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A tailored biocompatible neural interface for long term monitoring in neural networks

PER KÖHLER | FACULTY OF MEDICINE | LUND UNIVERSITY 2016



A tailored biocompatible neural interface for long term monitoring in neural networks

Per Köhler



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DOCTORAL DISSERTATION

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Faculty opponent

Thomas Stieglitz

Organization LUND UNIVERSITY Faculty of Medicine Department of Experimental Medical Science Neuronano Research Center Author Per Köhler	Document name DOCTORAL DISSERTATION	
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Abstract <p>Neural interface electrodes that can record from neurons in the brain for long periods of time will be of great importance to unravel how the brain accomplishes its functions. However, current electrodes usually cause significant glia reactions and loss of neurons within the adjacent brain parenchyma. To address this challenge, a novel, polymer-based neural probe, with protrusions tailored to the target tissue, was developed to investigate which probe properties affect the development of a glial scar and neuronal cell death surrounding probes. After many cycles of testing – refinements, promising recordings of neural activity were obtained in both cerebellum and cortex cerebri (papers I-III). In paper IV, we evaluated the importance of mechanical flexibility and demonstrated that probe flexibility has a significant impact on the astroglial scar, but not on the loss of neurons nearby. Moreover, by embedding the dummy probes in a gelatin matrix that dissolves shortly following implantation, neuronal cell death surrounding chronically (6 weeks) implanted electrodes was, for the first time, abolished. In paper V, sensory processing in primary somatosensory cortex during an episode of hyperalgesia was monitored using implanted neural interfaces in order to further evaluate the probe functionality and usefulness in neurophysiological research. By tracking the development of primary and secondary hyperalgesia as well as allodynia in the sensory cortex, we demonstrate the usefulness of our new neural interface and its capability to differentially and simultaneously record neural signals in different cortical laminae in awake freely moving animals.</p>		
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A tailored biocompatible neural interface for long term monitoring in neural networks

Per Köhler



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Introduction

Neural interfaces

The search for better neural recording devices started when du Bois-Reymond discovered the action potential of nerve cells in 1848 [1]. For more than a century, electrophysiological recordings from nerve tissue were almost exclusively performed in acute preparations *in vivo* or *ex vivo* and aimed at modelling the physiology or pathologies of the nervous system. This approach has produced some of the most profound insights into the working of brains to date, such as the Hodgkin-Huxley model [2] explaining the origin of the action potential and JC Eccles demonstration of chemical synaptic transmission [3], two concepts put forward by du Bois-Reymond himself.

After Strumwassers publication of chronic single unit recordings in 1958 [4], several attempts at chronic neurophysiological recordings were made in the 1960:s and -70:s, using iridium and gold microwires to study single unit activity in primate cerebral cortex. This is still a workable design, in principle, where soldered or welded wires arranged in a dense array can produce multiple single unit recordings over extended periods of time at a relatively low cost. However, Wise et al. recognized as early as 1970 that micromachined electrode arrays could be integrated with amplifier or other circuitry to further improve chronic recordings [5][6].

In their seminal 1970 paper, Wise, Angel, and Starr produced a simple two-lead silicon and gold microelectrode for long term neurophysiological recordings using standard micromachining techniques used to produce integrated circuits. The issues discussed in this paper remain central to

interfacing brain tissue to this day, like the matter of electrode impedances at the metal-electrolyte interface and how platinization och the electrode surface could theoretically solve this.

It should be noted that even these first attempts at chronic recordings in the central nervous system identified the interface between neural tissue and electrode as a major limiting factor in producing usable data. Schmidt et al. pointed out a drastic deterioration in recording quality in terms of number of single units and electrode impedance after about three weeks. They connected these findings to tissue reactions to the implanted wires, as indicated by simple cresyl violet stainings of explanted brains. Although considerations of the quality of the neural interface is of course inherent in any type of electrophysiological experiment, cellular reactions, migration and scarring are processes on a timescale exclusive to chronic recordings and has been a major concern for investigators in the field ever since its inception [7][8].

In wide sense, neural interfaces can be thought of as synonymous with brain-machine interfaces (BMIs) or brain-computer interfaces (BCIs). After all, any attempt at interfacing with neural tissue is bound to include electronics, particularly computers. In practice, however, the terms BMIs and BCIs are reserved for devices that facilitate direct neural control over a computer or computer-assisted hardware [9][10], either in the form of a cursor on a screen, a spelling program or various kinds of controllers for robotic limbs [11]. Tonet et al [12] defends this distinction in terminology by invoking the concept of human-machine interfaces (HMIs), denoting the interaction between able-bodied humans and tools. Within this framework, BMIs or BCIs are simply HMIs for those with bodies suffering disabilities. Put this way brain-machine interfaces exclude certain neural interfaces, notably any attempt at interfacing neural tissue with computers purely for research purposes, such as neurophysiological modelling or drug development. Notably, this view of BMI:s exclude open-loop stimulating devices for pain relief [13] and treatment of movements disorder symptoms [14]. For the purposes of this thesis, this inconsistency in nomenclature

will not be a problem, as the implant developed and presented here is for recording and not stimulating neural activity.

Our knowledge of neuronal behavior has, to a considerable degree, come from extracellular recordings obtained under general anesthesia. Excluding decerebrate and slice preparations, models with their own severe limitations, neuronal behavior observed under various types and degrees of sedation are far from naturalistic. Notably, most known sedatives affect NMDA receptor signaling [15][16][17][18] and hyperalgesia [19][20][21] signaling in the somatosensory cortex. For obvious reasons, sedation and anesthesia severely inhibits the studies of many other naturalistic behaviors beyond the scope of this thesis. Extracellular neural recordings in the unanesthetized animal enable studying fundamental brain processes without these pharmacological impairments.

Properties of current state of the art electrodes

The late 1980:s and early 90:s saw several efforts to develop more or less standardized multielectrode arrays for chronic neurophysiological recordings. It is convenient to divide the different approaches taken into a handful of groups, differing in material choices, shape and recording properties. Several of these designs have been very successful for very different reasons, and each will be presented in turn.

Tetrodes

The stereotrode, developed in 1983 [22], was essentially two single wire electrodes entwined and cut to form a single shaft with two measuring points. It, and its replacement the tetrode, offers the possibility to discriminate between detected units at with a higher degree of certainty than other implants, but had a lower yield in terms of recording channels or bandwidth. The close placement of the measuring points enable looking at individual spikes in up to four channels simultaneously, enabling higher precision clustering techniques to identify the sources of spikes. The tetrode, first published in 1993 [23], consisted of four teflon-coated 25 μm platinum-iridium wires, twisted together and cut with scissors to form a diamond-shaped pattern of recording sites, separated by the thickness of the 3 μm Teflon coatings. This design has since seen a number of variants on this simple concept. Key to its success is the ability to isolate features of single units in a noisy *in vivo* setting in modelling studies. This is mainly due to impedances at 1 kHz in the 0.2-3 $\text{M}\Omega$ range [24] and, it has been suggested, because of the placing of the measuring points at the tips of the implant shaft [25]. However, its low channel count is also the reason for the impopularity of the design in developing neural control of devices. The most advanced version of this design is the so-called optetrode[26] developed at Karl Diesseroths lab, combining the high precision nature of tetrode recordings with optogenetic stimulation for a behavioral paradigm in freely moving mice.

Microwire arrays

Chapin and Woodward started implanting 127 μm tungsten wires connected to a microelectrode driver, essentially enabling acute recordings from freely moving rats by injecting an electrode into the brain on the day of the experiment.[27] This method was used with some success in the 1980:s to obtain neurophysiological recordings during behavioral trials[28][29] Other groups developed similar, microdrive-based electrode techniques by adding more electrode channels to the setup.[30] By the

early 90:s, Chapin and Woodward had developed their recording techniques into microwire arrays with varying numbers of channels. In their landmark Nature paper of 1993 with Miguel Nicolelis, bundles of 8-16 teflon coated stainless steel wires 25 or 50 μm thick were implanted in the VPM thalamic nucleus.[31] Nicolelis is largely responsible for developing and standardizing this method further, and edited a book on general principles for microwire arrays in 2008,[32] on top of using this type of arrays very successfully to record neural activity for extended periods of time from large populations of neurons.[33] Wires used today are typically in the 0.1-1 $\text{M}\Omega$ range and in many respects quite similar to those used in tetrodes, as far as recording properties are concerned. However, wires in large arrays are prone to deviate during and after implantation and the geometry of the recording sites in relation to each other is therefore unknown.

The Utah Array

In the late 80:s, Normann et al. developed a 10 x 10 silicon array of microelectrodes, subsequently named after the University of Utah where development took place. A technical paper of 1991 describes what is today recognized as the Utah electrode array (or Blackrock array, since the purchase of the design by Blackrock a few years ago) [34]. The following year, acute recordings from cat cerebral cortex were published [35] with further characterization of electrode recording properties in 1996 [36]. The recording properties of the array has been tested many times since, and is comparable (but inferior) to tungsten or glass single electrodes used for acute recordings in terms of signal to noise ratio and response properties [37]. Interestingly, despite its standardized manufacture and appearance, impedances in individual electrodes of the array vary between 200 and 800 $\text{k}\Omega$, which is surprisingly similar to hand made wire arrays. Initially intended to record and stimulate areas of the visual cortex, the Utah array was shown to be viable in BMI applications in 1997 [38], mostly owing to the fact that it tended to pick up populations of neurons instead of the intended single units. Although the device is often referred to as a 3D-

array, the electrode sites of the bed of nails-like structure is actually arranged in a 2D sheet, either slanted or parallel to the surface of the brain. Discriminating the very large number of multiunits picked up difficult by triangulation is also very computationally demanding, although possible in theory, limiting the use of the Utah array in modelling where single unit identification and cortical anatomy is of interest. However, efforts have been made to develop a so-called optrode, where the Utah array is modified to include fiberoptics for optogenetic stimulation [39], thereby enabling both advanced closed loop BMIs and basic research paradigms. Surface modification of the Utah array has also been explored by several groups, using techniques such as electroplating [40] and carbon nanotubes to increase the surface area and charge injection capacity of implants [41].

Since the 2006 trial [11] where a BMI based on the Utah electrode array was successfully used by a paraplegic patient to control a cursor on a computer screen during consecutive test sessions, the Utah array has been used in human trials. The main focus has been alleviating paraplegia in locked in patients suffering from amyotrophic lateral sclerosis (ALS) or spinal cord injury, but there has also been opportunistic use of already implanted arrays for experimental work in areas such as anaesthesiology and basic neuroscience.[42]

Thin-film silicon arrays

The Michigan electrode array, developed at University of Michigan Ann Arbor in by Kensall Wise and first published in 1994, is similar to the Utah array in that it is a silicon-based device roughly shaped like a bed of nails [43]. Based on silicon etched through photostructured polymers, this was the first of the flat, rather sword-like types of microelectrode arrays. However, in contrast to the Utah array, it has multiple, flat recording sites along each silicon shaft, forming a true 3D rather than 2D grid of recording sites. In addition to being flat, these recording sites can also be varied in size and shape and are typically considerably larger than microwire or Utah array recording sites. Because of this three-dimensional arrangement of

relatively large recording sites, the Michigan array has been used to study populations of relatively large cells, notably pyramidal cells, either alone or in combination with higher-resolution recordings from tetrodes [44]. One notable characteristic of the Michigan array is its design flexibility, where electrode leads, shank geometry and coating has been manipulated for different neurophysiological purposes as well as for modelling tissue reactions and neural interface design itself [45][46][47]. The Moxon array, named after Karen Moxon of John Chapins group, has been a moderately successful competitor using the same basic design as the Michigan array since 2004 [48]. Similarly to other array designs, there are currently ambitious attempts to combine the Michigan electrode array with fiberoptics for optogenetic experiments, in collaboration with the lab of Györgi Buzsáki [49].

