Lunds Tekniska Högskola

Master's Thesis

Brain Tissue Response to Implanted SU-8

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Abstract

Brain-machine interfaces are expected to play an important role in medicine – if a sustainable solution can be found – and so this study was performed to determine the biocompatibility of the photoresist SU-8 as well as a thin film needle design. This was done by histochemical staining with a collection of antibodies and mass spectrometry. The staining was performed on the perfused brains of four rats that had had cortical, contralateral implants for six weeks and of four rats with six week old stabwounds where the needle was retracted after two minutes. Histology showed a marked damage response in the controls as well as the implanted animals, implying that SU-8 affects the tissue in an unfavorable way, possibly by leaking into the surrounds of the implant. It may also be as a result of fragmentation of the material at retraction causing an implantation-like control series. The hypothesis of leakage is supported by mass spectrometry using MALDI-TOF MS that shows a noticeable amount of material leakage with masses separate from the used matrix. The amplification of GFAP in the controls on the other hand imply that the needle design causes a greater damage than expected. Gathered results heavily suggest that SU-8 needs a more extensive study to pinpoint wherein the trouble lies before one would be able to discard it as a possibility for future implants.

Sammanfattning

"Brain-machine interfaces" förväntas spela en viktig roll inom medicinen – om en hållbar lösning kan hittas – så denna studie utfördes för att bestämma biokompatibiliteten hos fotoresisten SU-8 samt en nåldesign för tunna filmer. Detta gjordes genom histokemisk infärgning med ett antal antikroppar och genom masspektrometri. Infärgningen utfördes på de perfunderade hjärnorna av fyra råttor som haft kortikala, kontralaterala implantat i sex veckor och fyra råttor med sex veckor gamla sticksår där nålen drogs upp efter två minuter. Histologin visade ett tydligt skadesvar hos de implanterade djuren, såväl som hos kontrollerna vilket antyder att SU-8 påverkar vävnaden på ett oönskat sätt, möjligen genom att läcka ut i vävnaden runt implantatet. Det kan också vara ett resultat av fragmentering av materialet vid utdragandet av nålen vilket skulle ge en implantationsartad kontrollserie. Läckagehypotesen stöds av masspektrometri med MALDI-TOF MS som visar ett märkbart material-läckage med massor som är skilda från massorna i den använda matrisen. Uppregleringen av GFAP i kontrollerna kan å andra sidan innebära att nåldesignen orsakar större skada än väntat. De samlade resultaten ger stora indikationer att SU-8 behöver studeras närmare för att fastställa vari problemet ligger innan det kan förkastas som möjlighet för framtida implantat.

Acknowledgements

First I would like to thank my supervisor Lars Wallman at the Dept. of Measurement Technol. and Industrial Electrical Engin., for taking in a third year masters student and then curling the way to a thesis that involved biological as well as technical sciences. Also, thanks to Cecilia Eriksson-Linsmeier who took up a similar flag at the Neuronano Research Center.

I would like to extend a thank you to my roommate and project surgeon Per Köhler for doing the parts I was unable to perform. Also for Tanja Jensen who had time to laugh at the pressure we were both subjected to, albeit to different degrees. To Fredrik Ejserholm who tried his best to get the needles done in time, despite broken down machinery. Simon Ekström and Belinda Adler for taking the time to help me with the mass spectrometry. To Agneta SanMartin for all the help in the lab, and of course a special thank you to every one else who answered my endless questions that I am sure was repeated many times.

And naturally a huge thanks to the rock in my life, my boyfriend Carl Wolff. For the unwavering support of my choice of career and the honest attempts at understanding the contents of my report.

Contents

Chapter 1

Introduction

Neural devices may come to play an important role in diagnostics and therapies for a number of clinical conditions such as chronic pain and an assortment of motor symptoms. They may also be used for control of prosthetic limbs[2, 5], increasing the life quality of many disabled patients.

The last ten years has shown a definite increase in deep brain stimulation (DBS) for treating advanced Parkinson's disease, leading to marked improvements in motor function. In addition there is a potential benefit of applying DBS treatment for other movement disorders like dystonia or Tourette syndrome, as well as for migraines, depression and obsessive compulsive disorder[4].

The cochlear implant[20], where electrodes are inserted into the cochlea, is a brain-machine interface (BMI) that has been in use for several years and is meant to stimulate the membrane of the inner ear in accordance to the registered frequencies gathered from a connected microphone. Clinical studies where tetraplegic patients are to move a computer cursor through a BMI has also been conducted with promising results[7]. Further, experiments on cats have show that, in the near future, it may be possible for non-congenital blind patients to regain some or most of their eyesight with the help of a BMI[16].

Electrodes intended for neural implants must however fulfill certain criteria. They should be long-term stable, biocompatible and be able to stimulate as well as record signals from multiple nerve cells. If these criteria are not met, the resulting inconveniences may indeed lower the subject's life quality instead of increasing it.

There are mainly three types of devices currently used in science: Michigan and Utah electrodes, and insulated wires. The Michigan electrodes are silicon or polymer based, where several surface electrode sites are spread along

the shaft. The electrodes may also be arranged into arrays to increase the number of electrode sites[22]. Utah electrodes are silicon needles, where the needles are arranged in a two-dimensional format and the tip of each needle is the electrode site[15]. The advantage of insulated wires is that they can be arranged manually in a three-dimensional pattern[14].

Most neural devices in use sadly have several shortcomings. They are relatively large, have a low spatial resolution and most troublesome of all is that they are not fully biocompatible thus having little chance of long-term stability. Studies investigating the response of reactive astrocytes to the insertion and continuous presence of an implant have shown that a persistent sheath of cells surround the insertion site after as little as 2 weeks, keeping the probe isolated from the brain[19]. In the light of these results it is apparent that new materials are needed to minimize this response and make chronic implantations possible if BMIs are to have any further impact on medicine.

The study will make use of a particular, flexible, multi electrode BMI specifically created for recordings in the cerebellum[11]. The array is made of SU-8, an epoxy-based negative photoresist, carrying gold micro electrodes. Because of their small recording areas, the resulting impedance characteristics would be too high if only gold was used. To lower the impedance, and to increase biocompatibility (and possibly trial time) through nano-porous surfaces[12], the electrodes are coated with either PEDOT, a conjugated polymer, or Pt-black, a certain kind of platinum.

This thesis will deal with the biocompatibility of the main material of SU-8 partly by analyzing tissue reactions to the implants, and partly by analyzing SU-8 after exposure to a brain-like environment.

Two animal test groups of 4 rats each, with one test area in each hemisphere will be the basis of the tissue study. The first group will merely be a control group where the needles are used to create stab wounds. This to compare the actual damage response to the possible reaction against the implanted SU-8 needles of the second group. The tissues will then be stained with a collection of antibodies to investigate the tissue response to the implants.

The biocompatibility of SU-8, a material that though frequently used, is not widely researched. To further investigate SU-8 and its usefulness in bio-implants the material will be subjected to different hard baking times to see if the material itself is affected, at any hard baking temperature, by the brain's environment and if there is any leakage of material.

Chapter 2

Materials and Methods

Studies investigating the response of reactive astrocytes to the insertion and continuous presence of an implant have shown that a continuous, albeit loosely organized, sheath of cells surround the insertion site at 2 weeks. By 6 weeks the surrounding sheath is highly compacted and continuous, thereby isolating the probe from the brain. The perpetual presence of the probe also appears to result in a sustained response that both produces and maintains this compact sheath, keeping the probe isolated from the brain[19].

To test whether or not the preferred base material of SU-8 has a negative/positive effect relative other materials, the tissues will be evaluated by staining after 6 weeks. It is not only the tissue however that may be affected; the epoxy might be affected by the exposure to the brain environment. To assess the stability of SU-8 in a brain-like environment it will be subjected to a saline solution at 37 °C and then studied using mass spectrometry.

2.1 SU-8

The needles that are to be implanted are made of SU-8, see Figure 2.1, which is an epoxy-based negative UV-patternable polymer that is well suited for thick-film applications as it can be spun onto disks at high concentrations while at the same time showing low absorbance to near UV-light. The result being that layers of a few hundred micro meters can be spin-coated and patterned without much trouble using ordinary UV-exposure systems[1].

Studies investigating the biocompatibility of the epoxy are so far scarce, since the practice of using it as a bio implant is relatively new. One study suggests that biocompatibility may be reasonably good, using *in vitro* experiments to investigate the material[8].

