IN VITRO AND IN VIVO BIOCOMPATIBILITY TESTING OF SILICON CARBIDE FOR NEURAL INTERFACES

by

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A Dissertation
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree of
Doctor of Philosophy
Neuroscience

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Date: _________________________   Fall Semester 2014
George Mason University
Fairfax, VA
In Vitro and In Vivo Biocompatibility Testing of Silicon Carbide for Neural Interfaces

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at George Mason University

by

Gretchen L. Knaack
Master of Arts
George Mason University, 2011

Director: Joseph J. Pancrazio, Professor
Department of Neuroscience

Fall Semester 2014
George Mason University
Fairfax, VA
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DEDICATION

This is dedicated to my loving parents Boyd L. Knaack Jr. and Robin L. Chavez as well as my dog Payton who always cheered me up and reminded me to enjoy the little things in life.
ACKNOWLEDGEMENTS

I would like to thank my family and friends for their unwavering support both emotionally and sometimes even financially. I know that my stress was frequently displaced as anger or frustration, which did not always make me the most pleasant person. However, they tolerated me no matter what and encouraged me to persevere. I appreciate my committee members for their feedback and advice, which has helped me to mature into a better scientific researcher. I would especially like to express my gratitude to Joe Pancrazio for adopting me into the lab and providing the knowledge and resources to succeed. The experiences under his mentorship truly facilitated my career. I also have to thank Nathalia Peixoto for bridging this relationship because without her, I never would have been introduced to Joe and the NeuroEngineering lab.
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LIST OF ABBREVIATIONS AND SYMBOLS

Neuronal Nuclei ........................................................................................................ NeuN
Glial Fibrillary Acidic Protein .................................................................................. GFAP
Microelectrode Array ................................................................................................ MEA
Silicon ....................................................................................................................... Si
Silicon Carbide ......................................................................................................... SiC
Amorphous Silicon Carbide ..................................................................................... a-SiC
Microliter ................................................................................................................... μL
Millimeter ................................................................................................................... mm
Millimolar .................................................................................................................. mM
Micromolar ................................................................................................................ μM
Nanomolar .................................................................................................................. nM
Micrometer ................................................................................................................ μm
Calcium ..................................................................................................................... Ca²⁺
Poly-D-Lysine ............................................................................................................ PDL
Dulbecco’s Modified Eagle Medium ......................................................................... DMEM
Inter Spike Interval ................................................................................................... ISI
Inter Burst Interval .................................................................................................... IBI
Half Maximal Effective Concentration ...................................................................... EC₅₀
Microvolt ..................................................................................................................... μV
Analysis of Variance ................................................................................................. ANOVA
Standard Error of Mean .............................................................................................. SEM
Potassium .................................................................................................................... K⁺
Gold ............................................................................................................................. Au
Copper ......................................................................................................................... Cu
Polyethylene ............................................................................................................... PE
Polyvinyl Chloride ....................................................................................................... PVC
Cubic Silicon Carbide ............................................................................................... 3C-SiC
Titanium ...................................................................................................................... Ti
KiloHertz ..................................................................................................................... KHz
Hertz ............................................................................................................................. Hz
Celsius .......................................................................................................................... °C
Plasma-enhanced Chemical Vapor ............................................................................. PECVD
Paraformaldehyde ...................................................................................................... PFA
Phosphate Buffer Saline .............................................................................................. PBS
Immunohistochemistry ............................................................................................... IHC
ABSTRACT

IN VITRO AND IN VIVO BIOCOMPATIBILITY TESTING OF SILICON CARBIDE FOR NEURAL INTERFACES

Gretchen L. Knaack, Ph.D.
George Mason University, 2014
Dissertation Director: Dr. Joseph J. Pancrazio

This dissertation demonstrates the biocompatibility of amorphous silicon carbide for implementation with neural interfaces. To evaluate this capacity in vitro, frontal cortex networks cultured on microelectrode arrays were established and assessed pharmacologically by investigating the neurotoxic effects of ω-agatoxin. This valuable platform was then implemented as a functional compliment for the live/dead assay to test biomaterials in more sensitive manner. Finally, this assay was utilized to test the biocompatibility of different variations of silicon carbide for the possible use with neural interfaces. Since amorphous silicon carbide did not reduce spontaneous network firing rate in vitro, it was then examined in vivo. Standard silicon devices were coated with a thin film of amorphous silicon carbide and simultaneously implanted with silicon devices into the primary motor cortex of rats for either four or eight weeks. The neuroinflammatory reaction was then compared between the materials through device
capture immunohistochemistry. NeuN, GFAP, CD68 and DAPI all displayed an increase in labeling from four to eight weeks of implantation. The more intense fluorescence of CD68 and DAPI at eight weeks was only located at distances proximal to devices, whereas NeuN and GFAP exhibited an overall enhancement. However, a decrease in NeuN labeling was still observed within 0-30µm of the device irrespective of the implant duration. Although these findings were independent of material, tissue implanted with amorphous silicon carbide did have a reduction of GFAP labeling within 0-10µm compared to tissue implanted with silicon. This occurred regardless of implant time. The \textit{in vitro} and \textit{in vivo} data jointly support the notion that the addition of amorphous silicon carbide to the standard silicon probe is biocompatible and decreases the neuroinflammatory response to cortical implants by reducing the intensity of GFAP adjacent to the device.
CHAPTER ONE: INTRODUCTION

Implantable microelectrode arrays (MEAs) are gaining interest with the advancements in technology and material science. They are used for neural prosthetic devices to control artificial limbs (Hochberg et al., 2006; Velliste et al., 2008) by recording from neuronal tissue to create a brain machine interface. Although neural implants are promising, the obtained signals degrade over time until device failure (Rousche and Normann, 1998; Williams et al., 1999) and the reliability of this approach has been the subject of recent attention. Furthermore, there is significant variability in chronic electrode performance across research groups, animal models, and even within the same implant (Polikov et al., 2005). It is believed that this failure results from the brain’s immune response, which activates after penetrating the blood brain barrier (Fawcett and Asher, 1999; Winslow and Tresco, 2010), but the interaction between the implant and the tissue still lacks clarity in predicting device functionality. Device reliability measures are central to system operation and can provide complimentary information regarding the neuroinflammatory response, which consists of various interacting cellular processes.

Unfortunately, perturbing the brain starts with implantation of the device which causes shearing of vasculature and disturbances to the extracellular matrix, glia and neurons (Bjornsson et al., 2006; Polikov et al., 2005). This immediately initiates the
wound healing process and recruits macrophages as well as microglia to extend processes toward the site (Polikov et al., 2005, Potter et al., 2012, Stice and Muthuswamy, 2009; Winslow and Tresco, 2010; Woolley et al., 2013; Yoshida et al., 2012). Microglia usually exist in a ramified state until activated by breaking the blood brain barrier (Fawcett and Asher, 1999; Polikov et al., 2005; Potter et al., 2012; Winslow and Tresco, 2010; Yoshida et al., 2012). Once activated, they extend processes toward the probe, exhibit amoeboid morphology, and express leukocyte-associated molecules such as CD68, which make them virtually indistinguishable from macrophages (Polikov et al., 2005, Yoshida et al., 2012). Activated microglia also release numerous inflammatory, cytotoxic, and neurotoxic chemicals (Polikov et al., 2005). They further exacerbate the response by secreting factors that signal additional microglia/macroglia and astrocytes (Polikov et al., 2005; Stice and Muthuswamy, 2009; Szarowski et al., 2003).

Similar to microglia, a reactive state is also induced for astrocytes, which is primarily characterized by upregulation of glial fibrillary protein (GFAP) (Bardehle et al., 2013; Fawcett and Asher, 1999; Polikov et al., 2005; Stice and Muthuswamy, 2009; Winslow and Tresco, 2010). Although many have reported migration and proliferation of activated astrocytes to the probe site (Fawcett and Asher, 1999; Fitch et al., 1999; Polikov et al., 2005,) more recent live imaging did not observe any movement and only proliferation near vasculature, but did confirm elongated processes and hypertrophy (Bardehle et al., 2013). Within four weeks, the acute inflammatory reaction transitions into a chronic response. This is characterized by a glial scar encapsulating the device and is composed primarily of reactive astrocytes, although activated microglia can also be
observed (Fitch et al., 1999; Polikov et al., 2005; Potter et al., 2012; Stice and Muthuswamy, 2009; Szarowski et al., 2003; Woolley et al., 2011, 2013). This tissue sheath isolates the probe and is believed to increase impedance (Polikov et al., 2005; Prasad et al., 2012, Wang et al., 2013).

This is paired with a significant loss of neurons proximal to the device (Potter et al., 2012; Winslow and Tresco, 2010). However, the extent of this reaction varies across animals and is dependent on the size (Stice et al., 2007), shape and insertion rate of the implant (Bjornsson et al., 2006). Although elements of this response appear well characterized, others remain unclear, and a clear link between the tissue response and device performance remains to be drawn (Polikov et al., 2005).

