Bioelectronics: From Nanoscopic Intracellular Probe to
Macroscopic Brain-Electronics Interface

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Abstract

The structural and dynamical hierarchies of biological systems, especially the brain, spanning multiple spatiotemporal scales pose great challenges for fundamental research and biomedical study. Bioelectronics-based probes with detection resolution down to the subcellular scale and volumetric coverage up to the entire system have emerged as a promising approach to overcome the challenges. However, the invasiveness of conventional bioelectronics at both nano- and macro-scopic levels has limited their potential advances. Building upon recent progresses in nanoscience and flexible electronics, my PhD work has focused on the development of novel bioelectronics with structure and mechanics resembling biological systems for monitoring and modulation at different length and time scales with minimum perturbation. On the nano-scopic scale, I will describe the design and demonstration of a subcellular electrical probe by exploiting a unique three-dimension (3D) nanowire-nanotube structure, where a nanowire detector is synthetically-integrated with a nanotube probe. On the macro-scopic scale, I will introduce a novel concept—mesh electronics, a 3D electronic network with brain-like structure and mechanics, and its long-term interface with the nervous system in vivo. First, I will report the implementation of a syringe-injection paradigm to deliver this ultra-flexible mesh electronics into in vivo brain. Importantly, I will demonstrate that centimeter-scale mesh electronics could be
loaded into and injected through a needle as small as 100 µm. Besides, I will discuss an automated conductive ink printing method that can electrically link the injected mesh electronics to external recording instruments with quantitative connectivity. Second, I will demonstrate an alternative in vivo implantation paradigm for the mesh electronics by temporarily changing it to a rigid state through liquid nitrogen frozen. Third, I will present the results showing minimal gliosis and 3D neuronal interpenetration as well as stable tracking of the same neurons and neural circuits from mouse brains for at least eight months from chronically implanted mesh electronics. Significantly, these unique long-term brain-electronics interface have been further exploited to enable stable electrical stimulation and single neuron tracking in longitudinal studies of brain aging in freely behaving mice. Going beyond the brain, I will show the application of mesh electronics for in vivo electrophysiology from mouse retina, a highly curved structure that has been almost exclusively studied ex vivo. Specifically, I will demonstrate that the tissue-like mesh electronics enabled conformal integration with and chronic recording from retinal ganglion cells with characteristic light responses. Finally, I will describe about our recent efforts in increasing the density and total number of recording sites of mesh electronics to achieve stable long-term large-scale mapping of behavior related microcircuits and neural pathways. Together, the unique capabilities of the mesh electronics open up new opportunities to exploit the seamless interface between electronics and nervous systems at biologically relevant spatio-temporal scales for fundamental neuroscience research and translational applications. Looking to the future, I will briefly discuss the possibility of integrating nanoscopic multifunctional components into the tissue-like mesh electronics platform to go beyond current neuron-centric brain research by exploring glia-neuron interactions in live animals, which could potentially offer valuable insights into brain computation and glia-based therapies for neurodegenerative diseases.
Dedicated to my parents, Xinliang and Lanping, and my wife, Yiyi
Acknowledgements

Six years of PhD is like a long journey to climb a mountain. There were brushy hillsides and rocky gorge trails, there were steep ascent and serpentine descent, there were magnificent vista and intimidating cliff. Now the journey almost comes to the end, as I look back, I would not have had the courage and determination to get here without the guidance and encouragement of those people who have offered me their tremendous help at different stages.

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* Denotes equal contribution
Chapter 1

Overview

Brain, the most magnificent and mysterious miracle that has ever been designed by nature, has been studied since ancient Greek philosophers or even earlier (1). Significant progresses have been made during the past century with the progresses made in anatomy, psychology and advancements in microscopy and electrical recording technologies (2, 3). However, the central dogma of brain science, which links the dynamics of microscopic molecules and neurons inside the brain with the phenomena of macroscopic behaviors and emotions, remains elusive (4, 5). Indeed, the highly interconnected structure and strongly correlated dynamics of the brain may hinder the effectiveness of traditional reductionism approach, which focuses on isolated events and causal relationships. Instead, network-centered scientific and mathematical frameworks targeting patterns emerging out of underlying complex systems have received increasing awareness and emphasis (2, 5, 6). Further developments of network-centered frameworks to embrace the dynamic nature of nervous systems will be vital for laying down the theoretical foundation for understanding the brain (7). Nevertheless, an equally, if not more, important and urgent challenge is the development of new technologies capable of dissecting the structural and functional organizations of the brain with minimum perturbation to its natural state (8-10).
1.1 Challenges to probe the brain and existing neurotechnologies

**Figure 1.1 Spatiotemporal hierarchy of the brain.** Horizontal axis represents brain processes that happen at different temporal scales. From left to right: single neuronal action potential, synaptic potentiation, sensory input to motor output, short-term memory, circadian clock, seasonal modulation, long-term learning and memory and aging. Vertical axis shows the building blocks of the brain at distinct spatial scales. From bottom to top: neurotransmitters, synaptic connections, neurons, local neural circuit, visual pathway, the entire brain. Three major research fields are categorized based on the spatial scale of targeted objects: the molecular and cellular level, the circuit and system level and the whole brain level.

One of the central challenges that makes the brain extremely difficult to probe attributes to its spatiotemporal hierarchy (Fig. 1.1). Regarding the spatial dimension, the brain relies on communications of molecules, such as neurotransmitters, down to nanometer scale, whereas most behaviors involve collective network activities across the entire brain over meter scale (2-6). This hierarchical structure spanning nine orders of magnitude complicates the development of effective brain probes (8-10). On the other hand, while each action potential, electrical pulse
generated by individual neurons for information transfer and integration, lasts for only a few milliseconds, gradual brain changes, such as long-term memory and aging, can happen over years to decades (3). This hierarchy in temporal dynamics spanning nine to ten orders of magnitude poses further challenges (8-10).

**Figure 1.2 Spatiotemporal coverage and resolution of existing brain technologies.** Vertical and horizontal axes represent the spatial and temporal resolution and coverage of each technology, respectively. The bottom and left edges of each rectangle indicate the spatiotemporal resolution of each technology, while the right and upper edges denote the corresponding spatiotemporal coverage. This thesis focuses on the improvement of implantable electrical probe.

Existing technologies used to monitor and modulate the brain could be roughly divided into three classes based on their spatial scale (Fig. 1.1): (i) the molecular and cellular level, which ranges from the size of individual molecules to the scale of synaptic connections and up to the dimension of neuronal and glial cells; (ii) the system level, which covers individual neurons to local neural circuits and up to interconnected brain regions; and (iii) the whole brain level,
which spans from each brain region and all the way up to the entire brain and central nervous system.

Various approaches for monitoring and modulating the brain with different strengths and limitations have been developed and significantly advanced our understandings of the brain (Figs. 1.2 & 1.3) (8-11). For example, at the molecular and cellular level, whereas imaging technologies, such as optical and electron microscopes, have revealed the rich structural and molecular information of the brain network (10, 12-17), electrical probes, like patch clamp, revealed how information encoded in action potential and subthreshold events is processed and transferred among neurons (18, 19). At the whole brain level, noninvasive brain imaging, such as functional magnetic resonance imagine (fMRI) and positron emission tomography (PET), revealed the compartmentalization of the brain architecture (20-23), while surface electrical/magnetic probes uncovered the existence of distinct brain states when performing different behaviors (11, 24). To fill the gap in between, in other words to understand the circuit and system level, it becomes quite challenging because of the necessity to have measurement resolution down to cellular scale but at the same time have a coverage up to the whole brain level. Currently, there are two main complementary technologies: optical imaging of ion/voltage sensitive indicators and implantable electrical probes. Optical imaging methods, such as calcium imaging (10, 25, 26) and recently developed genetically encoded voltage sensitive indicators (10, 27-31), show advantages in cell type specificity, simultaneous mapping of large number of neurons and capability to correlate with anatomic connections (32). However, they also suffer from the limitations in terms of penetration depth, image acquisition rates and genetic incorporation of labels (Fig. 1.3) (31). On the other hand, implantable electrical probes, which can provide spatiotemporal mapping and modulation capabilities at the single neuron level from
deep brain regions by directly placing individual sensing units in close proximity to each individual neuron that comprises the local neural circuits, complement the challenges faced by optical imaging (33-36). Moreover, the label-free nature and accessibility to deep brain regions of implantable electrical probes provide huge translational potentials, including electroceutical treatment of neurological diseases, including Parkinson’s and Alzheimer’s diseases, development of brain-machine interface (BMI) for tetraplegia and paraplegia, as well as applications in neuroprosthesis for retina and spinal cord implants (37-44). Nevertheless, implantable electrical probes also faced several challenges (45, 46).

Figure 1.3 Comparison of existing technologies for brain monitoring and modulation. The three axes represent the spatial resolution/specificity, temporal resolution/response time and effective depth of corresponding brain recording (a) and stimulation (b) tools. This thesis focuses on the implantable electrical probe for both brain monitoring and modulation.

1.2 Implantable electrical probes for neural recording and stimulation

A central limitation faced by electrical probes is its long-term stability (45, 46). On the cellular level, disruptions of cell membranes and direct exposure of intracellular species to extraneous electrolytes might not only induce irreversible changes to the natural state and dynamics of targeted cells, but also prevent long-term and non-invasive cellular recordings (47-49). More importantly, on the system level, chronic failures of the implantable electrical probes
resulting in degradation of recording and stimulation capabilities over a time scale of days to weeks significantly limit the use of conventional brain probes for long-term neuroscience and clinic studies (45, 46, 50-54).

The chronic failure modes of conventional probes can be divided into two major categories (46): (i) material and manufacturing failure, including bio-incompatibility of coating materials, delamination and degradation of insulation layers as well as corrosion of sensing units; and (ii) mechanical and structural mismatch, which result induce blood-brain barrier breach, formation of glial scar, neuronal death and relative micromotion between the probes and neural networks. Over the past decades, significant progresses have been made towards selecting biocompatible materials and improving manufacturing process to overcome material and manufacturing related failures (46). For example, silicon probe based on biocompatible silicon substrate, inert metal interconnects, such as steel, gold and platinum, and polymer insulation combining with standard microfabrication process have been widely used for both fundamental neuroscience research and translational biomedical applications (33, 34). Nevertheless, the prominent mechanical and structural mismatch between existing implantable brain probes and neuronal tissues as evidenced by the striking gap (four to seven orders of magnitude) between their effective bending stiffness values (Fig. 1.4) substantially impede the applications of implantable brain probes to illuminate questions such as how existing neurons evolve into neural circuits with diverse dynamics through learning and developmental processes, and limit further developments of BMI with reliable decoding from individual neurons versus ensemble averages of large population activities for prosthetic applications (55, 56).

Indeed, progresses have been made towards overcome these challenges by either reducing the feature sizes of the probe or utilizing flexible materials to minimize deleterious
tissue response and improve neuron-probe interfaces (57-64). On the cellular level, for example, several groups have developed arrays of nano- and micro-meter sized protruding metal electrodes and demonstrated multiplexed intracellular/intracellular-like recordings from cardiomyocytes and neurons with assistance of electroporation for membrane penetration (58, 59). However, the large impedance at the metal electrode-electrolyte interface limits the ultimate size of these probes and can lead to a distortion and attenuation of recorded signals (49). On the system level, for example, Kozai et al. showed that composite carbon probes with diameter down to 5 μm could greatly alleviate the deleterious chronic gliosis and enable single-unit recordings up to 4 weeks (60). On the other hand, a recent study carried out by Khodagholy et al. demonstrated that the utilization of an organic material-based, ultraconformable and biocompatible substrate significantly improved probe-neuron interface (63). Despite these promising advances, mechanical and structural mismatch still exist between implantable electrical probes and the brain. Specifically, the bending stiffness of these probes are still orders of magnitude larger than that of a single-neuron layer brain slice, which limits the long-term stability of most existing probes to only a few weeks to a month (55, 64).
Figure 1.4 Mechanical and structural mismatch between existing implantable electrical probes and the brain. The horizontal and vertical axes represent the feature size and bending stiffness of existing implantable electrical probes, respectively. The green dashed line indicates the size of a neuron soma and the red dashed line denotes the bending stiffness value of a single neuron layer brain slice (~ 30 μm thick). The blue rectangle shows the characteristics of an ideal brain probe, which has a feature size smaller than the size of a neuron soma and possess a mechanical property softer than that of the brain tissue.
1.3 Our approach

To overcome the limitations of conventional electrical probes at both cellular and system levels, advancements in two fields—nanoscience and flexible electronics—have been combined. In this thesis, I will focus on our recent progresses made towards (i) the realization of one-dimensional semiconductor nanowire based functional devices with sub-10-nm feature size and minimum invasiveness to individual cells (65) and (ii) the integration of nano- and micro-devices onto flexible polymer substrates to form a three-dimensional macroporous electronic network to interface and interact with biological systems (55, 66-68).

Over the past two decades, one-dimensional semiconductor nanowires synthesized through the bottom-up paradigm have shown great promise for interfacing and interacting with biological systems (49, 56, 69). First, functional nanodevices built upon one-dimensional nanowires synthesized from primitive units with rationally controlled morphology, structure and property have provided an arsenal of building blocks for various applications (49, 69). For example, nanowire field effect transistor (FET) based electrical and chemical sensors have been demonstrated for detecting both localized and fast action potentials of individual cells (70) as well as relatively dispersed and slow local field potentials (LFPs) of brain slices (71) and protein diffusion (72). Second, the nanoscale feature size of nanowire based devices with uniquely engineered geometry and compositions allows for simultaneous large-scale, high density intracellular monitoring and manipulating of biological systems with much less invasiveness. Besides, the small feature size also enables more localized, higher spatial precision measurements, which is necessary for subcellular level interfacing, for example, in measurements from organelles or neurites (47, 49, 57). Last, several assembly methods have achieved integration of one dimensional nanowire based functional elements with nearly any
type of surface, including those are typically not compatible with standard complementary metal-oxide-semiconductor (CMOS) processing, such as flexible polymer substrates (73-75). In addition, sequential patterning and assembly steps further enable fabrication of devices with distinct functionalities on the same substrate (74, 75).

To integrate nanoscale devices into an array/matrix and interface with biological systems at the system level, a three-dimensional, free-standing, macroporous scaffold for synthetic tissue constructs and monitoring of cellular activity was recently developed (70, 76, 77). Significantly, three-dimensional nanowire FET-cardiac tissue hybrid scaffold with high density of cardiomyocytes in close contact with the nanowire sensing units and recordings of extracellular action potentials with amplitudes 2-3 mV were demonstrated (70, 76). In addition, multifunctional components, including pH, pressure and chemical sensors, were incorporated into the three-dimensional scaffold (77).

Further advances on nanowire based probes for minimum invasive subcellular interrogation (65) and three-dimensional ultra-flexible macroporous network integrating nano- and micro-scale probes for seamless in vivo brain interface (55, 66-68) will offer a potential platform to overcome the challenge to probe and modulate the hierarchical brain network and substantially advance our understandings for both fundamental neuroscience and translational biomedicine.
1.4 Thesis overview

**Figure 1.5 Overall goal of the thesis.** The main focus of this thesis is to present my Ph.D. works on the development of minimum invasive electrical probes and their integration into a three-dimensional macroporous network for stable long-term *in vivo* monitoring and modulation of the central nervous system.

This thesis mainly center on the development of novel nano- and flexible-materials with rationally controlled geometry, mechanical property, and multifunctionality to seamlessly integrate and interact with biological systems at different length scales. Specifically, I will focus on two main themes and their integrations: (i) Exploiting uniquely designed nanowire structures to develop bioprobe with size comparable to cell organelles for *in vitro* electrophysiology at subcellular level (Chapter 2); and (ii) Incorporating nanoscale devices into three-dimensional ultra-flexible macroporous mesh electronics for long-term *in vivo* brain mappings at system level (Chapter 3-8). These works aim at exploring the rich interface between material science and biology and blurring the distinction between man-made and living systems (Fig. 1.5).

In Chapter 2, I will focus on the miniaturization of bioelectronic probes to enable interrogation of subcellular structures that could impact significantly biology and medicine. Specifically, I will describe the design and fabrication of the first sub-10-nm intracellular
bioelectronic probes by exploiting a unique three-dimensional (3D) nanowire–nanotube heterostructure. I will show that these ultrasmall bioelectronic probes had sufficient temporal resolution to record fast action potentials in neurons and other cells. Moreover, systematic studies of the probe bandwidth in different ionic concentration solutions revealed the mechanism governing its temporal response. In addition, I will demonstrate its robustness and capability for intracellular electrophysiology by measuring cell transmembrane resting potentials \textit{in vitro}.

The behavior of complex biological systems could not be simply understood in terms of an extrapolation of a few cells, and may differ drastically from \textit{in vitro} studies. It is, therefore, important to integrate nano-scale devices into a three-dimensional macroscopic scale probe to innervate with living organisms at system level. In Chapter 3, I will introduce the development and implementation of a syringe-injection paradigm to deliver freestanding macroporous mesh electronics with brain-like mechanics and structure into \textit{in vivo} brain of live rodents. Importantly, I will demonstrate that this sub-micrometer-thick, centimeter-scale mesh electronics with nano-scale sensing elements could be loaded into and injected through a needle with inner diameter as small as 100 µm. Moreover, I will show that mesh electronics injected into the lateral ventricle can relax from the initial injection diameter to bridge the caudoputamen and lateral septal nucleus regions with seamless integration with the boundary cells.

To realize electrophysiology interrogations of brain circuits \textit{in vivo}, I will discuss the advancement of the injection technique and input/output connection method in Chapter 4. Specifically, I will show the development of a controlled injection paradigm that allows targeted delivery of the mesh electronics with single-neuron level spatial precision by matching the injection rate with the needle retraction speed. In addition, I will introduce an automated
conductive ink printing methodology that electrically connects each individual channel of the electronic network to external recording instruments with quantitative connectivity.

In Chapter 5, I will introduce an alternative scheme to deliver the ultra-flexible mesh electronics into the *in vivo* brain. Mesh electronics with built-in strains to control both global and local device structures can be implanted into the brains in a temporarily rigid state through liquid nitrogen frozen with minimal surgical damage. Multiplexed LFP and single-unit spike recordings from the somatosensory cortex area will be demonstrated. In addition, I will show that histology analysis revealed seamless probe-tissue interface, which can lead to opportunities for brain activity mapping and next generation brain-machine interfaces.

The aforementioned benefits of the mesh electronics, including tissue-like mechanical property, macroporous open structure, and precise targeting capability with quantitative input/output connectivity, allow seamless long-term integration with the brain. In Chapter 6, I will discuss the application of the developed mesh electronics for stable long-term *in vivo* chronic recording and stimulation of the brain. Specifically, I will show that multiplex single-unit spike recordings over a 8-month period revealed almost unchanged principal components and average spike waveforms, stable firing dynamics and phase-locking of spike firings/LFP oscillations, and thus suggest robust tracking of the same neurons over this period, which contrasted strongly with conventional rigid brain probes that usually suffer from signal shifting and degeneration over weeks due to relative micromotion and mechanical mismatch. Moreover, I will demonstrate the implementation of stimulation functionality into the mesh electronics and show stable single neuron level responses to chronic electrical stimulations. Furthermore, the capability for long-term cellular resolution recording was applied to longitudinal studies of brain
aging, where the firing dynamics and spike characteristics of the same individual neurons were followed.

The developed mesh electronics also provides a potential platform to interrogate and modulate neural systems that are challenging or inaccessible using conventional rigid probes. In Chapter 7, I will describe the application of syringe-injectable mesh electronics for chronic mapping of retina ganglion cells in live mice. I will first introduce the development of a non-axial injection methodology for precise delivery of the mesh electronics onto the surface of a bowl-shape retina and synchronized electrophysiology recordings with optical stimulations. I will then show that the injected mesh electronics can unfold onto the retina of intact eye with a superior conformability and record multiplexed single-neuron activities from retina ganglion cells, which has never been achieved by conventional neural probes. Responses to non-patterned and patterned light stimulations recorded chronically will also be discussed.

Finally, in Chapter 8, I will introduce recent progresses in scaling-up the recording density and spatial coverage of the mesh electronics for highly multiplexed chronic brain mapping. Two complementary approaches—increasing the number of recording sites on each mesh electronics for high density local circuit decoding and implementing multi-site injection into the same brain for simultaneous mapping of entire neural pathways—will be discussed. I will demonstrate 128-channel simultaneous chronic recordings from the same mouse brain over 4 months period.

Look to the future, the integration of multifunctional nanoscopic devices into the 3D ultra-flexible macroporous mesh electronics network could open up opportunities to ask questions beyond current neuron-centric brain research. In Chapter 9, I will discuss the possibility and potential approach to develop a novel 3D multifunctional brain probe that builds
on mesh electronics to provide a comprehensive understanding of contributions from both neurons and glia to learning and memory processes and to enable glia-neuron-oriented diagnosis and treatment paradigms for neurodegenerative diseases.
1.5 Bibliography


Chapter 2

Sub-10 Nanometer Intracellular Bioelectronic Probes from Nanowire-Nanotube Heterostructures

[The following chapter is derived in part from T.-M. Fu et al., Proceedings of the National Academy of Sciences, 111, 1259-1264 (2014).]

The miniaturization of bioelectronic intracellular probes with a wide dynamic frequency range can open up opportunities to study biological structures inaccessible by existing methods in a minimally invasive manner. Here, we report the design, fabrication and demonstration of intracellular bioelectronic devices with probe sizes less than 10 nm. The devices are based on a nanowire-nanotube heterostructure in which a nanowire field-effect transistor (FET) detector is synthetically-integrated with a nanotube cellular probe. Sub-10 nm nanotube probes were realized by a two-step selective etching approach that reduces the diameter of the nanotube free-end while maintaining a larger diameter at the nanowire detector necessary for mechanical strength and electrical sensitivity. Quasi-static water-gate measurements demonstrated selective device response to solution inside the nanotube, and pulsed measurements together with numerical simulations confirmed the capability to record fast electrophysiological signals. Systematic studies of the probe bandwidth (BW) in different ionic concentration solutions revealed the underlying mechanism governing the time response. In addition, the BW effect of phospholipid coatings, which are important for intracellular recording, was investigated and modeled. The robustness of these sub-10 nm bioelectronics probes for intracellular interrogation was verified by optical imaging and recording the transmembrane resting potential of HL-1 cells.
These ultra-small bioelectronic probes enable direct detection of cellular electrical activity with highest spatial resolution achieved to date, and with further integration into larger chip-arrays could provide a unique platform for ultrahigh resolution mapping of activity in neural networks and other systems.
2.1 Introduction

Intracellular electrical recording, which can reveal substantially greater details of cellular process compared with extracellular recording, is important for both biological studies and biomedical applications (1-8). Ideally, an intracellular probe should embody two essential characteristics: (i) it should be as small as possible to increase spatial resolution, which can provide access to small subcellular structures, and to reduce invasiveness; and (ii) it should have a sufficiently large frequency BW to record both fast signals related to opening/closing of ion channels (6, 9, 10) and slowly changing or DC signals associated with synaptic interactions (1, 2). Several approaches have been taken to achieve these goals (2, 11). Sharp microelectrodes, which access the cell interior by direct insertion of metal (1, 12) or carbon (13, 14) microelectrodes, and more recently chip-based arrays of vertical metal electrodes (11, 15-17), have been used to record intracellular and intracellular-like signals. Further development of these probes with respect to achieving the above miniaturization goals does, however, faces intrinsic limits related to increasing electrode impedance with decreasing size. The patch clamp micropipette technique also faces constraints for miniaturization related to increasing impedance with probe size reduction (2, 18).

An alternative approach that can overcome the above limitations of probe size reduction has focused on using active semiconductor nanowire FET detectors (7, 19, 20), which do not depend on interfacial impedance (21-23). The capability to control synthetically nanowire structure and morphology (24-31) also has enabled hierarchical design of several types of intracellular bioelectronic probes with sizes down to sub-100 nm regime (7, 19, 20). For example, branched intracellular nanotube field-effect transistors (BIT-FETs) (19), which are designed based on a nanowire-nanotube heterostructure, achieve intracellular recording by
penetrating the cell membrane with a silicon dioxide (SiO₂) nanotube thereby bringing the cell
cytosol into contact with the underlying (extracellular) silicon nanowire (SiNW) FET detector.

Here we exploit the synthetic flexibility of this bioelectronic probe design to investigate
reduction of the SiO₂ nanotube probe an order of magnitude to the sub-10 nm regime, which
could open-up opportunities to interrogate small subcellular structures and organelles (Fig. 2.1a)
and could provide high spatial resolution for mapping. To realize our sub-10 nm bioelectronic
probe, there are three challenges that must be addressed. First, nanotube probes and their
heterojunction with SiNW FETs will become mechanically less stable as diameter is reduced.
Second, the electrical sensitivity will be reduced with decreasing nanotube diameter since the
nanotube inner diameter (ID) defines the effective device gate area. Third, high frequency
dynamic response may degrade with decreasing nanotube ID due to increasing solution
resistance in the nanotube. Our synthesis, fabrication, and characterization studies described
below show how these challenges can be successfully met to realize a functional sub-10 nm
bioelectronic probe.
2.2 Results and Discussion

Figure 2.1. Schematics and scanning electron microscopy (SEM) images of the ultra-small BIT-FET. (a) Schematic illustration of an intracellular bioelectronic probe. Left panel: General scheme of a probe for intracellular electrophysiology recording. Right panel: A magnified view of the tip of a sub-10 nm bioelectronic probe and its related size to single ion channel. (b) Schematic structure of the ultra-small BIT-FET. Green, yellow, blue and grey colors represent SiO$_2$ layer, metal contact, SiNW and silicon nitride substrate, respectively. (c) SEM images of the ultra-small BIT-FET at different fabrication steps. A GeNW branch was first grown on top of SiNW (I), followed by a subsequent H$_2$O$_2$ etching of top part of GeNW to shrink its diameter down to sub-10 nm regime (II). A final view of an ultra-small BIT-FET with nanotube ID ~8 nm, and SiO$_2$ wall thickness ~10 nm is presented in (III). Top inset of (III) is the close-up image of the tip of the ultra-small SiO$_2$ nanotube. White dashed lines in (II) and (III) indicate the point below which the GeNW and SiO$_2$ is protected by photoresist during H$_2$O$_2$ and BHF etching, respectively. All scale bars are 100 nm.
Our target for the ultra-small BIT-FET incorporating a sub-10 nm nanotube probe (Fig. 2.1a,b) has several key features to enable sufficient mechanical strength and electrical sensitivity as follows. First, the nanotube probe has a long free-end with small ID and thin wall for facile and minimally-invasive cell penetration. Second, a much larger ID and thicker wall are used for the bottom part of the nanotube to provide mechanical stability and increase the effective gate area of the SiNW FET detector. This latter feature can also ensure high electrical sensitivity of the bioelectronic probe.

To realize this target device structure we developed an efficient multi-step synthesis and fabrication approach (Figs. 2.1c, 2.2 and 2.3; Chapter 2.4). First, SiNW FETs were fabricated on a substrate surface (Fig. 2.2a,b) using previously established methods (25). Second, germanium nanowire (GeNW) branches, which serve as sacrificial templates for the nanotube probes (19), were grown on top of the SiNW FETs via a gold-nanoparticle catalyzed method (26) with the nanoparticle position precisely defined by electron beam lithography between the source and drain (S/D) electrodes of the FET (Fig. 2.2c,d). A representative scanning electron microscopy (SEM) image shows a nearly vertical GeNW grown from the upper surface of the SiNW (Fig. 2.1c, I). Third, selective hydrogen peroxide (H₂O₂) etching was carried out to reduce the size of the upper portions of the GeNWs while the lower portions of the GeNWs where they connect to the SiNWs are protected by a thin photoresist layer with thickness about 20% of the entire GeNW length (Fig. 2.2e,f). A typical SEM image (Fig. 2.1c, II) shows that this selective etching step yields GeNWs with diameters <10 nm for the upper portions and diameters ca. 80-90 nm for the lower portions. The larger diameter for the lower portion enhances the mechanical strength of the structure, and together with the Ge shell deposited on the SiNW (simultaneously with GeNW growth), is also important to the overall device sensitivity as discussed below.
Figure 2.2. Schematics of the fabrication flow for the ultra-small BIT-FET. (a) SiNWs (blue) are dispersed on substrate (solid gray). (b) S/D contacts are defined by EBL followed by thermal evaporation. (c) Au nanodots are defined on SiNWs between S/D using EBL and thermal evaporation. (d) GeNWs (red) are grown on top of the SiNWs through nanocluster-catalyzed CVD process. (e) A thin layer of photoresist (transparent gray) is spin-coated on the chip to protect the lower GeNW part. (f) The resulting H₂O₂ etched GeNWs following photoresist lift-off. Only the GeNW above the photoresist in (e) is thinned by etching in H₂O₂. (G) SiO₂ is conformally deposited over the entire chip by ALD. (h) A thin layer of photoresist (transparent gray) is spin-coated to protect the lower region of chip. (i) The resulting BHF etched structures following lift-off. The region of SiO₂ above the photoresist layer in (h) is etched to ca.10 nm thickness. (k) Photoresist with thickness smaller than the GeNW heights is deposited. (k) The resulted structure following BHF etching of SiO₂, which exposes the tips of the GeNWs. Isotopic BHF etching yields a small taper with thinner SiO₂ at the topmost part of the structure. (l) The GeNW is removed by H₂O₂ etching to form an ultra-small nanotube connected to the bottom SiNW FET.
To form the nanotube structure, a thin ca. 30 nm SiO$_2$ layer was conformally deposited by atomic layer deposition (ALD) on the SiNW-branched GeNW heterostructures and entire device substrate (Figs. 2.2g and 2.3a). In addition to coating the GeNW, this ALD process deposits the same thickness SiO$_2$ on the SiNW FET and S/D electrodes, and thus serves to passivate (i.e., isolate) these structures from solution in our experiments. Selective buffered hydrofluoric acid (BHF) etching was then carried out to reduce the thickness of upper SiO$_2$ shell to $\sim$10 nm while the lower portion of the shell was protected by photoresist (Figs. 2.2h,i and 2.3a). To complete the device structure, a two-step etching process was carried out in a manner similar to that described previously for the conventional BIT-FET (20). First, the upper tip of the Ge core was exposed by removing the SiO$_2$ shell using BHF, and then the GeNW was removed using H$_2$O$_2$. A representative SEM image of an ultra-small BIT-FET (Fig. 2.1c, III) shows several key features. First, the upper portion of the SiO$_2$ nanotube has an ID of $\sim$8 nm and a wall thickness of $\sim$10 nm thick. Second, the very tip of the nanotube is tapered due to BHF etching yielding a relative sharp point for insertion. Third, the lower portion of the nanotube has much larger $\sim$80 nm ID and 30 nm wall thickness as expected based on our fabrication process. In addition, transmission electron microscopy (TEM) images (Fig. 2.3b) confirm the ultrasmall IDs of the upper portions of the SiO$_2$ nanotubes.

The basic electrical sensitivity of the ultra-small BIT-FET devices before and after GeNW etching was characterized using standard quasi-static water-gate measurements (19, 32) (see Chapter 2.4). Representative device conductance, $G$, versus applied water-gate voltage, $V_{wg}$, data (Fig. 2.4a) shows that $G$ changes very little as a function of $V_{wg}$ before GeNW etching, but exhibits a large change after GeNW etching to form the nanotube structure. Indeed, the magnitude of the transconductance (device sensitivity) increases from 0.24 to 2.75 $\mu$S/V after
etching. In addition, control measurements performed on SiNW FET devices without nanotubes or GeNW branches exhibit no increase in transconductance after etching similar to previous studies (19). Together, these results confirm that solution can fill a small SiO₂ nanotubes, and that the SiNW FET detector responds selectively to the solution inside the nanotubes versus outside.

![Figure 2.3.](image)

**Figure 2.3. Electron microscopy characterization of the ultra-small BIT-FET.** (a) Representative scanning electronic microscope (SEM) images of intermediate fabrication steps of the ultra-small BIT-FET. Left: Device after 30 nm ALD coating of SiO₂. Right: Device after first step of selective BHF etching of the upper 80% portion of the SiO₂ to ca. 10 nm (Fig. 2.2h,i). White dashed lines in (I) and (II) indicate the point below which the SiO₂ is protected by photoresist during BHF etching. Scale bars: 200 nm. (b) False colored transmission electron microscopy image of an ultra-small nanotube. This tube was fabricated following the same procedure as described in Chapter 2.4, and deposited onto lacey carbon grids from ethanol suspension. It has a tip ID ~7 nm, and bottom ID ~ 80 nm. False color is used here to distinguish the SiO₂ nanotube from background amorphous carbon. Scale bar: 50 nm.
Figure 2.4. Water-gate characterization and bandwidth (BW) measurements. (a) Conductance $G$ of an ultra-small BIT-FET with $\sim 7$ nm ID versus water-gate voltage $V_{wg}$ before (blue) and after (red) core GeNW etching. (b) A step water-gate pulse $V_{wg}$ with 0.01 ms rise time and 100 mV steady-state amplitude (black) and the corresponding response from a typical ultra-small BIT-FET with $\sim 10$ nm nanotube ID and 3.2 $\mu$m length (red). Response from metal contacts has been removed (Fig. 2.6). The recorded voltage signal is calibrated by the device transconductance measured from quasi-static water-gate experiment. $V_{wg}$ and $V_{signal}$ are offset by 50 mV for clarity. (c) Step pulsed water-gate responses from ultra-small BIT-FETs with different nanotube IDs. Same voltage calibration was adopted as (b). Traces are offset by 100 mV for clarity. (d) Measured BW of ultra-small BIT-FET devices versus the ID of the nanotube and comparison with numerically calculated data. Model 1 and 2 present the upper (the active gate area is defined by the nanotube ID) and the lower limit (the entire SiNW surface is active) of the device (Chapter 2.4). For comparison, all the BWs were normalized to a uniform nanotube length of 2.5 $\mu$m. Due to the equipment BW limit, the BW of the 22 nm device is extrapolated from its BW in 0.1X PBS solution by taking the relative conductivity of 1X PBS and 0.1X PBS into account (Chapter 2.4). The error bar corresponding to the standard error of the experimental data is $\pm 2$ nm induced by SEM measurement uncertainty. All the devices for experiment and modeling have a top nanotube wall thickness $\sim 10$ nm.
The enhanced sensitivity following GeNW etching to form the nanotube probe structure reflects two key contributions. First, the solution gating the SiNW FET via a much thinner (1-2 nm native oxide) SiO₂ versus 30 nm deposited layer before GeNW etching. Second, the Ge-layer deposited on the SiNW FET during GeNW growth was etched during nanotube formation process to yield an effective gate area that is much larger than that defined by the nanotube ID (Figs. 2.1b and 2.5).

We characterized the bandwidth (BW) of the ultra-small BIT-FETs through step water-gate pulse measurements in 1X phosphate buffered saline (1X PBS) solution (see Chapter 2.4) to define further their capabilities for electrophysiological recording in cells. The temporal response of a device with 10 nm ID nanotube following a step-like water-gate pulse (rise time = 0.01 msec, amplitude = 100 mV) is shown in Fig. 2.4b, where the capacitive coupling from the metal contacts was removed (Fig. 2.6) and the measured conductance change was converted to voltage using the device sensitivity (32). Qualitatively, the recorded data exhibits a slower time response (i.e., rounding with respect to water-gate voltage step), which indicates that the probe’s time response is slower than 0.01 msec rise time of the applied voltage step. Following previous studies (19), we can define the effective BW as,

\[
BW = \frac{0.35}{t_{90\%} - t_{10\%}}
\]  

(2.1)

where \( t_{90\%} - t_{10\%} \) is the time needed for the recorded signal to change from 10% to 90% of the steady-state amplitude of the applied \( V_{wg} \). Analysis of the data in Fig. 2.4b, where \( t_{90\%} - t_{10\%} = 58 \) μs, yield a BW of \( \sim 6.0 \) kHz.
Figure 2.5. Sensitivity of different device structures. (a), (b) Schematics of the ultra-small BIT-FET without and with Ge overcoating on the SiNW, respectively. Panels (I) and (II) correspond to the BIT-FET devices before and after Ge core etching. Panels (III) show schematically typical conductance ($G$) vs. water-gate ($V_{wg}$) measurements from these distinct structures.

To further elucidate the nanotube ID dependence of the ultra-small BIT-FETs BW, we carried out similar pulsed water-gate measurements for ultra-small BIT-FET devices with different nanotube IDs. The measured calibrated voltage signal as a function of time data for devices with nanotube IDs from 5 to 22 nm (Fig. 2.4c) show that the rise time for the recorded signal to reach 100 mV steady-state increases as the nanotube ID decreases. Quantitatively, the calculated BW of each probe based on Equation 2.1 are shown in Fig. 2.4d. These data show that BW decreases from ca. 88 kHz to 3.1 kHz as the nanotube ID decreases from 22 to 5 nm, respectively.
Figure 2.6. Dynamic water-gate responses from ultra-small BIT-FETs. (a) Pulsed (i.e., step function) $V_{wg}$ and corresponding conductance ($G$) vs. time responses from a 10 nm ID ultra-small BIT-FET in different concentration PBS solutions. The applied $V_{wg}$ with a 100 mV amplitude pulses had rise/fall times of 100, 10, 0.3 and 0.05 ms in (I), (II), (III) and (IV), respectively. The duration of the pulse is ten times the rise/fall time in all measurements. The peak and dip features associating with the rise and fall of the water-gate pulse is due to the capacitive coupling to passivated metal electrodes (19). Red arrows indicate the PBS solution concentration at which the ultra-small BIT-FET response can no longer respond to the applied water-gate pulse without time delay. (b) Removal of capacitive signals from the passivated metal electrodes. A 100 mV pulsed water-gate $V_{wg}$ with 0.3 ms rise/fall time was applied (I). Experimental responses from both ultra-small BIT-FET and passivated metal electrodes (II), and only the passivated metal electrodes (i.e., $V(S/D) = 0$) (III) are shown. Subtraction of (III) from (II) yields the pure field effect response from the ultra-small BIT-FET (IV).
To gain further insight into the measured data we also modeled the ultra-small BIT-FET BW (Fig. 2.7). The time evolution of the potential at the SiNW FET surface following a step-like pulsed water-gate potential was numerically evaluated (see Chapter 2.4) for two different models. The upper BW limit (Model 1) assumes the active gate area is defined by the nanotube ID (e.g., Fig. 2.5a), and the lower BW limit (Model 2) assumes that the entire SiNW surface is active (i.e., the deposited Ge layer is etched over the SiNW surface as shown schematically in Fig. 2.5b). The calculated BW results for the two models (Fig. 2.4d) are consistent with the experimental data, although somewhat overestimates BW at the smaller diameters. This difference can be attributed to a larger gap over the SiNW FET detector (Fig. 2.5b) than used in Model 2, although future studies will be required to understand fully these differences. Importantly, we noted that the measured and calculated BW values for the smallest 5 nm ID nanotube probes, 3.1 kHz, are sufficient to enable accurate recording of most fast dynamic cellular processes.

Figure 2.7. Device bandwidth circuit model. Equivalent circuit for the device bandwidth modeling of the BIT-FET.
We have further characterized the BW of the ultra-small BIT-FETs in different ionic strength PBS solutions. Representative data recorded following a water-gate potential step using an ultra-small BIT-FET with 10 nm ID nanotube (Fig. 2.8a) exhibit a clear increase of rise time with decreasing solution concentration. A plot of the BW versus solution concentration determined from this data (Fig. 2.8b) shows that the BW depends linearly on PBS concentration. Qualitatively, this behavior is consistent with our model given the expected dependence of BW on solution resistivity.

We have further explored this dependence by recording the time response from ultra-small BIT-FETs with different nanotube IDs in different concentration PBS solutions, where the resulting BW results are summarized in Fig. 2.8c. In addition, these results were rescaled by normalizing the solution resistivity to that in 1X PBS (Fig. 2.8d); that is, multiplying the BW by \( \rho_{\text{sol}}/\rho_{1\text{X PBS}} \), where \( \rho_{\text{sol}} \) and \( \rho_{1\text{X PBS}} \) are the resistivity of the solution and 1X PBS solution, respectively. The rescaled data exhibits a nearly universal dependence on nanotube ID, where we attribute deviations to contributions from the tip access impedance and capacitive coupling to the underlying SiNW FET. These latter factors become more prominent for larger ID nanotube devices, although we assume that the BW is dominated only by the nanotube in our analysis. We have also used a simplified analytical model to obtain additional insight into the device behavior (Chapter 2.4). Specifically, this analysis provides an explicit relationship for the BW as a function of solution resistivity inside the nanotube (inverse), the nanotube wall capacitance per unit length (inverse) and nanotube length (inverse square root), and thus also can be used to guide the design of ultra-small BIT-FETs to achieve required BWs.
Figure 2.8. Device bandwidth dependence on electrolyte concentration. (a), (b) Pulsed water-gate responses (traces offset by 100 mV) and corresponding BWs of a ~10 nm nanotube ID ultra-small BIT-FET in solutions with different PBS concentration. (c) Measured BWs of ultra-small BIT-FET devices versus nanotube ID in different concentration PBS solutions. (d) Rescaled device BWs versus nanotube ID. All BWs are rescaled by solution resistivity (Chapter 2.4).

The ultra-small BIT-FET nanotubes are coated with phospholipid layers to facilitate the cell membrane penetration for intracellular recording (7, 19, 20, 33-36). Because the smallest nanotube IDs investigated in our studies approach the thickness of phospholipid bilayer ~ 4.75 nm (37), we measured the BWs of several probes before and after phospholipid modification (Chapter 2.4). Analysis of data recorded with an ultra-small BIT-FET having a 10 nm ID and 2.8 µm long nanotube (Fig. 2.9a) shows that the BW dropped from 9.7 kHz to 3.0 kHz after phospholipid modification while the device sensitivity remained essentially unchanged (< 1% variation in transconductance).
To explain the observed reduction in BW in this and other measurements following phospholipid modification, we considered the three scenarios shown schematically in Fig. 2.9b: (i) the entire inner and outer nanotube surfaces of the nanotube are covered by a lipid bilayer; (ii) a phospholipid bilayer covers 50% of the inner and all of the outer surfaces of the nanotube; and (iii) the lipid bilayer is excluded from the inner surface but covers all of the outer surface of the nanotube. The calculated (Chapter 2.4) BW change expressed as a ratio after:before phospholipid modification for these three scenarios as a function of nanotube ID (Fig. 2.9c) highlights several key points. First, the BW ratio approaches zero for case-i and case-ii as the nanotube ID approaches the thickness of the bilayer coatings due to the increasing effective solution resistivity. Second and for case-iii, the ratio is >1 due to a reduction in effective capacitance of the nanotube. In addition, comparison of these calculations to experimental results for 15 and 10 nm ID nanotube probes (Fig. 2.9c) shows that the experimental BW ratios are close to case-i and case-ii, respectively. In the future, systematic experimental studies should give insight to the control of phospholipid modification to minimize coating of the inner nanotube wall while maintaining coverage of the outer surface to facilitate probe penetration through cell membranes, although we note that the BW drops on modification still yield sufficiently high BWs for 10 nm ID nanotubes to record sub-millisecond cellular processes such as neuronal action potentials (2, 3, 11).
Figure 2.9. Device bandwidth after phospholipid modification. (a) Pulsed water-gate responses from a ~10 nm nanotube ID ultra-small BIT-FET before (black) and after (red) phospholipid modification. The black and red arrows indicate the points when the curves reach steady-state amplitudes. Traces are offset by 100 mV for clarity. (b) Schematics of three possible scenarios for phospholipid modification: both (inner and outer) surfaces of the nanotube are fully covered (left), outer surface is fully covered and inner surface is only 50% covered (middle) and only outer surface is covered (right). The purple and green colors are phospholipid bilayer and SiO₂ nanotube, respectively. (c) Calculated ratio of the device BW after and before phospholipid modification versus nanotube ID under different scenarios depicted in (a) (Chapter 2.4). For comparison, two experimental data are presented (black square).
Last, we have investigated the capability of the ultra-small BIT-FET for intracellular recording in studies of isolated HL-1 cells (38). A series of differential interference contrast optical microscopy images recorded while moving a single HL-1 cell with a glass micropipette to/from a phospholipid modified ultra-small BIT-FET with 10 nm ID nanotube probe (Fig. 2.10a) shows several features. First, the substantial size difference between the ultra-small BIT-FET and glass micropipette is obvious in the images. Second, the nanotube probe can readily penetrate the cell membrane without affecting the overall cell shape, which suggests a biomimetic internalization assisted by lipid modification and hence a reasonably tight seal around the nanotube. Third, when the cell is retracted from the nanotube probe there is no change in cell shape indicating that the membrane readily reseals.

Significantly, measurement of the potential (mV) during the HL-1 cell approach/penetration/retraction process with an independent ultra-small BIT-FET (Fig. 2.10b) shows (i) a stable baseline prior to contact, (ii) a sharp ca. 59 mV drop when the HL-1 cell was brought into contact with the ultra-small nanotube, which corresponds to the intracellular potential of the cell, and (iii) abrupt (within ~300 ms) return to baseline coincident with retraction of the HL-1 cell from the device. These data demonstrate that the ultra-small BIT-FET can record intracellular potentials, and moreover, can record both low frequency (shown here) and high-frequency (described above) signals. The capability to record signals over a broad frequency range using 10 nm scale probe represents a clear advantage of the active ultra-small BIT-FET device over conventional passive electrodes.
Figure 2.10. Intracellular resting membrane potential recording. (a) Schematics (upper panel) and differential interference contrast optical microscopy images (lower panel) of an HL-1 cell manipulated by a glass micropipette to approach (I), contact (II), penetrate (III), and retract (IV) from a phospholipid-modified ultra-small BIT-FET probe. Red arrow indicates the position of the ultra-small nanotube tip. Since pure SiO$_2$ nanotube is optically-transparent, the GeNW template of this device was not etched for imaging. Scale bar: 2 μm. (b) Representative electrical recording results from a ~10 nm ID ultra-small BIT-FET device; in this case, the GeNW was etched to yield the ultra-small SiO$_2$ nanotube. Down and up pointing green arrows mark the beginning of cell penetration and withdrawal, respectively. The upper and lower horizontal dashed line indicate the extracellular and intracellular potentials. Quasi-static water-gate measurements made before/after cell measurements show <2% change in the device conductance and sensitivity.
2.3 Conclusion

We have designed, fabricated and demonstrated an ultra-small intracellular bioelectronic probe, the ultra-small BIT-FET, based on a nanowire-nanotube heterostructure with probe nanotubes having IDs as small as 5 nm. Systematic studies of the ultra-small BIT-FET BW through pulsed water-gate measurements and theoretical modeling showed the capability to record fast, sub-millisecond physiological signals with the smallest ID nanotube probes. Moreover, the effect of phospholipid coatings, which are important for intracellular recording, on probe performance has been characterized and modeled. Importantly, optical microscopy and electrical recording measurements demonstrate that ultra-small BIT-FET nanotube probes can penetrate and subsequently be retracted from HL-1 cells in minimally-invasive manner while continuously recording the transition between extracellular and intracellular rest potential. The ultra-small BIT-FET opens up unique opportunities for future electrophysiological researches, including intracellular recordings from small subcellular structures and intracellular organelles, such as dendrites, dendritic spines, and the cell nucleus. Integration of such probes into large-scale arrays could also enable mapping of electrical activity from neural networks with substantially greater spatial resolution and minimal invasiveness than possible with techniques now available.
2.4 Methods and Materials

2.4.1 Silicon nanowire (SiNW) synthesis

Boron-doped p-type silicon nanowires (SiNWs) were synthesized using a gold (Au) nanoparticle catalyzed chemical vapor deposition (CVD) methodology described previously (19). Briefly, 100 nm diameter Au nanoparticles (Ted Pella, Inc.) were dispersed on an oxidized silicon (Si) wafer (600 nm silicon dioxide (SiO₂), Nova Electronic Materials). Syntheses were carried out at 450-460 °C and 25 torr, with 2.5 standard cubic centimeters per minute (sccm) pure silane (SiH₄) as the silicon source, 3 sccm diborane (B₂H₆) (100 ppm in He) as the boron (B) dopant source and 10 sccm argon (Ar) as the carrier gas. Under these conditions, the resulting SiNWs have diameters of ca.100 nm and a delivered doping ratio of 4000:1 (Si:B). The total growth time was 40 min.

