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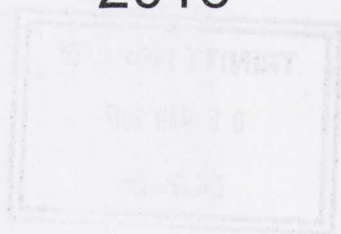
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Development of a microelectrode array biosensor platform for the study of neuronal function

Doctor of Philosophy
2016



PhD
medicine

Ayokunmi Ajetunmobi

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Abstract

The impact of brain disorders on the human population is significant, with neuropsychiatric illness, and in particular mood and psychotic disorders, contributing to over 25% of the 800 billion euro annual healthcare costs of neurological disorders across Europe. To combat this socio-economic challenge, novel therapeutic strategies are required that can identify sequences of events that underlie the processes of long-term synaptic function during post-natal development, in adult life and in the pathogenesis of neural diseases. Detecting this relationship between genes, synapse development and functional circuit maturation in disease models of neuronal development requires the analysis of multiple parameters in order to evaluate neurobiological activity, determine molecular biomarkers of the disease and identify novel therapeutic targets. However, current gold-standard techniques used to study the CNS have limitations that pose unique challenges to furthering our understanding of functional CNS development.

In order to bridge this gap, researchers require enhanced tools to study functional CNS development, both structurally and physiologically, in order to identify mechanisms that lead to devastating and debilitating disorders. Recent advancement in nanotechnologies for biomedical applications have seen the emergence of nanoscience as a key enabling technology for delivering a translational bridge between basic and clinical research. In particular, *in vitro* neuronal networks grown on nanoengineered devices can be used to investigate circuit physiology, with a particular focus on understanding the morphological and functional properties of neuronal development. This study presents the development of a nano-engineered biosensor platform designed for the growth, interaction and visualisation of neuronal network activity. The development of such systems may provide a unique opportunity for a multimodal platforms for modelling neuronal development and studying the cellular and molecular properties of neurodevelopmental disorders.

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1 Introduction

1.1 General Overview

The scale of neurological disorders on the human population is significant, covering hundreds of disorders with debilitating behavioural, social and cognitive deficits. Disorders in brain function are particularly insidious, affecting emotion, learning ability and memory, which unfold and regress as an individual gets older. These disorders not only have a major impact on individual lives but also place a severe strain on healthcare resources. In Europe alone, the impact of central nervous system (CNS) disorders on the population contributes to almost €800 billion in annual European healthcare costs. Yet, given the significant impact of these disorders on society, advances in drug development remain limited, with treatments for diseases such as depression and schizophrenia still largely based on therapies identified in the 1950's (Brandon and Sawa, 2011). The length of time for the clinical trial and approval phases of drugs targeting the central nervous system (CNS) is longer than for any other therapeutic area; 10 years for CNS vs. 7.8 and 7.6 years for cardiovascular and antineoplastic therapies respectively (Kaitin, 2010). Across the board, CNS drug discovery and development is associated with a longer and riskier development process, and given the high rate of attrition for pharmaceutical research into neural disorders, there is a clear and present need to identify novel approaches for driving innovations in how we study the brain to discover more sensitive diagnosis and efficacious treatment (Muglia, 2011).

Elucidating the mechanisms involved in regulating CNS development is critical for gaining new insights into the aetiology and progression of CNS disorders, leading to more sensitive diagnosis and potential treatment. Functional brain development requires synchronization of environmental signals with adaptive neuronal communication. Managing extracellular stimuli from early sensory experiences plays an important role in the ability of neural circuits to modulate synaptic connections and drive the structural and functional changes required for many aspects of post-natal development (Barnes and Finnerty, 2010). This has led to an increasing appreciation for the so-called 'critical period' of development represented by enhanced plasticity in post-natal life, whereby the mechanisms behind synapse development, acquired at an early age, are recruited for adult processes (Linkenhoker *et al.*, 2005). This phenomenon is synonymous with functional and morphological changes which include excitation-inhibition dynamics and structural rearrangements of dendritic branches accompanied by synapse formation or elimination. These synapse dynamics are extraordinarily flexible (Bourne and Harris, 2008) and are regulated by extracellular activity and visualised by variability in the size and number of spine heads at the synapse (Matsuzaki

et al., 2004). In neurodevelopmental and neuropsychiatric disorders, synapse dynamics can become impaired, illustrating the role of neuronal communication in the pathogenesis of these human diseases (Cohen, 2008).

Understanding the role of synapse dynamics in regulating CNS development requires a critical examination of how relationships between interacting neurons produce a collective functional output. This involves the study of the behaviour of locally interconnected neurons – microcircuits – and requires the identification of sequences of events that associate extracellular activity with synaptic function (Caroni *et al.*, 2012). Detecting these early stage mechanisms pose unique technical challenges, which are difficult to resolve with current technologies. The lack of direct access to the brain imposes obvious limitations to the study of human brain development and while functional brain imaging studies remain a powerful means of visualising regional brain structures and activity, it lacks the resolution required to probe neuronal activity at the microcircuit level. Cell culture studies using metal, glass or silicon electrodes has typically been the gold-standard for studying the cellular and molecular mechanisms of functional neuronal activity, permitting easy access to individual neurons for electrophysiological recording and stimulation, pharmacological interventions, and high resolution microscopic analysis. In particular, the introduction of novel assays for modelling brain disorders through induced pluripotent stem cell (iPSC) models, have provided innovative approaches for probing molecular mechanisms of neuronal development (Marchetto *et al.*, 2010) and high-throughput screening of novel therapeutic compounds (Lee and Studer, 2011). This approach presents a valuable diagnostic tool for understanding many of the properties of CNS function and the development of personalised therapeutic strategies (Chailangkarn *et al.*, 2012). However, classic neuronal culture preparations are not amenable to modelling microcircuit connections that can observe the physiological and morphological modifications regulated by extracellular activity and synaptic function. Firstly, connectivity testing is limited by random and uncontrolled synapse formation within the culture. Secondly, the number of cells available for recording is severely limited by the health of the culture over time and the technical difficulties of simultaneous electrophysiology recordings (Potter and DeMarse, 2001). These restrictions impede the ability to model how neurons in a local circuit develop functional outputs and limit the identification of novel biomarkers that can aid the diagnosis and treatment of CNS disorders.

The recent advancement in nanotechnologies for biomedical applications has seen the emergence of nanoscience as a key enabling technology for delivering a translational bridge between basic and clinical research. The advanced development of electrodes using thin-film manufacturing techniques and chemical synthesis has allowed for material properties to

become tailored for specific requirements in various biological processes. Given the improved physical properties of materials at the nanoscale, there has been increasing progress in the use of nanotechnology to expand bioelectrical contact with cells within the nervous system (Pancrazio, 2008). These advances in novel technologies are providing increasingly more powerful platforms for bioelectrical devices as high-quality neural interfaces for studying circuit physiology (Kipke *et al.*, 2008). In particular, neural biosensors are being developed to understand how the nervous system encodes information in response to activity-dependent stimulation and elucidate many of the mechanisms underpinning neural dysfunction (Ajetunmobi *et al.*, 2014).

In recent years, microelectrode arrays (MEAs) have emerged as a credible tool for shedding new light on the processes involved in activity-dependent circuit physiology. MEAs are planar glass structures mostly fabricated from standard photolithographic techniques implemented by the semiconductor industry. These bio-devices are able to provide a unique insight into the spatiotemporal patterns of activity in intact two-dimensional neuronal networks, a concept analogous to having petri dishes with electrodes embedded into the culture substrate and linked to a recording/stimulation system. MEA devices function by forming an electrical interface between the neurons and the microelectrodes embedded glass substrate, enabling researchers to monitor the circuit physiology of neuronal networks and providing a unique opportunity for multi-parametric analysis of microcircuit function via combined electrophysiological and molecular imaging modalities of high specificity and sensitivity. Critically, MEAs are non-invasive and potentially provide a much simpler and higher throughput approach than conventional electrophysiological techniques (Morin *et al.*, 2005). Since the development of MEA technology for monitoring neuronal responses (Pine, 1980), several studies have assessed how different activity-dependent stimulation paradigms act on target neuronal networks (Beom Jun *et al.*, 2005; He *et al.*, 2009; Marconi *et al.*, 2012; Scott *et al.*, 2012; Wagenaar *et al.*, 2005), in addition to how mechanisms of transcription-dependent plasticity affect neuronal architecture (Arnold *et al.*, 2005; Liu *et al.*, 2011). These studies investigated the effects of activity-dependent stimulation on developmental function and demonstrated the benefits of MEA technology for delineating the transcriptional activity of neuronal networks with high spatial and temporal resolution. However, maximising the potential of MEA technology requires the ability to individually monitor the activity of single neurons within a network and examine development-, activity-, and drug treatment-dependent changes in synaptic network and function (Jun *et al.*, 2007). Several new strategies have been incorporated into the design and development of MEAs, proposing innovative approaches to improving the interface between microelectrodes and neurons in culture and modelling physiological processes in order to define broader applications for MEA-based studies.

Replicating functional cellular development is a pivotal element to *in vitro* modelling of morphological and physiological features of living tissue. Nano-engineered devices are required to mirror desired physiological processes and allow functional development of cellular interactions. This is a major necessity for developing models that more accurately mimic the *in vivo* conditions of nervous system development and elucidate the pathophysiology of conditions that impact neural circuit physiology. For network modelling applications, thin-film polymers have been used to increase cell-device interactions providing a significant advantage for the study of basic neurobiology and the development of neural biosensors. Feature size has become an important characteristic for developing neural interfaces that more accurately mimic *in vivo* conditions. Nanostructured scaffolds have been used to increase cell-material interactions providing a significant advantage for the study of basic neurobiology and the development of neural biosensors. In particular, nanoscale grooves developed through photolithographic techniques have been successful in enhancing neuronal interaction with array substrates. Photolithography produces nanoscale and spatially defined topographical patterns on MEA surfaces, scaffolding neurite outgrowth for the investigation of *in vitro* neuronal networks and identifying developmental mechanisms associated with functional circuit physiology (Seidlits *et al.*, 2008). The fabrication of barriers, growth lanes, and pinning structures comprised of cross-linked proteins fabricated under conditions that do not compromise the viability of neurons has been shown to restrict neurite outgrowth within confined structures (Hill *et al.*, 2005; Kaehr *et al.*, 2004). Certain polymers have been evaluated for their effectiveness as interfaces that promote cell-cell and cell-substrate interaction. The SU-8 polymer has been extensively studied for its biocompatibility as a neural interface owing to its mechanical strength and biostability. Several studies have evaluated the biocompatibility of SU-8 surfaces using *in vitro* cell culture models (Gabi *et al.*, 2010; Kotzar *et al.*, 2002) and SU-8 has been used in the fabrication of biosensors for measuring electrical impedance in living tissues (Tijero *et al.*, 2009) and monitoring neuronal circuit physiology (Jones *et al.*, 2011; Scott *et al.*, 2012).

Neural biosensors are being developed to understand how the nervous system encodes information in response to activity-dependent stimulation and elucidate many of the mechanisms underpinning neural dysfunction. Developing an integrated platform combining MEA-based studies of circuit physiology with neuroimaging and electrophysiology tools requires an interdisciplinary approach (Figure 1.1), presenting new opportunities to develop a translational bridge between basic and clinical research and lead to new insights in our understanding of CNS physiology and the development of novel diagnostic modalities for therapeutic intervention.

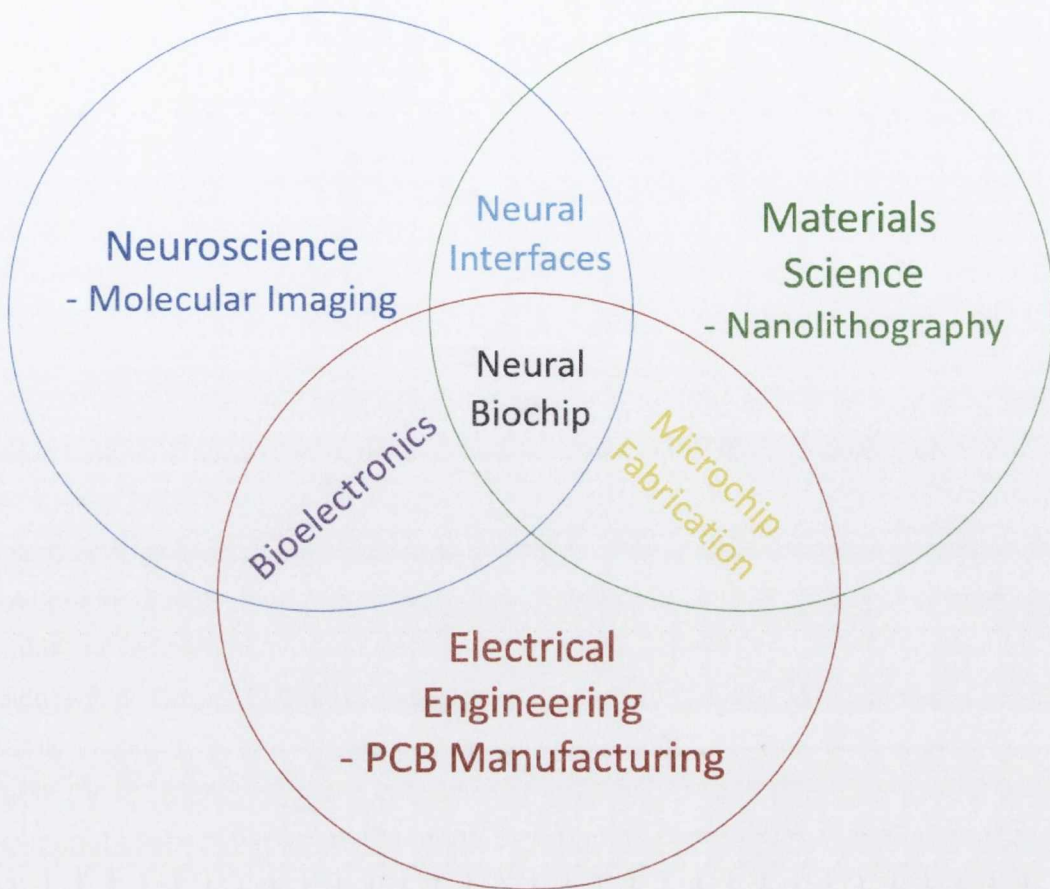


Figure 1.1 | Diagram of interdisciplinary approach to the development of nano-engineered devices.

2 Literature Review

2.1 Introduction

Modelling neurodevelopmental disorders and the impact of nanotechnology in neuroscience has been extensively covered in previous publications (Ajetunmobi *et al.*, 2014; Ajetunmobi and Tropea, 2015). Several elements of the following sections are derived from these publications and appropriate permissions have been sought and granted.

2.1.1 Synapse Dynamics in Neuronal Network Formation

From birth, the development of proper cellular function requires co-ordinated interplay between our genes and our environment. In the central nervous system (CNS), this interplay is particularly pertinent as extracellular sensory input manipulates the cellular and molecular mechanisms behind successful brain development. Functional brain development requires the synchronization of environmental signals with adaptive neuronal communication. Managing extracellular stimuli from rich sensory experiences plays an important role in the ability of neural circuits to modulate the strength of synaptic connections and drive the modifications required for many aspects of post-natal development (Barnes and Finnerty, 2010). It is now well understood that mechanisms which are incorporated during early synapse formation are critically important for laying down the foundations required for functional brain development, and that these mechanisms later underlie cognitive processes in various brain regions during adulthood (Hensch, 2005; Knudsen, 2004).

For decades neuroscientists have studied the role of sensory-evoked neuronal activity in CNS development, elucidating how regulatory inputs from sensory stimulation underlie the cellular mechanisms which incorporate the strengthening and stabilization of useful synapses, and the weakening or elimination of those deemed unnecessary for post-natal development. It is now widely accepted that during pre-natal development, elementary neuronal circuits are formed through genetically programmed molecular cues which control the proliferation, migration, and maturation of neurons, and that these nascent signalling networks lead to widespread implementation of connections within the cortex (Cohen, 2008). However, it is only through activity-dependent stimulation from spontaneous sensory experiences, that these early synaptic processes can be driven to provide modulatory information about the strength of the synapses formed, thereby conferring functionality (Katz, 1996). As such, the myriad of environmental stimuli experienced by the CNS require neurons to process thousands of synaptic inputs individually and co-ordinate their functional connectivity. This response to extracellular stimulation is mediated through synaptic activity, allowing for calcium-dependent signalling events which enable the expression of a group of genes involved in numerous

aspects of neuronal function (Cohen, 2008). This has led to an increasing appreciation for activity-dependent plasticity during post-natal life, whereby the mechanisms acquired at an early age are recruited in the adult under appropriate conditions (Linkenhoker *et al.*, 2005). This phenomenon is evidenced by physiological and morphological changes which include excitation-inhibition dynamics and structural rearrangements of dendritic branches accompanied by synapse formation or elimination. These synapse dynamics are extraordinarily flexible (Bourne and Harris, 2008) and are regulated by extracellular activity and visualised by variability in the size and number of spine heads at the synapse (Matsuzaki *et al.*, 2004), which is indicative of synaptic strength and stability (Holtmaat *et al.*, 2005). The plasticity of synapses at individual synapses is associated with enlargement of the spine head, an increase in synaptic efficiency and a switch in synapse stability. Recent findings suggest a role for protein kinases in the regulation of synaptic mechanisms directly implicated in LTP maintenance and behavioural learning (John *et al.*, 2012). Specifically, calcium/calmodulin-dependent protein kinase II (CaMKII) has been shown to be required for activity-mediated spine enlargement (Yamagata *et al.*, 2009), while protein kinase C (PKC) has been shown to contribute to *in vivo* spine stabilisation (Bednarek and Caroni, 2011). The local regulation of protein synthesis is also implicated in spine stabilisation. These include signalling cascades such as the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways and the mammalian target of rapamycin (mTOR) signalling complex. Other molecular factors involved in spine stabilisation include signalling pathways involved in actin cytoskeletal proteins. Interference with actin polymerisation has been shown to affect LTP maintenance and changes in spine size (Lorenzo and Yukiko, 2008). Studying the molecular pathways involved in communication between the synapse and the nucleus has made it possible to understand cellular events involved in both normal brain development and function, as well as investigate the role of susceptibility genes in the aetiology of disorders of cognitive function (Figure 2.1).

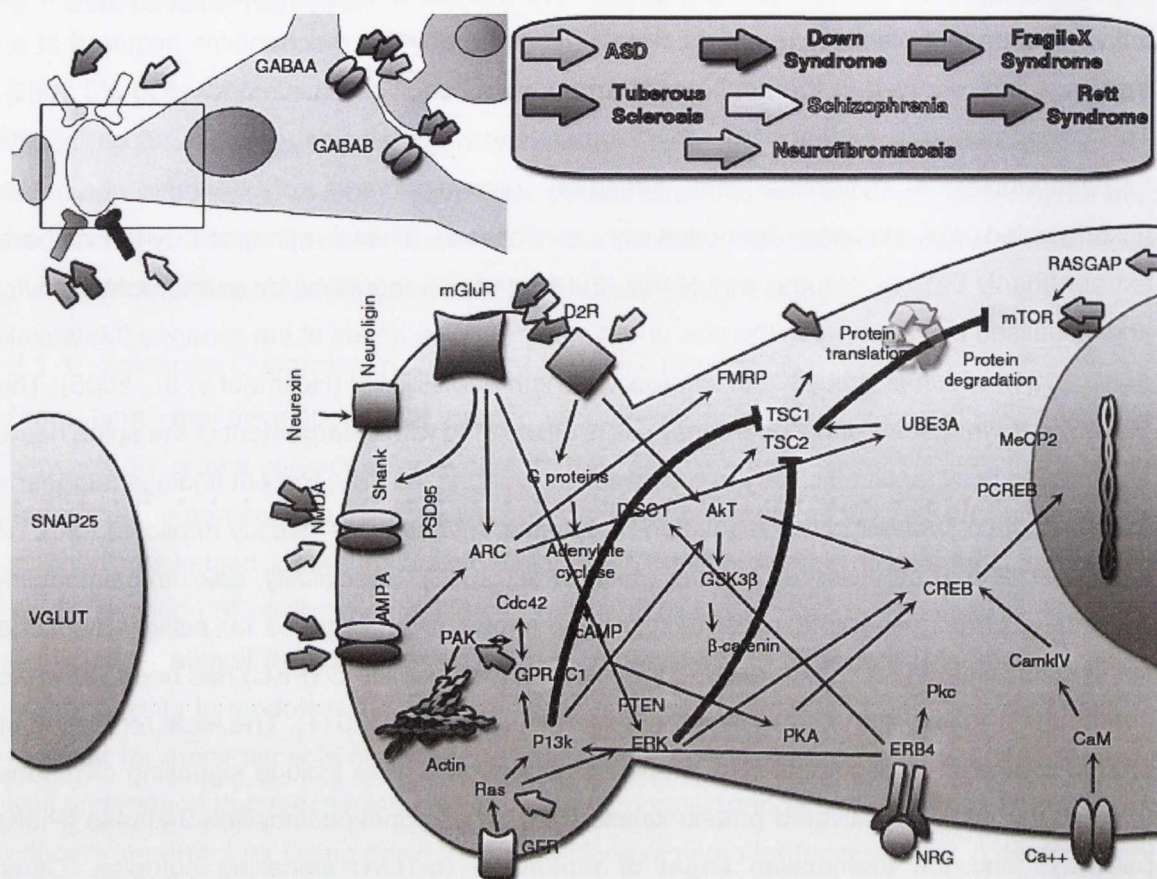


Figure 2.1 | Molecular targets of therapeutic interventions in neurodevelopmental disorders (NDDs). Schematic representation of circuits and pathways involved in NDDs and targets for therapeutic intervention. Adapted with permission from Ajetunmbi & Tropea, 2015.

Molecules such as SHANK3, PSD95, synapse-associated protein 97 and ubiquitin protein ligase E3A have been shown to be involved in excitatory synapse stabilization and implicated in diseases such as Autism and Alzheimer's disease (Penzes *et al.*, 2011). Others, such as DISC1 and the PAK family kinases which signal through the Rho GTPases are associated with cytoskeletal functions that regulate spine and synapse dynamics and have been shown to play a significant role in schizophrenia pathology (Brandon and Sawa, 2011; Kreis and Barnier, 2009; Wells and Jones, 2010). Additionally, MECP2 and other molecules of the neuroligin-neurexin complex have been shown to be involved in the regulation of excitation-inhibition dynamics that influence spine formation and are associated with Rett Syndrome and other intellectual disability disorders (Ropers, 2010). Further evidence of molecular mechanisms of synapse stabilisation is seen in the organisation of the postsynaptic density (PSD) that promote trans-synaptic adhesion and contact. Exclusive expression of PSD95 or in association with AMPA receptors enhances synaptic strength and synapse stability (Ehrlich *et al.*, 2007). Taken together, these studies indicate the importance of molecules associated with synapse function in learning behaviour and cognitive function. Dysregulation of these cytosolic and nuclear mechanisms required for the remodelling of developing synapses to activity-dependent stimulation, elicits a breakdown in communication between the synapse and the nucleus and elevates the risk of developmental disease. Interestingly, these studies highlight that these mutations not only predispose to neurodevelopmental disorders but also disorders of the adult brain such as Parkinson's disease and Alzheimer's disease. This suggests that improper synapse dynamics during the critical period of activity-dependent neuronal development may lead to aberrant neural circuit formation arising in deficits associated with both neurodevelopmental and neurodegenerative diseases (Stoeckli, 2012). Understanding the implications of synapse function in neural disorders requires the need to define how relationships between interacting neurons can collectively produce a particular functional output. This so-called 'microcircuit' involves the study of computations of locally interconnected neurons, for example within the pre-frontal cortex, in conjunction with long-range projections that connect different brain regions. At this level, there is a need to identify sequences of events that associate experience and learning with activity-dependent synapse development, including how excitation-inhibition dynamics can remodel neural circuits, affecting long-term plasticity regulation during post-natal development, in adult life and in the pathogenesis of neural diseases (Caroni *et al.*, 2012).

2.1.2 Synaptic Function in Brain Development

As described, nascent signalling networks lead to widespread implementation of connections within the cortex, however it is only through activity-dependent stimulation from extracellular sensory experiences that early synaptic processes can be driven to provide modulatory

information about the strength of the synapses formed, thereby conferring functionality. This is particularly evident in studies of learning and memory where circuit rearrangements can be confined to neurons involved in a particular learning process. The induction of major modifications in the numbers, arrangements and dynamics of synaptic connections has been shown to influence learning and memory outputs. Environmental enrichment is one such example, producing large increases in synapse turnover and number in multiple regions, with a causal link to improved learning (Bednarek and Caroni, 2011). Conversely, stress has been shown to reduce synapse numbers in some regions, for example in the hippocampus, while increasing them in other systems, for example, in the amygdala (Bruce, 2012). Most of our current understanding of plasticity regulation has been derived from studies involving young rodents where it is clear that the time windows for enhanced plasticity correlate with mechanisms that are important for adult function (Bavelier *et al.*, 2010; Hensch, 2005). This developmental period of enhanced plasticity, regulated by experience, can leave long-lasting structural traces that influence adult learning. This has been particularly evident in studies involving the visual and auditory cortex where the assembly and long-term retention of alternative extra circuits developed during the critical period can be recruited in the adult under appropriate conditions (Wang *et al.*, 2010). Such studies have uncovered a major role for maturing parvalbumin-positive inhibitory interneurons, in opening and closing plasticity windows during the critical period and are further recruited in the regulation of plasticity in the adult (Southwell *et al.*, 2010). As a result, recent studies of synaptic function have investigated the role of inhibitory inputs in circuit remodelling and physiology. These studies have revealed that inhibitory neurons become activated before excitatory neurons and may have a critical role in regulating plasticity during learning (Chen and Nedivi, 2010). Studying sensory deprived visual cortex connectivity provides evidence that regulation by inhibitory neurons may create amenable conditions for enhanced plasticity by excitatory neurons. In one study, loss of inhibitory inputs via visual deprivation decreased inhibitory tone and provided a permissive environment for structural adaptation, including addition of new synapse-bearing branch tips. The authors suggest the regulation of inhibitory inputs, when combined with an instructive stimulus, could facilitate restructuring of mature circuits impaired by damage or disease, improving function and perhaps enhancing cognitive abilities (Jerry *et al.*, 2011). As our understanding of plasticity in functional brain development continues to grow, so too does the role of circuit remodelling and synapse stabilisation in learning processes and memory retention take greater significance. As a result, defects in synapse dynamics and function can be implicated in several developmental psychiatric disorders arising from the development, organisation or specificity of synaptic networks.

2.2 Neurodevelopmental Disorders

2.2.1 Global Impact of Neurodevelopmental Disorders

Brain disorders involve profoundly debilitating behavioural, social and cognitive deficits, affecting emotion, learning ability and memory which unfold and regress as an individual gets older. These disorders not only have a major impact on individual lives but also place a severe strain on healthcare resources. An ambitious study by the European Brain Council estimated the total cost of disorders of the brain within the European Union at €798 billion in 2010 (Gustavsson *et al.*, 2011). Direct healthcare costs were seen to account for 60% of the total cost, with the remaining 40% associated with loss in production. In particular, neuropsychiatric and neurodevelopmental disorders (NDDs) were shown to contribute directly to over 25% of the costs detailed in the study with mood and psychotic disorders each ranking first and third most costly diseases respectively (Gustavsson *et al.*, 2011). Given the hugely significant impact of these disorders on society and after many decades of searching for cures to neural diseases, it is perhaps surprising that in 2013 we are no closer to treating these disorders than we were half a century ago. In many areas of drug development not much progress has been made in novel therapeutic approaches for diseases such as schizophrenia, where treatments are still largely based on substances identified in the 1950's (Brandon and Sawa, 2011). The length of time for the clinical trial and approval phases of CNS drugs is longer than for any other therapeutic area; 10 years for CNS vs. 7.8 and 7.6 years for cardiovascular and antineoplastic therapies respectively (Kaitin, 2010). Across the board, CNS drug discovery and development is associated with a longer and riskier development process and given the high rate of attrition for pharmaceutical research into neurological disorders, there is a clear and present need for identifying areas of research that have the best chance of driving innovation and providing a solid basis for the introduction of novel and more efficacious treatments (Muglia, 2011).

In an attempt to uncover the molecular aetiology of NDDs, the past decade has witnessed impressive efforts in genetic analysis studies, looking at both variability in repetitions of genomic sequences and single gene analysis. The outcome of these studies have revealed that most of the genes implicated in NDDs are not predicted from known biology, and that many of them take part in neurodevelopmental processes such as synaptic function and plasticity (Mitchell, 2011). The ability to sequence the human genome and characterise patterns of variation among populations and subpopulations has made it possible to conduct high resolution and large scale genome-wide association studies (GWAS) (Hall *et al.*, 2010), and more recently whole genome sequencing studies. Such hypothesis-free approaches have generated novel insights into the underlying molecular aetiology of neuropsychiatric and

neurodegenerative disorders (Engle, 2010; Lin *et al.*, 2009; Mitchell, 2011) for example, identifying shared genes involved in both circuit dysfunction of neurodegenerative disorders and calcium-channel dysfunction in psychotic disorders (Network, 2015). Studies in families have shown that genetic factors play a major role in the aetiology of NDDs and apart from few examples of monogenic disorders, in the majority of NDDs, it is not possible to link clinical outcome with mutations in a single gene. To complicate the picture, epidemiological studies show that environmental factors such as maternal infections and malnutrition contribute to the onset of the disorder, supporting the evidence for gene-environment interaction (Stolp *et al.*, 2012). While genetic analysis data has redirected efforts in the research community to develop novel targets for future drug developments, these studies have also revealed that genetic data alone cannot build a functional model of disease pathology, as there are further layers of complexity caused by interaction of functional gene expression with environmental stimuli (Figure 2.2). Thus, the key challenge for modern medicine is to further identify mechanisms behind brain function, from gene expression to physiological behaviour, and determine their implications in the aetiology and progression of CNS disorders (Stoeckli, 2012). As such, to discover new therapeutic approaches, there is a need to establish the cellular mechanisms that underlie each specific condition, identify new biomarkers and their clinical outcome, and establish the right model for understanding the neurobiology of the disorders and testing candidate treatments.

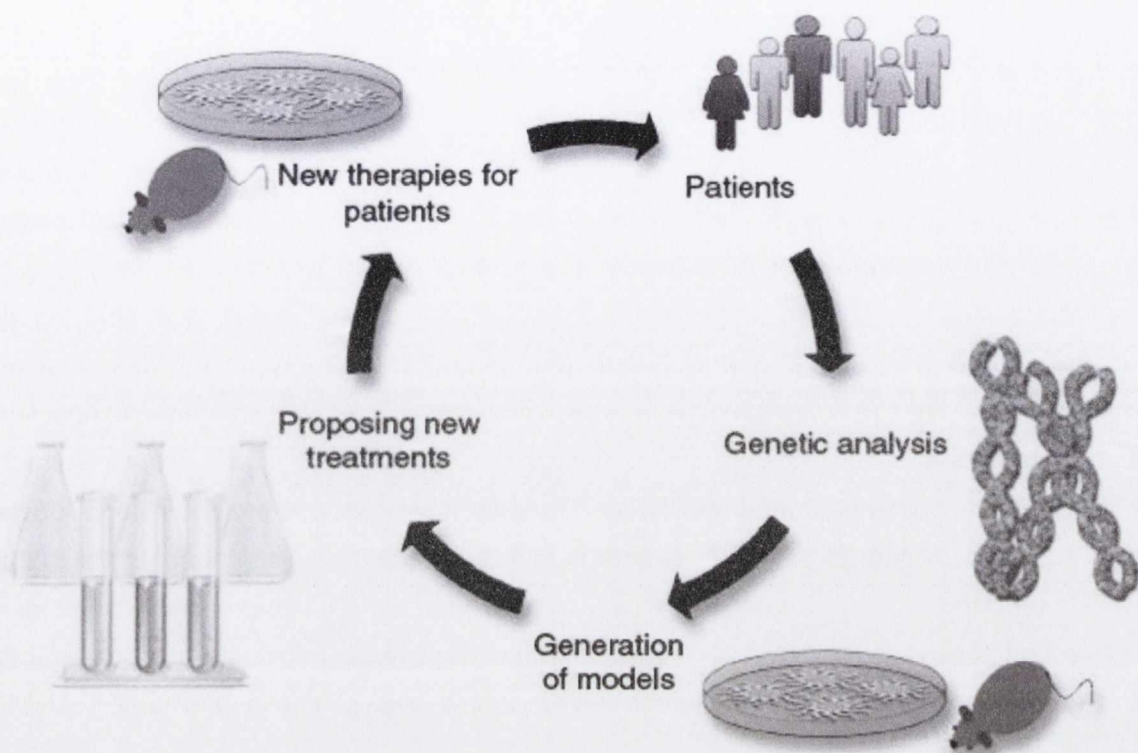


Figure 2.2 | The drug discovery cycle. Clinically relevant genetic information is derived from patients and used to model the disease *in vitro* and in animal models. Basic and translational research identifies molecular biomarkers and candidate treatments that can then be tested *in vitro* and in animal models. If found safe and efficacious, treatments are then tested in man. Adapted with permission from Ajetunmobi & Tropea, 2015.

2.2.2 Modelling Neurodevelopmental Disorders

2.2.2.1 *Animal Models of Neurodevelopmental Disorders*

Animal models have been useful to link the genetic mutations to the impaired neurobiology and to test candidate treatment. Several advances in implementing effective therapeutic strategies for NDDs have focused on the creation and characterisation of animal models specific for each disease state. These mutants have proven to be useful resources for identifying novel putative candidate genes involved in disease onset, and studying the biological functions of these genes. There are several advantages for the use of animal models; firstly, the structure of the nervous system is maintained, and so are the connections between areas and the action of neuromodulators. Secondly, it is possible to investigate the developmental progression of the disorders, including the onset and the possible prodromal signs of the condition. Finally, it is possible to test candidate therapies and to establish their effects on brain circuitry, together with the measurement at the peripheral level (blood tests, cardio-respiratory activity, brain activity, motor behaviour). This is particularly important for the use of new proposed therapies. One of the biggest discoveries from animal studies of NDDs is that, in some cases, a disorder can be rescued even within the adult animal, when the circuitry has already developed. This has shown that, in such cases, the genetic changes do not dramatically alter the basic development of the circuitry and that dysfunctional mechanisms can be corrected. These findings have opened up new avenues for the pharmacological treatment of these disorders and have been used for the pharmacological treatment of pathology and cognitive deficits specific to diseases such as, Rett syndrome, Fragile X syndrome, Tuberous Sclerosis, neurofibromatosis type 1, Schizophrenia and Autism Spectrum Disorders (Ajetunmobi and Tropea, 2015).

2.2.2.2 *Human *in vitro* Models of Neurodevelopmental Disorders*

Considering the diversity of genetic and environmental origins in individual cases of neurodevelopmental disorders, it is difficult to rely solely on the discoveries and therapeutic strategies developed on animal models of transgenic mutations. There is an increasing need to focus on more humanised models of these disorders. The SH-SY5Y human neuroblastoma cell line has been shown to be a credible candidate for modelling disorders of the central nervous system. Advantages over primary neuronal culture include higher yields of obtainable cells over a longer period of time due to their lack of susceptibility to replicative senescence (Blander *et al.*, 2003). Use of rodent cells (be it primary or immortalized lines, such as PC12) face the added problem of slight but relevant metabolic differences between rodents and humans (Herman, 2002). Human neuroblastoma cell lines are better suited for modelling neural diseases as they have biochemical properties of human neurons *in vivo*. Additionally,

as tumour-derived cell lines, they continuously divide and can produce the required quantity of cells for a multitude of experiments, without exhibiting large variability (Biedler *et al.*, 1973). However, they do not have all the characteristics of adult neurons in the brain and as cell lines they have a disadvantage of a short doubling time. One way to circumvent these shortcomings is through differentiation of these cells into dopaminergic neuron-like cells (Lopes *et al.*, 2010). Neuronal differentiation can be induced in vitro by exposure to different agents such as: tetradecanoylphorbol acetate, brain derived neurotrophic factor (BDNF), noradrenaline and retinoic acid (RA) (Encinas *et al.*, 2000). RA-induced differentiation in particular develops neurites whose length increases with time of exposure. Moreover, there is an increased synthesis of enzymes associated with neuronal maturations (such as acetylcholinesterase), neurotransmitters (catecholamines like dopamine), changes in the cytoskeleton markers (neurofilaments) and electrophysiological modifications as seen in normal neurons. These effects are primarily due to RA induction of numerous gene products, including transcription factors, structural proteins, neurotransmitters, neuropeptide hormones, growth factors, enzymes and cell surface receptors. After treatment with RA, cells arrest in the G1-phase of the cell cycle, DNA synthesis is inhibited and growth inhibition can be detected at 48 hours after treatment (Melino *et al.*, 1997). The ability for researchers to differentiate SH-SY5Y neuroblastoma cells into cells possessing a more mature, neuron-like phenotype through manipulation of the culture medium has afforded numerous benefits in the field of neuroscience research. Advantages of using the neuroblastoma model include the capacity for large-scale expansion prior to differentiation, with relative ease and low cost to culture compared to primary neurons. Since these cells are considered a cell-line, the ethical concerns associated with primary human neuronal culture are not involved. Additionally, since SH-SY5Y cells are human-derived, they express a number of human-specific proteins and protein isoforms that would not be inherently present in rodent primary cultures (Jane and Dianne, 2013). As a result, they are good candidates for studying various aspects of neuronal processes including the mechanistic properties of synapse dynamics and function.

While differentiated SH-SY5Y cells may present a good starting model for studying neural disorders there is an increasing appreciation of the need to develop personalised approaches with treatments tailored to the specific pathology and response of individual patients. The development of induced pluripotent stem cells (iPSCs) is rapidly advancing the cause of personalised medicine (Juopperi *et al.*, 2011). Several studies have indeed confirmed at the cellular level that it is possible to replicate some of the results observed in animals. In the case for cells derived from Rett syndrome patients (Marchetto *et al.*, 2010), the authors find decreased expression of markers for synaptic function and altered spontaneous electrical activity as found in animal models (Tropea *et al.*, 2009). Also, the authors find that potential

therapies proposed for patients (such as Insulin-like growth factor 1- IGF1) rescue the phenotypes described *in vitro*, therefore validating the hypothesis that iPSCs can be used to analyse patient phenotype and test candidate treatments. The very same conclusions have been reached for other NDDs such as schizophrenia (Brennand *et al.*, 2011), where alterations in cell morphology have been identified in iPSCs samples derived from patients, and psychotic drugs are able to rescue the phenotype *in vitro*. Additionally, in Fragile X syndrome, the iPSCs technique has been used to generate neuronal cultures derived from patients where a reduction of PSD95 immunostaining and in the number of synaptic puncta has been observed (Liu *et al.*, 2012).

Although this new technique has the potential to precisely address patient-specific issues, there are limitations that still need to be resolved in order to pursue the quest for personalized medicine. First, we need to be aware that the neurons derived in culture, even if they have the same DNA sequence, are likely to have different epigenetic modifications. This problem may have limited consequences for X-linked syndromes (i.e. Fragile X- and Rett- syndrome), when the X-chromosome inactivation is opportunely checked and taken into account (Cheung *et al.*, 2012), however, for mutations on autosomal chromosomes, the whole gene expression machinery can be altered and additional controls are required (Robinton and Daley, 2012). Second, the numbers of phenotypes that are visible in a dish are limited due to a still imperfect methodology used to derive more homogeneous classes of neurons (Brennand and Gage, 2011). Apart from the technical limitations, the *in vitro* preparation presents some drawbacks with respect to animal models; first, it is not possible to study events related to development; second, the cell types are not homogeneous and not organized in structures, third, it is not possible to evaluate the effects of systemic factors such as the effects of neuromodulators, and fourth it is not possible to study the succession of stages that eventually lead to dysfunction of neural systems. Still, iPSCs represent a powerful technology for the investigation and testing of the primary molecular biochemical defects leading to some cases of neuropsychiatric conditions and the continued integration of this platform with whole-genome analysis of individual patients suffering from neuropsychiatric illness will provide an excellent model to explore how risk susceptibility genes functionally interact to result in increased cellular vulnerability leading to disease onset. Such a system could ensure a high-throughput and reproducible means for the targeted investigation of neuronal function and provide valuable platform to facilitate the drug discovery process.

2.3 Developing Nano-based Technologies for Neuroscience Applications

2.3.1 Nanotechnology and Neuroscience

Current methods for characterising brain development and maturation involve the convergence of several diverse diagnostic methods, ranging from single-neuron observation at the intracellular level, to monitoring the principal activity of millions of neurons in synchrony. One fundamental modality for characterising brain development and activity has been the use of in vivo neuroimaging, a powerful technique for observing structural, biochemical, and functional changes within the brain. Significant advances in our ability to image the CNS at increasingly higher resolutions have elucidated several functional components of neuronal activity (Tropea *et al.*, 2010). In addition, the aetiology of disease pathologies has been better characterised, improving our understanding of these disorders and highlighting novel pathways for targeted treatment (Corvin *et al.*, 2012). Nonetheless, there are limitations in current approaches to neuroimaging such as artefact interference, lack of sensitivity, reduced half-life after intravenous administration and decreased permeation across the endothelial barrier surrounding the brain (Nunes *et al.*, 2012). In parallel, electrophysiological studies have also been critically important for understanding the functional connectivity of neuronal populations (Brown and Hestrin, 2009), combining major elements of CNS function from the molecular, to the cellular and behavioural. By using metal or silicon electrodes to record electrical signals caused by ion fluxes across neuronal membranes, electrophysiological approaches allow researchers to probe the mechanics of signal propagation and synaptic function at a high signal-to-noise ratio. However, as with neuroimaging, there remain drawbacks to current electrophysiological studies mainly associated with difficulties in multi-parametric studies of physiological models of brain development (Zhao *et al.*, 2008).

Given the struggles of these current approaches and techniques to the study of functional brain activity, the recent and rapidly advancing field of nanotechnology presents a unique opportunity to confront these challenges and provide a platform for innovation in the drug development process for neural diseases. The biomedical applications of nanoscale structures, materials, and devices are expansive and their diverse physical and chemical characteristics demonstrate a capacity for potentially novel technologies (Figure 2.3). These materials typically consist of solid colloidal particles on the nanometre scale (1-100 nm) composed of insoluble polymers. Within a biological context, they are comparable in size to viruses (20-300 nm) and significantly smaller than cells (30 μm to 300 μm). The small size of nanoparticles in relation to the size of cells allow them to be designed for many specific functions, including binding to cell membranes, delivery of agents into cells or across anatomical and physiological compartments, and monitoring of physiological events

(Provenziale and Silva, 2009). These advantages provide potential enhancements in neuroimaging techniques through higher contrast and better targeted molecular imaging probes. Additionally, nanomaterials can be implemented into advanced biosensor systems for probing the principles of microcircuit physiology and synaptic function within the brain. By exploiting the unique and often improved physical, chemical, and biological properties of nanomaterials, current methods for diagnosing debilitating CNS disorders can be significantly enhanced, and new insights into brain physiology can be applied to develop novel therapeutic strategies (Silva, 2006).

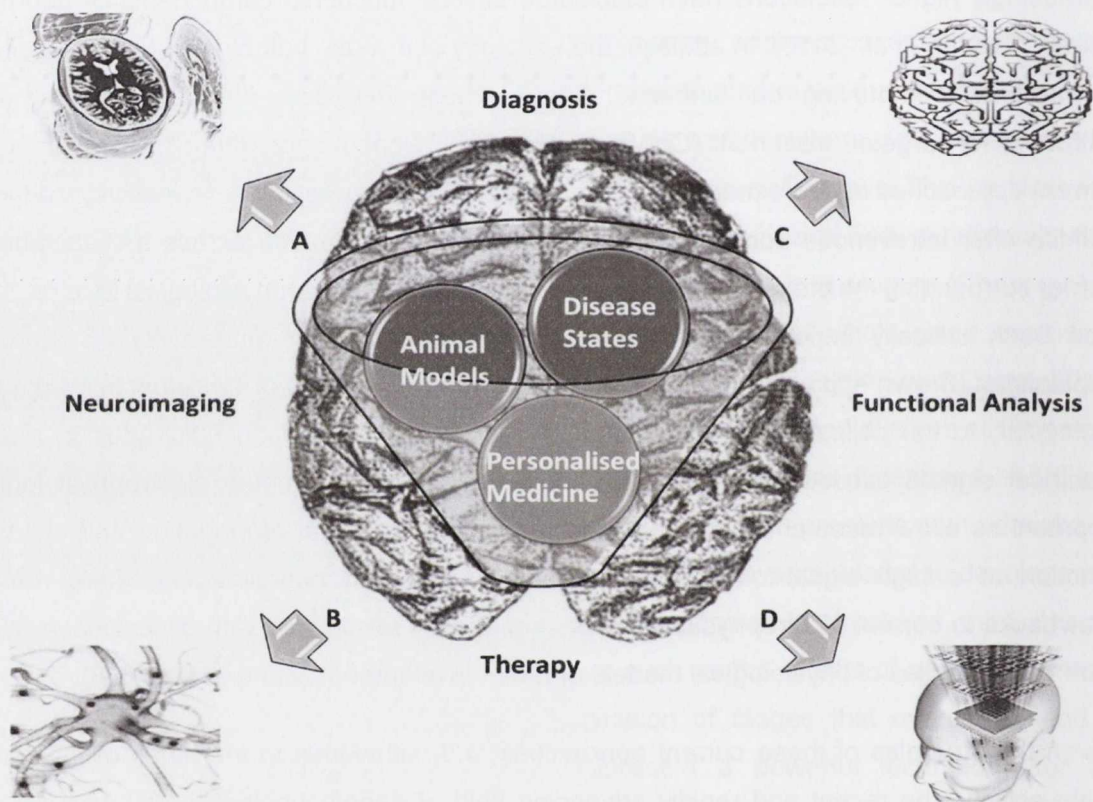


Figure 2.3 | Overview of nanotechnology application in neuroscience. A) Enhancing contrast agents. B) Targeting molecular pathways. C) Modelling circuit physiology. D) Recording neural activity. Adapted with permission from Ajetunmobi *et al.* 2014.

2.3.2 Molecular Imaging

The structural design of nanomaterials for biomedical imaging has sought to expand on the intrinsic advantages of multi-photon optical imaging by enhancing the properties of the fluorophores used to illuminate cellular activity. In recent years, a variety of nanoparticle carriers have been investigated to explore their potential use in molecular imaging, with emerging tools increasingly focused on novel approaches to improve the study and potential diagnosis of CNS disorders. There are several key parameters required for the effective delivery of optically active nanoparticles to the desired target. The most important of these include particle size, shape, charge, hydrophilicity and bioconjugation, with a plethora of nanomaterials under investigation for their molecular imaging properties, including solid lipid nanoparticles, liposomes, micelles, nanotubes, metallic nanoparticles, polymeric nanoparticles, dendrimers and quantum dots (Nune *et al.*, 2009). Several recent articles have been published detailing the use of a variety of nanoparticles as *in vivo* detection tools for optical fluorescence imaging in the CNS and these are discussed below.

2.3.2.1 Organic Nanoparticles

Tosi and co-workers described the development of targeted nanoparticles for optical *in vivo* brain imaging. Their study conjugated polylactide-co-glycolide (PLGA) nanoparticles with an opioid glycopeptide (g7) and a near-infra red (NIR) probe. Surface modification of the PLGA nanoparticles with the opioid g7 peptide sequence enhanced the ability of polymeric nanoparticles to cross the BBB and deliver previously impermeable molecular agents. The NIR-labelled nanoparticles were injected intravenously into mice tail veins and observed via *in vivo* time-domain optical imaging. Their results demonstrated that the nanoparticle conjugates could be easily visualised by optical imaging techniques due to the NIR probe and permeability across the BBB could be enhanced by the g7 glycopeptide, thus presenting a novel CNS-targeted *in vivo* tool for improving diagnostic neuroimaging capabilities (Tosi *et al.*, 2011). Another study looked at the use of biodegradable polysorbate 80-coated poly (n-butyl cyanoacrylate) (PBCA) nanoparticles to explore *in vivo* diagnostic imaging of dynamic CNS processes in living animals. In this work, Koffie *et al* investigated the potential of PBCA nanoparticles to deliver BBB-impermeable molecular imaging contrast agents into the brain of mice for *in vivo* multi-photon microscopy. They validated their approach through the visualisation of amyloid plaques in an *in vivo* mouse model of Alzheimer's disease using (traditionally) non-BBB permeable reagents. Their resultant 4-dimensional, real-time, two-photon imaging revealed PBCA nanoparticle uptake into the brain parenchyma and highlighted the potential of using a biodegradable nano-carrier system to deliver BBB-impermeable targeted molecular probes into the brain for diagnostic neuroimaging (Koffie *et al.*, 2011). Albertazzi and colleagues describe the use of modified polyamidoamine (PAMAM)

dendrimers to cross the BBB *in vivo* and penetrate living neurons without inducing apoptotic cell death or activating microglia. Dendrimers are highly branched molecules synthesised with a precise, well-defined structure and low polydispersity (Tomalia *et al.*, 2007). Due to their variable size, large numbers of reactive sites and hollow interior, dendrimers have proved a promising platform for neuroimaging. Their study investigated the diffusion kinetics of PAMAM dendrimers into the brain parenchyma using two-photon microscopy in living animals after direct intraparenchymal injection. Due to the low toxicity, low immune response, and diffusion ability, PANAM dendrimers were suggested by the authors to be potentially viable diagnostic nanomaterial for *in vivo* delivery to the CNS (Albertazzi *et al.*, 2013).

2.3.2.2 Quantum Dots

Quantum dots (QDs) are fluorescent semiconductor nanocrystals of variable size (1-100nm), with unique electrical and optical properties. In particular, they display superior imaging characteristics in comparison to organic dyes and fluorescent proteins, possessing near-unity quantum yields and far greater brightness (Smith *et al.*, 2008). These factors coupled with their broad absorption spectra yet narrow emission spectra characteristics, relatively long fluorescence lifetime, and negligible photobleaching over minutes to hours, make QDs advantageous for biomedical imaging applications after conjugation with specific bioactive moieties (Pathak *et al.*, 2006). By pairing QD imaging with *in vivo* targeting of specific cell populations, an increasing number of studies have validated the potency of bioconjugated QDs for molecular neuroimaging (Provenzale and Silva, 2009).

Santra *et al* demonstrated the rapid and effective labelling of brain tissue using TAT (a cell penetrating peptide) conjugated to CdSe:Mn/ZnS (cadmium-selenide:manganese/zinc sulphide) QDs intra-arterially delivered to a rat brain. Their experimental data showed that QD-loaded brain tissue could be grossly visualised (using handheld UV excitation) and cellular analysis of the brain parenchyma could be imaged using fluorescence microscopy. They were able to confirm the conjugation of the TAT peptide as a method to overcome the BBB and demonstrated the possibility of delivering a large amount of QD-based imaging agents into brain tissue (Santra *et al.*, 2005). Additionally, Palui *et al* recently developed highly luminescent and hydrophilic CdSe/ZnS QDs of various sizes, showing excellent colloidal stability with optimum spectroscopic properties. They validated the effectiveness of their QD conjugates by performing two-photon fluorescence *in vivo* imaging of mice brain vessels to visualise the brain capillaries of live mice for several hours without apparent adverse effects (Palui *et al.*, 2012). Another recent study reported the selective *in vivo* targeting of microglia by semiconductor QDs. Highly active microglia are the resident immune cells of the brain, continuously probing their vicinity and responding to nearby damage (Nimmerjahn *et al.*,

2005). However, they have also been implicated in brain injury and various neurological disorders, requiring greater knowledge of their behaviour in disease processes. Employing stereotaxic injections into mice hippocampus, Minami *et al* demonstrated an almost exclusive localisation of QDs to the brain microglia, providing a means of observing the dynamic interaction of microglia and neurons independently and uncovering new insights into their roles in the pathogenesis of CNS disorders (Minami *et al.*, 2012).

Several new studies have looked into conjugating QDs to other types of nanoparticles in order to improve stability and further enhance BBB permeability. Gao *et al* reported the development of a QD-based nanopatform for brain imaging by encapsulating QDs into the core of poly (ethylene glycol)-poly (lactic acid) nanoparticles and conjugating the agglomerate particles with wheat-germ agglutinin to improve their brain delivery. The resulting nanoparticles displayed high-payload capacity and were water soluble, showing considerable potential for the development of specific imaging agents for various CNS diseases (Gao *et al.*, 2008). Bardi *et al* similarly investigated the biocompatibility and imaging capabilities of amino functionalised CdSe/ZnS QD-doped silica nanoparticles administered to mice visual cortex via *in vivo* intracerebral injection. Their results demonstrated no toxicity associated with acute administration and supported their hypothesis for the use of QD-doped nanoparticles for biomedical imaging as a valuable tool for future nanomedicine applications (Bardi *et al.*, 2010). Additionally, QDs have been incorporated into liposomes to eliminate uptake by the liver and enhance brain targeting. Liposome-QD conjugates were injected intravenously through the tail vein of female nude mice to observe the localisation and time-course of fluorescent signals emitted. The results showed improved fluorescence intensity of liposome-QD conjugates in the brain when compared to free QDs and validated their potential use as a novel tool for the diagnosis of CNS disorders (Wen *et al.*, 2012).

