MED64 PRESTO

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MED64 Protocol

Axol Human iPSC-Derived Neural Stem Cells

Co-cultured with Human iPSC-Derived Astrocytes

axol

ALPHA MED SCIENTIFIC

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Contents

1. Introduction

Stem cell derived neurons and astrocytes can provide insights into mechanisms of the brain, behavior, and disease. Human iPSC-derived Neural Stem Cells are precursor cells which can be terminally differentiated into pure or heterogeneous populations of neurons and astrocytes. Protocols currently exist to generate dopaminergic neurons, motor neurons, sensory neurons, and astrocytes. During the differentiation process, which takes about 40-50 days, it is possible to study developmental neurobiology processes and characterize the electrical activity during that developmental process of synapse formation. Alternatively, it is possible to measure activity in mature neuron and astrocyte populations after circuits are formed, in vitro.

The MED64 is a high-fidelity microelectrode array (MEA) platform that is engineered to detect a broad range of action potentials from any neuron population. The MED64's broad acquisition bandwidth combined with superior signal-to-noise ratio and carbon nanotube technology affords impeccable extraction of basic electrophysiological variables. The combination of precise extraction of electrophysiological activity with the ease of culturing cells directly onto the MED Probe (Multi Electrode Dish) makes the MED64 ideal for pharmacological, drug safety screening, and basic scientific applications. The 64 electrodes on the MED Probe have the lowest impedance of any micro-electrode array, making the MED64 ideal for acquiring data from spontaneously spiking neurons or neurons that fire in response to drug applications. The MED64 also has high capacitance electrodes, enabling the MED64 to deliver high stimulating current, which is essential for evoked response studies.

The goal of this protocol is to describe how to set up experiments with Neural Stem Cell cultures, acquire relevant data, and extract the data for presentation or publication. This material has been prepared by scientists with expertise in neuroscience and electrophysiology. A complete protocol for plating and culturing neuron cultures has been prepared based on the users' experience.

1-1. Acknowledgement

Alpha MED Scientific would like to thank the MED64 users that have shared their knowledge,:

Ikuro Suzuki,PhD - Tohoku Institute of Technology
Aoi Odawara,PhD - Tohoku Institute of Technology
Zoe Nilsson,PhD - Product manager, Axol Bioscience
Nick Clare,PhD - Application scientist, Axol Bioscience
Ryan Arant,PhD - Application Scientist, Alpha MED Scientific

1-2. Disclaimer

This application note is a summary of information shared by MED64 users and is to be considered marketing material. These methods have been developed, tested, and verified in the course of projects published in peer-reviewed literature. However, Alpha MED Scientific does not guarantee that the information written in this document is correct and is free from all liabilities. Please refer to the scientific literature for further insight on these techniques, as well as the MED64 and Mobius manuals for detailed instructions on use of the MED64 System.

2. Pretreatment of the MEA Plate and plating iPSC-Derived Neural Stem Cells

2-1. Material to be prepared

Items	Supplifer	Cat. No	Note
Cell			
Human iPSC-Derivd Neural Stem Cells	Axol	ax0019	1.5 million cells / vial
Mature astrocyte	Axol	ax0084	
Reagents for coating			
SureBond+ReadySet	Axol	ax0052	
(SureBond)			Store at -80 °C
(ReadySet)			Store at 4 °C
Reagents for cell plating and cultureing			
Neural Plating-XF medium	Axol	ax0033	Store at -80°C
Neural maintenance media kit	Axol	ax0031a & b	Refer to Axol product manual
Neural differentiation-XF medium	Axol	ax0034-125	Store at -80°C up to 6 months.
Neural differentiation-XF medium	Axol	ax0031-125	Store at -80°C up to 6 months.
Differentiation Supplement A	Axol		
Differentiation Supplement B	Axol		
Differentiation Supplement C	Axol		
ROCK inhibitor		Y-27635 dihydrochloride	
Unlock XF	Axol	ax005	
Astrocyte maitenance medium	Axol	ax0086	
iMatrix-511 (Laminin)	Nippi	892011	Store at 4 degree. For coating of culture plate
Equipment			
MEA Plate24 eco	Alpha MED Scientific	MED-Q2430M	
CellSpotter24 eco	Alpha MED Scientific	MED-CRS24M	
Cloning ring	Iwaki	11-0162 (RING-05)	ID:3.4, OD:5, Height:10 (mm)
35mm culture dish	Various		
Conical tube (15, 50 ml)	Various		
Microtube (2.5ml)	Various		
Pipet, Petri dishes, kim wipes, waterbath, clean bench, CO2 incubator, Centrifuge, microscope			

2-2. Workflow

The Human iPSC-Derived Neural Stem Cell is plated onto the MEA plate, and the Human iPSC-Derived Mature Astrocyte is added into the MEA plates on the day 14. This section will describe how iPSC-Derived Neural Stem Cell is plated onto the MEA Plate.

