

**Graphene-based bidirectional neurotechnology
for treatment of movement disorders**

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Graphene-based bidirectional neurotechnology for treatment of movement disorders

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Thesis Framework

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Nicola

Who believes he can and who believes he can't, they are usually both right

Confucio

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Motivation

This work was centered in the development and optimization of a minimally invasive neurotechnology with high-resolution bidirectional electrodes for the treatment of movement disorders. The central motivation is to address some limitations of current neural interface technologies. In the last few decades, a variety of neural interfaces have been developed and tested for the treatment of different medical pathologies in both the central and peripheral nervous systems. Applications span from restoring motor and sensory function in limb loss,^{1,2} treatment of pain,³ and movement disorders⁴ such as Parkinson's disease (PD)⁵, essential tremor⁶ or dystonia⁷ to retinal and cochlear implants. However, translating these research efforts into clinical applications is slow due to technological challenges, such as electrode materials with poor recording and stimulation capabilities, the invasiveness of the implant, and low operational stability over time.

For this reason, this work was centered on the development and optimisation of a minimally invasive neurotechnology with high-resolution bidirectional electrodes made of reduced graphene oxide (rGO). This is a high-performing bidirectional material, characterised by high charge injection limits and low impedance. The electrodes are encapsulated in a flexible polyimide structure to reduce inflammation and tissue damage.

In Chapter 1, I introduce the technological requirements for the electrodes in terms of recording and stimulation capabilities, lead invasiveness and safety for the treatment of movement disorders.

Chapter 2 describes the fabrication protocol of the rGO technology and the electrochemical characterization of electrodes of different sizes to evaluate their performance both *in vitro* and *in vivo*. It then explores the safety of electrical stimulation in the mouse brain, at both the electrode and tissue levels, using electrochemical and histological analyses. These results establish guidelines for the safe operational use of this technology.

In Chapters 3 and 4, I present the applications of this technology for two different movement disorders, Parkinson's disease and spinal cord injury. Specifically, in Chapter 3, I show the advantages of having a high-performing material, such as rGO, which allows for the miniaturisation of the electrodes while keeping low noise neural recording and the capability to inject high current density. This enables high-resolution recording and modulation of Parkinsonian biomarkers.

In Chapter 4, I present the new advancement of rGO technology for chronic nerve applications. A new design of the lead and materials in the encapsulation layer was optimised to make a stable interface with the sciatic nerve. In a proof of concept application, the device demonstrated the capability to record compound action potentials for over a month and to selectively activate different nerve fibres with electrical stimulation to produce leg muscle contraction over 120 days.

Chapter 1

Background on neural interfaces

1.1 Neuroelectronic therapies for motor disorders

Motor disorders arising from nervous system malfunction or damage are among the most challenging areas of modern medicine. Conditions such as spinal cord injury (SCI), peripheral nerve damage, and neurodegenerative diseases like Parkinson's disease (PD) alter and/or disrupt the transmission of neural signals that control movement, leading to paralysis, tremor, spasms, and loss of coordination. Over the last few decades, a broad range of technologies has emerged to restore or compensate for these lost functions. These approaches include electrical and ultrasound stimulation, regenerative strategies guided by electric fields, and hybrid neuroprosthetic systems that target both the central and peripheral nervous systems.

Non-invasive neurotechnologies.

Non-invasive techniques, including transcutaneous electrical nerve stimulation and transcranial electrical or magnetic stimulation, further expand clinical applications by modulating nerve networks without surgery. Non-invasive brain stimulation, including transcranial magnetic stimulation and transcranial direct or alternating current stimulation, aims to modulate cortical activity without surgery (commercially available by Magstim, Nexstim, Brainsway). These techniques are used to enhance motor recovery after stroke, reduce spasticity, and support treatment of movement disorders and chronic pain. By gently altering the activity of motor and sensory networks, non-invasive stimulation can facilitate learning during physiotherapy and improve functional outcomes when combined with conventional rehabilitation.⁸ The main limitations are the relatively low stimulation penetration depth, variability in response between patients, and the need for repeated sessions to maintain benefits. Despite these constraints, non-invasive approaches offer a safe and accessible complement to implantable neurotechnology.

Peripheral nervous system-related motor disorders:

In the case of spinal or nerve injuries, the lesion can generate an interruption of the communication between the brain and the body below the level of the lesion. Traditional treatment has focused on rehabilitation and prevention, but recent technologies aim to actively re-establish neural signal connections⁹. Epineural^{10,11} (surface) or intraneural¹² (penetrating) electrodes implanted in the sciatic^{13,14}, femoral, or peroneal nerves can restore limb movement, grasping and foot drop correction (commercially available by Medtronic). This approach consists of bypassing the lesioned site, which

interrupts the input signal from the brain to perform a given movement, and activating intact nerve fibres with electrical stimulation to induce leg muscle contraction. Signals recorded from the motor cortex through implanted electrodes are intended to be decoded in real time and used to trigger stimulators placed below the injury, effectively bypassing the damaged segment. This closed-loop^{1,15} approach mimics natural motor pathways and offers hope for more intuitive control of paralysed limbs. To improve the efficacy of this approach and establish closed-loop systems capable of mimicking natural motor,¹⁶ it is necessary to establish a stimulation paradigm that can control the different muscles independently, and activate them in a smooth way and a specific sequence. Furthermore, to detect a high-fidelity signal from the brain, it is necessary to have a high-density array of small electrodes that can record with high resolution and systems that can decode it with precision.

In the case of limb loss, the use of neuroprosthetics helps to restore functional mobility and allow individuals to regain autonomy, social participation, and quality of life. However, an important function of prosthetics is not only to provide functional mobility of the limbs but also to restore tactile and thermal perception, which creates a sense of embodiment of our limbs.^{17,18} Somatosensory feedback plays a crucial role in object manipulation, since without the sense of touch, many everyday movements become significantly compromised. Therefore, neuroprosthetic systems that incorporate artificial somatosensory feedback in a natural and intuitive way are likely to enhance usability and ultimately improve the performance of brain-controlled bionic limbs. Electrical stimulation of the central¹⁹ or peripheral nervous system²⁰ represents an effective strategy to restore sensory feedback. Bidirectional neural prosthetics (e.g., commercially available by SensArs Neuroprosthetics), with electrodes implanted in the peripheral nerves,²⁰ in the spinal cord,^{2,21} or in the motor cortex¹⁹, can restore natural-like sensations^{15,22} by inducing with stimulation an intuitive and interpretable sensory feedback to the patient, overall improving the usability of the prosthetics.

Spinal cord stimulation for pain treatment

Spinal cord electrical stimulation (SCS) has become a major therapeutic option for the treatment of chronic pain (commercially available by Boston Scientific and Abbott), particularly when pharmacological approaches are insufficient or produce significant side effects. The injection of controlled electrical pulses induces the activation of large-diameter dorsal column fibres to modulate the abnormal sensory activity that characterizes neuropathic and centralized pain conditions²³. This activation inhibits the transmission of nociceptive signals at the spinal level and alters pain processing in supraspinal networks. Modern SCS systems employ high-frequency closed-loop stimulation modes that can provide effective analgesia without producing uncomfortable side effects such as paraesthesia.

Electrical strategies for neuroregeneration

An ongoing approach aims to regenerate damaged neural pathways. Nerves possess some intrinsic capacity for regrowth, and biomaterials that conduct electricity create microenvironments that

encourage axonal extension by guiding the growth. When combined with low current fields generated by low-amplitude DC stimulation, neurites tend to align along the electric gradient, accelerating reconnection with target muscles^{24,25}. In the spinal cord, regeneration is more limited due to inhibitory scar tissue. Experimental strategies combine electrical stimulation with underdura implantations²⁶ to penetrate deeper with the electric field inside the nerve or stem cell transplantation to overcome the fibrotic response over time. In the future, therapies could combine stimulation with stem cells or biomaterials so that the electric signals guide both nerve growth and cell development simultaneously, leading to more complete recovery.

Brain-related motor disorders

Brain-related motor disorders include a variety of neurological conditions that affect movement, muscle control, and coordination. An example is essential tremor, which causes rhythmic shaking of the hands; dystonia, marked by involuntary muscle contractions and abnormal postures; Huntington's disease, a genetic disorder leading to chorea and cognitive decline; cerebral palsy, resulting from early brain injury and affecting posture and muscle tone; multiple system atrophy (MSA), which combines parkinsonism with poor coordination and autonomic problems; progressive supranuclear palsy (PSP), causing stiffness, balance issues, and eye movement difficulties; and Tourette syndrome, characterized by motor and vocal tics. These disorders highlight the diverse ways the brain can influence motor function. Motor disorders can also arise from Parkinson's disease. The principal symptoms, tremor, rigidity, and bradykinesia, result from the loss of dopaminergic neurons in the substantia nigra, which leads to abnormal oscillatory activity within the basal ganglia circuits. In the early stages of the disease, symptom management relies mainly on pharmacological therapy, particularly levodopa, to restore dopamine levels. However, as the disease progresses, symptoms become more severe and it becomes increasingly difficult to maintain optimal medication dosing, with the risk of either insufficient doses and disorder control or overdosing, which can induce side effects such as dyskinesia. Because medications gradually lose efficacy, deep-brain stimulation (DBS) becomes the main therapeutic strategy²⁷⁻²⁹ in the later stages of the disease. DBS involves the implantation of leads with electrodes in specific brain targets, most commonly the subthalamic nucleus, to deliver high-frequency pulses with a pulse generator (clinical devices commercialized by Medtronic, Boston Scientific). A programmable touchpad can communicate with the pulse generator to adapt stimulation parameters and improve treatment efficacy. This can reduce or modulate pathological neural activity, such as increased beta,^{30,31} gamma^{32,33} and burst^{31,34,35} spiking activity, and significantly reduce tremor and bradykinesia. This approach also allows for substantially reducing medication dosage, thereby decreasing the associated adverse effects. Current research aims to improve the efficacy and safety of DBS technologies through adaptive^{33,36,37} or closed-loop systems^{32,38}. Instead of delivering constant stimulation, devices monitor neural biomarkers and adjust output in real time, reducing side effects and battery consumption.

Alternatives to implantable bioelectronics include ultrasound and optogenetic stimulation.

Ultrasound is emerging as a versatile tool for the treatment of motor disorders, with two main clinical uses, neuromodulation and focused ablation. Low-intensity focused ultrasound can modulate neural activity³⁹ in a non-invasive and highly localized manner by applying mechanical vibrations to modulate ion channels and synaptic transmission in targeted brain or peripheral nerve regions. This approach has been investigated to influence motor cortex excitability and enhance recovery after spinal cord or peripheral nerve injury. Ultrasound can reach deep structures with millimeter precision without invasiveness, and it offers an attractive alternative to implanted stimulators while avoiding surgical risks. At higher energy levels, focused ultrasound can produce tissue ablation. This is already an approved treatment⁴⁰ for conditions such as essential tremor and Parkinson's disease. Magnetic-resonance-guided focused ultrasound can create small, precise lesions in structures like the subthalamic region, interrupting pathological circuits to re-establish new healthy neural pathways with the motor cortex, and reduce the motor disorders associated. The main advantages are immediate clinical effect and the absence of implanted devices in the brain. Limitations include the irreversibility of the lesion and the current inability to adjust therapy over time as the disease progresses.

Another technology is optogenetics. Optogenetics has raised interest in the scientific community for investigating the nervous system, particularly in exploring neural circuit function^{41,42}, developing neuromodulation strategies, and extending control to peripheral and spinal networks. This technique allows neurons to be controlled with light after they have been genetically modified by viral vectors to express light-sensitive proteins called opsins. When specific wavelengths are delivered through optical fibers, targeted neurons can be activated or inhibited with millisecond precision. This method provides a level of cell-type selectivity that is not achievable with conventional electrical stimulation, even if the current is injected with small microelectrodes, due to the propagation of the generated electric field⁴³ for hundreds of μm in the tissue. This enables the study or manipulation of particular neurons responsible for a particular movement or symptom. Optogenetics has been widely used as a neuromodulation tool for deciphering the function of disease-relevant neural circuits⁴⁴ and testing targeted stimulation or inhibition strategies. In disorders such as Parkinson's disease, optogenetics has been used to dissect basal ganglia circuits⁴¹ and to suppress abnormal activity that generates tremor and rigidity. Importantly, optogenetic approaches are increasingly applied to the peripheral nervous system⁴⁵ and spinal cord. Studies now target motor neurons, sensory pathways, and spinal interneurons to investigate locomotor control, pain modulation, and recovery after nerve injury. Even if there is a consistent advantage in providing cell-type specificity in the activation or inhibition of the cells,⁴³ major limitations makes clinical translation challenging. The treatment requires the injection of a viral vector in the human body, which poses challenges from regulatory and safety perspectives. Moreover, long-term safety of such gene therapy is still uncertain, and implantable light sources must be developed for chronic applications. For these reasons, optogenetics is currently a powerful research tool rather

than a routine therapy, but it is shaping the design of future highly selective neuromodulation technologies.

In conclusion, the treatment of motor disorders comprises a wide range of medical strategies, including electrical and ultrasound stimulation, regenerative electric fields, and optogenetics, to restore function in the damaged brain, spinal cord, and peripheral nerves, although challenges remain regarding the long-term efficacy and safety of these treatments. Future therapies will likely combine multiple modalities, transforming conditions once considered irreversible into manageable or even curable disorders.

In this thesis, I will explore electrical-related neurotechnology for the central and peripheral nervous systems, for the treatment of movement disorders. Specifically, I will explore the detection and modulation of biomarkers in the brain of Parkinson's disease and the stimulation of the sciatic nerve to recover the movement of the leg after a spinal cord injury.

1.2 Technology requirements for neurostimulation

Electrical stimulation is applied to neural tissue to elicit a functional response. By depolarizing the membrane of the neurons, it is possible to inhibit or excite a certain population of cells in order to induce therapeutic treatments. In most neural applications, stimulation is delivered as a series of biphasic current pulses at a fixed amplitude and frequency. This current polarizes the medium, resulting in redistribution of anions and cations far from the equilibrium. The current can be purely capacitive or faradaic. Capacitive processes store charge at the electrode–electrolyte interface without transferring electrons across it. This can happen through electrostatic separation of charge, where ions and electrons align near the surface.⁴⁶ Faradaic processes, on the other hand, involve actual electron transfer between the electrode and chemical species in the medium. For this to occur, a molecule or ion either attached to the electrode surface or dissolved in the solution must change its oxidation state by gaining or losing electrons.

For stimulation applications, capacitive charge injection is generally preferred because it does not create or consume chemical species during the pulse, if they are irreversible. Irreversible Faradaic reactions can cause electrode oxidation or dissolution, and tissue pH can shift, inducing neuron death^{46,47}. However, the current that can be injected through a capacitive process is limited, due to the charge that can be stored in the double layer per unit area. To increase the total charge that can be delivered, electrodes are often made of porous materials to increase their active surface area or coated with nanostructured hydrated oxide layers functioning through a combination of capacitive and reversible faradaic reactions.

Different materials have been proposed in the research field to enhance charge-injection capabilities, which is important for neural modulation. These materials rely on different charge-transfer mechanisms and present distinct advantages and limitations.

- Titanium nitride (TiN) is a chemically stable and biocompatible metallic conductor. Its high charge-injection capacity^{48–50} mainly arises from its porous, rough structure, which greatly enlarges the double-layer capacitance. Charge transfer occurs primarily through capacitive mechanisms at the electrode–electrolyte interface. TiN coatings are mechanically robust and relatively stable over time.
- Iridium oxide^{51,52} (IrOx), particularly in the form of activated iridium oxide films (AIROFs), operates differently. Charge is injected largely through fast and reversible faradaic reactions involving transitions between Ir³⁺ and Ir⁴⁺ oxidation states within a hydrated oxide layer ($\text{Ir}^{3+} \leftrightarrow \text{Ir}^{4+} + \text{e}^-$). This redox-based mechanism can increase charge-injection capacity compared to purely capacitive materials. Applying a small positive voltage bias between pulses can further enhance performance by shifting the oxide toward the more conductive Ir⁴⁺ state, increasing the available charge.
- PEDOT (poly(3,4-ethylenedioxythiophene)), typically doped with poly(styrene sulfonate) (PSS), is a conducting polymer that supports both electronic and ionic transport. Mixed conductivity allows efficient charge transfer and significantly reduces electrode impedance. The porous and soft nature of PEDOT coatings makes them attractive for neural interfaces, especially for low-impedance recording^{53,54} and stimulation⁵⁵. However, polymers can degrade over time, especially under high-amplitude currents⁵⁶ or long-term stimulation, and their mechanical stability may be lower than that of metallic coatings.
- Planar platinum and platinum–iridium (PtIr) alloys are traditional noble metal electrodes used in neural stimulation. They transfer charge through a combination of double-layer capacitance and faradaic reactions. These materials are highly biocompatible and chemically stable, but their charge-injection capacity is generally lower^{57,58} than that of IrOx or PEDOT unless the surface is significantly roughened^{58,59} or made porous. Furthermore, in wet environments, platinum can dissolve⁶⁰, compromising electrode stability.

The choice of material depends on the required charge density, long-term stability, mechanical properties, and the specific demands of the neural interface. A key aspect is to consider the material's ability to inject sufficient charge to reach the tissue's neuromodulation threshold. This charge injection capability is evaluated by monitoring the interfacial polarization voltage produced by the flow of ionic current between the working and counter electrode. In a monopolar configuration, the counter electrode is connected to ground and serves as a return path for the current

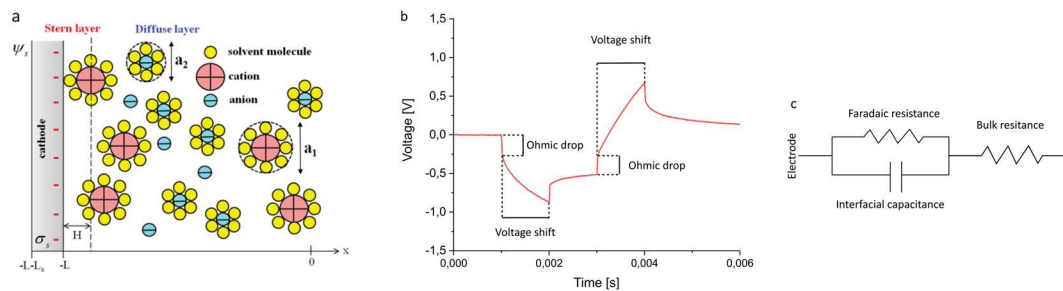


Figure 1.1 | Electrode/electrolyte interface. **a**, Schematic of the computational domain of the electric double layer structure consisting of Stern and diffuse layers close to an electrode (Figure adapted from⁶¹). **b**, Transient voltage generated by a biphasic current pulse of 10 μ A injected with an rGO microelectrode of 25 μ m in diameter, highlighting the ohmic drop and the interfacial polarization voltage. **c**, Schematic of the equivalent circuit model of an electrode-tissue interface.

The injection of a current pulse with the working electrode generates a transient voltage that can be modelled with the help of a Randles equivalent circuit; the transient voltage can be described by a resistive component, called ohmic drop, and a capacitive interfacial polarization at the electrode tissue interface, $V_{\text{pulse}} = V_{\text{interfacial}} + V_{\text{ohmic}}$ (Figure 1.1b). For a simple capacitive-dominant interface, the interface voltage is $V(t)_{\text{interfacial}} = \frac{1}{C_{dl}} \int I(t) dt$, where C_{dl} is the double-layer capacitance. This interfacial capacitance at the electrode interface ($\cong 2$ nm) arises from the charging and discharging of the electrical double-layer (Figure 1.1c). This layer comprises an inner layer of adsorbed water molecules, also called the Helmholtz layer, and an outer layer of hydrated ions that form a diffuse layer with a strong dependence on electrolyte concentration, as described by the Gouy-Chapman-Stern (GCS) model (Figure 1.1a). This interface voltage has a maximum operational range in order to avoid irreversible faradaic reactions and electrode deterioration. Each material has its own safe voltage window⁴⁶, which usually ranges from -0.6 to 0.8 V for metals and slightly larger for some carbon-based materials (-0.9V to 0.8V for example for graphene).

The resistive component arises from the current flow in the medium where the electrode is in (saline or tissue), between the working and counter electrode (Figure 1.1c), and it follows the ohms law, $V_{\text{ohmic}} = I \times R_{\text{bulk}}$. Materials with high charge injection limits can inject high current, in the medium while keeping the interfacial voltage low within safe limits. This facilitates reaching the stimulation threshold necessary to modulate neural activity.

Baseline drift assessment.

When stimulating, it is important to monitor not only the interfacial polarization voltage but also the slow DC polarization changes of the electrodes, as this voltage is also applied to the electrode-tissue interface. Variations in the voltage baseline during stimulation come mainly from two phenomena.

The first is the charge imbalance generated during typical biphasic current pulses. Because electrodes exhibit an asymmetric response, the charge accumulated during the anodic phase is generally different than the amount discharged during the cathodic phase, leading to accumulated charge and hence a drift of the voltage baseline (Figure 1.2a). The magnitude of this baseline shift depends on multiple factors, including electrode material, size, and impedance, the properties of the surrounding medium, and the electronic configuration used for current injection. This imbalance is generally small; it can be partially compensated by the natural redistribution of charge in the interval between consecutive pulses, depending on tissue conductivity and stimulation frequency. In most applications, however, the stimulation frequency is not low enough to allow complete discharge, and the residual charge accumulates, producing a progressive DC baseline drift (also called ratcheting)⁶².

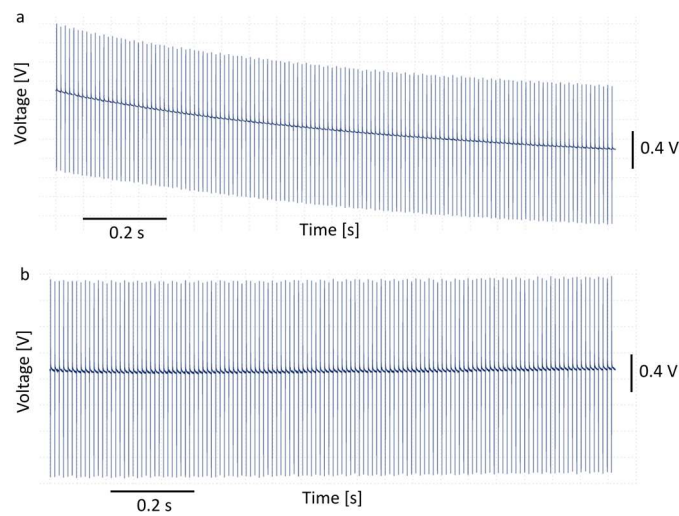


Figure 1.2 | Voltage drift and balancing. **a**, Transient voltage response resulting from the injection of non-balanced biphasic current pulses (100 μ s pulse width, 50 μ A amplitude, 130 Hz frequency) with a 25 μ m diameter rGO microelectrode; measurement conducted with a potentiostat. **b**, Transient voltage response resulting from the injection of a balanced biphasic current pulse (anodic phase: 100 μ s pulse width, cathodic phase: 92 μ s; 50 μ A amplitude, 130 Hz frequency)

A second issue is related to current leakage. Any stimulation circuitry has intrinsic limitations in controlling the exact current delivered to the electrode. As a result, a small DC current can flow during stimulation, shifting the voltage baseline. This leakage might depend on the current range selected in the stimulation settings; choosing the lowest range that still supports the required current is essential to minimize it.

The combination of pulse imbalance and current leakage can shift the baseline, eventually bringing the electrode potential outside the safety operation window. Approaches such as asymmetric pulses, with reduced current amplitude or shorter duration in one of the two phases, can improve charge balancing and mitigate this drift (Figure 1.2b). However, many variables influence this phenomenon and may also change over time, making manual adjustment difficult and poorly reproducible. Therefore, other

strategies are needed to minimize this phenomenon and improve the stability of the electrode response, a requirement that is particularly critical in long-term stimulation applications.

1.3 Compliance criteria for electrical stimulation safety

Charge injection limits (CIL) are important to achieve effective neuromodulation⁶³ while avoiding adverse tissue reactions and damage. These limits are typically defined by the charge density that drives the electrode potential to the limits of the water electrolysis and the electrode material itself; exceeding them can cause pH changes, gas formation, and electrode degradation^{64,65}.

However, CIL is not enough to determine stimulation safety. Multiple studies have shown that tissue injury can occur at charge densities well below material electrochemical limits; for instance, neural hyperactivity is a dominant mechanism underlying stimulation-induced damage^{66,67}. Designing an in vivo electrical stimulation study, with the associated experimental protocols, requires the adoption of established safety guidelines for selecting stimulation waveforms and amplitudes, to avoid excessive charge density that can result in irreversible neural tissue damage⁴⁶.

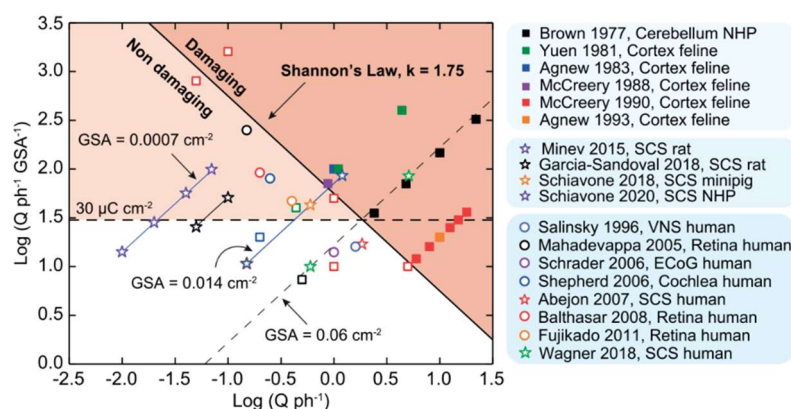


Figure 1.3 | Safe stimulation limits. Shannon's plot ($k = 1.75$) discriminating between damaging and non-damaging stimulation protocols with the references on the right. Figure extracted from.⁶⁸

To address this issue, the Shannon plot introduced an empirical safety guideline that defines the boundary separating damaging from non-damaging stimulation parameters⁶⁸. This limit can be defined by the relationship, $\log(D) = k - \log(Q)$, where Q represents the charge per phase of the stimulation pulse, D the charge density per phase (defined as $D = Q/A$), A the geometric surface area (GSA, in cm^2) of the electrode, and k is an empirical constant typically set to 1.5-2.0.

Shannon's limit is usually defined at $k = 1.75$ based on histological observations reported in several in vivo studies (Figure 1.3). Stimulation protocols above the equation line (solid data points) proved to be damaging to the tissue, while those below the line did not cause damage (hollow data points). Electrode

geometry is generally designed according to the specific application and the neural region to target. Once these parameters are predefined, the Shannon plot provides a practical tool for selecting stimulation pulse parameters that remain within safe charge limits. Taking into account the GSA of the electrode, the maximal D value that should be injected to avoid any tissue damage according to the previous equation is $D = \sqrt{\frac{10^k}{A}}$. This relationship does not scale linearly with electrode surface area, and it follows a $\text{GSA}^{-1/2}$ dependence.

For human applications, such as deep brain stimulation and cochlear stimulation, FDA guidelines indicate a maximum charge density of $30 \mu\text{C}/\text{cm}^2$. This limit reflects the typical surface area of commercial electrodes, which is approximately 6 mm^2 . For this electrode size, a charge density of $30 \mu\text{C}/\text{cm}^2$ intersects the Shannon line for $k = 1.75$, indicating the estimated safety limit. However, several clinical and preclinical studies have reported no observable tissue damage in patients subjected to stimulation parameters exceeding this nominal limit (round data points and red and green star markers in Figure 1.1). The Shannon plot highlights that there is a dependency also on the charge per phase, following the equation $Q = \sqrt{A * 10^k}$.

In the case of microelectrodes with GSAs typically ranging from 200 to $2000 \mu\text{m}^2$, at distances approximately 2–5 times the diameter of the electrode,⁴⁶⁶⁹ neuronal excitability of the neurons and tissue damage is strongly correlated with charge per phase rather than with charge density⁶⁸. Alternative guidelines propose a much lower charge-per-phase around 4 nC per phase as a vertical boundary in safety plots.^{66,69}

Importantly, neuronal injury is influenced by other stimulation parameters such as frequency, duty cycle, and the spatial separation between the electrode surface and nearby cells. Electrical parameters, however, represent only part of the picture. Tissue reactions after implantation are also shaped by non-electrical factors, including the surgical procedure, mechanical mismatch between the implant and neural tissue, stability of the device over time, and the physicochemical properties of the electrode surface.⁷⁰ Because these variables interact in complex ways, distinguishing damage attributable purely to electrical stimulation remains challenging. Moreover, it is still unclear how broadly existing safety models can be extended across different clinical applications, raising the question of whether stimulation safety can be reliably predicted in advance with high confidence.

1.4 Recording electrophysiological activity

In the nervous system, neurons transmit information through rapid electrical signals generated by the movement of ions across the neuronal membrane. In a non-active state, a neuron maintains a negative transmembrane potential (about -70 mV), due to differences in ion concentrations, mainly sodium (Na^+), potassium (K^+), and chloride (Cl^-), balanced by the permeability of its membrane. When a

stimulus depolarizes the membrane above a critical threshold (around -55 mV), voltage-gated sodium channels open, allowing Na^+ to flow into the cell. This causes a rapid depolarization, making the inside of the neuron temporarily positive. Shortly after, sodium channels close and voltage-gated potassium channels open, allowing K^+ to flow out of the cell, thereby repolarising and briefly hyperpolarising the membrane. The neuron then returns to its resting state through ion pumps and passive ion movement. This phenomenon is called an action potential.⁷¹

Communication between neurons is produced mainly via synaptic currents. These currents arise when presynaptic neurons release neurotransmitters that bind to ligand-gated channels on the postsynaptic dendrites, causing the channels to open and ions to flow. This ionic flow changes the postsynaptic membrane potential and, when occurring across many dendrites and neurons simultaneously, creates spatially extended dipoles. Because pyramidal neurons are often aligned, their currents sum, producing extracellular voltage fluctuations with high amplitude (~ 500 μV) that propagate through the surrounding conductive tissue. These summed, low-frequency signals, typically in the 1–200 Hz range, are detected as local field potentials (LFPs) and reflect the collective network activity of the neural tissue.

In contrast, action potentials (AP), also referred to as spikes, are high-frequency events generated by the rapid opening and closing of voltage-gated ion channels in individual neurons, typically at the axon initial segment or soma. As a consequence, when measured extracellularly, spikes are low-amplitude signals (< 100 μV) and highly localized, which amplitude decay rapidly with distance (~ 200 μm) between the neuron generating the AP and the recording electrode. This makes the recording of spiking activity particularly challenging, as it requires high-density electrode arrays capable of detecting action potentials in proximity to individual neurons, with electrode dimensions comparable to the neuronal scale.

Large-diameter electrodes (> 100 μm) detect spikes originating from multiple neurons simultaneously. As a result, the recorded signals are spatially averaged and attenuated, preventing the discrimination of individual spike events. For this reason, large electrodes are primarily used to record local field potentials and their associated biomarkers⁷². However, spiking activity provides critical information for identifying specific brain nuclei and disease-related biomarkers⁶³.

A key challenge in recording spikes is the requirement for electrodes with dimensions comparable to single neurons. Reducing electrode size, however, significantly decreases the geometric surface area (GSA), which in turn increases the electrode–tissue interfacial impedance. This increase can negatively affect recording quality⁷³ since it increases noise coming from different sources⁷⁴, thermal, and electrical.

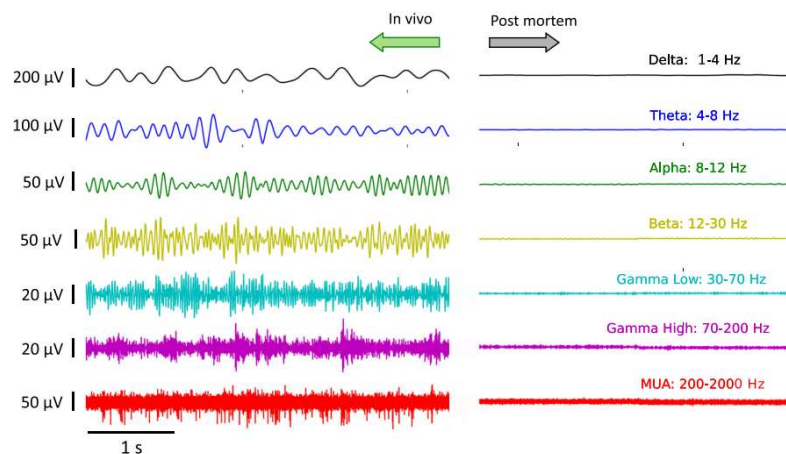


Figure 1.4 | Recording and noise. Representative brain activity recorded with an rGO microelectrode, filtered for different frequency bands, for in vivo (left) and post-mortem (right) conditions. This figure was extracted from.⁶³

Typically, a brain signal filtered in a frequency band suitable for recording action potentials (200-2000 Hz) will contain all the noise components previously described (Figure 1.4). For reliable recording, the signal-to-noise ratio should be at least 3, meaning the spike amplitude should be at least 3 times the baseline generated by the background firing activity combined with the noise level (Figure 1.4). High-frequency neural oscillations can contribute to the baseline as well, reducing spike-amplitude to baseline ratio. Electromagnetic noise is generated by ambient electromagnetic fields, produced by the electronics around, and can be dominated by the main AC component of the power mains (50 Hz in Europe) and its harmonics. This noise can be attenuated by grounding all the recording set-up and the animal itself, or by applying digital filters to exclude the signal centered at these frequencies. Thermal noise is produced by random voltage fluctuations arising from the thermal motion of charge carriers, with a magnitude proportional to the real part of the electrode impedance and to the temperature. It can be calculated using the Johnson–Nyquist equation $U_{rms} = \sqrt{4kT\Delta(f) \text{Re}(Z_{int})}$, where k is the Boltzmann constant, T is the absolute temperature, $\Delta(f)$ is the measurement bandwidth and $\text{Re}(Z_{int})$ the real component of the impedance of the electrode. Since a smaller electrode surface leads to a higher interfacial impedance Z_{int} , it increases the thermal noise. For this reason, many studies support a correlation between low impedance and a high signal-to-noise ratio in recording⁷³. However, for low-impedance electrodes, the thermal noise component has smaller contribution to the baseline, compared to the background firing activity⁷⁴. The contributions of electromagnetic and thermal noise can be distinguished from the biological background by examining the post-mortem recording baseline, which should be substantially lower than the in vivo one (Figure 1.4). For this reason, under certain experimental conditions, the presence of a low-impedance electrode might not substantially affect the total noise level and spike recording capability^{75,64}. However, ⁷⁶considering also the physiological increase of the tissue resistance over chronic implantations, having an electrode material with a high

active surface area, and smaller GSA in contact with the tissue, is overall still key, to keep impedance below certain threshold and record action potential with high signal- to noise ratio.⁷⁷

Many brain recording technologies are available nowadays⁷⁸, differing mainly in their spatial scale, temporal resolution, and invasiveness. Glass pipette (patch-clamp) recordings provide exceptional temporal resolution and signal fidelity at the single-cell level, but they are highly invasive and limited to very small spatial coverage. Microelectrode arrays (MEAs) enable simultaneous electrical recordings from multiple neurons with high temporal resolution and can be used chronically. EEG is non-invasive and suitable for whole-brain monitoring in humans, with excellent temporal resolution, but its spatial resolution is limited by signal attenuation through the skull. Optical techniques such as two-photon microscopy allow high spatial-resolution imaging of neuronal populations, including activity in deeper tissue layers, yet they require fluorescent indicators, have a limited field of view, and offer lower temporal resolution than electrophysiology. Wide-field imaging covers larger cortical areas with good spatial mapping but limited depth penetration. In contrast, fMRI provides whole-brain coverage with relatively good spatial resolution, but it measures indirect hemodynamic responses and therefore has poor temporal resolution compared to electrical methods.

Each recording technique has its preferred use depending on application, but for chronic implantable use for treating motor diseases, microelectrodes are one of the best technologies due to their spatial and temporal resolution and biocompatibility.

1.5 Open- versus closed-loop systems

Modern neuromodulation technologies typically comprise three core components: sensing, processing, and stimulation. Sensing refers to the acquisition of physiological signals from the nervous system, which can be obtained from the brain using surface or implanted electrode arrays, from the retina or optic nerve for visual information, or from the peripheral system. Stimulation encompasses technologies that can modulate the activity of the nervous system, for instance, by delivering electrical stimulation. The processor analyzes the sensed signals and adapts the output of the stimulation system based on the extracted information. Based on whether neural activity is used to adjust stimulation, two main neuromodulation paradigms can be distinguished (Figure 1.6): open-loop and closed-loop systems⁷⁹.

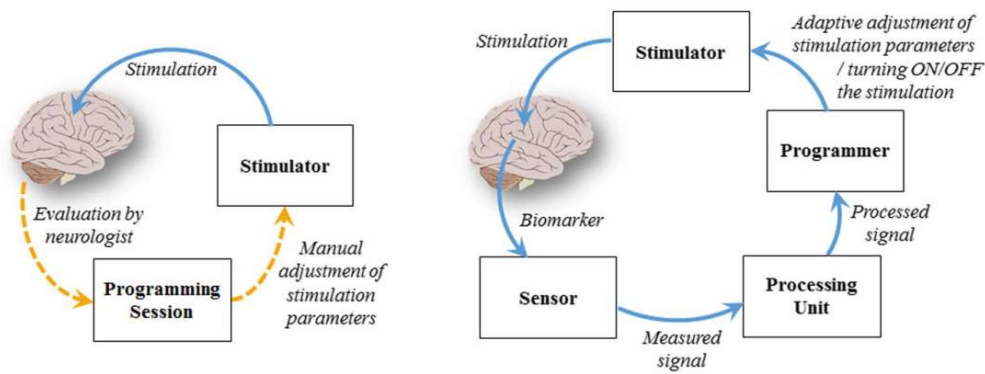


Figure 1.5 | Open- versus closed-loop neuromodulation. Overview of open-loop neuromodulation (left) versus closed-loop neuromodulation (right). In open-loop neuromodulation, stimulation parameters are manually adjusted (in the case of DBS, every 3–12 months after implantation). On the other hand, in closed-loop neuromodulation, the stimulation parameters can be adjusted automatically based on the measured biomarker. Pictures extracted from⁸⁰.

In open-loop neuromodulation strategies, stimulation is delivered according to predefined parameters without direct sensory feedback, although these parameters can be adjusted offline based on clinical outcomes. Most commercially available implanted stimulators currently operate in this mode, where the processor and stimulation electrodes form an open loop. These recording devices, such as motor prostheses, decode motor intentions from cortical activity and deliver control to robotic limbs, thereby restoring movement in paralyzed individuals. Similarly, unidirectional stimulation technologies, including cochlear implants, standard deep brain stimulation, and motor and visual prostheses, have demonstrated significant success in modulating neural activity for therapeutic or assistive purposes. However, in these systems, the absence of feedback prevents real-time adaptation of stimulation to the neural state.

Recent and emerging neurotechnology are increasingly focusing on closed-loop systems, in which neural feedback signals are directly incorporated to adjust stimulation parameters (Figure 1.5). This approach requires reliable monitoring of neural biomarkers associated with specific symptoms, allowing for adjustments to the control outcome to improve therapeutic efficacy³² while minimizing side effects. In Parkinson's disease, pathological neural biomarkers can be reduced during electrical stimulation; closed-loop systems allow these biomarkers to be monitored in real time and stimulation to be adjusted accordingly to maximize therapeutic efficacy⁸¹. In the restoration of arm or leg movement through the peripheral nerve, such as stimulation of the sciatic nerve, or spinal cord stimulation, sensory feedback signals can be used to adapt stimulation-induced muscle contractions⁸². This feedback enables smoother¹⁶, more coordinated limb movements by dynamically adjusting stimulation parameters in response to physiological conditions.

In other applications, stimulation must be delivered only during specific physiological states, an approach known as responsive stimulation. In this strategy, current is triggered after the detection of particular brain states or biomarker thresholds, thereby minimizing unnecessary stimulation periods, and side effects in the tissue while extending device battery lifetime. One example is the use of electrical stimulation to block spreading depolarizations⁸³ that occur before or after epileptic seizures or during stroke, transient neural events that require timely detection and fast control response.

In summary, closed-loop neurotechnological systems offer the potential to achieve improved therapeutic outcomes with greater precision and efficacy and to reduce side effects. However, several scientific and technological challenges remain, including an incomplete understanding of neural coding, restricted spatial resolution and coverage, and the lack of long-term stable neural interfaces capable of reliably tracking neural activity for feedback control.

Bidirectional electrodes: advantages and issues

In several neuromodulation applications, focal stimulation induces highly localized changes in neural signals and associated biomarkers, typically confined to a region within a few hundred micrometers of the active site. To accurately detect these changes and dynamically adapt stimulation parameters, it is essential to record and stimulate at the same location using a single device. Such electrodes are called “bidirectional” as they are capable of both high-fidelity neural recording and effective electrical stimulation. Moreover, some biomarkers are correlated to the activity of single neurons and require microelectrodes with dimensions comparable to individual neuron cell bodies, on the order of $\sim 25\ \mu\text{m}$. At these scales, achieving sufficient current injection to reach the neuromodulation threshold of neural tissue becomes challenging. Consequently, there is a critical need for high-performance bidirectional electrode materials that combine low impedance, to enable low-noise recordings, with high charge-injection capacity, to support safe and effective high-current-density stimulation.

An additional challenge in using electrodes of the same device for simultaneous recording and stimulation, as required for closed-loop deep-brain stimulation in Parkinson’s disease, is the presence of artefacts in the recording electrodes produced by the stimulation (Figure 1.6).

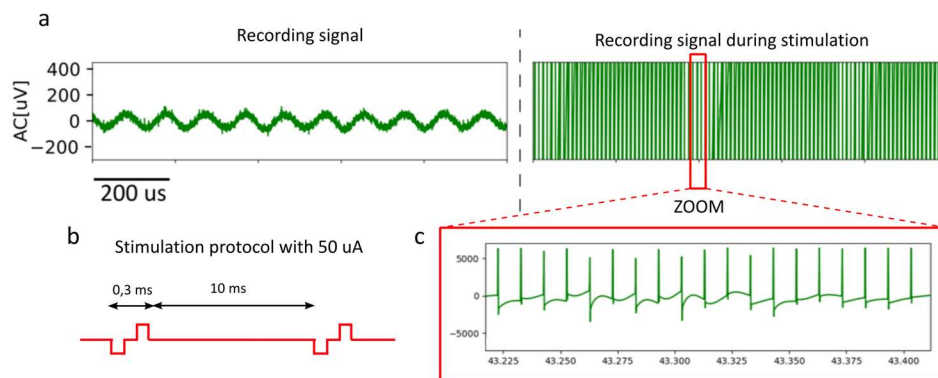


Figure 1.6 | Recording during stimulation. **a**, On the left, voltage trace recorded with an rGO microelectrodes in PBS; the recorded trace shows an artificial AC signal introduced in the saline medium with a signal generator. On the right, the recorded voltage trace corresponds to the same configuration during electrical stimulation. **b**, The stimulation protocol consists of biphasic current pulses of 50 μA and 100 μs pulse width at 100 Hz. **c**, Zoom of the subpanel a during stimulation, revealing the stimulation-induced artefacts.

Recording neural activity during electrical stimulation is challenging due to several fundamental factors. When an electrode delivers a biphasic current pulse, it induces a voltage polarization in the surrounding tissue that is detected by nearby recording electrodes. The amplitude of this polarization depends on the distance from the stimulating electrode. Beyond a certain distance, approximately 2–5 times the electrode radius, the electric field can be approximated as that of a point source⁷⁰, and the potential in each position in the tissue can be described as $V = \frac{1}{4\pi\sigma d}$, where σ is the tissue conductivity and d is the distance from the electrode. This distance assumption simplifies the model by neglecting spatial variations in the electric field caused by the electrode geometry, such as the increased field intensity at the electrode perimeter, commonly referred to as the edge effect⁴³.

As a result, a recording electrode will detect a stimulation-induced artefact whose amplitude scales inversely with distance ($\propto 1/d$). This artefact typically dominates the signal during the stimulation pulse, obscuring the underlying neural activity since its amplitude is orders of magnitude larger than that of physiological signals. In addition, the artefact amplitude may exceed the input voltage range of the recording amplifier, leading to amplifier saturation. Consequently, neural recordings are unavailable for a period following the stimulation pulse, corresponding to the time required for the tissue–electrode interface to rebalance the charge and for the recording system to recover from saturation (Figure 1.7b).

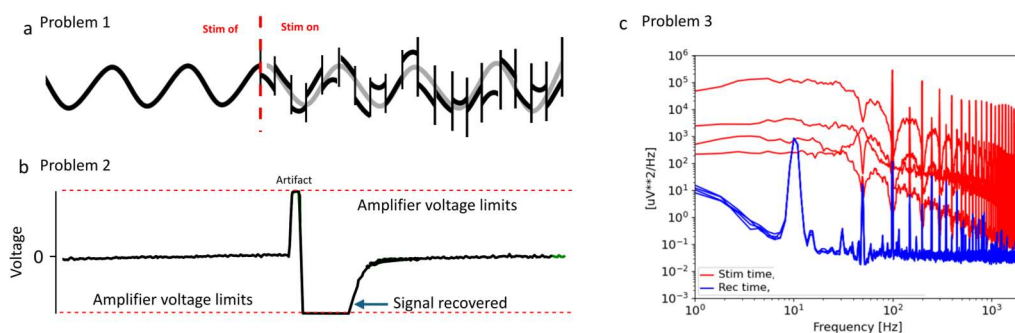


Figure 1.7 | Representative stimulation artefacts. **a**, Representation of the effect of stimulation on the recording of an artificial sinusoidal wave, revealing stimulation artefacts that do not induce saturation of the recording electronics. **b**, Example of a stimulation artefact inducing amplifier saturation. **c**, Power spectral density of signals recorded with several electrodes, with (red) and without (blue) stimulation with one of them.

Another problem is related to the fragmentation of the signal during stimulation. Brain activity is usually dominated by a low-frequency signal (0.5–100 Hz), which fluctuates. The stimulation frequency is usually faster than the brain signal frequency. Even if the voltage artefact doesn't saturate the amplifier, the starting point of the recording baseline will be slightly different after each artefact, due to variability in the response of the tissue to the stimulation. This will bring the recording signal up and down, producing a fragmentation that distorts the signal shape at a point that is not recognizable anymore (Figure 1.7a).

This issue is particularly critical for closed-loop neuromodulation systems that rely on neural biomarkers. Several neurological and psychiatric disorders, such as Parkinson's disease, schizophrenia, and epilepsy, are characterized by abnormal activity within specific frequency bands. These biomarkers are commonly quantified by analyzing the power spectral density (PSD) of the recorded signal and identifying peaks at frequencies associated with pathological activity. During stimulation, such pathological activity and consequently the corresponding spectral peak may be attenuated, indicating a therapeutic effect. Changes in the amplitude of these peaks can therefore be used in closed-loop systems to assess stimulation efficacy, optimize stimulation parameters, or, in the case of intermittent stimulation, disable stimulation when it is no longer required.

However, the PSD can be heavily contaminated by stimulation artefacts. Electrical stimulation introduces substantial spectral power at the stimulation frequency (F) and its harmonics (nF), generating sharp peaks that can obscure physiological activity. If the pathological biomarker lies at or near the stimulation frequency or one of its harmonics, it becomes indistinguishable from the artefact and thus unusable for closed-loop control. Moreover, coupling effects between the tracks of the device increase the recording noise level.

Several strategies have been proposed to mitigate stimulation-induced interference. Common hardware-based approaches involve modifying the recording amplifier during stimulation, such as temporarily disconnecting the sensing electrodes (blanking) or changing the amplifier bandwidth by increasing the low-frequency cut-off of the filters. However, blanking does not allow recording during stimulation and performs poorly, with long recovery times (tens of ms) after the stimulation pulse, while bandwidth changes limit the available sensed information.

Alternative approaches rely on post-processing techniques that attempt to remove stimulation artefacts and reconstruct the underlying neural signal with fitting processes. However, post-processing alone is generally insufficient to recover the neural activity, and combinations of hardware-based and software-based solutions are preferred⁸⁴.

1.6 Neural probe requirements for reliable chronic use.

Long-term performance of neural probes remains limited due to technological challenges related to device instability, as well as biological factors arising from tissue damage and inflammatory reactions triggered by probe implantation, and the mechanical mismatch between the probe and neural tissue^{50,85,86}. Understanding the origin of this damage and the role of probe mechanical properties is therefore critical for improving long-term neural interfacing. In the case of intraneural devices, tissue damage begins immediately after probe insertion. This procedure causes acute mechanical trauma, including rupture of blood vessels, disruption of neuronal processes, and direct cell death along the probe insertion track (Figure 1.9a). This injury initiates the so-called foreign body reaction, characterised by activation of microglia and astrocytes, which migrate to the implant site and release inflammatory molecules that amplify the immune response. This inflammatory environment promotes neuronal degeneration and reduces neuronal density near the electrode. Because spike amplitude depends on the proximity of neurons to the electrode, neuron loss or increased distance reduces signal amplitude and decreases the number of detectable neural units. It has been shown that the mechanical response of neural tissue to the insertion of penetrating implants depends strongly on both the probe geometry⁸⁷ and the insertion speed⁸⁸. Rapid insertion of sharp, stiff probes, such as silicon-based implants, at speeds exceeding 1 mm s^{-1} , is often preferred because it enables clean penetration through neural fibres and cell membranes, thereby minimising tissue deformation and vascular damage. On the other side, slower insertion speeds ($< 200 \text{ } \mu\text{m s}^{-1}$) may better accommodate the viscoelastic properties of brain tissue, allowing displaced capillaries and fibres to recover and reducing the risk of rupture⁸⁹. Additionally, the dimensions of the implant are critical: thin and compact devices produce less tissue damage compared to bulky ones. However, probes with small cross-sectional areas and made from flexible materials tend to bend during insertion due to their low mechanical stiffness. To address this limitation, several strategies have been developed to facilitate the implantation of flexible devices, including the use of insertion shuttles^{90,91} or needles⁹², dissolvable rigid carriers, and temporary stiffening of the probe backbone⁹³ to improve mechanical stability during insertion.

In addition to acute insertion-induced damage, chronic mechanical stress can play an important role in sustaining inflammation. The brain is a soft, viscoelastic tissue, whereas traditional neural probes made of silicon are rigid. This difference of several orders of magnitude creates a severe mechanical mismatch between the probe and the surrounding tissue. As the brain moves due to physiological processes such as respiration, heartbeat, and head motion, rigid intraneural probes do not deform with the tissue. Instead, relative motion occurs at the probe–tissue interface, producing repeated mechanical strain and micro-injury. This chronic micromotion leads to persistent activation of microglia and astrocytes. Over time, astrocytes form a dense encapsulation layer around the probe, known as a glial scar. This scar tissue acts as both a physical and electrical barrier between neurons and the electrode surface. The increased separation distance reduces recorded signal amplitude, since the low conductive

encapsulation layer increases impedance in particular at high frequencies⁹⁴ (> 1 kHz). Moreover, the presence of this low conductive layer in between the electrodes and the tissue, requires the injection of higher current to reach the activation threshold of the neural tissue.^{12,94} Furthermore, charge injection limits of porous materials can drastically decrease, since protein adsorption, cellular adhesion, and fibrotic tissue formation can partially occlude the pores, reducing ionic transport into the electrode and decreasing the electrochemically accessible surface area⁹⁵. This effect could potentially lead to a greater proportional reduction in capacitance and charge injection capacity compared to planar electrodes, whose performance depends primarily on their geometrical surface. As a result, the performance of porous electrodes is more sensitive to surface fouling and diffusion limitations.

