



PAPER

A 3D *in vitro* model of the device-tissue interface: functional and structural symptoms of innate neuroinflammation are mitigated by antioxidant ceria nanoparticles

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Abstract

Objective. The recording instability of neural implants due to neuroinflammation at the device-tissue interface is a primary roadblock to broad adoption of brain-machine interfaces. While a multiphasic immune response, marked by glial scarring, oxidative stress (OS), and neurodegeneration, is well-characterized, the independent contributions of systemic and local ‘innate’ immune responses are not well-understood. We aimed to understand and mitigate the isolated the innate neuroinflammatory response to devices. **Approach.** Three-dimensional primary neural cultures provide a unique environment for studying the drivers of neuroinflammation by decoupling the innate and systemic immune systems, while conserving an endogenous extracellular matrix and structural and functional network complexity. We created a three-dimensional *in vitro* model of the device-tissue interface by seeding primary cortical cells around microwires. Live imaging of both dye and Adeno-Associated Virus (AAV) - mediated functional, structural, and lipid peroxidation fluorescence was employed to characterize the neuroinflammatory response. **Main results.** Live imaging of microtissues over time revealed independent innate neuroinflammation, marked by increased OS, decreased neuronal density, and increased functional connectivity. We demonstrated the use of this model for therapeutic screening by directly applying drugs to neural tissue, bypassing low bioavailability through the *in vivo* blood brain barrier. As there is growing interest in long-acting antioxidant therapies, we tested efficacy of ‘perpetual’ antioxidant ceria nanoparticles, which reduced OS, increased neuronal density, and protected functional connectivity. **Significance.** Our three-dimensional *in vitro* model of the device-tissue interface exhibited symptoms of OS-mediated innate neuroinflammation, indicating a significant local immune response to devices. The dysregulation of functional connectivity of microcircuits surround implants suggests the presence of an observer effect, in which the process of recording neural activity may fundamentally change the neural signal. Finally, the demonstration of antioxidant ceria nanoparticle treatment exhibited substantial promise as a neuroprotective and anti-inflammatory treatment strategy.

1. Introduction

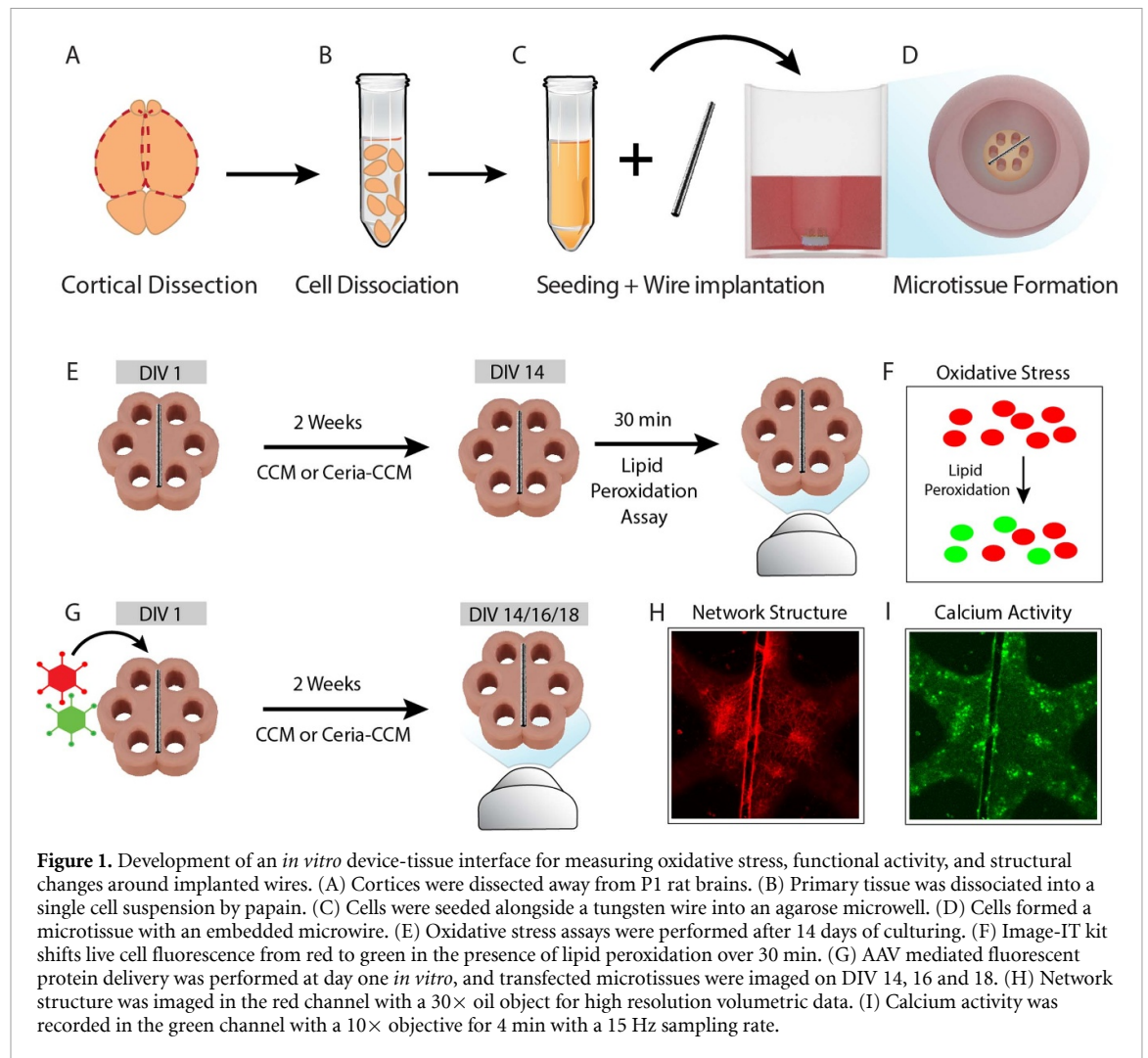
Today, neurotechnology is helping people with paralysis regain function through brain control of prostheses, communication interfaces, and even one's own limbs [1–4]. Facile control of effectors with single unit activity relies on physical proximity of the device to the local electric fields generated by neurons as they communicate with each other, necessitating probe implantation directly into the brain. Such invasive implantation results in acute tissue damage and chronic inflammation, which eventually disrupts the recording capabilities of the probe over weeks to months.

Inconsistent control of brain–machine interfaces is attributed in part to degradation of recording signal quality, initiated by inflammation at the device–tissue interface [5–7]. Inserting a microelectrode into neural tissue damages microvasculature, breaking the blood brain barrier (BBB) and introducing blood-born immune cells into the area [8]. These systemic immune cells initiate an acute wound healing process through recruitment of microglia, the brain's local 'innate' immune cell [8, 9], resulting in a combined immune reaction at the device–tissue interface marked by a glial scar that encapsulates the damaged tissue and electrode [10–16]. While glial scarring stabilizes at six weeks [13], reactive glia within the scar continue to attack the implanted foreign body through overproduction of reactive oxygen species (ROS) in a process called 'frustrated phagocytosis' [12]. This overproduction of ROS causes oxidative stress in the tissues surrounding the implant [17], inducing neurodegeneration and degradation of implanted materials [10, 18–20]. These combined effects contribute to a reduction of recording signal quality and eventual failure of the device [21, 22]. Chronic vascular instability caused by repeated micromotion injury [23, 24] and oxidative damage to the BBB [20, 25, 26] serves to prolong the acute neuroinflammatory response. As such, chronic neuroinflammation at the device–tissue interface is characterized by a complex interplay between the blood-born systemic immune system and the local 'innate' immune system. However, the independent role of the innate immune response in the absence of vascular instability has not been explored.

To better understand the role of the innate immune system in the neuroinflammatory response to recording implants, we engineered an avascular three-dimensional (3D) *in vitro* model of the device–tissue interface to decouple the innate immune response from vascular derived systemic immune drivers. A previously developed two dimensional (2D) *in vitro* model of the device–tissue interface exhibited histological changes around implanted wires which recapitulated some, but not all, hallmarks of the foreign body response to implants [27, 28].

Recent advances in culturing techniques have produced self-assembled 3D cultures which increase physiological relevance of *in vitro* paradigms compared to traditional 2D cultures [29–31]. Primary 3D neural cultures produce an endogenous extracellular matrix with multicellular functional and structural networks [30–36]. Such a 3D *in vitro* paradigm provides easy optical access for live longitudinal imaging of cellular behavior, making it particularly well suited for investigating progressive multicellular processes like neuroinflammation.

