Magnetic assistive and hydrogel technology for enhanced survival and function of neurons

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Abstract

Neurons are the targets of injury and disease in many neurological conditions, and achieving neuronal survival/repair is a key goal for regenerative medicine. In this context, genetic engineering of neurons offers a platform for (i) basic research to enhance our understanding of neuronal biology in normal, disease/and injury conditions; and (ii) for regenerative medicine to enhance the functionality of neurons. Although, a wide range of attempts have been made to promote gene delivery to primary neurons, these cells are still difficult to genetically engineer, and current methods rely heavily on viral vectors which pose safety considerations. Magnetic nanoparticles (IONPs) are currently of great interest in regenerative medicine including for non-viral gene delivery by the ‘magnetofection’ strategy, i.e when used with applied magnetic fields. This project aimed to examine (i) the influence of two novel uniaxial and biaxial oscillating magnetic field devices on primary neuronal transfection efficiency, and (ii) examine the safety of magnetofection using histological and electrophysiological studies. In order to do this, a robust protocol to derive primary cortical neurons was first established.

A second issue is that surgical delivery of Neurons results in low survival. Additionally, most basic research has relied on neurons grown on ‘hard’ substrates such as plastic, which do not mimic the mechanical properties of the in vivo microenvironment. To address these limitations, primary cortical neurons were grown in a 3-dimensional ‘soft’ collagen hydrogel construct which can serve both as a protective cell delivery system and a ‘neuromimetic’ substrate. The
safety of the established protocol was evaluated by electrophysiological analyses on neurons.

The findings demonstrate that the safety of magnetofection is magnetic field dependent, and at optimal conditions, electrophysiological properties of the nano-engineered neurons were normal. Secondly, I have shown that collagen hydrogels can support the 3D growth of neurons and electrophysiological studies can be carried out on the construct neurons; small differences were found between neurons grown on hard and soft materials. Finally, the amenability of genetic engineering of neurons within hydrogels using IONPs has been shown.
## Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td><strong>γ-Fe₂O₃</strong></td>
<td>Maghemite iron oxide nanoparticle</td>
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<tr>
<td>2D</td>
<td>2-dimensional monolayer culture</td>
</tr>
<tr>
<td>3D</td>
<td>3-dimensional construct</td>
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<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
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<tr>
<td>AraC</td>
<td>Cytosine arabinofuranoside</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CQ</td>
<td>Clioquinol(5-chloro-7-ido-8 hydroxyquinoline)</td>
</tr>
<tr>
<td>Cy3</td>
<td>Fluorophore-conjugated secondary antibodies</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>DIV</td>
<td>Days in vitro</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMEM:F12</td>
<td>Dulbecco’s Modified Eagle Medium: Nutrient Mixture-F12</td>
</tr>
<tr>
<td>DN</td>
<td>Dopaminergic neurons</td>
</tr>
<tr>
<td>DNase I</td>
<td>Deoxyribonuclease I</td>
</tr>
<tr>
<td>EBSS</td>
<td>Earle’s balanced salt solution</td>
</tr>
<tr>
<td>EPC7</td>
<td>Patch clamp amplifier</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td><strong>F</strong></td>
<td>Frequency</td>
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<tr>
<td>-------</td>
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<tr>
<td><strong>FBS</strong></td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td><strong>Fe₃O₄</strong></td>
<td>Magnetite iron oxide nanoparticle</td>
</tr>
<tr>
<td><strong>GFAP</strong></td>
<td>Anti-glial fibrillary acidic protein</td>
</tr>
<tr>
<td><strong>GFP</strong></td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td><strong>HA</strong></td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td><strong>HBSS</strong></td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td><strong>HD</strong></td>
<td>Huntington’s disorder</td>
</tr>
<tr>
<td><strong>HEPES</strong></td>
<td>N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid</td>
</tr>
<tr>
<td><strong>HFV</strong></td>
<td>Human foamy virus</td>
</tr>
<tr>
<td><strong>HSV</strong></td>
<td>Herpes simplex viruses</td>
</tr>
<tr>
<td><strong>HSV-1</strong></td>
<td>Herpes Simplex Virus-1</td>
</tr>
<tr>
<td><strong>IONPs</strong></td>
<td>Iron oxide nanoparticles</td>
</tr>
<tr>
<td><strong>iPSCs</strong></td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td><strong>Kcc2</strong></td>
<td>Potassium–chloride cotransporter</td>
</tr>
<tr>
<td><strong>LDH</strong></td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td><strong>LV</strong></td>
<td>Lentivirus</td>
</tr>
<tr>
<td><strong>MAG</strong></td>
<td>Myelin-associated glycoprotein</td>
</tr>
<tr>
<td><strong>MEF2</strong></td>
<td>Transcriptional factor protein</td>
</tr>
<tr>
<td><strong>MRI</strong></td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td><strong>MSC</strong></td>
<td>Marrow stromal cell</td>
</tr>
<tr>
<td><strong>NBM-1</strong></td>
<td>Neurobasal-1</td>
</tr>
<tr>
<td><strong>NBM-2</strong></td>
<td>Neurobasal-2</td>
</tr>
<tr>
<td><strong>NDS</strong></td>
<td>Normal donkey serum</td>
</tr>
<tr>
<td><strong>NGF</strong></td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td><strong>NSCs</strong></td>
<td>Neural stem cells</td>
</tr>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>NT-3</td>
<td>Neurotrophin-3</td>
</tr>
<tr>
<td>OMgp</td>
<td>Oligodendrocyte/myelin glycoprotein</td>
</tr>
<tr>
<td>OPCs</td>
<td>Oligodendrocyte progenitor cells</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin and streptomycin</td>
</tr>
<tr>
<td>PAMAM</td>
<td>Polyamidoamine</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PDL</td>
<td>Poly-d-lysine</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly glycolic acid</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly lactic acid</td>
</tr>
<tr>
<td>PMC</td>
<td>Primary motor cortex</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PS/F</td>
<td>Penicillin with streptomycin and amphotericin B</td>
</tr>
<tr>
<td>rpm</td>
<td>Round per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal cord injury</td>
</tr>
<tr>
<td>SMN</td>
<td>Survival of motor neuron</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
</tr>
<tr>
<td>TIA</td>
<td>Transient ischemic attack</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>Tuj-1</td>
<td>Anti-β III tubulin</td>
</tr>
<tr>
<td>UK</td>
<td>United kingdom</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>VGCC</td>
<td>Voltage gated calcium channel</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>ζ-potential</td>
<td>Zeta potential</td>
</tr>
</tbody>
</table>
# Table of Contents

Magnetic assistive and hydrogel technology for enhanced survival and function of neurons ................................................................. I  
Abstract ........................................................................................................... I  
Abbreviation .................................................................................................. III  
Table of Contents ............................................................................................ VII  
List of Tables .................................................................................................. XII  
List of Figures ................................................................................................. XIII  
Acknowledgment ............................................................................................ XVI  
Chapter 1 ......................................................................................................... 1  
General introduction ....................................................................................... 1  
1.1. Neuron loss is common in nervous system damage ........................................... 2  
1.2. Nerve cells ................................................................................................ 4  
1.3. Electrical signalling in neurons ..................................................................... 7  
1.4. Neuronal death mediators and regenerative failure in nervous system disorders .................................................................................. 14  
1.5. Strategies to replace lost neurons in neurological conditions .................... 16  
1.6. Combinatorial gene therapy is needed for CNS regeneration .................. 22  
1.7. Studies performed on genetic engineering of neurons ............................. 23  
1.8. Genetic engineering techniques ................................................................... 25  
  1.8.1. Viral transduction .................................................................................... 28  
  1.8.2 Non-viral transfection .............................................................................. 32  
1.9. Magnetofection ........................................................................................... 34  
1.10. Nanoparticle-based transfection ................................................................. 38  
1.10.1 Characteristics of IONPs ........................................................................ 38
1.10.2. IONPs as a gene delivery vector ................................................................. 40
1.11. Uptake mechanism .......................................................................................... 45
1.12. 3D hydrogel models for neurodegenerative medicine and .............................. 47
    basic research ................................................................................................. 47
1.13. What is the influence of substrate on cellular response and morphology? ..... 48
1.14. Cells interact with the extracellular environment .......................................... 50
1.15. Three–dimensional (3D) biological models ....................................................... 50
1.16. Aims .............................................................................................................. 52
Chapter 2 ............................................................................................................. 53
Materials and Methods ....................................................................................... 53
2.1. Reagents and Equipment .................................................................................. 55
2.3. Derivation of Primary Cortical cells ................................................................. 57
2.4. Primary cortical cell culture conditions ............................................................ 62
2.5. Magnetic nanoparticles mediated gene transfer to primary cortical cells .......... 65
    2.5.1 Magnetic arrays ....................................................................................... 65
    2.5.2. Magnetic nanoparticle characterization .................................................... 67
    2.5.3. Pmax plasmid preparation .................................................................... 67
    2.5.4. Magnetofection procedures .................................................................. 69
2.5.5. Cellular Uptake of IONPs ........................................................................ 71
2.6. Preparation of 3-dimensional (3D) hydrogel constructs containing neurons .... 72
2.7. Immunocytochemical procedures .................................................................. 76
2.8. 3D hydrogel construct immunostaining ......................................................... 77
2.9. Viability assessment ....................................................................................... 77
2.10. Microscopy and image analysis .................................................................... 78
2.11. Whole-cell electrophysiological recording

2.11.1. Processing 2D monolayer (PDL-Laminin) whole-cell recording

2.11.2. Processing 3D surface seeded whole-cell recording

Chapter 3

Primary cortical cell culture optimization

3.1. Introduction

3.2. Objectives

3.3. Experimental procedure and analysis

3.4. Results

3.5. Neuron distribution and characterization

3.6. The influence of culture incubation time on the proportions of neurons vs astrocytes

Chapter 4

Evaluating the safety of magnetofection for primary cortical neurons

4.1. Introduction

4.2. Objectives

4.3. Experimental procedure and analysis

4.4. Results

4.5. Neuron distribution and characterization

4.6. The influence of culture incubation time on the proportions of neurons vs astrocytes

Chapter 3
4.4.1. The influence of oscillating magnetic field on transfection efficiency of primary cortical cells
(Uniaxial VS Biaxial magnet) ................................................................. 122420

4.4.2. Confirmation of intracellular IONP internalization ............................. 126424

4.4.3. Safety assessment of magnetofection technology .............................. 129427
  4.4.3.1. Cell viability for magnetofected cortical cells .................................. 129427
  4.4.3.2. Histological evaluation of the safety of magnetofection for transfected neurons ...... 131429
  4.4.3.3. A comparative assessment of electrophysiological characteristics between transfected and non-transfected neurons ............................................................. 132430

4.5. Discussion .......................................................................................... 137435

Chapter 5 .................................................................................................. 143441
Hydrogels are a promising neuromimetic substrate for primary cortical cells. 143441

5.1. Introduction ......................................................................................... 144442
  5.1.1. The suitability of collagen-based hydrogels as a neuromimetic substrate .......... 146443

5.2. Objectives ........................................................................................... 150448

5.3. Experimental procedure and analysis .................................................... 151449

5.4. Results .................................................................................................. 151449
  5.4.1. Collagen as a substrate for growing cortical cells as a 2D monolayer .......... 151449
  5.4.2. Cell viability on 2D collagen coated coverslips ........................................ 154452
  5.4.3. Collagen as substrate for growing cortical cells as a 3D construct .......... 155453
    5. 4.3.1. Surface model ............................................................... 155453
    5.4.3.2. Entrapped model ............................................................ 157455
    5.4.3.2.1. Collagen gel concentration testing ...................................... 157455
    5.4.3.2.2. 3D construct purity and cell distribution at three and seven days in vitro 158456
Chapter 5: Hydrogels are promising neuromimetic substrate for primary cortical cells

5.4.3.2.3. Cortical cell distribution across the core and the extremity of the gel
5.4.3. Assessment of cellular viability of cortical cells in the 3D environment
5.4.4. Patch-clamp technique for evaluating the safety of the collagen hydrogel protocol
5.4.5. Genetic engineering of neurons on the surface of the hydrogel construct
5.4.6. Genetically engineering neurons within hydrogel construct

5. 5. Discussion

Chapter 6

Conclusions and Future Directions

6.1. Summary of key thesis findings

Chapter 3: Primary cortical culture optimization

Chapter 4: Evaluating the safety of magnetofection for primary cortical neurons

Chapter 5: Hydrogels are promising neuromimetic substrate for primary cortical cells

6.2. Future directions

Appendix

References
List of Tables

Chapter 1: General introduction

Table 1.1: Examples of viral vectors used for transducing neurons..............................31

Table 1.2: Examples of non-viral vectors used for transfecting neurons............................44

Table 2.1: Comparison of primary cortical neuronal culture protocols: Neurobasal-1 (NBM-1), DMEM:F12 (DMEM), and Neurobasal-2 (NBM-2). The table includes dissection and growth media in addition to some technical steps.........................................................61

Table 2.2: The examined parameters for optimizing primary cortical culture.....................64

Table 2.3: Formulae for calculating collagen solution formation for 2D and 3D.................75

Table 4.1: Comparison of voltage-dependent Na+ and K+ currents in GFP+ and GFP− neurons in voltage-clamp..................................................................................................................136

Table 4.1: Comparison of voltage-dependent Na+ and K+ currents in GFP+ and GFP− neurons in voltage-clamp..................................................................................................................136

Table 5.1: Amplitudes of Na+ and K+ currents, and membrane potential, from neurons grown on hydrogels.................................................................171

Table 5.1: Amplitudes of Na+ and K+ currents, and membrane potential, from neurons grown on hydrogels.................................................................171
List of Figures

Chapter 1: General introduction

Figure 1.1: Diagram of some structural features of neurons and its interaction with the other neural cells. .......................................................... 76

Figure 1.2 Action potential, plotting voltage measured across the cell membrane against time.................................................................................................................................. 10

Figure 1.3: Forms of the patch clamp technique. ........................................ 12

Figure 1.4: Schematic illustrating the factors that participate in neurodegenerative diseases. .................................................................................................................. 15

Figure 1.5: Schematic to illustrate the main gene delivery barriers. ........... 27

Figure 1.6: Schematic illustrates the magnetofection principle.................. 37

Figure 1.7: Schematic diagram which illustrates IONPs and interactions between functionalization layer and some biological entities. ......................................................... 43

Figure 2.1: A flow chart summarizing experimental sequences of thesis chapters, experiments conducted and parameters examin................................. 54

Figure 2.2: Diagram demonstrating the main dissection steps for derivation of cortical neuronal cultures. ................................................................. 60

Figure 2.3: Schematic showing cortical neuronal culture seeding technique. .......... 63

Figure 2.4: Schematic elucidating the experimental design for examining the effect of serum level, cell density and observation time point on generating reproducible primary cortical neuronal cultures. ‘Low’ refers to a cell density of 30x10^3 cells/cm^2 and ‘high’ refers to 60x10^3 cell/cm^2. .................................................................................................................. 65

Figure 2.5: Photographs and corresponding diagrams illustrating the two types of magnetic arrays. ........................................................................................................... 66

Figure 2.6: Schematic representation of plasmid map for pmax GFP................ 69

Figure 2.7: Schematic describing magnetofection process. ........................... 71

Figure 2.8: Schematic illustrate cell seeding models of hydrogel construct .......... 75
Figure 2.9: Schematic demonstrating fluorescence imaging location for the gel constructs.

Figure 2.10: Schematic exhibiting recording and blocking (passing TTX blocker through) electrode positions in relation to primary neurons during the electrical signal recording by patch-clamp technique.

Figure 3.1: Fluorescence micrographs show the validity of primary neuronal cultures derived according to three different protocols.

Figure 3.2: Cortical neuron distribution and growth.

Figure 3.3: Distribution and growth of the cortical neurons plated on the substrate coated with PDL and Laminin.

Figure 3.4: Serum in media influence on the proportion of cortical cells in culture.

Figure 3.5: Primary cortical cell proportions in cultures treated with serum free growth medium over three time points (3, 5 and 7 DIV).

Figure 3.6: Schematic representing the approved culture protocol for deriving neuronal primary cortical cells in addition to the optimal culture condition which chosen according to experimental criteria needed.

Figure 4.1: Magnetofection facilitates transfection of primary cortical cells.

Figure 4.2: Uniaxial magnetic field supported primary cortical cells transfection.

Figure 4.3: The susceptibility of primary cortical cells to internalize iron oxide magnetic nanoparticles.

Figure 4.4: Magnetic nanoparticles uptake is cell type dependent.

Figure 4.5: Uniaxial oscillating magnetic field is a safe technique.

Figure 4.6: Uniaxial oscillation magnetic field is a safe technique.

Figure 4.7: Uniaxial oscillating magnetic field is safe for transfecting primary cortical neuronal culture.

Figure 4.8: Representative ionic currents were seen in response to voltage steps from a holding potential of –60 mV from both magnetofected (GFP+) and non-magnetofected (GFP−) neurons.
| Figure 4.9: Representative current clamp recordings from magnetofected (GFP+) and non-magnetofected (GFP–) neurons showing spike-like activity. | 135433 |
| Figure 5.1: Collagen hydrogel supports primary cortical cell growth as a 2D monolayer. | 153451 |
| Figure 5.2: Image showing viable cells stained with Calcein (green) and red arrows pointing to dead cells stained with Ethidium homodimer (A). | 154152 |
| Figure 5.3: Three dimensional appearance of cortical cells grown on the surface of a collagen hydrogel construct. | 156454 |
| Figure 5.4: Collagen construct concentration affects cell distribution through the gel. | 157455 |
| Figure 5.5: Highly pure and homogenous neuronal 3D hydrogel construct at 3 days in vitro. | 160458 |
| Figure 5.6: Pure and homogenous neuronal 3D hydrogel construct at 7 days in vitro. | 161459 |
| Figure 5.7: Cortical cells distributed homogenously across the gel. | 163461 |
| Figure 5.8: Cortical cells exhibited high survival within the 3-dimensional environment. | 164162 |
| Figure 5.9: Collagen hydrogels support cortical cell survival in a 3-dimensional environment. | 165463 |
| Figure 5.10: Recording from cortical neurones on the surface of a hydrogel. | 169467 |
| Figure 5.11: Voltage-dependent Na⁺ and K⁺ currents, and membrane potential recordings, in primary cortical neurons grown on a hydrogel. | 170468 |
| Figure 5.12: Magnetofection based transfection of neurons seeded on the surface of the gel at 7DIV. | 172470 |
| Figure 5.13: Genetically engineered cortical cells in a 3-dimensional hydrogel construct. | 174472 |
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I would dedicate this work to the spirit of my father who is always in my heart and mind (Faiq Jameel Al-shakli).
Chapter 1

General introduction
1.1. Neuron loss is common in nervous system damage
The nervous system in mammals is complex in structure and function, and divided into two parts; the central nervous system (CNS, brain and spinal cord) and peripheral nervous system (PNS). The nervous system is vulnerable to various types of damage which can result in permanent neurological deficits, for example (i) structural disorders such as traumatic brain (TBI) or spinal cord injury (SCI) (Sciarretta and Minichiello, 2010), (ii) vascular disorders such as stroke, transient ischemic attack (TIA) and (iii) neurodegenerative diseases such as Parkinson’s disease (PD), Alzheimer’s disease (AD) and Huntington’s disorder (HD)(Asgharian et al., 2014), and amyotrophic lateral sclerosis (ALS).

Neurological disorders impact millions of people globally, for example around 2.5 million people suffer from SCI (Thuret et al., 2006), and the World Health Organization (WHO) has reported that annually, between 250,000-500,000 people worldwide survive SCI (World health organization, 2013). Furthermore, during the last 10 years, around 35 million persons worldwide have suffered from neurodegenerative diseases and this figure is predicted to reach up to 150 million by 2050 (Kiaei, 2013). All these disorders have a negative impact on the quality of life for patients who may experience long-term neurological deficit. As a consequence, people with neurological damage can require long term care which in turn can impact the economy. For instance, it has been reported that over £500 million is spent each year in the UK alone on treating SCI patients, and in the US the annual cost is in the order of US$7.7 billion (Adams and Cavanagh, 2004).
Many causative factors contribute to the damage of the CNS. For example, environmental and genetic defects contribute in developing neurodegenerative diseases, which differ according to the type of disease. However, they are strongly associated with advanced age (Sheikh et al., 2013). Traumatic CNS injuries are primarily due to mechanical trauma followed by biochemical mechanisms which will be discussed in (section 1.3). (Shoichet et al., 2008).

Despite the variation of etiologies of neurological deficit, the most common impaired/or damaged cells are Neurons. Both the brain and spinal cord are composed of two types of tissue (i) the gray matter which consists of Neuron bodies; and (ii) the white matter consisting of myelinated axons. Positioning of gray and white matter are different in the brain and spinal cord, as in the brain the gray matter is the outer tissue while white matter is at depth. However, the sequence of tissue is reversed in the spinal cord (Mak, 2007). The severity and location of the insult are the determinants of the degree of impairment and whether they include the cell body or the axon or both, resulting in continued dysfunction and prolonged degeneration (Ambrozaitis et al., 2006, Thuret et al., 2006). In neurodegenerative diseases, the death of several types of neurons within the cortical and hippocampal regions occurs in AD, loss of medium spiny neurons in cortical and striatal regions is characteristic of HD (Asgharian et al., 2014), loss of dopaminergic neurons DA in the substantia nigra results in PD, while ALS results from upper and lower motor neuron death and primary motor cortex (PMC) (Ross and Poirier, 2004, Rossi et al., 2010) CNS injury involves impairment and/or damage to neurons that is associated with destruction of neuronal circuits including axons and dendrites (Pekna and Pekny, 2012). This
neuronal loss leads to the major functional deficits. Depending on the mechanisms and timeframe of the pathology, cell death may occur through necrosis or apoptosis (Golstein and Kroemer, 2007). To address this major, growing, and currently incurable healthcare problem, researchers across the world are focusing on investigating CNS deficit etiologies and novel treatment options.

1.2. Nerve cells
The nervous system consists of two classes of cells; nerve cells (neurons) and glial cells (glia). Glial cells are reported to outnumber neurons about 10-50 times, and the role of these cells lies in supporting neuronal development and function. The major glial cells types include: (i) **Astrocytes** are the most abundant type in the brain and their supportive role for neurons is structural and protective, in addition to their regulatory function for neurite outgrowth and synapse formation and maintenance (Mak, 2007) (ii) **Oligodendrocytes** are involved with myelin production in the CNS; myelin is a lipid-rich white substance forming electrical insulation by spiral wrapping of the myelin sheath around axons of nerve cell in the central nervous system (White and Krämer-Albers, 2014) and (iii) Microglia are the primary immune cells of the nervous system, which transform into phagocytic cells when neuronal degeneration occurs (Gehrmann et al., 1995). In this project the focus will be on Neurons.

The neuron is the basic signalling unit of the nervous system. These cells are electrically excitable, and form the networks that detect, store and transfer information in the body as electrical and chemical signals that are essential to normal bodily function (Bray, 2017). Neurons are classified mainly as sensory,
motor or inter-neurons, according to their function. The main function of neurons is to process incoming signals, typically detected by structures known as dendrites, and to transmit signals to other cells using specialised projections referred to as axons (Figure 1.1). The typical regions of the neuron are: the cell body (soma), dendrites, the axon, and presynaptic terminals. Structural features of neuronal soma and the other cells in the body are similar. However, there are some differences that distinguish neurons. Neurons are distinguished by the presence of Nissl bodies (large granular bodies spread between the rows of endoplasmic reticulum combined with rosettes of free ribosomes and they are the site of protein formation). Neurons are rich in mitochondria because of the energy that is required for maintaining the transmembrane ionic gradients that are indispensable for neuronal signalling (Levitan and Kaczmarek, 2002).

Neurons are generally described as asymmetric cells because individual neurons may produce dendrites anywhere on their surface, but there is only a single axon extending from the cell body, or as recently suggested, axon can extend from other dendrites of the neuron as in hippocampal pyramidal cells (Thome et al., 2014). The axon varies in length from 0.1 millimetre to 3 meters and terminates with synapses. Synapses are highly specialized connection regions between two neurons. This connection can be electrical (transfer of electrolytes from one side of the cell to the other freely across small spaces called gap junctions), or chemical (connection occurs through the gap between the synapses of the pre and post synaptic neurons which is called the synaptic cleft), which facilitates signalling to other cells. These transboundary chemicals are called neurotransmitters. The first neuron (presynaptic) conveys information to the
second neuron (post synaptic) by these synapses, the nature of the connection is mostly chemical (Carnevale and Hines, 2006).

The axonal plasma membrane contains ion channels, which are specialized proteins used to convey electrical impulses rapidly along the axon. Ion channels called voltage gated ion channels are concentrated between the end segment of the soma and the initial segment of the axon which called axon hillock, where the action potential is believed to be initiated (Levitan and Kaczmarek, 2002, Carnevale and Hines, 2006). The dendrites are highly branched projections, originating from the cell body, specialized for receiving electrochemical inputs from the other cells by finger like projections called ‘spines’ at which the synaptic input region localized. These spines form part of a complex network called a dendritic tree. Dendrites are distinguished by having a cytoskeleton dissimilar to that of axons in terms of arrangement of the microtubules and actin filaments (Tamás et al., 2000). Dendrites like axons possess ion channels for conveying electrical information. It is worth mentioning that the dendrite structure is mutable according to the physiological condition (Kapitein and Hoogenraad, 2011).
1.3. Electrical signalling in neurons

What follows is a simple overview of electrophysiological properties and its importance in neuroscience.

In different brain regions and within each region neurons form neural circuits. Thus, neurons receive, modify, and transmit messages, including transferring information between two adjacent neurons and the different parts of the same cells. Studying and exploring the electrical activity, molecular and cellular processes that govern living neuron signalling is the discipline of neuroscience that is called electrophysiology.

The key elements of neuronal function (i.e. signal reception and transmission) are located at pre and post synapse, where localized excitatory events trigger an action potentials (Carnevale and Hines, 2006). These action potentials flow in one or two directions. In the case of two directional action potentials, there is backpropagation, which is a retrograde action potential that possesses the ability to modulate synaptic activity. Intracellular recordings in vitro suggest that neurons can respond to backpropagation action potential. For instance, facilitating BDNF release from synapsis (Kuczewski et al., 2008), also invade the dendritic tree to create global Ca\(^{2+}\) signals due to the forward direction of action potential down the axons to the synaptic endings and backwards. As global Ca\(^{2+}\) signals are playing a vital role in several biological processes including gene transcription and cell proliferation (Bootman et al., 2001). Generally, backpropagation initiation
is dendritic morphology, ion channel distribution, and synaptic input dependent (Murray and Holmes, 2011a).

Generally the plasma membrane has a **membrane potential** which refers to the difference between the voltage inside and outside a cell. A non-conducting neuron, when neurons at rest and the membrane potential here called **resting membrane potential**, is charged negatively on the inside with respect to the outside and has a typical value of -70mV. This charge variation results from the ion distribution across the plasma membrane. The most abundant ions on either side are chloride ions (Cl⁻), sodium ions (Na⁺) located outside the cell membrane, while, potassium ions (K⁺), and organic anions such as amino acids and proteins (A⁻) are highly concentrated within the cell. However, organic anions cannot move through the membrane due to their large size (Kandel et al., 2000). This negativity of the membrane potential results from the fact that at rest, there are more K⁺ channels open than Na⁺ channels, In another word, the membrane potential is principally determined by the distribution of K⁺ ions across the cell membrane.

The membrane potential can change, for example in response to an excitatory stimulus. Therefore the neuron can be more polarized (hyperpolarization) or less polarized (depolarization). Accordingly, when membrane potential is less negative than the resting potential the neurons is in a state of depolarization, when it is more negative the neuron is in a state of hyperpolarization. The **action potential** is a result of a large change in membrane potential, up to 100 mV. In other word, a rapid electrical change represented by rise and followed by fall in voltage or membrane potential across a cellular membrane. Action potential events start by a stimulus that causes the voltage change and leads to move the membrane
potential toward threshold (approximately -55 mV). Threshold is the level at which many Na\(^+\) channels open resulting in an influx of Na\(^+\), and further depolarisation (Platkiewicz and Brette, 2010). This potential continues to reach to +30 mV, at which Na\(^+\) channels are inactivating automatically and close, this event is associated with K\(^+\) channels opening and causes repolarization with an undershoot resulting from the absence of the depolarising influence of the background Na\(^+\) permeability (Hodgkin and Huxley, 1952).