To mitigate foreign body response produced by implants, the implanted device should be minimally invasive and be able to bend and adapt to the brain movements without creating a mismatch with the tissue. The bending stiffness can be calculated as $B = \frac{E t^3}{12(1-\gamma^2)}$, where E is Young's modulus, γ the Poisson's ratio and t the thickness of the device. The Young modulus can be calculated as $E = \frac{\varphi}{\sigma}$ where: σ is the stress (Pa) and φ the strain (dimensionless). The bending stiffness is dominated by the device's thickness; even rigid materials, with high Young modulus, can be semi-flexible if thin enough. However, Young's modulus remains important because it governs the device's local adaptability to the brain, while bending stiffness governs the device's overall response. The brain elastic modulus (Young modulus) is on the order of 1–10 kPa, whereas traditional neural probes made of silicon have an elastic modulus on the order of 100–200 GPa. Several materials that combine lower Young modulus and that can be fabricated in ultra-thin form, are emerging as interesting substrates for neural interfaces. The most commonly used are flexible materials like polyimide^{63,92,94}, parylene-C^{96,97}, SU8^{98,99}, and stretchable materials like PDMS^{100,101}, as well as soft hydrogels^{102,103} like polyethylene glycol (PEG) or polyvinyl alcohol (PVA), gelatine, and alginate. These materials are used by many established neurotech and spin-off companies and represent a promising new direction for neural interfaces (Figure 1.8).



Figure 1.8 | Companies working with advanced brain implants. Images of implantable devices used in some neurotech and spin-off companies.

Literature reports support the benefits of soft neural interfaces. Ultrasoft microwire electrodes made of PDMS have demonstrated a reduced inflammatory response compared to conventional tungsten electrodes⁸⁵. Flexible parylene-C microwire electrodes implanted in the rabbit cortex showed a reduced foreign body response and higher neuronal density surrounding the electrode compared to traditional microwires, even after long-term implantation up to 96 weeks¹⁰⁴.

Besides mechanical match, studies have shown that reduced implant dimensions lead to less initial tissue damage, lower neuronal loss near the implant, and diminished chronic tissue responses^{105,106}. Compared to multi-electrode silicon shank probes, ultra-miniaturised fibres induce a significantly reduced glial response, cause less disruption of the blood–brain barrier, and result in decreased tissue encapsulation around the implant site.^{105,107} Another promising strategy to reduce invasiveness involves the use of flexible mesh devices. In this approach, the implant exhibits not only extremely low bending stiffness but also a minimal footprint, high conformability¹⁰⁸ and promotes tissue integration within the open structure. These mesh devices are typically inserted *in vivo* using a cannula¹⁰⁹, or with the addition of temporary, water soluble coatings such as PEG and PVA to provide sufficient stiffness during implantation. Once the coating dissolves after insertion, the mesh unfolds and conforms to the surrounding tissue, forming a three-dimensional neural interface. Over the following weeks, cortical tissue progressively integrates into the microporous network, demonstrating excellent

biocompatibility^{89,109}. This intimate integration minimizes the distance between neurons and electrodes, thereby improving both recording and stimulation performance. As a result, mesh devices enable stable, long-term recordings of single-unit activity, maintaining reliable sorted spike detection for periods of one year or longer¹¹⁰, representing a significant improvement compared to conventional flexible devices⁶³.

Furthermore, by leveraging established cardiovascular technologies, this mesh architecture can be adapted into a stentrod. ⁸⁹ This innovative device combines a vascular stent with embedded electrodes and transmitters, which can be delivered through blood vessels using a catheter to interface with neural tissue in a minimally invasive way.

Some strategies integrate coatings with drugs, such as dexamethasone^{111–113}, to improve chronic neural recordings by locally suppressing inflammation and reducing glial scar formation, which helps preserve nearby neuronal populations and maintain signal quality over time. Similarly, hydrogel coatings provide a soft, tissue-mimicking interface that minimizes mechanical mismatch between the rigid implant and the brain, reducing tissue damage caused by micromotion. Hydrogels can also serve as carriers for neurotrophic factors or anti-inflammatory agents, promoting neuronal integration at the electrode surface and further enhancing long-term recording stability. Together, these strategies help create a more biocompatible electrode-tissue interface, improving both the reliability and longevity of intracortical implants. Bioactive¹⁰⁵ or biomimetic coatings, such as extracellular matrix proteins or adhesion-promoting peptides, encourage neuronal attachment while minimizing glial encapsulation, enhancing electrode-neuron coupling. Surface engineering, including nanopatterning or microgrooves, further directs neuronal growth and improves signal fidelity. Additionally, low-level electrical stimulation¹¹⁴ of surrounding tissue can reduce inflammatory responses and promote stable long-term recordings, creating a more functionally reliable implant interface.

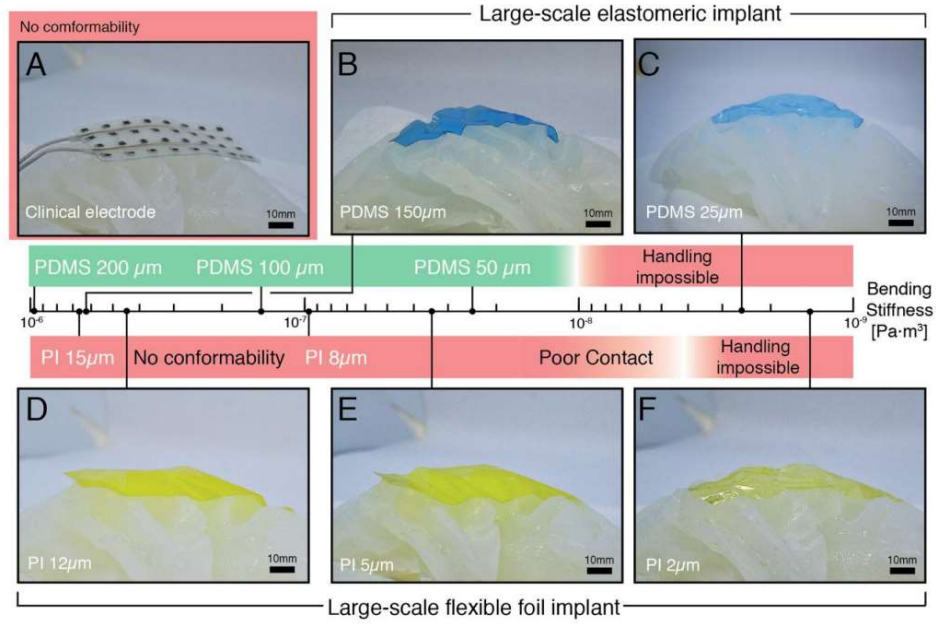


Figure 1.9 | Conformability of epicortical devices. The conformability of different mock-up devices (size of $4 \times 10 \text{ cm}^2$) is studied on an agarose gel brain model with the corresponding bending stiffness reported on the middle scalebar, along with a qualitative evaluation of the conformability and handleability. Tested materials: a, Clinical ECoG array. b-c, Silicone membrane. d-f, Polyimide foil. Figure extracted from¹¹⁵.

In epicortical devices, mechanical matching with brain tissue to reduce the chronic foreign body response (FBR) is less critical than in intracortical implants, because these devices are typically encapsulated in a rigid epoxy layer on the top side, in contact with the scalp and are not inserted into the tissue. Instead, the key requirement is the ability of the device to conform closely to the brain surface (Figure 1.10), or to the nerve anatomy in the case of epineural implants.

The brain surface exhibits natural curvature and roughness, which can reduce the contact between electrodes and neural tissue when the device lacks sufficient conformability. This limitation, combined with the presence of the pia mater and cerebrospinal fluid, increases the distance between the electrodes and neurons, thereby reducing the signal amplitude and signal-to-noise ratio¹⁰⁸ (SNR). As a result, recording single-unit activity from the cortical surface is more challenging compared to intracortical recordings, where the surrounding tissue provides mechanical support and maintains close electrode–neuron proximity.

Improving device conformability enhances electrode-tissue contact, enabling recordings from a larger number of electrodes and achieving higher SNR¹⁰⁸. To achieve high conformability, devices must have a low Young's modulus and minimal thickness. A device spontaneously conforms if it can bend to reach

the curvature radius of the brain $r_b = \sqrt{\frac{B}{2\sigma}}$, where B is the bending stiffness (previously defined as $B =$

$\frac{E t^3}{12(1-\gamma^2)})$, and $\mathcal{G}$ is the adhesion energy per unit area between the device and tissue. Fixed the brain conformability radius and the Young modulus of the material, it is possible to identify the device thickness to reach conformability¹¹⁶ $t < t_c$. Soft materials such as PDMS can conform well to the brain surface when fabricated below approximately 25 μm in thickness, while stiffer polymers such as polyimide can also achieve good conformability when reduced to thicknesses around 3 μm ¹⁰⁸ (Figure 1.9). However, when reducing device thickness, it is important to consider mechanical stability for handling and implantation procedures to avoid fragility and possible mechanical failures.¹¹⁵ Additionally, mesh structures provide superior adaptability to brain surface irregularities and have demonstrated the ability to record both local field potentials and single-unit activity from the cortical surface¹⁰⁸. An example is the NeuroGrid⁷⁷, an ultra-thin, highly flexible surface array that can conform precisely to the cortical folds and detect spikes from superficial cortical neurons, which is normally challenging with conventional rigid surface electrodes.

For applications in the spinal cord or peripheral nerves, stretchability^{68,117} is also important. Movements of the spinal cord or sciatic nerve during limb motion induce extension of the device, which over time can cause fracturing of metal traces or device encapsulation⁹⁴. Stretchable substrates such as PDMS^{68,101} or hydrogels¹⁰² exhibit shape-memory behaviour, allowing reversible deformation without failure. Flexible polymers such as polyimide can be engineered with kirigami-inspired patterns to enhance stretchability. Since metal traces are generally not stretchable, they can be patterned in serpentine¹¹⁸ geometries and encapsulated in polyimide to accommodate strain without fracturing. An alternative approach is the use of liquid¹¹⁹ or microcracked^{100,120,121} metals, which can elongate under strain while maintaining electrical continuity.

Overall, thin, flexible, and stretchable substrates have an important advantage for integration with soft neural tissue, reducing FBR. However, rigid probes, such as those made from silicon, offer excellent structural stability and advanced microfabrication techniques, enabling the integration of high-density electrode arrays and CMOS electronics directly on the probe shaft, such as the Neuropixel probe^{122–124}. This allows high channel counts, precise spatial sampling, and excellent electrical performance. Flexible probes may exhibit lower structural durability than rigid probes due to their higher water vapour transmission rates and weaker interlayer adhesion, which can promote degradation under humid conditions¹²⁵ and electromechanical stress.^{101,126} To overcome these limitations, hybrid organic–inorganic multilayer stacks have been developed, combining polymer layers with inorganic barrier materials such as HfO_2 ^{127–129}, SiO_2 ^{128,130}, SiN_x ¹³⁰, and Al_2O_3 .^{127,131} These multilayer structures achieve low water vapour transmission rates (WVTR) and demonstrate excellent barrier performance under accelerated ageing conditions. However, inorganic barrier layers such as alumina and hafnia, even at thicknesses below 100 nm, remain intrinsically brittle and lack stretchability. This limitation highlights

the need for stretchable yet hermetic neuroelectronic architectures^{103,118,132}, such as serpentine or mesh conductor layouts and elastomeric encapsulation incorporating embedded inorganic barrier layers.

Chapter 2

Reduced graphene oxide technology

Over the past decades, neural interfaces have been developed to treat disorders of the central and peripheral nervous systems, including movement disorders. Electrical stimulation of neural tissue is widely used in both basic neuroscience research and clinical neuromodulation therapies. As neural interfaces evolve toward high-density arrays with sub-millimeter contacts, conventional electrode materials increasingly struggle to maintain stable performance at the charge densities required for effective stimulation, thereby narrowing electrochemical safety margins and rising concerns about stimulation-induced tissue damage. During this thesis, I contributed to the development of a new neurotechnology based on reduced graphene oxide electrodes with high charge injection capacity and low impedance, integrated into flexible polyimide structures to improve performance and reduce tissue damage. Reduced graphene oxide (rGO) has emerged as a promising material for bidirectional neural interfaces owing to its electrochemical stability, high charge injection capacity, and long-term biocompatibility. In this chapter, I present the electrochemical performance of electrodes of different sizes both in vitro and in vivo.

Moreover, in this chapter I also investigated the short-term impact of acute stimulation delivered at charge densities of 30 and 90 $\mu\text{C}/\text{cm}^2$ using rGO microelectrodes with diameters of 150 μm and 300 μm implanted in the mouse brain.

Introduction

Electrical stimulation of neural tissue is a well-established strategy for modulating activity in the central^{33,81,133} and peripheral nervous systems^{16,134}, widely used in both fundamental neuroscience research and clinical therapies. Clinical neuromodulation technologies, including deep-brain stimulation⁸¹ (DBS) and neuroprosthetic interfaces⁸⁹, rely on implanted electrodes capable of delivering charge robustly for months to years while maintaining electrochemical stability and biocompatibility¹³⁵. As neural interfaces increasingly incorporate dense arrays of small electrode contacts, millimeter safe and effective stimulation becomes progressively more challenging. Decreasing the geometric surface area of electrode contacts increases impedance and limits charge injection capability, often requiring operation at elevated charge densities to achieve therapeutic efficacy.^{63,136}

Conventional electrode materials in the clinic, typically based on noble metals such as platinum or platinum iridium¹³⁷, are limited by their relatively low charge-injection capacity⁵⁸ and by long-term electrochemical and biological degradation processes that may be amplified during chronic stimulation.^{138,87,139} These limitations have motivated the exploration of alternative electrode materials and coatings to improve electrochemical performance and/or biocompatibility, including titanium nitride¹⁴⁰, porous platinum^{57,59,141}, iridium oxide^{51,52,142}, and mixed ionic-electronic conductors such as PEDOT:PSS^{26,53,55}. Despite substantial progress, simultaneously achieving high stimulation capability, stable recording performance, , and long-term in vivo durability remains a significant materials-engineering challenge^{79,143}.

A central constrain in stimulation design is the electrode's charge-injection limit, which is often defined by the charge density that drives electrode polarization toward the onset of water electrolysis or material-specific irreversible reactions. Exceeding these limits can induce pH changes, gas evolution, and electrode degradation^{64,65}. Importantly, in vivo electrochemical limits can be significantly lower than saline-based limits, emphasizing the need for in vivo benchmarking when establishing safe stimulation regime.^{144,145,146} Moreover, tissue injury may occur at stimulation intensities below electrochemical limits through biological mechanisms linked to overstimulation (e.g. hyperactivity-driven injury)^{66,67}, indicating that stimulation safety arises from coupled electrochemical and physiological processes.

As previously described in Chapter one, empirical safety frameworks, such as the Shannon relationship, have historically guided stimulation design⁶⁸. However, these guidelines were derived from macroelectrodes and may not accurately describe stimulation regimes relevant to microelectrodes^{147,66,69}, where tissue response depends strongly on charge per phase, waveform parameters, and electrode geometry. As neural interfaces transition toward dense arrays of sub-millimeter contacts, establishing material-specific and geometry-dependent safety limits becomes increasingly important.

Reduced graphene oxide (rGO) and related graphene-based materials have emerged as promising candidates for bidirectional neural interfaces due to their low impedance and high-charge injection capacity, chemical stability and compatibility with flexible and miniaturized device architectures^{12,148,7,29,17,18}. These properties suggest that rGO may support efficient charge transfer while maintaining stable electrochemical performance. However, despite these advantages, limited *in vivo* evidence exists regarding how high charge density stimulation using rGO microelectrodes affects post-stimulation electrode performance and surrounding tissue responses under conditions relevant to small-contact neural stimulation.

This chapter summarizes our study on the electrochemical stability and biological safety of rGO microelectrodes during controlled subcortical stimulation. By comparing tissue responses adjacent to active stimulating electrodes with contralateral sham implants, we isolated stimulation-specific effects from implantation-related damage. Using rGO microelectrodes of 150 and 300 μm diameters operated at charge densities of 30 and 90 $\mu\text{C}/\text{cm}^2$, we assessed post-stimulation electrochemical behavior as well as tissue response two weeks after stimulation using immunohistochemical markers of inflammation and glial activation. The stimulation paradigm has been designed to reflect a clinically relevant intraoperative pattern of device use, when stimulation is typically delivered only briefly to evaluate electrode placement or functional responses.^{150,151} Our results provide insight into the *in vivo* performance of rGO microelectrodes under high-charge stimulation conditions relevant to high-density neural interfaces.

2.1 Microfabrication of rGO thin-film electrodes

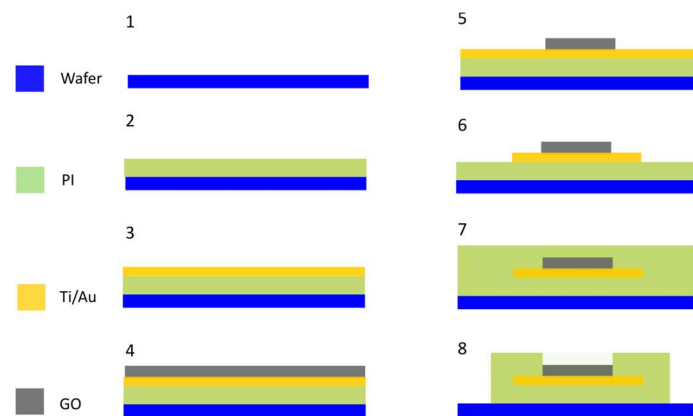


Figure 2.1 | Schematic fabrication process of flexible rGO microelectrodes. a, Schematic fabrication protocol. The legend displays reduced graphene oxide (rGO), polyimide substrate (PI), titanium and gold layers (Ti/Au) and wafer.

Graphene oxide membrane formation

The fabrication process starts by preparing a thin membrane of graphene oxide, by filtering a solution (0.15 mg/mL) of dispersed graphene oxide (GO) flakes in deionised water. The filtration allows the slow deposition of the flakes, while the water goes through the pores, in order to create a stacking of parallel sheets with nanochannels. The flakes interact with each other through van der Waals forces, hydrogen bonding and electrostatic interactions, creating a mechanically stable membrane with an approximate thickness of ~ 2 μm . By defining the solution concentration and quantity, it is possible to control the thickness of the membrane.

Metals and Polyimide deposition

Polyimide was chosen as a support substrate and also for encapsulation, since it is a biocompatible, flexible material, easy to integrate in the fabrication process. It offers good compatibility with standard microfabrication processes, including photolithography and metal patterning, and it can be deposited in thin, flexible layers with relatively good thermal and chemical stability. Compared to parylene, polyimide is generally easier to pattern and integrate in multilayer processes, but parylene provides superior conformal coating and pinhole-free encapsulation. Other materials like polydimethylsiloxane (PDMS) are also good candidates for encapsulation since they offer excellent elasticity; however, they suffer from lower mechanical stability.

In this study, I implemented a 7.5 μm layer of polyimide on a silicon wafer by spin coating (Figure 2.2). Then it was sputtered with 20/200 nm layer of Ti/Au (Figure 2.3). The wafer was treated with oxygen plasma for 1 min (PlasmaPro Cobra 100; Oxford Instruments, UK) to clean and activate the surface. A self-assembling monolayer was used to ensure the adhesion between the GO film and the Au substrate. A solution based on hydrazide-PEG-thiol is prepared by dissolving 24 mg of the compound in 12 mL of EtOH by sonication for 5 minutes (37 kHz, power 30%). The Au surface is cleaned using an ICP reactive ion etching (ICP-RIE) for 1 minute. Once the Au substrate is cleaned, it is immersed in a beaker with the SAM solution overnight (~ 16 h); afterwards, the Au substrate is rinsed with ethanol and dried with N_2 .

SAM formation is driven by the strong affinity of thiol ($-\text{SH}$) groups for gold surfaces, resulting in the formation of robust Au-S bonds with covalent-like character and high chemical stability, as widely reported in the literature.¹⁵² At the opposite terminal, hydrazide groups covalently bind to residual oxygen-containing functionalities on the GO surface through stable hydrazone linkages.¹⁵³ This dual-ended binding renders the SAM an effective molecular interface, significantly enhancing the mechanical adhesion of the GO film to the substrate. The PEG component, polyethylene glycol, works as a biocompatible glue, reinforcing the attachment of the membrane in the transfer process.

GO transfer and reduction

GO membranes were transferred onto the wafer using a wet-transfer technique (Figure 2.3). The membranes were left floating on the surface of deionized water, to fish them with a wafer forming a 45° angle with the water surface. Once alignment was achieved, the wafer was slowly raised from the water, completing the transfer (Figure 2.2a). Speed and angle of the fishing process are important to avoid wrinkle formation on the membrane and increase the yield of fabrication. Then the wafer is baked on a hot plate, first 10 minutes at 60 °C and then 10 minutes at 100 °C. Empirical observations showed that membranes filtered up to one day in advance and transferred with the rGO face that was in contact with the filter facing upward, improved adhesion to the substrate.

Since the membrane edges of the membrane are thicker, and they could leave residues during the RIE etching, they are thinned by gently brushing the perimeter with cleanroom paper wrapped around tweezers and moistened with DIW. The brushing should be gentle, to avoid scratching the gold and consequent cutting of the metal tracks. Furthermore, performing this process before autoclaving is important, because the material is softer and can be removed easily.

Then, the GO membranes are subjected to hydrothermal reduction in an autoclave (AH-21 N22; Raypa, Spain) at 134 °C for 3 h. This reduction step is critical, as pristine GO membranes are electrically insulating, exhibiting extremely high sheet resistance values (up to $10^{12} \Omega/\text{sq}$) due to the high density of oxygen-containing functional groups that disrupt the sp^2 carbon lattice. Hydrothermal treatment effectively removes a substantial fraction of these oxygen functionalities, partially restoring the π -conjugated carbon network and thereby enhancing electrical conductivity by several orders of magnitude¹⁵⁴.

The removal of the oxygen groups decreases the interlayer spacing from 7.8 Å in GO to 3.7 Å in reduced GO (rGO), with a total decrease of the membrane thickness from 2 µm to 1 µm, as confirmed by X-ray diffraction analysis.¹⁵⁵ Furthermore, Raman spectroscopy showed an increased D-to-G band intensity ratio in rGO, indicating a higher degree of structural disorder and defect density introduced during the reduction process¹⁵⁵.

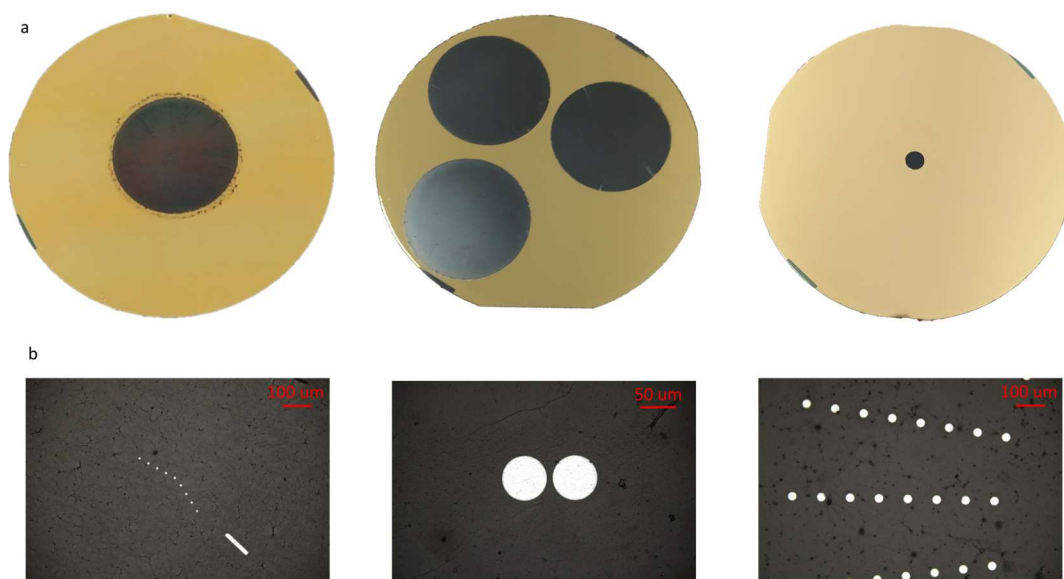


Figure 2.2 | Reduced graphene oxide transfer and patterning. **a**, Optical pictures of 3 wafers after the rGO membrane transfer for 3 different designs of devices (TIME, DBS, and Immuno study). **b**, Aluminum mask used to define the electrode shapes for the 3 different designs shown in panel a.

Patterning of rGO electrodes

To define the electrode's shape, the wafer was spin-coated with the image-reversal photoresist AZ NLOF 2070, and electrode geometries were defined using a KS MA6 mask aligner, with a positive chrome mask. A 80 nm aluminum layer was subsequently deposited by electron-beam evaporation, followed by lift-off in TechniStrip NI555. This process resulted in an Al protection disks, positioned over the place intended for the electrode sites (Figure 2.2b).

These Al disks function as hard masks for the subsequent reactive ion etching (RIE) step used to pattern the rGO. During this step, all the rGO will be removed, except in the areas covered, therefore defining the electrodes. Oxygen plasma etching was performed for up to 50 min (Figure 2.3b), and then the wafer was taken out for a gentle manual cleaning using a wet cleanroom tissue wrapped around the tweezers.

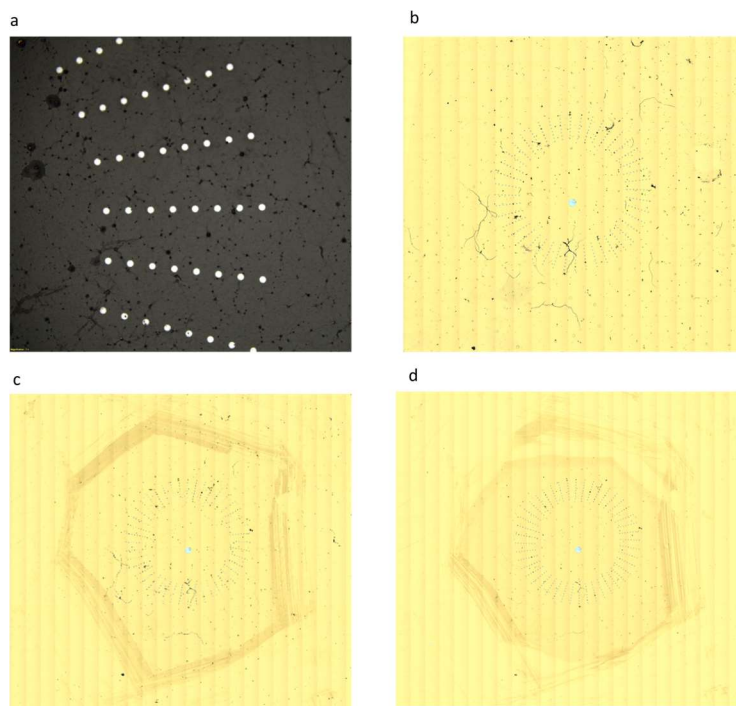


Figure 2.3 | Reduced graphene oxide etching. Optical pictures of a wafer, before etching (panel a) and after 30 (panel b), 40 (panel c) and 70 (panel d) minutes. The electrodes, located in the middle of the pictures, are protected by an aluminum mask.

The manual cleaning will remove part of the material in the area where the etching was less efficient, due to anisotropic gas flow, contamination, and difference of the membrane thicknesses. During this step is important to avoid the areas close to the electrode to avoid the removal of the Al mask. Then, the wafer is etched again in RIE for 10 additional minutes (Figure 2.3e), manually cleaned, and finally etched again for 10 min (Figure 2.3f), for a total of 70 minutes. Total etching time cannot be increased even further because the Al is slowly etched during this process, and the mask might not protect the rGO anymore. This step is critical to device yield, since any residual rGO can act as an electrical connector in between the gold tracks, leading to electrical shorts between electrodes.

Metal track patterning and passivation

Metal track definition was carried out using the positive photoresist HiPR 6512 (Fujifilm Electronic Materials, Japan) followed by standard photolithography with a positive mask design. In this layout, the electrode tracks, contact pads, and electrode areas were protected by the patterned photoresist. The developed resist acted as an etch-stop mask during subsequent wet etching steps, which were used to sequentially pattern the underlying Ti/Au layers. A second polyimide (PI) layer with a thickness of 2 μm was subsequently deposited on top of the patterned structures to serve as a passivation layer. Prior to PI deposition, the wafer underwent an oxygen plasma cleaning step (30 s, 200 W, 600 sccm O_2 , 20 $^\circ\text{C}$) to promote surface cleanliness and adhesion.

Three different photolithography steps were employed to open the electrode sites and contact pads, and the structure of the array. These photolithography steps utilized the positive photoresist AZ10XT (MicroChemicals GmbH, Germany) in combination with negative mask designs. Having a separate process for each opening is important to perform RIE for different etching times, since the opening of the PI is dependent on the dimension of the surface: we used 4.5 min for the electrodes. 6.6 min for the pads and 19 min for the structure. In the case of the electrodes' opening, the presence of the Al mask, remaining from the process of rGO electrode definition, allows the protection during RIE etching of the PI. This avoids the material from being etched and also contamination produced by the exposure to photoresists. At the end of the fabrication process, the Al hardmask is removed by wet etching using an acidic etchant solution. The completed devices are mechanically peeled off the carrier wafer.

2.2 Electrochemical performance in saline

Electrochemical characterization was performed to evaluate the functionality of the electrodes and ensure no broken active sites. It was performed by connecting the fabricated devices to a potentiostat by using a printed circuit board (PCB, Figure 2.4). The potentiostat was used in a three-electrode configuration with an Ag/AgCl electrode as the reference electrode and a platinum wire as the counter-electrode. Devices were immersed in a buffer solution containing 10 mM phosphate buffer, 137 mM NaCl, and 2.7 mM KCl at pH 7.4.

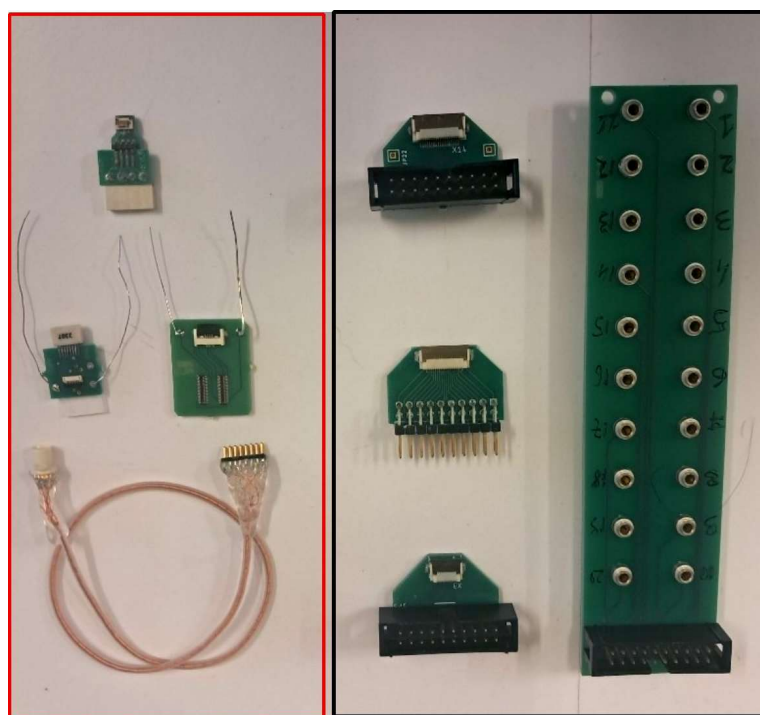


Figure 2.4 | Printed circuit boards as device connectors. Printed circuit boards (PCB) used to connect the flexible probes with the stimulators for in vivo experiments with animals (red box) and to connect with the potentiostat for electrode characterization and activation (black box).

Electrochemical characterization in PBS involved measuring impedance, cyclic voltammetry, and current pulses (Figure 2.5). Impedance represents the opposition that an electrode presents to the flow of alternating electrical current at a given frequency, and can help to assess how easily electrical signals can pass between the electrode and the medium. If the impedance is high, more voltage is required to inject the same current, the system becomes more sensitive to noise, and the signals may become distorted. On the other side, low impedance translates to lower noise and the capability to record with a higher signal-to-noise ratio, so it is measured to provide feedback about the recording capabilities of the electrodes.¹⁵⁶ Impedance was measured by applying a 10 mV sinusoidal wave over a frequency range from 1 Hz to 100 kHz, and measuring impedance magnitude and phase. At high frequencies (typically above 1 kHz), the impedance spectrum is mainly governed by a resistive element, which combines the medium resistance and contributions from the electronic circuitry. In the intermediate frequency range (approximately 10 Hz to 1 kHz), the response is typically dominated by capacitive effects associated with double-layer formation at the electrode–electrolyte interface. This behavior reflects factors such as the electrode’s surface area, intrinsic material properties, and any surface modifications. At low frequencies (below 10 Hz), impedance is increasingly affected by diffusion-limited processes and charge-transfer resistance, providing insight into the kinetics of electrochemical reactions occurring at the electrode surface. The phase angle indicates the phase shift between the applied voltage and the resulting current. A phase angle approaching 90° signifies capacitive dominance, meaning that the interface primarily stores charge with minimal contribution from faradaic reactions. On the other side, a phase angle near 0° indicates predominantly resistive behavior, typically associated with charge-transfer processes, and limited capacitive storage. Typical electrode behaviours are described by circuit elements like the Randles circuit presented in Chapter one (Figure 1.1c), which is composed of a capacitor or constant phase element (CPE) and a resistor in parallel, followed by a resistor for the spreading resistance of the tissue. The CPE accounts for non-ideal effects on the electrode-electrolyte interface that bring the electrode to phase values different than -90° .

Cyclic voltammetry (CV) is an electrochemical technique in which the voltage applied to an electrode is swept linearly forward and backwards between two set limits, while the resulting current is measured. It provides information regarding the presence of reduction or oxidation peaks, if these reactions are reversible or not, and about the electrochemical safety potential window to operate the electrode, to avoid irreversible reactions. Each electrode material has a different electrochemical safety potential window⁴⁶, which determines the maximum voltage excursion that can be generated during electrical stimulation. In the case of rGO, by looking at the oxidation and redox reactions produced at high excursion voltage, safety limits are established in a first approximation from 0.8 to -0.9V.¹⁴⁹ Cyclic voltammetry is also useful to quantify the capacitive behaviour of an electrode, since the integral of the CV can provide information on the charge storage capacity of an electrode. Chronopotentiometry was

used to evaluate the behaviour of electrodes during electrical stimulation. A current pulse is injected between the working and counter electrodes, and the resulting time-dependent voltage polarization of the working electrode is measured versus the reference electrode. The analysis of the voltage curve reveals the maximum current that can be injected, also known as the charge-injection-limit (CIL), without overcoming the voltage limits previously defined. Another feature that can be considered when assessing safety stimulation regime is the presence of a flexion point in the interfacial polarization voltage usually associated with the presence of faradaic reactions¹⁵⁶.

Before the *in vitro* electrochemical evaluation, the electrodes underwent an electrochemical activation process consisting of cyclic voltammetry cycles or electrical pulses to improve charge injection limit and impedance. This activation process further reduces the partially reduced graphene oxide even more, therefore enhancing the conductivity of the electrodes¹⁴⁹. During this process, oxygen groups (such as carbonyl or quinone-like groups) can be converted into hydroquinone-like species¹⁵⁷. These groups are electroactive and can undergo reversible redox reactions, contributing to pseudo capacitance, which increases the overall capacitance of the electrode. Furthermore, the reduction process, particularly at the edges of the graphene oxide flakes, increases the wettability and hydrophilicity of the nanopores within the film, which translate in reduced ionic diffusion resistance^{149,158}. This enables the electrolyte to access the smaller, nanometric cavities of the material, therefore increasing the electroactive area and the total capacitance. The reduction of the oxygen groups, generate also a decrease in the interflake distance, as observed by Raman analysis¹⁴⁹. After activation, the electrodes exhibit a pseudocapacitive dynamic response that depends on electrode bias. At negative potentials, the reversible electro-adsorption/desorption of the cationic species¹⁴⁹ enhances the electrode charge storage capability¹⁵⁹. The adsorption of positive ions promotes interflake stacking due to an electrostatic interaction between the adsorbed cations and the electron-rich π system of the vacancies in the graphene basal plane¹⁴⁹. Overall, increased reduction levels, ion confinement and electro-adsorption/desorption increase the total charge storage capacitance, thereby improving electrodes' performance.

Two different protocols have been used for activation: one consisting in applying 100 cycles of cyclic voltammetry from -0.9 V to 0.8 V at 50 mV/s, and one applying 70,000 biphasic rectangular current pulses (cathodic first) of 100 Hz, 1 ms pulse width, ramping the pulse amplitude, depending on the electrode size (Figure 2.5).

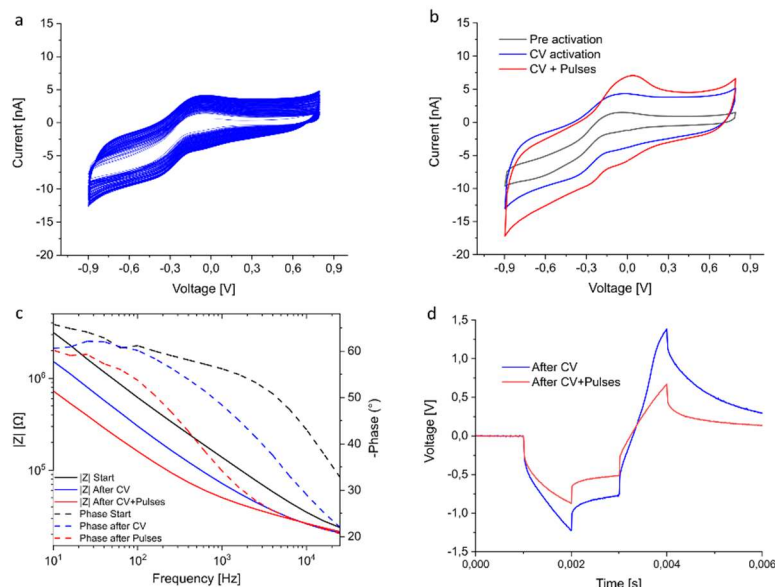


Figure 2.5 | Effect of activation process on electrode performance. **a**, 100 cycles of cyclic voltammetry from -0.9 V to 0.8 V at 50 mV/s with an electrode of 25 μ m diameter. **b**, Cyclic voltammograms recorded with an rGO microelectrode before (black), after CV activation (blue), and after pulse activation (red). **c**, Impedance recorded from the electrode activated with the conditions of panel b. **d**, Voltage transient curves generated in response to a biphasic current pulse of 10 μ A (pulse width and interphase width of 1 ms) recorded with an electrode after CV activation (blue) and after CV plus pulse activation (red).

It has been empirically shown that cyclic voltammetry offers more stable activation compared to electrical pulses, and the electrodes can stay in air for several weeks, with negligible loss of performance. However, to achieve optimal activation, electrical activation after CV is required, as it is possible to observe the further reduction of impedance, and interfacial polarisation voltage under electrical stimulation in Figure 5. The need for an extra activation process by current pulses is not fully understood; it can be tentatively attributed to the need for a larger excursion of both voltage and current range, which are limited when applying the cyclic voltammetry. After completing the activation, the electrochemical characterization provides important insights on the electrodes' behaviour and performance.

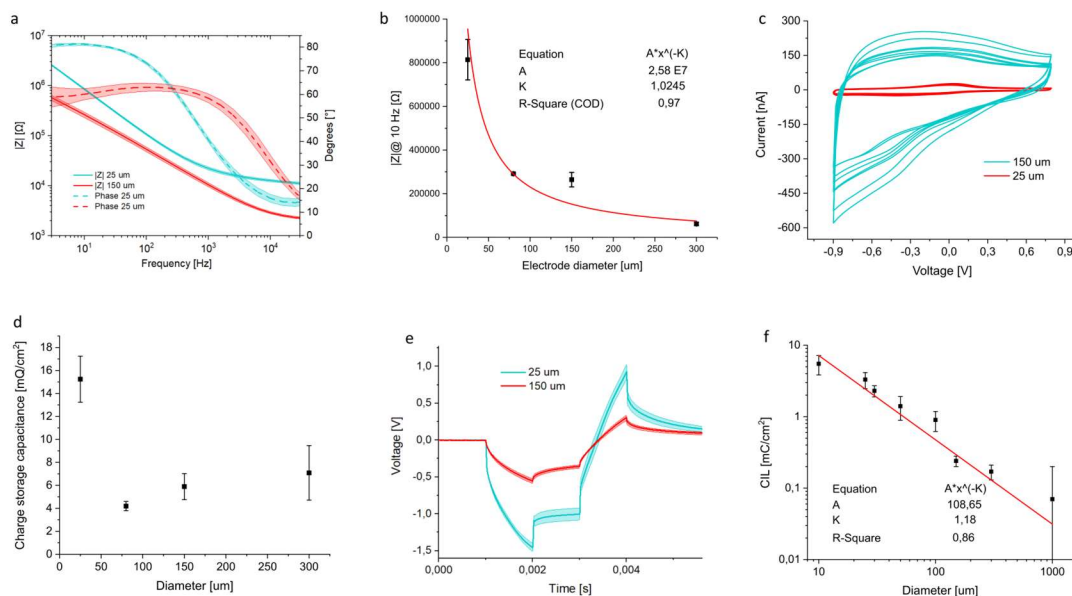


Figure 2.6 | Electrode performance and scaling factors. **a**, Average impedance magnitude and phase measured with microelectrodes of 25 μm (blue, n=8) and 150 μm (red, n=8) diameter. The shadowed area corresponds to the STD. **b**, Average impedance magnitude values extracted at 10 Hz for electrodes of different diameters (n=8 per each size). **c**, Cyclic voltammograms measured with microelectrodes of 25 μm (blue, n=8) and 150 μm (red, n=8) diameter, at 0.5 V/s scan rate. **d**, Average charge storage capacitance (normalized by geometrical area) (with STD whiskers) for electrodes of different diameters (n=8 per each size). **e**, Average electrode voltage transient recorded with microelectrodes of 25 μm (blue, n=8) and 150 μm (red, n=8) diameter in response to a biphasic current pulse (20 μA, 1 ms pulse width). **f**, Average charge injection limit (normalized by geometrical area) with STD whiskers, for electrodes of different diameters (n>20 per each size), with the fit in red

Electrodes exhibit a pseudocapacitive behaviour, as it is possible to estimate from the impedance phase that did not reach the ideal -90° (Figure 2.6a). This non-ideal capacitive nature of the electrochemical double layer is more prominent when increasing the electrode diameter. Impedance magnitude showed generally similar frequency-dependent patterns across electrodes of different sizes, with the absolute value decreasing by increasing electrode diameter (Figure 2.6a). The extracted values at 10 Hz for different sizes show that for both capacitive and resistive components, the response of the magnitude decreases as one over the diameter when scaling up the diameter size, highlighting a strong dependence on the perimeter, not the area (Figure 2.6b).

The cyclic voltammetry curves did not exhibit distinct redox peaks within the safe operating window of rGO, indicating the absence of significant faradaic reactions. Instead, the electrodes displayed predominantly capacitive behavior, as expected for a porous material with a high effective surface area (Figure 2.6c).

The interfacial polarization under electrical pulses shows lower negative interfacial polarization compared to the positive (Figure 2.6e); this is tentatively attributed to the higher capacitance of rGO

electrodes at negative bias voltages, resulting from reversible electro-adsorption/desorption of the cationic species, which is not present at a positive voltage¹⁴⁹. The calculated charge injection limit scales as $\sim 1/D^{1.18}$, when increasing the diameter of the electrodes, which indicates a dependence on the electrode perimeter rather than the area of the electrode (Figure 2.6f). Similar scaling of charge injection with electrode size has been previously observed, in particular in porous materials like IrOx⁴⁶, platinum nanorods¹⁶⁰ or rGO (Figure 2.7a,b), as well as for volumetric (PEDOT)¹⁶¹ (Figure 2.7).

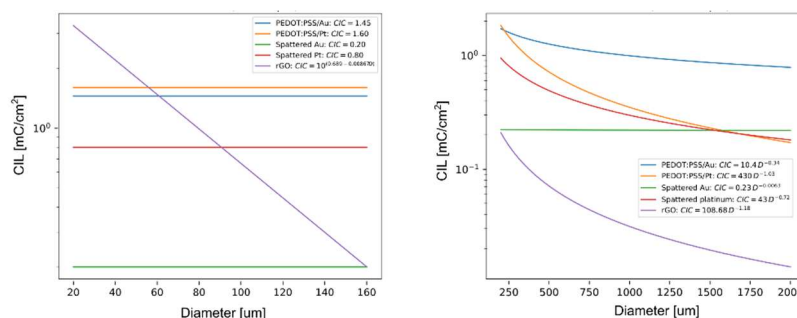


Figure 2.7 | Scaling factors for different materials and electrode sizes. On the left, linear fit of the average charge injection limits, for electrodes of different materials and diameters, up to 160 μm . On the right, fit for electrode diameters from 250 μm to 2 mm. Data extracted from¹⁶¹

2.3 Operational safety limits of rGO microelectrodes

The electrochemical and tissue safety limits of rGO electrodes were studied with devices containing four rGO electrodes with diameters of either 150 or 300 μm . The devices are 10 μm thin and 430 μm wide at the tip; the four electrodes are distributed two at the tip and two closer to the tail, at a distance of 1.5 mm from the electrodes in the tip. The spacing between electrodes, both in tip and tail, is of 360 μm (Figure 2.8a). All devices were electrochemically characterized in phosphate buffer saline (PBS) before implantation (Figure 2.8).

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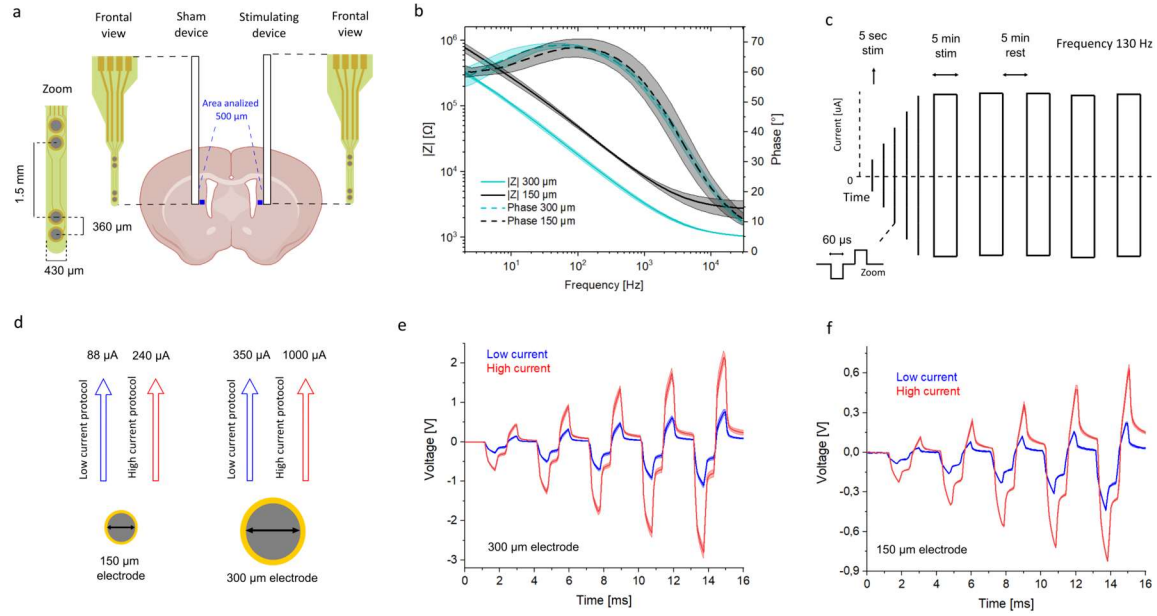


Figure 2.8 | Experiment design and electrode performance. **a**, Schematic of a device and the implantation strategy with a representative image of a brain slice. An active (stimulating) device and a sham device were implanted in the caudate–putamen of opposite hemispheres, while counter and reference electrodes were placed in the cerebellum. **b**, Average impedance magnitude (solid line) and phase (dashed line) measured in PBS for rGO electrodes of 150 μm diameter ($n=4$, black) and 300 μm ($n=4$, light blue); shaded regions indicate standard deviation. **c**, Schematic representation of the *in vivo* stimulation protocol. Biphasic current pulses (60 μs per phase) were injected for 5 s at progressively increasing amplitudes separated by 5 min rest intervals, followed by repeated cycles of 5 min stimulation at the maximum current amplitude alternating with 5 min rest periods. **d**, Description of current levels used during the 5 min stimulation cycles. Devices with 150 μm and 300 μm electrodes were stimulated using two protocols corresponding to low (30 μC/cm²) and high (90 μC/cm²) charge densities. **e–f**, Average voltage transients during the stimulation ramp for the high (red curves) and low (blue curves) current protocols, injected with the four 300 μm electrodes of one device (**e**) and for the electrode of 150 μm (**f**).

The Bode plot of the impedance for the two electrode sizes shows (Figure 2.8b) spectra characteristic of rGO microelectrodes, in which the high-frequency region is dominated by the series resistance of the PBS electrolyte, while the low-frequency response reflects the quasi-ideal capacitive response of the rGO interface, evidenced by an impedance phase that remains below -90° .^{136,149,162} Consistent with geometric scaling,¹³⁶ larger electrodes present lower impedance, with representative 1 kHz values of ~ 4 kΩ for 300 μm electrodes and ~ 8 kΩ for 150 μm electrodes.

The four stimulation protocols used *in vivo* were first tested in PBS to evaluate the electrode polarization generated at different current amplitudes. Biphasic current pulses (60 μs pulse width, 130 Hz) were delivered for 5 s at progressively increasing current levels, applied in five steps with the amplitude increased every 5 min (Figure 2.8c). This ramping sequence was followed by five continuous cycles consisting of 5 min of stimulation alternating with 5 min of rest (Figure 2.8c). The final current levels for the low- and high-intensity protocols were selected to produce charge densities of 30 or 90 μC/cm²

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for both electrode sizes (Figure 2.8d). Under these conditions, the interfacial polarization voltage measured during the current ramps in PBS remained within the electrochemical safe window of rGO (-0.9 to +0.8 V)¹⁴⁹ (Figure 2.2.8e–f). The stimulation paradigm investigated here was designed to approximate conditions encountered during intraoperative testing of neural implants, where stimulation is applied briefly to evaluate electrode placement or functional responses rather than delivered continuously over extended periods.