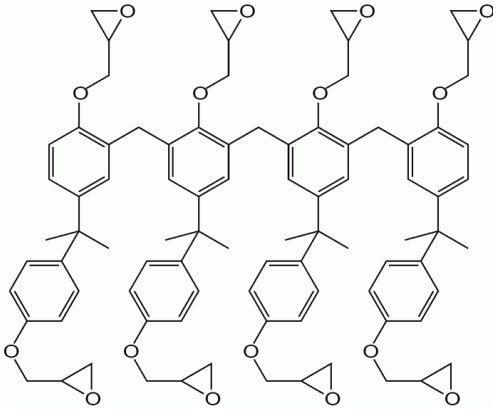


Figure 2.1: The molecular structure of SU-8

2.1.1 Fabrication

A thin layer of approximately 5 µm is spin coated onto an oxidized silicon wafer and then soft baked for 20 min at 90 °C. The wafer is then exposed to UV-light for 20 s through a mask to create the desired pattern, after which it is subjected to a post-exposure bake for 40 min at 90 °C. The wafer is developed using an SU-8 developer and since this developer is nonpolar, it is rinsed off using isopropanol. This is followed by a hard bake at 220 °C for 4 h where the temperature gradient is small to prevent tensions in the epoxy layer. To free the finished needles the silicon wafer is left overnight in hydrofluoric acid (1:5) and then rinsed several times with Millipore water. The freed needles, see Figure 2.2, are stored in ethanol until mounted. The exact measurements are displayed in Figure 2.3. The needles for mass spectrometry undergo the same protocol, but are freed after development to allow for different hard-bake temperatures.

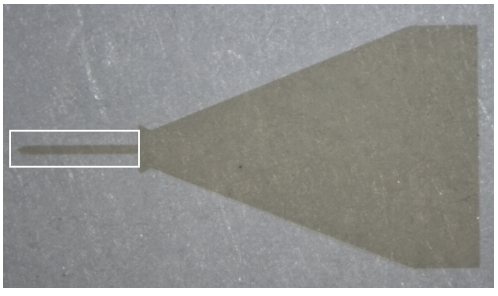


Figure 2.2: A freed electrode where the implantation part is marked in white

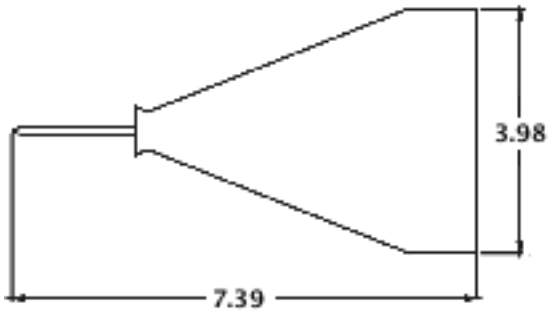


Figure 2.3: The needle design with measurements in millimeters

2.2 Mounting the needles

SU-8 is used partly because of its flexibility, but since the material is so flexible the needles are difficult to insert. Two strategies were evaluated: a gelatin coating which would give the needle a temporary rigidity, and a sucrose mounting of a wire for stability and penetration assistance.

2.2.1 Gelatin

Because the SU-8 is so flexible, the needle needed to be stabilized to be able to penetrate the brain surface. Pure gelatin would not do since it becomes soft and bendable as soon as it comes in contact with fluids. Instead the gelatin was cross-linked with EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) to create a stiff casing around the needle which would dissolve after implantation. It was empirically decided that a concentration of 0.1 mM would suffice.

The needles were first coated with a solution of 20 g gelatin and 80 g water heated to melt, by dipping them into the solution and then laid to dry horizontally. The gelatin was allowed to dry for 2 days. Cross-linking was started by making 10 mL of an ethanol (99%) and Millipore water solution of a 80:20 ratio mixed with 19.2 mg EDC to make a base solution of 10 mM. This was diluted to a concentration of 0.1 mM of which 1 mL was poured into a vial along with the gelatinized implants and incubated for 24 hours. The implants were then rinsed with a few milliliters of the original ethanol solution 3 times after which they were allowed to hang dry (to increase the possibility of straight, dry needles) for 48 hours.

This was rapidly discarded as a failure as, even with cross-linking, the gelatin proved too prone to water uptake and thus became pliant at contact with the cerebrospinal fluids (CSF). Another side effect of the cross-linking was the greater tensions in the material, causing the flexible needles to bend out of proportion, see Figure 2.4. Some were bent up to 180°, effectively eliminating approximately 90% of the gelatinized implants.

Figure 2.4: An electrode covered in gelatin

2.2.2 Sucrose

Since the SU-8 is not only flexible but also brittle, the base of the needles were encased in a piece of paper and set in a crocodile clip. With the aid of a microscope at 8 times magnification, a drop of sucrose solution (10 mL water and approximately 5.6 g sucrose, heated to 80 °C for a quick dissolution of the sucrose) was deposited onto the needle. In this case, the wooden part of a broken cotton swab proved useful to deposit the water based solution on the hydrophobic surface. A 5 mm long stainless steel wire of 50 µm width was carefully set in the drop of sucrose on the needle with a pair of tweezers and then its position modified to run parallel to the length of the needle, see Figure 2.5. The sucrose concentration allowed the solution some stickiness and therefore better adhesion to both wire and SU-8.

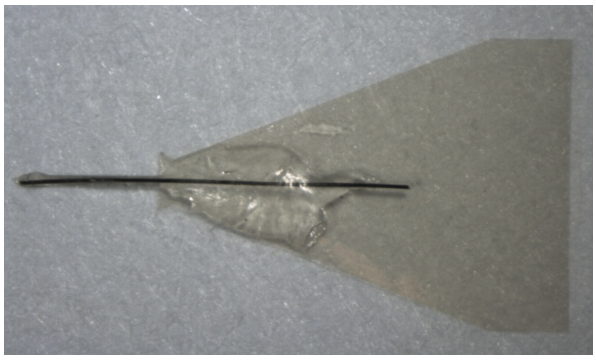


Figure 2.5: An electrode mounted with sucrose

2.3 The Implantation

The needles were implanted contralaterally in the primary motor cortex of the brain, one in each hemisphere, with the large areas facing one another, see Figure 2.6.

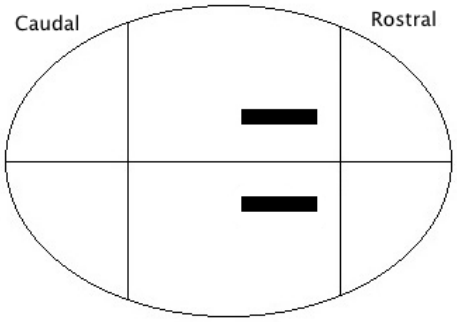


Figure 2.6: The basic model of implant placement

2.3.1 Animals & Surgery

Prior to surgery the animals were anesthetized by intraperitoneal (IP) injections of 6.3 mL/kg body weight of a mixture of Dormitor vet 1 mg/mL (medetomidin hydrochloride) and Fentanyl 5 µg/mL in 20:1 volume proportions.

The animals' heads were shaved and placed in a stereotaxic frame. An incision of approximately 3 cm was made and the skull bone exposed. Two

holes of about $0.5 * 1$ mm was drilled through the bone at the measurements of 1.5 mm laterally and 2.5 mm caudally, measured from bregma, see Figure 2.7.

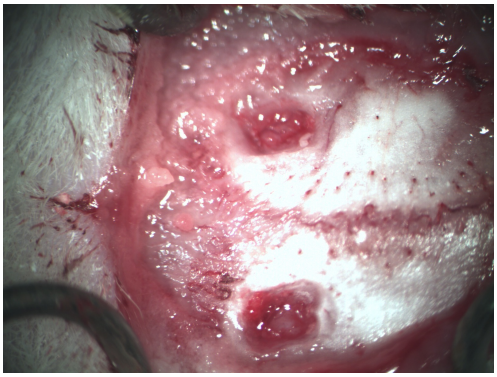


Figure 2.7: Holes drilled for implantation

The Dura Mater was removed from both sites and the needles were mounted on a micromanipulator to assure a straight alignment of the implant. When implant was confirmed, the protruding part of the needle was cut and the wire removed. The hole was then covered with bone cement, see Figure 2.8, fixing the needle for a tethered implantation, and the edges of the wound stapled together using surgical staples. For the control group, both wire and needle were left in for 2 minutes before retracted and the holes filled with bone cement.

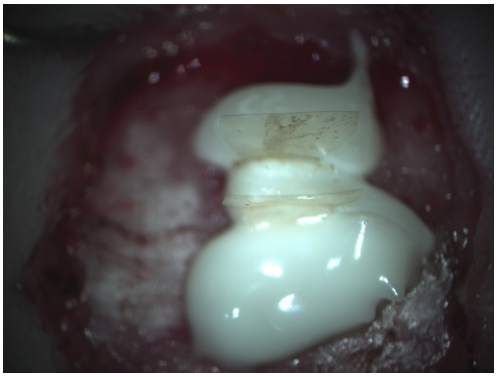


Figure 2.8: Implanted needles facing each other

After surgery the animals received subcutaneous injections of 1 mL/kg body weight of a mixture Antisedan vet 5 µg/mL (antipamezole hydrochlo-

ride) and sterile water, which serves as an antidote for the anesthesia. They also received analgesia subcutaneously.

