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An Investigation into the Effects of Magnetic Fields on Biological Cell Processes



A thesis submitted to the University of Dublin in partial fulfilment of the
requirements for the degree of

Doctor in Philosophy

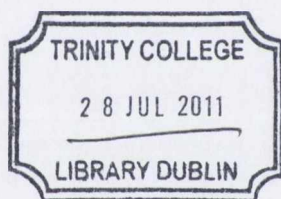
February, 2011

Darragh Crotty, B.Sc. (Hons)

School of Physics
Trinity College
Dublin

Supervisors

Prof. Mike Coey and Dr. Gavin Davey



THESIS
9217

I. Declaration

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II. Summary

The application of external magnetic fields to biological cells has received the attention of many researchers in recent decades and there have been countless reports of effects on a myriad of cellular processes. Despite this, there is in general a lack of consensus on the biophysical mechanisms responsible and independent replication of many effects has been unsuccessful. This field of research, often known as bioelectromagnetics, has many potential implications for human health, both in terms of therapeutic applications and concerns about adverse health effects due to fields associated power lines, electrical appliances and mobile telecom and Wi-Fi networks. Magnetic effects on cell processes could also shed new light on the fundamental biophysical/biochemical mechanisms involved. This thesis describes our investigation of a number of static and time-varying magnetic field effects at the cell (osteoblasts and neurons), organelle (mitochondria) and enzyme (creatine kinase) level.

Effects of static magnetic fields on mitochondrial superoxide production

Isolated rat brain mitochondria, treated with the electron transport chain inhibitors (ETC), rotenone and antimycin, were subjected to a number of static fields between 100 and 1000 mT. Using real-time enzyme-linked fluorescence measurements, it was found that a moderate, 148 ± 37 mT static magnetic field reduces, by 17%, the rate of mitochondrial superoxide production due to antimycin A treatment. This effect was only seen under toxic, high oxidative stress conditions, thus there is unlikely to be major implications for superoxide signalling, but the effect may offer some protection against oxidative damage caused by stroke and neurodegenerative diseases such as Parkinson's. The target of antimycin A is complex III of the ETC and so the most likely explanation for the effect is a radical pair mechanism involving some of the known paramagnetic species involved in electron transport at complex III.

Magnetic isotope effect in creatine kinase catalysed ATP formation

We have attempted reproduce the large magnetic isotope effect (MIE) that has been reported in a series of studies by Buchachenko and Kouznetsov (B&K). Their results indicate a 2-3 fold increase in ATP production by ADP phosphorylating enzymes such as creatine kinase, ATP synthase, pyruvate kinase and phosphoglycerate kinase when in the presence of the magnetic $^{25}\text{Mg}^{2+}$ isotope ($I = 5/2$) compared with non- magnetic $^{24}\text{Mg}^{2+}$ and $^{26}\text{Mg}^{2+}$. We examined this claim for a standard variety of creatine kinase (MM, bovine). Our results showed no increase in enzymatic ATP production when $^{25}\text{MgCl}_2$ (98% ^{25}Mg) is used instead of natural MgCl_2 (10% ^{25}Mg + 90% $^{24,26}\text{Mg}$). The absence of an isotope effect in our experiments suggests that the MIE does not

occur in MM creatine kinase and it casts doubt on the claim of B&K that the Mg isotope effect is a large (2-3 fold), robust and 'universally observable' effect exhibited by ATP-producing enzymes.

Effect of weak extremely low frequency electromagnetic fields on pre-osteoblast development

In this study we investigated whether the major processes of cellular development could be significantly altered by a 50 Hz, 2 mT AC magnetic field. Effects due to such a field would have many health implications and there is still considerable public concern over adverse effects due to the weak 50/60 Hz magnetic fields emitted by power lines and electrical appliances. Despite many studies showing cellular effects from fields of this magnitude and frequency, when we measured 16 cell developmental parameters via a high content screening system we found no significant changes in osteoblast progenitors due to a 50 Hz, 2 mT magnetic field and thus we have concluded that, over a 2 day exposure period, it is unlikely that such a field would induce significant developmental changes in osteoblastic cells and hence, also that it is unlikely that major toxic, carcinogenic or tumour promoting effects would occur in these cells under such conditions.

Magnetic Stimulation of Cultured Neurons *In vitro*

Magnetic Stimulation (MS) involves the use of strong pulsed magnetic fields to stimulate the electrically excitable tissue of the brain and peripheral nervous systems via an induced electric field. Despite over 20 years as a valuable tool in neuroscience research and the more recent development of clinical applications for diseases such as depression, Parkinson's and multiple sclerosis, demonstration of stimulation an *in vitro* cell culture model was only demonstrated for the first time in 2008 by Rotem and Moses. In a similar manner to Rotem and Moses, neurons were patterned via microcontact printing in configurations aligned with the magnetically induced electric field. Nevertheless, stimulation was not observed in otherwise electrically excitable neurons, when an electric field of 366 V/m was induced across them. As well as reproducing Rotem and Moses's findings we set out to investigate and develop the *in vitro* MS model in general. Based on our experience of using the magnetic stimulator *in vitro*, along with findings from FEM models, we conclude that applying electric fields directly via electrode arrays would provide a much more flexible, precisely controllable and time/cost efficient model system. Finally, electric field stimulation of neurons *in vitro* was demonstrated for the first time to be dependent on the orientation of the neurons to the electric field.

III. Acknowledgements

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V. List of abbreviations

2D	2 dimensional or 2 dimensions
3D	3 dimensional or 3 dimensions
A_B	Area through which uniform magnetic flux is passing
AC	Alternating current
ADP	Adenosine di-phosphate
ANOVA-1	One-way analysis of variance
ANOVA-2	Two-way analysis of variance
ATP	Adenosine tri-phosphate
B	Magnetic flux density
B_H	Cytochrome B (high spin)
BioEM	Bioelectromagnetics
B_L	Cytochrome B (low spin)
BSA	Bovine serum albumin
CI -V	Complex I to V (of electron transport chain)
CK	Creatine kinase
CoQ	Co-enzyme Q_{10} or Quinone cycle
Ctrl	Control
Cyt B_H	Cytochrome B (high spin)
Cyt B_L	Cytochrome B (low spin)
Cyt C	Cytochrome C
DC	Direct current
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E	Electric field
EAL	Ethanolamine ammonia lyase
E_{app}	Applied electric field
E_{cell}	Total electric field within a cell
E_{charge}	Electric field produced by charge accumulated at resistive boundaries such as the cell membrane
EDTA	Ethanediaminetetraacetic acid
$E_{effective}$	Total effective induced electric field
E_{ext}	Electric field induced by magnetic flux (B) through any loops traversing the area external to the cell
EF	Electric field
$E_{Faraday}$	Component of induced electric field that is governed by Faraday's law
EFS	Electric field stimulation
EGTA	Ethylene glycol tetraacetic acid
E_{ind}	Induced electric field
E_{int}	Electric field induced by magnetic flux through any loops only traversing the interior area of the cell
ELF-MF	Extremely low frequency electromagnetic

EPR	field
ERK	Electron paramagnetic resonance
ETC	Extracellular-signal-regulated kinase
FBS	Electron transport chain
FEM	Fetal bovine serum
GSH	Finite element method
GSP _x	Glutathione
HCS	Glutathione peroxidase
HEPES	High content screening
	4-(2-hydroxyethyl)-1-
	piperazineethanesulfonic acid (buffer)
HFI	Hyperfine interaction
HMF	High magnetic field
HRP	Horse radish peroxidase
HS	Horse serum
ICP-MS	Inductively coupled plasma mass spectroscopy
IgG	Immunoglobulin G
IMS	Intermembrane space (in mitochondrion)
IPR	Ion parametric resonance
ISP	Iron sulphur protein
JNK	c-Jun N-terminal kinase
LMF	Low magnetic field
MF	Magnetic field
MFE	Magnetic field effect
MIE	Magnetic isotope effect
MS	Magnetic stimulation
Myxo	Myxothiazol
NAD ⁺	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NBMA	Neurobasal medium A
NMDA	N-Methyl-D-aspartic acid
NMR	Nuclear paramagnetic resonance
OTS	Octadecyltrichlorosilane
ox	Oxidised
PBS	Phosphate buffered saline
PDMS	Polydimethylsiloxane
PMF	Pulsed magnetic field
Q	Quinone
Q [•]	Ubisemiquinone
QH ₂	Quinol
red	Reduced
ROS	Reactive oxygen species
RP	Radical pair
RPM	Radical pair mechanism
SMF	Static magnetic field
SOD	Superoxide dismutase
STE	Sucrose-Tris-EDTA (buffer)
Stig	Stigmatellan
tDCS	Transcranial direct current stimulation
TM	Transmembrane (inner membrane of

TMS	mitochondrion
Tris	Transcranial magnetic stimulation
Tris-HCl	Trizma base (buffer)
TVMF	Trizma-hydrogen chloride (buffer)
WHO	Time-varying magnetic field
α -MEM	World health organisation
	α -modified minimum Eagle's medium

1 General Introduction

1.1 Effects of magnetic fields on biological processes - Bioelectromagnetics

Bioelectromagnetics is the study of how magnetic and electric fields influence biological systems, thus it is focused primarily on the application of exogenous fields rather than the measurement of naturally produced endogenous fields in living systems. The latter is often distinguished by the term bioelectromagnetism. Our focus will be on the application of magnetic fields (MFs) to processes occurring in biological cells. Although there has long been an historical association with quackery, a survey of the literature will reveal thousands of studies reporting a myriad of biological effects from many types of magnetic field along with many hypothetical and not so hypothetical theoretical mechanisms [1-10]. Nevertheless results are often conflicting and difficult to replicate independently [11]. It can be hard to find a clear pattern to the effects in the literature and although, potential mechanisms are many and varied, there are few well accepted cause-effect relationships between theory and experiment.

Nevertheless there are exceptions where mechanisms are far less shaky. These include biological/biochemical effects caused by moderate (10 - 1000 V/m), magnetically-induced electric fields, forces due to diamagnetic anisotropy where applied fields are strong (> 1 T) [12-15] and radical pair effects [8, 16-21]. The

mechanism of action and hence the biological effect depends on the mode of the magnetic field which can be very broadly categorised, based on its temporal dynamics, as a static or time-varying magnetic field (SMF or TVMF).

1.2 Static magnetic fields

SMFs are non-time varying MFs. In bioelectromagnetic studies they are most commonly produced by permanent magnets made from ferromagnetically ordered materials like NdFeB. They may also be produced by DC coils and electromagnets. Those used in the bioelectromagnetics literature are usually in the range of 1 – 500 mT, but some studies have used up to 10 T produced by superconducting coils.

SMFs can exert cellular effects by a number of means. Some organisms such as bees, salmon, turtles, pigeons and magnetotactic bacteria contain ferromagnetic submicron-sized particles of magnetite (Fe_3O_4), which experience a torque (rotational force) in an SMF and a translational force in a magnetic field gradient. Movement of these relatively large particles (0.05 - 1 μm) inside the cell can cause structural changes in cytoskeleton and membrane that lead to various biochemical signals, thus allowing detection of and response to external magnetic fields. Very small amounts of magnetite have been found in human brain tissue but whether it has any functional role is unknown [22] and contamination from magnetite present in plastic and glassware remains a concern [11].

Para- and diamagnetically anisotropic materials will tend to align with or against an SMF respectively, particularly the long polymer chains of the cytoskeleton and extracellular matrix. Studies have shown that tubulin [14], F-actin [23] and collagen [15] chains show significant alignment with strong applied SMFs > 2 T and this alignment increases with field strength. Such mechanisms have been proposed to explain alignment of whole populations of cells [5]. Magnetically anisotropic

materials will also experience a translational force in an MF gradient, moving towards the region of lowest field magnitude if diamagnetically anisotropic and towards the opposite if paramagnetically anisotropic.

At the quantum level the angular momentum of electrons and nuclei in biomolecules will be altered in a magnetic field, so that the probability of their spins being aligned parallel with the field increases with field magnitude under certain conditions. This is ultimately the source of the larger scale effects mentioned above. Such effects on spin are at the heart of the radical pair mechanism (RPM), which provides the potential for MF effects, once a pair of unpaired electrons plays a rate-determining role in a biochemical reaction. The presence of such radical pairs is known or possible in a huge range of reactions involved in many key cellular processes, such as energy production, DNA replication, oxidative stress, cell signalling, toxin breakdown and apoptosis. The RPM responds differently to fields of different magnitude and over a very wide range of magnitudes from μT to T. As such, the RPM is a general bioelectromagnetic mechanism with the potential to explain the wide range of MF bio-effects that have been reported for an equally wide range of field strengths. Coupled to this are sound roots in a theoretically and experimentally well established area of magnetochemical research [24-26]. For these reasons our research work has focused on the RPM in investigating SMF effects and it will be outlined in more detail below (1.2.2).

1.2.1 Reported cellular effects

Many SMF effects on cellular processes have been reported in the literature and a selection are briefly described here. Strong inhomogeneous (gradient) SMFs of with an maximum field strength of 8 T have been used to enhance differentiation, but not proliferation, and to induce alignment with the MF in MC3T3 pre-osteoblasts [5]. This was explained by a torque on the cells due to the diamagnetic anisotropy of structures, such as the cytoskeleton and cell membrane, in elongated cells. A field gradient of 41.7 T/m was reported to increase expression of the proto-oncogenes, c-myc and c-fos in HL-60 cells, while the same study indicated that homogenous 6 and 10 T fields had no effect [27]. It has also been found that alignment of cultured glial cells can be controlled by aligning collagen fibres in a homogenous 10 T SMF and using this as a substrate for glial cell growth [7]. The effect could have potential clinical applications in the healing of damaged nerve, bone and muscle tissue whose microstructure is often oriented in a specific direction. Yamamoto et al. demonstrated increased differentiation and formation of calcified matrix (bone) in osteoblasts exposed to 160 mT MFs [28]. This effect was not altered by increasing MF strength up to 280 or 340 mT. Expression of the bone differentiation marker, BSP, was greatly increased by an 80mT SMF after 1 day exposures. Prina-Mello et al. have reported increased activity of the signalling kinases, ERK and JNK, in cortical neurons [29]. The effect was dependent on field strength, which ranged from 0.75 to 5 T. Changes in NMDA receptor subunit expression, leading to increased differentiation was seen in hippocampal neurons cultured in a 100 mT SMF [30]. In U937 cells, it has been reported that reactive oxygen species production and

apoptosis were increased by a 6 mT SMF [31]. Studies showing SMF effects, where the RPM is cited as the mechanism, include those on B12 ethanolamine ammonia lyase [17, 18], reactions at photosynthetic sites in plants [24] and the enzymatic phosphorylation that occurs during ATP production [21]. Approximately 2-fold increases in ATP production were reported for isolated mitochondria [16, 32] as well as the enzymes creatine kinase [19, 21] and phosphoglyceraldehyde kinase [20] when treated with a magnetic magnesium isotope (^{25}Mg). This effect was also shown to provide up to 80 % protection from ischemic damage to cardiac cells in rats [33-35].

1.2.2 Radical pair mechanism

The RPM has been studied in chemical systems for the past 30 years and has found most utility in spin resonance research (EPR and NMR) [25, 36]. It is a purely kinetic mechanism, allowing even low energy ($\ll \text{kT}$) weak magnetic fields ($< 1\text{mT}$) to affect the rate of certain types of chemical reaction. These reactions must contain a step that involves a pair of spin correlated unpaired electrons. These electrons can be provided by radicals, which give the mechanism its name, as well as paramagnetic d-metal complexes. Such a 'radical pair' (RP) state can occur when a bond breaks to form two radicals or when an electron is transferred to a neighbouring molecule to form radical ions or when a molecule is raised to an excited state by absorption of a photon. The correlated or coherent RP state is a superposition of two stationary states, singlet (S, anti-parallel) and triplet (T, parallel). In the anti-parallel ($\alpha\beta$) state

the spin angular moments cancel. A total spin angular momentum of 0 can only be achieved by a single arrangement of spins and this is the singlet state. For the parallel ($\beta\beta$) state total spin angular momentum is non-zero and this can be achieved by 3 arrangements of 'parallel' spins making up the triplet state. The T state consists of 3 sub-states/levels, T_0 , T_{+1} , T_{-1} . See Figure 1.1 for vector representations of the S and T states. After a short time (ps to μ s), during which oscillation between S and T can occur, this coherent state relaxes to a stationary state, being either S or T [24-26].

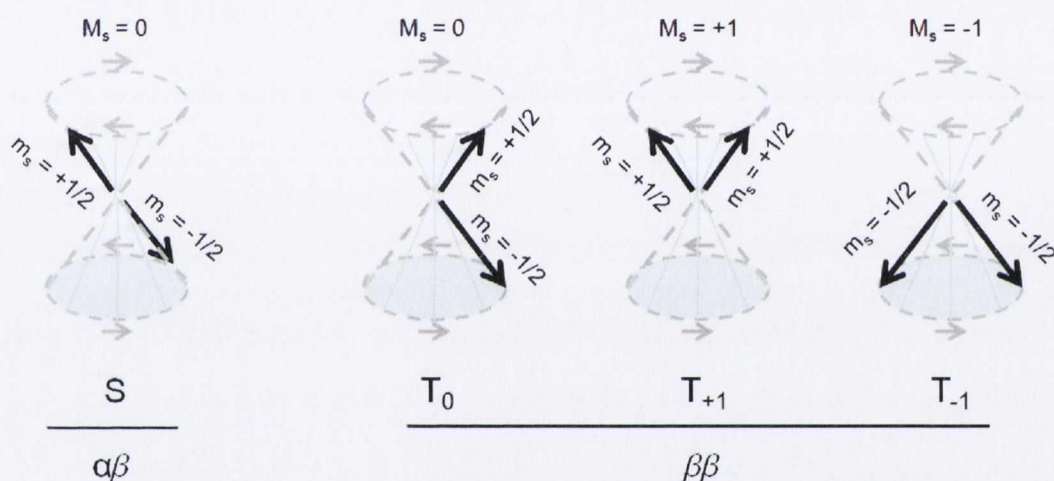


Figure 1.1: Vector representation of singlet (S, $\alpha\beta$) and triplet (T, $\beta\beta$) states of a pair of electrons, where m_s is the spin magnetic quantum number and M_s is the total m_s of the two electrons. The S state has total spin angular momentum of zero, while that of the T states is non-zero. There is only a single state that satisfies the anti-parallel (spins) singlet condition, while 3 possible states exist for the parallel triplet condition.

If after relaxation the stationary state is S, two things can happen, the radicals can diffuse apart and react elsewhere to form products or they can recombine to form a bond or the two initial pre-electron transfer species. Since the electron spins are anti-parallel, they are permitted to do so by Pauli exclusion. If T is the resulting state then a parallel spin configuration will bar recombination by the same principle and diffusion followed by product formation is the only available option. This is

illustrated at the top of Figure 1.2 and it explains how a reaction rate could be modified by altering the ratio of S to T states, since modifying the S population means changing the probability of the product-reducing recombination step. Magnetic fields can have significant influence over the ratio of S to T under certain conditions thus, magnetic field effects (MFEs) and magnetic isotope effects (MIEs) can occur when chemical reactions proceed via a rate determining step that involves an RP [20, 21, 24, 25, 37-40].

electrons in the pair to re-phase or flip its spin before the correlated (non-stationary) state is lost. The initial state depends on the process which it has resulted from. If diffusion leads to an encounter between two paramagnetic species then probability determines that that 25 % of encounters will produce S states while 75 % will produce T states. RPs formed this way are often referred to as F-pairs. The electrons making up geminal RPs, which are formed from the breaking of covalent bonds or by electron transfer, will retain the spins they had in the original molecule(s), as spin is conserved during such processes. Thus, in the case of a broken bond the resulting RP will always be S as electrons in a bonding orbital will always be anti-parallel due to Pauli exclusion.

Spin evolution of a two electron system in an external magnetic field is governed by the Hamiltonian below.

$$\begin{aligned}
 H = \mu_{\beta} B (g_1 S_1 + g_2 S_2) + \left[\sum_i I_i a_i S_1 + \sum_j I_j a_j S_2 \right] & \quad (1.1) \\
 + \left[\sum_i I_i D_i S_1 + \sum_j I_j D_j S_2 \right] - J(1/2 + 2S_1 S_2)
 \end{aligned}$$

The first term describes the Zeeman interaction with the external field where μ_{β} is the Bohr magneton, B is the externally applied magnetic flux density, g is the Lande g-factor and S is the operator for the spin of the electron. The square bracketed terms describe the hyperfine interactions (HFI) between each electron and any magnetic nuclei present, where a is the component of the hyperfine coupling

constant due to the Fermi contact interaction while D is the classical dipolar interaction component of the hyperfine coupling constant. The final term is the exchange interaction, where J is the exchange constant. For evolution or re-phasing to occur, spin correlation and sufficient but not excessive proximity must exist for at least as long as the spin re-phasing time (τ). The first of these conditions is destroyed by spin relaxation (T_1 and T_2) and the second by diffusion or recombination. τ is inversely proportional to the difference in the precession or Larmor frequencies (ω) of the two electrons of the radical pair (1.2). This can be seen in equations (1.2) to (1.4) for $S - T_0$ evolution, where J (discussed further below) is small compared to other influences on $\Delta\omega$.

$$\tau = (\omega_1 - \omega_2)^{-1} = \Delta\omega^{-1} \quad (1.2)$$

$$\omega = (1/2)(g\mu_B B + \sum_i a_i m_i + \sum_i D_i m_i) \quad (1.3)$$

$$\tau = (1/2)(\Delta g\mu_B B + \Delta A) \quad ; \quad A = \sum_i a_i m_i + \sum_i D_i m_i \quad (1.4)$$

Here m is the nuclear spin magnetic quantum number and A is the total hyperfine coupling constant i.e. the sum of the Fermi contact and dipolar components. It can be seen from this that there must be a difference between precession frequencies (energies) of the electrons in the pair for spin re-phasing/flipping and hence the S to T transition to occur. This generally means that there must be a difference between hyperfine constants (ΔA) or g factors (Δg) of the pair and increases in this difference

increase the probability of spin evolution by reducing the time required (τ). An explanation of why the *difference* in A or g is the driver of spin evolution is that a change in the radical pair state from anti-parallel to parallel (S to T) or vice versa requires a change in one member of the pair *only*. If both electrons flip their spins then there will be no net change in the pair's spin state. Thus, if A or g is large for one electron but small or zero for the other, then there is a probability of spin evolution occurring that is proportional to the difference (A or g) between the electrons. If A or g is the same for both electrons then spin evolution will not occur (assuming J is negligible – see below).

The ΔA or hyperfine (nucleus-electron) interaction mechanism of spin evolution operates with or without an external magnetic field and can be explained as follows: If an electron in a radical pair orbits a nucleus that has a net spin and hence a magnetic moment, then the hyperfine interaction (HFI) will cause a proportion of the electrons to flip their spin from the high energy nucleus-electron spin state to the low energy state (parallel or anti-parallel, depending on the atom or molecule). When this spin flip occurs in one electron of the pair but not the other then we get evolution from the initial S state to the T state or vice versa. This occurs when the HFI is strong for one nucleus but weak or zero for the other, i.e. ΔA is high.

Unlike the HFI, The Δg mechanism operates only in the presence of an external magnetic field ($B > 0$ in (1.4)). In an applied magnetic field, an electron with its magnetic moment anti-parallel to the field will have a lower energy than one with its moment parallel to the field. Hence, since the spin of the electron is always anti-parallel to its magnetic moment, it is energetically more favourable for an electron to

have its spin parallel to the field. Thus, an applied magnetic field will cause a proportion of the electrons to flip their spin from the high energy electron spin state to the low energy state (parallel). The energy difference between spin states in the field is proportional to the g value of the electron (1.1) so if one electron in a radical pair has a significantly higher g value than the other ($\Delta g \neq 0$), spin flipping will be more energetically favourable (probable) for it compared to its partner, thus, in the same manner as the HFI mechanism, the probability of spin evolution is increased.

At zero field the three T states (T_0 , T_{+1} , T_{-1}) are close to degenerate. If a magnetic field is applied, Zeeman splitting occurs and T_{+1} and T_{-1} states/levels are raised and lowered in energy respectively. If the magnetic field strength is increased further, these energy gaps can become so large that they are inaccessible from the S level. This means that spin evolution can only occur between S and T_0 and thus, overall S to T conversion is reduced.

At short inter-electron distances the briefly aforementioned exchange interaction, J , becomes very important. Either the S or T state will be strongly favoured (determined) by J (1.5) which primarily arises from the coulombic interactions of electrons and protons as well as the Pauli exclusion principle.

$$J = \frac{J_{ex} - C\sigma^2}{1 - \sigma^4} = \frac{1}{2}(\Delta E_{S-T}) \quad (1.5)$$

where J_{ex} is the exchange integral, C is the Coulomb integral and σ is the overlap integral. The energy gap between S and T states (ΔE_{S-T}) when B and A = 0 is 2J, so that J generates a baseline ΔE_{S-T} that is insensitive to magnetic influences. J is very

strong over short distances and will dominate over magnetic interactions in determining whether an S or T state is favoured, but it is generally assumed that the interaction decays exponentially with electron separation distance [41] so that,

$$J = J_0 \exp(-\beta r) \quad (1.6)$$

where r is the separation distance. J_0 may be positive or negative and $\beta > 0$. Once r between a pair of electrons goes beyond ~ 1 nm J becomes weak enough, that magnetic effects can become significant [24, 26, 41, 42].

As well as competing with magnetic field effects, the presence of a significant and constant J can also cause such effects if Zeeman splitting leads T. or T_+ energy levels to cross with that of S. This is illustrated in Figure 1.3. Thus, under conditions where two unpaired electrons are held at a relatively constant, close distance (e.g. a biradical) for a sufficient period of time magnetic field stimulated spin evolution may occur at small windows of B [24, 26].

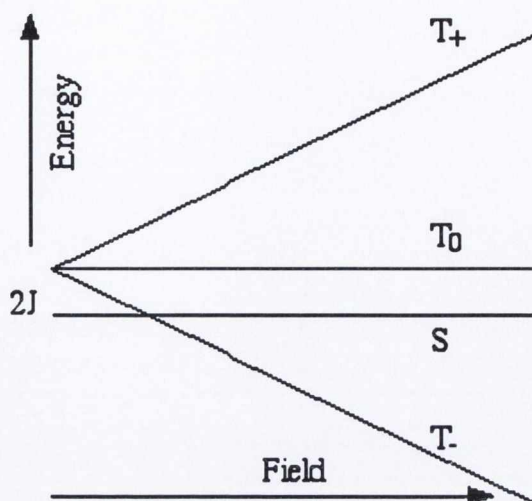


Figure 1.3: Crossing of singlet (S) and triplet (T) energy levels in an applied magnetic field due to Zeeman splitting and a significant exchange interaction, J . Taken from Brocklehurst, 2002 [26]. The occurrence of crossing of S/T energy levels allows for spin evolution from S to T levels or vice versa. This can be induced by external magnetic fields and is one of the ways magnetic fields can alter chemical reaction rates via the radical pair mechanism. The other major routes to magnetic field effects via this mechanism are outlined in Figure 1.2.

The other major field-sensitive drivers of spin evolution in radical pairs are hyperfine interactions (HFI) due to the presence of magnetic nuclei (ΔA) and inter-electron g -value differences (Δg). In the case of the former, weak to moderate magnetic fields, up to the magnitude of A (10 μ T - 25 mT), can further increase spin evolution by removing the degeneracy of nuclear spin states and thus splitting S and T energy levels in accordance with whether the nuclear spin is parallel or anti-parallel with the external field. This creates extra pathways from the S state to the T states (see Figure 1.2). If fields are increased much further spin evolution will be reduced due to large Zeeman splitting of T energy levels. The Fermi contact interaction (Δa) component of ΔA is isotropic and will always be subject to magnetic influence, but the dipolar interaction (ΔD) is anisotropic so that on average it will have no effect unless the interactions of the electron pair are geometrically constrained. Thus, ΔD would be expected to influence spin evolution at many enzyme active sites whose

geometry tightly constrains that of the reactants, but no influence would be expected where freely diffusing radicals encounter in a bulk liquid. Although ΔD could promote spin evolution at such constrained enzyme sites and thus contribute to an MIE, it is unlikely that this effect would be significantly influenced by externally applied fields, since on average each enzyme would have a random orientation in relation to the external field. This MF insensitivity means that ΔD generally competes against MFEs, while Δa enhances these effects [41, 42].

Another major spin evolution driver is Δg . This will generally be small for freely diffusing organic radicals, but it can be very large where the electronic environment of the RP is highly anisotropic, such as is the case when one of the unpaired electrons is belonging to a ligand coordinated d-metal atom [17, 24, 43, 44]. Such transition metal coordination complexes are found at the active sites of many important enzymes involved in energy production, protection against oxidative stress, oxygen transport, nitric oxide signalling and DNA transcription. Unlike the HFI mechanism, Δg only increases $S \leftrightarrow T_0$ transitions and it is proportional to B , with relatively large magnetic fields necessary for significant effects, e.g. > 100 mT for coordinated d-metal atoms and > 1 T for the small Δg values of most organic radical pairs [17, 24, 26, 43, 44].

1.3 Time-varying magnetic fields

TVMFs whose magnitude changes slowly over time compared with the rate of the cellular process in question will exert essentially the same effects as those previously described for SMFs. Since the TVMFs being investigated for bio-effects are most commonly at frequencies of <100 Hz, the above SMF mechanisms could also explain many of the cellular effects seen with TVMFs. Nevertheless, unlike SMFs, TVMFs will induce an electric field and this is their major distinguishing feature.

1.3.1 Induced electric fields

An electric field, $\vec{E}$ will be induced by a TVMF and this can be described using Faraday's law of induction, according to which, the magnitude of $\vec{E}$ along a closed loop, L (with radius r), is equal to the rate of change of magnetic flux, ϕ , through the area, A, enclosed by L:

$$\int_L \vec{E}(t) \cdot d\vec{l} = - \frac{\partial \phi(t)}{\partial t} = - \frac{\partial}{\partial t} \int_A \vec{B}(t) \cdot d\vec{A} \quad ; \quad \phi = \vec{B}(t) \cdot d\vec{A} \quad (1.7)$$

From this it can be seen that $\vec{E}$ is proportional to the area through which the time varying magnetic flux ($\vec{B}(t)$) is passing and as a result the induced electric fields

experienced by cells are limited by the size of the object containing the cells, whether it be an organism, organ, tissue sample or cell culture vessel. The field is also dependent on the shape and symmetry of the magnetic field itself. Then, the geometry and varied electrical properties of the exposed biological materials need to be taken into account, particularly resistive and capacitive boundaries which lead to charge accumulation. Charge accumulation plays a large role in determining the actual $\vec{E}$ in the cellular environment and the total effective $\vec{E}$ induced by TMVF is in fact a sum of the field induced via Faraday's law and that produced by regions of accumulated charge density [45, 46].

$$\vec{E}_{effective} = \vec{E}_{Faraday} + \vec{E}_{charge} \quad (1.8)$$

$\vec{E}_{charge}$ will oppose $\vec{E}_{Faraday}$ at some locations and add to it at others thus altering the magnitude and direction of the effective electric field. All this means that the simple formulation of Faraday's law above (1.7) is only applicable under certain simple centro-symmetric conditions and solving the full set of Maxwell's equations is usually necessary to get a realistic estimation. This has been done for in vitro cell exposure in chapter 5 where induced electric fields will be discussed in more depth.

The electric fields induced by TVMFs applied to biological systems ($\vec{E}_{Faraday}$) are usually weak, being in the range of 0.05 – 5 mV/m for continuous AC fields and 1 – 100 mV/m for pulsed fields, but for magnetic stimulation of neurons much larger

pulses induce $\vec{E}_{Faraday}$ from 50 – 500 V/m. With such strong pulsed magnetic fields the mechanism of action clearly operates via the induced electric field, but for weaker, slower changing fields like those used in continuous AC field studies the role of the electric field is still up for debate.

1.3.2 Cyclotron Resonance

Another potential mode of biological action for MFs is via the Lorentz force (1.9) which acts on moving charged particles such as the many ionic species involved in a cell's internal and external biochemistry.

$$F_L = q(\vec{v} \times \vec{B}) \quad (1.9)$$

where F_L is the Lorentz force, q is the charge of a particle (e.g. ion), $\vec{v}$ is the velocity of this particle (e.g. due to diffusion) and $\vec{B}$ is the magnetic flux density. The force acts perpendicular to the directions of both the applied MF and the current (moving charge). This causes paths taken by currents to form circles. The angular frequency ($\omega = 2\pi f$) of this motion is given by

$$\omega = \frac{ze\vec{B}}{m} \quad (1.10)$$

where z is the particle's charge, e is the elementary charge, m is the mass of the particle and $\vec{B}$ is the magnetic flux density. This is known as the cyclotron resonance

frequency and if an electric field is applied at this frequency, resonance will result in acceleration of the moving charged particle. The oscillating electric field required to satisfy this resonance condition can be generated via magnetic induction by AC magnetic fields. This mechanism involving a combination of SMFs and TVMFs serves as the framework used to explain many studies where cellular processes were altered by application of ELF-MFs at the cyclotron resonance frequencies of various biochemically relevant ions [1, 47-50]. Theoretical objections usually focus on the large gyration radius (metres) of the cyclotron motion of ions in the weak low frequency fields under study, when compared to the tiny (1nm to 1 μ m) distances being travelled by ions in cells [51]. Coupled with the huge amount of thermally induced random molecular collisions occurring in this liquid environment and the fact that the energy differences producing the Lorentz force and induced electric fields of the MFs studied are far less than kT at physiological temperatures (37°C), it would seem very unlikely that cyclotron resonance effects would influence cellular processes.

Nevertheless, many more complex treatments of the theory have found ways around the aforementioned problems [3, 51, 52] and the large number of studies showing positive effects, particularly on calcium ions lends the theory support. On the other hand, a number of studies have failed to detect any such effects on either calcium or potassium ions. One of the most commonly reported cyclotron effects is on the binding of calcium to calmodulin proteins such as myosin light chain kinase. Both Lednev [53] and Markov et al. [54] reported field-induced changes in phosphorylation of myosin light chain in two different calmodulin-dependent cell-

free systems. A detailed attempt at replication of these results detected no effect of a 20.9 mT sinusoidal field over a frequency range centred on 16 Hz, the cyclotron frequency of the unhydrated Ca^{2+} ion [55]. The same study also failed to detect an effect of combined static and AC magnetic fields on myosin light chain kinase activity over the same frequency range. A further direct attempt to replicate the findings of Markov et al. [56] has also proved negative. Another study showing Ca^{2+} cyclotron resonance effects was found to be irreproducible by Prasad et al. [57].

1.3.3 Reported cellular effects

Studies investigating such effects can generally be divided into two categories, continuous AC fields and pulsed magnetic fields (PMFs).

1.3.3.1 Continuous AC magnetic fields

Amongst the literature reports, such fields are generally weak to moderate (0.01 – 5 mT) in amplitude and fall within a frequency range of 1 – 100 Hz. These fields are commonly referred to as extremely low frequency magnetic fields or ELF-MFs. There are many reports suggesting that ELF-MFs affect neuronal development and that evidence from PC-12 and neuroblastoma cell lines seems to suggest that low strength AC fields from 5 – 250 μT inhibit differentiation and promote proliferation and thus possibly tumour development, but only within specific frequency windows

[4, 6, 58, 59]. On the other hand higher strength fields 0.3 – 1 mT seem to have the opposite effect, inducing differentiation by increasing influx of calcium ions into the cell [59-61]. Lisi et al. also showed a 30% increase in cell death due to glutamate induced excitotoxic Ca^{2+} influx. A 60Hz, 0.7mT ELF was seen to protect against oxidative stress induced by 3-nitropropionic acid in rat brains [62, 63]. ELF-MF studies on osteoblasts have demonstrated effects on differentiation, morphology and proliferation [64-66]. Effects on these cellular parameters have also been seen in keratinocytes [67] using 50 Hz, 2 mT fields.

Although there are many more similar reports of ELF-MF effects in many more cells types, it is important to bear in mind that a number of studies have shown no effect [68-74] and also that many attempts at independent replication of ELF-MF effects have failed [11], e.g. examples involving cyclotron resonance (section 1.3.2). Also, more recently there have been a large number of studies investigating the effects of much higher frequency fields in the radio (MHz) microwave (GHz) region. This has been largely on account of the new found ubiquity of mobile phone and Wi-Fi related MFs. Overall, many studies have shown effects while many have also shown no effect [75].