Generally, the shape and frequency of the action potentials varies among neuronal cell types. For instance, narrower spikes can be seen in the cortex and hippocampus, GABA (\(\gamma\)-aminobutyric acid)-releasing interneurons comparing to the recorded spicks of glutamatergic pyramidal neurons (Bean, 2007) according to the location in the cell. Additionally, the action potential of cell body of neurons is different in shape than that of axon and dendrite trees. Basically, recording action potential from mature neurons describe as isopotential during the spike. However, there is a current flow between the cell body, dendrites and axon of the cell that change action potential shape to non-uniform of voltage. While, dendrites can influence the form of cell body action potentials, partly by serving as a capacitive load that slows and truncates fast spikes, this variation is due to the variability in types of voltage-dependent ion channels which in turn allows neurons to encode information by generating action potentials with a wide range of shapes, frequencies and patterns (Figure 1.2) (Bean, 2007).
Figure 1.2 Action potential, plotting voltage measured across the cell membrane against time. Diagrams illustrating the action potential steps at left, (1) a stimulus that depolarizes the cell and shifts the membrane potential from rest state at -70 mV, toward the threshold, (2) then when the threshold of excitation is reached, all Na⁺ channels open, (3) the membrane depolarizes until the peak of action potential is reached, (4) here Na⁺ start to close and K⁺ channels open and K⁺ begins to move outside the cell (repolarize). The continuous movement of K⁺ to the outside cell membrane resulting in hyperpolarization. (5) The membrane resting potential restore due to K⁺ channels close. This figure derived from (cnx.org, 2018). At right real action potentials recorded from different neurons derived from mouse (Bean, 2007).

Historically, nerve conductance were discovered by Luigi Galvani in association with electricity discovery in the middle of 18th century, when electricity was applied by metal wire inserted across the vertebral canal of frog, which showed that nerves and muscles themselves could produce electricity (Piccolino, 1997, Kazamel and Warren, 2017). This was followed by series of experiments by which the scientists tried to describe the conductance of nerve cell. Hence, in 19th century Cole and Curtis managed to demonstrate that there is increment in ionic permeability during propagation of an action potential (Hille, 1984). After equipment capable of amplifying and recording the fine bioelectric potentials was
invented in the early 20th century, Alan Hodgkin and Andrew Huxley in the 1950s, conducted a group of experiments on a squid giant axon and provided a quantitative description of membrane currents by voltage clamp (Hodgkin and Huxley, 1952). This was followed by the invention of patch-clamp technique by Erwin Neher and Bert Sakmann at late in the 20th century (Verkhratsky et al., 2006).

In that context, the electrophysiological properties of neurons can be recorded both *in vitro* or *in vivo* at multiple sites by a technique called **microelectrode array** such as extracellularly in slice cultures (Obien et al., 2015) or for a single cell recording using a **patch-clamp** technique which is based on measuring the tiny currents through ion channels in the cell membrane (Molleman, 2003). The focus in this project will be on single cell recording via patch clamp, as it based on inserting long glass capillaries filled with saline to attach tightly to the cell membrane, causing an electrical isolation of a small patch of the membrane to reduce the current leakage, then measure the current flowing through ionic channels (Hodgkin and Huxley, 1952, Zhao et al., 2008). Current measurement can be through a single ion channel or the whole cell. Figure 1.3 demonstrates the different forms of patch clamp and the purpose of each form.
Figure 1.3: Forms of the patch clamp technique: (a) the cell-attached to the glass pipette; (b) patched membrane ruptured which allows access to the interior of the cell to measure the currents from all the ion channels (whole-cell recording); (c) outside-out form, due to pulling a patch of membrane from the whole-cell mode, this can be used for studying the extracellularly ligand gated ion channels; (d) inside-out form, due to pulling the pipette away from the cell in the cell-attached mode. This can be used for studying ion channels that are regulated by intracellular ligands. This figure is derived from (Zhao et al., 2008).

The whole-cell recording is the form of patch clamp technique that is mostly used in research. This technique has been used for studying the electrophysiological properties of several type of neurons whether in culture or in slices. For example, it is used for investigating the membrane potential of pre and post-natal rostral nucleus of the solitary (rNST) tract neurons in rat. Where, Suwabe, et al. (2011) have monitored the electrical events associated with the morphological changes of rNST from embryonic day 14 up to post-natal day 20, and they found that soma size, dendritic branch points, neurite endings, and neurite length all increase prenatally, however resting membrane potential decreased. Additionally, neurons
at prenatal stage appeared a low level of sensitivity to tetrodotoxin (TTX) which is a Na\(^+\) ion channel blocker. Thus the Na\(^+\) influx was low prenatally comparing to the postnatal stage (Suwabe et al., 2011). Also the action potential responses of neocortical cells in vivo was recorded and showed considerable threshold variability, which demonstrated by the variability of timing and rate. This variability results from variation in the relation between the timing of synaptic activities as well as the patterns of action potentials that resulted from these activities. These events results from the backpropagation of action potential spikes throughout the neuron after the initiation of action potentials in the axon (Yu et al., 2008).

Wang, et al. 2008 studied the electrophysiological properties of cultured cortical progenitor neurons and in rat brain slices following intracerebral injection (Wang et al., 2008). They found that some of the transplanted neurons express Na\(^+\) current which is a characteristic of a mature neuron.

Investigating the electrophysiological properties of neurons is an indicator for the functionality of neurons both in vitro and in vivo. For instance, electrophysiological study was conducted for mouse embryonic stem cells induced by transgene (proneural gene Neurog1) to generate glutamatergic neurons. This study was designed to establish the timeline for acquiring a functional neuronal phenotype in Neurog1-induced cells exhibiting a neuronal morphology associated with mature neurons functional properties. This study demonstrated the advantage of utilizing Neurog1 in neural repair process that require stimulation of functional neurons from pluripotent stem cells (Tong et al., 2010).
1.4. Neuronal death mediators and regenerative failure in nervous system disorders

The common result of CNS disorders is death of neurons which is noted in (section 1.1.) neuron loss mechanisms vary according the cause of disorder and there are multiple mediators that lead to neuron death. In traumatic injuries, several mediators participate in neuron death, including electrolyte abnormalities, free radical formation, vascular ischemia, edema, posttraumatic inflammatory reaction, apoptosis or genetically programmed cell death (Sekhon and Fehlings, 2001). In regard to neurodegenerative diseases, neuron death mediators include; protein mis-folding and aggregation, oxidative stress and free radical formation, metal dyshomeostasis, mitochondrial dysfunction, and phosphorylation impairment, all occurring at the same time (Figure 1.4) (Sheikh et al., 2013). Correspondingly, neurons lose the coordination with reactive gliosis (astrocytes react post injury) due to the abnormal neuron to glia association (Przedborski et al., 2003, Silver and Miller, 2004), undergo morphological changes due to process attrition, with soma and nucleus shape alteration, cytoplasmic vacuolation (formation of vacuoles in the cell cytoplasm that is synchronous with the presence of a cytotoxic stimulus) (Shubin et al., 2016), and chromatin condensation (Przedborski et al., 2003). All these events can lead to disease /injury type dependent neuronal death which can be either apoptotic or necrotic cell death (Kanduc et al., 2002).
Neurons have limited potential for recovery post damage, because of two factors: (i) intracellular (i.e. inefficiency of neuronal regrowth mechanisms) as they are post-mitotic cells and they cannot divide and proliferate, and (ii) extracellular such as inhibitory factors of neurite outgrowth found in CNS myelin such as myelin-associated glycoprotein (MAG) and oligodendrocyte/myelin glycoprotein (OMgp) and myelin glycolipid sulfatide (Silver et al., 2015). Therefore, the CNS has limited self-renewing capacity. Despite the evidence produced by the scientific community regarding occurrence of neurogenesis in adult mammalian brain and presence of proliferating neural stem cells (NSCs), the regenerative capacity of the brain is still insufficient for repairing damaged neurons (Magavi et al., 2000).
Based on these reasons scientists have investigated different treatment options. Hence, much effort has been made to treat nervous system disorders. Some new therapeutic options were effective in alleviating some symptoms, but none cure the disease, and there are often limits to the duration of their effectiveness, with efficacy also decreasing gradually (Wu et al., 2010). As an example, this includes treatments by administrating L-DOPA and dopamine receptor agonists (Costa et al., 2008) for movement disorders in PD (Mizuno, 2014, Chen and Pan, 2015). This leaves an unmet need for truly regenerative therapies to treat neurological diseases. Therefore, the strategies to replace damaged neurons is currently a key goal of experimental neurology.

1.5. Strategies to replace lost neurons in neurological conditions

There are many clinical considerations associated with neurological injury and disease: (i) irreversible loss of neurons related to neurological disorders, (ii) persistent functional deficits, and (iii) the lack of treatment strategies, which are currently limited to saving the remaining healthy neurons and alleviating symptoms. There is a need therefore to conduct investigations for finding more sophisticated treatment strategies that can be the hope to achieve complete repair. Therefore, scientists have directed research towards adopting new therapeutic strategies that lie in replacing the damaged or injured cells with new ones; this strategy is termed “cell replacement therapy”.

With regard to replacing neural cells, many studies report that the provenance of cells used for replacement is a critical determinant for the success of the treatment. For instance, cell lines (immortal tumour cells) which are used widely in research (Zhou et al., 2006, Gordon et al., 2013b) are not a suitable strategy as
transplants for clinical use because of the potential for the carcinogenic effects (Freshney, 2002). The second option is the direct use of the embryonic tissue for transplantation. It has been reported that there is a clinical improvement in PD patients who received embryonic cells derived from mesencephalic tissue from aborted embryos (Lindvall et al., 1990). Despite these promising results, there are technical and ethical obstacles that limit the use of this source of tissue for clinical use.

In this context, the low survival rate of implanted cells is a critical obstacle. For example, not more than 3–5% of implanted DA neurons survive (Björklund et al., 2002). This can be as a consequence of several factors including hypoxia, hypoglycaemia, mechanical damage during tissue preparation for transplantation, mainly the mechanical dissociation in order to prepare the tissue for injection, free radicals, growth factors deficiency in addition to the excitatory amino acids in the host tissue (Brundin et al., 2000) and mechanical trauma to cells during surgical transplantation procedures. This highlights the need to improve graft cell survival. Several procedures have been used for improving graft survival. One of them is increasing the number of embryonic tissue donors, but this triggers ethical concerns regarding the use of this strategy (Dunnett et al., 1997).

Neural stem cells (NSCs) can offer a solution. The role of these cells in treatment of nervous system disorders lies in two main categories: (i) endogenous strategy and (ii) exogenous strategy. The endogenous category consists of recruiting cells from neurogenic niche (i.e. recruiting neurons generated from either NSCs from neurogenesic microenvironments including subventricular zone (SVZ) or dentate gyrus (DG) of the hippocampus (Taupin, 2006), or from regional cells). The
second is reprogramming of local cells and converting them into neurons by
inducing transcriptional factors (gene expression regulatory proteins). The
exogenous strategy includes grafting exogenous neurons which can be derived
from neuronal lineage (Grade and Götz, 2017). The ultimate goal is reducing the
functional deficit and restoration of lost neurons and functional networks.

In this section (General introduction), the focus will be on TBI and
neurodegenerative diseases rather than SCI because the attention of scientists in
the latter condition is in finding therapeutic approaches related to glial cell
replacement and axonal regeneration.

The success of any of these strategies relies on the tools of success. Success
outcomes are long–term survival, differentiation, synaptic integration and
behaviour. These strategies are applicable at different developmental stages (i.e.
can be performed at any developmental stage of the cells). A brief overview of
each strategy and its limitation is now given.

**The endogenic recruitment of neurons:** This process occurs naturally, where
the activity of neurogenic mechanisms starts at the early developmental stages
and continues to the early postnatal. Neurogenesis then undergoes a reduction
throughout life, and this reduction increases during aging (Donega et al., 2013).
Neurogenesis can persist in specific brain niches. The SVZ of mouse produces
neuroblasts that migrate to the olfactory bulb and integrate into the bulbar circuitry
as granule or periglomerular interneurons (Lazarini and Lledo, 2011). It has been
reported that the main function of this process is to accelerate difficult odor
discrimination learning and memory improvement. In humans, interneurons
migrate from the SVZ to the striatum however the functional significance of this process is still unrevealed. These processes occur in normal conditions but the inflammatory environment caused by injury can impact neuronal integration and neurodegeneration leading to challenges for the newly integrating neurons. Following injury or diseases such as stroke, trauma and HD, experiments in animal models have shown that neuroblasts can migrate to the injury site, form synaptic connections and have normal functional characteristics and survive for several months or they experience apoptotic cell death (Darsalia et al., 2006).

**Reprogramming endogenous cells:** Glial cells such as astrocytes that are involved in scar formation post injury in neurodegenerative diseases, can *in vivo* generate neurons. The newly generated neurons can be used for transplantation to reduce disease conditions (Berninger et al., 2007). Pericytes in the adult human brain can be transformed into neurons *in vitro*. For example, in stab injuries in the mouse neocortex, astrocytes and oligodendrocyte progenitor cells (OPCs) in the injury site can be reprogramed by forcing Neuro D1 expression leading to transformation of both cells into glutamatergic neurons generate, but only OPCs generated GABAergic neurons (Guo et al., 2014).

Another important endogenous strategy for repairing CNS disorders, relies on stimulating *in vivo* NSCs by trophic factors i.e. growth factors, hormones, and other signalling molecules for example by expression of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) involved in growth and maintenance of nerve fibres (Danzer et al., 2002, Murray and Holmes, 2011b).
However, these strategies are still impeded by lack of information regarding the output and input connectivity of the induced neurons, and how they participate in behavioural improvement. In general, the main obstacle to these strategies is their restriction to specific brain regions. Due to the brain region restriction, scientists have turned to find exogenous sources by which lost neurons can be replaced with new ones from external sources, which can be utilised in both injury and diseases conditions and are widely applicable to nervous system disorders. There are limitations for transplantation strategies such as their suitability for some pathologies versus others, in addition to the influence of the source of cells used. Despite that, this strategy has received wide attention in recent times, for both basic science and clinical trials.

Determining the neuronal type needed depends on the type disorder, for example in PD the required cells for replacement are DA neurons, while in HD, striatal medium spiny neurons are the target (Grade and Götz, 2017). In injury pathologies, many neuronal types may be affected, and providing multiple neuron types will be very challenging. In addition, the presence of the glial scar might have an inhibitory effect on neurite outgrowth (Silver and Miller, 2004). The importance of choosing the appropriate cell source relies on ensuring the long survival, expandability and connectivity with the host tissue, ease of differentiation into the desired neurons, and reducing the immunogenic reaction of the host tissue.

For this purpose, a variety of stem cells have been considered as the source for replacement therapy such as embryonic NSCs-derived neurons (Björklund et al., 2002), multipotent or pluripotent stem cells (Dantuma et al., 2010), and induced
pluripotent stem cells (iPSCs) (Lindvall and Kokaia, 2010). These cells have demonstrated promising outcomes in both animal models and clinical trials. Another source of cells used for transplantation therapy is the primary fetal neurons which are derived from the brain region that corresponds to the area subjected to disease. This strategy has been tested in animal models, for instance, fetal midbrain DA neurons transplanted into the striatum of PD animal models (Grealish et al., 2010). The same strategy was tested in a clinical trial (Barker et al., 2013). In the two studies, transplanted cells displayed survival and complete maturation into desired neuronal subtype (DA neurons) within the host tissue. However, these results are associated with inconsistency and unpredictability relating to the source of cells harvested for transplantation which is non uniform in relation to both the number and type of neurons generated, and their integration to the host tissue (Donaldson et al., 2005).

The idea of transplanting neurons is to deliver new neurons that have been grown in vitro (in a laboratory dish) into the damaged or injured area. Cells can be delivered in two forms, either by implanting NSCs directly where the internal signals from the host tissue guide them to mature and differentiated neurons. Alternatively, they can be delivered after differentiation of NSCs in culture into the desired neuronal type before implantation (National Institutes of Health (NIH), 2017). The process of cell transplantation so far still suffers from difficulties in cell handling and immunological rejection. Furthermore, cell preparation for transplantation depends on differentiating cells in 2D culture, which in turn has its limitations related to cell expansion and the loss of clonal and differentiation capacity due to long-term passaging (McKee and Chaudhry, 2017).
There is a need to minimize or overcome the accompanying obstacles of tissue transplantation in general, and for neurons specifically (Rossi et al., 2010). There is also a need to investigate and understand biomolecular mechanisms relating to neuronal development and physiology, for instance, forming fully functioning connections of grafted cells within the native tissue. Regenerative medicine has therefore become directed towards gene and tissue engineering strategies for this purpose which will be addressed in the next sections.

1.6. Combinatorial gene therapy is needed for CNS regeneration

Despite the positive outcomes from experimental and clinical trials, it is desirable that the repair capability of implants is improved in order to enhance the regeneration of the CNS. This can be achieved by combining two or more strategies (Combinatorial therapy). Specifically, if implemented in the right sequence and at the right time, in some conditions it can be considered to be more promising than a single therapy approach (Suter et al., 2006). In this context, many studies have used animal models to support this idea. For instance, Lohara, et al. and Lu, et. al have demonstrated the benefit of combinatorial treatment for repairing lesioned dorsal-column sensory axon projections in an animal model (Lu et al., 2004, Lohara et al., 2013). A group of sixty adult female F344 rats were included in this study, divided into control group injected with PBS and treated groups either with cyclic adenosine monophosphate (cAMP) or neurotrophin-3 (NT-3) 5 days before lesion. Another group was treated with both, then at day 5, bone marrow stromal cell (MSC) suspensions were applied in the lesion site. The dual combination of one of the treatments with the lesion site resulted in promotion of dorsal-column sensory
axon growth, while combining the three treatments (cAMP and NT-3 with the grafted cells) produced a significant axonal growth beyond the lesion site (Lu et al., 2004) which occurred only when the three treatments were combined.

Gene therapy also is emerged as a promising approach, using genetically engineered transplanted cells which termed ex-vivo gene delivery. Here, the transplants act as vehicles for delivery of therapeutic biomolecules to the area of interest (Rose et al., 2012). This approach covers research to enhance the understanding of various biological and physiological phenomena specifically related to degenerative and regenerative CNS cells. In this regard, neurons have had wide and special attention, particularly because they are the main cells affected in CNS, and due to their inability to proliferate and differentiate are not replaced. Furthermore, neurons are challenging in terms of being both hard to transfect as post mitotic cells and showing high sensitivity to alterations in their microenvironment (Karra and Dahm, 2010b).

1.7. Studies performed on genetic engineering of neurons
Neuron engineering has received wide attention recently, in both the clinical and basic science research fields. In terms of the clinical benefits of engineering neurons, as discussed earlier, the main objective of transplanting neurons or neuronal precursors is to replace damaged neurons, and for grafted cells to become structurally and functionally integrated into the host brain. Immature neurons at the developmental stage when they have not formed an extensive axonal connection, have the ability to survive and integrate within either fetal or adult host brain. The probability of formation of neural circuitry is greater during the fetal period than at the adulthood (Bjorklund and Lindvall, 2000, Falkner et al.,
In this context, establishing functional connectivity in the adult brain can be enhanced if the host circuitry is damaged, wherein host neurons may release axonal growth factors that stimulate axons to regenerate (Lindvall and Björklund, 2004, Geral et al., 2013). Therefore, neurotrophic or neuroprotective factors can be produced by transplanted cells by genetically engineering these cells. Thus, degeneration can be counteracted, or regeneration can be promoted (Géral et al., 2013). Several studies demonstrate the benefit of genetically engineering neurons by introducing genes encoding neurotrophic factors such as brain derived neurotrophic factors (BDNF), nerve growth factor (NGF), and neurotrophin-3 (NT3), in animal model and clinical trials. For example, an in vitro study on a rat model was conducted in order to investigate the effect of BDNF on axonal regeneration in a spinal cord injury model which resulted in promoting rubrospinal axonal regeneration and functional recovery (Koda et al., 2004). Another study reported that BDNF released from dendrites and soma of neurons transfected with the BDNF gene results in an increase in dendrite branching of neighbouring neurons, which are no more than 4.5 µm away (Horch and Katz, 2002). Genetic engineering has also been used to express Nurr1 transcription factor important for midbrain DA neurones development, or in embryonic stem cells (ES) leading to promotion of DA cell differentiation (Chung et al., 2002).

For research, transfection of neurons in culture is important for studying biological and physiological processes in neurons. An example is the study of protein function in neurons, such as the investigation of the function of transcriptional factor MEF2 protein in neurogenesis, specifically in controlling maturation by transfecting cortical neurons with a plasmid containing a gene
encoding a dominant-interfering form of MEF2 (MEF2CR24L) (Mao et al., 1999). It can also be used for studying gene function in normal and disease status, such as determining the role of methylated-CpG-binding gene (Mecp2) encoding MECP2 protein that recruits additional factors such as histone deacetylase to repress transcription gene deficiency, critical for normal function of mature neurons, and to delay maturation of hippocampal neurons by reducing presynaptic protein expression and dendritic spine density (Smrt et al., 2007).

1.8. Genetic engineering techniques

Naked DNA can be delivered to the target cell or tissue when directly applied or injected to various cell types (Luo and Saltzman, 2000b). However, it is inefficient for gene delivery due to low DNA concentration at the cell surface and it is appropriate just for some applications, such as DNA vaccination (Nayerossadat et al., 2012).

Generally, in a gene delivery system, the access of the genetic material to the site of action either for gene therapy or a genetic engineering system requires crossing several barriers. These barriers are extracellular barriers which include; (i) degradation of free DNA in blood by serum nuclease when injected intravenously for gene therapy (Niven et al., 1998), (ii) the barrier imposed by the plasma membrane, as the gene needs to penetrate the cell membrane. Then, there are intracellular barriers, once the gene is internalized, and the plasmid travels through the cytoplasm by diffusion or active transport to reach the next barrier (iii) which is the nuclear membrane, then the transcription, translation and modification process starts (Vaughan et al., 2006).
For overcoming these barriers, various techniques can be introduced; (i) enhanced targeting process to increase gene uptake, (ii) in case of the uptake through endocytosis mechanisms, maintain the intracellular availability by enhancing the endosomal release, (iii) enhancing nuclear delivery (Ziello et al., 2010, Varkouhi et al., 2011) (Figure 1.5). In this project the focus is going to be on enhancing targeting system by enhancing gene delivery techniques.

Accordingly, several genetic engineering techniques have been developed, some of them viral and the other non-viral techniques. Nevertheless, so far there is no ideal gene delivery system effective for all cell types’ specifically post mitotic cells, both *in vitro* and *in vivo*, without restrictions to the type of cells or side effects.

Consequently, there is no established transfection technique for neurons that is suitable for both therapeutic and research applications. In that context, the ideal gene delivery technique for cells and tissue specifically neurons should have the (i) capability to transfect all type of cells and tissues with high efficiency, (ii) facilitate transfection of genes of varying sizes, and co-transfection with multiple genes.(iii) low cellular toxicity (iv) be easy and safe to perform (Washbourne and McAllister, 2002). Accordingly, researchers have made an effort for the purpose of developing safe, reliable, highly efficient strategies for genetically engineering cells. As the viral vector was at the forefront of potential vectors, as they evolved to efficiently deliver their genetic material to a cell before magnetic nanoparticles being used as a vector. This goes back to the biology of different viruses that offers promising solutions to the challenges of gene engineering strategy, such as cell targeting, transgene expression processes (figure 1.5). However, there is
consideration regarding the natural biology of a vector that prevents them from being the most effective for a specific cells.

Figure 1.5: Schematic to illustrate the main gene delivery barriers. (1) is the cell membrane barrier, the endocytic mechanism is proposed for delivering nucleic acid-IONPs complex inside the neuron, so complex internalization occurs via cell membrane invagination then (2) forming intracellular vesicle, (3) endosome that process the complex and form materials that will release into the cytoplasm to make their own way to their target. Then (4) some of the materials will be degraded by fusion with the lysosome. (5) And some of them will reach to the nucleus membrane.
1.8.1. Viral transduction

Currently, delivering therapeutic nucleic acids into the cells using viral vectors is
termed transduction, which consists of recombining the gene of interest with the
bacterial plasmid, as a vector, and forming copies then transferring them into the
nucleus of the host cells, in a highly successful gene delivery approach (Lentz et al., 2012b). This approach has been used since the early 1970s (Friedmann and
Roblin, 1972). Several types of viruses have been used such as retrovirus,
adenovirus (types 2 and 5), adeno-associated virus, herpes virus, poxvirus,
human foamy virus (HFV), and lentivirus (Huang et al., 2011). The popularity and
wide range of usage of this method are due to the robust transduction efficiency
reached (up to ca. 95%) mainly for the most challenging cells such as the post
mitotic neurons (Karra and Dahm, 2010b, Washbourne and McAllister, 2002). For
example, adeno-associated viruses, lentiviral vectors, and herpes simplex viruses
have been used to transduce different types of neurons such as the neuron line
PC12 (Sun et al., 2018), or primary neurons including hippocampal and cortical
immature (Royo et al., 2008) and mature neurons (Dong et al., 2003), some
examples of transducing are neurons listed in Table 1.1.

Viral vectors have specific traits that make them attractive to scientists as a gene
delivery system. The virions which envelope the viral genetic information provide
protection and stability during passage through the human body. The virions can
target the cell that the virus will enter by binding to external receptors of the host
cell. Gene expression in the host cells can be controlled by viral genetic material
(Lentz et al., 2012b). Another advantage of viral vectors includes the capability
to infect post mitotic cells such neurons both in *vitro* and *in vivo* (Karra and Dahm, 2010a).

Despite the advantages of viral vectors for gene expression strategy, they have disadvantages that limit their usage. Firstly, transduction protocols are time consuming due to the multiple complex steps including the production of infectious virions in cell lines and then virus purification (Gardlík et al., 2005). Viruses can also have cytotoxic effects associated with a high expression such as the toxicity of Herpes Simplex Virus-1 (HSV-1) which is *“a neurotropic virus that can establish lifelong persistence in sensory neurons”* (Epstein, 2009, White et al., 2002, Thomas et al., 2003). Secondly, the virions can sometimes be pathogenic because they are derived from viruses that are a source of disease, and the viruses possess the machinery to access the host cells and exploit their machinery to facilitate replication and toxicity (Thomas et al., 2003). During the replication cycle of herpes viruses, cells lyse as a part of the normal process (Lentz et al., 2012a), with immunogenicity and toxin production (Blömer et al., 1997). Finally, some viruses (e.g. adenovirus) show preferential infection for some cell such as glial cells and limited transduction in neurons. The late onset of gene expression, which can take up to two weeks after infection to show transgene expression, can impact the experimental and therapeutic time window. Further, there is also a limitation on the DNA insert sizes of ~5 kb, limiting the usage to a smaller transgene as in AAVs (Karra and Dahm, 2010a, Royo et al., 2008). An example of the impairment effect of the viral technique of gene delivery is the suppression of sodium current of mammalian neurons up to 80% by herpes simplex virus-1 (HSV-1) helper virus 5dl1.2 (White et al., 2002). Many efforts have
been made to optimize gene delivery techniques for neurons. However, no technique has yet been suitable for all applications. For these reasons the hopes are directed towards the non-viral approaches.
Table 1.1: Examples of viral vectors used for transducing neurons.

