Focused Ion Beam Lithography to Etch Nano-architectures into Microelectrodes

Shreya Mahajan¹, Jonah A. Sharkins^{2,4}, Allen H. Hunter⁵, Amir Avishai⁶, Evon S. Ereifej^{2,3,4}

¹Department of Electrical Engineering and Computer Science, University of Michigan

²Veteran Affairs Ann Arbor Healthcare System

³Department of Neurology, School of Medicine, University of Michigan

⁴Department of Biomedical Engineering, University of Michigan

⁵Michigan Center for Materials Characterization, University of Michigan

⁶Carl Zeiss SMT, Inc.

Abstract

With advances in electronics and fabrication technology, intracortical microelectrodes have undergone substantial improvements enabling the production of sophisticated microelectrodes with greater resolution and expanded capabilities. The progress in fabrication technology has supported the development of biomimetic electrodes, which aim to seamlessly integrate into the brain parenchyma, reduce the neuroinflammatory response observed after electrode insertion and improve the quality and longevity of electrophysiological recordings. Here we describe a protocol to employ a biomimetic approach recently classified as nano-architecture. The use of focused ion beam lithography (FIB) was utilized in this protocol to etch specific nano-architecture features into the surface of non-functional and functional single shank intracortical microelectrodes. Etching nano-architectures into the electrode surface indicated possible improvements of biocompatibility and functionality of the implanted device. One of the benefits of using FIB is the ability to etch on manufactured devices, as opposed to during the fabrication of the device, facilitating boundless possibilities to modify numerous medical devices post-manufacturing. The protocol presented herein can be optimized for various material types, nano-architecture features, and types of devices. Augmenting the surface of implanted medical devices can improve the device performance and integration into the tissue.

Keywords

Bioengineering; Issue 155; focused ion beam lithography; intracortical microelectrodes; nano-architecture; electrophysiology; neuroinflammation; biocompatibility

Correspondence to: Evon S. Ereifej at ereifej@aptcenter.org.

Video Link

The video component of this article can be found at <https://www.jove.com/video/60004/>

Disclosures

The authors have nothing to disclose.

Introduction

Intracortical Microelectrodes (IME) are invasive electrodes which provide a means of direct interfacing between external devices and the neuronal populations inside the cerebral cortex^{1,2}. This technology is an invaluable tool for recording neural action potentials to improve scientists' ability to explore neuronal function, advance understanding of neurological diseases and develop potential therapies. Intracortical microelectrode, used as a part of Brain Machine Interface (BMI) systems, enables recording of action potentials from an individual or small groups of neurons to detect motor intentions that can be used to produce functional outputs³. In fact, BMI systems have successfully been used for prosthetic and therapeutic purposes, such as acquired sensorimotor rhythm control to operate a computer cursor in patients with amyotrophic lateral sclerosis (ALS)⁴ and spinal cord injuries⁵ and restoring the movement in people suffering from chronic tetraplegia⁶.

Unfortunately, IMEs often fail to record consistently over time due to several failure modes that include mechanical, biological and material factors^{7,8}. The neuroinflammatory response occurring after the electrode implantation is thought to be a considerable challenge contributing to electrode failure^{9,10,11,12,13,14}. The neuroinflammatory response is initiated during the initial insertion of the IME which severs the blood brain barrier, damages the local brain parenchyma and disrupts glial and neuronal networks^{15,16}. This acute response is characterized by the activation of glial cells (microglia/macrophages and astrocytes), which release pro-inflammatory and neurotoxic molecules around the implant site^{17,18,19,20}. The chronic activation of glial cells results in a foreign body reaction characterized by the formation of a glial scar isolating the electrode from healthy brain tissue^{7,9,12,13,17,21,22}. Ultimately, hindering the electrode's ability to record neuronal action potentials, due to the physical barrier between the electrode and the neurons and the degeneration and death of neurons^{23,24,25}.

The early failure of intracortical microelectrodes has brought about considerable research in the development of next generation electrodes, with emphasis on biomimetic strategies^{26,27,28,29,30}. Of particular interest to the protocol described here, is the use of nano-architecture as a class of biomimetic surface alterations for IMEs³¹. It has been established that surfaces mimicking the architecture of the natural in vivo environment have an improved biocompatible response^{32,33,34,35,36}. Thus, the hypothesis compelling this protocol is that the discontinuity between the rough architecture of the brain tissue and smooth architecture of the intracortical microelectrodes may contribute to the neuroinflammatory and chronic foreign body response to implanted IMEs (for a full review refer to Kim et al.³¹). We have previously shown that the utilization of nano-architecture features similar to the brain's extracellular matrix architecture reduces astrocyte inflammatory markers from cells cultured on nano-architected substrates, compared to flat control surfaces in both in vitro and ex vivo models of neuroinflammation^{37,38}. Furthermore, we have shown the application of focused ion beam (FIB) lithography to etch nano-architectures directly onto silicon probes resulted in significantly increased neuronal viability and lower expression of pro-inflammatory genes from animals implanted with the nano-architecture probes compared to the smooth control group²⁶. Therefore, the purpose of the protocol presented here is to describe the use of FIB lithography to

etch nano-architectures on manufactured intracortical microelectrode devices. This protocol was designed to etch nano-architecture sized features into silicon surfaces of intracortical microelectrode shanks utilizing both automated and manual processes. These methods are uncomplicated, reproducible, and can certainly be optimized for various device materials and desired feature sizes.