2.4.2 SiNW field-effect transistor (FET) fabrication

SiNWs were suspended in isopropanol solution by gentle sonication (2-3 s, 30 W, Crest Ultrasonics) and then dispersed onto the silicon nitride (Si₃N₄) surface of a Si wafer (100 nm thermal SiO₂, 200 nm Si₃N₄, n-type, 0.005 V·cm, Nova Electronic Materials) with predefined outer electrodes (Ti/Pt/Ti, 5/50/30 nm) and markers (Ti/Pt, 5/50 nm). The dispersed SiNWs were spin-coated (4000 rpm for 40 s, each layer) with resists (MMA (8.5), MAA (EL9) and PMMA (950, C2), MicroChem Corp.), and each layer was baked at 185 °C for 5 min. Electron-beam-lithography (EBL, JEOL-7000F) was used to define source/drain (S/D) contacts on individual SiNWs. The typical width of the contacts was 400 nm and the separation between S/D was 600-800 nm. A step of BHF (Buffered HF Improved, Transene) was carried out to eliminate natural SiO₂ on SiNW before thermal evaporation (Sharon Thermal Evaporator) of Ti contact (140 nm thick). A schematic of SiNW FET fabrication is presented in Fig. 2.2a,b.
2.4.3 Germanium nanowire (GeNW) synthesis

Germanium nanowires (GeNWs) were grown on top of the resulting SiNW FETs as follows: First, 35 nm thick Au nanodots with ca. 80 nm diameter were defined by EBL and thermal evaporation on top of SiNW FETs between predefined S/D (Fig. 2.2c). Second, the chip was placed in the CVD reactor, and GeNW growth was initiated by nucleation at 315 °C, and 300 torr for 1 min with 20 sccm germane (GeH₄) (10% in hydrogen (H₂)) as the germanium source, 200 sccm H₂ as the carrier gas, followed by elongation step at 285 °C, and 100 torr for 20-40 min (gas flow same as for nucleation). The resulting GeNWs have lengths ca. 3-4 μm and a slight taper with bottom diameters ca. 80-90 nm and top diameters ca. 50-60 nm (Fig. 2.2d).

2.4.4 GeNW diameter reduction

The upper 80% portion of the GeNWs were etched in hydrogen peroxide (H₂O₂) solution to reduce their diameters: (i) A diluted photoresist protection layer (Shipley S1805: Thinner Type P=1:1, MicroChem Corp) with a thickness of ca. 20% of GeNW length was spin-coated to cover the bottom part of GeNWs (Fig. 2.2e) and baked at 115 °C for 5min. (ii) The chip was then placed in 0.17% H₂O₂ solution at 0 °C, and etched for 2-2.5 min.; the calibrated H₂O₂ etching rate was 10-12 nm/min. (iii) After etching, the photoresist layer was removed in acetone (without drying), subsequently transferred to 200-proof ethanol, and dried with critical point dryer (Auto Samdri 815 Series A, Tousimis). The resulting GeNWs had 5-10 nm diameters (controlled by etching time) for the upper 80% portion and 80-90 nm diameters for the lower 20% portion of the GeNWs (Fig. 2.2f).

2.4.5 Nanotube fabrication

To fabricate thin but mechanically robust SiO₂ nanotubes, we combined atomic layer deposition (ALD) and a two-step BHF etching as follows: First, a uniform 30 nm SiO₂ layer,
which serves as both the nanotube wall and metal electrode passivation, was conformally deposited by ALD (Savannah-S200, Cambridge NanoTech) at 250 °C (Fig. 2.2g). Second, a photoresist protection layer (~ 20% of GeNW length) was spin-coated and baked (Fig. 2.2h), and then the upper unprotected part of the SiO2 layer was etched in BHF (Buffered HF Improved, Transene) to ~10 nm (Fig. 2.2i); the etching rate was ~1.5 nm/min and was calibrated. Third, following lift-off of the former protection resist layer, a thicker photoresist layer (Shipley S1813 or S1818, MicroChem Corp.) was spin-coated and baked at 115 °C for 5min as shown in Fig. 2.2j. A second step of BHF etching was used to remove the exposed SiO2 layer at the GeNW tip; the SiO2 shell is tapered during this step due to etching along the axial and radial direction (Fig. 2.2k). After photoresist lift-off, the chip was transferred to H2O2 solution (30%, Sigma) to etch selectively the Ge (60 °C, 60 min), which produces the nanotube probe, and then the chip was dried in the critical point dryer. The resulting nanotubes have an inner diameter 5-10 nm and 10 nm tapered SiO2 wall for the upper 80% portion and an inner diameter 80-90 nm and 30 nm SiO2 wall for the lower 20% part (Fig. 2.2l).

2.4.6 Device electrical characterization

The behavior of the ultra-small BIT-FET devices in aqueous solution was characterized in two distinct ways in order to determine their quasi-static and dynamic responses. (i) Standard quasi-static water-gate measurements were carried out in 1X PBS to characterize the device sensitivity as follows: The water-gate potential, $V_{wg}$, was varied at 50 mV/s (via Ag/AgCl electrode) while monitoring the SiNW FET current for fixed 100 mV S/D voltage; the FET current was amplified (1211, DL Instruments) and digitized at 100 kHz sampling rate (Axon Digidata 1440A Data Acquisition System, Molecular Devices, Inc.). The resulting current versus $V_{wg}$ curves are used to calibrate the sensitivity (transconductance) for the devices. (ii) A quasi-
step-function water-gate pulse was used to characterize the bandwidth (BW) of the ultra-small BIT-FET devices in different concentration PBS solutions. In short, a 0.01 ms rise-time 100 mV amplitude $V_{wg}$ step was applied (Axon Digidata 1440A Data Acquisition System, Molecular Devices, Inc.) while simultaneously recording the corresponding current variation of the ultra-small BIT-FET, which was amplified, filtered at 30 kHz (CyberAmp 380, Molecular Devices, Inc.), and then digitized at a 100 kHz sampling rate. A 100 mV DC source voltage was used in all of the measurements.

2.4.7 Device bandwidth model

The calculation of the ultra-small BIT-FET bandwidth is based on the model described for conventional BIT-FETs in (19), and the equivalent circuit is shown in Fig. 2.7. With a applied step water-gate pulse (i.e. $V_{out} = V_0 \theta(t)$ with $V_0$ being the pulse amplitude and $\theta(t)$ is the step function equals 1 for $t > 0$ and 0 for $t < 0$), this circuit can be described by the following partial differential equation,

$$\frac{\partial^2 V_{in}}{\partial z^2} = \rho_s \rho_C \left( \frac{\partial V_{in}}{\partial t} - \frac{\partial V_{out}}{\partial t} \right) = \rho_s \rho_C \left( \frac{\partial V_{in}}{\partial t} - V_0 \delta(t) \right) \quad (2.2)$$

where $V_{in}, V_{out}, \rho_s, \rho_C, z$ and $t$ correspond to the potential inside the ultra-small nanotube, the potential outside the nanotube, the linear resistivity of the solution inside the tube, the capacitance of the ultra-small nanotube wall per unit length, the distance from the nanotube opening, and time, respectively.

For numerical simulations (Model 1 and 2 in Fig. 2.4d), we used a 1D finite element method to evaluate the potential change at the end of the SiO$_2$ nanotube as a function of time following Equation 2.2. We fixed the length of SiO$_2$ nanotube $L=2.5 \ \mu$m and the thickness as $d=10$ nm. Two models corresponding to the upper and lower bandwidth limits were considered.
In model 1, the active channel is limited to the area defined by the GeNW base, and in model 2, the active channel corresponds to the entire active SiNW surface. These two models represented the scenarios of no Ge overcoating and complete Ge overcoating (we used a 10 nm Ge overcoating thickness based on experimental data; this Ge is removed during GeNW etching to produce the larger active area), respectively, on the SiNW.

Previous simulation results (19) revealed that the device bandwidth is mainly limited by the small diameter nanotube, and thus, that the tip access impedance and capacitive coupling to underlying SiNW FET can be ignored. We used these simplifications and the initial condition of $V_{in}(z,0)=0$, and applied Laplace transform to obtain an analytical solution for Equation 2.2 as,

$$\frac{V_{in}}{V_0} = \frac{1}{\sqrt{\pi}} \int_0^{\infty} \frac{\exp\left(-\frac{1}{x}\right)}{x^{3/2}} dx$$

(2.3)

Since bandwidth (BW) is inversely proportional to the time needed for $\frac{V_{in}}{V_0}$ to increase from 0.1 to 0.9, we obtain

$$BW \propto \frac{1}{t_{0.9} - t_{0.1}} \propto \frac{1}{\rho_R \rho_C l^n} \propto \frac{\ln(1 + \frac{2t_{so2}}{d})}{\rho_{sol} l^2}$$

(2.4)

where $\rho_R, \rho_C, d, t_{so2}, l, \rho_{sol}$ are the solution linear resistivity, effective nanotube wall capacitance per unit length, nanotube inner diameter, nanotube thickness, nanotube length and liquid resistivity, respectively. Based on this relation, we can rescale measured bandwidth results by nanotube diameter, length or liquid resistivity (conductivity).

2.4.8 Effect of phospholipid modification on device bandwidth

From Equation 2.4, we can estimate the effect of phospholipid modification on device bandwidth by considering the change of $\rho_R$ and $\rho_C$. For example, if phospholipid bilayers
modify both inner and outer surfaces of the tube, $\rho_R$ increases to $\left( \frac{d}{d-2t_{lipid}} \right)^2 \rho_R$ resulting from the reduction of the effective nanotube inner diameter and $\rho_C$ decreases to

$$\frac{\rho_C}{\ln(\frac{d}{d-2t_{lipid}}) + \ln(\frac{d+2(t_{SiO_2} + t_{lipid})}{d+2t_{SiO_2}})}$$

since the lipid bilayers act as new capacitors connected with the nanotube wall capacitor in series. Here, $t_{lipid}$, $\varepsilon_{SiO_2}$ and $\varepsilon_{lipid}$ are double layer phospholipid thickness (ca. 4.75 nm (37)), relative dielectric constant of SiO$_2$ (ca. 3.9 (39)) and relative dielectric constant of bilayer phospholipid (ca. 5 (40)). From Equation 2.5, the ratio of the bandwidth after to before modification can be expressed as

$$\frac{BW_{after}}{BW_{before}} = \left( \frac{d-2t_{lipid}}{d^2} \right)^2 \left[ 1 + \frac{\varepsilon_{SiO_2}}{\varepsilon_{lipid}} \frac{\ln(\frac{d}{d-2t_{lipid}}) + \ln(\frac{d+2(t_{SiO_2} + t_{lipid})}{d+2t_{SiO_2}})}{\ln(\frac{d+2t_{SiO_2}}{d})} \right]$$

(2.5)

This ratio versus nanotube inner diameter is plotted in Fig. 2.9c (100%, red line) of the main text. Similarly, we obtain expressions for bandwidth ratio for the cases where the inner surface is only half covered and not covered at all. The nanotube inner diameter dependence of these ratios is plotted in Fig. 2.9c (50%, green line and 0%, blue line).
2.5 Bibliography


Chapter 3

Syringe-Injectable Electronics

[The following chapter is derived in part from J. Liu, T.-M. Fu et al., *Nature Nanotechnology*, 10, 629-636 (2015).]

Seamless and minimally invasive three-dimensional interpenetration of electronics within artificial or natural structures could allow for continuous monitoring and manipulation of their properties. Flexible electronics provide a means for conforming electronics to non-planar surfaces, yet targeted delivery of flexible electronics to cavities remains difficult. Here, we overcome this challenge by demonstrating the syringe injection (and subsequent unfolding) of sub-micrometer-thick, centimeter-scale macroporous mesh electronics through needles with a diameter as small as 100 μm. Our results show that electronic components can be injected into man-made and biological cavities, as well as dense gels and tissue, with > 90% device yield. We demonstrate several applications of syringe injectable electronics as a general approach for interpenetrating flexible electronics with three-dimensional structures, including (i) monitoring of internal mechanical strains in polymer cavities, (ii) tight integration and low chronic immunoreactivity with several distinct regions of the brain, and (iii) *in vivo* multiplexed neural recording. Moreover, syringe injection enables delivery of flexible electronics through a rigid shell, delivery of large volume flexible electronics that can fill internal cavities and co-injection of electronics with other materials into host structures, opening up unique applications for flexible electronics.
3.1 Introduction

The emergence of flexible electronics has significantly extended the applications of electronics by forming an intimate interface between electronic units and non-planar surfaces of structures for better monitoring and manipulation of their properties (1-3). For example, researchers have integrated a variety of electronic devices, including light-emitting diodes (4), mechanical sensors (5, 6) transistors (1-3, 7, 8) on flexible and stretchable substrates to enable applications from foldable display to electronic skin (3-8). Three-dimensional (3D) interpenetration of flexible electronics within existing structures could further broaden and open up new applications by directly interfacing devices with the internal structures of man-made and biological materials.

Recent work has shown that flexible electronics can be placed into existing 3D structures through surgical processes (9-12) or by being attached to and subsequently released from a rigid delivery substrate (13-14) for biological and biomedical applications, yet direct 3D interpenetration of electronic units within the structures is still limited by the existence of thin film or solid (14) supporting substrates. We have introduced the concept of macroporous mesh structures that allow electronics to be combined, for example, with polymer precursors and cells to yield on-chip 3D interpenetration (15, 16), although controlled delivery and non-surgical placement of these extremely flexible open electronic networks into objects and structures with seamless 3D integration and interpenetration has not yet been possible.

Here, we describe the structural design and demonstration of macroporous flexible mesh electronics that allow electronics to be precisely delivered into 3D structures by syringe injection and subsequently relax and interpenetrate within the internal space of man-made and biological materials. Distinct from previous reports (3, 17, 18), syringe injection requires complete release
of the mesh electronics from a substrate, and moreover, sub-micron thickness so that the electronics can be driven by solution through a needle. The concept of syringe injectable electronics is shown schematically in Fig. 3.1a-c and involves (i) loading the mesh electronics into a syringe and needle, (ii) insertion of the needle into the material or internal cavity and initiation of mesh injection (Fig. 3.1a), (iii) simultaneous mesh injection and needle withdrawal to place the electronics through the targeted region (Fig. 3.1b), and (iv) delivery of the input/output (I/O) region of the mesh outside of the material (Fig. 3.1c) for subsequent bonding and measurements.

![Figure 3.1 Syringe injectable electronics.](image)

(a) to (c) Schematics of injectable electronics. The
Figure 3.1 (Continued): red-orange lines highlight the overall mesh structure and indicate the regions of supporting and passivating polymer mesh layers; the yellow lines indicate metal interconnects between I/O pads (green filled circles) and recording devices (blue filled circles). (d) Schematic of the mesh electronics design (upper image), where the orange and red lines represent polymer encapsulated metal interconnects and supporting polymer elements, respectively, and $W$ is the total width of the mesh. The dashed black box (lower image) highlights the structure of one unit cell (white dashed lines), where $\alpha$ is the angle deviation from rectangular. (e) Longitudinal mesh bending stiffness, $D_L$, and transverse mesh bending stiffness, $D_T$, as a function of $\alpha$ defined in (d). (f) and (g) Images of mesh electronics injection through a glass needle, ID = 95 µm, into 1X PBS solution. Bright-field microscopy image (f) of the mesh electronics immediately prior to injection into solution; the red arrow indicates the end of the mesh inside the glass needle. 3D reconstructed confocal fluorescence image (g) recorded following injection of ca. 0.5 cm mesh electronics into 1X PBS solution. The blue and white dashed boxes correspond to regions shown in Fig. 3.4a,b. (h) Optical image of an injectable mesh electronics structure unfolded on a glass substrate. $W$ is the total width of the mesh electronics. The red dashed polygon highlights the position of electrochemical devices or FET devices. Green and black dashed boxes highlighted metal interconnect lines and metal I/O pads, respectively. (i) and (j) Yields and change with ±1 standard deviation (±1SD) in properties post-injection for single-terminal electrochemical and two-terminal field-effect transistor (FET) devices. (i) Yield (blue) and impedance change (red) of the metal electrodes from the mesh electronics injected through 32, 26 and 22 gauge metal needles. Inset: bright field image of a representative metal electrode on mesh electronics, where the sensing electrode is highlighted by a red arrow. Scale Bar: 20 µm. (j) Conductance change (red) and yield (blue) of silicon nanowire FETs following injection through 32, 26, 24, 22 and 20 gauge needles. Inset: scanning electron microscopy (SEM) image of a representative nanowire FET device; the nanowire is highlighted by the red arrow. Scale bar: 2 µm.
3.2 Results and Discussion

3.2.1 Implementation of electronics for syringe injection

The mechanical properties of the free-standing mesh electronics are important to the injection process. The basic mesh structure (Figs. 3.1d and 3.2a,b) consists of longitudinal polymer/metal/polymer elements, which function as interconnects between exposed electronic devices and I/O pads, and transverse polymer elements. The mesh longitudinal and transverse bending stiffness, $D_L$ and $D_T$, are determined by the mesh unit cell and corresponding widths and thickness of the longitudinal and transverse elements, and the angle, $\alpha$ (15, 16). A simulation of $D_L$ and $D_T$ as a function of $\alpha$ (Fig. 3.1e) shows that $D_T$ ($D_L$) decreases (increases) as expected for increasing $\alpha$. These results show that increasing $\alpha$ facilitates bending along the transverse direction (reduced $D_T$) and could allow for rolling-up of the mesh electronics within a needle constriction, while at the same time reducing bending and potential buckling along the injection (longitudinal) direction through an increase in $D_L$.

The mesh electronics were fabricated (details see, Chapter 3.4.2 and Fig. 3.3) and fully-released from substrates using reported methods (15, 16), and were then loaded into glass needles (Fig. 3.4a). Images of the injection of a 2 mm wide mesh electronics sample through a 95 μm inner diameter (ID) glass needle show the compressed mesh ca. 250 μm from the needle opening (Fig. 3.1f), and then injected ca. 0.5 cm into 1X Phosphate-buffered saline (PBS) solution (Fig. 3.1g), where the latter 3D reconstructed confocal fluorescence image highlights the unfolding of the mesh structure from the point of the needle constriction (blue dashed box). Higher resolution images from the region around the needle and several millimeters into solution (Fig. 3.5a,b) show clearly the continuity of the mesh structure as it unfolds in solution. Similar results were also obtained for injection of a 1.5 cm overall width mesh electronics through a 20
gauge (600 μm ID) metal needle (Fig. 3.5c) demonstrating generality of our approach for injection through common glass and metal syringe needles.

Figure 3.2. Structure of injectable electronics. (a) Schematic of an injectable mesh electronics structure. The red-orange lines highlight the overall mesh structure and indicate the regions of supporting and passivating polymer mesh layers, the ca. horizontal black lines indicate metal interconnects between input/output (I/O) pads (black filled circles) and recording devices (blue filled circles). The red dashed box highlights two different types of devices—either electrochemical or field-effect transistors (FETs) (insets, Fig. 3.1i,j), the green dashed box highlights mesh network metal interconnects, and the black dashed box highlights I/O pads. (b) shows a schematic (upper panel) of the zoomed-in region of mesh network highlighted by green dashed box in (a), where black horizontal lines correspond to metal interconnects, red-orange lines highlight the transverse polymeric elements of the mesh. Lower panel corresponds to a single unit cell of the mesh (green box in upper panel) with the same color nomenclature as in (upper panel). The unit cell (white dashed lines) is defined by the following parameters: $L_1$ and...
Figure 3.2 (Continued): $L_2$ are the unit lengths in the longitudinal and transverse directions, respectively; $w_1$ and $w_2$ are widths of the longitudinal and transverse mesh elements, respectively; and $w_m$ is the width of metal interconnect lines. (e) Optical image of an injectable mesh electronics structure unfolded on a glass substrate. $W$ is the total width of the mesh electronics. The red dashed polygon highlights the position of metal or nanowire FET sensor devices (insets, Fig. 3.1i,j). Metal interconnect lines (green dashed box) and metal I/O pads (black dashed box) are shown in detail in (d) and (e) respectively. (d) Bright field microscope image of the mesh structure (green dashed box in (e)) showing metal interconnects (horizontal, golden color lines), and polymer structural/passivation elements (greenish color lines). (e) Bright field microscopy image from region of the black dashed box in (e) showing metal I/O pads (golden colored filled circles) supported by polymeric structural elements (greenish features).

Figure 3.3. Schematics of mesh electronics fabrication. Components include silicon wafer (cyan), nickel relief layer (purple), SU-8 polymer ribbons (red), metal interconnects (black) and
Figure 3.3 (Continued): exposed metal electrodes (green). For each step a top-view and side-view are shown, where the side-view corresponds to a cross-section taken at ca. the midpoint of top-view image and indicated by the dashed white line in (a); the cross-section image (right panel) of (g) corresponds to the position of the black dashed line in the top view (left panel). The polymer ribbons defined by photolithography were 5-20 µm in width and 350-400 nm in thickness for each layer. The metal interconnects defined by thermal evaporator are Cr/Au, 2-10 µm in width and 5/100 nm in thickness. The exposed metal sensor electrode was Pt with 20 µm diameter and 50 nm thickness. Refer to Chapter 3.4 for details of the fabrication steps.

To test further the electrical continuity and functionality of the mesh electronics post-injection, we used anisotropic conductive film (ACF) (19) to connect the I/O pads of the mesh electronics post-injection to flexible cables that are interfaced to measurement electronics (Fig. 3.6a-d). Measurements of the change in electrical performance and yield of devices following injection into 1X PBS solution through ca. 100-600 µm ID metal needles (Fig. 3.1i,j) highlight several key points. First, the average device yield for metal electrochemical devices (Fig. 3.1i), which each uses a single ca. 3 cm long metal interconnect line from I/O pad to device end, was greater than 94%. In addition, the average device impedance, which represents an important characteristic for voltage sensing applications (20, 21), changed <7% post injection (Fig. 3.1i). Second, measurements of the yield of silicon nanowire FET devices, which each requires two ca. 3 cm long metal interconnect lines, was >90% for needle IDs from 260 to 600 µm and only dropped to 83% for the smallest 100 µm ID needles (Fig. 3.1j). The FETs also showed <12% conductance change on average post injection (Fig. 3.1j). Taken together, these results demonstrate the robustness of our mesh electronics design and the capability of maintaining good device performance following injection through a wide-range of needle IDs.
Figure 3.4. Loading and injection of mesh electronics. Schematics illustrating method used to load the mesh electronics into a glass needle and subsequently inject into a medium. (a) The tip of the glass needle (blue) was connected to a syringe by a plastic tube (pink). The injectable mesh electronics (yellow) suspended in solution (green) was pulled into the needle from the large end of the glass needle such that the device end of the mesh enters the tube first. Black and blue dots represent the I/O pads and devices on the mesh electronics, respectively, where the two ends can be readily distinguished optically during the loading process. (b) After the mesh electronics was loaded into the glass needle, the tubing was removed from the needle end and placed over the large end of the glass tube, and then the syringe was used to ‘push’ the mesh to the tip (inset, (b)). (c) The glass needle was mounted in the x-y-z manipulator for injection into solution, gel/polymer or tissue (red). Red arrows indicate the direction of the fluid flow during loading and injection.
Figure 3.5. Injection of mesh electronics into aqueous solution. (a) and (b) Images showing the mesh electronics was injected into 1X PBS solution by a glass needle with ID of 95 μm corresponding to the 3D reconstructed fluorescence image in Fig. 3.1g. Bright-field microscopy images show the mesh electronics with limited unfolding structure near the needle region (a) corresponding to the blue dashed box highlighted region in Fig. 3.1g and completely unfolding structure (b) corresponding to the white dashed box highlighted region in Fig. 3.1g. (c) Optical image of a 15 mm total width mesh electronics partially injected through a 20 gauge (ID = 600 μm) needle into 1X PBS solution. The device end of the mesh (lower-left to the middle of the image) is fully unfolded at this stage of injection with metal interconnect lines visible in the reflected light. The dashed red line corresponds to the air/solution interface, with the needle tip ~3 mm below the interface (upper-right of the image). The steps used for loading mesh electronics when injecting through metal needles are as follows: (i) The suspended mesh electronics was loaded into a glass pipette starting from the I/O end; (ii) The mesh electronics was then transferred to the syringe from plunger side with device region close to the needle/syringe connection and the mesh extended in the longitudinal (injection) direction. At this point, the mesh electronics could be injected into the desired medium or structure through the metal needle. The mesh electronic structural properties are summarized in Table 3.1, entry-1.
We have characterized the structures of different mesh electronics within glass needle-like constrictions to understand design parameters for successful injection. A schematic (Fig. 3.7a,b) highlights our approach in which a pulled glass tube with controlled ID central constriction is positioned under a microscope objective for bright-field and confocal fluorescence imaging, and the mesh electronics are injected partially through the constriction. Representative bright field microscopy images of mesh electronics with different structural parameters recorded from the central region of different ID glass channels (Fig. 3.7c) highlight two important features. First, mesh electronics with $\alpha = 45^\circ$ and total widths substantially larger than the constriction ID can be smoothly injected. Relatively straight longitudinal elements are seen in Fig. 3.7c, I and II, where the 5 mm 2D mesh widths are 11- and 20-times larger than the respective 450 and 250 μm ID needle constrictions. Second, indeed, even 1.5 cm width mesh electronics (Fig. 3.7c, III) can be injected smoothly through a 33-times smaller ID (450 μm) constriction. The density of longitudinal and transverse elements in the image makes it more difficult to trace through the needle, although approximately straight longitudinal elements can still be seen.
Figure 3.6. Flexible cable to I/O bonding. Schematic (a) and corresponding photograph (b) of the overall process of bonding a flexible cable to the I/O pads of the mesh electronics. In both the schematic and photograph, the flexible cable, anisotropic conductive film (ACF) and I/O region of the mesh electronics are indicated by (I), (II) and (III), respectively. (c) Photograph shows the flexible cable bonded to the I/O pads of the mesh electronics. The red arrow indicates the direction of flexible cable connection to the external hardware. (d) Photograph shows another end of the flexible cable connected to the amplifier for electrical recording. Red arrow indicates the direction of flexible cable connection to the I/O pads. (e) Connection resistance of the cable/ACF/mesh bonded by a commercial flip-chip bonder (red) and home-made bonder (blue). (f) Histogram summary of the connection resistance data in (e) showing the average value ±1 standard deviation (1SD).
Figure 3.7. Imaging mesh electronics structure in needle constrictions. (a) Schematic illustrating the structure of a pulled glass tube (blue) with mesh electronics passing from larger (left) to smallest (center) ID of tube, where the red arrow indicates the direction of injection and x-y-z axes indicate coordinates relative to the microscope objective for images in (c) to (e). (b) Schematic of the mesh structure from the region of the constriction indicated by the blue dashed box in (a). (c) Bright-field microscopy images of different design mesh electronics injected through glass channels. (I) and (II), total width, $W = 5$ mm, $\alpha = 45^\circ$ mesh electronics injected through 450 and 250 $\mu$m ID, respectively, glass channels. (III) $W = 15$ mm, $\alpha = 45^\circ$ mesh electronics injected through a 450 $\mu$m ID glass channel. (IV) $W = 10$ mm, $\alpha = 0^\circ$ mesh electronics injected through a 450 $\mu$m ID glass channel. The injection direction is indicated by red arrows in the images; the orientation relative to the axes in (a) are indicated in (I) and the same for panels (I) to (IV). (d) 3D reconstructed confocal images from the dashed red box.
**Figure 3.7 (Continued):** regions in the respective panels (I) to (IV) in (c); the x-y-z axes in (I) are the same for panels (II) to (IV). Horizontal, small white arrows in (c) and (d) indicate several of the longitudinal elements containing metal interconnects in the mesh electronics. (e) Cross-sectional images plotted as half cylinders from positions indicated by the vertical white dashed lines in (d). The white dashed curves indicate the approximate IDs of the glass constrictions.

Further insight into mesh electronics injection was obtained from higher-resolution fluorescence confocal microscopy images (see Chapter 3.4.4.2) recorded at the same time as the above bright-field microscopy images. The corresponding 3D reconstructed confocal images of $\alpha = 45^\circ$ mesh electronics samples with mesh width/constriction ID ratios from 11 to 33 (Fig. 3.7d, I-III) highlight several important points. First, the longitudinal elements maintain a straight geometry without substantial bending through the constriction even for the 33:1 width: ID ratio (Fig. 3.7d, III). Second, these images show that the transverse element bend with a curvature that appears to match the needle ID. This latter point and further structural details can be seen clearly in cross-sectional plots of these 3D images (Fig. 3.7e, I-III), which show that all of the transverse and longitudinal elements are uniformly organized near the ID of the glass constriction in tubular structures. Third, there is no evidence for fracture of $\alpha = 45^\circ$ design mesh elements in these images. Indeed, simulations of the strain versus needle ID show that upper limit strain value for the mesh in a 100 $\mu$m ID needle, $\sim 1\%$, is less than the calculated critical fracture strain (see Chapter 3.4.5.2.3). Last, from a series of cross-sectional images we can identify the longitudinal elements and estimate the number of rolls that these mesh electronics make at the glass needle constriction are $3.4 \pm 0.2$, $6.0 \pm 0.4$ and $9.5 \pm 1.0$ for Fig. 3.7e, I-III, respectively, which are comparable to a geometric calculation assuming that the 2D meshes roll up inside the different ID channels (see Chapter 3.4.5.1).
Figure 3.8. Injection of thin film electronics into needle-like constrictions. (a) Bright-field microscopy image of an attempted injection of a 5 mm total width thin film electronics sample through a 400 μm ID glass channel (I), and a 1.5 mm wide thin film electronics being injected through a 350 μm glass channel (II). Red arrows indicate the direction of injection. The continuous thin films have the same polymer and metal thicknesses as the mesh electronics. White arrows in (I) indicate the ends of metal lines where the thin film ‘jams’ and cannot pass through the needle. (b) 3D reconstructed confocal fluorescence microscopy images recorded from the regions in (a) highlighted by the dashed red boxes. (c) Cross-sections of through images in (b) at the positions of the vertical white dashed lines. The white dashed circles in (c) indicate the approximate IDs of the glass constrictions. The coordinate axes for images are shown in (I) of (a) to (c) and refer to the schematic for imaging in Fig. 3.7a. They are the same for (II).

In addition, we have investigated mesh electronics injection as a function of the fluid flow rate for a constant 400 μm ID needle. Significantly, we found smooth mesh electronics injection for flows from 20 – 150 mL/hr as long as the needle retraction speed matched the speed
of the injected fluid. The lower limit for smooth injection, 20 mL/hr, is believed to be restricted by the smallest fluid drag force relative to the friction force between the rolled-up mesh electronics and the inner needle surfaces. The maximum flow, 150 mL/hr, was limited by the needle retraction speed of our set-up.

![Figure 3.9. Calculated mesh electronics strain versus needle size.](image)

**Figure 3.9. Calculated mesh electronics strain versus needle size.** Plot of the upper bound for the strain of the mesh as a function of the inverse needle radius, 1/R. Simulations were made for single unit cell. The black and red circles represent meshes with 20 and 10 µm width transverse ribbons, respectively. The black and red lines correspond to linear fits to the respective simulation points. The inset is a representative simulation shows the strain distribution of one unit cell in a 200 µm ID needle. The red dashed circle highlights the point with highest maximum principle strain. Black dashed circle and black arrow show the inner boundary and radius of the needle, respectively.

### 3.2.2 Injection of electronics into synthetic structures

We have investigated several applications of our syringe injectable electronics, including delivery of electronics to internal regions of man-made structures and live animals. First, mesh electronics incorporating addressable silicon nanowire piezoresistive strain sensors (16) were co-injected with polymer precursors through a small injection site ([Fig. 3.10a, Chapter 3.4.3.5.1](#)).
into poly-dimethylsiloxane (PDMS) cavities, with the I/O pads ejected outside the structure. Visual inspection, micro-computed tomography (µCT) images and photographs (Figs. 3.10b and 3.11b) demonstrate that the mesh electronics unfolds and smoothly follows the internal cavity structure with continuous metal interconnects.

Figure 3.10. Syringe injection of mesh electronics into 3D synthetic structures. (a) Schematic of a mesh electronics injected with uncured PDMS precursor into a PDMS cavity
Figure 3.10 (Continued): (blue) with I/O pads unfolded outside the cavity. The injected PDMS precursors were cured after injection. The red lines highlight the overall mesh structure and indicate the regions of supporting and passivating polymers and the yellow lines indicate the metal interconnects between I/O pads (yellow filled circle) and devices (dark blue filled circle). (b) μCT image shows the zoomed-in structure highlighted by the black dashed box in (a) and Fig. 3.11b. False colors were applied with metal lines (yellow) in PDMS (purple). (c) 4 nanowire devices response to pressure applied on the PDMS. The blue downward and upward pointing triangles denote the times when the strain was applied and released, respectively. The purple downward and upward arrows show the tensile and compressive strains, corresponding to the minus and plus change of conductance, respectively. (d) to (f) (upper images) 3D reconstructed μCT images of a mesh electronics injected into 75% Matrigel™ after incubating for 0 h (d) 24 h (e) and 3 weeks (f) at 37 °C. The x-y-z axes are shown in (d) and the same for panels e and f, where the injection direction is ca. along the z-axis. In (d) to (f) false colors were applied with metal lines in the mesh (yellow) and the Matrigel™ (purple) (lower images). Corresponding cross-section images at z = 10 mm with 500 µm thicknesses; the positions of the cross-sections are indicated by white dashed lines in the upper images. The maximum extent of mesh electronics unfolding was highlighted by white dashed circles with diameter, D, in each image. (g) Time dependence of mesh electronics unfolding following injection into 25% (black), 75% (red) and 100% (blue) Matrigel™; the measured diameter, D, was normalized by the 2D width, W, of the fabricated mesh electronics. D was sampled from five cross-sections taken at z =5, 7.5, 10, 12.5 and 15 mm to obtain the average ± 1SD.

We monitored the response of the internal silicon nanowire piezoresistive strain sensors as the PDMS structures were deformed. Plots of strain recorded simultaneously from four typical calibrated devices (Fig. 3.10c, d1-d4) versus deformation with a point load along the z axis shows compressive (d1, d3) and tensile (d2, d4) local strains recorded by the nanodevices. Mapping the strain response onto the optical image of the electronics/PDMS hybrid shows the nanowire sensors separated by up to 4 mm (Fig. 3.11c), with the compressive and tensile strains consistent with expectation for the point-like deformation. These data suggest that syringe
injection of mesh electronics with piezoresistive devices could be used to monitor and map internal strains within structural components with gaps/cracks in a manner that is not currently possible, and more generally, to simultaneously monitor corrosion and strain within internal cavities or cracks by using nanowire devices to also measure pH and other chemical changes (22).

Second, we investigated 3D gel structures without cavities as representative models of mesh electronics injection into soft materials and biological tissue. Images recorded versus time following the injection mesh electronics into Matrigel™, a scaffold widely used in neural tissue engineering (Fig. 3.10d-f) (23), show that the mesh unfolds ~80% in the radial direction over a three-week period at 37 ºC. As expected, the degree of unfolding of the mesh electronics within the Matrigel depends on the gel concentration for fixed mesh mechanical properties (Fig. 3.10g), with ~90 and 30% unfolding for 25 and 100% Matrigel™, respectively. The ability to inject and observe partial unfolding of the electronics within gels with tissue-like properties also suggests that co-injection with other biomaterials (24) and/or cells (25) could be another application of injectable mesh electronics. Indeed, preliminary experiments show that co-injection of mesh electronics and embryonic rat hippocampal neurons into Matrigel™ leads to 3D neural networks with neurites interpenetrating the mesh electronics (Fig. 3.11d,e). These co-injection results highlight potential opportunities for tissue engineering and stem cell therapy (25).
Figure 3.11. Injection of mesh electronics into 3D cavities and gels. (a) Photograph of a typical polydimethylsiloxane (PDMS) cavity before injection, where the cavity has a stepped internal structure. (b) shows the hybrid structure of a mesh electronics embedded in a similar PDMS internal cavity after injection. The black dashed box highlights the region where the micro-computed tomography (µCT) image shown in Fig. 3.10b was recorded. (c) Image of the mesh electronics injected and unfolded within a PDMS cavity. The positions of the four nanowire sensors are indicated by d1, d2, d3 and d4. The strain field was applied to the PDMS/mesh electronics hybrid at the position indicated by white vector arrow. The strain field was color mapped (relative to the point force was applied) for the nanowire strain sensors using the calibration of the nanowire devices corresponding to the data in Fig. 3.10c. The detected strains are labeled in the PDMS/mesh electronics microscopic image at the device positions. (d) Schematic of the co-injection of mesh electronics with cells. The red lines highlight the overall mesh structure and indicate the region of supporting and passivating polymers and the yellow lines indicate the metal interconnects for the devices (blue filled circles). Green spheres highlight
Figure 3.11 (Continued): the structure of cells co-injected with mesh electronics. (e) Projection of 3D reconstructed confocal image from 100 μm thick, 635 μm long and 635 μm wide volume shows the interpenetration between neurons and mesh structure of injectable electronics after co-injected into Matrigel™ for 14 days. The red and green colors in this correspond to SU-8 and β-tubulin, respectively.

3.2.3 Injection of mesh electronics into brains of live animals

Finally, we investigated the behavior of mesh electronics stereotaxically injected into the lateral ventricle (LV) and hippocampus (HIP) regions of live rodents (Fig. 3.12a-d and Chapter 3.4.3.6), where the capability to deliver electronics with width on the scale of millimeters through needles with outer diameter (OD) on the scale of hundreds of micrometers allows for a much smaller window in the skull than the usual width of electronics, thereby reducing the invasiveness of surgery. Confocal microscopy images recorded from tissue slices from the LV region prepared five weeks post-injection of the mesh electronics (Figs. 3.12e-g and 3.13) demonstrate several important points. First, the mesh electronics relaxes from the initial ~200 μm injection diameter to bridge the caudoputamen (CPu) and lateral septal nucleus (LSD) regions, which define the boundaries of the cavity in this slice (Fig. 3.12e). Second, higher-resolution images from the boundary between the electronics and the CPu/subventricular zone (Figs. 3.12f and 3.13a) show that the mesh electronics interpenetrates with the boundary cells and that cells stained with neuron marker NeuN associate tightly with the mesh. Third, control images recorded from the same tissue slice but the opposite hemisphere (without injected mesh electronics; Fig. 3.13b,d-f) show that the level of glial fibrillary acidic protein (GFAP) expression is similar with and without the injected mesh electronics, indicating little chronic tissue response to the mesh electronics. Fourth, images recorded of the mesh electronics in the middle of the LV (Figs. 3.12g and 3.13c) show a large number of 4', 6-diamidino-2-phenylindole
DAPI stained cells bound to the mesh structure. These images indicate that (i) the mesh expands to integrate within the local extracellular matrix (that is, the mesh is neurophilic), (ii) cells form tight junctions with the mesh, and (iii) neural cells migrate hundreds of micrometers from the subventricular zone along the mesh structure (27). Notably, these results suggest using injectable electronics to mobilize and monitor neural cells from the LV region following brain injury (28) as well as delivering mesh electronics to other biological cavities for recording and stimulation.

We also injected mesh electronics into the dense tissue of the HIP (Fig. 3.12d). Bright-field images of coronal tissues slices, prepared five weeks post-injection (Figs. 3.12h and 3.14a,b), demonstrate that the electronics is fully extended in the longitudinal direction. The mesh only relaxes a small amount with respect to the initial injection diameter (red dashed lines in Fig. 3.12h), because the force required to bend the mesh (16) is comparable to the force needed to deform the tissue (23, 29). In addition, an overlay of bright-field and DAPI epifluorescence images (Fig. 3.12i) shows that the injection did not disrupt the CA1 and dentate gyrus (DG) layers of this region. The confocal microscopy images (Figs. 3.12j, 3.14, 3.15 and 3.16) highlight several unique characteristics of the injectable mesh electronics in dense neural tissue. First, analysis of GFAP fluorescence shows that there is a limited or no astrocyte proliferation near the mesh, although the full image (Fig. 3.12j) indicates a reduction in cell density at the central region of injection. Significantly, analyses of similar horizontal slice samples prepared from three independent mesh injections (Figs. 3.15 and 3.16a,b) also show limited or no astrocyte proliferation around our electronics, and quantitative analyses (Figs. 3.15b II and 3.16b) demonstrate that GFAP values versus distance from and along the mesh electronics surface are similar to background level. Second, these images show many healthy
neurons (NeuN signal) surrounding and close to the SU-8 ribbons of the mesh (Figs. 3.12j, 3.14c and 3.16c). Data and analyses from three independent samples (Figs. 3.15 and 3.16d I) quantitatively support this observation. Specifically, quantitative analyses (Figs. 3.15b I and 3.16d) demonstrate that the NeuN signals versus distance from and along the mesh electronics surface are enhanced or similar to background levels. These observations, which are similar to our results for injections into the LV, show the capability of the mesh electronics to promote positive cellular interactions, in contrast to the reported chronic responses of neural tissue following the insertion of silicon (30), metal (13), polyimide (31)/SU-8 (32), and ultrasmall carbon (33) electrical probes, which reduced the neuron density and enhanced astrocyte density near the probes/tissue interface.

We attribute the unique biocompatibility of our syringe-injectable mesh electronics to their ultra-small bending stiffness and micrometer-scale features. The bending stiffness of the injected mesh electronics (0.087 nN·m) is four to six orders of magnitude smaller than the values reported for previous implantable electronics, such as silicon probe (4.6 × 10^5 nN·m) (30, 34), carbon fibers (3.9 × 10^4 nN·m) (33) and thin-film electronic probes (0.16-1.3 × 10^4 nN·m) (12, 13, 35) (Chapter 3.4.5.2.2). The flexibility of the injected electronics is closer to the flexibility of the tissue, which has been demonstrated to minimize mechanical trauma caused by the relative motion between the probe and surrounding tissue (33, 35). In addition, the feature sizes of our injected mesh electronics (5-20 µm) are less than or equal to those of single cells, which can also reduce chronic damage, even when the probe stiffness is much greater than that of the tissue (33).
Figure 3.12. Syringe injectable electronics into *in vivo* biological system. (a) Schematic shows *in vivo* stereotaxic injection of mesh electronics into a mouse brain. (b) Optical image of the stereotaxic injection of mesh electronics into an anesthetized 3 months old mouse brain. (c,d)
Figure 3.12 (Continued): Schematics of coronal slices illustrating the two distinct areas of the brain that mesh electronics were injected through the cerebral cortex (CTX) into the lateral ventricle (LV) cavity adjacent to the caudoputamen (CPu) and lateral septal nucleus (LSD) (c) and through the CTX into the hippocampus (HIP) (d). Red lines highlight and indicate the overall structure of mesh and dark blue filled circles indicate recording devices. The blue dashed line in (e) indicates the direction of horizontal slicing for imaging. (e) Projection of 3D reconstructed confocal image from 100 µm thick, 3.17 mm long and 3.17 mm wide volume horizontal slice 5 weeks post-injection at the position indicated by blue dashed line in (e). Red dashed line highlights the boundary of mesh inside LV, and the solid red circle indicates the size of the needle used for injection. The red, green and blue colors in this correspond to GFAP, NeuN/SU-8 and DAPI, respectively, and are denoted at the top of the image panel in this and subsequent images. (f) 3D reconstructed confocal image from the dashed red box in Fig. 3.11a at the interface between mesh electronics and subventricular zone (SVZ). (g) 3D reconstructed confocal image from dashed red box in Fig. 3.11c at the ca. middle (of x-y plane) of the LV in the slice. (h) Bright-field microscopy image of a coronal slice of the HIP region 5 weeks post-injection of the mesh electronics at the position indicated in schematic (d). Red dashed lines indicate the boundary of the glass needle. The white arrows indicate longitudinal elements that were broken during tissue slicing. Black dashed lines indicate the boundary of each individual image. (i) Overlaid bright field and epi-fluorescence images from the region indicated by white dashed box in (h). Blue corresponds to DAPI staining of cell nuclei, white arrows indicate CA1 and dentate gyrus (DG) of the HIP. (j) Projection of 3D reconstructed confocal image from 30 µm thick, 317 µm long and 317 µm wide volume from the zoomed-in region highlighted by the black dashed box in (i). (k) Acute in vivo 16-channel recording using mesh electronics injected into a mouse brain. The devices were Pt-metal electrodes (impedance ~950 kΩ at 1 kHz) with their relative positions marked by red spots in the schematic (left panel), and the signal was filtered with 60 Hz notch during acquisition. The dashed red rectangle indicates the part for spatiotemporal mapping of multichannel-LFP recordings (Fig. 3.16d) (l) Superimposed single-unit neural recordings from one channel after 300-6000 Hz band-pass filtering. The red line represents the mean waveform for the single-unit spikes.
Figure 3.13. Chronic histology of interface between mesh electronics and subventricular zone of mouse brain. Mesh electronics were injected into the LV as shown schematically in Fig. 3.12c and the animals were returned to the animal facility. Five weeks (post injection) mice were euthanized and their brain tissue was fixed and sliced for staining and imaging. (a) Projection of 3D reconstructed confocal image from 30 µm thick, 635 µm long and 635 µm wide volume at the region indicated by the dashed white box in Fig. 3.12e. The dashed red box corresponds to the higher-resolution image shown in Fig. 3.12f. In this and following images: blue corresponds to DAPI staining of cell nuclei, green represents SU-8 ribbons and NeuN staining of neurons and red highlights GFAP staining of astrocytes. (b) Control image recorded from the LV of the opposite hemisphere without injected mesh electronics. The tissue slice was the same as used to obtain data panels (a) and (c). (c) Projection of 3D reconstructed confocal microscopy image 80 µm thick, 635 µm long and 635 µm wide volume for the region highlighted by the white box in Fig. 3.12e. The dashed red box indicates the higher-resolution image shown in Fig. 3.12g. (d) and (e) GFAP fluorescence channels from (a) and (b) respectively. (f) the integral of fluorescence intensity (mean ± 1SD) along the interface between the electronics and subventricular region (d) was analyzed and compared with that in control sample (e). The fluorescence intensity was averaged and analyzed for 20 µm × 20 µm regions (n=10) along the interface between mesh electronics/subventricular zone and the natural boundary of subventricular zone in the control sample.
Figure 3.14. Chronic imaging of mesh electronics injected into the hippocampus. (a) Composite bright field image of a mesh electronics injected through the mouse cortex into the HIP. The mesh electronics was fully extended along the injection/longitudinal direction and partially unfolded in the transverse direction. Black dashed lines indicate the boundary of each image. (b) Image from the white dashed box in (a) shows the HIP region of the hemisphere opposite to the hemisphere where the injection was carried out. (c) Projection of 3D reconstructed confocal microscopy image from dashed red box in Fig. 3.12j. Mice were euthanized 5 weeks post injection, and then brain tissue was fixed and sliced for staining and imaging.
Figure 3.15. Chronic histology of the interface between mesh electronics and the hippocampal region of a mouse brain. (a) Projection of a 3D reconstructed confocal image from 10 µm thick, 1.2 mm long and 1.2 mm wide volume at the region indicated by the dashed blue line in Fig. 3.12d. The dashed red box indicates the position of the injected mesh electronics. Blue corresponds to DAPI staining of cell nuclei, yellow represents SU-8 ribbons, green represents NeuN staining of neurons and red highlights GFAP staining of astrocytes. White dashed line highlight the region and direction where the fluorescence signals were averaged and analyzed for comparison of NeuN, GFAP and SU-8 signal intensities. (b) Fluorescence intensity plotted corresponding to the region in (a) where fluorescence intensity in each channel in 70 µm × 1600 µm area was averaged and normalized. (I), (II) and (III) corresponds to signals from NeuN, GFAP and SU-8 channels, respectively. Solid lines and shaded areas represent average intensities and s.e.m. values, respectively. The normalized factors are 47 for NeuN signal, 27 for GFAP signal and 48 for SU-8 signals (i.e., the NueN and SU-8 raw signals are ca. 2× larger than the GFAP signal).
Finally, we verified the ability of the injected mesh electronics to record brain activity in the HIP of anaesthetized mice (Figs. 3.12k,1 and 3.17). Representative multichannel recordings using mesh electronics with Pt-metal electrodes (Fig. 3.12k) yielded well-defined signals in all 16 channels. The modulation amplitude (200-400 μV) and dominant modulation frequency (1-4 Hz) recorded are characteristic of δ-wave local field potentials (LFPs) in anesthetized mouse. Moreover, spatiotemporal mapping of the LFP recordings (Fig. 3.17d) reveals a characteristic HIP field activity for the rodent brain (36, 37). Standard analysis (see Chapter 3.4) of the sharp downward spikes (Fig. 3.12l) showed a uniform potential waveform with average duration of ~2 ms and peak-to-peak amplitude of ~70 μV characteristic of single-unit action potentials (33). In the context of long-term chronic recording, our histology results and previous work (12, 13, 15) demonstrate the biocompatibility and long-term stability of using SU-8 passivated interconnects, and the long-term stability of metal oxide passivated silicon nanowire sensors (15, 40). Hence, we believe these results, together with the ‘neurophilic’ chronic response, offer substantial promise for implantation and long-term brain activity mapping (41).
Figure 3.16. GFAP and NeuN density vs. distance from and along implanted mesh electronics. (a) Confocal microscopy fluorescence images of GFAP from injectable mesh electronics-1 sample. Red and yellow colors correspond to GFAP and SU-8 structures from mesh electronics, respectively. Solid white arrows highlight the ribbons from mesh electronics. (II) and (III) are zoomed-in higher resolution images corresponding to the green and yellow dashed boxes, respectively, in (I). (b, I) GFAP fluorescence intensities and standard error of the mean (s.e.m.) 4 weeks post implantation versus distance from the interface of two different injected mesh electronics samples. The fluorescence intensity was normalized with the background value for each sample (value = 1, green dashed line) for comparison. (b, II) Normalized fluorescence intensity of GFAP (red points/line) and s.e.m. plotted along the outer curved surface of the mesh
Figure 3.16 (Continued): electronics highlighted by the white dashed box in (II); the normalized background fluorescence intensity level was determined from (III) (480 µm away from the electronics surface). The direction of GFAP plot is top to bottom along the curved surface (white dashed arrow in (II)). (c) Confocal fluorescence image of mesh electronics injected into the hippocampus (Fig. 3.12j), where blue, green and red correspond to nuclei, NeuN and SU-8 auto-fluorescence, and GFAP. The yellow box highlights the neuron region analyzed in (d, I). NeuN fluorescence intensity and s.e.m. 4 weeks post implantation versus distance from the interface of two different injected mesh electronics samples. Fluorescence intensity was normalized to the background value for each sample, where normalized background has value of 1. (d, II) Normalized fluorescence intensity and s.e.m. of NeuN plotted along the probe surface highlighted by the white dashed box in (c), and the normalized background fluorescence intensity level (black dashed line, value = 1) from red dashed box in (c). The white dashed arrow in c highlights the direction of plots in (d, II). The red box/background region in (c) represents a higher background than that obtained from averaging the entire image, and thus yields a lower limit on enhanced normalized NeuN signal at the injected mesh probe surface. All the data were analyzed by Matlab.
3.3 Conclusion

In summary, we have introduced a new strategy for delivering electronics to the internal regions of 3D man-made and biological structures that involves the syringe injection of submicrometer-thickness, large-area macroporous mesh electronics. We have shown that mesh electronics with widths more than 30 times the needle ID can be injected and maintain a high yield of active electronic devices. *In situ* imaging and modeling show that optimizing the transverse and longitudinal stiffness enables the mesh to ‘roll-up’ when passing through needle constrictions. We have demonstrated that injected mesh electronics with addressable piezo-resistive devices are capable of monitoring internal mechanical strains within bulk structures, and have also shown that mesh electronics injected into the brains of mice exhibit little chronic immunoreactivity, attractive interactions with neurons, and can reliably monitor brain activity. Compared to other delivery methods (9-18), our syringe injection approach allows the delivery of large (with respect to injection opening) flexible electronics into cavities and existing synthetic materials through small injection sites and relatively rigid shells. In the future, our new approach and results could be extended in several directions, including the incorporation of multifunctional electronic devices (7, 13, 16, 42) and/or wireless interfaces (13, 43) to further increase the complexity of the injectable electronics. Additionally, recent reports (42, 44-46) have demonstrated that novel electrophysiological recordings enabled by nanoelectronic units require an intimate nanoelectronics/cellular interface. In this emerging direction, our syringe-injectable electronics could serve as a unique yet general platform for building direct neuron-nanoelectronics interfaces for a variety of nanoelectronic units in *in vivo* studies. Finally, syringe injection brings the opportunity to co-inject mesh electronics with a polymer precursor or cells into host systems for unique engineering and biomedical applications.
Figure 3.17. Acute in vivo recording with mesh electronics. (a) The mouse was fixed in the stereotaxic frame, its skin was retracted and a hole was drilled through the skull plate and a ceramic scaffold (black arrow) was placed on top of the mouse skull with opened centered over the hole in the skull. The mesh electronics was injected into the mouse brain through a glass needle, where the red arrow indicates the position of the glass needle. (b) A flexible flat cable was bonded to the mesh I/O pads, where the blue arrow indicates the unfolded I/O region of the mesh electronics. (c) Electrophysiological recording, where black and blue arrows indicate Ag/AgCl reference electrode and unfolded I/O region of the mesh electronics, respectively. (d) Map of the acute in vivo mouse brain recordings from the region in Fig. 3.12k highlighted by the dashed red box. The y-axis represents the depth beneath the brain surface of each sensor electrode whose spatial distribution is shown in Fig. 3.12k. The x-axis shows the recording time. Colors indicate the amplitude of the recorded LFP amplitudes.
3.4 Methods and Materials

3.4.1 Mesh and control structure designs

3.4.1.1 Open mesh electronics

The overall structure and relevant parameters of the macroporous mesh electronics are illustrated in Fig. 3.2. The key design and fabrication parameters are as follows: \(W\), the total mesh width; \(w_1\), width of longitudinal ribbons along injection/long axis of mesh, \(w_2\), width of transverse ribbons, that cross and connect to the longitudinal ribbons with an angle, \(\alpha\), relative to the longitudinal ribbons; \(L_1\), the mesh unit cell length in the longitudinal direction; \(L_2\), the mesh unit cell length in the transverse direction; and \(w_m\), the width of metal lines, which run along the longitudinal ribbons. The longitudinal and transverse ribbon widths ranged from 5-40 µm, and \(\alpha\) was 45 or 0°. The embedded metal (SU-8/metal/SU-8) interconnects run along longitudinal ribbons; the metal contacts to nanowire transistor and bend-up passive metal sensors also have a metal line component embedded in the transverse ribbons. The specific parameters for injectable mesh electronics designs used in our studies are summarized as sample #1-6 in Table 3.1.