Day	Neural Stem Cells	Astrocyte
-1	Thawing **Surebond** Thawing **Plating XF Medium** Thawing **Neural Maintenance Medium (ax0031b,ax0031a)**	
0	Pre-coating MEA Plate	
	Plating neurons onto the MEA Plate	
1	Full Medium change **B**	
2	Thawing **Neural differentiation XF medium (ax0034-125), Differentiation Supplement A and B**	
3	Half medium change **C**	
4		
5	Half medium change **C**	
6		Thawing Supplement **B** and **C** overnight at 4 C
7	Half medium change **C**	Make **Astrocyte Maintenace Medium (Astrocyte Basal Medium + A +B+C)**
8	Thawing **Differentiation Supplement D**	
9	Half medium change **D**	Half medium change
10		
11	Half medium change **D**	Half medium change
12		Half medium change
13	Half medium change **D**	Thawing **Unlock (ax0044)**
14	Full medium change to **BrainPhys**	Adding the cultured Astrocyte to MEA plate where iPSC-neurons were cultured
15		
16	Half medium change **BrainPhys**	
17		
18	Half medium change **BrainPhys**	
19		
20	Half medium change **BrainPhys**	
21		

2-3. Preparation of Regents (Day -1)

- Please read product manual and protocol by Axol or other suppliers.

Neural Plating-XF Medium

- Thaw Neural Plating–XF Medium (Axol Cat# ax0033)overnight at 4°C in the dark.
- Store the Neural Plating–XF Medium at 4°C in the dark until use.

SureBond Coating solution

- Thaw the Surebond (Axol Cat# ax0041+) at 4 °C overnight.

Neuron Maintenance Medium

- Thaw an aliquot of Neural Maintenance Supplement (Axol Cat## ax0031a) overnight at 4°C in the dark.

2-4. Pretreatment of the MEA Plate (Day 0)

CAUTION:

- Avoid contact with the electrodes in all of following procedures as they are extremely fragile.

Pretreatment of the MEA plate is the most critical step to succesfully culture neurons onto the electrodes of the Presto MEA Plate. The surface of a new Presto MEA Plate is hydrophobic. Therefore, hydrophilization of the Presto MEA plate is necessary to enhance the adhesion of neurons to the electrodes of each well. If cells do not adhere to the electrodes, you will not be able to record from them. It should be noted that some coating agents can affect neural activity, viability, degree of neurite outgrowth, extent of migration, and longevity. Improper coating techniques can cause large-scale clumping and/or the death of neurons even if all other cell culture steps are performed properly. The following section contains recommendations for appropriate pretreatment of the MEA Plate.

Sterilizing the MEA Plate

1. Rinse a new MEA Plate with sterilized distilled water (SDW) at least three times. Clean it with 70% ethanol several times (or immerse it in 70% ethanol for 15 minute).
    - It is strongly recommended to immerse the MEA Plate in 70% ethanol for 15 minutes when a Presto MEA Plate is reused.
    - Higher-grade ethanol is recommended to avoid deposits of organic substances onto the MEA Plate after drying.

2. Rinse the Presto MEA Plate with sterilized distilled water (SDW) at least three times.

3. Let the Presto MEA Plate dish dry under ultraviolet light to irradiate for 15-30 min. Store and handle the Presto MEA Plate in a sterilized container.

Pre-coating the MEA Plate

- Thaw the Surebond (Axol Cat# ax0041) at 4 ºC overnight the night before plating.

1. Add 230 μL of Readyset (250 uL / Cm2) into the MEA 24well Plate-comfort, and incubate at 37ºC for 45 minutes.

2. Remove the Readyset solution, and immediately rinse the MEA Plate with sterilize water 4 times. Fill the wells in the MEA Plate with sterilize water.

    - Pay attention so that the plate surface won't dry during this process.