Following validation of the stimulation protocols in PBS, devices were stereotactically implanted into the mouse caudate-putamen ($n = 4$ -5 per group), with bilateral implantation of one device per hemisphere (see Methods in Annex 1 for surgical details). This region was selected because of its relatively homogeneous cytoarchitecture over a broad area. The device implanted in the left hemisphere was used for stimulation, while the contralateral device served as an electrically inactive sham control. After implantation, electrodes were electrochemically characterized *in vivo* using electrochemical impedance spectroscopy, cyclic voltammetry, and chronopotentiometry to confirm correct functionality following insertion. Based on these measurements, one distal electrode was selected for stimulation. Electrical stimulation was performed under anesthesia, after which the stimulated electrode was characterized again *in vivo*. Devices were subsequently explanted and characterized once more in PBS. Animals were allowed to recover and were euthanized 13-15 days later by transcardial perfusion. Brains were extracted, sectioned, and processed for immunohistochemistry targeting GFAP and Iba-1 to assess astrocytic scarring and microglial activation, respectively, in both hemispheres.

2.4 Comparison of electrochemical response in saline and in the brain

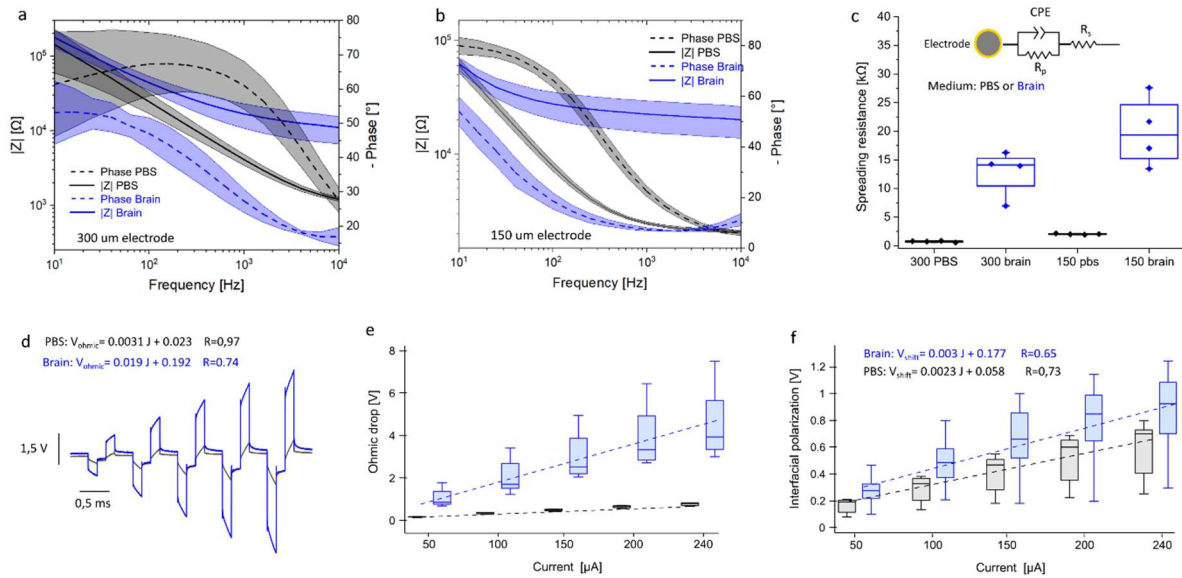


Figure 2.9 | Electrochemical characteristics of nanoporous graphene electrodes in saline and in vivo. **a**, Average impedance magnitude (solid lines) and phase (dashed line) for 300 μm electrodes ($n=4$, from 4 different devices) measured in PBS (black) and in vivo after implantation (light blue); shaded regions indicate standard deviation **b**, Average impedance spectra for 150 μm electrodes ($n=4$)

measured under the same conditions as in panel a. **c**, Spreading resistance extracted from the fits for the electrodes in panels a and b. Box plots indicate the interquartile range (25th-75th percentiles), with whiskers representing the 5th-95th percentiles. The inset shows the equivalent Randles circuit used to fit impedance spectra. **d**, Representative voltage transients recorded from a 150 μm electrode during the high-current stimulation ramp in PBS (black) and in brain tissue after implantation (blue). **e**, Ohmic drop extracted from voltage response measurements obtained from four 150 μm electrodes across four implants at each current amplitude of the high-current stimulation protocol. Box plots indicate the interquartile range (25th-75th percentiles), with whiskers representing the 5th-95th percentiles. **f**, Interfacial polarization (including both cathodic and anodic phases) extracted from the electrodes shown in panel d.

Excessive interfacial polarization beyond the limits of water electrolysis or the intrinsic stability window of the electrode material can induce irreversible faradaic reactions and tissue damage. To design stimulation protocols that comply with established safety limits, it is therefore essential to compare the *in vivo* electrochemical response of the electrodes with their behavior in saline. Electrochemical characterization (Figure 2.9), including cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), and chronopotentiometry, was performed for both electrode sizes before implantation in PBS and after implantation in brain tissue (black and blue curves, respectively, in Figure 2.9).

Both rGO electrode sizes showed asymmetric CV curves in PBS and in brain tissue (Figure 2.10a,b), consistent with previously reported asymmetric ion transport within the porous structure of rGO electrodes.^{149,162} In addition, charge storage capacitance (CSC) increased by approximately 30% in brain tissue relative to PBS. Although the mechanism underlying this increase remains unclear, it may reflect differences in ionic composition and transport properties between PBS and the *in vivo* extracellular environment.

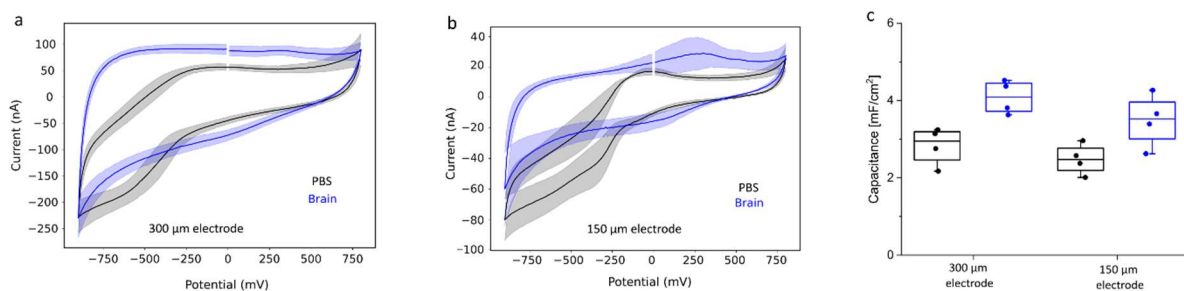


Figure 2.10 | Cyclic voltammetry experiments conducted in PBS and in brain tissue. a-b, Average cyclic voltammograms (50 mV/s scan rate) recorded with 300 μm (panel a) and 150 μm (panel b) electrodes in PBS (black) and in brain tissue after implantation (blue); shadow area indicates the STD measured with four electrodes of 4 different devices. c, Charge storage capacitance calculated in PBS (black) and in brain tissue (blue) for 300 μm (n=4) and 150 μm (n=4) electrodes, extracted from the cyclic voltammetry experiments shown in panels a and b.

Impedance spectra (Figure 2.9a,b) show that at low frequencies (~ 10 Hz) the response is dominated by electrode capacitance, with slightly lower impedance magnitude values in PBS compared to the brain tissue, in agreement with the response of the capacitance extracted from cyclic voltammetry (see Figure 2.10). At higher frequencies (20 Hz–20 kHz), however, the impedance magnitude is consistently higher in the brain. Although $1\times$ PBS and the brain extracellular space have comparable osmolarity¹⁶³ ($\sim 295 \pm 15$ mOsm), structural, heterogeneity and the presence of cellular components substantially increase the effective resistance between the electrode surface and the surrounding medium in brain tissue, thereby increasing the high-frequency impedance.^{68,72}

To further interpret these results, the EIS spectra were fitted using a Randles equivalent circuit model. The double-layer capacitance at the electrode–tissue interface showed frequency-dependent behavior and was modelled as a constant phase element (CPE) in parallel with a resistor, in series with the spreading resistance between the working and counter electrodes (Figure 2.9c). This spreading resistance R_s is inversely proportional to the conductivity of the surrounding medium (σ) and the electrode radius (r), according to¹⁶⁴ $R_s = \frac{1}{2\sigma r}$. Consequently, higher spreading resistance values are expected for smaller electrodes and for media with lower conductivity. Consistent with this prediction, the average spreading resistance extracted in PBS was 0.7 ± 0.1 k Ω for the 300 μ m electrodes and 2.0 ± 0.1 k Ω for the 150 μ m electrodes. In brain tissue, these values increased to 12.9 ± 4.1 k Ω and 19.2 ± 6.1 k Ω , respectively (Figure 2.9c), reflecting the lower conductivity of brain tissue¹⁶⁵ compared to PBS. The calculated conductivities were 2.38 S/m and 1.67 S/m in PBS, and 0.129 S/m and 0.174 S/m, respectively, for 300- and 150- μ m electrodes.

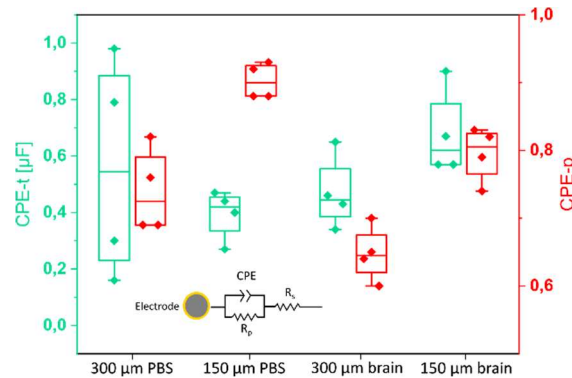


Figure 2.11 | Comparison of constant phase element (CPE) parameters for the different electrode size and experimental conditions. Values of CPE-t parameter (green) and CPE-p parameter (red) of the CPE element used to fit the impedance spectra of the 300 μ m ($n=8$) and 150 μ m ($n=8$) electrodes, in the brain and in PBS. Box plots indicate the interquartile range (25th-75th percentiles), with whiskers representing the 5th-95th percentiles. Inset: schematic of the Randles model, with the CPE element, used to fit the impedance spectra.

The extracted CPE-p values indicate pseudocapacitive behavior, with slightly higher values in PBS (0.7 ± 0.3 and 0.9 ± 0.03) than in brain tissue (0.6 ± 0.02 and 0.8 ± 0.04) for the 300 and 150 μ m electrodes, respectively (Figure 2.11), suggesting behavior closer to an ideal capacitor (CPE-p = 1) in PBS. Finally,

the voltage response during electrical stimulation was strongly influenced by the surrounding medium (Figure 2.9d). Using the Randles model as a first approximation, the measured voltage transient was decomposed into the interfacial polarization associated with double-layer capacitance and the ohmic drop arising from the effective resistance of the medium¹⁶⁰. Both components scaled linearly with the injected current (Figures 2.9e, f). Linear fits showed that interfacial polarization in brain tissue was 1.8 to 7.2 times larger than in PBS, depending on current amplitude and electrode size, consistent with values reported for other electrode materials^{140,166,167} (Figure 2.9f). This increase is attributable to the lower effective capacitance in brain tissue at the stimulation frequency (16.6 kHz) compared with PBS. Despite this increase, the interfacial polarization remained within the electrochemical safety window of rGO electrodes (0.8 to −0.9 V) for all conditions except the highest-current protocol with 300 μm electrodes (Figure 2.12 and 2.13). The ohmic drop, related to the spreading resistance of the surrounding medium, showed an even more pronounced difference, with substantially larger values in brain tissue than in PBS (Figure 2.9e, 2.12 and 2.13).

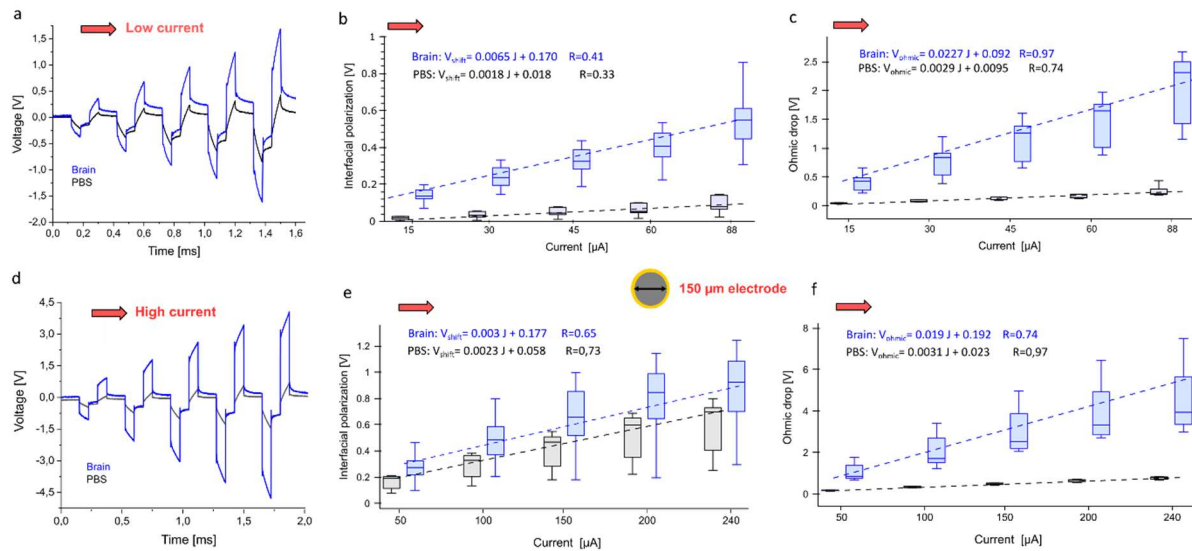


Figure 2.12 | Comparison of in vitro and in vivo responses of rGO microelectrodes of 150 μm diameter. **a.** Exemplary voltage transients recorded from a 150 μm electrode during the low-current stimulation ramp in PBS (black) and in brain tissue after implantation (blue). **b.** Interfacial polarization extracted from voltage response measurements obtained from four 150 μm electrodes across four implants at each current amplitude of the low-current stimulation protocol, in PBS (black) and in brain tissue after implantation (blue). Box plots indicate the interquartile range (25th-75th percentiles), with whiskers representing the 5th-95th percentiles. Dashed lines are linear fits of the data. **c.** Ohmic drop of the electrodes in panel b. **d-e-f.** Measurements conducted for the high-current protocol, under the same conditions described for panels a-c.

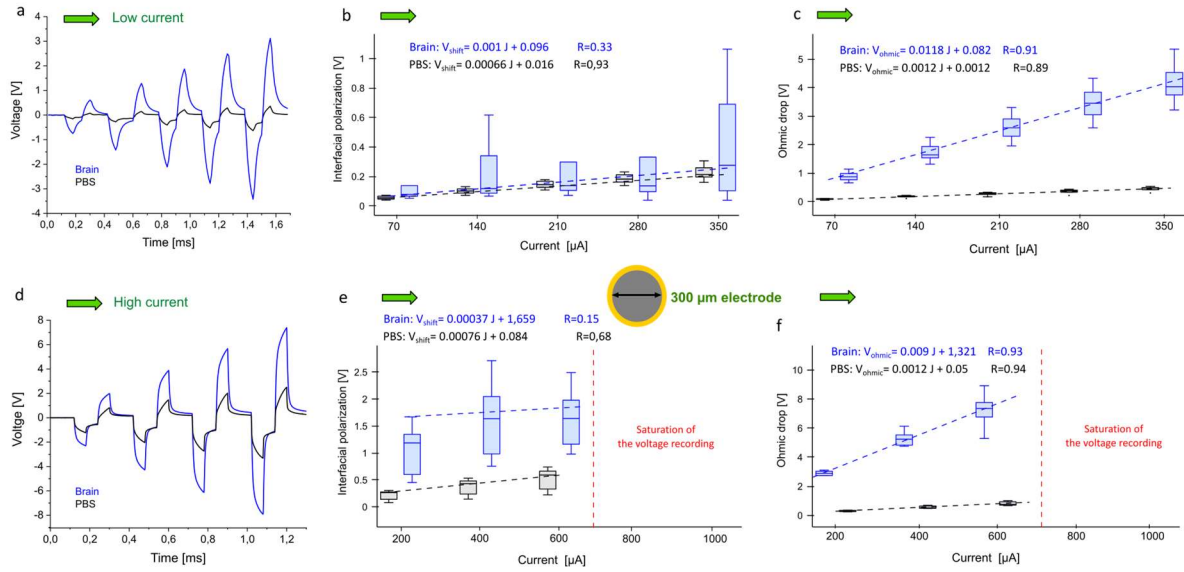


Figure 2.13 | Comparison of in vitro and in vivo responses of rGO microelectrodes of 300 μm diameter. **a.** Exemplary voltage transients recorded from a 150 μm electrode during the low-current stimulation ramp in PBS (black) and in brain tissue after implantation (blue). **b.** Interfacial polarization extracted from voltage response measurements obtained from four 150 μm electrodes across four implants at each current amplitude of the low-current stimulation protocol, in PBS (black) and in brain tissue after implantation (blue). Box plots indicate the interquartile range (25th-75th percentiles), with whiskers representing the 5th-95th percentiles. Dashed lines are linear fits of the data. **c.** Ohmic drop of the electrodes in panel b. **d-e-f.** Measurements conducted for the high-current protocol, under the same conditions described for panels a-c.

2.5 In vivo electrochemical stability after electrical stimulation.

Electrochemical characterization was repeated after stimulation and compared with measurements obtained prior to stimulation for both 300 μm (Figure 2.14, top panels) and 150 μm electrodes (Figure 2.15, bottom panels) to assess the impact of electrical stimulation on the electrode-tissue interface. Representative impedance spectra recorded from a 300 μm electrode before and after stimulation showed minimal variation across the measured frequency range, indicating stable electrochemical behavior (Figure 2.14a).

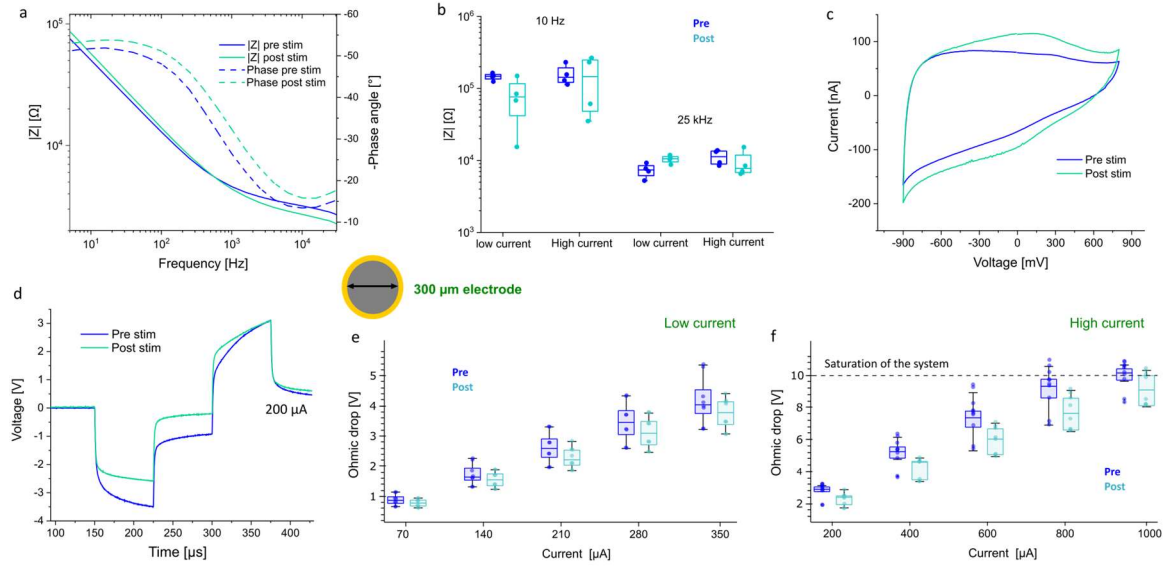


Figure 2.14 | Effect of in vivo electrical microstimulation on the electrochemical characteristics of rGO microelectrodes with 300 μm diameter. **a**, Representative impedance magnitude (solid line) and phase (dashed line) measured in brain tissue before (blue) and after (light blue) application of the low stimulation protocol. **b**, Impedance magnitude extracted at 10 Hz and 25 kHz for electrodes from different implants measured before (blue) and after (light blue) under low- (n=4) and high-current (n=4) stimulation protocols. Box plots indicate the interquartile range (25th–75th percentiles), with whiskers representing the 5th–95th percentiles. **c**, Representative cyclic voltammograms (50 mV/s scan rate) recorded in vivo before (blue) and after low stimulation protocol (light blue). **d**, Representative voltage transient electrode response of an electrode to a biphasic current pulse (60 μs pulse width, 200 μA amplitude) measured in vivo before (blue) and after stimulation (light blue). **e**, Ohmic drop extracted from voltage response measurements obtained from 4 electrodes across four implants at each current amplitude of the ramp used in the low-current stimulation protocol. Measurements were performed immediately after implantation (blue) and after electrical stimulation (light blue). Box plots indicate the interquartile range (25th–75th percentiles), with whiskers representing the 5th–95th percentiles. **f**, Ohmic voltage drop measured under the high-current stimulation protocol using the same analysis as in panel e. The dashed line indicates the potentiostat voltage compliance limit beyond which measurements are saturated.

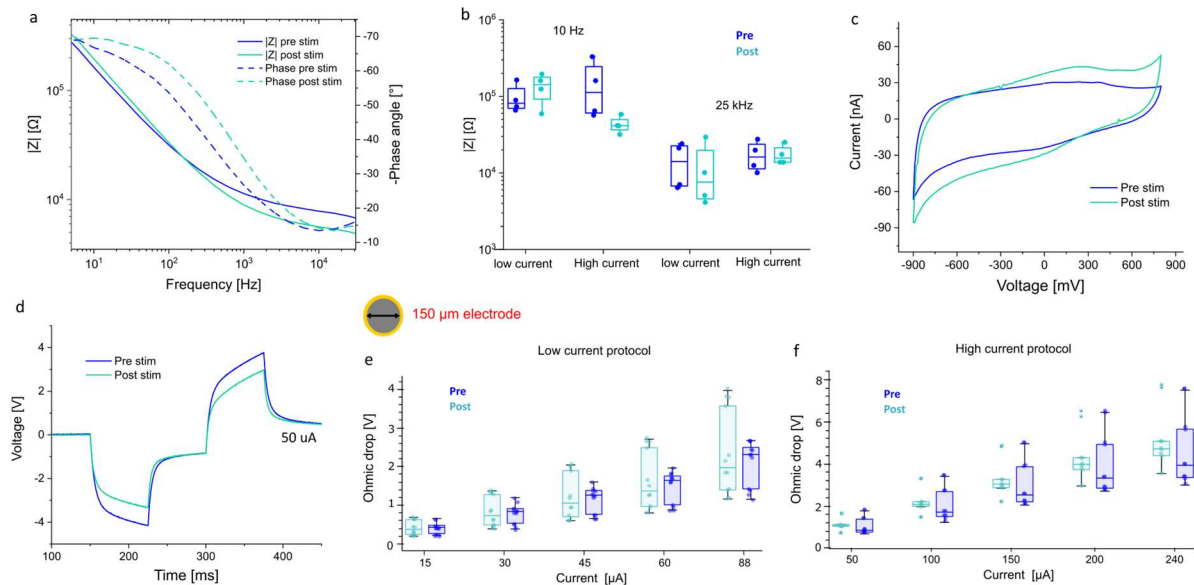


Figure 2.15 | Effect of in vivo electrical microstimulation on the electrochemical characteristics of rGO microelectrodes with 150 μm diameter. **a**, Representative impedance magnitude (solid line) and phase (dashed line) measured in brain tissue before (blue) and after (light blue) application of the stimulation protocol. **b**, Impedance magnitude extracted at 10 Hz and 25 kHz for electrodes from different implants measured before (blue) and after (light blue) under low- ($n=4$) and high-current ($n=4$) stimulation protocols. Box plots indicate the interquartile range (25th–75th percentiles), with whiskers representing the 5th–95th percentiles. **c**, Representative cyclic voltammograms (50 mV/s scan rate) recorded in vivo before (blue) and after low stimulation protocol (light blue). **d**, Representative voltage transient electrode response of an electrode to a biphasic current pulse (60 μs pulse width, 200 μA amplitude) measured in vivo before (blue) and after low stimulation protocol (light blue). **e**, Ohmic drop extracted from voltage response measurements obtained from 4 electrodes across four implants at each current amplitude of the ramp used in the low-current stimulation protocol. Measurements were performed immediately after implantation (blue) and after electrical stimulation (light blue). Box plots indicate the interquartile range (25th–75th percentiles), with whiskers representing the 5th–95th percentiles. **f**, Ohmic voltage drop measured under the high-current stimulation protocol using the same analysis as in panel e. The dashed line indicates the potentiostat voltage compliance limit beyond which measurements are saturated.

Quantitative analysis of impedance magnitude at 10 Hz and 25 kHz revealed no significant differences between pre- and post-stimulation measurements across implants (Figure 2.14b, h). Representative cyclic voltammograms obtained after stimulation did not show additional oxidation or reduction peaks for either electrode size (Figure 2.15c, i), suggesting the absence of stimulation-induced electrochemical degradation. However, the average charge storage capacity increased following stimulation, with values ranging from 1.7- to 4.5-fold higher than pre-stimulation measurements (Figure 2.16a,b), consistent with electrochemical activation of rGO electrodes and/or increased ionic accessibility within the porous structure.¹⁴⁹

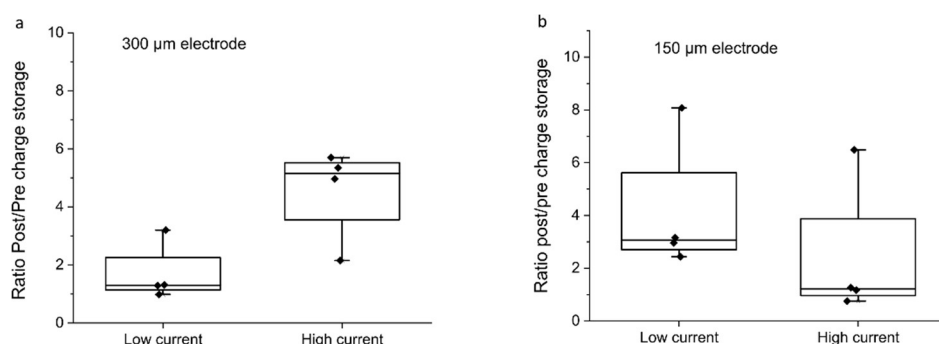


Figure 2.16 | Charge storage ratio pre/post-implantation. **a-b**, Ratio of charge storage capacitance in the brain, post-pre stimulation, for four different 300 μm (panel a) and 150 μm (panel b) electrodes, obtained with the low-intensity and high-intensity current levels. Box plots indicate the interquartile range (25th–75th percentiles), with whiskers representing the 5th–95th percentiles

To evaluate potential stimulation-induced changes in the surrounding tissue environment, voltage transients during electrical pulsing were analyzed before and after stimulation. Representative voltage transient traces exhibited a similar shape under both conditions (Figure 2.14d and 2.15d). The ohmic drop (Figure 2.14e-f and 2.15e-f) and interfacial polarization components (Figure 2.17) were extracted

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across the five current amplitudes of the stimulation ramps for all experimental conditions. No systematic differences were observed between pre- and post-stimulation measurements, although moderate variability was present across implants.

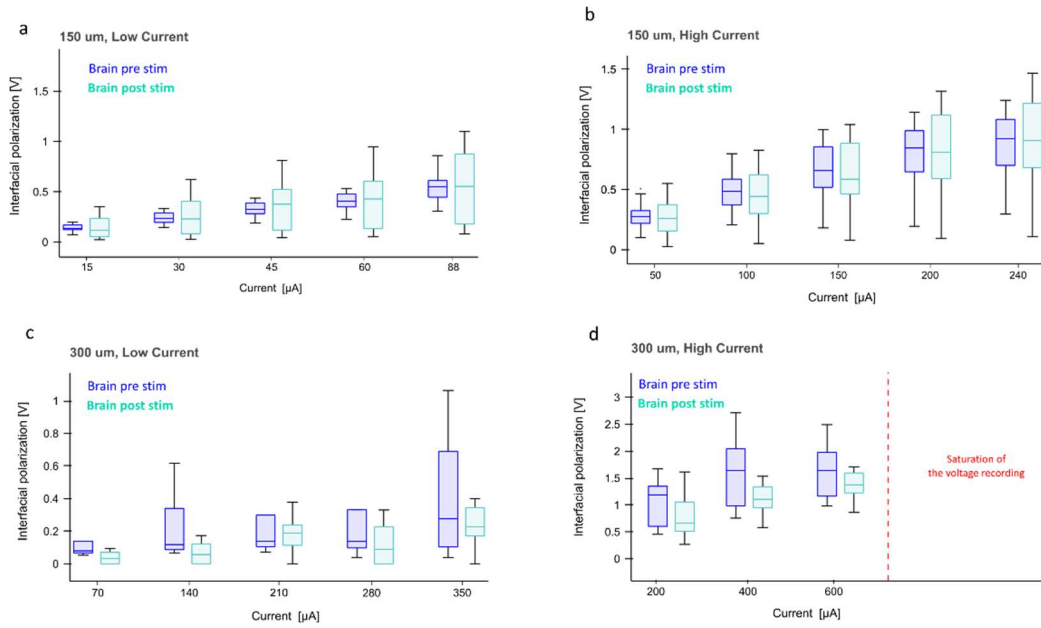


Figure 2.17 | Interfacial polarization in the brain pre-post stimulation. **a.** Interfacial polarization at the interface electrode tissue extracted from the voltage response measured with 4 electrodes of 150 μm diameter of four different implants, at each current amplitude of the ramp designed for the low stimulation protocol. The measurement is performed in the brain before (blue) and after (light blue) stimulation. The box plot represents the 25 and 75 interquartile with 95-5 interquartile for the whiskers. The dashed line is the linear fit of the data. **b.** Measurement with four different implants, for the high current ramp, in the rest of the conditions as subpanel a. **c.** Measurement with four different implants, with 300 μm electrodes, in the rest of the conditions as subpanel a. **d.** Measurement with four different implants, with the high stim protocol, in the rest of the conditions as subpanel c.

Following acute in vivo stimulation, devices were explanted and recharacterized in PBS to verify electrode integrity. Comparison of voltage transients measured before implantation and after explantation (Figure 2.18a,b,d,e) revealed an increased ohmic drop, consistent with higher interfacial resistance likely caused by residual biological material adhering to the electrode surface. Optical inspection of explanted electrodes supported this interpretation (Figure 2.18c,f). Overall, these results demonstrate that rGO microelectrodes maintain stable electrochemical performance in vivo following high-charge stimulation, with no evidence of stimulation-induced degradation of the electrode interface.

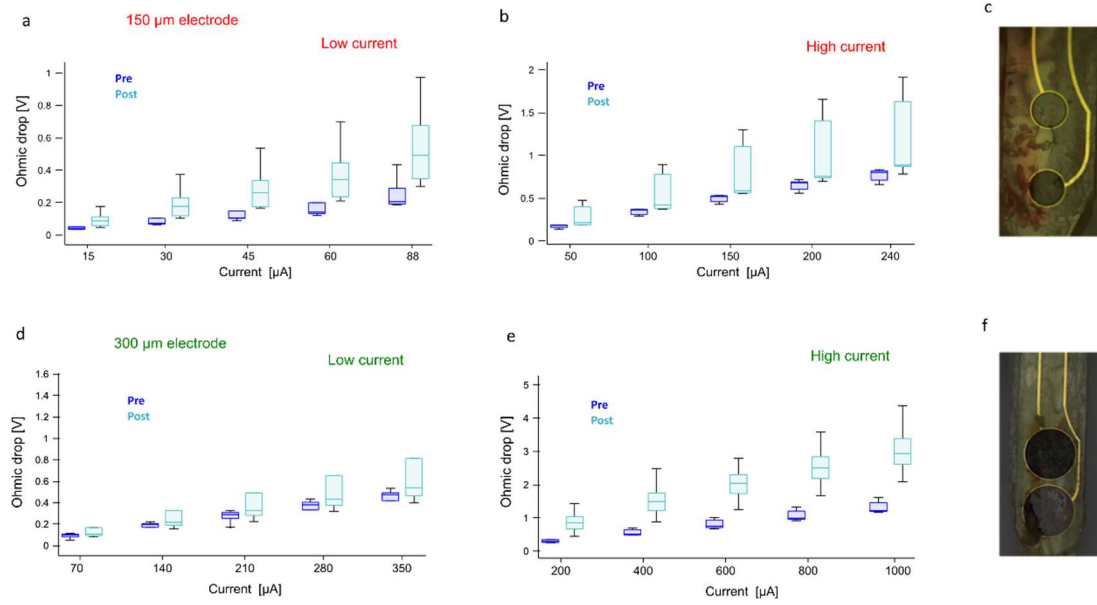


Figure 2.18 | Pre/post-implantation device characterization. **a-b**, Ohmic drop extracted from voltage transients measured with four 150 µm electrodes at each current amplitude of the low-stimulation (panel a) and of the high-stimulation (panel b) protocols. The measurements were performed in PBS before implantation (blue) and after explantation (light blue). Box plots indicate the interquartile range (25th-75th percentiles), with whiskers representing the 5th-95th percentiles. **d-e**, Ohmic drop extracted from voltage transients measured with four 300 µm electrodes at each current amplitude of the low-stimulation (panel a) and of the high-stimulation (panel b) protocols. The measurements were performed in PBS before implantation (blue) and after explantation (light blue). Box plots indicate the interquartile range (25th-75th percentiles), with whiskers representing the 5th-95th percentiles. **c,f**, Representative photographs of devices explanted after stimulation, in panel c a device containing 150 µm electrodes and in panel f a device containing 300 µm electrodes.

2.6 Immunohistochemical evaluation of brain tissue after stimulation

Cellular responses of glial cells, including astrocytes and microglia, were examined two weeks after completion of the stimulations in anesthetized animals to assess potential tissue effects associated with electrical stimulation. Reactive gliosis represents a hallmark response to central nervous system injury and constitutes a major component of tissue reactions to external perturbation. Astrocyte responses were assessed through immunolabelling of glial fibrillary acidic protein (GFAP), an intermediate filament whose expression level and morphological organization correlate with the severity and spatial extent of neural damage¹⁶⁸. Microglial activation, a key indicator of neuroinflammation, was evaluated using ionized calcium-binding adapter molecule 1 (Iba-1), a cytoplasmic protein selectively expressed in microglia and widely used to quantify inflammatory activation states¹⁶⁹. Accordingly, sub-chronic tissue responses (13-15 days post-stimulation) in the caudate-putamen were evaluated by quantifying GFAP and Iba-1 expression as complementary markers of astrocytic and microglial activation.

To distinguish tissue response caused by electrode implantation from that specifically associated with electrical stimulation, glial reactivity surrounding the active stimulating electrode was compared with tissue adjacent to an electrically inactive sham device implanted in the contralateral hemisphere. For

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each brain section, two regions of interest (ROIs; $500\ \mu\text{m} \times 500\ \mu\text{m}$) were analyzed, one in the stimulated hemisphere and one in the sham hemisphere. ROIs were positioned approximately $100\ \mu\text{m}$ from the center of the electrode implantation tracks to exclude tissue directly affected by mechanical implantation damage (Figures 2.2.19b and 20b). Within each ROI, the number of labelled cells, total fluorescent area, and mean fluorescence intensity were quantified for GFAP (astrocytes in green), Iba-1 (microglia in red), and DAPI (cell nuclei in blue).

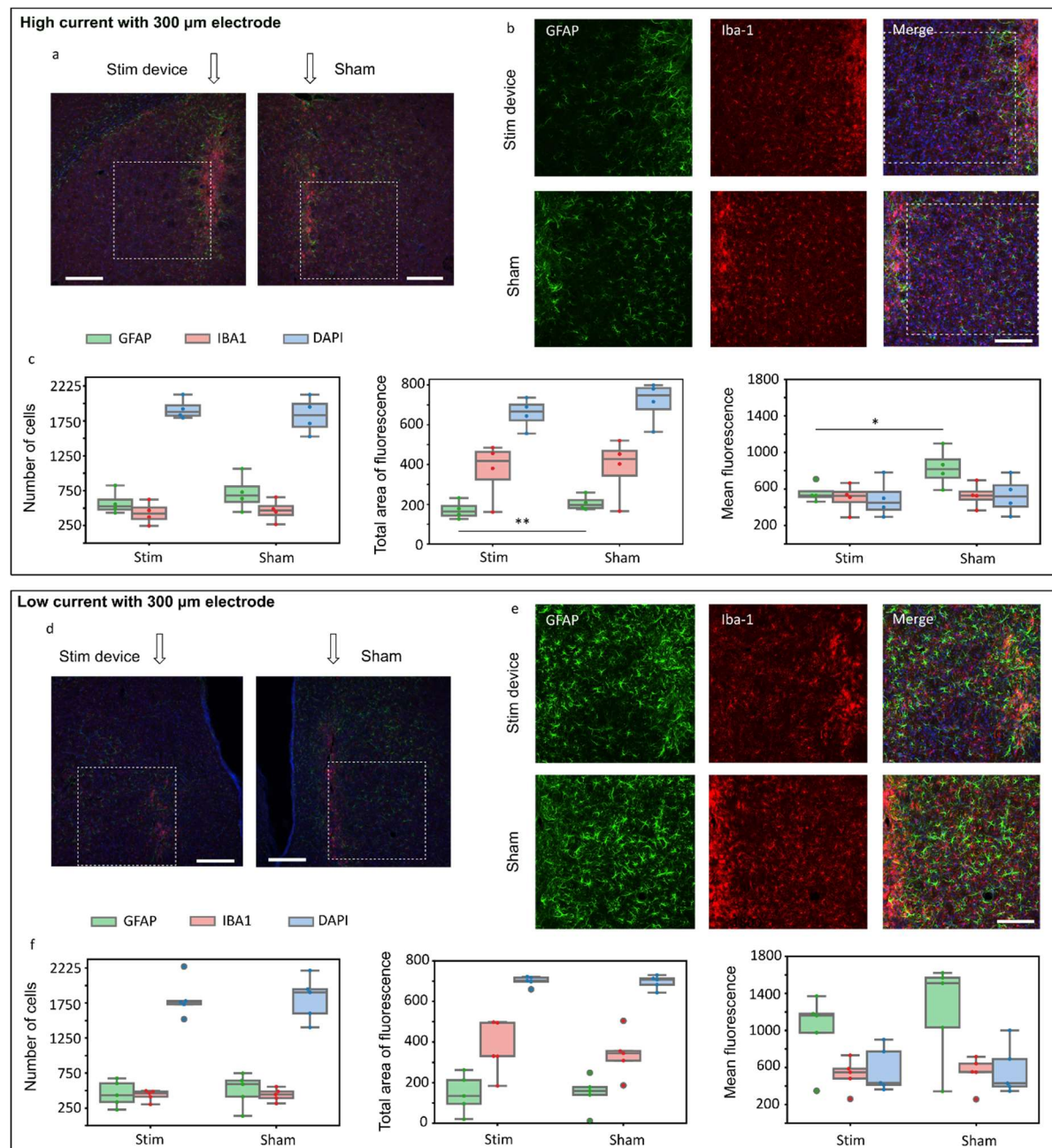


Figure 2.19 | Tissue histology following electrical stimulation with 300 μm -diameter rGO electrodes. **a**, Representative immunofluorescence images of the caudate-putamen after high-intensity stimulation (Stim) or sham implantation using 300 μm -diameter rGO electrodes. Sections were stained for astrocytes (GFAP, green), microglia (Iba-1, red), and nuclei (DAPI, blue). The dashed square (600

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x 600 μm) indicates the region shown at higher magnification in panel b. Images were acquired at 10x. Scale bar: 250 μm . **b**, Higher-magnification images showing individual GFAP and Iba-1 immunoreactivity corresponding to the regions shown in panel a. Dashed squares (500 x 500 μm) define the region on interest used for cell counting and fluorescence quantification. Images were acquired at 20x. Scale bar: 150 μm . **c**, Quantitative analysis of GFAP, Iba-1, and DAPI signals showing the number of labelled cells, total fluorescent area (μm^2), and mean fluorescence intensity of the total area (a.u.) measured in the stimulated (left) and sham (right) hemispheres. Four male mice were included in these analyses. **d-f**, Quantitative analysis performed as in panels a-c for the low-intensity stimulation group. Two female and three male mice were included in these analyses. Data are presented as mean $\pm$ SD. Statistical significance: * $p < 0.05$, ** $p < 0.01$.

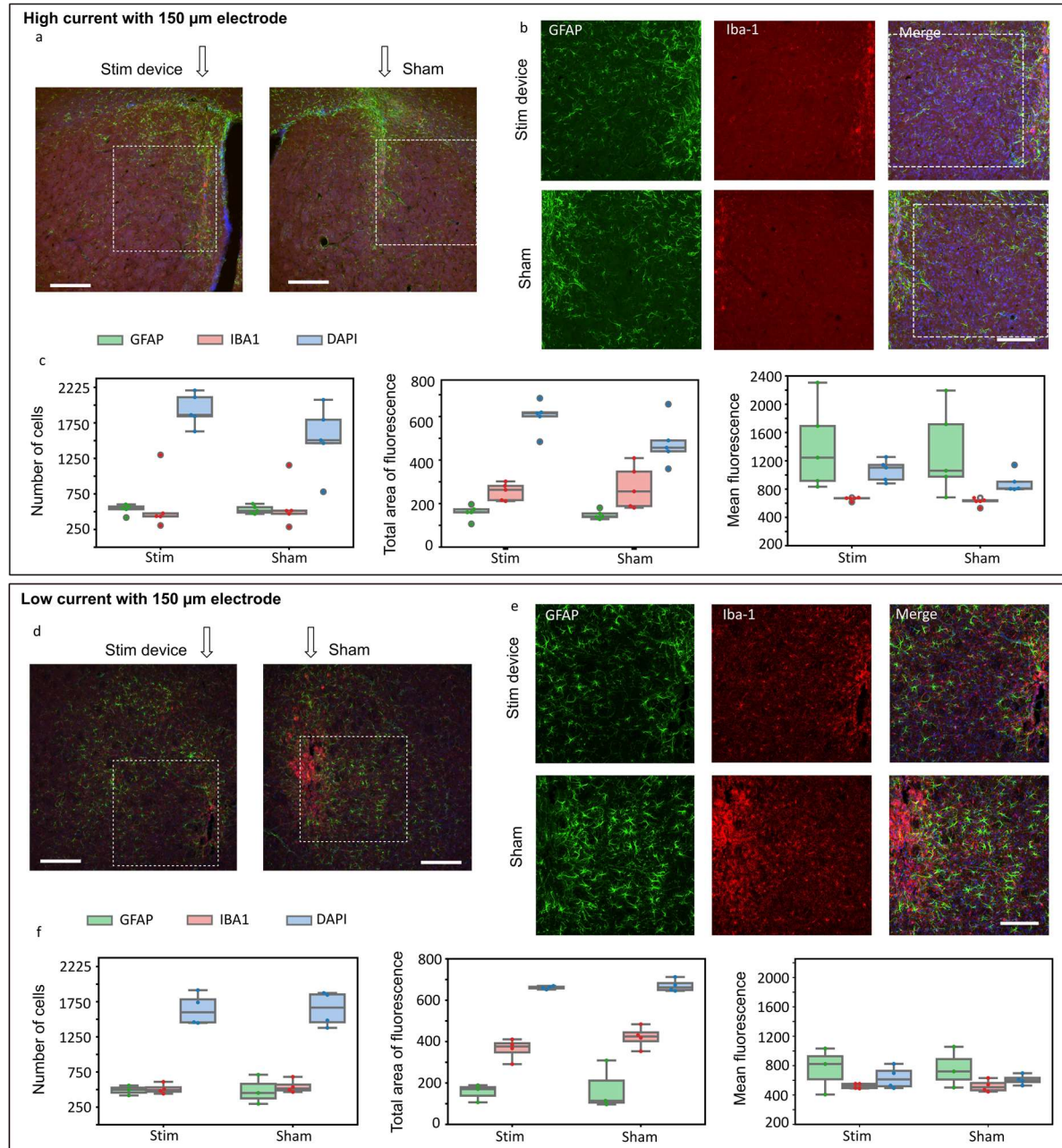


Figure 2.20 | Tissue histology after electrical stimulation with 150 μm -diameter rGO electrodes. **a**, Representative immunofluorescence images of the caudate–putamen after high-intensity stimulation (Stim) or sham implantation using 150 μm -diameter rGO electrodes. Sections were stained for

astrocytes (GFAP, green), microglia (Iba-1, red), and nuclei (DAPI, blue). The dashed squares (600 x 600 μm) correspond to the regions shown at higher magnification in panel b. Images were obtained at 10x. Scale bar: 250 μm . **b**, Higher-magnification images showing individual GFAP and Iba-1 immunoreactivities corresponding to the regions shown in panel a. Dashed squares (500 x 500 μm) define the regions of interest used for cell counting and fluorescence quantification. Images were obtained at 20x. Scale bar: 150 μm . **c**, Quantitative analysis of GFAP, Iba-1, and DAPI signals showing the number of labelled cells, total fluorescent area (μm^2), and mean fluorescence intensity of the total area (a.u.) for the stimulated (left) and sham (right) hemispheres. Two female and three male mice were included in these analyses. **d-f**, The same results as above for the low-intensity stimulation group. Two female and two male mice were included in these analyses.

Immunofluorescence analysis revealed localized reactive gliosis surrounding implantation tracks that decayed within approximately 150 μm from the insertion site across all experimental groups (Figures 2.19 and 2.20). For the high-current stimulation protocol delivered through 300 μm electrodes, quantitative analysis showed significantly lower total and mean GFAP fluorescence in the stimulated hemisphere compared to the sham hemisphere ($n = 4$ male mice; paired Student's t -test, $p = 0.005$ and 0.033 , respectively). Under this condition, electrode polarization exceeded the electrochemical safety window of the electrode, suggesting the occurrence of electrochemical stress at the electrode-tissue interfaces^{68,140}. Notably, for this condition, the reduction in GFAP fluorescence in the stimulating site occurred without changes in astrocyte or microglial cell density. While elevated GFAP expression is typically associated with reactive astrogliosis, several studies have reported that reduced GFAP immunoreactivity can reflect astrocytic cytoskeletal remodelling or functional stress responses in astrocytes under metabolically demanding conditions.^{168,170} In this context, the observed decrease in GFAP signal may indicate early tissue stress when stimulation parameters approach electrochemical safety limits rather than overt tissue damage. The absence of accompanying microglial activation further suggests that these effects remain localized and non-inflammatory¹⁷¹. No significant differences in GFAP, Iba-1, or DAPI immunofluorescence were detected between stimulated and sham hemispheres for the remaining stimulation protocols (Figures 2.19d-f and 2.20a-f).

Overall, these results indicate that electrode implantation induced a spatially confined glial response limited to the immediate vicinity of the implantation track. Importantly, cellular responses measured two weeks post-stimulation were not exacerbated by electrical stimulation under all experimental conditions. Collectively, these findings indicate that the stimulation protocols employed here produce minimal additional tissue disruption beyond implantation-related effects, with only limited protocol-dependent modulation of astrocytic reactivity. Taken together, these results support that short-term electrical stimulation using rGO electrodes is well tolerated in vivo and does not induce detectable gliosis or neuroinflammation in surrounding brain tissue two weeks after stimulation.

2.7 Conclusion

rGO is a promising technology for minimally invasive neural interfaces, enabling high-resolution neural recording and stimulation. The fabrication process involves multiple steps. First, a solution with dispersed GO flakes is filtered to create a membrane that is transferred to a wafer spin-coated with a polyimide layer and Ti/Au metallization. A hydrothermal reduction process of the membrane generates oxygen vacancies and basal plane defects to increase the material porosity and conductivity. Metal tracks are subsequently defined using photolithography followed by wet etching. A second polyimide layer is then spin-coated on top of the wafer. Finally, a sequence of photolithography and reactive ion etching (RIE) processes is used to define the device geometry and open the electrode sites.

Electrochemical characterization in PBS revealed a predominantly pseudocapacitive behavior, attributed to the porous structure of the rGO, which increases the electrochemically active surface area. Impedance spectroscopy showed a characteristic frequency-dependent response with low impedance magnitude across the measured range. Cyclic voltammetry confirmed the absence of irreversible faradaic reactions within the established safe potential window, indicating that the electrodes primarily operate through pseudocapacitive charge storage mechanisms. Chronopotentiometric measurements demonstrated low interfacial polarization and high charge injection limits for microelectrodes ($CIL > 1 \text{ mC/cm}^2$). Interestingly, when increasing the electrode diameter, the charge injection limit scaled with the electrode perimeter rather than with its surface area.

Overall, the rGO microelectrodes exhibited high electrochemical performance, combining low impedance and high CIL values, particularly for diameters below $100 \text{ }\mu\text{m}$. These results highlight the strong potential of rGO for electrode miniaturization while maintaining the capability to record neural signals with low noise and deliver sufficient charge to reach neuromodulation thresholds in neural tissue.

The second part of the chapter involved evaluating the electrochemical stability in the brain and biological safety of rGO microelectrodes during electrical stimulation across clinically relevant charge densities and electrode dimensions. The stimulation paradigm investigated here was designed to approximate conditions encountered during intraoperative testing of neural implants, where stimulation is delivered briefly to evaluate electrode placement or functional responses rather than continuously over extended periods. The two-week post-stimulation histological evaluation, therefore captures potential delayed inflammatory or glial responses arising from these acute exposures.

Short-term stimulation delivered through rGO electrodes with diameters of 150 and $300 \text{ }\mu\text{m}$ at charge densities up to $90 \text{ }\mu\text{C/cm}^2$ demonstrated stable electrochemical behaviour while maintaining favourable tissue compatibility under sub-chronic conditions, two weeks after stimulation. These findings further support graphene-based materials as promising platforms for neural stimulation technologies requiring high-charge injection capability together with biocompatibility and electrochemical stability.^{12,63,136,155}

Comparison of electrode behaviour in PBS and brain tissue revealed substantial differences in effective electrical performance despite similar bulk ionic concentrations. The structural complexity and composition heterogeneity of brain tissue increased both interfacial polarization and ohmic drop, resulting in 1.8–7.2-fold higher polarization voltage and up to tenfold greater resistive losses *in vivo*. These observations demonstrate that electrochemical limits derived from saline measurements substantially overestimate safe operational ranges in neural tissue and emphasize the need for *in vivo* validation when defining stimulation boundaries for neural interfaces.

Under the highest-current condition applied through 300 μm electrodes (1 mA), polarization voltage exceeded the electrochemical safety window of rGO *in vivo*, indicating the onset of faradaic processes at the electrode-tissue interface.^{68,70,144} Notably, this effect was observed in brain tissue but not in PBS, further illustrating the limitations of saline-based safety assessments¹⁴⁰. The dependence of stimulation limits on electrode geometry further indicates that charge density alone is insufficient to predict safe operation, particularly for sub-millimeter electrodes used in emerging high-density neural interfaces.

Electrochemical characterization performed before and after stimulation confirmed preservation of electrode functionality, with stable impedance spectra and electrode polarization responses following stimulation. Variability observed across measurements is consistent with the dynamic electrochemical environment of brain tissue rather than material degradation. Post-explant characterization further supported electrode integrity, with increased resistive behavior attributable to residual biological material adhering to the electrode surface rather than intrinsic changes in the rGO electrode.