The development of functionalised QDs with reduced toxicity profiles provides potentially clinically-relevant tools for evaluating specific brain processes. However, investigations of QD applications in diagnostic neuroimaging have been primarily limited to pre-clinical studies. This is mainly due to the varied and contradictory reports on the toxicity profile of QDs resulting from the release of cadmium heavy metal Cd^{2+} ions which has been shown to be cytotoxic to cells (Valizadeh *et al.*, 2012). For QDs to be realised as a valuable diagnostic medical technology, further studies are needed to investigate toxicity in a case-by-case manner, thereby improving study quality and leading to the potential identification a QD formulation that is safe for human use (Tsoi *et al.*, 2013).

Despite these significant advances in our ability to image the CNS at increasingly higher resolutions, current imaging platforms still lack the ability to effectively discriminate the

synapse dynamics of neuronal activity at a single-cell level. For that, electrophysiological measurements of neuronal populations using metal or silicon electrodes, remains the gold standard. By measuring electrical signals caused by ion fluxes across neuronal membranes, electrophysiological approaches allow researchers to probe the mechanics of signal propagation and synaptic function at a high signal-to-noise ratio.

2.3.3 Neural Biosensors

2.3.3.1 Microelectrode Array Technology

One of the major requirements for studying CNS disorders and discovering potential diagnostic biomarkers is to examine the relationship between cellular function and network structure, i.e., the integration of sensory physiology and synapse dynamics to develop functional CNS structure and activity (Lee and Reid, 2011). Primary neuronal culture has typically been the gold-standard for studying the cellular and molecular mechanisms of functional neuronal activity, permitting easy access to individual neurons for electrophysiological recording and stimulation, pharmacological interventions, and high resolution microscopic analysis. In particular, the introduction of more accurate assays for modelling brain disorders through induced pluripotent stem cell (iPSC) models, have provided innovative approaches for probing molecular mechanisms of neuronal development (Marchetto *et al.*, 2010) and high-throughput screening of novel therapeutic compounds (Lee and Studer, 2011). These humanised disease models are derived from peripheral cells isolated in affected individuals and differentiated into human neurons to study disease specific pathogenesis (Ming *et al.*, 2011). This approach presents a valuable diagnostic tool for understanding many of the properties of CNS function and the development of personalised therapeutic strategies (Chailangkarn *et al.*, 2012). However, classic neuronal culture preparations are not amenable to modelling microcircuit connections that can observe the physiological and morphological modifications regulated by neuronal activity and synaptic function. Firstly, connectivity testing is limited by random and uncontrolled synapse formation within the culture. Secondly, the number of cells available for recording is severely limited by the health of the culture over time and the technical difficulties of simultaneous electrophysiology recordings (Potter and DeMarse, 2001). These restrictions impede understanding of how neurons in a local circuit carry out specific computations and limit the identification of biomarkers that can aid the diagnosis of CNS disorders for therapeutic intervention.

In recent years, advanced development of electrodes using thin-film manufacturing techniques and chemical synthesis has allowed for material properties to become tailored for specific requirements in various biological processes. Given the improved physical properties of

materials at the nanoscale, there has been increasing progress in the use of nanotechnology to expand bioelectrical contact with cells within the nervous system (Pancrazio, 2008). These advances in novel technologies are providing increasingly more powerful platforms for bioelectrical devices as high-quality neural interfaces (Kipke *et al.*, 2008). The promising approach of new nano-patterning techniques gradually being implemented for the manufacturing of advanced biosensor systems, present a formidable toolset for producing novel technological platforms for investigating the principles of physical and functional connectivity within neuronal networks. Recently, the advent of planar microelectrode arrays (MEAs) has emerged as a credible tool for shedding new light on the processes involved in activity-dependent neural circuit formation. Since the first report by the Pine lab (Pine, 1980) demonstrating the usefulness of the technology for monitoring neuronal responses, microelectrode arrays have increasingly been appreciated as an essential tool in both basic and pharmaceutical research (Johnstone *et al.*, 2010). MEAs are planar glass structures mostly fabricated from standard photolithographic techniques implemented by the semiconductor industry. These bio-devices are able to provide a unique insight into the spatiotemporal patterns of activity in intact two-dimensional neuronal networks, a concept analogous to having petri dishes with electrodes embedded into the culture substrate and linked to a recording/stimulation system (Figure 2.4).

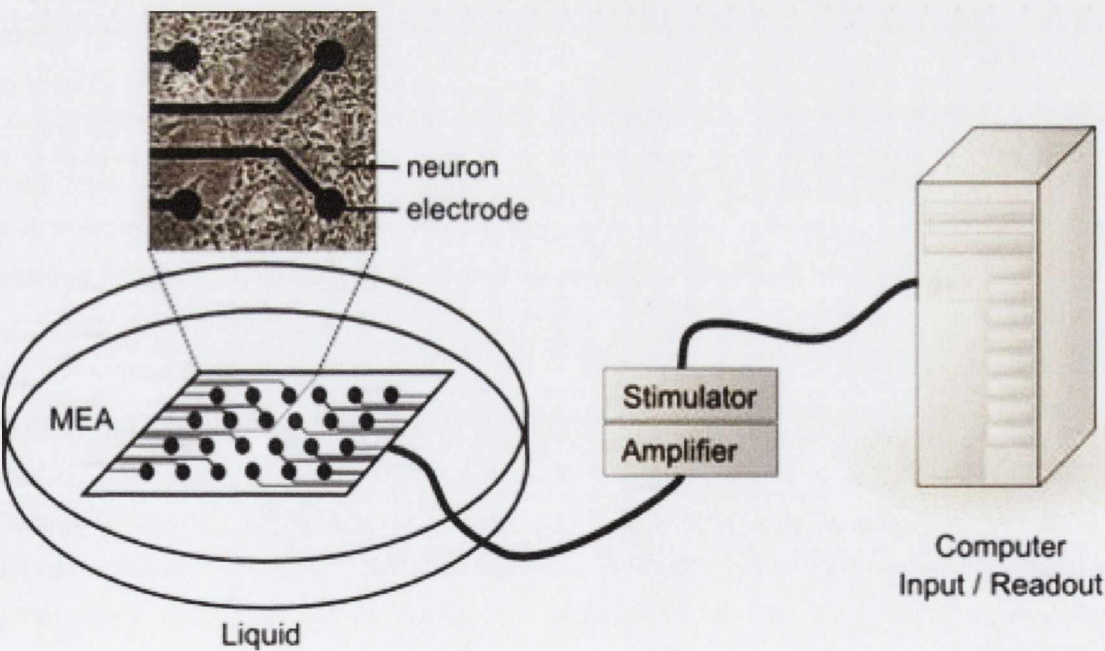


Figure 2.4 | Schematic overview of MEA platform. Consisting of the planar device, electronic circuitry for signal transduction/amplification, and a computer for signal processing software and data logging.

MEA devices function by forming an electrical interface between the neurons and the electrode embedded glass substrate, enabling researchers to monitor microcircuit physiology and synaptic function of neuronal networks and providing a unique opportunity for multi-parametric analysis of microcircuit plasticity via combined electrophysiological and molecular imaging modalities of high specificity and sensitivity. Critically, MEAs are non-invasive and provide a simpler and higher throughput approach than conventional electrophysiological techniques.

The set-up for MEA experiments typically comprise the planar device, electronic circuitry for signal transduction/amplification, and a computer for signal processing software and data logging. The most common substrate of choice for the MEA platform is glass, with microelectrodes generally made from gold and titanium to lower impedance and interconnects made of indium-tin oxide (ITO) which is transparent and allows the direct observation of neuronal cells with an inverted microscope. A layer of insulating material is required to form a barrier between the connection traces and the culture medium and can also serve as the culture substrate (Morin *et al.*, 2005). These characteristics require the insulating material to be both stable and biocompatible, in particular when considering the capacity for long-term studies. The negative photoresist SU-8 is commonly used as insulating material, preferentially treated with poly-L-lysine to promote cell adhesion and obtain healthy, long-term neuronal cultures (James *et al.*, 2000). Typically, MEA dimensions range from 10µm to 100µm, arranged in a grid that covers about 0.2 – 2mm², and a single electrode may record signals singularly or from several neurons. As such, these systems offer unparalleled flexibility regarding neuronal culture preparation and experimental design. Based on the experimental conditions, electrophysiological activity can either be studied after immediate isolation (tissue slices), or after an initial growth phase (1-3 weeks) in culture. Spontaneous electrical activity generated by action potentials elicited within the cell bodies, axons and dendrites of neurons create extracellular field potentials that are within the receptive field of the electrode array. Signal transduction primarily involves microelectrode detection of local variations in the extracellular field potentials created by spontaneous cellular activity; however a myriad of additional information can also be extracted by analysing the spatiotemporal aspects of the activity generated. Basic measurements typically assess the rate of action potentials ('spikes') and the groups of action potentials ('bursts') recorded from each electrode, in addition to the overall network spike and burst rates. The opposite terminus of each electrode extends to the periphery of the array and makes contact with an external amplifier, which relays the electrical signals to a computer for analogue to digital conversion, filtering, signal detection, storage and analysis (Johnstone *et al.*, 2010). When recording from cultures of dissociated cells plated at a low density (e.g. 0.5×10^6 cells/cm²), single cell activity of unitary events can easily be obtained, thus allowing for high spatial resolution and the precise investigation of network

function. By observing and maintaining the pattern of electrical activity associated with network structure and function, MEAs demonstrate their capacity as high-content platforms providing important detailed information that is often impossible to obtain using standard electrophysiological techniques. In addition, coupling the MEA device to stimulus generators makes it possible to create stimulating electrodes which confer the ability to measure the effects of evoked activity and allow for greater insight into the principles of synaptic function (Hoffman, 2006).

2.3.3.2 *Microelectrode Array Applications*

One of the first and most widely used application of MEA technology in neuroscience has been with neuropharmacological and neurotoxicity assessment. Neurons grown *in vitro* retain many of the basic processes underlying *in vivo* physiological behaviour. Thus *in vitro* assessment has been extensively used to screen chemicals for potential neuroactive/neurotoxic function. Typically this has involved the use of patch-clamp recordings to investigate membrane-bound ion channel activity and the membrane re-uptake transporters that control and modulate electrical excitability in neurons. However significant drawbacks have remained, in that patch-clamp recordings only allow for the assessment of one channel at a time and critically, only in individual cells rather than interconnected networks. Conversely, MEAs provide a valuable alternative to conventional techniques, using dissociated neuronal cultures which are very sensitive to neuroactive compounds in culture medium to classify the activity profile of known and unknown substances on neuronal networks. By recording the changes in spike activity of individual neurons on an MEA device, the network-level characteristics of substances such as GABA (Suyama *et al.*, 2004), strychnine (Harsch *et al.*, 1997), ethanol (Xia and Gross, 2003), dopamine (Eytan *et al.*, 2004), fluoxetine (Xia *et al.*, 2003), bicuculline (Arnold *et al.*, 2005), and acetylcholine esterase inhibitors (Keefer *et al.*, 2001) have been evaluated. The rapid characterization of chemical actions on electrical activity has highlighted MEA potential for biosensor applications in pharmaceutical research, illustrating their use as a high throughput and rapid screening method for toxicity testing (Gramowski *et al.*, 2004). This is particularly useful in the screening of chemicals that potentially cause developmental neurotoxicity by developing assay screens for important developmental processes such as proliferation, differentiation, neurite outgrowth, and synaptogenesis. Such MEA based studies on the processes involved in action potential generation and propagation, synaptic function and plasticity, and network formation and function, could provide a neuropharmacological platform for the functional screening of new drugs in the treatment of neurodevelopmental and neurodegenerative diseases. Measuring electrical activity outputs such as network spike and burst rates in addition to structural modifications associated with synaptic plasticity, could highlight how and where the proposed therapeutic target adjusts the electrophysiological

profile of activity-dependent neuronal development at an early pre-clinical stage (Johnstone *et al.*, 2010).

Understanding the cellular and molecular mechanisms involved in regulating these initial synapse dynamics is critical for gaining new insights into disease aetiology and providing the best chance for driving innovation in the introduction of novel treatments. GWAS (genome-wide association studies) and linkage analysis studies have presented a kaleidoscope of candidate genes implicated in dysfunctional neuronal network integration and there is now an urgent need to assign their functions in molecular pathways which underlie specific processes of developmental plasticity. In this endeavour, there is a need to demonstrate whether neuronal networks cultured on MEAs retain any capability to usefully process information as a paradigm of learning and memory. Some researchers have looked into how spontaneous spike charges are maintained as a function of developmental activity, establishing the means by which developing cortical networks grown on MEAs display consistent burst discharge patterns with age-dependent firing rate profiles (Jimbo *et al.*, 2000; Stegenga *et al.*, 2008; Van Pelt *et al.*, 2004). However such studies are incomplete, since a necessary pre-requisite for establishing learning paradigms on neuronal networks is through measuring how stimulation induces lasting changes to the network.

The earliest studies on the use of MEAs for assessing activity-dependent neural circuit formation have looked into how various stimulation protocols act on target networks. A study by Jimbo and colleagues attempted to elucidate the effects of sensitization (as measured by the evoked number of spikes) on network activity. They were able to show that electrical activity in response to probe stimuli could be depressed or potentiated as an indication of activity-dependent modification synonymous with developmental plasticity (Jimbo *et al.*, 1999). Similarly, Shahaf and Marom described how certain aspects of a network response could be programmed by pre-conditioned stimulus using an MEA device. They focally stimulated the network at low frequencies (0.3-1 Hz) until a pre-defined response was observed. By repeating this learning protocol they were able to achieve a target stimulus response and generate a learning curve across the network driven by functional changes in the neural circuitry (Shahaf and Marom, 2001). Eytan and colleagues used multi-site electrical recordings from *ex vivo* cortical networks grown on MEAs to demonstrate how neurons adapt selectively to different stimuli acting upon the network. Their findings illustrated that when stimulated at two sites; neurons in a network amplified their response to the rarely delivered stimulus and attenuated their response to the more frequent input. Additionally, they showed that the observed amplification of the rare stimulus required the presence of the frequent stimulus, whereas depression of the frequent stimulus occurred regardless of the rare

stimulus. They suggested that these phenomena arose as a result of changes in synaptic transmission and are inherent features of organising cortical networks (Eytan *et al.*, 2003). A further study by the Potter lab demonstrated the possibility of controlled activation and deactivation of burst activity, made possible by synchronous electrical stimulation of multiple sites in the culture. Using a closed-loop multi-electrode stimulation paradigm, the authors suggested that externally applied stimulation might be a validated means of creating an environment in cell culture that relates to aspects of network activity *in vivo* (Wagenaar *et al.*, 2005). This concept was further corroborated by a study that looked into the effects of different conditioning stimuli on LTP induction in anterior cingulate cortex (ACC) slices. Long and short train theta burst stimulation paradigms were applied in ACC layers V-VI and the LTP induction effects were compared across different layers of the cortex. Their results indicated that theta burst number and train duration significantly accounted for the resultant LTP induction and that a long train of theta burst stimulation may be more appropriate in facilitating LTP induction in the ACC area. Moreover, their results demonstrated the prominent superiority of MEA recordings in revealing the spatiotemporal properties of synaptic activities in cortical networks (He *et al.*, 2009).

Given these benchmarks set for adapting MEA studies for suitable models of *in vitro* network activity, focus has shifted in recent times to utilising MEA technology as a unique tool for studying transcription-dependent plasticity in neuronal networks. As mentioned earlier, the need to understand the effects of gene networks and molecular pathways on developmental plasticity has taken on greater significance since the advent of GWAS and linkage analyses studies, and as such basic research requires better tools to enhance our understanding of brain development in health and disease. Arnold and colleagues presented interesting findings on the use of MEA recordings on cultured hippocampal networks in revealing a simple model for transcription and protein synthesis-dependent plasticity. In their study, they exposed the neuronal network to the GABA_A receptor antagonist bicuculline and induced an increase in synaptic efficacy, changing from uncoordinated neuronal firing to a highly organised and synchronous burst pattern. Additionally they demonstrated that induction of the burst pattern lasted for several days and was dependent on NMDA receptor activation and extracellular signal-regulated kinase (ERK) 1/2 gene transcription. They concluded that the non-invasive nature of MEA recordings was an effective tool for studying synaptic connectivity and facilitated the search for activity-regulated genes critical for synaptic plasticity (Arnold *et al.*, 2005). A more recent study on pain-evoked synaptic plasticity sought to determine the roles of various mitogen-activated protein kinase (MAPK) family members in mediating spatiotemporal plasticity in hippocampal formation. Using MEA recordings, they found that pharmacological inhibition of the ERK- and c-Jun N-terminal kinase (JNK)-mediated signalling

pathway significantly decreased pain-enhanced LTP maintenance, whereas blocking the p38 MAPK pathway dramatically increased the potentiation. Their results suggested differential roles of the MAPK family members in regulating pain-dependent plasticity within the hippocampus and once again demonstrated the benefits of MEA technology in delineating the transcriptional activity of neuronal networks in both space and time (Liu *et al.*, 2011).

Despite these advances, one of the major limitations to studying *in vitro* dissociated cortical networks is that they are generally randomly organised on culture surfaces. To interpret any meaningful information, sophisticated analysis methods are required to identify individual neurons and describe the dynamics of electrophysiological activity within the network. As such, an inability to resolve the activity of individual neurons makes it difficult to understand their contribution to network-wide functions (Makarov *et al.*, 2005). In order to fully understand the function of individual synapses and their encompassing neuronal network on MEA devices, accompanying technological, electrophysiological and morphological approaches have been developed to augment the data output from MEA-based studies.

2.3.3.3 Modified Microelectrode Arrays

Several new strategies have been incorporated into the design of MEAs, proposing innovative concepts to improve the interface between microelectrodes and neurons in culture and defining broader applications for MEA based studies (Figure 2.5). The recent trend in higher density electrode arrays on MEA devices has led to the implementation of novel nanotechnology-based approaches to enhance MEA performance for neuronal recording and stimulation and new features have been developed to enable precise cell growth and guidance on novel nano-enhanced biosensor arrays (Heim *et al.*, 2012).

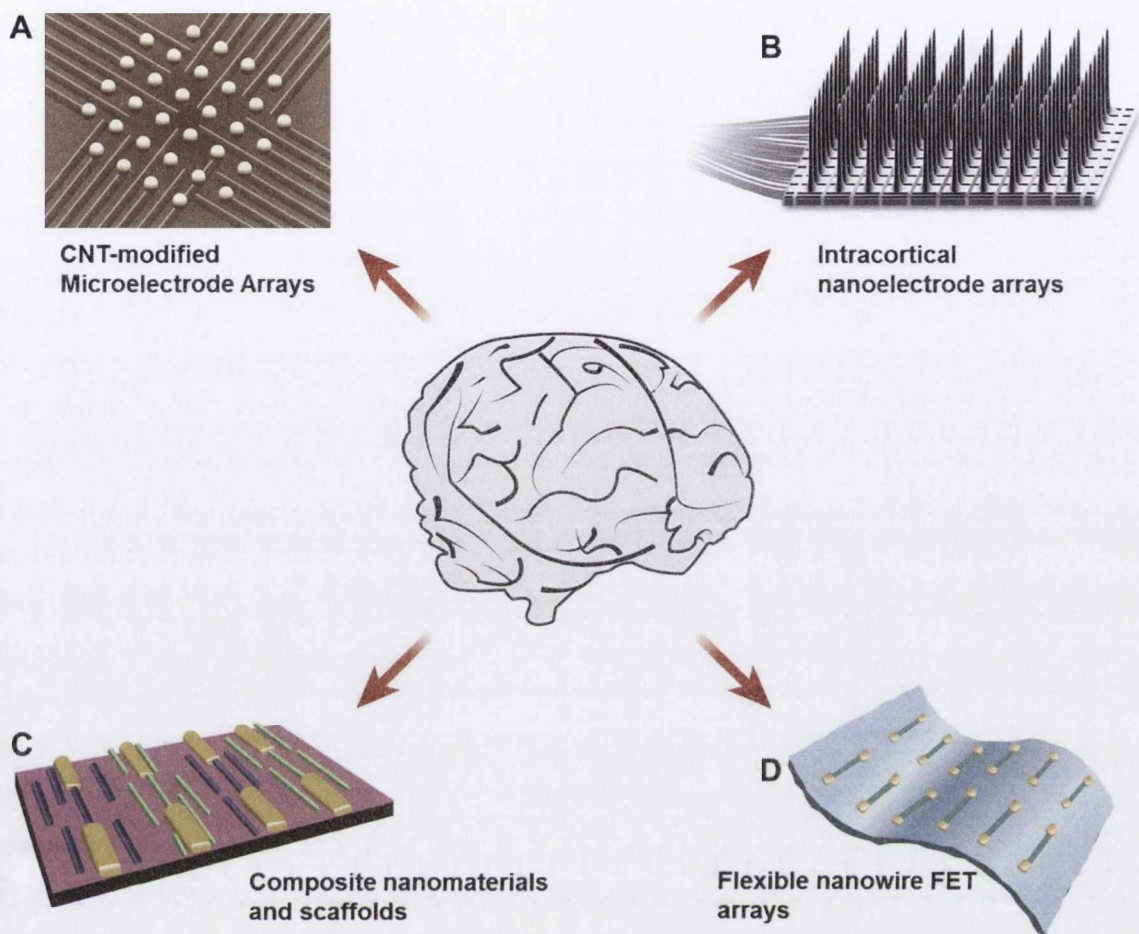


Figure 2.5 | Nano-modified neural interfaces. A) Carbon nanotube (CNT) films coated onto microelectrode arrays for studying neuronal networks. B) Intracortical nanoelectrode arrays for in vivo recording and stimulation of brain tissue. C) Assembly of composite nanomaterials and nanoscaffolds on the same interface for controlling neurite outgrowth. D) Flexible nanowire field effect transistors for intracellular electrical recordings. Adapted with permission from Ajetunmobi *et al.* 2014.

Feature size has become an important characteristic for developing neural interfaces that more accurately mimic *in vivo* conditions. For network modelling applications, nanostructured scaffolds have been used to increase cell-material interactions providing a significant advantage for the study of basic neurobiology and the development of neural biosensors. In particular, nanoscale grooves developed through photolithographic techniques have been successful in enhancing neuronal interaction with array substrates. Photolithography produces nanoscale and spatially defined topographical patterns on array surfaces, scaffolding neurite outgrowth for the investigation of *in vitro* neuronal networks and identifying developmental mechanisms associated with functional microcircuit physiology (Seidlits *et al.*, 2008). In a study of axonal outgrowth on nano-imprinted patterns, Johansson *et al* developed nanoscale grooves with depths of 300 nm and varying widths of 100-400 nm on polymethylmethacrylate (PMMA)-covered silicon chips. They monitored the growth of mouse sympathetic and sensory ganglia cultured in medium containing 25 ng/ml of nerve growth factor to stimulate axonal outgrowth. Using immunocytochemistry and scanning electron microscopy, they found that axons displayed contact guidance on the patterned surfaces but grew preferentially on ridge edges and elevations in the patterns rather than in grooves. Additionally, they found that grooves less than 100nm wide were less effective at promoting parallel neurite alignment (Johansson *et al.*, 2006). In a similar study, Gomez *et al* combined nanoscale topographical cues with chemical stimulation by nerve growth factor. Their results demonstrated the role of narrower and shallower grooves in the induction of neurites to grow across the grooves and align perpendicular to the long axis of the grooves, whereas wider and deeper grooves resulted in the growth of the neurites parallel to and along the grooves. They concluded that topography primarily dominated polarization mechanisms, whereas the synergy of immobilized NGF plus topography, dominated axon length (Gomez *et al.*, 2007). Another approach has been to use photo-crosslinking of proteins to focally excite non-cytotoxic photosensitizers that promote protein crosslinking, such as BSA, into matrices having sub micrometre feature sizes. The fabrication of barriers, growth lanes, and pinning structures comprised of cross-linked proteins fabricated under conditions that do not compromise the viability of neurons has been shown to restrict neurite outgrowth within confined structures (Hill *et al.*, 2005; Kaehr *et al.*, 2004).

Other studies have investigated the use of polydimethylsiloxane (PDMS) microfluidic chips for patterning adhesion molecules on the surface of MEA devices. Microfluidics refers to the ability to manipulate small volumes of liquid in micron-sized channels, capillaries and other geometries. Physically guiding cultured cells through controlled fluid delivery forces has advantages in providing more user input into the control over spatial and temporal gradients and patterns (Young and Beebe, 2010). A study by Morin and colleagues looked into

microfluidic approaches to constraining the connectivity of neuronal networks cultured on MEAs. They developed three-dimensional microfluidic systems using PDMS coupled to MEA devices and successfully reported the culturing of dissociated cortical cells on their microfluidic MEA device in addition to the recording of electrical activity over several weeks. Their technique demonstrated the ability for long-term cell culture studies in PDMS micro-chambers linked by microfluidic channels (Morin *et al.*, 2006). Another recent study developed an integrated microfluidic device for dissociated neuronal cultures, coupling a reversibly bonded PDMS microfluidic chamber with an MEA substrate. They reported obtaining reliable and reversible bonding of their microfluidic bioreactor to an MEA device through magnetic interactions. They posited that their method had benefits in reducing costs of experimental set-up in addition to lending itself to well-defined neuropharmacological assessment (Biffi *et al.*, 2012). Furthermore, efforts have recently turned to combining both aspects of microfluidic devices and microcontact printing. Takayama and colleagues created a PDMS-based culture device, consisting of arrays of U-shaped microwells, and placed it onto a chemically (polyethyleneimine) micropatterned glass substrate. They reported successful growth of cortical neurons, after 6 DIV, with extended neuritis along the medium flow direction and micropatterned regions and established a promising tool to produce user-defined morphologically and functionally regulated cultured neuronal networks (Takayama *et al.*, 2011).

2.3.3.4 *Light-based Electrophysiology*

A further avenue of interest for novel approaches to MEA-based studies of neuronal networks is in determining more accurate methods of probing neuronal function. The use of metal electrodes to record electrical activity has been the typical gold standard, allowing researchers direct insight into functional communication between neuronal populations. However the sensitivity of recording through such an approach necessitates direct physical contact between the electrode and cells under investigation. This limits the ability for single neuron delineation in complex networks and makes functional analysis of network function difficult. The use of light increasingly represents a better approach for probing neuronal function as it avoids the need for direct physical contact and does not interfere with neuronal activity. In addition, light presents further advantages including; improved spatial resolution, meaning signals in even the smallest neuronal structures can be resolved (Ji *et al.*, 2008), simultaneous measurement for a plethora of spatial locations (Wilt *et al.*, 2009), and the use of genetically encoded and targetable probes to access specific cellular subtypes and subcellular domains (Luo *et al.*, 2008). Investigating neuronal function using light requires a reporter molecule to convert membrane potential into an optical signal. Although an indirect form of measurement, such methods in conjunction with new methods of microscopy are being used to make a wide

range of discoveries about nervous system function (Scanziani and Häusser, 2009). These include the analysis of the structure and function of dendritic spines (Nimchinsky *et al.*, 2002), the principles of dendrite plasticity in the hippocampus (Losonczy *et al.*, 2008), and the spatial organisation of individual neurons within the cortical architecture (Ohki *et al.*, 2005). Such studies have raised the bar for the scope of using optical approaches to elucidate several of the mechanisms of neuronal activity. The most successful reporters have been the classic fast voltage-sensitive-dyes (VSDs) such as the merocyanine dyes which generate fluorescence depending on the electric field across the membrane and have been used to isolate single-trial action potentials in small neuronal compartments such as distal dendrites (Djurisic *et al.*, 2004), in addition to delineating the excitatory and inhibitory network activities of specific neuronal populations (Städele *et al.*, 2012). Another approach utilises calcium as a reporter of neuronal activity. Calcium ions present versatile reporters for the intracellular signals that determine many of the complex morphology of neurons. Pre-synaptically, calcium influx triggers exocytosis of neurotransmitter-containing synaptic vesicles, post-synaptically, calcium level increases induce the mechanisms of activity-dependent synaptic plasticity and at the intracellular level; calcium signals can regulate gene transcription. Organic calcium-sensitive dyes recorded through two-photon microscopy are now used extensively in many laboratories for the analysis of the mechanisms associated with synaptic function and plasticity in specific neuronal populations and several studies have demonstrated their effectiveness in monitoring the occurrence of both action potentials and synaptic input to spines, allowing even single-vesicle events to be reliably detected and analysed (Grienberger and Konnerth, 2012). Combining the descriptive capabilities of calcium imaging with the targeted approach of MEA-based studies presents the opportunity for precise elucidation of cellular and molecular events associated with neuronal development as indicated by a recent study which utilised simultaneous calcium imaging with extracellular recordings from cultured cortical neurons to directly map the efficacy of extracellular recordings on an MEA device. Their results demonstrated a correlation between electrical signals and calcium response and suggested the employment of this technique for researching neural systems by acquiring more detailed information than is possible through either MEA recordings or calcium imaging alone (Herzog *et al.*, 2011).

Optical approaches also present an alternative valid approach for sensory and electrical stimulation and with optogenetics even surpass the specificity and versatility of stimulating metal electrodes (Zhang *et al.*, 2007). Light-activated molecules such as channelrhodopsin or halorhodopsin allow for targeted stimulation of neurons and their compartments with greater spatial, temporal and genetic specificity (Scanziani and Häusser, 2009). Specifically, optogenetics provides an advantage to conventional neuronal stimulation by allowing the

individual study of activity-dependent expression of distinct neuronal populations on network development (Zhang *et al.*, 2008). Such advantages can allow researchers to precisely determine connectivity patterns and circuit dynamics of functional network formation and provides a novel avenue for convergence with MEA-based studies. This was demonstrated in a recent study of the role of synchronised bursting phenomena in the pathophysiology of neural disorders such as epilepsy, Parkinson's disease and schizophrenia. In the study, electrical recording using MEAs was combined with optical stimulation of cultured channelrhodopsin-2 rodent hippocampal neurons to study and manipulate network burst synchronisation. They found that low frequency photo-stimulation protocols are sufficient to induce potentiation of network bursting, modifying bursting dynamics, and increasing interneuronal synchronization. Overall their study showed that mild stimulation protocols that do not enforce particular activity patterns onto the network can be highly effective inducers of network-level synaptic function and presented a simple yet effective stimulation-recording platform for modifying and analysing collective neuronal dynamics (Hady *et al.*, 2013).

2.4 Moving Towards Personalised Diagnostics

2.4.1 A Multimodal and Multiplexed Platform

The convergence of nanotechnology and neuroscience is leading to the development of novel neurotechnologies with the potential to impact the translation of basic research into clinical application. Advancements in nanodiagnostic neuroimaging have shown significant potential to revolutionise how the brain is visualised. The ability to track the progression of CNS disorders at cellular and molecular levels has considerable benefit for clinical disease monitoring. In addition, there have been recent developments in molecular imaging techniques using organic nanoparticles and quantum dot applications for visualising *in vivo* molecular pathways. The combination of these nanoplatforms will significantly enhance how researchers and clinicians can quantitatively monitor the structure and function of CNS activity as well as molecular pathways associated with CNS disorders, pioneering the discovery of novel biomarkers for better diagnosis and more targeted treatment opportunities (Blumling Iii and Silva, 2012).

Additionally, nanotechnology can play a significant role in the development of next-generation biosensors for neuronal modelling and electrophysiology. Feature size is critical to sensor functionality; from surface properties affecting cell adhesion and network formation, to the development of CNT-based MEAs and flexible nanoelectronic arrays for *in vivo* applications and engineered *in vitro* neuronal networks grown on nanomaterial scaffolds have applications in the investigation of synapse dynamics and function. This capacity for highly sensitive nanoscale probes to record neuronal activity provides a unique opportunity to have a profound

impact on basic and clinical research (Duan *et al.*, 2013). The integration of these nanosensors with genome analysis, iPSC disease models and advanced imaging of individual patients may provide an excellent translational approach to explore the dynamics of gene expression with synapse development and circuit physiology. While still in relatively early stages of development, the field is growing fast and it is possible that new next-generation sequencing devices will take advantage of these nanobiotechnology platforms. By accelerating the growth and application of innovative nanotechnological solutions for neuroimaging and electrophysiology, researchers will be able to fully construct dynamic pictures of brain development and physiological function. This has the potential to open up vast new territories in the search for more effective treatments for CNS disorders driven by multidisciplinary research and a high demand in the neuroscience community for improved tools with better diagnostic precision. The recent announcements of the Human Brain Project and BRAIN Initiative in Europe and the USA respectively, point to an awareness of the need to refocus efforts on utilising such advancements in technology to help understand the fundamental processes underlying brain function (Devor *et al.*, 2013).

2.4.2 Project Objectives

The overarching goal of this project was to apply nanotechnological approaches to the study of CNS development and function. Developing a biosensor for interrogating neural activity, combined with molecular imaging for studying neuronal network behaviour presents an interfacing neurotechnology for understanding neuronal function, the efficacy of pharmacological intervention and the implementation of intelligent neuroprosthetic devices. The advantages of this approach could be fully realised when coupled with patient-specific models of diseases through induced pluripotent stem cells (iPSCs). Combining cellular reprogramming techniques with MEA technology presents significant advantages over traditional animal models of human diseases. Patient-derived neurophysiological assessment is an important and necessary step in the drug development process and the ability to predict the effect of new therapies on specific targets could allow for accurate patient stratification at a much earlier stage during pre-clinical analysis. The screening of drugs targeted at CNS development through the use of assay screens for important developmental processes such as proliferation, differentiation, neurite outgrowth and synaptogenesis, could open up vast new territory in the search of effective personalized treatments for neurodevelopmental and neurodegenerative disorders.

2.4.3 Rationale and Aims of the Thesis

The translation of basic research into clinical applications requires scientists to have a deep understanding of disease pathophysiology; this is enabled by robust disease models that accurately mimic the *in vivo* conditions of the disease. Brain disorders provide a unique challenge due to the inaccessibility of the tissue and the complexity of cellular function. In recent years, *in vitro* microelectrode array (MEA) technology has emerged as a novel and effective means of studying cultured neuronal networks non-destructively, in comparison to more established techniques such as the patch-clamp technique with glass electrodes. This technology has the potential for developing more accurate disease models; combining electrophysiological and molecular imaging modalities in a specific and high-throughput setting to study the cellular and molecular mechanisms of synapse dynamics and circuit physiology.

Presented in the following chapters is the development of an MEA technology platform that combines photolithography, PCB manufacturing and molecular imaging modalities to investigate neuronal development. The thesis proposes the development and application of this neural biosensor to support *in vitro* modelling of neural circuit physiology. This approach can enable the analysis of molecular and cellular mechanisms involved in neuronal network regulation and functional CNS development.

This results of this project are presented under three main sections:

- Design and development of a nano-engineered biosensor for interfacing with cultured neuronal networks.
- Establishment of biosensor biocompatibility and characterization of cell-device interaction properties.
- The calibration of these devices for proof-of-concept bioassays demonstrated through electrochemical detection of ions caused by redox reactions due to neuronal activity.

Presented herein is the proof-of-concept study of an *in vitro* MEA-based biosensor for investigating SH-SY5Y human neuroblastoma cell activity measured by molecular imaging and electrochemical detection. Overall, this project proposes this platform as a potential diagnostic tool for elucidating novel properties of CNS function towards the development of personalised therapeutic strategies.

3 Materials and Methods

3.1 Materials

Table 3.1 | Materials

Material	Catalogue Number	Supplier
100 mm cell culture dish	10581873	Thermo Fisher Scientific
25 cm ² Culture Flask	156367	Thermo Fisher Scientific
488-rabbit anti-mouse Secondary Antibody	A-11059	Thermo Fisher Scientific
96-well Microplates	2205	Thermo Fisher Scientific
Anti-guinea pig Synapsin Primary Antibody	106004	Synaptic Systems
Anti-mouse MAP2 Primary Antibody	MA5-12823	Thermo Fisher Scientific
Borosilicate Glass B	NEXTERION Glass B	SCHOTT
CellTiter 96® Aqueous	G3582	Promega
CY3-goat anti-guinea pig Secondary Antibody	A-21435	Thermo Fisher Scientific
Dulbecco's Modified Eagles Medium	D6046	Sigma-Aldrich
Dulbecco's Phosphate Buffered Saline	D8662	Sigma-Aldrich
Fetal Bovine Serum	F2442	Sigma-Aldrich
Fluorescent Mounting Medium	S3023	Agilent Technologies
Formaldehyde Solution	252549	Sigma-Aldrich
Hoechst Stain Solution	H6024	Sigma-Aldrich
Human TNF- α	430202	BioLegend
Impedance Measurement Circuit	AD5933	Analog Devices
MF319 Developer	DEM-10018042	Dow
NSG10 Silicon Tip	NSG10/50	NT-MDT
Poly-L-lysine	P8920	Sigma-Aldrich
Potassium Chloride Solution	P9327	Sigma-Aldrich
Retinoic Acid	R2625	Sigma-Aldrich
S1813 Photoresist	DEM-10018349	Dow

SH-SY5Y Human Neuroblastoma Cells	86012802	ECACC
SU-8 Epoxy Resist	SU-8 2000	Micro Chem
Sylgard 184 Elastomer Kit	DCC000001477	Dow Corning
TrypLE®	12604021	Thermo Fisher Scientific

3.2 Development of Planar Microelectrode Array Biosensors

3.2.1 Manufacture of MEA Biosensor

MEA biosensors were developed using thin-film polymer deposition and ultra-violet (UV) lithography techniques (detailed description of fabrication process can be found in Appendix 8.1). Microelectrode and interface patterns were previously designed and printed onto chromium lithographic photomasks using mask layout software. Layer 1 of the mask was fabricated as a non-inverted mask (Figure 3.1a) whereas Layer 2 was fabricated as an inverted mask (Figure 3.1b).

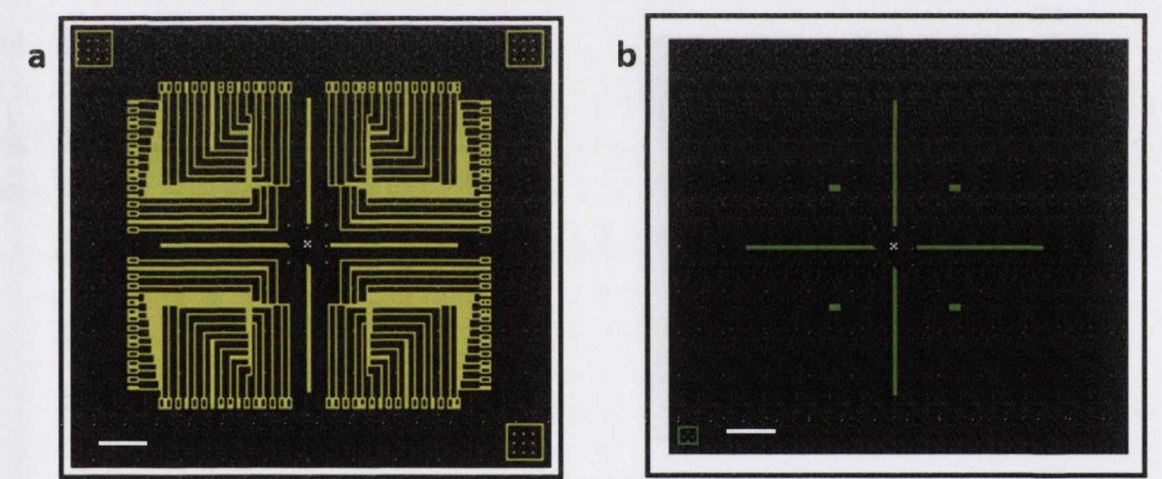


Figure 3.1 | Photomask Patterns for Biosensor Fabrication. a) Design of photomask layer 1 showing microelectrode contacts and traces. b) Design of photomask layer 2 showing neuronal interface layer with four microelectrode sites. Scale bar is 500 μm .

The fabrication process of custom planar MEA device was carried out in a clean room environment (Centre for Research on Adaptive Nanostructures and Nanodevices, CRANN Institute, TCD) using combined chemical and physical thin-film processes (Figure 3.2). Borosilicate glass substrates (SCHOTT, NEXTERION® Slide Glass B; 50 mm x 46 mm x 1 mm, Germany) were cleaned firstly by sonication for 30 minutes in acetone at 37°C, followed by sonication for 30 minutes in isopropanol at 37° C. Following the sonication process, substrates were rinsed in deionised water for 1 minute and dried using a nitrogen gun. The substrates were then baked on a hotplate for 5 minutes at 150°C and allowed to cool to room

temperature for 10 minutes. S1813 (Dow®, Microposit, UK, Appendix 8.3) was spin-coated onto the substrates at a speed of 5000 rpm for 45 seconds. S1813-coated substrates were then soft-baked at 115°C for 75 seconds and allowed to cool to room temperature for 10 minutes. After baking, mask layer 1 was patterned onto the resist by UV exposure at 365 nm wavelength and 14mW/cm² for 5.5 seconds (OAI Model 800 Mask Aligner, USA), to define bonding pads, wiring and microelectrode patterns (Figure 3.2a). Resist was then developed using MF319 (Dow® Electronic Materials, UK, Appendix 8.3) developer for 55 seconds before being rinsed in deionised water for 15 seconds and dried with a nitrogen gun (Figure 3.2b). Metal deposition was carried out by initial sputtering a 20 nm thick titanium seed layer, followed by a 100 nm thick gold layer. Lift-off of the photoresist was achieved by dipping in acetone in an ultrasonic bath for 30 minutes (Figure 3.2c). Following lift-off, the substrates were baked on a hotplate for 5 minutes at 150°C and allowed to cool to room temperature for 10 minutes. SU-8 (2015, MicroChem, USA, Appendix 8.3) was spun onto the glass substrate at a speed of 1000 rpm for 30 seconds. The SU-8 coating was then soft-baked on a hot-plate for 2 minutes at 65° C, followed by 95°C for 5 minutes and then allowed to cool to room temperature for 10 minutes. After baking, mask layer 2 was patterned onto the resist by UV exposure at 365 nm wavelength and 14 mW/cm² for 5.5 seconds to create microwell and microtrench patterns for interfacing with neuronal network (Figure 3.2d). Resist was then developed using MF319 developer for 55 seconds before being rinsed in deionised water for 15 seconds and dried with a nitrogen gun (Figure 3.2e). Finally, the biosensor devices were ready for testing following further cleaning in acetone and a last rinse in ultra-pure water.

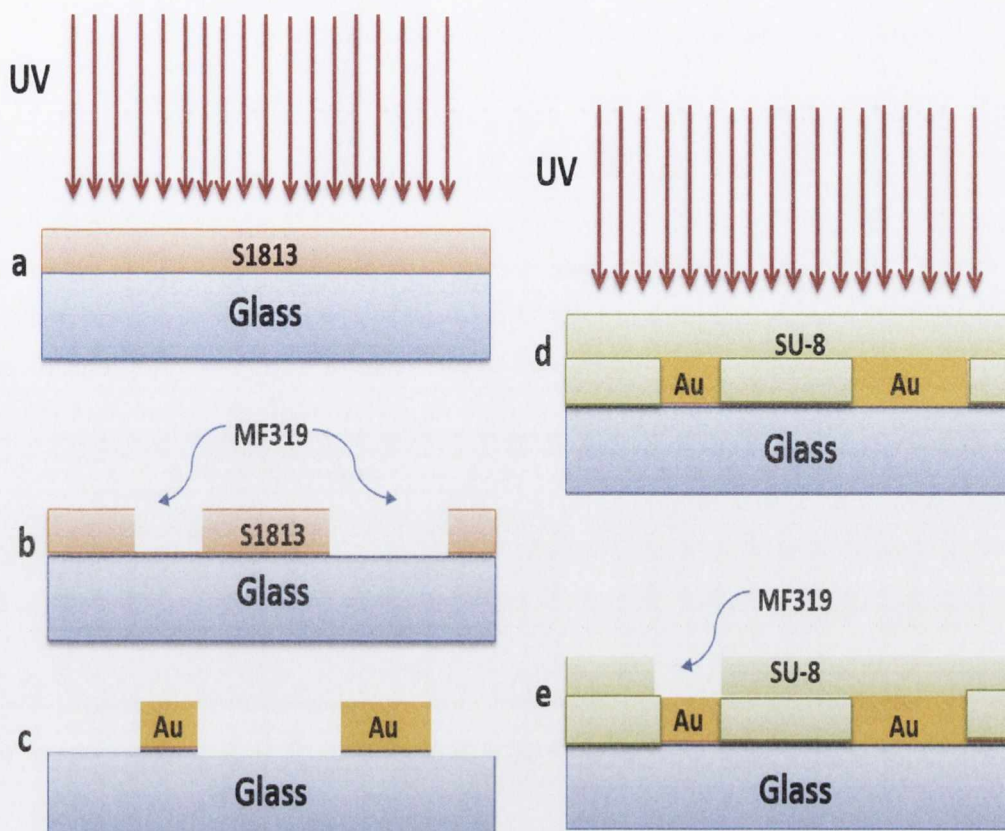


Figure 3.2 | Planar MEA Fabrication Process. a) Spin-coating of S1813 photoresist onto glass and UV exposure through chromium mask. b) Development of the photoresist in MF319 developer to define bonding pads, tracks, reference electrode and electrode sites of the array. c) Evaporation of gold (Au) onto glass and subsequent lift-off of the photoresist. d) Spin-coating of SU-8 photoresist onto glass and UV exposure through chromium mask. e) Development of the photoresist in MF319 developer to define microwells and microtrenches for neuronal interface layer.

3.2.2 Physical Characterisation of MEA Biosensor

3.2.2.1 Surface Profilometry

Stylus profilometry (DekTak, Bruker Corporation, USA) was used to measure the surface topography characteristics of S1813 and SU-8 spun photoresists (Figure 3.3). Specifically, the thickness of S1813 positive photoresist applied onto borosilicate glass was determined. Using a semi-sharp tool, thin scratches less than 1 mm in width were made through the resist coating to the surface of the glass substrate. S1813-spun substrates were placed onto the stage of the tool and the stylus was dragged across the surface of the 1 mm scratch to determine the depth of the applied coating. Additionally, stylus profilometry was also used to the uniformity of the SU-8 interface profile. SU-8-spun substrates were placed onto the stage of the tool and the stylus was dragged across the surface of developed micro-wells and micro-channels. Measurements were calculated using Veeco Dektak surface profiler software (DekTak, Bruker Corporation, USA).



Figure 3.3 | Image of Dektak, Bruker stylus profilometer.

3.2.2.2 Surface Roughness

Surface roughness values were determined using an MFP-3D standalone AFM (Asylum Research, USA) in tapping mode. Silicon cantilevers with a nominal resonance frequency of 300 kHz, force constant 40 N/m, tip radius < 10 nm (BS-TAP300, Budget Sensors, Sofia, Bulgaria) were used in the dynamic AFM experiments (Figure 3.4). For each tip, proportional (p_{Gain}) and integral (i_{Gain}) feedback parameters were optimized individually. The feedback parameters were adjusted to provide maximum possible feedback performance while avoiding ringing due to feedback instability. Such ringing would cause additional noise in the

measurement which would lead to an increased apparent surface roughness. Image processing was carried out using Gwyddion 2.37 software. Data was first levelled by mean plane subtraction to remove any tilt in the instrument, followed by calculation of Root Mean Square (RMS) roughness values for each surface.

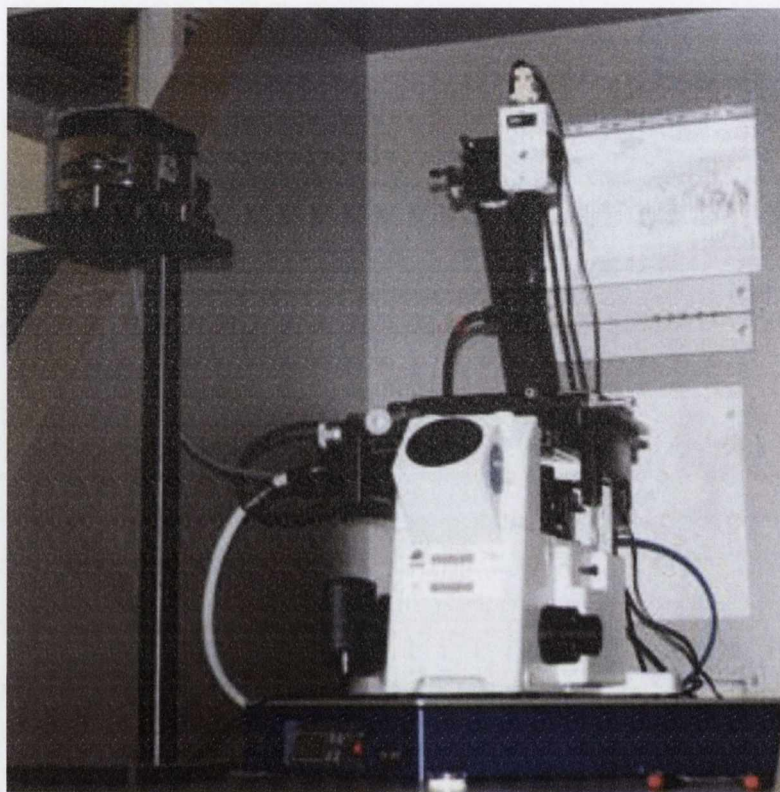


Figure 3.4 | Image of MFP-3D standalone AFM.

3.2.2.3 Nanoindentation Testing

Nanoindentation testing is used to characterise the mechanical properties of polymeric materials. Specifically, the Young's Modulus and subsequent hardness data for very thin layers can be deduced simply from load displacement data, providing insightful information on the viscoelastic and plastic behaviour naturally inherent to polymeric materials. Nanoindentation tests were carried out on glass and SU-8-spun substrates to characterise the mechanical properties of the substrates (Nano Instruments, Nanoindenter XP, USA). Quasi-static measurements were carried out in a 3 x 3 square array, with 20 μm spacing between each point using a diamond Berkovich-shaped indenter tip (Figure 3.5). Six indent measurements were taken from each surface at room temperature. Plots for the force/displacement curves in nanometers (nm) and micronewtons (μN) scale were plotted and analysed using the Oliver/Pharr method to calculate the hardness and the Young's modulus of the material.

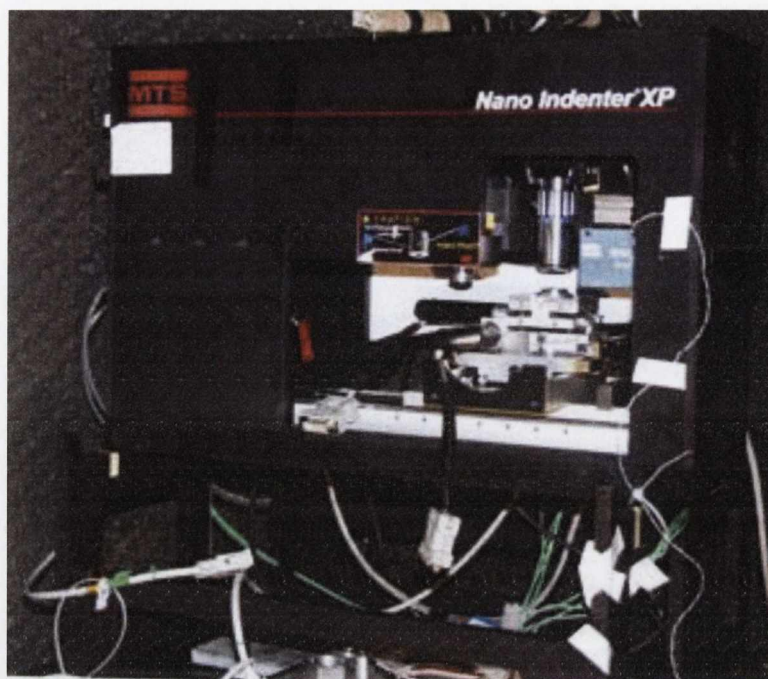


Figure 3.5 | Image of Nanoindenter XP.

3.2.3 Electrochemical Characterisation of MEA Biosensor

3.2.3.1 Cyclic Voltammetry

Cyclic voltammetry (CV) is a technique used to study electrochemical reaction mechanisms that give rise to electroanalytical current signals. It involves applying a voltage to an electrode immersed in an electrolyte solution, and measuring response. In a typical CV measurement, a linear sweeping voltage is applied to an aqueous solution containing the compound of interest. After the voltage reaches a pre-determined maximum value, the potential is reversed. The process can then be repeated in a periodic, or cyclic manner. A typical cyclic voltammogram recorded for a reversible single electrode transfer reaction is shown below in Figure 3.6. The forward sweep plots current as a function of voltage; the scan begins from the left hand side of the current/voltage plot where no current flows. As the voltage is swept from V_1 , a current begins to flow and eventually reaches a peak before dropping whereby at V_2 the scan is reversed and current flow is identical but inverse to the forward sweep. This process is driven by electron transfer following a potential difference above the electrode as the voltage sweeps between the values. To determine the electrochemical stability of microelectrodes, cyclic voltammetry was carried out in three separate solutions, deionised water, Dulbecco's Phosphate Buffered Saline (PBS, Sigma-Aldrich, Ireland) and Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, Ireland). Measurements were carried out between adjacent contact pads using the 4200-SCS Parameter Analyser (Keithley, Tektronix, USA) along a fixed potential range of -5 V to +5 V with 5 mV intervals based on steady-state potential limits.