Protocol

NOTE: Do the following steps while wearing the proper personal protective equipment, such as a lab coat and gloves.

1. Mounting Non-functional Silicon Probe for Focused Ion Beam (FIB) Lithography

NOTE: For the complete procedure describing the fabrication of the SOI wafer with the 1,000 probes, please refer to Erefej et al.³⁹.

1. Isolate a strip of 2–3 silicon probes from the silicon on insulator (SOI) wafer containing 1,000 probes. Do not make strips containing more than three silicon probes. This may increase the chances for loose mounting and may cause misalignment resulting in the FIB to etch incorrectly.

NOTE: Strips/probes not firmly sitting on the aluminum stub can cause two complications: 1) when the stage moves to work on the next section, there will be vibrations and the milling will not be accurate until the probe settles and 2) it can cause a high variation and be out of the focus plane.

1. While wearing gloves, use fine forceps to put pressure around the probes to break off a small section containing two to three probes.
2. Carefully clean the silicon probe of all dust and debris prior to FIB etching. Prepare a 6 well polystyrene plate by pipetting 3 mL/well of 95% ethanol into three wells.
 1. Carefully pick up the cut strip of silicon probes using fine tip or vacuum forceps and place it into cell strainer. Place only one strip of silicon probes per strainer to prevent breaking the probes. Place the strainer containing the silicon probes strip into the first well containing 95% ethanol for cleaning. Keep the strainer in the first well for 5 min.
 2. Move the strainer containing the silicon probes from the first well and place it into the second well containing 95% ethanol for another 5 min. Repeat once more in the third well.
 3. Place the strainer containing the cleaned silicon probes onto a polytetrafluoroethylene plate to air dry. Do this step in a sterile hood to avoid contamination from dust.
3. Place the air-dried strip of silicon probes in a sealed container for transport to the SEM-FIB. Wrap the strainer containing the air-dried samples with a plastic or aluminum foil wrap for transport and/or storage to maintain cleaning.

4. Use fine tipped or vacuum forceps to carefully pick up the clean strip of silicon probes and place them onto a clean aluminum stub (used for SEM-FIB imaging/etching) to prepare for mounting.
5. Use a toothpick (or other fine tipped instrument like a thin electrical wire), to place a tiny drop (~10 μ L) of silver paint on the edge of the silicon substrate surrounding the probes. Secure the strip down by spreading the silver paint around the sides of the silicon substrate surrounding the probe. Allow the silver paint to dry completely before placing the aluminum stub into the SEM-FIB.

NOTE: Be careful not to get silver paint on the shank of the electrode because that is the part that will be etched. If the strip of probes is not securely anchored to the aluminum stub, the strip may move during processing or have a different focal plane, thereby resulting in incorrect milling by the FIB. Several strips of silicon probes can be mounted onto the same aluminum stub, making sure there is ample space between the strips to allow for removal from the stub after etching. This will allow more efficient etching of multiple probes using the automated feature described below.

2. Aligning the FIB to the Silicon Probes

1. Click on **vent** button in the beam control tab to vent the chamber. Press **Shift+F3** to perform home stage. Confirm the selection by selecting the **Home Stage** button in the popup window.

NOTE: Running the home stage operation is a preventative step to ensure the stage axis are read correctly by the software and the microscope is in good condition.

2. After the Home Stage is complete, move the stage to coordinates X = 70 mm, Y = 70 mm, Z = 0 mm, T = 0°, R = 0°. Once the chamber is vented, put on clean nitrile gloves and open the chamber door.

NOTE: Depending on the previous user's application, it may be necessary to change the stage adapter. The standard stage adapters (e.g., FEI style) can be removed by unscrewing the central bolt counterclockwise and installed by screwing clockwise into the rotation plate of the stage.

3. Insert the aluminum stub holding the probes into the top of the stage adapter. Secure the aluminum stub by tightening the set screw on the side of the stage adapter. Use the 1.5 mm hex wrench for this task.
4. Adjust the height of the stage adapter by turning the adapter clockwise to lower it or counterclockwise to raise it. Secure the stage adapter to the rotation plate by turning the locking cone nut clockwise until the nut is secure against the stage rotation plate. Hold the stage adapter with the other hand to prevent the rotation of the adapter and samples while tightening the locking cone nut.

