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Protocol

A novel organotypic long-term culture of the rat hippocampus on substrate-integrated multielectrode arrays

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Abstract

Spatiotemporally coordinated activity of neural networks is crucial for brain functioning. To understand the basis of physiological information processing and pathological states, simultaneous multisite long-term recording is a prerequisite. In a multidisciplinary approach we developed a novel system of organotypically cultured rat hippocampal slices on a planar 60-microelectrode array (MEA). This biohybrid system allowed cultivation for 4 weeks. Methods known from semiconductor production were employed to fabricate and characterize the MEA. Simultaneous extracellular recording of local field potentials (LFPs) and spike activity at 60 sites under sterile conditions allowed the analysis of network activity with high spatiotemporal resolution. To our knowledge this is the first realization of hippocampus cultured organotypically on multi-microelectrode arrays for simultaneous recording and electrical stimulation. This biohybrid system promises to become a powerful tool for drug discovery and for the analysis of neural networks, of synaptic plasticity, and of pathophysiological conditions such as ischemia and epilepsy.

Themes: Other systems of the CNS

Topics: Limbic system and hypothalamus

Keywords: Correlation analysis; Hippocampus; Multielectrode array; Organotypic long-term culture; Paired pulse facilitation; Photolithography; Rat; Plasticity

1. Type of research

- Synaptic potentiation, paired pulse facilitation; long-term potentiation; network analysis [2,3,14].
- Extracellular recording; multi site recording; data acquisition and reduction [10,13,21].
- Organotypic explant culture; roller technique; stationary interphase culture; histological preservation in vitro [8,12,20].

2. Time required

The time required to run the total protocol is 2–4 weeks depending on the desired period of the long-term culture. The total time is subdivided into five distinct periods:

- A week is required to manufacture microelectrode arrays together with the attached culture chamber. This time can be saved, if arrays are purchased from a specialized vendor (see Section 3).
- Five hours are necessary for tissue dissection and initiation of the organotypic hippocampal interface cultures.
- A period of 1–4 weeks of incubation is required to perform long-term culturing depending on the desired experiments. During this period about 45 min per week are required to exchange culture medium.
- A period of 5 min to 5 h per MEA-culture is required to stimulate/record depending on the scientific aspects to be investigated.
- Fifteen minutes (DAPI) or 1 h (toluidine blue), respectively, plus the time required for microscopic documentation are necessary for histological staining of the specimens.
- A period of 1–3 days are necessary for data analysis depending on the software available and the complexity of the analysis.

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3. Materials

3.1. Microelectrode arrays

- Ultrasonic cleaner (T 112 BH; Bandelin Electronic, Berlin, Germany).
- Physical vapor deposition apparatus (PVD) (L 560; Leybold, Hanau, Germany).
- Sputtering apparatus (Z550; Leybold, Hanau, Germany).
- Spin coater (1001/146; Convac, Wiernsheim, Germany).
- Hot plate (CHM 450; Convac, Wiernsheim, Germany).
- Plasma enhanced chemical vapor deposition/reactive ion etching apparatus (PECVD apparatus) (Plasmafab 310; Electrotech, Ulm, Germany).
- Mask aligner (MJB; Suess KG, Munich, Germany).
- Quarz-chromium mask (MZD; Dresden, Germany).
- Positive photoresist/developer/remover (Microposit S1818 SOP16 and Microposit remover 1165; Shipley, Esslingen, Germany).
- Negative photoresist/developer/remover (OCG HNR 80; OLIN Microelectronic, Munich, Germany).
- Glass slides (5×5, Corning 7059).
- Silicone rubber; Sylgard 184 (WPI, Berlin, Germany).
- Glass rings, height: 6 mm, i.d.: 19 mm, o.d.: 23 mm, (Labortechnik Griesinger, Kirchheim/Teck, Germany).

3.2. Cell biological tools

- All chemicals were purchased from Merck; biological reagents were of analytical grade and from Sigma–Aldrich (Deisenhofen, Germany), if not stated otherwise.
- GBSS + (Gey's balanced salt solution, # 24260-028; Gibco BRL Life Technologies, Eggenstein, Germany) contained 0.5% D-glucose (# 8337; Merck, Darmstadt, Germany). Chicken plasma (# 30-0390L; Cocalico Biologicals, Reamstown, USA), thrombin (50 NIH-E/ml) (# 12 374; Merck), Triton X-100 (# X-100; Sigma), bovine serum albumin (# A-2153; Sigma), phosphate buffered salt solution (PBS, # 010270; Eurobio, Les Ulis, France).
- Culture medium was composed of 50% basic Eagle medium (# 21370; Gibco BRL), 25% Hanks' buffered salt solution (# 24020; Gibco BRL) 25% horse serum (# 26050; Gibco BRL), 33 mM D-glucose (# 8337, Merck), and 1 mM L-glutamine (# 25030; Gibco BRL). No antibiotics or antimitotics were added.
- Paraformaldehyde (# 1.04005) from Merck.
- Histoclear (Plano; Plannet, Marburg, Germany).
- PVC foil (type 48.4-0015-250 my gl/gl; Vereinte Kunststoffwerke, Staufen, Germany).
- Razor blades for tissue chopping (# 10-096-00; ARNOLD Medizintechnik, Seitingen, Germany).
- Flat bottom Nunclon-Delta tubes (# 156 758; Nunc, Wiesbaden-Bieberich, Germany).
- Glass coverslips (12×24 mm, St. 1; Kindler, Freiburg, Germany).
- Incudrive-S incubator (Schütt Labortechnik, Göttingen, Germany).
- Tissue chopper (McIlwain/Bachofer, Reutlingen, Germany).
- Centrifuge tubes, (50 ml, #610261, Greiner, Nürtingen, Germany).
- UV-source (Min UVIS; Desaga, Heidelberg, Germany).
- Cellulose nitrate filter (Schleicher and Schuell, Dassel, Germany).
- Slide racks, commercially available slide racks with lids for 5×5 cm slides.

3.2.1. Electrophysiological tools

Amplifiers, data acquisition, and temperature control units were purchased from Multi Channel Systems (Reutlingen, Germany): MEASystem, a 60-channel amplifier system (single-ended amplifiers, bandwidth 10–3.4 kHz, amplification $1200 \times$, amplifier input impedance: $> 10^{10}$ Ω, output impedance: 330 Ω) with a

- PC-based data-acquisition (sampling rate 25 kHz/channel, 12 bit resolution, 60 channels for neural signals, four channels designed for data such as TTL pulses for monitoring stimulus events, online high-pass filtering). Data were stored on harddisk (2×3 GB storage capacity) as either continuous data, event-triggered sweeps or spike-waveform cutouts with 1 ms pre- and post-trigger time.
- Oscilloscopes (HP 54601 A and HP 54501 A; Hewlett-Packard, Böblingen, Germany).
- Reference electrode Ag–AgCl pellet (type E205; Science Products Trading, Hofheim, Germany).
- Stimulation electrodes (type SNEX-200 semi-micro, electrode tips 250 μm apart, contact diameter 100 μm; Rhodes Medical Instruments, Woodland Hills, CA, USA).
- Audiomonitor, commercial speaker and audio amplifier (HK 610, Harman Kardon, Northridge, CA, USA).
- Inverted Microscope (Zeiss Axiovert 100; Carl Zeiss, Göttingen, Germany).
- Stimulus generator (Master-8-cp; A.M.P.I., Israel).
- Stimulus Isolation Units (WPI A 365, World Precision Instruments, Berlin; and Iso-flex; A.M.P.I., Israel).
- Software, (Matlab V. 4.3; The MathWorks, Prime Park Way, Natick, MA 01760-1500, USA; STRANGER V. 2.1; Biographics, Winston-Salem, NC 27104, USA).
- Power supply for heated window (Voltcraft TNG 35, Conrad electronic, Hirschau, Germany).
- Heated plug for the screwtop tube, bulk material delrin; produced in the NMI workshop (Fig. 4).
- Vibration isolated table (Vibraplane 1201-05-12; Kinetic Systems, Roslindale, MA 02131, USA).
- Micromanipulator (DC3K; Märzhäuser Wetzlar, Wetzlar, Germany).
- Additional female 64-channel flat-ribbon-cable.

