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Investigation of Rhabdomyosarcoma Cell- Cell Electrofusion

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ABSTRACT

Cell-cell fusion is an important natural and engineered process for in-depth studies into hybridomas, developmental biology, immunology and various cellular therapies. It is also a powerful tool for analysis of gene expression, chromosomal mapping, antibody production, cloning mammals and cancer immunotherapy. However, research so far has primarily focused on cell models such as *C.elegans*, drosophila, myoblasts, spleen-myeloma cell hybrids and various plant protoplasts. Rhabdomyosarcoma cells are a rare form of musculoskeletal cancer cells found in the head/neck, and other less skeletal areas of the human cancer patient's body. These cells do not normally undergo fusion naturally. Therefore, if these cells can be engineered to fuse, the technique is expected to be effective for other cell types. Among all the techniques of cell fusion, electroporation was chosen due to its high versatility, yield and viability. By coupling the cells with this technique, the effects on cell proliferation, growth pattern and hybridoma count were investigated. Overall, the experimental results showed that an adequate electrical stimulation successfully helped to facilitate the fusion and proliferation of the cells, and the DC current produced the highest fusion yield and proliferation. Under DC current, a fusion yield of 27% and 38% was obtained, compared to 4% and 13% with no electrical current respectively. The work done proved that high yield and proliferation rate could be achieved using a design with two parallel thin gold electrodes sputtered onto the flask, making this an efficient and effective cellular engineering system.

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Chapter 1 INTRODUCTION

1.1 *Background*

Cell-cell fusion (cell fusion) is an important and fundamental process in the normal biology of humans, animals and plants. Even though it has a history of over a century, it is still growing widely and expanding rapidly due to its diverse applications in the major disciplines of biology, biotechnology and medicine. [1], [2]

Cell fusion was first proposed in the 1800s by a German biologist named Johannes Muller and has since attracted a lot of scientific interest over the decades that followed. Its first roots lie in the pioneering work of Theodore and Marcella Boveri [3] on sea urchin eggs in 1902 and the insights of German pathologist Otto Aichel [4] on tumor progression in 1911. Theodore and Boveri's work revealed that cell fusion contributed to the rapid acceleration of the evolution of life on earth. However, the first cell fusions were non-specific, and any two membranes could fuse together, provided the conditions were right. Today, cell fusion is highly specific and controlled, most often involving artificial engineering methods and specialized molecular proteins.

In 1982, Zimmermann and Kuppers [5] showed that the intensity of lightning during a thunderstorm was more than sufficient to induce a breakdown in membranes, and in turn induce cell fusion. They did an experiment to support this hypothesis: An electrically-conducting ore-containing rock sample was laid on electrically non-conducting sand. A second electrically-conducting ore-containing rock sample was connected to earth at a distance of 200 μ m away without an insulating sand layer. The rock samples served as electrodes (receivers), whereby the rock sample on the sand acted

as the receiving wire of an electric dipole. When electromagnetic waves acted upon pieces of ore, voltages were induced between the earthed and insulated pieces of ore. Under these conditions, it was possible to observe the electrical method of fusion of plant cells. This was just one of the experimental proofs that fusion can be re-created in nature just by using the elements.

There are two major milestones in the history of cell fusion. The first major milestone in cell fusion was its use in creating chimeras (combination of one species and another). This was a topic of interest for many in the early 20th century, with notable examples of that between animal (mouse cells) and man (HeLa cells) by Harris et al [6] in 1965 and animal (hen erythrocytes) and plant (yeast cells) by Cocking et al [7] in 1975. The second milestone in the history of cell fusion was the advent of hybridoma technologies or production of monoclonal antibodies (see Section 2.7.1) in immunology by Milstein and Kohler [8] in 1975. Since then, its impact in cancer research has been phenomenal. However, during the 1990s, the interest shifted towards mammalian cells due to rise of genetic engineering and the race towards mapping the Human Genome Project by the year 2000.

By the time of the 21st century, cell fusion studies began to shift towards developmental cell biology, seeing the universal use of nematodes like *C. elegans*, and more recently myoblast fusion in *Drosophila*, in a concerted effort to understand the molecular mechanisms behind fusion. This was milestone by the discovery of the fusogen (protein responsible for initiating and mediating fusion, also known as fusogenic protein) **EFF-1** [9], [10] in 2002, with some researchers claiming in 2005 that it alone was sufficient for cell fusion [11].

1.2 Objectives and Scope

The objective of this master's project is:

- To conceptualize and develop a system for enhancing cell-cell fusion in a controlled environment.

The scope of work comprises of the following:

- 1) To investigate the influence of various parameters, such as electricity, on cell-cell fusion growth, proliferation and physiology.
- 2) To perform theoretical modeling on this system, after factoring for these parameters. And thereafter, to modify these models with experimental data.
- 3) To demonstrate a more efficient and effective cell fusion system using this new system.

In this report, **Chapter 1** covers the background and objectives. **Chapter 2** provides a thorough review of what has been studied and covered in the literature, including its possible applications. This section describes recent progress and explains how it motivates and shapes the work that the author does in this direction. **Chapter 3** places the study of the fusion of these rhabdomyosarcoma cells in a mathematical perspective, covering the modeling of the cells. **Chapter 4** covers the materials and methods used to conduct the experiments and data acquisition. **Chapter 5** presents the experimental results, including observations, analysis and discussion. **Chapter 6** wraps up the report with a conclusion of all the findings, and highlights the remaining work left to complete this project.

Chapter 2 LITERATURE REVIEW AND THEORY

2.1 *History of cell fusion*

Throughout history, cell fusion had been carried out based on several approaches. Chemical and virus-mediated fusion methods were developed in the earlier years based on the application of chemical fusogens such as polyethylene glycol (Section 2.5.1) or fusogenic viruses (i.e. Sendai virus, *not covered in this report*) These methods have been associated with limitations such as toxicity to cells, batch-to-batch variability, and low efficiency. Various other problems of chip-based cell fusion devices include difficulties in aligning the cells in the direction of the electric field sufficiently for fusion to occur readily, difficulties to achieve a one-to-one pairing ratios, and inability for the device to be used for cells of different shapes and sizes. There were also substantial quantities of cell death due to electrical or chemical effects. Lasers and optical equipment used in the designs were also very expensive. The chemical method described in Section 2.5.1, such as adding the surfactant polyethylene glycol (PEG), had been the gold standard for cell fusion during the 1970s. The chemical method described in Section 2.5.1, such as adding the surfactant polyethylene glycol (PEG), had been the gold standard for cell fusion during the 1970s. However, its disadvantages were that it was slow, low-yielding, and cytotoxic (harmful to the cell). On the other hand, the electrical method described in Section 2.5.2 turned out to be the most widely used method in the 1980-90s. This was not only because it had minimal disruption to the cell integrity, but also because it produced a yield of more than 80 times that of the chemical method. Its ability to fuse single cells in a tightly controlled manner, under minimal voltage, represented a technique whereby the long-term genetic identity could be preserved, and where the behavior of each selected

cell could be precisely manipulated. [12] However, its disadvantage was that it could only be done in bulk (i.e. the number of cells in each run is massive), which creates the problem of unwanted cell fusion (i.e. fusions between same type of cells). These are some of the pros and cons that have been taken into consideration in designing this MEng project.

The impact of creating a novel cell fusion device is great and has applications that extend far downstream. For example, one advantage of such a device is its ability to customize the variety of parent cells, sometimes even to two or more, each selectively chosen based on the user's objectives. Among some of the many applications of this project are more specialized ones such as in-vitro fertilization (IVF) and reproductive and therapeutic cloning. Cloning poses an ethical issue in Singapore's biomedical industry, but the potential of this project in this aspect makes it border around the realm of science-fiction. Other examples of the project's potential are in the somatic hybridization of plants, creation of novel food crops, hybridoma technologies, and so on. All the possible applications of this project would be covered in greater detail in Section 2.7.

2.2 Terminology

There are several possible outcomes of cell fusion, as shown in Figure 2-1 [13].

Figure 2-1 Cell fusion terminology [13]

From Figure 2-1, if fusion occurs between two cells of the same type, the product is called a *homokaryon*, but when the two pre-existing nuclei fuse, the product is called a *synkaryon*. Thus if two nuclei of the same cell type fuses, the product is called a *homotypic synkaryon*. Similarly, if fusion occurs between two cells of different types, the product is called a *heterokaryon*, and vice-versa, the product is called a *heterotypic synkaryon*. If fusion happens between more than two cells, the multi-nucleated mass of cytoplasm is called a *syncytium* (*pl*: syncytia). On the other hand, ploidy (n) refers to the number of sets of chromosomes in a biological cell. In short, $2n$ refers to a diploid cell, $1n$ to a haploid cell and $4n$ to a tetraploid cell. [13] Cell fusion occurs naturally in nature (in-vivo) as shown in Figure 2-2.

Figure 2-2 Examples of naturally-occurring cell fusion [13]

Fertilization occurs when the haploid sperm fuses with the haploid egg (oocyte) to form a diploid zygote. Placental development occurs when trophoblast cells (cells forming the outer layer of a blastocyst, which provide nutrients to the embryo) fuse to form a syncytiotrophoblast, allowing faster transport of nutrients, hormones and wastes across the maternal-fetal boundary. Skeletal muscle formation occurs when myoblasts (a type of embryonic progenitor cell that gives rise to myocytes) fuse to form myotubes and myofibres, which have increased ability to contract. Bone formation occurs when osteoblasts fuse to form bone-reabsorbing multi-nucleated osteoclasts. Liver development occurs when bi- and tri-nucleated hepatocytes are found in mammalian cells, suggesting natural cell fusion. And finally, immune response takes place when macrophages cannot eliminate intracellular pathogens, fusing to form giant cells that are surrounded by activated T-cells.

2.3 History of engineered cell fusion

It was around the year 1958, that deliberate cell fusion began to be used as a tool for studying its own natural phenomenon, when Okada [14] first induced the fusion of Ehrlich's tumor cells by using the Hemagglutinating Virus of Japan (HVJ) virus *in vitro* and set up an engineering method for cell fusion around it. Ten years later, a much improved device (Figure 2-3) for engineering cell fusion was developed by Pojnar and Cocking [15] and was published in *Nature* in 1968, though this device was rather simple and rudimentary. Two protoplasts (plant cells with cell walls removed) are to be placed in the chamber shown in Figure 2-3, with one cell in the sliding piece - which mechanically forces the cells to merge with each other, thereby causing fusion.

