Published in final edited form as:

J Neurosci Methods. 2013 August 15; 218(1): . doi:10.1016/j.jneumeth.2013.06.001.

A cranial window imaging method for monitoring vascular growth around chronically implanted micro-ECoG devices

Amelia A. Schendel¹, Sanitta Thongpang¹, Sarah K. Brodnick¹, Thomas J. Richner¹, Bradley D.B. Lindevig¹, Lisa Krugner-Higby², and Justin C. Williams¹

¹Department of Biomedical Engineering, University of Wisconsin-Madison, 1550 Engineering Drive Madison, WI, USA 53706

²Department of Surgical Science, University of Wisconsin-Madison, 2015 Linden Drive Madison, WI, USA 53706

Abstract

Implantable neural micro-electrode arrays have the potential to restore lost sensory or motor function to many different areas of the body. However, the invasiveness of these implants often results in scar tissue formation, which can have detrimental effects on recorded signal quality and longevity. Traditional histological techniques can be employed to study the tissue reaction to implanted micro-electrode arrays, but these techniques require removal of the brain from the skull, often causing damage to the meninges and cortical surface. This is especially unfavorable when studying the tissue response to electrode arrays such as the micro-electrocorticography (micro-ECoG) device, which sits on the surface of the cerebral cortex. In order to better understand the biological changes occurring around these types of devices, a cranial window implantation scheme has been developed, through which the tissue response can be studied *in vivo* over the entire implantation period.

Rats were implanted with epidural micro-ECoG arrays, over which glass coverslips were placed and sealed to the skull, creating cranial windows. Vascular growth around the devices was monitored for one month after implantation. It was found that blood vessels grew through holes in the micro-ECoG substrate, spreading over the top of the device. Micro-hematomas were observed at varying time points after device implantation in every animal, and tissue growth between the micro-ECoG array and the window occurred in several cases. Use of the cranial window imaging technique with these devices enabled the observation of tissue changes that would normally go unnoticed with a standard device implantation scheme.

Keywords

Micro-Electrocorticography; Tissue Response; Brain Computer Interfacing; Vasculature; Neural Electrode; Cranial Window

1. Introduction

The formation of scar tissue around chronically implanted neural micro-electrode arrays can significantly decrease the quality of the recorded signals, often rendering the devices

© 2013 Elsevier B.V. All rights reserved.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

unusable (Szarowski et al., 2003; Williams et al., 1999; Woolley et al., 2011). This well-known problem has led to a push towards less invasive neural implants, such as electrocorticography (ECoG), and more recently, micro-ECoG (Figure 1), which sit on top of the cortical surface rather than penetrating into it (Gierthmuehlen et al., 2011; Kitzmiller et al., 2006; Thongpang et al., 2011; Viventi et al., 2011; Wang et al., 2009). These devices are thought to strike a potential balance between the spatial resolution necessary for performing brain computer interfacing (BCI) tasks and the long-term stability required for human implantation.

Due to their minimally invasive nature, it is thought that the tissue surrounding surface electrode arrays elicits very little response to their presence. However, although there has been extensive research into *in vivo* biological responses to penetrating neural micro-electrode arrays (MEAs) (Williams et al., 2007; Woolley et al., 2011), there has been little investigation into tissue responses to MEAs implanted on the surface of the cerebral cortex. The assumption that these devices elicit little tissue response is based on results from traditional histological studies of brains implanted with surface electrode arrays (Henle et al., 2011). In order to perform these types of studies, however, the brain must be removed from the skull, and in the process, the electrode array is also removed from the cortical surface, resulting in disruption of the dura mater and any blood vessels and tissues that have grown around the device. Fong et al have reported vascular changes occurring around clinically implanted macro electrocorticography grids for mapping of seizure onset zones (Fong et al., 2010). In order to verify whether similar tissue changes occur around micro-ECoG devices, an imaging technique that does not require explantation of the brain and device would be advantageous.

The cranial window imaging method has been used extensively for other *in vivo* biological studies, particularly for imaging of tumor formation and vascular dynamics (Brown et al., 2010; Fukumura et al., 2001; Villringer et al., 1994). This technique employs a glass coverslip, chronically implanted on the surface of the cerebral cortex, through which the cranial tissue can be observed over extended time periods, from weeks to months. Since micro-ECoG devices sit on the surface of the cerebral cortex, their implantation is amenable to this imaging approach.

The objective of this study was to use a cranial window imaging method to study the tissue reaction to implanted micro-ECoG devices. By placing a glass coverslip over the top of the micro-ECoG device during implantation, a cranial window model was developed for imaging the tissue surrounding the implanted device. Use of this technique makes it possible to view the vasculature and other soft tissues that are often destroyed during traditional histological experiments, and also allows for observations of the tissue response at many different time points per animal, since the tissue can be imaged longitudinally *in vivo*. In this study we concentrate on the imaging of vascular responses to implanted micro-ECoG devices, as a first step towards chronic imaging of a multitude of different cell types.