4. Detailed procedure

4.1. Microelectrode array fabrication

The sequence of 14 preparative steps necessary to manufacture microelectrode arrays (MEA) is depicted in Fig. 1. Instead of producing the devices yourself, MEAs are commercially available (Multi Channel System, Markwiesenstr. 55, 72770 Reutlingen, Germany). The production of MEA with titanium nitride (TiN) electrodes is based on photolithography, reactive ion etching processes, physical vapor deposition (PVD) and plasma enhanced chemical vapor deposition (PECVD) [15]. The basic design consists of gold leads with TiN electrodes on a glass substrate. The insulating layer is composed of silicon nitride.

(1) Clean glass slides in alkaline detergent (10 min), rinse in distilled water followed by isopropanol and blow dry with nitrogen. Deposition of metals (gold, titanium, TiN) on the glass substrate is based on the following sputtering principle: in a vacuum tube within a high voltage field ions (plasma) are accelerated towards the metal target. Subsequently, metal atoms are released from the solid target and spread randomly into the vacuum. Metal ions hitting the nearby glass slide form a stable homogenous metal film.

(2) Deposit Ti to increase the subsequent adhesion of Au. Do not break the vacuum during this process as it leads to oxidation of Ti and compromises the adhesion of the gold.

(3) Spread 1 ml of positive photoresist onto the rotating surface of the metallized glass substrate by spin coating and dry by prebaking on a hot plate.

(4) Overlay the substrate with a quartz-chromium mask of your choice in the mask aligner (UV exposure microscope, Osram mercury HBO 100). The mask represents the negative or positive image of the MEA design. Expose the photoresist to UV light.

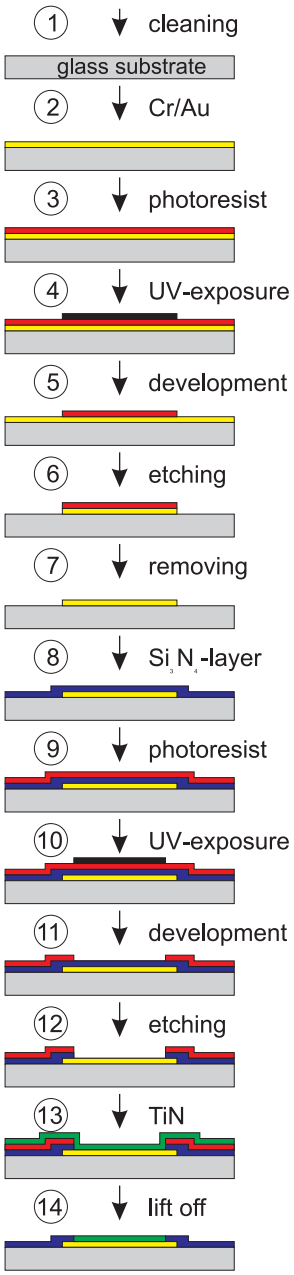

Fig. 1

Fig. 1. The sequential steps for fabrication of microelectrode arrays (MEAs). MEAs are produced by photolithography, reactive ion etching processes, physical vapor deposition and plasma enhanced chemical vapor deposition. The basic design consists of gold strip conductors (yellow) with TiN electrodes (green) on a glass substrate (grey). The insulating layer is composed of silicon nitride (Si_3N_4; blue). Photoresist (red); photo mask (black). Gold strip conductors are microstructured first (steps 1–7), covered with insulating Si_3N_4 except for electrodes and contact pads (steps 8–12) and the gold electrode tips are covered with TiN (steps 13–14). For details see Section 4.

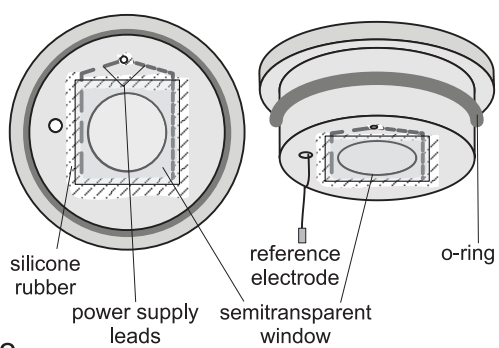

Fig. 2

Fig. 2. MEA chamber lid. Schematic representation of the MEA chamber lid used during recording sessions. Bottom view (left) and lateral view (right). The window fixed with silicone rubber allows continuous microscopic inspection. The lid is heated by a thin semitransparent layer of chromium to prevent condensation. The plug seals the screwtop tubes and positions the Ag–AgCl reference electrode in the culture medium. Sealing the chamber stabilizes temperature, prevents evaporation and contamination, and thus stabilizes the recording situation.

(5) Develop the specimen in Microposit developer.

(6) Eliminate unwanted Au areas by reactive ion etching to form the MEA leads. To do so, the substrate is used as target in an argon plasma.

(7) Remove the remaining photoresist in remover, rinse and dry as above.

(8) In the next step deposit the insulator Si_3N_4 as a continuous layer employing plasma enhanced chemical vapor deposition (PECVD). Place the substrate onto the base electrode and initiate Si_3N_4 deposition by addition of SiH_4 (silane gas) and NH_3 into the vacuum chamber. Under these conditions silan and ammonia form free radicals, which combine on the substrate to form an Si_3N_4 insulation layer. Typically, it does not contain any pinholes.

(9)–(11) During the following steps the Au electrodes are uncovered from the overlying insulator Si_3N_4. Add (9), UV expose (10), and develop (11) the photoresist as indicated during step (3)–(5) with two exceptions. First, the chromium mask used at this point covers only the microelectrode tips and peripheral contact pads. Secondly, use a negative-type photoresist, meaning that areas exposed instead of shielded from UV illumination are stabilized.

(12) To remove the insulating Si_3N_4 coating from the electrodes, insert the substrate into the PECVD apparatus and etch Si_3N_4 in the presence of fluoride ions from Freon gas plasma. Si_3N_4 covered by photoresist remains unaffected.

(13) Deposit a 1-μm thick layer of TiN onto the substrate by reactive sputtering in an argon/nitrogen atmosphere. The impedance as well as the charge transfer capacity of the microelectrode is drastically improved by covering the gold electrode with a columnar TiN layer.

(14) Lift off the TiN layer beside the electrode tips by incubating the substrate for 10–20 min in OCG microstrip remover in an ultrasonic bath; rinse and dry as indicated above.

4.2. MEA chambers

The culture chamber is formed by a 2.5 cm long segment of the top of a centrifuge tube.

- Center a glass ring on the recording area and glue it to the MEA with silicone rubber.
- Slip the tube-segment over this ring, fill the resulting gap between them with silicone rubber, and allow to cure.
- Sterilize the MEA with UV-light (30 min, 254 nm). (Vapor sterilization would cause the tubes to shrink so the lids would not seal tightly).

