MEA Application Note: Primary Hippocampal Neurons from *Rattus norvegicus*

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A major part of this information is based on the instructions provided by the laboratory of Dr. Steve Potter and Dr. Daniel Wagenaar. We also thank Dr. Frank Hofmann from the University of Heidelberg.

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1 Introduction

1.1 About this Application Note

The intention of the MEA Application Notes is to show users how to set up real experiments with the MEA-System on the basis of typical applications that are used worldwide.

The documents have been written by or with the support of experienced MEA users who like to share their experience with new users.

This application note includes a complete protocol for the isolation and cultivation of primary neurons, suggestions for long term cultures, suggestions for MEA-System configurations, and references.

1.2 Acknowledgement

Multi Channel Systems would like to thank all MEA users who shared their experience and knowledge with us, especially the following persons.

Daniel Wagenaar

Steve Potter

Frank Hofmann

2 Material

2.1 Biological Materials

- 5 Rat hippocampus slices, 350 µm thick (please see application note “Acute Hippocampus Slice” for details)

2.2 Technical Equipment

- MEA-System (with amplifier and data acquisition, see chapter “Suggested MEA-System”)
- Stimulus generator (for USB-MEA60-Systems, in MEA2100-Systems a STG is already included)
- MEAs (microelectrode arrays)
- Sterile workbench
- Incubator set to 35 °C, 65 % relative humidity, 9 % O_2, 5 % CO_2
- Water bath at 37 °C
- Stereo microscope
- Inverted microscope
- Micropipettes and pipette tips (20 µl and 1000 µl)
- 15 µl BD Falcon tubes
- 40 µm nylon mesh cell strainer (BD Falcon)
- Sharp forceps
- Curved forceps
- Small scissors
- Teflon membranes (ALA Scientific Instruments)

2.3 Chemicals

- NaOH
- $MgCl_2$
- $CaCl_2$
- HEPES
- Phenol Red
- Na_2SO_4
- K_2SO_4
- Kynurenic acid
- DL-2-amino-5-phosphonovaleric acid (APV)
- Polyethylenimine (PEI)
- Laminin
- Sodium pyruvate
- Insulin
- Glutamate
- Phosphate buffered saline (PBS)
- Bovine serum albumin (BSA)
- Protease from *Streptomyces griseus* 4,9 units/mg (Sigma)
- Papain suspension (Roche Applied Science, catalog No. 10108014001)
- DNAse (Sigma)
- Horse serum (Donor Equine Serum from HyClone)
- Dulbecco's Modified Eagle Medium (DMEM) (Gibco/Invitrogen)
- L-Alanyl-L-Glutamine (GlutaMAX from Gibco/Invitrogen)
- Hanks' Balanced Salt Solution (HBSS) without Calcium / Magnesium (Gibco/Invitrogen)
- Earle’s balanced salt solution (EBSS)

2.4 Media

2.4.1 Culture Medium for Primary Cultures

- DMEM (may contain GlutaMax, depending on the supplier)
- 10 % horse serum
- 0.5 mM GlutaMax (final concentration)
- 1 mM sodium pyruvate
- 2.5 µg/µl insulin

Note: Some work groups use trypsin, other papain for the enzymatic digestion of the tissue.

2.4.2 Enzyme Solution (Protease from *Streptomyces griseus*)

- Protease 2 mg/µl
- Earle's balanced salt solution (EBSS)
- 1 mM $MgCl_2$
- 0.5 mM $CaCl_2$

2.4.3 Alternative Enzyme Solution (Papain)

- 2 µl Segal's medium (see below)
- 200 µl papain suspension
- NaOH for adjusting the pH to 7.3

2.4.4 Segal's medium

(Banker & Goslin, p309ff.)

