2007

Cellular electrofusion utilizing corona fields and DC pulse technology

Joshua Stein
University of South Florida

Cellular Electrofusion Utilizing Corona Fields and DC Pulse Technology

by

Joshua Stein

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Biomedical Engineering
Department of Chemical Engineering
College of Engineering
University of South Florida

Co-Major Professor: Mark Jaroszeski, Ph.D.
Co-Major Professor: Don Cameron, Ph.D.
Richard Gilbert, Ph.D.

Date of Approval:
April 9, 2007

Keywords: direct current, cell-cell contact, cell fusion, NT2 cells, fusion chamber, 1:1
fusion

DEDICATION

To my parents, who have always stood behind me in everything I have done.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CELLULAR ELECTROFUSION UTILIZING CORONA FIELDS AND DC PULSE TECHNOLOGY

Joshua Stein

ABSTRACT

Cell fusion is an important technique that is used in the field of medicine and biomedical research. For instance, fusion can be used to create hybridomas [1] and novel types of secretory hybrid cells. It may also be used to engineer cultured insulin-secreting pancreatic B-cell lines for the treatment of diabetes [2]. Historically, the applications listed above have been accomplished by a number of methodologies including dielectrophoresis, centrifugation, polyethylene glycol (PEG) and viral fusion proteins. However, these approaches often fail to produce the desired results due to poor cell viability, lack of 1:1 fusion, and use of non-physiological environments. It is proposed that the application of an electrical field generated by corona charge (corona fields) and subsequent treatment with direct current (DC) pulse technology will overcome these deficiencies.

Isolated and pre-labeled neuronally committed human teratocarcinoma (NT2) cells in monoculture or co-culture, were seeded in chambers, constructed in the laboratory, and allowed to adhere to the chamber bottom prior to corona treatment. A corona generator, also constructed in the laboratory, was used to expose cells to positive and negative electrical charges to induce cell-cell contact. The cells were then pulsed

with DC voltage to induce fusion. During the experiments, cells were photographed sequentially to record cell movement/contact and fusion.

The project was designed to identify optimal corona-based electrofusion parameters for viable, 1:1 cell fusion. Optimal results for cell-cell contact were obtained using a cell density of 2.35×10^4 cells/µl Dulbecco's Modified Eagle Medium (DMEM) in a grounded circular plate corona chamber following at least 3 minutes of settling time. Corona charges from (+) 6.1 kilivolt and (-) 5.5 kilivolt potentials were determined as being most favorable for cell movement and viability. Fusion was best achieved by first exposing either a circular or square ungrounded corona chamber configuration to 3 minutes (+) corona charge followed by 3 minutes (–) corona charge; disturbing the cells in the chamber with mechanical force; and then exposing them to 8-15 sequences of a 2,500 Volts/cm DC pulse at 100 microseconds.

CHAPTER 1: INTRODUCTION

1.1 Cell Fusion Applications

Fusion between biological cells is an important procedure that is frequently used in the field of medicine and biomedical research. The applications for cell fusion have grown significantly since its discovery in the late 1970's [3]. For instance, it has grown from generating somatic cell hybrids [4] and homokaryon production to the production of tumor cell/dendritic cellular hybrids for cancer immunotherapy [5]. In addition, fusion applications have also progressed from creating hybridomas and novel types of secretory hybrid cells to engineering cultured insulin-secreting pancreatic B-cell lines for the treatment of diabetes. In recent years, [6] fusion techniques have been utilized to create novel hybrids for the facilitation of drug delivery. Cell fusion has even been investigated to be used as a means for bioengineering novel heterohybridized cell constructs for cell transplantation therapies [2]. Additionally, many of the ongoing clinical studies initiated by companies such as Genzyme and Dendreon are using a form of fusion as their primary method for producing cell hybrids [7]. In essence, it is quite easy to visualize how influential this methodology is in the field of medicine and how promising it can be for the future of biomedical research.

1

1.2 Methods of Cell Fusion

There are many different methods currently being utilized in scientific studies to bioengineer viable hybridized cellular constructs. While a variety of these methods are currently being used (some more popular than others), each respective method has its own deficiencies. This dilemma prompts the need to investigate a novel fusion technique that may overcome these deficiencies.

1.2.1 Viral Fusion Proteins

The first few studies involving cellular fusion were performed *in vitro* using either an inactivated virus or chemical fusogen. Inactivated particles from the Sendai virus were used to induce nuclei and micronuclei into a cell's cytoplasm [3]. Unfortunately, many limitations existed which restricted the feasibility of producing a pre-determined quantity of viable hybridized cells on a consistent basis. The main limitations for using chemical and viral agents, such as the Sendai virus, as stated by Zimmermann [8] are listed below:

- The desired number of fused cells cannot be pre-selected.
- The process of cellular fusion between two different cell species cannot be viewed under a microscope.
- The cell viability is jeopardized by a loss of intracellular substances.
- There is a presence of exogenous reagents during the fusion process, which in some cases may be toxic to the cells.
- The optimal fusion conditions for a set of species have to be pre-determined empirically as they vary from species to species.

2

1.2.2 Polyethylene Glycol (PEG)

Apart from viral fusogens, chemicals such as polyethylene glycol (PEG), its derivatives and lysolecithin [9] have been used to promote cell hybridization, although at a low frequency [10]. A major drawback for using PEG based methods is that the PEG has been shown to be cytotoxic to cells and can subsequently generate cell debris which can be taken up by unhybridized cells rendering the identification of true hybrids difficult [11].

1.2.3 Centrifugation

Centrifugation is another method that has been used as a means for achieving tight intracellular contact as part of cellular fusion procedures. One way of performing this has been to centrifuge the cells and then apply direct current (DC) pulses to induce reversible electrical membrane breakdown of the contacting surfaces [12]. The alternate way of performing this takes advantage of the relatively long lived (minutes) fusogenic state that exists after the cells have been pulsed. So, cells are first pulsed and then centrifuged into contact with each other [12, 13]. These methods have matched the success of traditional methods (polyethylene glycol, viral fusion proteins, dielectrophoresis, etc.). One drawback, particularly of the second centrifugation method, is that there is no control to insure that the cells contact each other in the polar regions that have induced membrane defects. Another drawback of using centrifugation as a means for cellular contact is that there is also no control for achieving a desired fusion ratio, because there is no way of predicting how many cells will fuse with their counterparts.

3

1.2.4 Electrofusion

The methodology involving the electrical treatment of living cells and nonliving membrane vesicles in a manner that will induce fusion (electrofusion) has been investigated for quite some time. The earliest published observation of this phenomenon was in 1983 [14]. The experiment was conducted by Bouchard and Teissie. It involved growing hamster ovary cells in monolayers on a Petri dish followed by exposure to square wave electric pulses to induce the formation of a large amount of fused mammalian cells. As demonstrated in Bouchard's study as well as later studies [15], electrofusion was effective in producing a higher yield of viable hybrid cells than the other methodologies discussed above. Unfortunately, during the earlier years, the mechanism of electrofusion was not completely understood. However, as time progressed, a number of scientists helped to contribute additional knowledge to this novel methodology which opened the door for further optimization. For instance, a couple of investigations conducted by Zimmermann revealed that electrofusion in strongly hypo-osmolar or isotonic solutions could enhance hybridoma production [16, 17]. In addition, Teissie was able to demonstrate that electrofusion is a two step process consisting of the creation of cell contact and subsequent reversible electropermeabilization [18]. Furthermore, a scientist from Rockville, MD (Sowers) was not only able to provide convincing evidence that electrofusion yield is partially controlled by biologically relevant membrane factors [19], but that fusion of dissimilar membrane partners depends on additive contributions from each of the two different membranes [20].

There have been many practical applications over the years that have established the use of electrofusion since the first observation. For instance, electrofusion has been

used for the production of monoclonal antibodies [21], transfer of membrane components [22-24], and the production of hybrid cells [25-27].

As discussed above, electrofusion was discovered to be a two step process. The first step is the creation of tight intracellular contact between the cells. The second step is the application of very brief but intense DC pulses to the cells resulting in a temporary membrane destabilization of the contacting surfaces. During this destabilization period, molecules that ordinarily would not be able to enter the cell's membrane can gain access to the cytosol for a time that is on the order of minutes after electrical treatment ceases [28]. In addition, if the cells remain in contact during their destabilized/fusogenic state, membrane fusion can occur. Once tight cellular contact has been obtained, reversible permeabilization (or fusion) can be achieved by delivering 6-15 fusogenic DC pulses to the cells by way of electrodes. The field strength ranges from 900 V/cm - 3000 V/cm and the interval between pulses can range anywhere from $10 - 100\mu s$ depending on the type of cells being fused. The method of delivering pulses to the cells in order to induce cell-cell hybridization is similar in all of the known fusion methodologies regardless of the application. However, the method of achieving cell-cell contact prior to electrofusion is where the methods differ. Unfortunately, forcing cells into contact with each other is the most challenging aspect of electrofusion due to a variety of limitations which conversely leaves room for improvements.

1.2.4.1 Dielectrophoresis

Dielectrophoresis is currently one of the most popular methods for obtaining close intracellular contact prior to fusion. Dielectrophoresis involves the directional movement of cells (neutral particles) towards the region of highest field intensity in a non-uniform electric field [29]. The field that is created during the process of dielectrophoresis is generated by a source of alternating current (AC). Cells normally do not attract one another due to their net negative outer surface charge. However, they become dipoles in the AC field and are forced into contact with each other due to a net force resulting from the non-uniform AC field. The cells undergo translational movement towards the region of highest field intensity when exposed to the non-uniform field. In addition, the cells move into close proximity of each other in their polarized state, causing them to attract to each other (due to an enhancement of the local field divergence and increase in field strength near the cell). The localized increase in field strength near each cell is strong enough to overcome the weaker electrostatic repulsions generated from the outer cell membranes, thereby resulting in the formation of pearl chains of cells [30].

One of the limitations of dielectrophoresis as a means for achieving cellular contact is that the cells need to be placed in a non-conducting medium. The presence of electrolytes in conductive media leads to Joule heating, turbulence and subsequent disruption of the pearl chains. This hinders the cell alignment process [31]. On the other hand, using a non-physiologic medium could jeopardize the cellular integrity and viability. Additionally, another limitation with using the associated technology is that only a small number of cells can be treated.

6

1.2.5   Other Cell Fusion Methods

There have been some other methods developed in an attempt to further optimize cell-cell contact for electrofusion. The technique of producing hybridomas by using laser radiation was investigated by Onkohci N. and Itagaki H. [32, 33]. In this study, the investigators were able to focus pulse laser beams on the contacting surfaces of select target cells and cut small perforations for mutual communication between the cytoplasm. Although this method proved to be very effective for producing hybridomas from a small number of cells, including fragile cells, the technology did not show the ability to produce a large number of hybrids at one time. Another fusion technique involving the cultivation of a monolayer of anchorage dependent cells was investigated by Finaz [34] and Teissie [35]. The main problem associated with this technology is that it requires anchorage dependent cells.

It is evident from the cell-cell fusion methods described above that there is significant room for technological improvement. Many of the techniques listed above have proven to be reasonably efficient in generating hybrids; however, each respective technology has its own limiting factors. Additionally, even if a technique is said to be satisfactory for generating hybrids from a specific set of cells, it does not equate to that technique being suitable for other cell types. Furthermore, some methods provide very positive results for generating viable hybridized cells on a consistent basis, but the associated technology may not have the ability to either produce viable hybridomas in mass quantity at one time or a small select target of viable hybrids.

Exploring the prospect of using electrical charges to induce cellular contact would be a good starting point towards finding a more generalized protocol for efficient cell-cell

contact and subsequent electrofusion. Therefore, the use of corona charge (a type of electrical charge) as a means for achieving cellular contact prior to DC mediated fusion should be investigated. Successful development of this technology would most likely be translated to produce select quantities of hybrids in a controlled environment irrespective of the cells adherent properties.

CHAPTER 2: BACKGROUND AND MOTIVATION

2.1 Corona Charge

Corona charge is a self-sustained current that is generated by strong electric fields that are associated with a highly curved electrode containing a high potential gradient. Corona charge may be positive or negative, depending upon the voltage applied to the highly curved electrode [36].   Corona discharge usually involves two asymmetric electrodes; a highly curved electrode (such as the tip of a needle, or a small diameter wire) and an electrode of low curvature (such as a plate, or the ground) [36]. During the process of corona discharge, a current is produced between the two asymmetric electrodes in a neutral fluid (usually air).   This current is produced by ionizing the fluid to form plasma around the highly curved electrode [36].   The ions created from the plasma formation event help to close the circuit by carrying the charge to the other electrode.

2.2 Concepts for the Generation of Corona

When an object with a sharp tip becomes charged it has a very high potential gradient nearby. As a result, the neutral fluid (air in this case) around that sharp tip has a much higher gradient than elsewhere. If a sufficiently high voltage is applied to the sharp object, the potential gradient may become large enough at a point in the fluid so as to

ionize the fluid, thereby creating plasma around the sharp tip [36]. From general physics [37], plasma is nothing more than a gas in its ionized state. When the fluid becomes ionized, the electrons are stripped from the atoms (leaving each atom with a net positive charge) and the ions are free to move about. The free electric charges that are present make the plasma electrically conductive so that it responds strongly to electromagnetic fields. The electrons that are freely moving throughout the plasma will continually collide with neutral atoms located outside the plasma to initiate further electron dissociations. These newly ionized atoms will then help to seed further events such as this. This process is known as an electron avalanche. Both positive and negative corona discharges rely heavily on electron avalanches [36]. Ion species that are created from an electron avalanche will naturally attract themselves to the low curved electrode (ground, where they are neutralized), thus completing the circuit and sustaining the current flow. If conditions of the geometry and gradient are such that the ionized region continues to grow instead of gradually coming to a halt at a certain distance, a completely conductive path may be formed. The result is a momentary spark or continuous arc. This event essentially follows the same mechanism presented during a lightning strike. As with the case of a lightning strike, the general notion is that a spark or continuous arc is simply a flow of current from negative to positive [38]. In the case of corona discharge, it is the ion species that are created from the electron avalanche that carry the current to the ground. If sufficient voltage is passed through a highly curved electrode containing a negative corona potential, the free flowing electrons and secondary electrons in free space will accelerate more quickly towards the opposite low curved electrode/ground due to the increase of the repulsive force, resulting in a completely conductive path or spark.

In the case of a highly curved electrode containing a positive corona potential, the ionization region will greatly increase when a high enough voltage is passed through the highly curved electrode, resulting in the immediate cascade of an electron avalanche and the formation of a completely conductive path. This process is more gradual and occurs at a higher potential than the process involving a highly curved electrode with a negative potential. This is due to the simple fact that the electrons are closer to the low curved electrode in the negative highly curved electrode process than they are with the positive highly curved electrode process and would thus require less voltage to reach the ground.

As mentioned in the preceding section, there are two different types of corona charge: positive and negative. The type of corona charge is dependent upon the polarity of the highly curved electrode. If the highly curved electrode is positive with respect to the flat electrode, then there is a positive corona potential. If the highly curved electrode is negative with respect to the flat electrode, then there is a negative corona potential. The physics between positive and negative coronas differ. This is a result of the great difference in mass between electrons and positively charged ions, as well as the verity that only the electrons have the ability to undergo a certain degree of ionizing inelastic collisions. When using a pointed electrode with a negative potential during corona discharge, the strong electric field located near the highly curved/pointed electrode will generate a force that will act upon the free electrons pushing them away from the sharp electrode [39]. These free electrons contribute to the ionization process by facilitating the dissociation of more electrons (secondary electrons), thereby creating an electron avalanche in the direction away from the sharp electrode. Since the electric field decreases as a function of distance from the pointed electrode, the electrons that continue

to move away from it will eventually arrive in a region that lacks the necessary energy for

ionization [39]. As a result, the free electrons will drift slowly in space creating a

negative space charge, and will easily attach to neutral oxygen molecules. The lack of

electrons near the sharp electrode (or presence of the negative space charge), due to the

presence of the repulsive force, drastically reduces the ionization region (plasma region)

and the ionization process eventually stops. The ionization process restarts when the

negative ions reach the positive electrode [39]. As a result, negative corona is observed

as bursts of ionization. Figure 1.1 depicts the motion of the electrons (a) and the

reduction of the ionization region (b) when using a sharp edge with a negative corona

potential.

Figure 1.1 Model of Sharp Electrode Containing a Negative Corona Potential (a) and Plot
of Distance from Sharp Electrode vs. Strength/Size of Electric Field (b) [39]

When the pointed electrode switches polarity to a positive corona potential, the

free electrons will accelerate towards the point and cause further ionization. The result is

a positive space charge. In addition, the flux of electrons towards the high potential

electrode drastically increases the ionization region, extending it all the way to the other

electrode. Thus, in contrast to the sharp edge at negative potential, the ionization is

enhanced by the space charge, and not decreased [39]. It is important to note that even

though the ionization region is increased, there are far fewer free electrons in free space

with a positive corona than there are with the negative corona configuration. However,

since the electrons in the positive corona case are heavily concentrated close to the

surface of the curved electrode (in a region of high-potential gradient), the electrons have

a very high energy. In contrast, for the negative corona case, many of the electrons are

located in the outer, lower field areas. As a result, a hissing sound is typically associated

with the positive corona. In addition, the positive corona will emit a bluish/white color

due to the generation of secondary ions described above. Figure 1.2 below illustrates the

motion of the electrons (a) and the extension of the ionization region when using a sharp

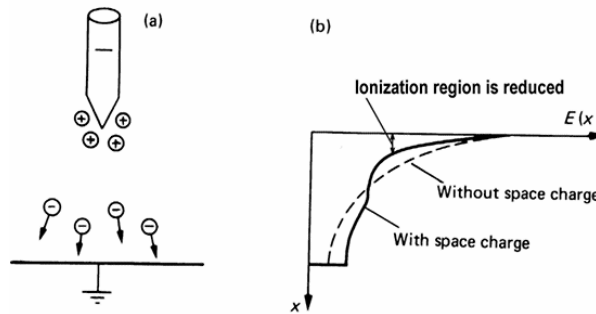edge with a positive corona potential.
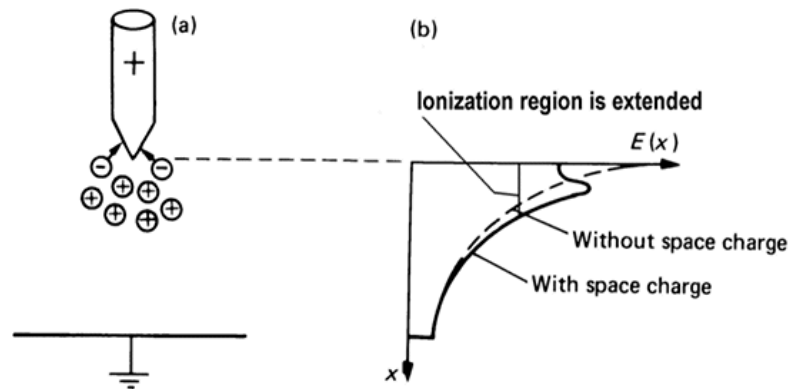
Figure 1.2 Model of Sharp Electrode Containing a Positive Corona Potential (a) and Plot
of Distance from Sharp Electrode vs. Strength/Size of Electric Field (b) [39]

2.3 Corona Applications

Corona discharges are currently used in a wide variety of commercial and industrial

applications. They are commonly used to generate charged surfaces for the application of

electrostatic copying or photocopying [36]. They have been known to be used as air

ionizers for possible health benefits [36]. Corona discharges are also used for high

voltage contact print photography called Kirlian photography [36]. Other applications of corona discharges are scrubbing particles from air in air-conditioning systems [36]. They accomplish this by removing the particulate matter from the air stream and then passing the charged stream through a comb of alternating polarity, to deposit the charged particles onto oppositely charged plates [36]. In addition, the free radicals and ions generated from corona reactions can be used to scrub the air of certain noxious products [36]. Corona discharge can also be utilized for the manufacturing of ozone [36]. A few studies have examined using corona for immunotherapy and biomedical research. One such study by Kwark and Lee involved a real-time corona discharge imaging system as a future biomedical imaging device [40].

