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London**

Single-cell nanobiopsy for spatial and temporal profiling of living cells

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Declaration of originality

I hereby declare that the work presented in this thesis is original and my own work. Any work performed by others or in collaboration with others is explicitly stated and appropriately acknowledged.

Annpreet Sahota

January 2024

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Abstract

Single-cell analysis is a rapidly growing field that is reshaping our understanding of fundamental biological processes and complex diseases. Subcellular sampling techniques advance from previous single-cell methods as they maintain cells in their standard microenvironment and do not require cell lysis or fixation. These have the potential to profile the single-cell omics of living cells and aid our understanding of intracellular mechanisms. However, many existing subcellular sampling tools involve the aspiration of cytoplasmic fluid, raising questions regarding the viability of cells with repeat sampling. In this work, minimally invasive dielectrophoretic nanoscale tweezers (nanotweezers) are employed for precise sampling of RNA and single mitochondrion in living neurons. With this nanobiopsy tool, biomolecules are trapped directly from cells, avoiding the need to remove large cytoplasmic volumes.

The nanotweezer platform is developed to provide spatial and temporal measurements of biomolecules in live single cells. Following optimising the dielectrophoretic trapping capabilities of the nanotweezer, we show that mRNA can be successfully isolated from different compartments of live rat hippocampal neurons with high spatial resolution. Repeat sampling from the same cell with minimal impact on cell viability and the detection of RNAs from individual nanobiopsies is achieved. As a result, the tool is used to study dendritic localisation of mRNAs, including measuring dynamic changes in gene expression in the same living cell.

Single mitochondrion extraction from the neurites of single neurons is also presented, alongside developing methods for single mitochondrion analysis. Finally, the effects of α -synuclein on single-cell mitochondrial expression are investigated, where we demonstrate the removal of mitochondria from the same living neuron before and after treatment. This work shows promising results for future expansion of the tool for dynamic and precise spatial quantification of biomolecules in single cells which could ultimately have significant implications in spatial transcriptomics, deciphering cellular mechanics, and tracking disease.

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Abbreviations

3'UTR – 3' untranslated region

AD – Alzheimer's disease

AFM – atomic force microscopy

AIS – initial axon segment

ALS – amyotrophic lateral sclerosis

ATP – adenosine triphosphate

BSA – bovine serum albumin

CAMK2A – Calcium/calmodulin-dependent protein kinase type II

cDNA – complementary DNA

cLTP – chemical long-term potentiation

CM factor – Clausius Mossoti factor

Cq value – quantification cycle

DAPI – 4',6-diamidino-2-phenylindole

DENT – dielectrophoretic nanotweezer

DEP – dielectrophoresis

DIV – day-in-vitro

DNA – deoxyribonucleic acid

DNase – deoxyribonuclease

ELISA – enzyme-linked immunosorbent assay

emCCD – electron-multiplying charge-coupled device

FACS – fluorescence activated cell sorting

FISH – fluorescent in situ hybridisation

FluidFM – fluid force microscopy

FTD – frontotemporal dementia

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

GFP – green fluorescent protein

LCM – laser capture microdissection

LSV – linear sweep voltammetry

LTP – long-term potentiation

MALDI-MS – matrix assisted laser desorption/ionization-mass spectrometry

MAP1A – microtubule-associated protein 1A

MAP2 – microtubule-associated protein 2

MERFISH – multiplexed error-robust fluorescence in situ hybridization

miRNA – microRNA

mRNA – messenger RNA

mRNP – messenger ribonucleoproteins

mtDNA – mitochondrial DNA

mtRNA – mitochondrial RNA

NGS – next-generation sequencing

NMDA – N-methyl-D-aspartic acid

NMDAR – N-methyl-D-aspartate receptors

OXPHOS – oxidative phosphorylation

PAGE – polyacrylamide gel electrophoresis

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

PD – Parkinson's disease

Q-score – quality score

qPCR – quantitative polymerase chain reaction

RBP – RNA binding protein

RNA – ribonucleic acid

RNA-seq – RNA sequencing

ROS – reactive oxygen species

rRNA – ribosomal RNA

RT-qPCR – reverse transcription quantitative polymerase chain reaction

s.d – standard deviation

s.e.m – standard error of the mean

scDNA-seq – single-cell DNA sequencing

sCMOS – scientific complementary metal-oxide-semiconductor

scRNA-seq – single-cell RNA sequencing

SEM – scanning electron microscopy

SeqFISH – sequential FISH

SICM – scanning ion conductance microscopy

smFISH – single-molecule fluorescent in situ hybridisation

TIRF – total internal reflection fluorescence

tRNA – transfer RNA

WGA – wheat germ agglutinin

α -syn – α -synuclein

λ -DNA – lambda phage DNA

Chapter 1

Introduction

Synopsis

This chapter introduces neuronal biology and the various neuronal processes that are relevant to the work discussed in this thesis. The field of single-cell analysis is presented, with particular attention on single neuron analysis. Recent state-of-the-art methods for subcellular analysis, including the nanotweezer technology which is the focus of this work, are reviewed and their limitations are discussed. Finally, the objectives of this thesis are outlined.

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1.1 Neurons in the brain

1.1.1 Structure and function

The brain is the most complex organ in the human body. Despite making up only 2 % of the body's mass, it takes up approximately 20 % of its metabolic energy demands.^{1,2} Of the cells that make up the brain, neurons have the highest energy demands.³ This energy is primarily used for the major functions of neurons, such as action potential propagation,⁴ synaptic transmission,³ synaptic plasticity⁵ and calcium regulation.⁶ Much of this energy is produced through the metabolism of glucose during oxidative phosphorylation (OXPHOS) in the mitochondria, or glycolysis in the cytosol.⁷

The intricate structure of neurons, with their unique cellular compartments, plays an essential role in directing information in the central nervous system.⁸ Neurons are extremely polarised cells with morphologically distinct compartments such as the soma (or cell body), axon, and dendrites (**Figure 1.1**). The soma makes up the core of the neuron and contains the genetic information of the cell, responsible for coordinating cellular activities and the synthesis of proteins. The dendrites serve as the primary sites for synaptic input, where they receive signals via the post-synaptic terminals. The axon, with its long projection, facilitates the rapid and efficient transmission of action potentials to the pre-synaptic terminal. Within the expansive neuronal structure are also sub-compartments. The dendritic spines are small protrusions on the dendrites which form synaptic contacts with the axons of other neurons. They contain most post-synaptic terminals and are regions of synaptic plasticity, during which, the size and shape of dendritic spines dynamically change in response to neuronal activity.⁹ The initial axon segment (AIS) is present at the base of the axon in close proximity to the soma and is where axon potentials are generated.¹⁰ The axon terminals, or synaptic boutons, are small swellings present at the end of axons which contain neurotransmitter-containing vesicles and form synapses with post-synaptic neurons.¹¹

Neurons are post-mitotic cells, meaning that they suppress the cell cycle and are rarely replaced through cell division.¹² This makes them highly vulnerable to functional disruptions such as those from mitochondrial dysfunction,¹³ protein abnormalities,¹⁴ reactive oxygen species build-up,¹⁵ DNA damage,¹⁶ and failed RNA regulation and translation mechanisms.¹⁷ These impairments are key features of many neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD).¹⁸

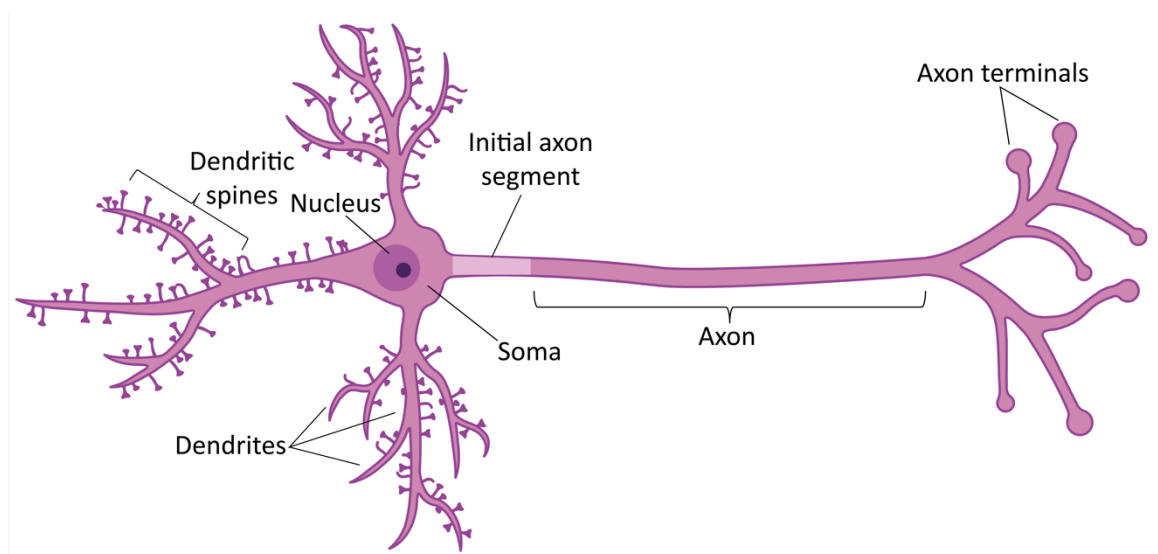


Figure 1.1: Structure and compartmentalisation of neurons. Schematic illustrating the basic structure of a neuron, with its various subcellular compartments labelled. The figure was created using Biorender.com.

1.1.2 mRNA regulation

Over the past few decades, understandings of the flow of information in biological systems, the process where DNA is transcribed to RNA which is then translated into proteins centrally in cells, has been transformed due to the discovery that mRNA can be localised in certain cells.¹⁹ mRNA localisation is the directed delivery of mRNA transcripts from the nucleus, where DNA is transcribed, to distally localised sites of cells before their translation into proteins.²⁰ This is in contrast to the conventional mechanism of exiting through the nuclear pores to the cytoplasm for immediate translation.²¹ mRNA localisation occurs by binding to RNA binding proteins (RBPs) at specific sequences, often on the 3' untranslated regions (3'-UTR) of mRNAs.^{22,23} These sequences are recognised by RBPs, where they assemble into messenger ribonucleoproteins (mRNP) to enable RNA processing.^{22,23} Each RBP can be associated with hundreds of mRNAs and each mRNA can be bound to multiple RBPs.²⁴ The self-assembly of mRNPs, made up of various protein complexes, form large granules that transport RNA along neurons through interactions with kinesins, dyneins and myosins motor proteins.²⁵ Kinesins, KIF5 in particular, transport mRNA along axons and dendrites to the plus ends of microtubules, whereas cytoplasmic dyneins transport molecules to the minus end towards the soma.^{22,25}

In neurons, mRNA localisation is a key post-transcriptional process and method of RNA regulation; it is recognised as the primary mechanism of protein localisation and accounts for approximately half of neurite-specific proteins.^{26,27} Many cellular processes regulated by extrinsic signalling, such as memory formation, synapse formation, axon steering and dendrite branching, require highly localised dynamics and translation of mRNA.^{19,28} Localisation enables the accumulation of signalling proteins at particular subcellular sites for specific functions and faster response times.^{19,20} This is important for neurons, where protein synthesis components must travel great lengths from the soma to reach parts of the axon, dendrites and synaptic terminals for protein synthesis.²⁸ This contributes to the rapid signalling and response times of neurons, where defects or disruptions in RNA localisation and local protein production machinery have been correlated with functional impairments.^{29–31}

Defects in RNA regulation and metabolism have been observed and linked to many neurological disorders.³² This can involve up- or downregulatory changes in transcript abundance in localised regions.^{33–36} For example, AD post-mortem brains were shown to have more frequent mRNA and protein localisation of the transcription factor ATF4 in the axons of neurons, where knockdown of axonal ATF4 mRNA was shown prevent neurodegeneration from acutely applied amyloid- β (1-42) in the forebrain of mice.³³ Changes in transcript localisation can also arise from mutations that reduce or silence the expression of RBPs, or from other disruptions of RBP homeostasis.³⁷ For example, mutations in the RBPs FUS and TDP-43 are linked to amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) and have been shown to cause axonal mRNA trafficking deficits and neurite dysfunction.³⁸ Many RNA targets of disrupted RBPs are involved in synaptic plasticity and transmission, suggesting that synaptic dysregulation may be involved in the early progression of neurodegenerative diseases.

The structural complexity of neurons suggests that tight control over RNA regulation and protein synthesis is required to maintain homeostasis.¹⁹ It is also evident that impairment of protein synthesis machinery plays a role in the pathogenesis of neurodegenerative diseases.²⁸ Based on emerging therapeutics that target disrupted post-transcriptional processes,^{39–41} it is plausible that dysfunctional RNA dynamics may drive neurodegeneration. Whilst significant progress has been made in understanding RNA regulation and localisation in neurons, a more comprehensive and detailed understanding of the molecular mechanisms involved at the cellular level is still required, particularly in neurodegeneration. Emerging tools with high

spatial and temporal resolution may help elucidate the key RNA regulatory drivers of neurodegeneration and provide opportunities for possible therapeutic interventions.

1.1.3 Mitochondria

Mitochondria, the primary source of cellular energy, are crucial organelles for meeting the high energy demands of neurons for neuronal function and survival.⁴² Most neuronal energy comes from ATP production via OXPHOS in the inner mitochondrial membrane.⁴³ This is opposed to glycolysis which occurs in the cytosol without oxygen and has a reduced capacity for generating ATP compared to OXPHOS. During OXPHOS, electrons, derived from the breakdown of glucose, are transferred via the electron transport chain through a series of protein complexes (complexes I to V) on the inner mitochondrial membrane.⁴⁴ As electrons move through the chain, protons are pumped across the inner mitochondrial membrane from the mitochondrial matrix to the intermembrane space. This creates an electrochemical gradient with a higher concentration of protons in the intermembrane space. The final protein complex of the chain, known as ATP synthase or complex V, utilises the energy stored in the proton gradient to catalyse the synthesis of ATP from ADP and inorganic phosphate. Due to the neurons' strong reliance on their mitochondria for cellular energy, disruptions in OXPHOS can have significant consequences for neuronal homeostasis and have been associated with various pathologies, such as mitochondrial disorders and neurodegenerative diseases.^{45,46} Additionally, reactive oxygen species (ROS) are produced as a by-product of the electron transport chain, where neurons are particularly vulnerable to oxidative damage from ROS due to having low levels of antioxidants and their limited regeneration capacity.^{47,48} The overproduction of ROS can lead to oxidative stress which can cause DNA damage, lipid peroxidation and protein oxidation, features observed in neurodegenerative diseases.⁴⁹

Mitochondria are present in distinct subpopulations in neurons (**Figure 1.2**), which differ in their morphology,⁵⁰ calcium buffering,⁵¹ dynamics,⁵² proteomic profiles⁵³ and enzymatic activity.⁵⁴ Mitochondria are highly abundant in the soma to support the overall function of the cell. Axonal mitochondria are small, discrete units sparsely distributed throughout the axon.^{55,56} Dendritic mitochondria are larger, often forming an extensive mitochondrial network with the soma and exhibiting less motion than axonal mitochondria.⁵⁵ Synaptic mitochondria localise at the synapses to support the high energy demands required for synaptic transmission.^{57,58} It has been reported that synaptic mitochondria may be more vulnerable to damage and

neurodegenerative diseases, where dysfunctions have been observed in synaptic mitochondria prior to non-synaptic mitochondria and cellular damage.^{51,59–61} Mitochondria are highly dynamic organelles which move along the microtubule network of neurons and undergo cycles of fusion and fission.⁵⁸ These cycles, combined with mitophagy and mitochondrial biogenesis, play a key role in mitochondria quality control, where dysregulation of these processes has been implicated in neurodegenerative diseases.^{62,63}

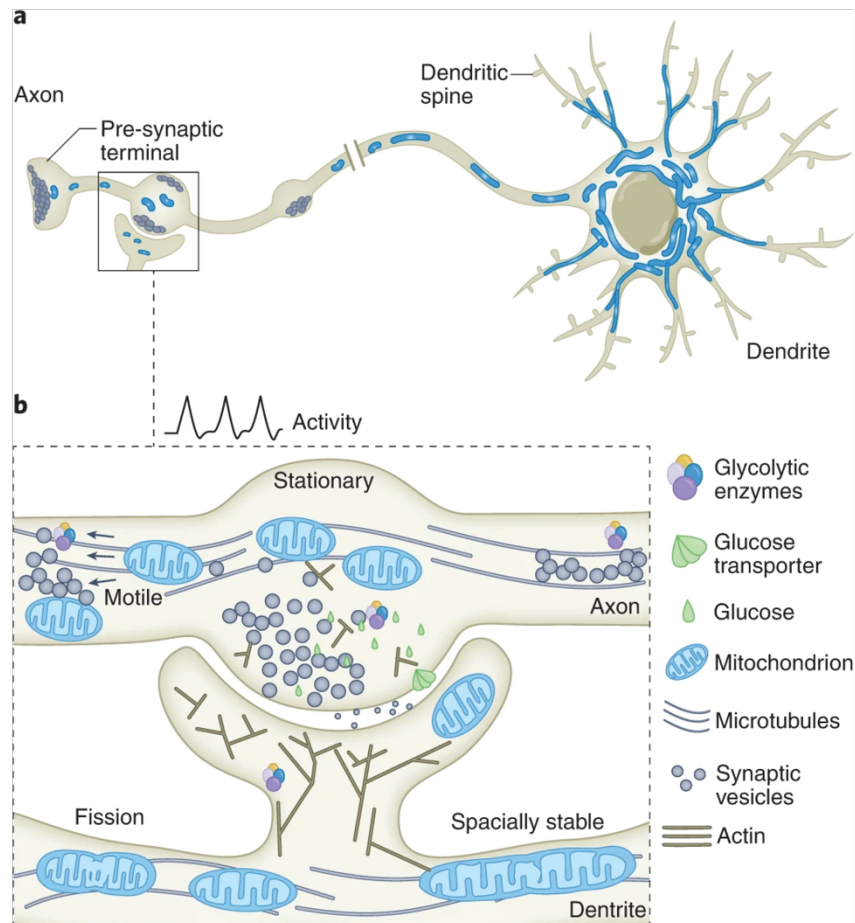


Figure 1.2: Mitochondrial morphology and localisation in neurons. a) Schematic of a neuron, showing its elongated mitochondrial network in the somatodendritic compartments and smaller discrete mitochondria in the axon and pre-synaptic terminals. b) The synapse between a presynaptic terminal of one neuron and the post-synaptic terminal of another neuron. Mitochondria localise to the synapses via the microtubule network and generate ATP for sustained synaptic activity. Figure reprinted with permission.⁵⁸ Copyright 2022, Springer Nature.

Mitochondria have their own circular genome that is approximately 16.6 kb in length in humans.^{64,65} It encodes 13 protein complexes, 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) which are vital for oxidative phosphorylation and ATP production.⁶⁵ Mitochondrial DNA (mtDNA) is present in multiple copies per mitochondrion and each cell

has up to 10,000 copies of mtDNA as opposed to only two copies of the nuclear genome.^{66,67} The mutation rate of mtDNA is much higher than that of nuclear DNA due to its limited DNA repair mechanisms, lack of protective histones and its proximity and susceptibility to ROS.⁶⁶ Taken together, this can lead to different proportions of mutant and wild-type mtDNA in each cell and even each mitochondrion, known as mitochondrial heteroplasmy.⁶⁸ The variation of mtDNA increases as it replicates, where it clonally expands and leads to an increase in somatic mutations within cells as we age, particularly in post-mitotic cells.^{64,68,69} This explains why ageing is a significant risk factor in the development of neurodegenerative diseases. There is also evidence that inherited mtDNA contains small numbers of mutations which may then expand with ageing.^{70,71}

Cells with high levels of mtDNA mutations have poorer respiratory functions, eventually leading to mitochondrial dysfunction.^{70,72} Mitochondrial defects, impaired mitochondrial function and mtDNA mutations exist in a wide range of neurodegenerative diseases such as PD, ALS, and AD, in addition to being linked with ageing.⁷³ Heteroplasmy levels are thought to correlate with the severity of a diseased phenotype in patients.⁶⁸ It is estimated that approximately 80-90 % point mutations or 50-60 % mtDNA deletions are required to manifest into disease at the cellular level, where the mutational burden of deletions appears higher than that of point mutations.⁷⁰ However, there are conflicting reports regarding whether specific variants contribute to neurodegenerative disease.^{74,75} mtDNA copy number has also been found to be decreased in neurodegenerative diseases^{74,76-78} and is proposed as a potential biomarker of PD.^{79,80} A decrease in mtDNA copy number may lead to a higher mutational burden of any mutations in the cell or mitochondrion, potentially leading to deleterious effects.⁷⁴ Further work studying mitochondrial heteroplasmy and mitochondrial copy number at single-cell resolution will aid our understanding of the expansion of mutations in cells and the relationships between mtDNA, cellular function and disease. This could provide crucial insight into the contribution of mtDNA on disrupted phenotypes in disease, or rather, the contribution of pathology on mitochondrial heteroplasmy and copy number.

1.2 Single-cell analysis

Single-cell analysis, the investigation of individual cells in populations (**Figure 1.3**), is a rapidly expanding field that has transformed our understanding of complex biological systems in health and disease. The development of single-cell technologies has progressed our

understanding from studying cell populations in bulk to understanding the molecular diversity of seemingly similar cells.⁸¹ Bulk analysis only provides an average profile of an ensemble of cells and does not reveal distinctions between the unique transcriptome of similar cell types and individual cells within populations.⁸² On the other hand, single-cell analysis can provide novel information on cell-to-cell variability and the stochasticity of gene expression which could be vital for understanding biological mechanisms, mutational threshold levels in disease, and the role of transcripts on cellular function.^{81–84} This can provide unprecedentedly detailed information on cellular landscapes and could lead to the development of more targeted disease treatments.⁸⁵ For example, single-cell analysis has led to the discovery of heterogeneous cell populations, which may explain why treatments, designed to target the average or bulk cell-types in populations, may not be completely successful due to the presence of heterogeneous subpopulations which are unresponsive to treatment.^{86,87} This understanding could lead to the identification of more specific biomarkers and the development of precision drugs and personalised medicine, ultimately leading to improved patient outcomes.

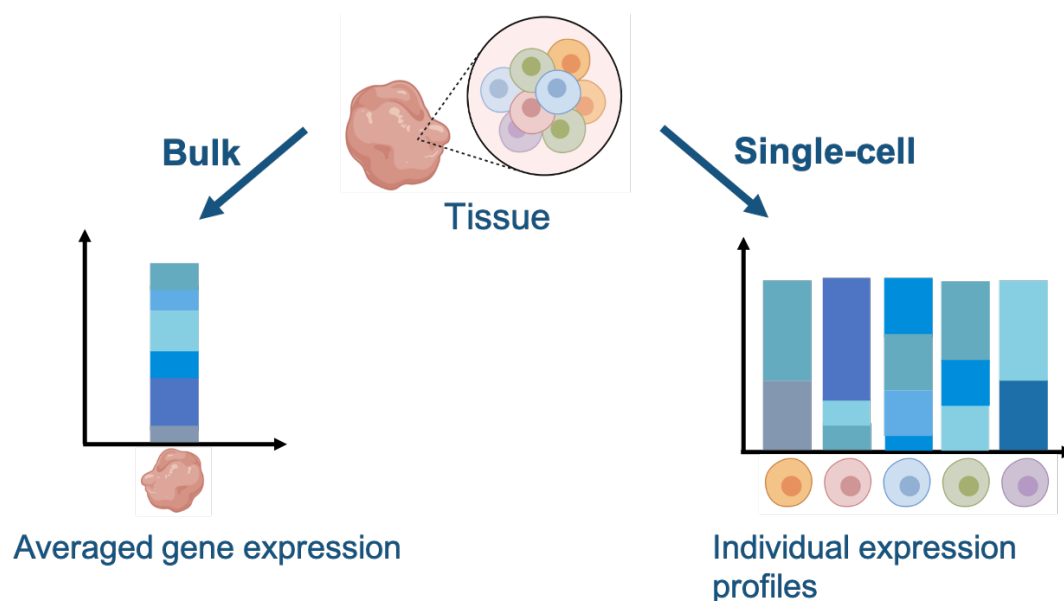


Figure 1.3: Single-cell analysis compared to bulk analysis. Tissues or collections of cells can be analysed together, providing bulk, ensemble averages of gene expression profiles (left). Alternatively, single cells can be analysed to provide individual expression profiles and highly resolved information. The figure was created using Biorender.com.

In neuroscience, single-cell analysis has enabled novel cell types and subtypes to be identified and characterised based on their transcriptomic profiles,^{88,89} the identification of rare transcripts in disease,⁹⁰ and improved understandings of tissue heterogeneity and the diversity of

seemingly similar cells.⁹¹ This has helped shed light on the cellular diversity of the brain,^{92,93} selective neuronal vulnerability,⁹⁴ novel biomarkers and molecular mechanisms in neurons.^{95,96}

A number of high-throughput methods have been incorporated to isolate single cells.⁹⁷ Isolation of neuronal cells, however, is more challenging due to their morphologically complex structures and sensitivity to standard enzymatic dissociation steps when applied to brain tissue.^{82,98} Single-nuclei isolation is therefore often used as an alternative for delicate cells like neurons as it does not require digestion or heating, which single-cell methods employ to access the contents of cells, and which may alter the gene expression or function of cells.^{99,100} Nevertheless, methods have been employed to analyse single neuronal cells via fluorescence-activated cell sorting (FACS),^{89,101} laser capture microdissection (LCM),^{102,103} and microfluidic platforms to isolate dissociated neurospheres.^{104,105} However, with methods such as FACS and LCM, large neuropil-containing debris can remain and the neurites may not be effectively isolated, meaning that often only the contents of cell bodies are available.¹⁰⁶

Following single-cell isolation, cells have been analysed by single-cell DNA sequencing (scDNA-seq), single-cell RNA sequencing (scRNA-seq), and multi-omics approaches where a combination of these analysis methods is used.¹⁰⁷ In particular, scRNA-seq has enabled massive clustering of cell populations based on their transcriptomic profiles and is the leading technology for the Human Cell Atlas project, aimed at mapping all cells in the human body based on their gene expression profiles, spatial organisation and functional relationships.¹⁰⁸

One of the challenges with single-cell analysis methods is the lack of spatial-temporal context of cells; cells become isolated from their environment and then lysed or destroyed for downstream analysis. This removes information regarding the cell's spatial environment and the potential position of the cell within a trajectory of its dynamic behaviour.¹⁰⁹ This is particularly apparent for neuronal cells which comprise a vast neuronal network that interlinks with various cell types and subtypes crucial for their function. Spatial transcriptomics, the combination of scRNA-seq with single-cell mapping within complex tissues, addresses some of these limitations and allows for gene expression to be mapped in the tissue microenvironment.¹¹⁰ This has been pivotal for recent developments in developing the human brain atlas.^{111–113}

1.3 Subcellular analysis

The spatial information of cells should be preserved to truly characterise single cells, as has been shown with the development of single-cell spatial transcriptomics, to prevent any alterations occurring as a result of removing the cell from its microenvironment. Additionally, it is imperative to analyse the intracellular make-up of single cells to understand the organisation of biomolecules in cells, along with their interactions, trafficking and localisation, and responses to cellular changes and treatments. This will aid in unravelling the complex molecular mechanisms at the single-cell level, potentially uncovering novel therapeutic targets and facilitating treatment monitoring.

The development of technologies to analyse the subcellular makeup of neurons is vital to map transcripts and combine spatial correlations with quantitative measurements of RNA and subcellular functions. This could aid our understanding of RNA localisation, regulation and local protein synthesis in neurons. Similarly, mitochondria are known to be distinct in different subcellular compartments of neurons, and so characterising these subpopulations may further our understanding of their roles in the cell and how they respond to cellular demands. Additionally, analysing mtDNA mutations on the tissue scale does not explain how these mutations are present in the mitochondria or cells. For example, mutations may be due to high levels of a specific point mutation within a single cell with lower levels of mutations within other cells, or they may be due to many different mutations present at low levels within each cell. Both mechanisms would provide the same result from the bulk analysis of cells, yet higher levels of mutations in single cells may be more deleterious and lead to degeneration. Similarly, we do not know the mutational load within single neurons, for example, if mitochondria located in specific sub-compartments have higher levels of mutations than other areas of the cell. One may hypothesise that the synaptic mitochondria may exhibit higher mutational levels due to synaptic mitochondria having been shown to have dysfunctions before non-synaptic mitochondria in ageing and AD models.^{60,61} Understanding the mutational landscape at the subcellular level may therefore aid our understanding of how mutations spread within single cells and highlight possible regions that are more vulnerable to mutations or copy number changes, potentially uncovering new targets to minimise this.

1.4 Subcellular imaging techniques

Fluorescent imaging techniques provide an exceptional route towards single-cell subcellular mapping, with enormous contributions to spatial transcriptomics and the development of the single-cell atlas.¹⁰⁸ In particular, fluorescence in situ hybridisation-based techniques and live-cell imaging provide exceptional resolution of individual RNAs in single cells. These methods are discussed in this section, along with their limitations for the subcellular analysis of neurons.

1.4.1 Fluorescent in situ hybridisation methods

Fluorescent in situ hybridisation (FISH) methods have been used to map mRNAs in fixed single cells, facilitating understanding of the locations and behaviour of mRNAs in neurons.^{26,28} These methods involve hybridising fluorescent probes that are complementary to the target of interest. Single-molecule FISH (smFISH) has allowed for single mRNA transcripts to be visualised in single cells and has increased the sensitivity of FISH for expression level quantification.¹¹⁴ This has been particularly useful for identifying low-abundant transcripts and providing accurate measurements of transcript abundance and localisation in subcellular compartments of single neurons.^{115,116} With the development of state-of-the-art smFISH methods such as multiplexed error-robust FISH (MERFISH), which can multiplex thousands of mRNAs in the same sample via combinatorial barcoding, and sequential FISH (seqFISH), which can multiplex mRNAs through sequential rounds of probe hybridisations, comprehensive profiling of the transcriptome of single neurons can now be performed.^{117,118} This has aided the identification of distinct subregions of cells and tissues based on their transcriptional profiles and led to the discovery of novel distribution patterns of mRNAs in single neurons. These methods have also provided a vital resource for spatial transcriptomics in the brain and will have significant contributions to the human brain atlas.

In addition to being an extremely useful tool for characterising the spatial organisation of neurons and the brain, FISH-based methods have also provided novel insights into the localisation characteristics of transcripts.^{119–121} Through dual-labelling of β -actin mRNA and ribosomes, Buxbaum *et al.* observed a dormant state of β -actin mRNA packaged with the ribosomes.¹²² Upon neuronal stimulation, they observed that these mRNAs become unmasked, showing higher abundances of mRNA in the dendrites followed by increased protein translation. This work provided key findings related to the possible mechanisms of RNA translocation during synaptic plasticity, where they may disassemble from their masked state

and become activated.¹²³ Other novel findings from smFISH have reported that some mRNAs travel singly in distal dendrites rather than collectively with other mRNAs,¹¹⁵ and that the 3' UTR region of transcripts may control mRNA localisation in neurons,^{120,121} where localised transcripts have longer 3' UTR regions and longer half-lives than non-localising transcripts.¹²⁰

1.4.2 Live-cell imaging

Live-cell imaging marks an advancement from FISH-based methods as it allows the behaviour of RNAs in the same cell to be observed, meaning that the true dynamics of molecules can be tracked in single cells. A number of methods have been employed to monitor the dynamics of mRNAs in living neurons. Microinjection or transfection of molecular beacons, for example, has been used to track specific mRNAs for up to 20 min under conditions of long-term potentiation and long-term depression, showing an enrichment of mRNA at the dendritic spines.¹²⁴ Additionally, and similarly to what was reported using smFISH, a molecular beacon live imaging approach found that β -actin mRNA travels singly in axons.¹²⁵ The authors also observed distinct motion-types of mRNA in different axonal sub-compartments, providing superior spatial resolution of mRNA trafficking within a subcellular compartment. However, the use of molecular beacons for live-cell imaging has been reported to be prone to low signal and high background.¹²³ Alternatively, the MS2-GFP bacteriophage system is an extremely powerful method to follow single-molecule RNA dynamics.¹¹⁹ This involves expression of mRNAs which have multiple unique hairpin sequences (or stem-loops) that a specific fluorescent bacteriophage protein binds to.¹²⁶ Using this method of live-cell imaging, similar findings to that of Buxbaum *et al.* were observed in live mouse neurons, where β -actin mRNA disassembles upon neuronal depolarisation, providing further insights into RNA dynamics in neurons.¹²⁷

1.4.3 Outlook and limitations

Innovations in live-cell imaging, fluorescent in situ hybridisation (FISH) and single-molecule tracking techniques have contributed significantly to our understanding of the motion of mRNAs within single neurons and to defining their spatial distribution within neurons and the brain. The high multiplexing capabilities of techniques like MERFISH enable massively parallel characterisation and quantification of mRNAs in the same sample, identifying possible interactions between mRNAs and distinct subcellular localisation patterns.

FISH-based methods involve the chemical fixation of cells or tissues, which are fixed at a singular timepoint and only provide a ‘snapshot’ of the transcriptome. This limits analyses of the dynamic behaviour and motion of mRNAs within single neurons, where this can only be inferred from imaging separate fixed cell samples at different timepoints which may show variable behaviour based on the heterogeneity of single cells. Temporal studies can therefore not be performed to elucidate the dynamic and molecular behaviour of transcripts in the same cell. Fixation of cells may also cause alterations in the functions of RNA, meaning that their observation may be less representative of a cell under physiological conditions.¹²⁸ Additionally, methods that involve multiple hybridisation rounds of probes, such as MERFISH or seqFISH, can become very costly with time-consuming sample preparations.

Although the multiplexing capabilities of live-cell imaging does not reach that of FISH, live-cell imaging methods advance from FISH methods for single-cell subcellular studies in that they offer exciting opportunities to monitor RNA dynamics in single cells with high spatial resolution. This has uncovered novel findings regarding RNA localisation in neurons with changing neuronal activity and the travels of mRNAs.

With both live-cell imaging and FISH-based methods, knowledge of the target sequence is required for the probe design and binding. This limits the analysis to known targets and features of mRNAs. Many of these methods therefore begin via RNA-seq of mechanically isolated compartments of cells to identify transcripts of interest, followed by the design of probes to target and image these.^{26,120,129} For live-cell imaging, delivering exogenous probes or reporters into living cells may lead to physiological changes in the cell and toxicity.¹³⁰ Phototoxicity may also occur upon imaging, particularly during prolonged exposures for monitoring subcellular dynamics, and possible artefacts may also interfere with biological function.^{131,132} Label-free methods remain the most desirable, particularly for long-term studies on living cells.

1.5 Subcellular sampling techniques

Many single-cell isolation methods are widely available for high-throughput analysis of single cells. These include FACS, optical tweezers, LCM, electrokinetic single-cell focusing, and microfluidic platforms that can manipulate cells via microwell-based docking. However, these methods require the removal of the cell from its standard microenvironment, combined with its lysis, to access the subcellular contents. Not only does this lose the contextual information

of the cell, but it may also lead to analyte modifications during the removal and lysis procedure. Lysis of the whole cell also means that the same cells cannot be studied over time, for example, to examine the effects of a treatment on a particular cell subtype which is necessary given the heterogeneity of cell populations and their responses.

For polarised cells like neurons, it is particularly important to analyse the contents of specific compartments to better understand their functions and roles in the cell. Subcellular compartment isolation techniques have been employed to address this through the use of micropipettes,¹³³ microdissection,^{26,121,129,133–135} microfluidic chambers,¹³⁶ and microporous membranes¹³⁷ to remove mRNA from different compartments of neurons for RNA sequencing and qPCR. Using these methods, the localisation behaviour of a vast number of transcripts in neurons has been identified and quantitatively measured.^{26,133,135} For example, 2,550 mRNA's have been discovered to be localised to the neurites of rat hippocampal neurons through bulk microdissection of the hippocampus and deep sequencing, followed by FISH in single neurons to confirm the localisation findings from deep sequencing.²⁶ A limitation of isolation techniques like microdissection for assessing subcellular transcript distributions is that, similar to LCM, they risk contamination from other subcellular compartments and glial cells due to the high precision required.^{138,139} Using microporous membranes can also lead to difficulties in completely separating the axons and the dendrites and therefore lead to incorrect conclusions for each location.¹⁴⁰ Additionally, all compartment isolation methods involve the destruction of the neuron, meaning that there is a loss of interconnection and only static omics analyses from dead neurons can be made. The physical destruction of the cell during compartment isolation may also trigger stress responses and transcriptomic changes in the cell, providing a less representative view of the cell's transcriptome.

To address the limitations of high-throughput cell isolation techniques and subcellular compartment isolation methods, there have been many recent developments in single-cell subcellular sampling techniques. These are techniques that can isolate and remove biomolecules from living cells to analyse the intracellular contents of single cells. Extracting molecules from single cells enables their molecular basis to be fully characterised and would enable the discovery of novel transcripts, proteoforms, and post-transcriptional and -translational modifications.¹⁴¹ These methods involve the non-destructive insertion of sampling probes or devices into living cells to remove biomolecules of interest. Often, these methods maintain cells in their standard cultured environment, with minimal modifications or

disturbances to the microenvironment of the cells. These advance from previous single-cell manipulation techniques, with many providing high spatial subcellular resolution and showing the potential to perform dynamic studies on living cells.

This section outlines the state-of-the-art technologies for subcellular sampling from live cells (**Figure 1.4**), including those based on micro/nanopipettes and atomic force microscopy (AFM) tips. Many involve nanobiopsy, the extraction of minute amounts of material from a small region within a single cell. This section features much of that reviewed by Sahota *et al.*,¹⁴¹ with some updates on more recent developments.

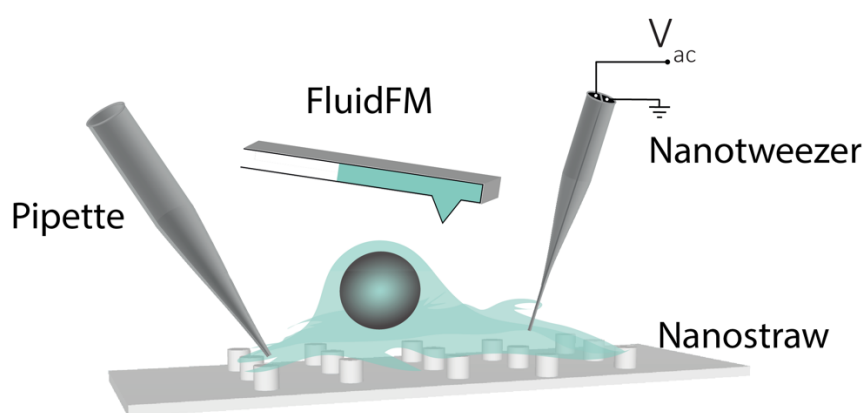


Figure 1.4: Summary of single-cell subcellular sampling methods. Reprinted with permission.¹⁴¹ Copyright 2023, Royal Society of Chemistry.

1.5.1 Pipette-based

Micropipette- and nanopipette-based methods have been successfully employed for subcellular sampling. Pipettes are fine, needle-like structures formed from laser pulling quartz capillaries, typically with pore sizes between 50 nm–5 μm for sampling which can be finely tuned based on the laser pulling parameters. A nanopipette is defined as a pipette which has a pore size <100 nm.¹⁴² Typical pipette-based systems have been incorporated for subcellular sampling via electrowetting combined with scanning ion conductance microscopy (SICM).^{143–147} This has enabled high-resolution imaging of cells and precise targeting of subcellular regions, combined with aspirations of cytoplasmic volumes to access cellular contents.

A subcellular ‘cookie cutter’, based on a 5 μm -diameter micropipette integrated with an SICM setup has been proposed as an alternative method to LCM for subcellular biopsy of tissue

samples.¹⁴⁸ This method was capable of dissecting regions sized $>20\ \mu\text{m}$ in diameter from human skeletal muscle tissue to isolate mitochondria, followed by quantification of mtDNA copy number. More recently, next-generation sequencing has been performed on mitochondria samples from human mitochondrial disease patient tissue to assess mtDNA mutational loads in skeletal muscle tissue.¹⁴⁹ Although the cookie cutter is not a single-cell subcellular method, it could be used to compare heterogeneity within tissues and in clinical samples or 3D cell cultures, as has begun in this work. Nevertheless, as with LCM, this is a disruptive technique that essentially cuts away parts of tissues, likely leading to the destruction of living cells within the tissue and only allowing for single timepoint characterisation of tissues.

On the single-cell level, micropipettes have been used to directly sample and extract single mitochondrion from mouse and human neuronal cells.¹⁵⁰ Mounted micropipettes, with a pore size of $0.7\ \mu\text{m}$, extracted mitochondria from neurons via exertion of pressure, followed by removal of this pressure with suction to collect the mitochondria for analysis. Individually extracted mitochondria were then exposed to whole genome sequencing to analyse mtDNA, revealing high levels of heteroplasmic variants within and between single cells. This method showed a potential approach for investigating the spread of mutations in a single cell as it preserved the spatial origin of the mitochondria for evaluating mitochondrial heteroplasmy. Despite the micropipette isolating mitochondria from the dendrites of neurons, the size of the micropipette may mean that it is too large to access thin regions of the dendrites without isolating part of the dendritic structure simultaneously. This raises questions regarding the viability of neurons during the procedure, where significant cellular disruptions will limit the technique to static timepoint analyses and induce possible phenotypic alterations in the cells.

Nanometre-sized disruptions in the plasma membrane have been shown to spontaneously reseal *in vitro* following their disruption, as opposed to larger disruptions which require the recruitment of active repair machinery.¹⁵¹ Nanopipettes therefore offer a less invasive method of subcellular sampling due to their smaller pore size and minimal disruptions to plasma membranes. Several nanopipette-based strategies have been employed to remove the contents of living cells. Actis *et al.* integrated electrowetting with SICM to extract material from human fibroblasts and HeLa cells.¹⁴³ This method involves the application of a negative voltage across a dichloroethane-filled nanopipette ($50\ \text{nm}$ pore size), causing aqueous solution, or cytoplasmic content from cells, to fill the nanopipette. Reversing the bias allowed the material to be removed from the pipette for downstream analysis. The integration with SICM means that

topographical maps of cells can be generated with optical imaging, allowing for automated nanometric precision in locating and positioning the nanopipette to the desired region of the cell. This same method has also been applied to study compartmentalised mRNAs in neurons, where nanobiopsies, making up aspiration volumes of approximately 1 % of the cell's total volume, were performed from the soma and neurites of human iPSC-derived neurons.¹⁴⁶ This led to the discovery of novel neurite-localising transcripts. Overall, cellular contents extracted using these methods have been analysed by RT-qPCR,¹⁴³ RNA-seq,^{143,146} whole genome sequencing of mtDNA,^{143,149} and matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS).¹⁴⁷

The pipette-based techniques discussed thus far incorporate a single-barrel pipette, a pipette in which there is a singular pore throughout the length of the pipette. An alternative nanopipette-based technique has incorporated double-barrel nanopipettes.^{144,145} As part of this technique, one barrel is filled with an electrolyte solution for SICM, and the other is filled with an organic solvent for electrowetting and aspiration of cellular contents. One of these studies collected the cytosol from different locations of the same live cell (mouse fibroblasts) and analysed the mRNA by RT-qPCR.¹⁴⁵ More recently, a similar double-barrel nanopipette system, with each barrel being approximately 150 nm in size, was able to longitudinally profile the transcriptome of single glioblastoma brain tumour cells in vitro over 72 h with and without standard treatment.¹⁴⁴ This included extracting volumes ≥ 200 fL from cells and following the sequencing method developed in 'Live-seq'¹⁵² (described below in **1.5.3**) to dynamically track the transcriptome of single cells. This development, combined with the Live-seq method it was inspired by, has greatly enhanced the capabilities of subcellular sampling for single-cell tracking. It would be interesting to see if this method and pore size could be used on more sensitive cell types like neurons for sequential nanobiopsies and time-dependent analyses.

1.5.2 Nanostraws

Nanostraws are polycarbonate membranes containing hollow 150 nm alumina-coated cylindrical tubes which protrude approximately 1-3 μm from the membrane surface.¹⁵³ This provides a surface for cells to be cultured on, where a fixed number of nanostraws contact the cells but do not penetrate them. Sampling is performed via electroporation, allowing the subcellular contents of cells to diffuse out into a buffer. Cao *et al.* found that 5-10% of proteins and mRNAs were extracted from individual human-derived cardiomyocytes and astrocytes

using nanostraws, exhibiting a 5 % reduction in cell viability.^{153,154} These volumes correspond to approximately 7 % of the cell's analytes. Isolated proteins were analysed by ELISA and mRNAs were analysed by RT-qPCR.¹⁵³ The technique was successfully used to study cardiomyocytes over 5 days, with sampling performed every 24 hours and a heat-shock perturbation induced at day 2. Levels of a heat shock protein were successfully monitored over the 5-day period, with an increase detected on day 3. Notably, the authors found that mRNAs detected using nanostraws were relatively consistent with the mRNAs detected from bulk lysed cells. This highlights that this subcellular sampling may provide meaningful and representative transcriptome analyses of whole cells. Nevertheless, larger molecules made up the small number of mRNAs that were not detected compared to bulk cells, which the authors suggested may be due to their slower diffusion rates into the sample buffer.

Nanostraws enable high-throughput longitudinal profiling of the omics of single cells. This is the most high-throughput method of all subcellular sampling techniques discussed here, where the contents of living cells can continuously be extracted over time with multiple nanostraws per cell. However, due to the structures being fixed on a substrate, there is little control over the spatial positioning of the nanostructures. This restricts the targeting of specific subcellular locations or compartments within cells, and so they may be more suited to whole single-cell monitoring rather than spatial subcellular analysis. Additionally, care should be taken to ensure that the electroporation method of removing the contents of cells does not influence the cell's transcriptome or phenotype.¹⁵⁴

1.5.3 FluidFM

Atomic force microscopy (AFM) is a high-resolution imaging technique that uses a sharp tip attached to a flexible cantilever to scan the surface of a sample in aqueous solution.¹⁵⁵ The interaction forces between the tip and the sample surface are measured, providing information about the topography and properties of the material being studied.^{155,156} Subcellular sampling using AFM has been previously demonstrated by making use of the AFM tip's multifunctionality: its ability to scan an aqueous surface for high-resolution imaging of living cells under physiological conditions, combined with its ability to probe cells at the subcellular level to extract their contents or deliver exogenous substances such as drugs, nanoparticles and organelles.^{157,158} This was initially achieved via non-specific adsorption of mRNAs in contact with the AFM tip after insertion into cells.^{159,160} This was the first subcellular sampling method

to detect mRNAs inside living cells without removing them from their physiological environment or showing significant damage to the cell. However, the isolation was solely based on non-specific adsorption of mRNAs, meaning that the amount of material available for detection was limited. Additionally, using standard AFM to manipulate or deliver molecules into cells may lead to mechanical indentation or scraping of the AFM probe onto the sample.¹⁶¹

FluidFM¹⁶¹ is a technique that addresses some of the problems with AFM for sampling from cells.^{152,162–165} FluidFM combines AFM with a pressure-driven micro-channelled silicon nitride probe for fluid-controlled extractions from single cells.¹⁶² This is achieved by incorporating a micro-sized channel into the AFM cantilever with a nanosized tip aperture, forming a continuous fluidic channel.¹⁶¹ For single-cell sampling, the standard probe comprises a pyramidal tip with a tuneable 400 nm diameter triangular aperture.¹⁶² For subcellular sampling, the tip of the probe is lowered into a cell so that the aperture is completely inserted, a negative pressure is then applied which causes the channel to fill with intracellular fluid, and the tip is withdrawn from the cell for dispensing and molecular analysis.¹⁶²

FluidFM initially demonstrated nuclear and cytoplasmic extractions from HeLa cells.¹⁶² Extraction volumes up to 4 pL and 0.6 pL in the cytoplasm and nucleus, respectively, could be taken from cells with 82 % and 86 % cell viability after a single extraction. The authors highlight that these volumes represented at least 90 % of the total cytoplasmic volume and 20 % of the total nuclear volume of the largest native volumes measured. Extraction volumes larger than this resulted in cell death. Despite this, the high extraction volumes showed that the cells could withstand a large loss of their native cytoplasmic volumes after the extraction procedure. The authors assessed cell viability up to 5 days after extraction and showed that extracted cells remained viable and behaved similarly to non-extracted cells, including undergoing cell division. This was a key finding in portraying the minimally-invasiveness of the technique, showing that removing these relatively large volumes did not affect the cells' normal behaviour in culture. However, extracting 90% of cytoplasmic volume could lead to cell viability issues in some cell types and will lead to morphological changes which may alter gene expression programs in cells.¹⁵¹

Recently, FluidFM technology was advanced by combining sampling with transplantation by tuning the aperture size and probe geometry to allow for the aspiration of mitochondria from cells.¹⁶⁴ Using an alternative slanted cylindrical probe with a larger aperture size (1.6 μm^2),

whole mitochondria and parts of the mitochondrial network were aspirated from HeLa cells and transplanted into U2OS and primary human endothelial keratinocytes cells. This involved extraction volumes of approximately 0.8 pL and injection volumes between 0.5-2 pL. The authors demonstrated an impressive 95 % success rate of mitochondrial transplants into the cytosol of living cells, with 80 % viability of the receiver cells after injection. Interestingly, mitochondria from the donor cell were shown to fuse to the host's mitochondrial network in 18 out of 22 cells, observed by differential fluorescent labelling of donor and host mitochondria, where the remaining 4 cells showed donor mitochondrial degradation. The authors found that fusion into the host's mitochondrial network occurred within 30 minutes for most cells. Long-term tracking of transplanted mitochondria showed up to 2.5 % of mtDNA from donor U2OS cells was detected in HeLa host cells 14 days after transplantation, during which time the host cells would have expanded in culture. This showed maintenance of cell viability following transplantation and demonstrated that 'foreign' mtDNA could be incorporated and replicated in new cell types. This work greatly expanded the potential of subcellular sampling technologies, where the transplantation benefit could have wide implications in understanding mitochondrial quality control for maintaining cell homeostasis. This could also have great potential in mitochondrial regeneration therapies, where impaired mitochondria have been shown to be key features of many diseases such as cancer¹⁶⁶, stroke¹⁶⁷, and neurodegenerative diseases^{70,168}. This new method, therefore, fills a technological gap in subcellular sampling technologies and opens new possibilities for studying mitochondrial replacement therapies and mtDNA selection and expansion.

Overall, a range of downstream analyses have been performed on subcellular samples from FluidFM, including RT-qPCR,¹⁶² enzymatic assays,¹⁶² MALDI,¹⁶³ and notably, RNA-seq.¹⁵² A recent seminal paper demonstrated that FluidFM could perform cellular extractions on live cells and the aspirated volume could be subjected to Smart-seq2,¹⁶⁹ a highly sensitive RNA-seq method for detecting low amounts of RNA in the picogram range, for transcriptome analysis.¹⁵² They coined this method 'Live-seq'. The sensitivity of Smart-seq2 was optimised to detect 1 pg of total RNA, and an average volume of 1.1 pL was taken from cells which corresponded to 40-70 % of the total cell volume. Remarkably, cells were shown to recover these volumes within 100-320 minutes, followed by continuing the same growth pattern as shown in controls. This shows that cells may withstand loss of a large portion of their cytoplasm and be able to recover back to normal function. The time taken to recover the cell volume, however, may delay cell growth and likely incur changes in gene expression until the volume

is fully recovered. The cells extracted using this method may therefore not be a true representation of a non-extracted cell and the delay time may need to always be considered when studying cells over time. Some transcriptomic changes detected using single-cell RNA-seq were shown 1 h and 4 h after Live-seq sampling compared to non-extracted controls, where 12 genes were shown to be significantly differentially expressed between Live-seq and control samples. This highlights the possible invasiveness of the technique. Nevertheless, the minor transcriptomic changes are still impressive with the amount of cytoplasmic volume that is removed from the cells. In its current state, however, Live-seq may not be suitable for short-term transcriptomic studies due to the risk of the extraction procedure inducing stress and changes gene expression in response to the loss of cytoplasmic volumes.

Two dynamic studies on the same single cells were reported using Live-seq: 1) application of a lipopolysaccharide (LPS) stimulation in macrophage-like cells to detect a fast molecular change 4 hours post-stimulation, 2) adipogenic differentiation of primary mouse adipose stem and progenitor cells (ASPCs) to detect a slower change after 2 days. Live-seq data obtained from cells that passed quality control measures mapped correctly to their cell state clusters and showed an expected transition after each model based on single-cell RNA-seq data. After differentiation, ASPCs were monitored for 7 days after the second extraction and notably demonstrated no change in viability compared to controls. They also maintained their differentiated state over this time period, highlighting that the cells maintained their functionality after two extractions from the same cell. For both time-dependent studies, however, the efficiency of cells that passed the quality control measures from the RNA-seq reads was relatively low; 40 % for the first study and 18 % for the second. Nevertheless, Live-seq has greatly expanded the scope of single-cell transcriptomics and proves to be an extremely exciting method for monitoring transcriptomic dynamics in the same cell over two timepoints.

1.5.4 Dielectrophoretic systems

1.5.4.1 Dielectrophoresis

Dielectrophoresis (DEP) is the movement of an electrically polarisable object in the presence of non-uniform electric field.¹⁷⁰ It was first devised by Pohl in 1951, demonstrating the use of DEP for selective separation of electrically neutral solid suspensions.¹⁷¹ Pohl's fundamental work established the basis of DEP for concentrating cells and particles upon application of non-uniform electric fields, highlighting critical physical and biological parameters that could

be exploited to separate biological molecules: electric field strength, field uniformity, electrode shape, frequency, conductivity, relative permittivity, concentration, thermal treatment, colony age, cellular shape and physiological state.^{172–176} The use of DEP has since expanded to a wide range of biomolecule manipulation applications including biosensing, drug development and clinical diagnostics.^{177,178}

If particles are overall neutral in charge and placed in a uniform electric field, the dipoles will polarise but result in no net force due to equal opposite forces being exerted on the particle (**Figure 1.5a**). In an inhomogeneous electric field, however, the particle experiences unequal forces at the terminals of its dipole, resulting in the migration of the particle (**Figure 1.5b**). The magnitude and direction of the force depend on the polarizability of the particle and its surrounding medium. The DEP force, F_{DEP} , acting on a uniform, spherical particle is given by:^{170,179}

$$F_{DEP} = 2\pi\epsilon_m r^3 \text{Re}[K(\omega)] \nabla |E|^2 \quad \text{Equation 1.1}$$

Where ϵ_m is the absolute permittivity of the medium, r is the radius of the particle, $\nabla |E|^2$ is the square of the gradient of the electric field (given in $V^2 m^{-3}$) and $K(\omega)$ is the Clausius-Mosotti (CM) factor.

Electric field strengths have a $V^2 d^{-3}$ dependency, where V is the applied voltage and d is the distance between the electrodes.¹⁸⁰ This means that:

$$F_{DEP} \sim \frac{V^2}{d^3} \quad \text{Equation 1.2}$$

Therefore, greater voltages and smaller electrode-electrode distances can lead to high dielectrophoretic forces, where the impact of reducing the distance between the electrodes is more significant. The real part of the CM factor is given by:

$$K = \frac{\epsilon_p^* - \epsilon_m^*}{\epsilon_p^* + 2\epsilon_m^*} \quad \text{Equation 1.3}$$

Where ε_p^* and ε_m^* are the conductivity- and frequency-dependent complex permittivities of the particle and the medium, respectively. These can be defined as:¹⁷⁰

$$\varepsilon_{p,m}^* = \varepsilon + \frac{j\sigma}{\omega} \quad \text{Equation 1.4}$$

where ε is the permittivity, σ is the conductivity, ω is the angular frequency of the applied electric field and $j = \sqrt{-1}$.