Here, we demonstrate the utility of a physiologically relevant 3D *in vitro* model of the device–tissue interface. An *in vitro* device–tissue interface was engineered by seeding cells around a tungsten microwire to model innate neuroinflammation at the exposed recording contact of an implanted electrode. Such an implantation method creates a device–tissue interface without inducing a confounding stab wound. This culturing system can be used as a paradigm for studying the mechanisms of immune responses to neural devices, as well as a platform for screening immunomodulating drug treatments. We treated the *in vitro* device–tissue interface via direct application of an antioxidant to investigate the potential therapeutic efficacy as well as the role of ROS in mediating innate neuroinflammation. *In vivo*, short-acting antioxidant treatments have been shown to prevent oxidative damage to neural tissue by directly scavenging free radicals. However, long-term stabilization of the device–tissue interface with conventional antioxidants is limited by the temperature instability and high dose requirements of small molecules [20, 25, 37–40]. Here, we investigate the use of a long-acting and thermally stable antioxidant made from cerium oxide (Cerium, CeO_2). This rare earth metal oxide exhibits 'perpetual' antioxidant properties in its nanoparticle form [41], effectively lengthening the time course of treatment at physiological temperatures and decreasing dose requirements. The antioxidant behavior of these materials is derived from the oxygen vacancy clusters present at the cerium(III)-rich nanoparticle surfaces [42], features that have been shown to be neuroprotective by neutralizing ROS and preventing oxidative damage to neurons [43, 44]. Further, ceria nanoparticle treatment has increased neuronal survival in several neuroinflammatory disease states, including Alzheimer's Disease and traumatic brain injury [45–48]. In the current study we demonstrate for the first time the potential for antioxidant ceria nanoparticles to treat neuroinflammation at the device–tissue interface. Furthermore, this study reveals that the innate immune system, via ROS-mediated neuroinflammation, plays a critical role in the foreign-body response to neural implants, independent of an initial implantation injury and subsequent systemic immune response.



2. Results

2.1. Development of a 3D *in vitro* model of the device-tissue interface

We developed and characterized a 3D *in vitro* model of the device-tissue interface by utilizing recent advances in primary 3D cortical culturing platforms [49]. Briefly, cortical tissue was collected from post-natal day 1 rats (figure 1(A)) and dissociated into a single cell suspension (figure 1(B)). To visualize a larger area of the wire implant surface, cells were seeded along with wire into an agarose microwell (figure 1(C)). At day *in vitro* (DIV) 1, cells produce an endogenous extracellular matrix, forming a microtissue (figure 1(D)). The placement of the agarose microwell close to the bottom of the plate placed the microtissue within the working distance of a confocal 30× objective, allowing for live imaging of oxidative stress, functional calcium activity, and structural neuronal networks in microtissues. Oxidative stress data was collected at DIV14 by measuring oxidation over a 30 min incubation period (figure 1(E)) using a fluorescent lipid peroxidation reporter (Image IT Lipid Peroxidation Kit, figure 1(F)). Functional and structural data was obtained through the application

of a genetically encoded calcium indicator with an mRuby control tag. AAV mediated delivery of fluorescent proteins was performed at DIV 1 (figure 1(G)), producing stable fluorescence by DIV 14. 3D neuronal network structure (red, figure 1(H)) Calcium activity (green, figure 1(I)) and were imaged at DIV 14, 16 and 18.

2.2. Local lipid peroxidation occurs at the device-tissue interface

Oxidative stress around implants is an important hallmark of the *in vivo* chronic foreign body response[17], and thus, an important feature to evaluate *in vitro*. To characterize oxidative stress around the implant *in vitro*, we used a fluorescent reporter of lipid peroxidation (Image-iT Lipid Peroxidation Kit) in which a fluorescent reagent shifts from red to green when the dye undergoes oxidation. A lower red/green fluorescence ratio indicates a higher degree of lipid peroxidation. Lipid peroxidation of control microtissues was assessed by measuring the red-/green fluorescence ratio over the entire center region of the tissue by using the 'whole-tissue' region of interest (ROI) (gray circle, figure 2(A)). Lipid peroxidation of wire implanted microtissues was assessed

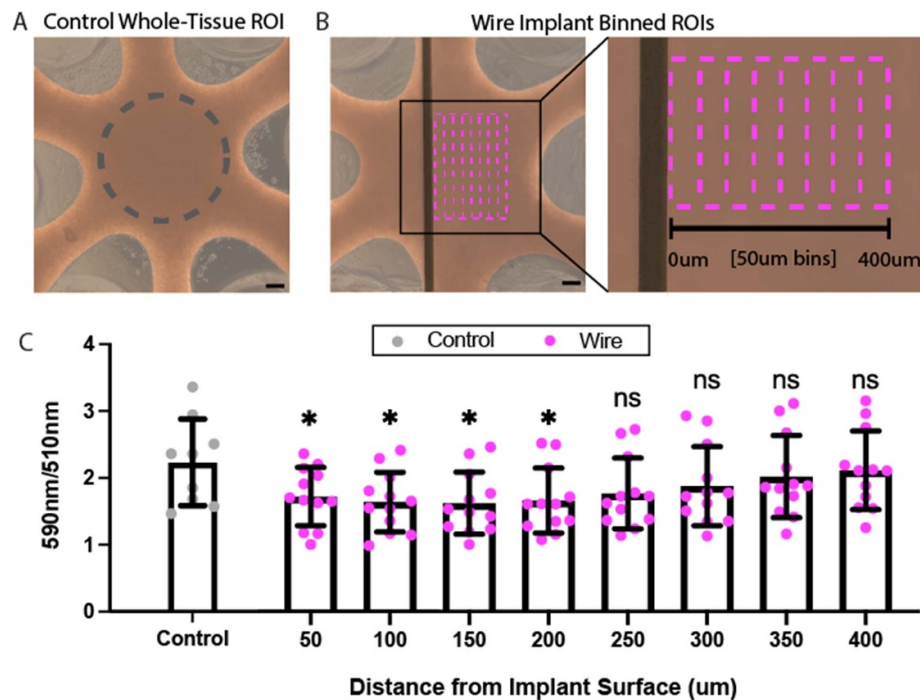


Figure 2. Lipid peroxidation occurs locally around implants. (A) Lipid peroxidation of control microtissues was determined by measuring the red/green ratio over the entire center of the microtissue (gray dashed circle). (B) Lipid peroxidation of wire implanted microtissues was determined by measuring the red/green ratio as a function of distance from the surface of the wire (0–400 μm from the wire in 50 μm bins). (C) The red/green ratio within 200 μm of the wire was significantly lower than the whole-tissue control, indicating a significant increase in lipid peroxidation around the implant compared to unimplanted tissue (50 μm , $p = 0.408$; 100 μm , $p = 0.0207$; 150 μm , $p = 0.0198$; 200 μm , $p = 0.0306$; 250 μm , $p = 0.0825$; 300 μm , $p = 0.1973$; 350 μm , $p = 0.4378$; 400 μm , $p = 0.6462$). Scale bars = 100 μm . * $p < 0.05$.

by measuring the red/green ratio as a function of distance from the implant surface by using 50 μm binned ROIs placed from 0–400 μm from the implant surface (pink, figure 2(B)). The red/green ratio within 200 μm of the surface of the wire was significantly lower than the control, indicating increased lipid peroxidation at the device-tissue interface (50 μm , $p = 0.408$; 100 μm , $p = 0.0207$; 150 μm , $p = 0.0198$; 200 μm , $p = 0.0306$; 250 μm , $p = 0.0825$; 300 μm , $p = 0.1973$; 350 μm , $p = 0.4378$; 400 μm , $p = 0.6462$; figure 2(C)). These results suggest that oxidative stress occurs within 200 μm of the device-tissue interface *in vitro*, without an implantation wound or chronic vascular instability associated with oxidative stress in the *in vivo* environment.