2.4 Motivation for Using Corona Fields as a Means for Cell Contact

There were five key determining factors for investigating the use of corona discharge as the methodology for obtaining tight intracellular contact prior to electrofusion. One such factor was the low current (μA) due to the ions and electrons. With this range of current, the discharge could be applied without compromising cell viability and integrity. Furthermore, the use of corona discharge as a means for achieving cell-cell contact does not directly affect the choice of electrofusion medium. As discussed in the previous chapter, some of the more traditional cell contact methods required the use of a non-physiologically balanced medium. Thus, using corona charge would help rid the concern involved with this discrepancy. In addition, the ability to view the electrofusion process under a microscope and the ability to move the cells in monolayers helped to provide further incentive towards using this methodology. Moving the cells in monolayers would

enable us to control the desired quantity of intracellular contact for the approximation of a 1:1 fusion event. Lastly, the possibility of having the technological flexibility to either create hybrids in great quantities or to target a small amount of cells for hybridization is a tremendous enticement for using corona discharge as a method for achieving cell contact.

CHAPTER 3: RESEARCH GOALS

Over the past 10 years, there has been a great increase in the growth of research surrounding cell transplantation therapy as a means for alleviating the devastating symptoms for diseases including stroke, Alzheimer's disease, spinal cord injury, cirrhosis of the liver, factor 8 hemophilia, Type I diabetes, and Parkinson's disease.

Parkinson's disease remains one of the foremost health issues world-wide, and although many advances have been made to treat the symptoms of this devastating disease, little has been accomplished in actually curing the disease. In recent times, the transplantation of isolated NT2 cells (dopamine producing neuronally committed teratocarcinoma cell line) into the Parkinsonian host has shown a 100% success rate in Parkinsonian patients that received NT2 allografts [41]. As promising as this is, major problems persist. To allow for prolonged NT2 engraftment, there is a need to continue the chronic use of immunosuppressive medications introducing significant side effects that range from sustained discomfort to devastating life threatening infections.

Recently, a number of studies have investigated the idea of using Sertoli cells in allograft cell transplantation protocols in order to achieve prolonged NT2 engraftment in the absence of systemic immune suppression. Sertoli cells are terminally differentiated cells found in mammalian testes that provide a dynamic trophic factor-rich microenvironment for developing spermatids in a sequestered testicular compartment

devoid of blood and lymphatic vasculature. They also play an essential role in preventing the individuals from rejecting their own highly antigenic mature germ cells [42]. It is this localized immunoprotection (provided by Sertoli cells) that has prompted such investigations. One such study was examined by Willing and Cameron [43], where they were able to show that isolated Sertoli cells (iSCs) can create a testis-like immune privileged site outside of the testis and that allo- and xenogenic neurons can be transplanted there (by co-transplantation with iSCs) without requiring systemic immunosuppression of the host rat. They also concluded that iSCs induce localized immunoprotection. It is, however, unclear how iSCs immunoprotect cell and tissue grafts, although a number of theories have been offered [42, 44, 45]. In general, the ability of iSCs to cause a significant reduction or even elimination of allo- or xenograft rejection appears to be related to their close proximity to the co-transplanted cells and tissues. This localized effect, while in close juxtaposition, suggests that there is a need for the Sertoli cells to maintain close contact with the co-transplanted cell or tissue graft, possibly by fusion. In order to obtain the desired cell therapy for Parkinson's, the Sertoli cells would have to be fused with the NT2 cells in a way so as to not affect their viability, integrity or functionality. This idea suggests the investigation of a protocol that would produce the heterohybrids on a 1:1 basis in mass quantity, with the hope that we would be able to isolate and subculture the hybridized cell with the desired genetic characteristics (local immune protection and secretion of dopamine).

This investigation is a step towards finding a novel cell contact method that will grant us the greatest opportunity towards obtaining 1:1 hybridization in great quantity without the deficiencies experienced when using traditional methods. As discussed in the

17

previous chapter, the use of electrical charge produced by corona discharge has many advantages over some of the other more traditional cell contact methods. In an attempt to exploit some of these potential advantages, this study was designed with the following specific aims:

- To determine whether corona discharge can be used as a method for achieving tight intracellular contact.

- To provide evidence of ability to control cellular movement of the cells in monolayers while suspended in a physiologically balanced medium.

- To determine optimal corona-based parameters for approximation of a 1:1 hybridization event.

- To provide evidence of ability to generate large quantities of hybridized cells and attempt to use optimal corona-based parameters to generate 1:1 fusion.

- To determine and confirm that corona does not compromise the hybrid cell viability, integrity, and functionality.

- To finalize a fusion chamber design and electrofusion method that will incorporate the use of both cell-cell contact and cell electrofusion to produce high hybrid yields.

CHAPTER 4: MATERIALS AND METHODS

4.1 Cell Preparations

4.1.1   Cell Line and Culture Methods

Neuronally committed dopaminergic human teratocarcinoma cells (NTera-2 cl.D, or NT2; ATCC #CRL-1973: American Type Culture Collection, Rockville, MD) were used throughout the experimental work performed in this study (Figure 4.1). The cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) (Cellgro Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (Cellgro Mediatech, Inc.) and 0.05 mg/ml of gentamicin (Cellgro Mediatech, Inc.). The cells were cultured under aseptic conditions in 21 cm^2 polystyrene petri dishes (Corning Incorporated, Corning, NY) and were incubated in 5% CO_2 at 37°C (CO_2 Water Jacketed Incubator, Forma Scientific, Inc., OH).
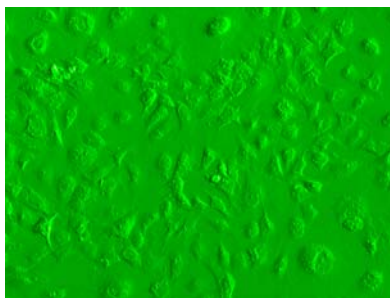

Figure 4.1 Human Neuronally Committed Teratocarcinoma Cell Line (NT2, 100X, Phase Contrast with a Green Filter)

NT2 cells were grown as adherent monolayers and required medium renewal and/or sub-culturing every 3-4 days. NT2 cells are non-terminally differentiated cells that require sub-culturing at about 90% confluence to insure the recovery of a large quantity of cells and to prevent contact inhibition from occurring. Before sub-culturing, cell monolayers were washed three times with DMEM supplemented with 0.05 mg/ml of gentamicin. Cells were detached from the dish using a non-enzymatic cell dissolution solution (Cell Stripper; Cellgro Mediatech, Inc.). In order to further facilitate cell detachment, the cells were placed in the incubator at 37°C for approximately 2-3 minutes. The Cell Stripper solution was neutralized with supplemented DMEM (containing 10% FBS and 0.05 mg/ml gentamicin) following incubation and prior to aspirating the suspended cells. Once the cells were recovered from the dish, they were centrifuged for 5 minutes at $220 \times g$ and 20°C in a 50 ml conical tube. Following centrifugation, the supernatant was aspirated, and the cells were re-suspended in DMEM solution supplemented with 0.05 mg/ml gentamicin. The sequence of centrifugation followed by re-suspension was repeated twice to wash the cells. The NT2 cells were then sub-cultured with a ratio of either 1:4 or 1:5 depending upon whether the cells were to be sub-cultured 3 or 4 days later again.

4.1.2 Cell Counting

Harvested cells were prepared for counting by washing with Dulbecco's Phosphate-Buffered Saline (DPBS 1X w/o Ca and Mg; Cellgro Mediatech, Inc.) three times. Cells were centrifuged (5810R, Eppendorf, Westbury, NY) at $220 \times g$ for 5 minutes at 20°C and suspended in approximately 5 ml of DPBS for each wash. A sample of the cells was then diluted in 0.9% sodium chloride (APP, Schaumburg, IL) and 0.4% trypan blue stain (Cellgro Mediatech, Inc.). Trypan blue penetrates the membranes of the dead cells and causes them to turn blue. A hemacytometer (Hausser Scientific, Horsham, PA) was used to count viable and non-viable cells at 100x using light microscopy. The concentration of the cells was determined using the following formula:

$$\frac{\#Cells}{mL} = \frac{\#Cells}{mm^2} \times D \times 10,000$$

Where D = dilution (if used) and
10,000 = conversion factor for 0.1 μl to 1 ml

The percent viability of the cells was also determined after counting. Only those cell cultures that were 85% - 100% viable were used for experimentation. Figure 4.2 and 4.3 show the hemacytometer used for counting, and a microscopic view of a 1 mm^2 square (counting space) on the hemacytometer respectively.
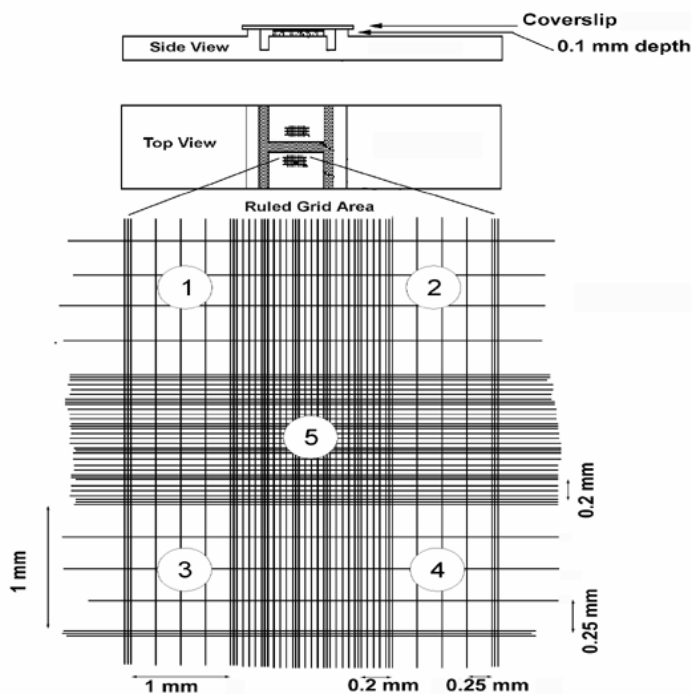
Figure 4.2 Hemacytometer [46] at 40X
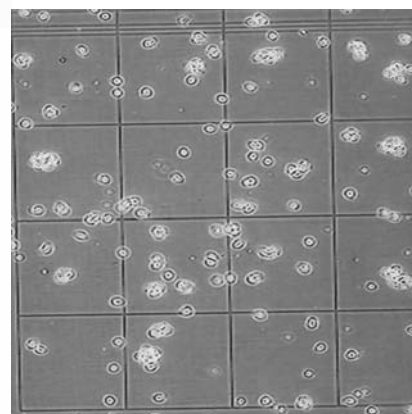
Figure 4.3 Scope View of a Hemacytometer at 100X[46]

4.2 Cell Staining

In some experiments the cells were stained to assist with visual distinction between fused cells and non-fused cells under fluorescent microscopy.

4.2.1 Stock Solution of Dyes

Stock solutions of fluorescent dyes were prepared in advance using the procedure discussed by Jaroszeski [47, 48]. The fluorescent dyes used for this study were 5-(and-6-)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine (CMTMR (red fluorescent dye); Molecular Probes, Eugene, OR) and 5-chloromethylfluorescein diacetate (CMFDA (green fluorescent dye); Molecular Probes). Both dyes were supplied by the

manufacturer in 1 mg aliquots. Stock solutions of 5 mM concentration of both dyes were prepared in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO). CMTMR (M_rW 554) stock solution was made by mixing the supplied 1 mg aliquot of CMTMR with DMSO to yield a final volume of 360 μl. Correspondingly CMFDA (M_rW 465) stock solution was made by mixing the supplied 1 mg aliquot of CMFDA with DMSO to yield a final volume of 430 μl. Both dyes were easily dissolved in DMSO at room temperature. The DMSO stock solutions were divided into single-use aliquots (usually 3 aliquots for CMTMR and 5 or 6 aliquots for CMFDA) and stored at ≤ -20°C, protected from light. This division into single-use aliquots avoided freeze-thaw cycles of the stock solutions in order to increase shelf life and ensure consistent results.

4.2.2   Staining Technique

For all experiments that used stained cells, NT2 cells were harvested from two 21 cm^2 polystyrene cell petri dishes. These cultures were 3-4 days old and the cells had reached the desired confluence (90%). One aliquot of each CMTMR (75 μl) and CMFDA (50 μl) were removed from storage and defrosted to room temperature. All staining was performed under aseptic conditions in a biological safety cabinet. The growth media in both petri dishes was reduced to 6 ml (just enough to cover the monolayer of cells adhered to the petri dishes). One dish was stained with 75 μl of CMTMR while the other dish was stained with 50 μl of CMFDA. The cultures were then incubated at 37°C for two hours. After incubation, the cells were then harvested and counted by using a hemacytometer.

4.2.3 Fluorescent Microscopy

A fluorescent microscope (Leica DM IL, Leica, West Germany) was used to visualize the contact between CMTMR and CMFDA stained cells, as well as the resulting dual fluorescing (fused) cells following direct current (DC) application.

Under fluorescent light there was a clear visual distinction between the fused and un-fused cells. The un-fused CMTMR stained cells appeared red, the un-fused CMFDA stained cells appeared green and the fusion products of the two were easily identified due to their yellowish/orange color and irregular shape (larger in size and non-spherical circumference). Figure 4.4, below, shows the resulting NT2 monohybrid (white arrow) when two NT2 cells stained with both CMTMR and CMFDA fuse. The result is a yellowish/orange, irregularly shaped cell.
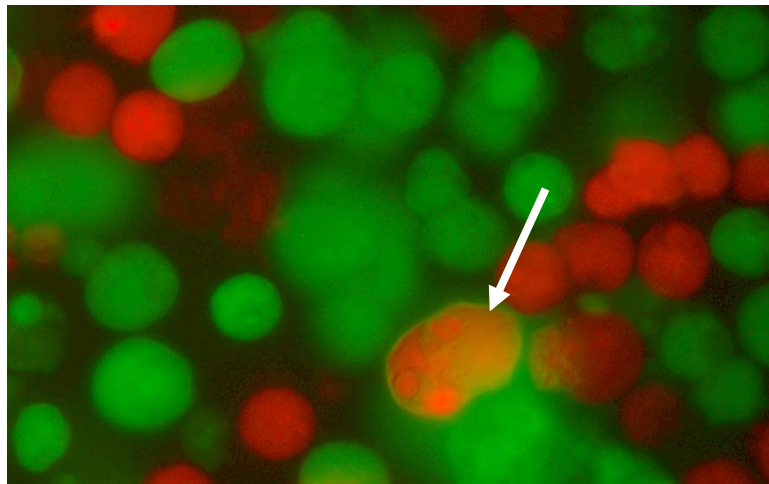

Figure 4.4 NT2 Monohybrid Under Fluorescent Microscopy (400X)

4.3 Media for Electrofusion

The media in which cell-cell electrofusion was conducted in was DMEM (Cellgro, Mediatech, Inc.) supplemented with 0.05 mg/ml gentamicin. The media was not supplemented with fetal bovine serum. To elaborate, the presence of serum would act as a blockade by preventing the cell membranes from contacting each other. The harvested cells (either stained or unstained) were counted and the DMEM supplemented with 0.05 mg/ml gentamicin was then added to the cell solution to adjust the concentration of cells as per the requirement of the experiments.

