Published in final edited form as:

J Neural Eng. 2012 December ; 9(6): 066001. doi:10.1088/1741-2560/9/6/066001.

***In vivo* two-photon microscopy reveals immediate microglial reaction to implantation of microelectrode through extension of processes**

Takashi D Yoshida Kozai^{1,3}, Alberto L Vazquez², Cassandra L Weaver¹, Seong-Gi Kim², and X Tracy Cui^{1,3}

¹Department of Bioengineering, University of Pittsburgh, PA, USA

²Department of Radiology, University of Pittsburgh, PA, USA

Abstract

Objective—Penetrating cortical neural probe technologies allow investigators to record electrical signals in the brain. Implantation of probes results in acute tissue damage, and microglia density increases around implanted devices over weeks. However, the mechanisms underlying this encapsulation are not well understood in the acute temporal domain. The objective here was to evaluate dynamic microglial response to implanted probes using two-photon microscopy.

Approach—Using two-photon *in vivo* microscopy, cortical microglia ~200 μm below the surface of the visual cortex were imaged every minute in mice with green fluorescent protein-expressing microglia.

Main results—Following probe insertion, nearby microglia immediately extended processes toward the probe at $(1.6 \pm 1.3) \mu\text{mmin}^{-1}$ during the first 30–45 min, but showed negligible cell body movement for the first 6 h. Six hours following probe insertion, microglia at distances $<130.0 \mu\text{m}$ ($p = 0.5$) from the probe surface exhibit morphological characteristics of transitional stage (T-stage) activation, similar to the microglial response observed with laser-induced blood–brain barrier damage. T-stage morphology and microglia directionality indexes were developed to characterize microglial response to implanted probes. Evidence suggesting vascular reorganization after probe insertion and distant vessel damage was also observed hours after probe insertion.

Significance—A precise temporal understanding of the cellular response to microelectrode implantation will facilitate the search for molecular cues initiating and attenuating the reactive tissue response.

Introduction

Penetrating microelectrodes are on the cusp of clinical relevance for chronic human cortical prosthetics. However, the reactive tissue response caused by the implanted cortical microelectrodes results in a degradation of functional performance over time limiting the device capabilities (Rousche and Normann 1998, Biran *et al* 2005, McConnell *et al* 2009). This reactive tissue response degrades the functional performance of the electrode sites over time (Polikov *et al* 2005, Williams *et al* 1999, Rousche and Normann 1998, Ward *et al* 2009, Schwartz *et al* 2006, Nicolelis *et al* 2003, Liu *et al* 1999, 2006). A deeper cellular and

molecular understanding of the tissue response at neural interfaces is necessary for improving neural technology to enable the long-term use of human cortical prosthetics.

Normal adult microglial cells in the brain are usually in a ramified state (R-stage) at rest. During this time, microglial cells radially extend and retract their processes to continually survey their surroundings at an average of $2.2 \pm 0.2 \mu\text{m min}^{-1}$ (Nimmerjahn *et al* 2005). They are known to play key roles in mediating responses to brain tissue injury (Kreutzberg 1996), Hanisch and Kettenmann 2007). The initial inflammatory response following a probe implantation is characterized by the activation of ramified microglia into the amoeboid state within the first 24 h, which leads to the formation of a thin cellular sheath encapsulating the device over the following week (Szarowski *et al* 2003, Purcell *et al* 2009).

After a major insult, such as electrode insertion or cutting the brain for brain slice studies, ramified microglial cells enter a withdrawal stage (W-stage) in which they retract their extended processes (Stence *et al* 2001, Kreutzberg 1996, Raivich *et al* 1999). These cells then enter into a motility stage (M-stage) involving cycles of extension and retraction of new processes as the cell body remains stationary. Other cells enter into a transitional stage (T-stage), generating a limited number of new protrusions that rapidly extend while most processes are withdrawn. In slice culture, these T-stage microglia only last 5–20 min before entering the M-stage (Stence *et al* 2001). Microglia can enter into a locomotory state (L-stage) as well, enabling the cells to move through the tissue rapidly. Following brain slicing, ramified microglial cells enter the amoeboid L-stage roughly 12 h post-insult (Stence *et al* 2001). Chronic slice cultures have shown that amoeboid microglia eventually revert back to the ramified state 1–2 weeks after slicing (Hailer *et al* 1996, 1997).

In cortex chronically implanted with a probe, increased density of amoeboid microglia is found as early as 24 h and persists for the entire implantation period. Retrieved chronic implants are covered with microglia that release pro-inflammatory factors such as monocyte chemoattractant protein (MCP-1) and tumor necrosis factor- α (TNF- α) (Biran *et al* 2005). This microglia activation is accompanied by peak glial fibrillary acidic protein (GFAP) activation of astrocytes during the first week, resulting in the formation of a compact glial sheath approximately three weeks later (Szarowski *et al* 2003, Biran *et al* 2005, McConnell *et al* 2009). Moreover, this activation of the glial inflammatory response correlates with modification of the extracellular matrix (Kim *et al* 2004) and neuronal loss in the microenvironment surrounding the implanted electrode (Biran *et al* 2005) through neurodegeneration (McConnell *et al* 2009).

Despite these findings, many details concerning the mechanisms underlying this tissue response remain poorly understood, including specific molecular pathways and the precise timing of these dynamic cellular changes. Understanding gained from conventional post-mortem histology is limited by extensive tissue processing. In traditional confocal histology, the tissue is excised from the native environment and reduced into small fragments, often after removal of the implant. While new histology techniques aim to capture three-dimensional changes around these devices (Woolley *et al* 2011), discrete endpoint post-mortem histology is still limited by inter-animal variability and the degree to which temporal effects can be investigated. *In vivo* two-photon microscopy (TPM) is an emerging technique for study of the progression of biological processes in deep tissue beyond the range of conventional confocal microscopy (Abdul-Karim *et al* 2003, Chauhan *et al* 2009, Majewska *et al* 2000, Lowery *et al* 2009, Tremblay *et al* 2010, Dunn and Sutton 2008, Svoboda and Yasuda 2006, Helmchen and Denk 2005). The ability to track dynamic progression of the tissue response may help us identify cause and effect relationships behind this inter-animal variability, for example by establishing or refuting the relationship between vascular damage and tissue reaction (Kozai *et al* 2010).

Here we report the use of TPM to visualize the neural probe/brain tissue interface in live animals. This is the first step toward gaining a more precise temporal understanding of the cellular response to microelectrode implantation. Such understanding will allow investigators to narrow their search for molecular cues initiating the reactive tissue response and observe how different intervention strategies lead to divergence from the typical inflammatory response. Specifically, we examine the cellular microglial response to microelectrode insertion over the order of minutes to hours. Process migration and cell body displacement were measured and morphological changes and activation characteristics quantified. Additional observations suggest that vascular reorganization following probe insertion damage and distant vessel damage can occur hours after probe insertion. In summary, microglia cells immediately begin to extend processes toward the implanted electrode, but show minimal cell body movement over the first 6 h following probe insertion.

Methods

Probe preparation

Acute 3 mm long four-shank Michigan silicon electrodes with $177\ \mu\text{m}^2$ iridium recording sites on $15\ \mu\text{m}$ by $100\ \mu\text{m}$ shanks, and $125\ \mu\text{m}$ center-to-center shank spacing (Neuronexus Technologies, Ann Arbor, MI) were used for this work. In order to better visualize the probe, fluorescent molecules were loaded into a conducting polymer polyethylenedioxythiophene (PEDOT) coating electrodeposited on the recording sites (Cui and Martin 2003). Specifically, PEDOT was electropolymerized from a 0.1 M poly(sodium-p-styrenesulfonate) (PSS) (Acros Organics; Morris Plains, NJ) aqueous solution containing 0.01 M monomer 3, 4-ethylenedioxythiophene (EDOT) (Bayer, Germany) and $0.1\ \text{mg ml}^{-1}$ Rhodamine B (Sigma- Aldrich Co.) under a constant current of 1 nA for 300 s.