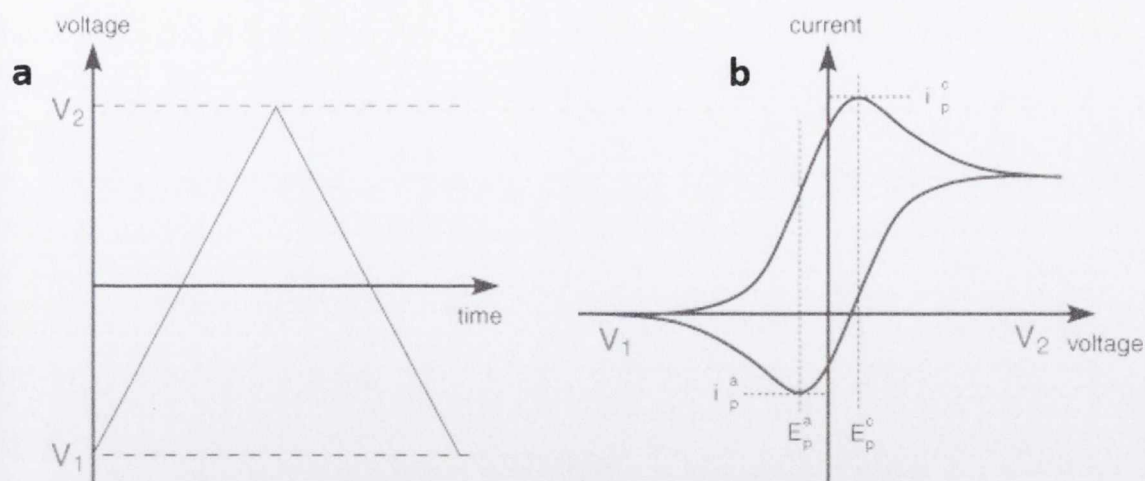


Figure 3.6 | Principles of Cyclic Voltammetry. a) Voltage is swept from V_1 to V_2 and back to V_1 through a series of voltage steps. b) Resulting voltammogram of a typical voltage sweep showing reversible current flow.

3.2.3.2 Impedance Measurement

The electrode impedance is an important parameter for MEA detection of neuronal activity. During stimulation, electrical activity is determined by the resistance of the material to the flow of current. A high impedance results in a large applied electrode voltage leading to undesirable electrochemical reactions that may be harmful to neuronal cultures. Preliminary calibration measurements of biosensor impedance were carried out by partners in Grenoble (Iven Technology, France) to determine the impedance values of the manufactured microelectrodes. Measurements were taken between adjacent contact pads using an AD5933 impedance measurement circuit. 2V P-P (volts, peak-to-peak) was applied at a sweeping frequency from 10 KHz to 110 KHz by a step of 1 KHz. The system was calibrated to a reference of 1 MOhms pure resistor equivalent to the reference impedance, against no impedance when the circuit is open and when the PCB is connected but without contact with the chip. Impedance values were tested for PCB contact with the chip and a connection between two adjacent pads against a drop of water at the microelectrode site.

3.3 Biocompatibility Assessment

3.3.1 PDMS Microenvironment Fabrication

In order to provide a suitable cell culture environment for neuronal cell growth on the MEA device surface, polydimethylsiloxane (PDMS) wells were prepared using Sylgard 184, Silicone Elastomer Kit (Dow Corning, UK) as per the manufacturer's procedure (Appendix 8.3). The lab oven was pre-heated to approximately 100°C. Elastomer and curing agent were mixed at an approximately 10 to 1 ratio to optimise the moulding process and polymer elasticity for a cell culture environment. 23.4 g of elastomer was measured into a clean graduated tube and after allowing time to settle, 2.6 g of the curing agent was gently added to elastomer, ensuring

enough PDMS to create 24 moulds. To homogenise the crosslinking of elastomer and curing agent, the mixture was stirred thoroughly with a spatula and allowed to settle until almost all the bubbles disappeared from the solution. The PDMS mixture was then applied to the sample mould with a surface area of 2 cm^2 and volume of 2 cm^3 , designed to hold up to $500\text{ }\mu\text{l}$ of media. This was baked in the oven for approximately 45 minutes. After baking, the PDMS samples were removed from the oven and placed in a freezer for 5 minutes to shrink the PDMS slightly and ease peeling of the sample from its mould. PDMS culture environments were separated from the mould using a scalpel blade and bonded reversibly to SU-8 substrates by gentle pressure of the PDMS onto the surface of the substrates (Figure 3.7).



Figure 3.7 | Image of PDMS bond to biosensor surface.

3.3.2 SH-SY5Y Cell Culture

SH-SY5Y human neuroblastoma cells (ECACC, UK) were routinely maintained in 25 cm^2 tissue culture flasks (Nunc, Thermo Fisher Scientific, UK) with the growth medium in a 5% CO_2 humidified air at 37°C . The growth medium was made with Dulbecco's Modified Eagles Medium (DMEM, Sigma-Aldrich, Ireland) supplemented with 15% heat inactivated fetal bovine serum (FBS, Sigma-Aldrich, Ireland), 4500 mg glucose/L , 2 mM L-glutamine and 2.2 g/L sodium bicarbonate. To prepare for seeding onto SU-8-spun glass substrates, SH-SY5Y cells were detached at 80% confluence using TrypLE (Thermo Fisher Scientific, UK) and re-suspended in growth medium for plating. Before plating, SU-8-spun glass substrates were sterilized with 70% ethanol overnight, washed twice with phosphate buffered saline (PBS, Sigma-Aldrich, Ireland) and twice with growth medium and then coated with 0.01% solution poly-L-lysine (PLL, Sigma-Aldrich, Ireland) for 30 minutes. The substrates were then washed twice with PBS and twice with growth medium. Approximately $2.5 \times 10^4\text{ cells/cm}^2$ were plated

into each PDMS culture well in growth medium, which was contained in a 100 mm cell culture dish (Corning, Thermo Fisher Scientific, UK).

3.3.3 SH-SY5Y Cell-Substrate Interaction

Atomic force microscopy was used to map cell-substrate interaction of SH-SY5Y with the SU-8 polymer. To prepare for imaging, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, Ireland) for 20 minutes at room temperature and washed in PBS three times, 5 minutes each. The samples were subsequently dehydrated through ascending grades of ethanol (from 60% up to 100%) and dried in room temperature overnight. The SU-8-spun glass substrate was mounted onto the microscope and images were taken using an Ntegra Spectra (NT-MDT, Russia) AFM/Raman system. Imaging was carried out courtesy of Dr. Namrata Jain in dry-phase, semi-contact AFM with a silicon-nitride tip (NSG10, "Golden" Silicon Probes; NT-MDT). The resonance frequency of the tip was found to be 280 KHz.

3.3.4 SH-SY5Y Neuronal Differentiation

SH-SY5Y human neuroblastoma cells (ECACC, UK) were routinely cultured in medium composition as described above. At 80% confluence, the cells were detached using TrypLE (Thermo Fisher Scientific, UK) and re-suspended in growth medium for plating. Twenty-four hours after plating, cells were pre-differentiated with 10 μ M RA (retinoic acid, Sigma Aldrich, Ireland) for 1 week with a full media change at day 3 and half media change at day 5. Following 1 week pre-differentiation, RA media was replaced with 50 μ M brain-derived neurotrophic factor (BDNF, Sigma-Aldrich, Ireland) enriched serum-free media.

3.3.5 Flow Cytometry of SH-SY5Y Cell Viability

Flow cytometry was carried out for quantitative assessment of SH-SY5Y cell viability following growth on glass and SU-8-spun substrates. Cells to be analysed are aspirated from a reservoir and hydrodynamically focused so that they pass one by one through a light source (Figure 3.8). Particles are introduced into a column of pressurized sheath fluid, and as they emerge from the nozzle, they pass through one or more laser beams. At this point, the moment of analysis, the cytometer gathers information about the fluorescence characteristics of the particle. At 48 and 72 hours respectively, cells were detached from glass and SU-8-spun substrates using TrypLE and re-suspended in PBS (Sigma-Aldrich, Ireland). Analysis of cell viability was carried out using the BD Accuri C6 (BD Biosciences, USA) based on a decrease in forward scatter and an increase in granularity.

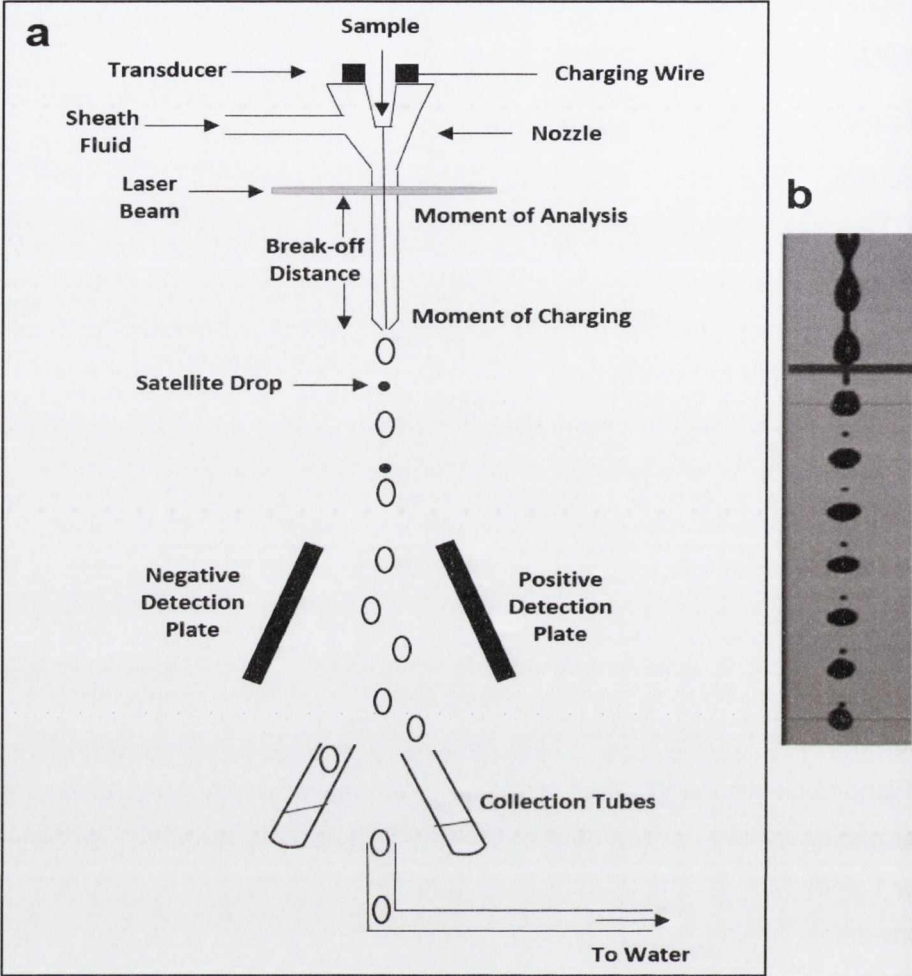


Figure 3.8 | Schematic of principles of flow cytometry. A single cell suspension is hydrodynamically focused with sheath fluid to intersect an argon-ion laser. Signals are collected by a forward angle light scatter detector, a side-scatter detector (1), and multiple fluorescence emission detectors. The signals are amplified and converted to digital form for analysis and display on a computer screen.

3.3.6 Spectroscopic assessment of SH-SY5Y Cell Proliferation

Absorbance spectroscopy was carried out for quantitative assessment of SH-SY5Y cell proliferation following growth on glass and SU-8-spun substrates. At 48 and 72 hours respectively, cells were detached using TrypLE and re-suspended in growth medium. 100 μ l of cell solution was transferred into a 96 well assay plate and 20 μ l of CellTiter 96® Aqueous Assay Reagents (Promega, UK) was added into each well containing 100 μ l of cells in culture medium. The plate was incubated for 1.5 hours at 37°C in a humidified, 5% CO₂ atmosphere. The absorbance was recorded at 490 nm using an ELISA (enzyme-linked immunosorbent analyte) plate reader (Figure 3.9).



Figure 3.9 | Image of Epoch absorbance spectrophotometer.

3.3.7 ELISA-based Analysis of SH-SY5Y TNF α Expression

SH-SY5Y cells were cultured on glass and SU-8-spun substrates and at 72 hours, cell supernatants were harvested and TNF- α levels were quantified by ELISA (Human TNF- α ELISA MAX™ Standards Sets, Biolegend, MSC, Ireland). Briefly, flat bottom 96-well plates (Nunc, Thermo Fisher Scientific, UK) were coated with diluted capture antibody for 16-18 h at 4 °C prior to commencement of the assays. Plates were washed three times with 400 μ l/well wash buffer (PBS supplemented with 0.05% Tween-20). Non-specific binding to uncoated regions of the 96-well plates was eliminated by incubating 200 μ l/well of 1x assay diluent for 1 h at room temperature with gentle agitation. Three further wash steps were completed before the addition of diluted cell culture supernatants in 1x assay diluent according to the supplier's instructions with standards and samples completed in duplicate and incubated at room temperature for 2 h. 100 μ l/well of diluted detection antibody was added next and incubated

for 1 h with gentle agitation. After the addition of avidin-HRP solution for 30 min all plates were washed 4 times. TMB substrate was added to each well and incubated in the dark until a positive blue colour was observed in TNF- α standards. The reaction was stopped by the addition of 2N H₂SO₄. The optical density for each well was recorded at 450 nm as recommended in the manufacturer's instructions using an Epoch microplate reader (Biotek, USA). Plates were also read at 570 nm to determine the optical interference of the 96-well plastic plate, values recorded were subtracted from standards and samples tested.

3.3.8 Immunofluorescence Microscopy

Immunofluorescence imaging was carried out for qualitative and quantitative assessment of SH-SY5Y morphology and function following growth on glass and SU-8-spun substrates. At 80% confluency, cultures were fixed for 20 minutes in 4% PFA at room temperature and washed in PBS three times, 5 minutes each. They were then blocked and permeabilised in 10% normal goat serum and 0.1% Triton X-100 in PBS for 1 hour at room temperature, shaking at 250 rpm. The cultures were then incubated overnight at 4°C with primary antibodies to MAP2 (microtubule associated protein-2), synapsin and PSD95 (post-synaptic density), diluted in 10% normal goat serum and 0.1% Triton X-100 in PBS. Following overnight incubation, cultures were washed three times for 5 minutes in PBS, shaking at 250 rpm and incubated with secondary antibodies diluted in 0.05% Triton X-100 in PBS for 2 hours. Following incubation, cultures were washed in PBS three times, 5 minutes each, stained with the nuclei stain Hoechst (Sigma-Aldrich, Ireland) and mounted using Fluorescent Mounting Media (Dako, UK).

To double-label the cultures, the following primary antibodies were used; anti-mouse MAP2 (1:200), anti-guinea pig synapsin (1:1000). Secondary antibodies used were Alexa-Fluor 488-rabbit anti-mouse (1:500, green) and Cy3-goat anti-guinea pig (1:500, red). Samples were observed through either a 20x, 0.70 N.A. objective or a 63x, 1.40 N.A. oil immersion objective. Parameters (laser intensity, pinhole, gain and offset) were set to avoid saturation and they were maintained constant across each experimental group to ensure semi-quantitative comparison. Images obtained by confocal microscopy (Figure 3.10) were analysed for morphological and functional characteristics using the ImageJ software package (<http://imagej.nih.gov/ij/>) according to the following four parameters; average nucleus area, average length of neurites and intensity of synapsin marker staining. Nuclei area measurements were conducted on Hoechst stained cultures. In order to distinguish between neurite projections and non-neuronal outgrowth projections, only those projections positive for MAP2 were considered to be neurites. The length of neurites were measured and quantified by manual tracing along neurites and synapsin intensity within the cell bodies and neurite

processes were measured by CTCF (corrected total cell fluorescence; the integrated density minus the area of the cell multiplied by the mean fluorescence of the background). All measurements were conducted blind to prevent possible bias. Data plots and statistical analysis were performed with a two-tailed T-Test assuming unequal variance (Mann-Whitney U-Test). Statistical significance between groups is denoted in the figures, where *** $p \leq 0.001$, ** $p \leq 0.01$, and * $p \leq 0.05$.

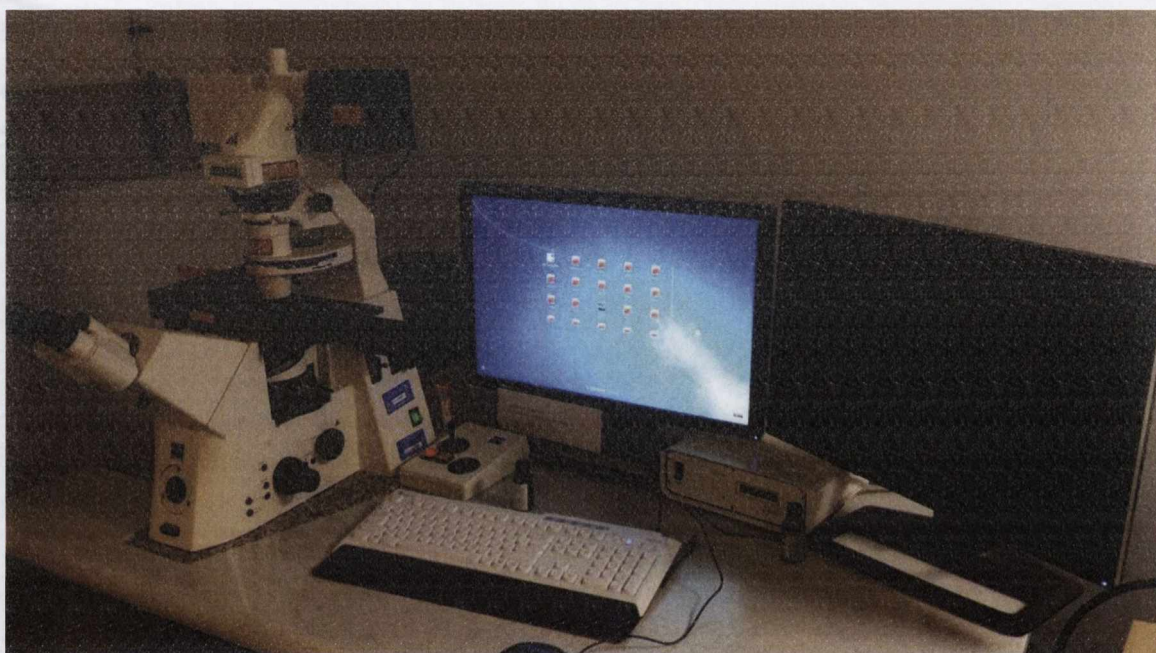


Figure 3.10 | Image of confocal microscopy set-up.

3.4 Bioassay Development for Neuronal Network Modelling

3.4.1 Potassium Chloride Treatment of SH-SY5Y Cells

Potassium chloride (KCl, Sigma-Aldrich, Ireland) was used to induce depolarisation of SH-SY5Y cells following growth on glass and SU-8-spun substrates as well as on the MEA biosensor. SH-SY5Y human neuroblastoma cells (ECACC, UK) were routinely cultured in 25cm² tissue culture flasks (Nunclon, Thermo Scientific, USA) with the growth medium in a 5% CO₂ humidified air at 37°C. At 80% confluence, the cells were detached using TrypLE (Thermo Fisher Scientific, UK) and re-suspended in growth medium and plated onto glass, SU-8 and MEA surfaces. Membrane depolarization was induced by treating the cells with 75 mM KCl in calcium dye solution.

3.4.2 Calcium Imaging

For Calcium (Ca²⁺) imaging, SH-SY5Y cells were cultured on glass and SU-8-spun substrates and incubated in a solution containing 5 μ M Fluo-4AM (Molecular Probes, UK) in a 37 °C incubator for 30 min before analysis. Substrates containing dye-loaded cells were visualized directly at an excitation wavelength of 488 nm filtered through an emission long pass filter (LP 515, Carl Zeiss). The confocal inverted microscope (LSM 510, Carl Zeiss) was equipped with a 40x objective lens (Carl Zeiss), a helium/neon laser (543 nm, 633 nm), and a second blue-enhanced argon laser (488 nm). To determine Ca²⁺ concentrations, the scan speed was set to 1.08 s for a 512 x 512 pixel image, and the pinhole was set to achieve a full-width, half-maximum z resolution of approximately 1 μ m.

3.4.3 MEA Biosensor Response Calibration

A frame compatible with Zeiss microscope manual stage was designed to hold the chip in place combined with a hinge system and an interconnected PCB. The dedicated PCB was fixed with a STM32F0 discovery board integrating the popular STM32F072B microcontroller. The circuit was composed of two main functions; the generation of voltage pulses between two pads with current recording, a second part measuring impedance between two pads by frequency sweep. The interconnected PCB contained spring loaded pins which are in contact with pads of the chip and 4 x 32 pins headers. The hinge system applied a pressure on the PCB to have good contact between pads of the MEA chip and spring loaded pins. Voltage-controlled stimuli were generated using a custom digital-to-analogue converter (DAC) and the resistance resulting from the stimulus, was measured according to the circuit diagram depicted in Figure 3.11.

We tested monophasic (positive and negative) and biphasic (positive-then-negative and negative-then-positive) pulses of various amplitudes (100 – 1000 mV) and duration (100 –

1000 μ s) on 32 electrodes from multiple MEAs. These parameters were chosen based on a publication by Wagenaar *et al.*, 2004 showing effectiveness at evoking neuronal activity (the stimulus repertoire is summarized in Table 3.2). Calibration measurements were carried out in deionised water, Phosphate Buffered Saline (PBS, Sigma-Aldrich, Ireland) and Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, Ireland).

Table 3.2 | Summary of voltage-controlled pulses tested

Pulse shape	Amplitudes (mV)	Pulse widths (μ s)
Monophasic, negative	100 – 1000	1000
Monophasic, positive	100 – 1000	1000
Biphasic, negative-first	100 – 1000	100 – 1000
Biphasic, positive-first	100 – 1000	100 – 1000

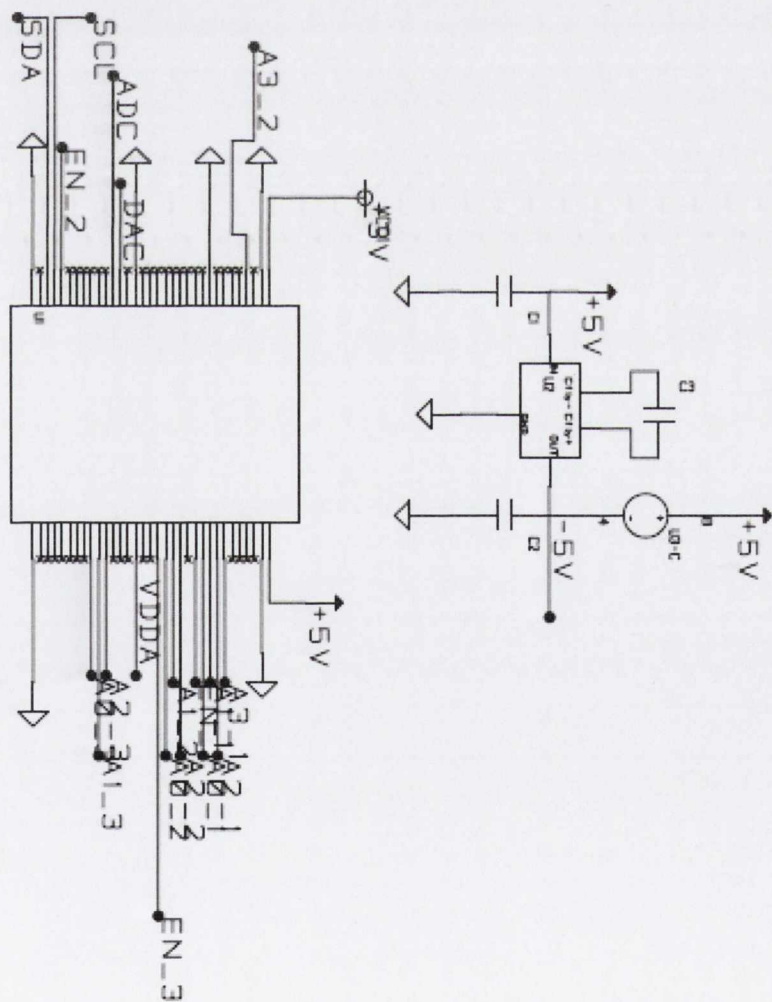


Figure 3.11 | Circuit diagram of stimulus generation/recording circuit.

Electrodes were manually selected, according to their adjacent positions. All stimuli were relative to a large electrode embedded in the MEA substrate which also served as a reference electrode for recording responses. Experimental setup can be visualised in Figure 3.12. Stimulation was fully automated using custom software controlling the hardware (Appendix 8.4).

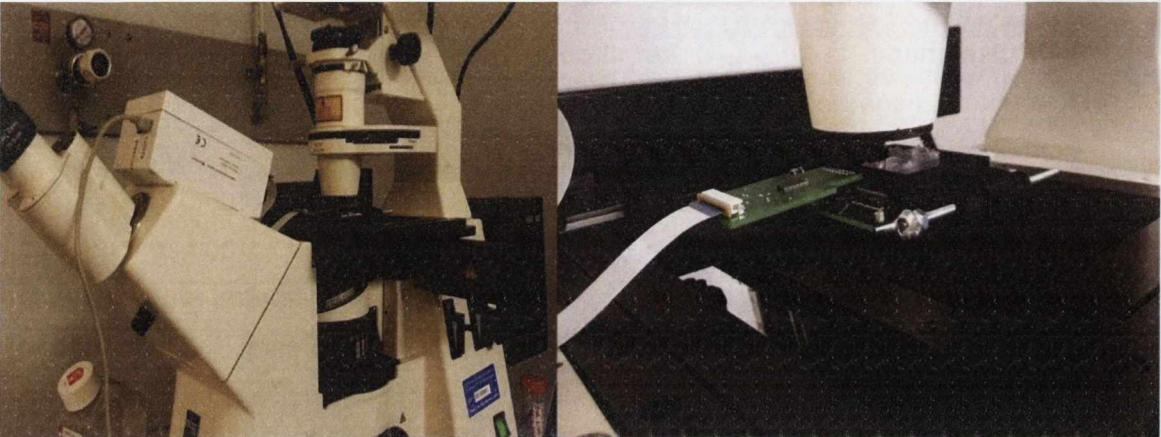


Figure 3.12 | Experimental setup for MEA biosensor response calibration.

4 Results

4.1 MEA Biosensor Development

4.1.1 Introduction

The current state-of-the-art for MEA experiments typically comprise the planar device, electronic circuitry for signal transduction/amplification, and a computer for signal processing software and data logging. The most common substrate of choice for the MEA platform is glass, with microelectrodes generally made from gold and titanium to lower impedance and interconnects made of indium-tin oxide (ITO) which is transparent and allows the direct observation of neuronal cells with an inverted microscope. A layer of insulating material is required to form a barrier between the connection traces and the culture medium and can also serve as the culture substrate (Morin *et al.*, 2005). These characteristics require the insulating material to be both stable and biocompatible, in particular when considering the capacity for long-term studies. The negative photoresist SU-8 is commonly used as insulating material, preferentially treated with poly-L-lysine to promote cell adhesion and obtain healthy, long-term neuronal cultures (James *et al.*, 2000). Typically, MEA dimensions range from 10 μm to 100 μm , arranged in a grid that covers about 0.2 – 2 mm^2 , and a single electrode may record signals singularly or from several neurons. As such, these systems offer flexibility for neuronal culture preparation and experimental design.

Developing devices based on electrochemical detection of activity has become a popular method due to its simplicity, ease of use and the huge potential as a label-free biosensor with high sensitivity. Several amperometric or electrochemical impedance techniques have been tried to detect cell viability and cellular functions by discovering the electrochemical dynamics at cell-electrode interface. A living cell can be described as an electrochemical dynamic system, due to various reduction-oxidation (redox) reactions and changes of ionic composition and concentration in biological processes. Cellular activities are accompanied with electron generation and charge transfer, which can be exploited using electrochemical methods to reveal information about changes in cell function as well as cell growth and development. In this case, cell biochemical parameters, such as concentrations of inorganic ions (H^+ , K^+ , Na^+ , Ca^{2+} , Cl^- , etc.), morphological change, membrane potentials and redox potentials, can be detected electrochemically. These methods have been shown to be suitable for specific materials which have well-known redox properties with fast electron transfer characteristics. Electrochemical detection utilising cell-based biosensors can be developed for cell analysis and evaluation through measurement of electrochemical signals such as current, potential, impedance, conductivity, capacitance, etc., and have become a focus of research in the

biosensor field. In recent years, with the development of nanotechnology and enhancement of interface between sensors and cells, a series of electrochemical cell-based biosensors are increasingly used to study cell type, concentration, viability, proliferation, apoptosis and molecule distribution inside and outside cells.

Feature size has become an important characteristic for developing neural interfaces that more accurately mimic *in vivo* conditions. For network modelling applications, nanostructured scaffolds have been used to increase cell-material interactions providing a significant advantage for the study of basic neurobiology and the development of neural biosensors. In particular, nanoscale grooves developed through photolithographic techniques have been successful in enhancing neuronal interaction with array substrates. Photolithography produces nanoscale and spatially defined topographical patterns on array surfaces, scaffolding neurite outgrowth for the investigation of *in vitro* neuronal networks and identifying developmental mechanisms associated with functional microcircuit physiology (Seidlits *et al.*, 2008). The fabrication of barriers, growth lanes, and pinning structures comprised of cross-linked proteins fabricated under conditions that do not compromise the viability of neurons has been shown to restrict neurite outgrowth within confined structures (Hill *et al.*, 2005; Kaehr *et al.*, 2004). The design of cell-based biosensors which employ immobilized living cells as sensing elements, can elicit specific reactions between stimulus and cells to reveal the intracellular and extracellular microenvironment condition, which can be applied in drug screening and even environmental monitoring.

Presented is the development of a biosensor device designed combining planar microelectrode array technology and microwell scaffolds for the study of synapse dynamics and function within cultured neuronal networks. This approach aims to support *in vitro* modelling of neural circuit physiology and allow for the analysis of molecular and cellular mechanisms involved in neuronal network regulation and functional CNS development.

4.1.2 Results

4.1.2.1 MEA Biosensor Fabrication

Optimised planar microelectrode arrays were developed using thin-film polymer deposition techniques, involving S1813 positive photoresist, titanium-gold sputtering and SU-8 negative photoresist. Biosensor devices were configured into 20 μm tracks extending from one array of 4x8 rectangle shaped electrode sites (40x40 μm each) and connected to 32 bonding pads (500x1000 μm each) as shown in Figure 4.1.

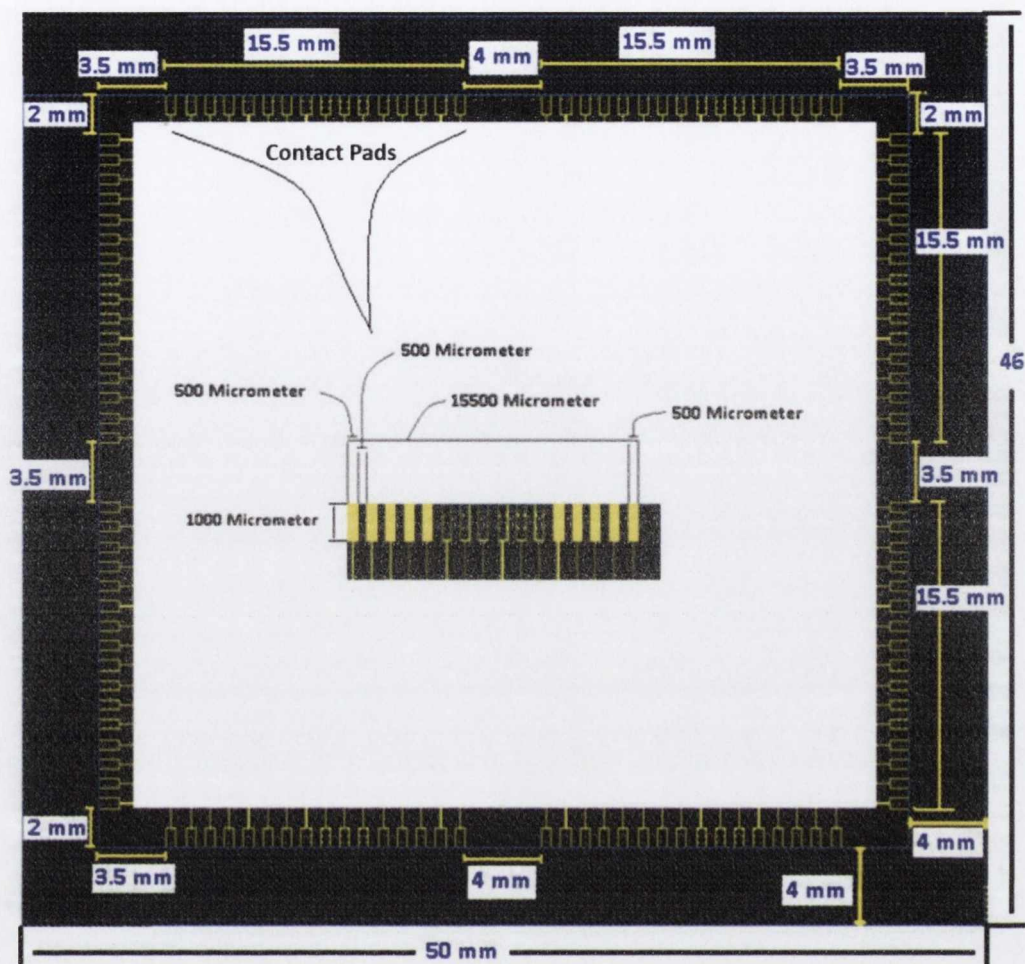


Figure 4.1 | Configuration of Array Contact Pads. Outline of the connection pads of the mask with associated ruled parameters. Surface size of glass substrate (50mm x 46mm) shaded in dark blue around array contact pads.

There were three major requirements for optimisation of the microelectrode array fabrication. Firstly, the gold electrodes fabricated needed to be relatively equal in terms of height and width across the glass substrate surface. Secondly, the width and gap between the contact pads, traces and microelectrodes needed to be equal. Finally, the insulating SU-8 layer needed to adhere well on the substrate and gold electrodes so that creeping of electrolyte under the layer and onto the gold traces could be eliminated.

Process settings outlined in the Materials & Methods resulted in 1 micron line resolution of the S1813 photoresist. This was used to define bonding pads, tracks, reference electrode and electrode sites of the array followed by thermal sputtering of a 20 nm titanium seed layer and 100 nm gold layer to create microelectrodes on the glass substrate (Figure 4.2a-b). One key feature to avoid was the presence of “lift-off” ears, rough edges arising at the edges of the gold patterns, which is particularly important when a thin film layer has to be deposited on top of the microelectrodes. This was achieved by optimisation of the lithography process in terms of exposure time and exposure mode (Appendix 8.1).

In order to monitor electrochemical reactions only at the site of the microelectrodes and not the connecting traces, it was necessary to insulate the microelectrodes from each other, leaving openings only at the active electrode surfaces. SU-8 negative photoresist was developed onto the substrate as an insulating polymer in addition to creating a neuronal interface layer designed into microwell and microtrench networks (Figure 4.2c). Each microwell was 20 μm in diameter and connected to its neighbouring wells via 5 μm wide and 100 μm long microtrenches to accommodate individual neuron cell bodies of same or smaller size (Figure 4.2d). The 4 x 8 microwell pattern was positioned above each electrode site of the array to support direct neuronal network recording and stimulation (Figure 4.2e-f). Measurements of optimised biosensors were carried out in triplicate using AFM and profilometry and are summarised in Table 4.1.

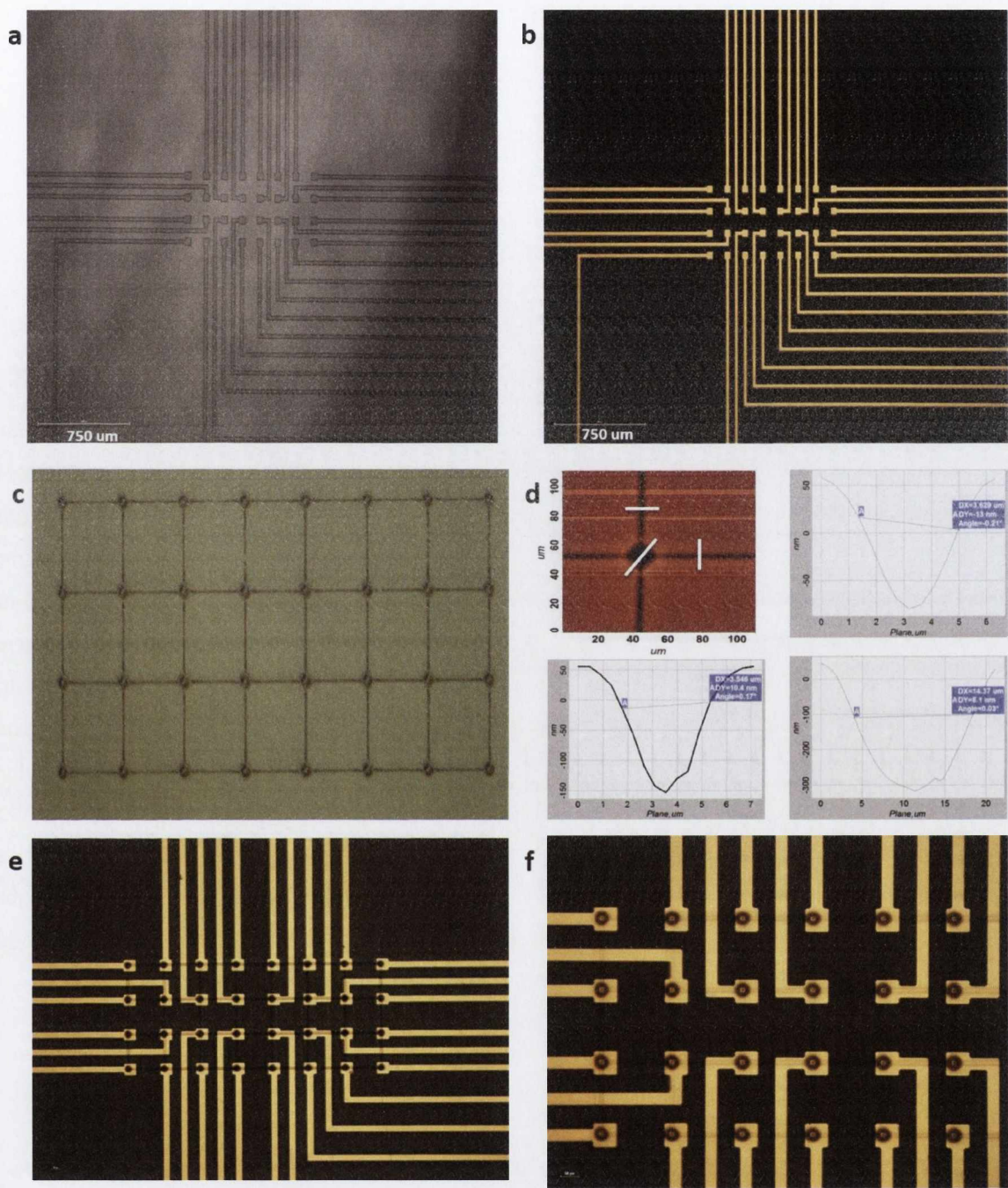


Figure 4.2 | Photoresist development, Microelectrode Deposition & SU-8 patterning of MEA. a) Post-development S1813 photoresist pattern for single 4x8 electrode site. b) Post lift-off Ti-Au metal deposition onto photoresist pattern. c) Post-development SU-8 photoresist pattern for neuronal interface layer. d) AFM profile measurements of SU-8 microtrenches and microwell. e) Stacked layers of combined SU-8 and microelectrode site. f) Magnified image focusing on microwell positioned over each microelectrode to promote preferential cell positioning for stimulation and resistance measurements.

Table 4.1 | Measurements of optimised biosensors by AFM and profilometry.

	AFM	Profilometry
Microtrench Length	99.7 ± 2.1 µm	99.3 ± 2.5 µm
Microtrench Width	5.4 ± 0.4 µm	5.7 ± 0.3 µm
Microtrench Depth	149.8 ± 0.3 nm	150.6 ± 3.8 nm
Microwell Diameter	19.6 ± 1.5 µm	20.1 ± 1.2 µm
Microwell Depth	300.3 ± 0.4 nm	300.3 ± 3.2 nm

4.1.2.2 Electrochemical Characterisation of MEA Biosensor

Cyclic voltammetry was carried out on the biosensor device microelectrodes using the 4200-SCS Parameter Analyser to measure microelectrode function in deionised water, PBS and DMEM media. This is a standardised method for characterising the behaviour and stability of fabricated microelectrodes by studying current flow associated with electron transfer kinetics caused by potential differences between the electrode and the solution. Characterisation involved a voltage sweep from a forward sweep of -5 V to +5 V and back to -5 V over 5 mV intervals. Measurements were taken from contact pads corresponding to adjacent microelectrodes in the array. Initial measurements demonstrated a reversible electrochemical reaction between the microelectrode and deionised water (Figure 4.3). Similar microelectrode measurements were carried out in saline (PBS) and biological medium (DMEM). The resulting voltammogram for PBS (Figure 4.4) and DMEM (Figure 4.5) demonstrated identical electrochemical behaviour at potential differences applied between -5 V and +5 V. These results indicate microelectrodes are stable and have a well-defined performance profile, although given the variability that can arise during fabrication steps, performance tests need to be carried out for each fabricated biosensor.

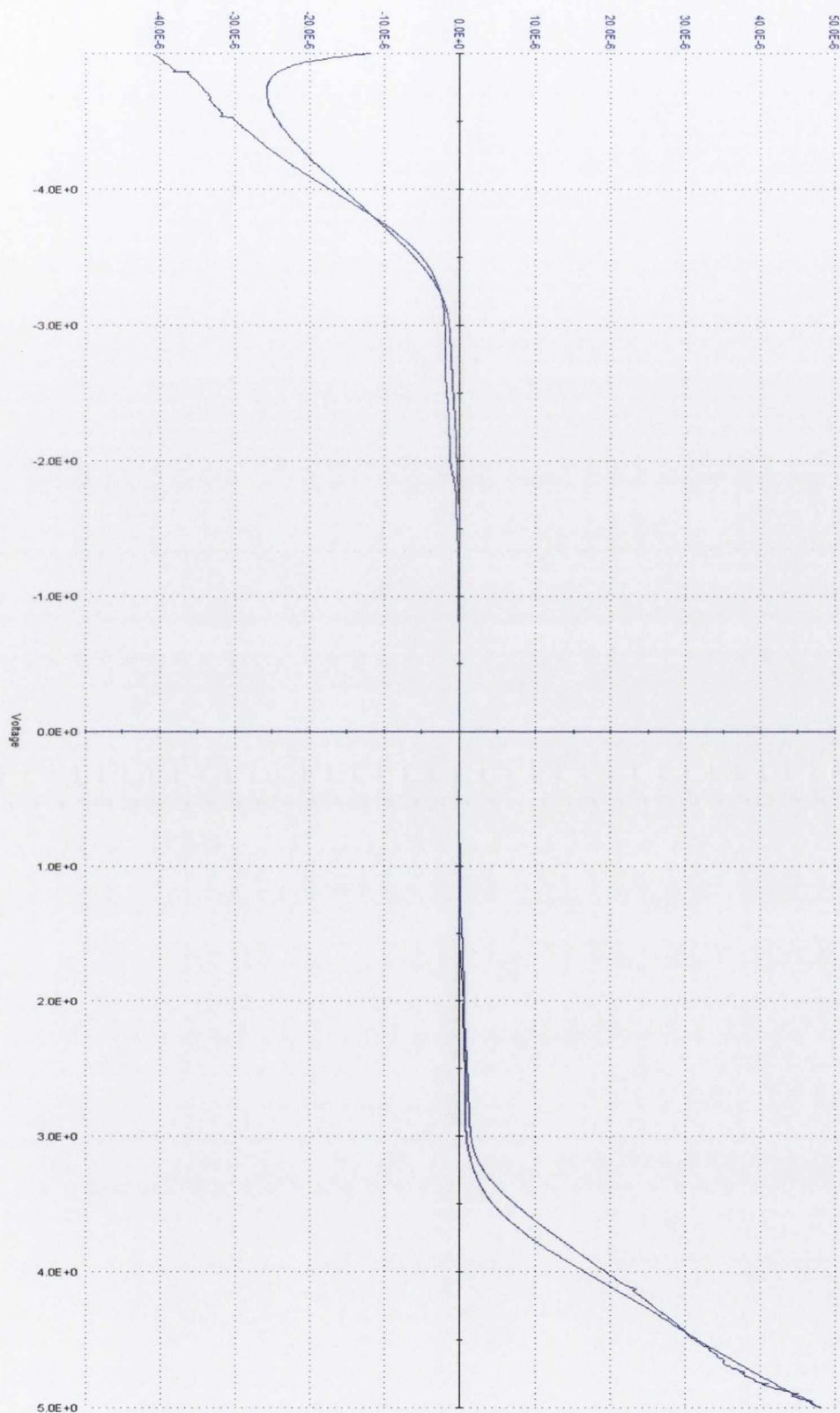


Figure 4.3 | Electrochemical characterisation of microelectrodes in deionised water. Schematic of measurements taken from contact pads corresponding to adjacent microelectrodes. Voltammogram of cyclic sweep of microelectrodes in deionised (Di) water between -5 V and +5 V.

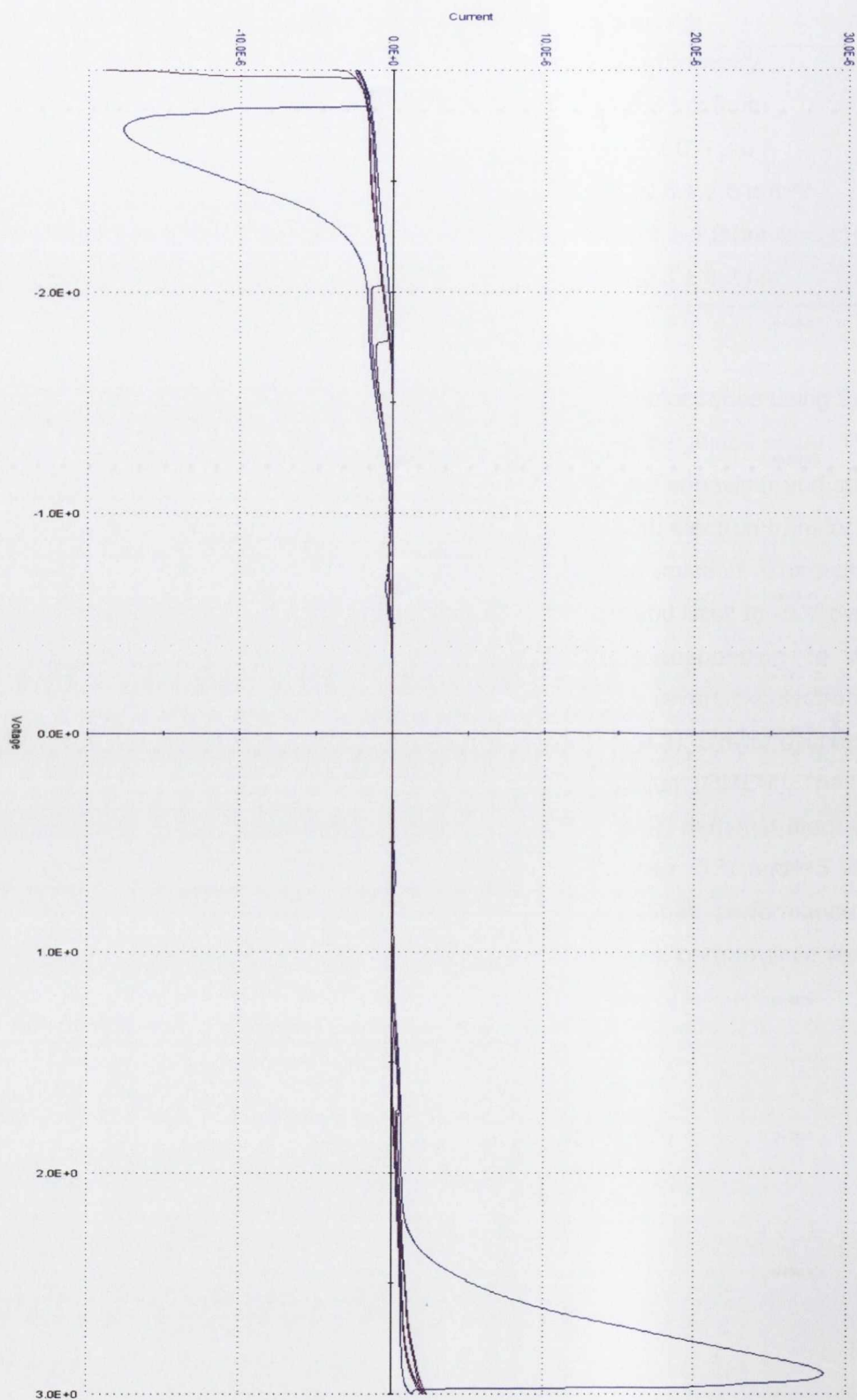


Figure 4.4 | Electrochemical characterisation of microelectrodes in PBS. Schematic of measurements taken from contact pads corresponding to adjacent microelectrodes. Voltammogram of cyclic sweep of microelectrodes in PBS between -5 V and +5 V.

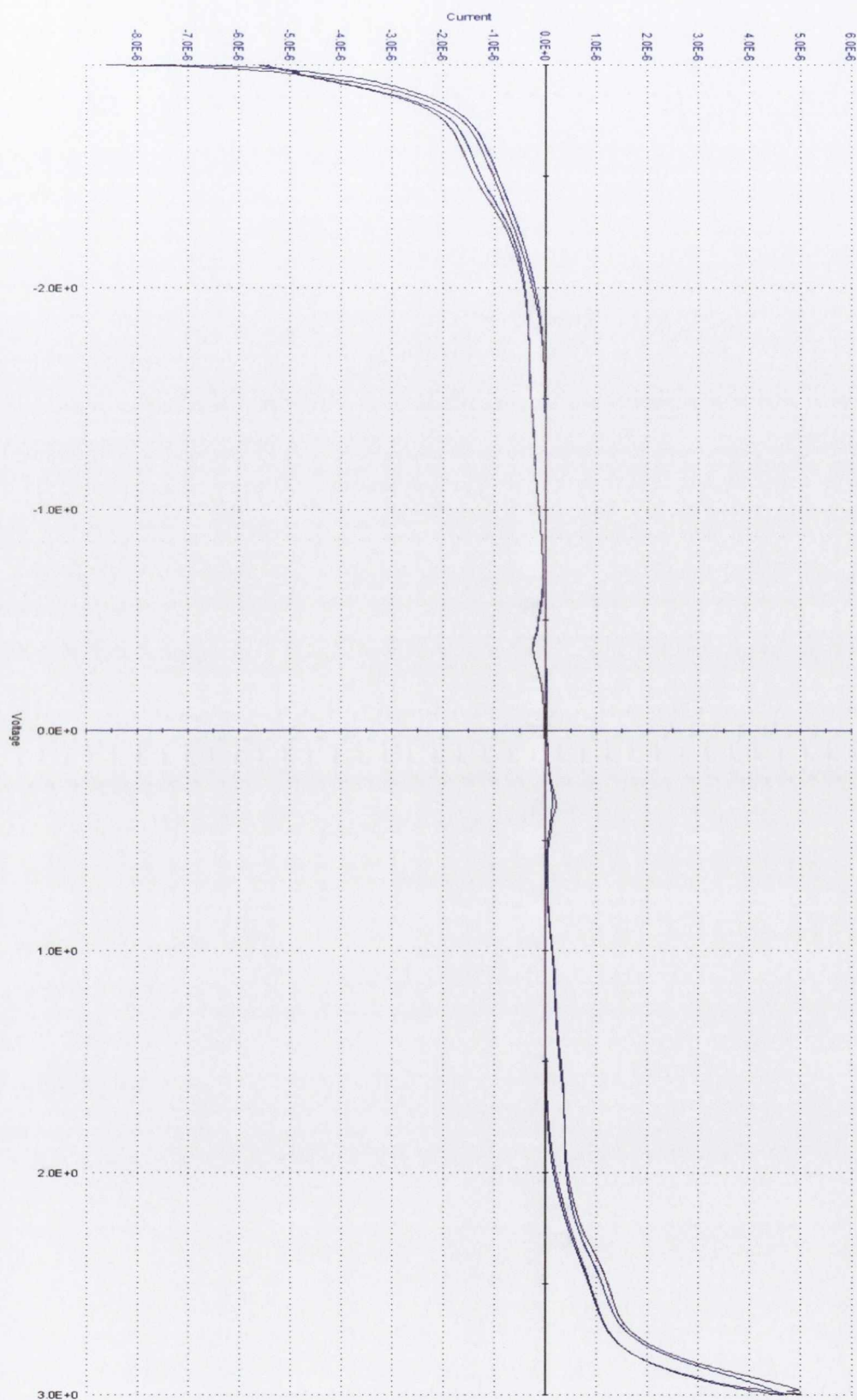


Figure 4.5 | Electrochemical characterisation of microelectrodes in DMEM. Schematic of measurements taken from contact pads corresponding to adjacent microelectrodes. Voltammogram of cyclic sweep of microelectrodes in DMEM between -5 V and +5 V.

