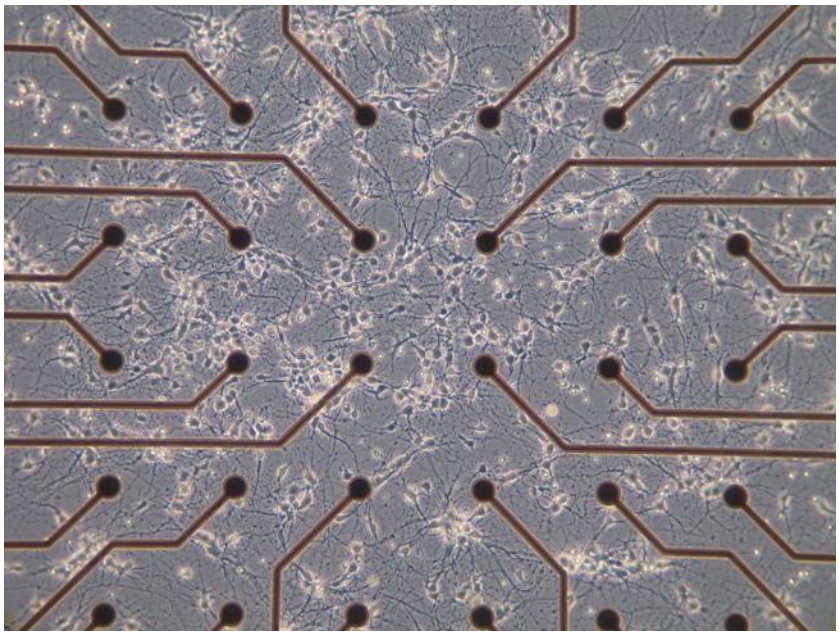
Cell electrophysiology of dissociated embryonic hippocampal neurons

1. Purpose

This protocol describes the platform used to characterise the *in vitro* network electrophysiology of embryonic hippocampal neurons carrying strategic genetic modifications specified by the 'Genes to Cognition' gene targeting pipeline. Primary cultures of hippocampal neurons from mouse embryos are cultured on multi-electrode arrays (MEAs) and maintained for one month, during which time, periodic recordings of spontaneous electrophysiological activity are acquired. In such a way, a time course of the development of spontaneous neuronal activity can be constructed. Numerous analyses are applied to the data to explore how network activity is altered by the mutation of interest.



Example of hippocampal neurons (7 days in vitro) growing on a multi electrode array

2. Procedure

2.1 Preparation of multi-electrode arrays

Residual medium from previous experiments is aspirated from MEAs. Zero-evaporation lids are washed in 70% ethanol. MEAs are run under a stream of reverse osmosis-purified (RO) water to lyse and remove any residual cells from the previous experiment. MEAs are sprayed with 70% ethanol to sterilize and allowed to air dry in a tissue culture hood. Once dry, MEAs are treated in a plasma cleaner for one minute then each is transferred to a sterile plastic dish, filled with 1ml sterile RO water and stored at 4 degrees C. Zero evaporation lids are allowed to air dry under ultra-violet illumination in a tissue culture hood.

For culturing, MEAs are removed from the refrigerator and the sterile water aspirated. MEAs are treated in a plasma cleaner for 1 minute. The central-most regions of the MEAs are coated with 8µl poly-D-lysine solution, which is aspirated after 2 minutes. 8µl sterile RO water is then added to the centre of each MEA, which is aspirated after 2 minutes. 30µl full Neurobasal® medium is added to the circumference of the culture compartment of the MEA. Laminin is slowly thawed on ice to prevent polymerisation before application to surface, and 3µl 1mg/ml applied to the centre of the MEA. A sterile zero-evaporation lid is affixed and the MEA is placed in the incubator until cells are plated.

2.2 Primary culture of embryonic hippocampal neurons

Timed matings are set up between parent mice homozygous null for the gene of interest. Pregnant female mice are killed by cervical dislocation, in accordance with Schedule 1 of the U.K. Animals (Scientific Procedures) Act 1986. The mouse is sprayed down with 70% ethanol to minimise the airborne spread of contaminants. Embryos are exposed by making a Y-shaped incision of the abdomen. Embryos are removed, sprayed with 70% ethanol and transferred to ice-cold DPBS containing 1% v/v streptomycin/penicillin solution. Embryos are separated from uterine membranes and placentae then transferred to a separate dish of DPBS where they are decapitated. Each head to be dissected is stored in a separate well of a 6-well plate containing ice-cold DPBS.