3.4.1.2 Thin film electronics

Control samples with the same thickness as the mesh electronics but comprising a standard flexible thin-film structure were also designed and fabricated. The metal line patterns, thickness and widths are the same as design #1 of tilted mesh electronics (Table 3.1). The overall widths, \(W\), of thin film electronics were 0.1-5 mm. The parameters of the thin film electronics designs used in our studies are summarized as samples #7 and 8 in Table 3.1.
Table 3.1. Dimensions of mesh electronics. Samples #1-3 are mesh electronics used for imaging and injection experiments. Samples #4-5 are mesh electronics for \textit{in vivo} experiment. Samples #6, and #7-8 are control rectangular mesh and flexible thin-film samples, respectively.

<table>
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<th>No.</th>
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<th>(w_1) (µm)</th>
<th>(w_2) (µm)</th>
<th>(L_1) (µm)</th>
<th>(L_2) (µm)</th>
<th>(w_m) (µm)</th>
<th>(\alpha) (°)</th>
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3.4.2 Free-standing mesh electronics fabrication

3.4.2.1 Initial fabrication steps

The overall fabrication of the syringe injectable electronics is based on methods described previously (15, 16). Key steps (Fig. 3.3) are as follows: \(i\) 100 nm nickel metal, which serves as a final relief layer, was deposited on the silicon fabrication substrate (600 nm SiO2, n-type 0.005 \(\Omega\cdot\text{cm}\), Nova Electronic Materials, Flower Mound, TX) by thermal evaporation; \(ii\) A 300 to 400 nm layer of SU-8 photoresist (2000.5; MicroChem Corp., Newton, MA) was spin-coated on the fabrication substrate, prebaked (65 °C/2 min; 95 °C/2 min), and then \(iii\) patterned by photolithography to define the bottom SU-8 layer of the injectable mesh electronics structure. \(iv\) After post baking (65 °C/2 min; 95 °C/2 min), and developing by SU-8 Developer (MicroChem Corp., Newton, MA), the SU-8 pattern was cured at 180 °C for 20 min. At this point, either of two distinct types of device elements, silicon nanowire transistors or passive
metal electrodes, was integrated in the fabrication process; these are described separately, followed by common steps used to complete fabrication of the free-standing mesh electronics.

3.4.2.2. Nanowire transistor elements

(va) A 300 to 400 nm layer of SU-8 photoresist was deposited on the fabrication substrate, prebaked (65 °C/2 min; 95 °C/4 min), and then (vb) silicon nanowires3 were aligned on the SU-8 layer by contact printing as described previously4. (vc) Photolithography was used to define the nanowire device regions, and after post-baking (65 °C/2 min; 95 °C/2 min), the pattern was developed by SU-8 Developer washed with isopropanol (2 times, 30 s per wash) to remove nanowires outside of the device regions. (vd) The new SU-8 pattern was cured at 180 °C/20 min. (ve) Nanowire device element contacts were fabricated as described previously (16, 44). Briefly, the substrate was coated with 300 nm LOR 3A and 500 nm S1805 (MicroChem Corp., Newton, MA) double layer resist and patterned by photolithography. Sequential Cr/Pd/Cr (1.5/50–80/1.5 nm) metal layers were deposited by thermal evaporation followed by metal lift-off in Remover PG (MicroChem Corp., Newton, MA) to define the minimally-stressed nanowire contacts.

3.4.2.3 Metal electrode elements

(vi a) The substrate was spin-coated with LOR 3A and S1805 double layer resist with similar thicknesses as described above. (vi b) 20 μm diameter sensor pads (Cr/Pt, 5/50 nm) were defined by photolithography and electron beam evaporation followed by metal lift-off in Remover PG. (vi c) The substrate was then spin-coated with LOR 3A and S1805 double layer resist with similar thicknesses as described above again. (vi d) For sensors designed to bend-out from the mesh plane, nonsymmetrical Cr/Pd/Cr (1.5/50-80/30-50 nm) metal lines (200 μm long)
were patterned by photolithography and subsequent thermal deposition followed by metal lift-off in Remover PG.

3.4.2.4 Completion of free-standing mesh electronics fabrication

(vii) The substrate was coated with LOR 3A and S1805 double layer resist with similar thicknesses as described above and patterned by photolithography. Unstressed, symmetrical Cr/Au/Cr (1.5/50–100/1.5 nm) metal lines were sequentially deposited followed by metal lift-off in Remover PG to define the minimally stressed interconnects/address lines (16, 44). All metal lines were defined such that they are on top of and smaller in width than the SU-8 mesh pattern described in steps 1-5. (viii) A 300 to 400 nm layer of SU-8 photoresist was deposited on the fabrication substrate, pre-baked (65 °C/2 min; 95 °C/2 min), and then patterned by photolithography to match the lower SU-8 mesh structure and serve as top encapsulating/passivating layer of the metal contacts/interconnects (except for active device regions). The structure was post-baked, developed, and cured as described above. (ix) In the case of nanowire transistor devices, 300 and 500 nm thick layers of LOR 3A and S1805 photoresist were deposited and defined by photolithography to protect the device region during release of the mesh from the fabrication substrate. (x) The syringe injectable mesh electronics were released from the substrate by etching the nickel layer (40% FeCl3:39% HCl:H2O = 1:1:20) for 3 - 4 hours at 25 °C and then transferred to deionized (DI) water by glass pipette (5 mL, Disposable Pasteur Pipets, Lime Glass, VWR International, LLC, Radnor, PA). (xi) The photoresist protection was removed from nanowire device meshes by exposure to ultraviolet light (430 nm, 120 s) and immersion in developer solution (MF-CD-26, MicroChem Corp., Newton, MA) (49).

3.4.3 Injection of electronics

3.4.3.1 Surface modification of mesh electronics for aqueous injection
Freestanding mesh electronics structures were transferred by glass pipette sequentially to (i) DI water for 5 min., (ii) aqueous poly-D-lysine (PDL, 0.5-1.0 mg/ml, MW 70,000-150,000, Sigma-Aldrich Corp., St. Louis, MO) solution for 2-12 hours at 25 °C, and (iii) 1X PBS (HyClone™ Phosphate Buffered Saline, Thermo Fisher Scientific Inc., Pittsburgh, PA) at 25 °C for storage (time limited for storage: 1-2 days).

3.4.3.2 Glass needles for injection and imaging

Glass needles for injection and imaging were prepared by using a commercial pipette puller (Model P-97, Sutter Instrument, CA). To prepare channels for imaging, the pulling was halted and suspended in the middle without breaking the glass tube. The channel sizes were characterized by confocal fluorescence microscopy, where rodamine-6G (Sigma-Aldrich Corp., St. Louis, MO) solution was filled into the channel for imaging. For a channel inner diameter (ID) smaller than 300 µm, epoxy glue was used to increase stability during imaging. Clean-cut needles were prepared by scoring (#CTS, Sutter Instrument, CA) and mechanical breakage followed by optical microscopy examination.

To introduce the mesh electronics into glass needles, the tip end of a glass needle was connected to a syringe, and then the large end of the glass needle was used to suck the mesh electronics in towards the sharp needle tip. The correct orientation of the mesh electronics (i.e., recording devices at the needle tip) is readily achieved given visual asymmetry of the structures (see Fig. 3.4a). The glass needle was removed from the plastic tube/syringe and the large end (Fig. 3.4b) connected to a conventional micropipette holder (Q series holder, Harvard Apparatus, Holliston, MA). A microinjector was connected to this holder by plastic tubing. The injection process was controlled using a microinjector (NPIPDES, ALA Scientific instruments Inc.,
3.4.3.3 Injection through metal needles

After surface modification, the mesh electronics was transferred by glass pipette into a syringe (Pressure Control Glass Syringes, Cadence, Inc., Cranston, RI) fitted with a metal needle (18-32 gauge, Veterinary Needles, Cadence, Inc., Cranston, RI). The syringe was assembled and the plunger carefully pressed to drive the region containing devices into the needle, and then to inject the mesh into aqueous solutions (Fig. 3.5c).

3.4.3.4 Input/output (I/O) bonding with anisotropic conductive film (ACF)

The I/O connection pads at the end of the mesh electronics structure (Fig. 3.2) were bonded to a flexible cable post-injection for measurements. First, the I/O region was allowed to unfold in solution layer outside of the injected materials, and then rinsed with ethanol and dried. Second, a piece of ACF (ACF, CP-13341-18AA, Dexerials America Corporation, San Jose, CA), 1.5 mm wide and 15 mm long was over the I/O pads and partially bonded for 10 sec at 75 ºC and 1 MPa using a homemade or commercial bonder (Fineplacer Lambda Manual Sub-Micron Flip-Chip Bonder, Finetech, Inc., Manchester, NH). Third, a flexible cable (FFC/FPC Jumper Cables PREMO-FLEX, Molex, Lisle, IL) was placed on the ACF, aligned with I/O pads and bonded for 1-2 min at 165-200 ºC and 4 MPa (Fig. 3.6a,b).

3.4.3.5 Injection of mesh electronics

3.4.3.5.1 Co-injection into polymer cavities with a polymer precursor

Cavities for injection (Fig. 3.11a) were formed from two pieces of cured polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning Corporation, Midland, MI). The PDMS cavity was designed with a step-like internal corrugation (4 steps, 0.1 cm drop/step, and
projected cavity area of 2 cm × 4.8 cm). Key steps for the co-injection are as follows: (i) mesh electronics were transferred from DI water to ethanol after etching. (ii) PDMS pre-polymer components were prepared in a 10:1 (base: cure agent; Sylgard 184, Dow Corning Corporation, Midland, MI), diluted by hexane 1:3 PDMS:hexane volume ratio, and then (iii) the mesh electronics was transferred to the PDMS/hexane solution and the resulting homogeneous suspension loaded into a glass syringe. (iv) The device region of mesh was injected through a 16 or 18 gauge metal needle into the cavity (Fig. 3.11b), and the I/O region was positioned outside the cavity on a silicon wafer or glass slide. (v) The I/O region was washed with hexane to remove PDMS residue and bonded to a flexible cable interface as described above. The PDMS cavity with the mesh electronics was left at room temperature for 2-4 hours to allow for evaporation of hexane, and then undiluted PDMS precursors were injected into the cavity to fill the entire volume and cured at room temperature for 48 h.

3.4.3.5.2 Injection into Matrigel™

PDL modified mesh electronics were transferred to 1X PBS solution, autoclaved for 1 hour, transferred into Neurobasal™ medium (Invitrogen, Grand Island, NY) by glass pipette, and then loaded into glass syringe as described above. 100% Matrigel™ (BD Bioscience, Bedford, MA) alone or diluted with Neurobasal™ medium to 75 and 25% (v/v) was polymerized for 20 min at 37 °C in an incubator. Mesh electronics were injected into the 100, 75 and 25% polymerized Matrigel™ samples, and the hybrid structures were incubated at 37 °C and imaged (Fig. 3.10d – f) at different times to investigate mesh unfolding in the gel.

3.4.3.5.3 Co-injection of mesh electronics with neurons

Hippocampal neurons (Gelantis, San Diego, CA) were prepared using a standard protocol described previously (15). In brief, 5 mg of NeuroPapain Enzyme (Gelantis, San Diego, CA) was
added to 1.5 ml of NeuroPrep Medium (Gelantis, San Diego, CA). The solution was kept at 37 °C for 15 min, and sterilized with a 0.2 µm syringe filter (Pall Corporation, MI). Day 18 embryonic Sprague/Dawley rat hippocampal tissue with shipping medium (E18 Primary Rat Hippocampal Cells, Gelantis, San Diego, CA) was spun down at 200 g for 1 min. The shipping medium was exchanged for NeuroPapain Enzyme medium. A tube containing tissue and the digestion medium was kept at 30 °C for 30 min and manually swirled every 2 min, the cells were spun down at 200 g for 1 min, the NeuroPapain medium was removed, and 1 ml of shipping medium was added. After trituration, cells were isolated by centrifugation at 200 g for 1 min, and then resuspended in 5-10 mg/ml Matrigel™ at 4 °C. Matrigel™ with neurons were mixed with electronics at 4 °C and then loaded into syringe with metal gauge needle. The electronics and neurons were co-injected into 30% (v/v) polymerized Matrigel™ in culture plate and then placed in incubator to allow Matrigel™ to gel at 37 °C for 20 min. Then 1.5 ml of NeuroPure plating medium was added. After 1 day, the plating medium was changed to Neurobasal™ medium (Invitrogen, Grand Island, NY) supplemented with B27 (B27 Serum-Free Supplement, Invitrogen, Grand Island, NY), Glutamax™ (Invitrogen, Grand Island, NY) and 0.1% Gentamicin reagent solution (Invitrogen, Grand Island, NY). The in-vitro co-cultures were maintained at 37 °C with 5% CO₂ for 14 days, with medium changed every 4-6 days. After incubation, cells were fixed with 4% paraformaldehyde (Electron Microscope Sciences, Hatfield, PA) in PBS for 15-30 min, followed by 2-3 washes with ice-cold PBS. Cells were pre-blocked and permeabilized (0.2-0.25% Triton X-100 and 10% feral bovine serum (F2442, Sigma-Aldrich Corp. St. Louis, MO) for 1 hour at room temperature. Next, the cells were incubated with primary antibodies Anti-neuron specific β-tubulin (in 1% FBS in 1% (v/v)) for 1 hour at room
temperature or overnight at 4 °C. Then cells were incubated with the secondary antibodies AlexaFluor-546 goat anti-mouse IgG (1:1000, Invitrogen, Grand Island, NY).

3.4.3.6 In vivo rodent brain injection

3.4.3.6.1 Mouse preparation

(i) Adult (25-35 g) male C57BL/6J mice (Jackson lab) and Adult (25-35 g) male transgenic mice FVB/N-Tg (GFAPGFP)14Mes/J (Jackson lab) were group-housed, given access to food pellets and water ad libitum and maintained on a 12 h: 12 h light: dark cycle. (ii) All animals were held in a facility beside lab 1 week prior to surgery, post-surgery and throughout the duration of the behavioral assays to minimize stress from transportation and disruption from foot traffic. All procedures were approved by the Animal Care and Use Committee of Harvard University and conformed to US National Institutes of Health guidelines.

3.4.3.6.2 Stereotaxic surgery

(iii) After animals were acclimatized to the holding facility for more than 1 week, they were anesthetized with a mixture of 60 mg/kg of ketamine and 0.5 mg/kg medetomidine (Patterson Veterinary Supply Inc., Chicago, IL) administered intraperitoneal injection, with 30 µL update injections of ketamine to maintain anesthesia during surgery. A heating pad (at 37 °C) was placed underneath the body to provide warmth during surgery. Depth of anesthesia was monitored by pinching the animal’s feet periodically. (iv) Animals were placed in a stereotaxic frame (Lab Standard Stereotaxic Instrument, Stoelting Co., Wood Dale, IL) and then (v) a 1 mm longitudinal incision was made, and the skin was resected from the center axis of the skull, exposing a 2 mm by 2 mm portion of the skull. (vi) A 0.5 mm diameter hole was drilled into the frontal and parietal skull plates using a dental drill (Micromotor with On/Off Pedal 110/220, Grobet USA, Carlstadt, NJ). (vii) The dura was incised and resected. Sterile 1X PBS was
swabbed on the brain surface to keep it moist throughout the surgery. A stereotaxic arm was used to hold and position the needle containing the injectable mesh electronics.

3.4.3.6.3 Stereotaxic injection

(viii) Mesh electronics were autoclaved for 1 hour in 1X PBS solution before injection, and then transferred into Neurobasal™ medium and loaded into the autoclaved glass needle as described above. (ix) The glass needle (with diameter of 100-200 µm) was mounted to a micropipette setup for injection. (x) The needle was lowered into the exposed brain surface approximately 1-2 mm into the skull (Interaural: 6.16 mm, Bregma: -3.84 mm) to test the effects of deep brain and superficial layer injections. A syringe or microinjector was used to inject the mesh electronics into the brain. The needle was retracted during injection using a linear translational stage on the stereotaxic frame. The mesh is injected concomitantly with retraction of the needle so that the electronics is extended in the longitudinal (injection) direction. For targeting cortex/hippocampus region and lateral ventricle, sample #4 and #5 in Table 3.1 were used, respectively. (xi) After injection, the needle was withdrawn from the brain tissue and the I/O region was ejected on the surface of the skull and recording scaffold.

3.4.3.6.4 Acute recording

(xii) A ceramic plate/scaffold with a 0.5-1 cm diameter hole was fixed above the mouse brain, and (xiii) silicone elastomer (World Precision Instruments Inc., Sarasota, FL) was used to seal the gap between the mouse skull and the scaffold to form a chamber that was kept filled with 1X PBS solution. (xiv) After injection of electronics as described in steps 10-11, the I/O region of electronics was unfolded on the surface of the ceramic scaffold. (xv) I/O pads were bonded to a flexible cable by ACF as described above. (xvi) A 32-channel Intan RHD 2132 amplifier evaluation system (Intan Technologies LLC., Los Angeles, CA) was used for acute
electrophysiology recording with an Ag/AgCl electrode acting as the reference. A 20 kHz sampling rate and 60 Hz notch were used during acute recording. A 300-6000 Hz band-pass filter was applied to original recording data for single-unit spikes analyses. Superposition of single-unit spikes was conducted by Clampfit (Molecular Devices, Sunnyvale, CA).

3.4.3.6.5 Chronic testing

(xvii) After injection, the skin that was retracted from the center axis was replaced and the incision was sealed with C&B-METABOND (Cement System, Parkell, Inc., Edgewood, NY). (xviii) Anti-inflammatory and anti-bacterial ointment was swabbed onto the skin after surgery. A 0.3 mL intraperitoneal injection of Buprenex (Patterson Veterinary Supply Inc. Chicago, IL, diluted with 0.5 ml of PBS) was administered at 0.1 mg/kg to reduce post-operative pain. (xiv) Animals were observed for 4 hours after surgery and hydrogel was provided for food, and heating pad was on at 37 °C for the remainder of post-operative care. All procedures complied with the United States Department of Agriculture guidelines for the care and use of laboratory animals and were approved by the Harvard University Office for Animal Welfare.

3.4.3.6.6 Incubation and behavioral analysis

(xx) Animals were cared every day for 3 days after the surgery and every other day after the first 3 days. (xxi) Animals were administered 0.3 mL of Buprenex (0.1 mg/kg, diluted with 0.5 mL 1X PBS) every 12 hours for 3 days. Animals were also observed every other day for behavioral changes. Animals, which were surgically operated on, were housed individually in cages with food and water ad libitum. The room was maintained at constant temperature on a 12-12 h light-dark cycle.

3.4.3.6.7 Brain tissue preparation for chronic immunostaining
Key steps for brain tissue immunostaining are as follows: (i) 4-5 weeks after the surgery, mice underwent transcardial perfusion (40 mL 1X PBS) and were fixed with 4% formaldehyde (Sigma-Aldrich Corp., St. Louis, MO, 40 mL) (33). (ii) Mice were decapitated and brains were removed from the skull and set in 4% formaldehyde for 24 hours as post fixation and then 1X PBS for 24 hours to remove excess formaldehyde. The mesh electronics remained inside the brain throughout fixing process. (iii) For samples with mesh electronics injected in the cortex/hippocampus region, brains were blocked, separated into the two hemispheres, and (iib) mounted on the vibratome stage (Vibrating Blade Microtome Leica VT1000S, Leica Microsystems Inc. Buffalo Grove, IL). (iii) 50-100 µm thick vibratome tissue slices (horizontal and coronal orientations) were prepared for staining. (iva) For samples with mesh electronics injected in lateral ventricle, brains were blocked and then fixed in 1% (w/v) agarose type I-B (Sigma-Aldrich Corp., St. Louis, MO) to fix the position of mesh electronics in the lateral ventricle cavity and then (ivb) mounted on the vibratome stage. (ivc) 100 µm thick vibratome tissue slices (horizontal orientations) were prepared. Coronal slices allowed for cutting in a direction along the long axis of the injection on the frontal plane and horizontal slices allowed for cuts in a direction perpendicular to the long axis of injection. (va) Sample prepared for cryosectioning were transferred to sucrose solution (30%) overnight, and then (vb) transferred to Cryo-OCT compound (VWR, International, LLC, Chicago, IL) with frozen at -80 °C. (vc) Frozen samples were mounted on the stage of a Leica CM1950 cryosectioning instrument (Leica Microsystems Inc., Buffalo Grove, IL) and sectioned into 10 µm thick horizontal slice.

3.4.3.6.8 Immunostaining

(vi) Slices >30 µm thick were then cleared with 5 mg/mL sodium borohydride in HEPES-buffered Hanks saline (HBHS, Invitrogen, Grand Island, NY) for 30 minutes, with 3-times
following HBHS washes at 5-10 minute intervals. Sodium azide (4%) diluted 100x in HBHS was included in all steps. (vii) Slices were incubated with 0.5% (v/v) Triton X-100 in HBHS for 30 min at room temperature. (viii) All slices were blocked with 5% (w/v) FBS and incubated overnight at room temperature. (ix) Slices were washed four times, 30 min intervals, with HBHS to clear any remaining serum in the tissue. (x) Slices were then incubated overnight at room temperature with the GFAP primary antibody (targeting astrocytes, 1:1000, #13-0300 Invitrogen, Grand Island, NY) and/or NeuN primary antibody (targeting nuclei of neurons, 1:200, #ab77315 AbCam, Cambridge, MA) containing 0.2% triton and 3% serum. (xi) After incubation, slices were washed 4-times for 30 min with HBHS. Slices were incubated with secondary antibody (1:200; Alexa Flour® 546 goat anti-rat secondary antibody, 1:200, Alexa Fluor® 488 goat anti-rabbit secondary antibody and/or 1:200, Alexa Fluor® 647 goat anti-chicken secondary antibody (for GFP labeled mice), Invitrogen, Carlsbad, CA) and counterstained with Hoechst 33342 (nuclein stain 1:150, #46C3-4, Invitrogen, Carlsbad, CA) with 0.2% Triton and 3% serum overnight. (xii) After the final washes (4-times, 30 min each with HBHS), slices were mounted on glass slides with coverslips using Prolong Gold (Invitrogen, Carlsbad, CA) mounting media. The slides remained covered (protected from light) at room temperature, allowing for 12 hours of clearance before imaging. When the antibody solutions were first prepared, they included 0.3 Triton X-100 and 5% FBS.

3.4.4 Characterization

3.4.4.1 Structure characterization

Scanning electron microscopy (SEM, Zeiss Ultra55/Supra55VP field-emission SEMs) was used to characterize the mesh electronics structures. Confocal, bright-field and epi-fluorescence imaging was carried out using an Olympus Fluvview FV1000 confocal laser
scanning microscope or Zeiss LSM 780 confocal microscope (Carl Zeiss Microscopy, Thornwood, NY). Confocal images were acquired using 405, 473 and 559 nm wavelength lasers to excite components labeled with Hoechst 33342, Alexa Flour® 488, Alexa Flour® 546, GFP, and Rodamine-6G fluorescent dyes. A 635 nm wavelength laser was used for imaging Alexa Flour® 647, and imaging metal interconnects in reflective mode. Epi-fluorescence images were acquired using a mercury lamp together with standard DAPI (EX:377/50,EM:447/60), GFP (EX:473/31,EM520/35) and TRITC (EX:525/40,EM:585/40) filters. ImageJ (ver. 1.45i, Wayne Rasband, National Institutes of Health, USA) was used for 3D reconstruction and statistical analysis of the confocal images, and overlapping epi-fluorescence images and bright-field images. Matlab was used for statistical analysis of fluorescence intensity of the confocal images for immunostained tissue slices.

3.4.4.2 Imaging of mesh electronics in glass channels

Mesh electronics and thin film control samples with different width and structure (sample #1-3, 7-8 in Table 3.1) were injected into the glass channels following the same injection process described above except that process was stopped so that the mesh remained in part in the constriction of the ‘needle’. Confocal fluorescence microscopy was used to image the 3D structure of mesh electronics and thin films in different diameter glass needles. 3D reconstructed images were obtained using Image J. Cross-section images of the samples were obtained using ImageJ to re-slice 3D reconstructed images in transverse direction with 1 µm steps along the longitudinal direction.

3.4.4.3 Micro-computed tomography

Structures of injected mesh electronics cured in PDMS and Matrigel™ were imaged using a HMXST μCT X-ray scanning system with a standard horizontal imaging axis cabinet
(model: HMXST225, Nikon Metrology, Inc., Brighton, MI). Typical imaging parameters for electronics in PDMS were 75 kV acceleration voltage and 120 µA electron beam current; for electronics in Matrigel™, 80 kV acceleration voltage and 130 µA electron beam current were used. In both cases, shading correction and bad pixel correction were applied before scanning to adjust the X-ray detector; no filter was applied. CT Pro (ver. 2.0, Nikon-Metris, UK) was used to calibrate centers of μCT images. VGStudio MAX (ver. 2.0, Volume Graphics GMbh, Germany) was used for 3D reconstruction and analysis of the calibrated μCT images.

3.4.4.4 Electrical measurements

3.4.4.4.1 Yield of injection

The yield of working devices after injection was determined by measuring the impedance of passive metal electrodes and conductance of nanowire devices before and after injection as follows: (i) As-made 2D mesh electronics were partially immersed in etchant solution as described above to release only the I/O region of mesh electronics and then mesh electronics was transferred to DI water and then dried in ethanol, while the released I/O region was unfolded on the substrate. (ii) Next, the remaining nickel layer was etched and the sample transferred to DI water and dried in ethanol such that the device region was unfolded on the substrate. This two-step etching process allows the mesh electronics to fully unfold on the substrate in a manner that it can be subsequently re-suspended for injection. (iii) Mesh electronics were modified by PDL as described above. (iva) For passive electrodes, the impedance ($Z_0$) at 1 kHz, and impedance-frequency ($Z$-$f$) data were recorded in 1X PBS using an Agilent B1500A semiconductor device parameter analyzer (Agilent Technologies Inc., Santa Clara, CA) with B1520A-FG multi-frequency capacitance measurement unit (Agilent Technologies Inc., Santa Clara, CA). Electrodes with impedance at 1 kHz below 1.5 MΩ were taken as suitable passive metal
electrodes with total number, \( N_0 \). \( (ivb) \) For nanowire devices, the conductance \( (G_0) \) for each device was measured using a probe station (Lake Shore Cryotronics, Inc., Westerville, OH). Current-voltage (I-V) data were recorded using an Agilent 4156C semiconductor parameter analyzer (Agilent Technologies Inc., Santa Clara, CA) with contacts to device through probe station. Devices with conductance above 100 nS were taken as suitable nanowire devices with total number, \( N_0 \). \( (v) \) After impedance/conductance measurements, mesh electronics were immersed in DI water for 4 - 6 hours to suspend them, \( (vi) \) mesh samples were transferred by glass pipette to PDL aqueous solution for surface modification as described above, and then \( (vii) \) loaded into syringes fitted with ID needles from 100 to 600 \( \mu \)m and into a chamber with I/O unfolded on a substrate adjacent to the chamber. \( (viii) \) Ethanol was used to rinse and dry the I/O. \( (ixa) \) The impedance \( (Z_1) \) of the passive electrodes was measured as in step 4a, and the total number of electrodes meeting above criteria, \( N_1 \), post-injection was recorded. Yield and impedance changes in Fig. 3.1h were calculated as \( N_1/N_0 \) and \( (Z_1-Z_0)/Z_0 \), respectively. \( (ixb) \) The conductance \( (G_1) \) of nanowire devices was measured again, and the total number, \( N_1 \), meeting the above criteria (step \( ivb \) above) was determined. Yield and conductance changes in Fig. 3.1i were calculated as \( N_1/N_0 \) and \( (G_1-G_0)/G_0 \), respectively. All measurements have been repeated for 16 different devices.

### 3.4.4.4.2 Test of ACF bonding

The connection resistance of ACF was measured to investigate the influence of bonding on electrical properties of devices (Fig. 3.6e,f). The conductance of each device (connected metal wires) was measured by probe station as \( R_0 \) and \( R_1 \) before and after ACF bonding, respectively. The connection resistance for each I/O pad (100 \( \mu \)m diameter) was calculated as \( (R_1-R_0)/2 \), illustrated in Fig. 3.6e. The calculated connection resistance after ACF bonding with
commercial (~21.2 Ω) and homemade (~33.7 Ω) instruments (Fig. 3.5d), was <0.05% of the typical nanowire resistance and <0.01% of the typical metal electrode impedance at 1 kHz. The insulation resistance between I/O pads without circuits was over 10 GΩ. These measurements and analyses demonstrate that ACF bonding had little influence on electrical properties of injectable mesh electronics, which ensured reliable measurements with injectable mesh electronics devices in the applications described in the text. Comparison of the connection resistance values obtained using a standard flip-chip bonder and custom set-up suitable for bonding in restricted environments, including in vivo measurements, (Fig. 3.6e,f) shows similar values that are also comparable to reported contact resistances for ACF (19).

3.4.4.4.3 Piezoresistance measurements

The piezoresistance response of strained nanowire devices was measured as conductance change of device subject to the deformation of PDMS structure. In brief, the I/O pads were bonded to a flexible cable as described above, and connected to a multi-channel current/voltage preamplifier (Model 1211, DL Instruments, Brooktondale, NY), filtered with a 3 kHz low pass filter (CyberAmp 380, Molecular Devices, Sunnyvale, CA), and digitized at a 1 kHz sampling rate (AxonDigi1440A, Molecular Devices, Sunnyvale, CA), with a 100 mV DC source bias voltage. Pressure was applied along z-axis for 20 sec using a homemade linear translation stage.

3.4.4.4.4 SU-8 passivation characterization

The effectiveness of our SU-8 passivation was characterized following immersion in Neurobasal™ medium at 37 °C for 6 weeks using impedance-frequency (Z-f) measurement. A PDMS chamber 2 mm in longitudinal direction and 5 mm in transverse direction was positioned over the interconnect lines (without exposing the sensor electrodes), filled with 1X PBS solution, and then Z-f data were recorded using an Agilent B1500A semiconductor device parameter
analyzer with B1520A-FG multi-frequency capacitance measurement unit. Significantly, impedance measurements from 1 to 10 kHz for 16 different SU-8 passivated metal interconnect lines showed average values above 10 GΩ. The large impedance demonstrates that there is no obvious leakage through our thin SU-8 polymer passivation. In addition, the impedance at 1 kHz of the SU-8 passivated region, ~30 GΩ, is $10^4$-$10^5$ larger than the typical values for our Pt-metal sensors.

3.4.5 Structure analysis and mechanical simulations

3.4.5.1 Number of rolls of mesh electronics inside glass needles

The mesh electronics rolls up in a scroll-like structure when injected through a glass needle. Theoretically, the number of circumferential rolls, $N_{rolls}$, can be calculated by dividing the total width, $W$, of the mesh with the perimeter of the tube, $\pi D$, with $D$ the tube ID, as $N_{rolls} = W/\pi D$ with values of 3.5, 6.3, and 10.5 for Fig. 3.7c-e (I), (II) and (III), respectively. Experimentally, the number of circumferential rolls was estimated from the cross sections of 3D reconstructed confocal images as follows: First, we count the number of longitudinal ribbon (LR) features, $K_{LR}$, in images of the scroll structure. Second, the number of LRs from a half circumference roll can be estimated as $n_{LR} = \pi D/2s$, where $s$ is the distance between LRs. Finally, the total number of circumference rolls is $N'_{rolls} = 2sK_{LR}/\pi D$. Using this method, the numbers of circumference rolls in Fig. 3.7c-e are 3.4 ± 0.2, 6.0 ± 0.4 and 9.5 ± 1.0 for (I), (II) and (III), respectively. The uncertainty arises from the identification of longitudinal elements from 8 random cross-sections for each case; small deviations from geometric analysis above may be arise in part from a failure to count some longitudinal elements due to low fluorescence intensity.

3.4.5.2 Mechanical simulation

3.4.5.2.1 Bending stiffness simulation
We estimate the bending stiffness of the mesh electronics with different structures by finite element software ABAQUS. A unit cell is used for the simulation, where the tilt angle $\alpha$ is defined in Fig. 3.1d and mesh electronics are modeled with shell elements: A homogeneous single shell section with 700 nm thick SU-8 is assigned to the transverse ribbons; a composite section with three layers of 350 nm thick SU-8, 100 nm thick gold and another 350 nm thick SU-8 is assigned to the longitudinal ribbons. Both SU-8 and gold are modeled as linear elastic materials, with Young’s modulus 2 and 79 GPa (50) respectively. To calculate the longitudinal and transverse bending stiffnesses, a fixed boundary condition is set at one of the ends parallel with the bending direction, and a small vertical displacement, $d$, is added at the other end. The external work, $W$, to bend the device is calculated. We define the effective bending stiffness of the device as the stiffness required of a homogenous beam to achieve the same external work $W$ under the displacement $d$. Therefore, the effective bending stiffness per width of the device can be estimated as (51)

$$D = \frac{2Wl^3}{3d^2b}$$  \hspace{1cm} (3.1)

with $b$ the width of the unit cell parallel with the bending direction, and $l$ the length of the unit cell perpendicular to the bending direction (Fig. 3.2b).

### 3.4.5.2.2 Effective bending stiffnesses of implantable probes.

The effective bending stiffness per width of the three-layer longitudinal ribbon, $D_1$, (longitudinal ribbon) in the mesh can be estimated as (51)

$$D_1 = \frac{E_s}{w_1} \left( \frac{h_1^3w_1}{12} - \frac{h_m^3w_m}{12} \right) + \frac{E_m}{w_1} \frac{h_m^3w_m}{12}$$  \hspace{1cm} (3.2)
where $E_s$ is young’s modulus of SU-8, $E_m$ is young’s modulus of gold, $h$ is the total thickness of ribbon, $h_m$ is the thickness of metal, $w_1$ is the total width of ribbon and $w_m$ is the width of metal. When $E_s = 2$ GPa, $E_m = 79$ GPa (16), $h = 800$ nm, $h_m = 100$ nm, $w_1 = 20$ µm, $w_m = 10$ µm, $D_1 = 0.086$ nN·m.

The effective bending stiffness per width of standard silicon probes, $D_2$, can be estimated as (51)

$$D_2 = E_{\text{silicon}} \frac{h_{\text{silicon}}^3}{12}$$

(3.3)

where $E_{\text{silicon}}$ is the young’s modulus of silicon, $h_{\text{silicon}}$ is the thickness of the probe. When $E_{\text{silicon}} = 165$ GPa, $h_{\text{silicon}} = 15$ µm (34), $D_2 = 4.6 \times 10^5$ nN·m.

The effective bending stiffness per width of ultrasmall carbon electrodes, $D_3$, can be estimated as (51)

$$D_3 = E_{\text{carbon}} \frac{\pi d^3}{64}$$

(3.4)

where $E_{\text{carbon}}$ is the young’s modulus of carbon fiber, $d$ is the diameter of carbon fiber probe. When $E_{\text{carbon}} = 234$ GPa, $d = 7$ µm (33), $D_3 = 3.9 \times 10^4$ nN·m.

The effective bending stiffness per width of planar shape probe, $D_4$, can be estimated as (51)

$$D_4 = E_s \frac{h_s^3}{12}$$

(3.5)

where $E_s$ is the young’s modulus of polyimide, $h_s$ is the thickness of probe. When $E_s = 2-2.73$ GPa, $h_s = 10-20$ µm (12, 13, 35), $D_4 = 0.16-1.3 \times 10^4$ nN·m.

3.4.5.2.3 Simulation of mesh electronics strain

The data in Figs. 3.1, 3.5a, b and 3.7 show that mesh electronics can be injected in a rolled-up geometry through needles to 95 µm ID without breaking. We further quantified the
importance of the rolled up geometry during injection by using simulations to estimate the strain distribution (Fig. 3.9) versus needle ID the rolled-up geometry. The simulation treats a unit cell of the mesh bent with a radius of curvature, R, where a fixed boundary condition sets the strain of one longitudinal ribbon at zero and the maximal principal strain, εm, value then occurs at the junction between the transverse and second longitudinal element of the unit cell (red dashed circle, inset, Fig. 3.9). This strain value represents an upper limit given that other edge of the unit cell was set to zero for the simulation. The plot of this upper limit strain value versus 1/R (Fig. 3.9) shows that strain increases linearly, where the black and red points/lines correspond to mesh electronics structural parameters summarized in Table 3.1 (entries #2 and #5, respectively). The upper limit strain values extrapolated for a 100 µm ID needle for these two mesh structures, ~1.0%, are both smaller than the fracture strain, 5%, reported for a 20 µm thick SU-8 beam (52). In addition, the stress intensity factor, K, for a thin film under pure bending exhibits a square root dependence on thickness (53), K~Eε√h, where E is the Young’s modulus of the material, ε is the strain and h is the thickness of ribbon. The ε reaches the fracture strain of ribbon, εc, when K reaches the toughness of the material Kc. Since the thickness of SU-8 in our mesh structures is 700 nm (vs. 20 µm) the fracture strain of ribbon can be expected to be larger than 5%.
3.5 Bibliography


Chapter 4

Syringe Injectable Electronics: Precise Targeted Delivery with Quantitative Input/Output Connectivity

[The following chapter is derived in part from G. Hong, T.-M. Fu et al., Nano Letters, 15, 6979-6984 (2015).]

Syringe-injectable mesh electronics with tissue-like mechanical properties and open macroporous structures is an emerging powerful paradigm for mapping and modulating brain activity. Indeed, the ultra-flexible macroporous structure has exhibited unprecedented minimal/non-invasiveness and the promotion of attractive interactions with neurons in chronic studies. These same structural features also pose new challenges and opportunities for precise targeted delivery in specific brain regions and quantitative input/output (I/O) connectivity needed for reliable electrical measurements. Here, we describe new results that address in a flexible manner both of these points. First, we have developed a controlled injection approach that maintains the extended mesh structure during the ‘blind’ injection process, while also achieving targeted delivery with ca. 20 μm spatial precision. Optical and micro-computed tomography results from injections into tissue-like hydrogel, ex vivo brain tissue and in vivo brains validate our basic approach and demonstrate its generality. Second, we present a general strategy to achieve up to 100% multi-channel I/O connectivity using an automated conductive ink printing methodology to connect the mesh electronics and a flexible flat cable, which serves as the standard ‘plug-in’ interface to measurement electronics. Studies of resistance versus printed line width were used to identify optimal conditions, and moreover, frequency-dependent noise
measurements show that the flexible printing process yields values comparable to commercial flip-chip bonding technology. Our results address two key challenges faced by syringe-injectable electronics, and thereby pave the way for facile in vivo applications of injectable mesh electronics as general and powerful tool for long-term mapping and modulation of brain activity in fundamental neuroscience through therapeutic biomedical studies.
4.1 Introduction

Syringe-injectable electronics represents a paradigm-shifting approach for seamless three-dimensional (3D) integration of electronics within man-made materials and living systems, for example, for in vivo interrogation and modulation of brain activity (1-5). In particular, unlike traditional and relatively rigid implantable brain probes based on metal, silicon and 10’s μm thick polymer films (6-10), the syringe-injectable electronics builds upon a submicron thickness macroporous mesh structure (11, 12) with tissue-like mechanical properties (1). Specifically, the syringe-injectable electronics have a bending stiffness 4-6 orders of magnitude smaller than traditional implantable probes (1), mesh widths features on the 10 μm scale similar to neuronal soma and axons (1), and ~90% free area structure that allows for facile neuronal interpenetration (12). These unique structural and mechanical properties of syringe injectable electronics have yielded minimal damage and immune response post implantation in brain tissue as well as unprecedented attractive or ‘neurophilic’ interactions with neurons thereby allowing for 3D interpenetration with intact neuronal networks (1).

The ultra-flexibility of the mesh electronics, which is central to the above advantages, also presents new challenges associated with the injection and input/output (I/O) connection processes. For example, during syringe-assisted injection into brain tissue the mesh electronics may crumple due to the extremely low bending stiffness of the structure. Such crumpling would displace the recording electrodes from expected stereotaxic injection coordinates and could thereby yield uncertainty in specific location from which signals are recorded. In addition, the small syringe needle diameters preclude injection of mesh electronics with pre-bonded I/O connectors, and the mesh thickness and flexibility make it incompatible with conventional semiconductor bonding methods such as wire bonding or soldering. Although anisotropic
conductive film (ACF) is a widely used approach for I/O bonding of flexible electronics (1, 10, 13), the relatively high pressure and temperature required for ACF bonding can damage the thin (< 1 μm) and ultra-flexible mesh electronics. In addition, post-injection unfolding of the ultra-flexible mesh electronics on the FFC cables prior to interconnection can yield orientation and spatial distributions of I/O pads that cannot be bonded by ACF. Although these aforementioned challenges are not associated with conventional rigid probes, it is noteworthy that the ultra-flexibility of syringe-injected electronics does not impose forces post-injection with respect to brain tissue and thus does not yield stresses at or motion with respect to targeted sites. Moreover, the intrinsically small and flexible nature of mesh electronics eliminates the bulky vertically-protruding I/O interface typically associated with rigid brain probes, and thus could benefit significantly chronic recording studies of animals.
4.2 Results and Discussion

The controlled injection elements of the stereotaxic surgery station used for \textit{in vivo} brain probe implantation consist of a syringe pump and a motorized stereotaxic stage (\textbf{Figs. 4.1a I} and 4.2a). The syringe pump typically injects 1X phosphate buffered solution (PBS) through a needle loaded with mesh electronics (\textbf{Fig. 4.2b}) at a fixed volumetric rate (20-50 mL/h), while a motorized linear translation stage withdraws the “vertical” arm of the stereotaxic stage at a constant velocity (0.2-0.5 mm/s) that matches the ejection rate of the mesh electronics from the needle. In general, the fluid shear force that drives the mesh electronics out of needle must overcome the friction between the mesh electronics and the needle inner wall, and thus mesh structures with different designs and different needle inner diameters (IDs) will require different flow conditions to balance electronics injection and needle retraction rates necessary to achieve full extension of the mesh electronics (\textbf{Fig. 4.1a II}). The I/O bonding components of our setup, which are also compatible with the stereotaxic surgery station, consist of a motorized and computer-controlled microprinter (\textbf{Fig. 4.2a,c}) that prints conductive ink in a programmable two-dimensional (2D) pattern that links I/O pads on the mesh electronics to corresponding channel lines of the flexible flat cable (FFC) (\textbf{Fig. 4.1b}), which then provides standard serial communication interface with the recording/control instrumentation.
Figure 4.1. Overview of mesh electronics injection and I/O bonding. (a) Schematics (I) of the controlled injection setup for precise targeted delivery of mesh electronics into a live mouse brain using a syringe pump (indicated by the red arrow) and a stereotaxic frame equipped with a motorized linear translational stage (golden). (II) Zoom (dashed red box in I) of mouse head showing the extended mesh electronics inside the mouse brain after injection with needle outside the skull. (b) Schematic showing the I/O of the mesh electronics unfolded on a flexible flat cable (FFC); electrical connections between individual channels of the mesh (red arrow) and FFC are made by printed conductive ink (yellow arrow). The white arrow indicates the connection of the FFC cable to external instrumentation. (c, I) Image showing mesh electronics being injected from a glass needle into 1X PBS solution. The mesh electronics expands to a size larger than the needle ID (400 μm) in solution. Longitudinal metal interconnect lines are prominent in the image (due to good light reflection). (II) Magnified portion of mesh highlighted by red dashed box in (I) shows the full mesh structure. The red arrow highlights one of the longitudinal SU-8/metal interconnect/SU-8 elements, and the yellow arrow denotes a transverse SU-8 element (see Fig. 4.3 for the structure of mesh electronics). (d) Schematics showing extended mesh electronics (left) and crumpled mesh electronics (right) post injection into dense tissue or gel.
Figure 4.2. Experimental mesh injection and I/O bonding set-ups. (a) Overview of the entire setup showing the relevant instrumentation for controlled injection (yellow dashed box) and conductive ink printing (blue dashed box). The yellow arrow indicates the syringe pump used for controlling the volumetric liquid injection rate, and the white arrow highlights the linear translational motor that drives the stereotaxic stage. (b) Zoomed-in view of the controlled injection setup, where the white arrow indicates the glass needle loaded with mesh electronics for injection. (c) Zoomed-in view of the conductive ink printing setup, where the white arrow indicates the motorized and computerized micromanipulator, and the blue arrow indicates the printer head loaded with conductive ink.