Polymer electrode arrays

Micromachined polymer electrode arrays for chronic neural recordings were shown to be viable in the early 1990:s [50] and have spawned a number of iterations from different groups [51][52][53][54]. Common features are shanks made of photostructurable polymers, predominantly polyimide, flat, variable geometries similar to Michigan probes and relatively high flexibility. No one thin-film design has had a breakthrough similar to the silicon and wire arrays of the same period, and many consider polymer-based electrodes to be unreliable in chronic neurophysiological settings. Nonetheless, polymer electrodes have many desirable properties for interfacing with neural tissue and have been extensively used for *in vitro* designs. This thesis will argue the strengths of a novel type of polymer electrode arrays with respect to their adaptable geometry, favorable tissue responses and mechanical characteristics.

Limitations of current state of the art electrodes

The longevity and failure modes of chronically implanted microelectrodes has been a field of interest since the 1980:s. Initially, research focused on the failure of the implants themselves [55], with faulty insulation and lead breakage being issues under investigation to this day [56]. As implant breakage (often termed *abiotic failure*) has become increasingly manageable, many groups have investigated failure due to immune reactions and neuronal death (referred to as *biotic failure*) [56][57]. As pointed out by Patrick Tresco in a talk at the IEEE Conference on Neural Engineering in 2013, the problem of biotic failures increases as animal size decreases. For this reason, electrodes suites for use in rodents, such as tetrodes and microwires, have been more extensively characterized immunohistochemically. Conversely, arrays used in larger animals, such as macaque and cat, have been evaluated mostly from an abiotic failure point of view. Ward et al. compared the electrical properties of and tissue reactions in rat towards wire, Utah, Michigan and Moxon arrays in 2009 [58]. As expected, the only array approved for human use (Utah) showed the most stability over time in terms of variance and absolute values of impedance at 1 kHz. Also expected, electrodes with lower impedance (notably the Moxon silicon array and Tucker-Davis wire array) appeared to record more single units over time, due to higher signal to noise ratios. However, the neurophysiological data from studies like these are hardly comparable, as the arrays record from different structures and cell types of the central nervous system and are clearly adapted to different animal types. Consequently, Ward et al:s limited histological comparisons of the implants are skewed against Utah arrays with very large connectors adapted for larger animals.

As an illustration of the scope of the problem, Nicolelis et al [59] reported a 40% drop in working electrode sites between 1 and 18 months, Nolte et al [60] reported 10 of 30 implanted rats yielded single units after 4 weeks and only 2 after 12 weeks, Rousche and Normann [61] saw a drop from 7 of 11 working probes to 4 of 11 from implantation to months post

implantation in cat cortex, and Williams et al [62] reported 3 of 8 microwire arrays breaking or falling off within 15 weeks. To a large degree, implant longevity is a measure of surgical skill and rigorous implant procedures, so it is remarkable that these groups, representing several of the most cited and respected in the field of BMI:s, evidently have failure rates this large.

As with the Utah array, the stiffness of individual electrode shanks is both an asset and a weakness. Both designs are helped by their rigidity and relative robustness during implantation, where speed and accuracy is improved compared to wire or polymer-based arrays. This comes at the cost of size, which has been correlated to tissue inflammation and neuronal death [63][64][65].

McConnell et al [57] implanted Michigan arrays in rats and found ED1 (a marker for activated microglia cells) responses in the 100 μm region of interest (ROI) increasing over time, up to 16 weeks post implantation, and GFAP (a marker for activated astrocytes) reactions of various magnitudes in the 300 μm ROI during the same time window.

Prasad et al [56] investigated 50 μm Tucker-Davis microwire biotic and abiotic failure modes in rat at time points up to 9 months post-implantation. Arrays explanted within 3 weeks all yielded single unit activity on >50% of channels at the day of explantation, compared to the “chronic” group of rats explanted at 9 months where yield was generally below 50%, sometimes in the single digits. Implantation time correlated with increases in electrode impedance and visible corrosion and insulation breakage on explanted wires, confirming that abiotic failure is a problem of very long-term implantations. Immunohistochemical analysis showed a microglial response peaking around the second to third week and, interestingly, spreading to the contralateral (control) hemisphere in the 9 month-group. Winslow and Tresco analyzed the role of increased blood-brain barrier permeability following microwire array implantation but could not connect this to a progressive inflammation similar to that found by Prasad et al [66]. Skousen and Tresco recently demonstrated that the astrocytic foreign body

response to Utah-type arrays implanted in rat cortex correlated with recording performance [60]. In this experiment, almost all devices had failed four weeks post implantation. >50% of implants failed due to hardware failure, such as wire breakage or head stage loosening, with another 40% of implants failing due to the foreign body response. Most importantly, both signal to noise-ratio and number of single units recorded correlated with markers for astrogliosis and blood brain barrier leakage.

Edell et al. showed that bending of electrodes during implantation increased gliosis around implants and that the tips at the penetrating edge of electrodes were the region most susceptible to gliosis in the chronic setting.[67] The latter finding can be interpreted in two ways. First, it could be that gliosis accumulates around any edge of a neuronal implant, regardless of position. This has been touched upon in two studies. Szarowski et al. implanted silicon shanks of varying cross sections and analyzed GFAP reactions qualitatively.[65] They found no difference between shanks with diamond-shaped, square or flat cross-sections. Similarly, Seymour and Kipke implanted similar probes with outlying edges an order of magnitude smaller than the shanks and compared the reactions surrounding the outliers to that around the shanks.[68] The large shanks generated a much larger astrocytic response, in addition to larger neuronal loss, than the outlier. These studies are insufficient to reject the possibility that edges in themselves promote gliosis, but do cast doubt over its viability. The second interpretation of Edell et al.'s finding is that micromotions between the implant and the tissue are driving gliosis. In this view, micromotions of the brain are translated into stress at the interface between tissue and implant. Gilletti and Muthaswamy [69] quantified micromotions around implants in rat somatosensory cortex and found displacements of 2-4 μm secondary to vascular pulsatility and 10-30 μm due to respiration. Subbaroyan and Kipke [70] modelled strain due to micromotions at the electrode interface and compared simulation results with an *in vivo* experimental setup. They found GFAP reactivity closely followed modelled micromotions induced by connecting a stiff penetrating to a stiff rather than flexible connecting wire, thus communicating micromotions between brain and skull into the shank. Together, these

findings support the hypothesis of micromotions as responsible for chronic gliosis around leading edges in chronically neural implants. The role of tethering implants to the skull in driving gliosis around chronically implanted neural interfaces has been further investigated by this group [71] as well as ours [63], demonstrating the importance of implants with at least some degree of freedom between skull and implant.

These findings lead us to formulate the following hypotheses concerning optimization of electrode array design:

- 1) The long-term recording capabilities of mechanically and chemically robust implants are limited primarily by the tissue response directed at the implant.
- 2) Increasing implant mechanical flexibility by size and reducing material stiffness should lead to a decreased glial response and neuronal loss.
- 3) Placing electrode leads at the tips of structures protruding through the glial scar surrounding the shank should enable recording from relatively unaffected tissue.
- 4) An implant designed to reduce micromotions at the neural interface should be surrounded by less gliosis due to decreased shear stress on immunoreactive cells.

Animal models of pain using implanted electrodes in awake rats

As mentioned above, the possibility of recording from neurons in the awake animals has a great potential to revolutionize the understanding of how the brain accomplish its functions. Pain research is an example of a field that can be expected to greatly benefit from the upcoming neural interface technology allowing recordings in awake, freely moving animals, since pain is strongly affected by anesthetics. Furthermore, pain perception

is likely to be a cortical phenomenon that could be monitored by electrode implants providing they don't cause significant cortical injury.

Routinely, animal models play an important role as an early step in the development of new analgesic pharmacotherapies. However, translating basic pain research findings to human applications has been notoriously difficult. Specifically, the importance of pain responses in the primary sensory cortex [72][73][74] to the perception of pain is routinely ignored in drug development where different nociceptive withdrawal reflex setups in awake or sedated animals are the standard measures of pain perception (see, for instance, [75], [76] and [77]), in spite of even the most reliable human withdrawal reflex being of at best limited practical use [78]. As might have been expected, developing new analgesics for use in humans has seen little progress despite decades of efforts [79].

As an illustrating example, AstraZeneca researchers recently identified a chemokine receptor 2 antagonist as a potentially novel analgesic agent after in vitro experiments on dorsal root ganglion cells showed reduced synaptic transmission and in vivo experiments showing CCR2 antagonists reduced nociceptive withdrawal reflexes following tactile and thermal stimulation [80]. However, the subsequent randomized controlled trial in humans by Kalliomäki et al [81] showed no significant effect on posttraumatic neuralgia, despite the promising preclinical trials. Thus, the reduction of nociceptive withdrawal reflexes is clearly inadequate as an indicator of meaningful analgesia.

The primary somatosensory cortex is known to receive somatotopically organized nociceptive input from A δ [82][83] and C fiber [84][85] and contains neurons encoding painful stimulus intensity [86][87]. One possibility, then, is that nociceptive input measured in the primary sensory cortex of relevant animal pain models is more relevant to translational pain research than using standard nociceptive withdrawal reflex models. Supporting this hypothesis, recent findings in our lab shows lamina 5 neurons in primary sensory cortex in the freely moving rat replicate changes in pain perception in humans during development of primary and

secondary hyperalgesia [88]. However, in view of that the rat S1 cortex process also other types of sensory information such as tactile information there is a need for simultaneous recordings throughout this cortical area to clarify the changes in cortical processing during hyperalgesia. Hence, a biocompatible multichannel electrode array tailored to the cortical laminae would be very useful in pain research.

Aims

Thus, the aims of this thesis have been stated as follows:

- 1) To test the impact of implant flexibility and gelatin embedding on the foreign body response leading failure of implanted electrodes.
- 2) To develop an implantable device with the aforementioned features, that minimizes the foreign body response, capable of recording neurophysiological signals in freely behaving animals.
- 3) To test this device in a realistic neurophysiological experimental setup.

Original papers

Paper I

Köhler, P., Eriksson Linsmeier, C., Thelin, J., Bengtsson, M., Jörntell, H., Garwicz, M., ... Wallman, L. (2009). Flexible multi electrode brain-machine interface for recording in the cerebellum. In *Proceedings of the 31st Annual International Conference of the IEEE Engineering in Medicine and Biology Society: Engineering the Future of Biomedicine, EMBC 2009* (pp. 536–538).[90]
doi:10.1109/IEMBS.2009.5333498

Paper II

Ejserholm, F., Kohler, P., Bengtsson, M., Jorntell, H., Schouenborg, J., & Wallman, L. (2011). A polymer based electrode array for recordings in the cerebellum. In *2011 5th International IEEE/EMBS Conference on Neural Engineering* (pp. 376–379).[91]
doi:10.1109/NER.2011.5910565

Paper III

Ejserholm, F., Kohler, P., Granmo, M., Schouenborg, J., Bengtsson, M., & Wallman, L. (2014). μ -Foil Polymer Electrode Array for Intracortical Neural Recordings. *IEEE Journal of Translational Engineering in Health and Medicine*, 2, 1–7.[92]
doi:10.1109/JTEHM.2014.2326859

Paper IV

Köhler, P., Wolff, A., Ejserholm, F., Wallman, L., Schouenborg, J., & Linsmeier, C. E. (2015). Influence of probe flexibility and gelatin embedding on neuronal density and glial responses to brain implants. *PLoS One*, *10*(3), e0119340.[93] doi:10.1371/journal.pone.0119340

Paper V

Köhler, P., Thorbergsson, P.T., Ejserholm, F., Thelin, J., Granmo, M., Bengtsson, M., Wallman, L., Schouenborg, J. Altered nociceptive and tactile input to primary somatosensory cortex during an episode of TRPA1 mediated hyperalgesia – implications for assessments of pain in animals. (to be submitted).

