

**APPLICABILITY OF CHRONICALLY IMPLANTABLE AND
TRANSPARENT MICROECOG DEVICES FOR
SIMULTANEOUS MEASUREMENT OF SPONTANEOUS
CALCIUM AND ELECTROPHYSIOLOGICAL ACTIVITY IN
AWAKE MICE**

PhD thesis

Madarász Miklós

János Szentágothai Doctoral School

Semmelweis University



Supervisor:

Dr. Rózsa Balázs, PhD

Official reviewers:

Dr. Dávid Csaba, PhD

Dr. Borbély Sándor, PhD

Head of the Final Examination Committee: Dr Alpár Alán, DSc

Members of the Final Examination Committee:

Dr. Zelles Tibor, PhD

Dr. Fiáth Richárd, PhD

Budapest

2025

1. Introduction

Neuroimaging methods are extensively combined with other experimental techniques in the field of neuroscience in recent years, to study neural activity and map brain connectivity. Among these techniques, electrophysiology is still the gold standard, despite the advent of optical tools like optogenetics and calcium- or voltage imaging.

Neuroimaging techniques can provide much desired spatial information that complements the information obtained by high temporal resolution electrophysiology, and the development of transparent neural interfaces that enable the application of both modalities were catalyzed by the need for multimodal measurements. Such interfaces designed specifically for experimental and clinical studies are promising candidates for brain-computer interfacing technology and may help in understanding brain mechanisms and pathological states.

1.1 Electroencephalography

Electrical activity may be measured from a small segment of a dendrite of a single neuron, the extracellular space among neurons, on the surface of the brain or from the scalp. The source of the gathered information can scale from only a small membrane segment of a single cell to the summed activity of an immense population of neurons. The characterizing advantage of electrophysiology over other investigation methods that record neuronal activity is its yet unchallenged temporal resolution.

Electrocorticography is one of the methods to measure the potential changes in a living brain, with arranged electrodes that measure the field potential on the surface of the brain as a function of time. ECoG signals are rich in frequency components that are difficult to record in a reliable manner with EEG due to the filtering properties of the skull, the subcutaneous tissue and the scalp. Although ECoG devices are invasive probes that require microsurgical implantation, they offer considerable benefits over EEG devices in terms of signal quality, longevity, reliability, spatial and temporal resolution and frequency bands.

1.2. Two - photon microscopy

Two-photon calcium imaging is an efficient way to monitor the activity of neurons through their calcium-binding kinetics with a highly precise morphological image in intact neural tissue. The near-infrared excitation light allows measurements deep in the brain with reduced off-focal fluorescence and lower energy photons that are less harmful. Neurons can be labeled with calcium- or voltage sensitive indicators by local viral injection into the tissue or by the expression of genetically encoded fluorescent proteins.

1.3. Transparent microECoG devices

MicroECoG devices are miniature recording systems designed to measure the electrical activity on the surface of the brain, composed of a conductive layer insulated by the structural substrate layer. Recording

quality, transparency, flexibility, biocompatibility and overall size are a few of the crucial aspects in their design. For transparent neural interfaces, high conductivity (low impedance) is desired for high SNR, however, conventional materials are non-transparent and block access of optical methods. Instead, alternative materials such as graphene, polymers or metal oxides such as indium-tin-oxide (ITO) may be used. For substrate materials, transparency, flexibility and biocompatibility in particular are key. Compressive and tensile forces acting on implanted neural interfaces (breathing, heartbeat) induce cyclic stress, compromising the integrity of the structure. Moreover, the implantation of microECoGs induce neuroinflammation, which may result in glial scar formation, encapsulation of the implant and unreliable electrical recordings chronically. Flexible interfaces that conform better to the curvilinear surface of the brain may reduce the mechanical mismatch between rigid implants and soft brain.

1.4. MicroECoG devices in the thesis

In my thesis, I introduce two microelectrode devices that enable multimodal measurements of neuronal activity by combining ECoG and 2P calcium imaging. These devices are based on the substrates Parylene HT and Thiol-ene/acrylate, and the conductive materials ITO and SIROF. Parylene HT has several advantages that justify its choice for transparent microECoGs. In terms of optical properties, its high transmittance (>90%), low autofluorescence and high UV light stability elevates

Parylene HT among other transparent substrate materials. It can be deposited at low ambient temperature in a conformal layer, and allows the patterning of high melting point conductive materials up to 350 °C. Among the four Parylene variants, it has the lowest coefficient of friction, beneficial for biocompatibility and the lowest permeability to moisture, decreasing the risk of delamination due to swelling.

