

Automated Image-Based Recognition  
and Targeted Laser Transfection Techniques  
for Drug Development and Stem Cell Research



**Clarence Han-Wei Yapp**

Department of Engineering Science

University of Oxford

*A thesis submitted for the degree of*

*Doctor of Philosophy*

Trinity Term, 2011

# Automated Image-Based Recognition and Targeted Laser Transfection Techniques for Drug Development and Stem Cell Research

CLARENCE HAN-WEI YAPP, WOLFSON COLLEGE  
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY, TRINITY TERM 2011

## Abstract

Advances in several areas of scientific research is currently hampered by the slow progress in developing a non-viral, high precision technique capable of safely and efficiently injecting targeted single cells with impermeable molecules. To date, one of the most promising techniques employs the laser to temporarily create a pore in the cell membrane to allow the entry of exogenous molecules. This technique has potentially wide applications.

In this thesis, I utilised the precision of laser transfection, also known as optoporation, to deliver two histone demethylase inhibitors (8-hydroxyquinoline and FMF1293) of the JmjC-domain protein JMJD3 into vital cells. The enzyme, JMJD3, demethylates histone H3 lysine K27, the methylation state of which has been shown in previous studies to regulate genes in such a way as to play a key role in the formation of tumours and even maintenance of stem cell pluripotency. The research here shows proof of principle that optoporation can be employed to quickly screen and test the efficacy of novel drugs by delivering them into cells at significantly low concentrations while still maintaining inhibition activity. I also used optoporation to deliver relatively large proteins such as bovine serum albumin (BSA), phalloidin and novel synthetic antibodies into living cells without fixatives. This offers the possibility of using reporter systems to monitor living cells over time. Finally, an attempt was made to generate iPS colonies by optoporating plasmid DNA into somatic cells, however, I find that this technique was unable to efficiently transfect and reprogram primary cells.

Two automated image-based systems that can be integrated into existing microscopes are presented here. First, an image processing algorithm that can quickly identify stem cell colonies non-invasively was implemented. When tested, the algorithm's resulting specificity was excellent (95 – 98.5%). Second, because optoporation is a manual and time consuming procedure, an algorithm to automate optoporation by using image processing to locate the position of cells was developed. To my knowledge, this is the first publication of a system which automates optoporation of human fibroblasts in this way.



## Acknowledgements

This thesis would not have been possible without the kind assistance of numerous people who, in one way or another, have contributed to its preparation and completion. I would like to take this opportunity to acknowledge those who deserve special mention.

First and foremost, I owe my deepest gratitude to Professor Udo Oppermann for his steadfast support and encouragement especially during my many difficult moments. His motivation and guidance inspired me to finish my DPhil. studies with renewed focus and determination. His mentoring has indeed enriched my experience as a student, researcher and scientist.

I am also equally indebted and most grateful to my joint-supervisor, Professor Alison Noble, for her role in providing technical expertise in image processing, and for patiently proof reading my drafts. Her keen insight, constructive comments and words of wisdom have made the writing process less daunting.

I am honoured to have worked alongside many senior members of the Botnar Research Centre as well as scientists/researchers from other departments. Many thanks to Philippa Hulley, James Dunford, Stan Ng, Peter Cain, Martin Philpott, Cameron Brown, Phil Tait, Paul Raju, Nuala Trainor, Tim Davies, Frances Brook and Lee Carpenter for their invaluable advice, research assistance and provision of materials.

In particular, my sincere gratitude to Harvey Che for his enormous help, insight and invaluable experimental advice involving the inhibition of histone demethylases, and to Michelle Daniel who worked tirelessly purifying the actin antibody. Likewise my labmates at the Botnar

especially Sharif Hala, Anneke Kramm, Minsi Chen and Alice Grabbe for their advice and moral support. I would also like to make a special reference to James Fisk, Roger Lewis and Selina Blades and her administrative team at the Institute of Biomedical Engineering for their support.

Many thanks to my industrial collaborators Graham Brown and Paul Sheppard from Carl Zeiss Ltd for their assistance and dedication in co-writing the automation program; Mark Day from Coherent Inc. for his technical expertise with the Ti:Sapphire laser; and Laurens Kruidenier and his team from GlaxoSmithKline plc for their input on the BacMam transfection system.

I would like to express my utmost gratitude to the Man Group plc for their kind financial support for the last three years. Their generosity has allowed the pursuit of scientific knowledge which has the potential to advance the development of novel healthcare solutions.

In addition, I would like to thank my parents, Yg. Bhg. Dato' Ralph and Datin Tracy Yapp, and my sister Tiffany, for their loving support, unwavering belief in my capability and never letting me give up. Last but not least, I would like to thank all my relatives and friends for their prayers and warm wishes.

# List of Abbreviations

| <b><u>Abbreviation</u></b> | <b><u>Definition</u></b>         |
|----------------------------|----------------------------------|
| 3D                         | Three dimensional                |
| $^3\text{H}$               | Tritium                          |
| 4D                         | Four dimensional                 |
| 5'AZA                      | 5'azacytidine                    |
| 8HQ                        | 8 hydroxyquinoline               |
| AOM                        | Acoustic optical modulator       |
| AP                         | Alkaline phosphatase             |
| bFGF                       | Basic fibroblast growth factor   |
| BSA                        | Bovine serum albumin             |
| $\text{Ca}^{2+}$           | Calcium ions                     |
| CCD                        | Charge coupled device            |
| CHO                        | Chinese hamster ovarian          |
| $\text{CO}_2$              | Carbon dioxide                   |
| CpG                        | Cytosine – phosphate – guanine   |
| cw                         | Continuous wave                  |
| DAPI                       | 4',6-diamidino-2-phenylindole    |
| ddH <sub>2</sub> O         | Double distilled water           |
| DMEM                       | Dulbecco's modified Eagle medium |
| DMSO                       | Dimethylsulfoxide                |
| DNA                        | Deoxyribonucleic acid            |
| DNMT                       | DNA methyltransferase            |
| DPhil                      | Doctor of Philosophy             |
| DPSS                       | Diode pumped solid state         |
| DsRed                      | Discosoma red                    |
| EOM                        | Electro-optic modulator          |
| FCS / FBS                  | Fetal calf/bovine serum          |
| FITC                       | Fluorescein isothiocyanate       |

|                  |                                                    |
|------------------|----------------------------------------------------|
| fs               | femtosecond                                        |
| G/GFP            | Green fluorescent protein                          |
| H3K27            | Histone 3 lysine 27                                |
| HBSS             | Hank's buffered salt solution                      |
| HD               | High density                                       |
| HDAC             | Histone deacetylase                                |
| HDFa             | Human dermal fibroblast adult                      |
| HEK293           | Human embryonic kidney 293                         |
| HeLa             | Henrietta Lacks                                    |
| HeNe             | Helium neon                                        |
| HEPES            | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| hESC             | Human embryonic stem cells                         |
| HIV-1            | Human immunodeficiency virus -1                    |
| HP1              | Heterochromatin protein 1                          |
| IC <sub>50</sub> | Half maximal inhibitor concentration               |
| iPS              | Induced pluripotent stem                           |
| JHDM1            | Jumonji histone demethylase 1                      |
| JHDM2A           | Jumonji histone demethylase 2A                     |
| JMJD3            | JmjC domain-containing protein 3                   |
| K/KLF4           | Kruppel-like factor 4                              |
| K <sup>+</sup>   | Potassium ions                                     |
| KCl              | Potassium chloride                                 |
| KO               | Knock out                                          |
| LB               | Lysogeny broth                                     |
| LIF              | Leukaemia inhibitory factor                        |
| LSD1             | Lysine-specific demethylase 1                      |
| LSM              | Laser scanning microscope                          |
| MEF              | Mouse embryonic fibroblasts                        |
| Mg <sup>2+</sup> | Magnesium ions                                     |
| MIP              | Maximum intensity projection                       |
| miPS             | Mouse induced pluripotent stem                     |
| M <sub>r</sub>   | Molecular weight                                   |
| mRNA             | Messenger ribonucleic acid                         |

|                 |                                                   |
|-----------------|---------------------------------------------------|
| NA              | Numerical aperture                                |
| NaCl            | Sodium chloride                                   |
| Nd:YAG          | Neodymium-doped yttrium aluminium<br>garnet       |
| $N_{\text{fn}}$ | Number of false negatives                         |
| NIR             | Near infrared                                     |
| NLOI            | Non-linear optical imaging                        |
| NN              | Nupherin neuron                                   |
| NSHP            | Naturally supercharged human proteins             |
| $N_{\text{tp}}$ | Number of true positives                          |
| O/OCT4          | Octamer-binding transcription factor 4            |
| OKS-2A          | Single plasmid containing OCT4, KLF4,<br>and SOX2 |
| PBS             | Phosphate buffered saline                         |
| Pc              | Polycomb                                          |
| PI              | Propidium iodide                                  |
| PMT             | Photomultiplier tube                              |
| R               | Resolution                                        |
| S/SOX2          | SRY (Sex determining region Y) - box2             |
| SAHA            | Suberoylanilide hydroxamic acid                   |
| SSEA            | Stage-specific embryonic antigen                  |
| Ti:Sapphire     | Titanium:sapphire                                 |
| TPE             | Two-photon excitation                             |
| TSA             | Trichostatin A                                    |
| TXR             | Transcriptional regulator protein                 |
| UV              | Ultraviolet                                       |
| VB              | Visual basic                                      |
| VPA             | Valproic acid                                     |
| YFP             | Yellow fluorescent protein                        |

# Table of Contents

|                                                                  |               |
|------------------------------------------------------------------|---------------|
| <b>1 Introduction .....</b>                                      | <b>1</b>      |
| 1.1 Aims of the Thesis .....                                     | 3             |
| 1.2 Thesis Overview .....                                        | 5             |
| <br><b>2 Background and Literature Review .....</b>              | <br><b>6</b>  |
| 2.1 Optoporation .....                                           | 6             |
| 2.1.1 Background on microinjection .....                         | 7             |
| 2.1.2 Current applications of optoporation .....                 | 9             |
| 2.1.3 Current techniques of optoporation .....                   | 13            |
| 2.1.4 Choice of medium .....                                     | 18            |
| 2.1.5 Fully automated optoporation system .....                  | 19            |
| 2.2 Possible Roles of Optoporation in Medical Applications ..... | 21            |
| 2.2.1 Epigenetics .....                                          | 22            |
| 2.2.2 iPS generation .....                                       | 28            |
| 2.3 Microscopy Techniques .....                                  | 33            |
| 2.3.1 Light microscopy .....                                     | 34            |
| 2.3.2 Confocal laser scanning microscopy .....                   | 35            |
| 2.3.3 Two-photon excitation .....                                | 36            |
| 2.4 Summary .....                                                | 39            |
| <br><b>3 General Materials and Methods .....</b>                 | <br><b>41</b> |
| 3.1 General Cell Culture .....                                   | 41            |
| 3.1.1 Cell culture .....                                         | 41            |
| 3.1.2 Cell passaging .....                                       | 42            |
| 3.1.3 Immunostaining .....                                       | 42            |
| 3.1.4 Viability staining .....                                   | 42            |
| 3.1.5 Maximum intensity projection .....                         | 43            |
| 3.2 Optoporation using LSM710 (Zeiss) .....                      | 43            |

|          |                                                                                      |           |
|----------|--------------------------------------------------------------------------------------|-----------|
| 3.2.1    | System setup.....                                                                    | 43        |
| 3.2.2    | Non-invasive TPEF and SHG imaging .....                                              | 44        |
| 3.2.3    | Optimisation of laser parameters for optoporation .....                              | 45        |
| <b>4</b> | <b>Generation of iPS Cells with Electroporation and Lipofection .....</b>            | <b>47</b> |
| 4.1      | Materials and Methods .....                                                          | 48        |
| 4.1.1    | Plasmid amplification through bacterial transduction.....                            | 48        |
| 4.1.2    | Lipofectamine LTX and PLUS reagent (Invitrogen) .....                                | 49        |
| 4.1.3    | Electroporation with Neon transfection system .....                                  | 49        |
| 4.1.4    | A combination approach of electroporation and lipofection<br>using STEMcircles ..... | 51        |
| 4.1.5    | Induced pluripotent stem cell culture .....                                          | 52        |
| 4.1.6    | Mouse feeder layer preparation.....                                                  | 53        |
| 4.2      | Optimisation of Electroporation Parameters .....                                     | 53        |
| 4.2.1    | STEMcircles.....                                                                     | 53        |
| 4.2.2    | GFP.....                                                                             | 57        |
| 4.3      | Optimisation of Lipofection Parameters.....                                          | 58        |
| 4.3.1    | Immunostaining of HDFa lipofected with OCT4, SOX2, and KLF4 .....                    | 60        |
| 4.3.2    | OCT4, SOX2, KLF4 .....                                                               | 61        |
| 4.4      | Optimisation of Small Molecule Concentration.....                                    | 62        |
| 4.4.1    | Control.....                                                                         | 63        |
| 4.4.2    | SAHA .....                                                                           | 63        |
| 4.4.3    | 5'AZA .....                                                                          | 64        |
| 4.4.4    | SAHA + 5'AZA .....                                                                   | 66        |
| 4.5      | Results : Derivation of iPS colonies .....                                           | 67        |
| 4.5.1    | iPS-like colonies from Lipofectamine LTX and PLUS reagent .....                      | 67        |
| 4.5.2    | Electroporation of MEFs.....                                                         | 70        |
| 4.5.3    | Electroporation of human fibroblasts.....                                            | 73        |
| 4.5.4    | Electroporation with STEMcircles.....                                                | 79        |
| 4.5.5    | Supplementation with small molecules.....                                            | 83        |
| 4.6      | Discussion.....                                                                      | 87        |
| 4.6.1    | Reprogramming efficiency.....                                                        | 87        |
| 4.6.2    | Starting cell type.....                                                              | 87        |
| 4.6.3    | Completion of reprogramming.....                                                     | 88        |
| 4.6.4    | Reprogramming time.....                                                              | 89        |
| 4.7      | Summary.....                                                                         | 89        |

## **5 Optoporation of Nucleic Acids .....91**

|       |                                                                                          |     |
|-------|------------------------------------------------------------------------------------------|-----|
| 5.1   | Optimisation of Laser Parameters for Optoporation in OptiMEM.....                        | 91  |
| 5.1.1 | Materials and methods .....                                                              | 91  |
| 5.1.2 | Result of optoporation with PI .....                                                     | 92  |
| 5.1.3 | Determining viability after optoporation in OptiMEM.....                                 | 94  |
| 5.1.4 | Optoporation with GFP plasmid in OptiMEM.....                                            | 96  |
| 5.2   | Re-Optimisation of Parameters with PI in Hank's<br>Buffered Saline Solution (HBSS) ..... | 97  |
| 5.2.1 | Optoporation with GFP plasmid in Hank's buffered saline solution.....                    | 105 |
| 5.3   | Re-Optimisation of Laser Parameters for Optoporation Using DAPI.....                     | 107 |
| 5.4   | Optoporation with Nupherin-Neuron .....                                                  | 109 |
| 5.5   | Discussion.....                                                                          | 110 |
| 5.6   | Summary.....                                                                             | 112 |

## **6 Optoporation with Small Molecules .....115**

|       |                                                                  |     |
|-------|------------------------------------------------------------------|-----|
| 6.1   | Single Cell Optoporation with DAPI.....                          | 115 |
| 6.2   | Shockwave Optoporation with DAPI.....                            | 117 |
| 6.3   | Shockwave Optoporation with Propidium Iodide .....               | 119 |
| 6.4   | Optoporation of Demethylase Inhibitors for Drug Development..... | 124 |
| 6.4.1 | Overexpression of JMJD3 by BacMam virus .....                    | 124 |
| 6.4.2 | Overexpression of JMJD3 by Fugene HD .....                       | 125 |
| 6.4.3 | Optoporation of 8-hydroxyquinoline (8HQ).....                    | 128 |
| 6.4.4 | Optoporation of FMF1293 .....                                    | 131 |
| 6.5   | Discussion.....                                                  | 133 |
| 6.6   | Summary.....                                                     | 137 |

## **7 Optoporation of Proteins into Fibroblasts .....140**

|       |                                                                                                        |     |
|-------|--------------------------------------------------------------------------------------------------------|-----|
| 7.1   | Optoporation of BSA-FITC.....                                                                          | 140 |
| 7.1.1 | Materials and methods .....                                                                            | 141 |
| 7.1.2 | Low power and short exposure time on cell membrane.....                                                | 141 |
| 7.1.3 | High power and long exposure time on cell membrane.....                                                | 142 |
| 7.1.4 | Low power and long exposure time on cell membrane<br>with supplementation of K <sup>+</sup> ions ..... | 143 |
| 7.1.5 | Shockwave optoporation in OptiMEM .....                                                                | 148 |



|          |                                                              |            |
|----------|--------------------------------------------------------------|------------|
| 7.2      | Optoporation of Phalloidin-FITC .....                        | 151        |
| 7.2.1    | Materials and methods .....                                  | 151        |
| 7.2.2    | Result of optoporation .....                                 | 151        |
| 7.3      | Optoporation of Antibodies .....                             | 154        |
| 7.3.1    | Materials and methods .....                                  | 154        |
| 7.3.2    | Result of optoporation .....                                 | 154        |
| 7.4      | Discussion.....                                              | 158        |
| 7.5      | Summary.....                                                 | 159        |
| <b>8</b> | <b>Automated Colony Recognition Program.....</b>             | <b>160</b> |
| 8.1      | Automation of Colony Recognition .....                       | 160        |
| 8.1.1    | Current techniques to identify colonies.....                 | 162        |
| 8.2      | Design of Colony Recognition Program .....                   | 166        |
| 8.3      | Method 1 – <i>If-then</i> Statements.....                    | 172        |
| 8.3.1    | Justification of conditional statements .....                | 172        |
| 8.3.2    | Evaluation of conditional <i>if-then</i> statements.....     | 175        |
| 8.4      | Method 2 – Shape Classification with Fuzzy Logic.....        | 177        |
| 8.4.1    | Fuzzy logic system .....                                     | 178        |
| 8.4.2    | Rules.....                                                   | 184        |
| 8.4.3    | Classification of colonies .....                             | 186        |
| 8.5      | Comparing Performances between Method 1 and Method 2 .....   | 190        |
| 8.5.1    | Stringency.....                                              | 191        |
| 8.5.2    | Yield (number of true positives) .....                       | 192        |
| 8.5.3    | Number of false negatives.....                               | 193        |
| 8.5.4    | Computational time .....                                     | 193        |
| 8.6      | Discussion.....                                              | 194        |
| 8.7      | Summary.....                                                 | 195        |
| <b>9</b> | <b>Optoporation with Automated Transfection Routine.....</b> | <b>196</b> |
| 9.1      | Design of Automated Transfection Routine .....               | 197        |
| 9.1.1    | Image segmentation.....                                      | 200        |
| 9.1.2    | Determination of coordinates for optoporation .....          | 202        |
| 9.2      | Results .....                                                | 204        |
| 9.3      | Evaluation of Script Robustness.....                         | 205        |
| 9.4      | Validation of Script Usefulness .....                        | 207        |

|           |                                                                 |            |
|-----------|-----------------------------------------------------------------|------------|
| 9.5       | Discussion.....                                                 | 208        |
| 9.6       | Summary.....                                                    | 210        |
| <b>10</b> | <b>Conclusions .....</b>                                        | <b>212</b> |
| 10.1      | Summary of Achievements .....                                   | 212        |
| 10.2      | Suggestions for Future Work.....                                | 217        |
| 10.2.1    | Improving yield of optoprated demethylase inhibitors .....      | 217        |
| 10.2.2    | Quantifying amount of drug optoprated into cells .....          | 218        |
| 10.2.3    | Improving reliability of automated optoporation system.....     | 218        |
| 10.2.4    | Increasing the accuracy of the colony recognition program ..... | 219        |
| 10.2.5    | Future experiments .....                                        | 219        |
|           | <b>References .....</b>                                         | <b>223</b> |

# List of Figures

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |    |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 2-1: (A) Brightfield image of CHO cell after optoporation with GFP plasmid. (B) Green fluorescence from GFP expression after transfection of the same cell shown in (A). Lightning bolt shows site of optoporation. Reprinted by permission from Macmillan Publishers Ltd: [NATURE] (Tirlapur, U. K. and K. Konig (2002). "Targeted transfection by femtosecond laser." Nature 418(6895): 290-291), copyright (2002).                                                    | 10 |
| Figure 2-2: Calcein –AM (green) stained cells (A) before and (B) after optoporation with propidium iodide (red) (Adapted from Uchugonova, A., K. Konig, et al. (2008a). "Targeted transfection of stem cells with sub-20 femtosecond laser pulses." Opt Express 16(13): 9357-9364).                                                                                                                                                                                             | 12 |
| Figure 2-3: Brightfield image of cell (A) before and (B) after optoporation at 20 $\mu$ m away from cell membrane. With kind permission from Springer Science+Business Media: Applied Physics A, Nanoparticle injection to single animal cells using femtosecond laser-induced impulsive force, 93(1), 2008, 41, Yamaguchi et al., Fig. 2.                                                                                                                                      | 14 |
| Figure 2-4: Effect of optoporation at different distances. (A) before optoporation, (B-D) after optoporation. (C) optoporated cells after uptake of TexasRed, (D) Oregon green staining of cells that have remained viable.                                                                                                                                                                                                                                                     | 16 |
| Figure 2-5: Graphs showing (A) viable cells remaining after optoporation, (B) % cells loaded with TexasRed after optoporation, (C) fluorescence from cells after loading with fluorescent dye, as a function of distance from site of optoporation. Reprinted (adapted) with permission from (Soughayer, J. S., T. Krasieva, et al. (2000). "Characterization of cellular optoporation with distance." Anal Chem 72(6): 1342-1347). Copyright (2000) American Chemical Society. | 16 |
| Figure 2-6: Illustration comparing effects of a cw, fs, and Bessel beam when subject is (A) exactly in focus, (B) out of focus. The Bessel beam successfully irradiates the subject at any focal depth. Brown, C. T. A., D. J. Stevenson, et al.: Enhanced operation of femtosecond lasers and applications in cell transfection. Journal of Biophotonics. 2008. 1(3). 183-199 Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.                          | 17 |

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |    |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 2-7: Image processing filters used by Cappelleri et al. (2010) to segment cells in MATLAB. Reprinted from Journal of the Association for Laboratory Automation, 15/4, David J. Cappelleri, Adam Halasz, Jai-Yoon Sul, Tae Kyung Kim, James Eberwine, Vijay Kumar, Toward a Fully Automated High-Throughput Phototransfection System, 1-13, Copyright (2010), with permission from Elsevier.                                                                                                                      | 20 |
| Figure 2-8: Successfully irradiated and viable cells (shown with arrows and circle). (A) Brightfield image, (B) TexasRed (red) nuclear staining from optoporation, (C) Calcein –AM (green) staining from viable cells (Adapted from Marchington, R. F., Y. Arita, et al. (2010). "Optical injection of mammalian cells using a microfluidic platform." Biomed Opt Express 1(2): 527-536).                                                                                                                               | 21 |
| Figure 2-9: (A) microfluidic setup, (B) schematic diagram showing entry of fluid (two left arrows), output of fluid (right arrow), and point of irradiation (microscope objective). (Adapted from Marchington, R. F., Y. Arita, et al. (2010). "Optical injection of mammalian cells using a microfluidic platform." Biomed Opt Express 1(2): 527-536).                                                                                                                                                                 | 21 |
| Figure 2-10: Illustration showing DNA wrapped around nucleosomes allowing it to be packed into higher order structures. Reprinted by permission from Macmillan Publishers Ltd: [NATURE] (Felsenfeld, G. and M. Groudine (2003). "Controlling the double helix." Nature 421(6921): 448-453), copyright (2003).                                                                                                                                                                                                           | 23 |
| Figure 2-11: Illustration showing the action of SUV39H1 catalysing methylation of a histone for the formation of heterochromatin by HP1 binding. Reprinted by permission from Macmillan Publishers Ltd: [Nature] (Bannister, A. J., P. Zegerman, et al. (2001). "Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain." Nature 410(6824): 120-124), copyright (2001).                                                                                                                    | 25 |
| Figure 2-12: Illustration of (a) acetylation versus deacetylation and (b) methylation versus demethylation. In both modifications, a transcriptional regulator protein (TXR) is required to 'read' the acetyl or methyl groups, which leads to either activation or suppression of a gene. Reprinted from Current Opinion in Cell Biology, 13/3, Rice J. & Allis C., Histone methylation versus histone acetylation: new insights into epigenetic regulation, 263-273, Copyright (2001), with permission from Elsevier. | 27 |
| Figure 2-13: Schematic of a widefield fluorescence imaging setup showing the excitation light path (red) and the resultant emission signal (green). A confocal setup would include a pinhole in front of the detector.                                                                                                                                                                                                                                                                                                  | 36 |

|                                                                                                                                                                                                                                                                                                                                                                |    |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 2-14: Illustration comparing the excitation conical volumes (green) of linear, as in confocal imaging, (left), and non-linear two-photon imaging (right). Reprinted by permission from Macmillan Publishers Ltd: [NATURE METHODS] (Helmchen, F. and W. Denk (2005). "Deep tissue two-photon microscopy." Nat Methods 2(12): 932-940), copyright (2005). | 38 |
| Figure 3-1: LSM710 (Zeiss) multiphoton/confocal microscope setup to be employed for optoporation and monitoring of cells.                                                                                                                                                                                                                                      | 44 |
| Figure 4-1: Graph showing % efficiency of electroporating HDFa cells with STEMcircles based on GFP fluorescence 1 day later. The graph is based on data from Table 4-1.                                                                                                                                                                                        | 54 |
| Figure 4-2: Simultaneous imaging of human fibroblasts electroporated at 1400 volts, 20 ms, 1 pulse. (A) Hoechst nuclear staining, (B) GFP fluorescence (green), (C) merged of (A & B), (D) transmission image. Image is representative from 1 experiment.                                                                                                      | 55 |
| Figure 4-3: Simultaneous imaging of human fibroblasts electroporated at 1400 volts, 20 ms, 2 pulse. (A) GFP fluorescence (green), (B) Hoechst nuclear staining, (C) merged of (A & B), (D) transmission image. Image is representative from 1 experiment.                                                                                                      | 56 |
| Figure 4-4: Simultaneous imaging of human fibroblasts electroporated at 1400 volts, 20 ms, 2 pulse. (A) GFP fluorescence (green), (B) Hoechst nuclear staining (blue), (C) merged of (A & B), (D) transmission image. Image is representative from 1 experiment.                                                                                               | 57 |
| Figure 4-5: Diagrammatic representation of a 24 well showing GFP plasmid DNA amounts and LTX volumes added to each well for optimisation.                                                                                                                                                                                                                      | 58 |
| Figure 4-6: Simultaneous imaging of human fibroblasts lipofected with 750 ng GFP plasmid, 0.75 $\mu$ l PLUS reagent, 1.5 $\mu$ l LTX. (A) GFP fluorescence (green), (B) Hoechst nuclear staining, (C) merged of (A & B), (D) transmission image. Image is representative from 1 experiment.                                                                    | 59 |
| Figure 4-7: Simultaneous imaging of human fibroblasts electroporated with 0.25 $\mu$ g of OCT4 plasmid. (A) Hoechst nuclear staining (blue), (B) immunostained OCT4 (red), (C) merged. Image is representative from 1 experiment.                                                                                                                              | 61 |
| Figure 4-8: Simultaneous imaging of human fibroblasts electroporated with 0.25 $\mu$ g of SOX2 plasmid. (A) Hoechst nuclear staining (blue), (B) immunostained SOX2 (green), (C) merged. Image is representative from 1 experiment.                                                                                                                            | 61 |