NOTE: Use the provided height gauge to determine the appropriate height. The top of the aluminum stub should be the same height as the maximum line shown

on the height gauge. Over tightening the cone nut can cause damage to the stage and adapter. Only use enough force to secure the samples.

5. Acquire a navigation camera image. Carefully swing the navigation camera arm open until it stops. The microscope stage will automatically move to a position beneath the camera. Watch the live image shown in Quadrant 3 of the microscope user interface (UI).
 1. Once the brightness level auto adjusts to an appropriate level, acquire the image by pushing the button down on the camera bracket. Be sure to wait for the entire image acquisition to finish, which is indicated by a pause symbol appearing in Quadrant 3 and the illumination of the camera turning off. This takes approximately 10 s. Swing the camera arm back to the closed position. The stage will return to the original position.
6. Carefully close the microscope chamber door. Watch the CCD camera image in Quadrant 4 while closing the door. Ensure that the samples and stage are a safe distance away from any critical component in the microscope chamber.
7. Select the down arrow next to the Pump button in the beam control tab. Select **Pump with Sample Cleaning** button in the UI software to start the chamber vacuum pump and built in plasma cleaner. Ensure the door is sealed by gently pushing on the face of the door while the pump is running. Wait for approximately 8 min for the pumping time and plasma cleaning cycle for the microscope chamber to be completed. NOTE: A vacuum seal can be confirmed by gently pulling on the chamber door, which should remain closed if the system is under vacuum.
8. Once the icon in the bottom right corner of the UI turns green, press the Wake-Up button in the beam control tab which turns on the electron and ion beams. Select quadrant 1 and set the beam signal to electron beam (if not set already), set quadrant 2 to ion beam (if not set already).
 1. Set SEM voltage to 5 kV, set SEM beam current to 0.20 nA, set SEM detector to ETD, set detector mode to Secondary Electron. Set FIB voltage to 30 kV, set FIB beam current to 24 pA, set FIB detector to ICE detector, set detector mode to the secondary electron.
9. Double click on the **silicon probe** in the navigation camera image, quadrant 3 to move the stage to the approximate location of the probe. Click on **quadrant 1** to select it as the active quadrant and hit the pause button to start SEM scanning. Set the scan dwell time to 300 ns and turn off **scan interlacing**, **line integration**, and **frame averaging**. Set scan rotation to 0 in the beam control tab and right click on the beam shift 2d adjuster and select **zero**.
10. Adjust the magnification to the minimum value by turning the magnification knob counterclockwise on the MUI panel. Adjust the image brightness and

contrast using the knobs on MUI panel or the **Auto Contrast Brightness** toolbar icon.

11. Move the stage by either double-left-clicking the mouse on a feature to center it, or by pressing down the mouse wheel and activating the joystick mouse mode. Move the desired silicon probe to be patterned into the center of the SEM image.
12. Locate an edge or other features such as a dust particle or scratch. Increase magnification to 2,000x by turning the magnification knob clockwise. Adjust the focus of the SEM by turning the coarse and fine focus knobs on the MUI until the image is in focus. Once the image is in focus, select the **Link sample Z to working distance** button in the toolbar.
13. Confirm that operation was completed by looking at the Z-axis coordinate in the navigation tab. The value should be approximately 11 mm. Type in 4.0 mm in the Z axis position and push the **Go To** button with the mouse or hit the enter key on the keyboard and the stage will move to 4 mm working distance.
14. Move the stage in X and Y to locate the shoulder of the silicon probe. Position it as close to the center of the SEM as possible. Change the stage tilt to 52° by typing in “52” in the T coordinate and hitting enter. Observe whether the shoulder of the probe appears to move up or down in the image. Use the Stage Z slider to bring the shoulder of the probe back to the center of the SEM image. Only adjust the Z position, do not move X, Y, T, or R axis.
15. Run the built in “**xT Align Feature**” command located in the stage drop down menu. Use the mouse to click on two points parallel to the edge of the probe. Make sure the horizontal radio button is selected in the popup window and click finish. The stage will rotate to align the probe with the X axis of the stage. Adjust the stage in X,Y using the mouse to put the lower shoulder of the probe in the center of the SEM image again.

NOTE: The first point should be towards the probe’s grip and the second point should be towards the probe’s point.

16. Select the FIB in quadrant 2 and make sure the beam current is still 24 pA. Set the magnification to 5,000x and the dwell time to 100 ns. Type **Ctrl-F** on the keyboard to set the FIB focus to 13.0 mm. In the beam control tab, right click in the **stigmator 2d adjuster** and select **zero** and, also, right click in the **Beam Shift 2d adjuster** and select **zero**. Set the scan rotation to 0° and push the auto contrast brightness button in the toolbar.
17. Look for an image of the probe shoulder in quadrant 2. Use the **snapshot tool** to acquire an image with the FIB. Confirm the probe shoulder is in the center of the FIB image, if not, double click on the probe shoulder to move it to center. Move the stage to the left by pushing the left arrow key on the keyboard approximately 10–15 times. Take another snapshot and observe whether the probe side is still in the center of the FIB.