The indium-tin-oxide conductive layer is formed by doping In_2O_3 with Sn impurities, which increases its conductivity. The conduction mechanism of ITO is based on oxygen vacancies that feature a large electron bandgap (2.6–3.65 eV), requiring more energy to excite electrons. Photons from the visible and infrared spectrum have less energy than this bandgap, which keeps photovoltaic effects at a low level and results in >80% transmittance, both valuable benefits for two-photon imaging.

Thiol-ene/acrylate is a mechanically responsive shape memory polymer that is engineered to soften in response to body temperature. Softening reduces the mechanical mismatch between the brain and the implanted neural interface, which may improve the longevity of implants in multimodal neuroimaging schemes by minimizing the inflammatory response. Furthermore, this softening is realized with less than 3% fluid uptake, beneficial for structural integrity. Together with its high transparency (>85%), these properties make Thiol-ene/acrylate a good candidate for fabrication of transparent neural interfaces designed for cortical electrophysiology and simultaneous two-photon imaging.

2. Objectives

First and foremost, I aimed to characterize the transparent Parylene HT / ITO microECoG device by:

- 1.1 Implanting the Parylene HT / ITO device into a craniotomy in a way that enabled chronic in vivo ECoG and 2P imaging in mice.
- 1.2 Investigating its limitations to its in vivo use, focusing on photodegradation and photovoltaic effects.
- 1.3 Demonstrating simultaneous ECoG and 2P calcium imaging, particularly in long term measurements.
- 1.4 Evaluating the immunohistological response to implantation.

Building on these results, we designed an improved, smaller device based on the shape memory polymer Thiol-ene/acrylate that emphasized brain conformability, aiming to accomplish the following:

- 2.1 Cortical implantation of the Thiol-ene/acrylate / SIROF device into a cranial window in a way that enabled ECoG and 2P calcium imaging.
- 2.2 Demonstration of chronic ECoG and 2P calcium imaging through cortical measurements.
- 2.3 Utilizing the improved adaptability of the Thiol-ene/acrylate / SIROF device for simultaneous hippocampal ECoG and 2P calcium imaging.
- 2.4 The immunohistological characterization of the cortical and hippocampal implantations.

3. Methods

3.1. Device structure and fabrication

Fabrication of the devices employed photolithography and standard MEMS technologies. The Parylene HT / ITO device holds 32 recording sites with a diameter of 150 μm and 500 μm inter-site distance in a 4.5 by 5.5 mm area. The 100 nm thick conductive indium-tin-oxide film is embedded between two 8 μm layers of Parylene HT and connected to a data microconnector. The Thiol-ene/acrylate based device holds 31 symmetrically arranged recording sites in a 2.4 by 3 mm area with a diameter of 115 μm and 400 μm inter-site distance. The 400 nm thick gold and 300 nm thick SIROF conductive layer is embedded in a 4 μm Parylene C layer and two layers of 7 μm Thiol-ene/acrylate.

3.2. Animals and surgery

Mice implanted with microECoG devices in vivo were wild type FVB/Ant or C57BL6/J. Anesthesia was induced by FMM mixture with reversal, local analgesia and hydration. To achieve local expression of GCaMP6f under the syn or mDlx promoter, 2x300 nl adeno-associated virus was microinjected in the visual cortex. For implantation of microECoG devices, part of the skull was removed for a cranial window, devices were placed on the cortical or hippocampal surface then glass coverslips sealed the devices in the craniotomy. Data connectors, reference electrodes and headbars were placed on the skull with cyanoacrylate glue and dental adhesive.

3.3. In vivo electrophysiology and two-photon imaging

In vivo impedance and ECoG data was recorded at 2-20 kHz with a 32-channel RHD 2132 headstage (INTAN Technologies). Sharp wave-ripple events were detected between 150-250 Hz. Events exceeding 25 ms and 4 SD over baseline were analyzed with Data Analysis Suite (github.com/BenceSzmola). 2P imaging measurements were performed and analyzed with MES software (Femtonics) running on MATLAB 2020b (Mathworks). In vivo imaging was performed with a Femto2D - DualScanhead (cortical, resonant scanning at 31 Hz) or a FEMTO3D Atlas Plug & Play acousto-optical microscope (hippocampal, chessboard scanning at 63-111 Hz) at 910-920 nm illumination with a 16x/0.8NA objective (CFI75 LWD). During measurements, awake mice were head-fixed on a circular treadmill but were otherwise freely moving.

