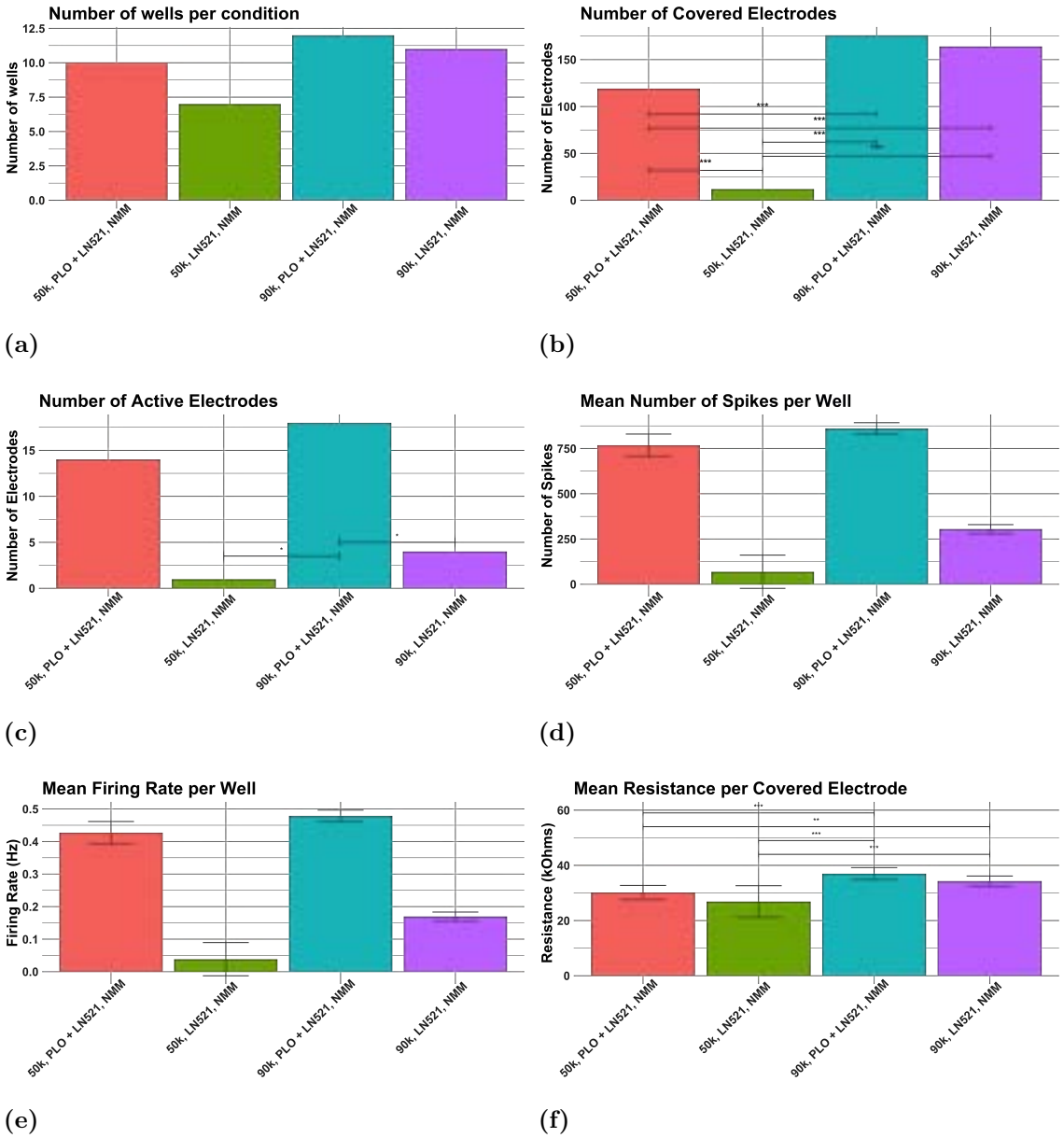


Figure 3.14: Results from MEA recording on day 52. Sub-figure (a) depict the number of wells with at least one covered electrode for each condition. Each condition has 12 wells. Sub-figure (b) depict the total number of covered electrodes per condition, with each well containing 16 electrodes. Similarly, sub-figure (c) shows the total number of active electrodes per condition. Sub-figure (d) shows the mean number of spikes per well that were measured during the entire length of the recording, while sub-figure (e) shows the mean firing rate per well, i.e. spikes per second. Finally, sub-figure (f) depict the mean resistance per covered electrode within each group. Significance levels are indicated in the plots with asterisks: * if $P \leq 0.05$; ** if $P \leq 0.01$; and *** if $P \leq 0.001$.

3.2.2 Day 54 - Day 73: 8 Conditions

Two days after replacing half of the NMM with BP in three wells for each condition, another MEA recording was made. This was day 54, and the results are found in Figure 3.15. The mean number of spikes and mean firing rate are significantly higher for [50k, PLO + LN521, BP] and [90k, PLO + LN521, BP] compared to almost all other conditions, as can be seen in Figures 3.15 (d) and (e). Moreover, [90k, PLO + LN521, BP] has a significantly higher number of active electrodes compared to all other conditions. While all condition with LN521 coating have few active electrodes in comparison to the conditions with PLO + LN521 coating.

On day 64, another recording was made that is found in Figure 3.16. There seems to be a correlation between the number of wells per condition and the number of covered electrodes, which is expected. This can be seen in Figures 3.16 (a) and (b). But although [90k, PLO + LN521, NMM] and [90k, LN521, NMM] have the highest number of covered electrodes, they have very little activity. In Figures 3.16 (c), (d) and (e) it can be seen that [50k, PLO + LN521, BP] has a significantly higher number of active electrodes, mean number of spikes and mean firing rate than all other conditions. Moreover, [50k, PLO + LN521, NMM] also have some active electrodes and some activity, although significantly lower than [50k, PLO + LN521, BP]. There are also significant differences in the mean resistance, where [90k, PLO + LN521, BP] has the highest value, followed by [90k, PLO + LN521, NMM].

The results from the measurement made on day 73 are found in Figure 3.17. The distribution is similar to the previous measurement, but with less significant differences. As can be seen in Figures 3.17 (c), (d) and (e), [50k, PLO + LN521, BP] still has the highest number of active electrodes, mean number of spikes and mean firing rate, although not as high as in day 64. Furthermore, even though [90k, PLO + LN521, NMM] and [90k, LN521, NMM] have the highest number of covered electrodes, neither condition have any activity.

In summary, the addition of BP had rapid effect on the activity, as both [50k, PLO + LN521, BP] and [90k, PLO + LN521, BP] had significantly higher activity than [50k, PLO + LN521, NMM] and [90k, PLO + LN521, NMM] respectively after two days. However, on day 64, the activity in [90k, PLO + LN521, BP] had decreased, while it had increased in [50k, PLO + LN521, BP]. On day 73, [50k, PLO + LN521, BP] still had the highest activity, although not as high as on day 64.

3.2.3 Day 75 - Day 87: 12 Conditions

Two days after adding psilocin to twelve of the wells, another MEA recording was made, i.e. on day 75. The results are found in Figure 3.18, and it shows no significant differences between the conditions. However, Figure 3.18 (c) shows that only the wells coated with PLO + LN521 have any active electrodes. Moreover, only [50k, PLO + LN521, BP] and [90k, PLO + LN521, BP] have a high mean number of spikes and mean firing rate, as can be seen in Figures 3.18 (d) and (e).