	Conc. (mM)	FW (g/mol)	mg (for 500 µl)
$MgCl_2 \cdot 6H_2O$	5.8	203.31	590
$CaCl_2 \cdot 6H_2O$	0.25	147.02	18.4
HEPES	1.6	238.3	191
Phenol Red	0.001 %		5
$Na_2SO_4 \cdot 10H_2O$	90	322.21	14500
K_2SO_4	30	174.26	2610
Kynurenic acid	1	189.2	95.6
APV	0.05	197.1	4.92

Note: Kynurenic acid takes a **lot** of stirring to dissolve. It is also recommended to add more NaOH while dissolving to keep the pH reasonable.

1. Use 0.1N NaOH (about 1 µl) for adjusting the pH to 7.3 before adding APV and Kynurenic acid.
2. Again, use 0.1N NaOH (about 1 µl) for adjusting the pH to 7.3 after having added APV and Kynurenic acid.
3. Bring up to 500 µl after the final pH adjustment.
4. Sterile filter, aliquot, and freeze in liquid nitrogen.

2.4.5 BSA / PBS

- Phosphate buffered saline (PBS)
- Bovine serum albumine (BSA)

3 Methods

3.1 MEA Coating

Depending on the type of selected MEA, various coatings may be applied. Standard MEAs should be coated with Polyethylenimine or Laminin. Suggestions for coating methods can be found in the MEA manual available in the "Download section" of the MCS web site.

3.2 Dissection

1. Euthanize the animal with carbon dioxide.
1. Decapitate the animal with large sharp scissors or with a guillotine.
2. Open the skull carefully with a scissor and remove the brain.
3. Cut the brain in an either sagittal or sagittal horizontal plane and trim the hippocampal slices of the surrounding tissue. Prepare hippocampus slices.

3.3 Enzymatic Digestion

1. Cut the slices into 4 – 5 pieces and transfer the pieces into a Falcon tube with 2 – 3 µl protease solution pre-warmed at 37 °C.
2. Digest the pieces for 25 min in protease solution at 37 °C in a carbogen atmosphere in an incubator. Gently swirl the suspension every 5 min.
3. Gently wash the fragments in the culture medium twice: Gently swirl or invert the suspension of brain pieces in medium to wash away the protease, allow the pieces to settle, remove the supernatant with a pipette, and add 2 – 3 µl fresh medium. After the second wash, add 1 µl medium to the fragments.
4. Gently triturate the fragments by passing the preparation five times through the 0.78 mm wide opening of a 1000 µl pipette tip. The majority of cells should now be in suspension.
5. Transfer the supernatant containing the suspended cells into a fresh Falcon tube.
6. Add 1 µl medium to the remaining fragments, and triturate the remaining fragments once more.
7. Combine the supernatants from the two triturations in one tube, giving 2 µl cell suspension.
8. Remove the debris by gravity flow filtering the cell suspension through a 40 µm nylon mesh cell strainer (Falcon) into a 15 µl DB Falcon tube filled with BSA/PBS.

3.4 Plating und Culturing the Cells

1. Centrifuge the cell suspension at 160 g for 5 min.

2. Discard the supernatant and resuspend the cells in approximately 0.5 µl culture medium.

3. Count the cells under a stereo microscope to determine the cell density by using a Neubauer chamber or an automated cell counter.

4. Plate the cells in a density of 1000 – 5000 cells per mm^2 (depending on your application) onto the recording field of the MEA.

5. Maintain the cells for 4 – 5 days in an incubator set to 35 °C, 65 % relative humidity, 9 % O_2, 5 % CO_2 before recording, depending on your application.

