

LTCC-Based Multi-Electrode Arrays for 3D *in Vitro* Cell Cultures

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Abstract

Current technologies to monitor neuronal cultures *in vitro* are based on 2-dimensional (2D) multi-electrode arrays and cell cultures. The complexity of actual high-level neurobiological systems requires 3-dimensional (3D) cultures and 3D electrode arrays to improve our understanding of such systems. The realization calls for smart multilayer and packaging technology. Our approach uses low-temperature cofired ceramics (LTCC) for the design of a 3-dimensional multi-electrode array (3D MEA). An LTCC multilayer board with gold electrodes forms the basis of the system. The layout of the 3D MEA is designed to fit into widely used measurement adapters for 2D signal recordings, enabling data processing identical to that of established chips. Design and manufacturing of the new 3D device as a basic tool for the investigation of 3D cell cultures are described. Features of thick-film gold electrodes are characterized by means of microscopic and spectroscopic tools complemented with complex impedance measurements. Possible biological applications for *in vitro* electrophysiological measurements were evaluated based on cell cultures of primary neurons, seeded directly to the chip surface. It was shown that activity can be measured over six months.

Keywords: Low-temperature cofired ceramics (LTCC), three-dimensional hybrid multi-electrode array (3D MEA), *in vitro* cell culture, thick-film gold electrode, primary neuron culture

I. Introduction

Multi-electrode arrays (MEAs) are devices for the detection of electrical activity in both tissue slices and *in vitro* cell cultures. A wide range of 2-dimensional (2D) devices has been fabricated to fulfil the purpose of extracellular stimulation and recording. Typical electrode materials are gold, titanium nitride and platinum^{1,2}. All existing devices use 2D thin-film electrodes for the monitoring of electroactive *in vitro* cell cultures, usually arranged on glass or silicon substrates. Because of this limitation to the dimensionality, neurobiological concepts derived from *in vitro* measurements have so far lacked the complexity of actual high-level neurobiological systems. Two key advances are needed to improve the understanding of such systems: *in vitro* 3-dimensional (3D) neuronal cell cultures and 3-dimensional multi-electrode array (3D MEA) systems for measuring these 3D cultures³. A concept for the realization of 3D MEAs based on thin-film technology has been proposed previously⁴, but the realization of this concept requires a challenging assembly technology, which must allow the mounting of micro-scale multilayers in a very confined space. Low-temperature cofired ceramic (LTCC) multilayer technology seems to be an applicable platform for this purpose. Bioreactors based on these ma-

terials have started to appear in recent years^{5,6}. Positive results of cell culture compatibility tests with the material DuPont™ 951 Green Tape™ provided by DuPont de Nemours (called 951 in the following) and screen-printed gold⁷, both part of the DuPont™ 951 Green Tape™ System, encourage their use for the design of bio-impedance measuring devices. Essential advantages for the construction of LTCC 3D MEAs are: (1) the facile realization of 3D hybrid devices based on complex multilayer assemblies; (2) excellent solvent resistance and chemical stability; (3) stability against sterilization protocols necessary for biological applications, thanks to their excellent temperature resistance. The present work introduces a first 3D MEA prototype and describes its manufacturing and biological testing. Thick-film electrode properties are investigated, including electrode topography, surface composition, electrochemical stability, impedance and long-term stability in real biological conditions. The suitability of the prototype for *in vitro* electrophysiological measurements was evaluated based on cell cultures of primary neurons.

II. 3D MEA Design and Manufacturing

Neuroelectronic hybrids include three elements important for the measurements: a neuron, a cleft formed between the neuron and the substrate surface and the electrode. Neurons are electrically excitable cells, which ad-

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here to MEA structures as a result of electrostatic or chemical interactions. The cleft between cell membrane and MEA electrode is filled with ionic solution and its size and form has significant influence on the hybrid's performance⁸. The dimensions of the electrodes should match the biologic dimensions, established 2D electrodes preferentially have a circular profile and vary in size from 4 μm to 100 μm . While larger electrodes capture the cumulative signal of cell clusters, smaller electrodes provide close to single-cell resolution but with limited signal-to-noise ratio². 2D MEAs typically use inter-electrode distances between 100 μm and 500 μm ⁹. The height of a 3D cell culture can exceed several hundreds of micrometers; therefore the electrodes should cover this range vertically to achieve a sufficient spatial resolution^{10,11}. Our layout uses round electrodes arranged at a distance of 400 μm from each other in perpendicular direction (3D). The round shape was adopted from proven 2D systems to accommodate the signal analysis. 3D electrodes were positioned on perpendicular fingers to capture neuronal signals from cells at different vertical levels. Further, round 2D electrodes were

arranged at the bottom. They provide a basis for comparison with existing 2D systems.

The layout of the 3D MEAs follows the design of 2D chips from Multi Channel Systems GmbH (MCS, Germany)⁹ in terms of contact pads and cell culture ring position (see Fig. 1).

The hybrid 3D MEAs consist of four LTCC parts. A schematic cross-section is depicted in Fig. 2, while dimensions and geometries are presented in Fig. 3. The base plate (Fig. 3a) contains the 2D electrodes with a diameter of 130 μm (punched with a round 150- μm tool), the wiring between 2D electrodes and 3D solder electrodes, and the outer pads for spring contact. Three miniaturized conductor circuits in form of bars carrying three fingers each provide the platform for the 3D electrodes (Fig. 3b). The 3D electrodes were designed as small as possible; a round punching tool with 100- μm diameter was used. On every finger, three electrodes were arranged and the bars were mounted into the slots of the base plate. The base plate consists of eight layers of 951 (green layer thickness 254 μm). All electrodes (on the base plate and the

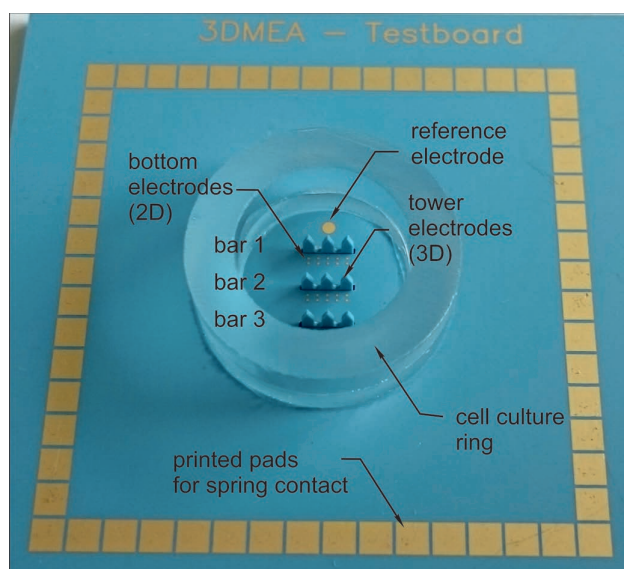


Fig. 1: View of the 3-dimensional hybrid MEA.