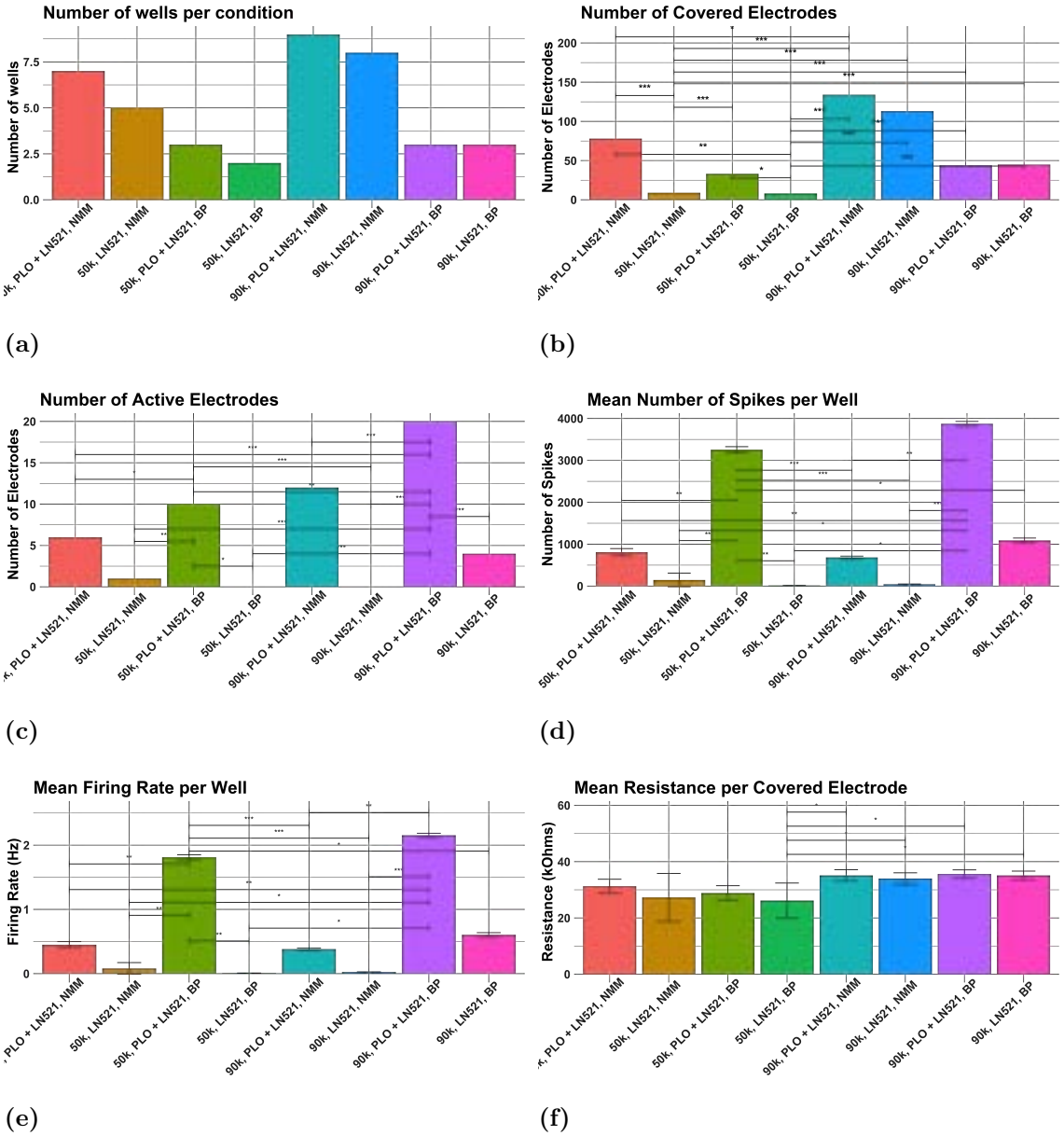


Figure 3.15: Results from MEA recording on day 54. Sub-figure (a) depict the number of wells with at least one covered electrode for each condition. Conditions [50k, PLO + LN521, NMM], [50k, LN521, NMM], [90k, PLO + LN521, NMM] and [90k, LN521, NMM] have 9 wells each, while the remaining conditions have 3 wells each. Sub-figure (b) depict the total number of covered electrodes per condition, with each well containing 16 electrodes. Similarly, sub-figure (c) shows the total number of active electrodes per condition. Sub-figure (d) shows the mean number of spikes per well that were measured during the entire length of the recording, while sub-figure (e) shows the mean firing rate per well, i.e. spikes per second. Finally, sub-figure (f) depict the mean resistance per covered electrode within each group. Significance levels are indicated in the plots with asterisks: * if $P \leq 0.05$; ** if $P \leq 0.01$; and *** if $P \leq 0.001$.

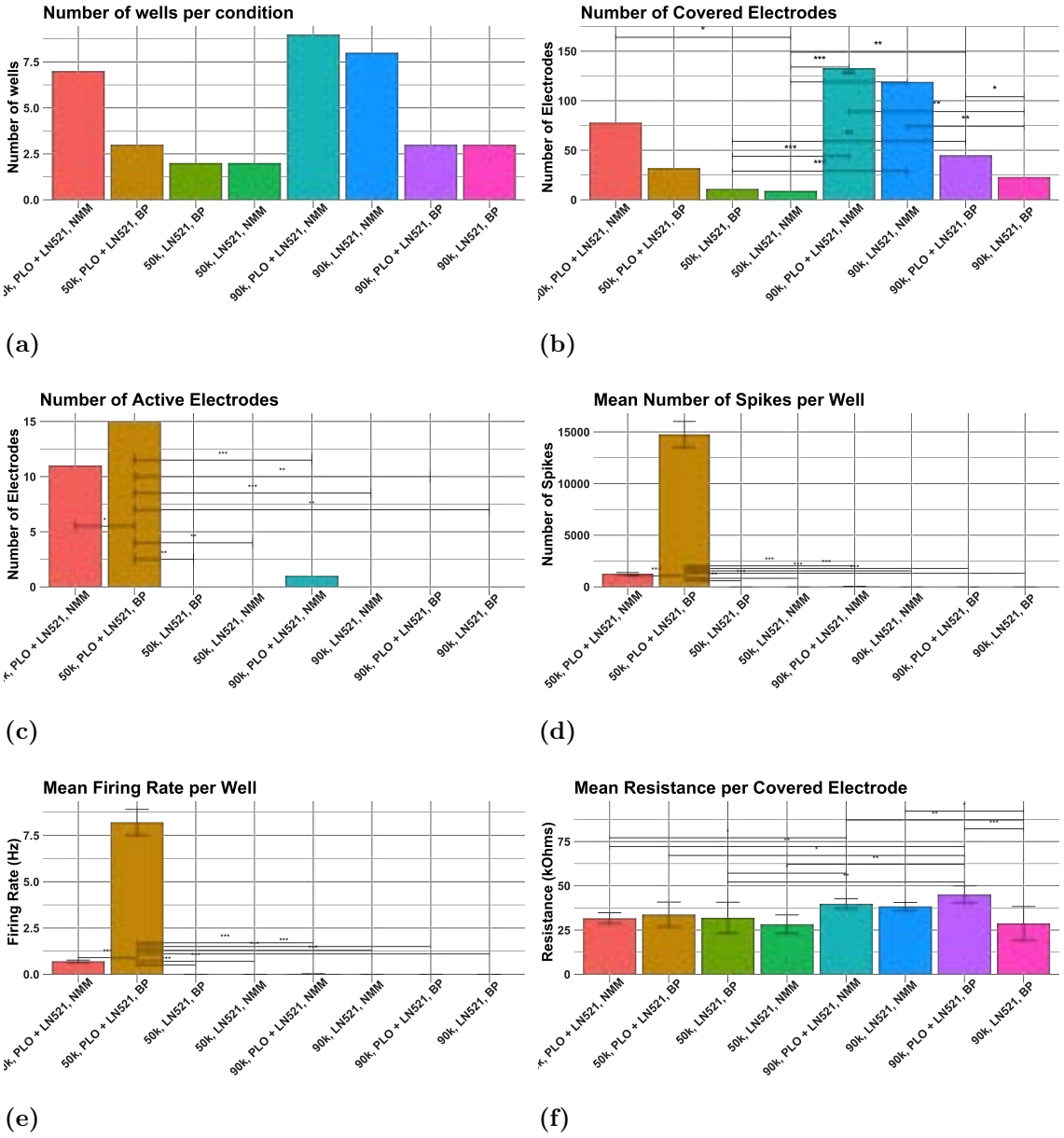


Figure 3.16: Results from MEA recording on day 64. Sub-figure (a) depict the number of wells with at least one covered electrode for each condition. Conditions [50k, PLO + LN521, NMM], [50k, LN521, NMM], [90k, PLO + LN521, NMM] and [90k, LN521, NMM] have 9 wells each, while the remaining conditions have 3 wells each. Sub-figure (b) depict the total number of covered electrodes per condition, with each well containing 16 electrodes. Similarly, sub-figure (c) shows the total number of active electrodes per condition. Sub-figure (d) shows the mean number of spikes per well that were measured during the entire length of the recording, while sub-figure (e) shows the mean firing rate per well, i.e. spikes per second. Finally, sub-figure (f) depict the mean resistance per covered electrode within each group. Significance levels are indicated in the plots with asterisks: * if $P \leq 0.05$; ** if $P \leq 0.01$; and *** if $P \leq 0.001$.

