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**DEVELOPMENT OF FLEXIBLE ELECTRODE ARRAYS FOR CHRONIC
STABLE AND SCALABLE NEURAL RECORDING**

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Zhengtuo Zhao

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Abstract

DEVELOPMENT OF FLEXIBLE ELECTRODE ARRAYS FOR CHRONIC STABLE AND SCALABLE NEURAL RECORDING

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The brain is a massively-interconnected and constantly-evolving network of specialized circuits; a systematic understanding of the circuits requires a probe-tissue interface that can record and modulate brain activities at diverse spatial and temporal scales. Implanted electrodes provide a unique approach to decipher brain circuitry by allowing for time-resolved electrical detection of individual neuron activity. However, conventional intracortical recordings are often sparse, and importantly, unstable over long term. These pose limitations on their scientific and clinical applications. Through the use of less-rigid polymer materials and the ten-to-a-hundred fold reduction on probe size, the flexibility of a neural probe can be improved by four orders of magnitude, which results in a friendly probe-tissue interfaces, long-term recording performance, and greater potential for scaling up in implantation density. Here we present our progress on the development of a novel neural recording platform including 1. A stable neural interface named nanoelectronic threads (NETs) which demonstrated long-term stable recording over four months, seamless chronic probe-tissue integration and easy compatibility with optical imaging. 2. A facile implantation method to apply the flexible NETs in scalable, reliable neural recording in rodent brain. 3. A dense and high-bandwidth NET platform towards volumetric mapping and large-scale distributed recordings in the neocortex and subcortical structures, and 4. A low-cost and versatile multifunctional neural probe platform to achieve optogenetic stimulation and controlled drug infusion with simultaneous, spatially resolved neural recording. These capabilities will drive new long-term studies of brain circuits across different spatiotemporal dimensions and modalities.

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1. Introduction

1.1. Background

As one of the most important and complex parts of the human body, the brain is responsible for generating consciousness, perceiving the world via sensations, forming memories, making decisions, and controlling locomotion. Therefore, studying the brain holds paramount importance for us to understand ourselves at both behavioral and physiological level [1]. This requires a thorough understanding of the large variety of neural circuits underlying different brain functions. Importantly, a better understanding of the brain can shed light on the mechanisms behind neurological disorders, and thus guide us to effective therapies [2]. In addition, advances in neural engineering are making possible neuroprosthetics for restoring disabilities through brain-machine interface [3, 4].

To study the functional brain, neuroscience relies on technologies that monitor and modulate neuronal activity. The brain consists of massively interconnected and dynamically evolving networks containing a large number of neurons, synapses and supporting glial cells. The neurons are densely packed with a density of $\sim 100\text{k}$ per mm^3 in the cortex and massively interconnected both locally and globally with 3k-10k synaptic contacts per neuron [5]. On the other hand, neural circuit activity occurs across diverse time scales. The activity in the form of action potential takes place on the timescale of milliseconds. However, changes in the activity patterns that support adaption, learning and related functions occur throughout the lifetime with timescales ranging from seconds to years. Therefore, to study the brain, we need tools with fast sensitivity and long-lasting functionality to cover a broad range of time scales, high spatial resolution to resolve the intrinsically dense structures, wide spatial coverage to interrogate anatomically distributed yet functionally related networks, and capabilities to differentiate neuron activities from different sources due to the inhomogeneity of the neuronal system.

There are currently three major modalities to record neuron activity [6]. Optical recording methods, such as 2-photon calcium imaging, measure activity-dependent light emission from neurons, typically generated by fluorescent indicator proteins. It offers sub-cellular spatial resolution, but the temporal resolution is restrained by slow molecular kinetics, high-frequency

saturation and beam scanning speed, etc. This methodology is also limited to shallow recording depths due to light scattering in tissue. Optical microscopy can currently record $\sim 100,000$ neurons at a 1.25 s timescale in behaving larval zebrafish using light-sheet illumination [7], and $\sim 12,000$ neurons at up to 17 Hz in mouse cortex using hybrid multiplexed two/three-photon microscopy [8]. Functional magnetic resonance imaging (fMRI) measures brain activity by detecting changes associated with brain hemodynamics. By utilizing magnetic fields, fMRI non-invasively records at a 1 s timescale throughout the entire brain, but currently limited by spatiotemporal resolution in part due to the use of hemodynamic contrast. Extracellular electrical recording utilizes implanted electrodes to measure the voltage changes in the neural tissue due to the activity of local neurons. This method can record at the resolution of single neurons with sub-milliseconds temporal resolution, but it is limited by the low multiplexing capacity of electrodes and their lack of intrinsic spatial resolution. Current state-of-the-art multi-electrode arrays enable simultaneous recording of ~ 700 neurons at sub-millisecond temporal resolutions across multiple different brain regions [9]. In addition to these neural recording methods, there are emerging techniques such as ultrasonic sensing [10] and molecular recording [11] which are potential venues for valuable neural recording, but require much additional development for effective implementation in neuroscience research.

Extracellular electrophysiology is the method of inserting electrodes to the neural tissue to sense the voltage changes generated by nearby neurons. It has been one of the most important and widely used neural techniques as it affords single-neuron sensing resolution at non-superficial layers in the living brain, and with sub-millisecond temporal resolution. Extracellular electrophysiology has contributed tremendously to the both basic and applied neuroscience by increasing the understanding of specific functions of neurons in throughout the brain [12, 13], providing clinically relevant neuroprosthetic devices to help patients with severe paralysis to regain independence, and treating diseases such as Parkinson [2, 14] and mental disorders [15].

In contrast to the optical method, which directly images individual neurons, electrophysiology recording data needs to be analyzed to separate the collective signals measured by electrodes into the individual signals produced by different local neurons. This process relies on the development of spike sorting algorithms [16], which typically use the features of spike waveforms to distinguish spikes coming from different neurons. In addition, there are difficulties for electrophysiology to resolve different cell types and structures of nerve cells due to the

heterogeneity of our nervous system, where the optical systems could be a very useful and complementary tool.

Conventional extracellular electrodes use either silicon-based microfabricated electrodes [9, 17-19] or array of microwires [20-23] to detect neural activities and have been powerful tools for neuroscientific research. Despite many advantages, challenges such as functional reliability and invasiveness are limit the application of conventional neural electrodes. The recordings made with these devices are often unstable for both the short term due to the movement of recording sites relative to the neighboring neurons and the long term due to tissue reactions manifested by blood brain barrier (BBB) leakage, neuronal degeneration, and glial scar [24-27]. Additionally, the spatial resolution is limited due to the conflict between the current sizes of the probe, its intrinsic invasiveness, and the physical constraints on tissue displacement, which is empirically thought to be less than 1% in order to keep good tissue integrity. This constraint also poses difficulties to the scalability of the current recording system.

A neural electrode would ideally feature 1. Long-lasting recording to stably monitor the evolution of neural activity patterns associated with specialized circuits across days, months and even years, 2. High density and large scale recording capability to resolve both the densely entangled local circuits and the distributed but parallel circuits across many distributed brain regions associated with the studied behaviors. 3. Compatibility with optical modalities to take advantage of its strength of resolving cell-type and/or cell-projection specificity.

1.2. Problem statement

The existence of the neural electrodes in the central nervous system (CNS) while necessary for interfacing with the nervous system can generate foreign tissue responses including persistence leakage on BBB, neural degradation, and glial scar encapsulation [23, 28-30] which in turn contribute to the chronic degradation of neural recording conditions. At current, there is growing evidence to attribute these responses to the mechanical mismatch between the stiff material that used to construct the conventional probes and the soft tissue of brain tissue. To solve the problem, many efforts have been made in the development of flexible devices, where biocompatible polymers with Young's modulus ranging from 100 kPa to 10 GPa have been used as the supporting material of the probes [26, 30, 31]. Despite improvements in mitigating foreign body reactions, significant tissue response and unstable long-term recording are still often reported [32]. The

hypothesis was that the flexibility of the implemented devices was still insufficient to mitigate the immune reaction when implanted in the relatively soft brain tissue whose Young's modulus was ~0.1 to 0.4 kPa. Additionally, there was also a dimension mismatch between current polymer electrodes and the cells and capillaries in brain, which inevitably causes damage and disruption to the local tissue environments. This has been supported by pioneer works in the field, Both carbon fiber electrodes with cellular cross-section [33, 34] and ultra-flexible macromesh electrode [35] with cellular flexibility both showed greatly suppressed tissue response.

The flexibility of an object can be characterized as its bending stiffness, which is the force required to bend and deform an object. The bending stiffness for a beam is defined as $k = Ewt^3/12$ where k is the bending stiffness, E is the materials' Young's modulus, w is the width and t is the thickness of the object. Notably, the bending force is proportional to the third order of thickness versus the first order to the material's Young's modulus. This indicates that adjusting the thickness of a probe has a larger effect on the flexibility compared to softening the material of the probe. By tuning the geometry factor, the flexibility of a neural probe can be further reduced by orders of magnitude [35-39], which results in better probe-tissue interfaces and long-term performance.

Another aspect to consider for neural electrode design is the scalability; one goal is to increase the number of recording sites in an electrode array without increasing the physical dimension of the array so that more neurons can be resolved in the same volume. By increasing the recording density, each neuron can be potentially recorded from multiple contact sites, which greatly benefits spike sorting [20, 40]. Another goal is to increase the spatial coverage of recording sites implanted throughout the brain. Both goals rely on massive reductions in the size of the current electrodes. As the state-of-the-art neural implants work toward this goal, the densely packed silicon array [9] achieve oversampled recording on 2D planes from different brain region along the probe track. This offers a clear advantage in depth-resolved recordings, but the lateral implantation density is limited due to large cross-sections outside the brain. There is also a dearth of data showing very long-term recordings, and the glia scars were seen in histological sections which indicate long-lasting tissue reactions. The large-scale polymer array [32] can record from hundreds to a thousand channels at distributed brain regions. However, a larger-scale platform with finer spatial resolution and chronic stability is still strongly desired.

This study aims to systematically develop a neural recording platform that integrates 1. The chronic recording stability enabled by the one-micron thin geometry profile of the probe and the flexibility that comes with it (Chapter 2). 2. Facile implantation method to apply the flexible neural probes in scalable, reliable neural recording (Chapter 3). 3. Large-scale and high-density recording capabilities to enable simultaneously interrogation of a large population of neurons in the brain and a variety of electrode designs that optimize the recording outcomes for different brain structures (Chapter 4). 4. Multi-functionality realized by applying thin nanoelectronic coating on the surfaces of conventional microscale devices such as optical fibers and micropipettes (Chapter 5).

2. Long-term stable neural electrophysiology enabled by Nanoelectronic thread (NET)¹

2.1. Introduction

Chronically implanted neural electrodes [1, 10-12] constitute the only means to electrically interface with intact and functioning brains at sub-millisecond and single neuron resolution [2-4]. Although they have contributed tremendously to neuroscience and neurotechnology, traditional electrodes suffer from a number of drawbacks that limit their scientific and translational use. Conventional neural implants have volumes and surgical footprints considerably larger than cells and capillaries, inducing substantial damage and disruption to local cellular and vascular networks [13]. Moreover, these probes are significantly more rigid than the host brain tissues, whose natural micro-movements induce intense stress at the interface. In the short term, the consequential electrode displacement from the targeted neurons leads to sudden waveform changes in time scales as short as hours that prevent reliable tracking of individual neurons over days and longer [6, 7]. In the long term, the presence of implants causes reoccurring cellular and vascular damage, elicits sustained inflammation and tissue response, and eventually leads to neuronal degradation and glial scar formation near the implants [13-15]. These chronic deteriorations are manifested in electrical recordings as degradations in recording fidelity, including increasing impedance, elevated noise levels, decreasing signal amplitudes and diminishing unit recordings [4, 10-12, 15].

It has been demonstrated by extensive effort in the past decade that reducing the neural probe dimension [16] and rigidity [15, 17, 18] improves neural interface. Recent work has shown that macroporous electronics [19] and ultra-small microelectrodes made of carbon fibers [20] greatly reduced tissue response. However, reliable brain probes that detect and track activities from the same neurons for extended periods with no aforementioned chronic detrimental effects require seamless bio-integration and are yet to be demonstrated.

¹ Luan, L., Wei, X., Zhao Z., Siegel J., Potnis, O., Tuppen, C., Lin S., Kazmi, S., Fowler R., Holloway, S., Dunn A., Chitwood, R., Xie, C., *Ultraflexible nanoelectronic probes form reliable, glial scar-free neural integration*. Science Advances, 2017. 3(2). My contribution to this work includes fabricating the device, performing the surgery and electrical recording, preparing and testing the shuttle devices and performing the histology.

Considering previous efforts in improving neural-electrode interface, we identify the following key aspects for establishing reliable and glial scar free neural-probe interface: (i) the dimension of the probe is comparable to or smaller than that of average cells and capillaries, so that its perturbation to the host biological matrix is minimal[13, 16]; (ii) the probe has sufficient flexibility to ensure complete compliance to tissue micro-movements and to reduce probe-tissue interfacial force to the range of cellular force (nanonewtons) [19, 21]; (iii) the surgical damage during implantation is less than ca. 100 μm across to allow for tissue recovery [22, 23]; (iv) the probe is mechanically and electrically robust for long-term functioning in physiological conditions. To meet these stringent requirements, we developed flexible nano-electronic thread (NET) brain probes and an implantation strategy with cellular-size surgical footprints. We verified the effectiveness of our approach in a rodent (mouse) model by chronic electrical recording and comprehensive characterizations of the probe-tissue interface.

2.2. Results

Two types of the NET brain probes were designed, NET-50 and NET-10, using a multi-layer, substrate-less architecture and specialized photolithography (Methods). Both designs feature a total thickness of 1 μm . NET-50 has an average width of 50 μm , hosting a linear array of eight electrodes. NET-10 has a cross-section of 10 $\mu\text{m} \times 1.5 \mu\text{m}$, the smallest among all reported neural probes to the best of our knowledge, hosting four electrodes on two opposite surfaces in a 7-layer layout. The electrodes and the interconnects were placed onto different layers to minimize volume and maximize flexibility. The electrical connection was made by “vias” through the insulation layers. (Fig. 1)

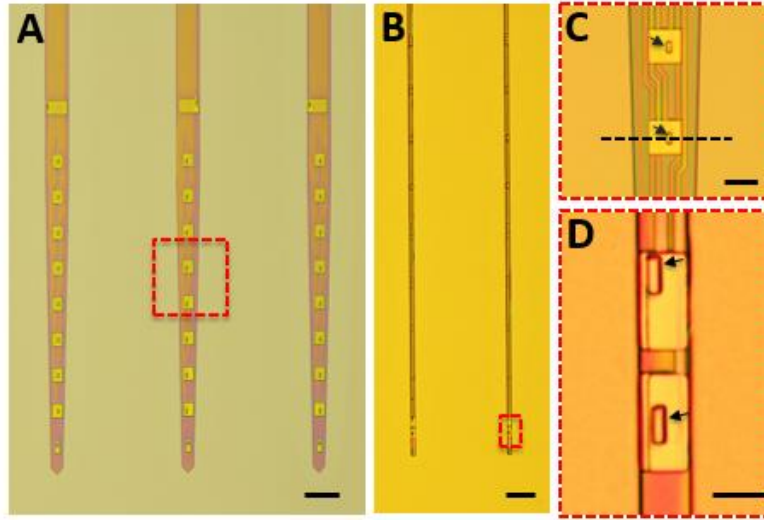


Fig. 1. Structures of two types of NET neural probes. A,B, As-fabricated NET-50 and NET-10 probes on substrates. C,D, Zoom-in views of two electrodes as marked by the dashed boxes in a and b respectively. Arrows denote vias. E, Schematics of the probe cross-section in A (top) and B (bottom), highlighting the multi-layer layout. Color code: grey, insulation; orange, interconnects; blue, electrodes. F, Height profile of the NET-50 probe along the dash line in c measured by atomic force microscope (AFM) .

The NET probes are designed to offer similar recording characteristics as conventional silicon probes and tetrodes [10, 12] but with aggressively reduced dimensions. The cross-sectional area of the NET probes was compared with other representative neural probes including tetrodes (1), microwires (2), silicon probes (3, 4), and carbon fiber probes (21), as well as the soma of a granule cell, which represents the smallest neurons.

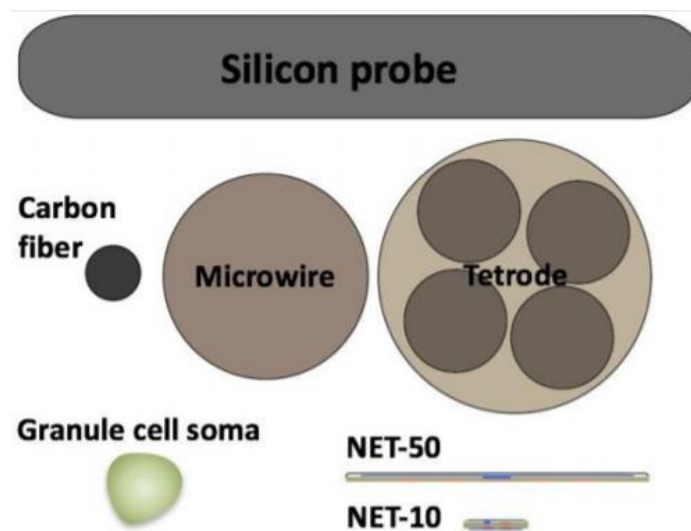


Fig. 2. Sketch showing the cross-section areas of the NET and representative neural probes

As shown in Fig. 3, comparing with the conventional electrodes, the drastically reduced size enabled the NET probes orders of magnitude improvement on effective bending stiffness and tissue displacement per electrode. Both are the key parameters for improved bio-compatibility. Specifically, the bending stiffness is reduced to $10^{-15} \text{ N}\cdot\text{m}^2$, which brings down the probe-tissue interfacial force to nanonewton range, on par with the single cell traction force [25]. The bending stiffness K is calculated using geometrical parameters listed in the reference. For shaft-shaped probes, $K_s = E_s w h^3 / 12$ (eq. S1), where E_s is the Young's modulus of the shaft material, h and w are the thickness and the width of the shaft. For cylindrical probes, $K_w = E_w \pi d^4 / 64$, where E_w is the Young's modulus of the wire/fiber material, d is the diameter of the wire.

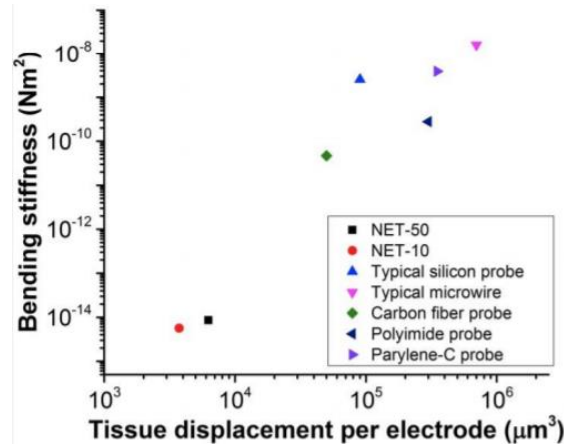


Fig. 3. A comparison of the bending stiffness and tissue displacement of the NET with other representative neural probes

The bending force of the NET probe was measured using atomic force microscopy (AFM). The NET-50 and NET-10 were suspended in air with the suspended section length about $400 \mu\text{m}$. For measurements, the AFM tip was moved on top and applied force onto the probe to slowly deflect them. The displacement and force was recorded. Using the bending stiffness K we obtain in Fig. 3, we calculate the deflection force with equation $F = 8eK/l$ [3, Fig. 4], where e is the deflection distance and l is the length of the probe. The forces were 10.6 nN and 6.8 nN for NET-50 and NET-10 probes respectively, which is in good agreement with the AFM measurements.

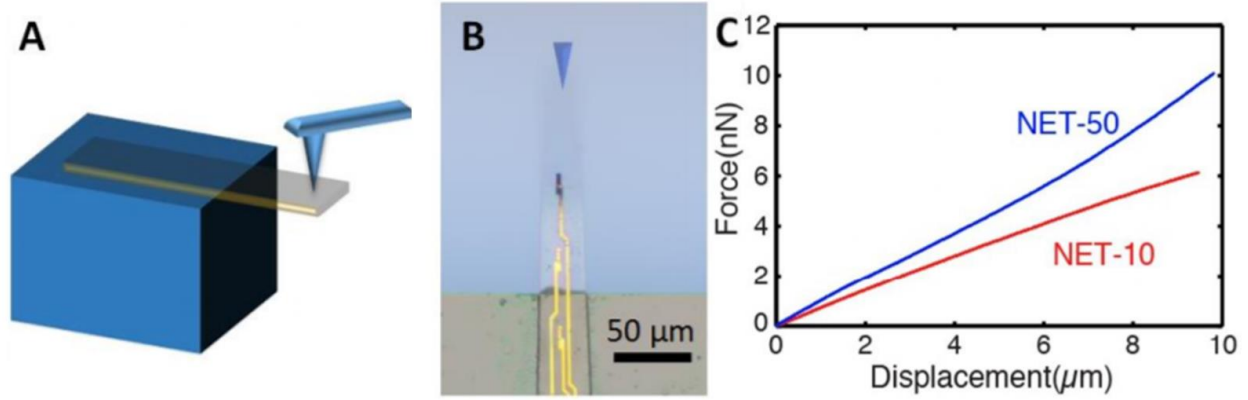


Fig. 4. In vitro measurement of the bending force from suspended NET probes. (A) Schematic of the measurement using atomic force microscopy (AFM). (B) Photograph of a suspended NET-50 probe in air. Blue cone illustrates the position of the AFM tip in the measurement. Scale bar: 50 μm . (C) Force-displacement plots for both NET probes measured

Due to the flexibility of the NET probes, self-supported penetration through brain tissue become impossible. Previously, researchers has employed approaches such as temporarily altering the probe's rigidity [19] and temporarily attaching to a rigid shuttle device [17, 18, 21] to deliver flexible probes. However, they often induce wounds significantly larger than the NET probes and results in persistent glial scar [22, 23]. In this study, we came up with a temporary engaging mechanism (Fig. 5A) enabled by a micro-post at the end of each shuttle device fabricated using focused ion beam (FIB). The shuttle devices were made of carbon fibers and tungsten microwires with diameters as small as 7 μm (Fig. 5B) to assist the probe delivery while maintaining minimal disruption to the tissue environment.

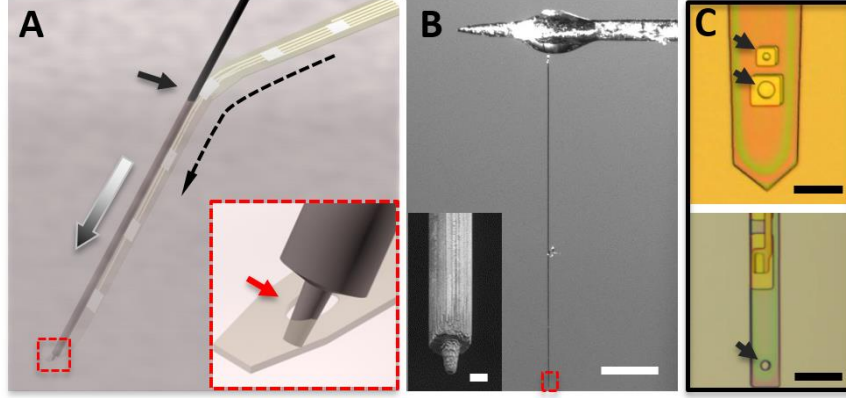


Fig. 5. Temporary engaging mechanism for delivering NET probe. A. Schematic showing the temporarily engaging mechanism. B. Photograph of a typical carbon fiber shuttle device in, with the diameter of 7 μm and length of 3 mm, mounted at the end of a micromanipulator. Scale bar: 500 μm . Inset: scanning electron microscopy (SEM) image of the micro-milled post 2 μm in diameter and 5 μm in height at the shuttle device tip. Scale bar: 2 μm . C. Optical micrographs showing engaging holes in NET-50 (top) and NET-10 (bottom) probes with slightly larger diameter than the post, as denoted by the arrows.

During delivery, the micro-post engages into the micro-hole (Fig. 5C) and pulls the NET probe to the desired depth, after which the shuttle device disengages and retracts (Methods). The overall insertion footprint is as small as 10 μm across, which leads to subcellular surgical damage as evidenced by little bleeding (Fig. 6A, B) and small insertion force.

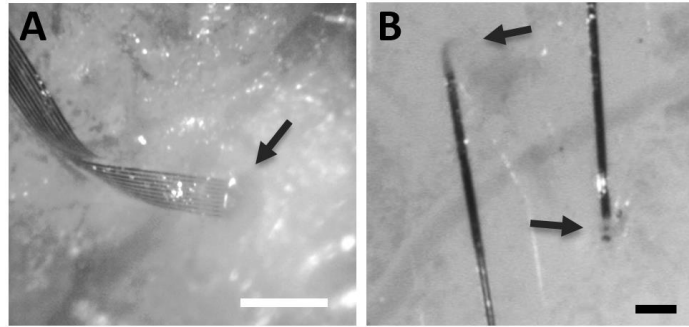


Fig. 6: Micrographs showing that both NET-50 (A) and NET-10 (B) probes were successfully delivered into the living mouse brain with minimal acute tissue damage. Scale bars, 100 μm (A) and 50 μm (B)

Taking advantage of the flexibility and the small dimensions, we accommodate chronic optical access with NET implants (Fig. 7 A,B), which allows for monitoring the tissue-probe interface by *in vivo* two-photon (2P) imaging. The electrical integrity is maintained through surgery and the implanted electrodes can readily detect unit activities (Fig. 7C).

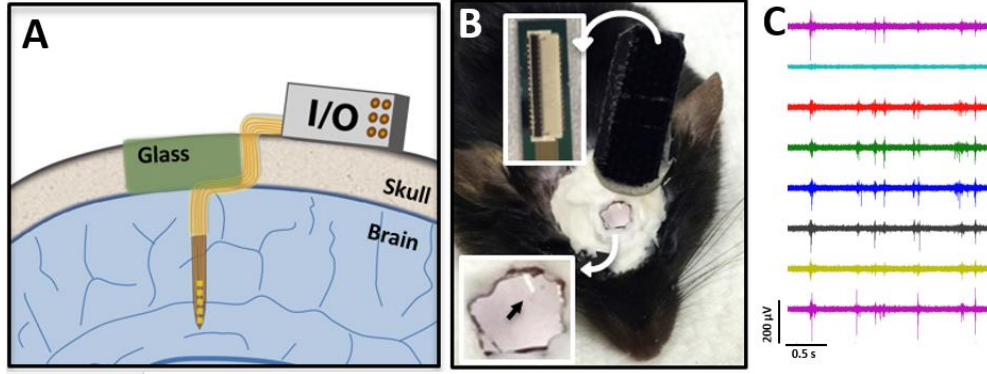


Fig. 7: Implantation procedure for the NET probes. A. Schematic of skull fixation that accommodates both connectors for the neural probes and a glass window allowing optical access. B. Photograph of a typical post-surgery mouse with implanted NET probes and a glass window mounted atop. Insets: top, B. photo of a cable connector mounted on the skull; bottom, zoom-in view of the glass window in which the arrow denotes an implanted probe. C. Typical unit activities recorded by eight electrodes on an implanted NET-50 probe. 300 Hz high-pass filter was applied.