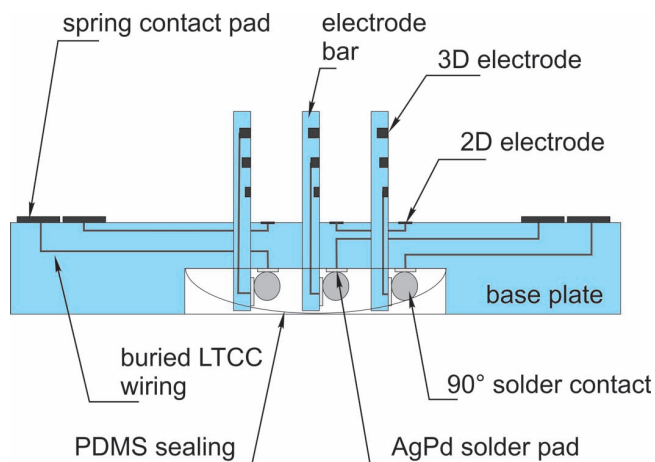


Fig. 2: Schematic view of the 3D MEA assembly.

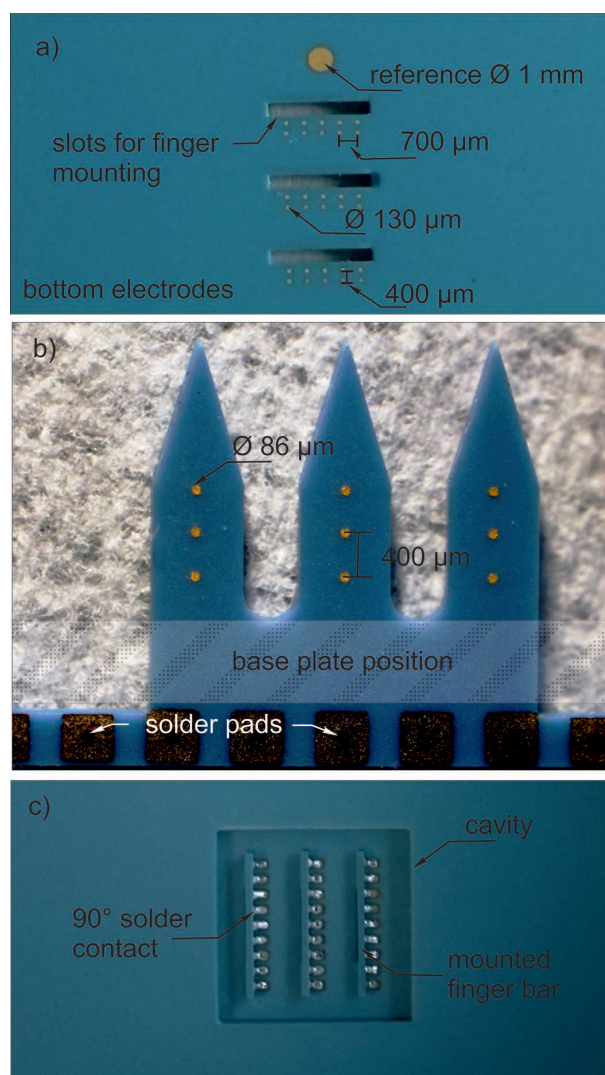


Fig. 3: a) Base plate with bottom electrodes (2D); b) Finger conductor circuit with tower electrodes (3D); c) 90° solder contacts at the backside.

fingers as well) and conductive paths were screen-printed with the DuPont 5740A Au co-fire conductor (DuPont de Nemours), called 5740A in the following. Vias were filled with the 5738 Au cofireable via fill (DuPont de Nemours), called 5738 in the following, using an extrusion via filler (VF-1000, PTC Pacific TriNetics Corporation). The pressure was adjusted between 0.2 MPa and 0.4 MPa. Via electrodes were overprinted with the composition 5740A. The solder pads were printed with 6146 Pd/Ag co-fire conductor (DuPont de Nemours), called 6146 in the following. The precise geometry of the slots is guaranteed with the use of carbon tape inlays with a thickness of 254 μm (tape TCS-CARB-1, C12 Advanced Technologies, formerly Thick Film Technologies) during the lamination at 70 °C for 10 minutes. The carbon tape burns out at 600 °C leaving cavities behind after sintering at 875 °C. Both tapes (951 and carbon tape) were laser-cut with a UV laser (Nd:YAG, 355 nm, LPKF Laser&Electronics AG). A power of 6.6 W was applied with a forward feed of 10 m/s for cutting the 951 green tapes; the carbon tape is cut applying 3 W at a forward feed of 15 m/s. For both tapes (951 and carbon) the structures were designed with the same contour size, which ensured that the carbon inlay fitted well into the tape slots. The clearance produced by the laser spot resulted in a width of approximately 40 μm . The produced slots have a width of 480 μm ($\pm 11 \mu\text{m}$) after firing.

The cavity at the bottom was also sustained by carbon tape inlays. Process details of the cavity forming using carbon tape are described in ¹². This process was applied to form the cavities. At the edges a slight rounding was observed, the affected zone in plane is smaller than 100 μm and the deviation from an ideal edge was found to be less than 30 μm . These tolerances fulfilled the requirements of an even support completely.

The miniaturized conductor circuits for the fingers were made out of four layers of 951 (green layer thickness of 113 μm) with a fired total thickness of 400 μm . The filling of small vias at the towers is a delicate process. The paste 5738 is not optimally adapted and produces indentations. Especially tiny vias are affected. Overprinting of the vias with the paste 5740A largely compensates for this effect. 45 finger bars were arranged on one sheet. The whole stack was laser-cut after lamination with a power of 6.6 W and a forward feed of 10 m/s. After the first cut, the cutting head was shifted downwards by 0.15 mm and the cut was repeated to divide the layer stack completely. The single bars were separated after sintering by dicing. Subsequently, they were mounted into the slots and soldered at an angle of 90 ° (see Fig. 2). For this purpose, the bars were inserted into the slots and the whole assembly was heated up to 110 °C on a hot plate which has openings for the fingers. The solder paste Sn62 15 gr Sn62Pb36Ag2 20 (EFD Nordson) was manually dosed with a syringe onto the pads and the flux TF 38 (Heraeus) was applied. The soldering was carried out manually with a fine solder tip. The combination of solder paste and flux yielded an excellent soldering contact; the liquid metal concentrates accurately on the solder pads and does not wet the ceramic surface, thus avoiding short-circuiting (see Fig. 3c). After soldering, the MEA was cleaned in an ultrasonic bath with ace-

tone to remove flux residues. Then it was rinsed with 2-propanol and dried with air blow. The obtained contacts offer good reliability. 40 MEAs was produced in total and no solder contact failure has been experienced up to now.

The MEAs were sealed from the backside with Polydimethylsiloxane (PDMS) using Silguard 184 (Dow Corning). The material was prepared with a ratio of 1:10 wt%, mixed and applied on the backside cavity. It was cured for 24 h at room temperature.