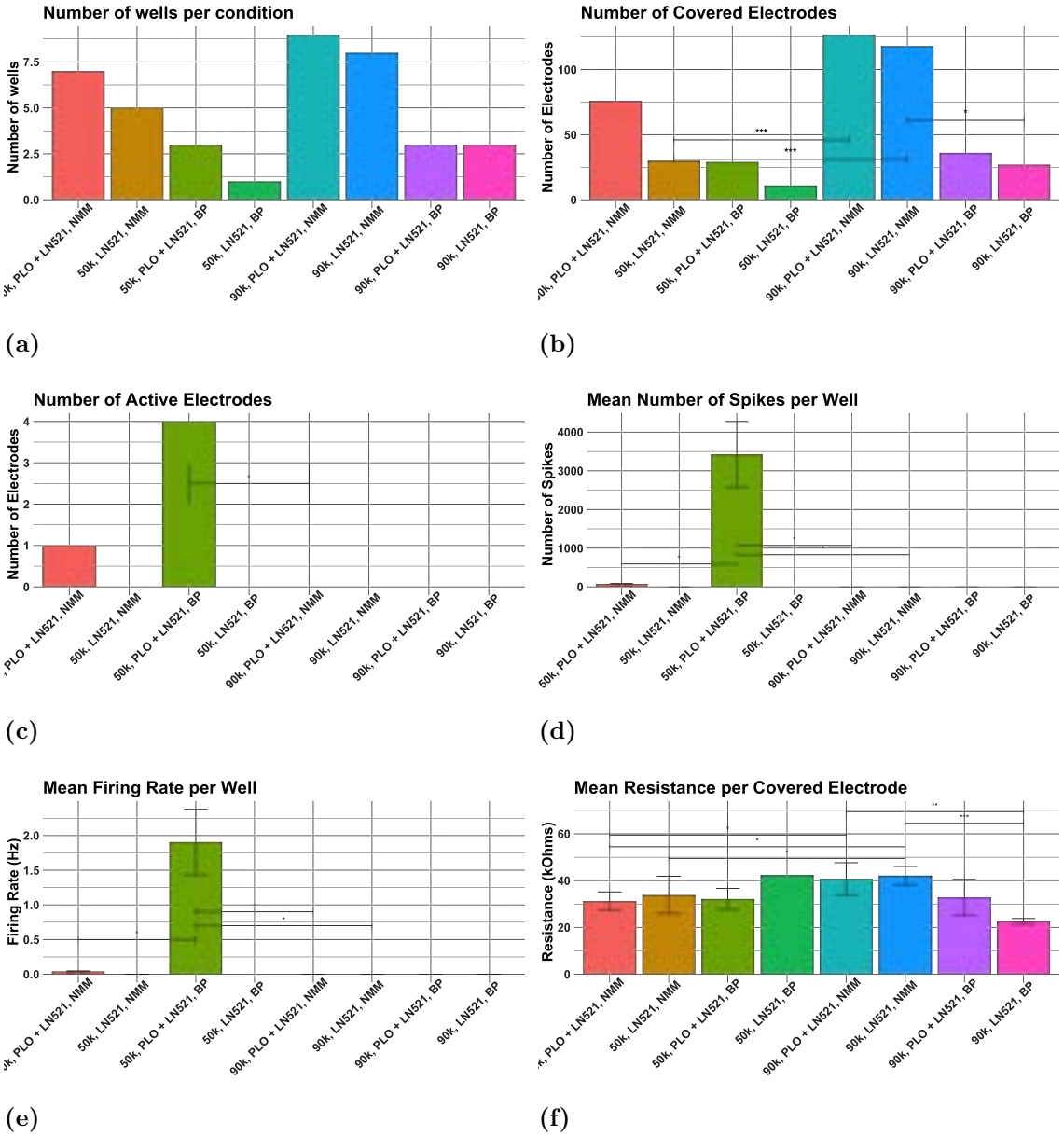


Figure 3.17: Results from MEA recording on day 73. Sub-figure (a) depict the number of wells with at least one covered electrode for each condition. Conditions [50k, PLO + LN521, NMM], [50k, LN521, NMM], [90k, PLO + LN521, NMM] and [90k, LN521, NMM] have 9 wells each, while the remaining conditions have 3 wells each. Sub-figure (b) depict the total number of covered electrodes per condition, with each well containing 16 electrodes. Similarly, sub-figure (c) shows the total number of active electrodes per condition. Sub-figure (d) shows the mean number of spikes per well that were measured during the entire length of the recording, while sub-figure (e) shows the mean firing rate per well, i.e. spikes per second. Finally, sub-figure (f) depict the mean resistance per covered electrode within each group. Significance levels are indicated in the plots with asterisks: * if $P \leq 0.05$; ** if $P \leq 0.01$; and *** if $P \leq 0.001$.

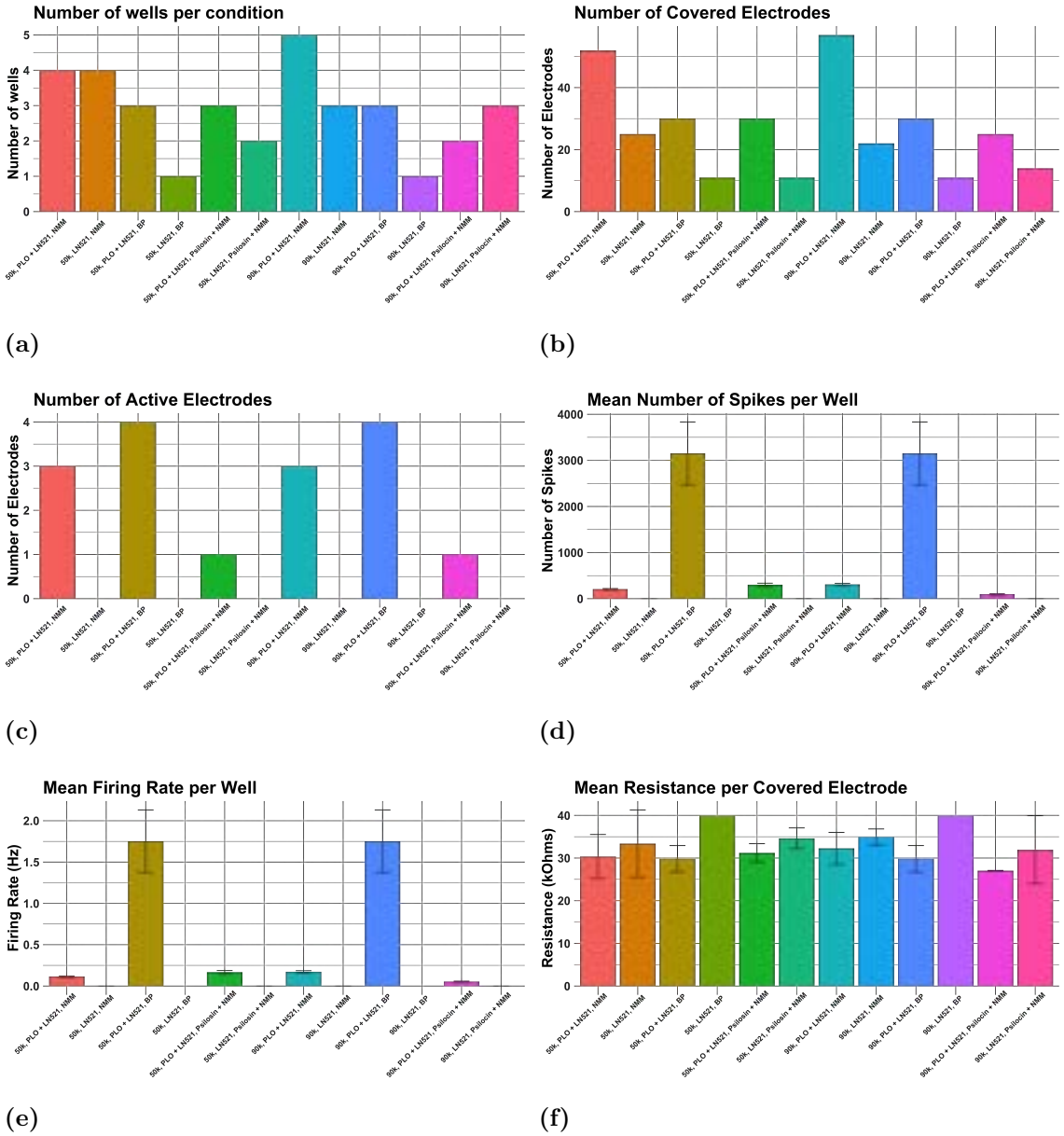


Figure 3.18: Results from MEA recording on day 75. Sub-figure (a) depict the number of wells with at least one covered electrode for each condition. Conditions [50k, PLO + LN521, NMM], [50k, LN521, NMM], [90k, PLO + LN521, NMM] and [90k, LN521, NMM] have 6 wells each, while the remaining conditions have 3 wells each. Sub-figure (b) depict the total number of covered electrodes per condition, with each well containing 16 electrodes. Similarly, sub-figure (c) shows the total number of active electrodes per condition. Sub-figure (d) shows the mean number of spikes per well that were measured during the entire length of the recording, while sub-figure (e) shows the mean firing rate per well, i.e. spikes per second. Finally, sub-figure (f) depict the mean resistance per covered electrode within each group. Significance levels are indicated in the plots with asterisks: * if $P \leq 0.05$; ** if $P \leq 0.01$; and *** if $P \leq 0.001$.

The results from the recording made on day 87 are found in Figure 3.19. It can be seen in Figures 3.19 (c), (d) and (e) that [50k, PLO + LN521, NMM] has the highest number of active electrodes, mean number of spikes and mean firing rate, followed by [50k, PLO + LN521, NMM + Psilocin].

3.2.4 Visual comparison

The four most active conditions were [50k, PLO + LN521, NMM], [50k, PLO + LN521, BP], [90k, PLO + LN521, NMM] and [90k, PLO + LN521, BP]. Images of one well from each conditions are found in Figures 3.20, 3.21, 3.22 and 3.23 respectively. The images are all captured at three different time points, day 52, day 75 and day 89, thus creating a timeline.

The comparison between wells containing 50k cells and 90k cells shows a clear difference in cell densities. Wells with 90k cells exhibit higher mean resistance, which is consistent with the images. In the images, distinguishing network formations is more challenging in wells with higher cell density. However, Figure 3.23 shows patches of cells on days 75 and 89, suggesting cell detachment from the surface. This observation aligns with the decreasing number of covered electrodes over time, as illustrated in Figures 3.14 (b), 3.18 (b), and 3.19 (b). Similar cell clumping is observed in the condition [50k, PLO + LN521, BP].

To summarize experiment 2, the conditions with 50k cells per well have consistently had more activity than the wells with 90k cells. The peak of activity was on day 64 for the condition [50k, PLO + LN521, BP]. Furthermore, the conditions with the PLO + LN521 coating has consistently had more activity than the conditions with only LN521. Moreover, the conditions with 90k cells per well have had a higher number of covered electrodes and a higher mean resistance than the conditions with 50k cells per wells. Additionally, while BP temporarily increased the activity, it caused the cells to detach from the electrodes after some time.