The electrical and recording performance of NETs were evaluated over 4 months. We implanted 16 probes in 7 mice, containing a total of 80 connected electrodes out of 96 (83.3% fabrication yield). From bi-weekly electrical measurements over four months (Methods) we observed that recording performance improved during the initial 1.5 months, and then remained stable for at least 2.5 months in the following aspects. First, the average impedance and the noise level of all 80 electrodes decreased in the first 1.5 months and remained stable (Fig. 8A). Second, the number of electrodes that detected unit events and sortable single-unit action potentials (APs) increased in the first 1.5 months and then remained at the same level (Fig. 8B); The yields of multi-unit (75%) and sortable single-unit recording (23.8%) are on par with conventional silicon probes [6], but with unprecedented chronic stability. Third, we detected sortable AP waveforms from 19 electrodes with average amplitude and the signal-to-noise ratio (SNR) stable throughout the four months period (Fig. 8C).

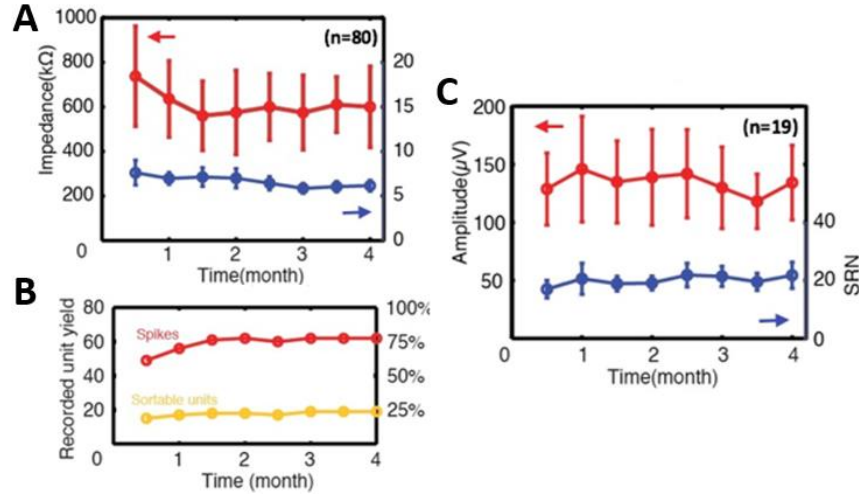


Fig. 8: Chronic recording and electrical characterization of implanted NET electrodes. A, Impedance (red) and the noise level (blue) of 80 implanted electrodes as a function of time. Error bar marks the standard deviation. B, The number (left) and percentage (right) of electrodes that recorded unit activities (red) and sortable single-unit Aps (orange) as a function of time. C, Average peak-valley amplitude (red) and signal-to-noise ratio (blue) of single-unit Aps recorded by n=19 electrodes as a function of time. Error bars indicate the standard deviation.

We followed all 80 electrodes for 4 months and summarized their recording performance over time in Fig. 9. Although there were variations in the number of functional electrodes and the unit detection yield among different animals, stability and consistency in unit detection over time were observed in all animals.

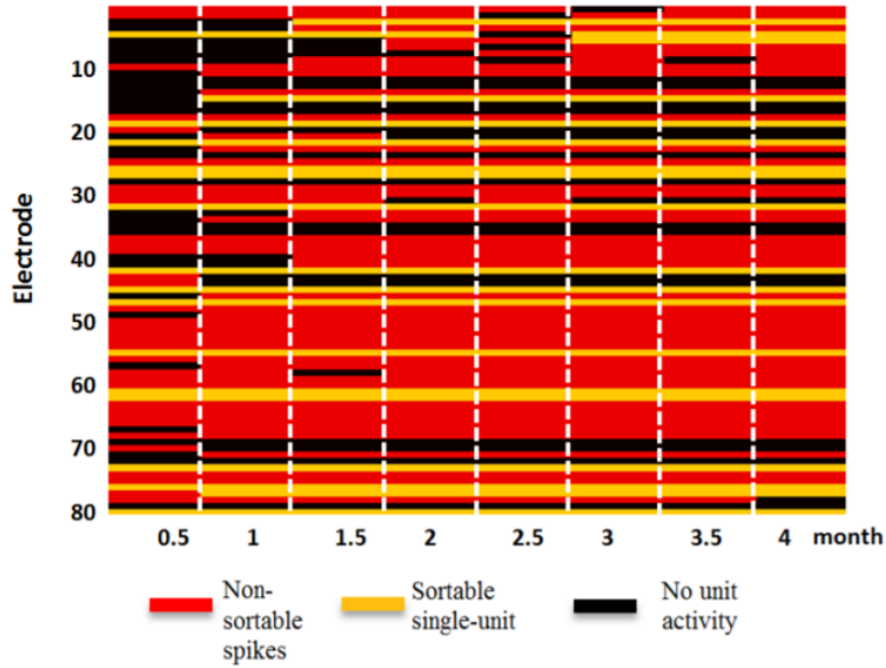


Fig. 9. Block chart illustrating non-sortable (multi-unit) spikes and sortable single-unit APs detected by the 80 implanted electrodes as a function of time

As an example, Fig. 10A shows the bimonthly measurements from the electrode that detected typical spike events and sortable AP waveforms with the highest SNR (>30) among all electrodes. The persistently high SNR of the sortable AP waveforms indicated that the firing neuron remained in close proximity to the electrode for the entire duration of the chronic recording. Combined principal component analysis for all measurements showed largely overlapping clusters with cluster centers' average position shifts between consecutive measurements equivalent to 0.88σ of the average cluster distribution (Fig. 10B), suggesting that the waveforms were generated by the same neuron.

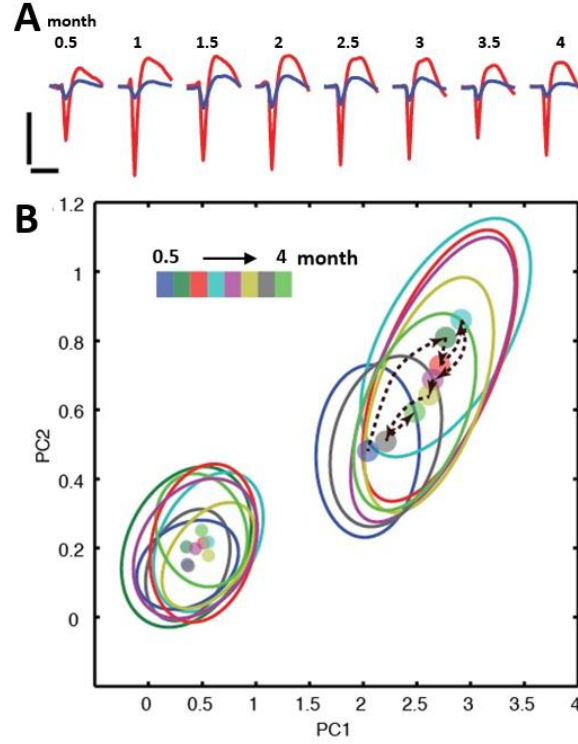


Fig. 10. Example stable recording from one representative electrode A, Bi-weekly measurements of the non-sortable spikes (blue) and sortable AP waveforms (red) from the electrode. The waveforms are isolated and averaged from 3-9 minutes recording segments. Vertical bar: 200 μ V; horizontal bar: 1 ms. B, PC analysis of all the waveforms in d for which the evolution of the single-unit waveforms over time is highlighted by dashed arrows. Dots: center of the PC. Ovals: 2 σ contour of PC distribution. Colors code the time stamps.

In addition, the measurements from all electrodes that recorded sortable single-units Aps over 4 months were shown in Fig. 11. Persistent characteristics in AP waveforms were observed for the duration of the experiment, whereas minor trackable changes occurred for all except one electrode (18 of 19, 94.7%). Significantly, in contrast to previous neuron probes where decreasing amplitudes were typically observed over time, most of the NET electrodes recorded AP waveforms with nonmonotonic changes in peak-valley amplitude. The recording evidence strongly indicates that the NET probes form a nondegrading, slowly evolving interface with neurons.

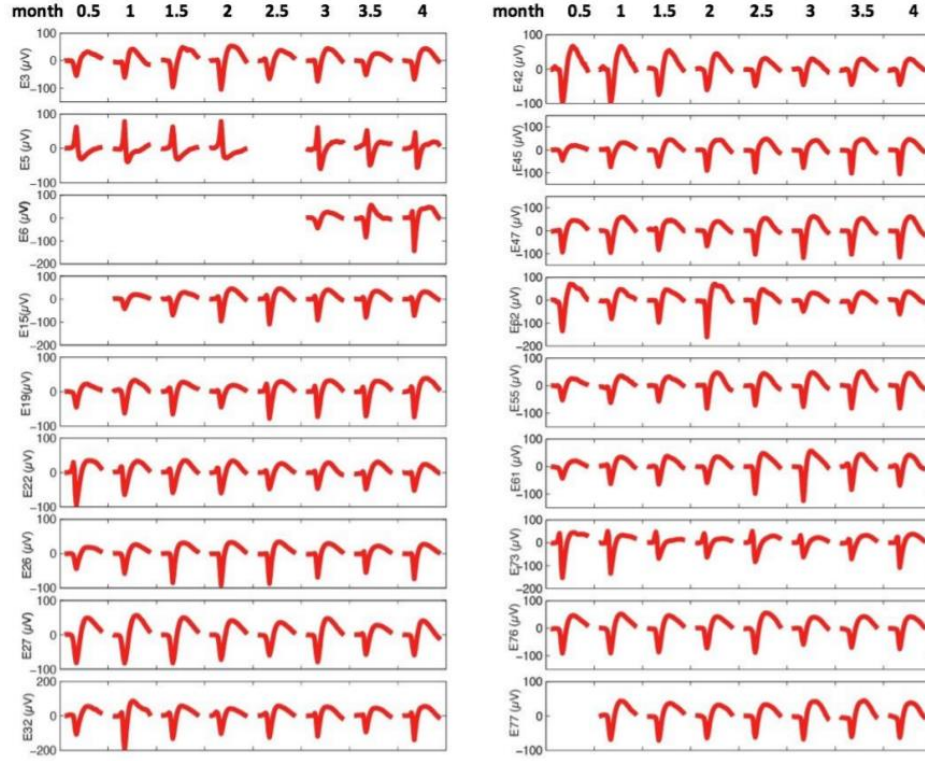


Fig. 11. Twice-a-month measurements for 4 months from all electrodes that recorded sortable single-unit APs

To directly investigate the nature of the probe-tissue interface, we monitored capillaries, astrocytes, and neurons around the NET probes by in vivo 2P imaging at the implantation sites for up to 3.5 months after surgery (Materials and Methods). Figure 12 shows the evolution of the vasculature, showing that the surgical damage led to minor local leakage of the blood-brain barrier (BBB) upon implantation, which lasted for at most 1 month and was fully repaired in association with vascular remodeling.

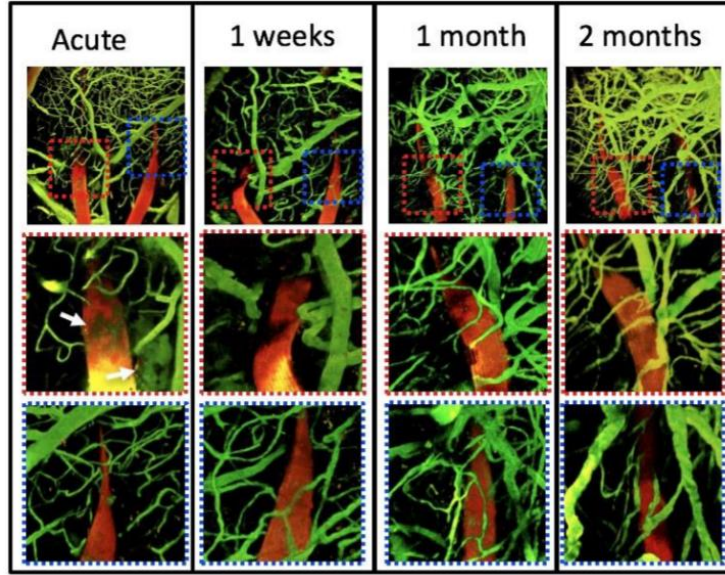


Fig. 12: 3D reconstruction of 2P images of the capillary networks (green) surrounding two NET-50 probes (red) at acute, 1 week, 1 month, and 2 months after implantation.

As shown in Fig. 13 (A and B), both types of NET probes were embedded in capillaries with normal density, morphology, and an intact BBB by 2 months. We measured the blood flow rate by line scans (Fig. 13, C and D) and obtained similar values next to ($420 \pm 180 \mu\text{m/s}$) and away from ($450 \pm 210 \mu\text{m/s}$) the probe (Materials and Methods), confirming that capillary perfusion immediately next to the probe was normal. This is in strong contrast to conventional microelectrodes where continuous BBB leakage was observed in chronic in vivo imaging at the interface.

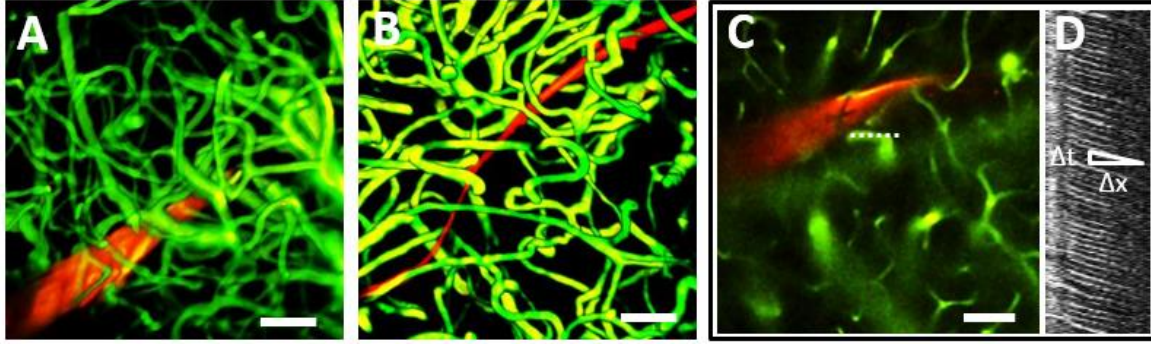


Fig. 13: Imaging of the cellular and vascular structures at the chronic probe-tissue interface a & b, 3D reconstruction of vasculatures by *in vivo* 2P microscopy around NET-50 (A) and NET-10 (B) probes (red), two months post-implantation, highlighting fully recovered capillary networks (green). Image stack: 0- 400 μm (A) and 100-320 μm (B) below the brain surface, 2P image at 200 μm deep marking the position of a capillary (dashed line) for the line-scan in D. D, Matrix of line scans showing movement of red blood cells as dark stripes the slope of which gives the blood flow speed.

We imaged the astrocytes by *in vivo* staining (Methods) 3.5 months post implantation around a NET-50 probe (Fig. 14A). We note that the imaged segment was unintentionally folded during implantation with a sub-100 μm curvature, which sets the upper limit of tissue-probe stress for NET probes. We observed that the astrocytes near the probe surround only capillaries with normal density and morphology. We also performed postmortem histology (Methods) five months post-implantation and observed resting microglia (Fig. 14B) near the probe. Remarkably, direct contact with the probe did not affect neurons' viability or activate microglia [22]. These results show that the NET probes have unprecedented bio-compatibility as they maintain normal vascular and cellular structures, and elicit no observable chronic tissue reactions at the interface.

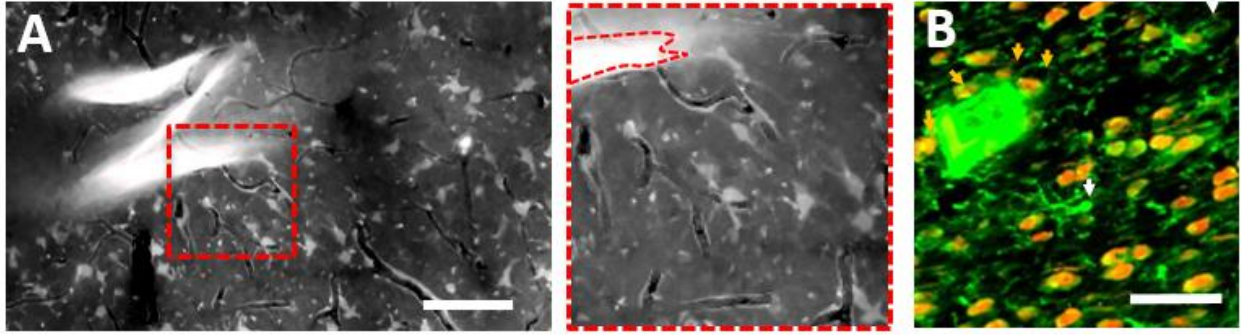


Fig. 14: Imaging of immune biomarkers and neurons at the chronic probe-tissue interface. A, Projection of in vivo 2P images at 210- 250 μm below the brain surface at 3.5 months post-implantation showing normal astrocytes and capillaries. The bright 'z'-shaped object is the folded NET-50 probe. The capillaries are visible as dark lines. Right: zoom-in view of the dashed area. B, Projection of confocal micrographs of an immunochemically labelled cross-section slice (30 μm thick, five months post-implantation). False colour code: orange, NeuN, labelling neuron nuclei; green: Iba-1 labelling microglia. White arrows denote microglia. Orange arrows denote neurons in contact with the NET probe.

To reveal the evolution of the neural interface, we repeatedly imaged neurons in the vicinity of both types of NET probes up to three months post-implantation and compared their locations relative to a few representative neurons and capillaries as positions markers (Fig. 15A-B). We observe that all neurons initially imaged near the probe were identified in later imaging sessions, which further proves that the NET probes induce no chronic neuronal loss.

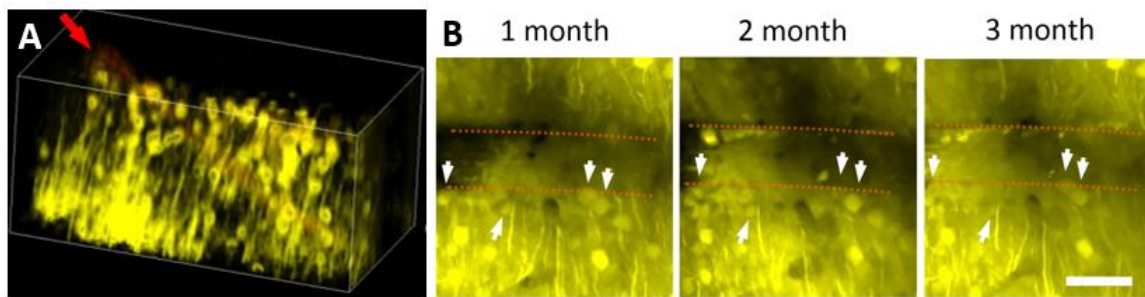


Fig. 15. Imaging and tracking of the neurons at the chronic probe-tissue interface. A, 3d reconstruction of in vivo 2P images of neurons (yellow, Thy1-YFP) surrounding a NET-50 probe 2 months post-implantation. The probes are in red and denoted by arrows. Imaging depth: A, 130-330 μm below the brain surface. Scale bar: 50 μm .

Further, while most neurons are relatively stationary, some show slow migrations (up to ca. 10 μm) over time course of weeks (Fig. 16). Importantly, we observed no preference in their migration trajectories of moving towards or away from the NET probes.

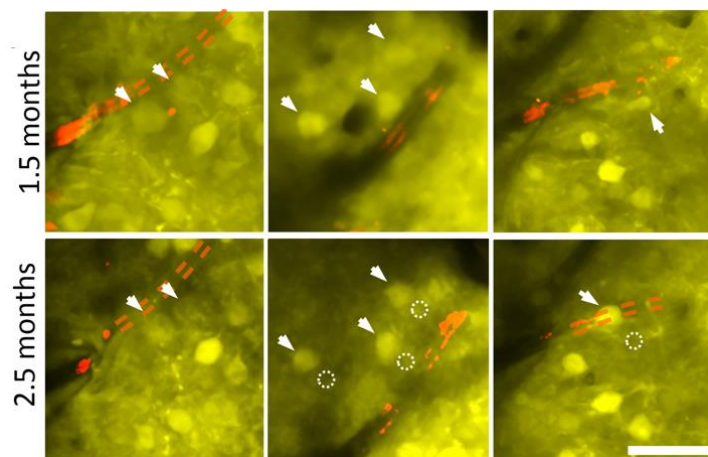


Fig. 16. In vivo 2P images of neurons surrounding a NET-10 probe. The neurons are repeatedly identified at different times post-implantation. Red dashed lines mark the edge of the probes. Arrows and dashed circles highlight the current and previous locations of neurons. Scale bar: 50 μm .

2.3. Discussion

The flexibility of the NET probe prohibits conveniently advancing to and recording from more than one brain region using schemes similar to microdrives that are widely applied in silicon probes and tetrodes. Instead, recording from deeper brain regions or from different brain tissue depths can be realized by implanting NET probes patterned with more electrodes spanning a larger depth, especially given the unprecedented tissue compatibility demonstrated here. Accommodating more electrodes on one probe does not necessarily increase the probe's dimension, as we can further increase the fabrication resolution by using electron beam lithography techniques.

We have thoroughly examined our full fabrication process and identified that the loss of yield is mainly due to fabrication defects. Our fabrication involves seven photolithography steps using a manual mask aligner and three metal deposition steps, which makes it difficult to completely avoid microparticles and scratches. Furthermore, our design has narrow metal lines (the interconnects, 2 to 3 μm wide) routed over 5 mm, which is vulnerable to microdefects. We expect that one can significantly improve the fabrication yield by using automated photolithography tools that can eliminate most of the defect sources.

The NET probes can be readily fabricated in linear arrays, similar to the conventional silicon probes. We estimate that we can deliver up to 12 NET probes within 40 min on the basis

of the demonstrated average delivery time of 3 min per probe. The closest interprobe spacing we have achieved is about 200 μm , as shown in Fig. 2G. The accuracy in interprobe space is currently determined by the accuracy in the initial positioning of the NET probe (under a stereomicroscope) on the brain surface before delivery, which has an uncertainty of up to 30 μm .

The NET probes performed stably in mice for at least 4 months, as evidenced by the intact device structure and the stable impedance and recording performance (Fig. 4), which are sufficient for most scientific studies and warrant further research on their performance in longer-term implantation (on the order of years). We attribute the stable performance of NETs to their unconventional dimensions and design, which can be explained by the following aspects: (i) The most prevalent failure modes in conventional devices are structural degradations, such as cracking, blistering, and delamination, none of which were observed in chronically implanted NETs. This can be attributed to the fact that the ultrathin thickness of NETs (1 μm) greatly reduces the strain on the device upon deformation, which prevents the formation of structural defects. (ii) Another major failure mode in conventional polymer implants is delamination due to insufficient bonding at interfaces. In NET probes, the SU-8 insulating layer adheres mainly with another SU-8 layer by thermal curing and cross-linking, which eliminated this failure mode. (iii) Most polymers absorb water under physiological conditions, which may lead to electrical leakage that results in signal attenuation. However, the possible water absorption-induced leakage did not affect our long-term recording performance, mostly because it did not induce structural damage of the NET as shown above, likely due to the improved adhesion between layers and reduced stress. Furthermore, NET probes were soaked for several days in phosphate-buffered saline (PBS) and other water solutions before implantation, which preconditioned the SU-8 film to a saturated water absorption of 3.3%, such that no signal attenuation was observed after implantation.

A recent study reported stable long-term recording in mice using mesh electrodes on a similar device platform. The consistency between their study and ours strongly supports the validity of using ultraflexible devices, particularly polymer-based devices with an overall thickness of about 1 μm , for long-term neural activity mapping. Here, we further reduced both the device width and the surgical footprint to cellular dimensions, such that no persistent glial scar was observed near the implants in our study. In addition, we characterized the interface not only by postmortem histology, which gave a “snapshot” assessment, but also by repeated *in vivo*

imaging of thick tissue stacks, which unambiguously revealed the evolution of vascular and cellular networks around the implanted NET probes.

Combining the results from chronic recording and in vivo imaging, we gain a comprehensive understanding of the neural interface formed by the NET probes. In vivo imaging and histology studies revealed a chronic tissue response free interface, which provides the biological foundation for nondegrading recording performance. Moreover, because the interface is dynamically evolving, which involves remodeling of vasculature and other changes in the microbiological environment, both the neurons and the NET probes are not completely stationary over a time frame of a few months. Correspondingly, we observed neuron migrations at a few micrometers per month, which led to slowly evolving waveforms in chronic recordings that could be reliably tracked by measurements twice a month.

We demonstrated that the NET probes form reliable, glial scar-free integration with the living brain by chronic recording, in vivo 2P imaging, and postmortem characterization of the tissue-probe interface. The unprecedented chronic reliability and stability are expected to fundamentally advance both basic and applied neuroscience, as well as lead to substantial improvement in the brain-machine interface which can be applied to neuroprosthetics. Furthermore, the subcellular dimension probes provide new opportunities for high-density electrical recording by overcoming current physical limitations.

2.4. Methods

NET brain probe fabrication and preparation

The NET brain probes were fabricated using specialized fabrication methods similar to those previously reported. The multilayer probes were fabricated using photolithography on a nickel metal release layer deposited on a silicon substrate (900 nm SiO₂, n-type 0.005 V·cm, University Wafer). An SU-8 photoresist (SU-8 2000.5, MicroChem Corp.), which offers excellent tensile strength, ease of fabrication, and demonstrated durability in ultrathin structures, was used to construct the insulating layers. We used the minimum thickness of the dielectric layer necessary for preventing more than 1% signal attenuation through the capacitive coupling between the interconnects and the conductive medium surrounding the probe, which was determined to be about 500 nm for our probe geometry and material. Platinum or gold was used for electrodes (size:

30 μm $\times$ 30 μm for NET-50 and 10 μm $\times$ 20 μm for NET-10) and interconnects, respectively, both with a thickness of 100 nm. The different sizes and materials used for the electrodes contributed to most of the variation in impedance, as shown in Fig. 4A. After fabrication, a 33-pin flexible flat connectors (FFC) (series 502598, Molex) was mounted on the matching contact pads on the Si substrate. The implantable section of the probe was then soaked in nickel etchant (TFB, Transene Co. Inc.) for 2 to 4 hours at 25°C to release the free-standing portion of the probe, while the contact region remained attached to the substrate. The substrate was cleaved to the desired length before implantation.

Shuttle device fabrication and assembly

A straight segment of a carbon fiber or tungsten wire was attached to a stainless steel microneedle (prod# 13561–10, Ted Pella Inc.) for convenient handling. It was then cut to the designed length (2 to 3 mm) using FIB. An anchor post was micromilled at the tip of the shuttle device using FIB to shape a well-defined micropost (3 μm in diameter and 5 μm in height).

Animals and surgery

Wild-type male mice (C57BJ/6, 8 weeks old, Taconic) and male transgenic strain [B6.Cg-Tg(Thy1-YFP)16Jrs/J, to which yellow fluorescent proteins were expressed in neurons; The Jackson Laboratory] were used in the experiments. Mice were housed at the Animal Resources Center at the University of Texas (UT) at Austin (at a 12-hour light/dark cycle at 22°C, with food and water ad libitum).