Methods

Probe design

Considerable work has gone into designing and testing prototype arrays aimed at meeting the optimization criteria. Probe fabrication was conducted at the clean room of the Department of Biomedical Engineering of Lund Institute of Technology under the supervision of Lars Wallman and Martin Bengtsson. Photolithographical thin film techniques used in manufacturing microelectromechanical systems (MEMS) were used consistently during probe development. For a historical overview of thin film techniques, see Willson et al[94]. Photostructurable thin films of metal, silicon and various polymers have been used extensively in biomedical application for the past few decades[95][96][97] with well documented effects on tissue *in vitro* and *in vivo*. Several of the previously mentioned electrode arrays, including the Utah and Michigan type probes, are produced using thin film techniques. During the course of this work, we have selected a number of standard MEMS materials, including silicon oxide[98][67], gold[99], and various polymers such as SU-8[100][101] and polyimide[102][103][104][105] in the process of developing prototype probes for testing. These materials were selected for their relatively well described biological properties. (For a description and comparison of *in vivo* biocompatibility of these materials, see Voskerician et al[106].) In brief, early etched silicon dioxide and polymer prototypes were succeeded by photostructured SU-8[90], [91] and polyimide [ref smärtmanus]. Early stage probes were plain shanks without conducting material evaluated only for their geometrical and tissue penetrating properties, with incremental additions of gold leads from 1 to 14 electrode channels. During

development, probe thickness has also decreased from 20-30 μm to the current 7-8 μm , with a subsequent increase in probe flexibility. Beside these developments toward a working electrode array, a mock polymer probe was developed for our biocompatibility paper to evaluate the impact of probe flexibility and gelatin embedding on tissue response.

Probe fabrication

The fabrication process in all published studies started with creating a sacrificial layer of either metal (chromium or titanium) or polymer (LOR 20b©, Microchem USA or Durimide©, FujiFilm Belgium) on a silicon wafer, or by oxidizing the wafer to form a sacrificial layer of silicon dioxide. On this, the bottom half of the shank was patterned in the material selected for that prototype. Patterning was typically done directly, in the case of probe shanks made of negative photoresist materials. Patterning of photoresists was done using UV-light (365 nm) and chromium masks, thus defining probe shank, anchors, and connecting. A conductive gold layer was added to probes intended for actual recordings by evaporation and patterning using standard lift-off processes. In several early prototypes, the evaporated gold layer was etched using a positive photoresist instead, but this method was found to be inferior in resolution to lift-off. For adhesion purposes, the gold conduction layer was combined with a titanium or chromium adhesion layer in later prototype models. This is a common approach, as gold adheres poorly to most non-metals, in particular polymers, and many variations on adhesion layers have been published[107][96]. Finally, another layer of shank material was added to cover the conducting gold leads, leaving only measuring points and contacts for the preamplification hardware. In most designs, adhesion between the layers was enhanced by treating the structures with oxygen plasma for 3 to 5 minutes between steps. Removing the probes from the silicon wafer was done by dissolving the bottom sacrificial layer with an etch suitable for the sacrificial material used (Chrome etch, MicroChem USA, hydrofluoric acid, or potassium iodide, for chrome, silicon dioxide

and gold, respectively). For an overview of fabrication steps for the published probe models (figure 1).

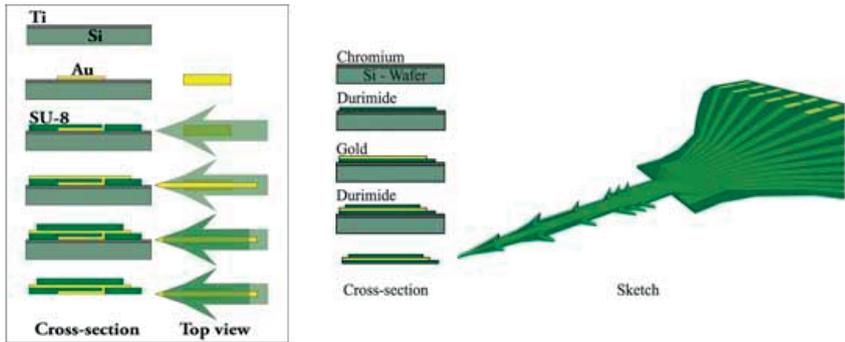


Figure 1. Schematic representation of SU-8 (left) and Durimide (right) probe fabrication. © 2009] IEEE

For papers I and II, the connecting cable end of the polymer probes were soldered onto print circuit board wiring corresponding to Kyocera Elko 5605 and 6206 connectors, respectively. For paper III and V, a Hirose Electric BM10 connector was used with the same circuit board arrangement, in order to reduce movement artefacts from the contact.

In addition to the general fabrication procedure, probes used in papers III and V had their recording sites enlarged post-fabrication by electroplating platinum black onto them. Electrodes were immersed in a solution consisting of chloroplatinic acid, lead acetate and hydrochloric acid together with a platinum wire anode, and platinized using a potentiostat. For a detailed description of the platinizing procedure, see paper III.

For papers III, IV and V, probes had become too thin and flexible to reliably penetrate the surface of the brain, even if the pia mater was cut using a 30 gauge (0.3112 mm) syringe. Extensive experimentation was done on methods to stiffen the probes during implantation with the use of gelatin. In brief, the generations of gelatin stiffened probes prior to published versions were:

- Dip-coated. Dip coating a hydrophobic surface like SU-8 with a hydrogel like gelatin required adding a solvent, like ethanol or glycol, or a detergent, like Triton X-100 (Sigma-Aldrich). After baking the hydrogel-coated probe overnight, this resulted in a sharp, needle-like structure with a roughly round cross-section. However, beside the unwanted potential toxic effects of the solvents and detergent, the shrinking of the gelatin resulted in breaks in the embedded probes.
- Embedding probes in a gelatin film sandwiched in a stainless steel mold removed the need for solvents or detergents and reduced failure due to breaks. This, however, requires cutting the film into a suitable shape without damaging the embedded probe in the process. Initially, this was done using a Model 70018 Laser Mill (New Wave Research, USA), which produced a reasonably sharp sword-like structure that could be implanted in brain tissue. However, this was excessively time consuming, as the laser mill required several hours to cut just one of these, and the method was abandoned.
- Polyurethane molds similar in shape to the sword-like structure described above were used to produce a similar result as the laser milled gelatin embedding. This speeded up production considerably and had most of the advantages of the milled gelatin film, but the resulting product was deemed too blunt to penetrate brain tissue. This was confirmed in both in vitro agar and in vivo implantation experiments.

For papers III and IV, probes were coated in a sucrose solution (MilliQ-water saturated with D-saccharose, KEBO Lab Sweden, at 50° C), forming a droplet on one side of the shank. Saccharose have been used in conjunction with polyimide probes of a similar design previously by Hassler et al.[108] and was deemed a safe choice of material in this context. A 75 μm tungsten wire was immersed in the droplet, aligning it with the shank of the probe. After drying, the wire-probe arrangement adhered together tightly and could also be coated with a thin layer of gelatin for histological analysis in

paper IV. For paper III, the tungsten wire was also sharpened using NaOH in order to decrease implantation trauma.

Surgical materials

Considerable effort has gone into developing durable transcutaneous fixation sites of the probes. In order to successfully record from freely moving animals, a head stage with preamplification hardware must be connected to wiring attached to the skull of the animal. In our experiments, this proved to be the most common source of device failure. Consequently, several materials and surgical approaches were tested or considered. Briefly, some notes on securing transcutaneous connectors:

- A fast hardening bone graft substitute (Stratec Medical, Germany), used in previous studies by our group[63], was used for the first generation of chronic implants. This was a cement mix forming a brittle polymer material intended for bone grafting, meaning a very biocompatible but fragile fastening of the implant. Cemented structures frequently crumbled after a period of days to a few weeks, and use of the product was discontinued. However, one positive feature of this cement was that it could be cut using an infrared CO² laser, which improved explantation procedures drastically.
- Different epoxy resins were tested on post mortem specimens but rejected for producing inconsistent and largely substandard results, mostly due to long hardening times and low viscosity.
- Dental cement (GC FujiCEM Automix), frequently referred to as “silastic”, was used for most implantations. This material has several strong points. First, application was easy with the dispensing system, which improved the detail and precision of attachments. Second, the resulting material was considerably harder and more durable than the bone graft substitute previously used. Device failure due to dental cement wear was consistently

rare. However, cracking of the cement has not been uncommon, and adhesion to the implant connectors and/or wiring was strong enough that cracks reaching these structures could propagate through the probe itself. There are also concerns regarding the biocompatibility of the glass ionomer used in the product. Although used extensively by dentists with good results, questions remain regarding the heat developed during hardening of the material, as well as cytotoxic fluoride release of some FujiChem products[109]. No such cytotoxic effects have been described for the specific ionomer used in our experiments, however.

- Adhesion between dental cement and the skull was generally the weakest point of all implantations. To counter this, the skull was washed with hydrogen peroxide before drilling, and anchoring titanium screws were fastened to the skull in a triangle around the hole. In spite of this, several implants failed due to screws and cement detaching from the skull. Usually, this was accompanied by considerable fibrous tissue wedged between skull and dental cement, indicating that invading inflammatory cells was at fault rather than the material itself. In such cases, the experiments were terminated immediately.

Surgical procedures

All animal experimental procedures were approved by the local Lund/Malmö ethical committee on animal experiments, which is subject to the code of regulations of the Swedish Board of Agriculture. These regulations, including directives of the European Union, follow the law on animal welfare legislated by the Swedish Riksdag. The County Administrative board governs the implementation of the legislation.

For paper I, recordings using the first working prototype array were done acutely in the cerebellum of decerebrate cat. Experiments were performed on cat preparations intended for another procedure [110] using the ethical

approval of that study. In brief, animals were anesthetized using profol and decerebrated at the intercollicular level after which the anesthesia was turned off. Animals were fixated in a stereotactic frame and SU-8 probes were mounted on micromanipulators and connected to external preamplifiers. For a detailed description of surgical procedures, see Ekerot and Jörntell [111].

For paper II, female Sprague-Dawley rats were implanted with 9-channel SU-8 probes with platinum black measuring sites. Animals were sedated (100 mg ketamine/20 mg xylazine per kg bodyweight) with a bolus injection intraperitoneally. The rat skull was fixed in a stereotactic frame and an incision in the skin was made rostrocaudally over bregma, and the skull covering the left hemisphere of the cerebellum was exposed after clearing the surrounding tissue. A hole was made in the skull using a dental drill and the dura mater of the cerebellar cortex was cut. After implanting the probe into the cerebellar cortex, it was covered by a stabilizing layer of 40° C 3% agarose solution (Sigma-Aldrich, Germany), and the array was attached to the skull with silastic. Finally, the wound was closed with surgical staples and the animals received subcutaneous injections of postoperative analgesia (Temgesic, buprenorphine, 50 µg/kg body weight).

For papers III, IV, and V, probes were implanted in the right primary somatosensory cortex of Sprague-Dawley rat and anaesthesia was achieved by intraperitoneal injections of fentanyl (0.3 mg/kg body weight) and Domitor vet (medetomidine hydrochloride, 0.3 mg/kg body weight). While ketamine/xylazine anaesthesia enables recording of neural activity during implantation, fentanyl/medetomidine is easier and safer to administer and can be cancelled after surgery using a subcutaneous injection of antidote (Antisedan, atipamezole hydrochloride, 0.5 mg/kg body weight). An antidote to the effect of xylazine, yohimbine, does exist, but is no longer on sale in Europe.

For papers IV and V, probe prototypes had become too thin and flexible as to make implantation without some form of guide or stiffening impossible. For paper IV, probes were glued to 50 µm tungsten wires using a saturated

sucrose solution and subsequently dip-coated in gelatin to prevent the sucrose from being dissolved too fast during implantation. Using the tungsten wires as guides, the probes could easily be implanted into the desired area of cortex, after which the guides were retracted using forceps with the probes still in place. There was concern that this implantation technique might damage neural tissue excessively or that removal of the guide might damage or displace the probe. Histological results from paper IV showed no such tissue damage, but removal of the guide from live recording probes showed that manual removal was highly dependent on the skill of the surgeon and could in some cases produce recording artefacts. Since it could not be ruled out that the production of transient artefacts in this manner was also associated with displacing the probe, a different implantation technique was developed for paper V, where probes were dip-coated in a mixture of gelatin, polyethylene glycol, glycerine and water that provided enough rigidity for implantation after drying.