2. Materials and Methods

2.1. Device Fabrication

The micro-ECoG array is shown in Figure 1(a). The device consisted of 16 electrode sites encapsulated in a Parylene C substrate. 12 holes were present through the device substrate, between the electrode sites. The electrode sites were 200 microns (μm) in diameter and the spacing between the site centers was 750 microns. The devices were fabricated following the process described in Figure 1(b) (Thongpang et al., 2011). A Parylene C layer was deposited onto a blank silicon wafer using a vacuum deposition system (PDS 2010 Labcoter 2, Specialty Coating Systems Inc.). Next, photolithography was used to define the electrode

sites and traces onto the layer of Parylene. The wafer was then placed in a metal evaporation system and a chrome adhesion layer, followed by gold and platinum was deposited onto its surface to form the conductive layer. After the metal deposition, lift-off techniques were used to remove unwanted metal from the surface of the wafer, leaving behind only the desired electrode sites and traces. Another layer of Parylene was then deposited on top of the metal layer, insulating the traces. Next, reactive ion etching with oxygen plasma was used to define the outline of the device and open holes through the Parylene substrate, as well as to remove the Parylene over the electrode sites, revealing the metal beneath. Finally, the wafer was soaked in water, releasing the devices from the silicon surface. After the devices were released from the wafer, printed circuit board (PCB) connectors (Imagineering inc., IL) were attached so that neural signals could be recorded from the electrode sites (Thongpang et al., 2011). The devices were then bench tested to verify electrode patency, and sterilized with ethylene oxide gas prior to implantation.

2.2. Ethics Statement

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin - Madison. All surgical procedures and *in vivo* imaging sessions were performed under isoflurane gas anesthesia. All efforts were made to minimize animal discomfort.

2.3. Surgical Implantation Procedure

Male Sprague Dawley rats ($n = 7$, Charles River) weighing 250-300 grams were implanted with micro-ECOG devices and cranial windows. Prior to surgery, animals received subcutaneous injections of dexamethasone (2 mg/kg body weight, AgriLabs) to prevent swelling of the brain during surgery, buprenorphine hydrochloride (0.05 mg/kg, Reckitt Benckiser Healthcare Ltd.) for pain management, and ampicillin (50 mg/kg, Sage Pharmaceuticals) to prevent infection of the implantation site. Animals were anesthetized with isoflurane gas and held in a stereotaxic frame for the duration of the surgical procedure. Heart rate and blood oxygen level were monitored throughout the surgery using a pulse oximeter.

The micro-ECOG implantation scheme is diagrammed in Figure 2. A craniotomy was made on one hemisphere of the rat skull, over somatosensory cortex, using a #107 engraving cutter. Through this craniotomy, the micro-ECOG device was implanted epidurally, and a circular glass coverslip, 5 mm in diameter and 0.15 mm thick, was placed over the top of the electrode array. An epidural implantation scheme was chosen in order to minimize trauma to the tissue underlying the device. Once the device and coverslip were in place, the PCB connector and coverslip were affixed to the skull using UV curable dental acrylic (Fusio dental acrylic, Pentron Clinical). A ground wire was run from the PCB connector to two ground screws (stainless steel, 00-80 $\times$ 1/8 inch) drilled into the back of the skull using a #56 drill bit. A reference wire was run from the PCB connector to a reference screw affixed to the front of the skull, contralateral to the micro-ECOG device. The screws and wires were then covered in dental acrylic and the skin was sutured around the acrylic headcap. After surgery, animals received another dose of buprenorphine as well as ampicillin injections twice daily for one week.

In addition to the animals implanted with the micro-ECOG devices, four rats were implanted with control windows. For these animals, the surgical procedure followed that described above, except that no micro-ECOG devices were implanted and instead the glass coverslips were placed directly on top of the dura-mater. Screws were implanted in the control animals, just as in the animals that received devices, for headcap stability purposes as well as to mimic as closely as possible the surgical procedure for the micro-ECOG array implantation.

Once affixed to the skull, the screws were covered in dental acrylic to form a smooth headcap.

To determine whether the tissue reaction could be a response to the presence of the window itself touching the dura mater, one of the control animals was implanted with a second control window, which sat up on the edge of the skull (with the diameter of the craniotomy smaller than the that of the window), such that the surface of the glass was not at all in contact with the dura mater. The gap between the window and the brain was filled with sterile saline before the edges of the window were sealed. This window sitting up on the skull edge was implanted in addition to the standard control window that sat directly on the surface of the dura mater (with the diameter of the craniotomy larger than that of the glass window). The standard control window was located on the left hemisphere of the rat's brain, over sensory-motor cortex, while the control window sitting up on the edge of the skull was located over the sensory-motor cortex on the right hemisphere. The rest of the surgical procedure followed that described above.

2.4. Vascular Imaging

The brain vasculature was imaged through the cranial window, using an upright fluorescent microscope (Leica MZ 16F, Leica Microsystems). For each imaging session the animals were anesthetized using a combination of isoflurane gas and subcutaneously injected dexmedetomidine hydrochloride (0.1 mg/kg, Orion Pharma). Ear bars were inserted in order to hold the animal still and prevent breathing artifacts. Fluorescein isothiocyanate labeled dextran (FITC-dextran) (30 mg/kg, 2,000,000 average molecular weight, Sigma Aldrich product #52471) was administered via tail vein injection so that the blood vessels would fluoresce under blue (460-500 nm, GFP2 filter) light, as shown in Figure 3. FITC-dextran is commonly used for visualizing microcirculation *in vivo* (Foley et al., 2012; Garkavtsev et al., 2004; Kleinfeld et al., 1998). It is advantageous, because it remains in circulation unless there is a breach in the wall of the blood vessel, thus allowing one to discriminate between old blood that has congealed outside the vessel walls prior to FITC injection, and new blood that is actively leaking out of the vessels after the time of injection (Mayhan and Heistad, 1985). After the imaging sessions were complete, the animals received subcutaneous injections of atipamezole hydrochloride (0.3 mg/kg, Orion Pharma) to reverse the anesthesia.