1.3.3.2 Pulsed magnetic fields

PMFs are approved by the FDA for treatment of chronic non-union bone fractures [76, 77] and a number of encouraging, clinical studies of magnetic stimulation (MS) in the treatment of depression [78] and Parkinson's [79, 80] and the diagnosis multiple sclerosis [81-83] as well as other neurological and psychological conditions have been carried out - see Wasserman et al. [84] and Hovey et al. [85] for reviews. Such effects are generally considered to be mediated solely through induced electric fields and most effects have been observed in neural and bone cells where electric (ionic) currents are known to play a major role in the functioning of the cell [86-89]. In the case of bone repair, weak to moderate ($B_{\max}$ 0.1- 10 mT, $dB/dT = 0.5-5$ T/s) strength PMFs are used. Much stronger pulses ($B_{\max}$ 1- 5 T, $dB/dT = 1000 - 5000$ T/s) cause firing (action potentials) in neurons *in vivo* and this effect is probably the only entirely undisputed and accepted magnetic field effect on a cellular process. Some neuronal effects have been reported for pulses on the order of those used on bone. For example, such fields have been shown to enhance neuronal differentiation as represented by increased and directed neurite outgrowth [90], but have also been shown to inhibit this in response to the differentiation inducing agent nerve growth factor (NGF) [91].

PMFs have been shown to be effective in enhancing differentiation [92] in MC3T3 pre-osteoblasts (bone cells), but also they have been shown to reduce this process and increase proliferation [93]. In this case, again it was seen that a lower field strength (0.1 mT) inhibited differentiation and increased proliferation, while a

higher one (7 mT) did the opposite. Another study by [94] using a 1.8 mT PMF has shown decreased proliferation and increased differentiation as evidenced by increases in the expression of the differentiation marker proteins, alkaline phosphatase, osteocalcin and collagen type I. All of these PMF studies have used various pulse trains at 15 Hz, which is the frequency used in many clinical bone stimulators [92].

2 Effect of static magnetic fields on mitochondrial superoxide production

2.1 Introduction

Mitochondria are the cell organelles responsible for energy production (Figure 2.1). They generate this via the movement of electrons down a chemical potential gradient populated by a chain of protein complexes. These electrons are finally transferred to O_2 and $4 H^+$ which combine to form water and terminate the chain (Figure 2.2). This electron transport chain (ETC) forms part of the mitochondrion's inner membrane and it uses the energy from electron motion to pump protons across the membrane, thus forming a steep proton gradient which causes protons to flow back across the membrane mainly through Complex V (ATP synthase) where the current drives a nanoscale protein rotor which eventually converts the mechanical energy to chemical energy in the form of adenosine triphosphate (ATP). The original electrons and therefore, energy, come from the by-products of food breakdown e.g. glutamate, malate, succinate.

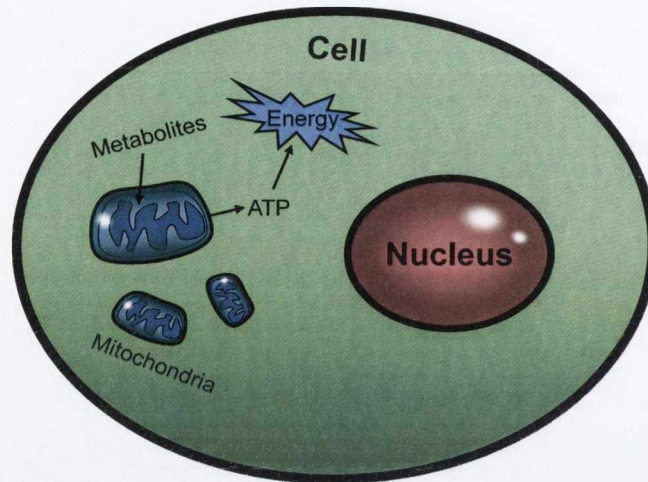


Figure 2.1: Mitochondria are organelles within all eukaryotic cells that convert metabolites from food breakdown into stored chemical energy in the form of adenosine triphosphate (ATP). This is then used as a fuel for energy demanding cellular processes.

If electron transport along the chain is blocked or slowed down (e.g. by chemical inhibitors or damage to protein complexes) these electrons have the chance to be transferred to O_2 thus forming the free radical, superoxide ($O_2^{\cdot-}$). Even at optimal ETC rates there is leakage of electrons onto O_2 , so superoxide is an inevitable by-product of mitochondrial energy production. This normal level of superoxide, which is generally not toxic and has a role in cell signalling [95], is greatly increased by ETC inhibitors, many diseases like stroke and Parkinson's [96-98] and in aging [99-101]. Here, the high levels of superoxide lead to interactions with endogenous anti-oxidants, iron ions and nitric oxide to produce highly reactive and damaging species such as $OH^{\cdot-}$, $ONOO^{\cdot-}$ and H_2O_2 (Reactive oxygen species or ROS) – see Figure 2.3. The result is termed oxidative stress and it leads to damage of many cellular components particularly lipid membranes and DNA.

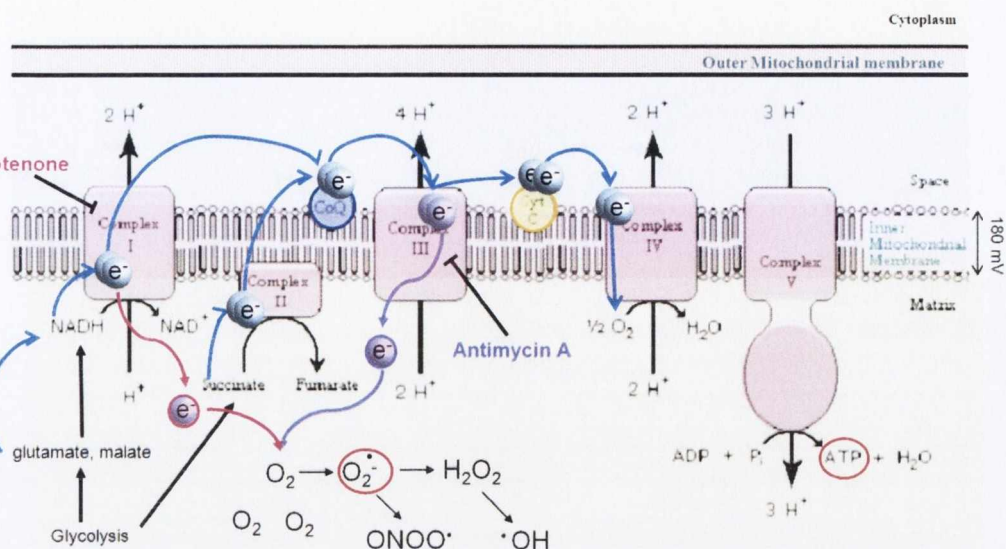


Figure 2.2: Mitochondrial electron transport chain (ETC) showing electron transport, Hydrogen ion pumping, inner membrane potential ($\Delta\psi_m$), inhibitor (rotenone, antimycin A) sites of action, superoxide production and ATP production. Adapted from Miller, Karl J. Overview of the Pathway. <http://www.gwu.edu/%7Empb/oxidativephos.htm>, 1998

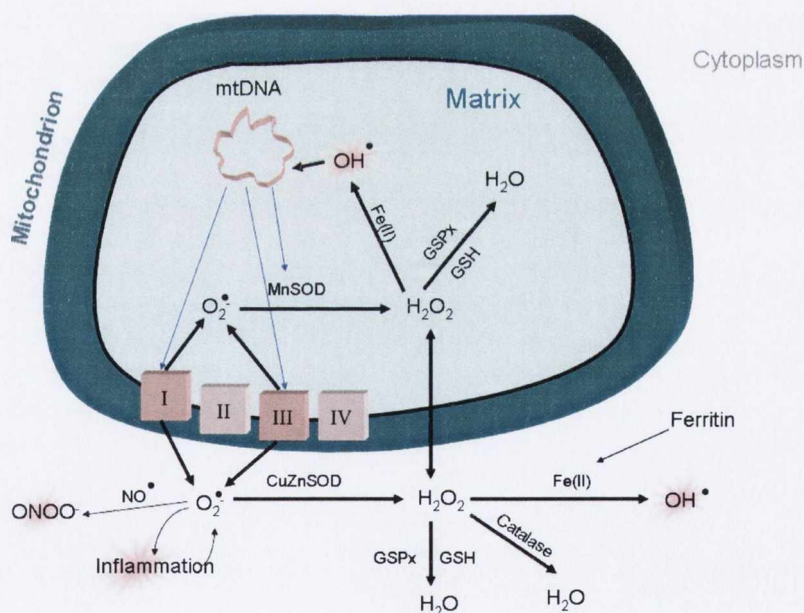


Figure 2.3: Pathways to reactive oxygen species (ROS) production in mitochondria. These species can lead to damaging oxidative stress, which is a feature of a number of diseases such as heart disease and Parkinson's disease.

ELF-MF effects on electron transfer between electron transport chain (ETC) enzyme, complex IV, and Cytochrome C have been studied by Goodman and Blank [102, 103] and they demonstrated increased reaction rates which were maximised at frequencies correlating with the enzyme's turnover number, while Nossol et al. [104] demonstrated up to 2 fold increases in rates but only in specific field strength windows, at around 0.3 and 10 mT. These effects were found for both 50 Hz AC fields and static fields varied continuously over time from 0.05 – 100 mT. Although complex IV is not known as a site of superoxide production it is part of the ETC and therefore could influence other ETC superoxide producing sites. A number of studies have reported MF effects on ROS and oxidative stress [31, 105-107]. In U937 cells reactive oxygen species production and apoptosis were increased by a 6 mT SMF [31]. A 60Hz, 0.7 mT AC MF was seen to protect against oxidative stress induced by 3-nitropropionic acid in rat brains and synaptosomes [62, 63]. Decreased oxidative damage due to experimentally induced stroke was seen in rabbits treated with 2.8 mT, 75Hz, PMF [108]. Finally, in *E. coli* bacteria exposed to a static field of 9 T for 24 hrs, an 80% increase in superoxide production was reported [109]

In this set of experiments static MFs, with field strengths in the range of 100 mT to 1 T, were examined for their ability to alter the rate of mitochondrial superoxide production under toxic (inhibited) and normal non-toxic conditions. ETC inhibitors, rotenone and antimycin were used to induce oxidative stress in isolated rat brain mitochondria. Acting at complex I and III respectively, these inhibitors, particularly rotenone are often used to model the dysfunctional mitochondrial states typical of neurodegenerative diseases like Parkinson's [110, 111]. They act by

inhibiting electron transfer between redox sites within the target ETC complex, thereby reducing electron flow along the ETC. This leads to an increase in the proportion of reduced (electron-rich) redox sites upstream of the inhibitor's site of action. This, in turn, increases the probability of an electron being transferred from one of these reduced sites to dissolved molecular oxygen to form the superoxide radical. See the discussion section (2.4) of this chapter for a detailed account of antimycin A's mechanism of action.

2.2 Methods and materials

2.2.1 Isolation of rat brain mitochondria

Non-synaptic brain mitochondria (i.e. those from all parts of the neuron except the axon terminals) were prepared/isolated using a discontinuous ficoll gradient, according to the method of Lai and Clark [112]. The procedure was as follows. Before the preparation began, all buffers were placed on ice and all instruments and centrifuge tubes rinsed with the appropriate buffer. For each preparation, two female Wistar rats (Bioresources Unit, Trinity College Dublin) were first stunned and then killed by cervical dislocation. All animal procedures were performed according to Irish and European Union regulations. They were quickly decapitated, their brains extracted and placed in ~40 ml STE buffer (320 mM sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4). The brains were chopped into small pieces using a scissors and allowed to settle to the bottom. The bloody buffer was decanted and replaced with fresh buffer, and the process was repeated 2-4 times. When the blood had been removed from solution and the brains were finely chopped, the pieces were placed in a glass Kontes homogenising tube with ~30 ml STE buffer and homogenised with at least 8 down and up strokes of a tight-fitting glass pestle. The homogenate was poured equally into two 50 ml centrifuge tubes and topped up to and balanced at 40 ml with STE buffer before centrifugation at 3000 rpm for 3 min at 4°C to remove all unwanted material. The supernatant was decanted and spun at 10,000 rpm for 10 min. The remaining crude mitochondrial pellet was carefully removed, leaving any

traces of blood in the centrifuge tube, topped up to 9 ml with STE buffer and homogenised using the tight-fitting pestle. The homogenate was placed on top of a discontinuous Ficoll gradient (9 ml of 7.5% Ficoll w/v in dH₂O on top of 18ml of 10% Ficoll w/v in dH₂O) and centrifuged at 28000 rpm for 45 min at 4°C. This resulted in 3 layers. The uppermost layer of myelin was removed by aspiration and the middle synaptosome layer was used by other lab members. The bottom layer which consisted almost entirely of non-synaptic mitochondria was homogenised using the tight-fitting pestle and re-suspended in approximately 1ml of STE buffer. This mitochondrial suspension was kept on ice for approximately 2 hours until the time of the experiment. During this time the total mitochondrial protein concentration of the suspension was determined (see below) and the experimental reagents and exposure equipment were prepared.

Mitochondria were quantified by the concentration of protein/ml. Total protein concentration in the mitochondrial suspension was determined by the Bradford assay [113]. A range of bovine serum albumin (BSA) concentrations (standards) were set up in 1.5 ml Eppendorf tubes as shown in Table 2.1. 10 µl of mitochondrial suspension was diluted 1:100 to 1ml with dH₂O and 100µl of this added to 700 µl of ultra pure H₂O in triplicate. 200 µl of Bio-rad dye (Bio-rad Laboratories, CA, USA), a colourimetric protein indicator, was added to each tube and these were then thoroughly mixed before being incubated at room temperature for 30 min.

Tube Contents	Mito	BSA Standards					
<i>Protein [BSA or mito] (µg/ml)</i>	x	0	2	5	10	15	20
<i>dH₂O (µl)</i>	700	800	780	750	700	650	600
<i>BSA (0.1 mg/ml) or mito added (µl)</i>	100	0	20	50	100	150	200
<i>BioRad dye added (µl)</i>	200	200	200	200	200	200	200
<i>Total vol. (ml)</i>	1	1	1	1	1	1	1

Table 2.1: Contents of solutions used in the Bradford assay. This is a colourimetric assay indicating the total protein concentration of a sample. The assay was carried out to quantify the concentration of mitochondria present in the initially prepared suspension.

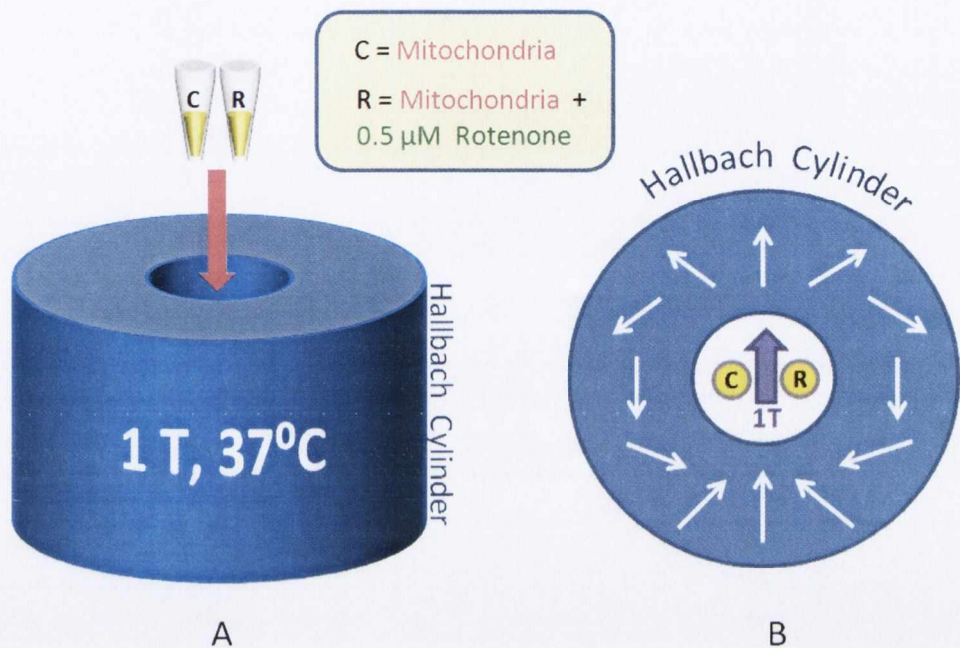
The contents of each Eppendorf were transfered into 1ml cuvettes and the absorbances read at 595 nm by a spectrophotometer (Agilent, CA, USA). Using the slope of the resulting standard curve, the mitochondrial protein concentration could then be determined.

2.2.2 Application of static magnetic fields

2.2.2.1 Study I

Two suspensions of brain mitochondria, with or without 0.5 µM of the complex I inhibitor rotenone, were placed in a homogenous 1 T static field produced by a Hallbach cylinder array of NdFeB permanent magnets (Magnetic Solutions, Ireland)for 30 min (Figure 2.4). During this time the mitochondrial suspensions were held in a sealed aluminium container connected via rubber tubing to a standard laboratory water bath. Continual circulation of heated water from the bath kept the temperature inside the container at 37°C. Measurements of mitochondrial

superoxide production took place 5 min (time for addition of reagents and transfer to 96 well plate) after completion of the MF exposure.



2.4: Schematic showing front view (A) and top down view (B) of exposure of untreated control (C) and rotenone treated (R) mitochondrial suspensions to a 1 T static magnetic field. This field was produced by a Hallbach cylinder made up of an array of NdFeB segments arranged with their magnetisations directed as shown by the white arrows in B [114]. This produces a homogenous 1 T field (perpendicular to cylinder axis; B) in a $\sim 100\text{ cm}^3$ region at the centre of the cylinder bore. The two suspensions were placed in this region and maintained at a temperature of 37°C during the 30 min exposure period via circulation of temperature controlled water. A pair of identical suspensions were kept at 37°C in a water bath but were not exposed to the field. The rates of superoxide production by field exposed and non-exposed groups were compared.

2.2.2.2 Study II

In the second case, a static magnetic field was applied to the brain mitochondria during measurement of superoxide production. This was produced via an array of permanent NdFeB magnets (Neotexx, Germany) arranged within an adapted black 96 well plate (see Figure 2.5 for setup). The plate consisted of a number of regions each

experiencing one of three distinct magnetic field conditions, $60 \pm 8 \mu\text{T}$ (no MF), a relatively inhomogeneous MF with a high B_{average} ($\pm \text{SD}$) of $273 \pm 82 \text{ mT}$ (HMF) or a slightly more homogenous MF with a lower B_{average} ($\pm \text{SD}$) of $148 \pm 37 \text{ mT}$ (LMF). These fields were modelled in 3D using the COMSOL Multiphysics (COMSOL, Sweden) FEM package (Figure 2.6 and Figure 2.7) since accurate experimental determination was not possible due to size of the hall probe relative to the microplate well. For each magnetic condition there were 3 chemical treatment conditions, $0.5 \mu\text{M}$ rotenone (Rot), $1 \mu\text{M}$ antimycin A (AntiA) or untreated (Ctrl). The modified plate was inserted into a microplate spectrofluorometer (Gemini XPS, Molecular Devices, USA) and ROS production during MF exposure was monitored over a period of 30 min.

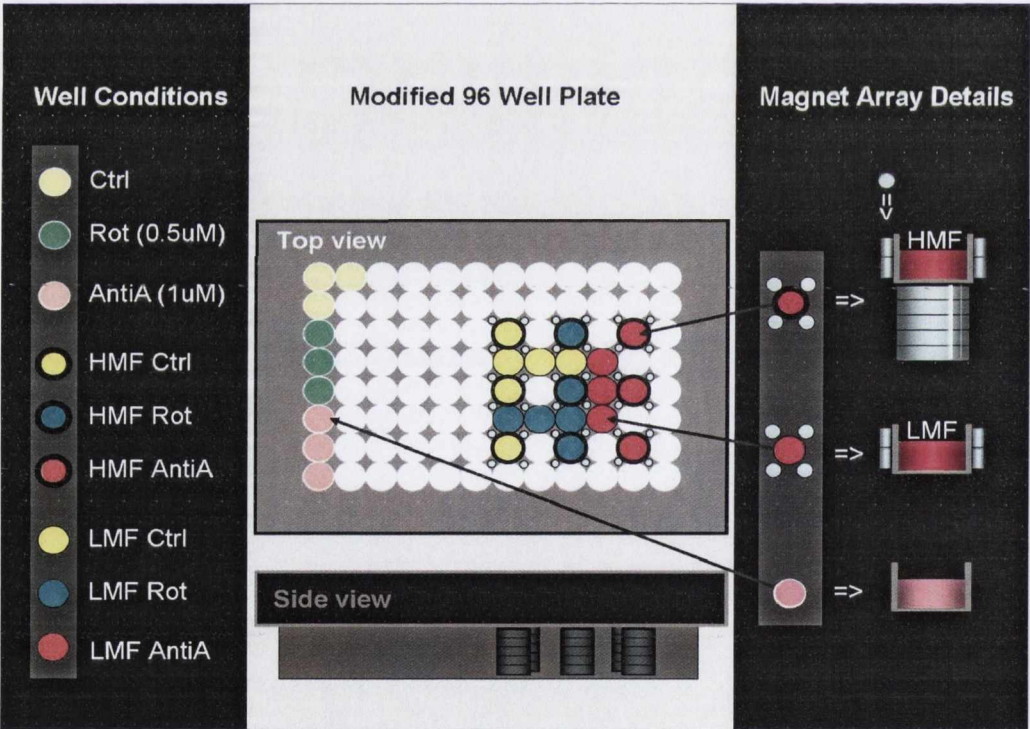


Figure 2.5: Modified 96 well plate with array of NdFeB magnets. Used for exposure of mitochondria to magnetic field simultaneous with fluorescence measurement. Ctrl = untreated; Rot = Rotenone; AntiA = antimycin A.

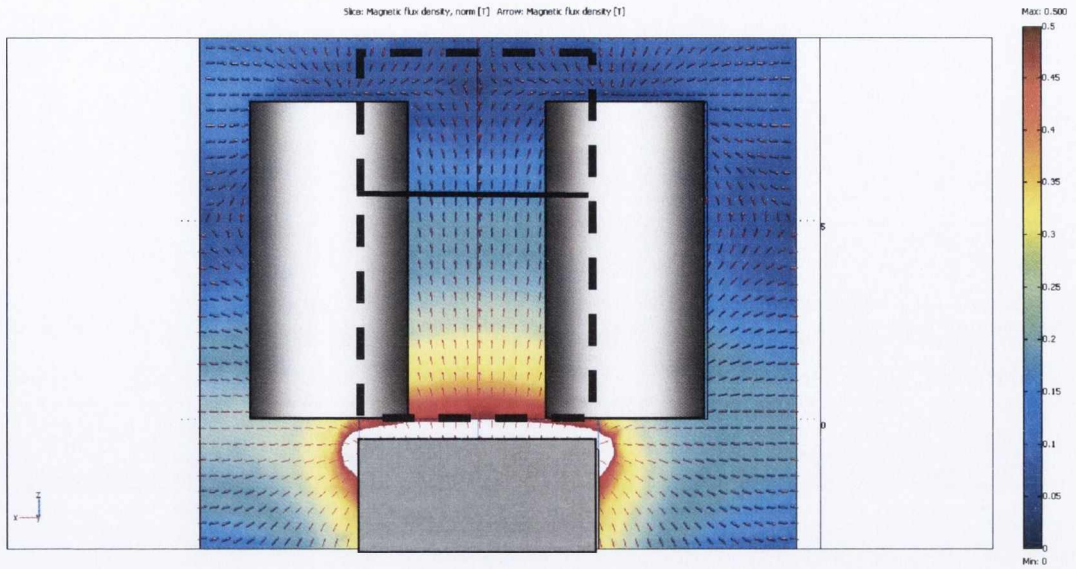


Figure 2.6: Simulated magnetic field profile of HMF in a vertical cross-section through the centre of the well. The well, magnets and liquid surface are represented by the dashed lines, grey shapes and solid line respectively. Colour map represents flux density in T, while arrows indicate the field direction. White area below the well is above the maximum value of the flux density range.

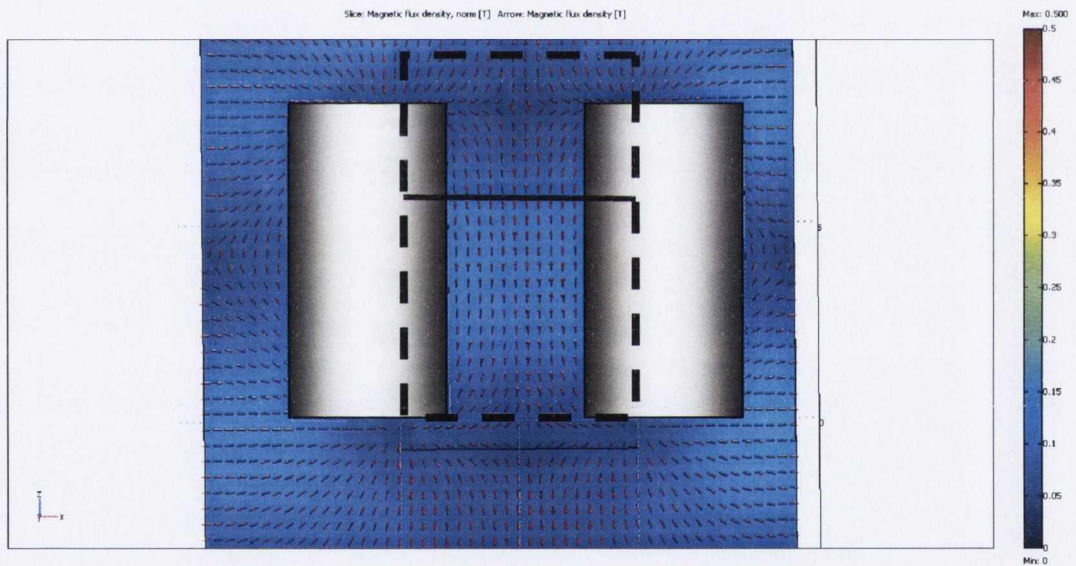


Figure 2.7: Simulated magnetic field profile of LMF in a vertical cross-section through the centre of the well. The well, magnets and liquid surface are represented by the dashed lines, grey shapes and solid line respectively. Colour map represents flux density in T, while arrows indicate the field direction.

2.2.3 Measurement of superoxide

Superoxide reacts extremely fast once it has been generated so it is very difficult to measure directly by chemical means. Most of it is converted almost immediately to hydrogen peroxide (H_2O_2) by the mitochondrial antioxidant enzyme superoxide dismutase (SOD) (Figure 2.3). Thus, H_2O_2 can be used as a proxy to measure superoxide production. We used the extremely sensitive amplex red assay for H_2O_2 . In the presence of horseradish peroxidase (HRP), the amplex red reagent reacts with H_2O_2 in a 1:1 stoichiometry to produce the red fluorescent oxidation product, resorufin. This allowed the rate of mitochondrial H_2O_2 production to be assessed using fluorescence measurements performed on a microplate spectrofluorometer (Gemini XPS, Molecular Devices, USA) in a black 96 well plate (Greiner) maintained at 37°C. Mitochondria were added to a standard incubation buffer that contained: 125 mM KCl; 2 mM K_2HPO_4 ; 5 mM MgCl_2 ; 10 mM HEPES (pH adjusted to 7.0 with KOH); 10 mM EGTA. Substrates, 5 mM of glutamate and 5 mM malate were added separately. Hydrogen peroxide was measured using 2 μM amplex red (Invitrogen, OR, USA) and 1 U/mL of HRP (Sigma-Aldrich, MO, USA). Measurements were carried out at excitation/emission wavelengths of 560/590 nm. For all experimental groups (conditions) there were 3 replicates and the concentration of mitochondria was diluted to 0.2 mg/mL of protein based on previous measurement of the protein concentration in the original mitochondrial suspension. The addition of superoxide dismutase to the assay medium did not further increase the signal (data not shown) indicating that the endogenous dismutase capacity was sufficient to metabolise all of the superoxide produced in this system.

2.2.4 Statistical analysis

Data were expressed as mean $\pm$ SEM. A one-way analysis of variance (ANOVA-1) was employed followed by a Bonferroni test to compare magnetic field vs. no magnetic field data (Prism; GraphPad Software Inc., USA). $p < 0.05$ was considered statistically significant. 4 independent replicate experiments were carried out. For more information on statistical methods used see appendix 0.

2.3 Results

2.3.1 Superoxide production

As expected, the complex I inhibitor, rotenone, greatly increased the rate of H_2O_2 (superoxide) production (> 2 fold), but it can be seen in Figure 2.8 that the strong homogenous 1 T static field had no significant impact on this rise. Also, comparing the control mitochondria, which had not been treated with the toxin, it was found that the magnetic field had no significant effect on superoxide production. These rates being measured here were those occurring post-exposure to the MF, so although the results indicate that a homogenous 1 T MF does not induce an effect on mitochondrial superoxide that can outlive its presence, it does not rule out the possibility of the effect existing during the exposure.

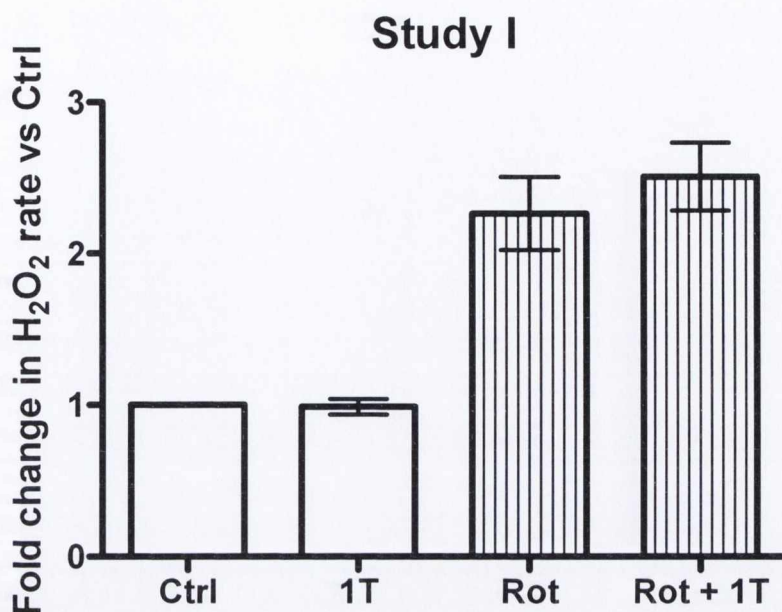


Figure 2.8: Rate of H₂O₂ (superoxide) production in isolated mitochondria after 30 min exposure to a homogenous 1 T field $\pm$ 0.5 μ M rotenone (Rot). The charts represent the average ($\pm$ SEM) of 4 independent experiments, where in each case the rate of H₂O₂ production was normalised to that of the control (Ctrl), thus indicating the fold change in the treated groups relative to this. No significant changes were seen in response to 30 min magnetic field exposure. ANOVA-1; Bonferroni test significant if $p < 0.05$; $n = 4$.

This limitation was overcome in the second set of experiments since we measured superoxide production rate during MF exposure, but this meant having the mitochondria inside a fluorometer and thus only an array of small magnets could be used. Instead of the very strong homogenous field produced by the Hallbach magnet, two distinct, relatively inhomogeneous weaker fields, the lower strength, LMF (148 ± 37 mT) and the higher strength HMF (273 ± 82 mT) were produced in the wells (Figure 2.6 and Figure 2.7). The LMF was seen to have a significant effect ($p < 0.01$) on antimycin-A induced superoxide, causing a 17% decrease (Figure 2.11 and Figure 2.12). On average, all groups exhibited a decrease in the LMF, but the changes were very small and only the aforementioned antimycin A LMF result was of statistical significance (Figure 2.9, Figure 2.10 and Figure 2.11).

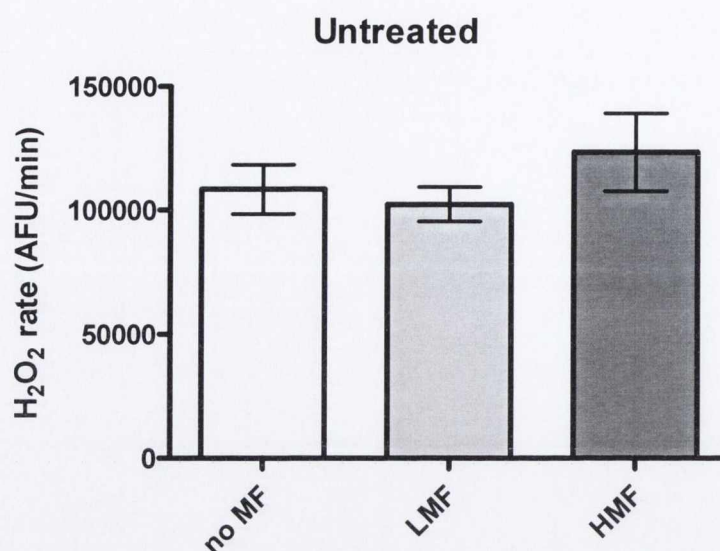


Figure 2.9: Rate of H₂O₂ (superoxide) production in untreated mitochondria during a 30 min exposure to 3 magnetic field conditions, no MF, LMF and HMF with average values of B were 0, 148 ± 37 mT and 273 ± 82 mT. No significant field effects were seen. ANOVA-1; Bonferroni test; significant if $p < 0.05$; $n = 4$.

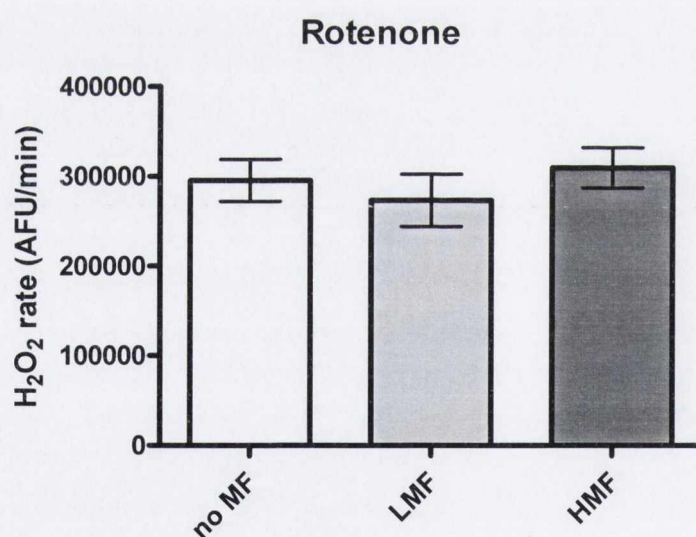


Figure 2.10: Rate of H₂O₂ (superoxide) production in rotenone treated (0.5 µM) mitochondria during a 30 min exposure to 3 magnetic field conditions, no MF, LMF and HMF with average values of B were 0, 148 ± 37 and 273 ± 82 mT. No significant field effects were seen. ANOVA-1; Bonferroni test; significant if $p < 0.05$; $n = 4$.

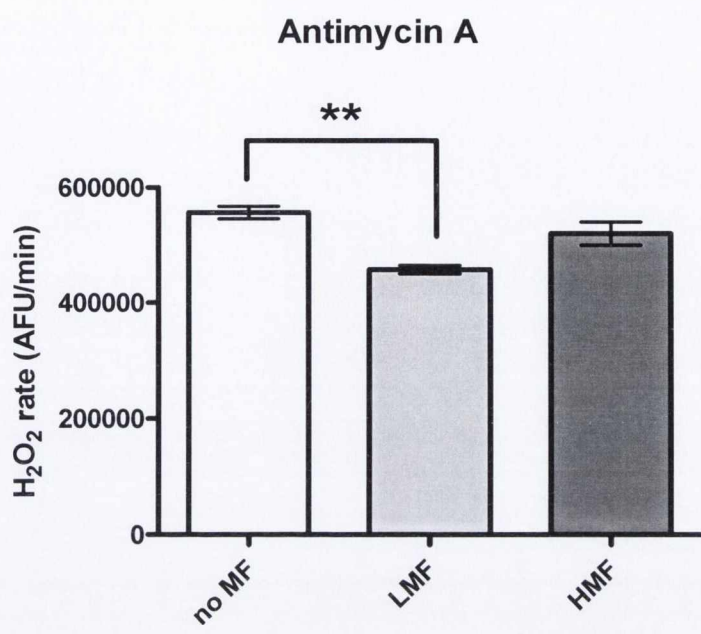


Figure 2.11: Rate of H₂O₂ (superoxide) production in antimycin A treated (1 μ M) mitochondria during a 30 min exposure to 3 magnetic field conditions, no MF, LMF and HMF with average values of B were 0, 148 $\pm$ 37 and 273 $\pm$ 82 mT. A significant decrease (** p < 0.01) was seen in antimycin-A induced H₂O₂ in the presence of the 148 mT field. ANOVA-1; Bonferroni test; significant if p < 0.05; n = 4.