The ultra-flexible nature of the mesh electronics, which comprises the sensors, interconnects and I/O pads (Fig. 4.3), is readily evident upon injection into aqueous solution (Fig. 4.1c), where the mesh spontaneously expands to a size substantially larger than injection needle and appears to ‘float’ within the solution. In synthetic gels or dense tissue such as the brain, matching the mesh electronics injection and needle retraction rates is critical for achieving
precise targeted delivery with a controlled and extended conformation (Fig. 4.1d, left) versus, for example, a crumpled conformation (Fig. 4.1d, right), which results from the needle being retracted more slowly than the mesh injection rate. The latter crumpled configuration yields poorly defined sensor device positions.

**Figure 4.3. Structure of syringe-injectable mesh electronics.** (a) Schematic of the mesh electronics structure, where the red network corresponds to SU-8 polymer, which defines the overall mesh structure and encapsulates the metal interconnect lines in the three-layer SU-8/metal/SU-8 structure, the green dashed box highlights the sensor electrodes (dark green dots), the red dashed box highlights the metal interconnect lines, and the blue dashed box highlights the I/O pads (dark blue circles). (b) Optical image of a fabricated mesh electronics probe, where the green, red and blue dashed boxes highlight the sensor electrodes, the metal interconnects and the I/O pads, respectively, as in (a).

A general attribute of syringe injection is the ability to deliver materials to hidden or opaque regions, such as tissue within the brain, in a minimally invasive manner. As discussed above for the specific case of syringe injection of mesh electronics, it is important to have visual guidance and feedback to match mesh injection/needle retraction rates to ensure the mesh electronics is delivered into a targeted brain region with extended conformation. Because direct visualization of mesh electronics inside an opaque material such as the brain cannot be carried out during injection, we devised a general method based on visualization and real-time tracking of the upper I/O end of the mesh electronics in the field of view (FoV) of an eyepiece camera.
The FoV method dictates that, if the mesh electronics remains fully extended along the longitudinal direction during the injection process without displacement, then the absolute spatial location of the mesh electronics remains the same and an image of the upper end of the mesh should be fixed in the camera FoV. In other words, precisely targeted delivery of the mesh sensor electrodes can be achieved by ensuring that the mesh stays stationary in the FoV while the needle moves upwards in the FoV (Fig. 4.4a, top). Correspondingly, although the bottom end of the mesh electronics remains invisible to the operator, the mesh remains stationary in the injected medium with sensing electrodes at the predefined target positions (Fig. 4.4a, bottom), while the needle is retracted.

Figure 4.4. Field-of-view (FoV) controlled delivery of mesh electronics. (a) Schematics illustrating controlled injection by the FoV method. The top row shows the mesh I/O pads
Figure 4.4 (Continued): remain stationary (red dashed arrow) within the FoV (red box) while the needle is retracted upwards (blue dashed arrow following the black dash marked on the needle’s exterior), resulting in fully extended mesh electronics structure inside the injected medium during needle withdrawal (bottom row). (b) Photographs showing the FoV injection process into 0.5% agarose hydrogel. The top row shows the needle moving upwards (blue dashed arrow) with the mesh I/O pads remaining stationary (red dashed arrow) in the FoV. The bottom series of images recorded at same time points shows the same injection process of an independent mesh structure obtained at the end of the mesh electronics in the gel. Images of the lower part of the mesh electronics in the agarose hydrogel post injection are shown in the far right panels for each experiment.

The capabilities of the FoV method were explored by injecting mesh electronics in 0.5% agarose hydrogel. This composition hydrogel is a good mimic of brain tissue since both the Young’s modulus and shear modulus are similar to those of brain tissue (14-18). In addition, the optical transparency of the hydrogel allows for direct imaging of injected mesh. In experiments carried out with fluid injection rates of 20-50 mL/h and needle retraction speeds of 0.2-0.5 mm/s, it was possible to meet the stationary FoV conditions (Fig. 4.4b, top) as evidenced by the stationary I/O pads (red dashed arrow, Fig. 4.4b, top) as the needle was withdrawn at a constant speed (blue dashed arrow). An independent balanced injection/retraction rate experiment with the camera set to image the mesh injected in the hydrogel (Fig. 4.4b, bottom) reveals that the bottom edge of the mesh electronics remains stationary (red dashed arrow, Fig. 4.4b, bottom) as the needle was withdrawn upwards (blue dashed arrow). Last, both injections resulted in fully extended mesh structures in the longitudinal direction at the completion of the injection process (Fig. 4.4b, far right image panels).

Analyses of the above results and additional experiments highlight several important points. First, the total volume of liquid delivered into the hydrogel during injection of a ~5 mm length of mesh electronics is typically 10 to 100 μL. Significantly, this volume is similar to the
volume of liquid introduced during intracranial injection of virus vectors and enzymes, 1~100 μL (19-23). Second, the final positioning precision of the mesh electronics in hydrogel measured during the injection process from the camera images is ~20 μm from the original target coordinates at $t = 0$ s (see Chapter 4.4). This relatively small positioning uncertainty suggests that our FoV injection approach can achieve precise targeted delivery of mesh electronics with tolerance smaller than the thickness of key subfields/layers of the mouse brain: for example, the CA-1 subfield of the hippocampus is ca. 620 μm thick, CA-3 subfield is ~230 μm thick (24) and cortical layer V is ~300 μm thick (25). Third, the importance of matching mesh injection/needle retraction rates was confirmed by control experiments (Fig. 4.5). Specifically, when the rate of needle retraction was slower than needed to exert sufficient kinetic friction force and balance the expulsion force due to fluid injection, we observed crumpling of the ultra-flexible mesh electronics (Fig. 4.5a), and when the needle retraction rate was faster and thus exerted larger kinetic friction force than the expulsion force, the mesh was displaced upwards from the initial targeted position during injection (Fig. 4.5b). Last, studies of FoV injection into tissue-like hydrogel for angles up to 45-degrees off vertical (Fig. 4.6) demonstrate similar targeting capabilities as vertical injection, and thus show that controlled targeted delivery to brain regions that are typically difficult to access using vertical injection alone (26) will be accessible with our approach. We have applied the FoV method to investigate the potential for controlled injection of mesh electronics into opaque ex vivo fixed brain tissue and in vivo live mouse brain. A schematic for the in vivo injection (Fig. 4.7a) emphasizes the general experimental protocol of making 2 or more mesh injections at distinct sites prior to analysis. We injected four mesh electronics samples at different sites in the ex vivo brain tissue, where three injections were using our balanced FoV method and one was injected manually. Because the opaque nature of the
brain tissue precluded direct optical imaging, we used micro-computed tomography (micro-CT; see Chapter 4.4) to visualize the mesh electronics structure post-injection, where the high X-ray attenuation contrast of metal interconnects compared to tissue allows for clear contrast of the mesh electronics. A 3D reconstruction of the *ex vivo* mouse brain following the above mesh injections (Fig. 4.7b) reveals several key points. First, the mesh electronics injected by the balanced FoV approach exhibited the desired fully extended morphology (yellow arrows). Second, the manually injected sample showed a crumpled structure (blue arrow) in the brain tissue. We note that it was not possible to distinguish these differences in internal morphology by optical visualization of the exterior of the brain (Fig. 4.7b inset) as all injections produced minimal visual damage/bleeding.

![Figure 4.5: Injection processes with mismatched injection rate and needle retraction speed.](image)

(a) Time course white-light optical photographs of the mesh electronics injection process when the needle is withdrawn at a speed slower than the injection rate, resulting in crumpled mesh electronics structure and inaccurate delivery of mesh electrodes into the medium. (b) Time course photos of the mesh electronics injection process when the needle is withdrawn at a speed faster than the injection rate, resulting in partial withdrawal of the mesh electronics structure from the medium. In (a) and (b) the medium was 0.5% (wt/vol %) agarose hydrogel.
In addition, *in vivo* injection of two mesh electronic structures into the left and right cerebral hemispheres was carried out using the balanced FoV controlled injection setup under a stereotaxic stage and through pre-drilled holes in the cranial bone (Fig. 4.7c; see Chapter 4.4 for stereotaxic coordinates of the two injections). Analysis of the 3D reconstructed micro-CT obtained post-injection (Fig. 4.7d) demonstrates the fully extended mesh morphology positioned at the chosen brain coordinates for both injected mesh electronics samples. The capability to achieve well-controlled mesh electronics injections into *ex vivo* whole mouse brains and *in vivo* live mouse brains highlight several key points. First, synchronized and balanced mesh injection and needle retraction, which are difficult to achieve in manual injections, enable the mesh electronics to be extended and kept stationary with respect to the optically-opaque brain tissue. Second, micro-CT imaging verified the effectiveness of the FoV method by proving the extended morphology of injected mesh electronics and the precise positioning within the brain using the stereotaxic stage.

![Figure 4.6](image.png)

**Figure 4.6. Controlled injection of mesh electronics at different angles.** White-light optical photographs are shown for controlled injections of mesh electronics at 15° (a), 30° (b) and 45° (c) to normal direction (black dashed lines) before (left) and after (right) injection. The medium in all of the experiments was 0.5% (wt/vol %) agarose hydrogel.
Figure 4.7. Blind injection of mesh electronics using the FoV method into brain tissue. (a) Schematic showing blind injection of multiple mesh electronics samples into the brain of a live mouse. The mesh electronics on the left is already injected with its I/O (left) unfolded on the skull, while the mesh electronics on the right is in the middle of the injection process. (b) Micro-CT reconstructed image of an ex vivo mouse brain (gray) blind-injected with 4 mesh electronics samples (red). Inset is white-light optical image of the brain surface. Blue arrows indicate the mesh electronics injected manually, and yellow arrows indicate the mesh electronics delivered via the balanced FoV controlled injection process. (c) White-light optical photograph showing live mouse skull following blind injection of two mesh electronics samples (indicated by yellow arrows), where both were injected using the balanced FoV controlled injection method. (d) Micro-CT reconstructed image of the same mouse head in (c) showing the extended mesh electronics structures (red, indicated by yellow arrows) inside the skull (gray).

Last, we have achieved quantitative I/O connectivity of the multiplexed mesh electronics through an automated conductive ink printing method (Fig. 4.8a). The conductive ink used in our work was comprised of surfactant-solubilized carbon nanotubes (CNT) in an aqueous solution (see Chapter 4.4 for detailed information). The CNT suspension was loaded into a glass capillary tube with tapered tip (ID = 150 μm) and subsequently printed as droplets on the FFC.
surface (Fig. 4.8b). For a 16-channel mesh electronics structure with recording electrodes injected into dense tissue as described above, the I/O pads at the other end of the mesh structure (Fig. 4.3) were unfolded on the surface of an FFC interface cable to expose the electrical connection pads for all 16 channels of the mesh electronics. The spatial coordinates of the mesh I/O pads and all FFC electrodes were taken as inputs into the automated microprinter, which then computed the shortest path for each connection and carried out conductive ink printing to make electrical connections between all available channels in the mesh and the FFC with a bonding yield of 100% (Fig. 4.8b). Another example in Fig. 4.9 shows imperfectly unfolded mesh I/O pads, which are not lined up in a straight line but in a curve (red dashed line, Fig. 4.9) with varying pitch distance between neighboring I/O pads (blue labels, Fig. 4.9). This non-ideal I/O alignment cannot be bonded by ACF but are readily connected in 100% yield by our printing method, thus highlighting the clear advantage compared to the ACF method. The typical time scale for unfolding the I/O pads on the FFC cable is ca. 10 min, and that for completing the 16-channel I/O connection through conductive ink printing is ~30 min.

![Figure 4.8](image)

**Figure 4.8. Conductive ink printing for I/O connectivity.** (a) Schematic showing the automated conductive ink printing approach used to achieve high-yield I/O bonding of the mesh
Figure 4.8 (Continued): electronics to an FFC cable. The red arrow highlights the connection from the FFC to external recording instrumentation. (b) White-light optical image showing all 16 channels in the mesh electronics (lined-up along the top red dashed line) bonded to the 16 metal lines of the FFC cable (lower red dashed line) by the conductive ink printing method, where the printed CNT lines are between the two red dashed lines. The red arrow indicates the glass capillary tube loaded with conductive ink. (c) Resistance of conductive ink printed lines as a function of line width. All lines are printed to a total length of 5 mm. (d) Noise spectra for mesh electronics sensor electrodes immersed in 1X PBS solution and recorded following bonding of mesh electronics I/O by standard ACF bonding (black curve) and our conductive ink printing (red curve) methods.

The resistance of the printed CNT lines was characterized using four-point measurements as a function of line width for a fixed length of 5 mm, where the 5 mm limit was longer than lines typically used in practice. These results (Fig. 4.8c) illuminate several key points. First, the resistance of the printed CNT lines decreased as a function of line width with fixed length as expected with an estimated resistivity of $1.04 \pm 0.15 \times 10^{-2} \ \Omega \cdot \text{m}$. Second, from these data we estimate that a typical CNT line with average width of $\sim 150 \ \mu\text{m}$ and average length of $\sim 3.5 \ \text{mm}$ will have a resistance of $\sim 4.2 \ \text{k}\Omega$, which is much smaller than the typical interface impedance, $100 – 1000 \ \text{k}\Omega$) between metal sensing electrodes and physiological solution. To further validate the utility of the conductive ink printing method, we compared the noise spectra recorded from mesh electronics sensor elements following bonding by either (i) standard flip-chip anisotropic ACF bonding, which is the standard method for flexible electronics (1, 10, 13) and (ii) our new conductive ink printing method. Notably, these data shown in Fig. 4.9d exhibit comparable noise-frequency dependence and thus validate our new approach. We are currently using this bonding method to carry out long-term chronic recording measurements in awake mice.
Figure 4.9. Conductive ink printing method provides 100% connectivity for a mesh electronics with imperfectly unfolded mesh I/O pads. The mesh I/O pads were spatially distributed in a curve (red dashed line) with varying distances between neighboring pads (two examples of inter-pad distances labeled in blue). It is straightforward to bridge these I/O pads to the regular pitch lines on the FFC cable (dark vertical lines, lower quarter of image) using the conductive CNT ink printing method. In contrast, it would not be possible to connect all of the I/O pads bonded to the FFC using the ACF bonding method reported previously (1).
4.3 Conclusion

In conclusion, we have reported controlled injection and conductive ink printing techniques to address the challenges associated with the ultra-flexible nature of syringe-injectable electronics. Controlled injection was achieved by balancing the electronics injection and the needle retraction rates, resulting in a mesh electronics structure that remains stationary and fully extended in the dense medium, thus allowing for targeted delivery of mesh electronics in any specific brain region with ca. 20 μm targeting precision. Optical and micro-CT imaging results from injections of mesh electronics into tissue-like hydrogel, *ex vivo* brain tissue and *in vivo* brains demonstrate the FoV controlled injection as a general method to achieve precise targeted delivery of mesh electronics without crumpling or displacement during injection. In addition, up to 100% I/O connectivity was demonstrated using computer-controlled hands-free conductive ink printing, which allows for customized patterns to accommodate different orientations of the mesh electronics I/O pads and pre-positions of FFC interface. Notably, frequency-dependent noise measurements show that our conductive ink printing process is comparable to commercial flip-chip bonding technology. These advances in controlled injection and I/O bonding of the mesh electronics together with previous studies showing minimal or the absence of chronic tissue response (1) now open up many opportunities for chronic brain recording using injectable electronics, including elucidating changes in neural circuits as a function of learning (27, 28) and neuropathologies (29, 30).
4.4 Methods and Materials

4.4.1 Fabrication of injectable mesh electronics

The geometrical design of injectable mesh electronics is similar to our recent report (1), with its key parameters as follows: total width $W = 4$ mm, longitudinal ribbon width $w_1 = 20$ μm, transverse ribbon width $w_2 = 20$ μm, angle between longitudinal and transverse ribbons $\alpha = 45^\circ$, longitudinal spacing $L_1 = 333$ μm, transverse spacing $L_2 = 250$ μm, metal interconnect line width $w_m = 10$ μm and total number of channels $N = 16$. Key steps used in the fabrication of the mesh electronics are given as follows (1): (i) A 100 nm layer of Ni, which was used as the sacrificial layer, was thermally evaporated (Sharon Vacuum, Brockton, MA) onto the pre-cleaned Si wafer (n-type 0.005 Ω·cm, 600-nm thermal oxide, Nova Electronic Materials, Flower Mound, TX). (ii) The Si wafer was spin-coated with 500 nm negative photoresist SU-8 (SU-8 2000.5; MicroChem Corp., Newton, MA) and pre-baked at 65 °C on a hot plate for 1 min and then transferred to a 95 °C hot plate for 4 min, before photolithography (PL) patterning (ABM mask aligner, San Jose, CA). The exposed SU-8 photoresist was post-baked at 65 °C for 3 min and 95 °C for 3 min. (iii) After post-baking, the SU-8 photoresist was developed (SU-8 Developer, MicroChem Corp., Newton, MA) for 2 min, rinsed with isopropanol, and hard-baked at 185 °C for 1 h. (iv) Subsequently, the wafer was spin-coated with MCC Primer 80/20 and LOR 3A lift-off resist (MicroChem Corp., Newton, MA), and baked at 185 °C for 5 min, followed by spin-coating Shipley 1805 photoresist (Microposit, The Dow Chemical Company, Marlborough, MA), which was baked at 115 °C for 5 min. The resist was patterned by PL and developed (MF-CD-26, Microposit, The Dow Chemical Company, Marlborough, MA) for 90 s. (v) A 1.5-nm Cr layer and a 100-nm thick Au layer were deposited by electron-beam evaporation (Denton Vacuum, Moorestown, NJ) followed by lift-off (Remover PG, MicroChem Corp., Newton, MA). (vi) Steps
and \( v \) were repeated for lithographically patterning and depositing the Pt sensing electrodes (Cr: 1.5 nm, Pt: 50 nm). (vii) Steps \( ii \) and \( iii \) were repeated for lithographically patterning the top SU-8 layer, which serves as the top encapsulating/passivating layer. (viii) The Si wafer with fabricated mesh electronics was transferred to a Ni etchant solution comprising 40% FeCl\(_3\): 39% HCl: H\(_2\)O = 1:1:20 to release the mesh electronics from the fabrication substrate. Released mesh structures were rinsed with deionized (DI) water, transferred to an aqueous solution of poly-D-lysine (PDL, 1.0 mg/ml, MW 70,000-150,000, Sigma-Aldrich Corp., St. Louis, MO) for 24-48 h, and then transferred to 1X phosphate buffered saline (PBS) solution (HyClone\textsuperscript{TM} Phosphate Buffered Saline, Thermo Fisher Scientific Inc., Pittsburgh, PA).

4.4.2 Controllable injection into dense materials and biological tissues

4.4.2.1 Loading injectable mesh electronics into glass needles

Glass capillary needles (Drummond Scientific Co., Broomall, PA.) with inner diameter (I.D.) of 400 \( \mu \)m and outer diameter (O.D.) of 650 \( \mu \)m were used for injection tests. To load the free-standing mesh electronics, the glass needle was inserted in a micropipette holder (Q series holder, Harvard Apparatus, Holliston, MA), which was connected to a 1-mL syringe (NORMJECT\textsuperscript{®}, Henke Sass Wolf, Tuttlingen, Germany) through an Intramedic\textsuperscript{TM} polyethylene catheter tubing (I.D. 1.19 mm, O.D. 1.70 mm, Becton Dickinson and Company, Franklin Lakes, NJ). The syringe was used to manually draw the mesh electronics into the glass needle.

4.4.2.2 Preparation of hydrogel

0.5 g agarose (SeaPlaque\textsuperscript{®} Lonza Group Ltd., Basel, Switzerland) was mixed with 100 mL DI water in a glass beaker. The beaker was covered with a piece of aluminum foil (Reynolds Wrap\textsuperscript{®} Reynolds Consumer Products, Lake Forest, Illinois) to prevent evaporation and heated at boiling on a hot plate until the solution was clear; the final mass concentration was ca. 0.5%. The
solution was allowed to naturally cool to room temperature where it exists as a hydrogel with mechanical properties similar to those of dense brain tissue (14-18).

### 4.4.2.3 Vertebrate animal subjects

Adult (25-35 g) male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used as vertebrate animal subjects in this study. All procedures performed on the vertebrate animal subjects were approved by the Animal Care and Use Committee of Harvard University. The animal care and use programs at Harvard University meet the requirements of the Federal Law (89-544 and 91-579) and NIH regulations and are also accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC). Animals were group-housed on a 12 h: 12 h light: dark cycle in the Harvard University’s Biology Research Infrastructure (BRI) and fed with food and water *ad libitum* as appropriate.

### 4.4.2.4 Preparation of *ex vivo* mouse brains

C57BL/6J mice were euthanized via intraperitoneal injection of Euthasol (Virbac Corporation, Fort Worth, TX) at a dose of 270 mg/kg body weight in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (31). After euthanasia, mice were decapitated and brains were removed from the skull and placed in 4% formaldehyde for 24 h for fixation. Excess formaldehyde was removed by rinsing the fixed brain in 1X PBS for 24 h and the brain tissue was stored in fresh 1X PBS solution before controlled mesh electronics injection tests.

### 4.4.2.5 Controlled injection of mesh into hydrogel and *ex vivo* mouse brains

Either 0.5% agarose hydrogel as a brain tissue mimic or the *ex vivo* fixed brain tissue was placed in a petri dish. The glass needle loaded with mesh electronics was inserted in the micropipette holder, which was connected to a 5 mL syringe (Becton Dickinson and Company,
Franklin Lakes, NJ) through an Intramedic™ polyethylene catheter tubing (I.D. 1.19 mm, O.D. 1.70 mm). The 5 mL syringe was pre-filled with 1X PBS and mounted on a syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA). The micropipette holder was mounted on a stereotaxic stage equipped with a motorized linear translation stage (860A motorizer and 460A linear stage, Newport Corporation, Irvine, CA) that could move the stereotaxic arm in $z$ direction with constant preset velocity ranging from 0.05 to 0.5 mm/s. The needle was positioned at the surface of the 0.5% hydrogel or the *ex vivo* fixed mouse brain samples, and liquid was injected through the mesh-loaded glass needle at a volumetric flow rate of 10 ml/h to expel air bubbles from the entire injection system. The needle was then inserted into the injection medium to the desired depth and x-y coordinates. Controlled injection was carried out by synchronizing the syringe pump with the motorized linear translation stage, with a typical liquid injection rate of 20-50 mL/h and a typical translational stage retraction velocity of 0.2-0.5 mm/s. In the field of view (FoV) method, the liquid injection rate and the needle retraction velocity were independently adjusted such that the upper part of the mesh electronics, which was visualized through an eyepiece camera (DCC1240C, Thorlabs Inc., Newton, NJ), remained stationary in the FoV of the camera. The optimum volumetric flow rate and the needle retraction rate were determined through experimental optimization to achieve fully extended mesh morphology with minimum motion relative to the injected medium, and a general rule of thumb dictates that a greater volumetric flow rate is needed for wider and thicker mesh designs, smaller needle diameter and higher needle retraction rate. Typical solution volumes injected into the medium with 4 mm length mesh were 10-100 μL, on the same order of magnitude as the volume of liquid introduced during intracranial injection of virus vectors and enzymes in saline and artificial cerebrospinal fluid into rodent brain (ranging from 1-100 μL) (20-23). After the glass needle
was fully retracted from the injection medium, the volumetric liquid injection rate was increased to 100 mL/h to fully expel the mesh electronics from the needle onto the outer surface of the injection medium or a support used for making input/output (I/O) connections for external recording instruments. The extended morphology of the mesh in 0.5% hydrogel was verified by lowering the eyepiece camera to cover the lower part of the mesh electronics inside the transparent hydrogel. The targeting precision was estimated by tracking the motion of the bottom end of mesh electronics during the injection process using the same eyepiece camera, which had a pixel resolution of \(~4.2\) μm. For \textit{ex vivo} brain tissue, the morphology of the injected mesh was verified by micro-computed tomography (micro-CT) given the optical opacity of the tissue.

4.4.2.6 Controlled \textit{in vivo} injection of mesh electronics into mice brains

For \textit{in vivo} injection experiments, all metal tools in direct contact with the mice were autoclaved for 1 h and all plastic tools in direct contact with the mice were sterilized with 70% ethanol and rinsed with sterile DI water and sterile 1X PBS before use. Mesh electronic samples were sterilized by 70% ethanol followed by rinsing with sterile DI water and transferring to sterile 1X PBS. C57BL/6J mice were anesthetized by intraperitoneal injection of a mixture of 75 mg/kg of ketamine (Patterson Veterinary Supply Inc., Chicago, IL) and 1 mg/kg dexdomitor (Orion Corporation, Espoo, Finland). A heating pad (Harvard Apparatus, Holliston, MA) was set to 37°C and placed underneath the mouse to maintain body temperature. The depth of anesthesia was monitored via the toe pinch method (32). In a given experiment, a mouse was placed in the stereotaxic frame (Lab Standard Stereotaxic Instrument, Stoelting Co., Wood Dale, IL) with two ear bars and one nose clamp used to fix the head in position. Hair removal lotion (Nair®, Church & Dwight, Ewing, NJ) was used for depilation over the mouse head and iodophor was applied to sterilize the depilated scalp skin. A 5-mm longitudinal incision was made in the scalp, and the
scalp skin was resected over the sagittal sinus of the skull, exposing a 1 cm × 1 cm portion of the skull. Two 0.5-mm diameter burr holes were drilled using a dental drill (Micromotor with On/Off Pedal 110/220, Grobet USA, Carlstadt, NJ) according to the following stereotaxic coordinates: left burr hole: anteroposterior: -1.20 mm, mediolateral: -1.25 mm; right burr hole: anteroposterior: -1.20 mm, mediolateral: +2.45 mm. The dura was carefully incised and resected using a 27-gauge needle (PrecisionGlide®, Becton Dickinson and Company, Franklin Lakes, NJ). Sterile 1X PBS was swabbed on the surface of the brain to keep it moist throughout the surgery. The same injection process as described in “Controlled Injection of Mesh Electronics into Hydrogel and Ex Vivo Mice Brains” was used for injection of mesh electronics into the live mouse brain through the two burr holes. Typical solution volumes injected into the brain with 4 mm length mesh were 10-100 μL. After the two injections, the mice were euthanized via intraperitoneal injection of Euthasol at a dose of 270 mg/kg body weight and decapitated. The mouse head was fixed on a user-made stage for micro-CT imaging.

4.4.2.7 Micro-computed tomography

The morphologies of injected mesh electronics in opaque ex vivo brain tissue and decapitated mouse head after in vivo injection were imaged using an HMXST Micro-CT X-ray scanning system with a standard horizontal imaging axis cabinet (model: HMXST225, Nikon Metrology, Inc., Brighton, MI). Typical imaging parameters were 80 kV and 121 μA (no filter) for scanning the ex vivo brain tissue, and 115 kV and 83 μA (with a 0.1-mm copper filter for beam hardening) for scanning the decapitated mouse head with cranial bones. In both cases, shading correction and flux normalization was applied before scanning to adjust the X-ray detector. The CT Pro 3D software (ver. 2.2, Nikon-Metris, UK) was used to calibrate centers of rotation for micro-CT sinograms and to reconstruct the images. VGStudio MAX software (ver.
2.2, Volume Graphics GMbh, Germany) was used for 3D rendering and analysis of the reconstructed images. False colors were added using the *VGStudio MAX* software to differentiate the soft tissue, bones and the metal interconnect lines in the mesh electronics due to their different contrasts to X-ray.

### 4.4.3 Implementation and characterization of high-yield I/O bonding.

#### 4.4.3.1 Preparation of conductive ink

Carbon nanotubes (Stock No.: P093099-11, Tubes@Rice, Houston, TX) were received as slurry in toluene. The toluene was evaporated at 100 °C on a hot plate to carbon nanotube powders. 100 mg of carbon nanotube powder and 400 mg of sodium dodecylbenzenesulfonate (Sigma-Aldrich Corp., St. Louis, MO) were mixed with 4 mL DI water. The mixture was sonicated using a bath sonicator (Crest Ultrasonics Corp., Model 500D, Trenton, NJ) for 1 h at its maximum power (power setting = 9, power = 120 W) with replacement of the sonication bath every 20 min to maintain a bath temperature < 40 °C. Following sonication the concentrated carbon nanotube suspension could be stored at room temperature for 3 months without significant precipitation. A brief, 5-min sonication at power setting of 9 was performed immediately prior to using as conductive ink for I/O bonding.

#### 4.4.3.2 I/O bonding by conductive ink printing

The carbon nanotube-based conductive ink was loaded into pulled glass capillary tube (I.D. 400 μm, O.D. 650 μm), which serves as the printer head. After pulling (Model P-97, Sutter Instrument, CA), the tapered tip of the glass capillary tube was ground to yield the optimal 150 μm I.D.. The printer head was fixed with an electrode holder (Warner Instruments, Hamden, CT) and dipped into the freshly sonicated carbon nanotube conductive ink; capillary forces draw the conductive ink to height of ~1 cm in the printer head. The ink-loaded printer head was mounted
onto a motorized micromanipulator (MP-285/M, Sutter Instrument, Novato, CA) controlled by a rotary optical encoder (ROE-200, Sutter Instrument, Novato, CA) and controller (MPC-200, Sutter Instrument, Novato, CA). After the I/O part of the mesh electronics was unfolded and dried to expose all I/O pads on a 16-channel flexible flat cable (FFC, PREMO-FLEX, Molex Incorporated, Lisle, IL), a user-written LabVIEW program was used to take the desired start position (the position of the mesh I/O pad) and end position (the position of the electrode in the FFC cable) for each channel as input coordinates and compute the minimum path between the two positions. Then the LabVIEW program drove the printer head to print the conductive ink along each computed path automatically in a ‘hopping’ motion with a typical step size of 150 μm. After the 16 independent connections (between mesh I/O pads and FFC cable lines) each channel of the mesh electronics could be individually addressed.

4.4.3.3 Resistance characterization of I/O connections using conductive ink

Multiple 5 mm lines were printed using the above method with widths between 80 and 300 μm. The resistance of each line was characterized using four-point probe resistance measurement with the inner two probes recording the voltage and the outer two recording the current on an Agilent 4156C semiconductor parameter analyzer (Agilent Technologies Inc., Santa Clara, CA) to minimize contact resistances.

4.4.3.4 I/O bonding using anisotropic conductive film (ACF)

The I/O part of the mesh electronics was unfolded and dried on a glass slide to expose all I/O pads. A piece of ACF (CP-13341-18AA, Dexerials America Corporation, San Jose, CA) with a length of 15 mm and width of 1.5 mm was placed over the I/O pads and partially bonded at 75 °C and 1 MPa for 10 s using a commercial flip-chip bonder (Fineplacer Lambda Manual
Sub-Micron Flip-Chip Bonder, Finetech, Inc., Manchester, NH). Then an FFC cable was placed on top of the ACF, aligned with the mesh I/Os and bonded at 165-200 °C, 4 MPa for 1-2 min.

**4.4.3.5 Noise spectrum characterization of I/O connections**

The sensing electrodes of two identical sets of mesh electronics were immersed in 1X PBS and their I/O pads bonded using either the conductive ink printing or ACF methods. The FFC cable, which was bonded to the mesh I/O pads, was connected to an Intan RHD 2132 amplifier evaluation system (Intan Technologies LLC., Los Angeles, CA) through a home-made printed circuit board (PCB). Ag/AgCl electrode was used as a reference. For noise evaluation, electrical recording measurements were made with a 20-kHz sampling rate and a 60-Hz notch filter. The recorded traces were analyzed, and corresponding noise-power spectra were plotted after fast Fourier transform *(Fig. 4.8d).*
4.5 Bibliography


Chapter 5

Three-Dimensional Macroporous Nanoelectronic Networks as Minimally-Invasive Brain Probes

[The following chapter is derived in part from C. Xie, J. Liu, T.-M. Fu et al., *Nature Materials*, 14, 1286-1292 (2015).]

Direct electrical recording and stimulation using micro-fabricated silicon and metal micro-wire probes have contributed extensively to basic neuroscience and therapeutic applications; however, the dimensional and mechanical mismatch of these probes with the brain tissue limits their stability in chronic implants and decreases the neuron-device contact. Here, we demonstrate the realization of a three-dimensional macroporous nanoelectronic brain probe that combines ultra-flexibility and subcellular feature sizes to overcome these limitations. Built-in strains controlling the local geometry of the macroporous devices are designed to optimize the neuron/probe interface and to promote integration with the brain tissue while introducing minimal mechanical perturbation. The ultra-flexible probes were implanted frozen into rodent brains and used to record multiplexed local field potentials and single-unit action potentials from the somatosensory cortex. Significantly, histology analysis revealed filling-in of neural tissue through the macroporous network and attractive neuron-probe interactions, consistent with long-term biocompatibility of the device.
5.1 Introduction

At present, there is intense interest in the development of materials and electronic devices that can extend and/or provide new capabilities for probing neural circuitry and afford long-term minimally invasive brain-electronics interfaces (1-4). Conventional brain probes have contributed extensively to basic neuroscience (5, 6) and therapeutic applications (7-10), although they suffer from chronic stability and poor neuron-device contacts (4, 11-13). Recent studies of smaller (14, 15) and more flexible (16, 17) probes suggest that addressing size and mechanical factors could help overcome current limitations.

The most common neural electrical probes are fabricated from metal (18) and silicon (19, 20), materials that have very different structural and mechanical properties from those of brain tissue (21). Evidence suggests that mechanical mismatch is an important reason leading to abrupt and chronically unstable interfaces within the brain (4, 22). For example, motion of skull-affixed rigid probes in chronic experiments can induce shear stresses and lead to tissue scarring (13, 23), thereby compromising the stability of recorded signals on the timescale of weeks to months (4, 24, 25). More recent work has shown that flexible probes fabricated on polymer substrates (12, 17) and smaller-sized probes (11, 14) can reduce deleterious tissue response. More generally, there has also been effort developing flexible bioelectronics (26-28) and nanoscale devices for single-cell recording (29, 30). We have also shown that three-dimensional (3D) macroporous electronic device arrays can function as scaffolds for and allow 3D interpenetration of cultured neuron cell networks without an adverse effect on cell viability (31), and such networks can be injected by syringe through needles into materials, including brain tissue (32). In the latter case, it remains challenging to make the electrical input/output (I/O) connections needed for recording signals as the conventional I/O cannot pass through the injection needles.
5.2 Results and Discussion

Figure 5.1 Macroporous nanoelectronic 3D neural probes. (a) Schematic of the probe implanted in the brain. The macroporous and flexible probe (yellow lines) is implanted in the brain and connected to the cranially mounted I/O connector. (b) Schematic of the microscopic interface of the macroporous nanoelectronic brain probe with the neural circuit. The probe is constructed of polymer-encapsulated metal interconnecting and supporting elements (pink, purple and light blue), and arms (orange) that support and connect sensors (green). (c) Schematic of the mechanism of probe geometry controlled by built-in strain after the removal of the sacrificial layer (grey). Compressive strain elements (transverse blue lines) shape the probe into a cylindrical structure. Tensile strain elements (red lines) cause the sensor-supporting arms to bend outwards from the probe surface. (d) Schematic of an assembled macroporous probe with an I/O connector. The front end of the probe (pink lines) is suspended in buffer and the back end (orange pads) is attached to a carrier substrate and connected to the I/O connector. (e) Photograph of a
Figure 5.1 (Continued): typical macroporous nanoelectronic brain probe suspended in buffer with a cylindrical shape. Its back end is attached to the carrier substrate (the dark piece in the back) at the top of the image. Scale bar, 500 μm. (f) Micrograph of the sensor area of the probe outlined by the red dashed box in (e). The self-organization of the probe geometry, including global scrolling and outward bent supporting arms are visible. Scale bar, 200 μm. (g) Zoomed-in view of the outward bent supporting arm and sensor outlined by the yellow dashed box in (f). The black dashed box highlights the sensor element. Scale bar, 50 μm. (h) Dark-field micrograph of a typical nanowire FET voltage sensor at the end of the supporting arms. The arrow points at a nanowire as the sensor unit. Scale bar, 5 μm. (i) Bright-field micrograph of two typical Pt electrode voltage sensors each with 4 μm × 20 μm area. Scale bar, 5 μm.

Taking the above facts into consideration, we define an ideal implantable neural probe as possessing a stiffness similar to brain tissue to minimize/eliminate mechanically induced scarring, a high degree of porosity and cellular/subcellular feature sizes to allow interpenetration and integration of neurons and neural projections with the electronics, a means for implantation of the resulting extremely flexible structure, and facile I/O to allow multiplexed recording. Our strategy to meet these constraints focuses on implementing 3D macroporous nanoelectronic networks (31, 33), where the macroporous nanoelectronic probe has a mesh-like structure designed to promote interpenetration and close integration with neural tissue (Fig. 5.1a,b). The mesh design is unique in having a two-dimensional (2D) open area of about 80%, feature sizes to sub-10 μm scale, and, importantly, a high flexibility with an effective bending stiffness of <0.64×10^{-15} N m^2 (Supplementary Information) four to seven orders of magnitude smaller than conventional Si (34), carbon fiber (14) and thin polyimide (16, 35) neural probes. The exceptionally small bending stiffness yields mechanical interactions with tissue in the range of cellular forces. For instance, the force to deflect the two sensor-supporting arms by 10 μm (scale of a cell) is estimated to be about 10 nN, which is comparable to the single-cell migration force (36) (see Chapter 5.4). Our fabrication exploits conventional planar 2D lithography with a
sacrificial layer that is etched to yield the free-standing macroporous nanoelectronic probe (Fig. 5.1c). The overall design of the mesh probe (Figs. 5.1c and 5.2; Table 5.1) consists of longitudinal metal interconnects that are sandwiched between SU-8 polymer layers for passivation and transverse SU-8 polymer structural elements. In addition, transverse compressive strain elements are incorporated to generate positive transverse curvature and yield a cylindrical global probe structure, and local tensile strain elements in the supporting arms of each sensor device are incorporated to produce negative curvature, bending the devices away from the surface of the cylinder. All key materials and feature sizes of the macroporous nanoelectronic probe are summarized in Table 5.1. Facile I/O between the probe and measurement electronics is achieved by bonding a printed circuit board (PCB) connector to the remaining portion of the substrate attached to the free-standing macroporous probe following fabrication (Figs. 5.1d and 5.3a).

The macroporous probes were fabricated on standard silicon wafers with a nickel release layer using photolithography for multi-layer patterning (Fig. 5.3 and Chapter 5.4). Optical images at various resolutions of a representative probe structure before etching the nickel release layer (Fig. 5.3) highlight all key probe features: the entire probe structure bonded to the PCB interface connector (Fig. 5.3a); the portion of the probe supported on the Ni layer and the silicon wafer that remains bonded to the PCB (Fig. 5.3b); the lower part of the probe that will be released from the substrate and the overall locations of 19 sensor elements in this probe design (Fig. 5.3c); and images of several addressable sensor elements, including one specific nanowire detector (Fig. 5.3d,e).
Table 5.1. Key design features of a typical macroporous brain probe are summarized. All features are denoted in Fig. 5.3b-e.

<table>
<thead>
<tr>
<th>Key structural elements</th>
<th>Materials</th>
<th>Dimension</th>
<th>Thickness</th>
</tr>
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<tr>
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<td>6.5 mm, total length</td>
<td>Total ≤1 µm</td>
</tr>
<tr>
<td>ii. device region of the probe</td>
<td>SU-8 and metal</td>
<td>1 mm, total width</td>
<td>Total ≤1 µm</td>
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<tr>
<td>iii. vertical spacing of the devices</td>
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<td>250 µm</td>
<td>N/A</td>
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<td>iv. longitudinal spacing of interconnects</td>
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<td>v. longitudinal interconnects</td>
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<td>1.5/100/1.5 nm</td>
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<tr>
<td></td>
<td>SU-8</td>
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<td>400 nm/metal/400 nm</td>
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<tr>
<td>vi. transverse scrolling elements</td>
<td>Cr/Pd/Cr</td>
<td>3 µm, width</td>
<td>10-20/80/1.5 nm</td>
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<td></td>
<td>SU-8</td>
<td>10 µm, width</td>
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<td>vii. device bend-up arms</td>
<td>Cr/Pd/Cr</td>
<td>4 µm, width</td>
<td>1.5/80/30-50 nm</td>
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<td>SU-8</td>
<td>6 µm, width</td>
<td>400/metal/400 nm</td>
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<td>viii. sensor metal contact</td>
<td>Cr/Pd/Cr for FET</td>
<td>4 µm, width</td>
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<td>Cr/Pt for electrode</td>
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<td>SU-8</td>
<td>5 µm, width</td>
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<td>400 nm*, electrode</td>
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* At electrode sites, only bottom SU-8 layer was defined, and the top SU-8 layer was absent to expose the electrode contacts.
Figure 5.2. Major layers of a typical macroporous nanoelectronic brain probe design. Bottom-to-top correspond to the sequence defined in our fabrication flow (i.e., starting with global scrolling lines); details of fabrication steps provided in Chapter 5.4. The purple lines in the global scrolling layer correspond to Cr/Pd/Cr metal ribbons with 10-20/80/1.5 nm thicknesses, respectively, and 3 µm widths. Green lines in Bottom and top SU-8 layers indicate 400 nm thick, 7 µm wide SU-8 ribbons. Brown lines in the Interconnects layer represent 5 µm wide Cr/Au/Cr (1.5/100/1.5 nm) interconnect lines. Red lines in the Bend-up layer arms represent 3 µm wide Cr/Pd/Cr (1.5/80/30-50 nm) metal lines.
Figure 5.3. **2D precursor of the macroporous nanoelectronic brain probe.** (a) Photograph of a fully fabricated probe with attached and wire-bonded I/O connector before etching the sacrificial layer under the macroporous electronics structure. The carrier substrate (gray-black rectangle visible in lower-center 2/3’s of image) was mounted on a custom socket/PCB connector (upper 1/3 of image), with I/O to recording instrumentation made by connections to the socket. Electrical connections between the carrier chip and PCB board were made by wire bonding (position highlighted by the black arrow). Scale bar: 1 cm. (b) Zoom-in of the mesh electronics area of the probe from the dashed white box in (a). The region below the red arrow is on the nickel sacrificial layer. Scale bar: 1 mm. (c) Micrograph of the white dashed-box in (b), which is released from the substrate following dissolution of the nickel sacrificial layer. The
**Figure 5.3 (Continued):** whole probe is designed with an open mesh structure to promote interpenetration and integration with the neural tissue. The individually-addressable sensors are located at design-specified positions in the mesh electronics; for the design shown the sensor elements define two edges of an inverted triangle (bottom 1/3 of the image). Scale bar: 200 μm.

(d) Zoomed-in view of the sensor area of the probe (black dashed-box in (c)). The strains, which define global scrolling of the mesh electronics and bend-out of the individual sensor elements, are represented by blue and red pseudo-colored regions, respectively. The compressive strain applied in the blue regions generates positive curvature along the transverse direction of the probe, and the tensile strain applied in the red regions generates negative curvature on supporting arm of the sensor (shown in Fig 5.1c, in main text). Scale bar: 100 μm. (e) Zoomed-in view of an individual sensor element (nanowire FET sensor, position highlighted by the black arrow), outlined by the black dashed box in (d). The sensor is located at the tip of the supporting arms. Scale bar: 20 μm. In the panels, (i) – (viii) denote key design elements of the probe; the relevant materials and dimensions of these elements are summarized in Table 5.1.

Significantly, optical images of the probe structure following removal of the sacrificial nickel layer and underlying wafer (see Chapter 5.4), and immersion of the free-standing portion in aqueous buffer (Fig. 5.1e–i), highlight key features of our design. First, lower-resolution images (Fig. 5.1e) show that the transverse compressive strain elements produce self-organization of the probe into a global cylindrical shape as designed. This cylindrical geometry distributes the electronic sensor elements around the probe surface. Second, higher-resolution images (Fig. 5.1f,g) demonstrate that the local tensile strain introduced in the supporting arms of each sensor element bends these arms outwards such that each of the sensor elements is about 100 μm away from the cylindrical probe surface. The average leakage impedance of the SU-8-encapsulated metal interconnect components (Fig. 5.4) was >10GΩ at relevant frequencies, which demonstrates the effectiveness of the SU-8 passivation used in the probes. For sensor elements, we have incorporated either silicon nanowire field-effect transistors (FETs, Fig. 5.1h) or micrometer-scale platinum metal electrodes (Fig. 5.1i) into our probes during fabrication (see
Chapter 5.4). Characterization of device performance and neural recording with these different sensor elements are discussed below.

![Image](image.png)

**Figure 5.4. Frequency dependent leakage measurements of the SU-8 encapsulated interconnect components.** The impedance was measured between 8 pairs of interconnect metal lines in a nanowire FET probe submerged in 1X PBS, at 100 mV bias and 1-10 kHz. The area of each interconnect line in the 1X PBS solution was 2 mm (length) × 20 µm (width). The solid line indicates the average of the measurements and the error bars indicate ± 1-standard deviation.

The high flexibility of our macroporous probes precludes direct insertion (14, 20) into neural tissue, and invasive surgery, which could allow placement of macroporous probes in specific brain regions, would largely eliminate many potential advantages of our design. To overcome this conundrum, we exploited the combination of built-in strain and liquid surface tension as probes are moved from liquid to air, as shown schematically in Figs. 5.5a and 5.6. Specifically, on withdrawing probes from liquid to air, liquid surface tension increases the global curvature to yield straight cylinders with diameters of about 100-200 µm (Fig. 5.5b). In addition, surface tension causes the supporting arms of the sensors to be ‘pulled back’ to yield a smooth probe surface on moving from liquid to air. Notably, the supporting arms return to the outward bent position with the sensors away from the probe surface when the probe is returned to an
aqueous environment (Fig. 5.5b). We recently reported a syringe injection approach for 2D mesh structures, although in this case all I/O connections must be introduced after injection.

Figure 5.5. Probe preparation and robustness. (a) Schematic of the probe geometry control before implantation. The global curvature of the probe increases and the supporting arms are flattened as the probe is pulled from liquid to air. (b) Four time-lapse images of the probe front-end geometry while it was moving in and out of the buffer. The dashed line marks the buffer surface (air above; buffer below). The black arrows indicate the direction of motion. Scale bar, 200 μm. (c) Photograph of the probe in the ‘frozen’ state held in air by the carrier substrate. Scale bar, 500 μm. (d) Sensor yield test following liquid nitrogen freezing and room-temperature thaw cycles. Left: Number of active nanowire FET sensors versus freeze–thaw cycle number. Right: Nanowire FET transconductance versus cycle number for 6 out of the 14 sensor elements.
Figure 5.6. Schematic of probe preparation and frozen insertion. (a) The fabricated macroporous neural probe on silicon carrier chip (prior to release) is attached to an I/O connector and electrical contacts are made by wire bonding. (b) The Ni sacrificial layer is etched and the macroporous neural probe was partially released and freestanding in liquid. (c) Excess silicon chip below the connector is trimmed off. (d) When the device assembly is removed from the buffer solution, a small amount of buffer is trapped inside the cylindrical probe. Removal was carried out manually with a vertical speed of ca. 1-2 mm/s. (e) The probe assembly is then slowly submerged in liquid nitrogen (LN$_2$) until ca. thermal equilibrium (5-10 seconds; greatly reduced LN$_2$ boiling). (f) The probe is immediately mounted in a custom-holder on a linear translational stage and rapidly inserted into the brain. The insertion step was driven manually with a speed of ca. 5mm/s. The entire process should be carried out within 10 s following removal from the LN$_2$, with the insertion into the brain taking no more than ca. 1 second.
This cylindrical probe structure has mechanical advantages compared to a flat structure, and although it remains too soft to penetrate brain tissue as removed from solution, rapid freezing in liquid nitrogen (Fig. 5.5c) provides sufficient rigidity to allow controlled insertion into hydrogel that has mechanical properties similar to dense neural tissue (37). In particular, rapid insertion of the frozen probe into 0.5% agarose gel (Fig. 5.7) yields an extended configuration >2 mm into the hydrogel, whereas an unfrozen probe would simply be deformed at the gel surface without penetration. To determine whether freezing could be a reliable insertion and measurement strategy, we also studied the electrical performance of fully assembled probes when subjected to repeated cycles of exposure to liquid nitrogen freezing and room-temperature aqueous solution. Significantly, characterization of a nanowire FET-based probe following 150 freeze/thaw cycles (Fig. 5.5d) showed that 12 out of 14 (86%) FET sensors on the probe remained connected, and that 14/14 devices remained active up to 21 cycles. In addition, the device sensitivities showed <14% change on average after the 150 cycles, thus confirming the reliability of this approach. The observed robustness of the FET sensors is particularly encouraging because each device requires continuity of two interconnect lines, versus a single line for metal electrode sensors.