Furthermore, the tissue reaction may in turn degrade the materials used for these devices, which could exacerbate the inflammatory immune response. Several studies report structural changes in commonly used materials like silicon (Si), in vitro (Alexander et al., 1954) and in vivo (Barrese et al., 2013; Prasad et al., 2012). A hermetic, or protective, coating could prevent foreign bodies from penetrating the materials and prevent outward dissolution of materials (Cogan et al., 2003; Zorman, 2009). Silicon carbide (SiC) is a robust material that is able to withstand high temperatures and adverse chemical conditions (Zorman, 2009). It is produced in several different forms, each with slightly different material properties. One of these types, amorphous SiC (a-SiC) is additionally beneficial because of its diffusion barrier property, low temperature deposition, direct binding to Si (Zorman, 2009), slow dissolution rate, and biocompatibility (Cogan et al., 2003; Iliescu et al., 2008). It has already been utilized as a
protective coating for orthopedic implants (Sella et al., 1993), and coronary stents (Amon et al., 1996). It is therefore feasible that a-SiC would be a valuable addition to Si or other SiC variations as a hermetic surface coating (Zorman, 2009) that may restrict the feedback loop at the neural interface and impede device failure.

To evaluate this capacity, the biocompatibility should be assessed in vitro first. Neuronal networks cultured on substrate integrated MEAs provide a valuable platform for toxicology (Defranchi et al., 2011; Johnstone et al., 2010; Martinoia et al., 2005; O’Shaughnessy et al., 2004; Pancrazio et al., 2003, 2001), neuropharmacology (Hascup et al., 2010; Kulagina et al., 2004; Novellino et al., 2011; Piet et al., 2011; Xia, 2003), and computation (Berry et al., 2012; Dockendorf et al., 2009). Neural tissue derived from primary dissection can be cultured to form functional networks where action potentials, or spikes, can be monitored non-invasively. After neuronal networks mature and stabilize in vitro, spontaneous spike activity exhibiting varying degrees of coordination across multiple neurons, or units, can be readily observed (Gross et al., 1995; Selinger et al., 2004; Wagenaar et al., 2005) and is largely influenced by functional synapses (Gross et al., 1995; Johnstone et al., 2010; Wagenaar et al., 2005). With modulation of spike activity associated with exposure to a putative neuroactive compound, this approach may be a valuable tool to complement pre-clinical testing of therapeutics (Johnstone et al., 2010; Shimono et al., 2002; Xia et al., 2003) as well as to screen for environmental toxins (Kulagina et al., 2004; Pancrazio et al., 2003, 2001). As such, we established this technique and evaluated it pharmacologically by examining the neurotoxic effects of ω-agatoxin (Knaack et al, 2013).
This valuable platform was then implemented as a functional complement for the live/dead assay to evaluate the biomaterials in a more sensitive manner. The international standard ISO10993 provides regulatory guidance aiming to ensure that new biomedical devices consist of materials that are biocompatible. The current \textit{in vitro} test consists primarily of cytotoxicity through the characterization of live or dead. However, a material may be deleterious to a cell without completely killing it. This is especially true in the case of neurons where functionality is a key concern. As such, we developed a functional method to assess biocompatibility of materials that follows the ISO while providing more sensitive measurements (Charkhkar et al., 2014).

Finally, this assay was utilized to test the biocompatibility of different variations of SiC for the possible use with neural interfaces. Since a-SiC did not reduce spontaneous network firing rate \textit{in vitro}, it was then examined \textit{in vivo} compared to the standard Si probe.
CHAPTER TWO: USE OF MURINE FRONTAL CORTEX DERIVED NEURONAL NETWORKS IN NEUROPHARMACOLOGY

Given the utility of spinal cord (Gramowski et al., 2004; Johnstone et al., 2010; Keefer et al., 2001; Morefield et al., 2000) and frontal cortex (Hascup et al., 2010; Novellino et al., 2011; Piet, et al., 2011; Xia et al., 2003) derived neuronal networks for neuropharmacological applications, we wanted to establish the technique for in vitro biocompatibility test of materials. To examine the dependence of network activity to functional synapses, we investigated the effect of ω-agatoxin-IVA on these networks.

ω-Agatoxin-IVA, derived from the venom of the funnel web spider, is a well-known P/Q-type voltage dependent calcium (Ca^{2+}) channel blocker in vertebrates (Mintz et al., 1992). There are five functionally and pharmacologically classified groups of Ca^{2+} channels, which vary in location and role across the nervous system (Bean, 1989; Mintz et al., 1992; Verderio et al., 1995). The P/Q-type is expressed in presynaptic nerve terminals and plays a vital role in controlling fast release of neurotransmitters such as GABA and glutamate (Johnston and Wu, 1995; Verderio et al., 1995; Zaitsev et al., 2007). Regulating these neurotransmitters affects synaptic function, which influences action potentials (Borst and Sakman, 1996; Katz, 1969; Takahashi and Momiyama, 1993). Therefore, block of P/Q-type Ca^{2+} channels by ω-agatoxin may affect action potentials and can be investigated through changes in network level activity in vitro.
Based on the key role of P/Q Ca\(^{2+}\) channels in controlling neurotransmitter release, we expected that ω-agatoxin exposure would produce an overall decline in network activity. Our results show that unit specific effects could be readily observed and statistically discriminated in frontal cortex networks. This unit specific response in may be related to ω-agatoxin action on both excitatory and inhibitory transmission in the heterogeneous network.

**Methods**

**MEA Preparation**

MEAs were purchased from the Center for Network Neuroscience (University of North Texas, Denton, TX). The procedures for fabrication and preparation have been previously described (Gross et al., 1985; Gross, 1979). Briefly, arrays were sterilized in 70% ethanol for 20 minutes. Autoclaved rubber gaskets were adhered to the array with sterile grease to form a well. The surface was made more hydrophilic by a brief exposure to a butane flame. The arrays were coated with 50µg/mL of poly-D-lysine (PDL) (Sigma-Aldrich, St. Louis, MO, CAS No. 27964-99-4) overnight, followed by three wash (five minutes each) with sterile water. Lastly, the arrays were coated with 20µg/mL of laminin (Sigma-Aldrich, St. Louis, MO, CAS No. 114956-81-9) for 1 hour and then removed without washing.

**Cell Culture**

All animal procedures were in accordance with and approved by George Mason University IACUC (protocol # 0221). Frontal cortex was extracted from E17 ICR mice. After enzymatic dissociation with DNase (Worthington Biochemical Corp., Lakewood, NJ, Cat. No. LK003172) and papain (Worthington Biochemical Corp., Lakewood, NJ,
Cat. No. LK003178), cells were plated on MEAs at a density of 150,000 in a 100μL droplet concentrated over the electrode matrix. All networks were incubated at 37°C with 10% CO₂ and maintained in Dulbecco’s modified eagle medium (DMEM; Life Technologies, Grand Island, NY, Ref. No. 11965-092) for frontal cortex supplemented with 5% horse serum (Atlanta Biologicals, Lawrenceville, GA, Cat. No. S12195H), 5% fetal bovine serum (Life Technologies, Grand Island, NY, Ref. No. 10082-139), 2% B-27 (Life Technologies, Grand Island, NY, Ref. No. 17504-044), and 0.2 % 4mg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO, CAS No. 50-81-7) for the first two days. At day 3, serum was removed and networks were maintained by a 50% media exchange twice a week.

**Reagents**

ω-Agatoxin-IVA (CAS No. 145017-83-0) was purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions were prepared by dissolving with dH₂O to a concentration of 20 µM. ω-Agatoxin was stored in 100µL aliquots at -80°C until thawed and diluted in recording media to final concentrations of 1, 5, 10, and 50 nM. Aliquots were not re-frozen.

**Extracellular Recordings**

After three to four weeks *in vitro*, the cultured neurons on MEAs have matured into active networks. The multichannel extracellular recordings from these networks were performed with an OmniPlex microelectrode recording system (Plexon Inc, Dallas, TX), where each of the 64 channels was digitized at a sampling frequency of 40KHz. Media containing DMEM enhanced with 25 mM glucose (Sigma-Aldrich, St. Louis, MO, CAS
No. 50-99-7), 40 mM HEPES (Sigma-Aldrich, St. Louis, MO, CAS No. 7365-45-9), and 26 mM sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, CAS No. 144-55-8) was perfused at a constant rate of 1 mL per minute. Temperature was maintained at 37°C throughout the experiment. Following the establishment of baseline activity for 90 minutes, increasing concentrations of ω-agatoxin were administered to the perfusion medium for 30 minutes each. The summation of which generated a concentration response curve for each network. For all neuronal networks, drug exposure was followed by a 1 hour washout with fresh recording media perfused at a constant rate of 1 mL per minute to determine if the effects were reversible.

**Data Analysis**

During extracellular recordings from the neuronal networks, mean noise level was calculated for each individual channel and a threshold was set at six times the standard deviation of this mean. A spike was then detected if it passed the threshold. The recorded spikes from each channel were sorted offline into well-resolved units (Figure 1A) determined by Scanning K-Means in Offline Sorter V.3 (Plexon Inc, Dallas, TX) and followed by visual inspection (Hill et al., 2011). We assumed that each sorted unit corresponded to a signal from a distinct neuron and the same unit was never observed on multiple electrodes.