Surgery

Four-shank Michigan electrodes were implanted into the cerebral cortex of five transgenic mice that express green fluorescent protein (GFP) in brain microglia under the direction of the CX3CR1 promoter (CX3CR1-GFP). Four to eight week old CX3CR1-GFP mice obtained from Jackson Laboratories (Bar Harbor, ME) and weighing 20–25 g were prepared for cortical implants using previously established methods (Kozai *et al* 2010). The animals were anesthetized with a mixture of $90\ \text{mg kg}^{-1}$ ketamine and $9\ \text{mg kg}^{-1}$ xylazine administered intraperitoneally (IP) with regular updates of $17.5\ \text{mg kg}^{-1}$ every 30 min or as needed. The depth of anesthesia was observed by monitoring heart rate. In two animals, $0.02\ \text{cc}$ of $1\ \text{mg ml}^{-1}$ sulforhodamine 101 (SR101) was injected IV as an optical vascular label (red), and then $0.01\ \text{mg ml}^{-1}$ SR101 was administered IP periodically as needed. Updates of ketamine and SR101 were given through an IP line so that they minimally impact imaging. After the animal was placed in a stereotaxic frame, the skin and connective tissue on the surface of the skull was removed. A thin layer of Vetbond (3M) was placed over the skull and a 1–1.5 mm tall well was constructed around the edges of the skull using dental cement. A 4 mm by 6 mm craniotomy was made by thinning the skull over the somatosensory and visual cortex using a highspeed dental drill and then removed with forceps. The skull was periodically bathed in saline to ensure that the underlying cortex did not experience thermal damage from drilling. Care was taken to prevent vascular damage during drilling and the removal of the bone. All experimental protocols were approved by the University of Pittsburgh, Division of Laboratory Animal Resources and Institutional Animal Care and Use Committee in accordance with the standards for humane animal care as set by the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Probe insertion

After the craniotomy, microglia morphology appeared uniform, especially over the targeted area for insertion. The electrode was aligned with a stereotaxic manipulator over the cortex away from major vasculature at a 30–35° angle under the two-photon microscope using a 2 ×, 0.08 numerical aperture air objective (Olympus, Tokyo, Japan) (figure 1). After alignment, the objective was carefully replaced with a 16 ×, 0.8 numerical aperture water immersion objective lens (Olympus, Tokyo, Japan). Probes were inserted using a z-axis automated microdrive (MO-81, Narishige, Japan) at 50 $\mu\text{m s}^{-1}$ for 500 μm such that the tip of the electrode was approximately 200–250 μm (~layer 3) below the surface of the brain. Little or no bleeding was observed during insertion.

Two-photon imaging

A two-photon laser scanning microscope was used for *in vivo* imaging. The microscope consisted of a scan head (Prairie Technologies, Madison, WI) and a Ti:sapphire laser (Mai Tai DS; Spectra-Physics, Menlo Park, CA) providing 100 fs pulses at 80 MHz tuned at a wavelength of 920 nm for this study. Fluorescence was detected using non-descanned photomultiplier tubes (Hamamatsu Photonics KK, Hamamatsu, Shizuoka, Japan) in whole-field detection mode. Images of 1024 × 1024 pixel (407.5 × 407.5 μm) were acquired over ~4.8 s using Prairie View software. The imaged volume was determined considering the desired image resolution and inter-stack interval. Because of the image size, only 2–3 shanks could be imaged at once (figure 1(a): probes were 475 μm wide outer edge-to-outer edge). Stacks were continuously acquired along depth (*ZT* stacks) every 2 μm from 180–202 μm below the surface of the cortex immediately before electrode insertion. After the first *Z* stack scan was completed, the probe was inserted using the microdrive motor while *ZT* scans continued. Each *Z* stack took approximately 1 min. Images were scanned for 7–12 h after insertion. For high quality 3D reconstruction, *Z* stacks were also taken 0.4 μm apart after 6 h in some animals.

Data analysis

For each animal, *ZT* stack images from the same plane were analyzed to determine process velocity. These images were first corrected over time for drift and motion with a custom-written MATLAB script (Math Works, Boston, MA). Inter-stack motion was corrected using a rigid-body translation only algorithm based on cross-correlation. Then, a maximum intensity projection was done for each stack acquired in the experiment and the 2D rigid-body translation-only algorithm was used again to align the images. The aligned images were visually inspected to ensure that this algorithm behaved as expected. All of the corrections were performed using the GFP microglia images and applied to images from other channels in the same stack. Sulforhodamine was administered prior to electrode insertion and anesthesia updates were given between acquisition periods so that motion from these administrations did not impact imaging. Under 600–1200% digital zoom, microglia process migration toward the probe and cell body displacement toward the nearest probe surface were measured using the ‘Measure’ function in ImageJ (National Institutes of Health). For these measurements, the initial position of the cell was taken from the first frame after probe insertion, so the cell body movement caused by tissue displacement from the accommodation of the probe’s volume does not become classified as migration. Time series were created by extracting the timestamps from individual frames. At 6 h, microglial cells were counted and binned as ramified (1) or in the activated transitional stage (T-stage) (0), characterized by retraction of most processes with extension of others toward an insult or injury (Stence *et al* 2001). Microglia cell distance from the probe was measured and correlated to binomial ramification state (0 or 1) using binomial logarithmic generalized linear regression.

The activated state of microglia was also assessed by calculating a T-stage morphology index and microglia directionality index. T-stage morphology index was calculated by measuring the length (n) of the longest microglia process from the hemisphere facing the probe and the length (f) of the longest process facing away from the probe at 6 h post-implantation. Similarly, microglia directionality index was calculated by measuring the number of processes (n) extending from the cell body in the hemisphere facing the probe and the number of processes (f) extending from the cell body in the hemisphere facing away from the probe at 6 h. Index values were calculated using the following formula:

$$\text{Index} = \frac{(f - n)}{(f + n)} + 1. \quad (1)$$

In both cases, an index value of 1 represents an inactive and ramified microglia cell ($n = f$), while an index of 0 represents a fully active T-stage microglia cell extending all processes toward the implant, similar to the microglia ramification state rating method. Note that if a microglia reduces ' f ' by 50% and increases ' n ' by 50%, the resulting index will be 0.5. The distribution of the index values as a function of cell body distance from the implant was then generated. Binning was performed to compute error bars as a function of distance for the indices, and the size of each bin was determined considering the number of samples available (ten samples per bin). This distribution was fitted to a dual sigmoidal function using a custom MATLAB script to capture the plateau feature between normal ramified microglia and T-stage active microglia, which corresponds to 1 and 0, respectively. The sigmoidal function was parameterized by amplitude (a), shoulder location (d_1 and d_2 in μm), and shoulder width (w_1 and w_2 in μm) and the fit was constrained to indexes between 0 and 1:

$$y(d) = \frac{a}{1 + e^{(d - d_1)/w_1}} + \frac{1 - a}{1 + e^{(d - d_2)/w_2}}. \quad (2)$$

3D reconstruction

To qualitatively capture the response of microglia to the implant, high-resolution stacks were rotated along x , y and z to place the implant horizontally and parallel to the y -axis by linear interpolation. This was performed using ImageJ's (NIH) built-in '3D Project' function. Visualization of microglia response adjacent to the implant was done by setting the function's interior depth-cueing parameter to zero. An average intensity projection was then performed parallel and perpendicular to the electrode penetration orientations.

Laser ablation of blood–brain barrier

To mimic rupture of blood vessels by electrode insertion, laser disruptions of vessels were carried out in mice that did not receive probe implants by continuously scanning along a line positioned over a blood vessel lumen with a wavelength of 860 nm for 60 s.

Results

Care was taken to prevent vascular damage during drilling and the removal of the bone. No changes in microglia shape, ramification, or migration were observed in the areas imaged within the craniotomy before probe insertion when compared to the contralateral thin-skulled hemisphere control (Drew *et al* 2010). Electrode implantation was targeted over an area of the cortex with relatively low vessel density. Prior to probe insertion, microglia morphology appeared uniform, especially over the area targeted for insertion (figure 1(d)). Imaging through thinned skull in the contralateral hemisphere showed no notable microglia

morphology differences compared to the clean open craniotomy. Because it is common practice to insert these electrodes through an open craniotomy, this study was designed to image through a craniotomy rather than through a thinned skull preparation. The dura was left intact to preserve the integrity of the environment and reduce motion. Leaving the dura intact did not impede the penetration of the electrodes used. Insertion locations were picked by targeting areas of the cortex with relatively low vessel density (Kozai *et al* 2010). Little or no bleeding was observed during insertion. Electrodes were able to penetrate through the dura without damaging the recording sites. This was verified through visual inspection and impedance spectroscopy after the experiment. 3D projections show that at 7 h post implant, microglia cells have reacted to the implanted electrode (figure 2). Microglial cells adjacent to the probe focus their processes toward the probe while cells distant to the probe maintain ramified morphology.