Mice were anesthetized using isoflurane (3% for induction and maintained at 1 to 2%) in medical-grade oxygen. The skull was exposed and prepared by scalping the crown and removing the fascia and then was scored with the tip of a scalpel blade. A 3-mm $\times$ 3-mm square craniotomy was performed with a surgical drill over the somatosensory cortex. Dura mater was carefully removed to facilitate the delivery. After NET probe implantation (described below), the remaining flexible segment of the NET probe, which connected the bonding pad on the substrate with the electrodes inside the brain, was routed to the edge of the cranial opening. The exposed brain was then protected by artificial cerebrospinal fluid (ACSF) and coverslip #1 (Fisher Scientific) fit into the cranial opening. The space between the coverslip and the remaining skull was filled with Kwik-Sil adhesive (World Precision Instruments). After the skull was cleaned and dried, a layer of low-

viscosity cyanoacrylate was applied over the skull. An initial layer of C&B-Metabond (Parkell Inc.) was applied over the cyanoacrylate and the Kwik-Sil adhesive. A second layer of Metabond was used to cement the coverslip and the NET carrier chip to the skull. All procedures complied with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the UT Institutional Animal Care and Use Committee.

Neural probe delivery

In typical procedures, a flexible neural probe was placed on the brain surface where dura mater was removed. The shuttle device was vertically mounted on a micromanipulator (MP-285, Sutter Instrument) and positioned atop the engaging hole at the end of the probe. As the shuttle device traveled downward, the anchor post entered the hole and pulled the neural probe into the brain tissue. Once the neural probe reached the desired depth, the shuttle device was retracted, and the neural probe was released and left embedded in the brain tissue. Because the volume of both the shuttle device and the neural probe was minimized to reduce tissue damage, the delivery process was delicate. A few experimental details are important to the success of the delivery. First, the anchor post of the shuttle device needs to be microfabricated precisely according to the well-defined cylindrical shape as designed, so that it can fit into the engaging hole on the probe, can have sufficient mechanical strength for the delivery, and can be retracted out of the brain without pulling the probe out with it. Second, the axial direction of the shuttle device needs to be aligned with the insertion direction to minimize buckling. Third, ACSF needs to be added to the brain surface as the shuttle device drags the probe into the brain to float the remaining flexible segment and to minimize friction between the probe and the brain surface. By controlling the engaging location and the insertion depth, we delivered NET probes to specific regions of interest. Multiple-probe delivery was achieved by repeating the same process sequentially.

2P imaging

2P imaging was performed using a laser scanning microscope (Prairie Technology) equipped with a 20 $\times$ water immersion objective (numerical aperture, 1.0; Zeiss) and a Ti:sapphire excitation laser (Mai Tai DeepSee, Spectra-Physics) from acute to up to 3.5 months after implantation. The laser was tuned to 810 to 910 nm for 2P excitation (power, 3.0 to 50 mW; dwell time, 4.0 to 6.0 μ s). Fluorescence emissions were detected simultaneously by two standard photomultiplier tubes with a 595/50-nm filter (Semrock) for “red” fluorescence emission and a

525/70-nm filter (Semrock) for “green” fluorescence emission. Mice were anesthetized using isoflurane (3% for induction and 1.5% during experiment) in medical-grade oxygen to maintain full immobility during imaging and placed in a frame that stabilized the head on the microscope stage. For 2P imaging on capillaries, anesthetized animals were given fluorescein isothiocyanate (FITC)–dextran (0.1 ml, 5% w/v, Sigma-Aldrich) retro-orbitally to label blood vessels before imaging. For 2P imaging on astrocytes, anesthetized animals were given sulforhodamine 101 (0.1 ml, Sigma-Aldrich) and Optison (GE Healthcare, 0.1 ml) retro-orbitally. Immediately following the injection, focused ultrasound with a center frequency of 2.25 MHz in the tone burst mode (rate, 20 Hz; burst duration, 10 ms; duty cycle, 20%) was applied to the regions of interest for 45 s to temporarily break the BBB. Animals were imaged 2 hours after ultrasound treatment to ensure sufficient dye diffusion and staining. To facilitate the imaging of the probe-tissue interface beyond superficial cortical layers, we doped the probes with sulforhodamine 6G (Sigma-Aldrich) in the insulating layers and delivered them at about 45° with respect to the skull.

Line scan and blood flow speed

We inferred the blood flow speed from the motion of red blood cells (RBCs) shown as stripes in line-scan measurements, that is, repetitive scans of the laser along the central axis of a capillary. The animal was given FITC-dextran (0.1 ml, 5% w/v, Sigma-Aldrich) retro-orbitally before imaging. After obtaining a 2P image of capillaries at the desired depth, we identified a capillary of interest and electromechanically oriented the direction of the scan along it. We typically scanned a distance of 30 to 40 μm with 128 pixels per line, repeating 128 to 256 times at 1 to 2 ms per scan. We imaged nine capillaries in the vicinity of a NET-50 probe and six capillaries from the contralateral hemisphere of the same mouse. The blood flow speed was $420 \pm 180 \mu\text{m/s}$ near the probe, similar to $450 \pm 210 \mu\text{m/s}$ away from the probe.

Histological sample preparation

Mice were given lethal intraperitoneal injections of 0.15 ml of ketamine mixed with xylazine [xylazine (10 mg/ml) in ketamine (90 mg/ml)] and then perfused intracardially with oxygenated, cold ($\sim 4^\circ\text{C}$) modified ACSF (2.5 mM KCl, 1.25 mM NaH_2PO_4 , 25 mM NaHCO_3 , 0.5 mM CaCl_2 , 7 mM MgCl_2 , 7 mM dextrose, 205.5 mM sucrose, 1.3 mM ascorbic acid, and 3.7 mM pyruvate) followed by 4% paraformaldehyde in 0.02 M PBS. Brains were cryoprotected in a 30% sucrose/4% paraformaldehyde solution overnight. Tissue was sectioned into 20- to 50- μm slices

perpendicular to the probe using a Leica CM1950 cryostat (Leica Microsystems). The slices were washed (3×5 min) and incubated in hot sodium citrate solution (85°C to 95°C , 0.01 M in H_2O) for 30 min for antigen retrieval. Then, the slices were washed (3×5 min), incubated in blocking solution and permeabilized [0.5% Triton X-100 and 10% normal goat serum (Sigma-Aldrich) in PBS] for 3 hours at room temperature, washed (4×5 min), and incubated in fluorophore-conjugated antibodies for 24 hours at 4°C .

Reagents used for different cell types are as follows (Millipore): neurons: Alexa Fluor 488–conjugated anti-NeuN antibody, clone A60; microglia: Alexa Fluor 647–conjugated anti–glial fibrillary acidic protein antibody, clone GA5.

Neural recording

Voltage signals from the neural electrodes were amplified and digitized using a 32-channel RHD2132 evaluation system (Intan Technologies) with a bare Ag wire inserted into the contralateral hemisphere of the brain as the grounding reference. The animal was anesthetized with 1.5% isoflurane in medical-grade O_2 during the measurement. The sampling rate was 20 kHz, and a 300-Hz high-pass filter and a 60-Hz notch filter were applied for single-unit recording. The impedance of the electrodes was measured using the same equipment at 1000 Hz. Variation in impedance was mostly attributed to the different sizes and materials of the electrodes (materials: Pt/Au; sizes: $30\ \mu\text{m} \times 30\ \mu\text{m}$ for NET-50 and $10\ \mu\text{m} \times 20\ \mu\text{m}$ for NET-10). Spike detection and spike sorting were performed using Offline Sorter (Plexon).

3. Parallel, minimally-invasive implantation of the NET arrays²

3.1. Introduction

The recent progress on flexible neural electrodes [1] with drastically reduced probe dimension and mechanical compliance showed seamless tissue integration [2] and great promise of long-term stable recording [2, 3]. There is an intrinsic conflict on the requirement of a probe's rigidity between minimal invasiveness and facile insertion into the brain with minimal surgical injury. To eliminate chronic tissue reactions, it is essential to reduce a neural probe's rigidity so that the deformation force of the probe is comparable to the cellular forces in the nervous tissue [2]. However, such flexibility mechanically precludes the probe's self-supported penetration through brain tissue. Implantation techniques that meet the following requirements simultaneously are highly desired: i) to be minimally invasive, having surgical footprint as small as possible to minimize the surgical injury [4-6]; ii) to be scalable and high throughput, so that a large number of electrode contacts at high density can be implanted within a short surgery duration; and iii) to be able to target specific brain regions and depths.

Prior strategies to deliver flexible probes include temporarily altering the probe's rigidity prior to insertion [1, 7, 8], and delivering with a separate rigid shuttle device that is later decoupled from the probe [9-13]. To temporarily alter the probe's rigidity, biodegradable materials, such as polyethylene glycol (PEG) [14] and silk [15], were used to encapsulate and stiffen neural probes to support penetration into the brain tissue, which were then dissolved by the cerebrospinal fluid (CSF) after implantation. Temporarily freezing the probe attached by a small amount of solution was also demonstrated for stereotaxic insertion [1]. Alternatively, novel substrate materials such as mechanically adaptive nanocomposites [16] and shape memory polymer [17] were used to reduce stiffness after implantation. For the shuttle device strategy, a variety of temporary

² Zhao, Z., Li, X., He, F., Wei, X., Lin, S., Xie, C., *Parallel, minimally-invasive implantation of ultra-flexible neural electrode arrays*. J Neural Eng, 2019. **16**(3): p. 035001. My contribution to this work includes conceiving the idea and designing the experiments, fabricating the device, performing the surgery and electrical recording, performing the histology, analyzing the data and writing the manuscript with inputs from all authors.

attachment mechanisms such as biodegradable adhesives [9, 11, 12], geometrical anchor [18], and syringe injection [13] have been used. However, most of these implantation methods were designed for sparse implantation of flexible probes that have cross-sectional areas of about $1000\text{ }\mu\text{m}^2$ or larger, and had limited possibilities to aggressively scale down in dimensions to accommodate increasingly smaller neural probes and denser implantations. Our laboratory has demonstrated flexible nanoelectronic threads (NETs) neural probes with cross-sectional areas ranging from $10 - 100\text{ }\mu\text{m}^2$ [2, 19]. Therefore, it is critical to develop implantation strategies that offer comparable surgical footprints to the dimension of neural probes. A “needle and thread” mechanism using a microscale shuttle device made of tungsten microwires or carbon fibers successfully delivered NETs at about $200\text{ }\mu\text{m}^2$ surgical footprint [2], but offered limited throughput and ease of operation, because NET probes were inserted in serial, and each delivery required manual alignment with $1\text{-}\mu\text{m}$ accuracy.

In this work, we demonstrate a versatile implantation strategy using microwire arrays as the shuttle device, which allows high throughput, parallel insertion of multi-shank NETs with surgical footprints as small as $200\text{ }\mu\text{m}^2$ per shank (Fig. 17). A typical multi-shank NET probe hosts $32 - 128$ contacts on $4 - 8$ shanks at the inter-shank spacing of $150 - 400\text{ }\mu\text{m}$ and an overall thickness of $1\text{ }\mu\text{m}$ [2]. Our implantation scheme aims at delivering all shanks in parallel into the target brain region and depth, while maintaining the mechanical and electrical integrity. To achieve this goal, we design and fabricate a variety of guiding structures such as microtrenches and microconduits to construct tungsten microwire arrays with desired spatial arrangements, and attach the NET probes on the microwire array using a thin layer of PEG. We then stereotactically insert the assembly into the targeted brain region, and retract the microwire array after the PEG dissolves in the CSF. By using PEG of high molecular weight (35 kDa), we achieve sufficient time duration of attachment that allows for targeting deep brain structures such as hippocampus. Because the PEG adhesion layer used in the procedure has a sub-micron thickness, the surgical footprint is mostly contributed by the volume of the microwires. We demonstrate successful implantation of multi-shank NETs and reduced tissue damage using tungsten microwires with diameters as small as $12\text{ }\mu\text{m}$. We present three methods of assembling microwire arrays, using either microfabricated or commercially available guiding structures (Fig. 17). The delivery methods reported in this work use easy-to-access, low-cost supplies and can be readily adapted to the implantation of other flexible neural implants.

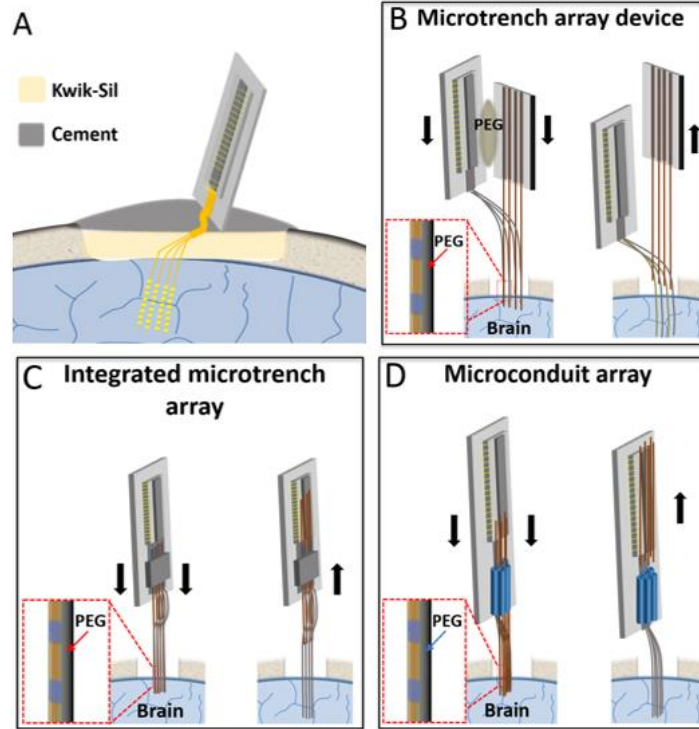


Fig. 17. Schematics of the implantation methods for NET probes. A. Sketch showing an implanted multi-shank NET and the skull fixation of the back-end carrier chip. B – D. Sketches showing the implantation procedures for three methods described in the paper, all using pre-aligned microwires as the shuttle devices and a temporary attachment mechanism by dissolvable adhesive.

3.2. Results

Implantation of NET arrays

To accurately implant multi-shank NETs with minimal surgical injury, the microwires must be highly straight, parallelly aligned, and as thin as possible. Using the methods described above, we constructed linear microwire arrays as shuttle devices for parallel implantation of NET probes, shown in Fig. 18 and 19. We fabricated microtrenches on a stand-alone silicon chip (Fig. 18A) as aligning structures for the microwires. The pitch of the microtrenches matched the inter-shank spacings of NET probes at 400 μm , 250 μm or 150 μm . We found that tungsten microwires at the diameter of 50 μm , among all commercially available microwires we have tested, had the best straightness and yielded the most predictable etching results, from which we generated straight microwires with thinned segments at the diameters of 25 μm and sharpened tips (Fig. 18B).

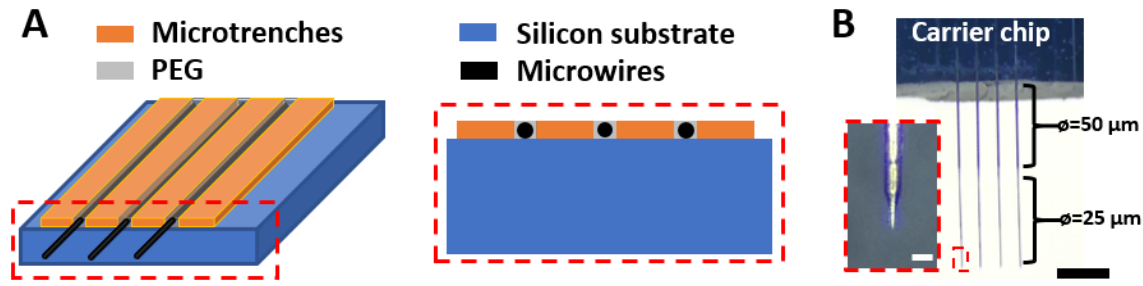


Fig. 18. Stand-alone microwire arrays. A. Sketches of the shuttle device showing the microfabricated trenches and the aligned microwires placed in between. B. Photograph of a shuttle device composed of a linear array of microwires aligned to the microtrenches. The last segment of the microwire was etched from a diameter of 50 μm to 25 μm . Inset shows the zoom-in view of the dashed box. Scale bars: 500 μm and 25 μm (inset).

We used a surface-tension assisted manual process (see methods) to achieve nearly conformal attachment of NET shanks on the microwires with PEG working as a water-soluble adhesive (Fig. 19A-B). Fig. 19C-D illustrate typical implantation of NETs in mouse brains with the microwire-shuttle device.

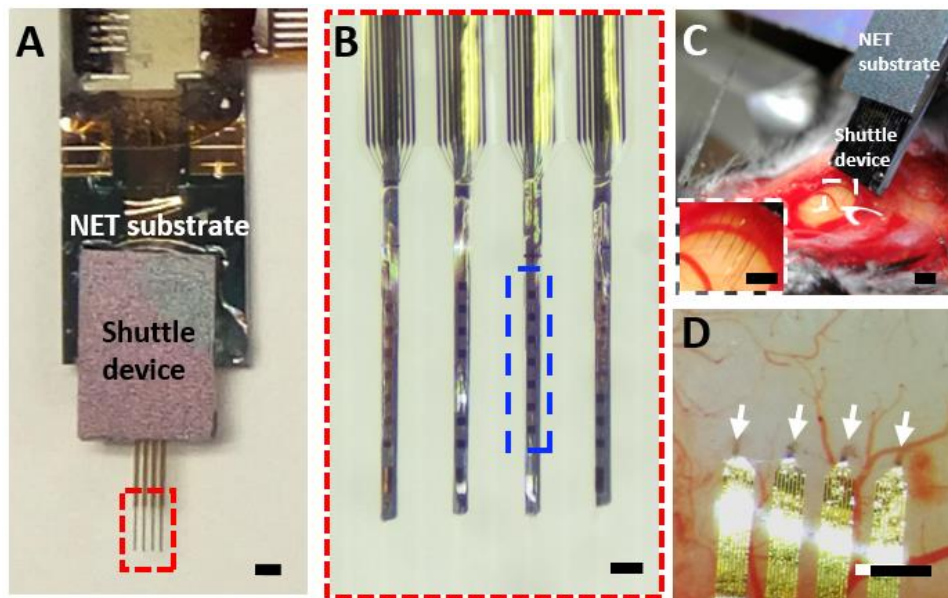


Fig. 19. Stand-alone microwire arrays assisted NET implantation. A. Photograph of assembled NET-shuttle device. B. Zoom-in view of the dashed box in A, showing the alignment of NETs on the microwire array. C. Photograph showing the stereotaxic insertion of the NET-shuttle device pair. D. Photograph of the implanted multi-shank NETs at inter-shank spacing of 400 μm . The arrows point to the four implantation locations. Scale bars: 500 μm (A, C and D); 100 μm (B).

As an alternative solution, we fabricated microtrenches directly on the NET substrate (Fig. 20A) and used them to guide the microwire alignment. We show aligning (Fig. 20B) and attaching (Fig. 20C) of eight-shank NETs onto microwire linear arrays with matching pitches.

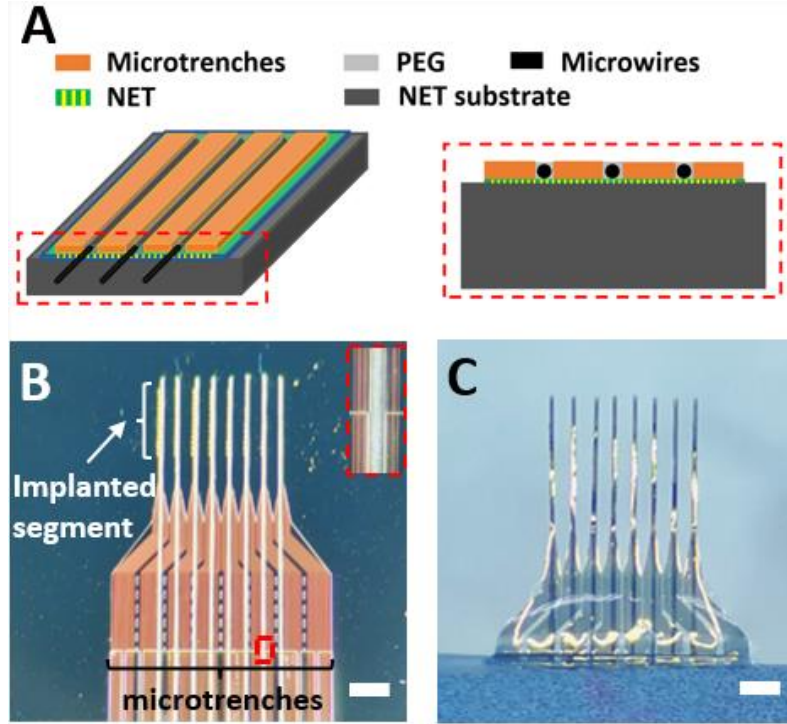


Fig. 20. Integrated microwire array. A. Sketches showing the microfabricated trenches on top of the NET substrate (left) and the cross-sectional view (right). B. Photograph of integrated microtrenches and microwires aligned on top of 8-shank NET probe on the substrate at the inter-shank spacing of 250 μm . Inset shows the zoom-in view of the dashed box. C. Photographs showing the back side of assembled NET-microwire arrays after NETs were released from the substrate.

Using simplified stereotaxic insertion and retraction procedures (see methods), we demonstrated the implantation of 8-shank NETs in the mouse cortex at 150 μm spacing (Fig. 21A, B). The procedures yielded repeatable assembly of NET-shuttle device with predictable arrangement. We used the NET probe with 150 μm shank pitch to quantify the spatial regularity, which has the smallest shank spacing among all our designs and is the most susceptible to wire deformation. We assembled 24 NET shanks with shuttle devices, from which we determined the discrepancy from expected outcome. The angle deviation of the shanks from the guiding structures measured $0.15 \pm 0.11^\circ$, and the averaged center-to-center distance measured $147 \pm 13 \mu\text{m}$. (Mean and standard deviation were used for all statistical analysis in this work.) The spatial arrangement of the shanks in the microwire-NET assembly was mostly preserved after the implantation. For

two groups of 24 shanks per group at the shank spacing of 400 μm and 150 μm , the average values of the shank spacing after implantation were $400 \pm 22 \mu\text{m}$ and $156 \pm 18 \mu\text{m}$, respectively.

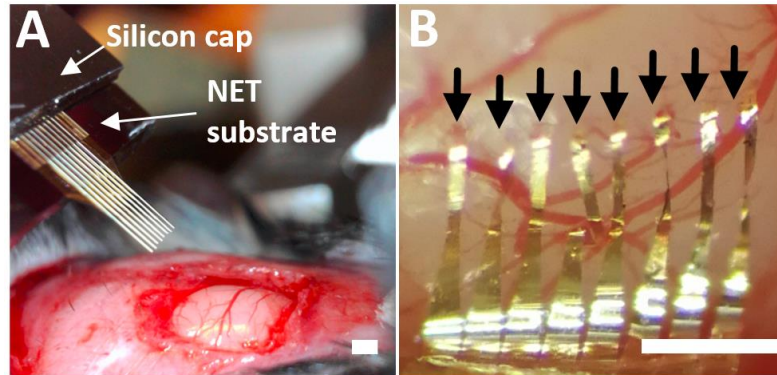


Fig. 21. Integrated microwire array assisted NET implantation. A. Photograph showing the stereotaxic insertion of the NET-microwire array. B. Photograph of the implanted NET probe after microwires were retracted. The arrows point to the implantation sites of eight NETs at pitch of 150 μm . Scale bars: 500 μm .

Besides in-line implantation of multi-shank NETs, we further demonstrated that a microconduit shuttle device made from off-the-shelf components can facilitate the implantation of NETs in various arrangements (Fig. 22). To construct the shuttle device, we stacked microtubes in a variety of patterns, such as linear, triangular or rectangular arrays, and inserted microwires in all or a selection of them (Fig. 22A-C). We then attached NETs onto the microwires (see methods). These versatile arrangements allowed NETs to be implanted in parallel with customized site and depth arrangement, which help optimize the recording coverage and/or avoid major surface blood vessel (Fig. 22D).

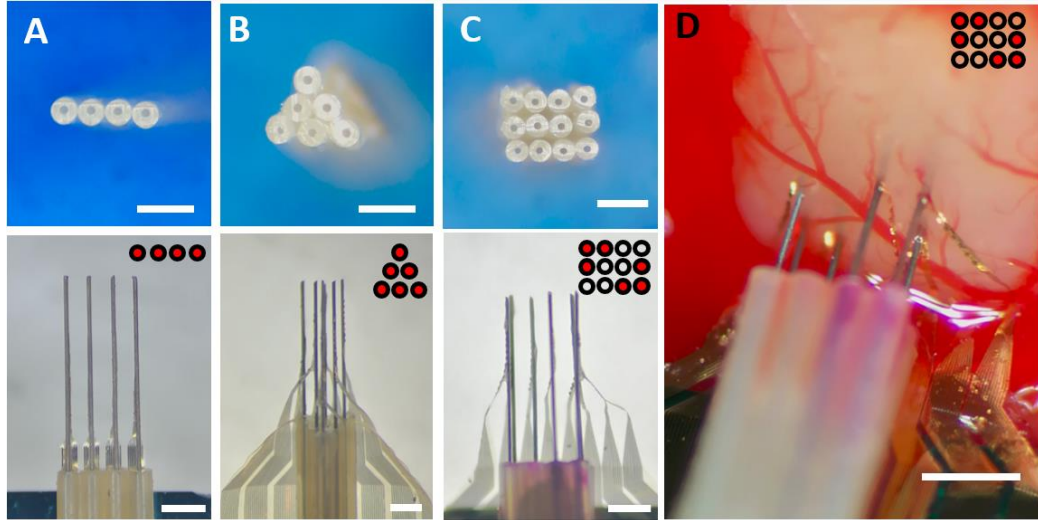


Fig. 22. Versatile implantation using shuttle devices made by microtubes. A-C. Photographs showing various arrangements of microwires aligned by microtube stacks. Examples of linear (A), triangular (B) arrays, and a customized selection in a rectangular structure (C) were shown. Top: microtubes stacked in desired patterns. Bottom: NETs attached to the shuttle device and ready for implantation. D. Photograph of a representative implantation using tube guided microwire array, for which the arrangement of microwires was customized to avoid a major blood vessel in the primary visual cortex (V1) of mouse brain. Insets for all panels illustrate the patterns of the microtubes (black circles) and the ones with inserted microwires (red dots). Scale bars: 500 μm .

3.2 Minimized surgical injury

To reduce the surgical injury during implantation, we minimized the overall cross-sectional footprint of the NET-shuttle assembly, which is determined by the diameter of the microwire and the thickness of the PEG adhesive in addition to the size of NETs. We chose to use PEG solution at 5% w/v and the molecular weight of 35 kDa for its desired balance of sufficiently long dissolving time in tissue (discussed later), relatively low viscosity for the ease of handling, and sub-micron coating thickness. Scanning electron microscopy (SEM) images (Fig. 23A-C) confirmed that the PEG coating added negligible volume to the NET-shuttle assemblies.