III. Features of Screen-Printed Gold Electrodes in their Function as Extracellular Recording Electrodes

MEAs for *in vitro* recordings enable the simultaneous capturing of electrophysiological activity of large populations of excitable cells for days and months by extracellular electrodes. The extracellular electrodes reflect the activity (field potential) of individual neurons or the superposition of fast action potentials, synaptic potentials and slow glial potential in time and space. Field potential amplitudes range between 10 μV and 1 mV. The signal passes from the cell membrane through a cleft filled with cell culture medium to the extracellular electrode ⁸. The electrode itself is coated with promoting proteins related to the extracellular matrix (e.g. laminin or gelatine), which are necessary to get a good environment for neuron growth ¹³. A good signal coupling requires a tight contact between the cell structures and the electrode. Screen-printed gold electrodes are different from thin-film electrodes on account of their surface structure and chemical composition ¹⁴. Topography, surface structure and composition have a strong influence on neuron adhesion and growth ¹⁵. They define the geometry of the cleft and should therefore be investigated.

(1) Electrode topography

The electrode topography was inspected with a laser-scanning microscope (OLS 4100, Olympus). The spot of the optical scanner has a diameter of 200 nm and allows precise mapping of the topography. The accuracy of the height measurement is better than 10 nm. A representative height profile of the 3D thick-film electrodes with fired diameter of 86 μm is depicted in Fig. 4. The image is plotted with an increased zoom factor for the z-scale (5x) to emphasize the topography. A trench with a maximum depth of 12 μm (measured from the highest electrode point) surrounds the gold electrode. It results from via filling and shrinking mismatch of paste and tape. The overprinted electrode surface itself features a maximum height deviation of 4 μm (Sp, maximum peak height, measured with the Olympus LEXT 4100 software). The roughness of the electrode was determined from the laser scanning data. Ten radial lines with a length of 50 μm were defined on five electrodes to obtain a sufficient confidence level and the data were analysed using the Olympus LEXT 4100 software. The mean value was calculated from the rms value (root mean square) of all ten lines and it was found to be 0.3 μm .

The surface structure of the thick-film gold electrode is depicted in Fig. 5, this SEM micrograph gives an undistorted impression of the electrode surface. The metal grains have dimensions of 3–5 μm , and small indentations and hollows are visible.

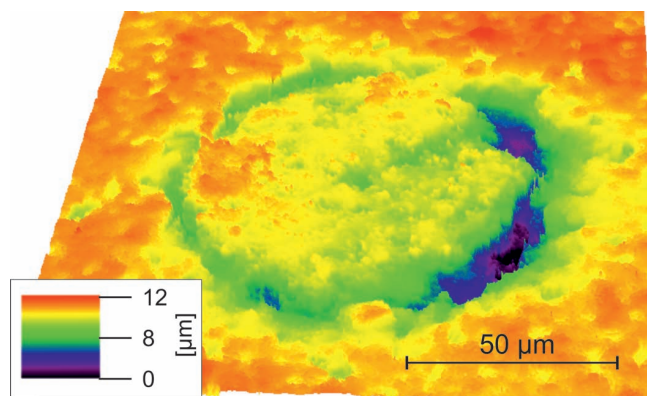


Fig. 4: Height profile of a 3D thick-film gold electrode (diameter of 86 μm). The z-scale is increased by a factor of 5.

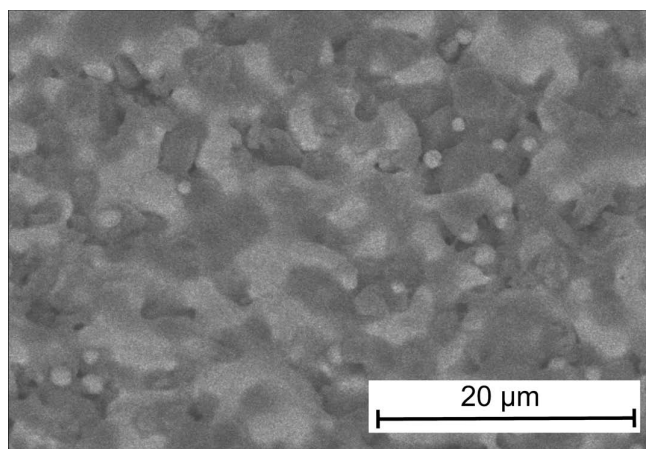


Fig. 5: SEM topography contrast image of the thick-film gold electrode (5740 A), screen-printed on Green TapeTM 951.

(2) Surface composition of the thick-film gold electrode

The surface composition of the electrode material was investigated based on analysis of large-area samples printed with the 5740A paste. For this purpose, X-ray photoelectron spectroscopy (XPS) was performed using monochromatic $\text{AlK}\alpha$ radiation and a SPECS Phoibos 150 hemispherical electron analyser. Fig. 6 includes representative survey spectra of the gold electrode after firing and after removal of the surface top layer (sputtered thickness 30 nm – 50 nm) by means of Ar^+ sputtering (5 min, 3 keV) in order to distinguish between surface and bulk composition. Based on the respective core level intensities, the elemental compositions were calculated, in both cases assuming a homogeneous distribution of the detected elements (see Table 1).

The printed gold surface is mainly composed of gold (Au) with hydrocarbon (C) and oxide-containing (O) surface adsorbates. Furthermore, trace amounts of lead (Pb), chlorine (Cl) and iodine (I) were detected. After Ar^+ sputtering the adsorbates were removed including the removal of surface chlorine and iodine, but still significant amounts of C and O are detected and the Pb concentration is unchanged. Consequently, significant amounts of additives in the gold paste are still incorporated in the printed gold-film matrix after firing at 875 $^{\circ}\text{C}$, also affecting the electrical contact properties. In addition, we have analysed the

effect of an oxygen plasma treatment on the properties of the Au contact layers. This step also reduces the amount of chlorine, iodine as well as hydrocarbon adsorbates and slightly oxidizes the Au film at the surface and might be a technological approach to improve the contact properties of printed gold electrodes in operating devices.

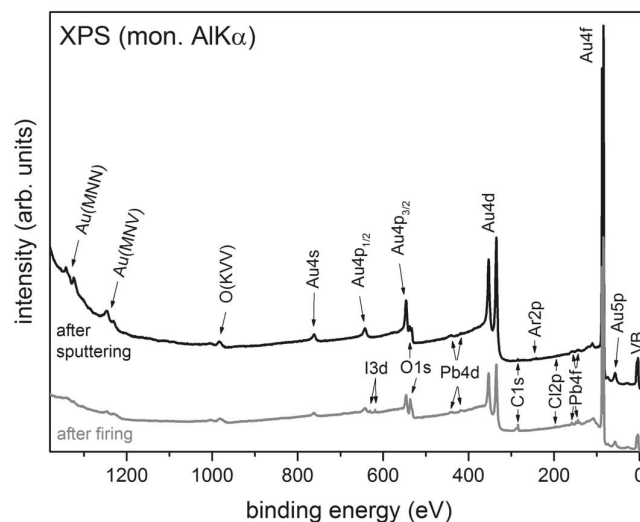


Fig. 6: Comparison of the XPS survey spectra of the screen-printed gold film after firing and after removal of the surface top layer by Ar^+ sputtering.