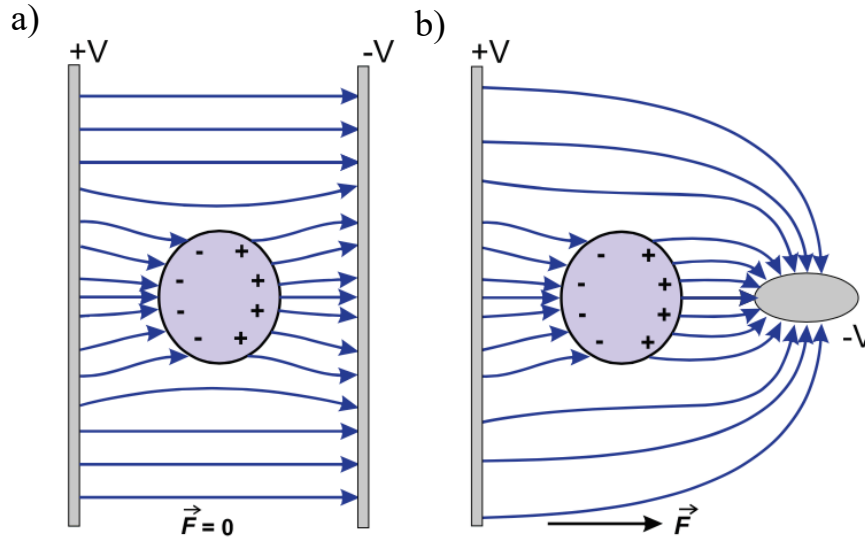


Figure 1.5: Schematic showing the forces experienced on a particle in a) uniform electric field (no net forces acting) and b) non-uniform electric field (overall net force due to the interaction of the dipole charges with the local electric field). Reprinted with permission.¹⁸¹ Copyright 2011, John Wiley and Sons.

The direction of the DEP force is based on the CM factor and therefore dependent on the polarizability of the particle compared to that of the medium. Generally, when the particle's polarizability is greater than that of the medium, $\text{Re}[K(\omega)]$ is positive and the force acts in the direction of high electric field strength, which means that the particle moves in this direction. This is referred to as positive DEP (pDEP).¹⁸² On the other hand, negative DEP (nDEP) is where $\text{Re}[K(\omega)]$ is negative, and the particle gets repelled by the region of high electric field strength and migrates towards the low electric field.

Not only is the DEP force voltage-dependent based on the electric field strength term, but the force and direction of DEP depend on the frequency employed with the conductivity and permittivity properties of the particle and surrounding medium.¹⁸³ This is illustrated in **Figure 1.6**.¹⁸⁰ At low frequencies, there is sufficient time for the charges to respond to the non-uniform field, and the CM factor is determined by the conductivity of the particle and the medium. At

higher frequencies, however, there comes a point where the charges no longer have enough time to respond to the field and the permittivities, or polarisation of charges, of the particle and medium dominate. This is because charges take a few milliseconds to equilibrate to the non-uniform field.¹⁷⁰ This leads to a crossover frequency, which is the frequency at which DEP transitions from positive to negative DEP. This represents the frequency at which the polarizabilities of the particle and the medium are equal and there is therefore no net dipole or F_{DEP} . After this frequency, particles exhibit negative DEP due to the particles not being capable of being polarized under high frequencies.

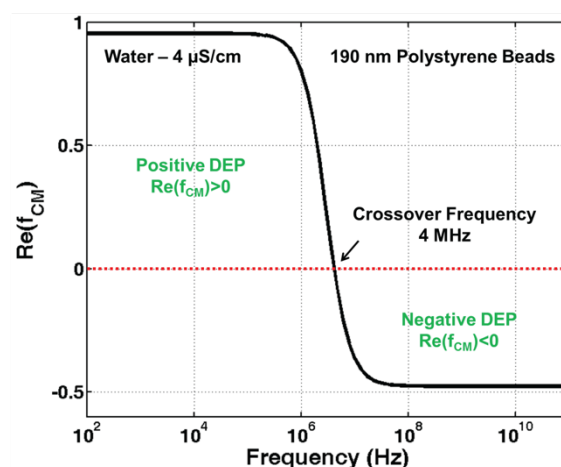


Figure 1.6: Frequency dependency of the CM-factor for DEP on a dielectric spherical particle. CM-factor plot for a 190 nm polystyrene bead in water. Reprinted with permission.¹⁸⁰ Copyright 2016, American Chemical Society.

When a polarisable particle in a medium is subjected to a non-uniform electric field, in addition to the DEP force, a number of other forces are experienced by the particle. These include: electrophoretic forces from the interaction of particle charges with the electric field; electrothermal forces which form as a result of Joule heating and temperature gradients in the medium; electroosmotic forces or fluid motion from the electric field interacting with electrical double layer of the particle; hydrodynamic forces arising from viscous drag on the particle; sedimentation forces from gravity pulling particles out of the solution phase; and Brownian motion, the random movement of particles in a medium.^{182,184,185} The particles' overall motion is influenced by all of these forces where, in order to trap a particle by DEP, the F_{DEP} needs to overcome the counteractive drag forces, namely electrothermal forces, hydrodynamic forces and Brownian motion.¹⁷⁰

The lower size-limit for dielectrophoretic manipulation of particles was once thought to be 1 μm due to electrothermal effects and Brownian motion becoming significant and competing with DEP forces at the sub-micron level.¹⁸⁶ However, a wide range of particles and sub-micron molecules such as cells, polystyrene beads, microorganisms, viruses, DNA RNA and proteins have now been manipulated with dielectrophoretic methods.¹⁸⁴ As the particle's volume reduces, Brownian motion becomes significant and the particle experiences larger rates of diffusion. This challenges movement by DEP, meaning that high DEP forces need to be generated to overcome this. This can be achieved by creating high electric field strengths by reducing the distance between the electrodes and increasing the voltage (**Equation 1.2**). Additionally, F_{DEP} is reduced further for smaller-sized particles (**Equation 1.1**), meaning higher electric field strengths are also necessary to compensate for this. This means that for trapping and isolating small sub-micron particles such as biomolecules by DEP, highly precise design of the electrode system is required. This is particularly important for living systems like cells, where high voltages will need to be minimised to minimise Joule heating and possible harm to the cell.

1.5.4.2 DENT

Dielectrophoretic nanotweezers (DENT) were developed by modifying silicon AFM probes.^{187,188} In initial work, SiO_2 -coated AFM probes were modified by electron-beam evaporation of a 10 nm titanium adhesion layer, followed by a 20 nm film of platinum onto the side of the cantilever polish the end of the Pt-coated tip until the silicon tip is exposed.¹⁸⁷ The tip surface was then modified with gene-specific primers complementary to the mRNAs of interest via biotin-streptavidin chemistry. The modified AFM tips were then positioned and inserted into rat fibroblast cells, and an AC voltage between the platinum layer and the silicon was applied to create a dielectrophoretic force for trapping mRNA from cells. Using finite element analysis, it was estimated that electric field strengths of up to $8 \times 10^{15} \text{ V}^2\text{m}^{-3}$ were generated at the end of the DENT probe. Immersion of the tip in cold water for 45 min released the captured mRNA into solution. Extraction of mRNA was confirmed by RT-qPCR analysis of high-, medium- and low-expressing genes. DENT was further developed by integrating microfluidics with an AFM tip coated with chromium and gold, where DEP could be generated by applying an AC voltage between the silicon core and Cr/Au layer.¹⁸⁸ Microfluidic chips were used to trap single cells, followed by the AFM tip penetrating the PDMS membrane of the chip to isolate mRNAs from specific HeLa cells. RT-qPCR was then performed on

extractions and copy numbers of the same 3 mRNAs as their previous publication were deduced.

By being based on DEP, DENT and the nanotweezer (described below) differ from those previously discussed in that they do not require electrowetting or aspiration of cytoplasmic fluid to access a cell's contents. They therefore offer a more direct method to trap and isolate biomolecules without changing the volume of the cell. As with other techniques based on AFM and SICM, precise positioning of the DENT AFM probe was also possible.

1.5.4.3 Nanoscale tweezers

A huge advance in DEP-based subcellular sampling methods was the development of nanoscale tweezers, otherwise known as nanotweezers.¹⁸⁹ Nanotweezers are double-barrel nanopipettes formed by laser pulling quartz capillaries, with carbon pyrolytically deposited at the end of the nanopipette tip to form two carbon nanoelectrodes that are separated by a septum. The size of the whole tip of the nanotweezer was approximately 70 nm which could be inserted into living cells to trap biomolecules by dielectrophoresis upon application of an AC voltage. Using finite element analysis, electric field strengths of up to $10^{28} \text{ V}^2\text{m}^{-3}$ were generated at the electrode gap, significantly higher than that reported for DENT.¹⁸⁷ These high electric field strengths were due to the nanometre-sized gap between the electrodes being extremely small, between 10-20 nm, meaning that high electric field strengths could be generated without the use of high voltages. Additionally, it was shown that the trapping volume extends 300 nm from the nanotweezer tip, meaning that trapping could occur from very precise locations of a cell for high spatial resolution. DNA and mRNA were successfully isolated from the nucleus and cytoplasm of cells, respectively, with their detection confirmed via qPCR. A single mitochondrion was also isolated from the axon of a mouse hippocampal neuron, demonstrating that single organelle extraction was possible. No downstream analysis of the extracted mitochondrion was performed, however. In more recent work, the nanotweezer was applied to study the localisation of mRNAs encoding beta-1 adrenergic receptor ($\beta_1\text{AR}$) and beta-2 adrenergic receptor ($\beta_2\text{AR}$) in rat cardiomyocytes.¹⁹⁰ This work provided novel findings regarding the differential localisation behaviour of these mRNAs under healthy and failing cardiomyocytes and demonstrated the nanotweezer's ability to differentially detect mRNAs in precise locations of a single cell.

The nanotweezer has numerous advantages as a single-cell sampling technique, particularly due to its high electric field strengths to trap biomolecules in precise subcellular regions. The fabrication procedure is also simple and inexpensive, the nanobiopsy procedure only requires a micromanipulator and simple fluorescent imaging setup, cells can be maintained in their standard culture conditions, it achieves a high spatial resolution of sampling, and is biocompatible for its use in living systems. This makes it a highly promising tool for minimally invasive subcellular sampling from precise regions of living cells.

1.5.5 Outlook and limitations

The non-destructive nature of the state-of-the art subcellular sampling techniques described here advance from previous single-cell analysis techniques by minimising alterations to cells and maintaining cells in their standard microenvironments. The techniques differ in terms of their fabrication, throughput, size, spatial resolution, cost and physiological impact on living cells.

Nanostraws are only statically positioned inside single cells but have extremely high throughputs as individual cells can be cultured on top of multiple nanostraws, allowing for parallel sampling of the same cell over multiple timepoints. However, cells need to be grown on specific substrates rather than a standard culture dish or cover glass, and the manufacturing process can be time-consuming and expensive due to the need for etching and photolithography. Other techniques based on micro- and nanopipettes or AFM probes can be positioned to specific subcellular regions of interest and controlled by micro- or nanomanipulators, providing nanoscale resolution. However, these all lack throughput as only single serial extractions can be performed. Nanopipette-based systems are often integrated with SICM which allows for precise and controlled positioning of the nanopipette inside a cell; the feedback mechanism ensures that appropriate insertion into the cell is achieved. FluidFM also allows force-controlled sub-picolitre volumes to be removed from cells for highly controlled sampling. Nanopipette and FluidFM sampling, however, require the removal of cytoplasmic fluid from the cell, which raises questions regarding the viability of cells and the impact on cellular function after the extraction procedure. The nanotweezer is distinct in that it isolates polarisable molecules directly from specific regions of cells, negating the need to remove cytoplasmic fluid, which may otherwise trigger local changes or stress responses. The nanotweezer may therefore be less invasive than aspirating methods and allows for tighter

control over the location of the desired material in the cell, increasing the spatial resolution of extracted material from the cell. The nanotweezer biopsy procedure, however, is manual which means that the throughput is relatively low.

Since it is now known that seemingly similar cells in a population can still reveal high levels of heterogeneity, it is becoming more important to access the same single cell over time rather than obtaining multiple static measurements of different cells which would only represent the average behaviour of a population. This has begun with the development of Live-seq.¹⁵² Given that seemingly similar cells may respond differently to external stimuli, accessing the ground state of a particular cell and ideally a subcellular compartment of a cell, and tracking it over time would enable us to fully understand the impact of cellular responses. This has the potential to widen our knowledge of the true molecular mechanisms at the single-cell and subcellular level, and understand the heterogeneity of responses in seemingly similar cell populations. Thus far, neurons have been successfully sampled from using micropipettes,¹⁵⁰ nanopipette-based techniques¹⁴⁶ and the nanotweezer,¹⁸⁹ with the pipette-based technique performing multiple sampling events on the same cell.¹⁴⁶ Time-dependent studies on the same living neuron have not yet been performed, which would open opportunities for tracking single-cell and subcellular responses. With further development of these technologies past the proof-of-principle stage and applied to targeted biological questions, the full potential will be realised. This could have major contributions in deciphering precise subcellular mechanics, mechanisms of disease and ultimately the development of improved therapeutics.

1.6 Thesis objectives and outline

The capabilities of the nanotweezer for trapping and isolating biomolecules will be expanded to a delicate primary cell type to probe the true minimally invasive nature of the technology for subcellular sampling. The polarised structure of neurons, made up of distinct compartments such as the axons, dendrites and soma, alongside sub-compartments such as the dendritic spines and axon terminals, make them ideal candidates for studying compartmentalised omics. This work aims to achieve spatially and temporally resolved measurements of mRNA and mitochondria in living neurons using the nanotweezer technology. This would establish the technology as a comprehensive tool for isolating and mapping biomolecules in precise compartments of living neurons. It would also allow for true single-cell dynamics to be

uncovered and allow links to be made between the spatial location of specific biomolecules and (sub)cellular function.

In **Chapter 2**, the materials and methods employed in this thesis are described, including the cell culture conditions and treatments, the nanotweezer fabrication and nanobiopsy procedures, and the analysis techniques implemented.

The initial characterisation of the nanotweezer and its ability to trap polarisable molecules in solution is outlined in **Chapter 3**. A particular focus on trapping RNA is presented, where the DEP trapping parameters are optimised in conductivity conditions similar to those of cells.

Following the validation of trapping RNA, these conditions are taken forward for trapping and isolating RNA from live hippocampal neurons in **Chapter 4**. Isolated RNAs are successfully detected using RT-qPCR and cells are shown to maintain viability following multiple biopsies from the same neuron. The method is then applied to study mRNA localisation in compartments of the same cell, where differential mRNA abundance between the soma and dendrites of the same neurons is quantified and compared to neurons at different levels of maturity. Finally, spatial and temporal measurements are combined to demonstrate that the response of a cell and its mRNA localisation mechanics can be tracked at precise locations and compared to its ground state.

In **Chapter 5**, the work is developed to isolate single mitochondrion from living neurons. Single organelle trapping and isolation are built upon, and the viability of mitochondria is validated based on the maintenance of an active membrane potential. Methods are developed to analyse mtDNA from individually isolated mitochondria, demonstrating the foundations for single mitochondrion copy number analysis and mtDNA sequencing.

Finally, in **Chapter 6**, a method for the novel detection of mtRNAs from individual mitochondria is developed. This is then applied to a temporal study of mitochondrial gene expression in the same living cortical neurons upon application of aggregated α -synuclein over a 24 h period, highlighting the potential for single-cell gene expression tracking in living cells.

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Chapter 2

Experimental setup and methodology

Synopsis

In this chapter, the experimental setups and methodologies for the thesis are discussed in detail. The chapter begins with the overall cell culture conditions, treatments, and labelling. This is followed by the experimental procedures required for bulk isolation work and for imaging fixed cells to optimise and validate the single-cell experiments. Nanotweezer fabrication and characterisation are then outlined, followed by the procedures used to perform and analyse single nanobiopsies.

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2.1 Cell Culture

2.1.1 Rat Hippocampal and cortical neurons

E18 rat hippocampal and cortical neuron isolations were performed by Dr Suzanne Claxton at the Francis Crick Institute, and Dr Flavie Lesept and Dr Jack Howden at University College London. Attachment medium was made up of Minimum Essential Medium (Gibco), 10% horse serum (Gibco), 1 mM pyruvic acid (Sigma-Aldrich) and 0.6% glucose solution (Sigma-Aldrich). Maintenance medium was made up of Neurobasal (Gibco), 2% B27 (Gibco), 1% Glutamax (Gibco), 0.6% glucose (Sigma-Aldrich) and 1% Penicillin-Streptomycin (Gibco). For time-dependent single-cell experiments, maintenance medium was made up of Neurobasal Plus (Gibco), 2% B27 Plus (Gibco), 1% Glutamax (Gibco), 0.6% glucose (Sigma-Aldrich) and 1% Penicillin-Streptomycin (Gibco).

35 mm glass bottom dishes (Greiner Bio-One), 35 mm gridded glass bottom dishes (Ibidi) or 13 mm coverslips (VWR) were coated overnight with poly-L-lysine hydrobromide (Sigma-Aldrich, molecular weight 30,000-70,000) at a concentration of 2.5 mg/mL in sterile water. Cells were diluted in attachment medium and attached overnight (37 °C, 5% CO₂) on coated glass dishes or coverslips at a density of 25,000 cells/cm². Attachment medium was then replaced by maintenance medium for cells to grow (37 °C, 5% CO₂). Half media changes were made every 3-4 days.

2.1.2 Transfections and labelling

Cells were transfected with 1 µl Lipofectamine 2000 (Invitrogen) and 0.25 µg mtDsRed DNA (obtained from Prof. Josef Kittler) in 50 µl plain Neurobasal to label mitochondria in cells. Cell maintenance medium was replaced by 300 µl pre-warmed Neurobasal with 0.6% glucose. The lipofection mixture was incubated for 25 min at room temperature before being added to cells for 3 h (37 °C, 5% CO₂). The lipofection mixture was replaced by the previous pre-warmed conditioned medium. Cells were imaged 48 h after transfection.

Cells were transfected with 2 µl Lipofectamine 2000 and 1 µg meGFP-C1 DNA (obtained from Dr Mike Devine) in 250 µl plain Neurobasal for cell-fill labelling. Cell maintenance medium was replaced by 1 mL pre-warmed Neurobasal with 0.6% glucose. The lipofection mixture was incubated for 20 min at room temperature before being added to cells for 1.5 h (37 °C, 5%

CO₂). The lipofection mixture was replaced by the previous pre-warmed conditioned medium. Cells were imaged 48 h after transfection. For calcium imaging, the same lipofection protocol was followed using GCaMP8s DNA (obtained from Dr Mike Devine).

Cells were loaded with NucSpot Live 488 (Biotum) according to the manufacturer's protocol for nuclear labelling. To visualise the extraction of RNA from cells, SYTO RNASelect dye (Invitrogen) was added to the cell medium at a concentration of 500 nM. Cells were incubated with the labelling solution for 20 min (37 °C, 5% CO₂) before washing with PBS (Gibco) and replacing the labelling solution with maintenance medium. Cells were incubated for 5 min before imaging.

2.1.3 Viability

The viability of cells was determined using Calcein AM cell-permeable dye (Invitrogen). Cells were incubated with 2 µM dye diluted in cell maintenance medium for 20 min at 37 °C. Cells were then washed with warm PBS and replaced with conditioned maintenance medium and incubated for 5 min before imaging.

For mitochondrial membrane potential detection, neurons were incubated with 20 nM tetramethylrhodamine methyl ester (TMRM, Invitrogen) diluted in cell maintenance medium for 30 min at 37 °C. Cells were washed with PBS and replaced with maintenance medium.

2.1.4 Treatments

For calcium imaging controls, 90 mM KCl diluted in cell maintenance media was slowly added to cells maintained in 1 mL media to make a final KCl concentration of 30 mM for neuron stimulation. Images were acquired before, during and after addition of KCl.

Glycine and N-methyl-D-aspartic acid (NMDA) were applied to DIV 14 hippocampal neurons to induce chemical long-term potentiation (cLTP). 50 µM NMDA and 10 µM glycine were applied to cells in Mg²⁺-free buffer (126 mM NaCl, 2 mM CaCl₂, 2.5 mM KCl, 5 mM HEPES, 11.1 mM glucose, 1 µM strychnine (Sigma-Aldrich), 20 µM bicuculline (Sigma-Aldrich), 0.5 µM tetrodotoxin (Abcam), pH 7.4) for 3 min, followed by replacement of this with conditioned media.

2.2 Total RNA Isolation

Total RNA was isolated from 100,000 rat cortical or hippocampal neurons using RNeasy Plus Mini kit (Qiagen) according to the manufacturer's protocol. RNA was eluted in nuclease-free water and the concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). RNA was either used immediately or stored at -80 °C before the downstream application.

2.3 Bulk Mitochondrial Analyte Isolation

2.3.1 Isolation of mitochondria

Bulk mitochondria were isolated from up to 1 million rat cortical or hippocampal neurons using the Mitochondrial Isolation Kit for Cultured Cells (Thermo Scientific) according to the manufacturer's reagent-based method. Isolated mitochondria were either lysed immediately or stored in 500 µl 'Reagent C' from the kit at -80 °C.

2.3.2 Mitochondrial chemical lysis

The mitochondrial pellet isolated from cells was resuspended in 100 µl lysis buffer (10 mM Tris [pH 7.4], 10 mM EDTA, 1% SDS) and heated at 70 °C for 5 min. The lysis solution was cooled to room temperature, and 1 µg Proteinase K (Invitrogen) was added and heated at 37 °C for 5 min to complete the lysis. The lysates were diluted with 100 µl nuclease-free water and the Proteinase K was then inactivated by heating at 95 °C for 10 min. Mitochondrial lysates were stored at -80 °C before the downstream application.

2.3.3 Mitochondrial sonication lysis

The bulk mitochondrial pellet was resuspended in 500 µl Reagent C from the Mitochondrial Isolation Kit for Cultured Cells (Thermo Scientific) and diluted 100x in nuclease-free water. 10 µl of this was stained with MitoTracker Green FM (Invitrogen) at a concentration of 100 nM and imaged to confirm the isolation of intact mitochondria. The diluted mitochondrial pellet was then further diluted 10x and 2 µl of this was used for sonication lysis in a total volume of 9 µl. The diluted mitochondria were sonicated (Vibra-cell VCX 750, 400 W, 25% amplitude) using 2 s pulses for 2 min under various conditions: 1) Sonication followed by

heating at 95 °C for 10 min, 2) Sonication with 0.05 µg Proteinase K (Invitrogen) followed by heating at 95 °C for 10 min, 3) Proteinase K treatment followed by heating at 95 °C for 10 min.

2.3.4 DNase treatment

Mitochondrial DNA removal from bulk mitochondrial lysate was attempted using DNase I treatment (Thermo Scientific) according to the manufacturer's protocol unless otherwise stated.

2.4 α -Synuclein oligomer preparation and characterisation

2.4.1 Preparation and aggregation

N-acetylated α -synuclein (α -syn) monomers were expressed by Dr Xiangyu Teng from Imperial College London using recombinant expression in *Escherichia coli*. The concentration was estimated from the absorbance at 275 nm by using a molar extinction coefficient of 5600 M⁻¹·cm⁻¹. The aggregation conditions were based on an established protocol reported previously.¹⁻⁸ Briefly, 800 µl of α -syn monomers (70 µM, 25 mM Tris, 100 mM NaCl, pH 7.4) were ultracentrifuged for 1 h at 90,000 rpm and 4 °C (Sorvall MTX 150 Micro-Ultracentrifuge, Thermo Scientific) to remove insoluble aggregates. The top 600 µl of the supernatant was collected to collect the monomers, while the remaining volume was discarded. 400 µl of this monomer sample was mixed with 0.01% NaN₃ to prevent bacterial growth during aggregation. The mixture was then used for aggregation by shaking at a constant speed of 200 rpm and incubating at a temperature of 37 °C (Digital Heating Shaker Drybath, Thermo Fisher). During initial optimisation experiments, 50 µl aliquots were taken at different time points (8 h and 24 h). In later experiments, the aggregation was stopped at 24 h. Aggregated samples were stored at 4 °C before use and the sample were used within 2 days.

2.4.2 Native PAGE

Aggregated α -syn samples were characterised by a native PAGE using native sample buffer (Thermo Fisher Scientific) and running on a Novex Tris-glycine gel (Thermo Fisher Scientific) in native running buffer (Thermo Fisher Scientific) for 70 min at 120 V. Native PAGE was run with Sian Allerton from Imperial College London.

2.4.3 Atomic force microscopy (AFM)

α -syn samples were characterised by AFM as reported previously.^{1,9} Briefly, α -syn samples were placed on mica sheets and dried with a nitrogen gun. AFM imaging was performed using an Asylum MFP-3D microscope in tapping mode. Nanosensors PPP-FMR tips (res $\sim$ 75 kHz, nominal tip radius: 7 nm, nominal spring constant: 2 N/m) were used and tuned to a target tapping amplitude of 1 V. Scans were sized 90 μm^2 , 256-512 points per line, with a scan rate of 0.5 Hz. AFM was performed by Dr. Victoria L. Bemmer from Imperial College London. AFM images were analysed by Dr Giulia Campolo and Dr Yaxian Liu from Imperial College London using Gwyddion software.

2.5 Single-molecule fluorescent in situ hybridisation (smFISH)

smFISH was used to image RNA target molecules in fixed cells. RNAscope® Multiplex Fluorescent Reagent Kit v2 (Bio-Techne) was used according to the manufacturer's protocol for cultured adherent cells. Briefly, cells were fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature and stored in 1 x PBS. If the RNAscope protocol was to be performed on a different day to fixation, instead of storing in PBS, cells were dehydrated via subsequent incubations with 50%, 70% and 100% ethanol for 5 min, and stored in 100% ethanol at -20 °C. Cells were then rehydrated before the RNAscope protocol via subsequent incubations in 70% and 50% ethanol followed by 1 x PBS for 5 min.

Cells were washed 3 times in 1 X PBS and incubated with hydrogen peroxide for 10 min at room temperature. Cells were permeabilised for 10 min at room temperature using a 1:15 dilution of protease III (Bio-Techne). Protease was washed off using 1 X PBS, and cells were incubated with hybridisation probes (18S (Rn-18S), MAP1A (Rn-MAP1A-C3), CAMK2A (Rn-CAMK2A-C2)) or positive and negative control probes for 2 hours at 40 °C in the HybEZ™ II Hybridization System (Bio-Techne). After probe hybridisation, samples were washed 2 times in 1 X RNAscope buffer for 2 min and were stored overnight in 5 X sodium chloride and sodium citrate solution buffer. The next day, the SSC buffer was washed twice using 1 X RNAscope buffer for 2 min. The signal was then amplified using the manufacturer's amplification reagents, followed by conjugating the fluorophores (Opal 520 and Opal 690, Akoya Biosciences) to develop the signal, and then blocking the channels with HRP blocker (Bio-technne). For samples that were not incubated with the 18S probe, cells were stained with Wheat Germ Agglutinin (WGA) conjugated to Alexa Fluor 647 (Invitrogen) to stain the whole

neuronal structure. After all targets were amplified and conjugated with fluorophore, the samples were counterstained with DAPI (Bio-Techne) and mounted onto EpreDia™ SuperFrost Plus™ Adhesion slides (ThermoFisher Scientific) using ProLong™ Gold Antifade Mountant (ThermoFisher Scientific, P10144). smFISH was performed with Dr Zoe Kwan from Imperial College London.

smFISH imaging was performed on either a Leica Stellaris 8 confocal microscope by Dr Zoe Kwan, a Zeiss LSM-780 confocal microscope or a Zeiss Axio Observer widefield microscope. Deconvolution was performed on the Leica Stellaris 8 microscope's built-in Stellaris Lightening platform. For widefield images, deconvolution was performed after imaging using Huygens Essential software.

2.6 Immunofluorescence staining

Immunofluorescent staining was used to assess dendritic lengths and morphological changes in developing neurons, and the uptake of α -synuclein into cells after washing twice with PBS to remove any α -synuclein that had not been taken up by cells. Cells were fixed using 4% PFA for 10 min. If staining was performed at a later date, fixed cells were incubated with 1 x PBS at 4 °C. Fixed cells were washed three times in 1 x PBS and permeabilised for 15 min at room temperature with 0.1% Triton-X-100 (Thermo Scientific) in PBS. Cells were washed 3 times in PBS and then blocked for 1 h at room temperature in 5% Bovine Serum Albumin (BSA, Thermo Scientific) with 5% goat serum (Thermo Scientific) in PBS. MAP2 anti-guinea pig primary antibody (118 004, Synaptic Systems) was diluted 1:500 in blocking buffer and/or α -synuclein anti-mouse primary antibody (LB509, Invitrogen) was diluted 1:100 in blocking buffer and added to the samples overnight at 4 °C. The next day, samples were washed 3 times in PBS and 1:1000 Alexa Fluor 647 goat anti-guinea pig (#A-21450, Invitrogen) and/or Alexa Fluor 488 goat anti-mouse (#A-11001, Invitrogen) secondary antibodies in blocking buffer were added to the samples for 2-3 h. Cells were washed 3 times in PBS and then mounted using ProLong™ Gold Antifade Mountant with DNA stain DAPI (Invitrogen). Imaging was performed on a Zeiss Axio Observer widefield microscope.

2.7 Nanotweezer fabrication and characterisation

2.7.1 Nanopipette fabrication

Nanopipettes were fabricated from double-barrel quartz capillaries (Friedrich & Dimmock, WAR-QT120-90-100) which comprise two semi-circular channels separated by a septum of 0.1 mm. The outer diameter of the capillaries was 1.2 mm and the inner diameter was 0.9 mm. The length of each capillary was 100 mm. The capillaries were plasma cleaned (Harrick Plasma, PDC-001, 150 V) for 25 min before nanopipette fabrication to remove contaminants and dust.

Cleaned capillaries were loaded into a laser puller (Sutter Instrument, P-2000) by aligning the septum horizontally and parallel to the laser source to ensure consistent laser irradiation. The capillaries were then pulled to form a pair of double-barrelled nanopipettes according to the two-step protocol outlined in **Table 2.1**. The protocol in **Table 2.2** was tested initially but not taken forward for use in single-cell biopsies. This process consisted of heating the capillaries to form a taper, followed by heating the capillary further with pulling to form two nanopipettes (**Figure 2.1**), as outlined in numerous previous publications.^{10–13} The pulling is based on 5 parameters: heat, filament, velocity, delay and pull. These parameters are defined as follows:¹⁴

1. Heat – An arbitrary scale for the output of laser power and, therefore, the amount of energy supplied to the capillary. Typical values for quartz range between 700 and 900.
2. Filament – The scanning pattern of the laser beam that is used to supply ‘Heat’ to the capillary. The values represent the longitudinal length and the rate of the scan.
3. Velocity – The velocity at which the puller bar must be moving before the hard ‘Pull’ is executed. This is determined by the viscosity of the glass which is a function of the glass temperature. This allows for a precise glass temperature to be selected as the trigger for the hard ‘Pull’. Typically, lower values are for nanopipettes and higher values for micropipettes.
4. Delay – The timing of the start of the hard ‘Pull’ relative to the deactivation of the laser when ‘Heat’ is turned off. The higher the ‘Delay’, the cooler the glass will be when pulled, leading to lower pipette tapers and higher pore diameters. When the value is <128, the ‘Pull’ is initiated before the laser inactivation. When the value is >128, the ‘Pull’ is executed after laser deactivation.

5. Pull – The force of the hard pull exerted onto the capillary. The higher the value, the smaller the pipette tips. 1 ‘Pull’ unit represents a change of 4 mA of current to pull the solenoid.

It should be noted that the pulling parameters are instrument-specific and are susceptible to temperature and humidity changes.¹⁵ They should therefore be optimised for each puller and pore size requirement and reprogrammed where necessary if used over long time periods.

Table 2.1: Parameters for pulling double-barrel quartz capillaries to form nanopipettes.

	Heat	Filament	Velocity	Delay	Pull
Line 1	850	4	30	160	100
Line 2	875	3	20	120	165

Table 2.2: Initial parameters for pulling double-barrel quartz capillaries to form nanopipettes.

	Heating	Filament	Velocity	Delay	Pulling
Line 1	850	4	30	160	100
Line 2	860	3	20	140	160

2.7.2 Carbon electrode fabrication

The nanotweezers were formed by depositing carbon on the tips of the nanopipettes to form two carbon nanoelectrodes.¹³ This was achieved by pyrolytic carbon deposition, utilising the setup outlined in **Figure 2.2**: 1) Nanopipettes were mounted onto a holder, connecting one end to a butane source (Campingaz) via rubber tubing (0.15 L/min flow rate); 2) The tip of the nanopipette was inserted approximately 2 mm into a larger single-barrel capillary, of which was connected to an argon cylinder via rubber tubing; 3) The tip of the nanopipette was flushed with argon momentarily by turning up the regulator to a high argon flow (0.6 L/min), followed by turning it back down (0.1 L/min); 4) A gas blow torch (RS Components) was then used to heat the tip of the nanopipette through the single barrel capillary for 5 s, allowing for pyrolytic deposition of carbon inside the two channels of the nanopipette; 5) The heat source was then slowly moved up approximately half of the nanopipette to further fill the pipette and ensure electrical contact when inserting copper wires into the two channels. The flow of both the butane and argon was regulated by gas flow meters. It should be noted that the amount of

carbon deposited depends on the argon and butane flow rate, and the time spent heating the tip. This should be optimised for each user and experimental setup.

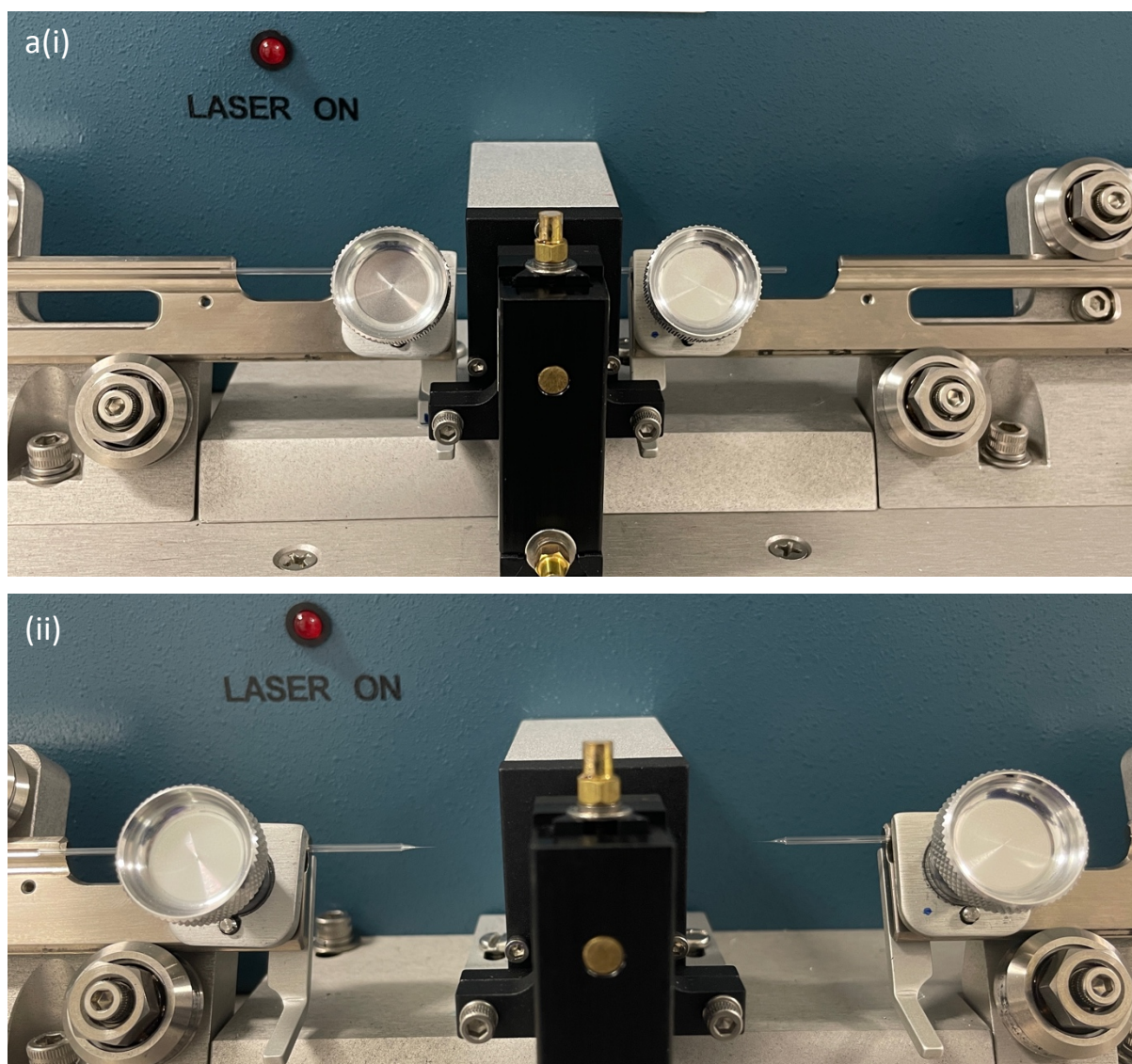


Figure 2.1: Laser pulling double-barrel quartz capillaries to form nanopipettes. a) Capillaries were positioned in the laser puller (i) and the relevant laser pulling protocol was performed to form two nanopipettes (ii).

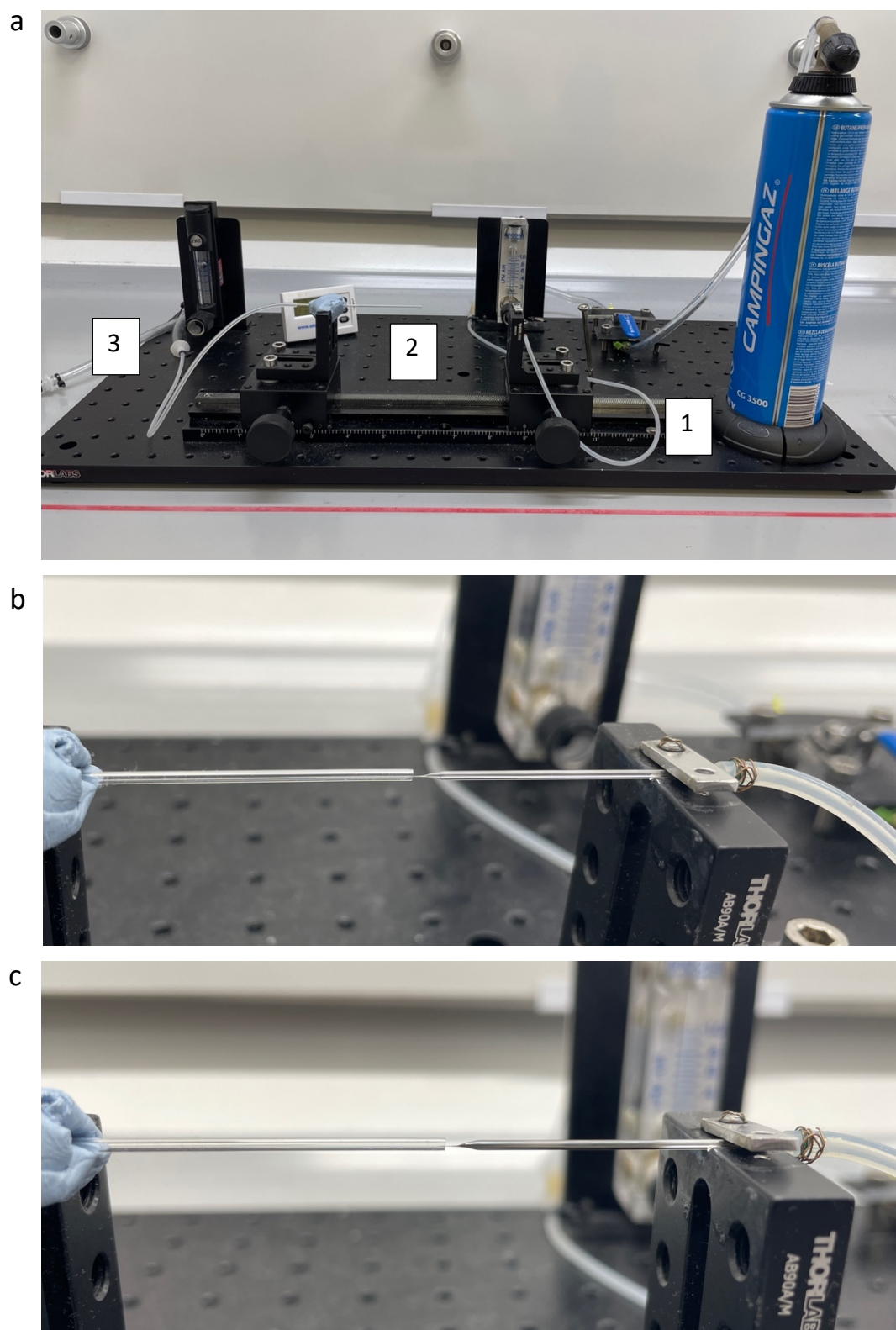


Figure 2.2: Experimental setup for pyrolytic deposition of carbon on nanopipettes. a) Nanopipettes were connected to the butane source (1) and then inserted approximately 1 mm into a larger quartz capillary (2), the nanopipette was flushed with argon followed by lowering the argon pressure (3), and the nanopipette tip was heated for 10 s followed by slowly heating half of the rest of the nanopipette. b) Nanopipette before carbon deposition. c) Nanopipette after carbon deposition.

2.7.3 Linear sweep voltammetry (LSV)

Linear sweep voltammetry (LSV) was performed using a potentiostat (CHI 760 C, CH Instruments). The size, symmetry and quality of the electrodes were estimated using LSV.

Ag/AgCl electrodes were fabricated by electro-oxidation of silver in a highly concentrated chloride solution, as shown by **Equations 2.1-2.2**.



Approximately 2 cm of 0.125 mm diameter silver wire (Goodfellow Cambridge) was immersed in 1 M KCl solution and a 2-V bias was applied over 2 min. The formation of AgCl was observed via bubble formation and darkening of silver at the working electrode. Ag/AgCl electrodes were prepared fresh each day before measurements.

The electrodes were characterised using Ru(NH₃)₆Cl₃ in LSV to confirm that the nanopipette was filled with carbon. Silver wire was inserted into one channel of the nanotweezer and connected to the working electrode. Ag/AgCl was used as the reference electrode during voltammetry measurements. A negative bias was applied to the electrodes, LSVs were run at a scan rate of 50 V/s, and the potential was swept from +0.2 V to -0.5 V to reduce Ru(III) to Ru(II). The process was then repeated for the other channel of the same nanotweezer.

The electrode size was approximated using **Equation 2.3**:

$$r = \frac{i_{ss}}{4.64nFCD} \quad \text{Equation 2.3}$$

where r is the radius of the electrode area, i_{ss} is the steady-state reduction current, n is the number of electrons transferred during the reduction ($n = 1$ in this case), F is Faraday's constant, D is the diffusion coefficient for Ru(NH₃)₆Cl₃ in 100 mM KCl ($8.5 \times 10^{-10} \text{ cm}^2/\text{s}$)¹⁶ and C is the concentration of the redox species (10 mM). As the electrode shape of the nanotweezer was semi-circular, and this equation assumes a circular shape, this calculation was only used as an approximation.

2.7.4 Scanning electron microscopy (SEM)

The size of the nanotweezer's tip, electrodes, and septum was confirmed by scanning electron microscopy (SEM). SEM images were acquired on a LEO Gemini 1525 (Carl Zeiss AG) high resolution-field emission gun scanning electron microscope. Samples were sputter coated with 10 nm chromium (Quorum Technologies, Q150 Sputter Coater) to reduce charging and improve imaging quality.

2.8 Dielectrophoretic trapping setup

2.8.1 Fluorescent labelling

0.1 μm FluoSpheres™ Carboxylate-Modified Microspheres (Yellow-Green, Invitrogen) were suspended in distilled water or 100 mM KCl solution to a final concentration of 0.002% for nanotweezer trapping.

YOYO-1 (Invitrogen) was diluted in 10 mM Tris and 1 mM EDTA to a concentration of 100 μM . Lambda DNA (New England Biolabs) was incubated with 100 μM YOYO-1 at a ratio of 1 YOYO-1 molecule per 7.5 base pairs of DNA to label the DNA. YOYO-1-labelled lambda DNA was further diluted to a DNA concentration of 100 pM for nanotweezer trapping.

Total RNA extraction and labelling for characterisation experiments in **Chapter 3** were performed by Dr Sung Pil Hong (Imperial College London). Total RNA was isolated and extracted from MCF-7 cells (provided by the Magnani group, Imperial College London) using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA labelling was achieved using ULYSIS Alexa Fluor® 488 Nucleic Acid Labelling kit (Thermo Scientific) according to the manufacturer's protocol.

2.8.2 Fluorescent and bright field imaging

Fluorescent and bright field imaging and videos for single-fluorophore samples were acquired using an inverted optical microscope (IX71, Olympus) with a 60X water-immersion objective (1.20 numerical aperture, UPLSAPO 60XW, UIS2, Olympus). Early characterisation and initial cell experiments were illuminated with a fibre-coupled 488 nm tunable argon ion laser (Model 35-LAP-431-230, Melles Griot) coupled to the microscope via a TIRF module (cellTIRF, Olympus) with single-mode optical fibre, and images were acquired with an

electron-multiplying charge-coupled device (emCCD) camera (Cascade II, Photometrics). Later experiments were illuminated with either a fibre-coupled 488 nm diode-pumped solid-state laser (Sapphire 488-50 CDRH, Coherent) or a fibre-coupled 561 nm diode-pumped solid-state laser (MGL-FN-561-100mW, Changchun New Industries Optoelectronics Tech.) coupled to the microscope via a TIRF module (cellTIRF, Olympus) with single-mode optical fibre, and images were acquired with a scientific complementary metal-oxide-semiconductor (sCMOS) camera (Marana 4.2B-11, Andor). In all cases, the TIRF module was used for illumination and imaging was performed in epifluorescence mode. **Figure 2.3** illustrates the general imaging setup. A dichroic mirror reflected the laser light towards the objective and into the sample. The emitted fluorescence was collected through the objective and transmitted through the dichroic onto the camera.

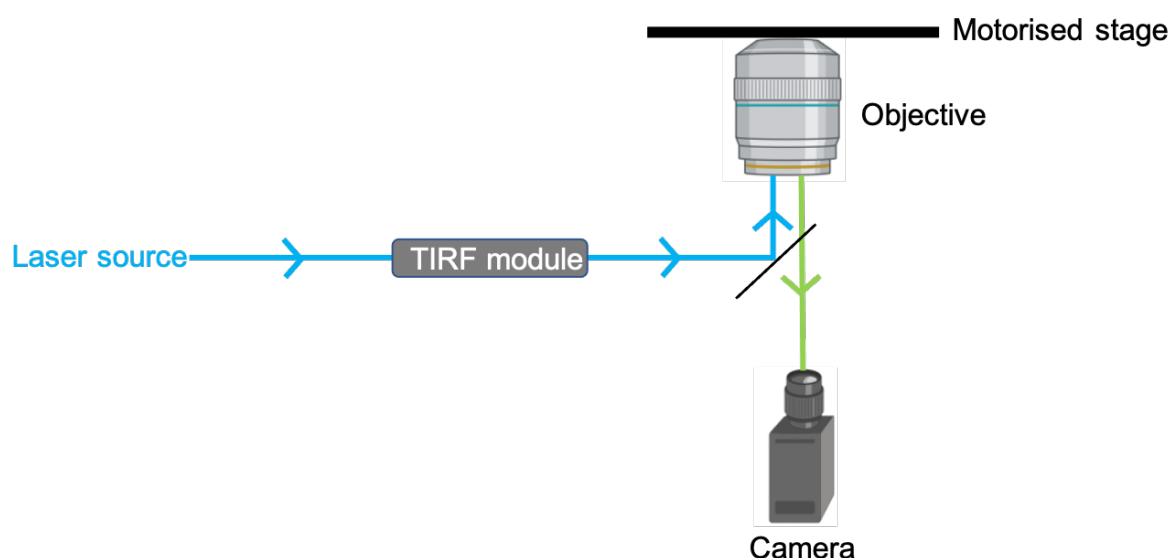


Figure 2.3: Fluorescent imaging setup. Schematic of the fluorescence imaging setup consisting of a fibre-coupled laser connected to a TIRF module and aligned to the back aperture of a 60x objective via a dichroic mirror. The emitted fluorescence was transmitted back through the objective and dichroic mirror and subsequently detected by either an emCCD or sCMOS camera. Figure created using Biorender.com.

2.8.3 Nanobiopsy

Using the optical setup above, the nanotweezer was mounted on a motorised stage using a micromanipulator (PatchStar, Scientifica) perpendicular to a glass cover glass (VWR) or cell culture dish on the fluorescent imaging setup. Electrical contact to each electrode was achieved by inserting copper wires into the top of each barrel through the pipette, to make contact with the carbon. The other ends of the copper wires were connected to a function generator

(TG2000, TTI, UK) which was used to apply DEP to the nanotweezer. The whole setup is shown in **Figure 2.4**.

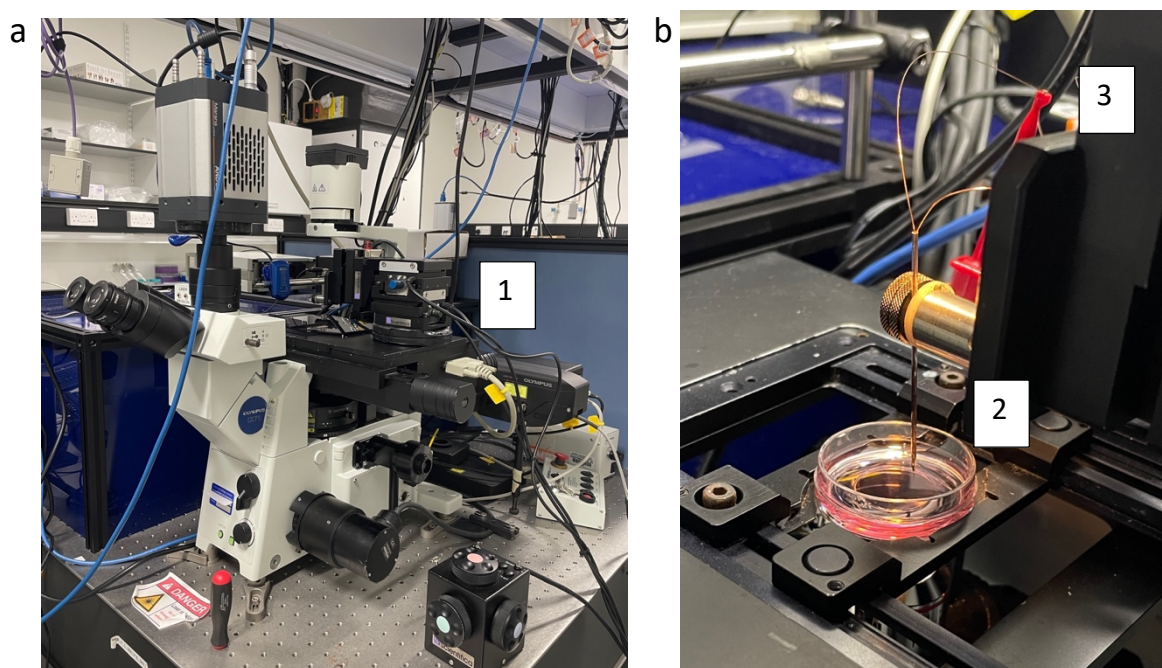


Figure 2.4: Nanobiopsy setup. a) Image showing the optical setup for the nanobiopsy with the micromanipulator mounted onto the stage (1). b) Close-up image of the stage, with the nanotweezer mounted onto the micromanipulator and placed into the cell culture dish (2), and copper wires connected to each barrel of the nanotweezer and connected via hook clips to a function generator (3).

The nanotweezer was vertically positioned towards the dish or coverslip in the absence of DEP or applied voltage using the x and y directions. The nanotweezer was approached towards the cell of interest or spotted solution using the micromanipulator in the z direction and brought close to the surface of the cell or coverslip using 5X and 10X objectives. Using a 60X water objective and epifluorescence mode to illuminate the sample, the nanotweezer was positioned above the region of interest within the cell and gently lowered into the cell to pierce the cell membrane. Under bright field or fluorescence illumination, DEP was applied by applying an AC voltage (1 MHz, 15 V_{pp} for cells) between the electrodes using the function generator and allowing polarisable molecules to get trapped at the nanotweezer tip. This was performed either label-free or fluorescently labelled to visualise increased fluorescence intensity at the tip. The nanotweezer was then retracted from the cell whilst maintaining the DEP force and the trapped material was transferred to a PCR tube by pressing the tip into the tube containing either 5 µl nuclease-free water (RNA biopsy) or 4 µl mitochondrial lysis buffer (mitochondrion biopsy). Trapped material was either used immediately for analysis or stored at -80 °C.

2.9 Single mitochondrion sample preparation

2.9.1 Lysis

Single mitochondria were lysed based on an adapted protocol from Morris *et al.*¹⁷ Single mitochondrion trapped at the nanotweezer were directly placed in PCR tubes containing 4 μ l mitochondrial lysis buffer (100 mM Tris-HCl [pH 8], 150 mM NaCl, 20 mM EDTA, 0.2% SDS). 0.05 mg Proteinase K was added to the buffer and the sample was lysed at 55 °C for 30 min followed by either inactivating Proteinase K (Thermo Scientific) at 95 °C for 10 min or directly using the inactivated sample for purification.

2.9.2 Magnetic bead purification

For mtDNA analysis, single mitochondrion lysates were purified using AMPure XP Bead-based reagent (Beckman Coulter) with a modified protocol. The reagent was vortexed before use to ensure homogenous distribution of beads. 7 μ l beads were added to 5 μ l mitochondrial lysate (1.4x) in a 0.5 mL tube, mixed by gentle pipetting 10 times and then incubated for 5 min at room temperature to allow for the DNA to bind to the beads. The tube was placed on a magnetic rack (#S1509S, NEB) for at least 2 minutes to allow for the beads to be separated from contaminants and lysis buffer components. The supernatant was removed, leaving approximately 2 μ l in the tube. Whilst still bound to the magnet, the beads were washed twice with 50 μ l fresh 70% ethanol in nuclease-free water. Full removal of the supernatant was performed with each wash step to allow for further removal of contaminants and lysis buffer components. Immediately after the last wash step, to minimise drying of the beads, 5-10 μ l nuclease-free water was added to the beads and mixed thoroughly away from the magnetic rack. Beads were incubated for 2 min to elute DNA from the beads. The tube was then placed back on the magnetic rack for 2 min, ensuring the solution was fully transparent, and the full eluate was transferred to a PCR tube.

For mtRNA analysis, surface-functionalised magnetic beads were generated to allow for specific binding to rat mtRNAs. Dynabeads™ MyOne™ Carboxylic Acid (#65011, Invitrogen) 1 μ m magnetic beads were functionalised with oligonucleotides complementary to mtRNAs via a carbodiimide coupling reaction. 2 types of primary amines were designed: one with a repeated sequence of 25 thymine nucleotides to bind to RNAs that are polyadenylated with a poly(A) tail; and a second with a sequence complementary to 12S mitochondrially-

encoded rRNA, an RNA which does not have a poly(A) tail. The sequences of these primary amines are summarised in **Table 2.3** and were purchased from Integrated DNA Technologies. Both sequences contained a C₆ spacer between the amine group and the target complementary sequence to minimise steric hindrance when binding to the target.

Table 2.3: Designed primary amines for EDC coupling with carboxylic acid-surface modified magnetic beads.

Target	Sequence (5'->3')
Polyadenylated mitochondrial RNAs	H ₂ N-C ₆ -PO ₄ ⁻ -oligo(T) ₂₅
12S mitochondrially-encoded rRNA	H ₂ N-C ₆ -PO ₄ ⁻ - oligo(TAATAAGGTTCGTGTGAAAGGTCAT)

The manufacturer's protocol for binding oligonucleotides was followed with the protocol scaled down to generate small volumes of 2 types of magnetic beads with the target sequences in **Table 2.3**. The reactions for these are summarised in **Figure 2.5**. Magnetic beads were vortexed to ensure homogenous mixing of the beads in solution. 100 µl beads (10 mg/mL) were placed on a magnetic rack for 2 min and the supernatant was removed. Beads were washed twice with 100 µl 2-(N-morpholino)ethanesulfonic acid buffer (MES, low moisture content, 100 mM, pH 4.8, Sigma-Aldrich), mixed by pipetting and incubated for 10 min each time. Beads were resuspended in 10 µl MES buffer. Immediately before use, 125 µl 1.25 M 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma-Aldrich) in 100 mM MES buffer was prepared (EDC-MES). 4 µl EDC-MES was added to 6 µl 830 µM primary amine with target sequence to give a final amine amount of 5 nmol. This 10 µl solution was then added to the 10 µl magnetic beads and vortexed for 10 s to activate the carboxylic acid. This was incubated on a roller mixer overnight to couple the amine group to the beads and form an amide bond with the beads. The next day, beads were washed 3 times with 100 µl TT buffer (0.25 M Tris pH 8 and 0.01% Tween-20), incubating on a rolling mixer for 30 min each time. Beads were then resuspended in 1xPBS with 0.1% Tween-20 to give a final concentration of 5 mg/mL. Surface-modified magnetic beads were stored at 4 °C prior to use. They maintain their binding capacity until the expiry date of the unmodified beads; however, fresh surface-modified beads were prepared every 6 months to ensure optimal stability.