2.5. Electrode Site Impedance Measurement

In order to test the hypothesis that changes in the tissue surrounding the device would result in changes in the electrical recording capability, the impedance spectrum of each electrode site was measured on the same days that the cortical vasculature was imaged. Impedance spectra were obtained for each electrode site using a potentiostat (Autolab PGSTAT12). Animals were tested bi-weekly for up to 10 weeks post implantation, after which they were euthanized by cardiac perfusion.

2.6 Histological Analysis

The cortical vasculature was examined post-euthanasia to determine whether the presence of the micro-ECoG device had any detrimental effects on the vasculature deeper within the cortex, which could not be seen through the cranial window. The vasculature was labeled following the protocol described by Li et al, in which a solution of lipophilic 1,1 - dioctadecyl-3,3,3,3 -tetramethylindocarbocyanine perchlorate (DiI) was injected into the circulatory system during cardiac perfusion of the animal (Li et al., 2008). After perfusion, the brain tissue was carefully dissected from the skull and sectioned into 200 µm thick coronal slices using a vibratome (Leica VT1200). The slices were then placed on slides and sealed following the method described in the Li protocol (Li et al., 2008). The slides were

imaged under green (541-551 nm, G filter) light using the same fluorescent stereoscope described in section 2.4.

3. Results

3.1 Longitudinal Tissue Response

The progression of vascular growth around an epidurally implanted micro-ECoG device is shown in Figure 4. Blood vessels began to grow through individual holes in the Parylene substrate of the device on day five, and continued to spread over the entire top surface of the micro-ECoG array over time. In this example, it appears that there had been a micro-hematoma on day 10. The dark areas in the image are indicative of old blood that had pooled beneath the window. On day 15, the bleed was still present, but 17 days after implantation it had begun to diminish, and by day 22 the hematoma had completely resolved itself. By 24 days after implantation of the micro-ECoG device, the vasculature had stabilized and no more significant blood vessel changes were observed. The stabilization of the vasculature approximately 24 days post implantation was consistent across all animals in the study. Additionally, the presence of micro-hematomas around the micro-ECoG device was observed in every animal; however, these hematomas did not always occur at the same time point after device implantation. For the animal shown in Figure 4, on days 3 and 15 the tail vein injection of FITC-dextran was not successful, so the images were taken in bright field.

Figure 5 shows examples of hematomas occurring in different animals at different time points after implantation. Generally, bleeds occurring within a few days of device implantation were more severe than those observed at later time points (15-20 days post implant). Most hematomas resolved themselves within one to two weeks, although some animals showed evidence of blood in the craniotomy for the duration of the experiment.

Animals were imaged for as long as their windows remained clear and the tissue vasculature was visible. In most cases this was for a period of one month. In some cases, however, the window was clear for longer than 2 months (Figure 6 (a) and (b)). In other instances, a white, highly neovascularized connective tissue grew between the micro-ECoG array and the window, obscuring the device (Figure 6 (c) and (d)). Upon removal of the brain from the skull after the animals had been euthanized, it was found that the connective tissue was mechanically solid when probed, which suggests that it is most likely ossified tissue or bone. This white tissue growth was found to occur anywhere from one to three weeks after implantation of the device.

Analysis of the data from the animals implanted with control windows revealed that white connective tissue began to form one week after surgery, even when there was no device present. Additionally, micro-hematomas were observed in each of the control animals at varying time points after the implantation procedure, just as in the animals implanted with micro-ECoG devices. The tissue response in an animal implanted with a control window can be seen in Supplementary Figure 1.

A comparison between the control window sitting directly on the dura-mater and that sitting up on the edge of the skull, such that it is not touching the dura-mater, is shown in Supplementary Figure 2. It seems that, in both cases, the opaque white connective tissue formed beneath the cranial window, but in the case of the window sitting up on the edge of the skull, the tissue growth appeared to initiate from the bone edge, whereas, in the case of the window resting on the dura-mater, the reaction seemed to begin more towards the center of the window, away from the bone edge.

3.2 Impedance Changes

Plots of the average change in impedance over time are shown in Figure 7 for both 1 kHz and 90 Hz frequencies. From these plots it is apparent that there is a general trend of a steep rise in impedance (between 150 and 300 kilo-Ohms) within the first 20 days after device implantation, after which the impedance appears to plateau. This impedance change seems to be occurring within the same time period as the rapid tissue changes observed through the cranial window. The change in the impedance at a frequency of 1 kHz was analyzed in this experiment because the magnitude of the impedance at this frequency has been cited in the literature as a good benchmark for measuring overall tissue resistivity (Cui et al., 2001; Norlin et al., 2002; Williams et al., 2007). The impedance change at 90 Hz was analyzed because this frequency is within the range of physiologically relevant frequencies recorded by the micro-ECoG device, as local field potentials (LFPs) occur between 1 and 300 Hz. Both frequencies show a similar impedance trend.