A fundamental feature of neuronal dynamics is the occurrence of bursts. A burst is defined by a series of at least $n$ action potentials occurring within some time threshold of one another. A burst was classified uniquely for each individual network by implementing the methodology used in Selinger et al (2007). Briefly, an algorithm was
utilized which considers the distribution of the log of the inter spike intervals (ISIs). For a network that is bursting, the log ISI distribution should be bi-modal, where the first peak represents the typical ISI of the network and the second peak represents the typical interval between bursts (termed inter burst interval or IBI). The minimum point between these two peaks is taken to be the network bursting threshold, or the maximum allowable ISI between action potential events in a burst. The ratio of the two peak areas defines the average number of spikes per burst. Burst rate, IBI, and burst duration were calculated and analyzed for frontal cortex networks. There was no significant change in IBI during pharmacological application; therefore, these data are not presented.

For all concentrations and the wash out period, mean spike rate and bursting parameters (frontal cortex only) were determined for each unit and normalized to its baseline. Outliers were removed if they were two standard deviations from the mean on at least half of the time points examined. These values were then averaged within a network to create mean network rates. To detect potential unit specific effects of ω-agatoxin exposure in frontal cortex networks (n = 6), normalized mean spike rate for each distinguished unit (n = 168) was calculated for all concentrations as previously mentioned. These unit responses were then classified utilizing the TwoStep Clustering Analysis (IBM SPSS Statistics), which is a type of clustering method that can automatically determine the number of distinct responses or clusters. Based on this analysis, there were two different effects (C₁=79 units and C₂=89 units) and the resulting clusters were examined separately. Results were analyzed with a repeated measures
ANOVA proceeded by planned contrasts to compare each concentration and washout to baseline.

The concentration dependence of ω-agatoxin induced decrease in spike rate was fit to the following logistic equation to calculate the half maximal effective concentration, or the effect half way between baseline and the maximum effect:

**Equation 1: Dose Response Curve Fitting**

\[
y = y_{\text{min}} + \frac{y_{\text{max}} - y_{\text{min}}}{1 + \left(\frac{D}{K_D}\right)^{n_H}}
\]

where \(y_{\text{min}}\) is the minimum spike rate observed, \(y_{\text{max}}\) is the maximum spike rate observed, \(K_D\) is the concentration for half maximal block (EC\(_{50}\)), \(D\) is the drug concentration, and \(n_H\) is the slope factor or Hill coefficient (Pancrazio et al., 1998).

**Results**

**Extracellular Recordings from Neuronal Networks**

Under our culture conditions, neuronal networks on MEAs typically show a carpet of cells (Figure 1B) and exhibit consistent, well resolved single units after 21 days in vitro. Frontal cortex data consist of responses from 168 total units over six networks cultured from three different mice. Frontal cortex networks displayed 28.6±5.5 active electrodes with an average amplitude of approximately 100 μV peak to peak. Of these active channels, 7.3±2.1, or about 25%, demonstrated multiple units (Figure 1A) with a firing rate of 3.9±0.9 Hz. Consistent with prior work, unit activity occurred in both single spikes and bursts (Gross et al., 1995; Selinger et al., 2004; Wagenaar et al., 2005) where,
coordinated bursting was readily apparent. Figure 1C displays representative raster plots of neuronal activity where every vertical line represents a point in time when an action potential was detected and each row is an individual unit.

![Raster plots](image)

**Figure 1: Representative Frontal Cortex Networks**

Raster plots depict spontaneous network activity where each vertical line represents a single detected action potential (spike) and each row illustrates spikes recorded from one sorted unit. A) Two well resolved units detected from a single electrode using Scanning K-means sorting method in Offline Sorter V.3 (Plexon Inc, Dallas, TX). B) Frontal cortex cells at 21 days *in vitro* plated on a microelectrode array. C) Raster plot from nine frontal cortex units for a 20 second period showing individual spiking and synchronous bursting.

**Unit Specific Effects of ω-Agatoxin IVA on Frontal Cortex Network Activity**

Exposure of frontal cortex cultures led to divergent effects at the level of discriminated units suggesting unit specific effects of ω-agatoxin IVA. Cluster analysis concluded that there were two types of responses. Units classified as cluster one demonstrated a significant decrease in mean network spike rate and burst rate, but those classified as cluster two only displayed a significant increase in burst duration (Table 1).
ω-Agatoxin did not appear to alter the amplitude or shape the extracellular potentials (data not shown).

Table 1: Comparison of Cluster 1 and Cluster 2 Neurons

Comparison of the changes observed between frontal cortex neurons classified as either cluster one or two based on their response to 200 nM ω-agatoxin IVA. Mean network spike rate and burst rate significantly decreased compared to baseline for units classified as cluster one (79 units), whereas burst duration significantly increased compared to baseline for units classified as cluster two (89 units). Spike rate was determined for each unit and normalized to its baseline. These values were classified into two groups (clusters) by using TwoStep Clustering Analysis (IBM SPSS Statistics). Normalized spike rate, burst rate, and burst duration were then averaged within a network to calculate mean network rate for each cluster. Data are from 168 total units over six networks cultured from three different mice. Values represent mean. 

- indicates a decrease
+ indicates an increase
* denotes significance compared to baseline, p < 0.05.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Spike Rate (%)</th>
<th>Burst Rate (%)</th>
<th>Burst Duration (%)</th>
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<tbody>
<tr>
<td>One</td>
<td>-53.1*</td>
<td>-46.7*</td>
<td>+11.4</td>
</tr>
<tr>
<td>Two</td>
<td>+17.5</td>
<td>+22.2</td>
<td>+100.5*</td>
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</table>

The effects on mean network spike rate, for cluster one, were concentration-dependent with statistically significant decreases at 50 nM, 100 nM, and 200 nM, $F(5, 25) = 21.51, p < 0.01$ as calculated with a repeated measures ANOVA (Figure 2). EC$_{50}$ was 33.7±9.9nM (mean±SEM; Equation 1), with the maximum decrease of 53.06% at 200 nM. Although activity recovered to 64.7% of baseline during washout, the change was still significantly different ($p < 0.05$). In contrast, cluster two did not indicate a statistically significant change in the spontaneous firing rate with exposure to ω-agatoxin ($p > 0.05$).
Cluster one additionally showed a significant reduction in burst rate at all concentrations, $F(5, 25) = 11.29, p < 0.05$, and this effect was not reversed during the washout. However, there was no change detected in burst duration ($p > 0.05$). Cluster two did not display a change in burst rate ($p > 0.05$), but did exhibit an increase in burst duration in a concentration-dependent manner with statistical significance at 100 nM and 200 nM, $F(5, 25) = 3.84, p < 0.01$. There was a maximum increase of 100.5% at 200 nM.

Figure 2: Response of Frontal Cortex Neurons to $\omega$-Agatoxin IVA
Spontaneous network activity significantly decreased with increasing concentrations of toxin for cluster one, but not cluster two. Spike rate was determined for each unit and normalized to its baseline. These values were classified into two groups (clusters) by using TwoStep Clustering Analysis (IBM SPSS Statistics) and then averaged within a network to calculate mean network spike rate for each cluster. Data are from 168 total units over six networks cultured from three different mice. Closed circles represent cluster one (79 units) and open squares represent cluster two (89 units). Values represent mean ± SEM. * denotes significance compared to baseline, $p < 0.01$. 
Discussion

Our results show that primary murine frontal cortex neuronal networks respond to ω-agatoxin IVA in a concentration-dependent manner as observed by a decrease in mean network spike rate, an effect that was not significantly reversed with a washout of fresh recording media.

ω-Agatoxin IVA blocks P/Q-type voltage dependent Ca\(^{2+}\) channels in vertebrates, a phenomenon that has been established in single cells (Mintz et al., 1992). Block of these Ca\(^{2+}\) channels prevents inward Ca\(^{2+}\) current, which inhibits evoked neurotransmitter release into the synaptic cleft (Turner et al., 1993; Verderio et al., 1995; Zaitsev et al., 2007). However, the density of these channels can change throughout development (Verderio et al., 1995) and differ between regions (Takahashi and Momiyama, 1993).

The neurotoxic effect of ω-agatoxin IVA on spike rate in frontal cortex is complicated by unit specific responses, which can be separated into two distinct classes that either show an inhibitory (cluster one) or excitatory (cluster two) effect. Cluster one also displayed a significant decrease in the burst rate, which parallels the decrease in spike rate. Conversely, the length of a burst did not change. This indicates that some of the units that originally displayed synchronous bursting were eliminated with the administration of toxin. However, there remained other units which continued to burst without changes to the bursting parameters.

Interestingly, cluster two did not show significant changes in spike rate or burst rate, but there was a significant increase in burst duration. Therefore, the number of units exhibiting bursting did not differ in response to ω-agatoxin IVA, but a characteristic of the burst, specifically the duration, was altered. One possibility is that that blocking
inhibition indirectly affects burst behavior. Reducing GABA causes an increased excitatory post synaptic potential (EPSP) via the glutamatergic NMDA receptor. This results in a reorganization of spike timing by increasing burst duration without changing firing rate (Murase et al., 1993; Overton and Clark, 1997). It has also been demonstrated that the calcium regulated potassium channel (K$^{+}_{Ca}$) regulates burst termination and is activated by Ca$^{2+}$ influx through P/Q-type or N-type Ca$^{2+}$ channels (El Manira and Wallén, 2000; Overton and Clark, 1997; Wikström and El Manira, 1998; Womack et al., 2004). It is possible that the P/Q-type calcium channel is more tightly coupled with the K$^{+}_{Ca}$ channel in those units receiving GABAergic input and were classified as cluster two. Future research should investigate the precise mechanism for burst termination in frontal cortex neurons that receive GABAergic input versus those that receive non-GABAergic input.