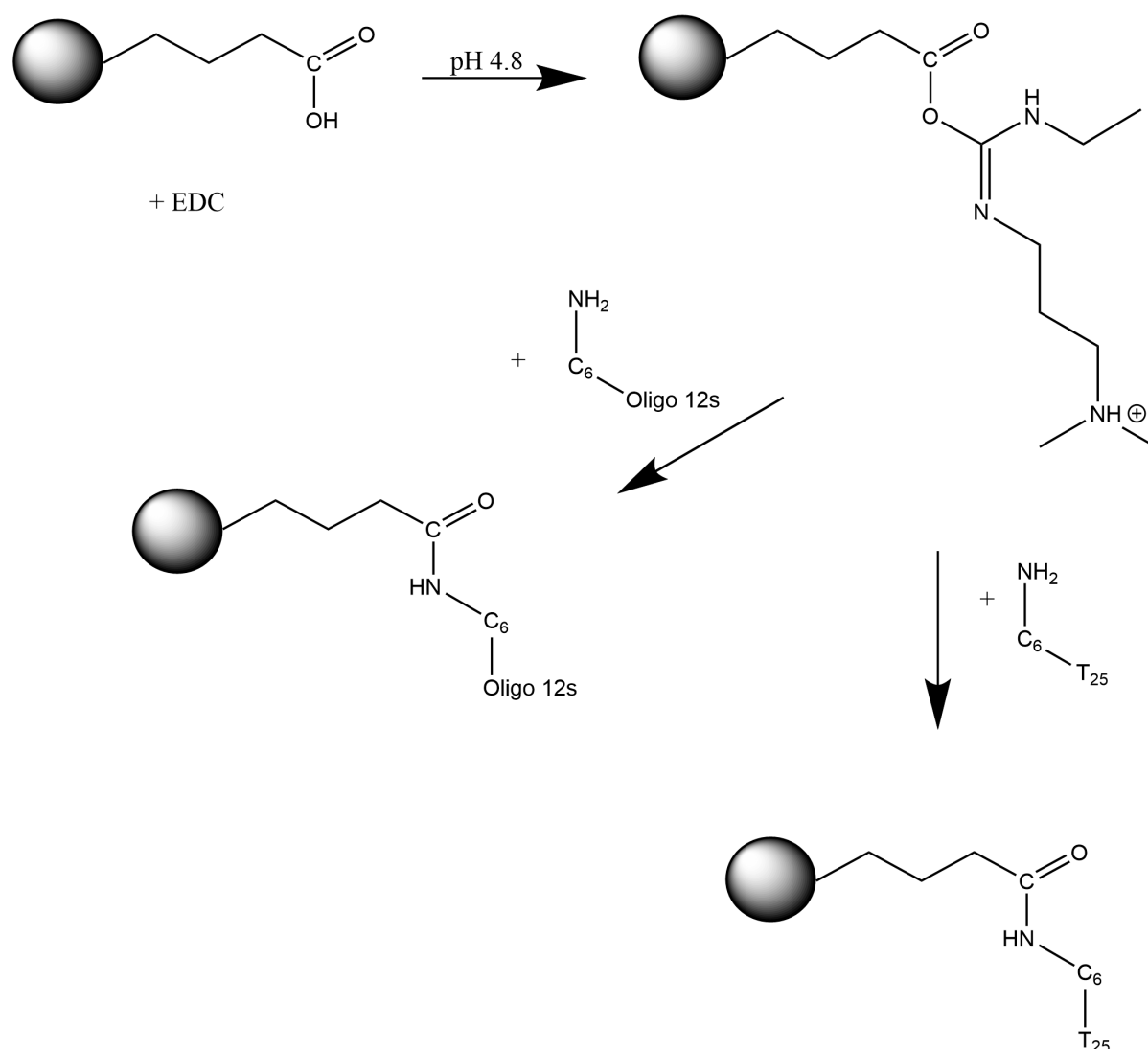


Figure 2.5: Workflow for surface-functionalisation of magnetic beads. Magnetic beads containing carboxylic acid groups on the surface become activated by the carbodiimide EDC to form an unstable intermediate which is readily displaced by a primary amine via nucleophilic attack to form an amide bond. The crosslinking reactions occur under acidic conditions. This schematic shows a single carboxylic acid group reaction, however, in practice, multiple carboxylic acid groups are present on the surface of the beads during the reaction.

2.10 Primers

Primers for PCR, qPCR and the generation of qPCR standards were either obtained commercially (Integrated DNA Technologies) or designed using Primer3 (<https://primer3.ut.ee>) and Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) online software. For mRNA, primers were designed to span exon-exon junctions where possible to limit amplification of DNA.

Table 2.4: Primer sequences used in this thesis. Primers were designed manually unless otherwise stated. Predesigned primers were obtained commercially from Integrated DNA Technologies. All primers were for the rat genome unless otherwise stated.

Target	Forward primer	Reverse primer	Amplicon size (bp)	Chapter
ACTB, human	CACCAACTGGGACGACAT	ACAGCCTGGATAGCAACG	189	3
18S	GGTAACCCGTTGAACCCCAT	TTCGACCGTCTTCTCAGCG	226	4
18S	CCAGTAAGTGCGGGTCATAA	GGCCTCACTAAACCATCCAA	95	4
MAP1A ex5-6 (commercial)	TGGAAATGACCCTGCCAATG	TCTGCTGATACCACTCACGA	143	4
MAP1A ex1-3 (commercial)	AGCCAATGCCTCCATGAG	GTGAACCTCTGAGCTGATACTG	129	4
GAPDH	GTAACCAGGCGTCCGATAC	TCTCTGCTCCTCCCTGTTC	120	4
CAMK2A (commercial)	GTGCTACACTGTCACCAGATG	TGCTCTCCCTCAACCTCTATG	134	4
MT-CO1	ATCAAATGATCCCCCGCCAT	GTACGATCCCTGTTAGGCCC	79	5,6
MT-CO2	CACAAGCACAATAGACGCC	GGGAGGGAAGGGCAATTAGA	86	5,6
MT-CO3	ACAGGAGCCCTATCAGCTCT	GGATGTTTGTCAGGAGGCCTAA	100	5,6
MT-RNR1	TACCGCCATCTTCAGCAAAC	TGTAGCCCATTTCTTTCCGCT	108	5,6
MT-RNR1 (sequencing)	TACCGCCATCTTCAGCAAAC	TTCCGCTTCATTGGCTACAC	94	5

For multiplexed reactions, predesigned primer/probe assays were based on a 2:1 primer:probe ratio and are listed in **Table 2.5**. 18S and mitochondrial gene primers and probes were designed manually using Primer-BLAST and the Integrated DNA Technologies PrimerQuest™ Tool (<https://eu.idtdna.com/pages/tools/primerquest>) to refine the desired parameters for the multiplexed reaction. The compatibility of primer/probe combinations was checked using the OligoAnalyzer™ Tool (<https://eu.idtdna.com/pages/tools/oligoanalyzer>) and Eurofins Genomics Oligo Analysis Tool (<https://eurofinsgenomics.eu/en/ecom/tools/oligo-analysis/>).

Table 2.5: List of primers and probes used for multiplexed RT-qPCR.

Target (bp)	Forward primer	Reverse primer	Probe	Amplicon (bp)	Chapter
MAP1A ex5-6 (commercial)	TGGAAATGACCCCTGCCAATG	TCTGCTGATACCACTCACGA	TCAGGTGAC TCTGATTCCCACTCATGA	143	4
MAP1A ex1-3 (commercial)	AGCCAATGCCTCCATGAG	GTGAACCTCTGAGCTGATACTG	CACCAGAGT GAGATCCTCGAGACCA	129	4
CAMK2A (commercial)	GTGCTACACTGTCACCAGATG	TGCTCTCCCTCAACCTCTATG	CGCGACCTG AAGCCTGAGAATCTG	134	4
18S	CCAGTAAGTGCGGGTCATAA	GGCCTCACTAAACCAATCCAA	TGCGTTGATTAAAGTCCCTGCCCTT	95	4
MT-CO1	ATCAAATGATCCCCCGCCAT	GTACGATCCCTGTAGGCC	TGAGCCTTAGGGTTTATCTTATTACAGT	79	6
MT-RNR1	TACCGCCATCTTCAGCAAC	TCCGCTTCATTGGCTACAC	AGGCACTAAAGTAAGCACAAAGAAACA	108	6
MT-ND1	TCTTACGAAAGTCACAATAGCCA	AGTCAGATATGTTCTTGTTAGTGA	CCGTCCTCCTAATAAGCGGCTCCTT	101	6
MT-ATP6	ATGCATCTAATCGGAGGAGCTA	TTAAGGCTACGGGCAAAATTCAG	AGACATCAGCCCCACCAACCGCTACA	115	6

2.11 Gel electrophoresis and extraction

For the production of PCR standards, cDNA or mtDNA was PCR amplified using a high-fidelity PCR master mix (Q5, New England Biolabs) following the manufacturer's protocol in a 25 µl reaction. Gel electrophoresis was performed on the amplified products with a 1.5-2.5% TBE agarose gel (Sigma-Aldrich) at 80 V for 90-105 min alongside a 50 bp DNA ladder (#N3236S, NEB). Staining was performed with SYBR Safe DNA stain (Invitrogen) diluted 1:10,000 in 1 x TBE buffer for 1 h and visualised under UV light. Pure PCR products were isolated and purified using the Monarch® DNA Gel Extraction Kit (NEB) following the manufacturer's protocol and the concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). qPCR standards were produced by converting this concentration to copy number and making 10-fold serial dilutions down to a single copy.

2.12 Reverse transcription

All types of RNA (bulk, nanobiopsy, single mitochondrion) were converted into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad) using a thermal cycler (Techne TC- 3000, Bibby Scientific). 1 µl reverse transcription enzyme was added alongside 4 µl iScript reaction buffer to individual samples containing up to 1 µg RNA. These reactions were then made up to 20 µl total volumes using nuclease-free water. For multiplexed RNA nanobiopsies, the total reaction volumes were made up to 10 µl.

2.13 qPCR

2.13.1 SYBR Green dye-based qPCR

qPCR reactions were performed using a StepOnePlus Real-Time 96-well PCR system (Applied Biosystems) in MicroAmp™ optical strip 0.1 mL tubes (Applied Biosystems) or MicroAmp™ optical 0.1 mL 96-well plates (Applied Biosystems). For two-step qPCR reactions, the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used on cDNA produced via reverse transcription, following the manufacturer's protocol for cycling conditions over 40-45 cycles. 20 µl qPCR reactions were run using 250 nM of each primer. For bulk reactions, 2 µl cDNA was used per qPCR reaction. For nanobiopsies, 5 µl cDNA was used. Nuclease-free water was used to make up the remaining volume. For one-step qPCR reactions directly on

RNA samples, the Luna Universal One-Step RT-qPCR kit (New England Biolabs) was used following the manufacturer's protocol. For all SYBR Green qPCRs, a melt curve stage was run after the amplification protocol to measure the conversion of double-stranded DNA into single-stranded DNA and determine the specificity of the reaction. This was performed by increasing the temperature at a rate of 0.5 °C/s from 60 to 90 °C.

For both dye-based and probe-based qPCRs, standard curves were frequently run in the same qPCR plate as the samples of interest for absolute quantification. In cases where this was not feasible, such as when there were too many samples to fit on the same plate, or multiple qPCRs of the same targets were run in the same week, the most recent standard curve was used, and the threshold of the sample run was adjusted to that of the standard curve run. Copy numbers were calculated based on the proportion of the total cDNA sample that was used per well. No RT controls and negative controls were run in each qPCR plate. Nanobiopsy samples were excluded and assumed to be unsuccessful if no amplification was observed in all targets.

2.13.2 Probe-based qPCR

Probe-based qPCR was used for multiplexed reactions where more than one target was analysed in the same reaction well. The PrimeTime™ Gene Expression Master Mix (Integrated DNA Technologies) was used for all probe-based reactions, using a primer concentration of 500 nM and a probe concentration of 250 nM in a final reaction volume of 20 µl. The manufacturer's recommended cycling conditions were followed for 40-45 cycles. qPCR reactions were performed using a StepOnePlus 96-well Real-Time PCR system (Applied Biosystems) in MicroAmp™ optical 0.1 mL plates (Applied Biosystems), except for single mitochondrion α -synuclein gene expression analysis (**Chapter 6**) which was performed using a QuantStudio™ 6 Flex Real-Time 384-well PCR System (Applied Biosystems) with MicroAmp™ optical 384-well reaction plates (Applied Biosystems).

2.14 Sanger sequencing

mtDNA regions were amplified using high-fidelity PCR (Q5 High-Fidelity 2x Master Mix, New England Biolabs) according to the manufacturer's protocol. The PCR products were purified using the PureLink PCR Micro Kit (Invitrogen) and the concentration was determined using a Nanodrop 2000 spectrophotometer. 6-20 ng purified PCR product was then sequenced using DNA Sequencing & Services (MRC I PPU, School of Life Sciences, University of

Dundee) using forward and reverse primers. The sequences were processed using UGENE (Unipro) software and alignment was carried out using BLAST Global Align tool (NCBI).

2.15 Image analysis

Image analysis was performed using Fiji. Cell images in **Chapter 4** had the ‘Green Fire Blue’ LUT applied to them and cell images in **Chapters 5-6** were subject to the ‘Fire’ LUT in Fiji. The SNT toolbox, as part of the Neuroanatomy ImageJ plugin, was used to trace and measure dendritic lengths.¹⁸ The MiNa Mitochondrial Network Analysis ImageJ plugin was used to estimate mitochondrial area in the regions of cells.¹⁹

2.16 Data analysis

Data analysis was performed using MATLAB 2020. Normality and statistical tests were performed using GraphPad Prism 8.

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Chapter 3

Nanotweezer fabrication and characterisation

Synopsis

The nanotweezer is a promising technology for the manipulation and extraction of biomolecules from living systems. This chapter outlines the development and modification of the procedures for nanotweezer fabrication. Previous work is built upon by presenting DEP trapping of biomolecules, including fluorescent polystyrene beads, λ -DNA and total RNA isolated from cells. These RNA characterisation experiments were conducted in salt solution, representing the conductivity of cells, and showed that the platform was capable of trapping RNA in physiological conditions for prior application to cells *in vitro*. The frequencies and voltages required for successful trapping and detection of RNA were assessed so that the optimal parameters could be taken forward for DEP trapping and isolation of RNA in cells.

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3.1 Introduction

Single-cell subcellular sampling methods have the potential to achieve precise profiling of single cells as they maintain cells in their standard environment and do not require cell lysis or fixation. Many of these methods, however, are costly and may be limited in performing multiple measurements of the same cell because they involve the aspiration of cytoplasmic fluid, potentially leading to cellular alterations or death. The nanotweezer presents an inexpensive, biocompatible method for trapping and extracting biomolecules, with the potential to achieve dynamic measurements of the same living cell due to its minimally invasive procedure of trapping biomolecules directly, rather than removing cellular volumes. The method is versatile and can be used on any basic microscope setup, either label-free or with fluorescence to target specific regions or biomolecules. In previous work, the fundamentals of the technology were introduced (**Figure 3.1**), characterising trapping of fluorescent beads and DNA in solution, in addition to proof-of-concept biopsies of DNA, RNA and single mitochondrion from living cells.^{1,2} This included 3D finite element modelling to measure the gradient of the electric field strengths ($\nabla|E|^2$) and heat generated at the nanotweezer tip with DEP applied at various conductivity and voltage conditions (**Figure 3.1d-e**).^{1,2}

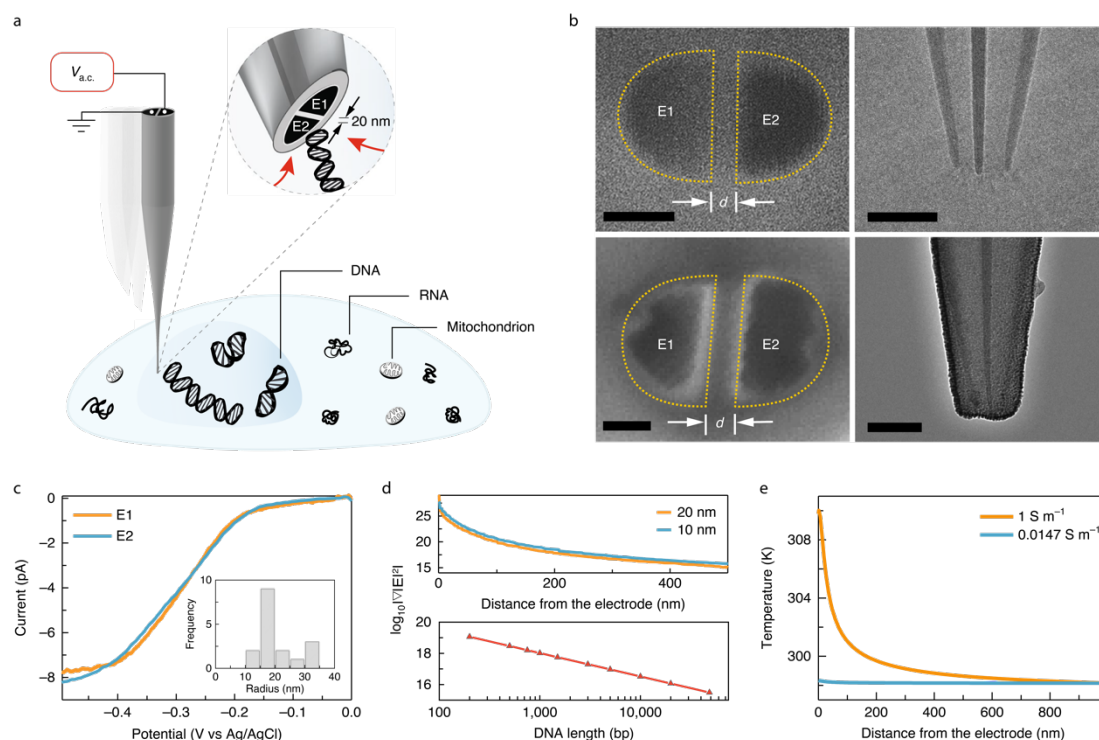


Figure 3.1: Original characterisation of the nanotweezer. a) Schematic of the nanotweezer extracting DNA from the nucleus of a single cell upon application of an AC voltage. b) SEM (left) and TEM (right) images of the bare nanopipette (top) and carbon-filled nanotweezer (bottom). Scale bars = 20 nm (left) and 100 nm (right). c) Linear sweep voltammograms of 2 electrodes from the same nanotweezer and size distributions of the electrode

(inset). d) Electric field gradient at the nanotweezer tip at 1MHz, 20 V_{pp} (top) and electric field gradients required to trap different lengths of DNA (bottom). e) Finite element model of the temperature distribution around the nanotweezer tip in different media ionic strengths. Figure reprinted with permission.¹ Copyright 2018, Springer Nature.

In this chapter, the previous fabrication and characterisation of the nanotweezer is reproduced, modified and expanded on. In particular, DEP trapping of RNA is characterised to determine the optimal frequency and voltage required for trapping RNA in physiological ionic conditions. The RNA characterisation work presented in this chapter builds on MRes work begun by the author,³ by following similar DEP trapping experiments that were previously presented by Nadappuram and Cadinu *et al.* for trapping DNA and polystyrene beads.^{2,4} The optimisation work in this chapter forms the basis for trapping RNA from live cells in **Chapter 4**.

For successful DEP trapping in cells, there are numerous factors and phenomena that need to be considered when designing and modifying the nanotweezer technology. These are evaluated throughout this chapter with the fabrication and characterisation of the platform for RNA isolation. These considerations are summarised below and are based on the theory of DEP (**Chapter 1.5.4**):

1. **Trapping force generated:** The electric field strength, and therefore F_{DEP} , is proportional to V^2d^{-3} . To achieve high electric field strengths for trapping sub-micron particles, the electrode-electrode distance must be small so that there is less reliance on using high voltages to achieve the necessary electric field strengths for trapping. This would therefore minimise the amount of heat generated from the use of high voltages which is crucial for cell survival and viability.
2. **Tip size for insertion into cells:** The nanopipette tip needs to be minimally invasively inserted into regions of live cells to avoid damage to the cell. Initial resealing of the plasma membrane has been shown to occur within 30 s of disruption, regardless of the size of disruption, with nanometre-sized disruptions shown to spontaneously reseal *in vitro*, compared to larger disruptions which require active repair machinery.⁵ The size of the nanopipette therefore needs to remain small to minimise the triggering of substantial repair mechanisms, phenotype alterations and possible long-term effects on cell viability. For sampling from precise subcellular compartments, such as the neurites

of neurons which can have varying widths of only 0.1-1 μm ,⁶⁻⁸ the tip size also needs to be small so that these compartments can be targeted and accessed.

3. **Joule heating:** Highly non-uniform heating can occur from Joule dissipation at the electrodes. Cells and physiological fluids have relatively high conductivities which can lead to increased current flow and Joule heating under applied electric fields. This increased thermal motion of molecules and electrothermal flow can inhibit DEP trapping, meaning that higher DEP forces would be required to overcome this. Joule heating also increases with increased voltages and leads to conductivity and permittivity gradients in the media.⁹ These heating effects can cause alterations and harm to cells when used *in vitro*. These effects therefore need to be carefully monitored and minimised for DEP trapping from living cells.
4. **Positive/negative DEP:** To trap molecules into regions of high electric field at the nanotweezer tip, positive DEP needs to be attained. This is based on the CM factor discussed in **Chapter 1.5.4** which is dependent on the frequency applied and the conductivity of the particle and the medium. The cytoplasm of cells has a high conductivity of around 1 S/m.¹⁰ It is therefore essential to establish the optimal frequency range to achieve positive DEP with this high medium conductivity. This would ensure that high enough DEP forces could be generated to allow for DEP trapping to occur. Understanding the frequency at which negative DEP occurs using the nanotweezer under cellular conditions could also be beneficial; it could be exploited for the release of biomolecules from the nanotweezer tip for use in downstream analysis.

3.2 Fabrication

Nanotweezers were fabricated by laser pulling double-barrel quartz capillaries into nanopipettes and filling the chambers with carbon to form two carbon electrodes at the tip (**Chapter 2.7**). The fabrication procedure of the nanotweezer had previously been established in the group.¹ This included the optimised laser pulling protocol on the designated laser puller, the argon and butane pressure used to deposit carbon onto the nanopipette tips, and the duration of heat applied to the nanopipette tip to deposit carbon. Many steps of this procedure, however, were prone to alterations which affected the size, shape and electrode formation of the

nanotweezer. One aspect was the laser pulling of quartz capillaries into nanopipettes, for which specific protocols needed to be established to achieve the desired pore size on each laser puller instrument. This is because factors such as temperature and humidity can affect the quality of the nanopipettes formed.¹¹ Additionally, the carbon deposition procedure was prone to person-to-person variation based on the pressure of the blow torch and the distance the blow torch was held from the nanopipette.

Table 3.1 summarises the initial protocol for nanotweezer fabrication (Protocol 1) and the optimised protocol used in this thesis (Protocol 2). The initial parameters for nanotweezer fabrication, based on previous work, were trialled (**Table 3.1**, Protocol 1) and the size of each electrode was estimated using linear sweep voltammetry (LSV). This was followed by SEM to confirm the deposition of carbon, the electrode size and the distance between the electrodes. To trap polarisable molecules at the electrodes, the forces generated need to overcome drag forces from fluid flow and thermal Brownian motion, which become significant for sub-micron particles.^{12,13} As discussed previously, $F_{\text{DEP}} \sim V^2 d^{-3}$ is based on the electric field strength term. For successful dielectrophoretic trapping of molecules in cells, it is therefore necessary for the gap between the electrodes to be small to achieve high dielectrophoretic forces, whilst minimising the use of high voltages to achieve these forces to minimise Joule heating in biological systems.

Table 3.1: Parameters employed for nanotweezer fabrication.

Protocol	Puller programme	Argon pressure (L/min)	Butane pressure (L/min)	Time spent heating the tip (s)
1	78	0.1	0.15	20
2	19	0.1	0.15	5

Each nanoelectrode was electrochemically characterised from LSV using the steady-state current for the one electron reduction of hexaammineruthenium (III) chloride. A negative bias was applied to the electrode, leading to the reduction of $\text{Ru}(\text{NH}_3)_6\text{Cl}_3$, given by the following half equation:



The size of each carbon electrode was calculated using the generated steady-state current and **Equation 3.2**.¹⁴

$$r = \frac{i_{ss}}{4.64nFCD} \quad \text{Equation 3.2}$$

where r is the radius of the electrode, i_{ss} is the steady-state reduction current, n is the number of electrodes transferred during the reduction (1 in this case), F is the Faraday constant, C is the concentration of the redox mediation (10 mM for $Ru(NH_3)_6Cl_3$) and D is the diffusion coefficient for the redox mediator ($8.2 \times 10^6 \text{ cm}^2\text{s}^{-1}$ for $Ru(NH_3)_6Cl_3$ in 100 mM KCl). It should be noted that this equation assumes circular disc-shaped electrodes, whereas these electrodes are semi-circular in shape, meaning that this calculation is only an approximation. LSV was therefore primarily used for determination of the quality of the electrodes, confirmation of carbon deposition, and for size optimisation purposes before confirming the true size with SEM.

Using the initial fabrication parameters, LSV gave a characteristic smooth sigmoidal curve (**Figure 3.2a**) which confirmed that current flowed through the electrodes and that carbon had been successfully deposited. The average estimated electrode diameter, however, was 91.1 nm with size distributions shown in **Figure 3.2b**. These electrode sizes were larger than the previously reported LSV estimated electrode diameter of $21 \pm 6 \text{ nm}$.¹ As LSV only provides an estimate of the electrode diameter for the nanotweezer, the size of the electrodes was then confirmed by SEM. SEM showed that with these conditions, the electrode diameters were $94 \pm 50 \text{ nm}$ ($n = 6$ electrodes), and the distance between the electrodes were $52 \pm 8 \text{ nm}$ ($n = 3$ nanopipettes), giving an average overall tip size of $240 \pm 60 \text{ nm}$. This is larger than the reported size of the nanotweezer electrodes and the reported distance between the electrodes ($23 \pm 6 \text{ nm}$ and $10\text{-}20 \text{ nm}$ respectively).¹

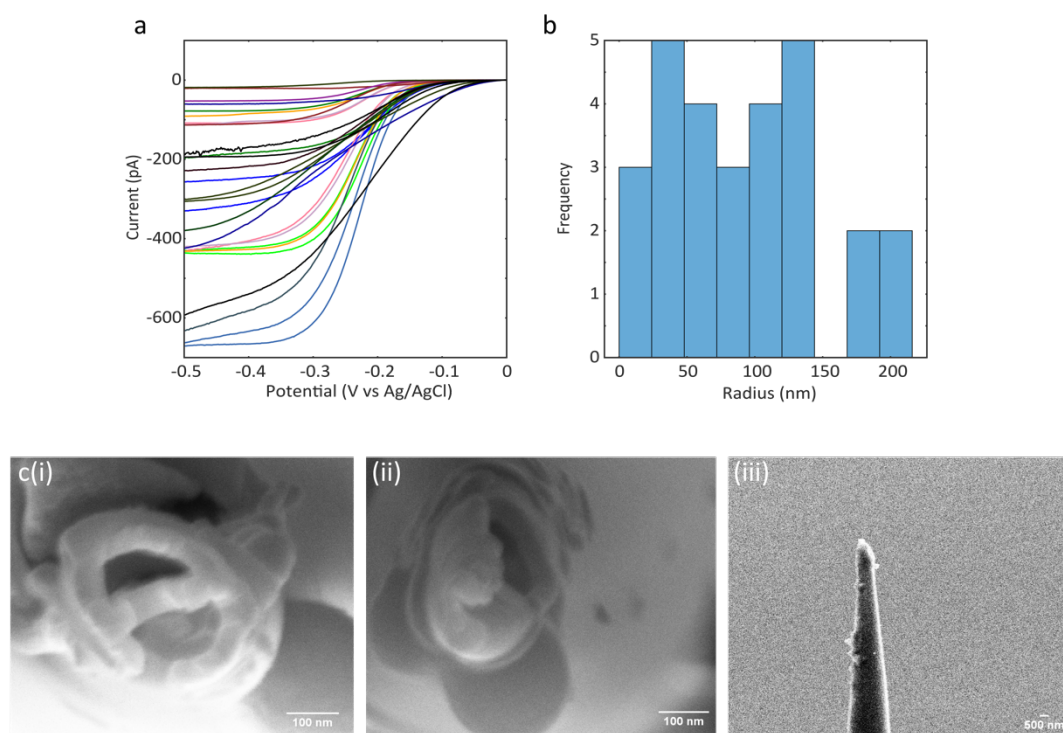


Figure 3.2: Characterisation of the nanotweezer tip using initial fabrication parameters. a) Linear sweep voltammograms for the reduction of 10 mM hexaammineruthenium (III) chloride at the carbon surface of multiple nanotweezer electrodes. The same colours represent electrodes from the same nanopipette. b) Histogram of the calculated carbon electrode diameters from LSV currents. c) SEM images of the tip view (i-ii) and the side view (iii) of nanotweezers, showing an excess of carbon around the tip.

This larger size may be due to the laser puller being moved to a different location, where changes in humidity and temperature would have occurred, in addition to the programme not being updated for a number of years. It was essential to reduce the nanotweezer tip size before performing trapping experiments so that sufficient DEP forces could be achieved at low voltages for later use *in vitro*. Excess heat pressure from the blow torch may also have caused the tip to break which would lead to larger electrodes. In addition to problems with the size, the SEM images showed that perhaps too much carbon was being deposited on the tip of the nanopipettes, shown by excess carbon present on the outside of the nanopipette (**Figure 3.2cii**). If too much carbon is deposited, this would lead to a build-up of carbon on the outside of the tip and the electrodes may become connected by a layer of carbon, meaning that DEP could not be achieved. It was therefore also important to alter the carbon deposition conditions to minimise this excess carbon deposition and ensure that the electrodes were well-separated.

Firstly, the programme for pulling double-barrel capillaries into nanopipettes was altered to form smaller pores (created by Dr Xiaoyi Wang, Imperial College London). The time spent

heating the tip during the carbon deposition was also reduced to minimise tip breakage and excess carbon forming around the tip. **Figure 3.3a** shows LSV voltammograms for nanotweezer electrodes using the new pulling programme. The estimated mean electrode size, calculated using **Equation 3.2**, was determined to be 29.4 nm, with distributions shown in **Figure 3.3b**. The new programme was therefore found to create smaller electrodes, close to that previously reported for the nanotweezer.¹ The symmetry of the two electrodes of the nanotweezer was determined by comparing the LSV curves of the two electrodes of the same nanotweezer as shown in **Figure 3.3c**. Symmetry was observed between many of the electrodes of the same nanotweezer which would be necessary for consistent and predictable DEP forces to be generated.

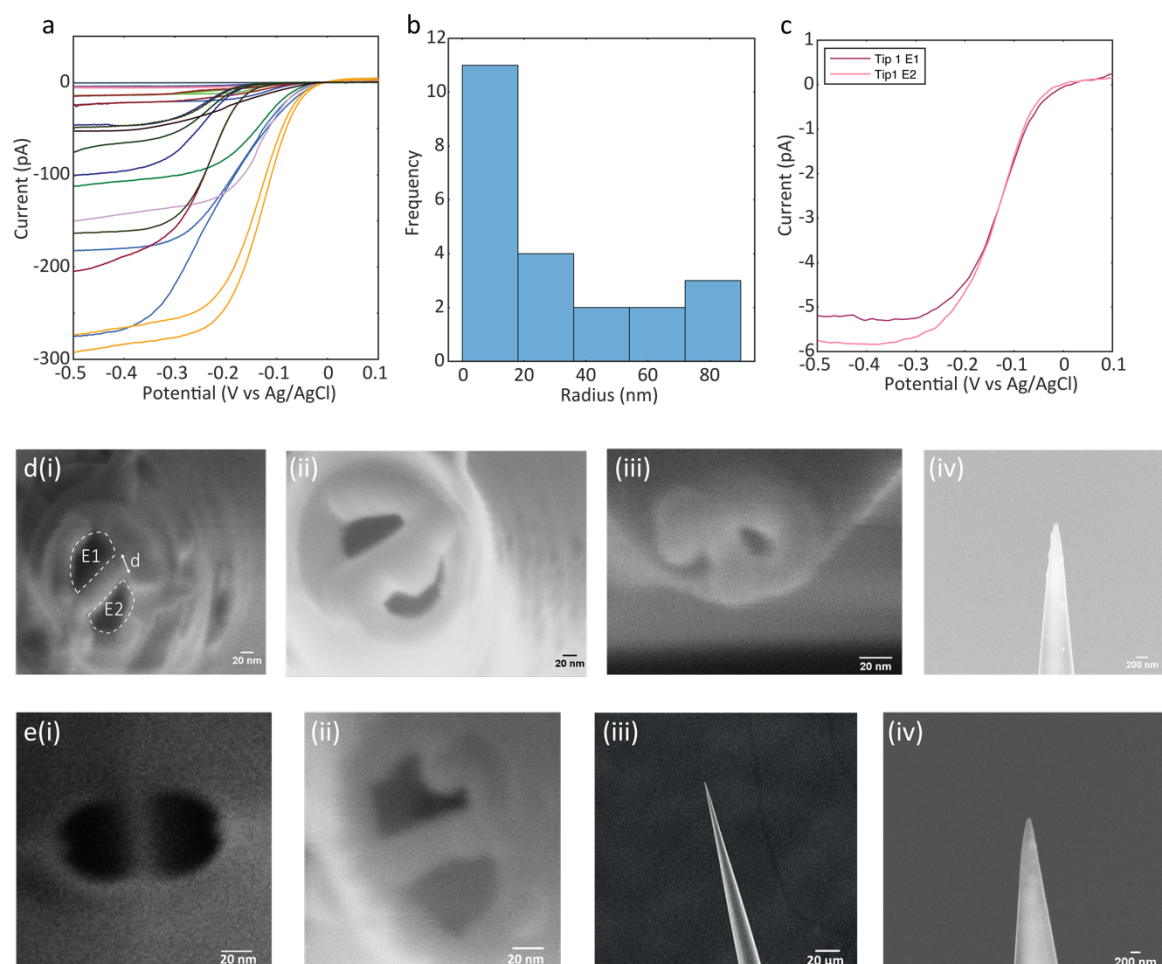


Figure 3.3: Characterisation of the nanotweezer tip using optimised fabrication parameters. a) Linear sweep voltammograms for the reduction of 10 mM hexaammineruthenium (III) chloride at the carbon surface of multiple nanotweezer electrodes. Same colours represent electrodes from the same nanopipette. b) Histogram of the calculated carbon electrode diameters from LSV currents. c) Example linear sweep voltammograms derived from electrodes of the same nanotweezer. d) Representative SEM images of the nanotweezer tip (i-iii) and side view (iv) after optimised carbon deposition conditions. E1 and E2 represent the two electrodes and d represents the septum or distance between the electrodes. e) Representative SEM images of the nanopipette tip before carbon deposition (i), the nanotweezer tip after carbon deposition (ii) and the side view of the tip (ii-iv) using finalised

fabrication parameters. The nanopipette SEM image before carbon deposition was performed by Dale Henseley at Oak Ridge National Laboratory.

The time spent heating the tip of the nanotweezer was optimised, showing a lower build-up of carbon at the end of the tip (**Figure 3.3d**). The optimised time for heating the tip that ensured sufficient carbon-filling of the electrodes for electrical contact, whilst minimising excess carbon formation outside the tip, was determined to be 5 s. **Figure 3.3e** shows typical nanopipette and nanotweezer SEM images fabricated using the optimised pipette pulling and carbon deposition conditions. The diameter of the electrodes and the electrode distance based on SEM were determined to be 34 ± 12 nm and 27 ± 7 nm respectively ($n = 6$ electrodes, 3 nanopipettes). Although care was taken to ensure consistent pulling of pipettes and carbon deposition by following the same programmes and protocols, small variations in the tip and electrode size can arise. This is due to the manual process of depositing carbon, which may lead to non-uniform carbon filling and variations in the surface area of the electrodes. Pulling capillaries into nanopipettes can also lead to asymmetry in the barrels, in addition to differences in pulling times which would therefore contribute to differences in the pore sizes produced.¹⁵ Although small, these differences may lead to changes in DEP trapping efficiency; checking the success of each nanotweezer, along with normalising for differences between each nanotweezer, is therefore paramount for assessing the quantity of analytes such as mRNAs.

3.3 Polystyrene bead manipulation

Previous work in the group presented extensive characterisation experiments of trapping fluorescent beads in solution.^{1,2} This included: the voltage and frequency dependence of trapping 100 nm fluorescent beads diluted in water; trapping and manoeuvring fluorescent beads in solution; and proof of spatial confinement at the nanotweezer tip upon application of DEP, compared to Brownian motion of particles in the absence of DEP.

Following on from previous work, to ensure the newly fabricated nanotweezers could generate DEP forces to trap polarisable molecules, DEP was initially tested in aqueous solution using 100 nm polystyrene fluorescent beads (FluoSpheres™) as a control. These acted as spherical particles that would be known to behave uniformly upon application of DEP, before later trialling the nanotweezer with more complicated biological molecules and systems.

Nanotweezers were mounted on a micromanipulator (Scientifica) positioned on the fluorescent microscopy setup described in **Chapter 2.8.2**. Copper wires were inserted into each barrel of the nanotweezer and connected to a function generator. Fluorescent images were acquired using an emCCD camera (Photometrics). The nanotweezer was slowly lowered into a droplet of fluorescent beads diluted 1:1000 in water (**Figure 3.4a-c**) and the fluorescent signal was measured by focusing on the nanotweezer tip (**Figure 3.4g**). Initially, no voltage was applied (DEP off) and there was no observation of fluorescence on the nanotweezer tip (**Figure 3.4d(i)-e(i)**). Upon application of a square wave AC voltage (1 MHz, 15 V_{pp}), a rapid accumulation of fluorescent beads at the nanotweezer tip was observed, confirming successful DEP trapping (**Figure 3.4d(ii)-e(ii)**). A square AC voltage was used, rather than a sine wave, to maximise the signal magnitude at the same voltage. It has previously been shown that a square wave leads to more than double the DEP trapping ability than a sine wave in carbon electrode systems.¹⁶ Additionally, 1 MHz was used as the frequency as previous work showed that frequencies between 500 kHz and 1 MHz gave the highest nanotweezer trapping efficiencies of 100 nm polystyrene beads.²

Maintaining the AC voltage led to an increase in fluorescent beads trapped at the tip. As more fluorescent beads were trapped, a higher signal was observed, illustrated by the step-like fluorescent signal in **Figure 3.4g** where each ‘step’ represented new fluorescent beads getting trapped at the tip. The smaller growth in fluorescence with each stepwise increase may be accounted for by the tip becoming saturated with fluorescent beads, where the fluorescence signal may be reaching its maximum at the area measured closest to the tip, and reaching a plateau, indicating that no more fluorescent beads can be trapped. As the trapping is only measured in a single plane, some beads will also be trapping around the tip, out of the main plane of focus, as more beads crowd around the tip. Additionally, photobleaching would have occurred over these timeframes, reducing the overall fluorescence signal detected. When the DEP was switched off, the fluorescent beads diffused away from the nanotweezer tip (**Figure 3.4d(iii)-e(iii)**), illustrating the trap and release capabilities of the technology.

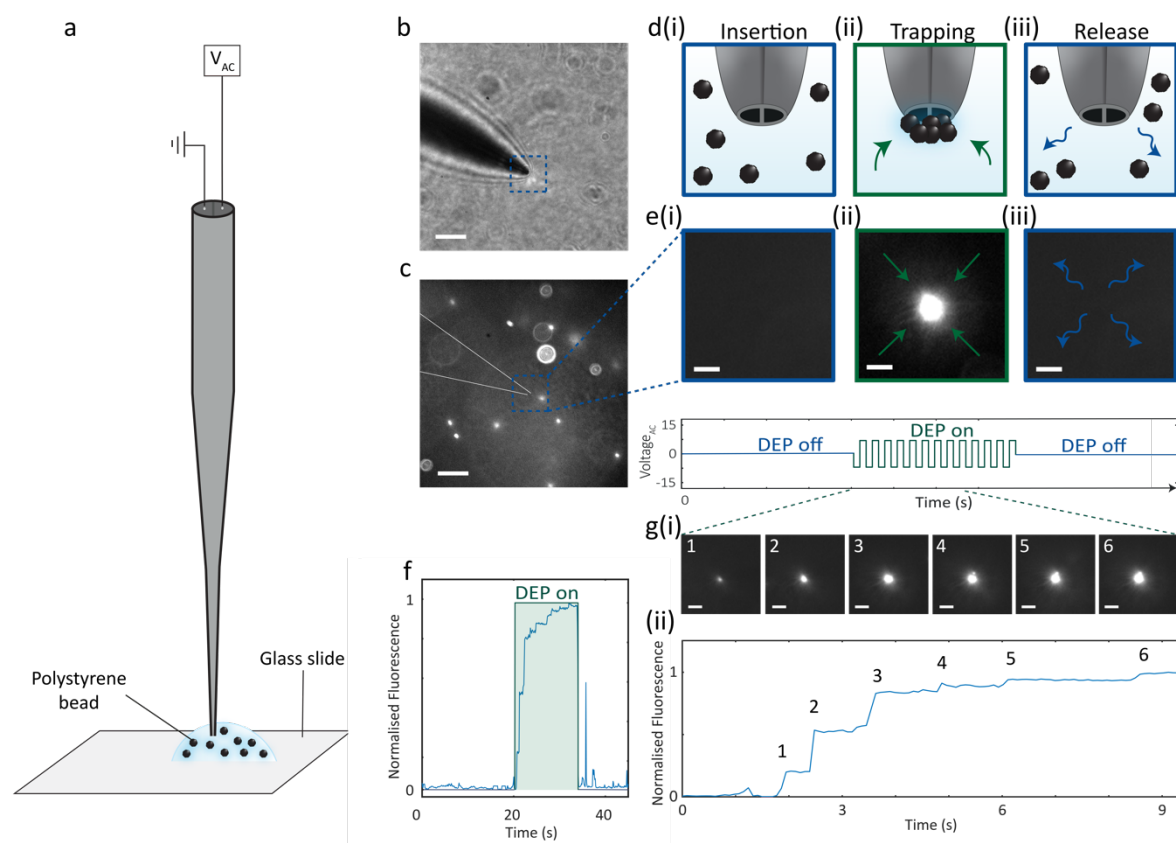


Figure 3.4: Polystyrene bead DEP trapping. a) Schematic of nanotweezer trapping of 100 nm fluorescent polystyrene beads diluted in water. The nanotweezer was positioned into a droplet of the solution placed on a glass slide and an AC voltage (1 MHz and 15 V_{pp}) was applied to initiate DEP trapping. b) Bright field image of the nanotweezer tip inserted into a solution of fluorescent polystyrene beads diluted 1:1000 in water. Scale bar = 20 μm. c) Corresponding fluorescent image of the nanotweezer in the fluorescent bead solution. White lines indicate the position of the nanotweezer. Scale bar = 20 μm. d) Schematic of DEP trapping of fluorescent beads where the nanotweezer is inserted into solution (i), DEP is applied to cause beads to become trapped at the tip (ii) and DEP is turned off, causing trapped beads to diffuse away from the nanotweezer tip. e) Corresponding fluorescent images for DEP trapping of fluorescent beads. Scale bars = 1 μm. f) Fluorescence-time trace of the nanotweezer tip, highlighting the time periods when the AC voltage was applied (DEP on). g) Fluorescent images of individual trapping events of fluorescent beads in solution (i), where each image represents an additional fluorescent bead trapped at the tip (scale bars = 5 μm) and corresponding fluorescence-time trace of each trapping event (ii), illustrating a stepwise signal representing each additional bead trapped at the tip.

It was also shown that once the beads were trapped at the nanotweezer tip and the DEP was maintained, moving the nanotweezer in its x and y directions moved the trapped beads along with it, showing that particles can be manipulated (**Figure 3.5**). This manipulation behaviour, combined with the trap and release capability, could have advantageous applications in ‘pick-and-place’ experiments to manipulate and isolate biomolecules, similarly to what has been demonstrated with the pick-and-place of single cells for screening cells for specific functions.^{17,18}

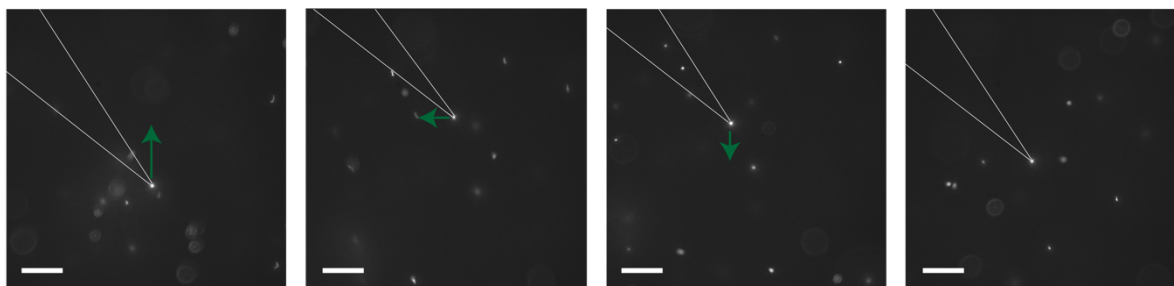


Figure 3.5: Single molecule manipulation. Fluorescent images of the manipulation of fluorescent beads trapped at the nanotweezer tip. The white lines represent the position of the nanotweezer and the green arrows illustrate the direction of manual manipulation of the nanotweezer in solution, showing that the trapped beads can be moved in solution. Scale bars = 5 μm .

3.4 DNA manipulation

As with polystyrene beads, extensive characterisation of trapping DNA in solution has previously been demonstrated.^{1,2} This included similarly determining the frequency and voltage dependency of trapping 10 kbp and λ -DNA (48.5 kbp), showing better trapping efficiencies at higher frequencies for longer length DNA, as well as pick-and-place demonstrations of both types of DNA.

It was essential to test and characterise trapping of biomolecules such as DNA and RNA in solution before going into biological systems such as cells to trap these molecules. This is because complications can arise due to the molecule type and the biological system. Therefore, initially testing the trapping of DNA and RNA in a controlled solution was required before adding the extra complexities of cellular systems. DNA and RNA will not behave as a uniform spherical particle such as a polystyrene bead. This is because, for heterogeneous particles, the DEP model becomes more complex due to the presence of several layers; free charges surround the surface of charged particles and form an electrical double layer. DNA and RNA have negatively charged backbones, leading to a cloud of positive counterions which form an electric double layer around the strand that can be readily polarised by an electric field.^{19,20} Application of an electric field on DNA and RNA may lead to the DNA or RNA strand and their cloud of counterions to become distorted, causing a charge separation and induced dipole.²¹ This results in the gradient force, causing the molecules to migrate to regions of high electric field strength in cases of positive DEP. Nevertheless, there is little consensus on the specific polarization models of complex biological molecules like DNA and RNA. The counterion fluctuation model has been proposed for DNA.²²

In this work, following confirmation of the nanotweezer's trapping ability using spherical polystyrene beads, the nanotweezer was tested for trapping DNA. λ -DNA was labelled with YOYO-1 intercalator dye and the DNA was diluted to a 100 pM concentration in 1 mM KCl, as employed in previously work.^{1,2} A droplet of the labelled DNA solution was placed on a glass slide and the nanotweezer was inserted into the droplet in the same manner described previously for trapping polystyrene beads (**Figure 3.6a-b**). λ -DNA was shown to be successfully trapped at the nanotweezer tip upon application of DEP (**Figure 3.6c(ii)-d(ii)**). This showed similar trap and release behaviour as was shown with polystyrene beads, where turning off the DEP leads to diffusion of DNA molecules in solution and release from the tip (**Figure 3.6c(iii)-d(iii)**).

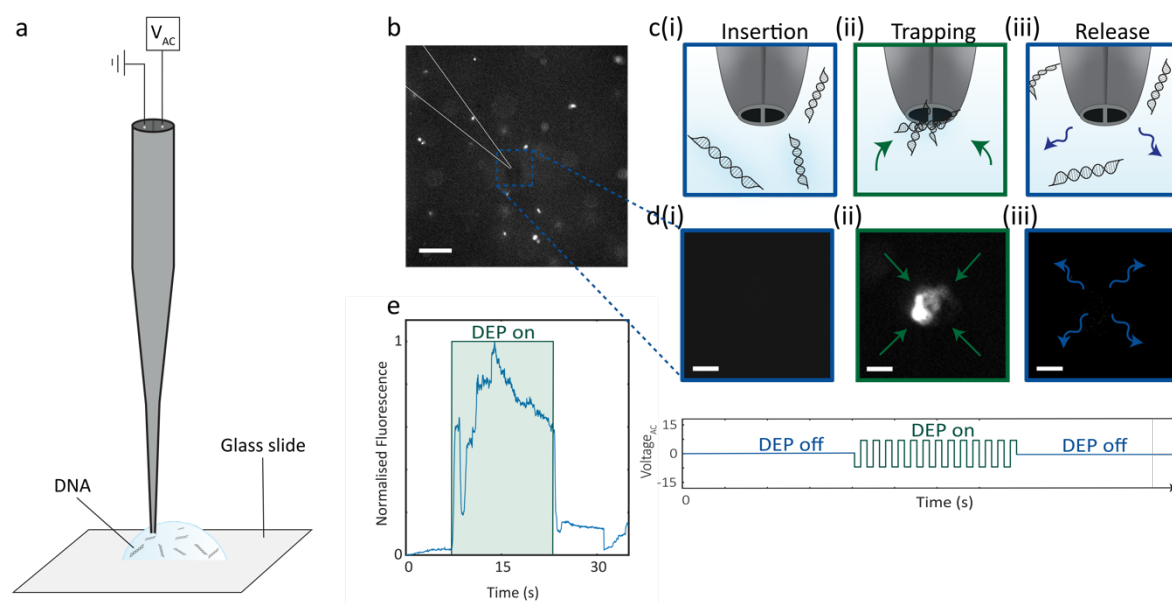


Figure 3.6: λ -DNA DEP trapping. a) Schematic of nanotweezer trapping λ -DNA diluted in 100 mM KCl. The nanotweezer was positioned into a droplet of the solution placed on a glass slide and an AC voltage (1 MHz and 15 V_{pp}) was applied to initiate DEP trapping. b) Fluorescent image of the nanotweezer tip inserted into a solution of λ -DNA. White lines indicate the position of the nanotweezer. Scale bar = 20 μm . c) Schematic of DEP trapping of DNA where the nanotweezer is inserted into solution (i), DEP is applied to cause DNA to become trapped at the tip (ii) and DEP is turned off, causing trapped DNA to diffuse away from the nanotweezer tip. d) Corresponding fluorescent images for DEP trapping of DNA. Scale bars = 1 μm . e) Fluorescence-time trace of the nanotweezer tip, highlighting the time periods when the AC voltage was applied (DEP on).

3.5 RNA manipulation and optimisation

3.5.1 Trapping

Extraction of RNA from live cells was one of the initial aims of this work. The polarizability of molecules like RNA strongly depends on the frequency applied and the ionic strength of the medium. The DEP parameters such as the frequency and voltage required for trapping RNA therefore needed to be optimised so that the conditions that led to the most RNA being trapped, under conditions suitable for use in cells, could be taken forward for isolation of RNA from neurons.

The cytoplasm of mammalian cells is composed of water, organic molecules and a number of ions such as K^+ , Na^+ , Ca^{2+} , Mg^{2+} and Cl^- .²³ As the CM factor, and therefore DEP, depends on the polarizability and permeability of the medium, which in turn is dependent on the conductivity of the medium, providing similar high medium conductivity conditions as cells was essential to determine the optimal frequency required for trapping in cellular conditions.

Total RNA was isolated from mammalian cells and fluorescently labelled with Alexa Fluor 488 dye following standard procedures summarised in **Chapter 2.8.1**. 100 mM KCl was selected as the medium for diluting isolated RNA in to create a conductive environment that resembles the cytoplasm of cells. 100 mM KCl provides a conductivity of 1.28 S/m^{24} which is close to that reported for the physiological conductivity of hippocampal neurons,²⁵ providing suitable and simple conditions for testing the nanotweezer prior to trapping within cells. These salt conditions were higher than what was previously reported for nanotweezer trapping in solution, altering the conductivity. Higher medium conductivities mean that there are smaller differences between the particle and the medium, leading to a weaker induced dipole and weaker DEP. It was therefore expected that trapping RNA in 100 mM KCl and from the cytoplasm of cells would be more difficult than what was previously observed in water or lower salt conditions.

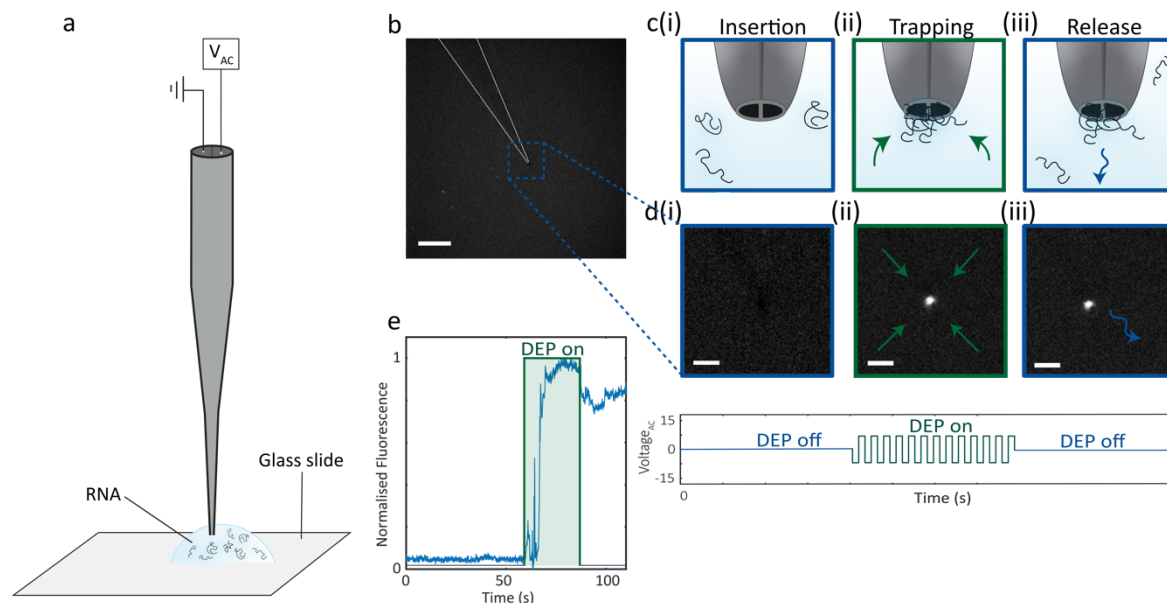


Figure 3.7: RNA DEP trapping. a) Schematic of nanotweezer trapping 64.5 pg/ μl RNA diluted in 100 mM KCl. The nanotweezer was positioned into a droplet of the solution placed on a glass slide and an AC voltage (2 MHz and 15 V_{pp}) was applied to initiate DEP trapping. b) Fluorescent image of the nanotweezer tip inserted into a solution of fluorescently labelled RNA. White lines indicate the position of the nanotweezer. Scale bar = 20 μm . c) Schematic of DEP trapping of DNA where the nanotweezer is inserted into solution (i), DEP is applied to cause RNA to become trapped at the tip (ii) and DEP is turned off, causing only some trapped RNA to diffuse away from the nanotweezer tip and the rest remained adsorbed to the tip. d) Corresponding fluorescent images for DEP trapping of RNA. Scale bars = 1 μm . e) Fluorescence-time trace of the nanotweezer tip, highlighting the time periods when the AC voltage was applied (DEP on).