2.4 Tissue Fixation

Tissues without life support start to decay rapidly unless they are stabilized in some way. In addition, any remaining blood in the tissue will create a highly unspecific staining. Which means that any study performed on such tissue will be basically impossible to gain any information from. Also, to see anything from the tissue it needs to be sliced into manageable sections. Animal tissues however are not very rigid and thus need to be hardened before any work can be done on them. That is why fixation is so important – it allows the experimenter a greater leeway with the tissues and makes it possible to perform the various tests needed to study the tissue on a molecular level. Paraformaldehyde (PFA) is a medium frequently used for tissue fixation.

2.4.1 Fixation Protocol

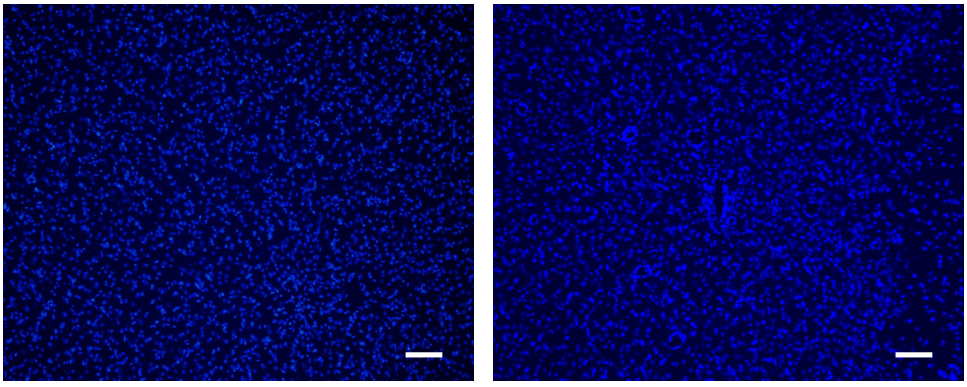
The animals were sacrificed after 6 weeks by an overdose of pentobarbital and then perfused transcardially with cold 4% PFA in 0.1 M phosphate buffered saline (PBS), pH 7.4. The brains were then dissected and submerged in PFA overnight, then cryoprotected in 30% sucrose until sunk to the bottom of the vial after which they were frozen using dry ice.

2.5 Histochemistry

The brains were serially cryosectioned horizontally in 30 µm sections by way of a microtome. The slides were then rinsed repeatedly in a 1:100 solution of PBS before blocking in goat serum to prevent unspecific binding. All samples were stained with DAPI through the coverslip solution *Vectashield*.

2.5.1 DAPI

DAPI (4',6-diamino-2-phenylindole) is DNA-specific and forms a fluorescent complex with A-T rich sequences in the DNA [10]. This makes it possible to visualize nuclear DNA in both living and fixed cells, making it feasible to count the number of cell nuclei, see Figure 2.9. Staining with DAPI also allows for multiple uses of cells thereby removing the need for duplicate samples.



(a) Uninjured tissue (b) Injured tissue

Figure 2.9: (a) and (b) shows the difference between normal and damaged tissue when stained with DAPI, scale bar at 100 µm

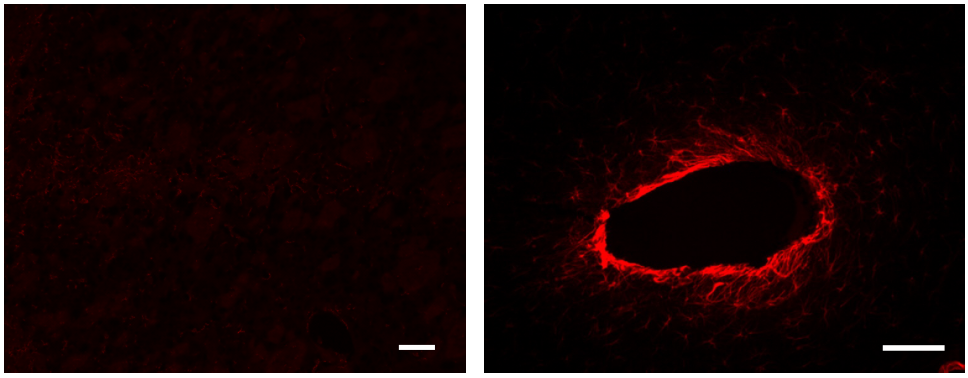
2.5.2 GFAP

Astrocytes (or astroglia) are characteristically star-shaped glial cells in the central nervous system (CNS). These cells carry out a number of duties, including biochemical aid of endothelial cells that form the blood-brain barrier, supply the nervous tissue with nutrients, maintaining the extracellular ion balance as well as the repair and scarring process of the CNS in response to trauma[18].

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein and is expressed specifically in astrocytes, satellite cells in peripheral ganglia, and in non-myelinating Schwann cells in the peripheral nervous system. Additionally, neural stem cells are known to express a large amount of GFAP. This makes antibodies against GFAP highly useful as markers of astroglia[23]. In Figure 2.10 the result of an injury compared to a healthy tissue is shown with GFAP stain.

2.5.3 ED1

Microglia can be found consistently throughout the CNS and accounts for up to 20% of the total populace of glial cells in the brain. Microglia exist mostly in a resting state but change into the activated state in response to a wide range of injuries. Activation occurs in a graded manner, in the first stage microglia becomes active but not phagocytotic. In a second stage microglia

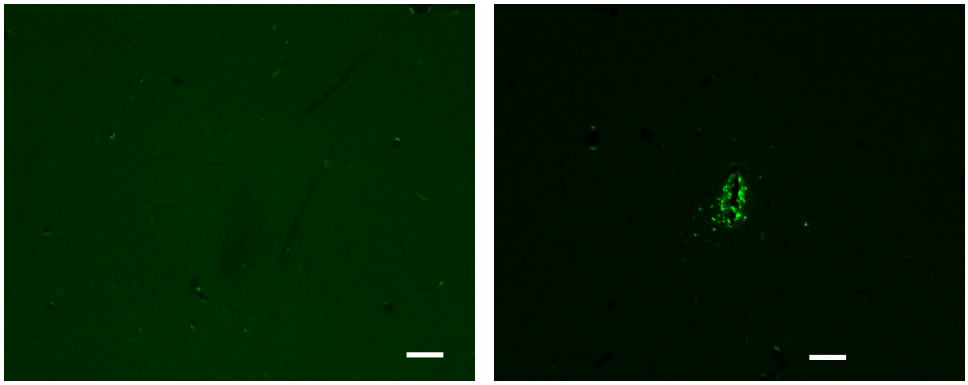


(a) Uninjured tissue (b) Injured tissue

Figure 2.10: (a) and (b) shows the difference between normal and damaged tissue when stained with GFAP, scale bar at 100 µm

undergoes a further transformation into phagocytotic cells[6] and engulf both foreign substances and dead cell waste.

The antibody ED1 (Ectodermal Dysplasia 1) recognizes a single chain glycoprotein, CD68 that is expressed chiefly on the lysosomal membrane of myeloid cells, i.e. young granulocytic cells. The antigen is expressed by the bulk of the macrophages (like microglia) in tissues and faintly by peripheral blood granulocytes[24]. The different responses are shown in Figure 2.11.

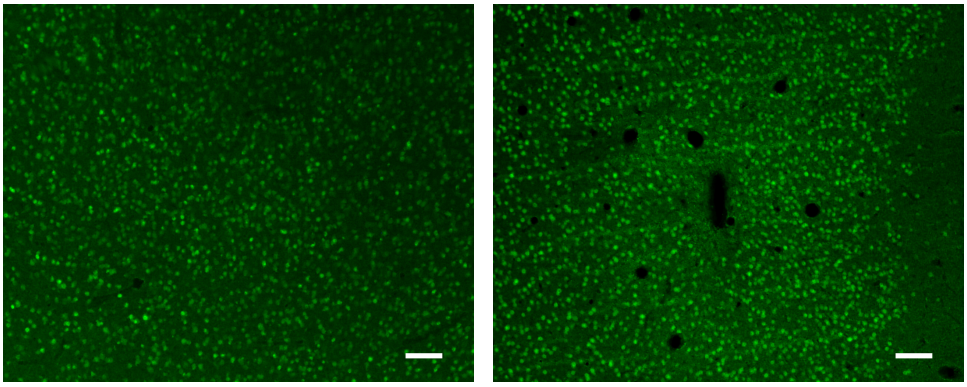


(a) Uninjured tissue (b) Injured tissue

Figure 2.11: (a) and (b) shows the difference between normal and damaged tissue when stained with ED1, scale bar at 100 µm

2.5.4 NeuN

The protein *neuronal nuclei* is expressed almost exclusively in the nervous system. It surfaces early in development and stays into adulthood. The expression of NeuN is limited to neurons and is type-specific to this kind of cell[13], thereby making antibodies against NeuN an effective way to stain neurons for counting. Figure 2.12a shows an example of uninjured tissue stained with NeuN and Figure 2.12b shows an injury stained with NeuN.