For hippocampal dissection, each head is transferred to the lid of a 50mm-diameter dish containing ice-cold DPBS and positioned under a dissection microscope in a flow hood. The skin covering the skull is peeled away and the skull opened up using two pairs of fine-tipped forceps. The whole brain can then be removed by sliding a micro-spatula along its ventral surface. Again using the micro-spatula, the brain can be divided along the midline into its constituent hemispheres, and residual midbrain can be removed from the inner surface of each hemisphere. The meninges are then removed with two pairs of fine mirror-finish forceps. Hippocampi can then be located and dissected from each hemisphere with two pairs of fine mirror-finish forceps. Hippocampi are transferred to a 35mm-diameter dish on ice containing ice-cold DPBS until the required number of heads has been dissected.

Hippocampi are transferred using a wide-nozzle sterile plastic dropper pipette to a 15ml Falcon tube containing 1ml of papain (10 U/ml) pre-warmed to 37 degrees C. The tissue incubated in this enzyme solution for 22 minutes at 37 degrees C. Excess enzyme is aspirated using a sterile plastic dropper pipette and residual papain inactivated by adding 500µl warm DMEM/FBS. Hippocampi are then manually disaggregated by gentle reverse pipetting with a P1000 pipette tip. Once a uniform cell suspension has been achieved, this is made up to 5ml in DMEM/FBS and the cells are pelleted by centrifugation at 0.4 x *g* for 3.5 min. DMEM/FBS is aspirated and the cells are again dispersed in 500µl DMEM/FBS, topped up to 5ml and spun at 0.4 x *g* for 3.5 min. After aspirating this medium, cells are resuspended in full Neurobasal® medium at a volume of 100µl per number of heads dissected. Cell yield is quantified by obtaining an average of two counts from a haemocytometer. $2x10^5$ cells are seeded over the centre of each MEA (20-30 µl, depending on yield)

2.3 Recording and maintenance of cultures on multi-electrode arrays

At 4 DIV, MEAs are fed by replacing 200µl (1/3 volume) with fresh pre-warmed full Neurobasal® medium. At 7 DIV, pairs of MEAs that have passed the quality control criteria

outlined in 4.1 are interfaced with duplex preamplifiers. External temperature controllers are set to 32 degrees C. A mixture of 5% CO_2/ 95% O_2 is applied. The central area of the preamplifier is covered by an upturned 90mm-diameter dish lined with copper mesh, into which the gas mixture is pumped.

Data from duplex MEAs is captured at a sampling rate of 25kHz using a 128-channel A/D converter card and MC_Rack software. Amplifier gain is 1100. After high pass filtering (100 Hz) action potentials are captured in a cutout recorded 1ms before and 2ms after crossing a threshold (-20µV relative to baseline, equivalent to approx. 6 x SD of baseline noise). 15 min recordings of spontaneous activity are acquired in accordance with the quality control criteria outlined in 4.2 and 4.3. Two recordings per week of each experimental pair of MEAs are taken while they are between 7 and 28 DIV. After each recording, cultures are fed by replacing 200µl of their medium with fresh, pre-warmed full Neurobasal® medium.

2.4 Cell harvesting for post-hoc genotyping

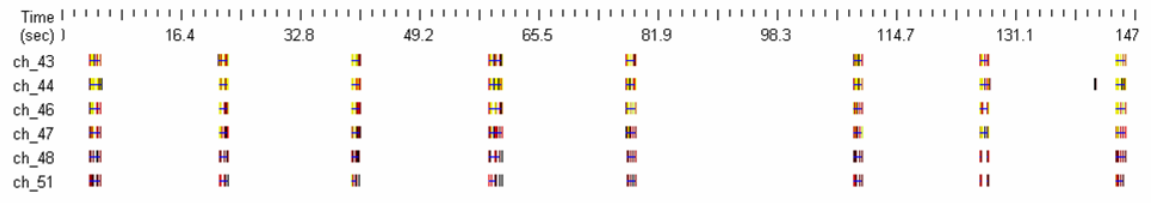
Medium from the experiment is aspirated and replaced with 500µl pre-warmed trypsin/EDTA solution after washing the culture with sterile DPBS. The MEA is returned to an incubator for 10 min then swirled gently to lift the layer of cells from the surface of the MEA. The cells are then transferred to a 15ml falcon tube and the volume made up to 5ml in DMEM/FBS. The cells are pelleted by centrifugation for 3.5 min at 0.4 x *g*, resuspended in 500µl lysis buffer and frozen down in advance of PCR-based genotyping.