Table 1: Elemental composition (at. %) of the screen-printed gold surface after firing and after Ar^+ sputtering based on quantitative XPS analysis.

Element	fired	sputtered
Au	35.3	67.3
C	25.6	8.1
O	36.8	22.5
Pb	1.7	1.8
Cl	0.3	0.1
I	0.3	<0.1

(3) Electrochemical stability of the thick-film gold electrode

Cyclic voltammetry was performed to evaluate the stability of the thick-film electrodes in sodium-based media. A sterile phosphate-buffered saline solution (further called PBS) for biological treatment was used as electrolyte. The base solution contains 80 g/l NaCl, 2 g/l KCl, 14.4 g/l Na_2HPO_4 and 2.4 g/l KH_2PO_4 dissolved in distilled water. The pH value was adjusted with HCl to 7.4. This base solution was diluted with distilled water in a ratio of 1:10. Silver chloride reference electrodes (called Ag/AgCl reference in the following) were fabricated by anodizing of screen-printed silver layers (6145 Ag co-fire inner conductor, DuPont de Nemours). The fired layer was plunged into 1 mol HCl and a current density of 1.9 A/dm² was applied for 2 min.

The electrochemical cell consisted of the gold thick-film electrodes as working electrodes and Ag/AgCl as reference. A platinum wire (purity degree 99.99 %) was used as the counter-electrode. A voltage ramp with a ratio of 0.1 V/s was applied starting from 0 V, the voltage maximum of the loop was 1.3 V and the minimum -0.5 V. Ten cycles were measured. Fig. 7 depicts the cyclic voltammogram.

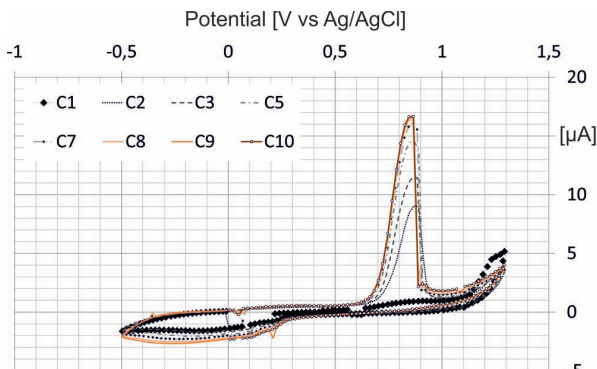


Fig. 7: Cyclic voltammogram of the thick-film electrode in PBS solution, C1 to C10 identify the respective number of cycles.

In the first cycle no current flow was observed up to a voltage of 0.3 V. The current remained below 5 μA , most probably owing to passivating adsorbates. An oxidation peak was detected at a voltage of 1.3 V. Starting from cycle 2, an irreversible oxidation peak appeared at 0.85 V. The peak height increased with the number of cycles, indicating that the active electrode area increased. The current remained stable at a value of 16 μA after 7 cycles. Voltage stress above 0.7 V should be avoided to prevent irreversible decomposition of the electrolyte or corrosion.

(4) Impedance Spectroscopy (EIS) of the thick-film gold electrode

The impedance spectra of the electrodes were measured with a potentiostat. A three-electrode setup was used for the measurements. The counter-electrode consisted of a platinum metal wire, an Ag/AgCl electrode was used as reference. PBS served as electrolyte. It was filled in the culture ring for the measurement. The potentiostatic measurement was carried out in the frequency interval between 30 Hz and 30 kHz with voltage amplitude of 35 mV, capturing 10 points per decade. The mean value and the deviation of the respective data for 2D (diameter 130 μm) and 3D (diameter 86 μm) electrodes are depicted in Fig. 8 and Fig. 9.

The impedance of 2D and 3D electrodes decreased with frequency, the plots of both electrode types have a typical course for gold electrodes. Smaller 3D electrodes showed in general higher impedances and a lower drop compared to the 2D electrodes. These characteristics of the phase response differed between 2D and 3D electrodes. The phase of 2D electrodes increased from an almost ideal capacitive phase shift at low frequencies. This behaviour corresponds to a charge-transport-limited system as described in ¹⁶. 3D electrodes exhibited different characteristics. The curve drops at lower frequencies, remains almost constant in an intermediate range and increases at higher frequencies. The characteristic can be explained by the smaller

area of 3D electrodes. The perpendicular orientation is assumed to have no influence. Fig. 10 depicts the equivalent Randles circuit, which is used to describe electrode-electrolyte interfaces by lumped elements. Diffusion (Warburg coefficient) was neglected because it should not have an influence at the measured frequency range as the time constants are too high. The phase angle function, which is linked to the model in this particular case, follows a polynomial function with a minimum in the intermediate frequency range. In the investigated frequency range the phase angle decreases with an increase of the electrode area and the minimum point is shifted to higher frequencies with decreasing area; this behaviour has already been described for metallic electrode-electrolyte combinations ¹⁷. In the intermediate frequency region, the resistance, mainly determined by the charge transfer resistance R_{CT} , is more dominant than the capacitance reactance of the double layer. Fig. 11 shows the serial capacitance as a function of the frequency. The strong influence of the charge transfer resistance is reflected in the nonlinear decrease of the serial capacitance for the smaller 3D electrodes. We conclude that the electrode area influences the charge transfer strongly. The working conditions and the electrode areas of the current system, especially in the case of 3D electrodes, are in a range where small surface changes lead to significant parameter changes of the lumped elements of the equivalent circuit and thus influence the transmission behaviour significantly.

(5) Impedance characterization with in vitro multi-electrode setup adapter

The recording of neuronal activity requires tailored measuring adapters, which allow parallel monitoring of numerous electrodes over a period of several weeks or even months. To get information about the spatial propagation of the signals it is necessary that all sensing electrodes of the MEA work reliably. Therefore it is important to characterize the reliability of the complete system. Impedance measurements were thus performed based on the data of six MEAs in order to judge the behaviour of thick-film electrodes in real biological conditions. Our measurement adapter uses the integrated circuit RHD2132 from Intan Technologies LLC ¹⁸. The device allows the capturing of all MEA electrodes simultaneously. In this way it is possible to identify treatment influences and to monitor long-term effects at reasonable effort. The adapter stands out for high-impedance bio-potential monitoring. The chip already contains a complete electrophysiology signal acquisition system. It includes arrays of low-noise amplifiers with analogue front-end, a multiplexed 16-bit analogue-to-digital converter (ADC) and a flexible electrode impedance measurement module, complete with a digital serial bus interface. Two RHD2132 chips are assembled on a printed circuit board, which is equipped with 60 spring-loaded contacts interfacing the standard 49 x 49 mm MEA60 format. The board is mounted on an aluminium frame, which provides the mechanical robustness for the amplifier board and MEA assembly. Electro-magnetic shielding is the additional purpose of the frame.