Microglia cell body movement over 6 h

Gross displacement of microglial cell bodies was not observed over the first 6 h (figures 3(a), (c) and (e)). The mean displacement of 167 microglial cell bodies in the first 6 h was $1.1 \pm 7.15 \mu\text{m}$ (figure 4). Additionally, cell body movement showed no correlation ($R^2 = 0.0072$) with respect to distance from the probe. While some cells appeared to have moved several microns, they maintained cell body shapes similar to those observed at the initial time points and did not exhibit an amoeboid morphology. Similar displacement in blood vessels was also observed suggesting that at least some of the cell body movement may be due to relaxation of tissue strain after probe insertion (figures 3(b), (d) and (f)). No proliferation of microglia was evident over the time course of the experiment. Severed and ruptured vessels can be observed near the probe following insertion as dark regions where blood cells interfere with fluorescent signals. In some cases, unsevered vascular features immediately surrounding the probe constricted or degenerated and lost the ability to perfuse over the first 6 h; these appear as dark bands (blood cells) in the fluorescent vasculature (figure 5). Ruptured, but not severed, vessels can be seen to re-perfuse over time.

Acute microglial response

Microglia cells reacted immediately following probe insertion into the cortex (figure 6; and supplementary movie 1, available from <http://stacks.iop.org/JNE/9/066001/mmedia>). Distant microglia cells ($>200 \mu\text{m}$) maintained radially projected processes and showed very few morphological changes (figure 7). These cells continuously moved their processes over time to survey their environment by constantly retracting and radially extending processes, while processes in the hemisphere facing away from the probe were maintained. On the other hand, microglial cells adjacent to the probe projected processes toward the electrode, entering into an activated T-stage morphology (figure 8). In order to quantify microglia activation morphology and correlate this to distance from the probe, 217 microglial cells from five mice were evaluated in images collected 6 h post-implant. The fitted binomial generalized linear regression showed that 50% of the microglial cells that were $130.0 \mu\text{m}$ from the probe expressed T-stage morphology (figure 8(c)).

T-stage morphology index

The morphology index data were plotted as a function of distance and, although variability is evident in this measurement, a significant relationship between index and distance was observed ($r = 0.47$, $p < 0.0001$) (figure 8(d)). Three general regions or clusters were observed in the distribution. One cluster was observed around zero (near the implant location) and included predominantly active microglia. Another cluster ($10\text{--}216 \mu\text{m}$) was observed at a distance of about $100 \mu\text{m}$ from the probe where microglia extended processes in all directions but showed preferential extension toward the implant. This middle cluster had an index of approximately 0.6, showing that these cells on average extended their processes

toward the probe by 60% and retracted processes facing away from the probe by 60%. Lastly, a more sparse population was observed relatively far away from the implant ($>216 \mu\text{m}$) and consisted of normal ramified microglia. A two-shoulder function (equation (2)) was used to characterize this distribution with the following parameter values: relative amplitude (a) of 0.60, near shoulder (d_1) and spread (w_1) of 10 and $13 \mu\text{m}$, respectively, and far shoulder (d_2) and spread (w_2) of 216 and $24 \mu\text{m}$, respectively. To determine the distance at which the microglia morphology is significantly different from 1, the index data were sorted by distance and divided into ten equally sampled bins. Two population t -tests were performed on the index data from each bin against the data from the farthest bin (mean distance of $280 \mu\text{m}$). Significant differences were observed for bin sub-populations with average distances of less than $51 \mu\text{m}$ from the nearest probe ($p < 0.05$ Bonferroni corrected).

Microglia directionality index

Similar to the T-stage morphology index, the microglia directionality index data were plotted as a function of distance (figure 8(e)). Three general regions or clusters were observed in this distribution. A two-shoulder function (equation (2)) was used to characterize this distribution with the following parameter values: relative amplitude (a) of 0.74, near shoulder (d_1) and spread (w_1) of 13 and $5 \mu\text{m}$, respectively, and far shoulder (d_2) and spread (w_2) of 159 and $10 \mu\text{m}$, respectively. One cluster was observed around zero ($<13 \mu\text{m}$) and indicative of active microglia committing all processes toward the implant. A second cluster was observed at a distance of about $13 \mu\text{m}$ to $159 \mu\text{m}$ from the probe where microglia extended processes in all directions but showed a preferential number of processes directed toward the implant. Lastly, a more sparse population was observed $>159 \mu\text{m}$ from the implant representing the normal ramified microglia. This observation is similar to T-state morphology changes (figure 8(d)).

Microglia process projection velocity

Microglia processes from cells within $130 \mu\text{m}$ (obtained from figure 8(c)) of the probe were measured to characterize their process velocity. Processes were extended toward the probe at approximately $(1.6 \pm 1.3) \mu\text{m min}^{-1}$ (602 data points; 34 cells; 5 mice). Process velocities were measured only from cells whose processes stayed within the Z-stack of the image. Within the first hour, processes reached the surface of the electrode, increased in surface area, and formed a lamellipodia sheath around the device. The final velocity reported described the process's extension toward the final contact point of the electrode, which was generally the closest surface of the probe shank. Although microglia process migration was observed both toward the probe shank and recording sites on the probe, there was no specific migration toward recording sites. Figure 8(f) shows that the majority of the processes reach the probe surface and stop around 30–45 minutes as indicated by the near zero migration velocity. Cells contacting the probe undergo substantial morphological changes, simultaneously extending nearby processes toward the probe surface and retracting many processes from the hemisphere facing away from the probe (figure 9). This can be observed initially as retraction of the small filopodia branches and then later by the retraction of the main processes facing away from the probe.

Discussion

It is known that activated microglial cells adopt an amoeboid morphology and increase in density around the implant site days after probe implantation. In this study, we examined a more precise temporal map (minutes to hours) of the microglial response to microelectrode implantation. After probe insertion, microglia cells immediately begin to extend processes toward the implanted electrode at a rate of $(1.6 \pm 1.3) \mu\text{m min}^{-1}$ until 30–45 min, but show very minimal cell body movement over the first 6 h following probe insertion. Six hours

after probe insertion, 50% of the microglia at a distance of 130.0 μm from the probe surface exhibited morphological characteristics of T-stage activation similar to that observed with laser-induced blood–brain barrier (BBB) damage. The transition band from inactive to active identification after 6 h of implantation spanned between 70 μm and 210 μm from the nearest probe surface. An understanding of the time dependence and variability of this response will allow investigators to more effectively probe the mechanisms of the acute tissue response. It will also facilitate the search for molecular cues that initiate inflammation. Additionally, the impact of different intervention strategies and how these lead to divergence from the typical inflammation response can be assessed.

Microglia cell bodies did not migrate to the probe in the first 6 h

Studies have shown that microglial cells aggregate around microelectrodes in an activated state 24 h post-implant (Szarowski *et al* 2003, Purcell *et al* 2009). This aggregation of microglia persists throughout the chronic time period. While microglial cell bodies showed some displacement ($1.1 \pm 7.15 \mu\text{m}$) during the first 6 h, they did not appear to have amoeboid shapes. In addition, many distant and relatively stable vascular structures showed similar displacement (figure 5). Note that cell body displacements were not normalized against blood vessel displacements, but were rather raw distances displaced in the image. The observation that none of the cells appeared to be in an amoeboid state suggests that microglia cell bodies did not migrate through locomotion. While studies in the retina have shown microglial locomotion in the first hour following injury (Eter *et al* 2008, Paques *et al* 2010), studies in the cortex have shown that microglial cell bodies do not move in the first couple of hours (Davalos *et al* 2005). The displacement of brain tissue has been observed after microhemorrhages (Rosidi *et al* 2011), including those caused by probe insertions (Kozai *et al* 2010). It has been observed in slice work that microglia take approximately 12 h to enter into the motile phase after a major insult, such as cutting the brain for *ex vivo* brain slice studies (Stence *et al* 2001). Other studies have also shown that microglia cell bodies have not been observed at higher densities until 24 h post-injury (Rosidi *et al* 2011, Purcell *et al* 2009, Szarowski *et al* 2003). These data suggest that the displacement observed in the first 6 h is due to mechanical strain such as changes in brain hydration/ionic tonicity level, or release of mechanical strain generated during the insertion of the probe, particularly during penetration of the dura.