(a) Uninjured tissue (b) Injured tissue

Figure 2.12: (a) and (b) shows the difference between normal and damaged tissue when stained with NeuN, scale bar at 100 µm

2.5.5 Oligodendrocytes

Oligodendrocytes are a variety of neuroglia whose main function is to insulate the axons in the CNS. One oligodendrocyte can extend to 50 axons, wrapping myelin of approximately 1 µm in sheets around each axon. The CNS depends heavily on the presence of myelin sheets since they radically increase impulse speed through the saltatory propagation of action potentials at the nodes of Ranvier. Myelin also decreases ion leakage thereby lowering the capacitance of the cell membrane. A normal tissue stained for oligodendrocytes is shown in Figure 2.13.

2.5.6 Staining protocol

The sections were incubated overnight with primary antibodies against GFAP (1:5000) coupled with antibodies against CD68 (1:100), NeuN (1:100) or oligo-

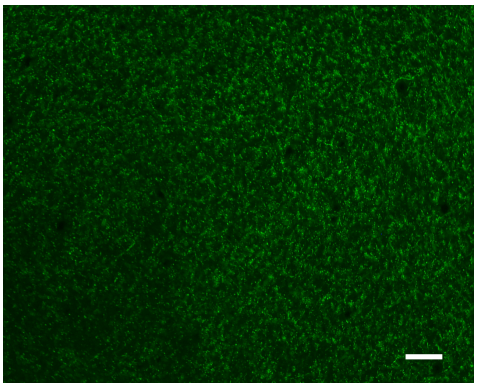


Figure 2.13: Uninjured tissue stained with antibodies against oligodendrocytes, scale bar at 100 µm

dendrocytes (1:750) and then rinsed repeatedly in a 1:100 PBS solution (tris-buffered saline in the case of oligodendrocyte staining). They were then incubated for two hours with Alexa594 goat-anti-rabbit and Alexa488 goat-anti-mouse (1:500) after which they were mounted onto glass slides and cover-slipped with Vectashield mounting media.

2.6 MALDI

Mass spectrometry can be used to determine the mass of volatile gasses and liquids by assessing how readily an ion is accelerated in an applied electric field. Since the force of the field is a known parameter, and the acceleration of the ion is measured, the mass is easily calculated using Newton's third law, $F = ma$. This is not applicable on peptides or proteins however, since they are not considered volatile. The same can be said for polymers. Instead, one must use for example the matrix-assisted laser desorption-ionization (MALDI) method. In the MALDI method the material of interest (be it peptide, protein or polymer) is coprecipitated with an organic compound that absorbs laser light of a specific wavelength. This compound is known as the "matrix". The flash of a laser on the sample expels molecules from the surface, which then capture electrons as they exit the matrix, thereby leaving negatively charged ions in their wake.

When gas-phase ions have been generated it is possible to determine their mass by performing a *time of flight* (TOF) analysis. In a TOF analysis, the ions are accelerated in an electric field toward a detector, much as in a

conventional mass spectrometer. The lighter ions will get a higher acceleration and thus a greater speed resulting in their quicker arrival at the detector after which, again, Newton's third law is used to calculate their mass[3].

2.6.1 ISET

Integrated selective enrichment target (ISET) is a downscaled sample processing platform for MALDI. It supplies a simplified solution to enrichments, cleanups and presentations of samples used in MALDI. The intrinsic function of this platform is to ensure a focusing effect of the sample that ultimately results in an amplification of the MALDI read-out signal. The ISET plate is both sample treatment tool and MALDI target and its surface is perforated with an array of nanovials that can be filled with reversed-phase beads. This way, the amount of sample transfers and the overall surface area free for unwanted adsorption of analytes is kept to a minimum and thus provides a high-sensitivity of the analysis[9].

2.6.2 Preparing the needles

The material of SU-8 was investigated by subjecting the photolithographically patterned needles to different hard-bake temperatures, since it is not known if the material is stable enough to be used as an implant medium. For this particular kind of needle it is preferable to have as supple a needle as possible, which translates to a hard-bake temperature that is as low as can be. The needles were baked for 2 hours in 7 different temperatures after which they were incubated for 1 week in a saline solution to mimic the cerebrospinal fluid of the brain. The temperatures for hard bake were: 90 °C, 125 °C, 150°C, 175 °C, 200 °C, 220 °C (which is the tabulated glass transition temperature for SU-8) and 250 °C.

After 1 week in a saline solution, it was assumed that any leakage that could occur had done so and the resulting solution was subjected to a MALDI test.

2.6.3 MALDI protocol

Two needles from each temperature group were placed in a glass vial previously cleaned with methanol and then rinsed with a saline solution (18 mg/mL). 1 mL of the same saline solution was added to the vial after which the needles were incubated for 1 week in 37 °C.

1.5 µL beads were added to an Eppendorf tube along with 5 µL of the needle-incubated saline solution. Approximately 15 min was deemed sufficient

for the beads to bind to any molecules in the solution. 4 similar tubes were made for each hard-bake temperature as well as 3 controls containing only beads. The entire volume of each tube was deposited into a nanovial on an ISET plate connected to a vacuum system, and then rinsed twice with 3 μL 0.1% trifluoroacetic acid and water. The ISET plate was dried off, put back in place and each vial was eluted twice with 0.4 μL matrix. The ISET was then investigated with MALDI.

Chapter 3

Results and Discussion

There does not seem to be a consistent difference in damage response between the rostral and the caudal directions. The samples that indicate any difference seem to have a greater GFAP response in the rostral than in the caudal direction. This kind of damage has been noted in previous studies[17] but the cause is yet to be explained.

The implant holes have been measured to be in the vicinity of 100 µm long and 25 µm wide. Needle parameters are: 100 µm wide and 5 µm thick. This is not necessarily due to the implant or its movements since the implantation was aided by a 50 µm thick wire. Insertion of this notably larger combination creates a larger wound and could be the cause of the larger width.

3.1 Histochemistry

From the histochemical staining, some results are clear while some are merely hinted at. None of the results achieved in this report are statistically viable.

3.1.1 GFAP

GFAP is markedly amplified in a consistent manner throughout both the control group and the 6 week group, see Figures 3.5 and 3.4. The reason for this is, as of yet, unknown but is believed to be caused by the implantation procedure as well as the design of the implants. The theory is that the needle is too sharp, causing it to slice through the brain tissue instead of pushing it to the sides as other, larger implants are thought to do. By doing this, these needles might cause so much damage that the response is sustained throughout

the 6 weeks of the study. To know for sure a wider range of timeframes is needed.

The presence of cells stained against GFAP shows the formation of a glial scar which, obviously, is something unwanted as this is one of the possible reasons for signal degradation. In Figure 3.1 it is apparent that the implant has caused some glial scarring.

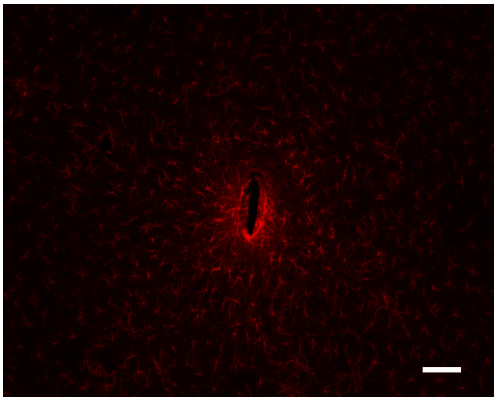


Figure 3.1: Showing a typical glial scar found in the study, scale bar at 100 μm

One way to possibly avoid this slicing behavior would be to produce a globule of gelatin to attach to the tip, making it blunter. Another could be to make the needle thicker, thus expanding the area penetrating the tissue and spreading the insertion force. However, the second suggestion would merely be for studying the damage response, since the needles meant for data acquiring need to be as small, and as flexible as possible. If one looks for a more permanent solution, the first suggestion would be applicable.

3.1.2 NeuN

If one looks closely at the images and put together a series comparing the GFAP and NeuN stains, one becomes privy to another pattern forming around the implant wounds. There appears to be a small area circling the wound where neurons seem to become scarce, see Figure 3.2.