<table>
<thead>
<tr>
<th>Virus vector</th>
<th>Neurons type</th>
<th>Transduction efficiency</th>
<th>Toxicity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adeno-associated viral (AAV1)</td>
<td>In vitro hippocampal &amp; cortical neurons</td>
<td>80%</td>
<td>Serotypes dependent (AAV 5&amp; 6) more toxic than the others.</td>
<td>(Royo et al., 2008)</td>
</tr>
<tr>
<td>Lentivirus Vector</td>
<td>Striatal cells</td>
<td>88.7%</td>
<td>Low</td>
<td>(Blömer et al., 1997)</td>
</tr>
<tr>
<td>Herpes Simplex Virus (HSV)</td>
<td>Multiple types of neurons</td>
<td>High</td>
<td>Low preclinical use, clinically remains a concern</td>
<td>(Karra and Dahm, 2010)</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Dissociated neurons, slice cultures and in vivo</td>
<td>Moderate</td>
<td>High with high doses</td>
<td>(Washbourne and McAllister, 2002)</td>
</tr>
</tbody>
</table>
1.8.2 Non-viral transfection

Any transfection techniques will have advantages and disadvantages, which are related to transfection efficiency, gene expression levels, cell survival, ease of use, reproducibility, cost, and applicability to a given experiment. Non-viral techniques are gene transfer methods which are an alternative to viral methods and they comprise of chemical, physical and electrical methods. These techniques have attracted researchers for gene delivery despite the presence of some extracellular and intracellular obstacles.

The following is a brief overview of some of the non-viral transfection techniques:

(i) Physical transfection methods: Microinjection: This is a direct technique based on injection of external genetic material cRNA into the cytoplasm or DNA into the nucleus of the host cell by a glass micropipette. The gene of interest recombines with its corresponding gene to express its function (King, 2004, Horii et al., 2014). This technique is a single cell dependent and can be used for mature neurons and cell lines (Karra and Dahm, 2010a). However, it is associated with a poor survival rate, as the survival rate relies on the experience and the appropriate controlling every microinjection in order to not cause physical damage of the neurons. Thus, much experience is needed in order to achieve 80% cell survival (Zhang and Yu, 2008). Accordingly, it requires a high skill specifically with mammalian neurons, and therefore has not been used routinely in the clinic.

(ii) Bio ballistics or Gene gun: In this technique, the gene of interest is gold coated and can be injected directly to the cytoplasm of the host cell by gunshot
(hand-held Gene Gun introduced by Bio-Rad ) (O’Brien et al., 2001) to find the nucleus and enters into it (Lo et al., 1994). Here, all brain cells types can be transfected but with low transfection efficiency, up to ca. 2%. This can be improved to ca. 10% of cultured neurons and reached up to ca 34% in brain slices (Karra and Dahm, 2010b). However, this technique faces the same problem of cell damage because of the particle size ranging between 40-180 nm, in addition to acceleration and high pressure of gold particles (Murphy and Messer, 2001, O’Brien and Lummis, 2011).

(iii) **Electrical transfection methods:** This is another direct gene entry technique where electrical stimuli are applied to the host cells which results in plasma membrane charge alteration. This facilitates the entry of the charged extracellular gene which crosses the cell membrane and enters the cytoplasm (Khattak et al., 2012). The transfection rate of this method is low specifically in post mitotic cells such as neurons (15-20%), and can be enhanced but at the expense of cell survival therefore, it is limited to embryonic and dissociated neurons. This method is termed ‘electroporation’ (Dib-Hajj et al., 2009). This technique has been developed to facilitate gene entry to the nucleus and is named ‘nucleofection’. Despite the high transfection rate that can be achieved by this technique, it is limited to freshly isolated cells and cells in suspension (Zeitelhofer et al., 2007, Zeitelhofer et al., 2009). Therefore, there is a necessity for developing techniques that can introduce a nucleic acid to the cell using a membrane-crossing vector, in addition to consideration of the safety problems (i.e. to ensure efficient gene delivery to the target cells safely).
(iv) **Chemical transfection methods:** Ca\(^{2+}\)-phosphate/DNA co-precipitation. Although this is considered to be one of the preferred techniques used for transfecting neurons at all developmental stages whether primary or cell lines, or mature neurons (post-mitotic), this is still challenging with cells difficult to transfect. Transfection efficiency in mature neurons ranges between 1-5% (Goetze et al., 2004). Its advantage relies on low cost with no specialist equipment needed. Its principle is a precipitation based method by assembling DNA crystals with the Ca\(^{2+}\) ions in the phosphate buffer. This buffer precipitates onto the cells (adheres to the cell membrane) and the crystals are then taken up via endocytosis (Washbourne and McAllister, 2002, Karra and Dahm, 2010b).

**1.9. Magnetofection**

This is a vector based delivering technique which uses applied magnetic fields to improve the transport of vector-plasmid complex, in order to interact sufficiently with the desired target structure (Figure 1.6). It is a recent technology that offers promise to overcome the limitations in nucleic acid uptake by the cell membrane. Magnetofection can be used for natural vectors (viral) (Table 1.1) and synthetic (non-viral nucleic acid) delivery (Table 1.2) (Plank et al., 2011b). Initially, paramagnetic nanoparticles (nanoparticles which have slight positive response to the magnetic field) were used for enhancing retroviral vectors delivery into the human erythroleukemia cell line (K562) (Hughes et al., 2001). Then Scherer and colleagues attached non-viral vector superparamagnetic iron oxide nanoparticles (IONPs) (smaller in size than paramagnetic nanoparticles and generate greater magnetization susceptibility; about 100 fold higher) (Herranz et al., 2011),which
were coated with polyethylenimine, for enhancing *in vitro* transfection efficiency in primary human peripheral blood lymphocytes (PBL) cells (Scherer et al., 2002).

This encouraged researchers to investigate and develop transfection techniques based on magnetofection specifically for primary cells. It is worth mentioning that enhancing the transfection efficiency should be associated with eliminating or reducing the associated toxicity which is an obstacle for both biological and electrophysiological studies.

During recent years the attention on developing magnetofection technology has been growing, specifically for studying the etiology of neurodegenerative diseases and treatment methods. For example, an *in vitro* model was used for studying the mechanisms underlying axonal growth and motor neuron disease. As in spinal muscular atrophy which is one of the neurodegenerative diseases, characterized by a selective degeneration of spinal cord motor neurons which results from a reduction in the ubiquitously expressed survival motor neuron 1 gene (SMN1) leading to the reduction in survival of motor neuron (SMN) protein. SMN functions with partner proteins in the assembly of nuclear ribonucleoproteins (Burghes and Beattie, 2009). Magnetofection technology was used in characterizing the interaction between the reduction in SMN and mRNA-Binding Protein which is required at multiple points during neuronal development (Akamatsu et al., 2005, Fallini et al., 2011).

The importance of magnetofection prompted scientists to develop this technology to test several cell types such as rat primary astrocytes derived from the cerebral cortex (Pickard and Chari, 2010a, Pickard et al., 2010), and neural stem cells
(Pickard and Chari, 2010b, Pickard et al., 2011). Additionally, to enhance the transfection efficiency metal-based nanoparticles and various magnetic fields (static, oscillating and pulsating magnetic) have been tested for transfecting different cell types and specifically for neuronal and neuroglial cells (Kamau et al., 2006, Zheng et al., 2014, Tickle et al., 2015, Subramanian et al., 2017). For more detail please see the introduction of chapter 4.

As described previously magnetofection is a promising technology that can contribute to achieving these goals in several cell types such as NIH 3T3 cells (Plank et al., 2003). Researchers are working on transfecting neurons and have using model neuron systems such as the SH-SY5Y cell line (Vernon et al., 2015) when it is difficult to generate primary neurons although the primary neurons are more mimetic to the in vivo environment than cell lines. So far, it is has been difficult and challenging to transfect primary neurons, which relates to their sensitivity to micro-environmental changes (Kim et al., 2006, Jiang and Chen, 2006). From this perspective, choosing the appropriate magnetic particle and transfection technique is an important concern. Various experiments have been conducted using magnetofection in order to enhance the transfection efficiency by applying different techniques and reagents (Ohki et al., 2001, Scherer et al., 2002), or gene expression time (Soto-Sánchez et al., 2015) and studying the uptake of magnetic nanoparticles (Petters and Dringen, 2015). However, little is known regarding the effect of magnetofection and nanoparticles on neuronal functionality, this needs further investigation.

In this project, the effect of increasing the physical activity of IONPs on primary neuronal transfection efficiency using oscillating magnetic assistive devices will
be investigated. Moreover, the safety of using magnetic nanoparticle-magnetofection technology for a transfecting primary cortical neuron will be evaluated in the terms of neuronal morphology viability and functionality.

Two terms will be used referring to nanoparticles. One is magnetic nanoparticles (MNPs) which refer to the different types of magnetic nanoparticles including iron oxide nanoparticles, the second term specifically referring to IONPs.

**Figure 1.6: Schematic illustrates the magnetofection principle.** The gene of interest is complexed with magnetic nanoparticle (vector), the effective ratio of gene/magnetic nanoparticles concentrations is cell type dependent. Gene-vector complex is applied to the desired cells, driven by magnetic force underneath the sample in order to enhance the complex attachment to the cell membrane.
1.10. Nanoparticle-based transfection

1.10.1 Characteristics of IONPs
Iron oxide nano-particles are nano-meter sized particles ranging from 1-100 nm (Zhang et al., 2007), which are classified according to their dimensions, shape, composition, uniformity and agglomeration, they are of great interest in many areas including as magnetic fluids (colloidal liquids made of nanoscale ferromagnetic, or ferrimagnetic, particles suspended in a carrier fluid), catalysis, magnetic resonance imaging, environmental remediation, biotechnology/ biomedicine and data storage. The best performance of IONPs is when their size ranges between 10-20 nm (Lu et al., 2007) which is below the critical limit (76 nm) (Li et al., 2017). They are characterized by being superparamagnetic (i.e. lose their magnetization when the external magnetic field is removed) therefore they play a vital role specifically in biomedical applications. Also, the risk of agglomeration under room temperature is very low (Gupta and Gupta, 2005).

Generally, the high chemical activity of naked metallic IONPs and their oxidization in air necessitates their protection via coating with organic layers such as surfactants or polymers, or inorganic layers like silica or carbon. Coating layers can be made up of cationic or anionic poly amino acids. An example of these coating layers are: polyethylenimine (PEI), phosphorylated starch, DEAE dextran or similar compounds, organic polymer or inorganic metallic (e.g. gold) or oxide surfaces (e.g. silica or alumina), biopolymers like collagen (Sinani et al., 2003, Schillinger et al., 2005, Bao et al., 2016) or any other molecules that make them
biocompatible such as poly ethylene glycol (PEG) and folic acid (Zhang et al., 2002).

This coating layer is not just acting to stabilize IONPs but also to functionalize them with other nanoparticles (e.g. iron oxide nanoparticles, coated with a silica shell that is functionalized with gold nanoparticles) or various ligands that can be varied according to the application. The coating layer also acts as an isolator to minimize the agglomerations that occur due to the hydrophobicity of the magnetic core (Lu et al., 2007).

The specific physicochemical properties of IONPs include their nano size, large surface area to mass ratio, and high reactivity which make functionalized IONPs of great interest in biological and clinical applications. They have been used as fluorescent biological labels, in drug/gene delivery and targeting, stem cell targeting, bio-detection of pathogens, protein detection, probing of DNA structure, tumour destruction via heating (hyperthermia), separation and purification of biological molecules and cells, diagnostic imaging (to improve the sensitivity of magnetic resonance imaging (MRI) (as contrast agents) (Zhang et al., 2007, Tietze et al., 2015). Moreover, the utility of IONPs is still growing specifically for a range of regenerative applications for example cell transplant imaging (Bulte et al., 1999), tissue engineering and gene delivery using the advanced magnetofection technology (Cheong et al., 2009, Adams et al., 2013) and diagnostic imaging in neurological injury (Muja and Bulte, 2009). During recent years, there has been an increased interest in the use of IONPs in regenerative medicine and cell therapy as gene delivery vectors in gene engineering technology. The importance of IONPs lies in their nanoscale size, their magnetic
properties that can be manipulated by applying external magnetic force, and their capability to carry various biological entities linked to the coating layer such as nucleic acids, antibodies or drugs. Attaching therapeutic DNA to IONPs can be used for treating damaged DNA in some diseases by replacing the damaged area of the gene, or down-regulate the oncogene overexpression which promotes carcinogenesis by RNA interference (RNAi) (Kievit and Zhang, 2011). Furthermore, attaching drugs to the IONPs coating layer to act as drug delivery system has a wide range of uses because this can overcome several drawbacks starting from limited effectiveness, poor bio-distribution, and a lack of selectivity of blood brain barrier for the transit of the drugs into the brain parenchyma.

1.10.2. IONPs as a gene delivery vector

Given the vast scope of using IONPs in nanomedicine, they have attracted great interest in this field with a vital role as vectors for gene delivery in gene engineering technology, specifically for transfecting neurons which will be the focus of this project.

IONPs can be natural compounds or they can be synthesized in laboratories (Cornell and Schwertmann, 2003). The formulation protocol for IONPs impacts their size and shape which in turn leads to heterogeneity in action (Gupta and Gupta, 2005). Generally, sixteen iron oxides have been recognised (Cornell and Schwertmann, 2003). The typical formulation of magnetic IONPs is an iron oxide core of either magnetite (Fe$_3$O$_4$) which is the most common usage specifically in the biomedicine field, or maghemite ($\gamma$-Fe$_2$O$_3$) and both have single domains of ferrimagnet (magnetization dose not vary across the magnet). They are characterized by possessing super magnetization properties and are widely
utilized in multiple applications that are listed in section 1.9.1. superparamagnetic IONPs (diameter ranges between 50–100 nm) and ultra-small superparamagnetic IONPs diameter <50 nm (Turcheniuk et al., 2013). Magnetic IONPs are characterized by their inexpensiveness to produce by either co-precipitation of ferric and ferrous iron salts, thermal decomposition of organometallic precursors and the microemulsion method in which iron salts form NPs within microdroplets (Petters et al., 2014), physical and chemical stability, biocompatibility, and environmental safety. The shape such as ultrathin nanowhiskers, nanoplates and nanoflowers, nanocubes, and single crystalline nanoworms (Bao et al., 2016) and size of IONPs have an impact on their magnetic properties. γ-Fe₂O₃ and Fe₃O₄ display ferrimagnetism at room temperature, with the saturation magnetization reaching to 92 emu g⁻¹ (Wu et al., 2015).

IONPs have been involved extensively in biomedical applications. Due to their super magnetization properties they have become popular for in vivo applications as they do not aggregate after exposure to a magnetic field versus large domain magnetic and paramagnetic materials (Bonnemain, 1998, Wang, 2011). Also the importance of utilizing IONPs lies in their stabilization by choosing the appropriate coating layer (Figure 1.5). Generally, IONPs undergo opsonization on entry into the bloodstream and tissue systems which leads to stimulation of the immune defence system (reticulo-endothelial system). This is a major obstacle in particle targeting to the site of injury. Therefore there is an effort to overcome this barrier by using the nanoparticle ‘stealth coating’ strategies such as using (PEG) that evade IONPs from immune clearance and prolong circulatory time thus facilitating
IONPs bioavailability and accumulation in the brain (Kim et al., 2007). Moreover, our group reported that PEGylated nanoparticles evade all the immune and non-immune cell populations in the brain (Jenkins et al., 2016). Stabilizing IONPs is necessary for targeting strategies and enhancement of their efficiency as delivery vehicles for various biomolecules (Figure 1.7). For example, several coating layers of IONPs have been tested and tailored in order to use IONPs as gene delivery vehicles. Polyethyleneimine (PEIs) is one of the first transfection grade agents that has demonstrated its ability to interact with positively charged amine groups electrostatically with the negatively charged phosphate backbone of DNA efficiently (Plank et al., 2003). IONPs as a gene delivery vehicle are of great interest in regenerative medicine and have been used to genetically engineer neural cells such as astrocytes (Tickle et al., 2016), oligodendrocytes precursor cells (Jenkins et al., 2011), and neurons (Petters and Dringen, 2015). Despite the advantages of using IONPs, there are concerns related to their potential toxicity. Specifically, using these particles in neurobiological applications can be a source of alterations in brain iron homeostasis which is strongly related to human neurodegenerative diseases such as AD and PD (Hare et al., 2013).

In general, IONPs toxicity depends on physiochemical parameters including particle size, shape, surface charge and chemistry, and composition (Li et al., 2012). The toxicity of IONPs is still under investigation in several cell types. One of the explanations related to IONPs toxicity is associated with particle uptake and metabolism in CNS cells. Particle uptake starts with internalization of the particles by endocytic mechanisms, which results in formation of intracellular vesicles containing IONPs which are directed to the lysosomal compartment in which iron
can be liberated from IONPs due to the acidic environment of lysosomes which can lead to toxic effects (Petters et al., 2014). Also, it has been reported that moderate concentrations of IONPs ranging from 0.15 to 15mM of iron can reduce cellular viability (Pisanic et al., 2007).

Figure 1.7: Schematic diagram which illustrates IONPs and interactions between functionalization layer and some biological entities. IONPs are often coated by organic polymers which generate charged surfaces that facilitate electrostatic interactions with components of the milieu.
Table 1.2: Examples of non-viral vectors used for transfecting neurons.

<table>
<thead>
<tr>
<th>Plasmid Magnetic nanoparticle</th>
<th>Neurons type</th>
<th>Transfection efficiency</th>
<th>In vivo or/ in vitro</th>
<th>Magnet type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLenti-Synapsin-hChR2 (H134R)-EYFP-WPRE NeuroMag</td>
<td>Visual cortex neurons</td>
<td>97% after 30 days</td>
<td>In vivo</td>
<td>Magnetoplexes</td>
<td>(Soto-Sánchez et al., 2015)</td>
</tr>
<tr>
<td>noDTS,SV40,and SMGA NeuroMag</td>
<td>Undifferentiated SH-SY5Y cell</td>
<td>Highest efficiency noDTS (over 50%) SV40 (4.5%)</td>
<td>In vitro</td>
<td>Oscillating magnet arrays</td>
<td>(Vernon et al., 2015)</td>
</tr>
<tr>
<td>EYFP fluorescenc CombiMag</td>
<td>Embryonic hippocampal neurons</td>
<td>5%</td>
<td>In vitro</td>
<td>Super magnetic plate</td>
<td>(Buerli et al., 2007)</td>
</tr>
<tr>
<td>EGFP-N1-RanBP9-FL Neuromag</td>
<td>Primary cortical neurons</td>
<td>30%</td>
<td>In vitro</td>
<td>Magnetic plate (Oz Biosciences)</td>
<td>(Wang et al., 2014)</td>
</tr>
<tr>
<td>mCherry fluorescent proteins, fluorescent green protein (GFP) NeuroMag</td>
<td>Primary motor neurons</td>
<td>45%</td>
<td>In vitro</td>
<td>Magnetic plate (Oz Biosciences)</td>
<td>(Fallini et al., 2010)</td>
</tr>
</tbody>
</table>
1.11. Uptake mechanism

Gene-vector internalization into the target cells relies on appropriate cellular interaction mechanisms which vary according to the vector characteristics. Further, delivering the genetic material into the target cell is a linked mechanism between uptake, intracellular trafficking and vector fate. Designing non-viral transfection techniques requires a comprehensive understanding of uptake pathways and the intracellular processing mechanisms. A brief overview of uptake pathways will be given here.

The first barrier to transfection is the cell membrane which is hydrophilic, hence internalization of entities large in size and hydrophilic molecules is limited (Khalil et al., 2006). Accordingly, endocytosis (active transport mechanism by which extracellular molecules enter the cell through vesicles formed from cell membrane), is considered the main mechanism for the non-viral uptake (Friend et al., 1996).

This mechanism encompasses two forms and is classified according to vesicle size; ‘pinocytosis’ which means cell drinking, and is a process used in internalizing fluids and small molecules, hence the vesicles formed are of small size. For the larger molecule such as cell debris and whole microorganisms, large vesicles form by a mechanism termed ‘phagocytosis/cell eating.’

In terms of the gene-vector internalization, the small size of complexes tends to evoke the pinocytosis mechanism. Clathrin, caveolae, macropinocytosis, and clathrin/caveolae-independent endocytosis are well characterized entities that can mediate pinocytosis (Ziello et al., 2010). Regarding IONP uptake mechanisms in
various neural cell types, Fernandes and Chari (2014) have reported that there is dramatic variation in particle uptake and astrocytes were the dominant population performing uptake compared to neurons and oligodendrocytes (Fernandes and Chari, 2014). With regard to neurons, endocytosis is considered as an essential mechanism which regulates intercellular signalling such as initiating signal transduction via ligand binding to tyrosine kinases and G-protein-coupled receptors (McPherson et al., 2001), nutrient uptake and synaptic transmission (Blanpied et al., 2002). The uptake process in neurons is either through forming clathrin-coated vesicles or a clathrin-independent mechanism which is still poorly understood (Cosker and Segal, 2014). These clathrin coated pits in immature neurons are spread throughout dendrites and at the tips of dendritic filopodia. They appear and disappear repeatedly and are present locally at active spots. While, clathrin-coats in mature neurons are stable in the dendritic spines, endocytic zones lie lateral to the postsynaptic density (PSD). This section has described a general overview of the main topics associated with genetic engineering of neurons. The subsequent sections will address the process of growing neurons in in vivo mimetic environments in order to be a useful platform for both scientific research and regenerative medicine.
1.12. 3D hydrogel models for neurodegenerative medicine and basic research

Cell or tissue engineering is an interdisciplinary field concerned with creating functional 3-dimensional combining scaffolds, cells and/or bioactive molecules for investigating the physiological and pathophysiological processes in vitro in addition to providing tissue mimicking constructs (Griffith and Swartz, 2006). Two main concerns have resulted in the need to develop neuronal 3-dimensional constructs.

Firstly, to meet the needs of basic research: For more than 100 years, in vitro studies have been conducted on 2D monolayer cultures grown on unnaturally flat substrates and associated standard cultivation conditions including temperature, sterilization technique, humidity and culture media (Khoruzhenko, 2011). Most of the previously described information of neuronal cellular biological and physiological processes, maturation, migration and proliferation, were obtained from experiments conducted in monolayer cultures that do not reflect the in vivo environment. Additionally, animal models cannot fully mimic or predict human responses and are costly, time consuming and ethically arguable. To that end, it has been necessary to develop ex vivo culture environments that can mimic the in vivo environment. In this context, the 3D approach is essential for reducing animal usage for testing drug effectiveness and toxicity screening such as in anticancer drugs and toxicology studies (Pampaloni et al., 2007).

Secondly, to meet the needs of regenerative medicine: This is to sidestep the problems related to present treatment strategies in relation to organ transplantation as there is potential for tissue rejection and lack of donors, and
mechanical devices cannot accomplish all the functions associated with the tissue; surgical reconstruction can also result in long term problems. The ultimate goal of regenerative treatment is reducing the functional deficit, restoration of lost neurons and functional networks. However, most treatment strategies rely on symptomatic treatments or slowing down disease development. Developing in vitro devices that can repair in vivo the damaged tissue can offer a solution to these issues.

Cells delivered in 3D hydrogels have been used as a potential and promising treatment for neurons damaged due to injury /disease (Lindvall et al., 2004, Thompson and Björklund, 2015). The ultimate goal of replacing the damaged neurons with intact ones is repairing the brain that can be achieved by integrating the neurons into the brain circuitry and reconstruction of the structural and physiological connectivity of the damaged neurons. Basing on this goal, grafted immature neurons or neural precursors derived from embryonic stem cells or induced pluripotent cells have been tested on rodents since the 1970s. These experiments recently have been translated into the clinic such as by transplanting cultured human motor neurons into patients with basal ganglia stroke and reported its feasibility by showing a positive improvement in motor function in six patients (3 to 10 points) and mean improvement up to 2.9 for all patients (Kondziolka et al., 2000).

1.13. What is the influence of substrate on cellular response and morphology?

The in vitro models used for expanding our understanding of cellular biological processes and pharmaceutical responses have been designated as two-
dimensional (2D) cultures (on a flat substrate). Polystyrene or glass substrates are the most common material used for manufacturing the substrates for cell culture (Freshney, 2005). Despite the tremendous benefits of this model of cell culturing over the years, which has resulted in thousands of published studies, these also have significant drawbacks, mainly as they are unrepresentative of the *in vivo* environment. Therefore, it necessary to develop a model that reflects the biological and structural characteristics of the cells *in vivo* that can be termed three dimensional (3D) culture. Tissue engineering and growing cells in 3-dimensional scaffolds is a promising strategy in order to bypass the limitations associated with current biological and medical studies and applications.

In the early 1970s, efforts were made to explore the different responses of cells on flat surfaces for monolayers and 3-dimensional scaffolds. There was a clear difference in responses between cells cultured as 2D monolayer and those in the 3D constructs. For example, it has been found that there is a significant variation in mammalian cell responses including cell polarity and enhanced migration along fibrils by providing contact guidance signal, cytoskeleton structure, distribution of receptors, response to a wide range of hormones, growth factors, and apoptotic factors (Tibbitt and Anseth, 2009, Kim et al., 2012).

In monolayer culture, biological responses such as receptor expression, transcriptional expression, cellular migration, and apoptosis in addition to the histological organization differ from that of the *in vivo* environment. The two-dimensional (2D) culture is simple and differs fundamentally from that of *in vivo* and 3D configurations, in terms of mechanical signals, access of nutrients, cell-cell and cell-matrix interaction, and cellular distribution (Cullen et al., 2007a). For
example, embryonic sensory neurons, *in vivo* undergo morphological transformations; at the early stages these cells express a bipolar morphology with two-opposed neurites, and then transform to pseudo-unipolar axonal arbour at the late developmental stages. The transformation process of these cells is delayed or disappears in cells cultured on flat substrates (Langer and Peppas, 2003). Embryonic DA neurons displayed more viability in 3D environments than the 2D ones (Lee and Mooney, 2001), and variability in differentiation patterns as they possessed longer neurites (Cullen et al., 2007a).

**1.14. Cells interact with the extracellular environment**

Cells in living tissues are embedded in the extracellular matrix (ECM) that provides spatial and mechanical signals. The interaction of cells with the surrounding domains has attracted wide interest. Cells *in vivo* interact with the environment by the ECM proteins. Additionally, adjacent cells interact with each other by the basement membrane, which is rich in nano-topography. Nanoscale features such as pores and fibres sized 30-400 nm, are fundamental for cell function such as adhesion, migration, proliferation and differentiation (Bettinger et al., 2009). The mechanism of cell-*in situ* interaction has been attributed to the presence of integrin receptors, which act as a bridge between the extracellular environment and intracellular cytoskeleton, that underpin its substantial role in signal transduction mechanism between inside and outside the cell (Bettinger et al., 2009, Fisher et al., 2014).

**1.15. Three–dimensional (3D) biological models**

A variety of biomaterial matrices have been developed for 3D neuronal cell studies. For instance, self-assembling peptide scaffolds have demonstrated the
capacity for neuron (PC12) attachment and differentiation as well as extensive neurite outgrowth. In addition there is evidence of functional synapse formation between the attached neurons (Holmes and de Lacalle, 2000). Another study reported the feasibility of growing cerebral cortex neurons with astrocytes in Matrigel™ and conducting electrophysiological studies (Irons et al., 2008). The approach has been used for studying the development of neurons derived from the rat superior cervical ganglion generated in methacrylamide chitosan with a gradient of a neurotrophic factor (Yu et al., 2007). These efforts are examples of the utility of hydrogel polymers as biomaterials for biological and medical applications.

Hydrogels can be classified into synthetic (chemically synthesized) such as poly glycolic acid) (PGA), poly (lactic acid) (PLA), and copolymers (PLGA), and natural (biologically-derived) and such as collagen, fibrin and hyaluronic acid (HA), alginate, agarose and chitosan (Lee and Mooney, 2001). Each has advantages and weakness. To select the appropriate type, the biomaterials should meet certain criteria to function appropriately. These criteria include biocompatibility with the host tissue, relevant mechanical properties that relate to the cell adhesion and gene expression, and degradation which can be due to hydrolysis (Lee et al., 2000, Lee and Mooney, 2001, Pathak et al., 2003).

Efforts have been made to develop hydrogels to grow neurons in 3D constructs such as the Puramatrix peptide hydrogel which was used for growing neonatal or prenatal rat brain cortical slices, or cultured primary neuronal-glial cells (Shivachar, 2008). Interestingly, this study reported that cellular viability was around 60%. Mixed cortical cells displayed neuronal aggregation associated with
cell promotion of neuronal outgrowth and extension of dendrite processes, and synapse formation with the neighbouring cells (Shivachar, 2008).