4.4 Corona Apparatus

4.4.1   Corona Generator

The corona generator (Figure 4.5 and 4.6 below) consisted of a corona generating element that emitted ions from a 25 mm diameter hole in a stainless steel ground plate. The wire plate geometry of the corona generating element consists of 9 needles (stainless steel acupuncture needles, gauge no. 30, SGAMAC, China) that were contained within a circular white teflon disk. The teflon disk was placed within a central hole of a larger white cylindrical teflon body. Eight of the needles were arranged in a circle of 9 mm diameter with the ninth needle located in the center. The height of the needles was adjusted to a height of 6.8 mm from the base of the central hole in the cylindrical teflon body. A circular ground plate was attached to the base of the cylindrical white teflon body, which was mounted on a micromanipulator. The micromanipulator enabled the corona generator to be lowered to a convenient distance of 8.0 – 9.0 mm from the cells in

preparation for corona exposure. It could also be raised in preparation for exposure of a new set of cells. In essence, the micromanipulator could be adjusted to move anywhere in 3-dimensional space. This was advantageous for the purpose of being able to simply position the corona element over a dish of cells located under a microscope in order to observe the cells during corona exposure. The entire set of corona generating needles in this element had a common connection to the voltage output of a high voltage DC power supply.
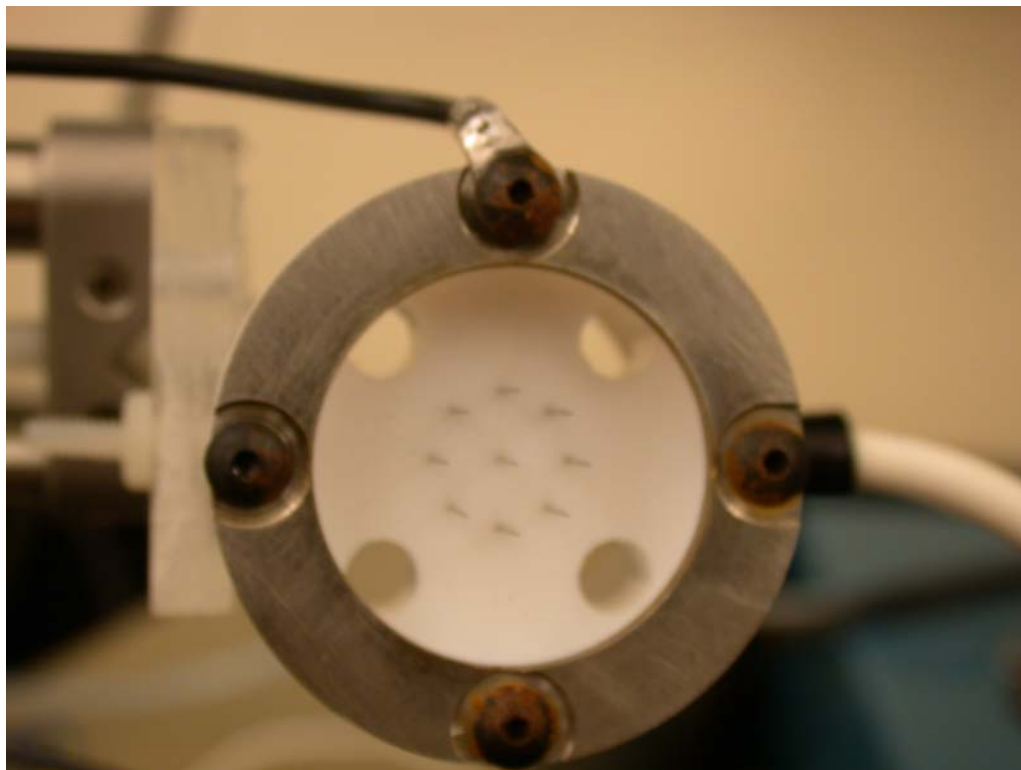

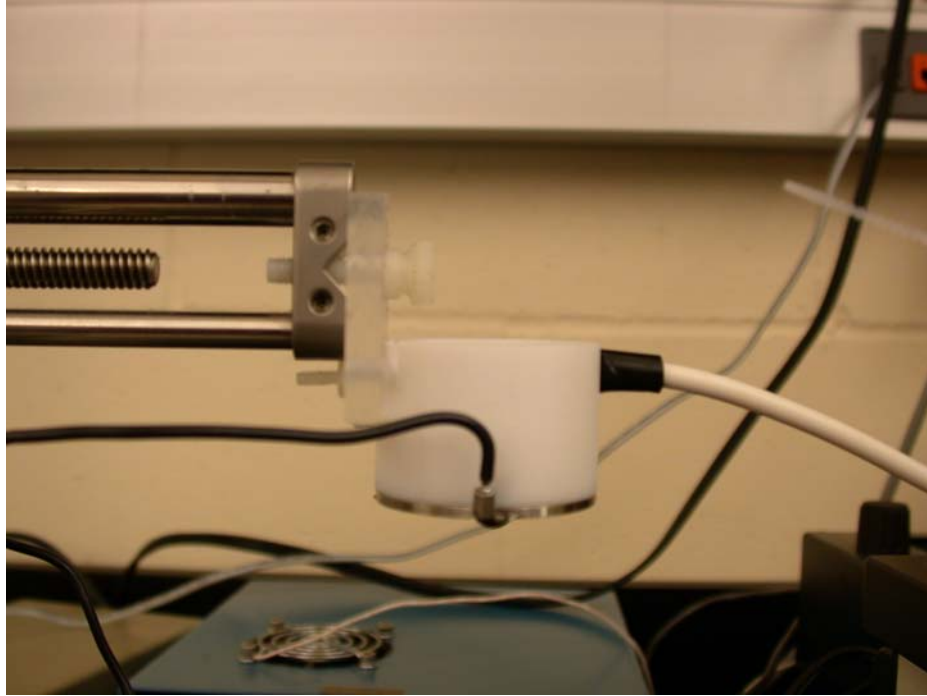Figure 4.5 Bottom View of Corona Generator

Figure 4.6 Side View of Corona Generator

4.4.2 Corona Experimental Setup

In order to investigate the effect of corona application on cell-cell contact, various pieces of equipment were used to not only generate the corona discharge but view the resulting cellular movement and hybridization. In order to manipulate and control the intensity level of the discharge, computer software was installed and utilized to help manage the equipment used in the experiment.

A corona generator was connected to a high voltage power supply (CZE 2000, Spellman High Voltage Electronics, Hauppauge, NY) in order to generate the corona discharge. The generator had both positive and negative leads. The positive electrical wire connected the nine acupuncture needles of the generator to the high voltage supply, whereas the negative electrical wire connected the ground plate (located on the bottom of

the white teflon body) to the ground. Additionally, the generator was attached to a

micromanipulator so that it could be suspended above the chamber containing the cells.

The chamber was placed on the microscope stage so that cell movement could be

observed during corona application. Furthermore, the microscope had capability to

simultaneously examine both red and green fluorescence which made it possible to view

the formation of hybrid cells. The input voltage, current and duration for corona

treatment was keyed into a program that was written in LabVIEW software (LabVIEW 7,

National Instruments, TX) on a computer (Dell Dimension 2400, Dell Inc. TX) that

controlled the entire system. The computer and software used a data acquisition card

(DAC) (PCI 6036 E, National Instruments, Austin, TX) to control the power supply using

the user input parameters. Figure 4.7 shows a diagram of the entire instrument system.

Figure 4.8 illustrates the LabVIEW computer software for corona generation as seen on
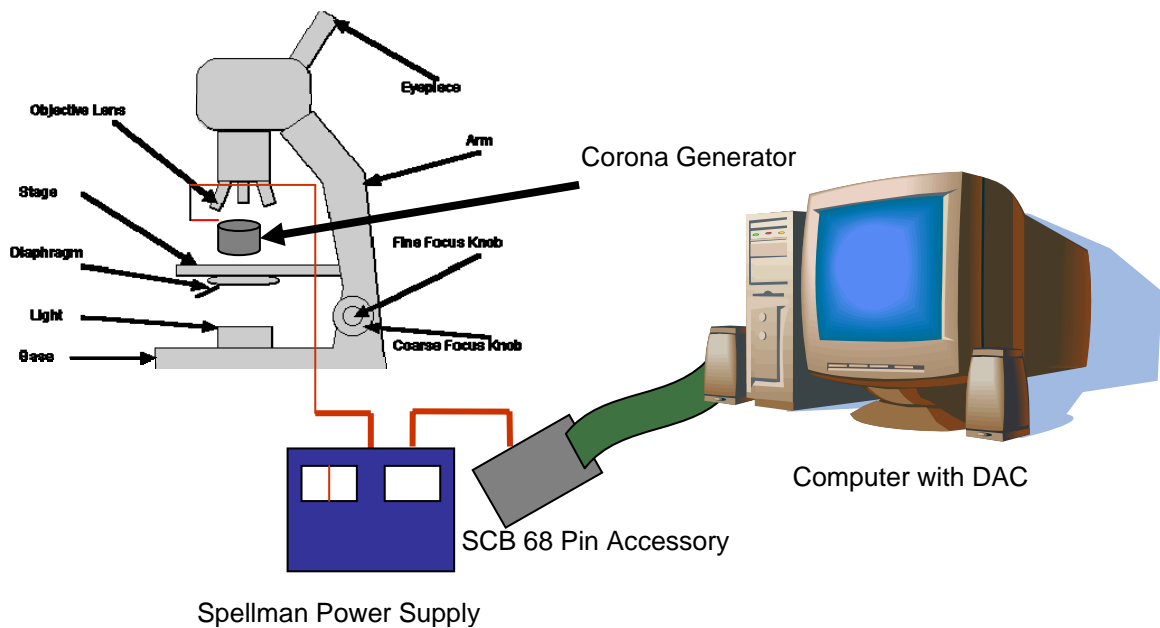
the computer monitor.

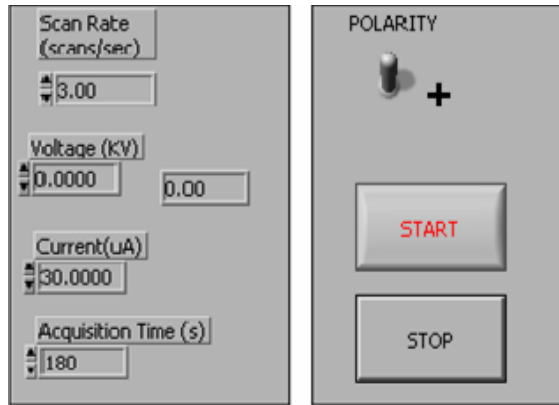
Figure 4.7 Corona Experimental Setup

Figure 4.8 LabVIEW Computer Program for Corona Generation

A reversible polarity switch was installed in the electrical line between the data acquisition card and the power supply to enable the user to switch from positive to negative corona (or vice versa). The polarity was manipulated from a signal sent through the DAC. In order to measure the temperature and humidity of the area surrounding the chamber during corona treatment, separate humidity and temperature probes were used. The probes were placed next to the chamber during the experiment to measure the effect of these two parameters on the corona generation process.

4.4.3 Fusion Chambers Investigated with Corona

Four separate fusion chambers were investigated for use with the corona generator. The first chamber (Figure 4.9) consisted of a circular outer stainless steel wire and an inner circular stainless steel plate having a thickness of 3 mm. The central plate was connected to an electrical wire from the bottom of the chamber. Both the outer stainless steel electrode and inner stainless steel electrode could be connected to a ground source during corona treatment and to the DC electroporator during electrofusion.
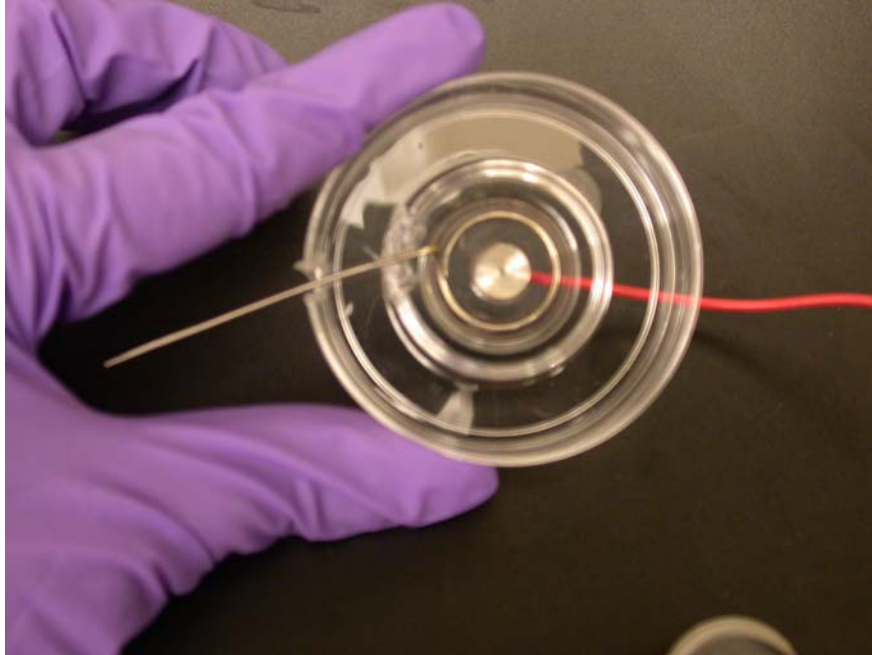
Figure 4.9 Circular Corona Chamber with Circular Electrodes

After observing the cell contact and fusion properties when applying corona discharge and subsequent DC pulses using this chamber, two more chamber configurations were investigated in order to better optimize the fusion properties. The next two fusion chambers that we examined were a circular corona chamber without electrodes (Figure 4.10) and a square corona chamber without electrodes (Figure 4.11). The circular corona chamber without electrodes matched the physical characteristics of the first fusion chamber investigated (See Figure 4.9), but this new chamber lacked the circular inner stainless steel electrode and the outer stainless steel electrode. The square corona chamber consisted of eight plastic square chambers mounted onto a plastic slide. Only four of the eight plastic wells as shown in Figure 4.11 were used during the investigation. The multiple chambers allowed for multiple fusion trials to be performed at the same time for comparison.
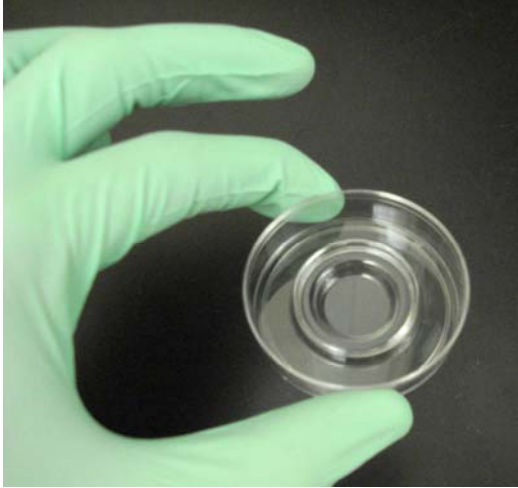
30

Figure 4.10 Circular Corona Chamber without Electrodes

Figure 4.11 Square Corona Chamber without Electrodes

A final chamber (Figure 4.12) was investigated in order to provide additional control on the intracellular contact during corona treatment. This chamber was identical to the square corona chamber stated above with one modification; two square stainless steel square plates were fitted against two of the walls of the chamber. Both square plates/electrodes could be connected to a ground source during corona treatment and then to the DC electroporator during electrofusion.
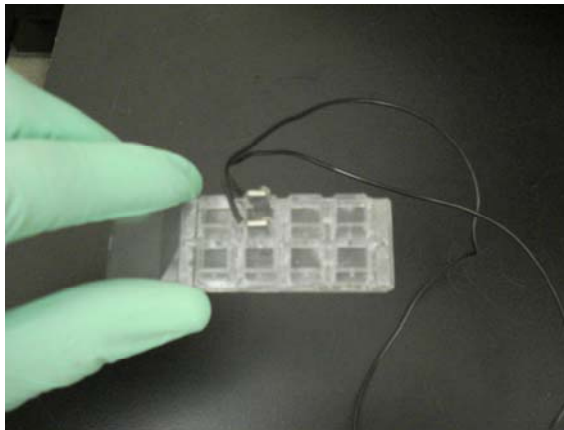

Figure 4.12 Square Corona Chamber with Square Electrodes

**4.5 DC Cell Fusion Apparatus and Experimental Setup**

For experiments involving the application of fusogenic pulses, an electroporation DC pulse generator (Figure 4.13) (ECM 830 BTX Molecular Delivery Systems, Harvard, MA) was used to obtain NT2 monohybridization. In this setup the positive and negative wires were used to connect the DC generator to the electrodes of a fusion chamber for the transfer of direct current to the cells. In other experiments involving the use of fusion chambers without electrodes, an ECM 800 pulse generator (Figure 4.14) was used to obtain NT2 monohybridization. In these cases, two stainless steel electrodes mounted on the end of a handle and connected to the ECM 800 were manually placed into the corona chamber for the transfer of direct current to the cell suspension.

Figure 4.13 ECM 830 Electroporation DC Pulse Generator

A) ECM (Electro Cell Manipulation) 800 Generator
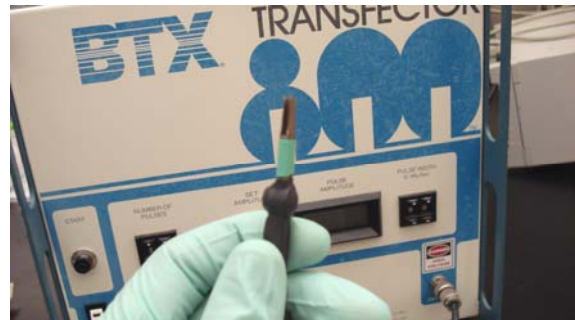
B) ECM (Electro Cell Manipulation) 800 Generator with Manual DC Pulse Generator (MPG) Connected

C) Manual DC Pulse Generator

Figure 4.14 ECM 800 Electro Cell Manipulation Instrument

CHAPTER 5: RESULTS AND DISCUSSION

5.1 Effect of Corona Charge on Suspended Cells vs. Non-Suspended Cells

In order to conduct a complete investigation for utilizing and optimizing corona discharge as a means for achieving intracellular contact, it was necessary to first gain insight as to the type of forces, in terms of magnitude and location in free space, that are present during corona treatment. Observation of cellular behavior exhibited under direct corona exposure, when in free suspension or affixed to the chamber floor, would help to provide valuable knowledge on the mechanisms acting in the suspension (not just at the surface). By gaining this insight, the probability of predicting the cell movement and thus optimizing the system for cell-cell contact would greatly increase.

NT2 cells were placed into a circular corona chamber containing circular electrodes (See Figure 4.9) at a concentration of 2×10^6 cells/175 μl. After introducing the cell suspension into the chamber, the cells were allowed to settle under the influence of gravity for a brief period (1-2 min.) to insure that a portion of the cells (not all) in suspension had settled to the chamber floor, affixed themselves, and were no longer in free suspension. In order to visually decipher between the suspended cells and the non-suspended cells, the chamber was mechanically perturbed following the settling time. The cells in suspension would move in response to the disturbance, while the non-suspended cells would not move. The outer and inner electrodes were both connected to

34

a ground source during this experiment. The cells were then exposed to 5 minutes of positive corona followed by 5 minutes of negative corona. The cells had to be inspected using a microscope during corona treatment in order to analyze the affinity or divergence in cellular behavior exhibited between the suspended and non-suspended cells.