4.3. Organotypic culture (OTC)

- Rapidly dissect brains from 1–3 postnatal day 6 (P6) rats (Wistar). Dissection of the brain should not exceed more than 60 s per animal. Place the brain tissue into ice cold GBSS + and excise the hippocampi.
- Position hippocampi on poly-vinylchloride foil (3×3 cm) on a tissue chopper and cut hippocampi into 425-μm thick slices. Transfer the tissue slices to a dish containing the buffer by putting the PVC foil upside down into GBSS. If the tissue slices adhere to the foil, carefully agitate the foil without harming the tissue.
- Incubate the hippocampi in 15 ml GBSS + 2×30 min, 4°C in order to wash out the proteolytic enzymes released from injured cells.
- Using a small spatula, transfer individual hippocampi onto the electrode field of MEA covered with 5-μl chicken plasma. Pay attention to transfer the least amount of GBSS + possible. Otherwise, subsequent fibrin network formation might be hampered or intimate tissue–electrode contact might suffer.
- Add 5-μl thrombin and carefully mix both solutions with the aid of a fragment of soft silicone tubing (3-mm wide) attached to a pipette tip. Leave the specimen for 10 min in the sterile hood to allow coagulation and then carefully add 1.5 ml culture medium.
- Air-seal the culture chamber by screwing the lid tightly onto the chamber and insert the MEA chamber into a slide rack. As long as the MEA chambers fit, any type of commercial rack is acceptable. Make sure that the chambers remain immobilized within the rack to prevent harmful rocking during culturing.
- Fasten the slide rack onto the roller drum (e.g., by elastic bands) and rotate continuously (10 rev/h; 36°C). No special gas supply is needed.
- Change medium after 3 and 7 days; thereafter, change it once per week.

4.4. Cytochemistry

4.4.1. DAPI staining

- To stain dead cells in explants, remove the culture medium, wash the tissue once with PBS and incubate it with DAPI (4,6-diaminido-2-phenylindol; 0.5 μg/ml PBS; 5 min).
- Wash once with PBS and immediately check for cell vitality using fluorescence microscopy (Zeiss, Axiophot, $20\times$ Neofluar/Ph2, excitation 365 nm, observation > 420 nm). Since the blue staining of cell nuclei by DAPI is dependent on disintegrated cell membranes, the number of stained cells reflects the degree of cell death.
- Thereafter, fix explants in 4% paraformaldehyde/PBS (1 h), wash once with PBS, incubate with 0.1% Triton X-100/PBS (30 min), wash and DAPI stain once again as indicated above. The detergent Triton X-100 permeabilizes cells, guaranteeing access of DAPI to all cell nuclei.
- Inspect microscopically.

4.4.2. Nissl staining

- Wash cultures twice in PBS 5 min each, fix in 4% PFA/PBS (1 h), dehydrate in 20%, 30%, 50% ethanol (10 min each) and 70% ethanol (30 min).
- Add 0.1% Toluidine blue solution (56°C, 10 min), wash once in 70% ethanol. Continue alcohol series: 80% and 95% (5 min each).
- Leave in Histoclear (30 min) before final mounting.

4.5. Electrophysiology

4.5.1. Preparations

- Switch on the heating system with a dummy MEA in place approximately 1 h before recording. To achieve approximately 36°C within the MEA chamber, set the temperature to 38°C.
- Adjust the power supply to the voltage necessary to warm the plug window to approximately 40°C.
- To minimize the data stream disconnect all *electrodes* not necessary for the experiment. Use the triggered-sweep mode for data acquisition whenever possible, e.g., with stimulation, and keep the sweep window short.

4.5.2. For spike recording

Note: italics are used to indicate virtual devices implemented in the MEASystem software.

- *High pass filter* cut-off frequency should be set to 200–400 Hz. Begin continuous recording and a 500 ms window setting.
- Connect the *spike detector* to the MEA.

4.5.3. For local field potential (LFP) [4] recording

- In order to reduce the CPU load the offset subtraction feature should be used.

4.5.4. Mounting the MEA and recording

- Select an OTC in which cells are visible in the plane of focus of MEA electrodes after 4–14 days in-vitro (DIV).
- Replace the screwtop-lid with the heated plug including the reference electrode (Fig. 2) and mount the MEA into the amplifier plate (Fig. 4). Now, the probe pins connect the MEA electrodes to the amplifier input stage. Keep this part of the process short to avoid evaporation of culture medium. Superfusion is not essential for successful recording.
- Connect the power supply to the heated plug.
- Verify and adjust the thresholds for spike detection found by the built-in threshold finder according to the waveform segments marked in the spike detector display. When the correct thresholds and the electrodes of interest have been determined, open an activity-monitor window to view overall activity on all channels on an extended timescale.

Activity can be recorded to harddisk when a *recorder* has been connected to

- the *spike detector* to record spike activity,
- the *MEA* to record LFPs.

Note that triggered sweeps and continuously recorded data can be reprocessed with a new rack offline, e.g. with a *spike detector* only.

4.6. Stimulation

- To stimulate via MEA electrodes, select the desired stimulation *electrodes* in the virtual *rack*. Connect the stimulus isolation unit (SIU) with a shielded cable and 1 mm banana plugs to the holes next to the probe pins contacting the MEA stimulation electrodes. All recording and stimulation can be done without using a vibration isolation table, and without contaminating the culture.
- MEA electrodes should be disconnected from the amplifiers for stronger currents to reduce the stimulus artefact resulting from amplifier overload, especially during LFP recording. Absolute current values depend on MEA impedance. Stimulation electrodes were connected to the SIU with separate wires.
- To use external stimulus electrodes remove the heated plug. Center the stimulus electrodes well above the tissue level. Note that the slice may be only a few cell layers in thickness. Touching the MEA may damage the electrodes and the insulation of the MEA. Without additional superfusion recording time is limited due to evaporation. Temperature should be set to 40°C to compensate cooling.

4.7. Selecting stimulus strength

Stimulus efficacy depends on electrode material, its impedance (largely determined by the effective electrode surface area), and the distance between electrode and tissue. The electrolysis potential of the electrode materials limits the maximum stimulus strength.

- Individual stimulus pulses should be 100–300 μs long; longer pulses facilitate electrolysis.
- For 30-μm MEA electrodes use stimuli only up to 100 μA to avoid electrolysis. Use weaker stimuli with 10 μm electrodes.
- Typical threshold values are 25–70 μA/150 μs. External electrodes with low impedance can be used with stronger stimuli, if necessary.

4.8. Data processing

LFP data and spike waveforms are best analyzed in detail offline with procedures written in MATLAB. Spike waveform segments were processed to extract voltage minima, maxima, and the timing of these values. If required, spikes can be sorted by separation of clusters

formed in plots of these values. The resulting time series can be analyzed with the program STRANGER.

5. Results

5.1. MEA hardware

The procedure described here provided MEAs of reproducibly high quality with electrodes 100 or 200 μm apart depending on the mask set used (Fig. 3a). The detailed inspection of electrode surfaces by scanning electron microscopy indicated that the plasma etching procedure resulted in well-structured electrode morphologies with equal diameters of 10 or 30 μm (Fig. 3b). Images of high resolution cross-sections revealed the columnar organization of TiN electrodes (Fig. 3c). Individual electrodes were composed of thousands of microcolumns of fairly uniform diameter (about 0.1 μm) and homogeneous height. This microstructure dramatically increased the total surface area of electrodes and consequently, reduced the impedance (80–250 kΩ) by about one order of magnitude compared to flat gold electrodes. Simultaneously, the charge transfer quality was greatly improved. A further advantage of TiN electrodes is the degree of mechanical stability being much higher than platinum black and other electroplated materials. This allows better cleaning of used MEAs and extends the life time of MEAs during repeated usage.

Fig. 4 depicts how electrical contacts are made to a MEA on a microscope stage. To allow the MEA to be used as culture device, the upper part of a screwtop tube was glued onto the MEA (Fig. 4a). While the tissue was cultured inside the tube directly on the recording area, electrical contact was realized outside the tube with the aid of 60 probe pins fixed to a preamplifier plate. The amplifier plate was lowered onto the MEA, while the tube was inserted into the open space in the centre of the amplifier housing (Fig. 4c). The tissue and recording area remained accessible to microscopic inspection and were not obscured by any component of the hardware (Fig. 4b).