Since P/Q-type Ca$^{2+}$ channels are responsible for fast neurotransmitter release such as GABA and glutamate (Johnston, 1995; Takahashi and Momiyama, 1993; Verderio et al., 1995; Zaitsev et al., 2007), it is possible that the units classified into the different clusters are receiving input from these two classes of neurons.

Our findings are in line with a recent study which examined the complex effects of cannabinoid activation on rat cortical networks in vitro. This receptor is located presynaptically and inhibits neurotransmitter release when activated (Piet et al., 2011). Piet et al. (2011) reported that administration of a cannabinoid receptor agonist had heterogeneous effects on spontaneous cortical network activity. Recognizing that cannabinoid receptor activation could affect both excitatory and inhibitory
neurotransmission, activity was additionally monitored with concurrent exposure of
GABA$_A$ receptor blocker picrotoxin. Similar to our observations with ω-agatoxin IVA,
block of inhibitory neurotransmission produced a homogenous response from the
networks to cannabinoid receptor activation. This was characterized by a decrease in
network activity.

Our observation of a 53-54% maximum reduction in network level activity is
consistent with the view that ω-agatoxin blocks P/Q-type Ca$_{2+}$ channels and affects
neuronal transmission at concentrations in the 100-200 nM range. ω-Agatoxin IVA has
previously been shown to reduce Ca$_{2+}$ entry by 71% in rat synaptosomes at 200 nM
(Minz et al., 1992). 100 nM of ω-agatoxin IVA inhibited glutamate release by 50-100%
in rat hippocampal cells (Verderio et al., 1995) and by 56% in rat synaptosomes (Turner
et al., 1992). Exposure to 200 nM ω-agatoxin IVA reduced inhibitory post synaptic
currents by 98% in the cerebellum and 92% in the spinal cord, while excitatory post
synaptic currents were decreased by 84% in the hippocampus (Takahashi and
Momiyama, 1993). Since ω-agatoxin inhibits presynaptic Ca$_{2+}$ entry, neurotransmitter
release, and post synaptic potentials, it could be surmised that this toxin would
additionally cause a change in extracellular action potentials as detected by the level of
network activity. Although the percentage of decrease seems to vary across the literature
it is important to note that some of the studies were examining presynaptic measures
(Minz et al., 1992; Turner et al., 1992; Verderio et al., 1995), while others detected
postsynaptic changes (Takahashi and Momiyama, 1993). These differences can also be
explained by the fact that they were observed across different brain regions, but the
density of P/Q-type Ca\(^{2+}\) channels is known to vary (Takahashi and Momiyama, 1993; Verderio et al., 1995) and their significance in neurotransmission may also fluctuate. Furthermore, single cell recordings may detect a larger change than what is displayed in a network because there are several cells types within a region (Degenetais et al., 2002; Kawaguchi and Kubota, 1997) and these may be affected differently. Therefore, it is important to examine a neurotoxin on the single cell and network level to better understand all of the possible effects and ensure nothing is missed (Novellino, et al., 2011).

**Conclusions**

These data show that there are unit specific neurotoxic effects for \(\omega\)-agatoxin IVA. Furthermore, the detection of network level changes in spontaneous activity confirm the establishment of neuronal networks on MEAs and the utility of this technique to assess functional changes resulting from pharmacological or material applications.

**Contributions**

Experimental design: Gretchen L. Knaack, Joseph J. Pancrazio, Thomas J. O‘Shaughnessy

Cell culture: Gretchen L. Knaack

Performed experiments: Gretchen L. Knaack

Data analysis: Hamid Charkhkar, Franz W. Hamilton

Statistical analysis: Gretchen L. Knaack

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Citation:

Although implantable cortical devices show promise, chronic use of such applications has been unreliable. Neuroinflammation at the tissue-device interface has been implicated in this shortcoming and different material designs are being investigated as a possible solution. SiC is a robust material that is able to withstand high temperatures and adverse chemical conditions (Zorman, 2009). It is produced in several different forms, each with slightly different material properties. One of these, or a combination, may be beneficial for neural devices.

The international standard ISO10993 provides regulatory guidance aiming to ensure that new biomedical devices consist of materials that are biocompatible. As described in ISO1099-5, consideration of new medical device materials typically begins with in vitro testing where cytotoxicity serves as the main endpoint. In general, cytotoxicity testing is considered to be a standardized and sensitive approach to assess whether or not a novel material, or residual reagent used in the fabrication process, can induce deleterious biological effects. While a negative cytotoxicity test result does not necessarily imply suitability of the material for in vivo use, these tests can be useful to identify reactive materials in vitro. The standard describes systematic methods of exposing materials or material extractions to cells for multiple cell viability assays.
In the present study, we examine the utility of living neuronal networks as functional assays for in vitro material biocompatibility. We describe a method for exposing networks, which provides 1) stable neuronal activity under control conditions, and 2) consistency with ISO10993-5 guidance. Lastly, this assay was utilized to test the biocompatibility of different variations of SiC for the possible use with neural interfaces.

Methods

Materials and Sample Preparation

We chose materials to evaluate our functional in vitro method for material biocompatibility testing based on the ISO 10993-12 standard as well as prior work on the cytotoxicity of implantable devices (Hooper and Cameron, 2007). These materials were divided into two categories: conductor and insulator. For each category a positive and negative control material was selected. A positive material is one that, when tested by a specific procedure, will cause a reactive response while a negative control will induce a non-reactive or minimal response under the same test procedure. For conductors, gold (Au) and copper (Cu) and for insulators, polyethylene (PE) and polyvinyl chloride (PVC) were chosen as negative and positive controls, respectively. After establishing the validity and sensitivity of the functional assay, it was additionally used to test three different variations of SiC: 1) a-SiC on Si 2) 3C-SiC on Si 3) a-SiC on 3C-SiC on Si.

For Au, W, Cu, 3C-SiC, and a-SiC thin films were deposited onto 8 mm x 10 mm rectangular silicon (Si) coupons using the following process. 100 mm diameter wafers of (100) Si (Wafer World, West Palm Beach, FL) were cleaned using a standard RCA clean process (Kern and Puotinen, 1970) and then placed in a 4-pocket E-beam evaporator and
loaded with ceramic crucibles containing titanium (Ti) and either Au, W, or Cu, respectively. 500 Å of Ti was evaporated onto the surface of the Si as it allows the target metals to attach to the surface of the Si. 5000 Å of the target metal was then evaporated onto the Ti film surface. The wafers were removed from the evaporator and diced into 8 mm × 10 mm rectangular coupons using a dicing saw equipped with a diamond blade. The samples were then solvent cleaned ultrasonically in acetone and isopropanol followed by a de-ionized water rinse.

A bulk high density PE sheet was purchased from Ridout Plastics Co. Inc. (San Diego, CA) and cut into the 8 mm x 10 mm rectangular coupons using a dicing saw. Tygon F-4040-A tubing designed for use with fuels and industrial lubricants, which consists of PVC with a plasticizer, was used as a positive control material (Hooper and Cameron, 2007).

All the samples were sterilized by ethylene oxide exposure for 12 hours before preparation with cell culture media.

**MEA Preparation**

Planar MEAs were purchased from ALA Scientific Instruments (Farmingdale, NY). Each MEA had 60 electrodes with an electrode diameter of 10 µm and inter-electrode spacing of 200 µm. Sterilization and preparation was similar to that described in chapter two.
**Primary Neuronal Culture**

The primary neuronal culture method was the same as described in chapter two, except that frontal cortex networks were seeded at a concentration of 100,000 in a 50µL droplet.

**Functional Assays and Analysis**

All recordings were performed after at least 3 weeks *in vitro* to ensure that the neural networks had reached maturity and consistency in activity. The multichannel extracellular recordings from these networks was performed by an OmniPlex data acquisition system (Plexon Inc, Dallas, TX), where each of the 60 channels of an MEA was digitized at a sampling frequency of 40 KHz. In order to increase the number of simultaneous experiments, another MEA data acquisition system, MEA2100-32 (Multi Channel Systems, Reutlingen, Germany), was also utilized. The MEA2100-32 had only 32 channels with sampling rate per channel of up to 50 KHz. In both recording systems, the culture temperature was controlled and set at 37 °C.

Extracts of materials were prepared by incubating 1 ml serum-free cell culture media for every 3 cm² of material sample (according to ISO 10993-12 and ISO 10993-5) in 35 mm diameter polystyrene petri dishes (Fisher Scientific) at 37°C for 24 hours under constant agitation using a laboratory shaker (Genemate; BioExpress, Kaysville, UT). Control extracts also underwent the same procedures except they were incubated with material-free cell culture media.

Complete media change was performed 24 hours before baseline recording to rule out any effects due to media replacement itself. The exposure paradigm, as depicted in Figure 3, consisted of baseline recordings followed by exposing the culture to the extracts
with 100% media change and measuring the activity again 24 hours later. The 24 hour period between the recordings was adapted from standard ISO 10993-5. The cultures were inspected under an optical microscope before every recording session to identify any obvious morphological changes or signs of media contamination.