Observations indicated that there was in fact a diverging effect for corona discharge on suspended vs. non-suspended cells. The suspended cells moved rapidly on the surface towards the inner circular electrode, while the non-suspended cells rolled slowly along the chamber floor in the same direction. This observation simply suggests that the electric field and the charges associated with it increase from the chamber floor to the suspension surface. As a result, it might be more beneficial, for the purposes of optimizing this methodology for cell-cell contact, to continue forward with the corona treatment of non-suspended cells being that it was more gradual and therefore easier to control.

5.2 Combined Negative and Positive Corona Treatment vs. Separate Treatment with Either Positive or Negative Charge

To obtain further knowledge of the effect of corona treatment on NT2 cells, a set of preliminary experiments were arranged to observe cellular movement resulting from various corona of both polarities.

NT2 cells were delivered into the circular corona chamber containing circular electrodes at a concentration of 2×10^6 cells/175 μl and allowed 3 minutes to settle as described above. At this time, approximately 95% of the cells had gravitated to the chamber floor. Both electrodes were connected to a ground source via electrical wires.

Three separate experiments were performed that used either positive corona, negative corona, or a combination treatment of positive followed by negative corona. In each test, cell movement and behavior were observed for the desired characteristics of achieving tight intracellular contact in the annular space between the two circular electrodes. These tests were repeated 3 times each for reproducibility. In addition, all three trials were executed with the same concentration of cells (2×10^6 cells/175 μl), at the same voltage (6.1 kilivolts for positive polarity, 5.5 kilivolts for negative polarity), and for the same amount of time (10 minutes). In the first test, the NT2 cells were treated with 10 minutes of positive corona polarity at 6.1 kilivolts (kV), in the second test the NT2 cells were treated with 10 minutes of negative corona polarity at 5.5 kV, and in the third test the NT2 cells were treated with 5 minutes of positive corona at 6.1 kV followed by 5 minutes of negative corona at 5.5 kV. The results showed that the combination treatment was the most successful.

5.3 Order of Combined Negative and Positive Corona Treatment

The order of the polarity (positive then negative or negative then positive) applied to the cells during the combination corona treatment was analyzed using the same corona chamber as in section 5.2 above. The NT2 cells were loaded into the corona chamber while suspended in DMEM, supplemented with 0.05 mg/ml gentamicin, at a concentration of 2×10^6 cells/175 μl. The cells were then allowed to settle for three minutes prior to corona application so that approximately 95% of the cells were attached to the bottom of the chamber. Next, the non-suspended NT2 cells were exposed to alternating positive and negative charge in the following manner. First, 1 minute positive

corona was applied followed immediately by 1 minute negative corona exposure (both electrodes grounded). Corona treatment was briefly suspended to allow for pictures to be taken with the intent of obtaining photographic evidence of cellular movement. This cycle of 1 minute of positive corona, 1 minute of negative corona, and photographs was performed a total of six times. The result was a cascade of pictures (Figure 5.1) showing the direction of cellular movement and the location of intracellular contact during 6 minutes of positive followed by 6 minutes of negative corona treatment. The aforementioned steps (6 cycles of alternating positive and negative charge involving photographs in between each cycle) were repeated a second time, but this time the cells were treated with 1 minute negative corona followed by 1 minute positive corona for each cycle (Figure 5.2).
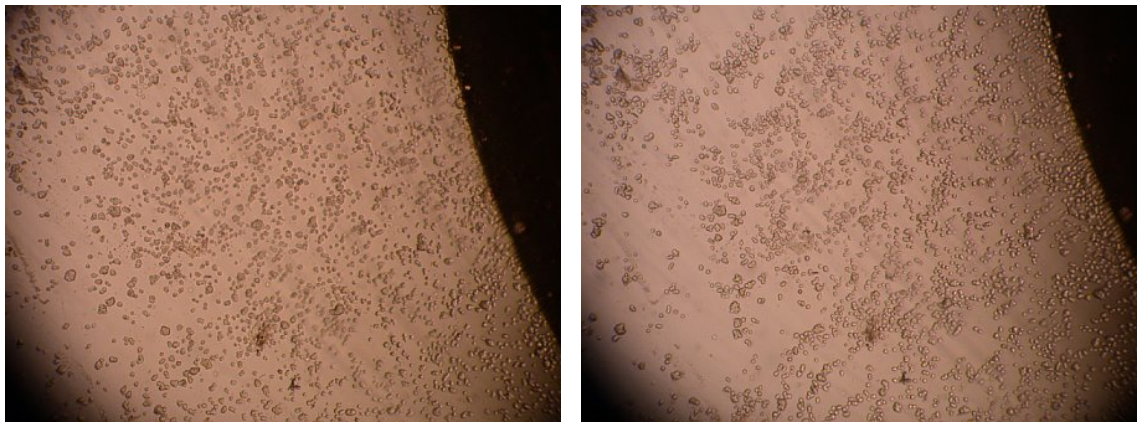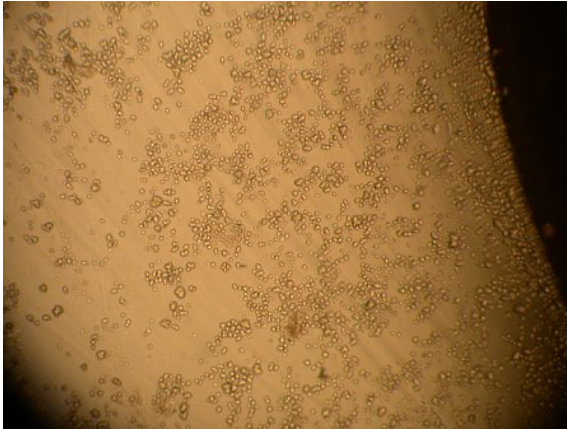

A) Before Corona Application   B) After 1 Cycle of 1 Minute Positive Corona
Followed by 1 Minute Negative Corona

Figure 5.1 Successive Corona Treatments of Positive Polarity Followed by Negative Polarity for Up to Six Minutes Each (40X)
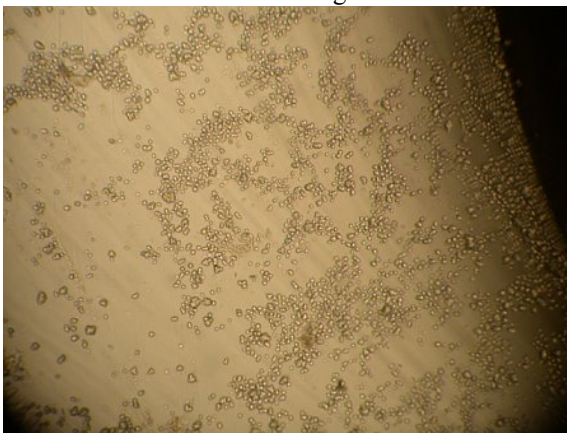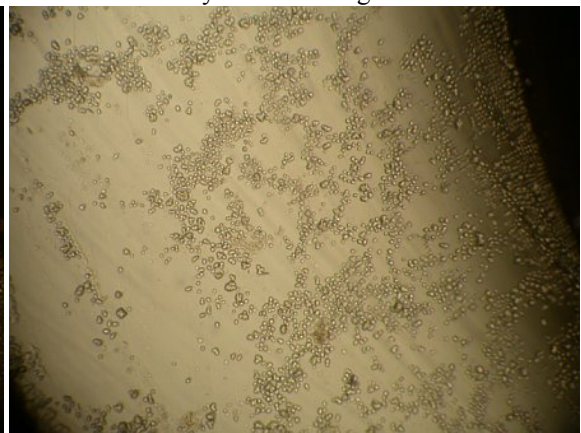
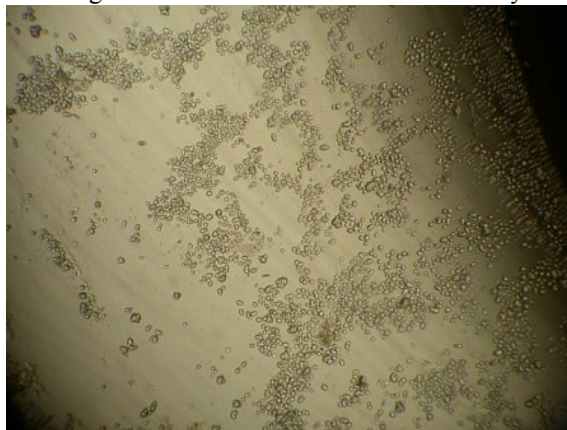C) After 2 Cycles of 1 Minute Positive Corona
Followed 1 Minute Negative Corona

D) After 3 Cycles of 1 Minute Positive Corona
Followed by 1 Minute Negative Corona

E) After 4 Cycles of 1 Minute Positive Corona
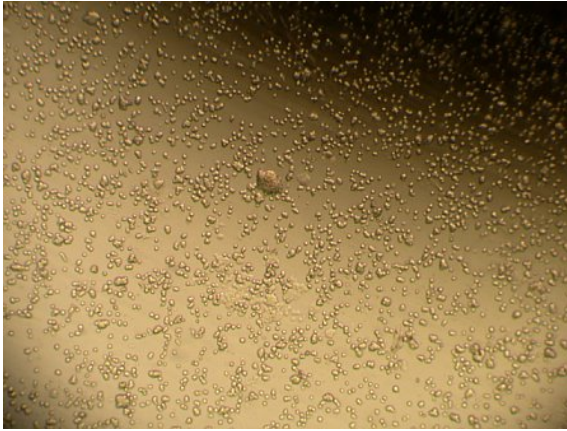Followed by 1 Minute Negative Corona

F) After 5 Cycles of 1 Minute Positive Corona
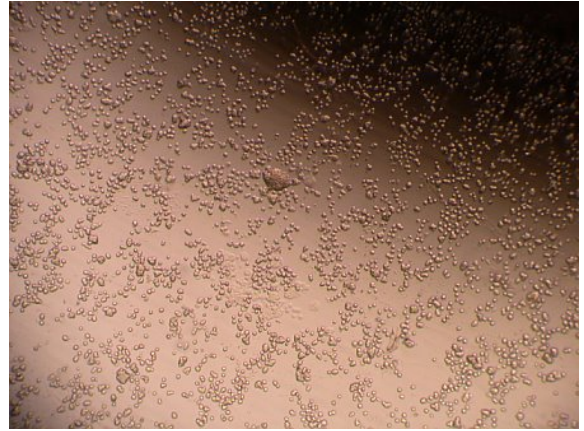Followed by 1 Minute Negative Corona

G) After 6 Cycles of 1 Minute Positive Corona
Followed by 1 Minute Negative Corona

Figure 5.1 Continued

A) Before Corona Application

B) After 1 Cycle of 1 Minute Negative Corona
Followed by 1 Minute Positive Corona

C) After 2 Cycles of 1 Minute Negative Corona
Followed by 1 Minute Positive Corona

D) After 3 Cycles of 1 Minute Negative Corona
Followed by 1 Minute Positive Corona

E) After 4 Cycles of 1 Minute Negative Corona
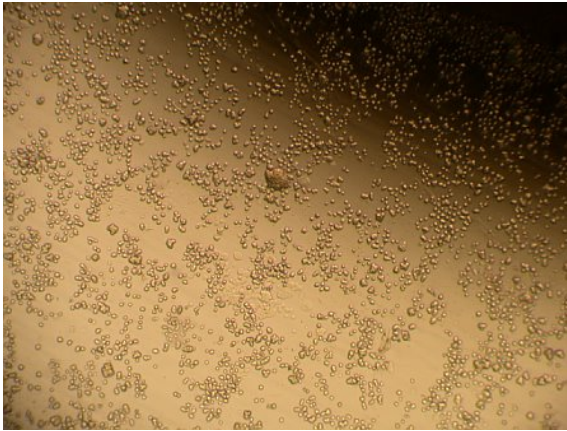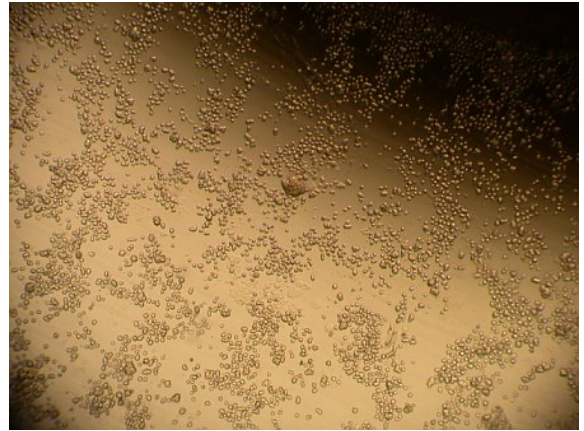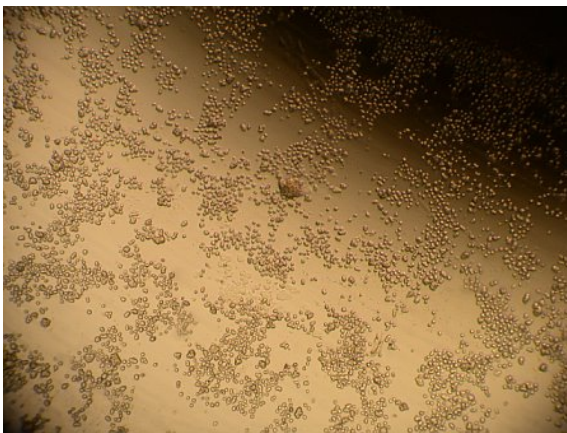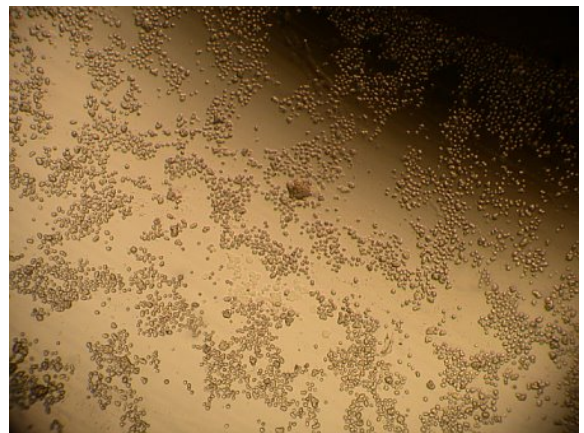Followed by 1 Minute Positive Corona

F) After 5 Cycles of 1 Minute Negative Corona
Followed by 1 Minute Positive Corona

Figure 5.2 Successive Corona Treatments of Negative Polarity Followed by Positive
Polarity for Up to Six Minutes Each (40X)

G) After 6 Cycles of 1 Minute Negative Corona
Followed by 1 Minute Positive Corona

Figure 5.2 Continued

The results revealed (Figures 5.1 and 5.2) that the order in which the different polarities were applied gave a similar result. In both experiments, the NT2 cells gradually gravitated towards one another in the middle space between both electrodes to form "small islands" of intracellular contact. Hence, any order of corona polarity would be adequate for the purpose of this investigation.

5.4 Effect of Grounding Variable Electrodes Under Corona Treatment

The effect of either grounding electrodes on the corona chamber or not grounding the electrodes was observed. This was done by performing four separate experiments in which the cells were prepared as discussed in Chapter 4, placed into the circular corona chamber with circular electrodes and treated with corona discharge while A) grounding both electrodes, B) grounding only the outer electrode, C) grounding only the inner electrode and D) not grounding either electrode. The reason for analyzing the effect of

grounding versus not grounding is that the experimentation would help to provide further insight as to what the electrical fields might look like within the suspension, as well as providing key information for the direction in which the forces are applied.

In each of the four experiments discussed above, NT2 cells were harvested, counted, and placed into the circular corona chamber containing circular electrodes at a concentration of 2×10^6 cells/175 μl. Results for Trial A (grounding both electrodes) were already obtained from Section 5.2. In Trial B, only the outer electrode was connected to a ground using a wire. The NT2 cells were then treated with 5 minutes of positive corona followed by 5 minutes of negative corona. A sequence of photographs was taken to record movement while under corona discharge (Figure 5.3). The cells were forced into contact near the inner electrode. Since the charge on the inner electrode was not displaced to a ground source, there was an accumulation of charge resulting in cellular attraction. Movement of cells close to an electrode is extremely unfavorable due to the arcing effects associated with the electrodes during the application of fusogenic DC pulses. The arcing could potentially jeopardize cellular integrity and/or viability. In addition, electrochemical products that may form at the electrodes during DC pulse application could adversely affect cell viability. For Trial C, only the inner electrode was connected to a ground source. Photographic evidence of the cellular movement (Figure 5.4) revealed that intracellular contact occurs much like Trial A (in the middle space between the two electrodes), although cell-cell contact is of a smaller quantity and occurs at a more gradual pace. This result is unfavorable because there might not be enough intracellular contact to obtain a large quantity of hybrids during electrofusion. Finally, in Trial D, neither electrode was connected to a ground source. A sequence of photographs

(Figure 5.5) was taken during Trial D to once again record cellular movement and behavior. As can be seen below, these photographs illustrate little to no cellular movement during corona application. Any motion at all was simply a result of the electrostatic repulsions/attractions between the cells and the ions present in the media. One can speculate that the evidence presented in Trial D points to the notion that in order for there to be any cellular movement there must be a current present within the solution that acts on the cells forcing them to a certain direction. The current is generated by the transport of charge from one or both of the electrodes to a ground source.
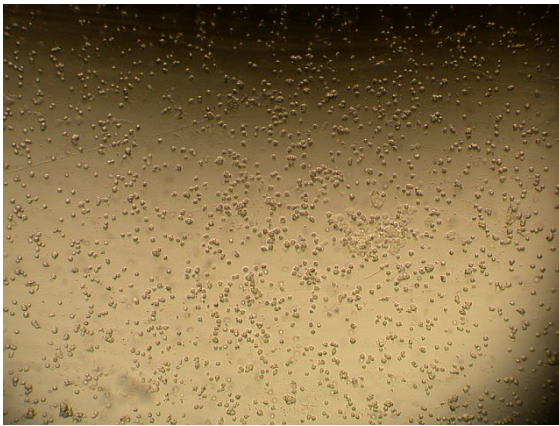
A) Before Corona Application

B) Trial B: After 1 Cycle of 1 Minute Positive Corona Followed by 1 Minute Negative Corona

Figure 5.3 Corona Treatment of Circular Corona Chamber Configuration with Only Outer Electrode Grounded (40X)