Histological analysis showed that tissue responses were primarily dominated by implantation-related mechanical injury, with reactive gliosis mostly confined to the immediate vicinity of the implantation track. Electrical stimulation did not exacerbate astrocytic or microglial activation across the different conditions, with the exception of the 300 μm high protocol, indicating that rGO-mediated stimulation induces minimal additional tissue disruption beyond implantation effects. These findings further suggest that stimulation above the historical FDA guideline of 30 $\mu\text{C}/\text{cm}^2$, originally derived from large-area platinum electrodes, may remain safe when electrode properties, geometry, and *in vivo* electrochemical behaviour are appropriately considered. Consequently, safety limits for modern neural interfaces should be interpreted within a material- and geometry-dependent framework rather than as universal charge-density thresholds.

A limitation of this study is the absence of histological analysis within the immediate electrode-tissue interface ($<100\ \mu\text{m}$), where peak stimulation-induced effects are expected, which would require a larger number of animals due to the high variability of lesions caused by the implants.

Overall, our results demonstrate that rGO microelectrodes support stable high-charge neural stimulation while maintaining favourable tissue compatibility *in vivo*. These findings highlight the potential of rGO-based microelectrodes for next-generation high-density and bidirectional neural interfaces.

Chapter 3

Graphene-based neurotechnology for the treatment of Parkinson's disease

Deep brain stimulation (DBS) is a neuroelectronic therapy for the treatment of a broad range of neurological disorders, including Parkinson's disease. Current DBS technologies face important limitations, such as large electrode size, invasiveness, and lack of adaptive therapy based on biomarker monitoring. In this chapter, I investigate the potential benefits of using nanoporous reduced graphene oxide (rGO) technology in DBS, by implanting a flexible high-density array of rGO microelectrodes (25 μm diameter) in the subthalamic nucleus (STN) of healthy and hemi-parkinsonian rats. The *in vivo* experiments were done in collaboration with Prof. Kostas Kostarelos and Dr. Rob Wykes from the University of Manchester. This chapter demonstrates that these microelectrodes record action potentials with a high signal-to-noise ratio, allowing the precise localization of the STN and the tracking of multiunit-based Parkinsonian biomarkers. The bidirectional capability to deliver high-density focal stimulation and to record high-fidelity signals unlocks the visualization of local neuromodulation of the multiunit biomarker. These findings demonstrate the potential of bidirectional high-resolution neural interfaces to investigate closed-loop DBS in preclinical models.

Most of the contents of this chapter have been published in:

Ria, N. *et al.* Flexible graphene-based neurotechnology for high-precision deep brain mapping and neuromodulation in Parkinsonian rats. *Nat Commun* <https://doi.org/10.1038/s41467-025-58156-z> (2025) doi:10.1038/s41467-025-58156-z.

Introduction.

Deep brain stimulation (DBS) is a widely used neuromodulation therapeutic method to alleviate symptoms and improve the quality of life in individuals with movement disorders such as Parkinson's disease (PD)⁵, essential tremor⁶ or dystonia⁷, and more experimentally, for some neuropsychiatric conditions¹⁷². Parkinson's disease is characterized by the excessive loss of dopaminergic neurons in the basal ganglia (BG)¹⁷³, a group of nuclei critical for movement control and execution. The subthalamic nucleus (STN), part of the BG, is the most common target for DBS in Parkinson's¹⁷⁴. DBS treatment involves the surgical implantation of a device with electrodes into the STN to deliver high-frequency electrical stimulation. By modulating neuron firing patterns, DBS aims to normalize excessive inhibitory output from the STN to the thalamus and motor cortex^{175,176}, thereby reducing motor symptoms such as tremor, bradykinesia and rigidity¹⁷⁷.

However, the use of deep brain implants presents several challenges, including the precise targeting of small deep nuclei in the brain¹⁷⁸, or the mitigation of the tissue damage and inflammation caused by the implantation. Miniaturization of the leads and electrode sites offers an attractive route for reducing the invasiveness and improving the resolution of DBS therapy. However, maintaining low impedance for high-fidelity recordings and the capability to inject high charge density to achieve tissue activation, remains challenging with the clinically-approved electrode materials.¹⁷⁹

Current clinical DBS technology, mainly based on Pt or PtIr metals, uses leads with electrodes on the millimeter scale (typical areas of individual electrodes are 6 mm² or 1.5 mm²). This does not allow to capture of action potential with high SNR, limiting the mapping of a small brain region for precise targeting of the lead and obscuring the observation of multi-unit-based Parkinsonian biomarkers.

Furthermore large electrodes offer poor resolution of the stimulation, causing in many cases side effects due to unwanted stimulation of neighbouring brain regions or structures¹⁸⁰. This limitation also affects the adaptability of the electrode array to the expected differences in structure and connectivity of the brain of different individuals¹⁷⁷, making the treatment less effective.

Another limitation of current DBS is its continuous, open-loop stimulation operation, which can lead to adverse effects, reduced efficacy over time and excessive battery consumption¹⁸¹. Closed-loop operation based on the monitoring of electrophysiological biomarkers is a promising strategy to improve DBS^{182–184}. Pilot studies have shown limited improvement in clinical outcomes so far, attributed to the lack of reliable biomarkers as feedback to close the loop and suitable electrode technology^{185,182}. Closed-loop DBS might benefit from bidirectional electrodes capable of sensing and selectively modulating pathophysiological brain activity at the resolution of single cells, for which new electrode materials and technologies should be developed.

As demonstrated in a previous study¹⁸⁶, rGO is a high-performing electrode material that exhibits high charge injection and low impedance, which translates into efficient stimulation and recording of brain activity with high fidelity. Aiming to explore the potential of this electrode technology in DBS, in this part of my thesis, I have developed a thin-film device (thickness of 10 μm and a width of 120 μm), equipped with a linear array of reduced graphene oxide (rGO) microelectrodes of 25 μm in diameter. The performance of the device for DBS applications was tested in hemiparkinsonian (induced with stereotactic 6-OHDA toxin administration)^{187,188} and healthy control rats. I validated the capability for electrophysiology-guided precise targeting of the STN during insertion, as well as the bidirectional capability for sensing a multi-unit-based Parkinsonian biomarker and for inducing local neuromodulation of the biomarker that lasts temporarily for tens of seconds after stimulation.

3.1 rGO thin-film devices for rat deep brain stimulation

In this study, I designed and fabricated flexible microelectrode arrays for recording and stimulation of deep brain structures. The lead is a thin-film device (with a thickness of 10 μm and a width of 120 μm), equipped with 8 microelectrodes (25 μm diameter) spaced 100 μm (Figure 3.2a).

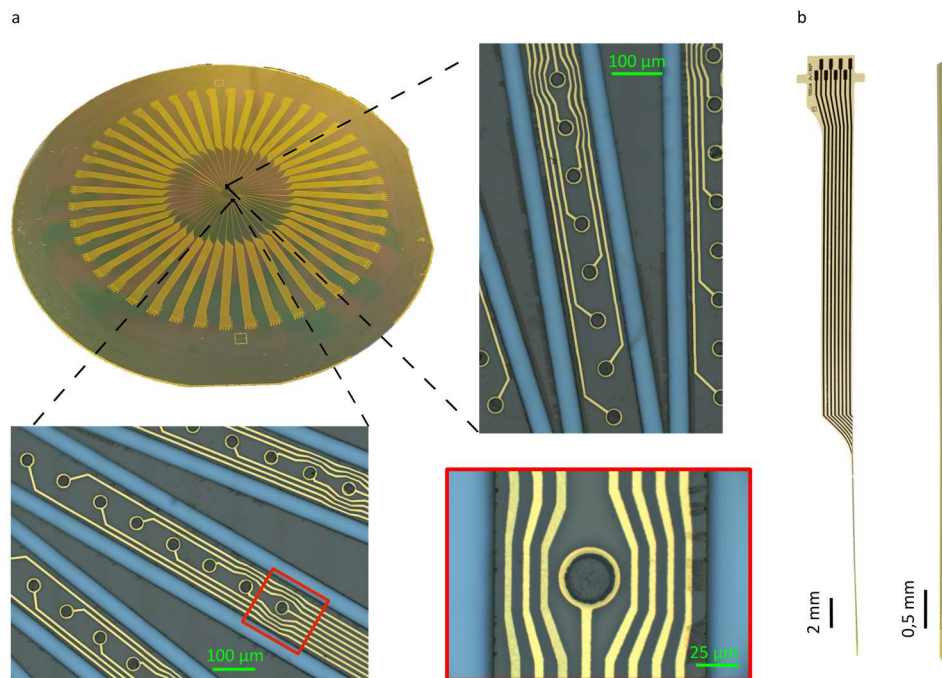


Figure 3.1 | Flexible thin film microfabrication on a 4-inch wafer. a, Photograph of a 4-inch wafer with 44 devices microfabricated. 2 zooms of the central part allow for visualization of the tips of the leads where the electrodes are placed. With the red square, a zoom of a single rGO electrode. b, Photographs of a device peeled off from the wafer with a zoom of the thin tip.

3. Graphene-based neurotechnology for the treatment of Parkinson's disease

The active material of the electrodes consists of reduced graphene oxide (rGO), connected by gold tracks, and encapsulated on a flexible substrate of polyimide. The stacking of rGO flakes together with oxygen-related vacancies and basal plane defects creates a nanoporous structure, which thus enables the preparation of microelectrodes with low impedance and high charge injection¹⁸⁶.

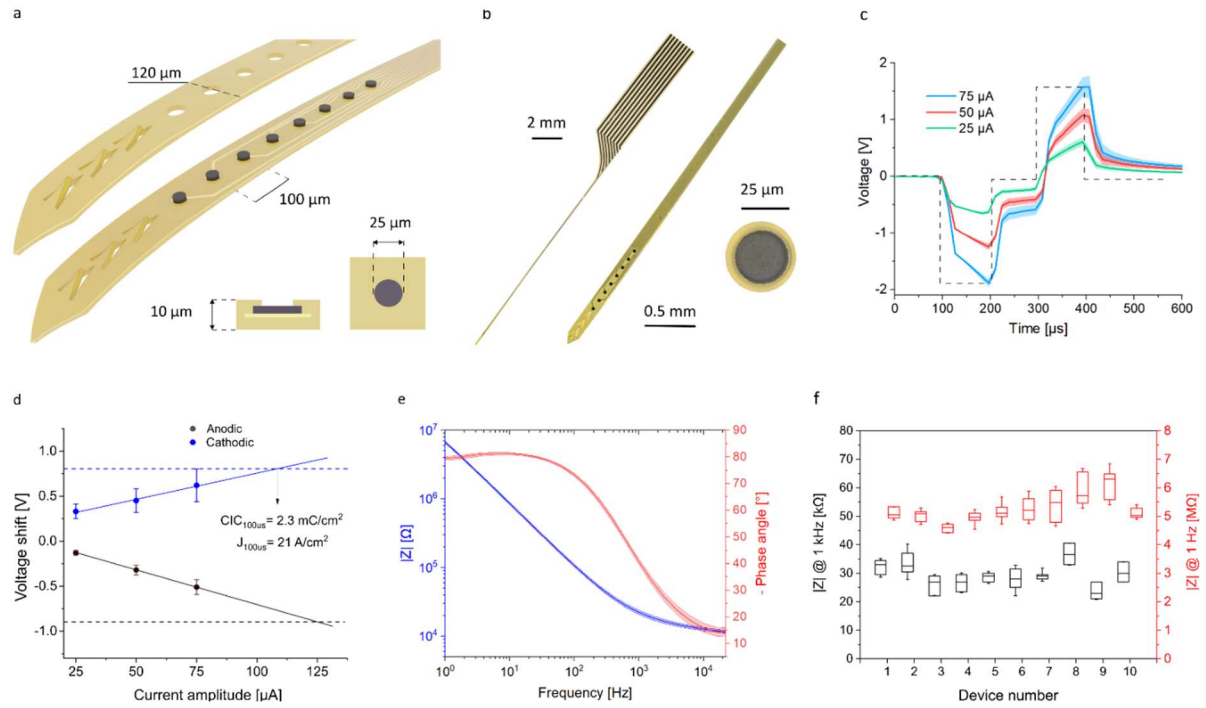


Figure 3.2 | Bidirectional graphene-based DBS technology. a, Illustration of microelectrode linear array at the tip of the device, with top and bottom polymeric layers. The device cross-section displays the layers of polyimide, gold, and reduced graphene oxide. b, Photograph of the device, with a zoom of the tip and of a microelectrode. c, Average voltage transient (solid lines), with shadowed area for standard deviation (STD) ($n=8$), of the DBS protocol, consisting of biphasic current pulses (100 μs pulse width) and increasing current levels. d, Average interfacial polarization at the electrode-electrolyte interface with STD ($n=8$). The dashed line represents the safe voltage limits for the anodic (black) and cathodic (blue) polarizations, from which the charge injection limit can be calculated ($\text{CIL}=2.3 \text{ mC}/\text{cm}^2$). e, Average impedance magnitude (blue) and phase (red) ($n=8$). f, Impedance magnitude at 1 Hz (red) and 1 kHz (black) of the microelectrodes for 10 different arrays; boxplots represent 25 and 75 percentile, with the median value line and whiskers for the STD (for each device $n=8$).

The fabricated devices were electrochemically characterized in phosphate buffer saline (PBS) electrolytes to evaluate their perfect functionality. The charge injection limit was determined by the maximum injected current that leads to voltage polarization within the rGO water window. Considering typical stimulation protocols used in DBS applications, biphasic current pulses of 100 μs were injected while recording the voltage at the electrodes (Figure 3.2c). For the 25 mm diameter rGO microelectrodes, currents as high as 100 μA can be safely injected (Figure 3.2d), resulting in a charge injection limit (CIL) of 2.3 mC/cm^2 . This value is significantly higher than for conventional metal microelectrodes¹⁸⁹, and within the range of CIL of novel electrode materials used for similar applications.^{190,191} Electrochemical impedance was also measured in PBS to determine the charge transfer properties of the electrodes. Figure 3.2e presents the full spectrum of the impedance, showing

the average values of magnitude and phase for the 8 electrodes of a single array. Figure 3.2f summarizes the average magnitude at 1 kHz and 1 Hz for the electrodes of ten different arrays ($29.4 \pm 5\text{k}\Omega$ at 1 kHz and $5.2 \pm 0.6\text{ M}\Omega$ at 1 Hz, total average $n=79$). Compared to flat metallic electrodes, rGO microelectrodes exhibit low impedance due to the increased electrochemically active surface area that results from their nanoporous structure¹⁹²; similar values are reported for microelectrodes based on PEDOT or IrOx, which are also characterized by a high electrochemically active surface area^{193,194}. The stability of the microelectrodes was assessed during continuous electrical stimulation with biphasic current pulses (100 ms pulse width and 50 μA current amplitude, corresponding to 1 mC/cm^2); after 100 million pulses, the microelectrodes did not exhibit any significant change in their characteristics (see Figure 3.3), in good agreement with previous work¹⁸⁶.

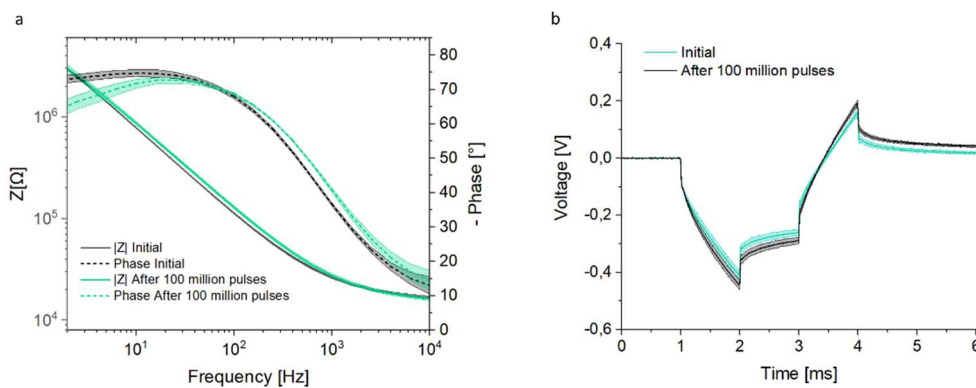


Figure 3.3 | Stability after long-term stimulation of PI-PI technology. a, Average impedance magnitude with STD (shadow area), and phase (for 3 electrodes) measured before and after injecting 100 million stimulation pulses (biphasic current pulses of 100 μs in pulse width and 50 μA amplitude). **b,** Average voltage polarization with STD (shadow area) in response to a biphasic current pulse of 1 ms in pulse width and 5 μA amplitude; the measurement is performed (with 3 electrodes) before and after the injection of 100 million pulses with the same protocol of subpanel a.

3.2 In vivo implantation strategy of flexible arrays

The use of neural interfaces based on mm-thin flexible substrates has been reported to be able to mitigate the foreign body response of the tissue^{195,196}. However, the implantation of such thin leads poses a significant challenge, particularly when inserting the lead deep into the brain. To address this issue, I used a strategy in which the flexible lead is temporarily attached to a rigid shuttle by a dissolvable adhesive polymer (Figure 3.4).



Figure 3.4 | Assembling of flexible probe onto silicon shuttle. On the left, setup used to glue the flexible device to the shuttle. The device is placed on a probe station that can be moved with manipulators. A vacuum pump connects with a plastic tube and a metallic tip that can hold the shuttle. After applying the adhesive polymer with a brush on the tip of the shuttle, the flexible device is aligned with the shuttle, looking under the microscope, and then assembled. On the right, a schematic of a device attached to the shuttle.

The rigid shuttle provides sufficient stiffness to penetrate the neural tissue during the implantation procedure and can be separated from the flexible array once the biocompatible adhesive polymer is dissolved (Figures 3.4 and 3.5a). The insertion is performed by using two stereotactic arms. The first one holds the PCB that connects the tail of the lead, while the second one holds the shuttle attached to the array. 2 The second arm is slowly lowered with a micro driver to insert the device in the brain. When the insertion is completed, the tail is encapsulated with Kwik seal, avoiding the insertion hall. PBS is then applied to dissolve the PVA that doesn't contact the brain fluids. The wires of the reference, ground, and the one implanted in the motor cortex (connected to the PCB) are fixed on the scalp using small screws. 3 After 20 min, when the adhesive polymer is dissolved, the shuttle is removed, and the device is kept in place thanks to the anchoring system. Everything is then encapsulated with epoxy.

In the case of the acute implantation, since the experiment is terminal, it is not necessary to remove the shuttle. In that case, a different approach was adopted, using only one stereotactic arm. The shuttle is fixed in a free space of the PCB below the ZIF connector. The PCB is then connected to the stimulator and the cable is fixed on a stereotactic arm controlled by the micro driver. The device, glued with a permanent adhesive polymer, is slowly inserted into the brain until reaching the STN.

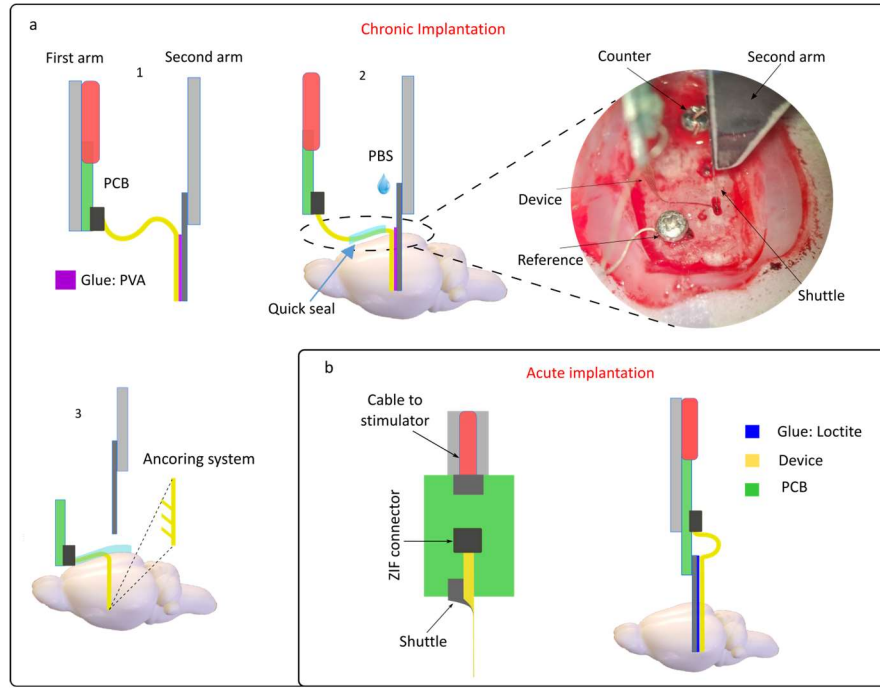


Figure 3.5 | Insertion strategy in chronic and acute. a, Schematic of the chronic implantation with a 2-arm strategy in 3 steps. **b,** Acute implantation with one arm insertion strategy; The shuttle is fixed in a free space of the PCB below the ZIF connector. The PCB is then connected to the stimulator and the cable is fixed on a stereotactic arm controlled by the micro driver. The device, glued with a permanent adhesive polymer, is slowly inserted into the brain until reaching the STN.

The silicon shuttle used was 80 μm in width and 60 μm in thickness. These dimensions were chosen to offer sufficient rigidity for a cm-deep insertion without fracturing, while minimizing tissue damage¹⁹⁷. To validate the penetration capability of the shuttle, I measured the buckling force generated by contacting the shuttle on a rigid surface (Figure 3.6). Experiments revealed a buckling force of 37.9 ± 1 mN, which is above the reported force threshold required to penetrate the rat dura with implants of similar dimensions¹⁹⁷.

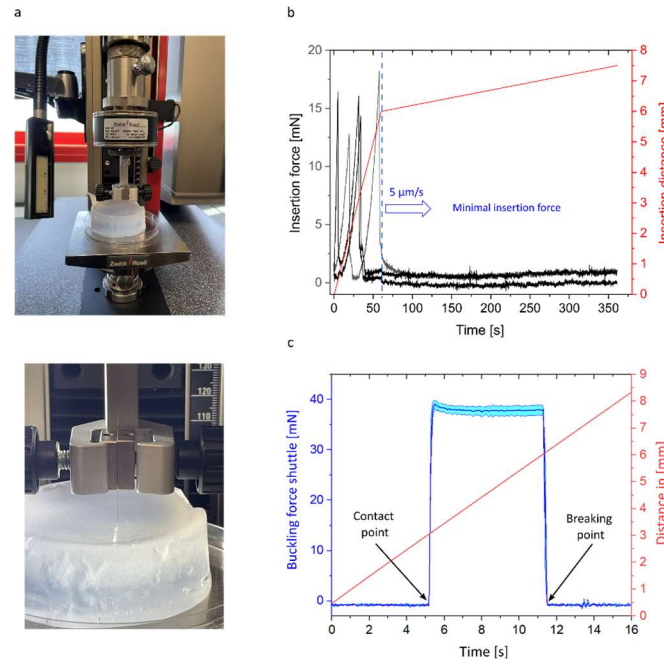


Figure 3.6 | In vitro test of the insertion force. **a**, Set-up for the measurement of the insertion force in the agar of a device glued with the shuttle. The device is inserted with the micro driver while a load force cell measures the force produced over time and the displacement. **b**, Insertion force (black) over time in agar for 3 devices glued with the shuttle, following the insertion speed used in vivo, 6 mm at 100 $\mu\text{m/s}$ and 1.5 mm at 5 $\mu\text{m/s}$. Displacement over time in red. **c**, Average buckling force with STD (shadow area) for 3 shuttles on a rigid surface. The first point around 5 s represents the moment when the shuttle touches the rigid surface. Then it starts to bend under a force of around 37 mN, until the breaking point. The displacement is at the constant speed of 100 $\mu\text{m/s}$.

The speed of insertion is another critical factor for mitigating brain injury, preserving nearby neurons, and ensuring the capacity to record single-neuron activity¹⁹⁸. A two-step method was used, involving an initial rapid insertion (100 $\mu\text{m/s}$) followed by a slow insertion (5 $\mu\text{m/s}$) around the structures of interest, starting at 6 mm deep. Measurements of the insertion force in agar (Figure 3.6) revealed that the first step at high speed is characterized by force peaks of 20 mN¹⁹⁹. However, during the second step, conducted at 5 $\mu\text{m/s}$, no stretching or compression forces could be measured (force resolution of the experiment is ~ 1 mN), considered relevant to reducing cell damage in the brain.¹⁹⁸

To allow the removal of the shuttle following the insertion of the probe to the desired depth, I investigated dissolvable adhesive polymers that could remain stable during insertion and then slowly dissolve by interacting with brain fluids. Given the constraints of the implantation procedure, the adhesive polymer shall be stable for at least 10 min (the insertion time), and its stability should not be overextended more than necessary (< 30 minutes), to minimize the surgery duration and facilitate post-implantation recovery of the animal. Polyethylene glycol (PEG) is a biocompatible adhesive polymer that has been used in previous studies^{200–202} to temporarily fix flexible probes to stiff shuttles for the implantation of intracortical devices. I tested PEG to glue our devices to the silicon shuttle, mimicking

the experimental in vivo conditions (agar phantom with an ion concentration of 290 mM, at 38 °C). I found an average dissolution time of merely 1 ± 0.6 minutes, which is considerably lower than the previously reported²⁰³, and which was attributed to the challenging conditions of our experiment, in particular, the very small width of the flexible array. For such narrow devices, the dissolution time of PEG, thus, is not enough for the deep and slow insertion required to reach deep brain structures such as the STN²⁰⁴.

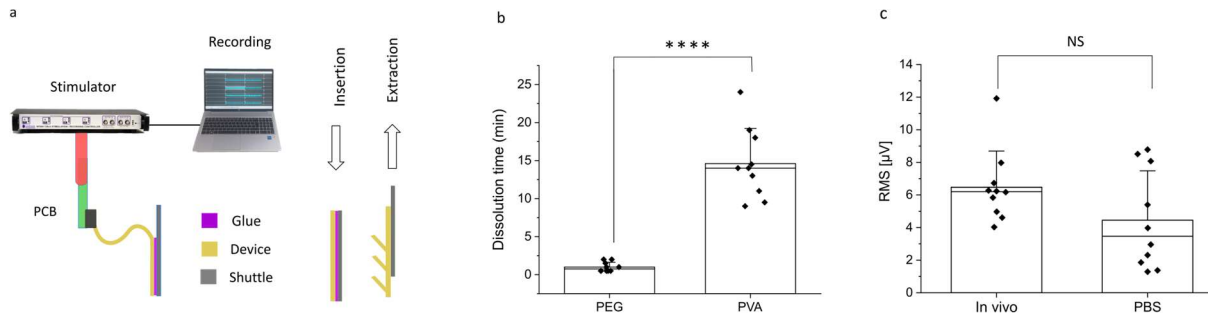


Figure 3.7 | Implantation strategy. **a**, Schematic of the in vivo set-up for bidirectional recording and stimulation. The flexible device is attached to a rigid shuttle with a dissolvable adhesive polymer. Once the insertion is completed, the flaps at the device's end keep the device in place during the extraction of the shuttle. **b**, Average dissolution time in agarose at 38 °C of the polyethene glycol (PEG, n=10) and polyvinyl alcohol (PVA, n=10). **c**, RMS of the voltage amplitude of the signal recorded (25 s, in the range 200-2000 Hz) in PBS before insertion and in the brain after reaching the STN. The plot displays the average values for the electrodes of 10 different devices (for each device n=8).

To meet these specifications, I prepared a biocompatible, water-soluble polyvinyl alcohol (PVA) adhesive polymer (see methods for details on the PVA preparation). The average dissolution time of the PVA-based adhesive polymers results in 14.6 ± 4.6 minutes (Figure 3.7b), which is compatible with the duration of our insertion protocol and, at the same time, with a short post-insertion dissolution time.

Shuttle removal introduces an additional challenge since friction at the shuttle-polyimide interface could eventually displace the implanted thin and flexible device. Given that the rat's STN is a very small structure (150-200 μm anteroposterior, 100-150 μm mediolateral and 200-250 μm dorsoventral), a relatively small displacement of the device could eventually move the microelectrodes outside the STN region. To prevent this, I implemented an anchoring system into the internal structure of the devices (Figure 3.2a), as previously used in similar nerve implants²⁰³. This system features internal flaps that flex during extraction but do not affect the insertion procedure (Figure 3.7a).

To validate that the insertion procedure does not affect the functionality of the microelectrodes, I compared the root mean square (RMS) of the signal recorded (200-2000 Hz) with electrodes before implantation (measured in PBS) and after completing the insertion of the devices in the brain (see Figure 3.7c). The results confirm that the microelectrodes maintain their functional integrity after implantation, and thus are not damaged by the implantation procedure.

3.3 Neural recording capability and high-precision mapping

The low impedance of the rGO microelectrodes is expected to translate into high-fidelity detection of neural signal²⁰⁵. To evaluate the recording capability in deep brain structures, linear arrays were implanted in anaesthetized rats, and neural activity was recorded with electrodes inside and outside the STN.

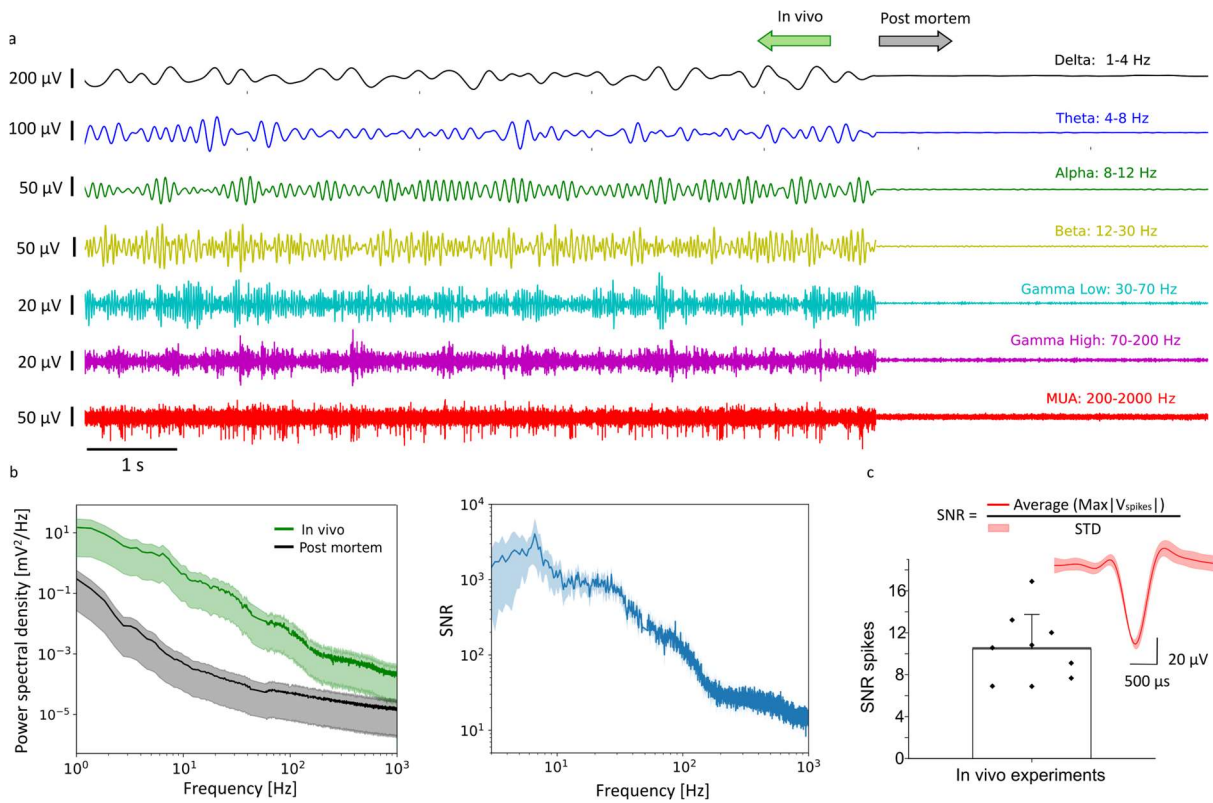


Figure 3.8 | Recording capability of rGO microelectrodes. **a**, Representative brain activity recording of one electrode located in the STN, filtered for different frequency bands, for in vivo (left) and post-mortem (right) conditions. **b**, *Left*: Average power spectral density (PSD), with STD, of the signal recorded with the electrodes of one array ($n=8$ electrodes), comparing in vivo (green) and post-mortem (black) conditions. *Right*: Average signal-to-noise ratio (SNR), with STD, calculated from the ratio between in vivo and post-mortem PSDs in the left panel. **c**, Average spike-to-noise ratio (SNR_{spike}) over 100 s, for the electrodes located in the STN. Each dot represents one acute experiment (PD=6 and control=3 rats). The inset shows an example of the average spike shape of a PD rat (the shadow area represents the STD of the spikes recorded over 100 s).

A representative signal recorded with an electrode inside the STN, depicting traces that correspond to the signal filtered in different frequency bands, shows the capability of the rGO microelectrodes to monitor high-fidelity neural activity from slow local field potentials (LFPs) up to fast multi-unit activity (MUA) (Figure 3.8a); for comparison, I show the postmortem recording of the same electrode. Figure 3.8b depicts the average power spectral density (PSD, $n=8$ electrodes) of the in vivo and post-mortem recording time shown in Figure 3.8a. The ratio between both PSDs, which can be used as a figure of

merit (performance metric) for the signal-to-noise ratio (SNR)²⁰⁶, is above 10 for all the frequency bands. In the 200-2000 Hz frequency range, where MUA is typically assessed, it was obtained (Figure 3.8c) an average spike-to-noise ratio (SNR_{spike}) of 10.4 ± 3.2 (n=9 rats), in line with what can be achieved by high-performance electrode materials^{207,208}.

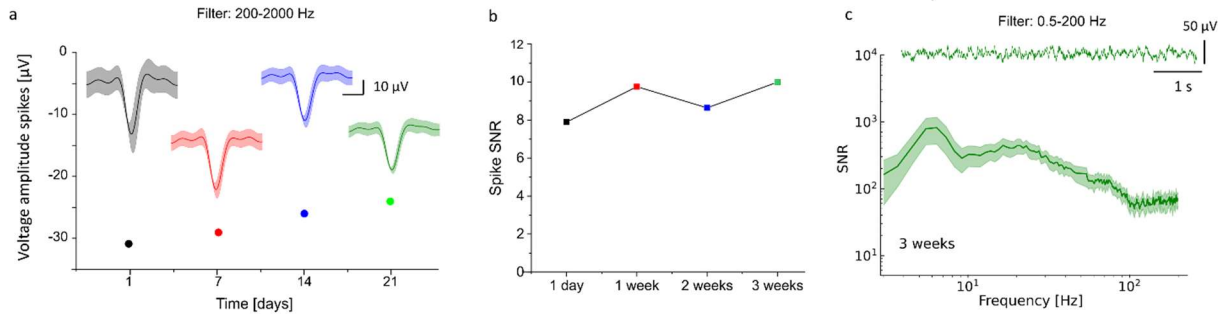


Figure 3.9 | Proof-of-concept in vivo chronic study of recording and stimulation capabilities. **a**, the spike amplitude was recorded over 3 weeks with one electrode in the STN. On top, the average voltage spike shapes with shadow area for the STD (over 100 s), are extracted at the corresponding time points. **b**, Spike to noise ratio over time of the experiment in the subpanel a. **c**, on top LFP recording at the third week with one electrode. Below is the average SNR spectrum with STD in the LFP range for the 8 electrodes of the array. In a proof-of-concept chronic study (Figure 3.9), both LFP and spike activity were recorded in an awake rat with SNR > 10 after 3 weeks of implantation. The capability of recording spiking activity with low noise can be correlated to the evolution of the electrode impedance over time. In vivo impedance measurements of the microelectrodes were recorded in chronically implanted arrays.

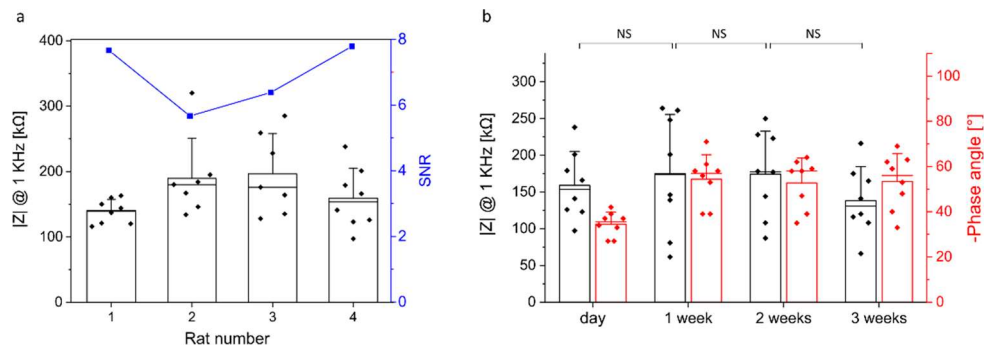


Figure 3.10 | Electrodes in vivo chronic stability. **a**, Average impedance magnitude in black (with median line and STD at the whisker) at 1 kHz at day one of 4 arrays implanted in vivo (for each array n=8), with the average spike-to-noise ratio in blue (over 100 s) of one recording electrode in the STN of each implant. **b**, Average impedance magnitude in black and phase in red with median line and STD at the whisker for the 8 electrodes of one array measured in a chronically implanted rat over weeks.

Figure 3.10a shows the average value of the impedance module measured at 1 kHz for electrodes in four different arrays implanted in the STN; the average impedance magnitude measured in vivo (n=31), around 169 ± 52 kΩ at 1kHz, is higher than the impedance measured in PBS (Figure 3.1f), due to the lower conductivity of the neural tissue²⁰⁹. In the proof-of-concept chronic study, the in vivo impedance remained stable over the course of three weeks (Figure 3.10b), suggesting no significant degradation of the electrodes or changes at the electrode/tissue interface²¹⁰.

The high-fidelity recording capability of the rGO microelectrodes, combined with the high density of microelectrodes in the linear array, allows the precise localization of the implanted device with respect to the STN during the implantation.

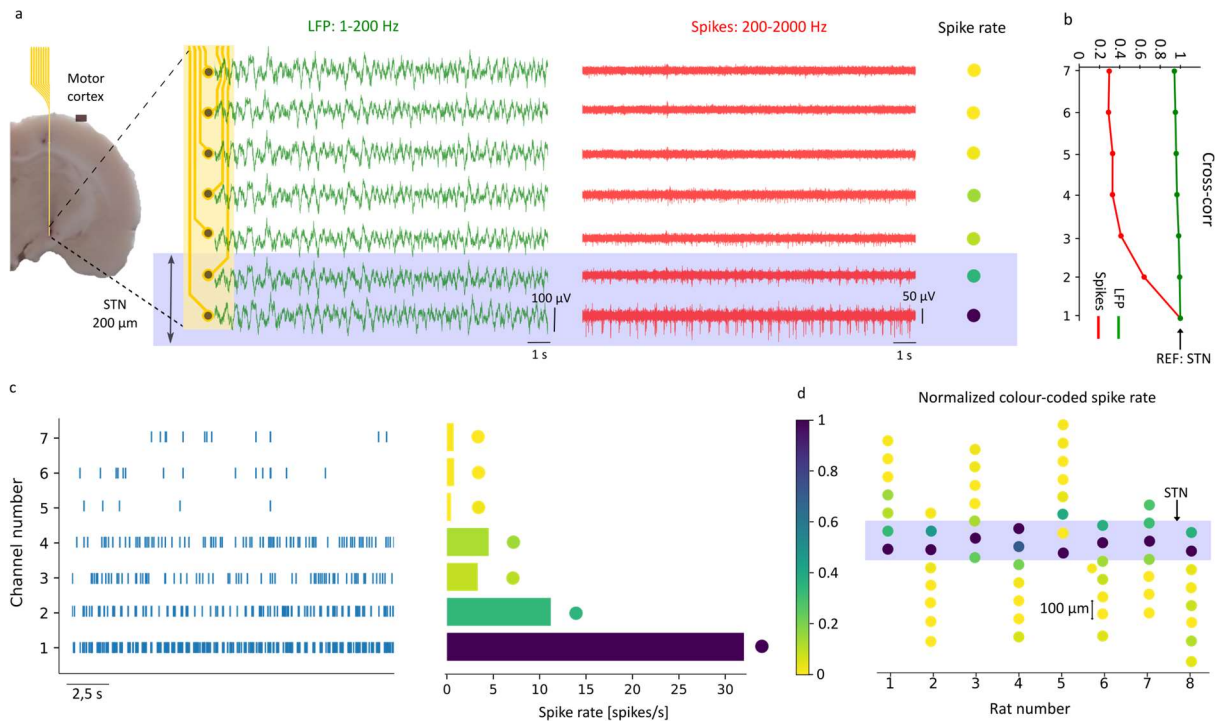


Figure 3.11 | STN localization by high-density microelectrode array. **a**, Representation of brain activity recorded with the 8 electrodes of one array, also depicting an image of the brain histology superimposed with a schematic of a device in the STN. The two groups of traces correspond to the recordings of each of the electrodes of the array, filtered in the LFP (green) and MUA (red) range. The blue rectangle highlights the traces corresponding to electrodes in the STN. The colour-coded spike rate quantifies the number of spikes for the different electrodes of the array. **b**, Cross-correlation between channel 1, located in the STN, and each one of the other electrodes, quantified at lag 0 for both LFP (green) and spikes (red). **c**, *Left*: Raster plot of the detected spike events over time for each electrode of the array shown in panel; *Right*: Colour-coded spike rate histogram of the raster plot, in which spike events are quantified over 100 s. **d**, The colour-coded dots represent the average spike rate (in the recording period of 100 s) of the electrodes of the arrays implanted in 8 different animals. The colour codes are normalized by the higher spike rate channel of each array. The blue rectangle represents the expected position of the STN.

This is enabled by the characteristic intense spiking activity of the STN compared to the activity of adjacent brain regions like the zona incerta (above the STN) and the internal capsule (below the STN), which are both relatively silent^{211,212}. A representative recording of brain activity obtained from an implanted array targeting the STN is presented in Figure 3.11a.

The activity recorded with the electrodes is presented in two sets of curves, corresponding to the signal filtered in the LFP band (1-200 Hz) and in the MUA frequency band (200-2000 Hz). The LFP signals appear uniform across all electrodes, both in time series and with spectral analysis (see Figure 3.12).

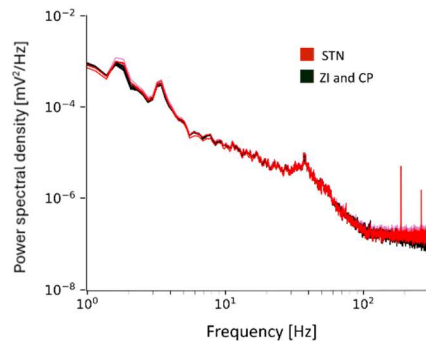


Figure 3.12 | Power spectral density. PSD through the electrodes of one array implanted in the STN. The red one represents the 2 electrodes in the STN while the black ones are placed in the Zona Incerta (ZI) and internal capsule (CP).

On the other side, the MUA signals reveal clear differences in the spiking activity recorded by the different electrodes. The two more distal electrodes in the array exhibit a significantly higher spike rate than the rest of the electrodes (see Figure 3.11a), indicating that these two electrodes are located within the STN. To assess the suitability of the two frequency bands (LFP and MUA) for localization of the STN, I computed the cross-correlation (see Methods) between the signal recorded with the more distal electrode in the array and each one of the others, for the two frequency bands. The analysis confirms a very high correlation (> 0.9) for all the electrodes in the LFP band; for the MUA band, on the other hand, the high correlation is only observed for the two more distal electrodes, whereas for the rest of the electrodes, the cross-correlation is below 0.4 (Figure 3.11b).

The raster plot (Figure 3.11c, left) illustrates the spike count for each electrode of an array, from which it can be generated a color-coded spike rate histogram (Figure 3.11c, right); These results suggest that 2 or 3 electrodes can be positioned (the ones with a high spike rate) within the tiny STN structure (200-250 μm along the dorsoventral direction), while the rest of the electrodes are located outside the STN. I conducted this analysis across multiple implantations by plotting the color-coded spike rate for each electrode in arrays implanted in eight different animals (Figure 3.11d). The shadowed region (violet) marks the position of the two electrodes with the highest spike rate of each array, which can be used to tentatively identify the position of the STN in each of the experiments.

3.4 Monitoring of biomarkers in hemi-Parkinsonian rats.

To explore the suitability of the rGO microelectrode technology for monitoring Parkinson's disease biomarkers, We used the well-established 6-hydroxydopamine (6-OHDA) rat model, in which dopaminergic neuron depletion is selectively induced by unilateral administration of 6-OHDA into the medial forebrain bundle¹⁸⁸.

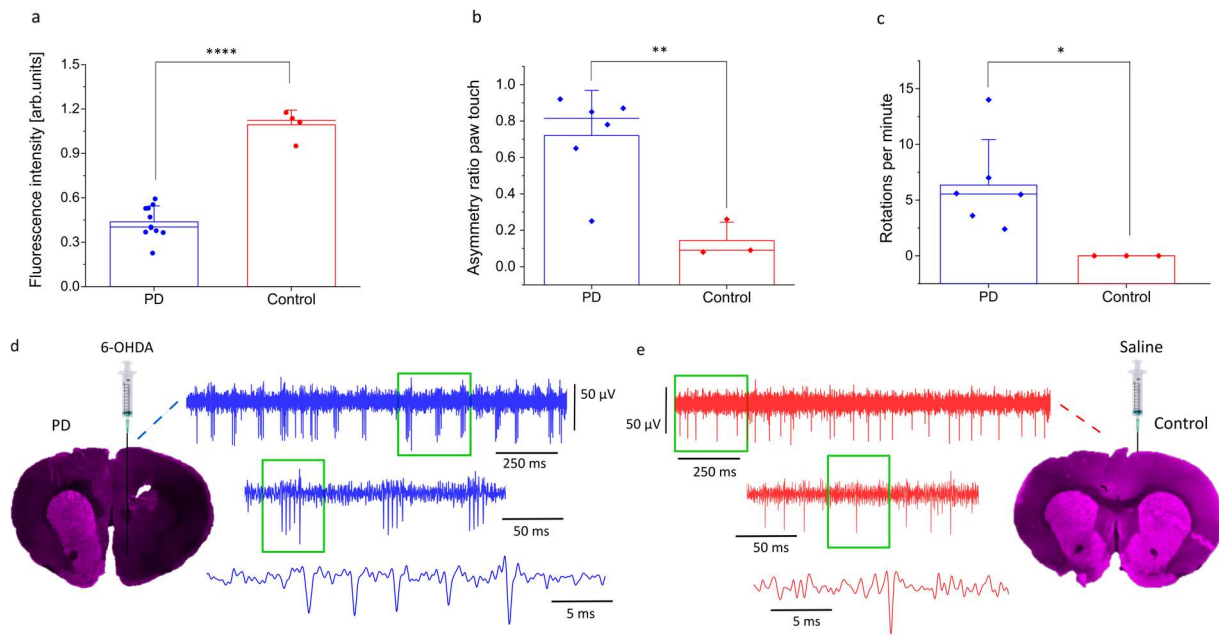


Figure 3.13 | Hemiparkinsonian rat model. **a**, Tyrosine hydrolase (TH) expression in the striatum as a measure of the extent of dopaminergic cell depletion. The average fluorescence intensity is expressed as a percentage of the contralateral side (animals analyzed: PD=10, control=4). **b**, Forelimb asymmetry test, quantifying the average asymmetric ratio in the use of the left and right paw (PD=6, control=3). **c**, Apomorphine-circling test, quantifying the average number of rotations per minute after apomorphine injection (PD=6, control=3). **d**, **e**, Exemplary recordings (MUA band, 200 Hz-2 kHz) of electrodes in the STN of the PD and control rats together with fluorescence images of brain tissue slices.

The generated asymmetric neurodegeneration can be evidenced by a marked reduction in dopamine fluorescence within the targeted hemisphere. Figure 3.13d visually demonstrates a dopamine deficit in the hemisphere injected with the neurotoxin, compared to the other brain, injected with saline, which is dopamine-intact. This dopaminergic cell loss was statistically quantified by computing the ratio of tyrosine hydrolase pixel intensity in the striatum of the injected side over the non-injected one (Figure 3.13a), and it results lower for the PD group (0.44 ± 0.11 , $n=6$) compared to the control one (1.09 ± 0.1 , $n=4$).

Before implantation of the devices, the animals underwent a series of behavioral tests to assess their response to the neurotoxin injection. In contrast to the control rats treated with saline, the rats injected with 6-hydroxydopamine exhibited a marked asymmetry in paw usage²¹³. Notably, PD rats were unable to effectively use the left paw when the right hemisphere was injected with the neurotoxin. This behavioral change was quantitatively evaluated by placing each rat in a transparent cylinder and recording the number of times it touched the cylinder wall with each paw (forelimb asymmetry test, Figure 3.13b). The analysis of the asymmetry ratio (see Method in Annex 1), revealed significant differences for the PD and control groups, 0.72 ± 0.24 and 0.14 ± 0.1 , respectively. The second

behavioral assessment involved administering apomorphine to the rats and observing their rotational behavior in the cylinder (apomorphine-circling test, Figure 3.13c). Typically, rats affected by the 6-OHDA injection display an excessive number of rotations, attributed to the asymmetry in their response to the movement induced by apomorphine. The average number of rotations per minute was significantly higher in the PD group 6.35 ± 4 , compared to the control one 0.1 ± 0 (Figure 3.13c). Both behavioral tests confirm that the animals treated with 6-OHDA exhibited sensory-motor anomalies characteristic of the Parkinsonian rat model, in clear contrast with the observations for the healthy control ones. Rats that completed more than 4 rotations per minute were considered hemiparkinsonian²¹⁴ and thus suitable for implantation.

Animals of PD and control groups were unilaterally implanted (see details in the Methods section on Annex 1) with the devices, with the objective of recording the neural signal from the electrodes in the STN and evaluate the capability of identifying electrophysiological biomarkers of the hemiparkinsonian rat model. Figures 3.13d and 3.3e show characteristic recordings (MUA band) for electrodes in the STN of a PD rat and of a control one, respectively. The recordings from the PD and control rats reveal clear neural spikes, but there are significant differences in the spike activity for both animal groups in terms of spike rate and firing pattern.