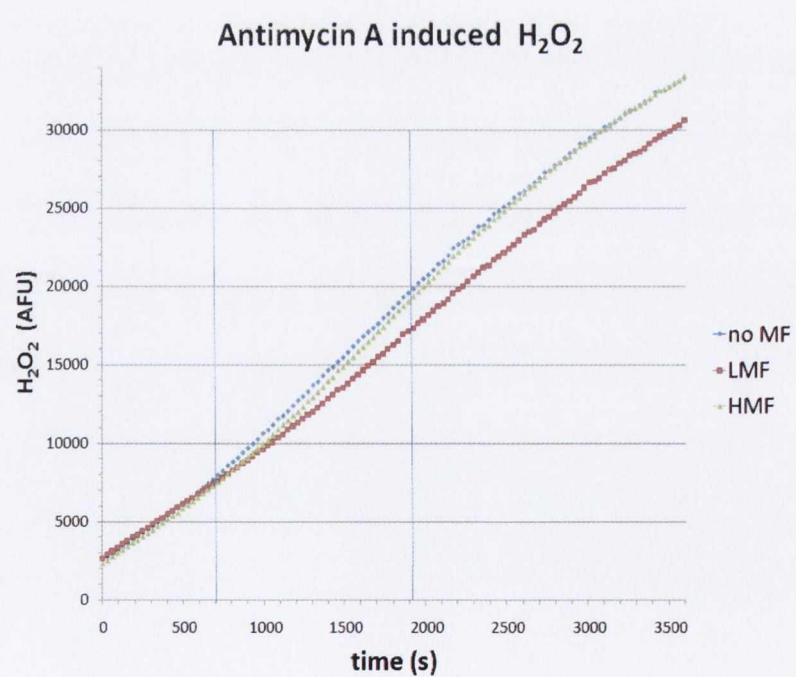


Figure 2.12: Representative fluorescence trace of antimycin A induced H₂O₂ production in isolated rat brain mitochondria. Each data point represents the average of 3 replicate wells. The Rate measurement was taken from the slope of the linear portion of the graph (between blue lines). The same region (time period) of the graph was used for all measurements within each experiment. A significant reduction in H₂O₂ production rate is seen for exposure to the low magnetic field (LMF; 148 $\pm$ 37 mT) but not for the high magnetic field (HMF; 273 $\pm$ 82 mT) where the rate differed little from the no magnetic field condition (no MF).

2.4 Discussion

Exposure of mitochondria to a homogenous static field of 1 T for 30 min (study I) does not seem to affect superoxide production in isolated rat brain mitochondria under normal conditions or with rotenone treatment. This suggests that such fields would not affect normal healthy mitochondria for better or worse and also that the superoxide generation at complex I is not a target of static magnetic fields of this magnitude. Nonetheless, only the post exposure rates of superoxide production were measured due to practical constraints, so it remains possible that these rates were altered during exposure, but then returned normal levels once the field was removed.

In the second part of the study this issue was addressed, but using lower field strengths and an extra ETC inhibitor, antimycin A. Using real time measurement of superoxide production in a static magnetic field, we have demonstrated that such fields can decrease superoxide production rates in isolated rat brain mitochondria (Figure 2.11). A statistically significant ($P < 0.01$), 17% decrease was recorded for antimycin A treated mitochondria when a 148 ± 37 mT magnetic field (LMF) was applied. As well as this significant effect, some statistically unreliable, yet interesting trends were observed. All groups of mitochondria, untreated, rotenone treated and antimycin treated, showed a decrease in average superoxide production in the LMF compared with zero field, while an increase was seen for the untreated and rotenone treated mitochondria in the HMF (273 ± 82 mT). In the same HMF a decrease was seen for the antimycin A group, but this decrease was smaller than that exhibited in

the LMF. This may indicate that the underlying mechanism is sensitive to field strength in a bi-phasic manner.

The radical pair mechanism (RPM) is known to be sensitive to a magnetic field's magnitude, and effects are often not monotonic with this magnitude [36, 39, 115]. In studies by Grissom and colleagues, the rate of product formation (V_{max}/K_m) in ethanolamine ammonia lyase (EAL) decreased with MF strength to a minimum of ~75% of the zero field value at ~100 mT. The rate then began to rise with field magnitude to a maximum of ~120 % at ~200 mT. Although in our study only two field strengths of 148 ± 37 mT and 273 ± 82 mT (relatively high inhomogeneity was also an issue) were applied, the size and direction of the effect (17 % decrease), the magnitudes of the effective field and its bi-phasic field strength dependence do bare surprising similarity to the EAL effect. This suggests that the observed mitochondrial effect may be due the RPM, since this was used by Grissom et al. to explain the EAL effects and then later, Eichwald and Walleczek [44] showed the results to be in relatively good agreement with their RPM-based model of the EAL reaction. It is suggested by these authors that the dip in product formation at around 100 mT is due to the Zeeman interaction reducing HFI induced spin evolution by energetically isolating the $T_{\pm}$ states, which leads to increased probability of the recombinative, product diminishing singlet (S) state. On the other hand the rise at higher fields is suggested to be due to the Δg mechanism increasing spin evolution from S to T_0 . Such an explanation is also plausible for the effect we have demonstrated, particularly since the chemical species involved in antimycin A induced superoxide production at complex III (CIII) include radicals as well as paramagnetic d-metal (Fe^{2+}

and Fe^{3+} -porphyrin complexes with similarities to the Co(III) -porphyrin complex of EAL.

The exact mechanism of antimycin A induced superoxide production at CIII is still debated [116-118], but the latest experimental studies of the mechanism, Yin et al. [118] and Yang et al. [117], suggest the two schemes shown below in Figure 2.13.

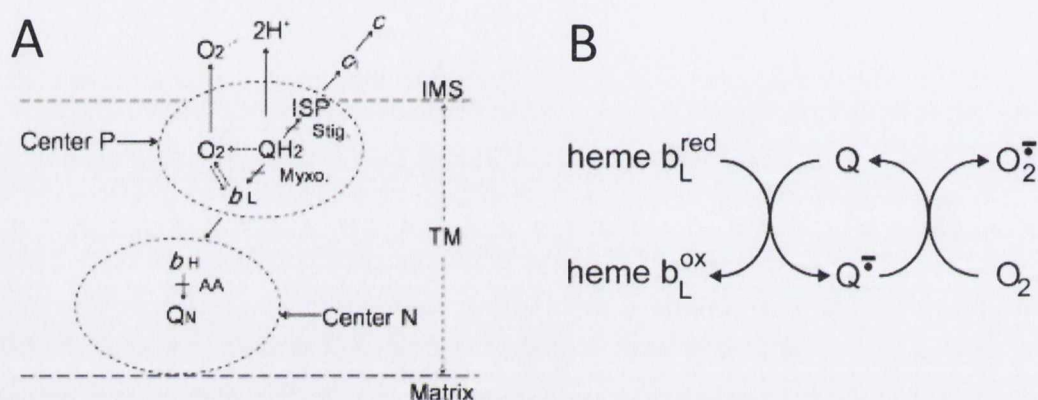


Figure 2.13: (A) Mechanism of antimycin A induced superoxide ($\text{O}_2^{\cdot-}$) radical production at complex III as proposed by Yin et al., 2010 [23]. (B) Mechanism of antimycin A induced superoxide production proposed by Droese and Brandt, 2008 [119]. These mechanisms provide a number of steps where pairs of radicals and other paramagnetic species exist. This, in turn, provides for the possibility of radical based magnetic effects on the superoxide production. Examples are most clearly seen in B, where ubisemiquinone ($\text{Q}^{\cdot+}$) could encounter superoxide or molecular oxygen (paramagnetic) to form a radical pair state. Symbols and acronyms are explained in the text of the discussion below.

According most proposed mechanisms [110, 116-118, 120, 121] of electron transport through CIII [116], including those in Figure 2.13, during normal electron transport, a bifurcated electron transfer from ubiquinol (QH_2) to an iron sulphur protein (ISP) and low spin cytochrome b moiety (cyt b_L or b_L) occurs [116]. Following the cytochrome b pathway, next an electron is transferred from the now reduced (Fe^{2+} centre) low spin cytochrome b to a nearby (0.21 nm) high spin cytochrome b (cyt b_H or b_H) and then

from here an electron is transferred to an ubiquinone (Q) molecule, forming $Q^{\bullet-}$ before reforming QH_2 via protonation. This redox cycle of ubiquinone is known as the Q-cycle and it is responsible for the pumping of protons across the inner mitochondrial membrane (TM in Figure 2.13) and hence also for the proton gradient necessary to drive oxidative phosphorylation of ADP at complex V.

Molecular oxygen dissolved in the lipid membrane can pick up an electron from along this transport chain to form the superoxide radical, $O_2^{\bullet-}$. Antimycin A increases the rate of this occurrence by inhibiting the electron transfer from cytochrome b_H to Q, thereby slowing down electron flow along the transport chain, which leads to increased 'leakage' of electrons onto O_2 [110, 116, 118, 119]. It is accepted that this occurs at the Q_p binding pocket (centre P in Figure 2.13) [116, 118, 119], but which species actually provides the O_2 reducing electron is not known for sure. The most widely suspected donor is the $Q^{\bullet-}$ radical which can be produced during either sequential or concerted mechanisms for cyt b_H reduction [116, 121]. Production of this reactive $Q^{\bullet-}$ could also occur by reverse electron (e^-) transfer from the ferric/reduced heme (Fe^{2+}) of cyt b_L as is proposed by Droese and Brandt [119] (Figure 2.13 B). Another possible single electron donor is cytochrome b_L itself. This is suggested as part of Yin et al.'s proposed mechanism [118] (Figure 2.13 A) and it is known that ferric/reduced heme (Fe^{2+}) reduces O_2 , binding it in the form of $O_2^{\bullet-}$ coupled to Fe^{3+} [122]. In fact, reduced heme is a potent pro-oxidant and its potential clinical application as a blood substituent has been limited largely by this fact [123].

From the above mechanisms of electron transport and reduction of O_2 , it is clear that there are numerous potential pairs of paramagnetic species involved.

These include: $Q^{\bullet-}$ and O_2 (paramagnetic) prior to e^- transfer; $Q^{\bullet-}$ and the paramagnetic heme (Fe^{3+}) of cyt b_L prior to e^- transfer; $Q^{\bullet-}$ and the paramagnetic heme of cyt b_H (Fe^{2+}) after e^- transfer; cyt b_L and cyt b_H ; cyt b_L or cyt b_H and O_2 ; cyt b_L or cyt b_H and $O_2^{\bullet-}$. All of these paramagnetic ('radical') pairs could be susceptible to magnetic field effects under the right conditions. Pairs consisting of combinations of $Q^{\bullet-}$ and cytochromes will be held in position for prolonged or indefinite (cyt b_L and cyt b_H) periods with separation distances on the order of 0.5 to 2 nm [116, 120] and these conditions are likely to provide the low exchange energies and long radical pair life times necessary for magnetic influence over spin evolution. Pairs involving $O_2^{\bullet-}$ coupled to $Q^{\bullet-}$ and cytochromes would likely have more variable separation distances and hence also exchange energies, but the geometric constraints of the binding sites and the fact at least one member of the pair is in a relatively fixed position could allow for long radical life times and favourable MFE conditions.

Despite the clear potential for an RPM much more information on the mechanisms and kinetics of superoxide production at CIII as well as the nature of our MF effect over a wide range of magnetic field strengths is required before a definite RPM based mechanism can be proposed. Investigation of the influence of different biochemical factors, particularly other CIII inhibitors such as myxothiazol (Myxo) and stigmatellin (Stig) should shed further light on the effect's mechanism and its specific locus of action. The acquisition and analysis of such data should be the focus of future studies on the subject.

As a final note, it is worth mentioning that RPM based magnetic field effects have in fact been reported for the horseradish peroxidase (HRP) enzyme that we

have used for our enzyme coupled fluorescence measurements (amplex red assay) [34, 124]. Nevertheless, these effects have since been shown to be non-existent and in actuality, the result of errors made during fitting of the data [125]. This has been acknowledged by the authors of the original studies. Thus, we consider our effects to be independent of the HRP measurement system used. This is further emphasised by the fact that a distinct effect was seen for antimycin A which targets CIII and not HRP.

In conclusion, it was found that a moderate, 148 ± 37 mT (inhomogenous) static magnetic field reduces, by 17%, mitochondrial superoxide production due to antimycin A treatment. This effect was only seen under toxic, high oxidative stress conditions, thus there is unlikely to be major implications for superoxide signalling, but the effect may offer some protection against oxidative damage caused by stroke and neurodegenerative diseases such as Parkinson's. The most likely explanation for the effect is a radical pair mechanism, but further investigation of its field strength dependence is necessary in order to give further insight into the mechanism involved as well as indicating whether the effect could be increased to a magnitude sufficient to provide any real clinical benefit as an anti-oxidant. Effects on untreated and rotenone treated mitochondria should also be investigated over a wider, more precise range of fields. This could result in some of the observed trends toward a magnetic field effect to rise above the errors and become significant. Methods of applying strong homogenous fields (up to $>1T$), while measuring radical production in multiple samples, would be of great benefit to any such future investigations.

3 Examination of the magnetic isotope effect in creatine kinase catalysed ATP formation

3.1 Introduction

The influence of magnetic fields on chemical reactions that proceed via the radical pair mechanism is well established, yet there are few biological examples [24, 26, 38]. Perhaps the most interesting example is found in a series of papers published over the past seven years by Buchachenko and Kouznetsov (B & K) with various co-workers [8, 16, 20, 21, 32, 126-130] who show that the production of ATP is very sensitive to the nuclear spin of the magnesium isotope that is involved in the phosphorylation reaction. The results were summarized in a sentence [131], 'It is universally observable that enzymes with the magnetic-isotopic nucleus of magnesium produce two to three times more ATP than the same enzymes with nonmagnetic nuclei under the same conditions'. We wished to test this striking observation.

Magnesium has three stable isotopes: Only ^{25}Mg (10% natural abundance) has a nuclear spin, $I = 5/2$. The nuclei of the other two, ^{24}Mg and ^{26}Mg have no spin, $I = 0$. B & K show huge increases (2-3 fold) in the rate of ATP production from ADP with a variety of enzymes. These are mitochondrial ATP synthase [16, 32, 130] phosphoglycerate kinase [20, 21, 128], pyruvate kinase [8] and creatine kinase [8, 21, 128, 129]. The effects increased linearly with the amount of ^{25}Mg in the isotopic

mixture [8, 126]. Furthermore, B & K demonstrate a large influence of magnetic field on ATP production (~ 50% further increase at 50 mT) when the magnetic isotope of magnesium was used [8, 21].

To explain this magnetic isotope effect (MIE), B & K propose a new radical pair mechanism involving Mg^{++} in the enzymatic synthesis of ATP [8, 20, 127, 132] (see Figure 3.1). According to the proposed mechanism the magnetic isotope exerts an influence since the radical ion pair (Mg^{++} and $ADP^{\bullet}$) initially exists in the singlet state but evolution to the triplet state can be increased by hyperfine coupling of the unpaired electron's spin to that of the magnetic nucleus. Recombination of the radical pair to reform the reactants can occur in the singlet state while it is forbidden by Pauli exclusion for the triplet state. All this means that increasing the % abundance of a magnetic nucleus (^{25}Mg) increases the triplet population via hyperfine coupling which in turn, leads to increased product (ATP) formation as the reaction can only go forward and not backward from this state.

Magnesium ions are well known to catalyze the reactions of adenosine triphosphate (ATP) producing enzymes (3.1) by altering the conformation and energies of reactants at the active site of the enzyme [133, 134].



This altered conformation promotes nucleophilic attack by the adenosine diphosphate (ADP) oxygen on the phosphorous of the phosphate (P). Depending on the enzyme in question, this P can be part of a phosphate donating compound like

phosphocreatine or simply take the form of an isolated (inorganic) phosphate molecule. According to B&K, at Mg concentrations greater than 50 times normal intracellular Mg concentrations [132], the reaction mechanism changes to one involving an ion radical pair. This new reaction mechanism, proposed by B&K, is shown below (Figure 3.1) for mitochondrial ATP synthase (Complex V). This same mechanism is also proposed for all other such enzymes exhibiting an MIE i.e. phosphoglycerate kinase, pyruvate kinase and creatine kinase. B&K find that the MIE does not operate on the reverse reaction involving energy release by ATP hydrolysis [8].

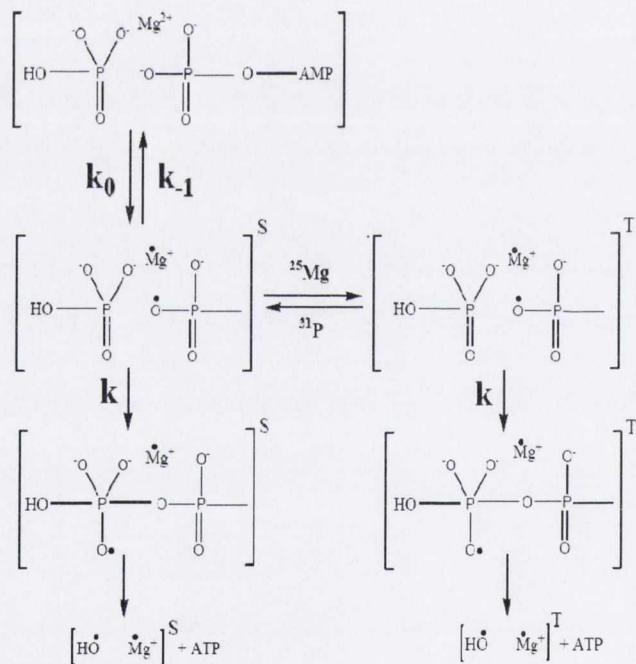


Figure 3.1: Mechanism of ATP synthase catalysed phosphorylation of ADP proposed by Buchachenko et al. [127]. The reaction involves the formation of a radical pair that can exist in both singlet and triplet states. Inter-conversion between these states is influenced by magnetic fields. Increased triplet formation leads to increased product (ATP) formation as the back reaction k_{-1} is spin-forbidden from this state. Taken from Buchachenko et al., 2007, *Chemical Physics Letters*, [127].

In the first step of the proposed mechanism we see that an electron is transferred from an O of the ADP molecule (AMP-P; P = phosphate) to a Mg^{2+} ion to form an ion radical pair in the S state. This S radical pair can recombine to form the reactants (k_{-1}) or progress with the forward reaction (k) thus forming ATP and reforming the catalytic radical ion. The unusually strong coupling ($a = 21$ mT) of a magnetic ^{25}Mg nucleus promotes spin evolution from S to T, thus opening a new channel for the forward reaction only since recombination can't occur. In this way the rate of ATP production is increased in the presence of purely ^{25}Mg ions compared to natural abundance Mg ions (79 % ^{24}Mg : 10 % ^{25}Mg : 11 % ^{26}Mg). Of its 3 isotopes only ^{25}Mg ($I = 5/2$) has a magnetic moment. Phosphorous occurs only as ^{31}P and so it is also magnetic ($I = 1/2$, $a = 3$ mT), but effects here are dominated by the much stronger magnetic field of the ^{25}Mg atom.

From a model of the above reaction [127] it was found that the increased ATP rates due to this MIE would be further increased by 50 % by applying an external field of 50 mT. The rate constants k_{-1} and k are not known at present, but by using 3 sets of values that fit with the experimental findings, the model was used to predict overall reaction (ATP production) rates in the presence of a range of magnetic fields (Figure 3.2).

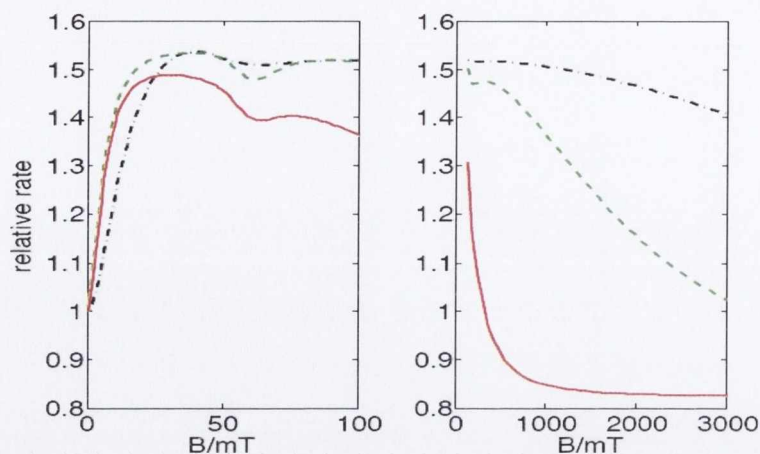


Figure 3.2: Magnetic field dependence of the rate of ATP production in presence of ^{25}Mg (MIE) relative to that in zero field (left). The parameter values for the intrinsic rate constants for the backward and forward electron transfer $\{k_{-1}, k\}$ are: red line (—) $\{10, 0.1\}$ ns $^{-1}$, green dashed line $\{100, 0.01\}$ ns $^{-1}$, black-dashed line: $\{1000, 0.01\}$ ns $^{-1}$. ATP production increases with field up to ~ 50 mT and levels off. Dependence over a much wider range of magnetic field values are shown on the right, where it can be seen that high field strengths cause ATP production to diminish towards zero field values and lower. Taken from Buchachenko et al., 2007, *Chemical Physics Letters*, [127].

From Figure 3.2 we can see that, at field strengths much higher than 50 mT, this magnetically induced rate increase diminishes and for one of the k_{-1} and k parameter sets (10, 0.1) the MIE is even inhibited by about 15 %. Inhibition of ATP production by about 8% under isotopically non-enriched conditions (natural Mg ions) was also predicted when fields of up to 100 mT were applied. These predicted magnetic field effects (MFE) were examined experimentally by B&K in another ATP producing enzyme, creatine kinase (CK) [21]. They tested fields from 0 to 80 mT and found that a 50 mT field augmented the MIE by ~ 50 % as predicted, although at higher fields (80 mT) the ATP yield increases even further rather than plateauing like the model.

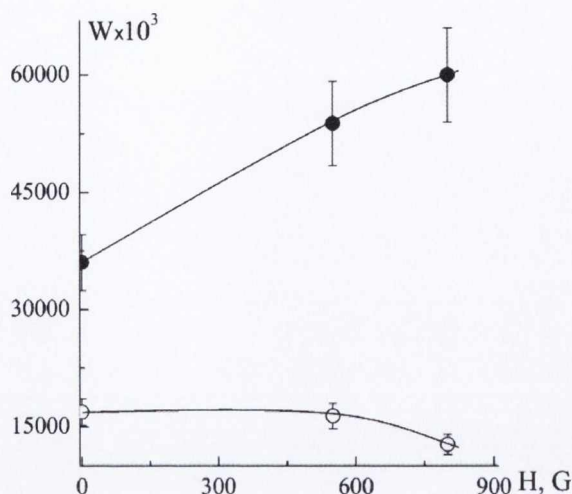
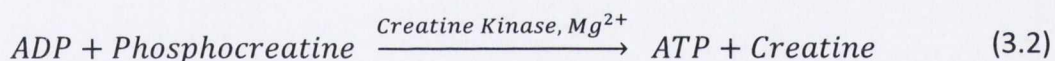


Figure 3.3: The yields of ATP produced by creatine kinase (from *V. Xanthia*) with $^{25}\text{Mg}^{2+}$ (black points) and $^{24,26}\text{Mg}^{2+}$ ions (open circles), respectively, as a function of magnetic field. Taken from Buchachenko et al., *JACS*, 2008 [21].

In this study we have attempted to reproduce this magnesium magnetic isotope effect in creatine kinase. The reaction under examination is as follows:



The production and hydrolysis of ATP are critical for metabolic processes in living organisms. Creatine kinase and ATP synthase are for the key enzymes involved in ATP production and regulation [133, 135] and the ability to modify their reactions via magnetic isotopes or magnetic fields would have significant implications for health. Of particular interest would be the treatment of ischemic conditions such as stroke and heart disease, where reduced oxygen supply to the affected tissue leads to a severe reduction in the cell's ability to produce ATP. Indeed B&K have been

involved in developing an ischemic therapy using a porphyrin adduct of C_{60} to deliver $^{25}\text{Mg}^{2+}$ ions to affected heart tissue. 80% decreases in ischemic damage have been reported in pre-clinical trials on mammalian heart muscle [33, 35, 136]. There are also potential ramifications if ATP production can be influenced by modest magnetic fields.

We are unaware of any independent corroboration of B & K's work since the publication of their first results in 2004. The claims are well suited for independent investigation because the effects are large, they appear to be general since they have been reported for a variety of enzymes and they have potentially wide significance. Here we have chosen to work with creatine kinase (CK), because it is a readily available enzyme which is reported to show a large effect – a 2 fold increase in the production of ATP when highly enriched ^{25}Mg is used instead of natural Mg (denoted as Mg).

3.2 Methods and materials

The experimental work covered in this chapter was carried out in co-operation with Soumya Poddar (summer internship with Prof. Coey).

3.2.1 Chemicals

Isotopically enriched magnesium oxide (^{25}MgO) was purchased from Isoflex (CA, USA). The luciferase based ATP determination kit (0.5 mM D-luciferin, 1.25 $\mu\text{g/mL}$ firefly luciferase, 25 mM Tricine buffer, pH 7.8, 5 mM MgSO_4 , 100 μM EDTA and 1 mM DTT ATP) was purchased from Invitrogen (cat # A22066, OR, USA). All other chemicals, including enzymes and MgO , were purchased from Sigma-Aldrich (MO, USA). Creatine kinase MM type I was from rabbit muscle and MM type III was from bovine heart. All water was purified using a Barnstead Nanopure (Thermo Scientific, MA, USA).

3.2.2 Preparation of Magnesium chloride

Enriched and non-enriched magnesium chloride salts ($^{25}\text{MgCl}_2$ and MgCl_2) were prepared by adding five times excess (0.1 ml/mg) concentrated molecular biology grade HCl to enriched and non-enriched MgO powders respectively. Both samples were then sonicated for 30 min to aid dissolution. Next, the acid was evaporated off

at 60°C to make MgCl₂ and ²⁵MgCl₂ salts. The resulting salt was washed 4 times with 0.5 ml of ultra-pure water i.e. water was added to the salt and then evaporated at 60-80°C. Inductively coupled plasma-mass spectroscopy (ICP-MS) was done to determine Mg concentrations in each sample and to verify their expected natural and enriched isotopic distributions (Table 3.1). It also allowed us to look at the concentrations of other potentially interfering trace metals (Table 7.1 of appendix). The concentrated MgCl₂ solutions, which were initially prepared by the aforementioned method, needed to be diluted 1:100,000 for ICP-MS. This put all trace metals but zinc below the detection limit of 1 µg/l (ppb) and zinc was just above this at 1.17 µg/l. Therefore, no contaminating metals were present in the concentrated solutions at levels higher than 117 µg/l (ppm) or 0.3 % of total mass of metal and that none were higher than 1.17 µg/l in the buffer used for the enzyme reactions. Magnesium levels were found to be 334 µg/l and 375 µg/l in the MgCl₂ and ²⁵MgCl₂ solutions respectively. This implies that the levels of other metals were always at least 300 times lower than those of magnesium. ²⁵MgCl₂ and MgCl₂ concentrations were then equalised by dilution before being used in the creatine kinase reaction.

<i>Isotope</i>	<i>Non-enriched (Known)</i>	<i>Non-enriched (measured)</i>	<i>Enriched</i>
²⁴ Mg	79 %	78.12 %	1.5324 %
²⁵ Mg	10 %	9.9 %	97.7 %
²⁶ Mg	11 %	12 %	0.8 %

Table 3.1: Isotopic distributions in enriched and non-enriched (natural) MgCl₂ solutions used for the creatine kinase experiments. Values were determined by inductively coupled plasma mass spectrometry (ICP-MS).

3.2.3 Creatine kinase reaction

The creatine kinase (CK) reaction was carried out in HEPES buffer, at room temperature at pH 6 in a 96 well plate (reaction plate) using quadruplicates for each reaction condition. The reagent concentrations and other reaction conditions used for the experiments can be seen in Table 3.2. For the sake of comparison this table also shows those conditions used by B&K, as reported in JACS, 2008 [21]. The reaction mixture minus the enzyme (10 mM HEPES, 8 mM ADP [$K_m \times 10$], 50 mM phosphocreatine [$K_m \times 10$], 15 μ M BSA [0.1% w/v], 14 mM MgCl_2 or $^{25}\text{MgCl}_2$) was made up first and then 90 μ l was added to the appropriate wells of the plate. Water was added to 2 sets of quadruplicate wells (8 in total) to give the blank/background ATP levels. One set contained the reaction mixture with MgCl_2 while other had $^{25}\text{MgCl}_2$. 10 μ l of enzyme was added to the remaining 16 wells to start the reaction. The reaction time was 30 min. A multichannel pipette was used to minimize time differences.

Two types, I and III, of the MM isozyme of creatine kinase were used in two separate reactions. This isozyme is a dimer of two muscle [M] subunits. The other common dimer CK isozymes are BB and MB which are made up of two brain [B] subunits or one B and one M subunit respectively. Final concentrations of enzyme were 0.28 nM for MMI and 4.9 nM for MMIII. Concentrations were chosen to ensure that final ATP concentrations were not too high or too low. ATP concentrations needed to be less than 10% of initial ADP concentration to maintain Michaelis-Menten kinetics and also to avoid significant occurrence of the reverse reaction from ATP to ADP. The concentration also needed to be above the background ATP levels

due to ATP present as an impurity in the ADP powder used (see Table 3.2) and also to be on the linear range of the luciferase standard curve (5 – 500 nM, Figure 3.4).

<i>Experiemental Condition</i>	<i>Our Study</i>	<i>Buchachenko et al., JACS, 2008 [21]</i>
Buffer	HEPES, 10 mM	Tris-HCl, NA*
pH	6.00	6.35
MgCl ₂ or ²⁵ MgCl ₂	14 mM	20 mM
ADP	8 mM (K _m x10)	0.16 mM
Phosphocreatine	50 mM (K _m x10)	0.16 mM
ATP	0.07 mM (impurity in ADP)	0.0125 mM
CK Enzyme (type; conc.)	MMI / MMIII; 0.28 nM / 4.9 nM	From V.Xanthia; NA*
Potassium Phosphate	0 mM	15 mM
Bovine Serum Albumin	0.015 mM	0 mM
Temperature	22°C	30°C
Reaction Duration	30 min	30 min
ATP measurement	Luminescence (Luciferase)	[³² P] ATP, HPLC

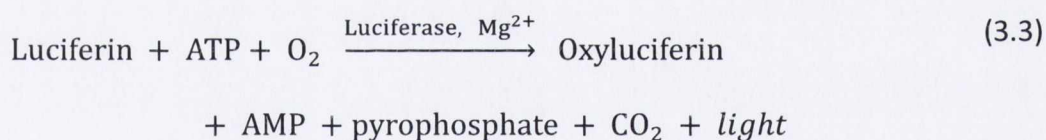
Table 3.2: Experimental conditions used for our study are shown in the second column. For comparison, those used by Buchachenko et al., JACS, 2008 are shown in the third column. *NA => Information not available in the literature.

The reaction was stopped after 30 min by the standard method of enzyme denaturation with perchloric acid (PCA) followed by neutralisation with K₂HPO₄ [137]. A white luminescence 96 well plate (Thermo Scientific, MA, USA) was put on

ice during the 30 min reaction and 2.5 µl of 6 M PCA was added to a set of wells corresponding to those of the reaction plate. After the 30 min reaction time, reaction well contents were rapidly transferred to the PCA wells of the luminescence plate using a multichannel pipette. After 10 min 125 µl of 1 M K₂HPO₄ was added to each well, again, using a multichannel pipette to minimise time differences. The plate was then kept on ice for a further 15 to 30 min before measuring the ATP yields.

3.2.4 Determination of ATP yield via luminescence (luciferase reaction)

Quantitative determination of ATP was based on the light producing (luminescent) reaction of luciferin with ATP and oxygen. The reaction is catalysed by luciferase and shown below:



An ATP determination kit (Invitrogen, OR, USA) based on this reaction was used according to the manufacturer's instructions and the correspondence between luminescence (arbitrary luminescence units, ALU) was found to be essentially linear ($R^2 = 0.996$) for known concentrations of ATP between 5 and 500 nM as shown by the standard curve in Figure 3.4.

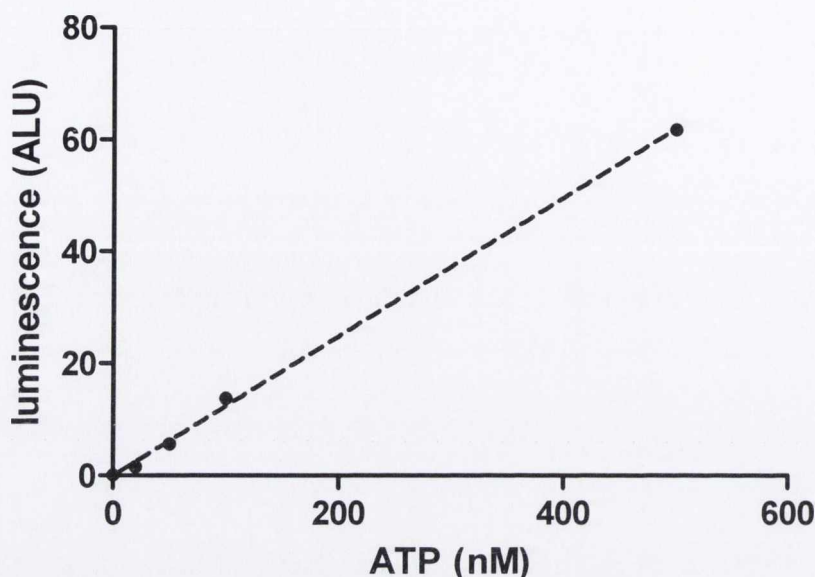


Figure 3.4: Luminescence intensity (ALU) versus ATP concentration (nM) standard curve for the firefly luciferase reaction. The data represent the average ($\pm$ SEM; not visible due to small magnitude) of 3 independent experiments ($N = 3$). Fitting to a linear regression plot ($R^2 = 0.9981$) demonstrates the direct proportionality of luminescence intensity to ATP concentration from 0 to 60 ALU or 0 to 500 nM ATP.

During the 30 min reaction, 95 μ l of the luciferase reaction mix was added to another set of wells on the luminescence plate. This set of wells corresponded to that of the reaction plate. 5 μ l from each CK reaction well was added to a corresponding well containing 95 μ l of the luciferase reaction mix. The resulting luminescence was measured at room temperature using a Luminoskan Ascent microplate luminometer (Thermo Scientific, MA, USA). Four independent experiments were carried out using the conditions described in section 3.2.3. Two additional experiments were also performed where conditions varied slightly in that PBS was used as the buffer and the CK MMIII concentration was 10 nM.

3.2.5 Statistical analysis

Within individual CK/ATP experiments, each condition was examined in quadruplicate. The results shown are averaged values for a number of independent experiments $\pm$ SEM, where in each case the luminescence intensity was normalised to that of the natural Mg sample, thus indicating the fold change in the ^{25}Mg sample relative to this. A paired student t test was used to compare Mg vs. ^{25}Mg values (Prism; GraphPad Software Inc., USA). $p < 0.05$ was considered statistically significant. For more information on statistical methods used see appendix 0.

3.2.6 Electron paramagnetic resonance spectroscopy

Electron paramagnetic resonance (EPR) spectroscopy is a magnetic resonance technique for studying chemical species that have one or more unpaired electrons. It was carried out to test for the presence of the radical pair involved in the mechanism of ADP phosphorylation suggested by the Buchachenko group (Figure 3.1).

45 μl aliquots of CK reaction mix with either MgCl_2 or $^{25}\text{MgCl}_2$ were prepared as in section 3.2.3. 5 μl of creatine kinase (MMI or MMIII) was added just prior to running the EPR spectrum. The final enzyme concentrations were 1.3 μM for MMI and 3.2 μM for MMIII. All enzymes and solutions were kept on ice until the time of the measurement. Water absorbs very strongly at the 9.8 GHz frequency used by the EPR machine (Bruker EMX, Bruker, MA, USA) so in order to prevent the water in these solutions from excessively broadening the signal and rendering cavity tuning

impossible, the sample diameter was minimized as recommended by Nesmelov et al. [138]. As narrower quartz EPR tubes were not available, a 29 μl sample was taken from the CK reaction solution using a glass capillary tube (inner D $\sim$ 1 mm) and then this was allowed to slowly slide to the bottom of the quartz EPR tube (inner D = 4 mm). EPR measurements were then carried out by Karl Ackland (research group of Prof. Mike Coey). The sample-containing quartz tube was placed in the cavity and after tuning the spectrum was acquired. The spectra of an empty quartz tube and a quartz tube containing an empty glass capillary were compared and no significant difference was seen, indicating the glass was not absorbing the microwave radiation above the level of the background noise. Glass capillaries have been used successfully in previous EPR studies [139, 140].

3.3 Results

3.3.1 Examination of magnetic isotope effect in creatine kinase catalysed ATP formation

We examined the effect of the ^{25}Mg isotope on the ATP yield of CK after 30 min in HEPES buffer (pH 6) with both MMI and MMIII types of the enzyme (0.28 and 4.9 nM respectively). After repeating the experiment 4 times we found there to be no significant changes in ATP yield due to the ^{25}Mg isotope (Figure 3.5). A similar experiment in PBS buffer was repeated twice and again no magnetic isotope effect was observed (Figure 3.6).