C) Trial B: After 2 Cycles of 1 Minute Positive
Corona Followed by 1 Minute Negative Corona

D) Trial B: After 3 Cycles of 1 Minute Positive
Corona Followed by 1 Minute Negative Corona

E) Trial B: After 4 Cycles of 1 Minute Positive
Corona Followed by 1 Minute Negative Corona

F) Trial B: After 5 Cycles of 1 Minute Positive
Corona Followed by 1 Minute Negative Corona

G) Trial B: After 6 Cycles of 1 Minute Positive Corona
Followed by 1 Minute Negative Corona

Figure 5.3 Continued

43

A) Trial C: Before Corona Application

B) Trial C: After 1 Cycle of 1 Minute Positive Corona Followed by 1 Minute Negative Corona

C) Trial C: After 2 Cycles of 1 Minute Positive Corona Followed by 1 Minute Negative Corona
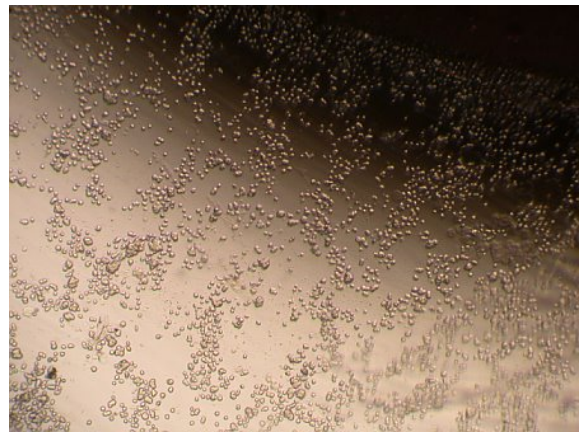
D) Trial C: After 3 Cycles of 1 Minute Positive Corona Followed by 1 Minute Negative Corona
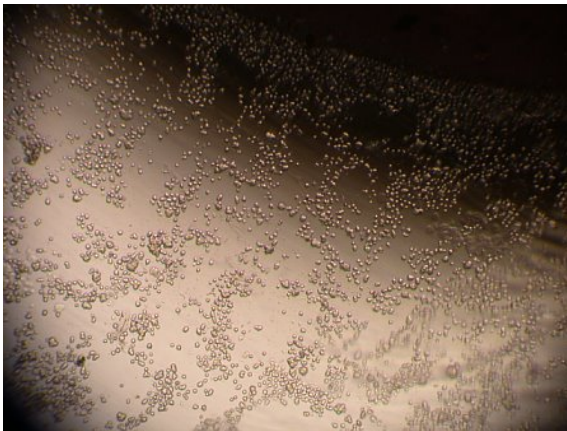
E) Trial C: After 4 Cycles of 1 Minute Positive Corona Followed by 1 Minute Negative Corona
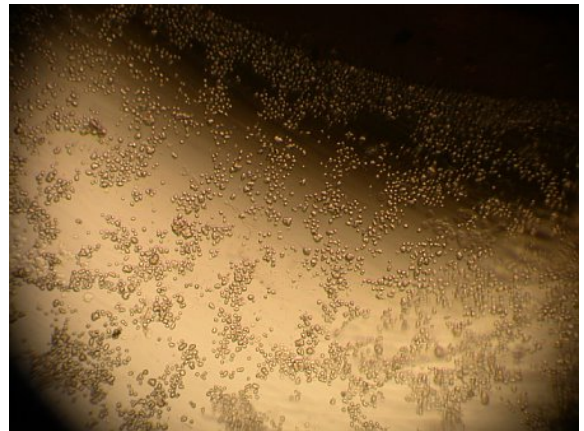
F) Trial C: After 5 Cycles of 1 Minute Positive Corona Followed by 1 Minute Negative Corona

Figure 5.4 Corona Treatment of Circular Corona Chamber Configuration with Only Inner Electrode Grounded (40X)
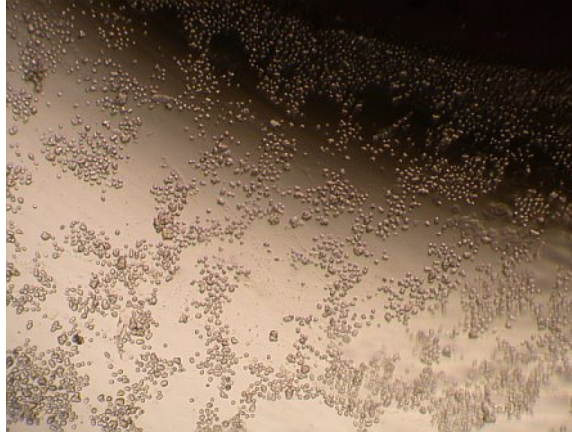
G) Trial C: After 6 Cycles of 1 Minute Positive
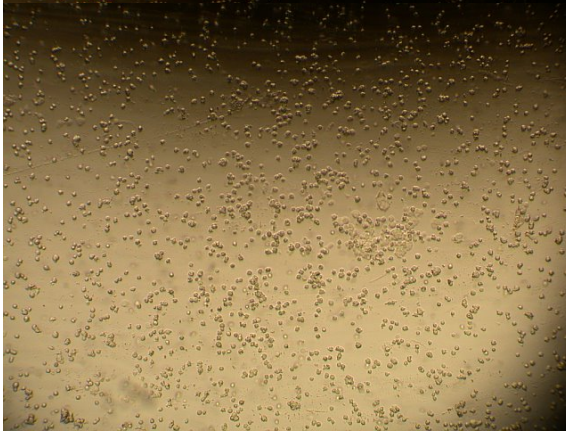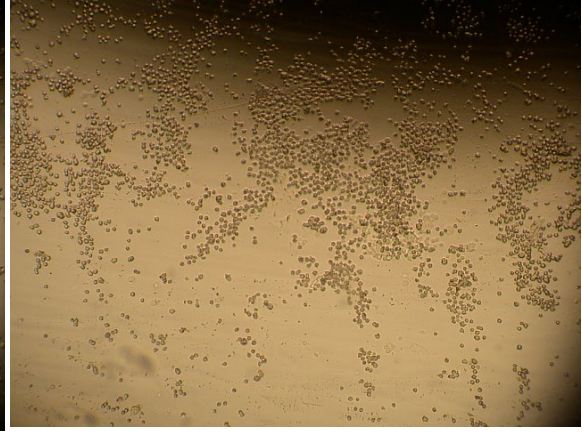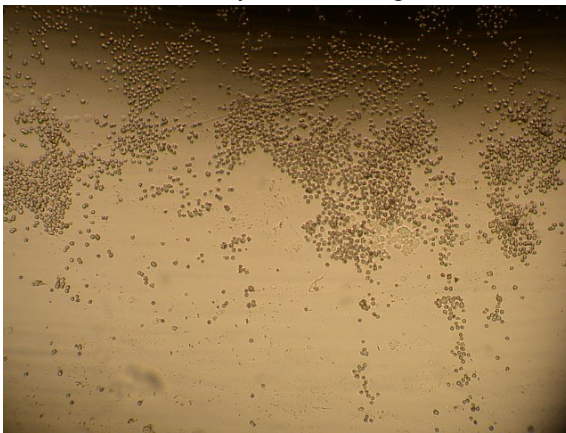Corona Followed by 1 Minute Negative Corona

Figure 5.4 Continued
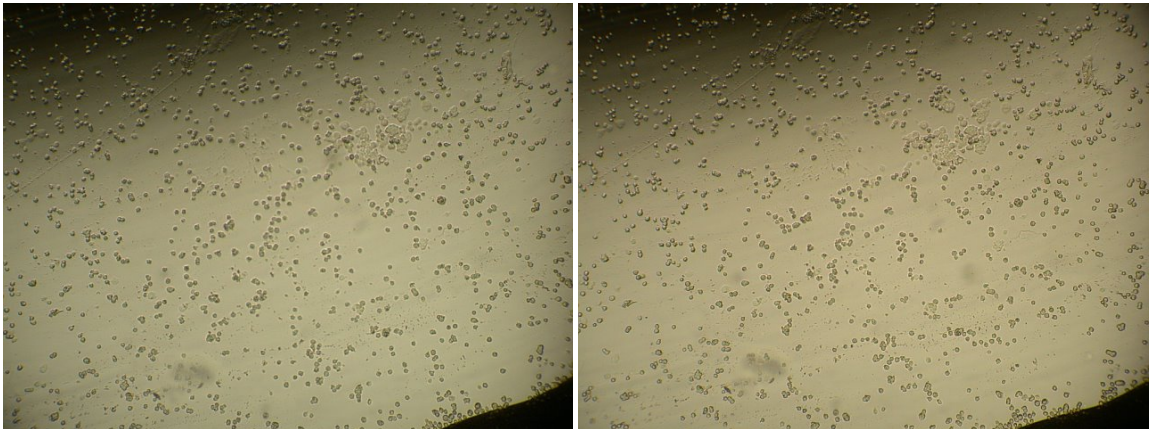
A) Trial D: Before Corona Application

B) Trial D: After 1 Cycle of 1 Minute Positive
Corona Followed by 1 Minute Negative Corona

Figure 5.5 Corona Treatment of Circular Corona Chamber Configuration with No
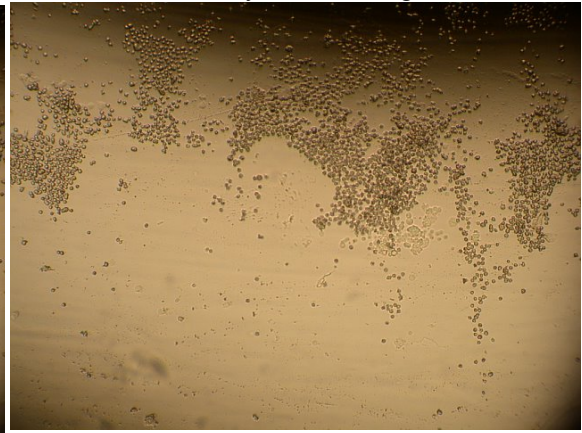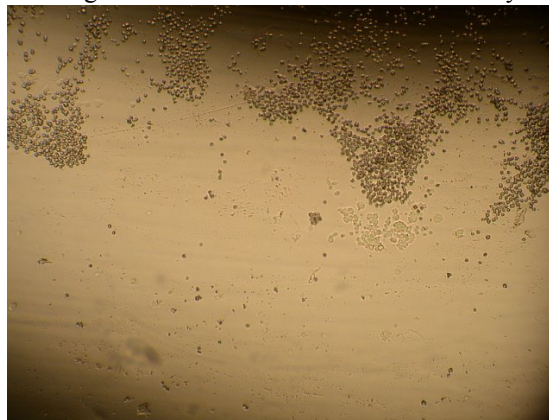Electrodes Grounded (40X)
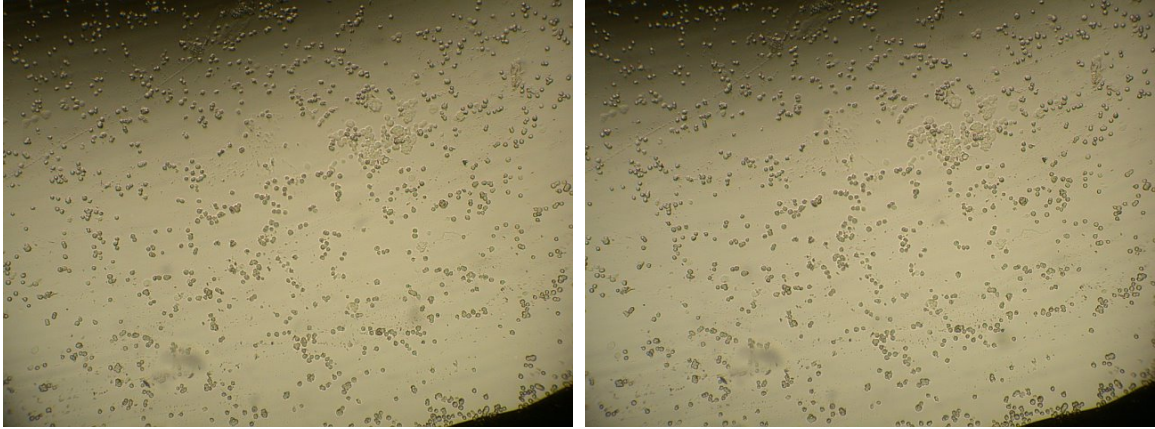
C) Trial D: After 2 Cycles of 1 Minute Positive
Corona Followed by 1 Minute Negative Corona

D) Trial D: After 3 Cycles of 1 Minute Positive
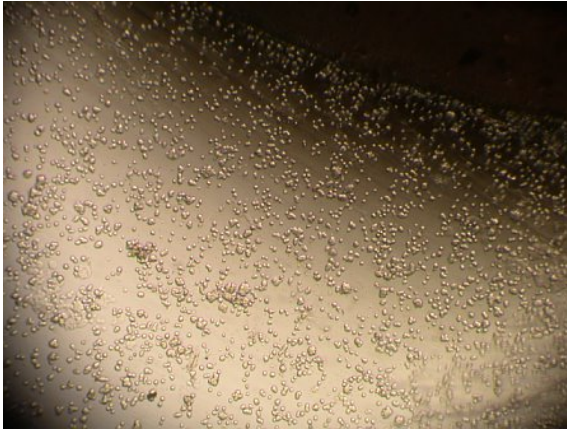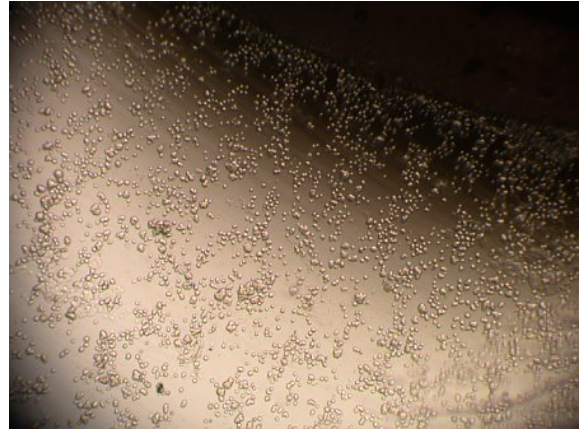Corona Followed by 1 Minute Negative Corona

Figure 5.5 Continued

In summary, grounding both electrodes (Trial A) provided the most favorable conditions for the purpose of this investigation. Not only do the cells aggregate in desirable quantities at a moderately controlled pace, but they contact each other in the middle space between the inner and outer electrodes, thereby preventing the risk of experiencing the deleterious effects of arcing and electrochemicals during subsequent electrofusion.

5.5 Determination of Optimal Corona Treatment Duration

In order to determine optimal conditions for cellular contact that were favorable for the generation of a large quantity of 1:1 hybrids, it was necessary to first define exactly what level of intracellular contact would be considered optimal for the purpose of this investigation. Optimal cell contact conditions were defined as those conditions that would provide the greatest probability for generating a large quantity of hybridized cells

on a 1:1 fusion basis. In order to insure that there would be a large quantity of 1:1 hybrids generated, it was determined that there must be moderate sized cell islands consisting of tight intracellular contact present in the corona chamber following corona treatment and prior to electrofusion.

NT2 cells were suspended in DMEM media (supplemented with 0.05 mg/ml gentamicin), and pipetted into the corona chamber at a concentration of 2×10^{6} cells/175 μl. The cells were then allowed to settle for 3 minutes by gravity to assure a uniform monolayer of cells in the chamber. The experiment in this section made use of the optimal conditions and parameters determined from the aforementioned sections in this chapter (combined corona polarity treatment in corona chamber with both electrodes connected to a ground source). In this experiment, NT2 cells were treated with six sequences of corona discharge. After each sequence (One sequence = 1 minute positive corona followed by 1 minute negative corona), a photograph was taken to observe the cell-cell contact properties (Figure 5.6). The particular sequence that matched the conditions for containing moderate sized cell islands consisting of tight intracellular contact was determined to be the optimal corona treatment duration.

As can be seen from Figure 5.6 below, the application of 3 cycles was optimal. Application of corona treatment for 2 minutes on each polarity was not enough time, because there was an insufficient amount of contact. As a result, there would not be enough hybrids generated during electrofusion. On the other hand, applying corona treatment for 4 minutes on each polarity generated too much intracellular contact and would not be good in virtue of obtaining hybrids on a 1:1 basis.

47
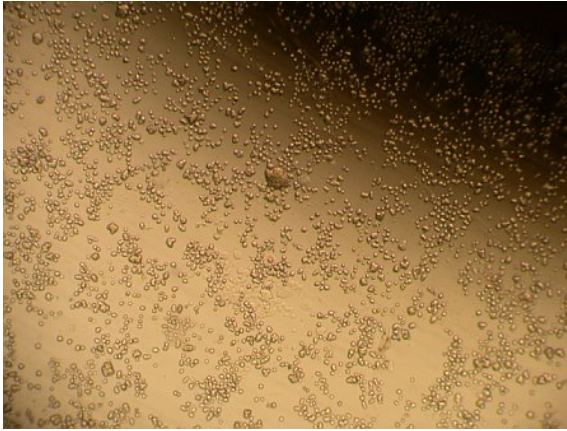
A) Corona Treatment of 2 Minutes Positive
Polarity Followed by 2 Minutes Negative Polarity
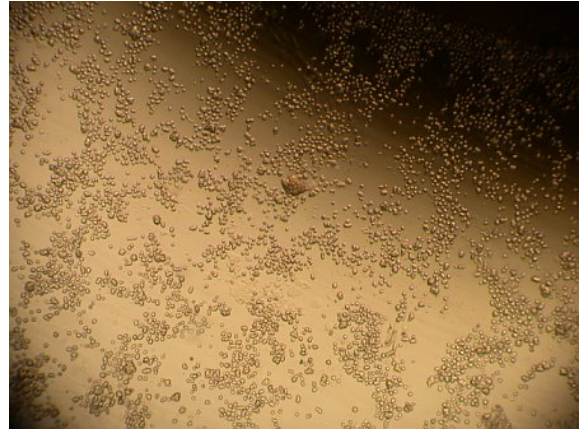
B) Corona Treatment of 3 Minutes Positive
Polarity Followed by 3 Minutes Negative Polarity
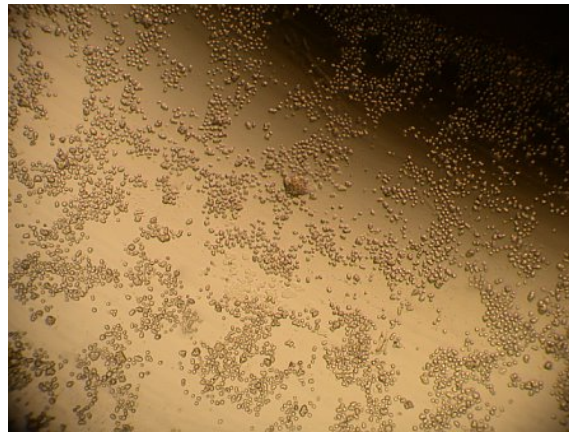
C) Corona Treatment of 4 Minutes Positive
Polarity Followed by 4 Minutes Negative Polarity

Figure 5.6 Corona Treatment of Circular Corona Chamber with Both Electrodes
Grounded for Determination of Optimal Corona Treatment Duration (40X)

5.6 Fusion Experiments with Circular Corona Chamber Containing Electrodes

The fusion experiments conducted over the course of this investigation were
designed to produce NT2 monohybrids at a 1:1 ratio by using optimal parameters
determined from the aforementioned experiments.