Electrode impedance (Z) is an important parameter for MEA detection of neural activity. This is critical for understanding parameters that are necessary to stimulate cells and study behavioural profiles caused by structural and functional modifications within a neuronal network. MEA functional response was additionally examined by impedance measurements of individual microelectrodes, which were carried out by our collaborators in Grenoble, France (IVEN Technology). Measurements were performed between two adjacent pads on the array using an AD5933 impedance measurement circuit. 2 volts, peak-to-peak was applied at a sweeping frequency from 10 KHz to 110 KHz by a step of 1 KHz (Figure 4.6). The system was calibrated to a reference of 1 MOhms pure resistor equivalent to the reference impedance, against no impedance when the circuit is open and when the PCB (printed circuit board) is connected but without contact with the chip. The resulting tests between two adjacent pads when the PCB was in contact with the chip and when 100 μ l of H₂O is placed on the chip illustrates a response curve synonymous with expected change in equivalent series resistance (ESR). The test with the chip shows an initial high impedance which decreases as the frequency increases. At 10 KHz impedance was found to be 0.5 M Ω which is an expected result for gold electrodes that have not been platinised. The introduction of 100 μ l H₂O exponentially reduced impedance values.

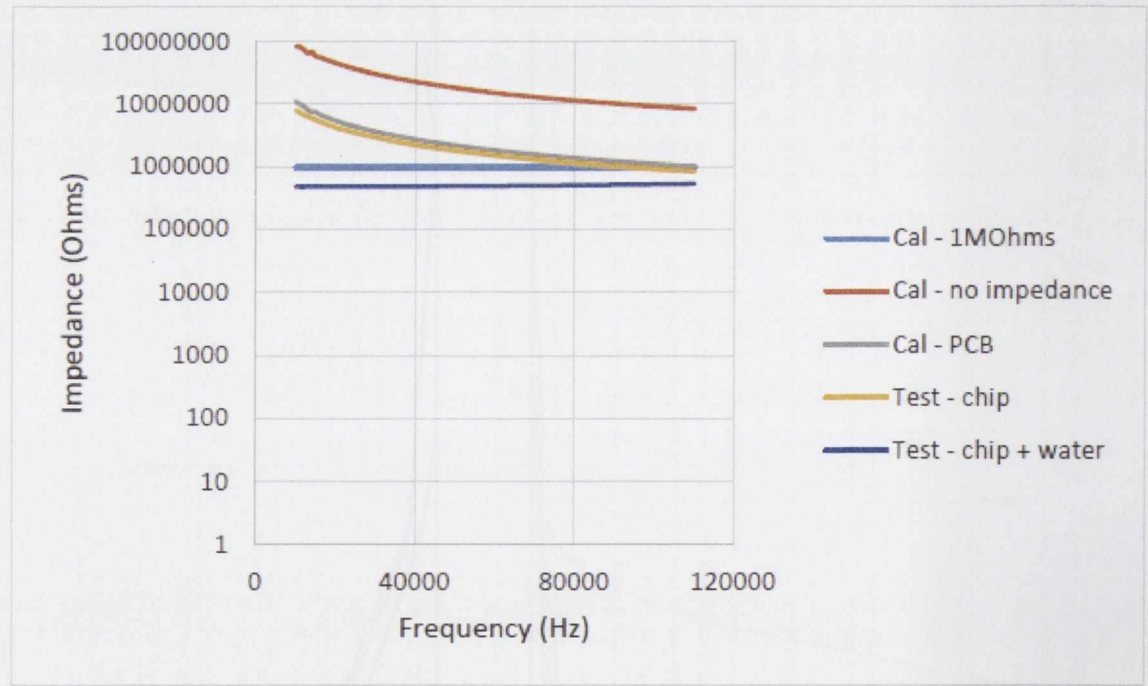


Figure 4.6 | Impedance measurement of microelectrodes. The system was calibrated to a reference of 1 MOhms pure resistor equivalent to the reference impedance, against no impedance when the circuit is open and when the PCB is connected but without contact with the chip. Impedance values were tested for PCB contact with the chip and a connection between two adjacent pads against a drop of water at the microelectrode site. Analysis carried out by IVEN Technology.

4.1.3 Discussion

Current commercial MEA models are designed to investigate the electrophysiological behaviour of high-density cultures randomly dispersed on MEAs and the examination of population attributes such as bursting frequency and bursting duration. While a lot of useful information can be gained, they are not amenable to testing conceptual frameworks of synapse dynamics and function within cultured neuronal networks. Techniques involving the integration of three-dimensional microstructures, created in an additional SU-8 layer, composed of microwells and microtrenches have been developed successfully (Jaber *et al.*, 2009). The implementation of these techniques to the study of neuronal development allows for a toolset that can potentially be used for investigating the principles of physical and functional connectivity within neuronal networks.

Presented is the fabrication process for developing a microelectrode array (MEA)-based biosensor for modelling and studying neuronal networks. Using combined physical and chemical thin film deposition techniques, reproducible devices composed of borosilicate glass, titanium-gold electrodes and SU-8 have been fabricated, as analysed by AFM and profilometry. These devices have undergone electrochemical analysis of microelectrode function, through cyclic voltammetry and impedance measurements. Cyclic voltammetry measurements have indicated microelectrode stability across multiple media and a reproducible behavioural profile.

However, there are limitations to the device in its current state. The high impedance of the electrode sites is expected and presents drawbacks for the induction of stimulatory protocols within a culture. Smooth microelectrodes of small geometric dimensions suffer from high electrode impedance, leading to high levels of thermal noise in extracellular recordings and limited charge injection for efficient stimulation of tissue. Nanostructuring of the electrode sites on MEAs presents an efficient way to overcome these problems by decreasing the impedance of the electrode/solution interface. Different techniques can be used to achieve this goal, including template assisted electrodeposition for generating macro- and mesoporous films, immobilization of carbon nanotubes (CNTs) and deposition of conducting polymers onto microelectrodes. When tested during *in vitro* and *in vivo* measurements, nanostructured MEAs display improved sensitivity during recording of neuronal activity together with a higher efficiency in the stimulation process compared to conventional microelectrodes (Heim *et al.*, 2012). These features were absent from our MEAs due to lack of necessary resources; however, it is intended to include them in future work.

4.2 MEA Biocompatibility Assessment

4.2.1 Introduction

Replicating functional cellular development is a pivotal element to *in vitro* modelling of morphological and physiological features of living tissue. Microdevices for cell-based assays are required to mirror desired physiological processes and allow functional development of cellular interactions. This is a major necessity for developing models that more accurately mimic the *in vivo* conditions of nervous system development and elucidating the pathophysiology of conditions that impact neural circuit physiology. For network modelling applications, thin-film polymers have been used to increase cell-device interactions providing a significant advantage for the study of basic neurobiology and the development of neural biosensors.

Several polymers have been evaluated for their effectiveness as interfaces that promote cell-cell and cell-material interaction. SU-8 polymer has been extensively studied for its biocompatibility as a neural interface owing to its mechanical strength and biostability. It is a high tensile strength polymer that has uses in a number of biomedical applications that include cell encapsulation and neuronal probes. Commercial SU-8 formulations comprise a monomer, containing 8 epoxy moieties, a solvent such as cyclopentanone, and a photoacid initiator such as triarylsulfonium hexafluoroantimonate. UV irradiation causes protonation of the epoxy moieties which react with neutral epoxy groups when heated, resulting in a cross-linked polymer network of high mechanical strength and thermal stability (Nemani *et al.*, 2013). Several studies have evaluated the biocompatibility of SU-8 surfaces using *in vitro* cell culture models (Gabi *et al.*, 2010; Kotzar *et al.*, 2002) and SU-8 has been used in the fabrication of biosensors for measuring electrical impedance in living tissues (Tijero *et al.*, 2009) and monitoring neuronal circuit physiology (Jones *et al.*, 2011; Scott *et al.*, 2012). The use of SU-8 based microwells integrated with human neuroblastoma cells for lab-on-a-chip applications is also well established (Nemani *et al.*, 2013). The evaluation of SU-8 as a potential neural probe material has been accomplished based on cytotoxicity assays using C6 rat astrocytes, whereby the mechanical strength, biocompatibility and biostability of SU-8 have been shown to be favourable for the fabrication of long-term implants such as neuronal probes (Ereifej *et al.*, 2011). Some studies have evaluated the effect of SU-8 based implantable neural probes using cells such as human skin fibroblasts, Schwann cells, and explanted neurons from dorsal root ganglia (Lee *et al.*, 2014).

While these studies have demonstrated SU-8 biocompatibility and indicated its usability as a substrate for neuronal interaction, there have been very few reports on the characteristic differences between cells cultured on SU-8 when compared to glass. However, this is of a

particular interest as the physical properties of thin film polymers are important features influencing the functionality of the polymer surface as potential interfaces for neural devices (Walther *et al.*, 2008). To assess this, we studied morphological and functional variations between SH-SY5Y human neuroblastoma cells cultured over SU-8 surfaces compared to control surfaces composed of borosilicate glass.

4.2.2 Results

4.2.2.1 Effect of Neural Interface on SH-SY5Y Cell Viability and Proliferation

Comparison of survival and proliferation rates of SH-SY5Y cells grown on SU-8-spun substrates was determined by flow cytometry and absorbance spectroscopy respectively and compared to glass controls. Figure 4.7 compares cell viability at 48 and 72 hours respectively. Growth of SH-SY5Y on SU-8-spun substrates indicated no significant changes to cell viability when compared to glass controls at 48 hours ($P > 0.05$; $n = 6$). In contrast, growth of SH-SY5Y on SU-8-spun substrates showed significantly increased viability at 72 hours when compared to glass controls. On SU-8-spun substrates, cell viability was found to be increased by $41\% \pm 6\%$ compared to glass controls ($P < 0.001$; $n = 6$). Additionally, the effect of SU-8-spun substrates on SH-SY5Y cell proliferation was analysed using absorbance spectroscopy and compared to glass controls. Once again, analysis was carried out at 48 and 72 hours respectively. In this case, SU-8 had no effect on SH-SY5Y cell proliferation compared to glass controls at each time-point measured. Taken together these results indicate that SH-SY5Y cells are more viable on SU-8-spun substrates with no demonstrable effect on cell proliferation (data not shown).

Flow cytometry analysis of SH-SY5Y cell viability on glass and SU-8 substrates

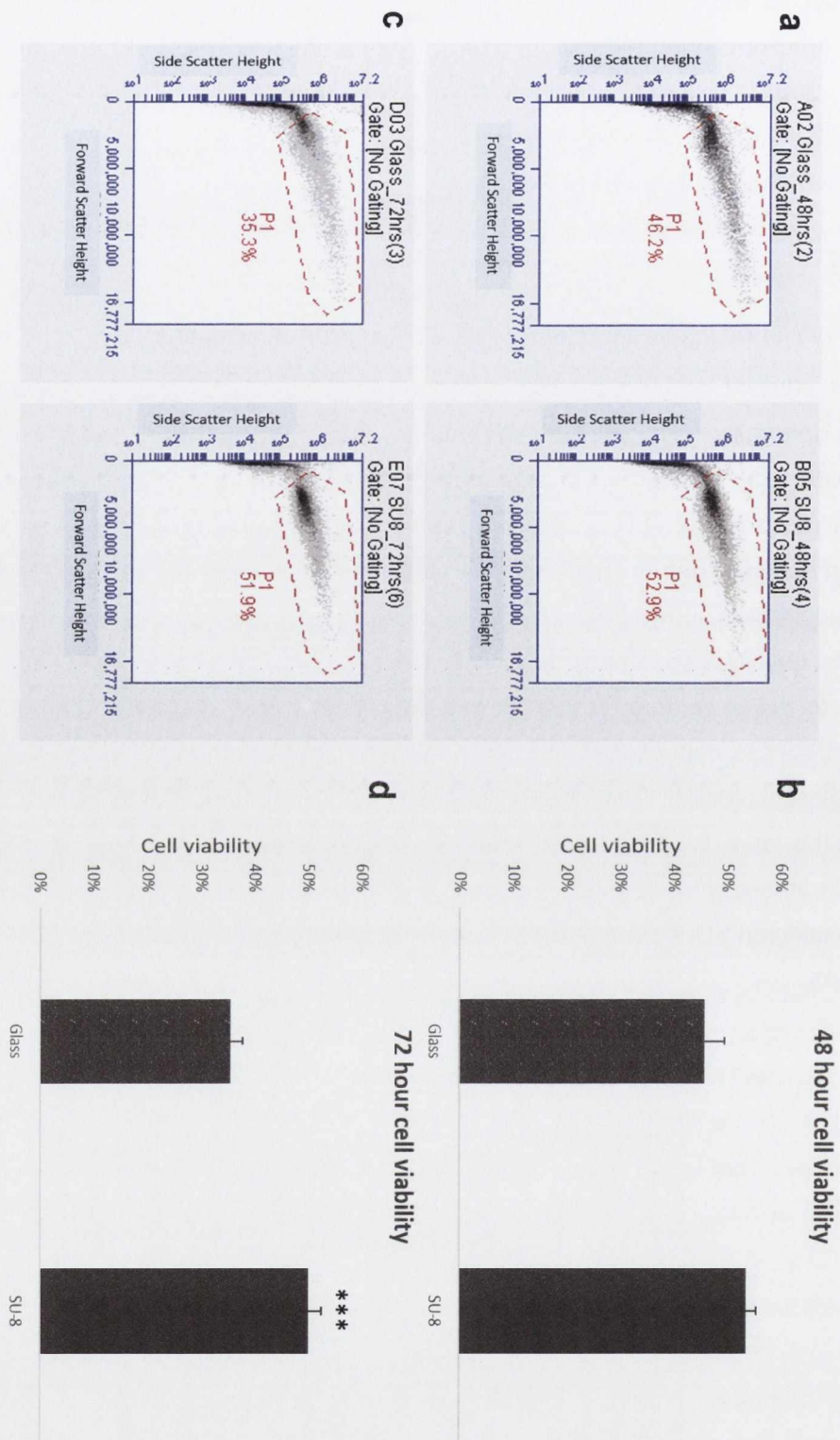


Figure 4.7 | Comparison of SH-SY5Y cell viability on glass and SU-8 at 48 and 72 hours. a) Flow cytometry analysis of SH-SY5Y cells on glass and SU-8-spun substrates at 48 hours. b) Cells grown on SU-8 show no significant changes to viability at 48 hours when compared to glass controls ($P>0.05$; $n = 6$). c) Flow cytometry analysis of SH-SY5Y cell on glass and SU-8-spun substrates at 72 hours. d) Cells grown on SU-8 substrates show $41\% \pm 6\%$ increase in viability at 72 hours compared to glass controls ($P<0.001$; $n = 6$).

4.2.2.2 *Effect of Neural Interface on SH-SY5Y TNF- α Expression*

ELISA-based analysis was carried out to determine the effect of SU-8-spun substrates on TNF- α expression. TNF- α is involved in many biological responses including apoptosis, cellular differentiation and activation of cellular pathways through NF- κ B. Figure 4.8 shows that cells grown over SU-8-spun substrates had a statistically significant decrease in average expression levels of TNF- α compared to glass ($34.30 \text{ pg/ml} \pm 0.05$ vs. $63.00 \text{ pg/ml} \pm 0.07$; $p < 0.01$; $n = 3$) and retinoic acid-differentiated cells, a positive control of TNF- α expression ($34.30 \text{ pg/ml} \pm 0.05$ vs. 127.70 ± 0.15 ; $p < 0.01$; $n = 3$). This result suggests that the culture of SH-SY5Y cells on SU-8 induces less toxicity than on glass.

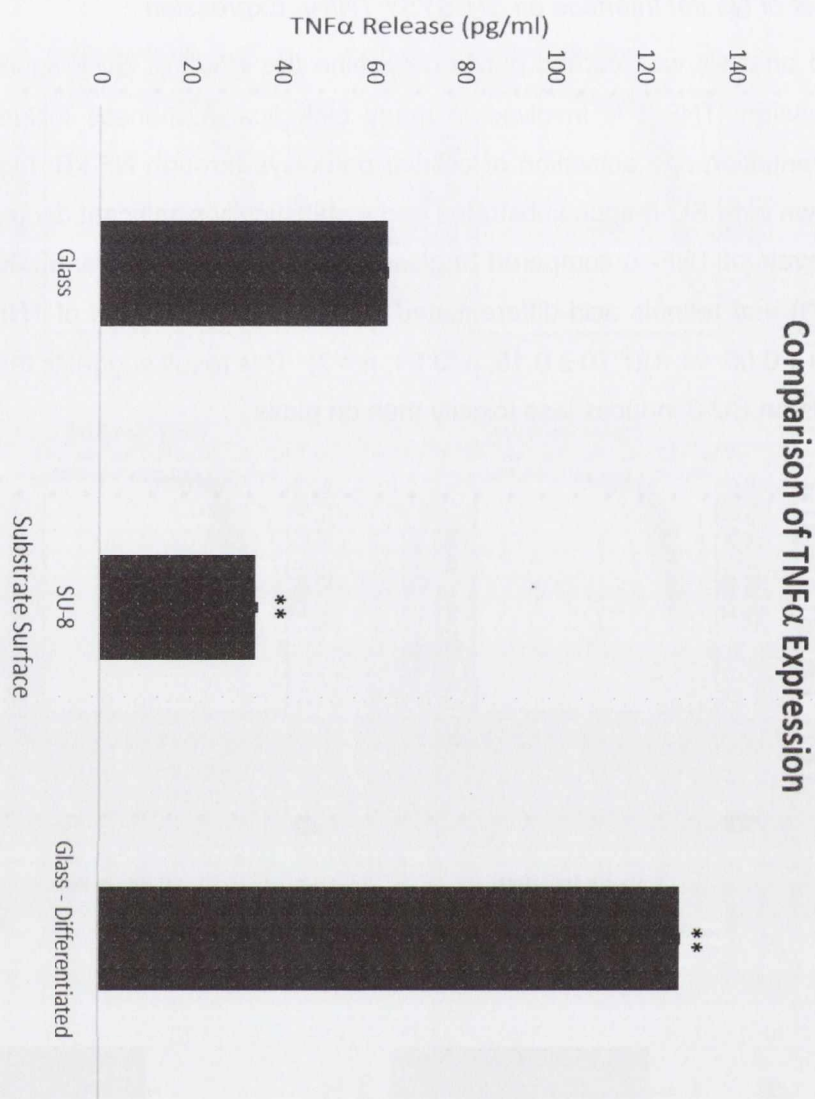


Figure 4.8 | Comparison of TNF- α expression on glass, SU-8 and RA-differentiated SH-SY5Y cells. SH-SY5Y cells grown over SU-8-spun substrates show a statistically significant decrease in average expression levels of TNF- α compared to glass (34.30 pg/ml $\pm$ 0.05 vs. 63.00 pg/ml $\pm$ 0.07; $p < 0.01$; $n = 3$) and retinoic acid-differentiated cells, a positive control of TNF- α expression ((34.30 pg/ml $\pm$ 0.05 vs. 127.70 $\pm$ 0.15; $p < 0.01$; $n = 3$).

4.2.2.3 Effect of Neural Interface on SH-SY5Y Cell Morphology and Function

Following observation of SU-8 exhibiting a significantly reduced toxicity profile at a biochemical level, AFM imaging of SH-SY5Y human neuroblastoma cells grown on glass and SU-8-spun substrates was carried out to visualise cell attachment to each surface. Qualitative assessment of images show comparable cell attachment between SH-SY5Y grown on SU-8 polymer comparable to glass and suggest an increase in affinity for SU-8 over glass as indicated by increased cell spreading (Figure 4.9).

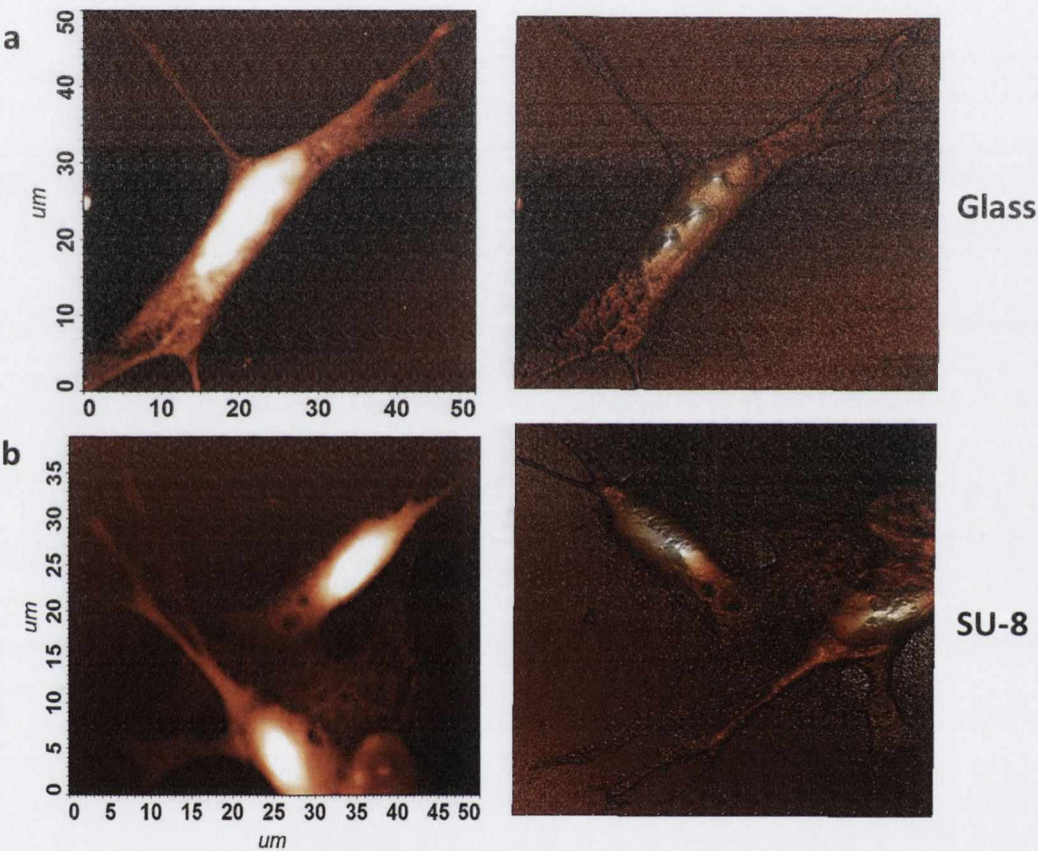


Figure 4.9 | AFM images of SH-SY5Y human neuroblastoma cells grown on glass and SU-8. a) Planar and 3D image of SH-SY5Y cell growth on glass. b) Planar and 3D image of SH-SY5Y cell growth on SU-8. Images courtesy of Dr. Namrata Jain.

In order to characterise the effect of the SU-8 surface polymer on SH-SY5Y cell growth compared to borosilicate glass controls in a quantitative manner, the expression of specific markers of neuronal morphology and function was investigated. To identify functional neuronal morphology, cells were stained for MAP2, a marker for neuronal differentiation and maturation as well as the Hoechst nucleus stain (Figure 4.10). Immunofluorescence images of MAP2 were acquired for SH-SY5Y cell growth on glass and SU-8-spun substrates and parameters for the area of the nucleus and average length of neurites were measured. Measurements began with a comparison of the average area of the nuclei between glass and SU-8 surfaces. Figure 4.10b shows that cells grown over SU-8-spun substrates had a statistically significant increase in average nucleus area, compared to glass controls ($28.09 \mu\text{m}^2 \pm 0.83$ vs. $20.62 \mu\text{m}^2 \pm 0.62$; $P < 0.001$; $n = 3$). Additionally, Figure 4.10d shows that cells on SU-8-spun substrates also had a statistically significant increase in average neurite length compared to glass controls ($19.45 \mu\text{m} \pm 1.59$ vs. $12.19 \mu\text{m} \pm 0.83$; $P < 0.001$; $n = 3$).

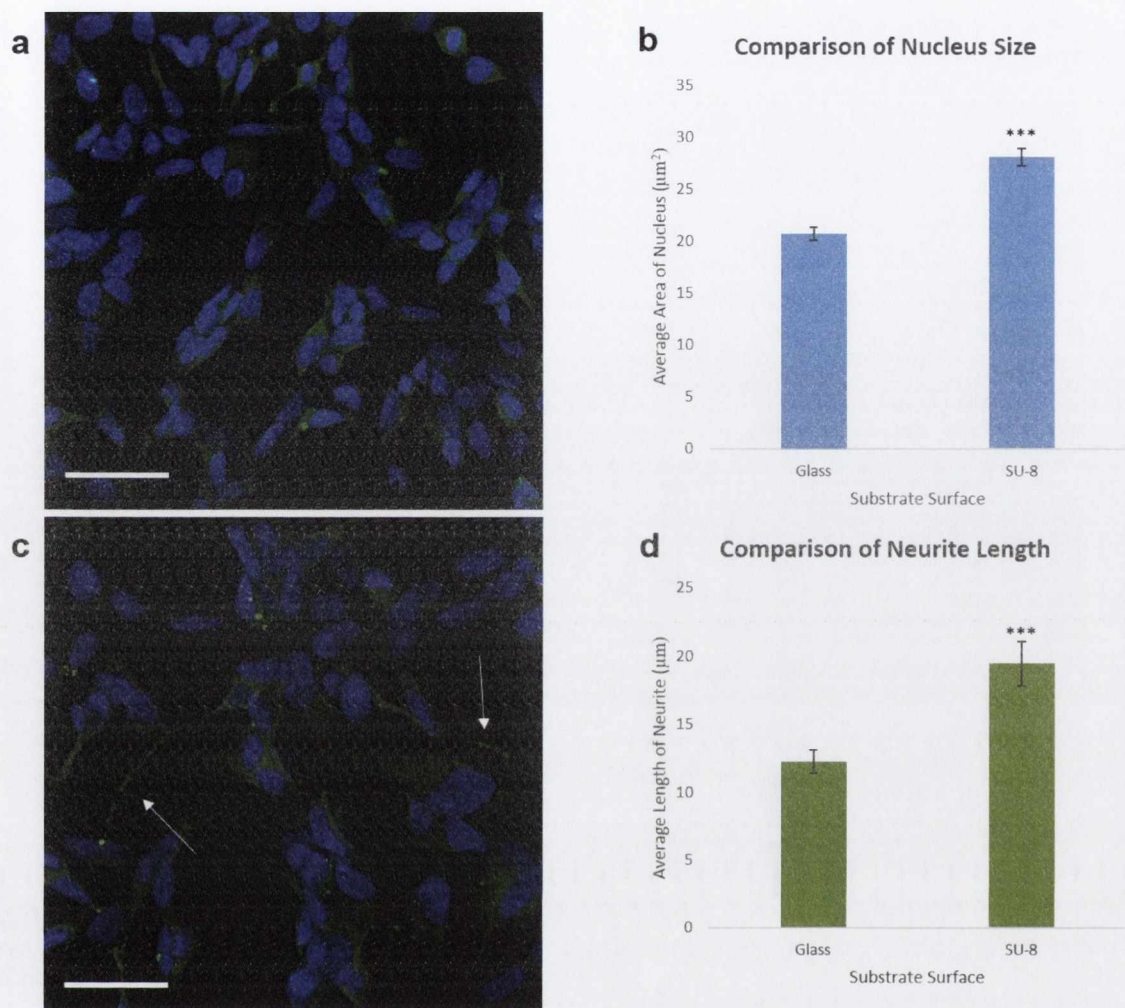


Figure 4.10 | Immunofluorescence analysis of SH-SY5Y nucleus size (Hoechst) and neurite length (MAP2) on glass and SU-8 surfaces. a) Representative confocal image of SH-SY5Y cells grown on glass surface. b) Graph comparing average nucleus size of cells grown over SU-8 surface and glass controls ($28.09 \mu\text{m}^2 \pm 0.83$ vs. $20.62 \mu\text{m}^2 \pm 0.62$; $P < 0.001$; $n = 3$). c) Representative confocal image of SH-SY5Y cells grown on glass SU-8 surface. d) Graph comparing average neurite length of cells grown over SU-8 surface and glass controls SU-8-spun substrates also had a statistically significant increase in average neurite length compared to glass controls ($19.45 \mu\text{m} \pm 1.59$ vs. $12.19 \mu\text{m} \pm 0.83$; $P < 0.001$; $n = 3$). Scale bar is $25 \mu\text{m}$.

Following morphological characterisation, the effect of SH-SY5Y cell growth on SU-8-spun substrates was determined by analysis of markers for synaptic function such as the presynaptic marker synapsin. Figure 4.11 shows that cells grown on SU-8 show a statistically significant three-fold increase in the level of synapsin expression when compared to glass controls ($p < 0.001$; $n = 3$) as measured by corrected total cell fluorescence (CTCF). Taken together, the data with suggest that SU-8-spun induces morphological and functional changes which may be indicative of a more neuron-like phenotype of SH-SY5Y cells.

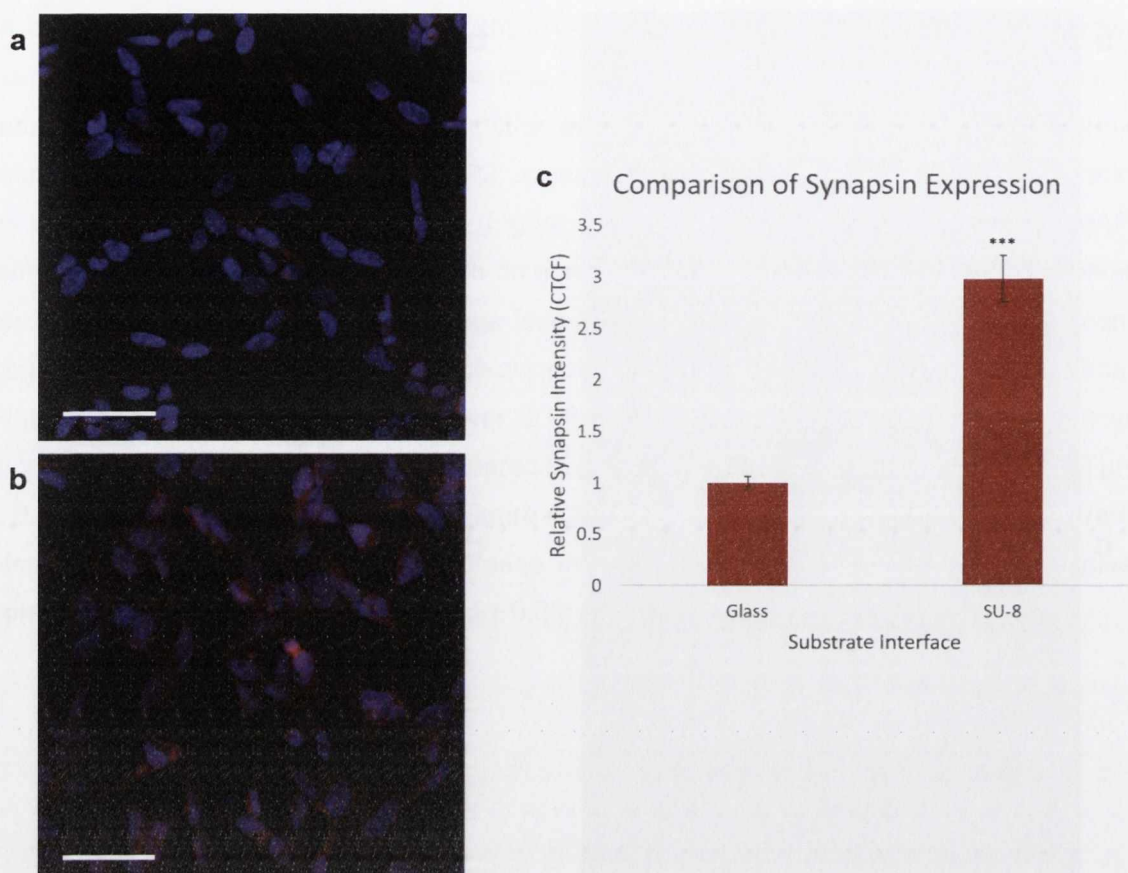


Figure 4.11 | Immunofluorescence analysis of SH-SY5Y synapsin expression on glass and SU-8 surfaces. a) Representative confocal image of SH-SY5Y cells grown on glass surface. b) Representative image of SH-SY5Y cells grown on SU-8 surface. c) Graph showing three-fold increase in relative expression levels of synapsin on SU-8 surface compared to glass controls ($p < 0.001$; $n = 3$). Scale bar is 25 μm .

4.2.2.4 Analysis of Physical Parameters of Neural Interface

Following on from results indicating that SU-8 may be preferentially suited to the growth and study of neuronal cultures, the physical parameters that may enable this phenotype were characterised. Figure 4.12a-b depicts AFM analyses of the RMS roughness value of peaks and valleys on the glass and SU-8 surfaces at multiple locations. Average roughness values were measured at ca 0.33 nm for SU-8 compared to ca. 1.27 nm for glass controls, indicating a decreased level of surface roughness at the nanoscale. Indentation tests were carried out to determine the surface hardness and Young's modulus of SU-8-spun substrates compared to glass controls. Measurements were repeated six times at different locations on each sample (Figure 4.12c). The average reduced modulus and hardness was 69.40 ± 3.50 GPa and 6.14 ± 0.47 GPa respectively compared to glass controls. By plotting the load on the sample against surface displacement, we see an exponential difference between SU-8 and glass stiffness characteristics (Figure 4.12d). Taken together, these results suggest SU-8 as a softer and smoother surface for cellular interaction, thus conferring a preferential interface for the growth of neuronal cultures.

Investigation of surface roughness and indentation on glass and SU-8 substrates

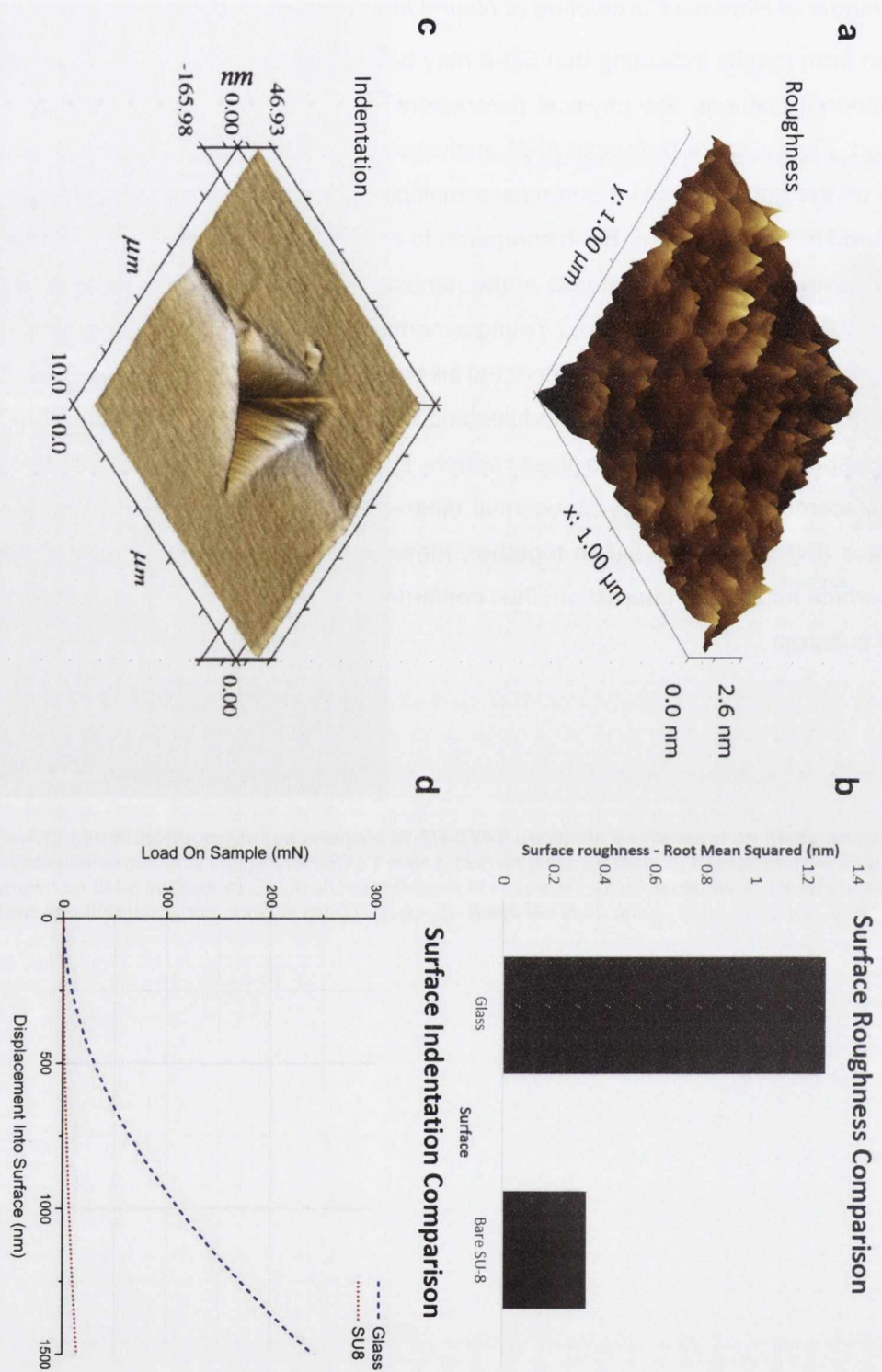


Figure 4.12 | Analysis of physical properties of SU-8 substrates compared to glass controls. a) Representative AFM image of surface roughness profile of SU-8 substrate. b) Graph comparing roughness root mean square (RMS) value of SU-8 surface and glass controls (0.25 nm vs. 1.00 nm respectively). c) Representative AFM image of nanoindentation profile of SU-8 substrate. d) Graph plotting load on sample against displacement into surface. Images courtesy of Owen Brazil.

4.2.3 Discussion

Replicating functional cellular development is a pivotal element for the *in vitro* modelling of morphological and physiological features of living tissue. Nano-engineered devices such as MEA biosensors are required to mirror desired physiological processes and to allow functional development of cellular interactions. This is a major requirement for models that are designed to more accurately mimic the *in vivo* conditions of nervous system development and to elucidate the pathophysiology of conditions that impact neural circuit function. For network modelling applications, thin film polymers have been used to increase cell-device interactions providing a significant advantage for the study of basic neurobiology and the development of neural biosensors (Ajetunmobi *et al.*, 2014). Different interfaces can exhibit surface properties that may negatively or positively impact on a culture environment and the use of SU-8 based microwells integrated with SH-SY5Y human neuroblastoma cells have implicated for biosensor applications (Nemani *et al.*, 2013). To assess the suitability of SU-8 as a neural interface, the morphological and functional variations between SH-SY5Y human neuroblastoma cells grown over SU-8 surfaces, compared to borosilicate glass control surfaces, was studied. The study began with an assessment of the viability of SH-SY5Y cells grown over 48 and 72 hours as a preliminary indication of neurotoxicity. The data showed no significant difference in cell viability at 48 hours, however at 72, a significant improvement in cell viability was observed with cells cultured on an SU-8 surface compared to glass. Notably, this improvement in cell viability was not associated with an increase in cell proliferation as has been reported previously (Hennemeyer *et al.*, 2008). To further investigate the potential neuroprotective effects of SU-8 for SH-SY5Y cell culture, we probed expression levels of $\text{TNF}\alpha$ as an indicator of apoptosis. It has been shown that $\text{TNF}\alpha$ induces apoptosis in SH-SY5Y cells (Pregi *et al.*, 2009). SH-SY5Y cells differentiated with retinoic acid (RA) were used as a positive control as RA is involved in the control of growth and death decisions in human neuroblastoma cell lines (Melino *et al.*, 1997) and is associated with production of high levels of $\text{TNF}\alpha$ expression (Encinas *et al.*, 2000). Results showed that culturing SH-SY5Y cells on SU-8 produced a significant decrease in $\text{TNF}\alpha$ expression compared to glass controls, indicating less induction of $\text{TNF}\alpha$ -mediated apoptosis and therefore less neurotoxicity. This result would seem to support the previous indication that growth of SH-SY5Y cells on SU-8 improves cell viability.

Following identification of the biochemical characteristics of SH-SY5Y growth on SU-8, the morphological and functional characteristics were next to be assessed. Hoechst and MAP2 staining was carried out to determine nucleus area and average length of neurite processes and our results showed an increase in both parameters. Intracellular architecture is commonly

studied in order to analyse the morphological health of different cell types, with analysis of SH-SY5Y in relation to nano-interfaces ranging from the localisation of Golgi apparatus and functional cytoskeletal orientation (Brunetti *et al.*, 2010) to more neuron-specific events like synaptic vesicular transport (Agholme *et al.*, 2010). Studies have also focused on distinct and specific patterns of nuclei orientation and distribution among different cell types. As such nucleus organisation has been indicated to be functionally linked to the regulation of transcription, and these differences are documented in neurons to include large nucleoli associated with high ribosome biogenesis, and specific differences in chromatin organisation such as lower distribution of histone H1 (Akhmanova *et al.*, 2000). This architecture is considered to be necessary for RNA synthesis and processing without affecting DNA replication and other mitotic effects of dividing cells and are likely to cause the observed larger size of healthier and more differentiated neuronal cells (Akhmanova *et al.*, 2000). This would support our hypothesis that SU-8 may be a superior interface for observing and studying neuronal characteristics such as those associated with synaptic function. This may be further validated by the fact that cells on the glass substrate showed high numbers of adherent cells in clusters. These clusters are known to occur, in addition to teardrop and round configuration observed in simultaneously occurring isolated cells, in cultures of undifferentiated SHSY5Y, but not in their differentiated counterparts (Kovalevich and Langford, 2013). These descriptions of cells clumped in close proximity match observed trends of undifferentiated cells cultured on glass substrates obtained in the study, which would be expected of cells undergoing mitosis. It is postulated that cell area can be affected by this clustering of cells and is reduced on cells grown on glass. Conversely, we observe less clustering of cells grown on SU-8 which would lead to an observed increase in cell area. As SU-8 has been shown to improve cell viability, this might be synonymous with an increased nucleus area when compared to the same cells grown on glass. Previous studies of cell viability in response to SU-8 provide evidence to support this possibility, such as the reduction of haemolytic activity to FDA-approved levels and a muted immune response observed in in vivo histocompatibility studies (Nemani *et al.*, 2013). Similar cell responses to nanostructuration have previously been described in a study which found that nuclei of induced pluripotent stem cells functionally aligned along nano/microstructure of the substrate, whereas they distributed randomly on flat surfaces. Additionally, the average length of neurite processes was also compared between glass and SU-8 substrates. Cells grown on SU-8 showed longer neurite length than cells seeded on glass and there is a wealth of recent investigation into the factors behind neurite elongation in SH-SY5Y cells. Typically, the markers commonly targeted in assays of neurite outgrowth are commonly the proteins upregulated during neurodevelopment. These include neurofilament proteins, neurotransmitter receptors (opioid, dopamine, muscarinic and

acetylcholine) and nerve growth factor receptors, along with upregulation of specific kinases such as p38, Focal Adhesion Kinase (FAK), Protein Kinase B (PKB or AKT) and extracellular signal-regulated kinases (ERKs) 1 and 2 (Ahmed *et al.*, 2014), and these processes have been confirmed in studies of retinoic acid induced differentiation of SH-SY5Y (Andres *et al.*, 2013). Many of the pathways of these components, notably FAK through focal adhesion signalling, are highly sensitive to substrate topography and cellular behaviour can be tuned through nanostructuration (Brunetti *et al.*, 2010). This observed increase in average length is a further indication of how cells grown on SU-8 exhibit improved morphological characteristics compared to glass controls. It is proposed that this observed increase in neurite length could be due to enhanced viability and subsequent adhesion, very possibly through focal adhesion-mediated signalling.

To assess the effect of SU-8 on SH-SY5Y neuronal function, the expression levels of synapsin were studied as it has been shown to influence the development of immature neurons, interacting with cytoskeletal and vesicular components and regulating synapse dynamics. It has also been demonstrated to be a reliable marker for the differentiation and maturation of neuronal cells. It has previously been whereby neurons cultured *in vitro* express synapsin at gradually higher levels as soon as neurites develop and depolarize and synaptic contacts are established. Synapsin can therefore be considered a functional marker of healthy neuronal growth and differentiation (Valtorta *et al.*, 2011). The intensity of synapsin within the cell body of the SHSY-5Y cells grown on both SU-8 and glass interfaces was measured using immunocytochemistry. Synapsin expression levels were shown to be increased on cells grown on an SU-8 interface compared to glass controls. Given that increased levels of synapsin are an indication of neuronal development, our results imply SH-SY5Y cell growth on SU-8 could be inducing a presynaptic phenotype as a result of increased adherence and viability on the surface. In neurons cultured *in vitro*, the expression of syn I and II has been consistently shown to gradually increase as soon as neurites develop and polarize and synaptic contacts are established (Camilli *et al.*, 1983). During development syn I undergoes changes in its intracellular localization and at early stages of differentiation, the protein is distributed in the cytoplasm. As soon as synaptic contacts are formed, syn I undergoes a rapid redistribution and accumulates at presynaptic contact sites (Fletcher *et al.*, 1991). Given the increase in synapsin expression on cells grown on SU-8 compared to glass, this suggests that these cells are in a more matured state, although the lack of specific accumulation in presynaptic contact sites would imply that they have not yet undergone a differentiated state. Nonetheless, the results support the morphological studies and provide a functional explanation to the increase in average neurite length observed with cells grown on SU-8 compared to glass. The increase in syn I/II expression is consistent with neurite elongation and early stage synapse formation

during neuronal development (Valtorta *et al.*, 2011) and this phenomenon is seen to be mirrored in the results obtained.

The identification of a correlation between cell viability, adherence and the morphological and functional phenotypes of neurodevelopment led to the investigation of physical properties that could account for the differences between SU-8 and glass interfaces. In particular, surface roughness, hardness and elastic modulus. It has been observed that micrometre-scale roughness may affect cell proliferation and morphology (Karuri *et al.*, 2004) and recent studies have shown SH-SY5Y cells specifically respond to roughness with nanometre sensitivity (Brunetti *et al.*, 2010). This is thought to be because the focal adhesion sites of the cell are in the range of 5 – 200 nm and these very small cellular components may be strongly influenced by nanoscale features rather than microscale structures (Arnold *et al.*, 2004). Nanofabrication techniques such as photolithography of an SU-8 neuronal interface may induce surface modifications that can achieve a precise control of the surface properties (e.g., controlled nano-texture), thus evaluating the mechanisms and spatiotemporal aspects of nanomaterial interactions with the cultured neuronal networks. While our results show a major reduction in nanoroughness parameters of SU-8 compared to glass controls, whether this is a true reflection of an effect is not clear given the sub-nanometre resolution levels observed. Surface stiffness and modulus elasticity was also studied. Nanoindentation testing method has become a popular tool for characterizing the mechanical properties of polymeric materials, where viscoelastic-plastic behaviour naturally inherent to polymeric materials, and the Young's modulus and hardness for very thin layers can be deduced simply from the load displacement data (Al-Halhouli *et al.*, 2008). Indeed, load displacement and modulus elasticity data illustrated SU-8 has a significantly decreased stiffness profile compared to glass, an indication of its soft-polymer characteristics. Mechanical rigidity or stiffness of the extracellular matrix (ECM) can profoundly alter cellular behaviour, including morphology, motility, and proliferation (Wells, 2008). For SH-SY5Y cells in particular, several processes such as differentiation, neuronal maturation, neurite extension, and the malignant potential and phenotype of tumour cells have all been shown to be influenced by ECM stiffness and other biophysical properties (Lam *et al.*, 2010). Studies characterising the effect of substrate stiffness on stem cell and primary neuronal cultures suggest that softer substrates are preferential for neuronal differentiation and neurite branching (Ali *et al.*, 2015; Flanagan *et al.*, 2002). This suggests that SU-8 exhibits a stiffness and elasticity profile optimal for the study of cultured neuronal networks which may account for the increased viability, adherence and morphological and functional characteristics of neurodevelopment (Appendix 8.2).

4.3 MEA Bioassay Development for Investigating Neuronal Network Activity

4.3.1 Introduction

Primary neuronal culture is the gold-standard for studying the cellular and molecular mechanisms of functional neuronal activity, permitting easy access to individual neurons for electrophysiological recording and stimulation, pharmacological interventions, and high resolution microscopic analysis. This approach enables investigation of neuronal connectivity and activity-dependent plasticity in the early stages of brain development. Analysis of the cellular and molecular mechanisms of synaptic plasticity in relatively small but active cell populations has been used to discover complex brain activity such as memory formation. Experimental analysis of connectivity with simultaneous multiple site recordings of functional neuronal networks is therefore a promising approach in neuroscience research.

To date, many of the mechanisms of synapse dynamics and function have been well characterised in cortical slice preparations and studied on MEA devices (Nam and Wheeler, 2011; Steidl *et al.*, 2006). However new insights have highlighted the greater need to understand comparable measures of synaptic function in dissociated neurons grown on MEAs, especially as dissociated neurons provide a more sensitive measure of function mediated by molecular pathways highlighted from genetic analyses, in addition to being more amenable to high-throughput approaches. Maximising the potential of MEA technology to study the functional dynamics of neuronal networks require dissociated cultures that provide identification of each neuron in the network, monitoring of electrical activities from each neuron in the network, and long-term network function in order to study development-, activity-, and pharmacology-dependent changes in synaptic network/function (Jun *et al.*, 2007).

Conventional microfabrication methods allow for the miniaturisation of electrodes and on-chip integration of electrochemical detection systems. These lab-on-a-chip platforms demonstrate reliability, selectivity and sensitivity for the detection of a wide range of analytes, e.g., DNA, amino acids, peptides, drugs, and cellular activity. Electrochemical sensing is carried out with a working electrode where the reactions of interest take place and a reference electrode in proximity to the working electrode to maintain a known stable potential, facilitating electron transfer and a current to pass through the electrolyte solution. This amperometric detection method is mediated by the movement of negatively charged ions in redox reactions when a voltage is applied between two electrodes. These electrochemical biosensors allow neuronal network analysis and evaluation through measurement of electrochemical signals from single cells such as current, potential, impedance, conductivity and capacitance (Wang *et al.*, 2012).

Presented are biosensor-mediated measurements of neuronal network activity. Calibration studies were first carried out to determine baseline microelectrode I-V characteristics. This involved calibration of MEAs according to voltage-controlled stimulation protocols in deionised water, PBS and DMEM. Following calibration, I-V characteristics comparing undifferentiated and differentiated SH-SY5Y cells were determined in spontaneous and evoked cell cultures to demonstrate proof-of-principle use of the MEA biosensor to detect variations in network signalling properties. Additionally, live-cell calcium imaging was carried out following KCL-induced neuronal depolarisation to demonstrate the capability for combined multimodal stimulation and molecular imaging on cultured neuronal networks.

4.3.2 Results

4.3.2.1 Current-Voltage Calibration on MEA Biosensor

Current-voltage characterization curves (I-V) were carried out using a customised signal generation circuit and multimeter for I-V recording, developed with collaborators in Grenoble, France (Appendix 8.4). A digital-to-analogue converter (DAC) was used to generate a voltage from 0 to 3 V based on the results of the cyclic voltammetry measurements. This voltage was then amplified to build ± 3 V signals. The microcontroller was designed to control the width of the amplified signal, which is adjustable by the software (Appendix 8.4). The current flowing through the working electrodes of the MEA were measured using a high sensitivity current monitor (Texas instruments INA286). The analogue voltage at the output of the monitor is directly proportional to the current flowing through a resistor in series with the two connection points. Monophasic (positive and negative) and biphasic (positive-then-negative/negative-then-positive) pulses of various amplitudes (100 – 1000 mV) and duration (100 – 1000 μ s) on 32 electrodes from multiple MEAs (previously summarized in Table 3.2).

Calibration tests were carried out in deionised water, PBS and DMEM. I-V curves were generated for each calibration test carried out to assess current output across a voltage range. Figure 4.13 shows I-V curves for negative pulses between 100 and 1000 mV. Current output is seen to range from 0.47 μ A to 0.64 μ A with a uniform curve shape between different test parameters. Figure 4.14 shows I-V curves for positive pulses between 100 and 1000 mV. Current output is seen to range from 0.42 μ A to 0.56 μ A with a relatively uniform curve shape between different test parameters. Figure 4.15 shows I-V curves for negative-positive biphasic pulses between 100 and 1000 mV. Current output is seen to range from 0.46 μ A to 0.67 μ A with a uniform curve shape between different test parameters. Figure 4.16 shows I-V curves for positive-negative biphasic pulses between 100 and 1000 mV. Current output is seen to range from 0.48 μ A to 0.55 μ A with a uniform curve shape between different test parameters. Given the high impedance previously observed in earlier tests, it is expected that the current

measured through our working electrodes is low and that is observed across all the tests. Nonetheless, the results demonstrate the possibility for reproducible stimulation parameters with a potential application for the interrogation of cultured neuronal networks.