We have implanted the macroporous nanoelectronic probes in rodents. In a typical implantation procedure (Fig. 5.8a), the frozen macroporous probe is stereotaxically positioned and rapidly inserted to a specific region of the brain of an anaesthetized rodent (Chapter 5.4). The positioning and inserting processes are kept within about 10 s and 1 s, respectively, to ensure successful insertion (Fig. 5.6). Images recorded post-insertion (Fig. 5.9a) highlight the high flexibility of our macroporous probe outside the brain, which allows positioning without moving the implanted portion within the tissue. In addition, a representative bright-field microscopy
image of a post-insertion fixed tissue sample sectioned along the longitudinal axis of the probe (Fig. 5.9b) verifies an extended linear structure within the brain tissue.

Figure 5.7. Probe geometry after insertion into hydrogel. Photograph showing a probe tethered to the carrier substrate following partial insertion into 0.5% agarose hydrogel with ~2 mm buffer solution on top. The 0.5% agarose gel provides a mechanical resistance during insertion similar to brain tissue (48). The insertion procedure is the same as discussed in the main text. The probe was frozen by immersion in liquid nitrogen, immediately followed by insertion, which typically took ca. 1 second. The dashed line indicates the gel/buffer boundary, and the arrow points the entry point. Scale bar: 2 mm.
Figure 5.8 Neural activity recording from rodent models. (a) Photograph of a typical rodent stereotaxic surgery. A rat was held in a stereotaxic frame, and a macroporous nanoelectronic probe was implanted into the brain through a cranial hole. The probe was attached to the carrier substrate for external electrical connections (Chapter 5.4). (b) Acute LFP recording by nanowire FET sensors from the barrel cortex area. Left: Schematic of the correlation between the neural activity in the barrel cortex and the rat whisker sensory behaviour. Inset: Schematic map of sub-areas in the barrel cortex. The red circle indicates the targeted sub-area. Right: Traces from four neighbouring sensors, where yellow areas mark stimulations applied to the whisker C1. Relative positions of the four sensors are marked in the schematic on the right. Scale bar, 200 μm. (c) Acute multiplexed LFP recording from 13 nanowire FET sensors following probe insertion into the somatosensory cortex. Relative positions of the 13 sensors are marked in the schematic on the left. Scale bar, 200 μm. (d) Top: Representative acute single-unit recording from Pt electrode sensors. Bottom: Zoomed-in view of nine single-unit events outlined in the top panel. (e) Superimposed 94 single-unit events from the recording in (d). The mean waveform of all traces is plotted in red.

We have exploited the capability to target specific brain regions via stereotaxic insertion of the frozen macroporous nanoelectronic probes to test their *in vivo* recording capabilities in
rodents, where all of the reported measurements are acute and performed within 0.5-2 h post-implantation. First, a probe was implanted in the barrel cortex area of the rat brain as it represents a somatosensory cortex region with well-defined mapping between cortical columns and facial whiskers (38). Signals recorded from four nanowire FET sensors showed strong signals in element-2 (6.3 ± 0.4 mV), corresponding to separate stimulations applied to the whisker C1 on the contralateral side of the implantation site (Fig. 5.8b), which was identified as the corresponding whisker by testing all whiskers (Fig. 5.7). In contrast, the neighboring FET sensor element-3 recorded a similar but much weaker signal pattern, whereas element-1 and -4, which are about 200 and 250 μm, respectively, from element-2, yielded no observable response.

Figure 5.9. Probe insertion into a rat brain. (a) Zoomed-in view of the implantation site in Fig. 5.8a. The arrow points the small and clean entry site into the brain. The part of the probe outside the rat brain (left of arrow) is relaxed and conformal to the brain surface due to its ultra-flexibility. Scale bar: 1 mm. (b) Bright field image of a brain slice cut along the probe insertion direction. The brain was fixed and sliced following the procedure described in Chapter 5.4 within 1 hour after implantation to reveal the probe geometry. The image shows clearly that the probe maintains a straight cylinder shape as designed, thereby yielding a predictable sensor distribution within the tissue. Scale bar: 500 μm.

Second, acute recording experiments made in the somatosensory cortex of an anaesthetized rat (Fig. 5.8c) demonstrate the capability for larger-scale multiplexed recording
with the macroporous nanoelectronic probes. Specifically, we recorded signals \((3.4 \pm 0.3 \text{ mV})\) simultaneously from 13 nanowire FET sensors on a single probe. The relatively large signal amplitude compared with that recorded by metal electrode sensors (typically <0.5 mV) is attributed to the active sensing nature of the FET sensors, which do not suffer from signal loss by shunt pathways (39). The dominant modulation frequency, 1-4 Hz, is characteristic of δ-wave local field potentials (LFPs) in anaesthetized rats (40). A spatial map of the recorded LFP is plotted in Fig. 5.7a. The similarity and coherence between channels is consistent with the fact that the LFPs spread beyond (41) the dimension of the macroporous probe recording region, about 100 μm laterally and 1 mm vertically (Fig. 5.10). Multiplexed recording experiments were conducted more than ten times using nanowire FET sensor probes, and all experiments resulted in >80% active sensor yield and similar recording performance in terms of potential shapes and amplitude. In addition, probes with platinum electrode sensors implanted in the somatosensory cortex region of a mouse brain (Fig. 5.8d) exhibited sharp millisecond spikes. Standard data processing and spike sorting (Chapter 5.4 and Fig. 5.8e) yielded a uniform potential waveform with an average duration of 1.8 ms and a peak-to-peak amplitude of 172 μV, characteristic of single-unit action potentials. The high signal-to-noise ratio (>7) of the single-unit recording suggests a close proximity between the sensor and the firing neurons (42), and thus provides at least comparable brain activity recordings to those of conventional probes (13), but with the advantage of being chronically much more biocompatible, as discussed below.
Figure 5.10. Identification of implanted sub-region of barrel cortex. (a). A photograph of whiskers on the contralateral side of the implantation side. Mechanical stimulations were applied to all whiskers to identify the sensor close to a sub-region of barrel cortex. In this implantation and recording, element-2 (Fig. 5.8b) had the strongest response during stimulation. Subsequently, stimulation of individual whiskers on both contralateral and ipsilateral sides were carried out while simultaneous recording from the implanted mesh probe. We observed that only stimulations applied to the C1 whisker (indicated by dashed circle) on the contralateral side could elicit a strong response from element-2 of the probe. (b) Three representative recordings from element-2. Red, while stimulating C1 whisker; blue, while stimulating the adjacent whisker C2; black, while stimulating whisker C1 after the rat was euthanized. (c) A 4-second map of the multiplexed LFP recording from the same experiment as shown in Fig. 5.8c. The vertical axis represents the depth beneath the brain surface. The horizontal axis indicates the recording time. Colors highlight the amplitude of the recorded LFPs.

The chronic response of neural tissue to our ultra-flexible macroporous probes has been addressed through histology studies carried out five weeks post-implantation. A schematic of a
macroporous probe inserted into the somatosensory cortex (Fig. 5.11a) highlights the perpendicular orientation (with respect to the implanted probe) at which the tissue was sectioned at different times post-implantation. In general, slices were prepared after fixing the brain tissue using standard procedures (Chapter 5.4) without removing the ultra-flexible macroporous nanoelectronic probes. Comparison of bright-field optical images recorded from similar acute (Fig. 5.11b, left) and chronic 5-week post-implantation (Fig. 5.11b, right) tissue slices highlights several key points. First, the acute slice exhibits a tissue void within the interior of the roughly hollow cylindrical probe structure, which is consistent with ablation or displacement of tissue during implantation of the frozen probe. Second, images from the tissue 5-week post-implantation shows no void, thus indicating that cells and/or neural projections interpenetrate through the macroporous probe over time to fill the acute void. In addition, analysis of chronic images (dashed white box, Fig. 5.12a) shows that at least some of the sensor arms can achieve the designed bend-out geometry post-implantation. This feature can facilitate positioning sensor devices away from any residual tissue damage resulting from implantation.

To evaluate more critically the chronic response of our macroporous nanoelectronic probes we used immunochemical staining (14) of cross-section slices containing our probes. Confocal microscopy images of a region including a macroporous probe (Fig. 5.11c) show a normal growth density of neuron cell bodies (NeuN) in close proximity, <50 μm, to the probe components (Fig. 5.12d), although the soma density inside the probe cylinder is lower than outside. The close proximity of neuron cell bodies is in contrast to typical chronic observations reported for other types of neural probes (4, 19, 25, 43), which we discuss further below. In addition, glial fibrillary acidic protein (GFAP) expression is slightly elevated at the center of the probe, but not at the outer edge of the probe or surrounding region. Indeed, the spatial
dependence of the GFAP signal encompassing this outer probe edge is similar to that in the control sample (Fig. 5.11d) prepared at the same time from the contralateral hemisphere without an implanted probe. These data suggest that the slightly elevated GFAP expression inside the probe cylinder is due to the acute tissue damage during implantation, and show, importantly, that our ultra-flexible macroporous probes do not elicit chronic immune response post-implantation.

Figure 5.11 Implanted macroporous nanoelectronic probe–tissue histology. (a) Schematic of brain slice sample preparation. The blue dashed line indicates the slice direction perpendicular to the implanted macroporous probe. (b) Bright-field images of the probe/tissue interface cross-section. The dark objects in the image are components of the probe. Left: Bright-field image of a 100-μm-thick acute slice. Scale bar, 100 μm. Right: Bright-field image of a 20-μm-thick cross-section slice five weeks after implantation. The white dashed box highlights the area imaged and shown in Fig. 5.11e. Scale bar, 20 μm. (c) Projection of 3D reconstructed confocal micrograph of immunochemically labelled cross-section slice in right-side panel in (b)
**Figure 5.11 (Continued):** (five weeks post-implantation). The pseudo colour coding is as follows. Blue: nucleus, Hoechst; green: NeuN, labelling neuron nuclei; white: SU-8; and red: GFAP, specifically labelling reactive astrocytes. The fluorescent intensity profiles of the red channel (astrocyte) along the long axis of the two dashed box areas are plotted in the right panel. Scale bar, 20 μm. Similar data from a slice obtained about 150 μm deeper in the brain on this same probe is shown in Fig. 5.12e. (d) Immunochemical staining image of the control sample for e obtained from the contralateral hemisphere of the same mouse. The fluorescent intensity profiles of GFAP along the long axis of the two dashed box areas are plotted in the right panel. Scale bar, 20 μm. (e) Projection of 3D reconstructed confocal micrograph of immunochemically labelled cross-section slice in (b). The pseudo colour coding is as follows. Blue: nucleus, Hoechst; green: β-tubulin-III; orange: SU-8; and red: GFAP. Scale bar, 10 μm. (f) Green channel (β-tubulin-III) fluorescence intensity plotted along the outer curved portion of the probe area outlined in the dashed area in (e) from left to right. Orange bars indicate the positions of the mesh components. The blue dashed line indicates the average of the β-tubulin-III fluorescence intensity for the entire imaged area in (e). All tissue slices were prepared post-implantation into the somatosensory cortex region of mice (Chapter 5.4), as shown schematically in (a).

To further characterize the robustness of these results and the details of the chronic distribution of neurons about the macroporous probe, we have stained tissue slices with β-tubulin-III, which can label both soma and neurites, from independent implantation experiments (Figs. 5.11e,f and 5.12). Significantly, confocal microscopy images recorded five weeks post-implantation show no significant drop in β-tubulin-III expression inside (9.8 a.u., average) versus outside (10.2 a.u., average) the probe. These results suggest that neural projections, unlike somas, have sufficient mobility to interpenetrate and partially fill the central void produced during initial implantation. Moreover, analyses of the fluorescent intensity in the region immediately outside the probe (Figs. 5.11f and 5.12) show no sign of suppressed neuron growth immediately adjacent to the probe structural elements; indeed, these data indicate that the
neuronal fluorescence signal within 10μm of the probe components (20–50 a.u., average) is more than two to five times the value for neurons averaged over the entire image. In addition, measurements made from a tissue/probe slice 150 μm deeper in the brain than shown in Fig. 5.11c and from an independent probe implant that partially collapsed during slow insertion (Fig. 5.12) exhibited similar results, and thus indicate that the macroporous probe structure is attractive to neurons (for example, neurophilic) and does not elicit the usual immune response. Similar results are observed in studies of syringe-injected mesh electronics (32), although conventional micro-wire (4, 25) and silicon (19, 43) neural probes as well as ultra-small but rigid (14) and flexible 2D polymer probes (16, 17) show enhanced GFAP/astrocyte proliferation and a reduction of neuron density near these probe surface. As chronic failure of conventional brain probes involves neuronal loss and the encapsulation of non-neuronal cells such as astrocytes up to several hundred micrometers from the probe surface (4, 43), our results suggest substantial benefits of the macroporous nanoelectronic probes for future chronic recording studies.
**Figure 5.12. Histology of the probe-tissue interface.** (a) Bright field images of a 20 μm thick slice showing the interface 5 weeks post implantation. The brain tissue has interpenetrated the probe with time (compared with acute cross-section images (e.g., Fig. 5.11b)). Dashed box indicates a pair of supporting arms with a sensor at the end. Scale bar: 50 μm. (b) The reconstructed confocal micrograph of immunochemically labeled cross-section slice shown in Fig. 5.11e, where the curved dashed box here encompasses tissue both inside and outside the indicated the curved probe surface. The pseudo color-coding is as follows. Blue: nucleus stained with Hoechst; green: neurons stained with β-tubulin-III; orange: SU-8, and red: GFAP. Scale bar: 10 μm. The fluorescence intensity in the dashed area is used to analyze the affinity of neurons to probe components. (c) The average neuron fluorescence intensity from green channel (β-tubulin-III, neuronal tissue) along the short axis of the outlined area in (b) is plotted, from inside of the probe to outside. The orange line indicates the position of probe. The blue dashed line indicates the average fluorescence intensity of the green channel in the whole image. These results demonstrate there is a higher density of neurons near the probe components, and thus suggest a tendency of neurons to form tight junctions with the probe components. The tissue slices were prepared 5-weeks post implantation into the somatosensory
Figure 5.12 (Continued): cortex region of a mouse (Chapter 5.4). (d) Zoom-in view of a bend-up nanowire sensor in Fig. 5.11c (indicated as dashed box area in (a)) illustrating the proximity of sensor arms and neurons as well as the intact SU-8/metal/SU-8 structure. Scale bar: 10 μm. (e) A reconstructed confocal micrograph of immunochemically labeled cross-section from the same mouse brain and brain probe sample as used in Fig. 5.11c. Scale bar: 100 μm. Inset: Zoom-in view of the white boxed region. Scale bar: 20 μm. The sample was ca. 5 slices/120 μm deeper in the brain relative to Fig. 5.11c; the same staining and imaging methods were used for both samples. (f) A “collapsed” probe resulting from slow implantation of the frozen probe (i.e. insertion time of the probe in the brain tissue longer >1 s). The probe becomes randomly folded due to thawing before the insertion was completed. This result demonstrates the importance of rapid insertion of the frozen probes to maintain the designed geometry. Although the probe did not hold the designed geometry, this probe also showed tight integration with neurons, which is consistent with the macroporous ultra-flexible nature of the structure. The tissue slices were prepared 5-weeks post implantation into the somatosensory cortex region of a mouse using the same the same staining method as in in Fig. 5.11c (Chapter 5.4). Scale bar: 20 μm.
5.3 Conclusion

In summary, our 3D macroporous nanoelectronic probes, which feature both ultra-flexibility comparable to neural tissue and open structures with subcellular feature sizes allowing neuron interpenetration, represent a new strategy to merge 3D nanoelectronic devices with the neural circuits in the brain. We have shown that the ultra-flexible macroporous probes can be stereotaxically implanted in a frozen state into rodent brains with minimal surgical and acute tissue damage, and demonstrated the capability of recording multiplexed LFPs and single-unit action potentials from the somatosensory cortex. Significantly, chronic histology studies revealed unique characteristics, including a filling-in of neural tissue through the macroporous network and attractive neuron–probe interactions, which is in contrast to results from other solid and more rigid probe designs (19, 20), and are consistent with a unique long-term stability and biocompatibility of the probe–tissue interface. Although it will be important in future studies to develop these probes further, for example by extending the chronic histology studies to shorter and longer times, and increasing the number of sensor elements available for multiplexed recording and/or introducing stimulation capabilities, we believe the present chronic histology and acute recording studies already show the unique advantages of our ultra-flexible 3D macroporous electronic probes and indicate the importance of exploring the stability of chronic neural activity mapping and implants for next-generation brain-machine interfaces in the near future.
5.4 Methods and Materials

5.4.1 Nanowire synthesis

Uniform 30 nm $p$-type single crystal silicon nanowires were synthesized using our reported gold nanocluster-catalyzed vapor-liquid-solid methodology (43). In a typical synthesis, the total pressure was 40 torr and the flow rates of $\text{SiH}_4$, diborane ($\text{B}_2\text{H}_6$, 100 p.p.m. in $\text{H}_2$), and hydrogen ($\text{H}_2$, Semiconductor Grade), were 2, 2.5 and 60 standard cubic centimetres per minute (SCCM), respectively. The silicon-boron feed-in ratio was 4000:1, and the total nanowire growth time was 30 min.

5.4.2 Macroporous nanoelectronic brain probe fabrication

The macroporous nanoelectronic brain probes were fabricated with key steps as follows: 

(i) photolithography and thermal deposition were used to pattern a 100 nm nickel sacrificial layer, where the nickel served as the final relief layer for the free-standing probe. (ii) Photolithography and thermal deposition were used to define the Cr/Pd/Cr (10-20/80/1.5 nm) non-symmetric metal ribbons to generate the strain for global scrolling. Typically, this layer consists of 3-$\mu$m wide parallel ribbons. (iii) A 300-500 nm layer of SU-8 photoresist was defined by photolithography as the bottom SU-8 passivation layer. Typically, this layer consists of 7-$\mu$m wide parallel ribbons. (iv) Either nanowire FETs or Pt electrodes were patterned as the voltage sensors (details in the following section). (v) Photolithography and thermal deposition were used to define the Cr/Pd/Cr (1.5/80/50-80 nm) double metal ribbons to generate strain for local bend-out from the global cylindrical structure. Typically, this layer consists of 3-$\mu$m wide metal interconnect lines. (vi) Photolithography and thermal deposition were used to define the non-strained metal contacts, Cr/Au/Cr (1.5/100/1.5 nm), to address each sensor and form interconnections to the input/output pads, which are patterned outside the Ni sacrificial layer. This layer usually consists of 5 $\mu$m
wide metal interconnect lines. \textit{(vii)} Another 300-500 nm thick layer of SU-8 photoresist was defined by photolithography as the top SU-8 passivation. This layer typically has the same pattern as the bottom SU-8 passivation layer in \textit{(iii)}.

It should be noted that Pd is a toxic metal and may cause side effect if exposed. In this work the Pd is well protected by the SU-8 encapsulation. However, in future long-term applications, other metals, such as Pt or Ti, which are known to make good contact with Si nanowire (44) and can provide controllable strain (45), can be used to replace Pd to eliminate possible toxicity.

5.4.3 Nanowire FET sensor patterning

(i) A 300 to 400 nm layer of SU-8 photoresist was deposited on the fabrication substrate, prebaked (65 °C/2 min; 95 °C/4 min), and then (ii) silicon nanowires were aligned on the SU-8 layer by contact printing as described previously (46). (iii) Photolithography was used to define the nanowire device regions, and after post-baking (65 °C/2 min; 95 °C/2 min), the pattern was developed by SU-8 Developer washed with isopropanol (2 times, 30 s per wash) to remove nanowires outside of the device regions. (iv) The new SU-8 pattern was cured at 180 °C/20 min. (v) Nanowire device contacts were defined by photolithography and Cr/Pd/Cr (1.5/50–80/1.5 nm) metallization.

5.4.4. Metal electrode sensor patterning

Cr/Pt (1.5/100 nm) electrodes are patterned by photolithography. The electrode size used in this work was 4 µm × 20 µm, with typical impedance of 600 ± 20 kohm at 1 kHz.

5.4.5 Calculation of the bending stiffness for different neural probes
The mechanical characteristics of the three-layer longitudinal ribbons make the dominant contribution to the probe-tissue interface. The bending stiffness of a single ribbon, $K_{1R}$, can be estimated as (47)

$$K_{1R} = E_s \left( \frac{h^3 w_1}{12} - \frac{h_m^3 w_m}{12} \right) + E_m \frac{h_m^3 w_m}{12}$$  \hspace{1cm} (5.1)

where $E_s$ is young’s modulus of SU-8, $E_m$ is young’s modulus of gold, $h$ is the total thickness of ribbon, $h_m$ is the thickness of metal, $w_1$ is the total width of ribbon and $w_m$ is the width of metal. When $E_s = 2$ GPa, $E_m = 79$ GPa, $h = 800$ nm, $h_m = 100$ nm, $w_1 = 7$ µm, and $w_m = 5$ µm, $K_{1R} = 0.64 \times 10^{-15}$ N·m².

The bending stiffness of another representative component of the probe, the sensor device support arm, $K_{1S}$, is calculated similarly taking $w_1 = 6$ µm, $w_m = 4$ µm. $K_{1S} = 0.54 \times 10^{-15}$ N·m².

The bending stiffness of standard silicon probes or planar thin film probes, $K_2$, can be estimated as (47)

$$K_2 = E \frac{wh^3}{12}$$  \hspace{1cm} (5.2)

where $E$ is the young’s modulus of the probe material, $h$ is the thickness of the probe, and $w$ is the width of the probe. When $E_{\text{silicon}} = 165$ GPa, $h_{\text{silicon}} = 15$ µm, and $w = 100$ µm, the bending stiffness of a typical silicon probe is $K_{2s} = 4.6 \times 10^{-8}$ N·m². When $E_{\text{polyimide}} = 2$ GPa, $h_{\text{polyimide}} = 10$ µm, and $w = 100$ µm, the bending stiffness of a typical polyimide probe is $K_{2P} = 0.16 \times 10^{-10}$ N·m².

The bending stiffness of ultrasmall carbon electrodes, $K_3$, can be estimated as (47)

$$K_3 = E_{\text{carbon}} \frac{\pi d^4}{64}$$  \hspace{1cm} (5.3)

where $E_{\text{carbon}}$ is the young’s modulus of carbon fiber, $d$ is the diameter of carbon fiber probe. When $E_{\text{carbon}} = 234$ GPa, $d = 7$ µm, $K_3 = 2.73 \times 10^{-10}$ nN·m².
5.4.6 Estimation of neural probes bending force

The force, $F$, to deflect a piece of three-layer longitudinal ribbons is estimated by (47)

$$F = \frac{8eK_{1R}}{l^2}$$

(5.4)

where $e$ is the deflection of the ribbon, $K_{1R}$ is the bending stiffness of the ribbon and $l$ is the length of the ribbon. For the two support arms of the sensors, take $e = 10 \, \mu m$, $K_{1R} = 0.54 \times 10^{-15}$ N·m², and $l = 200 \, \mu m$. $F = 10.8 \, nN$. 
5.5 Bibliography


16. Kim, T. I. et al. Injectable, cellular-scale optoelectronics with applications for wireless...


47. Javey, A., Nam, S., Friedman, R. S., Yan, H. & Lieber, C. M. Layer-by-layer assembly of


Chapter 6

Stable Long-Term Chronic Brain Mapping at the Single Neuron Level

[The following chapter is derived in part from T.-M. Fu et al., Nature Methods, 13, 875-882 (2016).]

Stable in vivo mapping and modulation of the same neurons and brain circuits over extended periods is critical to both neuroscience and medicine. Current electrical implants offer single-neuron spatiotemporal resolutions but face challenges of relative shear motion and chronic immune response, which yield signals shifting from targeted neurons and necessary probe position adjustments to break glial scarring. Here, we overcome these limitations by developing a chronic in vivo recording/stimulation platform based on ultra-flexible mesh electronics and demonstrate stable multiplexed local field potentials (LFPs) and single-unit recordings from mouse brains for at least eight months without probe repositioning. Data show almost unchanged principal components, average spike waveforms, stable firing dynamics and phase-locking of spike firings/LFP oscillations, thus suggesting robust tracking of the same neurons over this period. This platform also illustrates stable single-neuron responses to chronic electrical stimulation. The capability for long-term recording is applied to longitudinal studies of brain ageing, where the firing dynamics and spike characteristics of the same individual neurons are followed, and freely behaving mice. These demonstrated advantages could open up future studies in mapping and modulating changes associated with learning, ageing and neurodegenerative diseases.
6.1 Introduction

Highly stable mapping and modulation of the same neural network with cellular spatiotemporal resolutions over time periods from months to years could impact substantially work focused on illuminating fundamental neuroscience questions such as how existing neurons evolve into neural circuits with diverse dynamics through learning and developmental processes (1-5). Moreover, such advances could improve brain-machine interfaces (BMIs) by enabling reliable decoding from individual neurons versus ensemble averages of large population activities for prosthetic applications (6-10). In addition, this capability could enable longitudinal, rather than cross-sectional, studies of ageing-associated brain changes and abnormal cognitive declination caused by neurodegenerative diseases (11-16).

Approaches such as noninvasive brain imaging (17, 18), surface (19) and endovascular probes (20) can provide long-term monitoring of brain activity, although their low spatiotemporal resolutions preclude investigations of circuit dynamics at cellular level. Optical imaging techniques can achieve neuron-resolution mapping, but are limited in terms of penetration depth, image acquisition rates and genetic incorporation of labels (21). On the other hand, electrical implants, such as silicon probes and microwire tetrodes (22-25) can provide spatiotemporal mapping at the single-neuron level from deep brain regions. However, mechanical mismatch and chronic immune responses, which result in shear motion, glial scar formation and neuron depletion at probe/brain interfaces, lead to degradation of recording/stimulation capabilities typically over days to weeks (8-10, 26-28). Months- to year-long stable recordings from a small fraction of electrical implants have been claimed occasionally (29-31), although other researchers report intra-day to week instability using the same types of probes (10, 27). Practically, probes are repositioned to compensate for recording
instability (24, 25, 27, 28, 32), although this necessarily causes further tissue damage and precludes monitoring of the originally targeted neurons. These issues have thus limited implanted probes for long-term neuroscience and clinical studies.

Factors reported to contribute to chronic instability of implanted probes, include size and mechanical mismatch with neurons and neural tissue (8, 33-35). Indeed, studies focused on reducing probe size (34) have reduced deleterious immune response, although these probes still exhibit substantial mechanical mismatch with neural tissue. To address these structural/mechanical issues, we have recently described mesh electronics (36-38) with micrometer feature sizes comparable to neuron somata and effective bending stiffness values comparable to dense neural tissue. In this work, we demonstrate a chronic recording/stimulation mesh electronics platform that overcomes relative shear motion and chronic immune response limitations of conventional probes, and thereby enables consistent and reproducible recording from and stimulation of the same individual neurons in vivo for at least eight months.
6.2 Results and Discussion

6.2.1 Brain injection and recording interface for mesh electronics

Mesh electronics were fabricated using standard photolithography procedures (36, 37) (Fig. 6.1). The overall design (Fig. 6.2) consists of an array of 16 recording or stimulation electrodes at one end addressed individually by metal interconnects, and terminated with input/output (I/O) pads at the opposite end of the mesh structure. The interconnects are sandwiched by insulating and biocompatible polymer layers, leaving only the recording/stimulation electrodes in direct contact with brain tissue. The thicknesses and widths of the mesh elements (see Chapter 6.4) are ca. 800 nm and 20 μm, respectively, which yield

Figure 6.1. Schematic steps of mesh electronics fabrication. Components include silicon wafer (light green), nickel relief layer (dark green), polymer ribbons (blue), metal interconnects (black) and exposed metal electrodes (red). For each step (a-g) both top and side views are shown, where the side view corresponds to a cross-section taken at the position indicated by the white horizontal dashed line in the top view image of (a). (h) Zoomed-in views of regions highlighted by black (exposed Pt electrodes) and red dashed boxes (fully passivated interconnects) in (g).
ultra-low bending stiffness values of ~0.1 nN\,m, comparable with neural tissue (39) and correlate with a low immune response (36, 38).

**Figure 6.2. Schematic structure of mesh electronics.** (a) Schematic of the injectable mesh electronics. Blue lines highlight the overall mesh structure and indicate the regions of supporting and passivating polymer layers, the ~horizontal orange lines indicate Au metal interconnects between input/output (I/O) pads (orange filled circles, indicated by the magenta arrow) and Pt recording electrodes (green filled circles), respectively. The solid-line black box at left highlights several of the recording electrodes, and the dashed-line black box in the middle highlights several of the metal interconnects of the mesh electronics. (b) A zoomed-in view of the recording electrodes highlighted by solid-line black box in (a). (c) A zoomed-in view of the mesh electronics highlighted by dashed-line black box in (a). (d) A zoomed-in view of a unit cell of the mesh (dashed-line green box in (c)) with the same color codes as in (a, c). Polymer ribbons (blue) with and without metal interconnects (orange) correspond to longitudinal and transverse elements, respectively. L1 and L2 show spacing between longitudinal and spacing between transverse elements, respectively; W1 and W2 are widths of the longitudinal and transverse mesh elements, respectively; and Wm is the width of metal interconnect lines.
An overview of our approach (Fig. 6.3) highlights the flexible open mesh electronics and light-weight instrument interface. First, stereotaxic injection was used to deploy mesh electronics through a capillary needle into a targeted brain region (see Chapter 6.4) with positioning.
precision of \( \sim 20 \mu m \) (Figs. 6.4 and 6.5a,b), an extended morphology and integration of the mesh structure with neurons (Fig. 6.3a). Micro-computed tomography (micro-CT) post-injection (Fig. 6.3b) confirmed an extended morphology along the injection direction. Second, the I/O pads of mesh electronics were unfolded onto and electrically connected to a light-weight (~0.2 g) flexible flat cable (FFC) using printed conductive ink (37) (Figs. 6.3c and 6.5c,d). The ultra-flexibility of the electronics was visualized (yellow arrow, Fig. 6.3c) as the rolled-up mesh ‘sagging’ between the exit point on the brain/skull and the FFC. The FFC, which is plugged into recording instrumentation, was fixed to the mouse skull and folded to minimize its size (Figs. 6.3b,d and 6.5e,f). Finally, the positions of mesh electronics were not adjusted over the course of our chronic experiments following implantation.

Figure 6.4. Schematics illustrating method used to load the mesh electronics into a glass capillary needle and subsequently inject into a medium. (a) The injectable mesh electronics (dark blue) suspended in solution (light pink) was drawn into the glass needle such that the I/O pads (yellow dots, indicated by green arrows) of the mesh enter the tube first. (b) After the mesh electronics was fully loaded into the glass needle with mesh end at needle tip (magenta dashed box), the needle was removed from the solution. (c) The glass needle was mounted in an x-y-z manipulator for injection into solution, gel/polymer or tissue (light blue). Black arrows indicate the direction of the fluid flow during loading and injection.
Figure 6.5. Mesh electronics injection and chronic recording from mouse brain. (a) Image of a mouse fixed in a stereotaxic frame with scalp skin retracted, and a hole drilled through the skull plate. The glass needle (yellow arrow) loaded with mesh electronics is visible directly above the skull in this image. A flexible flat cable (FFC) is visible at the left of the image supported on a ceramic scaffold. A 0-80 grounding screw (white arrow) was positioned at the posterior side of the skull. (b) Image showing the relocation of the I/O end of the post-injected mesh electronics (red arrow) towards the FFC. (c) Image post-injection into the brain showing the input/output (I/O) region of the mesh electronics unfolded onto the FFC. Inset shows a zoom-in view of the red dashed box, which highlights the unfolded I/O part (red arrow) of the injected mesh electronics. (d) Image representing the unfolded I/O pads of the mesh electronics, which were electrically-connected to the FFC using the conductive ink printing process described in ref. 37. The red dashed box highlighted by the inset shows the details of the bonding with the red and cyan arrows indicating the mesh electronics and conductive ink connections, respectively. (e) Image showing the FFC fixed on top of the mouse skull using dental cement. (f) Image of a fully awake but restrained mouse during chronic in vivo brain recording. The white and black arrows highlight the grounding screw and the connector between FFC and external recording setups, respectively. See Chapter 6.4 for all experimental details of surgery and injection.
6.2.2 Long-term brain activity mappings at the single-neuron level

Initial long-term recording stability was assessed from 16-channel mesh electronics spanning the hippocampus (HIP) and somatosensory cortex (CTX) of a mouse. Representative multiplexed recordings from the same awake mouse at two and four months post-injection yielded well-defined LFPs in 16/16 channels with modulation amplitudes ~300 μV and single-unit spikes from 14/16 channels (Fig. 6.6a). Focusing on single-unit spikes, we found that different channels exhibited stable amplitudes and signal-to-noise ratios (SNRs) across this 2-month period (Fig. 6.6a). Cross-channel correlation maps of LFPs and single-unit spikes (Fig. 6.7) showed similar patterns over this time period. The constant single-unit amplitudes and similar spike firing patterns over time suggest that these data might correspond to signals from the same neurons and neural circuits. We address this central issue further below with studies extending to 8 months.

Multiplexed data recorded over at least 6-month periods from four mice (Figs. 6.6b and 6.8e Mouse 1-4) showed an initial amplitude increase followed by stable spike amplitudes ~6 weeks post-injection. Representative chronic single-unit recording traces from one electrode (Mouse4-ChannelA) highlighted several key points. First, peak-to-peak spike amplitudes increased from ~30 μV (1 week) to ~130 μV (2 months) and thereafter remained stable to at least 6 months post-injection, with overall firing rate approximately constant across the entire period (Fig. 6.8a). Second, two distinct clusters of sorted spikes with visually stable waveforms indicative of two neurons were observed (Fig. 6.8b). Waveform autocorrelation analyses (27) showed quantitatively a large percentage of similarity (see Chapter 6.4) both within the same recording session and across 6 months (Fig. 6.8c,d). Together these results suggest stable single-neuron recording during this extended time period. The electrode interfacial impedances (Figs.
6.6c and 6.8f) further showed relatively constant values (mean ~300 kΩ) over time, distinct from other brain implants with reported electrode impedance fluctuations attributed to chronic immune response (8, 26).

Figure 6.6. Long-term stable recording without signal degradation over six months and immunohistochemistry staining of mesh electronics/brain tissue interface. (a) Representative 16-channel local field potential (LFP) (heat maps) with amplitudes color-coded according to the
**Figure 6.6 (Continued):** color bar on the far right and single-unit spike (traces) mapping from the same mouse at two (left) and four (right) months post-injection. The x-axes show the recording time while the y-axes represent the channel number of each recording electrode with relative position marked by red dots in the schematic (leftmost panel). **(b)** Time evolution of average spike amplitudes of representative channels from four different mice. Mouse1 represents the recordings shown in (a) with Channel A and B denoting Channel 10 and 3, respectively. Mouse2-Channel A, B and Mouse3-Channel A were used for analyses shown in Figs. 6.10, 6.13, 6.16 and 6.17. Mouse4-Channel A represents the recordings shown in Fig. 6.8. **(c)** Time-dependent impedance values at 1 kHz of the channels shown in (b). **(d)** Immunohistochemical staining images of horizontal brain slices at 2 (left, hippocampus (HIP)), 6 (middle, cortex (CTX)) and 12 weeks (right, CTX) post-injection. Red, yellow and blue colors correspond to neurofilaments, NeuN and mesh electronics, respectively. Scale bar: 100 μm. **(e)** Neurofilament (red), NeuN (yellow), GFAP (cyan) and iba-1 (purple) fluorescence intensity normalized against background values (gray dashed horizontal lines) plotted versus distance from the interface (see Chapter 6.4). The pink-shaded regions indicate the interior of the mesh electronics. All error bars in this figure reflect ±1 standard error of the mean (s.e.m.).

To explore the mechanism of the observed long-term single-neuron signal stability and provide insight into the short-term amplitude increase, we carried out time-dependent histology studies (see Chapter 6.4). Representative confocal fluorescence microscopy images of horizontal brain slices with mesh electronics at 2, 6 and 12 weeks post-injection (Figs. 6.6d and 6.9) illustrate several important features. The 6- and 12-week images (Fig. 6.6d, middle and right panels) showed axonal projections and somata filling the mesh electronics interior, and quantitative analyses (Fig. 6.6e) demonstrated signals of axonal projections and somata close to and returning to background levels near the outer surface and interior of the mesh electronics, respectively. Notably, astrocyte and microglia data (Fig. 6.6e) showed signals close to and slightly below background near the outer surface and interior of the mesh, respectively.
The 2-week post-injection data provides insight into the initial increase in spike amplitude. Specifically, there was an interior depression in cell density (not evident at ~6 weeks) remaining from acute insertion damage, although quantitative analyses (Fig. 6.6e) demonstrated axonal projections, astrocytes and microglia in this region. These data also showed that astrocytes and microglia were somewhat enhanced up to 100-300 μm from the probe surface (returned to background, ~6 weeks). Hence, the observed amplitude increase can be associated with recovery from acute damage via gradual removal of astrocytes (and microglia).

Figure 6.7. Correlation maps of chronic multiplexed brain recording. (a,b) Correlation maps of 16-channel local field potential (LFP) recordings at two (a) and four (b) months post-injection. (c,d) Correlation maps of 16-channel extracellular action potential recordings at two (c) and four (d) months post-injection. Colors indicate the correlation coefficient between any two given channels according to the color bar shown on the far right. All the maps were calculated from 2 s long data traces at both time points. See Chapter 6.4 for details of correlation coefficient calculations.
Figure 6.8 Long-term stable recording without signal degradation over six months. (a) Representative extracellular action potential recordings from the same electrode (Mouse4-ChannelA shown in Fig. 6.6b) located in somatosensory cortex (CTX) at different time points post-injection. (b) Spike sorting results of the corresponding recordings shown in (a). All of the spikes from the corresponding time points in (a) are included with ca. 50 spikes at each point. (c) Autocorrelation histograms of average waveforms of sorted spikes from 30 1-min segments within a 30-min recording session for Mouse4-ChannelA at 3 months post-injection. (d) Autocorrelation histograms of average waveforms for each of the two identified clusters (color coded in magenta (neuron 1) and green (neuron 2)) for Mouse4-ChannelA at the 8 time points shown in (b). See Chapter 6.4 for details of waveform autocorrelation calculations. (e) Time
**Figure 6.8 (Continued):** evolution of normalized average peak-to-peak spike amplitudes across all channels with single-unit action potentials recorded (the average was done across 14, 5, 6 and 7 channels for Mouse 1 to 4, respectively) from four mice. The spike amplitude was normalized (value=1.0, gray dashed horizontal lines) against the average peak-to-peak amplitude values between 5 to 26 weeks post-injection for each channel. (f) Average impedance values at 1 kHz across all channels with recorded single-unit action potentials of the four mice shown in (e) plotted as a function of time over the same time period. The shaded areas in (e) and (f) indicate ±1 standard error of the mean (s.e.m.).

![Figure 6.8](image)

**Figure 6.9.** Immunohistochemical images of horizontal sectioned mouse brains containing mesh electronics from different times. (a) GFAP stained images of representative horizontal brain slices used for normalized fluorescence intensity plots (Fig. 6.6e) showing the interfaces between mesh electronics and astrocytes, and (b) iba-1 stained images of representative horizontal slices used for normalized fluorescence intensity plots (Fig. 6.6e) showing the interfaces between mesh electronics and microglia at 2, 6 and 12 weeks post-injection. All samples were 10 μm-thick. Blue, cyan and purple correspond to mesh electronics, GFAP and iba-1, respectively. Scale bars: 100 μm.
6.2.3 Chronic tracking of individual neurons

Figure 6.10. Consistent tracking of the same group of neurons. (a) Time evolution of representative single-unit spikes of Mouse2-ChannelA shown in Fig. 6.6 clustered by principal component analysis (PCA) over eight months post-injection. The x- and y-axis denote the first and second principal component, respectively, and the z-axis indicates post-injection time. The color scale bars show the corresponding post-injection time points from 5 to 34 weeks (8 months) of the 3D PCA plots. (b) Time course analysis of average spike waveforms from each PCA cluster shown in (a). (c) Time evolution of inter-spike interval (ISI) histograms of each of
Figure 6.10 (Continued): the 3 neurons identified in (a) from 3 to 34 weeks. Bin size: 20 ms. (d) Scatter plot with analysis of variance (ANOVA) of the firing parameter (n=32 for each neuron), $\lambda$, obtained by fitting each ISI distribution profile shown in (c) to an exponential decay. (e) Polar plots showing the phase-locking of single-unit spikes to theta oscillations (4-8 Hz) of LFPs in HIP for each of the 3 neurons in (a) at 3 and 34 weeks. (f) Scatter plot with ANOVA test of the locked phase angle (n=32 for each neuron). For (d) and (f), the open rectangles and bars indicate 25/75 and 0/100 percentiles, respectively; **** indicates p-value of <0.0001.

Statistical analyses were carried out to confirm the chronic stability of recorded neuron/neural circuit signals. Principal component analysis (PCA), which can define the number and stability of recorded single-neuron signals over time (32, 34), of representative sorted spikes (Fig. 6.10a) showed the same three clusters with nearly constant positions in the first and second principal component plane (PC1-PC2) from 5 through 34 weeks (8 months) post-injection. Time-dependent averaged spike waveforms (Fig. 6.10b), waveform auto-/cross-correlation (27) (Fig. 6.11a) and L-ratio analyses (40) (Table 6.1) further demonstrated good unit separation and high stability over this time period (see Chapter 6.4).

Figure 6.11. Autocorrelation and cross-correlation analyses of average spike waveforms across all recording time points. (a) Auto- and cross-correlation histograms for three identified neurons of Mouse2-ChannelA from week 3 to 34 post-injection shown in Fig. 6.10b. (b) Auto- and cross-correlation histograms for two identified neurons of Mouse2-ChannelB from week 1 to
Figure 6.11 (Continued): 34 post-injection shown in Fig. 6.13c. (c) Auto- and cross-correlation histograms for two identified neurons of Mouse3-ChannelA from week 1 to 26 post-injection shown in Fig. 6.13d.

Table 6.1. L-ratio of sorted spike clusters. L-ratio calculated with the same degree of freedom (df) of eight for each identified neuron in Mouse2-ChannelA, Mouse2-ChannelB and Mouse3-ChannelA at 2, 4 and 6 months post-injection. An L-ratio < 0.05 for a specific isolated unit among all sorted spikes is considered good separation/isolation as discussed previously (40, 53). See Chapter 6.4 for details of L-ratio calculations.

<table>
<thead>
<tr>
<th></th>
<th>Mouse2-ChannelA Neuron1</th>
<th>Mouse2-ChannelA Neuron2</th>
<th>Mouse2-ChannelA Neuron3</th>
<th>Mouse2-ChannelB Neuron1</th>
<th>Mouse2-ChannelB Neuron2</th>
<th>Mouse3-ChannelA Neuron1</th>
<th>Mouse3-ChannelA Neuron2</th>
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<tr>
<td>2 months</td>
<td>2.7 × 10^{-3}</td>
<td>2.2 × 10^{-3}</td>
<td>1.9 × 10^{-3}</td>
<td>4.2 × 10^{-4}</td>
<td>2.4 × 10^{-3}</td>
<td>3.2 × 10^{-3}</td>
<td>1.7 × 10^{-3}</td>
</tr>
<tr>
<td>4 months</td>
<td>3.1 × 10^{-3}</td>
<td>2.0 × 10^{-3}</td>
<td>4.7 × 10^{-3}</td>
<td>7.2 × 10^{-4}</td>
<td>6.6 × 10^{-3}</td>
<td>4.3 × 10^{-3}</td>
<td>3.6 × 10^{-3}</td>
</tr>
<tr>
<td>6 months</td>
<td>4.0 × 10^{-6}</td>
<td>1.1 × 10^{-3}</td>
<td>5.5 × 10^{-3}</td>
<td>4.2 × 10^{-4}</td>
<td>1.4 × 10^{-4}</td>
<td>1.8 × 10^{-2}</td>
<td>9.9 × 10^{-3}</td>
</tr>
</tbody>
</table>

The individual neuron firing dynamics was characterized by the inter-spike interval (ISI) distributions for the 3 identified neurons (Fig. 6.10c; see Chapter 6.4). Notably, these ISI histograms exhibited stable and distinct distributions with characteristic 2-3 ms refractory period (41) (Fig. 6.12) over 8 months. Analysis of the variance (ANOVA) on the firing parameter, λ (reflecting neuron firing rates (41)) obtained from exponential fits to each ISI histogram, showed a significant difference (p-value<0.0001) between any two neurons, thus confirming the same neurons were followed over this 8-month period. Similar analyses were carried out for another channel from the same mesh (Figs. 6.11 and 6.13, Mouse2-ChannelB) and one channel from another mouse (Figs. 6.11 and 6.13, Mouse3-ChannelA). Results showed unchanged principal components (Fig. 6.13a,b), L-ratios demonstrating good unit separation (Table 6.1), constant
spike waveforms supported by auto-/cross-correlation (Figs. 6.11b,c and 6.13c,d), and stable ISI histograms and firing parameters (Fig. 6.13e-h).

**Figure 6.12. Refractory periods of neuron firing.** (a-c) Interspike interval (ISI) histograms of the data shown in Fig. 6.10c at 26 weeks post-injection but replotted with a bin size of 1 ms. The data show clearly a 2-3 ms refractory period (orange-shaded regions) for neuron 1 (a), neuron 2 (b) and neuron 3 (c).

To test the potential for stable recording from neural circuits, we analyzed phase-locking between single-neuron firings and LFPs (see Chapter 6.4) with a focus on HIP data, which has been reported to show phase-locking (42). Rayleigh Z-test (see Chapter 6.4) of the recorded data (Fig. 6.10e) showed clear evidence for phase-locking at distinct angles from 3 to 34 weeks post-injection for each of the three neurons identified (Fig. 6.14) (43). Statistical analyses demonstrated stable and distinct phase-locked angles for all neurons over time (Table 6.2) with means of 330, 250 and 95-degrees (Fig. 6.10f), showing that mesh electronics can record from the same neural circuit over 8 months (44).
**Figure 6.13. Consistent tracking of the same group of neurons.** (a,b) Time evolution of representative single-unit spikes of Mouse2-ChannelB (a) and Mouse3-ChannelA (b) shown in Fig. 6.6 clustered by principal component analysis (PCA) over eight and six months, respectively. In each plot, the x- and y-axis denote the first and second principal component, respectively, and the z-axis indicates post-injection time. (c,d) Time course analysis of average spike waveforms from each PCA cluster shown in (a) from 1 to 34 weeks post-injection (c), and for each PCA cluster shown in (b) from 1 to 26 weeks (d), respectively. (e,f) Time evolution of ISI histograms of each of the 2 neurons identified from the PCA clusters in (a) and (b) from 3 to 34 (e) and 3 to 26 weeks (f), respectively. Bin size: 20 ms. (g,h) Scatter plot with analysis of
Figure 6.13 (Continued): variance (ANOVA) (n=32 and 26 for (g) and (h), respectively) of the firing parameter, $\lambda$, obtained by fitting each ISI distribution profile shown in (e) and (f) to an exponential decay. All plots for Mouse3 are shown up to 26 weeks post-injection instead of 34 weeks shown for Mouse2, because Mouse3 was older than Mouse2 when the initial mesh electronics injection was carried out, and thus exhibited ageing-associated changes (shown in Figs. 6.16 and 6.17) at an earlier post-injection time point.

![Image](image.png)

Figure 6.14. Rayleigh Z-test of neuron phase-locking behavior. (a-c) Rayleigh Z-test of all recording data for neuron 1 (a), neuron 2 (b) and neuron 3 (c) identified for Mouse2-ChannelA from week 3 to 34 post-injection (n=32). Each trial presents the recording data at a given week. The null hypothesis is: a given neuron does not have phase-locking behavior at a given week. The majority of trials on the right side of the vertical dashed lines suggest a >95% confidence interval (p<0.05) to reject the null hypothesis.

Table 6.2. Rayleigh Z-test of locked-phase alteration for each neuron shown in Fig. 6.14.
The null hypothesis is: The locked phase of a specific neuron has a random distribution through time evolution. A Ln(Z) value of >1.09 (i.e., Z>2.97) (sample size n=32) can reject the null hypothesis with a confidence interval >95% (p value of <0.05) (43). See Chapter 6.4 for details of Rayleigh Z-test.