2.3. Implants reduce neuronal density at the device-tissue interface

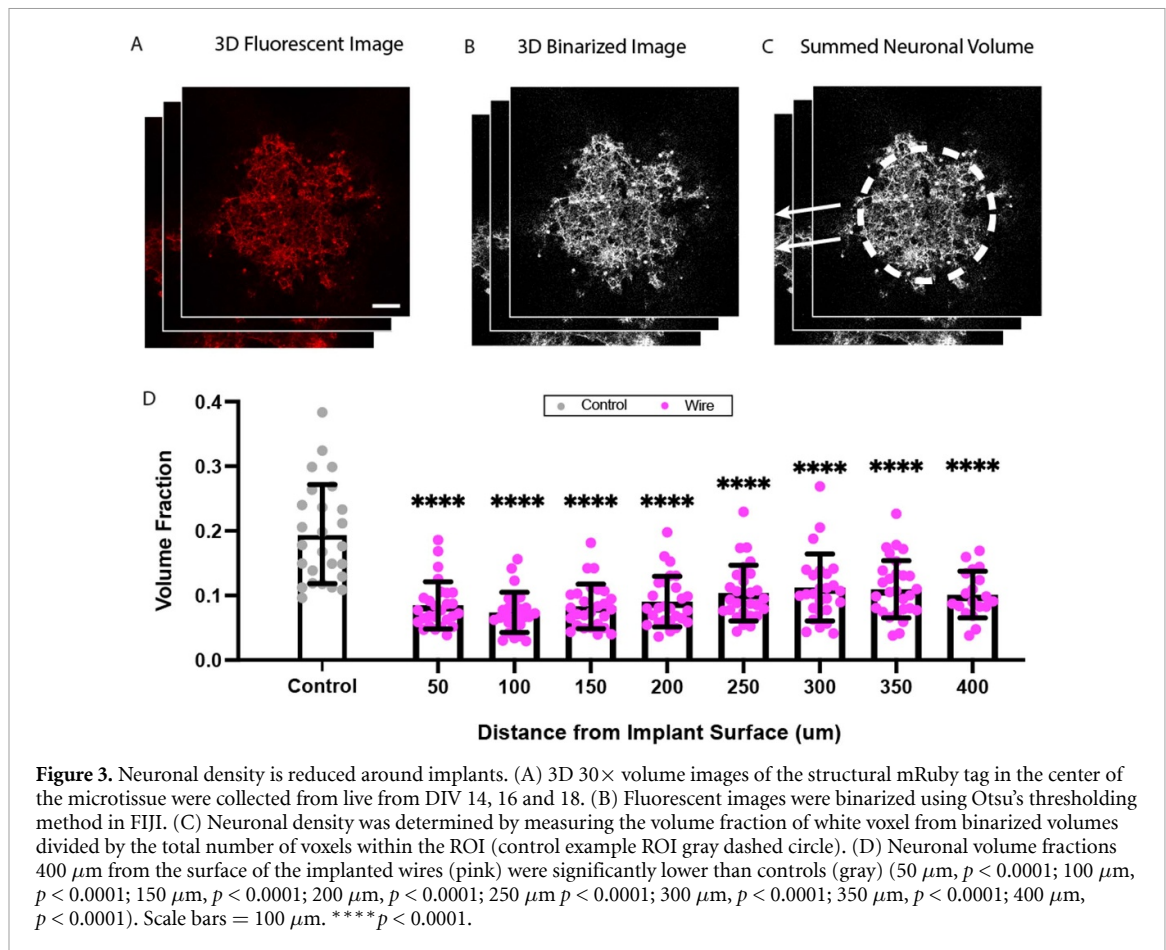
We further investigated changes at the device-tissue interface and the effects of ceria treatment by examining the live network structure. Live neuronal morphology was achieved by AAV-mediated transgene delivery of an mRuby fluorescent tag (AAV1-hSyn1-mRuby2-GSG-P2A-GCaMP6s-WPRE-pA, AddGene), which was expressed in neurons and imaged at DIV 14, 16, and 18 (figure 3(A)). Three-dimensional 30 $\times$ fluorescent images of the neuronal structure in the center of the microtissue were binarized using Otsu's thresholding method (figure 3(B))

and neuronal volume fraction was calculated by the sum of positive voxels/total voxels within the ROI (control ROI example image, figure 3(C)).

We found that local neuronal density, as measured by the neuronal volume fraction within each 50 μm bin from 0–400 μm from the edge of the wire implant, were significantly reduced from the neuronal density in control microtissues (50 μm , $p < 0.0001$; 100 μm , $p < 0.0001$; 150 μm , $p < 0.0001$; 200 μm , $p < 0.0001$; 250 μm , $p < 0.0001$; 300 μm , $p < 0.0001$; 350 μm , $p < 0.0001$; 400 μm , $p < 0.0001$; figure 3(D)). Overall, these data indicate that wire implants significantly disrupt neuronal network structure.

2.4. Implants increase functional connectivity and disrupt community structure of *in vitro* microcircuits

In order to assess the functional effects of wire implantations, we used AAV-mediated transgene delivery of a GCaMP6s calcium reporter (AAV1-hSyn1-mRuby2-GSG-P2A-GCaMP6s-WPRE-pA, AddGene) and recorded calcium activity in the tissue surrounding implants from DIV14–18. We used a mathematical method called graph theoretical analysis (GTA) to describe the complex functional interactions within a network [50, 51]. Application of GTA to a graph made up of single cell 'nodes' is a characterization of network behavior of 'microcircuits' [52,



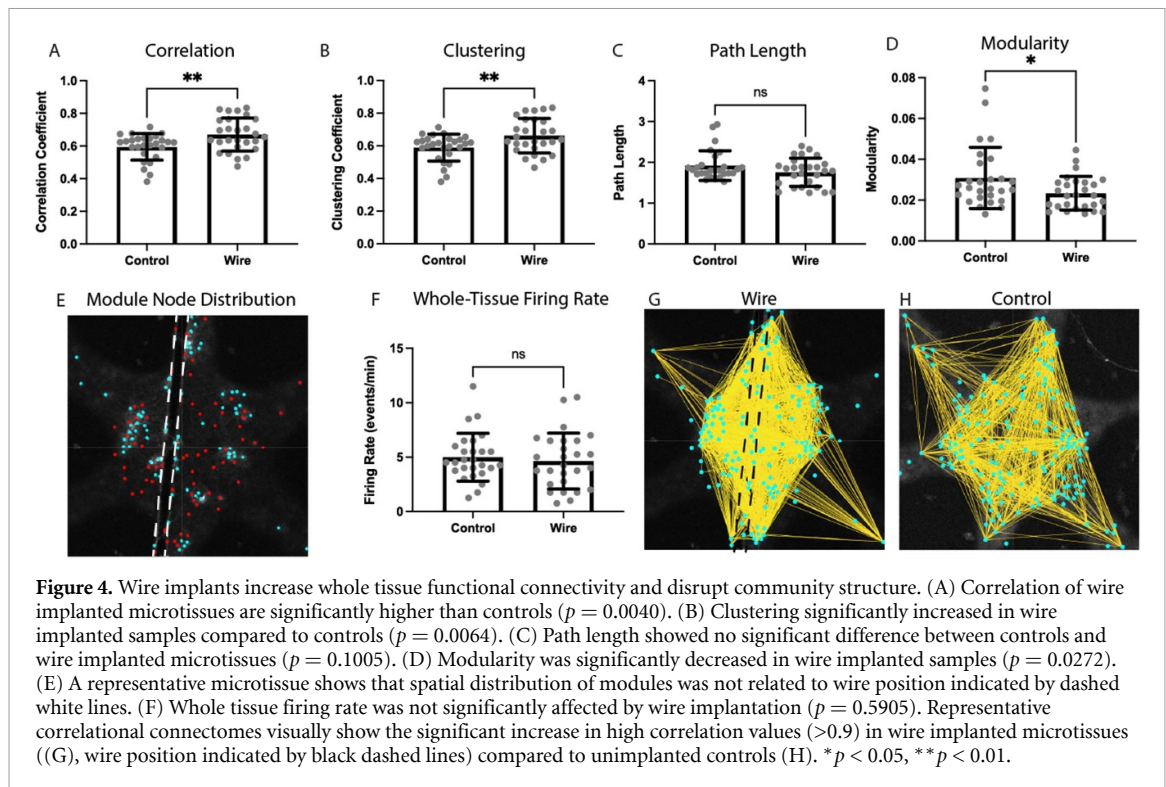
53], which are sensitive to subtle changes in network dynamics that precede gross histological and behavioral deficits in other neuroinflammatory and neurodegenerative disease states [54–57]. Examination of microcircuit dynamics is particularly well suited to the device-tissue interface, as the neuroinflammatory response is localized to the device and likely does not extend to multiple brain regions at the ‘mesocircuit’ level [58]. We applied GTA to time-series fluorescent calcium traces to characterize three metrics of node-pair interactions: correlation, clustering, and path length. Correlation, which represents the how similar calcium activity between two nodes are overtime, was calculated by averaging the Pearson cross correlation coefficient of every node-pair in the microtissue. Clustering is a metric of ‘small world’ network organization, which measures functional connectivity between triplets of nodes [31, 50, 59]. Path length, an indicator of the efficiency of transmission between nodes, is inversely proportional to correlation. Both path length and clustering were calculated using the network analysis toolkit from Dingle *et al* [31].