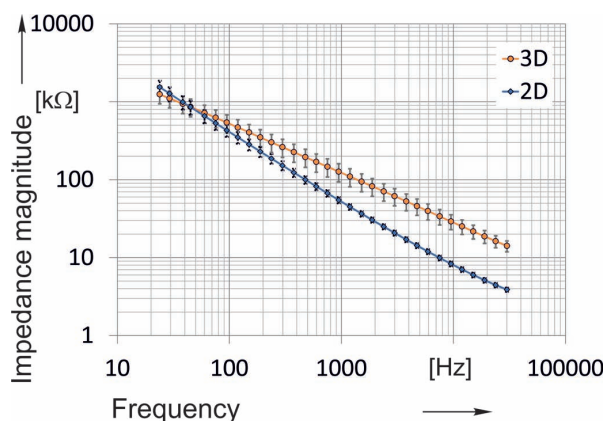


Fig. 8: Bode plot (impedance) between 30 Hz and 30 kHz for 2D and 3D screen-printed gold electrodes.

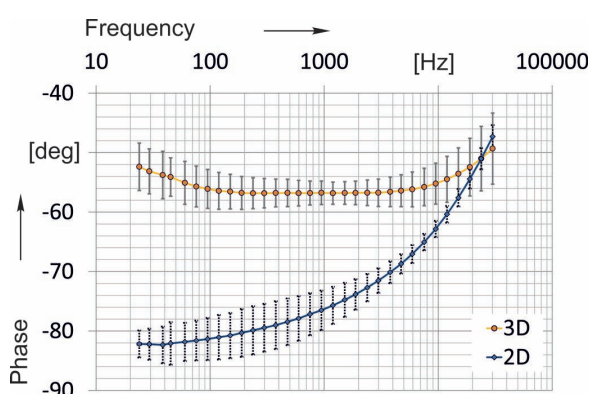


Fig. 9: Bode plot (phase angle) between 30 Hz and 30 kHz for 2D and 3D screen-printed gold electrodes.

This amplifier board is connected to the USB/FPGA interface board (Opal Kelly XEM6010), which is linked to the USB port of the control PC. The open-source software RHD2000 (provided by Intan Technologies LLC) controls the whole setup and delivers the measurement data to the computer for further processing.

PBS was used as electrolyte and a platinum wire served as reference. The MEAs were cleaned after fabrication with acetone and isopropyl alcohol in an ultrasonic bath for 10 minutes, respectively and finally rinsed with deionised water overnight. Their cell culture rings were filled with PBS and the measurements were conducted at a frequency of 1 kHz after an incubation time of 6 h at room temperature. Each measurement was conducted out three times to prove reproducibility, which turned out to be excellent. The measurement data were analysed with the statistical software Minitab 16.2.0.0.

The impedance of a random sample of six MEAs was measured, having 30 2D electrodes with a diameter of 130 μm and 27 3D electrodes with a diameter of 86 μm per chip. On each MEA some electrodes with high impedance (300 kW...1 MW) were detected. The number of these electrodes per chip was significantly different for the two electrode types: 3–7 electrodes of the 2D type and 8–15 electrodes of the 3D type were usually affected. Box plots of the measurement results grouped in electrode type (2D and 3D) and MEA are depicted in Fig. 12. The median of the impedance for 2D electrodes of all MEAs amounts to 74 kW and for 3D electrodes to 87 kW. The median

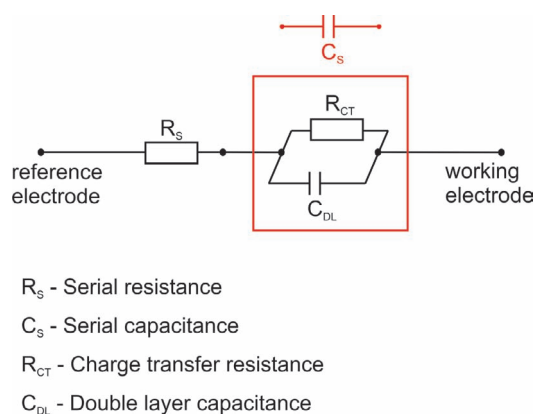


Fig. 10: Simplified equivalent Randles circuit for the electrode-electrolyte interface¹⁷.

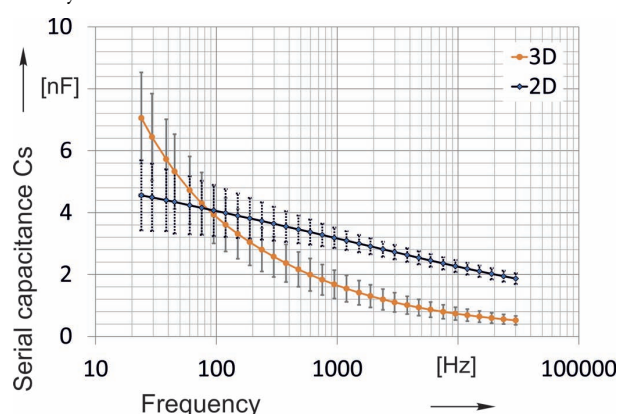


Fig. 11: Serial capacitance C_s versus frequency for 2D (130 μm) and 3D (86 μm) working electrodes.

of the phase angle for 2D electrodes amounts to -64° and for 3D electrodes to -40° . The measurements confirm the trend observed in EIS measurements in Fig. 8 and Fig. 9. Fig. 12 demonstrates that the impedances vary from MEA to MEA. A clear influence of the electrode size was observed for the median of the whole electrode random sample, but single chips show no clear relation between electrode size and impedance value. It is presumably a result of the surface adsorbates (section III(2)), which influence the wetting at the electrode interface significantly and may contribute to bubble forming. An additional test was conducted to underpin this assumption.

One MEA with particularly high standard deviation of the impedance was tested according to the reproducibility of the filling and the influence of surface treatment. The MEA was prepared as described above and the impedance was measured. Subsequently, the MEA was cleaned with deionized water and stored overnight. The measurement with PBS was repeated on the next day with the same MEA. Subsequently, it was cleaned with deionized water, dried and exposed to oxygen plasma cleaning at 200 W for 5 minutes. Then the MEA was filled with PBS again and the impedance measurement was performed after 6 h. Fig. 13 compares the data of these measurements. The impedance data of a commercially available 2D gold MEA (EcoMEA60, MCS GmbH) with electroplated electrodes are included for comparison. This 2D MEA was characterized after having undergone the same preparation procedure as the 3D MEAs.