The same protocol for trapping polystyrene beads and DNA was employed with RNA diluted to a concentration of 64.5 pg/ μl in 100 mM KCl (**Figure 3.7a**). Upon application of DEP, RNA molecules were trapped at the nanotweezer tip, showing that the technology could operate under high ionic strength (**Figure 3.7c(ii)-d(ii)**). Unlike what was observed with polystyrene beads and DNA, RNA did not get fully removed from the tip upon withdrawal of the DEP force (**Figure 3.7c(iii)-d(iii)**). In some cases, no loss of fluorescence was observed upon turning the DEP off. Additionally, upon inserting the nanotweezer into solution, sometimes some RNA was already observed on the tip before application of DEP. Nevertheless, this still led to a rapid accumulation of RNA at the nanotweezer tip upon application of DEP, compared to control nanotweezers where DEP was not applied (**Figure 3.8b**).

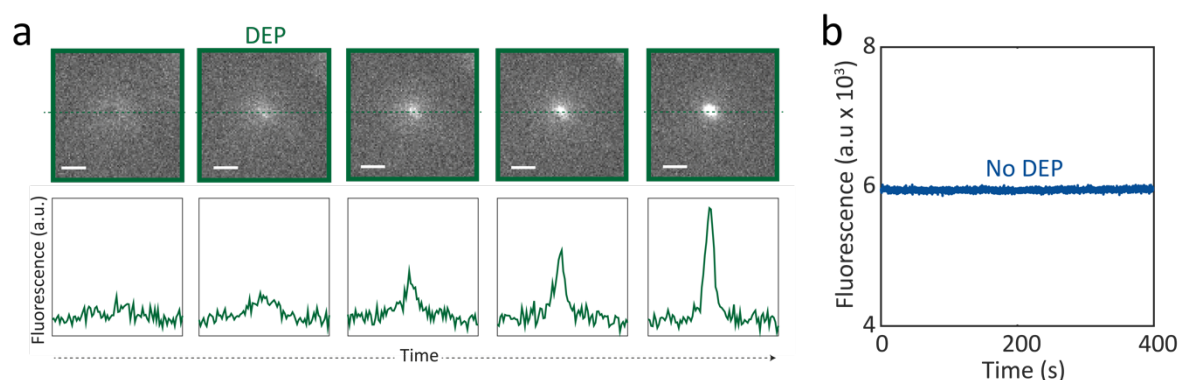


Figure 3.8: RNA DEP trapping and comparison to controls. a) A rapid accumulation of fluorescently labelled RNA was observed at the nanotweezer tip upon application of DEP. Green dashed lines illustrate where the fluorescence was measured over time at the nanotweezer tip. An increase in fluorescence at the nanotweezer tip is observed when DEP is applied. Scale bars = 5 μm . b) Representative fluorescence-time traces of the nanotweezer tip during control experiments when DEP is not applied.

It was apparent that RNA exhibited an adsorption behaviour that was not observed with beads or DNA. This could be explained by the single-stranded nature of RNA, where the aromatic bases of RNA would be exposed and in contact with the tip, leading to hydrophobic interactions and van der Waals forces forming between carbon on the nanotweezer and the bases of RNA. This could lead to adsorption interactions forming when the tip contacts RNA, including in the absence of DEP when placed in solution, and after trapping when DEP was no longer applied. Similar behaviour has been previously shown with single-stranded DNA adsorbing to graphene nanopores.^{26,27}

Simple removal of RNA from the nanotweezer tip, for example by allowing RNA to diffuse into solution upon removal of the DEP force, was desired so that the isolated RNA could be easily collected for downstream analysis. This would also allow the nanotweezer to be reused for multiple biopsies and increase the throughput of the technique. As RNA removal by turning off the DEP force was not possible due to the adsorption behaviour observed, other attempts to remove the RNA trapped on the nanotweezer tip were made. This included pulsing the DEP at high frequencies (5-10 MHz) as was previously performed for removing DNA wrapped around the tip,² and using high frequencies (up to 20 MHz) in attempt to attain negative DEP which, if successful, would cause the RNA to migrate away from the region of high electric field at the tip. However, removal of RNA from the tip was not possible using these methods. Submerging the nanotweezer tip in polar solvents such as ethanol and DMSO for various timeframes was then attempted in order to precipitate the RNA or disrupt its secondary structure, however this also did not remove RNA from the tip (**Figure 3.9**). Collecting the

trapped RNA for downstream analysis was therefore achieved following previous nanotweezer work: the nanotweezer tip was gently pressed into a PCR tube containing 5 μ l nuclease-free water.¹

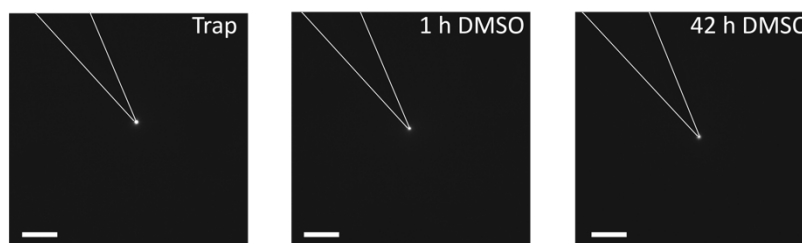


Figure 3.9: Attempts to remove trapped RNA from the nanotweezer tip. Fluorescent images of trapped fluorescently labelled RNA at the nanotweezer tip after trapping (left), after 1 h incubation of the tip in DMSO and after 42 h incubation with DMSO, showing that RNA remained trapped on the nanotweezer tip. The white dashed line illustrates the position of the nanotweezer. Scale bars = 20 μ m.

3.5.2 Frequency-dependence

Optimisation of the frequency for DEP under similar conductivity conditions as cells was required; different salt conditions would lead to an altered motion of molecules at the same frequency, which in turn would affect the trapping efficiency. For example, higher salt conditions, as incorporated in this work, would mean that the conductivity of the medium would be higher and there would therefore be increased Joule heating at the same frequency.⁹ Increased heating would cause a higher motion of molecules and electrothermal flow which would make trapping less efficient. This would mean that the DEP force would need to overcome this additional motion of molecules. Optimising the frequency to generate high DEP forces for trapping RNA under these conditions was therefore required to understand the frequency range at which the nanotweezer could overcome the motion of RNA molecules for positive DEP and to obtain the optimal frequency that trapped the highest amount of RNA.

A range of frequencies between 500 kHz and 20 MHz at a constant voltage of 20 V_{pp} were tested to determine the optimal frequency for trapping fluorescently labelled total RNA in 100 mM KCl. Frequencies below 500 kHz were not attempted as these may have led to undesired electrochemical reactions at the electrodes due to there potentially being more time for electron transfer at the electrodes before the field reverses. New nanotweezers were used for each repeat to account for tip-to-tip variation. The nanotweezer was inserted into 100 mM KCl solutions containing fluorescently labelled RNA in the picogram/ μ l range. These were relatively dilute

solutions so that, in cases where RNA had already adsorbed onto the nanotweezer tip from the insertion into solution, this would not be significant enough for there to be uncertainty as to whether trapping had occurred after DEP was applied.

To determine the trapping success, the fluorescence intensity was measured before, during and after application of DEP. The fluorescence determined for each trapping event was measured by measuring the fluorescence at a 3x3 pixel area around the nanotweezer tip 10 s after DEP was applied. This allowed enough time for RNA molecules to become trapped at the nanotweezer tip if DEP trapping was successful (**Figure 3.10a**). Trapping was successful at frequencies between 500 kHz and 3 MHz and exhibited the expected DEP frequency dependency in this range (**Figure 3.10b**). Between 500 kHz and 2 MHz, increasing the frequency led to a higher amount of fluorescent RNA trapped at the nanotweezer tip due to larger DEP forces being generated. After this point, the trapping efficiency decreased, where there were smaller increases in fluorescence at the nanotweezer tip upon application of DEP. This represented approach to the crossover frequency described in **Chapter 1.5.4**, where the behaviour of molecules was transitioning from positive DEP to negative DEP. Higher frequencies were expected to eventually lead to no positive DEP trapping and cause the RNA to move away from the regions of high electric field strengths at the nanotweezer tip.

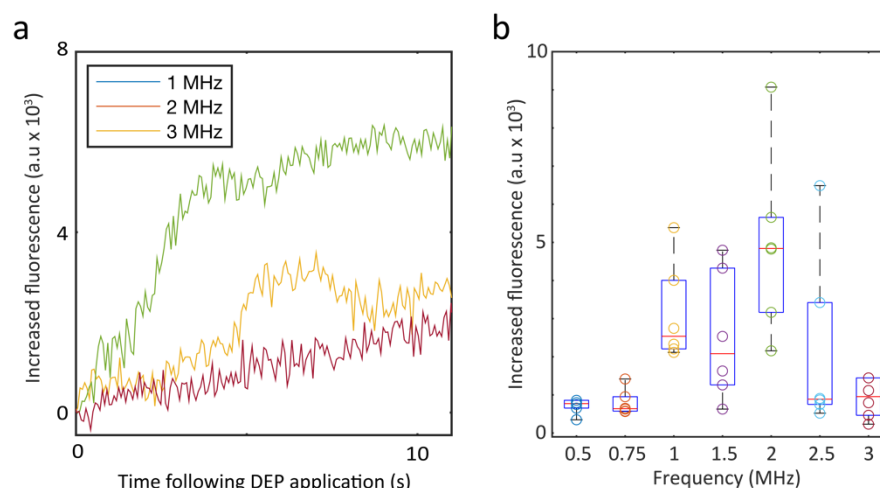


Figure 3.10: Frequency-dependence of DEP trapping of RNA. a) Representative fluorescence-time plots of the change in fluorescence measured at the nanotweezer tip upon application of DEP (1 MHz, 2 MHz and 3 MHz) in a solution of 645 pg/ μ l RNA diluted in 100 mM KCl. b) Overall increases in fluorescence measured at the nanotweezer tip using DEP frequencies between 0.5-3 MHz and a constant voltage of 20 V_{pp}. Outliers that were more than three local scaled absolute median deviations from the median were removed, n = 6 independent nanotweezers.

Based on these fluorescent measurements, 2 MHz was chosen as the optimal frequency for trapping RNA as this showed the highest median increase in fluorescence at the nanotweezer tip upon application of DEP. However, large variations between different nanotweezers were observed, shown by the spread in **Figure 3.10b** which may mean other frequencies, particularly between 1-2 MHz, may also be effective. This variation could be explained by slight manual differences in fabrication of each nanotweezer, as shown previously in **Figure 3.3** where variations in tip size, electrode distance and carbon deposition which would affect the DEP forces generated and therefore the amount of RNA collected. Nevertheless, the frequency optimisation work described here provided a strong foundation for effective frequencies to validate in cells.

3.5.3 Voltage dependence

As explained earlier, the strength of the electric field is proportional to the square of the voltage. Increasing the voltage therefore leads to higher DEP forces for trapping. Although the technology allowed high DEP forces to be generated by the nanometre-sized gap between the electrodes, it was essential to also test this with a range of voltages in order to define the lowest voltage that trapping could occur in this work, in addition to establishing the differences in trapping ability at higher voltages. This allowed a decision to be made based on a balance between achieving the highest DEP forces for trapping RNA, whilst minimising the use of high voltages that may affect cell viability.

In previous work, 20 V_{pp} (1 MHz) was used for effective trapping in cells which, from finite element models, was shown to cause approximately a 10 °C increase in temperature localised at the nanotweezer tip (< 5 nm), with DEP modelled at room temperature in a physiological conductivity of 1 S/m.^{1,2} This heating was negligible 700 nm away from the tip, showing the highly localised effects on the environment. As 20 V_{pp} was previously shown to be successful in cells, voltages between 1-20 V_{pp} were tested in this work at a constant frequency of 2 MHz for trapping fluorescently labelled RNA diluted in 100 mM KCl (**Figure 3.11**). Voltages below 5 V_{pp} were found not to be capable of trapping RNA under these conditions. The increase in fluorescence at a 3x3 pixel area on the nanotweezer tip 10 s after application of DEP was measured (**Figure 3.11a**). A new nanotweezer was used for each repeat to account for differences in tip sizes and trapping ability, representing the planned protocol for future cell experiments where a new nanotweezer would be used for each subcellular biopsy.

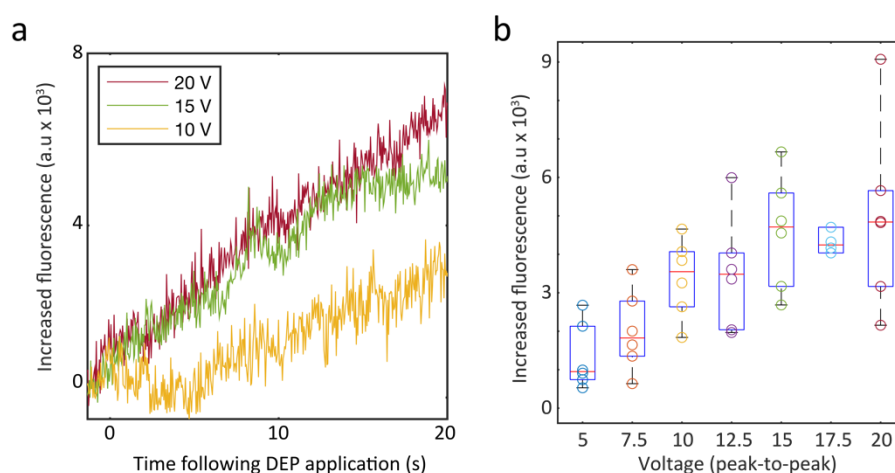


Figure 3.11: Voltage-dependence of DEP trapping of RNA. a) Representative fluorescence-time plots of the change in fluorescence measured at the nanotweezer tip upon application of DEP (10 V_{pp}, 15 V_{pp} and 20 V_{pp}) in a solution of 64.5 pg/μl RNA diluted in 100 mM KCl. b) Overall increases in fluorescence measured at the nanotweezer tip using DEP peak-to-peak voltages of 5-20 Vpp at a constant frequency of 2 MHz. Outliers were defined as more than three local scaled absolute median deviations from the median and were removed, n = 6 independent nanotweezers.

As expected, increasing the voltage led to an increasing trend of more RNA trapped at the nanotweezer tip and higher amounts of fluorescence detected at the tip over the same timeframe (**Figure 3.11b**). Generally, a linear trend was observed between 5-15 V_{pp}, where increasing the voltage led to a steady increase in the amount of trapped RNA. After this, however, a plateau was observed, where further increases in voltage led to fewer increases in the amount of RNA trapped. When 20 V_{pp} was used, bubble formation was sometimes observed as soon as the DEP force was applied. These likely formed as a result of Joule heating and a release of thermal energy at the nanotweezer tip upon application of DEP. Similar observations have been made in other DEP systems where high voltages were used in highly conductive media.²⁸ Although bubbles were only observed occasionally at this voltage, this suggested that 20 V_{pp} may not be suitable for use in cells as it would risk harming cells either via bubble formation physically disrupting the cells, or by heat generation that would harm cells.

From this, it was concluded that a voltage of 15 V_{pp} would be sufficient to take forward for initial trapping of RNA from cells as this could lead to similarly high amounts of RNA trapped at the tip, whilst minimising unnecessary higher voltages and additional heat generation in cells. As with the frequency characterisation, there were variations in different nanotweezers' trapping ability in solution under each condition which could be accounted for by variations in

the fabrication of individual nanotweezer, explained by the variations in electrode size and electrode distance.

3.5.4 Detection

In addition to demonstrating that RNA could be trapped at the nanotweezer based on fluorescence measurements, RT-qPCR was performed on extractions to additionally verify that viable RNA was trapped, along with showing that the amount of RNA trapped could be detected by RT-qPCR. RNA trapped from individual nanotweezer trapping events in 100 mM KCl using the optimised parameters of 2 MHz and 15 V_{pp} was transferred to an optical qPCR tube containing 5 µl nuclease-free water. Individual trapping events were then subjected to one-step RT-qPCR by adding the reagents directly to the qPCR tube. For this initial proof of concept study, β -actin mRNA, a relatively abundant mRNA and a typical RT-qPCR housekeeping gene, was analysed from the trapping events by using primers specific to the *ACTB* gene. Nanotweezer controls were also run where the nanotweezer was inserted into the RNA solution for the same amount of time but DEP was not applied, to account for any RNA that was non-specifically adsorbed onto the tip. β -actin mRNA was successfully detected in 5 out of 8 cases, with Cq values between 28-32 obtained (**Figure 3.12**).

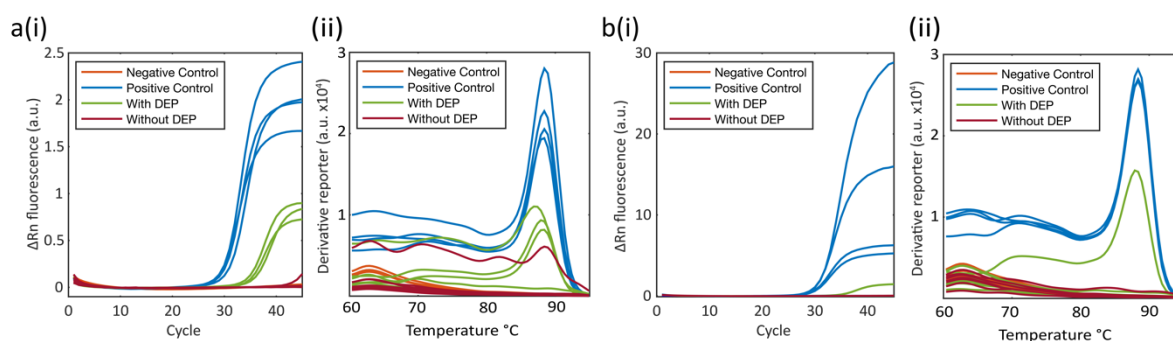


Figure 3.12: RT-qPCR detection of DEP nanotweezer samples. qPCR amplification plots (i) and melt curves (ii) for detection of *ACTB* mRNA in DEP nanotweezer samples (green) compared to controls where DEP was not applied (magenta). ΔRn fluorescence represents the normalised (Rn) and baseline-corrected fluorescence of the emission from the fluorescent reporter dye or probe. Derivative reporter represents the rate of change of fluorescence with respect to time. Positive (blue) and negative (red) controls were run alongside samples to confirm successful amplification using these primers, no non-specific products, and that no contamination was present in the RT-qPCR.

The successful detection of β -actin mRNA suggested that enough RNA was trapped at the nanotweezer under these conditions for analysis and the DEP employed under these conditions did not damage the RNA, providing promising results for future gene expression analysis in

cells using these parameters. In one case, a control nanotweezer which did not have DEP applied in solution showed small amplification of the target (**Figure 3.12a**). This is likely via non-specific adsorption of RNA at the tip, as observed with the fluorescence measurements earlier. Compared to the DEP samples run in the same qPCR, however, this gave a much larger Cq value (41 compared to 29 ± 1). A Cq larger than 40, particularly for SYBR Green chemistry, is typically discounted and not accepted as a true amplification,²⁹ meaning that any non-specific adsorption of this type of mRNA was insignificant. This validated that the DEP enhanced the amount of RNA isolated from solution compared to non-specific adsorption, consistent with what was observed with the fluorescent measurements. For the DEP samples where there was no β -actin mRNA detected, this could be explained by β -actin mRNA not getting trapped during the trapping events for those particular nanotweezers. mRNA typically only makes up $\sim 4\%$ of total RNA in mammalian cells;³⁰ it is feasible that specific mRNAs may not get trapped during a single trapping event in dilute RNA solution.

3.6 Conclusions

In this chapter, the nanotweezer technology has been characterised and optimised, building on the fundamental work previously performed. Adjusted fabrication methods were outlined, where the size of the electrodes, electrode-electrode distance and deposition of carbon were fine-tuned to produce nanotweezer electrodes of the expected size for optimal DEP trapping. Size characterisation was essential to ensure that the distance between the electrodes remained small to achieve high enough trapping forces for trapping small biomolecules. Electrode formation and electrical contact to the electrodes were confirmed via LSV before testing the trapping efficiency in solution.

Fluorescent imaging was used as the primary method for confirming successful DEP trapping of biomolecules. Trapping and manipulation of fluorescent polystyrene beads and λ -DNA was demonstrated which reproduced and validated the previous work. The focus of the work in this chapter, however, was primarily on DEP trapping of RNA for the future aim of isolating RNA from cultured neurons. RNA trapping in physiological conductivity conditions was characterised to determine the optimal frequency and voltage required to trap RNA under these conditions. The optimal frequency, which showed the highest median increase in fluorescently labelled RNA measured at the nanotweezer tip upon application of DEP, was determined to be 2 MHz. The voltage-dependency suggested that 15 V_{pp}, rather than 20 V_{pp} as previously

employed, may be sufficient for optimal trapping. This lower voltage would prove to be advantageous in cells as it would reduce heat generation from Joule heating which may otherwise affect cell viability. Under these conditions, the trapped RNA was successfully detected using RT-qPCR, demonstrating that enough RNA was trapped to be detected using gold standard molecular biology techniques, and that the process did not damage the RNA. Taken together, this work formed a solid foundation for the conditions required to trap RNA under physiological conditions using the nanotweezer. Although these parameters form the basis for highly effective DEP trapping of RNA from cells in the future, cells are heterogeneous and highly complex, so the phenomena observed in solution may not be fully replicated in cells. It is therefore essential that these parameters are tested in cells in proof-of-concept experiments prior to any comprehensive gene expression analysis. In particular, the viability and morphology of cells before, during and after the biopsy procedure, as well as any effects resulting from unexpectedly large tip sizes or Joule heating (bubble formation or whole cell trapping), should be carefully monitored.

It is also important to note that DEP trapping is size-dependent and these optimal trapping conditions would be based on the average size of single-stranded total RNA. Previous nanotweezer work showed that there is a larger field requirement for shorter length DNA,¹ suggesting that trapping shorter length nucleic acids are more challenging. This may imply that the RNA trapping described herein may have a bias towards longer length RNAs as these will be more readily trapped. The optimised frequency may therefore be more biased towards these longer length RNAs. As there is a particular interest in trapping mRNA from cells in this work, this bias would not be problem as mRNAs make up the longer proportion of RNAs, being on average 2000 nucleotides in length in mammals.³⁰ Nevertheless, the frequency employed and characterisation could be further modified if there was a desire to trap small RNAs. For example, microRNAs (miRNA) are typically 22 nucleotides in length and are of particular interest for their role in regulating gene expression and the localisation of certain mRNAs in different subcellular compartments of neurons.³¹ Through specific isolation and labelling of miRNAs, rather than total RNA as employed here, one could optimise the frequency and voltage for trapping these shorter length RNAs in cells and use the technique to map and study specific microRNAs in subcellular regions of neurons.

RNA was shown to adsorb readily when in contact with the nanotweezer tip, with enhanced RNA trapping upon application of DEP. Although attempts to remove the isolated RNA from

the nanotweezer tip were unsuccessful, this would greatly enhance the technology; it would offer possibilities to reuse the nanotweezer if RNA was washed from the tip, reducing waste and costs, and it could allow for pick-and-place of RNA molecules. This latter application could greatly enhance the throughput of the technique by minimising pipetting steps and manual handling of the nanotweezer. With automation, this could also lead to further improved throughput and smoother operation of the nanotweezer.

Overall, this chapter formed the basis for future application of the nanotweezer for isolating RNA from hippocampal neurons, as will follow in **Chapter 4**, via development of the electrode and tip size for efficient trapping, combined with the optimal DEP conditions to be taken forward for nanobiopsy in cells.

3.7 References

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Chapter 4

Spatially and temporally resolved nanobiopsy for differential mRNA regulation

Synopsis

The ability to analyse the transcriptome of subcellular compartments of living neurons is crucial for understanding RNA regulation and local protein production for maintaining neuronal homeostasis. This could help unravel the mechanisms of basic neuronal processes like synaptic plasticity and understand how dysregulation of these mechanisms may lead to neurodegeneration. However, many single-cell techniques do not allow for precise subcellular locations to be accessed or do not allow for dynamic measurements to be made on the same cell, limiting our ability to understand true subcellular single-cell responses. In this chapter, the nanotweezer is developed to trap and extract RNA from different subcellular compartments of the same living neuron to assess RNA localisation and compartmentalisation. The postsynaptic sites are also precisely accessed and changing RNA dynamics are measured in the same cell before and after induction of synaptic plasticity. The work highlighted in this chapter opens novel opportunities for measuring RNA dynamics in single cells with high subcellular spatial resolution and minimal impact on cell viability.

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4.1 Introduction

RNA localisation is the directed delivery of mRNA transcripts from the nucleus, where DNA is transcribed into RNA, to distally localised sites of cells before their local translation into proteins.^{1,2} In polarised cells such as neurons, RNA localisation is a key post-transcriptional process for neuronal homeostasis, where up to half of neuronally expressed mRNAs in the hippocampus are estimated to be enriched in the dendrites compared to the soma.³ This genetic regulation is required for a wide range of neuronal functions such as synaptic plasticity, synaptogenesis and axonal guidance.⁴

Many tools such as RNA FISH,^{5,6} deep sequencing,^{3,7} and live-cell imaging^{8–10} have been essential in revealing mRNAs that are differentially localised in neurons. However, the former two require cell fixation or destruction of the cell to assess the subcellular compartments of neurons, and the latter requires expensive sequence-specific probes. The nanotweezer offers an inexpensive method for performing live single-cell measurements of RNA on the same cell to understand RNA dynamics, with the potential to discover novel localised transcripts by incorporating an exploratory approach. Previous simulations reported that the DEP trapping forces achieved by the nanotweezer can reach 300 nm away from the nanotweezer tip.¹¹ This implies great potential for the tool to extract from precise subcellular compartments, or even smaller domains such as the synaptic contacts, with high spatial resolution. The nanotweezer therefore offers a novel method to precisely assess mRNA localisation in living cells by comparing subcellular regions within the same cell. This has been shown in recent work where the tool was used to detect differential mRNAs in the perinuclear region of rat cardiomyocytes, as well as differences in this localisation behaviour in a heart disease model.¹² Most importantly, the nanotweezer also opens possibilities for performing dynamic transcript analyses on the same living cell to assess how gene expression and RNA regulation are altered under different conditions such as with synaptic activity and neurodegeneration. This could help uncover novel transcripts and mechanisms to better understand basic neuronal biology, as well as provide potential treatment targets for neurodegenerative diseases.

In this chapter, the nanotweezer is applied to living primary rat hippocampal neurons to isolate and detect RNAs in the soma, dendrites, axons and dendritic spines of cells. The nanotweezer is validated as a tool to detect RNA localisation and show spatial differences in gene expression within the same cell via successful sequential biopsies in the same neuron. Additionally, RNA

is precisely extracted from the base of the dendritic spines without affecting cell viability, advancing the spatial resolution of other subcellular sampling techniques. Finally, differential localisation of gene expression is measured in the dendritic spines of the same cell before and after the induction of synaptic plasticity, demonstrating the potential of the tool for performing dynamic gene expression analyses of the same single cell under different cellular treatments and models.

4.2 RNA isolation from live neurons

4.2.1 RNA nanobiopsy

For DEP trapping of biomolecules from living primary rat neurons, the same procedure was followed that was previously reported for live cell nanobiopsies.¹¹ This is illustrated by **Figure 4.1**. The nanotweezer was inserted into the single cell or region of interest within a cell, the DEP force was applied for 10 s, then the nanotweezer was removed from the cell and inserted into a PCR tube containing 5 μ l of nuclease-free water.

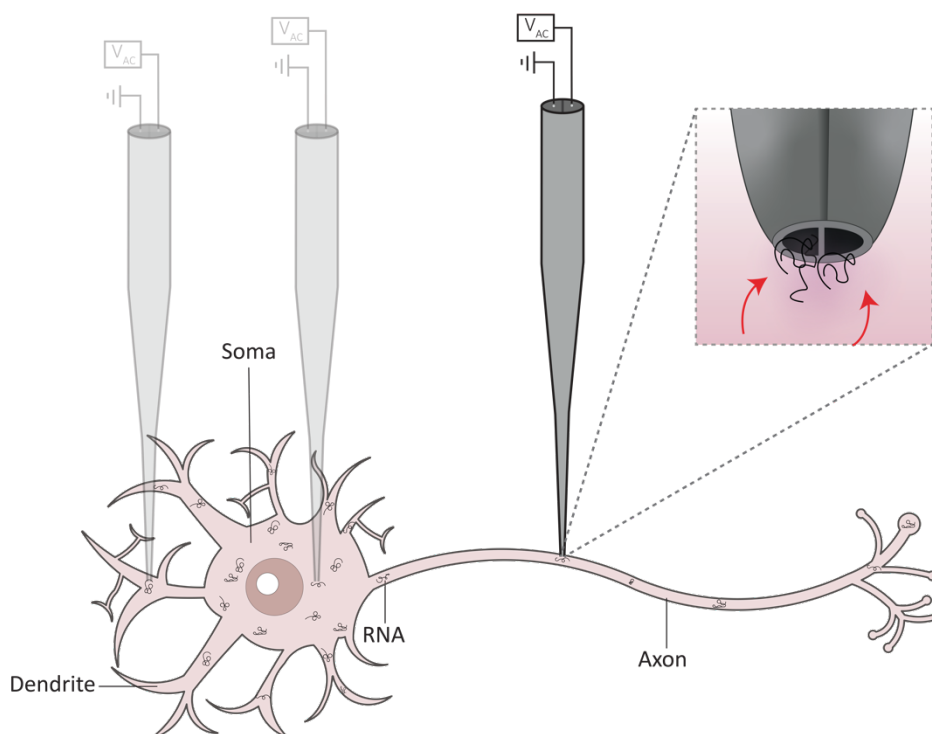


Figure 4.1: Schematic of RNA dielectrophoretic trapping from living neurons. The nanotweezer was inserted into a subcellular region of a single neuron and application of an AC voltage led to DEP trapping of RNA from a confined region within the cell. Different regions within neurons could be targeted, including the soma, axon and dendrites.

To initially confirm that RNA was trapped from neurons using the nanotweezer, the RNA of hippocampal neurons was labelled with SYTO RNASelect fluorescent stain. This dye is cell permeant and selectively binds to RNA in live cells, allowing for visualisation of RNA trapping using DEP. The nanotweezer was inserted into the soma of a single hippocampal neuron and the fluorescence was observed during the biopsy process. Upon application of DEP, an increase in fluorescence was observed at the location of the nanotweezer tip (**Figure 4.2c(ii)**), signifying RNA being trapped at the tip. Removal of the nanotweezer from the cell led to this signal being removed from the same plane of the cell (**Figure 4.2c(iii)**), indicating extraction of RNA from the cell. Refocusing on the nanotweezer tip outside the cell showed that a fluorescent signal was maintained on the nanotweezer tip outside of the cell (**Figure 4.2c(iv)**), representing the RNA trapped from the cell and confirming successful trapping and isolation of RNA using the technique. This demonstrated the proof-of-concept work for RNA isolation from living neurons using the nanotweezer.

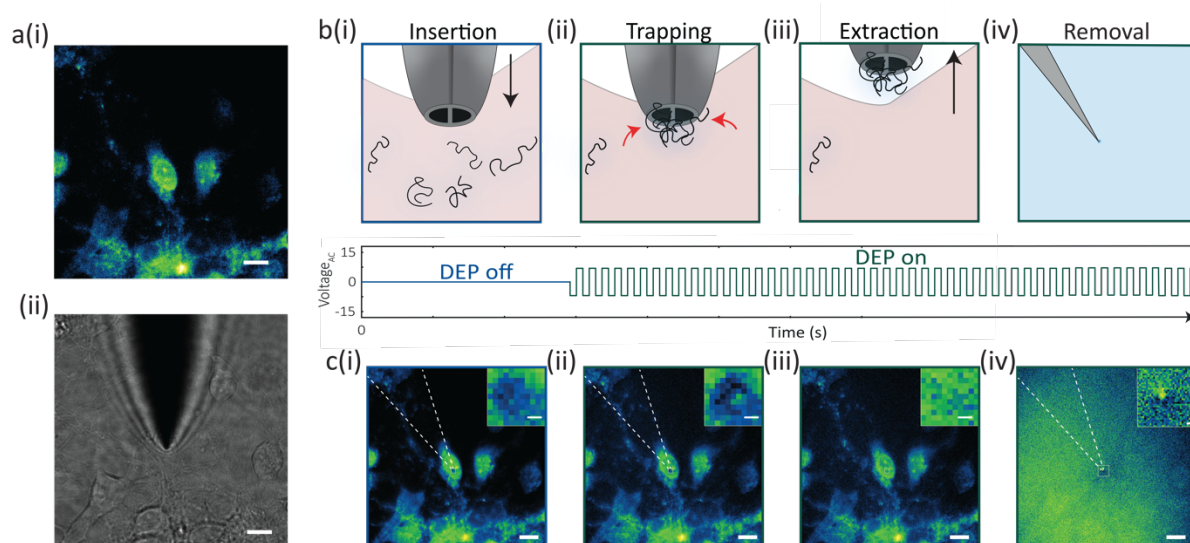


Figure 4.2: Labelled RNA nanobiopsy from a live single neuron. a) Imaging of hippocampal neurons labelled with SYTO RNASelect fluorescent stain (green) before insertion of the nanotweezer (i) and bright-field image of the nanotweezer inserted into the soma of the cell (ii). b) Schematics of the insertion of the nanotweezer into a neuron (i), DEP trapping of RNA upon application of an AC voltage at 1 MHz 20 V_{pp} (ii), extraction of RNA via removal of the nanotweezer from the cell (iii), and the nanotweezer outside the cell with RNA trapped at the tip (iv). c) Fluorescent imaging of the nanotweezer procedure with inset images showing the biopsy position: cell insertion (i), RNA trapping (ii), cell removal (iii), and confirmation of trapped RNA at the nanotweezer tip (iv). Scale bars = 10 μm, inset scale bars = 500 nm.

Not only was the nanotweezer capable of being inserted in the soma of neurons, similarly to cells with round morphologies,¹¹ the axons and dendrites of neurons could also be targeted.

This built on the spatial subcellular resolution of the technology by showing that specific and more restricted subcellular compartments of cells could be targeted for biopsy. To demonstrate this, cells were transfected to express green fluorescent protein (GFP) so that whole cells and their compartments could be visualised clearly. Using different nanotweezers for each biopsy, the nanotweezer was inserted into the soma, dendrites and axon of the same neuron and biopsied (**Figure 4.3**). The rest of the cell remained intact after each biopsy, suggesting that analysing multiple regions and compartments of the same cell could be possible via sequential biopsies.

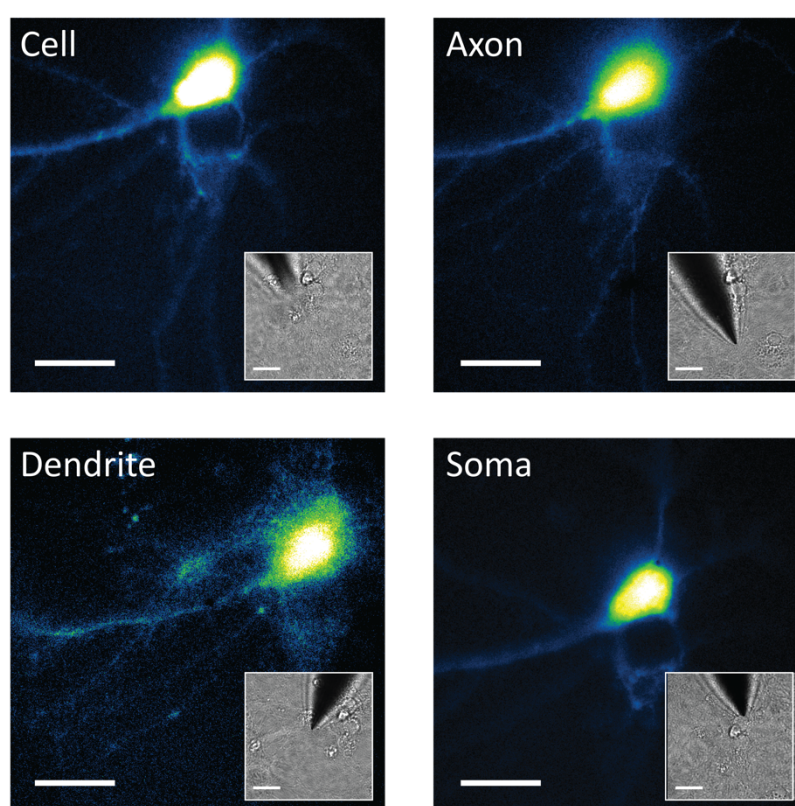


Figure 4.3: Subcellular and sequential biopsies from the same cell. Fluorescent imaging of GFP-labelled hippocampal neuron before biopsies (Cell), during axonal biopsy, dendritic biopsy, and soma biopsy. Inset: Corresponding bright-field images of cells and nanotweezer positioning. Scale bars = 20 μm .

As RNA is present throughout a neuron, labelling the total RNA in the cells was considered unnecessary for future RNA extraction work as RNA was expected to be trapped in all neuronal locations, assuming that the DEP application was successful. Studies have shown that fluorescent labelling can lead to phototoxicity,¹³ compromised cell viability,¹⁴ and physiological interferences.¹⁵ Label-free nanobiopsies were therefore advantageous for

minimising these effects on cells and providing more representative gene expression analyses. This would also lead to simpler and cheaper cell culture preparations, where cells could be maintained in their standard culture environment with minimal application of exogenous reagents. Where possible, label-free nanobiopsies were performed in the same manner as previously described, where the nanotweezer was approached to the cell and the tip was inserted (**Figure 4.4**), DEP was applied, and then the nanotweezer was removed from the cell for downstream analysis of the trapped RNA. Label-free extraction from the neurites of neurons was possible via careful positioning of the nanotweezer.

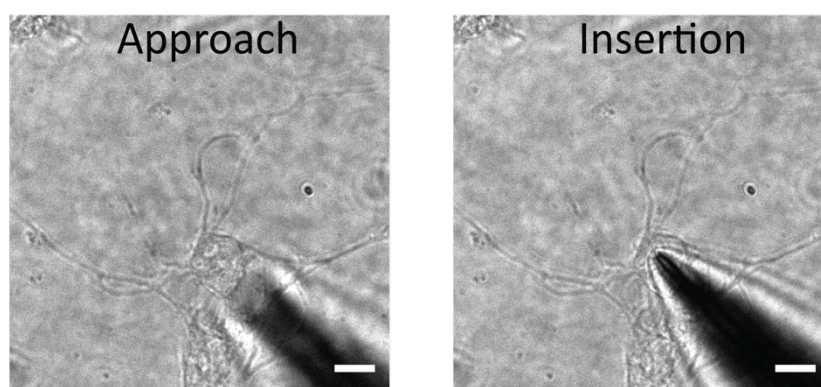


Figure 4.4: Label-free RNA nanobiopsy procedure. The nanotweezer was approached to a region of a live single hippocampal neuron using bright-field imaging (left) and slowly inserted into the cell (right). Scale bars = 10 μ m.

4.2.2 Single nanobiopsy detection

The nanotweezer was not specific for trapping RNA from cells; it trapped anything polarisable in the targeted region. Specific detection of RNA was therefore performed by designing primers to target the cDNA of the extracted RNAs of interest. Primers were designed to span exon-exon junctions to prevent amplification of any genomic DNA that may get trapped during application of DEP. This was particularly important as it reduced the need to deplete any genomic DNA contained in the sample, as is often performed with bulk RNA isolations using a DNase I treatment, minimising the processing steps prior to analysis which could otherwise lead to some loss of the RNA sample and poorer detection.

Initially, single biopsies were analysed for 18S rRNA. 18S rRNA is an extremely abundant RNA so was used to confirm isolation of RNA from neurons using the nanotweezer. Primers

were designed as outlined in **Chapter 2.10**. Primer specificity was validated using bulk rat cDNA via PCR followed by gel electrophoresis, as well as qPCR melt curve analysis for more sensitive product analysis, showing single bands of the expected amplicon size (228 bp) in the gel and a single distinct peak in the qPCR melt curve, with no amplification of negative controls or non-specific amplification melt peaks (**Figure 4.5**).

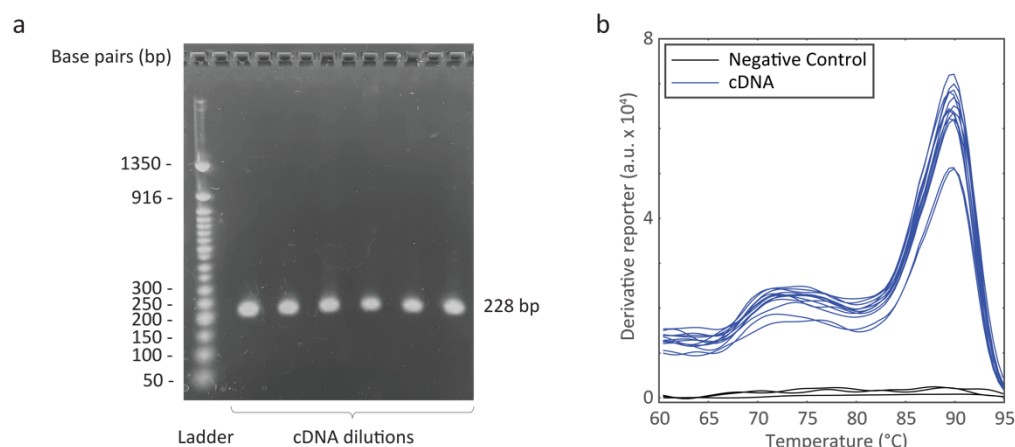


Figure 4.5: 18S primer design. a) Agarose (2.5 %) gel electrophoresis of PCR amplified bulk rat cDNA (10-fold dilutions from 50 ng to 50 fg total cDNA) using designed 18S primers (228 bp amplicon) alongside a 50 bp DNA ladder for size comparison. Gel showed a single amplified product and no other products. b) qPCR melt curve analysis of total cDNA dilutions and negative controls (water) using designed 18S primers, showing a single melt curve peak corresponding to the amplified product and no amplification, primer-dimer or non-specific products in the negative controls. Derivative reporter represents the rate of change of fluorescence with respect to time.

The previously optimised frequency of 2 MHz from **Chapter 3.5.2** was tested in live neurons by trapping from the soma of cells. As the work in this chapter suggested that frequencies between 1-2 MHz could be effective, and previous work used 1 MHz for trapping within live cells,^{11,12} a comparison of trapping at 2 MHz and 1 MHz was performed in neurons to determine which frequency gave enhanced trapping of RNA *in vitro*. Nanobiopsies were taken from the soma of neurons and 18S rRNA was analysed, where the amount of total RNA trapped at the nanotweezer tip was estimated using RT-qPCR. This was quantified by constructing a standard curve within the same qPCR run, made up of serial dilutions of known amounts of cDNA that had been converted from rat hippocampal RNA. It should be noted that the quantification of cDNA assumes a 100 % conversion of RNA to cDNA during reverse transcription. RT-qPCR on individual nanobiopsies performed at either 1 MHz or 2 MHz at 15 V_{pp} showed that trapping at 1 MHz isolated higher amounts of RNA in all biopsies compared to 2 MHz (**Figure 4.6a**). Although the optimisation in **Chapter 3.5.2** gave a larger median value at 2 MHz for trapping, the large variations in different nanotweezers meant that 1 MHz was also a prospective

effective frequency to use. The follow-on *in vitro* work in the current chapter therefore corroborated this. It is also important to note that the optimisation work in **Chapter 3.5** only provided conditions to match the conductivity of a cell, and, in practice, there will be many other complications in cells such as cytoplasmic drag,¹⁶ active transport¹⁷ and differences in viscosity¹⁸ which may affect the DEP force required for trapping.

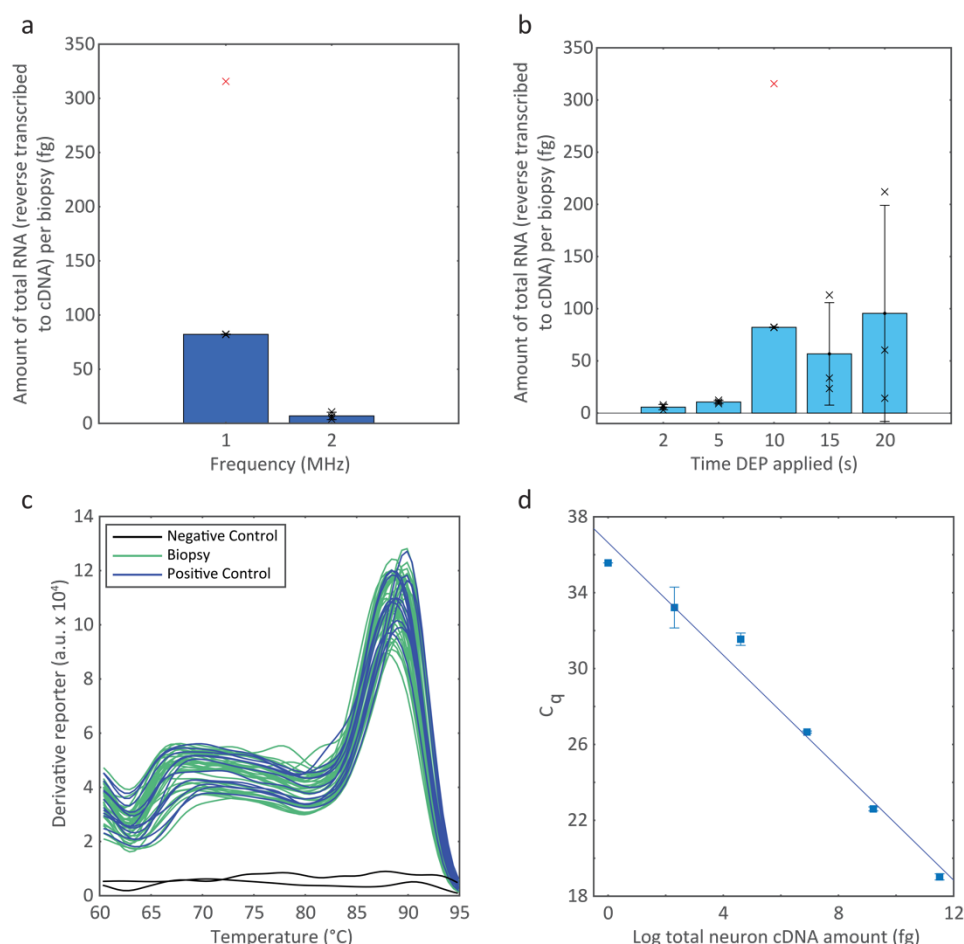


Figure 4.6: DEP trapping efficiency in cells under different DEP conditions. a) Estimated amount of RNA (cDNA) isolated per biopsy at 1 MHz and 2 MHz with DEP applied for 10 s at 15 V_{pp} (n = 3). Bars show the average amount of RNA (reverse transcribed to cDNA) detected in RT-qPCR and crosses show actual values. Outliers, defined as more than 3 scaled median absolute standard deviations from the median, were removed from calculations but are shown in red. b) Amount of RNA (cDNA) isolated per biopsy with DEP applied at 1 MHz 15 V_{pp} for 2 s, 5 s, 10 s, 15 s and 20 s (n = 3). Bars show the average amount of RNA (reverse transcribed to cDNA) detected in RT-qPCR and crosses show actual values. c) Melt curve analysis of RT-qPCR samples using primers for 18S rRNA, showing a distinct single curve for the PCR product, confirming primer specificity. Derivative reporter represents the rate of change of fluorescence with respect to time. d) Standard curve constructed for absolute quantification of RNA isolated using the nanotweezer. Pure rat hippocampal RNA of a known concentration was converted to cDNA and 10-fold dilutions were performed to construct a standard curve, with wells containing from 10 pg to 1 fg total cDNA. RNA to cDNA conversion was assumed to be 100 %. Primers for 18S rRNA were used to amplify nanobiopsy samples and positive control dilutions (total cDNA) alongside negative controls. The amount of total RNA isolated from cells using the nanotweezer was calculated by interpolating C_q values obtained from samples with the standard curve. Error bars represent s.d.

A comparison of DEP application over different timeframes (2 s, 5 s, 10 s, 15 s and 20 s) was also investigated in cells to evaluate the increase in trapping with longer applications of DEP (**Figure 4.6b**). Application of DEP for 2 s and 5 s led to smaller amounts of RNA collected at the tip compared to ≥ 10 s. With the sample size used in this work, little difference was observed between applying DEP for 10 s, 15 s and 20 s in cells. This, combined with the work in **Chapter 3.5** which showed that 10 s was sufficient for trapping fluorescently labelled RNA, suggested that 10 s timeframes were sufficient for trapping RNA in cells. This also allowed for the possible heating effects from DEP to be minimised by minimising the time spent in the cell which could therefore minimise possible effects on cell viability. From the quantification of total RNA isolated using the nanotweezer based on the standard curve of known dilutions of total rat hippocampal RNA (reverse transcribed to cDNA), it was estimated that the amount of total RNA isolated from neurons using the nanotweezer was in the femtogram region (≈ 10 -100 fg). The amount of total RNA isolated with the nanotweezer had not been previously quantified but could be useful for optimising future downstream analysis applications such as RNA-seq; this method of quantification could be used to support efforts to maximise the amount of RNA trapped at the tip for success in these applications.

Following from this work, single nanobiopsies from the axons, dendrites and soma of hippocampal neurons were performed using conditions of 1 MHz and 15 V_{pp}, and individual biopsies were analysed using RT-qPCR. The presence of 18S rRNA from individual biopsies was evaluated and showed successful amplification and detection in all biopsies (**Figure 4.7**), suggesting successful isolation and detection of RNA from these subcellular locations and using these DEP parameters.

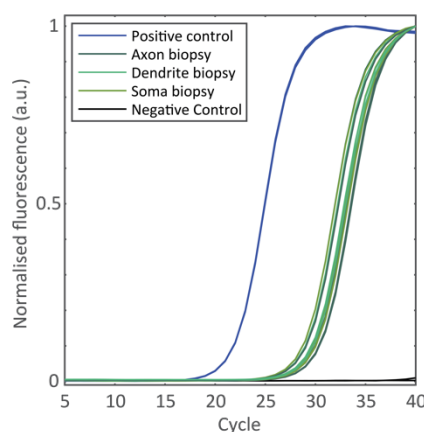


Figure 4.7: 18S rRNA detection in single nanobiopsies. RT-qPCR amplification of individual biopsies from the axons, dendrites and soma of live hippocampal neurons, showing successful detection in all biopsies.

4.3 Dendritic mRNA localisation

4.3.1 Target and multiplexed design

Having shown that RNA could be detected from different subcellular locations of single live neurons, the nanotweezer was used to establish if it could be used as a tool to study mRNA localisation in neurons. MAP1A mRNA was initially chosen as the target to assess dendritic localisation in the hippocampal neurons used in this work. MAP1A mRNA is an mRNA that has been shown to localise in the dendrites of hippocampal neurons in a number of studies.^{3,5–7,19,20} In particular, an extensive MERFISH study (pre-print) on DIV 18 hippocampal mouse neurons found MAP1A mRNA to have the highest amount of dendritic localisation compared to the soma out of the 950 genes they studied.⁵ They found that MAP1A was one of the few genes that showed more mRNA localisation in the dendrites than in the soma, with a dendrite-to-soma ratio of approximately 1.5. This gene was therefore used to validate the nanotweezer as a potential tool for assessing RNA localisation in specific subcellular compartments by corroborating the detection of a known localising mRNA.

As 18S rRNA was shown to be detected in all nanotweezer biopsies, it was chosen as a biopsy control, where sufficient detection of 18S would indicate a successful nanobiopsy. Previous work has also shown that 18S rRNA is present in both the soma and dendrites of neurons.²¹ RNA FISH was used to further assess the distribution of 18S rRNA in the hippocampal cultures used in this work (**Figure 4.8**). The presence of 18S throughout the neuronal structure, particularly in the soma and proximal dendrites, suggested that 18S could be an effective normalisation gene for accounting for differences in the DEP trapping efficiency of nanotweezers when comparing subcellular locations in single cells.

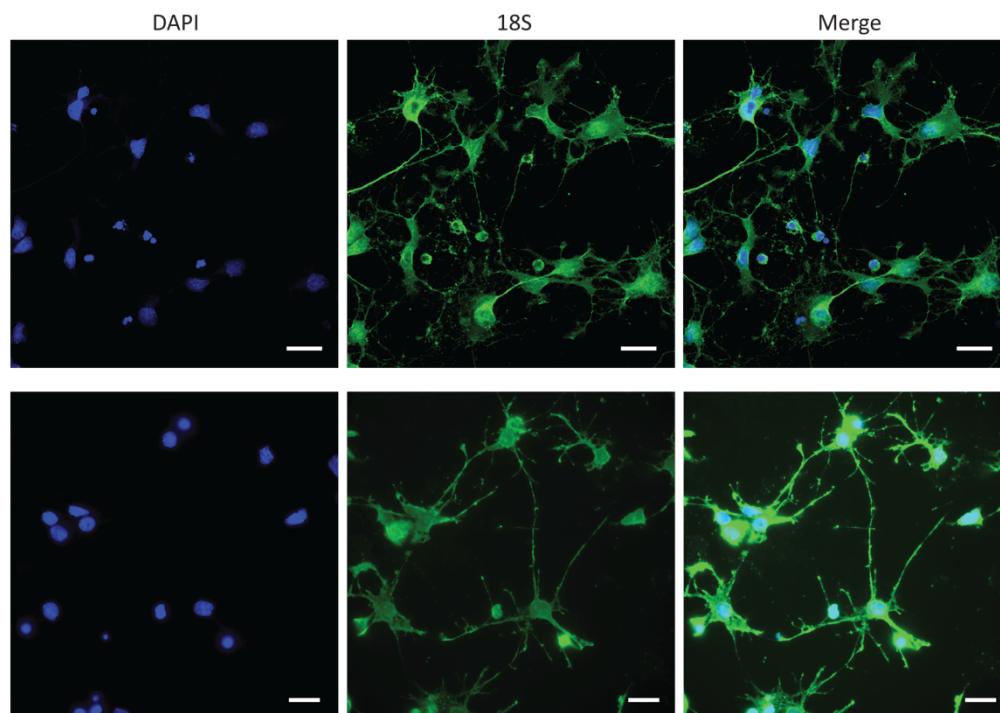


Figure 4.8: Distribution of 18S rRNA in hippocampal neurons. Representative RNA FISH images of hippocampal cultures labelled for 18S rRNA (green) with nuclear DAPI staining (blue). 18S rRNA was shown to be present throughout the neuronal structure. Scale bars = 20 μm . RNAscope was performed by Dr Zoe Kwan at Imperial College London.

As the amount of RNA extracted using the nanotweezer was extremely small and would potentially push the limits of detection of the qPCR, particularly for low abundant targets, maximising the amount of RNA used in qPCR analysis was imperative. This was performed by designing a multiplexed qPCR assay, so that MAP1A and 18S RNA could be quantified in the same limited sample. Multiplexing also allowed for increased accuracy of normalisation as the normalising gene and target gene would be analysed from the same volume of sample. A probe-based qPCR assay was developed by designing custom primers and probes for 18S that were compatible with pre-designed primers and probes for MAP1A. Although probe-based qPCR has the advantage of specific target detection, negating the risk of non-specific detection of targets like in a SYBR Green assay and improving the accuracy of target amplification, non-specific amplification may still affect the efficiency of reactions due to the reaction reagents being used up on non-specific products. The compatibility of the multiplexed design was therefore confirmed by gel electrophoresis of the qPCR products from bulk cDNA amplification in the multiplexed reaction (**Figure 4.9**), showing two clean bands of the expected amplicon sizes and no non-specific products. Single and duplex RT-qPCR reactions using the primer/probe sets were also compared using 28 ng of rat RNA. This gave the same

Cq values in the single reaction as the duplex reaction (18 for 18S and 26 for MAP1A) which further showed that the multiplexed reaction did not affect the efficiency of amplification of either target. The primer/probe design was therefore taken forward for analysing single nanobiopsies.

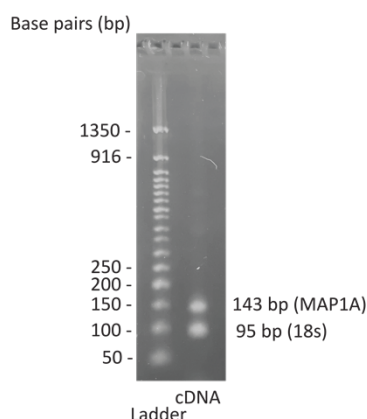


Figure 4.9: Multiplexed MAP1A and 18S qPCR design. Agarose (2.5 %) gel electrophoresis of qPCR amplified bulk rat cDNA in a multiplexed reaction using designed primers and probes for MAP1A (146 bp amplicon) and 18S (95 bp amplicon). Gel showed two distinct bands for each of the expected amplicons and no non-specific products.