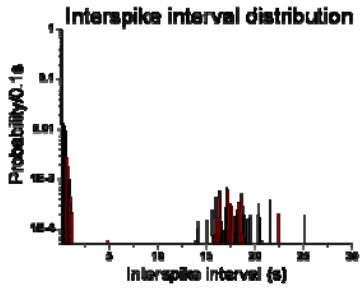
2.5 Data processing and analysis

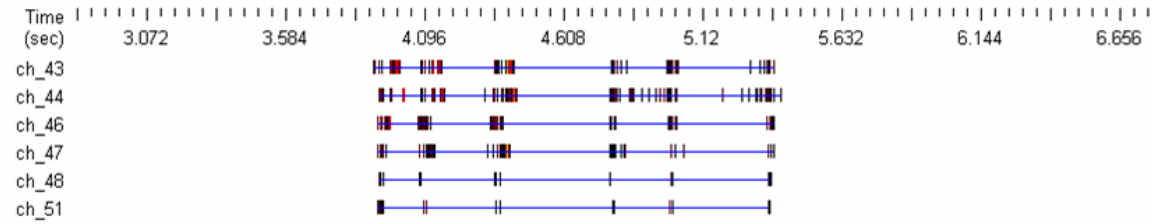
In MC_Rack, duplex recording files are replayed and recorded twice to extract the spikes data stream relating to each individual MEA. Directories of these files are then batch converted to text files containing timestamps of spike events in NeuroExplorer. These files are then processed by submitting them to custom-written analysis routines in the R mathematical programming environment. In an initial phase of analysis the following parameters are calculated:-

Number of active (spiking) channels (> 1 min^{-1})
Total number of spikes (action potentials)
Number of channels with bursts of spikes* (> 1 min^{-1})
Percentage of spikes occurring within bursts
Mean burst duration
Mean burst rate
Coefficient of variation of the inter burst interval

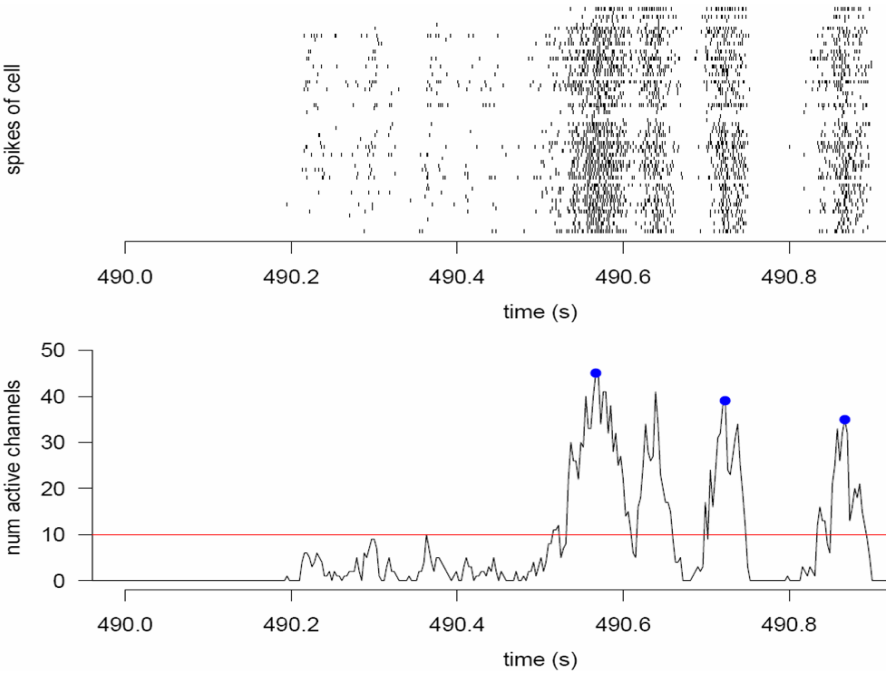
* Bursts are identified by defining an interspike interval below which spikes are considered to form part of a burst. Additionally, a minimum number of spikes (6) and duration (50 ms) is specified. (See below)







A second analysis phase measures the frequency, magnitude and duration of network spikes (defined as periods of near synchronous (< 3ms) activity on at least 10 electrodes of the array, see below, individual NSs marked with blue dots)