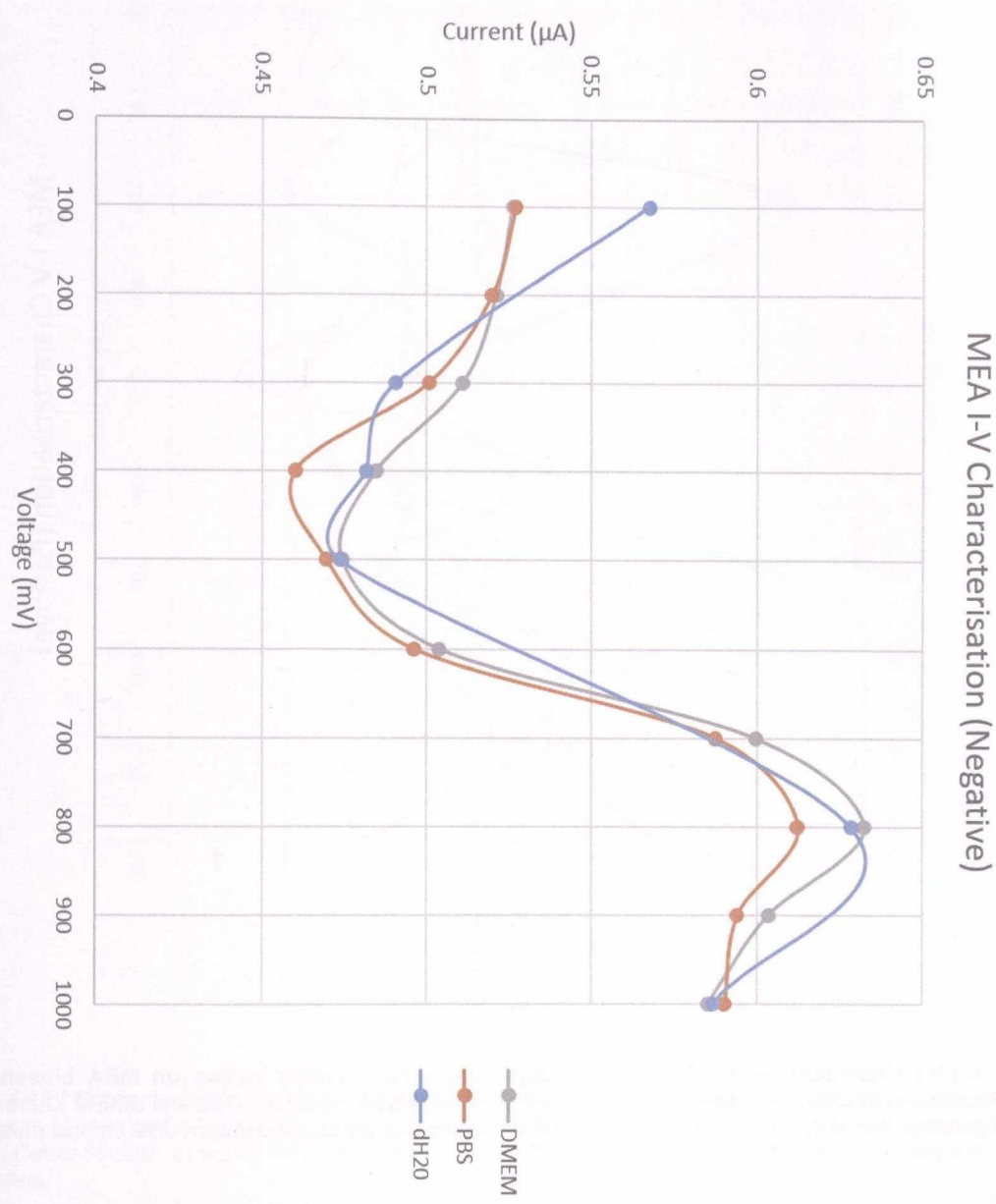


Figure 4.13 | Characterisation of current-voltage curves for negative pulses on MEA biosensor. a) I-V calibration curves for negative pulses between 100 and 1000 mV tested on dH2O, PBS and DMEM. Current output is seen to range from 0.47 µA to 0.64 µA with a uniform curve shape between different test parameters.

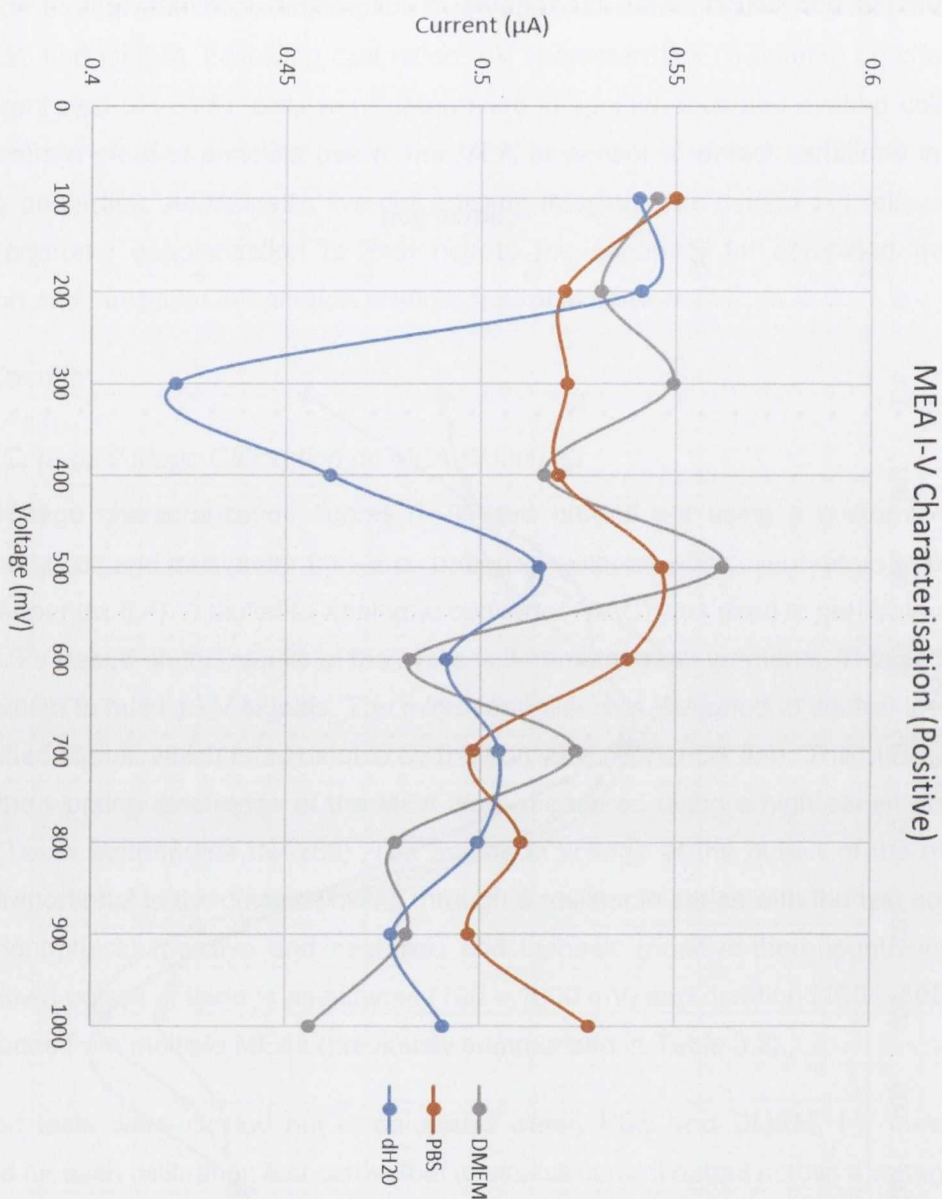


Figure 4.14 | Characterisation of current-voltage curves for positive pulses on MEA biosensor. I-V calibration curves for positive pulses between 100 and 1000 mV tested on dH₂O, PBS and DMEM. Current output is seen to range from 0.42 µA to 0.56 µA with a relatively uniform curve shape between different test parameters.

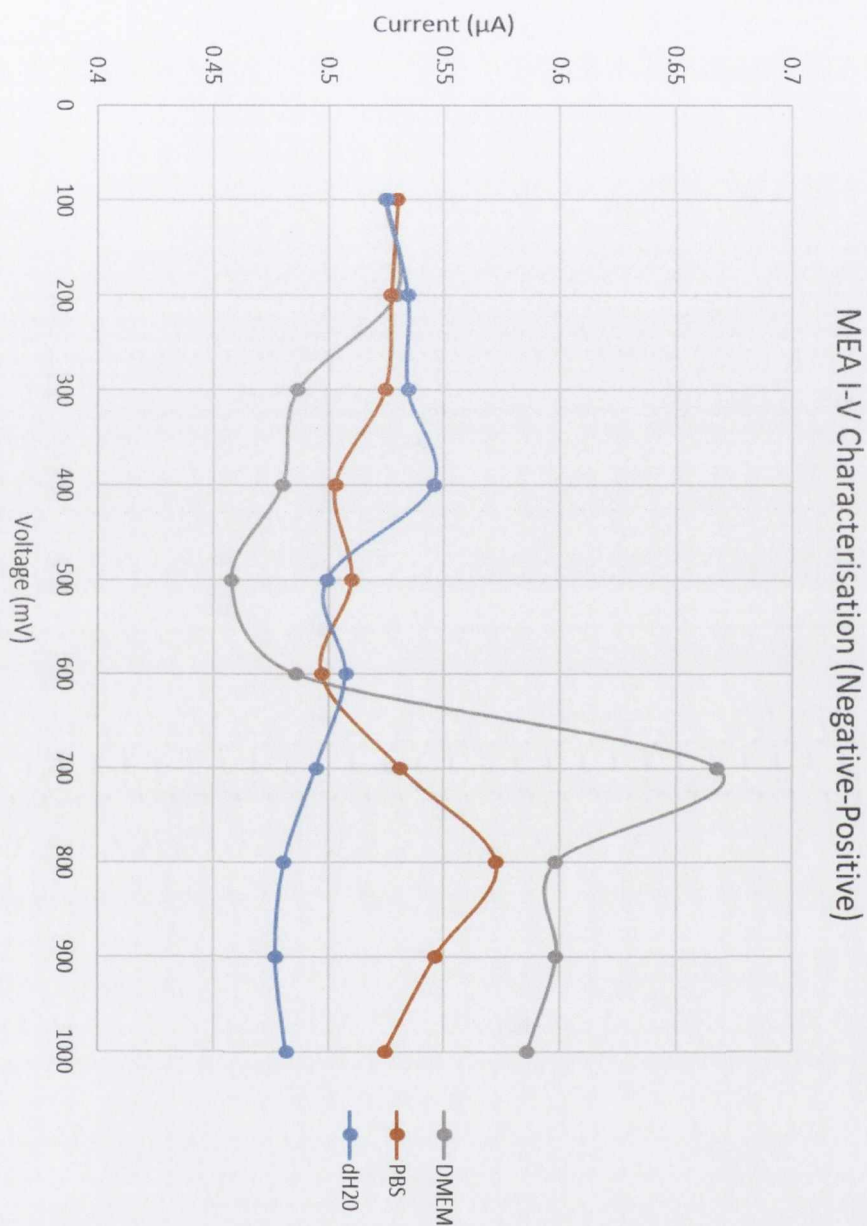


Figure 4.15 | Characterisation of current-voltage curves for biphasic negative to positive pulses on MEA biosensor. I-V curves for negative-positive biphasic pulses between 100 and 1000 mV tested on dH₂O, PBS and DMEM. Current output is seen to range from 0.46 µA to 0.67 µA with a uniform curve shape between different test parameters.

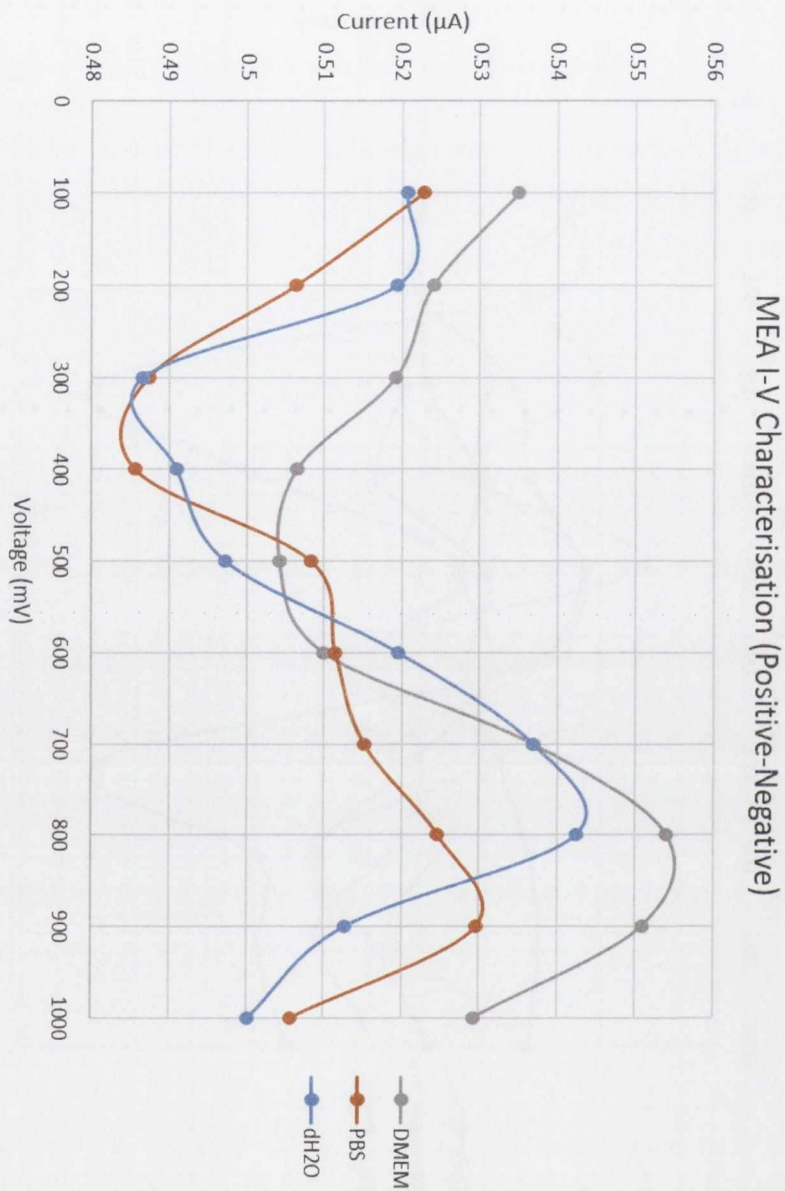


Figure 4.16 | Characterisation of current-voltage curves for biphasic positive to negative pulses on MEA biosensor. I-V curves for positive-negative biphasic pulses between 100 and 1000 mV tested on dH2O, PBS and DMEM. Current output is seen to range from 0.48 μ A to 0.55 μ A.

4.3.2.2 Evaluation of Spontaneous and Evoked SH-SY5Y Activity on MEA Biosensor

Based on the I-V calibration characteristics and evidence from literature, biphasic positive to negative voltages were applied to cultures of undifferentiated and differentiated SH-SY5Y neuroblastoma cells (Figure 4.17). MEA parameters were set up as previously described. Figure 4.18 shows I-V curves for positive-negative biphasic voltage pulses between 100 and 1000 mV. I-V measurements of spontaneous neuronal activity were taken from differentiated cultures and compared to undifferentiated cultures and medium controls. Similarly, Figure 4.19 shows I-V curves for positive-negative biphasic pulses between 100 and 1000 mV. In this scenario, I-V measurements of KCL-induced neuronal activity were taken from differentiated cultures and compared to undifferentiated cultures and medium controls. At an applied voltage range from 500 mV to 1000 mV spontaneous cultures (Figure 4.20a) show a 14% average increase in current flow in differentiated cells compared to undifferentiated cells and an 18% average increase compared to the control medium ($0.63 \pm 0.01 \mu\text{A}$ vs. $0.56 \pm 0.01 \mu\text{A}$ vs $0.53 \pm 0.01 \mu\text{A}$; $p < 0.001$; $n = 3$). Additionally at the same voltage range, evoked cultures (Figure 4.20b) show an 23% average increase in current flow in differentiated cells compared to undifferentiated cells and a 30% average increase compared to the control medium ($0.69 \pm 0.03 \mu\text{A}$ vs. $0.52 \pm 0.01 \mu\text{A}$ vs $0.53 \pm 0.01 \mu\text{A}$; $p < 0.01$; $n = 3$).

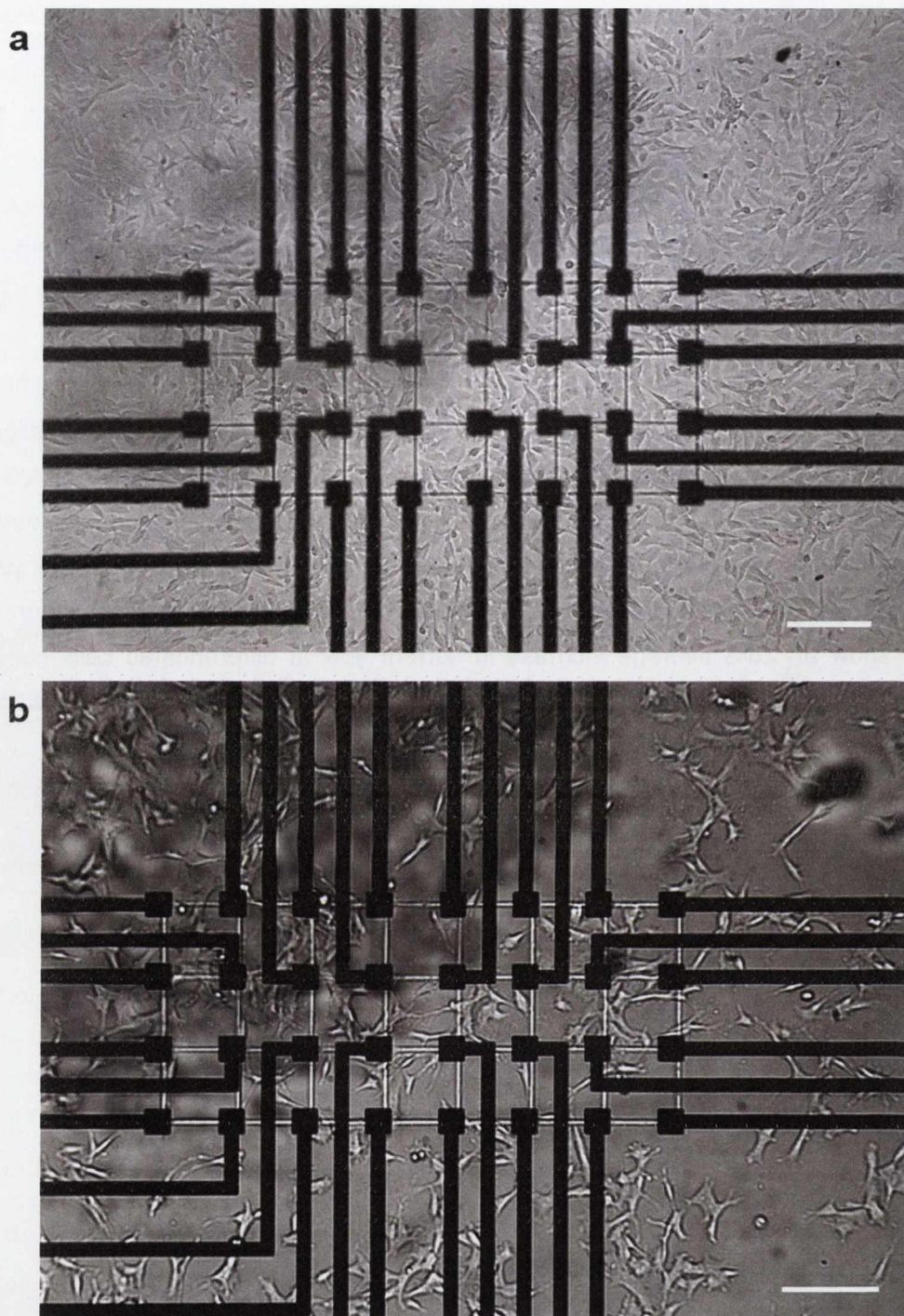


Figure 4.17 | Representative image of SH-SY5Y cells grown over MEA biosensor. Scale bar is 100 μm . a) Undifferentiated SH-SY5Y neuroblastoma cultures grown over microelectrode array. b) Differentiated SH-SY5Y neuroblastoma cultures grown over microelectrode array.

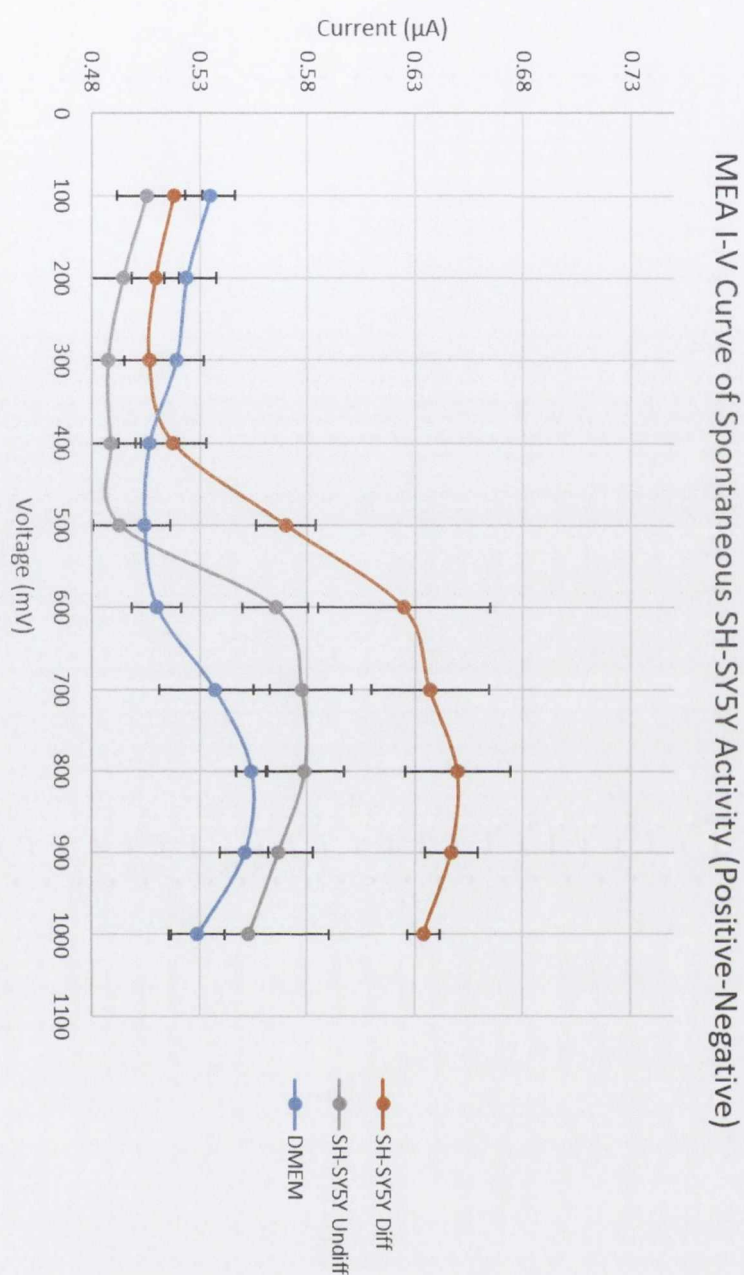


Figure 4.18 | MEA current-voltage curves for biphasic positive to negative pulses on spontaneous SH-SY5Y neuronal activity. I-V curves of spontaneous signalling in differentiated and undifferentiated SH-SY5Y neuroblastoma cultures compared to DMEM control. Differentiated cells (SH-SY5Y Diff), $0.63 \pm 0.01 \mu\text{A}$; Undifferentiated cells (SH-SY5Y Undiff), $0.56 \pm 0.01 \mu\text{A}$; DMEM medium (DMEM), $0.53 \pm 0.01 \mu\text{A}$.

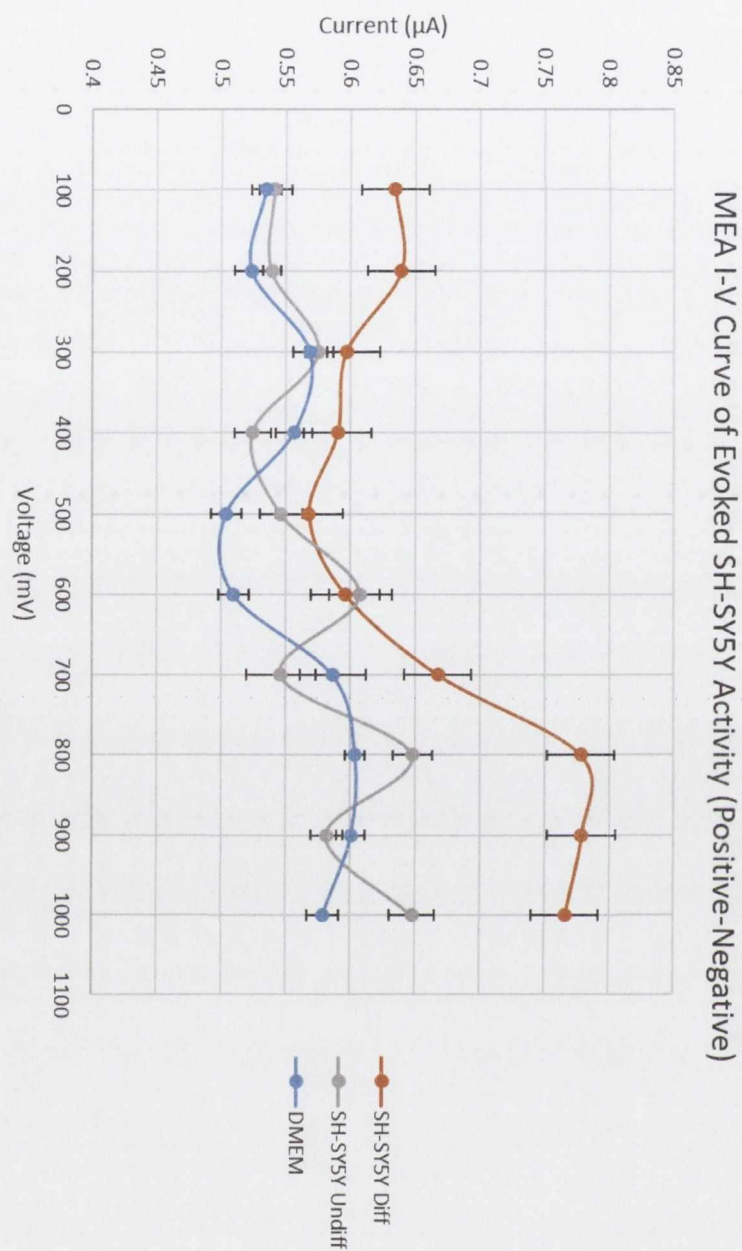


Figure 4.19 | MEA current-voltage curves for biphasic positive to negative pulses on evoked SH-SY5Y neuronal activity. I-V curves of evoked signalling in differentiated and undifferentiated SH-SY5Y neuroblastoma cultures compared to DMEM control. Differentiated cells (SH-SY5Y Diff), $0.69 \pm 0.03 \mu\text{A}$; Undifferentiated cells (SH-SY5Y Undiff), $0.52 \pm 0.01 \mu\text{A}$; DMEM medium (DMEM) $0.53 \pm 0.01 \mu\text{A}$.

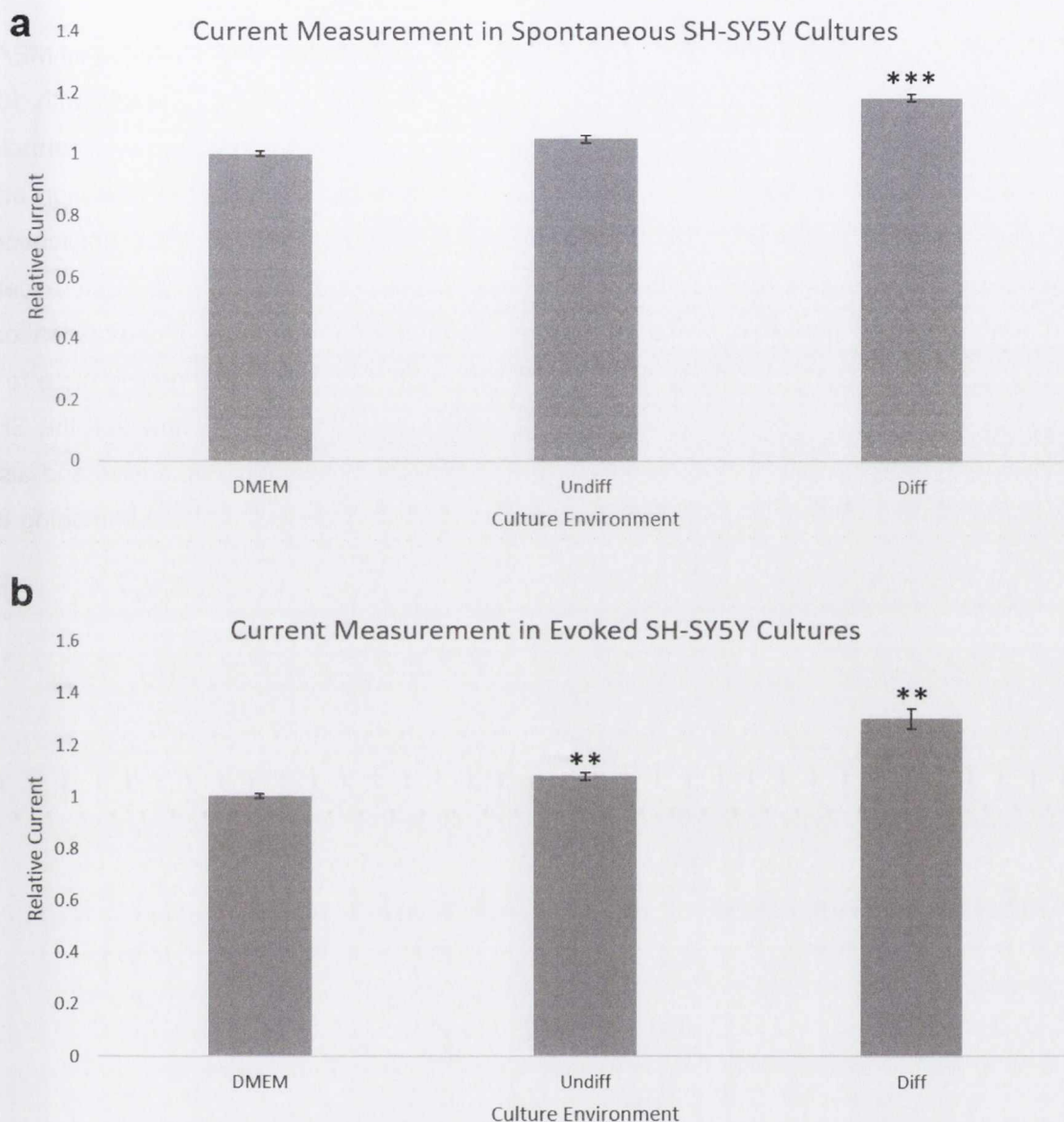


Figure 4.20 | Comparison of currents at microelectrode sites between differentiated and undifferentiated SH-SY5Y neuroblastoma cultures. a) Spontaneous SH-SY5Y cultures show 14% average increase in current flow in differentiated cells compared to undifferentiated cells and an 18% average increase compared to the control medium ($p < 0.01$; $n = 3$). b) Evoked SH-SY5Y cultures show 23% average increase in current flow in differentiated cells compared to undifferentiated cells and a 30% average increase compared to the control medium ($p < 0.01$; $n = 3$).

4.3.2.3 *Visualisation of SH-SY5Y Activity through Calcium Signalling*

Calibration analysis was further carried out via KCL-induced neuronal depolarisation on MEAs which was visualised using live-cell calcium imaging. SH-SY5Y cells were treated with 100 mM of KCl for 5 min to induce intracellular Ca^{2+} flow via voltage-gated calcium channels. Several regions of interest (Figure 4.21a) were selected to observe cell depolarisation and calcium response. Figure 4.21b illustrates the cell resting state prior to KCL application; following the application of KCL, there is a corresponding rapid rise in Ca^{2+} fluorescent intensity (Figure 4.21c) with a similarly rapid decline as the cells undergo hyperpolarisation. Figure 4.22 shows the Ca^{2+} activity profile of several cells as they go from resting state to a depolarised state and subsequent return to the resting state. This result show that the SH-SY5Y neuroblastoma cells cultured on the MEA device are functionally responsive and also illustrates the potential for combining stimulus generation with live-cell molecular imaging as a potential multimodal platform for studying cultured neuronal networks.

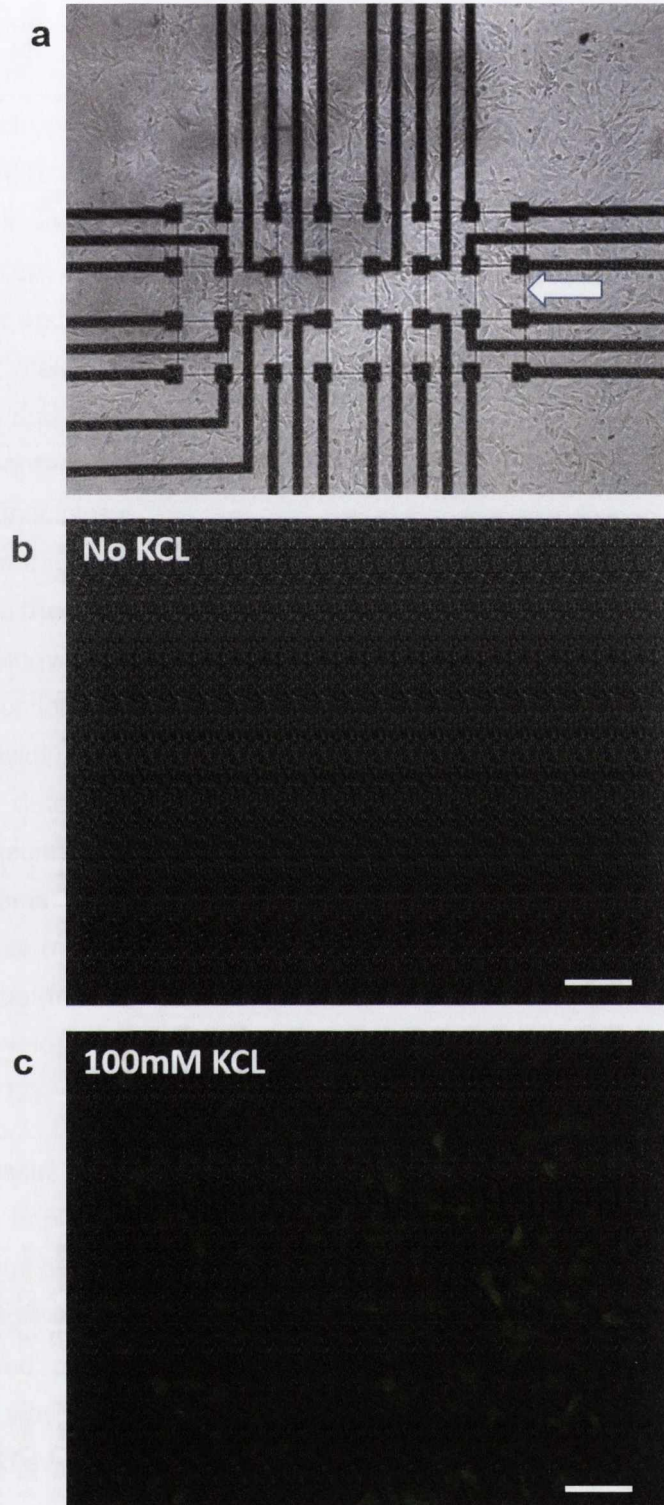


Figure 4.21 | KCL-induced depolarisation of SH-SY5Y cells on MEA biosensor. a) Growth of SH-SY5Y cells on MEA biosensor (arrow shows region of interest). b) Image of region of interest showing calcium levels at resting state. c) Image of region of interest showing calcium levels immediately following KCL-induced depolarisation. Scale bar is 10 μm .

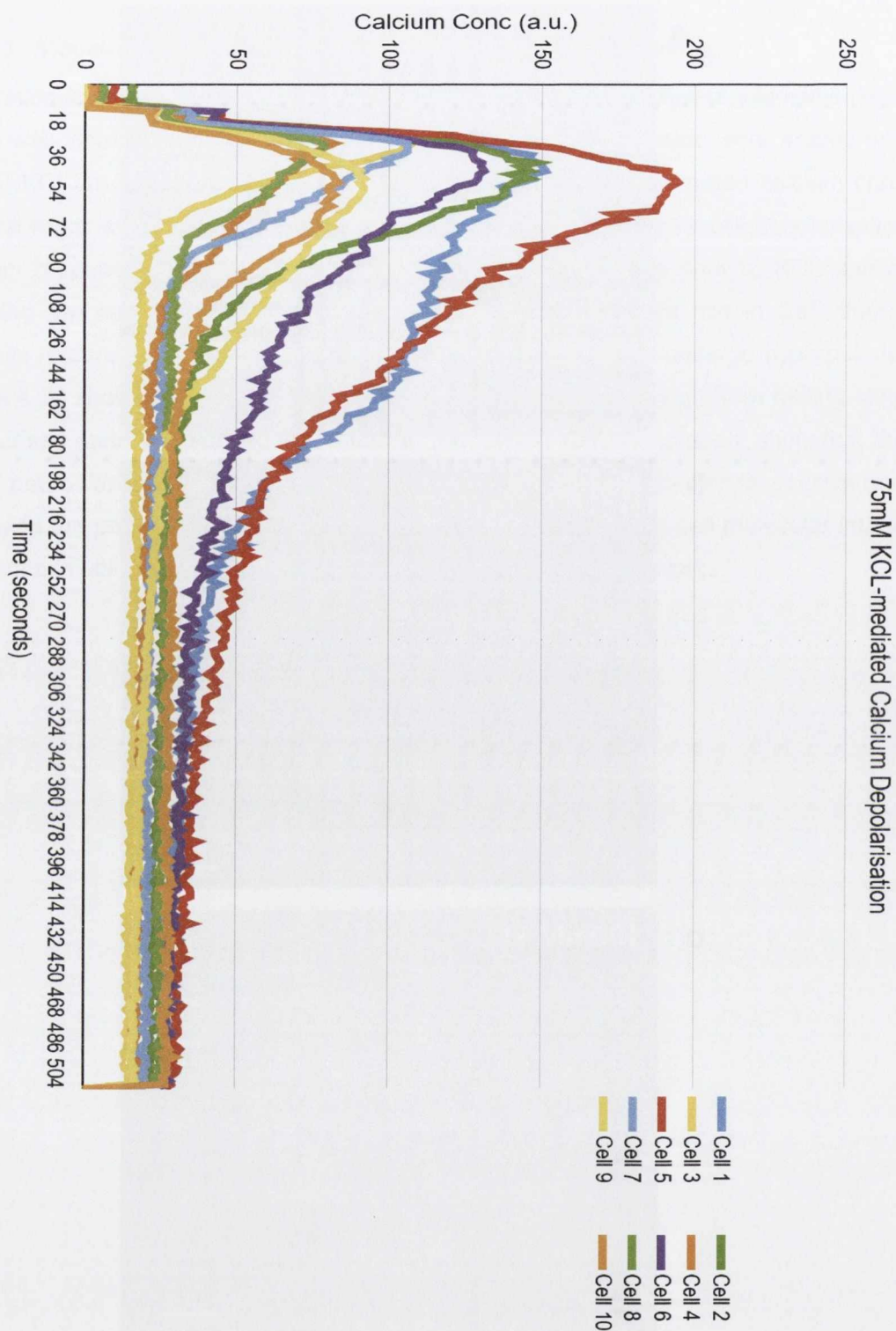


Figure 4.22 | Graph of calcium levels in individual cells following KCL depolarisation. Calcium influx was measured from ten randomly chosen cells. Average peak calcium concentration is 116.96 a.u.

4.3.3 Discussion

The use of metal electrodes to record electrical activity has been the typical gold standard for electrophysiological measurements, allowing researchers direct insight into functional communication between neuronal populations. MEA biosensors are able to provide unique insight into the behavioural activity of intact two-dimensional neuronal networks, a concept analogous to having petri dishes with electrodes embedded into the culture substrate and linked to a recording/stimulation system. Coupling the MEA device to a multimeter makes it possible to detect neuronal activity based on steady-state diffusion of ions and charge transfer within a network. This can be applied to measure the effects of spontaneous and evoked activity in neuronal cultures and provide greater insight into the principles of neuronal activity and synaptic function. The development of micro- and nanofabrication technology have allowed for the design of devices that more accurately mimic the *in vivo* conditions of nervous system development and elucidating the pathophysiology of conditions that impact neural circuit physiology. For network modelling applications, thin-film polymers have been used to increase cell-device interactions providing a significant advantage for improved device interaction and electrochemical detection by confining viable cells *in situ* (Massobrio *et al.*, 2015). Semiconductor technology stimulates the development of new cell-semiconductor hybrid biosensor systems utilizing the electrolyte-semiconductor interface for achieving biosensing. These miniaturized cell-based biosensor systems are capable of real-time, non-invasive, label-free measurements, biochemical analysis of living cells *in vitro* and facilitate the development of new analytical instruments based on these biosensors (Wang *et al.*, 2012).

As previously discussed, I-V characterisation curves portrayed a remarkable uniformity with favourable implications for neuronal interaction. Positive-then-negative biphasic voltage stimuli has been shown to be very effective at stimulating neuronal activity, while sufficiently strong negative-then-positive stimuli can also elicit response (Wagenaar *et al.*, 2004). Based on this evidence, I-V characterisation utilising positive-negative biphasic pulses was carried out on differentiated and undifferentiated SH-SY5Y cell in spontaneous and evoked cultures of neuronal activity. This was carried out to investigate the effect of cellular activity on current flow at our microelectrode sites and investigate its use in detecting variations in cellular response. Voltage-dependent recording of current within the different culture environments was elucidated by detecting changes in charge transfer mediated by neuronal membrane capacitance. Calibration measurements on biosensors were carried out to determine charge distribution profiles with the application of different voltage pulses. I-V curves across dH2O, PBS and DMEM

showed a similar profile as indicated from earlier cyclic voltammetry measurements in the same media. These calibration tests provided a profile for redox reactions taking place at the microelectrode sites. Following calibration, the biosensors were applied to distinguish spontaneous and evoked neuronal activity between differentiated and undifferentiated neuronal cultures. Using biphasic positive-negative voltammetry as a detection method, we see an increase in peak current between differentiated and undifferentiated cells in both spontaneous and evoked cultures. As a control, the response of microelectrodes in DMEM alone was recorded. This increase in detection of current at electrode sites is likely to be related to increased communication between differentiated cells caused by exocytosis-mediated neurotransmitter release (Zhao *et al.*, 2012). The detection of different neuronal responses provides proof-of-concept of the functionality of such a platform for studying network characteristics in different neuronal populations with potential applications in the study of disease behaviour.

Despite this result, recording physiological responses of neuronal activity through electrochemical detection requires direct physical contact between the electrode and cells under investigation in addition to sophisticated signal-sorting algorithms. This limits the ability for single neuron delineation in complex networks and makes functional analysis of network function difficult. The use of light increasingly represents an easier approach for probing neuronal function as it avoids the need for direct physical contact and does not interfere with neuronal activity. In addition, light presents further advantages including; improved spatial resolution, meaning signals in even the smallest neuronal structures can be resolved (Ji *et al.*, 2008), simultaneous measurement for a plethora of spatial locations (Wilt *et al.*, 2009), and the use of genetically encoded and targetable probes to access specific cellular subtypes and subcellular domains (Luo *et al.*, 2008). Investigating neuronal function using light requires a reporter molecule to convert membrane potential into an optical signal. Although an indirect form of measurement, such methods in conjunction with new methods of microscopy are being used to make a wide range of discoveries about nervous system function (Scanziani and Häusser, 2009). These include the analysis of the structure and function of dendritic spines (Nimchinsky *et al.*, 2002), the principles of dendrite plasticity in the hippocampus (Losonczy *et al.*, 2008), and the spatial organisation of individual neurons within the cortical architecture (Ohki *et al.*, 2005). Such studies have raised the bar for the scope of using optical approaches to elucidate several of the mechanisms of neuronal activity. Calcium ions in particular present versatile reporters for the intracellular signals that determine many of the complex morphology of neurons. Pre-synaptically, calcium influx triggers exocytosis of neurotransmitter-containing synaptic vesicles, post-synaptically,

calcium level increases induce the mechanisms of activity-dependent synaptic plasticity and at the intracellular level; calcium signals can regulate gene transcription. Organic calcium-sensitive dyes recorded through two-photon microscopy are now used extensively in many laboratories for the analysis of the mechanisms associated with synaptic function and synaptic plasticity in specific neuronal populations and several studies have demonstrated their effectiveness in monitoring the occurrence of both action potentials and synaptic input to spines, allowing even single-vesicle events to be reliably detected and analysed (Grienberger and Konnerth, 2012). Combining the descriptive capabilities of calcium imaging with the targeted approach of MEA-based studies presents the opportunity for precise elucidation of cellular and molecular events associated with neuronal development. Since *in vitro* MEA experiments are compatible with optical microscopy, the two techniques can be readily combined and used to elucidate the efficacy of extracellular MEA stimulation. The results indicate this possibility in a proof-of-concept model following KCL depolarisation, whereby SH-SY5Y cells grown over the MEA biosensor were functionally responsive and individual depolarisation characteristics could be recorded at specific regions on the biosensor interface. The employment of this technique might prove useful in researching neural systems, allowing the collection of more elaborate information than is possible using MEA recording or calcium imaging alone (Herzog *et al.*, 2011).

5 General Discussion

5.1 Characterising SU-8 in novel neurotechnologies for the study of neuronal development

A critical aspect of this project was the characterisation of SU-8 as an interfacing technology for the specific study of the neurodevelopmental features of cultured neuronal networks. Results have shown that the topographical properties of SU-8 can significantly change the viability, adherence, morphology and functional characteristics of SH-SY5Y neuroblastoma cell cultures and may be a preferential surface for inducing a more neuronal phenotype compared to standard glass interfaces. Given the malleability of SU-8 configuration and modification through UV lithography, it is proposed that SU-8 presents itself as a novel neurotechnology for specific applications in studying neuronal development.

The epoxy-based negative tone resist SU-8 has drawn a great deal of interest from the micro-fabrication community ever since its first introduction. Due to its high aspect ratio coupled with stable mechanical, thermal and chemical characteristics (Lorenz *et al.*, 1998), SU-8 has been developed for several applications including the development of micro gears, micro cantilevers for scanning force microscopy, microfluidic channels, optical waveguides and the fabrication of neural interfaces (Lee *et al.*, 2014). The application of SU-8 in the development of neural interfaces is of particular interest and several studies have demonstrated its use in the fabrication of microelectrodes for measuring electrical impedance in living tissues as well as monitoring neural spikes and intraocular pressure sensing. While these studies have demonstrated SU-8 biocompatibility and indicated its use as a biocompatible substrate for neuronal interaction, there has been little to no investigation of the effect of SU-8 on the structure and function of cultured neuronal networks. This is particularly important as the physical properties of thin film polymers are important features influencing the application of novel neurotechnologies to neuroscience research.

The biocompatible nature of SU-8 is evidenced by increased cell viability and a reduction in TNF- α expression of cultured SH-SY5Y neuroblastoma cells. Out of the almost 17 members of the TNF superfamily, TNF is the most potent inducer of apoptosis by activating both cell-survival and cell-death mechanisms simultaneously. TNF-induced apoptosis is mediated primarily through the activation of type I receptors, the death domain of which recruits more than a dozen different signalling proteins, which together are considered part of an apoptotic cascade (Rath and Aggarwal, 1999). By comparing

TNF- α expression levels in neurons cultured on SU-8 and glass, SU-8 was found to be a superior substrate for limiting apoptosis and improving cell survival, which is of critical importance to long-term studies of neuronal development (Potter and DeMarse, 2001). The role of SU-8 as an interface for studying neuronal development is further bolstered by examining morphological and functional markers of neurodevelopment in SH-SY5Y neuroblastoma cultures. In particular, neurite outgrowth and expression of synapsin are intrinsically linked to the differentiation and maturation of neuronal cells (Dwane *et al.*, 2013; Valtorta *et al.*, 2011). Neuronal differentiation involves a number of specific events (Figure 5.1), including formation and extension of neurite processes, increased electrical excitability of the plasma membrane, formation of synaptophysin-positive functional synapses, and induction of neuron-specific enzymes, neurotransmitters, and neurotransmitter receptors (Kovalevich and Langford, 2013). Results show that growth on SU-8 significantly increases both length of neurites and synapsin expression levels, which suggests an early-stage transition into a more mature neuronal phenotype. Cultures of SH-SY5Y cells on glass surfaces exhibited classic undifferentiated characteristics; clustered and neuroblast-like in morphology, with few, truncated processes. These cells are considered to model immature catecholaminergic neurons in their proliferation rate and lack of mature neuronal markers (Lopes *et al.*, 2010). Conversely, differentiated SH-SY5Y cells which are typically induced by RA treatment, become morphologically more similar to primary neurons with long processes and decreased clustering and proliferation rate (Encinas *et al.*, 2000). The combination of results which show that growth on SU-8 significantly increases SH-SY5Y viability, length of neurites and synapsin expression levels lead to the suggestion that SU-8 may induce an early-stage transition of these cells into a more mature neuronal phenotype (Appendix 8.2). The physical properties of SU-8 are thought to underlie this phenotypic response, particularly surface stiffness and elasticity. This has several potential applications to the study of the precise morphological and functional elements of neuronal development. The pliability of SU-8 allows for modifications to the surface chemistry and corresponding changes in neuronal interaction. Examples of this include plasma activation (Hennemeyer *et al.*, 2008), hydrophilic treatment (Wang *et al.*, 2005), and nano-modification (Kim *et al.*, 2015), impacting properties such as attachment, proliferation, migration and differentiation. Integrating nanoparticles onto the surfaces of neuronal interfaces has been shown to induce neuronal differentiation (Ahmed *et al.*, 2014) and the fabrication of nanoparticles with SU-8 interfaces is worth further study (Appendix 8.2). Overall, the characterisation of SU-8 as a biocompatible polymer with specific applications to the study of neuronal development in cultured networks has been previously undescribed. The results of this project illustrate the potential for SU-8 to be

uniquely developed into interfaces that can be used for studying neuronal development on cultured neuronal networks.

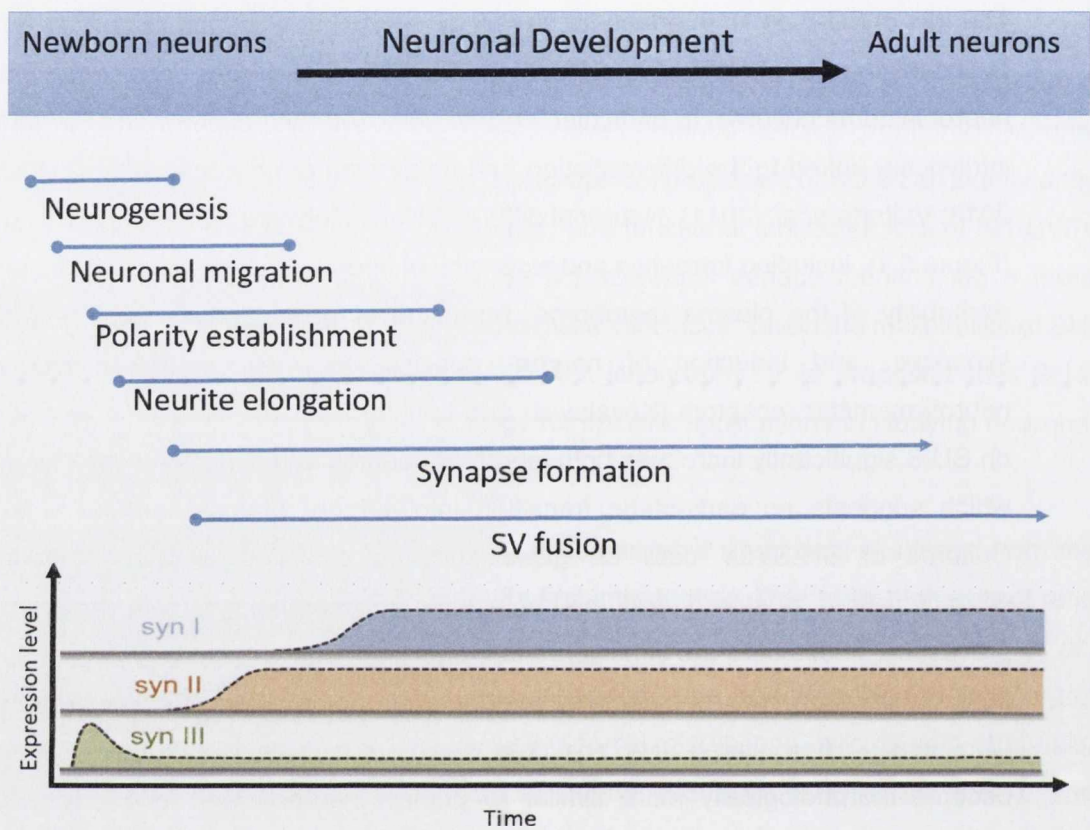


Figure 5.1 | Schematic representation of the temporal distribution of the key neurodevelopmental events and the expression pattern of syn isoforms. Adapted from (Valtorta *et al.*, 2011)

5.2 Developing a biosensor platform for studying cultured neuronal networks

Microelectrode array (MEA) technology has emerged as a credible tool for the functional analysis of activity-dependent circuit physiology. MEAs are planar multilayer sensors fabricated onto a glass surface by standard photolithographic techniques implemented by the semiconductor industry. They function by forming an electrical interface between neurons grown over electrode-embedded glass substrates. This enables researchers to monitor the circuit physiology of neuronal networks and provides a unique opportunity for multi-parametric analysis of microcircuit function via combined electrochemical and molecular imaging modalities of high specificity and sensitivity (Nam and Wheeler, 2011). Human neuroblastoma networks cultured over MEA setups have been used with success in hybrid systems and MEA setups have been designed for direct culturing neural cells over glass substrates, providing the capability to stimulate and record simultaneous populations of neural cells (Obien *et al.*, 2014).