Immunohistochemistry

Animals were anaesthetized with an overdose of pentobarbital intraperitoneally and transcardially perfused with 150-200 ml ice-cold 0.1 M phosphate buffer (PB), followed by 4% paraformaldehyde (PFA) in 0.1 M PB. Brains were dissected using standard dissecting tools and probe shanks were removed by pulling the silastic to which it was attached. In a few cases, probe shanks broke and left all or most of the penetrating part of the arrays still implanted in brain tissue. For paper IV, brains were sectioned as usual in these cases, resulting in sections of probe shanks in or around implantation sites in the affected micrographs. For paper V, this happened to one animal post dissection, and an attempt was made to section this brain parallel to the electrode to evaluate electrode placement in relation to cortical neurons. For most recording arrays, however, the protruding measuring sites worked as barbs on explantation, tearing out chunks of brain tissue when separating brain and silastic. While this could be viewed as an indication of anchoring effectiveness, it was also the

reason shanks for paper IV were designed without anchoring protrusions. All explanted brains were postfixed in 4% PFA overnight and then soaked in 30% sucrose for at least 24 hours for cryopreservation. They were then serially sectioned at 30 μm , either in the horizontal plane (paper IV) or coronal plane (paper V). Sagittal sections, prepared and mounted on glass slides in a cryostat, from brains explanted after recordings for papers I and II, but were excluded from analyses due to tissue damage from explantations. Attempts were made to explant brains with probes still in place by freezing the entire head of the rat following decapitation, PFA-preservation and cryoprotection using sucrose, followed by sectioning in a microtome and free floating immunohistochemical staining, but this too failed and was abandoned.

All sections were rinsed in phosphate buffered saline (PBS, pH 7.4) and preincubated in a mixture of 5% normal serum and 0.25% Triton X-100 (Sigma, Germany) in PBS. Following this, the sections were incubated with primary antibodies over night at room temperature. For paper IV, the primary antibodies used were rabbit polyclonal antibodies recognizing the astrocytic cytoskeleton protein glial fibrillary acidic protein (GFAP, 1:5000, Dako, Denmark), and mouse monoclonal antibodies recognizing either the microglial/monocyte marker CD68/ED1 (1:100, Serotec, USA) or the neuronal nuclei marker NeuN (1:100, Millipore, USA). After repeated rinses in PBS, the sections were further incubated in Alexa488-conjugated secondary antibodies for mouse IgG and Alexa594-conjugated secondary antibodies for rabbit IgG (1:500, Invitrogen, USA) for two hours at room temperature and rinsed again in PBS. Sections were then mounted on Superfrost Ultra Plus glass slides and cover slipped using Vectashield Hardset mounting media with the cell nuclei marker DAPI (Vector, USA). Several other primary antibodies were trialed prior to the experiments of paper IV, notably antibodies for calcium-binding proteins (calbindin-D28k and parvalbumin), the GABA_A-receptor-associated chloride-potassium symporter protein KCC2, glutamate decarboxylase, GABA, and the oligodendrocyte marker RIP. For various reasons, these were excluded from analyses, generally related to either unsuccessful staining or the

switch from cerebellum to primary somatosensory cortex, where imaging of inhibitory neurons was deemed unnecessary.

Electrical properties

Electrical impedance between frequencies of 100 Hz and 10 kHz against saline solution was measured (Gamry Reference 600, USA, with an Ag-AgCl reference electrode) for all recording electrodes to evaluate recording capabilities before implantation. This has long been accepted as the range of frequencies relevant to neurophysiological recordings[112][113][108]. Thermal noise is directly proportional to the impedance of the electrode leads and maintaining a low electrode impedance means a higher signal to noise ratio[114]. In vivo build-up of electrical impedance is a common source of failure for implanted stimulating and recording devices[115][116][56], so for papers III and V, the measuring sites were covered with platinum black using electroplating, to decrease impedances in vivo. Impedance measurements were also used to evaluate the effect of measuring site platinization, using the same method as above.

Neural recordings

For papers I and II, acute recordings with a sampling frequency of 27 777 Hz and a 0.5 Hz high-pass filter were obtained using custom built recording hardware, described by Ekerot and Jörntell [117]. For papers III and V, recordings from implanted probes were obtained using a OmniPlex Neural Data Acquisition System (Plexon, USA) sampling at 40 kHz with a 0.5 Hz high-pass filter with 1000X amplification. For paper III, an 8 kHz low-pass filter was added on wide-band recordings and a 400 Hz low-pass filter was added for evoked potentials.

For paper III, glabrous skin of the hind paw corresponding to the receptive field of the implanted area in primary somatosensory cortex was stimulated

intracutaneously with a train of single electric pulses (1 mA, 200 μ s, 1 Hz) using fine steel needle electrodes, as described in previous studies by our group [118].

For paper V, a CO₂ laser (Irradia, Sweden, model 315M Superpulse, wavelength 10.6 μ m, beam diameter 3.0 mm) with a 10W output power was used to elicit nociceptive neural activity and C fibre evoked potentials. This method is selective for cutaneous nociceptive A δ and C afferent fibres in the epidermis with precise temporal and spatial resolution [119]. Pain threshold was defined as the pulse duration of the CO₂ laser stimulation that elicited a withdrawal response in three out of five trials in each animal, usually between 20-30 ms. These stimulation energies have been shown to evoke late cortical field potentials in the rat primary somatosensory cortex through the activation of cutaneous nociceptive fibres in the normal [85], [120] and hyperalgesic [121] state. A pedal triggered manual probe was used for tactile stimulations.

Sprague-Dawley rats (n =9) implanted with our 13-lead multielectrode array modified with platinum black as described above were released into an open top chamber with mesh flooring. The plantar side of right hind paw was divided into two halves, heel and digits, and 40 nociceptive and tactile stimuli, respectively, were delivered to equally to these thought the mesh. After this preliminary stage, hyperalgesia was induced in the heel area by exposing the skin to 50% mustard oil in 99% ethanol for two minutes during isoflourane anesthesia. After waking and resting for 30 minutes, the rat was re-released into the chamber and tactile and nociceptive stimuli were again delivered to the heel and digit areas of the right hand paw. This stimulation protocol was repeated at 90 minutes after application of the mustard oil and finally 24 hours after application.

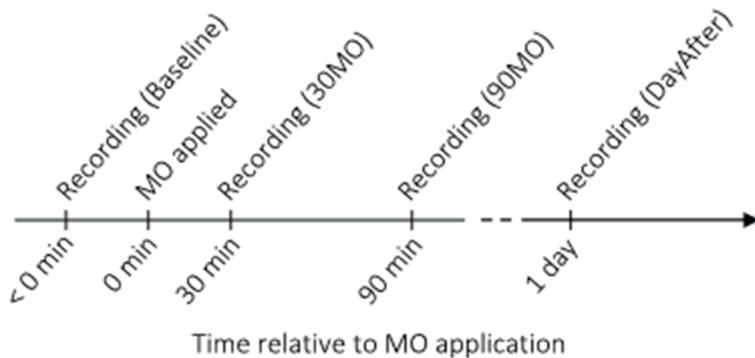


Figure 2. Timeline of experimental protocol and induction of hyperalgesia using mustard oil for paper V.

Results

Probe design and testing

For the sake of clarity, it is suitable to divide the different versions of prototype probes into three groups, with each corresponding to a stage of development in the project. The first generations of prototypes were all non-functioning by design, lacking connectors and often also conducting materials. Instead focus was on material choices, where SU-8 was the first bulk material settled on. A number of different SU-8 designs were produced in order to evaluate appropriate probe width, length and dimensions of the anchoring protrusions where electrode leads were to be placed. Micrographs of three different SU-8 probes developed at this stage are presented in figure 2.

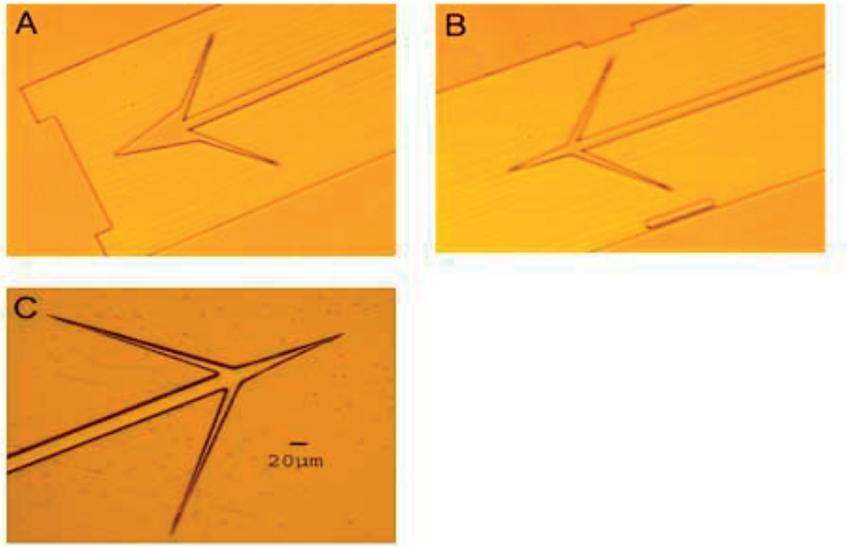


Figure 3. SU-8 prototype tips with variations on tip width and anchor length.

In the second stage of prototype development, gold conductors and connectors were added to the design in order to move on to impedance measurements and in vivo electrophysiology. The first round of probes with recording capability had a small (1-4) number of leads and intended for proof of principle experiments. Recordings were typically made in the cerebellar cortex of cat and Sprague-Dawley rats with the possibility of recording from inhibitory neurons in mind. For this reason, these probes were short in length (500 μm , see figure 4, left), which also helped curb the problem of penetrating the brain surface with extremely flexible probes.

The last stage of prototype development consisted of increasing the data yield per implanted probe. This was done both by gradually increasing the number of electrode leads (see figure 3) to a final number of 14 recording sites, and by improving the durability and impedance of individual leads. To this end, SU-8 was replaced with polyimide, which is less brittle and prone to tearing, with a Young's modulus of 2.5 GPa compared to 4.2 GPa

for SU-8. These probes had a thickness of 5-8 μm and a maximum width of 300-400 μm at the base of the penetrating part of the implant, tapering down to a very sharp tip.

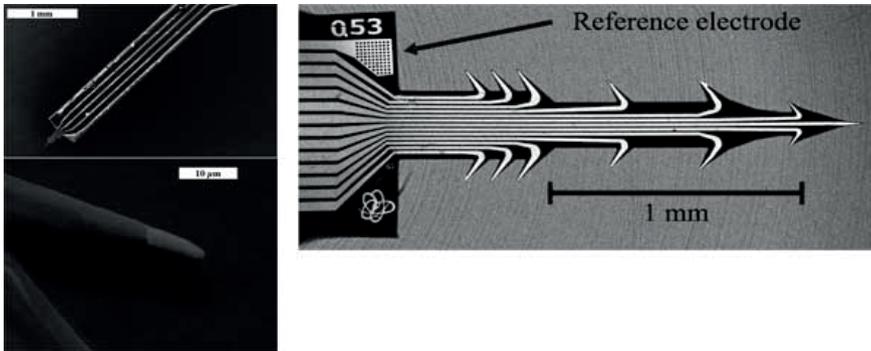


Figure 4. Early measuring prototype (left) with 3 recording channels and detail of anchor electrode lead, and 14 channel prototype (right) with serial number and lab logotype. © 2009 IEEE

The earliest probes with integrated electrode leads generally had impedances of several $\text{M}\Omega$ at 1 kHz, comparable to those of tungsten in glass electrodes used for acute in vitro and in vivo recordings by our group. This is adequate for recording extracellular potentials if recording sites are located close enough to neurons. However, at this stage the implants produced varied considerably in quality, with electrode impedances often exceeding 10 $\text{M}\Omega$. For a sample of electrodes with acceptable and unacceptable impedances, see figure 5.