Microglia rapidly responded to implants by directing processes toward it

Although significant microglial cell body movement was not seen prior to the 6 h time point, activation of these cells was observed immediately following probe insertion. These microglia cells responded by sending long projections toward the probe at a rate of $(1.6 \pm 1.3) \mu\text{m min}^{-1}$ while simultaneously retracting other processes. Similar rates and reaction have been observed for microglia processes toward local vascular injury in the CNS (Nimmerjahn *et al* 2005, Davalos *et al* 2005). These results suggest a chemical gradient is immediately established following probe insertion. Small molecules such as oxygen, bicarbonate, sugars, amino acids and adenosine triphosphate (ATP) have a diffusion coefficient (D) of $\sim 100\text{--}1000 \mu\text{m}^2 \text{s}^{-1}$. These molecules diffuse 200 μm in approximately 10–100 s. Albumin (66 kDa) is released as the BBB is disrupted from probe implantation and has a D of $16.3 \mu\text{m}^2 \text{s}^{-1}$ in cortex, diffusing 137 $\mu\text{m min}^{-1}$ (Tao and Nicholson 1996). Sugars and amino acids would diffuse with electrode insertion as cellular materials get disrupted. Therefore, it is possible that the resulting microglial response results from chemical gradients established by one or more biochemical molecules. For example, increased concentrations of ATP have been shown to moderate microglia response to brain injury (Davalos *et al* 2005). Considering the velocity peaked around 12–14 min, D of the slowest diffusion molecule contributing to the acute response is likely to be greater than $5 \mu\text{m}^2 \text{s}^{-1}$ ($r = 130 \mu\text{m}$; see results section: *microglia process projection velocity*). Larger

molecules may still play a role in the chemical gradient by activating biochemical pathways that release smaller signaling molecules in cells closer to the injury site. Further investigation will be necessary at higher temporal resolution to facilitate identification of molecules contributing to the chemical gradient affecting microglia morphology.

The processes tended to move in small zigzag motions toward the probe, presumably to navigate around other cellular and extracellular structures, such as neurites. Microglia in this form of activation are considered as being in the transitional stage (T-stage) (Stence *et al* 2001). Once they reach the probe surface, they form lamellipodia that encase the device (figures 2 and 8). Much of the microglial processes from cells at distances $<100\ \mu\text{m}$ reached their target in the first 30–45 min, and bulk activity appeared to decrease. Microglia at distances $>200\ \mu\text{m}$ from the interface appear to maintain a ramified morphology and continue to move processes to sample their microenvironment.

Acute microglia morphology changes occurred in the implant microenvironment

While figure 8(c) captures the probability of microglia exhibiting activated morphology with respect to distance from the nearest probe surface, it does not characterize a microglia's morphological preference as a function of distance from the probe. To capture these properties, T-stage morphology index and directionality index were calculated. Indices were normalized onto the same percentile scale as the microglia ramification values (1 = fully ramified morphology, 0 = fully polarized morphology). T-stage morphology index indicated that a majority of the microglia exhibit ramified morphology (index = 1) beyond $216\ \mu\text{m}$ 6 h after probe implantation. On the other hand, microglia $<51\ \mu\text{m}$ away from the probe showed statistically different T-stage morphology (index ≈ 0). Similarly, the microglia directionality index suggests that a majority of the microglia exhibit ramified morphology beyond $159\ \mu\text{m}$ at 6 h after insertion. The two shoulders of figures 8(d) and (e) show that microglia close to the probe exhibit polarized morphology while microglia at intermediate distances appear to maintain some processes in all directions while extending longer processes toward the probe.

Theoretically, a microglia cell cannot morphologically become more ramified than fully ramified (index > 1). Due to the image size ($407.5 \times 407.5\ \mu\text{m}$), the number of cell bodies $> 200\ \mu\text{m}$ from the probe surface captured in the images was substantially fewer in population to those $<100\ \mu\text{m}$, resulting in the larger variability in the T-stage morphology and directionality indices observed further from the probe. Additionally, the data points far away are impacted by normal microglia behavior where they extend processes in apparent random directions to sample their environment and would be manifested as increased variability to the index metric. It is also possible that the microglia are activating toward damage distant from the probe, such as microhemorrhages in the BBB. Zooming out or serially scanning, then stitching *ZT* stacks of adjacent regions (*ZTY* scan) can address this issue, but at the cost of spatial resolution or temporal resolution, respectively, hampering the ability to quantify microglia process movement.

The relatively high morphology index near the probe may be explained by the fact that microglia cells in these locations do not have much distance to project a process. Even if the cells retract processes from the opposite direction, the index may be high because extension toward the probe may be short due to the shorter distance to travel. For this reason, the directionality index was used to complement the T-stage morphology index because the number of processes is not limited by the available travel distance to the probe surface. However, it is also possible that microglia may be responding to other targets away from the probe surface, such as a distant probe shank, damaged cells, neurites for activity dependent remodeling or distant vascular damage (Nimmerjahn *et al* 2005, Derecki *et al* 2012, Paolicelli *et al* 2011, Schafer *et al* 2012, Tremblay *et al* 2010, Wake *et al* 2009). To try to

minimize this source of variability, the probe insertion method included previously established techniques to target regions of the brain with low densities of major blood vessels ($>5\ \mu\text{m}$ diameter) (Kozai *et al* 2010). Future studies should look to understand if and how variability might be related to vascular integrity by comparing electrodes purposefully implanted through a large vasculature and away from major vasculature.

Acute BBB microhemorrhage and reorganization to implanted electrode

Insertion of neural probes inevitably disrupts blood–brain barrier (BBB) integrity and causes microhemorrhages. The degree of microhemorrhages from probe insertion has been shown to be uncontrollable and difficult to reproduce across implants, mirroring the large variability in inflammatory tissue responses and chronic recording success. In *ex vivo* studies, compression and rupture of the transcranial BBB have been observed as far as 300 μm from the probe during insertion (Bjornsson *et al* 2006). An *in vivo* multiphoton imaging study has shown that blood cells and plasma infiltration into the central nervous system (CNS) is greatly influenced by the proximity of probe insertion location to large penetrating vasculature in the cortex (Kozai *et al* 2010). Furthermore, microhemorrhages have been shown to cause intracranial pressure and compression of brain tissue (Xi *et al* 2006, Yang *et al* 1994, Rosidi *et al* 2011). A combination of membrane impermeable dyes and microelectrode implantation show that tissue displacement from probe volume and vasogenic edema can cause damage to cell membranes multiple cell layers thick (Rohatgi *et al* 2009, Retterer *et al* 2008). As the probe punctures and tears the neural vasculature, the highly regulatory BBB is compromised, depositing molecules foreign to the CNS, such as albumin, immunoglobulins (IgG/IgM/IgA), fibrinogen, complement, and red blood cells (Cao *et al* 2007, Alafuzoff *et al* 1983, Winslow *et al* 2010, Gasque *et al* 2000, Fitch *et al* 1999), all of which could trigger CNS immune responses and neurodegeneration, ultimately leading to failures in neural recording.

In this study, we not only observed blood vessel damage during probe insertion, but that some intact capillaries around the probe also lost the ability to perfuse over time (figure 4). These capillaries appear to have thick dark bands where blood cells have stopped flowing. Over the 6 h time period, these capillaries decreased in diameter (figure 4) to prevent normal blood cell flow. This loss of perfusion and vascular reorganization might contribute to the ischemic microenvironment immediately surrounding the electrode (Zhang *et al* 2005, Zhang and Murphy 2007). Additionally, major vessels appear to become more dilated. This may be due to natural vasomotion, anesthesia, variation in animal condition, pressure build up from perfusion loss in capillaries, or vascular feedback to compensate for the local ischemic environment due to blood vessels severed by the probe. These vascular fluctuations and reorganizations near the electrode may intensify the tissue strain and drift.

Although studies have suggested that clotting from penetrating arterioles occurs on the order of seconds (Rosidi *et al* 2011), microhemorrhage can contribute additional cascades of injury chronically in the microenvironment immediately surrounding the probe through adsorption of inflammation molecules from the plasma and nearby cells, oxidative stress and free radicals from the plasma, red blood cell breakdown, vasogenic brain edema and cytotoxic swelling (Xi *et al* 2001, Jones *et al* 2002, Betz *et al* 1989, Klatzo 1967, Barzo *et al* 1997, Kimelberg 1995).

Mechanical strain induced chronic BBB leakage

Chronic leakage of the BBB has yet to be rigorously demonstrated. IgGs are a family of plasma proteins that are often associated with altered brain function when found in the neutrophil (Poser *et al* 1983, Hanly *et al* 1994, Wilson *et al* 1979, Azzi *et al* 1990, Seitz *et al* 1985, Aihara *et al* 1994, Somjen 2001). IgG is also expressed after probe implantation for

various periods of time (Winslow *et al* 2010), suggesting chronic leakage of the BBB. However, others have demonstrated that astrocytes sequester IgG and express IgG positivity for at least nine months following a stab wound in the CNS (Bernstein and Goldberg 1987, Bernstein *et al* 1993), indicating that continued IgG expression may not necessarily be due to chronic vascular leakage from implanted device.