NOTE: IF not, the stage rotation must be adjusted slightly. If the probe is above the image center, the stage must be rotated in the negative direction. If the probe is below center, the stage must be rotated clockwise. Enter a relative compucentric rotation of 0.01 to 0.2 degrees depending on which way is necessary to align the probe.

18. Repeat steps 2.16 to 2.17 as many times as necessary until the edge of the probe shoulder is perfectly aligned with the X axis of the stage, (the edge stays in the center of the FIB while moving left).
19. Using the FIB, move the stage back to the lower shoulder of the probe. Save the stage position in the position list by clicking the **Add** button. Change the FIB beam current to 2.5 nA and make sure the magnification of the FIB is still 5000x. Run the auto brightness contrast function and set the FIB dwell time to 100 ns.
20. Hit the pause button to start scanning. Adjust the FIB focus and astigmatism, as quickly and precisely as possible, using the Coarse and Fine focus knobs, and the X and Y stigmator knobs on the MUI panel. Hit the pause button to stop the FIB scanning.

3. Writing an Automated Process for Etching

1. Start the software by locating it in the Windows start menu (i.e., Start\Programs\FEI Company\Applications\Nanobuilder). Position the software window on the side monitor so the UI is not covered up. Open the file for patterning the silicon probes by clicking **file** and then **open**. Direct the windows browser to the location of the software script (Supplementary File 1 - the file name is "Case_Western_2000_micron_Final_11H47M_runtime.jbj").
2. Within the software, select the microscope dropdown menu and select **Set stage origin**. Within the software, select the microscope dropdown menu and then select **Calibrate Detectors**.
3. On the microscope UI, click in **Quad 1** once with the mouse to select Quad 1. Ignore the other instructions shown in the popup window, they are not necessary for this project. Click **OK** to start the calibration. The process will take about 5 min. Make sure the ETD and ICE detectors calibrate. It is ok if any other detectors have calibration failures.
4. Within the software, select the microscope dropdown menu and choose **Execute** to start the patterning sequence. When the pattern is complete, close the software.

NOTE: The software will take over quad 3 and 4 for the patterning and alignment functions. The script will take approximately 12 h to run. While the script is running, do not change any parameter on the microscope.

5. Hit "**Vent**" in the microscope UI beam control tab to shut down the microscope beams and start the vent cycle. While the chamber is venting, move the stage

to coordinates $X = 70$ mm, $Y = 70$ mm, $Z = 0$ mm, $T = 0^\circ$, $R = 0^\circ$. Once the chamber is vented, put on clean nitrile gloves and pull open the chamber door.

6. Loosen the set screw on the stub adapter using the 1.5 mm hex wrench. Remove the aluminum stub containing the patterned probe from the chamber. Carefully close the microscope chamber door. Watch the CCD camera image in Quadrant 4 while closing the door. Ensure that the stage adapter is a safe distance away from any critical component in the microscope chamber.
7. Select the **down arrow** next to the **Pump** button in the beam control tab. Select **Pump** button to start the chamber vacuum pump. Ensure the door is sealed by gently pushing on the face of the door while the pump is running.

NOTE: A vacuum seal can be confirmed by gently pulling on the chamber door, which should remain closed if the system is under vacuum. The pumping time will be approximately 5 min. Only one side of the probe can be etched during a single run.

8. If the front and back side of the probe requires etching, then carefully remove the etched strip of silicon probes after checking the final etch and imaging the front side (if images are needed). Dissolve the silver paint with acetone, by cautiously dabbing/brushing the acetone on the silver paint. Carefully turn the strip around to the backside, re-mount, align and etch following the steps described above.

4. Checking the Final Etch and Imaging

1. Once the milling is complete verify the uniformity of the different sections using SEM imaging at a higher magnification.

NOTE: Imaging at the tilted angle allows a better assessment of the variation in the milling depth. Special attention should be given to the transition regions between the milling locations.

2. Image the samples again after milling with an optical microscope.

NOTE: The periodic milled lines result in a refraction effect giving rise to different colors as a function of the imaging angle. If the color is not continuous along with the probe that is a clear indication of the disruption in the milled lines.

5. Mounting a Functional Silicon Probe for FIB Etching

1. Gently remove the functional silicon electrode from its packaging. Use forceps to carefully lift the plastic protective tab covering the head stage. Start lifting one corner of the tab up from the sticky glue holding it in place and keep lifting until the entire electrode is removed.
2. Carefully clamp the electrode with hemostats to prepare for mounting into the stereotaxic frame. While holding the covered tab with the forceps, gently place curved hemostats around the green shaft above the silicon shank, with the curved

part of the hemostats facing upwards towards the tab. Lock the hemostats in place to ensure the electrode will not drop out of the hemostats.