|                                                                                                                                                                                                                                                                                                     |    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 4-9: Simultaneous imaging of human fibroblasts electroporated with 0.25 µg of KLF4 plasmid. (A) Hoechst nuclear staining (blue), (B) immunostained KLF4 (green), (C) merged. Image is representative from 1 experiment.                                                                      | 61 |
| Figure 4-10: Simultaneous (A) GFP fluorescence (green) and (B) transmission imaging of human fibroblasts control group. Cells were electroporated at 1400 volts, 20 ms, 2 pulses. Medium did not contain small molecules. Image is representative from 1 experiment.                                | 63 |
| Figure 4-11: Simultaneous (A & C) GFP fluorescence (green) and (B & D) transmission imaging of electroporated human fibroblasts 24 hours after supplementation with (A & B) 1 µM and (C & D) 5 µM of SAHA. Image is representative from 1 experiment.                                               | 64 |
| Figure 4-12: Simultaneous (A & C) GFP fluorescence (green) and (B & D) transmission imaging of electroporated human fibroblasts 24 hours after supplementation with (A & B) 1 µM 5'AZA, (C & D) 10 µM 5'AZA. Image is representative from 1 experiment.                                             | 65 |
| Figure 4-13: Simultaneous (A & C) GFP fluorescence (green) and (B & D) transmission imaging of electroporated human fibroblasts 24 hours after supplementation with (A & B) 1 µM of SAHA and 1 µM of 5'AZA, and (C & D) 5 µM of SAHA and 10 µM of 5'AZA. Image is representative from 1 experiment. | 67 |
| Figure 4-14: Simultaneous GFP fluorescence (green) and transmission image of human fibroblasts 1 day after lipofection with 750 ng GFP plasmid, 0.75 µl PLUS reagent, 1.5µl LTX. 10x objective lens used.                                                                                           | 68 |
| Figure 4-15: Simultaneous GFP fluorescence (green) and transmission image of clusters 3 days after lipofection with 750 ng GFP plasmid, 0.75 µl PLUS reagent, 1.5 µl LTX. 10x objective lens used.                                                                                                  | 69 |
| Figure 4-16: Simultaneous GFP fluorescence (green) and transmission image of 2 clusters 3 days after lipofection with 750 ng GFP plasmid, 0.75 µl PLUS reagent, 1.5 µl LTX. 20x objective lens used.                                                                                                | 69 |
| Figure 4-17: Transmission imaging of iPS-like colonies (A & B) 10 days and (C) 16 days after lipofection with 750 ng GFP plasmid, 0.75 µl PLUS reagent, 1.5µl LTX. Objective lens used was (A & C) 10x and (B) 20x.                                                                                 | 70 |

|                                                                                                                                                                                                                                                                                                                                            |    |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 4-18: Simultaneous GFP fluorescence (green) and transmission imaging of mouse fibroblasts 1 day after electroporation at 1400 volts, 20 ms, 2 pulses. Objective lens used was (A) 4x and (B) 10x.                                                                                                                                   | 71 |
| Figure 4-19: Phase contrast images of 3 iPS-like colony derived from mouse fibroblasts 5 days after the 3rd and final electroporation at 1400 volts, 20 ms, 2 pulses.                                                                                                                                                                      | 72 |
| Figure 4-20: Simultaneous GFP fluorescence (green) and transmission imaging of 3 iPS-like colonies derived from mouse fibroblasts 1 day after the 1st electroporation of a new attempt at the same experiment. These were generated in a new experiment under the same conditions at 1400 volts, 20 ms, 2 pulses. 10x objective lens used. | 72 |
| Figure 4-21: Simultaneous imaging at day 23 of iPS-like colony derived from human fibroblasts after 4th electroporation at 1400 volts, 20 ms, 2 pulses. (A) phase contrast image, (B) GFP fluorescence (green), (C) merged image.                                                                                                          | 73 |
| Figure 4-22: Simultaneous imaging of a 36 day old iPS-like colony undergoing the alkaline phosphatase precipitation test. (A) phase contrast image, (B) GFP fluorescence (green), (C) merged image. 40x objective lens used.                                                                                                               | 74 |
| Figure 4-23: Images of a timelapse sequence of a true iPS colony undergoing the alkaline phosphatase precipitation test. Duration of timelapse was 10 minutes. 10x objective lens used.                                                                                                                                                    | 74 |
| Figure 4-24: Simultaneous imaging of an iPS-like colony stained with iPS surface marker antibodies. (A) phase contrast image, (B) SSEA4-Alexafluor 488 (green), (C) TRA-1-81-PE (red), (D) merged image. 20x objective lens used.                                                                                                          | 75 |
| Figure 4-25: Simultaneous imaging of a true iPS colony stained with SSEA4 antibody. (A) transmission image, (B) SSEA4-Alexafluor 488 (green), (C) merged image.                                                                                                                                                                            | 76 |
| Figure 4-26: Simultaneous imaging of a true iPS colony stained with TRA-1-81 antibody. (A) transmission image, (B) TRA-1-81 (red), (C) merged image.                                                                                                                                                                                       | 76 |
| Figure 4-27: Simultaneous imaging of human fibroblasts 1 day after electroporation at 1400 volts, 20 ms, 2 pulses with increased O,S,K plasmid. (A) phase contrast image, (B) GFP fluorescence (green), (C) merged image. 10x objective lens used.                                                                                         | 77 |
| Figure 4-28: Simultaneous imaging of human fibroblasts 3 days after electroporation at 1400 volts, 20 ms, 2 pulses with increased O,S,K plasmid. (A) phase contrast image, (B) GFP fluorescence (green), (C) merged image. 10x objective lens used.                                                                                        | 77 |

|                                                                                                                                                                                                                                                                                                    |    |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 4-29: Simultaneous GFP fluorescence (green) and phase contrast image of iPS-like colonies 20 days after second electroporation at 1400 volts, 20 ms, 2 pulses with increased O,S,K plasmid.                                                                                                 | 78 |
| Figure 4-30: Simultaneous GFP fluorescence (green) and phase contrast image of iPS-like colonies showing no significant change between (A) 20 days and (B) 24 days after 2nd electroporation at 1400 volts, 20 ms, 2 pulses with increased O,S,K plasmid.                                          | 78 |
| Figure 4-31: Simultaneous GFP fluorescence (green) and phase contrast image of 6 iPS like colonies 24 days after 2nd electroporation at 1400 volts, 20 ms, 2 pulses with increased O,S,K plasmid.                                                                                                  | 79 |
| Figure 4-32: Simultaneous imaging of human fibroblasts 1 (A&D), 3 (B&E), and 5 (C&F) days after electroporation at 1400 volts, 20 ms, 2 pulses with STEMcircles. (A – C) GFP fluorescence (green), (D - F) Hoechst nuclear staining (blue). Images are representative from 1 experiment.           | 80 |
| Figure 4-33: Simultaneous imaging of a 28-day old iPS-like colony derived using STEMcircles. (A) transmission image, (B) GFP fluorescence (green), (C) is merged image.                                                                                                                            | 81 |
| Figure 4-34: Transmission imaging of 2 38-day old partially reprogrammed iPS clusters (denoted by red arrows) seeded on feeders derived using STEMcircles. 10x objective lens used.                                                                                                                | 82 |
| Figure 4-35: Simultaneous imaging of human fibroblasts electroporated (A) once and (B) twice after 3 days at 1400 volts, 20 ms, 2 pulses showing GFP fluorescence (green). Image taken before supplementation of small molecules.                                                                  | 83 |
| Figure 4-36: Simultaneous GFP fluorescence (green) and transmission imaging of human fibroblasts electroporated at 1400 volts, 20 ms, 2 pulses second time. Image taken 1 day after addition of (A) 10 $\mu$ M of 5'AZA (B) 10 $\mu$ M of SAHA, and (C) 5 $\mu$ M of SAHA and 10 $\mu$ M of 5'AZA. | 85 |
| Figure 4-37: Simultaneous imaging of human fibroblasts electroporated at 1400 volts, 20 ms, 2 pulses third time showing (A) GFP fluorescence (green) and (B) merged with transmission image. Image taken 1 day after addition of 5 $\mu$ M SAHA and 10 $\mu$ M of 5'AZA.                           | 86 |



|                                                                                                                                                                                                                                                                                                                                    |     |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 5-1: Simultaneous timelapse (A) propidium iodide fluorescence (red) and (B) phase contrast image of a human fibroblast cell immediately after optoporation at 100 mW and 41 ms. Site of optoporation denoted by red arrow. Images are representative of 100 cells from 1 experiment.                                        | 92  |
| Figure 5-2: Simultaneous timelapse (A) propidium iodide fluorescence (red) and (B) phase contrast image of a human fibroblast cell immediately after optoporation at 150 mW and 41 ms. Site of optoporation denoted by red arrow. Images are representative of 100 cells from 1 experiment.                                        | 93  |
| Figure 5-3: Simultaneous timelapse (A) propidium iodide fluorescence (red) and (B) phase contrast image of another human fibroblast cell immediately after optoporation at 150 mW and 41 ms. Site of optoporation denoted by red arrow.                                                                                            | 94  |
| Figure 5-4: Simultaneous timelapse propidium iodide fluorescence (red) and phase contrast image of a human fibroblast cell (A) before and (B) immediately after optoporation at 100 mW and 41 ms. Site of optoporation denoted by red lightning bolt.                                                                              | 95  |
| Figure 5-5: Simultaneous imaging of the human fibroblast cell from Figure 5 4 surviving 2 hours after optoporation at 100 mW and 41 ms. (A) propidium iodide (red), (B) phase contrast image, (C) calcein-AM (green), (D) is merged image. Site of optoporation denoted by red lightning bolt.                                     | 95  |
| Figure 5-6: Simultaneous imaging of 2 human fibroblast cells surviving 1 day after optoporation at 100 mW and 41 ms. (A,D) phase contrast image, (B,E) GFP fluorescence (green), (C,F) is merged image.                                                                                                                            | 96  |
| Figure 5-7: Simultaneous imaging of a human fibroblast cell surviving 1 day after optoporation at 100 mW and 41 ms in another independent experiment. (A) phase contrast image, (B) GFP fluorescence (green), (C) is merged image.                                                                                                 | 97  |
| Figure 5-8: First and last frames of propidium iodide fluorescence (red) and phase contrast timelapse imaging of 4 human fibroblast cells after optoporation at (A) 80 mW, (B) 40 mW, (C) 20 mW, (D) 10 mW at 41 ms. Site of optoporation denoted by red lightning bolt. Images are representative of 100 cells from 1 experiment. | 98  |
| Figure 5-9: Start and end frames of propidium iodide fluorescence (red) and phase contrast timelapse imaging of a human fibroblast cell after optoporation at (A) 6 mW and 82 ms, (B) 12 mW and 82 ms. Site of optoporation denoted by red lightning bolt.                                                                         | 100 |

|                                                                                                                                                                                                                                                                                                |     |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 5-10: Simultaneous timelapse propidium iodide fluorescence (red) and phase contrast image of a human fibroblast cell (A) before and (B) immediately after optoporation at 6 mW and 82 ms. Site of optoporation denoted by red lightning bolt.                                           | 101 |
| Figure 5-11: Simultaneous imaging of the human fibroblast cell from Figure 5 10 surviving 2 hours after optoporation at 6 mW and 82 ms. (A) calcein-AM (green), (B) propidium iodide (red), (C) phase contrast image, (D) is merged image. Site of optoporation denoted by red lightning bolt. | 101 |
| Figure 5-12: Two frames taken from a timelapse image showing (A) propidium iodide fluorescence (red) and (B) phase contrast of a human fibroblast cell after optoporation at 20 mW and 20 ms. Site of optoporation denoted by red lightning bolt.                                              | 102 |
| Figure 5-13: Simultaneous propidium iodide fluorescence (red) and phase contrast image of a human fibroblast cell (A) before and (B) immediately after optoporation at 20 mW and 20 ms. Site of optoporation denoted by red lightning bolt.                                                    | 103 |
| Figure 5-14: Simultaneous imaging of the human fibroblast cell from Figure 5 13 surviving 2 hours after optoporation at 20 mW and 20 ms. (A) phase contrast image (B) calcein-AM (green), (C) propidium iodide (red), (D) is merged image. Site of optoporation denoted by red lightning bolt. | 104 |
| Figure 5-15: Simultaneous timelapse calcein-AM (green), propidium iodide fluorescence (red) and phase contrast image of a dead human fibroblast cell (A) before and (B) 2 hours after optoporation at 20 mW and 20 ms. Site of optoporation denoted by red lightning bolt.                     | 104 |
| Figure 5-16: Simultaneous imaging of GFP fluorescence (green) and transmission imaging of 3 GFP positive human fibroblasts 1 day after optoporation at 12 mW and 82 ms in the presence of 20 µg/ml GFP plasmid.                                                                                | 105 |
| Figure 5-17: Simultaneous imaging of 2 GFP positive human fibroblast cells from a population of cells that were optoporated at 6 and 12 mW throughout the dish for 82 ms in 20 µg/ml of GFP in HBSS. (A) GFP fluorescence (green), (B) is merged with transmission image.                      | 106 |
| Figure 5-18: Simultaneous imaging of several DsRed positive human fibroblast cells surviving 1 day after optoporation at 6-12 mW and 82 ms in 20 µg/ml of DsRed in HBSS. (A) transmission image, (B) DsRed fluorescence (red), (C) is merged image.                                            | 107 |

|                                                                                                                                                                                                                                                                                                                                                                                            |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 5-19: Simultaneous DAPI fluorescence (blue) and phase contrast imaging of 3 human fibroblasts surviving 1 day after optoporation at 50 mW at 12 ms in 20 µg/ml of GFP plasmid in OptiMEM. Site of optoporation denoted by red lightning bolt. (A) before optoporation, (B) immediately after optoporation, (C) overnight. Images are representative of 100 cells from 1 experiment. | 108 |
| Figure 5-20: Timelapse imaging of a dividing human fibroblast immediately after shockwave optoporation at 180 mW for 1 µs (site of optoporation shown with red arrow). Cell division occurred 3 hours after optoporation. Expression of DsRed occurred 2 hours after cell division.                                                                                                        | 110 |
| Figure 6-1: Timelapse imaging of DAPI nuclear uptake (blue) in (A-C) cell 1 and (D-F) cell 3. Red lightning bolt indicates location of optoporation. Images are representative of 3 cells from 1 experiment.                                                                                                                                                                               | 116 |
| Figure 6-2: Graph showing nuclear volume of 3 cells stained by DAPI normalised to final nuclear volume vs time (mins) after single cell optoporation.                                                                                                                                                                                                                                      | 117 |
| Figure 6-3: Timelapse imaging of DAPI nuclear uptake (blue) in (A & B) cell 3, (C-E) cell 4 (left cell) and cell 5 (right cell) and (F-H) cell 8 (right cell) and cell 9 (left cell). Red lightning bolt indicates location of optoporation. Images are representative of 11 cells from 1 experiment.                                                                                      | 118 |
| Figure 6-4: Graph showing nuclear volume of 11 cells stained by DAPI, normalised to final nuclear volume vs time (mins) after shockwave optoporation.                                                                                                                                                                                                                                      | 119 |
| Figure 6-5: Timelapse imaging of PI nuclear uptake (red) in (A-C) cell 1 and (D & E) cell 3 (bottom). Red lightning bolt indicates location of optoporation. Images are representative of 3 cells from 1 experiment.                                                                                                                                                                       | 120 |
| Figure 6-6: Graph showing volume of cell stained by PI, normalised to final cellular volume for 3 cells versus time (mins) after shockwave optoporation.                                                                                                                                                                                                                                   | 120 |
| Figure 6-7: Timelapse imaging of PI nuclear uptake (red) in (A-C) cell 4 (left cell) and 5 (right cell), (D-F) cell 7, (G & H) cell 10. Red lightning bolt indicates location of optoporation. Images are representative of 11 cells from 1 experiment.                                                                                                                                    | 121 |
| Figure 6-8: Graph showing volume of cell stained by PI, normalised to final cellular volume for 11 cells versus time (mins) after shockwave optoporation.                                                                                                                                                                                                                                  | 122 |
| Figure 6-9: Timelapse imaging of PI nuclear uptake (red) in (A-C) cell 1, (D-F) cell 2, (G-I) cell 3. Red lightning bolt indicates location of optoporation. Images are representative of 4 cells from 1 experiment.                                                                                                                                                                       | 123 |

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 6-10: Graph showing volume of cell stained by PI, normalised to final cellular volume for 4 cells versus time (mins) after shockwave optoporation.                                                                                                                                                                                                                                                                                                                                                                                                                | 123 |
| Figure 6-11: Expression of (A – C) eYFP (yellow) and the (D – F) resulting H3K27me3 stain (red) at (A & D) 0 hours (control), (B & E) 10 hours, (C & F) 18 hours after infection with JMJD3 BacMam virus. Images are representative from 3 experiments.                                                                                                                                                                                                                                                                                                                  | 125 |
| Figure 6-12: JMJD3 FLAG-tag transfected sample using Fugene HD. (A) phase contrast image, (B) DAPI nuclear stain, (C) H3K27me3, (D) JMJD3 FLAG-tag, (E) merged image. Red arrows indicate transfected cells undergoing demethylation. Images are representative from 1 experiment.                                                                                                                                                                                                                                                                                       | 126 |
| Figure 6-13: Control sample without transfection of JMJD3. (A) phase contrast image, (B) DAPI nuclear stain, (C) H3K27me3, (D) JMJD3 FLAG-tag, (E) merged image. Images are representative from 1 experiment.                                                                                                                                                                                                                                                                                                                                                            | 126 |
| Figure 6-14: Graph showing decrease in H3K27me3 and increase in JMJD3 expression over 24 hours in HeLa cells. Data is representative of about 600 – 1200 cells from 1 experiment.                                                                                                                                                                                                                                                                                                                                                                                        | 127 |
| Figure 6-15: Simultaneous imaging of 3 sets of HeLa cells transfected with JMJD3. (A-H) cells optoporated with 8HQ 6 hours after transfection with JMJD3. Cells were fixed and incubated with antibodies a further 6 hours later. (I-L) control samples cultured in presence of 8HQ but not optoporated. (A, E, I) are phase contrast images. (B, F, J) and (C, G, K) are fluorescence images of JMJD3-FLAG (red) and H3K27me3 (green) respectively. (D, H, L) merged images. Cells of interest are shown with red arrows. Images are representative from 3 experiments. | 129 |
| Figure 6-16: Graph showing change in intensity of H3K27me3 staining after treatment with 8HQ. A region of interest was drawn around the nuclei of optoporated cells and the average intensity measured. Data from Group 1 taken from 11 cells from 3 independent experiments. Other Groups represented by >15 cells. Standard error bars are shown. Statistical significance was calculated using Mann-Whitney test.                                                                                                                                                     | 131 |

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |     |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 6-17: Simultaneous imaging of 3 sets of HeLa cells transfected with JMJD3. (A-H) cells optoporated with FMF1293 6 hours after transfection with JMJD3. Cells were fixed and incubated with antibodies a further 6 hours later. (I-L) control samples cultured in presence of FMF1293 but not optoporated. (A, E, I) are phase contrast images. (B, F, J) and (C, G, K) are fluorescence images of JMJD3-FLAG (red) and H3K27me3 (green) respectively. (D, H, L) merged images. Cells of interest are shown with red arrows. Images are representative from 1 experiment. | 132 |
| Figure 6-18: Graph showing change in intensity of H3K27me3 staining after treatment with FMF1293. A region of interest was drawn around the nuclei of optoporated cells and the average intensity measured. Data from Group 1 taken from 6 cells in 1 experiment. Other Groups represented by >13 cells. Standard error bars are shown. Statistical significance was calculated using Mann-Whitney test.                                                                                                                                                                        | 133 |
| Figure 7-1: Simultaneous timelapse imaging of a human fibroblast cell optoporated at low power in the presence of BSA. Each slice was recorded at 0, 90, and 180 seconds. (A) BSA fluorescence (green), (B) corresponding phase contrast image. Images are representative of 20 cells from 1 experiment.                                                                                                                                                                                                                                                                        | 141 |
| Figure 7-2: Simultaneous imaging of a human fibroblast before (A-C) and 30 minutes after (D-F) optoporation with BSA-FITC. (A & D) phase contrast image, (B & E) FITC fluorescence (green), (C & F) is the merged image. Site of optoporation is shown by red lightning bolt. Yellow arrow denotes an unoptoporated cell. Images are representative of 20 cells from 1 experiment.                                                                                                                                                                                              | 142 |
| Figure 7-3: Examples of shrivelled fibroblast cells cultured in high potassium medium.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | 143 |
| Figure 7-4: Simultaneous imaging of a human fibroblast before (A-C) and 30 minutes after (D-F) optoporation with BSA-FITC at 36 mW for 82 ms. (A & D) FITC fluorescence (green), (B & E) phase contrast image, (C & F) is the merged image. Site of optoporation is shown by red lightning bolt. Images are representative of 20 cells from 3 experiments.                                                                                                                                                                                                                      | 144 |
| Figure 7-5: Simultaneous imaging of (A) fluorescein fluorescence (green) and (B) phase contrast of a human fibroblast cell 2 hours after optoporation at 36 mW at 82 ms. (C) is the merged image. Site of optoporation is shown by red lightning bolt.                                                                                                                                                                                                                                                                                                                          | 145 |
| Figure 7-6: Simultaneous imaging of (A) FITC fluorescence (green) and (B) phase contrast of the human fibroblast cell from Figure 7-5 1 day after optoporation at 36 mW at 82 ms. (C) is the merged image. Optoporated cell is shown by red arrow.                                                                                                                                                                                                                                                                                                                              | 146 |

|                                                                                                                                                                                                                                                                                                                                                                                  |     |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 7-7: Simultaneous imaging of a human fibroblast before (A-C) and 30 minutes after (D-F) optoporation with BSA-FITC. (A & D) FITC fluorescence (green), (B & E) phase contrast image, (C & F) is the merged image. Site of optoporation is shown by red lightning bolt. Images are representative of 20 cells from 3 experiments.                                          | 147 |
| Figure 7-8: Simultaneous imaging of (A) fluorescein fluorescence (green) and (B) phase contrast of a human fibroblast cell 1 day after optoporation at 100 mW at 80 ms. Optoporation cell is shown by red arrow.                                                                                                                                                                 | 148 |
| Figure 7-9: Simultaneous imaging of a human fibroblast undergoing optoporation with BSA-FITC. (A) phase contrast, (B) BSA-FITC (green), (C) merged image of (A) and (B), (D) the same cell stained with calcein-AM red (red) at 1 hour post-optoporation. Site of optoporation is shown with red dot. Images are representative of 20 cells from 1 experiment.                   | 149 |
| Figure 7-10: Simultaneous imaging of a human fibroblast (A & B) before, (C & D) after, and (E-G) 1-hour after optoporation. (A, C, E) show fluorescence from phalloidin-FITC (green). (F) shows fluorescence from calcein red-orange AM (red). (B, D, G) are merged images. Red dot indicates location of optoporation. Images are representative of 20 cells from 1 experiment. | 152 |
| Figure 7-11: Simultaneous imaging of a dead human fibroblast 1-hour after optoporation. (A) phase contrast image, (B & C) show fluorescence from phalloidin-FITC (green) and calcein AM red-orange(red) respectively. (D) is the merged image. Red dot indicates location of optoporation. Images are representative of 20 cells from 1 experiment.                              | 153 |
| Figure 7-12: Timelapse imaging of a human fibroblast before and after shockwave optoporation in presence of sAB-27 actin antibody (red). Site of shockwave labelled with blue dot. Images are representative of 20 cells from 1 experiment.                                                                                                                                      | 155 |
| Figure 7-13: Simultaneous imaging of human fibroblast after addition of phalloidin-FITC to optoporation cell. (A) Phalloidin-FITC (green), (B) sAB-27-CF633 (red), (C) merged image. Site of optoporation shown with red dot. Images are representative of 20 cells from 1 experiment.                                                                                           | 156 |
| Figure 7-14: Simultaneous imaging of a human fibroblast optoporation in the presence of (A) DAPI (blue), (B) phalloidin-FITC (green), and (C) sAB-27 conjugated with CF 633 (red). (D) is the merged image. Site of optoporation shown with pink dots. Images are representative of 20 cells from 1 experiment.                                                                  | 157 |

|                                                                                                                                                                                                                                                                                                                                                                                                                    |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 8-1: Tiled phase image of ES colonies cultured on a mouse feeder layer.                                                                                                                                                                                                                                                                                                                                     | 161 |
| Figure 8-2: Examples of 5 classes of joined clusters to be recognised by neural network: (A) false colony composed of fragments, (B-E) shows 1, 2, 3, and 4 joined colonies. (Adapted from Bottigli, U. M. C., Fiori, P. L., Golosio, B., Marras, A., Masala, G. L., and Oliva, P. (2007). "A New Automatic System of Cell Colony Counting." International Journal of Mathematical and Computer Sciences 3(4): 5). | 163 |
| Figure 8-3: (A) Single level wavelet decomposition, (B) two level wavelet decomposition. Reprinted from Pattern Recognition, 30/5, Lu C., Chung P., Chen C., Unsupervised texture segmentation via wavelet transform, 729-742, Copyright (1997), with permission from Elsevier.                                                                                                                                    | 165 |
| Figure 8-4: Brightfield images highlighting the different textures of an (a & c) – undifferentiated colony, and (b & d) differentiated colony. Adapted from Mangoubi et al., Non-invasive image based support vector machine classification of human embryonic stem cells, Biomedical Imaging: From Nano to Macro, 2007. ISBI 2007. 4th IEEE International Symposium on. © 2011 IEEE.                              | 165 |
| Figure 8-5: Line scans of (A) undifferentiated colonies, (B) differentiated colonies. Adapted from Mangoubi et al., Non-invasive image based support vector machine classification of human embryonic stem cells, Biomedical Imaging: From Nano to Macro, 2007. ISBI 2007. 4th IEEE International Symposium on. © 2011 IEEE.                                                                                       | 166 |
| Figure 8-6: Flowchart showing colony recognition algorithm using standard if-then statements and Fuzzy logic control.                                                                                                                                                                                                                                                                                              | 167 |
| Figure 8-7: The background was extracted by removing the cells within the colonies using morphological opening.                                                                                                                                                                                                                                                                                                    | 168 |
| Figure 8-8: Contrast enhancement allowed for improved visualization of colonies.                                                                                                                                                                                                                                                                                                                                   | 169 |
| Figure 8-9: Thresholding the contrast enhanced image with a value of 64.                                                                                                                                                                                                                                                                                                                                           | 170 |
| Figure 8-10: Morphological closing operation to fill holes in colonies.                                                                                                                                                                                                                                                                                                                                            | 171 |
| Figure 8-11: Routine repeated with only 2 criteria: area and eccentricity.                                                                                                                                                                                                                                                                                                                                         | 173 |
| Figure 8-12: Close up showing objects with low solidity and homogeneity (yellow arrows).                                                                                                                                                                                                                                                                                                                           | 173 |
| Figure 8-13: Routine repeated with only 2 criteria: area and fragment factor.                                                                                                                                                                                                                                                                                                                                      | 174 |
| Figure 8-14: Close up showing objects which are narrow and elliptical (yellow arrows).                                                                                                                                                                                                                                                                                                                             | 174 |

|                                                                                                                                                                                                                                                                                                                                                                        |     |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 8-15: The remaining objects after comparing their area, eccentricity, and solidity.                                                                                                                                                                                                                                                                             | 176 |
| Figure 8-16: Fluorescence image showing GFP positive colonies. Identified colonies shown in red bounding box. Yellow arrows show unidentified colonies.                                                                                                                                                                                                                | 176 |
| Figure 8-17: (A) enhancing contrast, (B) after removing fragments of less than 200 pixels in size.                                                                                                                                                                                                                                                                     | 177 |
| Figure 8-18: The Fuzzy Inference System (FIS) editor allows visualisation of inputs and output(s).                                                                                                                                                                                                                                                                     | 179 |
| Figure 8-19: (A) GFP fluorescence images of colonies with their area (pixels), (B) histogram of frequency vs area of colony, (C) Membership function of area input.                                                                                                                                                                                                    | 180 |
| Figure 8-20: (A) GFP fluorescence images of colonies with their partiality (missing area when compared to convex area in pixels), (B) histogram of frequency vs partiality of colony, (C) Membership function of partiality input.                                                                                                                                     | 181 |
| Figure 8-21: (A) GFP fluorescence images of colonies with their fragment factors, (B) histogram of frequency vs fragment factor of colony, (C) Membership function of fragment factor input.                                                                                                                                                                           | 182 |
| Figure 8-22: (A) GFP fluorescence images of colonies with their eccentricity values, (B) histogram of frequency vs eccentricity of colony, (C) Membership function of eccentricity input.                                                                                                                                                                              | 183 |
| Figure 8-23: Membership function output of ‘colony’ (ie. score of colony likeness).                                                                                                                                                                                                                                                                                    | 184 |
| Figure 8-24: Rule editor showing 7 rules used in distinguishing true and false colonies.                                                                                                                                                                                                                                                                               | 184 |
| Figure 8-25: Contrast adjusted phase image showing discovered colonies which are highly confident to be true (boxed in red), and less likely to be true but where manual verification is still recommended (boxed in yellow). Blue arrow denotes a colony that was missed by the algorithm.                                                                            | 187 |
| Figure 8-26: The algorithm repeated but with the results displayed on the GFP fluorescence image of the same colonies for validation purposes. Colonies marked with red and yellow boxes were identified by the algorithm as ‘true’ and ‘likely true’ respectively. Cells of interest are marked with blue arrows. (1 & 2) false positive, (3) missed positive colony. | 189 |
| Figure 8-27: Method 1 repeated with fewer colonies boxed in red on the GFP fluorescence image. False positives are marked with blue arrows.                                                                                                                                                                                                                            | 192 |



|                                                                                                                                                                                                                                                     |     |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 8-28: Comparison of colony segmentation by (A) method 1 and (B) method 2. Yellow arrows represent colonies that have been segmented properly using method 2 but not with method 1.                                                           | 193 |
| Figure 9-1: Flowchart showing how Visual Basic and MATLAB exchange data through the MATLAB server.                                                                                                                                                  | 197 |
| Figure 9-2: Flowchart showing MATLAB algorithm for locating cells and identifying coordinates for parking laser.                                                                                                                                    | 198 |
| Figure 9-3: Flowchart showing Visual Basic script to control microscope hardware.                                                                                                                                                                   | 199 |
| Figure 9-4: Monolayer of human fibroblasts imaged with a phase objective.                                                                                                                                                                           | 200 |
| Figure 9-5: Thresholding the grayscale image into a two-tone image.                                                                                                                                                                                 | 201 |
| Figure 9-6: Elimination of objects smaller than 1200 pixels using morphological opening. In this image, each identified object is labelled with a different intensity of grey.                                                                      | 201 |
| Figure 9-7: Furthest pixel from the centroid shown by a red dot overlaid (A) after edge detection, (B) and on the original raw image itself.                                                                                                        | 203 |
| Figure 9-8: (A) Phase contrast image of human fibroblasts, (B) edge detection of segmented cells with centroid, coordinate on membrane closest to centroid, and actual point of optoporation (marked with green, yellow and red dots respectively). | 204 |
| Figure 9-9: Phase contrast and fluorescence imaging of PI (red) uptake in human fibroblasts after shockwave optoporation (beam parked at locations shown by red circles).                                                                           | 205 |
| Figure 9-10: Gray scale image of high density culture. (A) original, (B) segmented objects. Each object is represented by a shade of grey.                                                                                                          | 206 |
| Figure 9-11: (A) Edge detection of clumps of cells, (B) close up of (A) marked by blue dashed rectangle.                                                                                                                                            | 207 |