3.4. Immunohistology

Coronal sections (40 μm , cryo) were prepared on a sliding microtome (Leica SM2010 R) from transcardially perfused and PFA fixated mice. Cell nuclei, astrocytes and the Nissl substrate was stained with dyes and antibodies (Invitrogen, DAPI, chicken anti-GFAP + goat anti-chicken Alexa Fluor 647, NeuroTrace). Images were acquired with Panoramic MIDI II (3DHISTECH) at 20X. Slices were referenced to a brain atlas. Evaluation was based on comparison of implanted and non-implanted hemispheres. For Parylene HT / ITO, 5 mice in three groups according to implantation duration were analysed by calculating the averaged and area

normalized intensity of selected areas from superficial (I-IV) and deep (V-VI) cortical layers. For cortical Thiol-ene/acrylate implantations, the average intensity of selected rectangular 500×500 μm areas was calculated in 3 mice 80 days after implantation. For hippocampal Thiol-ene/acrylate implantations, the number of cells (overall / in the pyramidal layer / astrocytes) were calculated with QuantCenter (3DHISTECH) based on rectangular, 400×300 μm areas in the CA1 area of the hippocampus of five implanted and one non-implanted mice 6 months after implantation. Custom analysis codes are available at github.com/mm90210/thesis.

4.1. Results - The Parylene HT / ITO device

4.1.1. Electrochemical stability

Long term electrochemical stability maintains signal quality, extends the lifetime of the chronic implant and increases the amount of obtainable data. In the accelerated aging test, devices were soaked in 0.01 M PBS at 67 °C for 4 days, which simulates 24 days of soaking at 37 °C and provide valuable predictions on in vivo stability. Impedance of channels below 500 k Ω at the start decreased only slightly from 369 ± 42 k Ω to 345 ± 130 k Ω at 1 kHz during the test, predicting stable and acceptable impedance magnitudes to record field potentials in the physiological environment in vivo.

4.1.2. Evaluation of photodegradation and photoelectric artefacts

Implanted microECoG devices may be vulnerable to photodamage or photoelectric artefacts caused by high power laser scanning, As this may affect long-term usability, the device was scanned directly ($Z = 0$) or closely below it with increasing power. Visible damage was registered at 13 mW (galvanic) and 33 mW power (resonant scanning mode) at $Z = 0$. Focusing below the device increased the limits of tolerance, to 34 mW at $Z = -30$ μm (galvanic) and 132 mW power at $Z = -20$ μm (resonant scanning). Exposition to power above these limits induced changes in autofluorescence and transparency, particularly around recording sites. Photoelectric artefacts are electric noise detected when the scanning laser beam passes through conductive layers, superimposed on real signals,

adding difficulty to analysis and decreasing SNR. Power density of generated artefacts revealed that at $Z = 0$, <12 mW resonant scanning does not evoke substantial artefact, resonant scanning is less sensitive than galvanic, and ITO is less sensitive than Ti/Au conductive traces. Scanning below the device suppressed the power density of artefacts greatly (galvanic, $10 \mu\text{m}$, 13 mW; resonant: $5 \mu\text{m}$, 33 mW).

4.1.3. In vivo two-photon calcium imaging

Combined ECoG and 2P imaging with the Parylene HT / ITO device was demonstrated by recording the spontaneous activity of GCaMP6f expressing V1 neurons in awake mice. The implanted device allowed the recording of 25 – 53 individual cells up until 51 days after implantation even under ITO recording sites. The clarity of neurons 17 and 51 days after implantation remained equally good. The event rate and decay time of neurons in implanted and unimplanted mice did not reveal significant differences (41.68 ± 2.68 / min and 39.38 ± 2.22 / min active bins, $n=25$ each, $p=0.51$; $0.42 \pm 0.03 \Delta F/F_0/s$ and $0.46 \pm 0.04 \Delta F/F_0/s$ decay time, $n=72$ and 42 events, $p=0.34$; two-sample t-tests, mean \pm SEM).