Although this does not seem to be very widespread, and in some cases, neuronal cell bodies can be found in very close proximity to the implant edge. The neuronal death is something the research groups strive to prevent, through needle design and implantation method. It was hoped that this particular needle design would cause so little damage that neuronal cell death would be

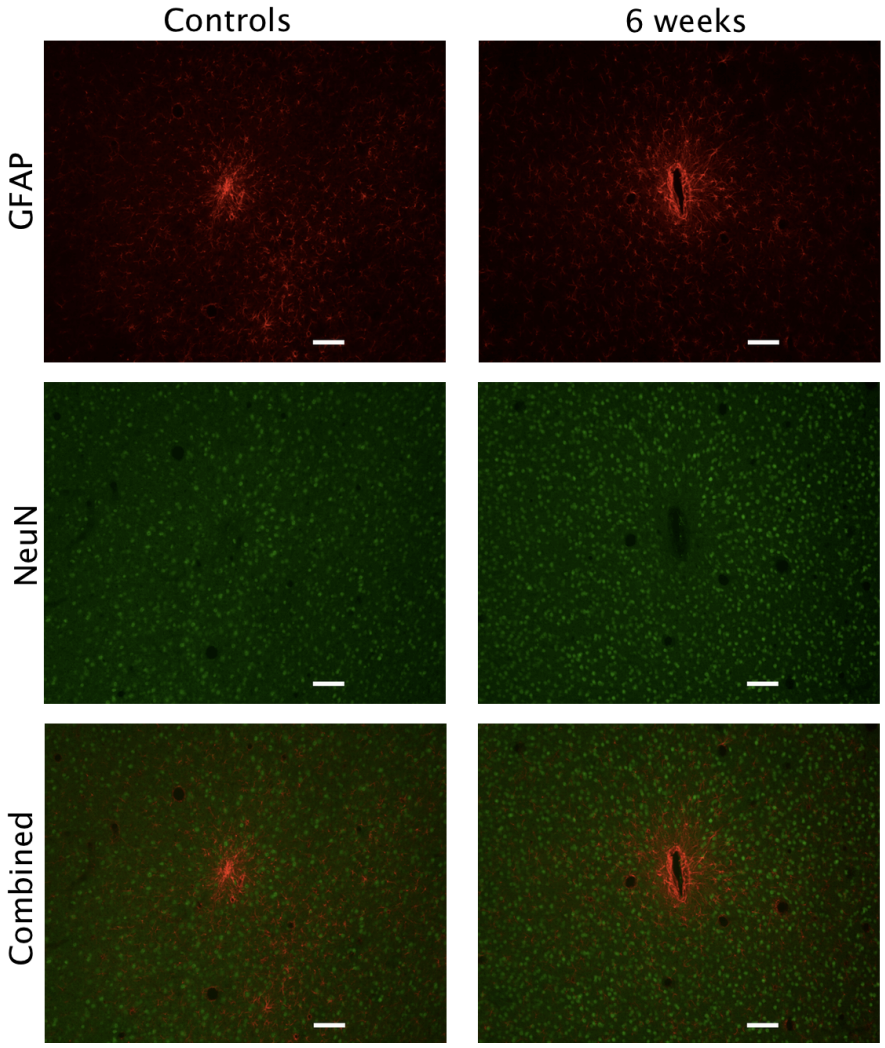


Figure 3.2: Comparing a 6 week implant with the control using NeuN and GFAP, scale bar at 100 µm

limited. However, one must also remember the odd response of GFAP in the controls, indicating a sustained damage response, perhaps the same thing is true for neurons and they were unable to cope with the trauma of insertion. Another element to consider would be the fact that the needle is meant to be rotated 90° and that this way may be preferable when it comes to cell survival since the largest motions of the brain are in the rostral and caudal directions.

For some implantation sites, see Figure 3.3, the characteristic halo of neurons can be guessed at. This has been noticed for other implants in previous studies[17]. It does seem however, that the resulting halo in this study is less pronounced and has a much shorter span than previously seen for tethered implants. It also is not clear for all implants, and can not be seen at all in the controls, suggesting a small impact on cellular orientation. No independent sources were asked to check the viability of this theory through a count, neither has any statistical calculations been performed to prove it. This will however be a necessary precaution in the follow-up study to be sure of statistical significance of the find.

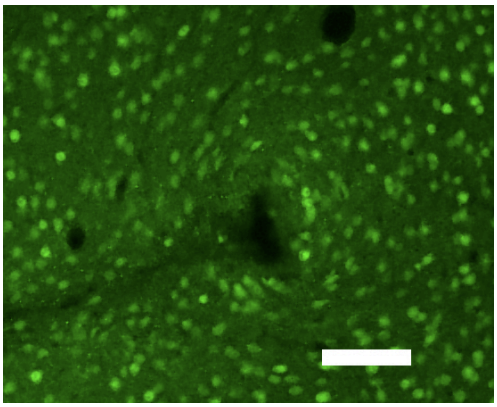


Figure 3.3: An implant site showing the typical halo-effect of neuronal cell bodies, scale bar at 100 μm

3.1.3 ED1

The microglia response does not look promising either. There is an unmistakable reaction against the implants. If one looks at Figure 3.4, it shows an active phagocytosis indicating a sustained attack against a foreign substance (the SU-8). This conveys the impression that the immune system of the brain does not approve of the substance it is exposed to or that the needles have created a hole in the blood-brain barrier. If the blood-brain barrier has been compromised, macrophages from outside of the brain's environment could migrate to the damage site and try to rid the brain of the contamination.

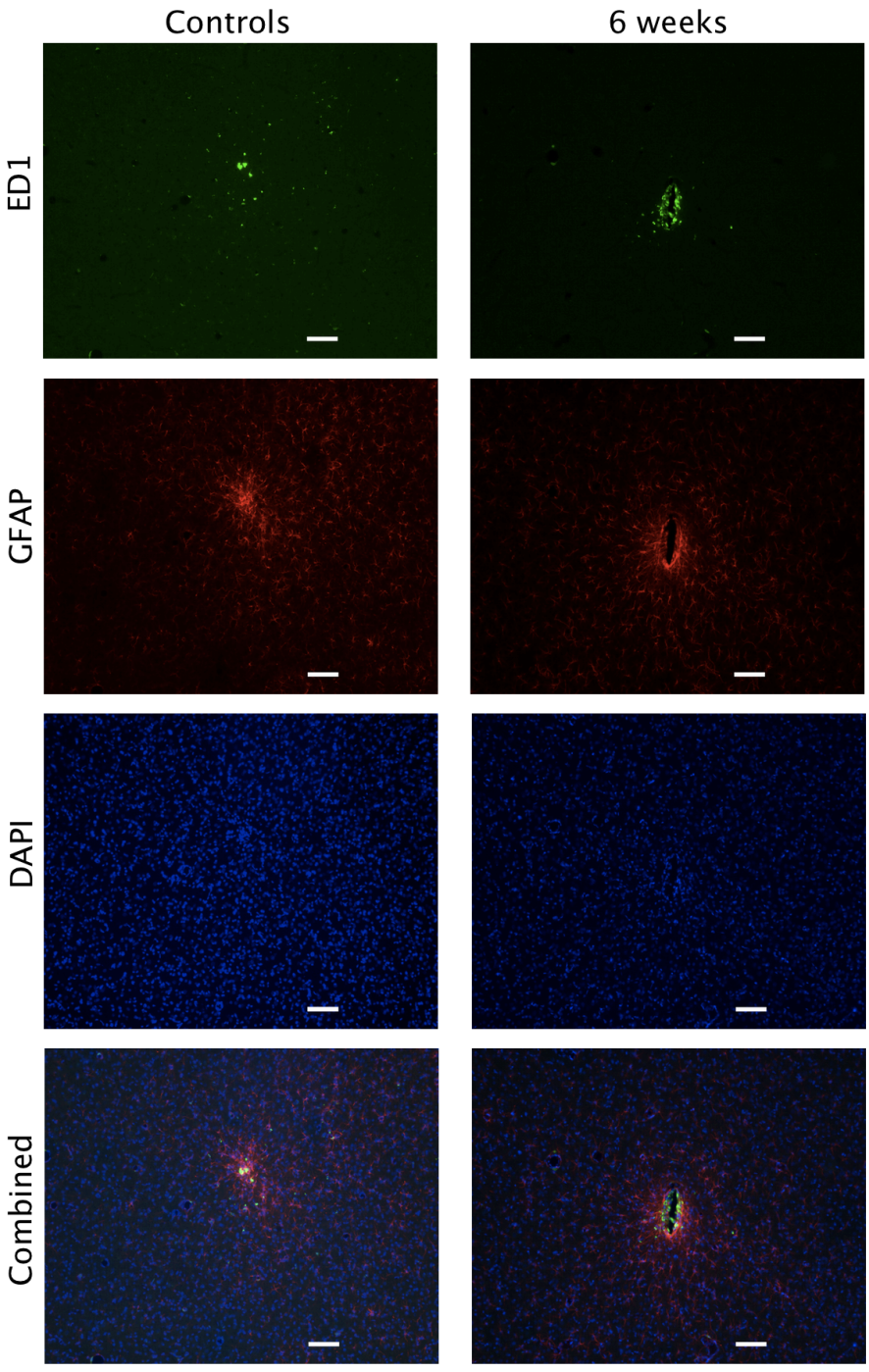


Figure 3.4: Comparing a 6 week implant with the control using ED1, DAPI and GFAP, scale bar at 100 µm