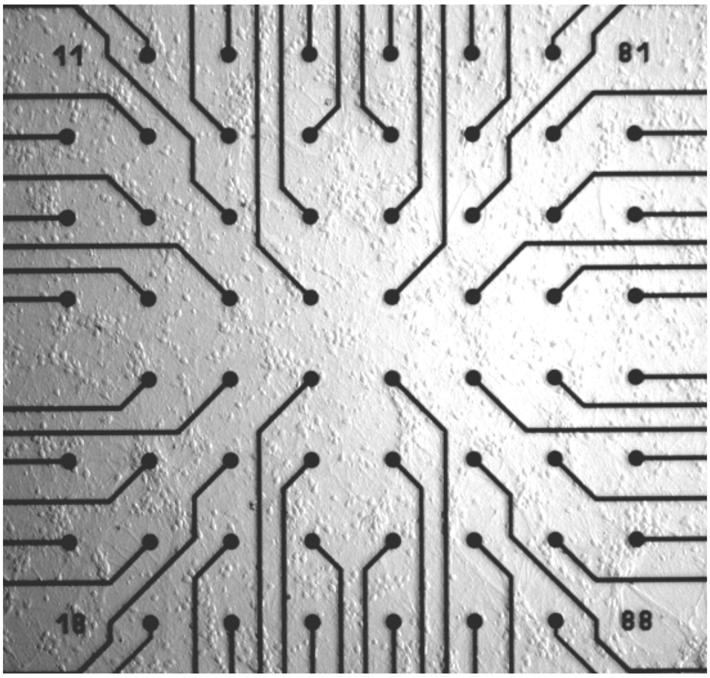
6. Check the pH of medium daily by eye, and change it as soon as the color indicates that the medium is going acidic (shifting from pink to orange color).

   Note: The choice of plating density is really up to the investigator. The denser, the sooner the activity will be observed (as soon as 4 days *in vitro*), but the more often the culture will need to be fed. 5000 cells/mm^2 is very dense, and would require feeding about every two days, while 1000 – 3000 may only require feeding weekly or every 5 days. The best time for recording depends on the application: Many studies may be aimed at the development of the cultures, and therefore require recording as soon as possible. For other applications, it may be better to keep the cells longer in culture before starting the experiment. Cells are still developing, but the culture is more stable after about one month in culture. The culture can be used for several months or years.

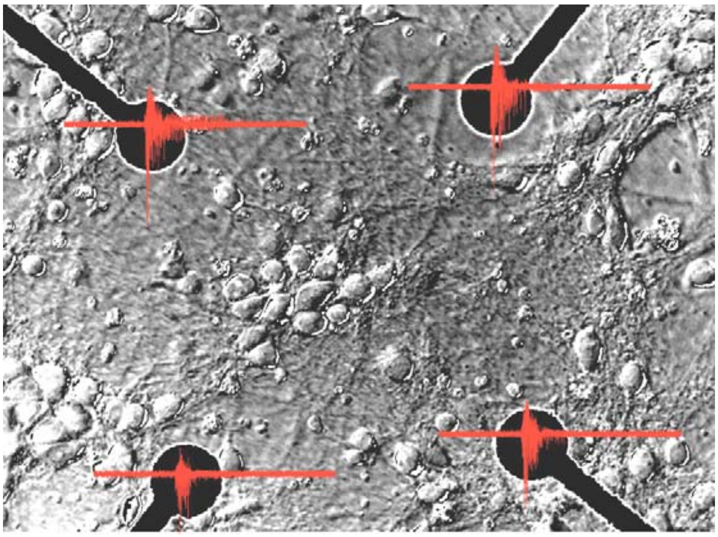
4 Application Example: Neuronal Plasticity

The following picture shows rat hippocampal cells (neurons and glia) per mm^2 plated onto a standard MEA after 12 days in culture. A typical plating density was about 500 +/– 20 % cells per mm^2. Less cells are generally better, but if the density is too low, they will not form a network.

In the following picture, you can see the cells on a typical electrode grid of 8 x 8 electrodes (electrode diameter 30 µm, interelectrode distance 200 µm).



The next picture shows a zoomed view of four electrodes overlaid with a typical highly organized, periodic and synchronous burst activity (about 1 s traces) of the hippocampal cells after stimulation with bicuculline, a GABA$_A$ receptor antagonist.