# List of Tables

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Table 4-1: List of parameters supplied by Invitrogen for optimising electroporation of HDFa with STEMcircles. Transfection efficiency was obtained by counting GFP positive cells divided by total cell number (indicated by Hoechst nuclear staining) from three 5x5 tile-scan images for each parameter. More than 15,000 cells were analysed for each parameter. Experiment was conducted once.                                                                | 54  |
| Table 4-2: Summary of transfection efficiencies for each transcriptional factor and GFP. Transfection efficiency was obtained by counting cells stained positively by appropriate antibody divided by total number of cells (indicated by Hoechst nuclear staining) from three locations in the same well. Approximately 30 cells were analysed. Experiment was conducted once.                                                                                   | 62  |
| Table 4-3: Concentrations of SAHA and 5'AZA used for optimisation.                                                                                                                                                                                                                                                                                                                                                                                                | 62  |
| Table 4-4: Approximate efficiencies of iPS generation for each method of transfection. Efficiency is the number of colonies divided by starting cell population. With the exception of Electroporation with OSK in human fibroblasts, experiments that yielded 0 colonies were excluded from the calculation. Electroporation of mouse fibroblasts yielded colonies in 2 experiments. In all other conditions, colonies were only generated in 1 experiment each. | 90  |
| Table 5-1: Comparison of laser parameters and different media for optoporation of DAPI and PI. Each condition was performed on 100 cells from 1 experiment. 'YES' in capitals denotes conditions which gave best results based on fluorescent molecule uptake and cell survivability.                                                                                                                                                                             | 113 |
| Table 5-2: Summarised results of GFP and DsRed transfection by optoporation. Each condition shows the result of an experiment where there were transfected cells. Experiments with no transfected cells were ignored. Except for when Nupherin Neuron was used, 100 cells were optoporated each time.                                                                                                                                                             | 114 |
| Table 7-1: Summary of parameters tested for optoporation of proteins. Each condition was tested on 20 cells from 1 experiment, except when high K <sup>+</sup> was used where 3 experiments were carried out. Survivability and BSA uptake were determined and compared between conditions by visual examination based on set imaging parameters.                                                                                                                 | 150 |

|                                                                                                                  |     |
|------------------------------------------------------------------------------------------------------------------|-----|
| Table 8-1: List of shape properties for classifying objects as colonies with if-then statements.                 | 172 |
| Table 8-2: List of shape properties for classifying objects as colonies with fuzzy logic system.                 | 178 |
| Table 8-3: Assessment of sensitivity of fuzzy logic system on five different images consisting of >100 colonies. | 190 |
| Table 8-4: Comparison between method 1 and 2.                                                                    | 191 |

# Chapter 1

## Introduction

The concepts of epigenetics and induced pluripotent stem (iPS) cells offer high therapeutic value in regenerative medicine. The ability to control transcription and suppression of genes using inhibitors of histone deacetylase and demethylase could be the answer to controlling the formation of tumours and other diseases. Hundreds of thousands of drug inhibitors exist but their specific functions and potency have yet to be verified, therefore, a screening method in living cells for such drugs in development would be beneficial. iPS cells offer the advantage of an autologous therapeutic transplant because of their embryonic stem-like nature. However, most iPS studies to date use retroviruses, which have a strong tendency to integrate directly with a host cell's genome, giving rise to the risk of insertional mutagenesis.

This thesis offers three main contributions to the above mentioned fields with regards to the methodology and application of laser-based optoporation, a technique that offers significant control for single cell transfection.

First, optoporation, electroporation and lipofection are compared in their ability to introduce plasmid DNA into cells for the purpose of reprogramming somatic cells into iPS cells without viruses. Here, only electroporation and lipofection are shown to successfully generate colonies which are morphologically similar to iPS colonies. Specific iPS surface marker staining reveals that these colonies are only partially reprogrammed. Optoporation is not able to efficiently transfect and reprogram primary cells possibly due to their slow-dividing nature. Nuclear

envelope breakdown, which would then allow plasmid DNA to enter the nucleus before it becomes degraded, is a rarer event in primary cells.

Second, optoporation is successful in delivering impermeable small molecules such as fluorescent DNA intercalating dyes (4',6-diamidino-2-phenylindole and propidium iodide) and potent histone demethylase inhibitors into living cells. The latter shows great promise in that this technique can be used in drug development platforms. Optoporation can help demonstrate *in vitro* the efficacy of novel drugs at lower concentrations, thereby reducing toxicity to cells. Here, 3D timelapse imaging is used to monitor the uptake of fluorescent dyes and help predict the resulting viability of the cell after optoporation.

Third, optoporation can also bring in relatively large proteins such as bovine serum albumin (BSA), phalloidin and novel synthetic antibodies into living cells without using fixatives. This offers the possibility of reporter systems in viable cells that may be used to monitor phenotype changes over time.

This thesis also discusses the development of two automated imaging-based systems that can be integrated into microscopes. First, an image processing algorithm was developed that can quickly identify stem cell colonies non-invasively. The current method to identify stem cell colonies is time-consuming, laborious, and possibly destructive due to antibody staining. When tested on five large tile-scanned images consisting of hundreds of colonies, the algorithm's resulting sensitivity was excellent (at least 95%). Second, this thesis presents a second algorithm to automate optoporation. By determining the locations of cells using image processing and edge detection, one can control the microscope stage and direction of the laser beam. Since optoporation is highly manual and can only permeate a small number of cells in any given time (unlike electroporation), such an algorithm could potentially increase the yield of optoporated cells. Current publications describe a manual 'point-and-click' interface or a fixed grid-like targeting pattern to quickly optoporate cells. To my knowledge, this is the first publication of a

system which uses image processing to define the exact locations of cells, and thus, the laser beam position for transfection of primary cells.

## 1.1 Aims of the Thesis

- The overall objective of this thesis is to develop optoporation and associated imaging methods, and apply these to investigate biological macromolecules and small chemical molecules in biological systems such as stem cells and chromatin modifications.

In this thesis, three types of molecules will be considered - nucleic acids, small molecules and proteinaceous material (peptides, proteins).

- *nucleic acids* - It has been extensively shown by other groups that plasmids such as green fluorescent protein (GFP) can be introduced into various cell types using optoporation. This project aims to extend this by introducing 3 reprogramming factors (OCT4, SOX2, KLF4), and a GFP reporter gene to generate human iPS cells from fibroblasts. From a clinical point of view, this work would be useful as it removes the need of using integrating retroviruses, which could potentially form tumours when transplanted back into patients. Thus, this goal can be divided further into two aims:

- *Evaluate the suitability of using optoporation in the context of iPS generation*- the traditional plasmid transfection methods of electroporation and lipofection will be used for comparison
- *Automate the identification of iPS colonies* –The goal is to automate the system to scan an entire dish to identify colonies with human iPS characteristics (compact, round, defined borders) as this would be less time consuming and less prone to operator error.

- *Small molecules* - A proof of concept in drug development whereby chemical compounds, which are known to be membrane impermeable, are tested for their efficacy *in vitro*. These compounds could be later modified to make them more permeable once they are proven to be highly potent when optoporated into cells. In this project, both 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) will also be used to simulate small molecules which have strong biological effects, such as in this case, intercalating to DNA and RNA. The work will be extended by substituting DAPI and PI with two membrane impermeable drug compounds, 8-Hydroxyquinoline (8HQ) and FMF1293 in order to quantify their effects as histone demethylase inhibitors.
- *Proteins* - A proof of concept that impermeable proteins (despite being larger than small molecules), such as bovine serum albumin (BSA), phalloidin, anti-actin antibody tagged with CF633, can also be optoporated into cells. These proteins can act as a reporter system for staining actin in living cells without the need to first fix them.
- *Automate the process of optoporation* - Because this method is currently manual, time consuming, and laborious, the yield of optoporation is much lower than electroporation. If optoporation is to produce the large number of cells necessary to overcome the problem of low iPS reprogramming efficiency, it should be developed into an automated system.

## **1.2 Thesis Overview**

This thesis is organised into 10 chapters beginning with this introduction. An overview of the current literature behind optoporation, iPS cell generation, histone modification by chemical compounds, and automation of colony recognition is presented in chapter 2. Chapter 3 briefly states the laboratory methods which are frequently used throughout this thesis. Because the focus of this research relies heavily on a laser-coupled microscope, this chapter also describes in detail the imaging setup and the principles behind the relevant light and confocal microscopy techniques used. Electroporation and lipofection are compared in terms of their ability to generate iPS cells in chapter 4. Chapter 5 describes the use of optoporation to introduce plasmid DNA into cells for the purpose of generating iPS cells. Chapters 6 and 7 present work in using optoporation to introduce impermeable small molecules and proteins respectively into living cells. An image processing algorithm is presented in chapter 8 that can identify stem cell colonies non-invasively and quickly. Chapter 9 demonstrates the integration of image processing and microscope control software to automatically locate and optoporate cells. Lastly, the findings of this thesis are summarised in chapter 10.



# Chapter 2

## Background and Literature Review

This chapter introduces relevant concepts of optoporation, epigenetics, and stem cells. The section on optoporation highlights past findings from other groups and how the technique of optoporation has been optimised and improved. To understand how optoporation can be used in the field of epigenetics, this chapter goes on to briefly outline the concepts of methylation and the use of small molecules for cancer therapeutics. Finally, current methods of generating induced pluripotent stem cells are discussed together with how optoporation may potentially circumvent the need for viruses, thus, making them more suited for clinical applications.

### 2.1 Optoporation

The plasma membrane normally acts as an impermeable barrier against molecules entering living cells. In optoporation, a laser temporarily permeabilises this membrane allowing exogenous material to enter into the cell (Yao et al., 2008, Stevenson et al., 2010). After irradiation, the pore has been reported to begin sealing immediately provided the power is not too high (Shirahata et al., 2001). The advantage of using laser-based transfection is the amount of control over which cell is transfected, thus, allowing for single cell transfection. The ability to target specific sites for the creation of a pore is also a unique feature of optoporation compared to other traditional methods such as electroporation. This means that in studies involving neurons, for example, scientists have the choice of introducing DNA at either the dendrites, cell body, or

axon. Because this technique requires the use of a microscope incorporated to the laser system, timelapse studies can be performed during and after the transfection process (Tirlapur & Konig, 2002). Unlike electroporation, however, single cells are optoporated one at a time meaning that the process yields low throughput. It is not a common technique as the equipment tends to be highly specialised and expensive (Yao et al., 2008). The remainder of this section will continue by giving a brief introduction into optoporation's closest equivalent, *microinjection*. Then, this section will go on to discuss the current applications of optoporation and specifically examine different strategies employed by others in an attempt to achieve higher efficiencies with this technique.

### **2.1.1 Background on microinjection**

Before exploring optoporation further, it is worth discussing traditional approaches that deliver exogenous molecules into cells. Electroporation and lipofection can be seen as transfection methods, which give high yield but with low cell specificity. The workflow of microinjection, on the other hand, is relatively similar to optoporation in that cells are laboriously manipulated in a single-cell fashion. Gordon et al. (2008) claim that only about 40 - 60 embryos can be microinjected in 1 - 2 hours. Microinjection introduces molecules into cells using a micromanipulator equipped with a glass pipette of diameter 0.5  $\mu\text{m}$  (Graessman and Graessman, 1976). This technique has been used to introduce anti-P53 antibodies (Mercer et al., 1984), anti-actin antibodies such as Fragmin (Scheer et al., 1984), Profilin (Valster et al., 1997), Utrophin (Burkel et al., 2007), Talin (Nuckolls et al., 1992), and Phalloidin (Cleary, 1995), as well as RNA (Graessman and Graessman, 1976), BSA, and the plasmids of GFP (Lu et al., 2009) and pBR322 (Gordon et al., 2008). One of the most exciting applications is *in vivo* microinjection. Brezenoff et al. (1969) microinjected histamine into the posterior hypothalamus of rats and monitored its effect on the arterial blood pressure. The researchers benefitted from the specificity

of microinjection as they were able to introduce drugs at a specific site and identify the drug's effect on that part of the brain. In the field of developmental biology, Davidson et al. (2010) microinjected a neural tracer into the brains of embryonic and neonatal mice. The specimens were then fixed to explore the neuroanatomy during development.

#### ***1.2.1.1 Comparison between optoporation and microinjection***

Lu et al. (2009) and Capecchi et al. (1980) exploited microinjection's sensitivity by introducing controllable volumes of DNA (10 - 20 fl in the case of the latter group) into either the nucleus or cytoplasm. Because the DNA was injected directly into cells, only small amounts had to be prepared. In optoporation, however, a transient pore is created in the membrane and the plasmid DNA from the medium diffuses in. It is not possible to directly control the amount that enters the cell.

Capecchi et al. (1980) and Stevenson et al. (2006) report that both microinjection and optoporation can give high transfection efficiency (50 - 100% in the case of microinjection) as well as cell viability. Capecchi et al. (1980) notes that there are also drawbacks such as the possibility of pipette clogging in the case of microinjection, in which, a new pipette will have to be fabricated. Optoporation only relies on lasers, which are generally long lasting and reproducible in terms of power. Microscopes are required for both techniques, which must be inverted for microinjection (Zhang & Yu, 2008). However, optoporation is more flexible and has been reported in both upright and inverted microscope settings.

Microinjection and optoporation both have lengthy preparation, operation, and optimisation procedures that require practice and skill. Thus, these techniques are not meant for running routine biochemical assays (Lu et al., 2009). Microinjection is also only suitable for adherent cells *in vitro* (Lu et al., 2009), thus, coating the substrate has been proposed. However, cells can be optoporated in suspension (Koller et al., 2004) or in a 3D setting (Uchugonova et al., 2008b). Both techniques can be performed *in vivo* though (Zeira et al., 2007 ; Brezenoff et al., 1969).

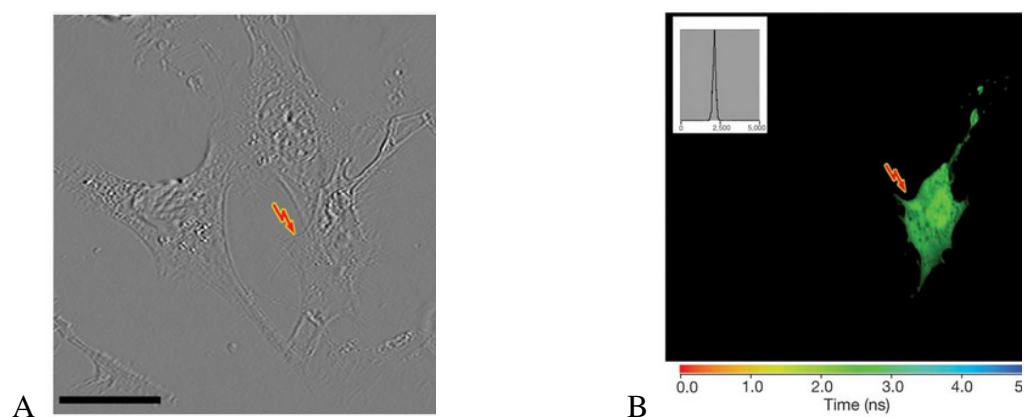
### **2.1.2 Current applications of optoporation**

Various forms of lasers have been used to deliver genes into cells. The lasers employed have been based on the pulsed nanosecond neodymium-doped yttrium aluminium garnet (Nd:YAG) (Tao et al., 1987, Souhayer et al., 2000, Mohanty et al., 2003, Satoh et al., 2005, Clark et al., 2006), argon ion 488 nm (Palumbo et al., 1996 and Schneckenburger et al., 2002), violet 405 nm (Paterson et al., 2005) and the more commonly used pulsed femtosecond Titanium: sapphire (Tirlapur & Konig, 2002, Stevenson et al., 2006, Baumgart et al., 2008).

The first published proof of concept study was in 1984 by Tsukakoshi et al. followed by their colleagues Kurata et al. (1986) with the Nd:YAG laser at the third harmonic of 355 nm with a pulse duration of 5 ns and energy of 1 mJ/pulse. The groups were able to introduce Eco-gpt into rat kidney cells, which only survived antibiotics treatment if they had been successfully optoporated. Expectedly, as the concentration of the external DNA increased, so did the efficiency of the transfection. However, it was interesting to observe that the efficiency saturated at just 0.6% when the concentration was increased from 10 µg/ml and beyond.

Optoporation was carried out using the ‘working laser’ pulsed at 10 Hz. Because the system was based on an upright microscope, the cells had to be tediously grown on coverslips which were then inverted onto a purpose-built vessel and filled with a large amount of culture medium. Using an electronic pen and TV monitor, Tsukakoshi’s group was able to prescribe the location where the laser was to be parked on a TV monitor which displayed realtime footage of the cells under the microscope from a TV camera. Once the location of the site had been decided, a signal was sent to the shutter controller, which allows a predetermined number of pulses to be sent through the microscope and down into the sample. This procedure is not vastly different from how manual optoporation is done today (see, for example, the ‘point and click’ system implemented by Tsampoula et al. (2007)).

Tirlapur & König (2002) were the pioneers in using a femtosecond Ti:Sapphire laser to optoporate Chinese hamster ovarian (CHO) cells with green fluorescent protein (GFP) plasmid. According to that paper, each cell that was irradiated with 50 - 100 mW for 16 ms expressed GFP the next day (Figure 2-1). Cells which were not irradiated did not fluoresce green. Tirlapur & König (2002) further speculated that by optoporating a dividing cell, the DNA could be transferred to the daughter cells as well.



**Figure 2-1: (A) Brightfield image of CHO cell after optoporated with GFP plasmid. (B) Green fluorescence from GFP expression after transfection of the same cell shown in (A). Lightning bolt shows site of optoporation. Reprinted by permission from Macmillan Publishers Ltd: [NATURE] (Tirlapur, U. K. and K. König (2002). "Targeted transfection by femtosecond laser." *Nature* 418(6895): 290-291), copyright (2002)**

Tirlapur & König's (2002) research made the use of the Ti:Sapphire laser for optoporation popular which spawned many more studies undertaken by other people such as Stracke et al. (2005), Tsampoula et al. (2007), Lei et al. (2008), Stevenson et al. (2008) and Marchington et al. (2010). Pulsed lasers such as Ti:Sapphire and the Nd:YAG operating at near infrared wavelengths and beyond have the advantage that they excited fewer cellular components and thus caused less damage (Yao et al., 2008). Yao et al. (2008) also stated that the femtosecond Ti:Sapphire has the further advantage that it causes less damage than the nanosecond Nd:YAG laser.

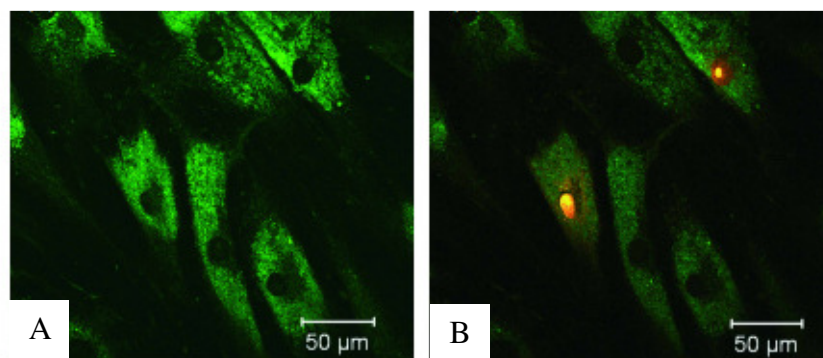
Typically, by using a high numerical aperture objective, the laser is tightly focused so that the power is highly localised to a sub-femtolitre volume at the site of interest (cell membrane) (Tirlapur & Konig, 2002, Stevenson et al., 2006, and Uchugonova et al., 2008a). Only then were the pores small enough to be repairable. The last two studies even go on to report that if a low numerical aperture (NA) lens had been used, the transfection efficiency dropped to as low as 50% due to the larger sized holes, said to be between 2.2 - 10  $\mu\text{m}$ . Only in one study, Palumbo et al. (1996), was the pore size reported to be as large as 6  $\mu\text{m}$  despite using a 100X / 1.0 NA objective. This could be due to the use of an argon 488 laser instead of a femtosecond Ti:Sapphire laser where, under the principles of multiphoton imaging, the effects of the laser are confined to a small volume where two or more photons must arrive simultaneously.

Using a shutter, the duration of the pulse can be varied from 10 - 250 ms (Stevenson et al, 2006) or up to seconds and minutes (Zeira et al., 2003 & 2008 and Barrett et al., 2006). When Stevenson et al. (2006) increased the exposure times beyond 100 ms at 80 mW, it was reported that a large irreparable pore formed in the cell membrane causing blebbing and ultimately death. Blebbing was described as an increase (as much as 21%) in size before the cell underwent apoptosis. Lower power levels of 10 - 30 mW meant that Zeira et al. (2003) and Barrett et al. (2006) required longer exposure times (2 - 3 minutes) as compensation.

In the above mentioned studies, a host of other cell types were attempted such as astrocytes (Lei et al., 2008), human melanoma (Stracke et al., 2005), rat gliosarcoma cells (Chakravarty et al., 2010), canine MTH53a (Baumgart et al., 2008) and the popular HEK293 cells (Marchington et al., 2010). The efficiency of optoporation is not only dependent on cell type; Schnekenburger et al. (2002) reported that the age of the cell population plays a factor. When transfecting CHO cells with GFP, they noticed that a younger population of cells gave rise to 30% efficiency whereas it dropped to just 10% for older cells. The researchers speculated that the membrane of

aging cells reacts differently when a pore is created (ie. where the membrane transforms from gel to liquid crystalline phase) compared to their younger counterparts.

Propidium iodide (PI) or ethidium bromide, both normally impermeable to living cells, were usually used to optimise the power settings for each cell type (Lei et al., 2008, Baumgart et al., 2008, Marchington et al. 2010) and sometimes performed after the incubation of calcein-AM to measure leakage of cytoplasm and to test the cell membrane's integrity after firing as shown in Figure 2-2. Mohanty et al. (2003) and Uchugonova et al. (2008a) first incubated cells with calcein-AM, which fluoresced green if they were alive (Figure 2-2 A). The researchers confirmed the survival of two cells after successful irradiation, (Figure 2-2 B), shown by the presence of both green cytoplasmic and red nuclear fluorescence. Importantly, the irradiated cells had similar green fluorescence intensities as the control cells nearby, which were not optopored. Apart from DNA and fluorescent probes, other substances could be introduced into cells including bovine serum albumin (BSA), (Chakravarty et al., 2010 & Yamaguchi et al. 2008), fluorescein isothiocyanate (FITC) labelled dextran (Stracke et al., 2005) and TexasRed labelled dextran (Souhayer et al., 2000).



**Figure 2-2: Calcein –AM (green) stained cells (A) before and (B) after optoporation with propidium iodide (red) (Adapted from Uchugonova, A., K. Konig, et al. (2008a). "Targeted transfection of stem cells with sub-20 femtosecond laser pulses." *Opt Express* 16(13): 9357-9364)**

Continuous wave single photon lasers have also been used for optoporation but only rarely and will only be discussed briefly here. Palumbo et al. (1996) were the first to use an argon ion laser at 488 nm for optoporation. They introduced beta-galactosidase and chloramphenicol-acetyl-transferase encoding plasmids into NIH-3T3 mouse fibroblasts, but did not disclose the efficiency. In their paper, they hypothesized that the phenomenon of optoporation was caused by the phenol red absorbing energy from the laser and converting it to heat energy. Thus, a 'melting' of the cell membrane occurred, which was reported to heal immediately without any irreversible effects. Other continuous wave lasers have been successfully attempted such as the violet 405 nm laser at just 0.3 mW for 40 ms (Patterson et al., 2005). Short wavelengths such as 350 nm (UV) and 405 nm have high energy photons and can induce DNA damage by introducing molecular lesions. Despite using such a short wavelength that can potentially and permanently damage membranes, cells, or DNA (Yao et al., 2008), the researchers claim that they did not observe any detrimental effects such as apoptosis.

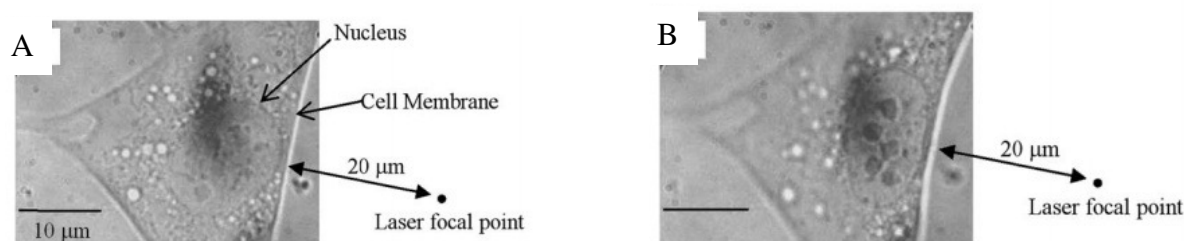
### **2.1.3 Current techniques of optoporation**

To increase efficiency of optoporation and viability of cells after irradiation, some researchers have devised different methods other than firing a single pulse at any random site on a cell's membrane. Peng et al. (2007) ensured that the image was focused to maximum sharpness (observed via a video camera) by adjusting the z-axis. With a two-photon system, the focus needs to be set even more carefully compared to a single photon system (Patterson et al., 2005) since the effect of two-photon excitation occurs in a localised area. With a single photon system, Patterson et al. (2005) required less precision during focusing.

Yamaguchi et al. (2008) proposed firing 20 pulses at 20  $\mu\text{m}$  away from the cell membrane, as represented in Figure 2-3. According to the researchers, a fluctuation in the cell membrane was observed as can be seen when Figure 2-3 A & B are compared. Because the focal size of the



laser was just 1  $\mu\text{m}$  and the focal point was 20  $\mu\text{m}$  away from the cell, this phenomenon was not caused by the laser light impacting the cell itself. Yamaguchi et al. (2008) described this effect as due to the multi-photon excitation of the medium, which then generated either a cavitation or shockwave, which increased in size with power. This theory was supported by the absence of perturbation when Palumbo et al. (1996) and Patterson et al. (2005) used a single photon 488 nm argon ion and 405 nm diode laser instead. Patterson et al. (2005) did not observe any change in the cell morphology after irradiation.



**Figure 2-3: Brightfield image of cell (A) before and (B) after optoporation at 20  $\mu\text{m}$  away from cell membrane. With kind permission from Springer Science+Business Media: Applied Physics A, Nanoparticle injection to single animal cells using femtosecond laser-induced impulsive force, 93(1), 2008, 41, Yamaguchi et al., Fig. 2.**

A similar effect, the presence of a vapour bubble, was recorded by Baumgart et al. (2008) and Peng et al. (2007). In fact, Baumgart et al. (2008) have relied on the occurrence of a vapour bubble as an indicator of the successful uptake of PI. If a bubble did not form, Baumgart et al. (2008) were confident that permeabilisation of the membrane had not taken place. Peng et al. (2007) have termed the formation of a vapour bubble as laser-induced breakdown and is evidenced when a vapour bubble 5 - 20  $\mu\text{m}$  in diameter forms. As the laser intensity increased, the size and the chances of occurrence of the bubble increased as well. The effect of the perturbation to the cell also increased with the size of the bubble, to a point where the cell exploded if the power was too high. Peng et al. (2007) and Baumgart et al. (2008) were not the only researchers to take advantage of these vapour bubbles. In the study by Chakravarty et al.