3.3 Histological Results

Histological analysis of the cortical vasculature after brain explantation revealed no significant vascular differences between brain regions away from the implantation site, brain regions beneath the control window, and brain regions beneath the micro-ECoG device (Figure 8).

4. Discussion

The goal of the current study was to develop a method for visualization of the tissue surrounding an implanted surface electrode array without disrupting the device-tissue interface. The cranial window imaging method proved very useful for this application. The majority of previous cranial window experiments have been carried out in mouse models and did not involve the use of implantable devices or devices placed on the cortical surface (Holtmaat et al., 2009; Trachtenberg et al., 2002). The mouse has been the preferred animal model for cranial window imaging because of the availability of many transgenic mouse strains which allow for the visualization of different cell types, including but not limited to microglia, astrocytes, and neurons. Additionally, because mouse bone is naturally thin and semitransparent, a thin-skull preparation can be used, in which the cranial window is placed on top of a thinned layer of the skull, reducing the amount of trauma inflicted during surgery, yet still allowing the brain tissue to be imaged (Drew et al., 2010; Marker et al., 2010). Discrepancies between the rat tissue response data reported in this paper and the findings from mouse cranial window imaging experiments could be due to a number of factors, including species differences, such as the skull and dural thickness, as well as experimental differences, such as the amount of dural trauma induced during surgery, the presence of the micro-ECoG device, and the duration over which the tissue response was monitored (Holtmaat et al., 2009). Although we are also working towards modifying the micro-ECoG arrays and the cranial window imaging method for a mouse model, the development of this technique for use in rats is beneficial for scaling up the dimensions of the micro-ECoG device towards the size that would be used in larger mammals and eventually in humans.

Administration of FITC-Dextran made it particularly easy to image the tissue vasculature; however visualization of different cell types is difficult with the current approach. Through use of the vascular imaging technique, rapid tissue changes were observed around the micro-ECoG devices following implantation, and we were able to track these changes over the course of the entire reaction cycle, after which the vasculature had stabilized. The observed tissue reaction was fairly consistent across all animals in the study, and the time-course of the rapid tissue changes seemed to align with the overall changes in the impedance, at both 1

kHz and 90 Hz frequencies. Although we are not correlating tissue changes and impedances around individual sites, there appears to be a general correlation between the overall device-level changes and the change in impedance. This is consistent with our previous results with penetrating devices that showed correlation between gross histological changes around implanted devices and their impedance at 1 kHz (Williams et al., 2007). Because there are many more factors involved in electrode site impedance than the vascular changes visualized in this experiment (Williams et al., 2007), more work is required to dissect the relationship between tissue growth and impedance at individual electrode sites.

Micro-hematomas were observed in every animal, including those implanted with control windows only, but the location of the hematomas and the time at which they occurred varied greatly. While the exact cause of these bleeds is unknown, a number of factors could have contributed to their development, including irritation of the dural blood vessels during electrode placement, or shifting or swelling of the brain which resulted in increased pressure on the dural or cortical vessels due to the presence of the micro-ECoG device or the cranial window itself. Although the amount of brain shift which normally occurs in rats is quite minimal compared to that in primates, even pulsing of the brain from normal heart beat could be enough to irritate meningeal blood vessels, especially if those vessels were in contact with a sharp edge on the cranial window. Additionally, it is well known that in angiogenesis, the immature blood vessels are inherently leaky, due to the presence of vascular permeability factor (VPF), commonly referred to as vascular endothelial growth factor (VEGF), which is necessary to induce angiogenesis, but also results in a hyperpermeability of the newly formed vasculature (Dvorak et al., 1995). Whatever their cause, these hematomas could account for some of the changes in the electrode site impedance and the neural signals recorded from implanted brain devices (Vetter et al., 2004; Williams et al., 2007). Without the use of the cranial window imaging technique it would be almost impossible to detect these bleeds, due to their random and transient nature.

Growth of vascularized, opaque, connective tissue was observed in both the control animals as well as animals implanted with micro-ECoG devices. In some cases, the connective tissue formed below the micro-ECoG devices, while in others the tissue grew between the devices and the cranial window. The formation of new blood vessels as well as this connective tissue, even in the animal which was implanted with a control window that was not in contact with the dura mater (Supplemental Figure 2), suggests that a tissue reaction occurs merely as a result of surgical trauma, and that no cortically implanted electrode array can be considered free from biological response (Xu et al., 2007). More investigation is necessary to determine the composition of the tissue and why it grows beneath the micro-ECoG device in some cases and above the device in others. At this point, it is postulated that this tissue formation is either in response to irritation of the dura mater or is a result of bone re-growth. The initiation of the connective tissue growth from the center of the craniotomy in some of the control cases was not unexpected, as the formation of “bony islands” in the center of cranial defects has been reported by several groups studying bone regeneration (Aronin et al., 2010; Levi et al., 2011; Oyama et al., 2010; Sohn et al., 2010). Additional histological studies comparing tissue re-growth in animals implanted with control windows to that in animals with no windows implanted may be useful in determining the extent of the tissue response that can be attributed to the presence of the cranial window.