![Image](image.png)

**Figure 3: Exposure Paradigm for Functional Toxicity Testing**

During extracellular recordings from the neuronal networks, the mean noise level was calculated for each individual channel and a threshold was set at 4.5 - 5 standard deviations from this mean. A spike was then detected if the signal passed this threshold. Recording sessions were 30 minutes long and channels with spike rates above 0.1 Hz were considered active. The recorded spikes from each channel were sorted off-line into well-resolved units using Offline Sorter V.3 (Plexon Inc.). The spike sorting method was based on the 2D principle component analysis of spike waveforms followed by scanning K-Means to find and separate between clusters. All the sorted units were also visually inspected to assure that the waveforms had bi-phasic physiological shapes (Hill et al., 2011). Each unit presumably corresponds to the signal from an individual neuron. Consistent with McConnell et al. (2012), our quantification for functional neurotoxicity focused on the determination of mean spike rate across each network. Exposure data were
normalized to the corresponding baseline levels measured before exposure to the material sample.

**Dose-Response Curve Fitting**

Dose-response curves for the positive controls (i.e., PVC and Cu extracts) were calculated for both assays. For each assay, the responses corresponding to different concentrations were fitted to the function described in Equation 1. For Cu extracts, in order to relate the EC$_{50}$ value to actual concentration of Cu ions in the extract, the molarity was determined by using a copper ion-selective electrode (Cole Parmer, Vernon Hills, IL) and a pH/ion meter (Fisher Scientific).

**Statistical Analysis**

All statistical analyses were performed using MATLAB Statistics Toolbox Version 7.10 (Mathworks, Natick, MA). Data are expressed as mean ± SEM. Student’s t-test was used to compare any two sets and $p < 0.05$ was considered statistically significant.

**Results**

**Functional Neurotoxicity of Positive and Negative Controls**

Sample-free extracts, which had no material samples at the time of preparation, were exposed to neuronal cultures to determine the effect of feeding and test procedure on possible changes in their activity. The normalized spike rate after exposure to such extracts was 1.11 ± 0.07 (n = 5 networks, mean ± SEM), indicating that the process of media exchange under the exposure protocol did not induce significant changes in spike activity. Note that the stability of this metric is dependent on network maturity. Initial
tests performed with networks younger than three weeks \textit{in vitro} showed larger fluctuations in mean spike rate with media replacement.

Exposure to extracts derived from negative control materials failed to alter the neuronal network spike firing rate. Application of extracts from Au and PE at 100% media extraction did not significantly affect neuronal network firing compared to baseline and cortical cultures maintained their typical synchronous bursting (Figure 4). For all of the experiments, network activity recorded 24 hours after extract exposure was normalized to the baseline (pre-exposure) activity. The normalized spike rate for Au and PE was $1.08 \pm 0.06$ and $0.93 \pm 0.07$, respectively ($n = 5$ for each material).
In contrast, exposure to extracts derived from positive control materials resulted in a significant reduction in the mean network spike rate compared to baseline. Cu and PVC extracts at 100% concentration completely blocked the activity (data not shown). Therefore, lower concentrations of positive extracts were tested on functional assays to determine if neuronal spiking could persist. For Cu, extract concentrations above 5% eliminated all spike activity whereas 0.1% or below had no observable effect on the activity. As shown in Figure 4, 0.5% Cu extracts resulted in a 52 ± 3% reduction of spike rate (n = 3 networks). For exposures to PVC extracts, the activity dropped by 63 ± 1%
after applying the 30% extracts (n = 3 networks, Figure 4), but no reduction was observed for 1% extracts. The raster plots shown in Figure 5 suggest that the overall reduction in spike rate with exposure to positive control material extracts is accompanied with changes in burst and synchronization dynamics.

Figure 5: Raster Plots of Positive and Negative Controls
Raster plots of 60s activity for 6 representative units after exposure to extracts derived from control materials. 100% extracts from negative controls (Au and PE) did not alter typical pattern of activity for frontal cortex network. However, spike rate was decreased after application of Cu and PVC extracts.

The dose response curves based on functional assay responses are shown in Figure 6. The EC$_{50}$ values derived from curve fitting for extracts from Cu and PVC were at 0.4% and 18%, respectively. For Cu extracts, the molarity of Cu$^{2+}$ ions at EC$_{50}$ concentration was found around 145µM which was consistent with previously reported
levels of Cu toxicity in primary cortical cultures (White et al., 1999) and cerebellar cultures (Brown et al. 1998).

To quantify the suitability of the functional assay, $Z$-factor as a measure of assay performance was calculated (Kuhn et al., 2013). For the $Z$-factor, a value between 0.5 to 1 is an indicator of an excellent assay whereas values between 0-0.5 reflect a marginal assay (Zhang et al. 1999). The estimated $Z$-factor for the functional assay was 0.65 which suggests the high quality of such an assay for screening purposes.

**Functional Neurotoxicity of SiC**
In addition to control materials, three variations of SiC on Si were examined by the functional neuronal network assay for *in vitro* biocompatibility. A one-way ANOVA
determined that there was no significant difference between materials on the change in spike rate after exposure compared to baseline (Figure 7).

![Figure 7: Silicon Carbide In Vitro](image)

**Figure 7: Silicon Carbide In Vitro**

Effects on neuronal network activity 24 hours after exposure to SiC material extracts. The spike rates were normalized to the baseline activity measured prior to adding the extracts. Data are shown as mean normalized spike rate and SEM for n = 3 Change in network spike rate after exposure to SiC variations normalized to baseline. Data are mean ± SEM

**Discussion**

The use of living neuronal networks cultured on MEAs for pharmacological and toxicological studies has been well established (Morefield et al., 2000; Johnstone et al., 2010; Hascup et al., 2010; Novellino et al., 2011). For the first time, the present study extends the utility of this approach to biomaterial testing applications, in particular for materials intended for implantable neural interfaces. Emerging applications of neural
interfaces include MEA technology where the ability to record spike activity is a key feature. By relying on the analysis of spike activity as an end-point, this approach to material testing provides a highly relevant and sensitive measure to the functionality of such devices.

The exposure paradigm utilizing extracts from culture media we have described is consistent in time and dilutions with the established ISO10993-5 methodology. While the positive and negative controls elicited effects in cultured neuronal networks that were in line with the L929 fibroblast cytotoxicity findings, the neuronal networks appeared to be more sensitive to Cu as a positive control. It has been shown that Cu ions lead to formation of reactive oxygen species in cellular microenvironment that cause oxidative stress and apoptosis (Franco et al., 2009). Comparing to other cell lines, neurons are highly sensitive to oxidative stress (White et al. 1999). Such higher sensitivity could be a reason for the large difference in response to Cu between the conventional L929 and functional neuronal assays.

A wide range of neural cell types have been previously used to assess the biocompatibility of materials in vitro. A common choice has been tumor derived neural cells such as neuroblastoma or primary neuronal cultures (Jain et al., 2013; He et al., 2009; Bardi et al., 2010) with endpoints that include neurite extension and the expression of neural specific proteins such as MAP2 and neurofilament 200. Relevant to our work, primary neuronal cultures have exhibited a markedly, albeit qualitatively, higher level of sensitivity over immortalized cell lines (Harry et al., 1998). In addition, primary derived
neurons offer the opportunity to include functional physiological measures of neural activity which are directly relevant to brain-machine interface applications.

A limitation to the approach we have presented is the expense and low throughput of MEA-based studies. Our assays were conducted in single well MEA dishes that, while reusable, are somewhat costly. Recent work by our group suggests that MEAs can be readily fabricated at low cost and of materials that are suitable for disposal (Charkhkar et al., 2012). Furthermore, multi-well MEA systems have become commercially available and offer the promise of increased throughput (McConnell et al., 2012).

Despite the possible shortcomings of this assay, we were able to utilize it to investigate the biocompatibility of different SiC variation in vitro. The addition of SiC to Si wafers did not significantly decrease spontaneous network spike rate, irrespective of the SiC variation. This finding is consistent with the literature (Iliescu et al 2008) where a-SiC membranes were successfully utilized as a substrate for fibroblast cell cultures. These data suggest that SiC is biocompatible in vitro and could be evaluated for in vivo applications.

**Conclusions**

Our findings suggest cultured neuronal networks on MEAs could be utilized to assess functional toxicity of materials for neural implants. In the functional assay, change in the spike rate is a measure of functional toxicity which is a relevant metric to brain-machine interface applications. Furthermore, compared to the conventional live/dead L929 assay, the functional assay showed more sensitivity in response to copper as a positive control. The estimated Z’ factor for the assay also reflects the high quality in the
performance for screening purposes. Considering the developed assay is consistent with the ISO standard in extract preparation and exposure time, the functional assay can be regarded as complementary method to the conventional cytotoxicity assays.

