

Action potential alterations induced by single neuron mechanical loading



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To my brothers.

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Abstract

Since the 1940s, an extensive body of empirical evidence has been gathered about non-electrical alterations accompanying the voltage depolarisation of a neuron during the generation and propagation of an action potential (AP). The prevailing traditional electrochemical models do not account for these changes, and are as a result incomplete. At the same time, multiple research programmes have demonstrated how ultrasound (US) –a mechanical wave– can non-invasively and reversibly mechanically perturb neuronal functions. Strikingly, the mechanisms of action remain largely unknown.

In this framework, this thesis takes the approach to focus on single cell studies to better understand and exploit the multiphysics of the neuronal action potential and aims at studying the effects of direct mechanical loadings on the shape and dynamics of the neuronal signal. First, a multiphysics platform combining nanoindentation and patch clamp systems, assembled in an inverted microscope, is designed to simultaneously measure mechanical and electrophysiological properties of single neurons while imaging the cell morphology. The functionality of the setup is first used to provide a full characterisation of the neuronal cell line chosen here, i.e., dorsal root ganglion-derived cells, and then to quantify the alterations in the magnitude and dynamics of single neuron spontaneous APs before, during and after quasi-static and dynamic mechanical loadings over a wide range of frequencies (up to 1 MHz).

Quasi-static indentation tests show transiently smaller, wider and slower depolarising APs during compressions within the cell's linear viscoelastic regime, which translates in slower AP dynamics, as well as sustained hyperpolarised cells with longer repolarisation rates after the indentation. Dynamic loading results, instead, show narrower APs with shorter depolarisation and repolarisation phases, i.e., faster induced AP dynamics, at increasing frequencies, particularly for stimulations at around 1 MHz. In addition, results at this frequency are more evident after indentation, hinting at a cumulative or lagged effect of mechanical stimulation at US frequencies. These observations are attributed to the stiffening of the membrane and membrane cortex as a result of the mechanical oscillations, as well as potentially related phase state alterations. Taken together, these findings highlight the importance of mechanical cues in shaping the neuronal AP, and the often overlooked role of the membrane and its mechanical properties in determining cell functionality.

In particular, the promoted faster AP dynamics that emerge after high frequency mechanical oscillations suggest a potential mechanism of US neuromodulation and support the assumption that direct mechanical cellular agitations induced by US stimulation protocols assist the observed neuromodulatory effects.

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List of Abbreviations

AFM		Atomic Force Microscope
ANOVA		ANalysis Of VAriance
AP		Action Potential
ATP		Adenosine TriPhosphate
Ca²⁺		Calcium ion
DRG		Dorsal Root Ganglion
FBS		Fetal Bovine Serum
FE		Finite Element
FFT		Fast Fourier Transform
FITC		Fluorescein Isothiocyanate
FP		Fabry-Perot
HEPES		4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF		High Frequency
HH		Hodgkin-Huxley
HIFU		High Intensity Focused Ultrasound
IC		Current Clamp
iPSC		induced Pluripotent Stem Cell
K⁺		Potassium ion
K_V		Voltage gated potassium channels
LIFU		Low Intensity Focused Ultrasound
LF		Low Frequency
LJP		Liquid Junction Potential
LVR		Linear Viscoelastic Regime
MS		MechanoSensitive
MSC		MechanoSensitive ion Channel

Na⁺		Sodium ion
Nav		Voltage gated sodium channel
NoR		Node of Ranvier
NPC		Nuclear Pore Complexes
OGB1		Oregon Green BAPTA 1
PBS		Phosphate Buffered Saline
P/S		Penicilin/Streptomycin
SD		Standard Deviation
SE		Standard Error
TTX		Tetrodotoxin
US		UltraSound
VC		Voltage Clamp

Chapter 1

Introduction and motivation

Since it was first proposed in 1952, the Hodgkin-Huxley model, that describes the evolution of the action potential (AP) in the squid giant axon membrane [1], has been one of the theoretical pillars of modern neuroscience. However, because of its phenomenological nature, while the model mathematically captures the cell membrane voltage evolution during an AP based on ion channel kinetics, it does not participate in explaining the underlying mechanistic details.

In parallel to the work of Hodgkin and Huxley, other efforts took a different approach and probed the mechanical behaviour of axons during AP propagation. In 1947 and 1955, both slow [2] and rapid [3] mechanical changes were initially observed to accompany AP propagation. While the former was attributed to long term reorganisation of cytoskeletal components, the latter pointed towards a mechanical vibration coupled to and in phase with the AP. Since then, and partly due to the development of new experimental techniques that allowed high resolution single cell measurements in physiological environments [4], a body of empirical observations of mechanical changes propagating through the neuronal membrane along with the AP have been gathered with various techniques [5–17], see Section 2.3.1. These results point towards the hypothesis that the AP is not merely an electrochemical pulse, but a more complex phenomenon that involves different physics and all components of the membrane.

Theoretical models have been proposed to explain these experimental results [14, 15, 18–20], all of which acknowledging the mechanical aspect of the neuronal signal and including the mechanical properties of the membrane in their theories, to completely describe the multiphysical nature of the neuronal AP.

In recent years, the role of mechanics in the neuronal spike has attracted significant attention for its potential in medical applications, such as ultrasound (US) neuromodulation. US are mechanical waves of frequencies above human hearing detection levels (>20 kHz) and have historically been applied in medical imaging, where it has been proven to be an exceptionally safe technique. In the context of neuromodulation, US can be used as a brain stimulation technique, as it can be non-invasively focused through the skull into virtually any brain region. This option presents many advantages over the existing either invasive Deep Brain Stimulation or Optogenetics [21], or superficial Transcranial Direct Current or Magnetic Stimulation approaches [22]. Among others, US neuromodulation allows for multimodal compatibility, submillimeter steerable focusing, multifocal, high temporal resolution, coregistration, and the ability to monitor delivered therapy and temperatures in real time [23, 24]. US brain stimulation has been proposed early on to treat essential tremor [25], and is now showing promise in the treatment of epilepsy and chronic neuropathic pain, in the promotion of neuroplastic repair mechanisms for post-stroke recovery, and even to enhance the delivery of drugs across the blood-brain barrier [26–30].

At the cellular scale, US has been shown to be effective in the modulation (by enhancing [31] or inhibiting [32]) of electrical activity since the 1950s [33]. High intensity focused US (HIFU) was first shown to suppress neuronal activity and the alterations were generally permanent [34], but it was later discovered that transient modulatory effects could also be achieved if lower intensities (low intensity focused US, LIFU) were used [28]. The latter allowed tuning brain cells signalling in a more physiological, controlled and reversible manner. In HIFU, acoustic absorption is the dominant phenomenon, and the response is mainly thermal [35], typically leading to tissue ablation, with applications in cancer tumour [34], essential tremor

[25, 36] or chronic pain [37] treatments. If, instead, low intensities and frequencies are employed, usually delivered in a pulsed fashion, transient temperature rises are prevented [38–40] and mechanical effects dominate [28, 29, 41–43]. The US-induced mechano-physiological interactions are highly dependent on the stimulation parameters and on the tissue under study [44]. Some of the main experimental observations of US neuromodulation on experimental models are summarised in Table 1.1, classified according to the effects observed.

US-induced effect	Experimental observations under US
Direct effects on neuronal activity	Enhancement [45–49] and decrease/inhibition [32, 50–53] of electrical activity
Motor and sensory responses	Motor cortex activation and increased tail or limb movements [31, 52, 54–59], as well as sensory responses [50, 60, 61]
General anaesthesia and conscious state	Decreased recovery from general anaesthesia [57], as well as early awakenings from these effects [62]. Awakening of a patient from a minimally conscious state [63]
Synaptic transmission and neurotransmitter release	Increased synaptic vesicle exocytosis [64], glucose uptake [65] and extracellular concentration of dopamine and serotonin, as well as decreased GABA [53, 66]
Plastic effects outlasting US stimulation	Suppressed somatosensory evoked potentials up to 10 min after stimulation [67], and altered brain activity up to 2 h after the stimulation [68, 69]

Table 1.1: Summary of the main US neuromodulation experimental observations, classified by the induced effect.

Because of the differences in the experimental setups, stimulation parameters and experimental models studied [44, 70], no consensus has been reached on the optimal US parameters leading to a successful neuromodulation, and the mechanisms by which US interacts with neurons and influences their electrical response remain largely unknown. Different theories of US modulation of neuronal activity have however been proposed and are briefly presented in Table 1.2.

US action mechanism hypothesis	Details and references
Membrane conformation and passive properties alteration	US-induced pressure waves locally modify the viscoelastic properties of neuronal membranes [71] and perturb lateral phospholipid distributions [48]. As a result, alterations in membrane conformational state and phase transitions [72–74] (see Figure 1.1-a), membrane fluidity and permeability [75], membrane area and capacitance [76–79] and changes in membrane resistance [48, 71, 79, 80], which resulted in faster membrane time constants [79], have been observed.
Thermodynamic waves	Induced pressure waves might elicit a mechanical wave and a coupled AP [19], interact with membrane density waves that accompany changes in membrane potential (see Section 2.3.2 and Figure 1.1-b), or interfere with the mechanical waves coupled to spontaneous APs (see Section 2.3.1) either facilitating or inhibiting them.
Direct flexoelectricity	The US wave can induce membrane curvature changes due to membrane bending, which create polarisation currents through the direct flexoelectric effect that flow across it [81] (see Section 2.3.2) and modify the transmembrane electrostatic potential. A strong enough strain gradient can trigger an AP [18]. Simulations of high-frequency oscillations induced APs even at weak displacement amplitudes through the direct flexoelectric effect [82], see Figure 1.1-c.
Acoustic radiation force	The transfer of momentum from US to the tissue creates a steady, time-averaged force known as acoustic radiation force [83]. The pressure step change caused by this force disrupts the balance between bilayer tension and hydrostatic pressure [77], as well as reversibly deforms cultured cells [46]. The induced membrane deformations result in capacitance changes that can excite APs [78, 84, 85]. This force was also the proposed mechanism for behavioural responses in <i>C-elegans</i> nematodes [86].
Ion channel opening	Not only primarily mechanosensitive (MS) ion channels [38], but also voltage-sensitive potassium [87–89], sodium [64, 90] and calcium [90, 91] ion channels respond to US stimulation, see Figure 1.1-d. Recently, cumulative effects leading to cell depolarisation have been observed [92, 93], and the involvement of calcium channels has also been suggested [93].
Thermal modulation	LIFU protocols are not likely to lead to temperature rises of more than a fraction of a degree [59, 94], too small to cause neuromodulation on its own [64, 95–97]. However, with higher intensities and sustained stimulation, a reversible thermal rise at the sonication site of one or a couple of degrees could transiently alter neuronal activity [25, 98, 99], see Figure 1.1-e.
Sonoporation through cavitation	Acoustic stimulation can lead to sonoporation, i.e., creation of physical pores in the membrane. These pores are lipid constructs that allow transport of ions throughout (see Section 2.3.2). Cavitation is the main hypothesised mechanism driving sonoporation, and occurs when the intensity of the US is sufficient to induce resonance, expansion (stable cavitation) and potential collapse (inertial cavitation) of gas bodies [83, 100], see Figure 1.1-f. These effects are prominent at high-intensity protocols, but are unlikely in LIFU [70]. The resulting mechanical oscillations might induce changes in the local curvature of the membrane, which could modulate membrane potentials [101] and interfere with mechanical waves coupled to APs (see Section 2.3.2).
Microtubule resonance	Hameroff suggested that the MHz frequencies employed in focused US stimulation protocols were in the range of intra-neuronal microtubuli resonance frequencies, and made them vibrate synchronously, in turn modulating the electrical signal [102], see Figure 1.1-g. Microtubules are the major component of the cell cytoskeleton and have an important role in regulating synapses, as they are connected to actin filaments, present in dendritic spines. Their oscillation could thus be coupled to the membrane's, as well as influence synaptic plasticity. Other authors also found cytoskeleton fluidisation and accelerated remodelling dynamics under LIFU [103].
Synaptic vesicle modulation	US-induced disruption of chemical synapses was suggested as a possible candidate for the immediate functional changes observed following US application [101], see Figure 1.1-h. Such a process involves increased neurotransmitter release [64], wider synaptic cleft and altered sizes of pre- and post-synaptic densities [104].
Auditory pathways activation	Recent studies suggested that US-induced brain activation can occur through the cochlear pathway [105, 106]. In these protocols, where the US was modulated at frequencies well within the audible range of the experimental animal, US elicited motor responses that were consistent with a startle reflex [105] and removal of cochlear fluids eliminated US-induced brain activation [106]. In a study with human participants, they confirmed hearing sound during the stimulation trials, although these effects could be masked [107]. In a different study, however, deaf mice showed identical motor responses as normal hearing mice [108].

Table 1.2: State of the art of the proposed mechanisms of action for US neuromodulation.

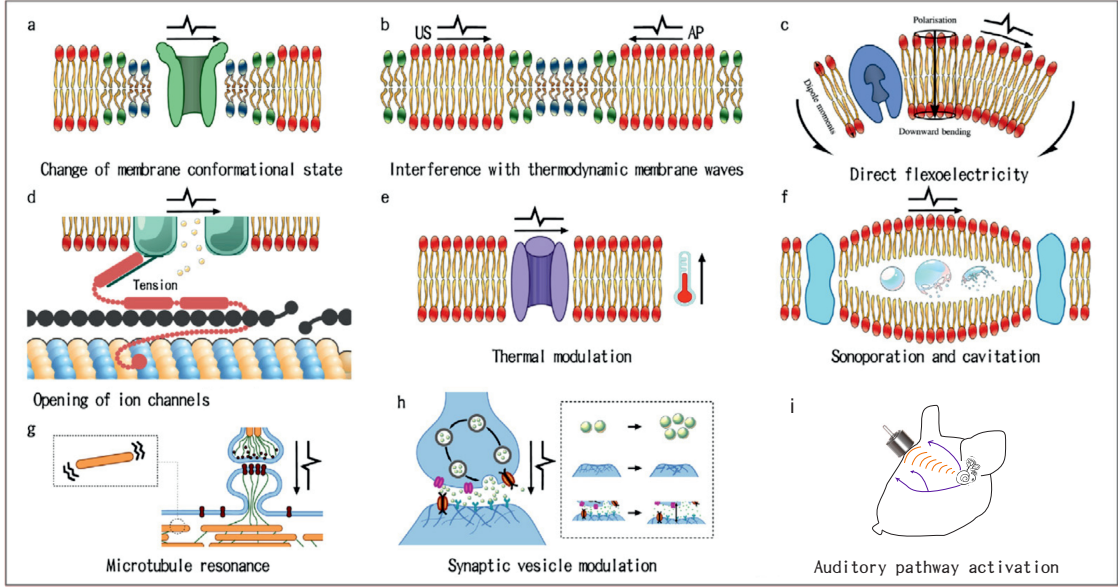


Figure 1.1: Hypotheses of US neuromodulation. Adapted from Ref. [109] with permission.

The wide variety of US neuromodulation results and proposed theories presented in Tables 1.1 and 1.2, sometimes even contradictory, reveals the lack of consensus on (1) the optimum stimulation parameters, (2) the achieved neuromodulatory results, and (3) the mechanisms of action at play.

To this end, a thorough understanding of the multiphysics of the neuron is needed to establish the link between US and the subsequent electrophysiological alterations at the cell level. In particular, single cell studies that examine the effect of specific mechanical vibrations (such as those induced by US) on the neuronal signal could help identify the relevant frequencies and amplitudes needed for a more universal US neuromodulation paradigm, as well as shed light on the action mechanisms behind this phenomenon.

This thesis thus aims at studying the effects of direct mechanical loadings (both static and vibrations, including US frequencies) on the generation and evolution of spontaneous APs at the single neuron level. Although different components of the cytoskeleton [102, 103] have been suggested as potential vectors for neuromodulation, most models point towards the polarised neuronal membrane leading the effects. In the following, the relevant literature on the multiphysics of the neuronal membrane is

first reviewed in Chapter 2. A multiphysics setup combining two discrete off-the-shelf nanoindentation and patch clamp systems, assembled in an inverted microscope, is then presented in Chapter 3. This setup facilitates the study of the mechano-electrical coupling at the single neuron level, where many open questions remain. Exploiting both individual techniques independently, a dorsal root ganglion-derived cell line is characterised both mechanically and electrophysiologically in Chapter 4. Alterations in the magnitude and dynamics of spontaneous APs before, during, and after a quasi-static mechanical load within the cell's linear viscoelastic regime, and dynamic mechanical indentations over US frequency ranges (up to 1 MHz) are examined in Chapters 5 and 6, respectively, and the underlying potential mechanisms are discussed. Conclusions, limitations of the study and future perspectives are finally given in Chapter 7.

Chapter 2

Literature review

Traditionally, neurons have been regarded as biological systems able to communicate with each other through electrochemical signals (see Section 2.1). In particular, the non-electrical properties of neurons have not typically been seen to influence their functionality (see Section 2.2), until an extensive body of evidence gathered over the last decades and reviewed in Section 2.3 showed that other physical phenomena were at play along with the electrical depolarisation, pointing towards the need to consider the neuron as a multiphysical system [109]. For a complete understanding of the biophysics of the neuronal signal, as well as to exploit the potential applications these insights entail, other physics thus need to be taken into consideration. This thesis focuses on the mechanical aspect of neurons and their signalling, and in particular in the role of the membrane as a mechano-electrical transducer.

In the following, the different physical properties of the neuronal membrane within the axon and neurites are reviewed. Although the membrane has further functional roles in growth cones [110] and at synapses [111], these are not the focus of this thesis. A lot of the materials presented below were published in Ref. [109].

2.1 The traditional picture: Electrophysiology of the neuronal membrane

The anatomy of a neuron consists of a cell body or soma enclosing the nucleus and multiple organelles, numerous branch-like extensions called dendrites, and a long tubular structure termed axon (see Figure 2.1). Neurons are functionally polarised [112]: dendrites read information from the microenvironment, which is gathered and processed through the soma, and then conveyed by the axon towards neighbouring neurons. The axon terminal of a cell forms synapses (nanometric gaps through which chemical vesicles are released) with other neurons' dendrites, thus creating networks, see Figure 2.1. When these vesicles, or neurotransmitters, reach the dendrites, they cause small, local and transient depolarisations in the membrane, that propagate towards the soma.

The cell membrane of a neuron separates the intracellular and extracellular media, which contain different concentrations of ions. Two of the most important ions involved in neuronal activity are sodium (Na^+) and potassium (K^+). When a neuron is at rest, there is a higher concentration of Na^+ outside the cell than inside, while the concentration of K^+ is larger in the intracellular fluid than in the extracellular one. This imbalance causes ions to move across the membrane. At the same time, the unequal distribution of charged particles across the cell results in a potential difference across the plasma membrane, where the value inside the cell V_{in} , is negative, typically around -60 mV [113] with respect to the one outside V_{out} . Under resting conditions, the chemical gradient (ion concentrations) balances the electrical gradient (ion charges), and the neuron is at its resting membrane potential, or V_m , defined as the difference of electrical potential between the inside of the cell and the outside: $V_m = V_{in} - V_{out}$. The value of V_m is driven by the membrane electrical properties, including the permeability of the membrane to the different ions, i.e., ion conductances [114].

The cell membrane is mainly composed of hydrophobic fatty acids that make it inherently impermeable to hydrated ions, and thus maintain the ionic gradient across the bilayer. However, there are specific proteins embedded in the membrane

that have hydrophilic cores, including ion channels acting as gates that, when open, allow the flow of ions across the membrane (see Figure 2.1).

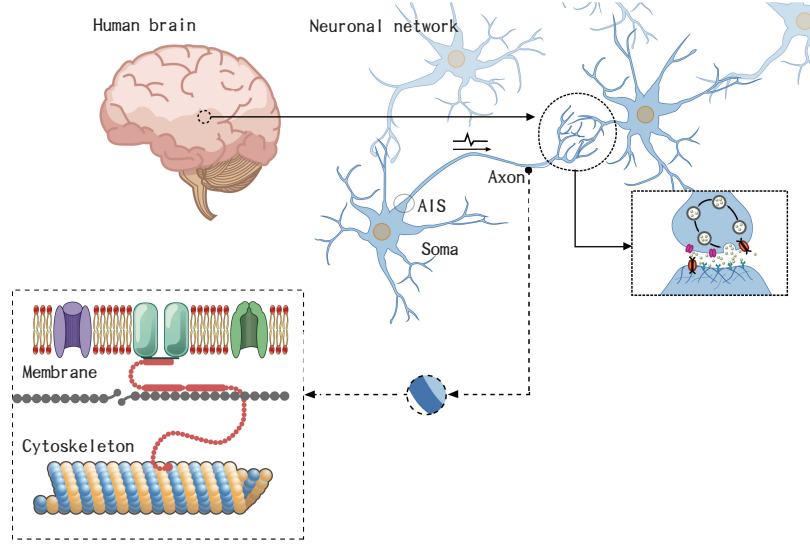


Figure 2.1: Neurons are the brain’s information processing units [112] that make connections through synapses and are organised into complex dynamic networks to accomplish their various functions. The neuronal signal is initiated at the axon initial segment (AIS) and propagates through the membrane of the axon, made of phospholipids, embedded ion channels, and linked to the cytoskeleton. Adapted from Ref. [109] with permission.

There are different channels with specific opening mechanisms, such as voltage-gated, ligand-gated or MS. There is, nevertheless, controversy about their specificity: voltage-gated potassium [115–118] and sodium [90, 119, 120] channel gating kinetics are known to be affected by membrane tension and composition, as well as purely MS Piezo channels have been shown to be modulated by voltage [121]. Regardless of the main mechanism, when these channels open, ions move down their concentration gradient, hence Na^+ move into the cell, while K^+ flow outward. The specific ion channels dynamics result in the neuronal membrane showing different permeabilities to different ions, with the resting membrane voltage being dominated by the ionic species that has the greatest conductance across the membrane, which, for most cells, is K^+ [113]. Membrane permeability for K^+ is much greater than that of other ions due to the presence of leaky K^+ channels [122], that make K^+ diffuse relatively freely out of the cell. If the total depolarisation is large enough,

an AP is triggered and propagated through the membrane (see Figure 2.2). If instead, a small cell depolarisation occurs, the outward K^+ current overwhelms the inward Na^+ current and the cell quickly repolarises back to its normal resting potential (see arrow in Figure 2.3).

2.1.1 The action potential

The electrical manifestation of an AP consists of a millisecond-lasting V_m change, caused by the rapid exchange of ions across the cell membrane, mainly Na^+ and K^+ for most cells.

The AP is generated at the AIS [123], adjacent to the axon hillock, where axon and soma meet and where depolarising signals coming from the numerous dendrites are gathered before being transmitted to the axon. The AIS membrane-cortex –as well as that of nodes of Ranvier (NoR) in myelinated axons– is rich in ankyrin G [124, 125], a protein that binds and immobilises membrane proteins to the cytoskeleton [126]. As a result, the AIS has a high density of voltage-gated ion channels [127–129].

If the net depolarisation signal propagated from the synaptic inputs through the dendrites to the AIS, is large enough, voltage-gated Na^+ channels begin to open, the inward Na^+ current increases more than the outward K^+ one, and a positive feedback is triggered: the more inward Na^+ current there is, the more the V_m depolarises, which in turn further opens Na^+ channels and increases the inward Na^+ current. If V_m reaches a threshold voltage V_{th} , ~ -45 mV for F11 cells, see Figure 2.2, all the Na^+ channels open and Na^+ rush into the cell and cause a sharp rise of V_m , triggering the AP. If this V_{th} is not reached, the resting membrane potential V_{res} is recovered (see arrow in Figure 2.3), meaning that the AP is an all-or-none event. As V_m reaches its peak voltage AP_{max} , Na^+ channels begin to inactivate and voltage-gated K^+ channels begin to open. The repolarisation phase starts and the K^+ efflux brings V_m back towards V_{res} . Slow K^+ channels dynamics cause a hyperpolarisation of V_m ($V_m < V_{res}$). While the cell is in this hyperpolarised state, with Na^+ channels in their inactivated state, the cell is unable to fire an AP: the so called neuron’s refractory period. Original ionic concentrations are restored

by the Na^+/K^+ pump, which pumps Na^+ out and K^+ in, against their gradients, and drives V_m back towards V_{res} . The resulting AP is shown in Figures 2.2 and 2.3.

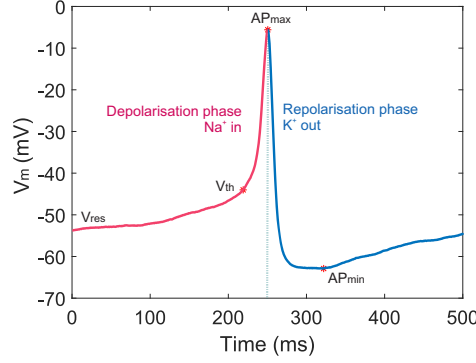


Figure 2.2: AP of a F11 cell. When a depolarising input reaches a cell at V_{res} , V_m depolarises as Na^+ enter the cell. If it reaches V_{th} , the AP is triggered, Na^+ rush into the cell until AP_{max} , when K^+ channels open. K^+ goes out the cell and the cell depolarises back to V_{res} . As K^+ channels take more time to close, the cell is hyperpolarised ($V_m < V_{res}$) for the so called refractory period.

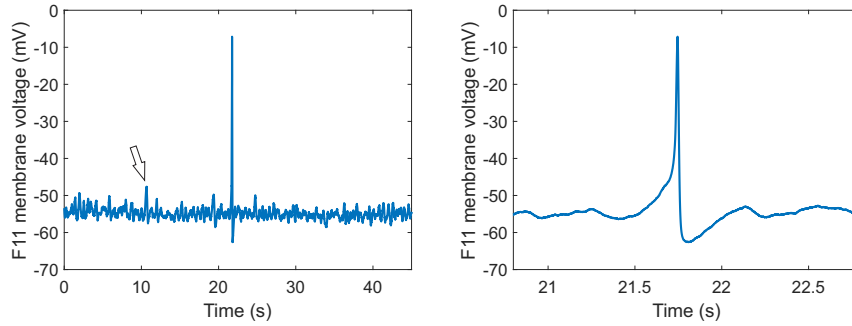


Figure 2.3: Intracellular recording of an spontaneous AP of a F11 neuronal cell, zoomed in on the right. The arrow on the left points to a sub-threshold membrane depolarisation that does not generate an AP.

2.1.2 Equivalent electronic circuit

The electrical nature of neurons can be illustrated by the properties of their equivalent electronic circuit. Understanding this is essential for a correct interpretation of patch clamp recorded results.

The mammalian cell membrane, consisting of a lipid bilayer composed of amphiphilic lipid molecules arranged in a 2D assembly, acts as an insulator that separates intracellular and extracellular conductive solutions. This sequence

conductor-insulator-conductor defines a capacitor. As such, the two leaflets of the lipid bilayer can be regarded as a parallel-plate capacitor (see Figure 2.4), whose capacitance value is:

$$C_m = \frac{\epsilon A}{d} \quad (2.1)$$

with ϵ being the dielectric permittivity of the insulating phospholipids, A the cell's surface area and d the distance between the monolayers, or membrane thickness. The specific capacitance of biological membranes (C_m/A) is considered to be $1 \mu F/cm^2$ [114].

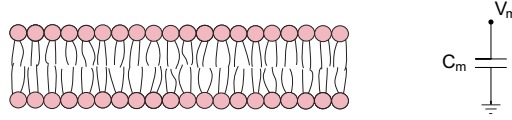


Figure 2.4: Bilayer lipidic membrane as a parallel plate capacitor. The inner part of the membrane, composed of carbohydrate tails, is hydrophobic, while the outer part, composed of charged phosphate heads, is hydrophilic.

The capacitance relates the stored charge Q to the potential difference V_m :

$$Q = C_m V_m \quad (2.2)$$

When V_m varies, charges rearrange within the dielectric, creating a capacitive current I_C , whose expression is obtained by deriving both sides of the equation with respect to time and assuming that C_m is constant [1]:

$$I_C = \frac{dQ}{dt} = C_m \frac{dV_m}{dt} \quad (2.3)$$

Current flow within a capacitor is then proportional to the rate of change of voltage. In other words, voltage across a capacitor only changes when there is current through it. With no current flow, the capacitor acts as a short-circuit. Initially, when a current flows through the circuit, most of it is used to charge the membrane capacitance. When fully charged, the capacitor then acts like an open circuit.

The cell membrane is not, however, a pure capacitor. Along with the major lipid content, the numerous embedded ion channels and their dynamics define the

permeability of the membrane. These channels represent a variable conductance (or resistance) in parallel with the cell capacitance (see Figure 2.5). The ionic current I_{ion} flowing through a resistance follows Ohm's law: $V_m = I_{ion}R_m$, where R_m is the membrane resistance.

As a result, the cell membrane can be represented by a RC circuit (see Figure 2.5-right). Membrane resistance R_m and capacitance C_m values determine the membrane time constant, $\tau_m = R_m C_m$, which dictates how fast the cell membrane potential responds to the flow or redistribution of ions through the membrane. With their specific R_m and C_m values (mainly dependent on the cell size, dictated by the cell-type), neurons thus act as low pass filters of neuronal signals with time constant τ_m . Typical τ_m range from less than 1 ms in cerebellar granule cells ($C_m = 2$ pF and $R_m = 600$ M Ω) to 30 ms in Purkinje cells ($C_m = 1,500$ – $2,000$ pF and $R_m < 100$ M Ω), one of the larger neuronal types in the brain [130, 131]. As a result, the values of these passive membrane properties are highly relevant to the generation of APs and define their characteristics. Cellular passive membrane properties can be measured by sending specific voltage pulses to a single cell and fitting the recorded current decay. The different approaches that allow to do that, as well as record single cell electrophysiology, are introduced in the next section.

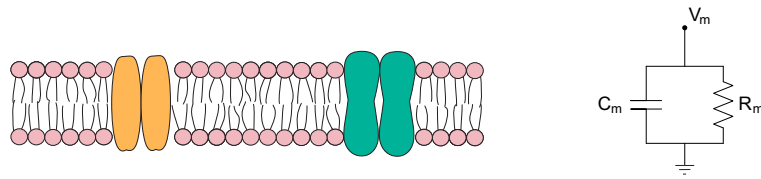


Figure 2.5: Electrical model of the properties of the neuronal membrane.

2.1.3 Experimental electrophysiology techniques

Measuring the electrophysiology of a neuron consists in recording the voltage changes across its polarised membrane. In 1939, the first intracellular recordings of APs were acquired simultaneously by Cole and Curtis [132] and Hodgkin and Huxley [133], by inserting a fine capillary electrode inside the giant (as large as 1 mm in diameter) nerve fibre of a squid. The mechanism underlying these

transmembrane potential changes remained elusive at the time, although Cole and Curtis demonstrated that the AP was associated with a large decrease in membrane resistance. To investigate how the membrane resistance depended on the transmembrane potential, in 1949, Cole and Marmont built a feedback controlled instrument able to clamp the membrane voltage at a specific value and record the ionic currents flowing across the membrane of the giant axon [134]. Alan Hodgkin and Andrew Huxley (half-brother of Aldous, author of *Brave New World*) improved this design with a two-electrode voltage clamp system that avoided the single electrode polarisation and compensated for the series resistance [135]. In 1952, making use of this technique on a squid giant axon, Hodgkin and Huxley published their mathematical model of the AP: the so-called Hodgkin-Huxley (HH) model, see Section 2.1.4 [1]. This was the first quantitative description of electrical excitability in nerve cells, and still remains nowadays as one of the pillars of neuroscience. The electrodes employed by Hodgkin and Huxley were, however, fine wires twisted around an insulating rod, which could only be inserted into large cells [136]. Years later, with the introduction of microelectrodes, the patch clamp technique was invented.

Patch clamp

In the late 1970s, Neher and Sakmann developed the patch clamp technique [137], that consisted of a micrometer-tip glass pipette filled with an electrolyte solution mimicking the cell cytoplasm's composition. With a ground electrode in the extracellular solution, ion currents across the membrane could be recorded, facilitating fundamental studies of neural activity. If a tight seal (the so called gigaseal) was achieved between the pipette and the membrane, single channel currents < 10 pA could be measured [137].

There are two operating modes for this technique: voltage clamp (VC) and current clamp (IC). The former measures the current while the voltage is fixed or clamped at a desired value, whereas in the latter, the current is fixed, and the induced voltage is recorded, including the possible induced APs. There is also the zero current mode (I0), in which the cell membrane potential is recorded with no current injection. This

IO mode is normally combined with gap-free recording, as opposed to the episodic stimulations of VC and IC protocols. A gap-free measurement consists of a recording of undefined length and no stimulation steps, used to detect spontaneous cell activity. Conversely, an episodic stimulation involves well-defined voltage or current steps of specific durations, step increments and number of repetitions or sweeps, chosen to study induced electrical activity or characterise ionic conductances.

There are different variations of the technique: whole cell, cell-attached, inside-out or outside-out (see Figure 2.6), which allow for a wide range of electrophysiological measurements, from excised membrane characterisation to entire cell response studies [138]. In this thesis, only whole cell is used, and hence it will be the one discussed from now on (see Section 3.4).

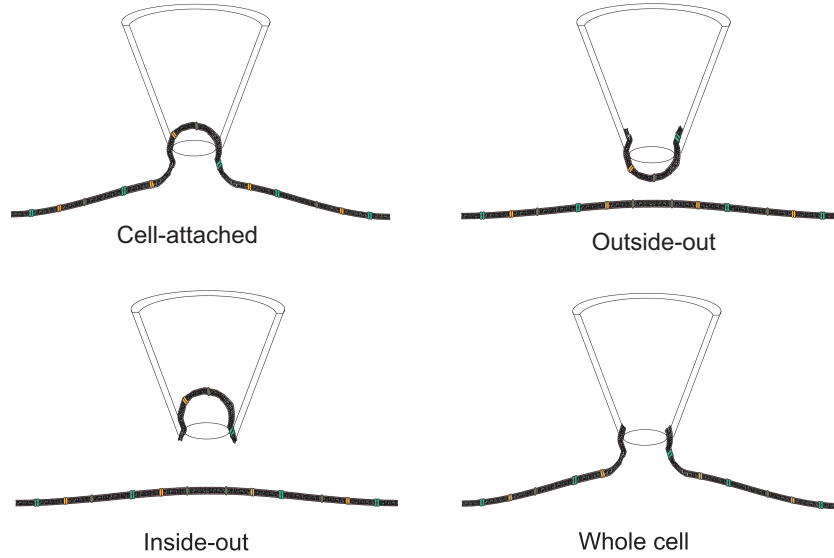


Figure 2.6: Different patch clamp configurations. The cell membrane is patched with the pipette. For the whole cell configuration, once a gigaseal is reached, the membrane is broken and the pipette has access to the intracellular ionic concentrations.

Fluorescent indicators of membrane voltage

As well as directly using electrodes to measure cell voltage, fluorescent voltage sensitive probes are a common option to measure electrophysiology [139]. They present several benefits when compared to micropipettes. They are considered less mechanically invasive than the pipettes and allow neuronal network studies

at different magnifications, as information from many connected cells can be gathered with a single measurement. In addition, they readout signals from very small structures such as dendritic spines, that pipettes are too large to patch, as well as from deep areas in tissues or *in vivo* that are not accessible to electrical measurements. Although less invasive than a micropipette, dyes inserted into cells to image membrane potential have been shown to alter their native mechanical properties [140, 141]. Two optical methods have been used in this thesis to optically image neuronal cell voltage: a calcium dye and a membrane voltage indicator. For results on the electrophysiological characterisation of the F11 cell line through these methods, as well as patch clamp, see Chapter 4.

Calcium imaging

Calcium ions (Ca^{2+}) play an important role in the neuronal AP. When the membrane depolarisation reaches the axon terminal, rich in voltage-gated Ca^{2+} channels, these open and Ca^{2+} rush into the cell, down its concentration gradient. As the concentration of free Ca^{2+} inside the cell is very low, this movement of ions creates a Ca^{2+} spike in the intracellular solution, easily detectable with Ca^{2+} indicators as a fluorescent spike (see Figure 2.7). Resting Ca^{2+} concentrations are then gradually restored by the $\text{Na}^+/\text{Ca}^{2+}$ pump, and so are the baseline fluorescence values.

This technique is commonly used for network analysis, as a single measurement records information from a population of neurons (all those in the microscope field of view), as well as individual cell activity. Ca^{2+} is involved in cell metabolism too, but the distinct Ca^{2+} spikes appearing at the end of the AP are known to be specific reporters of neuronal activity [142]. Ca^{2+} indicators are bright and sensitive, leading to good signal-to-noise ratios, and have the advantage to be non-invasively loaded into cells [139], although they have been proven to affect the cell mechanical properties [141].

Ca^{2+} spikes, however, take a few seconds to decay to baseline levels, hindering the resolution of single APs at high firing rates. As a result, a single Ca^{2+} peak

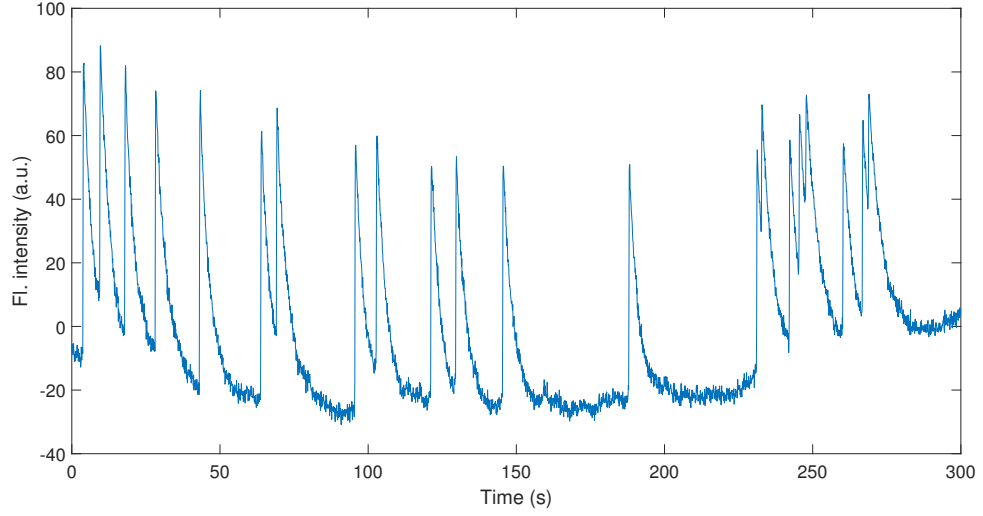


Figure 2.7: Fluorescence intensity, reporting the intracellular Ca^{2+} concentration of a single cell, over a 5 min recording. The Ca^{2+} spikes correspond to sudden increases in the Ca^{2+} concentration of the cell and are related to neuronal activity [142].

can be composed of several individual APs or AP trains, see Figure 2.7. Although deconvolution methods have been proposed to extract multiple APs from single Ca^{2+} spikes, based on the accumulated response induced by several APs close in time [143, 144], due to the complex Ca^{2+} dynamics and its numerous intracellular buffers, Ca^{2+} imaging is still regarded as an indirect and only qualitative reporter of neuronal activity.

Voltage sensitive probes

Voltage sensitive dyes measure the changes in the membrane potential of a neuron caused by the rapid redistribution of ionic charges across its membrane as a result of the opening and closing of ion channels. These ion fluxes transiently depolarise the cell tens of mV (up to 100 mV), and as the plasma membrane is only ~ 5 nm [145] thick, the associated electric fields are of 10^7 – 10^8 V/m [139]. These values decrease exponentially with distance from the membrane, for which it is essential that the voltage dye remains in the plasma membrane for effective measurements. For a review on the different voltage sensing mechanisms, see Ref. [139]. In any case, their response is much faster than that of Ca^{2+} indicators.

The voltage specificity of these probes has however been questioned, in favour of a more general environmental sensitivity. The electrostatic transmembrane potential is related to lipid order, as alterations in the order or packing of charged phospholipids influence the surrounding ionic distributions and as a consequence, the membrane potential [146]. This lipid order is further related to the phase state of the membrane and influences its hydration, and in turn the dynamics of its constituents [146]. Therefore, molecules that respond to electric fields, might well also be affected by the local pH or compression state of the membrane. Di-4-ANEPPDHQ, a probe initially designed and used as a voltage-sensitive dye, has also been applied to measure lipid packing [147] and instead referred to as an environmentally sensitive indicator for lipid membranes.

2.1.4 The Hodgkin and Huxley model

Making use of their two-electrode voltage clamp technique, Hodgkin and Huxley published in 1952 their conductance-based model (HH model) for AP initiation and propagation in the giant squid axon [1]. They measured ion currents in voltage clamp experiments and proposed that ions flowing through ion channels in the membrane changed the voltage across it. They identified Na^+ , K^+ and leak currents through the membrane. These currents altered the distribution of charges around the membrane, influencing its capacitance value, which they assumed to be constant. The expression of the total ionic membrane current is (HH equation):

$$I_m = C_m \frac{dV_m}{dt} + \bar{G}_{Na} m^3 h (V_m - E_{Na}) + \bar{G}_K n^4 (V_m - E_K) + G_L (V_m - E_L) \quad (2.4)$$

where $\bar{G}_{Na}$, $\bar{G}_K$ and G_L are the sodium, potassium and leak reference conductivities, m , h and n are the ion channels gating variables, and E_{Na} , E_K and E_L are the Na^+ , K^+ and leak reference potentials.

The HH model has been widely used to build ion channel models [148] and to construct biologically realistic neuron models. However, being phenomenological in nature, Equation (2.4) does not have any geometric or thermodynamic terms, for which this HH model is unable to explain a full range of non-electrical events of

mechanical, chemical, thermal and optical natures that have been shown to co-occur with neuronal excitation (see Section 2.3). As Hodgkin himself admitted, there was a need to “consider whether there are any unexplained observations which have been neglected in an attempt to make the experiments fit into a tidy pattern” [149], and mentioned the puzzling and unexplained observation made by Abbot, Hill and Howarth in 1958 of a positive, followed by a negative heat production associated with the nerve impulse [150]. Since then, many experimental results, outlined in Section 2.3, have recorded non-electrical phenomena accompanying the membrane voltage depolarisation during an AP. In that regard, a multiphysical model that couples all the different physics that happen simultaneously during an AP is still missing. Several models have been proposed and are presented in Section 2.3.2.

Among these non-electrical manifestations of the neuronal AP, this thesis is centred on the mechanical ones. In the following, then, the focus will be on the mechanical nature of neurons and its role in the AP signal.

2.2 The neglected picture: Mechanobiology of the neuronal membrane

The mechanics of neurons has, until recently, historically attracted little attention. However, starting from their anisotropic topology, their uniquely soft mechanical properties, and the number of subcellular mechanotransducer components they present [151], the role of mechanics cannot be set aside when establishing a thorough characterisation of the neuronal function. Neurons, with their many and extensive neurites, are constantly mechanosensing their microenvironments. Deformations in the membrane reach inner parts of the cell too, as the cell membrane is simultaneously connected to the cell cytoskeleton and the extracellular matrix through transmembrane proteins [152]. All these subcellular components are mechanically active structural networks. Their complex viscoelastic properties allow mechanical signals to propagate, be dampened or directed to specific cells [153]. Propagation of forces and mechanical interactions within these architectures influence several neuronal functions such as ion channel activation [154], synaptic vesicle clustering [155], synaptic plasticity [156], synapse morphology and maturation [157], remyelination of damaged white matter [158], neurotransmitter release [159], and axonal growth cone dynamics [160]. Furthermore, several medical conditions including stroke, traumatic brain injury, migraine and cerebral oedema have been associated with dysfunctional cellular mechanotransduction [161, 162].

Because they are the softest of the animal cells [163], neurons are sensitive to the smallest magnitude forces. Upon application of a mechanical load, such as that one induced by US when focused on to a cell or tissue, the lipidic membrane, a fluid and dynamic structure, easily folds and bends [164], altering the bilayer pressure profiles and thus the structure that supports the embedded proteins [165], in turn influencing their activity [151, 164, 165]. The latter is not only true for MS ion channels, known to specifically respond to mechanical cues, but also for voltage-gated K^+ [115–118] and Na^+ [90, 119, 120] channels, whose gating kinetics have been proven to be affected by membrane tension and composition. The opposite also holds: protein conformational changes induce deformation of the surrounding lipid

bilayer [166]. This mechano-electrical coupling is also manifested at the brain scale, with mechanical indentation responses varying with the imposed electric field [167], brain storage and loss moduli increasing by 26% and 60% within only three minutes *post mortem* [168] and lower brain stiffness values present in diseased state [169].

2.2.1 The dynamic neuronal membrane

The traditional representation of lipid bilayers as 2D fluid-like passive support matrices, with the functionality of cell membranes mainly attributed to membrane proteins (see Section 2.1), was gradually abandoned in favour of a more complex picture: an heterogeneous, anisotropic time-dependent viscoelastic biphasic material [170], with important roles in the distribution, organisation and function of transmembrane proteins [165, 171]. A more realistic description of the lipidic membrane as a highly specialised compartment, that, as well as maintaining the physiological electrochemical gradient explained in Section 2.1.2, acts as a multiphysics scaffold holding many essential cellular functions, including transport of molecules, communication, and metabolic properties, has gained advocates in the last decades.

The plasma membrane requires less energy to bend than to stretch or compress, in agreement with the neurons' ability to exocytose and recapture vesicles and to respond to mechanical forces experienced during cell growth and motility [151, 152]. Lipids and proteins in the membrane interact through hydrophobic coupling (the so-called hydrophobic mismatch) between the protein's hydrophobic domains and the bilayer hydrophobic core made of phospholipid tails. Membrane thickness fluctuations arise in lipid bilayers as a result of this mismatch, and are especially common phenomena in heterogeneous membranes with lipid components of different tail lengths and transition temperatures (T_m) [172]. At T_m , the hydrocarbon chains that form the tails of the membrane lipid components transition from their flexible and more coiled configuration (fluid) to their stiff and stretched configuration (gel). These stochastic [173] transitions induce fluctuations, some of which have timescales similar to those of conformational transitions in proteins [164, 172] that are relevant,

in particular, to neuronal activity [151]. These fluctuations, that vary with the bilayer material properties, modulate the energetics and kinetics of protein conformational transitions [165, 171], which in turn further perturb the surrounding bilayer [166].

Lipids do not only directly modulate protein function, but also indirectly through protein localisation and sorting. Lipid rafts, ordered microdomains of the plasma membrane highly enriched in cholesterol and sphingolipids [174], are thought to act as platforms on the membrane where proteins and lipids can be trafficked, compartmentalised and functionally clustered [175]. Together with the cytoskeleton [176], these dynamic structures anchor and restrict lateral distribution of membrane proteins [177], leading to accumulation of voltage-gated Na^+ channels in the AIS [127, 128] and in the NoR in myelinated cells, as well as voltage-gated Ca^{2+} channels in the axon terminal.