(2010), the culture medium was filled with carbon black nanoparticles, which, when irradiated with a femtosecond laser, heated up. The heat energy from the nanoparticles converted the surrounding medium to steam, which reacted with the nanoparticles to produce hydrogen and carbon monoxide gas in the form of vapour bubbles. When the bubbles collapsed, they created a shockwave which formed a pore in the membrane, thus, allowing exogenous materials to enter the cell. The pores have been reported to close within 1 second with no damage to the cell. Chakravarty et al. (2010) were able to introduce for example calcein, normally impermeable, into cancerous cells.

The above mentioned studies have focused on using a single shot per cell. Soughayer et al. (2000), on the other hand, focused a Nd:YAG laser onto the coverslip instead. This created a shockwave which permeabilised a large number of nearby rat basophilic leukemia cells. The researchers subsequently observed that the cells displayed different effects depending on how far they were from the centre of the shockwave. If they were within 30  $\mu\text{m}$ , the cells died and were washed away, as can be seen from the low bars in Figure 2-5 A and from the centrally located cells in Figure 2-4 D denoted by the symbol '^'. These cells were not stained with Oregon green as their membranes had ruptured beyond repair. Between '31 - 60  $\mu\text{m}$ ', the researchers reported that 60 - 90% cells survived and 30 - 80% of them were successfully optoporated with Texas Red (Figure 2-5 A & B). These cells can be seen at the top and bottom of Figure 2-4 C & D denoted by a '#'. Importantly, the graph in Figure 2-5 C shows that cells located in the '31 - 40  $\mu\text{m}$ ' range significantly had the highest fluorescence intensity than any other region. Cells in the third region which were beyond 60  $\mu\text{m}$  displayed low levels of optoporation but were all viable. This can be seen in Figure 2-5 B where the trend shown in bar chart B increased, tapered off at '41 - 50  $\mu\text{m}$ ', and then decreased as distance increased.

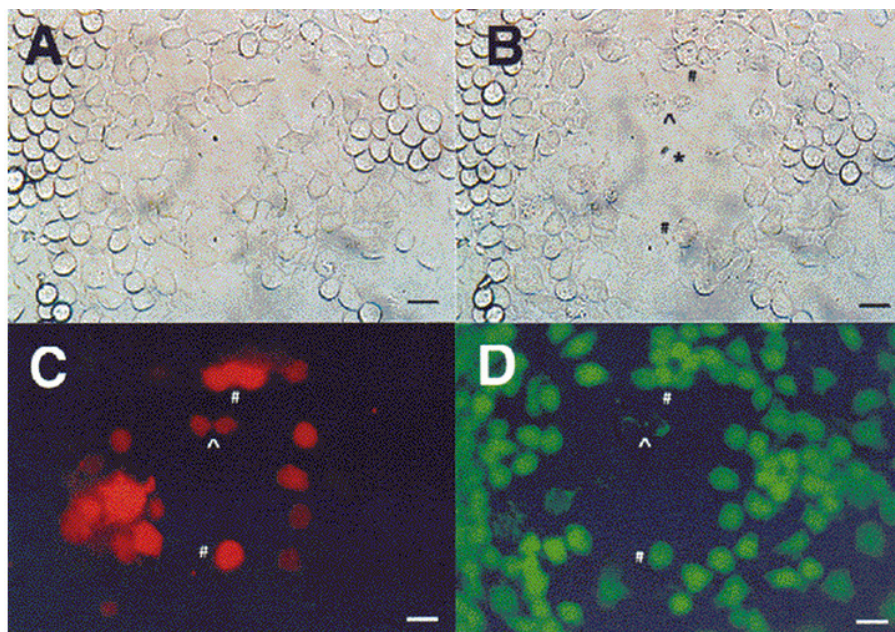


Figure 2-4: Effect of optoporation at different distances. (A) before optoporation, (B-D) after optoporation. (C) optoporated cells after uptake of TexasRed, (D) Oregon green staining of cells that have remained viable.

Reprinted (adapted) with permission from (Soughayer, J. S., T. Krasieva, et al. (2000). "Characterization of cellular optoporation with distance." *Anal Chem* 72(6): 1342-1347). Copyright (2000) American Chemical Society

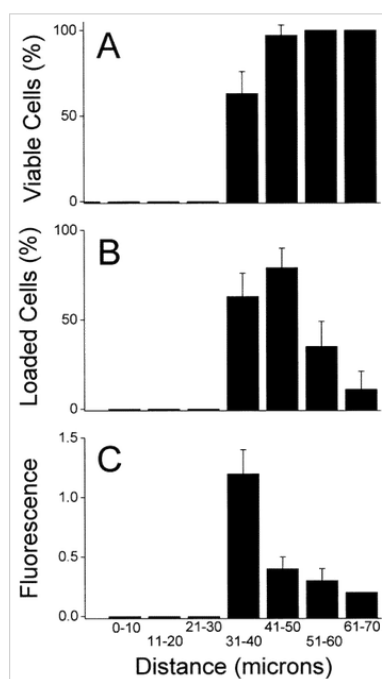
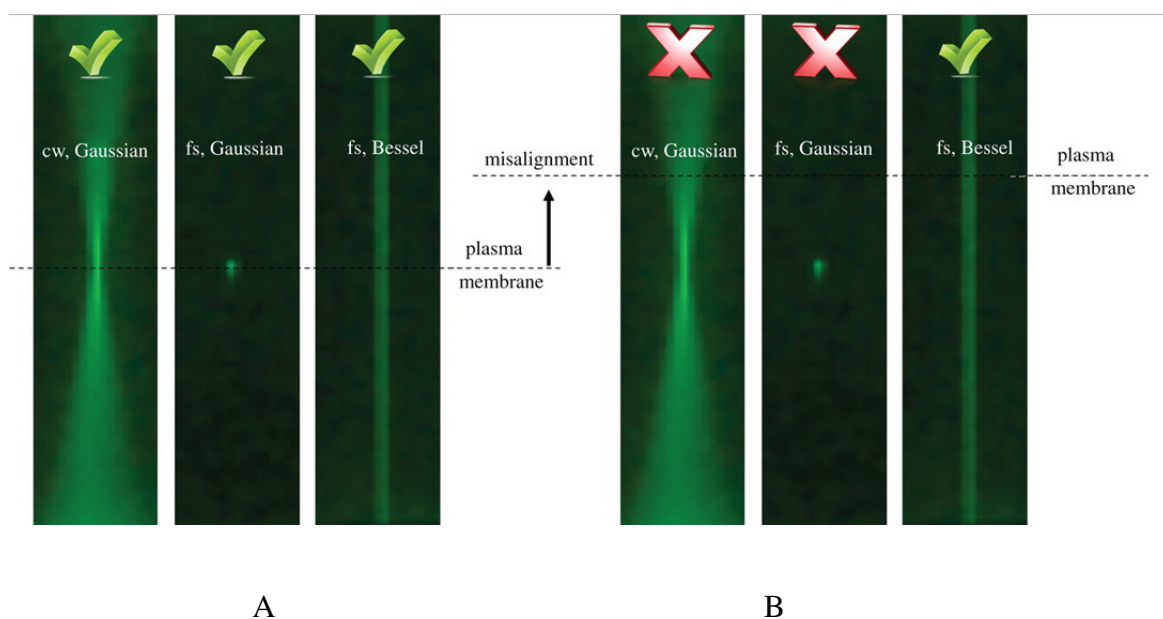


Figure 2-5: Graphs showing (A) viable cells remaining after optoporation, (B) % cells loaded with TexasRed after optoporation, (C) fluorescence from cells after loading with fluorescent dye, as a function of distance from site of optoporation. Reprinted (adapted) with permission from (Soughayer, J. S., T. Krasieva, et al. (2000). "Characterization of cellular optoporation with distance." *Anal Chem* 72(6): 1342-1347). Copyright (2000) American Chemical Society

In general, when a continuous wave laser is used, the laser power is distributed along two cones with the highest intensity located at the centre as shown in Figure 2-6 A (labelled cw, Gaussian). When out of focus, the sample is no longer located at the centre but is still nevertheless irradiated but at less power. Using a femtosecond two-photon laser (labelled fs, Gaussian in Figure 2-6), however, the power is concentrated at a highly localised region instead. According to Tsampoula et al. (2007), the efficiency of optoporation can drop by as much as 50% when the focus is out by a mere 3  $\mu\text{m}$ . Out-of-focus cells would experience virtually no irradiation. To overcome this problem, Tsampoula et al. (2007) devised a non-diffracting Bessel beam. As can be seen in Figure 2-6 A (labelled fs, Bessel), the laser intensity is uniformly distributed over the axial axis of the beam. Because of this, Tsampoula et al. (2007) optoporated cells within as much as 100  $\mu\text{m}$  in the focal axis, demonstrated in Figure 2-6 B. This greatly increased efficiency as it reduced the most time consuming task of focusing on each cell.



**Figure 2-6 : Illustration comparing effects of a cw, fs, and Bessel beam when subject is (A) exactly in focus, (B) out of focus. The Bessel beam successfully irradiates the subject at any focal depth. Brown, C. T. A., D. J. Stevenson, et al.: Enhanced operation of femtosecond lasers and applications in cell transfection. Journal of Biophotonics. 2008. 1(3). 183-199 Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.**

#### **2.1.4 Choice of medium**

Apart from using different lasers, researchers have also experimented with different types of culture media. Kurata et al. (1986) and Yamaguchi et al. (2008), for instance, used serum-containing culture medium for optoporation. However, serum contains DNase and RNase enzymes, which have the potential to interfere in transfection experiments by degrading plasmid DNA and RNA before being delivered into cells. Therefore, Palumbo et al. (1996) attempted to use serum-free culture medium when optoporating 3T3 cells, but it is unlikely that other cell types could be sustained in this for long periods of time. Thus, other researchers have resorted to the expensive OptiMEM due to its ability to keep cells viable without serum (Stevenson et al., 2006, Tsampoula et al., 2007, and Marchington et al., 2010).

The above mentioned media types took advantage of the commonly used phenol red as an energy absorber, which assisted in permeabilising the cell membrane. However, other researchers have also successfully used medium without phenol red. Peng et al. (2007) optoporated aortic endothelial cells in just phosphate buffered saline (PBS). Soughayer et al. (2000) and Barrett et al. (2006) used an interesting mixture of KCl, NaCl, Ca<sup>2+</sup>, Mg<sup>2+</sup>, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid ) with glucose. This combination was likely to have been more effective than PBS since calcium and magnesium ions are especially important for cells to remain adherent. Also, the presence of HEPES allowed the pH of the medium to remain relatively constant on a non-CO<sub>2</sub> regulated stage.

Guo et al. (1995) used a hypertonic solution with an increased concentration of calcium chloride at 0.75M to optoporate rice. The researchers were able to produce a pore in both the cell wall and membrane of the rice cell. Without the hypertonic solution, the contents would leak out through the opening and the cells would shrink to 80 - 90% of their original size.

### **2.1.5 Fully automated optoporation system**

Optoporation is a laborious and time consuming technique, with the process of positioning the laser over each cell taking up the majority of time (Tsampoula et al., 2007). Automating this technique will be necessary before researchers can fully harness its benefits. This section will discuss past methods developed to enhance the speed and throughput of optoporation.

#### ***2.1.5.1 Automatic cell finding and targeting***

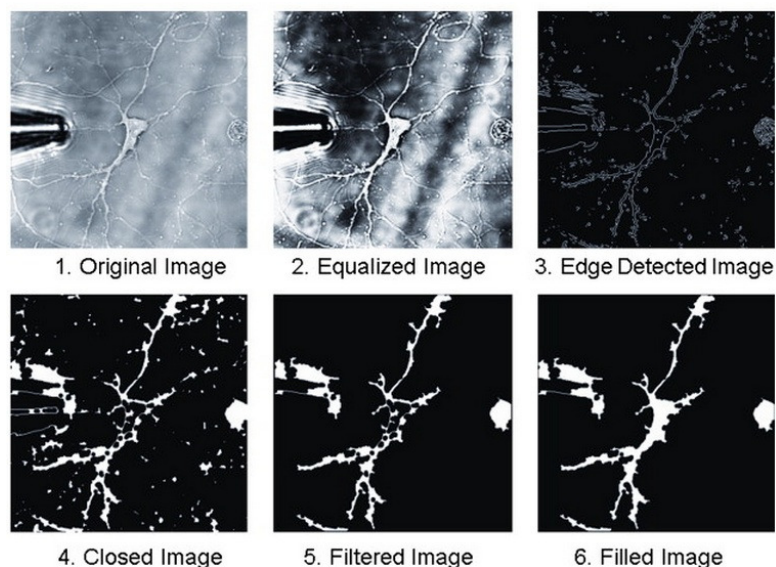
Cappelleri et al. (2010) designed software for the automatic recognition of cells, which then decided the most suitable site to position the laser. The overall objective of their system was to:

- Locate cells on a coverslip and record their positions
- Identify the cytosol and edges of cells
- Select a region on the cell membrane to irradiate with the laser
- Locally pipette mRNA to the target cell after irradiation
- Observe changes in cell morphology at different time intervals
- Repeat as needed

The researchers estimated that manual optoporation can yield transfected cells at just 20 cells/hour. Their goal was to complete 360 transfections/hour by using the automated software. Although the software was just a proof-of-concept and had not been implemented with a laser yet, the researchers proposed that 2,250 cells could be transfected in 1 hour assuming that there are 4 - 6 cells in the field of view at any time.

Their methodology of locating and segmenting each cell is summarised in Figure 2-7. The original image was first equalised which attempts to increase the contrast by re-distributing the pixel values so that all intensities (from 0 to 255) were used. This is followed by edge detection using the Sobel, Prewitt, or Canny edge detector. Closing the image is a morphological operator that performed a dilation followed by erosion. Filtering removed small objects and, finally, the

holes in the image were filled. From the segmented body, the cell's area, perimeter, major and minor axis length, eccentricity, and equivalent diameter could easily be obtained using MATLAB's image processing toolbox (Mathworks, 2010). The site for irradiation was set as simply the centroid of the segmented cell. However, Cappelleri et al. (2010) claim that other suitable sites could be identified and selected instead.



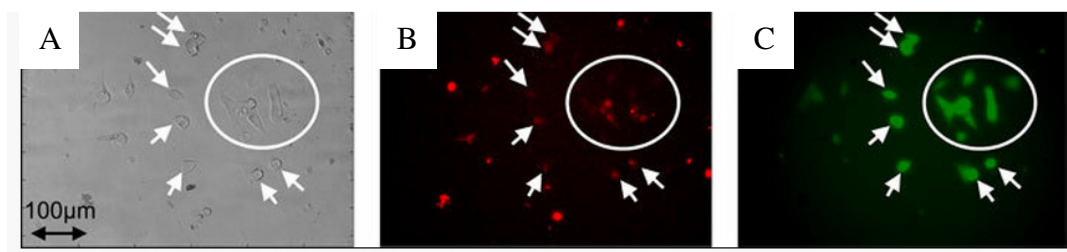
**Figure 2-7: Image processing filters used by Cappelleri et al. (2010) to segment cells in MATLAB. Reprinted from Journal of the Association for Laboratory Automation, 15/4, David J. Cappelleri, Adam Halasz, Jai-Yoon Sul, Tae Kyung Kim, James Eberwine, Vijay Kumar, Toward a Fully Automated High-Throughput Phototransfection System, 1-13, Copyright (2010), with permission from Elsevier.**

### **2.1.5.2 Microfluidic system**

Optoporation is time consuming because of the need to localise and focus the laser for each cell, and this can be laborious when working with 100 cells or more (Marchington et al., 2010). Instead of automating the positioning of the laser for each cell (as done by Cappelleri et al., (2010)), Marchington et al. (2010) devised a microfluidic system which pumped a single cell suspension of HEK293 cells through a femtosecond laser path. Each cell received 2 - 3 doses of power at 90 mW for 4 ms before taking up propidium iodide from the medium. Calcein –AM was used to test for viability after the cells were collected. Marchington et al. (2010) reported an efficiency of 28% for a 150-cell experiment while taking into account the viability of cells. The

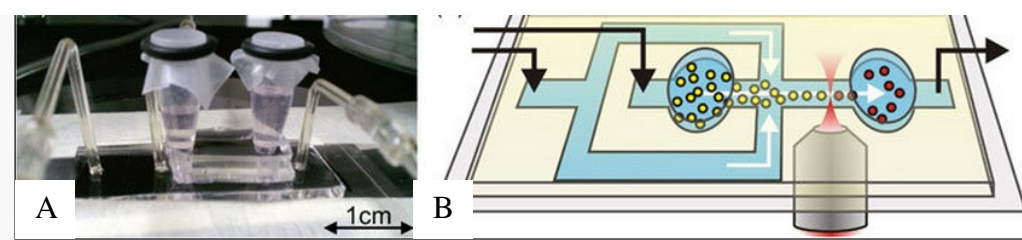


researchers speculated that several thousands of cells could be processed in 1 hour. Figure 2-8 shows cells (red and green fluorescence from PI and calcein-AM respectively; also denoted by arrows and a circle for more clarity) which had survived being optoperated.



**Figure 2-8: Successfully irradiated and viable cells (shown with arrows and circle). (A) Brightfield image, (B) TexasRed (red) nuclear staining from optoporation, (C) Calcein –AM (green) staining from viable cells (Adapted from Marchington, R. F., Y. Arita, et al. (2010). "Optical injection of mammalian cells using a microfluidic platform." *Biomed Opt Express* 1(2): 527-536).**

The researchers built their system using polydimethylsiloxane. In Figure 2-9 A, there are 2 tubes, where cells are forced from the left to the right with 2 syringes. One syringe forced the untreated cells out, and the other syringe drew cells out of the collection area. A third syringe injected buffer to the trail of cells in two opposing directions to force them into a single file. As shown in Figure 2-9 B, irradiation occurs between the two tubes.



**Figure 2-9: (A) microfluidic setup , (B) schematic diagram showing entry of fluid (two left arrows), output of fluid (right arrow), and point of irradiation (microscope objective). (Adapted from Marchington, R. F., Y. Arita, et al. (2010). "Optical injection of mammalian cells using a microfluidic platform." *Biomed Opt Express* 1(2): 527-536).**

## 2.2 Possible Roles of Optoporation in Medical Applications

The overarching goal of this thesis is to explore optoporation as a means to introduce biological macromolecules (nucleic acids, proteins) and small molecules into living cells, as detailed



further in chapters 5 - 7. Regarding nucleic acid optoporation, the aim is to develop, without using viruses, induced pluripotent stem (iPS) cells for therapeutic purposes. One method would be to use optoporation to introduce plasmids into somatic cells for reprogramming.

Another aim is to use optoporation to deliver membrane impermeable small molecules into living cells to test their efficacy as an inhibitor of DNA demethylation. This section will discuss the basic concepts of two important emerging fields of medicine, first, epigenetics (section 2.2.1) and followed by iPS cells (section 2.2.2). As iPS cells and embryonic stem cells are similar, there will also be a brief discussion on characterising the latter. This can be integrated with image analysis techniques to automate the recognition of stem cell colonies, which is also discussed in chapter 8.

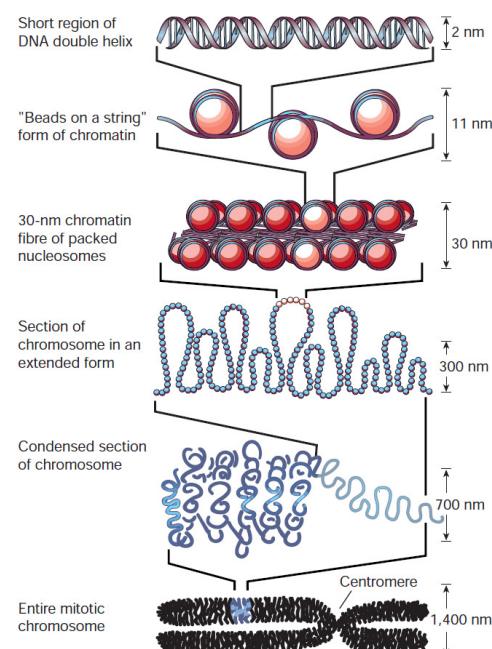
### **2.2.1 Epigenetics**

The human genome consists of 2 metres of  $3 \times 10^9$  base pairs of adenine, thymine, guanine, and cytosine. These base pairs form the 25,000 genes that are the ‘blueprints’ and instructions responsible for building and maintaining cells in the human body. Chromatin, a non-uniform repeating protein-DNA polymer is responsible for packing the DNA into a higher order structure and eventually into 23 chromosome pairs. This allows the genome to fit into the microscopic-sized nucleus of a cell. It has also been suggested that chromatin plays a role in indexing the genome by allowing certain genes to be accessible for transcription (referred to as euchromatin), while silencing others (heterochromatin). The timing and mechanisms of changes to the chromatin (but not to the DNA itself) that bring about expression or suppression of genes is the study of epigenetics. Holliday et al. (1994) defined epigenetics as the “nuclear inheritance which is not based on differences in DNA sequence”. The fact that this information is inheritable means that it can be passed on through cell division. A common example of epigenetics at work is when comparing the different phenotypes of identical twins. Although both individuals have the same

genome, subtle differences in their phenotype must therefore be caused by the genome being read differently. It is believed that stress-induction and environmental factors contribute to differences in the way genes are transcribed (Cooney et al., 2002; Fish et al. 2004). On a more cellular level, epigenetics has also been used to investigate how stem cells differentiate into the right somatic lineage based on signals around them, since stem cells and their differentiated lineages would still have the same genetic code.

### 2.2.1.1 Structure of chromatin

Kornberg (1974) was the first to suggest that the nucleosome was the fundamental unit of chromatin. Around 145 - 147 base pairs of DNA are spooled around histones into a repeating and flexible chain (Kornberg and Lorch, 1999; Luger et al., 1997) as shown in Figure 2-10. Each nucleosome consists of 4 pairs of histone proteins (H2A, H2B, H3, and H4). Each histone has extensions, called histone tails, which are made up of amino acids and allow the histones, and eventually chromatin, to be modified.



**Figure 2-10: Illustration showing DNA wrapped around nucleosomes allowing it to be packed into higher order structures. Reprinted by permission from Macmillan Publishers Ltd: [NATURE] (Felsenfeld, G. and M. Groudine (2003). "Controlling the double helix." *Nature* 421(6921): 448-453), copyright (2003).**

### **2.2.1.2 Modifying chromatin**

Chromatin can undergo several forms of covalent modifications, such as phosphorylation, acetylation, methylation, and ubiquitylation (Kouzarides, 2002). These modifications recruit only specific proteins through the recognition of particular motifs. The way these proteins alter the chromatin structure and hence, act to transcribe or suppress genes, is known as the 'histone code'. Acetylation and methylation are the most characterised modifications and will be briefly discussed here.

#### **2.2.1.2.1 Acetylation**

Acetylation is characterised by increasing the accessibility of the genome between two histones. Essentially, the chromatin is relaxed to form euchromatin and allows the DNA to be transcribed by DNA-dependent RNA polymerase. In histone acetylation, the attractive forces between oppositely charged histones and DNA are neutralised. Increased levels of acetylation generally result in increased transcription (Hebbes et al., 1988). Histone acetyltransferases (HAT) are enzymes which catalyse acetylation of histones by adding an acetyl group to histone lysine residues, while histone deacetylases (HDAC) reverse acetylation in a process called deacetylation. These two opposing processes are constantly occurring and their net effect dictates to what extent a histone has been acetylated (Rice and Allis, 2001; Kouzarides, 2007).

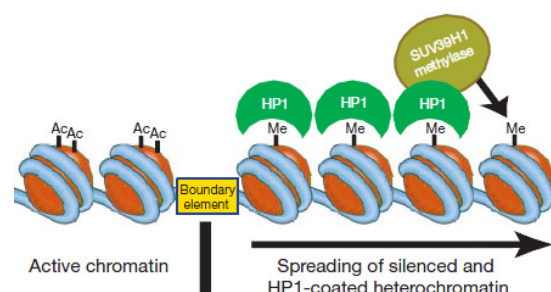
#### **2.2.1.2.2 Methylation**

Histone methylation, is brought about by the addition of methyl groups onto lysine and arginine residues on histone tails. Lysine methylation has only been found to occur on histones H3 and H4 (Martin and Zhang, 2005). Instead of neutralising the charge of histone tails, it increases the histone's affinity for specific proteins, which in turn, attract other proteins that may be involved in transcription of the gene (Rice and Allis, 2001). Methylation can occur when one (mono), two (di), or three (tri) methyl groups are added onto the lysine or arginine tails on histones (Kouzarides, 2007). Depending on where the methyl groups bind, methylation can either activate

or repress transcription, therefore, methylation is more complicated than acetylation. For example, histone methylation at H3K4, H3K36, and H3K79 activate transcription. However, methylation at H3K9, H3K27, and H4K20 suppress transcription (Esteller, 2005). Enzymes that encourage methylation are termed histone methyltransferases (Rice and Allis, 2001).

The most well-studied methylation modification is H3K9. The first enzyme found to methylate H3K9 was SUV39H1 (Rea et al., 2000), however, the methylation of H3K9 is most commonly catalysed by G9a (Tachibana et al., 2005). This important process is involved in recruiting Heterochromatin Protein 1 (HP1) to the promoter genes causing them to be repressed (Kouzarides, 2007). As shown in Figure 2-11, it can further bind to other nucleosomes, thus, enhancing its gene silencing effect. EZH2 is another enzyme that is commonly involved in breast and prostate cancer (Martin and Zhang, 2005). It methylates H3K27, which recruits the Polycomb (Pc) protein that is able to remodel chromatin in such a way as to silence genes such as tumour suppressors.

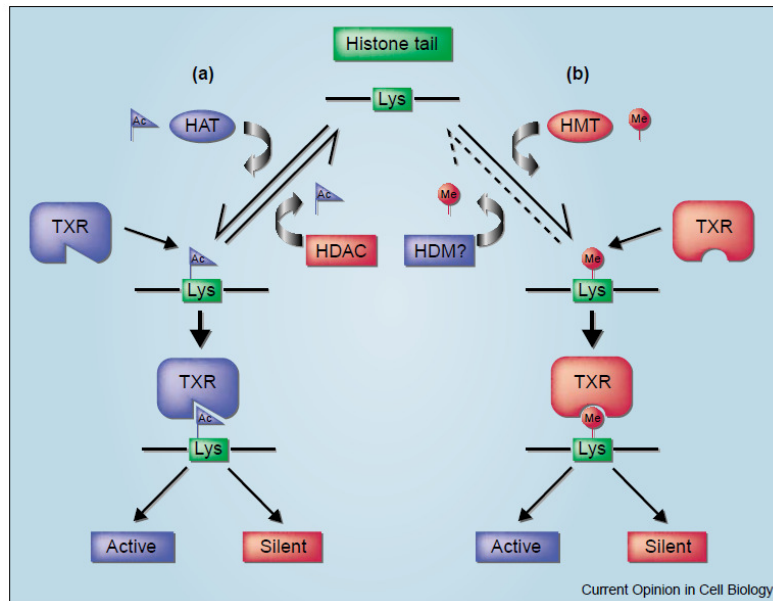
Demethylases, such as Lysine-Specific Demethylase (LSD1), act to remove methyl groups from histones. Other demethylases include JHDM1 for mono- and di-methylation of H3K36, JHDM2A for mono- and di-methylation of H3K9, and JMJD2A for tri-methylated H3K9 (Ng et al., 2007, Couture et al., 2007).



**Figure 2-11: Illustration showing the action of SUV39H1 catalysing methylation of a histone for the formation of heterochromatin by HP1 binding.** Reprinted by permission from Macmillan Publishers Ltd: [Nature] (Bannister, A. J., P. Zegerman, et al. (2001). "Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain." *Nature* 410(6824): 120-124), copyright (2001).

Methylation can also occur on DNA where a methyl group binds to cytosine to form 5' methyl cytosine catalysed by DNA methyltransferases (DNMT) (Bestor et al., 2000). The binding of methyl groups results in the suppression of genes by a mechanism which is still not well understood. DNA methylation can occur on CpG islands, promoter regions that contain abundance of cytosine and guanine bound by phosphodiester and signal the beginning of a gene. This suppresses transcription of the relevant gene (Esteller, 2005) by blocking specific transcription and nuclear factors from binding. For example, specific genes for chondrogenesis should be silenced in neuronal cells and would have their CpG islands methylated. DNA methylation outside of the CpG islands helps to silence parasitic DNA from endogenous retroviruses (Esteller, 2005).