3. Mix SureBond 120uL with D-PBS 6ml and pipette it.

4. Remove sterilized water from the MEA Plate and immediately fill the well with 200 uL of SureBond solution (#3).

5. Incubate in an incubator (37 ºC, 5%CO_2) for one hour.

2-5. Thawing and Preparing the iPSC-Derived Neural Stem Cell

- Please read product manual and protocol by Axol or other suppliers.

2-6.Plating the iPSC-Derived Neural Stem Cell onto the MEA Plate

CAUTION:

Be careful NOT to touch the electrodes.

Preparing the CellSpotter

The iPSC-neurons will be positioned onto the electrodes in a Presto MEA Plate using the CellSpotter24 eco + cloning rings. Prepare the CellSpotter as well as cloning rings before plating.

Figure 1. CellSpotter24 eco (left) and cloning rings (right). Cells will be amounted onto the recording electrodes through cloning rings placed in the central hole of the CellSpotter.

1. Autoclave the CellSpotter and cloning rings.
2. Place the cloning rings in a petri dish (Figure 2, Left).
3. Shake the petri dish. Select only cloning rings that do NOT move (Figure 2, Right).
    - Some cloning rings have an uneven edge, which can cause cell suspension to leak through the bottom. This step is necessary to select only the good cloning rings.

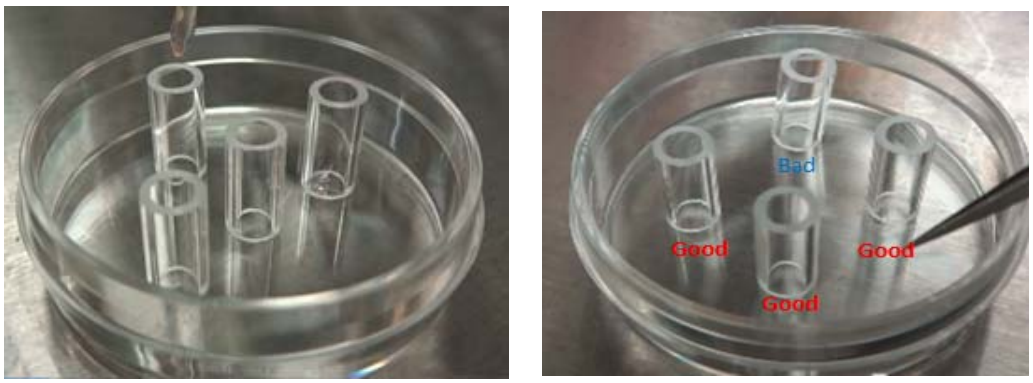


Figure2. Cloning ring placed in a petri dish (left). Shake it and select only cloning rings that do NOT move (right).

4. Moisten the inside of the cloning ring by pouring Neural Plating-XF Medium (Figure 3, left).
    - This process will help prevent the cell suspension from sticking in the middle of cloning ring (Figure 3, right).

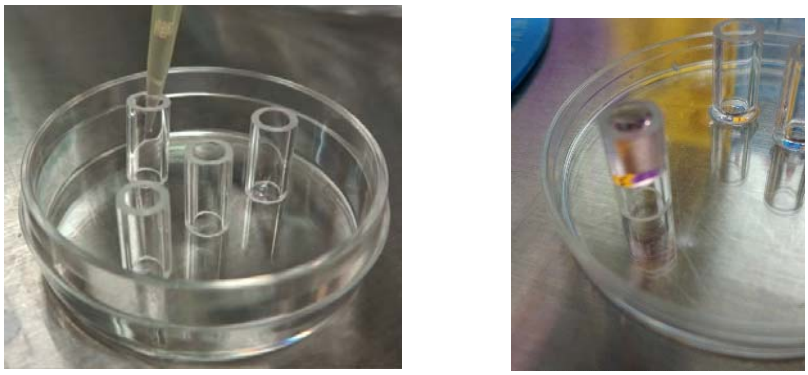


Figure 3. Moisten the inside of the cloning rings (left) and cell suspension sticking in the middle of cloning ring (right).

Plating cells onto electrodes using the CellSpotter

1. Place the CellSpotter onto the MEA plate (Figure 4, left)
2. Remove the SureBond from each well in the Presto MEA plate through the side-slot (Figure 4, right).