Ion channels affected by mechanics

MS ion channels (MSCs), present in all cells types and organisms [178–181], enable cells to detect a wide range of mechanical cues such as pressure, osmolarity, shear stress or stretch [182]. These channels open in response to a mechanical stimulus and generate mechanically activated currents, which are non-selective inward cation currents (MSCs are impermeable to anions [183]). There are two possible MSCs gating mechanisms: one is the bilayer mechanism or force-from-lipid [184], and the other one the tethered paradigm or force-from-filament [185]. The former implies that mechanical forces activate MSCs channels through the lipid bilayer alone, while the latter assumes that the forces come from the structural scaffolding proteins that form the cytoskeleton and the extracellular matrix. In practice, cell membrane cortex proteins bind the bilayer to the cytoskeleton, and transmembrane proteins such as integrins anchor the lipids to the surrounding matrix [152], so it is unlikely that a mechanical stimulus would independently affect the bilayer or the cytoskeleton. A more general mechanism that combines and reduces the two paradigms to a single basic principle of MSC mechanosensitivity remains to be addressed [186].

Some of the most notable MSCs are the Piezo channels, which are expressed in mammals in a wide range of MS cells and are found in abundance in the lungs, kidneys and dorsal root ganglia (DRG) neurons. Piezo1 has been identified as the mechanically activated ion channel involved in membrane indentation and membrane stretch-induced currents in the neuroblastoma cell line N2A [186]. Piezo1 is blocked by chemical agents such as gadolinium, ruthenium red and GsMTx4, a peptide isolated from tarantula venom, which is known to inhibit stretch-activated channels [187–189]. Furthermore, gadolinium has been proven to block MSCs by altering the order in the lipid membrane: gadolinium ions increase the lateral pressure within the lipids and “squeeze” the MSCs, shifting their equilibrium towards the closed state [190]. Piezo2, another related mammalian homolog, has been shown to be involved in a subset of mechanically activated currents in isolated DRG neurons [191]. It was previously demonstrated that heterologous expression of mouse Piezo1 and Piezo2, in addition to *Drosophila* Piezo, was enough to confer mechanically activated currents in naïve cells [192]. While Piezo1 is primarily expressed in nonsensory neurons exposed to fluid pressure and flow, Piezo2 is found in sensory tissues that respond to touch [193]. These Piezo channels, thought to be specifically MS, have been shown to also be modulated by voltage [121].

Equivalently, several classes of voltage-gated cation channels have also exhibited MS responses [194, 195]. The voltage-gated Na^+ channel $\text{Na}_V1.5$ is expressed in electromechanical tissues, including muscles, heart, and gut [196] and shows reversible accelerated activation and inactivation upon stretch stimuli [90]. Mechanosensitivity is not restricted to $\text{Na}_V1.5$, as $\text{Na}_V1.4$ (expressed in skeletal muscle) and $\text{Na}_V1.6$ are also MS, whilst $\text{Na}_V1.8$ and $\text{Na}_V1.9$ are selectively found in sensory neurons activated by painful pressure [120]. Bilayer elasticity alterations induced by membrane composition changes have also affected voltage-sensitive Na^+ channels gating kinetics [197, 198], see Figure 2.8.

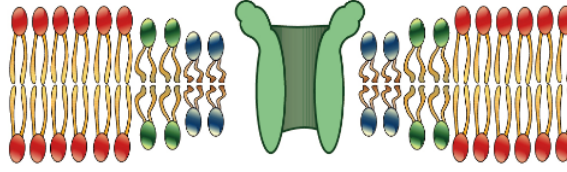


Figure 2.8: Membrane composition and lipid phase state influences embedded ion channels kinetics. Adapted from Ref. [109] with permission.

Membrane tension indirectly affects the activity of various voltage-gated K^+ channels (K_V) too, e.g., K_{2P} , TREK1, TREK2 and TRAAK [117, 199] and the classical voltage-gated K^+ Shaker channels [200–203]. Lipid membrane composition alterations also convert the functionality of K_V channels from non-inactivating delayed rectifiers to rapidly inactivating A-type channels, and vice versa [115]. Lipid membrane environment –lipid composition and mechanical state– determines the rate of K_V channel opening, the voltage dependence of the open probability, and the maximum achievable open probability [116]. Furthermore, the activity of the voltage sensor toxin VsTx1, which interferes with voltage-dependent gating by partitioning into the membrane and binding to the channel, also depends on the mechanical state of the membrane [116]. The authors hypothesised that voltage sensor toxins exert their effect by perturbing the interaction forces that existed between the K_V channel and the membrane [116], similar to the result of gadolinium altering the compression state of the lipids in the plasma membrane [190]. The voltage-gated Ca^{2+} channel, $Ca_V1.2$, expressed in cardiomyocytes, intestinal smooth muscle cells and knee joint afferents, has also shown to respond to mechanical stimuli [194].

Another important MSC is the glutamate-activated N-methyl-D-aspartate (NMDA) receptor [204], which is a ligand-gated Ca^{2+} channel that plays an important role in synaptic plasticity [205], neuronal excitotoxicity and cognitive complications due to pain and age [206–208]. NMDA response can be enhanced by the addition of arachidonic acid [209], membrane stretch, membrane phospholipids and osmotic pressure [210]. It was demonstrated that arachidonic acid and membrane induced stretch both alleviate the magnesium block of NMDA receptor channels [211], suggesting that there exists a complex mechanism whereby mechanical stretch of

the neuronal membrane alters voltage-dependent magnesium block and triggers intracellular Ca^{2+} entry through NMDA receptors.

Another family of channels that is crucial in sensory functions is the transient receptor potential (TRP) [212, 213], which have been described as polymodal sensors [214], as they are responsible for transducing a wide variety of physical stimuli, from stretch to osmosis, and are involved in sensory functions such as chemosensation, thermosensation, mechanosensation, and photosensation. TRPC1 channels, expressed in the brain, heart, smooth muscles, endothelium, salivary glands, liver, testis and ovaries, are directly activated by membrane stretching [213].

It has been proposed that all these findings of multiphysic gating of MSCs, together with their molecular diversity, reflect a common property of multiconformation membrane proteins such as ion channels: their inherent susceptibility to bilayer tension [200] and that mechanosensitivity, as initiated by changes in forces in the membrane, may be a more general feature of ion channels [199].

2.2.2 Membrane linked to cytoskeleton

The membrane is anchored to the cytoskeleton through specific proteins that form the membrane cortex, providing structural support and tension within the cell. In particular, the structural organisation of the cytoskeleton within neurons' long tubular axons is unique. In 2013, the 3D structural organisation of axons and dendrites was examined under superresolution fluorescence imaging [215], and exclusive neuronal cytoskeletal features were revealed in cultured rat hippocampal neurons: actin formed ring-like structures that wrapped around the circumference of axons and were evenly spaced with a periodicity of ~ 180 to 190 nm (see Figure 2.9). Adducin, an actin-capping protein, colocalised with the actin along the rings in axons. Spectrin exhibited periodic structures alternating with those of actin and adducin, and the distance between adjacent actin-adducin rings was comparable to the length of a spectrin tetramer. It was found that Na^+ channels in axons were distributed in a periodic pattern coordinated with the underlying actin/spectrin-based cytoskeleton. Similar periodic cytoskeleton organisation was

also found in dendrites and NoR in peripheral neurons [216] and in the AIS of rat hippocampal neurons [217]. These findings were further extended to a variety of neuronal and glial-cell types invertebrate and invertebrate animal species [218].

This cytoskeletal organisation was found to have functional implications, as it directed the periodical distributions of membrane proteins such as voltage-gated Na^+ channels, which were found to be more abundant halfway between the ends of the spectrin tetramers, presumably fixed to spectrin through ankyrin-G proteins [215], as is the case for the NoR [219]. Na^+ channels enriched AIS and NoR are crucial for the generation and fast saltatory AP propagation in myelinated neurons, respectively.

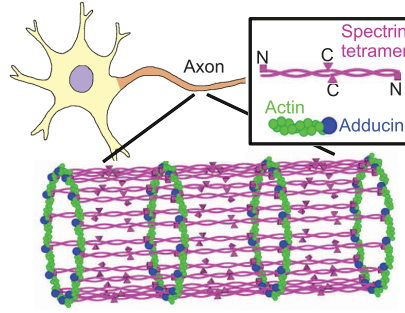


Figure 2.9: Periodic structure observed in axons. Actin filaments (in green), together with adducin (in blue) wrap around the circumference of the axon forming rings and creating a lattice with a periodicity of ~ 180 to 190 nm. Reproduced from Ref. [215] with permission.

2.2.3 Experimental mechanics techniques

Characterising or altering the mechanical properties of individual cells, particularly at subcellular length scales and under physiological conditions, became possible through the advancement of various techniques, including magnetic twisting cytometry [220], atomic force microscopy [221], micropipette aspiration [222], optical tweezing and stretching [223, 224] and microplate rheometry [225].

The most popular one, the atomic force microscope (AFM), has been used both to image and to take mechanical measurements through nanoindentation of biological samples under physiological conditions since 1989, when surface features of nonbiological samples were first resolved under buffer solutions [226]. The principle of nanoindentation consists of indenting the sample a certain nanoscale depth or

imposing a specific load with the hard tip of a cantilever of known mechanical properties. The cantilever is driven by a piezoelectric material with a feedback circuit that ensures the indentation profile is held. The resulting force response or the deflection of the cantilever, respectively, are recorded, and the material properties extracted from the force and indentation outputs. The AFM has been used to examine the response of neuronal cells [227] and their viscoelastic properties under dynamic mechanical measurements on single neurons, extracting the elastic storage and the viscous loss moduli from the force-displacement curves at different oscillating frequencies and small indentation depths [228]. Local or global cell mechanical properties can be probed with varying AFM tip sizes.

In this work, a nanoindentation instrument is used to explore and alter the micro-mechanical properties of cultured neurons: the Chiaro nanoindenter (Optics11). Similar to an AFM, the probe-sample interaction is measured through the bending of a well characterised cantilever. This nanoindenter, unlike an AFM, can be combined with an inverted microscope and a patch clamp setup (see Chapter 3.).

2.3 The emerging picture: Multiphysics coupling in the neuronal membrane

The advancement of the experimental techniques in the last decades has allowed the recording of the different non-electrical manifestations of the multiphysics of neurons or nerves through diverse and complementary methods, presented in Section 2.3.1, at the same time as theoretical models were developed to explain these empirical observations (see Section 2.3.2).

2.3.1 Empirical evidence of the multiphysical AP

In parallel to the development of the ion-based description of the AP (see Section 2.1.4), the first non-electrical manifestations observed to be coupled to the nerve signal, such as changes in mechanical properties, dye binding or light scattering, occurred in the late 1940s [229]. In the following, the historical optical and thermal manifestations of the neuronal AP are briefly presented, after which the mechanical ones are discussed in greater detail.

Optical changes associated with the AP

In 1947, an electrical stimulation of a squid giant axon was shown to induce increased turgidity and decreased the flow of axoplasm; both of which were attributed to viscosity changes during the AP [230]. Optical manifestations of the involved structural changes were discovered a year later, when Lüthy reported a slow, delayed alteration in ultraviolet light absorption of stimulated single myelinated nerve fibres [231], attributed to swelling or collapse of certain parts of the fibre as a result of the mentioned viscosity changes in the axoplasm. Soon after, reversible opacity changes were observed in a stimulated crab nerve, with increased scattered light during stimulation [232]. That same year, increased binding of a dye to the axonal membrane was reported to co-occur with the AP in non-myelinated nerves, indicating membrane permeability alterations [233, 234], followed years later by similar observations in myelinated nerves [229].

While all the above reported the result of trains of electrical stimuli, in 1968, Cohen *et al.* tracked the time course of light scattering changes in non-myelinated crab and squid nerve fibres after single stimuli [235], and found birefringence shifts in, or near, the membrane of the cell. The same authors further proved the membrane location of these optical alterations by showing that birefringence changes, with time courses very similar to those of the AP, were unaffected by the removal of the axoplasm and perfusion [236]. A wide variety of nerves and individual axons showed this birefringence variations: crab, lobster, crayfish and rabbit vagus nerves, eel electric organ and squid giant axon [229]. The birefringence alteration, termed as optical spike by von Muralt and colleagues, was stimulation dependent and its time course corresponded to that of structural changes in the axon membrane during excitation [237]. Further transient optical changes (fluorescence and turbidity) associated with crab, lobster and squid nerves excitation were recorded by Tasaki *et al.* [238], and were consistent with conformational changes in macromolecules of the nerve that did not involve the axoplasm.

More recent studies have corroborated and complemented these early results on optical manifestations of the nerve AP across the entire cell membrane [239–247], and suggested a travelling surface wave in the neuronal cell membrane as the responsible for the measured optical signal pulses [246].

Further optical results of imaged membrane mechanical displacements during AP firing are included in the following.

Thermal changes accompanying the electrical AP

As early as 1876, Bernard observed that there was no current flow when the two junctions of a thermopile were placed on a rabbit nerve at rest, but current appeared when the region of the nerve under one of the junctions was excited. Bernard concluded that the nervous activity was the source of the measured heat [248].

In 1926, an overall rise in temperature was detected when a frog sciatic nerve was stimulated repetitively [249]. Thirty years later, two distinct phases of this heat change were distinguished: a rapid release and a slower heat reabsorption were

observed in crab nerves [150] and subsequently in lobster and rabbit nerves [250]. This heat exchange was in phase with the AP [251], but even though Hodgkin himself knew about it [149], it was not studied thoroughly by most of the community [252]. Only a few authors investigated the origin of the transient heat production [251, 253], with the most satisfactory explanation being the “condenser theory”, in which the positive (emission) and the negative (absorption) heat represented the discharge and recharge of the free energy stored in the membrane capacity. However, when Howarth *et al.* studied the effects of altering the ionic concentrations of the solutions on the heat release, they observed that the condenser theory only accounted for half of the observed temperature changes, and they advocated for entropy changes of the nerve membrane when it was depolarised and repolarised back during an AP [251]. It was hypothesised that membrane polarisation changes during the AP induced molecular rearrangements in the dielectrics that form the membrane, which in turn altered the entropy during the AP [253]. The evolution of the heat pulse indicated that a decrease in entropy of the membrane dielectric was induced by depolarisation [254]. Only when the entropy change was taken into consideration, could the charging and discharging process of the membrane capacity quantitatively explain the observed thermal changes. For further references and explanations, see Ref. [255].

In 1989, Tasaki and colleagues observed thermal changes accompanying the propagation of an AP along the garfish olfactory nerve [256] and suggested that the heat changes were associated with the release and re-binding of Ca^{2+} in the nerve associated with AP production (further explained in Section 2.3.2).

Mechanical changes accompanying the electrical AP

The first evidence of mechanical alterations that accompany the AP propagation dates from 1947: an increased resistance of the nerve to twisting was observed during stimulation, as well as slow mechanical changes, the latter attributed to long term cytoskeletal reorganisation [2].

In 1950, Hill found that the observed reversible opacity changes of a stimulated crustacean nerve arose from a cumulative increase in fibre diameter upon electrical depolarisation (about $0.1\ \mu\text{m}$ for 10,000 impulses) [257], sometimes accompanied with fibre shrinkage [258]. Nerve shortening associated with activity was also found in frog nerves [259]. In 1955 [3], 1958 [5] and 1962 [6], rapid membrane mechanical changes were observed to be coupled to the AP propagation. In 1977, small rapid reductions in the diameter ($3\text{--}25\ \text{\AA}$) of a giant crayfish axon were detected by laser interferometry when an AP progressed along the axon, followed by a slow relaxation [8]. A $1.8\ \text{nm}$ contraction of the axon was also reported.

From the 1950s onwards, Tasaki and colleagues provided a vast amount of experimental evidence on the non-electrical manifestations of the AP and proposed their own theories for the origin of nerve excitation [260] (see Section 2.3.2). In 1980, Iwasa and Tasaki found that the conduction of the AP along a crab nerve was accompanied by a decrease in the length of the nerve [9]. Months later, the same authors observed a small, rapid upward movement ($50\text{--}100\ \text{\AA}$) of the surface of a crab nerve accompanying the nerve impulse, produced by a transient swelling of the nerve fibre [261, 262], see Figure 2.10. The peak of swelling during excitation was accurately in phase with the peak of the AP [261]. Furthermore, these rapid mechanical changes were suppressed with tetrodotoxin (TTX) and procaine [262], thus confirming the role of voltage-gated Na^+ channels in the generation of the mechanical transients. In 1982, Iwasa and Tasaki, observed that the squid giant axon also swelled when an AP was generated. The maximum swelling was reached at the peak of the AP and a shrinkage was found in the hyperpolarisation phase of the AP. Changes were present even if a major portion of the axoplasm was removed, and were dependent on the tonicity of the external solution and on the application of several chemical reagents [263]. The authors numerically proved that the process of Na^+/K^+ exchange associated with production of an AP could not solely account for the observed magnitude of swelling [264]. Seven years later, these same mechanical changes, together with thermal ones, were observed in the garfish olfactory nerve associated with the nerve impulse [256]. Again, not only

they observed membrane bulging (or radial swelling) during the AP but also a simultaneous marked longitudinal shrinkage of the nerves and found that these mechanical deformations existed even if a major portion of the endoplasm was removed from the axon without suppressing the cell excitability [256]. As they had already observed that repetitive stimulation of the axon enhanced the solubility of cytoskeletal protein molecules [264], and knew that the gel-to-solid transformation took place at the interface between the cytoplasm and the plasma membrane, they proposed that the swelling of nerve fibres during the AP was attributed to the enhancement of the solubility of protein molecules in the ectoplasm, the part of the cytoplasm adjacent to the plasma membrane [264].

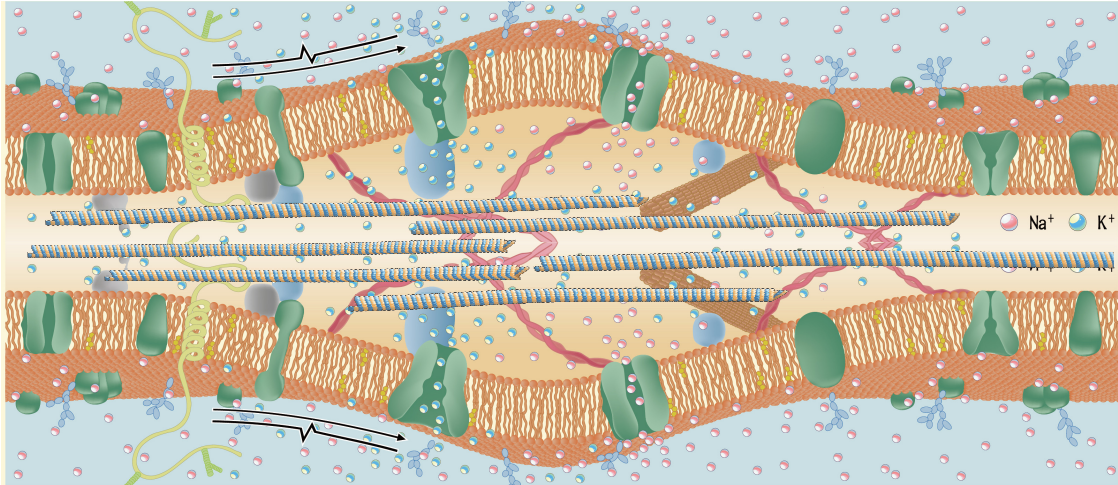


Figure 2.10: A mechanical deflection of the neuronal membrane accompanying the AP, as observed by Refs. [261, 262]. Adapted from Ref. [109] with permission.

Since the 1990s, improvements in the experimental techniques, especially interferometric, facilitated the detection of these nanometer-scale and millisecond-long deformations in cultured cells. In 1998, the depolarisation induced membrane movement was studied in voltage-clamped HEK293 cells with the AFM, which exhibited an outward movement during depolarisation with a magnitude approximately 0.5–15 nm normal to the plane of the membrane under a ± 10 mV peak–peak AC carrier stimulus [265]. The same authors recorded the membrane movement of voltage-clamped HEK293 cells in different ionic-strength solutions and observed that a depolarisation field could cause an outward membrane movement in normal

saline and an inward movement in saline with low ionic strength [14]. The amplitude of the movement was proportional to the voltage change in the ratio of about 1 nm per 100 mV. The membrane electromotility of these HEK293 cells was studied in real time by using a combined patch clamp-AFM setup by the same group, who proposed that the observed membrane deflections were the result of changes in interfacial tension [266]. Following these studies, Kim *et al.* observed changes in the nerve terminal volume of the posterior pituitary *via* AFM as nanometre scale movements of the cantilever [267]. The observed membrane swelling was believed to be related to water movement associated with the exchange of Na^+ and K^+ , although in other studies it was reported that the membrane displacement was about two orders of magnitude larger than the one measured for the exchange of Na^+ and K^+ [101]. Most recently, the AFM was also used by Heimburg's group to record axonal thickness changes in single giant axons of lobster nerves during AP propagation [268]. It was found that the membrane changes were in the order of 0.2–1.2 nm and lasted approximately 2–4 ms.

Gil *et al.* observed that prolonged depolarisation of a membrane patch of cultured rat hippocampal neurons in borosilicate pipettes resulted in a delayed slow displacement of the membrane into the pipette, with a magnitude of about 1 μm for each prolonged depolarisation [269]. The displacement was delayed by a duration ranging from tens of milliseconds to several seconds, in correlation with the activation of MSCs, for which it was hypothesised that the membrane tension associated to the voltage-induced displacement was what activated these channels.

Results with the non-contact coherence tomography technique, in which the cell deformation induced by membrane potential changes was measured as changes in the optical signals, confirmed that transient mechanical changes were associated with APs and could be altered under different environmental and physiological conditions, such as change of temperature or ionic concentration [270–274]. This technique also demonstrated that the magnitude of the measured membrane displacements was in the nanometre range.

Bronwell *et al.* measured cell membrane displacement and electromechanical force generation using optically trapped beads to detach the plasma membrane from the cytoskeleton and to form long thin cylinders (tethers) [275]. In this approach, electrical stimuli were delivered to the cell through the whole-cell voltage clamp, while membrane displacements and electromechanical forces were quantified by measuring the beads' displacements along the tether axis using a quadrant photo-detector. It was observed that hyperpolarising potentials increased (and depolarising potentials decreased) the force required to pull a tether. Both membrane displacements and tether forces were functions of the holding potential.

In addition, Nguyen used piezoelectric nanoribbons to measure the mechanical deformations of neuronal cells in response to electrical excitations, and reported that cells deflected by approximately 1 nm under a 120 mV voltage field applied to the cell membrane [276].

Fields *et al.* observed axon swelling of under $0.5\ \mu\text{m}$ with AP passage in embryonic DRGs with a combination of single-photon imaging of ATP release, imaging for intrinsic optical signals, Ca^{2+} imaging, time-lapse video, and confocal microscopy [277]. Superresolution imaging also confirmed wider axon diameters after high-frequency AP firing of mouse organotypic hippocampal slices [278]. For comparison, a study in the giant internodal plant cell of *Chara braunii* reported a range of swelling in cell diameter associated with the AP of 1–10 μm , as well as same velocities of propagation for the mechanical and the electrical pulses [279].

Previous setups measured the cell or nerve membrane displacement at a single point. Non-invasive optical imaging methods have tracked the membrane's displacement coupled to the neuronal signal of HEK-293 cells and rat hippocampus cells with sub-nm resolution: a fast brightfield image acquisition combined with a differential detection algorithm [16], a spike-triggered averaging of movies synchronised with electrical recordings [247] and a plasmonic imaging technique [280]. These methods tracked the displacements in larger areas of the cell and gave displacements of 0.2–0.4 nm [16], up to 3 nm [247] and 0.02 nm per 100 mV [280] at the cell boundaries, and 0.005 nm per 100 mV at the cell centre [280].

Recently, Ling *et al.* presented a high-speed quantitative phase imaging approach that allowed full-field imaging of cellular deformations accompanying the AP in mammalian neurons [17]. Results showed displacements of -1.8 to 1.4 nm in somas and -0.7 to 0.9 nm in neurites, corroborating that the time course of the optical phase changes closely matched the dynamics of the electrical signal. In particular, they observed a general trend of cells becoming more spherical during depolarisation, a result of the increase in cell surface tension, in line with the voltage-dependent membrane tension model [14] and an indication of the trend towards minimising the cell's surface area.

All these findings, summed up in Table 2.1, illustrate that rapid changes in neuronal membrane voltage, involving the opening and closing of ion channels, have mechanical consequences, and as a result there is a cell membrane deflection travelling in phase with the AP. These AP-accompanying membrane deformations appear to occur in a wide variety of excitable cells and tissues, including both invertebrate and vertebrate nerve fibres [101], and are affected by different environmental conditions such as the ionic concentration. This *a priori* conflicts with, or at the very least complements, the traditional understanding of the AP as a purely electrical phenomenon [1]. Given the pervasiveness of mechano-electrical mechanisms illustrated above, it therefore becomes reasonable to ask whether such deformation may itself play a role in the AP generation, and use it to modulate neuronal signals, as US neuromodulation does.

2.3.2 Proposed models for the multiphysical AP

Herein, the most popular models that include the observations presented in Section 2.3 are introduced. For more theoretical models, greater detail and specific numerical formulations, refer to Ref. [109].

Cation-exchange excitation system

One of the most documented nerve excitability models is the one proposed by Tasaki and colleagues [284]. After all his observations (see Section 2.3.1), Tasaki

Cell/Nerve type	Magnitude	Reference
Crab nerve	50–100 Å	[261]
Crayfish axon	3–25 Å	[8]
Crayfish nerve bundles	~1 nm	[281]
Crayfish and lobster nerve bundles	0.5–2.5 nm	[271]
Crustacean (crab, lobster and crayfish) nerves	10–20 nm	[262]
Lobster nerve	~5nm	[282]
Lobster nerve	<1 nm	[283]
Lobster nerve	0.2–1.2 nm	[268]
Squid axon	0.5–1 nm	[263]
Squid axon	<1 nm	[272]
Mouse neurohypophysis	5–10 nm	[267]
Embryonic DRGs cells	0.5 μ m	[277]
PC12 cells	1 nm per 120 mV	[276]
Rat hippocampus neurons	1 μ m per prolonged depolarisation	[269]
Rat hippocampus neurons	0.2–0.4 nm	[16]
HEK-293 cells	3 nm	[247]
HEK-293 cells	0.5–15 nm per ± 10 mV	[265]
HEK293T cells	0.02 nm per 100 mV	[280]
Primary rat cortical neurons	-1.8–1.4 nm	[17]
<i>Chara braunii</i> plant cell	1–10 μ m	[279]

Table 2.1: Mechanical deformation magnitudes accompanying neuronal AP in different cell and nerve models.

argued that the electrophysiological processes known as “nerve excitation and conduction” were, basically, manifestations of abrupt physicochemical or structural changes in the membrane, or membrane permeability changes, which specifically permitted the entry of Na^+ into the cell [285, 286]. This challenged the traditional notion that Na^+ entry through ion channels was the initiating event of the AP [1]. Tasaki’s hypothesis treats the membrane of the squid axon as a complex macromolecular system of proteins and phospholipids [284], whose external layers have an excess of fixed negative charges, that confer cation exchange properties to the system. The process of excitation is linked to the cation-exchange system [284]: nerve excitation consists of a rapid, reversible cation-exchange which involves transition between two stable conformational states of the membrane architecture [287, 288]. In the resting state, the anionic sites in the membrane are occupied by divalent cations (e.g., Ca^{2+}), whereas in the excited state, these sites are occupied predominantly by univalent cations (e.g., Na^+). The conformation of the membrane is mainly determined by the ratio of univalent to divalent cations. The process of

initiation and abolition of the AP is then interpreted as transitions between the two stable states of the membrane [260]. Recently, a transmembrane electrostatic proton localisation theory has been proposed for the neuronal AP, and the nonlinear curve of the localised protons/cations charge density during an AP shown to be an inverse mirror image to the AP [289].

Voltage-induced changes in membrane tension

It has been suggested that membrane displacement during polarisation occurs as a result of the cell attempting to alter their radii so as to maintain constant pressure across the membrane [14, 276, 290]. When membrane polarisation is induced by an applied external voltage field, it is hypothesised that it may change the surface tension of the membrane [14, 266]. The surface tension is defined as the free energy required to maintain a certain surface area, and therefore contains both chemical and electrical contributions. The chemical contribution is the natural surface tension of membrane interface in the absence of electric field, whereas the electrical contribution comes from the electrical energy stored in the membrane capacitance during polarisation induced by the applied voltage field. When the membrane is polarised, the change of membrane potential modulates the surface tension of the deformable cell membrane in contact with intracellular and extracellular fluids. In order to maintain a constant pressure across the membrane, differences in surface tensions between the intracellular and extracellular interfaces induce changes in membrane curvature, in turn leading to membrane displacement (see Figure 2.11-left). Consequently, changes in membrane potential result in an alteration of cell radii to maintain mechanical equilibrium across the membrane [152].

The direct and reverse flexoelectricity

Another proposed mechanism for the multiphysics of APs is biological flexoelectricity, which includes both direct and reverse flexoelectricity. Direct flexoelectricity is a property normally observed in dielectric materials, and refers to a spontaneous electric polarisation induced by an applied mechanical strain gradient [291]. The reverse flexoelectric effect refers to a strain gradient (usually in the form of bending

or distortion) induced by a substantial external electric field applied across the dielectric material [292, 293]. This concept differs from the piezoelectric effect in that piezoelectricity only occurs in non-centrosymmetric crystalline materials under a uniform mechanical strain, whereas all dielectric materials exhibit flexoelectricity under a non-uniform strain field (i.e., strain gradient) [294–297].

In lipid bilayers and cell membranes, the phospholipid molecules, that arrange themselves into two sheets (see Section 2.2.1), are charged and possess dipole moments on their surfaces. The fluid-like membrane can undergo a variety of mechanical deformations such as bending and thickness change [298] which alter its surface curvature, resulting in a redistribution of the dipoles and the development of a surface polarisation [299], i.e., the direct flexoelectric effect [296, 300], see Figure 2.11-centre. Flexoelectricity is found to play a significant role in a number of biological functions [301], such as ion channels kinetics, thermal fluctuation, vesicle equilibrium, and particularly in hearing [14, 81, 295, 302–305]. In neurons, both direct and reverse flexoelectric effects have been suggested to be closely related to membrane polarisation during AP propagation [304, 306].

Conformational changes of transmembrane proteins such as ion channels induce a deformation of the lipid bilayer in the vicinity of the channel (through hydrophobic coupling) [166, 307]. The presence of an external electric field, such as changes in the local ionic concentration gradients, may induce further curvature of the membrane due to the reverse flexoelectric effect. Alteration of the membrane curvature in turn exerts mechanical forces on the boundaries of ion channels, potentially altering their dynamics [165] or, in the case of MSCs, further promoting their activation. It may also change the local electric polarisation field due to the direct flexoelectric effect, which again influences the external electric field. As a result, this bi-directional action results in the membrane depolarisation that drives the ionic flux through the ion channels [296, 304, 306], accompanied by polarisation-induced membrane deformation [297].

The thermodynamic model of the AP

The above models are generally driven by the physics of interest. However, they suffer from a lack of generalisation. Thermodynamic frameworks that consider the different physics as intertwined energy contributing paradigms aim at bridging this gap. In such approaches, the state of the membrane, is characterised by its enthalpy and the different conformations (or states) it can take, i.e., its entropy. These approaches can be derived from first principles, and present the advantage to “capture” a phenomenon such as the AP as a simple thermodynamic wave intrinsically accounting for mechanics, electrophysiology, pH, temperature, etc.

One of the leading models was introduced in 2005 by Heimburg and coworkers [15, 308], and considers the AP as a localised multiphysics density pulse, see Figure 2.11-right. This pulse resembles a sound wave, as it is a nonlinear and dispersive solitary wave. It manifests itself, among other alterations (e.g., pH, phase change, etc.), as a mechanical pulse that maintains its shape with no attenuation while propagating at a constant velocity [309]: a soliton. This theory makes use of the fact that cell membranes have a melting point temperature above which the membrane consistency changes from liquid or fluid to solid or gel phase, and the temperature of the melting point is only slightly below the organism’s body temperature. As such, any perturbation of the membrane (not only temperature, but also hydrostatic pressure, lateral pressure in the membrane plane [310], electric or magnetic pulse, or pH) can influence the state of the membrane. Thermodynamically, the soliton model describes the AP propagation as an adiabatic process, in which the energy provided at the source of excitation is carried adiabatically through the plasma membrane: a result of the reversible release of heat. The measurement of a temperature pulse (see Section 2.3.1) and the absence of net heat release during an AP [251, 311] are some of the experimental support of the soliton model.

Ions can travel across the membrane through transient lipid pores or channels of discrete conductivity, first found in pure lipid bilayers by Kaufmann in 1989 [312]. These lipid channels are generated by thermodynamic fluctuations in the membrane

thickness, and their probability is controlled by any physics that perturbs the membrane state: membrane potential, pH, pCa, surface pressure or temperature.

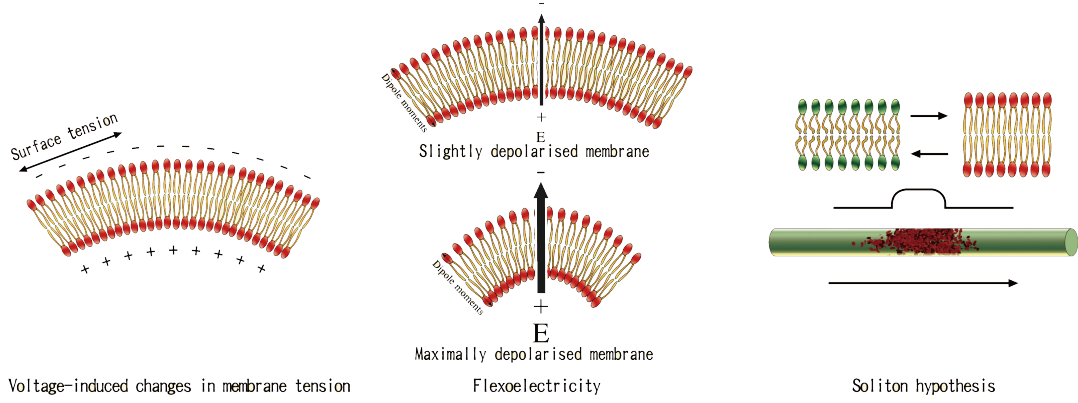


Figure 2.11: Potential mechanisms for membrane deformation during an AP. Reproduced from Ref. [109] with permission.

The mechano-electrophysiological coupling principles presented in this chapter, by which the AP has an inherent mechanical nature, imply that membrane displacements of particular characteristics could induce local electrophysiological responses in neurons, and influence the depolarisation and repolarisation during an AP, hence altering the AP shape and dynamics. However, no study has yet looked into the mechanism or degree by which a mechanical stimulus influences single cell electrophysiology. In order to bridge the notions presented in this chapter with US neuromodulation applications introduced in Chapter 1, the effects of single neuron direct mechanical loading on the characteristics of the AP are studied in this work. In particular, a multiphysics setup is introduced in Chapter 3, validated in Chapter 4 and used in Chapters 5 and 6 to apply static and dynamic direct mechanical stimuli to single neurons, respectively, while recording their electrophysiology.

Chapter 3

Multiphysics setup

The first milestone of this work was to design, build and optimise a multiphysics rig to study the influence of a mechanical load on single cell electrophysiology. As standard AFM sample stages do not generally allow room for conventional patch clamp systems, the Optics11's Chiaro nanoindenter was purchased (Figure 3.1-A) and directly mounted on an inverted microscope (Figure 3.1-B) with no specific chamber or sample stage, allowing its combination with the patch clamp technique (Figure 3.1-C). The ability to simultaneously image and mechanically and electrically manipulate single cells in physiologically relevant conditions makes this methodology particularly suitable for studying the influence of specific mechanical loading profiles on neuronal AP generation and propagation.

3.1 Cell culture

F11 cells (ECACC, UK), a hybrid between mouse N18TG-2 neuroblastoma and embryonic DRG neurons, commonly used as a functional DRG neuronal model [313, 314], were chosen for experiments for their mature neuronal features such as outgrowth of extensive neurite-like processes and spontaneous APs [315].

F11s were expanded and differentiated following published protocols [316]. For expansion, $\sim 3 \times 10^5$ cells were cultured in a T75 flask in high glucose

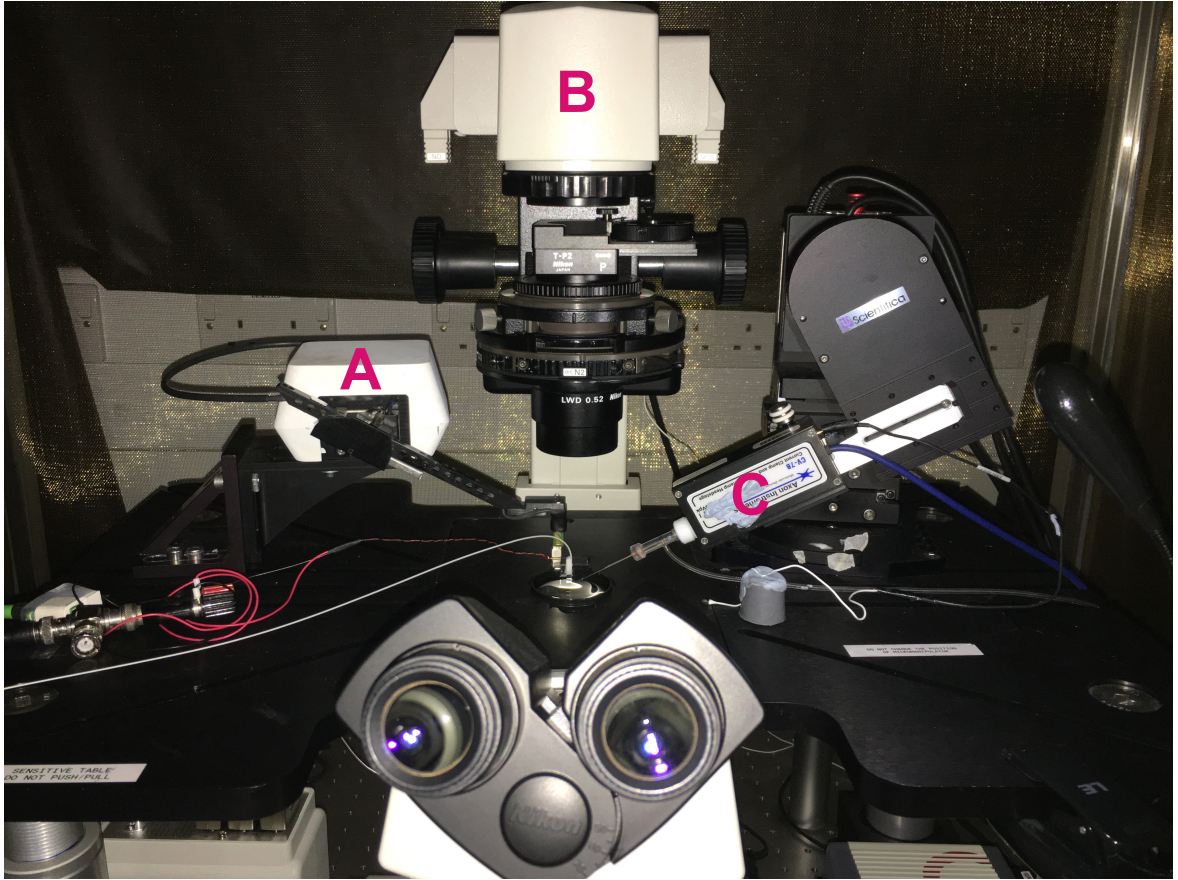


Figure 3.1: Designed multiphysics setup for single cell simultaneous mechanical and electrophysiological studies, combining A) nanoindentation and C) patch clamp on B) an inverted microscope.

DMEM (ThermoFisher) with 10% fetal bovine serum (FBS) (ThermoFisher) and 1% penicillin/streptomycin (P/S) (ThermoFisher) in a humidified incubator at 37° C and 5% CO₂, changing media every 3 days. For differentiation, $\sim 7 \times 10^4$ cells were seeded per glass bottom dish (HBST-5040, WillCoWells), in high glucose DMEM, supplemented with 1% FBS, 1% P/S, 0.5% insulin transferrin selenium (ThermoFisher), 10 μ M 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 50 ng/mL nerve growth factor (Peprotech), 2 μ M retinoic acid (Sigma-Aldrich) and 0.5 mM bromoadenosine 3,5-cyclic monophosphate, following Ref. [316]. Cells were differentiated for 5 days [313] prior to experiments, topping up the media every 2 days. The low confluence of cells seeded for differentiation was empirically optimised in order to obtain sparsely distributed yet connected cells, see Figure 3.2. Higher seeding densities resulted in less and shorter neurites, as well as cell bodies

in contact, preventing single cell indentation studies.

Undifferentiated F11 cells tend to cluster together and start forming extracellular matrix when $\sim 80\%$ confluent, and only show several short neurite-like processes. When differentiated, they exhibit a mature neuronal morphology with long neurites [313] (see Figure 3.2) and express voltage-gated K^+ and Na^+ channels [317], Ca^{2+} -activated K^+ channels [318], as well as temperature-sensitive TRPV2 channels [319].

Despite being a cell line, thus expecting all cells to be clones of the parent cell

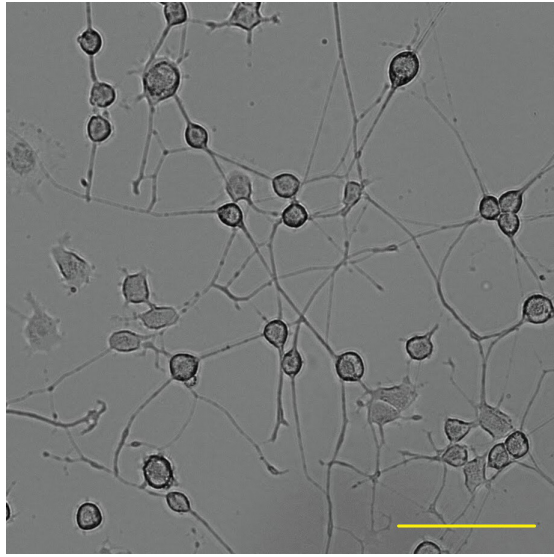


Figure 3.2: Brightfield image of differentiated F11 cells on a glass bottom dish. Scale bar is $100\ \mu\text{m}$.

line, there are multiple neuron phenotypes within F11 populations [316], as can be seen visually in Figure 3.2. This might be due to the fact that it is a hybrid cell line, as well as to the differentiation being chemically induced.

F11 cells are sensory neurons and hence show mechanosensitive traits. Differentiated F11s respond with small mechanosensitive currents to mechanical indentations [320] through endogenous mechano- and volume- activated channels such as TRPV4 [319, 321] and Tentonin3 (TMEM150C) [320]. They also present neuronal markers of A- and C-fibre mechanosensors and proprioceptors, including LDHB, NEFH, and TrkC [322]. Furthermore, ion channels with recognised roles in nociception such as ASIC1, CaV3.2, KV7.2, TRPM3 [322] and bradykinin and prostaglandin receptors [313] are expressed in F11s.

These cells are fully morphologically, mechanically and electrophysiologically characterised in the next chapter, Chapter 4, *via* the methods presented in this chapter, which include optical, mechanical and electrophysiological studies.

3.2 Inverted microscope

The experimental setup designed in this thesis includes an inverted Nikon Eclipse Ti microscope with multi-LED excitation (CoolLED) and a C2+ confocal module.

Cell diameter and height studies were performed from widefield fluorescence images of F11 cells stained with CellMask Green Plasma membrane live stain (ThermoFisher). Cells were incubated with the dye for 15 min at 1:1,000 dilution, then live imaged using a standard fluorescein isothiocyanate (FITC) filter. Z-stack images were acquired every 0.025 μm . Morphological characterisation results of the F11 cell line are presented in the next chapter.

In addition, confocal imaging was used to perform immunohistochemistry essays with NeuN (Abcam, ab177487, rabbit) antibody, a neuronal maturation marker, to characterise F11 cell maturation after 5 days in differentiation media. Cells were gently washed once with PBS (Porcine Bovine Serum, ThermoFisher), fixed with 4% Paraformaldehyde (Sigma Aldrich) for 15 min, then washed three times with PBS. Fixed cells were permeabilised with 0.1% Triton X-100 (Sigma Aldrich) in PBS for 30 min, and the non-specific binding sites blocked with 2% Bovine Serum Albumin (Sigma Aldrich) in PBS for 1h. Cells were left overnight on a 1:1,000 dilution of the primary NeuN antibody in PBS at 4° C, after which the second antibody (Alexa Fluor 568 goat anti-rabbit, A11011) was loaded at a 1:400 dilution for 1.5 h at room temperature and then washed six times with PBS.

The arrangement of the setup allows for continuous inspection and control of both the nanoindenter probe and the patch pipette positions, allowing for simultaneous brightfield and fluorescence (both widefield and confocal) imaging throughout the mechanical and/or electrophysiological experiments. In addition, when doing fluorescence studies, the probes do not obstruct vision within the field of view (see Figure 3.3).

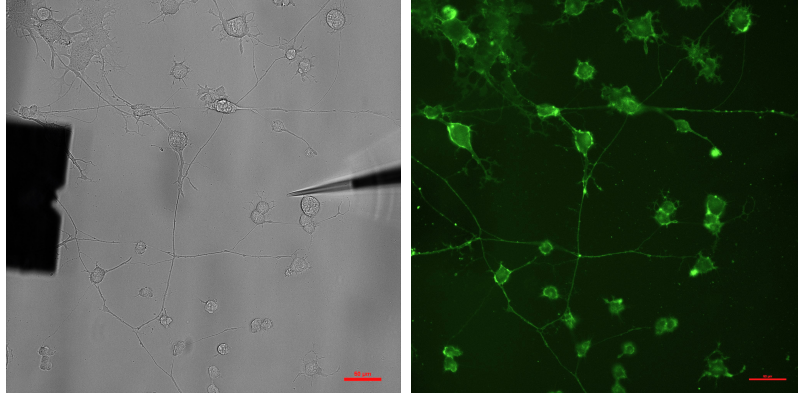


Figure 3.3: Brightfield and widefield fluorescence images of differentiated F11 cells on a glass bottom dish. Nanoindenter and pipette do not obstruct vision in fluorescence images. Scale bars are 50 μm .