It was initially unclear how the presence of the device would affect the ability to image the cortical tissue. However, it was found that the Parylene substrate was sufficiently transparent to allow us to view the vasculature beneath the electrode array for the first few days after implantation (before vessels grow on top of the device and obstruct the view below). Figure 9 shows an example of vessels imaged beneath the micro-ECoG device. It was also postulated that, due to its hydrophilic nature, the electrode array could potentially

stick to the glass surface instead of to the cortex, preventing some of the recording sites from contacting the neural tissue and precluding the growth of tissue around the entire device. The finding that the vasculature grows on top of the device, between the glass and the Parylene substrate, suggests that this was not the case.

Although, the observed neovascularization was similar to that reported by Arieli et. al from their studies in monkeys (Arieli et al., 2002) and was, to some degree, expected, the growth of blood vessels through the holes in the substrate of the device was not anticipated. These holes were originally incorporated into the device design for the purpose of inserting other types of neural probes through the micro-ECoG and into the cortex; however, the presence of the holes seemed to encourage tissue growth on top of the device. It is postulated that this may inhibit thickening of the dura mater and other tissues below the device, at the electrode-tissue interface, in turn helping to bolster recorded signal quality and longevity. Thus, a logical next step would be to investigate the effects of the Parylene substrate footprint on tissue growth around micro-ECoG devices. Additionally, since it is known that immature blood vessels are prone to leakage, it may be useful to explore the effects of anti-angiogenesis drugs applied at the implantation site on the tissue response to the micro-ECoG device and on the recorded signal quality (Brown et al., 1992). Small molecule inhibitors, such as thalidomide, are commonly used for angiogenesis prevention (Mabeta and Pepper, 2011; Segler and Tsimberidou, 2012).

It is important to note that the tissue reactions to the micro-ECoG devices studied in this experiment may not be directly comparable to the clinical ECoG grids currently used in humans. Current clinical grids consist of varying numbers of channels with a general spacing of 1 centimeter (cm) between sites and a general electrode site diameter of around 5 mm (Leuthardt et al., 2004). These grids are designed for mapping epileptic foci rather than brain computer interfacing applications, and thus require less spatial resolution. In addition, clinical ECoG arrays are not meant for long term implantation. They are generally only left in place long enough to determine the origin of a patient's seizures. Consequently, tissue integration may not be desirable in the case of these macro grids.

The purpose of this study was to determine the tissue response to micro-ECoG devices for brain computer interfacing applications, since these devices must be designed for long-term reliability *in vivo*. In the case of micro-electrode arrays for BCI applications, integration of the device and tissue may help to anchor the array in place, and thus minimize micro-motion which could potentially result in cortical trauma and signal instability. Therefore, the angiogenesis and overall tissue response observed in these window experiments could potentially prove to be therapeutic in the case of surface electrode arrays (Murphy et al., 2004). That being said, there have been some recent efforts to utilize micro-ECoG for human seizure detection (Stead et al., 2010), and the electrodes used in this study may prove clinically useful for this application, as the tissue in-growth could serve to stabilize the brain-electrode interface. It is not yet known what kind of damage would occur upon removal of the devices, but it may prove to be minimal, as the in-growing vessels are quite small. Evidence of similar small vessel damage has been found upon removal of macro-ECoG grids (Fong et al., 2012), and is well tolerated.

The cranial window imaging method has proven to be a useful tool for understanding the behavior of tissue surrounding implanted electrode arrays, but there are some limitations to this technique. Perhaps the most significant limitation is that we can very clearly image the tissue growing on top of the array, but cannot see what is occurring beneath the electrodes, at the electrode-tissue interface. In order to determine what is going on at the site of the neural recordings, other imaging techniques, such as traditional histological methods or multi-photon microscopy, which can penetrate more deeply into the tissue, would be

necessary. Histological analysis of the cortical vasculature of the animals used in this study revealed no significant differences between the vasculature directly beneath the micro-ECoG device and that below the control window or away from the implantation site altogether. This finding corroborates the theory that the majority of the tissue reaction to these surface electrode arrays is occurring in the meninges. However, the current setup is limited to observing the tissue vasculature, and further investigation is necessary into the response of different cell types, such as astrocytes and microglia. Although not apparent in the still photos, individual cells were visible through blood flow video recordings (see Supplementary Figures 3 and 4) suggesting that this technique could be easily modified to view individual cell types with the use of appropriate fluorescent dyes and transgenic animals. One additional shortcoming of this technique is that the presence of the cranial window itself appears to initiate a tissue response. Further histological comparison studies will be necessary in order to determine the time-course of the tissue response to the micro-ECoG device with and without the presence of the cranial window.

The cranial window imaging method provides insight into the transient changes occurring at the surface of the brain. Future studies, which incorporate the window imaging method in conjunction with electrophysiological recordings and supplementary imaging modalities, will help increase the understanding of why these tissue changes occur and how they correlate with variations in electrophysiological data.