Neuronal arrangement in the hydrogel is also a concern where it is important to simulate neuronal arrangement in the brain. Accordingly, Ning Zhang and his group demonstrated the possibility of forming a tissue like neuronal construct by growing neurons derived from iPSC in the layers of hydrogel, with the presence of electrophysiological activity of these neurons post 3 weeks of construct formation (Zhang et al., 2016).

**1.16. Aims**

From the previously described information, the benefit of the 3D hydrogels is in bridging the findings between lab and clinical applications. The use of 3D substrates offers greater knowledge relevant to an *in vivo* environment than monolayer 2D cultures. Moreover, the combination of neurons and hydrogels as implants need further development by promoting multiple subtype of neurons to grow, survive, fully integrate and form intact and functional networks. As this is an emerging field, until recently no ideal protocol has been developed for growing neurons in an *in vivo* like environment. This raises a number of questions which my thesis will attempt to address.

- How do primary cortical cells grow and distribute in a 3D environment compared to 2D flat substrates?
- Is there any influence of the 3D environment on the neuronal electrophysiological characteristics compared to neurons grown on 2D flat substrate?
Is it possible to genetically engineer cortical primary neurons on 2D and 3D substrates using the magnetofection technique? Can oscillating magnetic field devices assist in the process?

Chapter 2

Materials and Methods
Figure 2.1: A flow chart summarizing experimental sequences of thesis chapters, experiments conducted and parameters examined.
2.1. Reagents and Equipment

**Cell culture:** Culture grade plastics (24 well plates), 13 mm glass coverslips (round), Hank’s balanced salt solution (HBSS, calcium and magnesium free), B-27 serum-free supplement, GlutaMAX, L-glutamine, Earle’s balanced salt solution (EBSS) with MgCl₂ & CaCl₂, penicillin and streptomycin (P/S), penicillin with streptomycin and amphotericin B (PS/F), and TrypLE synthetic trypsin were obtained from Fisher (Loughborough, UK). Dulbecco’s Modified Eagle Medium (DMEM), Nutrient Mixture-F12, Neurobasal, insulin- and transferrin were purchased from Life Technologies (Paisley, Scotland, UK). N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) and poly-D-lysine (PDL) were from Sigma-Aldrich (Poole, Dorset, UK). Deoxyribonuclease I (DNase I) was from Roche (Welwyn, UK), normal horse serum was from A&B scientific (PAA), and foetal bovine serum (FBS) was from Dutscher Scientific, UK.

**Immunocytochemistry:** Rabbit anti-glial fibrillary acidic protein (GFAP) antibody was obtained from Dako Cytomation (Ely, UK), rabbit anti- β III tubulin (Tuj-1) antibody was from Covance (Princeton, NJ). Fluorophore-conjugated secondary antibodies (Cy3 and fluorescein isothiocyanate, FITC) were from Jackson Immunoresearch Laboratories Ltd (West grove, PA, USA). Phosphate buffered saline (PBS) was from Sigma-Aldrich (Poole, Dorset, UK). Normal donkey serum was from Stratech Scientific. Vectashield mounting medium with 4, 6-diamidino-2-phenylindole (DAPI, nuclear marker) was from Vector Laboratories (Peterborough, UK). All the experiments conducted have been summarized in (Figure 2.1).
Transfection experiments: pmax:GFP plasmid (3.5 kb in size; encodes GFP; (map shown in plasmid preparation section) was obtained from Amaza Biosciences (Cologne, Germany) and prepared using Endofree® Plasmid Maxiprep Kit which was from Qiagen (UK). Neuromag and FluoMag transfection-grade magnetic nanoparticles (IONPs) were from Oz Biosciences (Marseilles, France).

Hydrogel experiments: type I collagen, rat-tail, low concentrate (Corning–No.354236; 100mg) was obtained from (Tewkesbury, MA, USA). MEM (10x) solution was made by combining 10.17 g MEM alpha powder with 2.2 g NaHCO₃ sourced from Life Technologies, and dissolving them in 100 mL distilled water.

Magnetic arrays: Two oscillating magnetic array devices with a 24-magnet array (NdFeB, grade N42; field strength of 421 ± 20 mT) were supplied by Nano-Therics Ltd (Stoke-on-Trent, UK).

Safety experiments (live-dead) assay: Cell viability indicators were ethidium homodimer (high-affinity nucleic acid stain for dead cells) and calcein AM (fluoresces when metabolised in live cells), obtained from Invitrogen.

Whole-cell recording experiments: Borosilicate glass for patch electrodes was from Harvard Instruments. An Olympus BX51WI microscope fitted with an x40 objective (Olympus, NA = 0.8) was used, with images taken with a Watec 902B camera or Optimos camera. Signal software with a Power 1401 interface was from CED. Patch clamp amplifier (EPC7) was from HEKA. TTX was from Tocris (UK). The recording microscope was fitted with a filter block containing a dichroic
mirror and excitation filter suitable for fluorescein and GFP. The filter block and blue LED were purchased from Cairn Research (Faversham, UK).

2.2. Ethical Approval

The care and use of animals was in accordance with the Animals (Scientific Procedures) Act of 1986 (United Kingdom) with approval by the local ethics committee.

2.3. Derivation of Primary Cortical cells

For optimizing primary neuronal culture, three protocols were tested. In all protocols, cells were derived from CD1 mouse cortices at embryonic day 18-18.5 (E18-18.5; Figure 2.2 A). Embryonic day was set from the first day of positive vaginal plug seen (E0).

The dissection procedure was similar in all culture systems. Some adjustments have been made for each of: dissection medium, growth medium and cellular digestion process. The first protocol (Neurobasal-1) was tested alone. Thereafter, the second protocol (DMEM) and the modulated protocol (Neurobasal-2) were examined in parallel.

Cervical dislocation was used for sacrificing all pregnant female mice in this study. The surgical scissors were sterilized with 70% ethanol and an abdominal incision was made; the uterus was exposed and transferred into a 60 mm petri dish filled with ice. The embryos were rapidly decapitated into a 50 mL tube containing ice-cold dissection medium. Briefly, dissection medium used in the
Neurobasal-1 protocol comprised of EBSS, HEPES and PS/F, growth medium (Neurobasal, horse serum, B-27 supplement, L-Glutamine and P/S). For cellular digestion, the cells were incubated with trypsin (2.5%) in a 37°C water bath for 20 min.

For the DMEM protocol, dissection medium consisted of HBSS, HEPES and P/S, while growth medium was composed of basic medium (DMEM/F12, L-Glutamine and HEPES), insulin, transferrin and PS/F). Cells were digested using Tryple-DNase-I and incubated on a rotary shaker at 37°C for 20 min. Compositions of all media are detailed in Table 2.1.

The subsequent process is for the optimal protocol (Neurobasal-2). Dissection medium was comprised of 0.025 M HEPES, 97.5% HBSS (with MgCl₂ & CaCl₂) and 1% of Penicillin (50U/mL) streptomycin (50µg/mL).

For all three protocols, brain removal and cortices isolation was conducted aseptically in a laminar flow hood. One head at a time was transferred into a 35 mm Petri dish, which was placed on a stereomicroscope stage.

Each head was covered with ice-cold dissection medium, and then straight forceps were inserted into the eye sockets to hold the skull steady. A cross-shaped incision was made in the skull using fine surgical scissors (Figure 2.2 B & C), and the whole brain was removed by Chattaway’s spatula (Figure 2.2 D). The olfactory bulbs, brain stem and the hippocampus were removed (Figure 2.2 E) in order to obtain the cortex. Then cortical tissues were minced using a sterile scalpel (Figure 2.2 F); subsequently the tissue was incubated in digestion solution (5 ml working solution: 0.5 ml Tryple, 4.345ml HBSS, 125 µL of 0.2 M MgCl₂ and
40 µL DNase-I; 0.25 ml/brain). Tissue was incubated for 20 min at 200 rpm on an orbital shaker incubated at 37°C. 1 ml of FBS was added to stop enzymatic activity. Residual serum was eliminated by washing the cells three times with serum-free growth medium, followed by centrifugation for 3 min at 2000 rpm. The cells were then dissociated mechanically with a 10 ml pipette followed by 1 mL pipette for 5 mins. Dissociated cells were strained with 70-µm then 40-µm strainer. 0.5-1 ml of the medium was placed on the strainer in order to pre-wet the filter, then made up to the final volume of 3 ml.

Cell viability counts were performed prior to seeding by mixing 10 µl of cell suspension with 10 µl of 0.4% trypan blue, which stains dead cells blue, then transferring 10 µl of the cell mixture into a haemocytometer, which was examined microscopically. Cells were seeded in 24 well plates with 400 µL growth medium per well, and maintained in a standard humidified incubator (37°C, 95% air/5% CO2), with the cells allowed to adhere to the coverslip for less than 1 h. Subsequently, the medium was replaced with 600 µL fresh growth medium, with 50% medium changes every 2-3 days.
Figure 2.2: Diagram demonstrating the main dissection steps for derivation of cortical neuronal cultures. (A) 18-day embryo under the stereomicroscope. (B) Decapitated head and the cross-shaped incision in the skull for brain exposure. (C) Skull cut and exposed brain. (D) The brain released from the skull. (E) The cortex separated from the olfactory bulbs and brainstem. (F) The cortices collected in a 35 mm petri dish and minced into small pieces by a scalpel. (G) The tissue processed into a cell suspension in 50 mL tube. (H) Cells seeded in the plate and incubated for the required time. (I) Neurons immunostained with Tuj-1 antibody and astrocytes with GFAP antibody which will be detailed in immunostaining section.
Table 2.1: Comparison of primary cortical neuronal culture protocols: Neurobasal-1 (NBM-1), DMEM:F12 (DMEM), and Neurobasal-2 (NBM-2). The table includes dissection and growth media in addition to some technical steps.

<table>
<thead>
<tr>
<th>Dissection</th>
<th>NBM-1</th>
<th>NBM-2</th>
<th>DMEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals</td>
<td>E18-18.5/Rat</td>
<td>E18-18.5/Mouse</td>
<td></td>
</tr>
<tr>
<td>Enzymatic digestion</td>
<td>2.5% Trypsin (5ml) for 20 min in water bath at 37°C</td>
<td>Tryple-DNase-I (0.25 ml / brain) for 20 min / orbital shaker at 37 °C</td>
<td></td>
</tr>
<tr>
<td>Salt solution</td>
<td>Earle’s balanced salt solution (EBSS) 96.5%</td>
<td>Hank’s balanced salt solution (HBSS) 97.5%</td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td>0.021M</td>
<td>0.025M</td>
<td></td>
</tr>
<tr>
<td>Base media</td>
<td>Neurobasal 86%</td>
<td>DMEM-F12 (1:1) 96.24%</td>
<td></td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Penicillin-streptomycin amphotericin B</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50U/ml-50µg/ml</td>
<td>100U/mL-100 µg/mL 25 µg/mL</td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>Horse serum 10%</td>
<td>Foetal bovine serum 10%</td>
<td>None</td>
</tr>
<tr>
<td>Supplements</td>
<td>B-27 Supplement 2%</td>
<td></td>
<td>Insulin 100µg/ml</td>
</tr>
<tr>
<td></td>
<td>L-Glutamine 2mM</td>
<td>Glutamax 2mM</td>
<td>Transferrin 5 µg/ml</td>
</tr>
<tr>
<td>Techniques</td>
<td>NBM-1</td>
<td>DMEM</td>
<td>NBM-2</td>
</tr>
<tr>
<td></td>
<td>• Tryple-DNase-I</td>
<td></td>
<td>2.5% Trypsin (5ml)</td>
</tr>
<tr>
<td></td>
<td>• 0.25 ml / brain for 20 min / orbital shaker at 37 °C</td>
<td></td>
<td>for 20 min in water bath at 37°C</td>
</tr>
<tr>
<td></td>
<td>• 70 &amp; 40 µm Strainer used</td>
<td></td>
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</tbody>
</table>
2.4. Primary cortical cell culture conditions

Several factors govern the distribution of dissociated cells seeded onto coverslips and the purity of the culture (neurons /astrocyte proportions), which affect the reproducibility of culture conditions. An evenly dispersed culture is important in order to facilitate cellular identification and quantification. Therefore, cell density and coverslip coating technique were adjusted in these protocols. Several cell densities were examined (330, 250, 160, 80, 60 and 30 \( \times 10^3 \) cell/cm\(^2\)), and compared with different reported neuronal protocols as density references (Lesuisse and Martin, 2002, Xu et al., 2012)

In order to promote cellular adhesion on coverslips, as well as reduce cellular migration and neuronal cluster formation, two coating protocols were examined. The first protocol was as follows: the coverslips were washed with nitric acid then coated with PDL. The initial NBM1 protocol involved washing with 1% nitric acid (3 h on a shaker). The final protocol was changed to 65% nitric acid overnight was applied, then washed with distilled water (3 h; water was changed every 20–30 min), and sonicated (each time for 30 min: three times in distilled water, three times in 70% ethanol, 3 times in 95% ethanol). A PDL coating was applied (10 µg/ml working solution, 250 µL/well), incubated overnight at room temperature (RT), washed 3 times in deionised (dH\(_2\)O) and allowed to dry 1-2 min prior to cell plating. In the optimized protocol, PDL and Laminin were utilized. 10 µg/ml of PDL incubated on the coverslips at 37°C overnight as first coating layer, then 5µg/mL laminin was added as a second layer for 2 hr at 37°C, prior to adding the cells.

Another important factor in cell dispersion is the seeding technique. Neuronal cortical cells were seeded using a specific technique, namely dropwise in a spiral
pattern, starting from the middle of the slip ending at the edges (Figure 2.3.), in order to ensure even coverage of the entire coverslip.

Figure 2.3: Schematic showing cortical neuronal culture seeding technique.

Serum concentration in culture is a critical factor in determining the proportion of astrocytes that survive and proliferate (Price and Brewer, 2001). Accordingly, several serum levels were tested: 10%, 1%, 0.25% and 0% (serum-free medium). Cultures were examined at three time point [3, 5 and 7 days in vitro (DIV)]. Table 2.2 summarises the parameters (cell density, serum level and time points) examined, which are also stated in the experimental design (Figure 2.4).
Table 2.2: The examined parameters for optimizing primary cortical culture.

<table>
<thead>
<tr>
<th>Reproducibly derive primary cortical culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Neurobasal -1 (NBM-1)</td>
</tr>
<tr>
<td>• DMEM:F12 (DMEM)</td>
</tr>
<tr>
<td>• Neurobasal-2 (NBM-2)</td>
</tr>
</tbody>
</table>

<p>| Produce cultures amenable to analysis        |</p>
<table>
<thead>
<tr>
<th>(evenly distributed cells facilitating the quantification process)</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Seeding density of the cells (30x10³ &amp; 60X10³) cell/cm²</td>
</tr>
<tr>
<td>• Determine the appropriate substrate for coating coverslip</td>
</tr>
<tr>
<td>• (PDL &amp; PDL+ Laminin)</td>
</tr>
<tr>
<td>• Time in culture post-plating; 3, and 7 days in vitro (DIV) to evaluate neuronal dispersal and maturation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Obtaining a biologically balanced co-culture of neurons and astrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>• lowering the contamination of astrocytes by reducing the serum level (1 &amp; 0%)</td>
</tr>
<tr>
<td>• Evaluate cellular proportions over time (3, 5 and 7 DIV)</td>
</tr>
</tbody>
</table>
Figure 2.4: Schematic elucidating the experimental design for examining the effect of serum level, cell density and observation time point on generating reproducible primary cortical neuronal cultures. ‘Low’ refers to a cell density of 30x10^3 cells/cm^2 and ‘high’ refers to 60x10^3 cell/cm^2.

2.5. Magnetic nanoparticle mediated gene transfer to primary cortical cells

2.5.1 Magnetic arrays

Two oscillating magnetic array devices were tested, each with a 24-magnet array (NdFeB, grade N42; field strength of 421 ± 20 mT). The first device magnefect-nano oscillating magnetic array system (herein referred to as ‘uniaxial’) oscillates in one horizontal axis (X plane). The second version, which has an additional horizontal axis perpendicular to X, is referred to as ‘biaxial’ and oscillates
alternately in the X and Y planes (switching after each single oscillation). Both versions are adapted to fit 24-well culture plates. The oscillation frequency (F) and amplitude can be adjusted via a computerised program. Frequency in all transfection experiments was set to 4 Hz, as this has been reported to be the optimal frequency for neural cells including oligodendrocyte precursor cells, OPCs (Jenkins et al., 2011). The amplitude was set to 0.2 mm as this has been previously reported as effective for various neural cell types ((Tickle et al., 2015) (Figure 2.5).

Figure 2.5: Photographs and corresponding diagrams illustrating the two types of magnetic arrays. (A & C) photograph and corresponding diagram for uniaxial magnetic array, (B & D) photograph and corresponding diagram for biaxial magnetic array.
2.5.2. Magnetic nanoparticle characterization

NeuroMag and Fluo Mag particles are proprietary and patented by the company Oz Biosciences. Both particles are characterized positively charged, and relatively homogenous and round in shape. Neuromag particle size range reported by the company is between 140-200 nm, with average size 160 nm, associated with homogeneity in shape and size, ζ-potential +48.16 (Pickard and Chari, 2010a, Vernon et al., 2015). FluoMag size is 200 nm, polydispersity index is about 0.027, dynamic light scattering DLS is often expressed in terms of the Z-average. Which in turn is expressed as the intensity based harmonic mean, Z-average mean is 203 nm, and Z-average range 199-205 nm. Hydrodynamic diameter and zeta potential of Fluo Mag ζ-potential measured in our laboratory was ( +40.3 mV) (Pickard and Chari, 2010a, Fernandes and Chari, 2014).

2.5.3. Pmax plasmid preparation

Plasmid (pmax; 3486bp; Figure 2.6) encoding a reporter transgene (green fluorescent protein, GFP) was used for monitoring transfection efficiency. A bacterial colony was prepared by bacterial streaking technique using kanamycin-treated agar (50 mg/ml), incubated overnight at 37°C, then one colony per 15 ml tube containing 5 ml Luria-Brentani broth (LB broth) was transferred and incubated overnight (37° C, 180-200 rpm on an orbital shaker). Broth (1 ml) was transferred into an Eppendorf tube then centrifuged for 5 min /1000 rpm. 250 µl of P1 (reagent in Qiagen kit) was added to the pellet followed by 250 µl P2, with gentle mixing of the solution. Buffer N3 was added (350µl) and centrifuged for 10 min /13000 rpm. The resulting supernatant was poured into a filter column tube and centrifuged for 30-60 s. The supernatant was discarded and 0.5 ml of PB
buffer was added on top of the filter and centrifuged for 1 min/13000 rpm. The supernatant was removed again and 750 ml PE buffer solution was added. After 1 min the filter column was emptied and re-centrifuged for 1 min to evaporate the ethanol. The top part of the column was transferred to a new Eppendorf and 50 µl of EB buffer to the centre of filter and incubated at RT 1-10 min, followed by another 1 min centrifugation at 12-13000 rpm. The pellets in Eppendorfs were the DNA, which were stored for 2-3 months at -20°C.

The manufacturer’s instructions of Endofree® Plasmid Maxiprep Kit were followed for generating abundant amounts of plasmid DNA, similar to the process described above. Bacterial pellets were re-suspended and lysed by adding buffers P1 and P2 respectively. Buffer P3 was added in order to neutralize the lysis reaction. Clear lysate was obtained by filtering the previous mixture, then ER (endotoxin removal) buffer was added and incubated for 30 min. After that, the lysate was added to a DNA purification column, washed with buffer Q3 and eluted by buffer QN. DNA precipitation was carried out by adding isopropanol, then the DNA pellet was washed with 70% ethanol, and dissolved in buffer TE. This yielded highly purified plasmid DNA free of contaminating endotoxins from the Escherichia coli host.
2.5.4. Magnetofection procedures

The magnetofection protocol was performed using cells derived via the NBM-2 protocol. Cells were seeded at 60x10^3 cell/cm^2 in serum-free media, with particle-plasmid complexes (described below) applied at 3 and 7 DIV. All the experiments were conducted in 24 well plates. On the day of transfection, 1 h prior to transfection, the full volume of medium was replaced with 225 µL of Neurobasal-2 medium (free of P/S and serum, in order to reduce the adverse effect of antibiotic and serum on transfection efficiency) (Asgharian et al., 2014).

MNP-plasmid complexes were formed by diluting 178 ng pmax GFP plasmid in 75 µL base medium (DMEM: F12), adding 0.63 µL Neuromag or FluoMag, then incubating for 20 min (RT; volumes are for 1 well). These complexes were added drop-wise to the well, with gentle swirling of the plate, 75 µL (DMEM: F12)
medium free of complex were added to the controls. Plates were incubated using the standard incubation environment as mentioned earlier for 30 min on magnefect-nano oscillating magnetic array system (uniaxial and biaxial) with an oscillating frequency at 4 Hz (Figure 2.7). Cells were further incubated (without magnetic array) for 48 hr post-magnetofection, and then fixed, and assessed for transfection efficiency. Toxicity assessment (viability quantification) was also conducted 48 hr post-transfection. Electrophysiological studies were conducted at 24 hr post-magnetofection.

To evaluate culture purity, each DAPI-positive nucleus was identified as having a neuronal (Tuj-1⁺), astrocytic (GFAP⁺), or undetermined (Tuj1⁺/GFAP⁻) phenotype, and to determine transfection levels, each cell was also assessed for GFP expression. The immunostaining procedure was performed as detailed in section 2.7. Magnetofection toxicity was assessed by determining the percentage of viable, calcein-positive (live) cells, and by counting pyknotic nuclei (chromosomal condensation of necrotic or apoptotic cells) (Jenkins et al., 2016).
2.5.5. Cellular Uptake of IONPs

The capacity of primary cortical neurons to take up IONPs was determined by labelling cortical cells at 7 DIV with FluoMag in the presence of a uniaxial magnetic field. Assessment of uptake was conducted by visualizing FluoMag under a fluorescent microscope. The red channel was used for particle visualization, while GFP expression was visualized in the green channel.
Assessment was conducted by identifying fluorescent IONPs close to the cell body of either neurons or astrocytes, which were identified morphologically.

2.6. Preparation of 3-dimensional (3D) hydrogel constructs containing neurons

The material used for preparation of 3D neuronal hydrogel constructs was collagen I. It is a triple-helical protein formed of 67-nm periodic polypeptide chains with a total molecular weight near 300 kDa. Collagen bundled fibre diameter typically ranges between 12–120-nm, self-assemble at neutral pH, and can form crosslinks to produce a hydrogel in the presence of a water (Antoine et al., 2014). Collagen molecules are comprised of three α chains that assemble together, Collagen I molecular formula is α 1 (I) and α 2 (I) (Antoine et al., 2014).

The feasibility of applying a collagen hydrogel as a substrate for neuronal cultures was initially tested using a 2-dimensional (2D) model (i.e. on a coverslip coated with thin layer of hydrogel). The second model was 3D construct classified as (i) surface seeded model where cells were seeded on top of the gel construct and (ii) entrapped model where cells were mixed with the gel and forming internal multilayer seeded construct as illustrated in (Figure 2.8). The hydrogel synthesis procedure was as described in (Phillips and Brown, 2011).

On the day of conducting a gel experiment, coverslips were sterilized with 70% ethanol, then kept hydrated with dH₂O at RT and the ethanol was aspirated just prior to depositing the gel solution. Cells were derived as described in Section 2.3. All the hydrogel experiments were conducted in 24-well plates in sterile conditions. Coverslips were used for 2D and 3D cultures, as for 3D gels this facilitated lifting gels from the well for the purpose of imaging. In 2D-monolayer
experiments, coverslips were coated with 50 µL gel at concentration of 1 mg/mL, forming a thin layer. Regarding 3D constructs both surface seeded and entrapped hydrogel models were made up to the final volume of 400 µL/well. Collagen Hydrogel I post polymerization can be characterized as substrate with a multi-layered and porous fibrillary collagen network (Tickle, 2017)

For 3D cultures, several variations in gel stiffness were tested in order to determine the optimal construct capable of supporting even and homogeneous distribution of the cells. The varying stiffnesses were achieved by tuning the gel concentration (0.4, 1 and 2 mg/ml). The assembly of the gel solution was based on varying proportions of the following components. In 1 ml final volume (FV) 80% is collagen in acetic acid, 10% modified Eagle’s medium (MEM) alpha (10x) for biocompatibility and 10% cell suspension (cells in standard culture medium (NBM-2) for the entrapped model or medium free of cells for the gel construct used for the surface seeding model as the cells seeded post gel setting at the density 4x10^5 cell/cm^2. Finally, NaOH was added to neutralise the acetic acid. The formulas for calculating required volumes are listed in Table 2.3.

The collagen hydrogel components were kept at 4°C until use. The gel solution was prepared in a 25 ml tube, and swirled gently after each addition in order to ensure thorough mixing. The following sequence in adding the components is critical for gel formation: acetic acid for diluting the collagen, then MEM alpha was added, followed by NaOH dropwise with gentle swirling, to bring the solution to neutral pH. This is indicated via a colour change to red, due to phenol red, which is one of the MEM alpha components. Neutralized gel solution was applied to 24-
well plates immediately, and incubated at 37°C (5% CO₂ / 95% humidified air).

After 30-60 min, 600 µL serum-free medium was added on the top of the gel.
Figure 2.8: Schematic illustrate cell seeding models of hydrogel construct. (A) Cells plated on top of the gel, (B) cells mixed within the gel solution, dispersed throughout the construct.

Table 2.3: Formulae for calculating collagen solution formation for 2D and 3D.

<table>
<thead>
<tr>
<th>Collagen formation formulas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final volume (FV)</td>
</tr>
<tr>
<td>10xMEMα</td>
</tr>
<tr>
<td>Cell suspension</td>
</tr>
<tr>
<td>Final collagen volume (FCOV)</td>
</tr>
<tr>
<td>Final collagen concentration (FCOC)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Acetic acid (0.02M)</td>
</tr>
<tr>
<td>NAOH (1M)</td>
</tr>
</tbody>
</table>
2.7. Immunocytochemical procedures

For all experiments, the primary antibody used for detection of neurons was monoclonal mouse anti-β III tubulin (Tuj-1), while astrocytes were detected via polyclonal rabbit anti-glial fibrillary acidic protein (GFAP). Secondary antibodies were FITC (green)-labelled donkey anti-mouse or anti-rabbit IgG and Cy3 (red)-labelled donkey anti-rabbit or anti-mouse IgG. For culture characterization experiments, either in 2D-monolayer or 3D construct experiments, double staining was required. For transfection experiments, single staining with Tuj-1, for identifying neurons, was sufficient.

For immunostaining procedures in 2D-monolayer cultures (PDL-Laminin coated coverslips), cells were washed with PBS three times, fixed with 4% paraformaldehyde (PFA) at RT for 20 min, then washed again three times with PBS. The staining procedure started with incubating the cells with blocking solution (5% normal donkey serum (NDS) with 0.3% Triton-x100) for 30 min at RT.

Primary antibodies were diluted in blocking solution at the concentrations 1:1000 for Tuj1 and 1:500 for GFAP, both antibodies mixed in one tube with blocker, with 150 μl added per well, then incubated at 4°C overnight.

The following day, cells were washed three times with PBS. Cells were incubated with blocker solution (200 μL/well) at RT for 30 min, then secondary antibodies diluted in blocking solution to 1:200 for Cy3/FITC for 2 hr at RT and protected from light. Samples then washed three times in PBS for 5 min /wash. Nuclei were stained by adding vectashield mounting medium with 4',6-diamidino-2-
phenylindole (DAPI), then the coverslips were mounted on glass slides and sealed with nail varnish for imaging by fluorescence microscopy.

2.8. 3D hydrogel construct immunostaining
Hydrogel samples (2D collagen coated coverslip and 3D constructs) underwent the same stages of immunostaining for 2D culture as detailed in Section 2.7. However, there is a problem associated with imaging process post staining. Where, the stain diffuses throughout the gel which prevents obtaining clear images. Therefore, there were some modifications in the protocol were needed in order to facilitate imaging process. These modifications included; (i) the blocking solution was 5% NDS with 0.5% Triton-X100 (ii) the blocking solution volume was doubled at all immunostaining staining stages; (iii) all incubation times were doubled whether for fixation or with antibodies; (iv) hydrogels were transferred into 6 well plates during the immunostaining process, which allows for better removal of the residual primary and secondary antibodies from the hydrogel constructs; (v) hydrogels were washed with 2 ml of PBS on a shaker for 15 min.