3.1.4 Oligodendrocytes

It has been suggested that the reason for the signal degradation that occurs in neural implants may be caused by a reduction of myelin in the proximity of the implant[21]. A down regulation of myelin would indicate that the implant has an effect on either the oligodendrocyte cell body or its ability to envelop neural axons. This would cause a reduction in signal propagation through the destruction of the saltatory pathway but may be a result of axonal death of the neurons. If this is the case, neuronal cell death is the greatest problem to address when redesigning the implants and insertion method. Regardless, this has not been noted in any of the sections produced in this study, see Figure 3.5,

3.1.5 Special images

A few sections separate themselves from the others through their sheer deviation from the pattern seen previously. For some, the reason can be passably deduced while others are subject to wild speculation.

More Damage Zones

Some samples seem to have multiple damage zones: a stab wound and an implantation wound for the 6 week animals and double stab wounds for the controls, see Figure 3.6. This may be caused by the use of the stabilizing wire used for insertion. Sucrose is not the most resilient glue, but holds things together for a sufficient amount of time (the time it takes to penetrate the brain surface). The effect may be that the wire separates from the needle during insertion and so creates a separate stab wound.

Mirror images

In one of the section series, a strange occurrence is clearly visible. What looks like a mirror image of the same damage linked by some kind of canal, see Figure 3.7.

The cause of this appearance is unclear, but since there is a tissue response around both sites it is evident that the damage was not caused by the retraction of the needle. Neither does it seem as if, at implantation a double wound was created in the manner explained above since the cell response does not resemble that of the controls, see Figure 3.6. In light of this reasoning, coupled with the suspicion that the SU-8 fragments sometime during the study, the

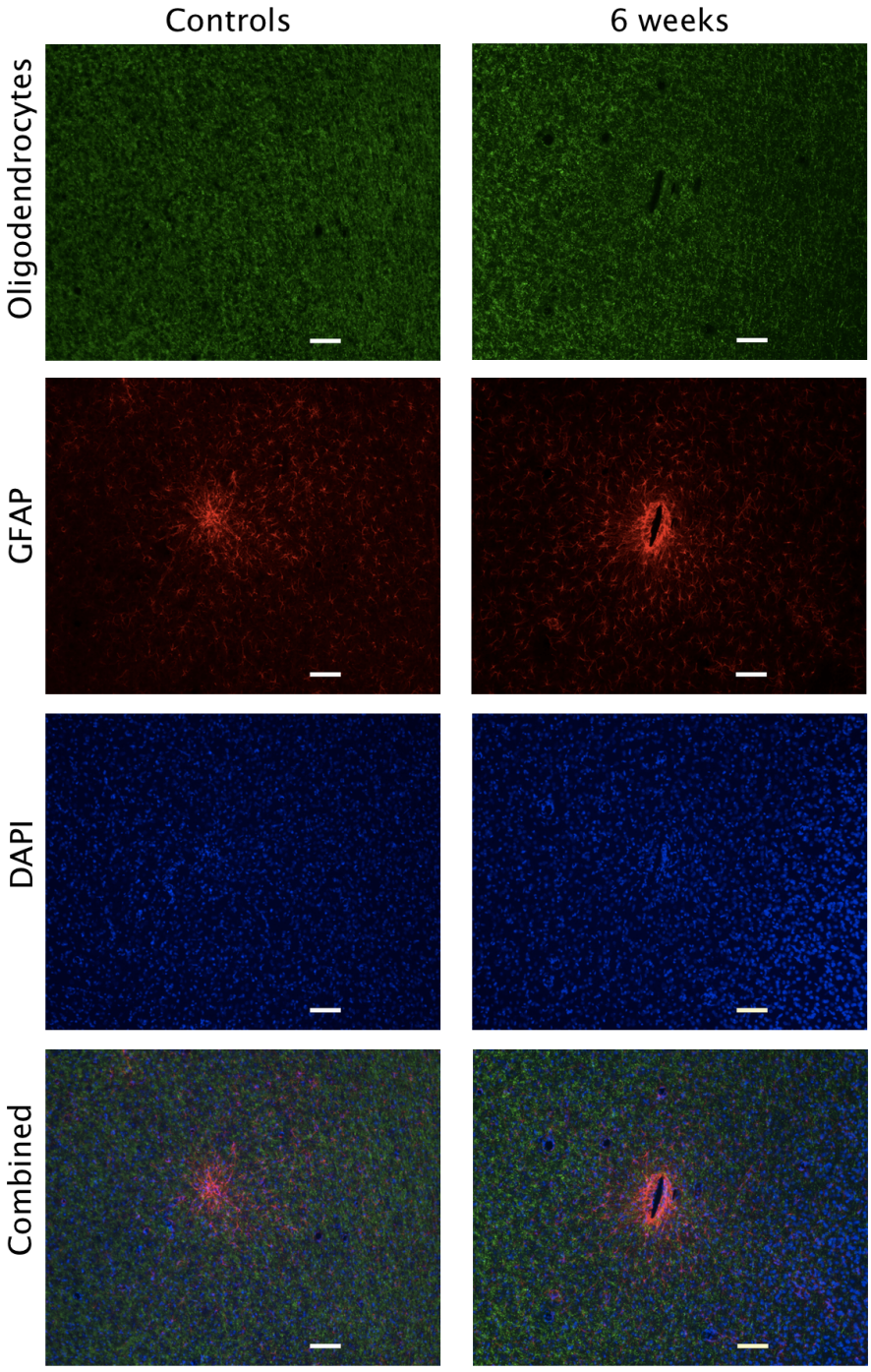


Figure 3.5: Comparing a 6 week implant with the control using an anti-oligodendrocyte stain, DAPI and GFAP, scale bar at 100 μm

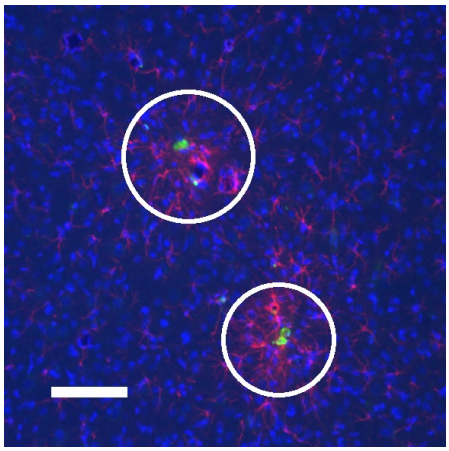


Figure 3.6: A control section showing a suspected double wound, scale bar at 100 µm

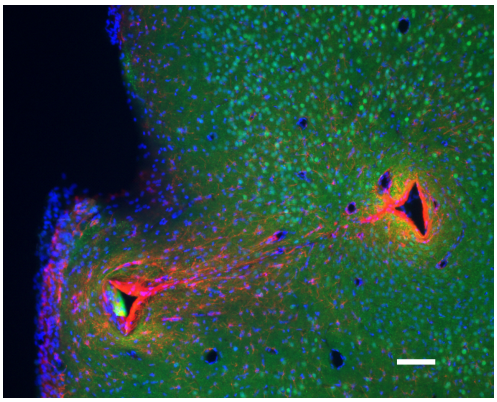


Figure 3.7: One of the sections stained for DAPI, GFAP and NeuN, scale bar at 100 µm

explanation that comes to mind would be that the needle fractured lengthwise during insertion, producing multiple damage zones.

Contaminants

When the needle is retracted from the perfused brain, it is not a precise art. Therefore the needle may fracture upon removal thus leaving fragments of SU-8 behind. During the 6 weeks' incubation period, some cells may have attached to the surface of the needle. They may then have been ripped out along

with the needle after perfusion, effectively removing tissue from the edges of the would-be cell response causing a thoroughly asymmetrical appearance of the wound, especially of the GFAP. These observations may at least partly be the explanation of the outlook of Figures 3.8 and 3.9, where the tissue was stained for GFAP, DAPI and ED1.