In this experiment, microglia processes were at times observed to advance toward and touch vascular structures up to 170 μm away from the probe surface hours after probe insertion (figure 10(a)). This activity may contribute to the variability in the index metrics used. It is unclear what signaled their activation at this time. While the animal and probe were secured through a stereotaxic frame and manipulator, it is possible that mechanical strain (insertion strain, probe volume strain, vasogenic edema, cytotoxic swelling), physiological motion (changes in hydration level/tonicity, physiological pulsing), and/or micromotion (IP anesthesia injections) might induce vascular leakage in vessels distant from the probe surface well after probe insertion. Even if blood cells were not observed escaping from the vasculature, it is possible that plasma and small plasma proteins might leak out from the strained BBB and activate microglia. Mechanical strain of the stiff devices tethered to the skull have been investigated and are thought to trigger the persistent chronic tissue reaction around the implant (Edell *et al* 1992, Biran *et al* 2007, Kim *et al* 2004, Subbaroyan *et al* 2005, Lee *et al* 2005, Gilletti and Muthuswamy 2006). These results suggest that the mechanical strain in the neural tissue may increase BBB damage and microhemorrhages during physiological motion or micromotion independent of the initial insertion injury. Future investigation should focus on quantifying cortical microelectrode induced interaction between microglia and vasculature, as well as interaction under normal conditions (Hanisch and Kettenmann 2007). If the strain from the mechanical mismatch between electrode and brain tissue causes chronic vascular leakage, it may be a source for perpetual inflammatory cascade.

Toward improving bio-integration of neural interfaces

In vivo two-photon microscopy is experiencing a large growth in biomedical research because it allows detailed observation of submicron features beyond the range of confocal microscopy (Helmchen and Denk 2005, Dunn and Sutton 2008, Zipfel *et al* 2003, Svoboda and Yasuda 2006). Traditionally, these types of investigations require discrete endpoint post-mortem histology or *ex vivo* brain tissue slice preparation. In both cases, the brain tissue must be excised from its native environment and extensively processed. This limits the degree to which dynamic changes can be studied during and after probe implantation and makes it more challenging to tease apart inter-animal variability. Multiphoton microscopy has allowed us to study dynamic changes in microglial cells and vasculature after microelectrode implantation over time. However, there remain some limitations and challenges to *in vivo* microelectrode multiphoton microscopy. *In vivo* imaging of electrode implantation is easier when the electrode penetrates at an angle simply due to physical constraints. An electrode can be fabricated so that it is inserted perpendicular to the surface and then flattened to run parallel to the cortex to avoid contact with the objective. Alternatively, the objective or the object could be rotated but physical constraints, particularly in the presence of a chronic chamber, limit perpendicular electrode penetration. One strategy for turning planar imaging into perpendicular imaging would be to implant a prism into the brain (Chia and Levene 2009), but this compresses the tissue significantly, causes massive bleeding and induces its own microglia reaction.

Understanding the timing, mechanism and source of variability of the chronic tissue response would allow investigators to focus on specific aspects of chronic probe design, such as bioactive coatings (Azemi *et al* 2011), stem cell seeding (Purcell *et al* 2009, Azemi *et al* 2010), drug delivery (Rohatgi *et al* 2009, Luo *et al* 2011), advanced probe architectures

(Seymour and Kipke 2007), flexibility (Kozai and Kipke 2009, Subbaroyan *et al* 2005, Gilgunn *et al* 2012) and navigating microstructures in the brain (Kozai *et al* 2010). This will be particularly interesting in understanding the influence of probe flexibility by examining if and how the elastic modulus and compliance of probes lead to a divergence in microglial response from silicon and metal devices. Additionally, mapping the dynamic tissue response will improve optimization of timing for triggered drug release (Luo *et al* 2011, Wadhwa *et al* 2006), delayed drug release (Kim and Martin 2006) and controlled drug release systems (Kim *et al* 2010). Another example is laminin and L1CAM coated electrodes. At four weeks, both laminin and L1 coated microelectrodes show reduced microglial activity around the implant, but show divergent neural health in the microenvironment around the devices (Azemi *et al* 2011, He *et al* 2006). Following dynamic changes and interaction of microglia with the coating and the surrounding tissue may elucidate why the neural population around the biomolecule coated electrodes differ. Identifying the temporal effects of new devices and new materials on the microglial response may help investigators understand inflammation chemical pathways in the CNS and improve treatments by targeting molecules in those specific pathways.

Microglia cells have been shown to rapidly encapsulate damaged blood vessels with their processes (Nimmerjahn *et al* 2005, Rosidi *et al* 2011). In our experiment, disrupting the BBB with a laser resulted in a similar microglial response to an inserted probe (figure 10(b); and supplementary movie 2 available from stacks.iop.org/JNE/9/066001/mmedia). Processes extended from microglia toward the damaged vessel surface. In addition, other processes that were facing away from the injury site were rapidly re-absorbed. Previous studies suggest that microglial response after laser-induced microhemorrhage is at least in part dependent on bleeding into the tissue (Rosidi *et al* 2011). Interestingly, T-stage activated microglia that advanced their processes toward these distant vascular structures tended to retract their processes over time (figure 10(a)). Further investigation should focus on understanding the relationship or decoupling the relationship between these observations and vascular integrity. If a relationship is established, focus should be placed on identifying and understanding the molecular signals that facilitate the retraction of microglial processes from damaged vasculature.

While this setup can facilitate the study of acute reactive tissue responses and rapid screening of intervention strategies, a chronic response may drastically diverge or an intervention strategy may only temporarily delay the onset of a chronic response. Furthermore, it is difficult to maintain anesthetized mice under good physiological conditions for periods exceeding 7–8 h. Instances of microglia migration were observed >12 h but none significant enough to report, longer recording times would be necessary to visualize cell migration. A chronic setup is necessary to reliably study the timing and characterization of *in vivo* M- or L-stage microglia migration. Chronic studies are underway using advanced setup combining omnetics packaged electrodes and a glass coverslip sealed craniotomy window for chronic multiphoton imaging and electrophysiology (Nimmerjahn 2012).

It may be impossible to prevent activation of microglia from the probe insertion-induced injury. However, the observation that microglia processes can withdraw after entering a transitional activated stage suggests that it may be possible to drive microglia to retract their processes once they reach the surface of the device through signaling molecules immobilized on the surface or released from surface coating. The feasibility of such treatments is further supported by the fact that microglia do not become motile for over 6 h post insertion injury, which provides a sufficient window for intervention. It is not well understood if this delay is a programmed delay or if the cell needs this time to undergo necessary structural changes to become motile. However, applying the proper molecular

signals before the microglia become motile may reverse the activation of T-stage microglia, induce a transition back into the normal ramified state and prevent the cascade of the reactive tissue response. One region where this cue may be found is on healthy or repaired BBB. A better understanding of cellular and molecular mechanisms of probe insertion injury and inflammation may lead to identification of cues to attenuate the reactive tissue response. These cues may be used or mimicked, such as in the form of surface modification or controlled drug release, to design bioinspired intervention strategies to improve chronic recording electrode performance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Ania K Majewska for helpful physiology discussions and Mitsuhiro 'Hiro' Fukuda for assistance with the experimental equipment. This work was financially supported by NIH R01 (5R01NS062019 and 5R01NS44589) and an NIH K-Grant (1K01NS066131). The authors would also like to thank Christi L Kolarcik and Noah R Snyder for assistance editing the manuscript.