3. Gently remove the plastic protective tab covering the head stage. While holding the electrode with the hemostats, carefully clip the electrode into the stereotaxic frame for cleaning.
4. Fill 3 Petri dishes with 95% ethanol (~10 mL per petri dish). Place the Petri dish under the electrode that is mounted into the stereotaxic frame for cleaning. Slowly lower the electrode by turning the micromanipulator downwards (100 $\mu\text{m/s}$) so that the shank is submerged into the 95% ethanol.

NOTE: Be careful not to turn the micromanipulator too fast or too deep, this can cause the electrode to break (i.e., the electrode should not touch the Petri dish).
5. Leave the electrode shank in the 95% ethanol for 5 min, and then slowly raise the electrode out of the 95% ethanol by turning the micromanipulator upwards (100 $\mu\text{m/s}$). Repeat this step two more times, for a total of three washes. Allow the electrode to air dry for five minutes.
6. Use the same technique for mounting the electrode into the stereotaxic frame, to remove the electrode from the stereotaxic frame. Carefully place the hemostats around the shaft of the electrode. Once the hemostats are clasped tight, release the electrode from the stereotaxic frame, return the plastic protective tab covering the head stage, and put the cleaned electrode back into its packaging.

6. Etching Functional Silicon Probe Using FIB

1. Mount the cleaned functional silicon electrode onto an aluminum stand. Carefully pick up the cleaned functional silicon electrode using forceps and remove the protective tab from the headstage. Place the electrode shank on the aluminum stub so it does not hang over any edge, then using a small piece of Cu or carbon conductive tape, pin the headstage securely to the aluminum stub.

NOTE: Alternatively, a low-profile clip holder can be used to hold the electrode down. Be careful not to touch the electrode shank.
2. Following the steps described above (Section 2), position the electrode at the eucentric height and make sure the electrode is at the coincidence point of the SEM and FIB beams. Align the shank with the “X” direction of the stage.
3. Set the FIB to the optimal current for milling the required nano-architecture and make sure the focus and stigmation are properly corrected. Prepare an array of lines with desired spacing and length to cover the field of view of the shank (500 μm sections). Adjust the line lengths as etching gets down the shank to the thinner sections.

NOTE: When etching the functional electrode, it is not possible to add fiducial marks to automate the process. Therefore, moving between the sub-sections (~500 μm) is done manually.

4. After the milling of the first section is complete, make sure to check the milling quality before moving on to the next section. Repeat step 6.3 to etch the next section of the shank. Align the milled lines from the previous section to the patterns used for the next section to prevent large gaps between runs.

Representative Results

FIB Etched Nano-architecture on the Surfaces of Single Shank Intracortical Probes

Utilizing the methods described here, intracortical probes were etched with specific nano-architectures following established protocols³⁹. Dimensions and shape of the nano-architecture design described in these methods were implemented from previous in vitro results depicting a decrease in glial cell reactivity when cultured with the nano-architecture design described here^{37,38}. The methods described here utilized a focused gallium ion beam (FIB) to etch nanoscale parallel grooves into the surface of non-functional single shank silicon microelectrode probes, as previously described³⁹. The nanoscale parallel grooves were etched along the shank of the backside of the probe using an automated script written in the software. The final dimensions of the etched nano-architecture were 200 nm wide parallel lines, spaced 300 nm apart, and had a depth of 200 nm (Figure 1). The use of FIB to etch nano-architectures into a device surface allows for etching of precise designs into manufactured devices.

Etched Nano-architecture into Intracortical Probes Effect on Neuroinflammation

In this previously reported data, the intracortical probes with etched nano-architectures were implanted into the cortex of rats for either two or four weeks (n=4 per time point) and compared to control animals implanted with smooth probes containing no nano-architecture etchings (n=4 per time point)³⁹. One of the mechanisms of failure impeding the clinical deployment of intracortical microelectrodes is the neuroinflammatory response induced from disrupting the brain parenchyma and blood brain barrier^{9,10,11,12,15}. A full description of the neuroinflammatory response observed after intracortical microelectrode implantation can be found in the following reviews^{13,14,22}. The ability of intracortical microelectrodes to record action potentials from neurons is dependent on the distance of the healthy neuronal bodies from the intracortical microelectrode recording site⁴⁰. Therefore, the previously reported study evaluated the neuroinflammation around the intracortical probe implantation site by quantifying histological markers for neuronal density, glial cell activation and gene expression of proinflammatory markers³⁹. Highlights from that study are presented below to represent the effects etching nano-architectures into the probe surface had on neuroinflammation.