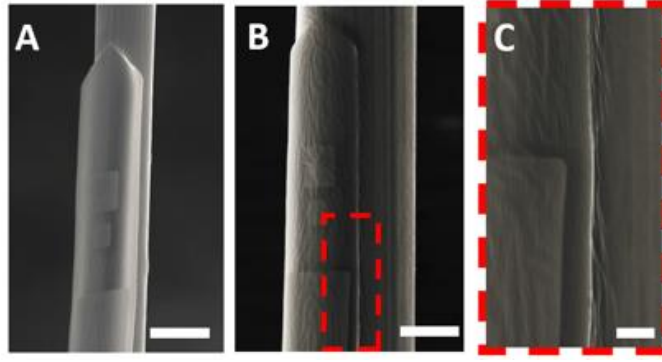


Fig. 23. SEM images showing NETs attached to microwires with diameters of 25 μm (A) and 50 μm (B, C) using PEG. C. is the zoomed-in view of the dashed box in B. Scale bars: 25 μm (A and B), 5 μm (C).

To further reduce the tissue displacement during implantation, we used microwires with reduced diameters of 12 and 25 μm that were electrochemically etched and sharpened from microwires 50 μm in diameter (Fig. 24A-C, see methods). The angle of the tips varies with the diameter of the shuttle wires, measured $8.5 \pm 1.5^\circ$, $11.3 \pm 2.1^\circ$, and $15.2 \pm 1.9^\circ$ for 12 μm , 25 μm , and 50 μm microwires respectively. Microwires of all dimensions supported parallel implantation of NET arrays in the mouse cortex (Fig. 24D-F).

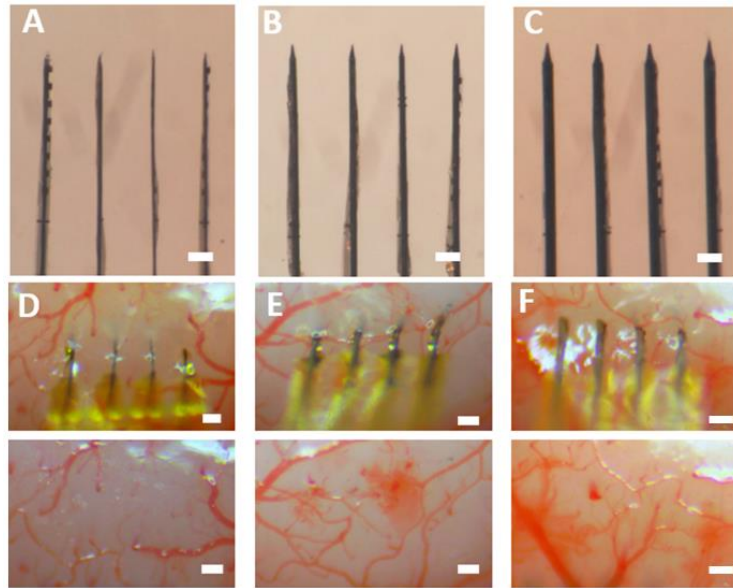


Fig. 24. Shuttle microwires of different diameters and their insertion damage. A– C. Photographs showing NET arrays attached on microwires with diameters of 12 μm (A), 25 μm (B), and 50 μm (C). D – F. Representative photographs showing in vivo insertion of NETs using microwires with diameters of 12 μm (D), 25 μm (E) and 50 μm (F), and the corresponding brain surface after the retraction of the wire. Scale bars: 100 μm

The surface bleeding induced by the implantation, which was measured by the area covered by blood after retraction of microwires, significantly decreased with the diameter of the microwires (Fig. 25).

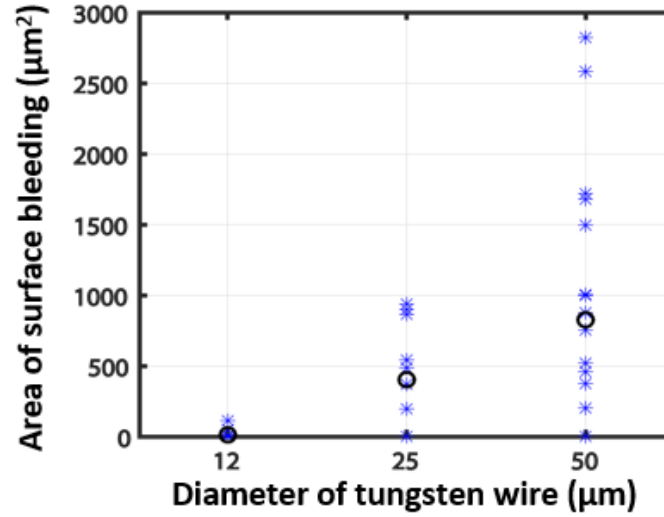


Fig. 25. The area of bleeding observed from brain surface for the three diameters of microwires. All trials (asteroid) and their averaged value (circle) are plotted.

3.3 Impedance and unit recording of implanted NET electrodes

The NETs are vulnerable to forceful manipulations. Our methods kept their structural and functional integrity during assembly and implantation. In a group 6 multi-shank NET probes with 140 contacts in total, we characterized the electrodes by measuring the impedance at 1 kHz before and after implantation (Fig. 26). All contacts remained connected and the average impedance had a minor increase of 50 – 300 kΩ from the in vitro measurements before implantation. We attribute this increase to the tissue-electrode interface impedance [21].

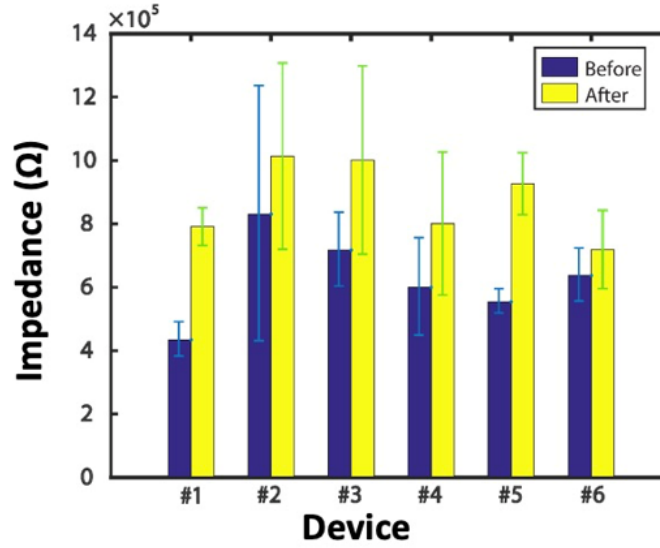


Fig. 26. Impedance before and after implantation for 6 32-channel NETs.

We show here the typical recording performance of a 32-ch NET probe implanted in mouse motor cortex using this method. All 32 channels were confirmed to be connected by impedance check. We yielded 33 single units from the recording with an average amplitude 107.15 ± 44.68 μ V and noise level 21.12 ± 1.67 μ V ten days post implantation.

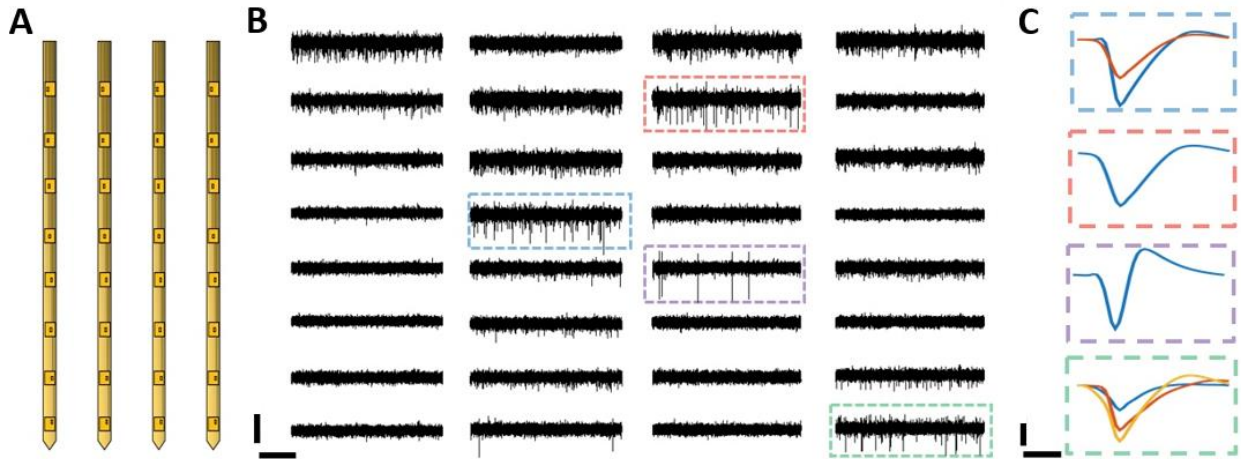


Fig. 27. Recording from the implanted NETs. A. Sketch of the 32-channel NET array hosting 4 shanks with 8 recording sites on each shank. B. 2-s recording traces from a NET array as sketched in B. Scale bar: 100 μ V (vertical) and 0.5 s (horizontal). C. Representative averaged waveforms from 4 electrodes indicated by dashed boxes in B. Scale bar: 50 μ V (vertical) and 0.5 ms (horizontal).

3.4 Control on the insertion depth

Precisely controlling the insertion depth of NETs is critical to reach the designated implantation target. We found that precise insertion depth could be achieved as long as the NET-shuttle assembly reached the targeted stereotaxic coordinates before the PEG was fully dissolved. Because we cannot directly observe the dissolution of PEG, we typically waited for a sufficiently long time (2 mins) before we proceeded. We performed in vitro and in vivo tests to determine the precision of targeting specific implantation depths. In 0.5% agarose gel, which had similar mechanic properties as mouse brain [22], we targeted the insertion depth of 2 mm at an insertion speed of 100 $\mu\text{m/s}$, and achieved $2.017 \text{ mm} \pm 34 \mu\text{m}$ ($n=10$) (Fig. 28).

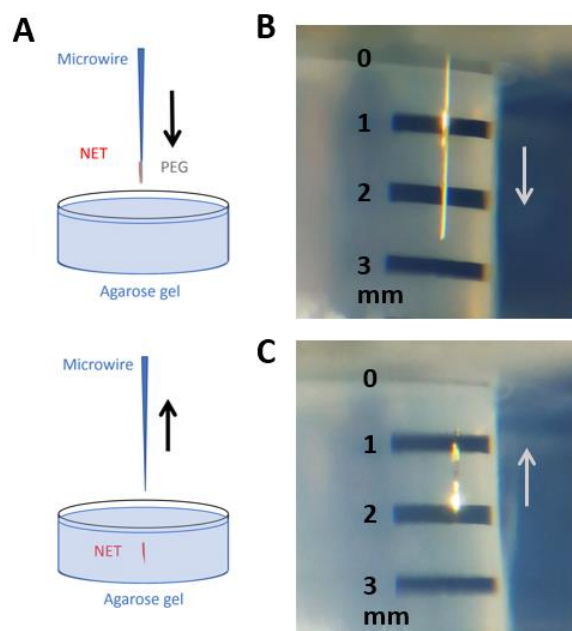


Fig. 28. In vitro tests of insertion depth accuracy in agarose gel. A. Sketch illustrating the in vitro test setup in the agarose gel. B and C. Insertion of NETs in the agarose gel targeting the depth of 2 mm.

In the in-vivo tests, we targeted the depth of 1 mm in the mouse primary somatosensory cortex (S1) at the speed of 300 $\mu\text{m/s}$. The experiment was conducted in two mice, with 3 and 4 shanks inserted in each animal respectively. We determined the implantation depth by postmortem histological tissue examination (Fig. 29A) and achieved $1.029 \text{ mm} \pm 55 \mu\text{m}$ ($n=7$). We also targeted the mouse hippocampus at depth of 2 mm and at the speed of 1 mm/s. We attempted to insert 20 shanks in total, and successfully implanted all with an average tip depth of $2.062 \text{ mm} \pm 108 \mu\text{m}$ ($N=20$). Fig. 29B shows a slice highlighting 5 shanks implanted in hippocampus

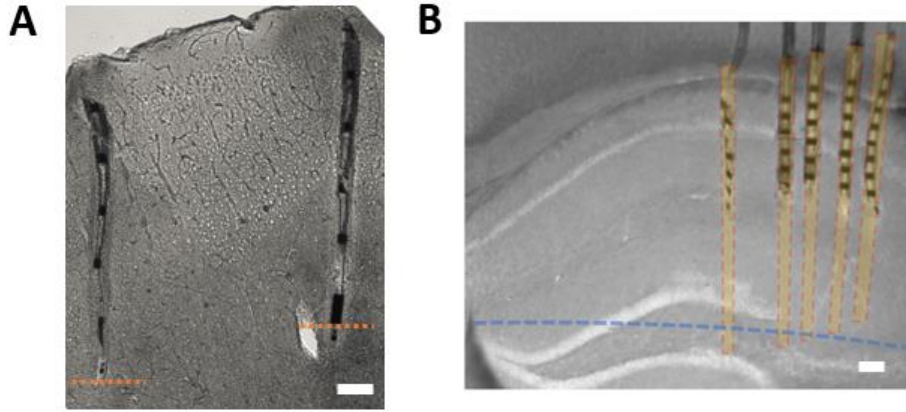


Fig. 29. In vivo tests of insertion depth accuracy. A. Stitched image of a brain slice showing two representative implanted NETs in the mouse somatosensory cortex (S1) at the designated depth of 1 mm. Orange dash lines mark the measured depth of 1mm from the brain surface. B. Bright-field image of a brain slice with implanted NETs in mouse hippocampus. The blue dash line marks the measured depth of 2mm from the brain surface. Part of the shanks were sliced off during sample preparation. Yellow shaded areas mark the estimated coverage of the recording regions. Scale bars: 100 μ m.

3.5 Post-Mortem tissue-probe interface

To further evaluate the implantation outcome, we examined the tissue-probe interface using postmortem histology two months after implantation (see Methods). Fig. 30 shows representative images of a tissue slice from the mouse primary somatosensory cortex (S1) at depth about 400 μ m where an 8-shank NET probe was implanted at the inter-shank spacing of 250 μ m. Normal neuronal density and morphology were observed in the close vicinity of the NETs, including regions between nearby NET shanks. These observations are qualitatively consistent with our previous studies on chronically implanted individual NET shanks [2] and NET linear arrays [19]. The long-term outcome of this implantation method will be further investigated in our future studies.

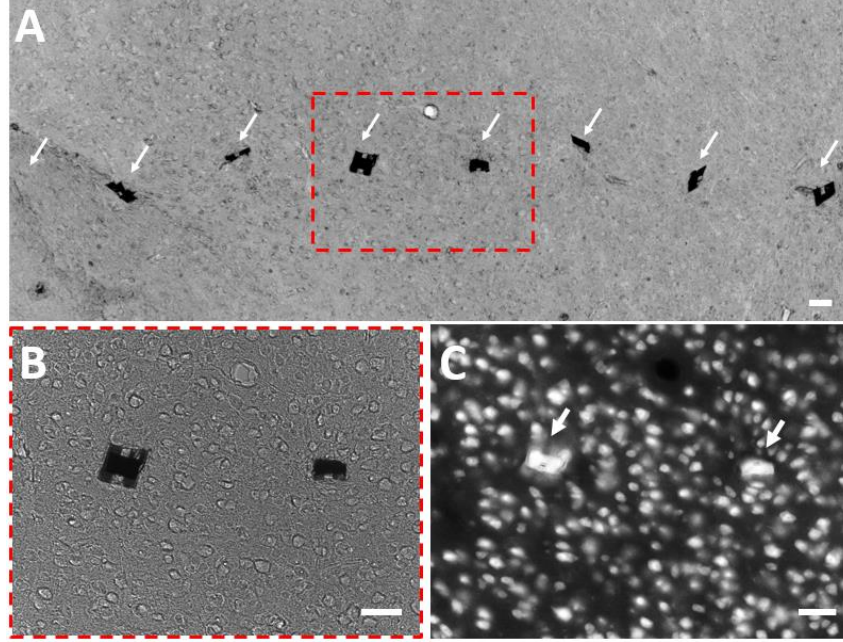


Fig. 30. Tissue-NET interface two months after implantation. A. Bright-field image of a tissue slice (20 μm thick) in the mouse cortex where an 8-shank NET probe was implanted. The arrows highlight the implanted locations of the NETs, in which one out of eight was dragged out during tissue slicing. B. Zoom-in view of the dashed box in (A), showing that the tissue morphology near the NETs was normal. C. Fluorescent image of the same tissue slice where neuron cell bodies were fluorescently labeled. Scale bars: 50 μm .

3.3. Discussion

We chose to use tungsten microwires to construct the shuttle devices in this work. First, they have sufficiently high Young's modulus (411 GPa), so that microwires with cross-sectional area as small as $110\text{ }\mu\text{m}^2$ (12 μm in diameter) have adequate mechanical strength to penetrate brain tissues, which allows for reduced surgical footprints (less than $200\text{ }\mu\text{m}^2$, shuttle-NET combined) comparing with other commonly used materials such as silicon [12]. Secondly, they have high tensile strength (1.5 GPa) and are robust during handling. Thirdly, they are commercially available at a variety of diameters and at low cost. The implantation techniques can be readily adapted using other high Young's modulus materials such as carbon fibers, SiC fibers, and diamond microbeams.

The smallest inter-shank spacing we demonstrated using the microtrenches guided microwire arrays was 150 μm , which is comparable to the closest spaced silicon neural probes [23, 24] and Utah arrays [23]. When using microconduit arrays to guide microwire alignment, the spacing is determined by the outer diameters of the tubes, which ranges from 150 μm to millimeters for off-the-shelf tubes.

The presented method involves considerable manual manipulations. On average, it took 20-30 min to assemble one device including microwire placement, NET attachment and additional packaging. The overall success rate was about 90%, and most of the failure came from mechanically tearing off the shanks when trimming the NET substrate and attaching NETs on the microwires. Once NETs are successfully assembled with shuttle devices, their implantation yield can reach nearly 100% by trained operators.

There are a few limitations in the current technique that can be improved in the future development. First, the PEG used in this method has relatively short dissolution time in tissues, which excludes extremely slow insertion speeds ($<5 \mu\text{m/s}$). Further, because the PEG coating encapsulated recording sites during insertion, it was difficult to record the neural activity during insertion, which was often used to target and verify the implantation depth. This limitation can be potentially improved by choosing adhesives with longer dissolution time such as silk, and applying it only at the tip of the probe instead of coating the entire shank. Second, the throughput, yield, and consistency of the assembly process can be improved by replacing some or all of the manual manipulation with automatic procedures.

3.4. Outlook

Despite the scalable and facile insertion with our current method, further development is strongly desired to allow for slow insertion at speed around $\mu\text{m/sec}$ to avoid tissue and blood vessel rupture which can hopefully reduce the acute insertion damage, and combining simultaneous recording for better targeting, especially for structures such as hippocampus whose thin, densely packed cell layers are difficult to target without guiding with the recordings. And for the cortical implantation, a controlled slow insertion speed would allow for precise targeting at different functional layers. To achieve it, we have designed mechanical anchors, which is a loop-hole design at the tip of the probe and used sharpened tungsten microwire to engage the probes and guide them into the brain tissue upon delivery. Fig. 31 shows the probe with the loop-hole design in A and the probe-wire assembly in B. Recording traces in C shows the bursting events at mouse hippocampus during slow insertion with the array shown in A and B. While significant amount of development are still demanded, this preliminary results has demonstrated the possibility to incorporate controlled slow insertion with flexible neural electrodes, which can potentially lead to better recording outcomes.

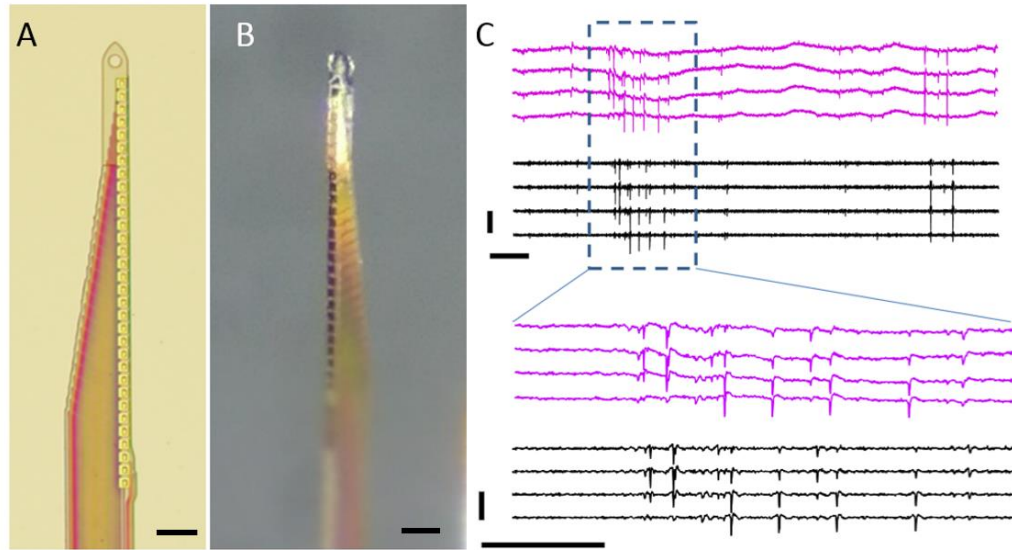


Fig. 31. Slow insertion of the NET probe. (A) Bright-field image the hippocampus NET probe with the loop-hole design on the tip. Scale bar: 50 μm . (B) Image of the NET-microwire assembly ready for implantation, showing that the microwire was engaged into the loop hole of the probe. Scale bars: 50 μm . (C) Recording traces showing the bursting events at mouse hippocampus during slow insertion with the array shown in A and B. Scale bars: 200 μV (vertical), 200 ms and 50 ms (horizontal).

3.5. Methods

Animal preparation and cranial surgery

Wild-type male mice (C57BJ/6, 8 weeks old, Taconic) were used in the experiments. Mice were housed in the Animal Resources Center at the University of Texas at Austin (UT Austin) at a 12-hour light/dark cycle at 22°C, food and water ad libitum. For cranial surgery, the mice were anesthetized with isoflurane (3% for induction, 1-2% for remainder of surgery) in medical-grade oxygen and mounted on a stereotaxic frame (Kent Scientific Inc.). A 3 mm by 3 mm cranial opening was created in the skull using a surgical drill. The dura was carefully removed at the insertion site to facilitate the delivery. After NET probe implantation (described below), the exposed brain and the remaining flexible segment of the NET probe, which connected the bonding pads on the substrate with the electrodes inside the brain, were protected by artificial cerebrospinal fluid (aCSF) and Kwik-Sil adhesive (World Precision Instruments). After the skull was cleaned and dried, a layer of low-viscosity cyanoacrylate was applied over the skull. An initial layer of C&B-Metabond (Parkell Inc.) was applied over the cyanoacrylate and the Kwik-Sil adhesive. A second layer of Metabond was used to cement the coverslip and the carrier chip to the skull (Fig.

1A). All procedures complied with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the UT Institutional Animal Care and Use Committee.

Electrochemical thinning and sharpening of tungsten microwires

Electrochemical etching in 0.8 M KOH solution was used to thin and sharpen tungsten microwires (W5607, Advent Research Materials). Tungsten microwires were cut into appropriate lengths of 4 – 6 mm and mounted on a strip of copper tape with silver epoxy. To thin the wires, a constant voltage of 2.5 V versus a graphite counter electrode (GR001CC, GraphiteStore) was applied to the wires to achieve an appropriate etching speed, while a segment (typically 2 mm long) of the wire was immersed in the etchant. The target diameter was controlled by the etching time. Empirically it takes approximately 1 min to reach the diameter of 25 μm and 2 mins for the diameter of 12 μm . After the wire is thinned to the designated diameter, a sharp tip was formed at the end of the microwire by positioning the tip at the solution-air interface and etching for an extra 3 mins at the same voltage.

Fabrication and preparation of stand-alone microwire arrays as shuttle devices

Microtrenches of matching spacing as the NET shanks were microfabricated on silicon substrates (4" wafer, 900 nm SiO_2 , n-type 0.005 $\text{V}\cdot\text{cm}$, University Wafer) using standard photolithography. SU-8 photoresist (SU-8 2075, MicroChem Corp.) was spun on the silicon substrate at 4000 rpm for 45 sec, followed by the standard SU-8 photolithography process which yielded microtrench structures with a depth of 60 μm and width of 50 μm . After hard baking (180 $^\circ\text{C}$, 3h), the 4" wafer was cut into rectangular pieces (3 mm $\times$ 4 mm) using dicing saw (7100 Dicing system, ADT), which were used as the carrier chip for shuttle devices. The etched tungsten microwires were placed into the microtrenches manually, resulting in a linear array of microwires at the pitch predefined by the carrier chip. The microwires protruded beyond the edge of the carrier chip by several millimeters depending on the implantation depth. A thin layer of super glue (Loctite) was used to fix microwires in the trenches on the carrier chip without overflowing. Small drops of epoxy (Loctite) were placed on all four corners of the carrier chip to act as spacers between the shuttle device and the NET probe when they were later attached together. The assembled shuttle device was then baked at 180 $^\circ\text{C}$ for 30 min for the epoxy to cure.

After the multi-shank NET was fabricated and partially released from the substrate as previously described [2], the shuttle device was aligned with of NET shanks under a stereomicroscope (A60S, Leica Microsystems), with the tip of the microwires protruded the tip of the NET shanks by 10 – 20 μm in length. A flat-end alligator clip was used to hold together the shuttle device and the NET probe, while a small gap was created between them by the epoxy drops at the corners of the shuttle device to prevent damaging the NET probe. The NET substrate was then trimmed to the desired length in deionized water. Subsequently, the NET-shuttle assembly was dipped into the PEG solution (5% w/v, molecular weight of 35 kDa, Alfa Aesar) and slowly pulled out. A 34-gauge syringe needle (O.D. 190 μm) was used to guild NET shanks to attach onto each microwire with the assistance of surface tension [20]. After the attachment, additional PEG was applied between the shuttle device carrier chip and the NET substrate to attach them together. The clip was then removed, and the assembled device was allowed to dry in air. The design and implementation of this procedure are shown in Fig. 18.

Stereotaxic implantation of NET probes with stand-alone microwire arrays as shuttle devices

The assembled NET-shuttle device pair was attached to a micromanipulator on the stereotaxic frame with the back of the NET substrate contacting the tape. The micromanipulator was manually controlled to insert the NET-microwire array into the designated region of the mouse brain. The three-dimensional (3D) coordinates of the arrays were measured (Digital lab standard, Stoelting Co.) during insertion for the control of depth and speed.

After the NET-microwire array reached the target position, a small drop of glue was gently placed on the shuttle device carrier chip. A customized stainless steel pole (1 mm in diameter) mounted on the second micromanipulator on the stereotaxic frame was manually controlled to approach the glue on the shuttle device carrier chip and stayed in contact as the glue cured. After the glue completely cured and the PEG between NET shanks and microwires was dissolved by the tissue fluid, sterile phosphate buffered saline (PBS) was applied to dissolve the PEG that attached the shuttle carrier chip with the NET substrate. The shuttle device was then carefully retracted from the tissue using the second micromanipulator, leaving the detached NETs in the implantation sites. The typical extraction speed was about 1 mm/s. In this study, NET probes were implanted in 3 mice with the stand-alone microwire array.