Richon et al. (1998) stated that such modifications only give potential for a gene to be actively transcribed. Modifications need to be read by proteins that cause downstream events such as activating or silencing genes (Strahl and Allis, 2000) as shown in Figure 2-12. A transcriptional regulator protein (TXR) with a particular motif will bind specifically to acetyl or methyl groups. For example, they can be read by bromo (Dhalluin et al., 1999), tudor (Lee et al., 2008), and chromodomains (Bannister et al., 2001). Domains allow proteins that have specific functions to recognise modified histone tails with an acetyl or methyl group. For example, HP1 (discussed earlier) contains a chromodomain and is able to specifically recognise methylation of H3K9 (Bannister et al., 2001). This protein is responsible for maintaining the heterochromatin state of chromatin by further recruiting the action of more histone methyltransferases to H3K9.



**Figure 2-12: Illustration of (a) acetylation versus deacetylation and (b) methylation versus demethylation. In both modifications, a transcriptional regulator protein (TXR) is required to ‘read’ the acetyl or methyl groups, which leads to either activation or suppression of a gene. Reprinted from Current Opinion in Cell Biology, 13/3, Rice J. & Allis C., Histone methylation versus histone acetylation: new insights into epigenetic regulation, 263-273, Copyright (2001), with permission from Elsevier.**

### 2.2.1.3 Epigenetics in cancer

Epigenetics plays an important role in diseases such as cancer. In healthy cells, an oncogene responsible for tumour formation is suppressed while a tumour suppressor gene is actively transcribed. When disease sets in, there is hypomethylation of the oncogene, thus, turning it ‘on’. Esteller (2005) and Feinberg & Vogelstein (1983a) observed high degrees of hypomethylation of various genes in tumour samples. The tumour suppressor gene may also be turned ‘off’ through aberrant DNA methylation or histone deacetylation. Small molecule compounds can effectively act as inhibitors of DNA methyltransferase (5’Azacytidine,  $M_r = 244.20$ ) and histone deacetyltransferase, such as suberoylanilide hydroxamic acid (SAHA,  $M_r = 264.32$ ) (Richon et al., 1998) and Trichostatin A (TSA,  $M_r = 302.37$ ) (Yoshida et al., 1990), which act to reactivate tumour suppressor genes.

It can be seen that epigenetics plays a significant part in the way cells work, whether it is differentiation from a progenitor to a differentiated somatic cell, or the formation of tumours.

The ability to reverse chromatin modifications by using e.g. demethylase and deacetylase inhibitors has allowed researchers to develop new drugs for cancer therapy. Although these inhibitors are small chemical compounds (< 500 Daltons), some may still not pass freely through the cell membrane due to the presence of e.g. a carboxylic acid functional group. Thus, some chemical compounds may actually be very potent but their efficacy cannot be confirmed unless they are administered at high toxic concentrations. Optoporation may be one method of permeating cells for a short duration to allow these inhibitors to enter. After, their efficacy can be determined by immunostaining for the appropriate chromatin modifications. In this thesis, optoporation will be used to introduce two potent impermeable lysine-demethylase inhibitors that act on the well-characterised lysine 9 and 27 of histone 3. These lysine residues were chosen for their importance in heterochromatin formation (Bannister et al., 2001, Barski et al., 2007) and therefore their possible roles in stem cell pluripotency maintenance and tumourigenesis.

## **2.2.2 iPS generation**

### **2.2.2.1 Stem cells**

Embryonic stem cells are derived from the blastocyst's inner cell mass during the first weeks of fertilisation (Evans & Kaufman, 1981; Thomson et al., 1998; Buttery et al., 2001). It has generally been accepted that stem cells (particularly embryonic stem cells, ESCs) have the ability to differentiate into all types of cell lineages in the ectoderm, endoderm, and mesoderm. In theory, the differentiated cells could be used for therapeutic purposes such as cell and tissue transplants. ESC-derived chondrocytes (Hwang et al., 2007), neurons (Fraichard et al., 1995) and pancreatic cells (Nakanishi et al., 2007) could be helpful for people with osteoarthritis, Alzheimer's disease and diabetes respectively.

#### 2.2.2.1.1 Characterisation of pluripotent cells

Human embryonic stem cells are easily identifiable by their morphology. The colonies are tightly compact and have a crisp border when cultured *in vitro*. Stem cell colonies can also be characterised by the presence of nuclear markers such as OCT4 and NANOG as well as the upregulation of alkaline phosphatase (AP) (O'Connor et al., 2008), TRA-1-81, TRA-1-60, and SSEA4 and downregulation of SSEA-1 (Thomson et al., 1998, Henderson et al., 2002).

hESCs can be injected into immune-deficient mice to form teratomas. These consist of tumours containing tissue from all three germ layers (endoderm, mesoderm, and ectoderm) (Evans & Kaufman, 1981, Thomson et al., 1998). Chimera formation is the most stringent test and is the most definitive property that mouse cell lines must possess to be considered as pluripotent embryonic stem cells (O'Connor et al., 2008). They have been shown to be highly successful in recolonising an early embryo to form chimeras (Bradley et al., 1984). In humans, such a test cannot be performed for ethical reasons.

#### **2.2.2.2 Approaches to make iPS technology safer**

Takahashi & Yamanaka (2006) were the first to show that it was possible to reprogram somatic cells into pluripotent-like stem cells. These were termed induced pluripotent stem cells. The scientists retrovirally infected mouse embryonic fibroblasts (MEFs) with 4 reprogramming factors Oct4, Sox2, Klf4, and c-Myc. Colonies began to form about 10 days after the transduction at an efficiency of just 0.02%. Takahashi et al. (2007), were the first to translate the iPS technology from murine to human somatic cells using the same transcription factors OCT4, SOX2, KLF4, and c-MYC. Interestingly, the efficiency reported was almost 10 times lower than in mice (0.001 - 0.002%).



Scheper and Copray (2009) stated that most iPS studies to date have used retroviruses because of the sustained high expression that they provide. Unfortunately, retroviruses have a strong tendency to integrate directly with a host cell's genome, which gives rise to the risk of insertional mutagenesis. It is possible that the virus could later upregulate or downregulate a certain combination of genes which may cause the cell to become cancerous (Okita et al., 2008). This clearly poses a problem if iPS cells are ever to be used for clinical applications. Below are summarised methods of surpassing the problems of integration:

#### 2.2.2.2.1 Minimisation of oncogenic transcription factors

Park et al. (2008) and Nakagawa et al. (2008) reported that when generating hiPS cells from fibroblasts using retroviruses, OCT4 and SOX2 were necessary, but either KLF4 or c-MYC can be eliminated but this, however, resulted in a much lower efficiency of 0.001% (Nakagawa et al. 2008). This was specifically possible when reprogramming cord blood cells and keratinocytes (Aasen et al. 2008, Giorgetti et al., 2009) as they already express high endogenous levels of KLF4 and c-MYC. Feng et al. (2009) mention that c-MYC is oncogenic and, therefore, have the potential of giving rise to cancerous cells. Thus, Yu et al. (2007) successfully used a different and safer set of transcription factors of OCT4, SOX2, LIN28, and NANOG. The efficiency when using fetal and newborn foreskin fibroblasts was 0.02%. Since NANOG is expressed endogenously by pluripotent stem cells, it is not surprising that NANOG can be used as a transcription factor. LIN28, on the other hand, is not required and therefore only assists in reprogramming (Welstead et al., 2008). Its role has not been well studied (Scheper & Copray, 2009), but it has been suggested that it acts by binding and stabilising mRNA transcripts from pluripotency-related genes (Welstead et al., 2008).

#### 2.2.2.2.2 Integration –free vectors

Stadtfield et al. (2008) tried using adenoviruses with Sox2, Klf4, c-Myc, and an Oct4 doxycycline inducible transgene. When the cells were cultured in doxycycline for 24 to 30 days, nine iPS colonies were observed. When Zhou and Freed (2009) used human fetal lung fibroblasts, 2 rounds of adenoviral infections carrying all four factors were necessary to give rise to just 1 iPS colony per 500,000 infected cells.

#### 2.2.2.2.3 Plasmid DNA

Okita et al. (2008), Kaji et al. (2009) and Gonzalez et al. (2009) devised plasmids that contained 3 or 4 transcription factors, which were then electroporated into mouse and human cells. Okita et al. (2008) admitted, however, that the efficiency (0.0001 - 0.001%) is significantly lower in mice when compared to using viruses, but is a step towards clinical applicability. Human iPS cells were also generated at an undisclosed efficiency (Kaji et al., 2009).

A vector designed by Jia et al. (2010) did not give rise to integration into the genome at all. They used ‘mini-circle’ DNA which contained the factors of OCT4, SOX2, NANOG, LIN28 and the reporter gene GFP. Due to its smaller size and lack of bacterial origin of replication and antibiotics resistance gene, the mini-circle offered higher transfection efficiencies in human lung fetal fibroblasts and adipose stem cells, but still suffered from low reprogramming efficiencies.

#### 2.2.2.2.4 mRNA and proteins

Generation of iPS cells is also possible by mRNA transfection (Yakubov et al., 2010; Plews et al., 2010, and Warren et al., 2010) and recombinant proteins of the reprogramming genes (Kim et al., 2009). The reprogramming efficiency was 0.05% and 0.001% respectively. The most important advantage is their inability to integrate into the genome (Kim et al., 2009; Yakubov et al., 2010). The drawback is that repeated transduction of mRNA and proteins are necessary. Kim et al. (2009) did not disclose the technique used to deliver proteins into cells, but, as many as 6 transductions over 6 weeks were required. For mRNA, Yakubov et al. (2010) implemented 5

rounds of transfection with Lipofectamine 2000 (Invitrogen) just 24 hours apart. This made reprogramming by proteins very lengthy and mRNA possibly toxic due to the frequency of transduction.

#### 2.2.2.2.5 Small molecules

Possibly the most promising method of translating iPS cell research for therapeutic use is replacing all factors with small molecules. Shi et al. (2008a) replaced Sox2 with G9a methyltransferase inhibitor BIX-01294 or a combination of both BIX-01294 and BayK8644 (an L-calcium channel agonist) in MEFs. Although the efficiency was reduced by half and the reprogramming time more than doubled, it showed proof of principle that small molecules may be able to replace other factors. Valproic acid (VPA) (a histone deacetylase inhibitor) replaced KLF4 in human dermal fibroblasts (Huangfu et al. 2008b). The efficiency dropped by 200 fold, but when it was used in conjunction with all 3 factors (OCT4, SOX2, and KLF4) the efficiency was 30 fold higher. Later, Mali et al. (2010) showed that sodium butyrate was more effective than VPA in human fibroblasts in upregulating pluripotent genes, especially OCT4 and NANOG). Other small molecules that have been reported to increase reprogramming efficiency are 5'azacytidine (5'AZA) (Wernig et al., 2008), SAHA (Huangfu et al., 2008a) and even vitamin C (Esteban et al., 2010, Cai et al., 2010). In all of these studies, small molecules were unable to totally replace all transcription factors.

#### **2.2.2.3 Limitations of iPS cells in clinical applications**

It was initially believed that iPS cells would be the cure-all method for all diseases. Theoretically, ESCs and iPSCs both have similar characteristics in that they are pluripotent and form teratomas. Being autologous, the implantation of iPS cells should pose no risk of immune rejection. However, it was recently shown by Zhao et al. (2011) that iPSCs are not so similar to ESCs after all. When mouse iPSCs were injected back into mice, they were rejected to the point

that the implanted tissue was necrotic. The authors performed global gene expression analysis and discovered that certain genes were over-regulated and this was the reason that there was an immune response by T-cell infiltration.

To date, it has not been possible to efficiently differentiate iPS cells to liver and insulin-producing pancreatic cells. Interestingly, both cell types are from the endoderm germ layer. Hayden (2011) suggests that this may be due to the need for more specific chemical signals which have yet to be determined. Another possibility is that the differentiation of iPSCs are inherently limited to certain cell types. To counter this, Huang et al. (2011) were able to generate hepatocytes directly from mouse tail tip fibroblasts by overregulating liver specific transcription factors. In this way, reprogramming to iPS cells was completely bypassed and not needed.

Another glaring issue is that iPS cells display different and unexpected DNA methylation patterns when compared to pluripotent ESCs (Kim et al., 2010; Polo et al., 2010; Lister et al., 2011). These authors reiterated that reprogramming should, in theory, reset genomic methylation, but in reality, this is incomplete. There are residual DNA methylation signatures that match the cell type which the iPSCs originated from. According to Kim et al. (2010), this ‘epigenetic memory’ effectively reduces the potential for differentiation towards certain cell lines. For example, Kim et al. derived iPSCs from blood cells and fibroblasts, but because the blood-derived iPSCs still contained DNA methylation patterns similar to blood cells, they were more efficient in differentiating to hematopoietic cells when compared to the fibroblast-derived iPSCs.

## **2.3 Microscopy Techniques**

This subsection briefly details the traditional and advanced forms of microscopy (such as light microscopy and two-photon excitation respectively) that are used in this thesis.

### 2.3.1 Light microscopy

Light microscopy allows the visualisation of tissue, cells and organelles using a lamp source such as a tungsten-halogen lamp. Light rays from the lamp source are transmitted through the main components of the microscope before the image is formed and observed by a charge-coupled device (CCD) camera or by eye. Light from the lamp source is first focused onto the sample through the condenser. The light is diffracted in the sample and is then collected by the objective lens, which then passes through a set of image filters depending on the type of contrast required.

Microscopes can be fitted with a variety of objective lenses with different magnifications and resolutions. The spatial resolution is defined as the minimum distance between two points that can be effectively resolved and is related to the numerical aperture (NA) by Equation 1:

$$R = \lambda / 2NA, \quad (\text{Equation 1})$$

where  $R$  is the resolution (nm),  $\lambda$  is the wavelength of the illuminating light (nm), and  $NA$  is the numerical aperture. An objective lens will have high resolving power if the  $NA$  is high and the wavelength of illuminating light is short. This also means that magnification is independent of the resolution.

The numerical aperture ( $NA$ ) is a measure of the light gathering ability of the lens. The  $NA$  can be defined by Equation 2:

$$NA = n(\sin\mu), \quad (\text{Equation 2})$$

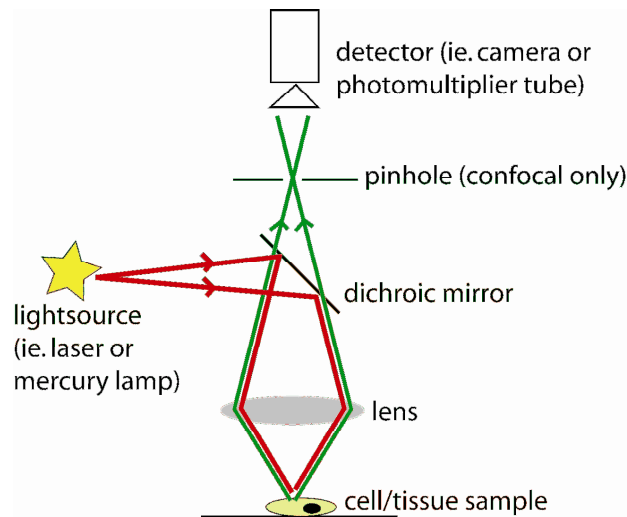
where  $n$  is the refractive index of the medium between the lens and sample (1.0 for air) and  $\mu$  is half the angle of the aperture angle. Therefore, a wide angular aperture (ie. 75 degrees) gives rise to a higher  $NA$ . It can be seen from Equation 2 that the theoretical maximum value of  $NA$  is 1.0.

By using a medium with a higher refractive index (such as immersion oil), even higher NA values can be obtained.

Thick tissue specimens attenuate the amplitude of the incoming light rays, which can be easily observed by eye. Using the microscope in this way is known as brightfield contrast. However, thin and unstained samples are not clearly visible as light easily penetrates them without much decrease in amplitude. Thin samples such as monolayers can be stained, but this usually requires fixing the sample. Phase contrast and Differential interface contrast are two methods that have been developed to overcome this, and were used extensively in this thesis.

### **2.3.2 Confocal laser scanning microscopy**

In a laser scanning microscope, a single photon continuous wave (a steady stream of photons) beam replaces the mercury lamp. The laser is emitted and focused through an objective onto a diffraction limited spot on the sample (Figure 2-13). A set of mirrors guides the excitation signal from the laser (red lines in Figure 2-13) so as to raster scan the sample which is in the field of view. The resulting emission signal (green lines in Figure 2-13) travels back through the same objective and is reflected by a beamsplitter through a pinhole onto a photomultiplier tube (PMT) detector. The image is subsequently formed by mapping the emission signal that arrives at the PMT to a pixel in an image. The pinhole, modified by Minsky in 1957, eliminated the out-of-focus signal commonly found in widefield fluorescence microscopy and allowed users to image samples with high axial resolution (Shotton, 1989; Christensen & Nedergaard, 2011).



**Figure 2-13: Schematic of a widefield fluorescence imaging setup showing the excitation light path (red) and the resultant emission signal (green). A confocal setup would include a pinhole in front of the detector**

### 2.3.3 Two-photon excitation

The concept of two-photon excitation (TPE) was first theorised by Goppert-Mayer (1931), where two photons could cooperatively cause excitation in one quantum event. Denk et al. (1990) were the first to apply two-photon excitation for non-linear optical imaging (NLOI) in a laser scanning microscope (LSM). Because it is a non-linear process, the image intensity increases in a quadratic manner with excitation power (Denk et al., 1990). Two long wavelength photons, when arriving at the sample simultaneously, are able to excite a fluorophore each with less energy than required by a single short wavelength photon. As an example, two near infrared (NIR) photons at 700 nm could substitute for a single 350 nm ultraviolet (UV) photon. There seems to be some disagreement as to how much time can be allowed between the arrival of two photons for TPE to occur. Zipfel et al. (2003) and Piston et al. (1999) give different estimates of  $10^{-16}$  s and  $10^{-18}$  s, respectively.

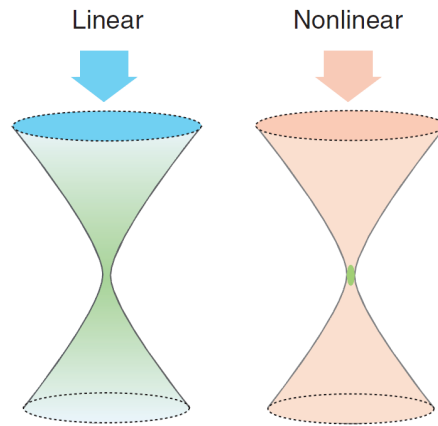
Because the theoretical probability of the almost simultaneous arrival of two photons is low, a femtosecond pulsed laser is normally used. The most common pulsed laser is the Ti:Sapphire laser pumped by a green 532 nm laser (Helmchen & Denk, 2005). Such a laser produces

sufficiently large numbers of photons in a short temporal window that the probability of two photons arriving simultaneously is high. Also, since the pulse width is in the femtosecond range, average powers are still low enough so that there is little damage to the sample.

The beam is focused through an objective lens. Piston et al. (1999) state that the photon density from a pulsed laser needs to be a million times higher than a single photon laser to produce the same fluorescence effect. Thus, two-photon imaging systems also employ high numerical aperture (NA) lenses to tightly focus photons at the focal plane giving rise to high local intensity only at the sample.

Once the sample is excited, the emission signal is collected through the same objective and is diverted to an array of non-descanned detectors each responsible for a specific range of wavelengths and highly efficient in collecting scattered photons from the sample. One can notice from Figure 2-14 that no pinhole is needed. There is no excitation anywhere but the focal spot, therefore, background emission from other focal layers is essentially eliminated, thus, giving a sharp image. Photobleaching is also restricted only to the focal site (2  $\mu\text{m}$  as reported by Denk et al., 1990) as opposed to the entire sample (wide-field and confocal fluorescence microscopy) as illustrated in Figure 2-14. It is true that confocal microscopy can minimise out of focus emission as well, however, this is through the use of a pinhole placed in front of the detector, which allows less light to pass through. The result is that longer exposure times are needed to gather sufficient light. Furthermore, a small amount of out of focus emission signal can be scattered through the pinhole giving rise to background noise (Christensen & Nedergaard, 2011).





**Figure 2-14: Illustration comparing the excitation conical volumes (green) of linear, as in confocal imaging, (left), and non-linear two-photon imaging (right). Reprinted by permission from Macmillan Publishers Ltd: [NATURE METHODS] (Helmchen, F. and W. Denk (2005). "Deep tissue two-photon microscopy." Nat Methods 2(12): 932-940), copyright (2005).**

Because of the advantages of two-photon microscopy (namely, the use of low-energy photons), this form of imaging has been adapted for many biological applications involving cell layers, thick tissue and even live animals (Zipfel et al., 2003). Short wavelength photons contain high energy which contributes to photobleaching and phototoxicity. Denk et al. (1990) compared the effects of excitation from two-photon lasers and a single photon mercury lamp on cells. After several minutes, the cells excited under the mercury lamp changed morphology drastically, whereas there was no visible change when cells were excited with two-photon excitation.

While it is true that electron microscopy can give even higher resolution images of cell structures than two-photon microscopy, it has the disadvantage that these cells need to be fixed. Thus, vital cell processes cannot be recorded in this way. In *in vivo* imaging, two-photon microscopy has been used to image *in vivo* human skin (Masters et al., 1998), mouse dermal (Tirlapur et al., 2006), and mitochondrial distribution in hamster and rhesus monkey embryos (Squirrell et al., 1999 & 2003). It was shown by Squirrell et al. (1999) that using a 514 nm single photon laser for imaging, damaged hamster embryos in such a way that they ceased to develop. However, embryos continued to grow when they were imaged with two-photon microscopy using a NIR

laser. The reason they found was that high energy lasers (such as 514 nm) produced peroxides which arrested development. Because of the reduced phototoxicity when using NIR lasers, these specimens can be imaged repeatedly.

Another advantage of two-photon excitation using near infrared wavelengths is the ability to image deeper because there is less scattering (Rocheleau et al., 2003) and there are fewer absorbers in tissue at these wavelengths (Christensen & Nedergaard, 2011) meaning more excitation light reaches the sample at deeper penetration depths. The fact that two-photon microscopy allows more light to be captured through the absence of a pinhole is another reason why it is suitable for deep tissue imaging.

Piston et al. (1999) lists some limitations to using two-photon microscopy relative to confocal microscopy:

- Increased complexity of the system – tunable pulsed femtosecond lasers are expensive and are less user friendly
- No significant improvement in resolution – a confocal microscope will give better resolution than a two-photon microscope using longer wavelengths
- No significant advantage over confocal microscopy in thin samples, as depth penetration not required.

## **2.4 Summary**

This chapter summarises the concept of optoporation and compares some of its aspects with traditional microinjection, which have similar abilities in manipulating single cells. Optoporation implements a focused laser to create a transient pore in the membrane in order to introduce small and macromolecules into cells. It requires intensive optimisation but has been claimed to offer high efficiency and viability. Because the procedure is slow, repetitive, and generates a low

yield, attempts have been made to automate optoporation using image-based cell tracking or microfluidics.

Epigenetics and stem cells were also briefly introduced as having potential therapeutic applications. The ability to inhibit histone demethyltransferases with drugs in the form of small molecules is generating much interest in the scientific and pharmaceutical fields. Likewise, reprogramming somatic cells to have pluripotent qualities is another potential area of therapeutic value. The goal of this thesis is to investigate the role of optoporation as a possible means to deliver small and macromolecules for the aforementioned purposes; the results in chapters 5 - 7 will present my findings.

# Chapter 3

## General Materials and Methods

This chapter summarises techniques which are frequently used throughout this thesis. Materials and methods specific to a particular experiment will be outlined in the relevant chapter.

### 3.1 General Cell Culture

Cells were cultured using regular culture medium and passaged using standard procedures as described below.

#### 3.1.1 Cell culture

Culture medium consisted of 176 ml of High glucose Dulbecco's Modified Eagle Medium (DMEM), 2 ml of GlutaMAX, 20 ml of fetal calf serum (FCS) (Invitrogen), and 2 ml antibiotic stock ( $5 \times 10^5$  units of penicillin and 500 mg streptomycin in 100 ml phosphate buffered saline (PBS) solution) (Sigma). The medium was filter sterilized with a membrane pore size of 0.22  $\mu\text{m}$  and stored at 4 degrees Celsius and used within 1 week.

### **3.1.2 Cell passaging**

When HeLa cells and fibroblasts were 70 - 80% confluent, they were split at a ratio of 1:4. This involved aspirating the old medium and washing the cells with 8 - 10 ml of PBS followed by incubation with 2 ml of 0.25% trypsin for 3 - 5 minutes. After 5 minutes, the flask was gently tapped to assist detachment of the cells. Subsequently, the trypsin was neutralised with serum-containing culture medium and the mixture was transferred to new flasks or onto glass-bottom dishes for optoporation.

### **3.1.3 Immunostaining**

Cells were first rinsed with PBS and fixed with 4% paraformaldehyde for 20 minutes. The cells were washed 3 times before being permeated with 0.5% Triton X-100 for 6 minutes. After 3 rinses with PBS, the cells were blocked in 20% FBS for 1 hour. This was followed by incubation with the primary antibodies at 1:500 in the blocking solution for another 1 hour. The cells were rinsed 3 times in PBS before incubating for 1 hour with the secondary antibodies at 1:500 in blocking solution. After 3 rinses with PBS, the cells were ready for imaging. Secondary controls consisted of samples without primary antibody incubation to test for unspecific binding.

### **3.1.4 Viability staining**

Calcein-AM (Invitrogen) is incubated in cells for approximately 5 minutes at 1:1000. Once inside cells with intact membranes, calcein-AM is hydrolysed by intracellular esterases into fluorescent and impermeable calcein. There are 2 color variants: green and red, and unless stated, calcein-AM green was used. Calcein-AM green and red are excited by the 488 nm and 561 nm laser respectively.

### **3.1.5 Maximum intensity projection**

In this thesis, where a fluorescence image is shown from a 3D dataset, a maximum intensity projection (MIP) was generated in ZEN2009. The intensity of each pixel in the MIP output image is determined by selecting the highest pixel intensity from the corresponding pixels from each plane of the dataset.

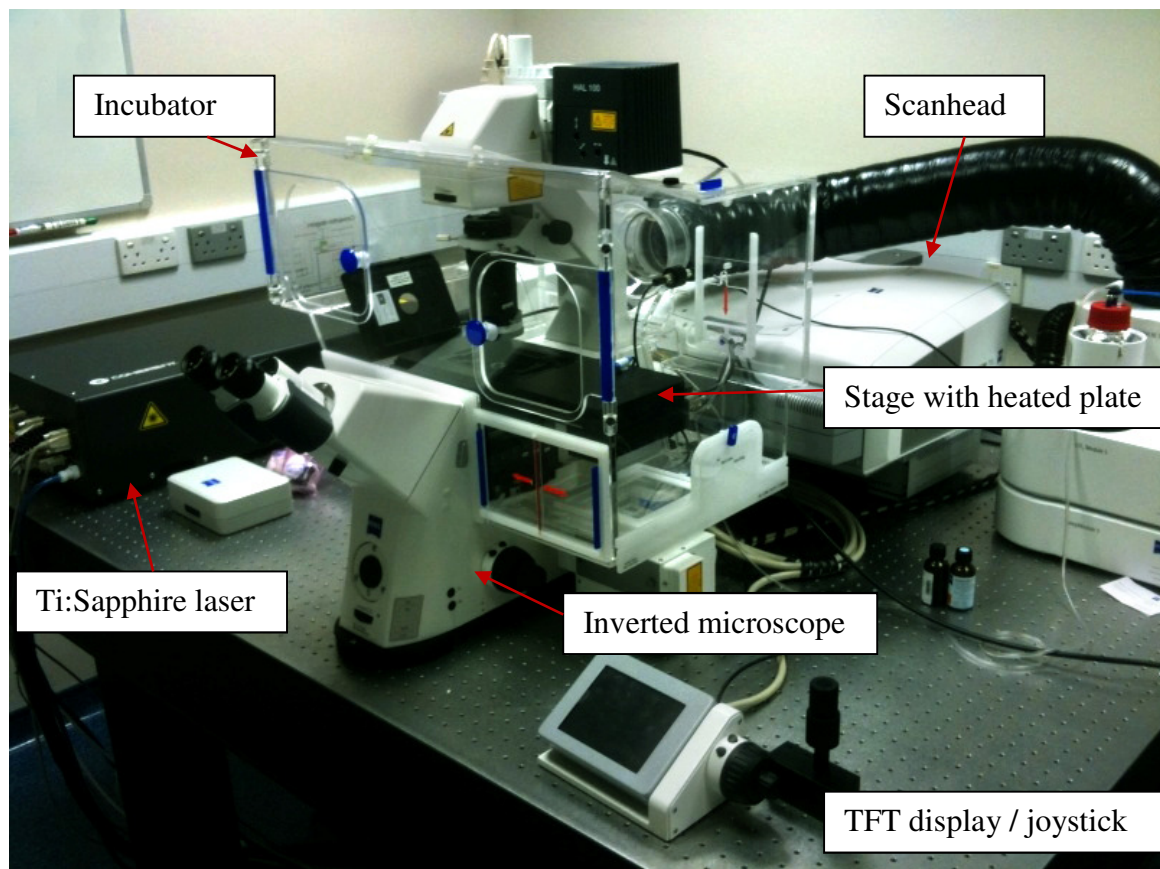
## **3.2 Optoporation using LSM710 (Zeiss)**

Sections 3.2.1 and 3.2.2 detail the system setup used in this thesis for imaging and optoporation.