2.9. Viability assessment
For assessing the proportion of viable cells, cultures were exposed to live/dead stains. At 48 hours post-magnetofection, samples of control and transfected 2D cultures (PDL-Laminin) were gently washed with PBS three times. To each well, 4 mM Calcein-AM (stains live cells green) and 2 mM ethidium homodimer-1 (stains dead cells red) were added, incubated for 5 min at 37°C, and then washed twice with PBS. Live imaging was conducted using fluorescence microscopy; the samples were kept in the 24 well plate during imaging.
For hydrogel samples (2D and 3D surface seeded and entrapped model), viability assays were performed at defined time points (3 and 7 DIV). The protocol was similar for surface seeded and entrapped models, although the final volume added to each well of 3D samples was doubled, as was the incubation time.

**2.10. Microscopy and image analysis**

Three different microscopes were used for 2D- (PDL-Laminin) and hydrogel (2D gel-monolayer and 3D gel constructs) samples imaging. A Leica DM IL LED inverted microscope equipped with a DFC 420 C digital camera and a pE-300 W Cool LED fluorescence unit, was used for cell viability counting prior to cell seeding. For images capturing from well plates for 2D-monolayer, the Leica Application Suite imaging software, version 3.3.1 was used. Fluorescence images from cell monolayers on coverslips were captured using an AxioScope A1 microscope equipped with an AxioCam ICc1 digital camera, and utilising Axiosvision imaging software by Carl Zeiss MicroImaging, GmbH (Germany). An Axio Observer.Z1 microscope equipped with an AxioCam MRm powered by Zen 2 was used for imaging of 2D gel-monolayer, 3D-surface seeded model and 3D-entrapped model.

For the purpose of analysing the results of 2D-monolayer samples, coverslip/hydrogel fluorescence images were captured at 20 x magnification, from three fields (locations): two at the edges and one at the centre of the sample. Three individual fluorescent images were merged for each field, using Adobe Photoshop CS2 (Version 9.0) for all 2D-monolayer samples, while 2D gel-monolayer and 3D constructs models were merged by Zen 2 (blue edition) software (Carl Zeiss MicroImaging GmbH, Goettingen, Germany).
Imaging of the 3D-Surface seeded model and entrapped model was as follows;

Each sample was transferred onto a rectangular glass coverslip (thinner than a standard microscope slide, to improve image quality), the sample was flipped upside down, and three imaging locations were captured for 3D-surface seeded model as mentioned previously.

The same process was used for 3D entrapped model. However, the images were captured as a series through the depth of the gel. This technique, called z-stack imaging, facilitates composition of a series of images within specified fixed distance. The beginning and ending points for imaging through the depth of the gel was determined manually, and then the microscope focus was shifted upward until the first cell nuclei became clear and in focus, and then continued to rise until the last nucleus went out of focus. Based on this, the vertical depth of gel was determined.

In 2D-monolayer and 2D gel-monolayer experiments, individual fluorescence images were captured and merged for quantification using ImageJ (v1.48). The cells in the 3D surface seeded and entrapped models were quantified manually.

To provide data relating to cellular distribution throughout the depth of the gel, three 'layers' were identified according to the equation:

\[ Gel\ layer = Gel\ final\ volume \div 3. \]

This result in measurements at an upper, middle and lower layer, and at either the edge or the centre of the gel, as shown in (Figure 2.9).
2.11. Whole-cell electrophysiological recording

Electrophysiological recordings were conducted by the assistant with Dr Michael Evans, Keele University using the single cell patch clamp technique (single-cell recording).

Whole-cell recordings were made at RT; the samples (grown either on coverslips or on hydrogels) were placed in the centre of lid of a 35mm cell culture dish that acted as a chamber. Patch pipette electrodes were pulled (using Narishige Vertical Puller) from borosilicate glass and the shanks coated with wax to reduce their capacitance. The back end of the pipette was gently fire-polished to preserve
the silver chloride coating of the silver wire used to establish electrical contact between the amplifier and the pipette filling solution. Pipettes had a resistance of 4 MΩ when filled. Voltage-clamp protocols were run using Signal software with a Power 1401 interface, a patch clamp amplifier (EPC7) and a standard laboratory computer. The pipette, filled with the K⁺-based intracellular solution contained (mM): KCl 140, Na₂ATP 2.5, MgCl₂ 3.5, EGTA 1, and HEPES 10, buffered to pH 7.4 with KOH., was advanced towards the cell body until touching, at which point a small dimple was observed in the cell membrane. Positive pressure was then released and negative pressure applied by mouth until a seal was obtained. The patch was then clamped at the holding potential (usually -70 mV) and the pipette capacitive transient was cancelled using the amplifier controls. A whole cell recording was obtained by additional pulsatile suction, and once established the cell capacitance and the series resistance were measured and noted. A series of voltage-clamp or current clamp protocols were then run in order to record voltage-dependent Na⁺ and K⁺ currents or spiking behaviour respectively. Occasionally the cell capacitance and series resistance were re-measured, as there was a tendency for the latter to increase over time. If necessary additional suction could be applied to reduce series resistance, but often at the expense of an increase in holding current.

Switching from voltage-clamp to current clamp was done by altering the amplifier controls. Hyperpolarizing current injections were applied from the resting potential. Spikes could then be observed at the end of the step, when the cell "jumped" back to its resting potential.
2.11.1. 2D monolayer (PDL-Laminin) whole-cell recording
Genetically engineered (GFP\(^+\)) and control non-engineered neurons (GFP\(^-\)) (e.g. unmagnetofected) were cultured on circular coverslips that were secured, using a small drop of Sylgard, in the centre of the lid. The lid was filled with Neurobasal basic medium and placed on the stage of the microscope. Neurons were identified by the presence of neurites extending from the cell body. The presence of fluorescence under blue excitation light identified individual neurons as GFP\(^+\) (transfected) or GFP\(^-\) (untransfected). The Na\(^+\) channel blocker, TTX was prepared as a 1mM stock solution in citrate buffer, frozen in aliquots, and dissolved in Neurobasal basic medium on the day of use. It was applied to the neuron from a second pipette positioned about 10 µm from the cell body as illustrated in (Figure 2.10), and ejected using a pico pump (PV820, WPI) either manually or via a trigger pulse.

2.11.2. 3D-surface seeded whole-cell recording
Gels were viewed in the chamber (lid of cell culture dish) under a binocular dissecting microscope (Leica). Neurobasal medium was gently added so that the gel was completely immersed. Small metal weights were used to hold down the gel. One U-shaped weight (cut from a paperclip) was positioned at the centre of the gel and four smaller straight weights were positioned roughly equidistant around the gel, pointing towards its centre. The chamber was then transferred to the recording microscope stage and was rotated so that the recording pipette was aligned to within about +/- 20 degrees of the long sides of the U-shaped weight and pointing down the U, although occasionally other angles were used. The weight tended to depress or indent the gel immediately beneath, which in turn
provided a convenient mound within the U along which to search for accessible
cell bodies, with the focus adjusted as required. When recording from un-
transfected cells, the microscope lamp was on continually and its brightness
adjusted to obtain a good image. When recording from transfected cells, the
microscope lamp was turned down (although still on) and the blue LED was
triggered to be on for about 0.5-1.0 s every 3-4 s, to allow identification of GFP
neurones while still observing the gel and its surface seeded cells.

Figure 2.10: Schematic exhibiting recording and blocking (passing TTX blocker
through) electrode positions in relation to primary neurons during the electrical
signal recording by patch-clamp technique.
2.12. Statistical analysis
All experimental data were analysed using Prism statistical analysis software (GraphPad, USA; version 7.0) and all data are presented as mean ± standard error of the mean (S.E.M). Data were analysed by one and two-way ANOVA with post-hoc analysis carried out using Bonferroni’s multiple comparison test (MCT). The statistical analysis used in electrophysiological studies was Student’s t-test.
Chapter 3

Primary cortical cell culture optimization
3.1. Introduction
Over the years, many advances in neuroscience have been based on the evolution of highly sophisticated neural cell culture systems. For example, understanding neural electrical activity (Bardy et al., 2015), expanded the perception regarding cellular maturation and interaction (Biffi et al., 2013), gene identification and modulation (Blömer et al., 1997), understanding the biological mechanisms of neurogenesis, maturation and the opposite mechanisms which related to deterioration and death (Branton and Clarke, 1999, Donega et al., 2013). The capacity to produce in vitro neurons and glia is indispensable for our understanding of the nervous system assembly and function at the molecular level.

Despite the long history of in vitro cell culture, originating with primary nerve cells by Harrison in 1907, primary neuronal culture is still a challenging technique since mature neurons are non-mitotic (do not undergo cell division). The development of neuronal cell lines, from neuron derived tumours, has been the predominant model and a valuable source for deriving ‘neurons' (Gordon et al., 2013a). The popularity of using cell lines in experimental neuroscience research is based on several factors such as the ease of growing these cells, cost efficacy, highly pure cultures, the production of an unlimited cell number that increases the probability of obtaining a continuous culture. These provide a reproducible result avoiding the ethical concerns linked with animal use (Kaur and Dufour, 2012).

Different types of cell lines have been relied on as a model to study several biological concepts related to the nervous system. These have been used in vaccine production, pharmacological research, such as testing drug metabolism
and cytotoxicity, antibody production, synthesis of some therapeutically usual proteins, gene function and neural cells engineering researches (Macdonald, 1990, Schurr et al., 2009, Gomez-Lechon et al., 2003, Pisanic et al., 2007). For example, SH-SY5Y neuroblastoma cells (Påhlman et al., 1984) and PC12 cells derived from pheochromocytoma of the adrenal medulla (Andrews, 1988) have been used. It is important to highlight the fact that some of these cells undergo manipulation by the addition of different agents such as retinoic acid, phorbol esters, or dibutyryl cAMP to display a neuronal phenotype. For instance, the SH-SY5Y line was originally derived from a human metastatic bone tumour biopsy of cell line SK-N-SH by June Biedler in the 1970’s (Biedler et al., 1973, Biedler et al., 1978) and can be used in two forms, (i) undifferentiated cells, which express immature neuronal markers (neuroblast-like morphology) (Björklund et al., 2002), and (ii) differentiated cells as they settle in G0 phase of the cell cycle and express mature neuronal markers (primary neuron morphology) (Pahlman et al., 1984). It is worth mentioning that although the cell lines are a useful tool in research, these are associated with considerable disadvantages and requires caution in relation to their use.

One of the problems of using cell lines is the contamination either with other cell lines or mycoplasma (microorganisms which are characterized by their lack of a rigid cell wall and resistance to antibiotics) such as penicillin and streptomycin which just work to mask but do not remove mycoplasmas) (Drexler and Uphoff, 2002). The identity of the cell line is a critical element for reliable results. Walter Nelson-Rees in the 1970s exposed the unfortunate truth that the majority of the cell lines that being used in research were misidentified due to the cross-
contamination with HeLa cell line which has been propagated by cell banks worldwide (Nelson-Rees et al., 1981). This problem extended over the next 40 years (Chou and Langan, 2003, Gstraunthaler, 2003). The contamination by HeLa cells can be detected after few passages while by mycoplasma can be undetectable for long time and can alter the cell behaviour and gene expression extensively (Nelson-Rees et al., 1981).

A great deal of research work has been conducted in misidentified cell lines (Hatton, 2002, Pisanic et al., 2007). Buehring and his group conducted a 2004 survey of 483 cell lines used by culture workers (Buehring et al., 2004). This survey indicated that 9% were accidentally using cell lines contaminated with HeLa cell line, 33% were not using verified cell lines, and 35% had obtained their samples from another laboratory. It has been reported that the incidence of contamination in primary cell cultures does not exceed 1-5%, while 15-35% cell lines were contaminated with mycoplasma (Peters and Palay, 1991, Drexler and Uphoff, 2002, Zille et al., 2012).

Generally, cell lines should possess structural and functional features as close to the corresponding primary neurons in vivo. This can be difficult to achieve, since there is no full understanding of the primary cells' functional properties. Further, cell lines can be obtained either from immortal cells (e.g. cancer cells) or a cell population that has been induced to become immortal by process called ‘transformation' (Freshney, 2002, Gstraunthaler, 2003, Ruponen et al., 2003). In either situation, cells are genetically altered and this can change their native phenotype and the functional properties. Once they are passaged, cells with the highest proliferative capacity predominate, and produce cultures characterized by
phenotypic and genotypic uniformity. This results in a culture unrepresentative of the biological variations that exist in the \textit{in vivo} environment (Oupicky et al., 2000). The phenotypic and genotypic alteration can be the source of misleading results, which impact the cellular responsiveness to stimuli and increase their resistance to toxins and cell death (Freshney, 2002, Hughes et al., 2007).

Despite all these considerations, cell lines are still a powerful tool in neuroscience research and widely used today. This does not preclude that primary cells, despite their complexity, are still preferable because they are not tumour cells and not genetically altered, therefore more likely to reflect the characteristics of neurons \textit{in vivo}. Their derivation is challenging, particularly for neurons as these are not proliferative hence limited cell numbers will be obtained, adding to that the necessity of obtaining ethical protocol approval for animal and human cells. Moreover, primary cells show difficulty in transfection in contrast to cell lines (Karra and Dahm, 2010a). The original tissue is heterogeneous (i.e. composed of several different types of cells) requiring separation of the cell type of interest to obtain purified cultures.

Many protocols have been described for isolating and cultivating primary neurons to pave the way for developing culture conditions that meet experimental needs (Braun et al., 2006, Xu et al., 2012, Todd et al., 2013, Chen et al., 2013, Hui et al., 2016). The majority of the methods focus on increasing the neuronal culture purity which is advantageous for cell-type specific biological interactions, such as in pharmacological studies. The purity of neuronal cultures in some studies has been reported to be up to 99% neurons and these are described as a neuron-enriched cultures (Ziello et al., 2010, Xu et al., 2012)
The first parameter to be considered in primary neuronal cultures is the choice of tissue age for cell derivation. The cell production schedule in the developing nervous system varies according to the cell type, and at the embryonic stages the production of neurons exceeds the production of glia (Jhon and Andrade, 1973). Glial production primarily occurs postnatally. For instance, mouse cerebral cortex formation begins around embryonic day 12 (E12) and reaches a peak around E15. Astrocyte genesis commences at E16 and oligodendrocyte production around birth (E 20), but the majority are generated during the first postnatal month (Qian et al., 2000, Jacobson, 2013). Furthermore, the age of donor tissue can be a determinant of cell type, for example, postnatal hippocampal tissue is composed of a high percentage of GABAergic neurons (Pathak et al., 2003) while the embryonic age group is composed mainly of pyramidal neurons (Ma et al., 2004).

A second avenue of research has been directed towards reducing the number of astrocytes, despite the neuro-supportive effect of these cells. Astrocytes in culture have a fundamental role in the regulation of extracellular fluid homeostasis via (i) recycling of neurotransmitters (Kimelberg and Nedergaard, 2010) and regulating extracellular potassium (Wong et al., 2006) (iii) secreting amino acids, neuropeptides and neurotrophic factors (Murphy and Messer, 2001). The aim is to generate biologically controlled co-culture. Approaches have been developed for reducing astrocytes and improving the purity of the neuronal culture, for example by adding glial cell inhibitors (i.e. eliminating dividing astrocytes by adding the DNA-synthesis inhibitor) such as cytosine arabinofuranoside (AraC). However, this increases the susceptibility of neurons to glutamate (Ahlemeyer et al., 2003),
and this approach has proved to be cytotoxic to neurons. Another approach considered safer than the approaches that use AraC, is where serum free medium has been used to suppress astrocyte proliferation. Astrocytes are proliferative cells, and serum deprivation can arrest them in the (G0) phase of the cell cycle (Aizenman and de Vellis, 1987).

Therefore, culture conditions have an important impact on neuron viability and behaviour. It should be noted here, that while efforts are focused on purifying neuronal cultures, a major point of view is that astrocyte presence is fundamental for neuronal survival and growth, to create an environment that closely mimics physiological conditions. This is because astrocytes play a critical role in neuronal protection against glutamate toxicity in mixed astrocyte/neuron cultures and astrocytic dysfunction promotes neuronal toxicity (Voloboueva et al., 2007). Stoppelkamp and his group have suggested that the reduction of astrocytes in culture has a negative impact on neuron excitability (Stoppelkamp et al., 2010).

Additionally, growing neurons optimally relies on basic environmental requirements, which include controlled temperature, appropriate growth medium, and the cell attachment substrate (Xu et al., 2012, Todd et al., 2013, Chen et al., 2013). A critical step in neuronal culture is selecting the appropriate growth medium: the source of nutrients and energy, in addition to its effectiveness in maintaining a balanced pH and osmolality. The most widely used media for primary neuronal culture currently are DMEM, DMEM:F12 and Neurobasal basic media, which was developed by optimization of DMEM:F12 (Brewer et al., 1994), and either serum-containing or serum free media (Arora, 2013).
Serum is one of the most important components of growth media, and is composed of albumin, growth factors and growth inhibitors (Lane and Miller, 1976). Using serum in media has advantages of supporting cell growth and function and it is important for cell attachment to the seeding surface, acting as a cell spreading and buffering agent. However, disadvantages of serum in the media also exist, such as the variability in serum composition necessitating the test each batch before use, it is likely to contain inhibiting factors, and it can be more susceptible to contamination (Arora, 2013).

To this end, development of new media and supplement components was required. Accordingly, Neurobasal medium was developed with optimized concentrations of alanine, asparagine, cysteine, glutamate, proline, and vitamin B12 which are found in DMEM:F12 composition but not found in DMEM alone (Brewer et al., 1993). Further, B27 supplement was developed as an alternative to the serum in media, consequently serum free media have become available for studies when there is no need for the presence of serum for avoiding its side effects (Brewer et al., 1994). B27 supplement can be used in combination with Neurobasal but not with DMEM or DMEMP:F12 media composition (Brewer et al., 1994). The variation in media composition showed an impact on the growth of neurons in culture (Harrill et al., 2015). Long term survival (four weeks) has been achieved when the growth medium used composed of Neurobasal medium supplemented with B27 reached up to 90% for hippocampal neurons and 80% for brainstem, in contrast to the media consisting of DMEM: F12 supplemented with B27 (Brewer et al., 1993, Kivell et al., 2000).
Neurons also have the tendency to aggregate and self-organise as clusters (Shefi et al., 2002). This is considered to be a problem that hampers the ability to image neurons for quantification purposes. This has prompted scientists to use automated software for imaging such as Matlab Boost Graph Library package and the Brain Connectivity Toolbox (de Santos-Sierra et al., 2014). Alternatively, previous studies have plated cultures of neurons at low densities (150,000 cells per 60-mm dish) to enable single cell study (Kaec and Banker, 2006)

My PhD required the development of a methodology to derive and maintain a biologically balanced culture (i.e. neuron/astrocytes) using rodent cells, to be used for further testing in subsequent experimental chapters. It should be noted that the culture methodologies for neuron derivation did not exist in the laboratory. Therefore, my first goal was to establish a reproducible technique for primary mouse neuron culture in the group.
3.2. Objectives

The main aim of the research described in this chapter is to obtain reproducible primary neuronal cultures.

The desired criteria for these cultures are (i) reproducibility (the ability to routinely obtain cultures with similar features), (ii) ability to distinguish morphological features of individual cells and (iii) support of the maturation of neurons (clear detection of neurite outgrowth).

In the first section of this chapter, the objective was to identify dissection and culture techniques that would reproducibly derive primary cortical co-cultures.

In the second section of the chapter, tailor the culture system to generate cultures that more closely meet the desired criteria, which are:

(i) Quantifiable cultures; in other words, obtaining evenly distributed cells in the culture to facilitate analysis of individual cells. For this, three variables will be adjusted:

(a) The seeding density of the cells.

(b) Different substrates.

(c) Time in culture, to evaluate neuronal dispersal and maturation.

(ii) Obtaining a balanced co-culture of neurons and astrocytes, by lowering the contamination of astrocytes by reducing the serum level and avoiding the toxic effect of chemical reagents (anti-mitotic agents), employed to limit proliferation of non-neurons.
3.3. Experimental procedure and analysis
2D primary cortical culture derivation and optimization protocols have been described (Section 2.3), as has dispersing primary neurons in culture at low and high cell densities (Section 2.4). Culture characterization was carried out by the immunolabeling procedure as described in (Section 2.7).

3.4. Results
Several protocols for primary neuronal culture were identified in the literature (Millet and Gillette, 2012), and the one selected for initial trials was obtained from another group termed NBM-1.

3.4.1. The NBM-1 protocol was unsupportive of primary cortical culture
Cell death associated with this protocol was high on every occasion, up to 90% ($C_{\text{max}}$ 10-15 attempts). Fluorescence microscopy of cortical co-culture post 7 days incubation time showed a low number of neurons and astrocytes in addition to poor neuronal network formation (Figure 3.1.A).

3.4.2. The DMEM protocol was unsupportive of primary cortical culture
Following NBM-1, the next protocol tested was termed DMEM. Pilot studies using the DMEM protocol showed unsatisfactory results. This included lack of genesis of neurons and poor immunolabeling that limited cell identification (Figure 3.1B).

3.4.3. NBM-2 optimized protocol was supportive of the primary cortical culture
Cortical culture generated by a NBM-2 protocol was feasible and met the desired criteria of the culture (In house made). Microscopic investigations revealed that
cell viability was elevated dramatically (up to Ca. 95%). Neurons were identifiable and often individually distinguishable post-immunostaining. The soma and neurites immunostained positively with TuJ-1 antibody, thereby the morphological characteristics of neurons could be assessed. Increasing the culture time to 7 days in vitro, showed a good maturation level that appears as an elongation in the neuron processes, and a complex network formation as shown in (Figure 3.1C). However, some of the neurons over time tended to aggregate and form clumps. These clusters limited the visualization and quantification of individual cells. Despite these minor limitations, NBM-2 media produced cultures suitable for analysis, and was utilized in all neuronal culture experiments conducted in the following experimental chapters. The outcomes of the primary cortical culture protocols in terms of media tested, are summarised in (Figure 3.1D).
Figure 3.1: Fluorescence micrographs show the validity of primary neuronal cultures derived according to three different protocols. (A) Neuronal cortical culture generated according to Neurobasal-1 (NBM-1) protocol, neurons stained for NMDA receptor subunit NR2A and DAPI. Neuronal cortical cultures generated according to (B) DMEM and (C) NBM-2 protocols, neurons stained with Tuj-1 antibody. (D) Schematic summarizing the criteria adopted to determine validity of the tested protocols.
3.5. Neuron distribution and characterization

This section represents the findings from optimization of primary cortical culture, conducted in order to facilitate analysis and quantification of cells.

Seeding density and substrate: Fluorescence imaging of cells plated on coverslips coated with just PDL as a coating layer at the density $1 \times 10^6$ cell/cm$^2$ revealed the tendency of neurons to aggregate and form clusters, limiting the identification of individual cells. However, reducing cell density to $60 \times 10^3$ cell/cm$^2$ and $30 \times 10^3$ cell/cm$^2$ resulted in better identification of cells but was still insufficient to be quantifiable specifically at the higher density $60 \times 10^3$ cell/cm$^2$. It has been reported that plating density affects the neuron maturation level, but not their distribution on the coverslip (Biffi et al., 2013). The two densities $60$ and $30 \times 10^3$ cell/cm$^2$ have been monitored along two incubation time points. After 3 DIV the observations revealed that Neurons at the higher density culture extended long neurites and formed complex networks. On the contrary, neurites extended from Neurons seeded at lower density were short and did not form any network at the same time points (Figure 3.2 A & C). Post 7 days of incubation time, neurons formed longer processes in cultures seeded at low density and assembled a complex network. Neurons in cultures at the higher density demonstrated more complex (greater density of neurites) network in comparison to the network of low density culture at the same time point (Figure 3.2 B & D).

The combination of cell density reduction and using PDL-Laminin as a second coating layer on coverslips, assisted in dispersing Neurons uniformly over the coverslip area. Figure 3.3 A, B, C & D display the cellular distribution and the extent of neuronal processing at the different time points and cell densities.
**Time in culture:** Cells were well dispersed at the time of seeding, and were spread evenly across the culture well. However, cultures always exhibited aggregation of neurons, to some extent. For the chosen conditions (3 and 7DIV) in cultures coated with PDL alone, and there was a neuronal aggregation that preventing analysis.

In, cultures that were incubated for 3 and 7 DIV and the coating was PDL-Laminin, the neurons did not aggregate or formed clusters that prevent quantification.

The extended culture time had a pronounced effect on the neuronal network formation and its complexity. The neurites were observed to be relatively short at 3 days post seeding compared to 7 days, at either the low seeding density (Figure 3.3 A & B) or higher seeding density (Figure 3.3 C& D).

With regard to cellular characterization, the fluorescence images of cultures revealed that there were two types of cells composed the primary cortical culture. Cells which were positive for Tuj-1 antibody (Tuj1+) and extended multiple processes (neurites) were identified as neurons. GFAP positive (GFAP+), flattened/star-shaped cells were identified as astrocytes. Some of the cells were of undetermined identity, as they were negative for both Tuj-1 and GFAP cellular marker and termed (Tuj-1-/GFAP-).
Figure 3.2: Cortical neuron distribution and growth. Neurons formed clusters when seeded on coverslips coated with PDL and showed highly branched and complex networks at the higher seeding density. (A & C) Neurons stained with Tuj-1 and DAPI at 30x10^3 cell/cm^2 and 3 days post seeding. (B & D) neurons seeded at 60 x 103 cell/cm² incubated for 7 days in vitro.
Figure 3.3: Distribution and growth of the cortical neurons plated on the substrate coated with PDL and Laminin. Neurons spread almost individually on PDL-Laminin coated coverslip for both densities, low density $30 \times 10^3$ cell/cm$^2$ and high density $60 \times 10^3$ cell/cm$^2$ and at the two-time points (3 and 7 days in vitro). The neurons showed a neurite extension regardless of the incubation time. However, the neurite length and complexity varies according to the length of incubation time and density of culture. At low density $30 \times 10^3$ cell/cm$^2$ and incubated for 3 and 7 days post plating (A & B) was less than their length and complexity at high density $60 \times 10^3$ cell/cm$^2$ (B & D). Neurons stained with Tuj1 and nuclei with DAPI.

Serum concentration in media: At 7 DIV incubated culture, the reduction in serum concentration in the growth media from 1% to 0% (Figure 3.4) resulted in no significant effect on cortical cells in culture with respect to (a) the proportions of neurons and astrocytes, as judged by the percentage of Tuj-1 and GFAP-labelled cells respectively, (b) number of cells per microscopic field, as judged by
number of neurons and astrocytes per microscopic field. The numbers of neurons/field were (86 ± 14) for cultures treated with serum free medium and (81 ± 15) for cultures treated with serum (1%) containing medium, while astrocytes were (67 ± 33) and (72 ± 6) per field respectively for both serum free and serum-supplemented media, the neurons and astrocytes appeared morphologically similar by visual inspection (Figure 3.4).

Figure 3.4: Serum in media influence on the proportion of cortical cells in culture. Tuj-1 labeled cells (Neurons) and GFAP labeled cell (astrocytes) proportions and morphology do not appear to be affected by the alteration in serum concentration in media, (A) 1% and (B) 0% serum in growth medium.

3.6. The influence of culture incubation time on the proportions of neurons vs astrocytes
At all incubation times tested, the proportion of primary cortical cells (Tuj-1+ and GFAP+) was calculated as the cell type out of the total number of healthy nuclei. There were no significant differences in the proportion of Tuj-1+ cells in culture over the three incubation times (Figure 3.5). However, the proportion of GFAP+ cells was significantly increased at day 7 of incubation time comparing to the 3
and 5 DIV (1 ± 1% and 6 ± 3 %) respectively (P <0.05) see (Figure 3.5). The findings also demonstrated presence of (Tuj-1+/GFAP+) healthy, unidentified cells.