Fig. 1. 'Perspex' culture chamber for the formation of cell aggregates from isolated tomato fruit protoplasts. (Constructed by Mr H. Hill.)

Figure 2-3 First rudimentary cell fusion device [15]

Since then, engineering cell fusion has been used extensively as a tool for plant hybridization by scientists such as Power and Cocking [16] in 1970, which marked the first step towards somatic hybridization of plants by working with plant tissues such as mature tomato fruit, radish storage root, and leaf tissues of tobacco. Similar parallel research led by groups such as Nagata and Takebe [17] focused on cell wall regeneration of isolated protoplasts, while others such as Kao and Michayluk [18] worked on standardizing the method of cell fusion by using the an agent called polyethylene glycol (PEG) to enhance fusion in 1974.

To date, many different methods have been developed for deliberately engineering cell fusion – ranging from chemical method (Section 2.5.1), electrical method (Section 2.5.2), optical trapping method (Section 2.5.3), molecular method (Section 2.5.4) to even the latest methods using technologies such as ultrasonic standing waves and surface acoustic waves (SAW). Among them, the electrical method (also known as electrofusion) stood out as the most widely used technique since U. Zimmermann [19] first developed a model in 1974 for the dielectric breakdown of cell membranes, and later went on to study its principles and industrial potential in 1983 in publications [20], [5]. In fact, physical cell manipulation methods such as electrostatic attraction, dielectrophoresis (DEP) and electroporation (Section 2.5.2) turned out to be better than chemical methods which disrupted normal cell functions.

Overall, electrofusion was an ideal choice in the 1980-90s due to its high efficiency, ease of implementation and the high viability of its post-fusion cells. [21] G.W. Bates was a researcher who invented the electrofusion device shown in Figure 2-4 that was published in *Planta* journal for fusion of tobacco (*Nicotiana Tabacum*) protoplasts in 1985. [22]

Figure 2-4 Engineered cell fusion chip – electrofusion [22]

Heterokaryons provide a way of mixing the components of two separate cells in order to study their interactions. The first direct evidence that membrane proteins are able to move in the plane of the plasma membrane came from an experiment in which mouse cells and human cells were fused. [1] Although the mouse and the human cell-surface proteins were initially confined to their own halves of the heterokaryon plasma membrane, they quickly diffused and mixed over the entire surface of the cell. Eventually, it was shown that a heterokaryon proceeded to mitosis and produced a hybrid cell in which the two separate nuclear envelopes have been disassembled, allowing all the chromosomes to be brought together in a single large nucleus. This showed that it was possible to produce heterokaryons with two nuclei for the purposes of studying the interactions between the components of the original two cells. Over time, the two nuclei would fuse, and heterkaryons eventually form a heterotypic synkaryon with a single fused nucleus.

2.4 Membrane science

Membrane interaction and deformation are basically two aspects of the same phenomenon. Because membrane interaction depends on structural features such as curvature, chemical composition, and surface organization, membrane interactions will change as the membranes are brought together. Deformation exists whenever there is any physical contact between two bodies. However, researchers have not readily understood the deformative response, and models which describe the extent of this deformation, upon experimental validation, would be useful in controlling any future cell fusion mechanisms. However, in order to develop a successful model, one must take into account the mechanical properties of the deforming bodies and the constraints under which this deformation takes place.

A biological cell is usually modeled as a conductive interior encapsulated by a thin insulating lipid bilayer [23], as shown in Figure 2-5.

Figure 2-5 Cell encapsulated by insulating lipid bilayer [23]

Two spherical cells (or vesicles) of opposite charges have an attractive force towards each other, causing both to flatten against each other. Therefore there are strong and weak interactions between two spherical bodies. To begin with, the basic relationship between

the minimum interaction energy G per unit area, the bilayer membrane tension T , and the contact angle θ is given by the Young's equation (Equation 2-1) below: [24]

Equation 2-1
$$\cos \theta = 1 + \frac{G}{2T}$$

The relationship between the contact angle θ (angle between the vertical dashed line of contact and the lines of vesicle tension T) of the two fused cells is shown diagrammatically by Figure 2-6.

Figure 2-6 Diagram showing relationship between T , G and θ [24]

This energy G , is balanced by the inter-membrane attractive and repulsive forces for a constant membrane tension T , and is dependent on the geometry of the cells. As the surface area-to-volume ratio decreases with increasing size, larger cells are more susceptible to relative deformation and to rupture. Furthermore, the principle of conservation of energy states that kinetic energy is required for fusion of the phospholipid bilayer membranes. Each phospholipid molecule requires a different level of activation energy. Once membrane-membrane contact is made, and the activation energy level threshold has been reached, a new membrane would be synthesized, and a fused cell

would be readily observed. However, besides the energy, the forces that exist between cells directly influence cell fusion. Examples of some of these forces are hydration forces (2.4.1), biochemical forces (2.4.2) and electropotential forces (2.4.3).

2.4.1 Hydration forces

Hydration forces are defined as repulsive forces that exist between two electrostatically charged phospholipids, when they are very close to each other in an aqueous media. These hydration forces pose a significant barrier to initiating inter-membranous contact, which is why the yield of unaided cell fusion is very low. (about 1 in 10^5) The change in molecular free energy with bilayer separation is the net repulsive force on one molecule acting in a direction perpendicular to the bilayer, and is given by:

Equation 2-2

$$F_R = \frac{\partial g}{\partial \left(\frac{d_w}{2} \right)} = PA$$

It is related to two forces, the repulsive force F_H and the van der Waals force F_A , such that $F_R = F_H - F_A$, where

Equation 2-3

$$F_H = F_0 \exp \left(-\frac{d_w}{\lambda} \right)$$

and

Equation 2-4

$$F_A = \frac{-A_H}{6\pi} \left[\frac{1}{d_w^3} - \frac{2}{d^3} + \frac{1}{(d + d_3)^3} \right]$$

where d_w is the intermolecular separation, A_H is known as the Hamaker coefficient, and $F_R=0$ at the equilibrium separation. If the van der Waals force is known at a particular bilayer separation, the calculation of the Hamaker coefficient is straightforward. For

charged lipid bilayers at the equilibrium separation with excess solution, the lamellae are at such great separations that the hydration force is negligible.

The logarithmic plot in Figure 2-7 shows the behavior of hydration forces, electrostatic forces (both in solid lines) and van der Waals forces (dashed line) between lipid bilayers at varying inter-atomic separations. A minimum threshold of activation energy must be imparted into the fusing cells in order to overcome these hydration forces and bring them into contact. According to the laws of physics, as cell-cell separation decreases, the hydration forces increases significantly, as shown in Figure 2-7. [24] In the figure, the van der Waals attractive forces are equal to the electrostatic repulsive forces at the equilibrium separation, at an inter-atomic separation of $10\text{\AA}$. After a certain threshold, the hydration forces overcome the van der Waals attraction forces, inter-atomic repulsions and gradients in the chemical potentials of the metabolites.

Figure 2-7 Relationship between hydration, electrostatic and van der Waals forces between the membrane bilayers with increasing inter-atomic separation of two cells [24]

2.4.2 Biochemical forces

In the discipline of biochemistry, a metabolic flux is defined as the rate of turnover of molecules through a metabolic pathway. The enzymes involved in the pathway regulate this flux. Regulation of the metabolic flux within cells is vital for the regulation of the metabolic pathways under different conditions. As two cells approach each other, two sets of individual metabolic flux profiles overlaps, and this overlap itself constitutes a force between the two cells, which can be attractive or repulsive.

A simple way of visualizing an intercellular force due to the metabolic flux is to understand that every living cell is surrounded by an osmotic pressure gradient (Figure 2-8) which can be radially positive or negative, depending on the balance of the metabolism according to Pennline et al. [25]

Figure 2-8 Electrical Double Layer [25]

Overlapping of two such gradients gives a net osmotic repulsion or attraction. However, there is no literature readily available on the quantitative calculations of such intercellular forces based on flux interactions. One of the few related calculations available shows that there is electrical double layer on the surface of the cell, which is a structure that appears on the surface of a charged object when it is placed into a liquid. This electrical double

layer can be overwhelmed by the diffusion potential resulting from realistic enzyme action involving the production of ions at the cell surface. [25]

2.4.3 Electro potential forces

Most cell membranes have a voltage across them, known as the *membrane potential*. This difference in potential exerts a force on any molecule that carries an electric charge. The cytoplasmic side of the plasma membrane is usually at a negative potential relative to the outside, and this tends to pull positively charged solutes into the cell and drive out negatively charged ones. For more details on electro potential forces, refer to Section 2.5.2 entitled Electrical method.

There is also an *electro chemical gradient*, defined as the net force driving a charged solute across the membrane. It consists of two forces: a force due to the concentration gradient (transport coefficient), and a force due to the voltage across the membrane (ionic flux). Furthermore, when the cells are in close proximity (apposition), short-range forces known as *stereo chemical forces* dominate. These forces are the basic cause of effects such as antibody reactions and type-dependent cell adhesion. In fact, upon contact, other membrane structures such as ion channels, aquaporins, gap junctions, and for plant cells, plasmodesmata come into play. All these concepts, however, are beyond the scope of this project.

2.4.4 Membrane proteins

Most membrane proteins play only a passive role in engineered fusion processes. Once the spatial hindrance of membrane proteins are removed, fusion of the bared lipid bilayers proceeds in a manner similar to that of fusion between model membranes. The composition of the lipids can then be a major determining factor in the fusion efficiency.

The molecules that take part in a fusion reaction can be divided into three groups according to their stages, as follows:

- 1) Molecules that function before fusion, known as *cell adhesion molecules* (CAMs).
- 2) Mediators of fusion that directly rearranges the lipid bilayers and lead to the formation of fusion pores. These are called *fusogens*.
- 3) Molecules that lead to the extension of the fusion pores, and to the complete disassembly of the membranes. These are called *extensors*.
- 4) Molecules that allow the passive movement of small water-soluble molecules into and out of the cell via the trans-membrane pores. These are called *channel proteins*.

For membrane proteins, there are three possible kinds of binding, namely: homophilic binding, heterophilic binding, and binding through an extracellular linker molecule as shown by Alberts et al [26] in Figure 2-9.