Microwire assembly using integrated microtrench arrays on the NET substrate

At the end of the NET fabrication, an extra photolithography process was used to define microtrench arrays using SU-8 photoresist (SU-8 2075, MicroChem Corp.) similarly as described in the previous sections. After connectors were soldered on the contact pads, etched tungsten microwires in diameters of 12, 25 or 50 μm and length of 4 – 6 mm were manually placed into the microtrenches. The front end of the microwires were aligned to the end of the NET shanks, while the rear end of the microwires were at least 2 mm exceeding the connector. The microtrenches were then partially filled with PEG solution to fix the microwires in place. PEG is used in this step to allow the microwires to be released and retracted after surgical insertion. A flat silicon chip was placed on top of the microtrenches and fixed on the NET substrate by epoxy, which acted as a cap to restrain the out-of-plane motions of the microwires during retraction. The NETs were then released and attached onto the microwires in the PEG solution using the same procedure as described in 2.3. The assembled device was ready for implantation after dried in air. The design and implementation of this procedure are shown in Fig. 19.

Assembling microwire arrays guided by microconduit arrays

Polytetrafluoroethylene tubes (Sub-lite-wall tubing, O.D. 200 μm , I. D. 100 μm , Zeus) of a length of 6 mm were manually stacked to form a variety of structures, including linear, rectangular and triangular arrays. Epoxy (Loctite) was applied to permanently fix the structures. Pre-cut straight tungsten microwires the same as used in the previous method were inserted into all or a selection of the conduits. The microwires protruded the tube edge by 3-5 mm on both ends while PEG solution was applied at the rear end of the conduits to temporarily fix the microwires. The microconduit array was then mounted on the NET substrate using Epoxy (Loctite), and aligned under stereomicroscope so that the lateral position of individual microwires best matched the positions of NET shanks and the microwires protruded the end of the NET shanks by 50 – 100 μm . The flexible segment of NETs was then released and attached onto the microwires using PEG solution as described in 2.3. The assembled device was ready for implantation after dried in air. The design and implementation of this procedure are shown in Fig. 20.

Stereotaxic implantation of NET arrays with integrated microwire arrays

The assembled NET device in 2.5 and 2.6 was attached onto a micromanipulator on the stereotaxic frame, with the back of the NET substrate facing the micromanipulator. The micromanipulator was manually controlled to insert the NET-microwire array into the designated region of the mouse brain similarly as described above. After the NET probe reached the target position and depth, sterile PBS was applied on the rear end of the assembly to dissolve the PEG and release the microwires from the microconduit or microtrenches, while the tissue fluid in brain dissolved the PEG and released NET shanks from the microwires. After PEG was fully dissolved on both ends, which typically took less than 2 minutes, individual microwires were picked up from the rear end by tweezers and manually retracted one at a time, leaving NETs implanted at the target positions. In this study, NET probes were implanted in 5 mice with the integrated microtrench array and 3 mice with the microconduit array.

Insertion injury test

The test of insertion tissue damage was performed at primary visual cortex with tungsten microwires' diameters of 12 μm , 25 μm , and 50 μm etched and sharpened as described in 2.2. The insertion speed was approximately 1 mm/s, and the insertion depth was 1 mm. For quantitative comparison, the surface area of the central bleeding spot was measured for each insertion site using ImageJ. NET probes were retracted with the microwires for the ease of the measurement. The insertions were repeated and the bleeding areas were averaged at 12 separate locations in two mice for microwires at diameters of 12 and 25 μm , and at 20 locations in two mice for microwires at the diameter of 50 μm .

Electrophysiological recording and impedance measurement

Multi-shank NET probes were implanted into the primary somatosensory cortex and primary motor cortex in the mouse brain at depths ranging from 700 μm to 1 mm at the tip. Intan 64-channel RHD2164 evaluation system (Intan Technologies) was used for neural signal acquisition using sampling rate of 20 kHz. The recording was performed from awake mice head fixed on a custom-made treadmill ten days post implantation. Common median referencing [25] was applied to the raw data to reduce the common mode noise such as motion artifacts, etc. Spike sorting was performed with Offline Sorter (Plexon). In vivo impedance was measured with the same system at 1 kHz prior to each recording session. The impedance was also measured with the same system in PBS solution prior to implantation. Single unit was identified based on the criteria

that the fraction of spikes with inter-spike intervals under 2 ms were less than 1% of the total firing. [26]

Histology

The mice were perfused intracardially using oxygenated aCSF at 4°C, then with a 4% paraformaldehyde solution in 0.02 M PBS. 30% sucrose/4% paraformaldehyde solution was used to soak the brains overnight. Brain tissue was sliced using a Leica CM1950 cryostat (Leica Microsystems) into 20 µm slices, which were then washed for three 5-minute sessions before incubated for 30 minutes in a sodium citrate solution (85°C to 95°C, 0.01 M in H₂O) for antigen retrieval. The slices were washed again for three 5-minute sessions, incubated in a blocking solution, permeabilized [0.5% Triton X-100 and 10% normal goat serum (Sigma-Aldrich) in PBS] for 3 hours at 25°C, washed again for four 5-minute sessions, then incubated with the one-step fluorophore-conjugated antibodies at 4°C for 24 hours. To immunochemically stain neurons, Alexa Fluor 568–conjugated anti-NeuN antibody (ab207282, abcam) was used. In this study, the evaluation of the insertion depth in cortex, in the subcortical region (hippocampus), and the examination of tissue-probe interface were performed in two, one, and one mouse brains, respectively

4. Large-scale electrophysiological mapping by NET arrays

4.1. Introduction

The brain is a highly-interconnected network composed of a large number of neurons. It contains many specialized circuits that regulate all the behaviors, such as locomotions, memory, learning and decision making. These physiologically and behaviorally relevant circuits span multiple orders of magnitude in both time and space. Moment-by-moment information processing takes place on the timescale of milliseconds, but changes in activity patterns that support adaptation, learning and related functions occur throughout the lifetime and on timescales ranging from seconds to years. Complex circuits consist of distributed neurons across multiple distant brain regions, while the neighboring neurons within hundreds of micrometers are responsible for local information processing. In order to observe and study these neural circuits, we need to achieve simultaneous measurements of large assemblies of neurons in both close-by and distributed regions in the brain. The advances in neural engineering has provided us with tools that are capable of these measurements with varying advantages [1]. Implanted electrodes [2-6] are one of the classic and widely adapted methods, it provides a unique approach to decipher brain circuitry by allowing for time-resolved electrical detection of individual neuron activity [7-9].

Representative conventional implanted electrodes include silicon-based microfabricated arrays [2, 5, 6, 10] and metal microwire arrays [3, 4, 11, 12]. Despite being widely used in neuroscience studies, they have significant limitations in their in-vivo recording stability. This is largely due to the mechanical mismatch of the devices and the soft brain tissues [13]. Also, the size of the implant and their volume occupation often causes perturbation to the delicate cellular and vascular networks in the brain [14]. Recent studies have shown that the use of less-rigid, polymer materials [15-20] and the reduction of the implant size, such as using carbon fibers [21, 22], have a significant impact on the health of the interfacing brain tissue by mitigating tissue reactions and thus improving recording stability. With further improvement in probe size and flexibility, the flexible nanoelectronic thread (NET) [23, 24] has demonstrated a glia scar-free tissue interface and marked long-term recording stability. However, the existing electrode technologies provide recording bandwidth and densities that are far less desirable to study the complex neural circuitry.

The recently developed Neuropixels probe [10] has enabled researchers high-density depth-recording of multiple connected brain regions along the probe axis. But these devices have limited lateral implantation density due to large cross-sections outside the brain that limits device packing. The electrode array made of polyimide [18] exhibits large-scale recording at multiple distributed brain regions and showed long-term tracking stability, but a clear tissue response was still found around the probe. Additionally, due to the diversity of cell types and anatomical structures in different regions of the brain, customized designs are required to optimize the recording outcomes for each region.

Building upon our flexible device platform, we systematically developed and expanded it to enable large-scale recording capabilities in conjunction with highly-customized probe designs. This platform was applied in different neuroscientific scenarios, including the long term stable interrogation of high-density local circuits, circuits distributed across the whole neocortex, and subcortical structures. These demonstrations show the capability of this platform to address more complex and challenging neuroscience issues across different spatiotemporal dimensions.

4.2. Results

In order to efficiently record from different brain regions, we designed and fabricated a variety of electrode patterns (Fig. 32A – C). For instance, a linear array was designed for recording in the neocortex where cells are relatively uniformly distributed. The dispersed contact arrangement allows for distributed coverage spanning a two-dimensional (2D) plane (Fig. 32A); a dense linear array for recordings with high-resolution coverage in regional structures (Fig. 32B); and a dense two-row array for recordings in highly inhomogeneous structures, such as hippocampus where neurons reside in thin, densely packed layers. The close contact spacing allows oversampling detection that enhances the capability to resolve single units (Fig. 32C). Despite the differences in electrode design, all probes had an overall thickness of approximately 1 μm , which allows for the unique ultra-flexibility of these devices, and affords a seamless probe-tissue integration. Importantly, these probes have cross-sectional areas of 30 – 100 μm^2 , which makes possible implantation of multiple probes at very high densities.

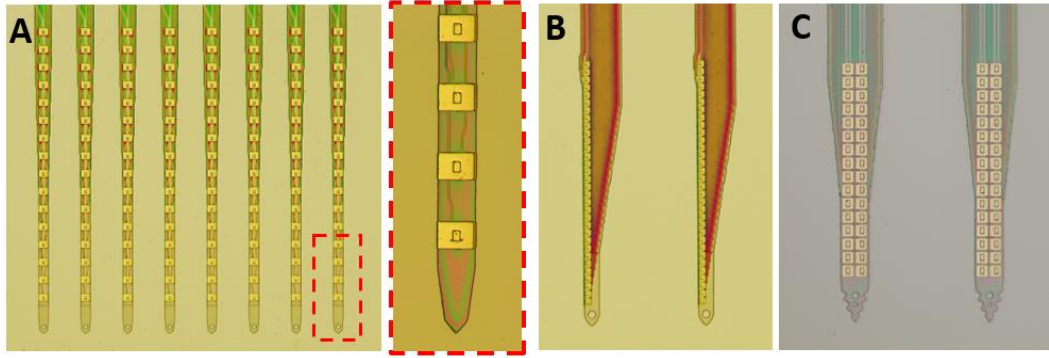


Fig. 32. Overview of the versatile electrode design. A, As-fabricated NET-cortex array on silicon substrate, with zoom-in view of four electrodes shown in the dashed box. B and C, As-fabricated NET-hippocampus and NET-oversample arrays on silicon substrates.

The backend of the probe make contact, through a 0.5-mm-pitch ball grid array (BGA), to a custom, 100- μ m thin flexible printed circuit board (Flex-PCB) (MicroConnex) (Fig. 33A). This compact interconnect design makes it possible to densely implant multiple 128-channel devices with acceptable head-mounted weight. Together, they constitute a high-density, highly scalable platform for neural recordings in behaving animals. A modular headstage system (Spikegadgets) was adopted for data acquisition with each module supporting 128 channels. The entire system can support up to 8 modules, with a total of 1024 channels (Fig. 33B). All channels can simultaneously stream out in real time for visualization and data storage with a single micro-HDMI cable. The use of the modular design not only increases the flexibility of the measurement by allowing researchers freely select a certain number of electrodes to implant or record from as needed and its light weight and compact size allows for the study of unrestrained, freely-moving animals (Fig. 33C, D).

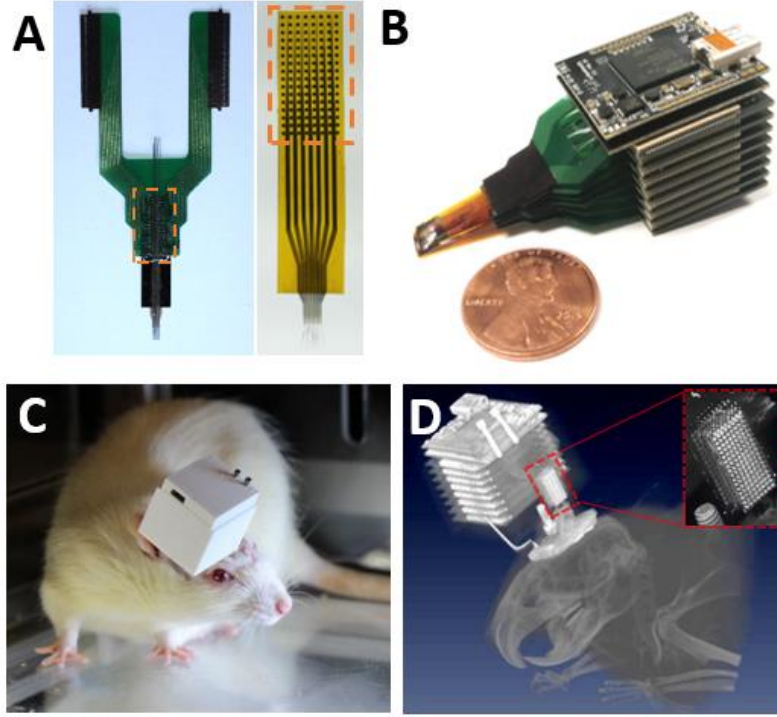


Fig. 33. Overview of the back end design and recording system. A, A ready-to-implant 128-channel NET array with a flexible printed circuits (FPC) connected to its backend through ball grid array (BGA). The 500 μm -pitch BGA was highlighted in orange dashed box. B, 1024-channel recording system with 8 128-channel NET probes interfaced through FPCs. C, As-implanted 1024-channel NET array in G in a free-moving rat. D, μCT scan of the upper body of the rat in H, zoom-in view of the red dashed box highlights the stack of BGAs.

To maximize the surgical and recording outcomes, we have adopted a hybrid implantation approaches to adapt to different brain structures. For large-scale implantation at neocortex, a temporary attachment mechanism with biodegradable glue previously developed by our lab [25] was used to increase the time efficiency of the implantation which shorten the surgical time, reducing the risk of infection. For implantation at subcortical structure such as the hippocampus, a “needle-and-thread” strategy [23] was used to slowly and precisely target the thin cell layer under the guidance of simultaneous neural recording to maximize the cell coverage and unit yield. The large-scale and high-density implantations were implemented through multiple insertions of single 128ch modules (Fig. 34A) to minimize the disruption to the surface vasculature system and reduce the tissue damage caused by dimpling through simultaneous multi-shank insertions. In addition, the ultra-flexibility of the probe and the use of separate wires as guiding structures enabled the

selective withdrawal of individual wires based on the distribution of surface blood vessels before insertion, further avoiding damage to the vasculature system (Fig. 34B).

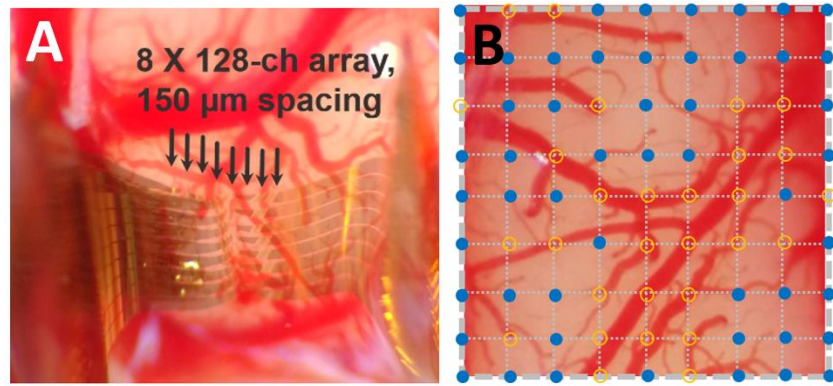


Fig. 34. Surgical photograph of the NET implantation. A, Photograph of the 8 x 128-ch NET arrays implantation during surgery, the arrows denote each layer of 128-channel NET array at pitch of 150 μm . B, Photograph of the surface vasculature at the implantation site. The imaginary position of each shank to be implanted (blue dot) and to be removed (hollow dot) were superimposed.

The large-scale NET array exhibits stable in-vivo electrical and recording performance. Electrode impedance was monitored for a total of 1653 connected electrodes (91% connection yield) from 14 128-channel modules implanted in the neocortex of two mice for two months. Specifically, one was implanted with 911 connected electrodes (8 128_ch modules) at primary visual cortex and the other one with 742 connected electrodes (6 128_ch modules) spanning across prefrontal cortex, primary motor cortex, somatosensory barrel cortex and primary visual cortex. All electrodes were initially coated with PEDOT (Poly(3,4-ethylenedioxythiophene)). The average impedance per contact site exhibited an increase from the beginning of the implant and gradually stabilized at ~ 550 kohm after the third weeks and remained stable for the duration of the experiment (Fig. 35).

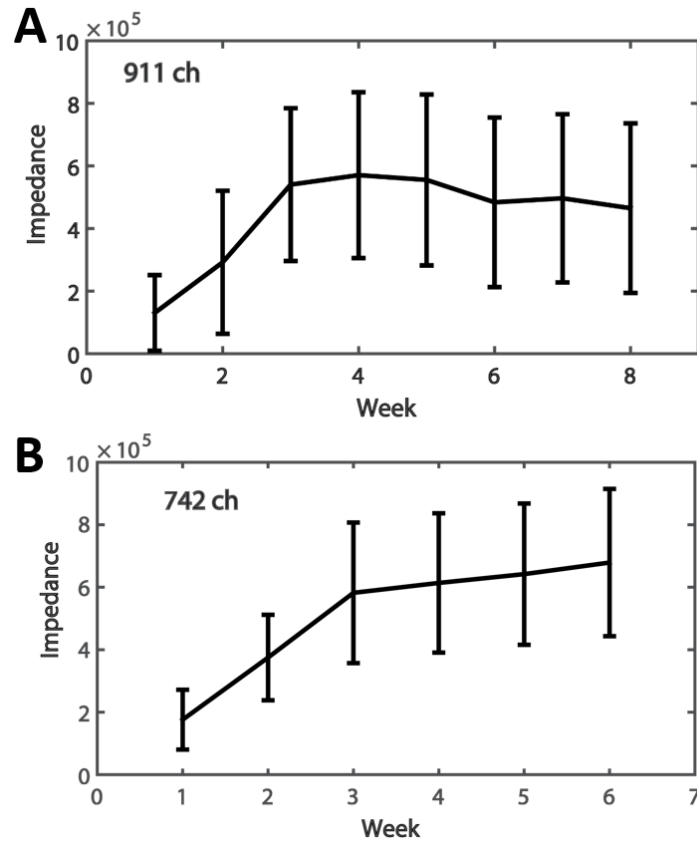


Fig. 35. Electrical characterization of implanted NET electrodes. A, Impedance of 911 densely implanted electrodes in visual cortex as a function of time. B, Impedance of 742 implanted electrodes in distributed cortical regions as a function of time. Error bars denote the SD.

The noise level and average signal strength were monitored for both implants weekly over two months. The average amplitude recorded from the densely implanted array experienced a slight drop in first month, and remained stable for the rest of the monitoring period, while the signal amplitude of the distributed array remained stable until the end of the experiment (Fig. 36A and B). The signal to noise ratio remained stable over the course for both cases (Fig. 36C and D).

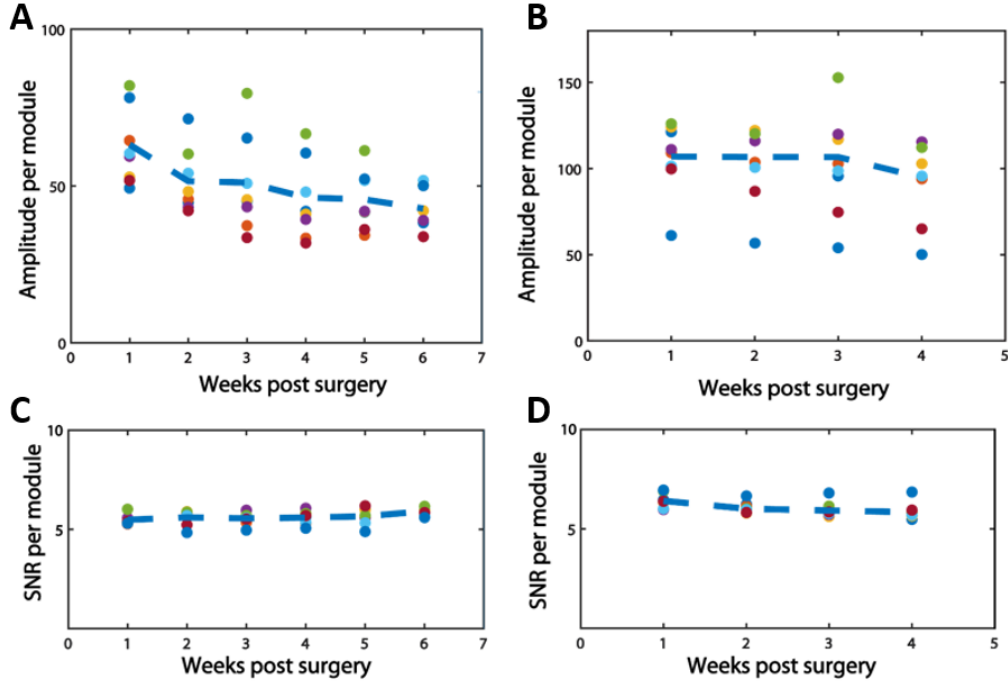


Fig. 36. In-vivo recording characterization of implanted NET electrodes. Average peak to valley amplitude (A and B) and SNR (C and D) of all units recorded by 912ch array (A, C) and 742ch array (B, D) as a function of time. Each dot was averaged from one 128-ch module.

The signal stability ensured a reliable recording of thousands of units from the large scale NET arrays over months of implantation. Here we show the changes in the unit yield per 128ch module and per electrode during the two-month monitoring period for both implants (Fig. 37). Mountainsort [26] plus manual curation were implemented here for spike sorting. The densely implanted array yielded an average of 0.7 units per electrode while the distributed array yield 1.2 units per electrode. These numbers were on par with the conventional silicon probes, but in contrast, there was no significant sign of degradation during long-term recordings. The above results confirmed a stable neural recording interface and laid the foundation for the chronic stable and large-scale electrophysiological recording in a number of different scenarios.

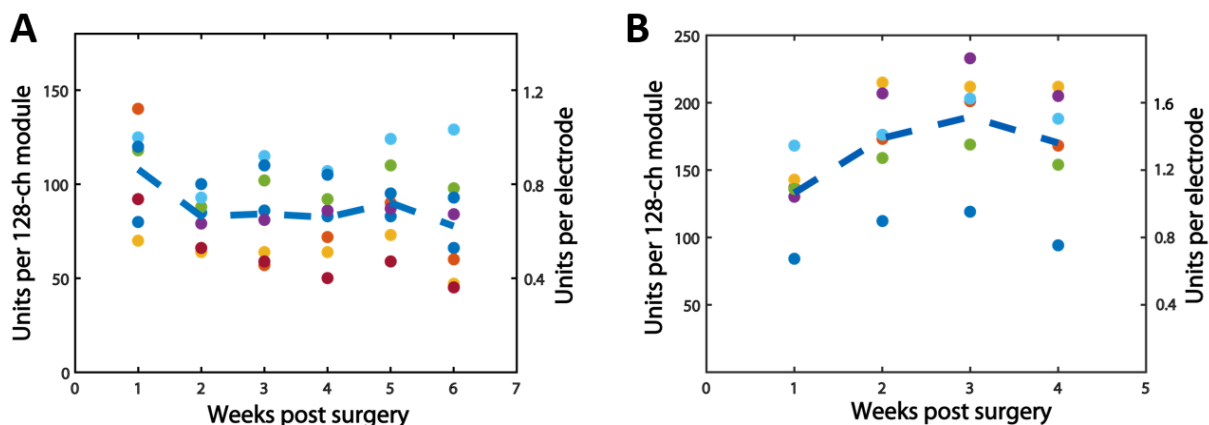


Fig. 37. Unit yields for all electrodes from the 912ch array (A) and 742ch array (B) as a function of time per 128-ch module (left) or per electrode (right) over time.

Through the locally dense arrangement of the NET array, a volumetric recording of a large number of neurons in the mouse visual cortex with an unprecedented level of details was achieved. Specifically, eight layers of the 128ch cortex module (Fig. 32A) were implanted sequentially into the mouse visual cortex at a layer-to-layer spacing of $150\ \mu\text{m}$. The total of 1024 contact sites was dispersed in $\sim 1\ \mu\text{L}$ of brain volume with approximately $150\ \mu\text{m} \times 150\ \mu\text{m} \times 60\ \mu\text{m}$ pitches in the lateral, radial and axial directions between each site. μCT image shows the entire electrode array in the brain and confirmed the nearly uniform distribution of all contacts throughout the measurement volume (Fig. 38).

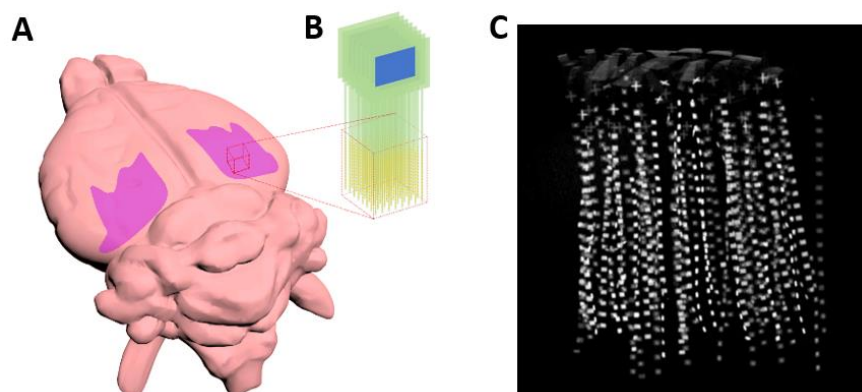


Fig. 38. A. Schematic of the mouse brain with targeted implantation region highlighted. B. The schematic of the 1024-ch NET array highlighting the spatially dense arrangement of the electrode contacts in the small implantation volume. C. 3D-reconstructed μCT scan of the implanted 1024-ch NET-cortex array in brain.

Average 650 units were recorded by the array in one hour recording session in the presence of visual stimuli from awake head-fixed mouse, which represents approximately 5% of the active neuronal population in the recording volume [27]. The number of resolved units remained stable for two months post initial recording despite a slight decrease in the first two weeks. (Fig. 39).

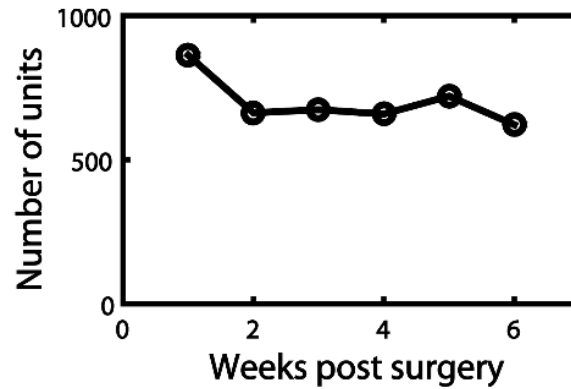


Fig. 39. Number of units recorded from the densely implanted arrays in Fig. 37C as a function of time.