We then assessed whole-tissue functional connectivity analysis, which included all cells in the microcircuit regardless of position in relation to the wire implantation. Wire implanted microtissues exhibited significantly higher whole-tissue correlations compared to controls (Wire, 0.6703 ± 0.1018 ;

Control, 0.5948 ± 0.08139 ; $p = 0.0040$; figure 4(A)). Wire implanted microtissues also showed a significant increase in clustering (Wire, 0.6617 ± 0.1053 ; Control, 0.5885 ± 0.08258 ; $p = 0.0064$; figure 4(B)), but not path length (Wire, 1.760 ± 0.3435 ; Control, 1.921 ± 0.3631 ; $p = 0.1005$; figure 4(C)). These data suggest substantial functional reorganization of neural networks surrounding implants.

Finally, we assessed modularity, a metric of network segregation associated with learning and memory processes. We calculated modularity using a hierarchical clustering method which identifies groups of highly correlated cells [31, 60]. Wire implantation significantly reduced modularity of the network compared to controls (Wire, 0.02340 ± 0.008260 ; Control, 0.03088 ± 0.01499 ; $p = 0.0272$; figure 4(D)). Interestingly, the spatial distribution of modules was not related to implant position (figure 4(E)). These results indicate that implantation disrupts critical community structure of microcircuits beyond the local network directly around the implant.

The increase in functional connectivity had no significant effect on whole tissue firing rate (Wire, 4.639 ± 2.564 ; Control, 4.991 ± 2.198 ; $p = 0.5905$; figure 4(F)). Such changes in functional connectivity can be visualized with a correlational connectome, which shows a clear increase in highly correlated



connections (>0.9) in wire implanted (figure 4(G)) compared to control (figure 4(H)) microtissues.

2.5. Optimization of ceria nanoparticle coatings for treatment of oxidative stress

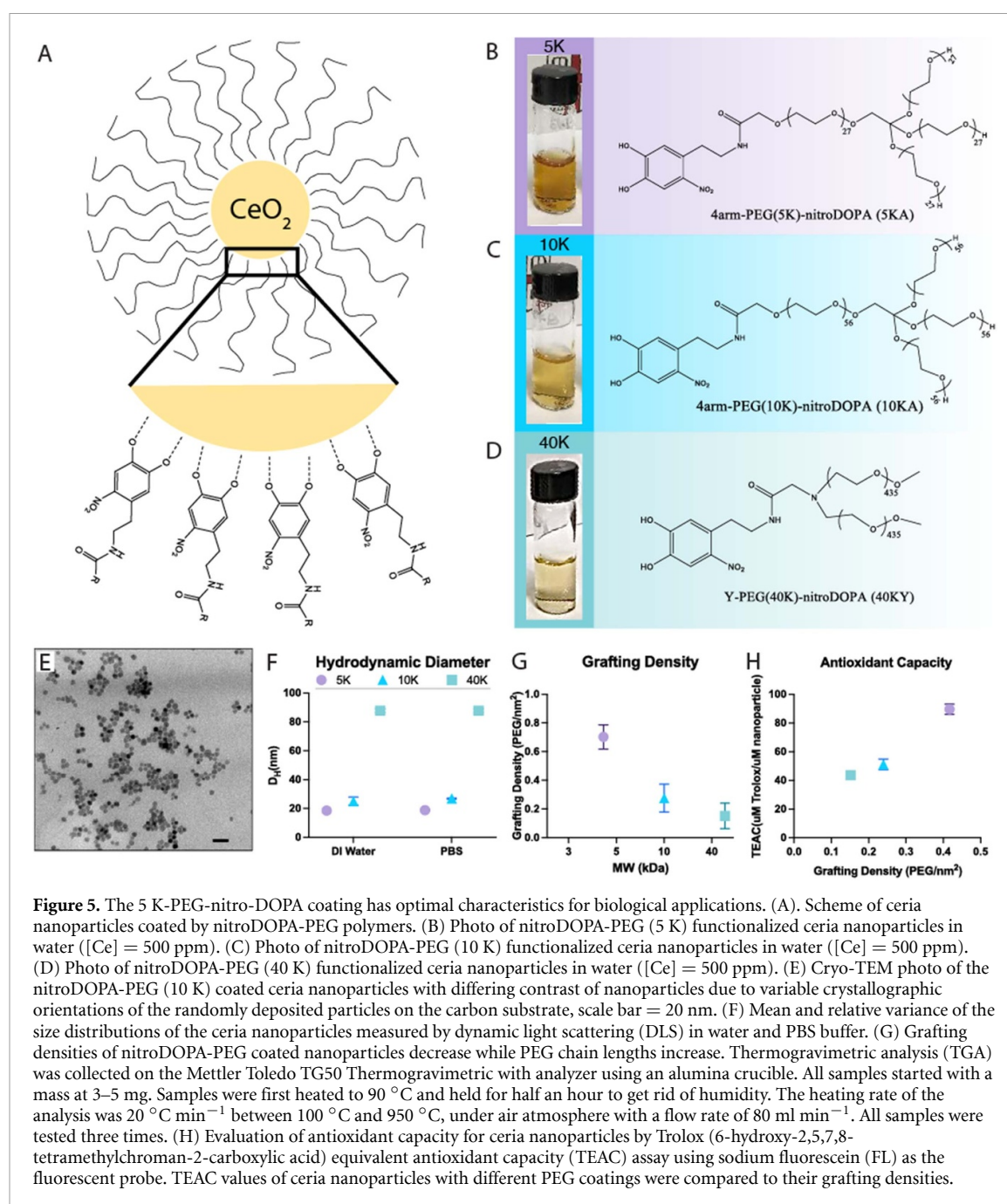
We then sought to investigate the efficacy of antioxidant ceria nanoparticles in protecting the device-tissue interface and, in turn, examine the role of ROS in mediating innate neuroinflammation. Ceria nanoparticles are cerium(III) rich at the particle interface, which subsequently results in more oxygen vacancies in smaller nanocrystals [41, 42]. This makes ceria nanoparticles excellent oxygen buffers, which can easily absorb and release oxygen during high temperature catalytic processes [61]. They are also potent antioxidants in water, capable of neutralizing large amounts of many types of ROS. During this process cerium(III) is oxidized to cerium(IV), but over days and weeks the fully oxidized particles eventually return to their cerium(III)-rich state [43]. Such perpetual, temperature-stable antioxidants are ideal for treating chronic neuroinflammation at the device-tissue interface. However, application of nanoscale ceria to biological tissues requires a polymer coating to ensure stability and prevent nanoparticle aggregation.

Here, we synthesized catecholamine-functionalized polyethylene glycol (PEG) and used it to coat uniform and crystalline ceria nanoparticles ($d = 5.1 \text{ nm} \pm 1.0 \text{ nm}$) prepared in organic media (figure 5(A)). We tested three catecholamine-functionalized PEG coatings with molecular weights

of 5 kDa (figure 5(B)), 10 kDa (figure 5(C)), and 40 kDa (figure 5(D)). The catecholamine bonds to the surface cerium atoms resulting in biocompatible ceria nanoparticles in water, which can be visualized by cryogenic transmission electron microscopy (cryo-TEM) (figure 5(E)). Increases in the PEG molecular weight resulted in increased hydrodynamic diameter in both water and PBS (figure 5(F)), as well as decreased graphing density (figure 5(G)). Additionally, we found that the attachment of dopamine (DOPA), a catecholamine, at the surface of the nanoparticle increased the antioxidant capacity, making the high graphing density 5 k PEG-nitro-DOPA coated sample the most effective antioxidant. MTT, or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, toxicity testing of 5 k PEG-nitro-DOPA coated ceria showed no acute toxicity to primary neurons at 14 ppm (supplemental figure S1 available online at stacks.iop.org/JNE/19/036004/mmedia). As such, we chose to use the 5 k PEG-nitro-DOPA coated ceria for all subsequent biological efficacy testing.