5. Conclusion

The cranial window imaging method has proven a useful technique for monitoring the tissue response to surface electrode arrays *in vivo*. Use of this technique has revealed the presence of micro-hematomas around every implanted device, but at varying time points. These hematomas could be contributing to changes in the recorded signal over the course of the implant lifetime, and would not be observable using traditional histological techniques. Moving forward, additional studies are necessary in order to determine the effect of the device substrate footprint on the tissue response, as well as to adapt this technique for visualization of different cell types and determine the relationship between cortical tissue changes and recorded signal quality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the National Institutes of Health (NIH NIBIB 1R01EB009103-01, NIH NIBIB 2R01EB000856-06, and NIH NIBIB 1T32EB011434-01A1) and the Defense Advanced Research Projects Agency (DARPA RCI #N66001-12-C-4025, DARPA HIST #N66001-11-1-4013).

References

- Arieli A, Grinvald A, Slovin H. Dural substitute for long-term imaging of cortical activity in behaving monkeys and its clinical implications. *Journal of Neuroscience Methods*. 2002; 114:119–133. [PubMed: 11856563]
- Aronin CEP, Sefcik LS, Tholpady SS, Tholpady A, Sadik KW, Macdonald TL, Peirce SM, Wamhoff BR, Lynch KR, Ogle RC, Botchwey EA. FTY720 Promotes Local Microvascular Network Formation and Regeneration of Cranial Bone Defects. *Tissue Eng Part A*. 2010; 16:1801–1809. [PubMed: 20038198]
- Brown E, Munn LL, Fukumura D, Jain RK. In vivo imaging of tumors. *Cold Spring Harb Protoc*. 2010; 2010.pdb.prot5452.

- Brown LF, Yeo KT, Berse B, Yeo TK, Senger DR, Dvorak HF, Water L van de. Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. *J Exp Med*. 1992; 176:1375–1379. [PubMed: 1402682]
- Cui X, Hetke JF, Wiler JA, Anderson DJ, Martin DC. Electrochemical deposition and characterization of conducting polymer polypyrrole/PSS on multichannel neural probes. *Sensors and Actuators A: Physical*. 2001; 93:8–18.
- Drew PJ, Shih AY, Driscoll JD, Knutsen PM, Blinder P, Davalos D, Akassoglou K, Tsai PS, Kleinfeld D. Chronic optical access through a polished and reinforced thinned skull. *Nat Meth*. 2010; 7:981–984.
- Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol*. 1995; 146:1029–1039. [PubMed: 7538264]
- Foley CP, Nishimura N, Neeves KB, Schaffer CB, Olbricht WL. Real-time imaging of perivascular transport of nanoparticles during convection-enhanced delivery in the rat cortex. *Ann Biomed Eng*. 2012; 40:292–303. [PubMed: 22009318]
- Fong J, Bingaman W, Alexopoulos A, Prayson R. Iatrogenic Pathological Findings Related to Invasive EEG Monitoring of Medically Intractable Epilepsy. 2010
- Fong JS, Alexopoulos AV, Bingaman WE, Gonzalez-Martinez J, Prayson RA. Pathologic Findings Associated With Invasive EEG Monitoring for Medically Intractable Epilepsy. *AJCP*. 2012; 138:506–510. [PubMed: 23010704]
- Fukumura D, Xu L, Chen Y, Gohongi T, Seed B, Jain RK. Hypoxia and Acidosis Independently Up-Regulate Vascular Endothelial Growth Factor Transcription in Brain Tumors in Vivo. *Cancer Research*. 2001; 61:6020–6024. [PubMed: 11507045]
- Garkavtsev I, Kozin SV, Chernova O, Xu L, Winkler F, Brown E, Barnett GH, Jain RK. The candidate tumour suppressor protein ING4 regulates brain tumour growth and angiogenesis. *Nature*. 2004; 428:328–332. [PubMed: 15029197]
- Gierthmuehlen M, Ball T, Henle C, Wang X, Rickert J, Raab M, Freiman T, Stieglitz T, Kaminsky J. Evaluation of μ ECOG electrode arrays in the minipig: Experimental procedure and neurosurgical approach. *Journal of Neuroscience Methods*. 2011; 202:77–86. [PubMed: 21896285]
- Henle C, Raab M, Cordeiro J, Doostkam S, Schulze-Bonhage A, Stieglitz T, Rickert J. First long term in vivo study on subdurally implanted Micro-ECOG electrodes, manufactured with a novel laser technology. *Biomedical Microdevices*. 2011; 13:59–68. [PubMed: 20838900]
- Holtmaat A, Bonhoeffer T, Chow DK, Chuckowree J, Paola VD, Hofer SB, Hübener M, Keck T, Knott G, Lee WCA, Mostany R, Mrsic-Flogel TD, Nedivi E, Portera-Cailliau C, Svoboda K, Trachtenberg JT, Wilbrecht L. Long-term, high-resolution imaging in the mouse neocortex through a chronic cranial window. *Nature Protocols*. 2009; 4:1128–1144.
- Kitzmler J, Beversdorf D, Hansford D. Fabrication and testing of microelectrodes for small-field cortical surface recordings. *Biomedical Microdevices*. 2006; 8:81–85. [PubMed: 16491335]
- Kleinfeld D, Mitra PP, Helmchen F, Denk W. Fluctuations and stimulus-induced changes in blood flow observed in individual capillaries in layers 2 through 4 of rat neocortex. *PNAS*. 1998; 95:15741–15746. [PubMed: 9861040]
- Leuthardt EC, Schalk G, Wolpaw JR, Ojemann JG, Moran DW. A brain–computer interface using electrocorticographic signals in humans. *Journal of Neural Engineering*. 2004; 1:63–71. [PubMed: 15876624]
- Levi B, Nelson ER, Li S, James AW, Hyun JS, Montoro DT, Lee M, Glotzbach JP, Commons GW, Longaker MT. Dura Mater Stimulates Human Adipose-Derived Stromal Cells to Undergo Bone Formation in Mouse Calvarial Defects. *STEM CELLS*. 2011; 29:1241–1255. [PubMed: 21656608]
- Li Y, Song Y, Zhao L, Gaidosh G, Laties AM, Wen R. Direct labeling and visualization of blood vessels with lipophilic carbocyanine dye DiI. *Nature protocols*. 2008; 3:1703–1708.
- Mabeta P, Pepper MS. Hemangiomas—Current therapeutic strategies. *International Journal of Developmental Biology*. 2011; 55:431. [PubMed: 21858768]
- Marker DF, Tremblay M, Lu S, Majewska AK, Gelbard HA. A Thin-skull Window Technique for Chronic Two-photon In vivo Imaging of Murine Microglia in Models of. 2010