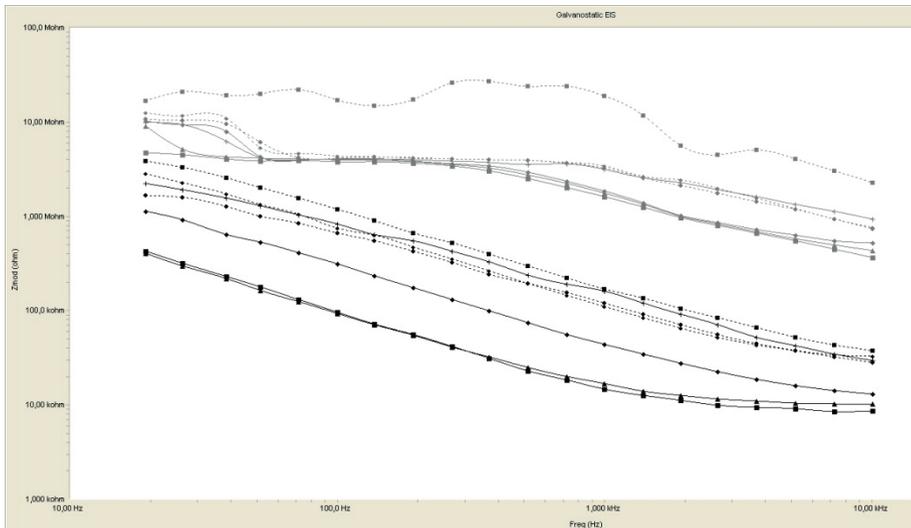


Figure 5. Impedances with acceptable (black lines) and unacceptable (gray lines) recording impedances.

To reliably lower electrode impedances, platinization of measuring sites was introduced as part of the third stage of prototype development. By electrodepositing platinum black, surface area and thus electrode impedance was lowered by several orders of magnitude (figure 6). This brought recording parameters into the range of competing multielectrode arrays on the market, even after redesigning recording sites to be smaller than previous prototypes.

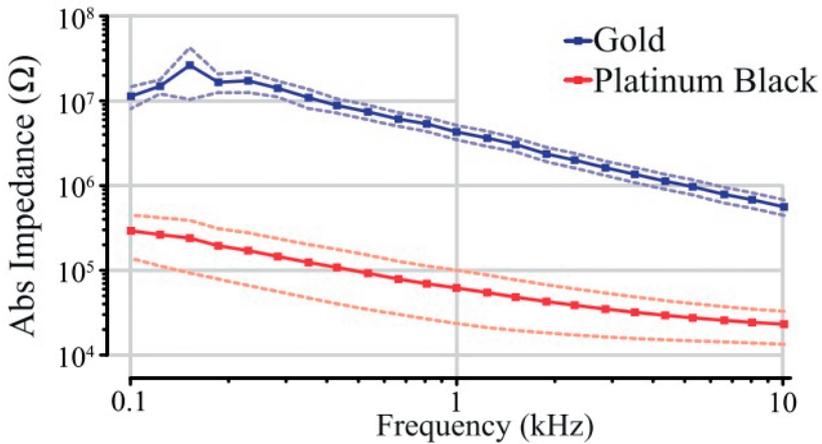


Figure 6. Result of platinization on electrode recording impedances.

Acute recordings

For paper I, extracellular recordings were obtained from decerebrate cat. The Kyocera connector regularly produced enough noise to drown out any biological signal, but in recordings where the connector worked as intended, noise levels were consistently low. For unknown reasons, most recordings where noise levels were judged to be acceptable produced only local field potentials but no extracellular spikes. In two animals, however, recognizable spikes with peak to valley amplitudes measuring 4-500 μV were recorded and one of these included in paper I. In it, complex spikes originating from two separate Purkinje cell dendrites could be identified together with spikes from two inhibitory cortical interneurons (figure 7). At the time, this was sufficient to conclude the general design was adequate to record neural signals in physiological conditions.

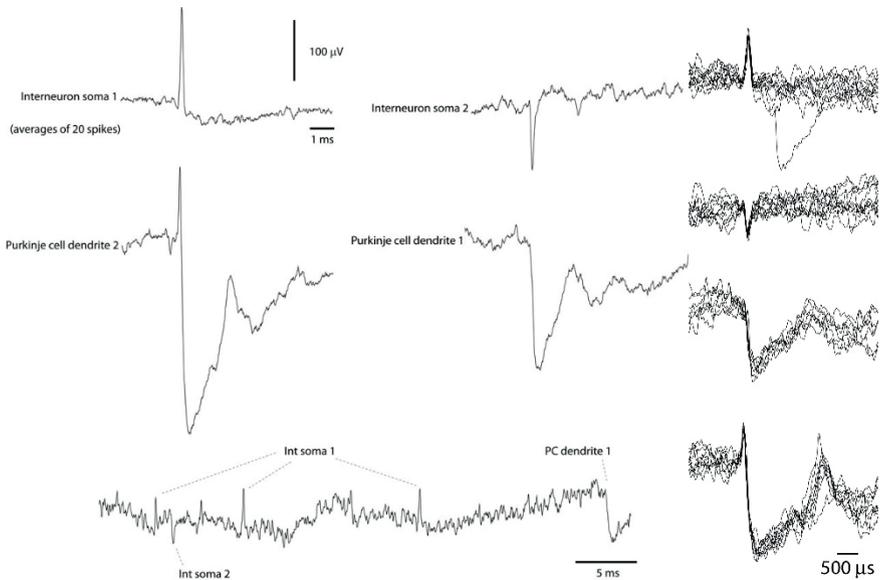


Figure 7. Inhibitory interneuron spikes (top) and Purkinje cell complex spikes (middle) recorded in the cerebellum of decerebrate cat, with a 50 ms sweep showing both spike types inlaid (bottom). Rightmost column shows sweeps of individual interneurons and Purkinje cells superimposed on themselves.

In paper III, the ability of our electrode array to accurately record evoked potential propagation through the different layers of rat somatosensory cortex was evaluated. The array was designed with investigating the different neuronal layers of the cortex in mind, using a fixed geometry to measure evoked potentials in all layers simultaneously. Acute recordings were obtained from the primary somatosensory cortex of three Sprague-Dawley rats during electrical stimulation of the glabrous skin of the hind paw using fine steel needle electrodes. Potentials evoked in this manner formed a depth profile with polarity inversion from surface downward and maximum negative amplitudes at electrode leads located 800 μm from the surface (figure 8). Importantly, we observed no cross talk between recording channels, a possibility stemming from the close proximity of the gold conductors along the probe shank.

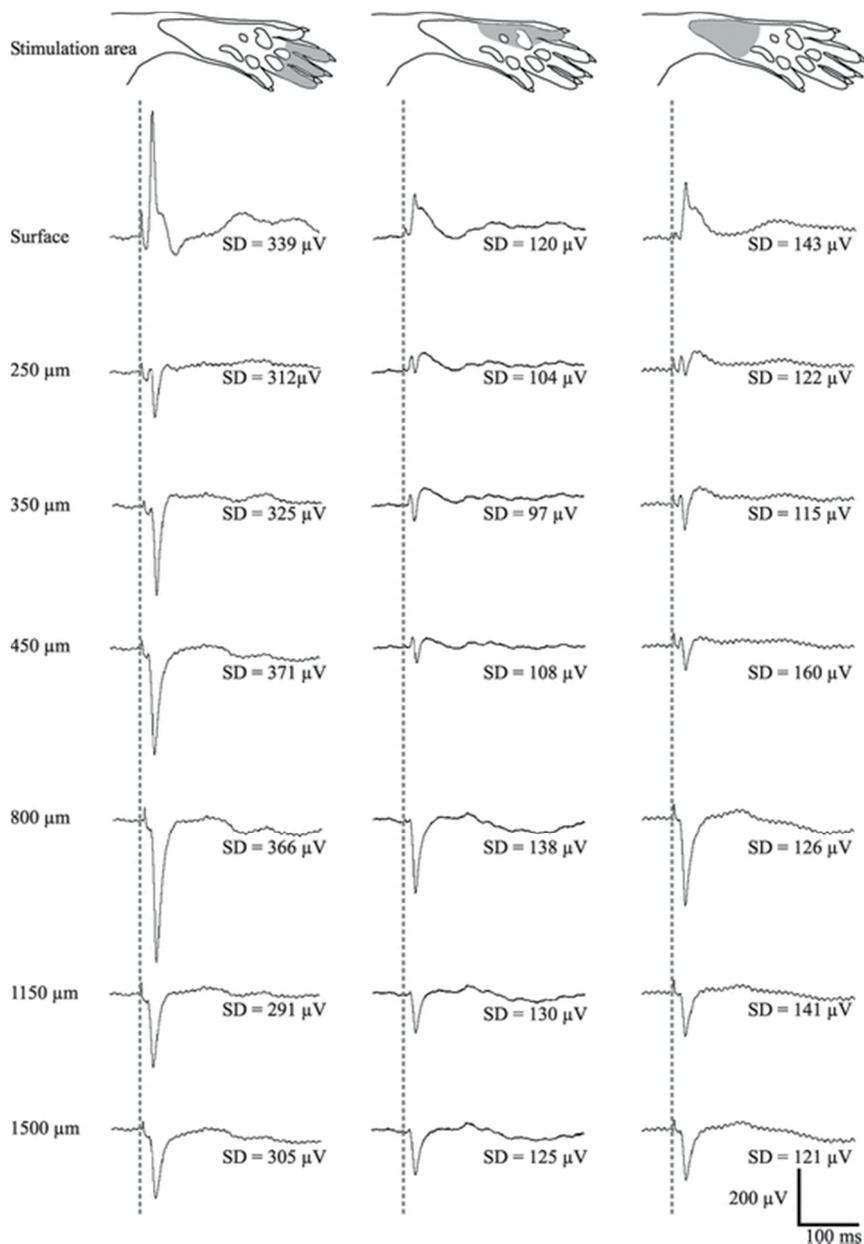


Figure 8. Depth profile from intracortical array following stimulations in three different receptive fields.

Chronic electrophysiological recordings

For paper II, evoked field potentials in the cerebellar molecular layer of Sprague-Dawley rats were recorded using a 9-lead prototype of our array. Potentials were evoked by stimulating the vibrissa region with a 2 mA current through fine intracutaneous needles. With this setup, climbing fiber potentials with amplitudes of several hundred μV were recorded, as well as mossy fiber input in the 100-200 μV range (figure 9).