References

- Abdul-Karim MA, Al-Kofahi K, Brown EB, Jain RK, Roysam B. Automated tracing and change analysis of angiogenic vasculature from *in vivo* multiphoton confocal image time series. *Microvasc. Res.* 2003; 66:113–125. [PubMed: 12935769]
- Aihara N, Tanno H, Hall JJ, Pitts LH, Noble LJ. Immunocytochemical localization of immunoglobulins in the rat brain: relationship to the blood–brain barrier. *J. Comp. Neurol.* 1994; 342:481–496. [PubMed: 8040362]
- Alafuzoff I, Adolfsson R, Bucht G, Winblad B. Albumin and immunoglobulin in plasma and cerebrospinal fluid, and blood–cerebrospinal fluid barrier function in patients with dementia of Alzheimer type and multi-infarct dementia. *J. Neurol. Sci.* 1983; 60:465–472. [PubMed: 6631444]
- Azemi E, Gobbel GT, Cui XT. Seeding neural progenitor cells on silicon-based neural probes. *J. Neurosurg.* 2010; 113:673–681. [PubMed: 20151783]
- Azemi E, Lagenaur CF, Cui XT. The surface immobilization of the neural adhesion molecule L1 on neural probes and its effect on neuronal density and gliosis at the probe/tissue interface. *Biomaterials.* 2011; 32:681–692. [PubMed: 20933270]
- Azzi G, Bernaudin JF, Bouchaud C, Bellon B, Fleury-Feith J. Permeability of the normal rat brain, spinal cord and dorsal root ganglia microcirculations to immunoglobulins G. *Biol. Cell.* 1990; 68:31–36. [PubMed: 2317595]
- Barzo P, Marmarou A, Fatouros P, Hayasaki K, Corwin F. Contribution of vasogenic and cellular edema to traumatic brain swelling measured by diffusion-weighted imaging. *J. Neurosurg.* 1997; 87:900–907. [PubMed: 9384402]
- Bernstein JJ, Goldberg WJ. Injury-related spinal cord astrocytes are immunoglobulin-positive (IgM and/or IgG) at different time periods in the regenerative process. *Brain Res.* 1987; 426:112–118. [PubMed: 3690308]
- Bernstein JJ, Willingham LA, Goldberg WJ. Sequestering of immunoglobulins by astrocytes after cortical lesion and homografting of fetal cortex. *Int. J. Dev. Neurosci.* 1993; 11:117–124. [PubMed: 8328295]
- Betz AL, Iannotti F, Hoff JT. Brain edema: a classification based on blood–brain barrier integrity *Cerebrovasc. Brain Metab. Rev.* 1989; 1:133–154.
- Biran R, Martin DC, Tresco PA. Neuronal cell loss accompanies the brain tissue response to chronically implanted silicon microelectrode arrays. *Exp. Neurol.* 2005; 195:115–126. [PubMed: 16045910]
- Biran R, Martin DC, Tresco PA. The brain tissue response to implanted silicon microelectrode arrays is increased when the device is tethered to the skull. *J. Biomed. Mater. Res.* 2007; A 82:169–178.

- Bjornsson CS, Oh SJ, Al-Kofahi YA, Lim YJ, Smith KL, Turner JN, De S, Roysam B, Shain W, Kim SJ. Effects of insertion conditions on tissue strain and vascular damage during neuroprosthetic device insertion. *J. Neural Eng.* 2006; 3:196–207. [PubMed: 16921203]
- Cao L, Chang M, Lee CY, Castner DG, Sukavaneshvar S, Ratner BD, Horbett TA. Plasma-deposited tetraglyme surfaces greatly reduce total blood protein adsorption, contact activation, platelet adhesion, platelet procoagulant activity, and *in vitro* thrombus deposition. *J. Biomed. Mater. Res.* 2007; A 81:827–837.
- Chauhan VP, Lanning RM, Diop-Frimpong B, Mok W, Brown EB, Padera TP, Boucher Y, Jain RK. Multiscale measurements distinguish cellular and interstitial hindrances to diffusion *in vivo*. *Biophys. J.* 2009; 97:330–336. [PubMed: 19580771]
- Chia TH, Levene MJ. Micropisms for *in vivo* multilayer cortical imaging. *J. Neurophysiol.* 2009; 102:1310–1314. [PubMed: 19494189]
- Cui XY, Martin DC. Electrochemical deposition and characterization of poly(3,4-ethylenedioxythiophene) on neural microelectrode arrays. *Sensors Actuators.* 2003; B 89:92–102.
- Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, Littman DR, Dustin ML, Gan WB. ATP mediates rapid microglial response to local brain injury *in vivo*. *Nat. Neurosci.* 2005; 8:752–758. [PubMed: 15895084]
- Derecki NC, Cronk JC, Lu Z, Xu E, Abbott SB, Guyenet PG, Kipnis J. Wild-type microglia arrest pathology in a mouse model of Rett syndrome. *Nature.* 2012; 484:105–109. [PubMed: 22425995]
- 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. Methods.* 2010; 7:981–984. [PubMed: 20966916]
- Dunn KW, Sutton TA. Functional studies in living animals using multiphoton microscopy. *ILAR J.* 2008; 49:66–77. [PubMed: 18172334]
- Edell DJ, Toi VV, Mcneil VM, Clark LD. Factors influencing the biocompatibility of insertable silicon microshafts in cerebral cortex. *IEEE Trans. Biomed. Eng.* 1992; 39:635–643. [PubMed: 1601445]
- Eter N, Engel DR, Meyer L, Helb HM, Roth F, Maurer J, Holz FG, Kurts C. visualization of dendritic cells, macrophages, and microglial cells responding to laser-induced damage in the fundus of the eye. *Invest. Ophthalmol. Vis. Sci.* 2008; 49:3649–3658. [PubMed: 18316698]
- Fitch MT, Dolfer C, Combs CK, Landreth GE, Silver J. Cellular and molecular mechanisms of glial scarring and progressive cavitation: *in vivo* and *in vitro* analysis of inflammation-induced secondary injury after CNS trauma. *J. Neurosci.* 1999; 19:8182–8198. [PubMed: 10493720]
- Gasque P, Dean YD, Mcgreal EP, Vanbeek J, Morgan BP. Complement components of the innate immune system in health and disease in the CNS. *Immunopharmacology.* 2000; 49:171–186. [PubMed: 10904116]
- Gilgunn PJ, Khilwani R, Kozai TDY, Weber DJ, Cui XT, Erdos G, Ozdoganlar OB, Fedder GK. An ultra-compliant, scalable neural probes with molded biodissolvable delivery vehicle. 25th Int. Conf. on Micro Electro Mechanical Systems (MEMS), IEEE 2012. 2012:56–59.
- Gilletti A, Muthuswamy J. Brain micromotion around implants in the rodent somatosensory cortex. *J. Neural Eng.* 2006; 3:189–195. [PubMed: 16921202]
- Hailer NP, Bechmann I, Heizmann S, Nitsch R. Adhesion molecule expression on phagocytic microglial cells following anterograde degeneration of perforant path axons. *Hippocampus.* 1997; 7:341–349. [PubMed: 9228530]
- Hailer NP, Jarhult JD, Nitsch R. Resting microglial cells *in vitro* analysis of morphology and adhesion molecule expression in organotypic hippocampal slice cultures. *Glia.* 1996; 18:319–331. [PubMed: 8972800]
- Hanisch UK, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat. Neurosci.* 2007; 10:1387–1394. [PubMed: 17965659]
- Hanly JG, Fisk JD, Eastwood B. Brain reactive autoantibodies and cognitive impairment in systemic lupus erythematosus. *Lupus.* 1994; 3:193–199. [PubMed: 7951305]
- He W, McConnell GC, Bellamkonda RV. Nanoscale laminin coating modulates cortical scarring response around implanted silicon microelectrode arrays. *J. Neural Eng.* 2006; 3:316–326. [PubMed: 17124336]