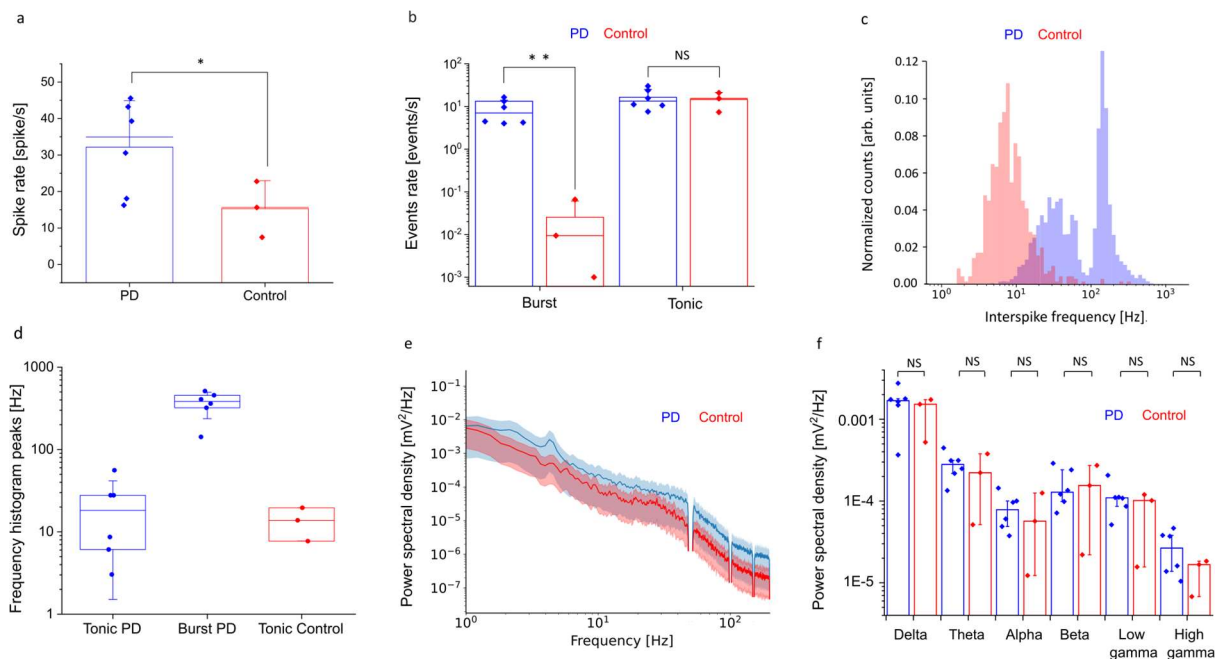


Figure 3.14 | Electrophysiological biomarkers recorded in the STN. **a**, Average spike rate (over 100 s) with of one electrode in the STN of the two groups (PD=6, control=3). **b**, Average number of spiking events, defined as bursts per second and tonic spikes per second, detected over 100 s (PD=6, control=3). **c**, Interspike frequency distribution, normalized by the total number of counts in 100 s, calculated from recordings of a PD (blue) and a control rat (red). **d**, Frequency peaks of the interspike frequency histograms for the 2 animal groups (PD=6 in blue, control=3 in red). The box plot represents the 25 and 75 interquartile. **e**, Comparison of the average PSD with STD (shadow area) of the signal recorded in the LFP range (1-200 Hz) over 55 seconds for the two animal groups (PD=6, control=3). **f**, Quantification of the total power over 100 s for each frequency band (delta 1-4 Hz, theta 4-8 Hz, alpha

8-12 Hz, beta 12-30 Hz, low gamma 30-70 Hz and high gamma 70-200 Hz), showing the median value with 25 and 75 interquartile whiskers (PD=6, control=3).

I calculated the total spike rate of the activity recorded by electrodes in the STN and found that the average spike rate for the control group was 15.3 ± 7.6 spikes/s, compared to 32.3 ± 12.7 for the PD group (Figure 3.14a), consistent with the expected enhanced neural firing activity of the hemiparkinsonian rats²¹⁵. Besides differences in the spike rate, the 2 groups present different firing patterns (Figures 3.13d and 3.13e): while the recording in the control rat exhibits a characteristic tonic spiking activity, the recording of the PD rat is characterized by a prominent bursty activity as previously reported^{215–217}. The analysis of all in vivo experiments was consistent with the exemplary recordings in Figures 3.13d and 3.13e, as illustrated in Figure 3.14b, which summarizes the calculated number of events per second for both groups (an event is defined as a single tonic spike or a burst of spikes). While the PD group exhibited an average rate of approximately 7.1 ± 3.5 bursts/s, in the control group it was lower than 0.1 bursts/s.

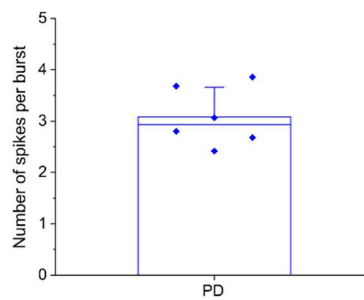


Figure 3.15 | Burst length. Average number of spikes per burst (with median line and STD at the whisker) recorded over 100 s in the STN of the PD group (n=6)

These bursts are groups of 3 ± 0.6 spikes on average (Figure 3.15), which fire very close to each other, usually around 400 Hz. In the case of the tonic spike pattern, similar rates were obtained for both groups, with values around 15 spikes/s (see Figure 3.14b). This indicates that while the tonic spiking pattern is consistent across both groups, the PD group exhibits an additional bursty component, which contributes to the overall difference in total spike counts. To further assess differences in the firing activity between the 2 groups, I calculated the interspike frequency (IF), which is defined as the inverse of the interspike time interval. Figure 13c plots the histogram of the interspike frequency for the experiments shown in Figures 13d and 13e, considering a normalized number of counts. The IF distribution for the PD rat is bimodal, with the low-frequency peak corresponding to tonic activity and the high-frequency peak associated with the burst-type activity (Figure 3.14c). In contrast, the IF distribution of the control animal is unimodal, characterized by a single tonic-related low-frequency peak. The frequency peaks of the IF distributions of the 2 groups are summarized in Figure 3.14d.

In contrast to the difference in spike firing patterns observed in the PD and control animals, the analysis of the recordings in the LFP bands did not reveal any significant difference between the two groups. The average power spectral density (PSD) of the signals recorded with electrodes located in the STN, shows that the PSDs do not allow the identification of any statistically significant difference between the PD and control groups (Figure 3.14e). A similar outcome can be obtained from the analysis of the median power of the recorded signal for each frequency band for both groups (Figure 3.14f). This analysis suggests that, contrary to the case of LFP signals, MUA is more effective in encoding PD disease-related biomarker information in anesthetized hemiparkinsonian rats.

3.5 Deep brain neuromodulation

After validating the suitability of the high-density linear array of rGO microelectrodes for localizing deep brain structures like the STN, as well as for identifying PD-related high-frequency electrophysiological biomarkers, I next investigated the effect produced by focal electrical stimulation on PD and control animals. To this end, electrical stimulation consistent with “standard” high-frequency DBS protocols was applied, and its effect monitored by comparing neural activity before and after stimulation.

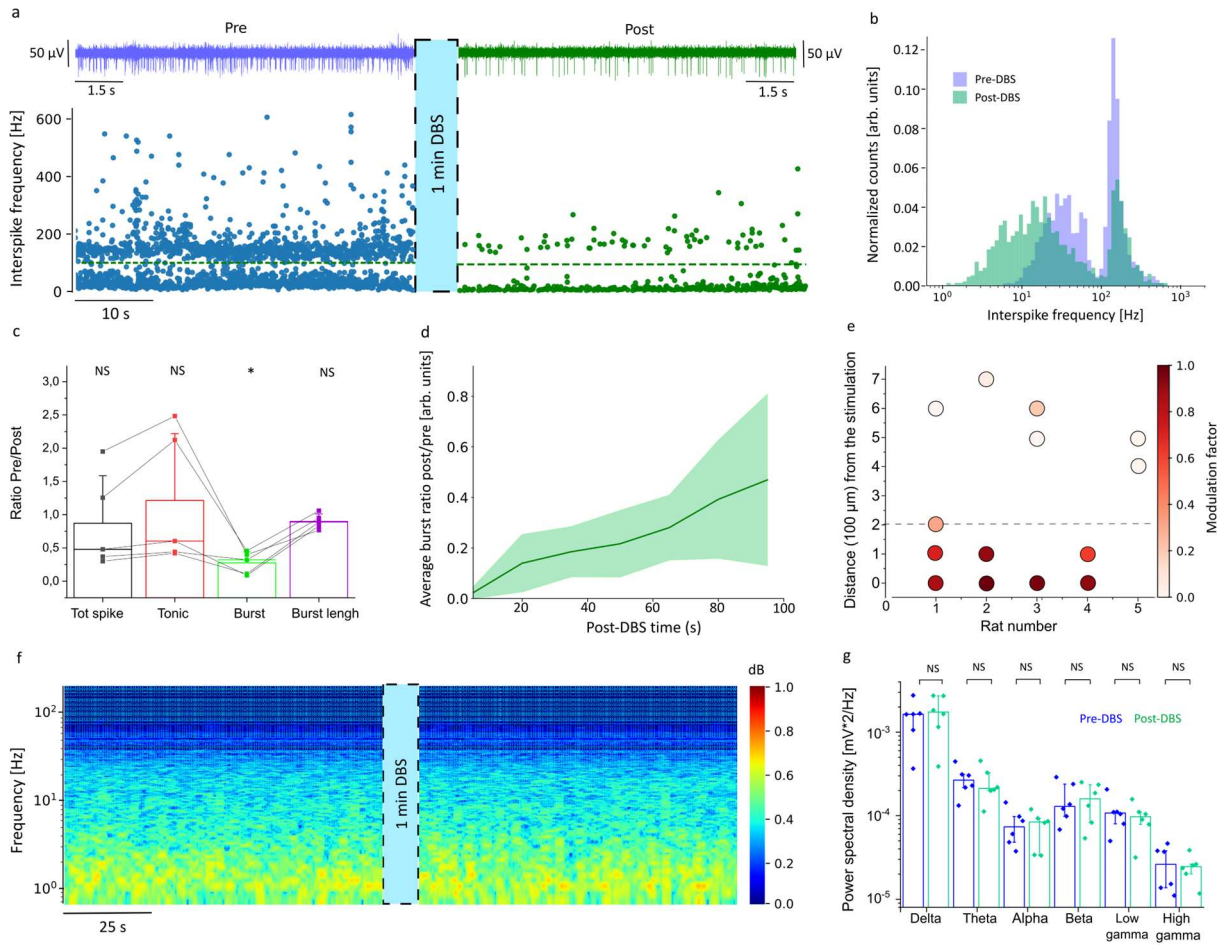


Figure 3.16 | Deep brain neuromodulation in the STN of Parkinsonian rats. **a**, Effect of microelectrode stimulation on the spike firing activity; the same microelectrode was used for stimulation and recording. The traces above correspond to the recordings (200 Hz-2 kHz) with a microelectrode in the STN of a PD animal, pre (blue trace) and post (green trace) stimulation. Below are shown corresponding time-dependent interspike frequency plots, pre and post-DBS; the green dashed line (at 100 Hz) indicates the division between the tonic and burst activity. **b**, Histogram of normalized IF spike distribution calculated pre- and post-DBS in a PD animal; each histogram quantified over 100 s. **c**, Average ratio (post- to pre-DBS) of different spiking-activity metrics: total spike rate, tonic spike rate, burst rate, and burst spike count (number of spikes per burst), calculated for the PD group (PD=5). **d**, Time evolution of the post-pre ratio of burst rates, depicting average (solid line) and STD (shadow color) for the PD group, quantified each 15 s. **e**, Color-coded modulation factor of the burst activity (definition in main text), displaying the impact of the distance of the stimulating microelectrode to the STN. The dashed line indicates the threshold distance at which the modulation factor drops to 0.5. Each dot corresponds to the modulation factor measured (over 100 seconds) in one electrode, for 5 different arrays (PD=5). **f**, Normalized spectrograms of LFP (1-200 Hz) activity of a PD rat, pre and post-stimulation. **g**, Total PSD of recorded signal, quantified (55 s) in different LFP frequency bands. Data were calculated for the PD group (n=6), showing the median value with the corresponding 25 and 75 interquartile error bars

In particular, I used biphasic current pulses of 100 μ s in pulse width, with various amplitudes and frequencies. A 1-minute stimulation protocol (current amplitude of 75 μ A and frequency of 130 Hz) changes the spike firing pattern in a PD animal (Figure 3.16a). Prior to the stimulation protocol, the

recorded signal exhibits the spike firing characteristic of the PD group, visible in the plot of the interspike frequency evolution over time (Figure 3.16a) and in the corresponding interspike frequency histogram (Figure 3.16b), revealing the previously discussed bimodal distribution, with tonic and burst spiking pattern. After stimulation (the stimulation was performed with the same microelectrode used for recording), the recorded activity shows a clear reduction of the burst activity: the interspike frequency distribution over time shows that the burst activity, represented by the high-frequency band, is significantly reduced and almost suppressed for several tens of seconds after the stimulation protocol; at this point, the brain activity looks close to the activity of a healthy control rat (Figure 3.17a,b).

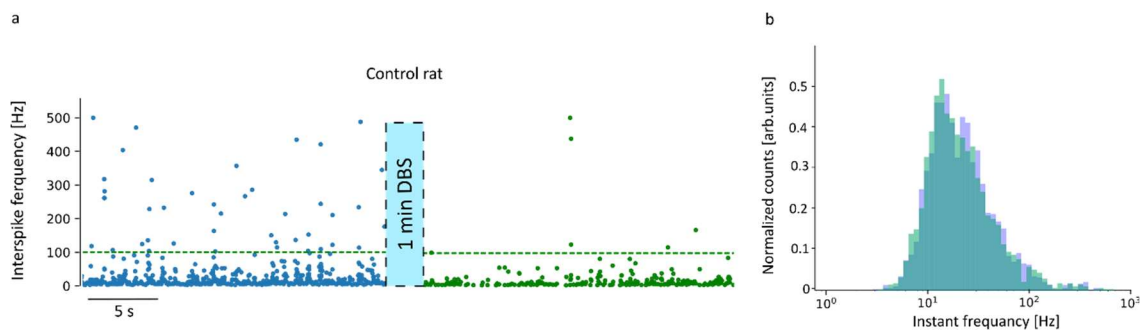


Figure 3.17 | Effects of DBS on control rat. **a**, Interspike frequency in the STN of a control rat, pre (blue) and post (green) DBS (75 μ A current amplitude, 100 μ s pulse width, and 130 Hz frequency). The green dash line highlights the cut-off frequency of the IF for the burst and tonic events. **b**, Normalized interspike frequency distribution over 100 s for the same control rat, pre and post-DBS.

The effect of stimulation on brain activity is better visualized by comparing the histograms of the normalized IF distributions pre- and post-DBS, as shown in Figure 3.16b. The normalization facilitates the evaluation of the change in frequency distribution, more than the variation of the total spike rate (not normalized IF distribution pre- and post-DBS for both PD and control groups are shown in Figure 3.18).

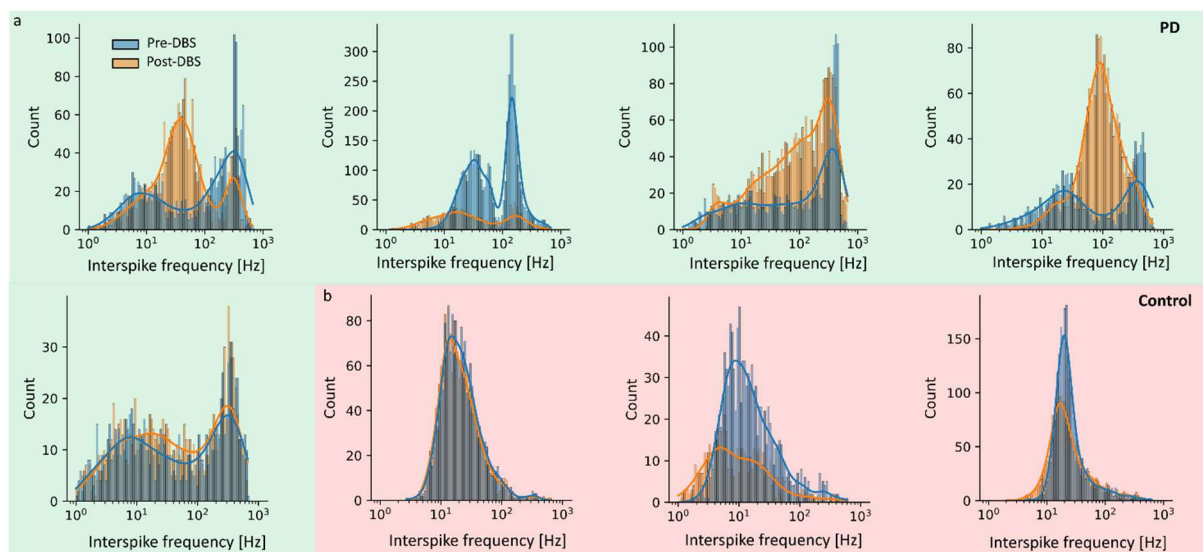


Figure 3.18 | Spike distribution in the PD-Control. **a,b** IF distribution pre- (blue) and post-DBS (orange) for the spikes recorded in the STN of 5 PD (green rectangle) and 3 control rats (red rectangle). The histograms are fitted with the kernel density probability **c**, Fano factor quantified over 100 s pre-DBS in the MUA range (200-2000 Hz) in the STN of the 2 groups, PD and Control (*p = 0,039 in the Welch T-test) **d**, Average number of spikes per burst recorded over 100 s in the STN of the PD group.

After stimulation, the amplitude of the burst peak is reduced to half its value before stimulation, while the IF distribution peak related to the tonic activity is shifted to lower frequencies (Figure 3.16b). When applying the same DBS protocol to a rat of the control group, no significant change in the spike firing pattern is observed (Figure 3.18a,b); the IF distribution associated with the tonic activity is unaffected both in its peak frequency position and width. mV^2/Hz .

I extended the experiment to the rest of the animals of the PD group; the summary of the analysis is depicted in Figure 3.16c. In particular, I calculated the ratio (R), post- to pre-DBS of different spiking-activity related metrics: the total spike rate, the tonic spike rate, the burst rate, and the burst spike count (Figure 3.16c). The total spike rate and the tonic spike rate show certain variability in the response to the electrical stimulation. This variability could be explained by the coexistence of excitatory and inhibitory neurons within the STN, which can be either excited or inhibited by the stimulation²¹⁸. Interestingly, while the tonic spike rate and total spike rate may increase in some cases after stimulation, the burst rate consistently decreases after stimulation for all investigated PD animals, evidenced by the median value of the burst rate ratio ($R = 0.29$). This finding suggests that the applied stimulation does not merely suppress neural activity but contributes to the desynchronization of the spike-firing activity.

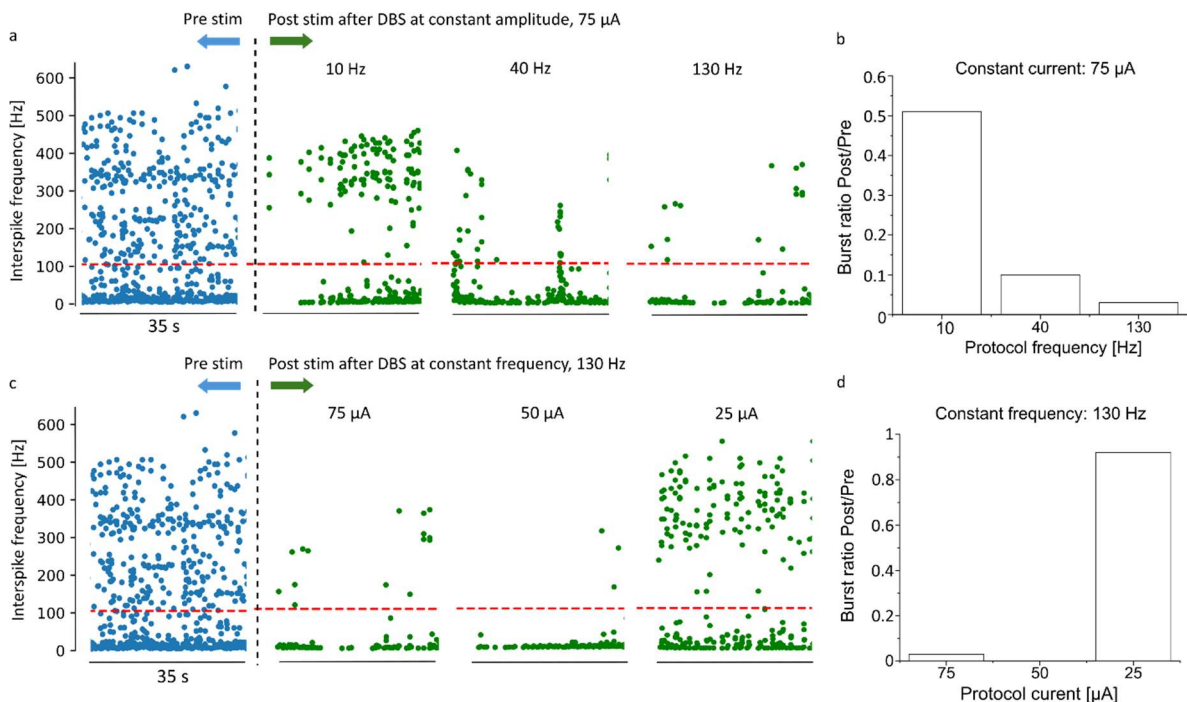


Figure 3.19 | Effects of DBS parameters on STN spiking activity. **a**, IF over time in the STN of a PD rat in period pre- (blue) and post-DBS (green), for biphasic current pulses of 100 μs and an amplitude of 75 μA . Different stimulation frequencies were tested, 10 Hz, 40 Hz and 130 Hz. The red

dash line highlights the cut-off frequency usually used to distinguish burst and tonic IF. **b**, Quantification over 35 s of the burst ratio post/pre for the 3 DBS protocols. **c**, The same effects are evaluated at a fixed frequency, 130 Hz and variable current, 25, 50 and 75 μ A. **d**, Quantification over 35 s of the burst ratio post/pre for the 3 DBS protocols of variable current.

Various stimulation protocols were explored to assess the capability of modulating burst activity at different frequencies and current amplitudes, as detailed in Figure 3.19. The analysis shows that, for the case of the 130 Hz stimulation protocol, a current amplitude of 25 mA is not sufficient to change the burst-type activity (10% burst reduction); in contrast, current amplitudes of 50 mA and 75 mA are effective in modulating neural activity (95% burst reduction, Figure 3.19c,d). The minimum current for which modulation of neural activity is observed corresponds to a charge density of 1 mC/cm² (considering the pulse width of 100 ms and the electrode diameter of 25 mm); this current level corresponds to a charge injection limit that cannot be reached by flat metal microelectrodes and is only compatible with a few electrode materials^{193,219,191,219}. I also studied the effect of the stimulation frequency (see Figure 3.19a,b). The current was maintained at 75 μ A while varying the stimulation frequency. No modulation was noted at 10 Hz, partial reduction of burst activity was observed at 40 Hz, and very effective modulation was achieved at 130 Hz (>95% reduction).

The long-term modulation capability of the rGO microelectrodes was demonstrated in a proof-of-concept experiment.

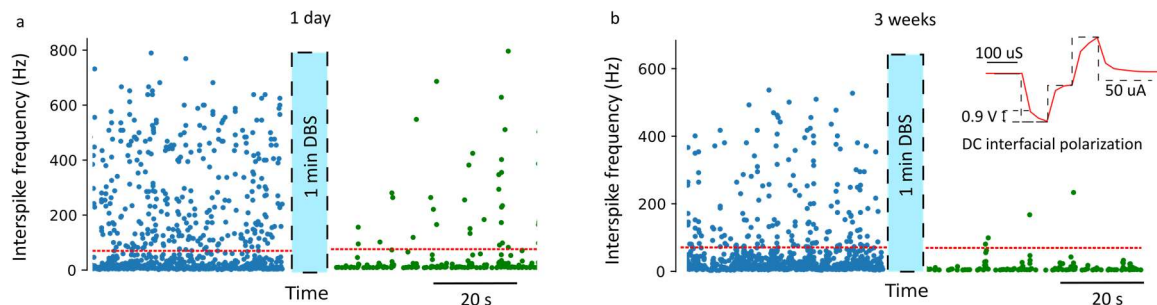


Figure 3.20 | Chronic modulation capability. **a**, IF distribution over time pre- (blue) and post-DBS (green), one day after implantation. **b**, IF distribution after 3 weeks. In red, the measurement shows the electrode voltage polarization measured in the brain during the DBS protocol (biphasic pulses of 50 μ A and 100 μ s pulse width).

After 3 weeks, the electrodes could effectively modulate high-frequency brain activity (see Figure 3.20a,b). Importantly, with a charge density of 1 mC/cm², the interfacial polarization at the electrode-tissue interface was still within the electrochemical safe limit of rGO. This result complements the data obtained in previous work,¹⁸⁶ showing the recording of auditory evoked potential in mice for 3 months and the capability to inject electrical stimulation in the sciatic nerve and induce muscle activation for 2

months. While these results indicate a stable electrode behaviour, further investigation is needed to understand the effect of high current density on brain tissue.

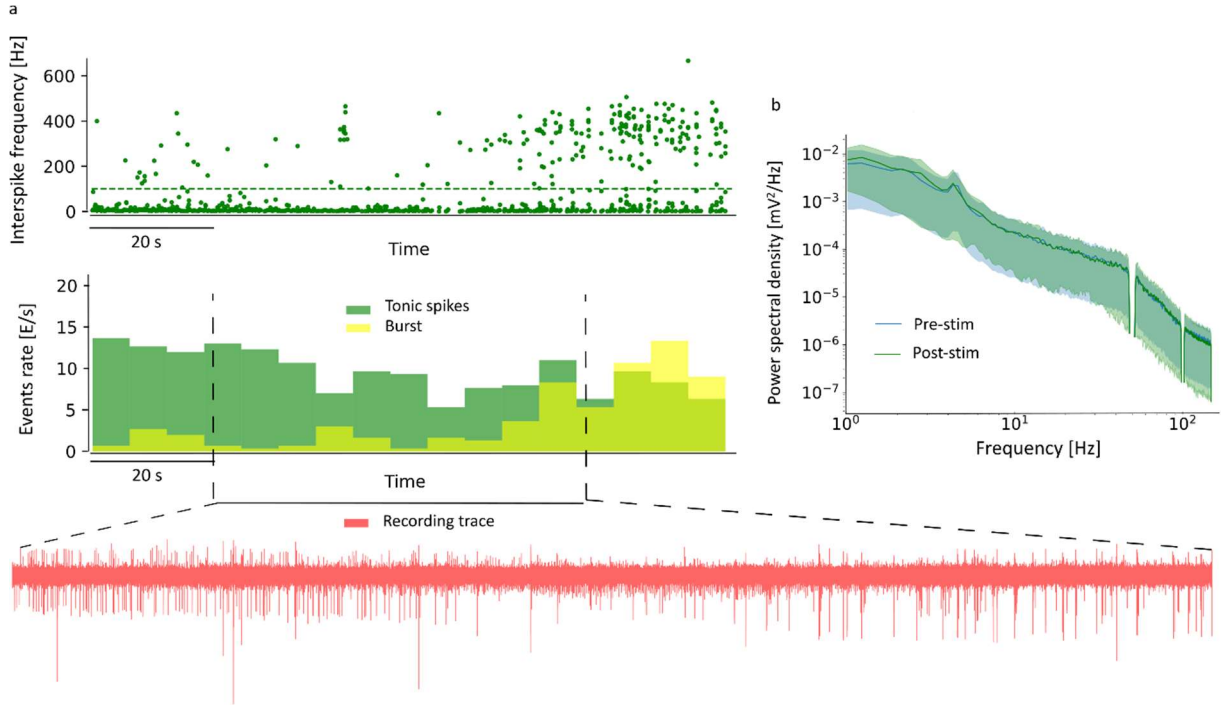


Figure 3.21 | STN Spiking activity evolution post-DBS. a, IF over time in the STN of a PD rat several minutes after DBS (biphasic current pulses of 100 μ s, 75uA at 130 Hz). The histogram below quantifies the burst rate (yellow) and tonic spike rate (green) in the same period in 6 s bins. Below is the corresponding zoom of the recording between the 2 dashed lines in the spike range (200-2000 Hz) to visualize the switch from tonic to burst activity. **b,** Average PSD with STD (shadow area) for the recording over 55 s with an electrode in the STD of the PD group (n=6), pre-DBS in blue and post-DBS in green.

Overall, it is important to consider that the observed modulation of neural activity, following stimulation, is not permanent and the burst activity is gradually recovered in the time scale of hundreds of seconds, indicating a reversible process. Figure 3.16d quantifies the recovery process, presenting the average burst rate ratio, post- to pre-DBS, over time for the PD group. This analysis reveals that immediately following DBS treatment (20 s after) burst activity is almost completely suppressed (less than 5% remains), slowly recovering 40% of its original level after 90 seconds. A more detailed description of the evolution of the burst recovery after stimulation is presented in Figure 3.21. The evolution of spiking activity post-DBS involves a gradual decrease in the tonic spike rate and a concurrent increase in the burst rate (Figure 3.21). Similar studies noted a general recovery process of the total spike rate after DBS²²⁰.

I then assessed the capability of the rGO microelectrodes for focal stimulation²²¹ by investigating the efficacy of modulating brain activity at increasing distances. To this end, I quantified a “burst modulation factor”, F_{burst}^{mod} , which represents the change in the burst activity induced by the stimulation,

defined as $F_{burst}^{mod} = \frac{Burst_{pre} - Burst_{post}}{Burst_{pre}}$, where $Burst_{pre}$ and $Burst_{post}$ correspond respectively to the burst rate before and after DBS. It is important to note that, while pre-DBS burst activity is relatively constant over time, post-DBS burst activity increases over time (Figure 3.16d), implying that F_{burst}^{mod} is an averaged estimation of a dynamic phenomenon. The F_{burst}^{mod} is quantified, with a color-coded scale, for several electrodes of different arrays (n=5 PD animals), considering an absolute distance from the stimulating electrode to the recording sites (Figure 3.16e). This analysis confirms that a very effective modulation is recorded in the electrode used for stimulation ($F_{burst}^{mod} \cong 1$). For microelectrodes located 100 μm “away” from the stimulating one, F_{burst}^{mod} already drops below 0.8. For microelectrodes located 500 μm away, F_{burst}^{mod} drops below 0.2, meaning almost no effect of modulation. These results suggest that focal modulation of the STN can be achieved, provided that the device is precisely positioned within the target area.

I also assessed the impact of stimulation on the LFP signal; the analysis did not evidence any significant change in brain activity for any of the frequency bands from 1 to 200 Hz, as illustrated by the spectrogram in Figure 3.16f. To confirm this result, I compare the power of the recorded LFP signal in each frequency band pre- and post-DBS, and it does not reveal any significant change in the LFP activity upon stimulation (Figure 3.16g). The lack of sensitivity of the LFP signal to the stimulation is attributed to the focal stimulation provided by the rGO microelectrodes²²¹, which are capable of modulating local neural activity without altering the less local, LFP-encoded neural activity, in particular under the anesthesia conditions of our experiments, characterized by highly synchronized LFP activity.

3.6 Conclusion

In order to expand DBS adoption, it is key to reduce the invasiveness of the therapy, minimize unwanted side effects by increasing stimulation precision, and enhance efficacy enabling adaptive modulation of brain activity. A new generation of electrode materials, offering better performance and bidirectionality, is needed to help resolve some of these challenges. As reported in a previous study¹⁸⁶ and described in Chapter 2, reduced graphene oxide is a high-performing material due to the nanoporous structure that increases the electrochemically active surface area, resulting in low-impedance and high-charge injection capacity. Besides its state-of-the-art electrochemical and mechanical properties, rGO is also a relatively low-cost material, can be integrated with flexible microelectronics and microfabrication processes, and it is highly biocompatible^{222,223} and chemically inert, which makes it promising for neural interfaces.

In this part of the thesis I investigated the potential benefits of using rGO technology for deep brain stimulation in Parkinson's disease. The devices were designed with a high-density linear array of 8 microelectrodes of 25 μm in diameter, spaced 100 μm , in a compact and lead 120 μm wide and 10 μm

thin. For the implantation of the flexible leads, I developed a methodology that uses an optimized dissolvable adhesive polymer (PVA) to temporarily attach a rigid and thin silicon shuttle, that enables a slow and controlled insertion while minimizing brain damage. The leads are equipped with an anchoring system to keep the electrodes in place during the removal of the shuttle, which is crucial to deliver electrical stimulation in the correct place and minimize the side effects of off-target stimulation. The structural and electrical properties of the rGO material allow for the miniaturization of the electrodes to microscale, while maintaining the capability to record single-neuron activity with high SNR and to inject enough charge to induce neuromodulation.

The combination of small electrodes with high-fidelity recording capability facilitated the precise localization of the STN by monitoring the specific pattern of spiking activity of these brain nuclei. Millimeter-size electrodes, as currently used in the clinic, do not allow to easily provide information about the lead position inside or outside the STN, since these large electrodes cannot monitor spiking activity, making targeting or localization based on electrophysiological recordings in the LFP range not possible with the same device.

This study with hemi-parkinsonian and control rats conducted under anesthesia reveals the presence of a multiunit-based Parkinsonian biomarker. The brain activity in the STN of Parkinsonian rats exhibits a unique spike distribution characterized by a combination of tonic and burst patterns. In contrast, the control rats display only a tonic pattern. This brings the possibility of using an additional biomarker for closed-loop, adaptive DBS in Parkinson's disease, different from the more standard beta peak in the LFP range²²⁴, whose use in the clinic has been recently questioned^{225,226,184}.

The bidirectional property of recording and stimulation with the same electrode allows us to investigate local effects produced by the electrical stimulation on this biomarker. I explored several high-frequency DBS protocols and monitored the effects on the neural activity of hemiparkinsonian and control animal groups. By analyzing STN spiking distributions before and after stimulation I confirmed a reduction of bursting activity in the STN of the PD animals, while the tonic spiking activity increases in some cases, indicating a modulation process, rather than a global suppression effect. Compared to other studies where no post-DBS modulation of the burst activity is reported^{227,228}, our study indicates that bidirectional high-resolution neural interfaces facilitate the visualization of local neuromodulation effects in the high-frequency activity that lasts temporarily for tens of seconds after stimulation. This modulation can be achieved only by injecting a high-density charge of 1 mC/cm² (for a "standard" DBS protocol of 100 μ s pulse width at 130Hz) which cannot be provided by flat metal microelectrodes and is possible only with few electrode materials^{191,193,219,229}. Moreover, delivery of DBS in the STN of hemiparkinsonian rats modulate the spiking activity to a state closer to the control rats one, suggesting a potential therapeutic effect that will be evaluated in future studies. Interestingly, no significant change was observed in the LFP range after DBS, presumably due to the high focality of the treatment²³⁰

(approximately 200 μm), which does not modulate LFP-encoded neural activity, especially under anesthesia, since the brain signal is highly synchronized.

In summary, this chapter demonstrates the benefits of using bidirectional rGO microelectrodes for improved acquisition of local electrophysiological biomarkers such as STN bursting activity and the capability to modulate them with high efficacy.

Chapter 4

Advances in rGO neurotechnology for nerve interfacing

Neurotechnology is being explored for restoring sensorimotor functions after paralysis or amputation, which requires peripheral nerve interfaces that are selective, bidirectional, and chronically stable. These devices use neural interfaces to record and stimulate nerve action potentials, creating a bidirectional connection with the nervous system. Most neural interfaces are based on standard metal microelectrodes. Reduced graphene oxide (rGO)-based microelectrodes offer low impedance and a high charge-injection limit, potentially enabling the stimulation of nerve fascicles to produce selective muscle activation. In a first-generation transverse intrafascicular multichannel electrode (TIME) device compound nerve action potentials (CNAP) elicited by electrical stimuli and electroneurography (ENG) evoked by mechanical stimuli were recorded with high resolution but their functionality decreased over time, presumably due to the limited durability of encapsulation.

To overcome these problems, I developed a new 10 μm -thick TIME with a hybrid polyimide- Al_2O_3 encapsulation engineered to improve fabrication yield, electrode-to-electrode uniformity, and device stability. In vitro, devices maintained near-ideal capacitive behaviour after accelerated ageing (3 months in PBS at 57 °C) and sustained 10^9 biphasic stimulation pulses without detectable electrochemical degradation. In vivo, the arrays recorded compound nerve action potentials after one month and enabled selective activation of distinct peripheral nerve fibres with comparatively low current thresholds during four months of follow-up, remaining below the device maximum injectable current. Together, these results demonstrate that combining graphene microelectrodes with a thin hybrid encapsulation improves chronic reliability of intraneural thin-film interfaces and helps to close the gap between laboratory prototypes and clinically relevant neuroprosthetic systems.

Most of the contents of this chapter have been published in:

1. Rodríguez-Meana, Bruno, et al. "Engineered graphene material improves the performance of intraneural peripheral nerve electrodes." *Advanced Science* 11.29 (2024): 2308689.
2. Ria, Nicola, et al. "Advances in Thin-Film Graphene Neurotechnology for Chronic Nerve Stimulation and Recording." *bioRxiv* (2026): 2026-01.

Introduction

Interfaces for recording and stimulating peripheral nerves are poised to enable a widening range of therapies and neuroprosthetic systems. Applications span restoration of motor and sensory function in limb loss^{1,2} and neuropathies, neuromodulation of organ function, inflammation via the vagus nerve^{103,231}, treatment of pain³ and movement disorders⁴ through targeted peripheral stimulation.

In the last few decades, a variety of peripheral nerve interfaces have been developed and tested^{232,233}. However, translation of these research efforts into clinical applications is rather slow due to technological challenges. To provide complex exchange of signals, as needed for producing natural-like sensations and accurate control of the movement of a prosthesis, it is mandatory to reliably and selectively stimulate and record small populations of afferent and efferent axons in a peripheral nerve¹⁵. Thus, improving selectivity and spatial resolution requires reducing electrode dimensions while increasing the number of electrodes interfacing with the nerve. However, when the size of metallic electrodes is scaled down to the micrometer range, the current required to elicit neural stimulation often exceeds the charge injection limit (CIL) of the electrode material. Operating beyond the CIL induces faradaic reactions at the electrode–tissue interface, which can compromise biocompatibility and degrade the conductive material, ultimately limiting long-term stability. In addition, decreasing the electrode surface area leads to higher interfacial impedance, resulting in recordings with reduced signal-to-noise ratio (SNR).

At present, most peripheral nerve interfaces rely on conventional metal microelectrodes (e.g., Au or Pt) fabricated on silicon-based films. To overcome the limitations of traditional metals, a new generation of neural interfaces is emerging in which metallic electrodes are replaced by reduced graphene oxide (rGO). This material exhibits high charge injection capacity due to its wide electrochemical potential window and large double-layer capacitance. These properties enable electrode miniaturization to the microscale while maintaining sufficient charge delivery for effective neural stimulation and low intrinsic noise during recording.

Beyond electrochemical performance, a major remaining challenge is to achieve the high functionality of advanced neuroprosthetic interfaces and the long-term stability and mechanical robustness required for chronic implantation. Different typologies of implants have been tested so far, including epineural and cuff devices,^{232,233} which are less invasive but often provide limited selectivity. Intraneural longitudinal⁸² (LIFE) or transverse (TIME) devices^{13,234} and Utah-style arrays (USEA) increase selectivity at the cost of more intimate tissue contact and greater mechanical demands. While currently explored clinical technologies have established the feasibility of nerve interfacing, their impact remains limited by invasiveness²³⁵, mechanical mismatch with tissue^{235,236}, and constraints in spatial resolution.

In this context, a new generation of flexible,^{92,237,238} thin-film technologies promise reduced tissue response^{89,235}, a smaller surgical footprint and finer spatial resolution⁶³ enabled by miniaturized microelectrodes capable of bidirectional operations^{136,155} (stimulation and recording). However, manufacturing small microelectrodes while maintaining low-noise recordings and delivering sufficient current density to activate fibers reliably remains a major challenge when using clinically approved materials.²³⁹ To this end, emerging electrode materials and associated fabrication strategies are being explored to raise charge-injection limits and reduce electrode impedance, while preserving the biotic/abiotic stability of the implant. Recent efforts have focused on PEDOT¹¹ and related conducting polymers, iridium oxide²⁶ (IrOx), and nanocarbon²⁴⁰ platforms. Within this landscape, reduced graphene oxide (rGO)-based microelectrodes combine chemical stability, a wide water window and favourable capacitive behaviour¹⁴⁹ with facile integration on flexible substrates, positioning them as promising candidates for high-density, bidirectional peripheral interfaces. In prior work, I showed in acute settings that rGO microelectrodes exhibit low impedance, a high charge-injection limit (CIL), low stimulation thresholds, and high signal-to-noise ratios (SNR) during neural recording^{12,155}.

However, most graphene microelectrode studies have been limited to acute or sub-chronic in vivo use. Translation to chronic use requires advances in thin-film encapsulation to prevent moisture ingress and interlayer delamination while maintaining mechanical compliance. Flexible, biocompatible polymers (PI^{63,155}, PDMS¹⁰⁰, Parylene C^{96,97}, SU-8⁹⁸) are widely used, but their relatively high water-vapour transmission rate and their poor interlayer-adhesion can drive degradation under humid conditions¹²⁵ and electromechanical stress^{101,126}. To address these limitations, hybrid organic–inorganic stacks have been developed, combining polymer layers with inorganic materials such as HfO₂^{127–129}, SiO₂^{128,130}, SiN_x¹³⁰, and Al₂O₃^{127,131}. These multilayer stacks offer low water-vapour transmission rates (WVTR) and have demonstrated excellent barrier performance in accelerated ageing, although they are often integrated into rigid structures²⁴¹, and their in vivo evaluation remains insufficiently explored.

In this work, I introduce a new generation of transverse intrafascicular multichannel electrode (TIME) technology engineered for chronic peripheral nerve use. I combine (i) a device layout with integrated anchoring features to mitigate displacements, (ii) a hybrid PI–Al₂O₃–PI encapsulation that improves moisture-barrier properties and metal–polymer adhesion, and (iii) via-under-electrode routing that isolates rGO residues from conductive tracks. I show that these advances result in high-yield devices with uniform electrochemical performance and robust in vivo function. In bench testing, arrays exhibited excellent electrode-to-electrode uniformity (e.g. CIL and impedance) and no detectable structural change by Raman spectroscopy after accelerated ageing and 10⁹ stimulation pulses. In vivo, rGO microelectrodes achieved selective activation of distinct groups of nerve fibers with thresholds remaining below the maximum injectable current over 120 days, and allowed recording nerve action potentials with robust signal-to-noise ratios. Collectively, these results contribute to advance thin-film rGO-based intraneural interfaces towards durable in vivo operation.

4.1 First-generation transversal intraneural multielectrode (TIME) arrays.

The first-generation transversal intraneural multielectrode (TIME) devices consisted of two linear arrays equipped with nine electrodes (diameter, 25 μm) along a 1.2 mm stripe (Figure 4.1). Before insertion, the device is bent to have electrodes facing both sides of the lead, to increase the number of active sites (AS) available for recording from different nerve fibers (increased spatial resolution) and improve stimulation directionality and focality.

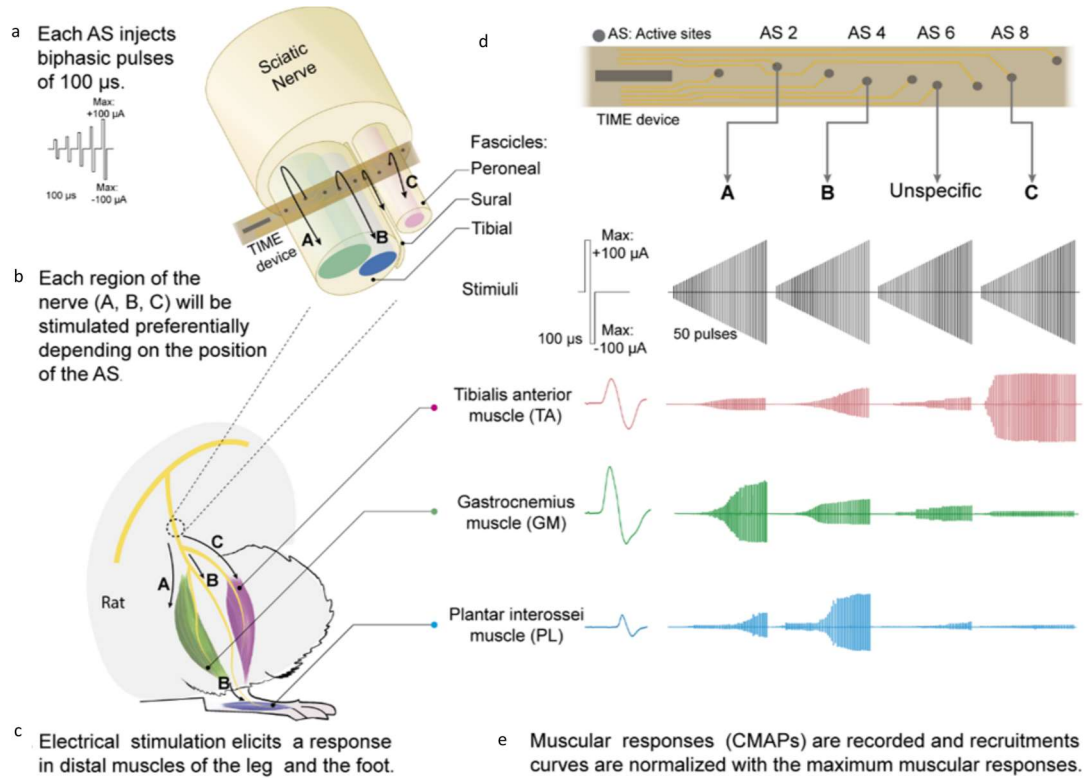


Figure 4.1. Protocol to assess stimulation capabilities of rGO electrodes. **a**, Active sites (AS) inject a sequence of 50 biphasic pulses of increasing current (0–100 μA), 100 μs each at 3 Hz. Each set of 50 pulses is repeated twice. **b**, The TIME is inserted transversally into the tibial and peroneal fascicles of the sciatic nerve. Hence, ASs interface different regions of the nerve (A, B and C) which innervate different muscles (plantar, gastrocnemius, tibialis anterior. **c**, Electrical stimulation through each of the ASs elicits a response in the distal muscles of the leg and the foot. Muscular responses vary depending on the AS that is used. **d-e**, Monopolar needles are placed in the belly of the studied muscles to record the CMAPs produced in each step of the stimulation protocol. CMAPs are normalized to the maximum amplitude for each muscle to build the recruitment curve: a representation of the progressive activation of the muscle as the current increases. The threshold currents needed to activate each muscle at 5%, 30% and 95% are used for further analyses. Figure adapted from¹²

Devices were transversely inserted (Figure 4.1a) into the sciatic nerve of the rats, to access the three different nerve fascicles of the sciatic nerve, peroneal, sural, and tibial, which control respectively the tibialis, the plantar and the gastrocnemius (Figure 4.1c). The application involved the selective stimulation of three different fibers to produce the contraction of the three muscles of one leg, to recover

the movement of the rat after a spinal cord injury. The final goal is to identify a paradigm to control in a specific sequence and intensity the activation of the muscle to induce a smooth and synchronized movement of the legs and recover walking. A bidirectional interface with the nerve is also required to record and detect compound nerve action potential (CNAP) and sensation-evoked potentials to set a closed-loop, where the stimulation is delivered in response to sensory feedback of the leg. The first phase to validate the technology was the evaluation under anesthesia of the capability to activate a determined percentage of the muscles with electrical stimulation, as well as to record signals of different amplitudes in the nerve.

Biphasic current pulses (100 μ s per phase) with increasing amplitude (0-100 μ A) were delivered while simultaneously recording compound muscle action potentials (CMAPs) from three target muscles using monopolar needle electrodes (Figure 4.1a). In the example shown in Figure 4.1d–e, the dense distribution of electrodes enables optimal positioning of individual active sites within specific nerve fascicles. In this case, AS8 is located within the peroneal nerve, as suggested by its high selectivity index (SI) for the tibialis anterior (TA) muscle. Conversely, AS2 and AS4 are positioned within the tibial nerve, with AS2 likely closest to the gastrocnemius medialis (GM) fascicle and AS4 associated with the fascicle innervating the plantar muscles. AS6 lies between the tibial and peroneal nerves and therefore exhibits a low ability to activate any of the recorded muscles. Overall, selective activation is achieved by choosing the appropriate electrode, allowing the induction of higher-amplitude CMAPs in a specific muscle while minimizing activation of the others.

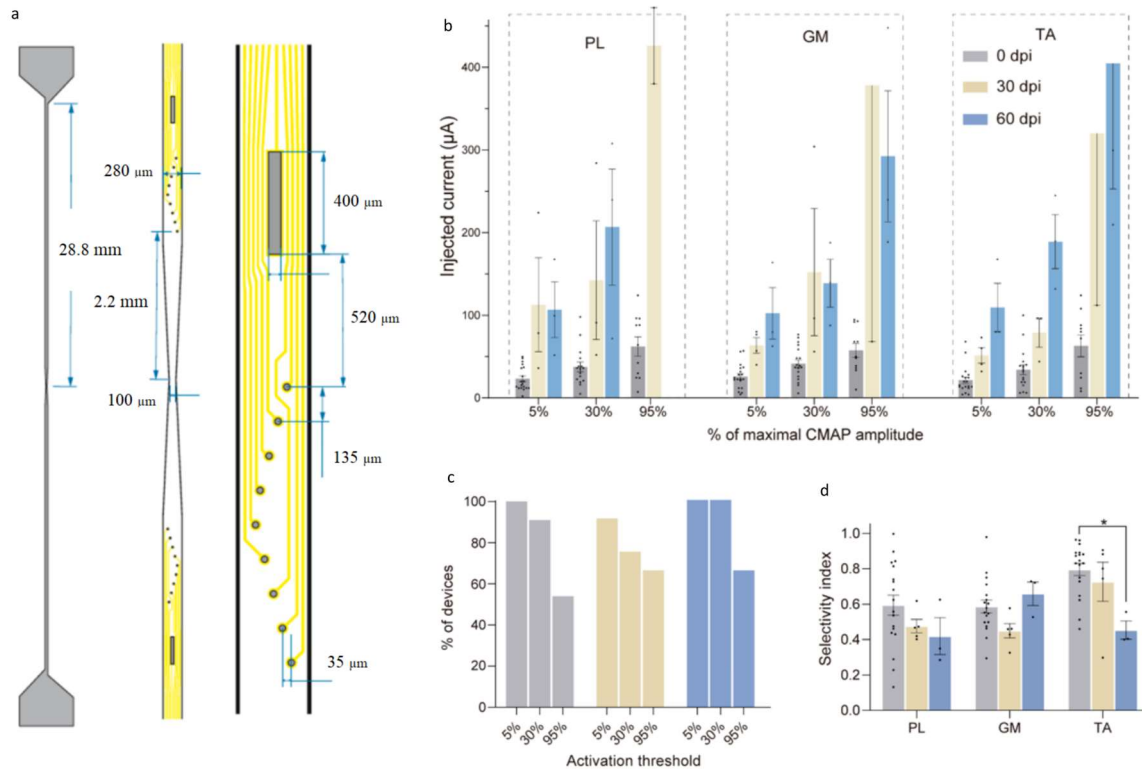


Figure 4.2 | Stimulation capability of 25 μm rGO electrodes. **a**, Schematic of a device, with two zooms, displacing a double arm implant with 2 arrays of 9 microelectrodes of 25 μm diameter (black dots) and a rectangular pad. **b**, Current threshold needed to elicit 5, 30, and 95% of the maximum CMAP amplitude in the PL, GM, and TA muscles over 60 days. **c**, Percentage of working devices able to elicit CMAPs of 5, 30, and 95% of the maximum CMAP amplitude in each of the three muscles tested. **d**, Selectivity index to activate by muscle at 5% and 30% respectively of the maximum CMAP amplitude. * $p < 0.05$, two-way ANOVA followed by Tukey's multiple comparison. Figure readapted from¹²

4.2 Acute and semi-chronic nerve stimulation.

Besides the capability to recruit the neuromuscular system, an important functionality is the possibility to activate the nerve fibres with selectivity, particularly in applications aiming to control the coordinated movement of the limbs to restore mobility²⁴² or sensation¹⁵. Dense, small intraneural electrodes delivering focal current can favour selective activation of specific fascicles. The selectivity index (SI) for the three tested muscles ranged between 0.6 and 0.9, similar to what was previously found¹⁵⁵ (Figure 4.2d, see Annex 1 for calculation details). Differences in the SI among the muscle groups can be attributed to the distinct spatial organization of the axons innervating each muscle within the sciatic nerve. The SI was calculated at the stimulation level required to evoke 30% of the maximal CMAP amplitude in one of the muscles. Variations in SI were observed over the course of chronic implantation. For the tibialis anterior (TA) muscle, selectivity decreased at 60 days post-implantation (dpi), while for the plantaris (PL) muscle a reduction in selectivity was already evident at 30 dpi. In contrast, selectivity for the gastrocnemius medialis (GM) muscle—although lower at baseline compared with the other two muscles—remained relatively stable throughout the follow-up period (Figure 4.2d).