Figure 2-9 Cell Adhesion Mechanisms [26]

Table 2-1 provides a brief summary of the molecular protein mechanisms:

Type of binding	Description	Example
Homophilic	A CAM binds to a same type of CAM in another cell	Cadherin-Cadherin binding
Heterophilic	A CAM binds to a different kind of CAM in another cell	Receptor-Ligand binding
Binding through extracellular linker molecule	Binding via an intermediate linker molecule that forms a bridge/link between the a CAM of one cell and the CAM of another cell	These linkers are called catenins

Table 2-1 Types of Cell Adhesion Molecules and Binding [26]

In a living tissue, cells adhere to each other and to the *extra cellular matrix* (ECM) through CAMs. Some CAMs are Ca^{2+} dependent, while others are Ca^{2+} independent. Generally, Ca^{2+} plays an important role in cell-cell adhesion, as it can either inhibit or promote binding and fusion. Once two cells are joined together by a CAM, a variety of events can lead to fusion, resulting in membrane components spreading across the “bridge” resulting in local chemical reaction shifts. For channel proteins, if they form large pores and exist between neighboring cells, they are known as *gap junctions*. If they form channels in the outer membrane of mitochondria and bacteria, they are known as *porins*. Most of these channel proteins are different and have narrow and highly selective pores. Almost all of these proteins are ion channels, concerned exclusively with the transport of inorganic ions, which are mainly Na^+ , K^+ , Cl^- , and Ca^{2+} ions.

2.5 Cell fusion techniques

2.5.1 Chemical method

Of all the methods of inducing cell fusion, the chemical method involving the addition of polyethylene glycol (PEG) treatment to plant cells is the oldest one. The addition of PEG to a suspension of protoplasts (plant cells after stripping of cell wall) alters the plasma membranes of the plant cells such that it induces them to fuse. The general formula for polyethylene glycol is $\text{HOCH}_2(\text{CH}_2\text{-O-CH}_2)_n\text{CH}_2\text{OH}$, and it is water soluble with a pH value of between 4.6 and 6.8 depending on the degree of polymerization. (Merck Index) [18] The working principle is that adding a surfactant such as PEG lowers the surface tension of the liquid bilayer of the two contacting 25nm-thick plasma membranes, causing the hydrophobic and hydrophilic lipids to “jump” and

re-position themselves to form a closed-loop heterokaryon. PEG destabilizes the lipid bilayer membranes of the cells, aiding them to breakdown and fuse. The ether linkages in PEG make the phospholipids slightly negative in polarity and capable of forming hydrogen bonds with water, proteins and carbohydrates in the suspension. When the chain of the PEG molecule is long enough, it binds to Ca^{2+} ions and acts as a molecular bridge between the negatively polarized groups of proteins and the PEG, thus enhancing adhesion. In addition, the mechanical pressure from the two micropipettes delivering the plant cells in a fusion experiment will enhance the formation of this new bilayer. A buffered suspension with surfactants and preservatives is normally used as the cell medium. Upon fusion of the two protoplasts, genetic transfer will occur readily to form a new hybrid cell (heterokaryon).

The first successful attempt at protoplast fusion was done by Roper [27] in 1952 on two species of filamentous fungi called conidia in the species *Aspergillus nidulans*. A strain of yellow conidia requiring lysine and a strain of white conidia requiring adenine were treated for 5 hours at 37°C with d-camphor vapor. Hoh et al [28], [29] also showed a successful binding of PEG to Ca^{2+} ions between two auxotrophic strains of *Aspergillus Niger*. His objective was to create new recombinant strains of *A. Niger* with improved activity of β -glucosidase, which is an enzyme present in the plant cell wall. This was needed because *Trichoderma spp.*, which is a fungus present mainly in soil, produces relatively low levels of extracellular β -glucosidase, resulting in limited hydrolysis of cellulose. Their papers showed that the overall rate of saccharification could be increased by culturing *Trichoderma spp.* with other fungi with higher β -glucosidase activities.

Another paper by Kao and Michayluk [18] in 1973 observed that protoplast fusion took a longer time to fuse as compared to animal or yeast cells. However, they proved

experimentally that the speed of fusion could be increased by adding concentrated PEG, which incidentally also increased the heterokaryon yield (number of fused cells). They conducted an experiment involving a mixture of soyabean and barley protoplasts in PEG solution and observed that it achieved a heterokaryon yield of 6%. Among the 721 living protoplasts examined, 44 of them were heterokaryons. Eleven out of 138 of the PEG-treated protoplasts (8%) divided in three days and formed colonies after more than ten days. From these experiments, it was shown that fused protoplasts could readily regenerate new cell walls and undergo cell division after PEG treatment.

The effects of calcium (Ca^{2+}) concentration, cyclic adenosine monophosphate (cAMP) analogs (a signaling molecule derived from ATP) and theophylline (a methylxanthine drug used in therapy for respiratory diseases such as COPD and asthma under a variety of brand names) were also proven to have a direct impact on cell-cell fusion, as shown by a paper by John Sauk in 1977 on the fusion of myoblasts of B.W. 10139 mouse rhabdomyosarcoma (RMS) cells. [75] As can be seen in Figure 2-10, the increase of Ca^{2+} ion concentration cause the change of the spindle-shaped cells into cells of round spherical shape, about $15\mu\text{m}$ in diameter.

Figure 2-10 (a) RMS cells at 72hr, $10\mu\text{M}$, Ca^{2+} - no evidence of cell-cell fusion (b) RMS cells at 72hr, $1500\mu\text{M}$, Ca^{2+} showing a small percentage of cells undergoing fusion

Thus Ca^{2+} concentrations of lower than $1000\mu\text{M}$ plus cAMP analogs and theophylline were noted to inhibit RMS cell-cell fusion. On the other hand, Ca^{2+} concentrations of more than $1000\mu\text{M}$ produced only limited multi-nucleated muscle fibers, implying that a majority of RMS cells lacked a signaling system for Ca^{2+} . In the same paper, the occurrence of surface microvilli and filopodia at various Ca^{2+} concentrations and after treatment with cAMP analogs were also discussed.

2.5.2 Electrical method

By applying an electric field of adequate strength and duration, the cell membrane may undergo some temporary perturbations and return to its normal state when the exposure to the electric field ends, making the electroporation reversible. However, if the exposure to the electric field is too long or the strength of the electric field is too high, the membrane does not reseal after the end of the exposure and electroporation becomes irreversible. Depending on the type of electroporation (reversible or irreversible), two groups of applications exist: (1) functional - where the functionality of the cells or tissues must be sustained, and (2) destructive - where the electric fields are used to destroy the plasma membranes of cells or microorganisms. [30] The irreversible electroporation can be used for non-thermal food and water preservation, mainly causing permanent destruction of microorganisms [31-33].

It was also shown that by electroporation, small and large molecules can be introduced into and extracted from cells, proteins can be inserted into the membrane, and cells can be fused. As a result of its high efficiency, electroporation has rapidly found applications in many fields of biochemistry, molecular biology, and medicine. Probably the most important functional application of electroporation is that it creates conditions

for the transport of small or large molecules to the cytoplasm through the cellular membrane [34], [35].

Dielectrophoretic force (or DEP force) is one of the key phenomena in the realization of electroporation in cell fusion. This technology has been widely accepted as the state of the art in a number of important areas of research such as plant breeding, biomedicine and biotechnology. Its proliferation has attracted enormous interest and attention and led to a wide variety of applications, such as hybridoma technologies and monoclonal antibodies production, nuclear transfer, gene transfection, cell sorting, and a wide variety of biophysical studies on membrane proteins. [36]

The working principle of DEP force is that unlike a cell that is placed in a uniform electric field (as shown in Figure 2-11) [37], there is net force acting on a cell that is placed in a non-uniform electric field due to the unequal distribution of charges on the cell. This force will cause the cell to move from a region of low-density electric field to a region of high-density electric field (right to left) in a phenomenon known as negative polarization. It is called negative DEP or n-DEP, as the cell moves towards the negative pole of the electric field.

Figure 2-11 (a) No net force in a uniform electric field (b) Net force due to negative polarization due to unequal surface charges on the cell (n-DEP) [37]

As most biological cells behave as polarized and ionized particles in non-uniform electric fields as shown in Figure 2-11 (b), they can be manipulated by the n-DEP force for a wide variety of applications, ranging from cell separation, orientation, rotation, to cell agglutination and fluidic flow control. In fact, this method is so versatile that it is the main method for cell alignment and cell pairing in traditional electrofusion and it remains the most widely used fusion technique since the early 1980s until now. [38] Many well-demonstrated applications of DEP force involving the separation of different cell types based on the differences in the dielectrical polarizabilities are present in literature [39-44].

Besides negative polarization (n-DEP), positive polarization (p-DEP) also exists such as in an example of a recent p-DEP force application by Yang et al [45] as shown in Figure 2-12.

Figure 2-12 (a) p-DEP experimental setup (b) Cell separation [45]

Yang's device comprises of a silicone rubber chamber and a set of inter-digitated microelectrodes on the floor of the chamber. The inter-digitated electrode has a total of 40 pairs of finger electrodes, consisting of a $25\mu\text{m}$ electrode and $25\mu\text{m}$ spacing. The yellow bars show the positive and negative DEP poles. The electric field is strongest at the edges of the finger electrodes, and weakest at the centers of the space between the finger electrodes. p-DEP moves some cells to the edges of the finger electrodes, while n-DEP moves the rest of the cells towards the centers of the space between the finger electrodes.

However, the disadvantage of most DEP-based cell-pairing methods is that many cells form clusters between the two electrodes. This makes control of the intended fusion outcome difficult. Furthermore, cell pairing via this method is random, resulting in lower pairing precision and efficiencies. Nonetheless, a group led by Cao et al [46], [47] managed to control clusters of cells effectively by using a microelectrode array shown in Figure 2-13.

Figure 2-13 Cell fusion chip using a microelectrode array [46]

Based on this DEP force, each electrode pair was used to control one cluster of cells. Each complementary electrode had opposite polarities, which attracted cells to form a string-like alignment (Figure 2-14). [47]

Figure 2-14 Cell fusion chip showing string cluster of cells just prior to fusion [47]

The advantage of this method is that cells of different sizes may be fused readily. The axis through the centers of the two contacting cells would be in the same direction as the electric field lines, thus fusion efficiency would be high. The DEP force may also be controlled by varying the distance between the electrodes and the magnitude of the membrane electric voltage V_m . Overall, this method was simple and of high pairing precision, and could be transplanted to many other on-chip cell electrofusion methods. [46], [47]

The mathematical modeling of DEP force remains an important consideration when studying cell fusion. As described above, when cells are placed in an electric field, they undergo polarization to form electric dipoles, which may cause the cell to move in a certain direction if the electric field is non-homogenous. The cell membrane is the only polarizable entity in the cell, as the cytoplasm is incapable of carrying any charge.