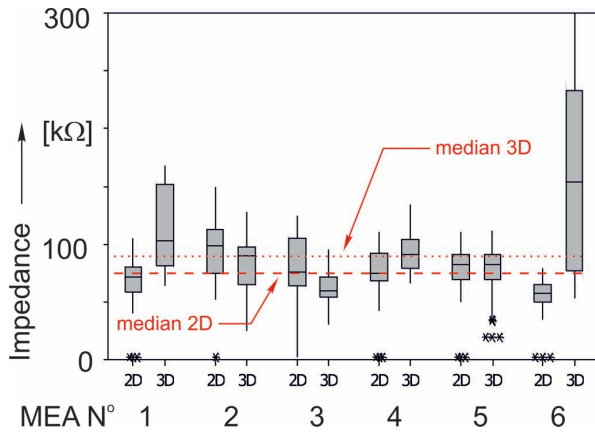


Fig. 12: Box plot of impedances measured at 1 kHz for a random sample of 6 3D MAEs. 2D designated the values for 2D electrodes with a diameter of 130 μm and 3D designates the values for tower electrodes with a diameter of 86 μm .

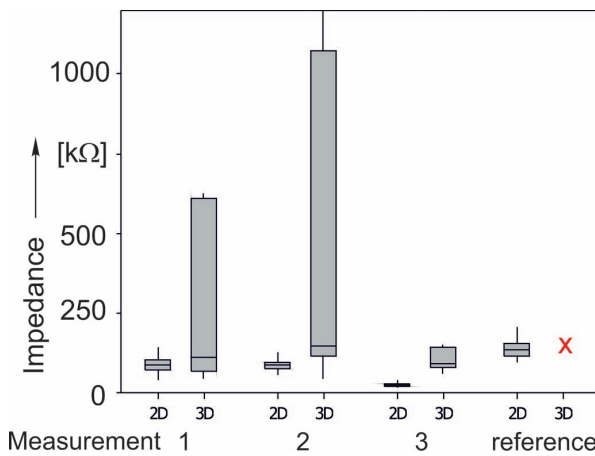


Fig. 13: Comparison of the MEA impedances measured after different treatments (1) After initial treatment (cleaning/6 h PBS); (2) Repeated treatment (cleaning after measurement 1, 6 h PBS); (3) After oxygen plasma treatment (5 min/200 W); (4) Reference (EcoMEA60) after initial treatment.

The repeated measurement without additional treatment shows similar results; the impedance values of the 3D electrodes scatter strongly in comparison with the values of the 2D electrodes. Random influences linked with the electrolyte filling, such as bubble forming or wetting conditions are observed: high-impedance electrodes vary in number, position and impedance value depending on the filling date. After plasma treatment the impedance scattering decreased significantly, the impedance median of the 2D electrodes dropped to 24 kW, comparable to the impedance of value 30 kW of electroplated gold electrodes with a diameter of 100 μm (reference)¹⁹. The distribution of the impedance values of the 3D electrodes was also significantly influenced. The impedance median of these electrodes decreased to 92 kW.

(6) Long-term impedance study in biological environment

All above-mentioned investigations used bare gold electrodes. Since the coating with promoting proteins influences the cleft and the linking of the neurons, and thus the signal transmission, it is necessary to investigate its effect on the electrode impedance and long-term stability. We therefore assessed the system stability in cell culture

medium similar to a real usage scenario over a period of six weeks. Impedance measurements with the *in vitro* recording adapter (see III(5)) at the frequency of 1 kHz were used for evaluation.

For the study, three MEAs were cleaned and prepared according to the procedure described previously. At day 01, the impedance was measured in PBS without additional coating after an incubation time of 24 h. Then the MEAs were cleaned and prepared for cell culture according to the following schedule.

All preparation steps were performed under sterile conditions in a laminar flow hood. Before coating, the 3D MEAs were sterilized for 15 minutes with 70 % ethanol followed by a rinsing step with distilled water. The MEAs were then allowed to air-dry under sterile conditions. Subsequently, a poly(ethylene imine) (PEI) solution in distilled water (PEI, 0.05% w/v, Sigma Aldrich, Taufkirchen, Germany) was pipetted onto the MEA and incubated overnight at +4 °C. After removing the PEI solution, the MEA was rinsed thrice with distilled water and allowed to dry. A 0.02-% solution of gelatin in distilled water was prepared and applied overnight at 4 °C for coating. The gelatin solution was removed and replaced by cell culture medium (Dulbecco's Modified Eagle's Medium supplemented with 10 % fetal calf serum (FCS), 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, called 10 % DMEM in the following). The ready-to-seed MEAs were filled with cell culture medium for the measurements on day 03 to 38. The medium was periodically exchanged at an interval of 2 days. Impedance measurements were conducted on day 03, 06 and 09, and subsequently at an interval of 7 days. The data for one representative MEA are depicted in Fig. 14a) and b).

The measurements without coating at day 01 confirmed the measurements presented in section III(5). The median of the impedance of the coated 2D electrodes on day 03 increased in comparison with the values measured in PBS, while the impedance of coated 3D electrodes decreased. The uncertainty band (25 % and 75 % quantile) of the 3D electrodes also decreased significantly. Later on, the impedance of 2D electrodes remained stable after day 06, while the impedance of 3D electrodes decreased continuously.

IV. Test with Primary Neurons

In order to test and characterize the developed 3D MEA chips under real conditions, primary neurons were seeded 2-dimensionally on the chips, which mean that no 3-dimensional supporting tissue or hydrogel was used to obtain a 3D cell culture. The cells were seeded and cultured according to the following protocol.

The day before cell plating the 3D MEA chips were cleaned in the sequential rinsing steps using purified water produced by an Elix 5 water purification system (Merck Millipore, USA), called Milli-Q in the following, followed by 0.01 % filtered sodium dodecyl sulphate (SDS) and Milli-Q. Then the chips were sterilized in ethanol (HPLC grade) for 15 minutes. Finally, the chips were rinsed again with the Milli-Q, blow-dried with nitrogen gas, then left in the laminar flow hood with the UV light on for overnight. For the filtration of SDS and ethanol, Minisart 0.2- μm filters were used, all purchased from Sigma-Aldrich, Switzerland.

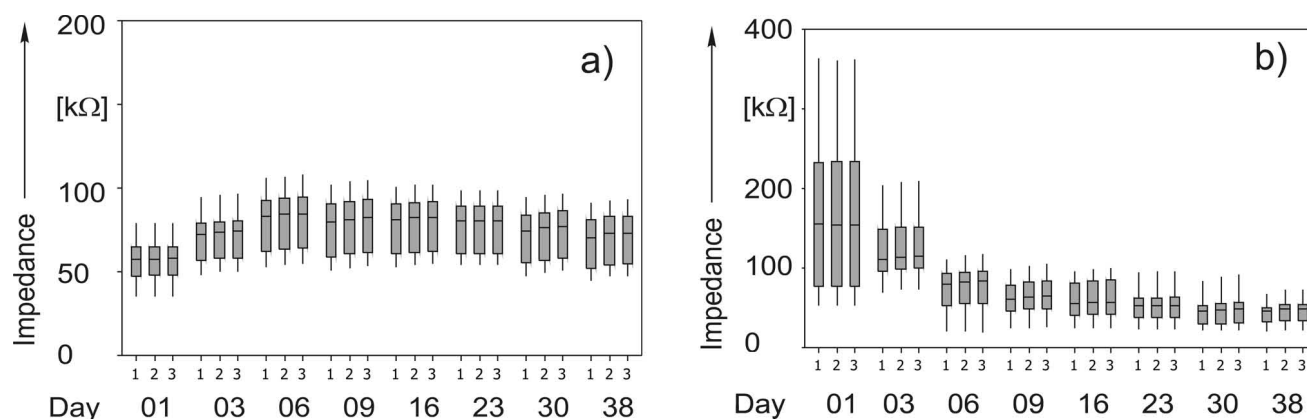


Fig. 14: Long-term impedance study of 2D bottom electrodes (a) and 3D tower electrodes (b). Initial measurements on day 01 were carried out in PBS without any additional coating, further measurements with protein coating in cell culture medium.