This project proposed the development and application of a novel MEA-based device as a neural biosensor to support *in vitro* modelling of neural circuit physiology and allow for electrochemical analysis of molecular and cellular mechanisms involved in neuronal network formation and functional CNS development. The progression of the project was divided into three main sections. Firstly, the design and development of an MEA-based device for interacting with cultured neuronal networks. Secondly, the establishment of the device biocompatibility and characterisation of cell-device interaction properties. Finally, the development of bioassays suitable for the investigation of synapse dynamics and circuit physiology in CNS development. In this endeavour, biosensors for interfacing with neuronal networks have been fabricated utilising combined physical and chemical thin film deposition techniques.

These reproducible devices are composed of borosilicate glass, titanium-gold electrodes and SU-8. They have been shown to be functional and reliable, using a simple and robust fabrication process to produce numerous batches of biosensors with consistent and reproducible results. In particular, the devices demonstrated a stable electrochemical profile, lending itself well for electrochemical detection of extracellular activity. The principles that underpin electrochemical analysis are based on charge transfer between the electrolyte and the surface of the microelectrode. Oxidation or reduction of a redox-active species at an electrode surface generates a concentration gradient between the interface and the bulk solution. This redox process requires electron-transfer across the electrode/solution interface. The rate at which electron transfer takes place across the

interface is described by the heterogeneous electron-transfer rate constant (Forster, 1994). According to the electric double layer (EDL) theory, when a metal is placed into ionic liquid, an equilibrium condition is established once the charge transfer between the metal and the solution is equal creating an electrical interface between the electrolyte and the electrode surface. Given that cells are effectively dynamic electrochemical systems, they exhibit electron generation and charge transfer due to redox reactions and the changes of ionic concentration following various biological processes. This allows for multiple types of detection capabilities including potentiometric, amperometric and conductimetric transduction of biological signals into electrical signals (Ding *et al.*, 2008).

Observation of high impedance of the microelectrodes remains a limitation which may affect suitability for generating long-term stimulatory protocols in the study of cultured neuronal networks. Nanostructuration of the electrode sites on MEAs could present an efficient way to overcome these problems by decreasing the impedance of the electrode/solution interface. This can be achieved by direct growth of carbon nanotube (CNT) films, from a thin catalyst layer, onto the electrode using a chemical vapour deposition (CVD) method; with growth conditions determining whether CNTs are deposited in a random manner (Su *et al.*, 2010), or vertically aligned along well defined parameters (Nguyen-Vu *et al.*, 2006). Due to their high surface-to-volume ratio, the growth of neurons on carbon nanotubes as possible substrate enhancements for recording and stimulating neuronal populations has gained popularity (Lovat *et al.*, 2005; Mazzatenta *et al.*, 2007; Zhang *et al.*, 2005), displaying higher capacitance and lower impedance values than when compared to bare metal electrodes (Keefer *et al.*, 2008). Recently, David-Pur *et al* investigated a full CNT-modified MEA for electrical recording and stimulation. These flexible devices were fabricated using standard lithographic processes and CVD for multi-walled CNT deposition onto a polymeric support. In their study, they analysed the electrical activity of embryonic chick retina (day 14) and measured clear spontaneous activity waves. Additionally, they were able to observe an electrical response following stimulation protocols achieved with currents as low as 4 μ A and a pulse width of 1ms. They concluded that flexible CNT-MEA devices could match classical rigid counterparts with added benefits for possible implantable applications. They further suggested their fabrication process for developing entangled yet porous bundles of CNTs make their device particularly optimal for neuronal applications and a promising advancement in MEA technology (David-Pur *et al.*, 2014).

While high impedance limit the capacity for stimulatory protocols for inducing specific neuronal responses, it is possible to detect neuronal activity based on steady-state

diffusion electrochemistry. It was necessary to calibrate the response of microelectrodes for studying cultured neuronal networks, with a particular focus on neuroblastoma cultures as a possible bioassay for studying neuronal development. SH-SY5Y neuroblastoma networks cultured on MEA setups have been investigated for their ability to respond electrically to external stimuli and modulate their neural firing by changing the stimulation parameters. Such cultured neuroblastoma networks exhibit dynamic physiological states, being able to develop and adapt functionally in response to external stimuli over a broad range of configuration patterns. The main interest in these experiments is to analyse if populations of neuroblastoma cells are able to process and store information, and if learning can be implemented over this biological structure (Cuadra *et al.*, 2012). Neuroblastoma cultures over MEA have a very low signal/noise ratio response to stimulation making it difficult to detect small changes in spike activity in the culture during experiments, nonetheless, the applied voltage parameters described earlier have been previously shown to be effective for measuring neuronal responses (Wagenaar *et al.*, 2004). In particular, monophasic and bi-phasic voltage pulses have been shown to lower the potential of the medium near the electrode, increasing sensitivity to charge transfer between neurons and the microelectrode surface following depolarisation. This was functionally demonstrated in amperometric measurement of differentiated and undifferentiated cells following spontaneous and evoked neuronal activity (Figure 5.2). A significant increase in current was observed at microelectrode sites of differentiated cells compared to undifferentiated cells in both spontaneous and evoked cell cultures. This would be expected as differentiated cells display a mature neuronal phenotype associated with increased synapse formation and neurotransmitter release (Korecka *et al.*, 2013). This demonstrates the capability of the biosensor to distinguish variable behavioural profiles in cultured neuronal networks which has potential applications in disease models of neurodevelopmental disorders.

Additionally, the ability to combine electrochemical analysis of neuronal activity with calcium imaging has been demonstrated. At rest, neurons have an intracellular calcium concentration of 50 – 100 nM that during electrical activity rise to exponentially higher levels (Berridge *et al.*, 2000). The balance of calcium influx and efflux, as well as the exchange of calcium within external stores maintains cytosolic calcium concentrations at homeostatic levels. Multiple mechanisms underlie calcium influx from the extracellular space, including voltage-gated calcium channels (VGCCs), ionotropic glutamate receptors, nicotinic acetylcholine receptors (nAChR), and transient receptor potential type C (TRPC) channels (Higley and Sabatini, 2008). Imaging dendritic spines, the postsynaptic site of excitatory connections in many neurons, is a critical biological

application of calcium imaging. This has been used to identify the calcium signalling mechanism of metabotropic glutamate receptor type 1-mediated transmission, involving calcium release from internal stores in dendrites and spines, which has been implicated in the induction of long-term synaptic depression (Wang *et al.*, 2000). The *in vitro* studies of neuronal populations in combination with targeted mutations of neuronal signalling proteins can provide highly quantitative information on the intracellular mechanisms involving calcium signalling in specific neuronal subdomains, like spines and nerve terminals (Grienberger and Konnerth, 2012). Combining the descriptive capabilities of calcium imaging with the targeted approach of MEA-based studies presents the opportunity for precise elucidation of cellular and molecular events associated with neuronal development. In this project, proof-of-concept of how such an integrated platform could be applied to the study of neurodevelopment in cultured neuronal networks has been demonstrated with the potential to provide more detailed information than is possible through either MEA recordings or calcium imaging alone.

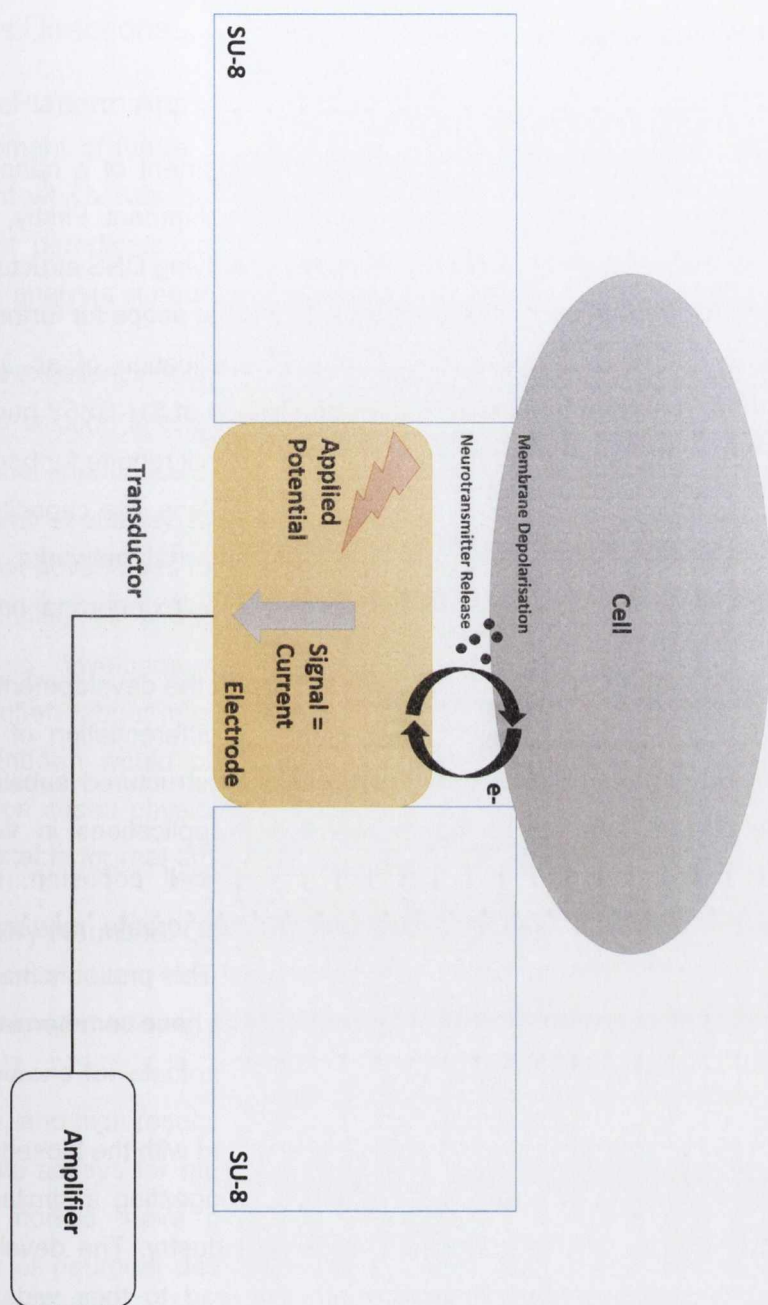


Figure 5.2 | Schematic of electrochemical detection of neuronal activity on MEA biosensor. Neuronal activity, leading to neurotransmitter release can be detected using a series of applied voltage steps inducing charge transfer across the surface of the microelectrode and eliciting signal detection of current flow.

6 Conclusions and Future Work

6.1 Core Findings from Thesis

This body of work has demonstrated the development of a nano-engineered neural biosensor and its use in the study of neuronal development. Firstly, the development of an *in vitro* biosensor platform for modelling and studying CNS structure and function has so far yielded some very positive results with a great scope for further development and application. Presented is a proof-of-concept application of an *in vitro* MEA-based biosensor to the growth, recording and visualisation of SH-SY5Y human neuroblastoma cell activity. While functional, this biosensor model will require further studies to optimise its use for clinically relevant applications. Nonetheless, the capacity for simultaneous, multisite and multi-modal study of cultured neuronal networks can open up new perspectives on the formation and dynamics of complex neuronal networks.

Additionally, we have elucidated the role of SU-8 in the development of novel interfaces specifically tuned to the growth, migration and differentiation of neuronal cells. By applying appropriate surface morphology, a nanostructured substrate may have the potential to be used for prolonged biological applications in the development of biomedical micro-devices to selectively control cell adhesion, differentiation, and mobility. In particular, soft interface technologies create new opportunities in the measurement and stimulation of biological tissues. This presents the capability for novel chemically-functionalised biomolecular sensors that have commercial applications in the analysis of investigative therapies.

The fabrication procedures and materials associated with the biosensor device reported here are seen as standard in the electronics, suggesting a similar rate of increased sophistication as with the consumer hardware industry. The development of devices targeted at specific research applications will lead to their widespread use in the neuroscience community. Critically, these technologies have significant potential utility in clinical medicine, ranging from *in vitro* diagnostic systems for patient stratification of disease types to *in vivo* modulatory systems for targeted therapeutic intervention. Accelerating commercial translation of these technologies will require well-defined applications solving specific questions in neuroscience research. Nonetheless, the scalability of embedded electronics with diverse, multifunctional operations present a large scope for future development driven by a continued multidisciplinary approach to expanding our knowledge of the brain (Jeong *et al.*, 2015).

6.2 Future Directions

6.2.1 MEA Platform Applications

The development of future applications for the platform will require further technical enhancement which was beyond the scope of this project. Several electrochemical measurement paradigms can be incorporated into the biosensor to add an extra dimension of analysis of neuronal network behaviour.

As cells have excellent insulating properties, they can affect local ionic concentrations at the electrode-solution interface which leads to a change of impedance. As such, the electrochemical impedance spectroscopic technique (EIS) can be used to study cellular behaviour, such as viability, morphology, adhesion and proliferation and has been shown to confer great advantages in drug screening (Xing *et al.*, 2006). Cell-based assays are used substantially as a substitution method for animal experiments in pre-clinical research, drug development and toxicological screening. Conventional cell-based assays are often labour-intensive, highly manual and time consuming. An MEA-EIS combined platform would present multi-parametric analysis the effects of different compounds on circuit physiology and synaptic function. Such an approach would be especially suitable for real-time and high-throughput monitoring of cellular responses.

6.2.2 Primary Neuronal Culture on Biosensor

Primary neuronal culture has typically been the gold-standard for studying the cellular and molecular mechanisms of functional neuronal activity, permitting easy access to individual neurons for electrophysiological recording and stimulation, pharmacological interventions, and high resolution microscopic analysis. In particular, the introduction of more accurate assays for modelling brain disorders through induced pluripotent stem cell (iPSC) models, have provided innovative approaches for probing molecular mechanisms of neuronal development (Marchetto *et al.*, 2010) and high-throughput screening of novel therapeutic compounds (Lee and Studer, 2011). These humanised disease models are derived from peripheral cells isolated in affected individuals and differentiated into human neurons to study disease specific pathogenesis (Ming *et al.*, 2011). The application of a biosensor platform such as the one developed for this project may present a valuable diagnostic tool for understanding many of the properties of CNS function and the development of personalised therapeutic strategies (Chailangkarn *et al.*, 2012). This capacity for highly sensitive nanoscale probes to model, stimulate and record neuronal activity provides a unique opportunity to have a profound impact on basic and clinical research (Duan *et al.*, 2013). The integration of these nanosensors with genome analysis, iPSC disease models and advanced imaging of individual patients

may provide an excellent translational approach to explore the dynamics of gene expression with synapse development and circuit physiology. While still in relatively early stages of development, the field is growing fast and it is possible that new next-generation sequencing devices will take advantage of these nanobiotechnology platforms.

7 References

- Agholme, L., Lindström, T., Kågedal, K., Marcusson, J., and Hallbeck, M. (2010). An in vitro model for neuroscience: differentiation of SH-SY5Y cells into cells with morphological and biochemical characteristics of mature neurons. *Journal of Alzheimer's Disease* 20, 1069-1082.
- Ahmed, D., BongWoo, K., Sangiliyandi, G., Hye, C., Gwangmo, Y., Subbroto, S., Dawoon, H., Jihae, H., Kyeongseok, K., Jin-Hoi, K., *et al.* (2014). Biologically synthesized silver nanoparticles induce neuronal differentiation of SH-SY5Y cells via modulation of reactive oxygen species, phosphatases, and kinase signaling pathways. *Biotechnology Journal* 9, 934-943.
- Ajetunmobi, A., Prina-Mello, A., Volkov, Y., Corvin, A., and Tropea, D. (2014). Nanotechnologies for the study of the central nervous system. *Progress in neurobiology* 123, 18-36.
- Ajetunmobi, A., and Tropea, D. (2015). Progress toward therapies and interventions. In *The Genetics of Neurodevelopmental Disorders*, K. Mitchell, ed. (Wiley).
- Akhmanova, A., Verkerk, T., Langeveld, A., Grosveld, F., and Galjart, N. (2000). Characterisation of transcriptionally active and inactive chromatin domains in neurons. *Journal of cell science* 113, 4463-4474.
- Al-Halhouli, A.T., Kampen, I., Krah, T., and Büttgenbach, S. (2008). Nanoindentation testing of SU-8 photoresist mechanical properties. *Microelectronic Engineering* 85, 942-944.
- Albertazzi, L., Gherardini, L., Brondi, M., Sulis Sato, S., Bifone, A., Pizzorusso, T., Ratto, G., and Bardi, G. (2013). In vivo distribution and toxicity of PAMAM dendrimers in the central nervous system depend on their surface chemistry. *Molecular pharmaceutics* 10, 249-260.
- Ali, S., Wall, I.B., Mason, C., Pelling, A.E., and Veraitch, F.S. (2015). The effect of Young's modulus on the neuronal differentiation of mouse embryonic stem cells. *Acta Biomaterialia* 25, 253-267.
- Andres, D., Keyser, B., M., Petrali, J., Benton, B., Hubbard, K., S., McNutt, P., M., and Ray, R. (2013). Morphological and functional differentiation in BE(2)-M17 human neuroblastoma cells by treatment with Trans-retinoic acid. *BMC Neuroscience*.
- Arnold, F., Hofmann, F., Bengtson, C., Wittmann, M., Vanhoutte, P., and Bading, H. (2005). Microelectrode array recordings of cultured hippocampal networks reveal a simple model for transcription and protein synthesis-dependent plasticity. *The Journal of physiology* 564, 3-19.

- Arnold, M., Cavalcanti-Adam, E.A., Glass, R., Blümmel, J., Eck, W., Kantlehner, M., Kessler, H., and Spatz, J.P. (2004). Activation of integrin function by nanopatterned adhesive interfaces. *Chemphyschem : a European journal of chemical physics and physical chemistry* 5, 383-388.
- Bardi, G., Malvindi, M., Gherardini, L., Costa, M., Pompa, P., Cingolani, R., and Pizzorusso, T. (2010). The biocompatibility of amino functionalized CdSe/ZnS quantum-dot-Doped SiO₂ nanoparticles with primary neural cells and their gene carrying performance. *Biomaterials* 31, 6555-6566.
- Barnes, S., and Finnerty, G. (2010). Sensory experience and cortical rewiring. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* 16, 186-198.
- Bavelier, D., Levi, D.M., Li, R.W., Dan, Y., and Hensch, T.K. (2010). Removing brakes on adult brain plasticity: from molecular to behavioral interventions. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 30, 14964-14971.
- Bednarek, E., and Caroni, P. (2011). β -Adducin is required for stable assembly of new synapses and improved memory upon environmental enrichment. *Neuron* 69, 1132-1146.
- Beom Jun, S., Hynd, M., Dowell-Mesfin, N., Smith, K., Turner, J., Shain, W., and June Kim, S. (2005). Synaptic connectivity of a low density patterned neuronal network produced on the poly-L-lysine stamped microelectrode array. *Conference proceedings : Annual International Conference of the IEEE Engineering in Medicine and Biology Society IEEE Engineering in Medicine and Biology Society Conference* 7, 7604-7607.
- Berridge, M.J., Lipp, P., and Bootman, M.D. (2000). The versatility and universality of calcium signalling. *Nature reviews Molecular cell biology* 1, 11-21.
- Biedler, J.L., Helson, L., and Spengler, B.A. (1973). Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer research*.
- Biffi, E., Menegon, A., Piraino, F., Pedrocchi, A., Fiore, G., and Rasponi, M. (2012). Validation of long-term primary neuronal cultures and network activity through the integration of reversibly bonded microbioreactors and MEA substrates. *Biotechnology and bioengineering* 109, 166-175.
- Blander, G., de Oliveira, R.M., and Conboy, C.M. (2003). Superoxide dismutase 1 knock-down induces senescence in human fibroblasts. *Journal of Biological*
- Blumling Iii, J.P., and Silva, G.A. (2012). Targeting the brain: advances in drug delivery. *Current pharmaceutical biotechnology* 13, 2417-2426.
- Bourne, J., and Harris, K. (2008). Balancing structure and function at hippocampal dendritic spines. *Annual review of neuroscience* 31, 47-67.

- Brandon, N., and Sawa, A. (2011). Linking neurodevelopmental and synaptic theories of mental illness through DISC1. *Nature reviews Neuroscience* 12, 707-722.
- Brennand, K., and Gage, F. (2011). Concise review: the promise of human induced pluripotent stem cell-based studies of schizophrenia. *Stem cells (Dayton, Ohio)* 29, 1915-1922.
- Brennand, K., Simone, A., Jou, J., Gelboin-Burkhart, C., Tran, N., Sangar, S., Li, Y., Mu, Y., Chen, G., Yu, D., *et al.* (2011). Modelling schizophrenia using human induced pluripotent stem cells. *Nature* 473, 221-225.
- Brown, S., and Hestrin, S. (2009). Intracortical circuits of pyramidal neurons reflect their long-range axonal targets. *Nature* 457, 1133-1136.
- Bruce, S.M. (2012). The ever-changing brain: Cellular and molecular mechanisms for the effects of stressful experiences. *Developmental Neurobiology*.
- Brunetti, V., Maiorano, G., Rizzello, L., Sorce, B., Sabella, S., Cingolani, R., and Pompa, P.P. (2010). Neurons sense nanoscale roughness with nanometer sensitivity. *Proceedings of the National Academy of Sciences* 107, 6264-6269.
- Camilli, D.P., Cameron, R., and Greengard, P. (1983). Synapsin I (protein I), a nerve terminal-specific phosphoprotein. I. Its general distribution in synapses of the central and peripheral nervous system demonstrated by immunofluorescence in frozen and plastic sections. *The Journal of cell biology* 96, 1337-1354.
- Caroni, P., Donato, F., and Muller, D. (2012). Structural plasticity upon learning: regulation and functions. *Nature reviews Neuroscience* 13, 478-490.
- Chailangkarn, T., Acab, A., and Muotri, A.R. (2012). Modeling neurodevelopmental disorders using human neurons. *Current opinion in neurobiology* 22, 785-790.
- Chen, J.L., and Nedivi, E. (2010). Neuronal structural remodeling: is it all about access? *Current opinion in neurobiology* 20, 557-562.
- Cheung, A., Horvath, L., Carrel, L., and Ellis, J. (2012). X-chromosome inactivation in rett syndrome human induced pluripotent stem cells. *Frontiers in psychiatry / Frontiers Research Foundation* 3, 24.
- Cohen, S., Greenberg, M. E., (2008). Communication Between the Synapse and the Nucleus in Neuronal Development, Plasticity and Disease. *Annu Rev Cell Dev Biol* 24, 183-209.
- Corvin, A., Molinos, I., Little, G., Donohoe, G., Gill, M., Morris, D., and Tropea, D. (2012). Insulin-like growth factor 1 (IGF1) and its active peptide (1-3)IGF1 enhance the expression of synaptic markers in neuronal circuits through different cellular mechanisms. *Neuroscience letters* 520, 51-56.

- Cuadra, J.M., Álvarez-Sánchez, J.R., Santos, D.d., Lorente, V., Ferrández, J.M., Paz, F.d.I., and Fernández, E. (2012). Response calibration in neuroblastoma cultures over multielectrode array. *Neurocomputing*.
- David-Pur, M., Bareket-Keren, L., Beit-Yaakov, G., Raz-Prag, D., and Hanein, Y. (2014). All-carbon-nanotube flexible multi-electrode array for neuronal recording and stimulation. *Biomedical microdevices* 16, 43-53.
- Devor, A., Bandettini, P.A., Boas, D.A., Bower, J.M., Buxton, R.B., Cohen, L.B., Dale, A.M., Einevoll, G.T., Fox, P.T., Franceschini, M.A., *et al.* (2013). The challenge of connecting the dots in the B.R.A.I.N. *Neuron* 80, 270-274.
- Ding, L., Du, D., Zhang, X., and Huangxian, J. (2008). Trends in Cell-Based Electrochemical Biosensors. *Current Medicinal Chemistry* 15, 3160-3170.
- Djurisic, M., Antic, S., Chen, W., and Zecevic, D. (2004). Voltage imaging from dendrites of mitral cells: EPSP attenuation and spike trigger zones. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 24, 6703-6714.
- Duan, X., Fu, T.-M., Liu, J., and Lieber, C. (2013). Nanoelectronics-biology frontier: From nanoscopic probes for action potential recording in live cells to three-dimensional cyborg tissues. *Nano today* 8, 351-373.
- Dwane, S., Durack, E., and Kiely, P., A. (2013). Optimising parameters for the differentiation of SH-SY5Y cells to study cell adhesion and cell migration. *BMC Research Notes* 6, 366.
- Ehrlich, I., Klein, M., Rumpel, S., and Malinow, R. (2007). PSD-95 is required for activity-driven synapse stabilization. *Proceedings of the National Academy of Sciences of the United States of America* 104, 4176-4181.
- Encinas, M., Iglesias, M., Liu, Y., Wang, H., Muhaisen, A., Ceña, V., Gallego, C., and Comella, J.X. (2000). Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells. *Journal of neurochemistry* 75, 991-1003.
- Engle, E. (2010). Human genetic disorders of axon guidance. *Cold Spring Harbor perspectives in biology* 2.
- Ereifej, E.S., Khan, S., Newaz, G., Zhang, J., Auner, G.W., and VandeVord, P.J. (2011). Characterization of astrocyte reactivity and gene expression on biomaterials for neural electrodes. *Journal of Biomedical Materials Research Part A* 99, 141-150.
- Eytan, D., Brenner, N., and Marom, S. (2003). Selective adaptation in networks of cortical neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 23, 9349-9356.

- Eytan, D., Minerbi, A., Ziv, N., and Marom, S. (2004). Dopamine-induced dispersion of correlations between action potentials in networks of cortical neurons. *Journal of neurophysiology* 92, 1817-1824.
- Flanagan, L.A., Ju, Y.E., Marg, B., and Osterfield, M. (2002). Neurite branching on deformable substrates. *Neuroreport* 13, 2411-2415.
- Fletcher, T.L., Cameron, P., and Camilli, D.P. (1991). The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture. *The Journal of Neuroscience* 11, 1617-1626.
- Forster, R.J. (1994). Microelectrodes: new dimensions in electrochemistry. *Chemical Society Reviews*.
- Gabi, M., Larmagnac, A., Schulte, P., and Vörös, J. (2010). Electrically controlling cell adhesion, growth and migration. *Colloids and surfaces B, Biointerfaces* 79, 365-371.
- Gao, X., Chen, J., Chen, J., Wu, B., Chen, H., and Jiang, X. (2008). Quantum dots bearing lectin-functionalized nanoparticles as a platform for in vivo brain imaging. *Bioconjugate chemistry* 19, 2189-2195.
- Gomez, N., Lu, Y., Chen, S., and Schmidt, C. (2007). Immobilized nerve growth factor and microtopography have distinct effects on polarization versus axon elongation in hippocampal cells in culture. *Biomaterials* 28, 271-284.
- Gramowski, A., Jügelt, K., Weiss, D., and Gross, G. (2004). Substance identification by quantitative characterization of oscillatory activity in murine spinal cord networks on microelectrode arrays. *The European journal of neuroscience* 19, 2815-2825.
- Grienberger, C., and Konnerth, A. (2012). Imaging calcium in neurons. *Neuron* 73, 862-885.
- Gustavsson, A., Svensson, M., Jacobi, F., Allgulander, C., Alonso, J., Beghi, E., Dodel, R., Ekman, M., Faravelli, C., Fratiglioni, L., *et al.* (2011). Cost of disorders of the brain in Europe 2010. *European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology* 21, 718-779.
- Hady, E.A., Afshar, G., Bröking, K., and Schlüter, O.M. (2013). Optogenetic stimulation effectively enhances intrinsically generated network synchrony. *Frontiers in neural circuits* 7, 167.
- Hall, J., Dennler, P., Haller, S., Pratsinis, A., Säuberli, K., Towbin, H., Walther, K., Walthe, K., and Woytschak, J. (2010). Genomics drugs in clinical trials. *Nature reviews Drug discovery* 9, 988.
- Harsch, A., Ziegler, C., and Göpel, W. (1997). Strychnine analysis with neuronal networks in vitro: extracellular array recording of network responses. *Biosensors & bioelectronics* 12, 827-835.

- He, Y., Liu, M.-G., Gong, K.-R., and Chen, J. (2009). Differential effects of long and short train theta burst stimulation on LTP induction in rat anterior cingulate cortex slices: multi-electrode array recordings. *Neuroscience bulletin* 25, 309-318.
- Heim, M., Yvert, B., and Kuhn, A. (2012). Nanostructuring strategies to enhance microelectrode array (MEA) performance for neuronal recording and stimulation. *Journal of physiology, Paris* 106, 137-145.
- Hennemeyer, M., Walther, F., Kerstan, S., Schürzinger, K., Gigler, A., M., and Stark, R., W. (2008). Cell proliferation assays on plasma activated SU-8. *Microelectronic Engineering* 85, 1298-1301.
- Hensch, T. (2005). Critical period plasticity in local cortical circuits. *Nature reviews Neuroscience* 6, 877-888.
- Herman, G.E. (2002). Mouse models of human disease: lessons learned and promises to come. *ILAR Journal*.
- Herzog, N., Shein-Idelson, M., and Hanein, Y. (2011). Optical validation of in vitro extracellular neuronal recordings. *Journal of neural engineering* 8, 56008.
- Higley, M.J., and Sabatini, B.L. (2008). Calcium signaling in dendrites and spines: practical and functional considerations. *Neuron* 59, 902-913.
- Hill, R., Lyon, J., Allen, R., Stevenson, K., and Shear, J. (2005). Microfabrication of three-dimensional bioelectronic architectures. *Journal of the American Chemical Society* 127, 10707-10711.
- Hoffman, F., Bading, H. (2006). Long term recordings with microelectrode arrays: studies of transcription-dependent neuronal plasticity and axonal regeneration. *J Physiol Paris* 99, 125-132.
- Holtmaat, A., Trachtenberg, J., Wilbrecht, L., Shepherd, G., Zhang, X., Knott, G., and Svoboda, K. (2005). Transient and persistent dendritic spines in the neocortex in vivo. *Neuron* 45, 279-291.
- Jaber, F., Labeed, F., and Hughes, M. (2009). Action potential recording from dielectrophoretically positioned neurons inside micro-wells of a planar microelectrode array. *Journal of neuroscience methods* 182, 225-235.
- James, C.D., Davis, R., Meyer, M., Turner, A., Turner, S., Withers, G., Kam, L., Banker, G., Craighead, H., Isaacson, M., *et al.* (2000). Aligned microcontact printing of micrometer-scale poly-L-lysine structures for controlled growth of cultured neurons on planar microelectrode arrays. *IEEE Trans Biomed Eng* 47, 17-21.
- Jane, K., and Dianne, L. (2013). Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. *Methods in molecular biology (Clifton, NJ)* 1078, 9-21.
- Jeong, J.W., Shin, G., Park, S.I., Yu, K.J., Xu, L., and Rogers, J.A. (2015). Soft Materials in Neuroengineering for Hard Problems in Neuroscience. *Neuron*.

- Jerry, L.C., Walter, C.L., Jae Won, C., Peter, T.S., Yoshiyuki, K., and Elly, N. (2011). Structural basis for the role of inhibition in facilitating adult brain plasticity. *Nature Neuroscience* 14, 587-594.
- Ji, N., Shroff, H., Zhong, H., and Betzig, E. (2008). Advances in the speed and resolution of light microscopy. *Current opinion in neurobiology* 18, 605-616.
- Jimbo, Y., Kawana, A., Parodi, P., and Torre, V. (2000). The dynamics of a neuronal culture of dissociated cortical neurons of neonatal rats. *Biological cybernetics* 83, 1-20.
- Jimbo, Y., Tateno, T., and Robinson, H. (1999). Simultaneous induction of pathway-specific potentiation and depression in networks of cortical neurons. *Biophysical journal* 76, 670-678.
- Johansson, F., Carlberg, P., Danielsen, N., Montelius, L., and Kanje, M. (2006). Axonal outgrowth on nano-imprinted patterns. *Biomaterials* 27, 1251-1258.
- John, L., Ryohei, Y., and Sridhar, R. (2012). Mechanisms of CaMKII action in long-term potentiation. *Nature Reviews Neuroscience*.
- Johnstone, A., Gross, G., Weiss, D., Schroeder, O., Gramowski, A., and Shafer, T. (2010). Microelectrode arrays: a physiologically based neurotoxicity testing platform for the 21st century. *Neurotoxicology* 31, 331-350.
- Jones, I., Livi, P., Lewandowska, M., Fiscella, M., Roscic, B., and Hierlemann, A. (2011). The potential of microelectrode arrays and microelectronics for biomedical research and diagnostics. *Analytical and bioanalytical chemistry* 399, 2313-2329.
- Jun, S., Hynd, M., Dowell-Mesfin, N., Smith, K., Turner, J., Shain, W., and Kim, S. (2007). Low-density neuronal networks cultured using patterned poly-L-lysine on microelectrode arrays. *Journal of neuroscience methods* 160, 317-326.
- Juopperi, T., Song, H., and Ming, G.-L. (2011). Modeling neurological diseases using patient-derived induced pluripotent stem cells. *Future neurology* 6, 363-373.
- Kaehr, B., Allen, R., Javier, D., Currie, J., and Shear, J. (2004). Guiding neuronal development with in situ microfabrication. *Proceedings of the National Academy of Sciences of the United States of America* 101, 16104-16108.
- Kaitin, K. (2010). Deconstructing the drug development process: the new face of innovation. *Clinical pharmacology and therapeutics* 87, 356-361.
- Karuri, N.W., Liliensiek, S., and Teixeira, A.I. (2004). Biological length scale topography enhances cell-substratum adhesion of human corneal epithelial cells. *Journal of cell*
- Katz, L.C., Shatz, C. J., (1996). Synaptic activity and the construction of cortical circuits. *Science* 274, 1133-1138.
- Keefer, E., Botterman, B., Romero, M., Rossi, A., and Gross, G. (2008). Carbon nanotube coating improves neuronal recordings. *Nature nanotechnology* 3, 434-439.

- Keefer, E., Norton, S., Boyle, N., Talesa, V., and Gross, G. (2001). Acute toxicity screening of novel AChE inhibitors using neuronal networks on microelectrode arrays. *Neurotoxicology* 22, 3-12.
- Kim, E., Yoo, S.J., Moon, C., Nelson, B.J., and Choi, H. (2015). SU-8-based nanoporous substrate for migration of neuronal cells. *Microelectronic Engineering*.
- Kipke, D., Shain, W., Buzsáki, G., Fetz, E., Henderson, J., Hetke, J., and Schalk, G. (2008). Advanced neurotechnologies for chronic neural interfaces: new horizons and clinical opportunities. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 28, 11830-11838.
- Knudsen, E. (2004). Sensitive periods in the development of the brain and behavior. *Journal of cognitive neuroscience* 16, 1412-1425.
- Koffie, R., Farrar, C., Saidi, L.-J., William, C., Hyman, B., and Spires-Jones, T. (2011). Nanoparticles enhance brain delivery of blood-brain barrier-impermeable probes for in vivo optical and magnetic resonance imaging. *Proceedings of the National Academy of Sciences of the United States of America* 108, 18837-18842.
- Korecka, J.A., van Kesteren, R.E., Blaas, E., and Spitzer, S.O. (2013). Phenotypic characterization of retinoic acid differentiated SH-SY5Y cells by transcriptional profiling. *Phenotypic characterization of retinoic acid differentiated SH-SY5Y cells by transcriptional profiling*.
- Kotzar, G., Freas, M., Abel, P., Fleischman, A., Roy, S., Zorman, C., Moran, J.M., and Melzak, J. (2002). Evaluation of MEMS materials of construction for implantable medical devices. *Biomaterials* 23, 2737-2750.
- Kovalevich, J., and Langford, D. (2013). Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. *Methods in molecular biology (Clifton, NJ)* 1078, 9-21.
- Kreis, P., and Barnier, J.-V. (2009). PAK signalling in neuronal physiology. *Cellular signalling* 21, 384-393.
- Lam, W.A., Cao, L., Umesh, V., Keung, A.J., and Sen, S. (2010). Extracellular matrix rigidity modulates neuroblastoma cell differentiation and N-myc expression. *Mol*
- Lee, G., and Studer, L. (2011). Modelling familial dysautonomia in human induced pluripotent stem cells. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 366, 2286-2296.
- Lee, J.B., Choi, K.H., and Yoo, K. (2014). Innovative SU-8 Lithography Techniques and Their Applications. *Micromachines*.
- Lee, W.-C.A., and Reid, R. (2011). Specificity and randomness: structure-function relationships in neural circuits. *Current opinion in neurobiology* 21, 801-807.

- Lin, L., Lesnick, T., Maraganore, D., and Isacson, O. (2009). Axon guidance and synaptic maintenance: preclinical markers for neurodegenerative disease and therapeutics. *Trends in neurosciences* 32, 142-149.
- Linkenhoker, B., von der Ohe, C., and Knudsen, E. (2005). Anatomical traces of juvenile learning in the auditory system of adult barn owls. *Nature neuroscience* 8, 93-98.
- Liu, J., Koscielska, K., Cao, Z., Hulsizer, S., Grace, N., Mitchell, G., Nacey, C., Githinji, J., McGee, J., Garcia-Arocena, D., *et al.* (2012). Signaling defects in iPSC-derived fragile X premutation neurons. *Human molecular genetics* 21, 3795-3805.
- Liu, M.-G., Wang, R.-R., Chen, X.-F., Zhang, F.-K., Cui, X.-Y., and Chen, J. (2011). Differential roles of ERK, JNK and p38 MAPK in pain-related spatial and temporal enhancement of synaptic responses in the hippocampal formation of rats: multi-electrode array recordings. *Brain research* 1382, 57-69.
- Lopes, F.M., Schröder, R., Júnior, M.L.C.d.F., Zanotto-Filho, A., Müller, C.B., Pires, A.S., Meurer, A.S., Colpo, G.D., Gelain, D.P., Kapczinski, F., *et al.* (2010). Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. *Brain Research* 1337, 8594.
- Lorenz, H., Laudon, M., and Renaud, P. (1998). Mechanical characterization of a new high-aspect-ratio near UV-photoresist. *Microelectronic Engineering* 41, 371-374.
- Lorenzo, A.C., and Yukiko, G. (2008). Actin in action: the interplay between the actin cytoskeleton and synaptic efficacy. *Nature Reviews Neuroscience*.
- Losonczy, A., Makara, J., and Magee, J. (2008). Compartmentalized dendritic plasticity and input feature storage in neurons. *Nature* 452, 436-441.
- Lovat, V., Pantarotto, D., Lagostena, L., Cacciari, B., Grandolfo, M., Righi, M., Spalluto, G., Prato, M., and Ballerini, L. (2005). Carbon nanotube substrates boost neuronal electrical signaling. *Nano letters* 5, 1107-1110.
- Luo, L., Callaway, E., and Svoboda, K. (2008). Genetic dissection of neural circuits. *Neuron* 57, 634-660.
- Makarov, V., Panetsos, F., and de Feo, O. (2005). A method for determining neural connectivity and inferring the underlying network dynamics using extracellular spike recordings. *Journal of neuroscience methods* 144, 265-279.
- Marchetto, M.C., Carromeu, C., Acab, A., Yu, D., Yeo, G.W., Mu, Y., Chen, G., Gage, F.H., and Muotri, A.R. (2010). A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. *Cell* 143, 527-539.
- Marconi, E., Nieus, T., Maccione, A., Valente, P., Simi, A., Messa, M., Dante, S., Baldelli, P., Berdondini, L., and Benfenati, F. (2012). Emergent functional properties of neuronal networks with controlled topology. *PloS one* 7.

- Massobrio, P., Tessadori, J., Chiappalone, M., and Ghirardi, M. (2015). In Vitro Studies of Neuronal Networks and Synaptic Plasticity in Invertebrates and in Mammals Using Multielectrode Arrays. *Neural plasticity* 2015, 196195.
- Matsuzaki, M., Honkura, N., Ellis-Davies, G., and Kasai, H. (2004). Structural basis of long-term potentiation in single dendritic spines. *Nature* 429, 761-766.
- Mazzatenta, A., Giugliano, M., Campidelli, S., Gambazzi, L., Businaro, L., Markram, H., Prato, M., and Ballerini, L. (2007). Interfacing neurons with carbon nanotubes: electrical signal transfer and synaptic stimulation in cultured brain circuits. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 27, 6931-6936.
- Melino, G., Thiele, C.J., Knight, R.A., and Piacentini, M. (1997). Retinoids and the control of growth/death decisions in human neuroblastoma cell lines. *Journal of neuro-oncology*.
- Minami, S., Sun, B., Popat, K., Kauppinen, T., Pleiss, M., Zhou, Y., Ward, M., Floreancig, P., Mucke, L., Desai, T., *et al.* (2012). Selective targeting of microglia by quantum dots. *Journal of neuroinflammation* 9, 22.
- Ming, G.-L.L., Brüstle, O., Muotri, A., Studer, L., Wernig, M., and Christian, K.M. (2011). Cellular reprogramming: recent advances in modeling neurological diseases. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 31, 16070-16075.
- Mitchell, K. (2011). The genetics of neurodevelopmental disease. *Current opinion in neurobiology* 21, 197-203.
- Morin, F., Nishimura, N., Griscom, L., Lepioufle, B., Fujita, H., Takamura, Y., and Tamiya, E. (2006). Constraining the connectivity of neuronal networks cultured on microelectrode arrays with microfluidic techniques: a step towards neuron-based functional chips. *Biosens Bioelectron* 21, 1093-1100.
- Morin, F.O., Takamura, Y., and Tamiya, E. (2005). Investigating neuronal activity with planar microelectrode arrays: achievements and new perspectives. *J Biosci Bioeng* 100, 131-143.
- Muglia, P. (2011). From genes to therapeutic targets for psychiatric disorders - what to expect? *Current opinion in pharmacology* 11, 563-571.
- Nam, Y., and Wheeler, B. (2011). In vitro microelectrode array technology and neural recordings. *Critical reviews in biomedical engineering* 39, 45-61.
- Nemani, K.V., Moodie, K.L., Brennick, J.B., Su, A., and Gimi, B. (2013). In vitro and in vivo evaluation of SU-8 biocompatibility. *Materials science & engineering C, Materials for biological applications* 33, 4453-4459.
- Network, T. (2015). Psychiatric genome-wide association study analyses implicate neuronal, immune and histone pathways. *Nature neuroscience* 18, 199-209.

- Nguyen-Vu, T., Chen, H., Cassell, A., Andrews, R., Meyyappan, M., and Li, J. (2006). Vertically aligned carbon nanofiber arrays: an advance toward electrical-neural interfaces. *Small* (Weinheim an der Bergstrasse, Germany) 2, 89-94.
- Nimchinsky, E., Sabatini, B., and Svoboda, K. (2002). Structure and function of dendritic spines. *Annual review of physiology* 64, 313-353.
- Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* (New York, NY) 308, 1314-1318.
- Nune, S., Gunda, P., Thallapally, P., Lin, Y.-Y., Forrest, M., and Berkland, C. (2009). Nanoparticles for biomedical imaging. *Expert opinion on drug delivery* 6, 1175-1194.
- Nunes, A., Al-Jamal, K., and Kostarelos, K. (2012). Therapeutics, imaging and toxicity of nanomaterials in the central nervous system. *Journal of controlled release : official journal of the Controlled Release Society* 161, 290-306.
- Obien, M.E., Deligkaris, K., Bullmann, T., Bakkum, D.J., and Frey, U. (2014). Revealing neuronal function through microelectrode array recordings. *Frontiers in neuroscience* 8, 423.
- Ohki, K., Chung, S., Ch'ng, Y., Kara, P., and Reid, R. (2005). Functional imaging with cellular resolution reveals precise micro-architecture in visual cortex. *Nature* 433, 597-603.
- Palui, G., Avellini, T., Zhan, N., Pan, F., Gray, D., Alabugin, I., and Mattoussi, H. (2012). Photoinduced phase transfer of luminescent quantum dots to polar and aqueous media. *Journal of the American Chemical Society* 134, 16370-16378.
- Pancrazio, J. (2008). Neural interfaces at the nanoscale. *Nanomedicine* (London, England) 3, 823-830.
- Pathak, S., Cao, E., Davidson, M.C., Jin, S., and Silva, G.A. (2006). Quantum dot applications to neuroscience: new tools for probing neurons and glia. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 26, 1893-1895.
- Penzes, P., Cahill, M., Jones, K., VanLeeuwen, J.-E., and Woolfrey, K. (2011). Dendritic spine pathology in neuropsychiatric disorders. *Nature neuroscience* 14, 285-293.
- Pine, J. (1980). Recording action potentials from cultured neurons with extracellular microcircuit electrodes. *Journal of neuroscience methods* 2, 19-31.
- Potter, S.M., and DeMarse, T.B. (2001). A new approach to neural cell culture for long-term studies. *J Neurosci Methods* 110, 17-24.
- Pregi, N., Wenker, S., Vittori, D., Leirós, C., and Nesse, A. (2009). TNF-alpha-induced apoptosis is prevented by erythropoietin treatment on SH-SY5Y cells. *Experimental cell research* 315, 419-431.

- Provenzale, J.M., and Silva, G., A. (2009). Uses of Nanoparticles for Central Nervous System Imaging and Therapy. *American Journal of Neuroradiology* 30, 1293-1301.
- Rath, P.C., and Aggarwal, B.B. (1999). TNF-induced signaling in apoptosis. *Journal of clinical immunology* 19, 350-364.
- Robinton, D., and Daley, G. (2012). The promise of induced pluripotent stem cells in research and therapy. *Nature* 481, 295-305.
- Ropers, H. (2010). Genetics of early onset cognitive impairment. *Annual review of genomics and human genetics* 11, 161-187.
- Santra, S., Yang, H., Stanley, J., Holloway, P., Moudgil, B., Walter, G., and Mericle, R. (2005). Rapid and effective labeling of brain tissue using TAT-conjugated CdS:Mn/ZnS quantum dots. *Chemical communications (Cambridge, England)*, 3144-3146.
- Scazziani, M., and Häusser, M. (2009). Electrophysiology in the age of light. *Nature* 461, 930-939.
- Scott, A., Weir, K., Easton, C., Huynh, W., Moody, W., and Folch, A. (2012). A microfluidic microelectrode array for simultaneous electrophysiology, chemical stimulation, and imaging of brain slices. *Lab on a chip*.
- Seidlits, S., Lee, J., and Schmidt, C. (2008). Nanostructured scaffolds for neural applications. *Nanomedicine (London, England)* 3, 183-199.
- Shahaf, G., and Marom, S. (2001). Learning in networks of cortical neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 21, 8782-8788.
- Silva, G., A. (2006). Neuroscience nanotechnology: progress, opportunities and challenges. *Nature reviews Neuroscience*.
- Smith, A., Duan, H., Mohs, A., and Nie, S. (2008). Bioconjugated quantum dots for in vivo molecular and cellular imaging. *Advanced drug delivery reviews* 60, 1226-1240.
- Southwell, D.G., Froemke, R.C., Alvarez-Buylla, A., Stryker, M.P., and Gandhi, S.P. (2010). Cortical plasticity induced by inhibitory neuron transplantation. *Science (New York, NY)* 327, 1145-1148.
- Städele, C., Andras, P., and Stein, W. (2012). Simultaneous measurement of membrane potential changes in multiple pattern generating neurons using voltage sensitive dye imaging. *Journal of neuroscience methods* 203, 78-88.
- Stegenga, J., Le Feber, J., Marani, E., and Rutten, W. (2008). Analysis of cultured neuronal networks using intraburst firing characteristics. *IEEE transactions on bio-medical engineering* 55, 1382-1390.
- Steidl, E., Neveu, E., Bertrand, D., and Buisson, B. (2006). The adult rat hippocampal slice revisited with multi-electrode arrays. *Brain research*.
- Stoeckli, E. (2012). What does the developing brain tell us about neural diseases? *The European journal of neuroscience* 35, 1811-1817.

Stolp, H., Neuhaus, A., Sundramoorthi, R., and Molnár, Z. (2012). The Long and the Short of it: Gene and Environment Interactions During Early Cortical Development and Consequences for Long-Term Neurological Disease. *Frontiers in psychiatry / Frontiers Research Foundation* 3, 50.

Su, H.-C., Lin, C.-M., Yen, S.-J., Chen, Y.-C., Chen, C.-H., Yeh, S.-R., Fang, W., Chen, H., Yao, D.-J., Chang, Y.-C., *et al.* (2010). A cone-shaped 3D carbon nanotube probe for neural recording. *Biosensors & bioelectronics* 26, 220-227.

Suyama, K., Daikoku, S., Funabashi, T., and Kimura, F. (2004). Effects of GABA and bicuculline on the electrical activity of rat olfactory placode neurons derived at E13.5 and cultured for 1 week on multi-electrode dishes. *Endocrine journal* 51, 171-176.

Takayama, Y., Kotake, N., Haga, T., Suzuki, T., and Mabuchi, K. (2011). Microfabrication- and microfluidics-based patterning of cultured neuronal network. Conference proceedings : Annual International Conference of the IEEE Engineering in Medicine and Biology Society IEEE Engineering in Medicine and Biology Society Conference 2011, 3613-3616.

Tijero, M., Gabriel, G., Caro, J., Altuna, A., Hernandez, R., Villa, R., Berganzo, J., Blanco, F.J., Salido, R., and Fernandez, L.J. (2009). SU-8 microprobe with microelectrodes for monitoring electrical impedance in living tissues. *Biosensors & bioelectronics* 24, 2410-2416.

Tomalia, D., Reyna, L., and Svenson, S. (2007). Dendrimers as multi-purpose nanodevices for oncology drug delivery and diagnostic imaging. *Biochemical Society transactions* 35, 61-67.

Tosi, G., Bondioli, L., Ruozi, B., Badiali, L., Severini, G., Biffi, S., De Vita, A., Bortot, B., Dolcetta, D., Forni, F., *et al.* (2011). NIR-labeled nanoparticles engineered for brain targeting: in vivo optical imaging application and fluorescent microscopy evidences. *Journal of neural transmission (Vienna, Austria : 1996)* 118, 145-153.

Tropea, D., Giacometti, E., Wilson, N., Beard, C., McCurry, C., Fu, D., Flannery, R., Jaenisch, R., and Sur, M. (2009). Partial reversal of Rett Syndrome-like symptoms in MeCP2 mutant mice. *Proceedings of the National Academy of Sciences of the United States of America* 106, 2029-2034.

Tropea, D., Majewska, A., Garcia, R., and Sur, M. (2010). Structural dynamics of synapses in vivo correlate with functional changes during experience-dependent plasticity in visual cortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 30, 11086-11095.

Tsoi, K.M., Dai, Q., Alman, B.A., and Chan, W.C. (2013). Are quantum dots toxic? Exploring the discrepancy between cell culture and animal studies. *Accounts of chemical research* 46, 662-671.