3.3 Experimental mechanics

Neurons are heterogeneous, nonlinear viscoelastic materials, and as such, their mechanical response depends on the time scale, magnitude and loading rate of the applied forces or indentations. This is due to the different nature and behaviour of the membrane, membrane cortex, cytoplasm, organelles and nucleus, as well as the characteristic anisotropy of neuronal cells. Furthermore, neurons are the softest cells in the human body [163], needing small forces in the nN or even pN range to characterise them. AFM is the most extensively used technique to characterise single cell mechanics. However, its combination with patch clamp, the single cell electrophysiology technique par excellence, is not straightforward, as standard AFM sample stages do not leave room for conventional patch clamp systems. Alternatively, nanoindentation, the technique of choice for the mechanical characterisation of biological tissues and biomaterials, can also be used for single cell characterisation, as the scales of indentation are typically cell length.

The Chiaro nanoindenter was used in this work, as it is compatible with both an inverted microscope and a patch clamp setup. It can be used with probes of different sizes, allowing for more global or more local studies, and stiffnesses. In addition, a customised dynamic module was purchased and developed in close collaboration with Optics11, to mimic the mechanical perturbations experienced by cells exposed to US stimulation.

In the following, the Chiaro nanoindenter is briefly presented, together with the available mechanical characterisation tests. For the complete explanation of its functioning principles and the high frequency module calibration, see Appendix A. The numerical methods implemented to analyse these tests on F11 cells, as well as the corresponding processed experimental results, are presented in Chapter 4.

Chiaro nanoindenter

The Chiaro nanoindenter was used to fully characterise the mechanical response of F11 cells (see Chapter 4). The instrument consists of the indenter head to which the probes are attached, a controller box and the OP1550 interferometer, all connected to a laptop running the Optics11's Piuma software (see Figure 3.4).

The probe consists of a ferrule-top gold cantilever [323] with a spherical glass

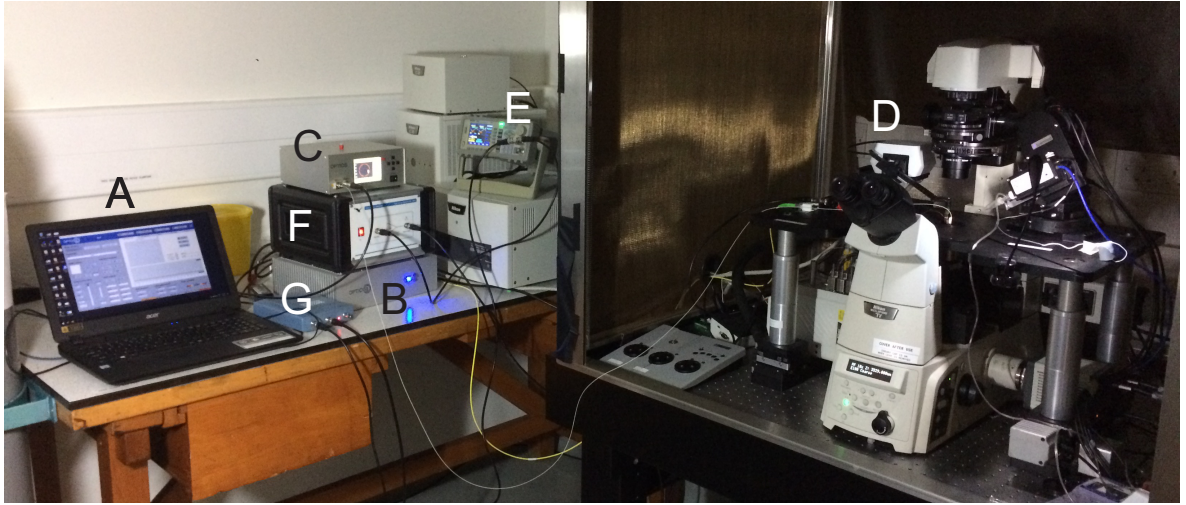


Figure 3.4: A) Piuma software. B) Chiaro controller box. C) Optics11 OP1550 interferometer. D) Chiaro nanoindenter head and arm. E) Signal generator. F) Voltage amplifier ($\times 50$). G) Picoscope oscilloscope. E), F) and G) are needed for the high frequency experiments presented in Chapter 6.

bead attached through a glass cylinder (see Figure 3.5). The bead radius and the stiffness of the entire probe can be selected upon purchase, and range from 3 to 50 μm tip radius and 0.01 to 50 N/m probe stiffness, enabling the study of a broad variety of deformation modes. Probes of 3 to 10 μm and 0.01 to 0.05 N/m were used in this project.

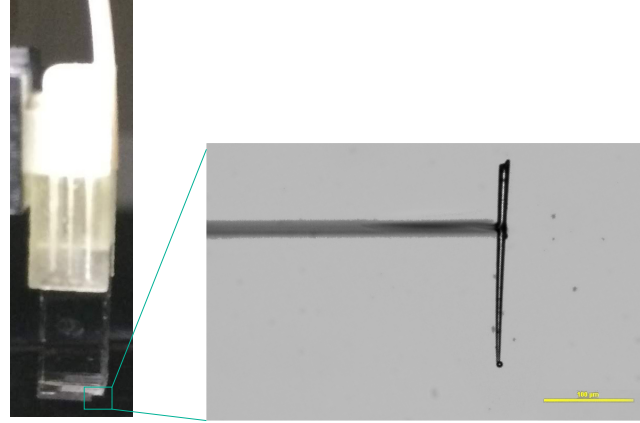


Figure 3.5: Left: Chiaro probe at the end of the Chiaro arm, with the optical fibre coming from above. Right: Sideways microscope view of a Chiaro probe. Scale bar is 100 μm .

A single-mode optical fibre connected to the interferometer guides the infrared laser-light ($\lambda = 1550 \text{ nm}$) through a cavity onto a cantilever and records cantilever bending as a result of sample deformation. The cantilever deflections are extracted from the phase of the interference signal between two reflected waves: one at the fibre-air interface (R1 in Figure 3.6), and the other one at the cantilever surface (R2 in Figure 3.6). The instrument's full working principle is described in Appendix A.

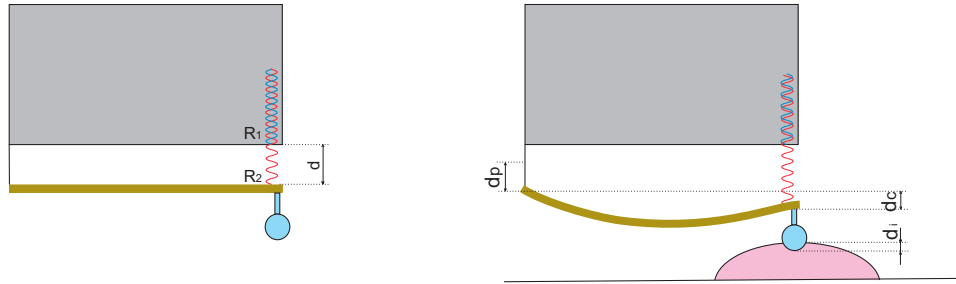


Figure 3.6: The phase of the interference pattern of the waves reflected at R1 and R2 changes as a result of the bending of the cantilever when it indents the sample. On the right, cantilever deflection d_c and indentation depth d_i as a result of a piezo displacement d_p .

Chiaro nanoindenter operating modes

The Chiaro nanoindenter can operate in three different modes: “Displacement”, “Load” or “Indentation”. The “Displacement” mode works in open loop: the piezo

moves as indicated in the input profile, and the corresponding cantilever bending is recorded. However, in “Load” and “Indentation” modes, the recorded cantilever bending is instantaneously fed back to the piezo displacement, so that the force and indentation depth, respectively, are fixed, while the other quantity is provided as an output. The relationship between piezo displacement d_p , cantilever bending d_c and indentation depth d_i is $d_p = d_c + d_i$, illustrated in Figure 3.6.

For each indentation, a *.txt* file is written with a (33 lines) header with the date and time of the experiment, the probe’s stiffness and tip radius, the input profile, and then in columns: (1) the time evolution (every 1 ms, 1 kHz sampling frequency), (2) the imposed piezo displacement, (3) the recorded cantilever bending, the calculated (4) indentation depth $d_i = d_p - d_c$ and (5) the load values. The load F is calculated from the recorded cantilever bending, taking into account the cantilever stiffness k , with a minus sign (Hooke’s law, $F = -kd_c$).

Mechanical characterisation tests

A single F11 cell indentation in “Displacement” mode is shown in Figure 3.7. The probe starts out of contact ($\sim 5 \mu\text{m}$), where the probe is recording zero load and the approaching phase starts (in blue in Figure 3.7). From the point of contact (indentation=0), the load starts to increase as the cell is indented, until the holding piezo displacement value is reached and maintained (in purple in Figure 3.7). Then, the probe starts to withdraw, recording smaller values of the force until the contact is lost again (in green in Figure 3.7).

The experimental force and indentation data over time can be analysed with a range of models to derive the material mechanical properties of the studied sample. Briefly, as the loading and unloading parts do not overlap (blue and green curves in Figure 3.7), it means that the sample is not purely elastic, but instead viscoelastic, and as a result not all the energy that is input is recovered after the indentation, i.e., there is an energy loss (equal to the difference of areas between the loading and unloading curves).

The mechanical behaviour of soft biological materials such as the cell studied in

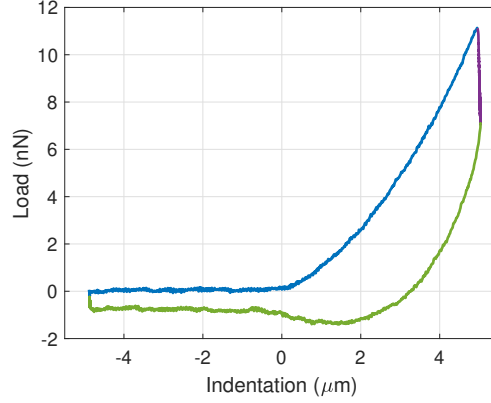


Figure 3.7: Force-indentation curve for a 10 μm piezo displacement in Displacement mode. Phases are differentiated with colours: in blue the approaching, in purple the holding and in green the retracting one.

Figure 3.7 is commonly extracted from the loading part of the force-indentation curve, which represents the properties of the virgin material, returning a constant modulus value regardless of the maximum load (or displacement) chosen for the measurements [115]. The most common theory to analyse the loading part of nanoindentation data acquired with a spherical tip is the Hertz model, which assumes that for small enough deformations, an isotropic material behaves as linear elastic (see Chapter 4).

To further characterise the viscoelastic response of solids, stress-relaxation and

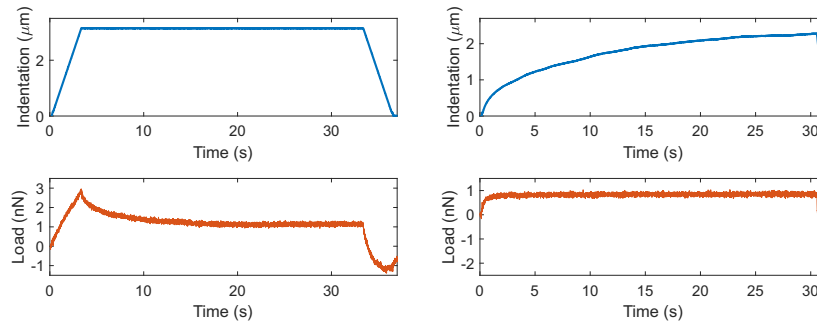


Figure 3.8: Stress-relaxation (left) and creep (right) experiments on a F11 cell body.

creep are standard tests that consist in maintaining a constant strain (or indentation depth), while recording the stress (or load) relaxation (Figure 3.8-left), or holding the load constant and measuring the displacement (Figure 3.8-right), respectively. By fitting the relaxations, both tests extract viscoelastic information that allow

defining an adequate mechanical model for the material under study. Most biological materials have a spectrum of relaxation times, corresponding to different subcellular components being excited and relaxed at specific indentation rates, hence the need to characterise cells at different indentation frequencies.

The complex viscoelastic behaviour of a material is commonly determined from its response to oscillations of small amplitude and varying frequency. Dynamic Mechanical Analysis (DMA) can be conducted in any of the modes, imposing indentations of constant small amplitudes at different frequencies (see Figure 3.9). By fitting the indentation and force time signals, DMA allows for the identification of the sample's complex modulus $E(f) = E'(f) + iE''(f)$, where $E'(f)$ and $E''(f)$ are the storage and loss moduli, respectively, as a function of the frequency f . These moduli are *a priori* frequency dependent, *i.e.*, the elastic and viscous behaviours of the material vary with the loading rate. For implementation details, see Chapter 4. Figure 3.9 shows the indentation and load responses of a F11 cell body indented 2 μm statically, with an additional 500 nm oscillating displacement at the frequencies of 0.1, 0.316, 1, 3.16 and 10 Hz, with the inset illustrating the phase lag δ between the imposed indentation and the recorded load response at 0.1 Hz. δ is 0° for a purely elastic material, 90° for a purely viscous material, and in-between for any viscoelastic material.

Chiaro nanoindenter experimental protocol

Depending on the mechanical characterisation test to be performed, the specific probe (stiffness and bead size), the indentation mode and whether the test is a static test or a dynamic test should be chosen. The indentation protocol is then as follows:

1. Wet the probe to be used in experiments, so that no bubbles are formed in the Fabry-Perot cavity (see Appendix A).
2. Carefully connect the probe to the Chiaro arm and gently immerse it in warmed bath solution in a glass bottom dish with no cells.

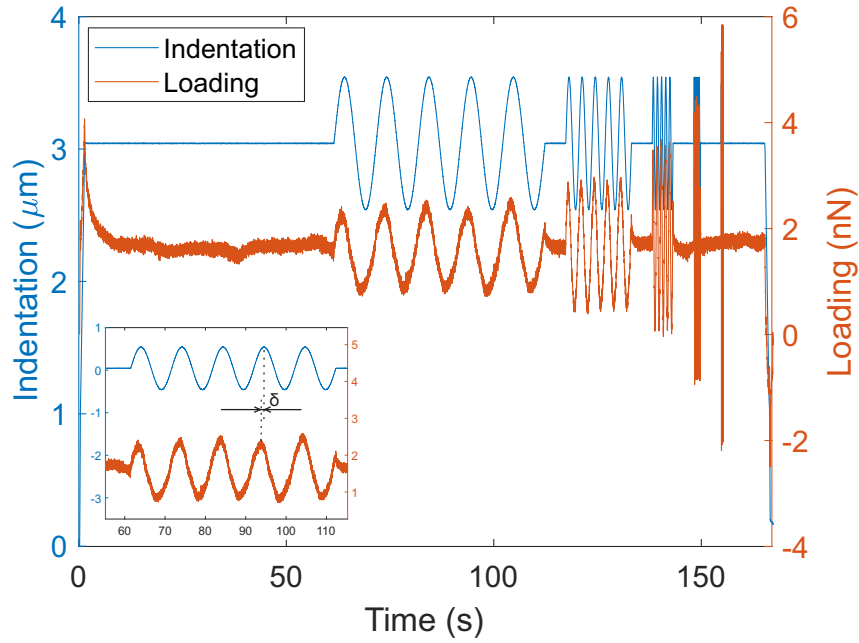


Figure 3.9: Imposed indentation (blue) and recorded load (orange) during a DMA test of a F11 cell body at 0.1, 0.316, 1, 3.16 and 10 Hz. Inset: 0.1 Hz frequency zoomed in, showing the phase lag δ between the imposed indentation and the recorded load.

3. Switch on first the OP1550, then the controller, and finally the PC. Open the Piuma software.
4. Introduce the probe stiffness and tip radius parameters in the Piuma software, select the indentation mode and define the indentation profile.
5. The calibration of the probe consists of 3 steps: (1) wavelength scan, in which a correction factor is calculated, depending on the medium's refractive index, (2) find surface, in which the probe approaches the glass surface and automatically retracts 3 μm when the contact is made, and (3) probe calibration, in which, through two consecutive indentations, the distance of the cavity and sensitivity are automatically calculated (see Appendix A). Now the probe is calibrated for the bath solution.
6. Retract the probe and replace the dish with one with F11s in 2 mL warm bath solution.

7. Select the cell of interest, approach the Chiaro probe and perform the indentation. From each indentation, a *.txt* file is recorded (and an additional one if a DMA is performed), which is then opened and processed in MATLAB.
8. The DataViewer software (Optics11) allows to load and visualise the recorded indentation curves, automatically fitting them and extracting the elastic storage and loss moduli.

High frequency dynamic module

The Chiaro nanoindenter was purchased with an extra customised high frequency (HF) module, consisting on a HF shear piezo (PL5FBP3, Thorlabs). The resonance frequency of this piezo is 1,900 kHz (above the maximum driving frequency of the signal generator used in this work) and the allowed maximum input voltage is 200 V. The shear configuration was adopted because in shear (longitudinal direction, see Figure 3.10), the system has a comparatively very high stiffness, and the cantilever bending response can be neglected in the probe-sample interaction. In other words, the nanoindenter bead horizontally pushes the cell as much as the HF piezo itself moves, which is in turn controlled by the signal generator. Longitudinal indentation depth is then the same as the HF piezo movement, as in this case there is no cantilever deflection. A HF piezo in the compression configuration was also available (see Figure 3.10-left), but never used, as in this configuration the cantilever displacement due to the HF piezo displacement could not be decoupled from the one arising from the sample interaction, so that the HF piezo could not be universally calibrated.

This HF module operates in open loop, hence a calibration is needed in order to identify the HF piezo's frequency-dependent response. As such, it is not possible to set a desired amplitude of displacement at a certain frequency; instead, the HF piezo's natural resonances need to be used. Knowing how the piezo moves is (because of the system's high stiffness in shear) equivalent to determining how the attached probe moves, and thus how the cell is being loaded in shear. Calibration of the HF shear probe is explained in Appendix A.

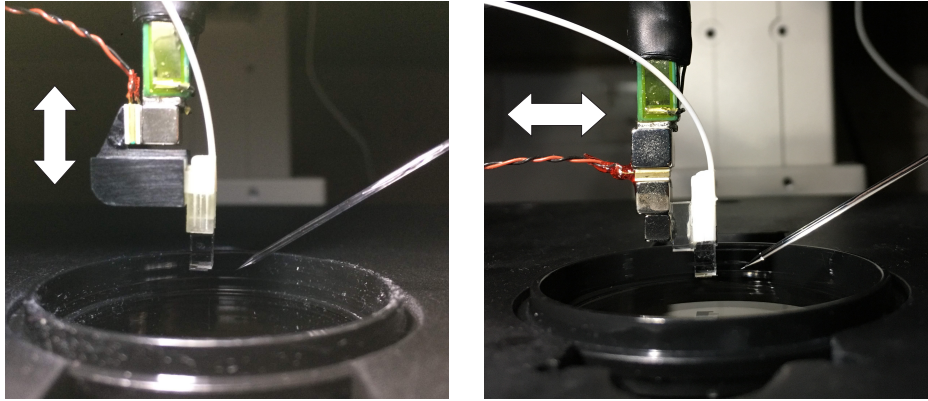


Figure 3.10: Low frequency compression (left) *vs.* high frequency shear (right) Chiaro probes. Even though the holder on the left also has a high frequency piezo in the compression configuration, it was never used and always remained disconnected.

3.4 Experimental electrophysiology

3.4.1 Patch clamp

Patch clamp is the technique of choice for single cell electrophysiology studies. It allows the detection of very small signals such as single ion channel currents or sub-threshold membrane voltage depolarisations in individual cells in culture, within a tissue, or even in artificial membranes. Spontaneous activity can be recorded, as well as induced. In this work, voltages were recorded with an Axopatch 700B amplifier and digitised with a Digidata 1550A data acquisition system, using the pCLAMP 10.5 software (Molecular Devices). The voltage signal was sampled at 20 kHz and low-pass filtered at 10 kHz.

A patch clamp setup consists of a hollow glass tube with a tip of a few μm in diameter, known as micropipette or patch pipette, filled with a solution that mimics the cell's native intracellular solution, and a recording metal electrode.

Whole cell electronics

Whole cell patch clamp involves recording currents through multiple (in theory all) channels simultaneously, over the membrane of the entire cell. This is achieved through cell membrane rupture after gigaseal ($>1\text{ G}\Omega$) formation between membrane and pipette, which provides direct access to the cell cytoplasm and its ions. Rupture of the membrane is usually achieved by mouth suction, through consecutive short

and sharp pulses. If this is not enough to break the patch, short current pulses can be sent *via* the “zap” function Multiclamp (the patch clamp amplifier software), that break the membrane by electroporation. Both methods could also be combined, but mouth suction is much gentler to the cell and hence recommended [114]. After membrane breakage, the cell’s cytoplasm and pipette’s electrolyte start to dissolve or dialyse, until a new equilibrium is established after a few min. In order to minimise alterations to the cell’s cytoplasm, the pipette solution must resemble as much as possible the cell’s intracellular one (the compositions of the intracellular and extracellular F11 solutions used in this project are given below). Through the electrode in the pipette, one now has access to the inside of the cell, and is able to measure and induce currents (and the corresponding voltage differences) from and into the cell, respectively.

It is important to note that currents sent and felt by the cell are ionic currents, which need to be transduced into electric or electron-based currents used by the electronic instruments. To do so, the patch clamp technique uses Ag/AgCl (silver-silver chloride) electrodes, consisting of a silver wire coated with a thin layer of silver chloride. The coating was achieved by dipping a pure Ag wire in bleach and leaving it overnight.

The Ag/AgCl electrode functions as a redox electrode [114]. When an ionic current reaches the electrode, Ag atoms in the electrode oxidise to Ag^+ (dissolution of the metal) and liberate electrons e^- , that travel up the wire. Ag^+ binds to Cl^- in solution to form AgCl:



On the contrary, when an electric current is sent through the Ag wire, Ag undergoes reduction: Ag^+ from AgCl in the coating accepts an electron, reduces to Ag (deposition of the silver ions in the wire) and liberates Cl^- to the electrolyte:



Ag is used, instead of other metals such as Au or Pt, due to its smaller redox potential, which facilitates the exchange from ions (charge carriers in the electrolyte)

to electrons (charge carriers in the electric wire) [114].

Figure 3.11 shows the equivalent circuit of a patched cell in whole cell configuration. Through the patch electrode, one should, in principle, be able to read the cell membrane potential V_m with a voltmeter, or the current coming from the cell I_m with an amperemeter.

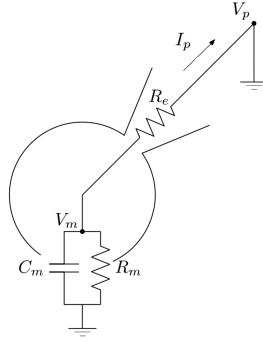


Figure 3.11: Equivalent electronic circuit of a patched cell. Adapted from [130] with permission.

However, cellular currents are very small and thus need to be amplified by a designated electronic circuit. Second, the recording electrode has a non-negligible resistance R_e , indicated in Figure 3.11, which causes a voltage drop $V_e = I_m R_e$ when the cell current passes through it, resulting in a voltmeter readout of $V_p = V_m - V_e$, instead of the true V_m . Equivalently, the cell is clamped at a reduced voltage, compared to the desired one. In addition, the glass pipette functions as a capacitor, separating the intra- and extracellular solutions, which introduces further errors in the measured or sent signals. In order to correct for these, different compensation electronic circuits are needed. Furthermore, as the main operating modes of whole cell patch clamp consist in fixing either the voltage or the current, a feedback control is needed. All these circuitry is based on an operational amplifier (the so-called patch clamp amplifier), commonly used to process analog signals. Its working principle, as well as the explanations behind all the necessary signal corrections (listed in the protocol below) are described in detail in Appendix B. Many of these corrections involve positive feedback circuits whose misuse can directly kill

the cell. The hardware connections and corresponding software configurations are also included in Appendix B.

Whole cell protocol

The complete patch clamp protocol, with the necessary compensations (thoroughly discussed in Appendix B) is as follows:

1. Thaw and 0.22 μm -filter 500 μL of intracellular solution (whose composition is listed below).
2. Pull fresh pipettes on the day (pulling parameters shown in Table 3.1), with 1–5 $\text{M}\Omega$ resistance values measured in the bath solution.
3. Back-fill the pipettes with intracellular solution to avoid bubble formation in the tip.
4. Remove the differentiation media from the cultured dish, wash with PBS once to remove the remaining serum (that would otherwise prevent gigaseal formation) and add 2 mL of warmed bath solution (composition listed below).
5. Place the ground electrode in the dish, immersed in the bath solution, and the pipette in the pipette holder.
6. Initialise Clampex and Multiclamp softwares, for the digitiser and amplifier, respectively.
7. Apply a slight positive pressure of 1-2 kPa and immerse the tip in the bath solution. This prevents tip contamination from cell debris. Pipette resistance should be between 1 and 5 $\text{M}\Omega$, as indicated in the “Membrane Test” mode, that sends voltage steps of known value and calculates pipette resistance values by recording and fitting the decay of the induced current responses.
8. Set a new experiment, zero the pipette offset and zero the pipette (C_p fast) capacitance. This should remove the fast capacity transients coming from the pipette capacitance (depicted in Figure 3.12).



Figure 3.12: Top: Pipette capacitance transients appear in the recorded current trace when a voltage step is held between the electrodes (“Membrane Test”). Bottom: These transients disappear when C_p fast is compensated. Adapted from [114] with permission.

9. Slowly approach the pipette to the cell soma, first using the coarse control of the manipulator, then switching to the fine one when close to the cell. When in contact, the pipette resistance increases slightly. The electrode is pressed against the membrane until the readout resistance experiences a 0.2 M Ω increase, after which the positive pressure is released and a small negative pressure or suction (2 to 5 kPa) is applied to pull the patch of cell membrane inside the electrode tip. The suction causes the cell to form a tight seal with the electrode, called a gigaseal when it reaches 1 G Ω .
10. When the resistance value reads 200 M Ω , release the negative pressure.
11. If a gigaseal (>1 G Ω) is not formed within the first two minutes, discard cell and pipette. If a gigaseal is formed, apply short and sharp negative pressure pulses by mouth suction. If these are not enough to break the patch, send 50 ms current pulses *via* the “zap” function of the Multiclamp software. When the membrane is ruptured, slow capacity transients appear in the recorded current trace (Figure 3.13), corresponding to the membrane capacitance, and indicating whole cell configuration.
12. Once in whole cell, the C_p slow (membrane capacitance) is compensated. For a VC experiment, C_p and R_e are compensated, the latter at least a 50%. For a IC experiment, the bridge is balanced and C_p compensated by the tuning

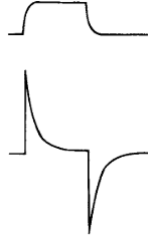


Figure 3.13: Membrane capacitance transients appear when the membrane is ruptured (bottom trace), indicator of whole cell mode. Adapted from [114] with permission.

function of the Multiclamp software (increasing C_p until extra peaks appear in the trace, coming from the oscillation of the positive feedback circuit).

Intra- and extracellular solutions and pipette pulling parameters

The intracellular or pipette solution contained 140 mM KCl, 5 mM NaCl, 0.1 mM CaCl_2 , 2 mM MgCl_2 , 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM guanosine-5'-triphosphate sodium salt hydrate, 2 mM adenosine triphosphate magnesium salt hydrate (mg-ATP) and 1 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), with pH adjusted to 7.3 by addition of KOH and osmolarity adjusted to 290 mOsm/L by glucose addition. The intracellular solution was aliquoted into 500 μL , stored at -20°C and filtered through a 0.22 μm filter after thawing, on the day of experiments.

The extracellular or bath solution contained 130 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM glucose and 10 mM HEPES, with pH adjusted to 7.4 by addition of NaOH and osmolarity adjusted to 300 mOsm/L by glucose addition. The bath solution was filtered through a 0.22 μm filter, aliquoted into 5 mL and stored at 4°C . All reactants were purchased from Sigma Aldrich.

When the pipette, filled with intracellular solution, is immersed in the bath solution, before patching the cell, as a result of the different concentration of ions in both electrolytes, a liquid junction potential (LJP) builds up at the tip of the pipette, which depends on the rate of diffusion of the ions from the more concentrated to the less concentrated solution and the ions' mobilities. However, this potential is zeroed when the pipette offset (voltage difference between recording and ground

electrodes) is zeroed at the beginning of the recording. After the gigaseal is formed and the membrane broken to reach whole cell mode, the LJP disappears as now the solutions in contact are both the intracellular ones, but as the LJP has already been zeroed previously, the recorded cell voltage values are now overestimated (more depolarised), and the value of the LJP has to be subtracted from them. The LJP between the solutions is experimentally measured by zeroing the pipette offset in I0 mode when dish and pipette both contain intracellular solutions, and then the dish solution is replaced with the extracellular one. After a few minutes of stabilisation, the new potential offset is the value of the LJP, -11.9 mV for the above solutions. The measured membrane potentials will then need to be corrected as: $V'_m = V_m - LJP$.

Pipettes between 2 and 5 M Ω were pulled from thick borosilicate glass capillaries (BF100-78-10, Sutter Instruments) through a 2.5 $\times$ 2.5 mm² box filament with a P-1,000 puller (Sutter Instruments), following optimised parameters from Ref. [324], see Table 3.1. The resultant low resistance pipettes allow a better electrical access to the inside of the cell.

Loop	Heat	Pull	Vel.	Delay	Pressure
1	500	0	20	255	500
2	500	0	20	255	500
3	500	0	20	255	500
4	500	0	20	255	500

Table 3.1: Patch pipette pulling parameters.

3.4.2 Fluorescent probes

Two different fluorescent probes were also used in this project to measure cellular electrophysiology: an intracellular Ca²⁺ imaging reporter and a membrane voltage sensitive dye.

Oregon Green BAPTA for calcium imaging

Oregon Green 488 BAPTA-1 AM (OGB1, ThermoFisher) was used to measure intracellular free Ca²⁺ concentration changes over time. The BAPTA part of the

dye is a Ca^{2+} chelator whose spectral performance changes depending on how many free Ca^{2+} bind to it (exhibiting an increase in fluorescence upon Ca^{2+} binding). The AM ester is a liposoluble molecule that allows the dye to go through the cell membrane and reach the cytoplasm. If incubated for too long, it can go through the membrane of the nucleus too and accumulate there, in which case it will not report intracellular Ca^{2+} concentration.

In this work, Ca^{2+} imaging was used to confirm the presence of spontaneous activity (indicator of neuronal maturation) of F11 cells differentiated for at least 4 days, and to determine the optimal differentiation day for electrophysiological experiments (see Chapter 4 for results). Because of previous successful results with this cell line, OGB1 was used as the Ca^{2+} probe for Ca^{2+} imaging experiments. A concentration of 4 μM OGB1 in Ca^{2+} -free PBS was directly added to the glass bottom cell culture dishes containing mature F11 cells and incubated for 30 min at 37° C and 5% CO_2 . The media was then replaced with 2 mL bath solution, and the sample incubated for another 30 min. OGB1 is excited at 494 nm (using a blue LED) and emits at 523 nm (green). Therefore, a standard FITC combination of filters was used in the Nikon Ti-E inverted microscope for widefield imaging. The recording consisted of images taken every 100 ms, which ensured a sufficient signal-to-noise ratio for Ca^{2+} spike detection [325], while cells were continuously illuminated at 494 nm.

FluoVolt as a voltage sensitive probe

The FluoVolt membrane potential dye (ThermoFisher) is a potentiometric probe, which chemically determines the concentration of specific components of an electrolyte solution, in turn being able to measure the electrical potential. This dye was used to determine the extent to which the patch was clamping a cell, the so-called space clamp problem: the voltage evolution of different parts of a patched cell subject to a voltage clamp episodic stimulation was tracked. A 1:100 PowerLoad concentrate (component B of the kit) and 1:1,000 FluoVolt dye (component A of the kit) loading solution was prepared in FluoroBrite-DMEM

imaging medium (FB-DMEM, ThermoFisher). 2 mL were added to a dish of F11 cells differentiated for 5 days and incubated for 30 min at room temperature, after which the loading solution was removed and replaced with 2 mL of warm FB-DMEM. Standard FITC settings, with a frame taken every 100 ms, were used to visualise the voltage evolution of different parts of a patched cell. Results are presented in Chapter 4.

3.5 Combined experimental mechanics and electrophysiology

The final arrangement of both nanoindenter and patch probes went through several optimisation steps until the last configuration was achieved. The Chiaro was initially installed in the microscope stage forming 90° with the patch pipette (see Figure 3.14), as this was the configuration for which both probes, as well as the microscope stage, had the largest freedom of movement, and so the widest field of view was covered.

However, depending on where the cell under study was located within the

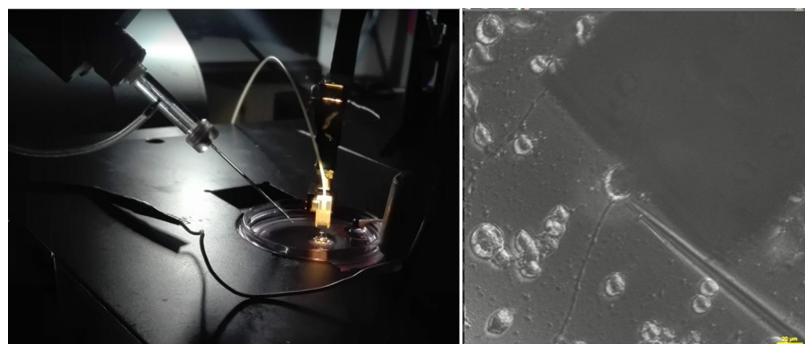


Figure 3.14: Nanoindenter and patch pipette initial 90° arrangement. Right: In some cases, depending on the cell's location within the dish, probes clashed at the level of the cell. Scale bar is $20\ \mu\text{m}$.

dish, the pipette holder and the Chiaro probe holder came into contact above the microscope stage, preventing their tips to come closer together and reach the cell, or their tips clashed at the level of the cell (see Figure 3.14-right). To circumvent this, as well as to maximise the distance between nanoindenter and pipette when both probes reached a cell body, the Chiaro was moved so that it faced the pipette. In the new arrangement, the probes approached the cell from opposite (180°) sides (see Figure 3.15-left and centre). Although this compromised the available working area for both probes, it promoted seal stabilisation during indentation.

A temperature and CO_2 control system for live cell imaging was used originally (H301-K-FRAME, Okolab), with the lid removed (see Figure 3.16). However, the CO_2 bubbling and the temperature feedback electronic system introduced

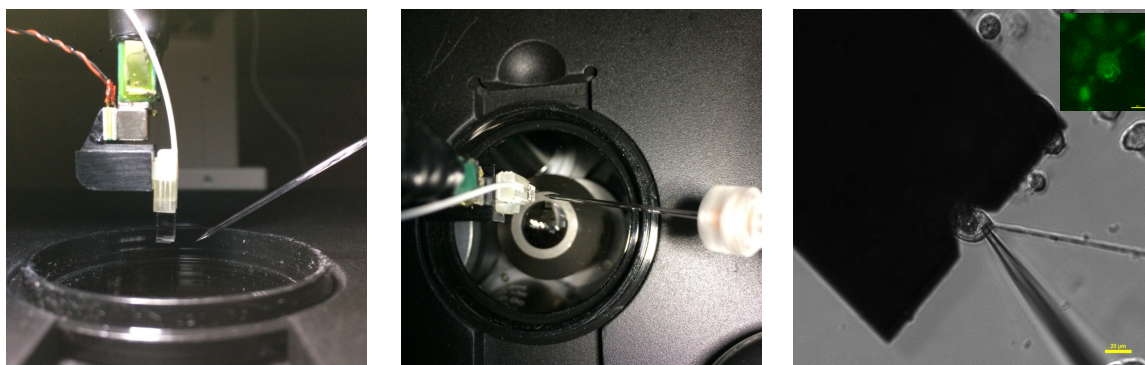


Figure 3.15: Left and centre: Nanoindenter and patch pipette final 180° arrangement. Right: both probes on the same F11 cell body. Inset: fluorescent image of a single cell with both probes. Scale bars are 20 μm .

mechanical and electrical noise in the nanoindenter and patch recorded signals, respectively, so it was decided to no longer use it.

The protocol for simultaneous mechanical and electrophysiological experiments



Figure 3.16: Temperature control chamber, eventually not used as it introduced electrical and mechanical noise.

is as follows:

1. Initialise all instruments. Nanoindenter (linearised or nonlinearised output, for low and high frequency experiments, respectively) and patch electrode signals are both connected to a PicoScope 4262 (Pico Technology) oscilloscope, sampled at 2 kHz.
2. Wet the Chiaro tip and calibrate it in a glass bottom culture dish without cells with 2 mL of warm extracellular solution.

3. Remove the calibrating dish and replace it with a dish with mature F11 cells in 2 mL warm extracellular solution that have been differentiating for at least 5 days.
4. Select a cell for study in the vicinity of the Chiaro: spherical and well-contrasted cell, with the soma isolated from those of other neurons and showing long neurites.
5. Approach the Chiaro close to the cell body.
6. Approach the pipette (coarsely first, then finely) and patch the cell at the corresponding edge of the cell body, leaving room for the Chiaro bead.
7. If a good seal is achieved and the cell shows the electrophysiological characteristics of interest (mainly spontaneous activity, see Chapters 5 and 6), start the PicoScope recording, bring the Chiaro into contact through the smallest possible steps (first 1 μm , then 0.25 μm) and start the indentation, while record the cell voltage in I0 mode.

Chapter 4

F11 cells characterisation

4.1 F11 cells morphological characterisation

Under microscope inspection, undifferentiated F11 cells show a spindle-like shape and few show short neurite-like processes (see Figure 4.1). They tend to cluster together and start forming extracellular matrix when $\sim 80\%$ confluent.

After 1 day in differentiation media, cells start to exhibit a mature neuronal

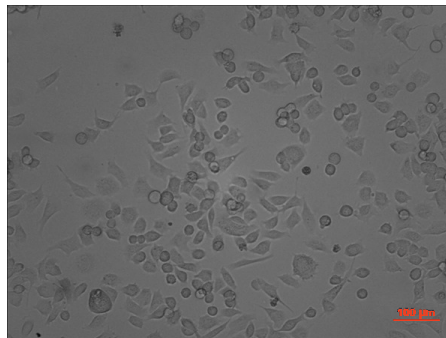


Figure 4.1: Undifferentiated F11 cells. Scale bar is 100 μm .

morphology and grow dendrites and neurites [313], see Figure 4.2. These neurites grow longer and increase in number, and after 5 maturation days the cells are well connected and form networks (see Figures 4.2, 4.3 and 4.4). After 7 days in differentiation media, some neurites first, and then their cell bodies, start detaching from the glass.

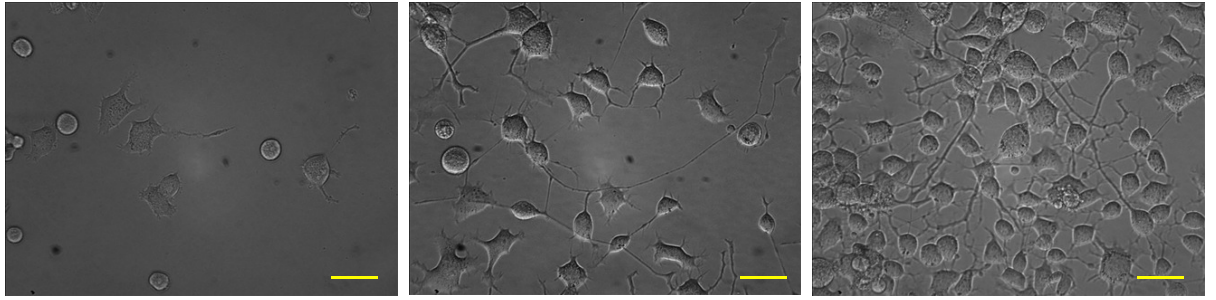


Figure 4.2: From left to right, F11s in differentiation media for 1, 3 and 5 days. At day 1, there are still some cells that have not yet spread out in the glass. Scale bars are 50 μm .

Brain tissue is the softest tissue in the human body [163], and so are individual

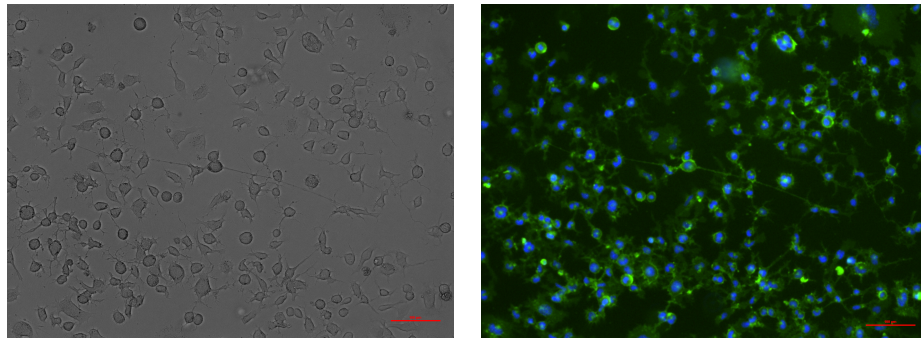


Figure 4.3: Bright field image and fluorescence image of the same population of F11s. Membrane shown in green and nuclei in blue (Cell Mask and NucBlue stainings, from ThermoFisher). Scale bars are 100 μm .

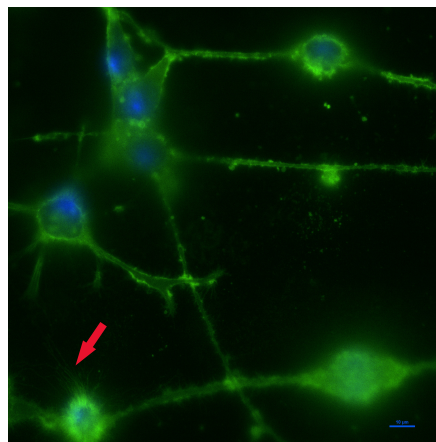


Figure 4.4: Single frame of a live z-stack image acquisition of F11 cells at 60 $\times$ magnification. Membrane (in green) stained with Cell Mask (ThermoFisher) and nuclei (in blue) with NucBlue (ThermoFisher). Dendrites of the bottom left cell are indicated by the red arrow. Scale bar is 10 μm .

neurons. When soft cells are cultured in stiff substrates such as glass, their cell

bodies adopt a more spherical configuration with low level of spreading [326], as confirmed by 3D-reconstructed cell bodies from live widefield fluorescence z-stacked images, see Figure 4.5. Live widefield fluorescence microscopy was used for this analysis in order to avoid possible cell morphology alterations induced by the cell fixation process, required for confocal imaging. Cells were incubated with CellMask Green Plasma membrane live stain (ThermoFisher) for 15 min at 1:1,000 dilution, then live imaged using a standard FITC filter. Z-stacks were acquired every $0.025\ \mu\text{m}$ with the NIS-element AR software (Nikon). The images of two cells were 3D-deconvoluted in AutoQuant x3 (Bitplane), their cell bodies cropped and their surface objects created with Imaris 7.6.5 (Bitplane), in order to extract their soma morphologies (see Figure 4.5). Their analysis confirmed visual observations results for mature (rounded somas showing extensive neurites) F11 cell bodies sizes: average cell diameter of $21.49 \pm 3.24\ \mu\text{m}$ ($N = 100$), and average cell height of $20.01 \pm 2.52\ \mu\text{m}$ ($N = 12$, from z-stack images).

Confocal imaging was used to perform immunohistochemistry essays with 1:400 NeuN (Abcam, ab177487) antibody, a neuronal maturation marker. Results confirmed NeuN expression of F11 cells showing spherical somas and long neurites from their 5th differentiation day, revealing F11 cell maturation after 5 days in differentiation media, see Figure 4.6.

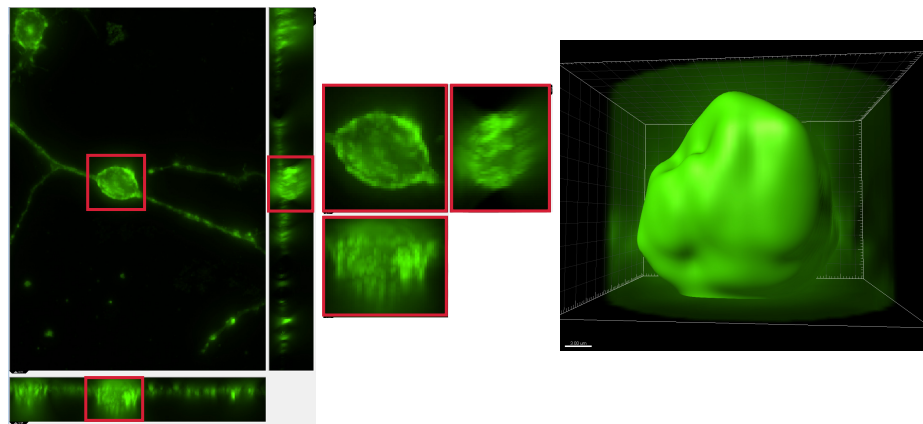


Figure 4.5: Live 3D fluorescence imaging of a F11 cell loaded with CellMask membrane dye, and surface reconstruction of its cropped soma. Scale bar is $3\ \mu\text{m}$.

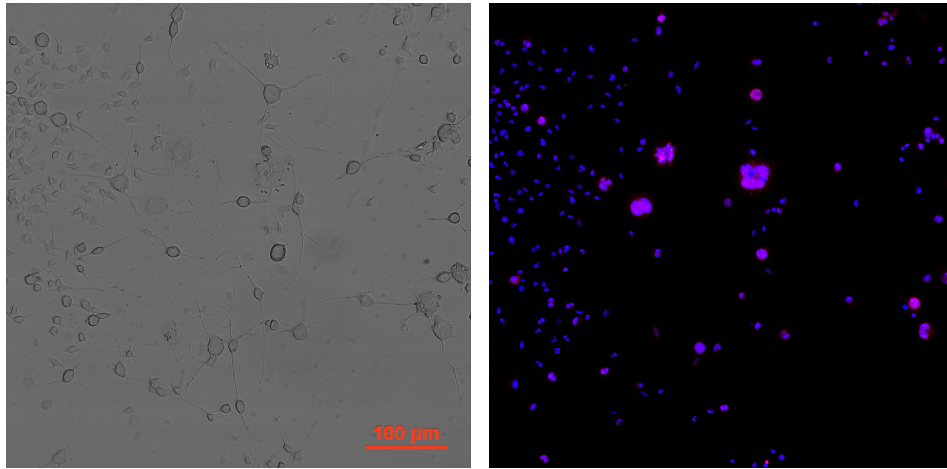


Figure 4.6: NeuN marker (in magenta) mainly colocalises with NucBlue (a nuclei dye, in blue) in mature F11 cells that show spherical cell bodies and long neurites, while it is not expressed in small cells with no neurites, see top left corner. Cells had been differentiated for 5 days. Scale bar is 100 μm .

4.2 F11 cells mechanical characterisation

F11 neuronal cells can be considered, as a first approximation, as nonlinear viscoelastic [327]. As such, their mechanical response varies with the indentation depth and rate, due to the different mechanical natures of the membrane, membrane cortex, cytoplasm, organelles and nucleus. However, for small enough strains, the cell viscoelastic response can be considered as linear as a first approximation [328].