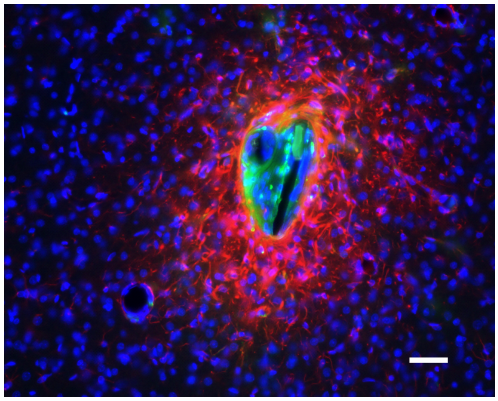


Figure 3.8: Suspected SU-8 contamination, scale bar at 100 µm

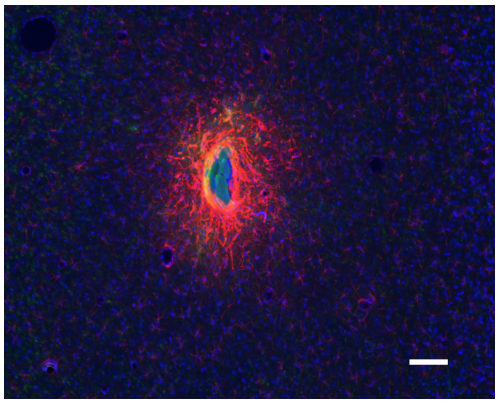


Figure 3.9: Suspected SU-8 contamination, scale bar at 100 µm

Strange ED1 Response

In a few cases the response of the microglia can be seen several hundred microns from the damaged area in a seemingly random manner, see Figure 3.10.

The cause of this is as of yet unknown, but may be related to the leakage of material from the epoxy.

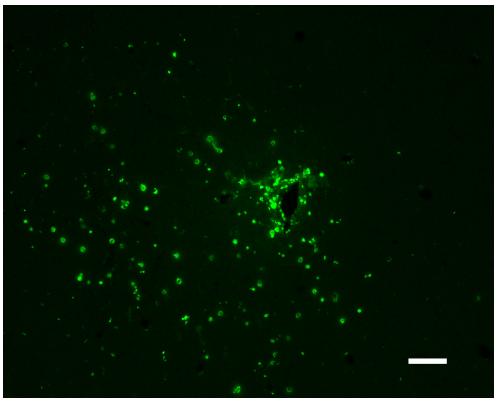


Figure 3.10: Strange microglia occurrence, scale bar at 100 µm

Blood Vessels

For all the sections there seems to be an increase in number of blood vessels something that could, again, be caused by the material leakage from the needles since it does not appear in the controls. For one implantation this seems extra clear as the presumed blood vessel is nearly twice as large as the implantation wound, seeFigure 3.11.

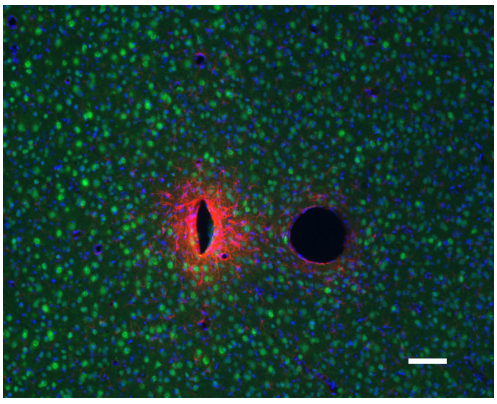


Figure 3.11: A suspected extreme blood vessel, scale bar at 100 µm

This extremism is not seen anywhere else, and is found relatively deep in the brain without any indication at the top layers of the sections.

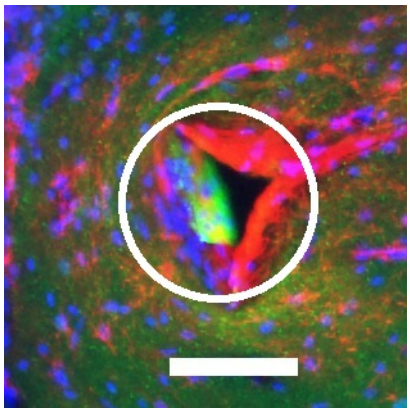
3.1.6 General Discussion

One gathers that SU-8 might not be as biocompatible as earlier studies have implied[8]. From the tissue sections examined histochemically, one can see suggestions that SU-8 might affect the tissue in ways that have not previously been considered. For instance, the epoxy might fragment already at insertion due to its inherent fragility, causing the prolonged damage response we see in the controls. Since amplification in GFAP response seems to happen for both the controls and the chronic implantations, the cause can be either a leakage of polymers from the SU-8 or fragmentation of the needle sometime during implantation. Although, for the controls, the needle is only left in the tissue for two minutes making the leakage an improbable cause, it is an interesting path to investigate. The leakage theory is strongly supported by mass spectrometry, but may only be relevant for the chronic implants. A theory that would fully support the increase of activated astrocytes in both groups would be that the needle design creates a wound that is so severe that the response to insertion can be seen for an extended time.

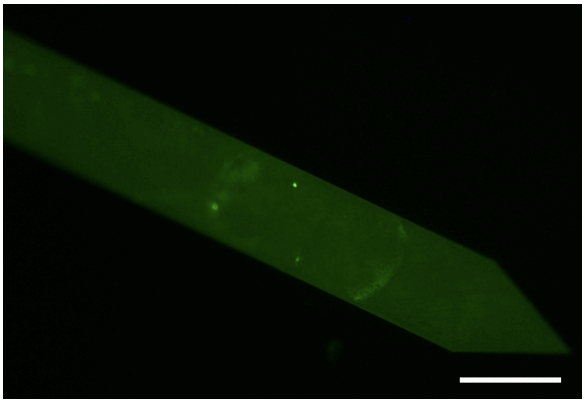
3.1.7 Needle

SU-8 autofluoresce at 356 nm and so should not be seen with the wavelengths used for the analysis of the histochemical staining (488 nm and 594nm). It seems however that the material binds to the ED1 antibodies and therefore fluoresce at the wavelength intended for microglia. This is hinted at in some sections, see Figure 3.12, where it is believed the needle has shattered sometime during insertion or retraction.

A quick test, where the needles were subjected to a similar staining protocol as described in Section 2.5.6 for ED1, was performed. It showed a tendency in SU-8 to bind to the antibodies, thereby fluorescing at the same wavelength as ED1 and NeuN, instead of staying dark as it should at that wavelength, hence its appearance in the images. The result of the staining can be seen in Figure 3.12b. Although it does not perfectly match the amount of fluorescence seen in the stained sections, it is an indication that the aberrations seen in the section may be due to SU-8 fragments.