2.6. Ceria treatment mitigates oxidative stress at the device-tissue interface

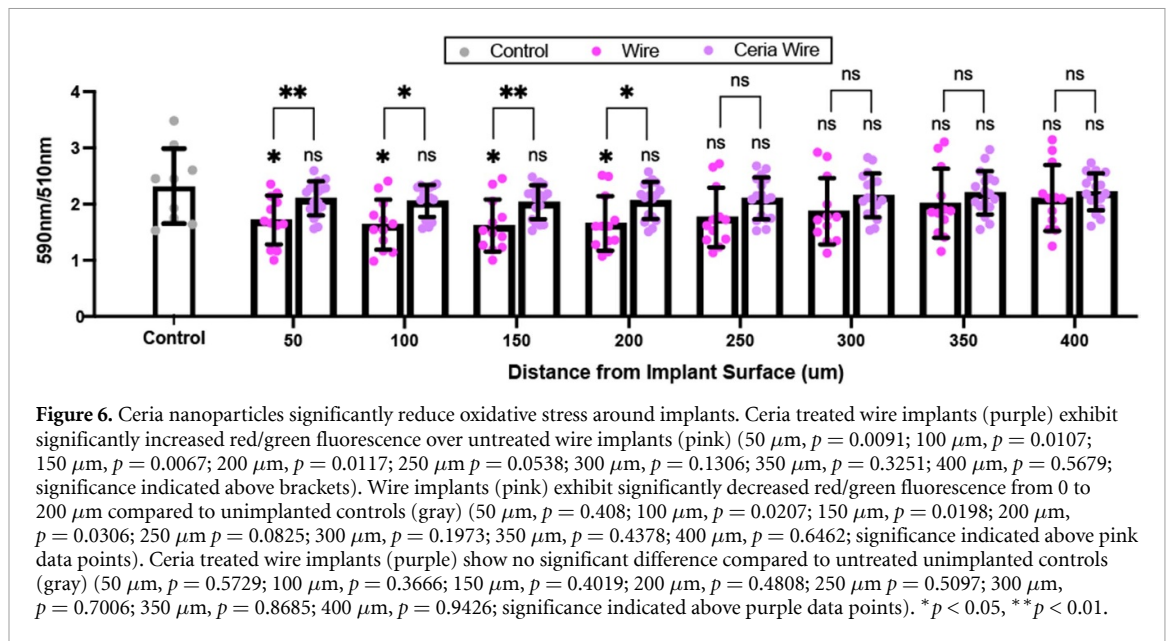
After optimization of the ceria formulation, we then characterized the effects of ceria nanoparticles on neuroinflammation around implanted wires. This investigation will provide insight into the role of ROS in the *in vitro* neuroinflammatory response, as well as an indication of biological efficacy of ceria in protecting the device-tissue interface. Cells were exposed to



a 14 ppm ceria solution diluted in media every other day for 14 days *in vitro* before performing the lipid peroxidation assay.

We found that ceria treatment mitigated oxidative stress in wire implanted microtissues, with a significant increase in the red/green fluorescence ratio in ceria treated wire implanted samples (purple) from 0 to 400 μm compared to untreated wire implants (pink) (50 μm , $p = 0.0091$; 100 μm , $p = 0.0107$; 150 μm , $p = 0.0067$; 200 μm , $p = 0.0117$; 250 μm , $p = 0.0538$; 300 μm , $p = 0.1306$; 350 μm , $p = 0.3251$; 400 μm , $p = 0.5679$; figure 6, significance indicated above brackets). While wire implantation significantly increased oxidative stress compared

to unimplanted controls with 200 μm of the wire (50 μm , $p = 0.408$; 100 μm , $p = 0.0207$; 150 μm , $p = 0.0198$; 200 μm , $p = 0.0306$; 250 μm , $p = 0.0825$; 300 μm , $p = 0.1973$; 350 μm , $p = 0.4378$; 400 μm , $p = 0.6462$; figure 6, significance indicated above pink data points), ceria treatment returned oxidative stress levels back to that of unimplanted controls within 400 μm of the wire surface (50 μm , $p = 0.5729$; 100 μm , $p = 0.3666$; 150 μm , $p = 0.4019$; 200 μm , $p = 0.4808$; 250 μm , $p = 0.5097$; 300 μm , $p = 0.7006$; 350 μm , $p = 0.8685$; 400 μm , $p = 0.9426$; figure 6, significance indicated above purple data points). These data indicate that ceria treatment mitigates oxidative stress at the device-tissue interface.



2.7. Ceria treatment reduces structural changes around implants

As oxidative damage can directly affect structural neural networks, we assessed the effect of ceria treatment on neuronal density. Ceria treatment of wire implanted samples (purple) showed significantly higher neuronal density around electrodes compared to untreated wire implants (pink) within 400 μm of the implant surface (50 μm , $p = 0.0002$; 100 μm , $p < 0.0001$; 150 μm , $p < 0.0001$; 200 μm , $p < 0.0001$; 250 μm , $p = 0.0001$; 300 μm , $p = 0.0003$; 350 μm , $p < 0.0001$; 400 μm , $p < 0.0001$; figure 7(A), significance indicated above brackets). While wire implants exhibited significant density loss within 400 μm of the implant compared to unimplanted controls (50 μm , $p < 0.0001$; 100 μm , $p < 0.0001$; 150 μm , $p < 0.0001$; 200 μm , $p < 0.0001$; 250 μm , $p < 0.0001$; 300 μm , $p < 0.0001$; 350 μm , $p < 0.0001$; 400 μm , $p < 0.0001$; figure 7(A), significance indicated above pink data points), neuronal density loss in ceria treated samples was reduced within 250 μm of the implant and fully mitigated from 250 to 400 μm (50 μm , $p = 0.0007$; 100 μm , $p = 0.0044$; 150 μm , $p = 0.0124$; 200 μm , $p = 0.0141$; 250 μm , $p = 0.0126$; 300 μm , $p = 0.2662$; 350 μm , $p = 0.5218$; 400 μm , $p = 0.1270$; figure 7(A), significance indicated above purple data points). Representative images of an unimplanted control (figure 7(B)), wire implanted sample (figure 7(C)), and ceria treated wire implanted sample (figure 7(D)) show a clear reduction in neuronal density around untreated wire implants and protection of neuronal density around ceria treated implants. Overall, these data indicate that ceria treatment prevents structural damage to neuronal networks caused by neuroinflammation around implants.

2.8. Ceria treatment restores functional connectivity and protects community structure in implanted microtissues

We characterized the effects of antioxidant ceria nanoparticles in protecting neurons against the functional connectivity changes caused by wire implantation. Such characterization of functional network activity around implants in after removal of excess ROS will indicate if disruption of functional connectivity is resultant from ROS-mediate neuroinflammation. To achieve this, microtissues were treated with ceria nanoparticles diluted to 14 ppm in media every other day for the 18 days *in vitro*, replacing 500 μl of liquid in each media change. Ceria treatment significantly decreased correlation in wire implanted microtissues (Ceria Wire, 0.5420 ± 0.1380 ; Wire, 0.6703 ± 0.1018 ; $p = 0.0002$; figure 8(A)).

Ceria treated microtissues similarly exhibited reduced clustering around wire implants (Ceria Wire, 0.5328 ± 0.1317 ; Wire, 0.6617 ± 0.1053 ; $p = 0.0002$; figure 8(B)), and increased path length (Ceria Wire, 2.266 ± 0.6559 ; Wire, 1.760 ± 0.3435 ; $p = 0.0008$; figure 8(C)). These results suggest that ceria nanoparticles reduce the functional connectivity changes at the device-tissue interface. Additionally, ceria treatment improved the strength of community structure around implants, with a significant increase in modularity (Ceria Wire, 0.03697 ± 0.02293 ; Wire, 0.2340 ± 0.008260 ; $p = 0.0056$; figure 8(D)). Remarkably, ceria treatment also significantly reduced whole tissue firing rate when compared to untreated wire implant samples (Ceria Wire, 2.963 ± 1.872 ; Wire, 4.639 ± 2.564 ; $p = 0.0083$; figure 8(E)).