Induced by this electric field, the electrolyte ions on both sides of the cell membrane are polarized to generate a membrane potential difference V_m given by:

$$\text{Equation 2-5} \quad V_m = \frac{3}{2} a E_0 \cos \theta \left[1 - \exp \left(-\frac{t}{\tau_s} \right) \right]$$

where θ is the zenith angle, and τ_s is given by:

$$\text{Equation 2-6} \quad \tau_s = a C_m \left(\rho_{in} + \frac{\rho_{out}}{2} \right)$$

where ρ_{in} and ρ_{out} are the intracellular and extracellular electric resistivities respectively, and C_m is the membrane electric capacitance per unit (F/m^2), which is given by:

$$\text{Equation 2-7} \quad C_m = \frac{\epsilon_m}{d_m}$$

where d_m is the thickness of the cell membrane, and ϵ_m is the dielectric permeability of the medium surrounding the cell. A biological cell may be approximated as a spherical particle and the strength of the DEP force (here denoted by F_{DEP}) acting on it is given by:

$$\text{Equation 2-8} \quad F_{DEP} = 2\pi\epsilon_m a^3 \text{Re}[K(\omega)] \nabla E_0^2$$

where ϵ_m is the dielectric permeability of the medium surrounding the cell, a is the cell radius, Re is the real part of a complex number, E_0 is the external electric field strength, ω is the angular frequency of the electric field, and $K(\omega)$ is the complex Clausius-Mossotti factor, which describes the strength of the effective polarization of the cell, and is given by:

$$\text{Equation 2-9} \quad K(\omega) = \frac{\hat{\epsilon}_c - \hat{\epsilon}_m}{\hat{\epsilon}_c + 2\hat{\epsilon}_m}$$

where $\hat{\epsilon}_c$ and $\hat{\epsilon}_m$ are the complex dielectric permittivities of the cell and medium respectively. The complex dielectric permittivity is defined as:

Equation 2-10

$$\hat{\varepsilon} = \varepsilon - i\left(\frac{\sigma}{\omega}\right)$$

where $i = \sqrt{-1}$ is the dielectric permittivity, and σ is the electric conductivity. Ceteris paribus, F_{DEP} is proportional to the electric field gradient ∇E_0 . When $K(\omega) < 0$, the medium is more polarizable than the cell, which means the cell travels from a region of high electric field to a region of low electric field, thus F_{DEP} is negative. Vice-versa, when $K(\omega) > 0$, the cell is more polarizable than the medium, which means the cell travels from a region of low electric field to a region of high electric field, thus F_{DEP} is positive.

The magnitude of the movement force F_{DEP} is also proportional to r^3 , and is usually significant for cells to the order of several microns. The real part of the Clausius-Mossotti factor, $\text{Re}[K(\omega)]$ determines the magnitude and directional sign of the movement force. This factor is bonded between $-\frac{1}{2} < \text{Re}[K(\omega)] < 1$ and depends upon the electrical properties of the cell and medium, and upon the frequency of the applied AC electric field. [48]

In summary, compared to other methods of cell fusion, dielectrophoresis or electrofusion has become a very popular method for cell fusion for the following reasons:

- 1) Fusion occurs in a more synchronized manner that allows the scientist to study the dynamic fusion intermediates. Such analysis is impossible with chemically or virally induced fusion.
- 2) Biological variability is less of a problem compared with other methods.
- 3) The fusion parameters are more predictable.
- 4) Control and monitoring of the fusion process can be done microscopically.

- 5) The results are more highly reproducible.
- 6) It has the ability to produce hybrids when input cell numbers are low/limited.
- 7) The yield is 80 times higher than the chemical method. In the PEG method, 1-2 cells fuse for every 10^5 protoplasts in the suspension, but in the electrofusion method, 1-2 cells fuse for every 10^3 protoplasts in the suspension.
- 8) It results in a controlled alignment of cells, compared to the random agglomeration of cells in the PEG method. This can be monitored under a light microscope, which would permit the scientist to identify the hybrid cells.

However, the disadvantages of electrofusion are:

- 1) The setup cost of the equipment and consumables is high.
- 2) It is impossible to select the individual cells to be fused.
- 3) Very often electrofusion leads to unwanted fusions between cells of the same cell type and product-selection protocols need to be implemented.

2.5.3 Optical trapping method

Stromberg et al [12], [49], [50] manipulated individual cells by optical trapping. Spatially selective fusion of cell-cell or cell-liposome pair was achieved by the application of a highly focused electric field through a pair of $5\mu\text{m}$ outer diameter carbon-fiber ultra-microelectrodes (Figure 2-15) onto a cell fusion chip (Figure 2-16). Figure 2-17 shows the variety of images of the Jurkat cells (immortalized line of T-lymphocyte cells), which were filled with different colored dyes prior to fusion. Bright-field images were obtained by a three-chip color CCD camera.

Figure 2-15 Optical trapping equipment setup [12], [49], [50]

Figure 2-16 Cell fusion chip [12], [49], [50]

Figure 2-17 Sequential pairwise fusion of different liposomes [12], [49], [50]

This method has a high pairing precision, but its efficiency is very low as only one pair of cells can be manipulated at each time.

2.5.4 Molecular method

For more than 30 years, *Caenorhabditis elegans* (*C. elegans*), a microscopic roundworm belonging to the family of nematodes, has been used as an animal model system in cell fusion research. They are often used to investigate what happens on a basic level before moving on to the more complex human system. As such, *C. elegans* has enabled researchers to learn a great deal about natural or developmental cell fusion. It is so small and simple that it is possible to mutate the gene and observe the effects directly, making it a valuable tool for researchers worldwide. *C. elegans* is responsible for producing a fusogen called EFF-1, which is essential and sufficient for fusion, as shown from literature by Mohler et al [9], Shemer et al [10], and Oren-Suissa et al [51].

For example, Shemer et al [10] examined developmental cell fusion in *C. elegans* and showed that EFF-1 mutants had implicated cell fusion while establishing its organs' shape and size, and EFF-1 was responsible for controlling cell migration. These authors led by Mohler, Shemer and Oren-Suissa found by mixing the cytoplasmic contents of the cells that ectopic expression of EFF-1, which encoded a membrane protein, could directly promote epithelial cell fusion. In pharyngeal muscle cells, they also showed that EFF-1 appeared to stimulate multiple micro-fusion events. However, this protein was not required for micro-fusion in other organs such as the uterus.

2.6 Cell-trapping and cell pairing designs

The current cell-trapping and cell-pairing designs of other research groups are an important consideration for the modeling and control of the micro-device. In 2009, Skelley et al [52] managed to solve the one-to-one selective cell pairing problem using device made by an array of strategically placed “cups” (Figure 2-18).

Figure 2-18 Three-step cell loading protocol device [52]

As shown in Figure 2-18, the procedural steps were as follows:

- 1) The first types of cells are loaded ‘up’ towards the smaller backside of the cup.
- 2) The direction of flow is reversed, and the cells are transferred ‘down’ into the larger front-side cup two rows below.
- 3) The second type of cell is loaded from the top, and they are captured onto the top of the first cell type. In this way, two different cell types of the same diameter as the large cup could be trapped, paired, and fused.

The advantage of this design for cell trapping and pairing was that the transfer was fast and highly efficient due to the laminar flow within the device. Furthermore, each cup was sized to only trap two cells, so additional cells could not enter the cups. However, the problems with the design were:

- 1) The design and fabrication of this chip was relatively complex
- 2) The two cell types must be of similar size as the width of the capture cups, or else the axis through their centers cannot be aligned with the direction of the electric field.

Another group of researchers led by Techaumnat et al [53] presented the use of electric field constriction created by a microfabricated structure to realize high-yield electrofusion of biological cells. Their method involved using an orifice on an electrically insulated wall (orifice plate) whose diameter was smaller than that of the cells. (refer to Figure 2-19)

Figure 2-19 Models of electrofusion device based on field constriction by an orifice [53]

Due to the field constriction created by the orifice as shown, the magnitude of the membrane voltage around the contact point could be selectively controlled, regardless of the cell size. The field constriction also ensured one-to-one fusion, even when more than two cells formed a chain at the orifice. However, the disadvantage of this device was that:

- 1) Precise cell pairing could not be accomplished because cells would flow freely through the orifice from one channel to another, thus cells from the same channel could also be paired inadvertently.
- 2) For each orifice, only cells of similar size could be paired and fused.
- 3) It was common for cell clusters to form at an orifice.
- 4) Many other cells that do not take part in the fusion would be aligned in a chain at the orifice, thus decreasing the overall fusion efficiency.

2.7 Possible applications of cell-cell fusion

Some of the current possible applications of this cell-cell fusion project include (but not limited to):

- 1) In horticulture, a successful fusion micro-device can create a system to hybridize plants of both inter- and intra-species, creating plants that are of higher aesthetic and commercial value.
- 2) In agriculture, cells from two different food crops can be fused and cultured into exotic food products with desired qualities. This method of producing novel food crops is superior to genetically engineered food crops as it is natural and safe, and will gain acceptance more readily by consumers over “genetically enhanced” fruits and vegetables.
- 3) In medicine, hybridization between myeloid (bone marrow) cells and tumor cells can be used explain the reduced immunogenicity (the ability of a substance to provoke an immune response) of metastatic cells, leading to breakthroughs in cell therapy and immunology.

For future possible applications of this project, an overview is illustrated in Figure 2-20, and this list is constantly growing as new developments in cell fusion science are being discovered, just as new micro-device technologies are constantly being developed.

Figure 2-20 Current and future possible applications

Details of these applications are given in the following sections.

2.7.1 Hybridoma technology

Hybridomas are products of cell fusion (or hybrid cell lines) created typically in a laboratory by fusing a specific antibody-producing spleen cell from a mouse with a myeloma cell that can grow uncontrollably in tissue culture (as shown in Figure 2-21) [26], in an absence of antibody chain synthesis. The mixture of the two cells is fused typically in a PEG medium (chemical method).

Figure 2-21 Hybridoma technology/Production of monoclonal antibodies [26]

The monoclonal antibodies (mAbs) produced by the hybridomas are all of a single specificity and can therefore be applied in the field of tumor immunology. Since their discovery in 1985, they have been widely employed to produce unlimited quantities of uniform monoclonal antibodies, were widely used to detect and purify cellular proteins or to defend against particular antigens. However, the efficiency of cell fusion using the chemical method (i.e. polyethylene glycol) is not good enough for efficient production of monoclonal antibodies. The alternative procedure for obtaining human monoclonal antibodies or hybrids of dendritic and autologous tumor cells, that can be more efficient than polyethylene glycol, is cell electrofusion (Section 2.5.2 and 2.7.4). [54]

2.7.2 Insights into metastasis

In 1911, the first formal application of cell fusion in oncology was well documented by a German pathologist named Dr. Otto Aichel. [55] From his studies, he claimed that fusion of leukocytes with tumor cells could lead to hybrids and the emergence of a malignant cell. [55], [56], [4], [57] Ever since that event, the theory of cell fusion between white blood cell (leukocytes) and tumor cells that developed was the most complete theory used to explain *metastasis* (the spreading of malignant, cancerous cells).