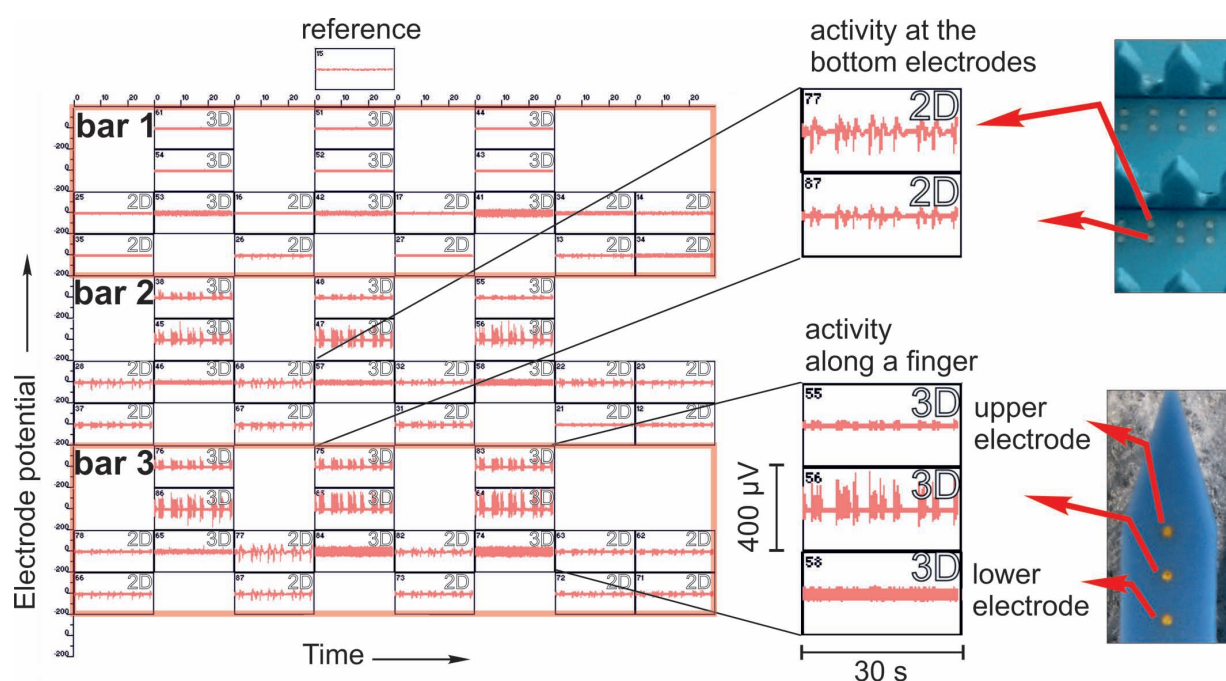


Fig. 15: Representative snapshot of neuronal long-term activity recorded with the 3D MEA using the MEA2100 system. Each window monitors the activity on a single electrode over time, the plotted time scale of one window shows a section of 30 s. The voltage axes depict a range of ± 200 μ V. The arrangement of the windows follows the layout of the electrodes on the MEA. Surface treatment for the cell culture has been applied to bars 2 and 3, restricting the cell growth to these areas. Typical activity patterns captured at 2D bottom electrodes and 3D finger electrodes are highlighted on the right.

The chip surface was prepared according to the protocol of Hales *et al.*²⁰ with the exception that here surface treatment was applied only to the close vicinity of the electrodes, not including the region around the reference electrode. Experiments were performed with primary rat hippocampal and cortical cell cultures prepared from E17 embryos, taken from time-mated pregnant Wistar rats (Harlan Laboratories, Netherlands). The animals were dissected by personnel of the Institute of Pharmacology and Toxicology, Morphological and Behavioral Neuroscience of the University of Zurich, Switzerland.

Neurons in solution were counted (Countess Automated Cell Counter, Life Technologies Europe B.V., Switzerland), then diluted to a seeding concentration of 200 000 cells/cm² with serum-free medium, called “cell culture medium” in the following. It is composed of neurobasal medium (#21103–049, Life Technologies Eu-

rope B.V., Switzerland) mixed with 2 % B-27 serum-free supplement (#17504–044), 1 % penicillin-streptomycin (#15140–148), and 1 % GlutaMAX (#61965–026). After seeding, the chips were stored inside an incubator (37 °C, 5 % CO₂), then one hour after seeding the cell culture ring was filled up to prevent evaporation. Two days after plating, the whole medium was replaced with fresh medium to remove any remaining cellular debris from the plating process. Later, half of the medium was removed and replaced regularly twice a week to maintain an optimal growing environment.

The measurement adapter of the MEA2100 system (Multi Channel Systems GmbH, Germany) was utilized to record the extracellular activity of the primary neuronal cultures. The cultures showed spontaneous activity typical for *in vitro* networks. Fig. 15 presents a snapshot representative to the recorded activity, showing active elec-

trodes both on the towers and on the base plate. Typical signal-to-noise ratio was found to be 10–20, comparable to that of commercial 2D MEAs¹³. Due to the localized surface preparation and seeding around bar 2 and 3, cultures did not extend beyond the borders of the treated regions even after several weeks *in vitro*. Therefore no activity was observed at bar 1. The cultures were active for over six months, indicating that no toxic processes harmed their growth.

V. Conclusions

A first prototype of a 3-dimensional multi-electrode array (3D MEA) is presented, which enables the monitoring of electrical activity of 3D cell cultures *in vitro*. The 3D MEA was designed to capture signals of cells and tissues with spatial dimensions of several hundred micrometres in height and 28 square millimetres laterally. Tests were performed to assess the suitability of the prototype for neuronal recordings.

Investigations of the solid-liquid interface of the MEAs based on impedance measurements resulted in wide distribution of the impedance values before cleaning, presumably because manufacturing-related features of the thick-film electrodes influence the behaviour of the MEA system. The microstructure of the surface (see Fig. 5) promotes the forming of fine bubbles, which can adhere especially in the fine hollows. Surface adsorbates contribute additionally to a significant variation of the surface state. All these factors affect the active electrode area and thus could cause the variations of impedance measurements from MEA to MEA in Section III(5). Correlations with the electrolyte filling date (Fig. 13) support this assumption further. An improvement of the surface state has been achieved by means of plasma treatment, which removes adsorbates. The cyclic voltammogram in Fig. 7 suggests that surface layers can also be removed by electrochemical cleaning.