4.3.2 Confirmation of enhanced RNA isolation

Continuing from the initial detection of RNA from subcellular compartments of neurons, the extent of RNA isolation using the DEP nanotweezer was evaluated by comparing nanobiopsies from cells to various controls. Firstly, a comparison of the nanotweezer in the absence of DEP was important to assess, as previous characterisation work (**Chapter 3**) showed that RNA could get non-specifically adsorbed onto the nanotweezer tip before DEP was applied. It was therefore imperative to determine if the RNA detected in the downstream analysis was due to the enhanced RNA isolation from DEP trapping, or due to non-specific adsorption of RNA when in contact with the nanotweezer tip. This would validate the DEP trapping ability of the nanotweezer in cells as a viable method for enhanced RNA extraction.

Nanobiopsies were initially performed on immature neurons at day-in-vitro (DIV) 3-4. Biopsies were taken from the soma and dendrites of cells (**Figure 4.10a-b**), using a new nanotweezer for each biopsy. RT-qPCR was then performed on these biopsies using the newly designed multiplexed reaction to target MAP1A and 18S in the same sample. The following controls were also performed: Insertion of the nanotweezer in cells without applying DEP to

account for any non-specific adsorption of RNA from the cell (**Figure 4.10c**); insertion of the nanotweezer into the cell's media and application of DEP to account for RNA trapping from the media and not the cell itself (**Figure 4.10d**); insertion of the nanotweezer into the cell's media without application of DEP to account for non-specific adsorption of RNA from the media (**Figure 4.10e**); and negative controls in the RT-qPCR where RNA was replaced with nuclease-free water to account for any contamination during the workflow.

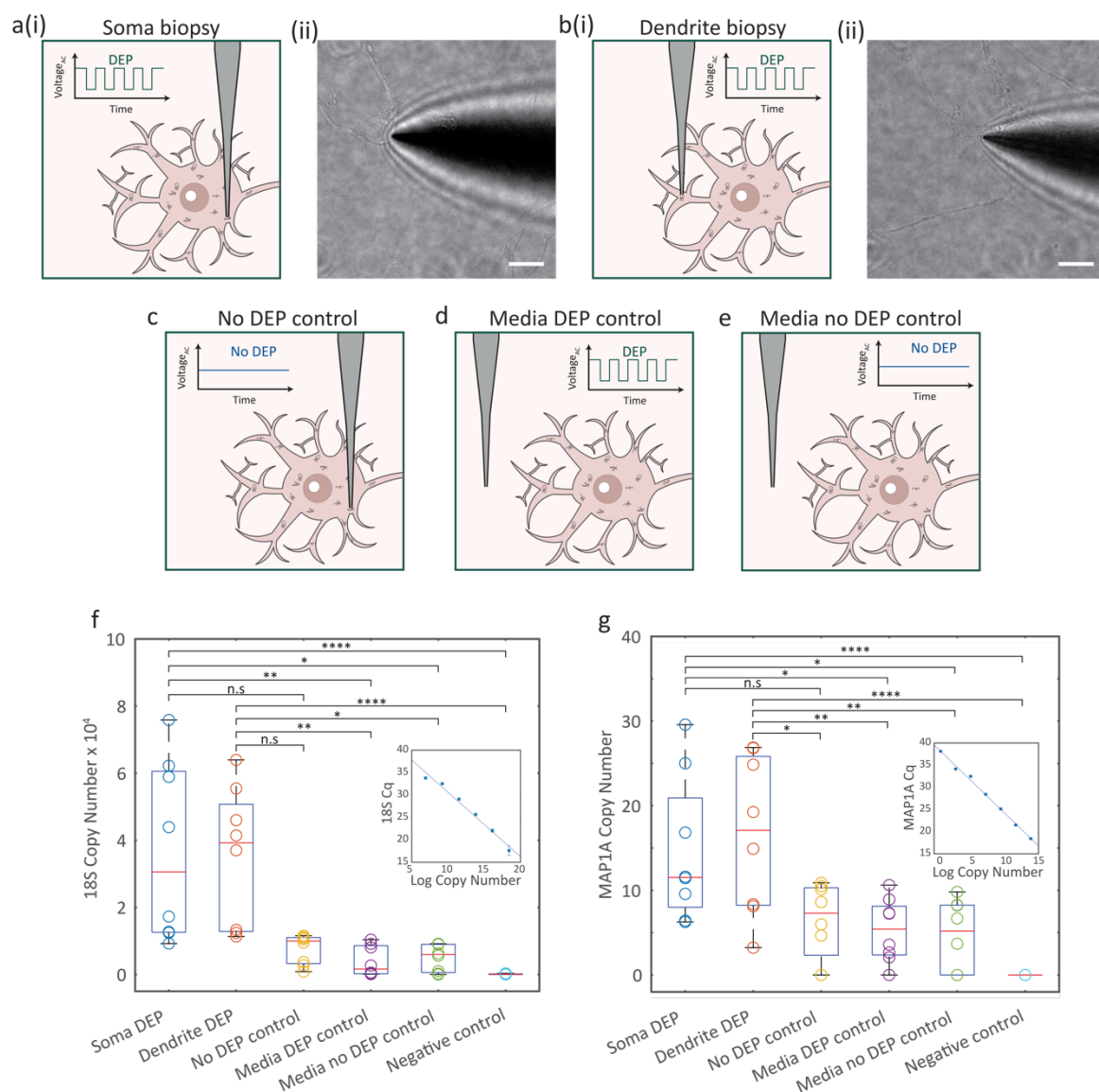


Figure 4.10: Nanobiopsy quantification and controls. a) Schematic of the soma biopsy procedure (i) and label-free bright-field image. b) Schematic of the dendritic biopsy procedure (i) and label-free bright-field image. Scale bars = 20 μ m. c) Schematic of nanotweezer biopsy control in the absence of DEP. d) Schematic of the DEP control from cell culture media. e) Schematic of the media control in the absence of DEP. f) Boxplot showing quantification of 18S copy numbers obtained from biopsy and control samples. Statistical analyses were performed using the Kruskal-Wallis non-parametric test and Dunn's multiple comparisons test between each DEP biopsy sample type and controls. g) Boxplot showing quantification of MAP1A copy numbers obtained from biopsy and control samples (n = 8). Statistical analysis was performed using one-way ANOVA followed by

Sidak's multiple comparisons test between DEP biopsy samples and controls. Shapiro-Wilk normality test was performed prior to statistical analyses. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s = not significant. Insets: Representative standard curves for qPCR absolute quantification with data represented as mean $\pm$ s.d.

MAP1A as well as 18S RNA was detected in soma and dendritic biopsies, showing the feasibility of mRNA extraction from compartments of living neurons using the nanotweezer. Some RNA was detected in the cell media either from non-specific adsorption or the application of DEP. These copy numbers, however, were significantly less than the copy numbers obtained from the DEP nanotweezer. This RNA could be extracellular RNA derived from extracellular vesicles from exocytosis.²² Work has shown that extracellular vesicle RNA has also been associated with neurodegeneration, where exosomes isolated from cerebrospinal fluid from Alzheimer's and Parkinson's disease patients contained several differentially expressed microRNAs and mRNAs, including for amyloid precursor protein and α -synuclein mRNAs.²³ This extracellular RNA could be explored using the nanotweezer, where the detection of certain extracellular RNAs could be used as potential biomarkers of disease progression. In some cases, there was no significant difference between the DEP biopsies and the no DEP controls that involved nanotweezer insertion into the cell in the absence of DEP. This may be due to the variations in individual nanotweezers, where some nanotweezers had poorer trapping ability than others. Although this does not impact the spatial results obtained from the nanotweezer when assessing RNA localisation in defined locations, future work should focus on ensuring consistent nanotweezer fabrication for high DEP trapping in order for the nanotweezer to be a reliable enhanced RNA isolation tool.

4.3.3 MAP1A localisation

For validating MAP1A RNA localisation in these cells using the nanotweezer, multiple biopsies were taken by sequentially targeting the soma and dendrites of the same live cell, using a new nanotweezer for each biopsy. MAP1A was quantified by absolute qPCR quantification followed by normalisation against 18S copy numbers to account for differences in nanotweezer trapping abilities. As cells are heterogeneous, it was important to make comparisons of the same cell as each cell has dynamic changes in their total RNA contents which may differ from others. The soma and dendrites of the same cell were therefore compared in order to unmask averages made with bulk measurements and observe true single cell expression levels of MAP1A. Multiple biopsies from same cell were performed over different development stages of the neurons from DIV 3 to DIV 11 by targeting the soma and

the proximal dendrites (**Figure 4.11a**). The proximal dendrites were used to ensure consistency between dendritic sampling of cells; previous work has shown that there is generally a decrease in abundance of mRNAs further away from the soma which further highlights the importance of subcellular analysis, even within subcellular compartments. This means that distal dendrites may show differing localisation behaviour to proximal dendrites and, as the number of biopsies from the same cell was limited, the location of dendritic sampling needed to be controlled. The proximal dendrites were therefore chosen as these would have higher abundances of mRNAs for detection, there would be improved ease of locating them in a label-free manner compared to distal dendrites, and the targeting would be simpler because proximal dendrites only make up a single protrusion from the soma as opposed to many branches.

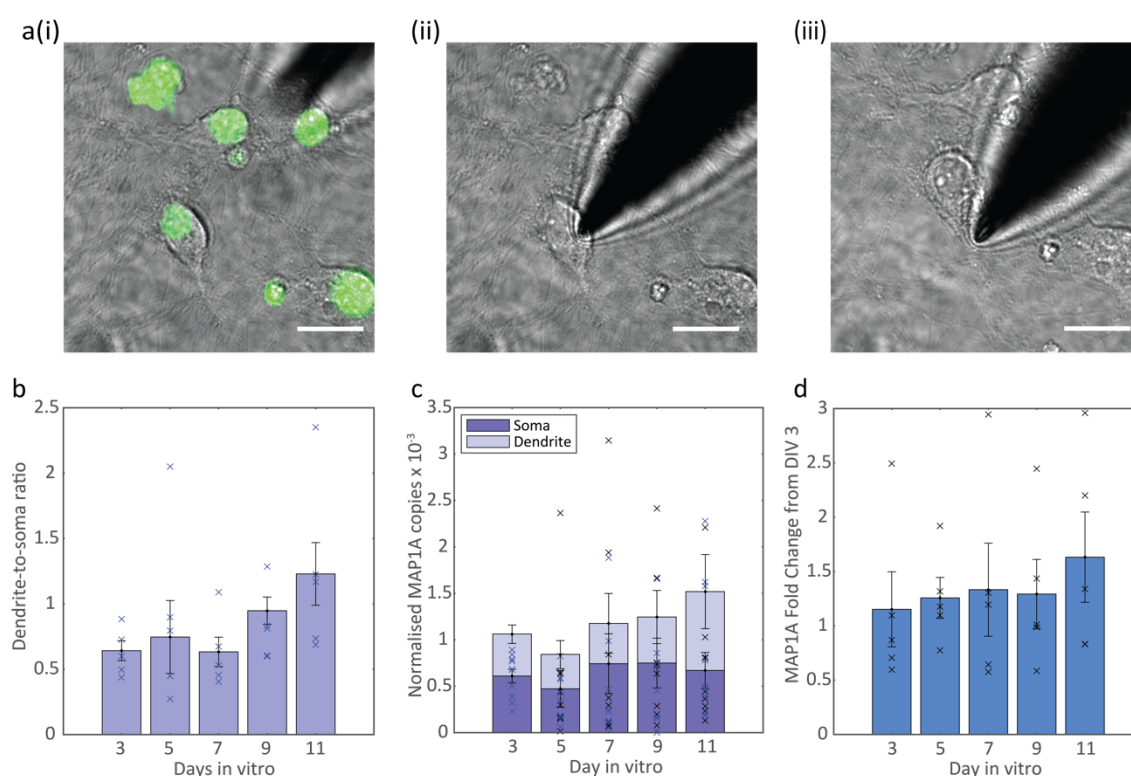


Figure 4.11: Spatially resolved MAP1A expression with neuronal development. a) Bright-field and fluorescent images showing nanobiopsies from the soma and dendrite of the same live hippocampal neuron, showing fluorescently labelled cells with NucSpot Live 488 dye to stain the nuclei (i), insertion into the soma for DEP trapping (ii) and insertion into a proximal dendrite of the same cell for DEP trapping (iii). Scale bars = 20 μm . b) Plot of the dendrite-to-soma ratios obtained from multiple soma and dendritic biopsies from the same cell at DIV 3-11 ($n = 5$ cells per DIV from 5 rats). MAP1A copy numbers were normalised against 18S rRNA and the dendrite-to-soma weighted means were calculated for each DIV based on the number of biopsies taken from each cell ($n = 20$ biopsies in total per DIV). Crosses represent dendrite-to-soma ratios for individual cells, error bars represent weighted s.e.m. c) Total combined copy numbers obtained from biopsies at each DIV normalised against 18S rRNA. Dark purple bars represent soma copies and light purple bars represent dendritic copies, black crosses represent soma values and blue crosses represent dendritic values per biopsy, error bars represent s.e.m. d) Bulk MAP1A expression from 100,000 cells relative to DIV 3 ($n = 5$ rats). Bulk expression was normalised against GAPDH, crosses represent bulk relative expression values per rat, error bars represent s.e.m.

Figure 4.11b shows the dendrite-to-soma ratios per cell obtained from cells at DIV 3, 5, 7, 9 and 11, with an average of 4 biopsies per cell performed. Although not significant, in most cases, an increasing trend was shown, where more average MAP1A mRNA was detected in the dendrites at DIV 11 compared to DIV 3, providing a dendrite-to-soma ratio of 1.2 ± 0.2 ($\pm$ s.e.m) at DIV 11. In comparison, Wang *et al.* demonstrated a dendrite-to-soma ratio of approximately 1.5 in DIV 18 mouse hippocampal neurons and determined that the majority of mRNAs showed upregulation in the dendrites with maturation when comparing DIV 10 to DIV 18.⁵ The nanotweezer dendrite-to-soma ratio results were therefore consistent with this work, where achieving a dendrite-to-soma ratio of 1.5 at DIV 18 was plausible. There could be numerous explanations for the increase in dendrite-to-soma ratio in terms of mRNA dynamics: 1) an increase in mRNA trafficking from the soma to dendrites during maturation; 2) degradation of somatic mRNA; 3) an increase in total mRNA expression in both compartments combined with increased trafficking in the dendrites; or 4) degradation of mRNA in both compartments with increased rates of degradation in the soma. To explore this further, the total amounts of MAP1A mRNA from all biopsies in each compartment at each DIV were investigated (**Figure 4.11c**). This generally showed an increasing trend in total MAP1A mRNA with maturation, where more MAP1A was extracted at higher DIVs, suggesting an increase in overall MAP1A expression with maturation. This suggested that with maturation, not only is there an increase in total MAP1A expression, but there is also an increase in MAP1A localisation rates in the dendrites.

The increase in MAP1A expression with maturation was then investigated with bulk RNA isolations from cells plated in the same manner as single-cell work. RNA was isolated from 80,000-100,000 hippocampal neurons at different stages of maturation in culture from 5 different rat isolations. The relative MAP1A expression from DIV 3 was determined using the relative quantification qPCR method (**Figure 4.11d**). This showed a slight increasing trend in the amount of MAP1A expression at DIV 11 compared to DIV 3, partly supporting the trend found in nanobiopsy work. Although the same amount of RNA was used in all bulk measurements and normalised against a common housekeeping gene for maturation,²⁴ large variations were apparent in bulk measurements. These may be accounted for by inter-prep variability, phenotypic differences in animals and variability in cell isolation procedures; all of which are common causes of variation with primary cells.²⁵ This, combined with cellular heterogeneity, could also explain the variations found in the single-cell biopsy work.

The increasing trend in MAP1A expression with maturation was further supported by the ‘Cortecon’, a repository of gene expression in the developing cortex which provides an extensive RNA-seq resource for the developing human cerebral cortex based on differentiated embryonic stem cells.²⁶ This extensive study showed that between DIV 0 and DIV 33, there is an almost linear upregulation in MAP1A expression. Although from a different region of the brain than the neurons studied in this work, this may further support the increase in overall MAP1A copies detected at DIV 11.

smFISH was then performed to visualise total MAP1A mRNA in single hippocampal neurons at the DIVs studied in this work. Initially, positive and negative controls were performed with a DAPI nuclear stain to assess background fluorescence levels and working protocols (**Figure 4.12**). Following successful controls, MAP1A mRNA in hippocampal neurons at DIV 3 and 11 were studied. Two methods were employed to visualise MAP1A expression and cell morphology to locate subcellular compartments: Multiplexing MAP1A with probes for 18S rRNA (**Figure 4.13a**) and using a WGA stain as a cell mask after the RNAscope protocol (**Figure 4.13b**). WGA staining was shown to be poorer at labelling the entire cell, where not all of the entire neuronal structure was visible, but this was employed as a cell marker control in the absence of multiplexing the MAP1A probe with an additional RNAscope probe.

smFISH showed that at DIV 3, very little MAP1A RNA was observed outside the soma. At DIV 11, however, MAP1A was observed in the dendrites of neurons. Additionally, there was an observed increase in the total amount of MAP1A RNA present per cell at DIV 11 compared to DIV 3. Overall, the bulk RNA work and smFISH studies described here, combined with localisation studies reported in the literature,⁵ supported the MAP1A RNA localisation trends from the nanotweezer. This work therefore begun validating the nanotweezer as an effective tool for detecting overall gene expression regulation in live single cells, as well as in specific subcellular compartments to study RNA localisation. This showed potential for the tool to be used to assess the spatial landscape of biomolecules in living cells to give true single-cell representations. Limitations from this work were also apparent, however, in the large variations in results between different cells and nanotweezer biopsies. Although variations between cells are to be expected due to cellular heterogeneity, further nanotweezer repeats should be incorporated in future to minimise variations in sampling efficiencies and obtain larger sample sizes for significance levels.

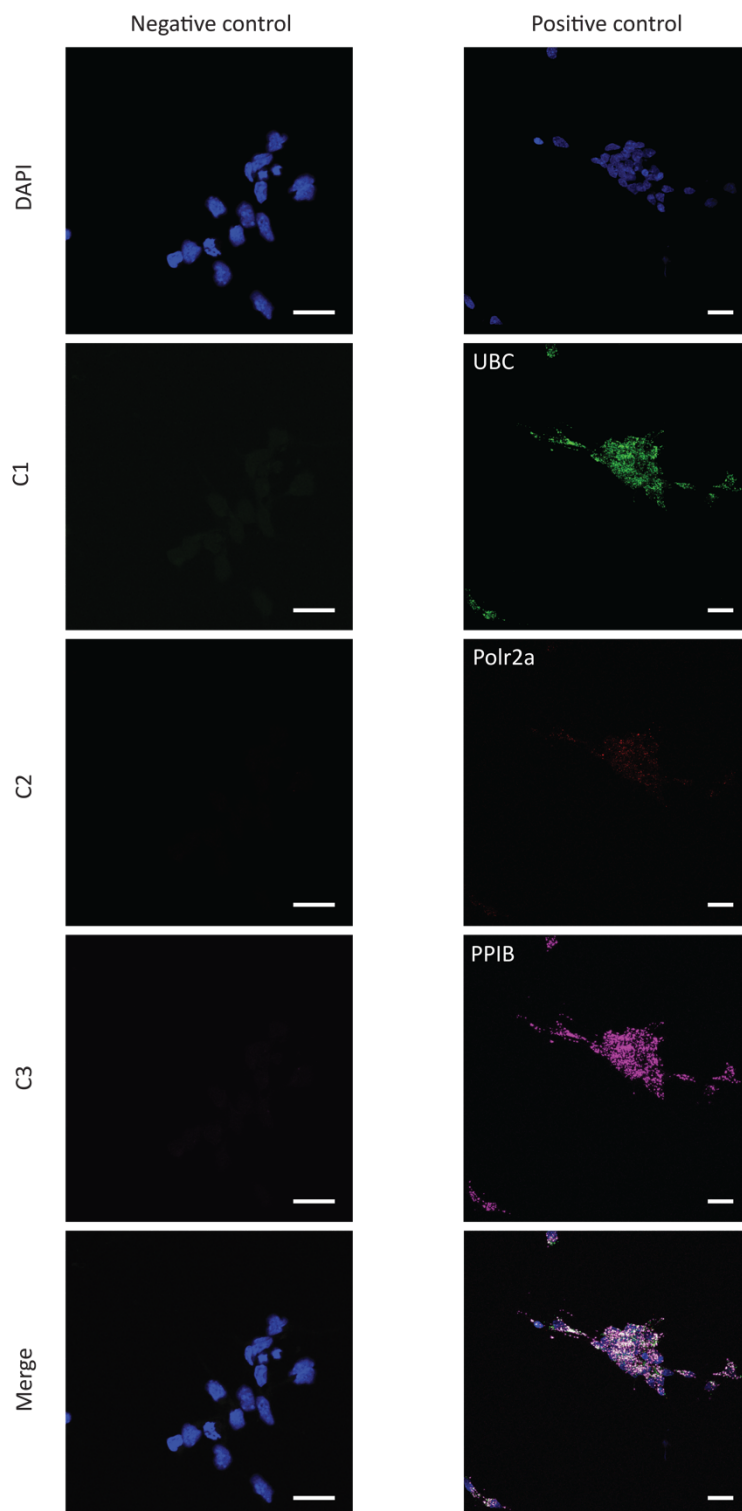


Figure 4.12: smFISH positive and negative controls. Representative images of negative (left) and positive (right) controls used to optimise protocols for smFISH using RNAscope. For negative controls, the full protocol was performed with negative control probes which do not hybridise to any RNAs, followed by nuclear counterstaining with DAPI (blue). Minimal background signal was observed. For positive controls, probes that hybridise to *UBC* (C1, green), *Polr2a* (C2, red) and *PPIB* (C3, magenta) RNAs were employed with the full protocol, followed by nuclear counterstaining with DAPI (blue). Distinct punctuate signals were observed in all

channels that corresponded to the expected abundancies of these RNAs. Scale bars = 20 μ m. RNAscope was performed with Dr Zoe Kwan, Imperial College London.

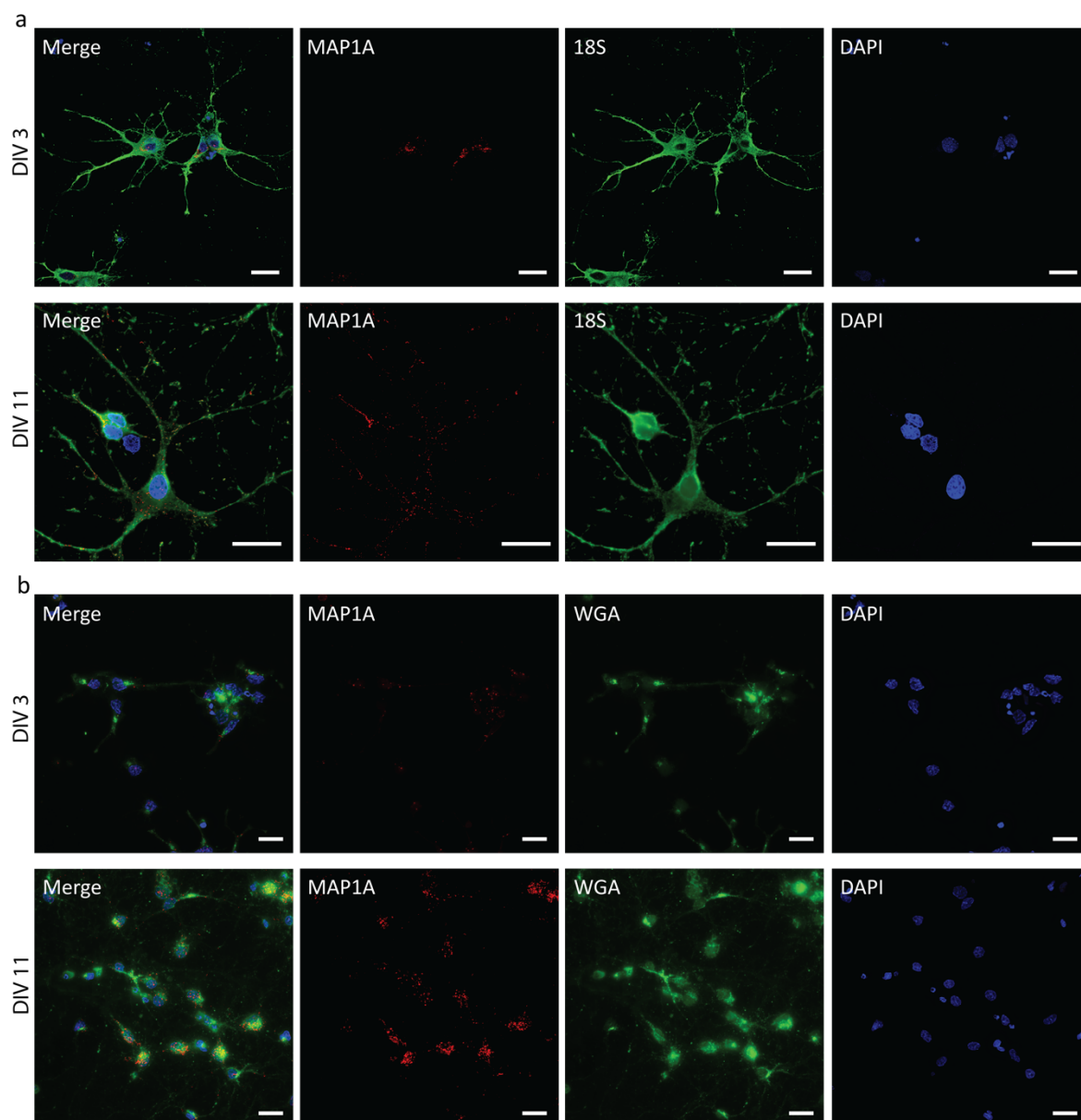


Figure 4.13: smFISH for MAP1A RNA. a) smFISH for DIV 3 (top) and DIV 11 (bottom) hippocampal neurons, labelling MAP1A (red) and 18S RNA alongside DAPI (blue) nuclear staining. b) smFISH for DIV 3 (top) and DIV 11 (bottom) hippocampal neurons, labelling MAP1A (red) RNA alongside WGA staining as a cell mask (green) and DAPI (blue) nuclear staining. Scale bars = 20 μ m. RNAscope was performed by Dr Zoe Kwan, Imperial College London.

Explanations for increased localisation and overall expression of MAP1A in maturing neurons may arise from its protein function; MAP1A is a brain-specific microtubule-associated protein that is important for microtubule stability and assembly.²⁷ As with other microtubule-associated proteins, this plays an important role in neurogenesis.²⁸ Increasing MAP1A expression with maturation and neuronal development would therefore be expected and has

been suggested to have a growth-related function.²⁹ This has been further supported *in vivo*, where MAP1A was found to be barely expressed at birth but increased levels were found at postnatal day 20.³⁰ In regard to its subcellular function, MAP1A has been reported to be an important gene for dendritic stability.³¹ MAP1A immunostaining has shown that the protein is present in somatodendritic regions, which becomes more enriched in the dendrites, in parallel with increased dendritic branching and synapse formation as neurons mature.³² They found that suppressing MAP1A expression in DIV 6-7.5 hippocampal cultures led to decreased branching, a lower number of proximal dendrites and, interestingly, a retraction of dendritic processes compared to controls. Overall, this protein work complements the RNA findings described here and in other studies, where the increased localisation of MAP1A RNA in the dendrites may correlate to increased protein production in the dendrites. This same study also showed that MAP1A expression was affected by the density of cultures, where high density cultures (10,000 cells/cm²) led to complete restriction of MAP1A in the dendrites after 2 weeks, whereas for cells grown in isolation, a delay of 4-6 days was observed for the accumulation of MAP1A in the dendrites. The authors deduced that cell-to-cell contact may be important for its subcellular distribution. It was therefore important to show that the densities and cells used in this work were showing morphological changes to complement the changes in gene expression observed. To show that the cells were changing structure in terms of their dendritic lengths and branching, immunostaining for MAP2, a microtubule-associated marker of the dendrites and soma,³³ was performed. The number of distal dendrites and overall dendritic lengths in 10 cells were calculated as described in **Chapter 2.15**. This showed a steady increase in dendritic length and branching with maturation up to DIV 11 (**Figure 4.14**), confirming that the cells were developing alongside the changes in dendritic RNA localisation observed in this work.

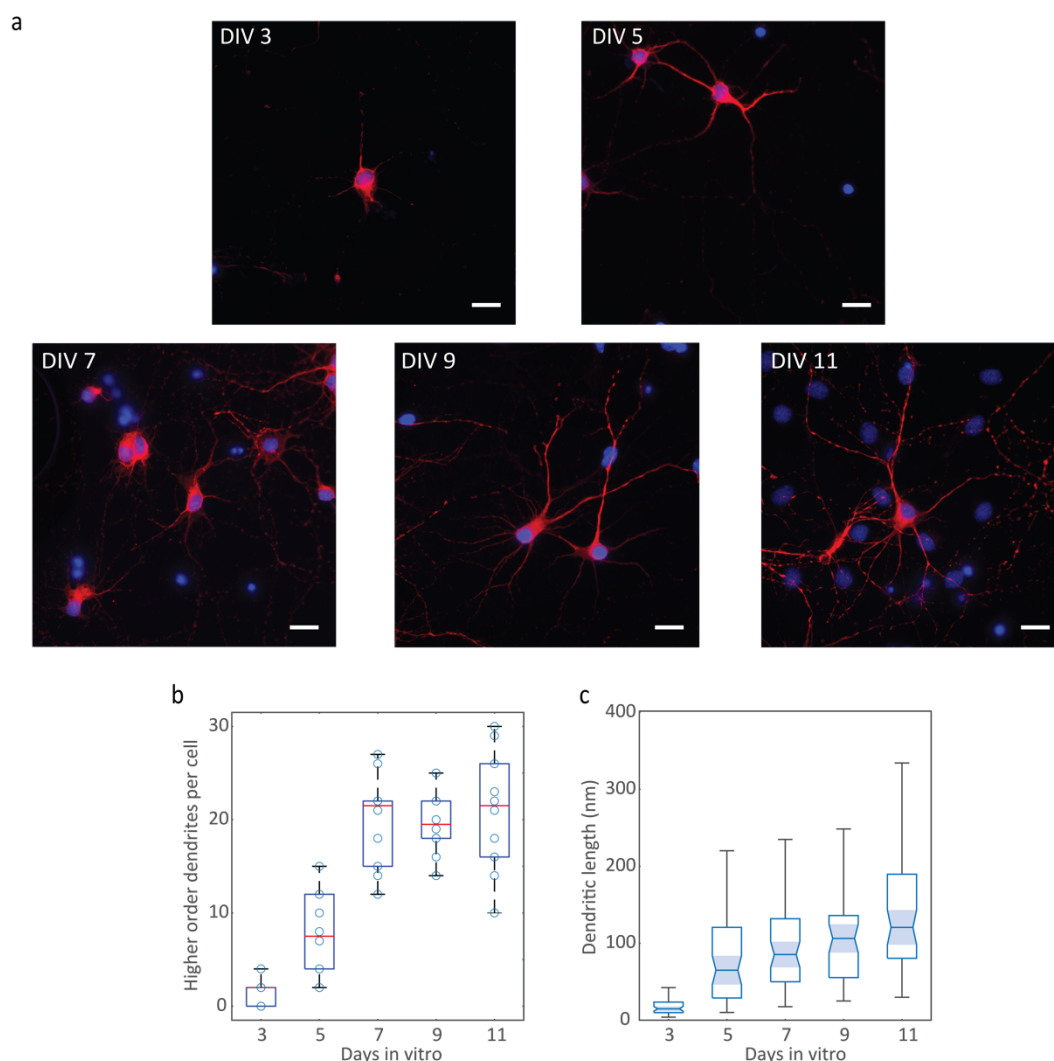


Figure 4.14: Changes in dendritic length and branching with maturation. a) Representative immunostaining images of MAP2 (red) in hippocampal neurons between DIV 3-11 to visualise dendrites, counterstained with DAPI (blue). Scale bars = 20 μm. b) Boxplot of the number of higher order dendrites (distal dendrites) per cell at DIV 3-11 (n = 10 cells). c) Notched boxplot of the dendritic lengths measured in 10 cells at DIV 3-11 (n = >47). Outliers that were more than 3 scaled median absolute standard deviations from the median were removed.

4.4 Dynamic and spatial RNA measurements

4.4.1 CAMK2A and synaptic plasticity

The work described in the previous section showed that RNAs could be detected in specific subcellular compartments of living neurons using the nanotweezer platform and this was validated with a known dendritically localised mRNA. Although this method has advantages in assessing the subcellular distribution of molecules in living cells with minimal alterations to the cells and their microenvironments, imaging techniques may still be favourable for this purpose due their ability to measure large numbers of cells and targets simultaneously. The

true benefit of the nanotweezer, however, would lie in its ability to make live cell measurements. Exploiting this via time-resolved measurements of gene expression, combined with the high spatial resolution of sampling, would allow for true dynamic changes to be observed in subcellular compartments of the same living cell. This would be highly desirable given the heterogeneity of single cells and their responses both *in vitro* and *in vivo*. It would broaden the information that can be obtained compared to static analyses, with particular importance for assessing cellular dynamics, treatment responses, or disease monitoring.

To explore the possibility of measuring dynamic changes in living neurons, calcium/calmodulin dependent protein kinase II alpha (CAMK2A) was chosen as an mRNA target to study. CAMK2A is an mRNA that has been highly studied for its localisation in the dendrites,^{3,7,20} where more than half of CAMKII α proteins, the protein derived from CAMK2A mRNA, have been found to be synthesised in the dendrites.³⁴ CAMKII α is the most abundant subunit of a major kinase (CAMKII) involved in calcium signalling in the brain. It has multiple roles in neurons, including regulating excitatory synaptic transmission, dendritic spine morphology and neuronal development.³⁵

The activation of CAMKII α is thought to be highly important for the induction and maintenance of long-term potentiation (LTP).³⁶ LTP is the most widely studied form of synaptic plasticity that involves the persistent strengthening of synapses following high-frequency stimulation, resulting in enhanced signal transmission between neurons.³⁷ This form of synaptic plasticity is widely recognised to form the basis of learning and memory formation, where deficits in LTP have been observed in Alzheimer's disease (AD) models and patients.³⁸ During LTP, N-methyl-D-aspartate receptors (NMDARs) are activated at the postsynaptic sites of excitatory neurons.³⁹ This leads to calcium entry at NMDARs and several cellular and molecular changes, such as alterations in dendritic spine morphology, activation of protein synthesis at synaptic sites, and signal transduction. It is well-reported that LTP induction and increased synaptic activity cause a rapid localisation of CAMKII at the dendritic spines of neurons due to its activation from calcium entry.⁴⁰⁻⁴⁴ The localisation of CAMKII protein has been suggested to be dynamically modulated by gene expression, where, following LTP, certain CAMK2A mRNA transcripts localise in the vicinity of dendritic spines for local *de novo* protein synthesis.^{45,46} This mRNA targeting is thought to be highly important for neural plasticity, where inhibiting the translocation of CAMK2A mRNA to the dendrites led to suppressed LTP.⁴⁷

4.4.2 LTP-induced mRNA localisation

As there is numerous evidence to support the localisation of CAMK2A mRNA in the dendrites,^{3,5,7,20,21} and its increased localisation at the dendritic spines with LTP,^{45–47} CAMK2A detection with LTP was chosen as a model to assess its change in localisation behaviour measured using the nanotweezer. There are several methods of inducing LTP in hippocampal tissues and cultures.^{39,48,49} Chemical LTP (cLTP) was chosen for this study due to the simplicity of incubating cells in the desired media without the need for electrode stimulation, which would otherwise lead to only a small fraction of synapses being activated compared to cLTP.⁴⁹ In this work, cLTP was induced by N-methyl-D-aspartic acid (NMDA), an NMDAR agonist which mimics the neurotransmitter L-glutamate in LTP.⁵⁰ Glycine, a co-agonist of NMDARs, was used with NMDA in Mg^{2+} -free media to further stimulate LTP and depolarisation of the neurons.^{49,51,52}

Previous work has shown that CAMK2A mRNA associates closer to the dendritic spines after the induction of LTP and that this may facilitate local translation to maintain plasticity.⁴⁵ In this work, the nanotweezer was used to measure CAMK2A mRNA at the specific sites of the dendritic spines before and after the induction of LTP. Initially, the precision of sampling was evaluated in hippocampal neurons that expressed GFP in order to visualise the whole cell, including the dendritic spines. Attempts to trap RNA within the heads of the dendritic spines led to full isolation of a single spine from the cell (**Figure 4.15**) where localised fluorescence at the nanotweezer was observed upon application of DEP, suggesting that some of the cellular structure, rather than unlabelled RNA, became trapped. This may be due to the small size of the spine heads which have been reported to have volumes of $0.06\text{--}0.081\ \mu\text{m}^3$.^{53–55} The small spine protrusion combined with the lack of control over the depth that the nanotweezer gets inserted meant that whole spines were easily trapped and pulled out if the nanotweezer was inserted too far or not inserted at all. This may particularly occur because spine heads are held to the cellular structure only by a single neck of $128.0 \pm 62.8\ \text{nm}$ in length,⁵⁴ unlike the neurites of neurons which are longer. For neurites, the adherence of the expansive neuronal network around these longer structures may contribute to holding the structures in place in the culture dish during the biopsy procedure, compared to spines which may only have one small protrusion attaching it to the neuronal network. Although isolating whole single spines could be extremely beneficial in evaluating the molecular makeup of dendritic spines, particularly if

performing RNA-seq on whole single spines was possible, isolation of whole spines often led to a rapid decrease in the cells' fluorescence after the biopsy. This was due cell death occurring after a spine was removed from the cell, leading to the cell no longer being able to express the GFP plasmid.

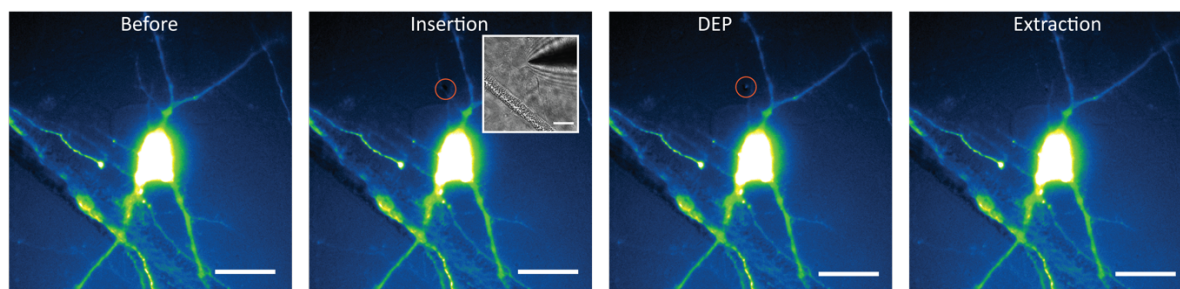


Figure 4.15: Nanotweezer extraction of dendritic spines. Fluorescent images of an attempt to remove RNA within the dendritic spines of a hippocampal neuron using the nanotweezer, showing the cell before insertion, during insertion of the nanotweezer, application of DEP and localised fluorescence at the tip, and extraction of the nanotweezer with a loss of localised fluorescence. Inset: Bright-field image of nanotweezer. Scale bars = 20 μm .

As the objective of this work was to achieve minimally invasive sampling for dynamic transcriptome measurements on the same cell over time, it was apparent that isolation of the dendritic spines may lead to a cascade of cellular and transcriptome changes as a result of the physical impact of the nanotweezer, rather than from a desired treatment application. Using the eGFP expression to clearly locate spines (**Figure 4.16a**), the base of the dendritic spines, located within the dendrites and next to a spine, was therefore used for nanotweezer targeting as the work in the previous section showed that this was possible without removing any cellular structure or harming the cell. Additionally, based on previous work, CAMK2A localised in closer proximity to spines rather than within the spines themselves.⁴⁵ Targeting the base of spines within the dendrites therefore still allowed for enhanced CAMK2A localisation to be detected in close proximity to the spines. Previous computational modelling of the nanotweezer found that biomolecules up to 300 nm away from the tip could be trapped,¹¹ which meant that some components of the spines may also lie within this distance from the base of the spines and allow for some RNA to be trapped. Nanobiopsies at the base of the dendritic spines were performed successfully without isolation of the spines themselves or of any other cellular structure (**Figure 4.16b**).

Precise sampling from the base of the dendritic spines of the same DIV 14 hippocampal neuron before and after cLTP induction was performed (**Figure 4.16c**). This included 3 biopsies before LTP, with cells maintained in their standard culture media, followed by 3 biopsies after the induction of cLTP and replacement with conditioned media (**Figure 4.16c**). All biopsies were performed within 1 h after LTP induction, where the effects from LTP should still be observed in neurons; previous work showed increases in CAMK2A translocation was observed up to 120 minutes after LTP before returning to baseline levels.^{46,56} The CAMK2A copy numbers from each biopsy of the same cell before and after LTP induction were determined using RT-qPCR and the fold change in copy number per cell was determined in order to evaluate the true single-cell change in CAMK2A expression at the dendritic spines. These biopsies were performed alongside a control dish of cells from the same rat isolation, with application of buffer in the absence of NMDA and glycine. Of the 3 cells measured for each treatment and control (each from 3 independent rat isolations), an increasing trend in CAMK2A copy number at the base of the dendritic spines was observed after LTP for all cells (**Figure 4.16**). This was not quite significant compared to control cells ($p = 0.0570$, paired t-test); however, only 3 cells were sampled in this work and so further repeats would be required to determine if there was a significant increase in CAMK2A mRNA at regions of the dendritic spines with LTP. This would minimise variations observed from the general heterogeneity of cells. Nevertheless, the trend observed was promising as it showed an expected increase in CAMK2A localisation in close proximity to dendritic spines after LTP using the nanotweezer. This confirmed that the nanotweezer is capable of biopsying from even more precise subcellular regions and obtaining higher subcellular resolution than described earlier and previously reported. It also showed that this could be combined with multiple biopsies from the same cell to measure dynamic localised changes in gene expression. This expands the capabilities of the technique, where it provides an inexpensive method to study precise subcellular compartments of living single cells over time.

Although this work requires further repeats and validation to understand changes in gene expression with LTP, it provides a promising model for deciphering the mechanisms of LTP and how this may be impacted in diseases such as AD. A comparison of healthy rat hippocampal neurons with those from AD rats could be made in future, as well as in different types of neurons. The effects of CAMK2A expression and number of other synaptic genes such as β -actin and Arc,^{45,57} could be studied for their localisation behaviour in healthy and diseased neurons, providing further insight into the contribution of transcript regulation in synaptic

plasticity and memory formation. With further improvements in throughput and trapping efficiencies, this work could contribute to the discovery of novel transcripts involved in LTP and potential targets for synaptic plasticity dysfunction.

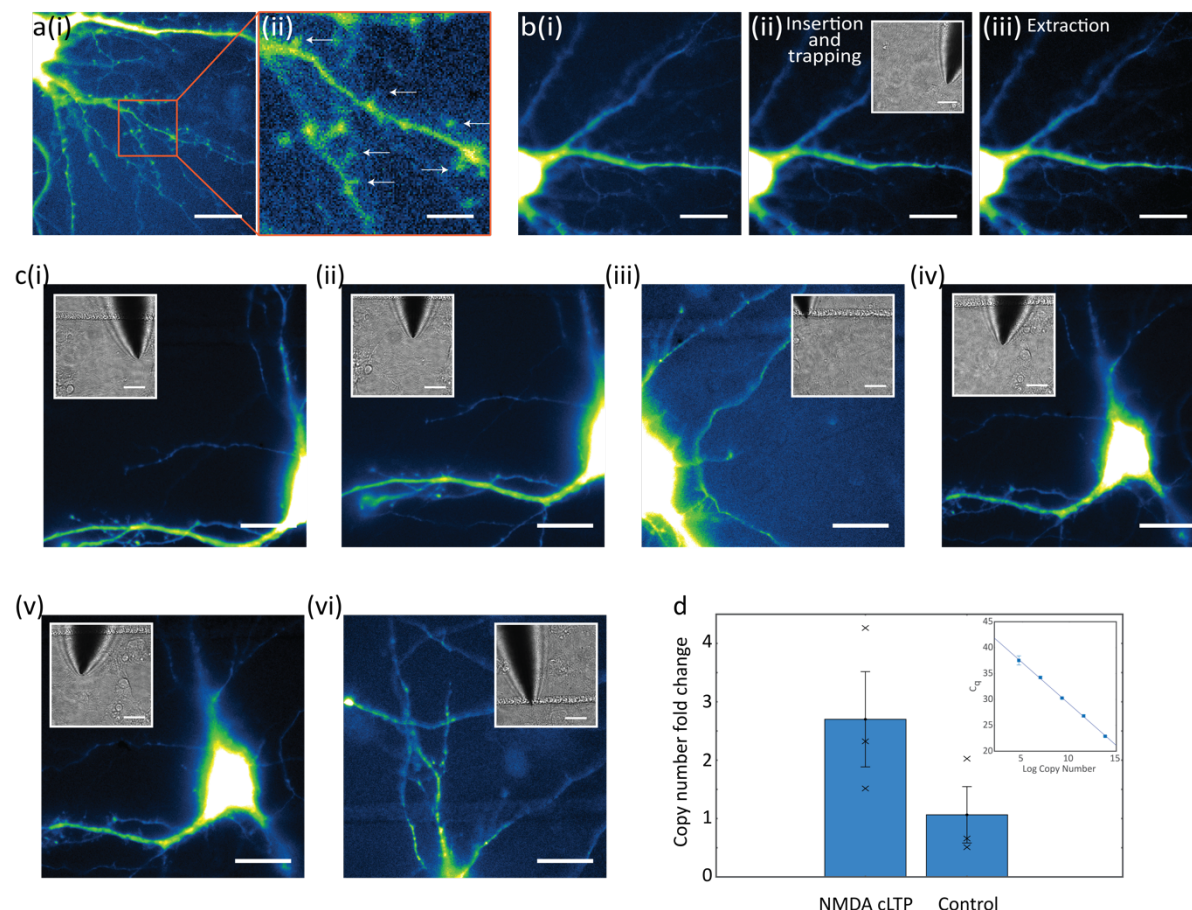


Figure 4.16: Time-resolved nanobiopsies with cLTP. a) DIV 14 hippocampal neurons were transfected to express eGFP (i) to visualise and target the dendritic spines on cells, where the white arrows show the dendritic spines (ii). b) Procedure for nanobiopsy from the base of the dendritic spines: Spines are targeted by fluorescence microscopy (i), the nanotweezer is inserted into the base of the dendritic spine within the dendrite (ii), DEP is applied and then the nanotweezer is extracted from the cell (iii). c) Example nanobiopsy positions taken from the same cell before induction of chemical cLTP (i-iii) and after the induction of cLTP (iv-vi). Insets: Bright-field images of the specific nanotweezer's position in the cell. Scale bars = 20 μ m. d) CAMK2A copy number fold change per cell at the dendritic spines of the same cell after cLTP induction (NMDA cLTP) compared to control cells with application of buffer without NMDA. Error bars represent s.e.m (n = 3 cells). Inset: CAMK2A standard curve used for absolute qPCR quantification comprised of known copy numbers of amplicons containing the target region.

4.5 Viability

4.5.1 mRNA integrity

The integrity of mRNA trapped at the nanotweezer was evaluated by designing a triplex qPCR reaction involving two primer/probe sets that bind to the same mRNA strand at different

regions, alongside 18S which was used as a biopsy control. The newly designed primer sets' compatibility in a triplex reaction was confirmed by gel electrophoresis (**Figure 4.17**). This included the same MAP1A primer assay used previously in this chapter which spans exons 5-6 on the mRNA strand. The additional MAP1A assay spanned exons 1-3, ensuring that there was no overlap of primers between pairs and that the primers and probes could bind to separate ends of the mRNA (cDNA) strand (**Figure 4.18a**).

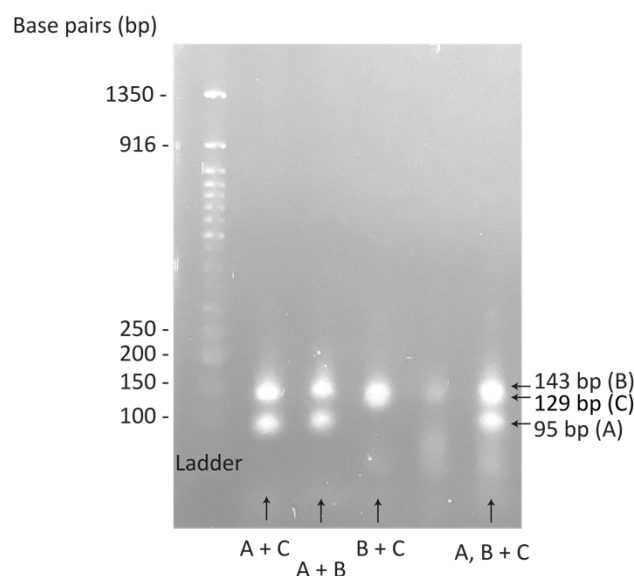


Figure 4.17: qPCR triplex reaction design. Gel electrophoresis (2.5 % agarose) of end points of multiplexed qPCR reactions amplifying 18S (A) cDNA, MAP1A cDNA at exons 5-6 (B) and MAP1A cDNA at exons 1-3 (C). Compatibility of the triplex reaction (far right well) was confirmed by distinct bands of the expected amplicon sizes and a lack of non-specific PCR products.

The triplex qPCR reaction was run on nanobiopsy samples and both MAP1A sets were quantified by absolute quantification (**Figure 4.18c**) using standard curves based on known copy numbers of both MAP1A amplicons (**Figure 4.18d**). The C_q values obtained from individual biopsies were consistent (**Figure 4.18b**), implying that both primer/probe sets were able to bind to the MAP1A cDNA strands. The copy numbers also showed similar trends (**Figure 4.18c**); although the second primer set gave slightly lower copy numbers, the distributions were very similar and each sample was detected with both types of primers. It is important to note that copy numbers obtained from absolute quantification are based on the accurate production of standards to construct the standard curves, but differences between standards and sample reactions will inevitably arise, particularly in a multiplexed reaction where primers may be competing. The results therefore strongly suggested that the mRNA trapped using the nanotweezer was intact and did not degrade: both regions of the MAP1A

cDNA strand were amplified in the same multiplexed reaction, very similar Cq values were obtained, and similar copy number distributions were shown.

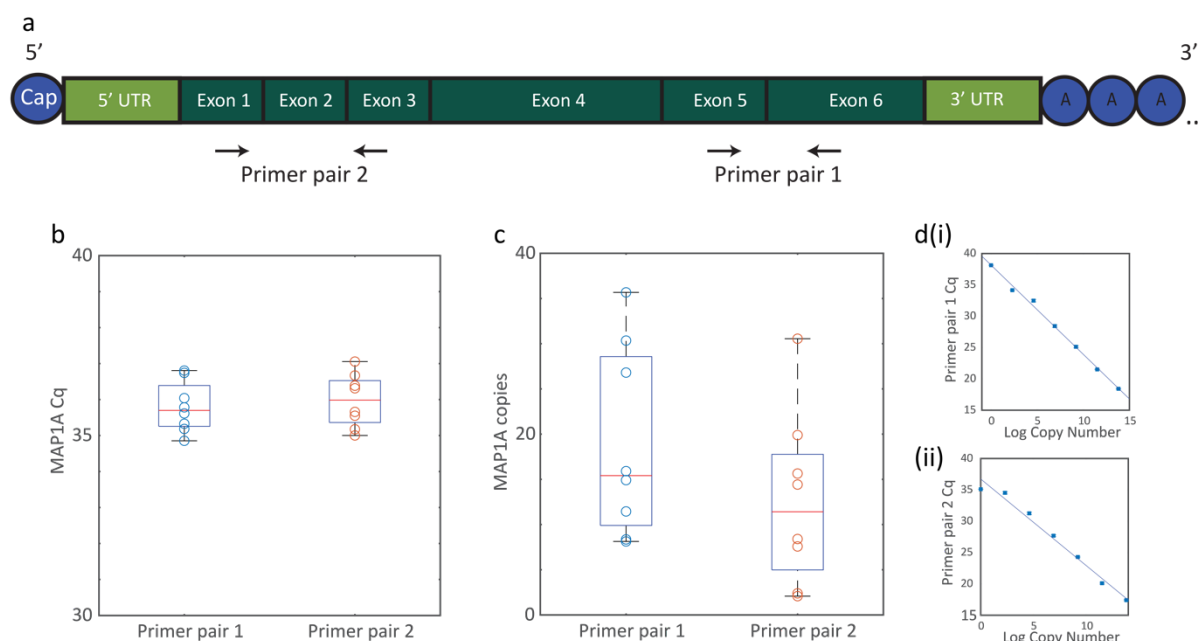


Figure 4.18: Assessment of mRNA degradation from nanobiopsy. a) Schematic to illustrate the primer design for targeting two separate regions of MAP1A mRNA. ‘Primer pair 1’ was designed to span exons 5-6 of the mRNA/cDNA strand, whereas ‘Primer pair 2’ was designed to span exons 1-3. This ensured that amplification of the same mRNA could be achieved with both primer sets to check for intact mRNA being isolated. b) Cq values obtained from individual biopsies for both primer pairs using multiplexed RT-qPCR. c) MAP1A copy numbers obtained from individual biopsies using both primer pairs in RT-qPCR. d) qPCR standard curves used for absolute quantification of MAP1A for ‘Primer pair 1’ (i) and ‘Primer pair 2’ (ii), constructed using known copy numbers of MAP1A amplicons.

4.5.2 Cell viability

In section 4.4.2, the expression of the eGFP plasmid was monitored to assess cell viability in the cells being biopsied, where a loss of fluorescence from the cell during the procedure would indicate a loss of cellular integrity or cell death via apoptosis or necrosis.^{58,59} This is because the GFP is held poorly in cells during cell death.⁶⁰

An additional viability study was performed using Calcein AM. Calcein AM is a non-fluorescent viability dye that is cell permeable.⁶¹ Upon internalisation into living cells with active esterase activity, the compound gets hydrolysed into fluorescent calcein which is membrane impermeable and excitable by blue light (497 nm), allowing for the visualisation of cells with active esterase activity. When cells die, their cytoplasmic contents, including esterases, leak from the cell and so dead cells do not allow fluorescent calcein to be produced

or retained in cells. After sequential biopsies from the same cell were performed, cells were incubated with Calcein AM and the fluorescence of the biopsied cell was monitored. A number of longer-term viability assessments were performed to complement the same-day viability monitoring with GFP. This included: 2 biopsies from the same cell, followed by assessing the viability 24 h later (**Figure 4.19a**); 2 biopsies from the same cell, followed by assessing the viability 48 h later (**Figure 4.19b**); and 8 biopsies in total from the same cell (4 on day 1, 4 on day 2), followed by assessing the viability 48 h after the initial biopsy (**Figure 4.19c**). In all these cases, cells were shown to be viable after these timeframes, indicated by the fluorescence of the cell after application of dye.