NT2 cells were independently stained with 50 μl CMFDA (green fluorescent dye) and 75 μl CMTMR (red fluorescent dye) and prepared for use in the chamber as described in Chapter 4. Aliquots of NT2 cells consisting of 2×10^6 cells/175 μl DMEM (supplemented with 0.05 mg/ml gentamicin) were pipetted into the corona chamber (circular configuration containing circular stainless steel electrodes). The cells were then allowed to settle for 3 minutes to insure that approximately 95% of the cells have confined themselves to the chamber floor. This resulted in the formation of a uniform cellular monolayer. Next, the cells were subjected to 3 minutes of positive corona followed by 3 minutes of negative corona. After corona application, the electrodes were connected to the DC electroporator in preparation for electrofusion. The cells were then pulsed with direct current pulses. Five separate electrofusion trials were performed. For the five trials, the cells were pulsed with six or more pulses of 750 Volts/cm, 1000 Volts/cm, 2000 Volts/cm, 2500 Volts/cm and 3000 Volts/cm (all using pulse durations of 100 μsec). In each of the five trials, little (< 1%) to no cell fusion was obtained, and cell viability ranged from little to no cell damage (750 – 2000 Volts/cm) to extreme cellular damage (3000 Volts/cm). The aforementioned trials were then repeated for 2 minutes of positive corona followed by 2 minutes of negative corona, and 4 minutes of positive corona followed by 4 minutes of negative corona respectively. These additional trials yielded very similar results to the first set of experiments involving 3 minutes of positive corona followed by 3 minutes of negative corona. The data recorded for the set of fusion experiments conducted in this section is shown in Table 5.1.

Table 5.1 Determination of Optimal Fusion Parameters for Circular Corona Chamber
with Circular Electrodes

| | | Voltage (V/cm) | | | | |
		750	1000	2000	2500	3000
# of sequences (1 min. (+), followed by 1 min. (-) and vice versa	2	Hardly any contact, No fusion, No cell damage	Hardly any contact, No fusion, No cell damage	Hardly any contact, No fusion, No cell damage	Hardly any contact, No fusion, A little cellular damage	Hardly any contact, No fusion, Severe cell damage
	3	Good cell contact (little islands), No fusion, No cell damage	Good cell contact (little islands), No fusion, No cell damage	Good cell contact (little islands), No fusion, No cell damage	Good cell contact (little islands), A little bit of fusion (barely any ~<1%), Very little cell damage	Very good cellular contact and overlap(more than before), A little bit of fusion (barely any ~<1%), Severe cell damage
	4	Very good cell contact (large islands), No fusion, No cell damage	Very good cell contact (large islands), No fusion, No cell damage	Very good contact (large islands), No fusion, No cell damage	Very good contact (large islands), A little bit of fusion (barely any ~<1%), Very little cell damage	Very good contact (large islands), A little bit of fusion (barely any ~<1%), Severe cell damage

Figure 5.7 depicts the NT2 cells stained with CMFDA and CMTMR in the corona chamber prior to electrofusion, as well as the pre-labeled NT2 cells, with a lack of yellow NT2 monohybrids, in the corona chamber following 6 pulses of 2500 Volts/cm DC at 100 μsec respectively. Figure 5.8 is a higher magnification (100X) of the NT2 cells following electrofusion.
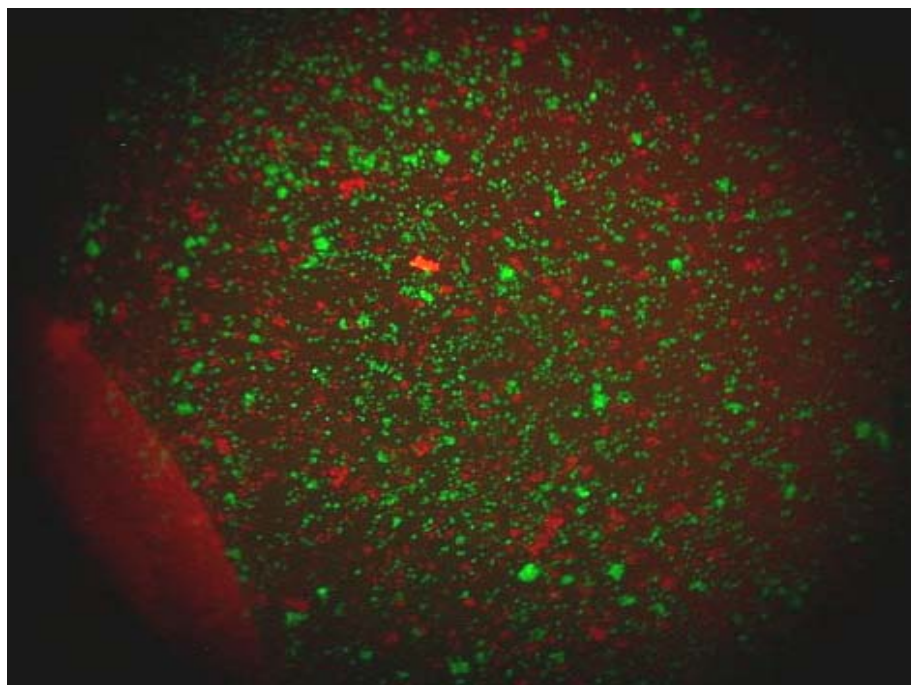
A) After 3 Minutes of Positive Polarity Followed by 3 Minutes of Negative
Polarity, but Prior to DC Electrofusion (40X)
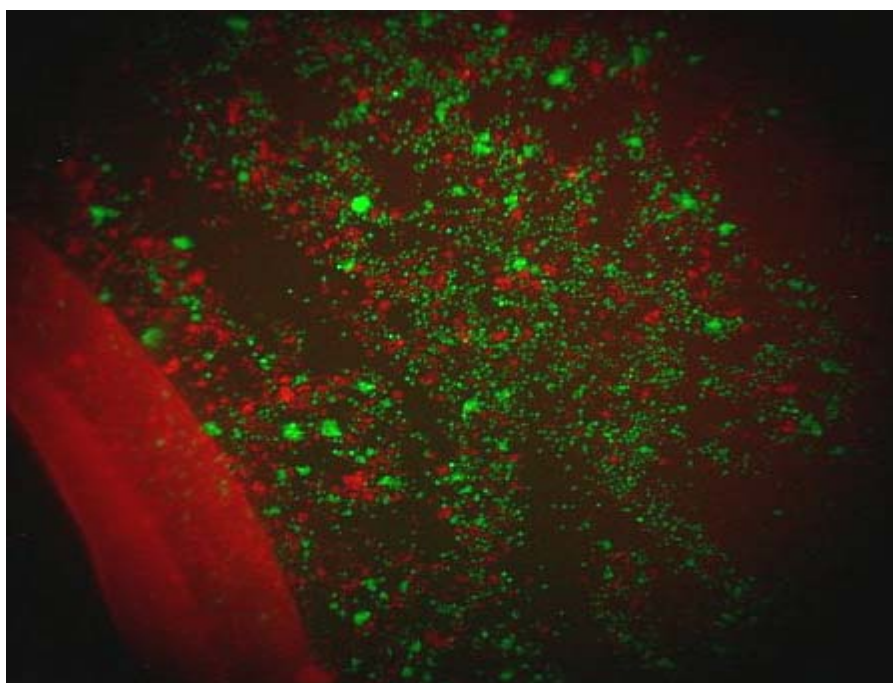
B) Following 6 DC pulses of 2500 Volts/cm at 100 μsec intervals (40X)

Figure 5.7 NT2 Cells Before and After DC Treatment Using Optimal Corona Exposure
Parameters in a Circular Corona Chamber Configuration (40X)
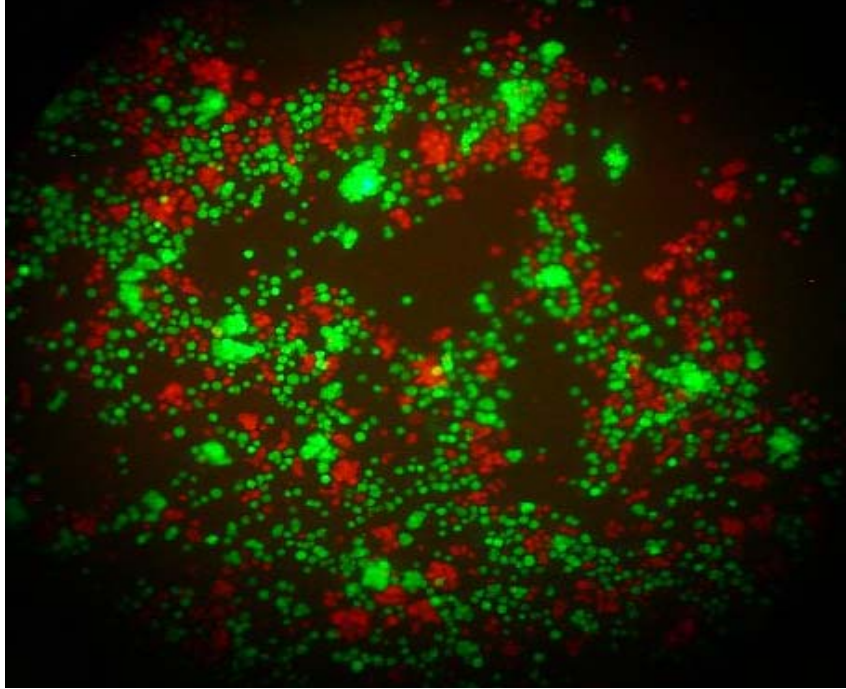
NT2 cells following 6 DC pulses of 2500 Volts/cm at 100 µsec intervals
(100X)

Figure 5.8 NT2 Cells Following DC Pulse (100X)

The inability to fuse may have been a direct result of obtaining a non-uniform DC field, due to the circular geometry of the chamber. For this reason, one would suspect that it might be beneficial to investigate alternative corona chamber geometries, such as a square. Despite this unexpected shortcoming, an interesting phenomenon was observed during electrofusion that provided additional promise for the investigation. During the electrofusion experiments, mechanical perturbations were delivered to the chamber, which subsequently caused the cells to release from the chamber floor and gravitate towards one another in free suspension. The reason for this sudden intracellular attraction has not yet been explained, but this unexpected phenomenon prompted an investigation for the effect of mechanical disturbance on the cells later in the study.

5.7 Results with Different Geometric Corona Chamber Configurations

The following set of experiments was designed to investigate the effect of mechanical perturbation on NT2 cells following corona application in alternative corona chamber geometries.

5.7.1   Investigation of Corona Treatment

5.7.1.1 Square Chamber without Electrodes

NT2 cells were harvested, counted and delivered at a concentration of 2×10^6 cells/175 μl DMEM (supplemented with 0.05 mg/ml gentamicin) into the square corona chamber (cells were pipetted into only one of the squares) illustrated by Figure 4.11. Unlike the previous corona chamber investigated, this chamber did not contain electrodes. The cells were allowed to settle for 3 minutes and were then exposed to 3 minutes of positive corona discharge followed by 3 minutes of negative corona discharge. As expected, results revealed little to no cellular movement, being that there was no connection to a ground source. However, after applying mechanical disturbance to the chamber, the cells exhibited a fascinating behavior; the cells released from the chamber floor and gravitated towards the periphery (chamber walls) in every direction, therefore leaving a void space in the middle of the chamber. Figure 5.9 below illustrates the cellular peripheralization that resulted from mechanical disturbance of the square chamber.
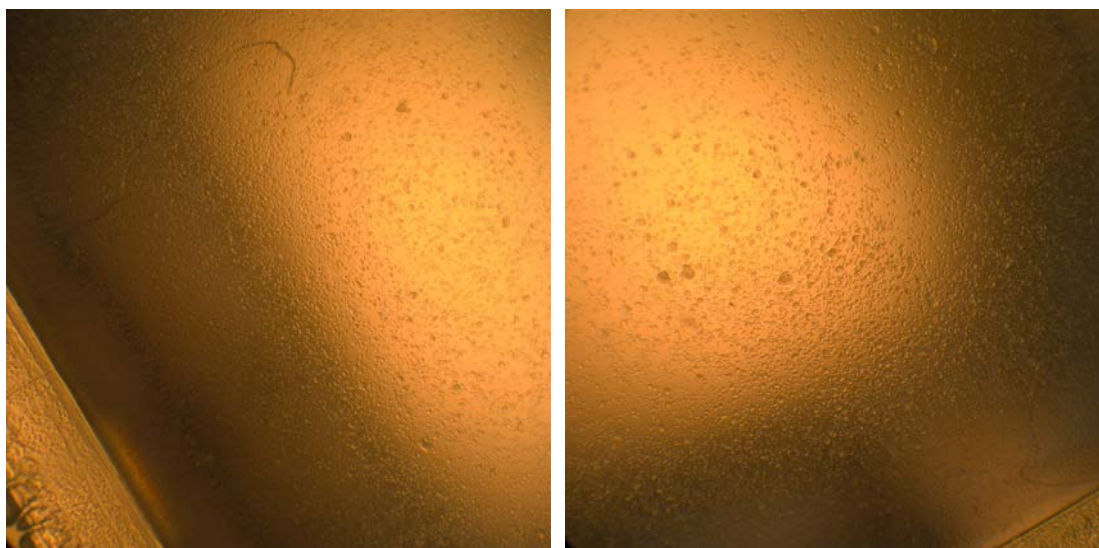
A) Left Wall of Square Chamber Following Corona Treatment and Prior to Mechanical Perturbation

B) Bottom Wall of Square Chamber Following Corona Treatment and Prior to Mechanical Perturbation
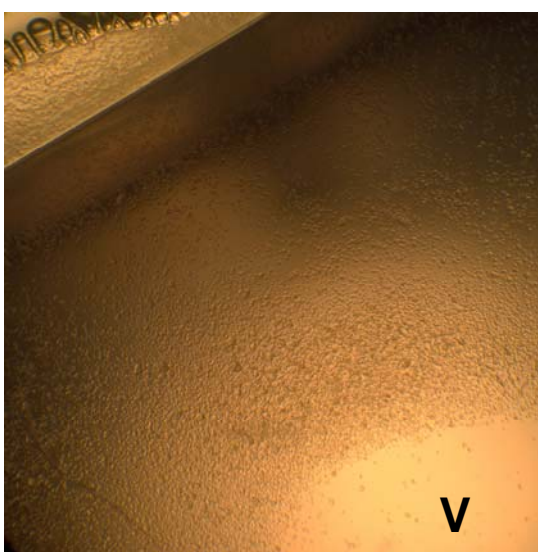
C) Void Space (V) Created in the Middle of the Square Chamber From Cellular Peripheralization Following Mechanical Disturbance

Figure 5.9 Effect of Mechanical Disturbance on NT2 Cells After Corona Treatment in a Square Corona Chamber

This method of mechanical perturbation following corona treatment, while difficult to quantify and control, resulted with extremely tight intracellular contact. After

achieving tight intracellular contact in a square corona chamber, it was plausible to obtain NT2 monohybrids in the square corona chamber by utilizing the electrofusion techniques discussed in the preceding section. Unlike the circular geometry investigated in the previous experiments, the square chamber was able to achieve a uniform DC field throughout the chamber while undergoing electrofusion. Thus, using a square configuration for the corona chamber provided a great chance for obtaining cell fusion.

5.7.1.2 Circular Chamber without Electrodes

The effect of mechanical disturbance on NT2 cells in a circular corona chamber (without electrodes) was examined in order to determine if chamber geometry had any effect on cell movement when experiencing mechanical disturbance.

NT2 cells were harvested, counted and delivered into the circular corona chamber illustrated in Figure 4.10 at a concentration of 2×10^6 cells/175 μl DMEM (supplemented with 0.05 mg/ml gentamicin). The cells were then allowed 3 minutes of settling time prior to corona treatment. Once again, the cells were treated with 3 minutes of positive corona followed by 3 minutes of negative corona and the chamber was subsequently disturbed. Results revealed that the cells exhibited the same movement as observed in the square corona chamber. Thus, it was concluded that the chamber geometry had no effect on cell movement following mechanical disturbance. Instead, cellular peripheralization was attributed largely to charge buildup on the chamber wall (there were no electrodes present to funnel the charge to a ground source), which subsequently attracted the positively/negatively charged cells. Figure 5.10 illustrates the cellular peripheralization from mechanical disturbance of the circular chamber.