5.2. Organotypic culture

For MEA cultures the roller technique developed for rat hippocampi was adapted [9,12]. The preparation of the tissue slices was modified [8] so as to eliminate antibiotics and antimitotics (see below). Since close contact between tissue and electrodes is crucial for electrophysiological investigations, the amount of the plasma clot was significantly reduced. The tissue slice thickness (425 μm) permits visual identification of the hippocampal formation without staining (Fig. 5a). During the first few days, a considerable percentage of cells died, which parallels very much the in vitro development in roller cultures on glass coverslips [8]. This might be due to the preparation trauma. Thereafter, the cultures stabilized. Explant thickness com-

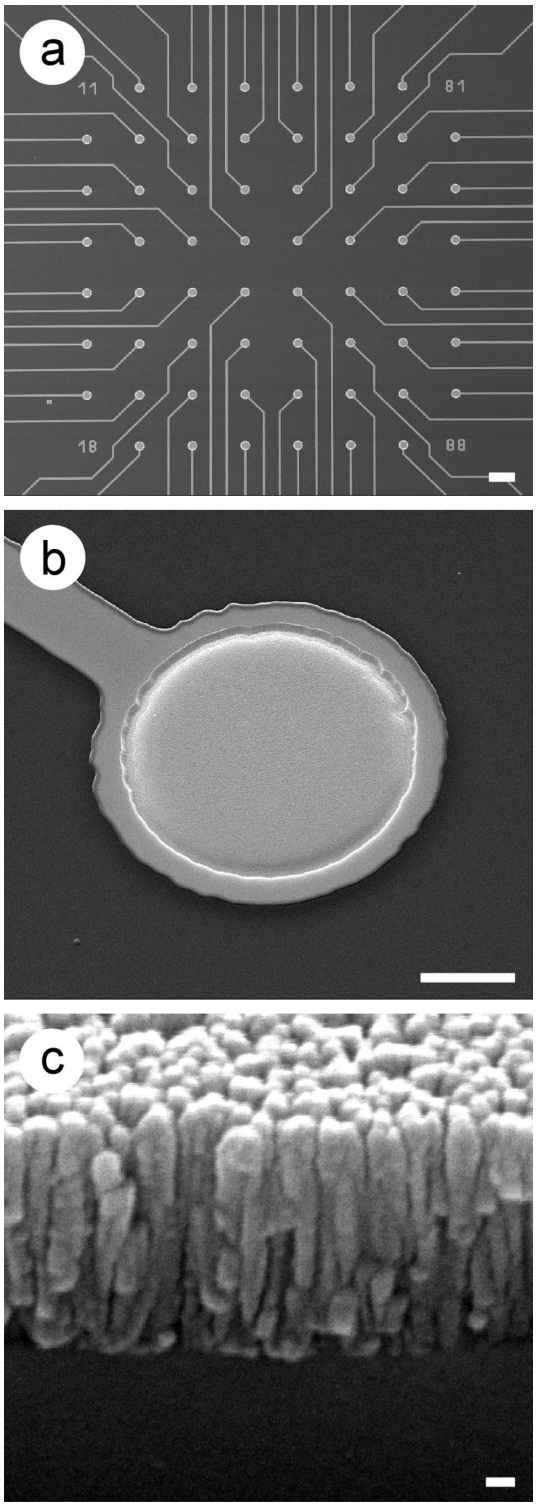

Fig. 3. MEA design. Microstructure of a MEA as revealed by scanning electron microscopy. (A) The recording field is composed of 60 electrodes connected to strip conductors. (B) The TiN electrode is well formed and freely accessible. (C) The nanostructure of the electrode consists of densely packed columns, resulting in a dramatic increase in surface area. Bars A: 100 μm, B: 10 μm, C: 0.1 μm.

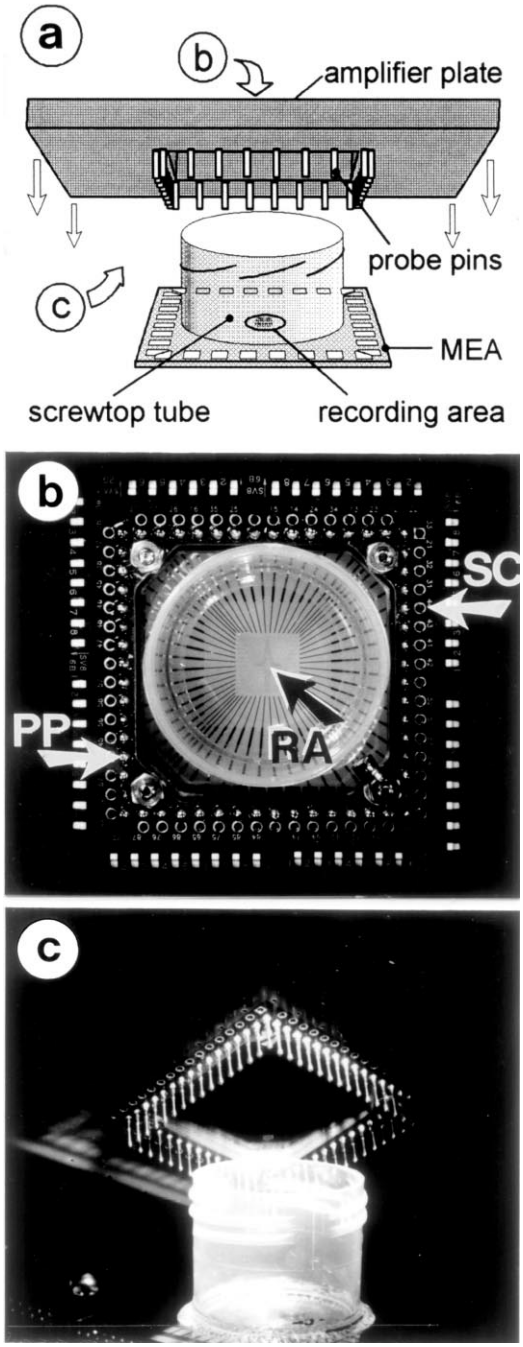

Fig. 4. MEA hardware. (A) Schematic representation of MEA chamber and amplifier plate. The plate is equipped with 60 probe pins at the bottom, which contact the outer MEA strip conductor pads when the plate is lowered onto the MEA. The screwtop tube is inserted into the central opening of the amplifier plate. Labels indicate views depicted in (B) and (C). (B) Top view of the MEA chamber attached to the amplifier plate. The set of 60 small holes (SC, stimulus connector) are designed to house pins to connect the stimulus isolation unit. Beside each stimulus connector the top of the corresponding probe pin (PP) is visible. The recording area (RA) is in the very center of the MEA. (C) Lateral perspective before lowering the amplifier plate with probe pins onto the MEA with attached screwtop tube. MEA dimensions are 5×5 cm.

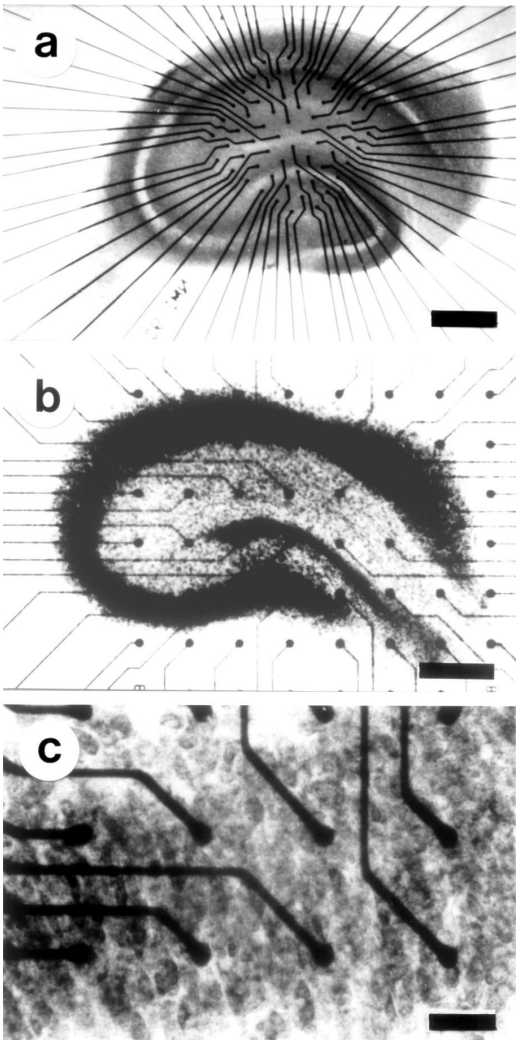

Fig. 5. Organotypic MEA culture. Postnatal day 6 rat hippocampal slices were immobilized on microelectrode arrays and kept for several weeks as roller culture. (A) Top view at the beginning of the incubation reveals the typical structure of the hippocampus formation. (B) Toluidine blue stained specimen after 10 days in vitro. (C) Magnification of neurons of the pyramidal cell layer of a toluidine blue stained specimen after 23 days in vitro. Sizes of the somata are in the range of the electrode diameter. Bars, A: 800 μm, B: 300 μm, C: 100 μm.