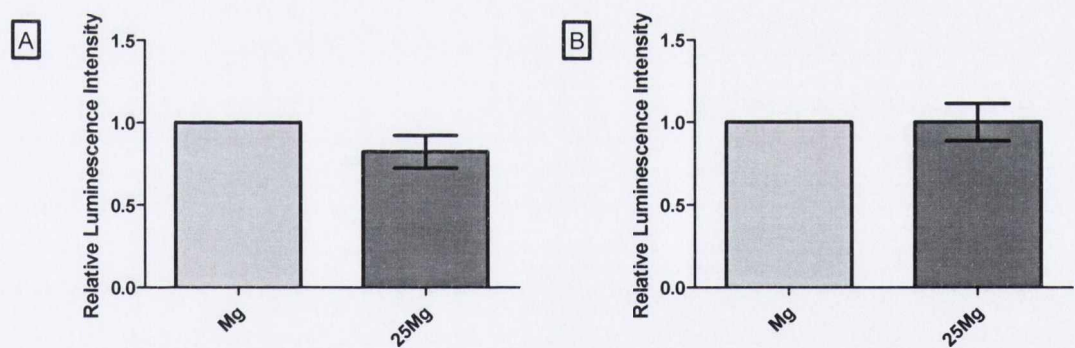


Figure 3.5: Luminescence intensity (proportional to ATP yield) of ^{25}Mg versus natural Mg creatine kinase reactions after 30 min in HEPES buffer. The reaction was examined for type I (A) and type III (B) of the MM isozyme. In both cases a significant change ($p < 0.05$) was not observed when comparing ATP yields in the presence of isotopically enriched MgCl_2 (98% ^{25}Mg) versus those in the presence of natural MgCl_2 (10% ^{25}Mg). The charts represent the average ($\pm$ SEM) of 4 independent experiments, where in each case the luminescence intensity was normalised to that of the natural Mg sample, thus indicating the fold change in the ^{25}Mg sample relative to this.

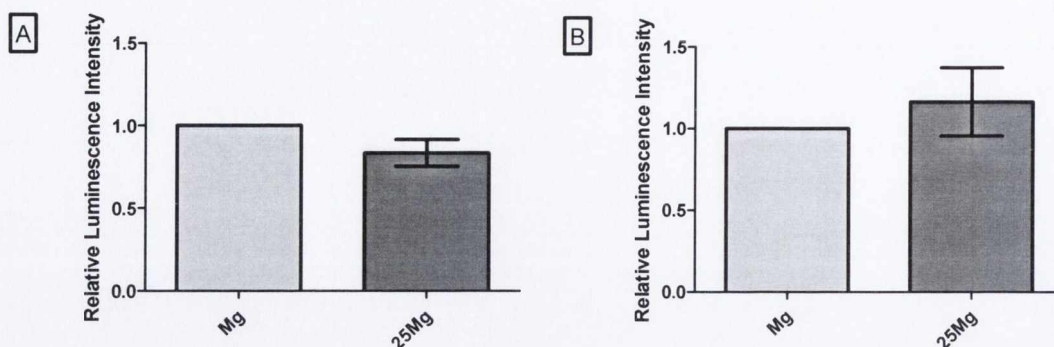


Figure 3.6: Luminescence intensity (proportional to ATP yield) of ^{25}Mg versus natural Mg creatine kinase reactions after 30 min in PBS buffer. The reaction was examined for type I (A) and type III (B) of the MM isozyme. In both cases a significant increase was not observed when comparing ATP yields in the presence of isotopically enriched MgCl_2 (98% ^{25}Mg) versus those in the presence of natural MgCl_2 (10% ^{25}Mg). The charts represent the average ($\pm$ SEM) of 4 independent experiments, where in each case the luminescence intensity was normalised to that of the natural Mg sample, thus indicating the fold change in the ^{25}Mg sample relative to this.

In both sets of experiments there is a small decrease (17%) in ATP (luminescence) for the ^{25}Mg sample with MMI CK. This is not statistically significant (t test; if $p < 0.05$) in the first case (Figure 3.5 A), while it is not possible to judge the statistical significance in the second case since N is only 2 (Figure 3.6 A). On the other hand there is a 16% increase when the 2 MMIII CK experiments are averaged (Figure 3.6 B). These experiments would need to be repeated more times to judge if real changes are occurring, nonetheless they do indicate that the 2 fold (100%) magnetic isotope induced increase in CK's ATP yield is not occurring.

3.3.2 EPR of creatine kinase reaction

The EPR spectra of both CK types reacting in the presence of either MgCl_2 or $^{25}\text{MgCl}_2$ were measured between 3050 and 4050 G and no peaks were observed. Spectra for CK MMIII are shown on in Figure 3.7 and Figure 3.8 for MgCl_2 and $^{25}\text{MgCl}_2$ respectively. Thus, the presence of paramagnetic species such as radicals was not indicated for this reaction.

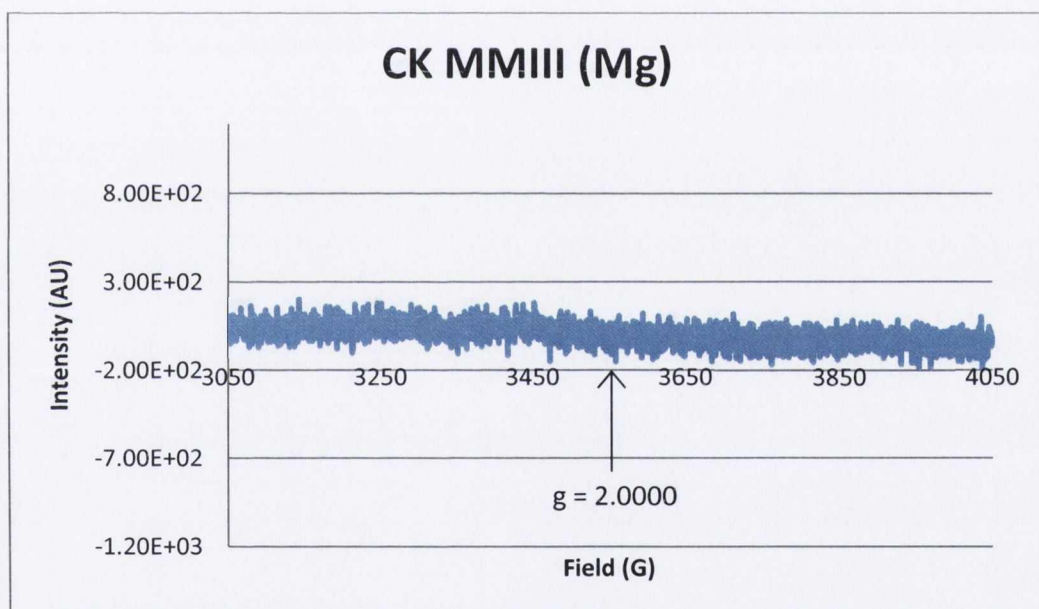


Figure 3.7: EPR spectrum CK MM type III reaction in the presence of MgCl_2 measured between 3050 and 4050 G. Microwave frequency was 9.785 GHz. No peaks were observed.

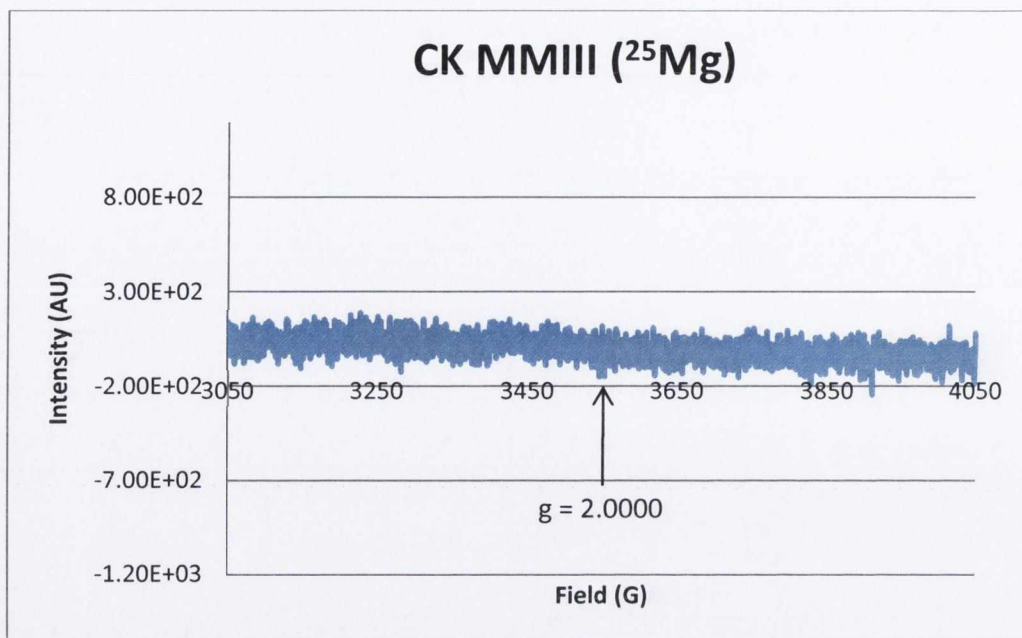


Figure 3.8: EPR spectrum CK MM type III reaction in the presence of $^{25}\text{MgCl}_2$ measured between 3050 and 4050 G. Microwave frequency was 9.780 GHz. No peaks were observed.

3.4 Discussion

3.4.1 ATP production

Our results display no magnetic isotope effect on the production of ATP by MM creatine kinase (Figure 3.5 and Figure 3.6). Potential reasons for this include the possibility that the effect is specific to certain CK isozymes or that reaction conditions such as substrate/enzyme concentrations or pH are different enough to alter the reaction mechanism and remove the effect.

The reaction mechanism at the active site of CK is generally regarded to be conserved across different CK isozymes (MM, MB, BB, Mt) [133, 141-143]. Furthermore, the magnesium isotope effect reported by B&K occurs in a number of different kinases including pyruvate kinase, phosphoglycerate kinase and ATP synthase so it would be surprising if the effect were to occur in one isozyme of CK, but not in either type of MM CK that we tested. The CK isozyme used by B&K is extracted from the venom of a snake *Vipera Xanthia* [8, 129] but details regarding the isozyme and type are not reported. The purified protein is a monomer [129], not a dimer like MM, MB and BB CK. The group also reports an MIE in mitochondrial creatine kinase (CK Mt) [8, 16, 32, 130] which has an octameric structure [133, 144]. It is possible that these non-dimeric varieties of CK operate via a different reaction mechanism to the more commonly studied dimeric forms, but this is not suggested by B&K.

In Table 3.2, the experimental conditions that were used in our study can be compared with those used by B&K [21]. Although conditions clearly differ between the studies, no significant change in the reaction mechanism of the enzyme would be expected due to these differences. The relatively small difference (0.35) in pH and the 8°C variation in temperature would undoubtedly result in differences in overall reaction rates but these differences would affect both isotopically enriched and non-enriched samples equally. The values of the two experiments are in a similar enough range that the reaction mechanism should not differ between them [133, 137, 145]. Thus, our pH and temperature conditions should not be an obstacle to the occurrence of the MIE.

We have used 14 mM $^{25}\text{MgCl}_2$ or MgCl_2 while B&K used 20 mM. In a recent publication [132] B&K state that the ion radical pair based mechanism responsible for the MIE does not operate at normal intracellular Mg^{2+} concentrations (0.21 – 0.24 mM), where the classical nucleophilic reaction dominates. The radical ion pair mechanism functions only when Mg^{2+} concentrations are in the range of 50 – 100 times these values, i.e. 10.5 – 24 mM. So this experimental difference in $^{25}\text{MgCl}_2$ concentration should not explain the lack of an effect since our concentration of 14 mM is within the stated range.

Another difference between the studies is the substrate concentrations (ADP, phosphocreatine and ATP) used. Different CK species and preparations will have different activities and substrate K_m values which determine the appropriate substrate concentrations, so it is expected that these would differ when different varieties of the enzyme are used. Since the specific activity [129, 146] and substrate

K_m [146] values for *V. Xanthia* CK are much lower than for MM CK [147] and the rationale behind their choice of concentrations is not explained, it is difficult to compare their values with ours. We have used 10 times the K_m values in order to ensure Michaelis-Menten kinetics and to minimise the reverse reaction. Again though, it seems unlikely that such changes would do anything other than alter the overall reaction rate without changing the reaction mechanism and this means that the MIE should be preserved despite these differences. On the other hand if ATP concentrations were high enough to compete with ADP then there would be a significant contribution from the reverse reaction ($ATP + Cr \rightarrow ADP + P-Cr$). According to B&K, this reaction does indeed have a different reaction mechanism, but they have reported it to be insensitive to the MIE [8] and thus, this would also not fit as an explanation, either for the presence of an effect in their reaction or its absence in ours. We have verified that ATP levels were less than 10 % of ADP levels throughout the 30 min reaction in our experiments.

When reporting magnesium isotope effects in CK, the enzyme concentration is not given by B&K [21, 146] so it is not possible to compare these, but again, they should not alter the reaction mechanism or the MIE. The final differences between the studies were the presence versus absence of potassium phosphate and bovine serum albumin (BSA) and the ATP determination method used. Potassium phosphate is not involved in the proposed active site reaction mechanism [8, 20, 127, 132] and it is assumed not to be of importance for the MIE since its role is not mentioned in any publications on the subject. Also our experiments in PBS (sodium phosphate) showed no effect (Figure 3.6). BSA is a protein that is necessary to stabilise the CK

enzyme at low concentrations. It is recommended by the supplier and it is commonly used for this purpose in many enzymes [137]. Interference with the reaction mechanism is not expected. Our ATP determination method was a commonly used commercial luminescence kit based on the firefly luciferase reaction. From the linear luciferase response to various known ATP concentrations (Figure 3.4) it can be seen this method should detect the large, 2 - 3 fold increases in ATP levels reported for the MIE.

Thus, it is difficult to suggest a reason for the lack of an MIE in our case other than a different catalytic mechanism occurring due to differences in the type of CK enzyme used. It seems unlikely that other differences in reaction conditions should negate the MIE for the reasons discussed above. Nonetheless, it remains a possibility and it would indicate a sensitivity to reaction conditions that needs greater emphasis in the literature concerning the effect.

3.4.2 EPR

The EPR spectra showed no peaks for the CK reaction with either CK type or with either variety of magnesium chloride. Thus, no evidence supporting the radical pair based reaction mechanism proposed by Buchachenko et al. was found. Nevertheless, these measurements far from refute this groups claims. It is probable that the radicals in question would be undetectable by conventional EPR due to the low concentrations of enzyme and particularly, the short lifetimes of these species. The proposed reaction rates for the back reaction k_{-1} (Figure 3.1) indicate lifetimes for the

initial radical pair of between 1 and 100 ps , while the other radical species involved in later steps (Figure 3.1) i.e. the magnesium radical ion and the hydroxyl radical are both extremely reactive and unlikely to last long. EPR using spin traps [148] or time-resolved fluorescence-detected magnetic resonance [149] has been used to detect many short-lived radical species and these techniques, coupled with the use of low temperatures during measurement, could offer potential avenues for future research into the phenomenon.

3.4.3 Conclusions

In conclusion, we find no evidence for an increase in enzymatic ATP production when ^{25}Mg is used instead of natural Mg. We worked with two common varieties of MM creatine kinase, type I from rabbit muscle and type III from bovine heart, which differed from the *V. Xanthia* derived CK used for the previous report of an effect by B&K. Accordingly, substrate concentrations also differed, but while these concentration differences may be expected to modify the overall reaction rate, they are unlikely to modify the reaction mechanism that B&K infer from their results. The absence of an isotope effect in our experiments suggests that the MIE does not occur in MM creatine kinase and it remains to be seen whether it may be specifically associated with non-dimerised varieties of the enzyme. As a result, our data cast doubt on the claim that the Mg isotope effect is a large (2-3 fold), robust and ‘universally observable’ effect exhibited by ATP-producing enzymes.

4 Effect of weak extremely low frequency electromagnetic fields on pre-osteoblast development

4.1 Introduction

In this study we investigated whether the major processes of cellular development (see Figure 4.1) could be significantly altered by a 50Hz magnetic field. Such changes due to magnetic fields may have implications for human health, since the 50 and 60 Hz alternating magnetic fields produced by power lines, generators and electrical appliances are now ubiquitous in our environment. They are most commonly encountered at flux densities of 0.01 - 100 μ T [11, 150], but can reach a few mT for those working in power generation/transmission facilities [151].

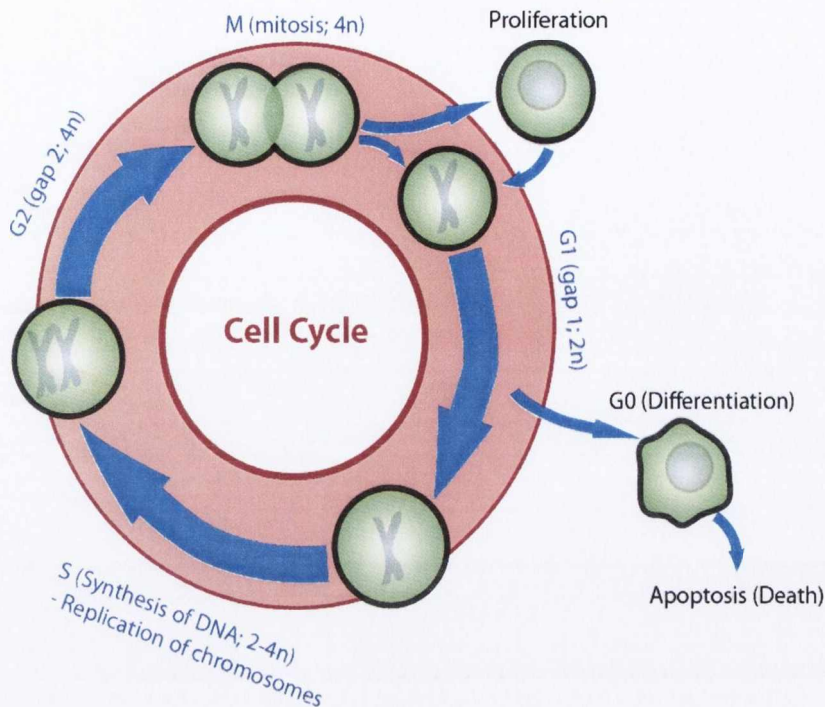


Figure 4.1: Overview of key phases and processes involved in normal cell development. Abnormalities in these processes are associated with cancer and other pathologies. n is the ploidy number representing the number of chromosomes in one complete set – $2n$ and $4n$ imply 2 sets of chromosomes respectively.

Such fields fall under the category of weak extremely low frequency magnetic fields or ELF-MF. Within the bioelectromagnetics literature ELF-MF generally covers a range of AC magnetic fields with frequencies from 10 to 100Hz and flux densities of 0.001 – 10 mT. ELF-MFs have been reported to affect cell developmental processes in numerous cell types. Examples include effects on proliferation and differentiation in neuronal [4, 59, 152, 153], fibroblastic [154], bone [64-66, 155] and skin [67] cells. Although environmental ELF-MFs rarely exceed 100 μ T, fears still persist concerning adverse health effects. The World Health Organisation (WHO) lists these fields as “possibly carcinogenic” [156] and a number of epidemiological studies have shown a correlation between cancer rates and residential proximity to power lines [157-161].

Nevertheless, the increase in risk was usually small and there are at least as many reports showing no correlation [11, 114, 162]. In vitro studies paint a similarly conflicted picture with some showing no effect on cell proliferation [69, 70, 72, 74] and no chromosomal alterations [72-74], while others have reported increased proliferation [154, 155] which is associated with tumour induction and promotion. Amongst the other evidence, Lai et al. [163] have shown increased DNA strand breaks due to 60 Hz 0.1 – 0.5 mT AC fields and Fatigoni et al. [164] observed a genotoxic effect of 50 Hz, 1 mT AC fields. De Nicola et al. reported that 50 Hz, 0.1 mT fields inhibited apoptosis, which, in turn could promote tumour formation [165, 166].

The main concern regarding ELF-MF tends to be cancer but other adverse health effects have been studied. Human studies have reported effects on the occurrence of Alzheimer's disease [167, 168], dementia [168], damage of immune cell DNA [169] and altered red blood cell counts [169]. Animal studies describe evidence for effects on immune cells counts [170] and activity [171]. Reports of toxic effects on cells *in vitro* that do not relate to DNA damage and cancer are few, but Lisi et al. [172] showed a 30% increase in cell death due to glutamate induced excitotoxicity in cortical neurons.

In this study we applied a 2 mT, 50 Hz AC magnetic field to osteoblast progenitor cells (MC3T3-E1). 0.1 - 5 mT is the range of flux densities that is most commonly used when biological effects due to ELF-MF are reported in the literature. 2 mT falls around the middle of this range and around the maximum of the range of human environmental exposures [11, 150, 151]. This field strength was also chosen to be substantially above that of ambient laboratory fields, in order to favour

consistency and repeatability. Osteoblast progenitor cells or pre-osteoblasts were used as they are developmentally located between undifferentiated stem cells and fully differentiated, mature osteoblasts (bone forming cells). Thus, they are ideal for studying shifts along the developmental scale. In the case of pre-osteoblasts, as well as most other cell types, such shifts would have implications, not only for disease, but also for applications where control of cell development is needed. Examples would be tissue engineering, tissue repair and stem cell therapy. ELF-MF studies on osteoblasts and their progenitors have described effects on differentiation, morphology and proliferation [64-66, 155, 173]. Heermeier et al. [64] showed increased differentiation (bone matrix formation) due to a 20 Hz, 6 mT AC field and Zhang et al. [155] also reported increased differentiation along with decreased proliferation in the presence of a 15 Hz, 5 mT ELF-MF. McLeod and co-workers reported that 60 Hz, 1 mT ELF-MFs affected MC3T3 pre-osteoblast morphology by causing a decrease in cell length and an increase in roundness [65], while differentiation was found to be inhibited by 30Hz, 2.54 mT ELF-MF [66].

Changes in cell development are often typical of transformation and tumour promotion. These include altered proliferation, altered cell cycle progression, impaired differentiation and impaired apoptosis. Such changes may be quantified by measurements of cell number (proliferation, apoptosis), nuclear DNA content (cell cycle, apoptosis), cell and nuclear morphology (differentiation, apoptosis) and cellular F-actin (apoptosis, differentiation, transformation). Measured changes in the aforementioned indicators could also signal more general toxic effects such as the cell death (necrosis or apoptosis) and reduced proliferation commonly associated

with toxic agents and disease. They could even demonstrate positive effects, applicable to tissue engineering, tissue repair and stem cell therapy particularly if increased differentiation was observed. In a study by Manni et al. [67], human primary keratinocytes were shown to be substantially more differentiated following exposure to a 50 Hz, 2 mT field when compared to controls. The associated developmental changes included reduced proliferation, altered morphology and altered F-actin distribution.

The broad, 'catch all' nature of our investigation, coupled with the nature of the measurements required, made high content screening (HCS) systems an ideal tool in this instance. These systems combine automated fluorescence microscopy with automated image analysis to produce large quantities of data from individual cells to whole wells of a multiwell plate. HCS systems are becoming increasingly important and powerful tools for the screening of drugs for both efficacy and toxicity [174-177]. In this study we used such HCS systems to examine the effect of a 50Hz, 2 mT magnetic field on pre-osteoblast development.

4.2 Methods and materials

4.2.1 Cell Culture

The MC3T3-E1 osteoblast progenitor cell line was purchased from ATCC (Manassas, VA, USA). The cells were cultured in α -minimum essential medium (α -MEM, Hyclone, Belgium), supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), 10% FBS, 2mM L-glutamine and 1 mM sodium pyruvate. This was replaced every 3 days. The cultures were kept in a humidified atmosphere with 5% CO₂ at 37°C. 75 cm² flasks were used to expand cell number. After the cells reached confluence, they were harvested and seeded at 800 cells/cm² into 96 well plates (Nunclon Δ , Nunc, Denmark). Then ascorbate (50 μ g/ml) was added to the medium of all wells. Ascorbate is a requirement for the development of fully mature osteoblasts, but it has little effect on the rate of differentiation up to day 9 or 10, after which proliferation stops [178]. Thus, over the relatively short time periods used for this study the cells were in a state where both differentiation and proliferation were occurring.

4.2.2 ELF exposure

Five hours after seeding, when the cells were fully adherent, the plates were placed in a solenoid (20 cm L x 12 cm d, 2000 turns/m) within an incubator, which received current from an external bipolar amplifier (Kepco, NY, USA) connected to a TG 1010A 10MHz DDS function generator (Thurlby Thandar Instruments, Cambridgeshire, UK). This produced a 50 Hz, 2 mT, sinusoidal AC magnetic field (Figure 4.2 A), which was characterised using a Hall probe gaussmeter (Lakeshore, OH, USA) - see Figure 4.2 B. The solenoid was fabricated and characterised by Seosamh O'Braonaigh (Research group of Prof. Mike Coey). The exposed cells were placed in the middle of the solenoid where the MF was uniform (Figure 4.2 A and B).

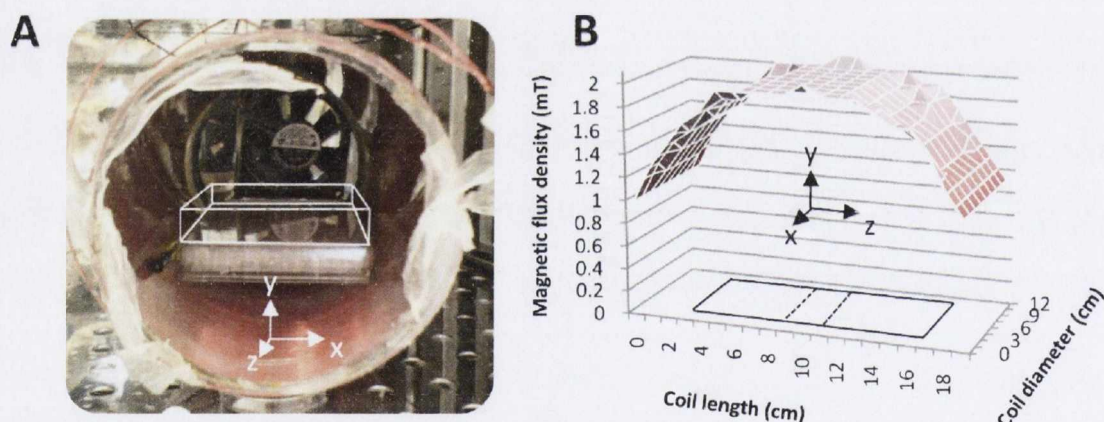


Figure 4.2: For A, B and C the z-axis is the long axis (length) of the solenoid. (A) Solenoid, 96-well plate and fan setup within incubator. (B) Profile of magnetic flux density mapped along the central horizontal plane of the solenoid using Hall probe gaussmeter. The 96 well plate's position with respect to the solenoid's field is shown by the solid rectangle. The analysed wells were located between the two dashed lines, i.e., within the area of homogenous magnetic flux density.

The maximum electric field induced within the medium in the wells was calculated from a finite elements model of our exposure setup using COMSOL Multiphysics (COMSOL Inc., LA, USA) (Figure 4.3). It was estimated that the maximum electric fields induced in the well medium were 1.7 mV/m (Figure 4.3) while those inside the cell were calculated to be around 45 μ V/m. The electric field in the medium decreased exponentially from the centre of the well to 8.5 % of its maximum at the edges (Figure 4.4). These simulations indicate that the same electric field was induced in the medium of all the wells of the plate (Figure 4.3 and Figure 4.4 B). For more detail on the methods used for these FEM models, see chapter 5.

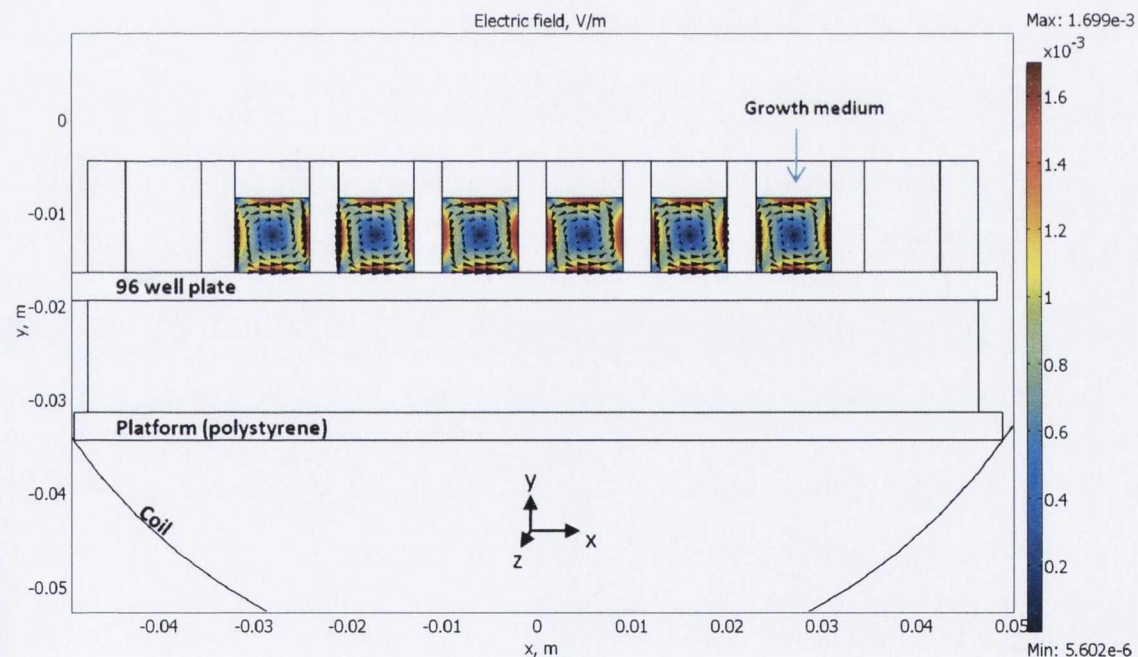


Figure 4.3: Finite elements model showing induced electric fields in the medium contained by a row of wells within our solenoid. The Colour map indicates electric field strength and the arrows indicate field direction.

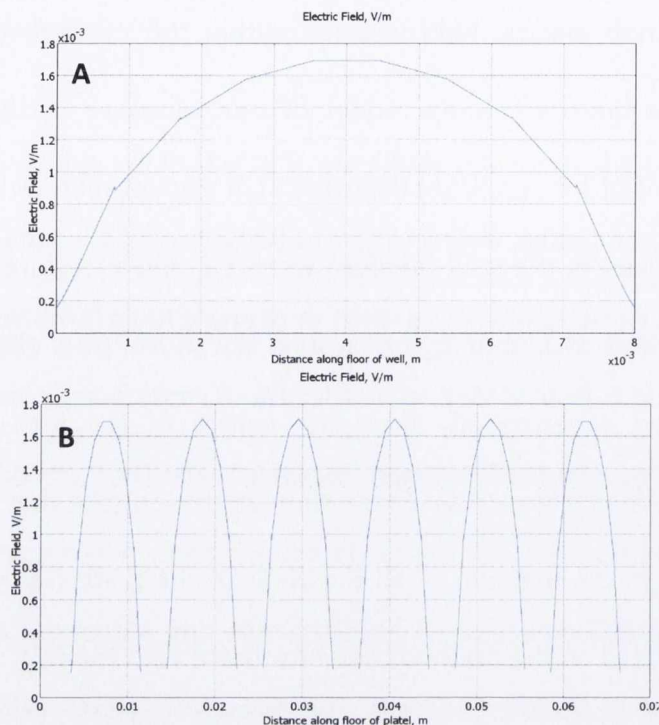


Figure 4.4: (A) Electric field induced along the floor of a well of a 96 well plate by a 50 Hz, 2 mT magnetic field that is directed parallel to the floor. (B) The same electric field shown across 6 adjacent wells.

Exposure times were for 24 and 48 hr for all measured parameters. For each experiment there were three plates, exposed (Exp), sham and control (Ctrl). The sham and exposed plates were placed in bifilar solenoids where the active exposure coil was connected in series to produce a 2.00 ± 0.01 mT field and the sham coil was connected in anti-series so that the magnetic fields cancel. The same current passed through both coils, but the sham did not generate a detectable magnetic field (± 0.01 mT). Thus, both sham and exposure coils generated the same amount of resistive heating so that the only parameter that differed between them was the presence of the magnetic field. The control plate was placed in the same incubator away from the solenoids and experienced effectively no AC magnetic field (± 0.01 mT). All fields were measured using a Hall probe gaussmeter (Lakeshore, OH, USA). Temperatures

in the medium in all 3 plates were stable at $37.0 \pm 0.1^{\circ}\text{C}$ as measured by a thermocouple connected to an electronic thermometer (TES 1316, Radionics, Dublin, Ireland). Heat generated by the current flowing through the two solenoids was dissipated using two small environmentally protected fans (Comair-Rottron, London, UK).

4.2.2.1 *Fluorescence staining*

After exposure, all cells were fixed with 4% formaldehyde for 30 min and washed twice with PBS. The actin cytoskeleton was stained with the fluorescent marker, phalloidin-BODIPY FL ($\lambda_{\text{ex}}=503\text{nm}$, $\lambda_{\text{em}}=512\text{nm}$; Invitrogen, OR, USA). The cells were first permeabilised with 0.1% Triton, followed by 20 min incubation in 165 nM phalloidin-BODIPY. After this period they were washed once with PBS, then the nuclei were stained with 2 $\mu\text{g}/\text{ml}$ Hoechst 33342 ($\lambda_{\text{ex}}=355\text{nm}$, $\lambda_{\text{em}}=465\text{nm}$; Invitrogen, OR, USA) for 10 min and finally, they were washed twice with PBS.

4.2.2.2 *Imaging and analysis*

Measurements were carried out using the GE IN Cell 1000 (GE Healthcare, WI, USA) and the Cellomics KineticScan (KSR) (Thermo Fischer, MA, USA) automated fluorescence microscope systems and their accompanying analysis software. To obtain the proliferation data (cells per well) entire wells were imaged with the KSR, followed by counting of the stained nuclei by the software. For the other measurements, 12 random images per well (200 - 600 cells) were taken using the IN Cell system. From these, again using the analysis software, the fluorescent staining

intensity of F-actin and DNA was isolated in each cell and quantified. To produce the morphological data the IN Cell Analyzer software sequentially scans each microscope field image and isolates and outlines the individual cells along with the nuclei inside them (Figure 4.5). This is achieved via a multi-scale top-hat segmentation algorithm. After this, further algorithms are applied to quantify each cell's shape and size parameters. Other algorithms measure the intensity and intensity distribution of pixels bound within the cell and nuclear outlines. A list of the measurement parameters used, along with explanations, is given in Table 4.1 and 4.2. Each microscope field image is sequentially analyzed by the software and measurements per cell, per field and per well are provided for the whole plate.

To verify that the automated imaging systems were capable of detecting changes, particularly toxic changes, in MC3T3-E1 cells, we treated them with the chemotherapeutic drug, paclitaxel (0.5 μ M, Sigma-Aldrich, MO, USA), for 48 hrs. Large and significant changes were seen compared with controls. For example F-actin spreading was 0.52 ± 0.05 of the control. Hoechst (DNA) intensity was 3.16 ± 0.10 and cell number was 0.57 ± 0.05 . Thus clear and significant changes in measured parameters resulted from treatment with a known cytotoxic agent. The experimental procedures along with the selection of parameters and image analysis algorithms were based on the previous experience of the group with toxicity testing using high content screening systems [179-182].