**Contributions**
Neuronal experimental design: Hamid Charkhkar, Gretchen L. Knaack, Stephan E. Saddow, Joseph J. Pancrazio

Primary neuronal culture: Hamid Charkhkar, Gretchen L. Knaack

Functional neurotoxicity experiments: Hamid Charkhkar

Data analysis: Hamid Charkhkar, Christopher Frewin

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Citation:

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The use of implantable microelectrode arrays for neural prosthetic devices is on the rise due to advancements in technology and material sciences. Although neural implants are promising, the obtained signals degrade over time until device failure. The brain’s immune response is a contributing factor to recording failure and it causes material degradation. A hermetic, or protective, coating could prevent foreign bodies from penetrating the materials and prevent outward dissolution of materials. SiC is a robust material that is able to withstand high temperatures and adverse chemical conditions (Zorman, 2009). It is produced in several different forms, each with slightly different material properties. a-SiC, a thin film type of SiC, is additionally beneficial because of its diffusion barrier property, low temperature deposition, direct binding to Si (Zorman, 2009), slow dissolution rate, and biocompatibility (Cogan et al., 2003; Iliescu et al., 2008). Therefore, the addition of a-SiC may impede device failure by restricting the auto-toxic loop. In this study, we aimed to assess the benefits of a-SiC coated probes compared to the standard Si probes.
Methods

Neural Implants
To determine if a-SiC improved chronic Si implants, single shank non-functional probes were inserted into the primary motor cortex of rats. Conventional Si probes (NeuroNexus, Ann Arbor, MI) were deposited with 0.5 μm a-SiC using plasma-enhanced chemical vapor deposition (PECVD). Si probes without a-SiC were also inserted as controls. Both probes were 5 mm in length, 15 μm thick, and 200 μm in width at the base and the widest part of the shank in contact with cortical tissue. To control for variability across animals, the plastic holders of each probe were glued together such that the needles were parallel with each other and could be inserted simultaneously. This combined device was sterilized with ethylene oxide gas for 24 hours.

Subjects
All animal procedures complied with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) at George Mason University, Fairfax, VA under protocol 0242. Adult female Long Evans rats (210-345 grams) were implanted with a chronic neural implant for either four weeks (n = 3) or eight weeks (n = 5).

Surgery
Rats was anesthetized with 5% isoflurane oxygen mixture at a rate of 1 L/min and maintained with 2%. Pain sensitivity was tested by paw pinches and breathing was monitored throughout surgery. Body temperature was maintained by a heating pad (Braintree Scientific, Braintree, MA). Surgical tools were sterilized with ethylene oxide gas and aseptic surgery technique was used.
After anesthetizing the rats, their heads were shaved from between the eyes to behind the ears and then placed in a stereotax frame (Kopf instruments, Tujunga, CA). Puralube eye ointment (PharmaDerm, Florham Park, NJ) was applied. Dexamethasone (Sparhawk Laboratories, Lenexa, KS) was injected subcutaneously at 2mg/kg and 2% lidocaine (Clipper Distributing Company, St. Joseph, MO) was also administered subcutaneously under the incision sight. The skin was disinfected with iodide before removing a small skin flap with sterile scissors. The fascia was removed and the skull cleaned with three alternations of 70% ethanol and 3% hydrogen peroxide, finishing with an additional application of ethanol. Compressed air was used to dry the skull in between each application.

Drilling and probe placement was visualized through a surgical scope (Seiler Instrument and Manufacturing Company, St. Louis, MO). Six skull screws were placed in burr holes drilled with a micro drill (Stoelting, Wood Dale, IL) to anchor the skull cap. After locating bregma, the bilateral craniotomy windows were drilled from -1.5 to + 1.5 mm anterior/posterior and ± 0.5 to ± 2.5 mm medial/lateral to target the primary motor cortex. Once detached, the bone fragments were removed. The dual probe device was placed in a holder attached to the stereotax arm and centered over the craniotomies paying special attention to avoid any surface vasculature. A hole was poked through dura just below the tips of the probes and then the device was lowered to the surface of the cortex. It was inserted using a micropositioner (Kopf instruments, Tujunga, CA) at a rate of 2mm/sec.
After insertion, the craniotomies were filled with silicon elastomere (kwik cast, World Precision Instruments, Sarasota, FL) and allowed to dry. A layer of Loctite prism 454 adhesive (Electron Microscopy Science, Hatfield, PA) was also applied and dried instantly with Loctite accelerant 7452 (Newark Element 14, Chicago, IL) to secure the probe to the skull and screws. Dental cement (Lang Dental Manufactures, Wheeling, IL) covered the screws and adhesive to create a robust head cap. To close the surgery site, skin was attached to the dental cement using gluture (World Precision Instruments, Sarasota, FL) and a triple-antibiotic cream was applied. To rehydrate the animals, 3 mL of 0.9% sterile saline was given subcutaneously. Ketaprofen (Fort Dodge Animal Health, Fort Dodge, Iowa) was administered subcutaneously at 5 mg/mL and continued twice daily for three days. Gentamicin (Clipper Distributing Company, St. Joseph, MO) was also injected subcutaneously at 8 mg/kg and continued once a day for one week.

**Immunohistochemistry**

To investigate the chronic response to a-SiC, implants remained in the brain for either four weeks or eight weeks. Rats were sacrificed by exsanguination from transcardial perfusion after anesthetizing with 5% isoflurane oxygen mixture (1 L/min) until no pain response was observed. Phosphate buffered saline (1X PBS, Fisher Scientific, Pittsburg, PA) flowed through the vascular system using a gravity pump to remove all of the blood from the circulatory vessels within the brain until fluid exiting the heart became clear. Then, 4% paraformaldehyde (PFA) was pumped to fix the tissue. The heads were placed in PFA for 48 hours and then transferred to PBS containing 90 mg/L sodium azide (Sigma-Aldrich, St. Louis, MO) at 4°C until head caps were removed.
Heads caps were removed such that the probes remained in the brain for device capture immunohistochemistry (IHC) in a method similar to Wolley et al (2011). Briefly, dental cement and adhesive were removed with a Dremel tool. The probes were separated at the base from the silicon elastomere using microscissors. The elastomere was then removed using forceps and the skull was carefully detached from the brain leaving the probes intact. All of these steps were performed under a surgical scope. Brains containing the probes were then placed in fresh to PBS containing sodium azide and stored at 4°C until slicing.

Brains were sliced sagitally, 200-250 μm thick with a vibratome, such that one slice contained the entire probe. Free floating sections were placed in a single well containing PBS with sodium azide. All slices were labeled for neurons (NeuN, Millipore, Billerica, MA), reactive microglia/macrophages (CD68, Millipore, Billerica, MA) glial fibrillary acidic protein of activated astrocytes (GFAP, Dako North America Inc., Carpinteria, CA) and cellular nuclei (DAPI, Life Technologies, Grand Island, NY).

Auto fluorescence was quenched two times with 6 mg/mL sodium borohydrate (Sigma-Aldrich) for 15 minutes each, followed by three washes with PBS containing sodium azide for five minutes each. A blocking buffer containing 4% normal goat serum (Life Technologies) and 0.3% Triton-X 100 (Sigma-Aldrich) in PBS with sodium azide was applied for one hour, followed by three rapid washes with PBS containing sodium azide. Slices were additionally blocked with Image-iT FX (Life Technologies) for 30 minutes followed by three rapid washes with PBS containing sodium azide. Slices were then treated with primary antibodies (CD68 1:1000, GFAP 1:500, NeuN 1:500) diluted in
blocking buffer containing 4% normal goat serum and 0.1% Triton-X 100 in PBS with sodium azide. Incubation occurred overnight at 4ºC and was followed by four washes with PBS containing sodium azide for 15 minutes each. Alexa Fluor conjugated secondary antibodies (1:1000, Life Technologies) and DAPI (0.6 µm) were diluted in the same blocking buffer as primary antibodies and incubated with slices for one hour followed by three washes with PBS containing sodium azide for 15 minutes each. To ensure homogenous penetration of all solutions slices were flipped half way through each step. Lastly, slices were mounted on slides with Fluoromount-G (Southern Biotech, Birmingham, AL).

Data Analysis
All slices were imaged using a confocal microscope at 20x objective (Nikon D-eclipse C1si; Nikon Instruments, Melville, NY). Laser intensity and gain were held constant for each label across slices. To obtain a broader field of view without losing resolution, each complete image contained 20 individual 20x images stitched together using Photoshop (Adobe Systems Inc., San Jose, CA). To control for naturally occurring variance in cell type density between cortical layers, complete images were cropped into individual layers according to Kandel (2000). For some slices, the implanted devices extended past the cortex and into the white matter. These images were cropped at the end of layer six and the white matter was not analyzed. Cropped images were then analyzed and normalized within each cortical layer. Fluorescence intensity was measured for NeuN, GFAP, CD68, and DAPI using MINUTE (Potter et al., 2012), a custom-written MATLAB program (Math Works, Natick, MA). An ellipse was used to outline the edge
of the device within each cropped image (Figure 8). Intensity values were determined as a function of distance from the probe edges every 10µm out to a final distance of 500µm. In order to combine fluorescence intensity across cortical layers, values were normalized to background within its layer as defined as the mean intensity value 400-500µm from the probe edge and then averaged across cortical layers to obtain a mean, normalized fluorescence intensity for each tissue slice. Outliers were removed if they were two standard deviations from the mean. For each label, differences in fluorescence intensity were compared using a three-way mixed ANOVA with Material (a-SiC, Si) and Time (4 weeks, 8 weeks) as between-subjects factors and Distance (0-100µm) as a within subjects factor using SPSS (IBM, Armonk, NY). A Huynh-Feldt correction was used if sphericity was violated and significance was determined as p < .05. Any significant main or interaction effects were followed up with planned contrasts due to apriori hypotheses where all distances were compared to baseline as defined as 100µm away. Data are presented as mean ± SEM unless otherwise stated.
Figure 8: Analysis Methods
Complete images were cropped into individual cortical layers to control for naturally occurring variance in cellular density. An ellipse was used to outline the device edges in each cropped image and fluorescence intensity was calculated every 10µm as a function of distance from the device edge.