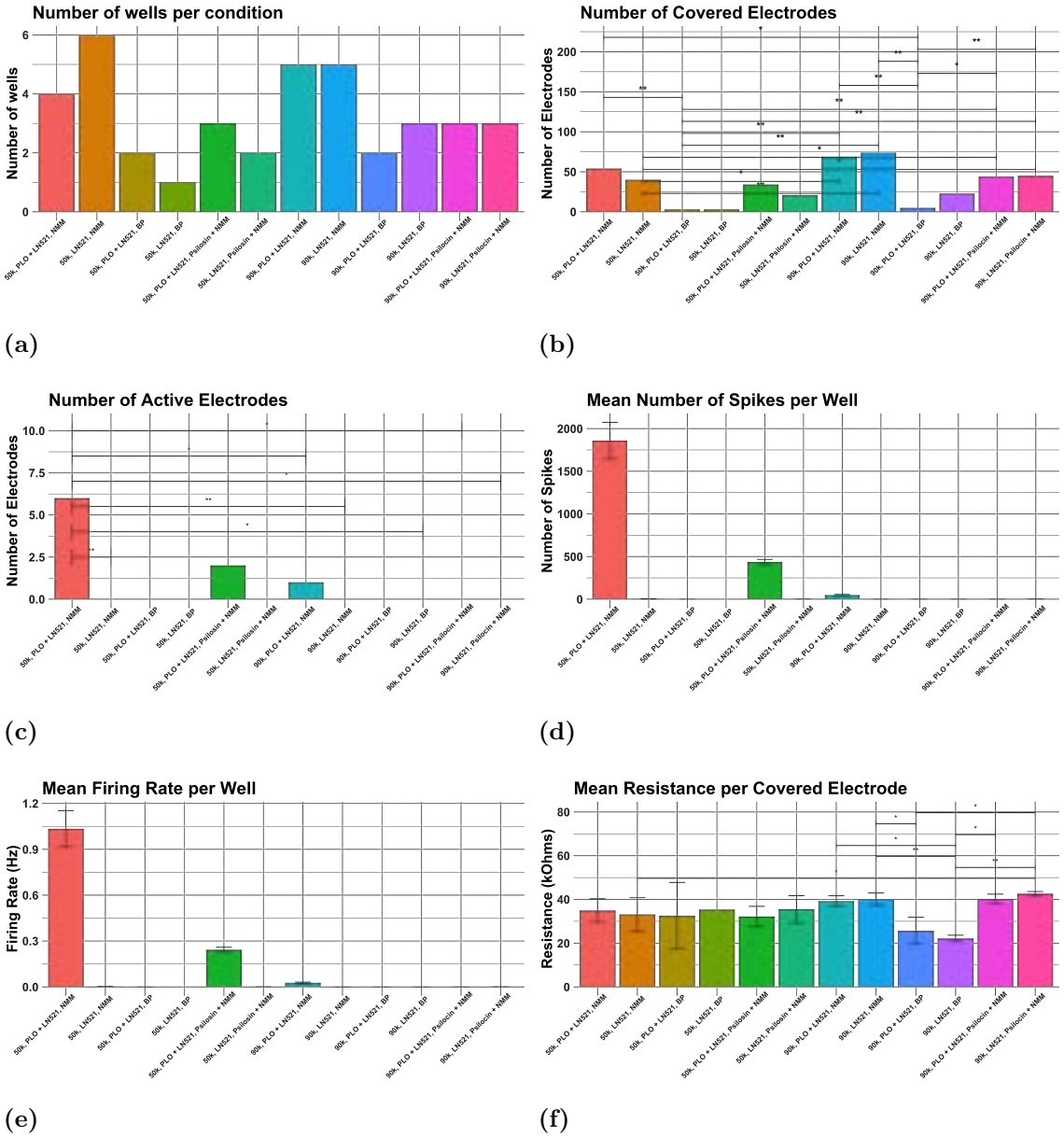
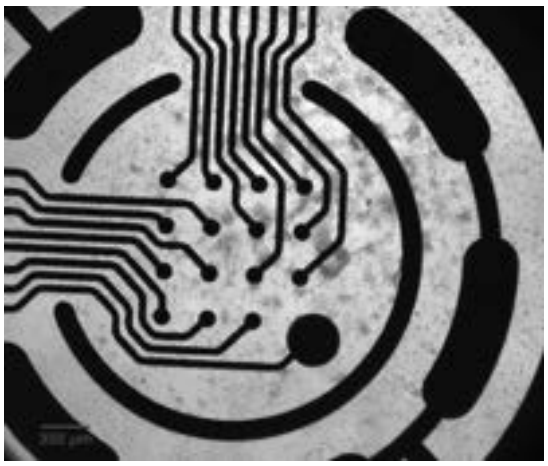
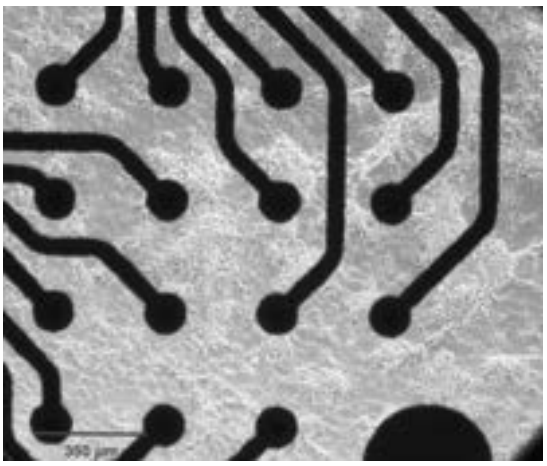


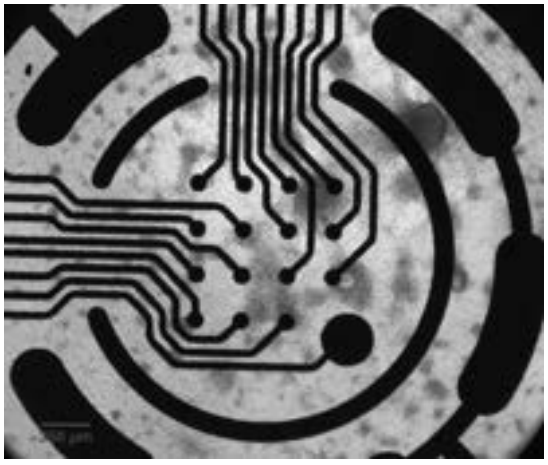
Figure 3.19: Results from MEA recording on day 87. Sub-figure (a) depict the number of wells with at least one covered electrode for each condition. Conditions [50k, PLO + LN521, NMM], [50k, LN521, NMM], [90k, PLO + LN521, NMM] and [90k, LN521, NMM] have 6 wells each, while the remaining conditions have 3 wells each. Sub-figure (b) depict the total number of covered electrodes per condition, with each well containing 16 electrodes. Similarly, sub-figure (c) shows the total number of active electrodes per condition. Sub-figure (d) shows the mean number of spikes per well that were measured during the entire length of the recording, while sub-figure (e) shows the mean firing rate per well, i.e. spikes per second. Finally, sub-figure (f) depict the mean resistance per covered electrode within each group. Significance levels are indicated in the plots with asterisks: * if $P \leq 0.05$; ** if $P \leq 0.01$; and *** if $P \leq 0.001$.