55

| A) Middle of Circular Corona Chamber Following Corona Treatment and Prior to Mechanical Perturbation | C) Void Space (V) Created in the Middle of the Circular Chamber from Cellular Peripheralization Following Mechanical Disturbance |

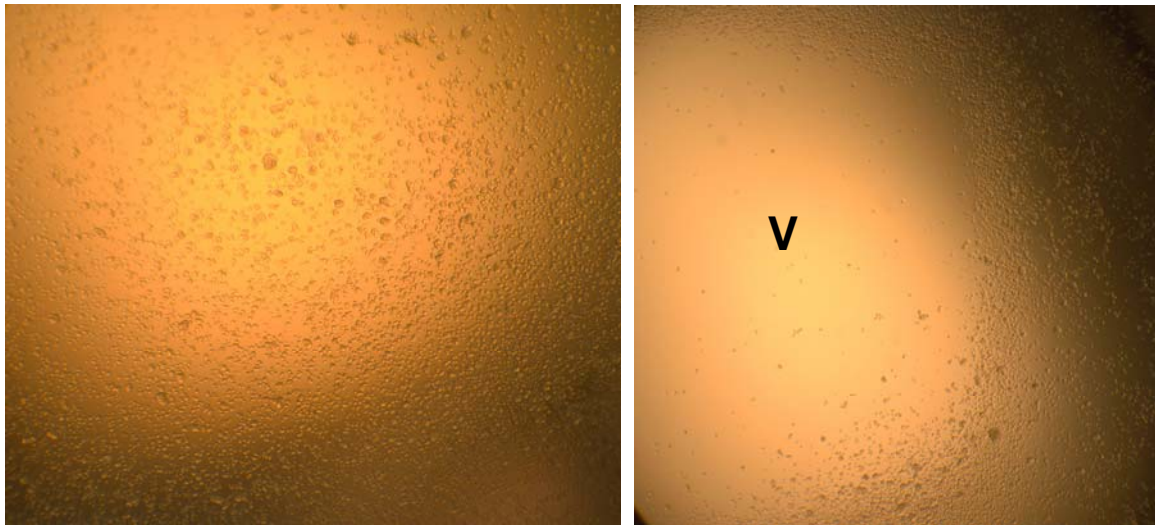Figure 5.10 Effect of Mechanical Disturbance on NT2 Cells After Corona Treatment in a Circular Corona Chamber

5.7.1.3 Square Chamber with Square Electrodes

Since it had been demonstrated from the previous section that it was possible to obtain tight intracellular contact in a square chamber using corona irradiation and subsequent mechanical perturbation, the next step was to attempt to control the level of cell-cell contact by adding electrodes to the current methodology. The reason for the addition of grounded electrodes to the existing chamber was that it was considered more plausible with the electrode technology to successfully quantify or approximate the ratio of fusion that resulted from subsequent electrofusion.

NT2 cells were prepared in accordance with the protocol discussed in Chapter 4 and pipetted into the square corona chamber (only one of the squares) containing two stainless steel square electrodes (Figure 4.12) at a concentration of 2×10^6 cells/175 µl

DMEM (supplemented with 0.05 mg/ml gentamicin). Once, the cells were allowed 3 minutes of settling time, they were treated with alternating positive and negative corona charges by the same protocol as discussed in Chapter 5, Section 3. Six cycles were completed, with each cycle representing an additional 1 minute of positive corona followed by 1 minute of negative corona. Corona application was briefly suspended in between each cycle to allow for photographs to be taken to record cell movement. This procedure was repeated for evidence of reproducibility. The first set of results (Figure 5.11 below) revealed a very positive outcome: the cells gradually formed tight cell islands in the annular space between the two electrodes before eventually migrating to the chamber walls in the form of tight intracellular aggregates. Unfortunately, these results could not be reproduced in subsequent trials, and therefore, the square chamber with electrodes must be further optimized and investigated before using the chamber to acquire hybrid cells during subsequent electrofusion. The reason for why the square corona chamber with electrodes was not able to achieve reproducible results has not yet been determined. Although, as a result, there is room for future investigations involving the optimization of the square chamber with electrodes so that it can be used as a novel method to achieve desired cell-cell contact and to quantify fusion ratios during subsequent electrofusion.

A) Before Corona Application

B) After 1 Cycle of 1 Minute Positive Corona
Followed by 1 Minute Negative Corona

C) After 2 Cycles of 1 Minute Positive Corona
Followed by 1 Minute Negative Corona

D) After 3 Cycles of 1 Minute Positive Corona
Followed by 1 Minute Negative Corona

Figure 5.11 Successful Cell Contact Using Optimal Corona Parameters in a Grounded
Square Chamber

E) After 4 Cycles of 1 Minute Positive Corona
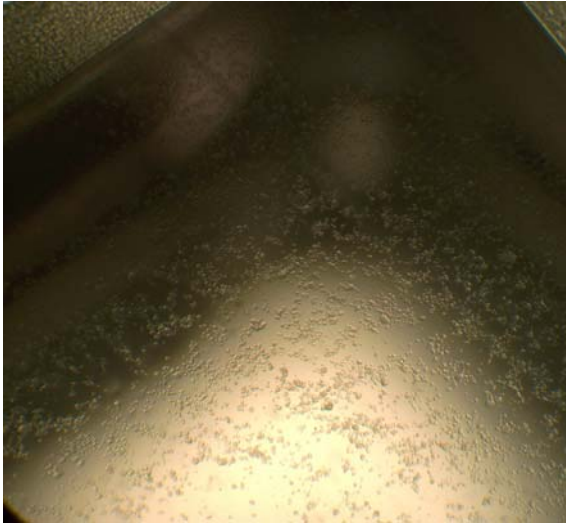Followed by 1 Minute Negative Corona

F) After 5 Cycles of 1 Minute Positive Corona
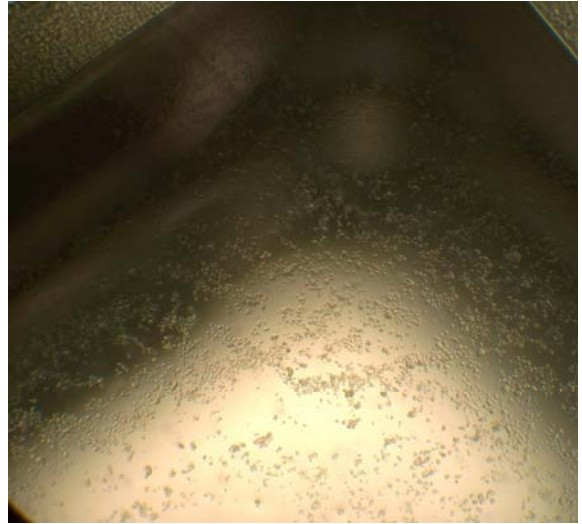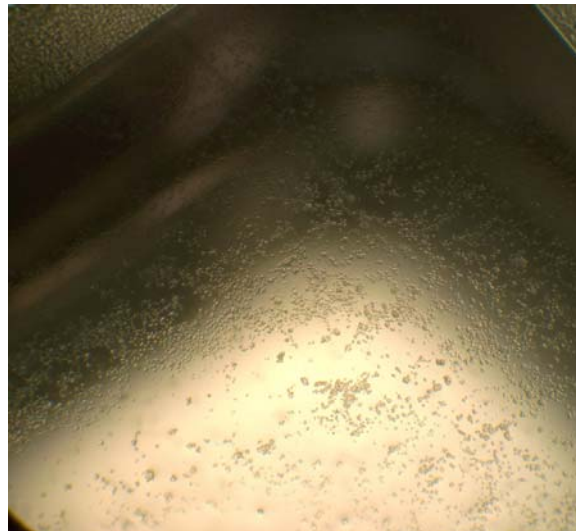Followed by 1 Minute Negative Corona

G) After 6 Cycles of 1 Minute Positive Corona
Followed by 1 Minute Negative Corona

Figure 5.11 Continued

5.7.2 Fusion Analysis

The square corona chamber (Figure 4.11) discussed in Section 5.7.1.1 was investigated for the purpose of obtaining NT2 monohybrids. The idea was to use a combination treatment involving corona application (3 minutes of positive corona followed by 3 minutes of negative corona (or in reverse)) and subsequent mechanical perturbation followed by exposure with fusogenic DC pulses in order to obtain hybridization. As discussed in the previous text, the aforementioned combination treatment of corona application and mechanical disturbance resulted in tight intracellular contact. Tight intracellular contact in addition to the application of a uniform DC field (due to the square geometry of the chamber) proved to be the right combination for the formation of NT2 monohybrids.

Two cultures of NT2 cells were prepared (grown, stained, harvested, and counted) in accordance with the protocols discussed in Chapter 4. The two cultures were pre-labeled with CMFDA and CMTMR respectively and pipetted into the square corona chamber (only one of the squares in Figure 4.11) at a total concentration of 2×10^6 cells/175 µl DMEM (supplemented with 0.05 mg/ml gentamicin) (individual concentration of 1×10^6 cells/87.5 µl DMEM each). The pre-labeled NT2 cells (red and green stained) were then allowed 3 minutes of settling time to insure that approximately 95% of the cells had gravitated to the chamber floor. Once again, the NT2 cells were treated with 3 minutes of positive corona followed by 3 minutes of negative corona and the chamber was subsequently disturbed. After the cells had achieved tight intracellular contact from cellular peripheralization, they were exposed to 8 fusogenic DC pulses of 2500 Volts/cm at 100 µsec intervals. The fusogenic pulses were applied using the hand

held electrode and ECM 800 pulse generator shown in Figure 4.14. The electrode was placed into the chamber containing the pre-labeled green and red NT2 cells so that the electrodes were in direct contact with the chamber floor prior to DC delivery. Then, the fusion parameters were entered into the ECM 800 and direct current was subsequently passed through the suspension containing the cells. The fusion parameters used for this investigation were selected because they were determined (when comparing with other parameters (Table 5.1)) from the experiment in Section 5.6 as being most optimal for cellular hybridization and cell viability.

As can be seen from Figure 5.12 (below), the pre-labeled NT2 cells accumulated between the MPG's electrodes and fused to form NT2 monohybrids. The NT2 monohybrids (Figure 5.12) were distinguished from the un-fused green or red cells by their characteristic yellowish/orange color, irregular oblong shape or large size. The presence of these NT2 monohybrids further validated the theory that the cells did not fuse in the circular chamber because they did not experience a uniform DC field during electrofusion.
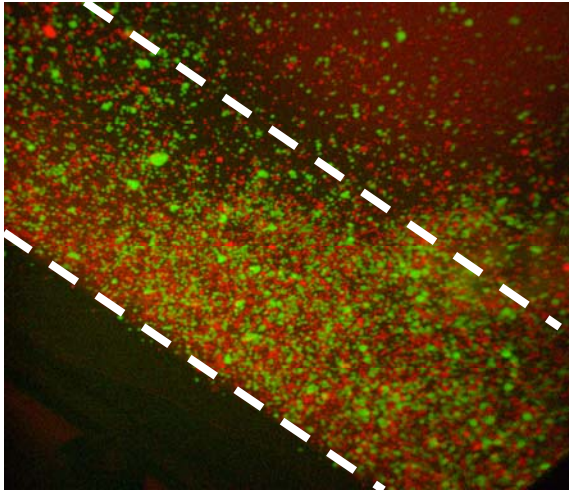
A) NT2 Cells Accumulating Between the Electrodes as Illustrated by the White Dotted Lines Following Electrofusion (40X)
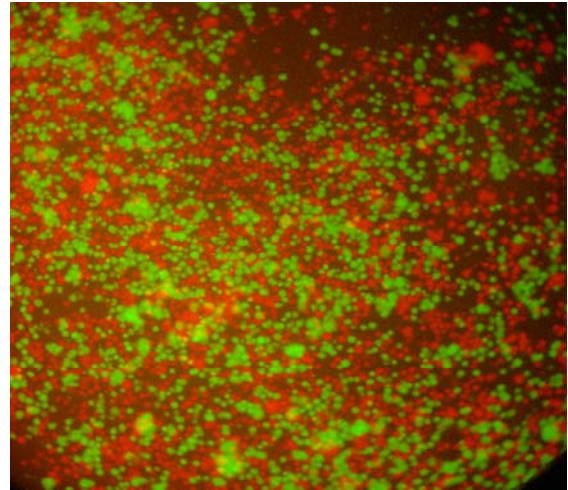
B) Pre-labeled NT2 Cells in the Square Corona Chamber Following DC application (100X)
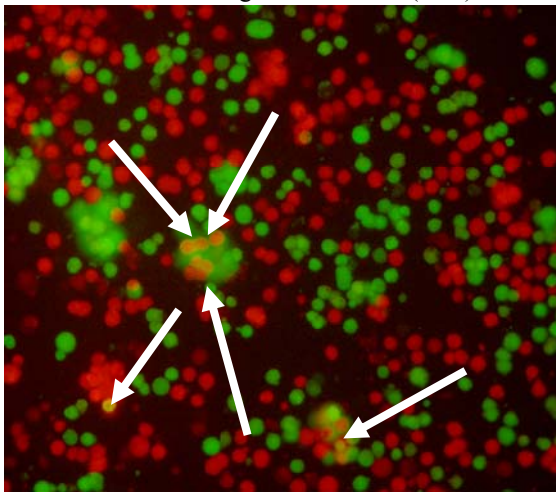
C) Fused NT2 Monohybrids in the Square Corona Chamber Following DC application (100X)
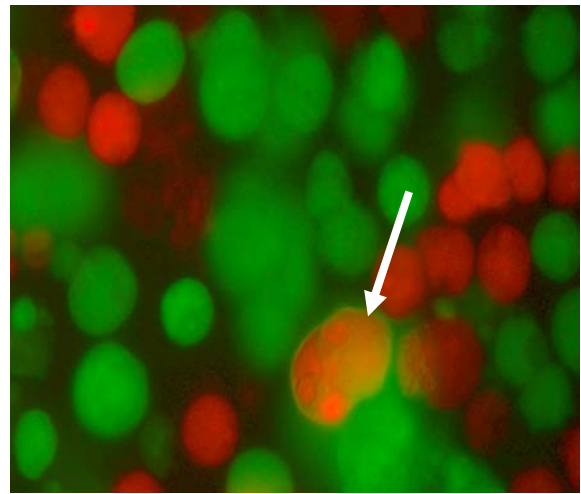
D) Fused NT2 Monohybrid in the Square Corona Chamber Following DC application (400X)

Figure 5.12 Resulting NT2 Monohybrids in a Square Corona Chamber After Successive Corona Treatment, Mechanical Disturbance, and 2500 DC Volts/cm
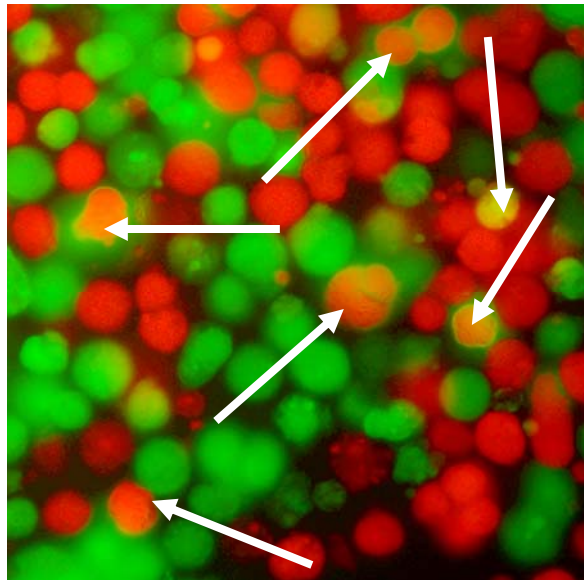
E) Fused NT2 Monohybrids Following DC
Application (400X)

Figure 5.12 Continued

In order to further verify that the NT2 Monohybrids were in fact fused cells and not the result of the two fluorescent dyes bleeding together, all of the NT2 cells in the corona chamber were transferred to a petri dish. As illustrated by the photographs taken in Figure 5.13, the yellowish/orange irregularly shaped cells observed in the corona chamber had the same appearance and morphology in the petri dish, which validated that these cells were in fact NT2 monohybrids.
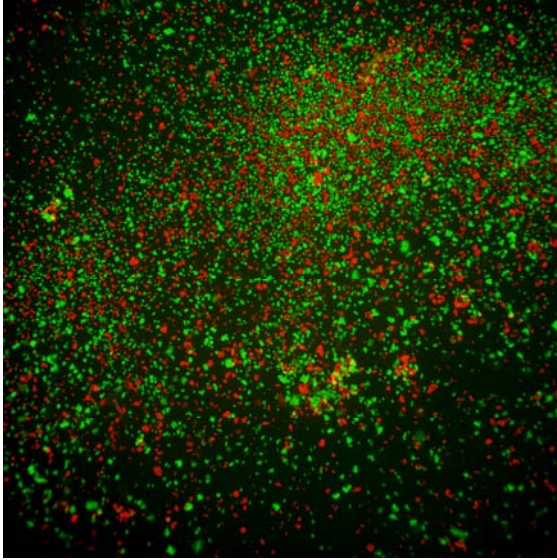
A) NT2 Cells in Petri Dish Following 2500 Volts/cm Fusogenic DC Pulse Application (40X)
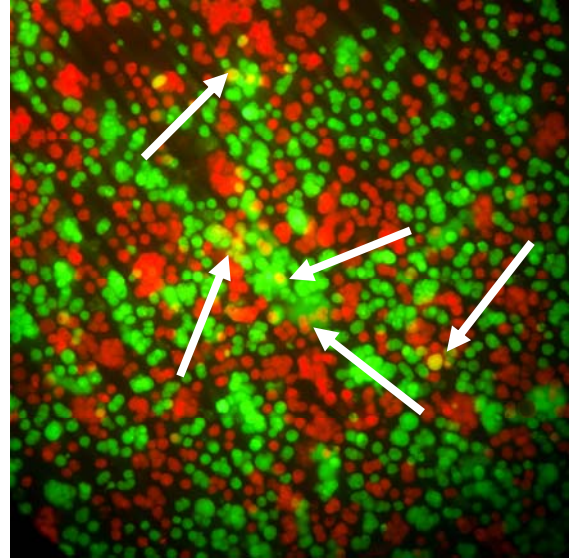
B) NT2 Cells in Petri Dish Following 2500 Volts/cm Fusogenic DC Pulse Application (Monohybrids indicated by white arrows) (100X)
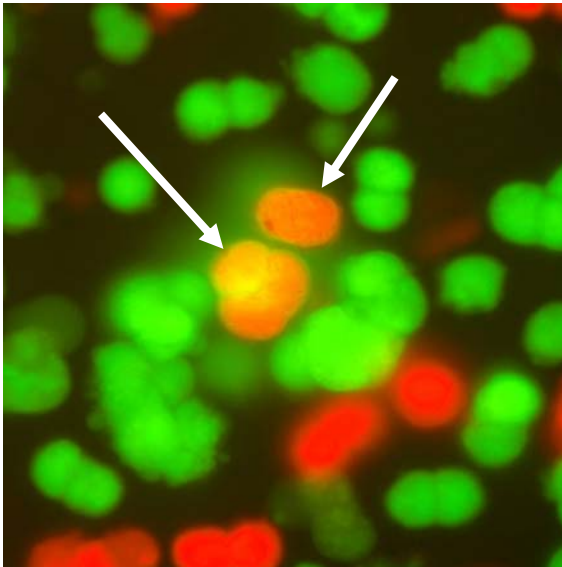
C) NT2 Monohybrids (Yellowish/Orange Cells Indicated by White Arrows) in Petri Dish Following Electrofusion (400X)
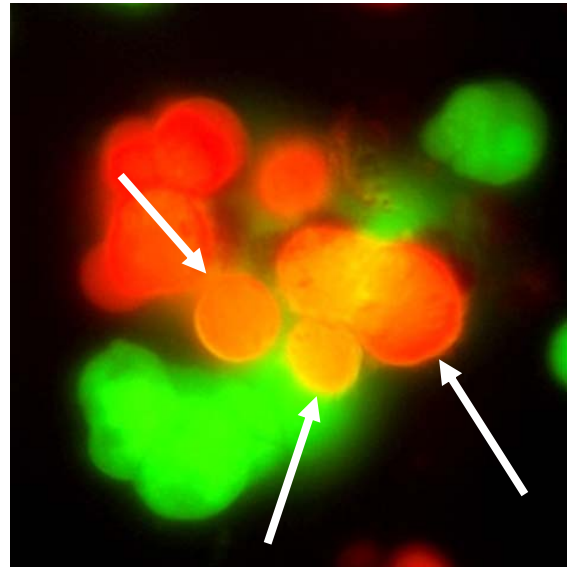
D) NT2 Monohybrids (Yellowish/Orange Cells Indicated by White Arrows) in Petri Dish Following Electrofusion (400X)

Figure 5.13 NT2 Monohybrids Once Transferred to a Petri Dish for Validation of Fusion

E) NT2 Monohybrid (Indicated by White Arrow)
(400X)

F) NT2 Monohybrids (Indicated by White Arrows)
(100X)

Figure 5.13 Continued

For comparison purposes, the experiment conducted in this section was repeated three more times using different fusion parameters each time: 15 pulses of 2500 Volts/cm, 8 pulses of 3000 Volts/cm and 15 pulses of 3000 Volts/cm. Table 5.2 below provides a summary of the results observed for cell viability and fusion in a square corona chamber when using the aforementioned electrofusion parameters and optimal cell contact techniques (3 or 4 cycles of alternating corona polarities followed by mechanical perturbation).