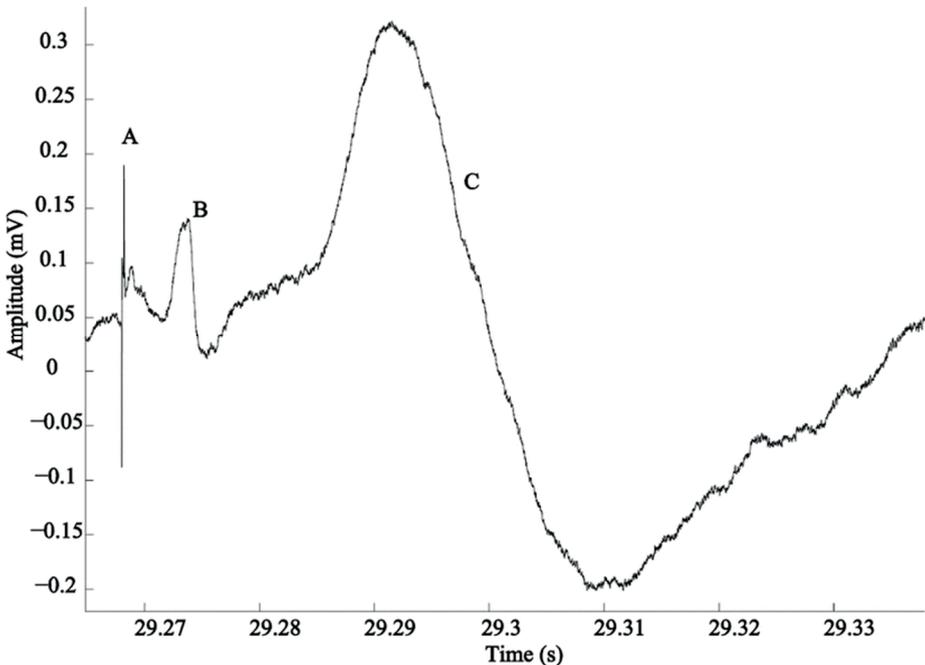


Figure 9: Evoked potential in the cerebellar cortex of rat. A indicates stimulus artifact, B climbing fiber potentials, and C mossy fiber input. © 2011 IEEE

The 9-lead prototype used for paper II was followed by a 13 lead version implanted in the same way. This yielded some very fine recordings in a few animals over time periods of up to 32 days, with simple and complex spikes as well as evoked potentials following electrical stimulation of the vibrissa area of the nose as described above. Three different animals

implanted with probes from the same batch yielded these recordings, leading us to believe other, less successful implantations faulted in fabrication in some way. Spike amplitudes measuring tenths of mV were recorded the first 2-3 weeks (figure 10). After this period, spike amplitudes diminished steadily until no spikes were detected or the skull cap failed.

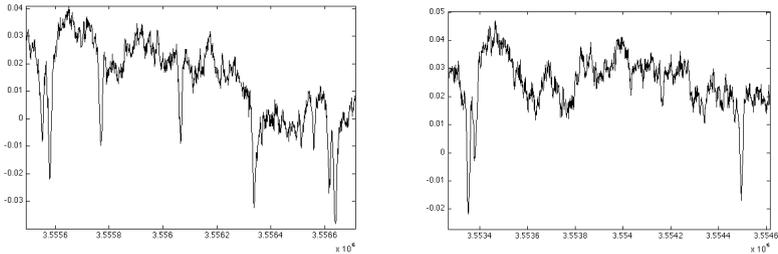


Figure 10: Multiunit spikes (top) from one animal recorded at 1 week (left) and 2 weeks (right) post implantation.

Biocompatibility

Several attempts have been made to visualize the impact of our recording probes on neural tissue. However, the anchoring protrusions in the array design have proven to function similar to barbs, almost invariably tearing large sections of brain with them upon explantation. Because the connecting cable extending from the surface of the brain, which is also used for handling and manipulating the probe during insertion, attaches to the skull upon closing the wound, explanting the brain from the skull post mortem without also explanting the probe from the brain has proved extremely difficult.

For paper IV, polymer needles of the same dimensions as our recording arrays but lacking the anchoring protrusions were implanted in rat primary somatosensory cortex. These could be explanted without tearing of the brain parenchyma and axial sections were analyzed by quantifying the astrocytic and microglial response as well as neuronal and gross cell nuclei

counts. By implanting probes either parallel or perpendicular to the principal direction of movement of the brain, termed rigid and flex mode respectively, we manipulated the flexibility of the implants selectively.

GFAP reactivity was found to be dependent on whether the probes were implanted sagittally or coronally (figure 11 B), where probes aligned coronally (termed flex mode) produced a significantly smaller astroglial reaction than probes aligned sagittally (termed rigid mode). This finding indicates micromotions are indeed responsible for the formation of a chronic astroglial scar around neural implants. Microglial reaction (figure 11, C) and neuronal cell loss was not affected by implant alignment, but was significantly decreased by embedding probes in gelatin (figure 12). This dissociation between microglial and astrocytic response is interesting, as they are frequently described together and often assumed to have a common or similar cause. These findings suggest the microglial reaction is distinct from the astrocytic response induced by micromotions. No significant effects on DAPI counts were seen. In summary, flexibility was found to decrease the formation of an astrocytic scar around the implant, and embedding the implant in gelatin brought neuronal survival to the point where it was indistinguishable from naïve tissue.

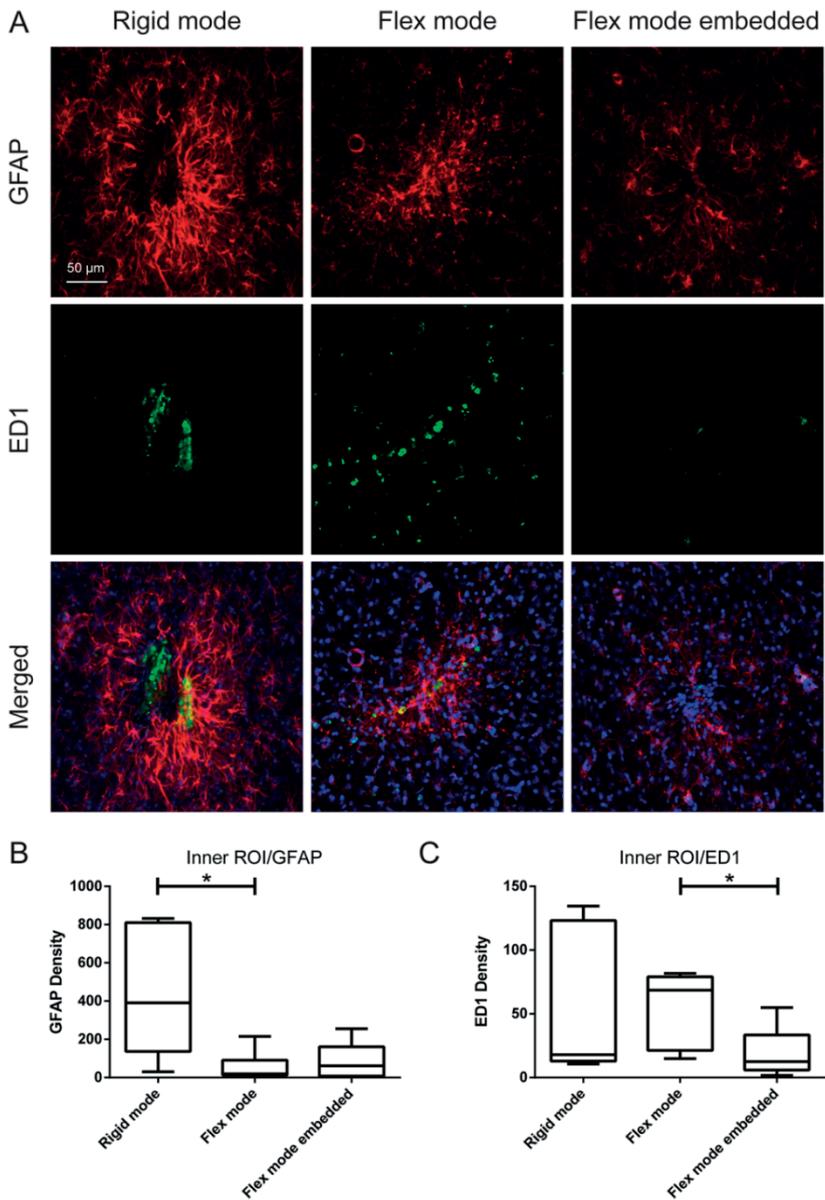


Figure 11: Astrocytic (red) and ED1 (green) responses to rigid mode, flex mode and flex mode embedded probes. In B, flex mode probes show significantly less astrogliosis compared to rigid mode. C shows a significant decrease in microglial response when probes were embedded in gelatin. [93]

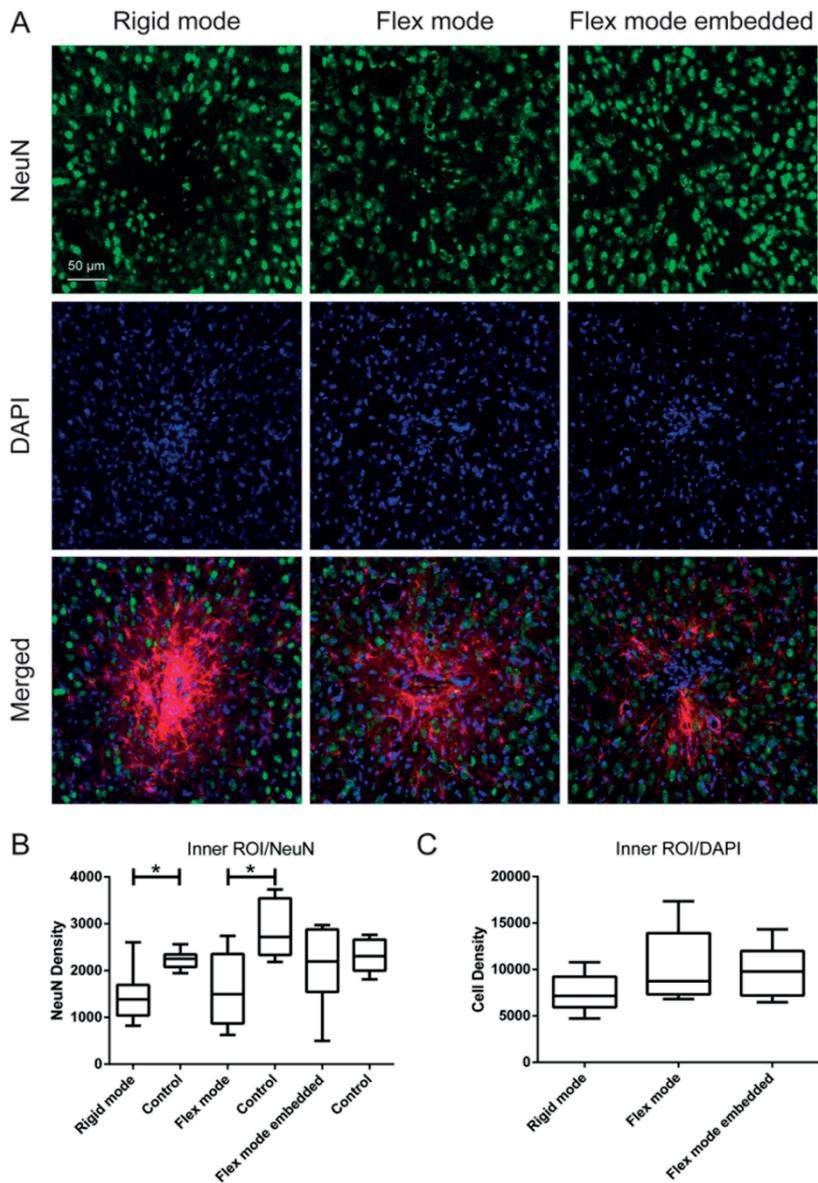


Figure 12: Neuronal survival compared to naive neural tissue was normalized when probes were embedded in gelatin and implanted in flex mode. [93]

A novel animal model of pain

As a final step in demonstrating the feasibility of the neural probe developed for this thesis, we therefore investigate the nociceptive A δ and C fiber potentials and multiunit activity in the primary sensory cortex of the freely moving rats following the induction of a hyperalgesia-like state using cutaneous application of a mustard oil solution. Mustard oil was chosen because it activates nociceptive afferents through TRPA1 receptors [89]. This leaves thermoceptive TRPV1 receptors unaffected by desensitization for our CO₂-laser stimulation protocol, used extensively by our and other groups for pain research.

Thus, in paper V, neural recordings from the primary sensory cortex in 9 female Sprague-Dawley rats were obtained before, during, and after induction of cutaneous hyperalgesia using the TRPA1 agonist mustard oil locally on the hind paw. After denoising and referencing (figure 13) the signal, both single- and multiunit activity (figure 14), and local field potentials resulting from tactile and nociceptive stimulations were recorded in for 1000 ms, starting at 200 ms before the stimulus event.