prised only a few cell layers from the second week on. While the position and orientation of pyramidal cell layer in the CA regions and the granule cell layer of the dentate gyrus were generally visible, histological details could not be identified directly by phase contrast or darkfield microscopy (Fig. 6a). Nevertheless, preservation of the principal cytoarchitecture with defined subregions such as CA1 and CA3 as well as nuclear and plexiform layers was confirmed by subsequent staining (Fig. 5b). Fig. 5c depicts a close-up of the pyramidal cell layer in CA1 of a 21 day old culture. As visualized by Nissl staining, pyramidal cells in our organotypic cultures resembled pyramidal cells in vivo. However, the number of cells in the pyramidal cell layer was increased. This finding may be due to a delayed

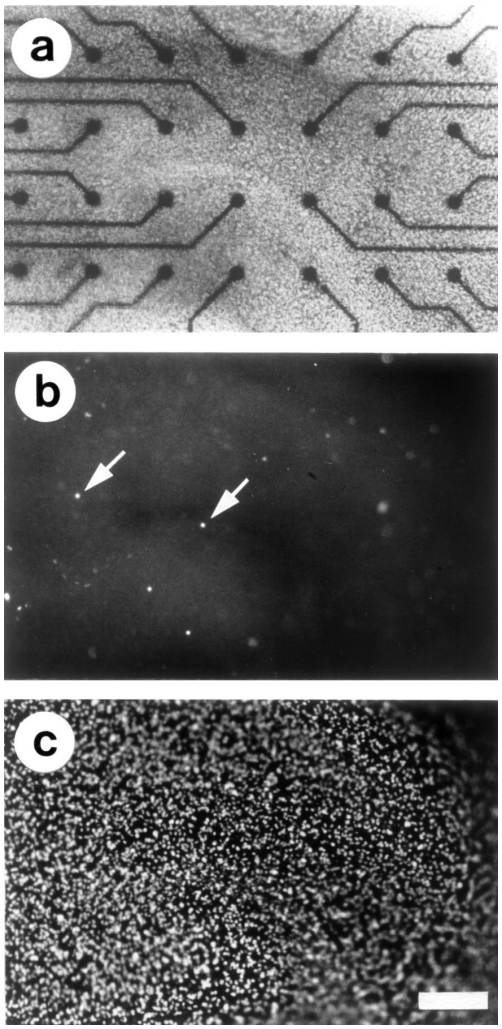

Fig. 6. Cell vitality in MEA culture. An organotypically cultured vital hippocampal slice was incubated with fluorescent DAPI, which stains only nuclei, and only in cells whose membranes are permeable. (A) Viable non-stained hippocampus organotypically cultured for 10 days. (B) Fluorescent micrograph after addition of DAPI. Note, that only very few cells (arrows) are stained, i.e., very few cells are dead. (C) Thereafter, the same specimen was permeabilized by paraformaldehyde fixation and stained with DAPI a second time, which illuminates all nuclei. Bar, 200 μm.

apoptosis program in the developing tissue [7]. Cultures tended to deteriorate after the fourth week of incubation.

Specific attention was paid to cell viability. To monitor cell vitality after electrical recording, specimens were incubated with the fluorescent nucleus stain DAPI, which does not penetrate cytoplasmic membranes. Therefore, in non-permeabilized cultures only dead cells appeared blue. Under these conditions, in 1- to 3-week old cultures only a small number of cells were found to be dead (Fig. 6b). In order to reveal the total cell population in the same explants, tissue was finally permeabilized by fixation and stained once again with DAPI (Fig. 6c). The second DAPI staining highlighted that only a minute fraction of cells had been dead in the first place. The data indicate that the viability of cells is of similar quality as in hippocampal explants cultured with the conventional roller technique [9,12]. The double use of DAPI staining after the final electrophysiological recording session permits us to assess cell viability directly, yielding insight into the histological preservation of the tissue explant.

5.3. MEA recording

Multiple single-unit spike activity and LFPs, either spontaneous or evoked, were recorded with MEA electrodes in OTC at 4–28 DIV.

5.4. Spike activity

Spontaneous spike activity (spike size up to 600 μV, typically 40–100 μV) was found predominantly at electrodes beneath the stratum granulosum of the dentate gyrus (30%), the hilus (18%), and stratum pyramidale in CA1 (Fig. 7a, upper trace) and CA3 (27%) ($N = 142$ positions in 14 experiments, average: 11 electrodes detecting spikes, maximum: 32 electrodes). Occasionally, spike activity was detected at electrodes beneath stratum radiatum, stratum oriens of the hippocampus and stratum moleculare of the dentate gyrus.

Successful recordings were more frequent, when cells were visible in the same plane of focus as the electrodes, which became more likely with increasing incubation time. This may indicate that the amount of plasma clot between cells and electrodes at the time of recording is an important determinant for successful recordings.

Overall spike rates depended on temperature, with highest overall rates at 36°C. The temporal characteristics of spike activity differed between the cultures with spontaneous LFPs and those, in which LFPs occurred only when evoked by stimulation. In the latter case, distributed, non-bursting activity prevailed, while in cultures with spontaneous LFPs periodic burst patterns resembled epileptic activity. The activity, however, was correlated between distinct neurons in both groups as is illustrated by the cross correlograms in Fig. 7b.

Stimulation through electrodes of the MEA or additional external electrodes using currents between 10–100 μA of 150–300 μs duration elicited spikes (Fig. 7a, lower trace) or LFP responses (Fig. 8), or both. Thresholds varied between experiments, probably depending on cell-to-electrode distance and variability of the tissue condition. Generally, thresholds for spike responses were lower than for LFP responses. Stimulus correlated spike activity showed a first response with a latency of 2–10 ms and a second response more than 200 ms after the stimulus, suggesting recurrent activation of cells at the recording site through the network (Fig. 7a, lower trace). Stimuli 3–4 times the threshold current induced burst sequences in some cultures, but not reliably in all.

5.5. Local field potentials

Stimulation in the dentate gyrus granule cell layer or the pyramidal cell layer elicited LFPs at downstream recording positions in CA3 and CA1 with onset-latencies of 4–10 ms. Latencies for population spike peaks corresponded to an approximate conduction velocities of 30 ± 0.41 cm/s ($N = 15$ stimuli in three experiments). LFPs were barely above detection threshold in the distal stratum radiatum, possibly because of relatively few cell bodies involved in synchronized activity in that area and the comparatively small signal generated in apical dendrites. LFPs were largest (up to 400 μV with population spikes) at the border between strata pyramidale and radiatum. However, the waveforms are complicated by overlapping population spikes originating from cells in the stratum pyramidale. The first peak of an LFP-response was positive in the stratum pyramidale of the CA regions and the hilus, while baseline and negative values were found in the stratum radiatum. Compared to acute slices, these stimulus-induced LFP waveforms are more variable and smaller in amplitude.