Among all units, 20% of them were modulated by visual stimuli and showed strong receptive field response. This was indicated by a higher firing rate when the bright grating stimuli was displayed in front of the animal in contrast to the uniform gray background when the stimuli was off. About 10% of the units that had receptive field response showed directional selectivity. A representative unit that had strong selectivity for 30 ° grating was shown in Fig. 40. The ratio of the firing times during the stimuli on and off was calculated as on/off ratio.

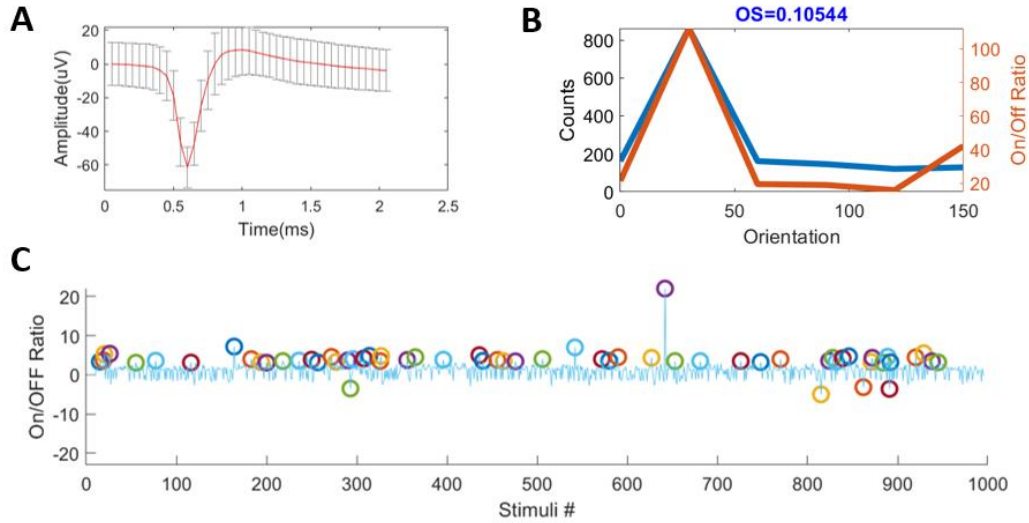


Fig. 40. A representative units that shows directional selectivity. A, Averaged waveform of this unit. B, The sum of counts for on/off ratio over 3 at different orientations. C. The on/off ratio for every stimuli during 30-minutes experiment. The point with on/off ratio higher than 3 was marked as a circle.

All units extracted from a one-hour recording session were individually plotted as a sphere color-coded by its average spike amplitude and superimposed on the contact site that the largest amplitude of the waveform was recorded from (Fig. 41A). Temporal spiking information can be used to generate a real-time firing sequence in which the active neurons were highlighted for every event to visualize the spatiotemporal pattern of firing. Also, the propagation of brain waves at different frequency bands can be visualized in space by extracting local field potential recorded from each contact, superimposing and interpolating based on the location of each contact (Fig. 41B).

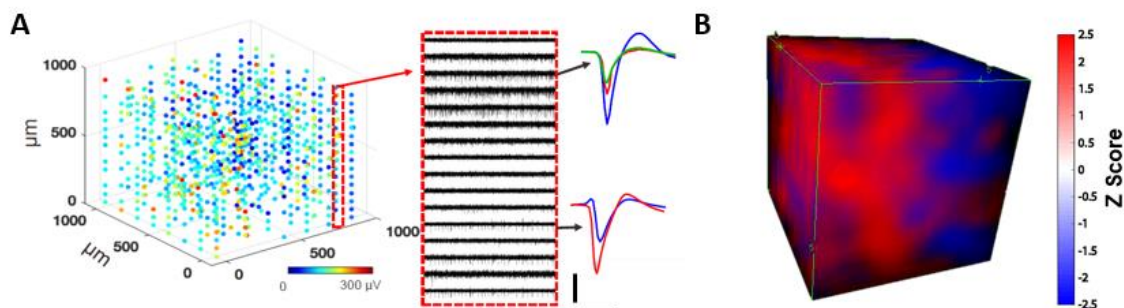


Fig. 41. Volumetric dense recording in awake head-fixed mice. A, Reconstructed units plotted at the locations of the contacts that recorded them. The spike amplitude was color coded. 5-s recording traces from one shank (16 electrodes) were shown in the dashed box. Representative averaged waveforms from two electrodes were plotted. B, Reconstructed spatial distribution of local field potential at 4-8Hz Theta band in the recording space with intensity color coded. The z-score of the 4-8 Hz LFP signal was superimposed and interpolated based on the location of each contact. Scale bar: 100 μ V (vertical) and 1 ms (horizontal).

Through the large-scale distribution of NET arrays, simultaneous monitoring of multiple distributed brain regions across the mouse neocortex has been achieved. Specifically, 6 layers of the 128ch cortex module shown in Figure 1A, a total of 742 contacts, were implanted across the neocortex on both hemisphere, including 142 contacts in prefrontal cortex, 200 contacts in the primary motor cortex, 200 contacts in the barrel cortex, and 200 contacts in visual cortex (Fig. 42A). The schematic shows the distribution of all the contacts in the brain (Fig. 35B). In average, 1100 units were recorded from the array during each of one-hour recording sessions from all these regions, and the number of sorted units remained stable after the improvement in first two weeks (Fig. 42B).

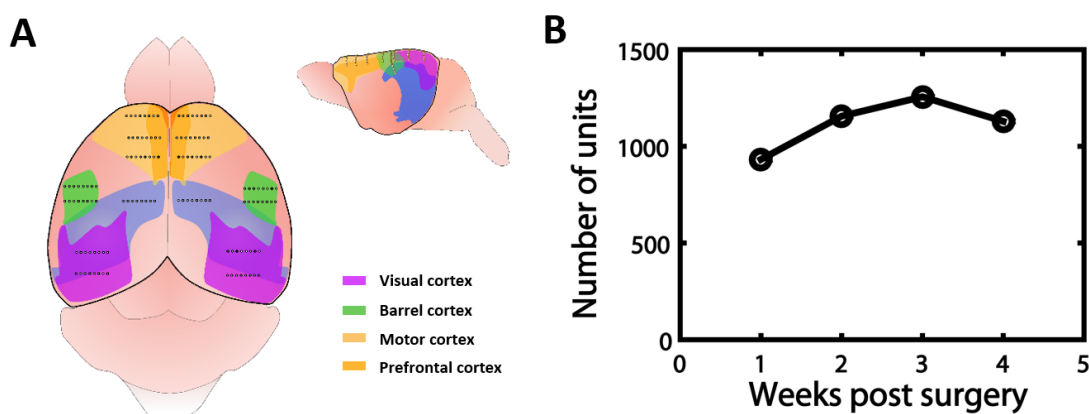


Fig. 42. A. Schematic of the mouse brain with targeted regions highlighted. B. Number of units recorded from the distributed NET arrays as a function of time.

In addition to the success in the cortex, we also achieved large-scale measurements from the hippocampus of free-moving rats with chronic stability. Specifically, four layers of the 128ch hippocampus module shown in Fig. 32B were implanted into the hippocampus at one hemisphere of the rat brain at a spacing of 300 μm from layer to layer. That is a total number of 512 contacts. With the insertion speed as slow as 10 $\mu\text{m}/\text{s}$ and the guidance of simultaneous recording, we precisely targeted the probe into the CA1 cell layer, and this is confirmed by the detection of a slow gamma band during sharp wave ripples and their spatial distribution among contacts. Majority of the units were recorded from multiple consecutive electrodes on each shank (Fig. 43). This is consistent with the spatially dense arrangement of the CA1 cell layer.

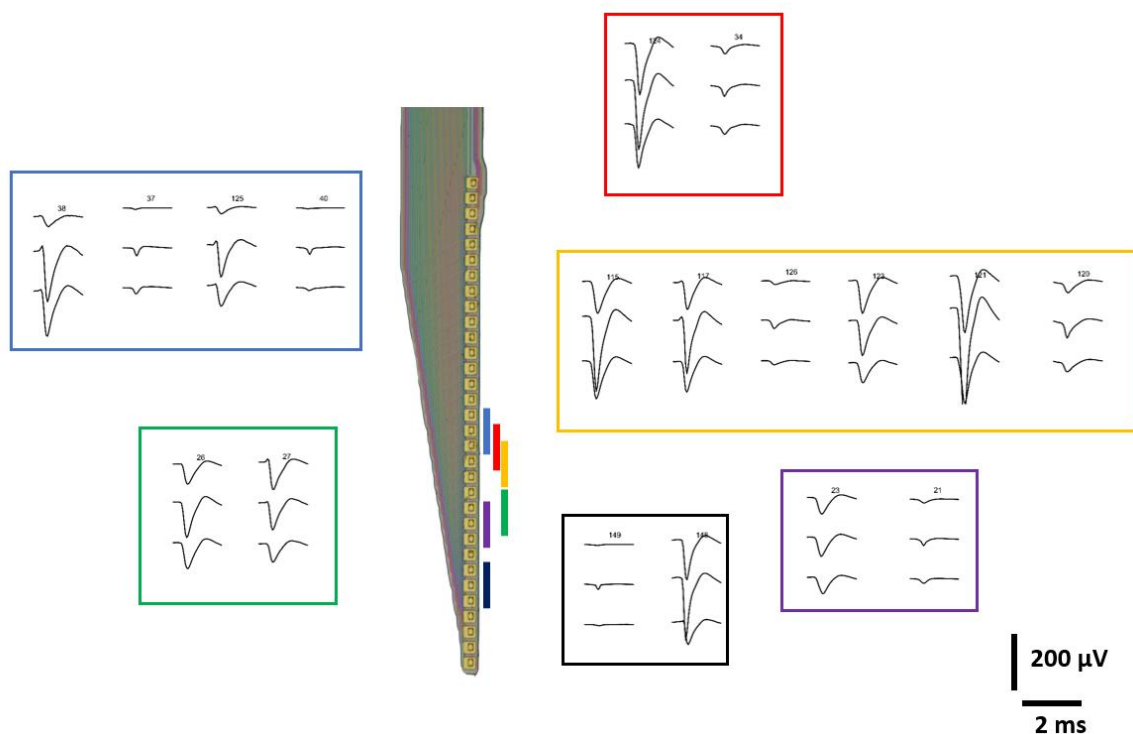


Fig. 43. Spike waveforms of the units recorded by one shank (16 electrodes) in the hippocampus of a freely-moving rat.

More than 100 units were recorded from the array during each of one-hour recording sessions. Among them, 50% had the characteristic of the pyramidal cells [28]. To show the

stability of recording, daily measurement was conducted 1 month after the implantation for a week, 10% of the total units could be stably monitored for the entire experiment duration (Fig. 44).

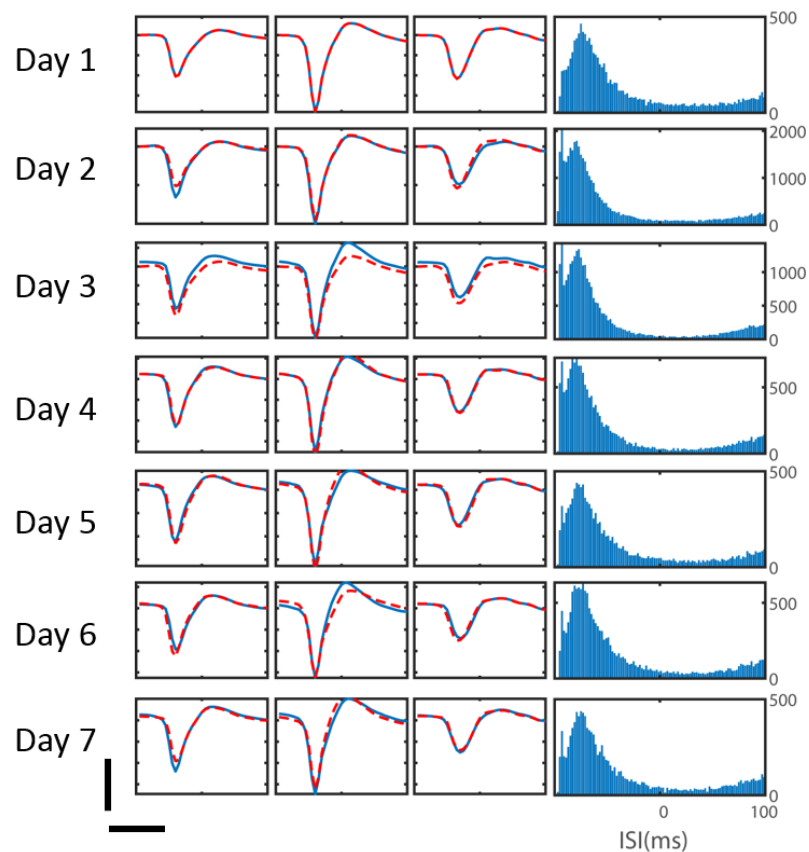


Fig. 44, Waveforms of one representative stable unit recorded daily for a week and histogram of their interspike interval (ISI). The waveforms are isolated and averaged from 1 hour recording session. Vertical bar, 200 μ V; horizontal bar, 1 ms. The waveforms from the previous day were plotted in dashed line for the ease of comparison.

It was found that 20% of the total units had encoded place fields. The place field of one representative cell was presented (Fig. 45) by plotting their firing rate map with respect to the recording arena.

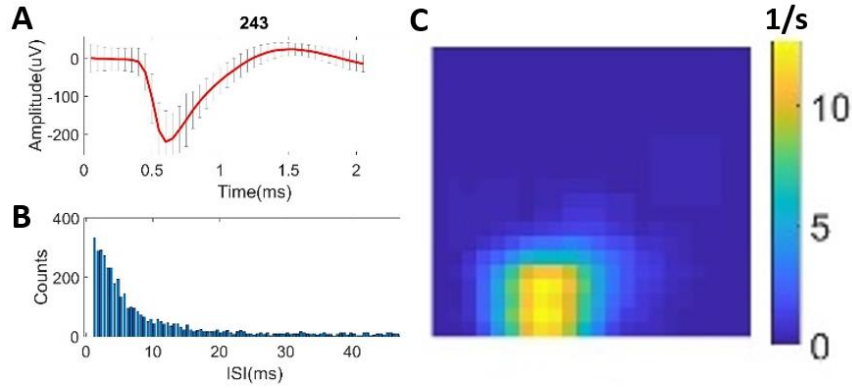


Fig. 45. The mapped place field of one representative cell. Averaged waveform (A) and the interspike interval (B) of the unit. C. Spatial map of the recording arena color coded by the local firing rate of the unit.

4.3. Discussion

The system we demonstrate here features flexibility from multiple aspects including electrode design, recording system and the surgical approaches. Different electrode patterns can be tailored to various local neuronal structures to optimize recording performance. The modular headstages allow for the selective implantation of individual probes and implementation of multiple probes at distributed locations according to specific research needs. The light-weight and compact design of the device back end permits simultaneously large-scale measurement in free-moving, unrestricted animals. Finally, the hybrid implantation approach allows researchers to balance the time efficiency achieved by PEG assisted parallel insertion and the insertion precision improved by loop-hole engaged slow insertion to ensure the best surgical outcome.

The NET platform has also shown remarkable extracellular recording performance, including long-term stable recording from thousands of channels with unit yield on par with the current state of the art electrode techniques, and at time of writing a record number of resolved units with implanted electrodes to the author's knowledge. Through demonstrations such as high-density volumetric mapping and large-scale distributed recordings in the neocortex and subcortical structures on both mouse and freely moving rats, this system proves itself a possible solution for studying challenging neuroscience problems at various spatial and temporal scales, such as the simultaneous information processing across the local and global, the superficial and deep circuits under specific brain functions, the massive neural computations within the nearby

nodes, and the circuit plasticity subject to adaptive behaviors over months or years, etc. The marked high bandwidth and long-term stability of the platform also highlight the advantages of the NET platform for applied neuroscience, such as neural prostheses and brain-machine interfaces.

Future research will optimize and test this platform on more complex animal models such as non-human primates (NHP) in order to increase its clinical relevance. In addition, density of contacts can be further increased by using electron beam lithography to resolve neural circuits to a greater degree of details, although it requires further development in miniaturization of the back end electronics and data acquisition techniques at the same time.

4.4. Methods

NET array fabrication and assembly

The NET-cortex array consists of a total of 128 $25\ \mu\text{m} \times 25\ \mu\text{m}$ gold contacts, distributed over eight shanks. The shank spacing is $150\ \mu\text{m}$ from the center to center. Each shank consists of 16 contacts at a pitch of $60\ \mu\text{m}$. The NET-hippo array consists of 128 $15\ \mu\text{m} \times 15\ \mu\text{m}$ gold contacts over four shanks spaced $300\ \mu\text{m}$ center to center with 32 contacts per shank at a pitch of $15\ \mu\text{m}$. The NET-oversampling array consists of 128 $20\ \mu\text{m} \times 20\ \mu\text{m}$ gold contacts over four shanks spaced $300\ \mu\text{m}$ center to center, each shank contains 32 densely spaced contacts in two neighboring rows at a pitch of $15\ \mu\text{m}$ both horizontally and vertically, with 16 electrodes on each row.

All these arrays were fabricated with a specialized microfabrication method similar to what was previously reported [19, 23, 29]. It has a multilayer structure with interconnects and electrodes patterned on different layers and connected through ‘via’ holes to reduce the lateral dimension. The probe was fabricated on a nickel metal release layer deposited on a silicon substrate. Micro-trenches were fabricated on top of the probe substrate to help align the guiding wire to the probe to facilitate assembly and confine the lateral movement during wire retraction. To minimize the size outside the brain, a $500\ \mu\text{m}$ -pitch ball grid array was patterned on the back of the probe and used to connect the electrodes to a $100\ \mu\text{m}$ -thin customized flexible printed circuits (FPC) by soldering, and the FPC was connected to a single 128ch headstage module for data acquisition. Before assembly, the probe was released from silicon substrate and coated with PEDOT through electroplating. The probe was then temporarily attached to the electrochemically sharpened tungsten wires with polyethylene glycol (PEG m.w. 35000) through a manual process [25] and

ready for implantation. For implantation that requires slow insertion speed, the tungsten microwires were engaged into the loop-hole design at the tip of the probe, and followed by applying a thin layer of PEG between the probe and the microwires to stabilize the assembly. The diameter of the microwires in both cases are 20 μm .

Animal and surgical procedure

Wild-type male mice from two different strains (C57B6 and CD1) and male rat (Sprague dawley) were used in the experiments. The animals were housed at the Animal Resources Center at the University of Texas (UT) at Austin (at a 12-hour light/dark cycle at 22°C, with food and water ad libitum).

For locally dense implant (8 module, 1024 channel) shown in Fig. 6, wild-type male mice (C57B6, 12 weeks old) were implanted with NET arrays that were targeted at primary visual cortex (V1, 1.5-3.5 ML, 1-3 AP, 0-1 DV, arrays were implanted normal to the brain surface). For distributed implants shown in Figure 4 (6 module, 768 channel), wild-type male mice (CD1, 12 weeks old) were implanted with NET arrays at a variety of targets including prefrontal cortex (PrL, 0.1 – 0.8 ML, 1.5 – 2.5 AP, 0.5 – 1.5 DV), motor cortex (M1, 0 – 1 ML, 1 – 2 AP, 0 – 1 DV), barrel cortex (S1BF, 3 – 4 ML, -1 – 2 AP, 0 – 1 DV) and primary visual cortex (V1, 2 – 3 ML, -3 – -4 AP, 0 – 1 DV), with all implanted normal to brain surface. For large scale implants at deep structure (4 module, 512 channel) shown in Figure 5, wild-type male rats (Sprague dawley, 12 weeks old) were implanted with NET arrays that were targeted at CA1 layer of the hippocampus (CA1, 2 – 3 ML, -3 – -4 AP, 2 – 3 DV, arrays were implanted normal to the brain surface).

The animal was anesthetized using isoflurane (3% for induction and maintained at 1 to 2%) in medical-grade oxygen. The skull was exposed and prepared by scalping the crown and removing the fascia and then was scored with the tip of a scalpel blade. The targets were then marked and titanium set screws (0-80) were placed around the perimeter of the exposed skull. Craniotomy was performed with a surgical drill over the target area. The skull was cleaned with PBS saline and allowed to dry before implantation. Durotomy was only performed for rats. The exposed brain was then cover with gel foam to keep the moisture.

NET arrays attached to the tungsten microwires were then inserted into the brain with stereotaxis [25]. A digital encoder (Digital lab standard, Stoelting Co.) was used to measure the

three dimensional coordinates. For targets at cortex, probe-wire assembly achieved by PEG were inserted at a speed of 1-3 mm/s. For targets at hippocampus, probe-wire assembly achieved by loop-hole engagement were quickly inserted to the depth about 200 μm to facilitate penetration of the brain surface, and then slowly advances to the targeted depth at a speed of 10 $\mu\text{m}/\text{s}$. Once at the final depth, the artificial cerebrospinal fluid (ACSF) was dripped onto the brain surface and the microtrenches to dissolve the PEG (30 seconds). The tungsten microwires were then slowly retracted individually with a surgical tweezer, leaving the probe in tissue. Insertions were repeated for all targeted locations.

After all NET arrays were affixed, a thin layer of silicone gel (Dow-Corning 3-4680) was applied to seal the craniotomies, and Kwik-Sil adhesive (World Precision Instruments) was used to fill up the gaps between the NET carrier chips and provide support for them. After the skull was cleaned and dried, a layer of cyanoacrylate was applied over the skull. A thick layer of C&B-Metabond (Parkell Inc.) was applied over the cyanoacrylate and the Kwik-Sil adhesive to cement carrier chips to the skull. For mice, a custom-made head plate was attached to the skull using extra cement. For rats, addition dental acrylic was applied around the carrier chips to reinforce mounting of the array. The modular headstages were connected to the FPCs, and enclosed by a 3D printed PLA cover box.

All procedures complied with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the UT Institutional Animal Care and Use Committee.

Impedance measurements and neural recording

In-vivo impedance of each module was measured once a week with Intan 128-channel RHD2164 evaluation system at 1 kHz with a bare Ag wire inserted into the brain as the grounding reference. The electrophysiological data was acquired with SpikeGadgets 1024-ch modular headstages at sampling rate of 20 kHz. The recording was performed from awake head-fixed mice on a custom-made running wheel, or the freely-moving rats in a customized recording arena

Visual stimulation

The visual stimulation was conducted on awake, head-fixed mice with implant in primary visual cortex. To measure the orientation selectivity, the animal was placed in front of a screen

monitor (Brand and model) where visual stimuli were generated in real-time using Psychtoolbox. The center of the monitor was positioned at the center of the eye of the mouse. The screen has dimension of 45cm x 35 cm and was viewed at a distance 15 cm, subtending 30 x 20 degree of visual angle. Gratings with 6 orientations equally spaced from 0 to 150° were employed as visual stimuli. The spatial frequency of the gratings was 5 cycles per degree with 100% contrast. Gratings were presented statically for 1s each followed by a uniform grey for 1s. The mean brightness of the gratings and grey are equal. Data recording started after stimuli run for 10mins and then continued for another 50 mins. A TTL pulse was generated using an Arduino at each stimulus transition and sampled by the SpikeGadgets system, providing the synchronization between visual stimulation and recording data.

Place field mapping

The place field mapping was conducted on free moving rats with implant in the hippocampus CA1 layer. Rats were placed in the recording arena of size 60 cm x 60 cm since day 7 after surgery and were allowed to free foraging. A recording cable was connected to a commutator and a tracking camera mounted 50 cm above the center of the arena transferring data to SpikeGadgets system. Data recordings were started when clear spikes were identified and then continued for 1 hr without interruption. The firing-rate maps were calculated by summing the total number of spikes that occurred in a location bin ($2 \times 2 \text{ cm}^2$) divided by the time the animal spent in that bin. The firing rate of bins where the rats were absent at all times were assigned null. The firing-rate maps were smoothed using a mean filter over a square of size 5-by-5 bins. For all data, a velocity filter was used to exclude periods when the rats had speeds below 5cm/s.

Spike sorting

Spike sorting was performed using Mountainsort [26]. Common median referencing [30] was applied to the raw data from each single shank to reduce the common mode noise such as motion artifacts, etc. The detection threshold was set to 4 times of standard deviation and the adjacency radius was set to 100 μm . A single unit was identified based on the criteria that the fraction of spikes with inter-spike intervals under 2ms were less than 1% of the total firing [31].

Data processing for Local field potential

To process the local field potential data, each channel was first filtered into N different frequency bands. Filtered data is standardized by subtracting channel mean and subsequently divided by channel standard deviation. Z-scored channels were clamped to be within -2.5 to +2.5. Each cluster is manually assigned to the corresponding recording channels on a given shank, the centroid location of each cluster was computed to represent electrode locations. A 3D volume fully covering the boundary of electrode coordinates having the size of X-by-Y-by-Z was interpolated from the original 1024 channel LFP z score at different time point separated by 4ms. Inspired by Visbrain, volume rendering is performed across time in Vispy [32].

Histological analysis

The mice were perfused intracardially using oxygenated aCSF at 4 °C, then with a 4% paraformaldehyde solution in 0.02 M PBS. 30% sucrose/4% paraformaldehyde solution was used to soak the brains overnight. Brain tissue was sliced using a Leica CM1950 cryostat (Leica Microsystems) into 20 µm slices, which were then washed for three 5min sessions before incubated for 30min in a sodium citrate solution (85 °C to 95 °C, 0.01 M in H₂O) for antigen retrieval. The slices were washed again for three 5min sessions, incubated in a blocking solution, permeabilized (0.5% Triton X-100 and 10% normal goat serum (Sigma-Aldrich) in PBS) for 3h at 25 °C, washed again for four 5min sessions, then incubated with the onestep fluorophore-conjugated antibodies at 4 °C for 24 h. To immunochemically stain neurons, Alexa Fluor 568-conjugated anti-NeuN antibody (ab207282, abcam) was used.

MicroCT scanning and image reconstruction

MicroCT images shown in Fig. 1I were acquired by scanning of the upper body of the perfused rat after fixation with 4% paraformaldehyde solution (Xradia, 10X objective, 90kV) and reconstruction of all the slices (Xradia Reconstructor, Voxels are 47.6 µm). Images in Fig. 3A, 4B, 5A were acquired by scanning of the dissected brain tissue (NSI scanner, 180kV) and reconstruction (Xradia Reconstructor, Voxels are 2.19 µm).

5. NET enabled multifunctional neural probes³

5.1. Introduction

Interrogation and manipulation of specific neurons in behaving animals are crucial to improve the understanding of the highly convoluted neural circuits. These studies can be enabled by implanted multifunctional neural probes that combine electrical recording in awake animals with modulation functionalities such as optical stimulation [1-5] afforded by optogenetics [6], or controlled infusion of pharmacological agents [7]. Previous strategies of fabricating multifunctional probes include attaching conventional electrical probes to optical fibers [3], and stacking different functional units on rigid [8, 9] or flexible substrates [10]. These approaches significantly increased probe dimensions to accommodate additional functionalities, which produces additional tissue damage upon implantation. Alternative approaches such as accommodating recording and microfluidic channels in fibers successfully integrated multimodality, but typically afforded one or only a few recording sites only at the probe tip [1, 5, 11]. As the effect of the stimuli is typically not localized [12], better understanding of neural activities often demands simultaneous recording of a group of neurons with spatial distributions determined by the problem of interests. Therefore, a new approach that conveniently offers versatile distribution of electrical recording sites in multifunctional neural probes is highly desirable.