Results
Device capture IHC was used to label neurons (NeuN), reactive astrocytes (GFAP), activated microglia (CD68), and cellular nuclei (DAPI) as a function of distance from the probe edge for cortical tissue implanted with Si and a-SiC probes for either four or eight weeks. All antibodies utilized labeled proteins of interest significantly more than background fluorescence (Figure 9).
**Figure 9: No Primary Antibody Control Tissue Slices**
Representative images from device capture IHC for cortical tissue. NeuN was used to label neurons (A), GFAP labeled activated astrocytes (C), CD68 labeled reactive microglia/macrophages (E), and DAPI labeled cellular nuclei (blue). All antibodies labeled specific proteins of interest more than background fluorescence in no primary controls (B, D, E).

**NeuN Labeling**
Tissue slices labeled with NeuN displayed a decrease in neurons near implanted devices, but an overall increase in neurons with time of implant (Figure 10). A three-way mixed ANOVA of NeuN indicated that there was a significant main effect of distance \((F_{(5.077, 55.85)} = 5.67, p < 0.001)\), such that there was less intensity near the probe edge regardless of implanted material or time point. Planned contrasts indicated that there was less NeuN labeling specifically 0-30\(\mu\)m from the probe compared to 100\(\mu\)m away (Figure 11A). There was also a significant main effect of time on NeuN fluorescence intensity \((F_{(1.11)} = 6.85, p < 0.05)\). Tissue implanted for eight weeks had more NeuN labeling in general compared to tissue implanted for four weeks (Figure 11B). There was no main effect of material or interaction effects between material, distance, or time on NeuN fluorescence intensity.
Figure 10: NeuN IHC Images
Representative images labeling neurons (NeuN) in cortical tissue implanted with Si (A & C) and a-SiC (B & D) for four (A & B) eight weeks (C & D). There was less NeuN intensity near implanted devices, but an overall increase in labeling from four weeks to eight weeks.
**Figure 11: NeuN Intensity**
Normalized NeuN fluorescence intensity as a function of distance from the device edge for tissue implanted with Si (red) and a-SiC (blue) probes for either four (dotted) or eight weeks (solid). There was a significant effect of distance such that tissue 0-30µm had significantly NeuN labeling compared to tissue 100µm away, regardless of material or time of implant (A). There was also a main effect of time on NeuN fluorescence intensity. Tissue implanted for eight weeks had more neurons overall compared to tissue implanted for four weeks regardless of material (B). Data are mean ± SEM, * denotes p < 0.05.

**GFAP Labeling**
Tissue slices labeled with GFAP illustrated a visually apparent increase surrounding implanted devices and the level of increase was material dependent. Additionally, there was an overall increase in astrocytes with time of implant (Figure 12). A three-way mixed ANOVA of GFAP determined that there was a significant main effect of distance \( (F(4.03, 48.36) = 3.69, p < 0.01) \), such that there was more GFAP intensity near the probe edge regardless of implanted material or time of implant. Planned contrasts indicated that the enhanced GFAP labeling was specifically 0-30µm from the probe compared to 100µm away (Figure 13A). There was an interaction effect between distance and material \( (F(4.03, 48.36) = 2.71, p < 0.05) \) where the intensity of GFAP labeling proximal to the device differed depending on the material. Planned contrasts specified that tissue implanted with Si devices had more GFAP labeling within 0-10µm than tissue implanted with a-SiC devices (Figure 13A). There was also an interaction effect between distance and time of implant \( (F(4.03, 48.36) = 2.56, p < 0.05) \). This indicates that the intensity of GFAP labeling decreased with distance from the device differently depending on the time of implant. Planned contrasts determined that tissue implanted for eight weeks had more GFAP labeling within 0-10µm than tissue implanted for four weeks (Figure 13A). Lastly, there was a main effect of time on GFAP intensity, such that tissue implanted for eight
weeks had more labeling in general than tissue implanted for four weeks, regardless of material or distance from the device (Figure 13B). There was no main effect of material on GFAP intensity.
Figure 12: GFAP IHC Images
Representative images labeling astrocytes (GFAP) in cortical tissue implanted with Si (A & C) and a-SiC (B & D) for four (A & B) eight weeks (C & D). There was a visibly apparent increase in GFAP intensity closer to implanted devices and this enhancement was larger for tissue implanted with Si compared to tissue implanted with a-SiC. Additionally, there was an overall increase in GFAP from four weeks to eight weeks.
Figure 13: GFAP Fluorescence Intensity
Normalized GFAP fluorescence intensity as a function of distance from the device edge for tissue implanted with Si (red) and a-SiC (blue) probes for either four (dotted) or eight weeks (solid). There was a significant effect of distance such that tissue 0-30µm had significantly more GFAP labeling compared to tissue 100 µm away, regardless of material or time of implant (A). There was an interaction effect between distance and material where tissue implanted with Si devices had more GFAP labeling within 0-10µm than tissue implanted with a-SiC devices (A). There was also an interaction effect between distance and time of implant such that tissue implanted for eight weeks had more GFAP labeling within 0-10µm than tissue implanted for four weeks (A). There was a significant effect of time of implant where tissue implanted for eight weeks had higher GFAP intensity in overall than tissue implanted for four weeks regardless of material (B). Data are mean ± SEM, * denotes \( p < 0.05 \).

CD68 Labeling
Tissue slices also exhibited an increase in reactive microglia/macrophages near implanted devices and the level of increase was dependent on the duration of implantation (Figure 14). A three-way mixed ANOVA of CD68 determined that there was a significant main effect of distance \( (F_{(2.66, 31.97)} = 14.22, \ p < 0.001) \), such that there were more CD68 labeling near the probe edge regardless of material or duration of implant. Planned contrasts denoted that the increase in CD68 intensity was specifically 0-80µm from the probe compared to 100µm away (Figure 15A). There was also an interaction effect between distance and time of implant \( (F_{(2.66, 31.97)} = 5.05, \ p < 0.01) \). Planned contrasts indicated that tissue implanted for eight weeks had more CD68 labeling within 0-10µm than tissue implanted for four weeks (Figure 15A). There was no main effect of material or time on CD68 fluorescence intensity (Figure 15B) or an interaction effect between material and distance.
Figure 14: CD68 IHC Images
Representative images labeling reactive microglia/macrophages (CD68) in cortical tissue implanted with Si (A & C) and a-SiC (B & D) for four (A & B) eight weeks (C & D). There was an increase in CD68 intensity proximal to implanted devices and this magnification was larger for tissue implanted for eight weeks compared to tissue implanted for four weeks.
Figure 15: CD68 Fluorescence Intensity
Normalized CD68 fluorescence intensity as a function of distance from the device edge for tissue implanted with Si (red) and a-SiC (blue) probes for either four (dotted) or eight weeks (solid). There was a significant effect of distance such that tissue 0-80µm had more CD68 labeling compared to tissue 100µm away, regardless of material or time of implant (A). There was also a significant interaction effect between distance and time of implant where tissue implanted for eight weeks had higher CD68 intensity within 0-10µm than tissue implanted for four weeks (A). There was no overall effect of material (B). Data are mean ± SEM, ** denotes p < 0.01.

DAPI Labeling
Tissue slices labeled with DAPI displayed an increase of cells near the device and the amount of increase was dependent on the time of implant (Figure 16). A three-way mixed ANOVA of DAPI denoted that there was a significant main effect of distance ($F_{(6.87, 82.38)} = 3.08, p < 0.05$), such that there was more DAPI intensity near the probe edge regardless of implanted material or time of implant. Planned contrasts indicated that the increased DAPI labeling was specifically 30µm from the probe compared to 100µm (Figure 17A). There was also an interaction effect between distance and time of implant ($F_{(6.87, 82.38)} = 2.43, p < 0.05$). Planned contrasts indicated that tissue implanted for eight weeks had more DAPI labeling within 0-40µm than tissue implanted for four weeks (Figure 17A). There was no main effect of material or time on DAPI fluorescence intensity (Figure 17B) or an interaction effect between material and distance.

Interestingly, there were DAPI labeled cells that did not co-localize with any other label. These cells were visibly apparent in all groups and were always located next to the device (Figure 18).
Figure 16: DAPI IHC Images
Representative images labeling cellular nuclei (DAPI) in cortical tissue implanted with Si (A & C) and a-SiC (B & D) for four (A & B) eight weeks (C & D). There was an increase in DAPI intensity near implanted devices and this amplification was larger for tissue implanted for eight weeks compared to tissue implanted for four weeks.
Figure 17: DAPI Fluorescence Intensity
Normalized DAPI fluorescence intensity as a function of distance from the device edge for tissue implanted with Si (red) and a-SiC (blue) probes for either four (dotted) or eight weeks (solid). There was a significant effect of distance such that tissue implanted 30µm had more DAPI labeling compared to 100µm away, regardless of material or time of implant (A). There was also a significant interaction effect between distance and time of implant where tissue implanted for eight weeks had higher DAPI intensity within 0–40µm than tissue implanted for four weeks (A). There was no overall effect of material (B). Data are mean ± SEM, * denotes p < 0.05.
Figure 18: Complete IHC labeling
Representative images from device capture IHC for cortical tissue implanted with Si (A & C) and a-SiC (B & D) for four (A & B) eight weeks (C & D). Neurons (NeuN) are yellow, activated astrocytes (GFAP) are green, reactive microglia/macrophages (CD68) are red, and cellular nuclei (DAPI) are blue. There was an increase of all labels for tissue implanted for eight weeks compared to tissue implanted for four weeks. Interestingly, there were DAPI labeled cells that did not co-localize with any other label. These cells were visibly apparent in all groups and were always located next to the device.