Herein, the results of quasi-static and dynamic indentations of F11 cell bodies are presented, as well as the numerical methods used to analyse the data. Taking as inputs the nanoindentation force and indentation curves, custom MATLAB scripts were developed to (1) determine the cell's Young's modulus, (2) its linear viscoelastic region, (3) fit the exponential decay of stress-relaxation experiments and (4) extract the storage and loss moduli at each indentation frequency from DMA experiments.

4.2.1 Quasi-static response

The mechanical behaviour of soft biological materials is commonly extracted from the loading part of the force-indentation curve. The most common theory to analyse the loading part of nanoindentation data acquired with a spherical tip is the Hertz model, which assumes that, for small enough deformations, an isotropic

material behaves as linear elastic, and the material responds with a force F to an indentation depth h following [329]:

$$F = \frac{4}{3} \frac{E}{(1 - \nu^2)} R^{1/2} h^{3/2} \quad (4.1)$$

where E is the Young's modulus, ν the Poisson's ratio (assuming that cells are incompressible: $\nu \approx 0.5$) and R the spherical indenter tip radius. A cell's Young's modulus is thus calculated by fitting the force-indentation curve of a quasi-static cell indentation with Equation (4.1).

The the force-indentation response of a 10 μm piezo displacement indenting a F11 cell $\sim 5 \mu\text{m}$ is shown in Figure 4.7. The loading region of the curve was fitted *via* nonlinear least squares regression (fit type $F = a \times h^{3/2} + F_0$, with F_0 being the value of the load at the point of contact) with a custom MATLAB code and the point of contact manually estimated from the curves, giving $a = 2.71 \times 10^{-8} \text{ Nm}^{-3/2}$, which, for a 10 μm spherical tip radius and a Poisson ratio of 0.5, results in a Young's modulus of 152.44 Pa for this cell (see Equation (4.1)). The obtained Young's moduli might be overestimated because of adhesion forces, but as this systematic error is present in all the samples and indentations, their direct comparison should not *a priori* be affected.

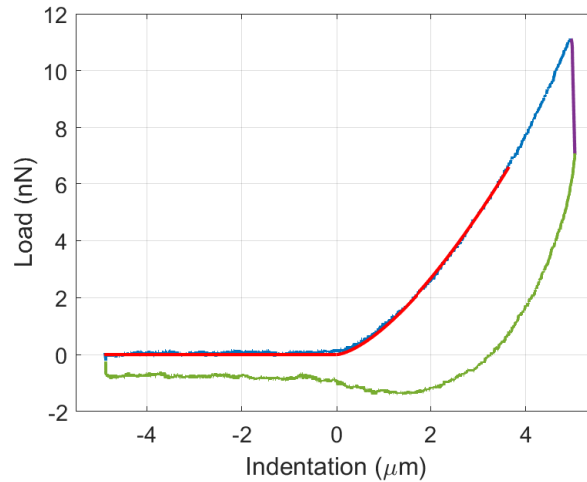


Figure 4.7: In red, the Hertzian fit of (the 60% of) a force-indentation curve for a 10 μm piezo displacement in “Displacement” mode. Phases are differentiated with colours: in blue the approaching, in purple the holding and in green the retracting one.

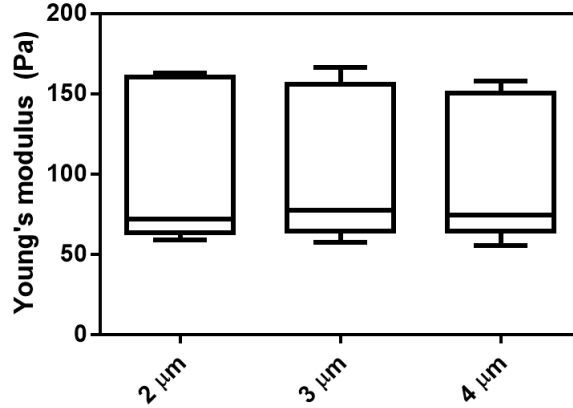


Figure 4.8: Boxplot showing the median, min and max fitted Young's moduli values for indentations of 2, 3 and 4 μm depth in N=9 cells.

If the indentation is too deep compared to the cell's height, substrate (glass) effects might be non-negligible, and the Hertz model does not apply. In addition, cell damage such as cytoskeletal components rupture [330] can occur during compression. In order to minimise these effects, the indentation needs to fall within the cell's Linear Viscoelastic Regime (LVR). The LVR of a material under strain is defined as that in which the Young's modulus can be calculated within a 10% variance. To identify F11s' LVR, cells were indented at increasing depths (2, 3 and 4 μm), giving them enough time (20 s) to relax after each indentation. For each case, the loading part of the force-indentation curve was fitted to the Hertz model. A boxplot of the fitted Young's moduli at indentations of 2, 3 and 4 μm for N=9 cells is shown in Figure 4.8. The Young's modulus of F11 cells at small indentations is between 55 and 166 Pa, in agreement with previous results on DRG elastic moduli values and other neuronal cell types [331, 332]. The obtained fitted curves and Young's moduli were in good agreement with those provided by the nanoindenter manufacturer's software for individual indentations. The variability on the measured Young's moduli comes from the different phenotypes present in F11s [316]. Of the studied N=9 cells, the Young's moduli at 3 and 4 μm fell within the 10% of the initial value at 2 μm , concluding that indentations up to 4 μm fall within the cell's LVR.

The viscoelastic behaviour of these cells needs to be studied by assessing their

relaxation responses. In Figure 4.7, during the holding phase indicated in purple, the indentation remains more or less constant, while the load decreases. As a result, the approaching (blue) and withdrawing (green) curves do not overlap, as it would happen for elastic materials. This is a typical viscoelastic response, that comes from specific cellular components relaxing at their characteristic time scales. Different cellular components are loaded at different strain rates, and hence obtaining a complete viscoelastic response of the cell requires a set of a dynamic tests at various indentation frequencies.

Stress-relaxation

In order to study the typical relaxation times of F11 cells, stress-relaxation experiments were performed. Similar viscoelastic information could be extracted from creep experiments, see Appendix C. In a stress-relaxation experiment, the stress (or force F , as measured by the Chiaro) exhibits an exponentially decaying response, see Figure 4.9 for the load relaxation responses of three F11 cells indented at 1.2, 2.4 and 3.4 μm depths for 30 s. By fitting this decay, the characteristic relaxation times corresponding to different viscoelastic relaxation mechanisms can be identified.

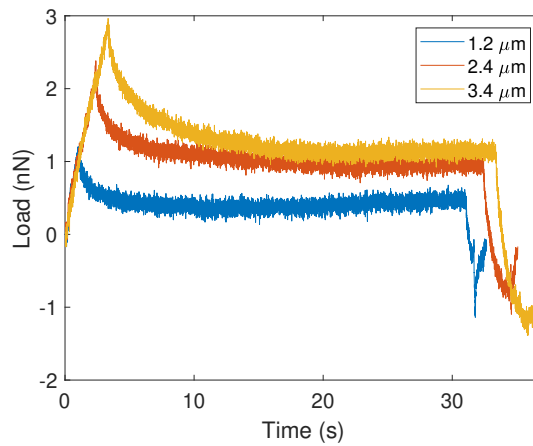


Figure 4.9: Stress-relaxation experiments on a F11 cell body indented 1.2, 2.4 and 3.4 μm for 30 s.

In this work, two methods were used to fit the stress-relaxation curves from constant strain (2 μm indentation depth) indentations in N=13 F11 cell bodies.

A single exponential decay was used to fit (*via* nonlinear least squares regression) the decay by fixing the peak and plateau values, and a double exponential [333, 334] fit (MATLAB’s “exp2”) was used by only fixing the plateau, see Figure 4.10. Results of the fits are included in Table 4.1. In the double exponential case, only τ_1 is physically relevant, as τ_2 essentially captures the drifting of the system.

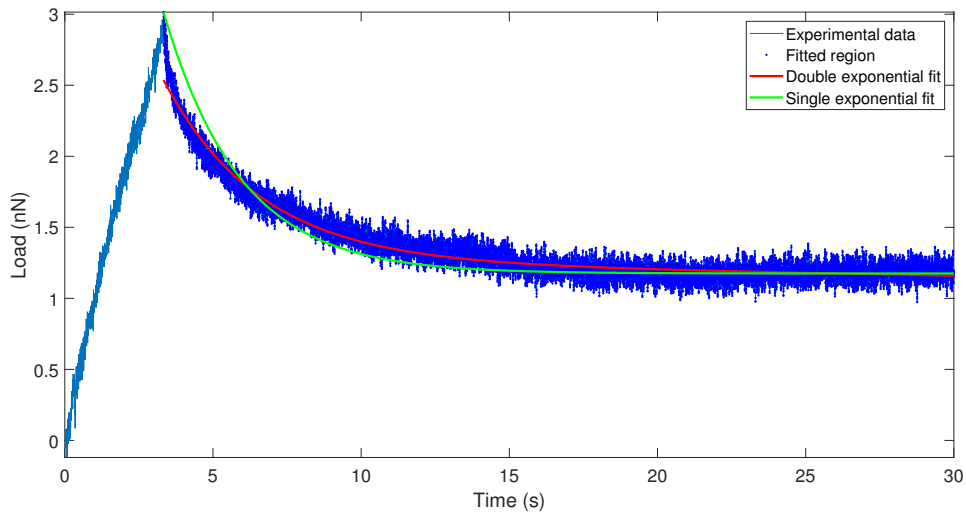


Figure 4.10: Single (in green) and double (in red) exponential decay fit of a stress-relaxation experiment from of a 2 μm F11 cell indentation for 30 s.

Fit type	τ_1 (s)	τ_2 (s)
Single exponential decay	2.77 ± 0.67	
Double exponential decay	2.29 ± 0.56	103.68 ± 94.98

Table 4.1: Fitted relaxation time values with one and two exponential decay functions.

4.2.2 Dynamic response

In order to mechanically characterise F11 cells for a wider frequency range, $N=7$ F11 cell bodies were studied through DMA, imposing indentations of oscillating amplitudes of 500 nm around a mean of 2 μm , at five different frequencies: 0.1, 0.316, 1, 3.16 and 10 Hz. The resulting force-indentation curves for each frequency f on a single cell are presented in Figure 4.11 and show larger forces (hence stiffer responses) and larger dissipations (area enclosed by the curves) at higher frequencies.

DMA allows for the identification of the material's complex modulus

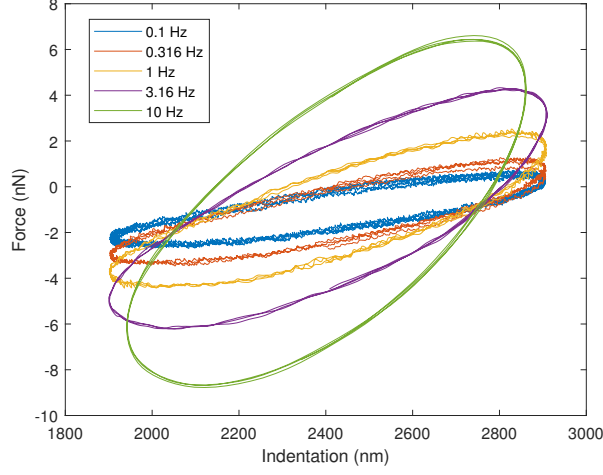


Figure 4.11: Force-indentation responses from DMA tests at 0.1, 0.316, 1, 3.16 and 10 Hz on a single F11 cell body. The cell responds with larger forces and dissipates more energy (area enclosed by the curves) at faster indentation rates.

$E = E' + iE''$, where E' and E'' are the storage and loss moduli, respectively. These moduli are *a priori* frequency dependent, i.e., the elastic and viscous behaviours of the material vary with the loading rate. E' and E'' at each indentation frequency can be calculated by fitting the experimental force F and indentation h curves at that frequency through the following relations [335]:

$$\frac{E'(\omega)}{(1 - \nu^2)} = \frac{F}{h} \cos(\delta) \frac{1}{2\sqrt{h_s R}} \quad (4.2)$$

$$\frac{E''(\omega)}{(1 - \nu^2)} = \frac{F}{h} \sin(\delta) \frac{1}{2\sqrt{h_s R}} \quad (4.3)$$

where δ is the phase lag between the indentation and force responses, h_s the static indentation depth and $\omega = 2\pi f$ the angular frequency.

Noting that the relation between a neuron's Young's modulus and shear modulus G follows:

$$G = \frac{E}{2(1 + \nu)} \quad (4.4)$$

for an isotropic material [336], one then obtains:

$$\frac{G'(\omega)}{(1 - \nu)} = \frac{F}{h} \cos(\delta) \frac{1}{4\sqrt{h_s R}} \quad (4.5)$$

$$\frac{G''(\omega)}{(1 - \nu)} = \frac{F}{h} \sin(\delta) \frac{1}{4\sqrt{h_s R}} \quad (4.6)$$

The force and indentation time signals shown in Figure 4.11 were fitted (with a custom-written MATLAB script through nonlinear least squares regression) to two sinusoids (see Figure 4.12) to obtain the fitted oscillation amplitudes for the imposed indentation and the measured load, as well as the phase of each signal to calculate the phase lag δ between them. The cell's storage and loss moduli and the phase lag between the signals were calculated at each frequency from these fitted parameters (mean values listed in Table 4.2). The dependence of the mean and standard deviation (SD) of the fitted moduli with the indentation frequency is presented in Figure 4.13, which shows that although the cell's viscous response increases with frequency, the elastic behaviour dominates over the studied frequency range. Note that the responses were independent of whether the indentations were performed in an increasing or decreasing frequency order, thus demonstrating that each frequency calibration is independent of the past history of the loading.

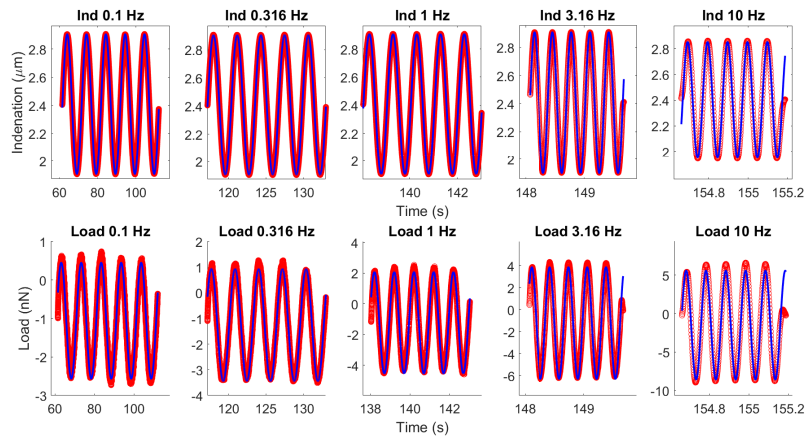


Figure 4.12: Sinusoidal fits (in blue) of the experimental (in red) indentation (top) and load (bottom) traces recorded at each frequency (f) for the DMA experiment of Figure 4.11. Signals fitted to $y(t) = A \sin(\omega t + \delta) + y_0$ by nonlinear least squares, with $\omega = 2\pi f$.

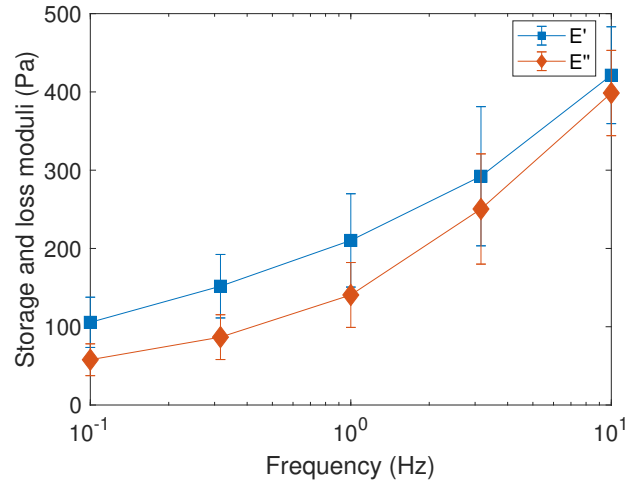


Figure 4.13: Mean and SD of N=7 F11 storage and loss moduli at DMA indentation frequencies of 0.1, 0.316, 1, 3.16 and 10 Hz.

Freq. (Hz)	E' (Pa)	E'' (Pa)	$\tan \delta$
0.1	105.63 ± 32.09	57.84 ± 20.33	0.55
0.316	151.79 ± 40.55	86.71 ± 28.62	0.57
1	210.29 ± 59.62	140.59 ± 41.46	0.67
3.16	292.29 ± 88.91	250.43 ± 70.43	0.86
10	421.36 ± 61.86	398.58 ± 54.48	0.95

Table 4.2: Storage (E') and loss (E'') moduli mean and SD, and $\tan \delta$ values at each DMA frequency.

4.3 F11 cells electrophysiological characterisation

This section first presents qualitative results of F11 functionality studied with two different fluorescent probes, after which quantitative electrophysiological measurements acquired with patch clamp are showcased. The numerical methods employed to analyse the recorded data are also explained.

4.3.1 Fluorescent probes

Two different fluorescent probes were used in this work to measure the cells' electrophysiology: an intracellular Ca^{2+} imaging reporter (Oregon Green BAPTA 1, ThermoFisher) and a membrane voltage sensitive dye (FluoVolt, ThermoFisher).

Oregon Green BAPTA for calcium imaging

Ca^{2+} imaging was used to confirm the presence of spontaneous activity (indicator of neuronal maturation) in differentiated cells. Due to previous successful results with this cell line, Oregon Green BAPTA 1 (OGB1) was used as the Ca^{2+} probe for Ca^{2+} imaging experiments.

Ca^{2+} imaging experiments were performed at differentiation days 4, 5, 6, 7 and 8 in order to study the cells' spontaneous activity on each differentiation day and determine the optimal day for further electrophysiological experiments. For each day, over 70 active cells were studied in a total of four different areas of the dish, and the experiment was replicated twice, resulting in over 150 cells per day.

In order to detect and count the spikes from their Ca^{2+} traces, several signal post-processing steps were developed and implemented in MATLAB. The raw fluorescent intensity trace recorded from a single F11 cell during a Ca^{2+} imaging experiment looks like that in Figure 4.14-A. As it can be observed, the OGB1 dye significantly photobleaches during a 5 min recording. This can be corrected with the “detrend” function from MATLAB, and the signal can be normalised. After a range of post-processing tests, the time derivative of the Ca^{2+} signal was found to be the most accurate way of identifying the Ca^{2+} spikes. In Figure 4.14-B, the

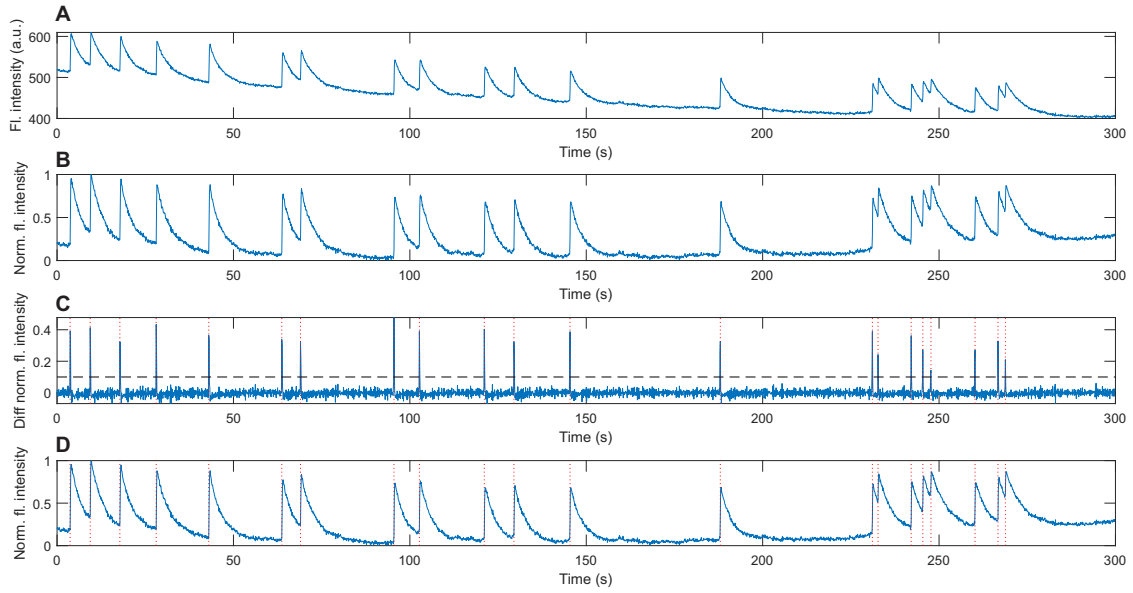


Figure 4.14: A) Raw Ca^{2+} imaging recording from a F11 cell, showing dye photo-bleaching. B) Normalised detrended Ca^{2+} signal. C) Time derivative of the normalised detrended Ca^{2+} signal, with the threshold set at 0.1 and the corresponding found peaks. D) Normalised detrended Ca^{2+} signal with the Ca^{2+} peaks detected.

time derivative of the normalised detrended signal is shown, together with the threshold at 0.1 and the peaks found through the “findpeaks” MATLAB’s function used with this threshold. In Figure 4.14-D, the normalised detrended signal is shown with its extracted Ca^{2+} peak locations.

The analysed average number of spikes per cell and per minute of the studied cells is shown in Figure 4.15, with their mean and SD values in Table 4.3. Statistical analyses of the post-processed Ca^{2+} spikes were performed with GraphPad Prism 6 (GraphPad Software). The normality of data distribution was checked with Shapiro-Wilk normality test, $\alpha=0.05$. As the data were not normally distributed, a non-parametric Kruskal-Wallis test with Dunn’s multiple comparisons *post-hoc* test was carried out to test the significance (defined with a threshold probability of 0.05).

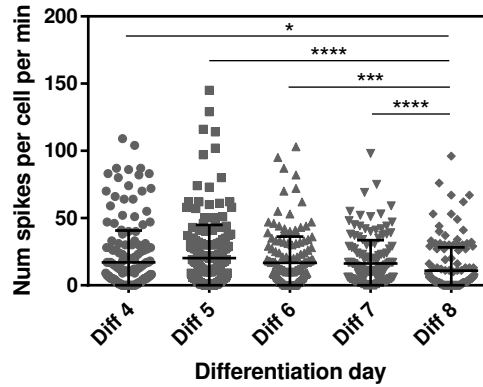


Figure 4.15: Number of Ca^{2+} spikes per cell per minute on differentiation days 4, 5, 6, 7 and 8. Adjusted p values are: *p = 0.0312, p*** = 0.0007 and ****p < 0.0001.

	Avg $N_{\text{Ca}^{2+}}/\text{cell}/\text{min}$
4 day diff	3.18 ± 4.17
5 day diff	4.02 ± 4.92
6 day diff	3.33 ± 3.93
7 day diff	3.26 ± 3.45
8 day diff	2.19 ± 3.46

Table 4.3: Mean and SD of the number of Ca^{2+} spikes per cell per minute in differentiation days 4, 5, 6, 7 and 8.

Figure 4.15 shows that mean F11 Ca^{2+} activity increases from day 4 to day 5, where it reaches a maximum, and then decreases. After 8 days in differentiation media, F11 cells show significantly less Ca^{2+} activity than the rest of the days. As these cells, especially long neurites, started detaching from the glass from day 7 onwards, cells on their 5th and 6th differentiation days (confirming protocols from Refs. [313, 316]) were used for further quantitative electrophysiological experiments (see Chapters 5 and 6).

Another experiment was conducted to test the time resolution of the OGB1 dye, combining Ca^{2+} imaging and patch clamp. The intracellular Ca^{2+} concentration of a patched cell was tracked while APs were induced by sending small (~ 40 pA) currents. Although Ca^{2+} dyes are commonly unable to resolve individual APs, Figure 4.16 shows single (albeit small) Ca^{2+} spikes generated from individual

induced APs in a patched cell body, demonstrating the potential of OGB1 to resolve single APs for firing frequencies below ~ 0.1 Hz.

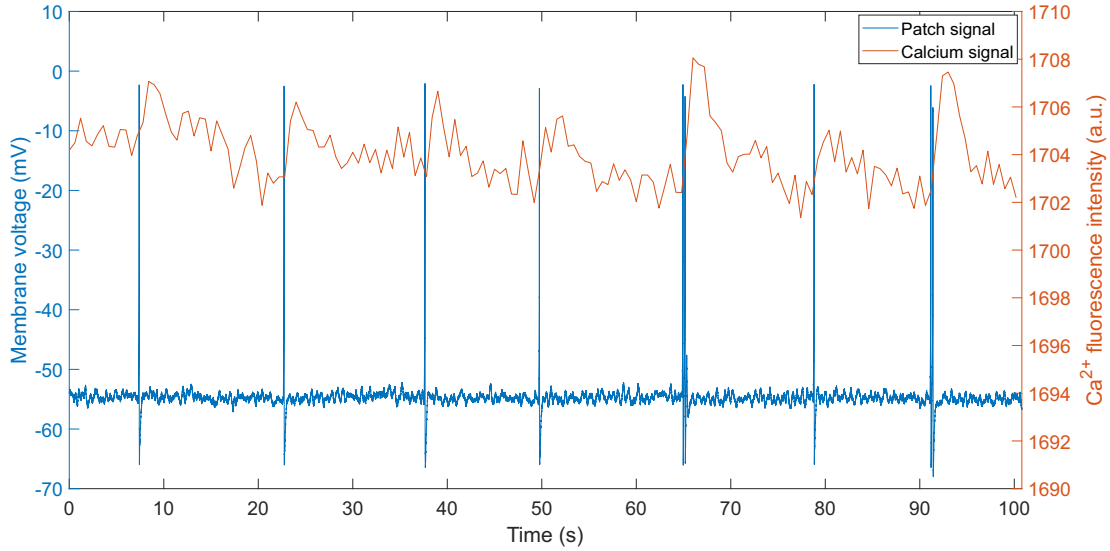


Figure 4.16: Ca^{2+} spikes (in orange) follow induced APs (in blue). Ca^{2+} spikes of individual APs are smaller than those of two consecutive APs (those at ~ 65 s and 91 s), but still distinguishable, especially if the first derivative of the fluorescence signal is calculated, as shown in Figure 4.14.

FluoVolt as a voltage sensitive probe

In order to determine the spacial extent to which the cell is clamped (the space clamp problem) in a whole cell experiment, the FluoVolt Membrane Potential Kit (ThermoFisher) fluorescent dye was used during a voltage clamp test. The episodic stimulation consisted of 10 voltage steps of increasing 20 mV amplitudes starting at -100 mV. The fluorescence intensity, used as the indicator of membrane voltage values, was quantified during hyperpolarisation and depolarisation in the soma and proximal regions of the neurites, see Figure 4.17. Results show that, even though a negative gradient in the voltage amplitude is observed, the activity remains the same in the soma within a 10%, and ionic currents occur, in phase, for all analysed regions.

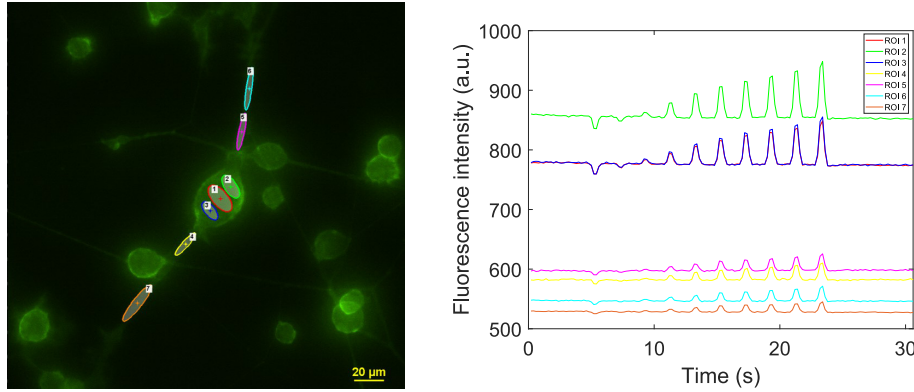


Figure 4.17: The voltage of all cell membrane regions follows a voltage clamp episodic stimulation of 10 sweeps of 20 mV increasing voltage steps, starting at -100 mV. On the right, the fluorescent intensities (FluoVolt Membrane Potential kit, ThermoFisher) of the regions selected on the left. Scale bar is 20 μm .

4.3.2 Patch clamp

Whole cell patch clamp

F11 cells on their 5th and 6th differentiation days were studied under patch clamp. Patch pipettes of 1–5 M Ω resistance values measured in the bath solution were used for experiments. Figure 4.18 shows a IC and a VC experiment on the same cell. Under IC, when a current step of sufficient amplitude and duration is sent to the cell, an AP is induced. For current amplitudes smaller than that, no AP is triggered. The top left graph in Figure 4.18 shows taller and narrower APs induced by larger amplitude currents, due to the opening of a greater number of Na⁺ channels in the cell. Larger currents, in addition, depolarise the cell faster, and the APs appear earlier in the trace. The cell of Figure 4.18 did only fire single APs for depolarisations sustained for 500 ms. The ionic current components of these APs can be studied in VC and are shown on the right of Figure 4.18. When the voltage is clamped at a sufficiently depolarised value, first fast inward (negative, mainly Na⁺) and then slower outward (positive, mainly K⁺) currents are induced. In between depolarisation steps, voltage and current are held at hyperpolarising values in order to close (from their inactive state) Na⁺ channels, so that they can be opened again. The single Na⁺ current peaks for each VC depolarisation correspond to single APs induced in IC. DRG cells do not commonly

show repetitive firing [337], a consequence of their channel dynamics, but these F11 cells are DRG hybrids, and some of them, such as the one presented in Figure 4.19, fire multiple APs under a single 500 ms long IC depolarisation. Equivalently, several inward currents arise during a single VC stimulation (see top right graph in Figure 4.19). The induced APs on the top left of Figure 4.19 are zoomed in Figure 4.20 in order to better observe the differences in height and width of the various APs induced within the same sweep, along with the dependence of the AP firing frequency on the magnitude of the injected current.

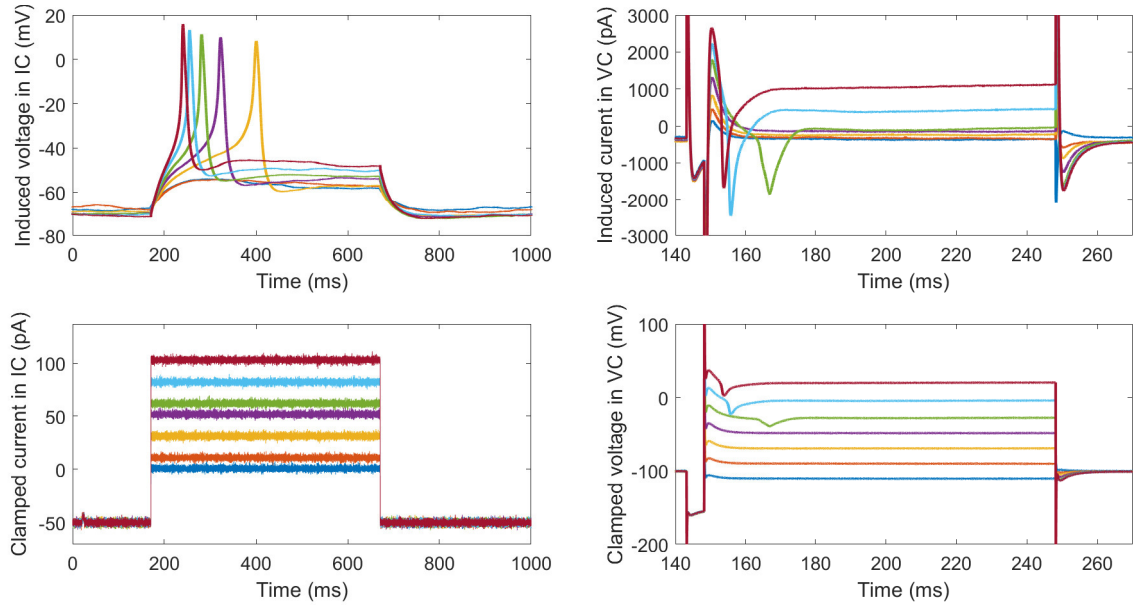


Figure 4.18: Left: current clamp experiment showing individual APs induced at different injected currents. Taller and narrower APs are induced with larger currents. Right: voltage clamp experiment on the same cell, showing single inward currents excited at more depolarised potentials. Na^+ currents are larger and faster at larger depolarisation voltages. Voltage traces on the right are corrected at 65% for the electrode resistance, which results in the fluctuations at the beginning of the trace.

Even with no injected current, these cells do not show a characteristic natural spiking frequency, as the recording of the spontaneous activity of a cell over a minute shows in Figure 4.21. Furthermore, two spontaneous APs from two different cells are compared in Figure 4.22 to illustrate the varied shapes spontaneous APs present in this cell line, naturally arising from the different cell phenotypes found in F11 cells [316].

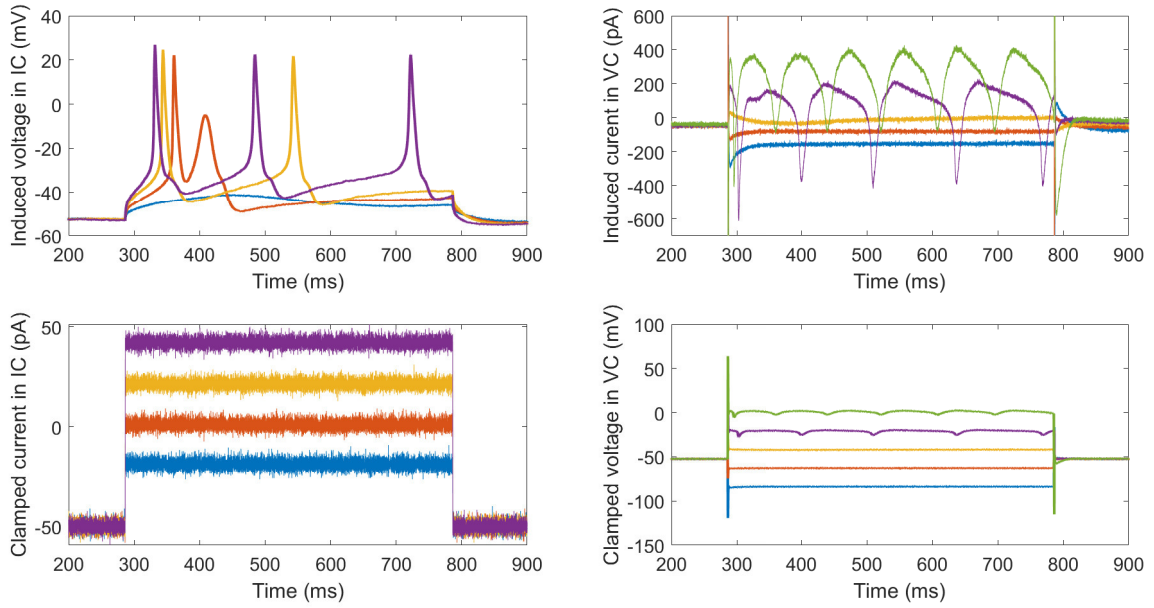


Figure 4.19: Current (left) and voltage (right) clamp experiments on a cell showing multiple induced APs and inward Na^+ currents, respectively. The first APs in the induced voltage traces, as well as the first Na^+ inward peaks in the induced current traces are larger in amplitude and narrower than the subsequent ones.

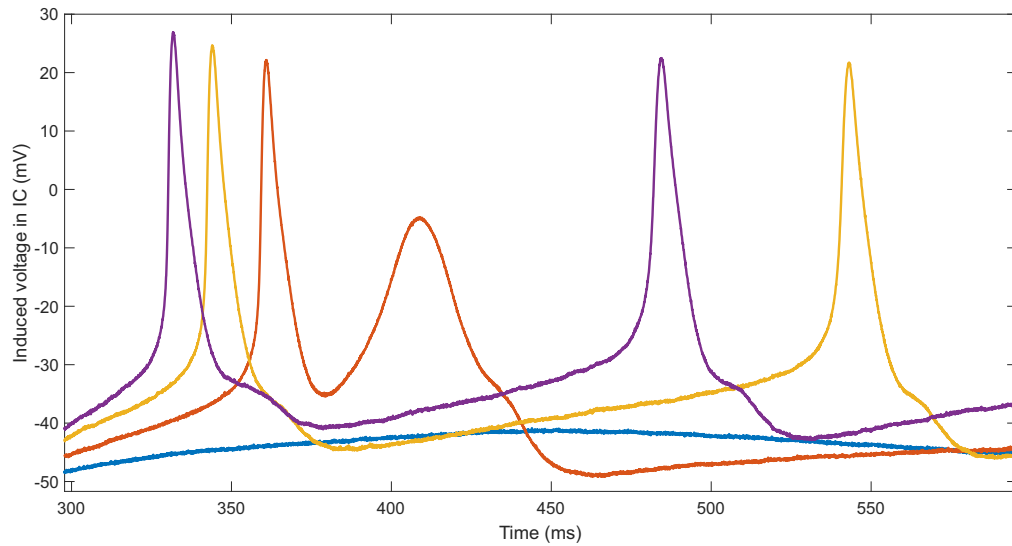


Figure 4.20: Zoomed induced APs in IC (top left graph in Figure 4.19). The first AP in the different traces is larger and narrower than the later ones, as the sustained depolarisation prevents Na^+ channels to fully close and thus not all Na^+ channels are available for subsequent APs.

Considering all the mentioned alterations introduced in the electrophysiological recordings when the activity is induced, it was decided that only spontaneous APs

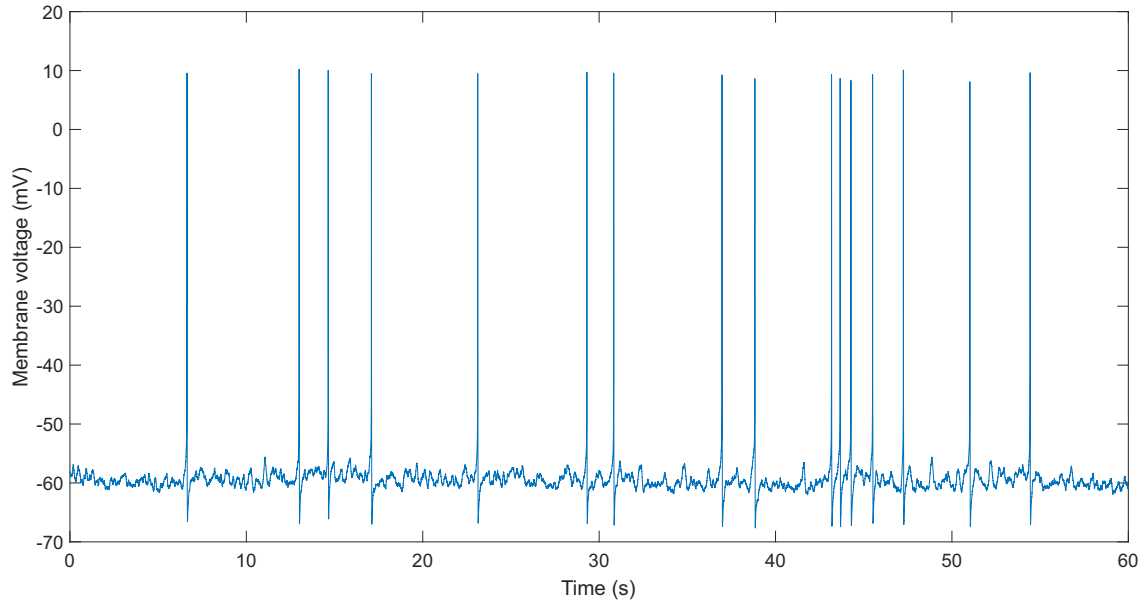


Figure 4.21: Spontaneous activity in a F11 cell does not show a regular firing pattern. Voltage values have been corrected for the LJP.

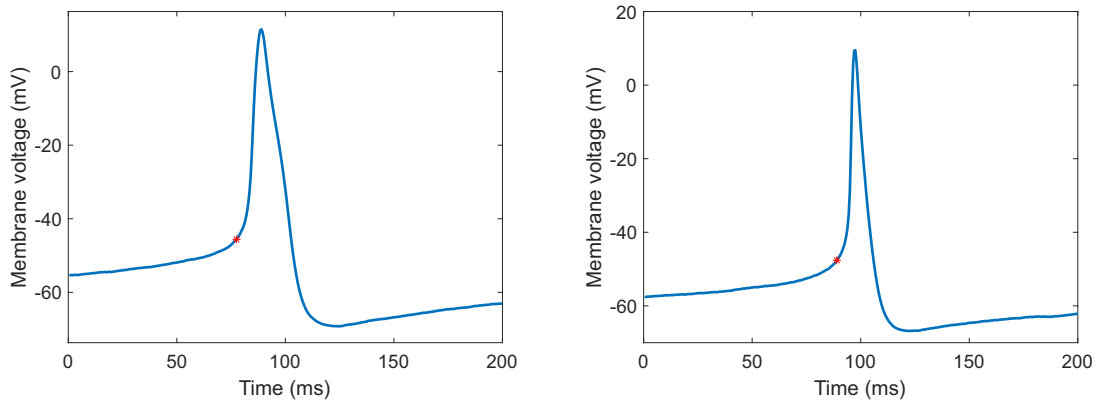


Figure 4.22: Spontaneous APs from two different F11 cells showing different widths but similar amplitudes. These F11s show AP threshold voltages, marked with red asterisks, at around -45 mV, and AP amplitudes of about 75 mV. Voltage values have been corrected for the LJP.

will be compared in this thesis (see Chapters 5 and 6). Although this minimised the variability otherwise introduced by the electrical stimulation, it also greatly reduced the number of cells viable for experiments.

Spontaneous APs were extracted from whole cell patch clamp recordings (*.abf* format). An *.abf* file was opened with MATLAB *via* the “abfload” MATLAB function [338], with which the current and voltage traces were extracted, and APs

were then detected with the “spike_times” MATLAB function [339], which detects the location of the peak of the AP. APs were defined and stored as the voltage recording for 250 ms before and 250 ms after that central peak value.

Action potential features extraction

Additional MATLAB codes were written to extract the following features from each detected AP: AP amplitude, AP width, membrane resting voltage V_{res} , AP threshold voltage V_{th} , rising and falling phases (see Figure 4.23), as well as AP firing frequency and maximum values of the first temporal derivative of the membrane potential, dV_m/dt . The AP amplitude is defined as the difference between the depolarisation peak (AP_{max} in Figure 4.23) and the maximum hyperpolarisation voltage (AP_{min}) values of the AP. The AP width is determined at half maximum AP amplitude. V_{res} is defined as the value of the voltage 250 ms before the AP peak. V_{th} is defined as the voltage at which dV_m/dt exceeded 0.35 mV/ms, as determined by visual inspection of the membrane voltage *vs.* time curve. The rising phase is defined as the time lapse between V_{th} and the peak of the AP, and the falling phase, as the time lapse between the maximum and minimum values of the AP.

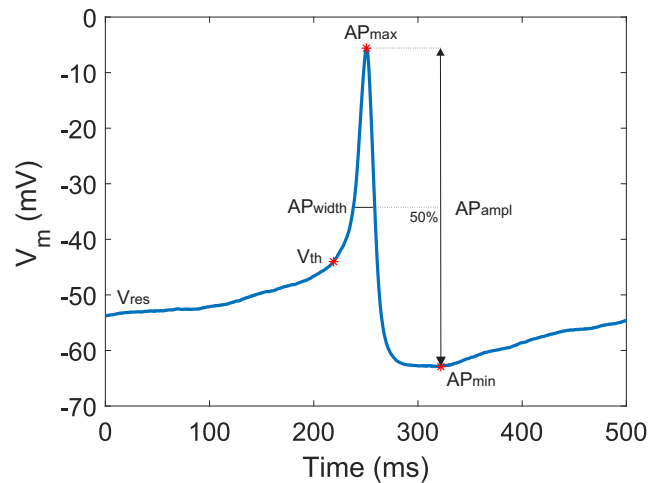


Figure 4.23: Analysed AP features.

Chapter 5

Action potential alterations under static loading

Neurons are constantly adapting to their microenvironment by exploring and mechanosensing their surroundings with their neurites' membrane receptors. This so-called mechanotransduction process allows the cell to translate mechanical forces into meaningful intracellular biochemical signals. These forces are essential, in neurons in particular [152], for proliferation, signalling and differentiation [151], and development [340].

While a lot of work exists on these processes, very little is known on how mechanical forces affect the electrophysiology of individual neurons. Previous studies have made use of open-loop indentation systems, mainly based on piezoelectric actuators, to push glass probes [187, 341, 342] or pillar arrays [343] into cells, while mechanically activated currents were recorded with patch clamp amplifiers. However, no study investigating the effects of a mechanical load on the AP at the single neuron level has yet been proposed.

To this end, a set of experiments is designed here to quantify the influence of mechanical loading on the generation and evolution of neuronal APs. In particular, the multiphysics setup presented in Chapter 3 is used for the measurement of spontaneous AP alterations induced by quasi-static indentation. To investigate the

mechanisms behind such alterations, a study making use of a finite element (FE) simulation of the 3D indentation coupled to an electrophysiologically based model is conducted. This analysis and the accompanying discussion conclude that the observed response can only be a coupled effect of the lipidic membrane deformation together with ion channel action alteration.

5.1 Materials and methods

5.1.1 Experimental methods

Cell line

F11s on their 5th and 6th differentiation days were used, with passages ranging from 14 to 23. Healthy F11s with sufficiently large round somas were selected for the study. While high cell density and well-connected neurons are desired for high spontaneous spiking rates, relative isolation of the cell is required to minimise the mechanical influence of neighbouring cells during indentation. As a compromise, cells whose somas were not in direct contact with those of other neurons, but still connected to other cells through their neurites, were chosen for experiments.

Mechanics

For the indentation, Chiaro nanoindenter probes of 0.05 N/m stiffness and 3 μm spherical tip radius were used. A fixed indentation of 3 μm was chosen to compare electrophysiological signals of F11s under mechanical loading, as it fell within the F11s' LVR (see Chapter 4). The indentation-controlled feedback loop ensured that this indentation was held constant throughout the experiment.

Electrophysiology

For the patch, fresh pipettes were pulled on the day and a whole cell patch was obtained following the protocol presented in Section 3.4.1. If the gigaseal was not formed in the first 2 minutes, cell and pipette were discarded. If after gigaseal formation the cell showed stable spontaneous activity, sometimes aided by very low (up to 50 pA) induced currents in IC, the nanoindenter was manually brought into contact with the cell soma, opposite the microelectrode, see Section 3.5.