Here we present a strategy to create multifunctional neural probes by conformal attachment of nanoelectronic coating (NEC) devices on the surfaces of conventional non-electrical probes (Fig. 1). We demonstrated that the NEC devices provided state-of-the-art multi-channel neural recording capabilities, which were easily transferred to a number of widely used non-electrical micro-probes in neuroscience such as optical fibers and micropipettes. Because the NEC devices were ultra-flexible and typically had thickness below 1 μm , they added minimal volume to the

³ Zhao, Z., Luan, L., Wei, X., Zhu, H., Li, X., Lin, S., Siegel, J., Chitwood, R., Xie, C., *Nanoelectronic Coating Enabled Versatile Multifunctional Neural Probes*. Nano Letters, 2017. **17**(8): p. 4588-4595. My contribution to this work includes conceiving and designing the study, designing and fabricating the NEC devices, assembling, characterizing the multifunctional probes and performing the surgical implantation, performing in vivo recording, analyzing the data and writing the manuscript.

host probes and enabled multifunctional neural probes with diameters as small as 30 μm . We further showed that these devices simultaneously recorded spatially resolved unit activities and stimulated neural activities through optogenetics and drug infusion in a mouse model. By combining conventional, off-the-shelf tools with NEC devices that can be mass-fabricated by planar photo-lithography with high resolution, we demonstrated a new approach for low cost, versatile multifunctional neural probes.

5.2. Results

In the efforts of developing NEC enabled multifunctional probes, the thickness and width of the NEC devices are the key design parameters. First, in order for NEC devices to readily conform to the micro-curvatures of these probes with negligible increase in the total volume, the NEC devices need to be sufficiently thin and flexible, which imposes stringent requirements on the thickness and material choice for the NEC devices. In addition, because the circumference of the hosting conventional probes spans from tens to a few hundreds of microns (Fig. 46), the width of the NEC devices and the electrode array size change accordingly. We chose to use SU8 photoresist as the insulating layers and adapted an unconventional substrate-less, multi-layer layout similarly to previously reported [13, 14]. We incorporated high level of integration to enable multi-channel recording with diverse design patterns to accommodate various applications.

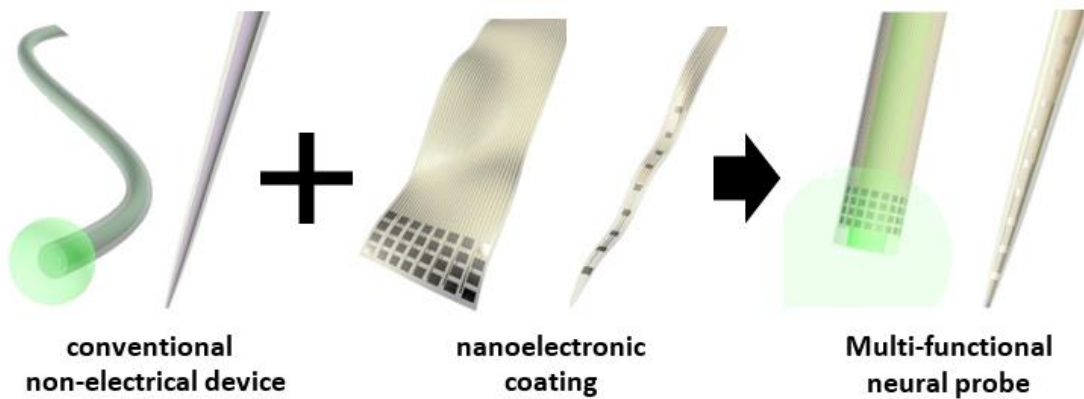


Fig. 46: Schematics of the NEC device enabled multifunctional probe. NEC devices were fabricated separately and attached onto the surface of conventional optical fibers and glass pipettes.

Figure 2A – E highlights a collection of as-fabricated NEC devices on substrate and demonstrated the versatile design of electrode arrays. NEC-a (Fig. 47A) hosts a linear array of 8 electrodes with minimal width of 30 μm and is designed for coating microscale probes such as micropipettes. The other NEC devices are designed for larger host probes. NEC-b and -c (Fig. 47B, C) have closely spaced electrode arrays (40 μm center to center separation) with overlapping detection range, which are designed to detect and resolve a cluster of neurons near the electrode array [15, 16]. NEC-d and -e (Fig. 47D, E) host multiple linear arrays along the probe, which can be used to study neural activity across cortical layers and beyond. All NEC devices have the same with total thickness of and below 1 μm , which is mainly determined by the thickness of insulating SU-8 layers.

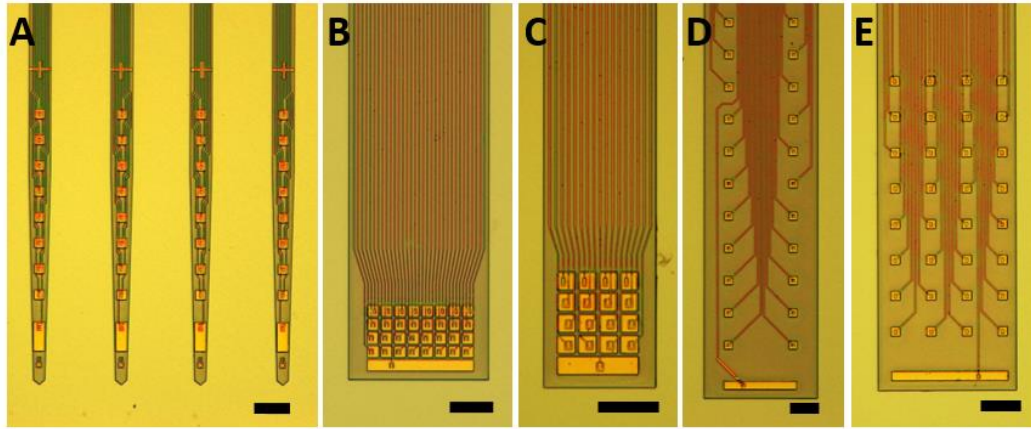


Fig. 47: Fabrication of NEC devices. A – E: photographs of as fabricated NEC devices type a – e on substrate, showing versatile design patterns for broad applications, including linear array of electrodes(A), 2D arrays of 32(B) and 16(C) closely spaced electrodes, 2D arrays of 2 $\times$ 16 electrodes(D) and 4 $\times$ 8 electrodes(E). Scale bar: 100 μm .

To facilitate attaching onto host probes, the NEC devices were released from substrates by etching off the sacrificial layer (Methods). The submicron thickness led to ultra-flexibility that can allow microscale deformation without damaging the embedded electronics (Fig. 48A, B). Inspired by Langmuir-Blodgett coating method [17], we designed a surface tension assisted mechanism [18, 19] to attach the NEC devices on the surface of host probes. As illustrated in Fig. 3C, this coating process was achieved by aligning the NEC device with the host probe in water and slowly lifting both devices across the water-air interface while keeping them in contact. Surface tension forced the flexible NEC devices to form conformal adhesion on the host probe's curved surface (Fig.

48D). We chose distilled water as the medium liquid for its high surface tension ($\gamma = 72.0 \text{ mN/m}$ [20]) and minimal residue after evaporation.

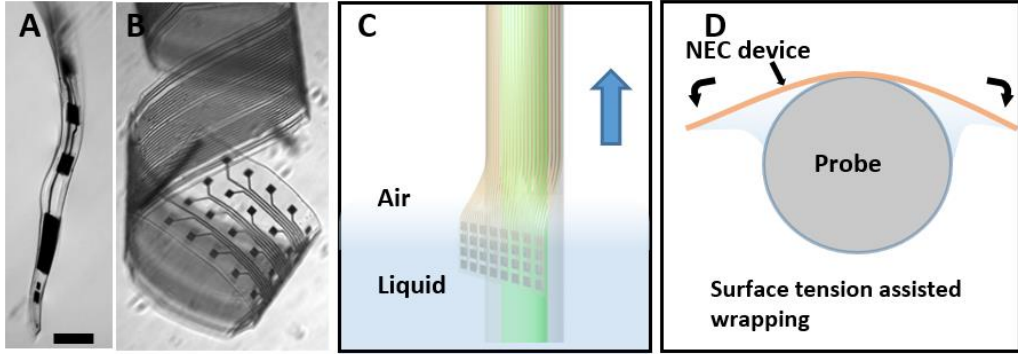


Fig. 48: Assembly of NEC devices enabled multifunctional probes. A, B: released NEC device type-a (A) and type-e (B) in water, showing the ultra-flexibility. scale bar: $100 \mu\text{m}$. C, D: Sketch illustrating that surface tension facilitates wrapping of a NEC device on the surface of a probe as both are pulled out of water.

Surface-tension assisted conformal coating is realized by reducing the surface energy at the liquid-air interface at the cost of increasing the elastic energy when the coating material is deformed. We analytically model the surface tension during NEC device coating by considering an elastic sheet of thickness h , in loose contact with a rigid cylinder of radius R (Fig. 48D). Both the sheet and cylinder are wetted with the same liquid. The sheet deformation reduces the liquid-air area A and therefore the surface energy γA (being the surface tension) at the cost of increasing the elastic energy. At the critical radius R_c , the increase of elastic energy $dE_{el} = B/R_c^2 dx$ equals the decrease of surface energy $2\gamma \cos \alpha dx$ where α is the contact angle of the liquid on the surface which we set to zero

$$R_c = \sqrt{\frac{B}{2\gamma}} \quad (\text{Eq. 1})$$

Where B is the bending modulus, a geometry and material related property describing the resistance of an elastic sheet against bending deformation, which dictates its ability to comply with a curved surface. B is determined by

$$B = \frac{Eh^3}{12(1-\nu^2)} \quad (\text{Eq. 2})$$

where E is the Young's modulus of the elastic material, h is the thickness of the elastic sheet, and ν is Poisson ratio of the material.

Combining Eq. S1 and Eq. S2, we obtain:

$$R_c = \sqrt{\frac{B}{2\gamma}} = \sqrt{\frac{Eh^3}{24\gamma(1-\nu^2)}} \quad (\text{Eq. 3})$$

For NEC devices which is mainly composed of SU-8, We use $E_{\text{SU-8}} = 2.02 \text{ GPa}$ and $\nu_{\text{SU-8}} = 0.22$, and $\gamma = 71.97 \text{ mN/m}$ for distilled water, we obtain the critical diameter $d_c = 2R_c$ at $h = 0.5 \mu\text{m}$ and $1 \mu\text{m}$ to be $24.7 \mu\text{m}$ and $70.1 \mu\text{m}$ respectively. The $d_c - h$ relation is plotted as the boundary between two regimes in Fig. 3e, in which for any h , the probes with diameters larger than d_c are in the “attach” regime. For $h \leq 1 \mu\text{m}$, this “attach” regime covers most conventional brain probes. For instance, optical fibers used in optogenetics studies typically have diameters larger than $200 \mu\text{m}$, which can be coated by all NEC devices demonstrated in the study. In other applications, fibers and glass pipettes may be sharpened with tip diameter around $20 \mu\text{m}$, in which case the NEC devices with $h = 0.5 \mu\text{m}$ will form conformal attachment.

By above analytically modeling the interplay between surface tension at the water-air interface and the bending force of SU-8 films [18, 21], we concluded that the sub-micron thickness of the NEC devices was necessary for the surface tension to surmount the flexural rigidity and facilitate conformal wrapping in micro-scale (Fig. 49).

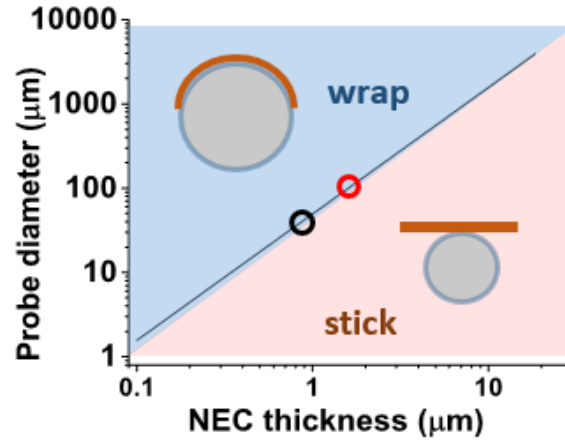


Fig. 49: Color-coded dimension dependence of the surface tension and the bending force of the NEC device, marking two regimes: blue, where the surface tension is larger than the bending force so NEC devices readily wraps on the probe surface; pink, where the surface tension is smaller than the bending force so that NEC devices do not fully comply with the probe surface. Critical probe diameters at thickness $0.5 \mu\text{m}$ and $1 \mu\text{m}$ are marked by open circles. Scale bar: $50 \mu\text{m}$.

A prerequisite of the above model is that both objects are completely wetted by the liquid to maximize the reduction in the surface energy, e.g. the contact angle of the liquid on the surface $\alpha = 0$. To make the surfaces of both NEC devices and target probes hydrophilic, we treated them with oxygen plasma before releasing, which significantly increased their surface energy and achieved contact angle $\alpha < 5^\circ$ as shown in Fig. 48D.

Several NEC enabled multifunctional brain probes were depicted in Fig. 50, including a micropipettes (diameter $< 30 \mu\text{m}$) coated with a NEC-a device to provide a linear array of electrodes along the probe (Fig. 50A), and optical fibers (diameters $150 \mu\text{m}$ to $200 \mu\text{m}$) coated with NEC-c and NEC-b devices (Fig. 50B, C) for different neural recording requirements. We also demonstrated aligning the electrode arrays onto the end of the host probe to provide multichannel electrical recording at the tip (Fig. 49C), which has similar geometry as previously demonstrated multifunctional probes [1, 5, 11].

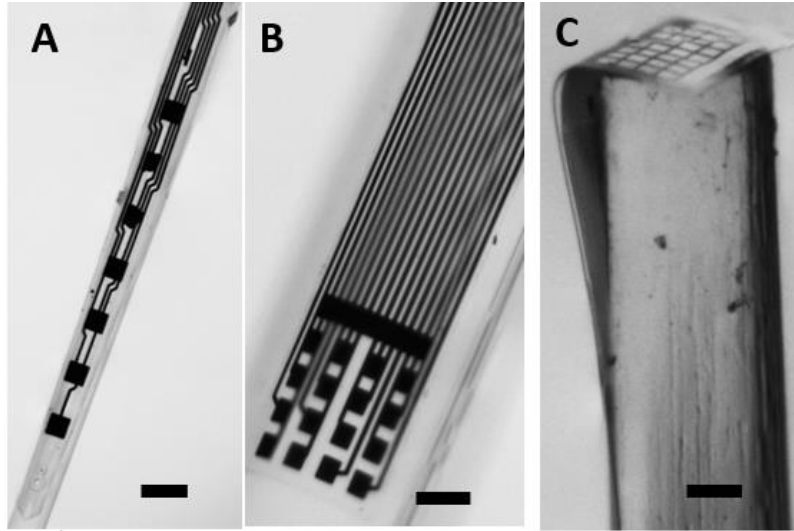


Fig. 50: Images of NEC devices enabled multifunctional probes. A – B: photographs of multifunctional probes made of NEC-a wrapped on a glass micropipette (A), NEC-c wrapped on the sidewall (B) and cross-section (C) of optical fibers. Scale bar: $100 \mu\text{m}$.

The NEC device was held in position by van der Waals interactions after drying in air. The bonding between NEC film and the hosting probe was enhanced by thermal curing (Methods) and was sufficient to survive multiple implantation procedures. In-vitro test showing that the NEC device remained conformal coating on the host probe after extensive insertion tests in 0.5% agarose gel. (Fig. 51)

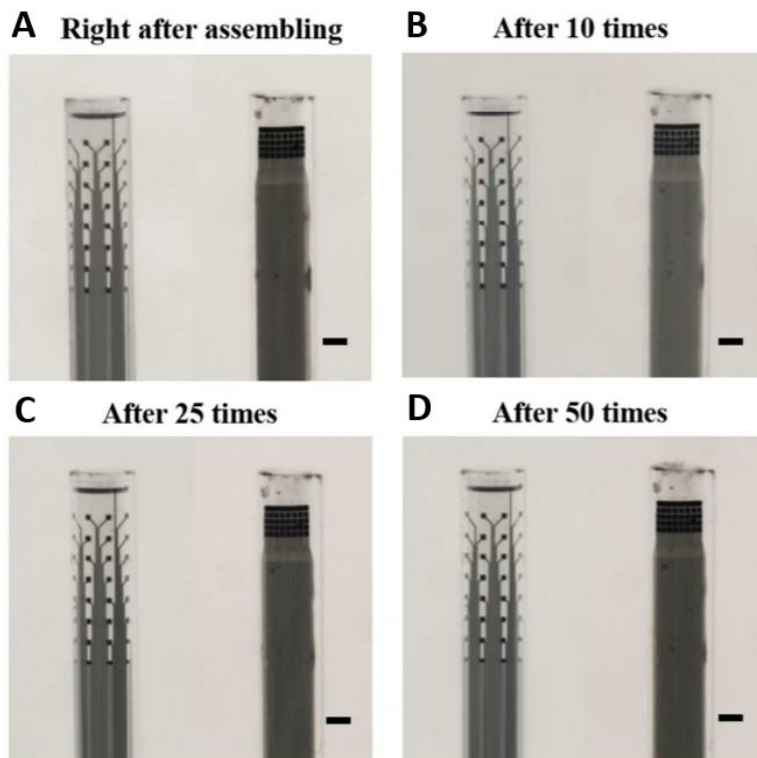


Fig. 51. In-vitro test of NEC devices adhesion. Photographs of two multifunctional probes at immediately after assembling (A), and after 10 times (B), 25 times (C) and 50 times (D) insertion-extraction cycles in agarose gel (0.5%). Scale bar: 100 μm .

To evaluate the electrical stability of the NEC devices, the averaged impedance of 38 electrodes on NEC devices and the number of connected channels were measured before and after wrapping onto hosting probes, as well as after 10, 25 and 50 insertion-extraction cycles in hydrogel. Both the impedance and the yield of connected electrodes remained unchanged during these procedures, showing the mechanical and electrical integrity of the NEC devices after extensive handling (Fig. 52).

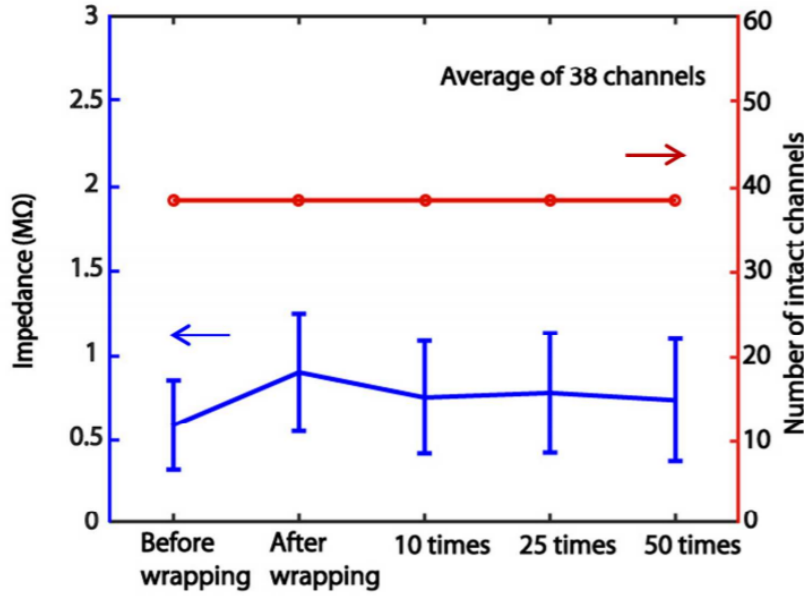


Fig. 52. In vitro measurement of impedance and the number of connected channels with extensive insertion test in agarose gel (0.5%). Error bars show the standard deviation of impedance among 38 electrodes.

To evaluate the electrical recording performance, we implanted NEC enabled multifunctional probes into somatosensory cortex of wild-type mice and recorded from awake, behaving mice while the animals ran on a custom made treadmill similar as previous reported [22] (Methods, an example of post-surgery mice is shown in Fig. 53A). Fig. 53B shows an example recording using a NEC-b device wrapped on an optical fiber with diameter of 250 μm , in which 26 out of 32 electrodes were functional (typical fabrication yield of this type is $\sim 80\%$). We detected extracellular action potential (AP) waveforms from all 26 functional electrodes (Fig. 53C).

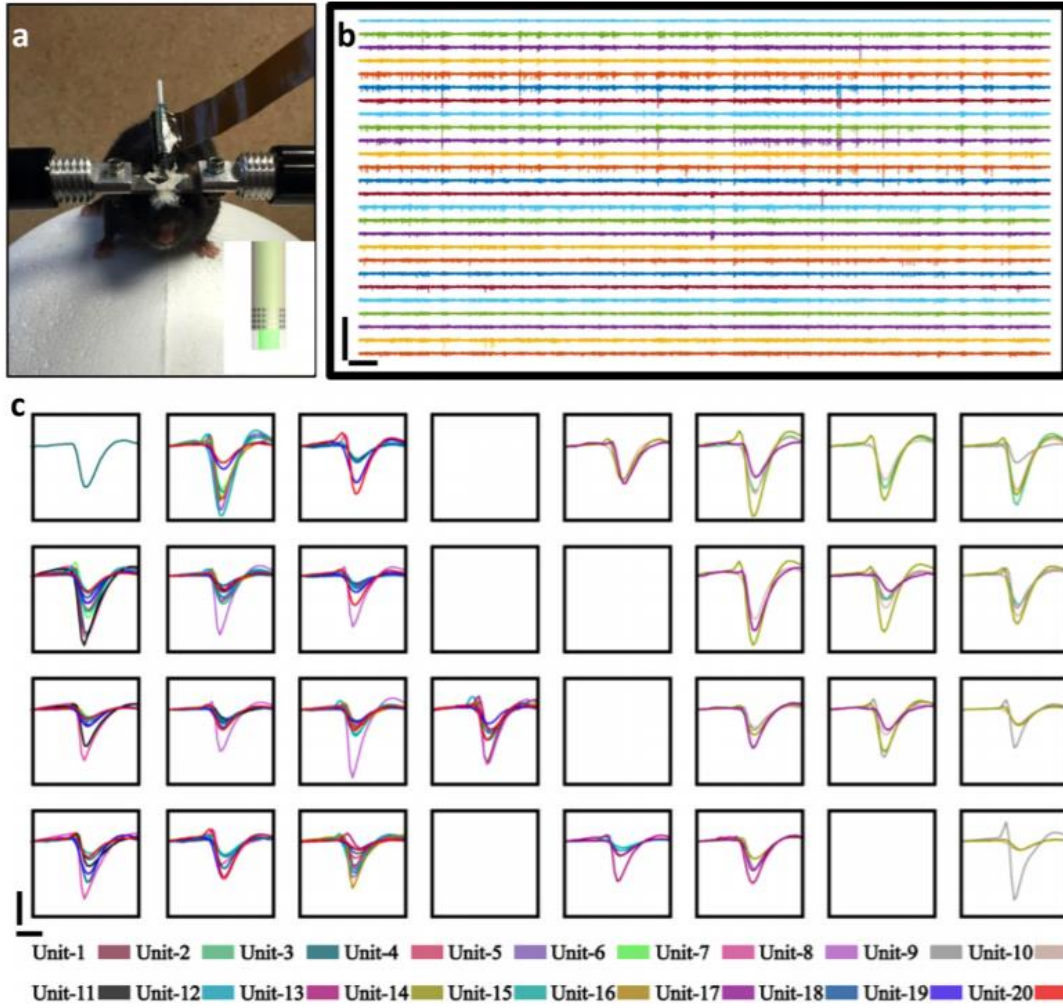


Fig. 53. Demonstration of the NEC device's electrical recording capacity. A: Photo of a mouse on the treadmill for awake measurement. The mouse was implanted with two multifunctional probes made of NEC-c devices wrapped optical fibers. Inset shows the sketch of the implanted probe. B: 5 s recording traces (300 Hz high-pass filter applied) from the 4×8 electrode array (left) of on a multifunctional probe showed in a. Scale bar: 500 μ V (vertical) and 0.2s (horizontal). C: Color-coded waveforms from all 20 sorted single-units plotted on the recording electrodes. Waveforms were averaged from all events recorded in 12 mins. Scale bar: 100 μ V (vertical) and 1 ms (horizontal).

As the electrodes were closely spaced with overlapping detection range, each individual neuron was detected by multiple neighboring electrodes, resulting in temporally correlated spikes from multiple recording channels. As a result, the yield and accuracy of spike sorting was greatly increased, because the identification of single units was realized not only by the characteristics of the spike waveforms but also by the spatial-temporal correlation among multiple recording sites [23]. We used a recently developed open-source platform, KlustaViewa [24], which provides semi-automatic spike sorting for large, dense electrode array. We resolved 20 single units, the

waveforms of which were color-coded and showed on the recording electrodes in Fig. 53C (Methods). Taking advantage of multiple recording sites for each unit, we estimated the positions of all sorted units relative to the 2D electrode array by triangulation [25] using the weighted center of the detection electrodes (Fig. 54, Methods). The oversampling capacity also allows for reliable detection and sorting of temporally sparse neural activity which is challenging for individual electrodes [26]. These results demonstrated that the NEC enabled multifunctional probes have electrical recording capacities on par with the state-of-art high-density neural electrical probes [27, 28].