- Valtorta, F., Pozzi, D., Benfenati, F., and Fornasiero, E.F. (2011). The synapsins: multitask modulators of neuronal development. *Seminars in cell & developmental biology* 22, 378-386.
- Van Pelt, J., Corner, M., Wolters, P., Rutten, W., and Ramakers, G. (2004). Longterm stability and developmental changes in spontaneous network burst firing patterns in dissociated rat cerebral cortex cell cultures on multielectrode arrays. *Neuroscience letters* 361, 86-89.
- Wagenaar, D., Pine, J., and Potter, S. (2004). Effective parameters for stimulation of dissociated cultures using multi-electrode arrays. *Journal of neuroscience methods* 138, 27-37.
- Wagenaar, D.A., Madhavan, R., Pine, J., and Potter, S.M. (2005). Controlling bursting in cortical cultures with closed-loop multi-electrode stimulation. *The Journal of neuroscience* 25, 680-688.
- Walther, F., Heckl, W.M., and Stark, R.W. (2008). Evaluation of nanoscale roughness measurements on a plasma treated SU-8 polymer surface by atomic force microscopy. *Applied Surface Science* 254, 72907295.
- Wang, B.-S.S., Sarnaik, R., and Cang, J. (2010). Critical period plasticity matches binocular orientation preference in the visual cortex. *Neuron* 65, 246-256.
- Wang, J., Wu, C., Hu, N., Zhou, J., Du, L., and Wang, P. (2012). Microfabricated electrochemical cell-based biosensors for analysis of living cells in vitro. *Biosensors*.
- Wang, S.S.H., Denk, W., and Häusser, M. (2000). Coincidence detection in single dendritic spines mediated by calcium release. *Nature neuroscience*.
- Wang, Z., Stangegaard, M., and Dufva, M. (2005). A simple hydrophilic treatment of SU-8 surfaces for cell culturing and cell patterning. *Systems for Chemistry*.
- Wells, C., and Jones, G. (2010). The emerging importance of group II PAKs. *The Biochemical journal* 425, 465-473.
- Wells, R.G. (2008). The role of matrix stiffness in regulating cell behavior. *Hepatology* 47, 1394-1400.
- Wen, C.-J., Zhang, L.-W., Al-Suwayeh, S., Yen, T.-C., and Fang, J.-Y. (2012). Theranostic liposomes loaded with quantum dots and apomorphine for brain targeting and bioimaging. *International journal of nanomedicine* 7, 1599-1611.
- Wilt, B., Burns, L., Wei Ho, E., Ghosh, K., Mukamel, E., and Schnitzer, M. (2009). Advances in light microscopy for neuroscience. *Annual review of neuroscience* 32, 435-506.
- Xia, Y., Gopal, K., and Gross, G. (2003). Differential acute effects of fluoxetine on frontal and auditory cortex networks in vitro. *Brain research* 973, 151-160.

- Xia, Y., and Gross, G.W. (2003). Histiotypic electrophysiological responses of cultured neuronal networks to ethanol. *Alcohol* 30, 167-174.
- Xing, J.Z., Zhu, L., Gabos, S., and Xie, L. (2006). Microelectronic cell sensor assay for detection of cytotoxicity and prediction of acute toxicity. *Toxicology in vitro*.
- Yamagata, Y., Kobayashi, S., Umeda, T., Inoue, A., Sakagami, H., Fukaya, M., Watanabe, M., Hatanaka, N., Totsuka, M., Yagi, T., *et al.* (2009). Kinase-dead knock-in mouse reveals an essential role of kinase activity of Ca²⁺/calmodulin-dependent protein kinase IIalpha in dendritic spine enlargement, long-term potentiation, and learning. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29, 7607-7618.
- Young, E., and Beebe, D. (2010). Fundamentals of microfluidic cell culture in controlled microenvironments. *Chemical Society reviews* 39, 1036-1048.
- Zhang, F., Aravanis, A., Adamantidis, A., de Lecea, L., and Deisseroth, K. (2007). Circuit-breakers: optical technologies for probing neural signals and systems. *Nature reviews Neuroscience* 8, 577-581.
- Zhang, F., Matthias, P., Florent, B., Satoshi, P.T., Joanna, M., Ofer, Y., Peter, H., and Karl, D. (2008). Red-shifted optogenetic excitation: a tool for fast neural control derived from *Volvox carteri*. *Nature Neuroscience* 11.
- Zhang, X., S, P., S, N., A, M., M, O., and C, O. (2005). Guided neurite growth on patterned carbon nanotubes. *Sensors and Actuators B: Chemical* 106.
- Zhao, H., Li, L., Fan, H.J., Wang, F., Jiang, L.M., and He, P.G. (2012). Exocytosis of SH-SY5Y single cell with different shapes cultured on ITO micro-pore electrode. *Molecular and cellular*
- Zhao, Y., Inayat, S., Dikin, D.A., Singer, J.H., Ruoff, R.S., and Troy, J.B. (2008). Patch clamp technique: review of the current state of the art and potential contributions from nanoengineering. *Proceedings of the Institution of Mechanical Engineers, Part N: Journal of Nanoengineering and Nanosystems* 222.

8 Appendices

8.1 Microfabrication: Lithographic Process

The following flow chart (Figure 8.1) describes the process of lithography for the fabrication of MEA biosensors. A detailed description of each step is explained below.

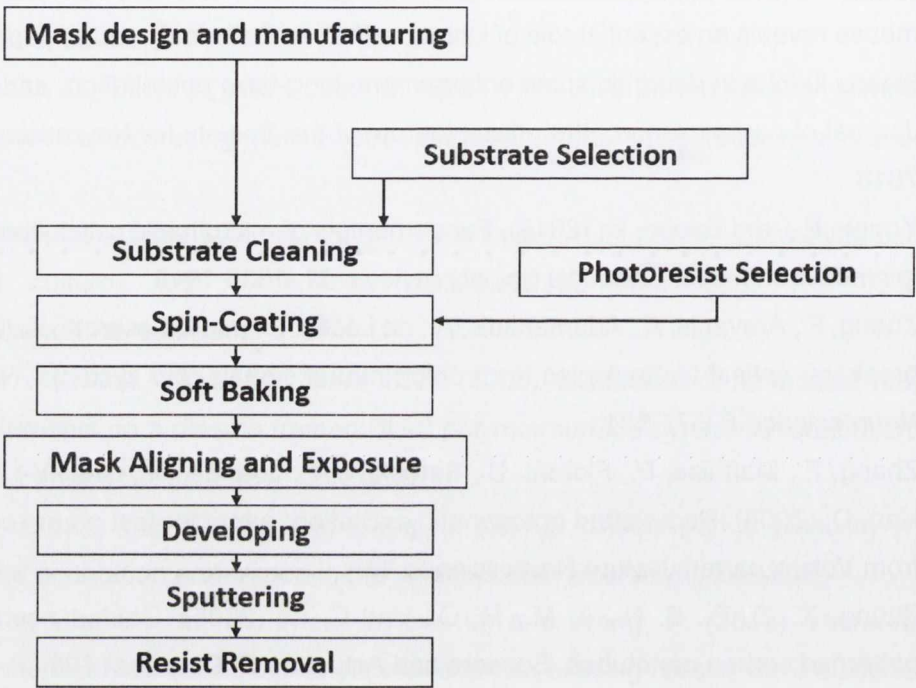


Figure 8.1 | Process of lithography for the fabrication of MEA biosensors.

8.1.1 Lithographic Mask

Lithographic mask contains the blueprints of the pattern that is to be fabricated on the selected substrate. A mask is usually made of borosilicate or quartz glass and is coated one side with chrome. The patterns that are needed for the microfabrication are etched onto the chrome using an e-beam technique. There are a few parameters to bear in mind when designing a mask: (i) pattern dimensions, (ii) pattern resolution, (iii) photoresist, (iv) lithographic process, and (v) etching techniques as indicated in the table below;

Table 8.1 | Table of parameters for mask design.

Parameter	Value
Pattern Dimension(s) (Max, Min)	1000 μm ; 5 μm
Pattern Resolution	$\pm 2 \mu\text{m}$
Photoresist	Positive/Negative
Lithographic Process	Spin coating open air system
Etching	Wet Etching

8.1.2 Photoresist

Two different photoresists were used in this work; (i) high resolution positive photoresist Microposit S1813 (Dow®, Microposit, USA), and (ii) high resolution negative photoresist SU-8 (2015, MicroChem, USA). With positive tone resists, the resist is exposed with UV light where the underlying material is to be removed. In these resists, exposure to the UV light changes the chemical structure of the resist so that it becomes more soluble in the developer. The exposed resist is then washed away by the developer solution, leaving windows of the bare underlying material. The mask, therefore, contains an exact copy of the pattern which is to remain on the wafer, as a stencil for subsequent processing.

For negative tone resists, they behave in the opposite manner. When exposed to the UV light, the negative resist becomes crosslinked/polymerized, and more difficult to dissolve in developer. Therefore, the negative resist remains on the surface of the substrate where it is exposed, and the developer solution removes only the unexposed areas. Masks used for negative photoresists, therefore, contain the inverse or photographic "negative" of the pattern to be transferred. The figure above shows the pattern differences generated from the use of a positive and negative resist.

8.1.3 Cleanroom

State of the art cleanrooms were used during the fabrication process, equipped to comply with the micro/nano fabrication processes carried out. Cleaning, spin-coating and soft-baking were carried out in a class 100 lithography cleanroom. Cleanrooms are classified

according to the number and size of particles permitted per volume of air, denoting the number of particles of size 0.5 μm or larger permitted per cubic foot of air. The class 100 room is designed to have a reduced level of contamination than the class 1000 cleanroom which was used for thin-metal deposition.

8.1.4 Substrate Cleaning

Deep cleaning was carried out using a solvent treatment technique to remove any contamination agents. A dipping technique was chosen with a mild stirring followed by a nitrogen spray cleaning and high temperature baking in order to dissolve and remove any unwanted trace of inorganic and organic contaminants.

Different reagents were used to clean the polymers and glass materials. Glass substrates were sonicated in acetone and isopropynol for 30 minutes and dipped in distilled water for two minutes to remove any organic and inorganic traces. They were then sprayed with nitrogen air for 30 seconds and dried with high-temperature baking (150°C) for 5 minutes. Polymers were dipped in isopropynol for 2 minutes and in distilled water for a further 2 minutes to remove organic and inorganic traces. They were then sprayed with nitrogen air for 30 seconds.

8.1.5 Spin Coating

The parameters for spin-coating substrates with the two types of photoresist are described in the table below:

Table 8.2 | Table of parameters for resist coating.

Resist	Resist Viscosity [g/cm3]	Spin Speed Range	Spin Time [seconds]	Room Temp. [°C]	Humidity [%]	Acc. Ramp [seconds]
Microposit S 1813	1.0	2500-5000	45	22 ±1	<40 ±2	10
SU-8	1.5	100-1000	45	22 ±1	<40 ±2	10

8.1.6 Soft Baking

Soft baking was carried out for all photoresist-spun substrates. Crucial variables were (i) baking temperature, (ii) baking time and (iii) baking atmosphere. From the S1813 data sheet, it was found that adequate soft baking required a baking temperature of 115°C for 75 seconds under room atmosphere condition of 22-24°C and 40-45% humidity to

achieve optimal resist evaporation and residual stress release. From the SU-8 data sheet, it was found that adequate soft baking required a baking temperature of 65°C for 2 minutes, followed by 95°C for 5 minutes under room atmosphere condition of 22-24°C and 40-45% humidity to achieve optimal resist evaporation and residual stress release. The soft baking was performed using a hot conductive plate (Baking stage model SB160-10427 Bibby Stuart, Sterlin Ltd, UK).

8.1.7 Mask Aligning and Exposure

The mask-aligning and exposure phase is the most important phase of microfabrication, ensuring a lithographic process with sub-micron resolution. A contact/proximity system was adopted and used for the aligning phase. The Karl Suss mask-aligner model MJB3 UV 400 had an accurate aligning stage and was equipped with a 400W Hg-UV lamp, allowing for sub-micron resolution

8.1.8 Developing

Shipley Microposit MF-319 developer, a non-ionic organic solution, was used to develop the two types of photoresist under the same ambient conditions used for spin-coating and exposure. Samples were placed in a beaker filled with the developer and manual agitation was carried out through gentle stirring of the beaker in order to have fresh developing solution reacting at the pattern site at all times. The main parameter associated with the developing process is developing time. Not controlling for development time can lead to the exposed pattern becoming under-developed or over-developed. Developing time can be influenced by the resist thickness, soft baking process and resist sensibility. Parameters for development of S1813 and SU-8 are indicated below:

Table 8.3 | Table of parameters for resist developing.

Photoresist	Developing Time	Resist Thickness	Developing Temp.
[Developer]	[seconds]	[μm]	[°C]
S1813 (MF319)	55	~ 1.3	21 ± 1
SU-8 (MF319)	55	~ 1.5	21 ± 1

8.1.9 Sputtering

Sputtering is a form of thin-film deposition that involves eroding material from a target source onto a substrate by high energy argon plasma particles. Sputtered atoms ejected into the gas phase are not in their thermodynamic equilibrium state and deposit on a substrate placed in the vacuum chamber. Glass substrates were coated with a 20 nm titanium seed layer and 100 nm gold layer using the Temescal FC-2000 evaporation system. The system comprises a 6-pocket electron beam evaporation source, a thermal

evaporation source, an ion source, a substrate infrared heater and liquid nitrogen cooler, a process gas controller and a fast-cycle load lock.

8.1.10 Resist Removal

Following the sputtering process, S1813 photoresist was stripped off using pure acetone on sonication for 30 minutes. The removal time depends on the resist thickness and 30 minutes was found to be an adequate length of time to ensure complete removal. Subsequently, the substrate was cleaned in a mild bath of deionised water and dried with compressed nitrogen gas. Pattern inspection was carried out by light microscopy.

8.2 Assay Optimisation Parameters

8.2.1 SU-8 Modification

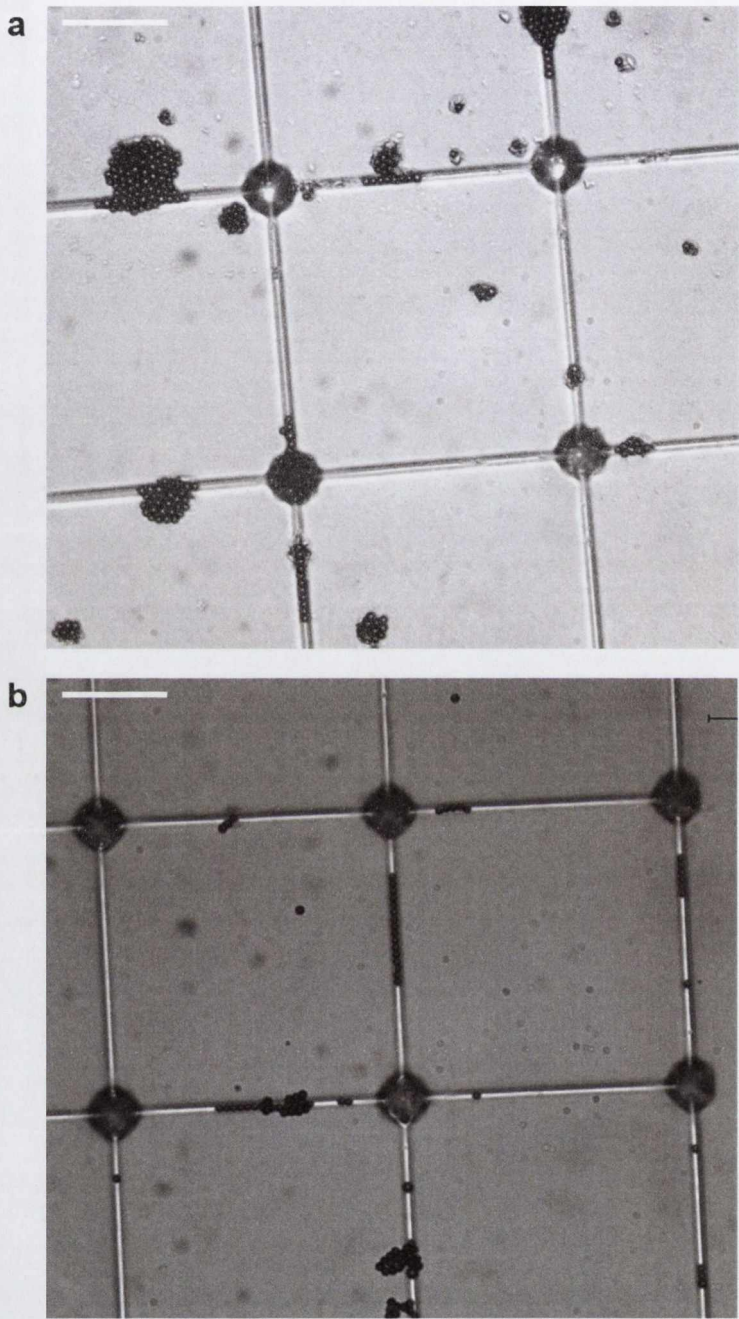


Figure 8.2 | Images of SU-8 modification with microbeads. Scale bar 50 μm

8.2.2 Neurite Outgrowth on SU-8 Interface

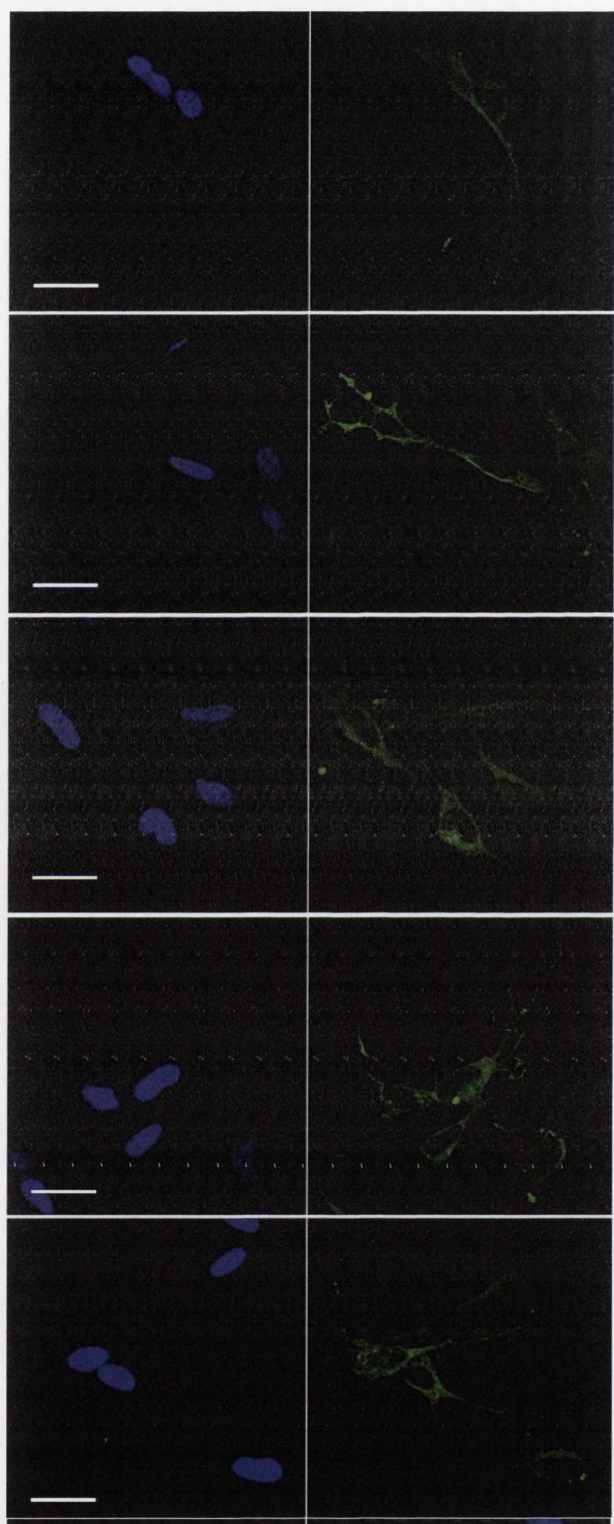
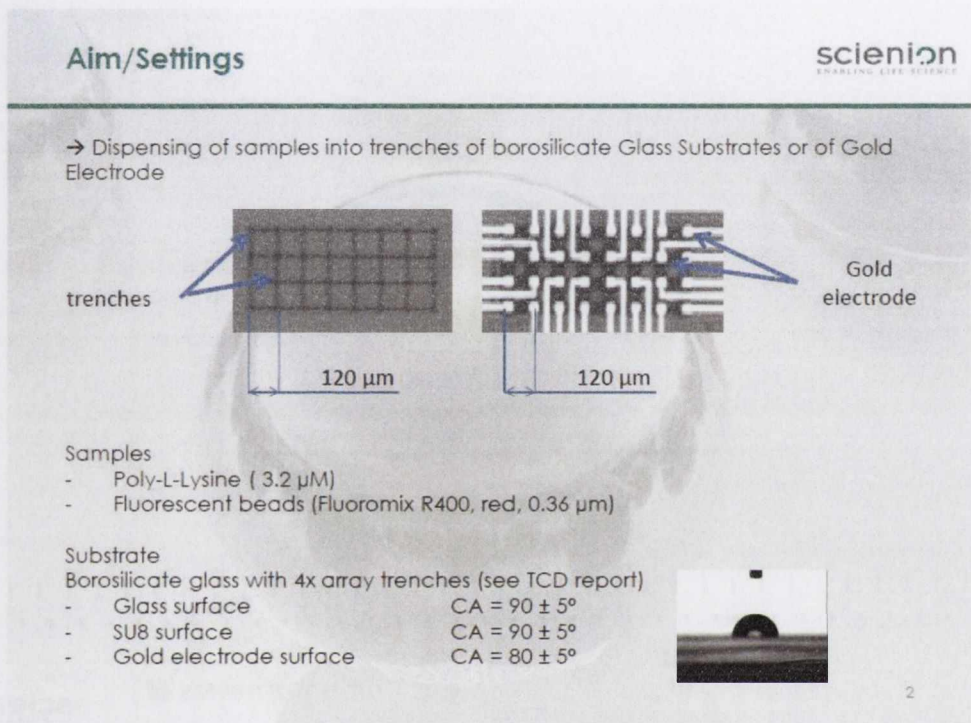
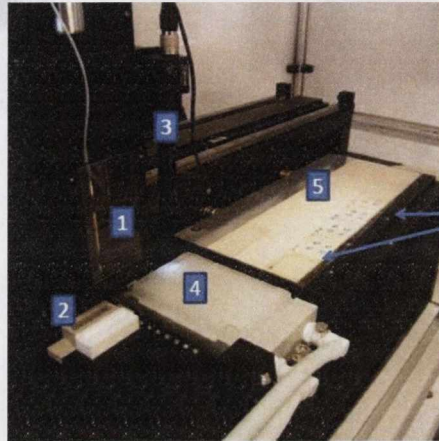


Figure 8.3 | Images of neurite outgrowth on SU-8 surfaces. Scale bar 25 μm

8.2.3 μ Drop Testing

Below are tests carried out in partnership with Scienion AG in attempt to use their μ Drop technology to precisely position neuronal cells over microelectrode sites. This approach is suggested as a further improvement to the application of MEAs to studying cultured neuronal networks.





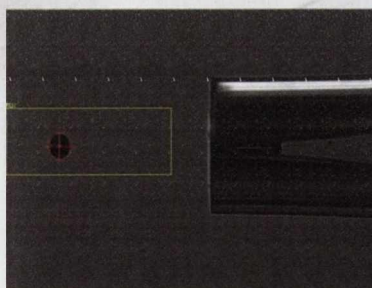
Substrate:
Borosilicate glass with trenches

- Glass surface
- SU8 surface
- Gold electrode surface

(1) Head with PDC
(2) Drop Camera
(3) Head Camera
(4) Source plate
(5) Working Area

3

Drop Camera Image - Measured parameters of drops with Trinity College samples



Drop camera image of Fluoromix solution

It was possible to generate stable printing with both solutions :

- Poly-L-Lysine (3.2 μM)
- Fluoromix R400 (beads 0.36 μm)

Measured drop volume is the same for both samples

$$V_{\text{drop}} = 210 \text{ pl}$$

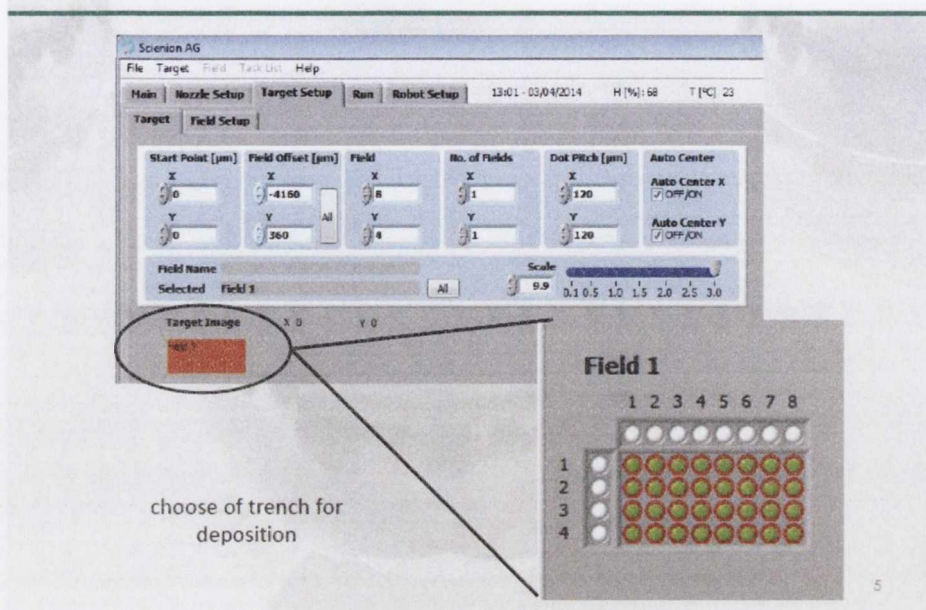
Measured diameter of drops

$$D_{\text{drop}} \approx 75 \pm 10 \mu\text{m}$$

4

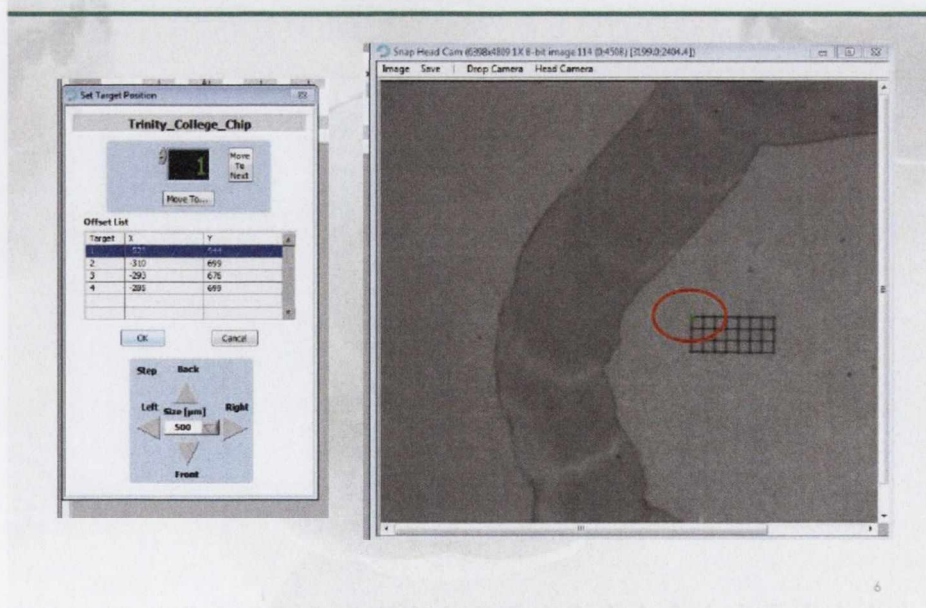
Target Setup

scienion
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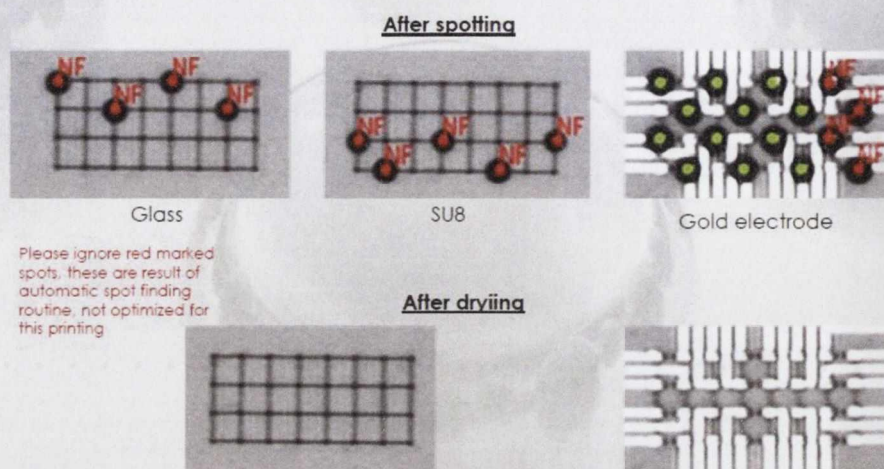
Manual Application Target Positioning

scienion
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Head Camera Images after Spotting of Poly-L-Lysine

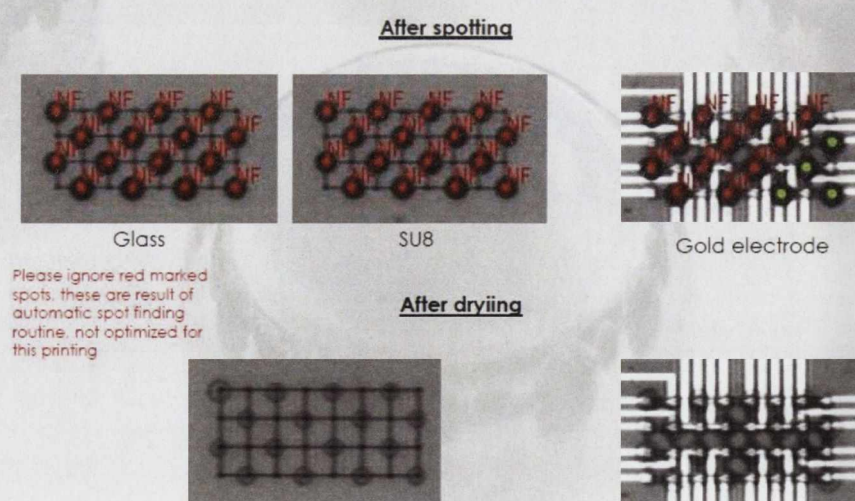
scienion
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7

Head Camera Images after Spotting of Fluoromix

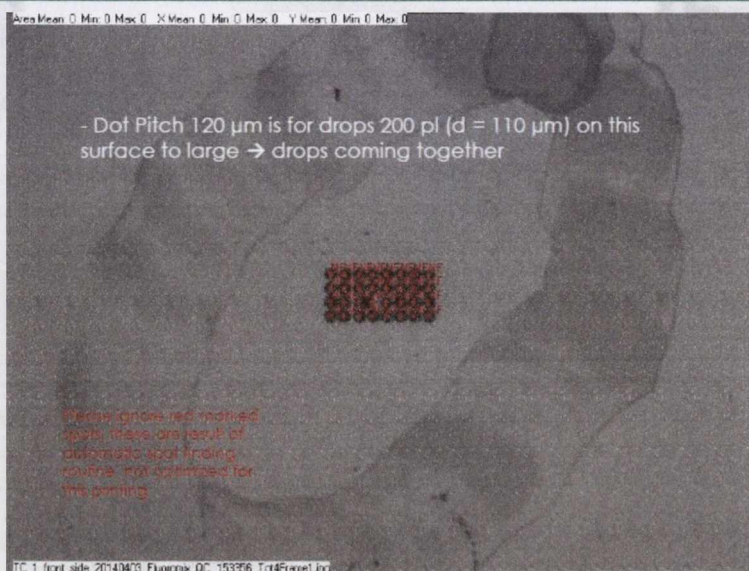
scienion
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8

Head Camera Images after Spotting of Fluoromix Full Array

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Summary

scienion
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- Stable drop generation of Poly-L-Lysine and Fluoromix bead solutions with sciFlexarrayer printing technology
- Printing of 8x4 Arrays with dot pitch of 120 μm and detection with CCD head camera
- Exact deposition in target trenches by application of sciFlexarrayer function "Target Position"
- Spots with drop volumes of 200 pl result in spot diameters of $110 \pm 10 \mu\text{m}$
- Smaller drop volumes, if required, could be realized by optimizing pulse parameter setting

→ Generation of different drop volumes with fluorescent beads (Fluoromix R400, red, 0.36 µm) from TCD for optimization of spot diameter

sciFLEXARRAYER S11

Samples

- Fluorescent beads (Fluoromix R400, red, 0.36 µm)

Substrate

sciCHIP M3

CA = 75 ± 1°

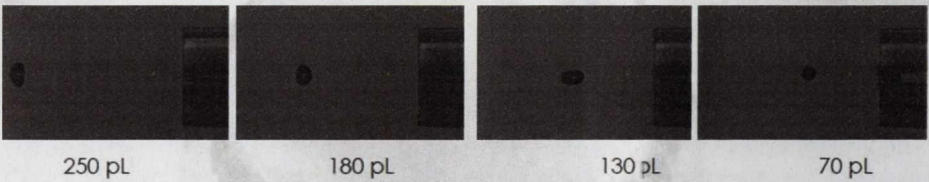
CA of substrates from TCD

- | | |
|--------------------------|--------------|
| - Glass surface | CA = 90 ± 5° |
| - SU8 surface | CA = 90 ± 5° |
| - Gold electrode surface | CA = 80 ± 5° |

2

Drop Camera Images of drops with different drop volumes

Fluoromix R400 (beads 0.36 µm)

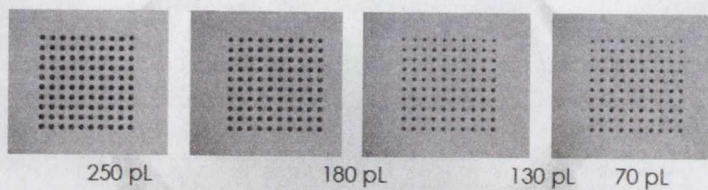


3

Head Camera Images of spotted arrays

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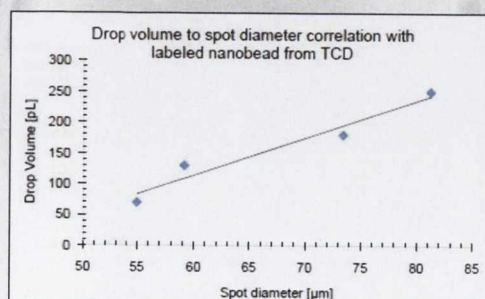
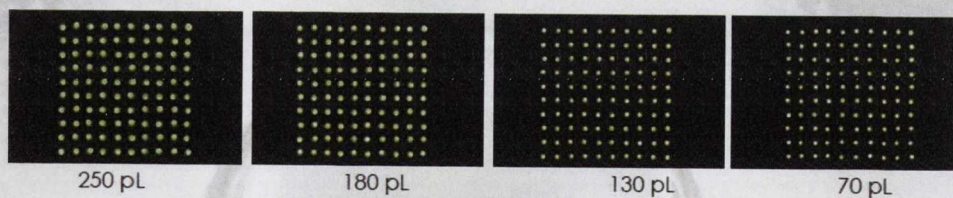
10x10 Array, Dot Pitch 250 μm
with Fluoromix R400 (beads 0.36 μm)



4

Fluorescence Images spotted arrays

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5

- sciCHIP M3 surface was chosen as substrate to have surface available with similar hydrophobicity as chips provided by TNC (CA = 75°)
 - Results show significant decrease of drop volumes by adjusting PDC parameters when printing TCD Fluoromix sample
 - The measured range of drop volumes ranges from 250 – 70 pL with resulting spot diameters between 80 – 55 μm on sciCHIP M3
- Reduced drop volumes will allow more precise loading of TCD chips with beads and cells compared to chip loading from the first test printing when 250 pL drops were spotted

8.3 Data Sheets

8.3.1 S1813

Technical Data Sheet



MICROPOSIT™ S1800™ G2 SERIES PHOTORESISTS

For Microlithography Applications

Regional Product Availability

- North America
- Europe, Middle East and Africa
- Latin America
- Asia-Pacific

Description

MICROPOSIT S1800 G2 Series Photoresists are positive photoresist systems engineered to satisfy the microelectronics industry's requirements for advanced IC device fabrication. The system has been engineered using a toxicologically-safer alternative casting solvent to the ethylene glycol derived ether acetates. The dyed photoresist versions are recommended to minimize notching and maintain linewidth control when processing on highly reflective substrates.

Advantages

Product Assurance

- Lot-to-lot consistency through state-of-the-art physical, chemical and functional testing
- Filtered to 0.2 μm absolute

Coating Properties

- Cellosolve™ acetate and xylene-free
- Striation-free coatings
- Excellent adhesion
- Excellent coating uniformity
- A variety of standard viscosities are available for single-layer processing

Exposure Properties

- Optimized for g-line exposure
- Effective for broad-band exposure
- Reflective notch and linewidth control using dyed versions

Develop Properties

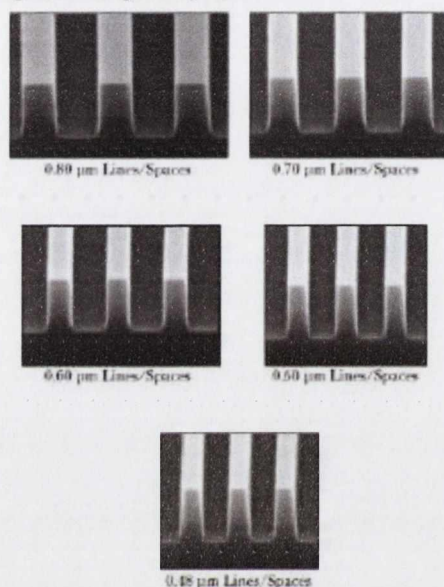
- Optimized for use with the MICROPOSIT MF™-319 metal-ion-free developer family
- Compatible with metal-ion-bearing MICROPOSIT developers

Removal Property

- Residue-free photoresist removal using standard MICROPOSIT removers

Table 1. Process Conditions (Refer to Figure 1)	
Substrate	Silicon
Photoresist	MICROPOSIT S1813 G2 Photoresist
Coat	12,302Å
Softbake	115°C/90 sec. Hotplate
Exposure	Nikon 1505 GDE, p-Line (0.54 NA), 150 mJ/cm ²
Develop	15-50 sec. Double Spray Puddle (DSP) @ 21°C

Figure 1. Masking Linearity SEMS



Instructions for Use

The following instructions cover the use of MICROPOSIT S1800 G2 Series Photoresists for all levels of microelectronic device fabrication. Exact process parameters are application and equipment dependent.

Substrate Preparation

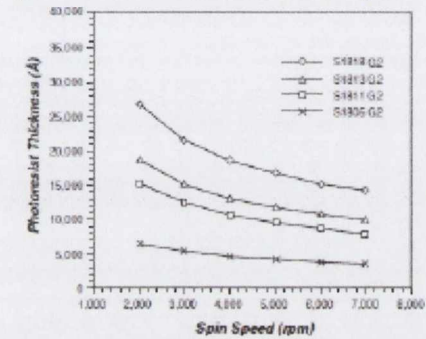
MICROPOSIT S1800 G2 Series Photoresist work well with the hexamethyldisilazane-based MICROPOSIT Primers. Concentrated MICROPOSIT Primer is recommended when vacuum vapor priming. Diluted primer is recommended for liquid phase priming applications.

Coat

MICROPOSIT S1800 G2 Series Photoresists provide uniform defect-free coatings over a wide range of film thicknesses. The film thickness versus spin speed plot displayed in Figure 2 provides the information required to properly select a MICROPOSIT S1800 G2 Photoresist version to meet process dependent thickness specifications. Maximum coating uniformity is typically attained between the spin speeds of 3,500–5,500 rpm.

Table 2. Process Conditions (Refer to Figure 2)	
Substrate	Silicon
Coat	SVG 61
Softbake	115°C/60 sec. Hotplate
Measure	Nanometrics 210

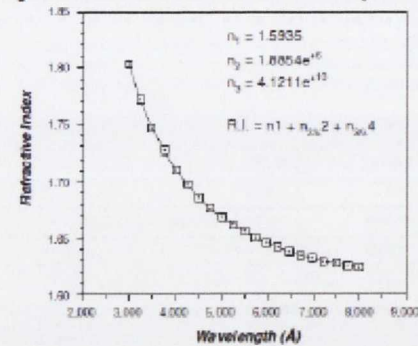
Figure 2. MICROPOSIT S1800 G2 Photoresist Undyed Series Spin Speed Curves



The dispersion curve and Cauchy equation displayed in Figure 3 describe how the refractive index of the photoresist film varies as a function of the wavelength of light incident upon the film. This information is required to program ellipsometric and other optically-based photoresist measuring equipment.

Table 3. Process Conditions (Refer to Figure 3)	
Substrate	Silicon
Coat	13,075Å
Softbake	115°C/60 sec. Hotplate
Measure	Prometric 8M300

Figure 3. MICROPOSIT S1800 G2 Photoresist Dispersion Curve

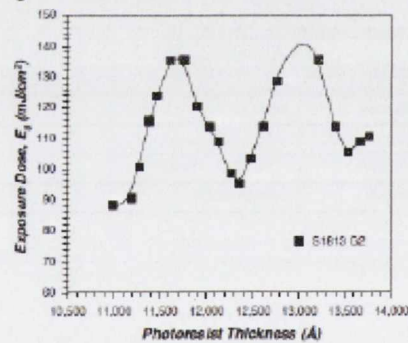


Exposure

Proper film thickness selection is critical in order to reduce photospeed and critical dimension variability. The interference curves displayed in Figure 4 illustrate the photospeed variability as a function of film thickness. The dyed version suppresses the interference effects which are more pronounced when exposing with monochromatic light sources and when using reflective substrates.

Table 4. Process Conditions (Refer to Figure 4)	
Substrate	Silicon
Coat	GCA 1006 WAFERTRAC™
Softbake	115°C/60 sec. Hotplate
Expose	GCA 8500 g-Line (0.35 NA)
Develop	MF-31/10 +30 D8P @ 21°C

Figure 4. MICROPOSIT S1813 G2 Photoresist Interference Curve



MICROPOSIT S1800 G2 Series Photoresists can be exposed with light sources in the spectral output range of 350–450 nm. The exposure properties have been optimized for use at 436 nm. Figure 5 shows the absorbance spectra for MICROPOSIT S1813 G2 photoresists.

Table 5. Process Conditions (Refer to Figures 5 and 6)	
Substrate	Quartz
Coat	12,300Å
Softbake	115°C/60 sec. Hotplate
Expose	Ortel Scanning Wedge
Develop	Hewlett Packard 8450A Spectrophotometer

Figure 5. MICROPOSIT S1813 G2 Photoresist Absorbance Spectrum

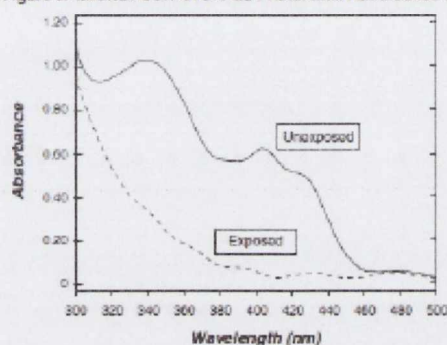


Table 6 summarizes the Dill parameters for MICROPOSIT S1800 G2 Series Photoresist. Dill parameters are used in optical exposure models such as SAMPLE and PROLITH.

Table 6. Dill Parameters				
Photoresist	365 nm		436 nm	
	A (μm^{-1})	B (μm^{-1})	A (μm^{-1})	B (μm^{-1})
S1813 G2	1.07	0.31	0.61	0.08

Figure 6 displays a contrast curve for MICROPOSIT S1813 G2 Photoresist developed with MICROPOSIT MF-321 Developer. In general, high contrast values correlate to higher wall angle profiles.

Figure 6. MICROPOSIT S1813 G2 Photoresist Contrast Curve

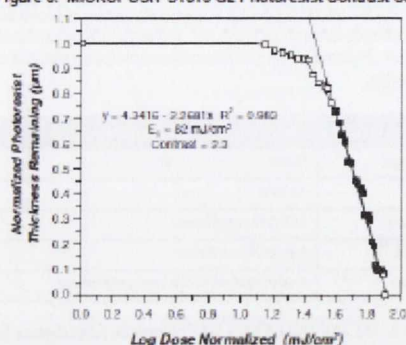


Table 7. Process Conditions (Refer to Figures 7)

Substrate	Silicon
Coat	12,300Å
Softbake	115°C/60 sec. Hotplate
Expose	GCA 8500 g-Line (0.35 NA)
Develop	MF-321/10 +30 D8F @ 21°C

Develop

MICROPOSIT S1800 G2 Series Photoresists are compatible with both metal-ion-free (MIF) and metal-ion-bearing (MIB) developers. A photoresist and developer system is dependent upon specific application requirements. Contact your local Dow Electronic Materials Technical Sales Representative for additional product information.

Figures 7-9 (next page) illustrate the lithographic functionality of MICROPOSIT S1813 G2 Photoresist using process parameters designed to maximize resolution while maintaining excellent exposure and focus latitude. The functional lithographic responses are summarized in Table 9.

Table 8. Process Conditions (Refer to Figures 8)

Substrate	Silicon
Coat	12,300Å
Softbake	115°C/60 sec. Hotplate
Expose	Nikon 1505 G6E g-Line (0.54 NA)
Develop	MF-321/10 +30 D8F @ 21°C

Figure 7. MICROPOSIT S1813 G2 Photoresist Masking Linearity Plot

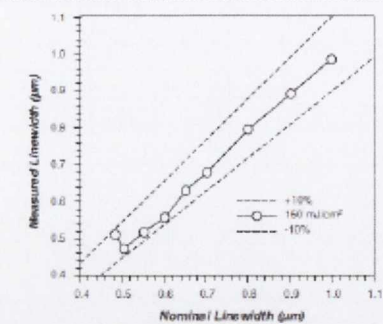


Figure 8. MICROPOSIT S1813 G2 Photoresist Exposure Latitude Plot

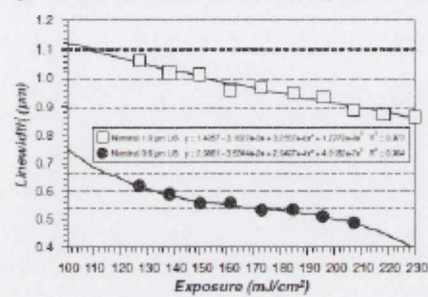


Figure 9. MICROPOSIT S1813 G2 Photoresist Focus Latitude Plot

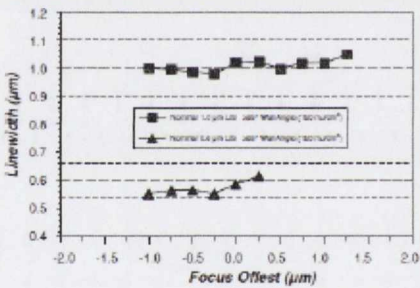


Table 8. Lithography Responses Summary		
Sizing Energy	150 mJ/cm ² (1.3 Ei)	
Resolution	0.48 μ m	
Masking Linearity ($\pm$ 10% CD)	0.50 μ m	
	1.0 μ m L/S	0.60 μ m L/S
Exposure Latitude ($\pm$ 10% CD)	65%	45%
Focus Latitude ($\pm$ 10% CD)	2.25 μ m	1.25 μ m
$\leq$ 85° Well Angle		

Handling Precautions

Before using this product, associated generic chemicals or the analytical reagents required for its control, consult the supplier's Material Safety Data Sheet (MSDS)/Safety Data Sheet (SDS) for details on material hazards, recommended handling precautions and product storage.

CAUTION! Keep combustible and/or flammable products and their vapors away from heat, sparks, flames and other sources of ignition including static discharge. Processing or operating at temperatures near or above product flashpoint may pose a fire hazard. Use appropriate grounding and bonding techniques to manage static discharge hazards.

CAUTION! Failure to maintain proper volume level when using immersion heaters can expose tank and solution to excessive heat resulting in a possible combustion hazard, particularly when plastic tanks are used.

Storage

Store products in tightly closed original containers at temperatures recommended on the product label.

Disposal Considerations

Dispose in accordance with all local, state (provincial) and federal regulations. Empty containers may contain hazardous residues. This material and its container must be disposed in a safe and legal manner.

It is the user's responsibility to verify that treatment and disposal procedures comply with local, state (provincial) and federal regulations. Contact your Dow Electronic Materials Technical Representative for more information.

Product Stewardship

Dow has a fundamental concern for all who make, distribute, and use its products, and for the environment in which we live. This concern is the basis for our product stewardship philosophy by which we assess the safety, health, and environmental information on our products and then take appropriate steps to protect employee and public health and our environment. The success of our product stewardship program rests with each and every individual involved with Dow products - from the initial concept and research, to manufacture, use, sale, disposal, and recycle of each product.

Customer Notice

Dow strongly encourages its customers to review both their manufacturing processes and their applications of Dow products from the standpoint of human health and environmental quality to ensure that Dow products are not used in ways for which they are not intended or tested. Dow personnel are available to answer your questions and to provide reasonable technical support. Dow product literature, including safety data sheets, should be consulted prior to use of Dow products. Current safety data sheets are available from Dow.

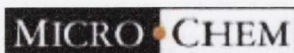
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8.3.2 SU-8



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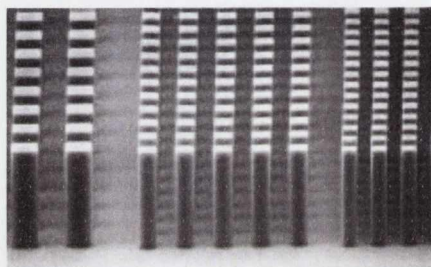
SU-8 2000

Permanent Epoxy Negative Photoresist

PROCESSING GUIDELINES FOR:

SU-8 2025, SU-8 2035, SU-8 2050 and SU-8 2075

SU-8 2000 is a high contrast, epoxy based photoresist designed for micromachining and other microelectronic applications, where a thick, chemically and thermally stable image is desired. SU-8 2000 is an improved formulation of SU-8, which has been widely used by MEMS producers for many years. The use of a faster drying, more polar solvent system results in improved coating quality and increases process throughput. SU-8 2000 is available in twelve standard viscosities. Film thicknesses of 0.5 to >200 microns can be achieved with a single coat process. The exposed and subsequently thermally cross-linked portions of the film are rendered insoluble to liquid developers. SU-8 2000 has excellent imaging characteristics and is capable of producing very high aspect ratio structures. SU-8 2000 has very high optical transmission above 360 nm, which makes it ideally suited for imaging near vertical sidewalls in very thick films. SU-8 2000 is best suited for permanent applications where it is imaged, cured and left on the device.



10 um features, 50 um SU-8 2000 coating

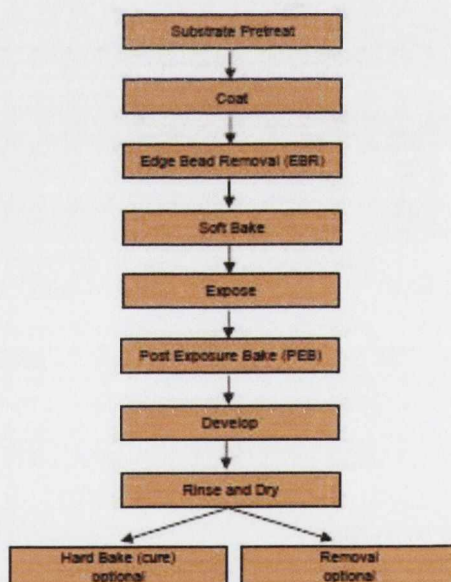
SU-8 2000 Features

- High aspect ratio imaging
- 0.5 to > 200 μm film thickness in a single coat
- Improved coating properties
- Faster drying for increased throughput
- Near UV (350-400 nm) processing
- Vertical sidewalls

Processing Guidelines

SU-8 2000 photoresist is most commonly exposed with conventional UV (350-400 nm) radiation, although i-line (365 nm) is the recommended wavelength. SU-8 2000 may also be exposed with e-beam or x-ray radiation. Upon exposure, cross-linking proceeds in two steps (1) formation of a strong acid during the exposure step, followed by (2) acid-catalyzed, thermally driven epoxy cross-linking during the post exposure bake (PEB) step. A normal process is: spin coat, soft bake, expose, PEB, followed by develop. A controlled hard bake is recommended to further cross-link the imaged SU-8 2000 structures when they will remain as part of the device. The entire process should be optimized for the specific application. The baseline information presented here is meant to be used as a starting point for determining a process.

Process Flow



Substrate Preparation

To obtain maximum process reliability, substrates should be clean and dry prior to applying SU-8 2000 resist. For best results, substrates should be cleaned with a piranha wet etch (using H_2SO_4 & H_2O_2) followed by a de-ionized water rinse. Substrates may also be cleaned using reactive ion etching (RIE) or any barrel asher supplied with oxygen. Adhesion promoters are typically not required. For applications that include electroplating, a pre-treatment of the substrate with MCC Primer 80/20 (HMDG) is recommended.

Coat

SU-8 2000 resists are available in twelve standard viscosities. This processing guideline document addresses four products: SU-8 2025, SU-8 2035, SU-8 2050 and SU-8 2075. Figure 1. provides the information required to select the appropriate SU-8 2000 resist and spin conditions to achieve the desired film thickness.

Recommended Program

- 1.) Dispense 1ml of resist for each inch (25mm) of substrate diameter.
- 2.) Spin at 500 rpm for 5-10 seconds with acceleration of 100 rpm/second.
- 3.) Spin at 2000 rpm for 30 seconds with acceleration of 300 rpm/second.