Combined nanoindentation and patch clamp

The recording of spontaneous or induced stable activity was then started and 1 min of cell activity was recorded before the indentation. The Chiaro was then manually positioned above the patched cell, opposite the microelectrode (see Figure

3.14-right). The nanoindenter was manually brought into contact with the cell through 0.25 μm z-steps and then retracted 1–2 μm above the cell surface. These small movements did not displace the micropipette, whose position was continuously monitored through brightfield imaging with the inverted microscope. A 3 μm indentation-controlled compression was then started and held for 80 s, and the activity recorded during loading, after which the probe was withdrawn a few tens of μm above the cell and the activity further recorded (after indentation) for another minute. A 80 s duration of the indentation was selected because it took a few seconds to start and finish the 1 min long recording before and after indentation, as approaching and retracting probe movements were required. This way, the different indentation stages had comparable times, as well as number of APs recorded, facilitating later statistical analyses. Nanoindenter and cell membrane voltage signals were simultaneously recorded with a PicoScope 4262.

5.1.2 Combined numerical mechanics and electrophysiology

If a combined nanoindentation and patch experiment was conducted, the corresponding oscilloscope recording was loaded into MATLAB, the APs of the patch trace detected by “spike_times.m”, vertical lines drawn at each AP location with the “vline” MATLAB function [344], and APs classified into before, during and after indentation by visual inspection together with the nanoindenter trace.

AP features analysis

The APs from the recorded voltage signal were detected and their features analysed *via* custom MATLAB codes. The following experimental AP features were compared between the three phases of the indentation (before, during and after): AP amplitude, AP width, membrane resting voltage V_{res} , AP threshold voltage V_{th} , rising and falling phases, as well as AP firing frequency and maximum values of the first temporal derivative of the membrane potential, dV_m/dt , all defined in Chapter 4.

Although the cells under study were *a priori* selected based on the similarity

of their phenotype, i.e., rounded cell bodies with neurites, the recorded APs had different cell-specific shapes and durations, even before indentation (see Chapter 4). This is however expected from the already mentioned multiple phenotypes found within F11s [316]. For comparison purposes, each averaged value of interest for each cell was normalised by dividing it by its counterpart before indentation. As voltage values during the APs remain always negative in the studied cells, both numbers are negative, the ratio is positive, and a normalised value >1 means that the cell hyperpolarised with respect to before the loading, while a normalised value <1 indicates a cell depolarisation during or after indentation. For temporal quantities, a ratio >1 illustrates a longer process, and <1 a shorter one.

In the following, results are classified into reversible (or transient) and irreversible (or sustained). Reversible results are those in which the normalised mean values increased (or decreased) from before to during, but was then followed by a decrease (or increase), see Figure 5.6, plotted together with the standard error (SE). If instead there were two consecutive increments or decrements, then the results are referred to as non-reversible, at least for the time-length of the study, see Figure 5.7.

Statistical analysis

The above features of the APs were analysed *via* custom MATLAB codes. Statistical analyses of the post-processed electrophysiological data were performed with GraphPad Prism 6. The normality of data distribution was checked with Kolmogorov–Smirnov test, $\alpha=0.05$. In order to test the significance (defined with a threshold probability of 0.05) of the observed differences between the three experimental phases in terms of normalised mean values of the analysed AP features, one-way ANOVA with Tukey’s multiple comparisons *post-hoc* tests were conducted if the data were normally distributed, and non-parametric Kruskal-Wallis test with Dunn’s multiple comparisons *post-hoc* tests were carried out otherwise.

Membrane area driven electrophysiology alteration

A modified surface area-dependent electrophysiological model [345] of DRG cells [337] is presented here to simulate the effect of the cell membrane area variation, as

a result of the compression, on the membrane capacitance and the voltage dynamics, when keeping the number of ion channels per unit reference area constant:

$$\begin{aligned} \frac{C_m A}{h_m} \frac{dV_m}{dt} = I_{inj} - & \left(\frac{\bar{G}_{Na} A_0}{h_m} m^3 h (V_m - E_{Na}) \right. \\ & + \frac{\bar{G}_{Na_s} A_0}{h_m} m_s h_s (V_m - E_{Na}) \\ & + \frac{\bar{G}_K A_0}{h_m} n (V_m - E_K) \\ & \left. + \frac{G_L A}{h_m} (V_m - E_L) \right) \end{aligned} \quad (5.1)$$

where C_m is the membrane electric constant, h_m is the membrane thickness, V_m is the membrane potential, A_0 and A are the undeformed and deformed total membrane areas, respectively, I_{inj} is the patch injected current, $\bar{G}_{Na}$, $\bar{G}_{Na_s}$, $\bar{G}_K$ and G_L are the fast and slow-inactivated Na^+ , K^+ and leak reference conductivities, m , m_s , h , h_s and n are the ion channels gating variables, and E_{Na} , E_K and E_L are the Na^+ , K^+ and leak reference potentials. The main assumption behind this equation is that the area change does not alter the number of ion channels, but alters the membrane leak conductance and capacitance, as proposed in Ref. [346]. The values of the parameters and the channels gating evolutions used to model the AP through Equation (5.1) were taken from the DRG model in Ref. [337] and are given in Appendix D.

The APs of a cell with membrane areas A_0 and A were simulated, and their features compared. To calculate the deformed area, a FE simulation of the $3 \mu\text{m}$ indentation on a real 3D F11 meshed soma (see Chapter 4) was conducted in ABAQUS (implemented by Dr. Haoyu Chen). The cell's viscoelastic properties were input as the parameters of a Generalised Maxwell model, fitted with the results of the quasi-static and dynamic indentations of F11 cell bodies presented in Chapter 4.

Equivalent Generalised Maxwell model

In Chapter 4, the force decays from stress-relaxation curves were fitted to extract the cells' relaxation times, and their storage and loss moduli were calculated by fitting the force and indentation time-evolutions at each frequency from DMA

experiments. In the following, a Prony series is fitted with these experimental parameters, that represents a third order Maxwell model describing the frequency-dependent viscoelastic behaviour of these F11 cells. The Prony series parameters are needed as inputs in ABAQUS to define the cell's mechanical properties for the 3D reconstruction.

The frequency-dependent mechanical response of viscoelastic materials can be modelled with the Generalised Maxwell model, which consists of a long-term elastic (spring) branch, in parallel with a set number of viscous branches (spring and dashpot) in series. Each viscous branch i represents one relaxation response of the material, where relaxation time is defined as $\tau_i = \frac{\eta_i}{G_i}$, G_i being the shear modulus (the spring) and η_i being the viscosity (the dashpot) of the branch. The elastic branch, with a shear modulus G_∞ , corresponds to the elastic response when the viscous branches are not loaded elastically, e.g., after a relaxation time much larger than $\max_i(\tau_i)$.

Using Prony series notation [347], the storage and loss shear moduli of a third

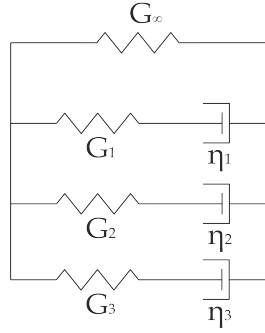


Figure 5.1: Third order Maxwell model.

order Maxwell model (Figure 5.1) are then given by:

$$G'(\omega) = G_0 \left[1 - \sum_{i=1}^3 g_i \right] + G_0 \sum_{i=1}^3 \frac{g_i \tau_i \omega^2}{1 - \tau_i^2 \omega^2} \quad (5.2)$$

$$G''(\omega) = G_0 \sum_{i=1}^3 \frac{g_i \tau_i \omega^2}{1 - \tau_i^2 \omega^2} \quad (5.3)$$

where $g_i = G_i/G_0$ and $G_0 = G_\infty + \sum_i G_i$.

Single and double exponential fits of stress-relaxation experiments gave largest relaxation values, τ_1 of 2.77 ± 0.67 s and 2.29 ± 0.56 s, respectively (see Section 4.2.1). As a compromise, the mean value of $\tau_1 = 2.5$ s was input into the model as the time constant of the first Maxwell branch. The parameters G_0 , and g_i and τ_i for each of the viscoelastic branches were fitted (numerical implementation done by Dr. Haoyu Chen) to match the storage and loss moduli from the experimental DMA results by using a nonlinear least squares algorithm in MATLAB, see Table 5.1 and Figure 5.2 for results. The 10 Hz frequency was not included in this study, as the experimental and simulated indentations happened at $3 \mu\text{m/s}$, so that no subcellular structure responding to faster indentation rates was excited.

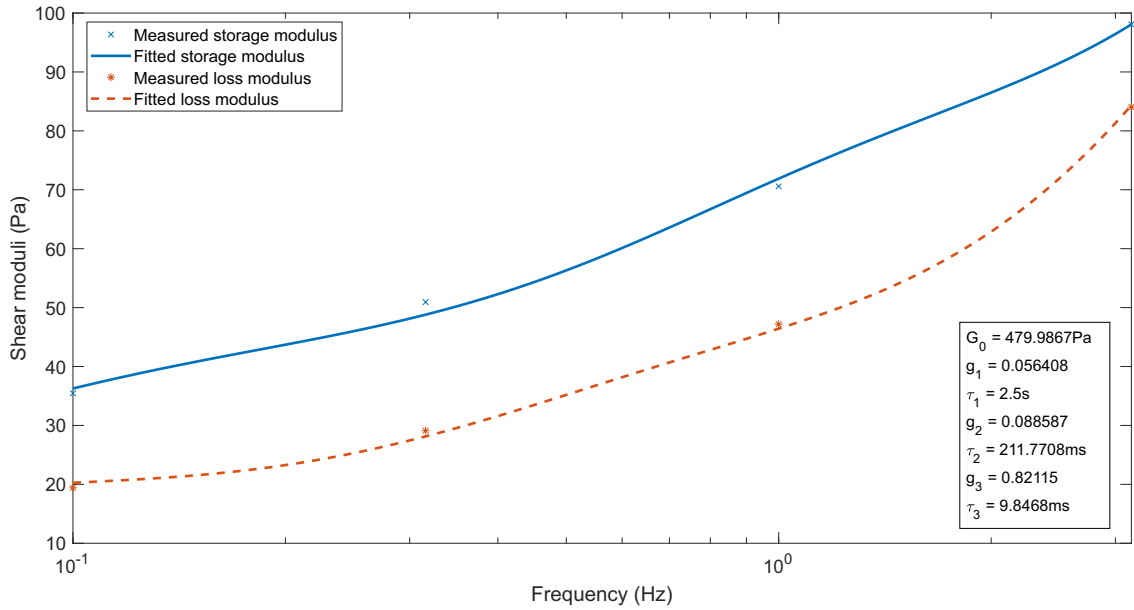


Figure 5.2: Measured (crosses and asterisks) and fitted (lines) viscoelastic cell parameters using a third order Maxwell model.

3D FE simulation of the indentation

A 3D finite element (FE) simulation of the $3 \mu\text{m}$ indentation was conducted by Dr. Haoyu Chen to study the cell deformation under compression. A surface object from the 3D cropped and deconvoluted cell body (see Chapter 4) was loaded into Autodesk Meshmixer software (Autodesk Inc), meshed with 8,512 tetrahedral elements and finally exported to ABAQUS (Dassault Systèmes SE) to perform a

Magnitude	Value
G_0	479.99 Pa
g_1	0.06
τ_1	2.50 s
g_2	0.09
τ_2	211.77 ms
g_3	0.82
τ_3	9.85 ms

Table 5.1: Calibrated viscoelastic parameters.

FE simulation of the indentation using the material properties defined through the parameters listed in Table 5.1.

5.2 Results

5.2.1 Controls

Patch clamp stability assessment

Patch stability under a 3 μm compression was tracked in several cells *via* the “Membrane Test” option of Clampex (Molecular Devices) in VC mode, by evaluating the access resistance R_a [348]. R_a is required to vary by less than 20% during electrophysiological recordings to be considered as stable [349]. A protocol was established for which R_a remained within the constrained values: seals were $>1\text{G}\Omega$, the nanoindenter was kept as far as possible from the micropipette, the smallest possible nanoindenter step of 0.25 μm was used when approaching the probe and the compression was carried out at small velocities (3 $\mu\text{m/s}$).

Loading of the cell capacitance

The 3 μm indentation could induce a capacitive load in the cell, that could contribute to the observed electrophysiological alterations. To quantify this effect, a control experiment was conducted in which the value of the cell capacitance was calculated before and during such indentation in three different cells, by fitting the current decay in a voltage clamp experiment.

In whole cell and voltage clamp, after correction of the pipette capacitance, a hyperpolarising step at -80 mV was held for 500 ms so that steady-state current was achieved. A hyperpolarising potential was chosen to minimise the activation of voltage-gated ion channels that would create ionic currents. As a result of a voltage step, the cell capacitance follows an exponential decay (with C_p compensated, the decay corresponds to C_m), which can be fit to extract the membrane time constant. As space-clamp tests showed that the entire cell was clamped (see Figure 4.17), an isopotential cell could be assumed, the cell could be depicted by a single RC circuit, and a single exponential was used to fit the decay [350–352]. An example of the current transients induced by a square voltage step of -80 mV held for 500 ms are shown in Figure 5.3, together with the single exponential fit of the current decay.

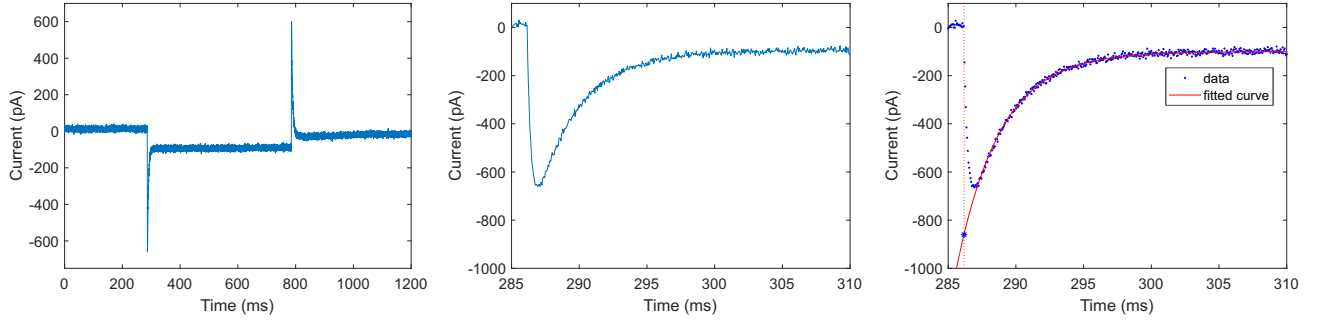


Figure 5.3: Left: Capacitive current transients corresponding to C_m charge and discharge in response to a square voltage pulse of -80 mV. Centre: zoomed current transient. Right: single exponential fit of the capacitive current, with the asterisk indicating the extrapolated peak of the transient, I_0 .

The peak of the current transient was reconstructed by extrapolating the exponential fit to the first sample point I_0 after the voltage change (see asterisk in Figure 5.3-right) [350]. This value I_0 , together with the voltage value at that time point, V_0 , gave the value of the series resistance R_s by Ohm's law: $R_s = \frac{V_0}{I_0}$. Then, the whole cell capacitance was calculated from R_s and the time constant τ of the fitted current decay from $\tau = R_s C_m$ (see Section 2.1), so that:

$$C_m = \frac{\tau}{R_s} = \frac{\tau I_0}{V_0} \quad (5.4)$$

Following this analysis, the capacitive current decays of N=3 cells clamped at -80 mV for 500 ms were fitted before and during a 3 μm indentation. Figure 5.4 compares the decays of one of these cells before and during indentation.

The fittings of the current decays of the cell shown in Figure 5.4 before and during indentation gave:

Magnitude	Before	During
I_0 (pA)	-854.32	-818.71
V_0 (mV)	-85.11	-85.17
τ (ms)	3.24	3.43
C_m (pF)	32.54	32.97

Table 5.2: Fitted capacitive current decay parameters.

The values of the membrane capacitance shown in Table 5.2 are in line with average rat DRG neurons capacitance values [353]. For the cell shown in Figure 5.4,

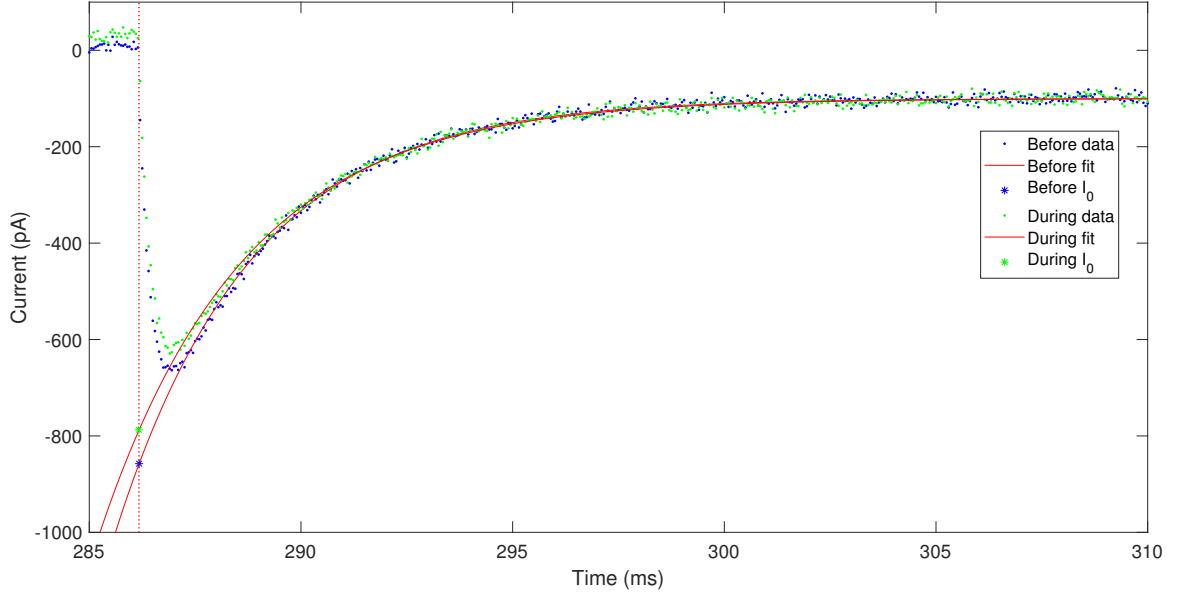


Figure 5.4: Single exponential current transients fits before (in blue) and during (in green) a $3 \mu\text{m}$ indentation.

with fitted parameters listed in Table 5.2, the $3 \mu\text{m}$ indentation introduced a capacitive load of a 1.33%. Identical analysis of two more cells gave a 2.28 and a 1.85% capacitance increases, with the variations potentially coming from differences in cell sizes. These small capacitive loading contributions are expected due to the deformation being localised ($3 \mu\text{m}$ radius probe in a $10 \mu\text{m}$ radius cell), hence only affecting a small portion of the entire cell membrane. As a result, the whole cell activity is not foreseen to be affected.

5.2.2 AP alterations during and after indentation

The features of a total of 284 spontaneous APs from $N=5$ F11 cells were analysed and compared. The altered characteristics of the APs before, during and after mechanical loading for one of these cells are shown in Figure 5.5. Comparisons were made for each cell in the three different compression regimes: before, during and after (with a total of 115 APs before, 80 during and 89 after).

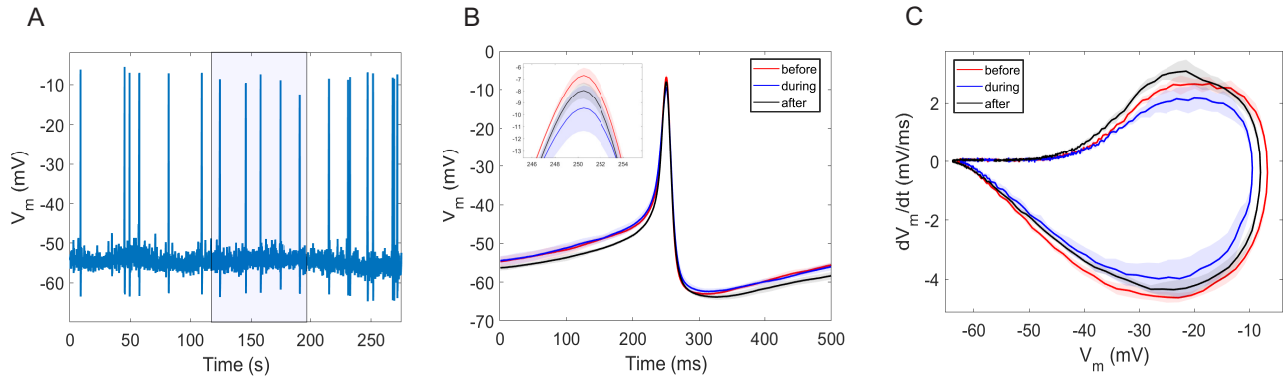


Figure 5.5: A) Spontaneous APs before, during (shaded) and after 3 μm indentation for one of the analysed cells. B) Mean and SD of the APs on A), with peaks in the inset. C) Mean and SD of the phase plots (dV_m/dt vs. V_m) for the APs on A).

Transient changes: Smaller, wider and slower rising APs upon indentation

The normalised mean amplitudes of the APs are significantly reduced under compression with respect to before (adjusted $*p = 0.0352$), recovering their original values after the 80 s indentation, see Figures 5.5-B and 5.6-A. APs during indentation are also significantly wider compared to before (adjusted $*p = 0.0347$) and show the same recovery trend when the applied pressure is released (Figure 5.6-B). The widening of the peaks is an indicator of slower dynamics of the electrical signals in cells under compression. To further investigate this, the rate of membrane voltage change over time (dV_m/dt) and the duration of the rising AP phases are analysed. The maximum normalised mean dV_m/dt value is significantly smaller under compression with respect to before (adjusted $*p = 0.0473$) and after (adjusted $*p = 0.0364$) indentation (Figure 5.6-C). Phase plots are obtained by plotting dV_m/dt against V_m , see Figure 5.5-C, which illustrates that the rate of voltage change is slower in a compressed cell (less pronounced depolarisation and repolarisation slopes), with the altered dynamics recovering once the loading is removed. Figure 5.5-C further shows that APs of loaded cells reach smaller maximum values, as does Figure 5.6-A. In agreement with the mentioned slower dynamics, although non-significant, the rising phases of the APs are longer under compression and tend towards recovery after indentation (Figure 5.6-D), indicating that a compressed cell

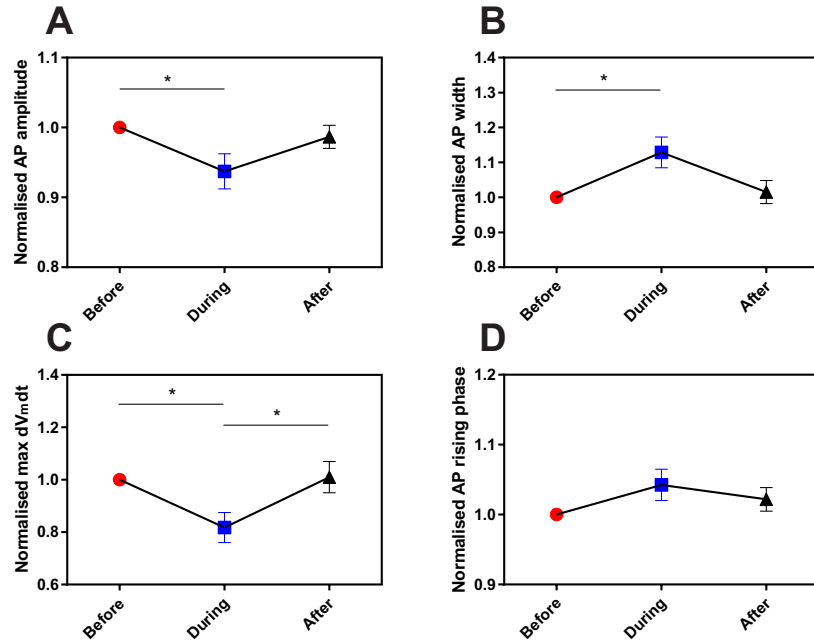


Figure 5.6: Mean and SE of the reversible or transient AP changes. A) Normalised AP amplitudes. B) Normalised AP widths. C) Normalised max dV_m/dt values. D) Normalised AP rising phases. In A), Kruskal-Wallis test was conducted with Dunn's multiple comparisons *post-hoc* test (* $p < 0.05$). In B) and C), one-way ANOVA was performed with a Tukey's multiple comparisons *post-hoc* test (* $p < 0.05$).

takes more time to depolarise (more gradual slope in Figure 5.5-C).

Sustained changes: Hyperpolarised cells with higher firing frequencies upon and after indentation

Non-reversible results show that cells remain hyperpolarised after indentation; at least for the minute recorded after the compression was halted. Normalised mean V_{res} values are significantly hyperpolarised after indentation when compared to before and during (adjusted ** $p = 0.0036$ and ** $p = 0.0065$, respectively, see Figure 5.7-A). The evolution of V_{th} reveals a similar result: normalised mean V_{th} values after indentation are significantly hyperpolarised with respect to before (adjusted *** $p = 0.0005$) and during (adjusted *** $p = 0.0002$) compression. In addition, although not significant, mean V_{th} values during compression are slightly depolarised (see Figure 5.7-B), which will be discussed in Section 5.3. Despite cells being hyperpolarised during and after compression, on average they also fire more APs per s (Figure 5.7-C). Finally, even though AP widths tend to recover

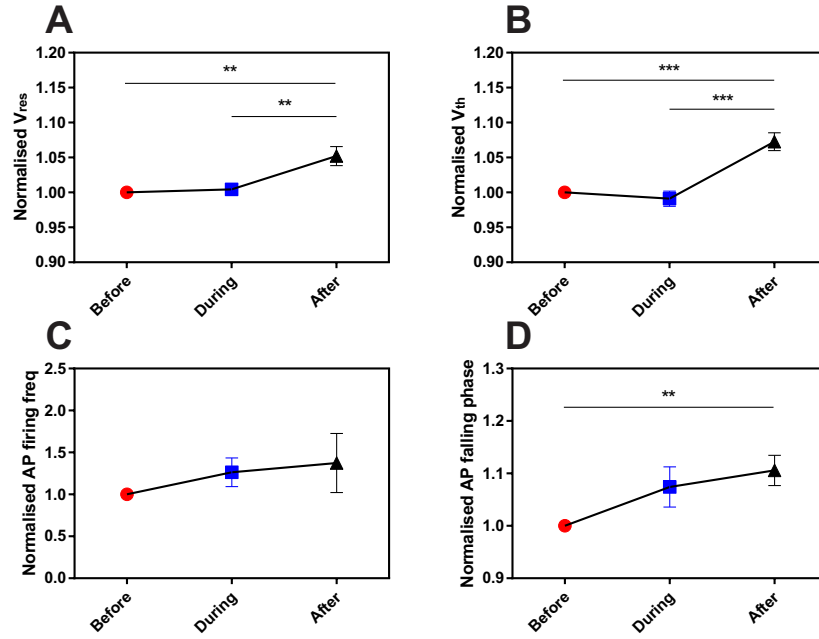


Figure 5.7: Mean and SE of the irreversible or sustained AP changes. A) Normalised resting membrane voltage values. B) Normalised threshold potential. C) Normalised AP firing frequencies. D) Normalised AP falling phases. In A) and B), one-way ANOVA was performed with a Tukey's multiple comparisons *post-hoc* test (** $p < 0.01$; *** $p < 0.001$). In D), Kruskal-Wallis test was performed, with Dunn's multiple comparisons *post-hoc* test (** $p < 0.01$).

once the indentation is released (Figure 5.6-B), normalised mean falling phases are significantly longer after indentation with respect to before (** $p = 0.0075$), see Figure 5.7-D. These longer repolarisation phases are in line with the more hyperpolarised voltage values the cell reaches after indentation.

5.3 Discussion

The normalised mean AP amplitudes during compression are significantly reduced when compared to those before indentation (Figures 5.5-B and 5.6-A). These values are reached more slowly, as shown by the significantly smaller maximum values of the dV_m/dt evolution (Figure 5.6-C) and longer rising phases (Figure 5.6-D) during loading. Furthermore, on average, cells take more time to repolarise back to their resting values during indentation, see Figure 5.7-D. Overall, this slower dynamics in turn results in slower voltage changes during the AP (Figures 5.5-C and 5.6-C), or equivalently, wider APs (Figure 5.6-B), during compression.

Similar results have previously been found in experiments and numerical simulations. Experimentally, a decrease in AP amplitude was observed in guinea pig spinal cord compression [354] and in patients with suspected median and ulnar nerve lesions [355] or carpal tunnel syndrome [356]. In many of these cases, AP conduction velocity was also found to decrease with compression. Modelling efforts have led to similar results. For instance, in Ref. [357], the authors simulated the AP shape along a compressed axon, showing that its amplitude was reduced upon application of a force, and its conduction velocity decreased as the compression increased. Identical results were obtained when the AP propagation was modelled in axons subjected to stretch [358, 359].

In the following, we investigate the possible causes for these findings. As the indentations are within the cell's LVR, we assume that mainly the membrane and membrane cortex are influenced by the compression. We first look at the more general contribution of the morphological nature of the membrane, accounting for the membrane geometry change and the corresponding induced voltage dynamics alteration. We then discuss the effects of the ion channels embedded in the membrane, focusing particularly on mechanosensitivity.

5.3.1 Geometrical contribution

An *in silico* FE neuronal soma was created from live widefield fluorescence images of a real F11 cell, segmented and meshed as described in Chapter 4. A simulation

of the cell indentation was performed in ABAQUS by Dr. Haoyu Chen (see Figure 5.8). The results showed an overall cell surface area increase of 0.4% for a 3 μm indentation. This increase is in line with the assumption that the shape of a quasi-incompressible volume with surface tension (e.g., arising from the cell membrane) will naturally tend towards a sphere so as to minimise its surface energy, and thus area. As such, any mechanical disturbance is indeed expected to increase the area. Note that comparable 0.26 and 0.16% peak and steady-state membrane area changes arose in artificial protein-free lipid membranes stimulated with 1 MHz US, as a result of the membrane displacement caused by the acoustic radiation force [77].

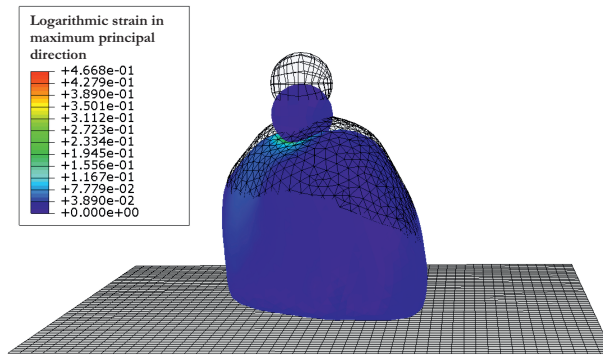


Figure 5.8: FE simulation (implemented by Dr. Haoyu Chen) of a F11 cell body compression showing both the undeformed and deformed configurations; colour field corresponds to the logarithmic strain in the maximum principal direction.

As Figure 5.8 shows, the higher values of the maximum principal strains in the compressed cell are localised at and around the area of direct contact with the glass bead. The three principal strain components of several elements, underneath the bead and in its direct neighbourhood, were extracted in order to evaluate their external surface area change. Results showed that elements below the bead increased their external area by $\sim 40\%$ when the cell was indented 3 μm , whereas those in the immediate vicinity of the nanoindenter experienced a $\sim 10\%$ area increase. This indicates that heterogeneous and localised electrophysiological changes can be expected upon indentation. The effect of these 10% and 40% area increments on the AP shape are given in Figure 5.9 for illustration.

These large area increments are significantly diluted (to 0.4% overall area change) when considering the entire cell membrane. A small area expansion was

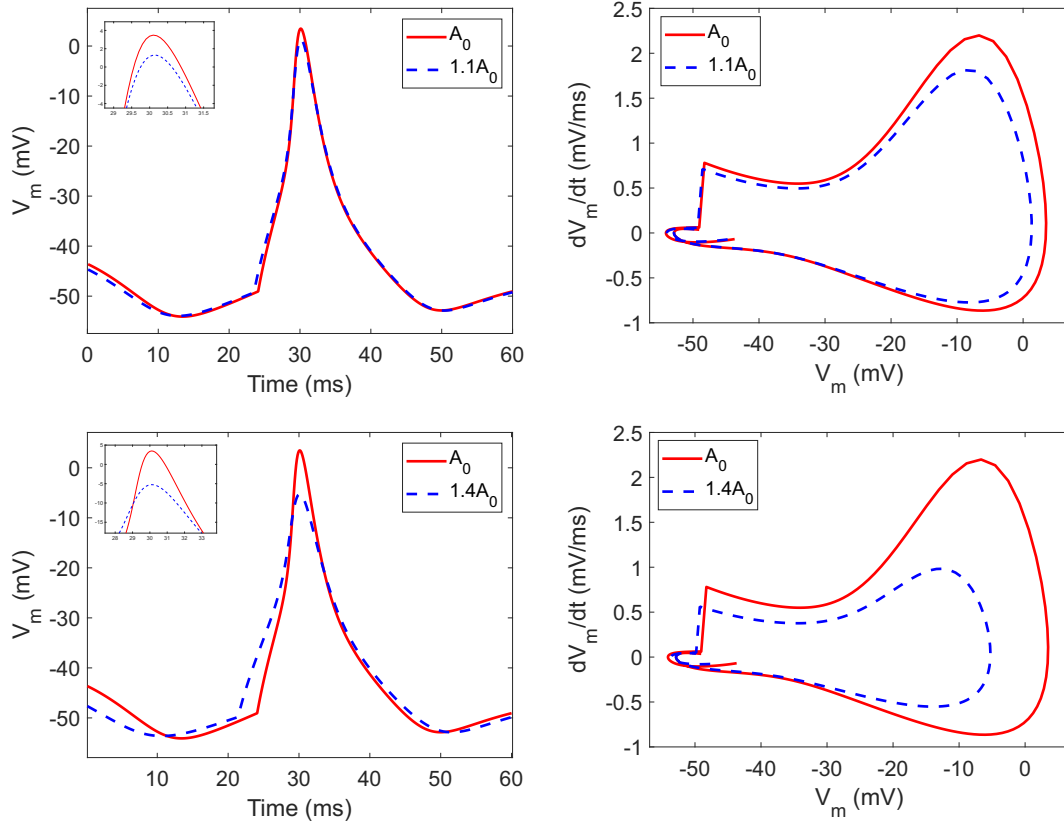


Figure 5.9: Left: prediction of APs through Equation (5.1) before and during a 10% (above) and 40% (below) area increase, as a result of cell compression during indentation. Insets: AP peaks zoomed in. Right: phase plots of the APs on the left.

expected, as cell membranes easily fold and bend but do not easily expand [164]. It is worth mentioning that, although in this simulation the cell area increased, a less spherical cell body indented in a particular way could have seen instead its area decrease. While the results discussed here are thus cell-specific, the order of magnitude of the predicted changes is not expected to vary drastically. Similar curvature effects connecting cell geometry and depolarisation have also been discussed in Ref. [17].

The resolution of Equation (5.1) was implemented in MATLAB and the APs were simulated for the following two cell area values: A_0 (the non-indented cell area) and $A = 1.004A_0$ the deformed configuration. While the simulated APs followed the same trend observed experimentally, i.e., smaller and wider (or slower) APs (see Figure 5.10), the AP peak reduction only accounted for a fraction of the one observed

experimentally (0.1 mV AP amplitude change, as opposed to the experimentally measured ~ 2.53 mV on average). In order to reach similar numerical values to those obtained experimentally in Figure 5.5, the overall cell area change had to be increased to $\sim 10\%$, as Figure 5.9-top shows. This discrepancy implies that other mechanisms are at play. In Equation (5.1), the membrane thickness h_m was considered constant during deformation. Nonetheless, in the indented area and potentially other parts of the cell, the tension induced in the membrane by the indentation can potentially make it thinner through Poisson effect, which would, similarly to a membrane area enlargement, increase the membrane capacitance ($C_m = \frac{\epsilon A}{h_m}$), and slow down the voltage dynamics during an AP. Myelin wrapped around axons has the opposite effect of increasing the total insulating lipidic membrane thickness, which decreases the membrane capacitance and increases the AP propagation speed. Whether driven by area or thickness changes, an overall cell membrane capacitance increase of $\sim 10\%$ is thus required to recover the time-changing dynamics of Figure 5.9-top. Previous authors have shown that cell capacitance increase is a mechanism by which, through different means such as infrared light [84] and thermal transients [85], one can influence neuronal electrophysiology (in these cases, by depolarising the cells). While the effect of capacitance changes cannot be discarded without further investigations, both area and thickness (and thus capacitance) changes are predicted to be very small for most of the cell, and other mechanisms are most likely involved.

5.3.2 Ion channels contribution

Ion channels and their gating kinetics can be expected to be affected in a compressed membrane [165]; especially those mechanically activated or MS. F11 cells are DRG hybrids, which are sensory neurons with MS traits. F11s respond with small currents to mechanical indentations [320] through endogenous mechano- and volume-activated channels such as TRPV4 [319, 321] and Tentonin3 (TMEM150C) [320]. They also present neuronal markers of A- and C-fibre mechanosensors and proprioceptors, including LDHB, NEFH and TrkC [322]. Furthermore, ion channels with recognised roles in nociception such as ASIC1, CaV3.2, KV7.2, TRPM3 [322]

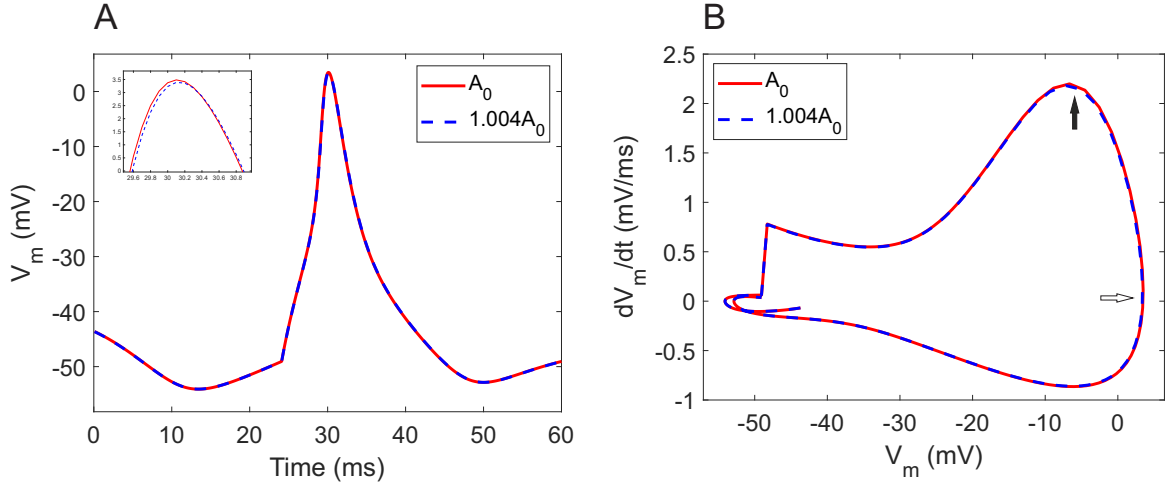


Figure 5.10: Modelled (through Equation (5.1)) DRG APs (A) and their phase-plots (B) before and during a 0.4% cell area increase, resulting from a 3 μm cell compression. Inset: AP peaks zoomed in. In B), unfilled and filled arrows point at smaller and slower APs, respectively, during compression (dashed blue curves).

and bradykinin and prostaglandin receptors [313] are expressed in F11s.

Simultaneous indentation and electrophysiological recordings have been conducted in F11 [320] and DRG [341, 342, 360] cells to characterise indentation-evoked MS currents. In particular, both rapid and slow adapting MS ion currents were recorded in DRGs under indentation [342]. These mechanically activated currents are non-selective cation inward currents that depolarise the cell membrane potential, in turn inactivating some Na^+ channels and contributing to smaller AP amplitudes and longer rising phases, in line with what was observed for F11 compression (Figure 5.6). The current from a single mechanically gated cation channel could trigger an AP in neocortical and hippocampal pyramidal neurons [361]. In addition, a previous result found intracellular Ca^{2+} increases, that lead to cell depolarisation, upon nodose sensory neurons mechanical deformation by a glass micropipette [362]. Although cell depolarisation (V_{res}) under compression was not observed in our experiments (Figure 5.7-A), normalised mean V_{th} values appear to be slightly reduced during loading (Figure 5.7-B), meaning that more depolarising current is needed to trigger an AP when the cell is being compressed.

After indentation, normalised mean V_{res} values are significantly hyperpolarised

(Figure 5.7-A) and the neuronal firing rate is higher (Figure 5.7-C). The repolarisation phase of the AP is mediated mainly by K^+ currents, which also regulate AP firing frequency [115]. The longer AP falling phases observed after indentation (Figure 5.7-D) point towards the activation of delayed rectifier K^+ channels, which permit high frequency AP firing [363]. In addition, hyperexcitability has been observed in chronically compressed DRGs, as a result of an upregulation of hyperpolarisation-activated currents [283]. These are, similarly to MS currents, inward non-selective cation currents that flow through hyperpolarisation-activated cyclic nucleotide-gated channels, known to be present in DRG somas and axons. While these DRGs were compressed between 5 and 7 days in order to induce such a chronic response, it may be that hyperpolarisation-activated currents could arise in F11 cells in a much shorter time (here, after only 80 s indentation) and contribute to the observed sustained enhanced hyperpolarisation and increased firing rates (Figure 5.7-A,C).

The observed sustained (for the minute recorded after indentation) electrophysiological alterations could be the result of a compound ion channels' response, potentially also including nociceptive signals. However, studies have shown that even the slow adapting MS currents in F11s and DRGs are only persistent for hundreds of ms, with their amplitudes considerably decaying after 4 s [342] and disappearing after 7 s [320]. Piezo1 and Piezo2 currents also decay within ms when subjected to a constant mechanical stimulus, most likely due to channel inactivation [193]. As the observed AP shape changes are maintained throughout the 80 s indentation (see Figure 5.5-A), an exclusively ion channels' response is yet again unlikely to be the sole driver behind the observed sustained 80 s lasting changes.

5.3.3 Multiphysics response

As discussed above, the sole contribution of either membrane topology or ion channel alterations is unlikely to account for the observed effects. A combined action, where membrane and ion channels (among other proteins) work concurrently and collaboratively through mechanical and biochemical changes, is proposed here instead. Such observation builds naturally on the growing body of evidence pointing

towards the need to redefine the cellular membrane as a coupled multiphysics system (see Chapter 2) [109]. In our experiments, the geometrical changes induced by the indentation alter the curvature of the membrane and affect the state of stress in the lipid bilayer, which under tension shows a significant decrease in lipid order [164], potentially altering the structure and function of the embedded ion channels [151, 165]. MS channels, TRP channels [214] *and* voltage-gated K^+ [115–118] and Na^+ [90, 119, 120] channels gating kinetics are known to be affected by membrane tension and composition, the latter altering bilayer elasticity [197, 198]. In fact, previous work demonstrated that these same F11 cells showed smaller magnitude Na^+ currents when subjected to uniaxial strain [364]. The altered rising (Figure 5.6-D) and falling (Figure 5.7-D) phases during and after compression observed here could indicate that voltage-gated Na^+ and K^+ channels kinetics, respectively, are altered by the applied mechanical load. Conversely, MS Piezo channels have been shown to also be modulated by voltage [121]. These results, together with the data provided here, indicate that the mechanics of the neuronal membrane is intrinsically coupled to its electrophysiology.

The contribution of the cytoskeleton to the results introduced here is not believed to be very significant due to the indentation depth falling within the cell's LVR, thus *a priori* pointing towards a limited biochemical reorganisation. A given degree of plastic deformation arising from bond ruptures within the cytoskeleton [365], in turn affecting the cell's electrophysiology [359] is still, however, possible. In Ref. [366], the authors argued that a negative (left-) shift found in the voltage-dependence of Na^+ channels was due to ruptures of sites of membrane attachment to cytoskeleton. Whether the irreversible AP characteristics observed here would recover over a longer period, or are the product of some mechanical damage, would need to be assessed in future work.

5.4 Conclusion

The results presented in this chapter on the quasi-static indentation of F11 cells and the induced AP changes provide evidence of the need to consider the effect of mechanics on the generation and propagation of neuronal APs. In particular, altered APs in compressed neurons appear as the result of the interplay between membrane and ion channels topological and conformational alterations. In the proposed paradigm, all physical processes need to be accounted for simultaneously and collaboratively to capture the mechanics-driven functional alteration of neurons in the nervous system.

5.5 Dissemination

The results and analyses presented in this chapter were published in Progress in Biophysics and Molecular Biology on 12th January 2021, under the Special Issue of Physics of Excitable Media, as a manuscript titled “Action potential alterations induced by single F11 neuronal cell loading”, DOI: 10.1016/j.pbiomolbio.2020.12.003.

Chapter 6

Action potential alterations under dynamic loading

While a number of US neuromodulation experiments have successfully achieved neuronal activity alterations in an extensive list of animal and cellular models [70], the involved mechanisms of action remain largely unknown. The wide variety of animal scales and models and experimental conditions employed prevent the identification of the optimal frequencies and intensities leading to specific results. Furthermore, there is no established convention on how to report the applied sonication magnitudes (pressure, different types of time or space averaged intensities, etc.), which complicates comparisons and challenges repeatability [367]. Even though a few studies have included simulation results to calculate the amount and characteristics of the mechanical energy that reaches the sonicated cells, especially important in the context of transcranial focused US stimulation tests [86], the vast majority solely reported the specifications of the US transducer, placed at the other side of the skull. Whilst this lack of characterisation has not precluded the development of the field, a deep understanding of the mechanisms involved is needed to fully establish US neuromodulation's efficacy and specificity [24].