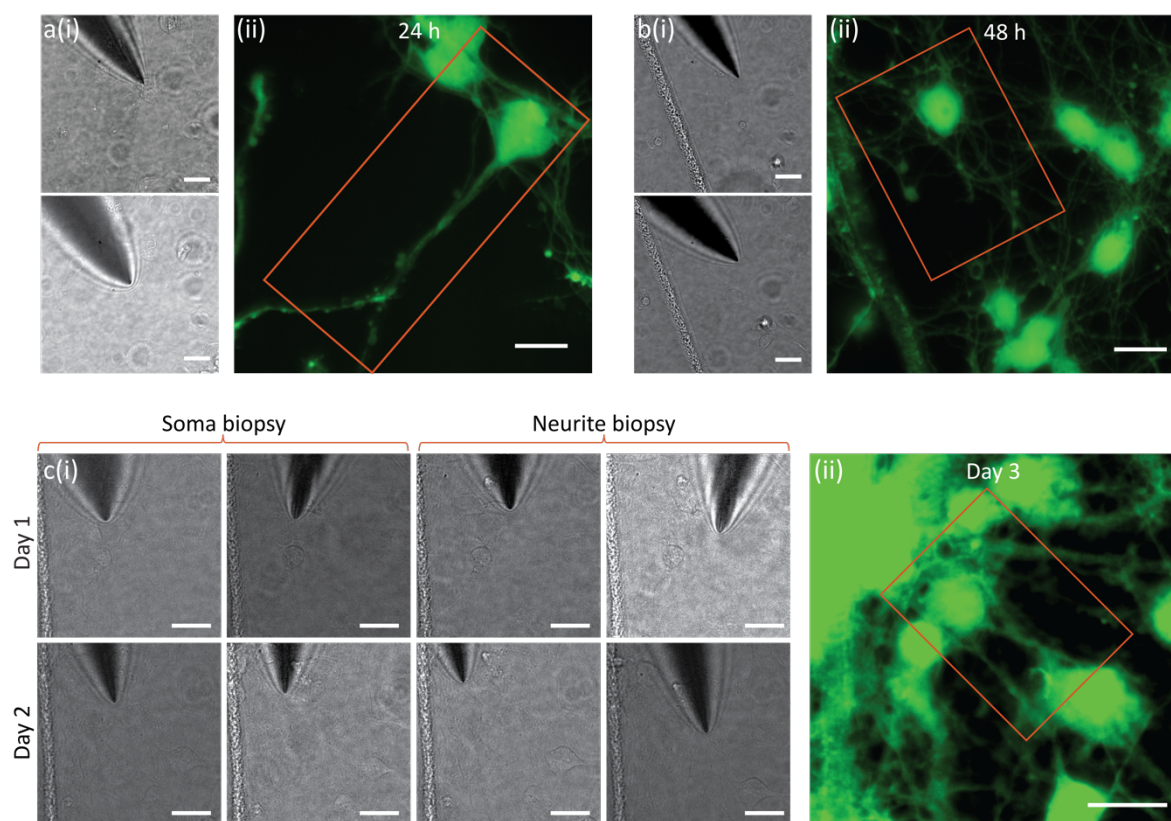


Figure 4.19: Cell viability after nanobiopsy. a) Bright-field images showing locations of 2 sequential nanobiopsies from the same cell (i) and the same cell 24 h later after application of Calcein AM (ii). b) Bright-field images showing locations of 2 sequential nanobiopsies from the same cell (i) and the same cell 48 h later after application of Calcein AM (ii). c) Bright-field images showing the locations of 4 sequential nanobiopsies from the same cell on day 1, followed by another 4 sequential nanobiopsies 24 h later (i) and the same cell a further 24 h later (48 h after first biopsy) after application of Calcein AM (ii). Cells with fluorescence showed that cells had active intercellular esterase activity, suggesting viability. Orange boxes represent the biopsied cell. Scale bars = 20 μm.

Overall, these initial viability results showed that longer term studies of cells, over several days, are possible with the nanotweezer platform, where individual cells were still viable up to

48 h after being biopsied multiple times from different subcellular locations. This further exhibits the dynamic live cell monitoring potential of the nanotweezer and widens the opportunities available for live cell measurements. For example, the application of both acute and long-term treatments could be used to monitor transcriptomic effects of drugs on single cells over time, or changes in gene expression could be monitored to support the differentiation of neural progenitors into mature neurons.

The use of Calcein AM complemented the transfection monitoring of biopsies and could be particularly important for assessing the viability of cells in the long term over periods of more than 48 h, where the expression from lipofectamine-based transfections may begin to decrease after this point.⁶² Nevertheless, as little is known about the long-term viability of using these dyes in cells, Calcein AM was only added after all biopsies were completed to ensure the dye did not unintentionally affect the viability of cells. Therefore, for continuous real-time monitoring of viability throughout the biopsy process, monitoring the expression of plasmids from transfections may be more suitable.

4.5.3 Calcium imaging

Calcium imaging was performed to understand if the DEP from the nanotweezer stimulated neurons, potentially triggering action potentials and leading to an influx of calcium in the cell. This stimulation could potentially induce alterations in gene expression,⁶³ diminishing the minimally invasive advantage of the nanotweezer. Neurons were transfected with jGCaMP8s constructs to act as a sensitive intracellular calcium indicator upon its expression.⁶⁴ The fluorescence of the soma of 3 cells were monitored upon application of DEP. No flash or sudden increase in fluorescence was observed, suggesting that DEP from the nanotweezer did not affect the neuronal activity of cells. **Figure 4.20a** illustrates an example of a cell in which two consecutive biopsies were taken, both showing no calcium influx or increase in fluorescence upon DEP application (**Figure 4.20d-e**). As discussed earlier, the expression of fluorescent constructs was also used as a measure of cell viability, where a decrease in plasmid expression would suggest the viability was being affected and a loss of fluorescence would suggest cell death was occurring with the cell no longer being capable of expressing the plasmid. The calcium imaging described herein therefore also supported maintenance of cell viability during biopsies.

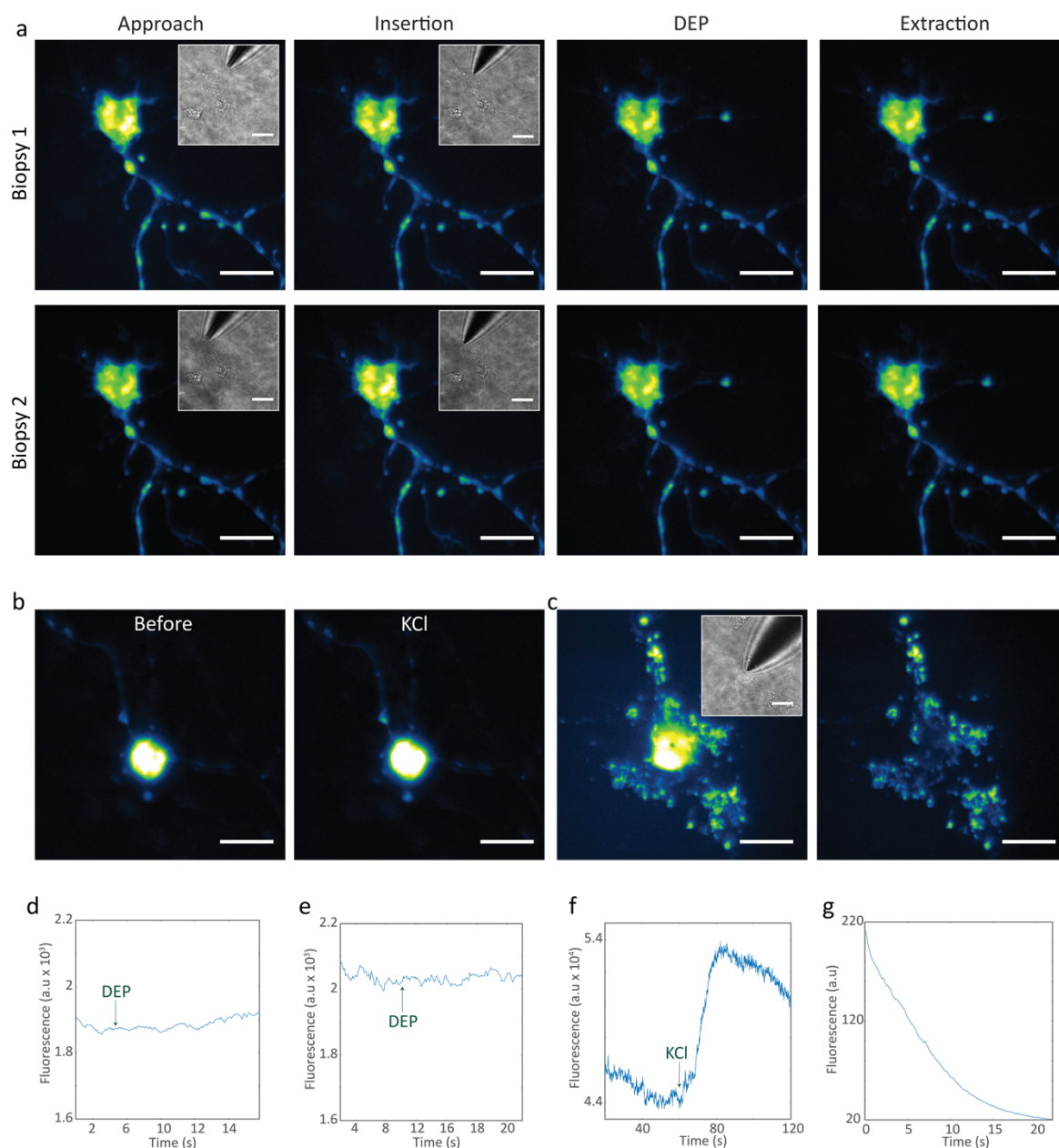


Figure 4.20: Calcium imaging. a) Fluorescent images showing two sequential nanobiopsies from the soma of jGCaMP8s transfected neuron to monitor changes in intracellular calcium levels. Insets show bright-field images of the nanotweezers' position during approach towards the cell and insertion into the cell. b) Calcium imaging of a neuron before and after the application of 30 mM potassium chloride in cell maintenance media. c) Calcium imaging of nanotweezer insertion through a cell to make contact with the cell's dish ($t = 0$). Inset shows a bright-field image of the nanotweezer's position. A rapid loss of fluorescence was observed after deliberate insertion of the nanotweezer through the cell. d,e) Fluorescence measured at the soma upon application of DEP using two sequential nanotweezers. f) Fluorescence measured at the soma upon application of 30 mM KCl. g) Fluorescence measured at the soma immediately after insertion of the nanotweezer through the cell. Scale bars = 20 μm .

Calcium imaging controls were run alongside DEP-calcium measurements to ensure that the calcium indicator worked as expected and that the cells were healthy and able to exhibit activity

changes. To ensure that changes in calcium could be detected in the cells, KCl was applied to the media of the cells (**Figure 4.20b,f**). A rapid influx of calcium and increase in fluorescence was observed immediately upon application of KCl, showing that the cell was able to be depolarised and that calcium ions were able to flow into the cell. A deliberate attempt to damage the cell was also performed to show that cell death could be monitored based on the expression of plasmid. The nanotweezer was inserted too far into the cell so that it would reach through the cell and make contact with the dish. Without application of DEP, this led to a sudden decrease in fluorescence immediately after the harsh insertion of the tip, leading to the cell no longer being overserved by fluorescence. This confirmed the ability of transfections to be used as a measure of cell viability.

4.6 Limitations and open challenges

The work described in this chapter expanded the capabilities of the nanotweezer for extracting RNA from precise subcellular locations of neurons and demonstrated its potential to study dynamic changes in living cells. Despite significant advantages in the progression of the technology and its application, some limitations regarding the platform were apparent.

The localisation work in sections **4.2-3** often involved label-free biopsies, or minimal labelling of the cell structure. Although this has benefits in minimising cellular changes and toxicity in cells to give more representative views of them, it meant that biopsying from regions far away from the soma, such as the distal dendrites and axon terminals, was not possible without labelling. Even with labelling, there was difficulty in biopsying from more mature neurons which have extensive networks. This was due to the neuronal networks of cells considerably overlapping with each other, making it difficult to ensure one was biopsying from the neurites of the cell of interest and not an adjacent cell. Transfection helped significantly due to the low transfection efficiency of neurons, meaning that single cells were observed. However, care still needed to be taken to ensure that the biopsy location was not overlapping or underneath an unlabelled, non-transfected cell. Hence, mature neurons older than DIV 14 were not studied in this work due to uncertainty on the precision of the targeted location. This may not necessarily be a cause for concern as DIV 14 is widely accepted as a timepoint when synapses are well-established.⁶⁵⁻⁶⁷ However, this technical limitation may still become challenging when studying and tracking the extensive biological processes of mature neurons, as many studies are based on neurons between DIV 14-28.⁶⁸⁻⁷⁰ Nevertheless, the culture density could be optimised and decreased to allow for more separation between neurons and minimise overlap

of cells. This could improve the targeting limitations and allow for more mature neurons to be studied. Of course, this needs to be evaluated for the relevant application as different densities have been shown to have differing RNA localisation behaviour and synaptic transmission.^{32,71,72}

The RNA localisation results from this work showed large variations between samples and cells which may be due to the heterogeneity of cells and primary cell isolation differences. Although understanding the heterogeneity of cells is useful to gain true gene expression analyses and to unmask averages from bulk analysis, which this system provides an ideal methodology to assess, a large enough sample size must be acquired to fully understand heterogeneity and single-cell responses to treatments. The limitation of obtaining a high sample size lies in the limited throughput of the technique. In future work, improving the sampling procedure, for example via automating the positioning of the nanotweezer, would greatly improve the throughput and allow for larger sample sizes to be obtained.

Finally, the estimated amount of total RNA isolated using the nanotweezer was calculated to be in the 10-100 fg region. This was too small to be used for RNA-seq. However, to fully understand RNA regulation and localisation in single cells to lead to the discovery of novel cellular mechanisms, RNA-seq is essential. This would enable the types of transcripts that show differential localisation behaviour to be fully examined, particularly as previous work has shown that the 3' UTR region of transcripts contains localising elements that may be key for RNA regulation, plasticity and the production of functionally diverse protein isoforms.^{6,73} Future work that aims to achieve RNA-seq on nanobiopsy samples should therefore be prioritised. This could be achieved by enhancing the DEP trapping capabilities for the nanotweezer, for example by minimising the gap between the electrodes further or by surface modification of the nanotweezer with an oligo(dT) strand to enhance mRNA extraction. Although these methods may improve trapping, samples may also need to be pooled to have sufficient RNA for sequencing. Pooling samples from the same subcellular compartment of a single cell,⁷⁴ which would still allow for rare transcripts to be uncovered compared to the possible masking of transcripts that one may acquire from larger whole single cell or bulk sequencing, could therefore be explored.

4.7 Conclusions

This chapter showed substantial development of the nanotweezer technology for single-cell studies, where precise subcellular compartments of living neurons could be targeted to extract and detect RNA. Multiple biopsies from the same neuron were demonstrated without affecting cell viability, opening the capabilities of the technique to assess RNA abundance in different subcellular compartments of the same cell, as well as time-resolved measurements. Both of these applications were explored in this work. RNA localisation of a known dendritic mRNA was studied and its relative abundance compared to the soma of the same live cell with maturity was evaluated, followed by dynamic measurements of the localisation behaviour of an mRNA in proximity to the post-synapses. This validated the nanotweezer as a tool for assessing RNA localisation in living neurons, with the dynamic application having particular biological relevance by demonstrating that this method was capable of measuring gene expression from precise locations in the same cell over time after application of a treatment. The work described in this chapter provides the foundations for in-depth biological studies of gene expression in precise compartments of living single neurons cultured in their standard conditions, negating fixation requirements, costly practices and cell lysis. With further technological developments and sequencing, this could have wide implications for a range of elusive biological applications, ranging from understanding basic neuronal function and synaptic activity to monitoring the treatment of a drug in a single cell for clinical development and diagnostics.

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Chapter 5

Single mitochondrion nanobiopsy

Synopsis

Mitochondrial heteroplasmy is a key feature of neurodegeneration and ageing. Characterising mtDNA copy number and mutational loads within single cells could improve understanding of clonal expansion, mutational thresholds in disease and mitochondrial quality control. In this chapter, the nanotweezer is used for single mitochondrion extraction from the neurites of living neurons and the viability of extracted mitochondria is established. Protocols are developed to analyse the nucleic acid content of a single mitochondrion, demonstrating potential for single mitochondrion copy number determination and mtDNA sequencing.

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5.1 Introduction

The disproportionately high energy demands of neurons compared to other cell types means they rely heavily on their mitochondria for ATP production. Neuronal mitochondria also play an important role in calcium buffering and signalling,¹ regulating axonal and dendritic development,² and activating cell death.³ Dysfunctions in mitochondria are key features of neurodegeneration and ageing, where they are linked to numerous diseases such as Parkinson's disease (PD),⁴ Alzheimer's disease (AD)⁵ and amyotrophic lateral sclerosis (ALS).⁶ One aspect of this dysfunction involves mitochondrial DNA (mtDNA), where decreased mtDNA copy numbers and high levels of mtDNA heteroplasmy have been identified in neurodegenerative diseases. Cells with high levels of mtDNA mutations, typically around 70 %, have been shown to cause a functional defect with lower respiratory chain activities.⁷

Understanding mitochondrial heteroplasmy is imperative to characterising the proportion of mitochondrial variants and their implications in the development and severity of disease. Analysing mtDNA mutations on the tissue scale does not fully explain how these mutations may be present. For example, mutations may be due to high levels of a specific point mutation within a single cell with lower levels of mutations within other cells, or they may be due to many of these mutations being present at low levels within each cell. Both mechanisms may provide the same result from bulk analysis, yet higher levels of mutations in single cells may be more deleterious and lead to cell death. Single-cell analysis technologies have enabled mitochondrial heteroplasmy to be characterised in neurons and show great potential for better understanding heteroplasmy at the single-cell level.^{8,9} Refining the spatial resolution further, however, by characterising mitochondrial heteroplasmy at the single mitochondrion level may provide additional insight into the spread and clonal expansion of mutations, enable rare disease-relevant variants to be identified, and allow possible trends in mutational load to be assessed in distinct subcellular locations. This work has begun via micropipettes and nanopipettes to isolate mitochondria, followed by sequencing methods.¹⁰⁻¹²

Previous work showed that a single mitochondrion from the axon of a mouse hippocampal neuron could be extracted using the nanotweezer.¹³ The extracted mitochondrion had an active mitochondrial membrane potential throughout the trapping procedure and after extraction from the cell. No further work or analysis of extracted mitochondria was performed, however, which would be necessary to explore the application of the technique for mitochondrial extraction. In

this chapter, the biomolecules extracted and analysed from live neurons using the nanotweezer are expanded to the mitochondria. The trapping procedure and viability of extracted mitochondria are confirmed, alongside optimisation of the lysis of a single mitochondrion and detecting its nucleic acids. The mtDNAs from single mitochondrion are successfully detected via copy number determination using qPCR. Additionally, work to achieve single mitochondrion mtDNA amplicon sequencing is outlined, demonstrating the potential to analyse the whole mitochondrial genome from a single mitochondrion to assess mitochondrial heteroplasmy in single cells.

5.2 Single mitochondrion isolation

5.2.1 Single mitochondrion nanobiopsy

In neurons, mitochondria are distributed throughout the whole cell. However, the morphology and composition of the mitochondrial network differ in subcellular compartments of neurons; mitochondria are densely populated in the soma, where the somatodendritic region can contain a long and connected network, whereas axonal mitochondria are generally singular and more sparsely distributed, occupying smaller volumes in the cell.¹⁴ These differences meant that the nanotweezer trapping procedure needed to be optimised accordingly to trap and isolate a single mitochondrion (**Figure 5.1**).

To ensure individual mitochondrion, not multiple mitochondria, were isolated using the nanotweezer, neurons were transfected with mtDsRed, a plasmid targeting the mitochondrial matrix to express a fluorescent protein (**Figure 5.1b**). This enabled precise targeting of specific mitochondria of interest for DEP trapping and enabled axonal and dendritic mitochondria to be distinguished based on their morphology. To achieve DEP trapping of single mitochondrion in living neurons, the dendrites and axons were targeted as these contained distinct single mitochondria along the length of the neurites, meaning that individual mitochondrion could be targeted more efficiently. As the mitochondria in the soma are too densely populated, targeting the nanotweezer in this compartment would lead to multiple mitochondria being trapped due to numerous mitochondria being present in the trapping volume. This could be useful for evaluating whole subcellular compartments of the same cell, for example, by extracting multiple mitochondria from each compartment and normalising against a housekeeping gene to account for differences in the number of mitochondria trapped. However, this was not explored in this work, and the focus was on single mitochondrion extraction.

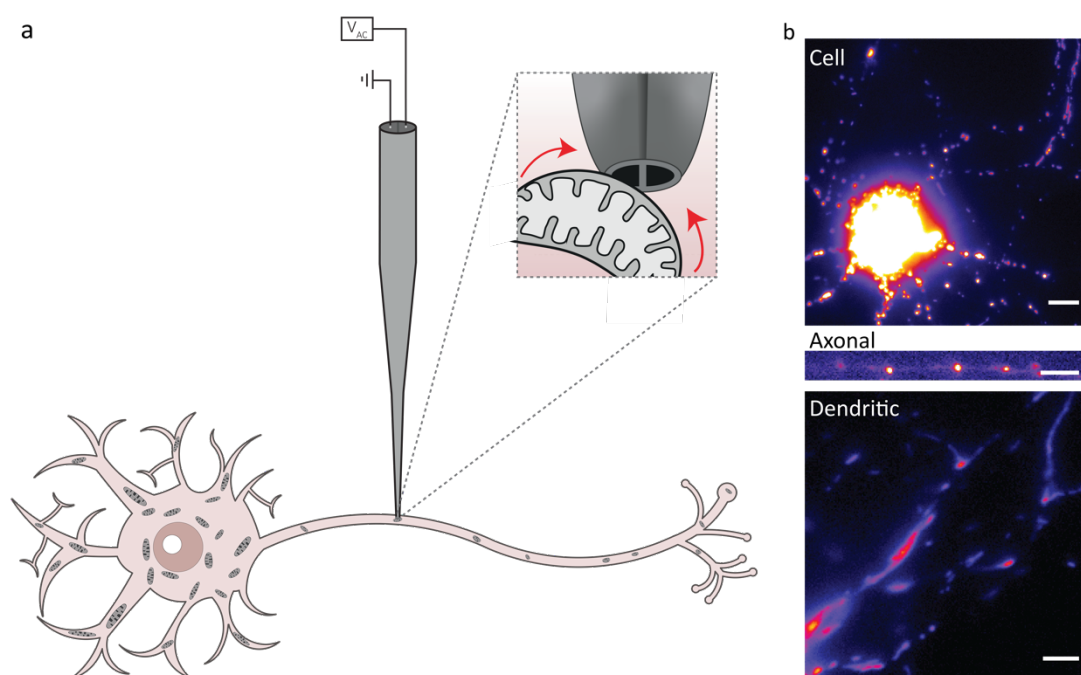


Figure 5.1: Single mitochondrion trapping schematic and labelling. a) Schematic illustrating trapping of a single mitochondrion from a neuron using the nanotweezer. b) Fluorescent images of hippocampal neurons transfected with mtDsRed to label and identify the mitochondria, showing a fluorescent image focused on the somata mitochondria and its extensions (top), and zoomed images of axonal (middle) and dendritic (bottom) mitochondria. Scale bars = 10 μm for cell and 5 μm for axonal and dendritic images.

Figure 5.2 shows the process for single mitochondrion trapping and extraction. Mitochondria of interest were targeted using fluorescent imaging, followed by manual insertion of the nanotweezer into the cell where the mitochondrion of interest was located. DEP was then applied and the nanotweezer was removed from the cell, extracting the trapped mitochondrion. Confirmation of the isolated mitochondrion was confirmed by fluorescent imaging (**Figure 5.2c-d**), where a loss of mitochondrial fluorescence from the cell was observed, and an addition of mitochondrial fluorescence was observed on the nanotweezer tip after removal from the cell (**Figure 5.2e**). Most previous mitochondrial isolation work has not isolated mitochondria from the neurites of neurons.^{12,15,16} It is likely that these methods, such as FluidFM which removes a large proportion of the cell's cytoplasm to access its contents, would be too invasive to remove mitochondria from these fine structures and may destroy the cell's structure. Micropipettes have been used to remove single mitochondrion from neurons, but the large size of these pipettes (0.7 μm) may mean that they are either close to, or larger than, the approximate width of certain neurites, raising questions about the cell's viability and the phenotypic impact during mitochondrion isolation from the neurites. The nanotweezer, therefore, offers a

minimally invasive method to extract a single mitochondrion from the neurites based on its nanometre size and direct trapping of biomolecules.

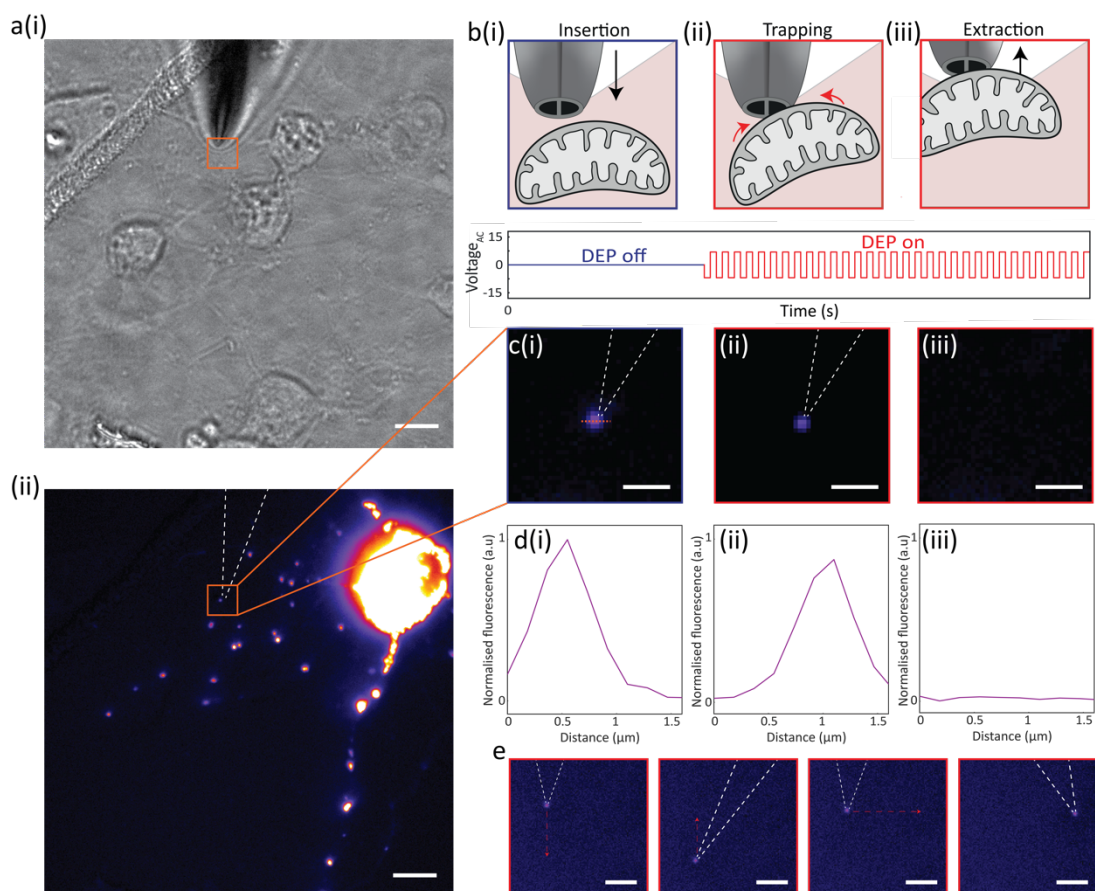


Figure 5.2: Single mitochondrion trapping procedure. a) Bright-field (i) and fluorescent (ii) images of nanotweezer targeting a single mitochondrion inside a hippocampal neuron. Scale bars = 10 μm . b) Schematic showing insertion of the nanotweezer (i), trapping via application of a DEP force (ii) and extraction of the mitochondria via removal of the nanotweezer from the cell. c) Corresponding fluorescent images of the targeted mitochondrion after insertion of the nanotweezer (i), DEP trapping at the nanotweezer tip (ii) and removal of the nanotweezer from the cell (iii), showing complete loss of the targeted mitochondria's fluorescence. Scale bars = 2 μm . d) Corresponding fluorescence traces of the targeted mitochondrion measured over the distance shown by the dashed line in (c), showing levels of mitochondrial fluorescence before application of DEP (i) trapping of the mitochondrion at the tip upon application of DEP (ii) and loss of mitochondrial fluorescence following removal of the trapped mitochondrion. e) Fluorescent images of the nanotweezer tip outside the cell after removal of the trapped mitochondrion, where the nanotweezer was moved in x and y directions and showed the fluorescent mitochondrion moving simultaneously, confirming trapped mitochondrion at the tip. Scale bars = 5 μm .

The trapping parameters of 1 MHz and 15 V_{pp} showed consistent single mitochondrion isolation, where voltages below 15 V_{pp} could not trap mitochondria. Nevertheless, some mitochondria could not be trapped, even when the voltage was raised to 20 V_{pp} . These included larger mitochondria ($\geq 2.5 \mu\text{m}$) and ones that were moving. For the former, one may assume that trapping larger mitochondria may be easier to trap as, for spherical homogeneous particles,

the DEP force generated is proportional to the cube of the particle's radius (**Equation 1.1**) and Brownian motion is less significant for micron-sized particles compared to submicron particles.¹⁷ This has also been shown by trapping DNA in solution with the nanotweezer, where smaller molecules of DNA had higher field requirements.¹³ However, trapping and isolating a mitochondrion from a cell provides a much more complex setting than the simple DEP model, which may lead to unpredictable trapping behaviour. For example, mitochondria are heterogeneous organelles made up of two membranes. They can also vary in shape, where larger mitochondria are typically elongated rather than spherical. This differs from the classic one-shell spherical model of DEP and may mean that the parameters for trapping different sized mitochondria need to be optimised.

Mitochondria are also dynamic organelles, part of a dynamic mitochondrial network in the cell where they regularly undergo fusion-fission cycles.^{27,28} Mitochondrion that are not isolated and are fused to other mitochondria may be more difficult to trap and may require higher DEP forces to separate them from other mitochondria in the cell. Their properties may also change if they are bound to other mitochondria or components in the cell and if they shrink or swell. In cases where mitochondria are fused, it may also be challenging to isolate a single mitochondrion rather than a collection of mitochondria that the targeted mitochondrion is fused to. For this reason, isolated mitochondria were targeted using the nanotweezer in this work to ensure that single mitochondrion were extracted.

The nanotweezer could not trap mitochondria that were moving, whether that was rapid anterograde or retrograde movements, or partial movement and 'wiggling' motions. This may be because even larger DEP forces may be required to trap these moving mitochondria as the DEP force will have an additional force to overcome for trapping. Voltages higher than 20 V_{pp} were not attempted to achieve these higher DEP forces due to concerns regarding the viability of cells with high voltages. Further optimisation of the nanotweezer, however, perhaps by minimising the gap between the electrodes further, may aid the removal of moving mitochondria. This could be advantageous as these types of mitochondria may be of particular interest to better understand mitochondrial trafficking, mitophagy and fusion-fission cycles, all of which have been shown to be impacted in neurodegeneration.^{29,30}

5.2.2 Mitochondrion viability

Assessing the viability of extracted mitochondria and confirming that intact mitochondria were isolated was necessary prior to downstream analysis. This was performed using TMRM, a fluorescent probe readily sequestered by mitochondria with active membrane potentials, to label the mitochondria instead of the mtDsRed transfection. Detecting mitochondria with active membrane potentials was used as a marker of mitochondrial viability. This showed that mitochondria were fully functional with electron transport occurring, where a loss or decrease in fluorescence would signify a loss of membrane potential and mitochondrial dysfunction.³¹ The nanotweezer was targeted to a labelled mitochondrion and extracted in the same manner described previously. The fluorescence of the mitochondrion was observed before and during DEP application, as well as after extraction from the cell, with the nanotweezer maintained in the cell's culture medium (**Figure 5.3a-c**). This confirmed trapping, as before, where an increase in fluorescence was observed at the nanotweezer tip upon application of DEP, showing that the targeted mitochondrion moved towards the nanotweezer tip (**Figure 5.3b**). It also confirmed that viable mitochondria were extracted using the nanotweezer, where no loss of mitochondrial fluorescence was observed during the trapping procedure and, after extraction from the cell, the fluorescence remained on the nanotweezer tip (**Figure 5.3c**). This showed that DEP did not affect the mitochondrial membrane potential and that the mitochondria remained functional even after removal from the cell. Further, this work confirmed that whole, intact mitochondrion were extracted from cells with the nanotweezer: if the nanotweezer procedure caused fragments of mitochondria to be extracted, this would destroy the mitochondria and lead to complete loss of fluorescence of the targeted mitochondria, however, this was not observed. This meant that whole viable mitochondria were extracted from the neurons which could be used for downstream single mitochondrion analysis.

Other stationary mitochondria within the same cell and adjacent cells were also monitored for effects on their membrane potential (**Figure 5.3d**). No change in the fluorescence of other mitochondria was observed during trapping of the targeted mitochondrion, confirming that they also maintained their viability. This further highlights the minimal impact that the nanotweezer has on living cells and their environment.

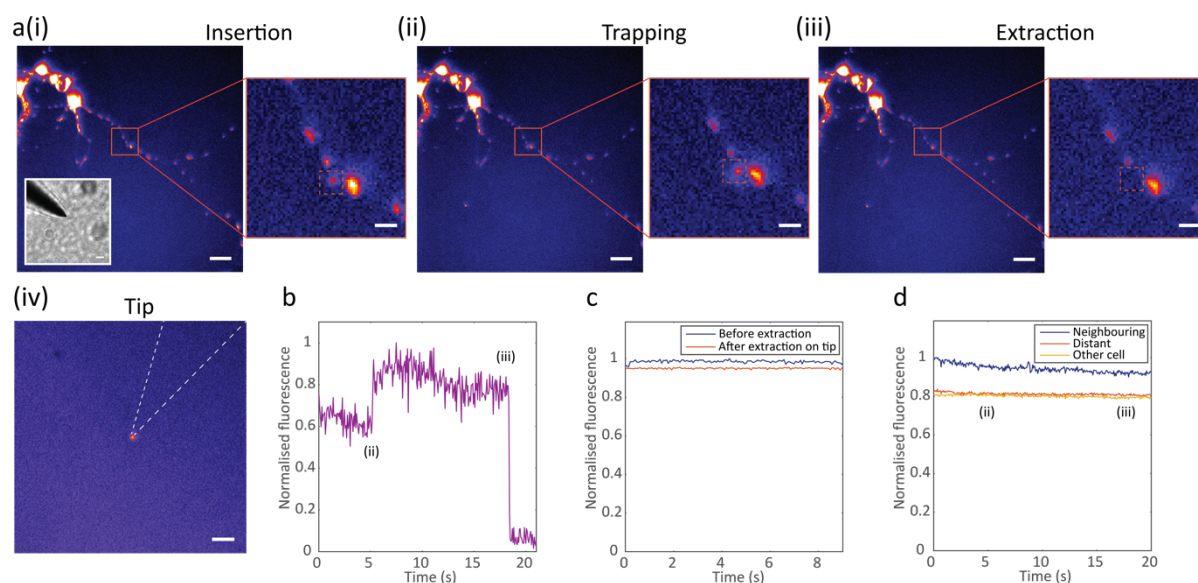


Figure 5.3: Mitochondrial viability during single mitochondrion isolation. a) Mitochondria were labelled with TMRM to monitor active mitochondrial membrane potentials. The nanotweezer was inserted into a neuron next to a labelled mitochondrion (i), DEP was applied to trap the mitochondrion, showing no loss or change in mitochondrial membrane potential (ii), the nanotweezer was removed from the cell to extract the mitochondrion (iii) and the tip of the nanotweezer was visualised outside the cell to confirm extraction of a viable mitochondrion (iv). Scale bars = 20 μm , zoomed scale bars = 10 μm , tip (iv) scale bar = 10 μm . b) Fluorescence-time trace of the nanotweezer tip during the trapping procedure, showing the timepoint where DEP was applied for mitochondrion trapping at the tip (ii) and when the nanotweezer was removed from the cell, leading to a loss of fluorescence from removal of the mitochondrion (iii). c) Fluorescence of the targeted mitochondrion before (blue) and after (orange) nanotweezer extraction, showing no loss of mitochondrion membrane potential. Fluorescence was normalised against the largest fluorescence value measured. d) Fluorescence of non-targeted neighbouring mitochondrion (blue), distant mitochondrion from the same cell (orange) and mitochondrion from a different cell (yellow) during the extraction procedure, with the timepoints of DEP application (ii) and extraction of the targeted mitochondrion (iii) highlighted. No significant change in fluorescence and membrane potential of other mitochondria was observed during the isolation procedure. Fluorescence was normalised against the largest fluorescence value measured across mitochondria.

The long-term fluorescence and viability of trapped mitochondria were also assessed by measuring the fluorescence of the mitochondrion on the nanotweezer tip after extraction from the cell (**Figure 5.4**). Interestingly, this work showed that trapped mitochondria remained viable with active membrane potentials when trapped at the nanotweezer tip outside the cell. The fluorescence was measured for over 30 min after extraction from the cell, showing no change or loss in mitochondrial membrane potential (**Figure 5.4c**). Due to photobleaching, the fluorescence could not be continuously measured over longer periods. Nevertheless, the fluorescence from the mitochondrion was still observed 90 min after extraction from the cell (**Figure 5.4d**). Not only did this work confirm the minimally invasive nature of the nanotweezer on mitochondrial function, but it also showed great promise for future mitochondrial transplantation applications. The long periods that mitochondria were viable after extraction from the cell would provide ample time for the mitochondrion to get transferred

to another cell, for example, by transplanting a healthy mitochondrion into a cell with dysfunctional mitochondria as a method of mitochondrial regeneration therapy.³² This could supplement ATP production in degenerated neurons, increase neuronal activity or alleviate the mutational load of mtDNA in disease.³³ FluidFM recently achieved this where transferred mitochondria from HeLa cells were able to fuse to the mitochondrial network of HEK293 cells and incorporate their mtDNA into the recipient mitochondrial genome.¹⁵ The nanotweezer may provide much more precise transplantation applications, where the high spatial resolution of the technique could be exploited for transplantation into specific subcellular compartments, such as the synaptic contacts, to monitor the dynamics of mitochondrial regeneration at specific locations and throughout a single cell.

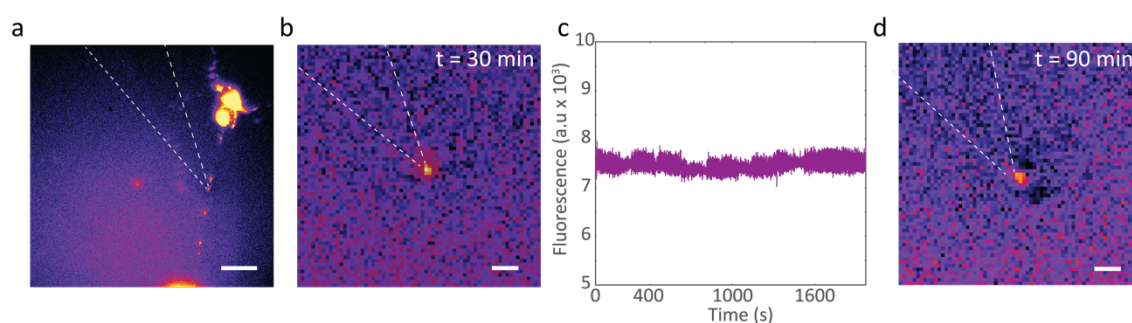


Figure 5.4: Isolated mitochondrion viability. a) Fluorescent image of TMRM-labelled mitochondria in live neurons with the nanotweezer targeted to a single mitochondrion for extraction. Scale bar = 20 μm . b) Targeted mitochondrion trapped at the nanotweezer tip 30 min after extraction from the cell, showing that the mitochondrion was still viable. Scale bar = 2 μm . c) Fluorescent-time trace of the trapped mitochondrion on the nanotweezer tip after extraction from the cell, showing that the fluorescence was maintained for over 30 min. d) The same trapped mitochondrion on the nanotweezer tip 90 min after extraction from the cell. Scale bar = 2 μm . Dashed white lines represent the position of the nanotweezer.

5.3 Mitochondria analysis optimisation

5.3.1 Primer design

To analyse the contents of a single mitochondrion using RT-qPCR or qPCR, an initial panel of primers was designed for genes encoding for cytochrome-c-oxidase (complex IV) subunits, MT-CO1, MT-CO2 and MT-CO3. Primers for MT-RNR1 were also designed to provide an abundant mitochondrially-encoded ribosomal gene. Designed primers were tested and optimised on bulk rat mitochondrial lysate isolated from cells as outlined in **Chapter 2.3**. The specificity of these was evaluated by gel electrophoresis (**Figure 5.5a**) and melt curve analysis using qPCR (**Figure 5.5b**). This showed that single amplicons were produced using the optimised primers, and no non-specific products were generated which would otherwise affect

the efficiency of the qPCR and the quantification. These primers were therefore optimal for downstream single mitochondrion analysis. Gel extraction and quantification of the purified amplicons were performed to generate positive control standards for absolute qPCR quantification, as outlined in **Chapter 2.11**.

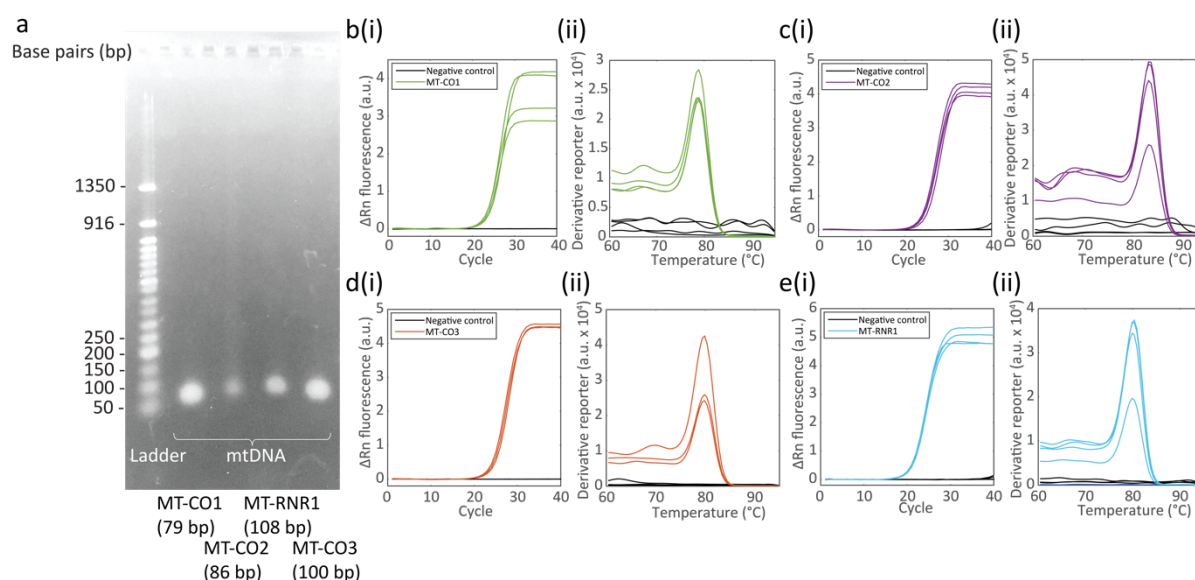


Figure 5.5: Mitochondria primer design. a) Agarose (2.5 %) gel electrophoresis of amplified mtDNA using optimised designed primers (rat) for MT-CO1, MT-CO2, MT-RNR1 and MT-CO3, showing single amplicons produced of the expected size. b-e) qPCR amplification curves (i) and melt curve analysis (ii) of 60 ng bulk rat mtDNA using optimised primers for b) MT-CO1, c) MT-CO2, d) MT-CO3, and e) MT-RNR1, showing single specific products produced. ΔRn fluorescence represents the normalised (Rn) and baseline-corrected fluorescence of the emission from the fluorescent reporter dye or probe. Derivative reporter represents the rate of change of fluorescence with respect to time.

5.3.2 Mitochondrial lysis

Analysis of the contents of isolated single mitochondrion using the nanotweezer had not previously been achieved. The work from Morris *et al.*,¹¹ in which micropipettes were used to isolate mitochondria from cultured neurons, demonstrated that a single mitochondrion could be chemically lysed using a lysis buffer incubation step followed by subjecting diluted lysates to two rounds of PCR (standard PCR followed by semi-nested PCR) prior to next-generation sequencing (NGS) to sequence the whole mtDNA genome. In the work described here, the same lysis buffer was used without diluting the lysates in order to maximise the concentration of nucleic acids present, followed by qPCR and RT-qPCR to attempt to analyse the nucleic acids present in single mitochondria. No detectable amplification was observed, however. It was deduced that some of the concentrations of components in the lysis buffer, such as SDS, sodium chloride and EDTA, were likely inhibiting the amplification. These are known to

inhibit DNA polymerase activity and affect PCR efficiency at certain concentrations.³⁴ This inhibition was confirmed by using a high amount of a purified positive control standard with the same volume of lysis buffer used in single mitochondrion analysis (**Figure 5.6**). This confirmed that even when large copy numbers of the sample were present, the lysis buffer components completely inhibited the amplification, where no amplification was observed. In comparison to positive controls without lysis buffer present, low C_q values were observed as expected for these high copy numbers. This inhibition perhaps also explained why lysates were diluted in Morris *et al.*'s work, to reduce the concentration of PCR inhibitors, and why two rounds of PCR was required, as perhaps little sample was successfully amplified after the first round due to inhibitors being present. Diluting the samples with lysis buffer into 10 separate qPCR reactions was attempted which led to some amplification. However, this gave poorer C_q values compared to the same amount of DNA present without any lysis buffer, showing that inhibition still occurred. Additionally, splitting individual samples up would not be practical when analysing single mitochondrion as ideally most of the mitochondrion's contents should be in the same well to maximise the detection. This therefore meant that a new lysis method needed to be employed to minimise PCR inhibition from individual samples.

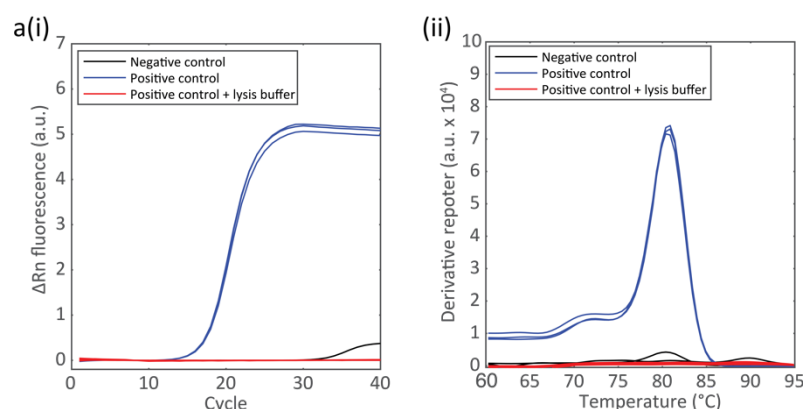


Figure 5.6: Lysis buffer PCR inhibition. a) qPCR amplification curve (i) and corresponding melt curve analysis (ii) of 10^6 copies of purified MT-RNR1 positive control with (red) or without (blue) lysis buffer present in the same reaction. Each positive control sample was amplified using MT-RNR1 primers, alongside nuclease-free water negative controls (black). ΔRn fluorescence represents the normalised (Rn) and baseline-corrected fluorescence of the emission from the fluorescent reporter dye or probe. Derivative reporter represents the rate of change of fluorescence with respect to time.

In other mitochondrial work, sonication has been used as a method of mitochondrial lysis.^{35–37} This has also been used for single mitochondrion analysis, where single mitochondria were isolated using optical traps and drawn into a micropipette, followed by the tip of the pipette being subjected to sonication.¹⁰ Sonication was therefore considered as a method of

mitochondrial lysis. Previous work employed a heating step (95 °C, 10 min) after the sonication step to disrupt mitochondrial membranes further.¹⁰ This was also employed in this work, along with a comparison of adding a Proteinase K treatment to the sonication step, as has commonly been employed after chemical lysis,^{11,38,39} to digest the mitochondrial membrane proteins and further disrupt the mitochondrial structure. **Figure 5.7a** shows the Cq values obtained from qPCR of the same volume of mitochondria sample without sonication and with various sonication conditions with heating and Proteinase K treatment. The most significant decrease in mean Cq value compared to mitochondria that had not been sonicated or treated was observed for a combination of sonication and Proteinase K treatment, suggesting that this method was optimal for mitochondrial lysis. Detection of mtDNA was observed in samples that had not been sonicated, which may be due to freeze-thawing isolated mitochondria and diluting mitochondria in nuclease-free water prior to analysis. Diluting the samples in water was performed to minimise the addition of any other salts or components that may inhibit the PCR reaction. However, this would likely have led to osmotic swelling and disruption of mitochondria which may explain the detection of mtDNA in the non-lysed mitochondria isolation. Nevertheless, bursting via the immersion of mitochondria in water was less disruptive than as sonication or Proteinase K treatment.

Although this sonication method presented a promising alternative mitochondrial lysis method to chemical lysis, practical considerations needed to be considered. Difficulties arose in preventing loss of sample volume on the sonicator probe, particularly when working with small volumes of 10 µl which would be necessary for maintaining the concentration of single mitochondria. Additionally, the detection of mtDNA was observed from water being sonicated. This cannot be attributed to contamination in the qPCR reaction because negative controls provided a mean Cq value of 35. The detection of mtDNA in water sonicated samples likely arose from cross-contamination of mtDNA from the sonicator probe, where, although care was taken to clean the probe in between samples, some mitochondrial content may have remained on the probe to have given such low Cq values of sonicated water. Despite the Cq values for the optimal sonication treatment being significantly different to those obtained from sonicating water, any amount of cross-contamination would likely significantly skew the data observed when going down to the single mitochondrion level and so this key aspect meant that sonication was not taken forward as a mitochondrial lysis method.

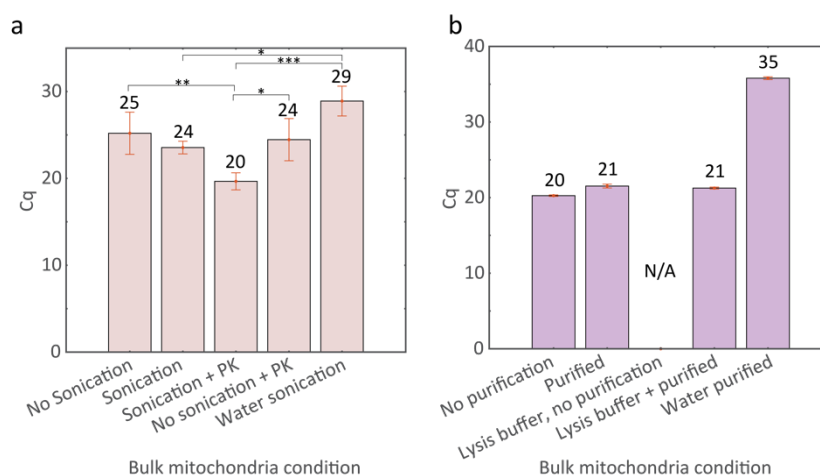


Figure 5.7: Bulk mitochondrial lysis assessment. a) Bar chart showing the Cq values obtained from qPCR of bulk mitochondria subjected to different lysis conditions. Bulk mitochondria were isolated from approximately 80,000 rat hippocampal neurons and diluted 1:5500 for sonication. The same dilution was used to compare different treatments. Mitochondria were subjected to: no lysis; sonication followed by heating at 95 °C for 10 min; sonication with Proteinase K treatment (0.05 µg) and inactivation for 10 min at 95 °C; Proteinase K treatment (0.05 µg) and inactivation for 10 min at 95 °C; and a comparison to water sonicated controls followed by heating at 95 °C for 10 min. Sonication was performed using 2 s pulses for 2 min at 400 W, 25 % amplitude. qPCR was performed on 5 µl of each sample using primers for MT-RNR1 (n = 4). Statistical analysis was performed using one-way ANOVA and Tukey's multiple comparisons test between all samples. Shapiro-Wilk normality test and Browne-Forsythe variance test were performed prior to statistical analyses. *p<0.05, **p<0.01, ***p<0.001, all other comparisons were not significant if not otherwise stated. b) Bar chart showing the Cq values obtained from qPCR of chemically lysed mitochondria subjected to different purification conditions. Bulk mitochondria were isolated from approximately 80,000 rat hippocampal neurons, chemically lysed following the protocol from Morris *et al.*,¹¹ and lysates were diluted 1:100 in nuclease-free water. The same dilution was used to compare different treatments. Mitochondrial lysates were subject to: No purification or treatment; purification with magnetic beads; the addition of 4 µl lysis buffer with no purification; the addition of 4 µl lysis buffer followed by magnetic bead purification; with a comparison to controls of water that was purified with magnetic beads. qPCR was performed on 5 µl of each sample using primers for MT-RNR1 (n = 2). The numbers on top of the bars represent the mean Cq value of each sample. Error bars represent s.d.

An alternative method was then trialled to remove PCR inhibitors. This involved chemically lysing mitochondria as before, followed by purification of mitochondrial nucleic acids after the lysis step. Solid-phase nucleic acid extraction using purification columns was not attempted as it was likely that much of the contents of a single mitochondrion would get held in the purification column during elution and limit the yields. Magnetic bead purification was trialled instead, however, as bead or particle binding are commonly used methods for single-cell nucleic acid isolation and allow for smaller elution volumes, which would be essential for concentrating nucleic acids from a single mitochondrion.^{40,41} AMPure XP Bead-based reagent was used on bulk mitochondrial lysate using the modified protocol outlined in **Chapter 2.9.2**. As the bulk mitochondrial lysate was diluted to ensure appropriate Cq values were obtained in the qPCR, an additional 4 µl of lysis buffer was added to samples to replicate the buffer conditions of a single mitochondrion, where one would not dilute the lysates. qPCR was run

on the same amount of mitochondrial lysate under various purification conditions to assess the effects of purification on qPCR detection (**Figure 5.7b**). These results showed that purification effectively removed the PCR inhibition observed with the lysis buffer components, where only a difference of 1 Cq value was observed when comparing the purified lysate sample with the unpurified diluted lysate due to sample loss during the purification procedure. Additionally, unlike sonication where one must reuse the sonication probe, fresh magnetic beads were used for each sample which minimised cross-contamination. The high Cq value of the purified water control demonstrated this (35) as it was very similar to the Cq value from the qPCR negative control (37), which was considered negligible for this SYBR green assay.⁴² Overall, magnetic bead purification was considered to be an effective method for removing PCR inhibitors from mitochondrial lysis and enabling the detection of mtDNA using qPCR

5.3.3 Sequencing

Following the detection of bulk mtDNA using qPCR, sequencing was performed on bulk mitochondrial lysate to confirm that this was feasible prior to transitioning to the single mitochondrion level. For this initial test, one region of the mitochondrial genome was amplified from bulk mitochondrial lysate using a high-fidelity polymerase to maintain accuracy. Inevitably, amplification will introduce bias even with high-fidelity polymerases. One study found that with the high-fidelity polymerase used in this work, Q5 polymerase, base substitution errors primarily arose from thermocycling-induced DNA damage which was two-fold higher than the rate of polymerase substitution errors.⁴³ This polymerase showed the highest fidelity out of the range of polymerases studied in the publication relative to Taq polymerase, but substitution errors were still observed at a low rate of 5.3×10^{-7} . In the work described here, however, amplification was used as it would be necessary for single mitochondrion genome sequencing due to the extremely low copy numbers of mtDNA per mitochondrion, estimated to be between 0-11.^{44,45} The bulk conditions therefore needed to replicate the single mitochondrion conditions accordingly during optimisation, where Q5 polymerase was used to minimise these amplification errors for sequencing.

An amplicon from bulk rat mtDNA was successfully sequenced and aligned to the reference genome (**Figure 5.8a**). The quality score (Q-score) or Phred score represents the accuracy of base calling and is defined by:

$$Q = -10\log_{10}(P)$$

Equation 5.1

where Q is the Phred quality score, and P is the probability of an error. Each base call has a Q -score associated with it, where $Q=20$ means that there is a 1 % chance that the base was called incorrectly. For this bulk sequencing result, the proportion of bases that had a Q -score ≥ 20 was 85 %, and the distribution of these Q -scores is shown in **Figure 5.8b**. **Figure 5.8a** shows the 6 mutations or unidentified bases observed in the alignment, which were as follows: substitution, substitution, insertion, substitution, deletion, unidentifiable, deletion. The Q -scores for each were 12, 20, 9, 18, 6, 10 and 7, respectively. The accuracy of many of these base calls was therefore low and could not be determined as true mutations. For the T->C substitution which had a higher Q -score of 20, however, the same substitution has been reported to be conserved in many strains of rat.⁴⁶ This corresponds to position 2170 in the rat mitochondrial genome and was suggested to have potential importance for mitochondrial function due to the position being in close proximity to the L1 binding domain.⁴⁶ This suggested that this observed substitution in bulk hippocampal rat mtDNA may be a true mutation. To investigate this further, a larger amount (54 ng) of rat hippocampal mtDNA from a different rat was amplified in the same manner, which also showed this T->C substitution. None of the other mutations observed in the sequence from **Figure 5.8a** were observed in this second sequencing run.