- Helmchen F, Denk W. Deep tissue two-photon microscopy. *Nat. Methods*. 2005; 2:932–940. [PubMed: 16299478]
- Jones LL, Yamaguchi Y, Stallcup WB, Tuszynski MH. NG2 is a major chondroitin sulfate proteoglycan produced after spinal cord injury and is expressed by macrophages and oligodendrocyte progenitors. *J. Neurosci*. 2002; 22:2792–2803. [PubMed: 11923444]
- Kim DH, Martin DC. Sustained release of dexamethasone from hydrophilic matrices using PLGA nanoparticles for neural drug delivery. *Biomaterials*. 2006; 27:3031–3037. [PubMed: 16443270]
- Kim DH, Wiler JA, Anderson DJ, Kipke DR, Martin DC. Conducting polymers on hydrogel-coated neural electrode provide sensitive neural recordings in auditory cortex. *Acta Biomater*. 2010; 6:57–62. [PubMed: 19651250]
- Kim YT, Hitchcock RW, Bridge MJ, Tresco PA. Chronic response of adult rat brain tissue to implants anchored to the skull. *Biomaterials*. 2004; 25:2229–2237. [PubMed: 14741588]
- Kimelberg HK. Current concepts of brain edema. Review of laboratory investigations. *J. Neurosurg*. 1995; 83:1051–1059. [PubMed: 7490620]
- Klatzo I. Presidential address. Neuropathological aspects of brain edema. *J. Neuropathol. Exp. Neurol*. 1967; 26:1–14. [PubMed: 5336776]
- Kozai TDY, Kipke DR. Insertion shuttle with carboxyl terminated self-assembled monolayer coatings for implanting flexible polymer neural probes in the brain. *J. Neurosci. Methods*. 2009; 184:199–205. [PubMed: 19666051]
- Kozai TDY, Marzullo TC, Hooi F, Langhals NB, Majewska AK, Brown EB, Kipke DR. Reduction of neurovascular damage resulting from microelectrode insertion into the cerebral cortex using *in vivo* two-photon mapping. *J. Neural Eng*. 2010; 7 046011.
- Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci*. 1996; 19:312–318. [PubMed: 8843599]
- Lee H, Bellamkonda RV, Sun W, Levenston ME. Biomechanical analysis of silicon microelectrode-induced strain in the brain. *J. Neural Eng*. 2005; 2:81–89. [PubMed: 16317231]
- Liu X, McCreery DB, Bullara LA, Agnew WF. Evaluation of the stability of intracortical microelectrode arrays. *IEEE Trans. Neural Syst. Rehabil. Eng*. 2006; 14:91–100. [PubMed: 16562636]
- Liu X, McCreery DB, Carter RR, Bullara LA, Yuen TG, Agnew WF. Stability of the interface between neural tissue and chronically implanted intracortical microelectrodes. *IEEE Trans. Rehabil. Eng*. 1999; 7:315–326. [PubMed: 10498377]
- Lowery RL, Zhang Y, Kelly EA, Lamantia CE, Harvey BK, Majewska AK. Rapid, long-term labeling of cells in the developing and adult rodent visual cortex using double-stranded adeno-associated viral vectors. *Dev. Neurobiol*. 2009; 69:674–688. [PubMed: 19551873]
- Luo X, Matrangola C, Tan S, Alba N, Cui XT. Carbon nanotube nanoreservoir for controlled release of anti-inflammatory dexamethasone. *Biomaterials*. 2011; 32:6316–6323. [PubMed: 21636128]
- Majewska A, Brown E, Ross J, Yuste R. Mechanisms of calcium decay kinetics in hippocampal spines: role of spine calcium pumps and calcium diffusion through the spine neck in biochemical compartmentalization. *J. Neurosci*. 2000; 20:1722–1734. [PubMed: 10684874]
- McConnell GC, Rees HD, Levey AI, Gutekunst CA, Gross RE, Bellamkonda RV. Implanted neural electrodes cause chronic, local inflammation that is correlated with local neurodegeneration. *J. Neural. Eng*. 2009; 6:56003.
- Nicolelis MA, Dimitrov D, Carmena JM, Crist R, Lehew G, Kralik JD, Wise SP. Chronic, multisite, multielectrode recordings in macaque monkeys. *Proc. Natl Acad. Sci. USA*. 2003; 100:11041–11046. [PubMed: 12960378]
- Nimmerjahn A. Optical window preparation for two-photon imaging of microglia in mice. *Cold Spring Harb. Protoc*. 2012
- Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma *in vivo*. *Science*. 2005; 308:1314–1318. [PubMed: 15831717]
- Paolicelli RC, et al. Synaptic pruning by microglia is necessary for normal brain development. *Science*. 2011; 333:1456–1458. [PubMed: 21778362]
- Paques M, Simonutti M, Augustin S, Goupille O, El Mathari B, Sahel JA. *In vivo* observation of the locomotion of microglial cells in the retina. *Glia*. 2010; 58:1663–1668. [PubMed: 20578032]

- Polikov VS, Tresco PA, Reichert WM. Response of brain tissue to chronically implanted neural electrodes. *J. Neurosci. Methods*. 2005; 148:1–18. [PubMed: 16198003]
- Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, Johnson KP, Sibley WA, Silberberg DH, Tourtellotte WW. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann. Neurol*. 1983; 13:227–231. [PubMed: 6847134]
- Purcell EK, Seymour JP, Yandamuri S, Kipke DR. *In vivo* evaluation of a neural stem cell-seeded prosthesis. *J. Neural. Eng.* 2009; 6 026005.
- Raivich G, Bohatschek M, Kloss CU, Werner A, Jones LL, Kreutzberg GW. Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. *Brain Res. Brain Res. Rev.* 1999; 30:77–105. [PubMed: 10407127]
- Retterer ST, Smith KL, Bjornsson CS, Turner JN, Isaacson MS, Shain W. Constant pressure fluid infusion into rat neocortex from implantable microfluidic devices. *J. Neural Eng.* 2008; 5:385–391. [PubMed: 18827310]
- Rohatgi P, Langhals NB, Kipke DR, Patil PG. *In vivo* performance of a microelectrode neural probe with integrated drug delivery. *Neurosurg. Focus*. 2009; 27:E8. [PubMed: 19569896]
- Rosidi NL, Zhou J, Pattanaik S, Wang P, Jin W, Brophy M, Olbricht WL, Nishimura N, Schaffer CB. Cortical microhemorrhages cause local inflammation but do not trigger widespread dendrite degeneration. *PloS One*. 2011; 6:e2661.
- Rousche PJ, Normann RA. Chronic recording capability of the Utah Intracortical Electrode Array in cat sensory cortex. *J. Neurosci. Methods*. 1998; 82:1–15. [PubMed: 10223510]
- Schafer DP, Lehrman EK, Kautzman AG, Koyama R, Mardinly AR, Yamasaki R, Ransohoff RM, Greenberg ME, Barres BA, Stevens B. Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron*. 2012; 74:691–705. [PubMed: 22632727]
- Schwartz AB, Cui XT, Weber DJ, Moran DW. Brain-controlled interfaces: movement restoration with neural prosthetics. *Neuron*. 2006; 52:205–220. [PubMed: 17015237]
65. Seitz RJ, Heininger K, Schwendemann G, Toyka KV, Wechsler W. The mouse blood–brain barrier and blood–nerve barrier for IgG: a tracer study by use of the avidin–biotin system. *Acta Neuropathol*. 1985; 68:15–21. [PubMed: 3901654]
- Seymour JP, Kipke DR. Neural probe design for reduced tissue encapsulation in CNS. *Biomaterials*. 2007; 28:3594–3607. [PubMed: 17517431]
- Somjen GG. Mechanisms of spreading depression and hypoxic spreading depression-like depolarization. *Physiol. Rev.* 2001; 81:1065–1096. [PubMed: 11427692]
- Stence N, Waite M, Dailey ME. Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. *Glia*. 2001; 33:256–266. [PubMed: 11241743]
- Subbaroyan J, Martin DC, Kipke DR. A finite-element model of the mechanical effects of implantable microelectrodes in the cerebral cortex. *J. Neural Eng.* 2005; 2:103–113. [PubMed: 16317234]
- Svoboda K, Yasuda R. Principles of two-photon excitation microscopy and its applications to neuroscience. *Neuron*. 2006; 50:823–839. [PubMed: 16772166]
- Szarowski DH, Andersen MD, Retterer S, Spence AJ, Isaacson M, Craighead HG, Turner JN, Shain W. Brain responses to micro-machined silicon devices. *Brain Res*. 2003; 983:23–35. [PubMed: 12914963]
- Tao L, Nicholson C. Diffusion of albumins in rat cortical slices and relevance to volume transmission. *Neuroscience*. 1996; 75:839–847. [PubMed: 8951877]
- Tremblay ME, Lowery RL, Majewska AK. Microglial interactions with synapses are modulated by visual experience. *PLoS Biol*. 2010; 8 e1000527.
- Wadhwa R, Lagenaur CF, Cui XT. Electrochemically controlled release of dexamethasone from conducting polymer polypyrrole coated electrode. *J. Control. Release*. 2006; 110:531–541. [PubMed: 16360955]
- Wake H, Moorhouse AJ, Jinno S, Kohsaka S, Nabekura J. Resting microglia directly monitor the functional state of synapses *in vivo* and determine the fate of ischemic terminals. *J. Neurosci*. 2009; 29:3974–3980. [PubMed: 19339593]
- Ward MP, Rajdev P, Ellison C, Irazoqui PP. Toward a comparison of microelectrodes for acute and chronic recordings. *Brain Res*. 2009; 1282:183–200. [PubMed: 19486899]