3. Materials

3.1 Preparation of multi-electrode arrays

- Multi-electrode arrays (59 titanium nitride electrodes, 30μm diameter, 200μm spacing, internal reference electrode; MultiChannel Systems)
- Zero-evaporation lids: Teflon lids machined to fit round the culture compartment of the MEAs and fitted with gas-permeable but water-impermeable membranes- ALA MEA-Sheet (ALA Scientific Instruments).
- Plasma Cleaner Diener electronic, Femto
- Neurobasal® medium - L-Glutamine (Invitrogen)
- B27 supplement (Invitrogen)
- Penicillin/Streptomycin solution (Invitrogen)
- L-Glutamine
- Full Neurobasal® medium: Neurobasal® medium, 1% v/v penicillin/ streptomycin solution, 2% v/v B27 supplement and L-Glutamine.
- Laminin, Natural, mouse, 1mg/ml (Invitrogen)
- Poly-D-Lysine Bromide (Sigma) diluted to 0.1 mg/ml in sterile water
- Sterile reverse osmosis-purified (RO) water

3.2 Primary culture of embryonic hippocampal neurons

- Papain (Worthington) dissolved in DPBS to 10 units/ml
- DPBS (Invitrogen)
- Penicillin/Streptomycin solution (Invitrogen)
- Full Neurobasal® Medium (defined in 3.1)
- Multi-electrode arrays (detailed in 3.1)
- Zero-evaporation lids (detailed in 3.1)
- Haemocytometer, improved Neubauer configuration (Hawksley)

3.3 Recording and maintenance of cultures on multi-electrode arrays

- Full Neurobasal® Medium (defined in 3.1)
- MEA-120 system (MullltiChannel Systems)
- MC_Rack (MultiChannel Systems)
- Axiovert 200 inverted microscope fitted with phase objectives and heated stage (Zeiss)

- Powershot G5 digital camera (Canon)

3.4 Cell harvesting for post-hoc genotyping

- Trypsin/EDTA (Invitrogen)
- SV Wizard Lysis Buffer (Promega)
- DMEM/F-12 (1:1) + L-glutamine + HEPES (Invitrogen)
- DPBS -CaCl2 -MgCl2 (Invitrogen)
- Foetal Bovine Serum (Invitrogen)
- Penicillin/Streptomycin Solution (Invitrogen)
- DMEM/FBS: 10% v/v Foetal Bovine Serum, 1% v/v Penicillin/Streptomycin Solution in DMEM/F-12 (1:1) DMEM/F-12 (1:1) + L-glutamine + HEPES

3.5 Data processing and analysis

- MC_Rack (MultiChannel Systems)* http://www.multichannelsystems.com/
- NeuroExplorer (Nex Technologies) http://www.neuroexplorer.com
- R (The R Foundation for Statistical Computing)* http://www.r-project.org/
  * Free software downloads available

4. Quality control

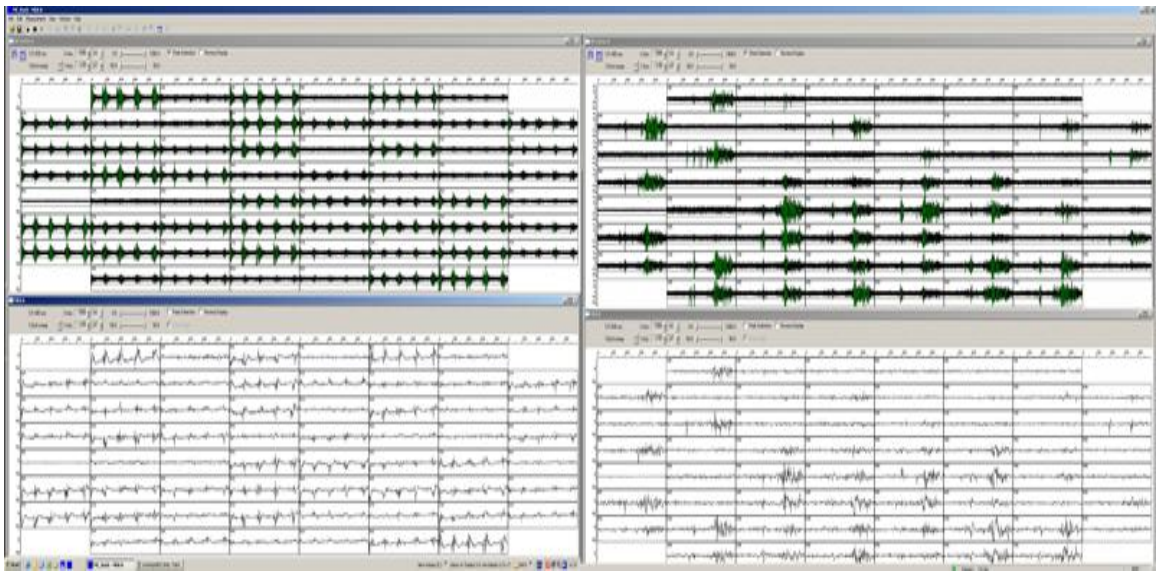
Selection of cultures based on number of adherent cells and uniform cellular distribution across electrodes.