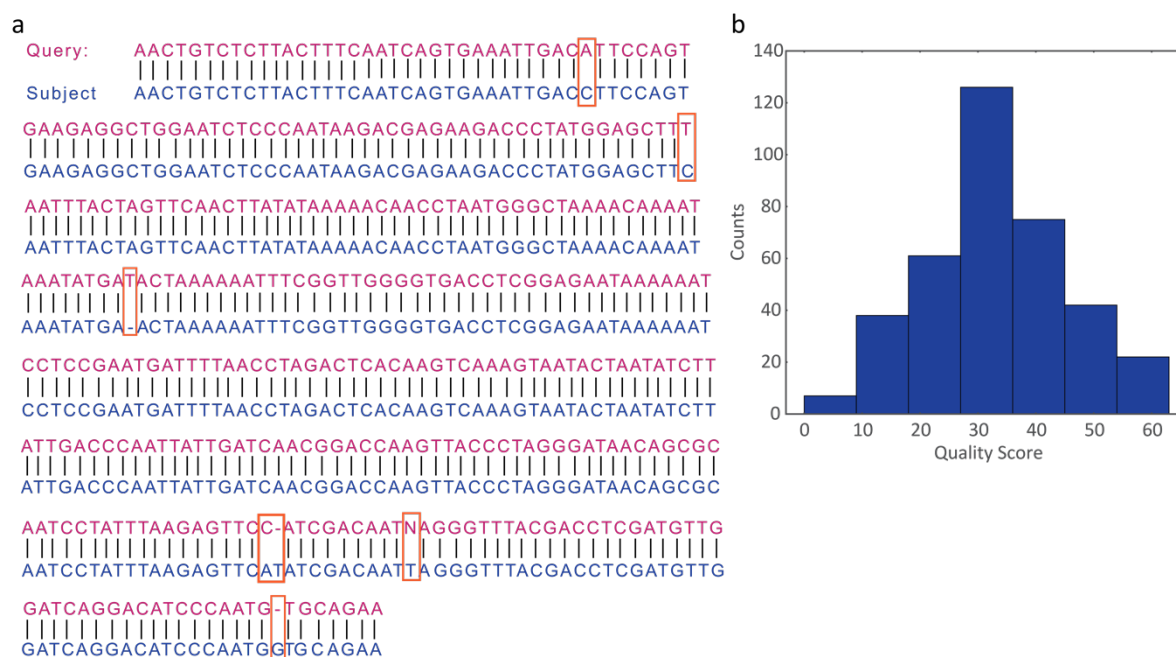


Figure 5.8: Sanger sequencing of bulk mitochondrial amplicon. a) Sequencing alignment of a region within the MT-RNR2 gene obtained from PCR amplification of 6 ng mtDNA. The sequencing result is shown by the ‘Query’ (pink), and the consensus sequence for that region is shown by the ‘Subject’ (blue). Orange boxes show

bases where no alignment was observed. b) Histogram presenting all the quality scores obtained for each base of the sequencing result.

5.4 Single mitochondrion analysis

5.4.1 mtDNA copy number

Having shown that magnetic bead purification of chemically lysed mitochondria effectively removed PCR inhibition and led to qPCR detection of targets, single mitochondrion isolated from hippocampal neurons were lysed, purified, reverse transcribed to cDNA and then amplified in qPCR with the aim to detect the nucleic acids present in single mitochondrial nanobiopsies. This successfully led to the amplification of nucleic acids contained in the extracted mitochondrion (**Figure 5.9a**). Absolute quantification was performed to quantify the number of nucleic acids detected in each mitochondrion by constructing a standard curve of known amplicon quantities (**Figure 5.9b**). The number of MT-RNR1 nucleic acids detected is shown in **Figure 5.9c**. It should be noted that mitochondrially-encoded genes in animals do not contain introns and that the maturation of mitochondrial RNA is much simpler than the maturation of nuclear-encoded mRNAs.⁴⁷ This means that designing primers that span exon-exon junctions would not lead to specific amplification of cDNA and exclude amplification of genomic DNA; splicing does not occur and much of the primary transcript remains in the mature transcript. Therefore, the copy numbers reported here represent a combination of mtDNA and MT-RNR1 RNA. As the number of mtDNA copies per mitochondrion varies, the contribution of mtDNA in the copy numbers cannot be ignored when assessing mitochondrial gene expression. This is discussed further in **Chapter 6**. Nevertheless, this work provided a significant step towards detecting nucleic acids contained in a single mitochondrion isolated from cells using the nanotweezer.

Changes in mtDNA copy number have been linked to a number of diseases, where decreases in mtDNA copies in both biological fluids and tissues have been suggested as potential biomarkers of neurodegenerative disease and ageing.^{48–53} Assessing mtDNA copies in single mitochondrion could, therefore, be extremely useful in assessing the variation in copy number in single cells, as well as how this might differ in disease models to further validate this as a marker of disease. Investigating if mtDNA copy number varies in different subcellular locations of neurons could also be interesting. For example, regions of a cell more prone to injury, such as long, thin, unmyelinated axons, may have different mtDNA copy numbers as

an adaptation or response to cellular stress and vulnerability. This vulnerability has been shown in PD and AD where sparsely myelinated or unmyelinated regions were more vulnerable to α -synuclein and tau aggregates than heavily myelinated regions.^{54–56} Additionally, it is unknown whether subcellular regions with high numbers of mitochondria and energy demands, such as the synaptic terminals, may have altered mtDNA copies per mitochondrion compared to regions with fewer mitochondria. Previous work has shown that sublethal rotenone exposure upregulates mtDNA replication in distal axons and that increased mtDNA replication occurs in the neurites before extending to the cell bodies.⁵⁷ This implies that compartment-specific changes in mtDNA copy number may occur in neurons rather than a global cellular change in mitochondrial replication. This highlights the importance of measuring mitochondrial content in specific compartments of neurons; these findings could provide key information on basic mitochondrial regulation and compartment-specific mechanisms, which may lead to novel insights into mitochondrial quality control.

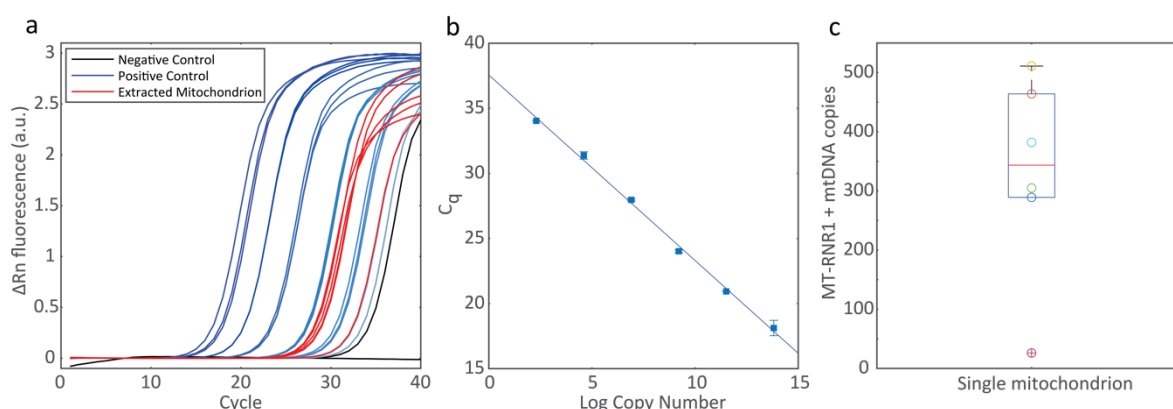


Figure 5.9: Single mitochondrion nucleic acid quantification. a) RT-qPCR amplification curves for the amplification of MT-RNR1 in single mitochondria isolated using the nanotweezer alongside negative RT controls and positive controls of known MT-RNR1 amplicon quantities. ΔRn fluorescence represents the normalised (Rn) and baseline-corrected fluorescence of the emission from the fluorescent reporter dye or probe. b) qPCR standard curve constructed from known quantities of MT-RNR1 amplicons for absolute quantification of MT-RNR1 in single mitochondria. c) MT-RNR1 and mtDNA copy numbers calculated per mitochondrion from RT-qPCR ($n = 5$). Each circle represents a single mitochondrion. Cross represents an outlier defined as more than 3 scaled median absolute standard deviations from the median.

qPCR was directly performed on purified single hippocampal mitochondria isolated with the nanotweezer to evaluate if the mtDNA copy number could be determined for an individual mitochondrion. Amplification was successfully observed in 9 out of 14 mitochondria (**Figure 5.10a-c**), providing copy numbers of ≥ 1 per mitochondrion. mtDNA quantification from single mitochondria was also compared to various nanotweezer controls performed on the same dish of cells, where no amplification was observed for these controls (**Figure 5.10d**). This showed

that the copy numbers obtained were derived directly from isolated mitochondria rather than from cell-free mtDNA in the cell, the cell's media, or the purification process. The overall mtDNA copy numbers measured per mitochondrion are shown in **Figure 5.10e**, providing copy numbers between 0 and 13. Considering that there may be some loss from the purification process to explain why mtDNA was undetected in some mitochondria, the copy numbers obtained were feasible based on the reported estimated mtDNA copy numbers of 0-11.^{44,45} One study estimated an average of 2.6 copies of mtDNA per mitochondrion, computed based on averages from larger mitochondrial isolates and enzymatic activity.⁴⁵ Another study reported copy numbers between 0 and 11, with a mean of 2, and was based on fluorescence-activated cell sorting of mitochondrial particles followed by qPCR analysis, similar to the analysis method described in this work.⁴⁴ It is important to highlight that absolute qPCR quantification is based on the construction of a standard curve, where, despite the standard curve constructed in this work having a high R^2 value (0.99) and the primers having good PCR efficiency (96 %), slight variations may affect the quantification, particularly when assessing copy numbers in the low range described here where one will be close to the limit of detection of the qPCR. This quantification may therefore be better suited to comparing samples rather than looking at absolute numbers, for example, by comparing the changes in copy number between mitochondria in different subcellular locations of healthy and PD neurons. Digital PCR may be more effective at measuring absolute mtDNA copy numbers in the future, however, as digital PCR is more sensitive in the low copy number range, does not require a calibration curve, and can provide more precise absolute quantities.⁵⁸

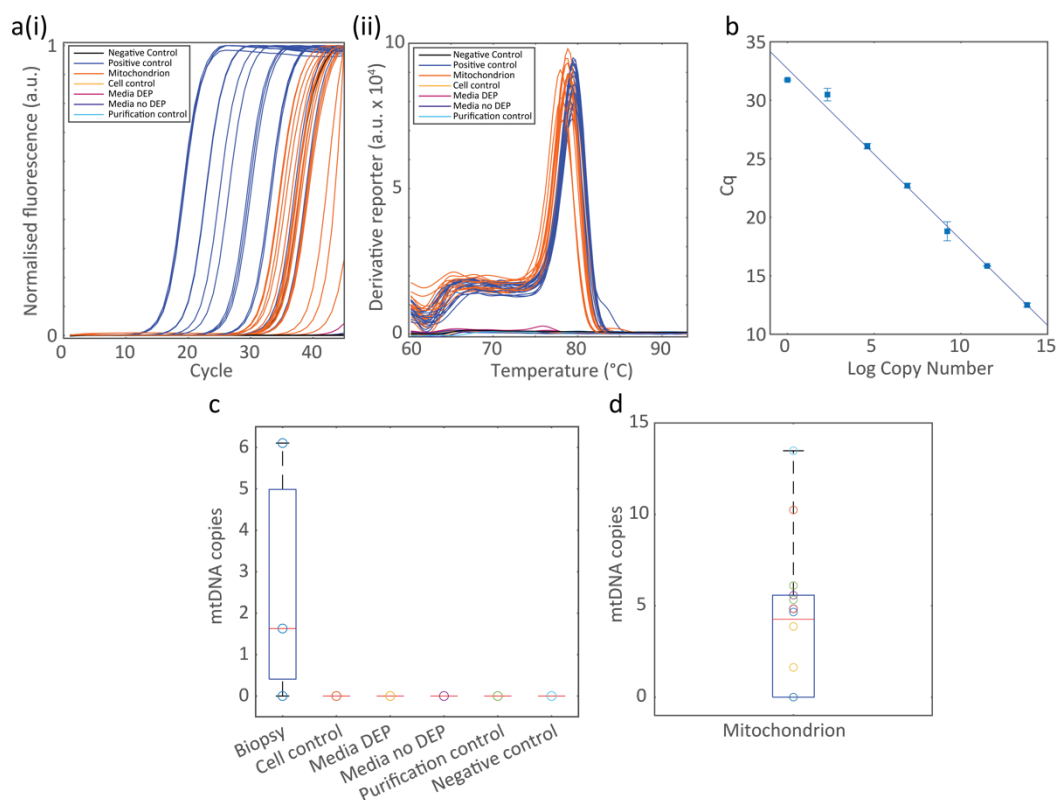


Figure 5.10: Single mitochondrion mtDNA copy number quantification. a) qPCR amplification curve (i) and melt curve analysis (ii) for mtDNA copies per single mitochondrion extracted from hippocampal neurons using the nanotweezer compared to controls to confirm the derivation of mtDNA from an isolated mitochondrion. Copy number quantification was based on the detection of the MT-CO1 gene. Derivative reporter represents the rate of change of fluorescence with respect to time. b) Standard curve for absolute quantification of mtDNA copy number, constructed with known quantities of MT-CO1 positive control amplicons. c) mtDNA copy numbers detected in single mitochondrion isolations and controls from the same cell and culture dish, confirming that the detection of mtDNA was not due to circulating free mtDNA. d) Single mitochondrion mtDNA copy numbers calculated from qPCR absolute quantification. Each circle represents a single mitochondrion ($n = 14$). Outliers that were more than 3 scaled median absolute standard deviations from the median were removed.

5.4.2 mtDNA sequencing

The same protocol for bulk Sanger sequencing in **Chapter 5.3.3** was used to attempt single mitochondrion sequencing. Initially, the same primers used for qPCR were employed to amplify the mtDNA prior to sequencing. The best alignment obtained from single mitochondrion sequencing is shown in **Figure 5.11a**, showing the exact position of the isolated mitochondrion from a dendrite of a live hippocampal neuron and its mtDNA alignment against the reported consensus sequence for that region of the mitochondrial genome. The only difference observed against the consensus sequence was one thymine base insertion. The Q-score for this insertion was 32, meaning there was a 99.94 % chance that the base was called correctly. An additional single mitochondrion sequencing result from a different region of the mitochondrial genome is shown in **Figure 5.11b**. From single mitochondrion sequencing of

short mtDNA amplicons, the proportion of bases that had a Q-score ≥ 20 was $60 \pm 5\%$ ($n = 3$). This work provided the foundations for single mitochondrion sequencing from mitochondria extracted using the nanotweezer, providing a proof-of-concept study to build on for analysing heteroplasmy within single cells. For sequencing the entire mitochondrial genome of multiple single mitochondria in the future, NGS or long-read sequencing should be employed for higher throughputs. This would also be useful for detecting low-frequency heteroplasmic variants in the mitochondrial genome which one would expect to observe at the single mitochondrion level.

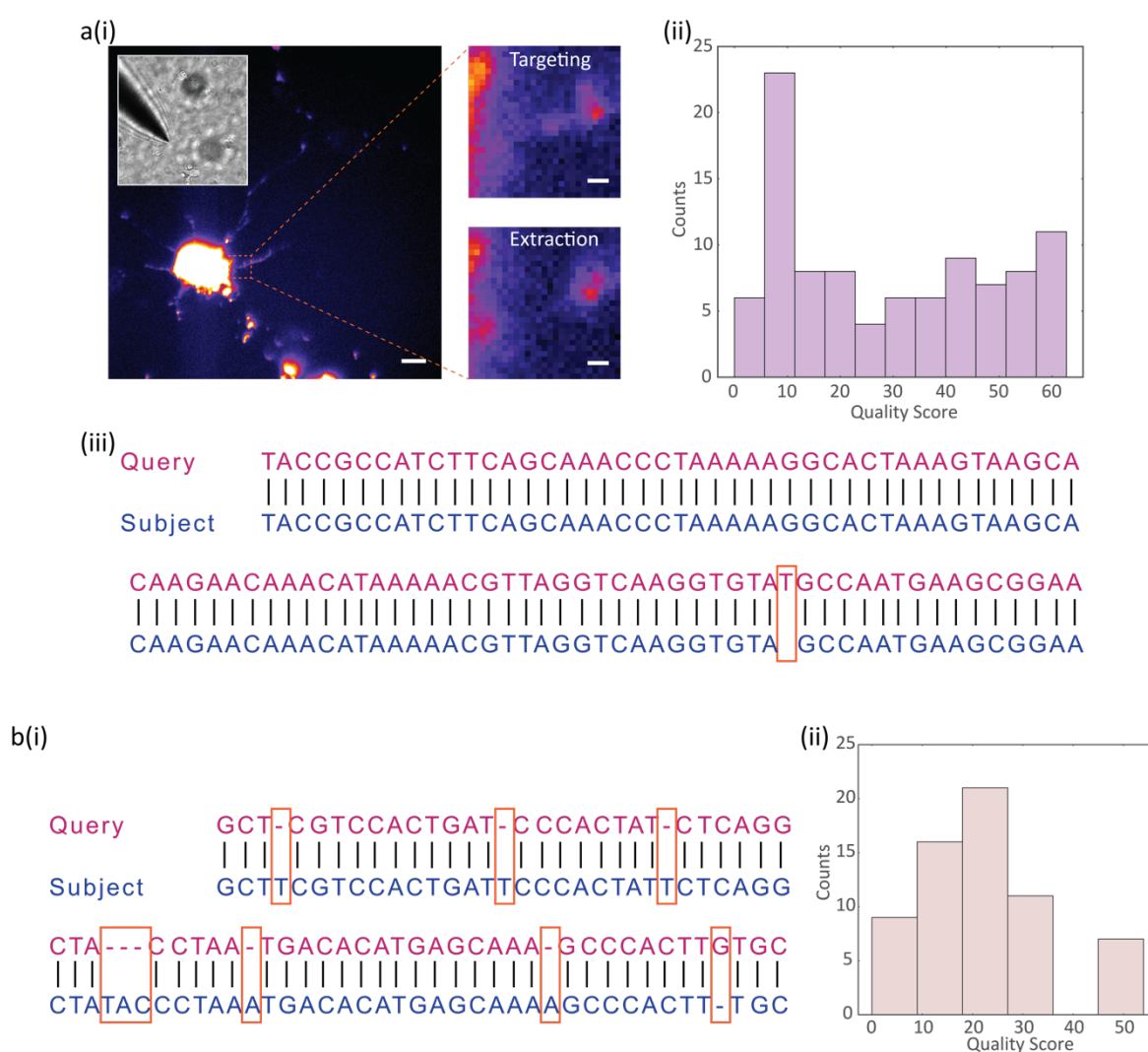


Figure 5.11: Single mitochondrion amplicon sequencing. a) Single mitochondrion sequencing for a region within the MT-RNR1 gene, showing the location of the trapped mitochondrion during and after extraction (i), the distribution of Q-scores for the base calling (ii), and the alignment of the sequencing result (Query) against the consensus sequence (Subject) for this region (iii). Whole cell scale bar = 10 μm , zoomed scale bar = 1 μm b) An additional single mitochondrion sequencing result for a region within the MT-CO1 gene, showing alignment if the result (Query) against the consensus sequence (Subject) for this region (i) and the distribution of Q-scores for the base calling (ii).

5.5 Limitations and open challenges

The work in this chapter outlines the nanotweezer's ability to isolate single mitochondrion from living neurons, followed by the detection of mtDNA from a single mitochondrion. In its current state, the isolation has a bias towards smaller mitochondrion. This may lead to some bias in the types of mitochondria sampled and analysed. For example, perhaps different sized mitochondria have differences in calcium buffering, capacities for energy storage, and their dynamics, similarly to how axonal mitochondria (small) have different motion and calcium buffering compared to dendritic mitochondria.^{59,60} This may lead to differences in the nucleic acid contents of the mitochondria sampled. However, a definite link between mtDNA content and size is unknown, but it could be interesting to explore with some optimisation of the technology to sample larger mitochondria. This could be achieved by increasing the size of the nanopipette so that larger disruptions in the cell membrane are formed to extract larger mitochondria. This, however, needs to be carefully tuned to ensure efficient trapping is maintained as larger nanopipettes would have larger distances between the electrodes, meaning smaller DEP trapping forces would be generated.

As discussed earlier, mitochondrion transplantation would be a fascinating application of the technology for highly precise cell or subcellular regeneration therapies. However, as with trapped RNA, single mitochondrion could not be readily removed from the nanotweezer tip, even when using frequencies up to 20 MHz to attempt to achieve negative DEP. This technological challenge limits the ability to perform transplantation. Additionally, compared to other techniques where mitochondria are isolated inside a pipette and are relatively protected, the mitochondria trapped by the nanotweezer are trapped on the outside of the nanopipette. Although the viability studies in this chapter have shown that mitochondrion isolation from a cell does not affect the viability of mitochondria, the mitochondria are still exposed on the tip which may mean some damage could occur when attempting to transplant it into another cell or region.

5.6 Conclusions

The work described in this chapter builds on the proof-of-concept single mitochondrion isolation work of Nadappuram *et al.*,¹³ evaluating the viability of mitochondrion extracted

using the nanotweezer and developing methods to detect the contents of single mitochondrion. The potential for assessing mtDNA copy number differences in mitochondria from single cells was outlined. This could have applications in understanding spatially resolved patterns of mitochondrial regulation in subcellular regions and differences in healthy and disease model neurons. Methods to sequence regions of the mitochondrial genome of single mitochondrion were developed, highlighting the potential for the nanotweezer to be used to analyse mitochondrial heteroplasmy in living single cells. This is within reach, with further development to analyse the whole mitochondrial genome, for example, using whole genome amplification methods or long-range PCR. This could have significant implications in understanding the dynamics and spread of mutations in single cells, as well as the mutational thresholds that lead to disease or cell death, providing key steps towards unravelling the complexity of mitochondrial biology, its role in pathology, and ultimately the development of therapeutic targets and strategies.

5.7 References

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Chapter 6

Single mitochondrion gene expression analysis

Synopsis

This chapter builds upon the single mitochondrion extraction and analysis work introduced in **Chapter 5** to focus on the specific and novel detection of mitochondrial RNAs from isolated mitochondria. Single mitochondrion analysis methods are optimised and summarised, prior to successfully implementing a dynamic study on mitochondrial expression in the same live cells over 24 hours. The impact of α -synuclein oligomers on single mitochondrion expression in live *in vitro* neurons are investigated, where an overall downregulation of mitochondrially-encoded targets was observed after incubation with aggregated protein. This work paves the way for future treatment and disease tracking at the single mitochondrion level in live cells which will combine true single-cell responses with the precise contextual information obtained with the nanotweezer.

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6.1 Introduction

The mitochondrial genome encodes a small but crucial set of transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), and polypeptides.^{1,2} These mitochondrially-encoded polypeptides are essential for the key proteins involved in oxidative phosphorylation (OXPHOS) and ATP production. As neurons rely heavily on their mitochondria for their high energy demands, regulation of mitochondrial gene expression and mitochondrial dynamics is vital for synaptic transmission, maintenance of membrane potentials, calcium buffering and apoptosis.³ Mitochondria also rely on the import of nuclear gene products for their function.¹ Studying mitochondrial gene expression is of particular importance as it could provide insight into the intricate interplay between nuclear and mitochondrial genomes, as well as shedding light on neuronal metabolism and how this changes under stress and in neurodegenerative disease. Mitochondrial subpopulations are apparent in neurons.⁴ For example, synaptic mitochondria are distinct in their morphology,^{5,6} proteomic profiles,⁷ enzyme activity,⁸ calcium buffering,⁹ and vulnerability compared to non-synaptic mitochondria.¹⁰ This, combined with the knowledge that specific compartments of a neuron may be selectively degenerated in neurodegenerative disease before the loss of the neuron,^{11,12} highlights the need to study the heterogeneity of compartmentalised mitochondria within single neurons. Understanding the spatial differences of mitochondria in neurons and the specific effects that pathology has on them could be advanced by analysing individual mitochondria, allowing for highly precise mechanisms to be unravelled and novel treatment targets to be identified with high precision. Steps towards this have been shown in a recent high-resolution imaging study, where mitochondrial translation products were observed in the axons, dendrites and synaptic regions of hippocampal neurons.¹³ Further advances in approaches like these have extraordinary potential to improve understanding of basic mitochondrial biology in single neurons and organelles in both health and disease, enabling the discovery of novel cellular mechanisms and improved therapeutic interventions against neurodegenerative diseases.

This chapter addresses the capability to study gene expression at the single mitochondrion level in live neurons using the nanotweezer platform. **Chapter 5** described the use of the nanotweezer for single organelle extraction and introduced methods to analyse the mtDNA from a single mitochondrion. Whilst single mitochondrion mtDNA analysis could be of great importance for understanding heteroplasmy and the mechanisms involved in the spread of mutations in single cells in ageing and disease, single mitochondrion mtRNA analysis could

be exceptionally interesting for measuring transient changes in gene expression in living cells and how this is linked to dynamic cellular phenotypes.

The challenge in achieving single mitochondrion mtRNA analysis, however, lies in the exclusive capture of mtRNA and removal of mtDNA from single mitochondrial lysates. This is because, unlike nuclear DNA, mtDNA can be present in varying copies per mitochondrion. The amount of mtDNA present in a mitochondrion can, therefore, not be ignored as it could lead to false positive results when analysing the cDNA of mtRNAs. Additionally, mammalian mitochondrially-encoded genes do not contain introns,^{1,14} so primers cannot be designed to span exon-exon junctions to exclude the amplification of DNA.

This chapter prioritises the analysis of dynamic mitochondrial gene expression in living neurons. Protocols are optimised to achieve single mitochondrion mtRNA analysis whilst excluding mtDNA. These are then applied to a study on the effects of α -synuclein oligomers, the toxic form of the protein implicated in Parkinson's disease (PD),^{15,16} on mitochondrial gene expression in living neurons. This work provides an innovative method to track mitochondrial gene expression in single cells, illustrated by a disease-specific model, and creates novel prospects to understand direct pathological implications on single organelles.

6.2 Mitochondrial RNA analysis optimisation

6.2.1 DNase treatment

In order to remove mtDNA from lysed mitochondria to achieve specific mtRNA analysis, a DNase treatment was attempted. DNase I is an endonuclease that digests double-stranded DNA by cleaving phosphodiester bonds, generating short 5'-phosphate-3'-OH oligonucleotides. Initially, the effectiveness of the treatment was trialled on low copy numbers of λ -DNA (10-1000 copies) to provide a control that exceeded the maximum copies of mtDNA per mitochondrion. qPCR was performed on the DNase treated products which showed effective elimination of λ -DNA and no Cq values generated over 40 cycles (**Figure 6.1a**). The same conditions were then applied to diluted bulk mitochondrial lysate to assess removal of mtDNA (**Figure 6.1b**). This was performed by including DNase-treated mitochondrial lysate, where one expected no amplification to be observed in the qPCR, and reverse transcribed DNase-treated mitochondrial lysate, where one would expect specific amplification of mtRNA in the absence of mtDNA. However, complete removal of mtDNA was not achieved, where Cq

values of 27-29 were observed for DNase treated lysate, compared to a Cq values of 22 for the same lysate that was untreated. This showed that the DNase treatment had some effect on the removal of mtDNA, but that it was unable to achieve complete removal. The bulk mitochondrial lysate used in this work was diluted 100x in water, meaning that the lysis buffer was assumed to have had minimal effect on the DNase I under these diluted conditions. However, this would be something to consider for single mitochondrion analysis where, if DNase treatment was effective, the treatment should be tested using the same volume of lysis buffer in the reaction to ensure that the buffer did not inhibit the enzyme.

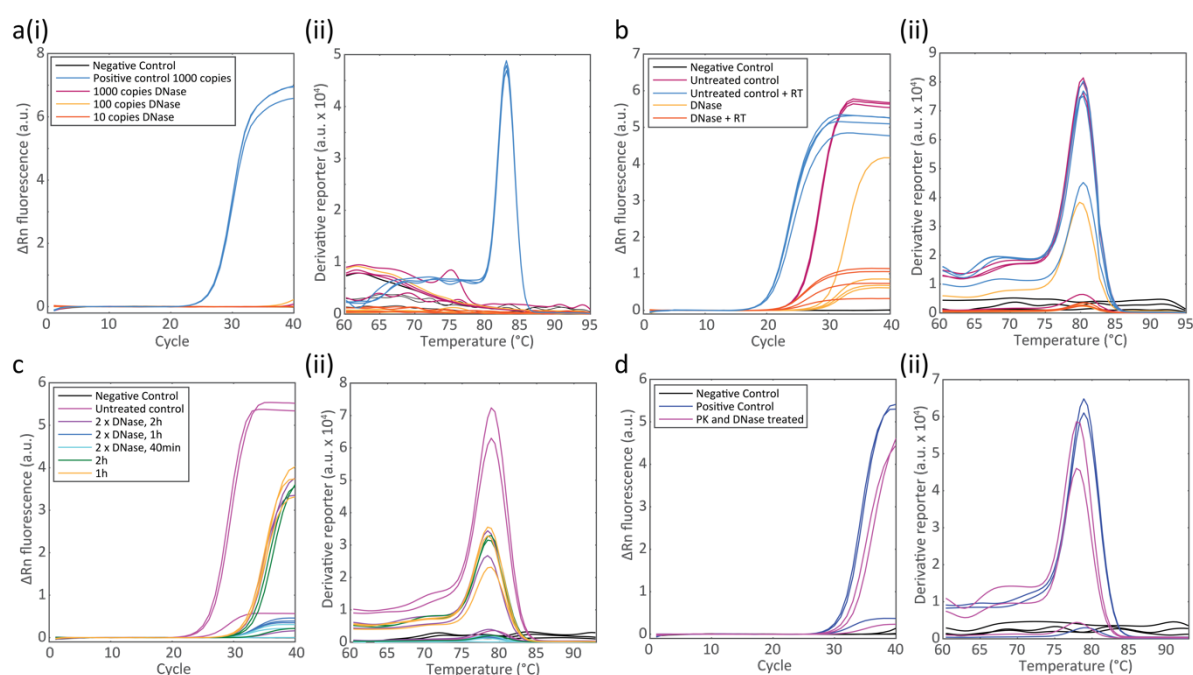


Figure 6.1: DNase treatment for mtDNA elimination. a) qPCR amplification (i) and melt (ii) curves for amplification of 10-1000 copies λ -DNA after DNase I treatment, showing effective removal of λ -DNA in treated samples compared to untreated controls. b) qPCR amplification (i) and melt (ii) curves for amplification of DNase treated mitochondrial lysate with and without reverse transcription performed, showing some, but not complete, elimination of mtDNA in DNase treated samples. c) qPCR amplification (i) and melt (ii) curves for amplification of mitochondrial lysate treated with either single or double units of DNase with incubation times between 40 min to 2 h, compared to untreated controls, showing incomplete mtDNA removal. d) qPCR amplification (i) and melt (ii) curves for amplification of mitochondrial lysate treated with Proteinase K or Proteinase K followed by 2 h DNase treatment, compared to untreated control, showing incomplete mtDNA removal. ΔRn fluorescence represents the normalised (Rn) and baseline-corrected fluorescence of the emission from the fluorescent reporter dye or probe. Derivative reporter represents the rate of change of fluorescence with respect to time.

Following this incomplete digestion of mtDNA, stronger treatments were performed, where the digestion was extended from 30 min to 40 min-2 h, and twice as much DNase I was incubated with mitochondrial lysate diluted 1000x in water to provide optimal DNase conditions (**Figure 6.1c**). This was still unable to achieve complete mtDNA removal, where

the largest Cq values (lowest copies) obtained were 31 ± 2 for a single unit of DNase incubated for 2 h, compared to the untreated control Cq of 24 ± 2 . This was an improvement of the DNase treatment and was considered as a potential method to take forward to the single mitochondrion level, where the Cq values would be much higher for single mitochondrion mtDNA and so the DNase treatment may effectively remove all mtDNA detection over 40 cycles. One possible reason for the incomplete removal of mtDNA with the DNase treatment compared to the λ -DNA control may be due to the structure of mtDNA in its native form. mtDNA is packed into discrete mtDNA-protein complexes called nucleoids, where several proteins bind to mtDNA for mtDNA maintenance and transcription.¹⁷ These nucleoid structures may potentially protect the DNA from digestion and explain why a standard DNase treatment is ineffective at completely removing mtDNA. In an attempt to disrupt these nucleoid structures to access the mtDNA, a protease treatment was performed to degrade some of the potentially protecting proteins bound to the mtDNA prior to DNase treatment. Mitochondrial lysate was diluted a further 10,000x in water to get closer to the low levels of mtDNA present in the sample, and 1 μ g Proteinase K was incubated with the sample for 1 h. This provided a second, stronger Proteinase K treatment compared to the initial mitochondrial lysis step. qPCR analysis showed that the Proteinase K combined with DNase treatment had a minimal effect on the complete removal of mtDNA, however (**Figure 6.1d**).

Although the stronger DNase treatment showed a large change in Cq value compared to the untreated control, it was important to consider the proposed conditions and number of steps required to analyse a single mitochondrion. The DNase buffer conditions, combined with the EDTA used to chelate magnesium ions and prevent the enzyme from being active, were likely to lead to a similar inhibition of PCR amplification as observed with the mitochondrial lysis buffer described in **Chapter 5**. Additionally, the mitochondrial lysis buffer, already containing EDTA and requiring minimal dilution of lysates at the single mitochondrion level, may provide suboptimal conditions for DNase to work effectively. This suggested that two possible purification steps, before and after DNase treatment, would be required to analyse the mtRNA from a single mitochondrion. This would lead to a significant loss of mitochondrial content alongside additional incubation steps, which may affect RNA integrity and further reduce the chances of detecting mtRNA from a single mitochondrion.

6.2.2 Surface-modified magnetic bead purification

An alternative method to exclude mtDNA from mitochondrial lysate was then tested prior to moving down to the single mitochondrion level. As outlined in **Chapter 5**, mitochondria required magnetic bead purification to remove the lysis buffer components prior to any amplification. The removal of DNA during the same procedure was therefore explored to minimise the sample preparation steps. This was performed by surface-modifying magnetic beads with complementary oligonucleotides to isolate mtRNAs from the lysate preferentially, as outlined in **Chapter 2.9.2**. This included an oligo(dT) repeated sequence to bind to mtRNAs with a poly(A) tail and a complementary sequence to 12S RNA (MT-RNR1).

Bulk mitochondrial lysate was initially used for purification using a mixture of both types of surface-functionalised beads. In the absence of reverse transcription, qPCR analysis was performed on the same volume of mitochondrial lysate with and without purification, which gave C_q values of 29 and 19, respectively, using primers for the MT-CO1 gene, and 28 and 19 using primers for the MT-RNR1 gene. This showed a significant reduction of mtDNA detection using the surface-functionalised beads. Due to the same number of sample preparation steps as the analysis of mtDNA in the previous chapter, this method was taken forward as a potential method to isolate and analyse mtRNAs from a single mitochondrion specifically. Firstly, single mitochondria isolated using the nanotweezer were purified using a mixture of both functionalised magnetic beads, followed by qPCR being performed without reverse transcription to detect any mtDNA present in isolated mitochondria (**Figure 6.2a**). No detection of mtDNA was observed in the absence of reverse transcription, confirming that the surface-functionalised beads effectively removed detectable mtDNA from the single mitochondrial lysate. This was then compared to single mitochondrion purification using the standard magnetic beads used in **Chapter 5** (**Figure 6.2b**), showing successful mtDNA amplification using the standard beads and no amplification using the surface-functionalised beads. This further confirmed the selectivity of the surface-functionalised beads for removing mtDNA from the lysate.

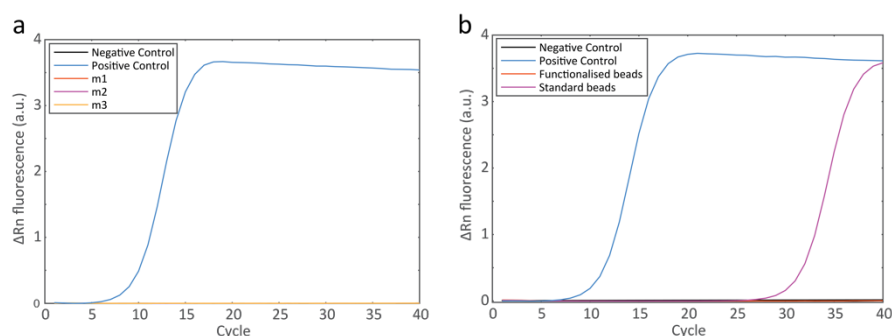


Figure 6.2: mtDNA detection of single mitochondrion purified using surface-modified and unmodified beads. a) qPCR amplification curves for single mitochondrion purified using surface-modified beads, showing no detectable amplification in the absence of reverse transcription. b) qPCR amplification of a single mitochondrion purified using surface-modified beads compared to using standard magnetic beads, showing successful detection of mtDNA using standard beads and no detection of mtDNA using surface-modified beads. ΔRn fluorescence represents the normalised (Rn) and baseline-corrected fluorescence of the emission from the fluorescent reporter dye or probe.

6.3 Single mitochondrion RNA analysis

Having confirmed the successful removal of mtDNA from single mitochondrial lysate, the isolation of detectable mtRNA from single mitochondria was validated. Multiple single mitochondria from the axon and dendrites of the same hippocampal neuron (**Figure 6.3a**) were extracted using the nanotweezer, lysed, and then purified with surface-modified magnetic beads. Due to the purification step, deactivation of proteinase K by heating for 10 min at 95 °C was no longer necessary during the lysis step, thus removing a step that may otherwise affect RNA integrity. The eluate from each single mitochondrion purification was reverse transcribed to convert any isolated RNA into cDNA. Using the primers designed in **Chapter 5** for MT-CO1, MT-CO2, MT-CO3 and MT-RNR1, qPCR absolute quantification was performed on each cDNA sample to quantify any isolated RNA (**Figure 6.3b-f**). Successful amplification of all genes was observed and quantified, demonstrating that mtRNA analysis of a single mitochondrion is possible without the inclusion of mtDNA.

The methods developed in this work revealed novel opportunities to analyse gene expression in individual mitochondria, which, to the best of our knowledge, has not been previously reported. This could have exciting applications for better understanding mitochondrial gene expression regulation in living cells, particularly for spatially and functionally distinct mitochondria in different subcellular locations of neurons in health and disease. The methods discussed in **Chapter 5-6** to analyse mtDNA and mtRNA from individual mitochondrion are summarised in **Figure 6.4**.

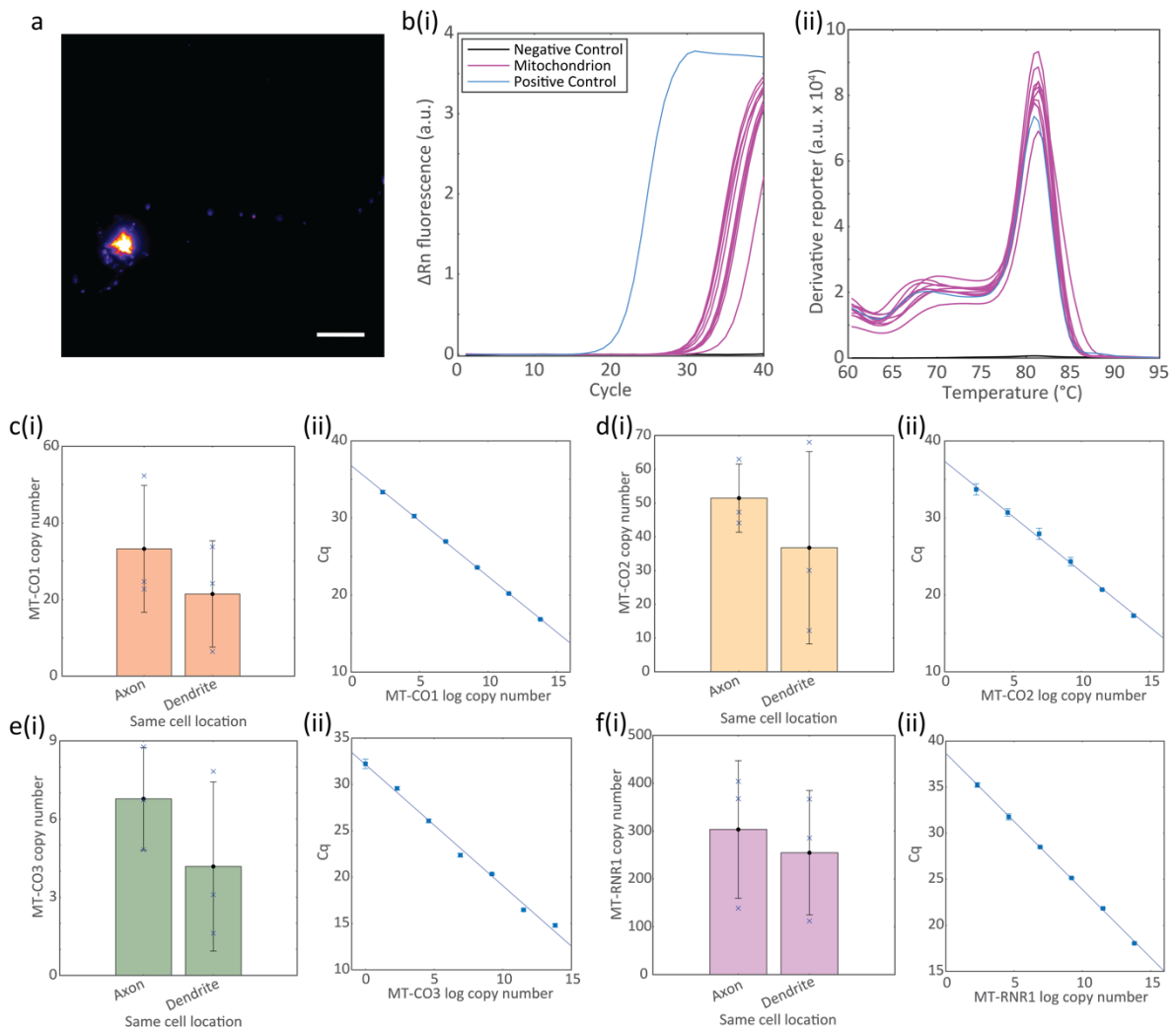


Figure 6.3: Single mitochondrion RNA analysis. a) Fluorescent image showing the mtDsRed-transfected hippocampal neuron which had individual mitochondria extracted from using the nanotweezer. Scale bar = 20 μm . b) Example amplification (i) and melt (ii) curve for MT-RNR1 amplification of single mitochondria alongside negative controls and a typical positive control. Amplification curves for each standard curve dilution are removed from the graphs and only a single positive control dilution is shown for clarity. ΔRn fluorescence represents the normalised (Rn) and baseline-corrected fluorescence of the emission from the fluorescent reporter dye or probe. Derivative reporter represents the rate of change of fluorescence with respect to time. c) MT-CO1 copy numbers obtained from isolated mitochondrion from the axon and dendrites of the same neuron (i) and standard curve constructed in the same qPCR run for absolute quantification (ii). d) MT-CO2 copy numbers obtained from isolated mitochondrion from the axon and dendrites of the same neuron (i) and standard curve constructed in the same qPCR run for absolute quantification (ii). e) MT-CO3 copy numbers obtained from isolated mitochondrion from the axon and dendrites of the same neuron (i) and standard curve constructed in the same qPCR run for absolute quantification (ii). MT-RNR1 copy numbers obtained from isolated mitochondrion from the axon and dendrites of the same neuron (i) and standard curve constructed in the same qPCR run for absolute quantification (ii). Error bars represent s.d (n = 3 mitochondria).

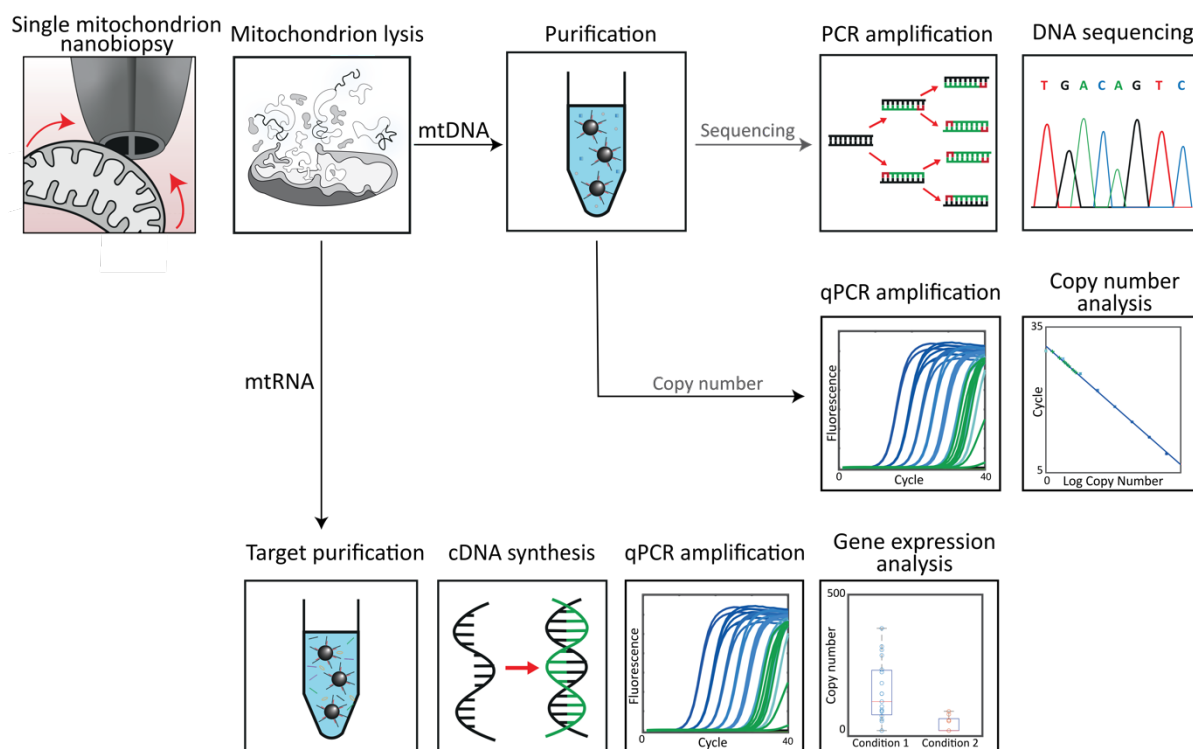


Figure 6.4: Summary of single mitochondrion analysis methods. Individual mitochondrion isolated from living neurons using the nanotweezer could be analysed for either their mtDNA content or gene expression.

Further primers for mitochondrially-encoded genes were then designed. This included MT-ND1, encoding a subunit of complex I in the electron transport chain, and MT-ATP6, encoding a subunit of complex V in the electron transport chain. These, in combination with the previously designed primers for MT-CO1 and MT-RNR1, would allow for a range of mitochondrially-encoded genes to be analysed to assess changes in OXPHOS and protein production. These primers were designed so that duplex reactions could be run in the qPCR, rather than the previous single target SYBR Green reactions, to increase the concentration of mitochondrial sample per well and improve chances of detection. The additional two designed primer pairs and the two duplex reactions for all 4 targets are illustrated in **Figure 6.5a**. Gel electrophoresis of PCR amplicons showed that single distinct bands were produced for the newly designed primers of MT-ND1 and MT-ATP6, demonstrating single PCR products and effective primer design. Additionally, duplex qPCR reaction products showed bands of the expected size for each amplicon. A small amount of non-specific product was observed in the gel for a duplex reaction between MT-CO1 and MT-RNR1, sized around 50 bp. However, when testing the single and duplex reactions in qPCR using bulk mtDNA, no difference between the copy numbers obtained in single and duplex reactions was observed (**Figure 6.5b**),

demonstrating that the multiplexed design did not affect the efficiency of the amplification and quantification.

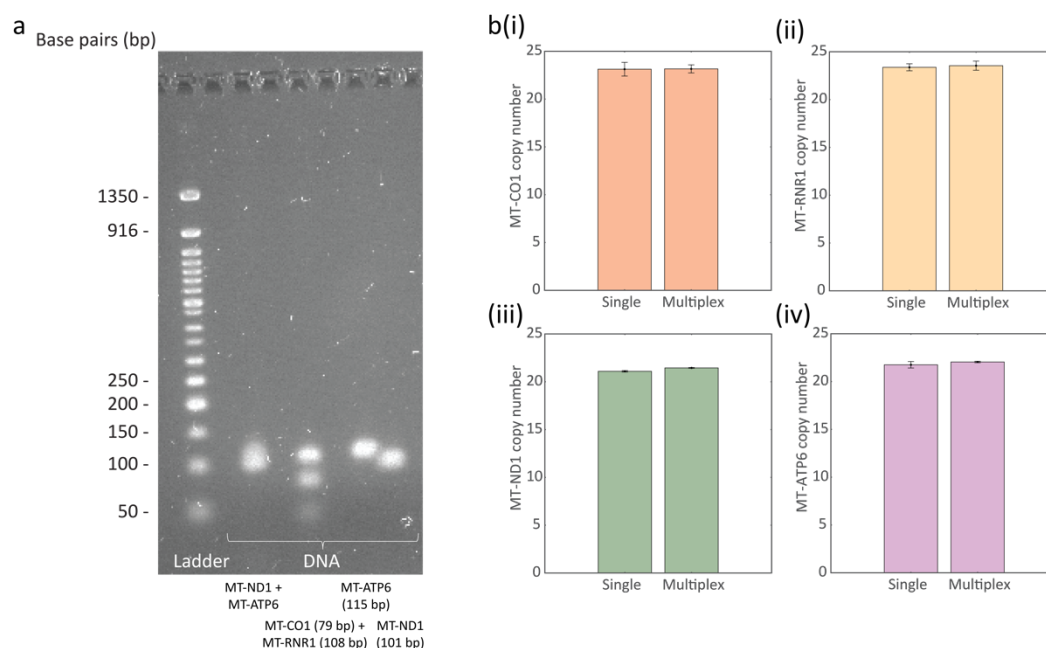


Figure 6.5: Mitochondrial multiplexed design. a) Agarose (2.5 %) gel electrophoresis of PCR amplified mtDNA using designed primers for MT-ND1 and MT-ATP6, alongside qPCR multiplexed reaction products for duplex reactions between MT-ND1+MT-ATP6 and MT-CO1+MT-RNR1, run next to a 50 bp DNA ladder for size comparison. b) Bar charts for the copy numbers obtained from qPCR amplification of purified bulk mtDNA (3.39 ng) for single and duplex reactions of MT-CO1 (i), MT-RNR1 (ii), MT-ND1 (iii) and MT-ATP6 (iv), showing no difference in copy numbers obtained in multiplexed reactions and therefore effective primer design. Error bars represent s.d (n = 3).

Although the isolation of single mitochondria was confirmed via fluorescence monitoring of labelled mitochondria during the nanobiopsy procedure, a comparison of nanobiopsies to controls was performed to further confirm that the mtRNAs detected derived from isolated mitochondria and not from the cell's media or sample preparation steps (**Figure 6.6**). Controls relating to the sample preparation steps included: a no RT control to confirm that detection derived from mtRNA and not mtDNA; a purification control, where water was used instead of mitochondria during the lysis and purification steps, to confirm that no mitochondrial contamination was present during these steps; and a negative control, where water was exposed to reverse transcription and qPCR amplification to confirm that there was no contamination in the qPCR steps. In all of these control cases, 0 copies of the mitochondrially-encoded genes studied were detected compared to the detected gene expression from isolated single mitochondria.

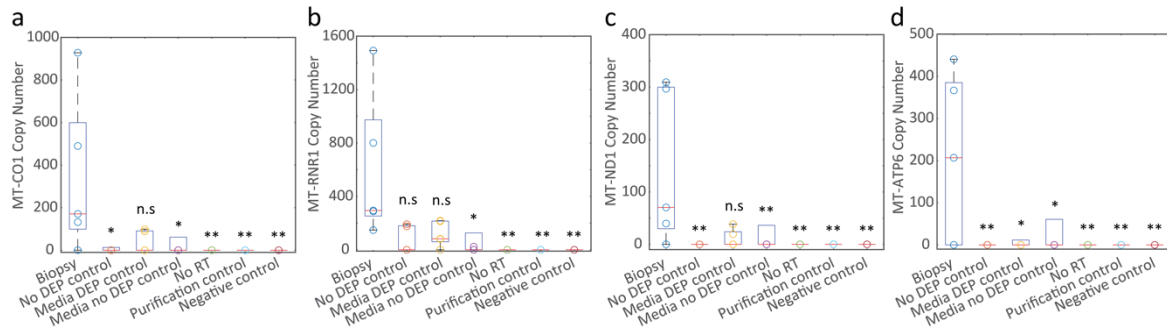


Figure 6.6: Single mitochondrion gene expression vs. controls. qPCR absolute quantification of mitochondria isolated from cortical neurons with the nanotweezer compared to controls ($n = 4$) for a) MT-CO1, b) MT-RNR1, c) MT-ND1, d) MT-ATP6. Outliers were defined as more than 3 scaled median absolute standard deviations from the median and were removed. Statistical analysis was performed using the Kruskal-Wallis non-parametric test between biopsy samples and all controls, followed by Dunn's multiple comparisons test. Shapiro-Wilk normality test was performed prior to statistical analyses. * $p < 0.05$, ** $p < 0.01$, n.s = not significant.

Comparisons were also made between single mitochondrial gene expression and various nanotweezer controls: nanotweezer insertion into the cell without DEP trapping to quantify any mitochondrial-free RNA in the cell that may get adsorbed onto the nanotweezer tip; nanotweezer insertion into the cell's media without DEP trapping to quantify any mitochondria or mitochondrial-free RNA in the media that may get adsorbed onto the nanotweezer tip; and DEP trapping in the cell's media to trap any possible cell-free mitochondria or mitochondrial-free RNA in the media that may get trapped during the biopsy process. Interestingly in some cases, mtRNAs were detected in the cell's media when DEP was applied. This may be a result of extracellular mitochondrial release or transcellular mitophagy, where mitochondria contained in endosomes get released from neurons via exocytosis and taken up by glial cells by endocytosis or phagocytosis.^{18,19} Similarly, there is some evidence for astrocytic transfer of mitochondria into injured neurons.^{20,21} Understanding mitochondrial trafficking between neurons and astrocytes has been of particular interest in recent years as it may reveal potential pathways that could be manipulated or targeted to enhance either healthy mitochondrial uptake or the removal of dysfunctional mitochondria in degenerated neurons. However, currently, there is little evidence and understanding of mitochondrial transfer between neurons and astrocytes and the circumstances in which these mechanisms may occur. Authors have suggested that mitochondrial transfer could be performed via a number of mechanisms, such as via the lysosomal pathway with the release of mitochondria from vesicles, mitovesicle fusion with the plasma membrane, or channel formation between neurons and astrocytes.²²

Based on the detection of mtRNAs in the culture media of this work, mitochondrial transfer could be explored with the nanotweezer in the future; vesicles containing mitochondria could be tracked and trapped from neurons, astrocytes, and the culture media to evaluate changes in mitochondrial gene expression. For example, levels of mitochondrially-encoded OXPHOS gene expression could be monitored in mitochondria taken up by astrocytes from neurons, validating if these mitochondria have low OXPHOS expression and are considered to be dysfunctional compared to the expression of other mitochondria in the same cell. Additionally, astrocytic mitochondria are known not to rely on OXPHOS for their energy production like neuronal mitochondria and, therefore, have lower expression levels of OXPHOS-related genes.^{23–25} Perhaps, if the transfer of astrocytic mitochondria into neurons does in fact occur, the transcriptomic identity of an astrocytic mitochondrion changes once it has entered a neuron and may begin to express higher levels of OXPHOS genes. Perhaps it may also integrate into the neuronal mitochondrial network, as has been shown previously with mitochondrial transplantation.²⁶ This could provide novel insight into mitochondrial quality control in both cell types and move us a step closer towards targeting these mechanisms for mitochondrial regeneration and cell repair.