<table>
<thead>
<tr>
<th>Neuron 1</th>
<th>Neuron 2</th>
<th>Neuron 3</th>
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<tbody>
<tr>
<td>Z-value</td>
<td>27.85</td>
<td>27.76</td>
</tr>
<tr>
<td>Ln(Z)</td>
<td>3.33</td>
<td>3.32</td>
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6.2.4 Multi-site recording from different brain regions

We also demonstrated stable chronic recording from two mesh probes injected into distinct brain regions. The I/O pads from the two meshes could be connected to the same FFC as shown schematically (Fig. 6.15a) and experimentally (Fig. 6.15b), and yielded insignificant increase in the interface weight. Multiplexed LFP recordings from mesh electronics implanted in motor CTX (red) and HIP (blue) of opposite cerebral hemispheres showed similar modulation within a probe but distinct signals between probes (Fig. 6.15c). Both probes exhibited stable LFP amplitudes across at least 2 months (Fig. 6.15c). Representative single-unit spike traces (Fig. 6.15d) demonstrated consistent chronic firing dynamics, and spike-sorting (Fig. 6.15d) showed stable amplitudes and consistent cluster waveforms (2 identified neurons in CTX and 4 in HIP) over this time period.

6.2.5 Multifunctional mesh for chronic stimulation and recording

We expanded the capabilities of mesh electronics by incorporating 150-μm diameter low-impedance stimulation electrodes (Fig. 6.15e). Chronic stimulation (see Chapter 6.4) and recording from 4 to 14 weeks post-injection highlighted key features. First, a peristimulus spike raster plot (Fig. 6.15f) showed an increased firing rate following stimulation. Second, histograms of first-spike latency following stimulation for two recording electrodes (Fig. 6.15g, channels 1&2) exhibited consistent distributions from 4 to 14 weeks in weekly stimulation trials. Control data recorded from a second mesh implanted in the contralateral hemisphere showed no stimulation-evoked response. Third, spike-sorting and PCA analyses of channel 1 and 2 (Fig. 6.15g, insets) confirmed stable recording of two unique neurons for both electrodes, although a third neuron was identified at ca. 14 weeks in channel 1.
Figure 6.15. Multi-site and multifunctional mesh electronics. (a) Schematic and (b) photo showing two mesh electronics (white arrows) injected into different brain regions (motor CTX of the right cerebral hemisphere and HIP of the left hemisphere) of the same mouse. The two mesh probes were bonded to the same FFC (red dashed box in b) (c) Multiplexed LFP and (d) single-unit recordings along with sorted spikes from the motor CTX (red traces) of one hemisphere and the HIP (blue traces) from the contralateral hemisphere. The three columns correspond to data recorded at 2 (left), 3 (middle) and 4 (right) months post-injection, respectively. The red and blue arrows in (c) highlight the channels corresponding to the representative spike trains shown in (d).
Figure 6.15 (Continued): (e) Photograph showing typical mesh electronics before releasing from substrate with unipolar stimulation electrodes (black arrow) and recording electrodes (red arrows). Scale bar: 200 μm. Inset: Zoomed-out photograph with yellow dashed box representing the area of (e). Inset scale bar: 1 mm. (f) Peristimulus raster plot showing spike events (black ticks) of 150 stimulation trials (red solid line: stimulation pulse). Inset: Representative recorded spike trains from -0.15 to 0.85 s. The red arrow indicates the stimulation pulse. (g) First spike latency distributions of stimulus-evoked firings recorded from two different electrodes (Channels 1 & 2) located in the same cerebral hemisphere but with progressively increasing distance from the stimulation electrode at 4, 6 and 14 weeks post-injection. Spike-sorting and PCA clustering results are displayed as insets. The color of each sorted spike cluster corresponds to that of each PCA group.

6.2.6 Longitudinal studies of ageing at single-neuron level

The long-term recording stability with mesh electronics can enable longitudinal studies of ageing-associated changes at single-neuron and neural-circuit levels. Previous research has been limited to longitudinal studies with low spatiotemporal resolution (11, 18) or high-resolution electrophysiology studies comparing different subjects due to chronic instability (11-13). We tracked the time evolution of firing dynamics and spike characteristics of individual neurons before and after two mice entered middle age, 10-12 months (45). These data (Figs. 6.16 and 6.17) revealed ageing-associated neuronal changes. First, analyses of ISI histograms showed firing rate declines for mice aged ~48 weeks and older with individual neurons exhibiting distinct time-dependent changes (Figs. 6.16a,b i and 6.17e,f). For example, neurons 2 and 3 of Mouse2-ChannelA (Fig. 6.16a, I) showed decreases in firing rate starting at ~48 weeks, while the firing of neuron 1 was relatively unaffected. Similar decreases were seen for Mouse3-ChannelA (Fig. 6.16b, I). Second, we found no systematic changes in electrode impedances (Fig. 6.17g), and histology study showed uniform distributions of neuronal somata, axonal projections, astrocytes and microglia through the mesh electronics interior at ~1 year post-
injection (Fig. 6.17h,i). These results suggest minimal or no degradation of recording electrodes, and correspondingly argue that the observed firing rate decreases are intrinsic to individual neurons. Third, quantitative analyses of sorted spike waveforms (Fig. 6.16a,b II) revealed a neuron-specific increase of peak-to-trough time, $\tau$, starting at $\sim$48 weeks of mouse age; that is, neurons 2 and 3 of Mouse2-ChannelA (Fig. 6.16a, II) both showed increases in $\tau$, with little or no increase observed for neuron 1. These increases in single-neuron peak-to-trough times coincided temporally with the firing rate decreases, and were especially prominent for neurons with larger firing rate decreases (Fig. 6.16a,b).

**Figure 6.16. Longitudinal study of brain ageing at the single-neuron level.** (a,b) Time evolution of average spike firing rate (I) and average peak-to-trough time $\tau$ (defined in upper left inset in (a, II)) with average spike waveforms shown as bottom right insets (II) of each neuron identified from the PCA clusters in Fig. 6.17 from representative channels of Mouse 2 (a) and 3 (b), respectively. The x-axes show the corresponding mouse ages in all panels. The error bars in (I) show fitting errors, and * indicates statistical significant ($p <0.05$, double-sided t-test, n=50) decrease of firing rate compared with that at age of 48 weeks. The error bars in (II) show $\pm$ 1 standard deviation (SD).
Figure 6.17. Tracking of the same individual neurons during brain ageing. (a,b) Time evolution of representative single-unit spikes of Mouse2-ChannelA (a) and Mouse3-ChannelA (b) shown in Fig. 6.6 clustered by PCA from 35 to 57 weeks of age. In each plot, the x- and y-axes denote the first and second principal components, respectively, and the z-axis indicates the corresponding mouse age. (c,d) Representative 2D (PC1-PC2 plane) plots of the PCA results.
Figure 6.17 (Continued): shown in (a,b) at week 52 and 57 of mouse age. (e,f) Time evolution of ISI histograms of each of the neurons identified from the PCA clusters in (a) and (b) from 35 to 57 weeks of age. Bin size: 20 ms. (g) Impedance values at 1 kHz of the two channels shown in (a-f) plotted as a function of mouse age over the same period. (h) Immunohistochemical images of a 10 μm-thick horizontal CTX brain slice showing the mesh electronics/brain tissue interface at 1-year mouse age. Red, yellow and blue colors correspond to neurofilaments, NeuN and mesh electronics, respectively. Scale bar: 100 μm. (i) Neurofilament (red), NeuN (yellow), GFAP (cyan) and iba-1 (purple) fluorescence intensity normalized against background values (gray dashed horizontal lines) plotted versus distance from the interface (see Chapter 6.4). The pink-shaded regions indicate the interior of the mesh electronics. All error bars in this figure reflect ±1 standard error of the mean (s.e.m.).

6.2.7 Chronic recording from freely behaving mice

Figure 6.18. Chronic recordings from a freely behaving mouse. (a) Photograph of a typical freely behaving mouse recording. Voltage-amplifier was directly positioned near the mouse head to minimize mechanical noise coupling. A flexible serial peripheral interface (SPI) cable was
**Figure 6.18 (Continued):** used to transmit amplified signals to the data acquisition systems. Inset: A zoom-in view showing the conductive ink (black lines), FFC (red arrow), Omnetics connector (yellow arrow) and the voltage-amplifier (blue-green rectangle). (b) Single-unit spike recordings at 5 weeks post-injection from five representative channels, two of which (Channels D & E, shown in red) are located in the somatosensory CTX, when the mouse was whisking food pellets (I) and foraging in the cage (II). (c) Bar charts summarizing the changes in firing rate for the same five channels as shown in (b) during whisking (black bars) and foraging (white bars) at 5 (top), 10 (middle) and 27 weeks (bottom) post-injection. The two channels (Channels D & E) with whisking-associated neuronal responses are highlighted with red borders. Error bars indicate ±1 s.e.m.. (d) Polar plots showing phase-locking of single-unit spikes recorded in the CTX barrel field (Channel D) to the theta oscillations (4-8 Hz) of LFPs in the HIP (Channel A) when the mouse was whisking (left column) and foraging (right column) at 5, 10 and 27 weeks.

Last, mesh electronics were directly bonded to a preamplifier (preamp) connector following injection (see **Chapter 6.4**) for chronic studies of freely behaving mice. The lightweight interface was only 1.0 g with the preamp plugged-in (0.35 g without preamp), allowing data acquisition through a highly flexible cable that did not restrict animal motion (**Fig. 6.18a**). The interface had minimal impact on the housed animal without preamp given its low profile and weight. Single-unit recordings from five channels at 5 weeks post-injection (**Fig. 6.18b**) were grouped into periods when the mouse was whisking food (I) or foraging (II) in a novel environment. The two channels located in CTX barrel field (Channels D&E) consistently showed behavior-related firing rate increases during whisking, while the other three channels exhibited no significant changes across the 27-week measurement course (**Figs. 6.18c** and 6.19). Analyses of sorted spikes within the same recording session (**Fig. 6.19a**) revealed comparable fluctuations in the intrinsic recording noise (**Fig. 6.19b; Table 6.3**) and stable unit isolation (**Fig. 6.19c**). Interestingly, phase locking analyses between single-unit firings in barrel CTX (Channel D) and theta-band LFP oscillations in HIP (Channel A) at different time points indicated
relatively constant locking at ~300 degrees during active whisking versus no identifiable phase coherence during foraging (Fig. 6.18d). These findings are consistent with a pathway linking the barrel field that receives vibrissa input and HIP with higher-order processing of texture information in agreement with recent reports (46, 47).

**Figure 6.19. Spike recordings from a freely behaving mouse.** (a) Sorted single-unit action potentials from chronic freely behaving mouse recordings. Each column represents the sorted spikes from an individual channel shown in Fig. 6.18 at 5, 10 and 27 weeks post-injection. (b)
Figure 6.19 (Continued): Noise distributions of all red unit clusters of Channels A-E shown at week 10 in (a, I), and the intrinsic recording noise of each corresponding electrode of the channel at the same week (II). See Chapter 6.4 for details of noise calculations. (c) Single-unit spikes of Channel D in (a) clustered by PCA when the mouse was actively whisking (I) and foraging (II) at week 5, 10 and 27.

Table 6.3. Noise distribution of sorted spikes. Standard deviation of noise distribution within an entire recording session of all sorted spike clusters at week 10 post-injection shown in Fig. 6.19a, and comparisons with intrinsic recording noise of each channel. Colors in column-1 correspond to the color-coded spike clusters in Fig. 6.19a. See Chapter 6.4 for details of standard deviation extraction.

<table>
<thead>
<tr>
<th>Standard deviation of noise distribution (μV)</th>
<th>Channel A</th>
<th>Channel B</th>
<th>Channel C</th>
<th>Channel D</th>
<th>Channel E</th>
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<tbody>
<tr>
<td>Red</td>
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<td>7.52</td>
<td>5.20</td>
<td>6.51</td>
<td>7.23</td>
</tr>
<tr>
<td>Cyan</td>
<td>NA</td>
<td>NA</td>
<td>10.79</td>
<td>7.83</td>
<td>9.13</td>
</tr>
<tr>
<td>Blue</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>8.49</td>
<td>9.27</td>
</tr>
<tr>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>9.42</td>
</tr>
<tr>
<td>Intrinsic recording noise</td>
<td>5.58</td>
<td>5.98</td>
<td>5.79</td>
<td>7.00</td>
<td>9.10</td>
</tr>
</tbody>
</table>
6.3 Conclusion

In summary, our chronic in vivo mesh electronics recording/stimulation platform has achieved stable multiplexed LFP and single-unit spike recordings from mouse HIP and CTX with tracking of the same neurons and neural circuits up to eight-month periods. These results contrast conventional brain probes that generally exhibit spike shape changes over days to weeks (8-10, 26-28), although occasionally long-term stability can be obtained from one or small numbers of electrodes (29-31). Stable characteristics of the mesh platform also were shown for simultaneous recording from distinct brain regions using multiple mesh implants and for stimulation and recording from neurons. We applied the platform for longitudinal studies of ageing-associated neuronal changes and long-term recording in freely behaving mice. To the best of our knowledge, consistent and reproducible stable chronic recording/stimulation from the same single neurons/neural circuits has been unattainable previously for more than several weeks.

Mechanistically, these findings correlate with the comparable bending stiffness values for the mesh electronics and neural tissue, which minimizes or eliminates relative shear motion of the electronics inside brain since the implant is effectively decoupled from the I/O fixed to skull. In addition, near natural distributions of neurons, axons and glial cells at the mesh electronics surface and interior shown for 6 weeks (Figs. 6.6d,e and 6.9) contrasts a 50-200 μm region of neuron depletion around conventional brain implants (26), substantiating the observed stable single-unit spike amplitudes for the same time periods.

Moreover, time-dependent histology (Fig. 6.6d,e) and waveform auto-/cross-correlation analyses (Fig. 6.20) provide insight into the amplitude increase seen at earlier times (Figs. 6.6b and 6.8e). First, the histology data suggest that amplitude increase was associated with recovery
from acute implantation damage. Second, representative waveform autocorrelation analysis during this period (Fig. 6.20) indicates the units identified at 1 week post-injection remained consistent through 8 weeks. The somewhat lower percentage of cross-week autocorrelation for some neurons (Fig. 6.20b) might suggest a contribution from axon/dendrite regeneration. Last, the observation of a new cluster (neuron 3, Fig. 6.10b) at 3 weeks indicates that tissue remodeling (also seen in histology data) also contributes.

Figure 6.20. Autocorrelation analyses of average waveforms from week 1 to 8 post-injection. (a) Autocorrelation histograms of average waveforms for neurons 1 (magenta) and 2 (green) for Mouse2-ChannelA from weeks 1 to 8 post-injection shown in Fig. 6.10b. (b) Autocorrelation histograms of average waveforms for neurons 1 (magenta) and 2 (green) for Mouse2-ChannelB from weeks 1 to 8 shown in Fig. 6.13c. Inset: Cross-correlation histogram of all raw spikes for neuron 2 between weeks 1 and 8 (light green, dashed line) and autocorrelation histogram of all raw spikes for neuron 2 on week 8 only (dark green, solid line). (c) Autocorrelation histograms of average waveforms for neurons 1 (magenta) and 2 (green) for Mouse3-ChannelA from weeks 1 to 8 shown in Fig. 6.13d. See Chapter 6.4 for details of autocorrelation calculations.

In addition, the gradually decreasing firing rate, which was found to negatively correlate with progressively increasing peak-to-trough time in individual neurons (Figs. 6.16 and 6.17), is consistent with impaired long-term potentiation (LTP), decreased [Ca^{2+}], baseline and increased
after-hyperpolarization (AHP) as suggested by cross-sectional rodent studies (14, 48, 49). Not only are our observations qualitatively consistent with population-averaged results discovered by cross-sectional studies (11-14), but they also reveal details on the evolution of individual neurons during ageing that has been previously inaccessible.

Last, it could be beneficial to increase the number of recording channels and to achieve full-amplitude spikes closer to initial implantation for our mesh platform. A combination of increasing the number/density of electrodes in each mesh probe and multi-site injection of several meshes into the same animal provides a feasible strategy for achieving higher multiplexing. On the other hand, initial tests of implantation with reduced needle diameters and injected solution volumes indicate that full-amplitude spikes can be observed in only ~3 weeks. Approaches involving co-injection of stem cells or neurotrophic factors will also be interesting to pursue. Furthermore, biochemical studies directed at better understanding the mechanisms accounting for chronic interpenetration of neuronal cells/projections and observed electrophysiological changes during brain ageing will be valuable future directions. However, the unique capability to record from and stimulate the same neurons and neural circuits over at least eight-month periods already opens up important neurobiology opportunities, including understanding fundamental neural circuit plasticity, reorganization and development during learning, memory formation and ageing-associated cognitive decline, as well as enabling closed-loop BMIs in freely behaving animals via stable single-neuron based decoding and communication.
6.4 Methods and Materials

6.4.1 Fabrication of syringe-injectable electronics

The syringe-injectable mesh electronics for chronic brain activity mapping used fabrication procedure and geometrical design similar to our recent reports (36, 37). Key steps involved in the fabrication of syringe-injectable mesh electronics are overviewed in Fig. 6.1, with the key mesh parameters (Fig. 6.2) as follows: total mesh width, \( W = 2 \text{ mm} \), longitudinal SU-8 ribbon width, \( w_1 = 20 \mu \text{m} \), transverse SU-8 ribbon width, \( w_2 = 20 \mu \text{m} \), angle between longitudinal and transverse SU-8 ribbons, \( \alpha = 45^\circ \), longitudinal spacing (pitch between transverse ribbons), \( L_1 = 333 \mu \text{m} \), transverse spacing (pitch between longitudinal ribbons), \( L_2 = 125 \mu \text{m} \), metal interconnect line width, \( w_m = 10 \mu \text{m} \) and total number of recording channels, \( N = 16 \). The with key fabrication steps (Fig. 6.1) are as follows: (i) A sacrificial layer of Ni with a thickness of 100 nm was thermally evaporated (Sharon Vacuum, Brockton, MA) onto a 3" Si wafer (n-type 0.005 \( \Omega \cdot \text{cm} \), 600-nm thermal oxide, Nova Electronic Materials, Flower Mound, TX), which was pre-cleaned with oxygen plasma. (ii) Negative photoresist SU-8 (SU-8 2000.5; MicroChem Corp., Newton, MA) was spin-coated on the Si wafer to a thickness of 500 nm, pre-baked sequentially at 65 °C for 1 min and 95 °C for 4 min, and then patterned by photolithography (PL) with a mask aligner (ABM mask aligner, San Jose, CA). After PL exposure the sample was post-baked sequentially at 65 °C for 3 min and 95 °C for 3 min. (iii) The SU-8 photoresist was then developed (SU-8 Developer, MicroChem Corp., Newton, MA) for 2 min, rinsed with isopropanol, dried in a \( \text{N}_2 \) flow and hard-baked at 185 °C for 1 h. (iv) The wafer was then cleaned with oxygen plasma (50 W, 1 min), spin-coated with MCC Primer 80/20 and LOR 3A lift-off resist (MicroChem Corp., Newton, MA), baked at 185 °C for 5 min, followed by spin-coating Shipley 1805 positive photoresist (Microposit, The Dow Chemical Company,
Marlborough, MA), which was then baked at 115 °C for 5 min. The positive photoresist was patterned by PL and developed (MF-CD-26, Microposit, The Dow Chemical Company, Marlborough, MA) for 90 s. (ν) A 1.5-nm thick Cr layer and a 100-nm thick Au layer were sequentially deposited by electron-beam evaporation (Denton Vacuum, Moorestown, NJ), followed by a lift-off step (Remover PG, MicroChem Corp., Newton, MA) to make the Au interconnect lines. (vi) Steps iv and v were repeated for PL patterning and deposition of the Pt sensing or stimulation electrodes (Cr: 1.5 nm, Pt: 50 nm). The diameter of Pt sensing electrodes was 20 μm and that of Pt stimulation electrode was increased to 150 μm (the larger diameter was used to afford lower impedance for electrical stimulation). (vii) Steps ii and iii were repeated for PL patterning of the top SU-8 layer, which served as the top encapsulating/insulating layer of the metal interconnect lines. (viii) Subsequently, the Si wafer was cleaned with oxygen plasma (50 W, 1 min) and then transferred to a Ni etchant solution comprising 40% FeCl₃:39% HCl:H₂O=1:1:20 to remove the sacrificial Ni layer and release the mesh electronics from the Si substrate. Released mesh electronics were rinsed with deionized (DI) water, transferred to an aqueous solution of poly-D-lysine (PDL, 1.0 mg/ml, MW 70,000-150,000, Sigma-Aldrich Corp., St. Louis, MO) for 24 h, and then transferred to 1X phosphate buffered saline (PBS) solution (HyClone™ Phosphate Buffered Saline, Thermo Fisher Scientific Inc., Pittsburgh, PA) before use.

6.4.2 Vertebrate animal subjects

Adult (25-35 g) male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were the vertebrate animal subjects used in this study. The total number of mice used for demonstrating chronic single neuron level recordings is 4, which was statistically determined by power analysis (50) by assuming a significance level of 5% and an average spike amplitude to variation ratio of
Moreover, a 5\textsuperscript{th} mouse with two meshes injected was used to show multi-site injection stability, a 6\textsuperscript{th} subject was used for stimulation studies, and a 7\textsuperscript{th} mouse was used for freely behaving mouse recordings, and 3 additional mice were used for immunohistochemical studies. Exclusion criteria were pre-established: animals with failed surgery or substantial acute implantation damage (>100 μL of initial liquid injection volume) were discarded from further chronic recordings. Randomization or blinding study was not applicable to this study. All procedures performed on the mice were approved by the Animal Care and Use Committee of Harvard University. The animal care and use programs at Harvard University meet the requirements of the Federal Law (89-544 and 91-579) and NIH regulations and are also accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC). Animals were group-housed on a 12 h: 12 h light: dark cycle in the Harvard University’s Biology Research Infrastructure (BRI) and fed with food and water \textit{ad libitum} as appropriate.

\subsection*{6.4.3 \textit{In vivo} mouse survival surgery}

\subsubsection*{6.4.3.1 Stereotaxic injection of mesh electronics in mouse brain}

\textit{In vivo} injection of mesh electronics into the brains of live mice was performed using a controlled stereotaxic injection method described previously (37). First, all metal tools in direct contact with the surgical subject were autoclaved for 1 h before use, and all plastic tools in direct contact with the surgical subjects were sterilized with 70% ethanol and rinsed with sterile DI water and sterile 1X PBS before use. Prior to injection, the mesh electronics were sterilized with 70% ethanol followed by rinsing in sterile DI water and transfer to sterile 1X PBS. The basic procedure for loading the mesh into glass capillary needles is shown schematically in Fig. 6.4.
C57BL/6J mice were anesthetized by intraperitoneal injection of a mixture of 75 mg/kg of ketamine (Patterson Veterinary Supply Inc., Chicago, IL) and 1 mg/kg dexdomitor (Orion Corporation, Espoo, Finland). The degree of anesthesia was verified via the toe pinch method before the surgery started. To maintain the body temperature and prevent hypothermia of the surgical subject, a homeothermic blanket (Harvard Apparatus, Holliston, MA) was set to 37 °C and placed underneath the anesthetized mouse, which was placed in the stereotaxic frame (Lab Standard Stereotaxic Instrument, Stoelting Co., Wood Dale, IL) equipped with two ear bars and one nose clamp that fixed the mouse head in position. Puralube ocular lubricant (Dechra Pharmaceuticals, Northwich, UK) was applied on both eyes of the mouse to moisturize the eye surface throughout the surgery. Hair removal lotion (Nair®, Church & Dwight, Ewing, NJ) was used for depilation of the mouse head and iodophor was applied to sterilize the depilated scalp skin. A 1-mm longitudinal incision along the sagittal sinus was made in the scalp with a sterile scalpel, and the scalp skin was resected to expose a 6 mm × 8 mm portion of the skull. METABOND® enamel etchant gel (Parkell Inc., Edgewood, NY) was applied over the exposed cranial bone to prepare the surface for mounting the electronics on the mouse skull later.

A 1 mm diameter burr hole was drilled using a dental drill (Micromotor with On/Off Pedal 110/220, Grobet USA, Carlstadt, NJ) according to the following stereotaxic coordinates: anteroposterior: -4.96 mm, mediolateral: 3.10 mm. After the hole was drilled, the dura was carefully incised and resected using a 27-gauge needle (PrecisionGlide®, Becton Dickinson and Company, Franklin Lakes, NJ). Then a sterilized 0-80 set screw (18-8 Stainless Steel Cup Point Set Screw; outer diameter: 0.060" or 1.52 mm, groove diameter: 0.045" or 1.14 mm, length: 3/16" or 4.76 mm; McMaster-Carr Supply Company, Elmhurst, IL) was screwed into this 1-mm burr hole to a depth of 500 µm as the grounding and reference electrode. Another 1 mm burr hole
was drilled for injection of mesh electronics according to the following stereotaxic coordinates depending on specific brain areas for activity recording:

1) Primary somatosensory cortex, barrel field: anteroposterior: -1.82 mm, mediolateral: -3.00 mm, dorsoventral: 0.75 mm.

2) Primary somatosensory cortex, trunk: anteroposterior: -1.70 mm, mediolateral: -2.00 mm, dorsoventral: 0.75 mm.

3) Hippocampal CA1 field: anteroposterior: -1.70 mm, mediolateral: -1.60 mm, dorsoventral: 1.17 mm.

4) Hippocampal CA3 field: anteroposterior: -1.70 mm, mediolateral: -2.00 mm, dorsoventral: 1.85 mm.

The dura was removed from the burr hole drilled for mesh electronics injection and sterile 1X PBS was swabbed on the surface of the brain to keep it moist throughout the surgery. The mesh electronics was injected into the desired brain region using a controlled injection method (37). In brief, the mesh electronics was loaded into a glass capillary needle with inner diameter (I.D.) of 400 μm and outer diameter (O.D.) of 650 μm (Produstrial LLC, Fredon, NJ). The glass capillary needle loaded with mesh electronics was mounted onto the stereotaxic stage through a micropipette holder (Q series holder, Harvard Apparatus, Holliston, MA), which was connected to a 5 mL syringe (Becton Dickinson and Company, Franklin Lakes, NJ) through a polyethylene Intramedic™ catheter tubing (I.D. 1.19 mm, O.D. 1.70 mm). Controlled injection was achieved by balancing the volumetric flow rate (typically 20-50 mL/h), which was controlled by a syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA), and the needle withdrawal speed (typically 0.2-0.5 mm/s), which was controlled by a motorized linear translation stage (860A motorizer and 460A linear stage, Newport Corporation, Irvine, CA).
Using the controlled injection method with field of view (FoV) visualization through an eyepiece camera (DCC1240C, Thorlabs Inc., Newton, NJ), the mesh electronics was delivered to specific brain regions with elongated morphology along the injection direction with ~20 μm spatial targeting precision. For successful long-term recordings, the total injection volume is usually between 10 and 100 μL. An unexpected large injection volume (>100 μL) could result in brain edema or failure of recovery from acute surgical damage, leading to expulsion of the subject from the study.

6.4.3.2 Electrical connection of syringe-injectable electronics for chronic recordings from awake and restrained mice

After the injection of mesh electronics into the desired region of a mouse brain, the stereotaxic stage was moved to reposition the glass capillary needle over a 16-channel flexible flat cable (FFC, PREMO-FLEX, Molex Incorporated, Lisle, IL), and then the remaining mesh electronics was fully expelled from the needle and unfolded onto the FFC to expose the input/output (I/O) connection pads. High-yield bonding of mesh electronics I/O pads to the FFC was carried out using our reported conductive ink printing method (37). In brief, the print head loaded with carbon nanotube solution (Stock No.: P093099-11, Tubes@Rice, Houston, TX) was driven by a motorized micromanipulator (MP-285/M, Sutter Instrument, Novato, CA) through a user-written LabVIEW program to print conductive ink automatically and connect each mesh I/O pad to each of the FFC lines to enable independently addressable sensor elements. Failure of mesh I/O unfolding could lead to potential low-yield electrical connection to the FFC interface cable. All printed conductive lines were passivated by METABOND® dental cement (Parkell Inc., Edgewood, NY), and then the entire FFC with mesh electronics bonded to the FFC was cemented to the mouse skull with METABOND® dental cement. The FFC was folded to reduce
its size on the mouse skull. The total mass of the bonded interface cable with mesh electronics is typically 0.2-0.3 g.

**6.4.3.3. Electrical connection of syringe-injectable electronics for chronic recordings from freely behaving mice**

After the injection of mesh electronics into the desired mouse brain region, the stereotaxic stage was manually moved to reposition the glass capillary needle to a 32-channel Omnetics male connector (A79024-001, Omnetics Connector Corp., Minneapolis, MN) with a weight of ~ 0.1 g glued on a nonconductive polyethylene terephthalate (PET) flexible substrate with a thickness of ~ 0.3 mm, and then the remaining mesh electronics was fully expelled from the needle and unfolded onto the flexible substrate with its I/O connection pads facing the horizontal mounting tails of the Omnetics connector. Conductive ink printing was used to bond the mesh electronics I/O pads to 16 horizontal mounting tails of the Omnetics connector as described above for the FFC cable. The 0-80 grounding screw was electrically connected to one of the four pre-installed grounding/reference pins of the Omnetics connector using silver conductive epoxy (MG Chemicals, Burlington, ON, Canada). All printed conductive lines were protected by METABOND® dental cement, before the entire packaged headstage was cemented to the mouse skull with dental cement.

**6.4.3.4 Postoperative care**

After surgery was complete, antibiotic ointment (WATER-JEL Technologies LLC, Carlstadt, NJ) was applied copiously around the wound, and the mouse was returned to the cage equipped with a 37°C heating pad and its activity monitored every hour until fully recovered from anesthesia (i.e., exhibiting sternal recumbency and purposeful movement). Buprenex (Buprenorphine, Patterson Veterinary Supply Inc, Chicago, IL) analgesia was given
intraperitoneally at a dose of 0.05 mg/kg body weight every 12 h for up to 72 h post brain surgery.

The overall success rate of our surgical procedure is around 70%, with the main causes for failure including (i) an unexpected large injection volume (>100 μL) resulting in brain edema, and (ii) failure of mesh I/O unfolding leading to low-yield electrical connection to the FFC interface cable. Further improvements on surgery success rate include: (i) better control of injection volume by further reducing the transverse bending stiffness of mesh electronics; and (ii) more reliable I/O unfolding/bonding through designs of I/O pads distributions with larger separation.

6.4.4 Micro-Computed Tomography

One mouse injected with mesh electronics, where the I/O was bonded to an FFC and then cemented to the mouse skull, was euthanized via intraperitoneal injection of Euthasol at a dose of 270 mg/kg body weight and decapitated. The decapitated mouse head was imaged using an HMXST Micro-CT X-ray scanning system with a standard horizontal imaging axis cabinet (model: HMXST225, Nikon Metrology, Inc., Brighton, MI). Imaging parameters were set as 115 kV and 83 μA (with a 0.1-mm copper filter for beam hardening) for scanning the decapitated mouse head. Before scanning, shading correction and flux normalization were applied to adjust the X-ray detector. The CT Pro 3D software (ver. 2.2, Nikon-Metris, UK) was used to calibrate centers of rotation for micro-CT sinograms and to reconstruct all 2D images. VGStudio MAX software (ver. 2.2, Volume Graphics GMbh, Germany) was used for 3D rendering and analysis of the reconstructed images.

6.4.5 In vivo chronic brain recording and stimulation in mice

6.4.5.1 Chronic brain recording from awake and restrained mice
Mice with implanted mesh electronics and FFC connector were recorded chronically on a weekly basis, starting from Day 7 post-injection and surgery. Mice were restrained in a Tailveiner® restrainer (Braintree Scientific LLC., Braintree, MA) while its head-mounted FFC was connected to an Intan RHD 2132 amplifier evaluation system (Intan Technologies LLC., Los Angeles, CA) through a home-made printed circuit board (PCB). The 0-80 set screw was used as a reference. Electrophysiological recording was made with a 20-kHz sampling rate and a 60-Hz notch filter, while the electrical impedance at 1 kHz of each recording electrode was also measured by the same Intan system.

6.4.5.2 Chronic brain recording of freely behaving mice

Mice with Omnetics connectors were recorded chronically on a weekly basis when they were freely roaming in the cage. For recording, an Intan preamplifier chip (RHD2132 16-Channel Amplifier Board, Intan Technologies LLC., Los Angeles, CA) with pre-installed female Omnetics connector was connected directly to the male Omnetics connector cemented on the mouse skull during surgery, and the mouse was allowed to roam in a cage environment not explored previously. Food pellets were placed at random positions inside the cage for each trial. Electrophysiological recordings were made using the same Intan evaluation system with a 20-kHz sampling rate and a 60-Hz notch filter, and were synchronized with video recording of the mouse’s motion inside the cage using a digital camera.

6.4.5.3 Chronic electrical stimulation of mouse brains

Mice injected with mesh electronics incorporating stimulation and recording electrodes were subject to electrical stimulation and simultaneous electrophysiological recording periodically to week 14 post-surgery. Similar to chronic electrophysiological recording described above, mice were restrained in the Tailveiner® restrainer with a head-mounted FFC connected to
the Intan RHD 2132 amplifier evaluation system for 12 recording channels, while the other 4 stimulation channels were connected to a homemade stimulator comprising a function generator (Model 33220A, 20 MHz Function/Arbitrary Waveform Generator, Agilent Technologies, Santa Clara, CA) that provided stimulus pulse trains with user-defined current, pulse duration and pulse interval. Typical currents used for stimulation ranged from 5-50 μA, followed by an inverted polarity with the same amplitude to provide capacitor-coupled and charge-balanced stimulation (51). The pulse duration was 1 ms for each phase (positive or negative) with two consecutive pulses spaced by 1 s. Neural responses to stimulus input through one of the 4 stimulation electrodes were recorded as both local field potentials (LFPs) and single-unit spikes from the 12 recording electrodes from the same injected mesh electronics. The 0-80 set screw was used as a reference for both stimulation and recording.

6.4.6 Data analysis of electrophysiological recording

6.4.6.1 Data analysis of LFP and single-unit action potential recording

The electrophysiological recording data was analyzed offline. In brief, raw recording data was filtered using non-causal Butterworth bandpass filters (‘filtfilt’ function in Matlab) in the 250-6000 Hz frequency range to extract single-unit spikes (36), in the 0.1-150 Hz range to extract LFP (36), and in the 4-8 Hz range to extract the theta rhythm of LFP (42). The intrinsic noise distribution of a specific channel was analyzed based on all recording traces bandpass filtered at 250-6000 Hz excluding any firing spikes. The correlation coefficient maps of single-unit spike recording traces shown in Fig. 6.7 were calculated based on the standard Pearson product-moment correlation coefficient for time series. Namely, for two spike traces, \( Y_1(t) \) and \( Y_2(t) \), the correlation coefficient between them is calculated as
\[
    Corr(Y_1, Y_2) = \frac{\int_{T_1}^{T_2} (Y_1(t) - \overline{Y}_1)(Y_2(t) - \overline{Y}_2) dt}{\sqrt{\int_{T_1}^{T_2} (Y_1(t) - \overline{Y}_1)^2 dt \int_{T_1}^{T_2} (Y_2(t) - \overline{Y}_2)^2 dt}} 
\]

where \(T_1\) and \(T_2\) indicate the starting and ending time of the recording traces (in Fig. 6.7, the time window is 2 s), and \(\overline{Y}_i = \int_{T_i}^{T_2} Y_i(t) dt / (T_2 - T_i)\) \((i = 1, 2)\) represents the averaged value of \(Y_i(t)\) over the time period between \(T_1\) and \(T_2\).

Single-unit spike sorting was performed by amplitude thresholding of the filtered traces by automatically determining the threshold based on the median of the background noise according to the improved noise estimation method (52). The average spike amplitude for each recording channel (Figs. 6.6b and 6.8e) was defined as the peak-to-peak amplitude of the spikes for a typical 1-min recording trace. All of the single-neuron spike analyses shown in Figs. 6.10, 6.13 and 6.17 were carried out based on a 30-min recording session. The peak-to-trough time for each recorded spike was defined as the time interval \(\tau\) between the major peak (which can be either positive or negative) and the following rebound with opposite polarity (Fig. 6.16a II, upper left inset). All sorted spikes were clustered to determine the number of single neurons and assign spikes to each single neuron using the WaveClus software that employs unsupervised superparamagnetic clustering of single-unit spikes (52). Spikes assigned to the same cluster were coded with the same color and plotted in the first and second principal components (PC1-PC2) plane. The noise distribution of sorted spikes was obtained by plotting the histogram of the difference between each raw spike and average spike waveform at every sampling point. The deviations of all the identified neuron noise distributions were computed by fitting each noise histogram to a Gaussian distribution.

The L-ratio for each cluster of spikes was calculated as follows (53),
where $N(C)$ denotes the total number of spikes in the cluster, $CDF_{\chi^2}$ presents the cumulative distribution function of the $\chi^2$ distribution in an eight-dimensional feature space, and $D^2_{i,C}$ is the Mahalanobis distance of a spike $i$ from the center of the cluster $C$. The summation goes over the entire set of spikes that do not belong to the cluster. An L-ratio of $<0.05$ is generally considered good cluster separation/isolation (40, 53).

The autocorrelation and cross-correlation of raw and average spike waveforms shown in Figs. 6.8, 6.11 and 6.20 were computed based on the standard Pearson product-moment correlation coefficient defined in Equation 6.1 for the 3 ms time series of each spike (27). A value of 1 indicates identical spike shapes, irrespective of absolute spike amplitudes.

The spiking times of all clustered single-unit action potentials assigned to each cluster (i.e., each single neuron) were used to compute the interspike interval (ISI) histogram under different bin sizes for verification of unit isolation (Fig. 6.12, bin size=1 ms) and extraction of firing rate by fitting the ISI histogram to a first order exponential decay (Fig. 6.10c, bin size=20 ms) (41). The instantaneous phase of the theta rhythm of LFP at the location of each single-unit spike that had been assigned to a certain cluster was determined by performing Hilbert transform of the filtered traces in the 4-8 Hz frequency range and phase locking behavior of single-unit spikes was investigated by plotting their phase distribution in a polar plot (44). All the extracted phases of individual spikes with respect to theta rhythm LFP in each recording session were subjected to a Rayleigh Z-test, and Ln(Z) values obtained from multiple recording sessions (across different weeks) for each identified neuron were used to test the statistical significance of each neuron’s phase-locking behavior (43, 54). A subsequent Rayleigh Z test was then applied to
the extracted locked phases from each week’s phase distribution from 3 to 34 weeks post-injection to test the chronic stability of each neuron’s phase-locking behavior.

### 6.4.6.2 Data analysis of freely behaving recording

For freely behaving mice, the raw recording data was taken synchronously with mouse video recording, and then bandpass filtered before single-unit spikes were sorted and clustered as described above. The video of mouse movement was analyzed with Gaussian blur filter and object tracking algorithm using Matlab to extract the mouse’s trajectory and the distance between its head and the food pellet in real time. The firing rate of the electrophysiological recording was then correlated with the mouse’s motion trajectory to derive the interaction-dependent firing behavior when the mouse whisked food pellets in its environment. Phase-locking analyses were performed using the same algorithm described above between the single-unit spikes from Channel D (located in the barrel field of somatosensory cortex) and the theta rhythm of LFP from Channel A (located in hippocampus) shown in Fig. 6.18 for data recorded when the mouse was whisking food pellets and foraging. These phase-locking results are presented separately for durations of active whisking and non-tactile foraging based on dynamic image processing of the video recording the mouse movement in the cage.

### 6.4.6.3 Data analysis of electrical stimulus provoked recording

For analysis of recording data with electrical stimulation, the onset time of each stimulus was determined by the large artifact peak due to stimulation input picked by all recording electrodes. All stimulation trials were aligned to that peak as \( t=0 \) s (where \( t \) is the peristimulus time, \( t<0 \) denotes before stimulation and \( t>0 \) denotes after stimulation), based on which peristimulus raster plot and post-stimulus first spike latency histogram were plotted using Matlab.
6.4.7 Chronic immunohistochemistry

6.4.7.1. Histology sample preparation

Mice with implanted mesh electronics at post-injection times of 2, 6 and 12 weeks were anesthetized with ketamine and dexdomitor, and then were transcardially perfused with 40 mL 1X PBS and 40 mL 4% formaldehyde (Sigma-Aldrich Corp., St. Louis, MO), followed by decapitation. The scalp skin was removed and the exposed skull was ground for 10-20 min at 10,000 RPM using a high-speed rotary tool (Dremel, Mount Prospect, IL). The brain was resected from the cranium and placed in 4% formaldehyde for 24 h, and then transferred to 1X PBS for another 24 hours at 4 °C to remove remaining formaldehyde. The brain was transferred to incrementally increasing sucrose solutions (10–30%) (Sigma-Aldrich Corp., St. Louis, MO) at 4 °C to cryoprotect the tissue, transferred to cryo-OCT compound (Tissue-Tek® O.C.T. Compound, VWR, Radnor, PA) and then frozen at -80 °C. The frozen sample was then sectioned into 10-µm-thick horizontal slices using Leica CM1950 cryosectioning instrument (Leica Microsystems, Buffalo Grove, IL).

6.4.7.2 Immunohistochemical staining and microscopic imaging

The brain tissue sections were rinsed three times in 1X PBS and blocked in a solution consisting of 0.3% Triton X-100 (Life technologies, Carlsbad, CA) and 5% goat serum (Life Technologies, Carlsbad, CA) in 1X PBS for 1 h at room temperature. Slices were then incubated with the primary antibodies, rabbit anti-NeuN (1:200 dilution, Abcam, Cambridge, UK), mouse anti-Neurofilament (1:400 dilution, Abcam, Cambridge, UK), rat anti-GFAP (1:500 dilution, Thermo Fisher Scientific Inc, Cambridge, MA) or rabbit anti-Iba1 (1:250 dilution, Abcam, Cambridge, UK) containing 0.3% Triton X-100 and 3% goat serum overnight at 4 °C. NeuN is a neuron-specific nuclear protein, and stains the neural somata. Neurofilament is intermediate
filaments found in neurons, and stains neural axons. GFAP is glial fibrillary acidic protein, and
stains astrocytes. Iba-1 is a 17-kDa EF hand protein that is specifically expressed in
macrophages/microglia, and is up-regulated by the activation of these cells. After incubation,
slices were rinsed 9 times for a total of 40 min with 1X PBS, before they were incubated with the
secondary antibodies, Alexa Fluor® 488 goat anti-rabbit (1:200 dilution, Abcam, Cambridge,
UK), Alexa Fluor® 568 goat anti-mouse (1:200 dilution, Abcam, Cambridge, UK), or Alexa
Fluor® 647 goat anti-rat (1:200 dilution, Abcam, Cambridge, UK) for 1 h at room temperature;
the specific choices of secondary antibodies were made based on primary antibodies used to stain
a given slice. Slices were rinsed 9 times for a total of 30 min after incubation with secondary
antibodies, before they were mounted on glass slides with coverslips using ProLong® Gold
Antifade Mountant (Life Technologies, Carlsbad, CA). The slides remained in dark at room
temperature for at least 24 h before microscopic imaging.

Confocal fluorescence imaging of the samples was acquired on a Zeiss LSM 880
confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). Confocal images were
acquired using 488 nm, 561 nm and 633 nm lasers as the excitation sources for Alexa Fluor®
488, Alexa Fluor® 568 and Alexa Fluor® 647, respectively. ImageJ software was used for image
analysis. The mesh electronics in each slice was imaged with differential interference contrast
(DIC) on the same microscope, and is shown with false blue color in the composite images of
Figs. 6.6d, 6.9 and 6.17h. Fluorescence intensities of Neurofilament, NeuN and GFAP were
based on the analysis of zoomed-out images of those shown in Fig. 6.6d, 6.9a and 6.17h with a
field of view of 1.2 mm × 1.2 mm. Iba-1 results were based on analysis of brain slices shown in
Figs. 6.9b and an adjacent brain slice of the one shown in Fig. 6.17h with the same field of view.
The fluorescence intensities were normalized (value=1.0, gray dashed horizontal lines in Figs.
6.6e and 6.17i) against the background values 500 μm away from the probe interface for each sample.
6.5 Bibliography


Chapter 7

Multiplexed Stable Chronic Electrophysiology of Retina Ganglion Cells in Live Animals

Understanding functional diversity of retinal ganglion cells (RGCs) has almost exclusively relied on electrophysiological recording and fluorescence-based calcium imaging of retinal explants. In contrast, in vivo RGC electrophysiology in live animal eyes can offer much greater opportunities to understand the interaction of retina with visual perception related brain regions in the visual pathway, but has been limited with significant damage to the eye, poor spatiotemporal resolution, low throughput with few recording channels and chronic recording instability. Here, we achieve chronically stable and multiplexed in vivo RGC electrophysiology at the single-neuron level with mesh-like electronics in the mouse eye. Mesh electronics is delivered into the vitreous humor of the eye with controlled syringe injection and followed by unfolding on the retina surface owing to both the internal built-in strain of mesh electronics and the hydraulic pressure applied during injection. Chronic study of blink and pupillary reflexes of the injected mouse eye reveals minimum interference to the normal eye functions by the injected mesh electronics over the course of two weeks. In vivo through-lens imaging reveals chronically stable interface between the injected mesh electronics and the retina with close proximity between the mesh and the RGCs evidenced from ex vivo confocal imaging. This chronically stable interface between mesh electronics and the retina allows for multiplexed recording of RGCs from 16 independent channels and chronic monitoring of the activity from the same RGCs.
based on recording the single-unit responses to light intensity modulation and moving grating stimulation.
7.1 Introduction

As an approachable and accessible part of the brain, the retina provides an excellent source of material for detailed anatomical and electrophysiological study and analyses of the neural mechanisms underlying elementary information processing, in particular visual information processing, in the central nervous system (1, 2). To understand the morphological, genetic and physiological diversity of retinal ganglion cells (RGCs) and the mechanisms by which RGCs process visual information, microelectrode array (MEA) (3-6), patch clamp (7-9) and calcium imaging (10-11) have been employed to study the electrophysiology and classify subtypes of RGCs in dissected retina explant based on their responses to different visual stimulation patterns (12). Despite the plethora of knowledge gained from ex vivo electrophysiology of retina explant, our understanding of the visual information pathway (13), interocular connection (14) and the interaction between retina and higher visual processing centers in the brain such as optokinetic reflex (15) and circadian neuromodulatory regulation (16, 17) can be significantly expanded by performing in vivo electrophysiology of RGCs in live animals.

Existing approaches for in vivo retina electrophysiology are limited with either poor spatiotemporal resolution for electroretinography (ERG) (18) or low throughput single- or few-channel recording of RGCs via invasive insertion of a rigid electrode into the eye (19). Neither approach is able to provide chronic mapping of single-unit activities of multiple RGCs in the eye of live animals over an extended time period, thus calling for new paradigms that can lead to precise delivery of minimally-invasive electronics onto the retina of live animals to form stable interfaces with the RGC layer suitable for chronic interrogation of retina function. Recently, we have developed a new form of nanoelectronics that can be delivered virtually into any part of soft tissue via a syringe, and form a chronically stable and seamlessly integrated interface with local brain tissue by making
electronics look like the neural network both structurally and mechanically (20, 21). The ‘mesh-like’
electronics with >99% macroporosity and exceptional flexibility allows chronic long-term recording
of brain activities in rodents by following the action potentials of the same single neurons for >8
months (22). In this work, we demonstrate the syringe-assisted intravitreal delivery of biologics-like
mesh electronics into the live mouse eye to form a chronically stable interface with the RGC layer
epiretinally, which allows multiplexed chronic single-neuron electrophysiology of RGCs in awake
mice receiving visual stimuli over 2 weeks.
7.2 Results and Discussion

7.2.1 Syringe-assisted intravitreal injection of mesh electronics in mouse eye

Figure 7.1. Intravitreal injection and epiretinal unfolding of mesh electronics in mouse eye. (a) Schematics showing the desired effect of epiretinal unfolding of mesh electronics that conformally coats the retinal ganglion cell (RGC) layer (I), after intravitreal injection in the eye (II) in a controlled manner that allows for non-coaxial positioning of mesh electronics on a curved surface horizontally (III). The inset in (I) shows the layout of mesh electronics, highlighting the 16 individually addressable recording electrodes (green arrow) connected to corresponding input/output (I/O) pads on the other end (red arrow). (b) Schematics (top row) with corresponding photographs (bottom row) of needle insertion and withdrawal during the intravitreal injection process. (c) Chronic in vivo through-lens images of the fundus of a mouse
Figure 7.1 (Continued): eye injected with mesh electronics on different days post-injection. (d) An \emph{ex vivo} confocal image after three-dimensional reconstruction of a mouse eye injected with mesh electronics, highlighting the interface between mesh electronics and the RGC layer. (e) A vertical cross-section of the interface between injected mesh electronics and the retina after immunohistological staining of different cell types in the retina. Red: bipolar cells; green: RGCs, horizontal cells and amacrine cells; blue: autofluorescence from SU-8 polymer comprising the mesh ribbons.