Fig. 8 gives an example of a bipolar biphasic stimulation experiment. A hippocampal slice was stimulated in CA3 via electrodes marked by red arrowheads in Fig. 8b and the field potential distribution was recorded. The temporal response at a defined electrode located beneath the pyramidal cell layer (yellow arrowhead in Fig. 8b) is depicted in Fig. 8a. The spatial potential distribution (Fig. 8c) and current source density (Fig. 8d) were reconstructed for a defined time point (9 ms after stimulation; red asterisk in Fig. 8a). To facilitate interpretation of electrophysiological data with regard to cytoarchitecture, values for voltage and current source density were color-coded as indicated and superimposed on the hippocampal structure. As should be expected, voltage values were smaller or even negative in the stratum radiatum, where neurites of pyramidal cells were located (Fig. 8c). In contrast, the stratum pyramidale was characterized by more positive voltages. The current source density analysis revealed the spatial distribution of current sinks and sources (Fig. 8d). The data showed excitatory synaptic activity in four foci of different CA regions (blue color) and passive current flow in plexiform hippocampal layers (red color) at this time point.

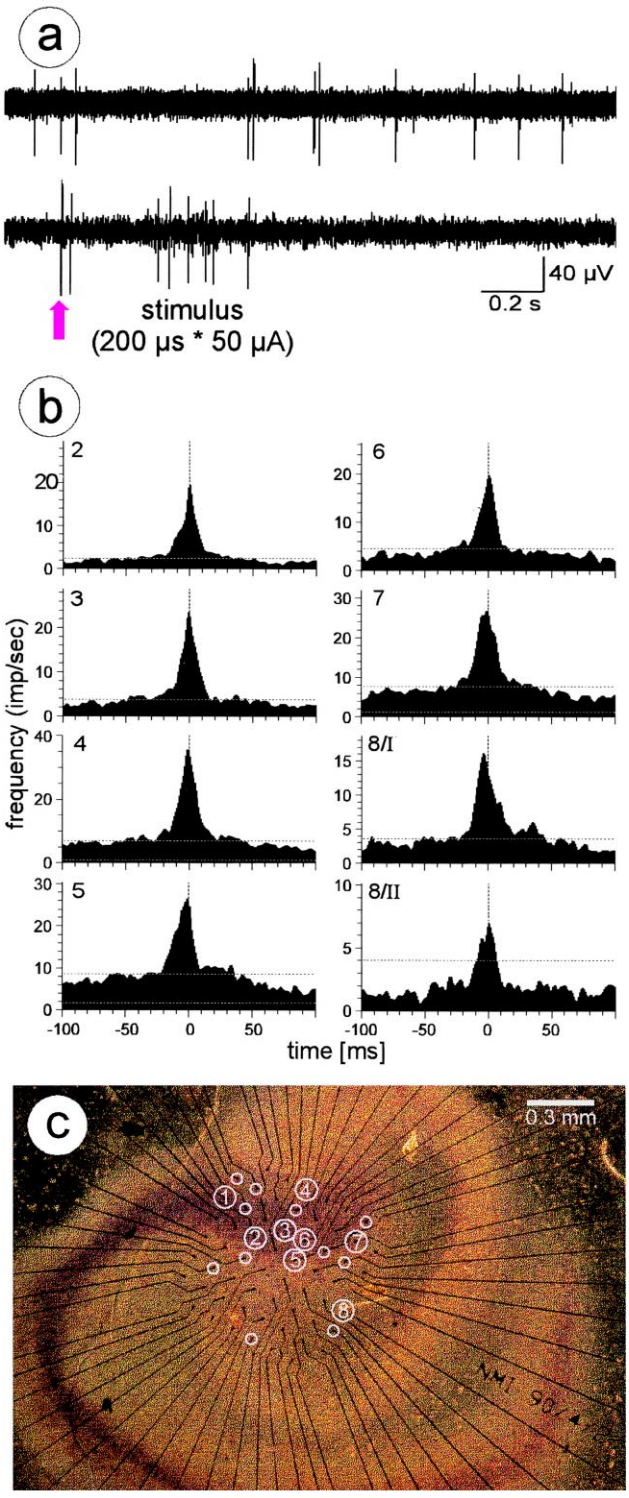

Fig. 7. Spike activity in MEA cultures. (A) Upper trace: spontaneous spike activity. Lower trace: stimulation induced spike response from the same experiment. Note the single spike shortly after the stimulus (arrow) and a multi-unit burst about 300 ms after the stimulus. The recording electrode was in the stratum pyramidale at the subicular end of CA1, while the external stimulus electrode was in the stratum pyramidale of CA3. The number of spikes in the fast response increased with stimulus current but the response latency did not change. (B) Cross correlograms of spontaneous spike activity in the hilus at positions 2–8 with reference to position 1 as shown in (C) (binwidth 1 ms) after 23 days in vitro. Activity is highly correlated even at positions 7 vs. 1 and 8 vs. 1, 610 μm and 728 μm apart, respectively. For position 8, two subpopulations of spikes (8I and 8II) with different spike sizes are shown. (C) Spontaneous spike activity was detected in the hilus of this OTC at all encircled electrode positions. Numbers within circles refer to the correlograms in (B).

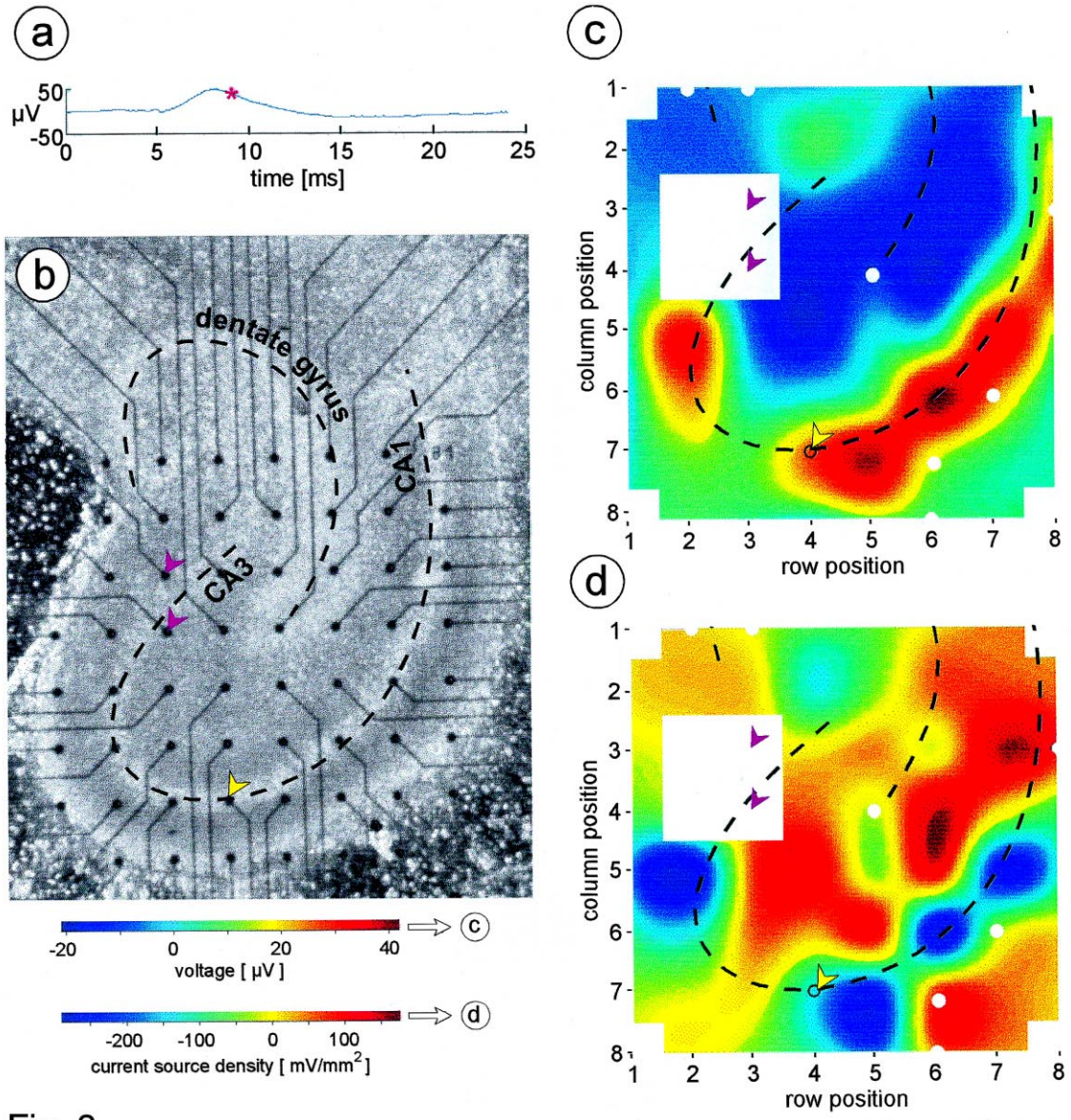
a
50
µV
-50
0
5
10
15
20
25
time [ms]
b
dentate gyrus
CA1
CA3
-20
0
20
40
voltage [µV]
c
-200
-100
0
100
current source density [mV/mm^2]
d
c
column position
row position
d
column position
row position