(a) The circle indicates the possible contamination, scale bar at 100 µm



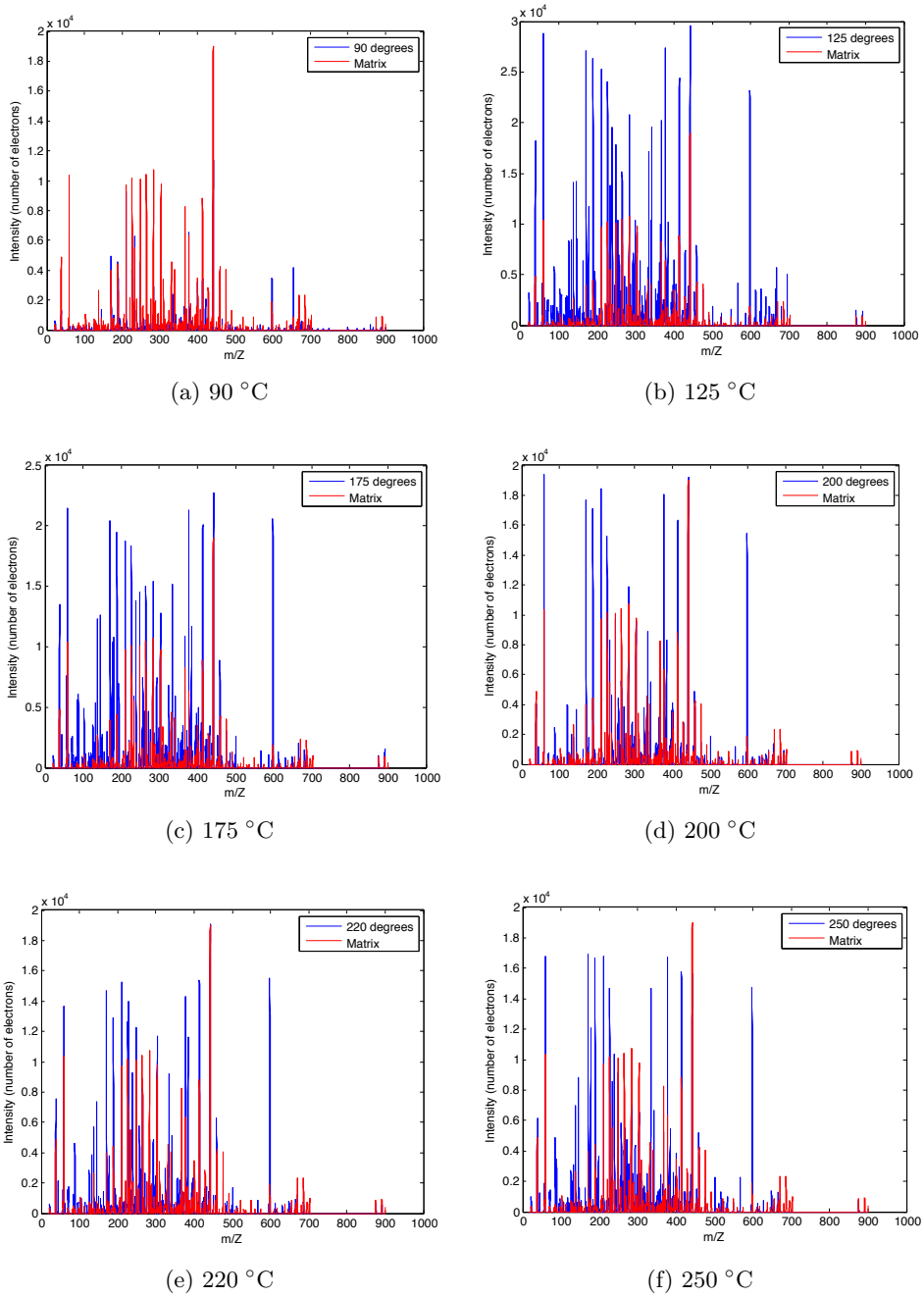
(b) Needle stained with ED1, scale bar at 100 µm

Figure 3.12: Images showing the possibility that SU-8 becomes fluorescent after a normal staining procedure

3.2 MALDI

The different mass spectra have been compared to the same matrix, thereby giving an approximation of material leakage for each hard-bake temperature, see Figure 3.13. It is obvious that some parts of the epoxy diffuse into the saline and from the broad range of different masses it would seem that the SU-8 is broken up into many different parts after hard-bake. The most striking difference is that between Figures 3.13a and 3.13b where it seems that the material undergoes a profound change, causing a great deal more contamination at 125 °C than at 90 °C. It is also apparent that no significant change takes place at the glass transition temperature (Figure 3.13e) as there is just as much leakage below it, see Figure 3.13c, and above it, see Figure 3.13f.

The result of this testing is of course not conclusive in the least as we do not know what materials have diffused and if they come from the SU-8 or if the samples were contaminated in any way. To be able to say anything definitive, the SU-8 would need to undergo a more thorough mass spectrometry where the elements released are mapped and where an attempt at identifying the contaminants are made. According to MicroChem the resin also contains a small amount of hexafluoroantimonate salt which is very harmful to the tissue. To ascertain whether this salt is washed away as it is assumed to be during one of the many rinses, a thorough analysis is even more necessary.



(a) 90 °C (b) 125 °C

(c) 175 °C (d) 200 °C

(e) 220 °C (f) 250 °C

Figure 3.13: Difference in leakage as a result of hard-bake temperature compared to a matrix

Chapter 4

Conclusions

None of the results given in this report have statistical significance, since only four animals were used for each group. The study was more intended as a pilot study for a more expansive investigation of the SU-8 needles. No definitive conclusions may therefore be drawn. However, the study suggests that SU-8 may not be as ideal as it was previously considered.

4.1 Limitations

If this study is to be believed, SU-8 is not the smash hit it was expected to be. According to the MALDI spectra, the needles may pose a threat to the sensitive environment they are meant for by releasing too many contaminants. This theory is supported by the unexpected upregulation of GFAP that can be seen through the entirety of the brain. However, if one looks at the controls, where the tissue was only exposed to the epoxy for approximately 2 min, one sees the same kind of upregulation of GFAP, indicating that it might not be due to material leakage. Instead it may be because of the slicing effect of the sharp tip, or because some SU-8 was left behind after needle retraction. None of this can be deduced from the performed tests documented in this thesis and so new tests need to be designed for this purpose.

4.2 Future

If the implant material is nontoxic, the acute reaction should have decreased to a level where stability is reached by 12 weeks. Therefore, this study cannot claim to investigate the permanent tissue response evoked by these implants. To simulate a more permanent implantation longer time frames are essential

but are beyond the scope of this thesis. However, we saw amplification tendencies of GFAP even in the control group which speaks of a sustained injury response. This may be due to the design of the needles, as they are very sharp, which could result in a more damaging cutting of tissue than other designs where the needle merely pushes the tissue out of harm's way.

The epoxy used in this study is being used in both acute and static trials by several different groups but seems to be harmful to the tissue. This makes it important to explore the material further, both by performing more detailed mass spectrometry and by doing more extensive histochemistry. It would also be necessary to look at the intended implantation direction of the needles, since they are made flexible to follow the brain's movements. A rotation of the needles of 90° would correspond more closely to the flow of the tissue and would hopefully lessen the damage.

It is preferable to have the needle as small and thin as possible. But if it is so that the needle is indeed so sharp it causes cut wounds in the tissue, one solution might be to return, at least partly, to the gelatin solution. If a drop of gelatin is deposited at the tip of the needle, this might suffice to blunt the tip temporarily, creating the pushing motion instead of the possible cutting and thus causing less damage.

To evaluate the histochemistry further one might also look at the possible amplification of blood vessels in the vicinity of the implant and what they contain by using an antibody against VEGF which is a growth factor active in, among other things, vasculogenesis and endothelial cell growth. It also induces permeabilization of blood vessels. There is also the question of leakage and if this would affect the blood vessels in the vicinity. This can be investigated by using IgG antibodies which are found in the body fluids of an animal, and any finding of this in the brain tissue would intimate a leakage from the blood vessels.

Since SU-8 appears to bind to certain antibodies, the material needs to undergo more testing in this area as well, to see if it does indeed stain. Another thing to factor in would be the environment to which the needles have been exposed. The needles that underwent the ED1 stain protocol were completely dry (lying in a petri dish for approximately 14 weeks) whilst the suspected SU-8 in the sections was not allowed to dry out after insertion. Further investigation of this disparity would be interesting, but not necessary.

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Marker-ab10062.html

[24] http://www.abcam.com/CD68-antibody-ED1-ab31630.html

Abbreviations

Blood-brain barrier	A separation of circulating blood and the brain extracellular fluid in the CNS
BMI	Brain-machine interface
Caudal	The posterior part of the body
CNS	Central nervous system
DAPI	A fluorescent stain that binds to DNA (detects cell nuclei)
DBS	Deep brain stimulation
ED1	A membrane-bound antigen (detects microglia)
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, used for cross-linking
GFAP	Glial Fibrillary Acidic Protein (detects astrocytes)
IP	Intraperitoneal injection (injection of a substance into the peritoneum (body cavity))
ISET	Integrated selective enrichment target
MALDI	Matrix-assisted laser desorption-ionization
Matrix	Organic compound, absorbs laser light of a determined wavelength, used in MALDI
Millipore	High purity water
NeuN	Antibodies directed against neural nuclei (detects neurons)
PFA	Paraformaldehyde a fixing solution used for perfusion
PBS	Phosphate buffered saline, a water-based salt solution
Rostral	The anterior part of the body
SU-8	An epoxy-based negative photoresist

Used Chemicals

Triton X-100	Sigma T-8787
Normal Goat Serum	Vector S-1000
Primary polyclonal antibody Rabbit-anti GFAP	DAKO Z0334
Primary monoclonal antibody Mouse-anti NeuN	Millipore MAB377
Primary monoclonal antibody Mouse-anti-rat CD68	Serotec MCA341R
Primary monoclonal antibody Mouse-anti-oligodendrocytes, IgM, clone CE1	Millipore MAB328
Secondary antibody Alexa Flour 594 Goat-anti-rabbit	Invitrogen A11037
Secondary antibody Alexa Flour 488 Goat-anti-mouse	Invitrogen A11029
Vectashield with DAPI, mountingmedium	Vector H-1500
Acetonitrile	
SU-8 2005	Microchem Y111045
Developer mr-Dev 600	Micro Resist Technology GmbH R815100
Isopropanol	
Hydrofluoric acid	
Trifluoroacetic acid	
CACH Matrix	