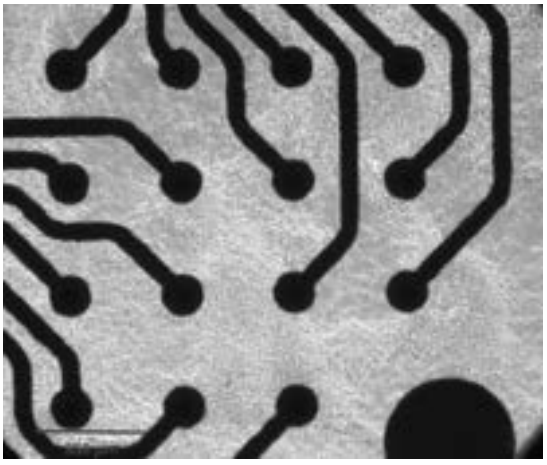
(a) Day 52, 4x



(b) Day 52, 10x

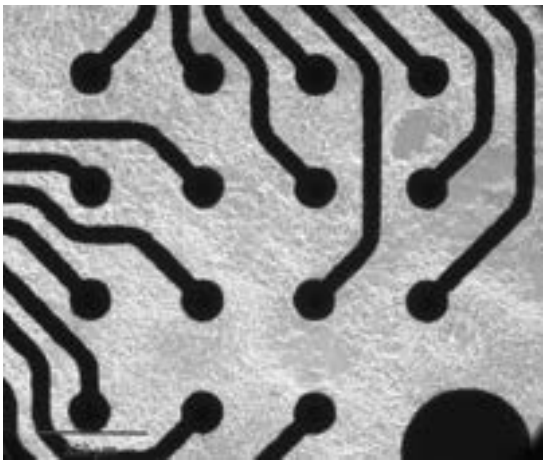


(c) Day 75, 4x



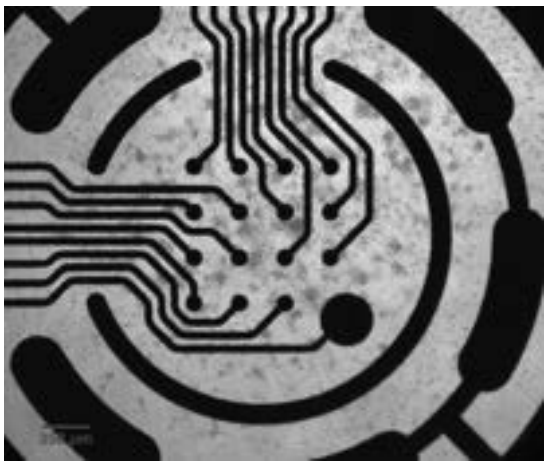
(d) Day 75, 10x

(e) Day 89, 4x

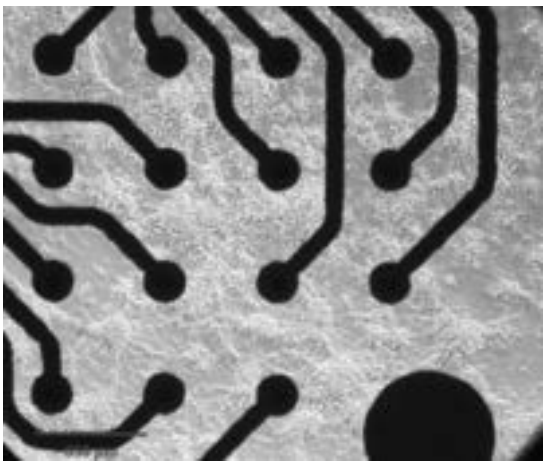


(f) Day 89, 10x

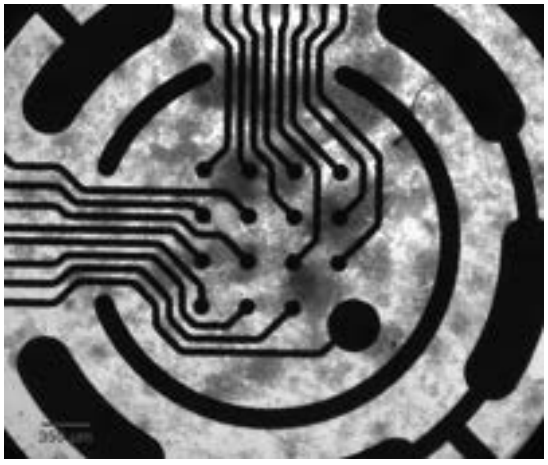
Figure 3.20: Brightfield images of well A3 with condition [50k, PLO + LN521, NMM] at three time points (day 52, day 75 and day 89). Images are captured at 4x and 10x magnification.



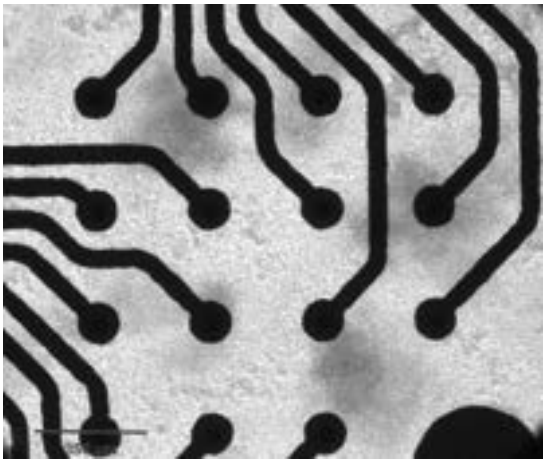
(a) Day 52, 4x



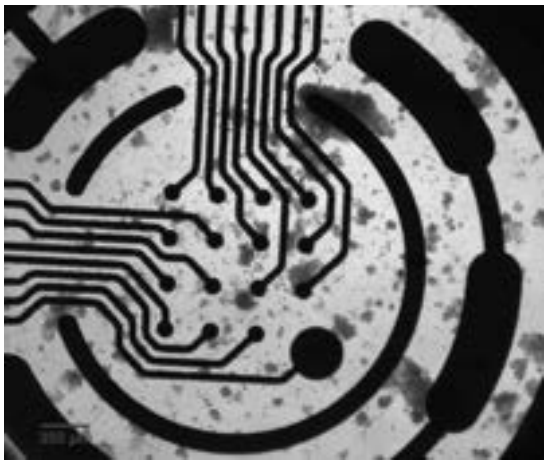
(b) Day 52, 10x



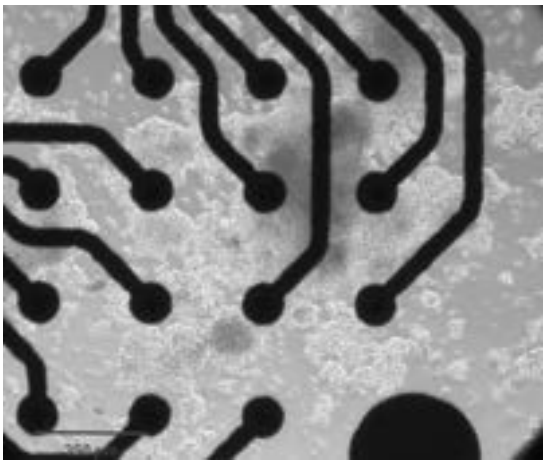
(c) Day 75, 4x



(d) Day 75, 10x

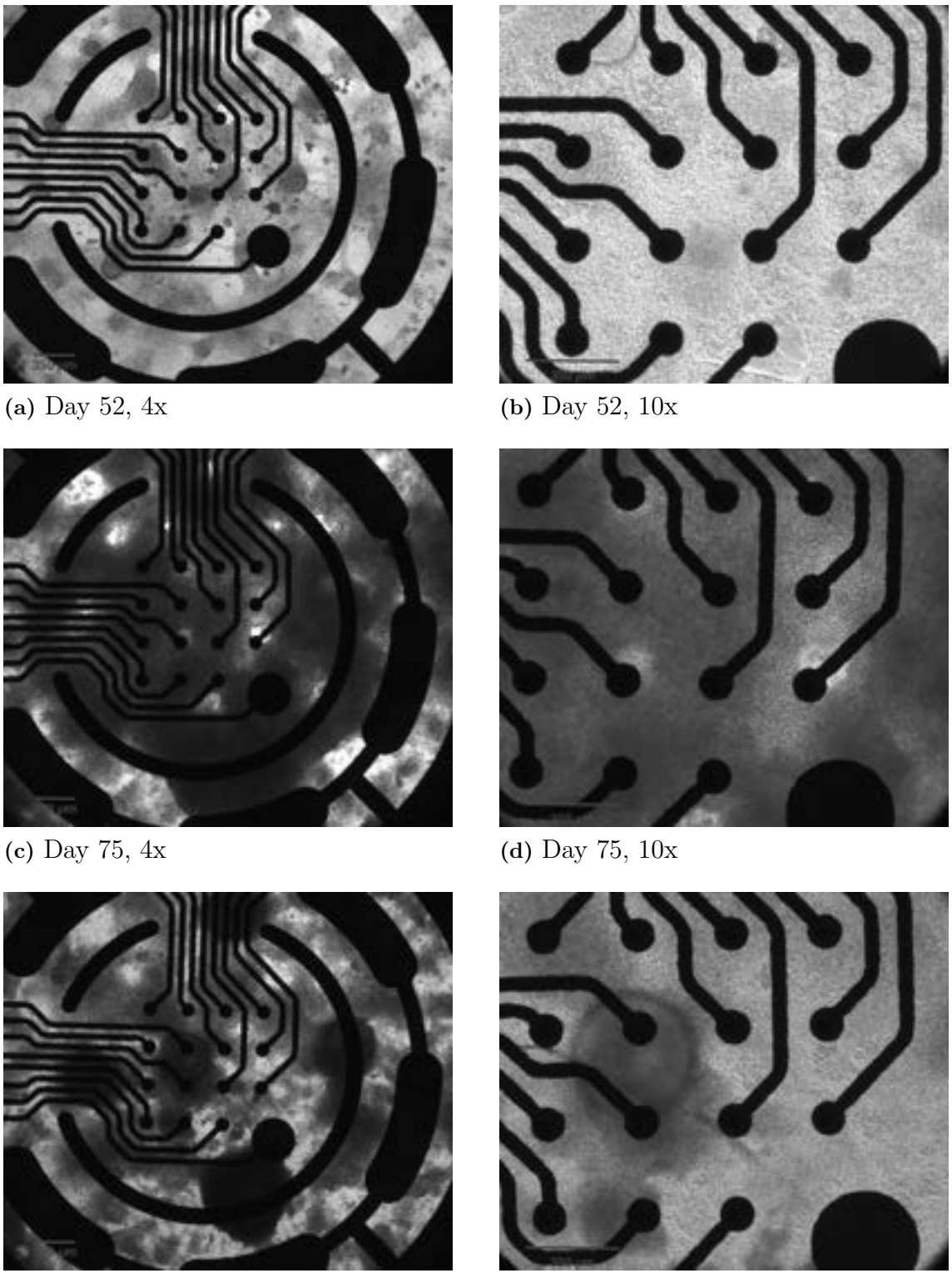


(e) Day 89, 4x



(f) Day 89, 10x

Figure 3.21: Brightfield images of well B3 with condition [50k, PLO + LN521, BP] at three time points (day 52, day 75 and day 89). Images are captured at 4x and 10x magnification.