We then compared functional connectivity around ceria treated wires to that of unimplanted controls. Correlations of ceria treated wire implanted

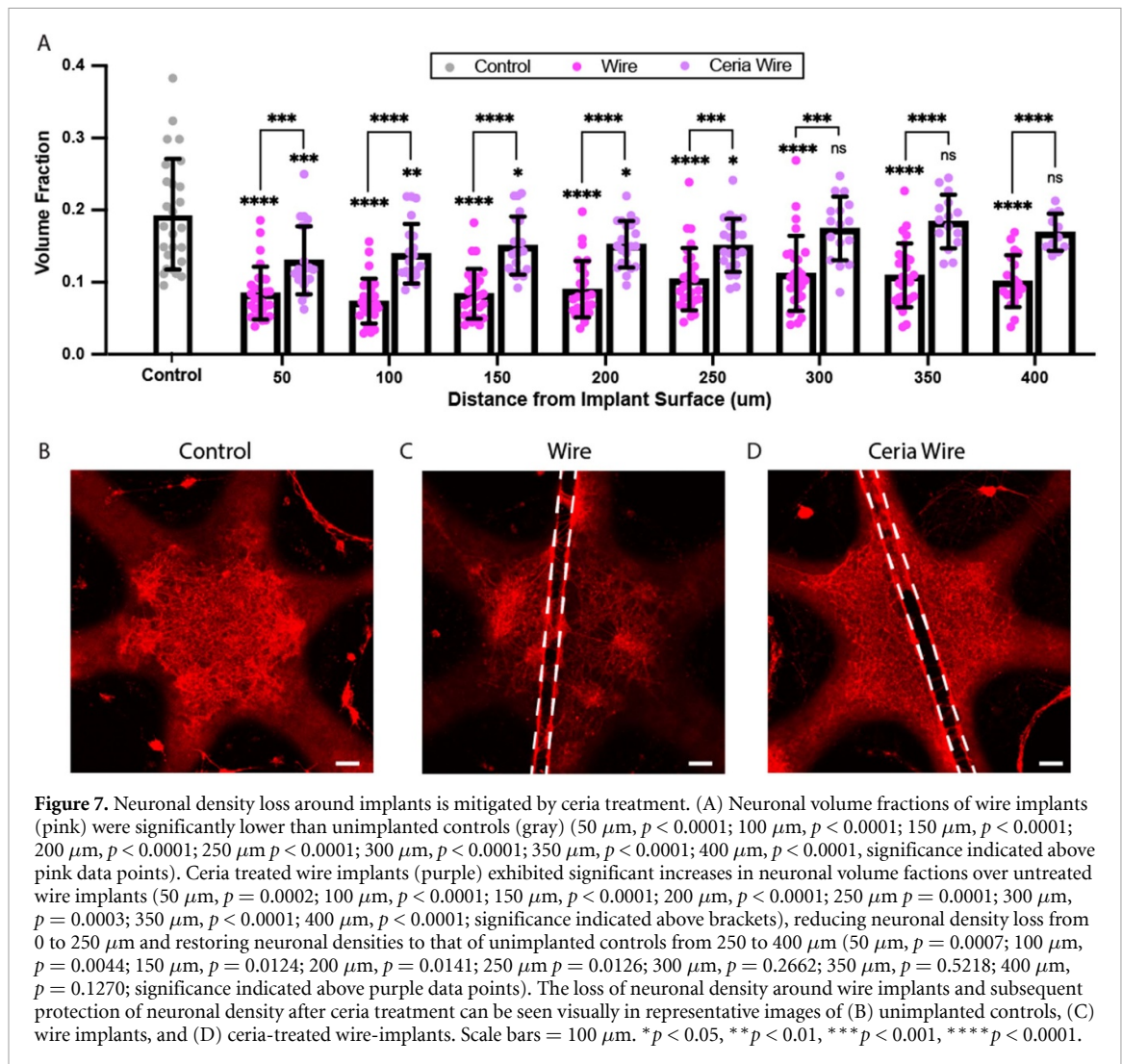


Figure 7. Neuronal density loss around implants is mitigated by ceria treatment. (A) Neuronal volume fractions of wire implants (pink) were significantly lower than unimplanted controls (gray) (50 μm , $p < 0.0001$; 100 μm , $p < 0.0001$; 150 μm , $p < 0.0001$; 200 μm , $p < 0.0001$; 250 μm , $p < 0.0001$; 300 μm , $p < 0.0001$; 350 μm , $p < 0.0001$; 400 μm , $p < 0.0001$, significance indicated above pink data points). Ceria treated wire implants (purple) exhibited significant increases in neuronal volume fractions over untreated wire implants (50 μm , $p = 0.0002$; 100 μm , $p < 0.0001$; 150 μm , $p < 0.0001$; 200 μm , $p < 0.0001$; 250 μm , $p = 0.0001$; 300 μm , $p = 0.0003$; 350 μm , $p < 0.0001$; 400 μm , $p < 0.0001$; significance indicated above brackets), reducing neuronal density loss from 0 to 250 μm and restoring neuronal densities to that of unimplanted controls from 250 to 400 μm (50 μm , $p = 0.0007$; 100 μm , $p = 0.0044$; 150 μm , $p = 0.0124$; 200 μm , $p = 0.0141$; 250 μm , $p = 0.0126$; 300 μm , $p = 0.2662$; 350 μm , $p = 0.5218$; 400 μm , $p = 0.1270$; significance indicated above purple data points). The loss of neuronal density around wire implants and subsequent protection of neuronal density after ceria treatment can be seen visually in representative images of (B) unimplanted controls, (C) wire implants, and (D) ceria-treated wire-implants. Scale bars = 100 μm . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

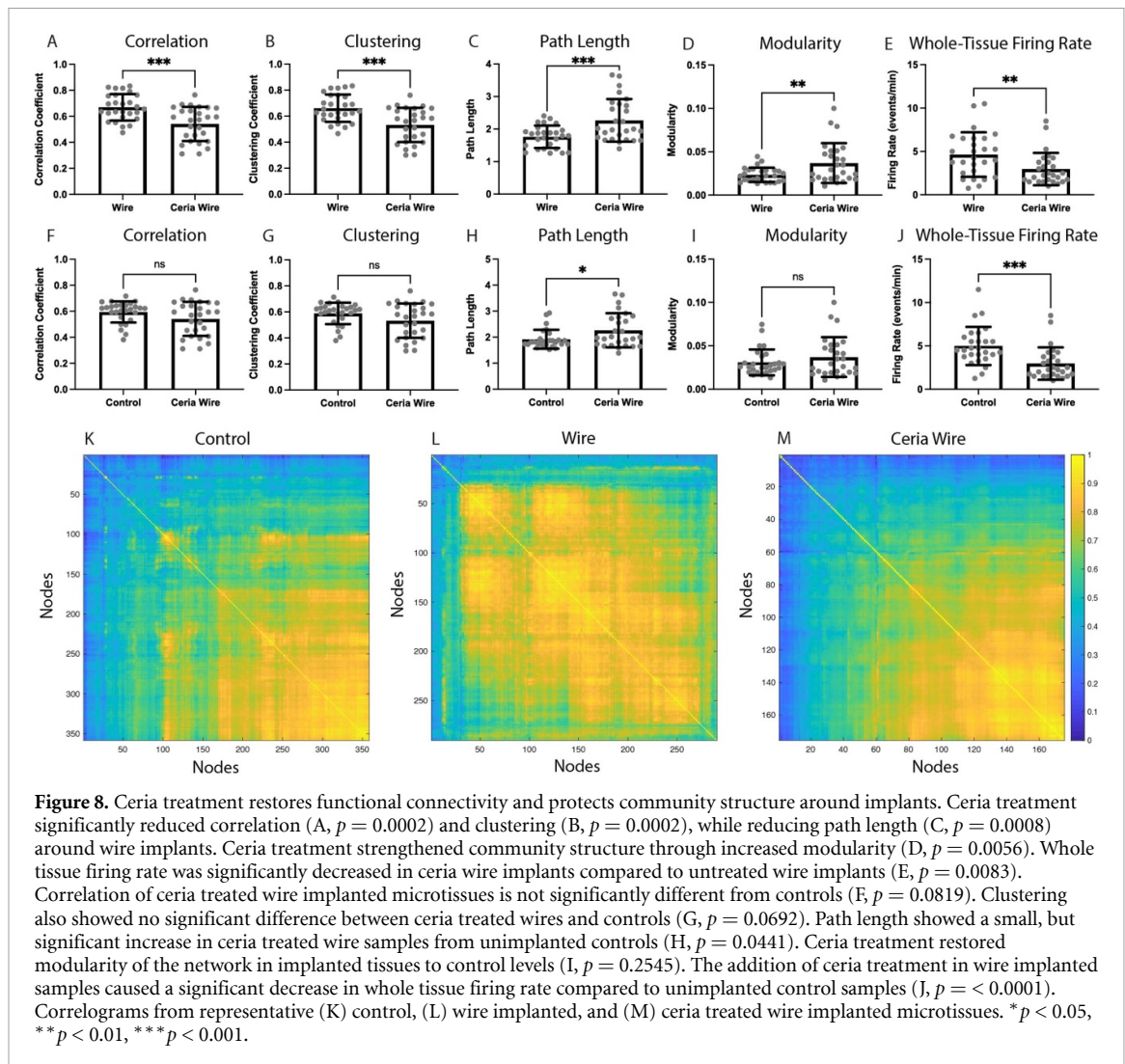
microtissues were not significantly different from correlations of unimplanted controls ($p = 0.0819$, figure 8(F)). Additionally, clustering of ceria treated wire implanted microtissues was not significantly different from unimplanted controls ($p = 0.0692$, figure 8(G)). Interestingly, path length of ceria treated wire implants showed a small statistical difference from controls ($p = 0.0441$, figure 8(H)). These data, while on the edge of significance, suggest that ceria treatment may restore functional connectivity of wire implanted microtissues closer to that of unimplanted controls.