Table 5.2 Determination of Optimal Fusion Parameters for Square Chamber

		Voltage (V/cm)			
		2500		3000	
		8 pulses	15 pulses	8 pulses	15 pulses
# of cycles (1 min. (+), followed by 1 min. (-) and vice versa	3	~20% Fusion, Minimal Cell Damage	~40% Fusion, Large Cell Damage	~10% Fusion, Very Large Cell Damage	~5% Fusion, Severe Cell Damage

5.7.2.1 Verification of Hybridized Cell Viability

Following electrofusion, cell death was distinguished by the visual membrane disintegration or by stain leaking from the cytosol into the surrounding media. Visual evidence with microscopy revealed that the NT2 monohybrids generated during this investigation did not share any of the above characteristics for cell death. In order to obtain even further confirmation that these hybridized cells were viable, the cells were centrifuged, washed, re-suspended in supplemented DMEM (with 0.05 mg/ml gentamicin and 10% fetal bovine serum) and subsequently cultured for a period of 2-3 days. At the end of this period, the cells were visually inspected for mitotic activity. As a result, the NT2 cells continued to show the ability to differentiate and grow, therefore providing confirmation of the notion that these hybridized cells were viable.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In an attempt to investigate corona discharge and subsequent direct current electrofusion as a method for obtaining tight intracellular contact and cell-cell hybridization respectively, a set of optimal parameters or conditions were determined.

Early in the investigation, it was demonstrated that not only can cells be placed into a physiologic-based media during corona treatment, but their level of contact can be controlled if the cells are placed into a circular chamber containing electrodes that are connected to a ground source. Even though this condition would most likely be best for obtaining 1:1 based hybridization, the geometry of the chamber was not favorable for achieving cellular fusion. However, later in the study it was demonstrated that by mechanically disturbing the chamber following corona treatment, tight intracellular contact could be obtained in both a circular and square shaped chamber. This discovery provided promise for obtaining large quantities of hybridized cells because unlike the circular chamber, the square chamber turned out to be more favorable for generating hybridized cell constructs during electrofusion. The reason for this difference has not been determined yet. One reason may be that the circular geometry resulted in an uneven distribution of charge during electrofusion due to the presence of a non-uniform DC field, charge was evenly distributed over the entire chamber in the square geometry.

The square chamber used in the investigation produced outstanding cell-cell contact when using the combination treatment of corona discharge and mechanical perturbation discussed above. In addition, when the aggregated cells were treated with 8-15 fusogenic DC pulses of 2500 Volts/cm at 100 μsec intervals (following corona treatment and mechanical disturbance), the chamber provided fusion yields as high as 40%. Throughout the investigation, it was clear that in order to obtain a substantial amount of cell contact (in the form of monolayers) and subsequent fusion, the cells needed 3 minutes of settling time followed by corona treatment of at least 3 minutes of positive corona followed by 3 minutes of negative corona (or in reverse). Furthermore, unlike other electrofusion methodologies, it appears that corona discharge and subsequent DC application, as described in the last set of experiments, does not compromise the hybridized cells' viability, integrity. Future studies, however, are necessary to verify this observation.

6.2 Recommendations

An attempt was made during the investigation to obtain 1:1 based fusion by using a square corona chamber that contained grounded electrodes in order to better control the level of intracellular contact. Unfortunately, these trials did not return any favorable results. For an undetermined reason, the cells were simply not induced to gravitate towards each other while under corona discharge. It was unclear as to whether this was a result of the placement of the electrodes on the chamber, the geometry of the electrodes used, or the chamber itself. The investigation of a suitable method or device that will not only induce cell-cell contact in a controlled environment but induce cellular fusion on a

near 1:1 basis is perhaps the first step towards optimizing this technology. In addition, a presentation of the mechanism or characterization of the electric fields present in the chamber during corona discharge can help in further optimizing the equipment to provide better cell contact and higher fusion yields. The ultimate goal would be to apply this technology to different cell types in order to engineer novel heterohybridized cell constructs that exhibit combined characteristics not observed in either of the cells being fused. Cell constructs designed in this way could be utilized in a number of cell therapies, such as therapeutic cell transplantation.

REFERENCES

1. Li, L.H.; Hensen, M.L.; Zhao, Y.L.; Hui, S.W. Electrofusion Between Heterogeneous-Sized Mammalian Cells in a Pellet: Potential Applications in Drug Delivery and Hybridoma Formation. *Biophys. J.* 1996, 71, 479-486.

2. Mclenaghan, N.H.; Flatt, P.R. Engineering Cultured Insulin-Secreting Pancreatic B-Cell Lines. *J. Mol. Med.* 1999, 77, 235-243.

3. Ege, T.; Krondahl, U.; Ringertz, N.R. Introduction of Nuclei and Micronuclei into Cells and Enucleated Cytoplasms by Sendai Virus Induced Fusion. *Exp. Cell Res.* 1974, 88, 428-432.

4. Finaz, C.; Lefevre, A.; Teissie, J. Electrofusion. A New, Highly Efficient Technique for Generating Somatic Cell Hybrids. *Exp. Cell Res.* 1984, 150, 477-482.

5. Sukhorukov, V.L.; Resuss, R.; Endter, J.M.; Fehrmann, S.; KatsenGloba, A.; Gessner, P.; Steinbach, A.; Muller, K.J.; Karpas, A.; Zimmermann, U.; Zimmermann, H. A Biophysical Approach to the Optimization of Dendritic-Tumour Cell Electrofusion. *Biochem. Biophys. Res. Commun.* 2006, 346, 829-839.

6. Hui, S.W. The Application of Electroporation to Transfect Hematopoietic Cells and to Deliver Drugs and Vaccines Transcutaneously for Cancer Treatment. *Technol. Cancer Res. Treat.* 2002, 1, 373-384.

7. Valone, F.; Small, E.; MacKenzie, M.; Burch, P.; Lacy, M.; Peshwa, M.V.; Laus, R. Dendritic Cell-Based Treatment of Cancer: Closing in on a Cellular Therapy. *The Cancer Journal*, 2001,7, S53-S61.

8. Zimmermann, U; Vienken, J. Electric Field-Induced Cell-to-cell Fusion. *J Membrane Biol*. 1982, *67*, 165-182.

9. Halfer, Carlotta ; Petrella, Lucia. Cell Fusion Induced by Lysolecithin and Concanavalin A in *Drosophila Melanogaster* Somatic Cells Cultured in Vitro. *Experimental Cell Research.* 1976, *100,* 399-404.

10. Hayashi, T.; Tanaka, H.; Tanaka, J.; Wang, R. ; Averbook B.J.; Cohen, P.A.; Shu, S. Immunogenicity and Therapeutic Efficacy of Dendritic-Tumor Hybrid Cells Generated by Electrofusion. *Clin. Immunol.* 2002, 104, 14-20.

11. Cheong, S.C.; Blangenois, I.; Franssen, J.D.; Servais, C.; Phan, V.; Trakatelli, M.; Bruyns, C.; Vile, R.; Velu, T.; Brandenbruger, A. Generation of Cell Hybrids Via a Fusogenic Cell Line. *J. Gene Med.* 2006, 8, 919-928.

12. Jaroszeski, M.; Gilbert, R.; Perrott, R.; Heller, R. Enhanced Effects of Multiple Treatment Electrochemotherapy. *Melanoma Research* 1996, 6, 427-433.

13. Heller, R.; Jaroszeski, M.; Atkin, A.; Moradpour, D.; Gilbert, R.; Wands, J.; Nicolau, C. In Vivo Gene Electroninjection and Expression in Rat Liver. *Fed. Europ. Biochem. Soc.* 1996, 389, 225-228.

14. Bouchard, Claude; Teissie, Justin. Homokaryon production by Electrofusion: A Convenient Way to Produce a Large Number of Viable Mammalian Fused Cells. *Biochem. Biophys. Res. Commun.* 1983, 114, 663-669.

15. Ramos, C.; Teissie, J. Electrofusion: A Biophysical Modification of Cell Membrane and a Mechanism in Exocytosis. *Biochem.* 2000, 82, 511-518.

16. Schmitt, J.J.; Zimmermann, U. Enhanced Hybridoma Production by Electrofusion in Strongly Hypo-Osmolar Solutions. *Biochem. Biophys. Acta.* 1989, 983, 42-50.

17. Vienken, J.; Zimmermann, U. An Improved Electrofusion Technique for Production of Mouse Hybridoma Cells. *FEBS Lett.* 1985, 182, 278-280.

18. Teissie, J.; Rols, M.P. Fusion of Mammalian Cells in Culture is Obtained by Creating the Contact Between Cells After their Electropermeabilization. *Biochem. Biophys. Res. Commun.* 1986, 140, 258-266.

19. Sowers, A.E. Evidence that Electrofusion Yield is Controlled by Biologically Relevant Membrane Factors. *Biochem. Biophys. Acta.* 1989, 985, 334-338.

20. Sowers, A.E. Electrofusion of Dissimilar Membrane Fusion Partners Depends on Additive Contributions from Each of the Two Different Membranes. *Biochem. Bioiphys. Acta.* 1989, 985, 339-342.

21. Heller, L.C.; Pottinger, C.; Jaroszeski, M.J.; Gilber, R.; Heller, R. In Vivo Electroporation of Plasmids Encoding GMCSF and Interleukin-2 into Existing B16 Melanomas combined with Electrochemotherapy Induces Long Term Antitumor Immunity. *Melanoma Research* 2000, 577-583.

22. Somiari, S.; Glasspool-Malone, J.; Drabick, J.J.; Gilbert, R.A.; Heller, R.; Jaroszeski, M.J.; Malone, R.W. Theory and In Vivo Application of Electroporative Gene Delivery. *Mol. Therapy* 2000, 2, 178-187.

23. Heller; Gilbert; Jaroszeski. Clinical Applications of Electrochemotherapy. *Advanced Drug Delivery Reviews* 1999, 35, 119-129.

24. Jaroszeski, M.J.; Gilbert, R.; Nicolau, C.; Heller, R. In Vivo Gene Delivery by Electroporation. *Advanced Drug Delivery Reviews* 1999, 35, 131-137.

25. Hyacinthe; Jaroszeski; Dang; Coppola; Karl; Gilbert; Heller. Electrically Enhanced Drug Delivery for the Treatment of Soft Tissue Scarcoma. *Cancer* 1999, 85, 409-417.

26. Mir, L.M.; Glass, L.F.; Sersa, G.; Teissie, J.; Domenge, C.; Miklavcic, D.; Jaroszeski, M.J.; Orlowski, S.; Reintgen, D.S.; Rudolf, Z. Effective Treatment of Cutaneous and Subcutaneous Malignant Tumors By Electrochemotherapy. *Br. J. of Cancer* 1998, 32, 53-64.

27. Heller, R.; Jaroszeski, M.J.; Reintgen, D.; Puleo, C.; DeConti, R.; Gilbert, R.; Glass, L.F. Treatment of Cutaneous and Subcutaneous Tumors with Electrochemotherapy using Intralesional Bleomycin. *Cancer* 1998, 83, 148-157.

28. Lewis, F.; Glass, M.; Jaroszeski, M.J.; Gilbert, R.; Reintgen, D.S.; Heller, R. Intralesional Bleomycin-Mediated Electrochemotherapy in 20 Patients with Basal Cell Carcinoma. *J. Am. Acad. Derm.* 1997, 37, 596-599.

29. Jaroszeski, M.J.; Gilbert, R.; Heller, R. Successful Treatment of Hepatomas with Electrochemotherapy in a Rat Model. *Biomchem. Biophys. Acta.* 1997, 1334, 15-18.

30. Abidor, I.G.; Sowers, A.E. Kinetics and Mechanism of Cell Membrane Electrofusion. *Biophy. J.* 1992, 61, 1557-1569.

31. Zimmermann, U; Vienken, J. Electric Field-Induced Cell-to-cell Fusion. *J Membrane Biol*. 1982, *67*, 165-182.

32. Ohkohchi, N.; Itagaki, H.; Doi, H.; Taguchi, Y.; Satomi, S.; Satoh, S. New Technique for Producing Hybridoma by Using Laser Radiation. *Lasers Surg. Med.* 2000, 27, 262-268.

33. Itagaki, H.; Doi, H.; Ohkohchi, N.; Satomi, S. Development of New Cell Fusion Technique by Laser Device and Application to Bio-medical Field. *Nippon Rinsho.* 1997, 55, 2780-2787.

34. Finaz, C.; Lefevre, A.; Teissie, J. Electrofusion: A New Highly Efficient Technique for Generating Somatic Cell Hybrids. *Exp Cell Res*, 1984, *150,* 477-482.

35. Bouchard, Claude; Teissie, Justin. Homokaryon Production by Electrofusion: A Convenient Way to Produce Large Number of Viable Mammalian Fused Cells. *Biochemical and Biophysical Research Communications*, 1983, *114*, 663-339.

36. Corona discharge <http://en.wikipedia.org/wiki/Corona_discharge>.

37. Plasma (physics) http://en.wikipedia.org/wiki/Plasma_%28physics%29.

38. Boating-Lightning Protection http://www.cdc.gov/nasd/docs/d000001-d000100/d000007/d000007.html.

39. http://blazelabs.com/1-intro.asp.

40. Kwark, C.; Lee, C.W. Experimental Study of a Real-time Corona Discharge Imaging System as a Future Biomedical Imaging Device. *Med. Biol. Eng. Comput.* 1994, 32, 283-288.

41. Betchen, S.A.; Kaplitt, M. Future and Current Surgical Therapies in Parkinson's Disease. *Lippincott Williams and Wilins, Inc.* 2003, 16, 487-493.

42. Willing, A.E.; Cameron, D.F.; Sanberg, P.R. Sertoli Cell Transplants: Their Use in the Treatment of Neurodegenerative Disease. *Molecular Medicine Today* 1998, 4, 471-477.

43. Willing, A.E.; Orthber, A.I.; Saporta, S.; Anton, A.; Sinibaldi, S.; Poulos, S.G.; Cameron, D.F.; Freeman, T.B.; Sanberg, P.R. Sertoli Cells Enhance the Survival of Co-transplanted Dopamine Neurons. *Brain Research* 1999, 822, 246-250.

44. Emerich, D.F.; Hemendinger, R.; Halberstadt, C.R. The Testicular-derived Sertoli Cell: Cellular Immunoscience to Enable Transplantation. *Cell Transplant* 2003, 12, 335-349.

45. Halberstadt, C.; Emerich, D.F.; Gores, P. Use of Sertoli Cell Transplants to Provide Local Immunoprotection for Tissue Grafts. *Expert Opin. Biol. Ther.* 2004, 4, 813-825.

46. http://www.nexcelom.com/Prod_Cellometer_Disposable.asp.

47.   Jaroszeski, M. J.; Gilbert, R.; Heller, R. Detection and Quantitation of Cell-Cell Electrofusion Products by Flow Cytometry. *Analytical Biochemistry,* 1994*, 216,* 271-275.

48.   Jaroszeski, Mark J.; Heller, Richard. Flow Cytometry Protocols. *Methods in Molecular Biology.* 1998, Humana Press, *91*, 149-157.

APPENDICES

Appendix A: Data for Calibration of the Corona Generator

Table A.1 Data for Calibration of the Corona Generator

Experimental Conditions Temperature Range: 22.7ºC – 23.0ºC Relative Humidity: 55.4 % - 57.4 % Height of Needles: 6.81mm		
Applied Voltage (kV)	Charge on Collector Plate For Positive Corona(μA)	Charge on Collector Plate For Negative Corona(μA)
3.0	0.00	- 0.01
3.5	0.00	- 0.75
4.0	0.02	- 2.45
4.5	0.02	- 5.26
5.0	3.59	- 10.32
5.5	7.35	- 16.04
6.0	11.86	- 23.81
6.5	16.89	- 32.98
7.0	25.28	- 44.20
7.5	36.70	- 56.40
8.0	59.00	- 70.30
8.5	-	- 86.80
9.0	-	- 104.50
9.5	-	- 109.50