The current thresholds required to elicit 5%, 30%, and 95% of the maximal CMAP amplitude are reported in Figure 4.2b. Both acute and chronic implantation experiments demonstrated that EGNITE microelectrodes are capable of selectively stimulating different axonal subpopulations within the sciatic nerve, depending on the active site configuration and the applied current amplitude. Considering the current compliance limit of 100 μA for 25 μm rGO electrodes, the average current required to reach near-maximal activation (95%) at day 0 remained within safe operating limits. Compared to cuff devices, having electrodes inserted transversally inside the nerve decreases the current threshold for muscle activation, due to the reduced distance between the active sites and the nerve fibers.⁵⁷

In contrast, to reach 30% and 95% activation after 30 days of implantation, higher injected currents were required, in some cases exceeding the safe limits (notably for the GM and PL muscles), thereby increasing the risk of electrode degradation and tissue damage (Figure 4.2b). Even exceeding the current limits, 40 % of the devices were not able to achieve responses higher than 95% (Figure 4.2c).

This pronounced ($\sim 4\times$) current increase in stimulation threshold can be attributed to both biological and technological factors. Biologically, the formation of a fibrotic encapsulation layer around the electrode—

nerve interface can increase impedance and reduce stimulation efficiency. From a technological perspective, long-term device stability represents a limiting factor, as the thin-film encapsulation can degrade under chronic exposure to a wet environment, leading to water ingress and subsequent electrode short-cutting and deterioration. Displacement of the electrodes outside the nerve could also explain the need how higher current to induce the same muscle contraction.

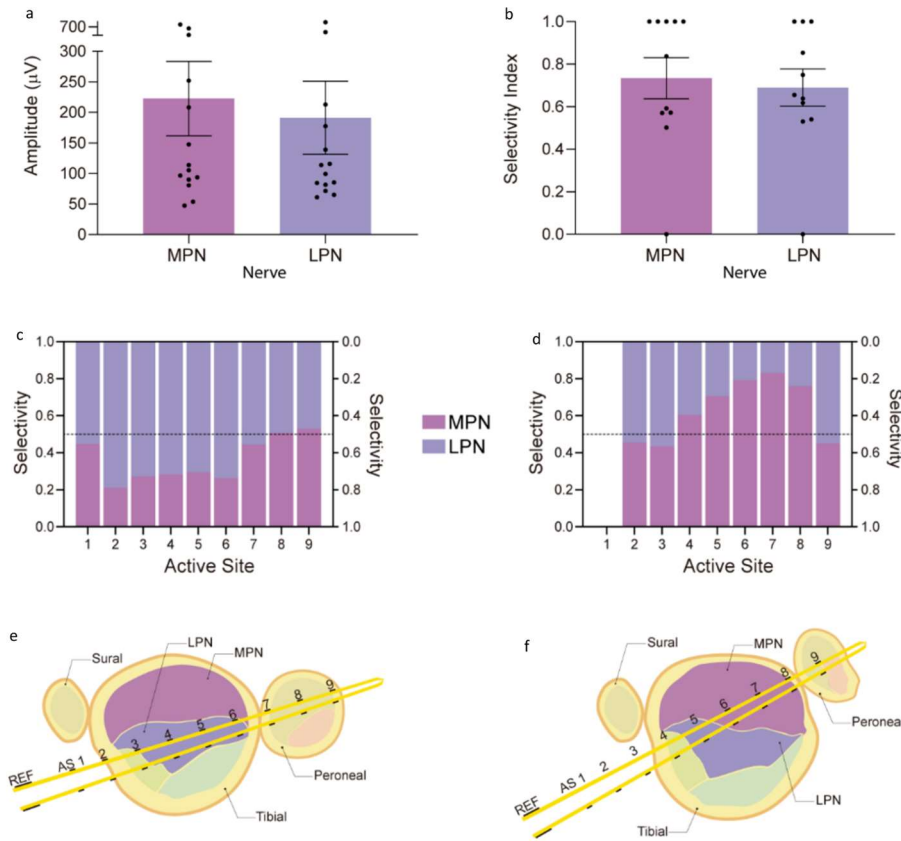


Figure 4.3. Recording capabilities of 25 μm rGO electrodes **a**, Plot of the maximal CNAP amplitude elicited by electrical stimulation of the Medial Plantar Nerve (MPN) or the Lateral Plantar Nerve (LPN), recorded with the implanted devices at 0 dpi. **b**, Selectivity index of the MPN and LPN CNAPs. The maximum selectivity value among all ASs in each device for both nerves was used for the analysis. **c-d**, Selectivity from each AS of the TIME 1 and TIME 2 calculated in the same step of the current increasing stimulation protocol. **e-f**, Representation of probable location of TIME 1 and TIME 2 devices within the nerve as deduced from the selectivity data. Figure readapted from¹²

4.3 Acute nerve recording.

Beyond the ability to effectively stimulate nerve branches and elicit muscle contractions, an additional key functionality is the selective recording of neural activity from sciatic nerve fibers, enabling bidirectional communication with the peripheral nervous system. To assess the recording performance of the rGO electrodes, compound nerve action potentials (CNAPs) were evoked by electrically stimulating either the medial plantar nerve (MPN) or the lateral plantar nerve (LPN). The devices were

able to record CNAPs with amplitudes ranging from a few microvolts, increasing progressively with stimulus intensity. However, not all electrodes on each device were able to record neural signals, either due to malfunction or because their position within the nerve was not optimal for signal detection. The mean maximal recorded amplitude was approximately 200 μ V (Figure 4.3a). Recordings at 30 days post-implantation (dpi) were particularly challenging, mainly due to limitations in the long-term stability of the devices under chronic implantation conditions.

The dense spacing of microelectrodes within the array, combined with the high signal-to-noise ratio achievable using rGO microelectrodes⁶³, enabled selective discrimination of CNAPs originating from the MPN and LPN, with an average selectivity of approximately 0.7 (Figure 4.3b). Six devices achieved 100% selectivity in at least one electrode, meaning that a specific electrode recorded CNAPs exclusively from one nerve branch and not the other. Conversely, two devices exhibited 0% selectivity for one of the nerve branches, which highlights performance variability

Two representative experiments demonstrated that the achieved selectivity (Figure 4.3c–d) depends not only on the intrinsic recording resolution of the electrode array but also on the positioning of the device after implantation (Figure 4.3e–f). Because the axons innervating both the MPN and LPN are located within the same tibial branch of the sciatic nerve, achieving high selectivity between fibers belonging to the same fascicle is particularly challenging. While the dense array of nine microelectrodes with 25 μ m diameter increases the probability of having at least one electrode positioned near each nerve fiber population, additional factors such as biological variability, surgical implantation accuracy, and device displacement during chronic use can reduce the selectivity index and overall recording performance.

4.4 Second-generation TIME device with hybrid encapsulation

In order to overcome the limitations of the first generation of TIME devices, I designed, fabricated, and tested in vivo a new TIME device optimized for stable nerve interfacing in chronic applications. The device is a thin-film PI ribbon (10 μ m thick, 280 μ m wide) comprising 2 arms with eight microelectrodes each of 80 μ m in diameter, centre-to-centre pitch is 160 μ m (Figure 4.4a).

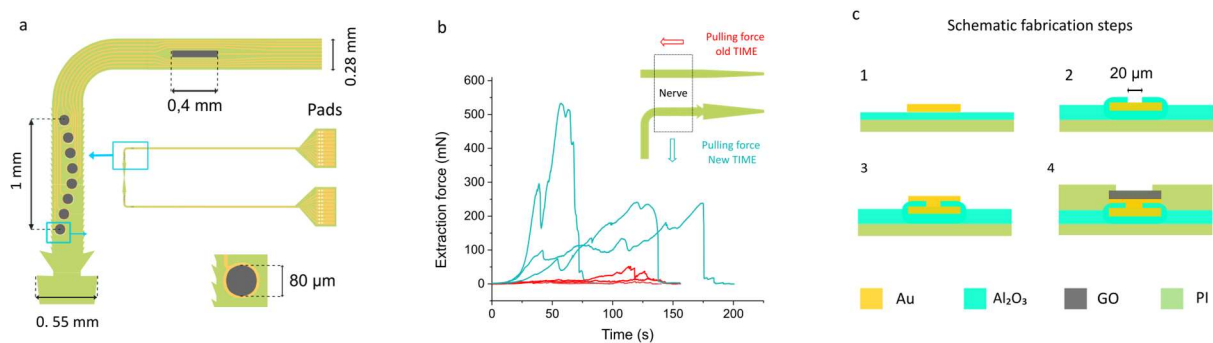


Figure 4.4 | L-shape improved TIME device, design and fabrication. **a**, Schematic of the new TIME design: each arm carries an array of eight 80 μ m-diameter microelectrodes and a rectangular reference electrode (0.4 mm x 60 μ m). The lead has an L-shaped layout and two 0.55 mm anchoring arrow-

shaped structures to improve fixation within the nerve. **b**, Extraction force for TIME devices pulled from an explanted sciatic nerve affixed to a petri dish. The graph shows three measurements made with the previous linear TIME design (red) and three with the new design incorporating the anchoring features (light blue). **c**, Cross-sectional fabrication workflow of the device, illustrating the four main process steps.

The dimension of the electrodes was increased to improve the current injection limits, while the center-to-center pitch was kept constant to maintain spatial resolution. The structure of each arm was designed with an L-shape layout (Figure 4.4a) to prevent longitudinal traction on the implant, which can otherwise displace the electrodes outside the nerve during movement, as previously reported¹².

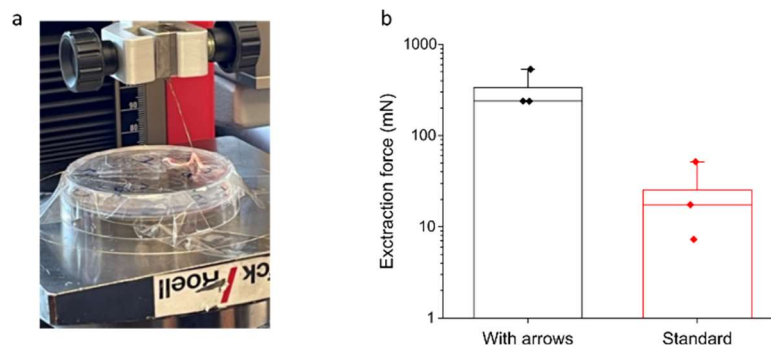


Figure 4.5 | Clamping force L-shape TIME. **a**, Set-up for measuring the force needed to extract a device from an explanted nerve from a beaker. The nerve is attached to the beaker, while the device is pulled outside the nerve with a micro driver. **b**, Extraction force needed to detach the PI device, for 3 dummies with and without the new anchoring structures in the design.

The layout also incorporates two arrow-like structures (0.55 mm at the base) placed at the centre of the device so that, upon implantation, they rest just outside the nerve to reinforce clamping (Figure 4.4b). The edges of the ribbon are also structured to increase friction with surrounding tissue and mitigate device migration. To evaluate the clamping capability of this design, three dummy PI devices with the new layout and three with the previous linear layout were inserted¹² into explanted rat sciatic nerves. Devices were then withdrawn at 100 $\mu\text{m/s}$ with a microcontroller while force was measured with a dynamometer (Figure 4.5). The mean extraction force was 337 ± 170 mN for devices with clamping arrows (Figure 4.4b and 4.5) versus 26 ± 23 mN for the standard device, more than 10-fold increase. In the context of in vivo applications, if explantation is necessary, the arrows can be cut, enabling the linear structure to be safely removed from the nerve.

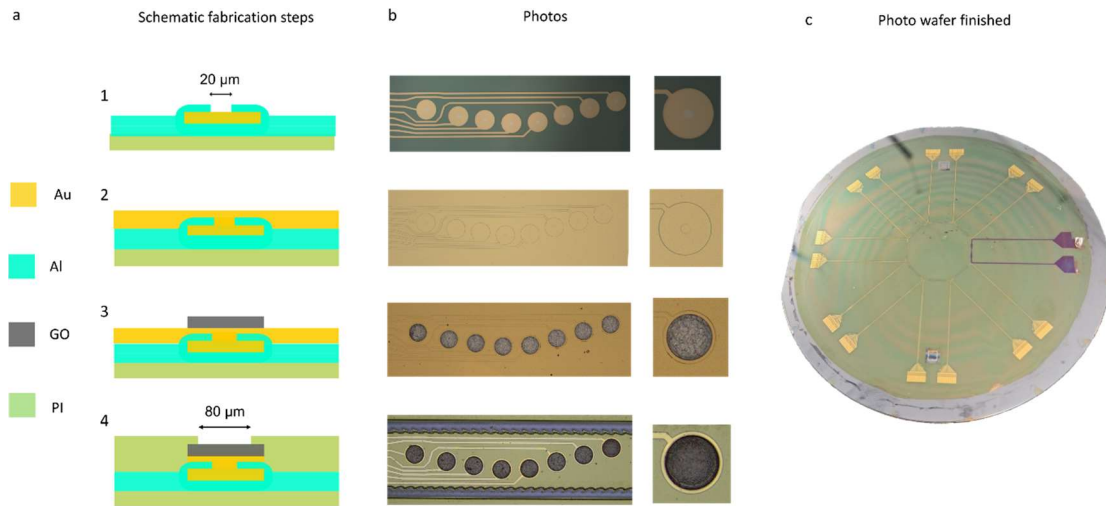


Figure 4.6 | Fabrication process of second-generation rGO technology. **a**, Schematic of the four main steps of the fabrication protocol in sequence: 1. opening of vias in the second alumina layer, 2. gold evaporation, 3. GO transfer, and electrode area definition, 4. deposition of the second PI layer with an opening at the electrode site. **b**, Optical images of the fabrication steps shown in panel a. **c**, Photograph of a completed wafer with one device delaminated.

To improve encapsulation hermeticity and interlayer adhesion, two 50 nm-thick layers of Al_2O_3 were incorporated between the metallic tracks and the PI (Figure 4.4c and 4.6a). rGO electrodes were contacted to the tracks through vias under the electrodes. Electrode shapes were subsequently defined by photolithography and reactive ion etching (RIE) (Figure 4.6).

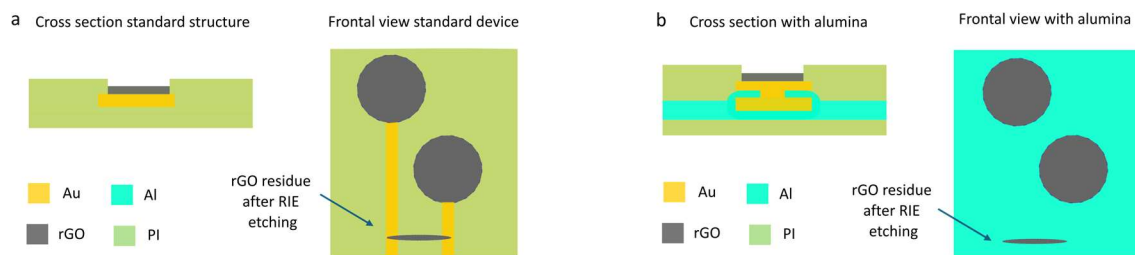


Figure 4.7 | Impact of rGO residues in the standard and the new encapsulation procedures. **a**, Schematic of the cross-section and frontal view of the standard fabrication. The rGO residues contact the gold, creating electrical shorts. **b**, Schematic of the cross-section and frontal view of the new layer stack. The rGO residues don't contact the gold tracks, since the tracks are packed between two alumina layers.

Previously this patterning step is particularly challenging because rGO residues can remain after RIE; in our new design, contacting the metal tracks to the electrodes from below and covering the remaining tracks with a thin, non-conductive layer prevents residual rGO from contacting the gold (Figure 4.7a, b). With this change, no inter-electrode short-circuits were observed post-fabrication, resulting in an

increased fabrication yield (+20%). A photograph of a finalized rGO array, with a magnified view of one electrode, is displayed in Figure 4.8a.

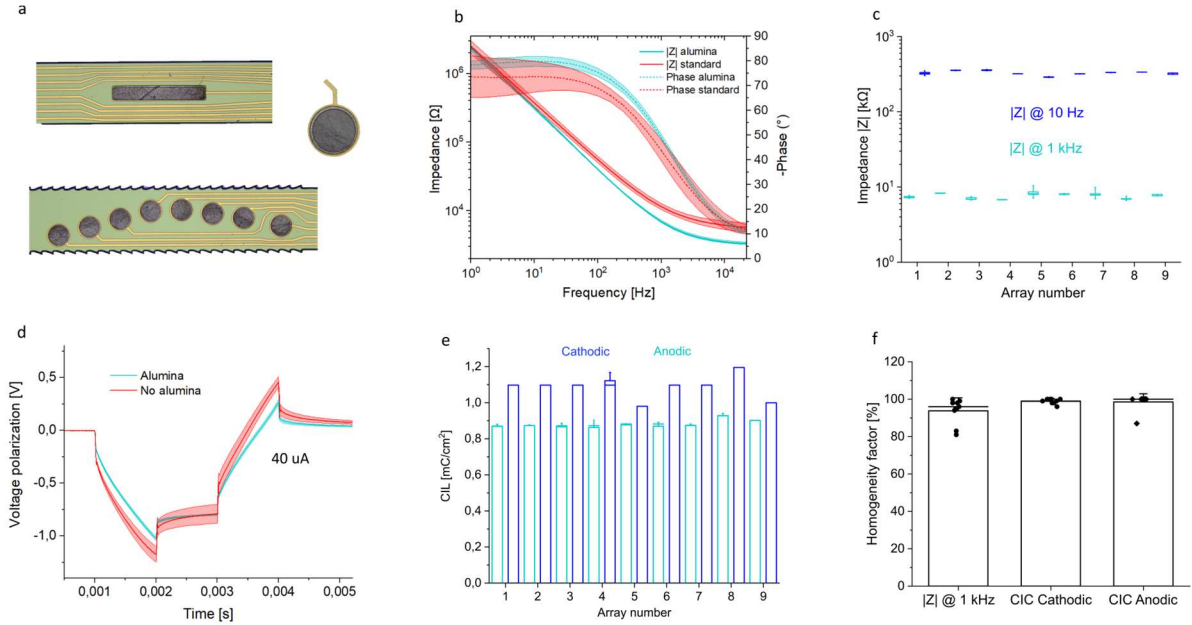


Figure 4.8 | Electrochemical performance L-shape TIME device. **a**, Optical image of a fabricated device showing the electrode array, with a magnified view of a single microelectrode and the rectangular pad electrode. **b**, Impedance spectra for a PI-PI device (red) and a device with alumina encapsulation (light blue): magnitude (solid line) and phase (dashed line), $n=16$ electrodes per device. **c**, Impedance magnitude at 10 Hz (blue) and 1 kHz (light blue) for nine arrays (each with eight microelectrodes). Boxplots represent the 25th-75th percentiles. **d**, Voltage polarization to biphasic current pulses (1 ms per phase): mean traces $\pm$ s.d. (shaded), comparing PI-PI (red) and alumina-encapsulated (light blue) devices ($n=16$ microelectrodes per type). **e**, Cathodic (blue) and anodic (light blue) charge injection limits for the eight electrodes across nine different arrays. The bar plot represents average values. **f**, Homogeneity factor for impedance magnitude at 1 kHz and for cathodic/anodic charge injection limits across the nine arrays shown in panels c and e. The bar plot represents the average value.

I next assessed whether the hybrid PI- Al_2O_3 encapsulation affected the performance of the electrodes by conducting electrochemical characterization in phosphate-buffered saline (PBS). Impedance spectra (mean $\pm$ standard deviation) from the two arrays of one device, with and without the new encapsulation, showed similar phase and magnitude at low frequency, consistent with near-ideal capacitive behaviour; at higher frequencies, electrodes with the new encapsulation exhibited a lower impedance (Figure 4.8b).

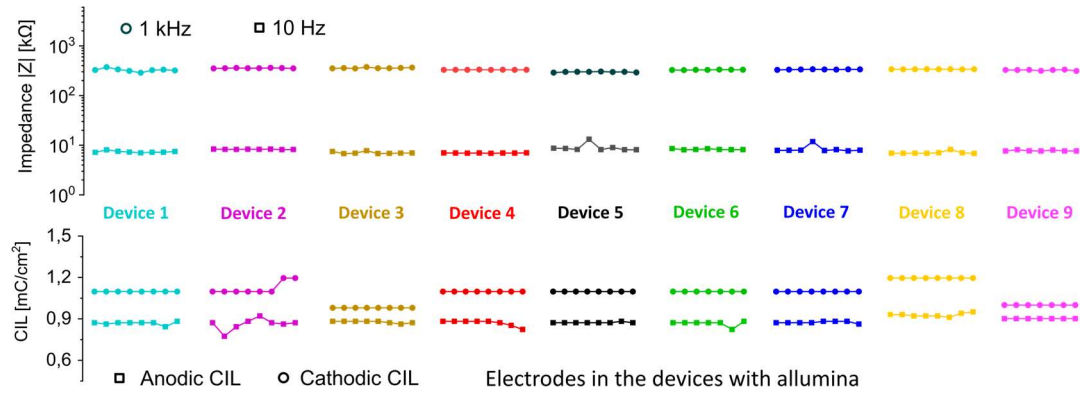


Figure 4.9 | Performance and homogeneity. **a**, Impedance magnitude values at 1 kHz and 10 Hz for all electrodes of 9 different arrays. **b**, Cathodic and anodic charge injection limits for the same electrodes shown in panel a.

The mean impedance values calculated for the eight electrodes of nine alumina-encapsulated arrays ($n=72$) were 329.27 ± 21.64 kΩ at 10 Hz and 7.71 ± 0.98 kΩ at 1 kHz (Figure 4.8c), with high uniformity (per-electrode values are shown in Figure 4.9). Biphasic current pulses (1 ms) were delivered while recording voltage polarisation at the electrode-electrolyte interface (Figure 4.8d). Devices with and without alumina exhibited similar interfacial polarisation, whereas the alumina design showed a smaller ohmic drop, consistent with the reduced high-frequency impedance (Figure 4.8d). The charge injection limit (CIL) was determined for both cathodic and anodic pulses across the eight electrodes from nine arrays, yielding average values ($n=72$) of 1.08 ± 0.06 mC/cm² (cathodic) and 0.88 ± 0.02 (anodic), with high homogeneity in each array (per-electrode values in Figure 4.9).

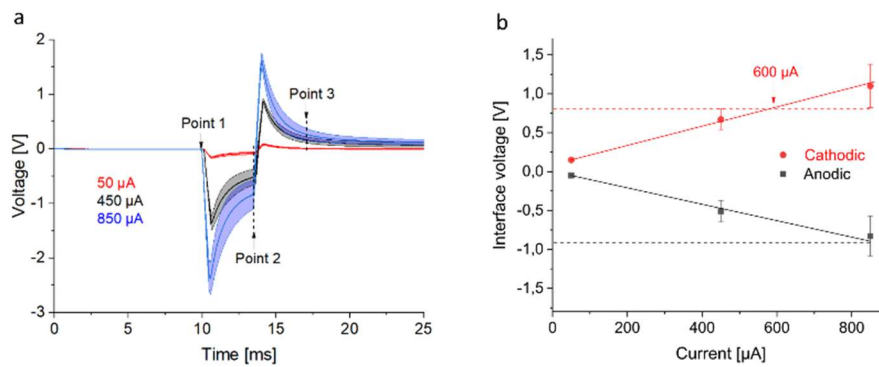


Figure 4.10 | Calculation of charge injection limit for short pulses. **a**, Average voltage polarization (solid lines), with shadowed area for standard deviation (STD) ($n=8$ electrodes), of biphasic current pulses of 50 μs in pulse width and 3 ms in inter-pulse interval, for 50 (red), 450 (black), and 850 (blue) μA current amplitude. **b**, Average cathodic (point 2 – point 1) and anodic (point 3 – point 2) at the electrode-PBS interface with STD ($n=8$) for the pulses of subpanel a. The dashed line represents the safe voltage limits for the anodic (black) and cathodic (blue) polarizations, from which the maximum current injectable (600 μA) is extracted.

Because CIL depends on pulse width, I also evaluated the stimulation protocol used in vivo (biphasic, 50 μ s per phase): the maximum safe current averaged $\sim 600 \mu$ A (n=8), corresponding to 0.6 mC/cm² (Figure 4.10a-b). In the case of small pulses of 50 μ s, since it was not possible to distinguish the interfacial polarization from the ohmic drop, a long interpulse interval of 3 ms was applied, to extract the maximum charge injection by considering the difference between the voltage baseline at the beginning and end (3 ms after) of the pulse (Figure 4.10a, point 1 and 3) and the voltage recovered at the end the interpulse interval (Figure 4.10a, point 2). In particular, the cathodic voltage was calculated as $V_{\text{point2}} + V_{\text{point1}}$, while the anodic $V_{\text{point3}} + V_{\text{point2}}$. In Figure 4.10b, the error bars are calculated from the standard deviation (σ) of the three voltage points $V_{\text{point1}} \pm \sigma_1$, $V_{\text{point2}} \pm \sigma_2$, $V_{\text{point3}} \pm \sigma_3$, as $E_{\text{cathodic}} = \sqrt{\sigma_1^2 + \sigma_2^2}$ and $E_{\text{anodic}} = \sqrt{\sigma_2^2 + \sigma_3^2}$.

Performance homogeneity for impedance and CIL was quantified (refer to Annex 1 for calculation of homogeneity factor, H) across electrodes of the nine arrays (Figure 4.8f). The electrodes show a high homogeneity factor for both impedance ($H = 93.7 \pm 6.9 \%$, at 1 kHz), and for CIL (cathodic: $98.8 \pm 1.3 \%$; anodic: $98.5 \pm 4.3 \%$).

Overall, these data indicate that this new fabrication method, developed to include the alumina encapsulation layer, yields high-performance devices with high yield and uniformity.

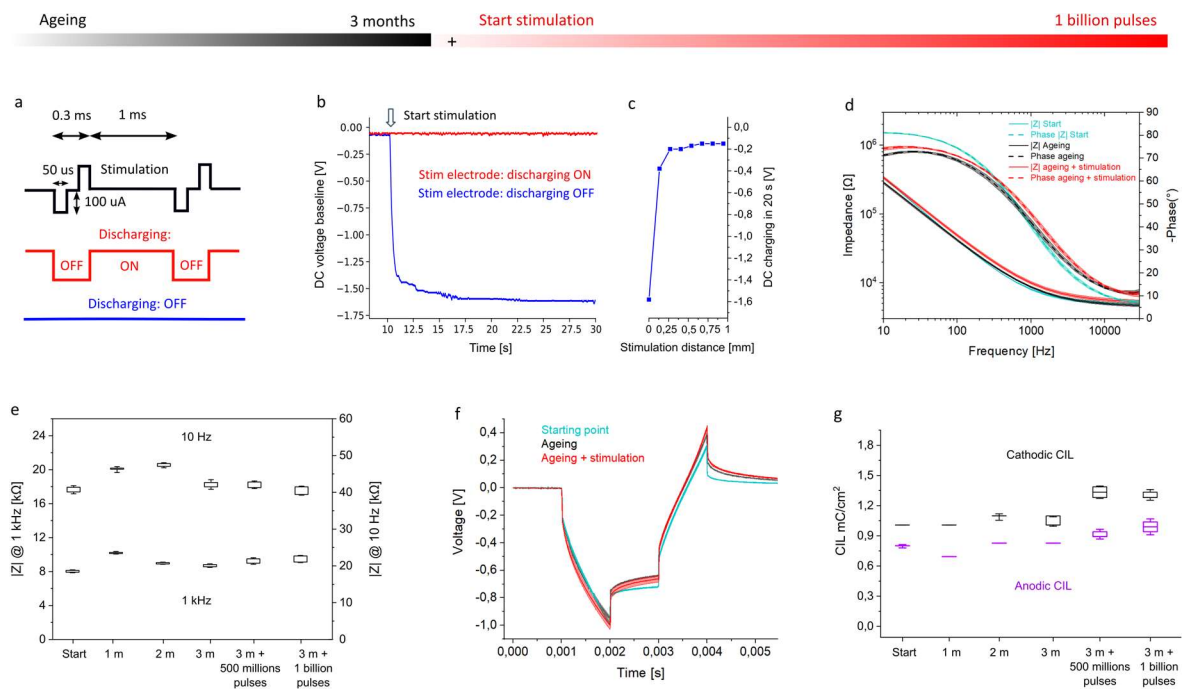


Figure 4.11 | Stability after ageing and long-term stimulation. Top bar, schematic timeline of the stress test: 3 months of immersion in PBS at 57° C (black), followed by long-term stimulation with 1 billion pulses (red). **a**, Stimulation protocol (black) used for long-term pulsing: biphasic current pulses

(50 μ s per phase, 100 μ A, 1 kHz) tested with passive discharge (red) and without discharge (blue). **b**, DC voltage baseline measured during stimulation at 1 kHz with one electrode in PBS. Stimulation starts after 10 s and is delivered with (red) and without (blue) inter-pulse discharge. **c**, Baseline voltage reached under the conditions in panel b as a function of the distance to the stimulating electrode ($n=8$ electrodes). **d**, Impedance spectra (magnitude, solid line; phase, dashed line) for one array at the starting point (light blue, $n=8$), after 3 months in PBS at 57 °C (black, $n=8$), and after 10^9 pulses (red, $n=4$). **e**, Impedance magnitude at 10 Hz and 1 kHz at the starting point ($n=8$), after every month of ageing at 57 °C ($n=8$), and after each 5×10^8 pulses ($n=4$). Boxplots represent the 25th-75th percentiles. **f**, Voltage transient for biphasic current pulses (1 ms per phase) in the same conditions as in panel d; the shadowed area corresponds to standard deviation (s.d.). **g**, Cathodic and anodic charge injection limits under the conditions in panel e.

4.5 In vitro evaluation of the hybrid encapsulation stability

To assess the effect of the hybrid PI-Al₂O₃ encapsulation on the devices' long-term functionality, I subjected them to accelerated ageing followed by prolonged electrical stimulation of the electrodes. To accelerate materials ageing^{126,156}, an array was soaked in PBS at 57° C for 3 months, corresponding to ~ 1 year at 37° C by standard acceleration models¹²⁷. To better approximate in vivo use, the same electrodes were then stressed electrically, as current stimulation can accelerate degradation mechanisms^{100,243}. I delivered 1 billion biphasic current pulses through 4 electrodes (the maximum simultaneous channels allowed by the software) using the same in vivo protocol (50 μ s per phase, 100 μ A amplitude, 1 kHz; Figure 4.11a), corresponding to a charge density of 0.1 mC/cm², higher than the maximum value approved clinically¹²⁶. Monitoring the electrode polarisation during stimulation (measured for all electrodes using the DC voltage recording capability of the Neuronexus stimulator) revealed that, even with nominally symmetrical pulses, charge accumulated owing to an imbalance in interfacial polarization between anodic and cathodic phases and slow charge balance in between pulses. At high stimulation frequency (1 kHz), the DC offset drifted to -1.6 V within ~20 s (Figure 4.11b), which could initiate faradaic reactions and degradation of the electrode⁴⁶. To mitigate this, I introduced a 1ms passive discharge interval between pulses, which stabilized the baseline near the open circuit potential throughout the stimulation protocol²⁴⁴ (Figure 4.11b). Measurements at neighbouring electrodes at increasing distances from the stimulation site indicated that charge build-up is highly localized around the stimulating site, decaying sharply with distance²⁴⁵ (Figure 4.11c).

The mean impedance spectra (including standard deviation) for one array at the starting point ($n=8$), after ageing ($n=8$), and after 1 billion pluses ($n=4$) indicated retained electrode functionality (Figure 11f). Impedance magnitudes at 10 Hz and 1 kHz for different time soaking periods and number of stimulation pulses supported the stability (Figure 4.11e) and uniformity of the electrode performance (>94%, 8f). The average voltage transients ($\pm$ s.d.) in response to biphasic current pulses of 30 μ A amplitude and 1 ms per phase remained consistent in shape and amplitude across all stress conditions (Figure 4.11f).

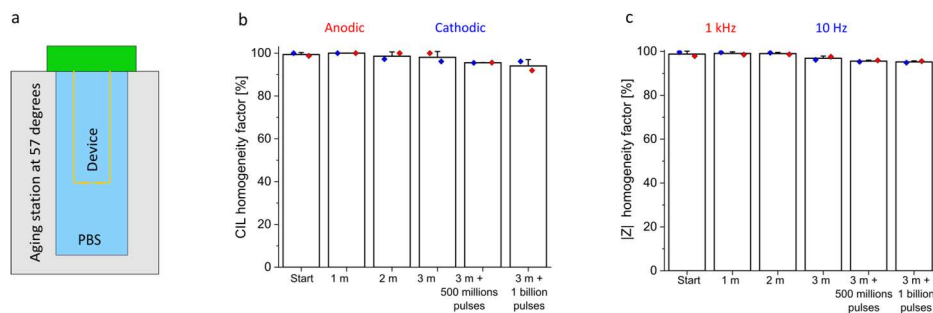


Figure 4.12 | Homogeneity factor during stress test (accelerated ageing and long-term stimulation. **a**, Schematic of the ageing set-up. A functional device is immersed in a beaker with 10 mM PBS in a controlled temperature station at 57 °C. **b**, Homogeneity factor, for the cathodic and anodic charge injection limits, after every month of soaking in PBS at 57 °C (n=8), and after 500 million pulses and after 1 billion pulses (n=4). **c**, Homogeneity factor for the impedance magnitude at 1 kHz and 10 Hz, for the conditions of panel b.

The quantification of the anodic and cathodic charge injection limits showed no major change between the starting point (n=8) and monthly ageing time points (n=8), indicating preserved capacitive properties of the electrodes and response uniformity (>95%, Figure 4.12b). During extended stimulation (n=4), the CIL increased by ~30 %, which can be attributed to an activation process of the electrodes related to an increase in effective surface area of the porous material, leading to enhanced charge storage and injection limits¹⁴⁹ (Figure 4.11g).

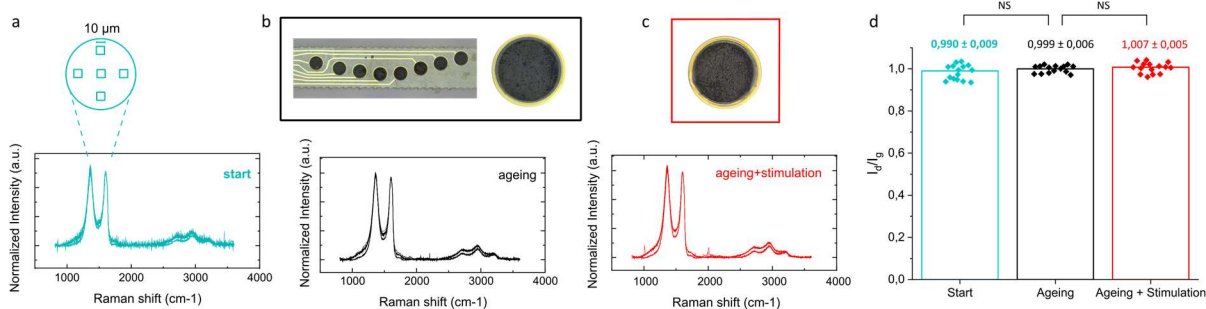


Figure 4.13 | Raman analysis of aging study. **a**, (Top) Schematic of the five 10 µm x 10 µm areas sampled for Raman spectroscopy on rGO electrodes. (Bottom) Averaged Raman spectra for 3 different electrodes at the starting point (each averaged over its five areas). **b**, (Top) Optical image of an array (with magnified electrode) after 3 months in PBS at 57 °C. (Bottom) Averaged Raman spectra for the 3 electrodes. **c**, (Top) Optical image of an electrode after 10^9 pulses. (Bottom) Averaged Raman spectra of the 3 electrodes after long-term stimulation. **d**, Ratio of D- to G-peak intensities from Raman spectra. Points represent the quantification of the ratio across each area of the three electrodes (n=15 per condition). On top, the total weighted mean with standard deviation in the three conditions. The bar plot displays the average value. Statistical tests are described in Annex 1.

To probe structural changes in rGO electrodes after stress testing, I measured Raman spectra over five 10 µm x 10 µm areas in the electrodes, spanning center and perimeter, to sample a substantial fraction

of the electrode surface (Figure 13a). For three electrodes measured at the starting point, after ageing, and after long-term stimulation, averaged Raman spectra showed no significant changes in peak positions or relative intensities (Figure 4.13a-c). The weighted mean ($\pm$ s.d.) of D/G intensity ratio across the 15 areas in the 3 conditions varied by $<1\%$, suggesting that there is no detectable change in the density or distribution of defects in the rGO electrode material²⁴⁶ (Figure 4.13d). Furthermore, optical images of the electrodes and the arrays did not evidence water ingress or metal oxidation after the ageing and long-term stimulation treatments (Figure 4.13a-c).

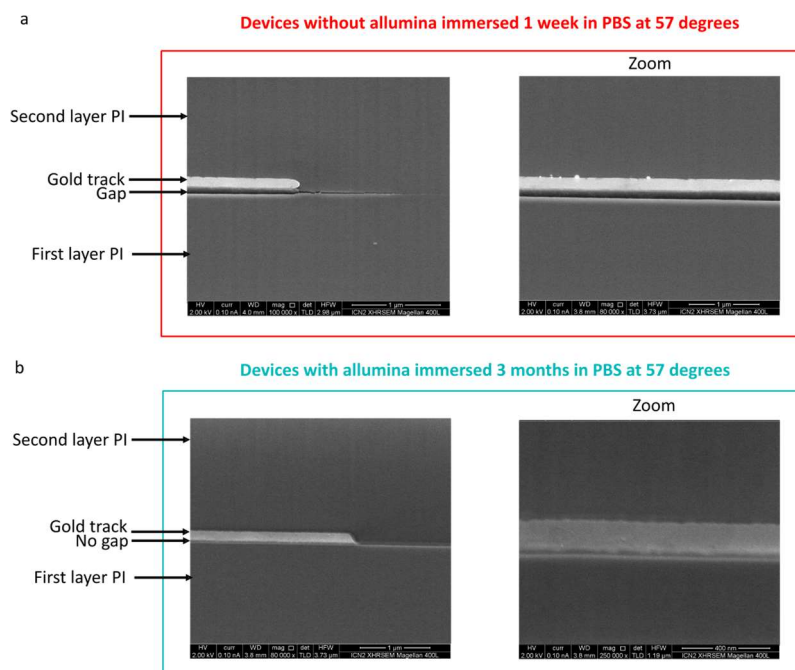


Figure 4.14 | Cross-section SEM to assess interlayer adhesion. a, SEM picture after FIB, to evaluate the cross section of a device without alumina, after 1 week soaking in PBS at 57° C. **b**, the cross section of a device with the hybrid encapsulation with alumina, after 3 months soaking.

Complementing the electrochemical characterization, I examined the encapsulation by SEM on FIB cross-sections (Figure 4.14a,b). I compared a PI-Al₂O₃-PI stack after 3 months in 57 °C PBS with a standard PI-PI stack soaked for 1 week. In the PI-PI device, a ~80 nm gap was observed between the first PI layer and the Ti/Au tracks; this gap was not present in the PI-Al₂O₃-PI device, indicating improved adhesion at metal-PI interface²⁴⁷.

Overall, the electrical and structural analyses indicate that the encapsulation combining PI and Al₂O₃ mitigates water ingress and delamination, while the nanoporous rGO electrode material supports stable charge injection limits over prolonged operation and tolerates extended electrical stimulation.

4.6 Improvements in chronic functionality for nerve stimulation

As in the case of the first-generation devices, the new-TIME devices were transversely inserted (Annex 1. Methods) into the tibial and peroneal fascicles of the sciatic nerve in rats to evaluate the capability of rGO electrodes to stimulate and record from peripheral nerve fibres over months of implantation. Biphasic current pulses (this time 50 μ s per phase) were delivered at increasing amplitude while simultaneously recording compound muscle action potentials (CMAPs) from three target muscles using monopolar needle electrodes, with a passive discharge interval in between the pulses to prevent the charge accumulation observed in vitro. The decrease of the pulse width from 100 to 50 μ s was due to the empirical observation that muscles activation requires less charge per phase.

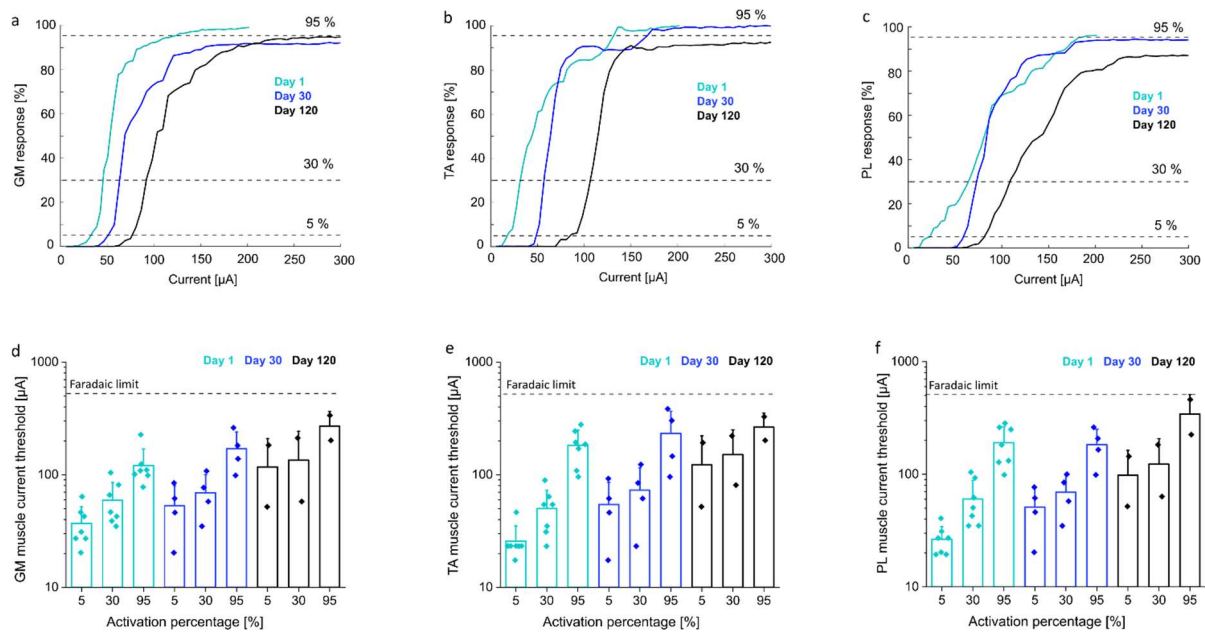


Figure 4.15 | Chronic stimulation of the sciatic nerve. a-c, Percentage muscle activation (based on CMAP amplitude) for gastrocnemius (GM; panel a), tibialis anterior (TA; panel b), and plantar (PL; panel c) muscles during stimulation with a single rGO electrode in the sciatic nerve (biphasic current pulses of 50 μ s at increasing current intensity). Curves shown for day 1 (light blue), day 30 (blue), and day 120 (black) post-implantation. Dashed horizontal lines mark 5%, 30%, and 95 % of maximal activation. d-f, Threshold currents needed to reach 5%, 30%, and 95 % activation for GM (panel d), TA (panel e), and PL (panel f) muscles on day 1 (light blue), day 30 (blue), and day 120 (black). The bar plot displays the average value, and each dot represents the current threshold of one electrode in different implanted animals. The dashed line denotes the maximum injectable current within the water window limits.

CMAPs recruitment curves were used to quantify neuromuscular activation for a given stimulating electrode. Curve shape at days 30 and 120 remained similar to day 1, with only a small shift towards higher currents (Figure 4.15a-c). From the responses produced by the electrodes in the array, I calculated the currents needed to achieve 5%, 30%, and 95% of the maximal CMAP amplitude (Figure 4.15d-f). On day 1 ($n = 7$ rats), the mean current to reach 95% activation was 121 ± 48 μ A for gastrocnemius (GM), 182 ± 66 μ A for tibialis anterior (TA), and 190 ± 74 μ A for plantar (PL) muscles, which is

comparable to what reported for platinum and iridium oxide electrodes of similar size^{10,12}. Activation thresholds are expected to drift during chronic implantation owing to the formation of a fibrotic capsule around the device, which lowers tissue conductivity²⁴⁸. For 95% activation, I observed mean increases of 21% at day 30 (n=12), and 51% at day 120 (n=12), a modest rise compared to prior reports^{12,20,249–252}. Importantly, current thresholds remained below the maximum injectable current ($\sim 600 \mu\text{A}$; dashed line in Figure 4.15d-f) even at day 120, thereby avoiding faradic reactions that can compromise biocompatibility and long-term functionality⁴⁶.

Previous failures of intraneural stimulation have been attributed to deterioration of the electrodes or the tissue at the interface²⁵¹, displacement of the device outside the nerve^{12,252}, or failure of the connection to the stimulator²².

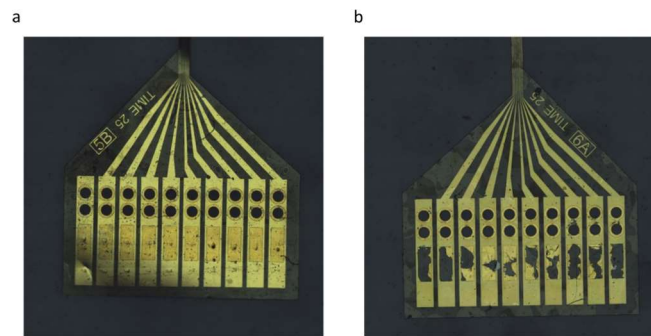


Figure 4.16 | Effect of improved interlayer adhesion on robustness of pad area. **a**, Images of the pads area for a device with alumina after 1 month of implantation. **b**, Image of the pads area for a device without alumina after 1 month of implantation.

Maintaining a stable connection with the electronics is very challenging due to the fragility of the contact pads of the device (Figure 4.4a), which can cause delamination with time and repeated handling. Here, optical inspection of all explanted devices with alumina (n=7; 30 or 120 days) showed no damage to the gold pads, consistent with the improved adhesion at the metal-PI interface (Figure 4.16a). By contrast, devices without alumina (n=9; explanted after 30 days) exhibited partial metal detachment at the pads (Figure 4.16b).

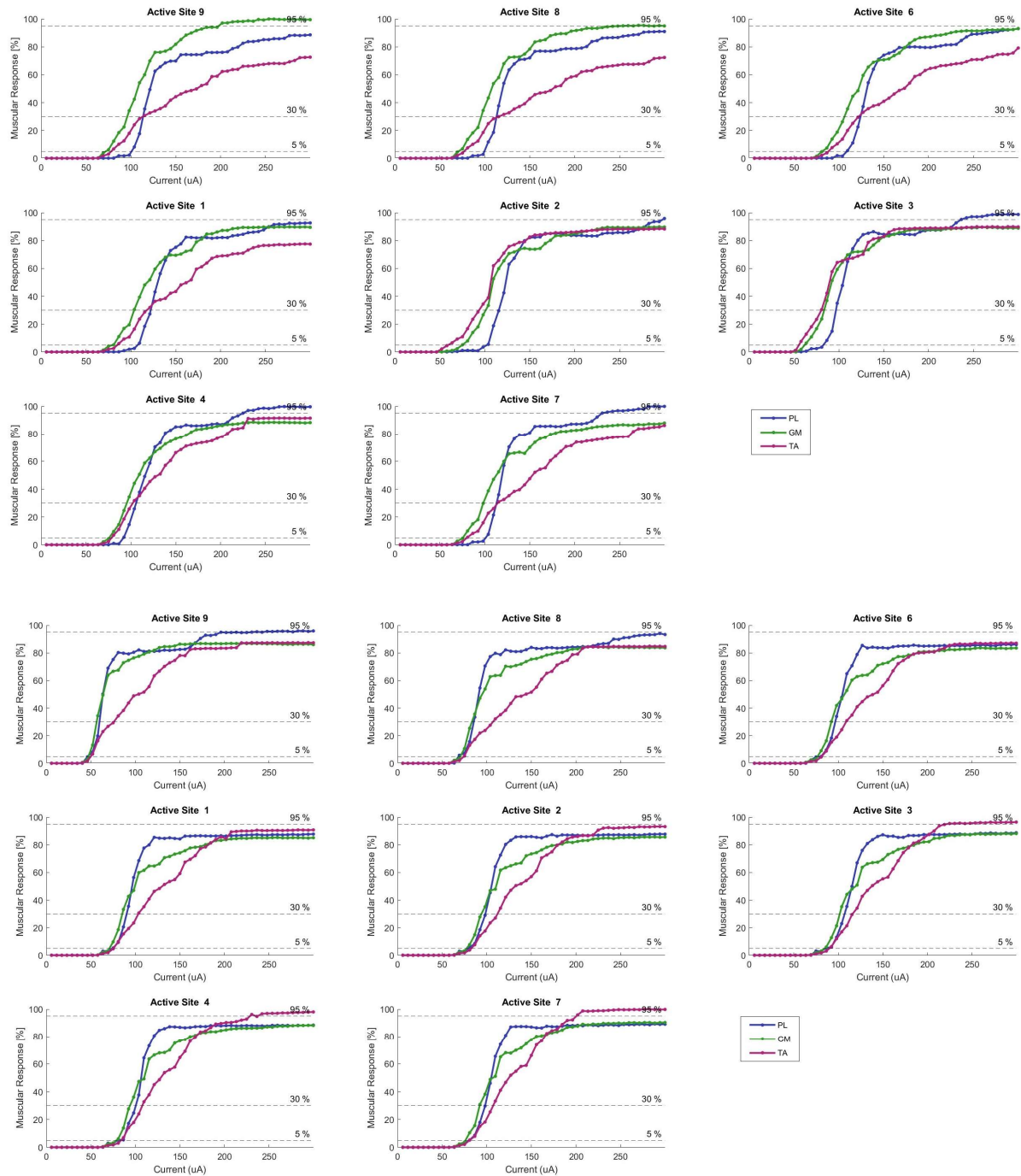


Figure 4.17 | Recruitment curves after 120 days of implantation. a, Percentage of plantar (blue), gastrocnemius (green), and tibialis anterior (red) muscle activation, during stimulation with the 8 electrodes of the 2 arrays of one device (array A on top, and array B below). Biphasic current pulses (50 μ s per phase) at increasing current amplitude were injected after 120 days of implantation. The dashed lines indicate the 5, 30, and 95% of the muscle activation.