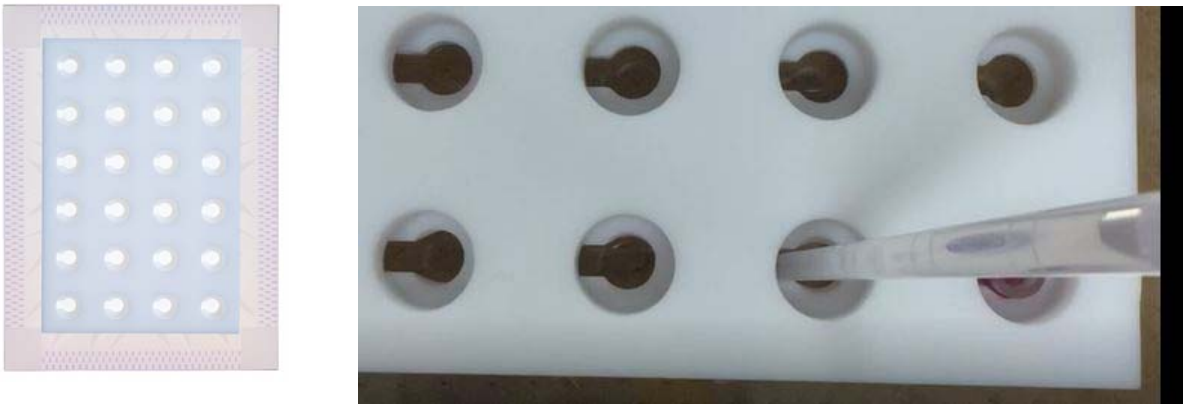


Figure 4. Placing the CellSpotter onto the MEA plate (left) and remove the SureBond from a well through the side-slot (middle, right).

3. Insert the cloning rings into the central holes of the CellSpotter.

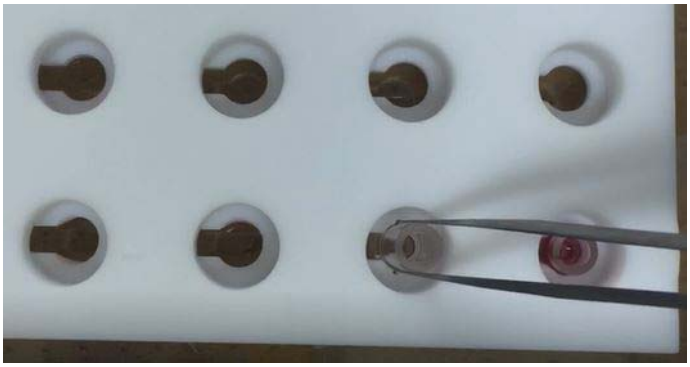
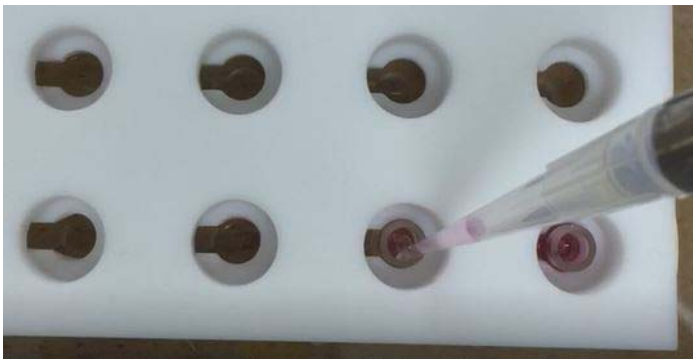


Figure 5. Place a cloning ring into a center-hole. Make sure the edge of cloning ring sits in the bottom of the MEA Plate.

4. Flood around 75 µL of cell suspension (containing around **75,000 cells**) into the cloning ring.

    - **Final cell density: 8.0 x 10^5 cells/Cm^2**



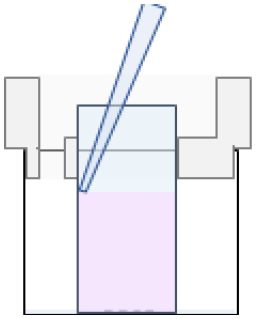
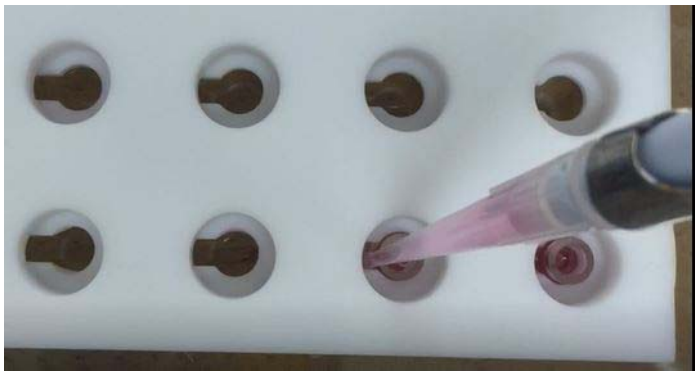


Figure 6. Flood the cell suspension into the cloning ring placed in the center-hole.