Effects of Etched Nano-architecture on Neuron Density

To determine how etching nano-architectures into the probe's surface effects the neuronal density immediately around the implant, the neuronal nuclei were stained and quantified utilizing previously describing immunohistochemistry methods^{39,41}. There were no significant differences of neuron densities around the nano-architecture and control probes at 2 weeks post implantation (Figure 2A). However, there were significantly more neurons around the nano-architecture probes at 100–150 μm distance from the

implant site compared to the smooth control implants ($p < 0.05$ vs controls) (Figure 2B) at 4 weeks post implantation. It was also found that there was an increased trend of neuronal density surrounding the nano-architecture probes over time, contrasting the decreased trend of neuronal density around the control implants (Figure 2). There is a direct relationship depicting a mitigated neuroinflammatory response coupled with a higher density of viable neurons surrounding the microelectrode, results in an increased ability for the microelectrode to provide quality recordings^{15,40,42}. Therefore, when interpreting neuronal density data, a higher density of neurons around the implant site may indicate a lessened neuroinflammatory response and potentially improved recording quality and stability from intracortical microelectrodes.

Effects of Etched Nano-architecture on Neuroinflammatory Molecular Markers

Histology is enough for identifying the cells around an implant site; however, it lacks the sensitivity and specificity for characterizing the phenotype of the surrounding cells. Hence, methods utilizing quantitative gene expression analysis were employed to quantify the relative gene expression of neuroinflammatory markers, in order to understand the effect nano-architecture has on the phenotype of the cells³⁹. Several neuroinflammatory markers were investigated in the previously reported study. Here only two will be highlighted that are specific to microglia cells, in order to discuss how their phenotype may have been altered. The cluster of differentiation 14 (CD14) is a pattern recognition receptor on the membrane of microglia that recognizes bacteria and signals the inflammatory pathway after injury/implantation^{43,44,45}. Nitric oxide synthase (NOS2), is an oxidative stress marker expressed in microglia/macrophages that is associated with an increased production of proinflammatory markers^{46,47}.

In the previously reported data, there were no significant differences of CD14 relative gene expression between nano-architecture and control implants at either two- or four-weeks post-implantation. Notably, there was a significant decrease ($p < 0.05$) of CD14 relative gene expression from two to four weeks around the nano-architecture implants' site, indicating a possible decrease in inflammation (denoted by * in Figure 3A). Similarly, there were no significant differences of NOS2 relative gene expression between nano-architecture and control implants at two weeks. However, there was significantly less ($p < 0.05$) NOS2 relative gene expression around the nano-architecture implant compared to the control implant at four weeks post-implantation (denoted by # in Figure 3B). Moreover, there was a significant increase from 2 to 4 weeks of NOS2 relative gene expression around the control implants (denoted by * in Figure 3B), and no differences observed around the nano-architecture implants over time, further indicating a potential decrease of inflammation around the nano-architecture implants. When interpreting this data, it is important to understand the function of the gene being quantified. For example, decreases of pro-inflammatory genes indicate a probable decrease in the inflammatory response around the electrode site, whereas an increase in these types of genes suggests a likely increase in inflammation.

FIB Etched Nano-architecture on the Surfaces of Functional Single Shank Microelectrodes

The previously reported study had promising results demonstrating a slight increase of neuron density and the potential decrease in microglia inflammatory phenotype around the nano-architecture probe implant site. To investigate the translation of these results to electrode functionality, a functional single shank silicon microelectrode was etched with the same nano-architecture design as the non-functional single shank silicon microelectrode probes, utilizing a similar FIB etching protocol. The only difference in the methodology for etching the specified nano-architecture was that the protocol for the functional electrodes could not be automated, as there was no extra substrate material to create fiducial markings. Thus, the functional electrode was manually etched using FIB by re-aligning the beam every 500 μm , as described in the protocol above. The final etchings were 200 nm wide parallel lines, spaced 300 nm apart, and had a depth of 200 nm (Figure 4).

Effects of Etched Nano-architecture into Intracortical Microelectrodes on Electrophysiology

Successful intracortical microelectrode recordings are reliant on the proximity of the neurons around the implant sites, the integrity of the device and the reliable transmission of single unit activity from the brain^{8,40,48,49}. Electrophysiological recordings were quantified utilizing recorded metrics collected twice per week over eight weeks. The metrics utilized in this study were, the percentage of channels recording single units, maximum amplitudes of recorded units, and signal-to-noise ratio (SNR). The Institutional Animal Care and Use Committees (IACUC) at the Louis Stokes Cleveland Veterans Affairs Medical Center approved all animal procedures. Sprague Dawley rats (8–10 weeks old and weighing ~225 g) were implanted with single shank silicon microelectrode, with the above-mentioned nano-architecture (n=1) or the smooth controls (n=6). No statistical analysis was performed on this data, as there was one nano-architecture microelectrode implanted for a proof-of-concept pilot study. Nonetheless, collective electrophysiological results showing an increased percentage of channels recording single units (Figure 5A), maximum amplitudes (Figure 5B) of recorded units, and SNR (Figure 5C) from the nano-architecture microelectrodes compared to the smooth control microelectrodes, are promising. These results indicate that the etching of nano-architecture into the surface of microelectrodes may potentially result in improved quality and increased longevity of electrophysiological recordings. Further evaluation with increased sample size is necessary to verify these preliminary findings.