- Williams JC, Rennaker RL, Kipke DR. Long-term neural recording characteristics of wire microelectrode arrays implanted in cerebral cortex. *Brain Res. Brain Res. Protoc.* 1999; 4:303–313. [PubMed: 10592339]
- Wilson HA, Winfield JB, Lahita RG, Koffler D. Association of IgG anti-brain antibodies with central nervous system dysfunction in systemic lupus erythematosus. *Arthritis Rheum.* 1979; 22:458–462. [PubMed: 375939]
- Winslow BD, Christensen MB, Yang WK, Solzbacher F, Tresco PA. A comparison of the tissue response to chronically implanted parylene-C-coated and uncoated planar silicon microelectrode arrays in rat cortex. *Biomaterials.* 2010; 31:9163–9172. [PubMed: 20561678]
- Woolley AJ, Desai HA, Steckbeck MA, Patel NK, Otto KJ. *In situ* characterization of the brain–microdevice interface using device capture histology. *J. Neurosci. Methods.* 2011; 201:67–77. [PubMed: 21802446]
- Xi G, Hua Y, Bhasin RR, Ennis SR, Keep RF, Hoff JT. Mechanisms of edema formation after intracerebral hemorrhage: effects of extravasated red blood cells on blood flow and blood–brain barrier integrity. *Stroke.* 2001; 32:2932–2938. [PubMed: 11739998]
- Xi G, Keep RF, Hoff JT. Mechanisms of brain injury after intracerebral haemorrhage. *Lancet Neurol.* 2006; 5:53–63. [PubMed: 16361023]
- Yang GY, Betz AL, Chenevert TL, Brunberg JA, Hoff JT. Experimental intracerebral hemorrhage: relationship between brain edema, blood flow, and blood–brain barrier permeability in rats. *J. Neurosurg.* 1994; 81:93–102. [PubMed: 8207532]
- Zhang S, Boyd J, Delaney K, Murphy TH. Rapid reversible changes in dendritic spine structure *in vivo* gated by the degree of ischemia. *J. Neurosci.* 2005; 25:5333–5338. [PubMed: 15930381]
- Zhang SX, Murphy TH. Imaging the impact of cortical microcirculation on synaptic structure and sensory-evoked hemodynamic responses *in vivo*. *PLoS Biol.* 2007; 5:1152–1167.
- Zipfel WR, Williams RM, Webb WW. Nonlinear magic: multiphoton microscopy in the biosciences. *Nature Biotechnol.* 2003; 21:1369–1377. [PubMed: 14595365]

Figure 1.

Experimental setup for *in vivo* multiphoton imaging of microenvironment surrounding implanted microelectrodes in mouse cortex. (a) A small dental acrylic well is made on the skull over the cortex region of interest. A craniotomy is made in the center of the well, and the probe is inserted through the dural tissue at a 30–35° angle. (b) Light microscope image of the probe implanted into cortex at a 30° angle. (c) Four-shank Michigan probe drawing overlaid on an *in vivo* multiphoton image of transgenic GFP labeled microglial cells ~100 μm below the surface of the cortex. The white square indicates the image domain of all following figures. (d) Normal ramified microglia morphology from an unimplanted brain. Scale bars indicate 100 μm.

Figure 2.

3D projection and 3D reconstruction 7 h post implant. (a) Z -stack projection of the cortex from the surface of the brain. Solid lines indicate the outline of the probe. Dotted lines indicate the region used for 3D reconstruction labeled as b and c . (b) Z - X image: Y -stack projection along the probe shank. Solid overlay indicates probe. (c) Z' - Y image: X' -stack projection such that the Z -axis is rotated 30° into the plane of the image so that the probe shanks are normal to the image. Solid overlay indicates probe. Scale bar indicates 100 μm .

Figure 3.

Tissue movement over the first 6 h. (a, b) Images taken at 1 h. (c, d) Images taken at 6 h. Plots (a) and (c) show microglia movement over the first 6 h. Plots (b) and (d) show displacement in blood vessels. Plot (e) shows cell bodies being displaced from initial implantation (red) and 6 h after implant (green) near the electrode, but cell bodies away from the electrode show minimal movement (yellow). Plot (f) shows similar displacement in blood vessels suggesting that at least some of the cell body movement may be due to relaxation of tissue strain after probe insertion. Blue/white transparencies indicate probe outline and recording sites. Cyan arrow indicates a point of reference for probe drift due to mechanical strain. Scale bar is 100 μm .

Figure 4.

Microglia cell body displacement toward the probe over the course of the first 6 h. Positive values indicate displacement toward the probe. Negative values indicate cell body displacement away from the probe. (a) Microglia cell body displacement sorted by distance from the probe in 20 µm bins. Error bars show standard deviation. Star indicates size of each bin (N). (b) Scatter plot of all microglia cell body displacements plotted against distance from the probe. Red line represents best fit line $y = 0.010x + 0.17$ with $R^2 = 0.0072$.

Figure 5.

Vasculature changes. 100 μm thick Z-stack projection of vasculature labeled with a dye. Blue outline indicates probe and recording sites. (a) 1 h post implant. Vasculature severed or ruptured during insertion can be observed (red left/right arrows). Green up arrows and yellow down arrows indicate perfused capillaries. Yellow right square bracket indicates diameter of perfused capillary. (b) 7 h post implant. In some cases, when the vessel is ruptured but not severed, reperfusion can be observed (red right arrow). While vasculature can be severed during insertion, additional blood flow damage can occur in unsevered blood vessels as they shrink (yellow down arrows) and lose the ability to perfuse (green up arrows) in the first few hours. Yellow left square bracket indicates diameter of constricted capillary. Loss of perfusion can be visualized as dark bands in fluorescently labeled vessels where blood cells have stopped flowing. Scale bar indicates 100 μm .

Figure 6.

Microglia processes advance toward the electrode immediately after insertion. Blue transparencies indicate location of implanted probes. Earlier time points (red) are overlaid with later time points (green). Time points are indicated in red (before) and green (after) above the top right corner of each panel. Green processes closer to the probe indicate microglial processes advancing toward the probe. Yellow cell bodies indicate that virtually no cell body movement occurs in the first hour. Scale bar is 100 μm . White boxes indicate region of interest for figures 7–9.

Figure 7.

Ramified microglia morphology. (a) Normal microglia $\sim 245 \mu\text{m}$ from the probe imaged every 30 min between 1 min and 2 h. (b) Image shows a differential projection (green) indicating areas sampled by creation and retraction of processes over time and static features (red). Scale bar is $10 \mu\text{m}$.

Figure 8.

Details of microglial process migration. (a, b) Zoomed in features of microglia process migration in two locations over 1 to 5 min, 5 to 10 min, 10 to 15 min, 15 to 30 min, 30 to 60 min, 60 to 90 min, and 90 to 120 min. Green indicates creation of new features and red indicates lost features. Yellow shows common features. Blue transparency indicates location of the probe. As processes reach the probe, they flatten along the surface and form lamellipodia. Scale bar is 100 μm . (c) Characterization of microglia ramification versus distance from the probe. 1 represents normal microglia ramification, while 0 represents loss of ramification and activation. Solid line is the best fit logarithmic binomial generalized linear regression curve $\log(p/(1-p)) = -7.63x + 0.0587$. $p = 0.5$ at 130.0 μm from the probe

surface. (d) T-stage morphology index where 1 is the ramified state and 0 is the transitional stage of activated microglia. (e) Microglia directionality index where 0 is the microglia state when all its processes are directed toward the probe, and 1 is when the cell is ramified. (f) Average velocity of 18 microglia processes migration (whose bulbous process ending remained in the plane of the image) toward the probe imaged continuously in a contiguous Z-plane over 70 min. Error bars show standard error.

\$watermark-text

\$watermark-text

\$watermark-text

Figure 9.

Morphological changes of microglia in response to probe insertion. (a) Morphological changes of a microglia $\sim 57 \mu\text{m}$ from the surface of the electrode at 1 min, 10 min, 15 min, 20 min, 22 min, 30 min, 60 min, and 90 min after implant. Blue arrows indicate new processes or re-adsorbed processes. (b) Differential overlay of (a). Green features indicate growth, while red features indicate retraction. White features are common features. Blue transparency at the top of the screen shows the location of the probe edge. Scale bar is $10 \mu\text{m}$.

Figure 10.

Distant microglia and vasculature interaction in the presence of an implant. (a) Occasionally, microglia (green) process movement toward vasculature (red) up to 170 μm from the probe (blue) can be observed over 2 h post implant (white arrows). Images are shown 105 min post implant and subsequently every 15 min. Over time, process density decreases. (b) The blood brain barrier wall of a large penetrating vasculature (red) was disrupted using a laser (white asterisk). Images show 2 min/frame time lapses as nearby microglia (green) extend processes over time toward the disrupted vasculature. Scale bar is 100 μm .