6.4 Time-resolved single-cell mitochondrial gene expression

6.4.1 N-acetylated- α -synuclein aggregation

α -synuclein (α -syn) is an intrinsically disordered protein that is present in neuronal cells and plays an important role in neurotransmitter release and synaptic function.^{27,28} Fibrillar aggregates of α -syn are key components of Lewy bodies, intracellular protein inclusions that are widely regarded as a neuropathological hallmark of PD.^{29,30} Soluble oligomers, an intermediate between monomers and insoluble fibrils, are considered to be the toxic form of the protein.^{15,16} In particular, smaller soluble aggregates sized less than 100 nm may be the most toxic, where they have been shown to cause a greater inflammatory response in cells.³¹ Oligomers have been shown to cause a range of disruptive changes in neurons such as mitochondrial impairment,^{32–34} lysosomal dysfunction,^{35,36} alteration of calcium homeostasis^{37,38} and ultimately neuronal cell death.^{15,37} α -syn, including oligomeric forms, has been shown to pass through cellular membranes and localise and interact with the mitochondrial membranes, including in close proximity to mitochondrial proteins.^{33,34,39–45} This localisation and import of α -syn into the mitochondria has been supported by identifying a mitochondrial targeting region on the N-terminus of the protein.⁴² α -syn oligomers have been

shown to interact with complex I and complex V, leading to reduced activities and increased production of ROS.^{33,42,43,46} Oligomers have also been found to bind to the mitochondrial outer membrane translocase TOM20, impairing the import of mitochondrial proteins and leading to loss of membrane potential, decreased mitochondrial respiration and mitochondrial senescence.⁴⁷ Overall, numerous studies strongly suggest that α -syn oligomers directly impact mitochondria in neurons rather than having a secondary spiralling effect from other neuronal defects.

In this work, the effects of α -syn oligomer-containing mixtures on single mitochondrial gene expression in the same living neurons was studied. The primary aim of this work was to dynamically measure mitochondrial gene expression in the same live cell over time to provide a true single-cell response to a treatment using the nanotweezer technology and the mtRNA analysis methods developed in this chapter. This would also allow for the heterogeneity of single mitochondrial responses in the same cell to be evaluated to understand global or localised effects on mitochondrial expression. This could then lead to further work investigating the direct role of α -syn oligomers on mitochondria in live neurons, taking full advantage of the highly precise nature of the technology to sample a range of mitochondria based on the presence and interactions of α -syn oligomers.

α -syn detected *in vivo* often has the N-terminus acetylation post-translational modification. This modification has been shown to trigger a helical conformation in the N-terminus region.^{48–51} As the N-acetylated form is considered to be the physiologically relevant form of the protein, it was used in the studies described in this chapter.

N-acetylated- α -synuclein oligomer mixtures (containing monomer and oligomers) were prepared using an established *in vitro* aggregation protocol.^{33,52,53} Based on previous work,^{33,34,54} α -syn monomers were aggregated for at least 8 h to form a mixture of mostly monomers with a small proportion of oligomers to apply to neurons. It is important to note that previous work incorporated aggregated wild-type α -syn into cells which would lead to different aggregation behaviour to the acetylated form. The N-terminus acetylation has been reported to lead to slower aggregation rates due to the increased helical folding enhancing its resistance to aggregation and the N-terminus disrupting intermolecular hydrogen bonds.^{51,55,56} Aggregation of monomers incubated for 8 h and 24 h was confirmed via atomic force microscopy (AFM), and the 24 h aggregation timepoint was also confirmed by native PAGE (**Figure 6.7**). AFM

showed an increase in equivalent disc radius size (R_{eq}) and wider size distributions with increasing aggregation time, as shown by the histograms in **Figure 6.7a**. Native PAGE of the 24 h timepoint showed the presence of oligomers amongst a larger proportion of monomers (**Figure 6.7b**). Some oligomers were shown to be present at the 0 h timepoint (monomer) in both AFM and native PAGE, but these were lower in quantity and smaller in size than in the aggregated samples.

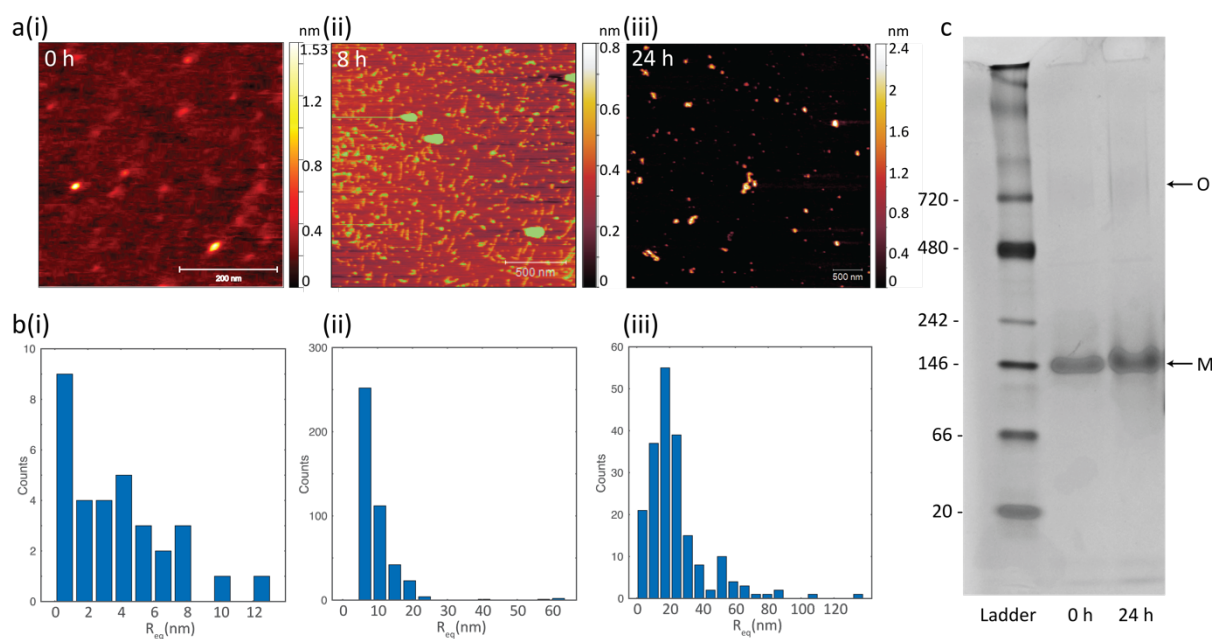


Figure 6.7: Characterisation of N-acetylated- α -syn aggregation. a) AFM images and b) histograms of the equivalent disc radius (R_{eq}) of α -syn monomers and aggregated mixtures at different incubation timepoints: 0 h (i), 8 h (ii) and 24 h (iii). AFM was performed by Dr. Victoria L. Bemmer (Imperial College London) and images were analysed by Dr Giulia Campolo and Dr Yaxian Liu (Imperial College London) c) Native-PAGE of unaggregated sample (0 h) alongside 24 h aggregated sample, with monomers (M) and oligomers (O) labelled. Native-PAGE was performed with Sian Allerton (Imperial College London).

6.4.2 Bulk mitochondrial expression and characterisation

Prior to nanobiopsy, the optimal treatment that led to a change in mitochondrial gene expression was investigated. This was performed by exogenously treating rat cortical neurons with 500 nM (monomer equivalent concentration) 8 h or 24 h aggregated mixtures for either 6 h or 24 h before isolating the total RNA from cells in bulk. It should be noted that further seeding and oligomerisation likely occurred *in vitro* after both monomeric and oligomeric α -syn uptake in culture and that the aggregates may be larger in size and quantity than the initial characterisation by the time of RNA isolation.^{33,34,57,58} As nanobiopsy experiments aimed to detect a dynamic change in the same cell from timepoint 0 h, a comparison relative to the

untreated cells at 0 h was made initially to analyse the different treatments. RT-qPCR analysis of MT-ND1 showed a decreasing trend in expression with longer aggregation and treatment times compared to untreated cells from paired rats at timepoint 0 h (**Figure 6.8**). Cortical neurons from 4 different rat isolations were included in the analysis to account for any variation from the individual animals. Results were inconclusive with regard to whether protein aggregated for 8 h led to a change in MT-ND1 expression in cortical neurons, due to the large variation in results from different rats for the 6 h treatment and the average for the 24 h treatment time being close to 1. However, a decrease in MT-ND1 expression relative to timepoint 0 h was observed for all rat isolations when protein that was aggregated for 24 h was applied, where each isolation gave a fold change value less than 1. This decrease in expression could be explained by α -syn potentially inhibiting complex I activity, as has been shown in previous work.^{33,42,46}

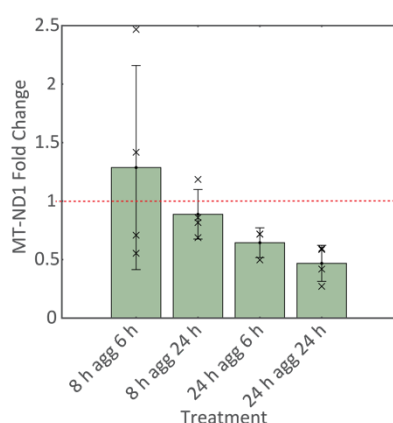


Figure 6.8: Fold change in MT-ND1 expression with application of α -syn. Fold change in bulk MT-ND1 expression in cortical neurons treated with 500 nM (equivalent monomer concentration) α -syn aggregated mixtures relative to untreated neurons at timepoint 0 (dashed red line). The timepoint before ‘agg’ represents the aggregation time of the protein and the timepoint after represents the duration of treatment. qPCR expression was normalised to the RPL13a gene and fold changes were calculated individually from timepoint 0 h of cells from the same rat ($n = 4$). Bars represent the mean of all fold changes, error bars represent s.d and crosses represent individual data points. Outliers were defined as more than 3 scaled median absolute standard deviations from the median and were removed.

The 24 h aggregated protein applied to cells for 24 h showed the largest average decrease in gene expression compared to timepoint 0 h and was therefore considered as a potential condition that may lead to changes in mitochondrial expression in single cells. This was explored further by analysing the expression of MT-CO1, MT-RNR1, MT-ND1 and MT-ATP6 in bulk cells with application of the 24 h aggregated sample for 24 h, compared to application

of the same monomer equivalent concentration of unaggregated monomers and a buffer control containing no protein (**Figure 6.9**).

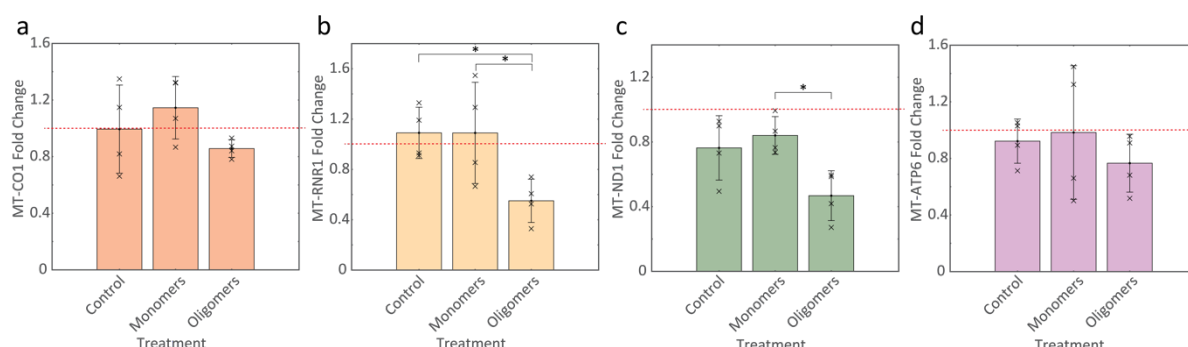


Figure 6.9: Fold change in mitochondrial expression with application of aggregated α -syn compared to monomer and controls. Fold change in bulk mitochondrial expression of DIV 14 cortical neurons treated with 500 nM unaggregated sample (monomers), 500 nM (equivalent monomer concentration) α -syn aggregated mixtures or the same volume of buffer without protein (control), relative to untreated neurons at timepoint 0 (dashed red line). qPCR expression for each of a) MT-CO1, b) MT-RNR1, c) MT-ND1 and d) MT-ATP6 were normalised to the RPL13a gene and fold changes were calculated individually from timepoint 0 of cells from the same rat ($n = 4$). Bars represent the mean of all fold changes, error bars represent s.d and crosses represent individual data points. Statistical analysis was performed by a one-way ANOVA followed by Sidak's multiple comparison test, comparing oligomer samples with monomers and controls. Shapiro-Wilk normality test was performed prior to statistical analyses. * $p < 0.05$, statistical analyses represent not significant if not indicated on the graphs.

In all samples for all genes, applying the aggregated sample to neurons led to a decrease in mitochondrial expression relative to timepoint 0 h, shown by each rat isolation providing a fold change less than 1. For MT-RNR1 and MT-ND1, the change in expression from application of the aggregated sample was significantly less than that from application of the unaggregated sample, suggesting that the decrease in gene expression compared to timepoint 0 h was due to the presence of oligomers. Hereafter, α -syn samples that were aggregated for 24 h for exogenous application to neurons are referred to as aggregated sample or oligomer mixtures.

Next, to investigate if the observed decrease in bulk expression from the exogenous application of this protein treatment was due to a decrease in mitochondrial expression, rather than due to cell death or mitophagy occurring in the cell culture, the viability of cells and mitochondrial quantity was investigated. The percentage viability of cortical neurons treated with oligomers was assessed by applying Calcein AM after 24 hours of treatment (**Figure 6.10a**). No difference in viability was observed between oligomer-treated cells and control cells, in which the same volume of buffer was applied to cells in the absence of protein, where cells remained

viable with the treatment. This suggested that the decrease in bulk mitochondrial expression observed with the application of oligomer mixtures was not due to cell death.

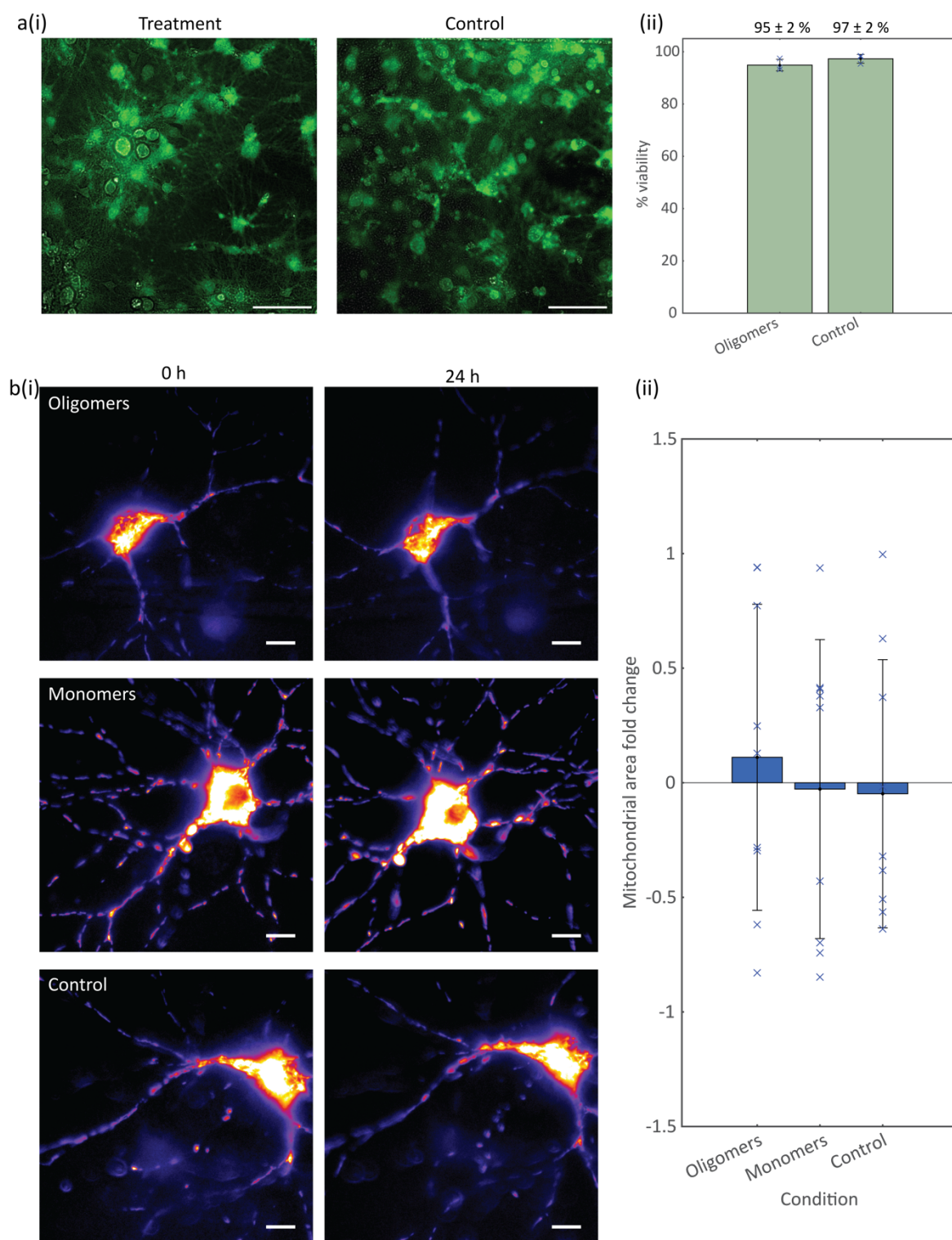


Figure 6.10: Viability after α -syn application. a) Calcein AM viability assay (i) and quantification (ii) of percentage cell viability of DIV 14 cortical neurons after treatment with α -syn oligomer mixtures (treatment) or buffer (control) for 24 h. Percentage viability was calculated based on the number of somas with distinct fluorescent signals. Scale bars = 100 μ m. b) Fold change of mitochondrial area in mtDsRed-transfected cells after

24 h application of aggregated α -syn (oligomers), unaggregated α -syn (monomers) and buffer (control) (i) The same areas of the neurites of neurons were measured in same cell before and after application, and the mitochondrial area was quantified using the MiNA ImageJ plugin (n = 9 regions from 3 cells). Error bars represent s.d. Scale bars = 10 μ m.

Mitochondrial mass (as a proxy measure of mitochondrial loss via mitophagy) was assessed by observing the same mtDsRed-transfected cells before and after treatment and measuring the mitochondrial area in the same regions of the same cells (**Figure 6.10b**). Due to the soma being highly condensed with mitochondria and difficulties in quantifying the mitochondria in this region, only areas of the neurites were measured to allow for more accurate quantification. Naturally, mitochondria are dynamic organelles, meaning they may have changed location in the cells and undergone fusion or fission during the 24 h treatment period. For this reason, the mitochondrial area was investigated as a measure of mitochondrial loss, rather than counting individual mitochondria which would be challenging for mitochondria that were part of a network. The oligomer mixture application showed no change in the overall mitochondrial area compared to the same cells before oligomer application. Similar trends were shown with control cells and cells with unaggregated α -syn (monomer) applied, demonstrating that the mitochondria showed similar changes in mitochondrial area during the 24 h period. The large variations in mitochondrial area measured in the same regions of cells for all conditions can be explained by mitochondria changing their dynamics over this period. Nevertheless, there was little net change in mitochondrial area measured over 9 regions of 3 cells. Both viability assessments showed that the decrease in mitochondrial expression observed with application of α -syn aggregates was not due to mitophagy or cell death and, therefore, likely due to a decrease in expression in the mitochondria of cells. This was therefore an interesting treatment to study at the single cell level with nanobiopsy.

Finally, immunostaining was performed as a control to show that α -syn was taken up by the cortical neurons used in this study (**Figure 6.11**). Monomeric, oligomeric and fibrillar α -syn has been shown to be taken up by neuronal cells via receptor-mediated endocytosis *in vitro* and *in vivo*.^{59–61} There is also evidence that monomeric but not aggregated α -syn can passively enter cells through cell membranes.^{59,62} In this work, strong signals and large aggregates of α -syn, amongst some smaller bright fluorescent punctae, were observed in neurons from 1 h after application of the aggregated sample (**Figure 6.11a-d**). These were amongst diffuse α -syn signals which were also observed in monomer (**Figure 6.11e**) and control (**Figure 6.11f**)

samples. This confirmed the uptake of exogenously applied α -syn with the cell type and treatment studied in this work.

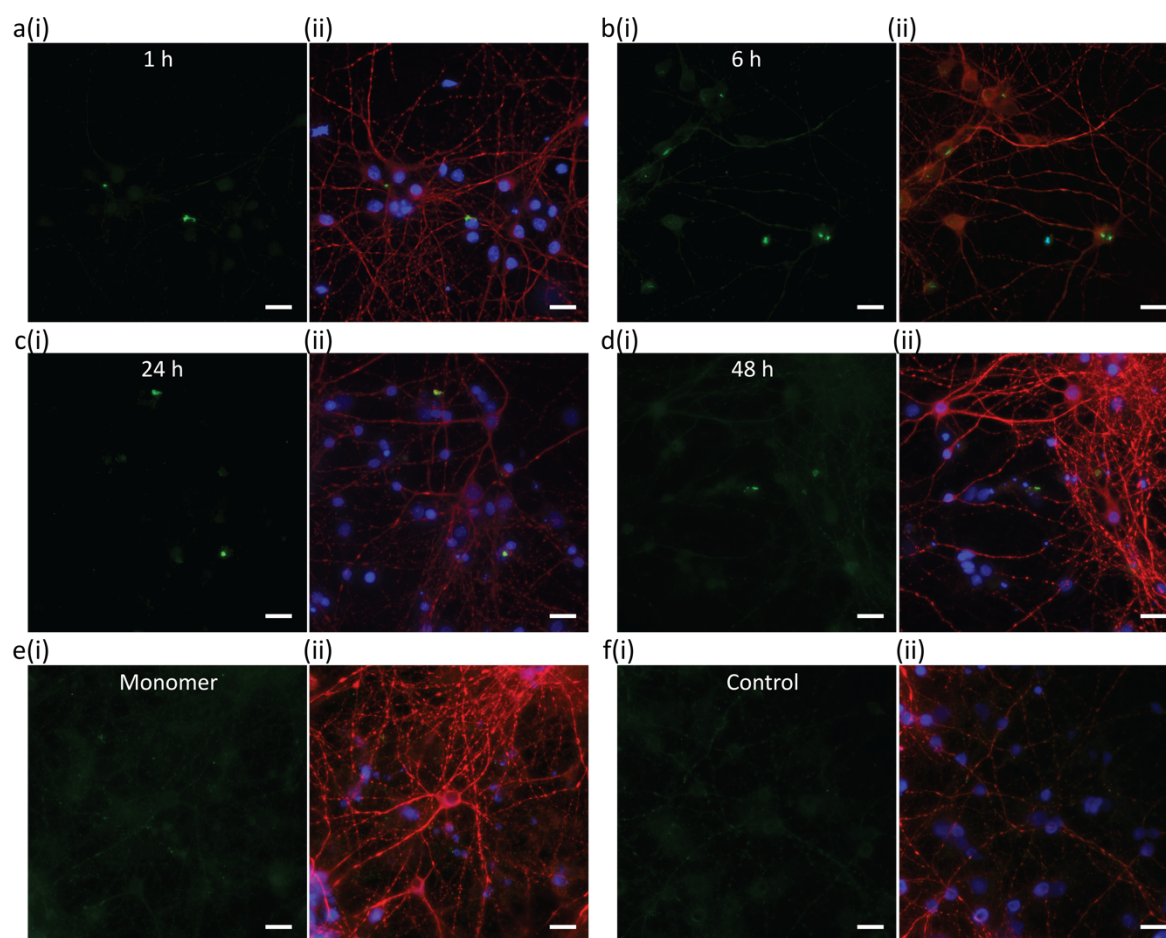


Figure 6.11: α -syn uptake in DIV 13-14 cortical neurons. Representative images of DIV 13-14 cortical neurons after exogenous application of α -syn or buffer, showing the α -syn channel (green, i) and merged images for α -syn (green), MAP2 (red) somatic and dendritic neuronal marker and DAPI (blue) nuclear stain (ii). Images show cells after application of 500 nM (monomer equivalent) aggregated sample at a) 1 h, b) 6 h, c) 24 h and d) 48 h timepoints after washing to remove any protein that had not been taken up by cells. This was compared to the application of e) 500 nM unaggregated monomer sample and f) control buffer. Scale bars = 20 μ m.

6.4.3 Dynamic single-cell, single mitochondrion expression

The nanotweezer was then applied to study the application of aggregated α -syn treatment on individual mitochondrial expression at the single neuron level. 4 single mitochondrion biopsies were performed on the neurites of a living cortical neuron maintained in standard cultured conditions. After this, 500 nM (monomer equivalent) aggregated α -syn was applied to the cell's media. 24 h after incubation with protein, a further 4 single mitochondrion biopsies were performed on the same living cortical neuron. This was repeated to obtain data from 5 single neurons derived from 5 different rats and was performed alongside control cells, in which the

same volume of buffer was applied to the cell culture media instead of protein. **Figure 6.12** shows an example of one live neuron that was studied in this work, with all the single mitochondrion biopsy locations shown based on the location of the nanotweezer tip.

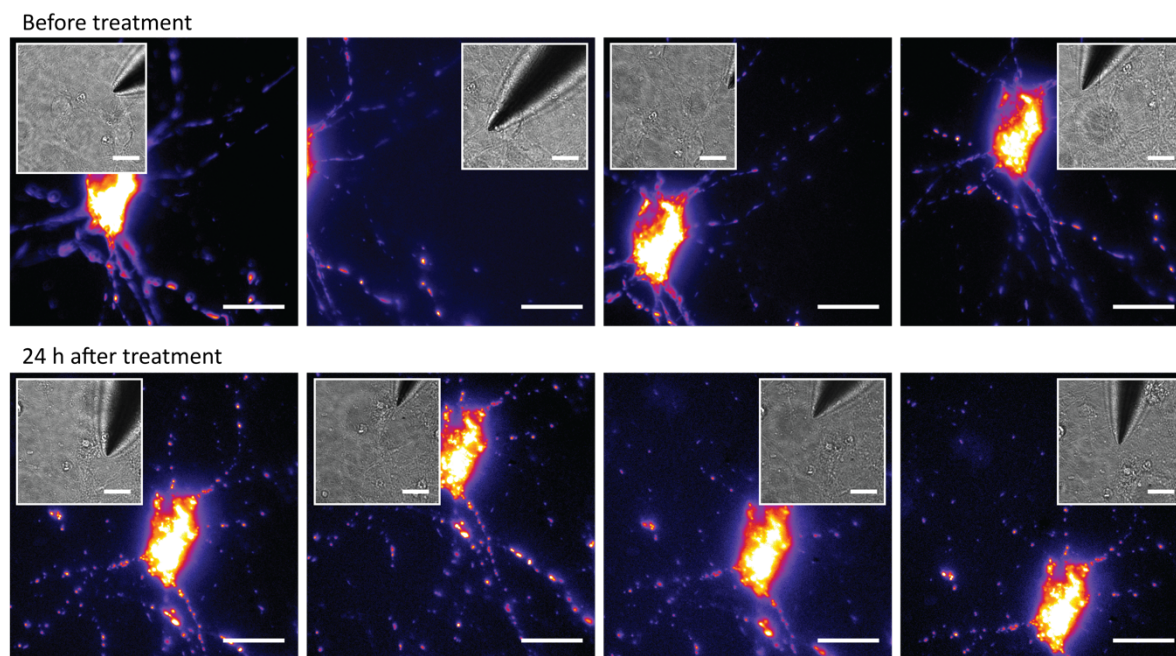


Figure 6.12: Single mitochondrion biopsies from the same living neuron for dynamic mitochondrial gene expression analysis with α -syn application. Fluorescent images showing DIV 13-14 cortical neurons transfected with mtDsRed and bright field images (inset) showing the location of the nanotweezer tip inserted into the cell for nanobiopsy. 8 single mitochondrion nanobiopsies were performed on the same cell; 4 prior to any treatment and 4 after application of aggregated α -syn for 24 h. Scale bars = 20 μ m.

After purification and reverse transcription, each mitochondrion was analysed for MT-CO1, MT-RNR1, MT-ND1 and MT-ATP6 in multiplexed qPCR reactions. The copy numbers of the RNA in individual mitochondrion were quantified using absolute quantification via the construction of standard curves in the same qPCR reaction (**Figure 6.13**). In cases of no amplification of all genes in a single mitochondrion, the sample was excluded and deduced as an unsuccessful isolation. When at least one target in a single mitochondrion was detected, this was accepted as a successful single mitochondrion isolation; no amplification was observed in all negative controls.

Dynamic gene expression analysis of multiple single mitochondria isolated from the same living cell was successfully achieved. The results and trends in single mitochondrion expression levels in the same single cell before and after application of aggregated mixtures or buffer are shown in **Figure 6.14**. In 4 out of 5 cells, a downregulation trend in the average

mitochondrial expression was observed for all genes analysed after the application of oligomers. No clear up- or downregulation trend was observed in the control cells for all genes. In all cells, treated or untreated, large variations in gene expression were observed between mitochondria of the same cell. This may be expected with the dynamic nature of gene expression, where mitochondria may perform different functions for the cell in each subcellular location. Nevertheless, these results successfully exhibit live cell mitochondrial expression tracking using the nanotweezer and the methods outlined in this thesis, where a living cell's dynamic state before and after a treatment or time period can be defined.

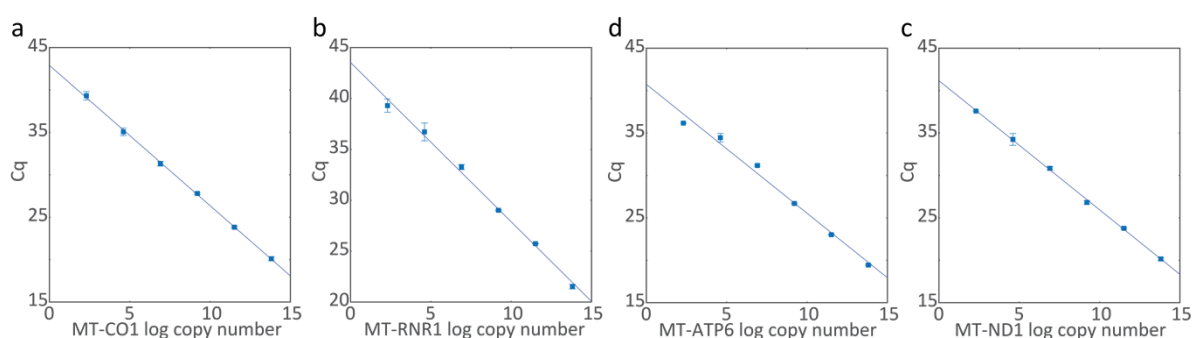


Figure 6.13: Standard curves for absolute qPCR quantification of single mitochondrion. qPCR standard curves for gene expression quantification of a) MT-CO1, b) MT-RNR1, c) MT-ND1 and d) MT-ATP6 in single mitochondrion. Standard curves were constructed by serial dilutions of known copy numbers of PCR amplicons. Each point represents the mean Cq value, error bars represent s.d (n = 3).

Figure 6.15 illustrates the expression data from all the mitochondria isolated with the nanotweezer before and after application of protein or buffer. In all genes analysed, a significant decrease in expression was observed between mitochondria before the application of aggregated sample and after 24 h incubation with the aggregated sample. In control cells with application of buffer, no significant difference was observed in mitochondria over this same time period. This supported the findings from bulk analysis, where a decrease in expression of all genes was observed with the aggregated protein treatment, whereas the fold change of controls remained relatively close to 1. The nanobiopsy results here, however, allowed for precise targeting of specific mitochondria in the neurons of the culture, omitting any gene expression from non-neuronal cells and allowing the location of specific mitochondrion to be mapped.

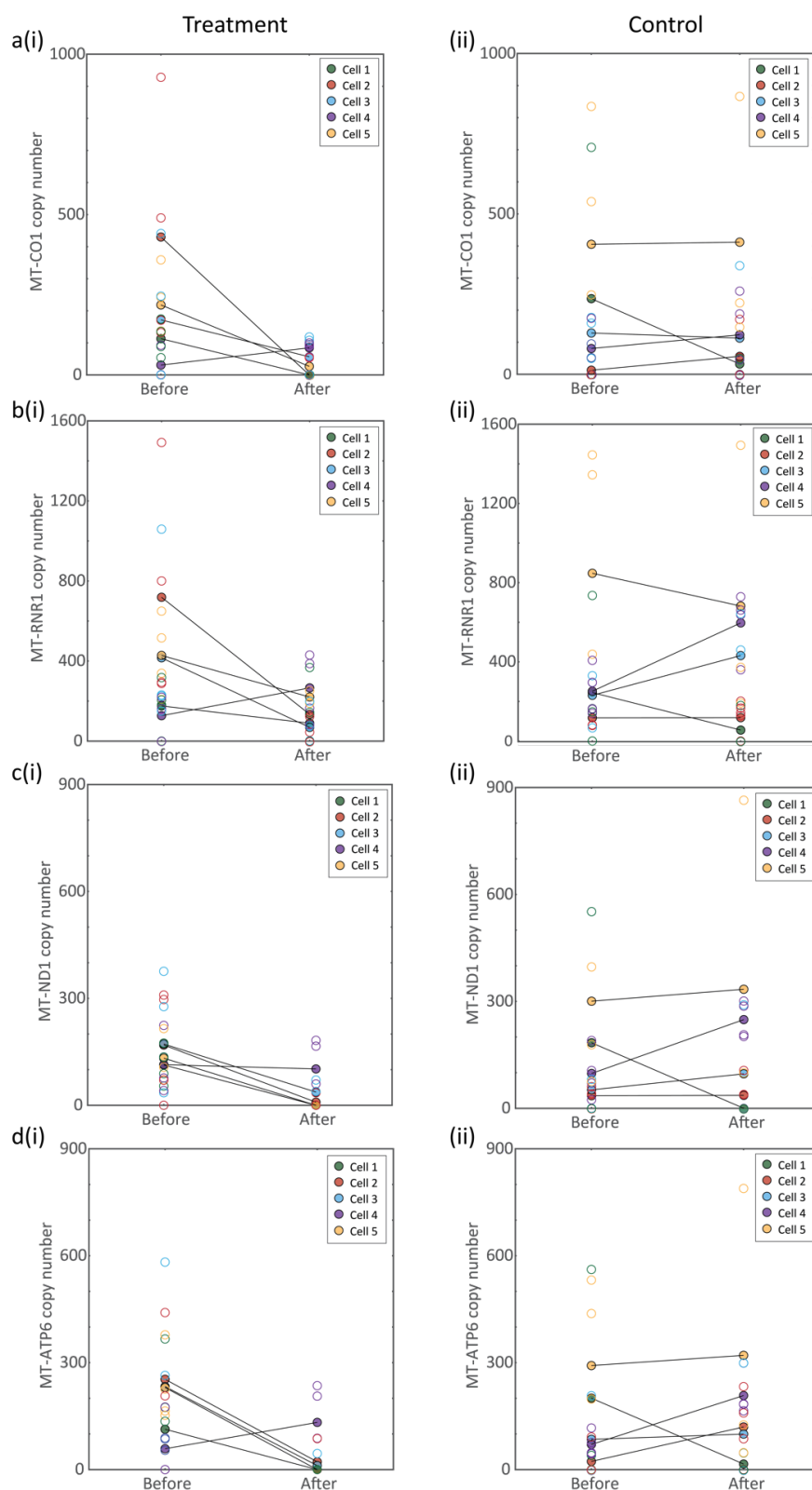


Figure 6.14: Same cell single mitochondrion gene expression. Mitochondrial gene expression for α -syn oligomer mixture-treated DIV 13-14 cortical neurons (i) and control cells (ii) for a) MT-CO1, b) MT-RNR1, c) MT-ND1 and d) MT-ATP6 expression. Mitochondrial expression from the same cell were tracked with each treatment. Each filled circle represents the mean mitochondrial copy number of all successful mitochondria isolations (unfilled circle) for that timepoint, black lines show the average copy numbers in the same cell before and after treatment. Isolations were assumed unsuccessful, and samples were excluded, if no amplification was observed in all targets. Mitochondria with at least one successful target amplification amongst clean negative control signals were included and confirmed as successful mitochondrion isolations.

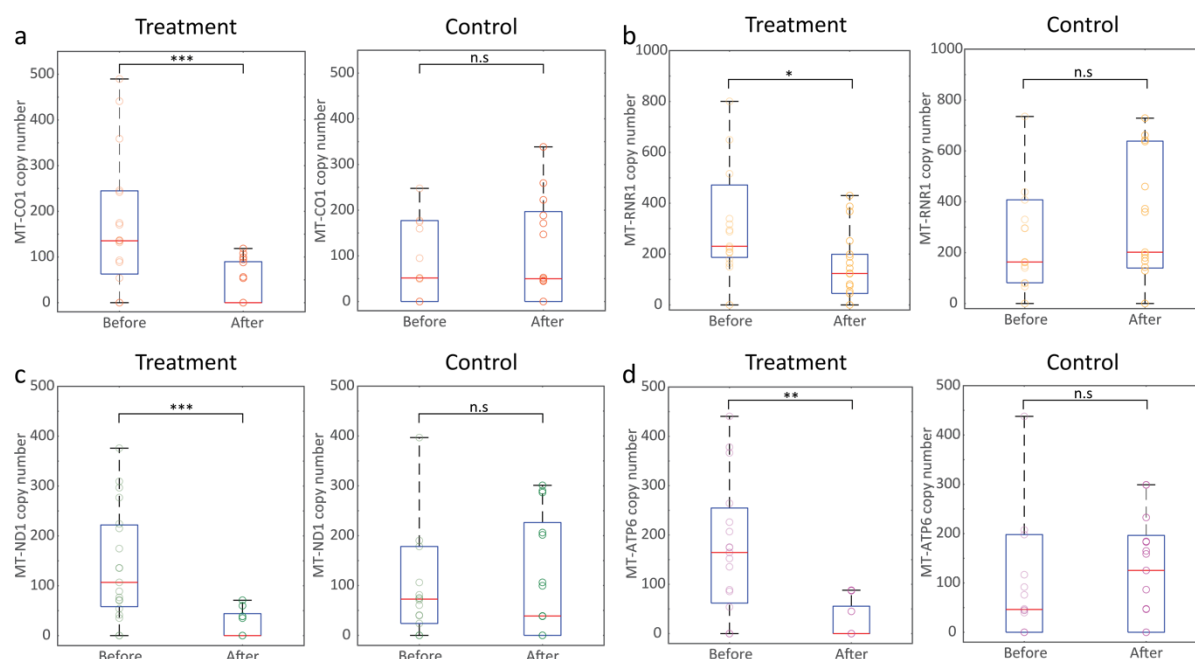


Figure 6.15: Total mitochondrial expression changes with application of α -syn oligomers. Boxplots comparing mitochondrial expression from individual mitochondria from 5 DIV 13-14 cortical neurons before and after application of aggregated α -syn (left) or control buffer (right) for a) MT-CO1, b) MT-RNR1, c) MT-ND1 and d) MT-ATP6 genes. Outliers were defined as more than 3 scaled median absolute standard deviations from the median and were removed. Statistical analysis was performed using the Mann-Whitney non-parametric test. Shapiro-Wilk normality test was performed prior to statistical analyses. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. = not significant.

The overall downregulation in mitochondrial expression observed with the treatment and the trends observed in single cells supported the premise that application of the oligomer mixture affected the mitochondria in neurons, including overall at the single-cell level, and that it may lead to possible hindrance of mitochondrial function. The decrease in expression observed for MT-RNR1 in **Figure 6.15** and **Figure 6.9**, encoding 12S rRNA, also suggested that reduced ribosome biogenesis and protein synthesis may be occurring in the cell in the presence of α -syn. This may be a result of cell damage and stress, as has been reported with the downregulation of other types of rRNAs in the substantia nigra of PD brains alongside the observation of oligomeric α -syn species.⁶³ The mechanisms of possible protein synthesis interference and the impacts on mitochondrial function in single cells should be explored in the future. For the former, this could be achieved via ribosome density measurements or ribosome profiling. For the latter, this could be achieved by measuring ROS production in live neurons and extracting mitochondria based on different ROS production rates to assess possible correlations between ROS production, single mitochondrion expression and the presence of aggregates.

The little change in mitochondrial expression observed in control cells showed that the nanotweezer extraction procedure had little effect on mitochondrial expression. This was further highlighted by calculating the change in mitochondrial copy number for each single cell, where the median changes in expression values for all genes in the control cells were close to 1 (**Figure 6.16**). One may hypothesise that removing multiple mitochondria from a neuron may impact the expression and function of the remaining mitochondria in the same cell due to the cell losing part of its energy resource. From these results, however, one may assume that the small proportion of mitochondria removed from the cell has little effect on this. Based on the sample of cells used in this work, significant differences in the change in expression of MT-CO1 and MT-ATP6 between cells treated with α -syn and control cells were observed. In future work, larger sample sizes should be incorporated, in addition to investigating the specific effects of oligomers compared to monomers in cultures.

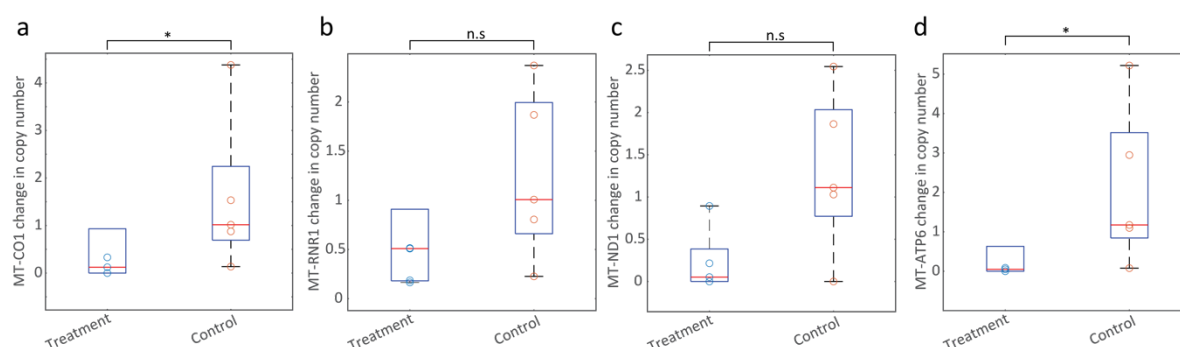


Figure 6.16: Single cell changes in mitochondrial expression with the application of α -syn vs. controls. Boxplots for mitochondrial change in copy numbers from 5 cortical neurons after application of aggregated α -syn for 24 h (treatment) or buffer (control) for a) MT-CO1, b) MT-RNR1, c) MT-ND1 and d) MT-ATP6 genes. Change in copy numbers was calculated by dividing the average copy numbers from mitochondria of the same cell after 24 h by the average copy numbers before treatment or control. Outliers were defined as more than 3 scaled median absolute standard deviations from the median and were removed. Statistical analysis was performed using the Mann-Whitney non-parametric test. Shapiro-Wilk normality test was performed prior to statistical analyses. * $p < 0.05$, n.s = not significant.

6.5 Limitations and open challenges

This chapter built upon the single nanobiopsy work in **Chapter 5** and presented a novel method to analyse gene expression from a single mitochondrion. As with all nanobiopsy work described in this chapter, improving the throughput and the number of cells sampled will be necessary to uncover significant biological findings. Additionally, although the method developed in this chapter enabled successful analysis of mtRNA from a single mitochondrion,

there will inevitably be a loss of mitochondrial content during the sample preparation which would limit the detection. This was particularly apparent in mitochondria that were extracted after α -syn oligomer application, where many mitochondrial genes were undetected in the qPCR, likely due to the mitochondria containing lower levels of these genes, making them undetectable at the analysis stage. Further improvements of the method to isolate mtRNAs from single mitochondria should therefore be prioritised in the future to maximise the measurable mitochondrial content.

For the single neuron α -syn study, this work provided the foundations for understanding the impact of α -syn on mitochondrial gene expression in single cells. Due to the application of both monomer and oligomers to cells, one cannot yet conclude whether the downregulation of expression is due to the presence of oligomeric species or monomer. Additionally, one cannot deduce the mechanisms of α -syn effects on the mitochondria, for example, whether it is due to direct protein-mitochondria interactions or whether the decrease in expression is a secondary effect of toxicity to the cell. Although the bulk mtRNA analysis comparison to monomer sample would suggest that the observed mitochondrial downregulation in cells with protein applied was due to the presence of oligomers, further work is necessary to determine this at the single cell level. This could involve fluorescent labelling of α -syn oligomers to specifically track their interaction with the mitochondria and its proteins. This could be followed by analysing mitochondria that interact with oligomers, compared to those which interact with monomers or do not have any α -syn in their vicinity. In this respect, the nanotweezer platform is an ideal tool to investigate this due to its precise sampling ability and capability to characterise and compare multiple mitochondria in the same live cell. This could also explain some of the unexpected trends observed in this chapter. For example, ‘cell 4’ in **Figure 6.14** showed an average upregulation of many genes after incubation with α -syn. Previous work has reported that monomeric wild-type α -syn interacts with and modulates the function of ATP synthase,⁶⁴ which has a subunit for the F_0 region of the protein encoded by MT-ATP6. One could hypothesise that monomeric α -syn may lead to an upregulation in the expression of genes for ATP synthase. Perhaps the mixture of monomeric and oligomeric forms may have opposing effects on mitochondrial gene expression, where a lack of oligomeric but strong monomeric interactions with a single mitochondrion may lead to some upregulation of genes. This, combined with assessments of the interactions with other mitochondrial membrane proteins, should be explored in future work.

6.6 Conclusions

The work described in this chapter outlines a novel approach to analyse mtRNA from a single mitochondrion isolated from a living single cell. Copy numbers of multiple key mitochondrially-encoded genes from the same mitochondrion were quantified, opening novel opportunities to characterise gene expression at the single mitochondrion level. This could contribute to an improved understanding of mitochondrial gene expression regulation in various cellular contexts such as stress, signalling and disease. Most importantly, this work was the first to show that multiple single mitochondria could be isolated from the same living neuron with their individual gene expression analysed before and after the exposure of cells to an exogenous treatment. In particular, the platform was used to study the effect of α -syn oligomer mixtures on single mitochondrion expression in cortical neurons. The results showed an overall downregulation of many mitochondrially-encoded genes in live neurons *in vitro*, consistent with previous studies that have reported possible toxic interactions between oligomeric α -syn and the mitochondria.^{32–34} This work also emphasised the highly resolved expression information that one can obtain with the technique, allowing for the heterogeneity of the mitochondrial landscape in a single cell to be evaluated. This could be particularly useful for analysing possible compartment-specific changes in mitochondrial expression or characterising mitochondria based on their spatial location. Future work studying α -syn oligomer interactions with mitochondria will take advantage of this precise feature of the nanotweezer; through the application of labelled oligomers,³⁷ or via the specific detection of oligomers in cells,^{65,66} oligomer-mitochondrion interactions and uptake will be observed, followed by nanobiopsy to compare the mitochondrial expression of mitochondria with and without oligomeric interactions.

6.7 References

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Chapter 7

Conclusions

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7.1 Conclusions

Single-cell technologies have unlocked exciting opportunities to profile the molecular basis of living cells. Recent advances in tools that probe and remove the contents of living cells, whilst maintaining them in their standard microenvironment, have the potential to monitor cells over time and understand true single-cell intracellular mechanisms with high spatial resolution. Many techniques, however, have complex fabrication procedures and involve the aspiration of cytoplasmic fluid which reduces cellular volumes, raising questions regarding the viability of cells with repeat sampling, particularly for sensitive cell types.

The nanotweezer addresses many of the limitations of other subcellular sampling tools; it allows for the direct trapping of biomolecules, with minimal disturbances to the cell, and is inexpensive to fabricate. For sensitive, compartmentalised cells like primary neurons, this provides a promising tool to access subcellular compartments and analyse their contents with high spatial resolution.

The primary aim of this work was to achieve spatial and temporal measurements of biomolecules in live neurons *in vitro*. To this end, two types of biomolecules were focused on for their extraction from living neurons: RNA to study mRNA localisation in specific sub-compartments of neurons, and single mitochondria because of their highly important role in neuronal homeostasis and function.

In **Chapter 3**, the fabrication and detailed characterisation of the nanotweezer were described and optimised from that reported previously, so that it could be used effectively under cellular conditions. The necessary DEP parameters required for trapping biomolecules from living cells were considered to ensure that biomolecules could be readily trapped and that negligible damage would be incurred on cells during the trapping process. Consequently, frequency- and voltage-dependencies were studied for DEP trapping of RNA in physiologically similar conductivity conditions as a cell.

In **Chapter 4**, the nanotweezer was applied to isolate RNA from living hippocampal neurons for the first time, where the previously determined conditions in **Chapter 3** were optimised and applied *in vitro*. It was shown that precise subcellular compartments of the same cell could be accessed and compared, demonstrated by the detection of a dendritic localised mRNA

relative to the soma of the same cell and analysing localisation changes with neuronal maturation. Importantly, cell viability was maintained following repeat sampling from the same cell over a 48-hour period, combined with no impact on neuronal stimulation and the integrity of the isolated RNA. This highlighted the minimally invasive nature of the technology for its use on sensitive cell types, opening opportunities for dynamic, long-term gene expression studies on living cells. Crucially, this was demonstrated via a dynamic study on the same live cells, where the highly precise sampling nature of the technology was combined with temporal gene expression profiling to map true single-cell subcellular responses in a model of synaptic plasticity.

Chapter 5 focused on single organelle extraction and analysis. Intact, individual mitochondria with active membrane potentials were successfully isolated from living neurons. It was shown that the copies of mtDNA from an individual mitochondrion could be quantified, and was proposed to be used as a comparison method between different cells, subcellular compartments, or phenotypes. This could contribute to verifying mtDNA copy number as a potential biomarker of disease and ageing. Endeavours to sequence mtDNA from a single mitochondrion were also demonstrated via proof-of-concept sequencing of small amplicons of the mitochondrial genome. This provides a platform for future work on single mitochondrion sequencing for measuring heteroplasmy in single cells.

Finally, in **Chapter 6**, the single mitochondrion work from **Chapter 5** was expanded to single mitochondrion RNA analysis. For the first time, mitochondria that were isolated from living cells were analysed to reveal their individual mitochondrion gene expression. Additionally, another temporal study was performed by biopsying from the same neurons over a 24-hour period, in which aggregated samples of α -synuclein were found to downregulate mitochondrially-encoded gene expression. This included the expression of three subunits of different OXPHOS proteins and one ribosomal RNA, suggesting possible impacts on mitochondrial protein synthesis and function. The results from this work are supported by numerous studies on the effects of α -synuclein oligomers on mitochondrial proteins and function.¹⁻⁵ Further spatial investigations of specific mitochondria of interest in single neurons, making use of the nanotweezer's precise sampling ability to directly target oligomer-interacting mitochondria, could provide novel insight into the direct effects of these oligomers on specific mitochondrial OXPHOS complex expression compared to other forms of the

protein. This could establish the nanotweezer as a significant tool for studying individual organelle interactions in living cells and tracking single-cell responses.

7.2 Future Directions

The nanotweezer offers a highly innovative route to subcellular sampling of living cells. However, several remaining limitations are apparent which should be addressed in future work.

Firstly, the technique is low throughput, with the tip manually positioned into regions of single cells using a micromanipulator, followed by manual DEP application and control. This means that one cannot precisely revisit the same biopsy location, for example, to track an exact location within a cell over time. With other techniques, such as those based on SICM, the positioning is automated and allows the user to return to the same location. Incorporating SICM with the nanotweezer method could be explored in future using a quad-barrel nanopipette. For this, two of the barrels could be filled with carbon for nanotweezer trapping, whereas the other two barrels could be filled with electrolyte solution for SICM. The SICM feedback would allow for much greater control and precision in sampling, combined with high resolution non-contact imaging of the cells. In addition to this precise sampling, incorporation with SICM could also enable more consistent penetration of the cell. Currently, there is no control over the penetration depth of the nanotweezer, and delicate manual operation is required from the user, combined with knowledge and practical experience of extracting from the cell type of interest. With SICM incorporation, the approach and penetration of the cell could be more precise, allowing for consistent and controllable penetration depth depending on the thickness of the cellular region of interest.

For both trapped RNA and mitochondrion, removal of biomolecules was not possible by turning the DEP off, nor was it possible when high frequencies were used (up to the limit trialled) to try to obtain negative DEP. Another improvement of the nanotweezer would be to enable the direct removal of biomolecules from the tip for pick-and-place operation. Combined with automation, this could lead to much smoother operation of the nanotweezer and the transfer of extracted material directly to reagent solution, such as for reverse transcription. It would also allow for investigations into the transfer of material between cells to be made which could have exciting applications in cell regeneration therapies. However, as discussed previously, the possible loss of integrity of trapped material during transfer would need to be

studied due to the lack of protection that the nanotweezer provides to biomolecules trapped on the surface of the tip.

Perhaps the most essential development of the technology would be achieving comprehensive transcriptomic analysis of biopsies. Although the nanotweezer offers advantages compared to live-cell imaging techniques, such as the potential to perform label-free RNA extractions from precise subcellular locations, alongside cheaper costs and simple equipment, sequencing the contents of nanobiopsies would truly set it apart from these techniques. This would allow novel transcripts and post-transcriptional modifications to be discovered, as opposed to imaging techniques which require prior knowledge of the target of interest. Live-seq was able to achieve this with living macrophage-like cells and ASCs, making tracking the transcriptome of single cells a reality at the expense of removing 40-70 % of cell volumes.⁶ Similar work was then followed by nanopipette-SICM-based technologies.⁷ For the nanotweezer, it was calculated that the total RNA extracted was in the 10-100 fg range which means that the estimated amount of mRNA ($\lesssim 1$ fg) isolated is extremely small for RNA-seq. Pooling samples from regions of the same subcellular compartment could be employed in future to reach the detection limits of RNA-seq. This would still allow for compartmentalised analysis of living cells and could maintain the high spatial resolution that the nanotweezer offers. To achieve this, further work determining the maximum number of biopsy samples that can be taken and pooled from compartments of the same cell without affecting cell viability should be performed, followed by testing this maximum amount as input for RNA-seq. Additionally, the continued development of lower input and highly sensitive next-generation sequencing methods may make investigating the small quantities of biomolecules isolated from spatially restricted cellular locations possible in the future.

Whilst strategies to achieve RNA-seq from nanobiopsies are developed, digital PCR could be used as an alternative method to qPCR for determining absolute quantities of RNA or DNA.⁸ Digital PCR is more sensitive for low copy numbers and can provide direct copy numbers without the need for standard curve construction, removing the possibility of inaccurate or unrepresentative standard generation. This could be particularly important for mtDNA copy number determination, where mtDNA is present in low copies per mitochondrion. This, combined with projecting the potential loss of content from purification, would provide a better estimate of true mtDNA copy numbers per mitochondrion and provide an updated method to directly quantify single mitochondrion in health and disease.

With the advantages that the nanotweezer provides for studying neurons, the potential future biological studies are numerous. In this work, the exogenous application of substances to cells was used to illustrate the ability to measure a direct single-cell expression change, which could have applications in studying synaptic signalling and plasticity, mitochondrial energy metabolism, and monitoring treatments in living cells. However, many other biological questions could be explored. Some examples include: mapping multiple mRNA transcripts of the same gene in different subcellular locations to understand the impact of localising elements, such as the 3' UTR region, on the distribution of transcripts under changing neuronal activity; tracking mitochondrial expression as mitochondria travel between astrocytes and neurons to understand the astrocytic or neuronal 'identity' of mitochondria when they are present in the alternate cell type; analysing the import of nuclear-encoded RNAs into individual mitochondrion and the possible disruptions of this during neurodegeneration; and compartmentalised approaches to characterising mitochondria such as extracting synaptic and non-synaptic mitochondria in hippocampal neurons upon application of amyloid- β , which has been shown to get taken up by mitochondria and affect synaptic mitochondria in the early stages of the disease prior to global mitochondrial and cellular damage,^{10,11} to track the effects on specific subtypes of mitochondria. With future expansion of the tool to address these biological applications via dynamic and precise spatial quantification of biomolecules in living neurons, this could ultimately have significant implications in single-cell spatial transcriptomics, cellular mechanics and disease tracking. Given the rapid rise in the prevalence of neurodegenerative diseases like AD and PD, a comprehensive understanding of these is vital to address the urgent and unmet need to develop effective treatments for neurodegenerative diseases.

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