Section 3.2.3 describes how optoporation was optimised and carried out on cells.

### **3.2.1 System setup**

A tunable (680 to 1080 nm) near infrared (NIR) Ti:Sapphire laser (Coherent, UK) was used to obtain a femtosecond pulsed (140 fs) laser beam that was directly coupled to a multiphoton imaging system. Briefly, it consisted of a Zeiss LSM 710 laser-scanning and control system (Zeiss GmbH, Jena, Germany) coupled to an inverted Zeiss Axio Observer.Z1 microscope (Zeiss GmbH, Jena, Germany), shown in Figure 3-1. The excitation beam for sub-femtoliter focusing was achieved using a high numerical-aperture (NA 1.2) 40x water immersion objective (Zeiss GmbH, Jena, Germany), with a working distance of about 0.28 mm. Stepper motor control of the objective lens focus enabled scanning along the optical z-axis with a minimum step size of less than 25 nm. Control of the stage in the x- and y- axis was motorized with a minimum increment of 1  $\mu\text{m}$ .



**Figure 3-1: LSM710 (Zeiss) multiphoton/confocal microscope setup to be employed for optoporation and monitoring of cells**

### **3.2.2 Non-invasive TPEF and SHG imaging**

The LSM 710 scanhead was coupled with 5 different lasers: diode laser (405 nm, 30 mW), diode laser (440 nm, 25 mW), Ar-laser (458 nm/488 nm/514 nm, 25 mW), DPSS-laser (561 nm, 20 mW), HeNe-laser (594 nm, 2 mW), and HeNe-laser (633 nm, 5 mW). Two high-transmission dichroic filter wheels directed fluorescence emission signal in the near-UV to visible wavelength range towards 2 PMT detectors and 1 array detector allowing for selectable configurations between 2, 3 or 34 QUASAR multi-alkaline PMT detectors.

Serial optical sections of cells were obtained using either NIR excitation at 800 or 850 nm through a depth of 150 to 400  $\mu\text{m}$  with a sub-micro stepsize of 0.9  $\mu\text{m}$ . The laser power for NIR (750, 800 and 850 nm) were regulated using an acoustic optic modulator (AOM) and were measured with a Fieldmaster FM power meter (Coherent, Ely, U. K.) at the back aperture of the objective lens. A transmission detection system consisting of a 0.55 NA condenser lens, coupled multi-alkaline PMT provided brightfield or phase contrast images.

### **3.2.3 Optimisation of laser parameters for optoporation**

Cells were seeded on uncoated glass Petri dishes from World Precision Instruments (Stevenage, UK) at  $5 \times 10^4$  cells per dish and left overnight to attach. The procedure for optimizing laser parameters was adapted from Marchington et al. (2010). Briefly, dishes containing cells were loaded with OptiMEM (Invitrogen) containing DAPI or PI (Sigma Aldrich) at a dilution of (1:1000). The dishes were placed in a heated insert on the microscope stage which kept the environmental conditions at 37 degrees Celsius and 5%  $\text{CO}_2$ . Cells were then optoporated using the NIR Ti:Sapphire two-photon laser at various power levels (5 mW – 200 mW) and exposure times (16 ms – 100 ms). Unless specified, the wavelength of the NIR laser was tuned to 800 nm, which gave peak power. After optoporation, calcein-AM was added to the cells and incubated for 30 minutes. The medium was changed to remove excess dye. The efficiency of each set of parameters was based on the number of cells expressing both DAPI and calcein-AM, since they had been optoporated successfully while still remaining viable.

Manual targeting and optoporation of human fibroblast cells was first performed without an automated routine. The microscope stage was first manually moved so that a group of cells were visible in the field of view. Optoporation was then carried out using either spot scan mode or bleach mode as outlined next.



### *Spot scan mode*

In spot scan mode, the laser is parked at a single point for a pre-defined duration. A cross hair was placed on the membrane of each cell and the laser was fired at 20% power (40 mW) for 40 ms. The cross hair was then repositioned on the next cell and the procedure was repeated. When all the cells in the field of view had been optoporated, the microscope stage was moved to another location and the new set of cells were optoporated as before. It is important to note that spot scan mode can only be used when lengthy exposure times in the order of milliseconds are needed. However, only one cell can be optoporated at a time making this technique very slow. This is in contrast to bleach mode (described next).

### *Bleach mode*

In bleach mode, the laser can scan several precise points/regions very quickly. More than one cell can be targeted at a time. Unlike spot scan mode which can achieve exposure times of the order of several milliseconds, bleach mode exposure time is of the order of microseconds and, therefore, higher power levels are required. Power and exposure times of 50-70% power (100 - 140 mW) and 0.5 - 6.0  $\mu$ s were used in my experiments. Although several regions of varying shapes can be drawn and scanned (unlike spot scan mode), optoporation was carried out on point regions so that the laser would remain stationary for the duration of the exposure time.

# Chapter 4

## Generation of iPS Cells with Electroporation and Lipofection

This chapter first presents the work undertaken in optimising the parameters for efficient transfection using electroporation and lipofection. Each transfection method required optimisation of reagent concentrations or electrical parameters, which when altered, gave a different result when transfecting GFP plasmid into human fibroblasts. The results were documented and are summarised here. Later, this chapter continues by presenting the results obtained using each technique with the focus of generating iPS colonies using combinations of the transcription factors OCT4 (O), SOX2 (S), KLF4 (K), OKS-2A, and STEMcircles as well as GFP/DsRed as a reporter gene. c-Myc was not provided by our collaborators and, therefore, will not be used. OKS-2A was designed by Okita et al. (2008), which combines all 3 transcription factors OCT4, SOX2, KLF4 into a single plasmid. STEMcircles was developed by Jia et al. (2010), which combines the transcription factors of OCT4, SOX2, NANOG, and LIN28 as well as a GFP reporter gene into a single plasmid. Also presented is the use of small molecules in an attempt to improve the efficiency and time required for iPS colony formation.

## **4.1 Materials and Methods**

### **4.1.1 Plasmid amplification through bacterial transduction**

1 µl of plasmid DNA was pipetted into an Eppendorf tube along with 40 µl of competent bacterial cells and left on ice for 20 minutes. The cells were then heatshocked by incubating in a waterbath at 42 degrees Celsius for exactly 45 seconds and then placed back on ice for at least one minute.

The mixture was incubated at 37 degrees Celsius while shaken in the presence of 300 µl of LB (lysogeny broth) medium for 1 hour. 200 µl of the mixture was plated onto a gel consisting of a 50:50 mixture of 2X LB medium and agar, which was allowed to solidify in a small petridish along with the appropriate antibiotics at 100 µg/ml in LB medium. Ampicillin was used for OCT4, SOX2, and KLF4 while Kanamycin was used for GFP, OCT4-GFP, DsRed, and yellow fluorescent protein (YFP) respectively. Control plates were also seeded with E.Coli but without plasmid DNA. The plates were incubated at 37 degrees Celsius overnight (15 hours), at which time, several colonies had appeared with the exception of the control plate. A single colony was picked and transferred to a starter culture tube containing a 50:50 mixture of 2X LB and double distilled water (ddH<sub>2</sub>O) with the appropriate antibiotics at 100 µg/ml dilution. This was incubated in a shaker for 12 hours until the mixture became cloudy. 1 ml of this was transferred to a large 2 litre flask containing 600 ml of LB medium with 500 µl of the appropriate antibiotics and shaken in an incubator for 8 hours. 400 µl was taken from this culture and mixed with 600 µl of 50% glycerol and frozen in cryotubes at -80 degrees Celsius. The remaining culture was centrifuged at 5,000 g for 20 minutes and the supernatant removed. The pellet was either frozen down or used immediately to prepare plasmid DNA using a GenElute Plasmid Maxiprep kit (Sigma). After determining the purity by measuring the absorbance ratio at 260 nm and 280 nm (A<sub>260</sub>/A<sub>280</sub>) to be between 1.8 and 2.0 using a NanoDrop, the purified plasmid DNA was diluted to a concentration of 1 mg/ml and frozen at -80 degrees Celsius.

#### **4.1.2 Lipofectamine LTX and PLUS reagent (Invitrogen)**

Experiments were carried out in 6-well plates coated with 0.1% gelatin. Cells were cultured in these plates overnight so that their confluency was approximately 50% the next day. Before transfection, the medium in each well was switched to antibiotics-free medium (DMEM, 1% GlutaMAX, 10% FCS). For each well, 2.5 µg of plasmid DNA was added to 100 µl of DMEM. When using individual factors (OCT4, SOX2, KLF4, GFP), these were added in the following proportions : 0.7 µg each for O, S, and K, 0.4 µg for GFP. When using OKS-2A plasmid and GFP, this was added as 2 µg for OSK, and 0.5 µg for GFP. 2.5 µl of PLUS reagent was then added to the plasmid mixture and allowed to incubate for 10 minutes at room temperature. After adding 6.25 µl of Lipofectamine LTX, the entire mixture was allowed to incubate for 30 minutes at room temperature. The mixture was then slowly added to each well and the plate was gently rocked to achieve a homogeneous distribution of the liposomes. The media was changed 6 hours later with the same culture medium without antibiotics. The next day, the medium was changed to sterile human embryonic stem cell (hESC) medium containing antibiotics and basic fibroblast growth factor (bFGF) at 4 ng/ml.

#### **4.1.3 Electroporation with Neon transfection system**

Electroporation was carried out with the Neon Transfection System (Invitrogen). The kit consists of an electroporator control unit, a docking station, pipette, R and E buffer. Additional reagents required are PBS, antibiotics-free culture medium, appropriate sized culture flasks/dishes, and a centrifuge.

Beforehand, a sufficient number of 6-well plates were coated with gelatin and each well was filled with 2 ml of antibiotics free medium (DMEM, 10% FCS, 1% GlutaMAX).

The cells to be electroporated were grown in flasks/plates until 80% confluent in fibroblast medium (DMEM, 10% FCS, 1 % GlutaMAX, 1% antibiotics). The cells were rinsed with PBS and then lifted off using trypsin. The trypsin was neutralized by serum-containing fibroblast medium and then centrifuged at 1,000 rpm for 5 minutes. The supernatant was removed and the pellet was resuspended in PBS. The mixture was centrifuged a second time where the pellet was resuspended in R buffer containing the appropriate amount of plasmid DNA required. For a 6-well format, a total of 4 µg of DNA was used. When using individual factors (O, S, K, GFP), these were added in the ratio of 3:3:2:1. When using OKS-2A plasmid and GFP, this was in the ratio of 3:1. One day after electroporation, the medium was changed to hESC medium with antibiotics and bFGF at 4 ng/ml.

#### ***4.1.3.1 Supplementation of small molecules for iPS generation enhancement***

Valproic acid, Sodium butyrate and 5'azacytidine (5'AZA) were purchased from Sigma-Aldrich. Suberoylanilide hydroxamic acid (SAHA) was purchased from Cambridge Biosciences. These compounds were tested as they have already been cited in literature for possessing the ability to increase the efficiency of iPS generation or replace certain transcription factors. For key references citing the use of these 4 compounds, please see section 2.2.2.2.5. For 5'Azacytidine, a fresh stock solution was made each time because of its instability. Each vial contained approximately 1 mg and was dissolved in 4.1 ml of regular culture medium, which resulted in a 1 mM solution. This was later diluted to 1 µM, 5 µM, and 10 µM in regular culture medium. SAHA was first dissolved in DMSO to give a 1M solution. This stock solution was divided into aliquots and frozen down. To make a working stock, 1 µl was serially diluted in 1 ml of culture medium to 1 mM. This was further diluted to the working concentration of 1 µM, 5 µM, and 10 µM. Controls contained the same volume of solvent used to dissolve the small molecules.

SAHA and 5'AZA were added 2 days after electroporation to allow the cells to recover. The medium, which was supplemented with small molecules was changed every second day.

#### **4.1.4 A combination approach of electroporation and lipofection using STEMcircles**

STEMcircles (StemCell Technologies, Vancouver, Canada) are plasmids containing a combination of the OCT4, SOX2, LIN28, Nanog transcription factors as well as a GFP reporter gene. The bacterial components have been removed so as to reduce the size to just 6.1 kb for more efficient transfection.

The protocol developed by Jia et al. (2010) consists of electroporation (day 1) followed by 2 rounds of lipofection (day 4 and 6) to maintain expression levels. Although the protocol has been optimised for the Nucleofector (Amaxa) electroporator, this particular system was unavailable so the optimisation had to be repeated with the Neon Electroporation system. A total of  $0.5 \times 10^5$  human fibroblast cells were first electroporated with the Neon Electroporator with 0.5  $\mu$ g of STEMcircles under various electrical parameters (shown in Table 4-1) and seeded into each well of a 24-well plate using 10  $\mu$ l tips. Optimisation was performed by trial and error as per manufacturer's instructions. The cells were incubated overnight in normal growth medium without antibiotics (DMEM, 10% FBS, and 1% GlutaMAX). The next day, cells were stained with Hoechst 33342 and incubated for 10 minutes. The cells were thoroughly washed 3 times with PBS before imaged with the LSM 710. Three 5-by-5 tile stacked images were acquired for each well and cell type (IMR90 & HDFa) for analysis later.

The datasets were loaded and processed in Imaris (Bitplane AG, Switzerland). GFP positive cells (green) and nuclei (blue) were separately surface rendered and counted. The efficiency was determined by the number of transfected cells (fluorescing green from GFP) divided by the total

number of cells in the well, which had their nuclei stained blue. The average was taken over the 3 datasets for each electroporation parameter.

The most optimal electroporation parameter was 2 pulses each of width 20 ms and 1400 volts. This was selected based on the highest transfection efficiency that maintained reasonable cell viability. Once the most efficient set of parameters were decided upon, the protocol developed by Jia et al. (2010) was then adapted for use. Cells were electroporated at  $3 \times 10^5$  with 0.75  $\mu\text{g}$  of STEMcircles and seeded into T25 flasks. The next day, day 1, the medium was changed to complete growth media containing antibiotics. Cells were cultured for 3 days where their confluency increased to 50 - 75% confluent before being lipofected with 10  $\mu\text{l}$  of PLUS reagent, 5  $\mu\text{l}$  of LTX, and 1.25  $\mu\text{g}$  of STEMcircles in 2 ml of antibiotics-free media on day 4. The medium was changed on day 5, and lipofection was repeated on day 6. The following day, cells were reseeded on a feeder layer in hESC media supplemented with bFGF. The media was changed every second day from there on.

#### **4.1.5 Induced pluripotent stem cell culture**

iPS-like colonies were generated from mouse embryonic fibroblasts between passage 4 - 5 or human adult dermal fibroblast (Invitrogen) between passage 5 - 7 and cultured in 6-well plates on a feeder layer of inactivated mouse fibroblasts. hiPS cell culture medium consisted of 160 ml high glucose DMEM, 40 ml KO serum replacer, 2 ml of antibiotics, 2 ml of non-essential amino acids, and 2-mercaptoethanol diluted in PBS. For miPS cell medium, ES cell qualified FBS was used instead of Knock-Out serum replacer. The medium was filter sterilised before adding basic fibroblast growth factor and used within 1 week.

#### **4.1.6 Mouse feeder layer preparation**

Mouse fibroblasts were harvested from 13.5 - 14.5 day old mouse embryos. Mouse fibroblasts were either frozen down at passage 2 or grown in T75 flasks and passaged up until passage 5 and then treated with 50 µl of mitomycin C in 5 ml of medium for 2 - 3 hours at 37 degrees Celsius. The cells were then washed with PBS at least 3 times and then lifted off with trypsin. The cells were neutralised with regular culture medium and then spun down at 1,000 rpm for 5 minutes. The cells were then replated at  $10^6$  cells per gelatinized 6- well plate and incubated overnight before use. Prior to seeding transfected cells onto the feeder layer, the medium was changed to hESC medium.

## **4.2 Optimisation of Electroporation Parameters**

### **4.2.1 STEMcircles**

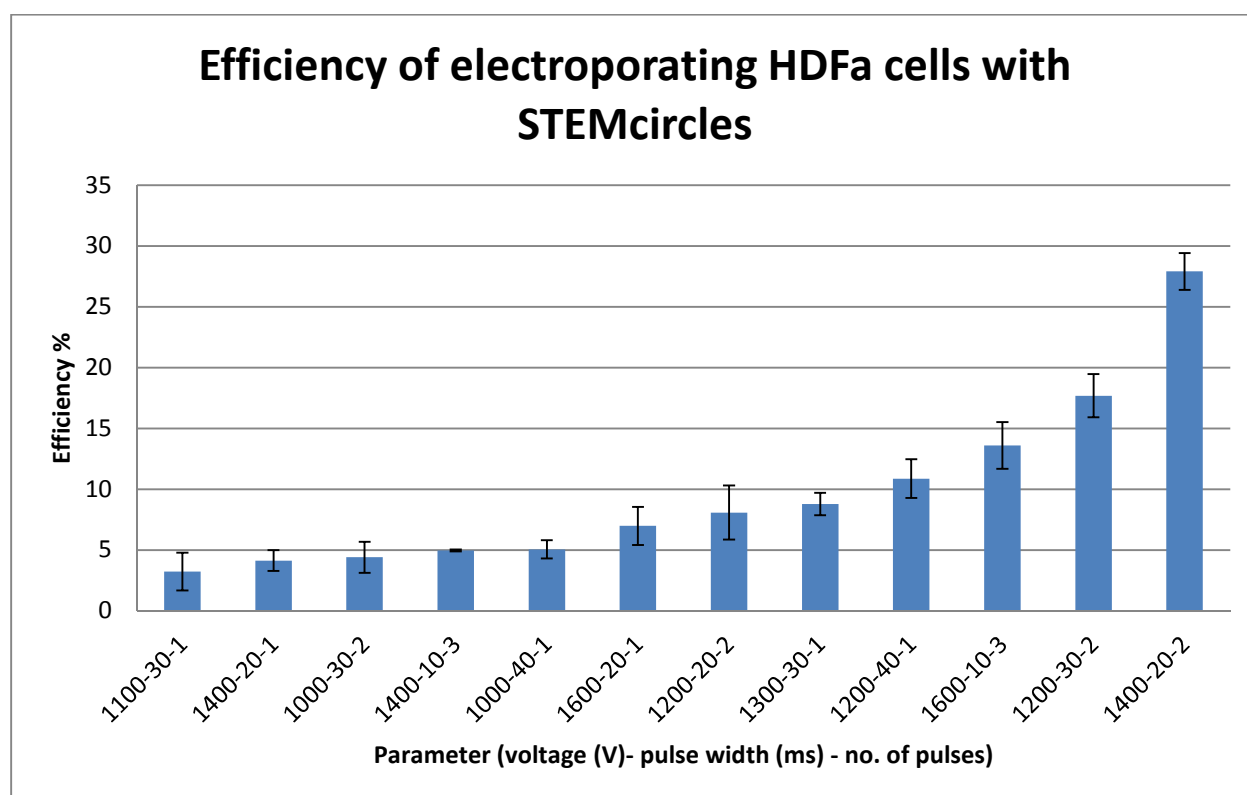
Human dermal adult fibroblast (HDFa) cells were electroporated at various parameters shown in Table 4-1. Each parameter consisted of electroporating 100,000 cells with 0.5 µg of STEMcircles and seeded into a well of a 24-well plate. The following day, the cells were stained with Hoechst 33342 and imaged under fluorescence microscopy for GFP expression.



**Table 4-1: List of parameters supplied by Invitrogen for optimising electroporation of HDFa with STEMcircles. Transfection efficiency was obtained by counting GFP positive cells divided by total cell number (indicated by Hoechst nuclear staining) from three 5x5 tile-scan images for each parameter. More than 15,000 cells were analysed for each parameter. Experiment was conducted once.**

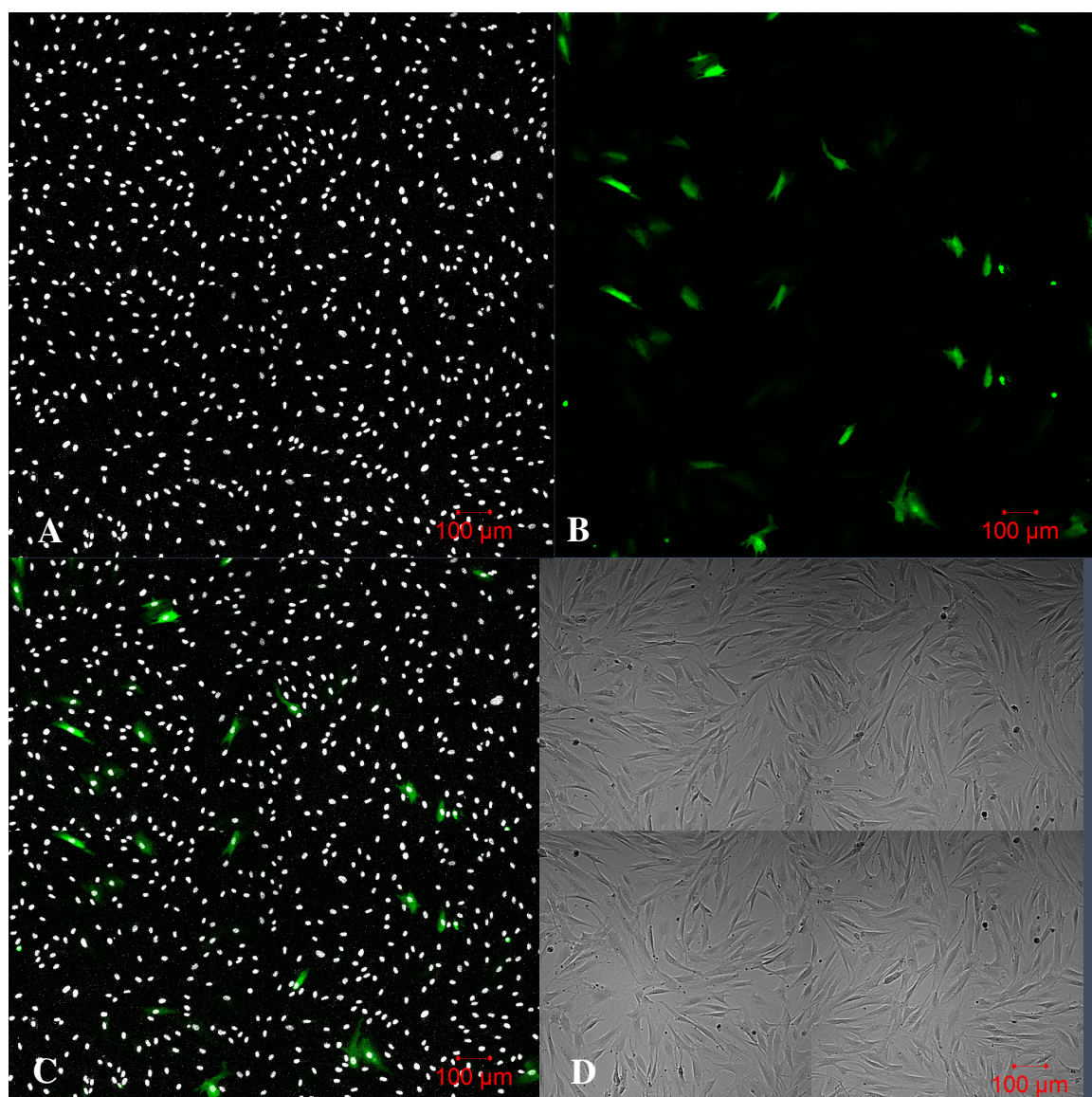
| <b><u>Well</u></b> | <b><u>Pulse voltage<br/>(V)</u></b> | <b><u>Pulse width<br/>(ms)</u></b> | <b><u>Pulse no.</u></b> | <b><u>Transfection<br/>efficiency (%)</u></b> |
|--------------------|-------------------------------------|------------------------------------|-------------------------|-----------------------------------------------|
| 1                  | 1400                                | 20                                 | 1                       | 4.1                                           |
| 2                  | 1600                                | 20                                 | 1                       | 6.9                                           |
| 3                  | 1100                                | 30                                 | 1                       | 3.2                                           |
| 4                  | 1300                                | 30                                 | 1                       | 8.8                                           |
| 5                  | 1000                                | 40                                 | 1                       | 5.1                                           |
| 6                  | 1200                                | 40                                 | 1                       | 10.9                                          |
| 7                  | 1200                                | 20                                 | 2                       | 8.1                                           |
| 8                  | 1400                                | 20                                 | 2                       | 27.9                                          |
| 9                  | 900                                 | 30                                 | 2                       | 4.4                                           |
| 10                 | 1100                                | 30                                 | 2                       | 17.6                                          |
| 11                 | 1400                                | 10                                 | 3                       | 5.0                                           |
| 12                 | 1600                                | 10                                 | 3                       | 13.6                                          |

The transfection efficiency was calculated by dividing the number of GFP cells by the total number of nuclei stained with Hoechst, the result is charted in Figure 4-1.



**Figure 4-1: Graph showing % efficiency of electroporating HDFa cells with STEMcircles based on GFP fluorescence 1 day later. The graph is based on data from Table 4-1.**

One of the least efficient parameters (1400 volts, 20 ms, 1 pulse) gave rise to just 4.1% transfected cells, shown in Figure 4-2.

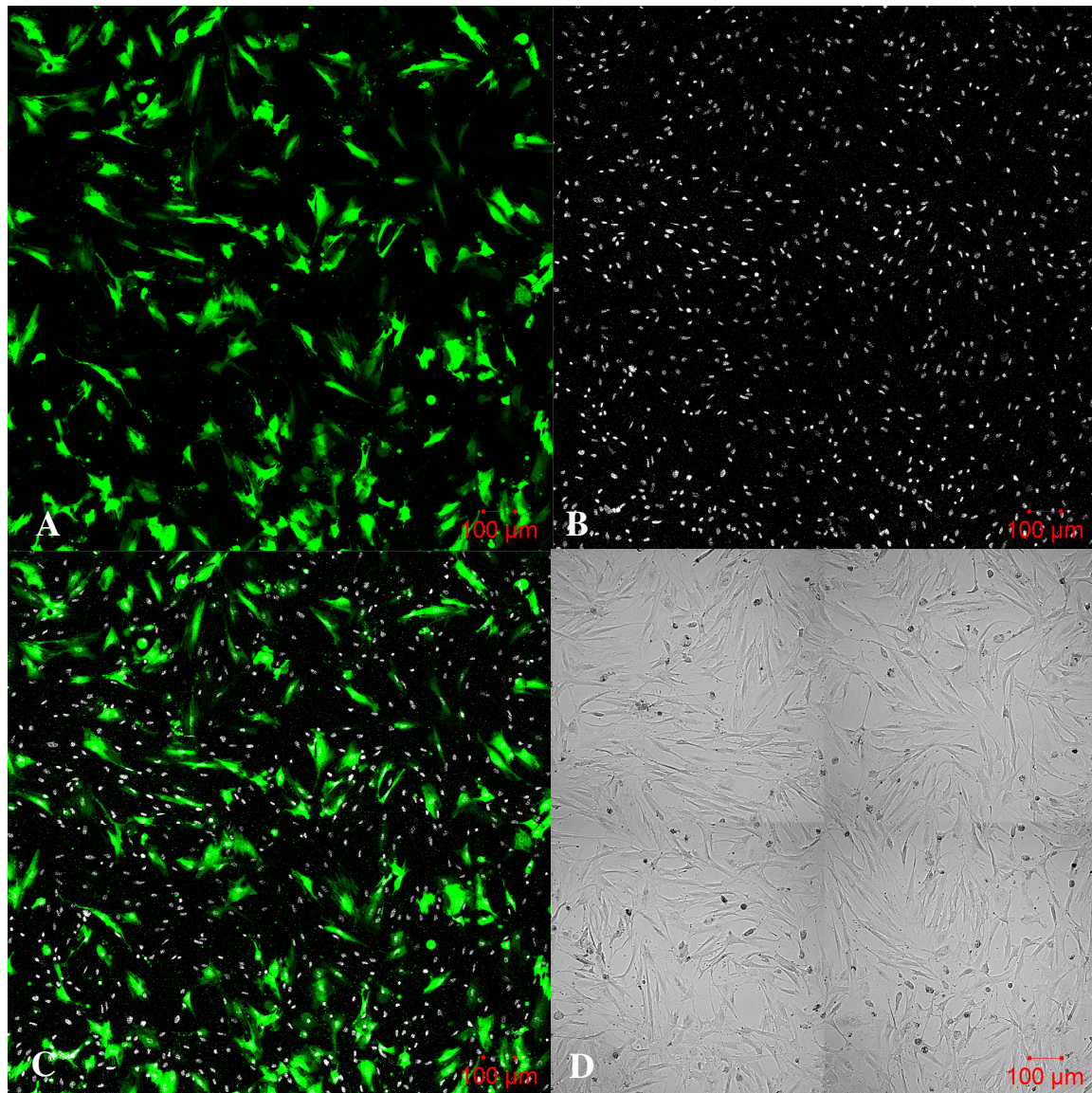


**Figure 4-2: Simultaneous imaging of human fibroblasts electroporated at 1400 volts, 20 ms, 1 pulse. (A) Hoechst nuclear staining, (B) GFP fluorescence (green), (C) merged of (A & B), (D) transmission image. Image is representative from 1 experiment.**

Interestingly, when the number of pulses was increased to 2 pulses, this became the most successful parameter with an overall efficiency of 27.9%. Not only was there a dramatic increase in the number of GFP positive cells, the expression of GFP protein (observed by green



fluorescence in Figure 4-3) was also higher. This suggests that more GFP plasmid may have entered the cells at this parameter.