4.1.4. Immunohistology

The impact of chronic histological changes post implantation (PI) was evaluated by analysing the fluorescent intensity of DAPI (cell nuclei), GFAP (astrocytes) and NeuroTrace (NT, informative of the physiological state of neurons) staining 80 days post implantation on the

implanted and contralateral hemisphere. Neuronal density (ND) and cortical thickness was also calculated. Deep (layer V-VI) ROIs were generally unaffected by implantation, the differences between control (C) and implanted (I) side were larger in superficial (layer I-IV) ROIs, suggesting a more localized impact on superficial layers. After 42 (Mouse 1) and 75-83 (M2-4) days PI, DAPI and NT were slightly higher in superficial (I) ROIs, while ND was non-different and the cortex was slightly thinner on (I). In M1, GFAP intensity was greatly increased in both superficial and deep ROIs ($187.78\% \pm 71.61\%$, $p < 0.0001$; $50.18\% \pm 38.45\%$, $p = 0.0361$), indicating the ongoing peak astroglial response. In M2-4, GFAP was only moderately higher in comparison (superficial: $11.21\% \pm 38.51\%$, $p = 0.0018$; deep: $7.56\% \pm 33.94\%$, $p = 0.0042$). Generally, results of M1 mirror those of M2-4 with a larger magnitude, suggesting an attenuation over time. In M5 (116 days PI), DAPI and NT were lower in superficial, but not in deep ROIs. GFAP was also lower in superficial (I) ($-8.13\% \pm 17.74\%$, $p = 0.0361$) and contrary to M1 and M2-4, ND on (I) was lower ($-2.32\% \pm 4.13\%$, $p = 0.015$), while the cortex was thicker ($31.26 \mu\text{m} \pm 33.21 \mu\text{m}$, $p = 0.0009$).

4.2. Results – The Thiol-ene/acrylate / SIROF device

4.2.1. Electrochemical stability

As part of EIS, impedance of devices immersed in 25 °C PBS was measured for 16 days. The initial impedance of SIROF devices was $7.33 \pm 1.93 \text{ k}\Omega$ ($n = 31$), which decreased minimally by the 6th day ($5.54 \pm 0.49 \text{ k}\Omega$, $n = 31$), then stabilized until the last day ($5.31 \pm 0.50 \text{ k}\Omega$, $n=29$). Similarly, phase angle shifted from the initial $-40.0^\circ \pm 6.9^\circ$ to $-18.5^\circ \pm 2.5^\circ$ by the 6th day, then stabilized. Equivalent circuit analysis suggested that electrode behavior and conductivity were dominated by resistance, and possible water uptake throughout the substrate layers. In vivo, pre-implantation impedance magnitude ($16.56 \pm 2.62 \text{ k}\Omega$, $n = 28$) increased to $73.10 \pm 27.81 \text{ k}\Omega$ ($n = 24$) on the 6th day, then to $127.72 \pm 52.73 \text{ k}\Omega$ ($n = 24$) after 12 days of implantation, then remained stable afterwards. Average SNR ranged from 1.04 to 5.74 on three different devices and did not show any trends except for two consecutive low outliers towards the end of the experiments, which recovered on the last measurement.

In hippocampally implanted mice, spontaneous SPW-Rs could be detected in LFP recordings. SPW-Rs detected on identical channels were comparable longitudinally 1, 4 and 8 weeks after implantation. SPW-Rs displayed a wide range in amplitude and in spatial involvement as well, appearing on a fraction or on the majority of the recording sites. Based on their distribution, events could be categorized as widespread (>10

channels) or spatially-restricted (7-10 channels), with a corresponding event frequency of 0.1 Hz and 0.33 Hz on average.

4.2.2. In vivo two-photon calcium imaging

Mice implanted cortically with the Thiol-ene/acrylate based device were imaged for neuronal calcium activity over a period of 22 weeks. Virally labeled GCaMP6f⁺ neurons were prominent on the initial measurements 1–2 weeks after implantation and this clear distinction from the background continued for the rest of the experiment. Neurons displayed spontaneous calcium activity often exceeding 50% increase in relative fluorescence, and the same cells were identifiable between measurements. Hippocampal in vivo calcium imaging of GCaMP6f expressing transgenic mice through the implanted Thiol-ene/acrylate / SIROF device allowed optical access to the dorsal hippocampus and provided ipsilateral electrophysiology signals. The transgenic mouse line showed a net-like expression pattern of GCaMP6f in the pyramidal layer, both in the implanted mice and in a non-implanted littermate. Leveraging the flexible 3D scanning capability of the AO chessboard scanning, multiple areas were scanned in different Z planes of the field of view, which picked up the sparse, stochastic activity of the densely populated CA1 neurons and allowed the concurrent measurement of spontaneous SPW-Rs and time correlated neuronal calcium activity from the same population.