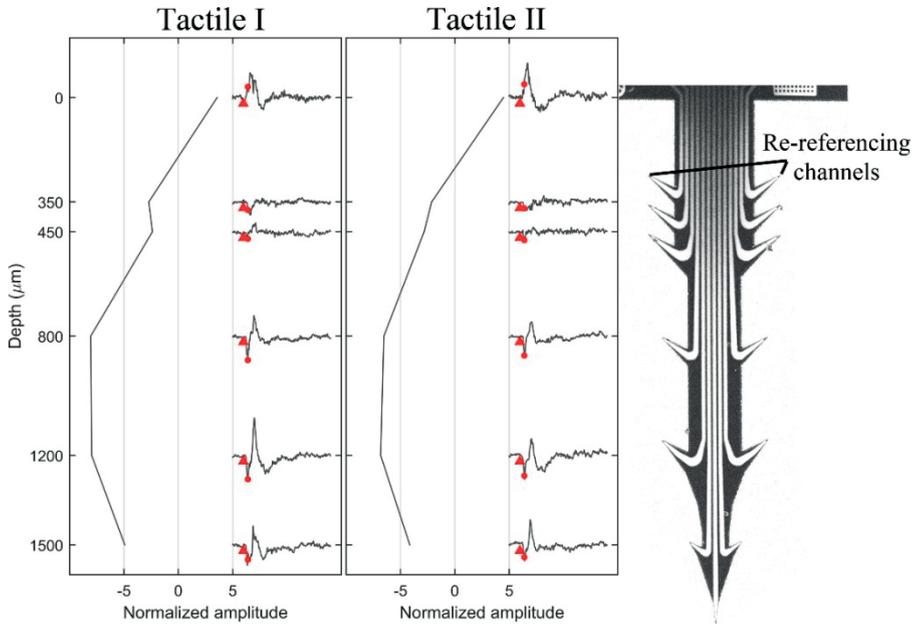


Figure 13: Re-referencing of the LFP signals. The median local field potential from the topmost intracortical electrode was subtracted from all signals analyzed, in order to mimic previous results [84][120]. This was necessary because subdermal reference electrodes had been excluded from the design after repeatedly failing, after which the large recording site at the base of the array (glimpsed at 0 in the diagram above) was used as reference during recording sessions. While functional, this corrupted all LFP:s with surface EEG signal, making intracortical potentials unrecognizable. Tactile responses showing primary (“Tactile I”, left) and secondary (“Tactile II”, right) hyperalgesia are shown, with normalized amplitudes between stimulation and baseline cases plotted to the left of individual channel traces. Red dots indicate baseline cases, 20 ms after stimulation, indicated by red triangles.

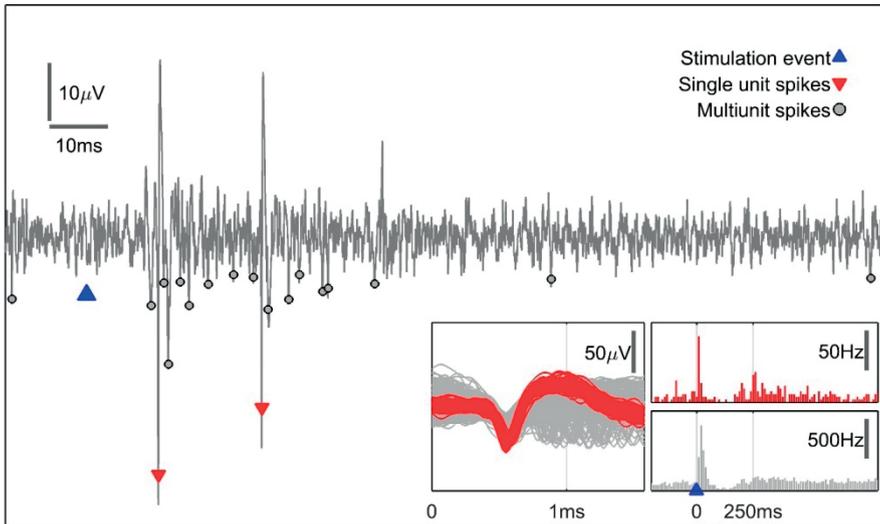


Figure 14: Sample 150 ms sweep following tactile stimulation (blue triangle) showing single- and multiunit spikes. Inset images show detected spike waveforms and peristimulus time histograms for sorted single units (red, top) and unsorted multiunits (blue, bottom).

To avoid problems caused by variations in latencies of evoked potentials we calculated the change in energy by rectifying, squaring and dividing LFP potentials by the total energy (sum of power) within the pre-stimulation interval. This yielded a normalized quantification of peristimulus LFP power response. The energy of the late component (200-500 ms) of the LFP evoked by CO₂ laser stimulation of the primary hyperalgesia area was significantly increased ($p < 0.05$ -0.001) in all the different layers of the cortical column compared to the baseline recording (fig 15). The highest increase (6.7-7.6 times the median energy compared to baseline) was observed in the deep layers (1200 to 1500 μm below the surface) 90 minutes after exposure and persisted for at least 24 hours after mustard oil application, where the component's median energy was about 2 times higher compared to baseline. Co₂-stimulation of the secondary hyperalgesia area resulted in an up to 2.8 times increase in median energy compared to baseline at the deep layers of the cortex, or 800-

1500 μm . Tactile input from the primary hyperalgesia area resulted in a significant ($p < 0.05-0.01$) increase in LFP 30-70 ms after stimulation at a depth of 1500 μm , while secondary hyperalgesia in the non-inflamed skin was observed mainly 24 hours post mustard oil application. Tactile stimulation of particularly the primary hyperalgesia area produced a new component in the LFP that was generally not present at baseline. In the primary hyperalgesia area, this component arrived with a latency of about 70-100 ms, had a duration of about 50-80 ms and gave rise to a significant increase ($p < 0.05-0.01$) in the LFP response energy between 70 and 175 ms in the recordings after mustard oil exposure.

There were no significant changes in multiunit firing rate due to tactile stimulation of either the primary or secondary hyperalgesia areas. In contrast, multiunit activity exhibited significant increase ($p < 0.05-0.01$) at more superficial layers, i.e. 350-1200 μm , peaking at 800 μm following CO_2 -stimulation of the primary hyperalgesia area, and CO_2 -stimulation of the secondary hyperalgesia area produced a significantly ($p < 0.05-0.001$) larger increase in MU-firing rate compared to baseline (up to about 24 Hz) already 30 minutes after mustard oil exposure and at depths of 350-1200 μm .

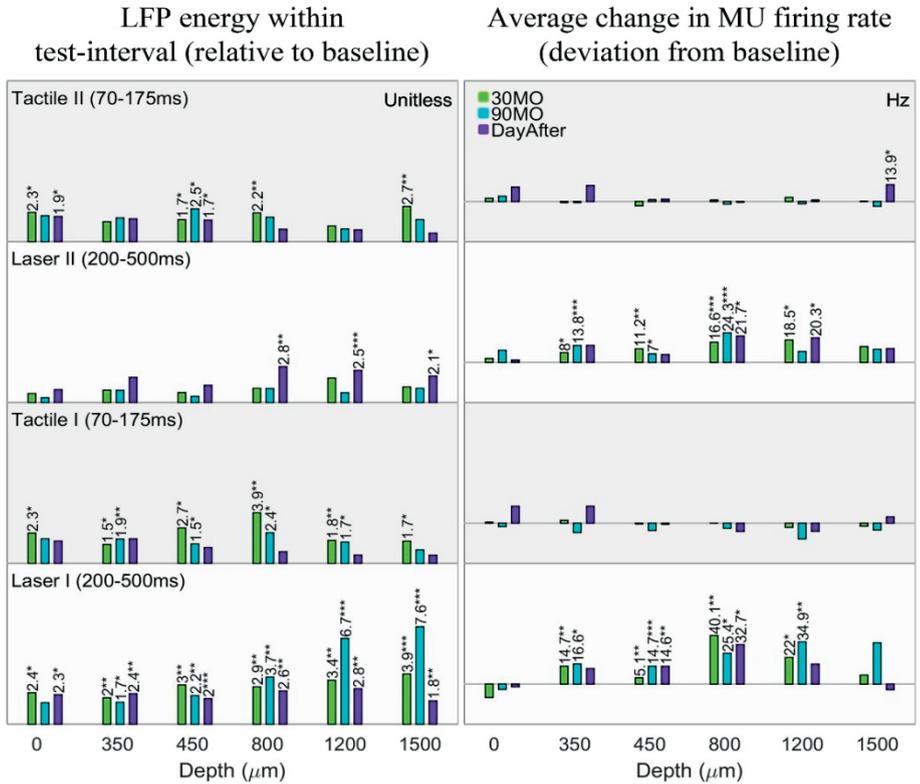


Figure 15: Mustard oil responses as indicated by LFP energy and multiunit firing rates relative to baseline. CO₂ laser and tactile stimulation of the primary hyperalgesia area (termed Laser I and Tactile I, respectively) produced the largest changes in LFP energies. CO₂ laser stimulation of the primary and secondary hyperalgesia areas (Laser I and II, respectively) also produced a consistent increase in multiunit firing rate.

Discussion

Thus, the aims of this thesis have been stated as follows:

- 1) To test the impact of implant flexibility and gelatin embedding on the foreign body response leading to failure of implanted electrodes.
- 2) To develop an implantable device with features that minimizes these factors, capable of recording neurophysiological signals in freely behaving animals.
- 3) To test this device in a realistic neurophysiological experimental setup.

In papers I, II, and III we demonstrate that our probe has electrical recording properties comparable to state of the art multielectrode arrays currently on the market and the feasibility of recording neural signals with a probe tailored to the cytoarchitecture of S1 cortex. In paper IV, we showed that a flexible probe, such as ours, causes a significantly reduced foreign body response when implanted into the brain parenchyma and tethered to the skull. We also showed that embedding such a flexible implant in gelatin brings neuronal survival around the implant up to the levels seen in naïve brain tissue. In paper V, the appearance of hyperalgesia and allodynia in the primary sensory cortex following chemical inflammation in freely behaving rats was studied over a period of days using the probe described in the above papers, demonstrating the potential of the design and the importance of tailoring the electrode array to the laminar structure of mammalian neocortex.

Probe characteristics

The first aim met in the work leading up to this thesis was the development of a novel implantable multielectrode array designed specifically to minimize tissue reactions and inflammatory response. The final design described here and in paper V is of a 14 lead, ultrathin polyimide probe for chronic recordings in rat somatosensory cortex. Probe characteristics hypothesized to be beneficial to this aim were small size, high flexibility, and protrusions with the dual purpose of placing the recording sites in less compromised tissue and to improve anchoring in the tissue in order to reduce mechanical stress on brain parenchyma, as well as electrical properties similar to those of existing wire- and micromachined arrays.

Regarding the need for anchoring, it is known that the size of the astrocytic scar correlate with the force needed to extract the probe from the brain [122] Reducing the astroglial response, as was one of the aims in this thesis, might therefore have the unwanted effect of reducing electrode anchoring in brain parenchyma. Indeed, probes devoid of protrusions like the ones developed for immunohistochemical analysis in paper IV could usually be explanted easily without tearing of the brain parenchyma (figure 16). Proximal parts of the probe shanks were regularly covered with considerable ED1 adhesions, but adhesions around the mid- and tip segments of the shank, where tissue sections for histological analysis were taken from, only had adhesions a few μm thick. Moreover, by comparing sections from brains where probes had been pulled out to those where probes were sectioned together with the brain we found no obvious difference in the scar surrounding the probe, suggesting that adhesion to probes without protrusions is indeed negligible.