A MEA1060 amplifier with a pass band of 10 Hz – 3.5 kHz, and a gain of 1200 was used for amplification and filtering. Sampling rate was 20 kHz per channel. Stimulations and recordings were performed after a culturing period of 10 to 14 days.

Before stimulation, network activity was recorded for three minutes; cultures with spontaneous bursting activity were excluded. Recurrent synchronous network bursting was induced by treatment of the neurons with 50 µM bicuculline dissolved in 0.05 % DMSO. After another three minutes of recording, bicuculline was washed out by changing the medium three times. Cultures were put back into the incubator and three minutes recordings were repeatedly performed at different time points following the washout of bicuculline.

Spikes were detected with the integrated spike detector of the MC_Rack software. Burst analysis was done with Neuroexplorer (NEX Technologies, www.neuroexplorer.com).

(Please see reference 1. Pictures kindly provided by Dr. Frank Hofmann, University of Heidelberg, Germany.)

5 Longterm Culturing

In order to allow long term cultivation and recording, Multi Channel Systems MCS GmbH recommends the use of teflon membranes (fluorinated ethylene-propylene, 12.5 microns thick) developed by Potter and DeMarse (2001). The ALA-MEA-MEM membrane is produced in license by ALA Scientific Instruments Inc., and distributed via the world-wide network of MCS distributors.

The sealed MEA culture chamber with transparent semipermeable membrane is suitable for all MEAs with glass ring. A hydrophobic semipermeable membrane from Dupont that is selectively permeable to gases (O_2, CO_2), but not to fluid, keeps your culture clean and sterile, preventing contaminations by airborne pathogens. It also greatly reduces evaporation and thus prevents a dry-out of the culture.

6 Suggested MEA-System

6.1 System Configurations

Depending on the throughput and the analysis requirements desired in your laboratory, different system configurations are recommended for the recording from cultured neurons.

- **MEA2100-System**: For the MEA2100-HS60(2x60)-System, the same MEAs can be used as for the MEA1060 amplifiers, with the same advantages and disadvantages. In addition, for the MEA2100-HS120 a 120-channel MEA layout is available (120MEA200/30iR), which provides a larger electrode field with a resolution identical to the 60MEA200/30iR. This might be interesting for a mapping applications. The MEA2100-System allow to operate a maximum of two headstages with up to two 60 channel MEAs each. If all those slots are used with 6 well MEAs 24 wells can be tested simultaneously.
- **MEA60-System**: **60-channel** MEA recording system for inverted or upright microscopes. The **temperature controller** TC02 regulates the temperature of the MEA and of the perfusion fluid via the **perfusion cannula** PH01. The peristaltic perfusion system PPS2 is provided respectively. A **MEA1060 amplifier** allows recording up to 60 channels from a 60-channel MEA. This is the standard configuration for low-throughput academic research.

6.2 Microelectrode Arrays

Available MEAs differ in electrode material, diameter, and spacing. For an overview on available MEA types please see the MEA Manual, or the Multi Channel Systems MCS GmbH web site or contact your local retailer.

The microfold structures formed by titanium nitride (TiN) result in a large surface area that allows the design of small electrodes with a low impedance and an excellent signal to noise ratio. For recording from cultured neurons, a medium spatial resolution with an electrode diameter of 30 µm and a spacing of 200 µm is generally sufficient.

Recommended MEAs include:

- **60MEA200/30iR-Ti** standard 8 x 8 layout, TiN electrodes for recording and stimulation, with substrate-integrated reference electrode.
- **60ThinMEA** for high-resolution imaging and combination with intracellular calcium measurements. ThinMEAs are only 180 µm “thick” and mounted on a robust ceramic carrier. Tracks and contact pads are made of transparent ITO.

Ring Options

The standard glass ring is best for running a perfusion, and will also allow using the ALA MEA-Insert. A plastic ring should only be used if parallel patch clamp experiments are planned.

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