**Figure 4-3: Simultaneous imaging of human fibroblasts electroporated at 1400 volts, 20 ms, 2 pulse. (A) GFP fluorescence (green), (B) Hoechst nuclear staining, (C) merged of (A & B), (D) transmission image. Image is representative from 1 experiment.**

### 4.2.2 GFP

GFP was optimized in human fibroblasts in the same manner as STEMcircles. The most optimal condition appeared to be 1400 volts, 20 ms, and 2 pulses in Figure 4-4 based on the number of green fluorescent cells as judged by visual inspection.

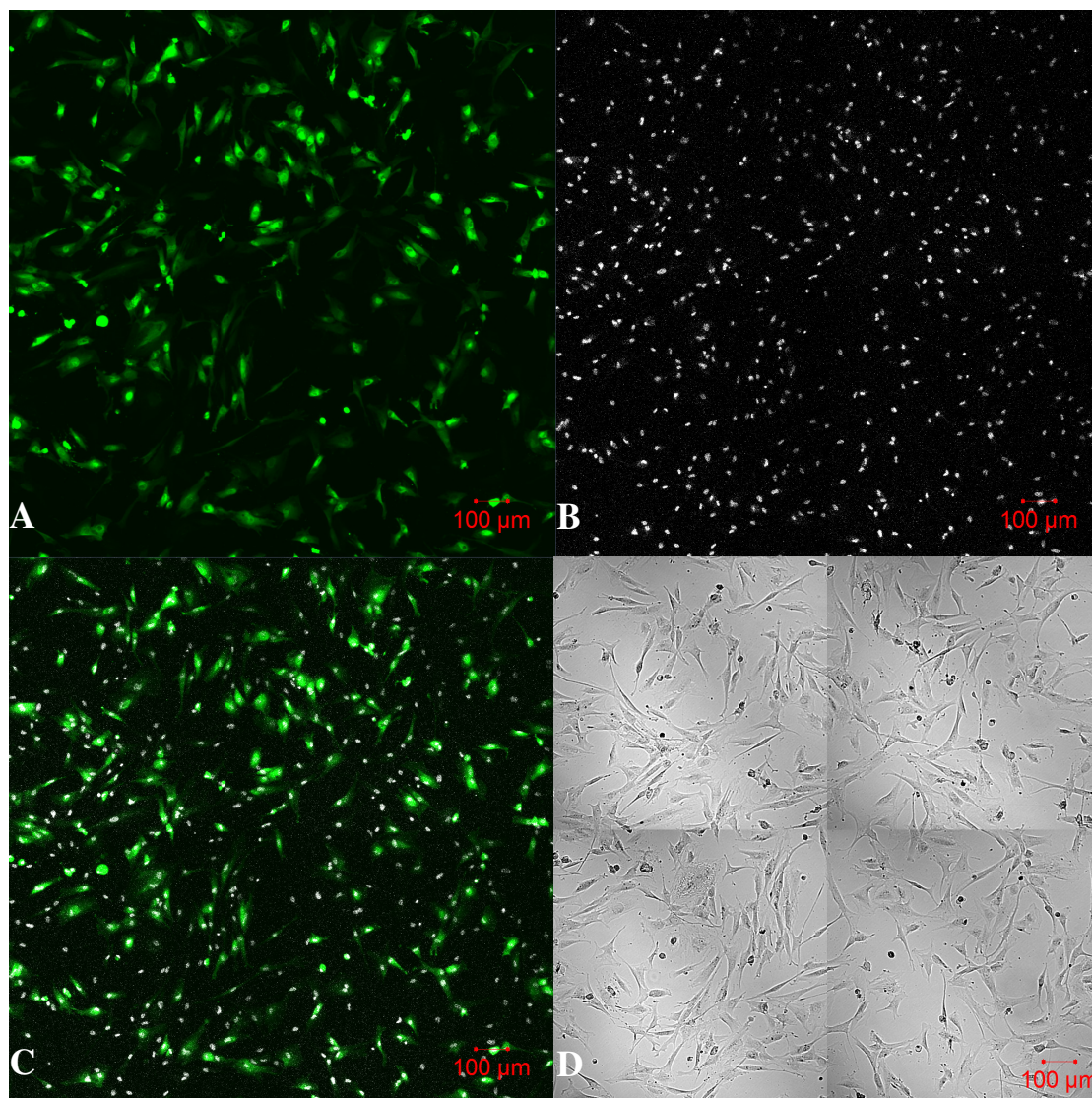


Figure 4-4: Simultaneous imaging of human fibroblasts electroporated at 1400 volts, 20 ms, 2 pulse. (A) GFP fluorescence (green), (B) Hoechst nuclear staining (blue), (C) merged of (A & B), (D) transmission image. Image is representative from 1 experiment.



To further optimise GFP transfection, the voltage was increased at 25 volt intervals from 1350 - 1450 volts. The pulse width and number of pulses were kept constant. The efficiency peaked at 1400 volts according to the number of GFP positive cells and dropped to just 10% at 1450 volts. Few cells also survived the electroporation and the resulting confluency was only half compared to when 1400 volts were used.

Increasing the concentration of GFP plasmid from 1 µg to 2 µg increased not only efficiency, but also cell death. According to the manufacturer's instructions, 2 µg is 4 times the recommended amount anyway and may have been excessive. Because of this fact as well as the decrease in cell viability as plasmid concentration increases, the use of 1 µg of plasmid were maintained for further experiments.

### 4.3 Optimisation of Lipofection Parameters

Lipofection was carried out with Lipofectamine LTX and PLUS reagent (both from Invitrogen). The parameters that can be altered are the amounts of DNA, LTX, and PLUS reagents. A diagrammatic layout of the parameters used is shown in Figure 4-5.

|            | Ratio of lipid to DNA in each column |          |          |          |          |          |
|------------|--------------------------------------|----------|----------|----------|----------|----------|
| DNA amount | 1x                                   | 2x       | 2.5x     | 3x       | 4x       | 5x       |
| 250 ng     | 0.250 µl                             | 0.500 µl | 0.625 µl | 0.750 µl | 1.000 µl | 1.250 µl |
| 500 ng     | 0.500 µl                             | 1.000 µl | 1.250 µl | 1.500 µl | 2.000 µl | 2.500 µl |
| 750 ng     | 0.750 µl                             | 1.500 µl | 1.875 µl | 2.250 µl | 3.000 µl | 3.750 µl |
| 1000 ng    | 1.000 µl                             | 2.000 µl | 2.500 µl | 3.000 µl | 4.000 µl | 5.000 µl |

**Figure 4-5: Diagrammatic representation of a 24 well showing GFP plasmid DNA amounts and LTX volumes added to each well for optimisation.**

Wells 6, 11, and 14 gave the most number of GFP cells with reasonable toxicity. The parameters are listed below:

Well 6: 250 ng DNA, 0.25  $\mu$ l PLUS reagent, 1.25  $\mu$ l LTX

Well 11: 500 ng DNA, 0.5  $\mu$ l PLUS reagent, 2.0  $\mu$ l LTX

Well 14: 750 ng DNA, 0.75  $\mu$ l PLUS reagent, 1.5  $\mu$ l LTX (shown in Figure 4-6)

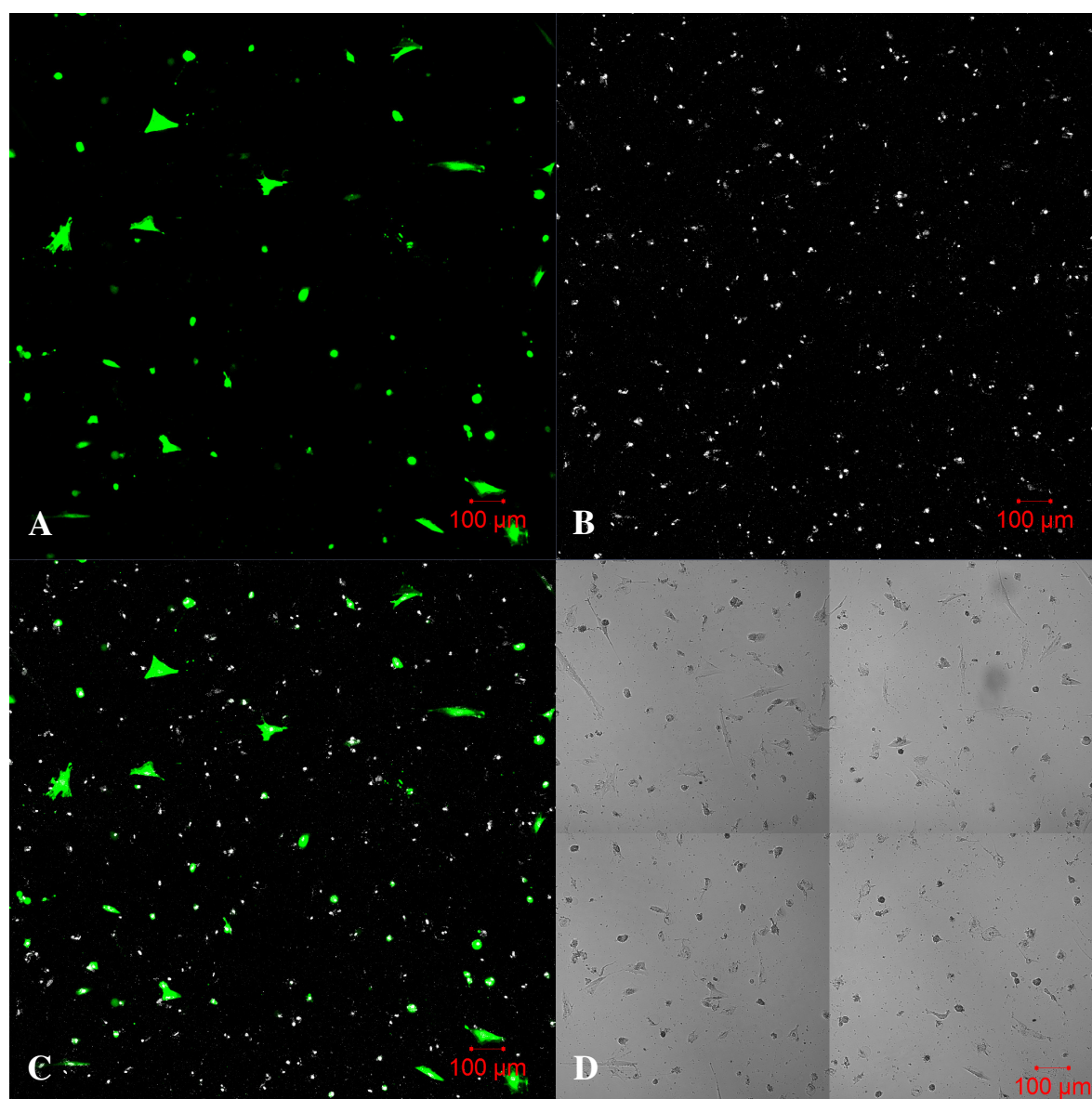


Figure 4-6: Simultaneous imaging of human fibroblasts lipofected with 750 ng GFP plasmid, 0.75  $\mu$ l PLUS reagent, 1.5  $\mu$ l LTX. (A) GFP fluorescence (green), (B) Hoechst nuclear staining, (C) merged of (A & B), (D) transmission image. Image is representative from 1 experiment.

The efficiency ranged from 22 - 29% (similar to electroporation with GFP). Well 11 had the most number of dead cells stained with propidium iodide. Furthermore, most of the transfected cells appeared to be dying as well. Wells 6 and 14 (Figure 4-6) both had fewer dead cells than well 11. However, well 14 had more GFP positive cells, whereas the cells in well 6 seemed healthier. Thus, the parameters for wells 6 & 14 were adopted for further experiments depending on whether efficiency or viability was more important.

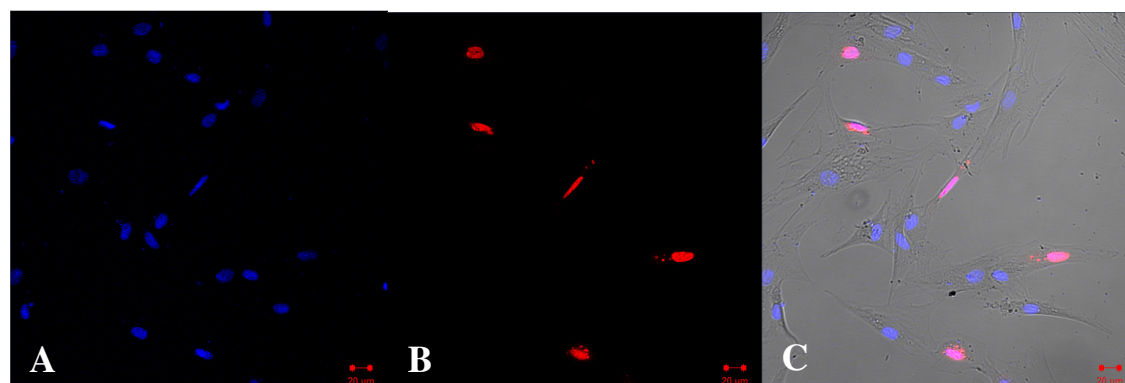
It could be observed that as the volume of LTX and PLUS reagent was increased, there was considerable cell death. The cells were healthiest in wells 1 and 2 where there was virtually no cell death (data not shown). The efficiency in these wells, however, was at the lowest.

#### **4.3.1 Immunostaining of HDFa lipofected with OCT4, SOX2, and KLF4**

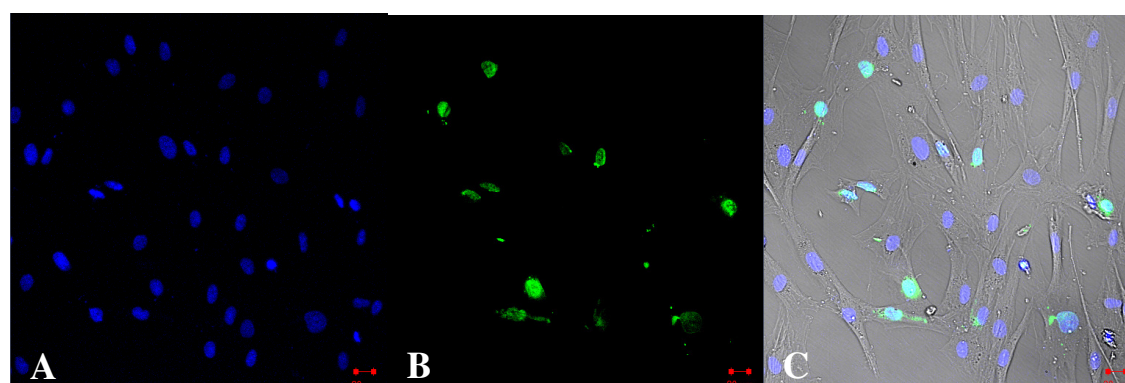
Using the parameters in well 6, HDFa cells were lipofected individually with the plasmids OCT4, SOX2 and KLF4. These parameters were chosen based on moderate efficiency but with low toxicity. 24 hours post transfection, the cells were stained with the appropriate primary and secondary antibodies and imaged. The primary antibodies (OCT4 mouse IgG2b; SOX2 goat IgG; KLF4 goat IgG) were purchased from santa cruz biotechnology. The secondary antibodies (Alexafluor 488 rabbit anti-goat IgG and Alexafluor 568 goat anti-mouse IgG2b) were purchased from Invitrogen. Negative controls were also setup with plasmid DNA but without transfection reagent. Secondary controls without primary antibody were also setup to show the absence of unspecific binding of the fluorescent antibody. A GFP control suggested that lipofection was not toxic to cells (figure not shown).

### 4.3.2 OCT4, SOX2, KLF4

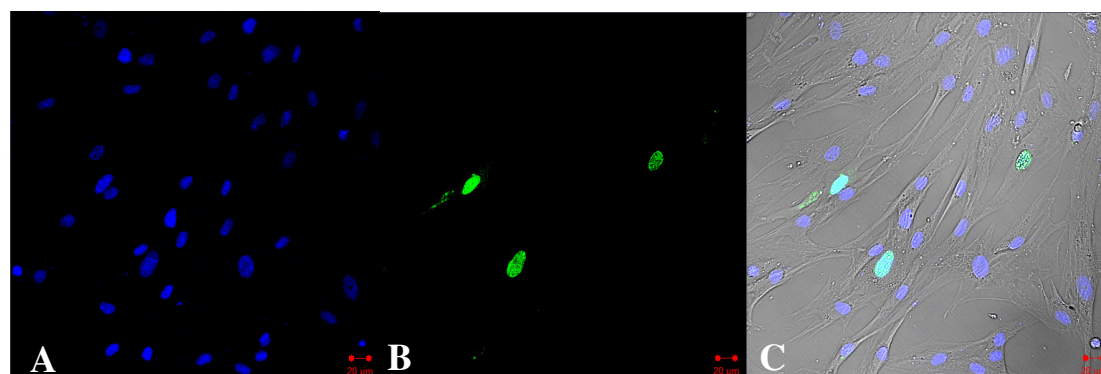
Immunostaining of OCT4, SOX2, and KLF4 are shown in Figure 4-7, Figure 4-8, and Figure 4-9 respectively. The staining co-localises with DAPI staining and is therefore nuclear. The transfection efficiencies are summarised in Table 4-2.



**Figure 4-7:** Simultaneous imaging of human fibroblasts electroporated with 0.25 µg of OCT4 plasmid. (A) Hoechst nuclear staining (blue), (B) immunostained OCT4 (red), (C) merged. Image is representative from 1 experiment.



**Figure 4-8:** Simultaneous imaging of human fibroblasts electroporated with 0.25 µg of SOX2 plasmid. (A) Hoechst nuclear staining (blue), (B) immunostained SOX2 (green), (C) merged. Image is representative from 1 experiment.



**Figure 4-9:** Simultaneous imaging of human fibroblasts electroporated with 0.25 µg of KLF4 plasmid. (A) Hoechst nuclear staining (blue), (B) immunostained KLF4 (green), (C) merged. Image is representative from 1 experiment.



**Table 4-2: Summary of transfection efficiencies for each transcriptional factor and GFP. Transfection efficiency was obtained by counting cells stained positively by appropriate antibody divided by total number of cells (indicated by Hoechst nuclear staining) from three locations in the same well. Approximately 30 cells were analysed. Experiment was conducted once.**

| <u>Plasmid</u> | <u>Transfection efficiency (%)</u> |
|----------------|------------------------------------|
| OCT4           | 15                                 |
| SOX2           | 20                                 |
| KLF4           | 14                                 |
| GFP            | 27                                 |

It is interesting to note that the transfection efficiency for GFP is consistently higher than all other plasmids, with KLF4 having the lowest range. This suggests that if GFP is simultaneously transfected into cells as a separate plasmid, it is not an accurate reporter gene for the transcription factors.

#### **4.4 Optimisation of Small Molecule Concentration**

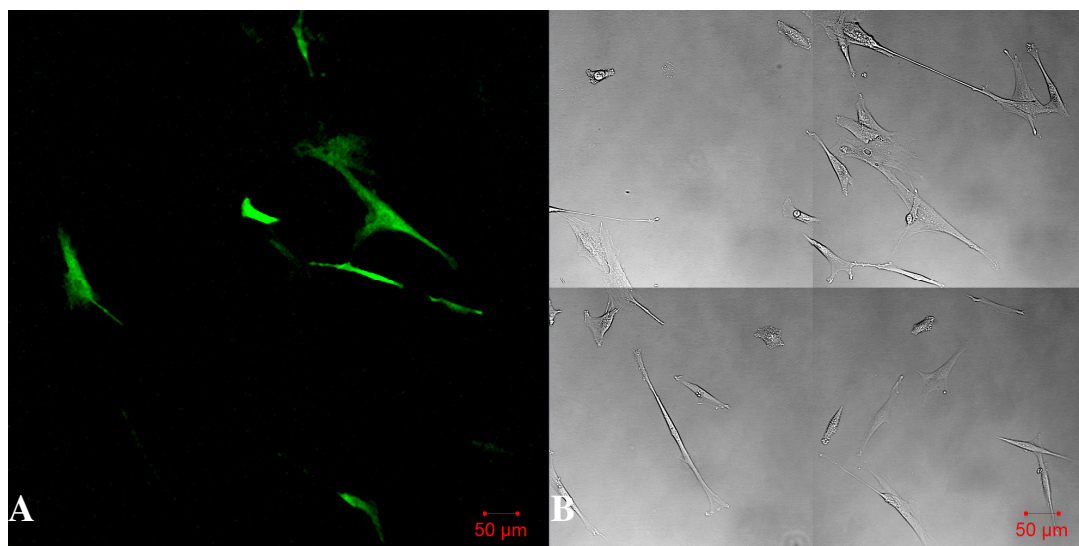
As mentioned in section 4.1.3.1, small molecules were added to the culture to increase reprogramming efficiency. Human fibroblasts were cultured in the presence of 5'AZA and SAHA at various concentrations (Table 4-3) 1 day after electroporation (1400 volts, 20 ms, 2 pulses). The plasmids used were OCT4, SOX2, KLF4 (1.25 µg each), and GFP (0.25 µg). The cells were then observed the next day for toxicity based on morphology. Control samples contained only the solvent (DMSO). As the cells were to be further cultured for iPS generation, the use of PI to determine cell viability was ruled out due to its toxicity.

**Table 4-3: Concentrations of SAHA and 5'AZA used for optimisation**

| <u>SAHA (µM)</u> | <u>5'AZA (µM)</u> | <u>SAHA+5'AZA (µM)</u> |      |
|------------------|-------------------|------------------------|------|
| 1                | 1                 | 1                      | 1    |
| 5                | 10                | 5                      | 10   |
| 10               | 500               | 10                     | 500  |
| 20               | 1000              | 20                     | 1000 |
|                  |                   | 20                     | 500  |

#### 4.4.1 Control

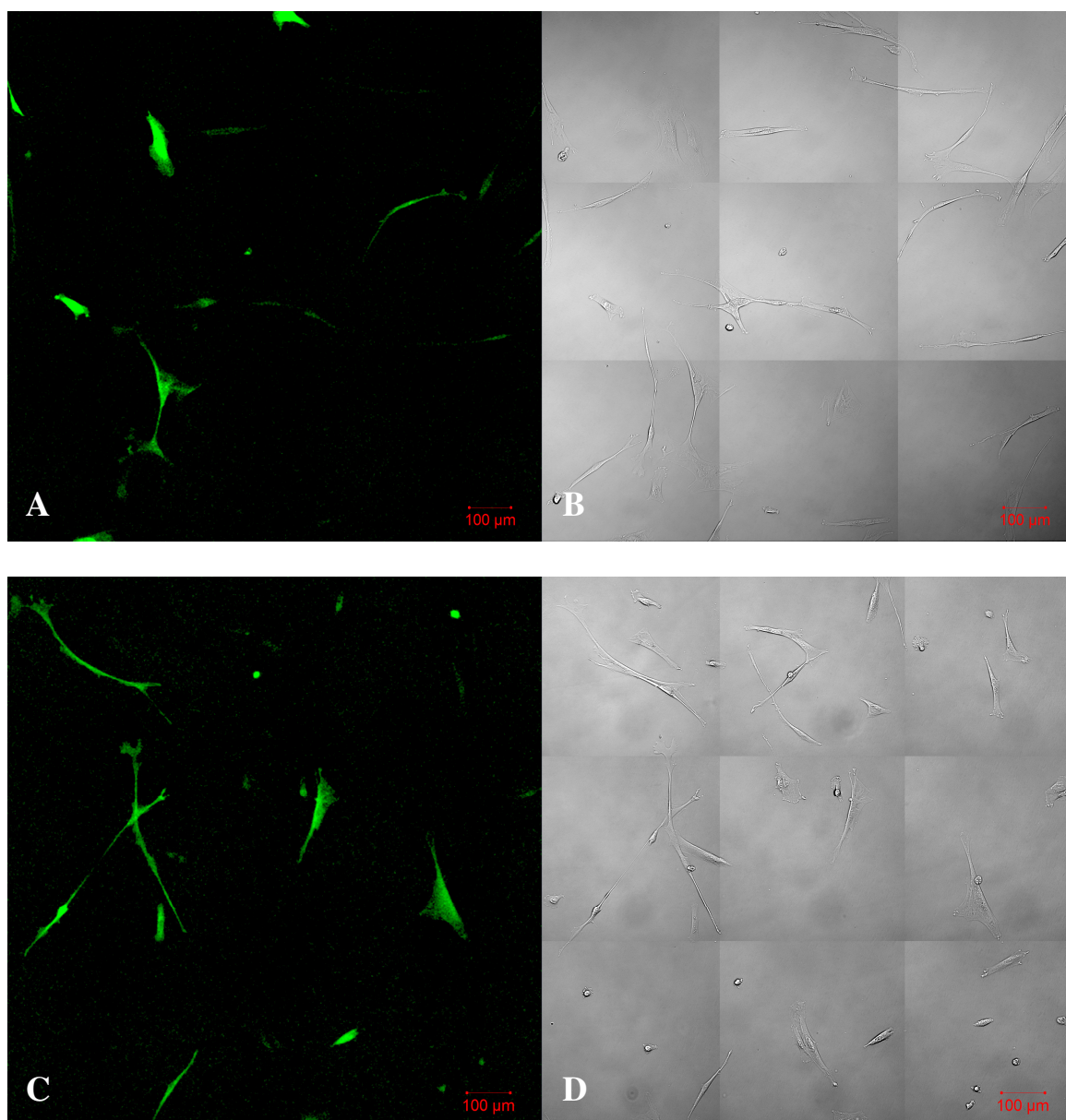
In the control sample, Figure 4-10, cells appeared normal and attached. As expected, there were a small number of detached cells.



**Figure 4-10: Simultaneous (A) GFP fluorescence (green) and (B) transmission imaging of human fibroblasts control group. Cells were electroporated at 1400 volts, 20 ms, 2 pulses. Medium did not contain small molecules. Image is representative from 1 experiment.**

#### 4.4.2 SAHA

Concentrations of 1  $\mu\text{M}$  and 5  $\mu\text{M}$  were least toxic. Cells did not round-up and lift off from the plate, which would signal cell death. Instead, they continued to attach and displayed fibroblast-like morphology after 24 hours in SAHA as shown in Figure 4-11 B. At 5  $\mu\text{M}$  (Figure 4-11 C & D), there were a small number of detaching cells, however, the healthy cells were still long, narrow, and expressing GFP. The level of expression was similar to the control sample in Figure 4-10A. At 10  $\mu\text{M}$ , the cells appeared similar to 5  $\mu\text{M}$ . All cells had died in 20  $\mu\text{M}$  of SAHA, thus, 10  $\mu\text{M}$  was optimal since there was little change between 5  $\mu\text{M}$  and 10  $\mu\text{M}$ .