Fig. 8

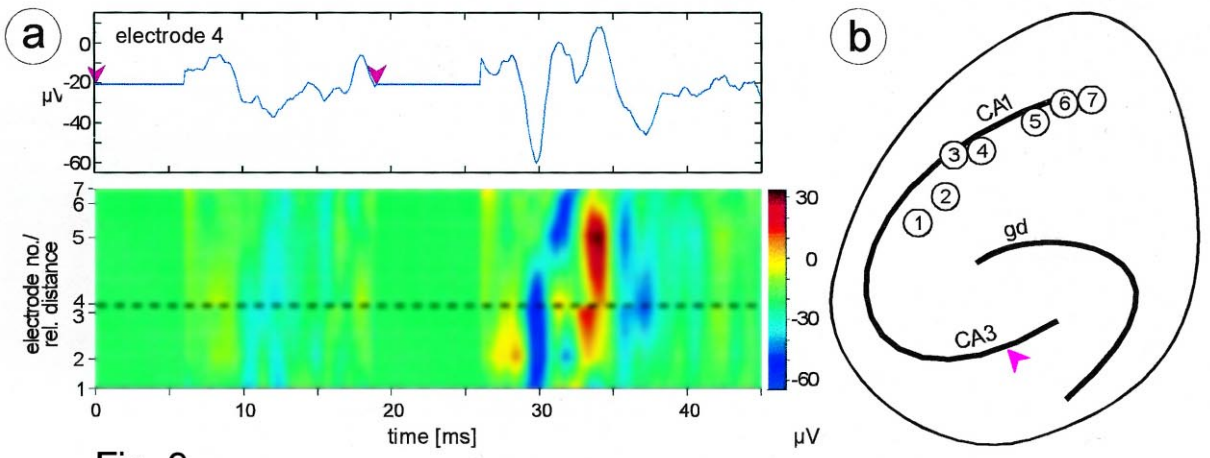
a
electrode 4
µV
0
-20
-40
-60
electrode no./
rel. distance
time [ms]
30
0
-30
-60
µV
b
CA1
gd
CA3

Fig. 9

Fig. 9 illustrates the phenomenon of paired pulse facilitation, in which the response to a second stimulus is greater than the response to the first stimulus. Here, the first weak stimulation in CA3 induced a barely recordable, propagating pulse in CA1, while a second stimulus of identical strength induced a much larger response. Intermediate stimuli elicited comparable responses, while stimuli 2–3 times the minimum needed to evoke the second response resulted in paired pulse depression. This suggests that the range of stimulus intensities covered by MEA electrodes is in the physiologically relevant range. Paired pulse interactions were observed at 20–100 ms intervals, while intervals >100 ms elicited responses of the same amplitude.

6. Discussion

6.1. Troubleshooting

6.1.1. MEA quality

If fabrication of the MEA results in an unacceptable number of pinholes in the layer of insulating Si_3N_4, a sandwich layer of Si_3N_4 and SiO_2 could be produced by PECVD. Alternatively, an additional insulating layer of Sylgard on top of the MEA could cover a larger area of the MEA. However, it is essential that the electrode field of about 2×2 mm remains accessible to cells of the explant.

6.1.2. Insufficient yield

The yield of electrodes at which activity can be detected depends on the distance between the tissue and the MEA surface. If none or only a few electrodes pick up spikes or LFPs, this distance may be too large, because of a fluid or plasma filled space between electrodes and the hippocampal explant. Tissue attachment can be easily improved by repeated oxygen plasma treatment (10 min).

6.1.3. Distorted explants

Considerable differences were observed between the batches of chicken plasma from different suppliers. In various instances plasma clots formed readily but started to disintegrate from the third day onwards resulting in distortion of the explant. This might be due to inadequate degradation of the fibrin network by proteolytic enzymes released from the tissue [8].

6.1.4. Infections

Infections are usually not a problem. If so, infection could originate from culture medium residues at the screw lid, which might get infected during medium exchange. Therefore, care should be taken at this point to solve this problem. If this should not suffice, antibiotics such as streptomycin could be added.

6.1.5. Oscillations in the recorded signal

Excessive oscillating voltages at an electrode other than line frequency may arise from insufficient contact between the probe pins of the amplifier inputs and the MEA contact fields:

(i) Check for dirt, fat or abrasion signs on the outer contacts and clean with alcohol or acetone and soft cloth or paper. Worn contact fields of the MEA are translucent and cannot be repaired reliably.

(ii) Damaged or deinsulated electrodes can be identified by impedance measurement at 0.1–10 kHz. Damage by

Fig. 8. The voltage distribution and current source density in response to electrical stimulation in CA3. The LFP trace shown in (A) was recorded in response to stimulation in an OTC at incubation day 14 at the position marked by the yellow arrowhead in (B). The stimulus was delivered at time 0 through the electrodes in CA3 marked with red arrowheads (disconnected, electrode spacing is 200 μm). Stimulation in CA3 resulted in a $+50$-μV response at this position starting about 5 ms after the stimulus. The trace was from a single stimulus trial and was filtered at 10–300 Hz. The voltage at 50 recording sites at 9 ms after stimulus onset (red asterisk in (A) was color-coded in (C) against the axes of the MEA. To visualize the voltage distribution with respect to the layers of the tissue, we approximated the voltage at sites that could not be recorded due to electrode defects (white dots) as the mean of the voltage at the nearest electrodes recorded. The data pattern thus generated was processed for visualization by 2-dimensional 'bicubic' interpolation with Matlab to reduce the dominating checkerboard effect. Voltage was then color-coded as indicated in the upper color bar. The resulting image reveals a spatial structure in the voltage distribution closely following the layers of the hippocampus (indicated by dashed lines). This spatial coherence is retained as the signal spreads throughout the time course of the response. Similarly, we approximated current source density (CSD) from the original voltages with a discrete Laplacian operator [16]. CSD is shown in (D) after processing as in (C). In this CSD-plot current sinks are shown in red and sources in blue (lower color bar).