- Mayhan WG, Heistad DD. Permeability of blood-brain barrier to various sized molecules. *Am J Physiol Heart Circ Physiol*. 1985; 248:H712–H718.
- Murphy WL, Simmons CA, Kaigler D, Mooney DJ. Bone regeneration via a mineral substrate and induced angiogenesis. *Journal of dental research*. 2004; 83:204–210. [PubMed: 14981120]
- Norlin P, Kindlundh M, Mouroux A, Yoshida K, Hofmann UG. A 32-site neural recording probe fabricated by DRIE of SOI substrates. *Journal of Micromechanics and Microengineering*. 2002; 12:414–419.
- Oyama Y, Kazama JJ, Fukagawa M, Arakawa Y, Ezuka I. Ectopic ossification in the cranial dura mater in dialysis patients with secondary hyperparathyroidism. *NDT plus*. 2010; 3:64–67.
- Segler A, Tsimberidou AM. Lenalidomide in solid tumors. *Cancer Chemotherapy and Pharmacology*. 2012; 69:1393–1406. [PubMed: 22584909]
- Sohn JY, Park JC, Um YJ, Jung UW, Kim CS, Cho KS, Choi SH. Spontaneous healing capacity of rabbit cranial defects of various sizes. *J Periodontal Implant Sci*. 2010; 40:180–187. [PubMed: 20827327]
- Stead M, Bower M, Brinkmann BH, Lee K, Marsh WR, Meyer FB, Litt B, Gompel JV, Worrell GA. Microseizures and the spatiotemporal scales of human partial epilepsy. *Brain*. 2010; 133:2789–2797. [PubMed: 20685804]
- Szarowski DH, Andersen MD, Retterer S, Spence AJ, Isaacson M, Craighead HG, Turner JN, Shain W. Brain responses to micro-machined silicon devices. *Brain Research*. 2003; 983:23–35. [PubMed: 12914963]
- Thongpang S, Richner TJ, Brodnick SK, Schendel A, Kim J, Wilson J, Hippensteel J, Krugner-Higby L, Moran D, Ahmed AS, others. A micro-electrocorticography platform and deployment strategies for chronic BCI applications. *Clinical EEG and neuroscience: official journal of the EEG and Clinical Neuroscience Society (ENCS)*. 2011; 42:259. [PubMed: 22208124]
- Trachtenberg JT, Chen BE, Knott GW, Feng G, Sanes JR, Welker E, Svoboda K, others. Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. *Nature*. 2002; 420:788–794. [PubMed: 12490942]
- Vetter RJ, Williams JC, Hetke JF, Nunamaker EA, Kipke DR. Chronic neural recording using silicon-substrate microelectrode arrays implanted in cerebral cortex. *Biomedical Engineering, IEEE Transactions on*. 2004; 51:896–904.
- Villringer A, Them A, Lindauer U, Einhaupl K, Dirnagl U. Capillary perfusion of the rat brain cortex. An in vivo confocal microscopy study. *Circulation Research*. 1994; 75:55–62. [PubMed: 8013082]
- Viventi J, Kim DH, Vigeland L, Frechette ES, Blanco JA, Kim YS, Avrin AE, Tiruvadi VR, Hwang SW, Vanleer AC, Wulsin DF, Davis K, Gelber CE, Palmer L, Spiegel JV, der Wu J, Xiao J, Huang Y, Contreras D, Rogers JA, Litt B. Flexible, foldable, actively multiplexed, high-density electrode array for mapping brain activity in vivo. *Nature Neuroscience*. 2011; 14:1599–1605.
- Wang W, Degenhart AD, Collinger JL, Vinjamuri R, Sudre GP, Adelson PD, Holder DL, Leuthardt EC, Moran DW, Boninger ML, Schwartz AB, Crammond DJ, Tyler-Kabara EC, Weber DJ. Human motor cortical activity recorded with Micro-ECOG electrodes, during individual finger movements. *Conf Proc IEEE Eng Med Biol Soc*. 2009; 2009:586–589. [PubMed: 19964229]
- Williams JC, Hippensteel JA, Dilgen J, Shain W, Kipke DR. Complex impedance spectroscopy for monitoring tissue responses to inserted neural implants. *Journal of neural engineering*. 2007; 4:410. [PubMed: 18057508]
- Williams JC, Rennaker RL, Kipke DR. Long-term neural recording characteristics of wire microelectrode arrays implanted in cerebral cortex. *Brain Research Protocols*. 1999; 4:303–313. [PubMed: 10592339]
- Woolley A, Desai H, Otto K. Imaging the Tissue Response Around Brain-Implanted Microdevices. *Microscopy and Microanalysis*. 2011; 17:146–147.
- Xu HT, Pan F, Yang G, Gan WB. Choice of cranial window type for in vivo imaging affects dendritic spine turnover in the cortex. *Nature Neuroscience*. 2007; 10:549–551.