Sub-micrometer-thick, millimeter-scale, 16-channel tissue-like mesh electronics were fabricated with standard photolithography procedures (see Chapter 7.4). The 16 recording electrodes at the one end of the mesh electronics to be injected into the eye (green arrow, Fig. 7.1a inset) are individually addressable through parallel metal interconnect lines to input/output (I/O) pads at the other end for connection to external recording instrumentations (red arrow, Fig. 7.1a inset). The 16 recording electrodes are distributed evenly over a 1.5 mm × 0.8 mm region in 4 parallel rows, ensuring large-area coverage and interrogation of the retina after injection. The structural design of mesh electronics with >90% porosity in two-dimension (2D) and small features sizes (<1 μm total thickness and 5 μm width for metal interconnect lines) contributes to the overall ultraflexibility and minimum blockage of light, allowing for loading and injection of mesh electronics through a syringe and minimally interfering interface with the retina.

To deliver the 16 recording electrodes of mesh electronics into the mouse eye, we used a controlled non-coaxial injection scheme to achieve lateral positioning of the array of recording electrodes on the retina (Fig. 7.1a,b). Several key features of the non-coaxial injection approach are highlighted: first, the entire injection procedure is compatible with a standard mouse stereotaxic frame commonly used for injection and implantation in mouse brain surgeries (Fig. 7.2a,b). Second, the ultraflexibility of mesh electronics enables loading and injection into the eye through the lateral canthus using a small needle with an inner diameter (ID) of 200 μm and an
outer diameter (OD) of 330 μm (Fig. 7.1b, left column), which is similar in size to needles used for intraocular injection of virus vectors and drugs (see Chapter 7.4) (23-26). Using a needle with a small diameter helped minimize damage to the eye, especially to the lens, and prevent excessive bleeding during sclerotomy (Fig. 7.1a, II). Third, during the controlled injection process, the volumetric flow of saline in the needle was synchronized with the lateral motion of the needle, affording lateral positioning of injected mesh electronics in the vitreous humor by tracking the trajectory of the top end of mesh electronics using the field of view (FoV) method (Figs. 7.1a, III and 7.2c) (21). Fourth, during the injection process a total of up to 20 μL sterile saline solution was expelled from the needle, applying hydraulic force to push the laterally positioned mesh electronics closer to the retina to afford an intimate interface while avoiding increase of intraocular pressure by draining the excessive liquid through a draining hole at the medial canthus (see Chapter 7.4). Both the hydraulic force and the built-in intrinsic stress in the mesh electronics enabled complete unfolding of recording electrodes to cover a large area of the retina (Fig. 7.1a, I) after the needle was withdrawn (Fig. 7.1b, right column). After injection, all individually addressable I/O pads left outside of the eye were connected to an interface cable (flexible flat cable, or FFC) via a well-established conductive ink printing method (21).
Figure 7.2. Intravitreal injection of mesh electronics into the mouse eye. (a,b) The non-coaxial injection setup (a) for intravitreal injection of mesh electronics to afford epiretinal unfolding, with a close-up view of the red dashed box in (b). (c) Non-coaxial injection test in 0.14% hydrogel (with similar mechanical properties as vitreous humor) to demonstrate the capability to position mesh electronics laterally (bottom row) by tracking the trajectory of the top end of the mesh marked by I/O pads via FoV visualization (top row). (d) Schematics and corresponding photographs showing the eye injection process, including needle approaching (I), needle insertion (II), needle withdrawal (III) and mesh left inside the eye (IV).
7.2.2 Optical imaging for characterization of mesh-retina interface after injection

To verify that mesh electronics could unfold to cover the retina with a close interface for epiretinal electrophysiology after injection, we employed both in vivo through-lens imaging using a home-made rodent fundus imager (Fig. 7.3a,b) at different time points post-injection and ex vivo three-dimensional (3D) confocal imaging for dissected retina immediately after injection.

**Figure 7.3. In vivo through-lens imaging of mouse eye fundus.** (a,b) The in vivo through-lens eye fundus imaging setup (a), with a close-up view of the red dashed box in b highlighting the air gap between the objective and flattened eye lubricant gel by a cover slip. (c) A schematic showing the construction of the ‘liquid Hruby lens’ by coating the eye with refractive index (RI) matching gel and flattening the gel/air interface with a cover slip. The black lines indicate emanating rays from an illuminated light source without the ‘liquid Hruby lens’ and the red lines indicate those with the ‘liquid Hruby lens’. (d) Stepwise illustration of mouse eye fundus imaging using the ‘liquid Hruby lens’, showing the visualization of eye fundus vessels after
**Figure 7.3 (Continued):** dilation, application of RI matching gel and interface flattening. (e) A high-magnification microscopic image showing the close proximity between the mesh and the retina in live mouse on the same day of injection and surgery, which is indicated by the focal plane where both the mesh ribbons and the retinal blood vessels are in focus (depth of focus = 20 μm).

We implemented *in vivo* through-lens imaging by making a ‘liquid Hruby lens’ comprising transparent gel that matched the refractive index (RI) of the cornea and conformally coated the curved surface of cornea and lens to neutralize the focusing power of the eye (see Chapter 7.4; Fig. 7.3c), allowing for visualization of eye fundus features independent of the lens power (Fig. 7.3d). Eye fundus images taken with the ‘liquid Hruby lens’ have several salient findings: first, unlike conventional, ‘solid Hruby lens’ that is customized for eyes with different radii of curvature (27, 28) or contact-type eye fundus imaging using medium-immersion objectives (29, 30), the ‘liquid Hruby lens’ had the advantages of easy implementation without special lens crafting and could be readily adopted to imaging systems with different working distances. Second, *in vivo* through-lens eye fundus images taken immediately after injection (Day 0, Fig. 7.1c) and on different days post-injection (Days 1, 7 and 14, Fig. 7.1c) allowed clear visualization of both mesh electronics and retinal blood vasculature, the latter of which provided a spatial context map for evaluation of the chronic mesh-retina interface over time. Third, time-dependent fundus images of mesh-injected eye revealed relatively stable locations of all 16 recording electrodes (shown as dots at the end of all metal interconnect lines) in the context of retinal blood vessels, and tracking a single recording electrode (indicated by the blue arrows in Fig. 7.1c) demonstrated almost identical location on top of the same retinal blood vessel over the course of 2 weeks. Finally, our home-made eye fundus imager with a ‘liquid Hruby lens’ afforded tunable magnification of imaging, and a higher-magnification image zoomed into part
of the mesh electronics clearly revealed both the metal interconnect lines of mesh electronics and retinal blood vessels in the same focal plane (Fig. 7.3e), suggesting their close distance in the z direction given a depth of focus of 20 μm at this magnification with a numerical aperture (NA) of 0.15.

Figure 7.4. Vertical section of the mesh-retina interface. (a) A schematic of the vertical section of layered retina structure showing the close interface of the mesh electronics with the RGC layer. (b) A vertical section from 3D confocal imaging of the ex vivo mesh-retina interface, which was resected immediately after intravitreal injection of mesh electronics into live mouse eye and reveals intimate interface with <10 μm distance between the mesh elements (indicated by blue arrow) and the RGCs (indicated by green arrows).

To afford 3D confocal imaging of the mesh-retina interface to reveal the close proximity between them, we also resected the mouse eye injected with mesh electronics to afford label-free confocal imaging and immunohistological staining and imaging after vertical sectioning. A TYW3 transgenic mouse line was used to afford endogenous expression of green fluorescent proteins (GFP) in certain subtypes of RGCs (31) and the autofluorescence of SU-8 polymer comprising longitudinal insulating ribbons and transverse supporting ribbons was collected in the same GFP channel to afford label-free imaging of mesh electronics. 3D retina whole mount image taken on dissected mouse eye with the lens removed exhibited the concave structure of the retina, on top of which the mesh electronics was also found to bend to a concave shape and
conformally coat the retina (Figs. 7.1d, I and 7.4). Moreover, all mesh ribbons were found to be located within ~20 μm distance from the closest RGCs, enabling recording of single-unit firing activities from nearby RGCs. In addition, immunohistological staining and imaging of a vertically sectioned retina sample along with injected mesh electronics revealed different layers of the retina and highlighted the close proximity of mesh ribbons (blue) to the RGC layer (green) that were both fixed in position in the frozen slice (Fig. 7.1d, II).

7.2.3 Chronic study of blink and pupillary reflexes of mesh-injected eye

Figure 7.5. Mesh electronics induces minimum interference to normal eye functions. (a) Representative near-infrared (NIR) images of a control mouse eye and the eye from another
Figure 7.5 (Continued): mouse injected with mesh electronics on different days post-injection before, during and after the air puff was given to the eye. The green arrow indicates the location of injected mesh electronics. (b) Quantification of number of blinks per air puff between the control and injected mouse eyes, showing no statistically significant difference between the two eyes and between different days post-injection. (c) Representative NIR images of the control and injected mouse eyes on different days post-injection during consecutive light on, off and on phases. The green arrow indicates the location of injected mesh electronics. (d) Pupil diameter of the control and injected mouse eyes on different days post-injection plotted as a function of time during alternating light intensity modulation. Red shared regions indicate the ‘light on’ phases while the gray shaded regions indicate the ‘light off’ phases. (e) A bright-field through-lens eye fundus image of the mesh-retina interface taken at normal incidence, showing minimum contrast of the mesh electronics and thus negligible blockage of incoming light to the retina (I), which was confirmed by UV-Vis-NIR transmission spectroscopy that reveals ca. 95% light transmittance of mesh electronics in the visible window (II).

To demonstrate that the injection and fixation of mesh electronics to the mouse eye did not interfere with the normal eye functions such as blink and pupillary reflexes, we used near-infrared illumination and imaging to track the responses of both a control mouse eye from an age-matched mouse with injection into neither eye and a mouse eye injected with mesh electronics during air puffs and ambient light intensity modulation (see Chapter 7.4). Chronic studies of blink and pupillary reflexes on different days post-injection reveal several key findings: first, despite the existence of mesh electronics that was previously injected and fixed to the lateral canthus of the eye, injected eye on Days 1, 7 and 14 post surgery all exhibited immediate and complete blinks in response to timed air puffs (Fig. 7.5a), with the number of blinks per air puff showing no statistically significant difference between the injected eye and the control eye and between different days post-injection (Fig. 7.5b). Second, in addition to normal blink reflex, the injected mouse eye also demonstrated similar response to ambient light intensity modulation by showing prompt and full-scale expansion and shrinking of the pupil size (Fig.
7.5c), which was also evidenced by the plots of fitted pupil diameter as a function of time with light on and off cycles (Fig. 7.5d). Third, it is noteworthy that owing to the high porosity of mesh electronics that occupied <5% space in 2D by metal features, the unfolded mesh electronics imposed minimum blockage of incoming light that reached retina, which was evidenced by both a bright-field through-lens image of the mesh-retina interface with near-normal light incidence showing near-transparent mesh features on top of the retinal blood vessels (Fig. 7.5e, I), and a UV-Vis-NIR transmission spectrum of the mesh electronics (Fig. 7.5e, II) with ca. 95% light transmittance in the spectral window visible to the mouse eye (ca. 400-600 nm) (32, 33). The minimum attenuation of incident light reaching the retina by the injected mesh electronics further justified the observed normal pupillary reflex of the injected eye. Taken together, both the normal blink and pupillary reflexes of injected eye suggest minimum damage of mesh electronics injection to the orbicularis oculi and iris dilator muscles and minimum interference of light perception of the eye.

7.2.4 Chronic 16-channel retina electrophysiology

With the 16-channel mesh electronics intravitreally injected into the mouse eye to afford epiretinal unfolding on the retina surface with chronically close interface, we carried out multiplexed electrophysiological recording from all 16 channels via the head-mounted interface FFC cable to external recording instrument when the mouse was restrained and its head fixed to reduce mechanically coupled noise. During recording the light intensity received by the injected mouse eye was modulated between the ‘on’ phase (23.95 μW/cm²) and the ‘off’ phase (0.24 μW/cm²) to identify different RGC subtypes. Chronic 16-channel retina electrophysiology reveals a few key features: first, all 16 channels remained connected, out of which 10 channels continued to measure single-unit firing activities from RGCs over the course of 14 days (Fig.
7.6a). Second, two representative channels, Ch 2 and Ch 8, demonstrated clear modulation of firing patterns in response to impinging light intensity, with Ch 2 exhibiting a significant increase in firing and Ch 8 showing a suppression of firing during the ‘on’ phase. Similar patterns of firing activity modulation were found for these two channels across different days post-injection and remained stable for 14 days (Fig. 7.6b). Third, spike sorting of these two representative channels clearly identified two neurons with distinct waveforms and amplitudes for each channel (Fig. 7.6c,d I), where the two neurons identified from Ch 2 showed greater firing for ‘on’ phase versus ‘off’ phase and thus suggested ON-type RGCs (Fig. 7.6c, II), while the two neurons from Ch 8 showed increased firing rate for ‘off’ phase versus ‘on’ phase and suggested OFF-type RGCs (Fig. 7.6d, II). The same two neurons for each channel were tracked over the course of 14 days as evidenced by similar modulation of firing rates by light intensity (Fig. 7.6c,d II). Fourth, analysis of the peak-to-peak spike amplitudes of neurons identified from Ch 2 and Ch 8 demonstrated relatively stable amplitude over time except for some fluctuation for the first two days after injection and surgery (Fig. 7.6c,d III), suggesting gradual improvement of the junction between mesh and retina for the first 2 days before forming a stable interface for recording and tracking the same individual neurons, similar to our previous publication (22) but with much shorter ‘annealing’ period owing to much reduced initial damage to the retina.
Figure 7.6. Chronic 16-channel *in vivo* epiretinal electrophysiology using mesh electronics. (a) Representative 16-channel recordings from the same mesh electronics injected into a mouse eye on Day 1, 7 and 14 post-injection. (b) Recording traces from two representative channels of the 16-channel recording data, showing light intensity modulated firing activity that remains stable for the course of 14 days. (c,d) Overlay of sorted and clustered single-unit spikes (I), analysis of firing rates (II) and time-dependent evolution of spike amplitudes (III) for each identified individual neuron from two representative channels, Ch 2 (c) and Ch 8 (d), on Day 1, 7 and 14 post-injection.
7.2.5 Chronic recording of direction selective ganglion cells (DSGCs)

Figure 7.7. Head-fixed in vivo retina electrophysiology setup for moving grating visual stimulation and simultaneous pupil tracking. (a) A white-light photograph showing a mouse that was injected with mesh electronics into its left eye and restrained in a Tailveiner® restrainer with the head-plate (black arrow) fixed for simultaneous in vivo retina electrophysiological recording through wired connection (red arrow), moving grating visual stimulation and NIR pupil tracking. (b) Photograph showing the same setup as a when visual stimuli of moving gratings were displayed in the dark with only deep red to NIR illumination (invisible to mouse eye) to avoid unwanted of RGC activity by ambient white light. (c) Setup for NIR illumination (green arrow) and video recording (blue arrow) of pupil dynamics. (d) An overview of simultaneous moving grating visual stimulation (right computer monitor) and NIR pupil tracking (left computer monitor) of mouse eye. (e) A representative NIR image of mouse eye showing the pupil (blue arrow) and mesh injection site at the lateral canthus (red arrow). (f) Representative traces showing the motion of the center of the pupil in two different directions (x: dorsal-ventral; y: nasal-temporal) in response to moving grating stimuli.
The chronically stable recording capability of mesh electronics for \textit{in vivo} retina electrophysiology also enabled us to track the same DSGCs in the retina over time. With the mouse restrained and its head fixed to restrict the visual field of the eye injected with mesh electronics, moving gratings in different directions were displayed on a computer monitor as the visual stimuli for identification of DSGCs with simultaneous electrophysiological recording through the interface FFC cable (Fig. 7.7a,b) and NIR imaging of the pupil (Fig. 7.7c,d; Chapter 7.4). Chronic recording followed by spike sorting allowed for identification of three putative DSGCs from a representative channel on both Day 7 (Fig. 7.8a) and Day 14 (Fig. 7.8b) post surgery, with the following key findings: first, on both days three distinct spike waveforms were identified from the recorded extracellular action potentials to suggest recording from three different RGCs, while both the waveform and amplitude remained relatively stable for each putative neuron from Day 7 to Day 14 post-injection (left column, Fig. 7.8). Second, firing times associated with each sorted and clustered putative RGC were plotted in a raster plot for 10 consecutive trials comprising gratings moving in 8 different directions (middle column, Fig. 7.8; see Chapter 7.4), clearly showing modulation of firing activities by both light intensity (moving grating vs. dark screen) and direction of the moving grating (indicated by arrows underneath all plots). The angular dependence of firing activity on moving direction of the grating was also shown in corresponding polar plots to confirm the identification of DSGCs (right column, Fig. 7.8) by computing the average firing rate over multiple trials of grating moving in the same direction. Third, both raster plots and polar plots showed similar firing patterns and angular preference for all three identified neurons from Day 7 to 14 post-injection, suggesting chronically stable recording from the same DSGCs.
Figure 7.8. Chronic in vivo recording and tracking of the same direction selective ganglion cells (DSGCs). (a,b) Spike overlay of sorted and clustered single-unit action potentials (left column) and their corresponding raster plots (middle column) and polar plots (right column) showing firing events in response to 10 repetitive trials of moving gratings in different directions on Day 7 (a) and 14 (b) post-injection.
7.3 Conclusion

In summary, we have demonstrated multiplexed, chronically stable recording of RGCs using syringe-injectable mesh electronics. The ultraflexibility of mesh electronics allowed for intravitreal injection of mesh electronics into the eye through a small needle like pharmaceuticals, while both the ultraflexibility and macroporosity of mesh electronics minimized the damage to the eye and attenuation/distortion of impinging light wavefront to the retina in the presence of an epiretinal implant, as evidenced by normal eye functions including blink and pupillary reflexes after injection. Mesh electronics after injection could unfold and form a close interface with the retina, allowing for chronically stable recording of the same RGCs based on single-unit firing analysis in response to different visual stimulation patterns. The minimally invasive delivery and chronically stable retina electrophysiology of mesh electronics have opened up new windows to understanding the functions and modulations of retina in the context of the entire organism, with future opportunities to quest how visual information is processed from the retina to higher-order brain centers such as the lateral geniculate nucleus, and how the activities of each individual RGC can be modulated by circadian rhythm, hormonal release, and optokinetic reflex.
7.4 Methods and Materials

7.4.1 Design and fabrication of mesh electronics

The syringe-injectable mesh electronics for intravitreal injection and epiretinal retinal ganglion cell (RGC) electrophysiology were fabricated according to geometrical design and fabrication procedure similar to our previous reports (20-22). Key parameters involved in the geometrical design of the mesh electronics are given as follows: (i) total mesh width, $W=1.5$ mm; (ii) longitudinal SU-8 ribbon width, $w_1=20$ μm, transverse SU-8 ribbon width, $w_2=20$ μm; (iii) angle between longitudinal and transverse SU-8 ribbons, $a=70^\circ$; (iv) longitudinal spacing (pitch between transverse ribbons), $L_1=333$ μm, transverse spacing (pitch between longitudinal ribbons), $L_2=100$ μm; (v) metal interconnect line width, $w_m=5$ μm, total number of recording channels, $N=16$; (vi) longitudinal ribbons have three-layer sandwich structure (SU-8 polymer/metal/SU-8 polymer) and transverse ribbons have two-layer structure (SU-8 polymer/SU-8 polymer). Key steps involved in the fabrication procedure are described as follows: (i) A 3" Si wafer (n-type 0.005 Ω·cm, 600-nm thermal oxide, Nova Electronic Materials, Flower Mound, TX) was pre-cleaned with oxygen plasma (100 W, 5 min), and a sacrificial layer of Ni with a thickness of 100 nm was thermally evaporated (Sharon Vacuum, Brockton, MA) onto the Si wafer. (ii) Negative photoresist SU-8 (SU-8 2000.5; MicroChem Corp., Newton, MA) with a thickness of ca. 420 nm was spin-coated onto the Ni-coated Si wafer, pre-baked sequentially at 65 °C for 1 min and 95 °C for 4 min, and then patterned by photolithography (PL) with a mask aligner (ABM mask aligner, San Jose, CA). After photolithographically patterned UV light exposure the Si wafer was post-baked sequentially at 65 °C for 3 min and 95 °C for 3 min. (iii) After post-baking, the SU-8 photoresist was developed in SU-8 Developer (MicroChem Corp., Newton, MA) for 2 min, rinsed with isopropanol, dried in a N$_2$ flow and hard-baked at 180 °C.
for 1 h. (iv) The wafer was then cleaned with oxygen plasma (50 W, 1 min), spin-coated with MCC Primer 80/20 and LOR 3A lift-off resist (MicroChem Corp., Newton, MA) sequentially, baked at 185 °C for 5 min, followed by spin-coating Shipley 1805 positive photoresist (Microposit, The Dow Chemical Company, Marlborough, MA), which was then baked at 115 °C for 5 min. The positive photoresist was patterned by PL to define the features of metal interconnect lines and developed in MF-CD-26 (Microposit, The Dow Chemical Company, Marlborough, MA) for 90 s. (v) A 1.5-nm thick Cr layer and a 100-nm thick Au layer were sequentially deposited by electron-beam evaporation (Denton Vacuum, Moorestown, NJ), followed by a lift-off step (Remover PG, MicroChem Corp., Newton, MA) to make the Au interconnect lines. (vi) Steps iv and v were repeated for PL patterning and deposition of the Pt recording electrodes (Cr: 1.5 nm, Pt: 50 nm). The diameter of Pt recording electrodes was 20 μm. (vii) Steps ii and iii were repeated for PL patterning of a top layer of the SU-8 polymer, which encapsulated and insulated the metal interconnect lines except for the exposed Pt recording electrodes. After hard-baking of top SU-8 layer at 180 °C for 1 h, the Si wafer with fabricated mesh electronics was hard-baked again at 200 °C to melt both top and bottom layers of SU-8 polymer and improve the fusion of both layers into a monolithic component. (viii) Finally, the Si wafer was cleaned with oxygen plasma (50 W, 1 min) and then transferred to a Ni etchant solution comprising 40% FeCl₃:39% HCl:H₂O=1:1:20 to remove the sacrificial Ni layer and release the mesh electronics from the Si substrate. Released mesh electronics were rinsed with sterile deionized (DI) water, transferred to a sterile aqueous solution of poly-D-lysine (PDL, 1.0 mg/mL, MW 70,000-150,000, Sigma-Aldrich Corp., St. Louis, MO) for 24 h, and then transferred to a sterile 1X phosphate buffered saline (PBS) solution (HyClone™ Phosphate Buffered Saline, Thermo Fisher Scientific Inc., Pittsburgh, PA) before use.
7.4.2 Vertebrate animal subjects

Adult (20-30 g) female CD-1 mice (6-8 weeks old, Charles River Laboratories, Wilmington, MA) were the vertebrate animal subjects used for chronic in vivo through-lens imaging and epiretinal electrophysiology post-injection in this study. Exclusion criteria were pre-established: animals with failed surgery (e.g., severe bleeding, cataract development, or clouding of vitreous body) or substantial acute damage to the eye (>20 μL of initial liquid injection volume) were discarded from further chronic recordings.

TYW3 transgenic mice were the vertebrate animal subjects used for acute ex vivo immunohistochemical staining and confocal imaging of the interface between injected mesh electronics and RGCs. The TYW3 transgenic mouse line was generated in the Sanes laboratory and characterized in a previous publication (31). Certain subtypes of RGCs were labeled by green fluorescent protein (GFP) in the TYW3 mouse line.

Randomization or blinding study was not applicable to this study. All procedures performed on the mice were approved by the Animal Care and Use Committee of Harvard University. The animal care and use programs at Harvard University meet the requirements of the Federal Law (89-544 and 91-579) and NIH regulations and are also accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC). Animals were group-housed on a 12 h: 12 h light: dark cycle in the Harvard University’s Biology Research Infrastructure (BRI) and fed with food and water ad libitum as appropriate.

7.4.3 In vivo mouse survival surgery for intravitreal delivery of mesh electronics

7.4.3.1 Intravitreal injection of mesh electronics in mouse eye

In vivo intravitreal injection of mesh electronics into the eyes of live mice was performed using a controlled non-coaxial injection method adapted from previously reported approach (21).
First, all metal and glass tools in direct contact with the surgical subject were autoclaved for 1 h before use, and all plastic tools in direct contact with the surgical subjects were sterilized with 70% ethanol and rinsed with sterile DI water and sterile 1X PBS before use. Prior to injection, the mesh electronics were sterilized with 70% ethanol followed by rinsing in sterile DI water and transfer to sterile 1X PBS. Mesh electronics probe was then loaded into a sterile borosilicate capillary tube with an ID of 200 μm and an OD of 330 μm (Produstrial LLC, Fredon, NJ) using the standard procedure described in previous publications (21, 22).

CD-1 mice were anesthetized by intraperitoneal injection of a mixture of 75 mg/kg of ketamine (Patterson Veterinary Supply Inc., Chicago, IL) and 1 mg/kg dexdomitor (Orion Corporation, Espoo, Finland). The degree of anesthesia was verified via the toe pinch method before the surgery started. To maintain the body temperature and prevent hypothermia of the surgical subject, a homeothermic blanket (Harvard Apparatus, Holliston, MA) was set to 37 °C and placed underneath the anesthetized mouse. GenTeal lubricant eye gel (Alcon, Fort Worth, TX) was applied on both eyes of the mouse to moisturize the eye surface throughout the surgery.

For intracortical implantation of the grounding screw for chronic electrophysiological recording from the retina, the mouse was placed in prone position and fixed with the stereotaxic frame. Hair removal lotion (Nair®, Church & Dwight, Ewing, NJ) was applied to the scalp for depilation of the mouse head and iodophor was applied subsequently to sterilize the depilated scalp skin. A 1-mm longitudinal incision along the sagittal sinus was made in the scalp with a sterile scalpel, and the scalp skin was resected to expose a 6 mm × 8 mm portion of the skull. METABOND® enamel etchant gel (Parkell Inc., Edgewood, NY) was applied over the exposed cranial bone to prepare the surface for mounting the grounding screw and interface cable on the mouse skull later. A 1 mm diameter burr hole was drilled using a dental drill (Micromotor with
On/Off Pedal 110/220, Grobet USA, Carlstadt, NJ) according to the following stereotaxic coordinates: anteroposterior: -4.96 mm, mediolateral: 3.10 mm (in the right hemisphere). After the hole was drilled, the dura was carefully incised and resected using a 27-gauge needle (PrecisionGlide®, Becton Dickinson and Company, Franklin Lakes, NJ). Then a sterilized 0-80 set screw (18-8 Stainless Steel Cup Point Set Screw; outer diameter: 0.060" or 1.52 mm, groove diameter: 0.045" or 1.14 mm, length: 3/16" or 4.76 mm; McMaster-Carr Supply Company, Elmhurst, IL) was screwed into this 1-mm burr hole to a depth of 500 μm as the grounding and reference electrode. METABOND® dental cement (Parkell Inc., Edgewood, NY) was used to fix the junction between the implanted grounding screw and the skull.

For intravitreal injection of mesh electronics into the mouse eye, the mouse was placed in right lateral decubitus position in the stereotaxic frame to expose its left eye for surgery. The GenTeal lubricant eye gel previously applied the eyes was gently removed only at the lateral and medial canthi of the left eye with sterile surgical spears (Braintree Scientific Inc., Braintree, MA), and the exposed area was swabbed with sterile 1X PBS. A sterile 27-gauge needle was used to puncture a hole for sclerotomy ca. 1 mm below the limbus at the lateral canthus (26) for subsequent intravitreal injection of mesh electronics and another hole at the medial canthus for draining the injected saline to reduce intraocular pressure during injection. The 200 μm ID and 330 μm OD capillary needle loaded with mesh electronics was mounted onto the stereotaxic stage (Fig. 7.2a,b) through a micropipette holder (Q series holder, Harvard Apparatus, Holliston, MA), which was connected to a 5 mL syringe (Becton Dickinson and Company, Franklin Lakes, NJ) through a polyethylene Intramedic™ catheter tubing (ID 1.19 mm, OD 1.70 mm). The sterilized capillary needle was allowed to advance through the pre-punctured hole at the lateral canthus of the left eye until its tip reaches the nasal part of the retina, taking special caution to
avoid damaging the lens (Figs. 7.1b, left column and 7.2d, left two columns). The mesh electronics was injected into the eye using a non-coaxial injection method adapted from our previously reported controlled injection approach (21, 22). Notably, the non-coaxial injection setup was built upon conventional stereotaxic frame used for brain probe implantation and is readily adaptable by other labs. In brief, controlled non-coaxial injection was achieved by balancing the volumetric flow rate (typically 3-7 mL/h), which was controlled by a syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA), and the horizontal needle withdrawal speed (typically 0.2-0.5 mm/s), which was controlled by a motorized linear translation stage (860A motorizer and 460A linear stage, Newport Corporation, Irvine, CA). During the controlled non-coaxial injection process, an eyepiece camera (DCC1240C, Thorlabs Inc., Newton, NJ) was used to ensure FoV visualization of the trajectory of the top end of mesh electronics that followed a pre-defined trajectory allowing horizontal placement of the bottom end of the mesh electronics during injection (Fig. 7.2c). After 2-3 mm length of mesh electronics was injected intravitreally, conventional coaxial injection was employed simultaneously with needle withdrawal, applying hydraulic pressure to push the external portion of mesh electronics with recording electrodes down towards the retina and allowing the capillary needle to exit the insertion hole at the lateral canthus (Fig. 7.2d, third column from left). For successful long-term epiretinal recording, the total injection volume is usually between 8 and 20 μL. An unexpected large injection volume (>40 μL) could result in significant clouding of vitreous body or bleeding at the corneal vessels, leading to expulsion of the subject from the study. An ample amount of water was injected extraocularly to fully expel the remaining mesh electronics from the capillary needle (Figs. 7.1b, right column and 7.2d, rightmost column). The junction between the mesh electronics and the lateral canthus of the eye was protected with a small amount of Kwik-Sil adhesive silicone.
elastomer (World Precision Instruments Inc., Sarasota, FL) while the draining hole at the medial canthus of the eye was sealed with a small amount of 3M™ Vetbond™ Tissue Adhesive (Santa Cruz Biotechnology Inc., Dallas, TX).

7.4.3.2 Electrical connection of intravitreally injected mesh electronics for chronic recording

After intravitreal injection of mesh electronics into the mouse eye, the fully expelled mesh electronics was unfolded onto a 16-channel flexible flat cable (FFC, PREMO-FLEX, Molex Incorporated, Lisle, IL) to expose the input/output (I/O) connection pads. High-yield bonding of mesh electronics I/O pads to the FFC was carried out using our reported conductive ink printing method (21, 22). In brief, the print head loaded with carbon nanotube solution (Stock No.: P093099-11, Tubes@Rice, Houston, TX) was driven by a motorized micromanipulator (MP-285/M, Sutter Instrument, Novato, CA) through a user-written LabVIEW program to print conductive ink automatically and connect each mesh I/O pad to each of the FFC contact electrodes to enable independently addressable recording elements. Failure of mesh I/O unfolding could lead to potential low-yield electrical connection to the FFC interface cable. All printed conductive lines were passivated by METABOND® dental cement, and then the entire FFC with mesh electronics bonded to the FFC was cemented to the mouse skull with METABOND® dental cement. Additional dental cement was applied to cover the silicone previously applied at the lateral canthus of the injected mouse eye for protection of mesh electronics without touching any part of the mouse eye or eyelids, resulting in a chronically stable interface for long-term retina electrophysiology. The FFC was folded to reduce its size on the mouse skull. A mouse head-plate made of poly(lactic acid) (PLA), which comprises an opening in the middle to fit the FFC and the grounding screw and two untapped free-fit holes
(hole diameter: 0.177" or 4.50 mm) in both wings for 8-32 screws, was also cemented to the skull using METABOND® dental cement for head-fixation during retina recording and pupil tracking.

7.4.3.3 Postoperative care

After surgery was complete, antibiotic ointment (WATER-JEL Technologies LLC, Carlstadt, NJ) was applied copiously around the wound, and the mouse was returned to the cage equipped with a 37°C heating pad and its activity monitored every hour until fully recovered from anesthesia (i.e., exhibiting sternal recumbency and purposeful movement). Buprenex (Buprenorphine, Patterson Veterinary Supply Inc, Chicago, IL) analgesia was given intraperitoneally at a dose of 0.05 mg/kg body weight every 12 h for up to 72 h post brain surgery.

7.4.4 In vivo through-lens imaging of mouse retina

In vivo through-lens imaging of the retina was performed on live mice before and after injection of mesh electronics to characterize the chronic interface between the injected mesh and the retina. We invented a ‘liquid Hruby lens’ by modifying the original design of the solid Hruby lens (6) widely used in ophthalmic fundus photography with transparent eye lubricant gel to counteract the intrinsic focusing power of the lens in mouse eye. This ‘liquid Hruby lens’ allows for microscopic imaging of eye fundus directly through the lens in a live animal with a conventional wide-field microscope.

To implement the ‘liquid Hruby lens’ for mouse eye fundus imaging, the mouse was anesthetized by intraperitoneal injection of a mixture of 75 mg/kg of ketamine and 1 mg/kg dexdomitor, before 1-2 drops of 1 wt% atropine sulfate ophthalmic solution was applied to the eye of interest. The ophthalmic solution was allowed to cover the surface of the eye for ca. 5 min
to afford complete dilation of the pupil before the eye was rinsed with 1X PBS. GenTeal lubricant eye gel (Alcon, Fort Worth, TX) was used to cover the dilated eye and a micro cover glass (25 mm × 25 mm, VWR International, Radnor, PA) was gently placed on the applied eye lubricant to flatten the gel/air interface. A ‘liquid Hruby lens’ was thus formed between the curved surface of the cornea and the flat cover slip with matching but opposite focusing power to the cornea and lens owing to similar refractive index of the lubricant gel to those of cornea and lens. A home-built white-light and wide-field microscope (Figs. 7.3a,b) was used to take eye fundus images in two different modes: in the bright-field mode, the optical axis of observation is within a range of 5° from that of illumination to afford the maximum contrast of retinal blood vasculature while the mesh electronics appears to have low contrast due to large porosity of mesh design and minimum blockage of illuminating/reflected light; in the pseudo-dark-field mode, the optical axis of observation is 30-40° from that of illumination to afford collection of scattered light off the longitudinal Au interconnect lines and Pt recording electrodes and improve the contrast for imaging. After imaging, the cover slip was removed and additional eye lubricant gel was applied copiously on both eyes, and the mouse was returned to the cage equipped with a 37°C heating pad and its activity monitored every hour until fully recovered from anesthesia.

7.4.5 Ex vivo imaging of the mesh-retina interface

For ex vivo confocal imaging based on the intrinsically expressed GFP fluorescence in certain RGCs, TYW3 transgenic mice were injected with mesh electronics in the left eye using aforementioned non-coaxial injection approach, and the degree of unfolding and area of coverage of mesh electronics on the retina were examined with the in vivo through-lens eye fundus camera to ensure successful intravitreal injection and epiretinal unfolding. Mice with good injections of mesh electronics in the eye were sacrificed by intraperitoneal injection of
euthasol at a dose of 270 mg/kg body weight, and injected eyes were carefully resected and fixed in fresh 4% paraformaldehyde in 1X PBS at 4 °C for 1 h without disturbing the mesh electronics in the vitreous humor. After fixation, the lens was carefully removed with spring scissors (15009-08, Fine Science Tools, Foster City, CA) to allow 3D confocal scanning of the mesh-retina interface without distortion of wavefront due to curvature of the lens. Retina whole mount images were obtained on a LSM 710 microscope at 20X (Zeiss, Jena, Germany) in the GFP channel using an excitation of 488 nm to collect both the GFP fluorescence from endogenously labeled RGCs and autofluorescence from the SU-8 polymer component of mesh ribbons. 3D reconstruction of confocal images was performed using ImageJ software with false green color for fluorescence signals.

For ex vivo confocal imaging of different retinal cell types with immunohistochemical staining, after mesh injection, eye resection and fixation, the injected eyeball was frozen and sectioned vertically to 20-µm slices in a cryostat. Retina sections were incubated in 1X PBS with 3% donkey serum and 0.3% Triton X-100 for blocking, followed by incubation with primary antibodies for at least 24 h at 4 °C and secondary antibodies for ~4 h. The primary antibodies used were: mouse anti-calbindin (1:100, for staining RGCs, amacrine cells and horizontal cells; Swant Inc., Marly, Switzerland); rabbit anti-PKC alpha (1:1000, for staining rod bipolar cells, Sigma-Aldrich, St. Louis, MO). Alexa647- and Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used to label the primary antibodies of mouse anti-calbindin and rabbit anti-PKC alpha, respectively. Samples were then washed with 1X PBS and mounted in Fluoromount G (Southern Biotech, Birmingham, AL). The vertical section image of the mesh-retina interface was obtained by the LSM 710 microscope at 40X with three different excitation channels: 568 nm, for excitation of Cy3-labeled bipolar cells (red) and
SU-8 polymer in the mesh ribbons (blue); 647 nm, for excitation of Alexa647-labeled RGCs, amacrine cells and horizontal cells (green).

7.4.6 In vivo eye imaging and pupil tracking

CD-1 mouse injected with mesh electronics into its left eye on different days post-injection and age-matched control CD-1 mouse without any injection of mesh electronics into either eye was restrained in a Tailveiner® restrainer (Braintree Scientific LLC., Braintree, MA). The same head-plate was cemented to the exposed skull of the control mouse in a similar manner as described above in “In vivo mouse survival surgery for intravitreal delivery of mesh electronics”. The skull-cemented head-plate was fixed to two Ø1/2" optical posts (Thorlabs Inc., Newton, NJ) through the two untapped free-fit holes in both wings with two 8-32 screws. The two optical posts were mounted to a frame assembled from standard optomechanical components prefixed on an optical breadboard (Thorlabs Inc., Newton, NJ). A near-infrared (NIR) light-emitting diode (LED) (emission wavelength: 850 nm, power density: 33.45 mW, M850L2, Thorlabs Inc., Newton, NJ) invisible to the mouse eye was used for illumination of the eye of interest, while a Watec CCD camera with sufficient quantum efficiency in the NIR range (WAT-502A, Newburgh, NY) was used for collecting dynamic video-rate (25 frames per second) NIR images of the mouse eye. For mice injected with mesh electronics in the left eye, the FFC interface cable and ground screw were connected to an Intan RHD 2132 amplifier evaluation system (Intan Technologies LLC., Los Angeles, CA) for simultaneous electrical recording of RGC activity during different stimuli to the eye (more details on recording in Chapter 7.4.7).

For imaging the blink reflex, a gentle air puff was given to the eye of interest every 15 s while the Watec CCD camera captured the NIR eye images continuously. For imaging the pupillary reflex, the white light power density impinging on the pupil was modulated between
23.95 μW/cm² for the on phase with 15-s duration and 0.24 μW/cm² for the off phase with 15-s duration while the Watec CCD camera captured the NIR pupil images continuously. For imaging the pupillary response to moving gratings, alternating white and black bars filling the entire computer screen (20.5"x12.5") in four different orientations (east-west, northeast-southwest, north-south and northwest-southeast) are programmed in Matlab to display on the computer screen and move in eight different directions (north, south, northwest, southeast, east, south, northeast and southwest; 2 s for each duration, 16 s for a complete trial, and 10 consecutive trials on a single day) with a randomized sequence of moving directions. All directions are with respect to the horizon, which was defined as the line connecting the medial and lateral canthi of the eye of interest. Location of the pupil was tracked and imaged continuously by the Watec CCD camera.

**7.4.7 In vivo chronic retina electrophysiology in mice**

Mice with mesh electronics injected into the left eye were recorded chronically on a daily basis, starting from Day 0 post-injection and surgery (i.e., ca. 5 h after surgery on the same day injection was performed). Mice were restrained in a Tailveiner® restrainer (Braintree Scientific LLC., Braintree, MA) with the head-plate secured to reduce mechanical noise during recording and restrict the visual field of the recorded eye during visual stimulation. Head-mounted FFC was connected to an Intan RHD 2132 amplifier evaluation system (Intan Technologies LLC., Los Angeles, CA) through a home-made printed circuit board (PCB). The 0-80 set screw was used as a reference. Electrophysiological recording was made when different visual stimuli were given to the mouse eye, including:

(i) Light on and off cycles: uniform illumination of white light with power density impinging on the pupil modulated between 23.95 μW/cm² for the on phase with 2-s duration and
0.24 μW/cm$^2$ for the off phase with 2-s duration was used for identification of RGCs with different polarities (ON- and OFF-center RGCs);

(ii) Moving gratings: gratings comprising alternating white and black bars and moving in different directions. A complete moving grating test set comprised 10 repetitive trials, where each trial comprised eight different directions (east, northeast, north, northwest, west, southwest, south and southeast) in a randomized sequence. The grating moved in each direction for 2 s, with a 2-s interval of dark screen between two directions. When moving grating was displayed on the computer monitor, it had an average power density of 10.1 μW/cm$^2$, in comparison to an average power density of 1.7 μW/cm$^2$ when dark screen was shown. The computer display was placed at a distance of 20 cm from the mouse eye, resulting in a lateral magnification of 0.015 (which means the image was minified on the retina) given the mouse eye diameter of 3 mm. The average power density for the images of moving gratings formed on the mouse retina was estimated as 1.26 μW/cm$^2$ based on the magnification, distance to the computer screen and size of the pupil. Each bar had a width of 5.4 mm on the computer screen, corresponding to a width of 81 μm on the retina based on the lateral magnification of 0.015. Grating moved at a speed of 16.96 mm/s in all directions on the computer screen, corresponding to a moving speed of 0.25 mm/s on the mouse retina given the same lateral magnification.

Electrophysiological recording was synchronized with light intensity modulation and moving grating stimulation for analysis of different types of RGCs. Data were acquired with a 20-kHz sampling rate and a 60-Hz notch filter, while the electrical impedance at 1 kHz of each recording electrode was also measured by the same Intan system.

7.4.8 Data analysis of pupil imaging and electrophysiological recording

7.4.8.1 Data analysis of pupil imaging
Dynamic NIR imaging of pupil was analyzed by a user-written Matlab software that recognizes the boundary of pupil based on contrast difference and fits the identified pupil boundary to a circle. Both the diameter and center of the fitted circle were extracted for each frame of the NIR video of pupil tracking, and plotted as a function of time with respect to timing of the light on/off cycles or moving gratings in different directions.

### 7.4.8.2 Data analysis of electrophysiological recording

The electrophysiological recording data was analyzed offline in a similar manner to our previous report (22). In brief, raw recording data was filtered using non-causal Butterworth bandpass filters (‘filtfilt’ function in Matlab) in the 250-6000 Hz frequency range to extract single-unit spikes of RGCs (6, 22). Single-unit spike sorting was performed by amplitude thresholding of the filtered traces by automatically determining the threshold based on the median of the background noise according to the improved noise estimation method (34). All sorted spikes were clustered to determine the number of single neurons and assign spikes to each single neuron based on principal component analysis (PCA) using the WaveClus software that employs unsupervised superparamagnetic clustering of single-unit spikes (34). For each sorted and clustered spike, a peak-to-peak spike amplitude and a firing time were assigned and all spike amplitudes and firing times belonging to the same cluster were used to plot the evolution of firing rates (Fig. 7.6c,d II) and spike amplitudes (Fig. 7.6c,d III) for each putative RGC on different days post-injection. Analysis of single-unit firing events was different for the two different visual stimulation protocols:

(i) Light on and off cycles: Firing rate was computed by taking the average inverse interval between two neighboring firing events of the same putative neuron during on or off phases.
(ii) Moving gratings: Firing events of each putative neuron after spike sorting and clustering were plotted as short vertical ticks in a raster plot (Fig. 7.8, second right column) that is aligned to the onset of the moving grating stimulation protocol. The number of firing events for a specific moving direction of the grating was averaged over 10 trials and repeated for all eight directions, and the angular distribution of firing rates were plotted in the polar plots to reveal direction selectivity of the recorded RGCs (Fig. 7.8, rightmost column).
7.5 Bibliography


Chapter 8

Multiplexed, High-Density Mesh Electronics for Stable Chronic Multi-Regional Brain Electrophysiology

The development of implantable electrical brain probes has significantly advanced fundamental neuroscience and translational neuromedicine. An ideal electrical probe should be capable of simultaneous monitoring the activity of large numbers of neurons across multiple local circuits that participate in the behavior of interest and stably tracking the evolution of these neuronal populations over the entire course of study. Silicon probes based on standard micro-fabrication technology offer large-scale, high-density brain recording but face challenges of chronic gliosis and instability due to mechanical and structural mismatch with the brain. Ultra-flexible mesh electronics, on the other hand, show negligible chronic immune response and stable long-term chronic brain monitoring and modulation at the single-neuron level, whereas only sixteen channel recordings have been demonstrated. Here, we present and integrate two complementary schemes—highly multiplexed mesh electronics design and multi-site implantation—that significantly increase the spatial coverage for brain mapping and the number of recorded neurons by mesh electronics. We demonstrate stable simultaneous 128-channel local field potentials (LFPs) and single-unit recordings from multiple regions of the same mouse brain over four months. This high-throughput integrated mesh electronics platform has also been applied to free-moving mice. This implemented scalability of mesh electronics together with demonstrated long-term stability provide an important progress towards the realization of an
ideal implantable electrical probes and could open up future studies in mapping the detailed circuit changes associated with learning, ageing and neurodegenerative diseases.
8.1 Introduction

Implantable electrical probes comprising microelectrodes capable of recording and/or stimulating the brain activity have proven to be not only a critical tool to decipher how information is encoded inside the brain (1-3), but also an effective technology to treat neurological diseases, including Parkinson’s and Alzheimer’s diseases (4-6), as well as to realize brain-machine interface (BMI) (6-9). On one hand, in contrast to non-invasive brain imaging methods (e.g., functional magnetic resonance imaging (fMRI)) (10) and surface recording probes (e.g., electroencephalography (EEG)) (11), implantable electrical probes offer spatiotemporal mapping and modulation capabilities at single-neuron level by directly placing sensing units in close proximity to individual neurons comprising local neural circuits (1). On the other hand, compared with optical techniques, electrical probes can access virtually any regions embedded deep inside the brain from freely behaving objects without the limitations of penetration depth, acquisition rates and incorporations of fluorescent labels (12, 13).

A central goal of implantable electrical probe development is to realize stable high-density large-scale brain mapping over long-term. High-density recording of local circuit activity compromising large numbers of neurons combined with large-scale sampling across multiple interconnected brain regions will decode how the brain processes information across various scales, from locally connected micro-circuits to long-range correlated macro-networks (14-16). In addition, robust tracking of these targeted neuron ensembles and circuits over time can illuminate how the brain gradually evolves in response to different behaviors or diseases (6, 18, 19). Therefore, an ideal implantable electrical probe with spatiotemporal resolution down to single-neuron level (micrometer, millisecond) and coverage up to whole-brain behavior level (meter, year) with simultaneous monitoring of a statistically representative fraction of neurons of
the behavior-related neural circuits/pathways could significantly advance understanding of brain computation and treatment of neurological and neurodegenerative diseases.

Important progresses have been made towards the realization of the ideal implantable electrical probes. Advancements in manufacturing process and circuit design have significantly increased the number of recording channels in a single probe and the number of implanted probes into the same object (14-16, 20, 21). For example, silicon probes comprising sixteen 128-channel shanks capable of targeting four spatially distinct neuroanatomical planes and measuring extracellular spikes and local field potentials (LFPs) from 1,024 electrodes were developed (16). More recently, a single shank silicon probe containing 384 channels and 966 recording sites with switches under each site has been tested (22, 23). While micro-fabrication based silicon probe offer great premise to achieve highly scalable electrical recordings of the brain, relative shear motion and chronic immune responses, in particular the formation of glial scars and the depletion of neurons surrounding the probes, cause degradation and instability of recorded signals over a time scale of weeks, which significantly limit its application for long-term neuroscience and clinic studies (24-28). On the other hand, mesh electronics, an ultra-flexible macroporous electrical probe that can be precisely implanted to targeted brain regions by syringe injection, shows a seamless three-dimensional (3D) interface with neural tissue post injection with absence of chronic gliosis (29-32). Moreover, robust tracking of the same neurons activities from mouse brains for at least eight months without probe repositioning has been demonstrated (33). Despite this unprecedented long-term stability of the mesh electronics, simultaneous recordings from only sixteen channels were realized.