A leukocyte is capable of migrating from its site of origin to various distant organs, while a tumor cell is capable of dividing uncontrollably. When fused together, the resultant hybrid adopts both of these two properties, characterizing what is known as metastasis, or the ability of the tumor cell to divide uncontrollably and spread to the rest of the body. [4] Currently, there are approximately 30 reports confirming cancer cell fusion in animal tumor models. Recent publications from Pawalek et al [57], [58] have fused healthy macrophages with weakly metastatic mouse melanoma cells in the laboratory to better understand the mechanisms of cell fusion and its relationship to metastasis.

2.7.3 Mechanobiology

Over the past 50 years, cancer research has produced over 1.6 million publications, but despite its numerous successes, it had little impact on reducing cancer mortality. [59-64] Looking back at the history of medicine, an explanation for this phenomenon is that in the strife for better cures and treatments, scientists have overlooked the primary cause of this complex disease, which is its cellular disease states. [65-68] Going into the micro-

and nano-scale level of the cell, the prevention and cure of many diseases, including cancer, may be the solution. Cellular/molecular engineering or mechanobiology of cells, and notably fusion studies of cells, lymphocytes and viruses looks promising in unlocking the keys to this problem. This application is just one of the many spin-offs that can result from cell fusion studies after a fusion micro-device has been successfully created.

2.7.4 Electrofusion

As explained in Section 2.5.2, under certain experimental conditions, a delivery of electric pulses could lead to the fusion of membranes of adjacent cells. Electrofusion has been observed between suspended cells [69-71] and even between cells in tissue [72]. For successful electrofusion in suspension, the cells must previously be brought into close contact, for example, by dielectrophoresis (DEP). [70] Electrofusion has proved to be a successful approach in the production of vaccines [73] and antibodies [74].

2.8 *Theory of project*

Based on the literature review, it was determined that cell-cell fusion is an important natural and engineered process for in-depth studies into hybridomas, immunology and various human cellular therapies. [1], [13] Rhabdomyosarcoma (RD) cells, of 20 to 40 μ m in dimension, are a rare form of musculoskeletal cancer cells found in the head, neck, and other less skeletal areas of the human cancer patient's body. As fusion of these cells is not a natural and automatic process, engineering it artificially via specially-designed chip-based microfluidic technologies is expected to be an effective tool that can be extended for other similar cell types.

In a standard Bio-Safety Level 2 (BSL2) laboratory passage experiment conducted by the author, upon the addition of 0.05% Trypsin-EDTA (3ml), the cells changed from the state shown in Figure 2-22 to the state shown in Figure 2-23 respectively.

Figure 2-22 RD cells in Minimum Essential Medium Eagle (MEM) culture (live cells are spindle-shaped, dead cells are spherical) (Image by author)

Figure 2-23 RD cells in suspension state (5 mins after the addition of 0.05% Trypsin-EDTA, “lifted off” for fusion studies) (Image by author)

From Figure 2-23, it is easy to conclude prematurely that cell-cell fusion may have happened if the cells appear to aggregate in the way as shown in Figure 2-23. However, one should not be misled by the natural tendencies of the cells to adhere to one another *in vitro* in the presence of trypsin as shown above, as there is no breakdown and fusion of the two separate membranes.

By definition, cell fusion is defined to have occurred if and only if the two membranes have been permeated and combined into one continuous structure. Thus a multi-nucleated cell would be considered a hybridoma, provided it (1) survives the 24-hour post culture period, and (2) can proliferate further post-culture. This proliferation rate can be determined by counting the number of live cells before and after the fusion treatment, and for this project, using a formula shown in Equation 3-1.

$$\text{Equation 2-11} \quad \text{proliferation_rate} = \frac{\#_live_cells_after_24hrs_of_electrofusion}{\#_live_cells_before_experiment}$$

The key advantage of this formula was that only the cells that survive are counted. This is important, as only the cells that survive are useful for obtaining hybrids for monoclonal antibody production. In addition, based on the paper by Trontelj et al (2008) [76], how effective and efficient the cell fusion would be was determined by Equation 3-2.

$$\text{Equation 2-12} \quad \text{fusion_yield} = \frac{\#_live_nuclei_in_multi-nucleated_cells}{total_\#_of_live_nuclei}$$

This formula gives more accuracy than the ones based just on bi-nucleated cells. However, in order to fully verify the formation of a hybridoma, one needs to outline of the membrane perimeter using a fluorescent dye or by running them through a Florescence-Activated Cell Sorting (FACS) device, or flow cytometer for short. The

latter is covered in the Future Work of this project (Section 6.2). *Note:* All the cells in this report were counted using a standard Neubauer hemocytometer.

According to the Gauss theory, the charge density ρ is related to the dielectric constant ε and electric field intensity ∇E by

$$\text{Equation 2-13} \quad \nabla E = \frac{\rho}{\varepsilon}$$

The relation between the potential Φ and the electric field E is given by

$$\text{Equation 2-14} \quad E = \nabla \Phi$$

Replacing E in Equation 2-13 with that in Equation 2-14 derives Poisson's Equation:

$$\text{Equation 2-15} \quad \nabla^2 \Phi = \frac{\rho}{\varepsilon}$$

If $\rho = 0$, Equation 2-15 can be simplified into Laplace's equation as

$$\text{Equation 2-16} \quad \nabla^2 \Phi = 0$$

Because the electrodes in this electrofusion microchip are conductors which attract free charges to their surface, the Maxwell's Equation is given by

$$\text{Equation 2-17} \quad e_n \times E = 0, e_n \cdot E = \rho_s$$

where ρ_s is the surface charge density, e_n is a unit vector in the normal direction of the interface from the conductor to the external environment, and D is the electric displacement vector, which can be described as εE . The electric field intensity ∇E in the conductive solution can be found by solving Equations 2-14 to 2-17.

Chapter 3 MODELING

The prediction of the behavior of each cell under an electrical field is an important factor in creating a high fusion yield. In this section, an introduction to the study of cell membrane electroporation would be presented using the (1) cell fusion model (**Figure 3-1**), (2) diffusive transport model (**Figure 3-2**) and (3) electrical conductance model (**Figure 3-3**), along with a variety of models proposed by previous researchers. These models are significant and relevant to the experiments of this project, and are validated by the results, which will be shown in Chapter 5.

The concept behind these models is that an infinitesimally thin spherical insulating membrane in conducting media would be highly representative of each cell, on which accumulation of charges occur when an external electrical field is applied. Firstly, a simplified spatial cell fusion model was created in one-dimensional (1D) space as shown in **Figure 3-1**.

Figure 3-1 Cell Fusion Model The concentration γ and electric potential ϕ of the cells varies in the x -direction. The n^{th} cell has a diameter of $x_{n,n+1} - x_{n-1,n}$ (created by author)

Due to the highly complicated structure of a lipid bilayer cell membrane, modeling it with real world accuracy would be a highly arduous task. Therefore, a sphere was chosen as the basic shape for the cell, based on the morphology displayed during the cells' trypsinized state as was previously shown in Figure 2-23. Based on this assumption, this simplified model would provide the required data after an input with key parameters, but most importantly it would work for an infinite number of cells of same or different sizes.

To reproduce all of the real life transport phenomena such as mass, thermal, convective, diffusive and electrical transport into the model would be impossible, but one can make the model as realistic as possible by introducing Fick's law, which states that the quantity's flux is proportional to the gradient of that quantity. Thus in a 1D system, the diffusive and electrical transport of this system is given by

Equation 3-1
$$\vec{J}_D = -D \frac{\partial \gamma}{\partial x} \hat{x}$$

and

Equation 3-2
$$\vec{J}_E = -\sigma \frac{\partial \phi}{\partial x} \hat{x}$$

where $\vec{J}_D$ and $\vec{J}_E$ are the diffusivity and electrical fluxes of the cells respectively, and D and σ are the diffusivity and conductivity of the medium respectively. In two and three dimensions, one must use the ∇ (del or grad) operator, obtaining

Equation 3-3
$$\vec{J}_D = -D \nabla \gamma$$

and

Equation 3-4
$$\vec{J}_E = -\sigma \nabla \phi$$

The assumption made here is a steady-state system and the transport medium is a dielectric. If that is not the case, then the electrical flux cannot be completely described

by Fick's law. A second term must be added to account for the electrical displacement flux, which converts the second term to

Equation 3-5

$$\vec{J}_E = - \left(\sigma \frac{\partial \varphi}{\partial x} + \epsilon_m \frac{\partial}{\partial t} \left(\frac{\partial \varphi}{\partial x} \right) \right) \hat{x}$$

where ϵ_m is the permittivity of the medium. Based on these equations, a further model was developed to describe the diffusive transport phenomena because the effect of diffusion mimics an electrical circuit as shown in **Figure 3-2**.

Figure 3-2 Diffusive Transport Model (created by author)

Just as a solute diffuses across the membrane from a region of higher concentration to a region of lower concentration, a representation of diffusion would be a current that flows across a resistor from a point of higher charge to a point of lower charge. Also, as charges can accumulate within the body of the cell membrane itself, this can be represented by a capacitor (holding a certain amount of charges) connected to a ground. In this diffusive transport model, we can use Kirchhoff's second law (KCL) to verify its validity. By Kirchhoff's second law, the sum of all the currents at any node i must be zero. This resulted in Equation 3-6 at the node γ_i .

Equation 3-6

$$I_D^{i-1,i} - I_D^{i,i+1} - I_D^i = 0$$

During electroporation, an applied electrical field produces a current through the cell suspension. As the cell membrane is composed of lipid bilayer with extremely low electrical conductance, the effective current passing through the solution is a direct function of the applied voltage. When the voltage is lower than the electroporation voltage, no current flows through the cell. As the voltage increases, reversible electroporation of the cell is expected and micro-aqueous pores start to form in the cell membrane. These micro-aqueous pores yield a dramatic increase in membrane conductance, thus measurable currents can flow through the cell membrane. When the voltage exceeds a certain critical value, namely the *transmembrane voltage* (or V_m), otherwise also known as *membrane breakdown voltage*, irreversible electroporation is triggered, which leads to the degeneration of cell membrane which then causes the cells to have zero electrical resistance. Thus, before this irreversible electroporation can occur, the trapped cell would be fully incorporated in the electrical circuit of the chip, and behaves like a non-linear resistor and capacitor connected in parallel as shown in **Figure 3-3**.