5. Add **250 µL o**f pre-warmed Medium A to the wells through the side-holes (Figure 7).



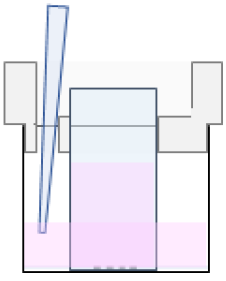


Figure 7. Add the Medium A to the well through one of the side holes.

6. Plate the neurons onto electrodes in the all wells using the same procedure (#2-5).

7. Incubate at 37ºC, 5%Co_2 for one hour.

8. Gently remove the cloning rings from all wells (Figure 8, left) and then CellSpotter (Figure 8, right).

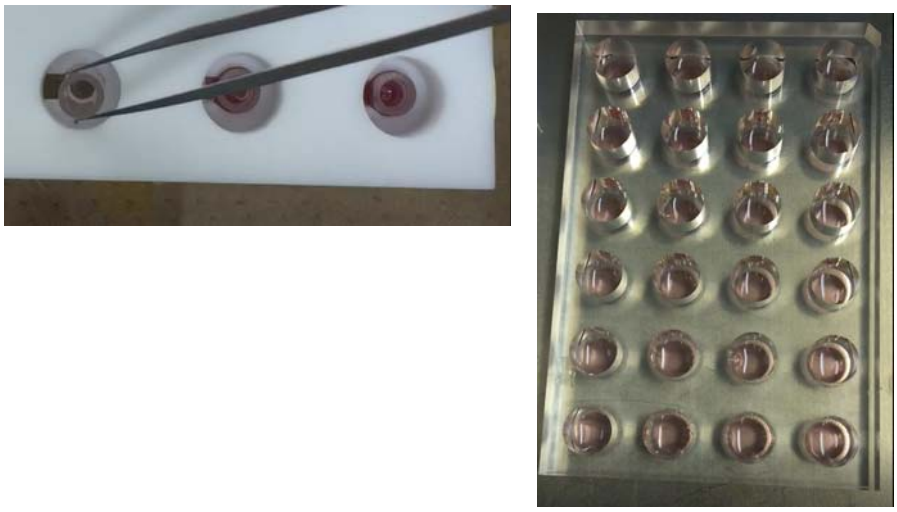


Figure 8. Remove the cloning rings (left), and then CellSpotter after removing all coning rings.

9. Add **450** µL of Medium A to each well.

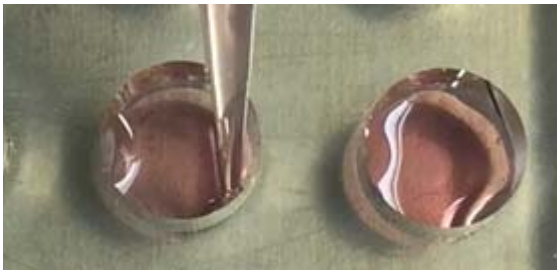


Figure 9. Add 450 µL of Neural Plating-XF Medium to each well.

On the following day (Day 1)

10. Replace the full medium with **700** µL of fresh pre-warmed (37ºC) Medium B.

On Day 3

11. Half medium change with pre-warmed Medium C. Change the half medium with Medium C every to days (at Day 7 and 9).

On Day 9

12. Half medium change with pre-warmed Medium D. Change the half medium with Medium D every 2 days.(up to Day 20)

3. Co-culturing with Astrocyte

3-1. Workflow

At Day 14, add the human iPSC-Derived Astrocyte into a plate that iPSC-neurons were cultured. Culture the Astrocyte in a culture plate for several days BEFORE plated into MEA.