![Proportion of primary cortical cells](image)

Figure 3.5: Primary cortical cell proportions in cultures treated with serum free growth medium over three time points (3, 5 and 7 DIV). Bar graph of the percentages of primary cortical cells in culture. Green bars represent the percentage of Tuj-1+ cells (neurons) which demonstrated negligible difference over the three incubation time points, while orange bars represent the percentage of GFAP+ cells (astrocytes), which displayed a significant increase at day 7 (P<0.05). (n=3)

The conditions that have been tested in order to generate primary cortical culture and the optimal conditions which will be utilized for the subsequent experiments in the following chapters are summarised in Figure 3.6.
Figure 3.6: Schematic representing the approved culture protocol for deriving neuronal primary cortical cells in addition to the optimal culture condition which chosen according to experimental criteria needed.
3.7. Discussion
As detailed in section 3.1, data from primary cultures will be of greater biological relevance than will data from cell lines. Primary neuronal culture derivation is known to be challenging, but a procedure to derive neuronal culture was successfully established, after careful optimization of the methods. From continuous observation during the experimental process for optimizing primary cortical culture, the success of growing the cells optimally relies on basic environmental requirements, which included mainly: (i) tissue processing reagents (medium and tissue digestion reagents) and appropriate technique, (ii) growth media, and (iii) adherent culture system of plating surface ((see materials and methods chapter). In the first part of this chapter, the optimal primary cortical culture protocol was established.

The stability of pH of the culture medium is a critical factor when propagating cells for a long time. Accordingly, the choice of media is critical for maintaining a stable pH. If the pH increases over 8, cell viability will decrease. Such an effect was shown by Eagle in 1973. In culture media that contain bicarbonate, the pH may increase to 8.5 after 1 hour of incubation (Lelong and Rebel, 1998). Poor cell survival results when the pH rises above 8 (Eagle, 1973). Therefore, choosing media containing low sodium bicarbonate is preferable specifically for cultures designated for pharmacological and toxicological studies. Hanks’ Balanced Salt Solution is one of these buffers that contains sodium bicarbonate (NaHCO₃) compared to Earle’s Balanced Salt Solution. Furthermore, buffering capacity can be improved by adding HEPES (Lelong and Rebel, 1998).

The cell dissociation protocol including enzymes contributes to determining the viability of cultures (Volovitz et al., 2016). According to the manufacturer's
instructions (See Chapter 2 Section 2.1), the utilized tissue dissociation enzyme (Tryple) is gentler on cells (protect cell’s surface proteins) than trypsin (Schwartz et al., 2011). Also, the digestion enzyme DNase I was used because it was necessary for removing the DNA traces of lysed cells that can hamper further cell digestion (Chen et al., 2011, Xu et al., 2012). Strainers were used to remove dead cells from surrounding medium. Tissue dissociation including enzymatic and mechanical dissociation can induce apoptotic cell death which is characterized by plasma membrane rupture leading to local inflammation (Branton and Clarke, 1999).

As a growth medium is designed to be the source of energy and cell cycle regulators (Arora, 2013), selecting the appropriate growth media for cultivating primary cortical cells should be built on a solid foundation. Providing nutrients (complement of amino acids, vitamins, inorganic salts, glucose, and serum as a source of growth factors, hormones, and attachment factors) and maintaining stable pH and osmolality are the main requirements for the culture (Arora, 2013).

NBM-2 medium was the optimal composition that supported cortical culture, in contrast to the NBM-1 and DMEM media as detailed in table 2.1. The basic elements in the media are Neurobasal or DMEM: F12. If we look closely at the composition of these media, we find that Neurobasal medium is developed from DMEM: F12 by optimising the concentration of alanine, asparagine, cysteine, glutamate, proline, and vitamin B12. Furthermore, the osmolality, glutamine and sodium bicarbonate were optimised (Brewer et al., 1993, Brewer et al., 1994). Our results confirmed Gregory J. Brewer’s findings in 1994 and showed that the Neurobasal in combination with B27 performance was superior to DMEM: F12 for survival and maintenance of Neurons. Brewer also reported that serum free
Neurobasal media complemented with B 27 supported the survival of Neurons in cultures and obtained survival up to 70%, however, that was brain region dependent (Brewer, 1995). That goes back to the idea that DMEM: F12 medium has potential excitotoxic amino acids such as glutamate and aspartate (Brewer et al., 1994, Price and Brewer, 2001). Where, the excitotoxicity is defined as “cell death resulting from the toxic actions of excitatory amino acids” (Dong et al., 2009). This toxic action results from prolonged exposure to glutamate which considered as the major excitatory neurotransmitter that causing over activations of receptors for the excitatory neurotransmitter glutamate, such as the NMDA receptor (Berliocchi et al., 2005) . This process associate with excessive calcium influx which casing the activation of enzymes that degrade proteins including phospholipases, endonucleases, and proteases such as calpain. These enzymes degrade proteins, membranes and nucleic acids and result in damage cell structures such as components of the cytoskeleton, membrane, and DNA (Dong et al., 2009).

Growth media using theNBM-1 protocol was improved to NBM-2 growth media by replacing, L-glutamine supplements to GlutMax-I and horse serum to foetal bovine serum. According to the manufacturer (Fisher), L-Glutamine is a supplement that spontaneously degrades, generating ammonia and pyrrolidine carboxylic acid as a product of the reaction (Ozturk and Palsson, 1990, Bray et al., 1949). GlutaMAX-I is a developed supplement that does not spontaneously degrade (Christie and Butler, 1994). The substituted supplements are recommended according to the literature, where FBS enhanced fibroblast cell passage number in addition to preserving the cellular morphology (Franke et al., 2014). For the purpose of experimental quantification, it was necessary to obtain
almost individually distributed cells; proceeding from this need, the cellular substrate was adjusted to control the neuronal dispersal in culture.

Cultivated Neurons on PDL-coated coverslip formed clusters at all the developmental stages examined regardless of the cell seeding density. The cellular interaction with the substrate relies on the ability of a family of transmembrane glycoproteins named integrins (mainly β1 family) to bind to the binding sites of the extracellular components such as collagen, fibronectin and Laminin (Clyman et al., 1990, Hynes, 1992). Therefore, poor coverslip coating can influence the neuronal cell distribution. Hence, PDL as one coating layer, was insufficient to provide the consistency for cellular distribution. This is in agreement with the study that reported the physical bounds between PDL and the surface was insufficient for neuronal cell adhesion enhancement and promoting neuronal growth and neurite outgrowth (Kim et al., 2011). According to this, adding laminin as a second coating layer enhanced cellular adhesion and reduced neuronal aggregation and cluster formation. On the basis that astrocytes are supporting cells to the neurons by protecting them from various forms of cytotoxicity (Desagher et al., 1996), maintaining a quantity of astrocytes in culture was deemed necessary.

Obtaining biologically controlled culture was achieved by serum starvation, in turn trying to reduce number of astrocytes but not totally eliminating them, avoiding by that means the toxicity that can result from the glial cells inhibitors, such as the anti-mitotic agents arabinosylcytosine C (AraC) (Geller et al., 2001, Ahlemeyer et al., 2003). The findings here demonstrated that the influence of serum starvation on the percentage of astrocytes was obvious when the serum level reduced from 10% to 1% as visually determined. However, there was no for
further effects on percentage of astrocytes following further reduction in serum from 1% to 0%. The findings here are inconsistent with those of Chou & Langan during their study on regulation of cell division cycle. As in their study they demonstrated the influence of serum starvation on the cell division mechanism, astrocytic cells treated with 10% bovine calf serum in DMEM medium first, then cells reached the desired confluence up to (30-50%), the second step was reducing serum in medium to 0.1%, by that astrocytes arrested in the G0 phase of the cell cycle (Chou and Langan, 2003). Then for re-entering astrocyte to the cell cycle the serum level up-shifted again to 10%. These findings suggested that the astrocytes can be arrested and exit the cell cycle by reducing the serum below 10% (i.e. astrocyte entering into G0 phase can be either stimulated with low serum or serum free medium).

Taken together, the findings from the three examined protocols for generating primary cortical neuronal cultures suggested that NBM-2 protocol for obtaining reproducible culture was the best. Furthermore, the optimal parameters which included seeding density (60 x10³cell/cm²), coverslip coating (PDL-Laminin), serum level in media (0%) and time points (3 and 7 DIV) suggested to serve the need for obtaining survive, mature, quantifiable, purified safely without using cytotoxic inhibitors neuronal culture. This optimization process was conducted in order to configure the neuronal culture to be used for the subsequent experiments which encompass gene engineering, electrophysiological analysis in addition to growing neurons in 3D constructs, which will be demonstrated in the next experimental chapters.
Chapter 4
Evaluating the safety of magnetofection for primary cortical neurons
4.1. Introduction
The limited capacity of the CNS for endogenous repair following injury or disease, in which the low capacity of neurons to regenerate is a major factor, has motivated researchers to develop basic research in this area (Bjorklund and Stenevi, 1979). Researchers have enhanced the understanding of many aspects of neuronal biology by investigating gene and protein function that can promote neuronal survival and function, in order to inform the development of novel therapeutic interventions (Tuszynski and Steward, 2012). In this respect several technologies to genetically modify neurons have developed as discussed earlier in the general introduction. However, there are two technical issues that need to be considered in delivering the gene to the neuronal population, namely, successful gene targeting and safety.

Neurons derived from E18 (embryonic) are known to be challenging in terms of introducing and expressing exogenous genes. This is related to their sensitivity to alterations in their microenvironment including temperature, pH, changes in osmolarity, and physical stress (Lelong and Rebel, 1998, Karra and Dahm, 2010a). Accordingly, choosing the appropriate neuronal gene engineering technique is critical for successful outcomes. Since the main obstacle to the process of gene delivery is the limited diffusion of the gene across the cellular barriers, several methodologies have been optimized and tested for gene delivery into Neurons including viral and non-viral approaches. Despite these techniques being efficient at delivering genes to cells they have their limitations (discussed previously in the General Introduction). Here, some examples for transfecting post mitotic Neurons using (i) viral and (ii) non-viral techniques, will be presented
together with a summary of viral versus non-viral techniques for genetic engineering of neurons, as discussed more widely in the General Introduction.

The herpes simplex virus (HSV) was the first viral gene vector used for transfecting neurons (Washbourne and McAllister, 2002), for example cultured rat superior cervical ganglia and dorsal root ganglia (Geller and Breakefield, 1988). This type of viral vector possesses special qualities that facilitates a high level of gene delivery and subsequent transfection of neurons as they can transfect neurons efficiently, and can be utilized as neuronal pathway tracers due to their ability to be transported across synapses in a retrograde way. Moreover, protein expression possesses a long lasting ability that can persist for up to a year. However, their major limitation is their high toxicity level in vitro and local immune response in vivo (Bergen et al., 2008).

The biolistic technique is a mechanical non-viral technique using a hand-held gene gun for transfecting neurons in organotypic mouse cerebellum slices (O'brien and Lummis, 2006). Nucleofection is a method developed from the biolistic technique, for the transfection of hippocampal neurons. Both techniques display a high transfection efficiency of up to 95%. However the main drawbacks are related to the type of targeted cells, in that the biolistic technique is limited to tissue slices, and nucleofection is successful only for immature cultured neurons that have not produced neurites (Zeitelhofer et al., 2007). Although these transfection techniques produce high transfection efficiency, each of these methods has a number of limitations.

The process of delivering gene to the target cells is limited by multiple barriers confronting the entry of the gene-vector complex into the cell to manipulate
nucleic acid in the targeted cells. These include (i) vector-cell contact; (ii) transport across the plasma membrane—which is considered a barrier for molecules that are not actively imported by cells; (iii) endo/lysosomal clearance; (iv) nuclear membrane, and in addition (v) the limited targeting ability of the gene-vector complex i.e. the non-specific interaction with non-target cells or organs and the probability of systemic spreading of the vector in in vivo application (Kim et al., 2002) and (vi) vector inactivation due to non-specific and rapid interaction with undesired components (i.e. defence complements or immune system) in in vivo milieu (Ogris et al., 1999). Toward enhancing transfection efficiency, research efforts focused on developing DNA carriers such as synthetic vectors that can mediate efficient gene delivery to the target cells or tissue such as polymer carriers, lipid, polypeptides and nanoparticles (Bergen et al., 2008) as discussed in the General Introduction. The other method was by designing physical assistive methods that accelerate vector-DNA complex in the direction of the target cells, and so enhance vector-cell contact.

Vector-cell contact is an accumulation of nanoscale particles such as viruses and IONPs within the cell which, to a great extent, relies on a diffusion-limited process. Efforts have been made to facilitate vector-surface bound biomolecule (virus or gene) delivery to the target cells/tissue instead of the random orientation. However, conventional transfection reagents such as polyamidoamine (PAMAM) are toxic to cells specifically with long time exposure. Consequently, just a few fractions of DNA could be internalized into the cells (Haensler and Szoka Jr, 1993). Luo and Saltzman have managed to enhance transfection efficiency about 8.5 fold over the conventional methods utilising a method designed to increase DNA concentration at the cell surface by relying on gravity.
of nanoparticle-association with vector–DNA complexes to facilitate sedimentation of DNA onto cell surface (Luo and Saltzman, 2000a). This offers an explanation of why large and heavy particles have demonstrated more efficient transfection levels than smaller particles (Luo and Saltzman, 2000a). In this respect, Bunnell and his group have successfully used centrifugal force for enhancing vector-cell contact (Bunnell et al., 1995). To that end ‘magnetofection’ - as defined previously within the General Introduction - has been considered as the most promising approach for enhancing neuronal transfection efficiency (Plank et al., 2011a). Accordingly, Furlani and Ng (2008) have developed a method using a magnetic field that is based on applying magnetic force that can attract the magnetic particles-surface bound gene to the target cells (Furlani and Ng, 2008). This technique offers the advantages of enhancing transfection efficiency via rapid and efficient gene transfer, protein expression level, production scalability, and reduction in cost, technical complexity and toxicity (Karra and Dahm, 2010a).

With respect to transfection of neurons using magnetofection, several neuron types have been tested, using both neuronal cell lines and primary neurons, for expression of reporter genes and physiologically relevant biomolecules. Examples include transfection of motor neurons with a plasmid encoding the F-actin reporter Lifeact-GFP for the purposes of inspecting any morphological changes associated with magnetofection technology, as well as delivering (GFP) reporter gene for investigating nanoparticle uptake (Fallini et al., 2010, Fernandes and Chari, 2014); expression of potassium–chloride cotransporter KCC2 in immature hippocampal neurons derived from P0 for studying KCC2’s role in GABAergic network formation (Chudotvorova et al., 2005); and study of the protein localization and transport of axonal proteins such as spinal muscular
atrophy protein (SMN) (Ang et al., 2011) in embryonic primary motor neurons (Fallini et al., 2010).

The magnetic carrier’s formulation and the mode of the applied magnetic field are the two determinants of magnetofection efficiency. Mah et al. (2000), have reported magnetically enhanced AAV vector transfec by linking the virus to magnetic microsphere mediated gene delivery to C12S cells (Mah et al., 2000).

The deployment of a static magnetic field (Scherer et al., 2002) through application of high–field /high-gradient magnets underneath culture plates can promote particle sedimentation over cells that leads to rapid and efficient transfection (Schillinger et al., 2005, Buerli et al., 2007). However, a non-uniform distribution of IONPs-gene complex on the surface of cultured cells was apparent when a static magnetic field was used. Therefore, some refinements have been made by different groups in order to achieve uniform particle distribution promoting targeting and efficiency specifically for Neurons. Baryshev and his group (2011) developed “DynaFECTOR” which is a dynamic gradient magnetic field, using a rotating platform assembled with magnets of the orbital shaker to provide the movement. Although this technique promoted transfection efficiency in human liver hepatocellular carcinoma cell line HepG2 over the static field, it has a limitation in a lack of precise particle directing toward cell surface (Baryshev et al., 2011). Hence, a series of attempts to develop oscillating magnetic fields have been made. Pulsating magnetic field termed “electromagnet” has also been developed and also produced high transfection efficiency within minutes of exposure. However, this method was associated with an increase in temperature up to 42.5 °C which in turn can lead to cell death (Fouriki et al., 2010b).
Magnetic actuator was another magnetic device developed that provides linear movement of the magnet producing variable magnetic fields (Oral et al., 2015). This device has been tested on different cell types but not yet on neurons. To this end, it has been reported that the overall transfection level using magnetofection is four times greater than that of other techniques like cationic-lipid based reagents (Lipofectamine 2000TM) (McBain et al., 2008). An oscillating magnetic array of cylindrical stacks of high-gradient NdFeB magnets utilises both frequency and amplitude to produce a lateral movement to the MNP-gene complexes (McBain et al., 2008). Use of specific frequency and amplitude parameters with this device has been reported to enhance transfection efficiency in oligodendrocyte precursor cells (Jenkins et al., 2011); neural stem cell suspension cultures (Adams et al., 2013); neuronal cell line SH-SY5Y and primary hippocampal neurons (Subramanian et al., 2017). Furthermore, fluorescence intensity was affected by the magnet distance. Thus, using an oscillating magnetic array with magnetic distance no less than 3 mm and no more than 5 mm from the cell culture plate demonstrated high fluorescence intensity in comparison to a static array (Fouriki et al., 2010b). The basic mechanisms believed to participate in enhancing transfection efficiency by applying magnetic fields are:

(i) Applying permanent magnetic field increase IONPs sedimentation rate on the cell surface and accelerates transfection in in vitro experiments. In vivo, utilization of magnetic fields not only increases transfection efficiency but enhances therapeutic gene targeting to the desired tissue or organ (Dobson, 2006). Regarding oscillating magnetic field, the lateral movement of magnetic field directs the magnetic vector (IONPs) to contact the cells in culture within a given
time. (ii) It is believed that an oscillating field can stimulate the endocytic mechanism of the cell membrane (Fouriki et al., 2010a). (iii) The other proposed mechanism of action of oscillating fields is the facilitation of the endosomal escape of IONPs by disrupting endosomal processing (McBain et al., 2008). A further advantage of enhancing transfection efficiency by using the oscillating magnetic field that is characterized by high field strength and gradient associated with limited heat production; the magnetic force results in a translational force on the particles acting in the direction of the applied field, resulting in rapid sedimentation of particles over the cell monolayer, versus other similar techniques, for example the electromagnetic system used by Kamau and others has weak field strength and gradient and produced significant heating which may have a negative effect on cell viability (Kamau et al., 2006, Pickard and Chari, 2010a).

One of the key factors that determine the suitability of the magnetofection strategy is safety. Despite the efforts made by a wide range of laboratories that investigated the adverse effects of magnetofection approach on Neurons, validation was based on simple histological read outs, phenotypic evaluation, live/dead staining, viable cell counts using flow cytometry, mitochondrial toxicity assays and lactate dehydrogenase (LDH) (Pisanic et al., 2007, McBain et al., 2008, Petters and Dringen, 2015). However, further assessment is needed to be related to neuronal functional properties. Rosen reported that the moderate-intensity static magnetic fields (SMF) altered membrane calcium ion Ca$^{2+}$ flux and Na$^{+2}$ channels in cultured GH3 cells which are cell line derived from rat pituitary cells. The effect was in the form of a delay in alteration of ion kinetics through the channels (increase in the activation time constant) due to altering
channel activation kinetics which results from ionic channels deformation (Rosen, 2003). In this project it was important to evaluate the effect of the oscillating magnetic field on the functional properties of neurons, in order to determine whether there is an adverse effect of magnetofection on neuron excitability and signalling.

It is important to address this issue as some nanoparticles can exhibit a neurotoxic effect in a particle type dependent manner and disturb neuronal electrical activity although the morphology is not affected. For instance, the effect of carbon black (CB), hematite (Fe₂O₃), and titanium dioxide (TiO₂) IONPs on primary murine cortical network activity cultured on a microelectrode array was concentration dependent (i.e. there was a reduction in the general electrical network activity, spiking and burst rate, in low dose associated with decomposition of the network oscillation at 20µg/cm²), but there were no features of damage or injury to Neurons (Gramowski et al., 2010). Also, whole-cell patch clamp recorded a significant alteration in action potential of CA1 hippocampal neurons in a concentration dependent manner when silver nanoparticles were applied (Liu et al., 2009). Therefore, it’s important to investigate the effect of magnetofection on neuronal excitability, as it includes a combination of IONPs and magnetic fields.
4.2. Objectives

From the previous overview of magnetofection developments and strategies it is necessary to:

1-Compare the effect of using two oscillating field devices (uniaxial versus biaxial) on MNP based transfection efficiency of neuronal and glial cells in primary cortical culture at two different time points. The two different oscillating field devices have been detailed previously (please see Materials & Methods), however to summarise, both devices have the same aim which is to increase the sedimentation rate of particles. The uniaxial employs a one-direction lateral movement while the biaxial imparts lateral motion in two axes which has the potential for wider distribution of the particles over the cell layer, thus enhancing particle: cell contact.

2-Examine the safety of using magnetofection as a bioengineering strategy by evaluating the electrophysiological activity of primary nanoengineered Neurons.
4.3. Experimental procedure and analysis

All experimental procedures included in this chapter were described in materials methods chapter. As magnetofection experiments has been described in (section 2.5.4), nano-particles internalization in (section 2.5.5), immunostaining in (section 2.7), and finally safety assessments including cell viability examination in (section 2.9) while electrophysiological properties assessment in (section 2.11).

4.4. Results

4.4.1. The influence of oscillating magnetic field on transfection efficiency of primary cortical cells (Uniaxial VS Biaxial magnet)

Investigation of the effect of incubation time points on the transfection efficiency of cortical cells transfected by uniaxial and biaxial was conducted in parallel. In any control cultures (plasmid only) reporter gene GFP expression was not observed. In cultures transfected with IONP-plasmid complex, GFP expression was observed in both Tuj1+ and Tuj1- cells. The timing of the peak of protein expression was at 48 h post-transfection. Application of uniaxial magnetic field resulted in no significant effect on the level of GFP expression in both Tuj1+/Tuj1- cells at 3 DIV incubation time point comparing to 7 DIV (3.2 % ±1.2 and 3.6 % ±1.4) and (4.8 % ±1.9 and 6.3 % ±2.9) respectively. Application of biaxial magnetic fields showed no significant differences in GFP expression level for Tuj1+ cells between the two time points. However, GFP expression level in Tuj1- cells enhanced about two folds at 7 DIV comparing to GFP expression in cells incubated for 3 DIV in culture (4.2 % ±1.1 and 14.5 % ±1.7) respectively (P <0.01) (Figure 4.1).
Investigating the effect of the magnetic field on the protein expression was at two different time points. Statistical analysis revealed that the variation in the nature of magnetic field oscillation had no influence on the percentages of transfected cells whether they were TuJ1⁺ / TuJ1⁻ at 3 DIV. This included TuJ1⁺ transfected at 7 DIV, however magnetic device effect appeared on TuJ1⁻ cells in cultures transfected at 7 DIV (P < 0.005) (Figure 4.1).

Figure 4.1: Magnetofection facilitates transfection of primary cortical cells. Bar chart showing the percentage of TuJ1⁺ and TuJ1⁻ cells transfected by magnetofection technique at 3 & 7 DIV using the uniaxial and biaxial magnet in parallel. Two factors (7 DIV time point and magnet) facilitate number of TuJ1⁻ cells expressed GFP protein elevation to approximately double comparing to TuJ1⁻ at 7 DIV transfected using a uniaxial magnet. (n=3), P < 0.01.

Magnetofected cortical cells immunostained with TuJ-1 antibody showed a typical Neuron morphology and similar to the morphology of the counterpart cells in un-
transfected cultures. Non-transfected neurons at 3 DIV expressed Tuj-1 (red) antibody and displayed healthy neuron body and extended short processes. Transfected neurons in addition to exhibiting healthy neuronal morphology, co-expressed both GFP (green) and Tuj-1 antibody to give yellowish colour that can be the indicator of transfected neurons. Moreover, Neurons had formed a complex network of cultures incubated for 7 DIV before transfection and 2 more days post transfection (Figure 4.2 A, B, C & D). Astrocytes showed a flattened, hexagonal morphology typical of astrocytes in 2D culture and as has been seen in the Non-transfected cells.
Figure 4.2: Uniaxial magnetic field supported primary cortical cells transfection. (A & B) Fluorescent micrographs show GFP expression of magnetofected primary cortical cells, white arrows point to Neurons expressing GFP at 3 & 7 DIV, maximum incubation time post transfection was 48 h. C & D) demonstrated the typical morphology of Neurons at the normal maturity level that has been seen in un-transfected cultures. Neuron processes demonstrated normal morphology and level of complexity according to the incubation time.
4.4.2. Confirmation of intracellular IONP internalization
Phase contrast microscopy of 7 DIV cortical culture treated with fluorescent IONPs suggested that IONPs were internalized into the neurons and astrocytes in both transfected and non-transfected cells (Figure 4.3 A & C). However, it was difficult to precisely determine particle internalization without using GFP protein expression in cortical cells as indicator. Accordingly, there is a need to more sophisticated technique such as transmission electron microscope to determine location of fluorescent particles for cell. The micrographs also revealed that fluorescent IONPs were distributed in culture, however they aggregated in some areas and formed different size aggregates (Figure 4.3 B).
Figure 4.3: The susceptibility of primary cortical cells to internalize iron oxide magnetic nanoparticles. (A,B & C) Phase contrast and fluorescent images demonstrate that the fluorescent Neuromag magnetic nanoparticles distributed in culture homogeneously despite particle aggregation in some areas. Those cells in culture that expressed GFP are circled in a white dashed line. Cells that took up particles without showing any gene expression are circled with a red dashed line.
The expression of GFP was not closely associated with the level of uptake of particles internalized by cortical cells. Low uptake of fluorescent nanoparticles by neurons was sufficient to result in high intensity GFP expression (Figure 4.4 A). Astrocytes that expressed GFP showed high uptake of fluorescent magnetic nanoparticles versus neurons (Figure 4.4B).

Figure 4.4: Magnetic nanoparticles uptake is cell type dependent. (A) Neurons can express high intensity GFP with a low level of particle uptake. However, level of magnetic nanoparticles taken up by astrocytes was higher, associated with a low intensity of GFP expression (B).
4.4.3. Safety assessment of magnetofection technology

4.4.3.1. Cell viability for magnetofected cortical cells

Applying a uniaxial magnet to cortical cultures at 7 DIV and incubation with IONPs-gene complex for 48h had no cytotoxic effects as judged by live/dead assay, versus incubation with a plasmid in medium alone (control) (59.7 % ± 6.2, 65.3 % ± 3.9, respectively). Applying biaxial magnet had an adverse effect on the cell viability, (57.7 % ± 1.5, 32.3 % ± 7.5) (Figure 4.5 & Figure 4.6 A & B).

No differences were noted between magnetofected and control cultures with respect to pyknotic nuclei for cultures transfected by applying uniaxial (38.7 % ± 2.1, 35.3 % ± 5.5), or biaxial magnet (51 % ± 5.5, 48 % ± 7.3).

Figure 4.5: Uniaxial oscillating magnetic field is a safe technique. The graph represents the safety of magnetofection is magnetic device type dependent, no alteration seen in the percentage of cell viability when uniaxial magnetic device applied however cell viability reduced when biaxial magnetic device applied. (n=3)
Figure 4.6: Uniaxial oscillation magnetic field is a safe technique. The graph represents the safety of magnetofection is magnet type dependent, micrographs for (A) uniaxial and (B) biaxial magnet. There was no cytotoxic effect of magnetofection (uniaxial magnet) on cortical cells comparing to the cytotoxic effect of a biaxial magnet on the cortical cells. (n=3)
4.4.3.2. Histological evaluation of the safety of magnetofection for transfected neurons

All assays exhibited no toxic influence on the cells (Figure 4.7 A, B, and C). Neurons also displayed a typical healthy morphology in comparison to neurons exposed to neither IONPs nor magnetic fields (Figure 4.6 D, E, and F).