Figure 3-3 Electrical Conductance Model (created by author)

From Figure 3-3, the circles in dotted lines represent infinitesimally thin spherical insulating membrane in conducting media, on which accumulation of charges occurs

when an external electrical field is applied. When the electric field is strong enough to trigger the formation of micro-aqueous pores on the cell membrane, the membranes' resistance decreases dramatically. The conductivity of the cell interior, being higher than that of the exterior, has a field concentration at the contact point between the two adjacent cells, as denoted by R_D and C_D . As time elapses, the membrane capacitance is charged, and the electrical field lines pass around the cell as shown in Figure 3-4 from Kinoshita et al [77]. The cell membranes shield the field from passing through the cell cytoplasm, which leads to the formation of charges (or ions), which accumulate on both sides of the opposite membrane surfaces in the direction of the charge.

Figure 3-4 “Electrical Field Effect” diagram [77]

This gives rise to induced surface potentials on both sides of the membrane, resulting in the trans-membrane voltage V_m as described earlier. The relationship between the applied voltage V and the transmembrane voltage V_m is given by

Equation 3-7

$$V = V_m \left[1 - \exp \left(-\frac{\tau}{t_p} \right) \right]$$

where τ is the duration of the pulse, and t_p is the characteristic polarization time given by

Equation 3-8

$$t_p = RC_m (r_i + 0.5r_0)$$

where R is the radius of the spherical membrane and δ is the angle between E and the radius vector, and their relationship is given by

Equation 3-9
$$V_m = 1.5ER \cos \delta$$

where C_m is the specific membrane capacitance; r_i and r_o are the resistivities inside and outside of the cell respectively. However, as only a portion of the membrane breaks down with an applied electric field, these effects cause an angular and radial dependence shown in Figure 3-5 according to Neil et al [78].

Figure 3-5 “Angular and Radial Dependence” diagram [78]

As the electrical potential builds up across the membrane in response to the external electrical field, it reaches its highest value at membrane sites oriented *parallel* to the field direction (poles) and progressively decreases toward the sites *perpendicular* to the field direction (equator). For membrane sites in perpendicular orientation to the field, the potential is zero. Therefore, the transmembrane voltage V_m is first reached at sites parallel to the field direction. It is only reached in membrane sites oriented at a certain angle α to the field direction if electrical field strengths greater than the breakdown field strength

are applied. For this model, since pore formation always takes place at the 0° pole regions (due to the angular and radial dependence diagram) of sphere-shaped cells facing electrodes where maximum V_m is established, only small patches of membrane material in pole regions are of relevance and interest in this research.

In summary, the transmembrane voltage V_m derived by the models presented (Figure 3-1 to 3-5) was later validated via experiments D02 and D03, where a 12V uniform DC field was introduced as the equivalent transmembrane voltage, yielding the results presented in Chapter 5. The FEM result of Chapter 5 (Section 5.5) showed that the electrical field was indeed uniform across the electrode gap where the cell-cell fusion of D03-C occurred, thus the models (Figures 3-1 to 3-5) and equations (Equations 3-1 to 3-9) apply.

Chapter 4 MATERIALS AND METHODS

All the experiments conducted in this project are summarized in Table 4-1. D01 was conceptualized but never tested experimentally as it was deemed unsuitable by the collaborators. Nevertheless, it has been included in this report for documentation reasons.

S/No.	Experiment (D=Design)	Brief Description
1	D02-01-A	Control Set
2	D02-01-B	DC (12V) electrical current
3	D02-01-C	AC (20V) electrical current
4	D02-02-A	Control Set
5	D02-02-B	AC (12V) electrical current
6	D02-02-C	DC (20V) electrical current
7	D03-01-A	Thick parallel electrode design
8	D03-01-B	Thin parallel electrode design
9	D03-01-C	Step electrode design
10	D03-02-A	Thick parallel electrode design
11	D03-02-B	Thin parallel electrode design
12	D03-02-C	Step electrode design
13	D03-03-A	Thick parallel electrode design
14	D03-03-B	Thin parallel electrode design
15	D03-03-C	Step electrode design

Table 4-1 Summary table of experiments

4.1 D01 - Overview

The majority of first few micro-devices used in the trial fabrications and experiments in this MEng project for studying the biological cells were fabricated via soft lithography, which consists of three basic stages – (1) rapid prototyping (2) replica molding and (3) sealing/bonding. A diagram of a typical soft lithography process is shown in Figure 4-1. [79]

Figure 4-1 Soft lithography process [79]

Soft lithography offers high spatial resolution for micro-patterning of the internal surfaces, and easy integration with the other downstream on-chip processes. During the initial stages, SU-8 was used because it was the standard for microfluidic devices for biological cellular studies. Features in SU-8 with a minimum feature size of $>25\mu\text{m}$ could be rapidly and reliably created using this method. The whole process revolves around using the patterned features of the SU-8 photoresist achieved by rapid prototyping as “masters” for molding channels in the PDMS elastomer cast and cured onto it.

For the first design in this project, a serpentine channel with interdigitated electrodes was created shown in Figure 4-2 (left and right perspectives). Four different electrode configurations were investigated as shown in Figure 4-3 using the experimental seen in Figure 4-4.

Figure 4-2 D01 (a) Wafer layout before dicing (b) Individual microchip configuration with alternating electrode design - electrode wall thickness=10 μm, channel width=100 μm

Figure 4-3 D01 electrode configuration (a) insulating posts trap the cells, different shapes were investigated to determine the optimum shape b) A serpentine channel was created to increase the surface area for electroporation

Figure 4-4 D01 experimental (a) photo (b) setup

From this design, the minimum membrane breakdown (transmembrane) voltage was tested and proven to be around 1V. TB Jones (1995) [81] proved that the minimum voltage required to break the membranes was typically within the range of 100V-1kV/cm.

However, this design had a limitation. Studying the rhabdomyosarcoma cells outside of their natural network would only provide a limited amount of information on how these cancer cells proliferate after the fusion process is over. Furthermore, over the course of this research project, it became important to minimize the disturbance and contamination to the cells as much as possible to ensure their post-electrical stimulation viability. It thus became necessary to create a new chip-based design to grow the RD cells *in situ*, in their original network-form. Furthermore, due to the complex nature of a microfluidics structure, other variables (such as viscous drag forces, wall-fluid interface frictional forces, flow-induced eddy currents, flow turbulences, and the chemical reactions between the PDMS and electrolyte) made the investigation of cell-cell fusion very complicated and problematic. Hence D02 was created to address these issues.

4.2 D02 - Overview

The design plan for D02 is shown in Table 4-2.

D02	Description
A	Control, without any electrical current
B	Set treated with a 12V-DC electrical current.
C	Set treated with a 20Vpp-AC (peak-to-peak AC voltage), 1MHz frequency electrical current

Table 4-2 D02 Design Plan

In Design D02, the key considerations were (1) transparency (able to be viewed from all angles and directions), (2) sterility (no cross-contamination) and (3) flexibility to add an electrical conductor to the device. To fulfill requirement (2), the best way would be to

control the electrical current from the outside of the cell culture, so as to avoid direct contact with the RD cells. This led to the design setup of the T25 flask shown in Figure 4-5.

Figure 4-5 D02 schematic diagram

As the first primary objectives of this project were to investigate the various electrical effects on the RD cells, both AC and DC electrical field was introduced through the setup as shown in Figure 4-6.

Figure 4-6 Electrical stimulation (a) diagram (b) photo

Three batches of cells from a common parent flask were left to culture in three T25 flasks at 37°C for three hours prior to electrical stimulation to change them from a suspension state to a quiescence state. The cells were left for 24 hours in as in (A) Control, (B) 12V-DC and (C) 20V-AC. Based on the models developed in Chapter 3 and the result from Smith et al [80], the transmembrane voltage used in the 12V-DC and 20V-AC experiments using D02 were high enough for electrical membrane breakdown to take place. Cells were counted using a Neubauer hemocytometer, which has a counting chamber as shown in Figure 4-7.

Figure 4-7 Neubauer hemocytometer counting chamber. Count the total number of live cells in the four large corner squares (1-4) plus the middle large square (5)

The number of cells needed in order to calculate the proliferation rate and fusion yield was obtained by counting the total number of live cells in the four large corner squares (1-4) plus the middle large square (5) for each chamber. (Total of 2 chambers) The cell count was obtained by finding the average number of live cells in the four large corner squares (1-4) plus the middle large square (5) of the two counting chambers.

For example, each large square has a surface area of 1mm^2 and a depth of 0.1mm , giving it a volume of 0.1mm^3 . For example in Table 5-1 (first row, first column), the total number of live cells in 10 large squares (5 large squares x 2 counting chambers) is 39.

Multiply this number by 1000 to get a cell count (per ml) of 39,000. Fusion Yield is calculated by counting the number of nuclei in multinucleated cells divided by the total number of nuclei from each of the 100x magnification images from Figure 5-9. Recall from Equation 2-11 that:

$$\text{Proliferation Rate} = \frac{\text{Number of live cells after 24 hours of electrofusion}}{\text{Number of live cells before the experiment}}$$

and from Equation 2-12 that:

$$\text{Fusion Yield} = \frac{\text{Number of live nuclei in multinucleated cells}}{\text{Total number of live nuclei}}$$

4.3 D03 - Overview

The project objective of “Conceptualizing and developing a system for enhancing cell-cell fusion in a controlled environment” was attained in D03, which was designed to achieve higher cell growth and proliferation rates during cell fusion processes. Based on the result of D02, the electrical setup that yields highest fusion yield and proliferation rate would be D02-B (refer to Section 5.2 - D02 Results). From that electrical setup, the D03 design plan was derived as shown in Table 4-3 below, with setups A, B, and C, as shown in Figure 4-8, Figure 4-9, and Figure 4-10. All the experiments were conducted in T25 flasks that were specially designed and fabricated to further enhance the fusion yield while maintaining high cell viability at the same time.