Day	Neural Stem Cells	Astrocyte
-1	Thawing **Surebond** Thawing **Plating XF Medium** Thawing **Neural Maintenance Medium (ax0031b,ax0031a)**	
0	Pre-coating MEA Plate	
	Plating neurons onto the MEA Plate	
1	Full Medium change **B**	
2	Thawing **Neural differentiation XF medium (ax0034-125), Differentiation Supplement A and B**	
3	Half medium change **C**	
4		
5	Half medium change **C**	
6		Thawing Supplement **B** and **C** overnight at 4 C
7	Half medium change **C**	Make **Astrocyte Maintenace Medium (Astrocyte Basal Medium + A +B+C)**
8	Thawing **Differentiation Supplement D**	
9	Half medium change **D**	Half medium change
10		
11	Half medium change **D**	Half medium change
12		Half medium change
13	Half medium change **D**	Thawing **Unlock (ax0044)**
14	Full medium change to **BrainPhys**	Adding the cultured Astrocyte to MEA plate where iPSC-neurons were cultured
15		
16	Half medium change **BrainPhys**	
17		
18	Half medium change **BrainPhys**	
19		
20	Half medium change **BrainPhys**	
21		

3-2. Preparation of Regents

One day before thawing the Astrocyte (Day 6)

- Thaw the Supplement B and C (Axol Cat# ax0084) at 4 ℃ overnight.

On the day of thawing the Astrocyte (Day 7)

- Make Astrocyte Maintenance Medium. Transfer the 25mL of Astrocyte Basel Medium (to a fresh 50 mL tube and add Supplement A, B and C according to the volumes outlined in the Table below.

Component	Volume
Basal Medium	25 mL
Supplement A	2.25 mL
supplement B	0.5 mL
Supplement C	25 µL

- Prepare the coating solution for culture plate (Laminin-511) with following procedure:

  1) Add 20 µL of Laminin511 (iMatrix) to 3980 mL of PBS.

  2) Add 1 mL of Laminin into each well in the culture plate, and incubate it at 37C, 5%CO2.

  3) Remove the Laminin. Add 3mL of Astrocyte Maintenance Medium into each well and incubate it before the Astrocytes are plated.

3-3. Thawing and preparing the Human iPSC-Derived Mature Astrocyte

Culture the Astrocyte for 7-10 days in a 6well culture plate before plated onto the MEA with following the instruction in the Axol Protocol: Human iPSC-Derived Mature Astrocytes (page 4).

3-4. Co-culturing with the Human iPSC-Derived Mature Astrocyte

Preparation

On the day before seeding (day 13)

- Thaw the Unlock-XF (Axol Cat# ax0054) at 4 ºC overnight.

On the day of seeding (Day 14)

- Prepare the BrainPhys with following procedure:

  1) Pour 10 mL of PBS into a 15mL conical tube, and warm it up to 37 ºC.

  2) Pour 5 mL of BraihPhys into the conical tube and warm it up to 37 ºC.

Plating the Astrocyte to the MEA Plate

1. Remove the all medium in the MEA plate where iPSC-neurons are cultured, and add **700 µL** of pre-warmed BrainPhys to each well.
2. Remove all the medium from the 6well culture plate where Astrocyte is cultured, and then immediately add 2mL of PBS into each well.
3. Remove the all PBS, and then add 1mL of Axol Unlock-XF into each well and incubate it at 37 ºC, CO2 5% for 3-5 minutes.
4. Confirm the cells are detached from the culture plate and suspended. If cell aggregation is observed, pipette it gently to disperse them. Transfer the cell suspension to 15mL conical tubes.(3ml / each 15mL conical tube)
5. Add 6mL of BrainPhys, and pipette it gently.
6. Centrifuge at 200 g (1200 rpm on most tabletop centrifuges)at room temperature for 5 min.
7. Carefully remove the supernatant.
8. Add 1 mL of BrainPhys and gently pipette it several times.
9. Mix the 10 mL of cell suspension with 10 mL of trypan blue solution. Count the number of cells.
10. Plate 20,000 cells into each well.
11. Half-change the BrainPhys every 2 days.

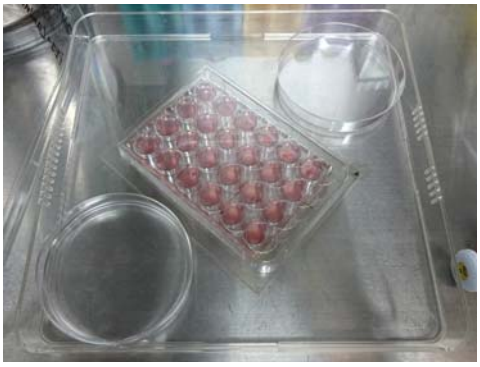


Figure 10. Cultured neurons plated in an MEA Plate.