Although mechanical effects have been suggested as the main factor dominating the interaction during LIFU sonications [70, 86, 101, 103, 368], mechanistic

studies of the influence of mechanical waves on neuronal electrophysiology at the tissue or cellular level remain scarce. *In vitro* studies have broadly studied the effects of mechanical stimulation in the context of touch or pain sensation [369], neuronal outgrowth [370] and traumatic brain injury [371, 372]. In regards to electrophysiological effects of dynamic mechanical stimuli, early experiments with frog and mouse sciatic nerves achieved brief and fully reversible enhancement and suppression in the amplitude of the compound AP by direct pressure pulses applied with transducer-driven glass and single US pulses [38, 373]. Another study combined microindentation and a high-density multi-electrode array to stimulate mice retinæ at 0.1, 1 and 10 Hz (far from US frequencies) and 10, 20, 30 and 60 μm indentation depths [374]. The stimulation evoked a plethora of different responses, with the main one being a prolonged increase in the firing rate lasting for a few seconds, suggesting that the retina perceived the mechanical stimulation as modulation of the visual input. The mechanical response of smooth muscle cells to LIFU was also studied, and the results showed fluidisation of the cytoskeleton and acceleration of its remodelling mechanics [103] as a consequence of US disrupting bonds within cytoskeletal proteins [375]. Recently, oscillating pistons at 60 Hz (again well below typical US ranges) and AFM were used to stimulate rat cortical neurons treated with synaptic activity inhibitors through global shear stress and local indentation, respectively [376]. These stimulations evoked transient and sustained Ca^{2+} activity in neurons, which was not fully inhibited by the blockage of MSCs, but was suppressed by the application of TTX and benipidine, blockers of voltage-gated Na^+ and Ca^{2+} channels, respectively. The authors proposed that subtraumatic forces/pressures applied to neurons evoked neuronal responses *via* nonspecific gating of ion channels, primarily voltage-gated. Further Ca^{2+} responses to mechanical stimulation have been reported by several groups. DRG cells seeded with magnetic nanodiscs and subject to weak alternated magnetic fields at 1 or 5 Hz (below US frequencies range) demonstrated the ability to trigger Ca^{2+} influx by magnetic torque [377]. Increases in intracellular Ca^{2+} concentration were also reported in early results of mechanical stimulation of nodose sensory neurons [362],

which were reversibly blocked by gadolinium, a MSC channel blocker that also modifies membrane lipid packing [190]. In two recent studies, continuous 250 kHz and 500 kHz US stimulation of transgenic mouse brain slices induced calcium signaling increases [378], and stimulation of leech ganglia with a 490 kHz and 8 to 20 kPa US transducer reported an accumulative prolonged depolarization and faster APs [92]. A theoretical model suggested that the selectivity of US excitation of different cortical neuron subtypes by changing the stimulation pattern, was driven by the response properties of T-type calcium channels [379]. Although many of these effects have been attributed to mechanosensitive responses, the effects of mechanical stimuli on voltage-gated ion channels have also been suggested.

To date, no study has performed intracellular recordings of single neurons under direct mechanical vibrations at varying US frequencies, nor quantified the AP characteristics alterations induced by such mechanical stimuli. In this chapter, the influence of dynamic loads at frequencies up to 1 MHz at two different indentation oscillating amplitudes directly applied to single neurons is studied. The proposed methodology aims at minimising the wave-induced thermal effects, while singling out the mechanical contribution, and eventually shed light on the effects of frequency and amplitude of US neuromodulation protocols in the modulated response, as well as give insights into the possible mechanisms of action involved.

6.1 Materials and methods

6.1.1 Cell line

F11 cells on their 5th and 6th differentiation day and passages from 18 to 25 were used for experiments. F11s were expanded and differentiated following protocols presented in Chapter 3. Isolated cells with contrasted spherical large somas, showing neurites elongations were studied. Their characterisation is included in Chapter 4.

6.1.2 Combined dynamic mechanical loading and electrophysiological recording

The whole cell configuration was obtained following the protocol detailed in Section 3.4.1. If the cell showed stable spontaneous activity, sometimes aided by very low (up to 40 pA) induced currents, the nanoindenter was manually brought into contact with the cell soma, opposite the microelectrode.

Two different dynamic loading regimes were studied in two different configurations: low frequency (LF) compression and high frequency (HF) shear, those accessible with the Chiaro nanoindenter standard and HF piezos (see Chapter 3 and Appendix A). For both regimes, probes of 0.05 N/m stiffness and 10 μm spherical tip radius were used.

For all the tests, a 500 nm static compression in indentation-controlled feedback loop was maintained, and two different oscillating indentation amplitudes were compared: 25 and 50 nm (see HF calibration in Section A.5 in Appendix A). The 500 nm static compression was chosen to maximise cortical membrane contact [380, 381]. The 25 and 50 nm correspond to shear strains $<0.3\%$ in these $\sim 20 \mu\text{m}$ diameter cells, thus ensuring that most of the mechanical effects are concentrated on the cortical membrane solely. Separated experiments were conducted for each frequency and oscillation amplitude combination.

For each test, the static load was maintained for 400 s. 160 s were recorded before the vibration started, 80 s during each LF or HF loading, and another 160 s after the oscillation was released. These durations were empirically determined so that enough spontaneous AP could be recorded at each stage, for subsequent substantial

statistical analyses. Continuous stimulation was chosen, as it has proven as effective as or more effective than pulsed stimuli in focused US neuromodulation protocols [54].

Low frequency compression

The standard piezo from the Chiaro nanoindenter can do dynamic indentations in compression mode of up to 10 Hz. Three frequencies were compared: 0.1, 1 and 10 Hz. As this piezo works under closed loop, no further calibration was needed: the piezo followed the defined indentation profile. Each combination of LF frequency and amplitude was repeated $n=5$ times in a total of $N=11$ cells (see Section 6.2).

High frequency shear

The HF piezo introduced in Chapter 3 is mounted on a different probe holder as standard compression probes, with an improved magnet design to ensure mechanical stability (see Figure 3.10 in Chapter 3). The HF module is an open loop system, whose movement needs to be calibrated to identify the probe's natural resonant frequencies. The calibration process is explained in detail in Section A.5, and one representative analysis of the HF piezo displacement with the input frequency and voltage is shown in Figure 6.1. In order to compare the effects of varying the frequency and the amplitude, three different pairs of adjacent frequencies (250-260, 370-380 and 960-970 kHz) were selected, as they reached two different amplitudes, 25 and 50 nm, indicated in green and magenta, respectively, in Figure 6.1. Table 6.1 shows the combination of input voltages and frequencies selected in the signal generator (see Figure 3.4 in Chapter 3) that drive the HF piezo at the resulting 25 or 50 nm amplitude movement. These amplitudes are comparable to the calculated 30 nm oscillatory displacement induced by LIFU stimulation of smooth muscle cells [103]. $n=4$ experiments were conducted per HF shear loading test: 25 and 50 nm at 250, 370, 960 and 260, 380 and 970 kHz, respectively, in a total of $N=9$ cells (see Section 6.2). The corresponding analysed AP features were then compared. In the following, and in order to facilitate reading, only the smallest frequency of each frequency pair will be used.

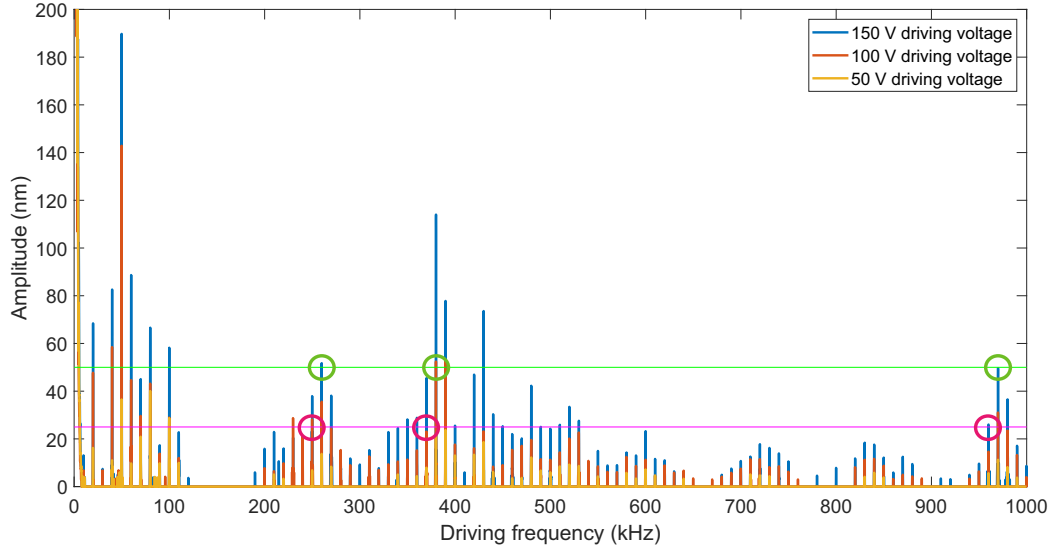


Figure 6.1: HF piezo calibration at three driving voltages of 50, 100 and 150 V and a frequency sweep from 0 to 1,000 kHz in 10 kHz steps. Three different pairs of frequencies (250-260, 370-380 and 960-970 kHz) were chosen to compare two different amplitudes, 25 nm (in magenta) and 50 nm (in green).

Voltage (V)	Frequency (kHz)	Amplitude (nm)
100	250	25
150	260	50
100	370	25
100	380	50
150	960	25
150	970	50

Table 6.1: Input HF piezo voltage and frequency pairs, with the resulting oscillating amplitudes directly applied to single neurons.

6.1.3 AP features extraction

For each combination of frequencies and amplitudes, the features of the spontaneous APs of $N=11$ cells in LF compression and $N=9$ cells in HF shear were compared. If a cell showed stable activity a minute after the first indentation, up to three consecutive loading experiments were conducted, randomly choosing the second and third loading frequencies and amplitudes.

The order effect was checked for all the frequencies, so that, for each frequency, the trend and significance of the normalised AP features from the second and third indentations did not significantly change from those of the first indentation. The

inter-cycle analysis of the highest energy protocol, 970 kHz and 50 nm amplitude, is given as an example in Appendix E, confirming that reproducible responses without significant attenuation were evoked by repeated mechanical stimulation of neurons, and that the analysed features did not significantly change within cycles (see Figures 6.3 and E.3). These results point towards the possibility to submit a cell to a series of tests, the results of which can be taken independently from each other. Similar non significant inter-cycle differences in neuronal activity responses were confirmed in previous mechanical stimulation studies [92, 376].

In addition, a sham experiment with no oscillatory load, but only the static 500 nm indentation held for the total 400 s was performed, and the analysed invariant APs are included in Figures E.1 and E.2 of Appendix E.

AP features were extracted as explained in Chapter 4: AP amplitude, AP width, membrane resting voltage V_{res} , AP threshold voltage V_{th} , rising and falling phases, AP firing frequency and maximum value of the first temporal derivative of the membrane potential, dV_m/dt . Similar to the analyses presented in Chapter 5, and as a result of the varied phenotypes found within F11s [316] and their APs (see Chapter 4), each averaged AP feature value of each dynamic stimulation test was normalised by dividing it by its counterpart before the dynamic indentation.

6.1.4 Statistical analysis

Statistical analyses of the post-processed electrophysiological data were performed with GraphPad Prism 6. The significance (defined with a threshold probability of 0.05) of the observed differences between the three experimental phases in terms of normalised mean values of the analysed AP features was evaluated by conducting a two-way ANOVA test followed by Tukey's multiple comparisons *post-hoc* test to obtain the multiple comparison p-values. The corresponding p values are indicated in figure captions.

6.2 Results

In all cases, cells preserved their spontaneous activity, stable V_{res} and morphology after the stimulation, as checked by microscope inspection, and functional neuronal injury was thus neglected as a first approximation.

6.2.1 LF compression

The AP features of N=11 spontaneously firing cells loaded at 25 and 50 nm and 0.1, 1 and 10 Hz were analysed, with a minimum of n=5 per combination of parameters. If the firing was stable after the indentation, up to three loading experiments were performed on the same cell. The reproducibility of the responses was assumed from the consistent DMA results during a frequency sweep (see Chapter 4) and the 160 s relaxation time given after each LF loading.

Normalised values of the AP amplitude, width, baseline and threshold voltages, falling phase, and maximum value of the first derivative of the AP do not show significant alterations over the studied ranges, see Figure 6.2-A, B, C, D, F, G. The rising phase, i.e, the depolarisation stage of the AP, appears to decrease with increasing frequency, and is significantly smaller after 1 Hz and 25 nm compression compared to during loading (adjusted *p = 0.0478, see Figure 6.2-E). In regards to the firing frequency, all combinations of loading show a tendency to decrease in AP firing both during and after oscillation, with a significant decrease at 1 Hz and 50 nm, both during and after loading with respect to before (adjusted *p = 0.0206 and *p = 0.0282 respectively, see Figure 6.2-H). Aside from the 1 Hz and 50 nm stimulus, the decrease in AP firing frequencies seems to be less pronounced at higher frequencies.

For the 1 Hz and 50 nm oscillation condition, data show that the APs tend to be narrower during the stimulation (Figure 6.2-B), V_{res} appears to be depolarised during and after (Figure 6.2-C) and V_{th} shows a decrease during the stimulation (Figure 6.2-D).

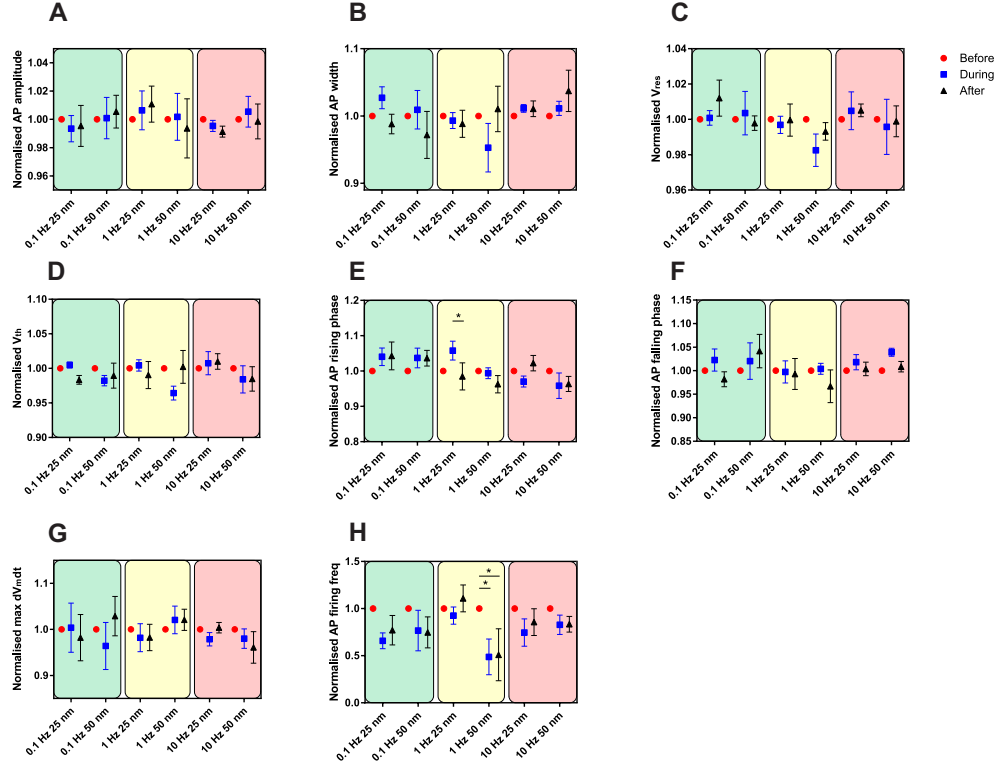


Figure 6.2: Mean and SE of the AP features analyses under LF compression. A) Normalised AP amplitudes. B) Normalised AP widths. C) Normalised resting membrane voltage values. D) Normalised threshold potential. E) Normalised AP rising phases. F) Normalised AP falling phases. G) Normalised max dV_m/dt values. H) Normalised AP firing frequencies. In E) and H), two-way ANOVA with Tukey's multiple comparisons *post-hoc* tests were performed (* $p < 0.05$).

6.2.2 HF shear

A total of $N=9$ cells were loaded in shear at 25 and 50 nm and 250, 370 and 960 kHz, with a minimum of $n=4$ per frequency-amplitude combination. Up to three tests were conducted if the cell showed stable voltage signal and spontaneous spikes after each loading. The reproducibility of subsequent shear indentations on the same cell was checked by performing a control experiment consisting in three repeated stimulations at 970 kHz and 50 nm on the same cell, see Figure 6.3. Results show that the analysed features within cycles did not significantly change within cycles, see Figure E.4 in Appendix E. As this is the highest energy configuration, similar behaviour was assumed for the stimulation at lower frequencies and/or lower amplitudes. An example of the AP shapes and dynamics before, during and after a 970 kHz and 50 nm is included in Figure 6.4.

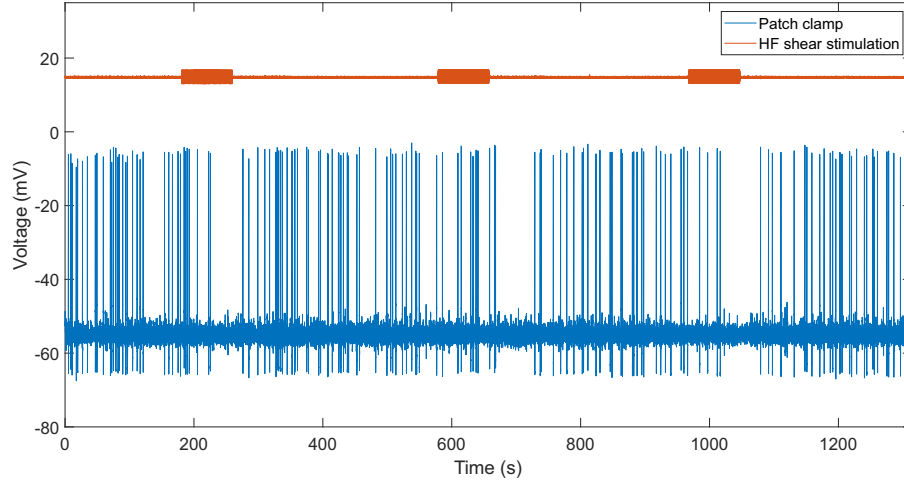


Figure 6.3: Spontaneous activity of a patched cell indented three times at 970 kHz and 50 nm. The analysed AP features showing no inter-cycle variability are included in Appendix E.

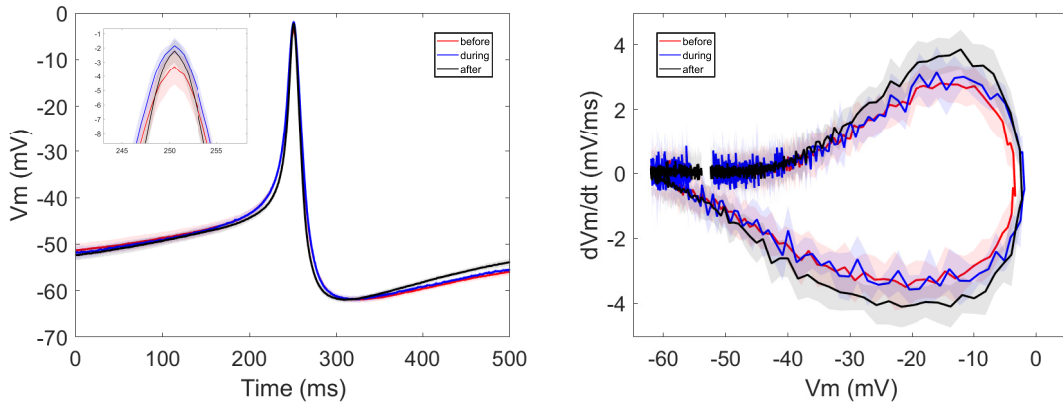


Figure 6.4: Left: Mean and SD of the APs on of a cell loaded at 970 kHz and 50 nm for 80 s, with peaks in the inset. Right: Mean and SD of the phase plots for the APs on the left. There is non significant amplitude changes during the loading, but faster AP dynamics appear after (black phase plot) the mechanical oscillation.

The analysis of the APs before, during and after all the HF loading experiments are shown in Figure 6.5. Normalised amplitudes, resting voltage values and firing frequencies do not show significant alterations under any of the studied conditions, see Figure 6.5-A, C, H. The highest frequency case (960 kHz) shows significant differences in normalised AP width, V_{th} , rising and falling phases and max dV_m/dt , see Figure 6.5-B, D, E, F, G. The widths of the APs of cells loaded at 960 kHz are significantly narrower after 25 and 50 nm loading compared to before and during (adjusted **** $p < 0.0001$, see Figure 6.5-B). For the 25 nm case, cells also show

significantly hyperpolarised V_{th} values after the shear load (adjusted $*p = 0.0408$ and 0.0195 with respect to before and during, respectively, see Figure 6.5-D). The depolarising phase after the 50 nm and 960 kHz loading is significantly shorter compared to before and during such loading (adjusted $***p = 0.0008$, $**p = 0.0013$, see Figure 6.5-E). The repolarisation phases for both amplitudes at the maximum frequency are significantly shorter after the shear indentation with respect to during (adjusted $**p = 0.0038$ for 25 nm, $**p = 0.0064$ for 50 nm, see Figure 6.5-F). Finally, the maximum values of the time-voltage change ($\max dV_m/dt$) are significantly larger after the 960 kHz oscillations at 25 and 50 nm amplitudes compared to before and during, respectively (adjusted $*p=0.408$, $p^*=0.0195$, see Figure 6.5-G).

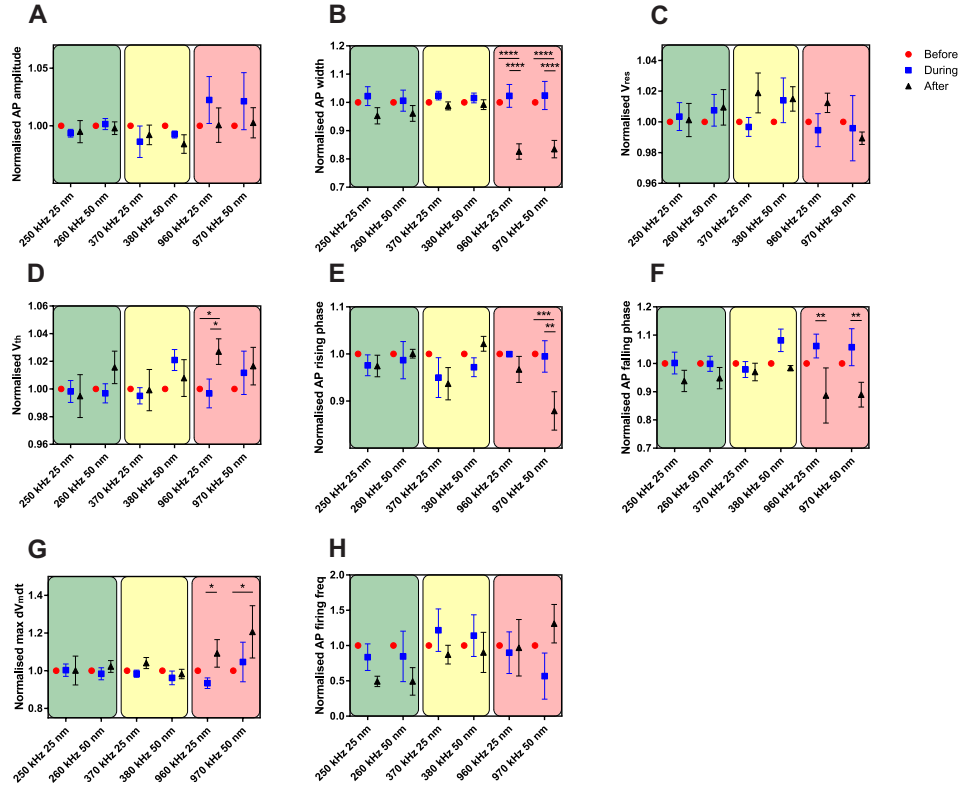


Figure 6.5: Mean and SE of the AP features analyses under HF shear. A) Normalised AP amplitudes. B) Normalised AP widths. C) Normalised resting membrane voltage values. D) Normalised threshold potential. E) Normalised AP rising phases. F) Normalised AP falling phases. G) Normalised $\max dV_m/dt$ values. H) Normalised AP firing frequencies. In B), D), E), F) and G), two-way ANOVA with Tukey's multiple comparisons *post-hoc* tests were performed ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$). Note that the during and after data in H) are not statistically significant.

6.3 Discussion

Within the frequency range investigated in LF, F11 cells responses are predominantly elastic ($E' > E''$), and both storage and loss moduli increase with increasing frequencies (see Chapter 4). At higher indentation frequencies, cells are thus stiffer and also dissipate more energy. The cellular response to a dynamic mechanical stimulus is dictated by how these energies perturb (transiently or in a sustained manner and locally or globally depending on the dissipation rate) subcellular structures, and how the cell actively reacts to them. Neurons subjected to LF compressions show an overall decrease in the rising phases of their APs with increasing frequency (Figure 6.2-E), indicating that faster compressions rates (here, 10 Hz) speed up the depolarisation stage of APs, whereas slower rates (0.1 Hz) elongate it. In addition, Figures 6.2-C and D indicate that larger oscillating amplitudes depolarise cells more (both V_{res} and V_{th}) at each frequency pair, especially when loaded at 1 Hz and 50 nm. Similar results of increased membrane potential depolarisation with larger pressures have been observed in leech ganglia subjected to US at 490 kHz and 8 to 20 kPa [92]. Despite showing more depolarised cells, firing rates are reduced during and after all combinations of stimulation parameters (Figure 6.2-H). The activity seems to be less suppressed at higher frequencies, and at larger amplitudes within the same frequency, and is significantly reduced during and after 1 Hz and 50 nm loading. The results shown in this study are in line with those presented in Chapter 5, where hyperpolarised cells after quasi-static loading showed an increase in the firing frequency, attributed to the higher availability of Na^+ channels. Na^+ channels, needed to trigger an AP, inactivate at depolarising potentials but are readily available at hyperpolarising ones. In a recent paper, studies on mechanical stimulation of DRG cells *via* magnetic nanodiscs subject to weak alternate magnetic fields also observed increased Ca^{2+} signal activation at 5 Hz as opposed to 1 Hz [377], as well as with larger amplitudes. During and after loading at the lowest frequency, 0.1 Hz, rising phases (Figure 6.2-E) and most of the falling ones (Figure 6.2-F) are longer. This phenomenon is accompanied by consistently reduced firing rates at this frequency (Figure 6.2-H). These observations point

towards a slowing down of AP dynamics and AP inhibition during and at least a few minutes after the slowest oscillations. The total duration of F11s' APs (from V_{th} to its minimum value) is around 20 ms (see Chapter 4), which corresponds to a 50 Hz voltage change. When considering the in-phase mechanical wave accompanying the AP (see Chapter 2), all these LF compressions could potentially slow down the dynamics of the APs and somehow hinder their generation through coupling mechanisms. This is in accordance with the decreased AP firing values found throughout the studied frequencies (Figure 6.2-H).

At HF, on the other hand, viscous effects approach the elastic ones, and might even overtake them at around 1 kHz [220] or 60 kHz [381]. Cellular viscoelastic responses at these kHz frequencies are scarce, but two distinct regimes have been identified and attributed to the fast and the slow dynamics of the cytoskeleton [220], whose individual filamentous components are excited at HF [381]. Although the indentations applied here are not expected to directly affect the main cellular cytoskeleton, they influence the membrane cortex, which the cytoskeleton is linked to. Prompt fluidisation and subsequent re-solidification of the cytoskeleton of smooth muscle cells was reported at 1 MHz stimulation [103], as a result of US disrupting bonds within cytoskeletal proteins [375]. The setup presented in this work does not allow the recording of the interference pattern resulting from the shear stimulation. Additionally, the HF piezo works in open loop, for which the specific HF stimulation-induced mechanical responses cannot be fully assessed. However, the electrophysiological changes induced by these mechanical alterations are presented in Figure 6.5.

Notable changes in the analysed AP features appear at the highest HF studied (960 kHz). Figure 6.5 shows faster AP dynamics at vibrations around 1 MHz: significantly narrower (in time) APs after loading (Figure 6.5-B), with shorter rising phases and repolarisation phases after indentation (Figures 6.5-E and 6.5-F). Furthermore, the maximum rate of depolarisation is also significantly larger after 960 kHz shear stimulation at both amplitudes (Figure 6.5-G), confirming the induced faster voltage dynamics. The threshold voltage is significantly hyperpolarised after

the 25 nm displacement at that same frequency (Figure 6.5-D). Within each frequency pair, hyperpolarized V_{th} values appear after larger amplitudes (Figure 6.5-D), hinting at less depolarizing current needed to fire an AP during deeper oscillatory indentations. These findings are in accordance with faster repolarisations found in mechanically stimulated cells at 60 Hz [376] and 490 kHz [92]. The rising and falling phases are shorter after the indentation frequencies from 1 Hz to 960 kHz, leading to narrower APs after these loadings, and corroborating the faster AP time changes promoted by higher mechanical frequencies. Regarding the normalised amplitudes, it appears that APs modulated at 960 kHz are larger during the stimulation (Figures 6.4 left and 6.5-A), but smaller during and after 370 kHz loading. Smaller APs were found upon 490 kHz stimulation of leech ganglia [92]. Although overall the firing frequencies decrease after HF stimulation with respect to before, similarly to the LF results, this effect seems to be less pronounced at higher frequencies, and the inverse is actually observed after 960 kHz and 50 nm oscillations (Figure 6.5-H). A reason for this could be that fast mechanical oscillations propagating through the membrane favour the AP-accompanying mechanical deflections (see Section 2.3.1) and facilitate cell depolarisation. Increased maximum firing frequencies were observed in rat brain slices stimulated with 43 MHz US [382]. V_{res} analyses show hyperpolarised cells (Figure 6.5-C) under 250 and 370 kHz, while cells loaded at 960 kHz show a more depolarising trend, in particular after the largest oscillation amplitudes. Whilst hyperpolarised membrane potentials –as well as narrower APs– were observed under continuous US sonication of rat brain slices at 43 MHz [382] and attributed to the activation of a sustained potassium conductance by US, prolonged and pressure dependent depolarisation was previously reported in cells under 490 kHz US stimulation [92].

All the above observations suggest that higher oscillatory indentation frequencies, both in compression and shear, promote faster AP dynamics with a threshold somewhere between 370 and 960 kHz. The effect of quasi-static indentations, presented in Chapter 5, lead instead to wider and slower APs, so there appears to be a threshold frequency above which the induced faster AP modulation effects emerge.

The AP time length being shorter while maintaining its amplitude translates in less electrical energy needed to fire an AP, hence indicating that a mechanical stimulus of sufficiently high frequency facilitates AP generation and makes the neuronal signal more electrically efficient. At kHz frequencies cell stiffness always increases with frequency ($E'(f_2) > E'(f_1)$ for $f_1 < f_2$). Here, the cellular membrane and the membrane cortex are the main structures sensing and transducing the mechanical stimuli. If the membrane becomes stiffer, the gating mechanisms of the embedded ion channels are affected [115, 116, 197, 198], which alters the generation and evolution of the AP. The induced 25 and 50 nm mechanical oscillations travel faster through the stiffer membrane at higher frequencies, potentially explaining the faster modulated functional dynamics observed at higher frequencies. These fast membrane oscillations could indeed induce membrane area and capacitance changes, found in artificial protein-free lipid membranes stimulated with 1 MHz US [77], that could contribute to the observed effects (see Equation (5.1) in Chapter 5).

In parallel, the AP has been referred to as a propagating membrane phase change (see Section 2.3.2). In order for this phase change to occur (from fluid to gel), a local decrease in temperature is needed. In polymer physics, the time-temperature superposition principle establishes that changing a material's temperature is equivalent to shifting its viscoelastic behaviour by time-dependent means, such as frequency. As such, decreasing the temperature is equivalent to increasing the frequency. This fundamental principle is applicable to brain tissue [383]. Accordingly, lipid membrane phase transitions could be favoured by mechanical vibrations of enough frequency, and these could, in turn, generate, or at the very least modulate, APs. In practice, the highly inhomogeneous neuronal membrane presents numerous dynamic domains (see Section 2.2.1), which are thought to correspond to different membrane phase states [384, 385], and compartmentalise proteins, such as ion channels [175]. The individual and collective responses of these domains are envisioned to be altered under direct mechanical stimulation protocols such as the ones presented here, with the corresponding functional alterations observed, especially at high frequencies.

Moreover, in many of the HF results presented in this chapter, the altered AP effects only appear after the load is removed, which is more evident at higher frequencies. This points towards a lag or an accumulation of the response, that has already been observed in US studies *in vitro* [92, 93], and attributed to the build-up of intracellular Ca^{2+} [93, 378], and *in vivo* (up to 10 min after sonication) [67–69], and that might be more pronounced at higher frequencies. Here, a stress-relaxation mechanical viscous effect coupled to electrophysiology by the membrane mechanical state could explain the observations.

Finally, thermal effects arise during loading of viscoelastic solids as a result of the dissipated mechanical energy, more prominent at higher frequencies and continuous stimulations such as the ones presented here. A numerical estimation of the heat generated during 970 kHz and 50 nm shear indentations is calculated in Appendix F. The numerical analysis shows that the induced maximum temperature increment for the highest energetic situation is negligible at the cell level (10^{-5} °C), and quickly diffuses over the surrounding extracellular solution.

6.4 Conclusion

This chapter presents the capability of the multiphysics setup introduced in Chapter 3 to mechanically load single neurons up to 1 MHz in frequency. The proposed methodology provides a quantitative framework to mechanically stimulate neurons at US frequencies, while cells are monitored *via* direct microscopy throughout. Results show that direct mechanical vibrations applied to the cellular membrane and membrane cortex modulate the neuronal AP shape. In particular, higher frequencies tend to speed up AP dynamics, and larger amplitudes emphasise the modulatory effects too. In addition, results are more evident once the oscillation is removed, pointing at a cumulative or lagged effect of US stimulation. These observations are attributed to the stiffening of the membrane and membrane cortex as a result of the mechanical oscillations and its subsequent relaxation, and emphasise the role of the membrane and its mechanical properties in the AP shape and firing process. These findings contribute to the understanding of how neurons respond to dynamic mechanical stimuli. In particular, the promoted faster AP dynamics that emerge after high frequency mechanical oscillations suggest a potential mechanism of US neuromodulation and assert the importance of direct mechanical cellular agitations arising from US stimulation protocols, whether or not other effects (e.g., thermal) are involved. Finally, most of these results appear to be strengthened at a frequency between 370 kHz and 960 kHz, in agreement with the literature [54, 70, 386, 387].

Future complementary works could be directed to study the influence of intermediate frequencies (especially those between 370 and 960 kHz, aiming at finding a threshold frequency for the observed effects) and larger amplitudes, different pulsed protocols, as well as explore the responses when the vibrations are sent to axons and dendrites, instead of cell somas. Furthermore, the activity of neighbouring neurons could be tracked, in order to infer the influence of modulated APs in single cells within a network. Finally, phase membrane indicators such as Laurdan, a membrane dye that changes fluorescence as a result of alterations

in the lipid packing density, could be used to identify possible membrane phase changes induced by high frequency indentations.

Chapter 7

Conclusions, limitations and future directions

7.1 Summary

Non-electrical manifestations of the neuronal AP have been observed in an ample range of cells. These include mechanical, optical and thermal changes that happen in phase with the electrical depolarisation. However, they have been mostly left aside by the neuroscience community, and the current leading electrophysiological models do not account for such changes. Chapter 1 identified US neuromodulation as a technique that validated the multiphysical nature of the neuronal AP, demonstrating that the AP could be modulated by mechanical waves. Paradoxically, despite successful results, the mechanisms behind US neuromodulation remain unclear. To this end, this thesis aimed at addressing how specific mechanical vibrations, such as those induced by US in tissue, influence the neuronal signal at the single cell level.

The leading hypothesis was that the neuronal membrane was responsible for the coupling effects. Its electrical and mechanical properties were reviewed in Chapter 2. Chapter 3 presented a setup that combined nanoindentation and patch clamp techniques with an inverted microscope and allowed for multiphysics studies at the single cell level. The validation of the individual techniques composing the setup was demonstrated in Chapter 4, where the F11 cell line was optically, mechanically and

electrophysiologically characterised. The proposed methodology was used to analyse the AP features (amplitude, width, rising and falling phases, etc.) under different mechanical loading conditions (up to 1 MHz oscillations), with the objective of identifying the effects of direct mechanical vibrations on single cell electrophysiology and shedding light on the mechanism of action of US neuromodulation. In Chapter 5, quasi-static mechanical loadings were applied to single neurons. Results showed transiently smaller, wider and slowly rising APs during the indentation, which translated into slower AP dynamics induced by the quasi-static load. In addition, loaded cells showed sustained hyperpolarisation and longer repolarisation rates after indentation. The quasi-static study emphasised the role of the membrane and its altered mechanical properties resulting from the indentation in shaping the neuronal signal. Chapter 6 delved into the effects of direct dynamic mechanical loadings, from 0.1 Hz to 1 MHz applied to single neurons, on the characteristics of their APs. Dynamic loads induced narrower APs with shorter depolarisation and repolarisation phases at increasing frequencies, translated into faster induced AP dynamics, in particular for excitations after around 1 MHz. The significantly faster AP dynamics appearing after ~ 1 MHz oscillation pointed towards a cumulative or lagged effect of mechanical stimulation at US frequencies. It was proposed that remarkable membrane properties changes were achieved above a threshold frequency, situated between 370 kHz and 960 kHz, which modulated the AP and were possibly related to membrane stiffening and potential phase state alterations.

7.2 Conclusions

The first contribution of this thesis is the versatile setup proposed in Chapter 3, which allows for simultaneous mechanical and electrophysiological measurements. It is relevant for fundamental multiphysics single cell studies such as the one undertaken here. Using this setup, evidence is provided for the modulation of the neuronal AP shape and dynamics under quasi-static and dynamic mechanical loads. APs of cells during quasi-static loadings are transiently smaller, wider and slowly rising, indicators of slower AP dynamics. This is accompanied by sustained cell hyperpolarisation and longer repolarisation rates after the indentation. On the contrary, low frequency compression and high frequency shear excitations up to 1 MHz speed the time signal of the APs up after the stimulation: narrower APs with shorter rising and falling phases at increasing frequencies, in particular for vibrations around 1 MHz. The results appearing after the oscillation hint at an already proposed cumulative or lagged effect of US stimulation tests. It is hypothesised that these AP modulation results arise as a consequence of the altered membrane properties achieved especially at the highest oscillatory frequencies, and that might be related to membrane stiffening and membrane phase state modifications.

The results presented in this thesis are summarised in Table 7.1 and Figure 7.1. They highlight the importance of mechanical cues in shaping the neuronal AP. In particular, the promoted faster AP dynamics that emerge after high frequency mechanical oscillations are suggested as a potential mechanism of US neuromodulation. This observation asserts the importance of the direct mechanical cellular agitations induced by US stimulation protocols in assisting the observed neuromodulatory effects. The work presented in this thesis underscores the need to take the mechanical properties of the neuronal membrane into account when characterising the AP or aiming at modulating it, and emphasises the role of the often overlooked membrane bilayer properties in determining neuronal functionality.

It is envisioned that the findings presented here for the various mechanical loading regimes tested could be directed to improve clinical applications such as the mentioned US neuromodulation, aiming at inducing controlled and well

characterised neuromodulatory effects. Furthermore, this knowledge could provide a better understanding and potential treatment avenues for the neural mechanobiology and the linked functional deficits during neuronal pathologies including traumatic brain injury [388–390], brain oedema and swelling [391], Alzheimer’s disease [392], chronic neuropathic pain [27], carpal tunnel syndrome [393] or spinal cord injury [394–396], see Table 7.1. All these conditions present functional deficits that have been linked to altered mechanical properties. Mechanical stimuli could then be used to tune these properties and help restore physiological conditions.

Indentation	Quasi-static	LF	HF
Frequency	0 Hz	0.1 Hz – 10 Hz	250 kHz – 970 kHz
Amplitude	3 μm	25 and 50 nm	25 and 50 nm
Duration	80 s	80 s	80 s
Main findings	Slower AP dynamics. Smaller and wider APs during indentation. Hyperpolarised cells after indentation.	Faster AP dynamics at higher frequencies.	Significantly faster AP dynamics at ~ 1 MHz, with shorter rising and falling phases.
Clinical relevance	Study of carpal tunnel syndrome [393], spinal cord injury [394, 396, 397] and brain swelling [388].	Promote neurite outgrowth [398] and neuroplastic repair [399].	Frequency and amplitude tuning of US neuromodulation tests. Explore treatments for Alzheimer’s disease [392] and brain ischemia [400]

Table 7.1: Summary of the AP alterations induced by single neuron mechanical loading presented in this work, together with their associated clinical relevance.

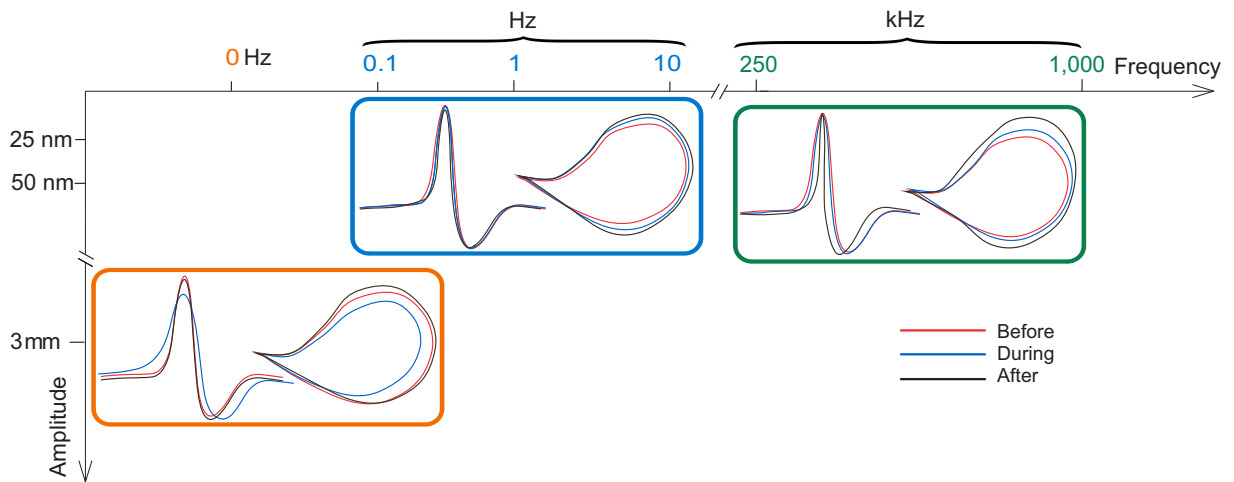


Figure 7.1: Qualitative schematic AP shape and AP dynamics alterations induced by the different loading conditions studied in this work (quasi-static in orange, LF in blue and HF in green). AP shape and evolution trends are exaggerated for better visibility.

7.3 Limitations and future directions

In the following, the limitations of this study are presented. First, the limitations for the extrapolation of the results achieved in Chapter 6 to the mechanisms of US neuromodulation are listed. Second, the limitations of the current setup and methodology are discussed, as well as future directions are proposed.

7.3.1 Limitations for extrapolation to mechanisms of US

Although the membrane mechanical properties alterations induced by the HF vibrations of Chapter 6 might be related to those induced by US in neuronal tissue, the extrapolation of these results to the mechanisms of US neuromodulation has several limitations:

- Whereas US stimulation protocols generate the same pressure over the entire cell surface (wavelengths of mm [24, 70]), the HF mechanical loading is localised to an area of the cell soma, within the μm length scale.
- With the methodology employed in Chapter 6, only the effects of shear waves in the specific direction of the HF indentation are studied, while US induces a combination of longitudinal and shear waves in multiple directions [23]. In addition, the setup designed in this work minimises the effects on the surrounding extracellular medium, whilst acoustic streaming (i.e., fluid flow in the US propagation direction), for instance, has been proposed to contribute to US neuromodulation results [378].
- The duration of the applied mechanical vibrations in this work (80 s) is two orders of magnitude longer than commonly used pulsed US protocols (hundreds of ms) [401].
- While the frequencies studied in this work (0.25–1 MHz) are in the range of those typically used in therapeutic US applications [29], other works have used sonications of tens of MHz [86, 402].

- The maximum shear force applied to the cell soma, corresponding to the 50 nm indentation and calculated from the force-indentation response in compression, see Figure 4.7, is 0.055 nN (from 0.166 nN in compression and applying Equation (4.4) with $\nu \approx 0.5$). The magnitude of this force (55 pN) is four orders of magnitude smaller than the 873 nN applied to *C. elegans* through sonication at 10 MHz, 1 kHz pulse repetition frequency and 50% duty cycle, calculated with the k-Wave toolbox [86]. The amplitude of the displacement (50 nm) is also between two and three orders of magnitude smaller than the observed 18-300 μm displacements induced by 1–5 MPa at 4 MHz in mice sciatic nerves [289].

7.3.2 Limitations on the proposed methodology and future directions

In this project, a hybrid sensory cell line was used, which presented MS traits. Using a non-sensory cell type, with well defined AP features and natural firing frequency, would be recommended for future studies, to further confirm the results. Human-derived induced Pluripotent Stem Cells (iPSCs) could be a good candidate, as they are relevant for literature and have a more direct clinical applicability. Cortical neurons were derived from human iPSCs in this work, but were not selected as good candidates for the proposed study as they present small somas (see Figure 7.2) that did not allow simultaneous mechano-electrophysiological recordings with the current setup. For future work, sharp electrodes could be used to overcome this limitation. They present smaller tips than patch pipettes and are less invasive, as they are used to impale the cell instead of patching its membrane. In addition, the effect of single cell stimulation could be investigated in neighbouring cells (network effects) with voltage sensitive fluorescent dyes such as FluoVolt, or *via* Ca^{2+} imaging.

Smaller bead sizes and a closed-loop HF module would also facilitate the study of intermediate frequencies and different amplitudes, as well as enable mechanical characterisation tests at HF. These characterisation tests could be combined with simultaneous fluorescence dyes (such as Laurdan) that report on the state of the

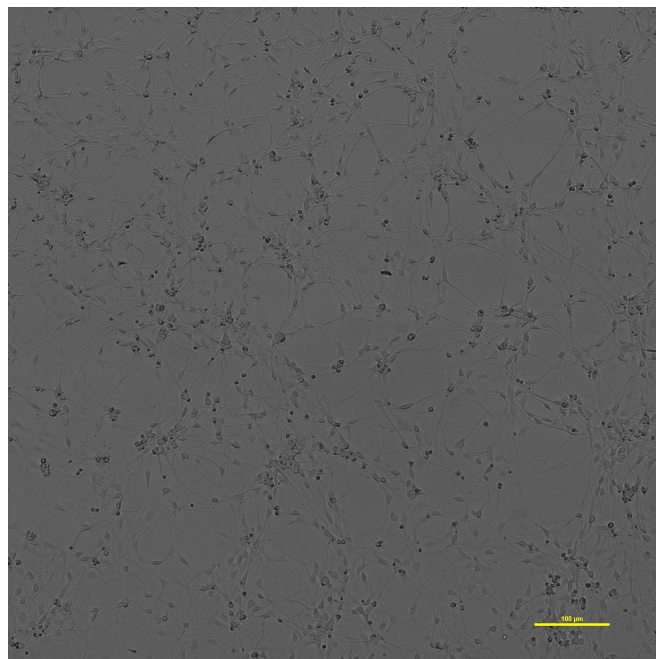


Figure 7.2: Human iPSC-derived cortical neurons differentiated for 16 days by dual inhibition of SMAD signalling. Their small somas did not allow to fit both pipette and nanoindenter in the same cell. Scale bar is 100 μm .

lipids in the membrane to relate the induced cellular mechanical responses to the state of the membrane under high and low indentation frequencies. These could be further related to the observed faster APs induced at ~ 1 MHz shear loadings (see Chapter 6). Furthermore, similar concurrent mechanical properties characterisation and membrane phase state assessments could be performed at different depolarisation and hyperpolarisation potentials on a voltage clamped cell to investigate the potential link between membrane mechanical properties, membrane phase state and membrane voltage.