Discussion

The fabrication protocol outlined here utilizes focused ion beam lithography to effectively and reproducibly etch nano-architectures into the surface of non-functional and functional single shank silicon microelectrodes. Focused ion beam (FIB) lithography allows for the selective ablation of the substrate surface by using a finely-focused ion beam^{50,51}. FIB is a direct-write technique that can produce various features with nanoscale resolution and high aspect ratio^{50,52,53}. In order to create the various sized features, the magnitude of the ion beam current can be optimized to change the ion beam spot size within a range of 3 nm to 2 μm ^{50,51}. Some general advantages of using FIB to etch features onto surfaces are: 1) it can

be used on a wide variety of materials, including silicon, metals, and polymers^{53,54,55,56}, 2) FIB can be performed on non-planar surfaces, and 3) FIB can be used for post-processing on individual devices⁵⁷.

Here, FIB was used in combination with an SEM microscope and software used to write a specialized automated script for etching specific features into non-functional single shank probes. The script included parameters required (2.5 nA ion beam current and 30 kV voltage) to etch the exact spacing desired (200 nm wide parallel grooves, spaced 300 nm apart and 20 nm deep). The dimensions of the electrode exceeded the field of view for the FIB, so the patterning was performed in multiple stage positions. In order to automate the process, fiducial marks were etched into the side of the silicon sheet holding the probes in place, to allow the software to precisely locate the patterned lines on the probe shank. The fiducials were necessary because the stage motion created a large (~5 μm) uncertainty in the location of the pattern area with respect to the ion beam field of view. Placement of the fiducials on the silicon sheet allowed for the FIB to locate the pattern areas without directly scanning the beam on the probe shank, which could potentially contaminate or damage the probe shank. The entire automated etching process for one shank took approximately 12 h to complete and required no operator intervention after the patterning was started. Collectively, the benefits of using FIB to etch features into the silicon probe shank were the ability to make nanometer sized features, automate the etching process, and the capacity to etch on a fabricated probe. Although FIB has copious benefits, one of the drawbacks of using this fabrication method is the slow throughput rate that ultimately limits the potential for mass production of devices with nano-architectures into their surface⁵⁸. Alternatively, other fabrication methods utilized to create feature sizes and geometries of interest, perhaps at faster rates and at mass productions, include electron beam lithography and nanoimprint lithography^{59,60,61,62,63,64,65}. However, these methods do not allow for etching of nano-architecture features into manufactured devices. Traditionally these methods are utilized during the fabrication process on a sheet of silicon or polymer, which can then be used in downstream processing steps to fabricate the final device.

The functional electrodes were unable to undergo an automated process due to not having any surrounding material around the electrode shank in which to include fiducial marks into the run. Therefore, the functional electrodes were manually aligned and etched at 500 μm sections along the shank, using the same ion current and voltage as the automated run to ensure the same feature sizes. The beam had to be manually realigned after completing each 500 μm interval and set up to etch the next section. The process of manual realignment of the patterns every 500 μm can potentially lead to damaged nanostructures or structures that do not match the intended geometry. This is due to longer exposure times the ion beam needs for manual alignment⁶⁶. This was one of the difficulties encountered with the manual etching. Due to this complication, two recording contacts were short-circuited and were unable to record neuronal action potentials (Figure 6). Figure 6 demonstrates an electrophysiological live recording segment from the animal implanted with the nano-architecture electrode. The blue boxes outline the channels recording strong action potentials, in comparison to the red boxes denoting the two dead channels. Hence, one of the challenges of using manual FIB etching on post-manufactured electrodes is that there is a chance that the beam can short-circuit contacts and prevent them from recording.

This challenge is enhanced when attempting to etch the front side of single shank silicon electrodes, in which the recording contacts and traces are along the entire shank of the electrode. Although it is feasible to etch the silicon around the recording contacts and traces, extra caution is advised to avoid damage and decreased performance of recording capabilities of the electrode.

As previously mentioned, FIB can be utilized on various materials to etch numerous feature geometries into the surface. However, it is important to note that the parameters to etch geometries, such as lines into the various materials, are complicated to predict. Particularly for line patterns, the line width and depth are strongly dependent on many parameters such as the accelerating voltage, beam current, dwell time, pixel spacing, lifetime of the aperture and material type. Another parameter that results from the optimization is total time to mill each line. Narrower and deeper lines could be achieved using smaller beam currents; however, the pattern time for an entire probe shank would extend to multiple days, which is not practical. Thus, although it is feasible to optimize the protocol presented here, it would be exceedingly difficult to describe the parameters for unknown materials. In troubleshooting the parameters for the silicon probes described in this protocol, numerous test cuts in silicon were made to evaluate how the changing conditions affected the line width and depths. As soon as the evaluated conditions were able to etch the specific feature size and geometry of interest (200 nm wide lines that were 200 nm deep), those parameters were used to write the software script.