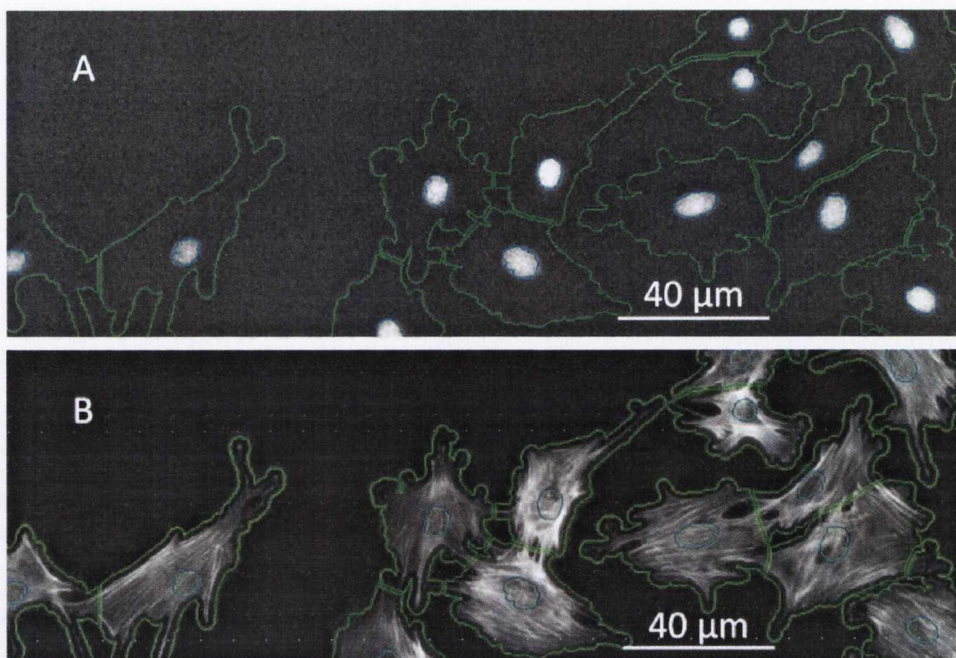


Figure 4.5: Typical screenshots showing automatic isolation and measurement of morphology and DNA and F-actin fluorescence intensities by the IN Cell 1000 Analyser software. A) Blue channel showing Hoechst 33342 stained DNA (nuclei). B) Green channel showing phalloidin-BODIPY stained F-actin (cell/cytoskeleton).

<i>Parameter</i>	<i>Description</i>
Cell elongation	The ratio of the short axis of the cell to the long axis of the cell. If the value =1 then the object is centre-symmetric (not elongated).
Cell area	The area of the cell body segmented in the cell marker channel.
Cell gyration radius	A measure of the spread of a cell. Gyration Radius is defined as the square root of the mean squared distance between cell's pixels and its centre of gravity.
F-Actin intensity	Average fluorescence intensity (gray level) of pixels within the cell.
F-Actin intensity spreading	Intensity-based descriptor allowing estimation of the extent of intensity concentration near the boundary of the object. If the value =1 then the object is uniformly bright. If the value >1 (<1) intensity concentrates near the border (near the centre).
Cell process count	Count of processes extending from a cell. Quantifies the average count per cell.
Cell process length	Average length of the processes extending from a cell. Quantifies the average process length per cell.

Table 4.1: HCS image analysis parameters for cell channel based on Green fluorescence due to Phalacidin-BODIPY staining of F-actin. These parameters are relevant to various processes associated with cell development and this is described in the discussion section of this chapter (0).

<i>Parameter</i>	<i>Description</i>
Nuclear intensity	Average fluorescence intensity of pixels within nucleus.
Nuclear area	The area of the nucleus segmented in the nucleus marker channel.
Nuclear displacement	Distance between the nucleus's centre of gravity and the cell's centre of gravity, divided by the gyration radius of the nucleus.
1/(form factor)	Roundness index. Value ranges from 1 to infinity, where 1 is a perfect circle.
Nuclear elongation	Ratio of the short axis of the nucleus to the long axis of the nucleus. If the value =1 then the object is centre-symmetric (not elongated).
Compactness	Compactness is another measure characterizing shape.
Chord ratio	Shortest chord to longest chord ratio. Both chords pass through the shape's centre of gravity.
Nucleus count	Number of nuclei identified per well. Equivalent to cell number per well.

Table 4.2: HCS image analysis parameters for nucleus channel based on blue fluorescence due Hoechst 33342 staining of DNA. These parameters are relevant to various processes associated with cell development and this is described in the discussion section of this chapter (0).

4.2.3 Statistical analysis

Data were expressed as mean ± SEM. A two-way analysis of variance (ANOVA-2) was employed followed by a Bonferroni test to compare ELF-MF vs. ctrl, sham vs. ctrl and ELF-MF vs. sham data (Prism; GraphPad Software Inc., CA, USA). *p* < 0.05 was considered statistically significant. The average of 3 - 5 replicate experiments ± SEM is presented for each parameter. For more information on statistical methods used see appendix 0.

4.3 Results

Pre-osteoblasts were exposed to a 50 Hz, 2 mT magnetic field for 24 and 48 hours and then various cell development related parameters were quantified using two HCS systems. After analysis of the large number of acquired images, exposed cells were compared to sham-exposed and control cells. No significant differences (ANOVA-1; significant if $p < 0.05$) were seen for any of the morphological parameters such as cell area, nuclear area, cell elongation and the number or length of processes extending from the cells (Figure 4.6 and Figure 4.7). There were also no significant changes in cell proliferation (Figure 4.8 A) , cell DNA levels (Figure 4.8 B), or in F-actin levels and distribution (Figure 4.9).

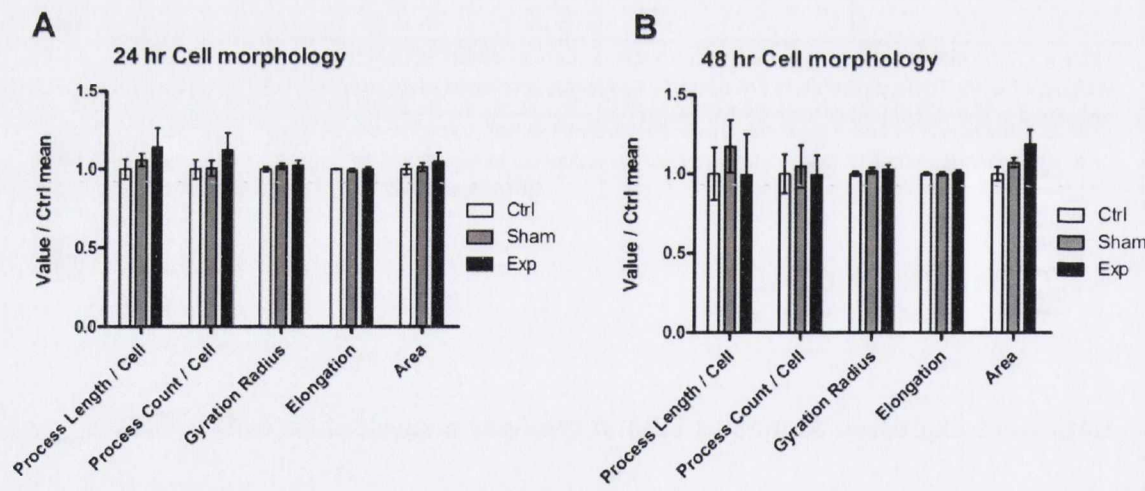


Figure 4.6: Effect of 24 (A) and 48 hr (B) exposure to 50Hz, 2mT AC magnetic field on pre-osteoblast cellular morphology. See Table 4.1 for parameter explanations. Each 24 hr experiment consisted of an average of 1023 individual cell measurements for each treatment group (Ctrl, sham or exposed) and this figure was 1297 for the 48 hr experiment. No significant (ANOVA-2; significant if $p < 0.05$) changes were seen.

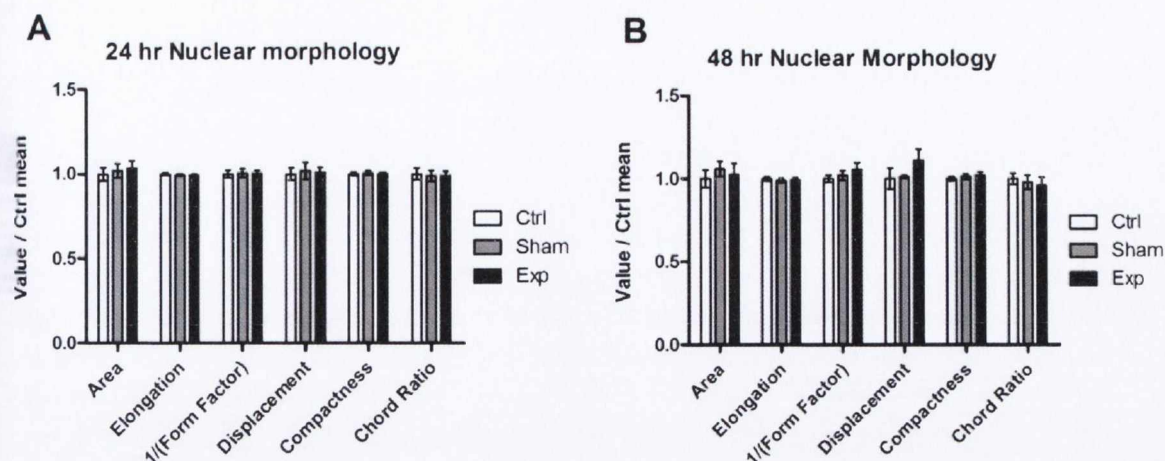


Figure 4.7: Effect of 24 (A) and 48 hr (B) exposure to 50Hz, 2mT AC magnetic field on pre-osteoblast nuclear morphology. See Table 4.2 for parameter explanations. Each 24 hr experiment consisted of an average of 1023 individual cell measurements for each treatment group (Ctrl, sham or exposed) and this figure was 1297 for the 48 hr experiment. No significant (ANOVA-2; significant if $p < 0.05$) changes were seen.

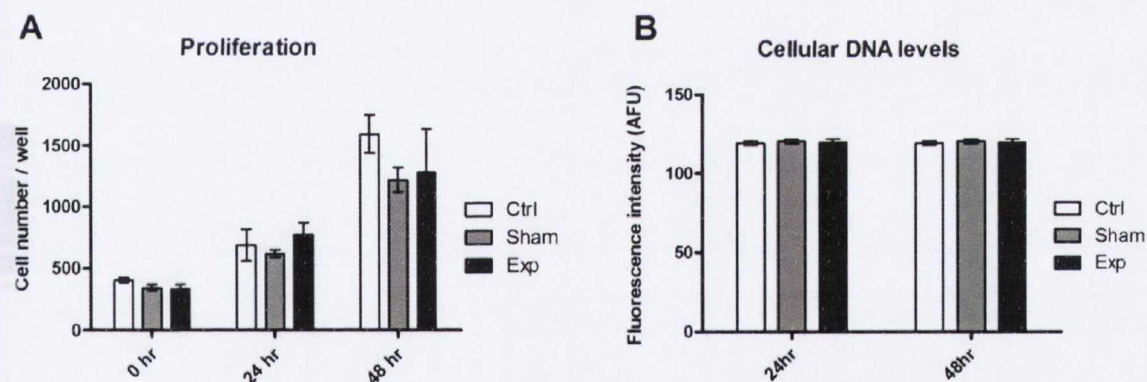


Figure 4.8: Effect of 24 and 48 hr exposure to 50Hz, 2mT AC magnetic field on pre-osteoblast proliferation (A) and DNA levels (B). For B Each 24 hr experiment consisted of an average of 1023 individual cell measurements for each treatment group (Ctrl, sham or exposed) and this figure was 1297 for the 48 hr experiment. No significant (ANOVA-2; significant if $p < 0.05$) changes were seen.

Cell cycle progression was also unchanged in either sham or exposed cells. A frequency distribution representing the % of the total population which falls within each of a number of DNA content (Hoechst intensity) bins (ranges) is shown in Figure 4.10. A cell's DNA content is indicative of its progress in the cell cycle, so that those in later stages (closer to dividing/proliferating) will have higher DNA levels. For example cells in the final G2/M stages which are about to divide should have double the DNA (4n) as those in G1 (2n). At both time-points in these experiments there were practically no cells at this stage and almost all were within a band of lower intensity ranges between 110 and 160 AFU. Thus, most were probably in G0/G1 with a small minority in S. This method of cell cycle analysis is commonly used in FACS studies [183, 184] but it is also possible with HCS systems.

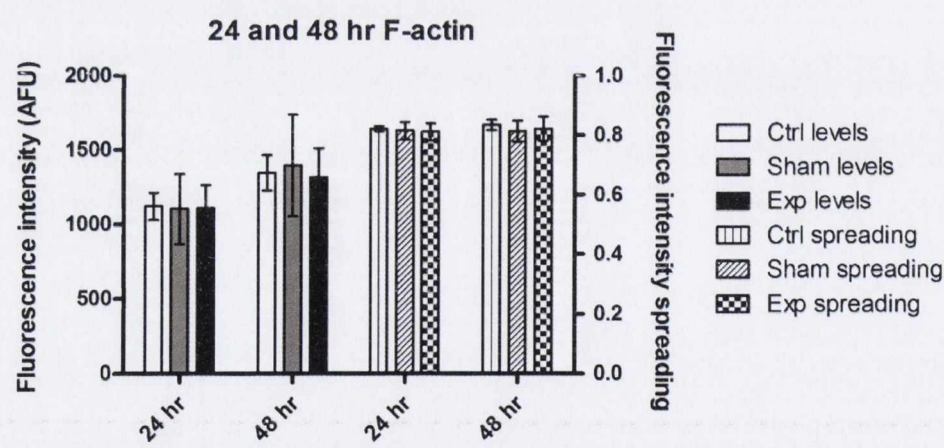


Figure 4.9: Effect of 24 and 48 hr exposure to 50Hz, 2mT AC magnetic field on pre-osteoblast F-actin levels and cell distribution (spreading). See Table 4.1 for parameter explanations. Each 24 hr experiment consisted of an average of 1023 individual cell measurements for each treatment group (Ctrl, sham or exposed) and this figure was 1297 for the 48 hr experiment. No significant (ANOVA-2; significant if $p < 0.05$) changes were seen.

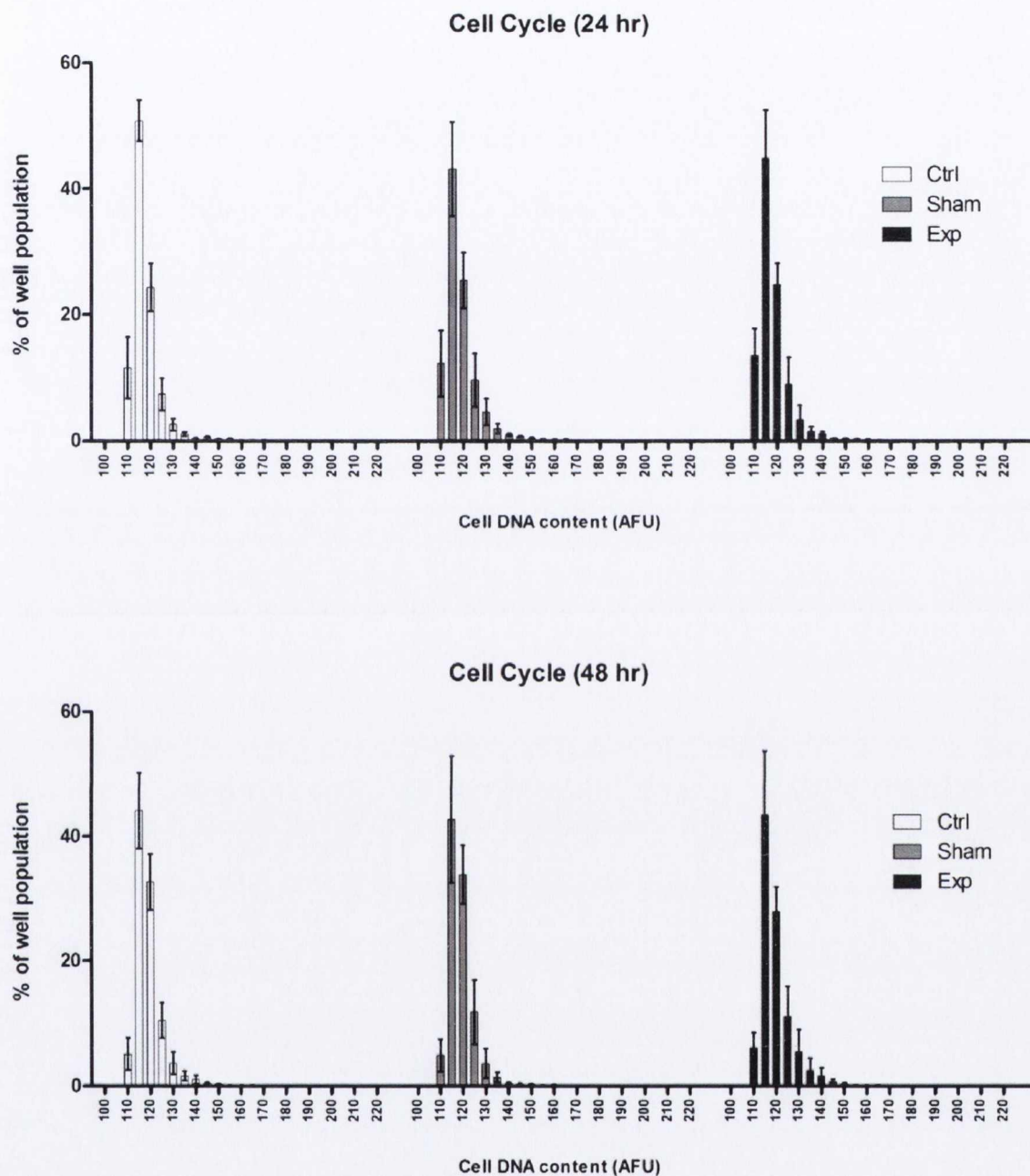


Figure 4.10: Effect of 24 (top) and 48 hr (bottom) exposure to 50Hz, 2mT AC magnetic field on pre-osteoblast cell cycle progression (frequency distribution of cell DNA levels). X-axis shows the range of observed cell DNA levels (arbitrary fluorescence units) divided in into bins of 5 units. Each 24 hr experiment consisted of an average of 1023 individual cell measurements for each treatment group (Ctrl, sham or exposed) and this figure was 1297 for the 48 hr experiment. No significant (ANOVA-2; significant if $p < 0.05$) changes were seen.

4.4 Discussion

In this study we measured a range of indicators of cell developmental change including many morphological parameters, as well as cell proliferation, DNA content, and F-actin content. We saw no change in F-actin levels or distribution (Figure 4.9). Increases in F-actin levels have been shown to be associated with transformation [183, 185, 186]. F-actin levels and distribution are affected by the onset of apoptosis [183, 187-190]. Altered F-actin distribution has also been associated with differentiation [67, 183, 191]. Thus, our results suggest that the 50 Hz, 2 mT AC magnetic field did not affect cell transformation, apoptosis or differentiation. Differentiation is also indicated by changes in cell morphology particularly a progression from the ragged-edged, spindly, fibroblast-like morphology of pre-osteoblasts to that of mature osteoblasts which are smaller with more even edges and a compact, almost trapezoidal shape [192]. Such changes would be detected by our morphological measurements, but no changes were seen in any of the 11 such parameters (Figure 4.6 and Figure 4.7), thus suggesting that no differentiation was being induced. Cellular and nuclear morphology as well as DNA staining/content (following permeabilisation) were also found to be unaltered after magnetic field exposure (Figure 4.6, Figure 4.7 and Figure 4.8). These parameters are indicators of apoptosis [187, 188, 190, 193, 194] so the absence of change suggests no major apoptotic effects during the exposure period. No changes due to the field were seen in cell proliferation which was quantified by measuring the cell number per well (Figure 4.8 A). A feature of differentiation is reduced proliferation while increased

proliferation, on the other hand, is associated with de-differentiation and tumour promotion. These proliferative processes related to (de-) differentiation and tumour promotion were also unaffected by the field over 48 hrs.

Finally we looked for changes in the cell cycle. Shifts in the number of cells at each stage of the cycle were not evident from our analysis (Figure 4.10), indicating that the field did not alter cell progression. Progression to a further stage in the cell cycle (see Figure 4.1), as determined by the osteoblast's DNA content ($2n \rightarrow 2-4n \rightarrow 4n \rightarrow 4n$), is another indicator of a shift towards proliferation and away from differentiation and apoptosis [183, 190]. Cancer cells exhibit a poorly regulated cell cycle, accelerated proliferation, reduced differentiation and apoptotic capability [Hanahan and Weinberg, 2000]. Thus, our cell cycle measurements correlate with the rest, suggesting no field effect relating to proliferation, tumour promotion, apoptosis or differentiation over the 48 hr period.

Our results indicated that 50Hz, 2 mT AC ELF-MFs do not significantly affect any of the 16 measured parameters over a 48 hr exposure period, thus suggesting that such fields would not significantly alter osteoblastic cell development over this period. This also suggests that strong toxic effects on osteoblastic cells, including transformation or tumour promotion, are unlikely as a result of exposure to ELF-MF radiation over a 48 hr period. Future studies should include additional time points up to 10 days or more to allow for a more conclusive toxicological verdict. The results also suggest that fields of this magnitude and frequency applied over one or two days would not be useful for the modification/control of cell development that is needed in bone tissue engineering and repair.

Our null result fits with a large body of the literature [11, 71, 156] but our findings seem to contradict two quite similar studies which is worth discussing briefly. The previously mentioned, Manni et al. [67] and McLeod et al. [65, 66] studies share common measured endpoints (proliferation, morphology, F-actin) with ours and the former uses the same field parameters as ours while the latter uses the same cell type and fields of similar frequency and magnitude (60 Hz, 1 mT_{pp} and 30Hz, 2.54 mT_{pp}). The absence of those effects seen by Manni et al. may be due to ELF-MF effects being cell specific, since keratinocytes were used here. Future work should include multiple cell types. The effects on pre-osteoblasts seen by McLeod et al. were small and only observed in subsections of the cell population that held specific orientations or positions relative to the induced electric field. At the whole well level used for analysis of our results there is agreement with the findings of McLeod et al. Future studies should use the excellent capabilities of HCS systems for analysis of sub-well (single cell and cell sub-populations) information to examine such effects further. It should also be noted that our use of a HCS system meant that each experiment analysed over 1000 cells on average compared to the 30 – 40 cells examined in the case of McLeod et al. and the 150 used for the Manni et al. Study. The automated and entirely consistent imaging and analysis across all wells and plates provided by the HCS system should also reduce the possibility of bias and human error, when compared to the conventional (non-automated) fluorescence and confocal microscopy used in these other studies.

Further research using other cell types, time points and magnetic field parameters will be required in order to draw robust and general conclusions

regarding the effects of ELF-MF on cell development, but these negative results, nonetheless, contribute to this on-going endeavour. This contribution is emphasized by recent findings concerning the vital importance of negative results and the chronic under-reporting of such data in the scientific literature [195].

4.4.1 Conclusion

We measured 16 cell developmental parameters via a HCS system but found no significant changes in osteoblast progenitors due to a 50 Hz magnetic field and thus we conclude that, over a 2 day exposure period, it is unlikely that such a field would induce significant developmental changes in osteoblastic cells and hence, also that it is unlikely that major toxic, carcinogenic or tumour promoting effects would result in these cells under such conditions.

5 Magnetic Stimulation of Cultured Neurons *In vitro*

5.1 Introduction

Magnetic Stimulation (MS) involves the use of strong pulsed magnetic fields to stimulate electrically excitable tissue in the brain and peripheral nervous systems via an induced electric field (E) – see Figure 5.1. Magnetic fields are not absorbed by biological tissue like externally applied electric fields. As a result deeper stimulation can be achieved and the pain associated with electrodes applied to the skin is avoided. It has been a valuable tool in experimental neuroscience for over 20 years, where it has been used to map brain regions by localised disruption of normal brain function [196, 197]. This allows for correlation of the physical brain region that is disrupted with the neurological/psychological function that is blocked. For the establishment of cause effect relationships between brain structures and brain phenomena this technique is superior to fMRI and such information was previously only available through the consequences of brain injury. Assessment of pharmacological effects on neuronal excitability is another common application in research [83]. More recently, MS has been used for clinical applications including the diagnosis of multiple sclerosis [81, 82] and the treatment of stroke [198] and Parkinson's disease [79, 80].

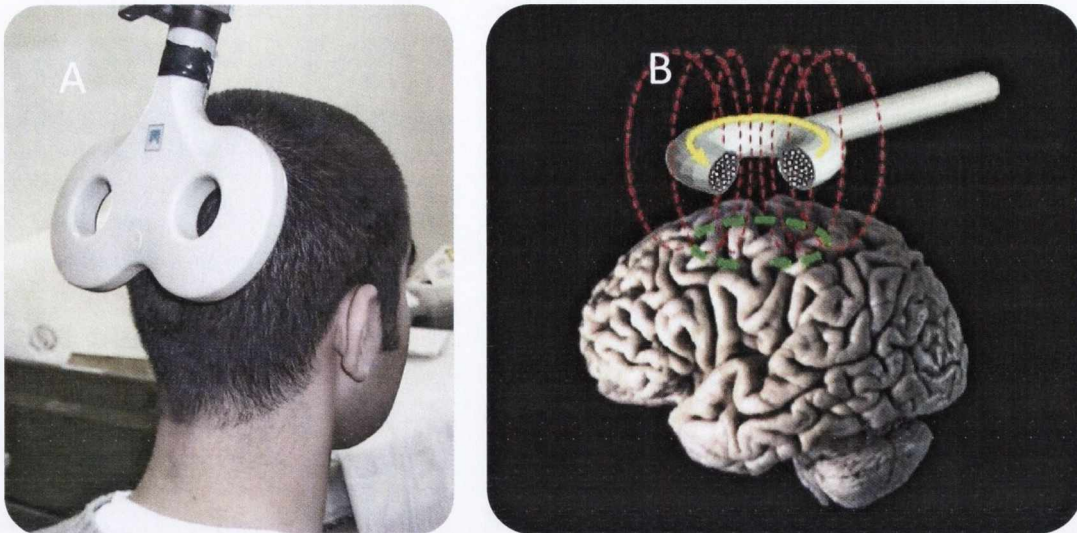


Figure 5.1: (A) Figure of 8 MS coil being applied to stimulate the brain of a patient. (B) A current pulse (yellow arrow) around a circular coil produces a magnetic field pulse (red dashed lines) that induces a circular electric field in the brain (green arrow). A is taken from <http://www.popsci.com/science/article/2010-03/bending-morality-magnetism>. B is taken from Wasserman et al., 2008 [196]

Despite these applications over the last two decades, *in vitro* magnetic stimulation in cultured neurons was only demonstrated for the first time in 2008 by Rotem and Moses [10] of the Weizmann Institute. Here, and throughout this thesis, *in vitro* refers to cell culture experiments as opposed to those on extracted tissue/organs (*ex vivo*) and those on whole, living multi-cellular organisms (*in vivo*). Of the three (to our knowledge) other *in vitro* MS studies carried out so far, one has shown neuroprotective effects [199], one has caused inhibition of spontaneous neuronal firing [200] and the other showed no effect [201], but none showed any magnetically induced stimulation of neuronal firing. According to Rotem and Moses, the main reason for the lack of an *in vitro* response in such studies is the fact that cell cultures generally exhibit random orientation and positioning in relation to the induced E. They argue that neurons must exhibit alignment of their axons with the induced E and also be positioned close to where E is strongest in order for

stimulation to occur via MS. When they used an *in vitro* system where neurons were grown in “1-dimensional” rings aligned with the circular E induced by the MS coil they found they could achieve repeatable neuronal excitation as long as the ring radius was large enough. Under these circumstances, E is proportional to the ring radius by Faraday’s Law (5.8). They could not, on the other hand, achieve excitation of standard unpatterned neuronal cultures (random orientation) covering the same radius. In this way they demonstrated their claims and formulated an *in vitro* method for MS in cultured neurons. They compared their experimental results to what is predicted by the 1-dimensional cable equation, the model used by most computational neuroscientists to describe the dynamics of the membrane potential in the axons and dendrites of neurons. Largely, good agreement was found between experiment and theory, allowing explanation of most of their findings.

Rotem and Moses also found that, unlike MS, direct electric field stimulation (neurons between two electrodes) was not dependent on the orientation of the neurons. This is surprising, since the induced E is generally regarded in the MS literature as having essentially the same properties as a direct E, at least for the purposes of stimulation [196, 202]. It is also a fundamental assumption of the cable theory used in most theoretical models of magnetic and electric field stimulation [89, 202-204]. It has been reported previously that stimulation via a direct DC electric field applied *ex vivo* to hippocampal brain slices is dependent on neuronal orientation relative to the field [205]. Nevertheless, the effects of the complex and inhomogeneous brain tissue in modifying the applied field via charge accumulation are difficult to predict and the CA1 neuronal pathways that were aligned parallel with

the field will have other connected pathways aligned perpendicular. All this leaves some doubt when it comes to the orientation dependence of electric field stimulation. A conclusive finding on subject is important for MS and also its sister electrical stimulation techniques such as transcranial direct current stimulation (tDCS).

The Rotem and Moses study is an important step in the investigation of MS effects since it should open up many routes of research previously slow, expensive or simply unfeasible *in vivo*. *In vitro* studies allow for much greater control of experimental conditions, provide cell-level information more easily and precisely and can offer much higher throughput acquisition of data. Where *in vitro* studies would have most obvious advantage would be studies looking at the effect of many different pulse trains frequencies on a cellular process or where many cellular processes and/or cell types need to be examined under highly controlled conditions. The detailed biophysics of MS should also be more easily examined under such controlled conditions.

Our work aimed to reproduce the *in vitro* MS of neurons seen by Rotem et al. and to further develop the *in vitro* model of MS experimentally, with the aid of finite element computational models based on solving Maxwell's equations in 2D rather than the cable equation in 1D. The orientation dependence of electric field stimulation of neurons has never been demonstrated *in vitro* and there is a lack of conclusive evidence for this in the literature, thus we have also examined such orientation dependence in patterned neuronal cultures.

5.1.1 Theoretical Background

Neurons transmit information both chemically, at the synapse between neurons, and electrically, within the neuron itself (see Figure 5.2). Thus, they are electrically excitable and they owe this excitability to the properties of their membrane. Like all cells, they exhibit a resting membrane potential (V_m), due mainly to differences in K^+ and Na^+ concentrations on either side of the membrane. The resting potential across a neuronal membrane (V_m) is much larger than that for non-excitable cells, being usually around -70 ± 10 mV, with intracellular (cytoplasmic) potential negative with respect to extracellular potential by convention. This resting potential is maintained via active, ATP (energy)-dependent, ion pumps/exchangers in the membrane.

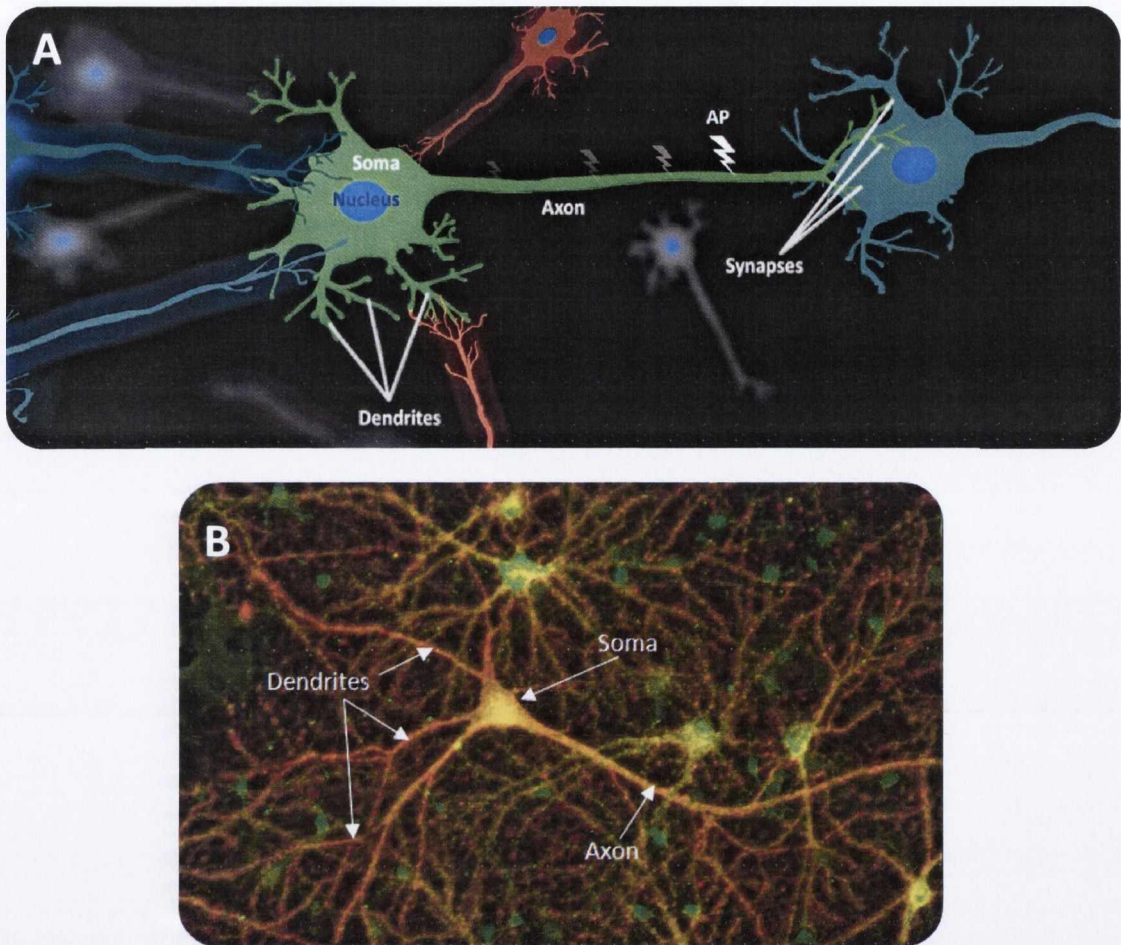


Figure 5.2: (A) Schematic of a neuron (green) interacting with other neurons (blue and orange). An action potentials (AP) travel along axons leading to release of neurotransmitters at the synapses with other neurons. (B) Fluorescence micrograph of real neuron interacting with other neurons. *B is taken from <http://stepitup2007.org/article.php?id=648>.*

Neuronal cell membranes also have a large number of embedded voltage-gated ion channels (VGCs) that are closed at resting potential but can open due to increases in the intracellular potential vs. the extracellular potential (i.e. decreased V_m), generally referred to as membrane depolarization. This is usually due to accumulation of positively charged ions at an area along the intracellular face of the membrane. This positive charge repels positively charged protein domains attached to the 'gates' of VGCs. At a certain threshold level, usually equivalent to a ~ 30 mV

rise in potential at the membrane, the force on the positive domains and their attached gates is sufficient to cause a change in conformation that brings about an opening of the channels [89]. This leads to a rapid influx of positive ions such as Na^+ and Ca^{2+} that is known as an action potential (AP). By depolarizing a patch of nearby membrane an AP can generate further APs and thus, an electrical signal can propagate along a neuron. Activation of voltage-gated Ca^{2+} channels at the nerve terminal is the final electrical event in the successful firing of a neuron and it is the primary cause of neurotransmitter release from the terminal into the synapse and thus, also for transmission of a stimulus to another neuron. This is the essential basis for transmission of information throughout the nervous system.

In the literature [10, 89, 202, 203] when describing the electrical behaviour of axons and other neuronal processes the problem is almost always considered as a long cylinder or cable with sealed ends (Cable theory) and a membrane that behaves as a leaky capacitor. The cable is considered 1-dimensional with a lumped axoplasmic resistance (r_a), membrane resistance (r_m) and membrane capacitance (c_m) measured per unit length of cable in units of Ω/m , $\Omega\cdot\text{m}$ and F/m respectively. If an electric field is applied along the axon (cable) a current, I_a , will flow along the inside of the axon until it meets the axon terminal or a sharp bend where positive charge accumulates and the current is reduced. Positive charge accumulation leads to a drop in current along the axon so that it depends on $-\frac{\partial I_a}{\partial x}$, i.e. the negative spatial derivative of the current along the axon. This accumulation of positive axoplasmic (intracellular) charge raises the intracellular potential, V_{in} , but the critical parameter, V_m , will

depend on charge accumulation (current drop) on the intra- and extra-cellular sides of the membrane. Most theoretical treatments of electric and magnetic stimulation set the extracellular potential, V_{ex} , to zero, essentially assuming that charge accumulation is minimal outside the cell so that V_m is entirely dependent on V_{in} (for axons, $V_{in} = V_a$). This is probably a fair assumption *in vitro* where accumulation on external cell surfaces is greatly reduced by free access of ions to the comparatively huge body of conducting medium around them. In the dense 3d networks of tightly packed neurons found in neural tissue, particularly the brain, this assumption would likely lead to inaccuracies. Nonetheless, the assumption will be held for the remainder of this text, unless specified otherwise.

The total current across the neuron membrane (I_m) consists of two elements: (1) The ionic membrane current per unit axonal length (I_{ion}) through passive (leakage) channels, voltage-gated channels and ATP (energy)-dependent ion pumps; (2) The current across the membrane capacitance per unit axonal length (c_m).

$$c_m \frac{\partial V_a}{\partial t} + I_{ion} = I_m \quad (5.1)$$

In the active cable model I_{ion} is governed by the Hodgkin-Huxley equations (see Appendix) and involves voltage-dependent conductances for Na^+ and K^+ which lead to non-linear membrane currents during firing of the AP. The passive cable model, used by Rotem and Moses [10], replaces I_{ion} with I_r which is the current across a fixed resting membrane resistance, r_m per unit length. r_m is essentially constant up to the point of firing so this is a fair approximation when one is not interested in

what occurs after stimulation of firing and just in the required electrical input to achieve it.