![Figure 4.7: Uniaxial oscillating magnetic field is safe for transfecting primary cortical neuronal culture.](image)

The graph shows no alteration in (A) cell viability, (B) percentage of pyknotic nuclei, (C) neuronal number. Fluorescence micrographs of cortical cultures after 7 days in vitro. (D) Neurons (Tuj1+) display small rounded soma with long processes, astrocytes (GFAP+) are flat, membranous, and unbranched. (E) Pyknotic nuclei show condensed chromatin in cortical co-culture. The inset shows more clearly a neuron with condensed chromatin (arrowed). (F) Neurons after magnetofection co-express Tuj1 and GFP. The inset shows a GFP+ astrocyte (orange arrow) and a Tuj1+/GFP+ neuron (white arrow). (n=3)
4.4.3.3. A comparative assessment of electrophysiological characteristics between transfected and non-transfected neurons

Magnetofected and non-magnetofected (control) neurons which were included in this experiment have been identified under blue light (excitation wavelength 495 nm). The morphological features of neurons were similar to what has been elucidated in section 4.3.1 (see Figure 4.8 E).

In voltage-clamp experiments, biphasic currents were generated by depolarizing voltage-clamp steps. These currents comprised of both early inward current followed by a delayed outward current. The inward current reached a peak at around 2ms after the step, while the outward current activated over 5–10 ms (Figures. 4.8 A & B). In order to validate that the inward current resulted from Na\(^+\) channels activity, the sodium channel blocker TTX (25 μM) was added that resulted in rapid blocking of the early inward current. This confirms that this inward current is a voltage-dependent Na\(^+\) current (Figure 4.8 D). The delayed outward current was maintained for the duration of the voltage step. The reversal potential of this current was negative to –60 mV, based on an outward tail current polarity at –60 mV. Therefore, it was believed that it was due to a K\(^+\) current (Figure 4.8 A). Currents carried by K\(^+\) would be expected to have a reversal potential of –83 mV (the calculated K\(^+\) equilibrium potential under our recording conditions). The membrane potential was held to –60 or –70 mV so that Na\(^+\) and K\(^+\) currents were visible in response to depolarizing steps. The currents started to be seen around –40 mV. After that rapid current activation (1-3 ms) occurred and reached a maximum at about –20 mV. A comparison between GFP\(^+\) neurons and GFP\(^–\) neurons revealed no differences between current amplitudes or resting membrane potential (Table 4.1). In current clamp experiments, following a
depolarizing current injection step, small spikes appeared, their amplitude ranged between 15 to no more than 30 mV (i.e. did not reached or exceed 0mV) (Figures 4. 9 A & B). Spontaneous depolarizations that increased in amplitude with hyperpolarization were noticed in a few cells (Figures 4.8 A & B).
Figure 4.8: Representative ionic currents were seen in response to voltage steps from a holding potential of –60 mV from both magnetofected (GFP+) and non-magnetofected (GFP–) neurons. (A) Voltage steps (top) and corresponding ionic currents are shown for GFP+ and GFP–neurons as indicated (bottom two rows). (B) Leak-subtracted records shown on a faster time scale to reveal early inward (Na+) currents followed by late outward (K+) currents. Holding currents have been subtracted. Same cells as (A). In (A) and (B) the horizontal (time) and vertical scales are shown at the bottom and on the left respectively. (C) I–V plots of early inward and late outward currents from GFP+ (top) and GFP– neurons (bottom). The late currents (squares) were measured from the records shown in (a) at the 40-ms time point, the early currents (circles) were measured as peak inward currents from the records in (B). (D) The inward (Na+) current seen in response to a 40 mV depolarizing voltage-clamp step, from a holding potential of –70 mV (left), is blocked by TTX (2 µM, right). The voltage steps are shown above the current records. (E) Micrographs of magnetofected neurons (GFP+) used for whole-cell recording taken under normal transmitted light conditions (left) and a combination of low transmitted light and fluorescence excitation (right), also showing the recording and drug-application pipettes. Scale bars = 10 µm.
Figure 4.9: Representative current clamp recordings from magnetofected (GFP+) and non-magnetofected (GFP−) neurons showing spike-like activity. (A) Current steps (top) and corresponding voltage responses (middle) from a GFP+ neuron. The arrows indicate spike-like voltage transients (blue arrows) and spontaneous depolarizations, probably reflecting synaptic activity (black arrows). The bottom panel shows the response to the largest depolarizing current injection on an expanded scale. A negative holding current was applied to hyperpolarize the neuron, and the injection begins at 50 ms. (B) An equivalent recording from a GFP− neuron.
Table 4.1: Comparison of voltage-dependent Na$^+$ and K$^+$ currents in GFP$^+$ and GFP$^-$ neurons in voltage-clamp. Current amplitudes were measured at the voltages indicated. For the measurements made at −30 mV, records were made using leak subtracted records. Membrane potential (Em) measurements are also shown, measured by linear interpolation of I–V curves at I = 0. There were no significant differences between the means for GFP$^+$ and GFP$^-$ neurons (Student’s t test). Values are mean ± SEM, given to the nearest whole number.

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<tr>
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<th>GFP positive (n = 8)</th>
<th>GFP negative (n = 6)</th>
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<tr>
<td>$E_m$ (mV)</td>
<td>-38 ± 1</td>
<td>-32 ± 3</td>
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<td>$I_{Na}$ at -30 mV (pA)</td>
<td>-271 ± 88</td>
<td>-385 ± 144</td>
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<tr>
<td>$I_{K}$ at -30 mV (pA)</td>
<td>70 ± 20</td>
<td>33 ± 9</td>
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<tr>
<td>$I_{K}$ at 0 mV (pA)</td>
<td>806 ± 235</td>
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4.5. Discussion

Adding lateral movement in one direction (uniaxial) for the nanoparticle/gene complex has been shown to be successful for improving the overall transfection efficiency in different cell types such as human airway epithelial cell line (McBain et al., 2008), astrocytes (Pickard and Chari, 2010a, Tickle et al., 2015), microglia (Pickard and Chari, 2010b), and neural stem cells (Pickard et al., 2011). Furthermore, some groups successfully enhanced neuronal cell line (SH-SY5Y cells) and primary neuronal transfection efficiency up to 10-15% with low associated cytotoxicity with this technique (Subramanian et al., 2017). Primary neuronal transfection efficiency using magnetofection is relatively low compared to other cell types such as oligodendrocyte precursor cells (OPCs) which ranged from 15.9-26.3% (Jenkins et al., 2011). Up to 80 % transfection efficiency, but low viability, of hippocampal neurons, was reported when the electroporation technique was used for gene delivery (Rathenberg et al., 2003).

Several studies have addressed several magnetic device parameters. Some measured range of frequencies/amplitudes (McBain et al., 2008), while others determined the optimal working distance of the magnetic field (field strength) which was 3 mm for oscillating magnetic fields (Fouriki et al., 2010a). However, to the best of our knowledge, our work is the first to introduce a bidirectional lateral motion (biaxial) to the particles to enhance MNP-gene complex delivery to cells of primary origin such as neurons.

In that respect, it was necessary to compare the efficiency and the safety of this developed device on transfecting primary cortical neurons and comparing it to the unidirectional oscillating lateral motion provided by a uniaxial device.
Multiple factors can be predicted to influence transfection efficiency, including: developmental stage, conditions of cell culture, for example type of media used, physicochemical structure of vectors, and vector: DNA ratio (Buerli et al., 2007, Fallini et al., 2010, Jenkins et al., 2013a) These factors are likely to account for the differences in observations between studies.

The findings of this study indicated that transfection efficiency was about 10% when the applied magnet was uniaxial and about 15% when the applied magnet was biaxial. These results are considered low in comparison to those reported by some groups for various neuronal types including primary cortical neuronal cultures, up to 46% using non-viral approaches combined with high DNA concentrations (Fallini et al., 2010). However these results are within the range reported by other groups for example Buerli et al (2007) have achieved ca. 5% and Subramanian et al (2017) ca.15% transfection efficiency.

With regard to cellular uptake, the experiments revealed that there is no apparent correlation between extent of particle uptake and GFP expression, (i.e. some cortical cells took up the nanoparticles but GFP expression was not noted). This might be related to a defect in the mechanisms that determine the fate of the gene post internalization inside the cells, which is discussed in the General Introduction.

Transfection-based bioengineering strategies for neural repair will require both efficient, preserving function and safe protocols. Therefore, further investigation which related to the neuron's signalling has been conducted here. To our knowledge, this is the first study investigating the electrophysiological properties of magnetofected primary neurons in comparison to non-magnetofected neurons.
Generally, the neuron’s membrane potential, and ability to spike (all functions of ion channels), and its ability to secrete chemicals or neurotransmitters is a prerequisite for normal neuronal function in any environment, including within the host tissue post transplantation and even in the absence of functional synaptic contacts. Studies have shown that metal or carbon-based nanoparticles influence spiking frequency and bursting patterns, based on neurochip extracellular recordings (Gramowski et al., 2010), and with alterations in Na\(^+\) current amplitude and activation range in whole-cell patch recordings. These effects are apparently concentration-dependent and are subtle at low concentrations of nanoparticles, including iron oxide core nanoparticles (Gramowski et al., 2010). However, our observations revealed that the Na\(^+\) and K\(^+\) channels in cortical neurons were not obviously influenced by magnetofection. Furthermore, there were spontaneous excitatory synaptic potentials, indicating that synaptic contacts are functional in these neurons.

Particle uptake into the cells is highly dependent on particle size and cell type, therefore it will be difficult to make comparison between studies in terms of metal or particle concentration. The IONPs employed here, at ~160 nm diameter (Pickard et al., 2015), and with an additional chemical envelope for plasmid attachment, are relatively large, but these and similar particles have a well-documented safety profile across a range of neural cell types (Pinkernelle et al., 2012, Pickard et al., 2015, Fernandes and Chari, 2016, Jenkins et al., 2016).

Interestingly, the findings confirmed the notion that the IONP internalization amount by cortical cells is cell type dependent. The relative amount of iron oxide nanoparticles taken up by Neurons was low compared to astrocytes. Cell specific particle uptake level is also dependent on the endocytic capability of the cells
(Ziello et al., 2010). Where clathrin-mediated endocytosis is the suggested pathway that participates in IONPs uptake by neurons (Petters and Dringen, 2015), these clathrin coats in neurons are abundant in the synaptic areas (pre and post synaptic) (Blanpied et al., 2002). Whereas, astrocytes which possess higher levels of endocytotic activity (Tickle et al., 2016, Ziello et al., 2010) can handle higher magnetite content than neurons (Bareford and Swaan, 2007, Cosker and Segal, 2014). However, the mechanism of IONP internalization by Neurons is still poorly understood. The high levels of endocytic activity in astrocytes in line with their homeostatic functions in the nervous system, results in documented ‘competitive uptake dynamics’ for nanoparticle uptake, which in co-cultures would limit neuronal transfection (Jenkins et al., 2013b, Jenkins et al., 2016, Jenkins et al., 2015). As discussed previously (please see Chapter 3), specific cell culture conditions were chosen to facilitate pure neuronal cultures i.e. that would limit astrocyte numbers and proliferation. To summarise, (i) by deriving cultures from embryonic tissue, astrocyte numbers can be kept low, as it is estimated that > 90% of cells at this developmental time point are neurons (Murphy, 1990, Bandeira et al., 2009). (ii), the use of serum free medium can enhance the purity of neuronal cultures by reducing astrocyte proliferation and provide greater definition of experimental conditions by removing confounding variation in serum composition (Langan et al., 1994, Evans et al., 1998) and therefore, eliminates the need to use chemical inhibitors of astrocytes such as arabinosylcytosine C (AraC) (Geller et al., 2001, Ahlemeyer et al., 2003). Nevertheless, in our hands it proved difficult to fully eliminate astrocyte contamination; approximately 35% of the astrocyte population can re-enter the
cell cycle after serum deprivation, likely accounting for the high proportions of astrocytes in these cultures (Murphy, 1990).

With respect to the safety of the two oscillating magnetic fields examined, the results indicated that using uniaxial magnetic field was a safe technique for transfecting primary cortical cells. This agrees with other studies conducted for primary neurons (Subramanian et al., 2017) and neurons derived from stem cells (Fernandes and Chari, 2014) in addition to variable cell types has been reported (Pickard and Chari, 2010a, Adams et al., 2013, Oral et al., 2015).

The new biaxial magnetic field did not enhance transfection efficiency. Furthermore, it displayed low levels of safety for transfecting primary cortical cells. The mechanism behind the inefficiency and lack of safety necessitates further study. The mechanism by which oscillating fields produce high transfection efficiency is not fully understood. However, the theory behind it is that the IONPs gain lateral motion resulting from oscillating magnetic fields (McBain et al., 2008). This motion transfers to the IONPs across the cell membrane, or leads to vibration of membrane-bound particles in order to facilitate internalization of IONPs, and/or may mechanically stimulate endocytosis (McBain et al., 2008, Pickard and Chari, 2010a, Adams et al., 2013). Accordingly, the author’s assumption here is that the exposure to additional lateral motion in the orthogonal direction (biaxial), may have impacted on the cell membrane integrity or caused cell detachment due to the physical stress that might result from the rapid sedimentation of IONPs-gene complexes.

In summary, we provide the first complementary electrophysiological and histological analyses supporting the concept that iron oxide nanoparticles and
uniaxial applied magnetic fields can be safely deployed for genetic modification of primary neurons for basic research and translational applications. However, further investigation should be directed towards more mature cells and to track changes post-transfection over more extended time periods.
Chapter 5

Hydrogels are a promising neuromimetic substrate for primary cortical cells
5.1. Introduction
The two disciplines, Neuron transplantation in regenerative medicine and neurons in basic research, have challenges that require the development and utilization of neuronal 3-dimensional constructs. This emerging strategy is vital for developing therapeutic and research applications.

On the one hand it is difficult to transplant post-mitotic neurons, which are generated in a 2D environment (Kondziolka et al., 2004), so the 3D environment is vital for overcoming the limitations that hinder the integral recovery process post-transplantation, and provide support and protection of transplant cells during the delivery into the host parenchyma. Further, it has been reported that neurite outgrowth and electrical signals of human pluripotent stem cell-derived neurons in scaffolds were improved by gene engineering them (Carlson et al., 2016). Thus, genetically engineering primary neurons maybe more beneficial as they are more relevant to the host environment. These efforts have focused on reducing the drawbacks of mechanical cell damage during delivery into the host tissue (Kondziolka et al., 2004).

On the other hand, most laboratories are conducting biological experiments to investigate neuronal survival, neurite outgrowth, network formation, synaptogenesis and functionality using monolayer cultures on 2D polystyrene or glass substrates (Flanagan et al., 2002, Ould-yahoui et al., 2009). The nature of cell attachment in 2D monolayer cultures is ‘one sided’, i.e. one side of the cells attach to the hard substrate and the other side faces the medium. Thus, 2D culture is un-representative of in vivo physiological conditions as the hard substrate is not representative of the extracellular matrix. In turn, cellular response in 2D, such as receptor expression, cell polarization, transcriptional
expression, cellular migration, and apoptosis, differ from that in the original in vivo environment (Khoruzhenko, 2011). Furthermore, 2D monolayer culture is very primitive regarding the anatomy, gene and protein expression, and diffusion of soluble molecules such as nutrients and growth factors (Smalley et al., 2006, Pampaloni et al., 2007).

It is also worth mentioning that animal model responses cannot fully mimic or predict human response, and are costly, time-consuming and ethically arguable (Sala et al., 2013). Therefore in order to reduce the use of animal models for biological testing purposes, it is essential to develop 3D cell culture models. This culture should possess, as far as possible, the features of the in vivo environment from an anatomical, morphological and physiological perspective. 3D animal models are considered to be a preliminary step to lay the foundations for a 3D model of human cells that can be usable in clinical trials, and to bridge the gap between 2D cell culture and animal models for basic research.

Several parameters need to be considered including the choice of material for the scaffold, the source of the cells, the formation method, and the design of the scaffold which are all crucial for recreating the in vivo environment (Pampaloni et al., 2007). Further, the scaffold should possess biophysical, biomechanical and biochemical cues that facilitate cellular proliferation, differentiation, maintenance and function (Dutta and Dutta, 2009). In this regard, it should be noted that electrophysiological responses of primary embryonic neurons to 3D hydrogel constructs currently has not been widely studied.
5.1.1. The suitability of collagen-based hydrogels as a neuromimetic substrate

The ECM is a net-like structure, consisting of amino acid and sugar-based macromolecules (i.e. proteins and glycoproteins). Its importance lies in supporting the physical adhesion of cells, acting as a biological scaffold, and controlling biomolecule diffusion such as nutrients and growth factors (Vecino and Kwok, 2016). As well biomechanical traits of the CNS are influenced by ECM composition and mechanical properties, and any defect in these two factors leads to loss of the regenerative capacity of CNS as this process is connected to the physiochemical property of the ECM (Haycock, 2011, Vecino and Kwok, 2016). Therefore, choosing a polymer scaffold is a critical step in assembling hydrogels that mimic the ECM found in tissues. Additionally, the nature of the polymer utilized for 3D matrix formation is a significant determinant of cell responses (O’Brien and Lummis, 2011, Baker and Chen, 2012).

Various polymer types are used in research and clinical applications (Liu and Ma, 2004, Karageorgiou and Kaplan, 2005). Synthetic polymers widely used in medical applications include poly-lactic acid (PLA), poly-glycolic acid (PGA), and copolymers (PLGA); however, there are limitations and restrictions on their use in these applications. Using a synthetic polymer in tissue transplantation has drawbacks including the need to create a relatively large incision to deliver the scaffold into the target site due to its mechanical properties (Lee and Mooney, 2001) and the risk of rejection. They can also be the causing factor for necrotic cell death due to degradation processes with polymers such as PGA being degraded by hydrolysis that results in carbon dioxide production and leads to a reduction in local pH causing cell death (O’Brien and Lummis, 2011). In order to
overcome these drawbacks, an alternative injectable polymer has been used. Collagen is a biologically-derived polymer as it is the main component of the extracellular matrix, and the most abundant protein in mammalian tissues (Drury and Mooney, 2003). It is considered optimal for developing *in vitro* models for the following reasons: (i) it can be modulated in response to ionic strength or temperature (Mahoney and Anseth, 2006), (ii) it can be injected into the body using a minimally invasive approach, (iii) it possesses low antigenicity, excellent biocompatibility, and can be degraded by collagenases and serine proteases facilitating its local degradation by the cells in the engineered tissue (Chevallay and Herbage, 2000, Han et al., 2010, Drury and Mooney, 2003). Its chemical structure, molecular architecture and morphology validated its use in medical and biological applications, in particular, the crosslinked form of hydrophilic polymers, and it is considered a biodegradable scaffold (Jhon and Andrade, 1973). Collagen hydrogels have many characteristics that mimic the ECM to facilitate cellular infiltration and nutrient transport. Moreover, hydrogels have an affinity to water without dissolving in it because of their chemical and physical cross-linked network (Langer and Peppas, 2003). They are highly porous biomaterials that allow the cells to grow and develop into tissue (Smalley et al., 2006). Collagen hydrogels have been used for regenerating various tissues such as liver, skin, blood vessels, bone (Peppas et al., 2006, Wang and Stegemann, 2010) and for spinal cord regeneration (Han et al., 2010). Additionally, it is utilized as a scaffold for generating neurons in a 3D environment (see section 1.14).

In general, extrinsic signals from the ECM have an impact on cell characteristics, both morphological and functional. Several studies demonstrated the influence of environmental stimuli on the functions of various cell types (Kiryushko et al.,
2004, Schindler et al., 2006). Accordingly, it has been reported that the cell behaviour in 3D constructs is more representative of the *in vivo* environment than cells cultured in 2D monolayers (Schindler et al., 2006). For example, voltage gated calcium channel (VGCC) function was examined in 2D and 3D cultures of superior cervical ganglion neurons and cells in the freshly dissected tissue. Cultures were exposed to high K\(^+\) to compare the increment in Ca\(^+\) concentration amongst these three models, concerning intracellular calcium increase in response to high K\(^+\) depolarization. The findings demonstrated that the calcium increases were identical for 3D-cultured and freshly dissected tissue, but significantly higher for 2D-cultured cells (Lai et al., 2012). In this context, the axonal growth cone of neurons has importance in axonal growth by sensing extracellular environment signals via integrin (a transmembrane protein) that transduces mechanical stimuli from the surrounding environment to the cytoskeleton. This stimulus results in changes in gene expression, in turn influencing the functional properties of neurons such as differentiation, migration, and survival (Li and Gundersen, 2008, Witte and Bradke, 2008). Consequently, extracellular mechanical properties are a critical determinant of cellular responses (Discher et al., 2005). To that end few studies have tested the electrophysiological properties of neurons in soft substrates.

The main aim of the research reported in this chapter was to develop a 3D neuronal construct within a hydrogel and test its safety via cellular viability and, specifically, neuronal functionality using the patch clamp technique on single cells. This 3D construct can be used for basic research studies and for transplantation of Neurons. Developing this model is considered a preliminary step before genetically engineering cortical neurons in 3D hydrogels where it will
be a vital tool with applications in regenerative medicine. In this respect, several challenges needed to be faced regarding developing such system.

The first challenge is adjusting the hydrogel stiffness as a tight matrix leads to cell death due to (i) inhomogeneity of the cells, (ii) lack of oxygen and nutrients penetrating throughout the matrix and removing waste products from within the hydrogel (Mertens et al., 2014) which results in necrotic cell death (Malda et al., 2004). The second challenge is resolving inhomogeneity within the cell graft following delivery (Pearse et al., 2007) which is considered to be one of the challenges within a 3-dimensional construct (Unsworth et al., 2003). Cellular inhomogeneity results in cell death which, in turn, inhibits cellular network functionalization and electrical conduction to the regenerative environment (Wakatsuki and Elson, 2002). The third challenge is studying neuron conductivity in a 3-dimensional environment (Xu et al., 2009) as delivering functional neurons is a major challenge for the regenerative environment. In order to meet these challenges, the objectives of this study were set as in the next section.
5.2. Objectives
Type I collagen gels were used to develop primary cortical neuron culture that mimics the *in vivo* environment in order to be used as a model for conducting biological studies and as a potential delivery system for neurons of the host tissue.

Therefore, the specific objectives of this chapter are:

1- Determine the feasibility of growing primary cortical neurons in a type I collagen hydrogel in two-dimensional (2D) cell monolayers.

2- Determine the capability of maintaining cortical cells within a complex three-dimensional (3D) microenvironment.

3- Form an evenly distributed cellular system in a 3D construct.

4- Examine the safety of using collagen hydrogels as the 3D microenvironment for supporting cortical cells growth.

5- Study the effect of collagen gel and the 3D microenvironment on the electrophysiological properties of neurons.

6- Genetically engineer primary cortical cells in a 3D construct.
5.3. Experimental procedure and analysis

Using collagen hydrogel as a substrate for neuronal cultures whither the culture was in the format of 2D monolayer or 3D construct described in (section 2.6). Immunostaining procedure detailed in (section 2.8.), while the safety assessment including cell viability has been described in (section 2.9) and neurons signalling and functionality in (section 2.11.2).

5.4. Results

5.4.1. Collagen as a substrate for growing cortical cells as a 2D monolayer

The feasibility of growing cortical cells as a monolayer when collagen was utilized as a coating substrate was compared to cells produced as a monolayer on a coverslip coated with PDL-Laminin (see Chapter Three).

The growth of cortical cells seeded on a coverslip coated with a collagen hydrogel was comparable regarding reproducibility and feasibility to cells seeded as a monolayer on coverslips coated with PDL-Laminin. Cell morphology in fluorescent micrographs revealed that neurons grew for 7DIV on a glass coverslip coated with collagen and demonstrated similar morphological characteristics to those grown on PDL-Laminin substrate. Although the morphology of neuron body was typical in both models, the neurites were more tangled for neurons grown on collagen compared to the neurons grown on PDL-Laminin coated coverslip.

Cortical cells on collagen coated coverslips were unevenly distributed. In general, the cells were concentrated in the centre and a few were spread on the edges
(sides) of the coverslips regardless of the protocol used for seeding (Figure 5.1 A & B) (Materials and methods figure 2.3).

Astrocytes displayed an alteration in their morphology and exhibited a small and multi-branched cell body, unlike typical astrocytic morphology in a 2D monolayer culture which showed a flattened membranous shape (Figure 5.1C & D).
Figure 5.1: Collagen hydrogel supports primary cortical cell growth as a 2D monolayer. Phase contrast images show cortical neurons at 7DIV dispersed as individual cells with extended long processes at the side of the coverslip (A), clusters of neurons and astrocytes were concentrated at the centre of the coverslip displaying maturity characteristics of neurons via high network complexity level (phase contrast image B and fluorescent image D), (C) Fluorescent image revealed that astrocytes possess asymmetric characteristics compared to typical astrocytes in a 2D monolayer culture and exhibit small soma in the form of branches instead of the typical flat shape.
**5.4.2. Cell viability on 2D collagen coated coverslips**

Statistical analysis demonstrated that the use of collagen hydrogel as coating substrate for monolayer culture was supportive of primary cortical cell survival. At day 7, there was no impact on the percentage of viable cells in comparison to 3DIV (73.7 % ± 3, 77.3 % ± 8.4, respectively) (Figure 5.2 A and B).

![Figure 5.2: Image showing viable cells stained with Calcein (green) and red arrows pointing to dead cells stained with Ethidium homodimer (A). (B) shows around 80% of cells were viable and no significant differences between 3 and 7 days of growth in vitro. (n=3)](image-url)
5.4.3. Collagen as substrate for growing cortical cells as a 3D construct

5. 4.3.1. Surface model

After 3 days of culture the percentage of cell survival was ca. 90 %. At day 7 it was slightly reduced to ca. 80 % (Figure 5.3 A & E). The volume of gel construct was (~640 µm) at both time pointes. The morphological features of neurons cultured in the 3D surface model was not comparable to the neurons grown on the 2D monolayer. At day 7, the neurons were closer to a spherical shape in comparison to those in 2D culture (Figure 5.3 D). In addition, neurites that extended from the cell body were highly tangled to the level that it was difficult to recognize their origin and directions. Astrocytes were similar in their morphology in 2D monolayer culture when collagen was the coating substrate. From the observations of cell localization, the fluorescent micrographs showed some of the cells penetrated 10-20 µm depth into construct (Figure 5.3 B & C).
Figure 5.3: Three dimensional appearance of cortical cells grown on the surface of a collagen hydrogel construct. (A) Bar chart exhibiting the high viability of cortical cells at the early (3DIV) and later (7DIV) incubation time points. (B) Cortical cells stained with calcein and ethidium homodime show high viability level. (C) Astrocytes distributed within a 3D construct and formed multi processed cells (neurons clumped together with extended long processes forming a complex network. (D) the cell body of neurons has a spherical morphology. (n=3)
5.4.3.2. **Entrapped model**

5.4.3.2.1. **Collagen gel concentration testing**

Three collagen concentrations (0.4, 1 and 2 mg/ml) were examined. The hydrogel with the lowest concentration was semi liquid and cells sank to the bottom of the gel. This resulted in the cells attaching onto the coverslip and growing as a monolayer (Figure 5.4 A). In contrast, the highest concentration tested (2mg/ml) exhibited a high stiffness level, judged by observing the differences in the hardness of the gel across the three concentrations and its motility during handling, and cells aggregated in different areas, forming clusters. This caused difficulty in cell type recognition and counting. Neurons produced few, short processes versus the usual length seen 7DIV (Figure 5.4 B). The concentration 1 mg/ml was the most suitable for cells to grow and disperse through all layers of the gel construct (Figure 5.5 A, B, C, and D).

![Image](image_url)

**Figure 5.4:** Collagen construct concentration affects cell distribution through the gel. Phase contrast images shows (A) the cells sink to the bottom in the low concentration gel (0.4mg/ml) with the red arrow pointing to the side of coverslip. (B) At a gel concentration of 2mg/ml, cells aggregated and formed clusters 7DIV.
5.4.3.2.2. 3D construct purity and cell distribution at three and seven days in vitro

Z-stack images captured throughout the gel construct at both the early time point (3DIV) and the later incubation time point (7DIV) revealed that the cells distributed homogenously throughout the gel construct.

Statistical analysis showed no significant differences in the percentages of both neurons and astrocytes at the side and the centre of each layer of the hydrogel, whether these cells were incubated for 3DIV or 7DIV. This indicated the high purity of the 3D construct. (Figure 5.5 E). Around 90% of cells were neurons throughout the depth of the gel and at each of the side and the centre of the gel, while astrocytes were rarely seen compared to neurons (Figure 5.5 E). Remaining cells where un-identified and did not stain with Tuj-1 or GFAP antibodies (i.e. Tuj-1^-/GFAP^-).