In this work, we implement two complementary schemes to (i) increase the density and number of electrodes for individual mesh electronics while keeping its brain-like mechanical and
structural properties and (ii) enable simultaneous recordings from distinct brain regions via multi-site injections of multiple mesh electronics, thereby achieves large-scale, high-density, long-term stable *in vivo* brain recordings at single-neuron level from freely behaving mice.
8.2 Results and Discussion

8.2.1 Highly multiplex design for mesh electronics

Figure 8.1. High-density multiplexed mesh electronics via standard photolithography. (a,b) Schematics (a) and photos (b) showing scaling up of channel number and recording site density via increasing the longitudinal element density (from standard (I) to 32-channel (II) mesh design) and fabricating multiple channels in a single longitudinal element (from 32-(II) to 64-(III) and 128-channel (IV) mesh design). Red circles, orange lines and blue mesh structures in (a) represent the Pt-microelectrodes, Au-interconnects and SU-8 mesh ribbons, respectively. The
Figure 8.1 (Continued): red arrows in (b) highlight the number of recording sites (Pt-microelectrodes) on a single longitudinal element. The insets in (b) present the zoom-in view of the white dashed boxes. Scale bar: 100 μm. (c) Simulated longitudinal (black, left y-axis) and transverse (blue, right y-axis) bending stiffness of standard 16-channel and newly developed 32-, 64- and 128-channel mesh. (d) Stacked confocal image of a Rodamine-6G labeled 128-channel mesh electronics injected into water via a 400 μm inner diameter (ID) needle (yellow dashed line). (e) A single plane confocal image (3 μm focal depth) showing a zoom-in view of the white dashed box in (d). Scale bar: 100 μm. Inset: A magnified image of the white dashed box in (e) shows four Au-interconnects (dark lines) in a single SU-8 ribbons (red).

A major goal of highly multiplexed mesh electronics design is to increase the number and density of recording sites on each mesh electronics to reveal dynamic details of individual neurons comprising local circuits while maintaining its brain-like mechanics and structure. We achieved this goal via implementation of the following approaches (Figs. 8.1-8.3). First, we doubled the density of longitudinal (parallel to the metal interconnects) mesh elements to increase the number of recording sites on each mesh electronics from 16 to 32 (Fig. 8.1a,b I, II). Simulations of the mesh longitudinal ($D_L$) and transverse ($D_T$) bending stiffness showed an increase of $D_L$ by ~2 folds and an increase of $D_T$ by ~1.1 folds after doubling the density and number of longitudinal mesh elements (Chapter 8.4.2, Fig. 8.1c). Importantly, the bending stiffness value of the new mesh design with doubled longitudinal mesh elements density is still smaller than that of a single-neuron layer brain slice, which has a bending stiffness value 3-6 orders of magnitude smaller than those of conventional implantable brain probes (Chapter 8.4.2, Fig. 8.3e) (27, 29). Second, to maintain the low bending stiffness and macroporosity of the mesh electronics, we further scaled-up the number of recording sites on a mesh electronics by defining multiple interconnects and microelectrodes on a single longitudinal mesh elements (Fig. 8.1a,b III, IV). Specifically, we fabricated mesh electronics using standard photolithography...
procedures (Figs. 8.2 and 8.3) and demonstrated 64 and 128 recordings sites/channels on a single mesh electronics by defining 2 and 4 gold (Au) interconnects (2 μm wide) and platinum (Pt) microelectrodes (20 μm diameter) on each longitudinal SU-8 ribbons, respectively (Fig. 8.1b III, IV). Mechanical simulation showed almost no change of the bending stiffness values by increasing the number of channels from 32 to 128 (Fig. 8.1c) due to the minimum contribution of thin Au interconnects (100 nm) to the overall mechanical property (Chapter 8.4.2).

Figure 8.2. Schematic steps of mesh electronics fabrication. Components include silicon wafer (light green), nickel relief layer (dark green), polymer ribbons (blue), metal interconnects (black) and exposed metal electrodes (red). For each step (a-g) both top and side views are shown, where the side view corresponds to a cross-section taken at the position indicated by the white horizontal dashed line in the top view image of (a). (h) Zoomed-in views of regions highlighted by black (exposed Pt electrodes) and red dashed boxes (fully passivated interconnects) in (g).
Figure 8.3. Manufacturing and mechanical property of highly multiplexed mesh electronics. (a-d) Photos and differential interference contrast (DIC) image showing fabricated meshes with various design on a silicon wafer (a), during Ni etching (b), being transferred to 1X PBS (c) and injected through glass needle (d). (e) Comparison of the bending stiffness of 128-channel mesh electronics (black square) with those of conventional implantable electrical probes. The purple rectangle shows the bending stiffness of a single-neuron layer brain slice (see Chapter 8.4.2 for detail).

Significantly, confocal (Fig. 8.1d,e) and optical images (Fig. 8.3b-d) of the newly designed 128-channel mesh electronics following removal of the sacrificial nickel layer and underlying wafer (Fig. 8.3b, Chapter 8.4.1) and immersion of the free-standing mesh electronics in aqueous buffer (Fig. 8.3c) highlight key features of our design. First, the 128-
channel mesh electronics maintains ultra-flexible mechanical property with > 98% porosity in 3D volume. Second, this highly multiplexed mesh electronics with a total width of ~2 mm could be loaded and injected through a glass needle with inner diameter (ID) of 400 μm without jamming or damaging the integrity of the mesh (Figs. 8.1d,e and 8.3d). In addition, we characterized the inter-channel crosstalk by measuring the impedance between two adjacent SU-8 encapsulated metal interconnect components at both wet and dry state (see Chapter 8.4.3). The average between adjacent channels (2 μm spacing) was 2-3 GΩ (vs. 200-300 kΩ of each microelectrode sensor) at relevant frequencies (Fig. 8.1e), which demonstrated the effectiveness of the SU-8 passivation and minimum crosstalk for this highly multiplexed mesh electronics design (31).

8.2.2 Multi-site injections into different brain regions

Figure 8.4. Multi-site injections of mesh electronics. Schematic (a), photo (b) and Micro-CT (c) showing four mesh electronics (yellow arrows) injected into the same mouse. Four FFCs were stacked both vertically and horizontally for I/O connections during surgery. A, P, D, V in (c) correspond to the anterior, posterior, dorsal and ventral directions, respectively. (d) Photo of a freely behaving mouse with four meshes injected.
We further implemented injections of multiple highly multiplexed mesh electronics into distinct regions of the same mouse brain (Fig. 8.4a). An overview of our approach (Figs. 8.4 and 8.5) highlights the stereotaxic surgical process and light-weight instrument interface. First, four injection holes with well-defined stereotaxic coordinates were drilled bilaterally on top of the motor cortex (CTX) and somatosensory CTX/hippocampus (HIP) of the same mouse (Figs. 8.5a and 8.6a-c) followed by the implantation of a grounding screw (Fig. 8.5b). Second, stereotaxic injections of four 32-channel mesh electronics through the injection holes were carried out sequentially to deploy the mesh electronics into targeted brain regions with positioning precision of ~20 μm (Fig. 8.4b and Chapter 8.4.5) (30, 33). Micro-computed tomography (micro-CT) post-injection confirmed an extended morphology of all four mesh electronics along the injection direction (Fig. 8.4c and Chapter 8.4.6). Third, video tracking and mean squared displacement (MSD) analyses (Chapter 8.4.5, Fig. 8.6d,e) of the mouse trajectory with two of the four mesh electronics bilaterally injected into the motor CTX showed moving trajectory and MSD similar to control mouse without mesh electronics injection since 3 days post-injection (Fig. 8.6d,e), which is indicative of the minimum damage caused during acute mesh electronics implantation. Fourth, the input/output (I/O) pads of mesh electronics were unfolded onto and electrically connected to corresponding light-weight (~0.2 g) flexible flat cables (FFCs) (30, 33), which were stacked both laterally and vertically (Figs. 8.4b and 8.5c). The FFCs, which are plugged into recording instrumentation (Fig. 8.5d), were vertically stacked, folded and fixed to the mouse skull to minimize their size (Fig. 8.4a,c). Importantly, the interface of multiple mesh electronics injections had minimal impact on the housed animal given its low profile and insignificant increase in weight (Fig. 8.4d). Finally, the positions of mesh electronics were not adjusted over the course of our chronic experiments following implantation.
Figure 8.5. Multi-site mesh electronics injection and chronic recording from mouse brain. (a) Image of a mouse fixed in a stereotaxic frame with scalp skin retracted, and four injection holes (yellow arrows) and one grounding hole (white arrow) drilled through the skull plate. (b) Image showing the implantation of a grounding screw (white arrow). (c) Image representing four FFCs stacked both vertically and horizontally on a ceramic scaffold. (d) Photo of a fully awake but restrained mouse during chronic in vivo brain recording from 128 channels. See Chapter 8.4 for all experimental details of surgery and injection.

Figure 8.6. Movement trajectory tracking of mice with multi-site mesh injections. (a-c) Schematics of sagittal (a) and coronal (b,c) slices showing the stereotaxic coordinates of four
Figure 8.6 (Continued): injected mesh electronics, where mesh 1 (red) & 2 (green) were injected into the motor CTX and mesh 3 (blue) & 4 (black) were injected across the somatosensory CTX and HIP. (d,e) Tracked mouse moving trajectory during a 10-min video recording session (d) and corresponding MSD analysis results (e) (see Chapter 8.4.5 for detail). (e, II) shows a zoom-in view of the first 100s MSD. The orange, green, red and purple lines represent the mouse MSDs at 1, 2, 3, 7 and 14 days post-injection. The black lines show the MSD of a control mouse without mesh injection.

8.2.3 128-channel long-term brain activity mappings

Figure 8.7. Simultaneous 128-channel chronic recordings. (a,b) Simultaneous LFP (a) and extracellular spikes (b) recordings from four 32-channel meshes injected into the same mouse at 2 months post-injection. Mesh 1 (red) & 2 (green) were injected into the motor CTXs and mesh 3 (blue) & 4 (black) in the HIPs. (c) Bar charts showing average spike amplitude from all channels at 2 (upper row) and 4 (lower row) months post-injection. The color codes of different meshes are consistent with (a,b). (d) Average firing rate maps of all channels at 2 (upper row) and 4 (lower row) months post-injection. Colors indicate firing rate based on the color bar on the right.
We demonstrated simultaneous multiplexed chronic electrophysiology from the same awake mouse recorded by four 32-channel mesh electronics (Fig. 8.5d). Representative LFP (Figs. 8.7a and 8.8a, b I) and single-unit extracellular spike (Figs. 8.7b and 8.8a, b II) recordings from mesh electronics implanted into the motor CTX (mesh 1 & 2) and HIP (mesh 3 & 4) of bilateral hemispheres at 2 and 4 months post-injection highlight several points (Figs. 8.7a,b and 8.8a,b). First, LFP recordings from motor CTXs and HIPs showed distinct characteristics (Figs. 8.7a and 8.8a). Specifically, LFPs recorded from motor CTXs were dominated by relatively slow oscillations with a frequency ~3 Hz (Figs. 8.7a and 8.8a), which is characteristic of the prominent delta wave (1-4 Hz) in CTX (11, 34). On the contrary, LFPs recorded from HIP exhibited relatively weak delta wave but stronger fast oscillations representative of theta wave (4-8Hz) (Figs. 8.7a and 8.8a). These observed difference in relative strength of delta and theta waves are consistent with literature reports (11, 34). Second, both the amplitudes and temporal patterns of the LFPs remained stable across these 2 months recording period. In addition, characteristic spike trains representing extracellular action potentials from individual neuron firings were observed from multiple channels on all four mesh electronics (Figs. 8.7b and 8.8b).

To further quantify the chronic stability of the recorded single-unit spikes from all channels, we have sorted spikes from each recording traces and calculated the corresponding average amplitude. Indeed, peak-to-peak spike amplitudes from the majority of recording channels remained stable from 2 to 4 months post-injection (112 out 124 channels showed < 10% variation in recorded spike amplitude) (Fig. 8.7c). Furthermore, analyses of the average firing rate of each channel, obtained from dividing the recording time with total number of spikes fired during this recording period, demonstrated consistent chronic firing dynamics over this 2 months period (Fig. 8.7d). In addition, cross-channel correlation maps of both LFPs and single-
unit spikes of these four 32-channel simultaneous in vivo brain recording traces showed similar patterns at two and four months post-injection (Fig. 8.8c). These quantitative analyses further confirmed stable chronic single-neuron recording from our newly developed highly multiplexed mesh electronics, which is distinct from other brain implants with reported signal degradation and recording instability for majority of channels over weeks (24-28, 33).

Figure 8.8. Analyses of simultaneous 128-channel recordings from the same mouse. (a,b) Simultaneous LFP (a) and extracellular spikes (b) recordings from four 32-channel meshes injected into the same mouse at 4 months post-injection. Mesh 1 (red) & 2 (green) were injected into the motor CTXs and mesh 3 (blue) & 4 (black) in the HIPs. (c) Correlation maps of LFP (I) and extracellular spikes (II) recordings at 2 (upper row) and 4 (lower) months post-injection. Mesh 1-4 are shown from left to right. Colors indicate the correlation coefficient between any two given channels according to the color bar shown on the far right. All the maps were calculated from 2 s long data traces at both time points. See Chapter 8.4.8 for details of correlation coefficient calculations.
8.2.4 Multiplexed chronic recording from freely behaving mice

Figure 8.9. Chronic recordings from freely behaving mouse. (a) Photograph of a typical freely behaving mouse with low profile FFC and PCB when housed in animal facility. (b) Photograph of typical freely behaving mouse during recording. Voltage-amplifier was directly positioned near the mouse head to minimize mechanical noise coupling. A flexible serial peripheral interface (SPI) cable (light purple) was used to transmit amplified signals to the data acquisition systems. Insets: Zoom-in views of the electrical connections on mouse head. (c) Representative 32-channel LFP (heat maps) with amplitudes color-coded according to the color bar on the far right and extracellular spikes (traces) mapping from the same mouse at 2 (left) and 4 (right) months post-injection. The x-axes show the recording time while the y-axes represent the channel number of each recording electrode. (d) Correlation maps of LFP (I) and extracellular spikes (II) recordings at 2 (left) and 4 (right) months post-injection. All the maps were calculated from data shown in (c). See Chapter 8.4.8 for details of correlation coefficient calculations.
Last, we demonstrated chronic studies of freely behaving mice using newly designed highly multiplexed mesh electronics (Fig. 8.9). A 32-channel mesh electronics was injected into the somatosensory CTX/HIP of a live mouse and the I/Os were bonded to a trimmed 32-channel FFC, which was inserted into a customer designed printed circuit board (PCB). The entire interface, including the 32-channel mesh electronics, FFC and PCB, were then passivated and fixed onto the skull using dental cement (Fig. 8.9a). During electrophysiology recordings, preamplifier (preamp) was directly connected with the PCB to minimize mechanical noise coupling (33), while the other end of the preamp was connected with data acquisition system through a highly flexible cable that did not restrict animal motion (Fig. 8.9b). The lightweight interface was only 1.3 g with the preamp plugged-in (0.65 g without preamp) and had minimal impact on the animal behavior.

![Figure 8.10](image)

**Figure 8.10. Representative 32-channel chronic recording from freely behaving mouse.** (a,b) LFP (a) and extracellular spikes (b) recordings at 2 and 4 months post-injection.

Representative multiplexed chronic recordings from the freely behaving mouse at two and four months (Figs. 8.9c and 8.10) post-injection yielded well-defined LFPs in 32/32
channels with modulation amplitudes ~300 μV and single-unit spikes from 26/32 channels. Importantly, both recorded LFPs and single-unit spikes exhibited stable amplitudes without signal degradations across this two months period (Figs. 8.9c and 8.10). In addition, cross-channel correlation maps of these 32-channel *in vivo* brain recording traces showed similar patterns at two and four months post-injection (Fig. 8.10d).
8.3 Conclusion

In summary, we have demonstrated a feasible strategy for achieving highly multiplexed stable chronic recordings from multiple brain regions of the same mouse via a combination of two schemes: (i) increasing the number of microelectrodes in individual mesh electronics for high-density sampling of local microcircuits and (ii) multi-site injections of several meshes for simultaneous mapping of functionally correlated brain regions. We have shown the effectiveness of both schemes by showing simultaneous stable chronic recordings of LFPs and single-unit extracellular spikes from four 32-channel mesh electronics from the same mouse brain, and highly multiplexed recordings from freely behaving mouse with low profile electrical connections. Importantly, what we have demonstrated for either scheme here does not represent a limit for what is achievable even under current technology. On one hand, the compatibility of our mesh electronics manufacturing process with standard microfabrication procedure leaves immense room for further scaling-up of number of channels for individual mesh electronics by defining narrower metal interconnects with smaller spacing. Indeed, state-of-the-art lithography can easily achieve sub-100-nm and even sub-10-nm patterning (35), which could give more than 10 to 100 folds reduction on interconnects line width than our current design. On the other hand, mechanical engineering of mesh electronics to reduce both the size of needle for injection and the amount of liquid introduced could further increase the number of meshes that can be injected into the same rodent brain. Indeed, initial tests of new mesh design with half the transverse stiffness could enable reliable injection of mesh electronics through 150 μm ID needle with < 20 μL liquid. In the future, the correlation of functional network mappings from the highly multiplexed mesh electronics developed in this work with 3D structural imaging of mesh-brain
interface could provide comprehensive understanding regarding the structural and dynamical
evolution of neural circuits responsive for different behaviors.
8.4 Methods and Materials

8.4.1 Fabrication of syringe-injectable mesh electronics

The highly multiplexed mesh electronics for chronic brain activity mapping used fabrication procedure similar to our recent reports (29-33). Key steps involved in the fabrication of syringe-injectable mesh electronics are overviewed in Fig. 8.2, with the key mesh parameters as follows: total mesh width, $W=2$ mm, longitudinal SU-8 ribbon width, $w_1=20$ μm, transverse SU-8 ribbon width, $w_2=20$ μm, angle between longitudinal and transverse SU-8 ribbons, $\alpha=45^\circ$, longitudinal spacing (pitch between transverse ribbons), $L_1=333$ μm, transverse spacing (pitch between longitudinal ribbons), $L_2=62.5$ μm, metal interconnect line width, $w_m=2$ μm and number of recording channels on each longitudinal ribbon $N_{\text{each}}=1, 2$ and 4 for mesh electronics with total number of recording channels $N=32, 64$ and 128, respectively. The with key fabrication steps (Fig. 8.2) are as follows: (i) A sacrificial layer of Ni with a thickness of 100 nm was thermally evaporated (Sharon Vacuum, Brockton, MA) onto a 3" Si wafer (n-type 0.005 Ω·cm, 600-nm thermal oxide, Nova Electronic Materials, Flower Mound, TX), which was pre-cleaned with oxygen plasma. (ii) Negative photoresist SU-8 (SU-8 2000.5; MicroChem Corp., Newton, MA) was spin-coated on the Si wafer to a thickness of 400 nm, pre-baked sequentially at 65 °C for 1 min and 95 °C for 4 min, and then patterned by photolithography (PL) with a mask aligner (ABM mask aligner, San Jose, CA). After PL exposure the sample was post-baked sequentially at 65 °C for 3 min and 95 °C for 3 min. (iii) The SU-8 photoresist was then developed (SU-8 Developer, MicroChem Corp., Newton, MA) for 2 min, rinsed with isopropanol, dried in a N$_2$ flow and hard-baked at 180 °C for 1 h. (iv) The wafer was then cleaned with oxygen plasma (50 W, 1 min), spin-coated with MCC Primer 80/20 and LOR 3A lift-off resist (MicroChem Corp., Newton, MA), baked at 180 °C for 5 min, followed by spin-coating Shipley 1805 positive
photoresist (Microposit, The Dow Chemical Company, Marlborough, MA), which was then baked at 115 °C for 5 min. The positive photoresist was patterned by PL and developed (MF-CD-26, Microposit, The Dow Chemical Company, Marlborough, MA) for 90 s. (v) A 1.5-nm thick Cr layer and a 100-nm thick Au layer were sequentially deposited by electron-beam evaporation (Denton Vacuum, Moorestown, NJ), followed by a lift-off step (Remover PG, MicroChem Corp., Newton, MA) to make the Au interconnect lines. (vi) Steps iv and v were repeated for PL patterning and deposition of the Pt sensing or stimulation electrodes (Cr: 1.5 nm, Pt: 50 nm). The diameter of Pt sensing electrodes was 20 μm. (vii) Steps ii and iii were repeated for PL patterning of the top SU-8 layer, which served as the top encapsulating/insulating layer of the metal interconnect lines. The final hard-bake temperature of the top SU-8 layer was set at 190 °C to cross-link the bottom and top SU-8 layers, which then formed a monolithic encapsulation. (viii) Subsequently, the Si wafer was cleaned with oxygen plasma (50 W, 1 min) and then transferred to a Ni etchant solution comprising 40% FeCl₃:39% HCl:H₂O=1:1:20 to remove the sacrificial Ni layer and release the mesh electronics from the Si substrate. Released mesh electronics were rinsed with deionized (DI) water, transferred to an aqueous solution of 1X phosphate buffered saline (PBS) solution (HyClone™ Phosphate Buffered Saline, Thermo Fisher Scientific Inc., Pittsburgh, PA) before use.

8.4.2 Mechanical simulation

8.4.2.1 Bending stiffness simulation

We estimate the bending stiffness of the mesh electronics with different structures by finite element software ABAQUS. A unit cell similar to Fig. 3.1d in Chapter 3 was used for the simulation, where the mesh electronics was modeled with shell elements: A homogeneous single shell section with 800 nm thick SU-8 is assigned to the transverse ribbons; a composite section
with three layers of 400 nm thick SU-8, 100 nm thick gold and another 400 nm thick SU-8 was assigned to the longitudinal elements. Both SU-8 and gold are modeled as linear elastic materials, with Young’s modulus 2 and 79 GPa (29) respectively. To calculate the longitudinal and transverse bending stiffnesses, a fixed boundary condition was set at one of the ends parallel with the bending direction, and a small vertical displacement, \( d \), was added at the other end. The external work, \( W \), to bend the device was calculated. We defined the effective bending stiffness of the device as the stiffness required of a homogenous beam to achieve the same external work \( W \) under the displacement \( d \). Therefore, the effective bending stiffness per width of the mesh electronics can be estimated as (34)

\[
D_{\text{mesh}} = \frac{2Wl^3}{3d^3b}
\]  

(8.1)

with \( b \) the width of the unit cell parallel to the bending direction, and \( l \) the length of the unit cell perpendicular to the bending direction.

### 8.4.2.2 Effective bending stiffnesses of implantable probes

The effective bending stiffness per width of standard silicon probes, \( D_{\text{silicon}} \), can be estimated as (36)

\[
D_{\text{silicon}} = E_{\text{silicon}} \frac{h_{\text{silicon}}^3}{12}
\]  

(8.2)

where \( E_{\text{silicon}} = 165 \) GPa, \( h_{\text{silicon}} = 15 \) µm (37) are the young’s modulus of silicon and thickness of the probe, respectively. This calculation gives \( D_{\text{silicon}} = 4.6 \times 10^5 \) nN·m.

The effective bending stiffness per width of ultrasmall carbon electrodes, \( D_{\text{carbon}} \), can be estimated as (36)

\[
D_{\text{carbon}} = E_{\text{carbon}} \frac{\pi d_{\text{carbon}}^3}{64}
\]

(8.3)
where $E_{\text{carbon}} = 234$ GPa is the young’s modulus and $d_{\text{carbon}} = 7$ µm are the diameter of carbon fiber probe (38), respectively. This calculation gives $D_{\text{carbon}} = 3.9 \times 10^4$ nN·m.

The effective bending stiffness per width of planar shape polyimide probe, $D_{\text{polyimide}}$, can be estimated as (36)

$$D_{\text{polyimide}} = E_{\text{polyimide}} \frac{h_{\text{polyimide}}^3}{12}$$ (8.4)

where $E_{\text{polyimide}} = 2$-2.73 GPa and $h_{\text{polyimide}} = 10$-20 µm (39) are the young’s modulus of polyimide and the thickness of probe, respectively. This calculation gives $D_{\text{polyimide}} = 0.16$-1.3 × $10^4$ nN·m.

**8.4.3 128-channel mesh characterizations**

Confocal imaging was carried out using a Zeiss LSM 880 confocal microscope (Carl Zeiss Microscopy, Thornwood, NY). Confocal images were acquired using 561 nm wavelength lasers to excite mesh electronics labeled with Rodamine-6G fluorescent dyes.

Impedance-frequency (Z-f) relationship between adjacent metal interconnects on the same longitudinal ribbon were recorded in both dry and wet (a portion of the SU-8 passivated metal interconnects with a length of ~ 4mm was immersed in 1X PBS) states using an Agilent B1500A semiconductor device parameter analyzer (Agilent Technologies Inc., Santa Clara, CA) with B1520A-FG multi-frequency capacitance measurement unit (Agilent Technologies Inc., Santa Clara, CA).

**8.4.4 Vertebrate animal subjects**

Adult (25-35 g) male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were the vertebrate animal subjects used in this study. Exclusion criteria were pre-established: animals with failed surgery or substantial acute implantation damage (>100 µL of initial liquid injection volume) were discarded from further chronic recordings. Randomization or blinding study was
not applicable to this study. All procedures performed on the mice were approved by the Animal Care and Use Committee of Harvard University. The animal care and use programs at Harvard University meet the requirements of the Federal Law (89-544 and 91-579) and NIH regulations and are also accredited by American Association for Accreditation of Laboratory Animal Care (AAALAC). Animals were group-housed on a 12 h: 12 h light: dark cycle at Harvard’s Biology Research Infrastructure (BRI) and fed with food and water ad libitum as appropriate.

### 8.4.5 *In vivo* mouse survival surgery

#### 8.4.5.1 Stereotaxic injections of multiple mesh electronics in mouse brain

*In vivo* injections of multiple mesh electronics into the brains of live mice was performed using a controlled stereotaxic injection method described previously (33). First, all metal tools in direct contact with the surgical subject were autoclaved for 1 h before use, and all plastic tools in direct contact with the surgical subjects were sterilized with 70% ethanol and rinsed with sterile DI water and sterile 1X PBS before use. Prior to injection, the mesh electronics were sterilized with 70% ethanol followed by rinsing in sterile DI water and transfer to sterile 1X PBS.

C57BL/6J mice were anesthetized by intraperitoneal injection of a mixture of 75 mg/kg of ketamine (Patterson Veterinary Supply Inc., Chicago, IL) and 1 mg/kg dexdomitor (Orion Corporation, Espoo, Finland). The degree of anesthesia was verified via the toe pinch method before the surgery started. To maintain the body temperature and prevent hypothermia of the surgical subject, a homeothermic blanket (Harvard Apparatus, Holliston, MA) was set to 37 °C and placed underneath the anesthetized mouse, which was placed in the stereotaxic frame (Lab Standard Stereotaxic Instrument, Stoelting Co., Wood Dale, IL) equipped with two ear bars and one nose clamp that fixed the mouse head in position. Puralube ocular lubricant (Dechra Pharmaceuticals, Northwich, UK) was applied on both eyes of the mouse to moisturize the eye.
surface throughout the surgery. Hair removal lotion (Nair®, Church & Dwight, Ewing, NJ) was used for depilation of the mouse head and iodophor was applied to sterilize the depilated scalp skin. A 1-mm longitudinal incision along the sagittal sinus was made in the scalp with a sterile scalpel, and the scalp skin was resected to expose a 6 mm × 8 mm portion of the skull. METABOND®, enamel etchant gel (Parkell Inc., Edgewood, NY) was applied over the exposed cranial bone to prepare the surface for mounting the electronics on the mouse skull later.

Multiple 1 mm diameter burr holes were drilled using a dental drill (Micromotor with On/Off Pedal 110/220, Grobet USA, Carlstadt, NJ) according to the targeted stereotaxic coordinates. After the hole was drilled, the dura was carefully incised and resected using a 27-gauge needle (PrecisionGlide®, Becton Dickinson and Company, Franklin Lakes, NJ). Then a sterilized 0-80 set screw (18-8 Stainless Steel Cup Point Set Screw; outer diameter: 0.060" or 1.52 mm, groove diameter: 0.045" or 1.14 mm, length: 3/16" or 4.76 mm; McMaster-Carr Supply Company, Elmhurst, IL) was screwed into this 1-mm burr hole to a depth of 500 μm as the grounding and reference electrode. Burr holes were drilled for injection of mesh electronics according to the following stereotaxic coordinates for activity recording (40):

1) Primary somatosensory cortex, barrel field: anteroposterior: -1.82 mm, mediolateral: 3.00 mm, dorsoventral: 0.75 mm.

2) Primary somatosensory cortex, trunk: anteroposterior: -1.70 mm, mediolateral: 2.00 mm, dorsoventral: 0.75 mm.

3) Hippocampal CA1 field: anteroposterior: -1.70 mm, mediolateral: 1.60 mm, dorsoventral: 1.17 mm.

4) Hippocampal CA3 field: anteroposterior: -1.70 mm, mediolateral: 2.00 mm, dorsoventral: 1.85 mm.
5) Primary motor area: anteroposterior: 1.34 mm, mediolateral: 1.50 mm, dorsoventral: 0.70 mm.

The dura was removed from the burr hole drilled for mesh electronics injection and sterile 1X PBS was swabbed on the surface of the brain to keep it moist throughout the surgery. The mesh electronics was injected into the desired brain region using a controlled injection method (30). In brief, the mesh electronics was loaded into a glass capillary needle with inner diameter (ID) of 400 μm and outer diameter (OD) of 650 μm (Produstrial LLC, Fredon, NJ). The glass capillary needle loaded with mesh electronics was mounted onto the stereotaxic stage through a micropipette holder (Q series holder, Harvard Apparatus, Holliston, MA), which was connected to a 5 mL syringe (Becton Dickinson and Company, Franklin Lakes, NJ) through a polyethylene Intramedic™ catheter tubing (ID 1.19 mm, OD 1.70 mm). Controlled injection was achieved by balancing the volumetric flow rate (typically 20-50 mL/h), which was controlled by a syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA), and the needle withdrawal speed (typically 0.2-0.5 mm/s), which was controlled by a motorized linear translation stage (860A motorizer and 460A linear stage, Newport Corporation, Irvine, CA). Using the controlled injection method with field of view (FoV) visualization through an eyepiece camera (DCC1240C, Thorlabs Inc., Newton, NJ), mesh electronics were delivered to targeted brain regions with elongated morphology along the injection direction with ~20 μm precision. For successful long-term recordings, the total injection volume was usually between 10 and 100 μL. An unexpected large injection volume (>100 μL) could result in brain edema or failure of recovery from acute surgical damage, leading to expulsion of the subject from the study.

8.4.5.2 Mouse moving trajectory tracking and mean squared displacement (MSD) analyses
The movements of mice while foraging around a pristine cage were recorded for a 10 min session everyday since acute mesh electronics injection. The videos of mouse movements were analyzed with Gaussian blur filter and object tracking algorithm using Matlab to extract the mouse’s trajectories. The extracted trajectories were then used for MSD, which is defined by Equation 8.5, analyses

\[
MSD(t) = \langle (d(t + \tau) - d(\tau))^2 \rangle, \tag{8.5}
\]

where \(d(t + \tau) - d(t)\) denotes the relative displacement of the mouse between time \(t + \tau\) and \(\tau\). The average is over all time points \(\tau\) that satisfies \(t + \tau\) smaller than the total recording time period (i.e. 10 min in our analyses) (41).

### 8.4.5.3 Electrical connection of mesh electronics for chronic recordings from awake and restrained mice

After the injection of mesh electronics into the desired region of a mouse brain, the stereotaxic stage was moved to reposition the glass capillary needle over four vertically and horizontally stacked 33-channel flexible flat cable (FFC, Molex Incorporated, Lisle, IL), and then the remaining mesh electronics was fully expelled from the needle and unfolded onto the FFC to expose the input/output (I/O) connection pads. High-yield bonding of mesh electronics I/O pads to the FFC was carried out using our reported conductive ink printing method (30, 33). In brief, the print head loaded with carbon nanotube solution (Stock No.: P093099-11, Tubes@Rice, Houston, TX) was driven by a motorized micromanipulator (MP-285/M, Sutter Instrument, Novato, CA) through a user-written LabVIEW program to print conductive ink automatically and connect each mesh I/O pad to each of the FFC lines to enable independently addressable sensor elements. Failure of mesh I/O unfolding could lead to potential low-yield electrical connection to the FFC interface cable. All printed conductive lines were passivated by
METABOND® dental cement (Parkell Inc., Edgewood, NY), and then the entire FFCs with mesh electronics bonded were vertically stacked and cemented to the mouse skull with METABOND® dental cement. The FFC was folded to reduce its size on the mouse skull. The total mass of the bonded interface cable with mesh electronics is typically 0.8-1.0 g.

8.4.5.4. Electrical connection of syringe-injectable electronics for chronic recordings from freely behaving mice

After the injection of mesh electronics into desired mouse brain regions, the stereotaxic stage was manually moved to reposition the glass capillary needle to a 32-channel trimmed FFC (FFC, PREMO-FLEX, Molex Incorporated, Lisle, IL), and then the remaining mesh electronics was fully expelled from the needle and unfolded onto the FFC. Conductive ink printing was used to bond the mesh electronics I/O pads to the FFC cable. The FFC was then inserted into a zero insertion force (ZIF) connector (Molex Incorporated, Lisle, IL) mounted on one side of a customer designed printed circuit board (PCB) with a thickness of ~ 0.5 mm (Advanced Circuits Inc. Aurora, CO), whose other side contains an Omnetics male connector (A79024-001, Omnetics Connector Corp., Minneapolis, MN) with through-hole connections to the ZIF connector. The 0-80 grounding screw was electrically connected to one of the two pre-defined grounding/reference pads on the PCB using silver conductive epoxy (MG Chemicals, Burlington, ON, Canada). All printed conductive lines were protected by METABOND® dental cement, before the entire packaged FFC and PCB were cemented to the mouse skull.

8.4.5.5 Postoperative care

After surgery was complete, antibiotic ointment (WATER-JEL Technologies LLC, Carlstadt, NJ) was applied copiously around the wound, and the mouse was returned to the cage equipped with a 37°C heating pad and its activity monitored every hour until fully recovered.
from anesthesia (i.e., exhibiting sternal recumbency and purposeful movement). Buprenex (Buprenorphine, Patterson Veterinary Supply Inc, Chicago, IL) analgesia was given intraperitoneally at a dose of 0.05 mg/kg body weight every 12 h for up to 48 h post surgery.

**8.4.6 Micro-Computed Tomography**

One mouse injected with four mesh electronics, where the I/Os were bonded to four FFCs and then cemented to the mouse skull, was euthanized via intraperitoneal injection of Euthasol at a dose of 270 mg/kg body weight and decapitated. The decapitated mouse head was imaged using an HMXST Micro-CT X-ray scanning system with a standard horizontal imaging axis cabinet (model: HMXST225, Nikon Metrology, Inc., Brighton, MI). Imaging parameters were set as 95 kV and 93 μA for scanning the decapitated mouse head. Before scanning, shading correction and flux normalization were applied to adjust the X-ray detector. The *CT Pro 3D* software (ver. 2.2, Nikon-Metris, UK) was used to calibrate centers of rotation for micro-CT sinograms and to reconstruct all 2D images. *VGStudio MAX* software (ver. 2.2, Volume Graphics GMbh, Germany) was used for 3D rendering and analysis of the reconstructed images.

**8.4.7 *In vivo* chronic brain recording and stimulation in mice**

**8.4.7.1 Chronic brain recording from awake and restrained mice**

Mice with four implanted mesh electronics and FFC connectors were recorded chronically on a biweekly basis, starting from Day 14 post-injection and surgery. Mice were restrained in a Tailveiner® restrainer (Braintree Scientific LLC., Braintree, MA) while its head-mounted FFC was connected to four Intan RHD 2132 amplifier evaluation systems (Intan Technologies LLC., Los Angeles, CA) through four home-made PCBs. The 0-80 set screw was used as a reference. Electrophysiological recording was made with a 20-kHz sampling rate and a
60-Hz notch filter, while the electrical impedance at 1 kHz of each recording electrode was also measured by the same Intan system.

**8.4.7.2 Chronic brain recording of freely behaving mice**

Mice with Omnetics connectors were recorded chronically on a biweekly basis when they were freely roaming in the cage. For recording, an Intan preamplifier chip (RHD2132 16-Channel Amplifier Board, Intan Technologies LLC., Los Angeles, CA) with pre-installed female Omnetics connector was connected directly to the male Omnetics connector cemented on the mouse skull during surgery, and the mouse was allowed to roam in a cage environment. Electrophysiological recordings were made using the same Intan evaluation system with a 20-kHz sampling rate and a 60-Hz notch filter, and were synchronized with video recording of the mouse’s motion inside the cage using a digital camera.

**8.4.8 Data analysis of electrophysiological recording**

The electrophysiological recording data was analyzed offline. In brief, raw recording data was filtered using non-causal Butterworth bandpass filters (‘filtfilt’ function in Matlab) in the 250-6000 Hz frequency range to extract single-unit spikes (33), in the 0.1-150 Hz range to extract LFP (33). The intrinsic noise distribution of a specific channel was analyzed based on all recording traces bandpass filtered at 250-6000 Hz excluding any firing spikes. The correlation coefficient maps of single-unit spike recording traces shown in **Figs. 8.8c** and **8.9d** were calculated based on the standard Pearson product-moment correlation coefficient for time series. Namely, for two spike traces, \( Y_1(t) \) and \( Y_2(t) \), the correlation coefficient between them is calculated as

\[
\text{Corr}(Y_1, Y_2) = \frac{\int_{\tau_1}^{\tau_2} (Y_1(t) - \bar{Y}_1)(Y_2(t) - \bar{Y}_2)dt}{\sqrt{\int_{\tau_1}^{\tau_2} (Y_1(t) - \bar{Y}_1)^2 (Y_2(t) - \bar{Y}_2)^2 dt}}
\]

(8.6)
where $T_1$ and $T_2$ indicate the starting and ending time of the recording traces (in Figs. 8.8c and 8.9d the time window is 2 s), and $\bar{Y}_i = \frac{\int_{T_i}^{T_2} Y_i(t)dt}{(T_2 - T_i)}$ ($i = 1, 2$) represents the averaged value of $Y_i(t)$ over the time period between $T_1$ and $T_2$.

Single-unit spike sorting was performed by amplitude thresholding of the filtered traces by automatically determining the threshold based on the median of the background noise according to the improved noise estimation method (42). The average spike amplitude for each recording channel (Fig. 8.7c) was defined as the peak-to-peak amplitude of the spikes with recordings carried out based on a 30-min recording session. The average firing rate of each recording channel (Fig. 8.7d) was obtained from dividing the recording time with total number of spikes fired during this recording period.
8.5 Bibliography


Chapter 9

Future Perspective: Beyond Neuron-Centered Brain Research

Current brain research centered on learning, memory and associated neurodegenerative diseases such as Alzheimer’s has focused primarily on neurons, although growing evidence suggests that non-neuronal glial cells likely maintain a significant role in these fundamental processes. Elucidating such contributions from glia is therefore critical in developing a comprehensive understanding of the brain. However, the reliance of glia on both chemical and electrical communications, their intrinsic three-dimensional (3D) organization, and aberrant activation in response to foreign bodies make glia extremely difficult to study in live animals using existing tools. The mesh electronics, which has a 3D open structure analogous to brain cell networks and tissue-like mechanical properties to minimize abnormal glia activation, offers great promise to overcome these challenges. Specifically, the following future directions building on top of nanoscopic cellular probes and macroscopic mesh electronics described in this thesis may not only provide a critical platform for understanding glia-neuron interactions in live animals but will also offer valuable insights into learning and memory as well as potential glia-based therapies for neurodegenerative diseases: (i) Development of a biocompatible brain probe that integrates electrical and chemical sensing with optical and pharmacological manipulation to allow long-term interrogation and modulation of glia-neuron interactions. (ii) Application of this probe to study how controlled regulation of glia alters neural circuit dynamics and topology in context-dependent learning and memory formation. (iii) Employment of this probe as a diagnostic and potential therapeutic tool for Alzheimer’s disease (AD), which is associated with progressive memory loss and glia-neuron dysfunction.


9.1 Introduction

Historically, brain research has focused on the computational role of neurons in brain function (1). Recently, glia, non-neuronal cells occupying more than half of the brain volume, have been implicated in learning and memory processes through both active local neural connectivity (synapse) mediation and global information integration (2-4) (Fig. 9.1). For example, a recent study showed that mice engrafted with human glia outperformed their mouse-brained counterparts in learning and memory tasks (5). Correspondingly, dysfunction of the glia has been linked to pathogenesis in several neurodegenerative diseases (e.g., Alzheimer’s) (3, 6-8) which is characterized by progressive cognitive impairment and memory loss. Shifting our focus from neuron-centric to glia-neuron-oriented research could be a new paradigm for understanding how the brain encodes, integrates and stores information and could offer much-needed guidance for treatment of neurodegenerative diseases suffered by millions of people.

The challenges of studying glia-neuron biology are not to be underestimated. Current mechanistic studies of glia-neuron interactions are based primarily on ex vivo two-dimensional (2D) cell culture or explant brain slices (9, 10). However, the intrinsically 3D nature of the glial network and the deviation from its in vivo physiological state upon explantation limit the generality of the findings inferred from these methods (8-10). In vivo approaches with cellular spatiotemporal resolution mainly rely on optical imaging (11) and electrical implants (12), both of which have limitations. Optical imaging is constrained in terms of penetration depth (13), and existing electrical implants, though capable of recording neuronal electrical activity from deep brain regions, are functionally incapable of capturing chemical signaling between glia and neurons (2, 4). Moreover, mechanical and structural mismatch between these rigid implants and
the brain triggers extrinsic glial immune activation (14) and causes chronic recording instability (15).

Figure. 9.1. Glia-neuron interaction. Different types of glia, including astrocytes (red), microglia (green), and oligodendrocytes (purple), actively interact with neurons (blue).

Syringe-injectable mesh electronics, a flexible electrical probe described in Chapter 3-8 (16-18), holds great promise as a tool to overcome these challenges. In particular, its brain-like mechanical properties and open 3D macroporous structure causes minimal perturbation to glia and enables a seamless interface with glia-neuron networks on timescales of up to a year (18). However, several key functions enabling regulation and recording of glia and neurons’ intrinsic dynamics and mutual interactions remain to be incorporated into this promising probe. The overall goal will be to develop a novel 3D multifunctional brain probe that builds on mesh electronics to provide a comprehensive understanding of contributions from both neurons and glia to learning and memory processes and to enable glia-neuron-oriented diagnosis and treatment paradigms for neurodegenerative diseases.
9.2 Future Directions

I propose the following future directions to shift our focus from current neuron-centric to glia-neuron-oriented research: (i) develop a 3D multifunctional brain probe as a general platform for the investigation of glia-neuron interactions with minimal disturbance to the brain’s normal physiological state, (ii) use this probe to study the contribution of glia-neuron interactions in context-dependent learning and memory, and (iii) employ this probe as a glia-centered diagnostic and therapeutic tool for AD (Fig. 9.2). For (ii) and (iii), the primary focus will be on astrocytes, the most abundant glia type, because there is strong evidence that they actively participate in learning and memory by modulating synaptic elimination and plasticity and integrating information from millions of neurons (2, 4, 19). The major role of astrocytes in brain immunity (20) and the difficulty of monitoring their activity make a novel approach - one that will not interfere with their normal activity - essential.

Figure. 9.2. 3D multifunctional tools for exploring glia-neuron interactions. Multifunctional brain probes (blue dashed box) with minimum disturbance to the glia-neuron network (inset) will be used to study glia-neuron interactions in learning and memory, and treatment of AD.
9.2.1 To develop an enhanced, minimally invasive 3D multifunctional brain probe

A major goal is to develop a multifunctional probe based on the syringe-injectable mesh electronics. In addition to the already-achieved electrical recording and stimulation functions, it will be important to integrate the following key components, each of which will be individually addressable, for simultaneous electrical, optical and chemical modulation and monitoring of the glia-neuron system (Fig. 9.2, inset).

Glia-neuron modulation: The capability to regulate the glia-neuron system is essential to infer its causal link with learning and memory and to interfere with its dysfunction. To enable this ability, it will be critical to combine (i) optical waveguides and (ii) microfluidic channels for in vivo cell-type-specific optogenetic and label-free pharmacological regulation, respectively. Optical waveguides and microfluidic channels could be implemented by standard microfabrication (21) and soft lithography (22), respectively. For verification, ex vivo light and drug delivery could be carried out and characterized on transparent “phantom brain” (0.5% agarose hydrogel) (23).

Glia-neuron monitoring: While glia communicate with each other mainly through calcium signaling (24), they interact with neurons via gliotransmitters and cytokines (25). Therefore, it will be desired to integrate (i) calcium ion (Ca\(^{2+}\))-selective sensors and (ii) microdialysis probes for in vivo real-time monitoring of glial dynamics and continuous gliotransmitter and cytokine measurement of glia-neuron interactions, respectively. Ca\(^{2+}\)-sensors could be fabricated by coating electrical sensors with ionophore-doped polyvinyl chloride (26). Microdialysis probes could be implemented by incorporating a semipermeable membrane into the microfluidic channels described above (27). Both components could be tested in high ionic strength solution to mimic brain conditions.
9.2.2 To apply the multifunctional brain probe in studies of learning and memory in mice

The multifunctional probe will be capable of examining how astrocyte-neuron interactions affect neural circuit evolution during context-dependent learning and memory processes (Fig. 9.2). Specifically, the primary focus will be on elucidating the astrocytic regulation of neural activity, especially individual dynamics and network topology, in inhibitory avoidance (IA) memory consolidation, a context-dependent memory associating an emotional aversive event with a given context (28). The probe based on mesh electronics platform is essential for this study because the long-term nature of IA memory (28) and necessity of modulating astrocyte activity preclude the use of conventional invasive electrical probes. Three major steps designed to facilitate this study are as follows:

First, two multifunctional probes will be implanted into the mouse amygdala and hippocampus, key brain areas for context-dependent learning and memory, respectively (29), followed by adenovirus injection via the microfluidic channel to drive astrocyte-specific expression of photo-activatable ion channels (AAV2.5-GFAP-ChR2-mCherry) (30) for optogenetic manipulation using embedded waveguides. Fear conditioning to evoke IA (29) will be performed on these mice and control groups comprising wild-type and astrocyte-deficient mice (31).

Second, from a macroscopic and phenomenological perspective, I propose to assess the influence of astrocyte activation on IA by comparing the retention latency, which reflects memory strength (29), of optogenetically manipulated mice with that of control groups at different time points after fear conditioning. Standard statistical tests (e.g., Wilcoxon test) could be performed to quantify the significance of behavioral variation.
Third, from a microscopic and mechanistic perspective, I propose to investigate the astrocytic regulation of IA by correlating the astrocytic activity with neural circuit changes. Specifically, it will be interesting to map the progressive evolutions in astrocyte calcium dynamics, neuron firing rates and neuron-neuron correlations using Ca$^{2+}$ and electrical sensors. Measured changes will be linked to corresponding astrocyte-neuron network structural rearrangements revealed by later histochemical study to build a comprehensive model correlating astrocyte-neuron interactions with IA memory.

9.2.3 To apply the multifunctional brain probe for diagnosis and treatment of Alzheimer’s disease

The strong link between astrocyte dysfunction and AD-associated synapse loss (6, 7, 32), which may begin twenty years before symptoms appear, suggests that targeting glia-neuron interactions using the probe developed in this work could provide a novel paradigm for early AD detection and pharmacotherapies beyond the current neuron-centric approaches (33). I propose to implement this therapeutic application via two complementary approaches: (i) a direct method relying on detection and modulation of astrocyte-neuron network dysfunction (7), and (ii) an indirect method based on monitoring and regulation of astrocyte-driven neuroinflammation (Fig. 9.2) (32, 34).

Diagnosis: Abnormal synapse loss caused by astrocyte-neuron network dysfunction is a powerful early predictor of AD (6, 7). For diagnosis, changes in neuron-neuron firing correlations and γ-band of local field potential reflecting ensemble synaptic kinetics (12) could be monitored over time in a mouse model of AD (3xTg-AD) (35) together with astrocyte calcium dynamics. In addition, irregular neuroinflammation driven by astrocyte malfunction, an
alternative early hallmark of AD (34), could be detected via *in vivo* microdialysis probing of inflammatory cytokines (e.g., IL-1) (27) to provide complementary diagnostics.

Treatment: Chronic delivery of gliotransmitter via microfluidic channels and electrical stimulation via embedded electrodes could be combined for pharmacological and electroceutical regulation of astrocyte-neuron interactions and synapse connections, respectively. Another strategy exploring delivery of drugs targeting inflammatory cytokines to restore normal astrocytic function (34) could also be tested. IA tests could be performed to evaluate the treatment outcome and compared with tests performed on untreated control mice.
9.3 Potential Outcome and Impact

The realization of these future directions will provide a critical new platform to advance glia-neuron-oriented research in the area of learning and memory and neurodegenerative diseases. Development of this platform will open up opportunities for future investigation of the role of other glial cell types (e.g., microglia) in brain processes and glia-neuron interactions in brain development and related disorders like autism and schizophrenia (36, 37).
9.4 Bibliography