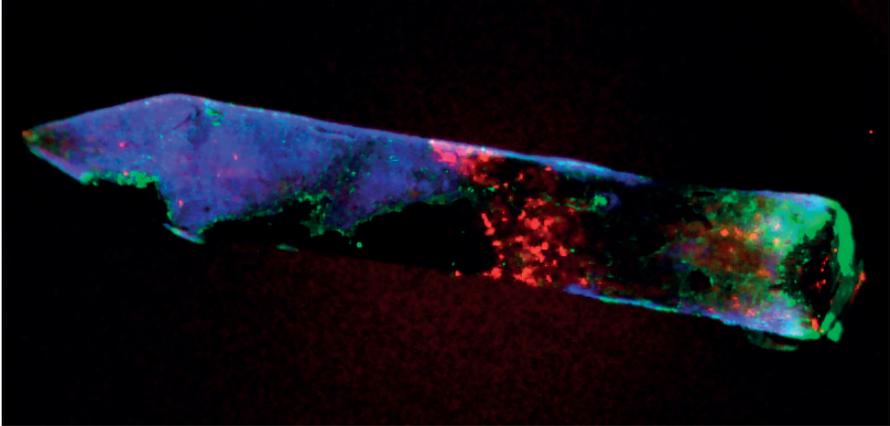


Figure 16: Explanted probe used for paper IV. DAPI (blue), ED1 (green) and GFAP (red) coating on the probe is very thin and absent in many regions, indicating adhesion to the probe is small. *Unpublished materials.*

Anchoring of ultrathin electrodes to the region of interest for recording, then, appear to be a necessary step towards stabilizing the electrode in the brain parenchyma and thus to reduce electrode migration in the tissue. The inclusion of anchoring structures in our design is such a step. Although no extraction force measurements have been made, the fact that extracting our probes was not possible without tearing the brain parenchyma suggests the anchors serves this purpose well.

Gilgunn et al [123] have produced a single channel probe very different from ours, but following some very similar reasoning. In Kozai et al [124] they expand on this concept, arguing that the flexibility of a neural probe is not just a function of material choice or geometrical cross section. They point out that a large enough probe can be very stiff even when using low elastic modulus materials, and that conversely, a thin enough probe can be flexible even if using a very high elastic modulus material. Interestingly, they point out that flexibility is also a function of probe length. Thus, the probe presented in Gilgunn et al is sinusoidal to increase length and flexibility although made of platinum, a relatively high elastic modulus material. While this method of increasing flexibility should reduce shear stress on the brain parenchyma and reduce the astrocytic response similarly

to our findings in paper IV, it is unclear if a sinusoidal wire-like probe provide an anchoring effect or if the matrix used also increases neuronal survival similarly to ours. In any case, this manner of increasing flexibility by increasing probe length is interesting and has a number of potential benefits, such as lessening the anchoring effect of the skull.

Impact of flexibility and embedding

To test the impact of probe flexibility on the foreign body response to a neural implant we utilized previous findings that the predominant movement of the brain relative to the skull is in the anterior-posterior direction and evaluated the difference in tissue response between same probe that depending on mode of insertion could either flex or not with the dominant movements. To our knowledge, this is the first time flexibility, hypothesized by several groups to be a desirable trait in neural implants, has been tested independent of other variables such as differences in size or in materials. We found that the astrocytic response, but not the microglia response or neuronal survival largely results from implant flexibility.

Notably, the microglial response decreased and the otherwise ubiquitous loss of nearby neurons was abolished after embedding the flexible probe in a gelatin matrix, while the astrocytic reaction did not change. This finding is in sharp contrast to what has previously been found and is, in fact, the first time neuronal survival following probe implantation has been preserved at normal levels. For example, Biran et al [125] demonstrated a considerable neuronal loss they termed “kill zone” around implanted Michigan probes. Neurofilament intensity as measured with line profiling showed a clear decrease more than 200 μm around the implant at 2 and 4 weeks post implantation that did not seem to be attenuating between the two measuring points. Similar results have been found for other commercially available electrodes [58].

Interestingly, Biran et al also concluded that the neuronal “kill zone” is the likely result of ED1 reactivity in the nearest 100 μm from the implant, as

these two variables showed a negative correlation to each other. This was mirrored in our study where embedding the probe in gelatin had the greatest effect on ED1 proliferation and normalized neuronal counts.

With respect to recording quality, the informational content of extracellular field potentials is inversely related to the distance between the current source and the recording site [126]. Retaining viable neurons as close to the electrode leads as possible should therefore be a priority in neurophysiological experiments as this is likely to be the single most important parameter for obtaining high signal quality.

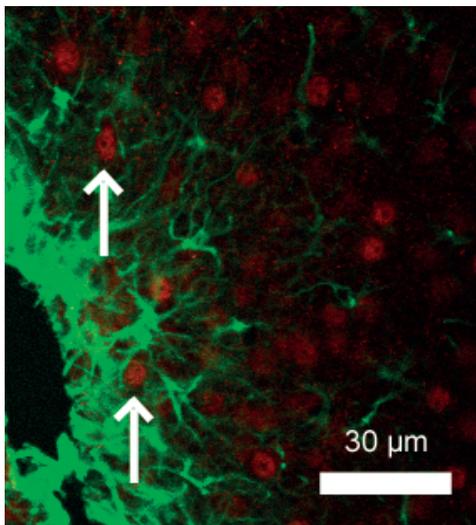


Figure 17: NeuN-positive cells (red) close to the hole left after explanting the probe used for paper IV. Apparently viable neurons are still detectable within the tens of microns from the probe.

Several different lines of evidence implicate astrocyte mechanosensitivity as a factor in inflammation following implantation trauma. Gao et al [127] observed a calcium ion influx leading to astrocyte activation in an in vitro mechanical scratch model. Using a similar model, Shi et al [128] demonstrated a proinflammatory upregulation of aquaporin 4 in scratch-injured astrocytes. Johnson et al [129] investigated extracellular matrix influence on astrocyte response to mechanical injury by scratching an in

vitro monolayer of astrocytes grown on different matrices. They demonstrated astrocyte activation is context dependent, where not only astrocyte regrowth varied between different substrates, but also astrocyte response to IL1 β signaling. There is also a growing literature on astrocyte contribution to neuronal signaling [130][131][132], as well as findings demonstrating that this contribution is significantly altered by astroglial activation [133][134]. In light of these findings, it is clear that the astrocytic foreign body response, at the very least partially depending on the physical properties such as flexibility of the implant, not only threatens the physical integrity and recording capability of the implant itself, but that it also affects the neural circuits recorded from.

Regarding the mechanisms underlying the beneficial effects of gelatin, evidence has been presented by other research groups suggesting that the blood brain barrier may play a key role for electrode function [135]. Following BBB disruption, albumin has been shown to bind to TGF β receptors and activate myosin light chain kinase reactivity in astrocytes [136] as well as astrocyte release of intracellular potassium into the brain parenchyma following TGF β downregulation of inward rectifying potassium channels [137]. Albumin release into the brain parenchyma has also been shown to promote neuroinflammation through activation of astrocytic and microglial IL1 β release by activation of the MAPK pathway [138]. BBB leakage of fibrinogen leading to recruitment of microglia has also been described in the study of neurodegenerative disease [139]. Spataro et al [140] reduced BBB leakage and astrogliosis following the implantation of a neural probe into rat cortex by administering the synthetic glucocorticoid dexamethasone. It is unclear if this effect was purely a general anti-inflammatory effect, or if the decreased BBB leakage contributed to the effect on astrogliosis. With this in mind, it may be suggested that gelatin may help restore BBB after the implantation. Previous work by our group [141] has shown that implanted gelatin needles implanted into rat cortex produced a significantly smaller glial reaction than a stab wound of the same size, indicating gelatin is not only more biocompatible than common biomaterials, but that it has some

beneficial effect on the wound itself. Possible mechanisms for this effect include a hemostatic effect of gelatin being derived from (mostly porcine) collagen [142] and temporary physical occlusion of BBB wounds or lessening of implantation trauma through reduction of friction. An alternative explanation not including the BBB is that gelatin serves as a temporary barrier to implant surface-immune cell interactions. Winslow et al [143] demonstrated a connection between BBB integrity as measured by IgG diffusion surrounding implant sites and disruption of oligodendrocytes in axon-containing sections of parenchyma adjacent to implant sites. One way to investigate the question of the importance of BBB penetration would be the simultaneous infusion of a hyperosmolar agent such as mannitol [144] into the blood stream during implantation, in order to reduce the amount of fluid carrying potential proinflammatory substances into the brain parenchyma. Reducing the water exchange rate following BBB disruption is an established clinical technique to prevent brain edema following traumatic brain injury.

Chronic recordings and physiological experiment

In paper 5, we utilized ufoil electrodes tailored to the cytoarchitecture of the cortex cerebri to perform a detailed study in the awake freely moving animal on the altered nociceptive input to different laminae during an episode of chemical inflammation. This study showed that nociceptive input from both inflamed (primary) and non-inflamed (secondary) skin areas to the primary sensory cortex in freely moving rat is enhanced and peaks in layer 5. We found that the spatial profile of potentiated input from primary and secondary skin regions to S1 cortex was similar with primary and secondary hyperalgesia peaking in lamina 5. However, the potentiation of nociceptive evoked LFP from the secondary skin region was notably delayed and less powerful as compared to that from the primary inflamed skin. Thus, while confirming data from UVB induced hyperalgesia in rats [88] that both primary and secondary hyperalgesia can be monitored in S1 cortex, the present findings in addition indicate a clear mechanistic

dissociation between primary and secondary hyperalgesia in the awake rat. Interestingly, during peak hyperalgesia, tactile input was also potentiated in lamina 5 and a delayed tactile input (onset 70-150 ms) peaking in more superficial laminae was observed. This could be related to allodynia produced by mustard oil application as observed in humans.

Notably, this study demonstrate that the probe developed and described in this thesis is quite useful and opens up for detailed studies of fundamental brain functions in the awake animal.

Limitations

The single most important problem, as measured by time invested into it if nothing else, is the relatively low yield in terms of neuronal spike activity. While early trials showed our design had the capacity to record action potentials with very high fidelity, subsequent trials failed to reproduce this for several generations of prototype development. Furthermore, the final design, while capable of recording both single and multiunit activity reliably, did not reproduce the outstanding recording quality of earlier designs. Although our histological data using simplified probes show large neurons viable very close to implanted probes, the most obvious prerequisite for recording neuronal spikes, individual neuron activity, has been relatively scarce. One conceivable reason is that the ufoil probe used for recordings was anchored to both the skull and the tissue for practical reasons. It is known that free floating electrodes cause less tissue reactions [63]. It is therefore possible that a free floating design of the ufoil would perform better.

The six week study period in paper IV was chosen based on our experience from previous studies on the neuroinflammatory response to probes of various designs [7], [63]. In line with results from other labs [66], a chronic response to implanted probes is usually present (assuming the probe is large enough to produce chronic gliosis in the first place) at around the four to six week mark. Studies with longer time frames show the progression and development of the chronic gliosis our probe design aims to avoid. While it is our opinion that six weeks is long enough for reliably detecting a chronic glial foreign body response, a longer perspective morphological study comprising both earlier and later time points would have undeniable benefits. First, it would confirm or contradict abovementioned findings that gradually progressing neuroinflammation is established relatively early post implantation. Second, and more importantly from a physiological stand point, the remodeling of neuronal circuitry following probe implantation trauma has not been studied to any satisfying degree. That the columnar organization of the neocortex will be disrupted to some degree

following implantation of any penetrating recording device is self-evident, but the quality and quantity of this disruption is currently unknown. Hence, it remains to analyse the detailed reorganization of neuronal circuits after electrode implantation. Nevertheless, our finding of a preserved neuronal density represent a key finding as it indicates that it will be possible to record from relatively undisturbed neuronal circuits. Finally, one obvious weakness of the probe design presented in this thesis is that the part of the array penetrating into the cerebral parenchyma is anchored to the cranium of the animal. As shown in paper IV of this thesis and in other publications from our group [63], [64], an ideal implant would be free to move with the brain parenchyma in order to minimize impact from the foreign body response. However, such a design would require incorporating a miniature scale telemetry system including a sufficient energy source small enough to fit inside the neurocranium of a rodent. To date, no such system exists, although fully integrated telemetric implants for larger animals and humans have been developed.

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