(a) Day 52, 4x

(b) Day 52, 10x

(c) Day 75, 4x

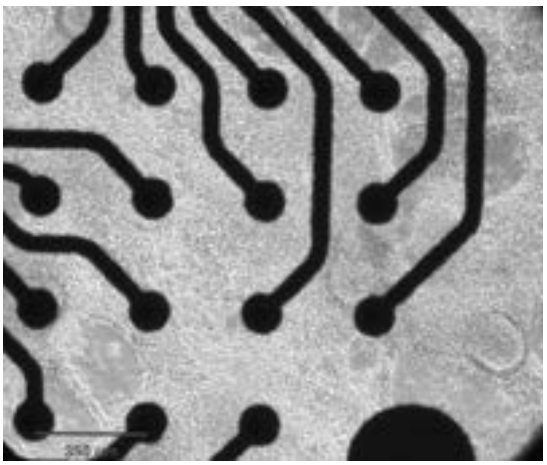
(d) Day 75, 10x

(e) Day 89, 4x

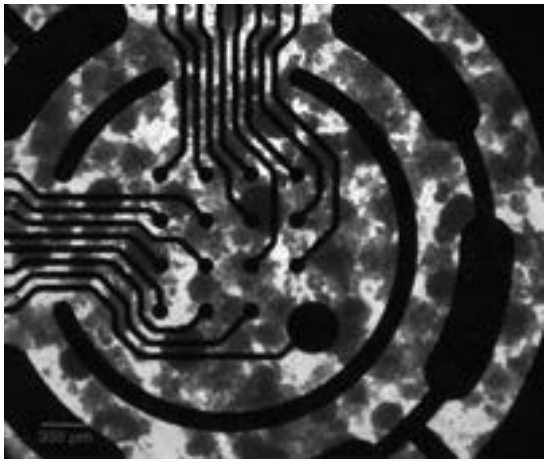
(f) Day 89, 10x

Figure 3.22: Brightfield images of well A3 with condition [90k, PLO + LN521, NMM] at three time points (day 52, day 75 and day 89). Images are captured at 4x and 10x magnification.

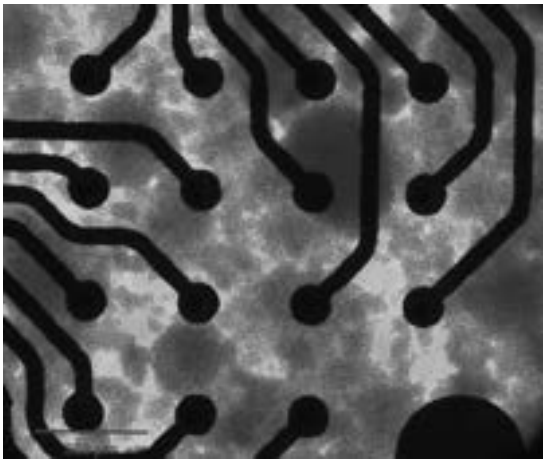
(a) Day 52, 4x



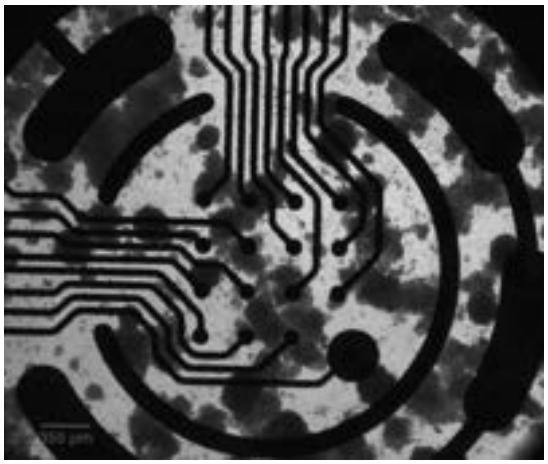
(b) Day 52, 10x



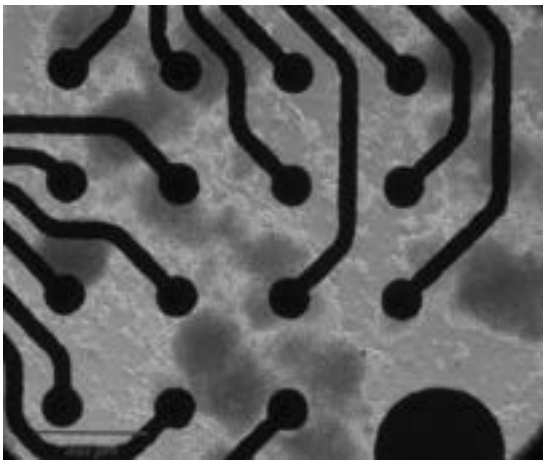
(c) Day 75, 4x



(d) Day 75, 10x



(e) Day 89, 4x



(f) Day 89, 10x

Figure 3.23: Brightfield images of well B6 with condition [90k, PLO + LN521, BP] at three time points (day 52, day 75 and day 89). Images are captured at 4x and 10x magnification.

4

Discussion

4.1 Experiment 1

Experiment 1 aimed to provide insight in what cell densities and coatings to use in the following, more extensive experiment. Although four different cell densities were tried, only three were present in the plots: 50k, 100k and 150k. This was because the 25k wells had no electrodes that were considered covered, and were thus filtered out in the data processing. It was hence concluded that the cell density 25k cells per well was too low, which is why it was not included in experiment 2. Moreover, no single cell density had a consistently significant effect on the activity than the others. However, on day 59, the wells with 50k cells had significantly higher mean firing rates than the other conditions, and almost three times as high as in the other recordings. This could suggest that 50k is the most suitable cell density.

Although the images of the wells with 100k and 150k showed more intricate network formation, there was no clear correlation between that and the activity of the cells. The decrease in activity observed in the 150k wells over time may indicate an interplay between cell density and neural network dynamics, possibly influenced by factors such as nutrient availability or network saturation. However, the increase in the number of covered electrodes across the cell densities over time suggests potential cell proliferation or migration. This indicates that although the wells may be densely populated, the cells remain viable but exhibit reduced activity.

The mean number of covered electrodes remained consistent for wells coated with LN521, whereas it exhibited a gradual increase for those coated with PLO + LN521. Although a significant difference was observed on day 47, this difference did not persist in following recordings. Despite consistently higher values for the LN521 coating, these differences failed to reach statistical significance. Moreover, over time, the differences between coatings decreased. As a result, definitive conclusions about the impact of coatings cannot be drawn from this experiment alone. Therefore, both coatings were included in the subsequent experiment for further evaluation.

Furthermore, it is important to note that inconclusive results were encountered and no clear patterns evolving over time were noticed. This suggests that the relationship between the variables that were studied might be more complex than initially thought. Another thing to consider is that this experiment lacked replicates, which makes the statistical analysis less reliable.

4.2 Experiment 2

Experiment 2 aimed to explore the impact of different culturing mediums on neural network activity, building upon the insights gained from Experiment 1. During the initial phase (Day 43 - Day 52), four conditions were tested, with notable differences observed in electrode coverage, activity, and resistance. While wells with 90k cells consistently exhibited higher electrode coverage and resistance, the levels of activity varied between each recording. Moreover, the conditions with LN521 coatings had lower activity than the conditions with PLO + LN521, although not always significantly lower.

The second phase (Day 54 - Day 73) introduced additional conditions, where the culturing medium was exchanged from NMM to BP in some wells. This intervention resulted in a rapid increase in activity, particularly in wells with PLO + LN521 coating. However, this heightened activity was not sustained uniformly across conditions, with fluctuations observed over time. Notably, the addition of BP led to significant increases in activity.

In the final phase (Day 75 - Day 87), the addition of psilocin to some of the wells did not yield significant differences in activity compared to previous conditions. However, wells coated with PLO + LN521 exhibited increased activity, suggesting a potential interaction between coating and chemical stimuli. Although previous research has suggested that a single dose of psilocin is enough to detect an increase in the denditric spine size and density, it was not observed in this project. However, more in-depth characterization of the cells would necessary to discover and confirm such results. Additionally, more long-term studies would be needed.

When comparing the wells with 50k cells and 90k cells, the difference in cell densities is evident. This is consistent with the higher mean resistance measured in the conditions including 90k cells. The high cell density also makes it more difficult to distinguish any network formations from the images. However, in Figure 3.23 patches of cells are formed on day 75 and 89, indicating that the cells have detached from the surface. This is consistent with the decreasing number of covered electrodes over time that are found in Figures 3.14 (b), 3.18 (b) and 3.19 (b). The same issue is found for condition [50k, PLO + LN521, BP], where the cells have clumped together. This might indicate that BP is not as sufficient as NMM for long-term culturing.

Overall, Experiment 2 showed that neural network activity is dynamic and influenced by factors like cell density, coating, and chemical treatments. Conditions with 50k cells and PLO + LN521 coating consistently showed higher activity, however they were not always statistically different from the other conditions. The relationship between these factors is complex and needs to be studies further and in more depth. The changes in activity over time highlight the importance of long-term studies to understand how neural networks behave over extended periods.

5
Conclusion

The combination of 50k cells and PLO + LN521 coating consistently showed higher neural activity, making it a promising candidate for future studies. BP appears to be more effective than NMM in promoting neural activity, suggesting that the choice of culturing medium is crucial for maintaining active neural networks. However, it caused the cells to detach from the surface in contrast to the wells with NMM, indicating that it is not as suitable for long-term culturing. The study also highlights the complex interplay between cell density, coating, and chemical interventions. These factors do not operate in isolation and can have varying effects on neural activity depending on the context.

Finally, the lack of replicates and clear patterns over time indicates the need for more rigorous experimental designs, including the use of replicates and long-term studies, to better understand the factors influencing neural network activity. These conclusions provide a foundation for refining experimental designs and exploring the interactions between various factors in neural network studies.

6
Future Studies

Although this project may act as a stepping stone towards studying the electrophysiological behaviour of stem cell-derived neurons, further research is needed to understand the intricate dynamics of neural networks. Thus, future studies could include co-culturing stem cell-derived neurons with astrocytes to enhance the stability and longevity of neural cultures. Astrocytes play crucial roles in supporting neurons, and by integrating them into the culture system could provide a more physiologically relevant environment, enabling long-term studies and more accurate modeling of neurodevelopmental and neurodegenerative processes.