Furthermore, no devices (n=7) were found outside the nerve after chronic implantation, which I attribute to the implementation of the new anchoring features. Together, the new mechanical design and alumina encapsulation provided a stable neural interface for at least 4 months.

Across the electrodes ($n=16$) in the two arrays of one representative device, all electrodes could activate at least one muscle up to 95% of maximal CMAP and exceeded 70% in the other two muscles, depending on the relative position of each electrode to the specific nerve fascicle (Figure 4.17). Although working electrodes showed high performance, some channels lost functionality to activate the muscles over time (43% non-functional at day 30; 71% at day 120), likely due to the limited mechanical stretchability of the device, which is challenged during the leg motion and repeated access for testing.

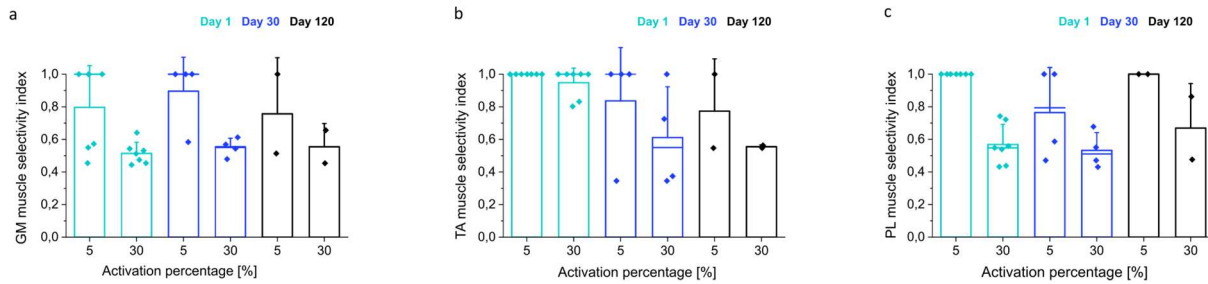


Figure 4.18 | Selectivity index over time. a-c, Selectivity index (SI) at 5% and 30 % activation for GM (a), TA (b), and PL (c) muscles on day 1, day 30, and day 120. The bar plot displays the average value, and each dot represents the current selectivity of one electrode in different implanted animals.

The selectivity index (SI) was calculated to quantify the selectivity to activate each of the three muscles. It showed, on average at day 1, values ($n=7$ for each muscle) of 0.8 and 0.5 for GM at 5% and 30% activation, 1 and 0.95 for TA, and 1 and 0.57 for PL. The SI was higher in the case of the TA, due to the separation of the tibial and peroneal fascicles by the perineurium, as shown by the high SI of the TA muscle, which is innervated by axons running in the peroneal fascicle. In contrast, selectivity was lower for muscles innervated by axons within the same tibial fascicle. Because these fibers are closer to each other and not separated by a perineurial barrier, achieving intrafascicular selectivity was more challenging, resulting in lower SI values for the gastrocnemius medialis (GM) and plantaris (PL) muscles. This index was also monitored at day 30 ($n=4$) and 120 ($n=2$); despite variability, no significant decreases were observed, indicating that the electrodes can stimulate different groups of nerve fibers with good selectivity over time (Figure 4.18a-c). Overall, despite the limited stretchability of the PI devices, the results indicate that these electrodes can interface stably and effectively with nerves, achieving low activation currents and good selectivity across subfascicles even after long-term implantation.

4.7 Chronic nerve recording

Bidirectional interfacing with the peripheral nervous system, needed to reproduce natural sensations and control complex prosthetic movements, requires both high-resolution stimulation and recording²⁵³.

Having established stable chronic stimulation capability with the new-TIME devices, I next assessed the recording capability of 80 μm rGO electrodes. Representative traces show increasing CNAP amplitude during the current ramp for each nerve (Figure 4.19a-c).

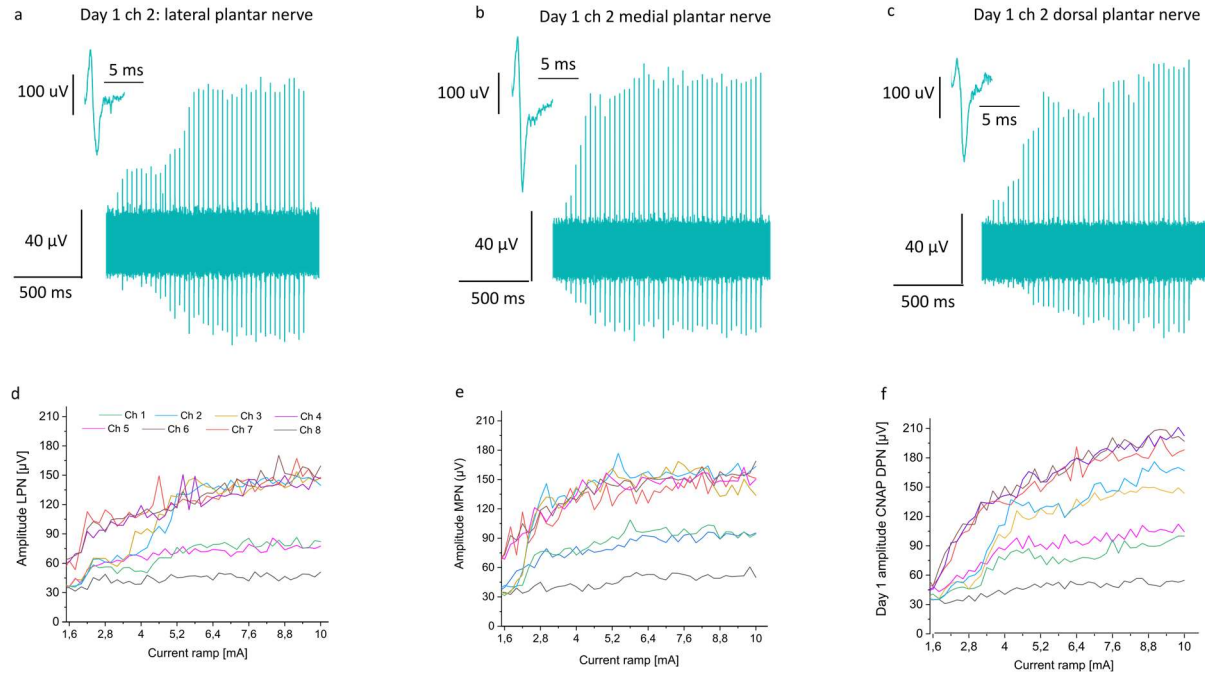


Figure 4.19 | Recruitment curves of the CNAPs. a-c, Representative recording, day 1, from a single rGO electrode showing CNAPs elicited by stimulation of the lateral plantar nerve (LPN) with biphasic current pulses (100 μs per phase) at increasing current intensity. Inset, example of CNAP. d-e, Recruitment curves of the peak-to-peak amplitude of the CNAPs recorded with all the electrodes of one array ($n=8$), elicited by stimulating with needles the LPN, the MPN, and the DPN with biphasic current pulses (100 μs per phase, 3 Hz) at increasing current intensity.

The peak-to-peak CNAP amplitude exceeded 40 μV for all electrodes in one array (Figure 4.19d-f), above the baseline noise (~ 30 μV). The average maximum CNAP recorded in 5 different animals were 320 ± 107 μV (MPN), 290 ± 81 μV (LPN), and 287 ± 102 μV (DPN) (Figure 4.4h, light blue). The average signal-to-noise ratio (SNR), calculated as the maximum CNAP amplitude divided by the root-mean-square (RMS) of the full signal, was 11.4 ± 2.4 (MPN), 10.4 ± 1.6 (LPN), and 10.7 ± 4.1 (DPN), comparable to high-performance electrode materials^{63,155} (Figure 4.20d, black).

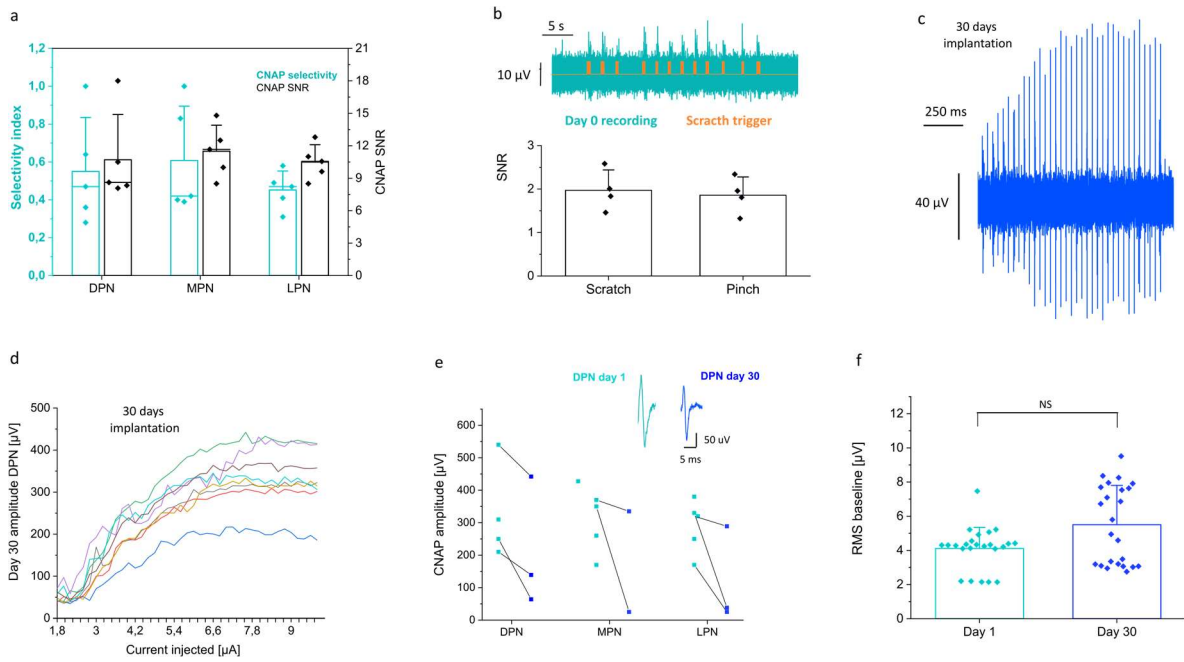


Figure 4.20 | CNAP recordings from the sciatic nerve. **a**, Selectivity index at 30% activation (light blue) and SNR (black) for MPN, LPN, and DPN across five implants. Bar plots represent the average value. **b**, (Top) Example of ENG recording (band-pass 100-3000 Hz, light blue) recorded from the sciatic nerve during repeated scratch stimuli to the plantar skin (orange ticks mark stimulus times). (Bottom) SNR for scratch and prick stimuli from one electrode in four animals. The bar plot displays the average value. **c**, Representative recording of DPN-evoked CNAPs from one rGO microelectrode at day 30, using the protocol in **a**. **d**, Recruitment curves of CNAP peak-to-peak amplitude for DPN stimulation recorded with all the electrodes of one array (n=8) at day 30. **e**, (Top) CNAPs recorded at day 1 (light blue) and day 30 (dark blue). (Bottom) Maximum CNAP amplitudes for MPN, LPN, and DPN from one rGO electrode per device at day 1 (light blue, n=4), and day 30 (dark blue, n=3). **f**, RMS baseline noise (1 s window) after stimulation of the three distal nerves at day 1 (light blue, n=24) and day 30 (dark blue, n=24) across eight electrodes per array. Statistical tests are described in Annex 1, Methods.

The dense microelectrode spacing in the array, together with the high SNR achieved with the rGO microelectrodes⁶³, enabled discrimination within the sciatic nerve (diameter ≈ 1 mm) of responses from a small branch relative the other two, with selectivity indices of 0.6 ± 0.28 (MPN), 0.45 ± 0.1 (LPN), and 0.55 ± 0.28 (DPN) (Figure 4.21).

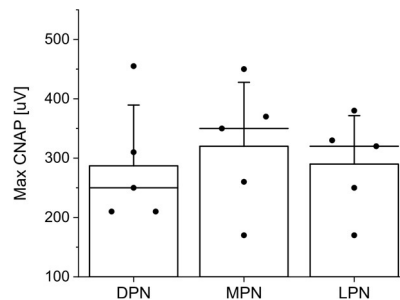


Figure 4.21 Maximum CNAP. The maximum voltage CNAP was recorded in the three fibers of the sciatic nerve. Each dot represents one different implant. The columns represent the average value, with the median line and STD whiskers.

To probe the capability to record bursts of single action potentials, brief mechanical stimuli was applied to the plantar surface of the paw. In 4 rats, stimulus-locked bursts of action potentials were detected with mean SNR of 1.97 ± 0.46 (scratch stimuli) and 1.86 ± 0.42 (prick stimuli) (Figure 4.20b), in acute as well as in an exemplary recording made at day 30 after implantation (Figure 4.22a-b).

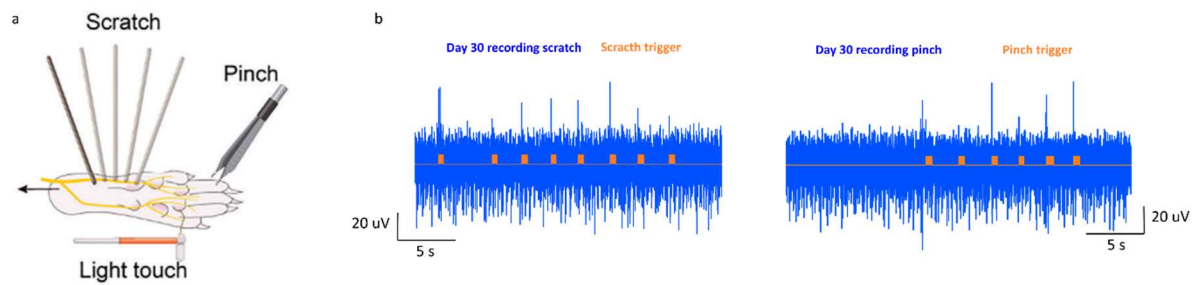


Figure 4.22 | Neural recordings in chronic implantation. **a**, Schematic of the experimental set-up to scratch, pinch and light touch the paw of a rat. **b**, Representative recording (light blue) in the sciatic nerve when the paw is scratched and pinched after 30 days of implantation. The trigger (orange) indicates the time when the stimulus is performed.

Because electrically evoked CNAPs are synchronous, their amplitudes exceeded those from asynchronous mechanical responses by $> 10x$, allowing reliable chronic detection in more animals ($n = 3$). Recruitment curves for the eight electrodes of one array showed that all electrodes detected CNAPs $> 200 \mu V$ after 30 days (Figure 20d). However, this maximum CNAP amplitude decreased relative to day 1, with reductions ranging from 10% to 93% (Figure 20e). As with stimulation, recording performance declined over time, in part due to fibrotic encapsulation, which increases resistive tissue around the implant and separates axons from the electrode¹¹¹.

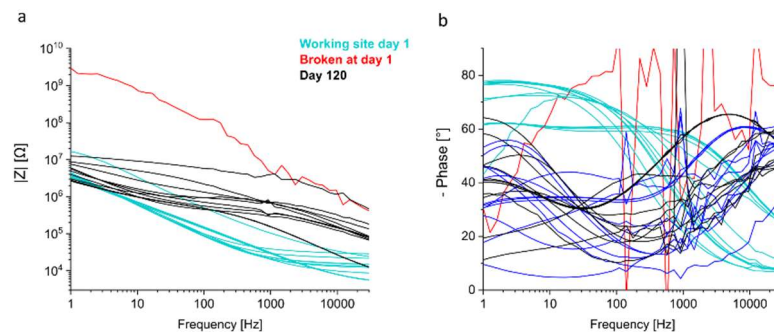


Figure 4.23 | In vivo impedance over time. **a**, In vivo impedance magnitude measured with one array of electrodes at day 1 (light blue), and after 120 days (black). The red trace indicates the impedance of a broken electrode at day 1 as a reference. **b**, Phase for the plots of panel **a**

Consistent with this interpretation, impedance spectra from the eight electrodes of one array measured on day 1 and day 30 showed an increase in the magnitude at high frequencies (indicative of higher series/tissue resistance) with similar capacitive behaviour at low frequencies, which is dominated by the electrode properties (Figure 4.23). Nevertheless, the magnitude remained well below that of a broken channel measured on day 1 (red trace in Figure 4.23), indicating that all the electrodes were still functional. This increase in impedance did not generate a significant increase in the recording noise level. The average RMS value ($n=24$) was 4.1 ± 1.2 at day 1 and 5.5 ± 2.3 at day 30 (Figure 4.20f). Despite the noise level being stable, SNR decreased due to smaller maximal CNAPs ($n=3$ rats) (Figure 4.20e).

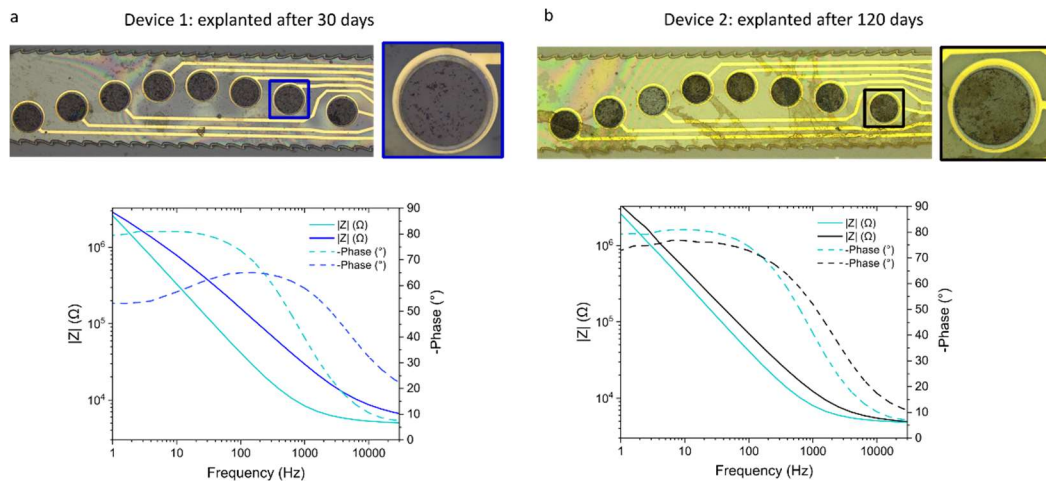


Figure 4.24 | Functionality after explantation. **a**, (Top) Optical image of an array explanted after 30 days, with a magnified view of one electrode. (Below) Average impedance magnitude (solid line) and phase (dashed line) of the electrode before (light blue) implantation and after explantation (blue). **b**, (Top) Optical image of an array explanted after 120 days, with a magnified view of one electrode. (Below) Average impedance magnitude (solid line) and phase (dashed line) of the electrode before (light blue) implantation and after explantation (black).

Device durability was further evaluated by comparing impedance before implantation and after explantation for two devices used for 30 and 120 days. Few electrodes per device survived the explantation procedure because stretching of the lead during removal broke some metal tracks. In two surviving electrodes, impedance showed no significant change before and after the implantation (Figure 4.24).

4.8 Conclusion

Complex bidirectional communication, essential for restoring natural-like sensory perception and fine motor control in prosthetic systems, depends on the ability to selectively stimulate and record from

small fibers of peripheral nerves. Consequently, higher selectivity and spatial resolution can be obtained by decreasing the dimensions of the electrodes and simultaneously increasing their density along the nerve interface. rGO microelectrodes are suitable for miniaturization, due to the high charge injection limit and low impedance. Electrodes of 25 μm were capable in acute experiments to produce the maximum muscle contraction in the rat leg with relatively low currents ($<70 \mu\text{A}$ for 95% at day 1), and to record CNAP with a high signal-to-noise ratio. However, translation to the clinics requires functional and stable neuroprosthetics over extended periods of implantation. The challenge in recording CNAP after one month, as well as the increase in the current threshold needed to produce muscle contraction over the limit allowed by the electrodes, limits the chronic use of the device. The presence of tissue encapsulation and the failure of the device encapsulation can compromise the stability of the nerve-electrode interface and, therefore, reduce functionality over time. A new generation TIME electrode was designed to overcome these limitations and enable the chronic use of the devices. Moreover, the implementation of an L-shaped layout and two arrow-shaped anchoring structures increased the clamping force to the tissue by an order of magnitude compared to the linear one. As a result, none of the implanted devices were found outside the nerve after 120 days.

Adding two 50 nm-thick Al_2O_3 layers of between the metal tracks and the PI, with a specific stack order, provides a simple route to improve fabrication yield, homogeneity, and robustness in wet environments. Compared with similar multi-layer designs that place tracks and electrodes on different planes separated by mm-thick insulators to compact the layout^{254–256} or to enhance stability,^{129,130,241,257} this approach addresses both aims without significantly increasing the total film thickness (10 μm). Routing the tracks through vias beneath the electrodes also prevents short circuits caused by rGO residues after dry etching, thereby increasing fabrication yield. This strategy is transferable to other applications where sensing sites are defined by dry etching, such as graphene transistors^{83,98} and carbon nanotubes²⁵⁸. The absence of fabrication defects and the protection of the tracks during dry etch and following steps yielded highly uniform electrochemical performance (CIL $> 98\%$ homogeneity; 1 kHz impedance magnitude $> 93\%$ homogeneity).

The hybrid organic/inorganic stack also improves the stability of the lead and the active contacts. Al_2O_3 contributes a low WVTR,^{93,259} while PI provides mechanical flexibility²⁶⁰. In contrast to stacks that place alumina in the external layers of the film stacking,^{127,128} having this material between PI-PI and PI-metal tracks enhances interfacial adhesion, as suggested by the intact gold pads of explanted devices; this is a common failure site in neural interfaces^{261,262}. After 3 months of accelerated ageing in PBS at 57 °C, cross-sections prepared by FIB and visualized by SEM confirmed good adhesion in the case of alumina-encapsulated devices, in contrast to the delamination observed for devices with no alumina.

The electrochemical performance of electrodes tested in vitro showed no significant change after combined accelerated ageing and long-term stimulation (10^9 pulses). Incorporating a passive discharge

interval in the stimulating protocol, in vitro and in vivo, prevented charge build-up and drift of the potential beyond rGO's safe water window. The combination of balanced-charge injection protocols and the intrinsic electrical properties of rGO effectively mitigated structural damage in the microelectrodes; Raman spectroscopy showed <1% change in the D/G intensity ratio. Optical inspection of the leads revealed no water ingress in-between the PI layers and no metal corrosion, consistent with the low moisture permeability of Al₂O₃ and the chemical resistance of PI^{260,263}.

Regarding the in vivo response, the 80 µm electrodes were able to elicit 95% muscle activation with current values comparable to those reported for platinum and iridium oxide electrodes of similar size^{10,12}. In relation to the selectivity of the activation with electrodes of different sizes, it was expected that smaller electrodes would improve the focalization of current and, as a consequence the stimuli in the target fibers. However, in acute studies higher selectivity was observed in the 25 µm rGO electrodes, compared to the 80 µm, as well as in larger metal active sites used in previous studies¹⁰. This is probably due to the anatomy of the sciatic nerve, since the GM and PL are located in the same fascicle and achieving intrafascicular selectivity is challenging. Moreover, considering the dimensions of the fibers that innervate the sciatic nerve, electrodes of 80 µm are already much smaller than what is needed to achieve high selectivity in this biological model. Trying to increase these selectivity values, in the case of the GM and PL could result in difficulty since they are located in the same fascicle and achieving intrafascicular selectivity is challenging.

The chronic evolution of the stimulation capabilities showed different behaviour between first- and second-generation devices. In the first-generation devices, the current threshold to achieve 95 % activation increased by approximately 650% after one month, whereas in the second-generation devices, the increase was limited to about 20%. Since the tissue response should be the same, this discrepancy is attributed to technological limitations like the failures in the encapsulation of the PI-PI devices, which can lead to electrical short-circuiting between electrodes and a reduction in effective current density, as the injected current spreads across multiple active sites simultaneously. Because muscle activation depends on current density, increasing the total injected current can partially compensate for this effect and restore muscle contraction. The potential failure of the PI-PI could also explain the reduction over time of the selectivity, which was more stable in the case of the hybrid encapsulation

The improved encapsulation of the new generation TIME electrodes allowed for the activation of all three muscles even after 120 days of implantation, with current thresholds slightly higher due to the fibrotic encapsulation (e.g. +51% at 95% muscle activation after 120 days) but below the safety current limit of the electrodes (~600 µA). The capability to chronically activate muscles while maintaining the current injection below the safe charge-injection limits of the electrode material is essential to avoid Faradaic reactions at the electrode-tissue interface that compromise long-term functionality. Here, this

was enabled by the high CIL of rGO microelectrodes together with improved device alumina-based encapsulation, which reduces water ingress and prevents inter-track short circuits.

In relation to the recording capability, the new generation TIME devices overcame previous limitations in the detection of sensory-evoked nerve activity and CNAPs after 30 days of implantation. Signal amplitude decreased over time, which is attributed to fibrotic capsule formation¹²; degradation of the electrodes would be expected to increase the noise levels of the recording, which was not observed. The increase in impedance at high frequencies after 30 days is consistent with a higher resistance generated by the fibrotic tissue. Notably, the explanted working electrodes exhibited an impedance comparable to values measured before implantation.

Overall, these advances contribute to increase the maturity of thin-film intraneural interfaces by improving homogeneity, stability, and fabrication yield. In vitro and in vivo results demonstrate substantial gains over previous generations, narrowing the gap between laboratory prototypes and clinical translation.

Chapter 5

Conclusions

Neural implants can treat many neurological disorders, such as Parkinson's disease, spinal cord injury, limb loss, and chronic pain. Commercial devices typically use millimetre-scale platinum-iridium electrodes for electrical recording or stimulation. These large electrodes capture mostly neural activity in the local field potential range (1-200 Hz) and can inject relatively low charge densities ($<30 \mu\text{C}/\text{cm}^2$ for electrode diameter $> 1 \text{ mm}$). Consequently, they lack the spatial resolution needed to detect and modulate disease-related biomarkers precisely. Furthermore, the charge spreads over the large electrode surface, making it challenging to selectively stimulate small brain nuclei or distinct nerve fascicles. This limitation reduces treatment efficacy and personalization, and it increases side effects caused by stimulation of untargeted regions. Commercial leads are semi-flexible and relatively thick ($\cong 1.3 \text{ mm}$), which increases chronic tissue response.

Over the last century, many technologies have trended toward higher sensor resolution (e.g., increasing TV pixel density or camera resolution) and toward thinner, more flexible devices enabled by new materials and compact electronics. Similarly, neural implants are moving toward miniaturization and higher-resolution neural recording. Recording action potentials from single neurons increases the information collected and enables the identification of complex brain patterns, pathological activities, and detailed neural mapping.

In this work, I have explored the potential advantages of using rGO microelectrodes embedded in a thin and flexible polymers. The rGO material was first characterized *in vitro* to evaluate performance against commercial devices and alternative high-performing electrode materials explored from the literature. The porous structure of rGO produced by the hydrothermal reduction process confers a high active surface area and the capability to inject charge through pseudocapacitive reactions. Cyclic voltammetry confirmed that within the defined safe material voltage window (0.8 to -0.9 V), there are no significant irreversible faradaic reactions and that the charge injection mostly occurs via pseudocapacitive processes. Small electrodes ($<100 \mu\text{m}$ diameter) can inject high charge densities ($> 1\text{mC}/\text{cm}^2$ for hundreds of microseconds or larger pulse widths) and have low impedance, thanks to the high active surface area. This allows electrode miniaturisation while maintaining the capability to inject current sufficient for neuromodulation and to record neural activity with low noise⁶³.

The electrodes' electrochemical response was also tested *in vivo* (Chapter 2) to evaluate differences between the actual brain tissue and the *in vitro* model used for characterization. Despite similar ionic

concentrations, the complex structure of neural tissue, and the presence of a large variety of biomolecules such as proteins, increase the impedance of the medium. This results in higher impedance magnitudes *in vivo* and larger voltage transients during electrical stimulation. These findings demonstrate that safe current limits derived from interfacial polarisation estimates in saline may substantially overestimate the operational boundaries of neural stimulation devices. They emphasize the importance of *in vivo* validation when defining truly safe stimulation parameters.

In this line, a second contribution of this work was the evaluation of the electrochemical stability *in vivo* and the biological safety of the rGO electrodes under stimulation conditions relevant to neural implants. Under the tested conditions, rGO electrodes maintained stable functionality and electrochemical performance. Histological analysis after neural stimulation showed that tissue responses were primarily dominated by implantation-related mechanical injury, with reactive gliosis mostly confined to the immediate vicinity of the implantation track. Electrical stimulation did not exacerbate astrocytic or microglial activation across different conditions, except for the protocol where interfacial polarisation exceeded the material's safety limits, suggesting a stress-induced tissue response. These findings further suggest that stimulation above the historical FDA guideline of $30 \mu\text{C}/\text{cm}^2$ (derived from large-area platinum electrodes $> 1 \text{ mm}$) may remain safe if electrode limits and *in vivo* electrochemical behaviour are appropriately considered. Consequently, safety limits for modern neural interfaces should be interpreted within a material- and geometry-dependent framework rather than as universal charge-density thresholds. While this study focused on short-term stimulation and recovery, future studies in larger animal cohorts should incorporate high-resolution spatial analysis together with chronic stimulation paradigms to evaluate long-term glial remodelling, neuronal viability, and neurovascular responses under sustained operation.

A translational consideration is that currents on the order of several mA may represent a practical operational limit for a single $300 \mu\text{m}$ rGO microelectrode under the stimulation parameters investigated here. This arises because capacitive materials with a large active surface area, like the porous rGO, tend to inject charge near the electrode edge at typical neural stimulation frequencies ($\sim 1 \text{ kHz} - 10 \text{ kHz}$). Therefore, when electrodes are scaled larger, both charge injection limit and impedance scale with the electrode perimeter rather than area, reducing the performance of macroelectrodes ($> 100 \mu\text{m}$ diameter). However, clinical neuromodulation often uses total currents in the multi-milliampere range, depending on therapeutic targets and side-effect threshold⁸¹. Since next-generation neural interfaces rely on high-density arrays composed of sub-millimetre contacts (rather than $\sim 1 \text{ mm}$ electrodes), total therapeutic current can be distributed across multiple microelectrodes simultaneously. This enables delivery of multi-mA currents while keeping per-contact charge injection and electrode interfacial polarization within electrochemical safety limits²⁷. Beyond enabling current steering and improved spatial selectivity, this distributed stimulation paradigm can reduce electrochemical stress at individual electrode-tissue interfaces. Translation of this approach will likely optimize electrode layout, more

complex current-source stimulation architectures, and it will require careful consideration of inter-electrode interactions and synchronisation effects.

Other strategies to increase current density should explore the effect of rGO flakes distribution (e.g. disordered stacking), varying reduction, and introducing defects to facilitate ion diffusion and reduce diffusion resistance in the porous structure. Additionally, micropatterning the encapsulation layer on the electrode can boost performance by increasing the perimeter-to-area ratio. However, more investigation is required to fully understand the scaling of the CIL in rGO and similar capacitive electrode materials and provide potential solutions.

With the electrodes' safety and operational range validated, Chapter 3 and Chapter 4 explore the use of this technology to treat two different motor disorders in preclinical models. In Chapter 3, I evaluated the ability to detect and modulate electrophysiological biomarkers in a Parkinsonian rat model as proof of concept of the use of DBS to reduce tremor. Initially, in vivo recording and stimulation capabilities of the rGO electrodes were evaluated in the brain. The electrodes recorded both local field potentials and spikes with a signal-to-noise ratio (SNR) greater than 10. This high-fidelity recording, along with the electrodes' small size, facilitated precise localization of small brain nuclei (e.g. the STN) by monitoring spiking activity. Millimetre-sized electrodes (as used in the clinic) do not readily capture this frequency range, so important information is missed.

Moreover, it was possible to detect a multiunit-based Parkinsonian biomarker in hemi-Parkinsonian rats that was absent in control rats. This biomarker was modulated thanks to the high charge injection capability: it required $\sim 1 \text{ mC/cm}^2$ in anaesthetized rats. This threshold exceeds the clinically approved $30 \text{ }\mu\text{C/cm}^2$ limit; however, the results shown in Chapter 2 and other studies^{264,265} suggest the need to revisit this limit for microelectrodes. The bidirectional capability to modulate and monitor local neural activity with the same electrode enables visualization of long-lasting biomarker modulation effects not observed before. This highlights the importance of bidirectional electrodes for improving neuromodulation therapies, facilitating the investigation of the mechanisms around DBS and implementing closed-loop therapies with stimulation parameters adaptive to the biomarker level. This could help enhance the effectiveness, safety, and personalization of neuroelectronic therapies for neurological disorders.

Future translation to humans will face important challenges, such as developing a new insertion strategy^{266,267} compatible with clinical procedures and designing leads adapted to the human brain. This will require somewhat larger electrodes (up to a few hundreds of μm) for higher current injection, while still using small electrodes for spike recordings. Additionally, chronic recording of spiking activity over years of implantation will be necessary, as achieved only in a few studies (e.g. mesh electronics).¹¹⁰

Clinically compatible insertion strategies, including approaches such as the BlackRock or Neuralink method, should be implemented, along with techniques to anchor the device securely to the tissue while still allowing its safe removal without causing significant damage.

Chapter 4 summarizes a study addressing the use of rGO neural interfaces for sciatic nerve stimulation, using transversal intraneural microelectrodes (TIME) devices. Restoring natural sensory feedback and fine motor control in prosthetic systems requires selective activation of small nerve fibers. This requires electrodes with small dimensions ($<100\text{ }\mu\text{m}$), high density, and stable long-term performance.

rGO microelectrodes of $25\text{ }\mu\text{m}$ and $80\text{ }\mu\text{m}$ were able to record compound nerve action potentials and to selectively activate the three nerve fascicles of the sciatic nerve with high selectivity (>0.6) and relatively low current thresholds ($<150\text{ }\mu\text{A}$ for 95% activation on day 1 after implantation). The selectivity achieved with the $80\text{ }\mu\text{m}$ electrodes was comparable to that of the smaller $25\text{ }\mu\text{m}$ electrodes. This highlights that miniaturization is not always advantageous when the biological structures involved are significantly larger than the electrode (nerve fibers are about an order of magnitude larger). Furthermore, electrode size must be considered for long-term implantation: over time, higher currents are often required to induce the same muscle contraction, and the electrode must remain capable of delivering these currents within its safe material limits.

Part of the work in this PhD thesis also focused on improving the long-term stability of the device. Previous TIME devices faced challenges in both recording and stimulation due to tissue response and device instability during chronic implantation. In comparison with rigid implants, thin-film flexible technologies reduce chronic tissue response thanks to their smaller footprint and better mechanical compliance with soft tissue; however, they are more fragile. Nevertheless, transitioning from bulky, rigid devices to thin, flexible ones appears to be the correct direction for improving neural interfaces integration in the body and enabling long-term communication with neural tissue.

Therefore, in Chapter 4, I presented a new hybrid PI- Al_2O_3 -PI encapsulation that improves moisture-barrier properties and metal-polymer adhesion. Moreover, a via-under-electrode routing was designed to isolate rGO residues from the conductive tracks, enhancing electrode yield and homogeneity. The devices demonstrated excellent performance and electrode-to-electrode uniformity (e.g., in CIL and impedance) and showed no detectable structural changes by Raman spectroscopy even after one year (in accelerated ageing time) and 10^9 current stimulation pulses. These results suggest that rGO, when embedded in a device with stable encapsulation, can sustain immersion in wet environments and long-term electrical stimulation without structural or electrochemical deterioration. This provides an alternative to other high-performance materials with lower long-term stability, such as porous platinum (which can dissolve⁶⁰ over time) or PEDOT:PSS (which degrades under prolonged electrical stress⁵⁶). This is only a first step toward a long-term stable, flexible neural interface, since commercial devices

must remain stable for the patient's lifetime and sustain electrical stimulation throughout their operational period.

In vivo results, consistent with in vitro tests, showed selective activation of distinct nerve fibers groups, with stimulation thresholds remaining below the maximum injectable current throughout 120 days of implantation. The devices also recorded nerve action potentials with robust signal-to-noise ratios after 30 days, overcoming previous limitations in recording sensory-evoked nerve activity and CNAPs. These results represent a substantial improvement over previous generations of TIME devices, suggesting that device stability is crucial for reliable chronic use. However, signal amplitude decreased over time, likely due to fibrotic encapsulation and limited long-term device–tissue integration.

Future strategies could explore: 1) hydrogel coatings to create a soft, tissue-like interface, 2) mesh structures to promote tissue integration inside the device^{110,3}) anti-inflammatory coatings (e.g. dexamethasone-functionalized PI^{111–113}) or low-level electrical stimulation¹¹⁴ to reduce tissue encapsulation¹¹³. These approaches may further improve long-term stability.

However, more effort is required to understand long-term stimulation effects. For example, it needs to be considered if body fluids can contact the metal below the rGO electrode through the porous structure, which might require using different metals such as platinum instead of gold. Also, it needs to be further studied potential delamination of the graphene flakes under prolonged implantation. Another limitation of these second-generation TIME devices was the low mechanical stretchability of the lead. Leg motion and repeated handling of the device can induce mechanical loads that fracture metal traces, leading to loss of electrodes over time. This highlights the importance of personalising devices for each application: intracortical devices require high flexibility to minimise neural damage during tissue pulsation, cuff or epicortical implants need conformability to fit neural structures, and spinal or peripheral nerve interfaces require stretchability to accommodate larger movements. Therefore, future work should explore stretchable yet hermetic neuroelectronic architectures for nerve applications^{4,73,74}. Possible solutions include elastic substrates (PDMS^{100,101} or hydrogels^{102,103}) that undergo reversible strain, patterned substrates (serpentine/kirigami designs) for enhanced stretchability, and conductive tracks made from liquid metals¹¹⁹ or micro-cracked^{100,120,121} metal films that maintain continuity under strain. To facilitate clinical translation, these solutions should be integrated into a highly automated and controlled industrial fabrication process, minimizing manual steps, as in the case of the membrane transfer and smoothing.

Finally, future work could explore applications beyond those presented in this PhD thesis. For instance, rGO's high capacitive response at low frequencies could benefit nerve regeneration through low-frequency stimulation. Cochlear and retinal prostheses could also gain from this technology, since these applications require very small electrodes ($\approx 30\ \mu\text{m}$) to improve spatial resolution in bidirectional interfacing with the nervous system.

5. Conclusion

In summary, this PhD work presents both the advantages and challenges of implementing rGO-based technology for treating movement disorders. rGO proved to be a high-performance, chemically inert, biocompatible material with strong potential for next-generation neural interfaces, potentially improving the safety and efficacy of neurological treatments.

ANNEXES

ANNEX 1. METHODS

CHAPTER 2

Fabrication of rGO microelectrode array.

A silicon wafer that had been spin-coated with a 7.5 μm layer of polyimide (PI-2611, polyimide precursors based on BPDA/PPD, biphenyldianhydride/1,4 phenylenediamine) and evaporated with 20/200 nm layer of Ti/Au with e-beam evaporation (Leybold Univex 400). An aqueous solution of dispersed graphene oxide flakes was filtered with a vacuum system through a filter with pores of 0,02 μm , to create a 2 μm -thin membrane. This membrane was then water-transferred onto a silicon wafer and partially reduced hydrothermally in an autoclave. The electrode areas were defined by using an aluminium hard mask patterned using optical lithography (nLOF 2070, Microchemicals) and lift-off. Then, the GO membrane was etched using RIE with 2000 W ICP and 40 sccm of O₂ and 20 sccm of Ar. The Ti/Au tracks were defined with photolithography using photoresist (AZ5214E, Merck) and wet etching. Then a second PI layer was spin-coated on top. A series of photolithography (AZ 10XT, Microchemicals) and RIE processes were used to open the pads, electrodes, and structure sides. RIE was performed using a HF power of 40 W, ICP power of 1200 W, with 60 sccm of O₂ and 50 sccm of Ar flow for 4.5 min to etch the top PI layers that opened the electrodes. Finally, the aluminium on the electrodes was chemically etched using an aqueous etching solution of orthophosphoric acid, nitric acid, and deionized water (74% v/v H₃PO₄, 2.5% v/v HNO₃, 2.5% H₂O). The completed devices were mechanically peeled off from the carrier wafer.

The devices were attached to a plastic sticker of 127 μm (ARcare 8259, adhesives research), which was used as a rigid support for the insertion. For the alignment with the device, a customized set-up described in a previous publication⁶³ and here summarized. A vacuum pump was connected through a plastic tube to a small metallic tip that held the adhesive suspended. Under an optical microscope, the adhesive was aligned using a micromanipulator to the device placed on a working base below. Then by moving up the base with the micromanipulator, the device is attached to the adhesive.

Electrochemical evaluation.

After fabrication. Electrochemical characterization was performed to evaluate the functionality of the electrodes using a potentiostat (Metrohm Autolab PGSTAT128N) in a three-electrode configuration. An Ag/AgCl electrode (FlexRef, WPI) was used as the reference electrode, and a platinum wire (Alfa Aesar,

45093) as the counter-electrode. An electrolyte solution containing 10 mM phosphate buffer, 137 mM NaCl, and 2.7 mM KCl at pH 7.4. in a 10 mMol PBS was prepared by dissolving one PBS tablet (Sigma-Aldrich, P4417) in 200 ml of DI water.

The electrodes underwent an electrochemical activation process consisting of 70,000 biphasic rectangular current pulses (cathodic-first with interpulse) of 1 ms pulse width, ramping from 10 to 40 μ A at 100 Hz for the 150 μ m electrodes and from 10 to 70 μ A for the 300 μ m. This was followed by impedance, cyclic voltammetry (CV), and current pulses measurements to evaluate functionality.

Impedance spectroscopy was measured using the previously mentioned three-electrode setup by applying a 10 mV sinusoidal signal in the frequency range 1 Hz - 100 kHz. Electrodes with impedance between 2 and 70 k Ω at 1 kHz were considered functional.

Before implantation. With the previously described set-up, and a different potentiostat (Ivium n-Stat), a gentle reactivation was performed in the surgical theater prior to in vivo implantation, consisting of 100 cycles of cyclic voltammetry from -0.9 V to 0.8 V at 50 mV/s, followed by the standard characterization (EIS, CV at 50mV/s and few current pulses of different amplitudes).

After brain implantation. Electrochemical characterization was performed after implantation to verify electrode and device integrity. A platinum wire serving as a counter electrode was placed in the cerebellum of the implanted hemisphere, while a Ag/AgCl wire used as reference was positioned in the contralateral cerebellum.

After stimulation in the brain. Identical characterization as in the previous paragraph was performed to evaluate electrochemical changes at the electrode-electrolyte interface. Finally, the device was extracted from the brain and characterized again in PBS.

In vivo experimentation.

Ethics. All experimental procedures were performed in accordance with EU directive 2010/63/EU and Spanish regulations (Laws 32/2007 and 6/2013 and Real Decreto 53/2013) and were approved by the University of Barcelona Animal Research Ethics Committee under the license number 285/23.

Animals and housing. Eighteen young adult female and male C57Bl6/J mice (2–3 months old, 20–30 g) were obtained from Charles River. Mice were housed under controlled environmental conditions, including a temperature of 22 ± 2 °C, relative humidity of 60%, and a 12:12 light–dark cycle (lights off from 7:30 p.m. to 7:30 a.m.). Food (standard pellet diet) and water were available *ad libitum* throughout the study, except during the surgeries and stimulation sessions, when the animals were anesthetized.

Surgical procedures. Adult mice were anesthetized with isoflurane (3–4% for induction, 1–2% for maintenance) in oxygen (0.8–1.0 L/min) and placed in a stereotaxic frame on a thermostatically controlled heating pad to maintain body temperature. Atropine (0.05 mg/kg, s.c.) was administered to

reduce salivary and bronchial secretions. Ophthalmic ointment was applied to prevent corneal drying, and anesthetic depth was assessed by the absence of pedal withdrawal reflex. The scalp was shaved and disinfected with alternating applications of povidone–iodine and 70% ethanol. A midline incision was made to expose the skull, and bregma and lambda were aligned in the same horizontal plane. Bilateral burr holes were drilled over the caudate putamen (anteroposterior +0.6 to +1.0 mm; mediolateral $\pm$ 1.5 to 2.0 mm from bregma; dorsoventral 2.7 mm). Two probes were slowly lowered into the caudate–putamen, one in each hemisphere. The left probe was used for stimulation experiments, whereas the right probe served as a sham control. Both probes were implanted along the sagittal plane with the rGO electrodes oriented outward. The probes were held in place using electrode holders mounted on the stereotaxic frame arms. A separate screw over the cerebellum served as a common ground. After completing the stimulation and probe characterization procedures (see below), both devices were carefully withdrawn, the surgical site was thoroughly rinsed with sterile saline, and the scalp incision closed using absorbable sutures. Antibiotic ointment was applied to the wound site. Postoperative analgesia was provided with buprenorphine (0.05–0.1 mg/kg, s.c.), and mice were allowed to recover in a warmed cage under close observation. Analgesic and anti-inflammatory treatments were continued for the first 48 h. All animals recovered uneventfully from surgery and stimulation sessions.

Electrical stimulation.

Electrical stimulation was delivered using a MCS stimulator (STG-4008, $\pm$ 1.6mA current range). Biphasic, charge-balanced symmetric square-wave pulses (60 μ s pulse width per phase) were applied through rGO microelectrodes with diameters of 150 and 300 μ m.

For the high-intensity stimulation protocol, current amplitudes of 240 μ A and 1000 μ A were applied to 150 μ m and 300 μ m electrodes, respectively, corresponding to a charge density of 90 μ C/cm². For the low-intensity protocol, current amplitudes of 88 μ A (150 μ m) and 350 μ A (300 μ m) were used, corresponding to a charge density of 30 μ C/cm².

Each stimulation session consisted of two sequential epochs. First, electrodes were stimulated using progressively increasing current amplitudes to reach the target stimulation level (ramp-up epoch). Then, stimulation was delivered at the maximum amplitude corresponding to either high- or low-intensity protocol (prolonged stimulation epoch).

Immunohistochemistry.

Thirteen to fifteen days after the *in vivo* experiments, mice were euthanized and transcardially perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Brains were post-fixed in 4% PFA for 24h at 4 °C, then cryoprotected by immersion in 30% sucrose at 4 °C overnight or until they sank. Subsequently, brains were frozen at –80 °C until processing. Prior to sectioning, the sham hemisphere (always the right hemisphere) was marked with a small lateral incision to allow unambiguous identification of the stimulated and sham sides in each brain slice. Brains were embedded

in OCT compound and coronally sectioned at 40 μm thickness using a cryostat. Sections were stored in 30% sucrose at $-20\text{ }^{\circ}\text{C}$ until use. Slices containing the implant tracks were selected, washed in PBS ($3 \times 10\text{ min}$), and incubated in blocking solution (5% donkey serum and 0.3% Triton X-100 in PBS) for 2 h at room temperature. Alternating sections from each brain were used to quantify astrocytes via glial fibrillary acidic protein (GFAP) immunoreactivity and microglia via ionized calcium-binding adaptor molecule 1 / AIF1 (Iba-1) immunoreactivity. Sections were then incubated overnight at $4\text{ }^{\circ}\text{C}$ with primary antibodies diluted in blocking solution: mouse anti-GFAP (1:500; Invitrogen, Waltham, MA, USA) and rabbit anti-Iba1 (1:500; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). After primary incubation, sections were washed in PBS ($3 \times 10\text{ min}$) and incubated for 2 h at room temperature in the dark with secondary antibodies: donkey anti-mouse IgG Alexa Fluor® 488 (1:1000; Invitrogen, Waltham, MA, USA) and donkey anti-rabbit IgG Alexa Fluor™ 594 (1:1000; Invitrogen, Waltham, MA, USA). Finally, sections were washed again in PBS ($3 \times 10\text{ min}$) and mounted with Fluoromount-G™ containing DAPI (REF 00-4959-52, Invitrogen) for nuclear counterstaining, then coverslipped. Fluorescence images were acquired at 10x and 20x magnification using a Dragonfly 200 confocal microscope system (Oxford Instruments Andor, Abingdon, UK) controlled by Fusion software (version 2.4.0.14, Oxford Instruments).

Data analysis.

Chronopotentiometry analysis. Voltage transient data obtained during electrical pulsing were organized according to stimulation condition. For each waveform, the inflection points corresponding to the rising and falling edge of both anodic and cathodic phases were identified, and individual pulses were isolated with a temporal padding of 40 μs on each side. Signals were baselined-corrected to 0 V. The effective ohmic drop was calculated by averaging the voltage within a time window defined as $t_0 + 40\text{ }\mu\text{s}$: $t_0 + 50\text{ }\mu\text{s}$, where t_0 corresponds to the onset of the pulse phase (rising phase for anodic and falling edge for cathodic). The starting point of each pulse was centered to 0. The interfacial electrode polarization was calculated by subtracting the ohmic drop from the absolute peak voltage of each pulse.

Because anodic and cathodic phases exhibited comparable statistical distribution and did not produce bimodal behavior, data from both phases were pooled for each stimulation condition. Linear relationships between injected current and voltage components were obtained using least-squares regression. All analyses were performed using custom scripts written in Python.

Capacitance extraction from CV. Capacitance values were extracted from cyclic voltammetry (CV) measurements by integrating the absolute value of the current response within the electrochemical potential window from -0.9 to +0.8. The integrated charge was normalized by the scan rate (50 mV/s) and the applied voltage range (1.7 V) to obtain the effective capacitance.

Extraction parameters from impedance. Impedance spectra were analyzed from Bode plot data by calculating the real and imaginary components. Equivalent circuit fitting was performed using Z-View (v4) employing a CPE-R circuit model to extract spreading resistance and interfacial capacitance parameters. All impedance spectra were fitted with a relative fitting error below 15%.

Immunohistochemistry. Images were analyzed using ImageJ software (version 2.14.0/1.54f). For each section, a $500 \times 500 \mu\text{m}$ region of interest (ROI) was analyzed. The ROI was positioned approximately $100 \mu\text{m}$ away from the electrode track to avoid effects associated with direct tissue damage from implantation. The number of labelled cells, total fluorescent area (μm^2), and mean fluorescence intensity (a.u.) were automatically quantified using ImageJ's *Threshold* and *Analyze Particles* functions. The images were processed to subtract half of the background fluorescence before thresholding and creating a binary image for the fluorescent area identification. Different particle sizes were applied when analysing to extract the number of cells (bigger particle size, IBA1: 10-infinity and GFAP: 2-infinity) and the total area of fluorescence and mean fluorescence (all fluorescent particles identified after thresholding) for each marker. When analysing DAPI, the tool *Watershed* was used in the thresholded image to automatically separate the different nuclei that may be detected together as one. The ROIs of the identified particles for mean fluorescence were used in the original unprocessed image to calculate the real fluorescence. All fluorescence comparisons were performed using images acquired during the same microscopy session and processed under identical conditions to minimize variability and prevent fluorescence-related bias. Statistical analyses were conducted using paired Student's *t*-tests.