To identify the transducers leading the mechano-electrical coupling, the effects of voltage-gated and MSC blockers (TTX, tetraethylammonium, gadolinium), or cytoskeletal proteins depolymerisation drugs (cytochalasin D, latrunculin) could be studied, as well as the lipid composition of the membrane altered with cholesterol depletion (β -cyclodextrin), for instance, to enquire the specific contributions that these subcellular structures have on the cellular mechanical response at different loading frequencies, and the corresponding coupled electrophysiological effects.

In this work, a 960 kHz and 50 nm shear stimulation has been shown to induce electrophysiological responses in a DRG cell model. For future work, and in order to scale up to tissue, I propose to use a local US probe [403] to first stimulate cultured neurons and then brain slices with US frequencies that include the mentioned 960 kHz. Figure 7.3 shows a 200 μm diameter optical fibre coupled to an infrared (IR) laser and coated with a carbon nanotubes and PDMS coating at the end. The coating absorbs 98% of the energy of a light pulse [403], and subsequently contracts and expands due to the so-called photoacoustic effect [404], hence creating US [289]. Varying the parameters of the initial IR pulse allows to control the characteristics of the generated US pulse. This proposed setup could be combined with Ca^{2+} imaging to quantify the activity of the cells in the field of view before, during and after specific US stimulations. Prior to experiments, the US field needs to be exhaustively characterised with the use of hydrophones whose position could be finely adjusted with micromanipulators. Moreover, simulations *via* the k-Wave MATLAB toolbox [405] could be run to evaluate the amount of energy reaching the cells.

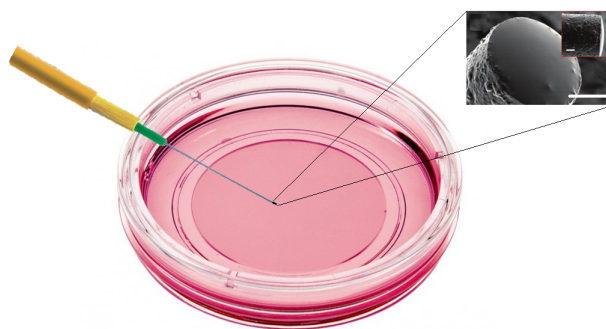


Figure 7.3: Proposed setup for local US stimulation of neuronal networks. Scale bar of the zoomed probe is 100 μm , and 50 μm for the inset.

For the analysis, a qualitative study of the effects of the different stimulation protocols on the activity could be performed by plotting the spiking activity (extracted as explained in Chapter 4) of all the neurons in the field of view over time in a raster plot. Figure 7.4 shows such an analysis for a preliminary study that was conducted in collaboration with Prof. Adrien Desjardins at University College of London, using the photoacoustic probe [403] shown in Figure 7.3. F11 cells were stimulated with US pulses of 1.5 MPa peak-peak pressure, 15–20 MHz

central frequency, 30 MHz bandwidth frequency at the -6 dB level [403] and 1 Hz pulsed repetition frequency for 2 min.

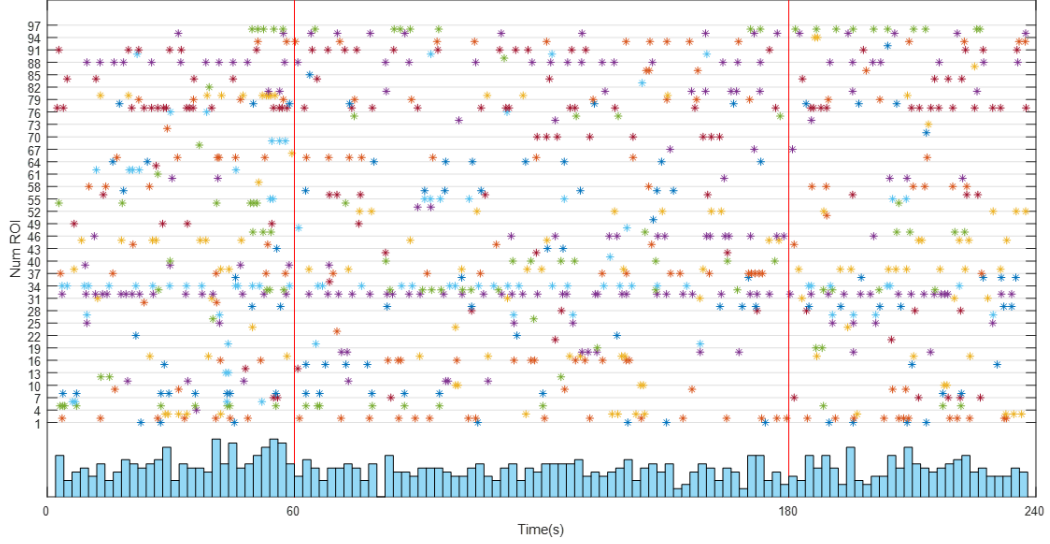


Figure 7.4: Time location of Ca^{2+} transients of each cell or Region of Interest (ROI) in the microscope field of view before, during and after US stimulation, indicated by the two red vertical lines.

In addition, the modulated increase or reduction of neuronal activity, the transience or permanence of these effects, as well as the different cell responses, could then be explored by classifying the evolution of the number of spikes per second or minute for the three stages (before, during and after US) for each cell into the patterns shown in Figure 7.5.

As an example, the most repeated patterns for the experiment shown in Figure 7.4 are presented in Figure 7.6. The most repeated activity patterns for each combination of US stimulation parameters could then be compared, leading to stimulation protocols classified into promoters or inhibitors of neuronal activity, for short or long terms.

Lastly, the correlation between cells showing specific activity patterns and their phenotype, location or orientation with respect to the US probe can then be studied. This would give more insight regarding US specificity and spacial effects. The activity of correlated cells could be also be compared to that in the raster plot in Figure 7.4, and connection studies inferred. Finally, the accumulated response

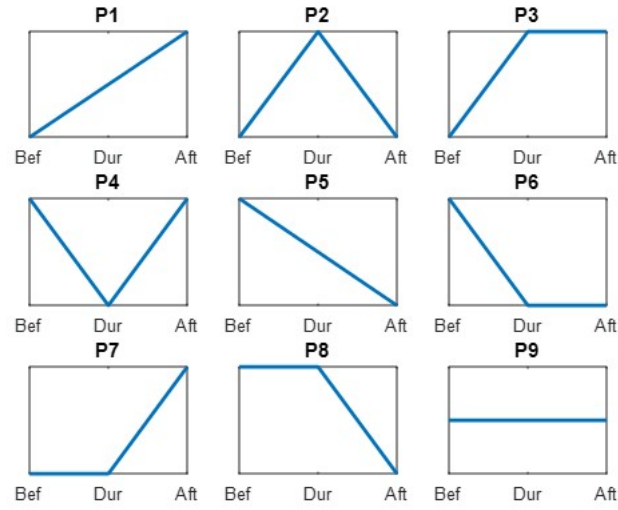


Figure 7.5: Classification of possible cell activity patterns (P). The evolution of the number of spikes per second before (Bef), during (Dur) and after (Aft) US stimulation of a single cell follows one of the presented patterns. By evaluating the most popular patterns appearing at specific US stimulation protocols, these could be classified into neuronal network activity enhancers or inhibitors, in a transient or sustained manner.

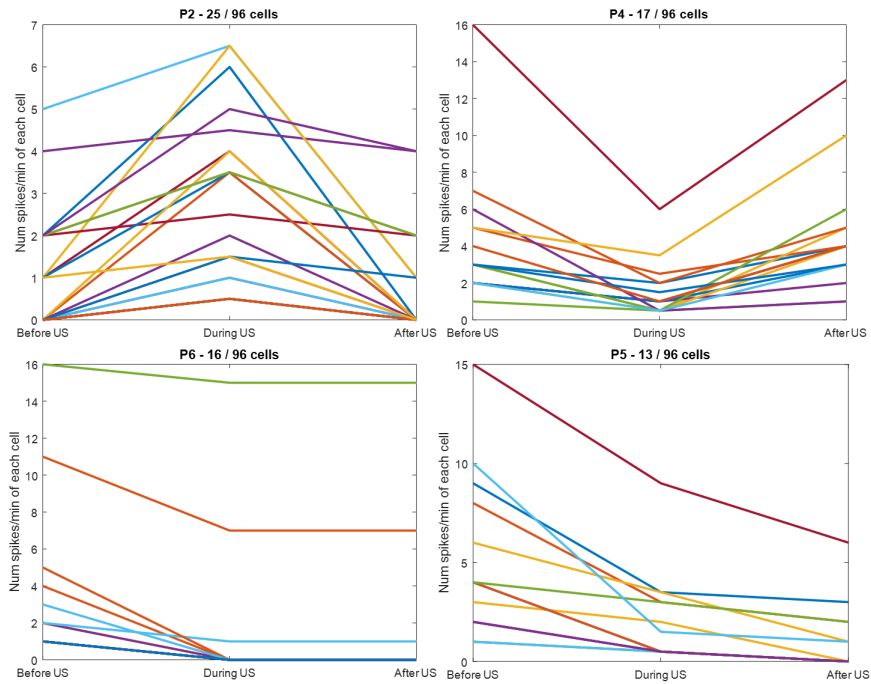


Figure 7.6: Most repeated activity patterns of cells under US stimulation preliminary experiment.

and subthreshold depolarisations, discussed in previous works [92, 93], could also be investigated for specific stimulation protocols. Results could then be compared to Jiang *et al.*'s work, which demonstrated successful activation *in vitro* and *in vivo* following a similar approach [406].

Appendices

Appendix A

Nanoindenter working principle and high frequency module calibration

The Chiaro nanoindenter is shown in Figure A.1. It consists of the indenter head (D) to which the probe is attached to, a controller box (B) and the OP1550 interferometer (C), all connected to a laptop running the Optics11's Piuma software (A).

The probe is a ferrule-top gold cantilever [323] attached to a spherical glass

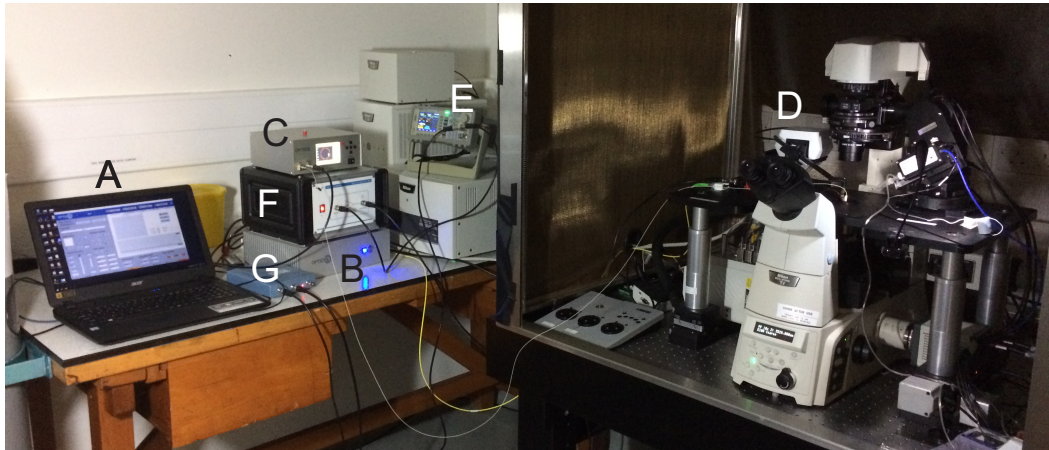


Figure A.1: A) Piuma software. B) Chiaro controller box. C) Optics11 OP1550 interferometer. D) Chiaro nanoindenter head and arm. E) Signal generator. F) Voltage amplifier ($\times 50$). G) Picoscope oscilloscope. E), F) and G) are needed for the high frequency module calibration.

bead. A single-mode optical fibre connected to the interferometer guides the

infrared laser-light ($\lambda = 1550$ nm) through a cavity onto the cantilever and records cantilever bending as a result of sample deformation. The laser is coupled to a fibre coupler or circulator such that the returning interference signal can be detected by a photodiode in the interferometer. No alignment is needed, since it is given by the design of the sensor [323].

A.1 Fabry-Perot interferometer

An interference occurs when two (or more) light waves of the same frequency interact. It can be constructive, if the resulting wave is of greater amplitude than the incoming ones, or destructive, when it has a lower amplitude. A Fabry-Perot (FP) interferometer consists of two parallel mirrors that form an optical cavity that allows for multiple light reflections [407]. In the Chiaro nanoindenter, the two mirrors or reflecting surfaces are the cleaved end of the optical fibre and the gold cantilever (R1 and R2 in Figure A.2).

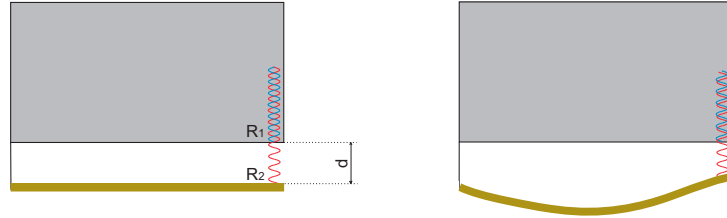


Figure A.2: Left: FP cavity: some light is reflected at the end of the fibre (R1, blue), and the rest at the gold cantilever surface (R2, red). Right: The interference pattern changes as a result of cavity distance d change.

Only light that is in resonance with the FP cavity can leave it, which allows one to determine the absolute size of the cavity by analysing the pattern of the resulting light of a known wavelength. In other words, any change of dimensions within the cavity can be detected by monitoring the interference signal (Figure A.2-right). In Figure A.2, part of the light is reflected at the end of the cleaved fibre (due to refractive index mismatch, illustrated in blue) and the rest exits the fibre (red wave), hits the reflective cantilever (R2) after a certain distance d , where it is reflected and coupled back into the optical fibre. The light reflected off the fibre-air interface and the light from the cantilever then interfere and propagate

back through the optical fibre to the OP1550 interferometer. These two waves of the same wavelength are shifted in phase when superimposed if the cantilever bends (Figure A.2-right). The phase changes 1:1 in relation to a cantilever bending due to external stress, which allows one to extract the cantilever displacement by analysing the phase of the interference wave. This is elaborated in the following.

The amplitude (shown in volts in the OP1550 display) of the interferometric readout or interference pattern coming out from a FP cavity can be described by [407]:

$$V = V_0 \frac{R_1 + R_2 - 2\sqrt{R_1 R_2 \cos(\phi)}}{1 + R_1 R_2 - 2\sqrt{R_1 R_2 \cos(\phi)}} \quad (\text{A.1})$$

where $V_0 = (V_{max} + V_{min})/2$ is the midpoint offset of the sinusoidal interference, with V_{max} and V_{min} being the maximum and minimum voltage values of the interference signal, R_1 and R_2 are the reflectivities of the two FP mirrors, and ϕ is the phase difference between the two beams, which is dependent on the cavity distance d and is defined by [408]:

$$\phi = \frac{2\pi L}{\lambda} = \frac{4\pi d}{\lambda} \quad (\text{A.2})$$

where L is the optical path length ($L = 2d$) and λ the laser wavelength.

For the Chiaro interferometry system, $R_1 \ll 1$ and $R_2 = 1$ (neglecting multiple reflections), and the intensity at the output of the interferometer is described by [409]:

$$V(d) = V_0 \left[1 + V_{vis} \cos \left(\frac{4\pi d}{\lambda} + \varphi_0 \right) \right] \quad (\text{A.3})$$

where φ_0 is a constant phase shift that only depends on the geometry of the cantilever, and $V_{vis} = (V_{max} - V_{min})/(V_{max} + V_{min})$ is the interference fringe visibility. This signal has a periodic nature due to consecutive constructive and destructive interferences. These interferences exist when $\phi = \frac{4\pi d}{\lambda}$ varies as a result of d or λ changes, in which case there is interference fringes. The periodicity of the interference (Equation (A.3)) is $\frac{4\pi d}{\lambda} = 2\pi$, hence $d = \frac{\lambda}{2}$.

A.2 Quadrature point

During an experiment, the laser wavelength remains constant, thus Equation (A.3) can be assumed to depend only on cavity distance d changes as a result of the cantilever bending. If the cantilever bends, the interference pattern follows a cosine wave centred at V_0 . When the cosine is 0, $V \simeq V_0$ and the interference signal is in its linear region, meaning that the readout voltage signal scales linearly with cantilever deflection. This point is referred to as quadrature point (Figure A.3). Around quadrature, $\cos(\frac{4\pi d}{\lambda}) \approx \frac{4\pi d}{\lambda}$ [409], the signal can be linearised and Equation (A.3) can be rewritten as [323]:

$$V(d) = V_0 \left(1 + V_{vis} \frac{4\pi d}{\lambda} \right) \quad (\text{A.4})$$

from where it follows that the minimum detectable change of d is given by [323]:

$$\Delta d_{rms} = \frac{\lambda}{4\pi} \frac{V_{rms}}{V_0 V_{vis}} \quad (\text{A.5})$$

where V_{rms} is the noise value out of contact.

Now, for a given λ , the relationship between the output voltage measured with the interferometer (V) and the actual cantilever bending displacement (nm) is given by the sensitivity S of the laser-based interrogation system:

$$S \left(\frac{V}{nm} \right) = \frac{V_{pp}}{\frac{4\lambda}{n}} \frac{1}{1.3} \quad (\text{A.6})$$

with $V_{pp} = V_{max} - V_{min}$ the peak-peak voltage of the interference signal and n the refractive index of the medium (see Figure A.3).

In order to optimise the sensitivity of the measurement to achieve high resolution measurements, the probe needs to be in close vicinity of the quadrature point, where the response in V from d is approximately linear (Figure A.3). When the signal reaches the peak or valley of the sinusoidal interference response, a slight change in voltage corresponds to a large change in d , which changes a lot the sensitivity and invalidates the readout.

As the periodicity of the interference signal (Equation (A.3)) is $d = \frac{\lambda}{2}$, every $\lambda/2$ (or any integer multiple of it) there is a new fringe, and a new quadrature

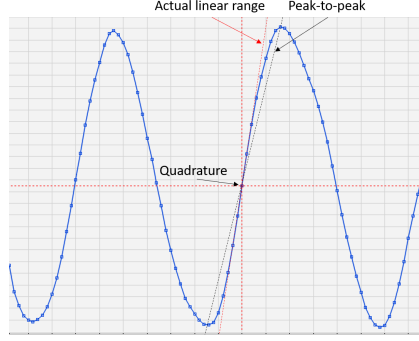


Figure A.3: V_{pp} corresponds to half a period of the interference wave, which is $\frac{\lambda}{4}$ (Equation (A.3) has a periodicity of $\frac{\lambda}{2}$). A correction factor of $\approx 30\%$ has to be applied to correct for the overestimated slope of the peak-to-peak value with respect to the actual linear range. Courtesy of Optics11.

point (red asterisks in Figure A.4-C), where the intensity of the readout signal is maximum. However, this also means that the linearisation and corresponding sensitivity readout is limited to cantilever deflections below $\frac{\lambda}{4} = 387.5$ nm. In many experiments, nonetheless, cantilever deflections in the μm range are required to be measured at high sensitivities. Wavelength modulation is a technique implemented in the OP1550, that allows to overcome this limitation. It is based on the premise that the interference pattern not only depends on the cavity distance d , but also on the wavelength λ of the light source (see Equation (A.3)). Hence by modulating λ , one can ensure that the sensitivity of the signal is optimised beyond 387.5 nm displacements.

A.3 Wavelength modulation

The laser source is tunable over a wavelength from 1528 to 1563 nm. If the wavelength is altered so that instead of a fixed λ , one uses a high frequency sinusoidal modulation $\delta\lambda = \Delta\lambda \sin(\omega_{mod}t)$, of amplitude $\Delta\lambda$ and angular frequency $\omega_{mod} = 2\pi f$, then Equation (A.3) changes to [409]:

$$V(d) = V_0 \left(1 + V_{vis} \cos \left(\frac{4\pi d}{\lambda + \delta\lambda} \right) \right) \quad (\text{A.7})$$

We can approximate Equation A.7 with a Taylor expansion around 0 to obtain:

$$V(d) \approx V_0 \left(1 + V_{vis} \left[\cos \left(\frac{4\pi d}{\lambda} \right) + \sin(\omega_{mod}t) \frac{4\pi d \Delta\lambda}{\lambda^2} \sin \left(\frac{4\pi d}{\lambda} \right) \right] \right) \quad (\text{A.8})$$

which gives us a high-frequency modulated signal of angular frequency ω_{mod} , whose amplitude follows $\sin(\frac{4\pi d}{\lambda})$, in addition to the normal $\cos(\frac{4\pi d}{\lambda})$ response. By using a lock-in amplifier (details below), one can extract the amplitude at that specific frequency, obtaining two sinusoidal signals for the cantilever displacement with a 90° (or $\frac{\pi}{2}$) phase shift (see Figure A.4). These orthogonal signals allow that, when the sensitivity is lost in the cosine signal, it is improved in the sine signal, as when one is at its minimum, the other one is in quadrature (see Figure A.4). The low-frequency (LF) signal still corresponds to the previous solution for interference intensity, Equation (A.3):

$$V_{LF}(d) = V_0 \left(1 + V_{vis} \cos \left(\frac{4\pi d}{\lambda} \right) \right) \quad (\text{A.9})$$

while the high-frequency (HF) signal follows:

$$V_{HF}(d) \approx V_0 V_{vis} \sin(\omega_{mod} t) \frac{4\pi d \Delta \lambda}{\lambda^2} \sin \left(\frac{4\pi d}{\lambda} \right) \quad (\text{A.10})$$

The amplitude of this HF signal thus depends on the cantilever displacement d . With Equations A.9 and A.10, the cantilever deflection can now be accurately determined over the full period or fringe $d = \frac{\lambda}{2}$.

A schematic simulation of a modulated interference response is given in Figure A.4, where the change in interference readout due to wavelength modulation is depicted. The cantilever is displaced linearly over time up to $3 \mu\text{m}$ A), which at a wavelength of $\lambda = 1550 \text{ nm}$ without modulation, gives a sinusoidal response in the interference readout B), corresponding to Equation (A.3). With the HF modulation in place, the recorded interference changes to the signal shown in C). The modulation frequency is set to 50 Hz, to clarify the effect of the response, although in reality it is 150 Hz. In D), the zoomed-in signal in C) shows that the amplitude of the modulated signal is largest in quadrature and smallest in the peaks and valleys of the total interference.

The low and high frequency components could be extracted offline after the measurement is recorded by frequency filtering methods. However, if this were to be the case, one could only drive the piezo with no direct control on cantilever

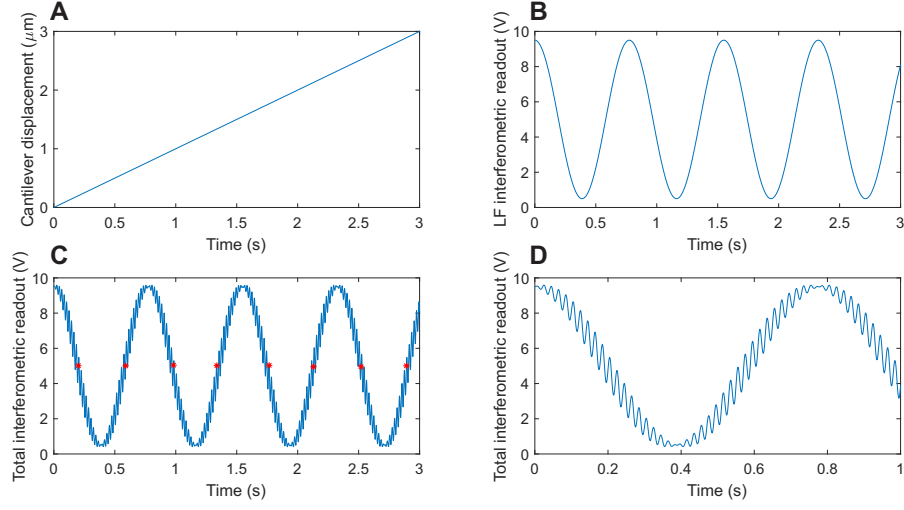


Figure A.4: The effect of wavelength modulation on the interference signal as the cavity distance d changes for an artificial oscillation at 50 Hz (for illustrative purposes, in reality it is 150 Hz). As the cavity distance changes $3 \mu\text{m}$ in 3 s (A), the LF (hence without modulation) interference readout changes sinusoidally, (B). When the 50 Hz modulation is implemented, (C) and zoomed in (D), the amplitude of the LF signal is sinusoidally modulated, being maximum at the quadrature points (red asterisks in C)) and minimum in the peaks and valleys of the interferometric signal.

deflection (and thus force) or indentation depth. In order to control cantilever deflection, a live or online readout is needed.

A.4 Lock-in amplifier

The LF response is measured as the direct interferometric readout, while a lock-in amplifier, given the reference frequency of the wavelength modulation, provides an immediate readout of the amplitude of the HF modulation from the interference signal, which is fed back to drive the piezo, so that the cantilever has the desired deflection (equivalent to a specific FP cavity distance) [409]. This ensures that over the full period $\lambda/2$, a unique solution for the cantilever deflection d as a function of interferometric voltage (coming from the LF component) and lock-in voltage (corresponding to the HF modulation) is obtained. The phases of the scaled interferometric and lock-in signals are then unwrapped (while keeping track of the number of periods), and the cantilever deflection is obtained as long as the visibility of both signals remain constant over the deflection.

Figure A.5 illustrates this process for the same cantilever displacement as in Figure A.4. A) and B) show the LF and HF components, respectively. In C), the amplitude of the HF component in B) is extracted, mimicking the effect of the lock-in amplifier. One can check that A) and C) are 90° or $\frac{\pi}{2}$ shifted, as per their cosine and sine in Equations (A.9) and (A.10). In D), signals in A) and C) are scaled (over -1 to 1 V) and the offset corrected, and the readout becomes a circle for which over one revolution gives a unique solution for the cantilever displacement d . The phase through this circle shows a linear relation to the cantilever deflection, with high sensitivity over small and large deflections [409]. In other words, by unwrapping the phase (adding one period after going through a full circle once), one can directly read out the cantilever displacement. This is the process by which each probe is calibrated, by measuring the cantilever displacement *vs.* phase unwrapping (in μm per rad) to obtain a live demodulation readout [409].

Since the lock-in amplifier can detect very small amplitude changes at the modulated frequency, it can also be used as a feedback control system (Proportional-Integrative-Derivative (PID) controller), able to adjust the piezo movement with very high accuracy. The cantilever deflection can be now set at a desired indentation (or its corresponding load), and the PID loop automatically adjusts the piezo movement accordingly to enable indentation and/or load-controlled measurements.

A.5 High frequency module calibration

In order to calibrate the HF shear piezo, two different Chiaro probes are needed: one is the HF moving probe (in the following, the HF probe), attached to the Chiaro arm by an improved two-magnet system, and the other one is a bare or naked measuring probe (a bare optical fibre, in the following, the recording probe), placed in front of it, as pictured in Figure 3.10, which records the movement of the former. The recording probe is connected to the OP1550 interferometer and remains still throughout the process. The position of the HF probe is adjusted by moving the standard piezo embedded in the Chiaro arm through the controller box. The height of the recording probe is adjusted so that it faces the mirror of the

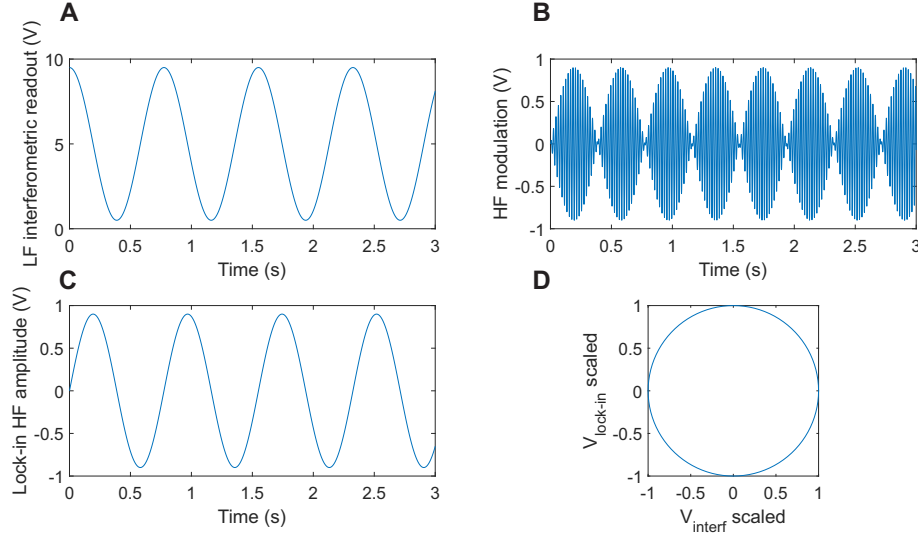


Figure A.5: A) LF component of the total interferometric signal. B) HF component of the readout. C) Amplitude of the HF component in B), as extracted by the lock-in amplifier. One can check that A) and C) are 90° or $\frac{\pi}{2}$ shifted, as per their cosine and sine in Equations (A.9) and (A.10). In D), signals in A) and C) are scaled (over -1 to 1 V) and the offset corrected, and the readout becomes a circle whose phase linearly corresponds to cantilever displacement d .

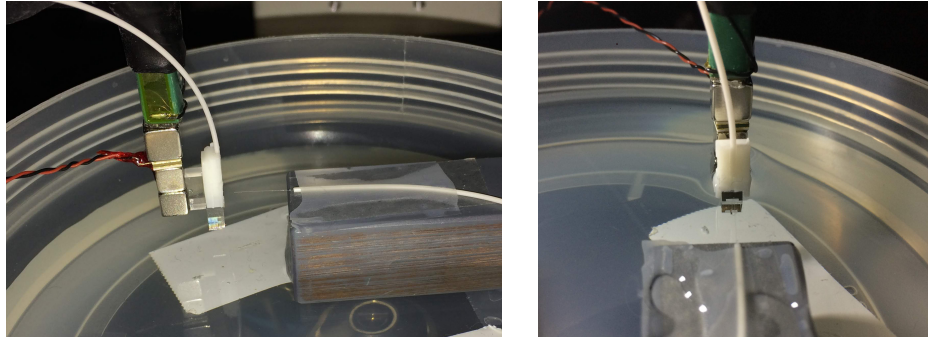


Figure A.6: The HF moving probe is connected to the Chiaro arm, while the recording bare fibre is placed in front of the mirror of the HF moving probe.

HF probe (see Figure A.6-right), which functions as the second reflecting surface, that together with the end of the bare measuring probe forms a FP cavity. The nonlinearised output (up to 1 MHz) of the OP1550 is connected to the Picoscope (see Figure A.1).

The input signal driving the HF probe (through the red and black cables attached to the HF piezo, see Figure A.6) is defined in the signal generator, which is amplified $\times 50$ after passing through the voltage amplifier (see Figure A.1). This voltage cannot exceed 200 V at any point, as that would irreversibly damage the

piezo.

At any frequency, the HF piezo and the connected probe constantly move at the same amplitude. However, it is only when the probe is in quadrature when the visibility of the readout is maximum and hence the true probe displacement amplitude is recorded, due to the interference phenomena explained above. Far from quadrature, the recorded amplitude is reduced, although the probe is still moving the same magnitude. To maximise visibility, the distance between the measuring and the moving probes has to be optimised, so that it matches the FP cavity distance designed for the system.

The measuring naked probe is manually positioned in front of the mirror of the moving probe, as perpendicular to it as possible to maximise the amount of light reflected back to the measuring fibre, see Figure A.6. From there, the distance is further adjusted by moving the Chiaro arm through the Piuma software and monitoring the amplitude of the interference fringes from the nonlinearised OP1550 output in the Picoscope oscilloscope. When these fringes reach the maximum peak-peak voltage amplitude, V_{pp} , no extra position adjustments are needed. This V_{pp} is used to calculate the sensitivity (V to nm factor) of the measurement, and has to be checked at various points during the calibration process to ensure that it remains constant. If V_{pp} changes, so does the relation of V to nm, and the calibration has to be started again. Throughout the measurement, the V_{pp} remained at 0.22 V, corresponding to a measuring sensitivity of 3.63×10^{-5} V/nm (with 1.33 the refractive index of water and 1550 nm the laser wavelength used, see Equation (A.6)).

The response of the HF piezo is then evaluated at three different input voltages: 50, 100 and 150 V, to avoid the piezo working at its maximum allowed voltage capacity. These corresponds to 1, 2 and 3 V in the signal generator passed through the $\times 50$ voltage amplifier. With the Picoscope in Fast Fourier Transform (FFT) mode, the y-axis set to dBV and the x-axis limit set at 1 MHz, several frequency sweeps of sinusoidal waves from 0 to 1,000 kHz in 10 kHz steps, with each frequency held for 3 s, are sent to the HF piezo, recording the moving probe's response until

the FFT signal does not change. During the recording, in order to ensure the probe goes through quadrature (so that the real amplitude is recorded), the measuring probe is subsequently moved $1\ \mu\text{m}$ in the X and Y directions (parallel to the mirror, to avoid visibility changes). After each input voltage frequency sweep, the maximum V_{pp} value is checked.

An unprocessed raw recording of this calibration experiment is shown in Figure A.7. From these data, the baseline noise signal (without HF stimulation) is subtracted, peaks detected *via* custom MATLAB functions, dBV units converted into V by $A_V = 10^{\frac{A_{dBV}}{20}}$, and these into nm by the calculated sensitivity factor. The resulting HF piezo displacements during frequency sweeps from 0 to 1 MHz in 10 kHz steps, held for 3 s, for the three input voltages are shown in Figure A.8.

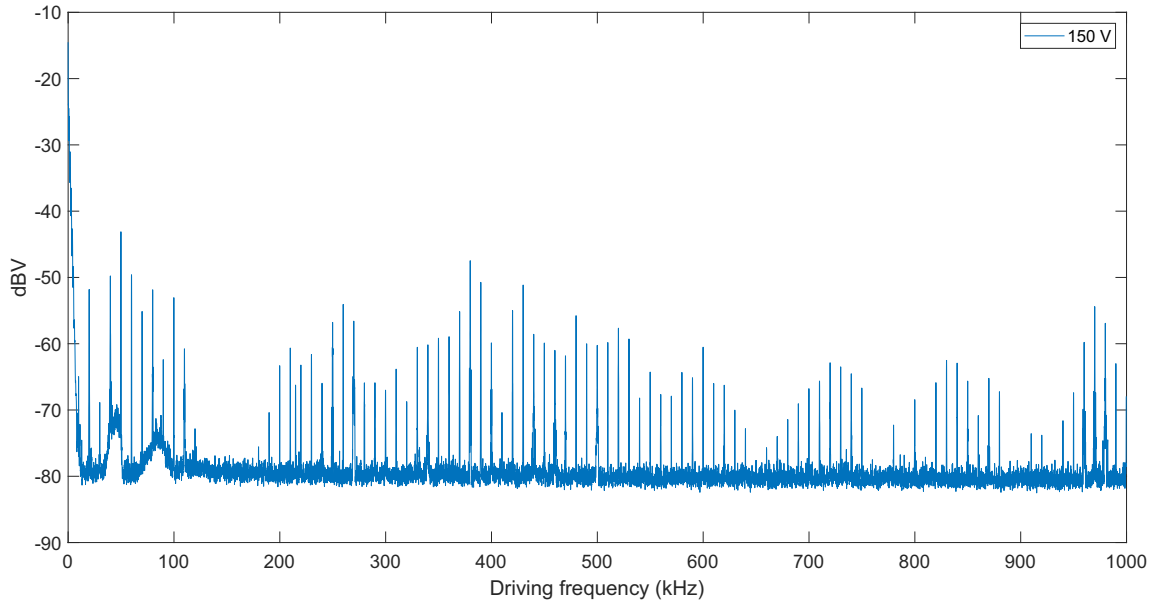


Figure A.7: Raw FFT response of the HF shear piezo for a frequency sweep from 0 to 1 MHz in 10 kHz steps with an input voltage of 150 V.

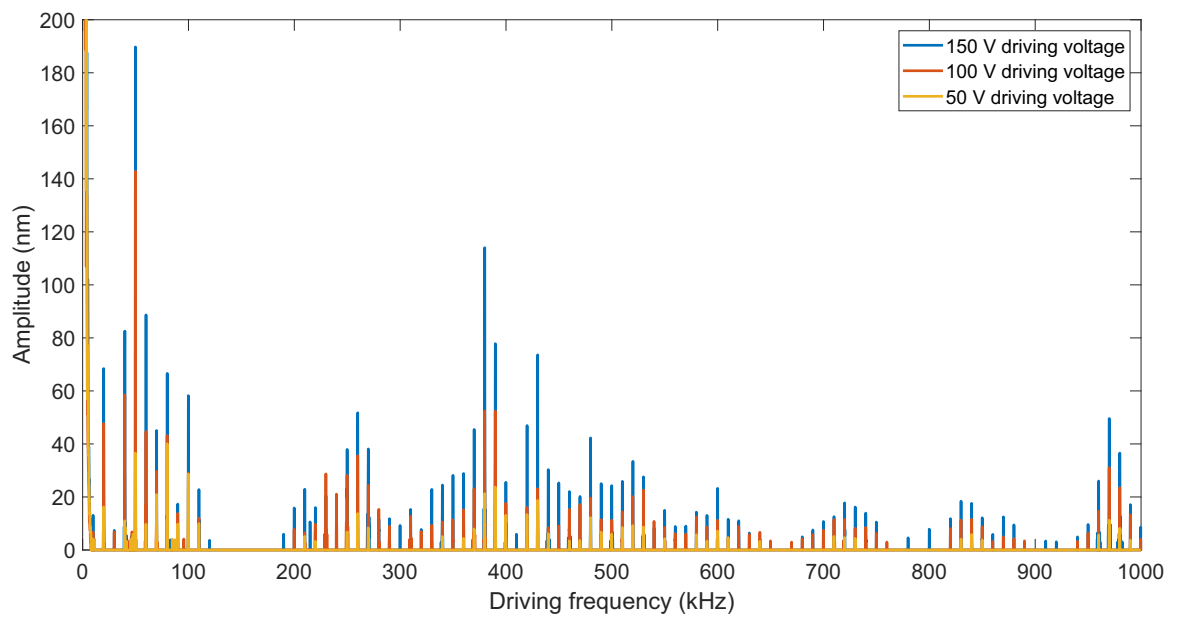


Figure A.8: HF piezo calibration at three driving voltages of 50, 100 and 150 V and a frequency sweep from 0 to 1,000 kHz in 10 kHz steps.

Appendix B

Whole cell patch clamp electronics

B.1 Operational amplifier

The most common type of operational amplifier (op-amp) is a differential amplifier with high gain, such as that pictured in Figure B.1, that amplifies the difference between its two inputs, inverting V_- and non-inverting V_+ . The output voltage is $V_{out} = G(V_+ - V_-)$, with G the op-amp gain, normally 10^5 or above. The

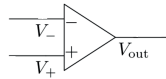


Figure B.1: Differential inputs and single output of an op-amp. Adapted from Ref. [114] with permission.

non-inverting input means that the output has the same polarity, while the output has opposite polarity to that of the inverting input.

If V_{out} is connected to the inverting input V_- via a resistor R_f (see Figure B.2),

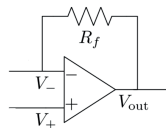


Figure B.2: Negative feedback circuit in the op-amp. Adapted from Ref. [114] with permission.

a negative feedback circuit is created that minimises the voltage difference between

V_- and V_+ inputs as follows: as V_{out} and V_- are connected, $V_{out} = V_-$, and

$$V_{out} = G(V_+ - V_{out}) \quad ; \quad \frac{V_{out}}{G} = V_+ - V_{out} \quad (\text{B.1})$$

As G is typically greater than 10^5 , $\frac{V_{out}}{G}$ is almost zero, which leads to:

- $V_{out} = V_+$, i.e. the output follows the input at V_+
- $V_- = V_+$

An important condition of op-amps is that the inputs draw no current (no current flows into the op-amp). This op-amp can be now set as an inverting or non-inverting amplifier.

In an inverting amplifier (see Figure B.3), also called current to voltage amplifier or current follower, $V_+ = 0 = V_-$ and the input current is equal to the feedback current: $I_p = -\frac{V_{out}}{R_f}$. This configuration of the op-amp is used in the patch amplifier to measure current or clamp the voltage.

In a non-inverting amplifier such as that in Figure B.4, V_+ is fixed at a command

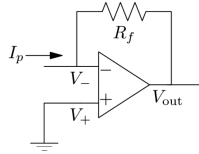


Figure B.3: Inverting op-amp or current follower. Adapted from Ref. [130] with permission.

voltage V_c , and the negative feedback ensures that the inverting input of the amplifier is always at the command voltage, $V_- = V_c$. In addition, as the inputs draw no current, any current entering the inverting node must be removed *via* the feedback resistor, and, by Ohm's law, the output voltage is therefore $V_{out} = V_- - I_p R_f$, or, $V_{out} = V_c - I_p R_f$. This op-amp operating mode, also known as voltage follower, is used in the patch amplifier to measure voltage or clamp the current.

In the following, the simplified whole cell equivalent electronic circuit of a patched cell presented in Figure B.5 will be subsequently corrected, in order to meet the demands that first, voltage recording and IC, and then current recording and VC, require.

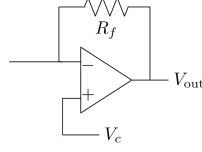


Figure B.4: Non-inverting op-amp or voltage follower. Adapted from Ref. [130] with permission.

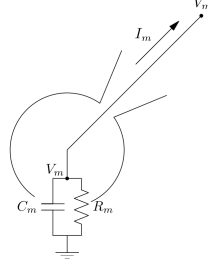


Figure B.5: Simplified (and incomplete) equivalent electronic circuit of a patched cell. Adapted from Ref. [130] with permission.

B.2 Measuring voltage in current clamp mode

In a patched cell in whole cell IC, the op-amp operates as a voltage follower when the cell membrane potential V_m arrives to the op-amp through the non-inverting V_+ pole, that is also grounded. However, part of V_m is lost in the electrode resistance R_e (also referred to as pipette, series or access resistance in literature), resulting in a voltage $V_p \neq V_m$ reaching the op-amp, see Figure B.6. The electrode resistance value mainly depends on the pipette's tip size: wider diameter tips facilitate ion fluxes throughout. In practice, R_e can be minimised by using low resistance electrodes: pipettes of 1-5 M Ω resistances are recommended for patch clamp. However small, R_e needs to be compensated by a designated circuitry in the patch clamp amplifier.

B.2.1 Electrode resistance compensation

$V_e = I_e R_e$ is an instantaneous voltage drop that appears as soon as any current is injected, while V_m increases gradually as a result of cell membrane capacitance charging, see Figure B.7. In order to determine the value of R_e , a current of known value I_p is sent through the electrode towards the cell, the instantaneous voltage drop V_e recorded, and R_e extracted by Ohm's law (see Figure B.7). The Multiclamp

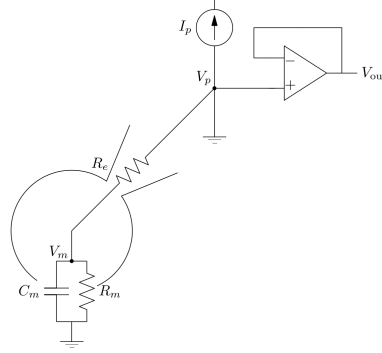


Figure B.6: IC configuration. An op-amp in the voltage follower configuration is connected to the cell *via* an electrode that has resistance R_e . A current source controls the injected current I_p . Adapted from Ref. [130] with permission.

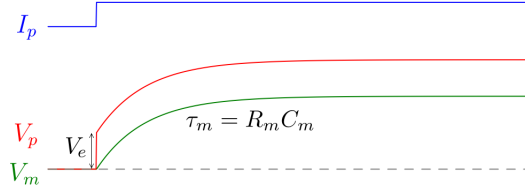


Figure B.7: Real cell (V_m) and measured pipette (V_p) voltages during a step current injection (I_p). The instantaneous voltage drop on the V_p trace corresponds to the error voltage V_e across the electrode R_e , which needs to be corrected as it does not come from the cell. The exponentially varying V_m traces result from the charging of the cell membrane capacitance C_m , until it reaches a steady value, determined by the membrane time constant, which is the product of the membrane resistance and capacitance, $\tau_m = R_m C_m$. Adapted from Ref. [130] with permission.

software does this automatically through the “Bridge Balance” circuit (see Figure B.22), which sends different current values I_p to the cell until no instantaneous voltage drop is observed. This compensation ensures that the real cell membrane potential is measured, $V_p = V_m$. Its name comes from Wheatstone bridge, as this resistor network was initially used for R_e compensation. Figure B.8 shows the different voltage traces recorded from a real cell after a current step injection, when the electrode resistance is compensated through the bridge balance (green), and when it is not (blue). One can see that the green curve is qualitatively similar to the curve of V_m in Figure B.7, corresponding to the true membrane voltage.

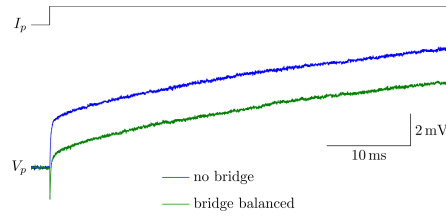


Figure B.8: Voltage traces from a Purkinje cell with the bridge balanced (green) and without (blue). The instantaneous voltage increase in the uncompensated V_p (blue) at the onset of I_p corresponds to the voltage across the electrode, $V_e = I_p R_e$. The drop in the compensated V_p (green) curve corresponds to the R_e value correction. Adapted from Ref. [130] with permission.