4.2.3. Immunohistology

The impact of chronic implantation was evaluated through DAPI, GFAP and NeuroTrace stainings 80 days post implantation, by comparing fluorescent intensity on the implanted and contralateral hemisphere. Compared to primary control, GFAP and NeuroTrace intensity was significantly different. The highly increased GFAP intensity was indicative of the repairing and scarring mechanisms still in progress 10 weeks PI. The increased intensity of NeuroTrace staining likely reflected a consistently high protein synthesis capacity of neuronal cells, as in injured or regenerating neurons, the Nissl substance is redistributed around the periphery of the cell body. The secondary control, one sham implanted mouse, supported these results.

Histological changes following 6 months of hippocampal implantation were examined as well. We found an increase in the number of astrocytes and in the total cell count on the implanted side compared to the intact side of the same mouse, while the number of cells in the CA1 pyramidal layer was lower, similarly to a sham implanted mouse. Next, the implanted hemisphere of mice were compared to the sham implanted hemisphere of the secondary control mouse. In this case, the number of astrocytes and total cell count was higher in implanted mice, while the number of cells in CA1 pyramidal cell layer was lower in the sham mouse, although significantly only in one case.

5. Conclusion

In my thesis, I introduced a transparent microECoG device composed of Parylene HT / ITO and presented its first use in chronic experiments of simultaneous ECoG and two-photon calcium imaging in awake mice. The device is simple, cost-effective and straightforward to fabricate. The recording sites exhibited impedance in the range of a few hundred k Ω -s in both accelerated aging tests and in vivo after implantation. Compatibility with two-photon imaging and safe to use was demonstrated by experiments aimed at photodegradation and photovoltaic effects. In vivo two-photon imaging and combined ECoG recordings demonstrated that cell bodies and dendrites were clearly visualized at $-250\ \mu\text{m}$ in the cortex, either under the substrate layer or under ITO metallization. In line with previous reports of immune responses after cranial surgery, we encountered increased GFAP and NeuroTrace labeling. We conclude that these changes do not suggest an additional, aggravating element introduced by the materials of the ECoG device, and recommend Parylene HT as a stable and biocompatible substrate for chronically implanted transparent neural interfaces.

In the second part of my thesis, I introduced a novel microECoG device featuring the shape memory polymer Thiol-ene/acrylate as an encapsulating substrate and gold/SIROF as a conductive layer in chronic experiments for the first time. The device was designed specifically for chronic recordings in awake mice, with the challenges in mind that arise

in combined two-photon imaging and electrocorticography experiments where a cranial window is also necessary, such as limited space and weight. Excellent recording quality was reflected throughout the course of the experiment by high SNR, impedance in the range of several hundred k Ω -s, detection of sharp wave – ripples and the recording of action potentials. This underlines the quality of electrocorticography that can be achieved with the Thiol-ene/acrylate / SIROF device. The optical performance of the microECoG device satisfied the requirements of chronic experiments aiming for the synergetic use of two-photon microscopy and cortical electrophysiology. Cortical two-photon calcium imaging were performed up to 22 weeks after implantation of the device, a period that is considerably longer than for other transparent ECoG devices. In the *str. pyramidale* of the hippocampus, we recorded neuronal level calcium events simultaneously with sharp wave – ripples, confirming the parallel use of the Thiol-ene/acrylate microECoG device. Although the device was not designed to record single unit activity, we also proved that recording of action potentials is possible at sufficient quality. While histological evaluation indicated increased GFAP levels in both cortically and hippocampally implanted mice, histology of secondary control, coverglass implanted mice as well as data in the literature suggested that the surgical procedure greatly influences cellular organisation, and the changes observed in implanted mice are unlikely to be caused solely by the implantation of the Thiol-ene/acrylate / SIROF microECoG device.

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