Current flowing out of the axon ($\uparrow I_m$), across the membrane surrounding a small axonal segment of length x , corresponds to a decrease in the axoplasmic current, I_a , in the two segments either side of this due to conservation of charge, so that,

$$I_m = -\frac{\partial I_a}{\partial x} \quad (5.2)$$

$$I_a = \frac{\Delta V_a}{r_a \Delta x} \quad (5.3)$$

where ΔV_a is the difference in axoplasmic potential (intracellular potential) between the two ends of the segment and $r_a \Delta x$ is the resistance of the path along a segment of length Δx . r_a is the longitudinal axoplasmic resistance per unit length measured in Ω/m . If we let $\Delta x \rightarrow 0$

$$I_a = -\frac{1}{r_a} \frac{\partial V_a}{\partial x} \quad (5.4)$$

Then, combining (5.1), (5.2) and (5.4) we get the cable equation,

$$c_m \frac{\partial V_a}{\partial t} + I_{ion} = \frac{1}{r_a} \frac{\partial^2 V_a}{\partial x^2} \quad (5.5)$$

Applying an external (e.g. magnetically induced) electric field, E_{app} , produces an electric field, E_x , directed along the length of the axon. This requires the addition of another source of axoplasmic current variation to the cable equation.

$$c_m \frac{\partial V_a}{\partial t} + I_{ion} = \frac{1}{r_a} \frac{\partial^2 V_a}{\partial x^2} - \frac{1}{r_a} \frac{\partial^2 V_{aE}}{\partial x^2} \quad (5.6)$$

Here, V_{aE} is the externally induced voltage along the axon due to E_x . Since $E_x = \frac{\partial V_{aE}}{\partial x}$ we can modify eq. 6.7 to include the electric field as follows:

$$c_m \frac{\partial V_a}{\partial t} + I_{ion} = \frac{1}{r_a} \frac{\partial^2 V_a}{\partial x^2} - \frac{1}{r_a} \frac{\partial E_x}{\partial x} \quad (5.7)$$

The induced current is directed into the neuron in that it increases the axoplasmic current rather than decreasing it like I_m . The term added to describe these currents is therefore negative with respect to I_m .

This final term, which often referred to as the field gradient driving force or function, allows us to relate E_x to $\frac{1}{r_a} \frac{\partial^2 V_{aE}}{\partial x^2}$ and hence also to the current drop or charge accumulation, $-\frac{\partial I_{aE}}{\partial x}$ (as in (5.4) and (5.5)), so that the electric field induced charge accumulation is maximal at regions of the axon where $\frac{\partial E_x}{\partial x}$ is greatest. This can lead to membrane depolarization and action potentials (firing/excitation). Since E_x is always directed parallel to the axon, if it is also parallel to the applied electric field, E_{app} , $\frac{\partial E_x}{\partial x} = 0$ and $E_x = E_{app}$, but if it then deviates from parallel, e.g. the axon bends, E_x will decrease along a gradient proportional to the axon's angle of

deviation. This means that gradual bends will cause low magnitude, sub-threshold charge accumulation while abrupt ones are likely to be sites where excitation would occur (see Figure 5.3). At the axon terminals E_x drops to 0 over a very short distance relative to the axon length. Here there is maximal charge accumulation. This is realistic in the sense that the terminal membrane is > 100 fold less conductive than the axoplasm so maximum charge accumulation would be expected here if currents were directed along the length of the axon.

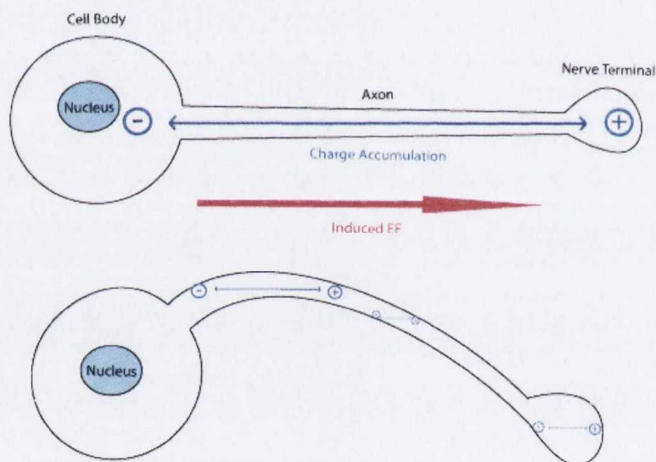


Figure 5.3: Charge accumulation in neurons due to induced electric field (EF). Longer regions of alignment with the EF result in greater charge accumulation and hence, a greater probability of excitation.

From the discussion of E_x above we can explain the orientation dependence of magnetic and electrical stimulation of neurons demonstrated theoretically or experimentally by a number of studies [10, 196, 202, 203, 205, 206] as well as clinical studies in human brains [196]. In order to have a large gradient driving function, it is clear that the axon must have a region closely aligned to E_{app} immediately followed

by a sharp bend or terminal. If no region is aligned with E_{app} then the maximum E_x will be low and thus only E_x gradients of limited magnitude will be achieved.

In modeling the axon we are only interested in $-\frac{1}{r_a} \frac{\partial E_x}{\partial x}$ or $-\frac{1}{r_a} \frac{\partial^2 V_{aE}}{\partial x^2}$ along its long axis (along x). This is because charge accumulation is the driver of E effects on V_m . This will occur so that the applied electric field is opposed to minimise energy. More charge is required to generate this opposing field over longer distances than short ones, since by Coulomb's law E is proportional to the magnitude of the total charge ($E \approx q$), but it is inversely proportional to the square of the distance from that charge ($E \approx q/r^2$). As a result, the sites where current flow is reduced along the long axis, which is 50 – 10,000 times longer than the short axis in real neurons, will almost always have much greater charge accumulation so that accumulation across the axon can be neglected. This idea also explains the length dependence of stimulation predicted and observed by many studies [10, 196, 202, 203, 205, 206], since the longer the distance over which an axon is aligned with E_{app} , the greater the accumulated charge necessary to oppose E_{app} .

All of the above taken together indicates that, to maximize the chances of stimulating via an applied electric field, neurons must have long axons aligned parallel to the field. Maximum depolarisation resulting in firing of action potentials would then be expected at the axon ends. For this reason we have used microcontact printing of cell adhesion proteins to control the growth of our cultured neurons to achieve this field aligned orientation.

E_{app} in our case will be induced by a magnetic field pulse and this can be described using Faraday's law of induction, according to which, the induced electric field E along a closed loop L (with radius r) is equal to the rate of change of magnetic flux, ϕ , through the area, A , enclosed by L :

$$\int_L \vec{E}(t) \cdot d\vec{l} = -\frac{\partial \phi(t)}{\partial t} = -\frac{\partial}{\partial t} \int_A \vec{B}(t) \cdot d\vec{A} \quad ; \quad A = \pi r^2 \quad (5.8)$$

Hence,

$$2\pi r E = -\pi r^2 \frac{dB}{dt} \quad (5.9)$$

$$E = -\frac{r}{2} \frac{dB}{dt} \quad (5.10)$$

Within a 2 cm radius of the coil axis and 2 cm above or below it, our MS coil (Figure 5.4) produces a spatially uniform centro-symmetric magnetic field pulse.

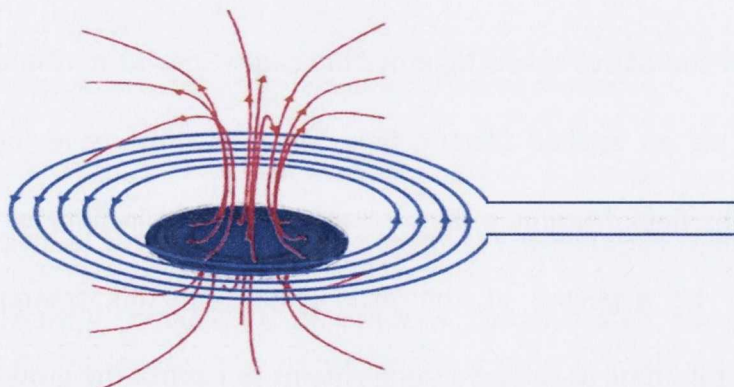


Figure 5.4: A pulse of electric current is discharged into a circular coil (blue rings). This induces a time varying magnetic field (red lines). This magnetic field induces an electric field in the neuronal culture dish (blue disk). The induced electric field is concentric with the coil current. *Taken from: Rotem and Moses, 2008 [10].*

This field is directed perpendicular to the coil plane and it induces an electric field (E) which is directed concentrically with the coil and increases with radius (r) from the coil centre due to Faraday’s law. This gives rise to the three major physical requirements for magnetic stimulation of neurons *in vitro* and these are described in Figure 5.5.

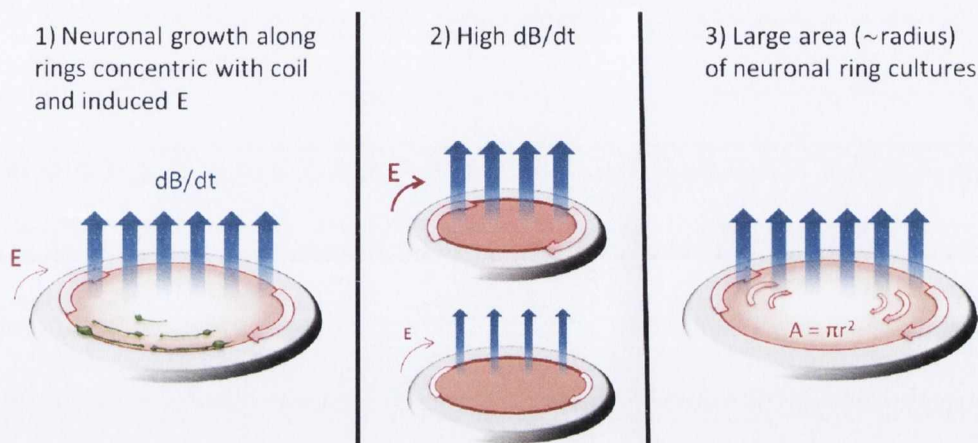


Figure 5.5: Three physical requirements for *in vitro* magnetic stimulation.

For these reasons, in our experiments we have cultured the neurons along a ring concentric with the coil that has a radius of similar dimension to that of the area of uniform MF direction around the coil centre (2 cm). This should allow us to minimise the threshold induced electric field, E_T , required to cause neuronal excitation. It also simplifies modelling of the experiment.

5.2 Methods and materials

5.2.1 Magnetic Stimulator

The device was designed and built in-house by Nigel Carroll with the help of Dr. Adriele Prina-Mello and Dr. Plamen Stamenov. It is similar in design and function to commercial transcranial magnetic stimulators such as the Magstim (UK) and MagVenture (Denmark) devices. The stimulator consists of two major components. A high voltage source is used to charge a capacitor bank. A switching circuit then discharges the capacitor to feed a current into a flat 90 mm coil (Figure 5.6). This coil current produces a magnetic field that is homogenous in strength and direction at 2 cm from the coil centre. This homogenous region extends from here to form a circular area with a radius of 2 cm. Other important specifications of the stimulator and the fields it generates are summarised in Table 5.1.

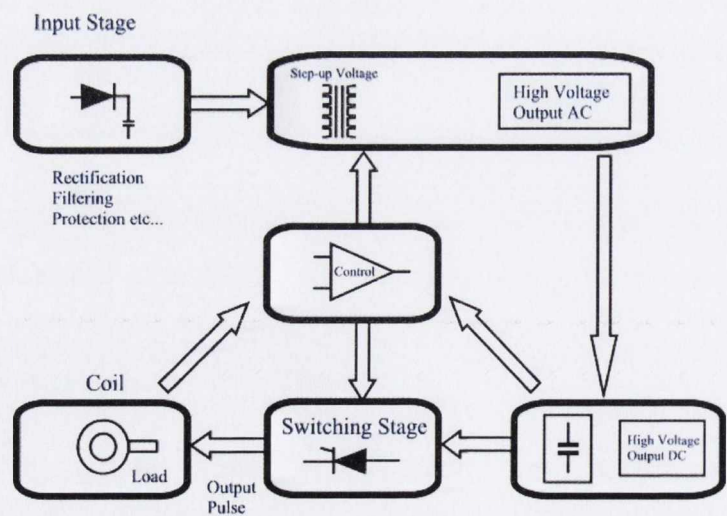


Figure 5.6: Block diagram of magnetic stimulator.

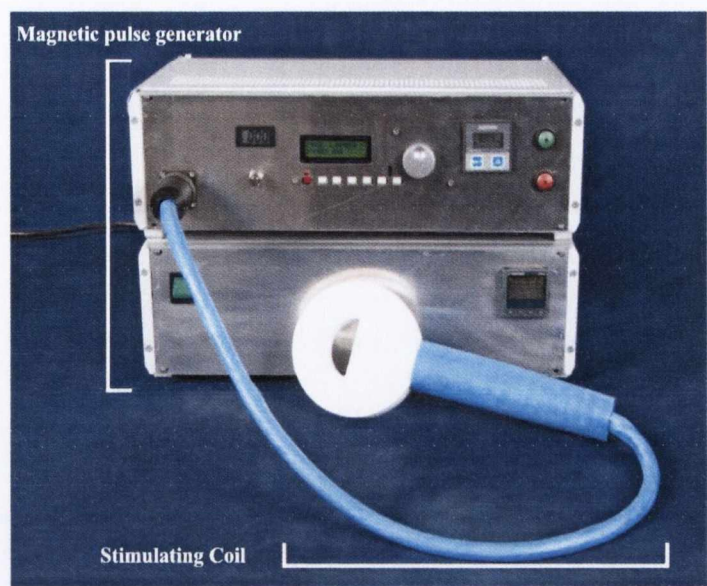


Figure 5.7: Magnetic stimulator with attached 90 mm flat coil.

<i>Pulse Generator</i>	
Capacitance	172 μ F
Capacitor Voltage	0 – 1.5 kV
<i>Coil</i>	
Inner Diameter	66mm
Outer Diameter	123mm
Turns	14
Inductance	23.5 μ H
<i>Output</i>	
Magnetic Field	0 – 2.1 T
Magnetic Pulse rise time	90 μ s
Average dB/dt	23 kT/s at 1.5 kV
Max dB/dt	36 kT/s at 1.5 kV
Peak Induced Electric Field	366 V/m at 2cm from coil axis
Homogenous Field	2cm from coil axis, 2cm above and below coil plane

Table 5.1: Key magnetic stimulator specifications.

5.2.2 Patterning of cell substrates

The microcontact printing (or soft lithography) method was used to pattern the cell adhesion promoter laminin (matrix protein) on glass substrates. See Figure 5.8 and Figure 5.9 below for an overview of the procedure.

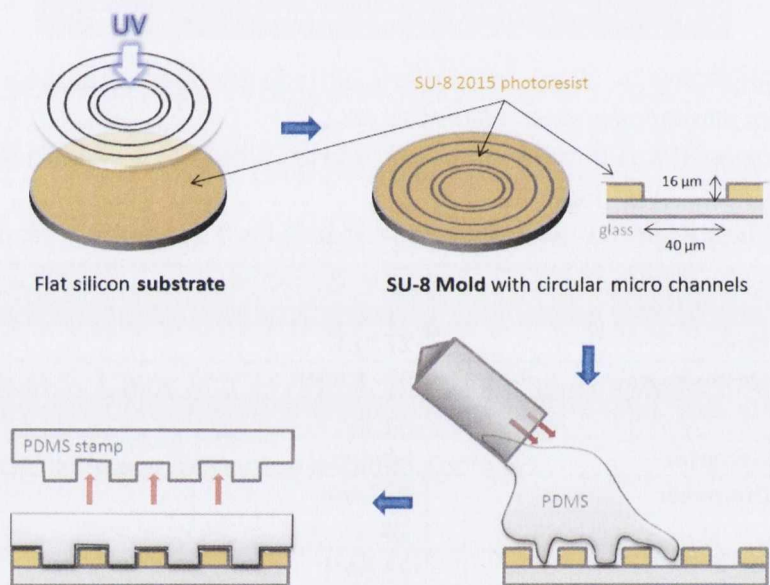


Figure 5.8: Schematic of the process used to make PDMS stamps for microcontact printing. Photolithography is used to make a patterned mold from the thick photoresist, SU-8 2015. Then the PDMS elastomer (+ crosslinking agent) is poured over the mold and after a few hours at 70°C it sets to form a removable stamp ready for printing.

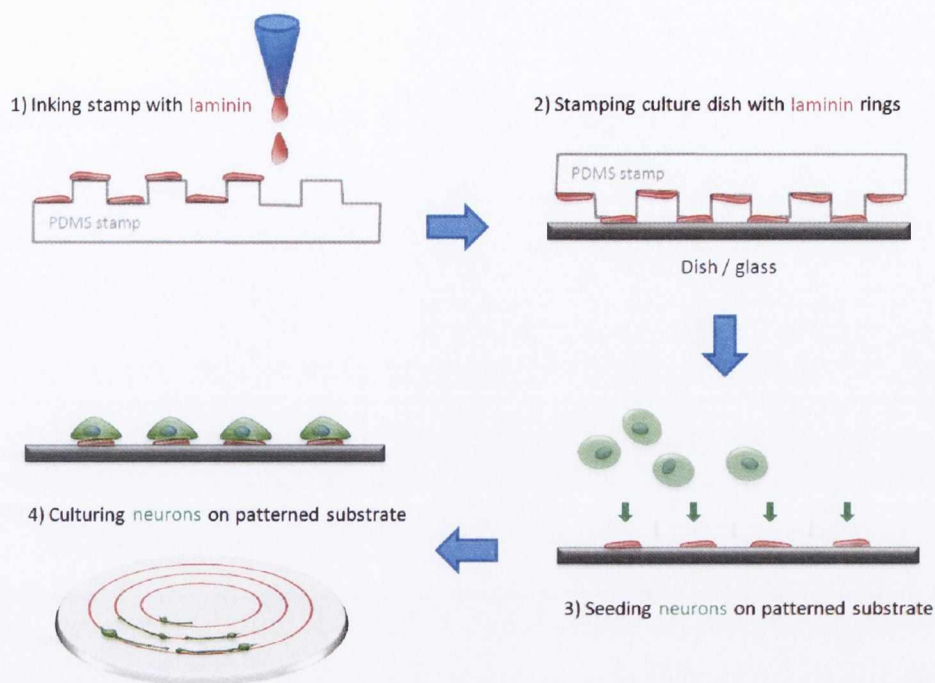


Figure 5.9: Schematic of the microcontact printing method used to produce patterned neuronal cultures.

5.2.2.1 SU8-2015 Mold Fabrication – UV Photolithography

All mask fabrication and photolithography steps were done in a class 100 cleanroom.

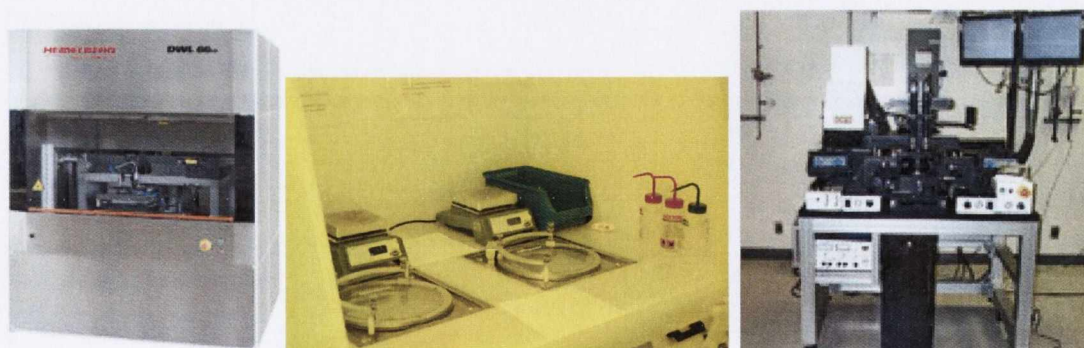


Figure 5.10: Clean room equipment used for the UV photolithographic fabrication of the SU-8 2015 mold later used to generate a PDMS stamp for microcontact printing. From left to right: Laser mask writer; spin coaters; UV mask aligner.

Mask Fabrication

UV masks patterned with 40 μm wide rings or lines and 40/80 μm spacing were designed using Layout Editor software (Jurgen Thies, Germany). These files were inputted into a Heidelberg DWL66 laser mask writer (Heidelberg, Germany; Figure 5.10) which reproduced the design on quartz masks coated on one side with chromium. Chemical etching (hydrofluoric acid) of the mask removed the chromium layer only where the pattern specified, leaving these areas transparent to UV light. The laser writing and etching procedure was carried out by Dr. Huseyin Kurt (research group of Prof. Mike Coey).

Mold Fabrication by UV photolithography

For each mold, a 4" silicon wafer was first cleaned in piranha solution (3:1 mix of concentrated sulphuric acid and 30% hydrogen peroxide) for 15 min followed by drying with compressed N_2 and then for 30 min on a hotplate at 200°C. The substrate was then spin coated with SU-8 2015 negative photoresist (Micro Chem, CA, USA) using a single spin cycle where the spin speed was ramped to 2500 rpm at 10000 rpm/s and held at this speed for 30 seconds (Figure 5.10). Due to the very high viscosity of SU-8 2015, fast acceleration was necessary to reduce formation of tendrils and to force bubbles to the edges. The wafer was then softbaked on a hotplate at 95°C for 4 min or until most visible surface wrinkles had disappeared. The wafer was next placed in UV mask aligner (OAI, CA, USA; Figure 5.10) along with the mask. The resist was exposed through the mask to UV light at 14 $\text{mJ}/\text{cm}^2/\text{s}$ for 9 s.

The UV light fully polymerises the exposed SU-8 2015 resist so that it becomes a hard stable structure which will not be removed by solvent during the development step (below). A post-exposure bake was then performed on the hotplate at 95°C for 5 min and after this, the pattern was developed by submerging the wafer in Micro EC solvent (Micro Chem, CA, USA) for 4 min (or until 10 s after the pattern initially appears), followed by rinsing with isopropyl alcohol (IPA; Sigma-Aldrich, MO, USA). Finally, the patterned wafer was hard baked at 150°C for 15 min. All this produced rings or lines of SU-8 2015 resist that were 16 μm high and 40/80 μm wide as determined by a Dektak 150 profilometer (Veeco, NY, USA).

5.2.2.2 PDMS stamp fabrication

The SU-8 2015 patterned area of the wafer (mold) was surrounded by a stack of heavy copper rings and this circular enclosure was sealed where it met the wafer with petroleum jelly. The elastomer, polydimethylsiloxane (PDMS; Sylgard 184 base, Dow Corning, MI, USA) was mixed in a ratio of 10:1 with the cross-linking agent (Sylgard 184 curing agent, Dow Corning, MI, USA) and poured into the enclosure, over the pattern to a height of 3 mm. This was then degassed and partially cured over night at room temperature. Next it was placed in an oven at 80°C for two hours to complete the curing (cross-linking) process. Finally, the PDMS was cut around the edge of the patterned region and the stamp was gently peeled off the mold.

5.2.2.3 Microcontact printing of laminin onto glass substrates

50 x 50 x 1 mm glass squares were cut into 16 0.9 x 0.9 mm square culture substrates using a DFD6240 dicer (Disco, Japan). These were then sonicated in ethanol for 20 min. They were then dried in a sterile laminar flow hood under sterile-filtered compressed air and laid out in position on a grid marked layer of PDMS which holds the glass substrates steady during printing due reversible adhesion to the glass.

The patterned PDMS stamp was treated with an O₂ plasma for 18 s to make it hydrophilic, thus greatly improving wetting of laminin. This produced more effective printing and reduced the amount of laminin required for inking by 60 – 70%. The stamp was then sonicated in ethanol and dried under filtered compressed air in a laminar flow hood. It was then adhered to a supporting glass square (50x50 mm) and inked with the minimum volume (~ 1ml) of 50 µg/ml laminin solution using a Pasteur pipette. The solution was very gently spread over the stamp using the pipette. The inked stamp was then placed in a sterile incubator for two hours, after which it was rinsed with sterile water and dried with compressed air. Directly after drying the stamp lined up over an array of glass substrates using marked guiding lines and the stamp was placed down on the substrates and light pressure was applied using copper weights for 10 min. After this, the stamp was carefully peeled back off the substrates and these were then placed in wells of a 24 well cell culture plate, ready for seeding with neurons. The stamp was left face down in sterile water until the next printing.

5.2.3 Preparation and maintenance primary rat cortical neurons

In a sterile laminar flow hood, a 1 day old Wistar rat pup (Bioresources Unit, Trinity College Dublin) was sacrificed by rapid decapitation and the two cortical lobes were dissected. All animal procedures were carried out in accordance with national and European Union regulations. The cortical tissue was cut into fine pieces with a sterile scalpel and then digested in 20 U/ml papain in serum free neurobasal medium A (NBMA) supplemented with 1% glutamax, 1% penicillin/streptomycin and 2% B27 at 37°C for 30 min. 3x excess supplemented NBMA with added 10% horse serum to deactivate the papain. This suspension was centrifuged at 200 G for 4 min and the pellet was resuspended in NBMA + 10% HS and then triturated x 10 with a pasteur pipette. Then the cell suspension was filtered through a cell strainer producing the final concentrated suspension of cortical neurons and glia. This was then diluted to 5×10^5 cells/ml and 0.5 ml was added to the wells of a 24-well plate each of which contained a laminin patterned glass substrate (from 5.2.2.3). The cells were left to adhere overnight and then the medium in the wells was replaced with NMBA + 1% HS to inhibit glial overgrowth. The cultures were maintained for 3 – 4 weeks with medium changes every 3 or 4 days. They were ready for use in experiments after 7 days.

5.2.4 Calcium imaging

When a neuron fires, i.e. is excited, voltage-dependent calcium channels open. When they do, there is an influx of calcium ions. This change in intracellular Ca^{2+} concentration can be measured via a fluorescent dye (fluo-4, $\lambda_{\text{ex}}=494\text{nm}$, $\lambda_{\text{em}}=516\text{nm}$; Invitrogen, OR, USA) which greatly increases its fluorescence intensity upon binding Ca^{2+} ions. Fluorescence imaging can then be used to detect such changes. The cells are loaded with the dye and are then imaged in a dye-free recording solution so that only changes in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) are monitored. Bursts of action potentials are represented by fluorescence peaks.

Neuronal cultures were loaded with 2.5 μM fluo-4 (+ 0.02% Pluronic F68) for 45 min at 22°C. They were then transferred to the recording buffer (4 mM KCl, 128 mM NaCl, 1 mM CaCl_2 , 10 mM HEPES, 10 mM glucose, 1 mM MgCl_2 , pH 7.4 at 37°C) used by Rotem and Moses [10]. Stimulation and imaging was performed using this Ca^{2+} containing buffer.

Imaging was performed by an Olympus CKX-41 inverted microscope (Olympus, Japan) (Figure 5.11). Videos were captured using a micropublisher 3.3 CCD camera (QImaging, Canada). Image-Pro software (Media Cybernetics, MD, USA) was used to display a real-time field of view on a computer screen at 3-5 fps. This field of view was captured using Debut Video Capture (NCH software). This method was chosen because Image-Pro cannot capture video above 1 fps. Analysis of calcium fluorescence intensity within regions of the neuronal culture over time was carried out using Image Pro software. Fluorescence peaks representing firing usually rose

over much less than one second and then fell over about 5 seconds. If a fluorescence peak rose in < 1 s, lasted > 1 s, began to diminish within one second and occurred within 1 s of a stimulation pulse it was considered as firing as long as it could be repeated at least once within the next 3 applied pulses. This procedure, which is similar to that used by Rotem and Moses [10], ensured we distinguished between induced and spontaneous firing. Spontaneous firing occurred infrequently and randomly and the resulting increases in Ca^{2+} fluorescence were either gradual over 10s of seconds or relatively fast rising to a constant plateau intensity.

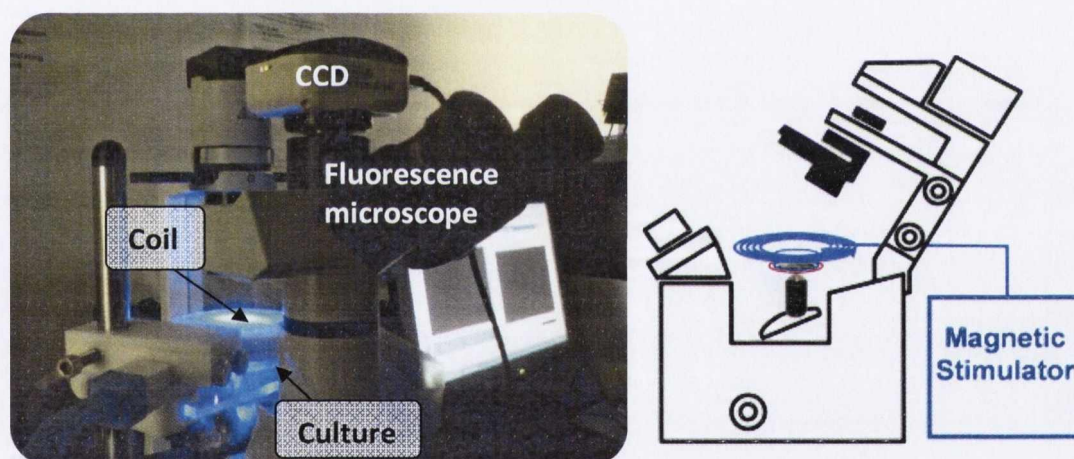


Figure 5.11: Our experimental setup for magnetic stimulation of cultured neurons with simultaneous measurement of fluorescence response (left). Schematic diagram of *in vitro* magnetic stimulation-measurement setup (right) - Taken from: Rotem and Moses, 2008 [10].

To test the excitability of the neurons and to optimise fluorescence imaging conditions a standard depolarizing agent, KCl was added to the recording buffer at a final concentration of 80 mM. This increases the extracellular K^+ concentration enough to depolarize the cell membrane and excite the cell. If neurons had been

unresponsive during a stimulation experiment this KCl test was performed to ensure the neurons were excitable.

5.2.5 Electrophysiology

Whole-cell patch-clamp recordings were carried out on the patterned neuronal cultures by Dr. Sarah Harney (research group of Prof. Roger Anwyl). Patch pipettes were pulled from thin-walled borosilicate glass (World Precision Instruments, Sarasota, FL, USA) and had a resistance of 3–5 M Ω when filled with intracellular solution containing (in mM) 140 K-gluconate, 10 HEPES, 0.2 EGTA, 20 phosphocreatine, 2 Mg₂ATP, 0.3 NaGTP (pH 7.3, 290–300 mOsm). Slices were maintained at 32°C during recordings. Recordings were made with an Axopatch 1D amplifier (Molecular Devices, CA, USA), and signals were filtered at 5 kHz using a four-pole Bessel filter and digitized at 10 kHz using a Digidata 1320A analog–digital interface (Molecular Devices, CA, USA).

5.2.6 Electric field stimulation

The neuronal culture was placed in a custom 1 x 1 x 1.5 cm PDMS chamber where two opposite walls had 1x1 cm (exposed area) inert platinum plates fixed so that a homogenous linear electric field was created throughout the recording buffer in the chamber once a voltage was applied across the plates (Figure 5.12). This voltage and

its time variation was determined by output of a Cakewalk UA-25EX (Roland, MA, USA) soundcard attached to an A303 (A.A. Lab systems, Israel) high speed amplifier. The bi-phasic square pulse waveforms were created using Audacity software (<http://audacity.sourceforge.net>). The voltage across the electrodes was constantly monitored using a TDS-210 oscilloscope (Tectronix, OR, USA).

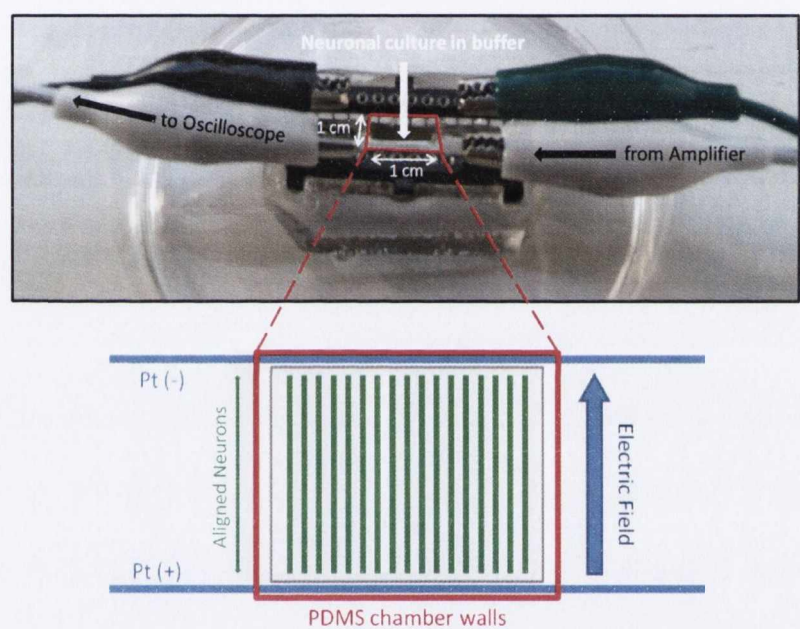


Figure 5.12: Electric field stimulation chamber. Two platinum (Pt) plates are embedded in PDMS surrounding a square (1 x 1 cm) chamber into which, the neuronal culture is placed along with recording buffer. Applying a voltage across the Pt plates generates a homogenous electric field across the chamber. The neuronal culture can be oriented either parallel (as shown) or perpendicular to the field.

5.2.6.1 Statistical analysis

A paired student t test was used to compare the threshold stimulation field of perpendicular and parallel cultures (Prism; GraphPad Software Inc., USA). $p < 0.05$ was considered statistically significant. For more information on statistical methods used see appendix O.

5.2.7 Magnetic stimulation

See Figure 5.11 for outline of setup. The metal microscope stage for holding the samples was replaced by a custom built perspex unit, in order to avoid unwanted induced electric currents and strong repulsive forces between coil and stage. Cultures were subjected to magnetic pulses ($B_{\max} = 2.1$ T) inducing electric fields of 366 V/m at 2 cm above and below coil centre along a ring with a radius of 2 cm. This value was calculated based on measurements of the voltage induced in a copper pickup coil.

In order to align the laminin arcs with the induced electric field, the glass square with the dye-loaded neurons was aligned with markings on an 86 mm petri dish. At this point, 20 ml of recording buffer was added. The circular petri dish was then held in place concentric with the coil so that the distance from coverslip to coil was 2 cm and the radii of the arcs were approximately 20 mm.

5.2.8 FEM modelling methods

2D models were constructed and solved using the AC/DC module of the COMSOL software package. This software uses the finite element method (FEM) to solve Maxwell's equations for regions (subdomains) with user defined geometrical, electrical and magnetic properties. In general, first, the geometry of the cell culture vessel, the contained medium and the cell/neuron (if appropriate) was generated using subdomains of appropriate shape and size. A subdomain of air was generally placed around this. See Figure 5.13. Then, the electrical and magnetic properties of the materials were defined. For all materials the only magnetic parameter was μ_r and this was set to 1 in all cases as this is essentially the case for almost all biological materials [51]. For the medium, σ was 1.7 S/m and ϵ_r was 80 in accordance with the findings of previous studies [207]. For cell membranes σ was 4×10^{-9} S/m and ϵ_r was 2.1 [89] and for the cytoplasm σ was 0.5 S/m and ϵ_r was 80 [89]. Since the 2 - 3 nm thickness of real membranes requires too large a number of mesh elements to be solved on our computer system (2.66 GHz Intel quad core, 4 GB RAM, Dell, TX, USA), its thickness was increased by a factor (generally 50). To compensate for resulting effects on the membrane capacitance and conductance, σ and ϵ_r were always multiplied by this factor. With this operation the correct $V_{(t)}$ drop across the membrane is calculated and the accuracy of the model is not altered once we are not interested in what is going on inside the membrane, which we are not. The σ of the plastic (polystyrene or polypropylene) vessel and surrounding air is very low, $\sim 0.5 \times 10^{-17}$ and $\sim 0.5 \times 10^{-14}$ S/m respectively, and $\epsilon_r = 1$ for these materials.

The boundaries of the model were then assigned conditions so that appropriate magnetic or electric fields were applied throughout. Next the subdomains were meshed using COMSOL's meshing algorithms. These meshes were then refined until re-meshing produced no significant change to the solver's output.