Figure 1. SU-8 2000 Spin Speed versus Thickness

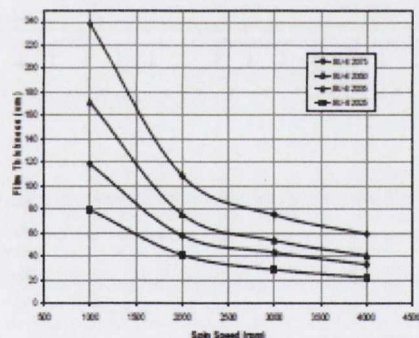


Table 1. SU-8 2000 Viscosity

SU-8 2000	% Solids	Viscosity (cSt)	Density (g/ml)
2025	68.55	4500	1.219
2035	69.95	7000	1.227
2050	71.65	12900	1.233
2075	73.45	22000	1.238

Edge Bead Removal (EBR)

During the spin coat process step, a build up of photoresist may occur on the edge of the substrate. In order to minimize contamination of the hotplate, this thick bead should be removed. This can be accomplished by using a small stream of solvent (MicroChem's EBR PG) at the edge of the wafer either at the top or from the bottom. Most automated spin coaters now have this feature and can be programmed to do this automatically.

By removing any edge bead, the photomask can be placed into close contact with the wafer, resulting in improved resolution and aspect ratio.

Soft Bake

A level hotplate with good thermal control and uniformity is recommended for use during the Soft Bake step of the process. Convection ovens are not recommended. During convection oven baking, a skin may form on the resist. This skin can inhibit the evolution of solvent, resulting in incomplete drying of the film and/or extended bake times. Table 2. shows the recommended Soft Bake temperatures and times for the various SU-8 2000 products at selected film thicknesses.

Note: To optimize the baking times/conditions, remove the wafer from the hotplate after the prescribed time and allow it to cool to room temperature. Then, return the wafer to the hotplate. If the film 'wrinkles', leave the wafer on the hotplate for a few more minutes. Repeat the cool-down and heat-up cycle until 'wrinkles' are no longer seen in the film.

THICKNESS microns	SOFT BAKE TIMES	
	(65°C) minutes	(95°C) minutes
25 - 40	0 - 3	5 - 6
45 - 80	0 - 3	6 - 9
85 - 110	5	10 - 20
115 - 150	5	20 - 30
160 - 225	7	30 - 45

Table 2. Soft Bake Times

Optical Parameters

The dispersion curve and Cauchy coefficients are shown in Figure 3. This information is useful for film thickness measurements based on ellipsometry and other optical measurements.

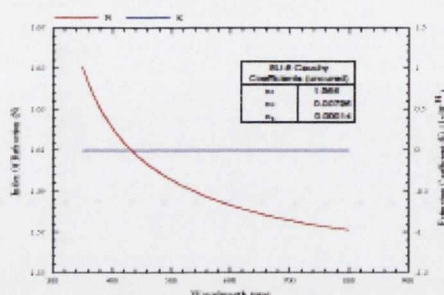


Figure 3. Cauchy Coefficients

Exposure

To obtain vertical sidewalls in the SU-8 2000 resist, we recommend the use of a long pass filter to eliminate UV radiation below 350 nm. With the recommended filter (PL-360-LP) from Omega Optical (www.omegafilters.com) or Asahi Technoglass filters V-42 plus UV-D35 (www.atgc.co.jp), an increase in exposure time of approximately 40% is required to reach the optimum exposure dose.

Note: With optimal exposure, a visible latent image will be seen in the film within 5-15 seconds after being placed on the PEB hotplate and not before. An exposure matrix experiment should be performed to determine the optimum dosage.

THICKNESS microns	EXPOSURE ENERGY mJ/cm ²
25 - 40	150 - 160
45 - 80	150 - 215
85 - 110	215 - 240
115 - 150	240 - 260
160 - 225	260 - 350

Table 3. Exposure Dose

	RELATIVE DOSE
Silicon	1X
Glass	1.5X
Pyrex	1.5X
Indium Tin Oxide	1.5X
Silicon Nitride	1.5 - 2X
Gold	1.5 - 2X
Aluminum	1.5 - 2X
Nickel Iron	1.5 - 2X
Copper	1.5 - 2X
Nickel	1.5 - 2X
Titanium	1.5 - 2X

Table 4. Exposure Doses for Various Substrates

Post Exposure Bake (PEB)

PEB should take place directly after exposure. Table 5. shows the recommended times and temperatures

Note: After 1 minute of PEB at 95°C, an image of the mask should be visible in the SU-8 2000 photoresist coating. If no visible latent image is seen during or after PEB this means that there was insufficient exposure, heating or both.

THICKNESS microns	PEB TIME (65°C)* minutes	PEB TIME (95°C) minutes
25 - 40	1	5 - 6
45 - 80	1 - 2	6 - 7
85 - 110	2 - 5	8 - 10
115 - 150	5	10 - 12
160 - 225	5	12 - 15

* Optional step for stress reduction

Table 5. Post Exposure Bake Times

Development

SU-8 2000 photoresist has been designed for use in Immersion, spray or spray-puddle processes with MicroChem's SU-8 developer. Other solvent based developers such as ethyl lactate and diacetone alcohol may also be used. Strong agitation is recommended when developing high aspect ratio and/or thick film structures. The recommended development times for immersion processes are given in Table 6. These development times are approximate, since actual dissolution rates can vary widely as a function of agitation

Note: The use of an ultrasonic or megasonic bath may be helpful when developing out via or hole patterns or structures with tight pitch.

THICKNESS	DEVELOPMENT TIME
microns	minutes
25 - 40	4 - 5
45 - 75	5 - 7
80 - 110	7 - 10
115 - 150	10 - 15
160 - 225	15 - 17

Table 6. Development Times for SU-8 Developer

Rinse and Dry

When using SU-8 developer, spray and wash the developed image with fresh solution for approximately 10 seconds, followed by a second spray/wash with Isopropyl Alcohol (IPA) for another 10 seconds. Air dry with filtered, pressurized air or nitrogen.

Note: A white film produced during IPA rinse is an indication of underdevelopment of the unexposed photoresist. Simply immerse or spray the substrate with additional SU-8 developer to remove the white film and complete the development process. Repeat the rinse step.

The use of an ultrasonic or megasonic bath will energize the solvent and allow for more effective development of the unexposed resist.

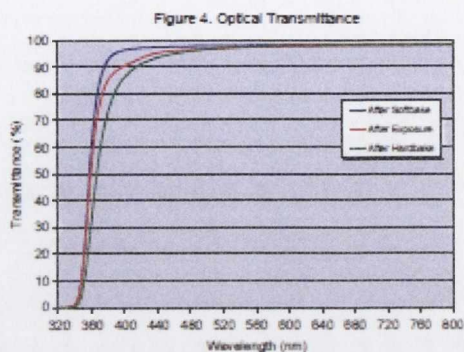
Physical Properties

(Approximate values)

Adhesion Strength (mPa) Silicon/Glass/Glass & HMDS	38/35/35
Glass Transition Temperature (T _g °C), tan δ peak	210
Thermal Stability (°C @ 5% wt. loss)	315
Thermal Conductivity (W/mK)	0.3
Coeff. of Thermal Expansion (CTE ppm)	52
Tensile Strength (Mpa)	60
Elongation at break (εb %)	6.5
Young's Modulus (Gpa)	2.0
Dielectric Constant @ 10MHz	3.2
Water Absorption (% 85°C/85 RH)	0.65

Table 7. Physical Properties

Optical Properties



Process conditions for Figure 4.

Softbake: 5 minutes at 95°C

Exposure: 180 mJ/cm²

Hardbake: 30 minutes at 300°C

Hard Bake (cure)

SU-8 2000 has good mechanical properties. However, for applications where the imaged resist is to be left as part of the final device, a hard bake can be incorporated into the process. This is generally only required if the final device or part is to be subject to thermal processing during regular operation. A hard bake or final cure step is added to ensure that SU-8 2000 properties do not change in actual use. SU-8 2000 is a thermal resin and as such its properties can continue to change when exposed to a higher temperature than previously encountered. We recommend using a final bake temperature 10°C higher than the maximum expected device operating temperature. Depending on the degree of cure required, a bake temperature in the range of 150°C to 250°C and for a time between 5 and 30 minutes is typically used.

Note: The hard bake step is also useful for annealing any surface cracks that may be evident after development. The recommended step is to bake at 160°C for a couple of minutes. This applies to all film thicknesses.

Removal

SU-8 2000 has been designed as a permanent, highly cross-linked epoxy material and it is extremely difficult to remove it with conventional solvent based resist strippers. MicroChem's Remover PG will swell and lift off minimally cross-linked SU-8 2000. However, if OmniCoat (30-100 nm) has been applied, immersion in Remover PG can effect a clean and thorough Lift-Off of the SU-8 2000 material. Fully cured or hard baked SU-8 2000 cannot be removed without the use of OmniCoat.

To remove minimally cross-linked SU-8 2000, or when using OmniCoat: Heat the Remover PG bath to 50-80°C and immerse the substrates for 30-90 minutes. Actual strip time will depend on resist thickness and cross-link density. For more information on MicroChem OmniCoat and Remover PG please see the relevant product data sheets.

To re-work fully cross-linked SU-8 2000: Wafers can be stripped using oxidizing acid solutions such as piranha etch, plasma ash, RIE, laser ablation and pyrolysis.

Plasma Removal

RIE 200W, 80 sccm O₂, 8 sccm CF₄, 100mTorr, 10°C

Storage

Store SU-8 2000 resists upright and in tightly closed containers in a cool, dry environment away from direct sunlight at a temperature of 40-70°F (4-21°C). Store away from light, acids, heat and sources of ignition. Shelf life is thirteen months from date of manufacture.

Disposal

SU-8 2000 resists may be included with other waste containing similar organic solvents to be discarded for destruction or reclaim in accordance with local state and federal regulations. It is the responsibility of the customer to ensure the disposal of SU-8 2000 resists and residues made in observance all federal, state, and local environmental regulations.

Environmental, Health and Safety

Consult the product Material Safety Data Sheet before working with SU-8 2000 resists. Handle with care. Wear chemical goggles, chemical gloves and suitable protective clothing when handling SU-8 2000 resists. Do not get into eyes, or onto skin or clothing. Use with adequate ventilation to avoid breathing vapors or mist. In case of contact with skin, wash affected area with soap and water. In case of contact with eyes, rinse immediately with water and flush for 15 minutes lifting eyelids frequently. Get emergency medical assistance.

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MICROPOSIT[®]

MF[®]-319

DEVELOPER

MICROPOSIT MF-319 DEVELOPER is designed for high resolution semiconductor device fabrication. It has been specifically formulated for use with MICROPOSIT S1400[®] and S1800[®] SERIES PHOTO RESISTS, and is recommended where it is desirable to avoid a potential source of metal ion contamination. Although MICROPOSIT MF-319 DEVELOPER can be used in the immersion and batch spray modes, ideal use is in spray and puddle processing on inline high resolution track equipment.

Ideal for Spray and Puddle Processing

- Low volume usage per wafer layer processed
- Reduced dependence on nozzle(s) positioning
- Ease of process development

Very Low Unexposed Resist Loss

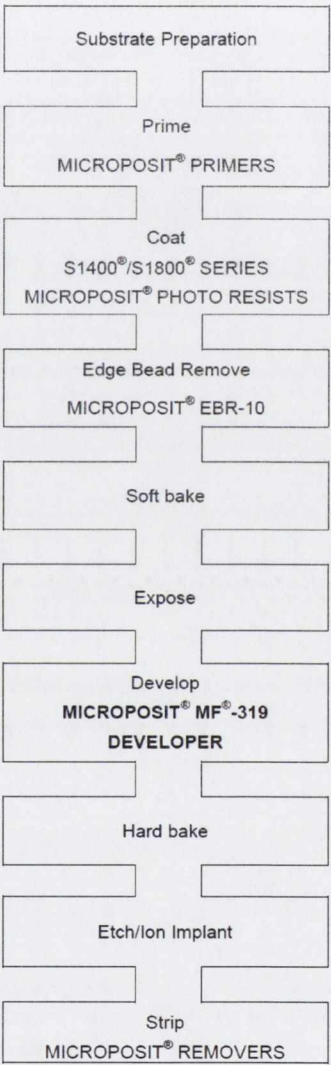
- Outstanding edge acuity
- High differential solubility
- Improved resist profiles

Clean Efficient Development

- No scumming
- Excellent resolution
- Wide process latitude
- Good exposure throughput

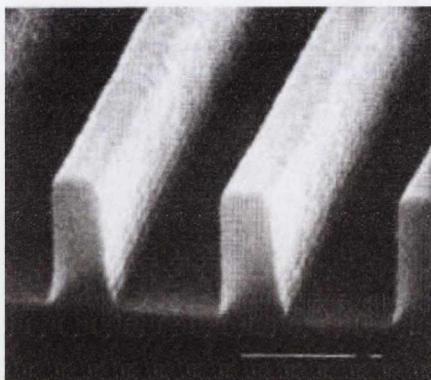
Lot to Lot Consistency

- Tight product specifications
- Complete systems functional testing
- Statistical quality control

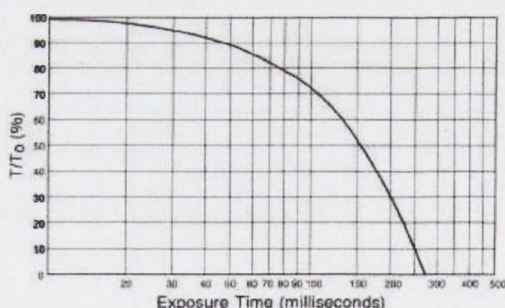


MD MF-319 B
0391

MICROPOSIT MF-319 DEVELOPER



1.23µm thick MICROPOSIT S1400-27 PHOTO RESIST was developed using MICROPOSIT MF-319 DEVELOPER on a Solitec developer track where the ultrasonic spray time was for 22 seconds. The resulting line/space patterns shown are 0.61µm photoresist structures (0.8µm nominal) separated by 0.99µm spaces. The coating process consisted of using an Eaton 6000 system, spinning at 5600 rpm, and hotplate softbaking at 110°C for 45 seconds. Exposure at 436nm for 0.175 seconds was on a GCA 8500 DSW stepper equipped with a 0.35 numerical aperture lens.



The above S1400-27/MF-319 contrast curve was generated using 1.23µm (±0.01µm) thickness of resist. Single puddle development on a GCA Wafertrac 1000 was used where the total active development was for 40 seconds at 20°C. The hotplate softbake was at 110°C for 45 seconds on a GCA Wafertrac 1000. Exposure mode was open frame at 436nm on a GCA6300 DSW stepper equipped with a Maximus 1000 illuminator, a 350W mercury lamp and a 0.30 numerical aperture lens. The coherence σ was equal to 0.5.

MICROPOSIT MF-319 DEVELOPER Page 2

Instructions for Use

I. Bath Make-up

MICROPOSIT MF-319 DEVELOPER is supplied as a ready to use solution.

II. Temperature

Recommended operation is between 15° and 20°C to ±1°C. Photospeed does decrease as temperature increases although contrast and unexposed loss remains basically constant. The rate of change of this decreasing photospeed has been found to be approximately 2% to 3% per °C.

III. Time

Immersion: 40-60 seconds

Spray and puddle: Varies with equipment.

Consult your Shipley Technical Sales Representative.

Longer development times permit the use of shorter exposure times. Shorter development times minimize developer attack on the unexposed photoresist. We recommend keeping the development time constant and adjusting the exposure time as necessary to meet critical dimension requirements.

IV. Agitation

Immersion: Keep agitation mild and consistent.

Spray and puddle: Contact your Shipley Technical Sales Representative.

V. Rinse

Immersion: A deionized water rinse is recommended using a cascade/overflow setup with rinsing continuing until a desired resistivity is reached.

Spray and puddle: An overlap deionized water rinse in conjunction with the developer cycle is recommended to prevent drying on the substrate surface.

VI. Bath Control

Immersion: For maximum process control, replace with a fresh bath at least once per shift. Cover when not in use.

Spray and puddle: Not applicable.

Batch Spray: As recommended by the equipment manufacturer.

VII. Determination of Total Alkaline Normality

Principle—The normality is determined by direct potentiometric acid-base titration.

A. Reagents and Apparatus

1. Hydrochloric acid (HCl), 1 N, standardized ± 0.001 .
2. Autotitrator (e.g. Metrohm Potentiograph or Titroprocessor) with 20.0 ml volume burette.
3. pH electrode and single junction reference electrode or combination pH electrode.
4. Magnetic stir plate.

B. Procedure (to be run in duplicate)

1. Pipette 50.0 mls of sample into a 250 ml beaker, add a magnetic stir bar and dilute to approximately 150 mls with deionized water.
2. Calibrate autotitrator with fresh pH buffer solutions (pH 4.0, 7.0, 10.0).
3. Place electrode and titrant dispenser into sample solution on magnetic stir plate. Stir rapidly without loss of solution.
4. Titrate sample with standardized 1 N HCl to approximately 2.0 mls past the inflection point.
5. Determine inflection point and calculate the volume of HCl to inflection in milliliters (mls).

C. Calculation

$$\text{NORMALITY} = \frac{\text{mls of HCl} \times \text{N of HCl}}{\text{Sample aliquot (50 mls)}}$$

Note: Reproducibility is dependent on accuracy from both operator and instrument. Analysis should be repeated until a constant value for inflection is assured.

D. Results

The normality of a fresh MICROPOSIT MF-319 DEVELOPER should be approximately 0.237 N.

Equipment

Use polypropylene, polyethylene, 316 stainless steel, polytetrafluoroethylene, or equivalent materials.

Storage

Store MICROPOSIT MF-319 DEVELOPER only in upright, original containers in a dry area at 50°-90°F (10°-32°C). Store away from acids. Do not store in sunlight. Store away from heat and sources of ignition. Keep containers sealed when not in use. MICROPOSIT MF-319 DEVELOPER has a limited shelf-life.

Waste Treatment

A used MICROPOSIT MF-319 DEVELOPER bath may be treated according to Shipley Waste Treatment Procedure WT 77-1. Contact your Shipley Technical Sales Representative for more information. It is your responsibility to verify that this procedure complies with federal, state, and local laws and regulations for wastewater discharge.

Due to the nature of MICROPOSIT MF-319 DEVELOPER, disposal of it, or residues therefrom, should be made in compliance with federal, state, and local environmental laws.

Properties as Delivered

MICROPOSIT MF-319 DEVELOPER is manufactured to the highest quality standards and is subjected to state of the art testing for physical, chemical and functional properties to assure the user of maximum lot to lot reproducibility.

MICROPOSIT MF-319 DEVELOPER is filtered to 0.2µm absolute directly into clean containers.

Certificates of Analysis will be supplied with each shipment upon request. Quality Assurance Material Specifications and Analytical Testing Procedures may be obtained upon request from your Shipley Technical Sales Representative.

MICROPOSIT MF-319 DEVELOPER has the following typical properties:

- Specific gravity at 20/20°C: Approximately 1.0
- Color: Water white to light yellow
- Turbidity: 2 NTU's maximum
- Total Alkaline Normality: 0.232 -0.242 N
- Sodium Content: 0.5 ppm maximum

Handling Precautions

DANGER! MICROPOSIT MF-319 DEVELOPER is an alkaline corrosive solution containing tetramethyl ammonium hydroxide. Contact with eyes causes burns. Contact with skin or mucous membranes may cause irritation. Handle with care. Do not get in eyes, on skin or on clothing. Avoid breathing vapors. Use adequate ventilation. Wash thoroughly after handling.

Wear chemical goggles, chemical gloves and suitable protective clothing when handling MICROPOSIT MF-319 DEVELOPER.

In case of eye or skin contact, flush affected areas with plenty of water for at least 15 minutes. Then contact a physician at once.

Consult product Material Safety Data Sheet before using.

FLUSH EMPTY CONTAINERS THOROUGHLY WITH WATER BEFORE DISCARDING.

CAUTION! When using immersion heaters, failure to maintain proper volume level can expose tank and solution to excessive heat resulting in a possible combustion hazard, particularly when plastic tanks are used.



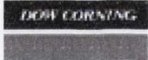
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Product Information



Encapsulants

Dow Corning®
184 Silicone Elastomer

FEATURES

- Flowable
- RT and heat cure
- High tensile strength
- Same as Sylgard 182 but with RT cure capability
- UL and Mil Spec tested

BENEFITS

- Rapid, versatile cure processing controlled by temperature
- High transparency allows easy inspection of components
- Can be considered for uses requiring UL and Mil Spec requirements

COMPOSITION

- 2-part
- 10:1 mix ratio
- Polydimethylsiloxane elastomer

APPLICATION METHODS

- Automated metered mixing and dispensing
- Manual mixing

Transparent encapsulant with good flame resistance

APPLICATIONS

- General potting applications
- Power supplies
- Connectors
- Sensors
- Industrial controls
- Transformers
- Amplifiers
- High voltage resistor packs
- Relays
- Adhesive/encapsulant for solar cells
- Adhesive handling beam lead integrated circuits during processing

TYPICAL PROPERTIES

Specification Writers: These values are not intended for use in preparing specifications. Please contact your local Dow Corning sales office or your Global Dow Corning Connection before writing specifications on this product.

Property	Unit	Value
Viscosity (Part A)	cP	5175
	mPa-sec	5175
	Po-sec	5.2
Viscosity (Mixed)	cP	3500
	mPa-sec	3500
	Po-sec	3.5
Specific Gravity (Uncured Base)	-	1.03
Specific Gravity (Cured)	-	1.04
Working Time at 25°C (Pot Life - hours)	hr	1.4
Cure Time at 25°C	hrs	48
Heat Cure Time @ 100°C	minutes	35
Heat Cure Time @ 125°C	minutes	20
Heat Cure Time @ 150°C	minutes	10

DESCRIPTION

Dow Corning® silicone encapsulants are supplied as two-part liquid component kits. When liquid components are thoroughly mixed, the mixture cures to a flexible elastomer, which is well suited for the protection of electrical/electronic applications. Dow Corning silicone encapsulants cure without exotherm at a constant rate regardless of sectional thickness or degree of confinement. Dow Corning silicone elastomers require no post cure and can be placed in service immediately following the completion of the cure schedule. Standard silicone encapsulants require a surface treatment with a primer in addition to good cleaning for adhesion while primerless silicone encapsulants require only good cleaning. Underwriters Laboratory (UL) 94 recognition is based on minimum thickness requirements. Please consult the UL Online Certifications Directory for the most accurate certification information.

MIXING AND DE-AIRING

The 10:1 mix ratio these products are supplied in gives one latitude to tune the modulus and hardness for specific application needs and production lines. In most cases de-airing is not required.

PREPARING SURFACES

In applications requiring adhesion, priming will be required for many of the silicone encapsulants. See the Primer Selection Guide for the correct primer to use with a given product. For best results, the primer should be applied in a very thin, uniform coating and then wiped off after application. After application, it should be thoroughly cured prior to application of the silicone elastomer. Additional instructions for primer usage can be found in the information sheets specific to the individual primers.

PROCESSING/CURING

Thoroughly mixed Dow Corning silicone encapsulant may be poured/dispensed directly into the

TYPICAL PROPERTIES, continued

Property	Unit	Value
Tensile Strength	psi	1025
	MPa	7.1
	kg/cm ²	71
Elongation	%	120
Tear Strength (Die B)	ppi	5
	N/cm	2
Durometer Shore A	-	44
Dielectric Strength	volts/mil	475
	kV/mm	19
Volume Resistivity	ohm*cm	2.9E+14
Dielectric Constant at 100 Hz	-	2.72
Dielectric Constant at 100 kHz	-	2.68
Dissipation Factor at 100 hz	-	0.00257
Dissipation Factor at 100 kHz	-	0.00133
Mil Specification	NA	Mil Spec
Agency Listing	-	UL 94V-0
Shelf Life at 25°C	months	24
Refractive Index @ 589 nm	-	1.4118
Refractive Index @ 632.8 nm	-	1.4225
Refractive Index @ 1321 nm	-	1.4028
Refractive Index @ 1554 nm	-	1.3997

container in which it is to be cured. Care should be taken to minimize air entrapment. When practical, pouring/dispensing should be done under vacuum, particularly if the component being potted or encapsulated has many small voids. If this technique cannot be used, the unit should be evacuated after the silicone encapsulant has been poured/dispensed. Dow Corning

silicone encapsulants may be either room temperature (25°C/77°F) or heat cured. Room temperature cure encapsulants may also be heat accelerated for faster cure. Ideal cure conditions for each product are given in the product selection table. Two-part condensation cure encapsulants should not be heat accelerated above 60°C (140°F).

POT LIFE AND CURE RATE

Cure reaction begins with the mixing process. Initially, cure is evidenced by a gradual increase in viscosity, followed by gelation and conversion to a solid elastomer. Pot life is defined as the time required for viscosity to double after Parts A and B (base and curing agent) are mixed and is highly temperature and application dependent. Please refer to the data table.

USEFUL TEMPERATURE RANGES

For most uses, silicone elastomers should be operational over a temperature range of -45 to 200°C (-49 to 392°F) for long periods of time. However, at both the low- and high-temperature ends of the spectrum, behavior of the materials and performance in particular applications can become more complex and require additional considerations. For low-temperature performance, thermal cycling to conditions such as -55°C (-67°F) may be possible, but performance should be verified for your parts or assemblies. Factors that may influence performance are configuration and stress sensitivity of components, cooling rates and hold times, and prior temperature history. At the high-temperature end, the durability of the cured silicone elastomer is time and temperature dependent. As expected, the higher the temperature, the shorter the time the material will remain useable.

COMPATIBILITY

Certain materials, chemicals, curing agents and plasticizers can inhibit the cure of addition cure adhesives. Most notable of these include: Organotin and other organometallic compounds, Silicone rubber containing organotin catalyst, Sulfur, polysulfides, polysulfones or other sulfur containing materials, unsaturated hydrocarbon plasticizers, and some solder flux residues. If a substrate or material is questionable with respect to potentially causing inhibition of cure, it is recommended that a small

scale compatibility test be run to ascertain suitability in a given application. The presence of liquid or uncured product at the interface between the questionable substrate and the cured gel indicates incompatibility and inhibition of cure.

REPAIRABILITY

In the manufacture of electrical/electronic devices it is often desirable to salvage or reclaim damaged or defective units. With most non-silicone rigid potting/encapsulating materials, removal or entry is difficult or impossible without causing excessive damage to internal circuitry. Dow Corning silicone encapsulants can be selectively removed with relative ease, any repairs or changes accomplished, and the repaired area reotted in place with additional product. To remove silicone elastomers, simply cut with a sharp blade or knife and tear and remove unwanted material from the area to be repaired. Sections of the adhered elastomer are best removed from substrates and circuitry by mechanical action such as scraping or rubbing and can be assisted by applying Dow Corning® brand OS Fluids. Before applying additional encapsulant to a repaired device, roughen the exposed surfaces of the cured encapsulant with an abrasive paper and rinse with a suitable solvent. This will enhance adhesion and permit the repaired material to become an integral matrix with the existing encapsulant. Silicone prime coats are not recommended for adhering products to themselves.

PACKAGING

In general, Dow Corning silicone 1:1 mix ratio encapsulants are supplied in nominal 0.45-, 3.6-, 18- and 200-kg (1-, 8-, 40- and 440-lb) containers, net weight. Dow Corning silicone 10:1 mix ratio encapsulants are supplied in nominal 0.5-, 5-, 25- and 225-kg (1.1-, 11-, 55- and 495-lb) containers, net weight. Packaging options may vary by product. Consult Dow Corning Customer

Service at (989) 496-6000 for additional packaging options.

USABLE LIFE AND STORAGE

Shelf life is indicated by the "Use Before" date found on the product label. Refer to the product label for storage temperature requirements. Special precautions must be taken to prevent moisture from contacting these materials. Containers should be kept tightly closed and head or air space minimized. Partially filled containers should be purged with dry air or other gases, such as nitrogen. Exposure to moisture could reduce adhesion and cause bubbles to form.

HANDLING

**PRECAUTIONS
PRODUCT SAFETY
INFORMATION REQUIRED
FOR SAFE USE IS NOT
INCLUDED IN THIS
DOCUMENT. BEFORE
HANDLING, READ PRODUCT
AND MATERIAL SAFETY DATA
SHEETS AND CONTAINER
LABELS FOR SAFE USE,
PHYSICAL AND HEALTH
HAZARD INFORMATION. THE
MATERIAL SAFETY DATA
SHEET IS AVAILABLE ON THE
DOW CORNING WEBSITE AT
WWW.DOWCORNING.COM, OR
FROM YOUR DOW CORNING
REPRESENTATIVE, OR
DISTRIBUTOR, OR BY
CALLING YOUR GLOBAL DOW
CORNING CONNECTION.**

HEALTH AND ENVIRONMENTAL INFORMATION

To support Customers in their product safety needs, Dow Corning has an extensive Product Stewardship organization and a team of Product Safety and Regulatory Compliance (PS&RC) specialists available in each area. For further information, please see our website, www.dowcorning.com or consult your local Dow Corning representative.

LIMITATIONS

This product is neither tested nor represented as suitable for medical or pharmaceutical uses.

LIMITED WARRANTY INFORMATION PLEASE READ CAREFULLY

The information contained herein is offered in good faith and is believed to be accurate. However, because conditions and methods of use of our products are beyond our control, this information should not be used in substitution for customer's tests to ensure that our products are safe, effective, and fully satisfactory for

the intended end use. Suggestions of use shall not be taken as inducements to infringe any patent. Dow Corning's sole warranty is that our products will meet the sales specifications in effect at the time of shipment. Your exclusive remedy for breach of such warranty is limited to refund of purchase price or replacement of any product shown to be other than as warranted.

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A PARTICULAR PURPOSE OR
MERCHANTABILITY.**

**DOW CORNING DISCLAIMS
LIABILITY FOR ANY
INCIDENTAL OR
CONSEQUENTIAL DAMAGES.**

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8.4 Biosensor Electronics Specifications

8.4.1 Scope

The PMEA chip is made of a glass substrate and 128 electrodes terminated by 128 pads. A frame compatible with a Zeiss microscope manual stage has been designed to hold the chip in place. It is also made of a hinge system and an interconnected PCB. The interconnected PCB contains spring loaded pins which are in contact with pads of the chip and 4 x 32 pins headers. The hinge system applies a pressure on the PCB to have good contact between pads of the PMEA chip and spring loaded pins. This document is a proposal for a design of an electronic circuit to drive specific signals to electrode to excite cells coated on the chip.

8.4.2 Circuit Architecture

The circuit architecture (Figure 8.4) is composed of two main functions:

- Generating voltage pulses between two pads with corresponding recording of current output
- Measuring impedance between two pads by frequency sweep

A dedicated PCB is fixed with a STM32F0 discovery board integrating the popular STM32F072B microcontroller.

Characterisation of current-voltage is composed of:

- A signal generation circuit which is used to generate a voltage from 0 to 3V. This voltage is then amplified to build a $\pm 3V$ signals. The microcontroller controls the width of the amplified signal, and the pulse width can be set by the software
- A current monitor which reads the current flowing through the points of connections pads of the MEA. This is measured using a high sensitivity current monitor (Texas instruments INA286). The analogue voltage at the output of the monitor is directly proportional to the current flowing through a resistor in serial with the two points of connections.

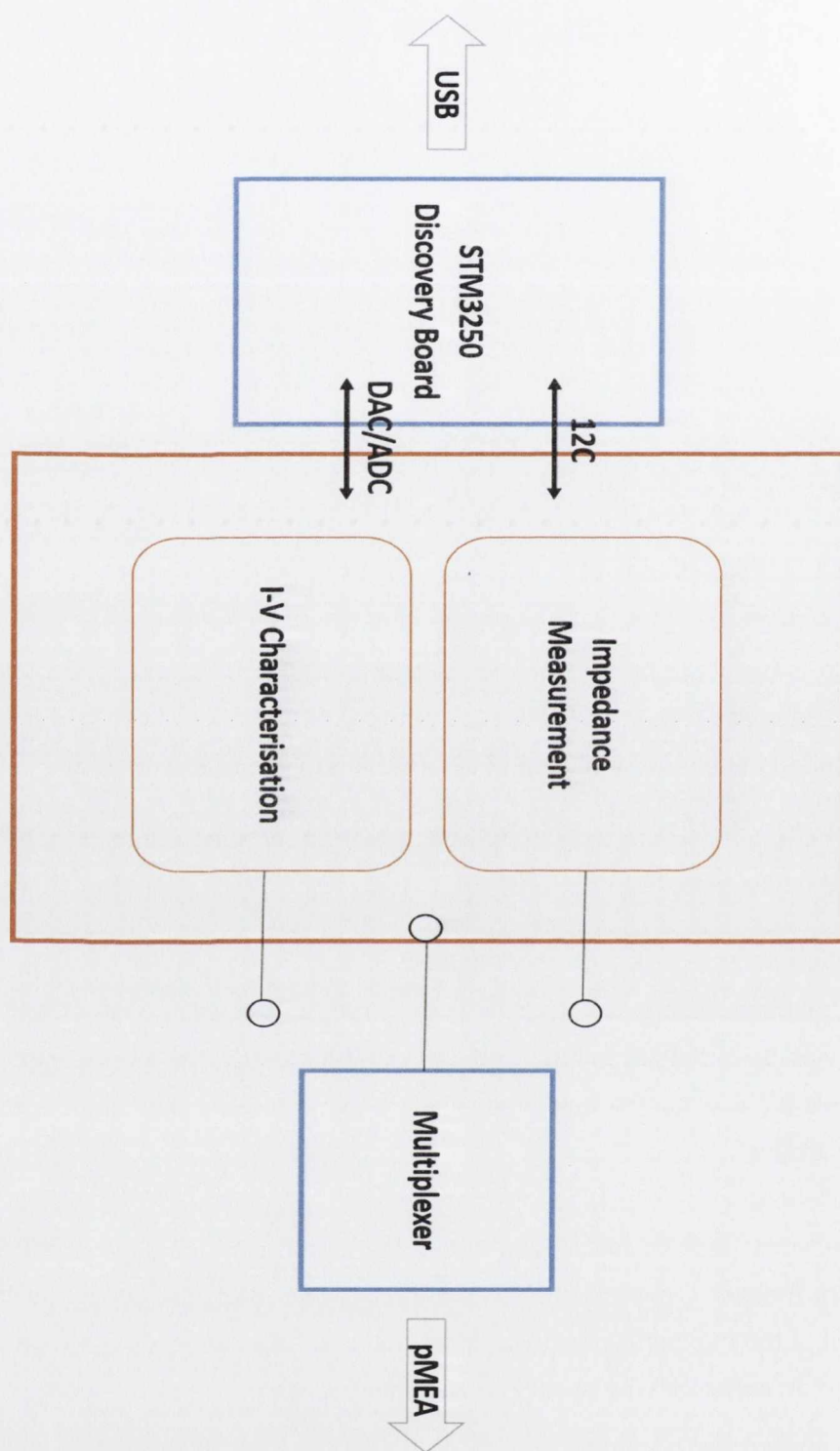


Figure 8.4 | Schematic of MEA biosensor circuit architecture.

8.4.3 Impedance Measurement

The impedance measurement is performed using the Analog Devices AD5933 impedance measurement kit. A set of reference resistors is used in conjunction with the integrated circuit for fine tuning of the impedance.

8.4.4 Multiplexing

A set of 1:16 demultiplexers are used to connect to a set of 32 contact pads. The addresses of the multiplexer are controlled by the microcontroller and the software.

8.4.5 Microcontroller

The microcontroller is connected to a computer using a USB (universal serial bus) connection. Firmware for the microcontroller is developed with a set of functions; to control communication with the computer, to control communication of the integrated circuit with the impedance meter, to control signal generation and current monitoring and to control the multiplexer.

8.4.6 Software

Custom software was designed to program the microcontroller with a set of parameters for both pulse generation and impedance measurement (Figure 8.5). The main functions allow the start of communication with the controller, selection of current-voltage or impedance measurement, pulse generation settings and commands and impedance measurement settings and commands.

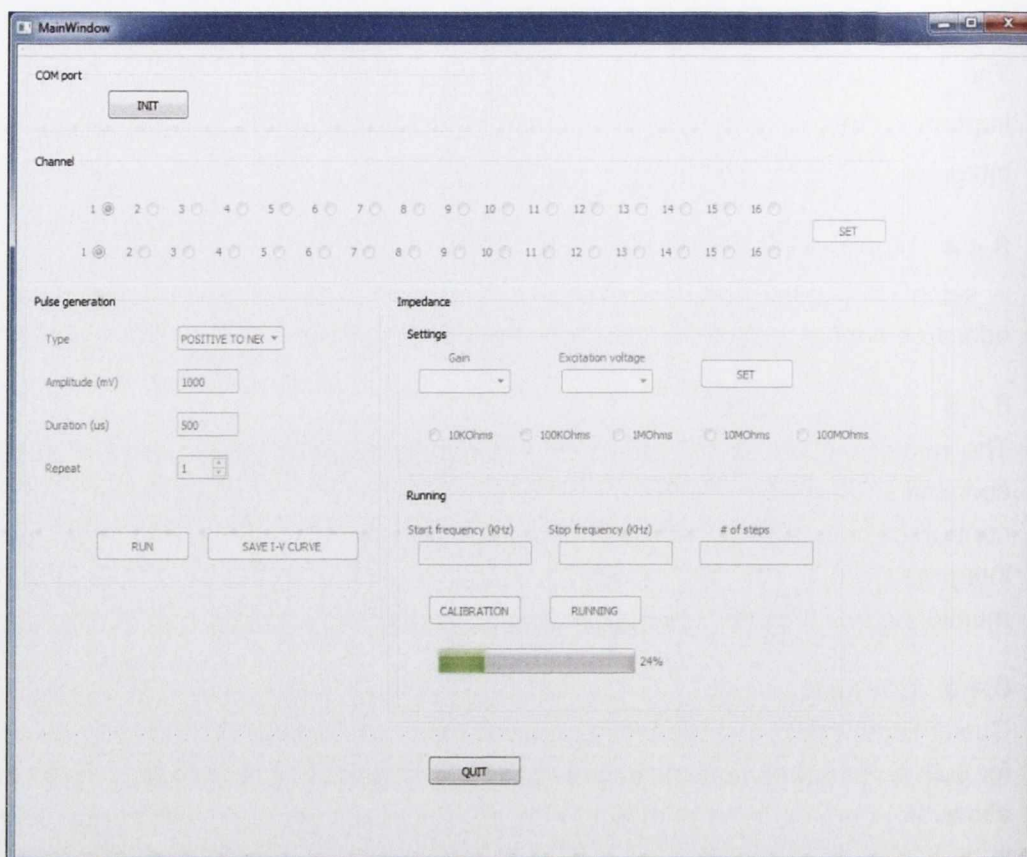


Figure 8.5 | Image of customised software for interacting with MEA biosensor.

8.4.7 Firmware

The firmware plays two major roles; receiving and acknowledging command from the computer and controlling pulse generation from the STM32 discovery board. The architecture of the firmware includes an initialising phase with clock settings, timer interrupt settings, UART (universal asynchronous receiver/transmitter) settings, GPIO (general purpose input/output) settings and an infinite loop.

8.5 Manual for Biosensor Platform

8.5.1 User Guide

The platform is supplied with:

- Software to control the generation of signals and the acquisition of data
- A controller with a USB port and two driving connectors (one small antenna connector and a large zero insertion force connector – flex cable)
- Multiplexer board for interconnection between the controller and the biosensor (spring loaded PCB)

To be able to run experiments, follow these instructions:

- Make sure the environment is clean and dry
- Plug the interconnect board to the spring loaded board

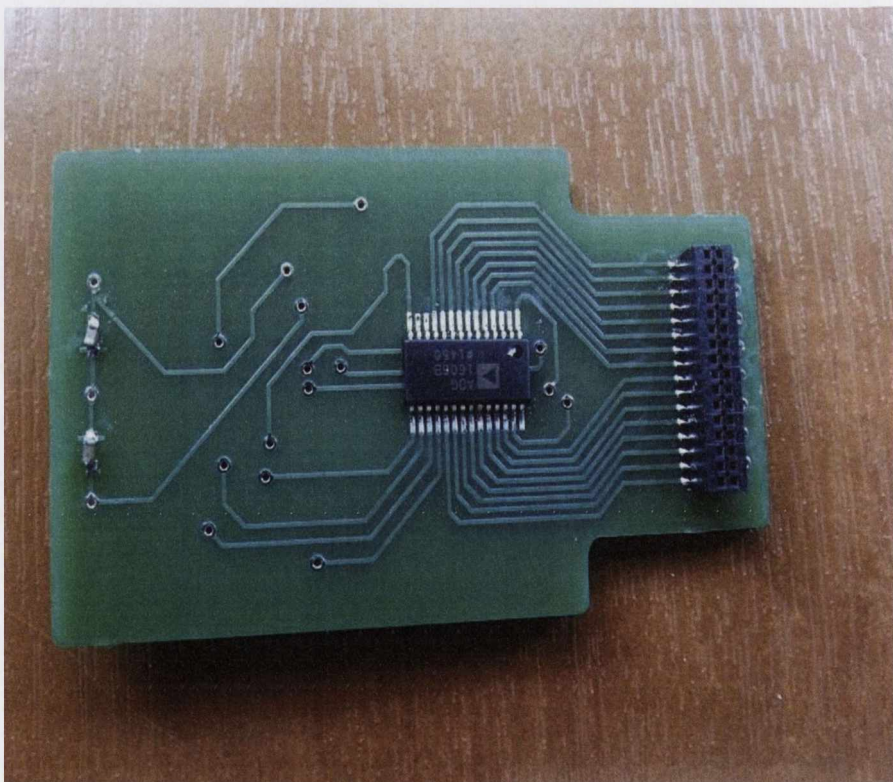


Figure 8.6 | Interconnector board.

- Plug the interconnect board to the controller using the flex cable or antenna cable

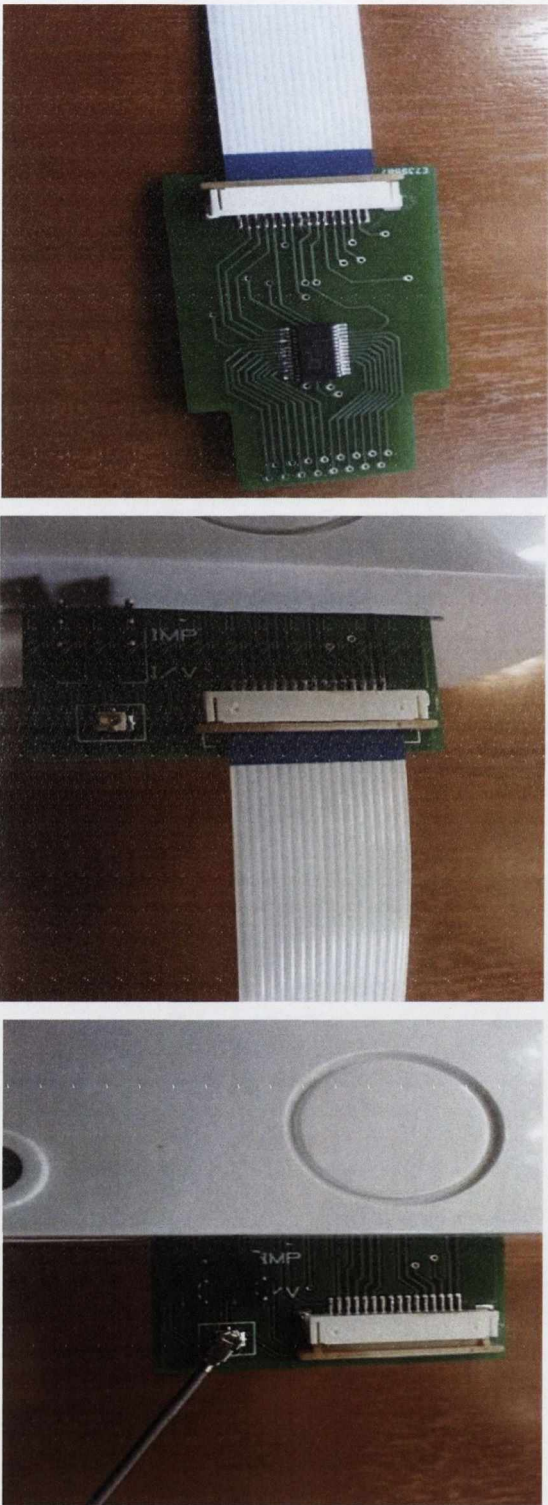


Figure 8.7 | Interconnector board with flex cable.

- Plug the USB cable to the USB port (not the PROG port – this is used to load new software to the controller)



Figure 8.8 | Microcontroller USB port.

- Plug the USB cable to the computer (red light flashes to show power is on) and start the application
- Click on "INIT" to begin interaction with the microelectrodes
- Select the channels to apply a pulse and record a signal and press "SET"
- Select desired stimulation parameters (type of signal, signal amplitude, signal duration) and click "RUN" to generate the signal directly
- Once the signal is applied, you can request the data by pressing "Save I-V Curve." The file is saved with a column for each parameter (*csv type) which can be opened by Excel

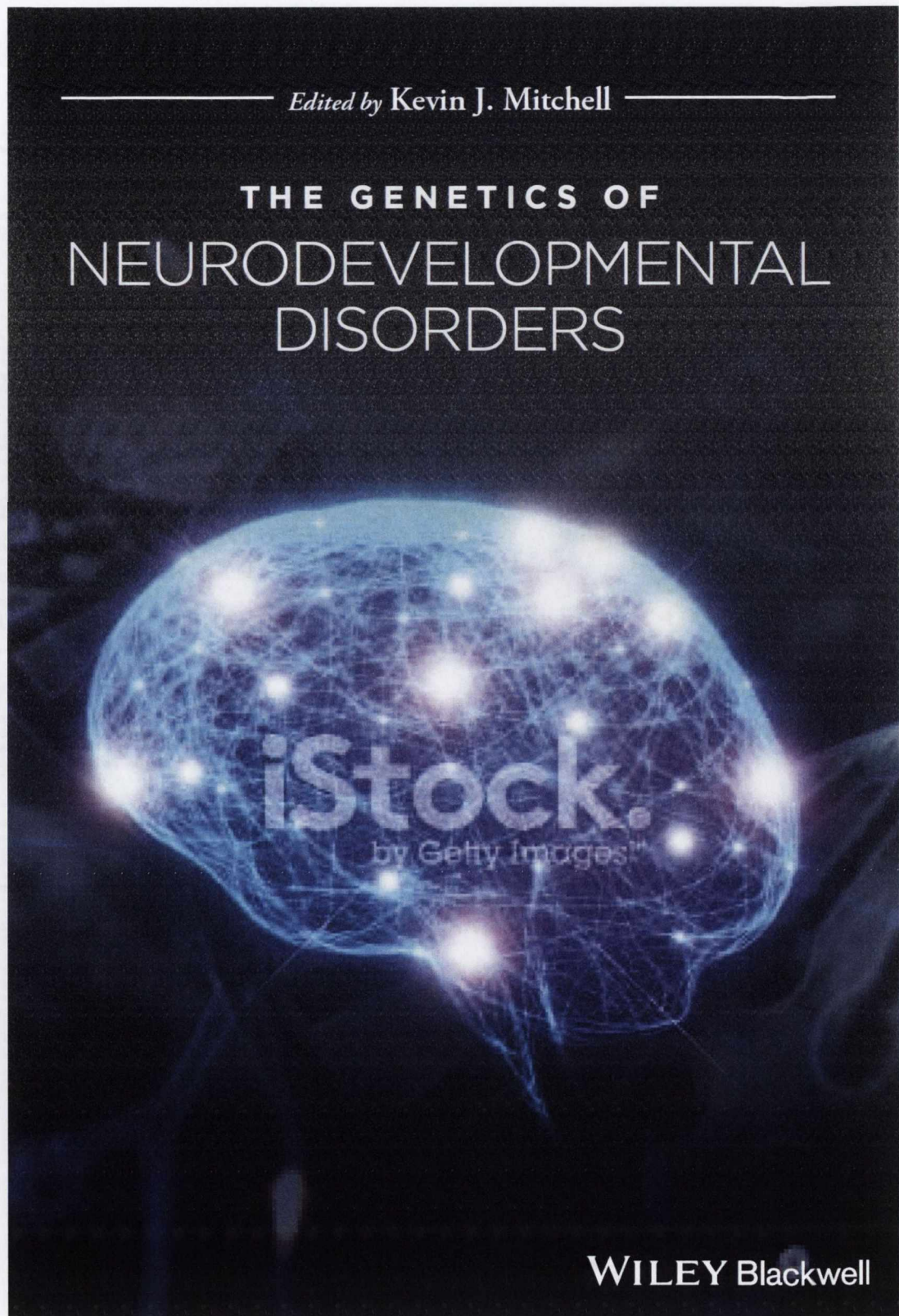
8.6 Nomenclature of PCBs

8.6.1 Interconnection Boards

Ref supplier	584- <u>ADG1606BRU</u> Z	584- <u>ADG1606BRU</u> Z	978-6147	180-6398	175-9016	175-9016
Supplier	Mouser	Mouser	Farnell	Farnell	Farnell	Farnell
Ref manufacturer	ADG1606	ADG1606	52271-1679	MLE-116-01-G-DV	MC0603B104 K160CT	MC0603B104 K160CT
Manufacturer	Analog devices	Analog devices	Molex	Samtec	Multicomp	Multicomp
Description	16:1 analogue multiplexer	16:1 analogue multiplexer	ZIF/FPC connector 1x16	Vertical header 2x32 1mm	Capacitor ceramic 100nF - 0603	Capacitor ceramic 100nF - 0603
Ref	U1	U2	J1	J3	C1	C2

8.7 Communications from this thesis

8.7.1 Book Chapter - Wiley



8.7.2 Review - Progress in Neurobiology

Progress in Neurobiology 123 (2014) 18–36



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Nanotechnologies for the study of the central nervous system



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ABSTRACT

The impact of central nervous system (CNS) disorders on the human population is significant, contributing almost €800 billion in annual European healthcare costs. These disorders not only have a disabling social impact but also a crippling economic drain on resources. Developing novel therapeutic strategies for these disorders requires a better understanding of events that underlie mechanisms of neural circuit physiology. Studying the relationship between genetic expression, synapse development and circuit physiology in CNS function is a challenging task, involving simultaneous analysis of multiple parameters and the convergence of several disciplines and technological approaches. However, current gold-standard techniques used to study the CNS have limitations that pose unique challenges to furthering our understanding of functional CNS development.

The recent advancement in nanotechnologies for biomedical applications has seen the emergence of nanoscience as a key enabling technology for delivering a translational bridge between basic and clinical research. In particular, the development of neuroimaging and electrophysiology tools to identify the aetiology and progression of CNS disorders have led to new insights in our understanding of CNS physiology and the development of novel diagnostic modalities for therapeutic intervention. This review focuses on the latest applications of these nanotechnologies for investigating CNS function and the improved diagnosis of CNS disorders.

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Abbreviations: Aβ1–42, amyloid-β peptide; AD, Alzheimer's disease; anti-IGFBP7, anti-insulin-like growth factor antibody; BAM10, amyloid-β monoclonal antibody clone; BBB, blood-brain barrier; BRAIN, brain research through advancing innovative neurotechnologies; Cd²⁺, cadmium heavy metal ions; CdSe:Mn/ZnS, cadmium selenide:manganese/zinc sulphide; CLIONS, cross-linked iron oxide nanoparticles; CNS, central nervous system; CNT, carbon nanotube; CT, computer tomography; CVD, chemical vapour deposition; DAQ, data acquisition card; DOX, doxorubicin; DSC-CBV, dynamic susceptibility contrast perfusion imaging of cerebral blood volume; EAE, experimental autoimmune encephalomyelitis; Gd, gadolinium; GWAS, genome wide association studies; MB, microbubbles; MEA, microelectrode array; MEP, magnetoelectroporation; MNPs, magnetic nanoparticles; MNP-PBP, iron oxide nanoparticle contrast agent targeted towards p-selectin; MRI, magnetic resonance imaging; NIR, near-infra red; PAMAM, polyamidoamine; PBCA, poly(n-butyl cyanoacrylate); PEG, polyethylene glycol; PET, positron emission tomography; PLGA, polylactide-co-glycolide; PMMA, polymethylmethacrylate; QD, quantum dots; SCINs, solid-conductor intracellular nanoelectrodes; Si-NWFET, silicon nanowire field-effect transistor; SiO₂, silicon dioxide; SPIONs, superparamagnetic iron oxide nanoparticles; SS-CBV, steady-state cerebral blood volume maps; TAT, trans-activator of transcription; Tf, transferrin; USPIOs, ultra-small superparamagnetic iron oxide nanoparticles; VCAM, vascular cell adhesion molecule; VSPIONs, very-small superparamagnetic iron oxide nanoparticles.

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8.8 Presentations

8.8.1 MMI Annual Scientific Meeting

Dublin (2015). Oral Presentation. "Modelling CNS Development on an in vitro biochip platform"

8.8.2 Cell Symposia in Translational Neuroscience

Washington DC (2014). Poster Presentation. "Morphological study of SH-SY5Y human neuroblastoma cells for neural interface development"

8.8.3 EuroNanoForum

Dublin (2013). Poster Presentation. "Development of a microelectrode array biosensor for modelling the central nervous system and targeted drug screening"