CHAPTER 3

Fabrication. The fabrication of the silicon shuttle has also been previously described²⁰¹, and it is briefly summarized here. The silicon shuttle was fabricated on a silicon-on-insulator (SOI) wafer. The structure was dry-etched using the standard Bosch process, which stops at the buried oxide layer. Finally, the shuttles were released by wet etching the buried oxide layer in 49% hydrofluoric acid. For the fabrication of the probe, refer to section Chapter 2 in this Annex.

Electrochemical activation.

Before in vitro electrochemical evaluation, the electrodes underwent an electrochemical activation process consisting of 70,000 biphasic rectangular current pulses (cathodic first with delay) of 1 ms pulse width, ramping from 5 to 20 μA at 100 Hz. Prior to in vivo implantation, a gentle reactivation was performed¹⁹², involving 100 cycles of cyclic voltammetry from -0.9 V to 0.8 V at 50 mV/s. Electrochemical characterization in PBS involved measuring impedance, cyclic voltammetry, and current pulses to evaluate functionality, ensuring no broken or electrically shorted electrodes.

Impedance spectroscopy. It was measured using the previously mentioned three-electrode setup by applying a 10 mV sinusoidal wave over a frequency range from 1 Hz to 100 kHz. Electrodes with impedance between 20 and 70 k Ω at 1 kHz were considered functional.

Charge injection limit (CIL). Biphasic current pulses were injected, and the voltage was measured against the reference. Cathodic and anodic voltage shifts were determined for each current level by visually distinguishing the interfacial polarization ($V_1 - V_2$) across the electrode-PBS interface from the linear voltage produced by the resistive ohmic drop²⁰⁹. To determine the charge injection limit, interfacial polarization values were plotted against the current for both the cathodic and anodic parts of the pulse at increasing current levels. In figure 2d, the errors (E_r) of the average interfacial polarization of the electrodes of a single array, were calculated from the standard deviation (σ) of the two voltage points $V_1 \pm \sigma_1$ and $V_2 \pm \sigma_2$, as $E_r = \sqrt{\sigma_1^2 + \sigma_2^2}$. From the linear fit of these points, iextrapolated the current values at 0.8 V and -0.9 V, representing the maximum currents safely injected within the GO water window. The CIL was then calculated by multiplying the maximum current by the pulse duration and dividing by the electrode area. Similarly, the maximum current density was calculated by dividing the maximum current by the electrode area.

Stability. To assess the stability of the electrodes after long-term stimulation, devices were tested in PBS with the Intan stimulator in the three-electrode configuration (refer to the chronic electronic setup). 100 million biphasic current pulses (100 μ s width, 50 μ A in amplitude, and 1 kHz frequency) were applied through 3 electrodes. A 1 ms passive discharge, integrated into the system, was implemented between consecutive pulses to prevent charge building up at the electrode/tissue interface. Impedance and chronopotentiometry were measured with the potentiostat before and after the stimulation protocol to ensure that the electrodes did not present significant changes in their electrochemical behavior.

Dissolution time of two adhesive polymeric coatings.

Two adhesive polymers were tested in vitro by attaching polyimide-only devices to a silicon shuttle. Polyethylene glycol (PEG, 10.000 Mmol, Aldrich Sigma) was prepared by melting 5 grams at 70 °C. Polyvinyl alcohol (PVA, Aldrich Sigma 9000/10.000 Mw) was prepared by stirring 20 grams in 50 ml of distilled water on a hot plate at 105 °C and 400 RPM. The mixture was boiled for 3 minutes and then allowed to cool overnight, covered with aluminum foil. The small tip of the dummy devices required a customized setup for alignment with the shuttle during attachment. A vacuum pump was connected through a plastic tube to a small metallic tip that held the shuttle suspended. The shuttle tip was coated with the adhesive polymer using a small brush. Under an optical microscope, the flexible lead was aligned to the shuttle by moving the stage with a micromanipulator. The dissolution time of each adhesive polymeric coating was tested by inserting assembled devices into agarose. The agarose (Duchefa Biochemie) was prepared by stirring 0.6 grams of agarose powder in 100 ml of 10 mM PBS at 400 RPM and 200°C until the complete dissolution. The agarose was then cooled down at room

temperature over several hours. The assembled devices were inserted in a beaker with agarose at 38°C, and the tail of each device was gently pulled out every 30 seconds to evaluate adhesion with the shuttle. In case of detachment, the shuttle remained inside the agarose while the flexible lead was removed, determining the dissolution time for each sample.

Insertion test in vitro

Measurement of insertion and buckling forces. I measured the insertion force using a load force cell (Zwick Roell, nominal force 50 N) by inserting functional devices attached to the shuttle with PVA into agar using a micro driver (Zwick Roell Z 0.5). The shuttle base was clamped to the arm controlled by the micro driver and inserted perpendicularly into agar positioned in a beaker below. The insertion speed was the same as in vivo: 100 µm/s for the first 6 mm and 5 µm/s for the next 1.5 mm. The buckling force of the shuttle was measured by contacting it against a rigid surface (the dynamometer probe station). The shuttle was clamped as previously described and moved toward the base at 100 µm/s. Upon contact with the base, the shuttle began to bend until it reached the breaking point.

Animals and surgery. Rats were housed in ventilated cages with free access to food and water, maintained at room temperatures between 23 °C and 25 °C, and subjected to a 12-hour light cycle. Animal experiments were conducted in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986 and approved by the Home Office (license PPLPP9890301).

Nine Sprague Dawley rats (250–400 g) were used for acute electrophysiological recordings and another four for awake recordings. Rats received a unilateral injection of either 6-OHDA (6-hydroxy dopamine) to induce Parkinson's disease (PD model) or saline into the medial forebrain bundle (MFB) as a control. Lesioning surgery was performed under general anesthesia with isoflurane (induction at 5% in 3 l/min O₂; maintained at 1.5-2% with 3 l/min O₂). Buprenorphine (0.03 mg/kg) was injected subcutaneously before surgery for pain management. Body temperature was maintained at ~37°C using a homeothermic monitoring system (Harvard Apparatus, USA). Burr holes were drilled over the STN and MFB using coordinates from the rat brain atlas (STN: AP -3.6 mm from bregma, ML -1.4 mm; MFB: AP -4.3 mm, ML -2.0 mm) using a dental drill. Histological analysis determined the placement of the device and the extent of 6-OHDA dopaminergic lesions.

Lesioning of the nigrostriatal pathways. To induce hemi parkinsonism, 4 µl of 6-OHDA (7.5 µg/µl in 0.2% ascorbic acid dissolved in saline) (Sigma-Aldrich, USA) was injected into the MFB (DV -7.5 mm) through a 33-gauge needle connected to a 10-µl Hamilton syringe at a rate of 1 µl/min, pausing for 1 minute after every 1 µl. The needle remained in place for 10 minutes after the injection was complete, and was then withdrawn in small steps initially, then larger steps, until fully removed. The 6-OHDA solution was freshly prepared on the morning of the lesioning surgery and kept in a cool, dark place

until use. Rats were allowed to recover from surgery and returned to their cages. Behavioural signs of hemi-parkinsonism typically appeared about 2-3 weeks post-surgery.

Behavioural tests. Fourteen or fifteen days after toxin injection, each animal was assessed using two robust behavioural tests, the Forelimb Asymmetry Test and the Apomorphine-circling test,²¹³ to determine if hemi parkinsonism was successfully induced. Not all rats that received 6-OHDA developed motor deficits; however, 75 percent of the rats that received 6-OHDA did develop motor deficits and were included in electrophysiological studies.

Forelimb Asymmetry test. Rats with hem-parkinsonism developed motor deficits in their forelimb contralateral to the lesioned hemisphere. They became less likely to use the affected forelimb (left) when exploring their environment compared to the intact side (right). This test involved placing the rat in a transparent cylinder and counting the number of times the rat used the intact vs. affected forelimb during rearing behaviour. The asymmetric ratio was calculated as $A_r = \frac{Right-Left}{Right+Left+Bot}$. Properly lesioned rats preferentially used the forelimb ipsilateral to the lesion when rearing, while otherwise, rats tended to use both forelimbs equally.

Apomorphine-Circling test. To perform this test, rats were placed in a transparent cylinder and allowed to habituate for 5-10 minutes before receiving a subcutaneous injection of apomorphine (0.05 mg/kg, Sigma-Aldrich, USA). After administration, rats were observed for 20 minutes. The number of contralateral rotations (rotations in the direction opposite to the lesioned hemisphere) was counted. Rats completing more than 80 rotations within this time window were declared hemiparkinsonian²¹⁴.

Acute in vivo experiments

Electronic Setup. The device was connected to a 9-contact Zero Insertion Force (ZIF) Molex connector (Mouser electronics, 0.3 mm pitch), which was attached to a custom small, printed circuit board (PCB) (WURTH ELEKTRONIK). A 36-channel header connector (DigiKey) connected the PCB to a 32-channel stimulation/recorder system (ME2100, Multichannel Systems). For the datasheet of the system: https://www.multichannelsystems.com/sites/multichannelsystems.com/files/documents/data_sheets/MCS_ME2100-System_Datasheet.pdf

Surgery. 2-3 weeks after the lesioning surgery, a male rat was used under isoflurane to evaluate the recording capability, while 7 female and 3 male rats were prepared for a terminal DBS experiment under urethane anesthesia (1 g/kg). Urethane was chosen as it minimally interferes with the expression of electrophysiological biomarker signals^{268,269} of PD, maximizing the chances of detecting these signals. The shuttle was pre-glued with permanent glue (Loctite), and then the rGO electrodes were electrochemically activated in PBS, as previously described¹⁹². The shuttle was secured with double-sided tape to the PCB, which was connected to the MCS head stage, and then clamped to the arm of a micromanipulator (Scientifica, UK) (Figure 4b) to perform the insertion (Figure 4b).

Four burr holes were drilled: one over the STN (AP -3,6 mm from bregma, ML -1.4 mm) for the device, one for a Pt wire to record from the motor cortex (M1: AP: 1.0 mm, ML -2.6 mm), one for an Ag/AgCl reference wire (AP: -5.5 mm, ML: 3mm), and one for a Pt counter electrode (AP: -5.5 mm, ML: -3mm) (Figure 4a). The micromanipulator enabled controlled insertion of the device at 3 $\mu\text{m/s}$ to minimize mechanical damage of the neurons. The STN was localized by looking at its characteristic spiking activity, which is quite intense compared to regions before and after that are both silent.

Recording and Stimulation. Neuronal activity was recorded at a 40 kHz sampling rate and high-pass filtered at 1 Hz. Once the STN was reached, DBS was delivered through the electrodes inside the STN, identified by their higher spiking activity, compared to the nearby areas. Biphasic rectangular pulses with a duration of 100 μs /phase were delivered at various amplitudes (25 μA , 50 μA , 75 μA) and frequencies (10 Hz, 100 Hz, 130 Hz) for 1 minute. Stimulation periods were preceded and followed by 2 minutes of recording to capture STN activity pre- and post-stimulation.

Chronic in vivo experiments

Electronic Setup. The device was connected to a small PCB (1.3 cm) through the same ZIF Molex connector. An 18-contact Omnetics connector (0,64 mm pitch) connected the PCB to an Intan head stage (RHS2116 16-channel, datasheet https://intantech.com/files/Intan_RHS2116_datasheet.pdf), managed by an Intan RHS 128-channel stimulation/recording controller and RHX software. This setup, integrating the amplifier directly into the chip connected to the PCB, reduced noise that would arise from cables if the amplifier were situated on the main stimulator. A 3D-printed plastic cover protected the head stage from animal scratches. Data recording and stimulation were managed with RHX Data Acquisition Software (version 3.3.1).

Surgery. 3-4 weeks after lesioning surgery, 4 rats underwent another surgery for permanent device implantation. Anesthesia was induced with 5% isoflurane in 70% NO_2 and 30% O_2 and maintained at 1-2% isoflurane. Buprenorphine analgesia (0.05 mg/kg) was administered subcutaneously at the beginning of the surgery. Videne and EMLATM cream were applied to the scalp. Four burr holes were drilled over the STN, M1, reference, and counter electrodes using the same coordinates as previously described for the acute experiment.

Compared to the insertion strategy used in the acute experiments, which involves just one arm, for the chronic one iperformed a double arm insertion (Figure 4a). The PCB was connected to the Intan RHS head stage and secured to the first stereotactic arm. The shuttle was attached with double-sided tape to a second arm controlled by the micromanipulator (Figure 4a). The device was implanted in the STN (approximately 7.5 mm deep) at an initial speed of 100 $\mu\text{m/s}$ for the first 6 mm and 5 $\mu\text{m/s}$ for the following 1.5 mm. When the insertion is completed, the tail is encapsulated with Kwik seal avoiding the insertion hall. 30 mMol PBS drops were applied to dissolve the PVA on the part of the device outside the brain, while brain fluids dissolved the internal coating. After sufficient time (20 minutes), the shuttle was removed, leaving the flexible device in the STN (Figure 4a). The entire assembly was

secured with dental cement attached to stainless steel bone screws anchored to the skull. STN activity was recorded before, during, and after dental cement encapsulation to ensure device functionality.

Recording and Stimulation. Recording and stimulation sessions began 24 hours after surgery to allow rats to recover. At the start of each session, rats were briefly sedated with 5% isoflurane to connect the PCB to the Intan head stage. Rats were placed in a transparent cylinder, and after regaining consciousness, DBS trials began. Data were stored at a 30 kHz sampling rate with a high-pass filter of 1 Hz. Stimulation protocols similar to those under anesthesia were delivered to compare recordings from awake and anesthetized states. Recordings were obtained at multiple time points post-implantation to assess electrode performance over time. Electrochemical impedance spectroscopy at 1 kHz in chronic settings was evaluated using the Intan built-in function.

Histology. At the end of the experiment, rats were deeply anesthetized and perfused transcranially with 0.1 M PBS followed by 4% paraformaldehyde in 0.1 M PBS. Brains were fixed in 4% paraformaldehyde overnight at 4°C and then transferred to 30% sucrose (4°C). The brains were cut into 50 µm sections using a cryostat (Leica Microsystems) and processed for staining. Tyrosine hydroxylase (TH) immunofluorescent staining was used to determine the degree of degeneration of dopaminergic neurons. Brain sections were rinsed with PBS, immersed in citric acid solution (pH 6), blocked for 1 hour in 10% goat serum, and incubated overnight at 4°C in anti-TH antibody (AB152; 1:100, Millipore) in 1% BSA solution. Sections were then incubated with anti-rabbit donkey (Alexa Fluoro 647, 1:300, Code A31573) secondary antibody in 1% BSA solution for 1 hour at room temperature. Sections were covered in mounting media and imaged using a slide scanner. Electrode placement accuracy was determined by obtaining sections at the level of the STN and staining with 0.2% cresyl violet (Sigma-Aldrich Co.).

Ethics. Every experiment involving animals has been carried out in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986 and approved by the Home Office (license PPLPP9890301).

Electrophysiological data analysis

Electrophysiological signals recorded in vivo were processed using Python packages including Numpy 1.21.5, Neo 0.11.1, pandas, seaborn 0.11.2, Matplotlib 3.5.1, Quantities 0.13.0, Elephant 0.10.0, and the custom library PhyREC 0.6.5. Origin 2018 was used for scatter plots, box and bar charts and statistical analysis.

Spiking Activity Analysis. The signal analysis involved band-stop filtering at 50 Hz and its harmonics to remove the noise of the electronics, followed by band-pass filtering in the 200 Hz-2 kHz MUA frequency range. For awake recordings, to remove the artifacts generated by chewing and rat movements, additional noise reduction was achieved by subtracting a silent channel's recording outside the STN. It was performed the channels reorder to match the actual spatial distribution of the array, with a mapping that depends on the recording system (Intan or MCS). Spike events were detected using a voltage threshold, with a minimum relaxing time of 1.5 ms between two consecutive events. Threshold

voltage levels, ranging from -20 to -30 μ V, were determined individually for each experiment, based on baseline amplitude assessment. The time of detection of each spike (spikes time) was collected, and the signal was sliced in a window of 2 ms per side around these events.

To assess the spike-to-noise ratio, it was calculated the average of the maximum absolute voltage of the spikes (in the recording period of 100 s), divided by the standard deviation of the total recording signal in the MUA frequency range after noise cleaning, $SNR = \frac{\frac{1}{n} \sum_{i=1}^n \text{Max}|V_i(\text{spikes})|}{STD_{\text{signal}}}$.

In the plot of Figure 8c, it was selected for each rat the electrode in the STN with a higher SNR value. The spike rate was determined as the ratio between the number of spike events detected over the recording time considered (100 s).

Color-Coded Plots. Color-coded plots, displaying spike rate and modulation factor, were normalized to the range [0, 1] by dividing each value by the maximum in the dataset. A scatter plot visualized the normalized data, using a colormap that encoded the intensity values.

Root Mean Square (RMS). For the RMS calculation, the signal was pre-filtered for noise-cleaning (50 Hz and harmonics), within the MUA frequency range (200-2000 Hz). Then it was computed the square root of the mean of the squared signal values.

Interspike frequency (IF) distribution. The interspike frequency (IF) distribution was calculated as the inverse of the interspike times (IT) between two consecutive spikes detected. To ensure comparability across recordings, the cumulated IF counts were normalized by dividing for the total number of counts in the recording period. The histograms were fitted with a kernel density probability function using the `displot` seaborn function with `kind='kde'` and default parameters. The resulting curve represents the density of interspike frequencies, normalized such that the integral over all possible values is equal to 1. By examining these fitted histograms, it was possible to classify the IF distribution as either unimodal (typical in control rats) or bimodal (common in PD rats) for each in vivo recording.

In bimodal distributions, it was identified for each experiment a cut-off frequency (threshold), typically centered around 100 Hz, which separated the two peaks in the histogram. By calculating the maximum values above and below this threshold, it was determined the frequency center of the two peaks for each rat. For unimodal distributions, the frequency peak corresponded to the value with the maximum counts in the histogram.

Burst and tonic events. For bimodal distributions, burst events were defined as sequences where interspike frequencies consecutively exceeded the cut-off frequency (denoted as IF_a). Each sequence of consecutive frequencies above this cut-off represented one burst count. The end of a burst was identified by the presence of at least one interspike frequency below the cut-off (denoted as IF_b), as slow-firing neurons do not contribute to bursts. The number of spikes per burst was calculated by

counting the number of $IF_a + 1$. Frequencies below the cut-off were considered tonic spike counts (tonic events).

Burst modulation. The evolution of the burst ratio (post/pre) over time (Figure 16d), was determined by first calculating the average burst rate during the post-DBS period for each 15-second interval. These values were then divided by the burst rate averaged in the total recording period pre-DBS, to obtain the post/pre ratio. The ratios were then averaged with STD across all rats. The modulation factor was calculated using the formula $F_{burst}^{mod} = \frac{Burst_{pre} - Burst_{post}}{Burst_{pre}}$, where $Burst_{pre}$ and $Burst_{post}$ represent the average burst rates over the total 100-second recording period pre and post-DBS. This factor was calculated only for channels that exhibited bursting activity.

Cross-Correlation. The cross-correlation between the reference electrode (number 1) in the STN and each one of the others, was calculated with the correlate function in both LFP and spike frequency range and then normalized. The resulting values at lag 0 were printed for each pair.

Local field potential analysis. The power spectral density (PSD) was computed using the Welch method. A minimum frequency threshold (FMin) of 0.5 Hz is set, and the specific number of points for the Fast Fourier Transform (nFFT) is determined based on the sampling rate and FMin. An overlap of 2 points between consecutive segments is applied to improve frequency resolution. For average PSD, the variability between different recordings is quantified by computing the standard deviation (shadow area) around the average PSD. This is achieved by first calculating the variance (squared differences from the average PSD divided by the number of recordings) and then taking its square root. To improve the visualization of the PSD, the frequency cuts in the plot produced by the notch filters are digitally removed

Total PSD power. The total power within different frequency bands of the PSD was quantified by integrating the PSD across each specified frequency band for each rat.

LFP-to-noise ratio. The SNR of the LFP signals was calculated by dividing the PSD of signals recorded in vivo over those recorded post-mortem for each working recording channel of the array. The average SNR across channels was then computed, along with its standard deviation, to assess the quality of the recorded signals.

Spectrogram. It was generated using a custom library (PhyREC) with default parameters, including a frequency range of 1-200 Hz and a resolution of 0.5 Hz and 0.5 s. The color map ‘jet’, was normalized in a range from 10^{-13} to 10^{-7} and it was performed a bilinear interpolation.

Fluorescence density analysis. To compare dopaminergic neuron density between sham and Parkinsonian samples, ImageJ was used to calculate striatal fluorescence intensity following TH staining. In coronal sections, striata were visually identified in each slice by morphological

characteristics, and a region of interest (ROI) was manually drawn around the structure in each hemisphere. Mean grey values (MGV) were calculated in each striatal ROI for each hemisphere. To account for variability in antibody staining and fluorescence intensity between samples, MGV from the right hemisphere (lesioned) was normalized to those from the left hemisphere (control). Normalized MGV values were then plotted and analyzed in Prism 9 (GraphPad software). A single coronal section between +1,5 and 0mm (anterior posterior relative to bregma) was used as a representative sample of dopaminergic neuron density in each animal.

Statistical Analysis. For each data set, it was first performed the Normality test to evaluate if the data followed a normal distribution ($P > 0.05$) or not ($P \leq 0.05$). In the first case, I quantified the statistical significance with the paired t-test for groups of the same size, while Welch's t-test for groups of different ones. In the second case, since the data are non-parametric (they don't follow a normal distribution), I performed the Mann-Whitney U Test. Significance levels were indicated as follows: NS (not significant, $P > 0.05$), * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$), **** ($P \leq 0.0001$). All the statistical tests are two-sided. Unless otherwise specified the bar plots are expressed with median line and STD whisker. All the tests were performed with Origin 2018.

Figure 3.7. b, **** $p = 10^{-5}$ in the Mann-Whitney U-Test. i, NS: $p = 0.14$ in the Mann-Whitney U-Test.

Figure 3.10. b, magnitude after 1 week NS $p = 0.62$, after 2 weeks NS $p = 0.98$, after 3 weeks NS $p = 0.19$, in the Welch T-test.

Figure 3.13. a, **** $p = 0.00004$ in the Welch T-test. b, ** $p = 0.0017$ in the Welch T-test. c, * $p = 0.012$ in the Welch T-test. f, * $p = 0.045$ in the Welch T-test. g, ** $p = 0.01$, tonic: NS $p = 0.73$ in the Welch T-test. k, Delta NS $p = 0.48$, theta NS $p = 0.59$, alpha NS $p = 0.68$, beta NS $p = 0.91$, low gamma NS $p = 0.43$, high gamma NS $p = 0.11$, in the Welch T-test for the power PD-control.

Figure 3.16. c, the statistics consider the post-pre values of each category in the X axis, burst *: $p = 0.015$, Tot spike NS: $p = 0.38$, Tonic NS: $p = 0.80$, Burst spike count NS: $p = 0.41$ in the paired T-test. d, delta NS $p = 0.23$, theta $p = 0.27$, alpha NS $p = 0.61$, beta NS $p = 0.87$, low gamma NS $p = 0.11$, high gamma NS $p = 0.62$, in the paired T-test.

CHAPTER 4

Microfabrication of TIME devices.

First-generation TIME device. For the fabrication protocol, refer to the description of Chapter 2 in this Annex.

Second-generation TIME device. The fabrication started by spin coating a 7.5 μm of PI (PI-2611, HD Microsystems) onto a Si/SiO₂ (5000 μm , 285 nm) wafer. The PI was then baked at 350°C in a nitrogen-rich atmosphere, and a 50 nm layer of Al₂O₃ was deposited at 200°C using plasma-assisted atomic layer deposition (ALD) (Picosun, R200 Advanced). Then, 20/200 nm thick Ti/Au metal tracks were defined with photolithography using an image reversal photoresist (AZ5214E, Merck) and e-beam evaporation (Leybold Univex 400) in a lift-off process. A second 50 nm layer of Al₂O₃ was deposited on top, and with photolithography using a positive photoresist (HiPR 6512, FujiFilm) and reactive ion etching (RIE), vias were opened at the electrode and pad positions. RIE was performed with a HF power of 50 W and ICP of 800 W for 9 minutes with 20 sccm of CHF₃ and 30 sccm of CF₄. A second 200 nm layer of gold was evaporated on top to contact the metals below the vias. A GO membrane was obtained by filtration of an aqueous solution of dispersed graphene oxide flakes (Global Graphene Group) and then transferred onto the previously prepared wafer. The membrane was partially reduced hydrothermally in an autoclave at 134° C for three hours. The electrode areas were defined by using an aluminium hard mask patterned using optical lithography (nLOF 2070, Microchemicals) and lift-off. Then, the GO membrane was etched using RIE with 2000 W ICP and 40 sccm of O₂ and 20 sccm of Ar. Both the aluminium on top of the electrode and the electrodes themselves were used as a mask to wet etch the gold from all the wafer except below the electrodes. Afterwards, a second layer (2.5 μm thick) of PI was spin-coated on top. A series of photolithography (AZ 10XT, Microchemicals) and RIE processes were used to open the pads, electrodes, and structure sides. RIE was performed using a HF power of 40 W, ICP power of 1200 W, with 60 sccm of O₂ and 50 sccm of Ar flow for 4.5 min to etch the top PI layers that opened the electrodes. For the pads and the structure, a second RIE process was applied to open the intermediate alumina using the conditions mentioned before. To open the device structure, an extra RIE process was performed to etch the bottom PI layer. Finally, the aluminium on the electrodes was chemically etched using an aqueous etching solution of orthophosphoric acid, nitric acid, and deionized water (74% v/v H₃PO₄, 2.5% v/v HNO₃, 2.5% H₂O).

Electrochemical characterization.

Set-up. The electrochemical characterization of the electrode was performed as previously reported⁶³; here below, I provide a brief summary. A potentiostat (Metrohm Autolab PGSTAT128N) was used in a three-electrode configuration. A platinum wire (Alfa Aesar, 45093), providing sufficient active area, was used as the counter-electrode, and an Ag/AgCl electrode (FlexRef, WPI) as the reference electrode. The TIME device was connected to a 10-contact Zero Insertion Force (ZIF) Molex connector (Mouser Electronics, 0,5 mm pitch), which was attached to a custom small printed circuit board (PCB) (WURTH ELEKTRONIK). A 20-channel header connector (DigiKey, 2.54 mm pitch) connected the PCB to the potentiostat. The device was immersed in a solution prepared by dissolving one PBS tablet (Sigma-Aldrich, P4417) in 200 ml of distilled water to reach the final concentration of 10 mM phosphate buffer, 137 mM NaCl, and 2.7 mM KCl at pH 7.4.

Electrochemical activation. Before in vitro electrochemical evaluation, the electrodes underwent an electrochemical activation process consisting of 70,000 biphasic rectangular current pulses (cathodic first with delay) of 1 ms pulse width, ramping from 5 to 40 μA at 100 Hz. Electrochemical characterization in PBS involved measuring impedance, cyclic voltammetry, and current pulses to evaluate functionality, ensuring no broken or electrically shorted electrodes.

Impedance spectroscopy. It was measured using the above described three-electrode setup by applying a 10 mV sinusoidal voltage excitation over a frequency range from 1 Hz to 100 kHz. Electrodes with impedance between 2 and 20 $\text{k}\Omega$ at 1 kHz were considered functional.

Chronopotentiometry. Biphasic current pulses were injected to individual electrodes while the interfacial potential was measured against the reference. For current pulses of 1 ms, the CIL is determined by looking at the cathodic and anodic voltage shifts. These values were determined for each current level by visually distinguishing the interfacial polarization across the electrode-PBS interface from the linear voltage produced by the resistive ohmic drop.⁴⁶ To determine the charge injection limit, interfacial polarization values were plotted against the current for both the cathodic and anodic parts of the pulse at increasing current levels. From the linear fit of these points, i extrapolated the current values at 0.8 V and -0.9 V, representing the maximum currents safely injected within the rGO water window.

Homogeneity factor (H). This parameter was calculated from the mean of the impedance magnitude at 1 kHz and the cathodic and anodic charge injection limits, for the electrodes of each array, and their standard deviation, by using the equation $H = 100 (1 - \text{STD}/\text{Mean})$

Raman spectroscopy. Raman scattering spectroscopy was performed at five evenly spaced regions ($10 \times 10 \mu\text{m}^2$, $\sim 1 \text{ point}/\mu\text{m}^2$) of three electrodes for each condition ('Initial', 'After ageing', 'After ageing + 1B pulses'). A WITec alpha300 R spectrometer was used for all the measurements, with a 600 gr/nm, and a laser excitation wavelength of 488 nm with a 50x objective over 10 s exposures and three accumulations per point. Each Raman map was acquired with ControlSIX software, analysed by fitting the bands of interest (D band $\sim 1350 \text{ cm}^{-1}$ and G band $\sim 1590 \text{ cm}^{-1}$) to 2 Lorentzian peaks, after performing a polynomial background subtraction, and averaged them for each condition. The amplitude intensity ratio between D and G bands was calculated to monitor changes in the defect density throughout the ageing process. In Figure 2K, the weighted mean is calculated as $\text{Mean} = \frac{\sum_{i=1}^{15} R_i w_i}{\sum_{i=1}^{15} w_i}$ with $w_i = \frac{1}{\sigma_i^2}$ and σ the STD.

In vitro tests

Charge building up. Biphasic current pulses (50 μs per phase, 100 μA , and 1 kHz) were applied through the first electrode of the array of one device in 10 mM PBS, using the Neuronexus stimulator (X-Series XDAQ Core Family neurostimulator and a XDAQ Smartbox Pro X3SR head stage which carries an

Intan RHS 2000 chip). The stimulation was performed with and without 1 ms discharging in between the pulses. From the DC recording of all the electrodes, one point was extracted between the pulses every 12 ms to plot the voltage baseline versus time. This baseline was smoothened from noise fluctuation with the Savitzky-Golay filter with a second-order polynomial fit in segments of 15 points.

Long-term stimulation. To evaluate the long-term stability of the electrodes, biphasic current pulses (50 μ s per phase, 100 μ A, 1 kHz) were delivered using the StimRG stimulator (Neurinnov, France). Stimulation was applied simultaneously through four rGO electrodes, which is the maximum number supported by the software. The setup used a multipolar push–pull configuration: the four rGO electrodes injected pulses in phase, while a platinum wire delivered pulses of the opposite phase. A second platinum wire was used as the counter electrode for the current return path. Charge-balancing, integrated in the stimulator, was applied between consecutive pulses to prevent charge accumulation at the electrode–tissue interface. Electrochemical stability was monitored by measuring impedance and chronopotentiometry with a potentiostat after every 500 million pulses.

Ageing test. Accelerated ageing test were performed to evaluate the lifetime of the hybrid PI-Al₂O₃ encapsulation. The devices were immersed in a beaker with 10 mM PBS, hermetically sealed with parafilm, and placed in a heating station at 57°C. By increasing the temperature, it is expected to accelerate the degradation of the polymers used in biomedical applications²⁷⁰, with an expected accelerated aging factor of 4, calculated using Arrhenius' law as reported in previous studies¹⁵⁶. Devices were removed from the beaker and characterized each month, with the standard setup.

Measurement of insertion and buckling forces. Dummy devices of only PI, of both standard (n=3) and improved (n=3) designs, were inserted in the sciatic nerve of rats, with the same procedure as the regular implantation in vivo (refer to in vivo surgery for more details). The nerve was explanted by cutting proximally and distally to the dummy devices. The nerve stumps were fixed with tape on a petri dish, and each device was clamped to the arm controlled by the micro driver (Zwick Roell Z 0.5). The extraction force was measured using a load force cell (Zwick Roell, nominal force 50 N) by pulling out the devices from the nerve at 100 μ m/s until complete extraction.

In vivo procedures.

Ethics. Animal experiments were conducted in accordance with protocols approved by the Ethical Committee of the Universitat Autònoma de Barcelona and in compliance with the European Communities Council Directive 2010/63/EU. Animals were housed at 22 ± 2 °C under a 12 h light/12 h dark cycle with food and water available *ad libitum*. Appropriate measures were implemented to minimize pain and discomfort during surgical procedures and throughout the postoperative period.

Implantation procedures. Surgical procedures were carried out under anaesthesia using ketamine/xylazine (90/10 mg/kg, i.p.) in female Sprague–Dawley rats (250–300 g, ~ 18 weeks old).

Prior to implantation, the TIME device was sterilized with ethanol 70% and carefully folded at the central point so that the two arms were perfectly overlapping. Implantation was performed using a straight needle attached to a 10–0 loop thread^{10,12} (STC-6, Ethicon), which guided the device transversally through the insertion across the tibial and peroneal branches of the sciatic nerve. The tibial fascicle innervates both the gastrocnemius (GM) and plantar interosseous (PL) muscles, whereas the peroneal fascicle innervates the tibialis anterior (TA) muscle. All manipulations of the nerve and devices were done using fine forceps under a dissection microscope to ensure precise positioning of the microelectrodes within the nerve fascicles; the ending clamp arrow was left just outside of the nerve to avoid possible pulling of the device. After completing the nerve stimulation and recording protocols, as detailed below, the ribbon of the device was routed through the muscle incision and the pad section positioned subcutaneously to allow easy access, and enclosed in a plastic envelope sealed with Kwik-Cast to protect it from damage and fibrotic encapsulation. The muscle layer was closed with silk sutures, and the skin incision with small surgical staples. In chronic experiments, the skin and the envelope were reopened, and the pad area was gently cleaned and reconnected to a ZIF connector to perform the required experiments. Throughout the procedure, the animal body temperature was maintained using a heating pad.

After completing the experiments, the devices were explanted. The arms of the device were carefully followed toward the nerve, gently removing any surrounding tissue. The nerve segment containing the implanted device was harvested and immersed in a collagenase solution to dissolve the tissue and release the electrode without applying additional mechanical force to the device.

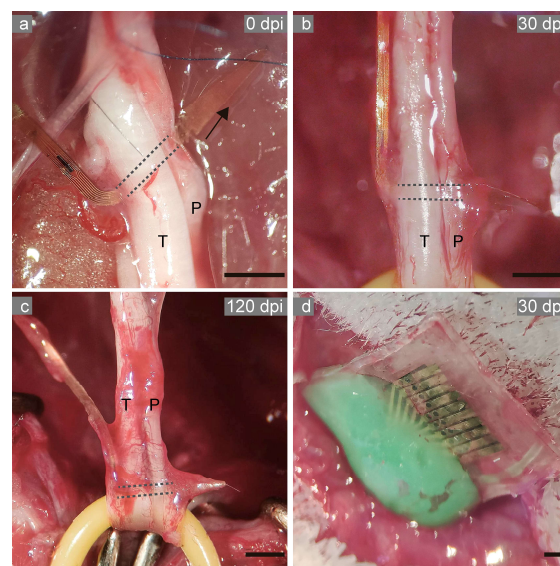


Figure A1.1 Implantation procedure | a–c, Representative images of the TIME device implanted transversally into the tibial (T) and peroneal (P) branches of the sciatic nerve. The tentative path of the TIME within the nerve is indicated by dashed lines. After implantation, fibrotic tissue covers the device,

as observed in panels b and c. **d**, A plastic envelope is used to protect the pad area of the TIME device, placed subcutaneously. Scale bar: 1 mm.

Impedance measurement in vivo. The device contact pad was connected (with the same connector and PCB used for the electrochemical characterization in vitro) to a PalmSens 4 potentiostat. The impedance was measured at each of the microelectrodes, using a stainless-steel needle electrode placed in the adjacent muscle as the counter electrode, and a reference electrode positioned at the base of the tail. The impedance was recorded across a frequency range from 50 kHz to 1 Hz in 47 steps.

StimRG stimulator. The stimulator was specifically developed to drive the new TIME electrode. The core technology is based on previous developments.^{271,272} The stimulator has 16 current-controlled outputs, among which at most 4 anodes and 4 cathodes are driven simultaneously to provide multipolar stimulation. The stimulation main features include: i) 4 balanced biphasic with a 100 μ s interphase waveforms (rectangular / ramp up, (a)symmetric), ii) 10-500 μ s pulse width with 2 μ s step, iii) 1-6000 μ A (sum on up to 4 simultaneous outputs), with a minimal step of 1 μ A. To ensure safe and reproducible stimulation, the stimulator operates at 20 V, capable of driving high-impedance loads with very precise current control and passive charge balancing, thanks to a high-precision ASIC design.

Stimulation procedure. First, the maximal muscle response was determined by delivering rectangular pulses (100 μ s duration, 1–10 mA) using a Grass S44 stimulator with a PSIU6 isolation unit and two small needles positioned near the sciatic nerve. The evoked CMAPs were recorded from PL, GM, and TA muscles using small stainless steel needle electrodes (13 mm length, 0.4 mm diameter; A-03-14BEP, Bionic). The active electrode was inserted in the muscle belly, while the reference electrode was placed near the distal tendon. Electromyographic signals were amplified (P511AC amplifiers, Grass), band-pass filtered (3 Hz–3 kHz), and digitized at 20 kHz using a PowerLab16SP system (ADInstruments).

Subsequently, nerve stimulation through each of the microelectrodes in the implanted TIME device was performed by applying two series of 52 biphasic current pulses (50 μ s per phase) at 3 Hz using the StimRG stimulator (Neurinnov, France). The current intensity was progressively increased from 0 μ A up to the threshold that evoked the maximal muscle response or to a maximum of 600 μ A, using each microelectrode as cathode, and the rectangular rGO electrode or a small needle in contact with the sciatic nerve as anode. A counter electrode was positioned at the base of the tail. The CMAP recordings were made as above.

Recording procedure. The CNAPs were recorded from each microelectrode, following electrical stimulation of three distal nerves in the hind paw: the medial plantar nerve (MPN), the lateral plantar nerve (LPN), and the dorsal peroneal nerve (DPN). In acute experiments, the reference electrode was the rectangular rGO electrode, whereas in chronic experiments, it was a small needle placed near the sciatic nerve. In both cases, the ground electrode was a small needle inserted into the skin at the mid-

thigh. Stimulation was delivered using 50 biphasic rectangular pulses (100 μ s duration, 0 to 10 mA; DS4 Stimulator, Digitimer) in bipolar configuration via two small needle electrodes inserted near each nerve, into the medial, lateral, or dorso-medial regions of the paw.

In a second protocol, evoked electroneurography (ENG) activity was recorded from the rGO microelectrodes implanted in the sciatic nerve in response to mechanical stimulation of the paw. The configuration of recording electrodes was the same as in the CNAPs recording. Brief scratches were applied with a thin probe, and pricks were delivered using a blunt 27-G needle. These stimuli were synchronized using a push-button trigger (ADInstruments) to facilitate analysis. The evoked CNAPs and ENG signals were amplified (P511AC, Grass), band-pass filtered (300 Hz–10 kHz), processed through a noise eliminator (HumBug, Quest Scientific), digitized at 20 kHz, and recorded using LabChart software (PowerLab System, ADInstruments).

In vivo data analysis

Analysis for stimulation experiments. The CMAP recordings were exported and analysed using custom MATLAB scripts (MathWorks, R2023b). The CMAP waves were identified within predefined 10.3 ms time windows, corresponding to each stimulation event. Since stimulation was delivered twice for each microelectrode, the peak-to-peak CMAP amplitude of the two repeated trials was measured and averaged to enhance reliability. These values were normalized as a percentage of the maximal CMAP of each muscle and animal, and used to generate recruitment curves, illustrating the relationship between stimulation intensity and neuromuscular activation. To assess the selectivity of stimulation, the selectivity index was calculated for each electrode configuration²⁷³. This index is computed as the ratio of the normalized response of an individual muscle to the sum of responses across all monitored muscles. Recruitment threshold and selectivity were evaluated at three relative activation levels: 5%, 30%, and 95% of the maximum observed CMAP amplitude. These levels were chosen to represent distinct functional activation scenarios. A 5% threshold represents the minimal activation of the muscle, considered to avoid potential crosstalk effect; the 30% threshold corresponds to typical muscle activity needed for everyday tasks (Paternostro-Sluga 2008), and the 95% threshold reflects near-maximal muscle activation.

Analysis for recording experiments. Electrophysiological signals recorded in vivo were processed using Python packages (Numpy 1.21.5, Neo 0.11.1, pandas, seaborn 0.11.2, Matplotlib 3.5.1, Quantities 0.13.0, Elephant 0.10.0), and the custom library PhyREC 0.6.5. Origin 2018 was used for scatter plots, box and bar charts and statistical analysis.

The recorded signal was first cleaned from voltage artefacts produced by the stimulation pulses. A voltage threshold of 0.4 V was implemented in the trigger channel to detect the time point of the artefact events. Then the signal in the real recording channel was set to 0 in a time window (from 1.1 to 2.1 ms) around the detection, depending on the specific experiment and current level. A refractory period of 15

ms was employed to ensure that the same event was not detected twice. A second threshold was employed to detect the time point of the actual CNAP, and the peak-to-peak amplitude was measured. In the ENG recording, the Root Mean Square (RMS) was calculated in 1 second recording pre-filtered for noise-cleaning (50 Hz and harmonics), in a frequency range of 200-2000 Hz, and then computing the RMS of the squared signal values. The SNR was calculated as the ratio between the maximum RMS detected by each microelectrode during each mechanical stimulus, and the respective RMS of 1 1-second recording baseline before the stimulation.

Statistics. For each data set, a normality test was first performed to evaluate if the data followed a normal distribution ($P > 0.05$) or not ($P \leq 0.05$). In the first case, I quantified the statistical significance with the paired t-test for groups of the same size, and Welch's t-test for groups of different size. For data not following a normal distribution, I used the Mann-Whitney U Test. Significance levels were indicated as follows: NS (not significant, $P > 0.05$), * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$), **** ($P \leq 0.0001$). All the statistical tests were two-sided. The bar and box plots are expressed with the median line and STD whiskers. All the tests were performed with Origin 2018 software.

Figure 4.13d, NS $p = 0.085$ start-aging; NS $p = 0.34$ ageing-ageing and stimulation, in the paired T-test,

Figure 4.20f, NS $p = 0.081$ in the Mann-Whitney U-Test. i, NS: $p = 0.14$ in the Mann-Whitney U-Test.

ANNEX 2. PATENT FILINGS

Patent filing 1. Electrode device with hybrid encapsulation and method for encapsulating an electrode device.

The technology presented in Chapter 4, consisting in the new hybrid encapsulation polyimide-alumina, was patented. This patent displays the integration of alumina in the rGO technology to improve the long-term stability of the leads for chronic applications.

All the information are available online under the patent number 4520251052 · Filed Nov 7, 2025

Patent filing 2. Artefact-reduction method for recording electrophysiological signals during electrical stimulation in close proximity sites.

The present invention relates to bio tissue stimulation systems, and more particularly, to a method for sensing information during an electrical stimulation procedure.

Sensing of electrophysiological activity during electrical stimulation is key for the development of true closed-loop systems and therapeutic interventions. This is, however, extremely challenging as stimulation-related artefacts generally interfere with the recording systems, deteriorating or completely blocking the recording of the tissue signals.

Several methods are used in the literature to minimize the interference caused by the stimulation artefacts. Some of the most commonly used methods are related to modifying the recording amplifier during stimulation, for example, by disconnecting it from the sensing element (also called blanking), or changing its bandwidth with the lower cut-off frequency of the low-pass filters. However, blanking does not allow recording during stimulation²⁷⁴ and performs poorly, with long recovery times (tens of ms) after the stimulation pulse²⁷⁵, while bandwidth changes limit the available sensed information.

Here, this method provides a solution for the recording of neural signals that does not involve blanking nor bandwidth modification and that results in a fast sub-millisecond recovery time after the stimulation pulse. The method cannot be presented since it is not filed yet, but I show the results obtained.

In vitro validation

The method was validated in vitro, in a beaker with 1 mM phosphate solution, to simulate the conductivity of the brain. An artificial signal of 10 Hz, that simulates brain activity, is injected with a signal generator through the reference inside the PBS to evaluate the capability of the electrodes to record it. Ground, reference, and our device with electrodes are connected to a standard potentiostat to perform recording and stimulation.

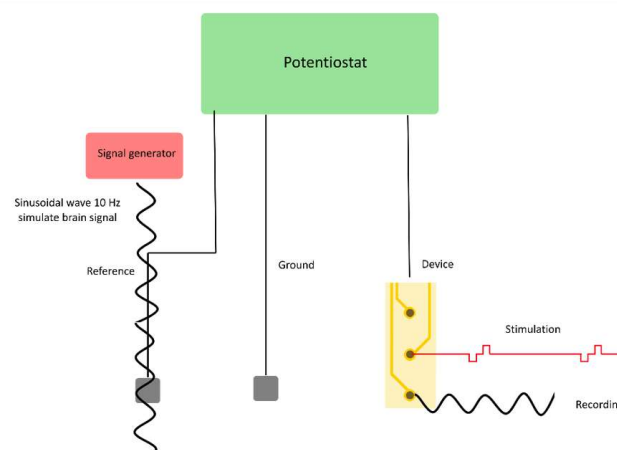


Figure A2. 1 | Set-up. Set-up used in vitro to validate the method of recording during electrical stimulation.

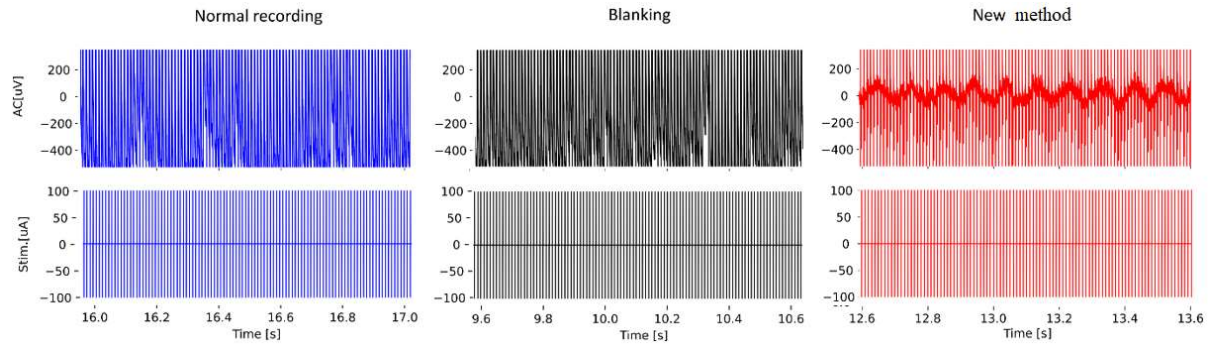


Figure A2.2 | Recording during stimulation in vitro. In blue the normal recording settings; in black, with the blanking and in red, with the new method; all measurements in PBS. The first row represents the recording in AC in the recording channel, and the second the stimulation pulses injected in the stimulating electrode. It is possible to visually record the artificial wave just with the third method.

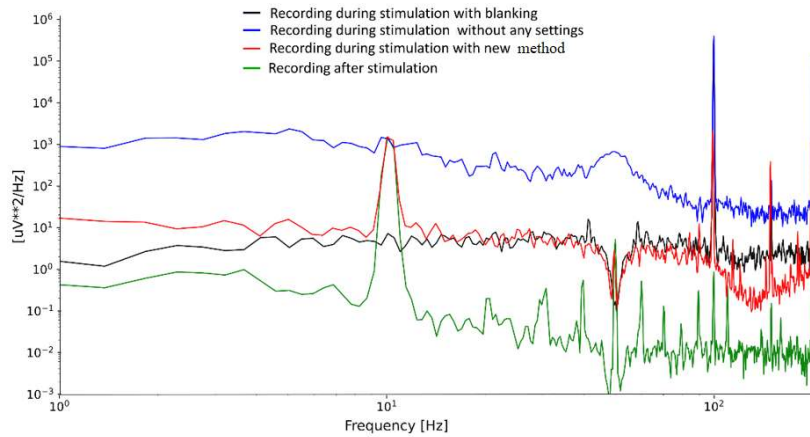


Figure A2.3 | Power spectral density. In blue, the normal recording settings; in black, with the blanking, and in red with the new method all in the stimulating period. In green the recording when any current is injected. It is possible to see a peak at 10 Hz in the recording in green and just in the recording during stimulation with the new method in red.

The recording confirms that the externally added signal can be recorded just with our proposed method (Figure A2.2). In Fig, we show that the 10 Hz peak of the artificial signal (10 Hz) in the power spectral density is visible in the period without stimulation, and during stimulation using this method (Figure A2.3). Importantly, the added noise of this method is below a few microvolts, so even if the stimulation current is high, it is possible to record microvolt-scale signals with a good signal-to-noise ratio.

In vivo validation during an experiment with a rat of the proposed solution.

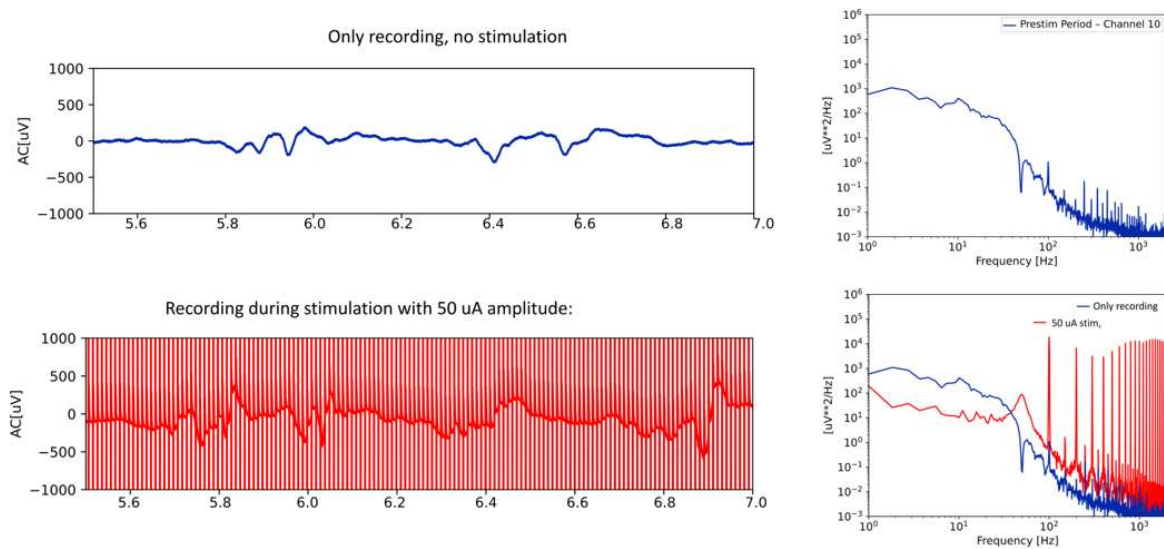


Figure A2.4 | Recording during stimulation in the brain. On the left, recording trace of the brain activity of a rat with a 300 μm diameter electrode at approximately 1.5 mm from a 300 μm diameter stimulating electrode, in the period without stimulation in blue, and during stimulation in red, with the new method. On the right, the corresponding PSD over 7 seconds of the recording with stimulation (red), compared with the PSD without (blue).

The in vivo recording confirms that during stimulation, it is possible to see the recording line of the brain activity. This line is comparable to the one in the non-stimulating period, meaning that it is not noise but biological activity. This method will be filed in the next few weeks.

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