Highlights

- Micro-ECOG devices were implanted epidurally beneath cranial windows
- Blood vessels grew up through holes in the substrate and spread laterally
- Micro-hematomas and opaque connective tissue growth was observed
- The observed tissue changes could detrimentally impact recorded signal quality
- We observed transient tissue changes not always detected with traditional histology

Figure 1. Micro-ECoG device and fabrication process

A. The micro-ECoG device consisted of a Parylene C substrate with 16 Cr/Au/Pt electrode sites. 12 holes through the Parylene substrate are visible between the electrode sites. Scale bar represents 750 μm . **B.** Micro-ECoG fabrication process. **A:** 15 μm of Parylene was deposited on a blank Si wafer. **B:** 1813 photoresist was used to pattern the electrode sites and traces onto the Parylene. **C:** 10 nm of chrome, 200 nm of gold, and 20 nm of platinum was deposited using an electron beam evaporator. **D:** Lift off techniques were used to remove the metal that was on top of the photoresist. **E:** 10 μm of Parylene was deposited onto the sample. **F:** Photolithography was used to create an etch mask. **G:** RIE was used to etch through the 1st 15 μm of Parylene, creating the holes and the outline of the micro-ECoG device. **H:** A second photoresist etch mask was patterned. **I:** RIE was used to etch the Parylene off of the electrode sites and finish etching through the holes and the MEA outline. **J:** The sample was soaked in water and the devices were released from the Si wafer.

Figure 2. Implantation scheme

A. Cross-sectional diagram of the window imaging implantation scheme, in which a glass cover slip was affixed over the top of an epidurally implanted micro-ECoG array. **B.** Top view of the cranial window implant setup. **C.** Rat implanted with a micro-ECoG array and cranial window.

Figure 3. Example of observed vascular growth

Blood vessels growing over the top of an epidurally implanted micro-ECOG array 30 days after implantation. Image was taken using an upright fluorescent microscope with broad spectrum light and a GFP-2 filter. Rat was injected with 6 mg of FITC-Dextran dissolved in 0.5 ml saline solution. Scale bar represents 750 μm .

Figure 4. The *in vivo* progression of vascular growth over an epidurally implanted micro-ECoG device

A. 3 days post implantation. **B.** 5 days post implantation. **C.** 8 days post implantation. **D.** 10 days post implantation. **E.** 15 days post implantation. **F.** 17 days post implantation. **G.** 22 days post implantation. **H.** 24 days post implantation. Scale bars represent 750 μm . At day 5 blood vessels begin to grow through a hole in the upper left corner of the Parylene substrate. Over time, the vessels spread out over the entire top surface of the device. After 24 days, the vasculature seems to have stabilized, no more significant vessel changes occur after this point. Images in **A.** and **E.** were taken in bright field because the tail-vein injection of FITC-dextran was unsuccessful on those days.

Figure 5. Micro-hematomas occurring in different animals at different time points after device implantation

A. 2 days post implantation, **B.** 3 days post implantation, **C.** 15 days post implantation, **D.** 20 days post implantation. Scale bars represent 750 μm .

Figure 6. Cranial windows remain clear for varying lengths of time

A and B. Images of the vasculature around a micro-ECoG device 2 months after implantation. **C and D.** Images of the micro-ECoG device 19 days after implantation. Scale bars represent 750 μm . Note the growth of opaque connective tissue over the majority of the cranial window.

Figure 7. Impedance changes over time

Plot of average change in impedance (from initial impedance measurement after implantation) at 1 kHz (A.) and 90 Hz (B.) for four animals and all viable channels (60 channels) over time. Error bars represent +/- one standard deviation.

Figure 8. Cortical vasculature labeled with DiI in coronal brain sections

A. Region away from implant. **B.** Region beneath control window. **C.** Region beneath Micro-ECOG device. Scale bars represent 500 μm .

Figure 9. Vasculature beneath the array

Blood vessels visible beneath the micro-ECOG device two days after implantation. Scale bar represents 750 μm .