At 24 hr post-plating, each MEA is inspected by phase-contrast light microscopy.

Photographs of a low magnification overview of the MEA (x10 objective) plus five separate fields of view unobstructed by the electrode wiring (x32 objective) are acquired. This allows cell density on the electrode to grid to be calculated. At this point, any MEAs in which few cells have attached to the substrate or in which neuritogenesis has failed are excluded. Exclusion of electrodes detecting excessive levels of noise.

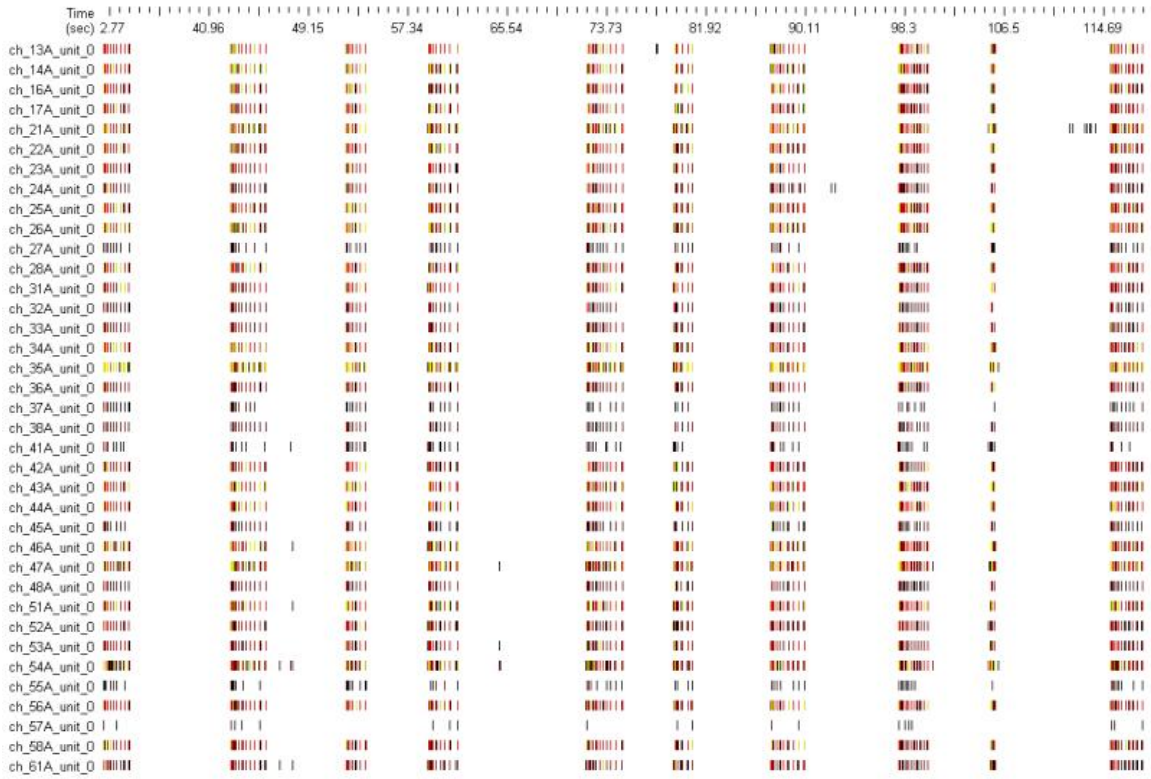
5. Example Data

5.1 Example screen capture during data acquisition



Example of real-time data acquisition simultaneously from duplex multi-electrode arrays using the MC_Rack application.

5.2 Example raster plot



An example raster plot produced by NeuroExplorer to provide a preliminary visual overview of the data from one MEA.

6. Supporting Information

MultiChannel Systems: http://www.multichannelsystems.com

The R Project for Statistical Computing: http://www.r-project.org

Nex Technologies: http://www.neuroexplorer.com

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Eytan D, Marom S. (2006) Dynamics and effective topology underlying synchronization in networks of cortical neurons. J Neurosci. 26(33):8465-8476. PMID: 16914671

Valor LM, Charlesworth P, Humphreys L, Anderson CN, Grant SG. (2007) Network activity-independent coordinated gene expression program for synapse assembly. Proc Natl Acad Sci U S A. 104(11):4658-63. PMID: 17360580

7. Document History

Created by Andrew Morton and Dr Paul Charlesworth, 27th February 2008.