Discussion
Intracortical microelectrode arrays record neuronal signals which are utilized for controlling prosthetic devices. However, these signals degrade over time until device failure. One possibility for the loss of neuronal signals is the brain’s immune response to the implant, consequently resulting in neuronal loss near the electrodes. To produce reliable brain machine interfaces for chronic use, a device that can overcome this problem needs to be designed. We present data supporting the notion that the addition of a-SiC to the standard Si probe is biocompatible and decreases part of the neuroinflammatory response to cortical implants by decreasing GFAP labeling near the device.

To assess the effects of chronic implants on neurons, NeuN intensity was calculated as a function of distance from the implant. Overall, fewer neurons were located within the first 30μm near the implant. NeuN values then became statistically indifferent from baseline as defined as 100μm away. This decrease in labeling occurred for both materials and time points. This finding is consistent with the literature (Biran 2005, McConnell et al., 2009; Potter et al., 2013; Winslow and Tresco, 2010) and may result from the formation of the glial scar encapsulating the device, from neuronal death caused during insertion, or from unhealthy neurons not expressing NeuN. In vivo two
photon imaging studies may be able to track neurons immediately after insertion to elucidate the outcome of neurons proximal to implants.

Additionally, there was significantly more NeuN labeling overall for tissue implanted for eight weeks compared to tissue implanted for four weeks and this result was independent of material. This change in NeuN labeling overtime differs from previous research (Potter et al., 2012). However, the devices were removed and NeuN labeling was measured as a function of distance from the remaining hole. It has been reported that at earlier time points such as two and four weeks, tissue tends to fall back into the hole, whereas by six weeks, this phenomenon is not observed and the tissue appears more compact around the hole (Polikov, 2005; Turner et al., 1999). It is feasible that some cells, including neurons, may have remained attached to the devices at the eight week time point and consequently removed from tissue slices and analysis. In contrast, we present data from device capture IHC, where the device remains in the tissue and the analysis is performed from the edge of the device. Furthermore, cortical layers naturally contain differing neuronal densities and this factor was not controlled for in the literature, where as our analysis was separated and normalized within each cortical layer to create values which could be collapsed across layers. Comparing across animals or time points without taking this confound into consideration could lead to unintentionally comparing between cortical layers.

In addition to neurons, markers of the brain immune reaction were also labeled and quantified. There was significantly more GFAP labeling within 0-30µm from the device edge and this increase was independent of implant time or material. This has been
previously shown and denotes the glial scar formation (McConnell et al., 2009; Polikov et al., 2005; Potter et al., 2012; Turner et al., 1999; Winslow and Tresco, 2010). There was an amplified GFAP labeling in tissue implanted for eight weeks compared to tissue implanted for four weeks and this was independent of material. Additionally, the intensity of GFAP decreased with distance from the device differently depending on the time of implant. This indicates that GFAP labeling extended further from the device for the eight week time point. Jointly, these data suggest that the astrocyte reaction is more intense adjacent to the device and extends further in tissue implanted for eight weeks. This is in line with previous studies that also found increased GFAP after four weeks of implantation and characterized it as a more compact sheath (McConnell et al., 2009; Turner et al., 1999, Szarowski et al., 2003). In contrast, one group detected less GFAP at eight weeks (Potter et al., 2012) and another group detected no difference in GFAP between four weeks and later time points (Winslow and Tresco, 2010). These discrepancies may again be attributed to device removal, where the eight week time point has more compact tissue surrounding the hole (Polikov, 2005, Turner et al., 1999) and may be more likely to remain adhered to the device. They could also be caused by differences in material, device size, or surgical technique. However, this finding was consistent across the two materials we compared while controlling for as many surgical variables as possible by implanting devices simultaneously.

Although there was not an overall effect of material on GFAP intensity, there was an interaction between material and distance. Tissue implanted with a-SiC coated devices actually had less intense GFAP within 0-10µm, than tissue implanted with Si devices.
This indicates that a-SiC significantly reduces astrocyte reactivity in tissue closest to the device and within the region where max intensity is observed. It is theorized that one role of the glial encapsulation is to insulate devices from nearby neurons and impede diffusion (Polikov et al., 2005, Roitbak and Sykova, 1999). Since diffusion barrier is one of the material properties of a-SiC (Zorman, 2009), it is possible that a-SiC acted as a preliminary hermetic coating and therefore, fewer astrocytes were needed to prevent diffusion to adjacent neurons.

Similarly to GFAP, there was enhanced CD68 labeling proximal to devices and the level of increase differed between lengths of implant. More reactive microglia/macrophages were specifically located 0-80µm from the probe edge. This finding aligns with previous studies (Biran et al., 2005, McConnell et al., 2009; Polikov et al., 2005; Winslow and Tresco, 2010; Woolley et al., 2011) and results from the inflammatory response. Additionally, tissue implanted for eight weeks had more CD68 labeling within 0-10µm than tissue implanted for four weeks. These findings were independent of material. An increase of activated microglia at eight weeks compared to earlier time points has also been detected by one group (McConnell et al., 2009). In contrast, another group reported a decrease from four to eight weeks (Potter et al., 2012) and no statistical differences between time points was reported by a third group (Winslow and Tresco, 2010). As with the other labels, variance across the literature may result from removal of the device before IHC or other variables across groups. In the case where no difference was detected, the integral or area under the curve from 0-100µm was compared, with a one-way ANOVA. This is similar to the main effect of implant time.
reported by a mixed ANOVA. Since we utilized the value of a three-way mixed ANOVA, we were able to parse out an interaction effect corresponding to statistical differences between time points specifically with 0-10µm even though a general effect of implant time was not apparent. A similar difference would have gone undetected by only comparing the integral with a one-way ANOVA.

There was also an increase in DAPI exhibited near devices and this enlarged signal was dependent on the time of implant, which parallels GFAP and CD68, but contrasts NeuN. Tissue implanted for eight weeks had more cells within 0-40µm than tissue implanted for four weeks and this was irrespective of material. Interestingly, when images of all cellular markers were examined, it was noted that there were numerous DAPI labeled cells that did not co-localize with NeuN, GFAP, or CD68. These cells were visibly apparent in all groups and were always located next to the device. In these regions, there was a much larger loss of NeuN and more intense CD68 labeling. Since we only labeled reactive microglia/macrophages it is possible that these cells could be resting/ramified microglia (Polikov et al., 2005) or M2-type macrophages (Kigerl et al., 2009). However, the pattern with CD68 and NeuN implies a proinflammatory cell type. A similar observation was reported in Wolley et al. (2013) after four weeks of implantation and these cells were presumed to be meningeal fibroblasts due to vimentin labeling. However, vimentin is not cell type specific and can also be expressed by astrocytes, microglia, and perivascular cells in general (Cui et al., 2003). In support of the fibroblast possibility, other groups have also detected connective tissue and the presence of the extracellular matrix in the glial scar surrounding transcranial devices (Kim et al.,
2004, Stensaas and Stensaas, 1976). In contrast, this phenomenon was only observed in the superficial layers of the cortex (Wolley et al., 2013), but in the current study the unidentified cells were located in all cortical layers. Other possible cell types include monocytes, which are blood cell precursor to macrophages, or mast cells, which are involved in the neuroinflammatory process by releasing cytokines and signal activated microglia (Skaper et al., 2012).

Although there was an increase in GFAP and CD68 from four weeks to eight eights, there was also an overall increase in NeuN at eight weeks. This suggests that the neuroinflammatory response of astrocytes and microglia is not likely responsible for the decrease of NeuN within 0-30µm. In contrast, other groups have observed an inverse relationship between microglia and NeuN labeling (Biran et al., 2005, Potter et al., 2012). Since the loss of NeuN detected in the current study was much larger in reactive regions containing unidentified cells and these cells were the closest to the device, they may be key to the decline in neuronal signal recorded over time with intracortical microelectrode arrays. Future research should investigate the identity of this cell type(s) and characterize its role in the neuroinflammatory response to cortical implants.

Since tissue implanted with a-SiC coated devices did not statistically differ in NeuN, CD68 and DAPI labeling from tissue implanted with standard Si devices, it can be considered biocompatible for the in vivo use with chronic neural implants. This is in line with other in vivo studies which detected no significant difference in capsule thickness for a-SiC coated discs implanted subcutaneously compared to Si and quartz controls (Cogan et al., 2003). Additionally, neuronal density seemed to return to baseline levels
20µm from the center of the device track for cortical tissue implanted with a-SiC coated microelectrodes (Cogan et al., 2003). Furthermore, we present data showing that the addition of a-SiC significantly reduced GFAP labeling adjacent to the devices, which suggests that a-SiC may reduce the neuroinflammatory response.

In summary, our findings jointly support the notion that a-SiC is biocompatible and reduces the neuroinflammatory reponse to standard Si probes used for chronic implantation. Therefore, a-SiC could be implemented with devices that require a hermetic coating to protect moisture sensitive components.

Contributions
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