D03-	Description
A	Thick parallel electrodes (Figure 4-8)
B	Thin parallel electrodes (Figure 4-9)
C	Step electrodes (Figure 4-10)

Table 4-3 D03 Design Plan

Figure 4-8 D03-A - Thick Parallel Electrodes Design

Figure 4-9 D03-B - Thin Parallel Electrodes Design

Figure 4-10 D03-C - Step Electrodes Design

The key advantage of D03 was that, similar to D02, there was minimal disturbance to the cells even after external stimuli was applied, as all the electrical components were located on the outside, thus direct contact with the cells was avoided. Thus sterility was never

compromised and the cell media was not contaminated. A magnetron sputterer was used to coat a layer of gold (Au) of thickness 100-200nm on the base as shown on the previous page. The conductivity of the electrodes was tested to be highly conductive via a conductivity probe, hence making them suitable to act as conductors for the electroporative currents.

Based on D02, the highest fusion yield and proliferation rate occurred in the electrical setup of D02-B. (Refer to Section 5.2 and 5.3 for more details) Based on the previous results, D03 was setup as shown in Figure 4-11.

Figure 4-11 D03 Experimental Setup

Using the experimental setup shown in Figure 4-11, the cell proliferation rate was then studied under the three different electrode configurations (Figure 4-8 to Figure 4-10). Finite element methods, which used systematic numerical calculation approaches to

obtain approximate solutions of partial differential equations based on the models presented in Chapter 3, was then used to calculate the respective electric fields. To calculate the electric field, the continuum was divided into a group of finite units that connected each other. By replacing all the differential equations in the solving domain with a series of algebraic equations, the required parameters was be solved by iterative numerical calculations and the profile of the potential and the electric field intensity was be determined. The results of these solutions would be presented in Section 5.5, under D03 Discussion.

Chapter 5 RESULTS AND DISCUSSION

5.1 Overview

The experimental results are summarized in Table 5-1 below.

S/No.	Experiment	Setup	Initial cell count	Final cell count after 24 hrs	Proliferation Rate	Fusion Yield
1	D02-01-A	Control	39,000	45,000	0.04	1.15
2	D02-01-B	DC (12V)	39,000	89,500	0.27	2.29
3	D02-01-C	AC (20V)	39,000	66,500	0.1	1.71
4	D02-02-A	Control	96,000	118,000	0.13	1.23
5	D02-02-B	AC (12V)	96,000	120,000	0.38	1.25
6	D02-02-C	DC (20V)	96,000	114,000	0.18	1.19
7	D03-01-A	Thick parallel	28,000	85,000	3.04	
8	D03-01-B	Thin parallel	28,000	94,000	3.36	
9	D03-01-C	Step	28,000	64,000	2.29	
10	D03-02-A	Thick parallel	75,000	308,000	4.11	
11	D03-02-B	Thin parallel	75,000	332,000	4.43	
12	D03-02-C	Step	75,000	252,000	3.36	
13	D03-03-A	Thick parallel	118,000	57,000	0.48	
14	D03-03-B	Thin parallel	118,000	125,000	1.06	
15	D03-03-C	Step	118,000	90,000	0.78	

Table 5-1 Summary of Experimental Results

The Neubauer hemocytometer method described in Section 4.2 was used to count the number of cells. A total of 15 sets of experiments, as shown in Table 5-1, were conducted. For D02, the influence of electrical treatment (AC and DC) on cell proliferation and fusion yield was studied over a 24-hour period, and these results were compared to a control set. For D03, the cell proliferation rate was studied under various electrode configurations. Based on the result of D03, the highest- fusion yield occurred in the D03-

B (Setting B). (Details are in Section 5.4) The results of all the experiments have been published in the journals *Advanced Materials Research* (Print: Vol. 254, pp. 207-210. DOI: 10.4028) and *IEEE* (Print ISBN: 978-1-4244-9276-3. DOI: 10.1109/DSR.2011.6026864). (Refer to AUTHOR'S PUBLICATIONS on page 78 for more details)

5.2 D02 - Results

First, a T75 parent flask was chosen, with more than 90% confluenceⁱ. Using an advanced inverted microscope, selectively controlled positions of the same co-ordinates were imaged throughout the flask using four different objective lens. (4x, 10x, 20x and 40x). *Note:* the actual magnification is 10 times of the objective, i.e. 4x lens gives 40x magnification, as the master lens has a 10x magnification in itself. The experiments were conducted in duplicates (D02-01 and D02-02) for statistical accuracy.

5.2.1 D02-01

Figure 5-1 shows the images of cell growth over a 24-hour period, and the corresponding magnification. The cells in the parent flask were also characterized to compare and contrast the differences before and after electrical stimulation.

ⁱ percentage of cells occupying each unit area

Figure 5-1 D02-01-0 Parent Set (before the addition of trypsin)

Figure 5-2 D02-01-A Control Set (without any electrical stimulation)

Figure 5-3 D02-01-B Under 12V DC current

Figure 5-4 D02-01-C Under 20Vpp, 1MHz sinusoidal AC current

5.2.2 D02-02

Figure 5-5 D02-02-0 Parent Set (before the addition of trypsin)

Figure 5-6 D02-02-A Control Set (without any electrical stimulation)

Figure 5-7 D02-02-B Under 12V DC current

Figure 5-8 D02-02-C Under 20Vpp, 1MHz sinusoidal AC current

5.3 D02 - Discussion

The first inference one can draw by studying the confluence and distribution of the cells would be that if the cells has a confluence nearer the electrodes, then areas of higher electrical field intensity are more conducive to cell proliferation since the electrical field lines are closer. Interestingly, the experimental results showed that the cells grew better

nearer the electrodes, proving that higher electrical field intensities provide a better milieu for cell proliferation. This is an important consideration in future designs. In this report, the electrical field strength in the DC setup is 12V and the AC setup is 20V peak-to-peak. Based on the summary of results shown in Figure 5-9, the cells in the DC setup display a 10% higher confluence than in the Control setup.

Figure 5-9 Summary of results from Exp 1 (top row) and Exp 2 (bottom row) – A 10% higher confluence is observed in [D2] compared to [C2] and [A2] after 24 hours

Using the methods described by Equation 2-11 to 2-12 (Section 2.8, page 38), the results of D02 are summarized in Table 5-1, and Table 5-2.

	Control	12V-DC	20V-AC
Cell count at 0 h (per ml)	39,000	39,000	39,000
Cell count at 24 h (per ml)	45,000	89,500	66,500
Proliferation Rate	1.15	2.29	1.71
Fusion Yield	10/238= 0.04	40/148= 0.27	28/232=0.1

Table 5-2 Table of Results for D02-01– Compared to the control flask without electrical field, the proliferation rate and fusion yield are much higher under the DC setting

	Control	12V-DC	20V-AC
Cell count at 0 h (per ml)	96,000	96,000	96,000
Cell count at 24 h (per ml)	118,000	120,000	114,000
Proliferation Rate	1.23	1.25	1.19
Fusion Yield	26/208= 0.13	50/133= 0.38	22/119=0.18

Table 5-3 Table of Results for D02-02 – Compared to the control flask without electrical field, the proliferation rate and fusion yield are much higher under the DC setting

Overall, the experimental results showed that an adequate electrical stimulation helped to facilitate the fusion and proliferation of the rhabdomyosarcoma cells. The cells also had a tendency to grow near the electrodes, which was not shown in this report. On analysis, the results from the two tables showed that the proliferation rate was notably higher in D02-01 than in D02-02 for all the three settings, possibly proving that an initial cell population of close to 40,000 before the experiment is more optimal than a higher cell population of near 100,000. It is also evident that under the DC setup, the (1) proliferation rate and (2) fusion yield (both highlighted in **bold**) were higher than both the AC setup and control, highlighting both its comparative safety and efficiency. Under the DC setting, a fusion yield of 27% and 38% was obtained, compared to 4% and 13% in the Control set with no electrical current respectively. After subtraction of the multinucleated cells in the control samples, the corrected Fusion Yield under the DC setup for D02-01 was $0.27 - 0.04 = 23\%$ and D02-02 was $0.38 - 0.13 = 25\%$. This gave a very close average of **24%**. Even though simple and limited resources were used in this study, this value is comparable to the fusion yield of 20% obtained by Trontelj et al (pp.128) [76] and that by other researchers [82-84] using the same technique. This result was successfully published in the journal *Advanced Materials Research* on 31st May 2011. (Vol. 254, pp. 207-210. DOI: 10.4028. www.scientific.net/AMR.254.207)

5.4 D03 – Results

The results of D03 are presented as follows:

5.4.1 D03-01

Figure 5-10 D03-01-0 - Cell morphology of the parent flask (initial cell count of 28,000 /ml)

Figure 5-11 D03-01-A - The black region is the electrode area - the cells tended to grow less densely near the edge of the electrodes. (Cell count: 85,000 /ml, Proliferation Rate: 3.04)

Figure 5-12 D03-01-B – This design produces a higher cell count of 94,000 /ml and a higher Proliferation Rate of 3.36 than the previous, making Design B the highest performer in this set.

Figure 5-13 D03-01-C – This design performs the poorest in this set in both aspects, with a cell count of 64,000 /ml and a Proliferation Rate of 2.29.

5.4.2 D03-02

Figure 5-14 D03-02-0 - Cell morphology of the parent flask (initial cell count of 75,000 /ml, higher than D03-01)

Figure 5-15 D03-02-A – This design gives a much higher cell count of 308,000 /ml than Set 1, due to the higher initial cell count of the Parent flasks. The Proliferation Rate of 4.11, and is also notably higher than Set 1. Overall, Set 2 tended to produce higher values than Set 1 owing to the higher initial cell count.

Figure 5-16 D03-02-B – This design gives the highest cell count of 332,000 /ml and the highest Proliferation Rate of 4.43, and is also the highest of all this set and of all the three sets added together.

Figure 5-17 D03-02-C – Even though the cell count of 252,000 /ml and Proliferation Rate of 3.36 makes it the lowest of this Set, the fact that both values are much higher than Set 1 of the same design shows that the initial cell count of the parent flask plays an important role.

5.4.3 D03-03

Figure 5-18 D03-03-0 - Cell morphology of the parent flask (initial cell count of 118,000 /ml, highest of all the three sets)

Figure 5-19 D03-03-A - A cell count of 57,000 /ml and proliferation rate of 0.48 is observed. Both values are so low that the conclusion one can draw is the initial cell count is too high for the cells to grow comfortably and healthily. Overcrowding has become a problem, though this cannot be seen by the images shown in this figure.

Figure 5-20 D03-03-B – This time a higher cell count of 125,000 and proliferation rate of 1.06 than Design A is achieved, but the values still falls short of the corresponding ones from Design B in Set 2. The images show signs of overcrowding - the cells are overlapping and crowding together due to lack of space. Design B still excels among the three designs even for this set.