Morphological characterization revealed that neurons possessed their typical morphology. However, very few neurites were extended with no evidence of network formation (Figure 5.5 A-D) in contrast to counterpart neurons in a 2D monolayer culture.

At seven days incubation, neurons still made up a large proportion of the cell population despite the reduction in their percentage to Ca. 70%. Astrocytes displayed an increase in number to 30%. This elevation in astrocyte percentage results in a reduction of purity. There were no significant differences between the percentages of neurons and astrocytes across all layers, except the middle centre (MC) layer (Figure 5.6 E).

Morphologically neurons, after 7 days in culture, displayed characteristics of more mature cells as the neurites were more elongated than neurites of neurons
incubated for 3 days. These penetrated through the layers of the gel forming a complex network with the neighbour neurons. Astrocytes also were more mature and displayed a multi branched cell body (Figure 5.6 A-E).
Figure 5.5: Highly pure and homogenous neuronal 3D hydrogel construct at 3 days in vitro. Fluorescent z-stack image series throughout the collagen hydrogel construct (distance between each image 10 µm) were divided into 3 groups: top of gel (upper layer), the middle of the gel (middle layer) and the bottom of the gel (lower layer). (A-D) Cortical cells were distributed equally through the depth of the gel. (E) Bar chart shows the high purity of neurons in the 3D hydrogel construct (P=0.0001). (n=3).
Figure 5.6: Pure and homogenous neuronal 3D hydrogel construct at 7 days *in vitro*. Fluorescent z-stack image series throughout a collagen hydrogel construct (distance between each image 10 µm), divided into 3 groups: top of gel (upper layer), the middle of the gel (middle layer) and the bottom of the gel (lower layer). (A-D) Cortical cells were distributed almost equally through the depth of the gel. (E) Bar chart shows the purity of neurons in the 3D hydrogel construct. (n=3) The hydrogel construct is displayed in a supplementary video in the supplied disc.
5.4.3.2.3. Cortical cell distribution across the core and the extremity of the gel

Quantification of cells across the width of the gel at 7 DIV demonstrated that the percentage of neurons at the sides was 93% and 96% at the core of the construct. In contrast, the percentage of astrocytes at the sides was 4% and 2% at the core. These findings demonstrated that the 3D construct was pure and highly homogenous, i.e. there was no effect of incubation time on the purity and homogeneity of the culture (Figure 5.7).
Figure 5.7: Cortical cells distributed homogenously across the gel. (A & B) Selective images of the core and side of the gel matrix at 7 DIV demonstrate the homogeneity and the cell purity of the gel construct. (C & D) Bar chart displaying quantification of cell percentage in the extremities and the core of the gel, percentages of astrocytes and neurons across the gel. (E) Schematic illustrates the image localization and quantification process: cells in the depth of the two sides of the gel were counted and averaged then compared cells in the core of the gel. n=3
5.4.3. Assessment of cellular viability of cortical cells in the 3D environment

The percentage of viable cells at 7 DIV were declined to about 70 % comparing to their percentage at 3DIV which was about 95% (Figure 5.8 A)&(Figure 5.9 A-C). Across all the layers and the depth of the gel, the average percentage of pyknotic cells was about 20 % at both 3 and 7 days (Figure 5.8 B) & (Figure 5.9 D & E).

**Figure 5.8: Cortical cells exhibited high survival within the 3-dimensional environment.** Bar charts represent cell health throughout the gel construct. Viable cell quantification was conducted on two sides (s) of the gel and at the centre (c) going down through the depth of the gel starting from upper layer down to the base lower layer of the gel (A). LIVE/DEAD assays show a high proportion of viable cells at 3 days post seeding in a 3D collagen hydrogel (blue bars), then cell survival declined at 7 days post gel construct setting (red bars). (B) Pyknotic nuclei were quantified in the same manner as in the cell viability quantification process and show low percentages of pyknotic cells at both time points.
Figure 5.9: Collagen hydrogels support cortical cell survival in a 3-dimensional environment. Fluorescent micrographs show dead cells stained with Ethidium homodimer (red) (A) and viable cells stained with Calcein (green) (B). Merged micrographs (C) reveal a high proportion of viable cells at 3 days post seeding in a 3D collagen hydrogel. D and E show examples of pyknotic nuclei indicated by white arrows, and some healthy neurons, stained with neuronal antibody Tuj-1, with extended long processes through the depth of the collagen construct. The hydrogel construct is demonstrated in a supplementary video in the supplied disc.
5.4.4. Patch-clamp technique for evaluating the safety of the collagen hydrogel protocol

To investigate the feasibility of our 3D construct hydrogel, it was important to determine the impact of the hydrogel upon neuron signalling.

Some technical modifications were required to facilitate the recording from the individual neuron (see Methods). These included: (i) angling the surface of the hydrogel, i.e. the plane of focus, to allow the observation and recording from neurons (Figure 5.10 A), (ii) adding fluorescein dye to the patch pipette which permitted precise identification of individual neuron bodies within a clump of cells (Figure 5.10 A), and, in some cases, neuronal processes were labelled in addition to the cell body (Figure 5.10 B).

During the process of whole-cell recording, occasionally the soma appeared to swell several minutes into recording (Figure 5.10 B). However, this did not impact on the recording process or the results.

The results of patch-clamp recording revealed that signalling in neurons grown on the surface of the hydrogel (Figure 5.11 E) were comparable to earlier recordings from primary cortical neurons grown on glass coverslips (Evans et al., 2017). The step depolarisations in voltage clamp resulted in a biphasic appearance of the current which was due to the sequential activation of voltage-gated Na⁺ and K⁺ currents, resulting from a fast inward (Na⁺) current followed by a maintained outward (K⁺) current (Figure 5.11 A, B). The potential was held at -70 mV, so the inward Na⁺ current was the first potential observed at -50 mV then reached -20 mV where the maximum amplitude was recorded (Figure 9.11 D), showing rapid activation, i.e. within 1 ms at -20 mV (Figure 5.11 B). Blocking Na⁺ channels with
Tetrodotoxin revealed that the current was as expected for Na$^+$ ions, i.e. the amplitude of this current became smaller at voltages positive to -20 mV.

This reduction in size of the current can be attributed to the joint effect of activation of the K$^+$ current and the reduction in the driving force of Na$^+$ influx (Figure 5.11 D). At about -30 mV, the outward K$^+$ current started to activate (Figure 5.11 D) and was maintained throughout the step, often with a slight decline (Figure 5.11 A).

On return to -70 mV, the outward tail currents returned rapidly to baseline (Figure 5.11 C). We examined the effect of voltage on Na$^+$ current amplitude by using the pre-pulse protocol (see Methods). Na$^+$ currents were strongest following a pre-pulse to -90 mV (at more negative voltages they saturated). At -70 mV they were approximately 90% of their maximum amplitude.

In the current clamp, hyperpolarising current injections revealed transient depolarisations which presumably arose from excitatory synaptic activity (Figure 5.11 E). These depolarisations usually did not elicit action potentials. Action potentials were evoked on return to the resting potential following cessation of the current injection (Figure 5.11 E and F). The amplitude of the action potentials was measured as the difference between the peak and the baseline (resting potential). It was typically around 30 mV and was dependent on the extent of the preceding hyperpolarisation (Figure 5.11 F), declining steeply positive to -70 mV (Figure 5.11 G). This decline is due to the increasing steady-state inactivation of the Na$^+$ channels as the membrane potential becomes more positive. The slight decline in amplitude at the most negative voltages (> -85 mV) probably results from a longer transition time to threshold for action potential initiation in this voltage range.
(Figure 5.11 F) allowing a greater fraction of the channels to inactivate before the membrane reaches threshold. The threshold value was not systematically investigated but was close to -50 mV (Figure 5.11 F).

The Na$^+$ and K$^+$ current amplitude and membrane potential (zero current potential) of voltage-clamped neurons grown on the surface of hydrogels are shown in Table 5.1. For comparison, equivalent measurements from cells cultured on glass coverslips taken from a subset of neurons included in an earlier study (Evans et al., 2017) are also shown. These data included neurons transfected with GFP using magnetic nanoparticles and their controls (non-transfected neurons). These neurons are included here as one group as there were no significant differences between sub-groups (transfected and non-transfected). We found that there was a significant difference in the size of the Na$^+$ currents between the two groups (hydrogels versus glass, Mann-Whitney U test, $U = 17.0, p = 0.002$). Other measured parameters (K$^+$ current at 0 mV and membrane potential at zero current) were not significantly different (K$^+$ current by Mann-Whitney U test, $U = 46.0, p = 0.215$; membrane potential by Student’s t-test, $p = 0.058$). The ratio of median currents (hydrogel/glass) was 6.4 and 1.2 for Na$^+$ currents and K$^+$ currents respectively. When data was randomly excluded from the larger hydrogel group to ensure equal group sizes ($n = 8$, see Methods), the ratios were 5.8 and 1.3 respectively. This manipulation did not alter any of the conclusions concerning the significance of differences between the two groups.
Figure 5.10: Recording from cortical neurones on the surface of a hydrogel. Examples of high (A) and low densities (B) of neurons within the culture. A transmitted light image is shown (left) together with a fluorescence image of the same field (right). A fluorescein dye was added to the pipette.
Figure 5.11: Voltage-dependent Na\(^+\) and K\(^+\) currents, and membrane potential recordings, in primary cortical neurons grown on a hydrogel. (A) Whole cell currents recorded in voltage clamp (bottom) in response to positive and negative voltage steps (top). Holding potential -70 mV. Horizontal bar indicates the section of the recording shown in B. (B) Leak-subtracted records shown on fast time scale (bottom) together with associated positive voltage steps (top). The inset shows the block of the Na\(^+\) current (red) by a short (0.65s) application of 2 µM TTX (black). Scale 0.5 nA, 5 ms. The voltage step from -70 mV to -30 mV is also shown. (C) Tail currents (leak-subtracted) at -70 mV following a step to 0 mV. The time constants to the double exponential fit were 0.13 ms and 1.45 ms. (D) I-V curve. Currents measured at 40 ms after the beginning of the voltage step (K\(^+\) currents), from records shown in A (black squares). Maximum inward currents measured within 2 ms following the start of the voltage step, from records shown in B (red squares). E. Current clamp records (bottom) in response to hyperpolarising current injections (top). Holding current = 0 pA. The horizontal bar indicates the section of the records shown in F on a faster time scale. F. Action potentials evoked by depolarization from a hyperpolarised voltage. The current injection stops at 0.85s. Same records as D. G. Graph showing action potential amplitude against membrane potential measured close to the end of the hyperpolarising step. The red line shows a linear fit to the data between -68 mV and -54 mV (gradient = -1.66 mV/mV). All data collected from the same neuron with the exception of the TTX experiment (B, inset).
Table 5.1: Amplitudes of Na\(^+\) and K\(^+\) currents, and membrane potential, from neurons grown on hydrogels. The holding potential was -70 mV. Currents were measured at -30 mV and at 0 mV for Na\(^+\) currents and K\(^+\) currents respectively. The Na\(^+\) current was measured at the peak of the transient inward current. The K\(^+\) current was measured towards the end of the 50 ms voltage step. Values quoted to nearest whole number.

<table>
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<th>Mean</th>
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<td>(I_{Na}) at -30 mV (pA)</td>
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<td>738</td>
<td>1593</td>
</tr>
<tr>
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<td>800</td>
<td>1051</td>
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<td>EM (mV)</td>
<td>-29</td>
<td>-26</td>
<td>16</td>
</tr>
</tbody>
</table>
5.4.5. Genetic engineering of neurons on the surface of the hydrogel construct

These proof of concept experiments have been conducted twice to show that it is possible to genetically engineer a cortical cell culture in a soft substrate.

Microscopic observations revealed that it is possible to genetically engineer neurons when they are seeded on the surface of a hydrogel construct. Neurons expressed GFP however the transfection efficiency was estimated to be less than 2%. The observations also revealed that neurons possess typical morphology as previously described in chapter 3 (Figure 5.12).

Figure 5.12: Magnetofection based transfection of neurons seeded on the surface of the gel at 7DIV. (a) Neurons are in the centre of the gel construct. (b) Neurons expressing GFP after transfection were seeded on the surface of the hydrogel construct and transfected by applying an uniaxial magnet for 30 min then incubated for 24hr post transfection. (n=2)
5.4.6. Genetically engineering neurons within hydrogel construct

Observation of Z-stack images revealed that the magnetic plasmid–IONPs complexes can penetrate inside the hydrogel construct and transflect the cortical cells in the different layers of the gel construct. Neurons displayed a healthy, typical morphology. Astrocytes also showed a healthy morphology however tended to have the flat shape which is similar to the 2D culture. Figure 5.13 shows evidence of transfection of cortical cells at the three different layers of the hydrogel construct.
Figure 5.13: Genetically engineered cortical cells in a 3-dimensional hydrogel construct. Series of z-stack images through the 3 layers of 3 different constructs of hydrogel (described as gel 1, gel 2 and gel 3). Hydrogel constructs were lifted from the coverslip and flipped over on to thin rectangle coverslip to facilitate the imaging process. Gel 1 shows the transfected neurons localized in the bottom layer of the hydrogel construct. Gel 2 demonstrates that the neurons in the middle of the hydrogel can be transfected while gel 3 shows transfected astrocytes localized on the surface of the hydrogel construct. Gels were stained with a – Tuj-1, b – DAPI and c – anti GFP. Channels were merged to produce D. You can see the hydrogel construct in a supplementary video in the supplied disc.
5. 5. Discussion
The results show it is possible to deliver neurons into a gel and form an in vitro model that mimics the in vivo environment as a platform for scientific research. To that extent, development of such a system required overcoming several technical challenges. This includes assessing the suitability of collagen I as a 3-D substrate for growing primary cortical cells and determining the functionality of the neurons via signal recording. Herein these challenges were addressed.

Growth of primary cortical cells in 2 and 3D constructs within collagen revealed that collagen provides an optimal substrate. These findings agreed with other studies that showed collagen enhanced cell survival and promoted growth (Carbonetto et al., 1983, Flanagan et al., 2002, Cullen et al., 2007b). However, it was observed that there is an issue related to cell distribution on a 2D collagen substrate, where cells were concentrated in the centre of the coverslip with only a few on the edges. In turn, neuronal network complexity varied according to cellular localization (i.e. the network was more complicated at the centre of the coverslip compared to the edges). The assumption was cell localization was affected by the nature of the distribution of collagen over the coverslip. The second challenge was obtaining a homogenous 3D construct, i.e. homogenously distributed cells throughout a hydrogel. 1mg/ml was shown to be the optimal concentration of collagen and, in agreement with other studies that tested lower collagen concentrations (0.4 and 0.5 mg/ml), cells settled close to the base of the hydrogel construct (O'Connor et al., 2001).

There was a fundamental impact on the morphological characteristics of cortical cells (neurons and astrocytes) grown on collagen hydrogels dependent upon whether they were grown in 2D culture or a 3D construct. This finding
demonstrated there was a modulation in cell morphology, where the soma of the neurons were more rounded with more elaborate extensions. Inversely, astrocytes displayed similar morphology, i.e. a small cell soma, stellate morphology with a diffuse network of fine extensive processes, to the typical flattened astrocytic morphology in glass 2D monolayer culture (Balasubramanian et al., 2016). Such modulation may relate to the variation in the extracellular mechanical properties of the collagen substrate (Cullen et al., 2007b). The cross-linking of hydrogel nanofibers are very important for maintaining their phenotypic shape and natural behaviour as in the in vivo environment by tethering the external nanofibers to the cellular cytoskeleton (Stevens and George, 2005). Accordingly, they possess the potential to mimic in vivo counterparts. These findings agree with other studies that demonstrated the variation in cellular morphology in 2D vs 3D microenvironments regardless the type of the 3D construct (Dillon et al., 1998, Irons et al., 2008, Xu et al., 2009, Balasubramanian et al., 2016, Tickle, 2017).

One of obstacles in cell transplantation is low cell survival which can be the cause of inhomogeneity of the grafted cells (Pearse et al., 2007). This study offers a promising 3D construct model that demonstrated high survival rate up to 90% that remained consistently high across the time course studied.

The success of forming transplantable neuronal 3D constructs relies on three major key factors; (i) cell survival, (ii) cell homogeneity, and (iii) the possibility of neurons assembling functional circuitry. The finding of this study demonstrated the feasibility of growing neurons on 2D and homogenous 3D collagen hydrogels in addition to the capability of neurons to form a functional network associated
with evidence of cellular communication. These findings agreed with findings of Tao Xu and his colleagues (Xu et al., 2009).

Ensuring the safety of our protocols was a major concern; therefore, to provide a complete picture on the safety of our 3D construct model, an electrophysiological study was conducted analysing surface seeded neurons using the whole-cell recording technique. Patching neurons on collagen gels presents several challenges, mainly that the patch electrode does not adequately penetrate the collagen matrix making it necessary to patch cells located at or near the surface of the gel (see Material and Method section).

A simple comparison was made between signals taken from 3D construct model (chapter 5) and neurons cultured on hard glass surfaces (chapter 4). Voltage-dependent currents appeared to be similar in both models however the size of the Na\textsuperscript{+} current was approximately 6 times larger in the hydrogel group, when comparing median values, at -30 mV. Medians provided an appropriate comparison given the non-normal distribution of the Na\textsuperscript{+} and K\textsuperscript{+} current data. Our findings related to Na\textsuperscript{+} current measurements in 3D constructs were similar to those reported in a number of studies using cortical brain slices in rodents. In early postnatal rats (0-4 days postnatal), peak Na\textsuperscript{+} currents were typically 0.5 – 0.7 nA (Luhmann et al., 2000). Bahrey et al., (2003) reported that the size of Na\textsuperscript{+} currents in mouse cortical neurons increase during the developing brain from around 0.3 nA at E18 to 0.8 nA at P6 (Bahrey and Moody, 2003). The results obtained in 3D hydrogel cultures, compared to 2D cultures, show a much larger amplitude change in the Na\textsuperscript{+} current compared to the K\textsuperscript{+} current. This might be due to acceleration in cell development within the 3D construct. This corresponds to a recent study where neurons developed at an accelerated rate in 3D
compared to 2D cell culture systems (Zhang et al., 2016). In voltage-dependent 
Na$^+$ and K$^+$ currents, neurons from the 3D hydrogel group had developed both 
Na$^+$ and K$^+$ currents by 3 weeks post-differentiation, whereas neurons from the 
2D group had a K$^+$ current but only a negligible Na$^+$ current (Zhang et al., 2016). 
An acceleration in development over this developmental period in 3D hydrogel 
cultures compared to 2D could explain the results described here. Further work, 
however, would be required to confirm how closely this represents the natural 
development of these currents in vivo. The reason behind the increased size of 
the Na$^+$ current is unclear. Most likely it relates to the presence of a higher 
number of Na$^+$ channels present in the membrane. In relation to channel 
numbers, and in particular to channel density, it is worth noting that L-type 
voltage-gated Ca$^{2+}$ channels have been reported to concentrate into lipid rafts in 
2D but not in 3D primary neuron culture from the superior cervical ganglion (Lai et 
al., 2012). This clustering of Ca$^{2+}$ channels produced a larger Ca$^{2+}$ increase in 2D 
cultured neurons in response to depolarisation by high external K$^+$, while 3D 
neuronal cultures provided a similar, smaller response to that found in native 
neurons. While this is the opposite result to that found here for Na$^+$ currents, it 
hints that the underlying mechanism may depend on the interaction of the 
neurons with the substrate and the effect this has on membrane architecture and 
properties (Lai et al., 2012). In the case of 2D cell culture, all cells are in contact 
with a hard substrate, usually plastic or glass, whereas in 3D cell culture the 
substrate is generally softer and, additionally, there is likely to be less contact with 
the substrate. Therefore contact with hard surfaces in cell culture systems could 
influence the membrane and subsequent distribution of ion channel proteins may 
alter, positively or negatively, channel-dependent ion flow across the membrane.
There did not appear to be a simple relationship between the size of the Na\(^+\) current and the chances of evoking action potentials in a neuron or even the size of the action potentials. The action potentials evoked in this study were relatively small (<30 mV) and they occurred as a single spike, as often found in immature neurons (McCormick and Prince, 1987) Within the hydrogel group, neurons with large Na\(^+\) currents (e.g. > 0.5 nA) invariably did not produce action potentials at the cells resting potential. The value of the resting potential appeared to be the key determinant influencing spiking since action potentials were not seen in cells with resting potentials greater than -35 mV which may reflect the extent of Na\(^+\) channel inactivation at depolarised voltages.

The presence of spontaneous membrane depolarisations in some recordings indicated that the neurons had formed synaptic connections. Other similar studies have also reported synaptic activity in 3D cultured neurons and spiking activity (Irons et al., 2008, Xu et al., 2009), although in these cases synaptic activity was observed after longer times in culture (21 and 14 DIV respectively). Morphological evidence of synaptic contact was also reported (Irons et al., 2008). We also observed synaptic activity in our 2D neuronal cultures (Evans et al., 2017), an unsurprising result in view of the complexity of the neurite outgrowth in both culture systems, however the other studies did not report synaptic activity in their 2D cultures (Xu et al., 2009). These authors recorded from E18 hippocampal neurones after 7 days (and other time points) within the hydrogel, whereas we confined our study to neurons on the surface of the hydrogel, but at an equivalent developmental age (7 DIV). Their values for mean Na\(^+\) current amplitude (2.6 nA for their hydrogel data), although larger than ours, could be attributed to the different neuronal type and more negative holding potential of -85 mV.
As previously discussed, it is important to be able to transfect primary neurons in 3D construct as it is more representative of the *in vivo* environment. It has previously been reported that transfection of neurons derived from pluripotent stem cells is possible using lentiviral constructs (Carlson et al., 2016). In regards to transfecting primary cortical cells in a soft substrate, our promising results demonstrated for the first time the possibility of genetically engineering cortical cells, despite the challenges encountered. The observations revealed a low transfection level irrespective of whether the cells were seeded on top of the construct or within the hydrogel. Neurons themselves are difficult to transfect, however the physio mechanical and chemical properties of hydrogels provided additional barriers against the delivery of IONPs to the cells inside the hydrogel. Further optimisation of this protocol is still needed.

Taken together, the findings from the morphological analysis of this study suggest that the collagen hydrogel can be used to generate healthy a neuronal 3D construct and highlights the importance of choosing the appropriate collagen concentration that supports the even distribution of cells throughout the construct. Furthermore, these findings confirmed the safety of the protocol used in generating healthy and highly pure neuronal constructs in terms of viability, morphology end functionality.
Chapter 6
Conclusions and Future Directions
6.1. Summary of key thesis findings
The work presented in this thesis has studied the utility of magnetofection as a non-viral gene delivery technique, and hydrogel technology for enhancing survival and function of primary neurons. The results obtained in this thesis can be summarised as follows:

Chapter 3: Primary cortical culture optimization
This chapter was undertaken to generate reproducible primary cortical neuronal cultures which to meet the need of the neuronal gene engineering experiments and the 3D hydrogel constructs. Therefore the focus in this chapter was on developing a successful protocol.

According to the findings, the NBM-2 protocol was the most suitable for obtaining 2D monolayer primary cortical culture routinely. Tailoring the culture characteristics for neuron distribution, showed that cortical cells were distributed evenly on coverslips coated with PDL-Laminin substrates regardless of the cell density and the extent of time in culture. There was an apparent influence of lowering serum concentration in media from 10% to 1%, in experiments to reduce astrocyte contamination. However, there was no noticeable effect of reducing serum from 1% to 0%.

The proportion of neurons in culture was constant over the three time points examined and was ca 60% while astrocyte proportions increased from ca 1% at 3DIV to ca 39 % at 7DIV.
Chapter 4: Evaluating the safety of magnetofection for primary cortical neurons

In this section, the safety of magnetofection as a technique for transfecting primary cortical neurons was investigated using morphological characterization, viability assays and cellular morphology) using two magnetic assistive devices. Neuron electrophysiological properties were also assessed using the single cell patch clamp technique (single-cell recording).

The results from this chapter showed that the transfection efficiency of neurons at the two time points 3 and 7DIV was not significantly different when uniaxial magnetic field applied comparing to that when biaxial was used as the magnetic field. The transfection efficiency of astrocytes was enhanced when exposed to biaxial magnetic field at 7 DIV. However, the safety analysis (live/dead) assay revealed that the uniaxial magnetic field is a safer magnetofection technique versus biaxial. Electrophysiological properties comparison between magnetofected versus non-magnetofected cells confirmed the safety of using the uniaxial device for magnetofection. As the results showed there is no significant difference between ionic current in both cells, this current represented by an early inward (Na⁺) currents followed by late outward (K⁺) currents when measured by using patch clamp technique.

Chapter 5: Hydrogels are promising neuromimetic substrate for primary cortical cells

The results of this chapter revealed that collagen hydrogel is a supportive coating substrate for growing primary cortical 2D monolayer culture. Further, collagen
hydrogels at the concentration of 1mg/ml supported the growth of cortical cells on both the surface and within the depth of 3D constructs.

Cortical cells within the 3D construct distributed evenly throughout the gel, and neurons were seen to extend their processes. This model showed a high percentage of neurons versus astrocytes > ca 90 % neurons. Live/ dead assays and electrophysiological analysis demonstrate that our protocol is safe for growing neurons in hydrogels. Furthermore, pilot show the feasibility of genetic engineering neurons in 3D hydrogel constructs.

6.2. Future directions
There are a number of avenues by which the work presented in this thesis could be expanded to enhance transfection efficiency of neurons by magnetofection in both 2D and 3D environments. A lot of labs have abandoned the use of primary neuronal culture due to the difficulty of dealing their sensitivity to the environment. Hence, most of the generated information for tissue engineering studies is based cell lines.

1-Relative to what was achieved during my PhD, the primary cortical culture could be improved. It is possible to obtain highly pure neuronal culture without the need to use antimitotic agents which can be toxic. Enhancing purity can be simply by choosing embryonic age 15-16 rather than E18, because astrocytes are produced after neurons and are more abundant at the later developmental stages and postpartum (Sanes et al., 2011).

2-In terms of genetic engineering of cells, neurons are the most challenging cells to transfected despite extensive research. The findings of this research demonstrated the difficulty of enhancing transfection efficiency of neurons even
using the efficient and safe magnetofection technique. The magnet field frequency (1-4 Hz) and amplitude between 100-1000 µm have an impact on transfection efficiency in different neural cell types (Pickard and Chari, 2010a, Tickle et al., 2015). Based on these findings, there are other avenues that could be explored. The influence of oscillation frequency and amplitude of magnetic fields on primary neurons has not yet been investigated for uniaxial and biaxial oscillating magnetic fields.

1) It is worth investigating if the transfection level is neuronal phenotype dependent (i.e. the differences in transfection level between GABA neurons and glutamatergic neurons?).

2) What is the uptake mechanism of IONP internalization into neurons and what is the subcellular localisation?

3) Is the uptake mechanism also neuron type dependent?

Testing various IONPs in terms of having various chemical (IONPs fuctionalized with various coating layers), and physical properties (size and shape) for neuronal transfection would also be valuable.

3-Growing neurons in 3-dimensional construct was quite challenging. However, we were able for the first time in our group to obtain evenly distributed neurons in a 3D-construct. Other areas could be further improved relating to stiffness of the collagen to match a range of reported brain tissue stiffness (ca. 0.5-3kPa) during neural development, and study its effects on neuronal development and physiology (Gefen and Margulies, 2004, Elias and Spector, 2012). Further, mimicking the CNS structure, there are various types of neurons
distributed as layers, so, delivering a multi-neuronal type 3D construct can be a sophisticated achievement in regenerative medicine. This achievement can provide a solid base for basic research as it will facilitate study of the interaction between different types of neurons.

Additionally, our findings regarding transfecting neurons in hydrogels also need to be developed further. Methods need to be involved to improve transfection efficiency potentially by using new classes of DNA vectors such as minicircles, eventually to deliver neurotrophic factors such as BDNF or NGF.
Appendix

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**Electrophysiological assessment of primary cortical neurons genetically engineered using iron oxidenanoparticles**

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Much of the data included in chapter 4.
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