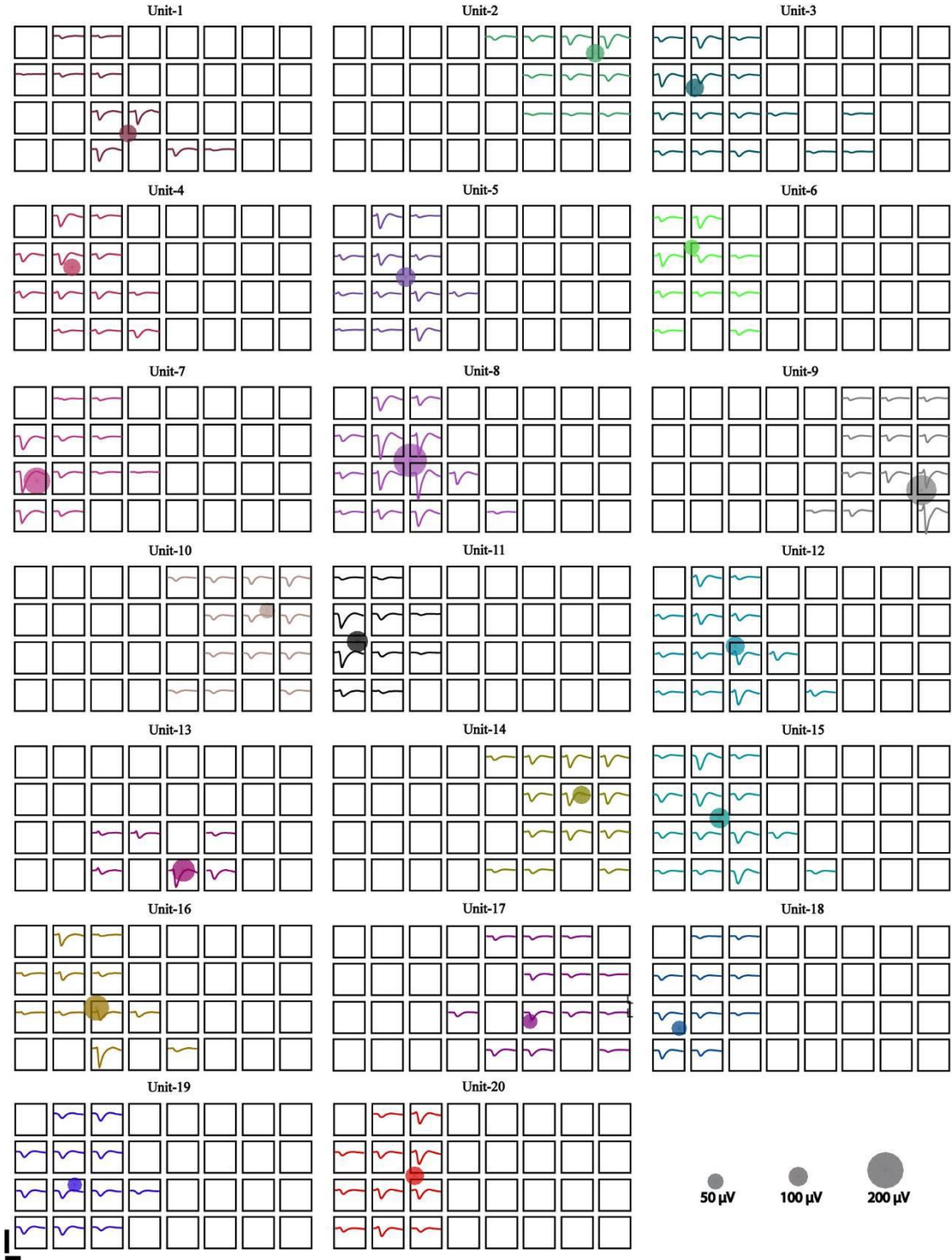


Fig. 54: Waveforms of Individual units (a total of 20) plotted on the recording electrodes, using the same color code as in Fig. 5c. Waveforms were averaged from all events recorded in 12 mins. Scale bar: 100 μV (vertical) and 1 ms (horizontal). Dots show the location of neurons inferred from triangulation where the size of the dots was scaled to the largest peak amplitude in waveforms of the corresponding unit.

We demonstrated the multifunctional neural probes enabled by NEC devices in awake, behaving mice. We delivered optical fibers coated by NEC devices (Fig. 55A) to medial prefrontal cortex (mPFC) of genetically engineered mice Thy1-mhChR2-YFP, where illuminating mhChR2-expressing neurons with blue light (450-490 nm) leads to rapid and reversible photostimulation of AP firing in these cells [29]. By simultaneous optical stimulation and electrical recording, we observed evoked neural activities from multiple electrodes by optical stimuli (Fig. 55B, C). The reliability of the optical stimulation was confirmed by the significant increase in AP firing rate (paired t-test with $p = 0.0007$, $n = 4$) consistently observed both from multiple electrodes (Fig. 55B, 7 ± 2 fold averaged from 4 electrodes) and in multiple repetitions of the duty cycles (10s/10s laser on/off).

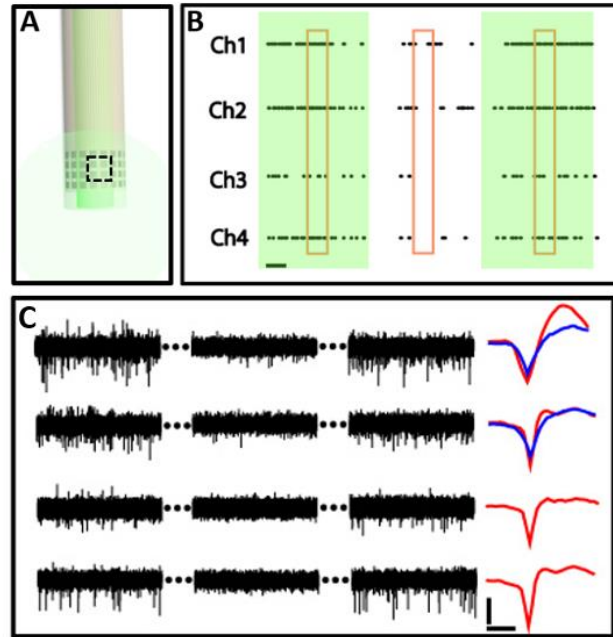


Fig. 55. Demonstration of optical stimulation and recording of the neural activities. A. Sketch of a multifunctional probe composed of an optical fiber and NEC-b device. Dashed box marks the electrodes used in B and C. B: Example recording from 4 channels marked in a implanted in a transgenic optogenetic mouse (Thy1-mhChR2-EYFP) in mPFC. Shade marks the presence of optical stimulation using a 473 nm continuous laser excitation. Dots indicate the occurrence of APs. Box marks the region shown in C. Horizontal bar: 2 s. c: 4 channel recording trace of 2×3 s (300 Hz high-pass filter applied) selected from 10×3 s recording as marked in B. Sorted single-unit waveforms (averaged over 200 spikes) are shown on the right. Scale bar: 50 μ V (vertical) and 0.5 ms (horizontal).

In addition, we observed little change in firing rate when the optical excitation was at 532 nm even at larger power than at 473 nm (Fig. 56), which excludes the possibility of thermal effect and other artifacts.

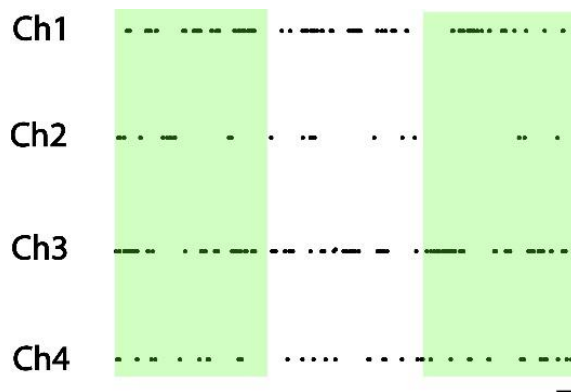


Fig. 56. Example recording from 4 channels implanted in a transgenic optogenetic mouse (Thy1-mhChR2-EYFP) in mPFC. Shade marks the presence of optical stimulation using a 532 nm continuous laser excitation. Dots indicate the occurrence of APs. Horizontal bar: 2 s.

We then tested the drug delivery modality by implanting multifunctional probes in the mPFC of wild-type mice (Fig. 57A). For this purpose we fabricated multifunctional probes from pulled glass micropipettes wrapped with NEC-a, NEC-b, and NEC-d devices (depending on the pipettes' diameter). During awake experiments, we injected CNQX at rates of 5 – 50 nl/s while recording neural activity (Methods). We observed a delayed suppression of firing rates from multiple electrodes (Fig. 57B, C). The firing rate became suppressed ~50 s after the drug infusion began and recovered ~60 s after the drug infusion was stopped. Consistent patterns were observed for repetition of drug infusion in the same mouse and on other mice under the same procedure, confirming the effectiveness of the drug delivery modality (paired t test with $p = 0.003$, $n = 12$).

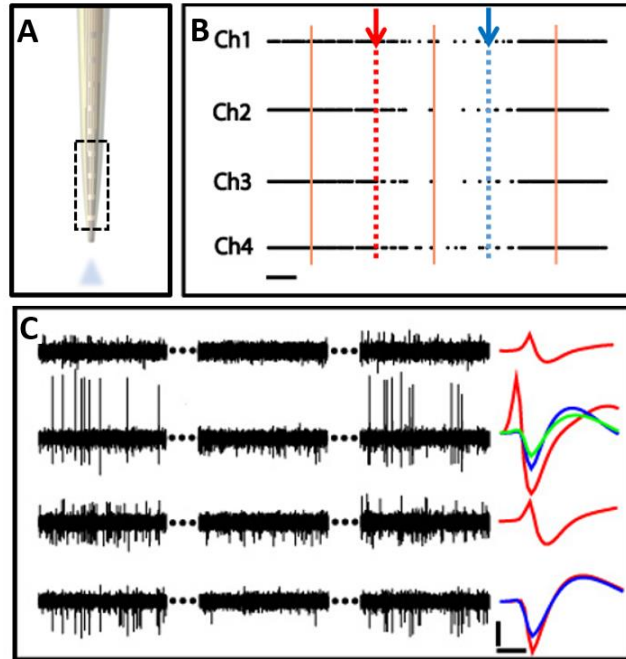


Fig. 57: Demonstration of chemical modulation and recording of the neural activities. A: Sketch of a multifunctional probe composed of a glass pipettes and NEC-a device. Dashed box marks the electrodes used in B and C. B: Example recording from 4 channels marked in a implanted in a wild-type mouse in mPFC cortex. Arrows mark the start and finish of the controlled infusion of CNQX at 50 nL/s. Dots mark the occurrence of APs, showing lagged decrease in firing rate with CNQX infusion. Vertical lines mark the region shown in C. C: 4 channel recording trace of 2×3 s (300 Hz high-pass filter applied) selected from B as marked. Sorted single-unit waveforms (averaged over 1000 spikes) are shown on the right. Scale bar: 50 μ V (vertical) and 0.5 ms (horizontal).

In addition, control experiment was conducted by injecting $1 \times$ PBS at 50 nl/s (Fig. 58), and no inhibition effect was observed.

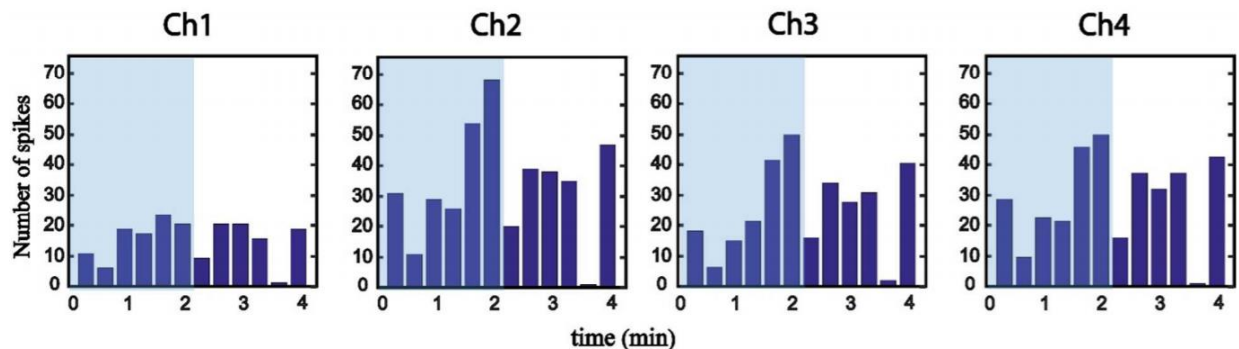


Fig. 58. Histogram showing little firing rate change of individual channels in response of $1 \times$ PBS injection. Shade marks the presence of PBS infusion at 50 nl/s. To exclude the latency effect of drug (60 s for CNQX injection), 120 s recording after PBS infusion is shown.

5.3. Discussion

The diameters of the NEC-enabled multifunctional probes in this study ranged from 30 – 200 μm , and were mainly determined by the host probes [4, 11]. As small implant dimensions are correlated with reduced tissue damage and more stable neural recordings [30], our study provides an approach to improved biocompatibility while achieving multi-functionality. The electrode pattern and the position of the NEC devices on the host probes can be adjusted according to the application to provide customized recording site arrangements, as demonstrated in Fig. 49b,c. The alignment was performed manually in this study with accuracy of 50 μm or less. We note that the surface-tension assisted wrapping mechanism is effective only for a small range of device thickness because the elastic rigidity increase sharply with thickness, e.g. only devices thinner than 2 μm can attach onto a surface with curvature of 100 μm . This stringent requirement precludes similar applications of previous flexible electronic devices with larger thicknesses [4]. The smallest thickness of NECs we have demonstrated is 0.5 μm (Fig. 47g), which can be coated on a probe with diameters less than 30 μm (Fig. 48e).

The NEC devices contain the following three functional components, similar to other multi-channel micro-fabricated neural electrical probes [31]: (i) electrodes, working as local recording sites; (ii) interconnects, metal leads individually connecting the electrodes and the contact pads for external electronics; (iii) insulating protection, a thin layer of insulating material protecting the interconnects. The fabrication resolution demonstrated in this study is ca. 1 μm , which is approaching the diffraction limit in research-grade photolithography. The fabrication yield is $\sim 80\%$ with loss mostly due to fabrication defects on the interconnects layer. We choose to use photoresist SU-8 as the insulating layers for its wide range of tunable thickness in spin coating, excellent tensile strength [32] and biocompatibility[33], the ease of photo-patterning, and extensive prior experience with fabrication using this material[13, 14].

The excellent tensile strength of SU-8 [32] provides sufficient durability of the NEC devices despite their sub-micron thickness. Our previous studies using neural implants of similar architect demonstrated that these devices maintained functional and structural integrity for at least four months in-vivo [14]. In addition, we enhanced the bonding strength between NEC devices and the hosting probe by hard baking the assembled probe before implantation (Methods). We tested the durability of these devices in-vitro by performing 50 insertion-extraction cycles in

hydrogel, which has similar mechanical properties as brain tissue [34]. We observed that the NEC devices maintained conformal adhesion on the host probe. Moreover, we compared the impedance of NEC devices before and after wrapping onto hosting probes, as well as after up to 50 insertion-extraction cycles in hydrogel. Both the impedance and the yield of connected electrodes remained unchanged during these procedures. These results suggest sufficient durability of NEC enabled multifunctional probes for experiments that require long-term implantation.

In this study, we presented a new approach for multifunctional neural probes by combining conventional microscale tools, such as optical fibers and pipettes, with conformally coated ultra-thin nanoelectronic devices. Because the NEC devices were fabricated separately, we can achieve high resolution, high level of integration and design versatility without being limited by the geometry and materials of the host probes. Unlike unconventional fabrication approaches that often require a specialized setup, the fabrication of NEC devices uses widely available planar photolithography techniques on silicon substrate that are optimal for massive production. More importantly, as the thickness of NEC devices are typically below 1 μm , the additional volume induced by the coating is minimal, which leads to significantly reduced tissue damage compared with conventional approaches.

5.4. Methods

NEC device fabrication and multifunctional probe preparation:

The NEC devices were fabricated using planar photolithography fabrication methods similar to that previously reported [13, 14]. After a nickel metal release layer was deposited on a silicon substrate (600 nm SiO_2 , n-type 0.005 $\text{V}\cdot\text{cm}$, University Wafer), SU-8 photoresist (MicroChem Inc, SU-8 2000.5) was used to construct the bottom insulating layer. Then interconnects were patterned by depositing 100 nm gold. After a second insulating layer of SU8, electrodes with dimension of 30 $\mu\text{m} \times 30 \mu\text{m}$ were patterned by depositing 100 nm platinum or gold, electrically connected with the interconnects through “via”s on the top SU8 layer. After fabrication, a connector (33-pin FFC/FPC, Molex) was mounted on the matching contact pads on the Si substrate. The flexible section of the devices was then released from the substrate by soaking in nickel etchant (TFB, Transene Company) for 2 – 4 hours at 25 $^{\circ}\text{C}$, whereas the contact region remained attached to the substrate. The substrate was cleaved in water to the desired length to facilitate conformal attachment on conventional probes. The flexible section of the NEC devices

was then aligned and attached to the surface of conventional probes by surface tension as discussed in the main text. The resultant probes were dried in air or in vacuum, followed by baking at 60°C in oven for 30 mins to improve the adhesion. Optional baking at 190 °C in oven can further improve the adhesion, but was not required for experiments performed in this study.

Animal care and surgery procedure:

Wild type male mice (C57BJ/6, 8 weeks old, Taconic) and transgenic strain Thy1-mhChR2-EYFP Line 20 (12 weeks old, The Jackson Laboratory) were used in the experiments. Mice were housed at the animal research center, UT Austin on a 12 h light/dark cycle, 22 °C, with food and water available ad libitum. All procedures complied with the National Institute of Health guidelines for the care and use of laboratory animals and were approved by the University of Texas Institutional Animal Care and Use Committee (IACUC).

For surgery mice were anesthetized using isoflurane (3% for induction and maintained at 1-2%) in medical grade oxygen. The skull was exposed and prepared by scalping the crown and removing the fascia, and then was scored with the tip of a scalpel blade. A 1.5 mm × 1.5 mm square opening was drilled using a 0.7 mm spherical stainless steel burr on a surgical drill over the medial prefrontal or somatosensory cortex. The multifunctional probes were then stereotactically inserted into the brain at the target depth at 200 – 500 µm. After implantation, Kwik-Sil (World Precision Instruments) was filled into the cranial opening. After the skull was cleaned and dried, a layer of low viscosity cyanoacrylate was applied over the skull. An initial layer of Metabond (Parkell) was applied over the cyanoacrylate and the Kwiksil, and additional Metabond was used to cement the carrier chip and the head plate to the skull. The head plate will be used for head-fixation during awake recording.

Awake electrophysiological recording:

Mice were allowed a 1 – 2 weeks to recovery after surgery. For recording, mice were head constrained on a custom made air supported spherical treadmill to allow walking and running, similarly to the setup previously used for optical imaging [22]. The treadmill was made of an 8 inch diameter Styrofoam ball (Floracraft) levitated by a thin cushion of air between the ball and a casting containing air jets. Voltage signals from the NEC devices were amplified and digitized using a 32-channel RHD 2132 evaluation system (Intan Technologies) with a bare Ag wire inserted

into the contralateral hemisphere of the brain as the grounding reference. The sampling rate was 20 kHz, and a 300 Hz high-pass and a 60 Hz notch filter were applied for single-unit recording. Mice and the amplifier were placed in a noise-attenuated, electrically shielded chamber.

Spike sorting:

Spike sorting was performed automatically using open-source KlustaViewa (<https://github.com/klusta-team/klustaviewa>). For automatic sorting, continuous raw recording data were imported into KlustaViewa and a 300 Hz high-pass filter was applied. Spikes were detected at the strong threshold of 6 std ($\sim 50 \mu\text{V}$) and the weak threshold at 3 std. Electrode spatial configuration was imported into KlustaViewa for automatic clustering at the maximum cluster number of 50. Obvious noise clusters were excluded. Pairwise cluster similarity was computed to guide semiautomatic verification and adjustment at the final step. Mask vectors with values ranging from 0 to 1 were computed from KlustaViewa for each sorted unit using rescaled peak spike amplitude on recording channels. The averaged mask vectors for all events from the same unit were used for further screening of the sorted units so that only clusters with more than 3 channels having mask values above 0.1 were selected as valid units to present in Fig. 5. In addition, only waveforms with mask values above 0.1 were used in triangulation, in which the locations of the firing neurons were estimated using weighted mean center method by weighing the coordinates of the recording channels with the peak-valley amplitude times the mask vector value.

Controlled drug infusion:

Multifunctional probes composed of pulled glass pipettes and NEC devices were tested *in vitro* before implantation. The glass pipette (Drummond Scientific) coated with an NEC device as previously described was connected through a needle (gauge 30, BD) to a syringe controlled by an injection system (PHD 2000, Harvard Apparatus) through ethylene-vinyl acetate tubing (0.5 mm inner diameter, McMaster-Carr). The injection rates were calibrated by injecting water and measuring the time and the output weight at the target rates of 1, 10, 20 and 50 nl/s. Before implantation, the needle was filled with 10 μL sterile PBS and sealed by a PDMS cap. Mice were allowed to recover for a week after surgical implantation of the probes. For drug infusion tests, the pre-filled PBS was exchanged by the same volume of 0.1 mM α -amino-3-hydroxy-5-methyl-4-

isoxazole propionic acid (AMPA) receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), followed by the injection of the same drug at 5 – 50 nl/s for 3 mins. Awake electrical recording were performed simultaneously to monitor the suppression and recovery of neural activities. The infusion was repeated after the neural activities recovered to the base line for multiple cycles.

Optogenetic stimulation:

Transgenic strain Thy1-mhChR2-EYFP Line 20 (Male, 12 weeks old, The Jackson Laboratory) was used for optogenetic stimulation of neurons. Multimode optical fibers (Ø200 µm) were used as the host probe. One end of the fiber was cleaved, inserted into a Ø1.25 mm ceramic ferrule (Thorlabs), glued in place and polished. The other end of the fiber was wrapped by NEC devices. The ferrule was mounted on the animal skull after surgical implantation. For measurements and stimulation, the ferrule was connected through a ceramic split mating sleeve to a ferrule patch cable (Thorlabs). Continuous laser excitation was presented at 473 nm at 10 mW (LRS-0473 DPSS, Laserglow) for 10s followed by 10 s laser off during neural recordings.

6. Conclusion

Implanted microelectrodes provide a unique means to electrically interface with individual neurons in vivo, but their lack of stable functionality, sufficient sampling density and recording bandwidth due to the intrinsic invasiveness pose limitations on scientific and clinical applications. There is growing evidence suggesting that substantially reducing the mechanical rigidity of neural electrodes promotes tissue compatibility and improves their recording stability in both the short- and long-term. We thus developed a neural electrode platform called nanoelectronic thread (NET) with subcellular dimensions, ultraflexibility, and cellular surgical footprints which formed reliable, glial scar-free neural integration. As the results, we demonstrated that NET electrodes reliably detected and tracked individual units for months; their impedance, noise level, single-unit recording yield, and the signal amplitude remained stable during long-term implantation. In vivo two-photon imaging and postmortem histological analysis revealed seamless, subcellular integration of NET probes with the local cellular and vasculature networks, featuring fully recovered capillaries with an intact blood-brain barrier and complete absence of chronic neuronal degradation and glial scar.

However, the miniaturized dimensions and ultraflexibility desired for mitigating tissue responses preclude the probe's self-supported penetration into the brain tissue. To efficiently delivered the probe into the brain with minimized surgical damage, we developed a parallel implantation method achieved by using arrays of tungsten microwires as shuttle devices, and bio-dissolvable adhesive polyethylene glycol (PEG) to temporarily attach a shank onto each microwire. We demonstrate the high-throughput implantation of multi-shank ultraflexible neural electrode arrays with surgical footprints as small as $200\ \mu\text{m}^2$ in a mouse model. We show the ability to simultaneously deliver electrode arrays in designed patterns, to adjust the implantation locations of the shanks by need, to target different brain structures, and to control the surgical injury by reducing the microwire diameters to cellular scale. These results provide a facile implantation method to apply ultraflexible neural probes in scalable neural recording.

While the long-term recording stability afforded by the NET and the inherent sub-ms temporal resolution of implanted electrodes allow us to study the dynamics of the constantly-evolving brain circuits at diverse temporal scales. The recordings were still quite sparse compared to the neuronal density and its massively-interconnected structure in the brain. On the other hand,

neural computations not only depend on local circuits but the distributed circuits within or across distant regions. Therefore, a systematic understanding of the brain also requires an interrogation at diverse spatial scales. We thus have massively expanded the NET platform towards both high-density volumetric mapping and large-scale distributed recordings in both neocortex and subcortical structures with > 1000 recording channels, and doing so with chronic stability. Specifically, we have demonstrated volumetric recording densities as high as ~ 1000 channels / μL in the visual cortex of head-fixed mice and freely moving rats. We show that > 600 neurons were simultaneously recorded from $\sim 1.3 \mu\text{L}$ of cortical tissue for over eight weeks. And we also demonstrated large spatial coverage of this recording platform with > 1000 neurons simultaneously recorded across the entire mouse cortex. These new capabilities will drive new long-term studies targeting regional and distributed brain circuits.

Brain function can be better studied by simultaneous measurements and modulation of the multifaceted signaling at the cellular scale. Extensive efforts have been made to develop multifunctional neural probes, typically involving highly specialized fabrication processes. Taking advantage of NET's $< 1 \mu\text{m}$ thin thickness and ultraflexibility, we have developed a novel multifunctional neural probe platform realized by applying ultrathin nanoelectronic coating (NEC) on the surfaces of conventional microscale devices such as optical fibers and micropipettes. Taking advantage of the surface tension, we can precisely align and coat the NEC devices on the surfaces of these conventional microprobes and enabled electrical recording capabilities on par with the state-of-the-art neural electrodes. We have demonstrated optogenetic stimulation and controlled drug infusion with simultaneous, spatially resolved neural recording in rodent model. This provides a low-cost, versatile approach to construct multifunctional neural probes that can be applied to both fundamental and translational neuroscience.

While the stable recording has been demonstrated in rodents with NET for more than half a year, the performance at a longer time frame remained to be untested, partially due to the suffice of this time duration to most of the current neuroscientific studies and the short life span of the animal model we are currently using. As the future step, we will further prove upon the functionality of current platform at an even longer time frame such as years, and on more complex animal models such as non-human primates (NHP) to meet more scientific needs. Also, optimization on the device structures and material selection will be carried out to satisfy the need

of translational applications for longer functional time and better robustness. A thorough test of different thickness of probes, and probes constructed with different materials such as polyimide will be carried out at longer term in the future.

Despite the scalable and facile insertion with the current method, further development is strongly desired to allow for parallel slow insertion at speed $\sim \mu\text{m}/\text{sec}$ for achieving better recording outcomes. A slow insertion speed can potentially reduce the tissue damage and blood vessel rupture during insertion, and enable simultaneous recording during implantation for better targeting, especially for structures like hippocampus where the thin, densely packed cell layer is difficult to target. For the cortical implantation, this will allow for precise targeting at different functional layers. For achieving it, a loop-hole can be integrated into the future's probe design and used to construct the mechanical anchors to assist the delivery.

In addition, the current probe assembly and surgical implantation still require a significant amount of manual input. An automated system can be developed to facilitate the process, simplify the operation procedures and reduce the human-factor involved failures. As an example, the implantation can be automated with the assist of computer vision and customized surgical robotics in the future. For the front end, the electrode density can be further increased by using electron beam lithography to resolve neural circuits to a greater degree of details, and the upscaling and miniaturization of the back-end electronics and data acquisition techniques need to be carried out at the same time.

This large-scale and long-term stable neural recording platform proves to be a possible solution for studying challenging neuroscience problems at various spatial and temporal scales, such as the activity structure that is not possible to resolve with fewer recorded neurons, the simultaneous information processing across the local and global, the superficial and deep circuits under specific brain functions, the massive neural computations within the nearby nodes, and the circuit plasticity subject to adaptive behaviors over months or years, etc. The marked high bandwidth and long-term stability of the platform also highlight the advantages of the NET platform for achieving neuroprosthesis for patients with paralysis, such as a more robust and dexterous control of robotic arms to regain the lost motor functions or a high-resolution cortical visual prosthesis to reconstruct vision with a higher definition for the blind. Additionally, the platform can provide an effective modality to better study the brain diseases, such as stroke and

neurodegenerative disorders by enabling chronic tracking of the neurological changes caused by the diseases.

7. Bibliography

Chapter 1

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