Figure 5-21 D03-03-C – The cell count of 90,000/ml is higher than its equivalent Design C in Set 1, but the proliferation rate of 0.78 is the second lowest of this set. The statistics show that Design C obviously pales in comparison to Design B. Design C has shown to be the worst among all the three sets.

5.5 D03 – Discussion

Based on the result of D02, the electrical setup that yields highest fusion yield and proliferation rate is D02-B. The correlation of the D03 results with the electrical field intensity ∇E proposed in Equation 2-13 would prove that the models in Chapter 3 are sufficient for the calculation of the complex profile of electric field present in the step electrode configuration of D03-C.

To simulate the electric field modeling described in Section 2.8, the voltage, conductivity and dielectric characteristics of the buffer used in the different models were assumed to be invariable. The first step in the analysis was the development of a computer-aided design model of a D03-C electrode configuration as shown in Figure 5-22 using NX6.0. Because the material of the microelectrodes was a conductor whose interior charge density was zero, Equation 2-16 $\nabla^2 \Phi = 0$ can be safely assumed to be exact. Furthermore, the buffer that filled the T25 flask was non-metallic, and the conductivity of the electrode was given as $r_m = 2.0 \, \Omega \cdot \text{m}$ and the conductivity of the suspension given as $r_b = 2 \times 10^5 \, \Omega \cdot \text{m}$. In particular, the distance and voltage between two

counter-electrodes used in D03-C was 5mm and 12V respectively. The electric resistivity of the buffer and the electrode were $2 \times 10^5 \, \Omega \cdot \text{m}$ and $2.0 \, \Omega \cdot \text{m}$ respectively. The electric current density was $2 \times 10^{-4} \, \text{A} \cdot \text{m}^{-2}$.

Figure 5-22 CAD model of electrode configuration in Design C

To run the FEM simulation, a 12V voltage difference was applied between the left and right electrodes as shown in Figure 5-22. The voltage on the left side of the model was positive and that on the right side was 0. The finite element analysis software in NX6.0 was used to simulate the electric field profile. It is a direct solution method of a set of partial differential equations not shown in this report. In the FEM, PLANE010 was selected as the solving element, which was a two-dimensional eight-node electro-

analytical unit with one degree of freedom of voltage on each node. Based on the above model and parameter, a simulated result of local electric potential Φ profile was obtained as shown in Figure 5-23.

Figure 5-23 (a) Local electric potential profile Φ of D03-C and (b) Change of electric potential $\Delta\Phi$ along the diagonal of a pair of electrodes

From the figure, the local electric potential profile (in V/mm) at various points between the gap of the two electrodes of D03-C can be seen clearly. From Figure 5-23(a), MX denotes the maximum positive potential, while MN denotes the maximum negative potential. Figure 5-23(b) showed that the voltage gradually decreased along the diagonal of electrode pair, from left to right. The relation between the voltage and the distance was approximated linear, which means that the electrical field is uniform, as predicted by

Similarly, the local electric field intensity profile Φ of D03-B was obtained as shown in Figure 5-24. Based on the electric field profile along the diagonal, the maximum local electric field intensity was about 2.0 V/mm when a 12V-DC voltage was applied. Zimmermann et al [69] showed that the electrical pulse generated by of 2.0 V/mm was large enough for the electroporation of cell membrane and subsequent cell-cell fusion to take place. This obtained experimental result of 2.0V/mm is in agreement

with the research of Zimmermann et al [69], who showed that the transmembrane voltage V_m predicted in the modeling section (Chapter 3) was sufficient to cause electroporation and cell-cell fusion.

Figure 5-24 (a) Local electric field intensity Φ of D03-C electrodes (b) Change of electric field intensity $\Delta\Phi$ along the diagonal of a pair of electrodes (c) Change of electric field intensity gradient $\Delta^2\Phi$ along the diagonal of a pair of electrodes

The electric field intensity was mapped by a multi-color scale (unit: V/mm) as shown in Figure 5-24(a), where MX showed the regions of highest electric field intensity, and MN the regions of lowest electric field intensity. The electric field intensity profile along the diagonal (Figure 5-24(b)) shows that it is high near the edges of the electrodes, but dips towards the middle of the gap. This suggests that the greater the distance from the tip of electrode, the weaker the electric field intensity would be. Referring to the electrical conductance model (Figure 3-3), the cell membranes are charged and shield the ambient electrical field from passing through the cell cytoplasm, which leads to the formation of charges (or ions), which accumulate on both sides of the opposite membrane surfaces in the direction of the charge. This results in the cells aligning along the lines of highest electrical field intensity Φ shown above as predicted by the cell-cell alignment and fusion model in Figure 3-1.

From Figures 5-23 to 5-24, it was shown that the highest electric potential (2-3V/mm) was near the right-angle corner protrusions of the electrodes, and the lowest

electric field intensity (0.16V/mm) was also located at the corners of the electrodes. These areas were also where cells grew the least, as could be seen in Figure 5-10. The conclusion drawn was the high electric potential was not favorable for cell growth. On the other hand, a strong electric field existed along the diagonals of two diagonal electrode peninsulas, where cell proliferation was high and fusion chains occurred readily. Based on the dielectrophoretic theory and electroporation principle of membrane described in Chapter 2 of this thesis (Section 2.4.3 and Section 2.5.2), cells would align along the diagonals where the electrical field lines were the highest. This was clearly seen in the experiments of D03-C. The formation of pearl-chains along the zig-zag region connecting the diagonally-facing electrode corners in the experiments correlate to the highest electrical field profile along the same diagonals. This showed coherence between theory, modeling and practical results. In fact, in the experimental results of D03, many cells were simultaneously aligned and fused along this diagonal-to-diagonal region, hence validating the cell fusion (and alignment) model and cell electrical conductance models presented in Chapter 3.

Chapter 6 CONCLUSION

The conclusions from all the experiments are summarized in Table 6-1.

No.	Experiment	Setup	Initial cell count	Final cell count after 24 hrs	Prolifn Rate	Fusion Yield	Conclusion
1	D02-01-A	Control	39,000	45,000	0.04	1.15	D02-B produces the highest proliferation rate and fusion yield. A cell count of above 100,000 is too high to display a good proliferation rate. An initial cell count of 40,000 to 80,000 /ml would yield the highest proliferation rate.
2	D02-01-B	DC (12V)	39,000	89,500	0.27	2.29	
3	D02-01-C	AC (20V)	39,000	66,500	0.1	1.71	
4	D02-02-A	Control	96,000	118,000	0.13	1.23	
5	D02-02-B	AC (12V)	96,000	120,000	0.38	1.25	
6	D02-02-C	DC (20V)	96,000	114,000	0.18	1.19	
7	D03-01-A	Thick parallel	28,000	85,000	3.04		
8	D03-01-B	Thin parallel	28,000	94,000	3.36		The best configuration to achieve the highest cell growth after 24 hours of electroporation.
9	D03-01-C	Step	28,000	64,000	2.29		The worst among performer in terms of cell count and proliferation rate for all the three sets.
10	D03-02-A	Thick parallel	75,000	308,000	4.11		
11	D03-02-B	Thin parallel	75,000	332,000	4.43		The best configuration to achieve the highest cell growth after 24 hours of electroporation.
12	D03-02-C	Step	75,000	252,000	3.36		The worst among performer in terms of cell count and proliferation rate for all the three sets.
13	D03-03-A	Thick parallel	118,000	57,000	0.48		
14	D03-03-B	Thin parallel	118,000	125,000	1.06		The best configuration to achieve the highest cell growth after 24 hours of electroporation.
15	D03-03-C	Step	118,000	90,000	0.78		The worst among performer in terms of cell count and proliferation rate for all the three sets.

Table 6-1 Table of Conclusions

The main objective of the “Conceptualization and development of a new system for enhancing cell-cell fusion in a controlled environment was achieved in D02. Furthermore,

the influence of various parameters, such as AC and DC electrical currents, on cell growth, proliferation and physiology were investigated in D02. The D02 results showed high cell growth and proliferation rate in setting B, which was used to design D03. Thereafter, the models proposed in Chapter 3 were validated by D03 as described in Section 5.5. D03-B proved to be the best configuration to achieve the project objective. The experimental results presented in this project are a good platform for other research scientists to build upon if they wish to study cell fusion in further detail, for example, if they wish to go into lipid bilayer membrane interactions, nuclear behavior, and biological material transfer, and mechanobiology.

AUTHOR'S PUBLICATIONS

JOURNALS:

- (1) Chong Xian Yeo, Kian Hwa Tan, Eng Lee Tan, Chu Sing Lim (2011) "Investigation of Rhabdomyosarcoma Cell Electrofusion", **Advanced Materials Research**, Vol. 254, pp. 207-210. DOI: 10.4028. www.scientific.net/AMR.254.207
- (2) Chong Xian Yeo, Ying Ying Woh Kham, Kian Hwa Tan, Eng Lee Tan, Chu Sing Lim (2011) "Optimization of electrofusion in vitro for rhabdomyosarcoma cells", **IEEE**, Print ISBN: 978-1-4244-9276-3. DOI: 10.1109/DSR.2011.6026864 (23rd Sep 2011)
http://ieeexplore.ieee.org/xpl/freeabs_all.jsp?arnumber=6026864

CONFERENCES:

- (3) Chong Xian Yeo, Kian Hwa Tan, Eng Lee Tan, Chu Sing Lim, "Investigation of Rhabdomyosarcoma Cell-cell Fusion Via Chip-based Electroporation" **International Conference of Materials and Advanced Technologies** (ICMAT 2011), 26 Jun – 1 Jul 2011, Suntec City Convention & Exhibition Centre, Singapore. (Oral Presentation)
- (4) Chong Xian Yeo, Kian Hwa Tan, Eng Lee Tan, Chu Sing Lim, "Optimization for electrofusion in vitro for rhabdomyosarcoma cells" **Defence, Science and Research Conference** (DSRA00149-00229P3), 3-5 Aug 2011, Suntec City Convention & Exhibition Centre, Singapore. (Oral Presentation)
- (5) Chong Xian Yeo, Kian Hwa Tan, Eng Lee Tan, Chu Sing Lim, "Investigation of Rhabdomyosarcoma Cell-cell Fusion via Chip-based Electrofusion" **Gordon Research Conference** (7-12 Aug 2011) & **Gordon- Kenan Research Seminar** (6-7 Aug 2011), University of New England, ME, United States. (Poster Presentation)

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