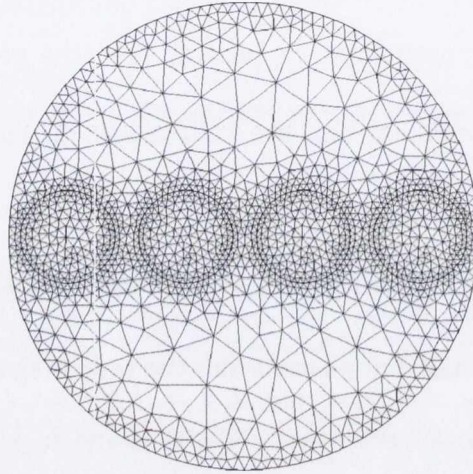


Figure 5.13: Example of a COMSOL mesh of 4 medium-filled wells of a 96 well plate inside a circular domain of air. The wells are represented by 4 circular subdomains with the electrical properties of cell culture medium.

With the geometric and material properties of the model in place, Maxwell's equations ((5.11) - (5.15)) were solved using either the full time dependent formulation or the quasi-static time-harmonic formulation. The quasi-static approximation is valid once the electrical size of the model is not smaller than 0.1 [208], i.e. that the largest spatial dimension of the model is less than $1/10$ of the electromagnetic wavelength. In our case we are looking at frequencies in the range of 1 Hz to 3 MHz which means wavelengths from 100 to 3.33×10^8 m. With a

maximum diameter of 12 cm our model is clearly of appropriate electrical size.

Maxwell's equations are as follows:

$$\nabla \times H = J + \frac{\partial D}{\partial t} \quad (5.11)$$

$$\nabla \times E = -\frac{\partial B}{\partial t} \quad (5.12)$$

$$\nabla \cdot D = \rho \quad (5.13)$$

$$\nabla \cdot B = 0 \quad (5.14)$$

; where $\nabla \times$ is the curl operator, $\nabla \cdot$ is the divergence operator, H is the magnetic field strength or magnetizing field, D is the electrical displacement, J is the current density, E is the electric field, B is the magnetic flux density and ρ is the charge density. The first two equations are also referred to as Maxwell-Ampère's law and Faraday's law, respectively. Equation three and four are two forms of Gauss' law—the electric and magnetic form, respectively. Another fundamental equation is the equation of continuity, which can be written as

$$\nabla \cdot J = -\frac{\partial \rho}{\partial t} \quad (5.15)$$

Finally the associated constitutive relationships are:

$$D = E \varepsilon_0 \varepsilon_r \quad (5.16)$$

where ε_0 is the permittivity of the vacuum and ε_r is the relative permittivity of a material, and ,

$$H = B\mu_0\mu_r \quad (5.17)$$

where μ_0 is the permeability of the vacuum and μ_r is the relative permeability of a material.

5.3 Results

5.3.1 Micro contact printing and culturing of patterned neurons

Neurons were successfully patterned along straight lines and arcs (Figure 5.14 and Figure 5.15). These cultures survived and responded to electrical stimulation for at least 3 weeks after seeding. Overgrowth of neural processes and glia lead to loss of pattern definition, but this was only a significant problem after 3 weeks. Treating substrates with 0.15% agarose prior to microcontact printing increased the degree to which neurons were restricted to laminin lines, but this improvement only became advantageous after 3 weeks when untreated cultures became significantly overgrown. Agarose treatment also reduced the overall level of cell adhesion (cell number) during seeding. Due to this fact and since it was not necessary over the culture periods used for stimulation experiments (< 2weeks), agarose treatment was not used for any of the stimulation experiments.

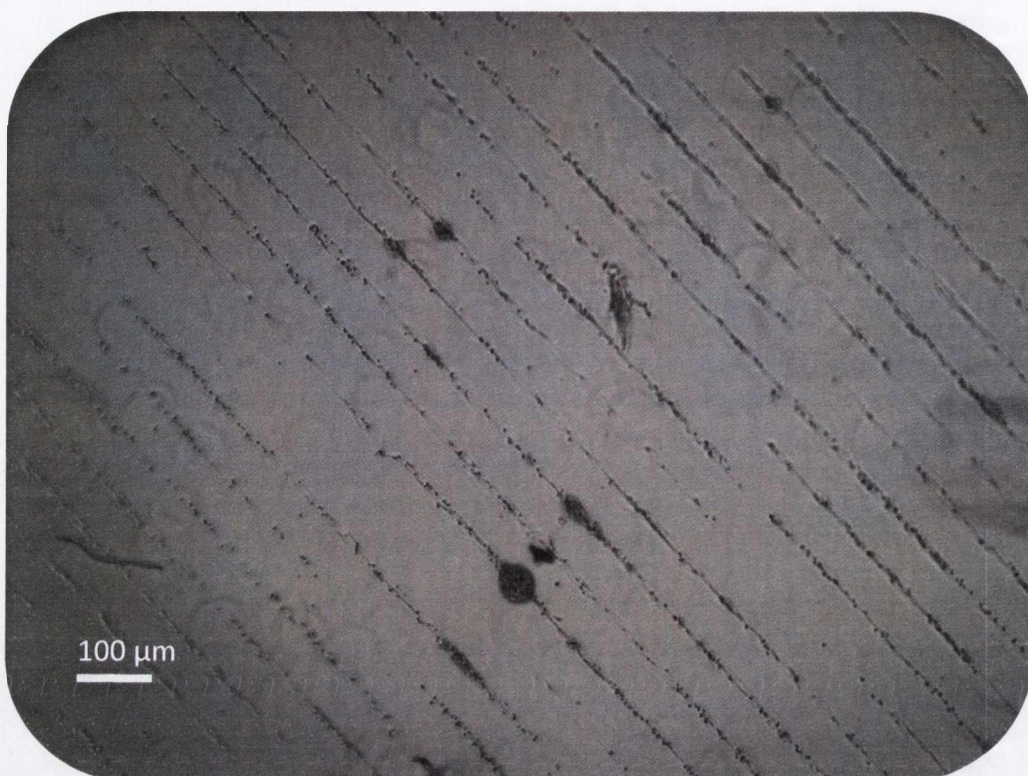


Figure 5.14: Cultured rat cortical neurons cultured along microcontact printed lines of laminin (40x total magnification).

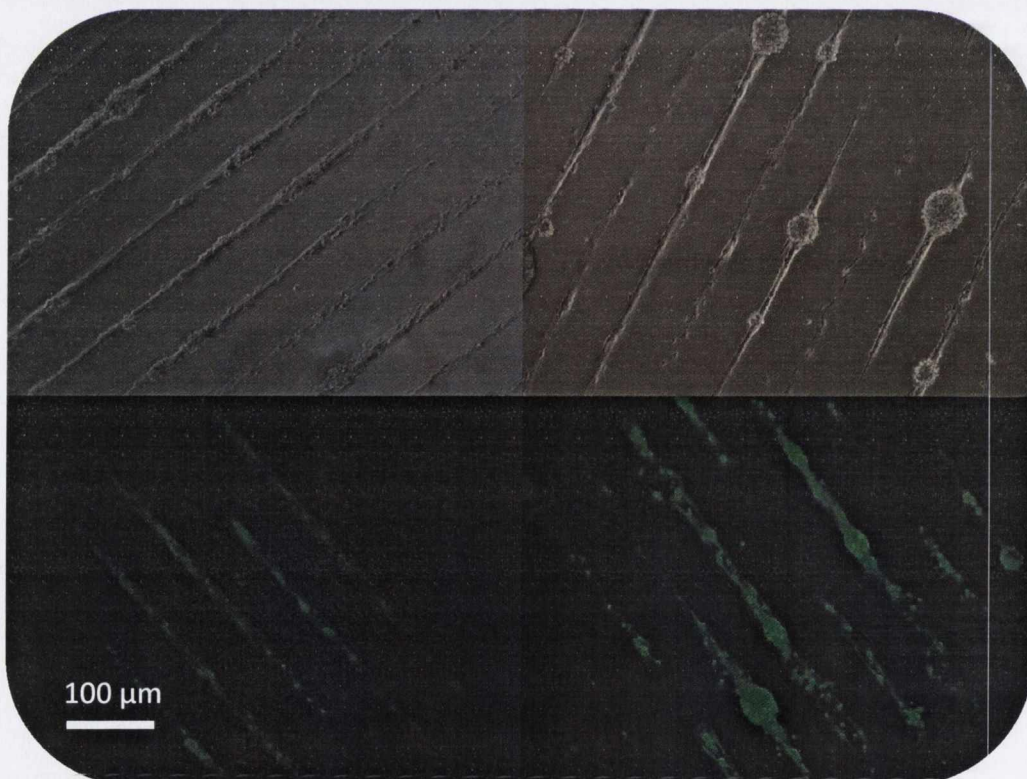


Figure 5.15: Cultured rat cortical neurons cultured along microcontact printed lines of laminin (100x total magnification). Green fluorescence due to Fluo-4 dye interaction with intracellular Ca^{2+} .

5.3.2 Magnetic Stimulation

Four experiments were run to test if a 366 V/m magnetically induced electric field (E) could stimulate firing of neurons growing along lines parallel to this. In no case were any Ca^{2+} spikes observed or recorded, indicating that firing of action potentials was not being induced. The same cultures could be excited via 80 mM KCl demonstrating that they had functioning voltage gated ion channels. Direct application of electric fields (Figure 5.19) as well as patch clamp measurements (Figure 5.17) of other cultures from the same rat brain indicated that the neurons used were excitable.

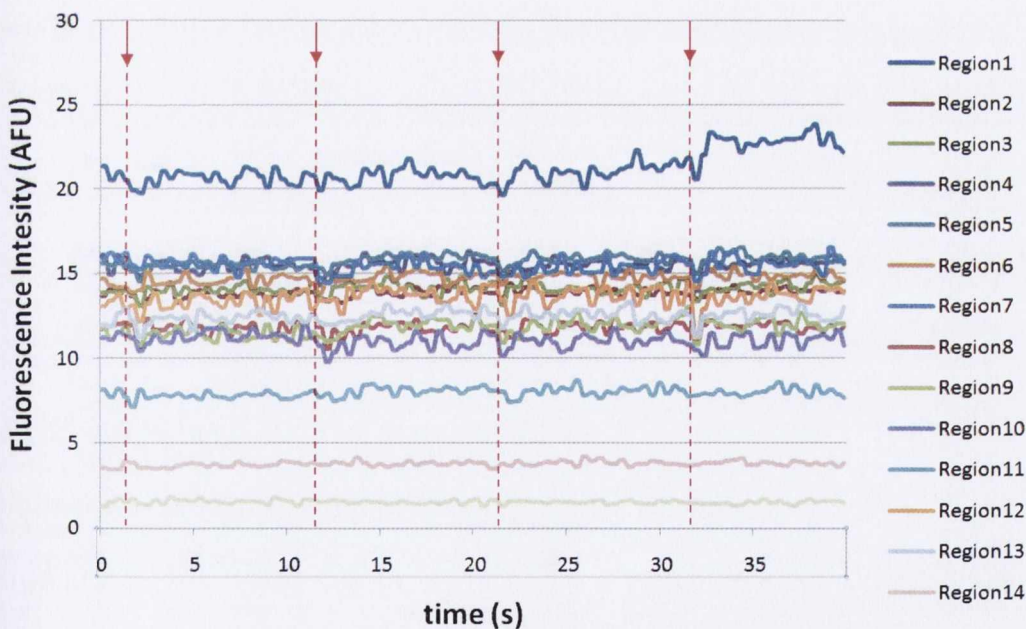


Figure 5.16: Representative fluorescence trace for regions of a neuronal culture aligned parallel to the 366 V/m electric field induced by pulses from our magnetic stimulator. Magnetic field pulses were applied at 10 s intervals as indicated by the red arrows and dashed lines. The y-axis corresponds to the fluorescence intensity of defined regions of the patterned culture, typically consisting of 5 to 50 neurons. Fluorescence intensity is proportional to the intracellular Ca^{2+} concentration.

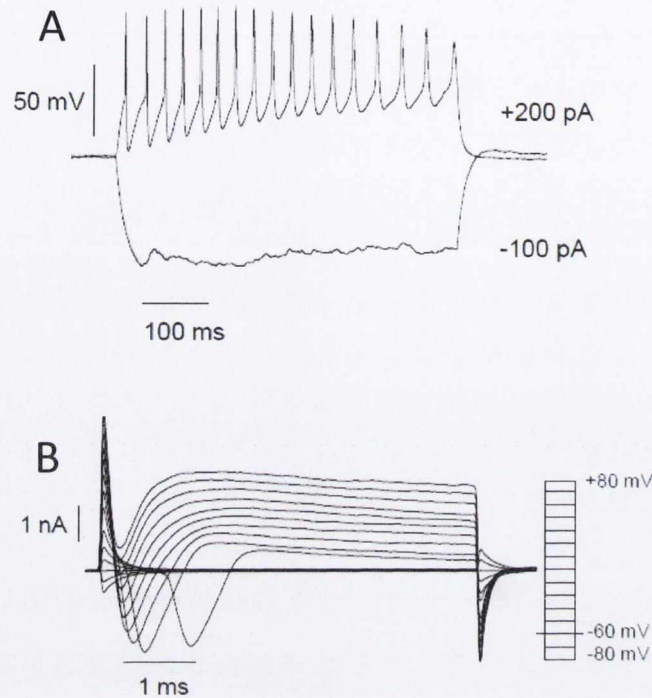


Figure 5.17: Patch clamp measurements at soma of cultured rat cortical neuron. (A) Current clamp mode showing membrane potential dynamics in response to 200 pA intracellular current. The result is a train of 1-2 ms wide spikes (action potentials). (B) Voltage clamp mode showing membrane current dynamics at fixed membrane potentials ranging from -80 mV to +80 mV in 20 mV steps. Large inward sodium currents (negative slopes) can be seen at potentials ≥ -40 mV.

5.3.3 Orientation dependence of electric field stimulation

The threshold for electric field stimulation of lines of neurons oriented parallel to the field was compared to that for those aligned perpendicular. After 4 experiments comparing the two orientations it was found that the average threshold field (E_T) was significantly lower ($P < 0.001$) for parallel cultures where the average E_T was 235.9 V/m versus 650.6 V/m for the perpendicular cultures (Figure 5.18). These averages were for recordings that were responsive at some E_T over the range of field magnitudes used (200, 300, 500, 600 and 800 V/m). See Figure 5.19 and Figure 5.20 for representative fluorescence traces of a parallel and perpendicular culture

respectively. A minority of the recordings showed no stimulation response even at 800 V/m (E_{max}). The percentage of the total number of recordings in each experiment where no response was seen is indicated by the right hand half of Figure 5.18. In keeping with their higher thresholds when stimulated, there was a significantly ($P < 0.05$) higher percentage of non-responsive recordings for the perpendicular cultures at an average of 23% compared with 2 % for parallel cultures.

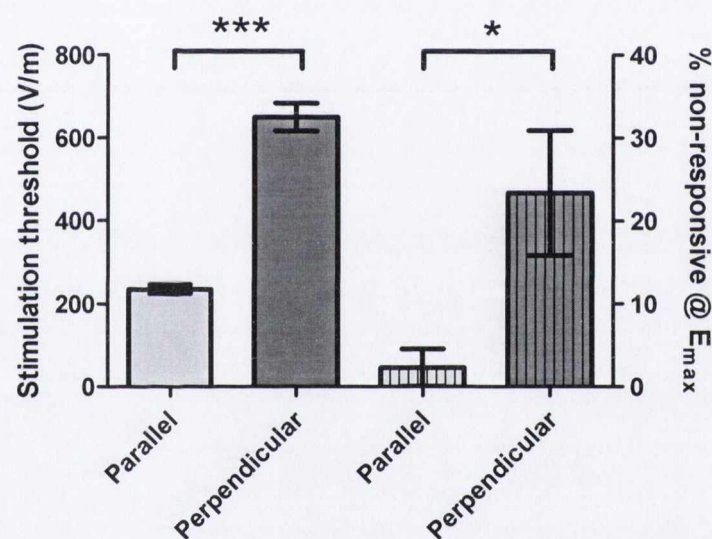


Figure 5.18: Threshold for electric field stimulation when neurons are aligned parallel or perpendicular to the applied field. The right hand Y-axis gives the percentage of recordings where no firing was detected at the maximum field strength tested ($E_{\text{max}} = 800 \text{ V/m}$). Chart represents the average of 4 experiments $\pm$ SEM. There was a significantly lower threshold for parallel vs. perpendicular neuronal cultures ($***, P < 0.001$, t-test) and also a lower percentage of recordings showing no response ($*, P < 0.05$, t-test).

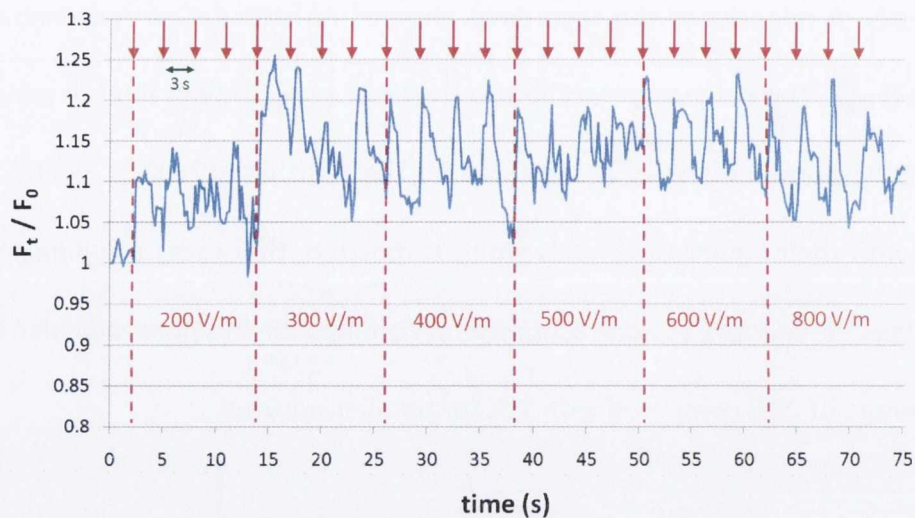


Figure 5.19: Representative fluorescence trace for a region of a neuronal culture aligned parallel to electric field. Bi-polar 10 ms square pulses were applied at 3 s intervals as indicated by the red arrows. The field was increased every 4 pulses as indicated by the dashed lines. The y-axis corresponds to the fluorescence intensity of the region at the given time divided by the intensity at $t = 0$ s (F_t / F_0). Regular peaks (firing of neurons) can be seen on the electric field pulse from 200 – 800 V/m.

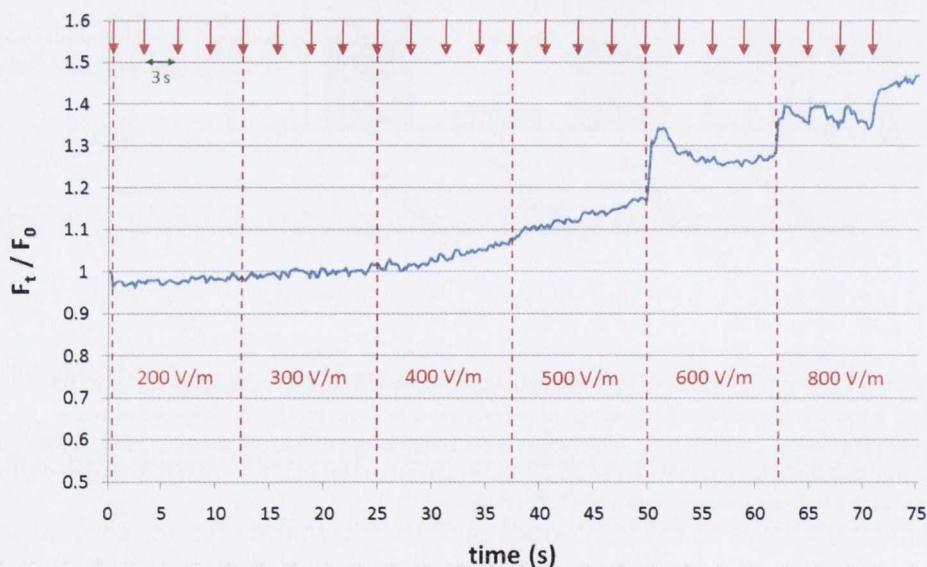


Figure 5.20: Representative fluorescence trace for a region of a neuronal culture aligned perpendicular to electric field. Bi-polar 10 ms square pulses were applied at 3 s intervals as indicated by the red arrows. The field was increased every 4 pulses as indicated by the dashed lines. The y-axis corresponds to the fluorescence. Regular peaks (firing of neurons) can be seen on the electric field pulse from 800 V/m. A single peak was induced by the 600 V/m pulse train.

5.3.4 FEM models of *in vitro* application of TVMFs

5.3.4.1 *Induced electric fields in cell culture medium*

For a large region of medium filling the entire area (A_B) of the passing uniform B , we get a circular, radially increasing induced E , as we would expect from Faraday's law (5.18) (Figure 5.21). This is essentially the scenario used in our MS experiment and when values of dB/dt and A equating to those of our setup were used, the simulated $E_{\max}$ of 366.776 V/m agrees well with the measured value of 366 V/m. The other scenario (Figure 5.22) examined was that of 4 wells of a 96 well plate subjected to the same dB/dt . Here the magnitude and shape of the induced field was not determined simply by the size and shape of A_B (determined by coil geometry) as might be expected from Faraday's law alone. Instead the field was proportional to the size (radius) of the well while circulating about the well centre rather than that of the coil.

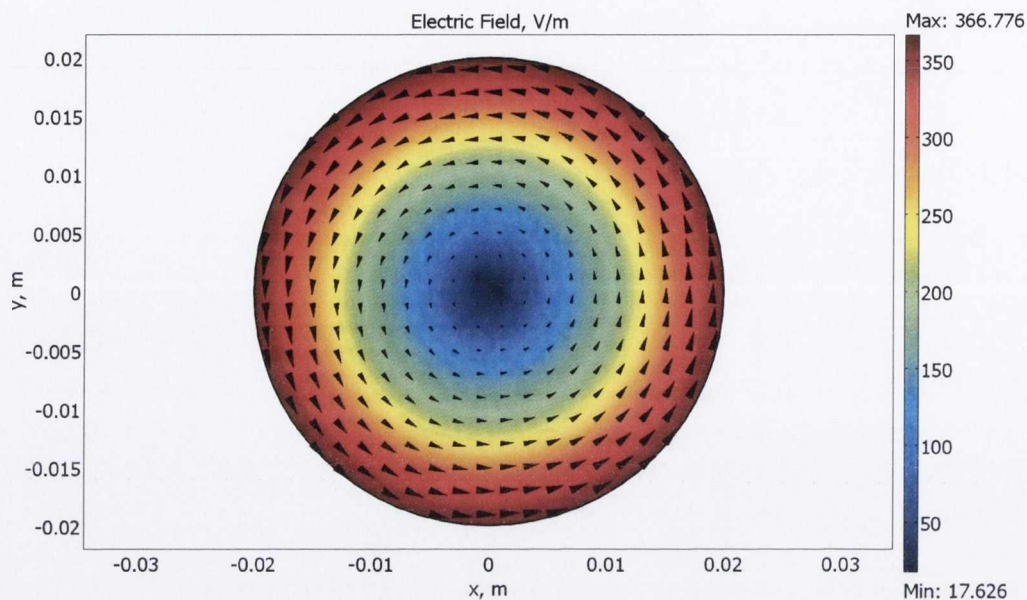


Figure 5.21: Electric field (E) induced in cell culture medium which is located in a circular region of spatially homogenous flux density ($B_{(t)}$) produced by a coil. B varies over time sinusoidally at 2778 kHz reaching a maximum of 2.1 T. The resulting induced E is proportional to the radius of the area of homogenous B and it increases radially from the well centre to a maximum of 367 V/m.

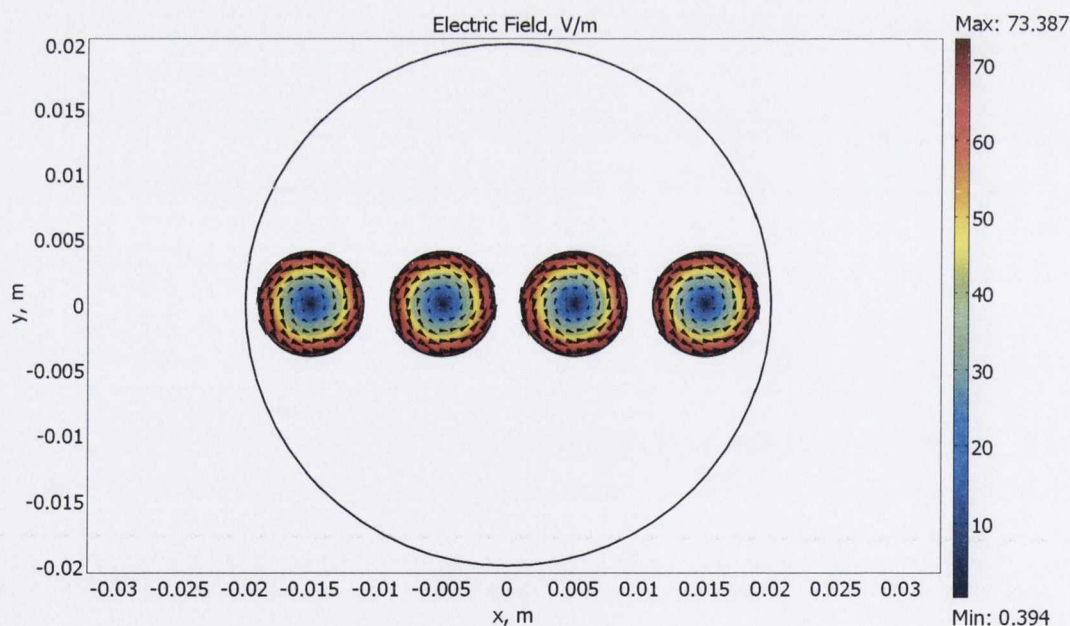


Figure 5.22: Electric field (E) induced in cell culture medium in 4 wells of a 96 well plate, which are located in a circular region of spatially homogenous flux density ($B_{(t)}$) produced by a coil. B varies over time sinusoidally at 2778 kHz reaching a maximum of 2.1 T. The resulting induced E is proportional to the radius of the well and increases radially from the well centre to a maximum of 73.4 V/m.

Faraday's law (5.8) simply gives the $\vec{E}$ around any loop within A_B . This includes those which are concentric with the well. The total electric field throughout the area of uniform B is the sum of all the possible loops. If conductivity is homogenous across the region then these will cancel and complement each other so that $\vec{E}$ will form large loops concentric with the A_B (or the coil) (Figure 5.21). But in the case where a region within A_B has a much higher conductivity than its surroundings, then charge will accumulate at the boundary so that the electric field causing the accumulation (that which is normal to the surface) is almost completely opposed. This leaves only fields with a zero normal component, i.e. the tangential $\vec{E}$ concentric with the well surface (Figure 5.22).

This means that the direction and magnitude of $\vec{E}$ in any culture medium containing well is dependent on the geometry of its boundaries and on its radius respectively and thus, the induced $\vec{E}$ in any cylindrical or cuboid well placed anywhere in a region of homogenous B can be calculated simply from Faraday's law applied to its specific geometry. It is independent of positioning and the geometry of the coil producing B .

5.3.4.2 Induced electric fields in cells

A cell in the medium within the well is similar to the well in an A_B of air described above, except that there is much less resistance to charge flow out of the cell. Resistance is still substantial due to the membrane resistivity ($2.5 \times 10^9 \Omega.m$), but its tiny thickness (3 nm) and the low resistivity (0.8 $\Omega.m$) of the surrounding medium make it much less than that experienced by charge exiting the well/air barrier. This means that $\vec{E}$ along the loops external to the well are no longer completely screened by charge accumulation and hence, $\vec{E}$ inside the well is no longer constrained to tangential paths. This translates into the following relation,

$$\vec{E}_{cell} = \vec{E}_{ext} + \vec{E}_{charge} + \vec{E}_{int} \quad (5.18)$$

where $\vec{E}_{cell}$ is the total electric field inside the cell, $\vec{E}_{ext}$ is the electric field that is magnetically induced along closed loops traversing areas external to the cell, $\vec{E}_{charge}$ is that due to charge accumulation, caused by $\vec{E}_{ext}$ (it will be directed in opposing directions to $\vec{E}_{ext}$) and $\vec{E}_{int}$ is the field induced along closed loops within the cell.

This relation was deduced from our FEM models of TVMFs applied to cells in media containing wells and this is demonstrated in Figure 5.23 and Figure 5.24. Figure 5.23 shows a rectangular region bound by a membrane that represents a cell located at the edge of a rectangular well. The sinusoidally varying B (50 Hz, 1 mT) is

directed out of the page and so the induced electric field (red arrows) loops around the media in plane with the page. This situation can be viewed as a horizontal cross section of a cell at the wall of a well whose floor is perpendicular to the applied B or as a vertical cross section through a cell on the floor of a well whose floor is parallel to B. The former scenario has been used for the limited number of *in vitro* neuronal MS studies to date, while the latter is most common for weaker B field studies, particularly ELF studies (see chapter 4). Thus this model applies generally to *in vitro* TVMF application.

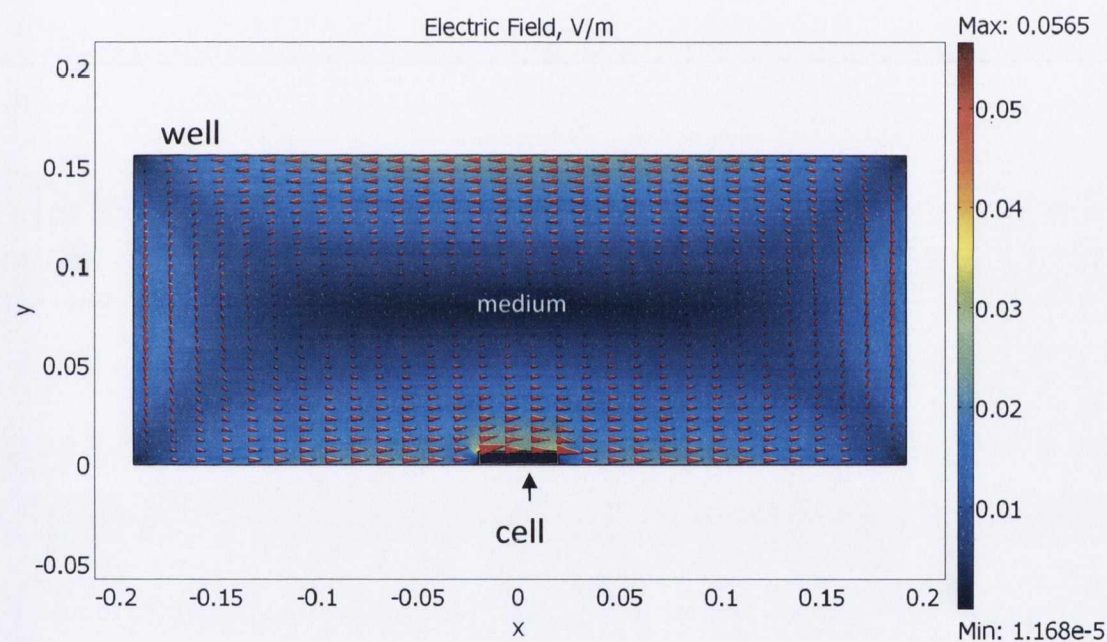


Figure 5.23: FEM simulation of the electric field (red arrows) induced by a 50 Hz, 1 mT TVMF in the medium in a well containing a cell.

It can be seen that $\vec{E}_{ext}$ around the vicinity of the cell is effectively linear from left to right. Without any charge accumulation $\vec{E}_{cell}$ will be equivalent to $\vec{E}_{ext} + \vec{E}_{int}$ with $\vec{E}_{ext}$ dominating over $\vec{E}_{int}$ since the area of the well (A_{ext}) is much larger than the cell (A_{int}). If charge accumulates, $\vec{E}_{charge}$

reduces $\vec{E}_{ext}$ so that $\vec{E}_{cell}$ takes on more of the character of $\vec{E}_{int}$ which loops concentric with the cell boundary. In Figure 5.24, this can be seen happening over time, with $\vec{E}_{cell}$ being largely linear from left to right ($\vec{E}_{ext}$) at 5.56 ns and then shifting gradually as charge accumulates at the cell membrane to a final equilibrium field exhibiting both a linear and circular character. Using this model, we also simulated the application of a linear electric field across the cell of the magnitude of $\vec{E}_{ext}$ and then added the resulting $\vec{E}_{cell} (= \vec{E}_{ext} + \vec{E}_{charge})$ to that produced in a separate model containing the same cell with no external $\vec{E}$ and only B applied over its area. This gave the same result in the timing, magnitude and direction of $\vec{E}_{cell}$.

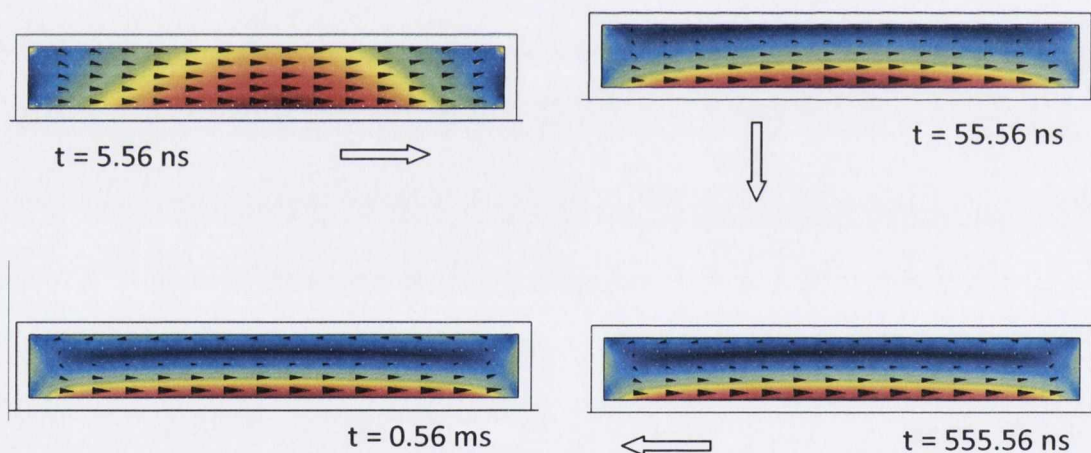


Figure 5.24: FEM simulation of the electric field (black arrows) induced by a 50 Hz, 1 mT TVMF in the cell depicted in Figure 5.23. The field changes initially over a short period of time until charge accumulation has reached its equilibrium state. A combination of the field due to closed loops external to the cell (linear left to right direction, e.g. $t=5.56$ ns) and that due the closed loops inside the cell (concentric with cell boundary) can be seen once charge accumulation has screened out some of the stronger externally induced field (e.g. $t= 555.56$ ns and 0.56 ms).

$\vec{E}_{cell}$ depends on the σ and ϵ_r of the membrane, cytoplasm and medium as well as the ratio of A_{ext} to A_{int} . In the model shown in Figure 5.23 the well was made unrealistically small for the sake of computability but for a real 96 well plate, for

example, $A_{ext} : A_{in}$ will be 200 – 1000, so even with charge accumulation $\vec{E}_{ext}$ will dominate. Thus, for simulating the effects of MS of neurons *in vitro*, we can safely neglect $\vec{E}_{int}$. We can remove magnetic fields entirely from our cell models by calculating $\vec{E}_{ext}$ from Faraday's law based on the dimensions of the medium in the well and choosing a value corresponding to the position of the cell in the well. The calculated $\vec{E}_{ext}$ can now be applied directly across the cell, simply by assigning appropriate voltages to nearby boundaries (act as electrodes). This reduces Maxwell's equations to just (5.13) and (5.15), which allows very high aspect ratio structures like neurons using time dependent equations to be simulated. Without this simplification such calculations require a huge amount of computational power. This method was used for our models of simple neurons being stimulated by magnetic/electric pulses.

5.3.4.3 Stimulation of neurons

Passive membrane properties were used in all cases since for this study we are only looking at the minimum applied electric field, $\vec{E}_{app}$, required to raise the membrane potential (V_m) by 30 mV and hence induce firing in a typical neuron [10, 89, 196, 202]. The neurons were modelled as long, narrow membrane-bound rectangles (300 – 600 μm long x 1 μm wide). Firstly we looked at the issue of alignment of the neuron with $\vec{E}_{app}$. Here, $\vec{E}_{app}$ is equivalent to $\vec{E}$ due to magnetic induction ($\vec{E}_{ext}$) or a voltage drop created by electrodes ($\vec{E}_{elec}$). It was found that the maximum increase

in V_m occurred at the end of neurons as is predicted by cable theory [10, 89, 202, 203] (Figure 5.25) and this was length dependent.