3-6. Cleaning the used MEA Plate

The MED64 Presto MEA Plate's electrical characteristics are best during the first use. High quality signals can be recorded and effective stimulation is possible with the Presto MEA Plate thanks to the electrode's characteristics, the lowest impedance in a commercially available microelectrode array. The electrode's impedance will increase with repeated use of the MEA Plate due to damage in handling and/or residual cellular debris and coating materials. However, the MEA plates can be re-used if they are handled and cleaned very carefully. The following are cleaning procedures recommended by MED64 users:

- **CAUTION:**

  ALWAYS avoid contact with the surface of the MEA Plate to preserve the microelectrodes and insulation layer.

1. Clean the MEA Plate with distilled water.
2. Fill all wells in the MEA Plate with 0.25% Trypsin, and incubate at 37°C for 1 hour.
3. Rinse the MEA Plate with distilled water.
4. Clean the MEA plate in a ultrasonic bath for 3 minutes.
5. Immerse the MEA plate in 70% ethanol for 15 minutes.
6. Store the MEA plate in a distilled water.

When re-used:

1. Immerse the MEA Plate in 70% ethanol for 15 minutes.
2. Rinse the MEA Plate with distilled water.
3. Let it dry under ultraviolet irradiation (Irradiate both sides for 30 min each).
4. Proceed to pre-coating as soon as possible.

4. Data acquisition

Cultured sensory neurons start firing 1-2 weeks after plating, but initial activity can vary depending on culturing technique and conditions. Once the neurons begin firing spontaneously, activity can be recorded using the MED64 Presto, however you may want to perform your experiments 4 weeks or later after synchronized firing is observed. The following section details the recommended environment for recording from sensory neurons.

4-1. Recommended experimental environment

37°C is the typical recording temperature recommended for recording from sensory neuron cultures. However, the set temperature can vary depending on samples and nature of your experiments.

The MED64 Presto Amplifier incorporates heater, that heats the MEA Plate from the bottom. Below are the recommended settings for data acquisition.

- Do NOT place the MED64 Presto Amplifier in an environment where temperature changes frequently, for example in the proximity of an air conditioner or heater.
- Keep the acrylic lid closed and secure during acquisition.
- It can take time for the set-temperature to stabilize. Power on the temperature controller at least 30 minutes before starting acquisition.

If/When the set-temperature is changed, wait for the temperature reported by the temperature controller to stabilize at the new temperature. It can take 5-30 minutes or more but is typically very fast.

4-2. Data acquisition and analysis

Each well in the MEA 24-well plate has 16 recording electrodes as well as 16 reference electrodes. The differences between the field potential acquired at each recording electrode is compared to the potential of the average for all reference electrodes. Acquired signals are amplified by x1000, digitized, and sent to an acquisition PC.

MEA Symphony software is available for data acquisition and analysis. The Symphony software always acquires extracellular signals with acquisition bandwidth of 0.1Hz - 5 kHz. However, acquired raw data can be filtered for analysis. The following parameters are typically recommended for analyzing spikes recorded from sensory neurons.

- Low-pass filter (2 pole): Bessel 3000Hz
- High-pass filter (2 pole): Butterworth 100Hz (Select 1Hz if you want to record slow wave)

4-3. MEA Symphony software

The MEA Symphony is an acquisition and analysis software for the MED64 Presto System.The MEA Symphony records raw extracellular signals at all 384 electrodes, extracts spikes, and analyzes the spike frequencies. The software also extracts synchronized bursts over every electrode in a well and analyze those synchronized bursts.

The MEA Symphony always records and **saves raw data (**0.1Hz - 5kHz acquisition bandwidth) no matter what filtering option is selected. However, filtered data is used for all display and analysis.

This section will quickly guide you capabilities of the Symphony software.

Figure 11. Main screen for the MEA Symphony software.

Oscilloscope

There are several ways to displayed acquired data such as:

- Raw data acquired from all 384 electrodes
- Raw data acquired from all electrodes in a selected well
- Raw data acquired from an electrode in a selected well (with spike extractions)
- Extracted spikes from all electrodes in a selected well
- Heat map for a selected well

Analysis

The MEA Symphony provides abundant of spike and burst analysis. All analysis are displayed as a graph in the Main screen, and can be included in your Analysis report as well as exported independently to CSV file.