Fig. 9. Stimulus-induced LFPs. Paired pulse facilitation was recorded in CA1 with stimulation in CA3 (external electrode). Red arrows mark stimulus onset times in (A) and their position (B). The waveform of the LFP response recorded at electrode 4 (A) and other electrodes varied considerably across different trials and experiments. This suggests that simple averaging of evoked responses is inappropriate, when LFP distributions are determined from sequential measurements at different positions with averaging across trials. The pseudo color plot generated from the original traces by bicubic interpolation represents voltages along a line drawn between the electrode positions. The y-axis is scaled to reflect relative distances between neighboring electrodes, whose identification numbers are shown. The total distance is 848 μm. The dashed line represents electrode 4. The stimulus artefact was removed computationally for both plots. The response latencies increase for positions more distant to the stimulation electrode for both stimuli. The plot illustrates that a response with low signal-to-noise ratio (e.g., to the first stimulus) is reliably identified by the spatiotemporal coherence of activity recorded simultaneously, although barely visible in the single trace. CA1 and CA3, hippocampal regions; gd, gyrus dentatus.

electrolysis is visible by cracks of the insulator as seen in reflectance microscopy (e.g., differential–interference–contrast or epi-fluorescence modes).
(iii) Inadvertently spilled buffer may corrode the tips, the internal surfaces, and the springs of the probe pins. While internal corrosion is evident when the probes are stuck, signs of tip corrosion may be visible only with a dissecting microscope. Sometimes lubricating the springs with silicone oil will help. Otherwise, corroded pins can be replaced.
(iv) Although unlikely, damaged input stages of the amplifiers (because of electrolyte spilling or excessive static voltage) could be another cause. As a short term solution the amplifier input from individual MEA-electrodes can be connected to ground with dip-switches (Fig. 4).

6.1.6. Line hum

This is far less of a problem than with conventional recording setups since the amplifiers are very close to the recording site and completely shielded, and leads are thin and short. Accessory instruments and external stimulus electrodes can, however, introduce noise and should be shielded or grounded using the ground connector at the amplifier casing.

6.1.7. Electrolysis / gas formation

The maximum stimulus current that can be applied through MEA electrodes is limited by the electrolysis potential of the electrode material, which is approximately 1.3 V for TiN. Driving potentials exceeding this limit will produce electrolysis and, subsequently, gas bubble formation. This will change electrolyte composition, and damage the tissue as well as the electrodes. With voltages just above the limit gas bubbles might not be seen readily with an inverted microscope but will accumulate after repeated stimulation. To avoid electrolysis, reduce stimulus currents, choose MEAs with larger electrodes (e.g., 30 μm), and check for increased impedance of damaged electrodes.

6.1.8. Epileptic activity

Occasionally, epileptic activity develops. If this is not desired, leave the entorhinal cortex attached to the hippocampus. It improves survival of inhibitory cells in the dentate gyrus which may reduce epileptic activity. Increasing Mg^{2+} concentration in the medium might also work.

6.2. Alternative and support protocols

In the present work the composition of the washing buffer was changed and application of antimitotics and antibiotics were omitted. In a previous approach the non-NMDA glutamate receptor blocker kynurenic acid was employed to reduce the potential neurotoxic effect of endogenous glutamate, which might be released during initial slice preparation [8]. Kynurenic acid was not used during incubation at 4°C in this study to minimize possible interference with neuronal network activity.

Other researchers employed penicillin and streptomycin as antibiotics [18]. We found that even in the absence of antibiotics the infection rate was low. This is probably due to the fact that MEA devices were tightly sealed throughout the incubation period, and only opened in the sterile hood to exchange medium. Consequently, we refrained from adding antibiotics.

In several studies antimitotics were added at 3 DIV for 24 h [5,11,17]. The omission of mitotic inhibitors, which in a previous formula comprised a mixture of 5-fluoro-2-deoxyuridine, cytosine-β-D-arabinofuranoside, and uridine, was intended to reduce proliferation of non-neuronal cells such as glia [8]. Granule cells are generated between day 14 of gestation and the third postnatal week. Autoradiographic studies in vivo indicate that in the rat up to 50,000 cells are born per day postnatally, resulting in some 600,000 cells in the adult [19]. Therefore, a considerable percentage of granule cells must be born during the culture period starting from postnatal day 7. Inhibition of this process is likely to interfere with regular histogenesis of the dentate gyrus and would interfere with the electrical activity in the culture.

In our cultures astroglia proliferated outside the explant and migrated onto the MEA surface away from the tissue. Within the tissue no obvious alteration of glia cell behaviour was evident. This was shown most recently in standard cultures with regard to two morphologically different populations of glia, namely fibrous and fusiform astrocytes [8]. The behavioral difference between cells within their native microenviroment and in isolation on synthetic substrates is most likely based on the lack of contact-inhibition and other regulatory cell–cell interactions, when cells migrate away from explant tissue [1]. Since no disadvantage is evident for regular cell interactions within the explant, we suggest to refrain from the use of antimitotic agents.

For some studies, especially of LFPs, optical recording using voltage-sensitive dyes could be an alternative approach. However, for a given experiment, the temporal and spatial resolution required, the signal quality, the observation time needed, and dye toxicity are critical parameters that limit the application of this technique. In contrast, MEA recording allows high temporal resolution independent of the area monitored by the electrodes. Especially for long-term studies the feasibility of non-invasive, sterile, and non-toxic recording is important. To our knowledge, the temporal resolution necessary for multi-channel spike recording is not yet state-of-the-art for voltage sensitive dyes.

For studies using pharmacological tools a superfusion system is necessary, which we are currently testing. Experiences with an acute retina preparation in our lab indicate that it is necessary to guide the buffer solution with a plastic insert. The meniscus produced by surface tension in

the small recording chamber might otherwise produce uncontrolled concentration changes of the drugs used. Furthermore, washout times would need to be extended.

A recent article [21] describes a device to record from organotypic slices in transfilter cultures with an array of 30 free-standing low impedance electrodes. Recording is realized by positioning the multielectrode device onto the slice after transfer to a special chamber. While it seems advantageous that the set of electrodes can be positioned with respect to the slice as a whole, this limits the stability of the electrode to tissue interface and the comparison of recordings from the same slice at different recording times. However, the device could be suitable for recording from acute slices, though the authors have not reported the recording of spike activity. Most recently, a paper by Thiebaud et al. [22] described a microelectrode array with holes of 40 μm to record from organotypic hippocampal slices. This array allows the perfusion of the system while recording with large embedded Pt-electrodes on top. However, the long-term survival of the tissue and, subsequently, the recording time, are still limited under these conditions. In a similar approach LFPs, but no spikes were recorded in acute slices with a substrate integrated array on a perforated, flexible carrier [6].

7. Conclusion

In summary, our data indicate that the biohybrid system of organotypically cultured hippocampi on MEA provides an extremely valuable tool for the analysis of the spatiotemporal structure of LFPs as well as of spike activity in tissue cultures. The feasibility of sterile recording lends the system the possibility to study developmental plasticity and long-term effects of neuropharmacological drugs. Moreover, network analysis will benefit from multi-site recording from different units of the network and open the possibility to correlate local spike patterns to the overall states of activity as represented by the distribution of LFPs.

8. Quick procedures

8.1. MEA culture

- Rapidly dissect hippocampi.
- Chop hippocampi into 425-μm thick slices and wash in GBSS + .
- Immobilize individual hippocampi onto the electrode field of MEA with chicken plasma and thrombin. Leave the specimen for 10 min to allow coagulation.
- Add 1.5 ml culture medium, air-seal the culture chamber.
- Rotate MEA chambers continuously (10 rev/h; 36°C).
- Change medium after 3 and 7 days, thereafter, once per week.

8.2. Electrophysiological recording

- Preheat the stage, adjust the external power supply, connect the stimulus monitor line to one of the analogue input channels, set up the virtual *rack*, configure the pulse generator and connect it to monitor channels.
- Exchange the screwtop lid for the heated plug and mount the MEA chamber into the MEA holder; connect the heated window to the power supply.
- Adjust filter/offset settings for the experiments intended.
- Select relevant *electrodes* and check the signal for signs of defects. In case spikes need to be recorded: verify/adjust threshold values of the *spike detector*. Connect the audiomonitor to selected channels.
- Select stimulation electrodes and contact the SIU.
- Connect a *recorder* to the *MEA* directly or to the *spike detector*.
- Stimulate with pulses of 100–300 μs duration and less than 100 μA amplitude.

9. Essential literature references

Refs. [8,10,13,15,16].

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