Manufacturing-related area tolerances (see Fig. 4) are also expected to add to the high variation. This affects mainly the 3D electrodes (see Fig. 12 and 13), whose transmission behaviour is sensitive to area changes owing to the nonlinear characteristics of the lumped elements of the equivalent circuit (see Section III(4)).

Impedance measurements with protein coatings on the electrodes and cell culture medium as electrolyte have shown that the impedance of 3D electrodes decreases and that of the 2D electrodes increases over time. While the previously discussed effects are mainly related to the electrode area, which is designed with different dimensions for 2D and 3D electrodes, this observation should be related to the electrode orientation. The reason for this behaviour is still not clear and requires further investigation. Summarizing all the results, we can derive the following conclusions:

- Topography and surface structure of the investigated thick-film electrodes differ significantly from commonly used thin-film electrodes. As a consequence of the via fill process, the area, especially that of the small electrodes with a diameter of 86 μm , can vary significantly. This variance influences the charge transfer resistance and the double layer capacitance at the solid-

liquid interface, resulting in a wide distribution of the measurement values.

- Surface adsorbates, which are present after firing, strongly affect the coupling of the electrodes. These layers can be removed by means of a 5-min oxygen plasma treatment at 200 W; additionally, it is also possible to apply electrochemical cleaning to remove this layer. The cyclic voltammogram indicated that adsorbates are removed after a run-in of seven cycles.
- The impedance and signal-to-noise-ratio of the thick-film gold electrodes were found to be comparable to that of commercial MEA systems. The electrodes remained stable in sodium-based media up to 0.7 V. Higher loads caused irreversible effects and should be avoided.
- Tests with primary rat hippocampal and cortical cell cultures prepared from E17 embryos provided the evidence, that the prototypes are suitable for extracellular recordings. Cells stayed viable and active over a period of more than six months.

Finally, we can state that LTCC technology provides tools for the manufacturing of biohybrid microsystems, which fulfil the requirements of *in vitro* monitoring of neuronal cell cultures.

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Author responsibilities

Jari Hyttinen and Andreas Schober designed and supervised the project, Heike Bartsch and Michael Fischer manufactured the 3D chips, Heike Bartsch carried out the measurements of section 3, except those of section 3.2, which were performed by Marcel Himmerlich. Laszlo Demko performed the cell related experiments and takes responsibility for the corresponding part of the manuscript, section 4.

References

- ¹ Gross, G.W.: Multielectrode arrays, *Scholarpedia*, **6**(3):5749 (2011) doi:10.4249/scholarpedia.5749

- ² Morin, F.O., Takamura, Y. *et al.*: Investigating neuronal activity with planar microelectrode arrays: achievements and new perspectives, *J. Biosci. Bioeng.*, **100/2**, 131–143, (2005).
- ³ Klefenz, F., Williamson, A. *et al.*: Modelling the formation process of grouping stimuli sets through cortical columns and microcircuits to feature neurons, *Computational Intelligence and Neuroscience*, 290358, 10 p <http://dx.doi.org/10.1155/2013/290358> (2013)
- ⁴ Schober, A., Hampl, J. *et al.*: Hybrid three-dimensional sensor array, in particular for measuring electrogenic cell assemblies and measuring assembly, U.S. Patent Application US 2012/0319705 A1 (2012)
- ⁵ Besecker, J., Cornell, K.A. *et al.*: Dynamic passivation with BSA overcomes LTCC mediated inhibition of PCR, *Sensor. Actuat. B*, **176**, 118–123, (2013).
- ⁶ Ciosek, P., Zawadzki, K. *et al.*: Monitoring of cell cultures with LTCC microelectrode array, *Anal. Bioanal. Chem.*, **393/8**, 2029–2038, (2009).
- ⁷ Bartsch de Torres, H., Rensch, C. *et al.*: Thick film flow sensor for biological microsystems, *Sensor. Actuat. A*, **160**, 109–115, (2010).
- ⁸ Spira, M.E., Hai, A.: Multi-electrode array technologies for neuroscience and cardiology, *Nat. Nanotechnol.*, **8/2**, 83–94, (2013).
- ⁹ MEA2100-Systems, data sheet, <http://www.multichannelsystems.com/products/mea2100-systems>
- ¹⁰ Ylä-Outinen, L., Joki, T. *et al.*: Three-dimensional growth matrix for human embryonic stem cell-derived neuronal cells, *J. Tissue Eng. Regen. M.*, **8/3**, 186–194, (2014).
- ¹¹ Frega, M., Tedesco, M., *et al.*: 3D engineered neural networks coupled to Micro-Electrode-Arrays: Development of an innovative *in vitro* experimental model for neurophysiological studies. In: Proceedings of 6th Annual International IEEE EMBS Conference on Neural Engineering, San Diego, California, 957–960 (2013).
- ¹² Schulz, A., Welker, T. *et al.*: Optimized cavities for microwave applications using the new low loss LTCC material Du Pont 9k7. In: Proceedings of the IMAPS/ACerS International Conference and Exhibition on Ceramic Interconnect and Ceramic Microsystems Technologies, Erfurt, Germany, 000258–000262 (2008).
- ¹³ Yu, L.M., Leipzig, N.D. *et al.*: Promoting neuron adhesion and growth, *Mater. Today*, **11/5**, 36–43, (2008).
- ¹⁴ Fakunle, E.S., Aguilar, Z.P. *et al.*: Evaluation of screen-printed gold on low-temperature co-fired ceramic as a substrate for the immobilization of electrochemical immunoassays, *Langmuir*, **22/5**, 10844–10853, (2006).
- ¹⁵ Micholt, L., Gärtner, A. *et al.*: Substrate topography determines neuronal polarization and growth *in vitro*, *PloS One*, **8/6**, e66170, (2013).
- ¹⁶ Gupta, C., Shannon, M.A. *et al.*: Mechanisms of charge transport through monolayer-modified polycrystalline gold electrodes in the absence of redox-active moieties, *J. Phys. Chem. C*, **113/11**, 4687–4705, (2009).
- ¹⁷ Ahmed, R., Reifsnider, K.: Study of influence of electrode geometry on impedance spectroscopy, *Int. J. Electrochem. Sci.*, **6**, 1159–1174, (2011).
- ¹⁸ Intan Technologies LLC, RHD2000 series digital electrophysiology interface chips, datasheet, Intan Technologies LLC (2013).
- ¹⁹ Multi Channel Systems MCS GmbH: Microelectrode array (MEA) manual, Multi Channel Systems MCS GmbH, Reutlingen, 2014, http://www.multichannelsystems.com/sites/multichannelsystems.com/files/documents/manuals/MEA_Manual.pdf. (2015, June 27).
- ²⁰ Hales, C.M., Rolston, J.D. *et al.*: How to culture, record and stimulate neuronal networks on micro-electrode arrays (MEAs), *J. Vis. Exp.*, **39**, 2056, (2010).