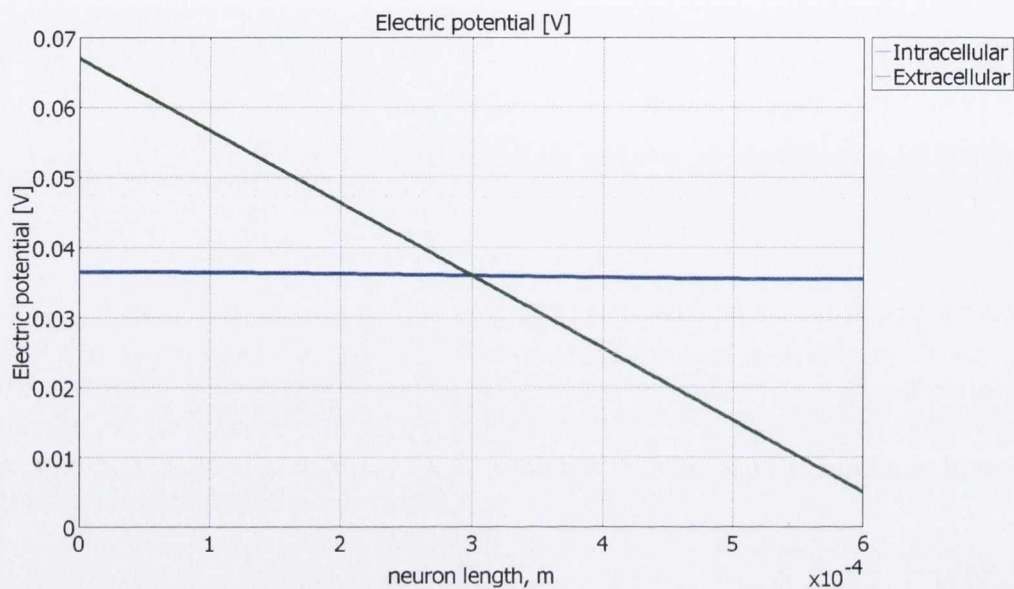


Figure 5.25: Intracellular (blue) and extracellular (green) potential along length of neuron (600 μm).

This maximum V_m was approximately 150 times larger for parallel neurons (Figure 5.26) versus perpendicular neurons (Figure 5.27). This result is in partial agreement with our experimental findings, where the parallel threshold was found to be lower but only by a factor of 3 or 4, not 150. Like cable theory, our FEM results indicate that it should essentially never be possible to stimulate perpendicular neurons. The threshold applied electric field, E_T , was 103 V/m for a 600 μm parallel neuron (Figure 5.26) and 200 V/m for a 300 μm parallel neuron which is not far from those found in our electric field experiments (Figure 5.19) for parallel neurons.

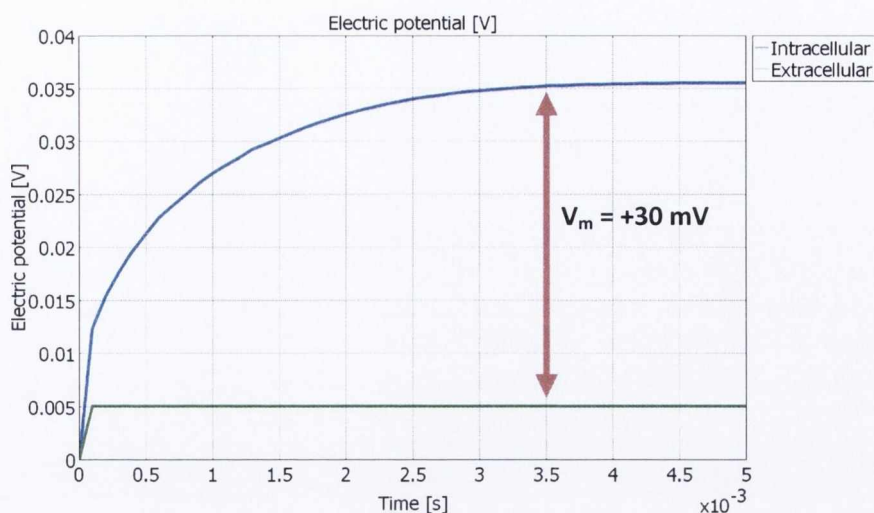


Figure 5.26: Simulated application of 103 V/m electric field perpendicular to 600 μm neuron. Traces show electrical potential on intracellular (blue) and extracellular (green) sides of the membrane at the end of the neuron. The difference (V_m) reaches the +30 mV threshold for firing an action potential after about 3.5 ms

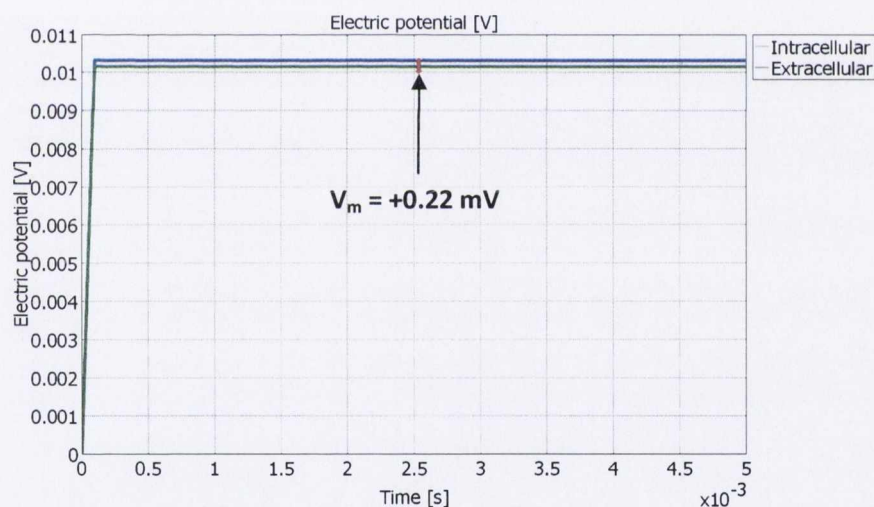


Figure 5.27: Simulated application of 103 V/m electric field perpendicular to 600 μm neuron. Traces show electrical potential on intracellular (blue) and extracellular (green) sides of the membrane at the end of the neuron. Very little difference (0.22 mV) between the two was seen in the perpendicular case as predicted once extracellular charge accumulation is neglected – see discussion.

When the electric field pulse induced by our MS coil (366 V/m, 2778 kHz) was applied parallel to a 600 μm neuron, the maximum rise in V_m was +18 mV (Figure 5.28) which is not enough to induce firing and as such this agrees with our

experimental findings. It was found that E_T for such a pulse was 700 V/m (Figure 5.29), well beyond the capabilities of our stimulator.

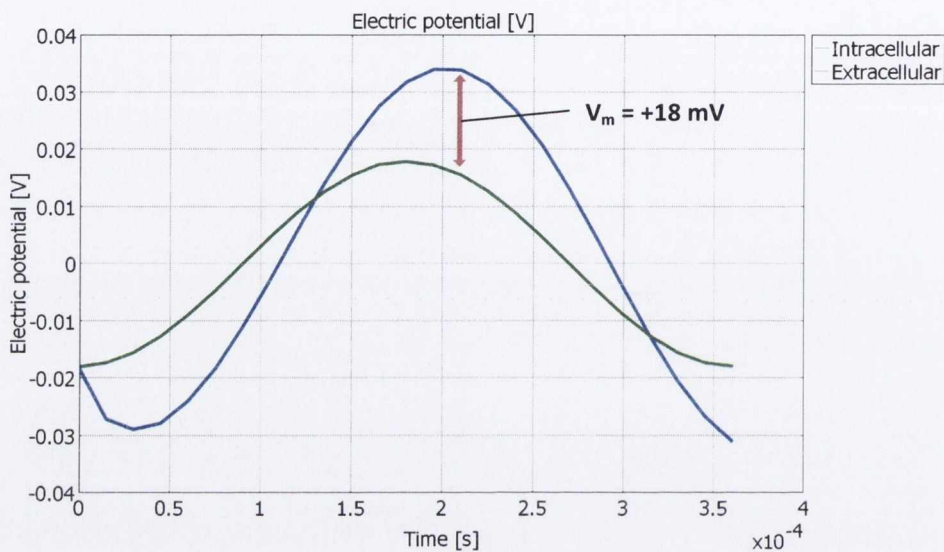


Figure 5.28: Simulated application of the electric field pulse induced by our MS coil (366 V/m, 2778 kHz) parallel to a 600 μm neuron. Traces show electrical potential on intracellular (blue) and extracellular (green) sides of the membrane at the end of the neuron. The difference (V_m) reaches the a maximum of +18 mV at about 200 μs , which is below the threshold +30 mV needed for firing.

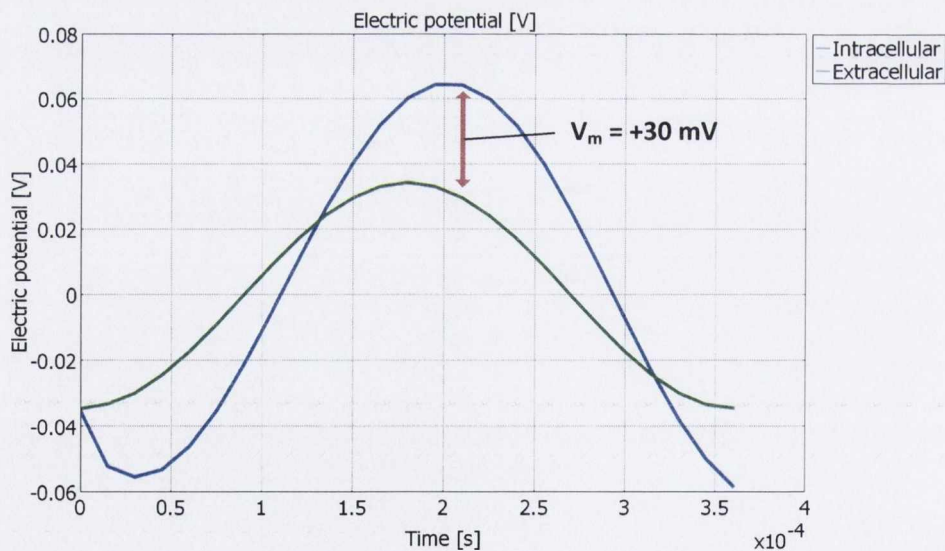


Figure 5.29: Simulated application of the 700 V/m, 2778 kHz electric field pulse parallel to a 600 μm neuron. Traces show electrical potential on intracellular (blue) and extracellular (green) sides of the membrane at the end of the neuron. The difference (V_m) reaches below the threshold +30 mV needed for firing at about 200 μs .

5.4 Discussion

As can be seen from Figure 5.14 and Figure 5.15 neurons were successfully patterned along straight lines and arcs. The cells were healthy and excitable for over 3 weeks, although overgrowth of neuronal processes and glia, leading to loss of pattern definition, did occur after 3 weeks. Pre-treatment of substrates with anti-adhesion agents like 0.15% agarose prior to microcontact printing aided the precision of patterning, but significant improvements were only seen when cultures were over 3 weeks old when overgrowth onto unpatterned areas became a significant problem. Thus, agarose treatment was not necessary for our purposes and since it also caused an overall reduction in cell adhesion (cell number) even in patterned regions, it was not used for any of the electric or magnetic field stimulation experiments. The fact that such substrate treatment is not necessary for reasonable culture lifetimes (weeks) would be advantageous were the technique to be used for patterning neurons on electrode arrays. This is because such pre-treatments increase the resistance between the cell/medium and the underlying electrode thus reducing signal strength – see later discussion of electric field only model of MS.

Rotem and Moses used a different patterning technique involving the use of a modified printer to scratch off patterns from a substrate surface treated with the hydrophobic anti-cell adhesion compound octadecyltrichlorosilane (OTS). We used the microcontact printing method to generate 40 μm wide neuronal lines that were at least 4 times narrower than those used by Rotem and Moses for their “1-dimensional” neuronal cultures (170 - 200 μm), thus we have achieved a higher

degree of precision and '1-dimensionality' in the patterning of our neurons. Nevertheless this increased 1-dimensionality could lead to a reduction in network connectivity that can affect the length and time constants of the neural pathway [209].

Electric stimulation of neuronal cultures was achieved using 10 ms bi-phasic pulses of no more than 200 V/m (Figure 5.18) and stimulation was also seen in one culture at 150 V/m, but not at 100 V/m. Despite our neurons being comparably excitable in direct electric fields to those in studies by Rotem and Moses [210], we were unable to detect any neuronal firing that was caused by the magnetic pulse, with Figure 5.16 showing an example of this. In their experiment, Rotem and Moses found the average E_T to be 300 ± 128 V/m. Our stimulator's E_{max} was in excess of this at 366 V/m so it is surprising that stimulation did not occur. Nevertheless, the magnitude of the errors (42%) in their experimental findings indicates that neurons can still vary substantially in their susceptibility to magnetic stimulation even when their geometry and location is highly controlled and that stimulation at our induced E was seen in some of their cultures so maybe a large number of repetitions would see the stimulator being matched with a particularly excitable culture by chance. Rotem and Moses do suggest that the stimulation is highly dependent on the presence of a small population of low threshold, "initiating neurons" so a large total number of neurons experiencing a pulse is necessary to make observation of stimulation statistically likely. Nevertheless, it seems necessary to increase the capacity of the magnetic stimulator and the excitability of the neurons if future attempts are to be successful. Tests with wider arcs should also be performed. Longer arcs or complete

circles should also increase the chances of stimulation by increasing the number of aligned neurons present. Nonetheless, larger cultures greatly increase the time and quantity of expensive culturing resources necessary for experiments and this was why the large patterned cultures (long arcs to full rings) were avoided in our study.

Because the length of the magnetic pulse is on the order of 0.36 ms compared to the 10 ms pulse applied during direct electrical stimulation, the E_T will be higher than for the electric pulses we have used unless the time constant ($\tau = c_m r_m \approx t$ at 63% of max V_m) is less than the rise time of the pulse (90 μ s). This was not the case in our FEM model where τ was between 0.5 and 1 ms depending on the length of the neuron, which is to the low end of values reported in the literature (0.5 to > 100 μ s), and hence E_T was 700 V/m for a 600 μ m parallel neuron. From our patch clamp measurements the actual τ values seem to be around 20 ms which is typical for many types of neuron. All this suggests that the short pulses used for MS would never be capable of stimulating neurons. Rotem and Moses suggest that axons have particularly low τ values < 300 μ s allowing stimulation. Also, the real ability of patch clamp measured and calculated τ values to estimate how fast V_m to a given stimulus has been called into question as real τ values can vary hugely over time and space depending particularly on the neurons interactions with other neurons [211]. Part of the reason for our inability to achieve magnetic stimulation may be on account of our cultures having few low time constant neurons compared to the Rotem and Moses study. This should be tested in more detail in future studies and in particular, short cosinusoidal electric field pulses similar to those induced by the magnetic stimulator should be examined for their ability to cause neuronal stimulation. Also, future

studies should test wider laminin arcs/lines as this may increase neuronal network connectivity and thus reduce time constants as mentioned previously.

Overall, it was found that using MS *in vitro* was difficult and awkward and necessitated using large (> 40 mm diameter), single well vessels. Thus, higher throughput studies looking at numerous physical and biological parameters would be difficult, if not, unfeasible due to their reliance on multiwell plates, which are incompatible with MS since they reduce the magnitude of the induced electric field greatly. As shown by our FEM model, the induced electric field becomes proportional to the radius of the small wells (Figure 5.22). Another limitation for MS *in vitro* is that only a single pulse rise time, duration and train frequency can be tested at any given time. One of the main benefits for clinical applications that an *in vitro* model should provide is the ability to test many different MS parameters in a single experiment, preferably simultaneously across numerous wells, so that such parameters can be examined for various cellular conditions, disease states and drug/toxin treatments. This should allow for much greater understanding of the MS parameters appropriate for a given *in vivo* clinical application and perhaps lead to, as yet unconsidered applications. It seems unlikely that an *in vitro* model using magnetic pulses will ever be able to achieve this goal in a practical, cost and time efficient manner. Even the use of an array of small coils with radii similar to those of multiwell plates wells (e.g. ~0.5 mm for 96 well) would only induce electric fields of the same magnitude as that of a large coil applying a magnetic flux over the entire area of the plate. Either way there is no way around the fact that for any cell culture inside the area of homogenous B (A_B), the maximum induced E can only be achieved if the vessel radius

is at least that of A_B (usually radius of coil bore), if the vessel radius is decreased to that of a small well the decrease in induced E will be directly proportional so that, for instance, in the case of a 96 well plate with our coil, the well radius is 4 times smaller than the A_B so the induced E will be 73 V/m (Figure 5.22) compared to the 366 V/m (Figure 5.21) for a large petri dish. In other words to achieve the same E we need 4 times the dB/dt and really we need 4 times the B since although reducing the rise time (dt) will increase the magnitude of E , it will also reduce its duration which is already low relative to τ for neuronal membranes. Having to generate such large and long (relatively) magnetic pulses would be difficult for most labs.

All this indicates that an alternative *in vitro* model would be of great benefit. Such a model could be provided by directly applied electric field pulses along patterned neuronal cultures. Electric fields of the required magnitude and direction can easily be applied across wells of any size either by some sort of insertable array of electrodes or an electrode array patterned along the floor of a modified multiwall plate. These electrodes could be connected to a specifically designed electronic circuit along with a multichannel function generator and amplifier to provide different stimulation parameters to different wells. Thus, testing many stimulation parameters simultaneously on one or numerous biological endpoints would be possible and allow for effective and efficient comparison of stimulation parameters. As well as the endless list of cellular processes that could be monitored by all the standard fluorometric and colourimetric assays applicable to cultured cells, electrical activity (firing) could also be monitored using microelectrodes patterned beneath the

cells. Thus it is predicted that, a truly high throughput, flexible and practical *in vitro* model of MS would be best achieved via an electric field only approach.

For an electric pulse model of MS to be applicable it must be clear that the magnetically induced and directly applied electric fields are equivalent once the geometric factors discussed above have been taken into account. The results of our finite element models (see section 5.3.4.2 of results) indicate that this is essentially the case for *in vitro* MS where the ratio of the area enclosed by the loop on which the neuron is located (A_{ext}) is always large ($>20 \times$) compared to that of the cell/neuron (A_{int}). Our FEM models indicated that electric field stimulation is also orientation dependent (Figure 5.26 and Figure 5.27) as has been shown by Rotem and Moses for magnetic stimulation. Despite this, the fact that Rotem and Moses did not find such alignment dependence with electric stimulation and the fact that there is no conclusive experimental evidence of the orientation dependence of electric field neuronal stimulation *in vitro*, all casts some doubt on this often assumed orientation dependence [202, 203, 212].

We have demonstrated experimentally that *in vitro* electric field stimulation is indeed orientation dependent (Figure 5.18, Figure 5.19 and Figure 5.20) as cable theory and our FEM models predict. Standard cable theory predicts neurons to be non-excitable via perpendicular electric fields and our model predicts them to be around 150 times less excitable (Figure 5.26 and Figure 5.27). Our experimental results, where cortical neurons were cultured along lines of laminin directed either parallel or perpendicular to applied electric field pulses from 200 V/m to 800 V/m, showed that 77% of the perpendicular cultures were excitable via E and that the

thresholds of these cultures were on average about 3 times higher than the parallel cultures (650.6 V/m versus 235.9 V/m). This much greater than predicted ability of perpendicular electric fields to stimulate cultured neurons is probably due to the unavoidable diversions that real neurons take along the straight laminin line in combination with the tight packing together of multiple neurons within each 40 μm wide line and also the presence of other lines of neurons either side of the line in question. This could make for non-negligible charge accumulation in the extracellular space leading to membrane depolarisation and firing. Our model actually showed that when the extracellular charge flow is highly constrained by resistive surrounding structures, the perpendicular field can cause greater rises in V_m than parallel fields. Such scenarios are not catered for by cable theory, but the FEM model could quite easily be extended to describe multiple side-by-side neurons and other geometric complications. This is an obvious avenue for future work on this FEM model.

5.4.1 Conclusions

In our attempts to reproduce the *in vitro* magnetic stimulation reported by Rotem and Moses we have been unsuccessful, while our efforts to further investigate and develop the *in vitro* model of MS have borne more fruit. In a similar manner to Rotem and Moses, rat cortical neurons were successfully patterned via microcontact printing in configurations aligned with the magnetically induced electric field. Nevertheless, stimulation was not observed in otherwise electrically excitable neurons, when an electric field of 366 V/m was induced across them. This was

probably due to a combination of under-excitability neurons, small culture sizes and a slightly underpowered magnetic stimulator. Future studies should attempt to increase all of these parameters.

It is generally assumed that MS effects are solely due to the induced electric field and this was confirmed by FEM models of *in vitro* TVMF application. Based on this, and our experience of using the magnetic stimulator *in vitro*, we conclude that applying electric fields directly via electrode arrays would provide a much more flexible, precisely controllable and time/cost efficient model system. The orientation dependence demonstrated experimentally by Rotem and Moses for magnetic stimulation was, for the first time, demonstrated with electric field stimulation *in vitro*. Nonetheless this dependence was not nearly as absolute in culture as is predicted by cable theory or our simple FEM model of a single neuron. Imperfect alignment of individual neurons along with constraint of extracellular charge flow was probably responsible for the digression in the experiment. Our 2D FEM model of neuron stimulation based on Maxwell's equations should have advantages over the standard 1D cable model in explaining effects seen in the geometrically more complicated scenarios appearing in real neuronal cultures, where extracellular charge accumulation can become significant. The model would also have applications in other areas such as the *in vitro* ELF exposure described previously in chapter 4 and indeed for *in vitro* TVMF application in general.

6 Final conclusions and future work

Magnetic field effects on a number of important biological cell processes have been investigated in this thesis. This investigation has been carried out at the molecular, organelle and whole cell level and from the results the following conclusions are drawn.

In isolated rat brain mitochondria, treated with the electron transport chain inhibitors, it was found that a moderate, 148 ± 37 mT static magnetic field reduces, by 17%, the rate of mitochondrial superoxide production due to antimycin A treatment. This effect was only seen under toxic, high oxidative stress conditions, thus there is unlikely to be major implications for superoxide signalling, but the effect may offer some protection against oxidative damage caused by stroke and neurodegenerative diseases such as Parkinson's.

The most likely explanation for the effect is a radical pair mechanism involving some of the known paramagnetic species involved in electron transport at complex III. Further investigation of the field strength dependence of the effect is particularly necessary in order to give further insight into the mechanism involved as well as indicating whether the effect could be increased to a magnitude sufficient to provide real clinical benefit as an anti-oxidant. In general, future studies should improve on the homogeneity of the applied fields and methods of applying strong homogenous fields (up to >1T), while measuring radical production in multiple samples, should be found and developed. The mechanism could be probed further in future using other

complex III inhibitors such as myxothiazol and stigmatellin. The trends toward a magnetic field effect observed under untreated and rotenone treated conditions should also be investigated further with an increased number of replicates to increase the statistical power. Efforts should also be made to improve the assay or to find a more sensitive detection method.

Of the few cellular processes that have been reported to be sensitive to magnetic fields via the radical pair mechanism, the series of studies by Buchachenko and Kouznetsov (B&K) with various co-workers stand out in that the effects are large and consistent across a variety of related biochemical reactions. Nonetheless, our attempt to reproduce their magnetic isotope effect showed that the 2-3 fold increase reported previously did not occur for a standard variety of MM creatine kinase. Our results showed no significant change in enzymatic ATP production when $^{25}\text{MgCl}_2$ (98% ^{25}Mg) is used instead of natural MgCl_2 (10% ^{25}Mg + 90% $^{24,26}\text{Mg}$). This suggests that the MIE does not occur in MM creatine kinase and it calls into question B&K's general conclusion that "It is universally observable that enzymes with the magnetic-isotopic nucleus of magnesium produce two to three times more ATP than the same enzymes with nonmagnetic nuclei under the same conditions".

Any future attempts to verify this magnetic isotope effect (MIE) should reproduce the experimental conditions used previously although this would be difficult owing to the rare variety of creatine kinase used here. Effects on one of the other ATP producing enzymes where effects have been reported e.g. phosphoglycerate kinase or pyruvate kinase could also be tested, as well as MIEs on

mitochondrial and cellular ATP. The magnetic field dependence reported by B&K could also be examined.

If there are future attempts at verifying the MIE in ATP producing enzymes, these researchers may want to note the dubious publication history of one half of the B&K partnership, Dmitri Kouznetsov. Severe criticism of his methodologies, conclusions and his use of fabricated references was reported in a letter to the International Journal of Neuroscience [213]. This concerned his study in the same journal that supposedly contradicted the “modern molecular-genetic concept of evolution” [214]. Concerning his study on the Shroud of Turin (religious relic - cloth that supposedly wiped the face of Jesus Christ) [215] another group of researchers again severely criticised his methodologies and also found his study to be irreproducible [216]. These two Kouznetsov papers along with another, concerning ancient Irish artefacts [217], have been investigated thoroughly by an Italian skeptic, Gian Marco Rinaldi [218]. This investigation was described in an article in the journal, Antiquity in 2007 [219]. It was found that all contained references to non-existent articles and that the two archaeological studies used samples of non-existent artefacts from non-existent sources (as indicated by the acknowledgements). Even the labs where the studies were reportedly carried out seem not to exist. Although the B&K literature on the MIE exhibits certain peculiarities such as a high level of recycled data and a sparseness of experimental details, no accusations of fraud have been or are being made regarding this work. Nevertheless, when combined with the fact that our work and that of Prof. Mike Wilson’s group at the University of Essex (results as yet unpublished) has found no MIE in CK, all these issues surrounding the

publication record of Kouznetsov are something for future researchers to take into account before large investments of time and resources are made.

For our ELF-EMF study, we investigated whether cellular development could be significantly altered by a 50Hz, 2 mT AC magnetic field. Significant alteration of processes related to cell development due to such a field would undoubtedly have many health implications and there is still considerable public concern over adverse effects due to the weak 50/60 Hz magnetic fields emitted by power lines and electrical appliances. Despite many studies reporting cellular effects from fields of this magnitude and frequency, when we measured 16 cell developmental parameters via a high content screening system we found no significant changes in osteoblast progenitors due to a 50 Hz magnetic field and thus we have concluded that, over a two day exposure period, it is unlikely that such a field would induce significant developmental changes in osteoblastic cells and hence, also that it is unlikely that major toxic, carcinogenic or tumour promoting effects would result in these cells under such conditions.

Future studies should include additional time points up to 10 days or more to allow for a more conclusive toxicological verdict. They should also use the excellent capabilities of HCS systems for analysis of sub-well (single cell and cell sub-populations) information to examine the relationship between any observed cellular changes and the distribution of the induced electric field. Finally, the results also suggest that fields of this magnitude and frequency applied over one or two days would not be useful for the modification/control of cell development that is needed in bone tissue engineering and repair.

We have attempted to reproduce the *in vitro* magnetic stimulation (MS) of cultured neurons reported by Rotem and Moses (2008). In a similar manner to Rotem and Moses, rat cortical neurons were successfully patterned via microcontact printing in configurations aligned with the magnetically induced electric field. Nevertheless, stimulation was not observed in otherwise electrically excitable neurons, when an electric field of 366 V/m was induced across them. This was probably due to a combination of under-excitability of neurons, small culture sizes and a slightly underpowered magnetic stimulator. Future studies should attempt to increase all of these parameters.

As well as reproducing Rotem and Moses's findings we set out to investigate and develop the *in vitro* transcranial MS model in general. It is commonly assumed that magnetic stimulation effects are solely due to the induced electric field and this was confirmed by FEM models of *in vitro* TVMF application. Based on this, and our experience of using a magnetic stimulator *in vitro*, we conclude that applying electric fields directly via electrode arrays would provide a much more flexible, precisely controllable and time/cost efficient model system.

The orientation dependence demonstrated experimentally by Rotem and Moses for magnetic stimulation was, for the first time, demonstrated for electric field stimulation *in vitro*. Nonetheless, this dependence was not nearly as absolute in culture as is predicted by cable theory or our simple FEM model of a single neuron. Imperfect alignment of individual neurons along with constraining of extracellular charge flow was probably responsible for the digression in the experiment. Our 2D FEM model of neuron stimulation based on Maxwell's equations should have

advantages over the standard 1D cable model in explaining effects seen in the geometrically more complicated scenarios appearing in real neuronal cultures, where extracellular charge accumulation can become significant. The model would also have applications in other areas such as *in vitro* ELF-EMF exposure.

The application of external magnetic fields to biological cells has received the attention of many researchers in recent decades and there have been countless reports of effects on a myriad of cellular processes. Despite this, there is in general a lack of consensus on the biophysical mechanisms responsible and independent replication of many effects has been unsuccessful. Thus, with the exception of our study of ELF effects, we have attempted to steer our research towards effects where biophysical mechanisms are better established, like those based on the radical pair mechanism or induction of moderate to strong electric fields. Despite this, much of our work has shown there to be no effect from the magnetic field in question, even when we have attempted to reproduce large effects seen by others. This is in keeping with the experience of many previous researchers in the bioelectromagnetics field and it emphasises the need for continued work on the independent replication of reported bio-effects due to magnetic fields. Following from this, increasing the publication of such negative findings is essential to give the scientific community a balanced and realistic view of magnetic field effects on biology. Published negative results also serve as an important guide for other researchers, potentially allowing them to save large amounts time and money otherwise spent on dead ends.

On the other hand we have found an important, possibly radical pair based magnetic effect on mitochondrial superoxide production and we have demonstrated, for the first time, the orientation dependence of *in vitro* electric field stimulation.

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8 Appendix

8.1 MgCl_2 solutions: Trace metal concentrations

<i>Trace metal</i>	<i>MgCl_2 solution</i>	<i>$^{25}\text{MgCl}_2$ solution</i>
Lithium, $\mu\text{g/l}$	<1	<1
Beryllium, $\mu\text{g/l}$	<1	<1
Sodium, $\mu\text{g/l}$	<1	<1
Magnesium, $\mu\text{g/l}$	<1	<1
Aluminium, $\mu\text{g/l}$	<1	<1
Potassium, $\mu\text{g/l}$	<1	<1
Calcium, $\mu\text{g/l}$	<1	<1
Vanadium, $\mu\text{g/l}$	<1	<1
Chromium, $\mu\text{g/l}$	<1	<1
Manganese, $\mu\text{g/l}$	<1	<1
Iron, $\mu\text{g/l}$	<1	<1
Cobalt, $\mu\text{g/l}$	<1	<1
Nickel, $\mu\text{g/l}$	<1	<1
Copper, $\mu\text{g/l}$	<1	<1
Zinc, $\mu\text{g/l}$	1.17	<1
Gallium, $\mu\text{g/l}$	<1	<1
Arsenic, $\mu\text{g/l}$	<1	<1
Selenium, $\mu\text{g/l}$	<1	<1
Rubidium, $\mu\text{g/l}$	<1	<1
Strontium, $\mu\text{g/l}$	<1	<1
Silver, $\mu\text{g/l}$	<1	<1
Cadmium, $\mu\text{g/l}$	<1	<1
Cesium, $\mu\text{g/l}$	<1	<1
Barium, $\mu\text{g/l}$	<1	<1
Mercury, $\mu\text{g/l}$	<1	<1
Thallium, $\mu\text{g/l}$	<1	<1
Lead, $\mu\text{g/l}$	<1	<1
Uranium, $\mu\text{g/l}$	<1	<1

Table 8.1: Trace metal concentrations in final (washed x4) enriched and non-enriched MgCl_2 solutions as determined by ICP-MS after 1:100,000 dilution.

8.2 Statistical Methods

All statistical calculations were done using the built-in algorithms of the PRISM 5 software package (GraphPad Software Inc., USA). This software is specifically designed for the analysis of biological experiments and is very widely used throughout the biological literature. All analyses were done based on the recommendations given in the PRISM 5 manual and interface that corresponded to particular experimental design in question. The main statistical procedures mentioned in the text will be briefly outlined below. For full explanations see PRISM 5 statistics guide [220] or Fundamentals of Biostatistics [221].

All of these procedures are based on rejecting/refuting *the null hypothesis* which states that differences in the mean values of different experimental groups/treatments are simply due to random variation (Gaussian distribution is assumed) inherent in the biological system. In other words, the true differences that are specifically induced by the treatment/action under examination are in fact zero (null). If the procedure (calculation) indicates that it is very unlikely that the null hypothesis is true, then we can say that our differences are 'statistically significant', i.e. that it is most likely a real change and not a false positive due to random variation. We can never be certain of having measured a true change, but conventionally, a change is said to be statistically significant (very likely true) when our calculations indicate that the probability of the null hypothesis being true is less than 5% ($p < 0.05$) or in other words, that we are 95% sure that the change is due to

the experimental treatment and not due to random variation. This $p < 0.05$ convention was used for all studies described in this thesis.

8.2.1 t-test

Paired (repeated measures) Student t-tests were performed to test for statistically significant changes in the results of chapters 3 and 5. This calculation is used when there are two independent sample groups being compared. Each group is made up of n number of independent replicate experiments (repeated measures) and the mean of these replicate values is used for the comparison. The output is the probability of the null hypothesis being true.

Before reaching this p-value, the t -ratio to test whether the means are different must be calculated as follows:

$$t = \frac{\bar{X}_D - \mu_0}{s_D / \sqrt{n}}$$

where

$\bar{X}_D$ is the average of the differences (seen in each experiment) between the two groups, μ_0 is the hypothetical difference being tested for (zero for the null hypothesis), s_D is the standard deviation of the differences between the two groups and n is the number of independent experiments in each group. Once a t -ratio is

determined, a p-value can be found using a table of t-ratios from Student's t-distribution. The degrees of freedom for this test is $2n - 2$.

8.2.2 ANOVA and post-test (Bonferroni)

A repeated measures analysis of variance (ANOVA) was performed to test for statistically significant changes in the results of chapters 2 and 4. An ANOVA is used when there are 3 or more groups being compared and it tests whether the variation seen amongst the means of these groups is likely to be down to random variation or real effects of the experimental treatments. An ANOVA rather than just a simple t-test is necessary when there are more than two groups being compared since the probability of a false positive due to random variation increases with the number of groups.

Rather than a t-ratio the ANOVA produces an F-ratio and the probability (p) of the null hypothesis being true is derived from a standard table of F-ratios in a similar fashion to the t-test (table of t-ratios). Calculation of the F-ratio is complicated, but essentially based on the ratio of group to group variation to the variation in the means from individual experiments within the group (residual variation) [220, 221]. An ANOVA can indicate that significant (non-random) differences exist across group means, but unlike a t-test it does not allow us say which groups are changing significantly with respect to others e.g. is treatment x producing a significant change relative to the control? In order to make such conclusions, comparisons between specified groups (usually control vs. treated) must be made via a post-test. Post tests

are variations on the t-test designed to incorporate the variation statistics from the ANOVA. We have used the Bonferroni test in all cases as this is the most stringent of the standard post-tests (least likely to give false positives) [220, 221].

8.3 Hodgkin-Huxley equations

The conductances of voltage-gated channels ($g_n(t, V)$) can be described using the Hodgkin-Huxley equations as follows:

$$g_n(V_m, t) = \bar{g}_n \varphi^\alpha \chi^\beta$$

$$\dot{\varphi}(V_m, t) = \frac{1}{\tau_\varphi}(\varphi_\infty - \varphi)$$

$$\dot{\chi}(V_m, t) = \frac{1}{\tau_\chi}(\chi_\infty - \chi),$$

and χ are gating variables for activation and inactivation, respectively, representing the fraction of the maximum conductance available at any given time and voltage. $\bar{g}_n$ is the maximal value of the conductance. α and β are constants and τ_φ and τ_χ are the time constants for activation and inactivation, respectively. φ_∞ and χ_∞ are the steady state values for activation and inactivation, respectively, and are usually represented by Boltzmann equations as functions of V_m .

8.4 Publications

Crotty D., Martinez G., O'Braonaigh S., Coey J.M.D., Prina-Mello A. Magnetic fields effect on development of osteoblast progenitors: high content screening approach. *Proceedings of the 16th Bioengineering Ireland*. The Centre for Bioengineering, Trinity College Dublin, Ireland, 2010, p.73.

Prina-Mello A., Crotty D., Byrne F., Davey G. and J.M.D. Coey. Cell alignment using a magnetic nanowire and external field gradient. *Joint European Magnetic Symposia*. Dublin, September 2008, Editors Coey, Sanvito, 2008, BM11.

Crotty D. Martinez G; Coey J.M.D., Prina-Mello A. Effect of 50Hz, 2mT AC electromagnetic field on proliferation, morphology and CBFa1 protein expression in pre-osteoblasts. *Proceedings of Bioelectromagnetics Society 30th Annual Meeting*, San Diego, 2008, p 21.

Crotty D., Morgan J.P., Coey J.M.D., and A. Prina-Mello. Osteoblast cell response to external stimulation after internalization of nickel nanowires. *Proceedings of the 13th Bioengineering Ireland*. The School of Mechanical and Aerospace Engineering, Queen's University Belfast, ISBN 1-905254-18-0, 2007, p.86.

8.5 Awards

3rd prize for a student poster presentation at Annual Meeting of the Bioelectromagnetics Society, San Diego, California, USA, 2008.

GE IN Cell image competition runner up – Image from my HCA work displayed on Times Square, New York City, USA, 2008 (see below).