Further investigation of the network revealed that ceria treatment restored modularity of wire implanted microtissue to control values ($p = 0.2545$, figure 8(I)), indicating that ceria treatment protected community structure around implants. Additionally, treatment significantly reduced the whole-tissue firing rate compared to untreated-unimplanted controls ($p = 0.0001$, figure 8(J)), suggesting that ceria treatment of the whole tissue via media delivery may interfere with synchronous network activities. Correlograms from representative microtissues display the clear increase in correlation from wire implantation

and subsequent mitigation of the functional changes with ceria treatment (figures 8(K)–(M)). Collectively, these results indicate that ceria nanoparticles protect neural function around the device-tissue interface in this *in vitro* model.

3. Discussion

We developed a 3D *in vitro* model of the device-tissue interface to better understand the independent contributions of the innate immune system on the neuroinflammatory response to devices. We found that an isolated innate immune response, in the absence of vascular derived immune drivers, was capable of recapitulating important hallmarks of the *in vivo* response, including increased oxidative stress and decreased neuronal density within 250 μm of the implant surface. These symptoms were accompanied by significant changes in functional connectivity and community structure. To understand the role of ROS-mediated oxidative damage in this innate neuroinflammatory response, we tested the effects of antioxidant ceria nanoparticles, optimized for hydrodynamic diameter and antioxidant capacity, on the



symptoms of neuroinflammation. We found that ceria nanoparticles reduced oxidative stress, increased neuronal density, and restored metrics of functional connectivity and community structure.

Increased oxidative stress and decreased neuronal density in an avascular model of the *vitro* device-tissue interface indicate the presence of a distinct innate immune response to microwire implants. These results suggest that, while vascular instability is a significant contributor to the chronic inflammatory response *in vivo*, it is not necessarily the only driver of chronic inflammation around implants. Our work is the first to show oxidative stress and neuronal density loss directly around implants in an *in vitro* environment, improving physiological relevance over previous 2D models [28]. Additionally, we found that tissue reaction occurs in the absence of an acute stab wound from implantation, suggesting that innate glia may be sensing and responding to chemical or mechanical cues from the implant, as demonstrated elsewhere [62–65]. Increases in functional connectivity and disruption of community structure in the wire-implanted microtissues were consistent with

LPS-induced neuroinflammatory responses both *in vitro* and *in vivo*, alluding to the fact that functional and structural changes to neurons and their synaptic behaviors may be mediated by glial dysregulation [49, 54]. Remarkably, we found that while symptoms of oxidative tissue damage occurred locally at the implant surface, changes in functional network dynamics affected the whole microtissue, revealing that localized neuroinflammation impacts the surrounding microcircuitry. The changes in neural network dynamics reveal a significant ‘observer effect’ from exogenous material implantation and has broad implications for the use of invasively recorded data in studying normal neural activity. Further, the influence of the multiphasic neuroinflammatory state on functional connectivity may contribute, in part, to the long-term signal drift of recordings that results in inconsistent decoding for prosthetic control [66, 67].

We demonstrate the use of ceria nanoparticles for reducing oxidative stress, increasing neuronal density, and reducing functional connectivity in wire implanted samples. The restoration of functional connectivity after antioxidant treatment suggests that

the structural and functional changes around electrodes are likely downstream symptoms of oxidative stress. *In vivo* antioxidant treatments have shown similar reductions in oxidative stress and increases in neuronal density. Additionally, these results indicate that antioxidant ceria nanoparticles may be an efficacious drug treatment for protecting neural function at the device-tissue interface. While these acute treatment results are promising, the true advantage of employing a recyclable antioxidant like ceria nanoparticles lies in the long-treatment capacity, which will require further testing.

While ceria treatment was neuroprotective under oxidative stress conditions, ceria treatment of unimplanted tissues showed significant changes in neuronal density and functional connectivity compared to untreated controls (supplemental figures S2–S5). As ROS is an important signaling molecule involved in normal synaptic regulation [68, 69], neutralization of ROS in healthy tissues with a normal oxidative load can interfere with functional connectivity. We found that ceria affected the whole tissue firing rate of microtissues, in both wire-implanted (figures 8(E) and (J)) and non-implanted microtissues (supplemental figure S4), which may be an example of antioxidant overdosing interfering with synchronized bursts. We hypothesize that the ideal antioxidant treatment strategy should utilize an adaptive local delivery method to treat only the local device-tissue interface when the oxidative load becomes too large. Future work will also explore other metrics of neuroimmune interactions, through live cell morphology imaging to assess real-time microglial activity, to a variety of implanted materials, including glass and parylene coated electrodes.

While the 3D *in vitro* microenvironments presented here closely resemble their *in vivo* counterparts, there are several key differences that are important to note. One such limitation is the gas environment of culture, which exposes cells to ambient oxygen levels (20%), much higher than normoxic levels of the brain (4%–9%) [70, 71]. Exposure to high oxygen can induce oxidative stress, requiring neuroprotective antioxidants to be added to cell culture media [72, 73]. As such, cultures are likely exposed to an oxidative load that is close to the oxidative stress threshold and, thus, more sensitive to increases ROS production than *in vivo* tissues. We hypothesize that this high oxygen environment is the reason for the accelerated time course for oxidative damage *in vitro* compared to the longer *in vivo* disease progression. Additionally, exposure to high oxygen can have downstream effects on gene expression, which have yet to be characterized in such a model. Finally, it is important to acknowledge that while the absence of vascular-derived systemic immune cells in this model allows us to isolate and understand the innate immune response, it limits the scope of the conclusions that can be drawn about

the *in vivo* microenvironment. Such *in vitro* testing helps to expand our understanding of disease states but does not preclude the necessity for *in vivo* experiments.

4. Conclusion

The development and characterization of a novel 3D *in vitro* model of the device-tissue interface revealed a significant innate immune response to neural implants. This study has broad implications for the role of the innate immune system and potential treatments strategies for the chronic foreign body response to neural implants. Furthermore, the novel application of graph theory analysis to microcircuit calcium activity at the device-tissue interface exposed a breadth of underlying connectivity changes under neuroinflammatory conditions. The mitigation of oxidative stress, neuronal density, and functional connectivity with antioxidant ceria nanoparticles offers significant supporting evidence to indicate that many symptoms of the tissue reaction are related to redox dysregulation. Successful treatment of the device-tissue interface with long-acting antioxidant ceria nanoparticles shows potential for a local long-term antioxidant treatment strategy in clinical application, which could stabilize neural recordings and facilitate long-term use of implanted neural devices.