Moreover, adding further metrics such as bursts and network bursts in the assessments might reveal other aspects of neuronal communication, such as synchronization and network connectivity. Furthermore, incorporating neural broadband measurements to assess local field potentials (LFPs) to analyze the amplitude and patterns of the signals could complement the spike and burst analyses. This could offer a more holistic understanding of the neural network behaviour.

By developing tissue models using iPSC-derived neurons from patients with SMI, biomarkers and therapeutic targets specific to SMIs could be identified. Researchers can investigate the underlying genetic and molecular mechanisms that influence treatment efficacy, by including cohorts of responders and non-responders to treatment. Comparative studies between SMI patients and healthy controls, as well as responders and non-responders, can help identify phenotypical differences detectable by MEA. These comparisons can lead to the discovery of distinct electrophysiological signatures associated with SMIs and treatment responses, aiding in the development of personalized medicine approaches.

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A

Appendix - Materials and Methods

A.1 Reagents

Table A.1: Table of reagents.

Reagents		
Chemicals, peptides, and proteins	**Source**	**Identifier**
DMEM/F-12 (1:1)(1X) + Glutamax	Gibco, Thermo Fisher Scientific	CAT31331-028
Matrigel hESC-Qualified Matrix	Corning	CAT354277
mTeSRTM PLUS Medium	Stemcell Technologies	CAT100-0276
Biolaminin 521	BioLamina	LN521
Poly-L-Ornithine 0.01%	Sigma Aldrich	CATP4957
FGF2	PeproTech	CAT100-18B
LDN193189	Stemcell Technologies	72149
SB431542	Stemcell Technologies	CAT72232
Insulin (9.5-11-5 mg/ml)	Sigma Aldrich	CATI9278
Beta-Mercaptoethanol (50mM)	Thermo Fisher Scientific	CAT31350-010
MEM-NEAA (100X)	Thermo Fisher Scientific	CAT11140-023
Sodium-Pyruvate (100mM)	Sigma Aldrich	CATS8636
Penicillin-Streptomycin (100X)	Hyclone	CATSV30010
DPBS (1X) +MgCl2/+CaCl2 (+/+)	Gibco, Thermo Fisher Scientific	CAT14040-083
Dispase	Thermo Fischer Scientific	CAT17105-041
Accutase	Thermo Fischer Scientific	CAT00-4555-56
EDTA	Thermo Fischer Scientific	CATAM9912
DMEM (1X)	Gibco, Thermo Fisher Scientific	CAT1190-044
Neurobasal medium (1X)	Gibco, Thermo Fisher Scientific	REF12348-017
B-27 supplement (50X)	Gibco, Thermo Fisher Scientific	REF17504-044
B-27 supplement without Vitamin A (50X)	Gibco, Thermo Fisher Scientific	REF12587-010
BrainPhysTM Neuronal Medium	Stemcell Technologies	CAT05790
NeuroCult™ SM1 Neuronal Supplement	Stemcell Technologies	CAT05711
N2 Supplement-A	Stemcell Technologies	CAT07152
BDNF	PeproTech	CAT450-02
GDNF	PeproTech	CAT450-10
Dibutyryl cAMP	Sigma Aldrich	CATD0627
L-ascorbic acid	Sigma Aldrich	CATA0278

A.2 Materials

Table A.2: Ingredients and amounts for Neural Maintenance Media (NMM).

Neural Maintenance Media (NMM)	
Ingredient	**Amount**
DMEM/F-12 (1:1)(1X) + Glutamax	250 ml
Insulin (9.5-11-5 mg/ml)	125 µl
β-mercaptoethanol (55mM)	500 µl
MEM-NEAA (100X)	2.5 ml
Sodium Pyruvate	2.5 ml
PEST	1.25ml
N2 supplement	2.5 ml
B27 Supplement	2.5 ml
Glutamax (100x)	2.5ml
Neurobasal media	250ml

Table A.3: Ingredients and amounts for BrainPhys Neuronal Media (BP).

BrainPhys Neuronal Media (BP)	
Ingredient	**Amount (50 ml)**
BrainPhys(TM) Imaging Optimized Medium	50 ml
NeuroCult^{T}M SM1 Neuronal Supplement	1 ml
N2 Supplement-A	500 µl
*BDNF (-> 20ng/ml)	10 µl
**GDNF (-> 20ng/ml)	10 µl
100 mg/ml dibutyryl cAMP (-> 1 mM)	250 µl
50 µg/ml L-ascorbic (-> 200 nM)	35 µl
PEST (100x)***	125 µl

A.3 Thawing of neuroepithelial cells during differentiation of iPS cells to cortical neurons

1. Pre-warm a Falcon tube with sterile water to 37°C.
2. Bring the cells to the cell culture on dry ice.
3. Place the cryovial in the pre-warmed water until all but a small fraction of the cells have thawed.
4. Spray the vial with ethanol.
5. Transfer the partially thawed cells to 10 ml of room-temperature neural maintenance medium.
6. Centrifuge the cells once at 400g (1800 rpm in large cell culture centrifuge) for 3 min and discard the supernatant.

7. Gently resuspend the cells in 2 ml of neural maintenance medium supplemented with 20 ng/ml FGF2, and plate into laminin-coated wells (1 cryovial to 1 6-well).
8. Change to neural maintenance medium without FGF2 the following day and thereafter every other day.

A.4 Freezing of neuroepithelial cells during differentiation of iPS cells to cortical neurons

Freeze media: Neural maintenance medium supplemented with 10% DMSO and 20 ng/ml FGF2.

1. Start with confluent 6-wells (or the cells will not grow properly after thawing).
2. Prepare 1 ml 1x Freezing media/6-well (= NMM with 10% DMSO and 20 ng/ml FGF2) and keep in the fridge. Place a CoolCell freezing container with pre-marked cryotubes in the fridge.
3. Wash the cells once with PBS.
4. Add 0.5 ml Accutase to each 6-well. Incubate at 37°C for 5 min.
5. Dissociate the cells by flushing them with the Accutase solution and transfer to a Falcon tube containing 10 ml DMEM/F12 media (wash media).
6. Centrifuge at 400 g (1800 rpm in large cell culture centrifuge) for 5 min.
7. Repeat the wash step once.
8. Discard the supernatant and dissolve the pellet in 0.8 ml cold Freezing media.
9. Quickly transfer the cell suspension from one tube to each cryovial. Place the vials in a pre-cooled CoolCell freezing container. Place the CoolCell freezing container in -80°C overnight.
10. Transfer the cryovials to the nitrogen tank after 24h for long-term storage.

A.5 Coating of cell culture for iPS cell differentiation

A.5.1 With Matrigel

Corning^{TM} Matrigel^{TM} hESC-Qualified Matrix, Fisher Scientific: 11573560. Thaw in fridge and aliquot on ice. Store at -20°C and thaw aliquots in fridge. Keep the Matrigel cold at all times to avoid polymerization.

1. Add 15 l of Matrigel to each 1 ml of cold DMEM media. See table below for volumes required per well. Add an appropriate volume of Matrigel/DMEM to cover the total surface area of the well.

2. Incubate for at least 1 h at 37°C (or room temperature).
3. Remove the Matrigel solution before adding cell suspension (no need to wash).

Culture vessel	Volume diluted Matrigel
6 well	1 ml
12 well	600 µl
24 well	300 µl
96 well	50 µl
8 well ibidi slide	300 µl
35 mm ibidi dish	400 µl

A.5.2 With Laminin-521

Laminin-521 (human) 100 µg/ml BioLamina, LN521.
Store at -20°C for up to 3 years or in fridge for up to 3 months. 0.5 µg/cm^2 is recommended for coating.

1. Thaw the laminin in fridge to avoid polymerization.
2. Dilute laminin stock in PBS +/+
   Calculate the amount of laminin used from the table below

Type of plate	Volume/well	Laminin 0.5 µg/cm^2 (µl/ml PBS+/+)
6 well	1 ml	50 µl (50 µl/ml)
12 well	500 µl	25 µl (50 µl/ml)
24 well	400 µl	15 µl (37.5 µl/ml)
96 well	70 µl	3.5 µl (50 µl/ml)
8 well ibidi slide	300 µl	5 µl (16.7 µl/ml)
35 mm ibidi dish	400 µl	20 µl (50 µl/ml)
T25 cm^2 flask	3 ml	150 µl (50 µl/ml)
T75cm^2 flask	8 ml	400 µl (50 µl/ml)

3. Add desired volume to each well to be coated
4. Incubate for 2 hours at 37°C or overnight in fridge, sealed with Parafilm.
5. Remove laminin solution before adding cell suspension (no need to wash).

A.5.3 With Poly-L-ornithine

Poly-L-ornithine 0.01% SigmaAldrich, P4957.

1. Add poly-L-ornithine to each well accordning to table below
2. Incubate for at least 4 hours (or over night).
3. Aspirate poly-L-ornithine solution and add laminin solution.

Type of plate	Volume/well	Dilute before?
6 well	1 ml	No
24 well	400 µl	Yes 1:2
96 well	50 µl	No
8 well ibidi slide	Yes 1:2	
35 mm ibidi dish	400 µl	No

A.6 Passaging of neural progenitor cells with Accutase

StemPro® Accutase® Cell Dissociation Reagent: A11105-01 (Life Technologies). Ready-to-use solution: Store aliquots at -20°C, thaw in fridge or RT. Store thawed aliquots in fridge (DO NOT refreeze!).