The script was used to control the spacing of each line, from center to center, which in this protocol is 300 nm. Future studies utilizing silicon substrates/devices requiring feature sizes in the hundreds of nanometers, can use the parameters described in this protocol as a starting point for troubleshooting the conditions needed to create the desired feature sizes. Further optimization and troubleshooting of the etching conditions will be required for metal and polymer substrates/devices. Overall, utilizing FIB for etching nano-architectures into material surfaces allows for ample control and flexibility in feature geometries, use of numerous compatible materials, and several surface types, including manufactured devices. Representative results presented herein demonstrated the potential benefits observed in our studies of utilizing FIB to etch nano-architectures into the surface of intracortical microelectrodes: 1) increased neuronal density and 2) reduction of neuroinflammatory markers around implanted devices with nano-architectures, as well as 3) preliminary findings depicting improved quality of electrophysiological recordings over time. Likewise, the employment and optimization of the described protocol etching nano-architecture features into the surface of a material can be utilized to improve the functionality of numerous medical devices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: FIB Etched Nano-architecture on the Surfaces of Single Shank Intracortical Probes. SEM images of the non-functional single shank silicon probes with FIB etched nano-architectures along the backside of the shank. **(A)** Composite images of the entire probe post etching shown at 120x magnification, Scale bar = 400 μm . The fiducial marks, (square box with a + symbol going through it), are etched along the silicon substrate surrounding the probe. Magnified SEM images of the probe tip are shown in **(B)** at 1,056x magnification (Scale bar = 40 μm), **(C)** at 3,500x magnification (Scale bar = 10 μm), and **(D)** at 10,000x magnification, Scale bar = 4 μm . This figure has been modified from reference³⁹.

Figure 2: Effects of Etched Nano-architecture on Neuron Density.

Neuronal survival is presented as a percentage of the background region from the same animals in distances of 50 μm bins away from the implant site. **(A)** No significant differences of neuronal survival were observed between the smooth surfaces (control) and nanopatterned implants at 2 weeks post-implantation. **(B)** There was a significantly higher neuronal survival around the nanopatterned implants at the 100–150 μm distance compared to smooth surfaces ($p < 0.05$) at 4 weeks post-implantation. Representative images of neurons (stained green), with the yellow outline depicting the implantation site, and the “P” denoting the etched side of the microelectrode, Scale bar = 100 μm . This figure has been modified from³⁹.

Figure 3: Effects of Etched Nano-architecture on Neuroinflammatory Molecular Markers. Tissue was collected around 500 μm radius of the implantation site for both the nanopatterned and control implants. Relative gene expression of inflammatory markers was quantitatively compared between both implant types (the differences are denoted by # on the graph; $p < 0.05$) as well as over time (the differences are denoted by * on the graph; $p < 0.05$). **(A)** Relative gene expression of CD14 significantly decreased around nanopatterned implants from two to four weeks (*). **(B)** There was a significantly lesser relative gene expression of NOS2 around nanopattern implant compared to control at four weeks (#) and there was a significant increase of NOS2 from two to four weeks around smooth control implants (*). This figure has been modified from reference³⁹.

Figure 4: FIB Etched Nano-architecture on the Surfaces of Functional Single Shank Microelectrodes.

The inset on the top right corner displays the microelectrode utilized in this study next to a dime to portray the size of the electrode shank (thin black line). SEM images of the microelectrodes shank with FIB etched nano-architectures along the backside of the shank. The entire shank is shown at the top at 600x magnification (Scale bar = 50 μm), while the inset depicts the nanopatterned surface at 25,000x magnification, Scale bar = 1 μm).

Figure 5: Effects of Etched Nano-architecture into Intracortical Microelectrodes on Electrophysiology.

Evaluation of electrophysiological metrics discovered a promising preliminary increased trend of (A) percentage of channels recording single units, (B) maximum amplitudes of recorded units, and (C) signal-to-noise ratio from the microelectrode etched with nano-architectures compared to the smooth control electrodes.

Figure 6: Electrophysiology Recording from Implanted Functional Single Shank Microelectrodes with FIB Etched Nano-architectures.

One of the challenges of using FIB to etch nano-architectures on manufactured microelectrode devices is the risk of short-circuiting recording contacts. The x-axis depicts the recording time in seconds, and the y-axis shows the electrode channels recording neuronal action potentials. Each numbered line on the y-axis represents a different electrode channel, with channel number 1 being the shallowest and 16 being the deepest. The red boxes outline the short-circuited channels, whereas the blue boxes outline channels with visible neuronal activity.