5. Materials and methods

5.1. Animals

Animal handling and procedures were performed in accordance with Institutional Animal Care and Use Committee (IACUC) protocols, which were approved by Brown University and the Providence VA Medical Center. Sprague Dawley timed-pregnant rats sourced from Charles River Laboratories were monitored until pups were born. All biological experiments were performed using equal numbers of male and female rats. All procedures are in accordance with ARRIVE guidelines.

5.2. Agarose gels

Nonadherent microwell were made with 2% agarose (16 500, Invitrogen) solutions using a custom injection molding system as described in Atherton *et al* [49]. Briefly, a 2% agarose solution in phosphate buffered saline was heated in the microwave and mixed in 30 s intervals until fully dissolved. Hot agarose was then drawn into a 5 ml syringe and injected through the custom mold and into the well of a glass bottom 24 well plate (P241.5HN, CellVis). Agarose was allowed to cool completely before removal of the injection mold. Gels were diffused with complete cortical media (CCM) by incubation at 37 °C, with two media changes at a minimum interval of 2 h.

5.3. Three-dimensional primary cortical cultures

Creation and maintenance of 3D primary cultured microtissues were performed as described in Atherton *et al* [49]. In brief, primary cells were isolated from postnatal day 1 rats by cortical dissection in cold Hibernate A medium (HA, Brainbits) with B27 supplement (17504-044 Invitrogen) (HA-B27), followed by chemical dissociation with papain (Brainbits) in hibernate solution (Brainbits) at 30 °C for 30 min with gentle inversion mixing every 5 min. A single cell suspension was made by trituration with a P1000 pipette in HA-B27. The cell suspension was filtered through a 40 µm cell strainer and then centrifuged at 150 rpm for 5 min. The HA-B27 supernatant was replaced with CCM, resuspending the cell pellet, and the filtering-centrifuging steps were repeated twice more. Live cells from an aliquot of the cell suspension were counted using a 1:1 mixture with Trypan Blue (T8154, Sigma-Aldrich), and then resuspended to a concentration of 2.7×10^8 cells in 10 µl of CCM. About 10 µl of suspended cells were seeded into each agarose microwell, and cells were allowed to settle in the incubator for 20 min before covering in 600 µl CCM. Implanted microtissues were made by placing sterilized tungsten microwires in the bottom of the microwell and seeding overtop of the wire. Any adjustments to the wire position were made with sterile forceps immediately after seeding. Cultures were maintained in CCM at 37 °C 5%CO₂ with 500 µl media changes performed every other day starting at DIV1.

5.4. AAV transduction

AAV transduction of the genetically encoded GCaMP6s calcium reporter and mRuby control tag (AAV1-hSyn1-mRuby2-GSG-P2A-GCaMP6s-WPRE-pA, AddGene) was performed at DIV1 as described in Atherton *et al* [49]. Briefly, replacement of CCM with 500 µl viral media, made by diluting viral stock in CCM to a concentration of 1×10^6 vg ml⁻¹, was performed at DIV1 and allowed to incubate for two days before replacement with CCM on DIV4. A half CCM media change was performed on DIV5, followed by a full media change every other day for the remainder of the study.

5.5. Lipid peroxidation assay and analysis

Microtissues undergoing the lipid peroxidation assay (Image-iT Lipid Peroxidation Kit) were cultured until DIV 14. CCM was removed and replaced by 500 µl of 10 µM BODIPY fluorescent reagent in CCM and allowed to incubate for 30 min. After incubation, excess dye was removed and three quick washes with warm phosphate buffered saline were performed. Microtissues were immediately imaged in PBS at 590 (red) and 510 (green) emissions with an Olympus FV3000 confocal microscope. About 10× image stacks of microtissues were collected and converted

to TIF stacks in ImageJ. Analysis of whole-tissue and local lipid peroxidation was performed by creating a summed z projection of each channel and measuring the summed fluorescence of the projections within the whole-tissue or local ROI. The lipid peroxidation was determined by dividing the total measured fluorescence in the red channel by that of the green channel. Images in which the wire was not in the same z plane as the tissue due to improper wire placement or subsequent explantation were excluded from analysis. Additionally, malformed microtissues which did not form around the electrode were excluded from analysis.

5.6. Live image acquisition

Live imaging of virally mediated fluorescence was performed with an Olympus FV3000 confocal microscope with a stage top environmental chamber attachment (WSKMX chamber, Tokai Hit) to keep cells at 37 °C with 5% CO₂. Calcium and control tag fluorescence was measured at DIV 14, 16, and 18. GFP calcium fluorescence was recorded using a 10× objective with an open aperture at 15 Hz. Laser power was adjusted each day to avoid overexposure. mRuby control fluorescent image stacks were collected with a 30× oil objective using a 1 µm step size and optimized aperture. Laser power was adjusted to avoid overexposure.

5.7. Calcium image processing and analysis

Calcium videos were converted to tiff stacks in ImageJ using the Olympus Plugin, and maximum z projections of the videos were then saved as tiff images. Microtissues that were malformed or did not have tissue clearly in the same plane as the wire implant were excluded from analysis. Fluorescent cell bodies were identified as ROIs from z projections and masked videos were created from ROI coordinates as described in Atherton *et al* [49]. Briefly, max projection images were smoothed using a Gaussian filter with a kernel size matching the cell body size in the 10× images. Binary images were created by adaptive thresholding and used to create a distance transform image. A Laplacian of Gaussian pyramid function was performed on the distance transform image to detect 'blobs' or cell bodies. Manual interactive correction was performed to remove any cell bodies outside the tissue edge. For wire implanted microtissues, neural extensions which wrapped around the wire were frequently misidentified as cell bodies due to being obscured by the wire and were manually removed from ROI identification. ROI positions were used to make a binary mask, which was overlaid onto calcium videos. Calcium traces were extracted from masked videos using the FluoroSNNAP application [74]. DF/F traces created using a baseline fluorescent signal, which was identified as the 20th percentile across a 10 s time window.

Graph theory analysis of calcium traces was performed as described in Atherton *et al* [49]. In brief, time-series data of calcium fluorescence from each cell body in the image was used to create a cross correlation matrix in MATLAB with the `corr()` function. The cross-correlation matrix was then used to calculate the average node-pair correlation for each microtissue. The average clustering coefficient was calculated using a threshold-free weighted graph to measure the intensity of local connections, a method developed by Onnela *et al* [75] and implemented in a MATLAB toolkit by Dingle *et al* [31]. The average pathlength of the weighted graph was determined by the shortest distance between nodes proportional to the network size, a method from Muldoon *et al* [76] and implemented with a MATLAB toolkit by Dingle *et al* [31]. Modularity was determined by identifying modules through hierarchical clustering of node-pair correlations and calculating the sum of the correlation coefficients within a module divided by the sum of the expected correlation coefficients in a random network, a method developed by Newman [60] and implemented with a MATLAB toolkit by Dingle *et al* [31].

5.8. Structural image processing and analysis

3D 30× images were converted to tiff stacks in Image J using the Olympus Plugin. 3D images were binarized in Image J using automated Otsu thresholding. Summed z projections were then created and whole-tissue or local ROI's were overlaid onto the image to measure the summed white voxels of the volume data. Volume fraction was calculated by dividing the measured summed white voxels by the total number of voxels. Microtissues that were malformed or did not have tissue clearly in the same plane as the wire implant were excluded from analysis. Several images from the oil objective acquisition were discovered to be blurred after collection due to an oil contamination issue and were excluded from analysis.

5.9. Statistical analysis

Statistical analysis was performed in GraphPad Prism. All biological data was collected from three independent replicate litters. All statistical tests performed were unpaired two-tailed t-tests. All data was tested for normality to determine the use of a parametric or non-parametric t-test. For all t-tests, each data point displayed represented a measured value from a single microtissue. All t-tests had a significance level of $\alpha < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$), and all error bars represent the standard deviation from the mean.

Data availability statement

All data generated or analyzed during this study will be made publicly available upon publication and can be made available upon request.

The data that support the findings of this study are available upon reasonable request from the authors.

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Author contributions

E A, V C and D A B conceived of the experiments; E A, Y H, S B, E P and V L performed the experiments; E A and S B performed image acquisition and data analysis; E P and E A performed the toxicity tests; Y H synthesized the ceria formulations; Y H and V L performed the ceria characterization; E A wrote the first draft of the manuscript; All authors reviewed and edited the final manuscript.

Conflict of interest

The authors declare no competing interests.

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