Preparations:

- Alt.1. Passage for expansion: Coat the wells with laminin (L521 from Biolamina) ON at 4°C or 2h at 37°C (see separate protocol).
- Alt. 2. Passage for Final seed-out: Coat the plates with Poly-l-ornithine ON followed by laminin for 4 h.
- Pre-warm PBS -/- for washing (2 ml/6-well).
- Pre-warm Accutase (0.5 ml/6-well) to room temperature.
  NOTE! Accutase is only active for 5 min at 37°C: DO NOT thaw or prewarm Accutase at 37°C).
- Prepare wash media (DMEM/F12) 20 ml/6-well
- Prepare neural maintenance media (NMM) needed for plating. 2 ml/6-well

- Remove old media from the well/wells.
- Add 2 ml of sterile PBS -/- to each 6-well.
- Remove PBS.
- Add 0.5 ml Accutase per 6-well (or 35 mm dish).
- Incubate at 37°C for 5 minutes.
- Pipette the cells up and down 3-4 times in the Accutase solution to dissociate cells into single-cells.
- Transfer each Accutase-cell suspension to a Falcon tube containing 10 ml of wash media.
  NOTE! If several wells are passaged simultaneously, they should all be washed separately.
- Centrifuge the cell suspension at 400g for 5 min (RT).
  (Approx. 1800 rpm in cell culture centrifuge).
- Discard the supernatant.
- Repeat the wash step once.
- Resuspend the cells in 2 ml of NMM for 1:1 passage or 4 ml for 1:2 passage.

- Remove Laminin from the wells. Plate the cells onto laminin-coated (Alt.1) (or laminin + poly-L-ornithine coated (Alt.2) plastic wells.
- Incubate overnight.
- Change neural maintenance media the day after, and thereafter every 2^{nd} day.

A.7 Passaging of neuroepithelial cells with dispase

Dispase solution: Dilute dispase (powder) to 10 mg/ml in PBS.Filter sterilize (0.22 µm filter). Aliquot into appropriate volumes and store in -80°C for 6 months.

Preparations:

- Coat the wells with laminin (LN521) (see separate protocol) at 37°C for 4 h → ON
- Alt.1. **Passage on day 10:** Prepare NMM with 20 ng/ml FGF2 (4 ml/6-well to render a 1:2 passage).
- Alt. 2. **Passage after day 10:** Prepare neural maintenance media (NMM) for plating (1:2 passage for expansion, 1:1 passage for cleaning the cultures from cells differentiated to other cell types).
- Thaw Dispase in room temperature.
- Prepare wash media (1:1 DMEM/F12:Neurobasal)

- Add 100 µl of dispase stock solution per ml media directly into the well containing a confluent neuroepithelial layer or rosette-containing sheet.
- Incubate at 37°C for 5 minutes (up to 30 min if needed).
- When the cell mat starts to detach around the edges, use a transfer pipette to carefully detach the cell mat from the bottom of the well.
- Pipette up and down 2-3 times to break up the cell mat in smaller pieces (but not too small!).
- Carefully transfer the colonies from each 6-well to a 15-ml Falcon tube containing 10 ml of pre-warmed wash medium (DMEM/F12)
  NOTE! If several wells are passaged simultaneously, they should all be washed separately.
- Allow the clumps to settle at the bottom of the tube, by leaving the tube for 3 minutes at room temperature (or in 37°C-heating block).
- Gently remove the supernatant using a transfer pipette (leave some media to avoid losing cells).
- Repeat this wash twice (the third time, let the cells sink for 5 minutes).
- Carefully resuspend the cells in 4 ml of NMM + FGF2(Alt.1)/NMM(Alt.2) per 6-well without breaking the colonies too much. The colonies should be clearly visible by eye.
  If cells are passaged in order to remove unwanted cells, a 1:1 split is enough and cells should be resuspended in 2 ml of NMM.
- Remove laminin from coated wells.

- Add 2 ml of cell suspension to each well.
- Incubate cells over night to re-attach.
- Alt.1. **Passage on day 10:** Change to neural maintenance media (NMM) supplemented with 20 ng/ml FGF2 (2 ml/6-well) the day after passage, then change to NMM + FGF2 every second day for a total of 4 days.
  Alt. 2. **Passage after day 10:** Change to neural maintenance media (NMM) the day after (2 ml/6-well), then change to NMM every second day.

A.8 Differentiation of iPS cells to cortical neurons

In mTeSR1 – Matrigel system

Seeding of iPS Cells:

- Dissociate iPS cells from **two** 70-90% confluent 6-wells using EDTA(or Versin) and seed on **one** Laminin-521 coated 6-well in mTeSR1 media with 10 µM ROCKi (2:1 passage).

Induction of neuronal differentiation

- The day after seed-out (day 0), start neural induction by:
  If the cells are not confluent it is OK to wait one more day, but not more.
  - Remove old media, wash the cells once using PBS -/-, and thereafter add 2-3 ml/well of neural induction media supplemented with Noggin (500 ng/ml) or Dorsomorphin (1 µM) and SB431542 (10µM).
- Change neural induction media every day for 8-12 days (usually 10 days) and monitor neural induction by the appearance of cells with neuroepithelial morphology.
- Dissociate cells using **dispase** and passage the cells 1:2 onto laminin coated (**L2020** (Sigma Aldrich)) plastic plates (**See separate protocol for laminin coating of plastic**) NMM supplemented with 20 ng/ml FGF2.

Expansion of neuronal progenitors

- The day after, change to fresh NMM supplemented with FGF2 (20 ng/ml).
- Incubate the cells with neuronal maintenance media supplemented with FGF2 for 4 days, with media change every 2^{nd} day (2 changes).
- After FGF2 induction (at around day 16-17 at the most) look out for the appearance of neuronal rosettes. When the rosettes start to appear, passage the cells once again with dispase (**See separate protocol for dispase**) and plate on laminin-coated plates (**See separate protocol for laminin coating of plastic**).
- Between day 20 and 30 from day of neuronal induction (usually around day 25), substantial neurogenesis should occur. This means that neurons start to grow out from the edges of the rosettes.

- When the outgrowing neurons appear, **preferably on day 25**, passage the cells using accutase (**See separate protocol for Accutase passaging**) onto laminin **L521** (Biolamina) At the first passage split the cells 1:1 (should be about 70% confluent).
- Change the medium the day after plating, and thereafter every 2^{nd} day. After 1-2 days, the cells should have reached about 90-95% confluency.
- Passage the cells every 2-3 days using accutase and a 1:2 split. The last split should be performed around day 35 (+/- 1 days) 1:4 split (at around 50'000 cells/cm). And then onto laminin (L521) + poly-l-ornithine coated plates (**See separate protocol for poly-L-ornithine coating of plastic plates**).
- Continue to culture the cells for another 50-60 days, changing media every 2^{nd} day (neural maintenance media).
- Spike the wells with laminin every 10^{th} day for long term culturing.
- Functional synapses appear around day 50.

Freezing of cells

- Cells can be frozen around day 26-30. Freeze the cells in NMM with 10% DMSO and 20 ng/ml FGF2. Freeze each 6-well in one vial and thaw in the same surface area.

A.9 Axion Biosystems Cell Culture Protocol - FCDI iCell GlutaNeurons [1]

Preparing the MEA plate

- Add 50 µl of 0.1% PEI solution to each well in the MEA plate.
- Incubate the PEI-coated MEA plate in a cell culture incubator at 37°C, 5% CO2 for at least 60 minutes.
- Rinse PEI from the culture surface with 200 µl of sterile DI water 4 times, then allow the MEA plate to air dry overnight.

Culturing iCell GlutaNeurons

- Prepare iCell GlutaNeuron media according to the FCDI iCell GlutaNeuron User's Guide. Aliquot media in functional 10-15 ml aliqouts, store at -80°C, and thaw at room temperature as needed. Laminin should be added to freshly thawed media as per User Guide recommended concentration.
- Thaw iCell GlutaNeurons according to the FCDI iCell GlutaNeuron User's guide.
- Remove a sample of the cell suspension and count the neurons using a hemocytometer to determine both the viability and total number of viable cells. Transfer the cell suspension to a 15 ml conical tube.
- Centrifuge the cell suspension at 180 x g for 5 min.
- Aspirate the supernatant, being careful not to disturb the cell pellet.

- Dilute the cell suspension in complete medium combined with laminin (20 µg/ml) to 12.000.000 neurons/ml.

Plating iCell GlutaNeurons onto the MEA

- Place a 10 µl droplet of iCell GlutaNeuron suspension over the recording electrode area of each well of the MEA.
- Incubate the MEA plate with the seeded neurons in a cell culture incubator at 37°C, 5% CO2 for 1 hour.
- Gently add 1/2 of the final volume of the medium to each well of the MEA. Adding the medium too quickly will dislodge the adhered neurons. Recommended final well volumes for each plate type are: 6- and 12-well = 1000 µl, 24-well = 500 µl, 48-well = 300 µl, 96-well = 200 µl.
- Repeat the previous step a second time to reach the final recommended volume of medium.
- Incubate in a cell culture incubator at 37°C, 5% CO2.
- For optimal cell health, exchange 50% of the media on day 1 and 2 post-plating, then every 2 days after that. Though neural spikes may be detectable within 4 days, optimal neural network structure is typically achieved after 18 days in culture.

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