Spike frequency analysis

The following spike frequency analyses are available:

- The total number of spikes (Per electrode, Per well, Per treatment).
- Spike frequency (Per electrode, Per well, Per treatment).
- Inter-spike interval (Per electrode, Per well, Per treatment).
- Time course of spike frequencies (per well).Number of spikes (Per electrode, Per well, Per treatment).

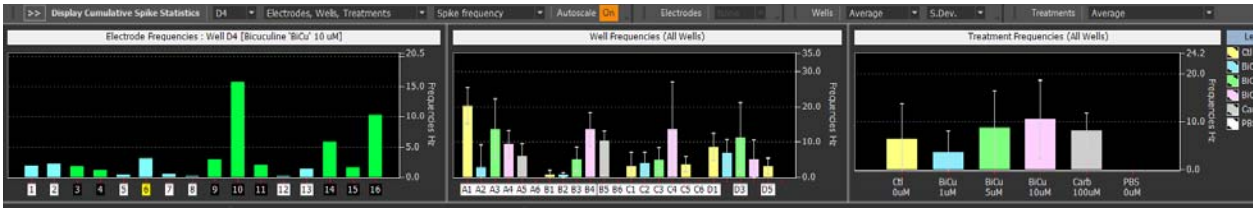


Figure12. Spike frequency analysis performed with the MEA symphony per electrode (left), well (middle), and treatment (right).

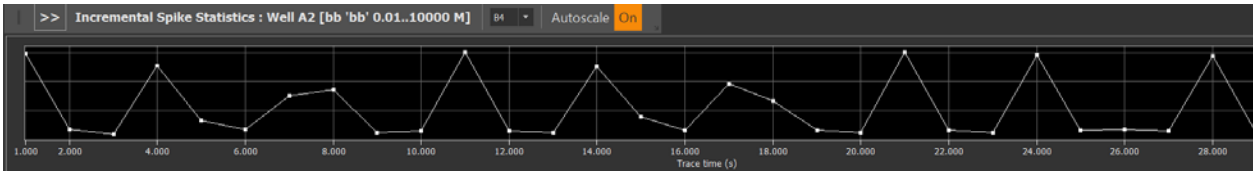


Figure 13. Time course of spike frequency analysis (per a well) performed with the MEA Symphony.

Burst analysis

Following analysis is available for synchronized bursts (bursts synchronized over electrodes in a well):

- Raster plots (with burst extractions) for a selected well
- Spike frequency histogram
- Burst analysis information:
  Number of bursts, Start time, End time, Burst duration, Inter-burst-intervals, Number of spikes in a burst (per a well)

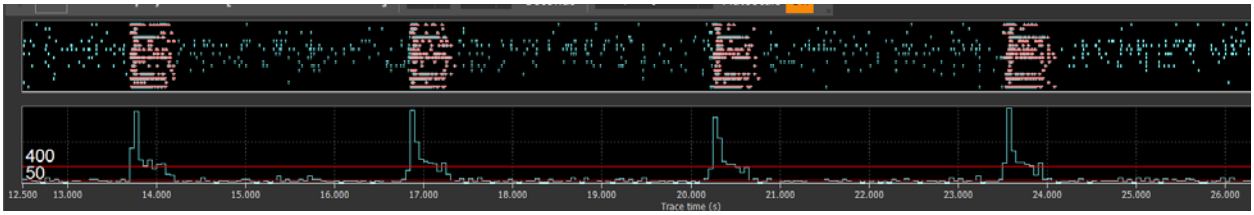


Figure 14. Raster plots (top) and Spike frequency histogram computed (bottom) and displayed in the MEA Symphony.

Exporting

Following data or/and analysis can be exported with the MEA Symphony.

	Format	
Raw data	CSV Text (Ascii)	
	Binary short 16-bit	
	Mobius (.modat)	
Spike	CSV Text (Ascii)	Time stamp only
	CSV Text (Ascii)	Time stamp and spike waveforms
Time course of Spike Frequencies	CSV Text (Ascii)	
Spike analysis per electrode	CSV Text (Ascii)	Number of spikes, Spike frequencies, Spike intervals
Spike analysis per well	CSV Text (Ascii)	Number of spikes, Spike frequencies, Spike intervals
Spike analysis per treatment	CSV Text (Ascii)	Number of spikes, Spike frequencies, Spike intervals
Burst analysis	CSV Text (Ascii)	Number of bursts, Start time, End time, Burst duration, Inter-burst-intervals, Number of spikes in a burst (per a well)

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