B.2.2 Pipette capacitance compensation

Apart from the artefact arising from the electrode resistance, the pipette introduces a parasitic capacitance effect on the measured V_p signal, that also needs to be compensated. The glass of the pipette is an insulating material separating two conductive electrolytes: the intracellular or pipette and the extracellular or bath solutions. Therefore, it introduces a capacitance C_p in the system, shown in Figure B.9, that results in slowed and distorted signals arriving at the op-amp (the electrode resistance and parasitic capacitance combine to form a low-pass RC filter), as part of the cell current is used to charge this capacitor. Compensating this C_p is equivalent to fully charging it, and the current needed to charge it should come from the op-amp and not the cell. A circuit called capacitance neutralisation is introduced (see red circuit in Figure B.9), functioning as a positive feedback circuit that injects an amplified version of the output, GV_{out} , back into the input V_p via a capacitor C_n whose value approximates that of C_p . When the gain of the feedback is correctly adjusted, the extra voltage needed to charge C_n is reintroduced into V_p as a current that charges C_p . This gain adjustment is manually done by the electrophysiologist by tuning the “Pipette Capacitance Neutralisation” value of the Multiclamp software in IC mode (see Figure B.22). It is important to note here that positive feedback circuits can cause oscillation and cell damage if incorrectly adjusted. Figure B.10 illustrates the slightly faster and taller APs when the electrode capacitance is neutralised in a Purkinje cell. In practice, pipette capacitance can be

reduced by using thick wall borosilicate pipettes, as well as by coating the pipette with a thick layer of Sylgard 184, an inert, hydrophobic, translucent elastomer resin rapidly cured by heating. The hydrophobic coat both thickens the wall of the pipette and reduces the area of the capacitor by preventing the bath solution from creeping up and wetting the pipette [114], see Figure B.11.

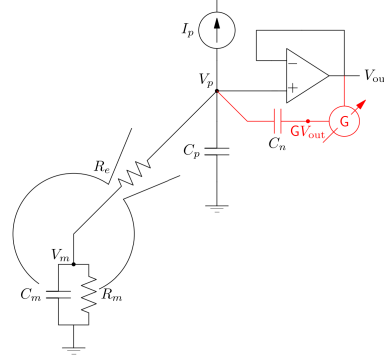


Figure B.9: Voltage recording with capacitance neutralisation. A positive feedback circuit (red) injects an amplified version of the output GV_{out} back into the input *via* a capacitor C_n . Adapted from Ref. [130] with permission.

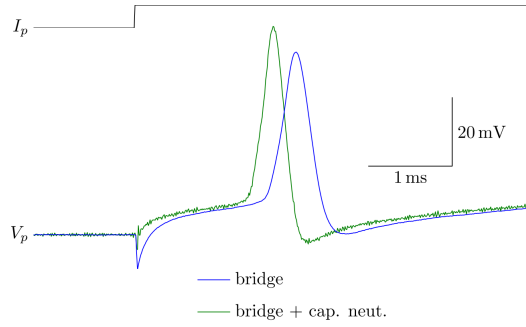


Figure B.10: Effect of capacitance neutralisation in a Purkinje cell. When C_p is neutralised, the true shape of the APs potential (narrower and taller, in green) is recorded. Adapted from Ref. [130] with permission.

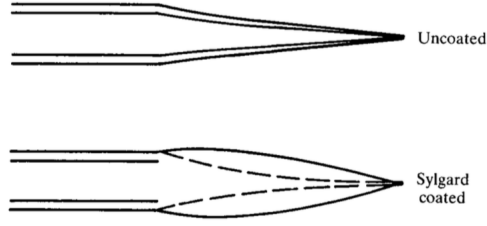


Figure B.11: Patch pipette before and after coating with Sylgard and curing. Reproduced from Ref. [114] with permission.

B.3 Measuring current in voltage clamp mode

If one instead wants to measure the current or clamp the voltage, the op-amp is operated on current follower configuration (see Figure B.12).

As for voltage recording, the properties of the electrode and the pipette

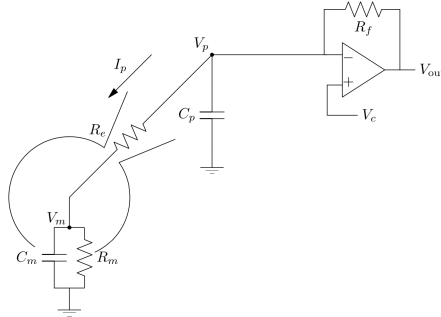


Figure B.12: VC configuration implemented using an op-amp in the current follower configuration. The inverting input is clamped to the command voltage V_c by the negative feedback, such that $V_p = V_c$. Adapted from Ref. [130] with permission.

introduce unwanted resistive and capacitive artefacts, respectively. As well as the mentioned voltage drop at R_e , on this configuration, the combination of the electrode resistance R_e and the cell capacitance C_m causes a strong low-pass filtering effect of time constant $\tau = R_e C_m$ on the measured or sent signals. If not corrected, a rapid voltage change in the cell is filtered by this time constant, as well as a voltage step at the amplifier is not instantly transmitted to the cell. The uncompensated current and voltage transients in response to a command voltage step can be observed in Figure B.13.

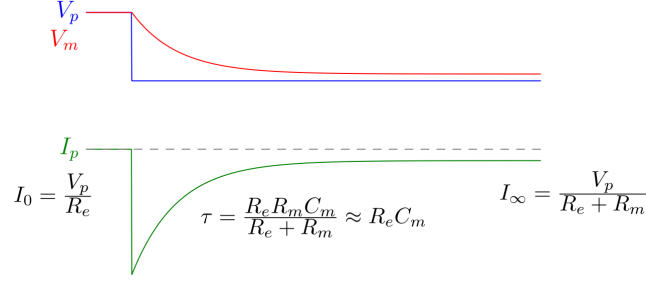


Figure B.13: After a voltage step, the current and membrane potential relax to their final values according to an exponential decay with a time constant $\tau = R_e C_m$. Adapted from Ref. [130] with permission.

B.3.1 Pipette capacitance compensation

In VC, the pipette capacitance is compensated before the electrode resistance, as otherwise the capacitance present at the inverting input of the amplifier causes it to oscillate. Pipette capacitance is compensated with the addition of an amplified command voltage that is coupled directly to the input V_p via a variable capacitor and resistor in series (see Figure B.14). This way, a capacity current exactly equal to the current required to charge C_p is added to the recorded signal. The Multiclamp software does this automatically through the “ C_p fast” option (see Figure B.21). As the cell remains connected to the inverting input, which is still always clamped at the command voltage, this compensation does not affect either the behaviour the cell, or the value of its membrane potential. The resulting corrected current traces are shown in Figure B.15.

In practice, the op-amp has several of these circuits of variable capacitors and resistors connected in parallel, which allows to not only compensate the capacitance transients coming from C_p , but also those from the capacitance of the cell, C_m . If not cancelled, C_m transients can saturate the op-amp during large voltage steps, hence preventing the amplifier to clamp the voltage or to correctly measure current, as under saturation there is no current flow within the feedback loop. The “ C_p slow” function in Multiclamp software automatically corrects for these C_m transients (see Figure B.21).

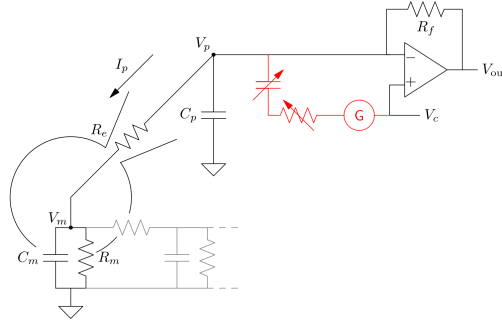


Figure B.14: Pipette capacitance cancellation circuit, shown in red, with a variable resistor in series with a variable capacitor, which generate the currents required to charge C_p . Adapted from Ref. [130] with permission.

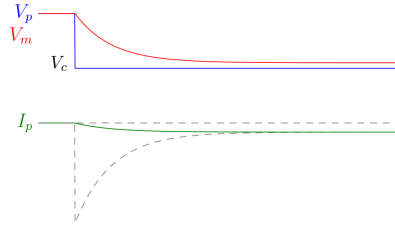


Figure B.15: Current response to a voltage step with compensated pipette capacitance (as opposed to uncompensated, shown by the dashed line). As the cell remains connected to the inverting input, which is still always clamped at the command voltage, the behaviour of the membrane potential is not affected by the compensation. Adapted from Ref. [130] with permission.

B.3.2 Electrode resistance compensation

The reason why the electrode resistance needs to be compensated in VC mode is twofold: (1) there is otherwise a voltage drop across R_e that leads to erroneous readout or imposed voltages, and (2) the combination of R_e and C_m create a RC circuit that low-pass filters (with time constant $\tau = R_e C_m$) the signal coming from the cell, as well as the one sent to the cell. This is electronically implemented by adding a positive-feedback circuit that adds a fraction of the output to the command potential (see Figure B.16), so that an extra current is driven through the electrode. This compensation is activated through the “ R_s Compensation” function of Multiclamp software (R_s corresponds to series resistance, electrode or pipette resistance), for which the user has to choose a percentage of compensation, which in practice depends on the cell size. As with all positive feedback, over-compensation

leads to oscillation and cell death. The recommended procedure is to increase the percentage of R_s compensation until some ringing is observed, and then to reduce the compensation slightly, resulting in corrections around 60%. When compensated, the transient current is larger and faster and V_m approaches the command value more quickly and more closely (see Figure B.17). If one is studying ion channel conductances in VC and does not compensate R_e , the recorded conductances are erroneous, as they do not correspond to the true voltage the cell is subject to. For instance, only part of the Na^+ channels open with a given V_p , which results in less depolarised V_m , as that of a real Purkinje cell shown in Figure B.18.

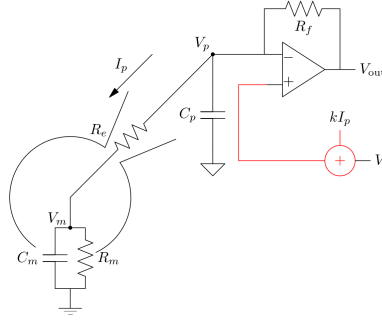


Figure B.16: Compensation of the electrode resistance. A positive feedback (shown in red) adds a signal proportional to the current (extracted from V_{out}) to the command potential. Adapted from Ref. [130] with permission.

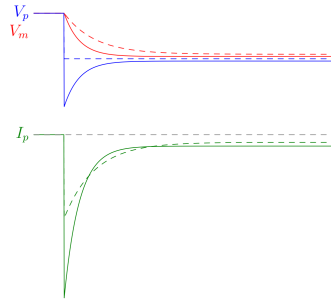


Figure B.17: Application of a 50% R_s compensation (shown as solid lines as opposed to uncompensated shown as dashed lines) increases the peak capacity current and the membrane potential approaches the command value more quickly and more closely. Adapted from Ref. [130] with permission.

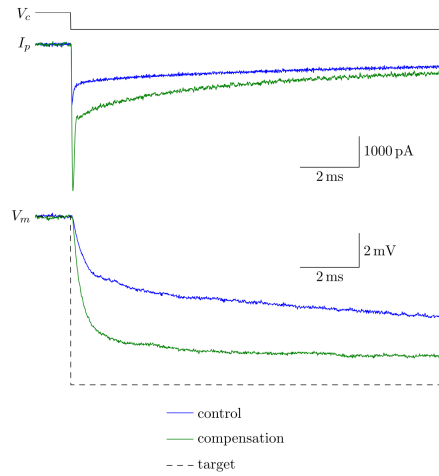


Figure B.18: Effect of electrode resistance compensation on a Purkinje cell. V_m is better clamped with a 90% R_s compensation, as the curve better approximates that of the command voltage, represented by the dashed line. Adapted from Ref. [130] with permission.

B.4 Hardware and connections

Patch clamp hardware consists of an amplifier and a data acquisition system. In the setup used in this work, the amplifier is an Axopatch 700B, controlled by the Multiclamp software, and the data acquisition system, a Digidata 1550A, controlled by the Clampex software, both from Molecular Devices. The signal is amplified and corrected by the Axopatch 700B, and digitised with the Digidata 1550A. The connection between both is as shown in Figure B.19: the analog output of the digitiser is connected to the command input of the Multiclamp software, and the analog inputs #0 and #1 of the Digidata are connected to the primary and secondary scaled outputs of the amplifier, respectively. These connections need to be specified in the “Telegraphed instrument” configuration of the Clampex software (Analog IN #0 as Primary output 1, Analog IN #1 as Secondary output 1, and Command as OUT #0, see Figure B.20).

In addition, the configuration options in the Multiclamp software for VC and IC modes need to match those specified in the “Lab Bench” in Clampex (see Figures B.21 and B.22).

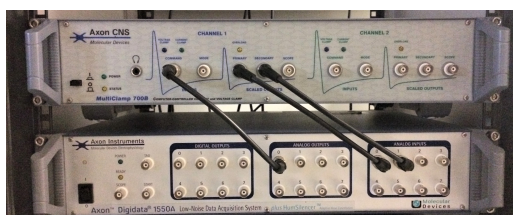


Figure B.19: Patch clamp electronics. Top: Axopatch 700B amplifier. Bottom: Digidata 1550A data acquisition system.

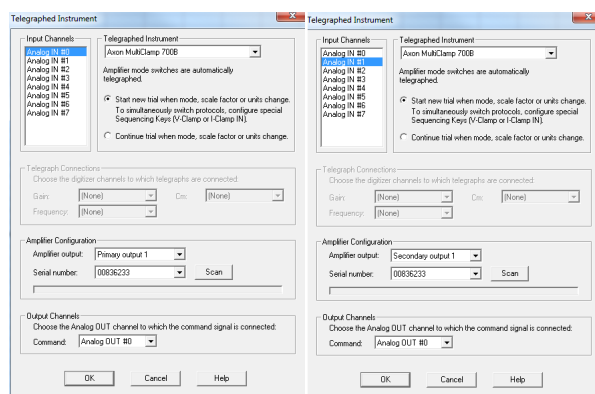


Figure B.20: Telegraphed instruments configuration

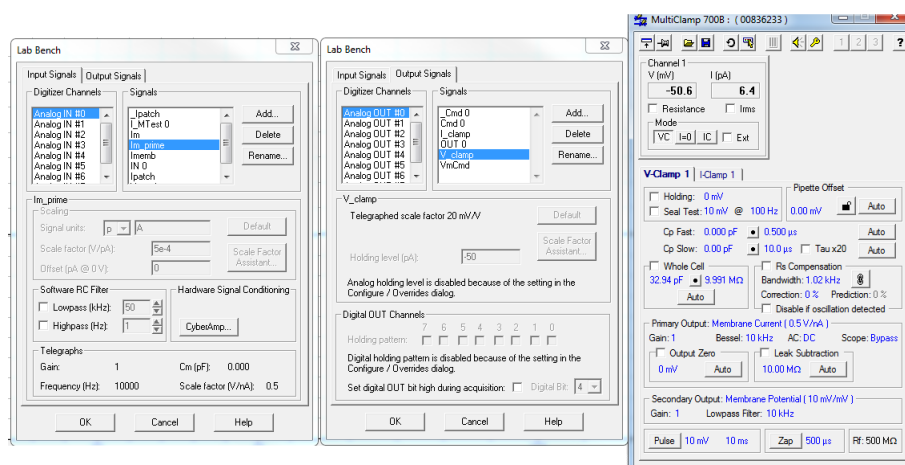


Figure B.21: Lab bench configuration for VC

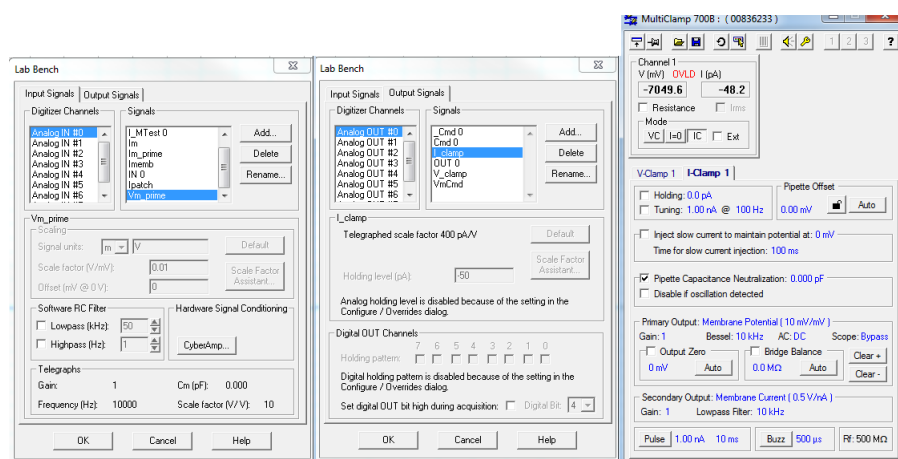


Figure B.22: Lab bench configuration for IC

Appendix C

Creep experiments results

A creep experiment consists in holding the load constant on an indentation, while measuring the displacement (or indentation) relaxation due to viscoelastic phenomena. Equivalently to how the force in a stress-relaxation experiment decays with time, the cell deformation increases with time in a creep experiment (see Figure C.1). The indentation needed to hold the constant applied force can be fit to a double exponential, see Ref. [333] for details.

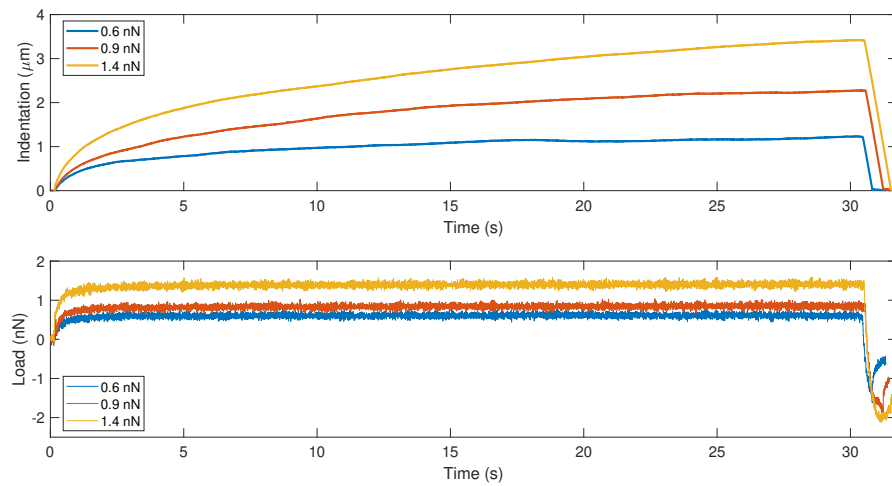


Figure C.1: Three creep experiments on three different F11 cells, showing the cell relaxation under a constant load.

Appendix D

Area dependent electrophysiological equation parameters

The values of the parameters and the Na^+ and K^+ channels' gating variables evolution equations used to model the AP through the modified surface dependent electrophysiological equation presented in Chapter 5, and taken from the DRG model in Ref. [337], are given in Tables D.1, D.2 and D.3, respectively.

Magnitude	Value
C_m	4.43×10^{-11} F/m
$\bar{G}_{Na}$	1.41×10^{-6} S/m
$\bar{G}_{Na_s}$	2.76×10^{-7} S/m
$\bar{G}_k$	8.40×10^{-8} S/m
G_l	5.60×10^{-9} S/m
V_m	- 49.1 mV
E_{Na}	62.94 mV
E_K	- 92.34 mV
E_l	- 54.3 mV
A_0	$1256.64 \mu\text{m}^2$
A	$1261.66 \mu\text{m}^2$

Table D.1: Parameter values used in the area dependent DRG electrophysiological model.

Na ⁺ channel	Slow-inactivated Na ⁺ channel
$\frac{dm}{dt} = \alpha_m(V)(1 - m) + \beta_m(V)m$	$\frac{dm_s}{dt} = \alpha_{m_s}(V)(1 - m_s) + \beta_{m_s}(V)m_s$
$\frac{dh}{dt} = \alpha_h(V)(1 - h) + \beta_h(V)h$	$\frac{dh_s}{dt} = \alpha_{h_s}(V)(1 - h_s) + \beta_{h_s}(V)h_s$
$\alpha_m(V) = \frac{11.49}{1+e^{\frac{V+8.58}{-8.47}}}$	$\alpha_{m_s}(V) = \frac{1.032}{1+e^{\frac{V+6.99}{-14.87115}}}$
$\beta_m(V) = \frac{11.49}{1+e^{\frac{V+67.2}{27.8}}}$	$\beta_{m_s}(V) = \frac{5.79}{1+e^{\frac{V+130.4}{22.9}}}$
$\alpha_h(V) = 0.0658e^{\frac{-(V+120)}{20.33}}$	$\alpha_{h_s}(V) = \frac{0.06435}{1+e^{\frac{V+73.26415}{3.71928}}}$
$\beta_h(V) = \frac{3.0}{1+e^{\frac{V-6.8}{-12.998}}}$	$\beta_{h_s}(V) = \frac{0.13496}{1+e^{\frac{V+10.27853}{-9.09334}}}$

Table D.2: Na⁺ channels' gating equations.

K ⁺ channel
$\frac{dn}{dt} = \alpha_n(V)(1 - n) + \beta_n(V)n$
$\alpha_n(V) = 0.001265 \frac{V+14.273}{1-e^{\frac{V+14.273}{-10}}}$
$\beta_n(V) = 0.125e^{\frac{V+55}{-2.5}}$

Table D.3: K⁺ channels' gating equations.

Appendix E

Analysed AP alterations under sham and subsequent HF shear loading

A sham experiment was conducted, in which the spontaneous activity of a cell was recorded for 400 s under 500 nm static indentation but no dynamic loading. Results are included in Figures E.1 and E.2 and show that no significant electrophysiology variation is induced by the quasi-static 500 nm compression alone.

In addition, the alterations in the shape and dynamics of spontaneous APs of a cell before, during and after three consecutive 970 kHz and 50 nm shear loading experiments (see Figure 6.3) are presented in Figure E.3, and analysed in Figure E.4, and confirm that the analysed features do not significantly change within cycles.

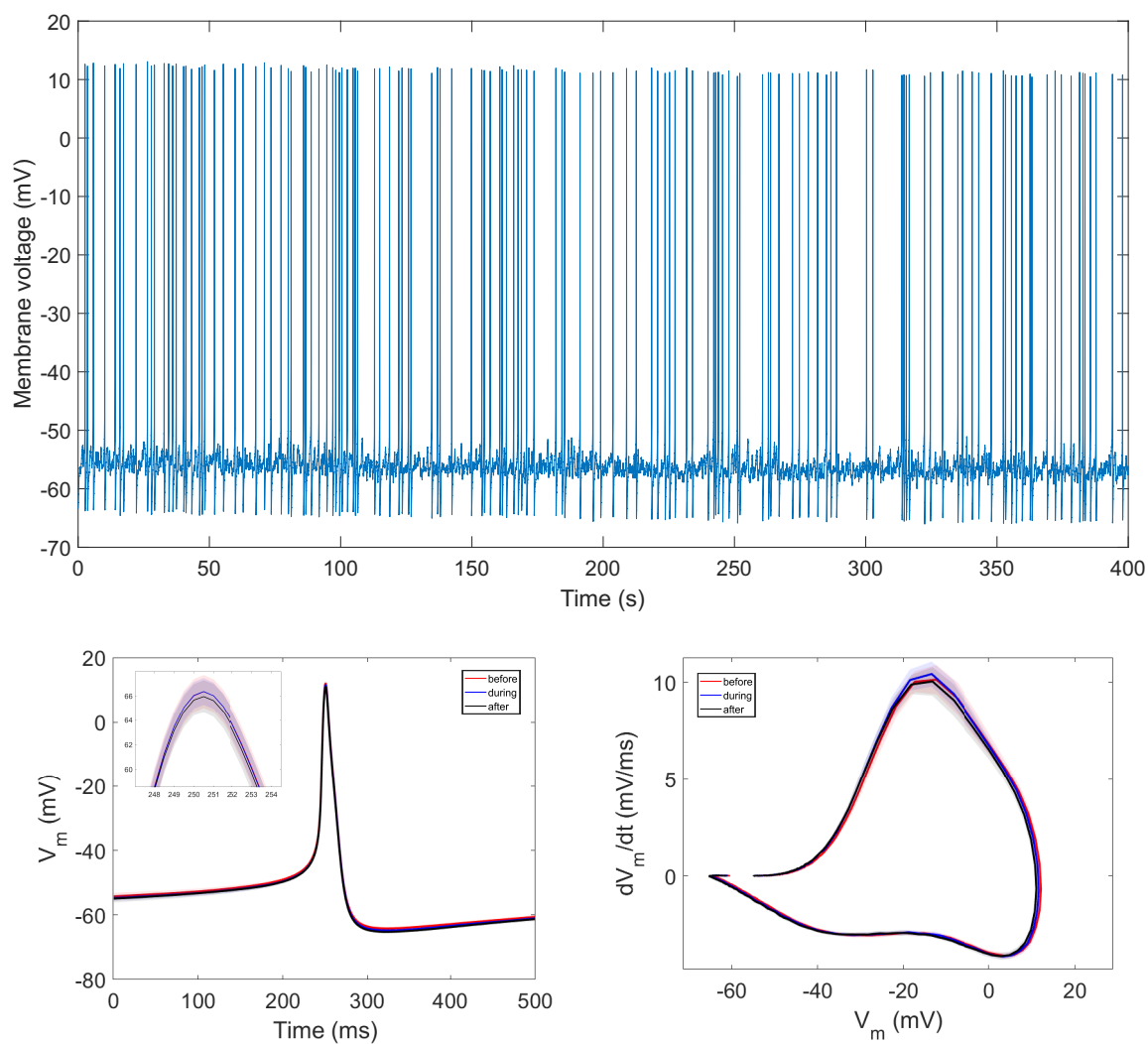


Figure E.1: Recorded voltage signal (above) and analysed APs (below) during a sham experiment of 500 nm quasi-static loading held for 400 s with no induced oscillation. For the analysis, before, during and after times were defined as in standard dynamic loading experiments: 160, 80 and 160 s durations.

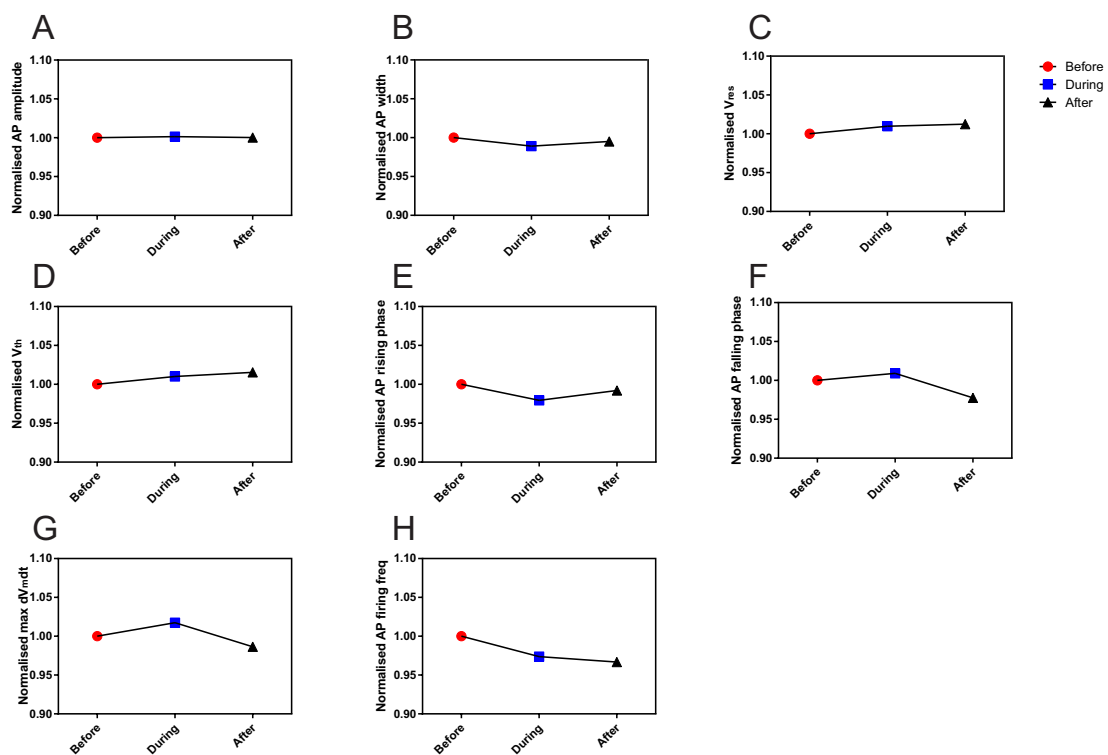


Figure E.2: Mean normalised AP features values during the sham experiment shown in Figure E.1. A) Normalised AP amplitudes. B) Normalised AP widths. C) Normalised resting membrane voltage values. D) Normalised threshold potential. E) Normalised AP rising phases. F) Normalised AP falling phases. G) Normalised max dV_m/dt values. H) Normalised AP firing frequencies. All normalised magnitudes remain within 0.95 and 1.05.

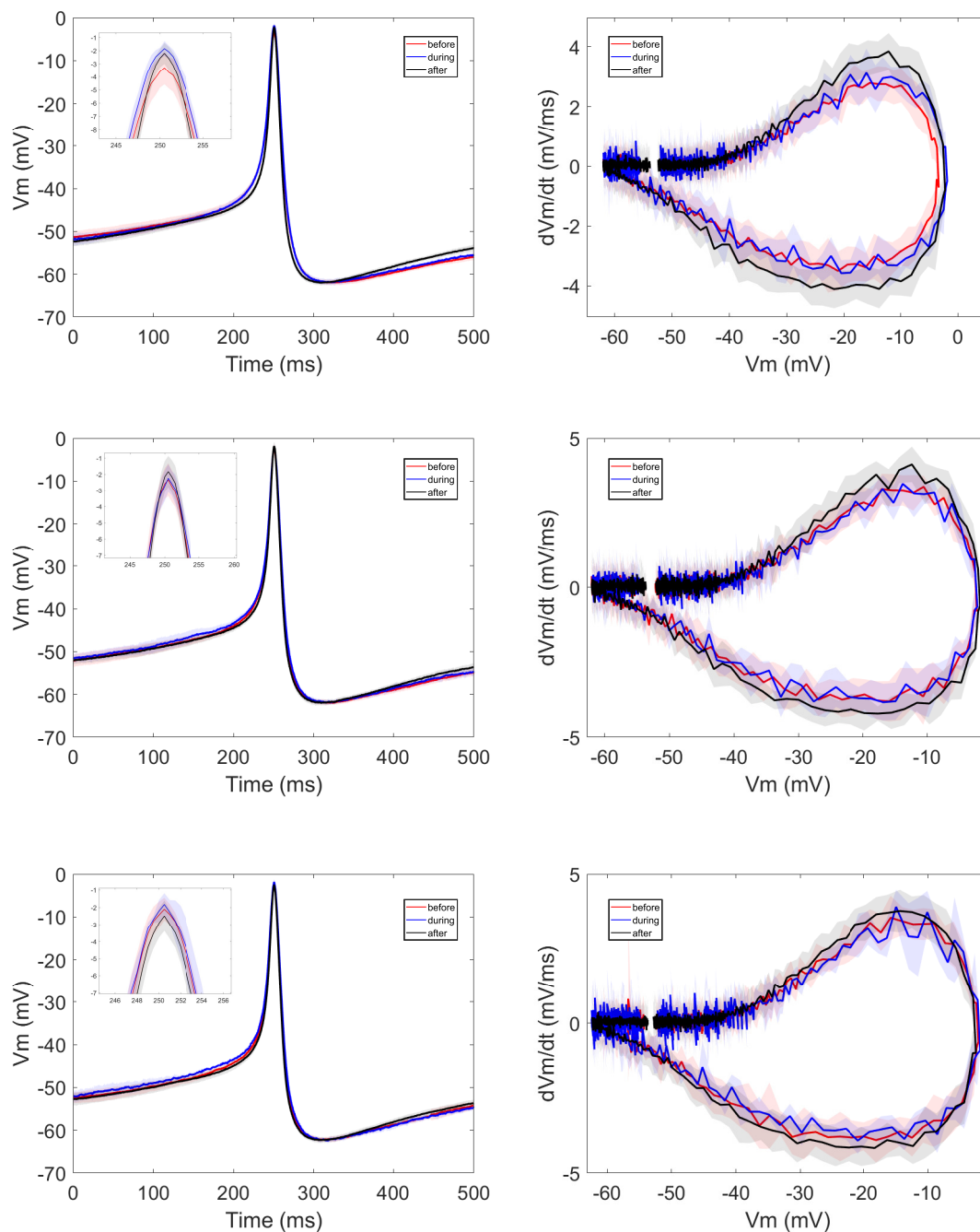


Figure E.3: Analysed APs during three consecutive HF shear indentations at 970 kHz and 50 nm. Insets show non significant amplitude changes during the loading. Phase plots show faster AP dynamics after (black curves) the mechanical oscillation. The statistical analysis of the AP features before, during and after such loads are shown in Figure E.4.

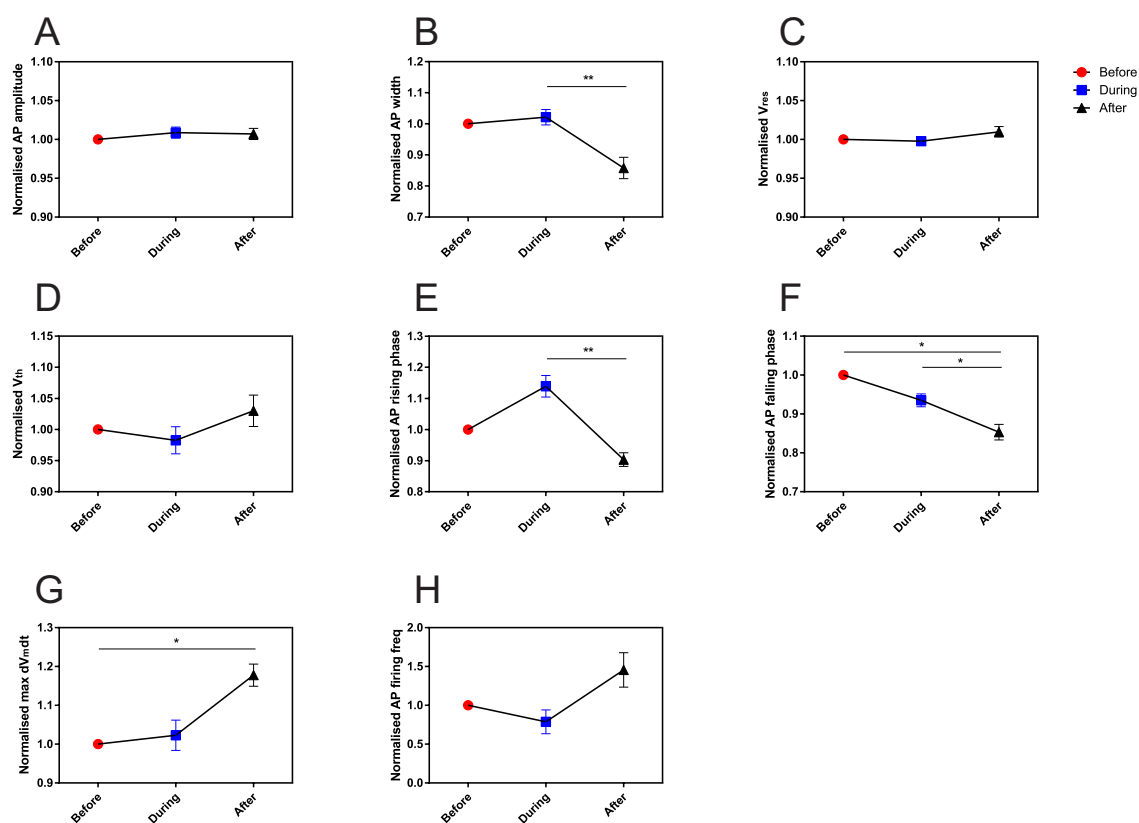


Figure E.4: Mean and SE of the AP features analyses under the three consecutive 970 kHz and 50 nm loadings shown in Figure 6.3. A) Normalised AP amplitudes. B) Normalised AP widths. C) Normalised resting membrane voltage values. D) Normalised threshold potential. E) Normalised AP rising phases. F) Normalised AP falling phases. G) Normalised max dV_m/dt values. H) Normalised AP firing frequencies. In A) and B), Two-way ANOVA with Tukey's multiple comparisons *post-hoc* tests were performed (*p < 0.05, **p < 0.01).

Appendix F

Estimation of the thermal effects induced by the shear loading

Deformations of viscoelastic materials such as soft biological tissue dissipate energy, especially at high indentation or deformation rates. At the HF shear indentations presented in Chapter 6, then, the energy dissipated by the cell in the form of heat might be non-negligible, and contribute to the observed electrophysiological effects, particularly significant at ~ 1 MHz (see Figure 6.5). The amount and rate of energy dissipated by the cell depends on the value of its shear loss modulus, G'' , at the loading frequency. As the maximum induced thermal effects are those at ~ 1 MHz oscillation, the first step towards calculating the energy dissipated as heat at this frequency is obtaining $G''(1 \text{ MHz})$ for neuronal cells.

In Chapter 4, the mechanical behaviour of F11 cells was studied up to 10 Hz. Under the studied frequency range, the elastic response dominated over the viscous one (see Figure 4.13). At higher frequencies, however, viscous effects tended to approach the elastic ones, and in airway smooth muscle and fibroblasts cells, even overtook them at around 1 kHz [220] and 60 kHz [381], respectively. Cellular viscoelastic responses at these kHz frequencies are scarce, as most cell characterisation studies are limited to the 100-200 Hz range [332, 410–413] due to technical limitations.

F.1 F11 shear moduli at 1 MHz

To the best of our knowledge, there is only one work in which the cellular mechanical response was characterised up to 100 kHz [381]. The cell type under study was fibroblast, and the work shared some of the frequencies at which F11s were studied under DMA (see Chapter 4), allowing the comparison of the storage and loss shear moduli of neurons (G'_n and G''_n) and fibroblasts (G'_f and G''_f) at those frequencies. Fibroblasts' shear moduli values were extracted from Figure 10f of Ref. [381] through the "WebPlotDigitizer" tool [414]. Table F.1 compares these moduli to those of F11 neurons at the same frequencies.

Freq (Hz)	F11 neurons		Fibroblasts	
	G'_n (Pa)	G''_n (Pa)	G'_f (Pa)	G''_f (Pa)
1	70.57	47.18	900	200
10	141.40	93.13	1800	450

Table F.1: Mean storage (G') and loss (G'') shear moduli for F11 neurons (from Table 4.2 and Equation (4.4)) and fibroblasts (from Ref. [381]).

Although neurons are softer and less viscous than fibroblasts at 1 and 10 Hz (see Table F.1), the increases in both storage and loss moduli from 1 to 10 Hz are comparable for both cell types:

$$\frac{G'_n(10Hz)}{G'_n(1Hz)} \sim \frac{G'_f(10Hz)}{G'_f(1Hz)} \sim 2$$

$$\frac{G''_n(10Hz)}{G''_n(1Hz)} \sim \frac{G''_f(10Hz)}{G''_f(1Hz)} \sim 2.12$$

As a result, the ratio increase for the shear moduli of fibroblasts per logarithmic frequency increase was used to extrapolate, from those values at 10 Hz, the F11 shear moduli up to 100 kHz. As a final step, and following the tendency curves for G' and G'' , the 1,000 kHz (1 MHz) data point was extrapolated. Results for F11s are shown in Figure F.1.

The approximated shear modulus ($G = \sqrt{G'^2 + G''^2}$) of F11s at 1 MHz is $G(1 \text{ MHz}) \simeq 9962.13 \text{ Pa}$, and the shear loss modulus $G''(1 \text{ MHz}) \simeq 8875.32 \text{ Pa}$.

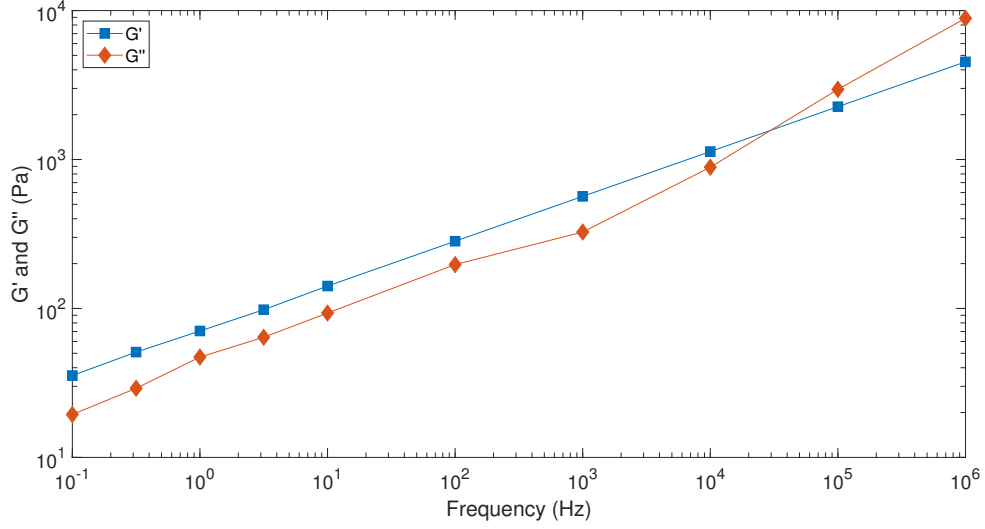


Figure F.1: Calculated F11 shear moduli at frequencies up to 1 MHz.

F.2 Energy dissipated at 1 MHz

In this subsection, an approximation of the generated energy dissipated as heat is calculated for the highest energetic shear indentation, i.e., that of maximum frequency and amplitude, 970 kHz and 50 nm, on top of the 500 nm quasi-static compression. The oscillatory shear strain induced in the cell is given by [415]:

$$\gamma(t) = \gamma_0 \sin(\omega t) \quad (\text{F.1})$$

with γ_0 the amplitude of the oscillatory shear strain and $\omega = 2\pi f$ the angular frequency. For the maximum amplitude case, 50 nm, in a F11 cell of average diameter $\sim 20 \mu\text{m}$ (see Chapter 4), $\gamma_0 = 0.0025$.

In steady-state, and assuming small deformations and linear viscosity, the total energy dissipated over a full cycle of the excitation is constant [415]. After much analysis, the energy dissipated per unit volume of the material over a cycle of deformation is given by [415]:

$$W_d(\omega) = \pi \gamma_0^2 G''(\omega) \quad (\text{F.2})$$

which for the herein studied case at 1 MHz, takes the value of:

$$W_d = \pi 0.0025^2 \cdot 8875.32 = 1.74 \times 10^{-1} \text{ J} \cdot \text{m}^{-3}$$

The increase in temperature this dissipated energy induces per unit volume and per cycle is given by [416, 417]:

$$\Delta T_{cyc} = \frac{W_d}{c_p \rho} \quad (\text{F.3})$$

with c_p specific heat capacity and ρ density of the medium. As the thermal properties of biological tissue can be approximated by those of water [418], the specific heat capacity and density values for water can be used: $c_p = 4.18 \times 10^3 \text{ J} \cdot \text{kg}^{-1} \cdot ^\circ\text{C}^{-1}$ and $\rho = 1,000 \text{ kg/m}^3$, respectively.

In steady-state, the increase in temperature per unit volume per indentation cycle is:

$$\Delta T_{cyc} = \frac{1.74 \times 10^{-1}}{4.18 \times 10^3 \cdot 1,000} = 4.17 \times 10^{-8} ^\circ\text{C}$$

Even though this value is small within a single cycle, if the time this temperature increment takes to decay significantly is larger than the period of a cycle of shear loading ($t_{cyc} = \frac{1}{970 \cdot 10^3} \simeq \frac{1}{1\text{MHz}}$), the dissipated energy will accumulate and the temperature in the cell, increase over time.

F.3 Dissipated heat conduction

The temperature evolution over time and space in a medium assuming constant thermal conductivity is given by the 1D homogeneous heat (or diffusion) equation [416]:

$$\frac{\partial T}{\partial t} = \alpha \frac{\partial^2 T}{\partial x^2} \quad (\text{F.4})$$

with $\alpha = \frac{\kappa}{\rho c_p}$ the thermal diffusivity, which for water is $\alpha = 0.143 \text{ mm}^2/\text{s}$. Here, heat removal by convection or radiation processes has been neglected for simplicity.

As thermal properties of cells and water are comparable, as a simplification, the generated heat equally diffuses through the cell and through the extracellular solution, so that an isotropic homogeneous finite body problem under an transient heat conduction can be assumed. Every cycle, ΔT_{cyc} is generated at $x = 0$, the loading site. Assuming the cell starts at ambient temperature T_{amb} , after a

cycle, $x = 0$ is at $T_0 = T_{amb} + \Delta T_{cyc}$. This temperature T_0 decays exponentially with distance from the heated surface. The problem now is how much heat the cell accumulates until this heat starts to diffuse away into the surrounding extracellular medium.

The analytical solution of the above problem is given by [419]:

$$T(t, x) = T_0 + (T_{amb} - T_0) \operatorname{erf} \left(\frac{x}{\sqrt{4\alpha t}} \right) \quad (\text{F.5})$$

with erf the error function, defined as:

$$\operatorname{erf}(z) = \frac{2}{\sqrt{\pi}} \int_0^z e^{-z^2} dz \quad (\text{F.6})$$

In order to solve Equation (F.5), the surface heat flow q (or rate of heat transfer) can be calculated by applying Fourier's law of conduction in the x -direction:

$$q_x = -\kappa \frac{\partial T}{\partial x} \quad (\text{F.7})$$

giving [419, 420]:

$$q_x(t) = \frac{\kappa \Delta T_{cyc}}{\sqrt{\pi \alpha t}} e^{-\frac{x^2}{4\alpha t}} \quad (\text{F.8})$$

Equation (F.8) describes a Gaussian spread of thermal energy, which decays exponentially with distance from the heated surface at $x = 0$.

Assuming the cell is a sphere of radius $R = 10 \mu\text{m}$, the time the heat takes to diffuse throughout its volume and attenuate to $1/e$ (a $\sim 68\%$) of the maximum value observed at the surface, is given by [416, 417, 419–422]:

$$\tau = \frac{R^2}{\alpha} \quad (\text{F.9})$$

which for this problem takes a value of:

$$\tau = \frac{(1 \times 10^{-5})^2}{1.43 \times 10^{-7}} = 6.99 \times 10^{-4} \text{s}$$

At $t = \tau$, the temperature at the opposite side of the cell has attenuated to $1/e$ of the maximum value at the loaded surface. This time is known as the characteristic

time or thermal (diffusion) time constant. Up to $t = \tau$, the energy dissipated through each cycle accumulates within the cell volume, generating a maximum temperature ΔT_{max} that depends on the number of cycles n_{cycles} accumulated up to τ :

$$\Delta T_{max} = \Delta T_{cyc} \cdot n_{cycles} = \Delta T_{cyc} \frac{\tau}{t_{cyc}}$$

of value:

$$\Delta T_{max} = 4.17 \times 10^{-8} \cdot \frac{6.99 \times 10^{-4}}{\frac{1}{970 \times 10^3}} = 2.83 \times 10^{-5} \text{°C}$$

The maximum temperature increase generated at the cell surface is therefore, negligible. It can then be concluded that at the cell level, there are no significant thermal effects. As the pipette is recording whole cell activities, electrophysiological alterations must come mainly from mechanically induced effects.

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