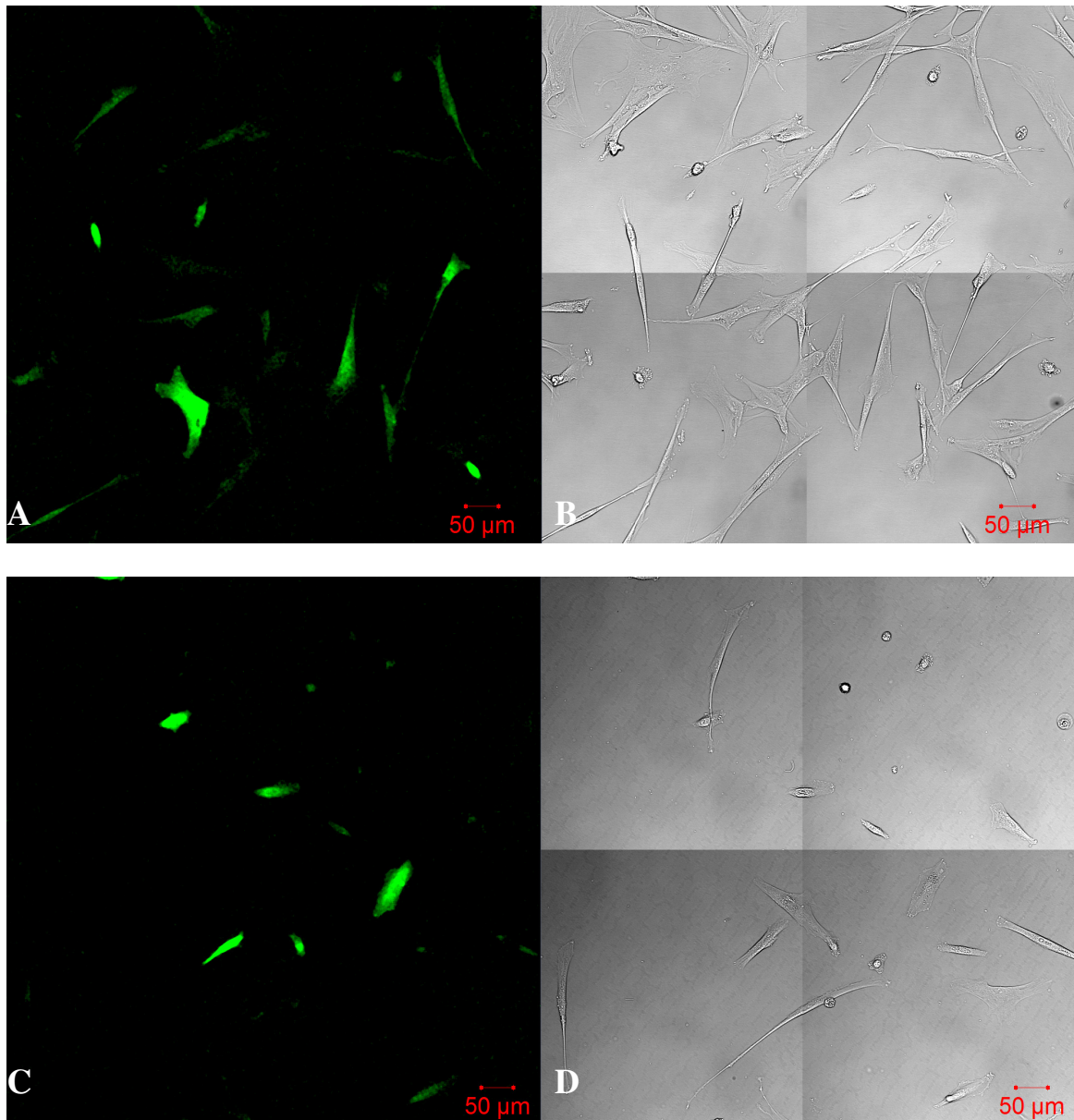


**Figure 4-11: Simultaneous (A & C) GFP fluorescence (green) and (B & D) transmission imaging of electroporated human fibroblasts 24 hours after supplementation with (A & B) 1  $\mu$ M and (C & D) 5  $\mu$ M of SAHA. Image is representative from 1 experiment.**

#### 4.4.3 5'AZA

At 1  $\mu$ M, the cells were most healthy, shown in Figure 4-12 B. The cells appeared to be more confluent, however, this is due to uneven seeding causing more cells to attach in this specific area. At 10  $\mu$ M, a larger number of cells started to die, shown in Figure 4-12 D. 0.5 mM and above resulted in high cell death. 10  $\mu$ M was chosen for further experiments as the number of

surviving GFP expressing cells was still high. It was assumed that the sample would recover and the surviving cells would divide and make up for the cells which had died.



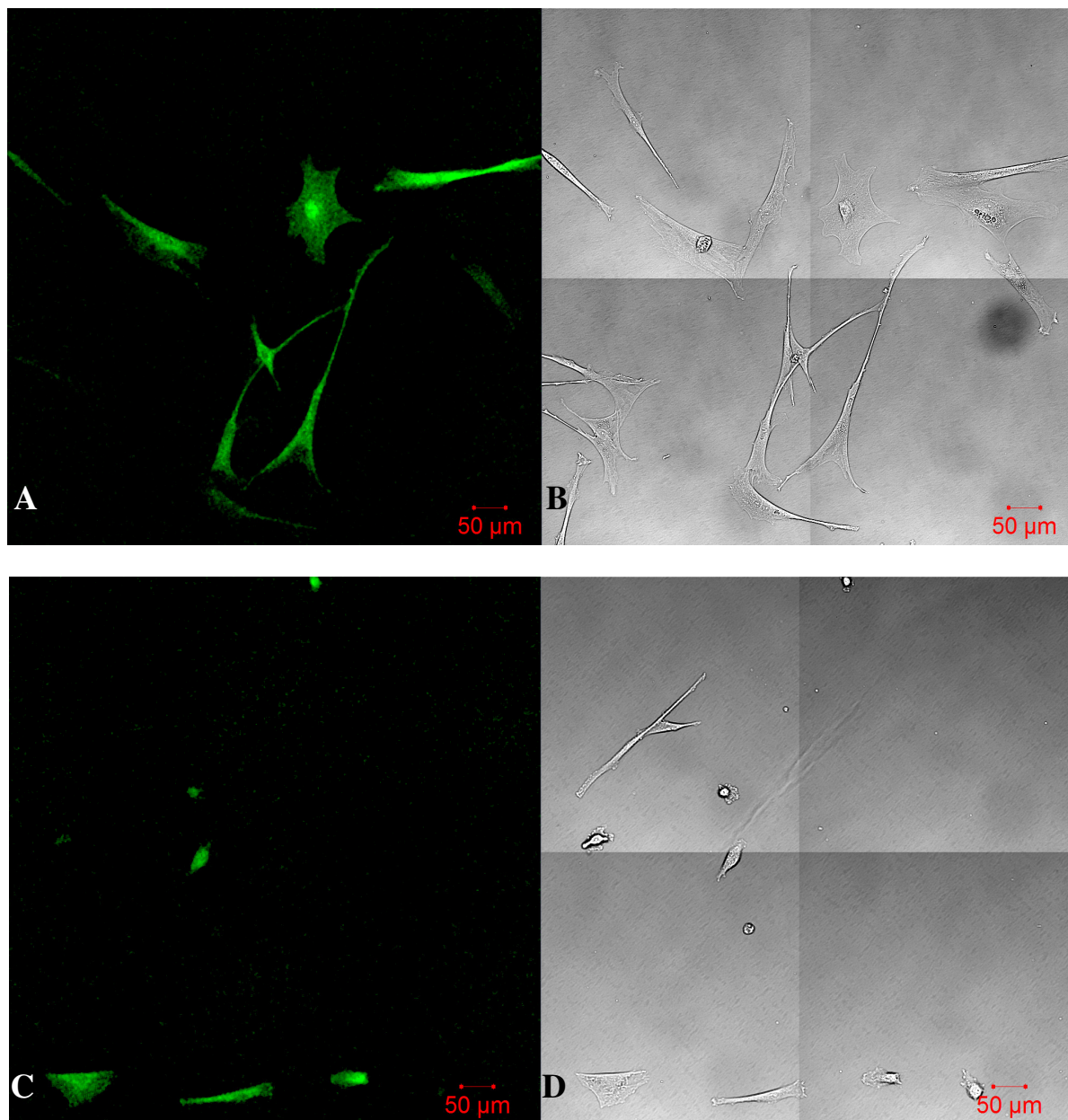
**Figure 4-12: Simultaneous (A & C) GFP fluorescence (green) and (B & D) transmission imaging of electroporated human fibroblasts 24 hours after supplementation with (A & B) 1  $\mu$ M 5'AZA, (C & D) 10  $\mu$ M 5'AZA. Image is representative from 1 experiment.**

#### **4.4.4 SAHA + 5'AZA**

When SAHA and 5'AZA were used individually at 1  $\mu$ M each (Figure 4-13 A & B), the samples were healthiest. When these compounds were combined at the same concentrations, the sample was nearly identical to when they were used individually. The expression levels of GFP were comparable in both samples.

If the concentrations of SAHA and 5'AZA were to be increased to 5  $\mu$ M and 10  $\mu$ M respectively (Figure 4-13 C & D), the cells appeared less healthy similar to when 10  $\mu$ M of 5'AZA was used on its own. A larger number of dead cells were present, however, the cells which were expressing GFP survived and remained attached. Increasing the concentration of 5'AZA to mM range caused massive cell death. Thus, using SAHA and 5'AZA concentration at 5  $\mu$ M and 10  $\mu$ M respectively was near optimal and was used for further experiments. Individually, SAHA at 10  $\mu$ M and 5'AZA at 10  $\mu$ M were also repeated later.





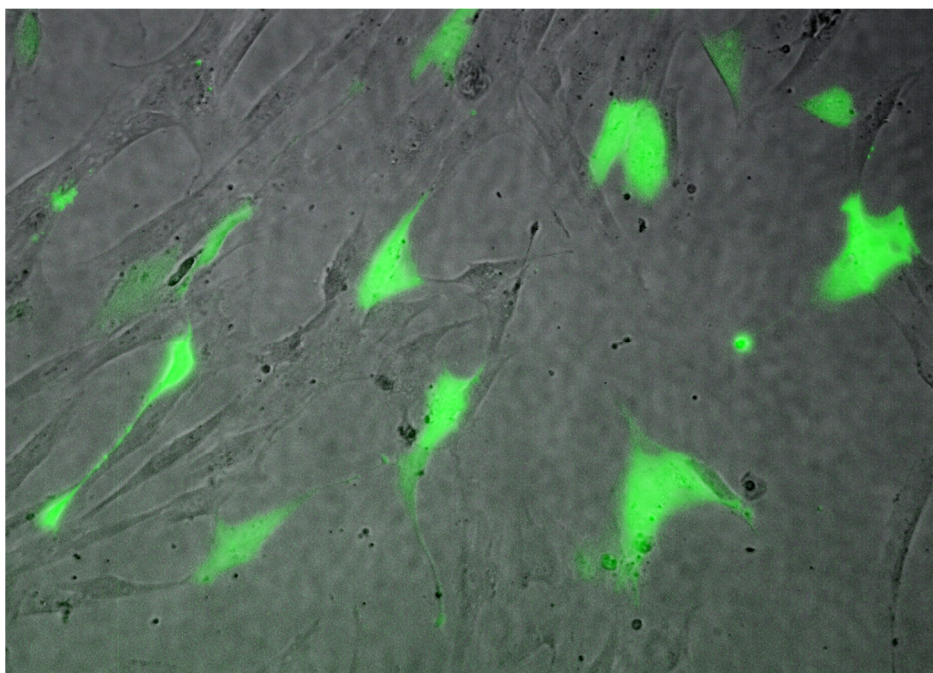
**Figure 4-13:** Simultaneous (A & C) GFP fluorescence (green) and (B & D) transmission imaging of electroporated human fibroblasts 24 hours after supplementation with (A & B) 1  $\mu$ M of SAHA and 1  $\mu$ M of 5'AZA, and (C & D) 5  $\mu$ M of SAHA and 10  $\mu$ M of 5'AZA. Image is representative from 1 experiment.

## 4.5 Results : Derivation of iPS colonies

### 4.5.1 iPS-like colonies from Lipofectamine LTX and PLUS reagent

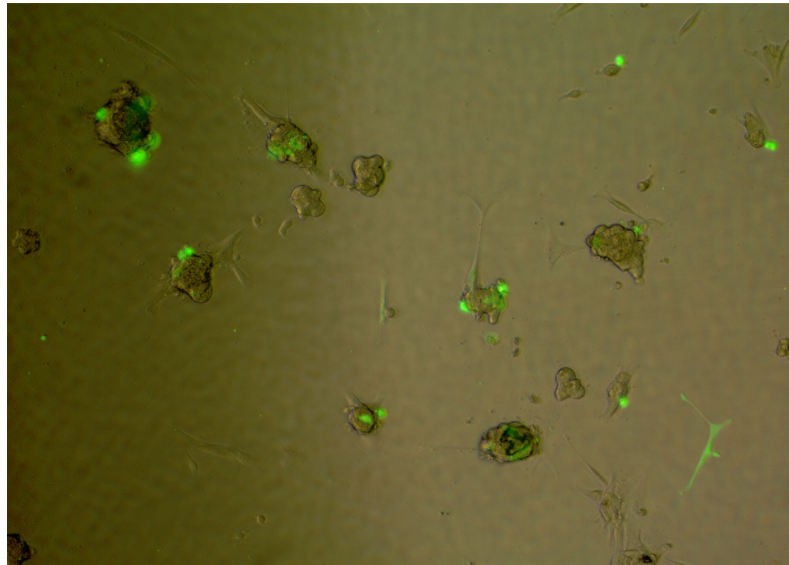
Adult human dermal fibroblast cells (HDFa) were transfected with lipofectamine LTX and PLUS reagent. According to the manufacturer's instructions, PLUS reagent improves efficiency

of transfection. When the cells were lipofected with OKS-2A (plasmid containing OCT4, SOX2, and KLF4 in single plasmid) and GFP plasmid, the majority of cells highly expressed GFP protein the next day (Figure 4-14). The cells appeared healthy and displayed the characteristic fibroblast morphology.

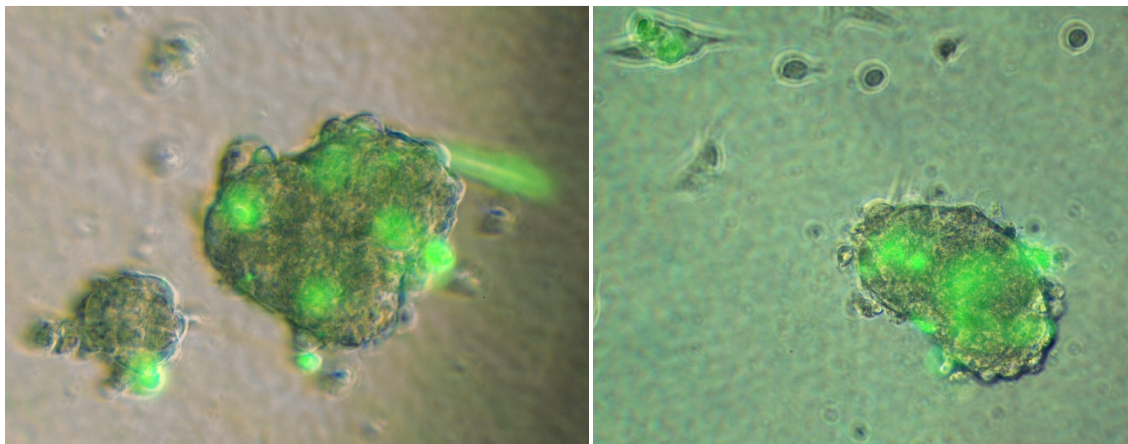


**Figure 4-14: Simultaneous GFP fluorescence (green) and transmission image of human fibroblasts 1 day after lipofection with 750 ng GFP plasmid, 0.75  $\mu$ l PLUS reagent, 1.5 $\mu$ l LTX. 10x objective lens used.**

By day 3, some of the cells had clustered together to form aggregates. A small number of cells within the clusters continued to express GFP. Figure 4-15 is an overview image of the number of clusters found in a particular area. Figure 4-16 shows a closeup of 3 of such clusters.



**Figure 4-15:** Simultaneous GFP fluorescence (green) and transmission image of clusters 3 days after lipofection with 750 ng GFP plasmid, 0.75  $\mu$ l PLUS reagent, 1.5  $\mu$ l LTX. 10x objective lens used.

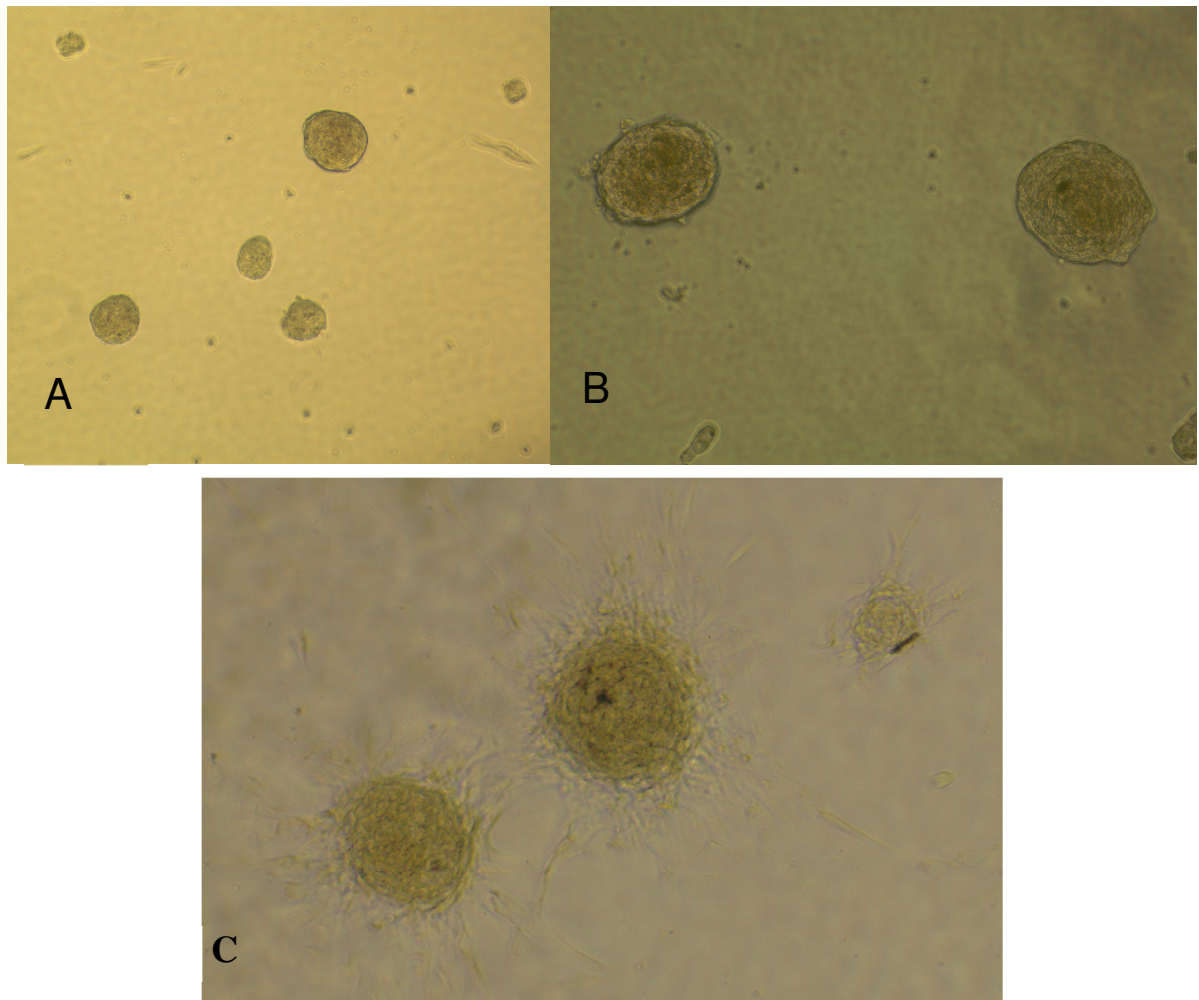


**Figure 4-16:** Simultaneous GFP fluorescence (green) and transmission image of 2 clusters 3 days after lipofection with 750 ng GFP plasmid, 0.75  $\mu$ l PLUS reagent, 1.5  $\mu$ l LTX. 20x objective lens used.

After 10 days, the colonies had further enlarged and increased in number. However, the cells in the colonies no longer expressed GFP, as observed in Figure 4-17 A and B. The colonies had a circular morphology containing cells which were tightly packed. The boundary of each colony was also very well defined without any signs of differentiation from the peripheral cells. This was in contrast to day 16 colonies (Figure 4-17 C) whose boundaries already showed signs of differentiating cells. The peripheral cells appeared fibroblastic with narrow and spindle-shaped



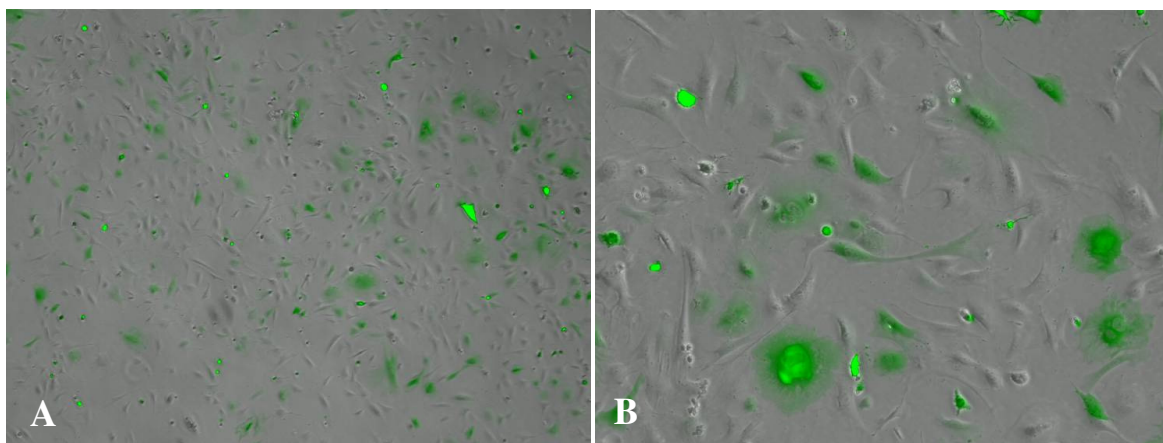
morphologies. The central cells, however, were still tightly packed, which is characteristic of ES and iPS cell colonies.



**Figure 4-17:** Transmission imaging of iPS-like colonies (A & B) 10 days and (C) 16 days after lipofection with 750 ng GFP plasmid, 0.75  $\mu$ l PLUS reagent, 1.5 $\mu$ l LTX. Objective lens used was (A & C) 10x and (B) 20x.

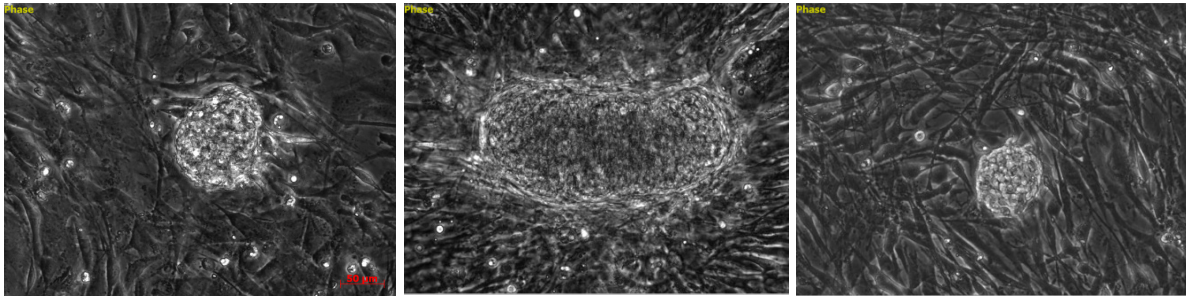
#### **4.5.2 Electroporation of MEFs**

Mouse embryonic fibroblasts at passage 4 were electroporated at 1400 volts, 20 ms, and 2 pulses and seeded into 6-well plates. Each well contained  $0.5 \times 10^6$  MEFs with 3  $\mu$ g of 3-in-1 plasmid (OKS-2A) and 1  $\mu$ g of GFP plasmid in antibiotics-free medium. Transfection efficiency ranged between 30 - 50%. When imaged, there was high intensity of GFP fluorescence (Figure 4-18).



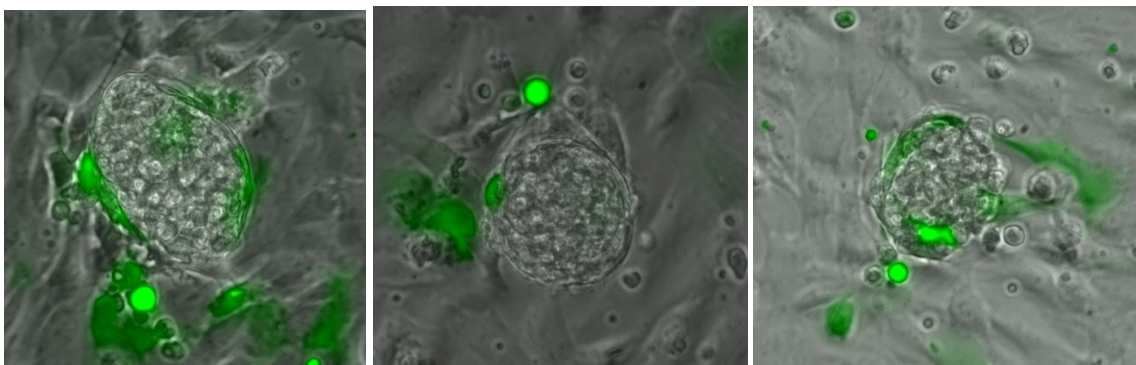
**Figure 4-18: Simultaneous GFP fluorescence (green) and transmission imaging of mouse fibroblasts 1 day after electroporation at 1400 volts, 20 ms, 2 pulses. Objective lens used was (A) 4x and (B) 10x.**

The protocol used was modified from Okita et al. (2008) where 4 transfections at 2 day intervals were needed before passaging. When this was tried in this thesis, the overall cell population declined dramatically due to the number and frequency of transfections. Instead, the protocol was changed so that cells were transfected 3 times at 3 day intervals to allow more time for recovery between electroporation. One day after the final transfection, the medium was switched to mESC medium (see section 4.1.5) supplemented with LIF. On day 11 (5 days after last transfection), 3 colonies appeared, shown in Figure 4-19. None of the colonies expressed GFP after 11 days in culture (Figure 4-19). Similar to the colonies obtained through lipofection with the OKS-2A plasmid, the cells in these colonies were tightly packed. The morphology of the colonies was round (except for one colony which was long and tubular Figure 4-19 B) with a distinct boundary that was easily identifiable against the monolayer of fibroblast cells beneath the colonies.



**Figure 4-19: Phase contrast images of 3 iPS-like colony derived from mouse fibroblasts 5 days after the 3<sup>rd</sup> and final electroporation at 1400 volts, 20 ms, 2 pulses.**

These colonies did not grow larger even after several more days in culture, thus, **the experiment was repeated again with a new fresh batch of cells** (passage 3). Unexpectedly, 8 colonies had formed the next day after just 1 transfection. Several cells within the colonies were expressing GFP, shown in Figure 4-20. Individual cells within the colonies could be discerned from the transmission image alone. The morphology of these colonies was similar to the previous experiment (Figure 4-19). Another similarity was that the colonies did not grow despite being cultured for at least another week in mESC medium with LIF.

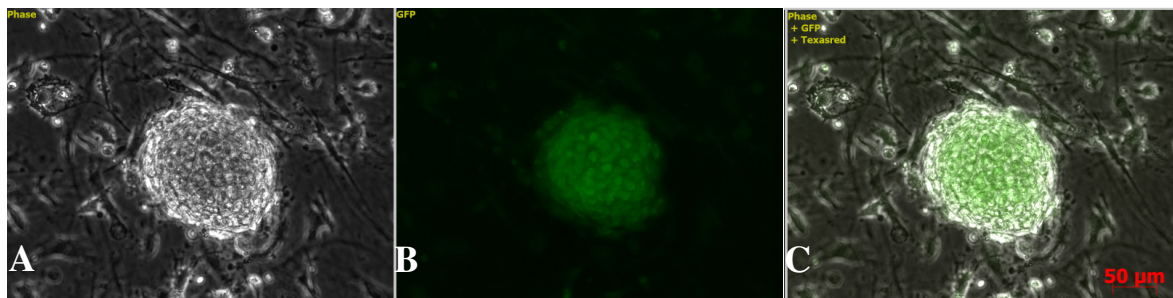


**Figure 4-20: Simultaneous GFP fluorescence (green) and transmission imaging of 3 iPS-like colonies derived from mouse fibroblasts 1 day after the 1<sup>st</sup> electroporation of a new attempt at the same experiment. These were generated in a new experiment under the same conditions at 1400 volts, 20 ms, 2 pulses. 10x objective lens used.**

### 4.5.3 Electroporation of human fibroblasts

Human fibroblasts (HDFa) were electroporated and then seeded into 6-well plates with  $0.5 \times 10^6$  cells per well. Each well was electroporated with either 1  $\mu\text{g}$  each of individual plasmids (OCT4, SOX2, KLF4, and GFP) or 3  $\mu\text{g}$  of OKS-2A plasmid and 1  $\mu\text{g}$  of GFP plasmid. Since different cells may require different protocols, HDFa may be able to withstand 4 transfections rather than just 3 (as in the case of MEFs). Cells were electroporated four times on days 1, 4, 7, and 13 in antibiotics-free fibroblast medium. After the last transfection, the medium was switched to hESC medium containing 20% KO serum (see section 4.1.5).

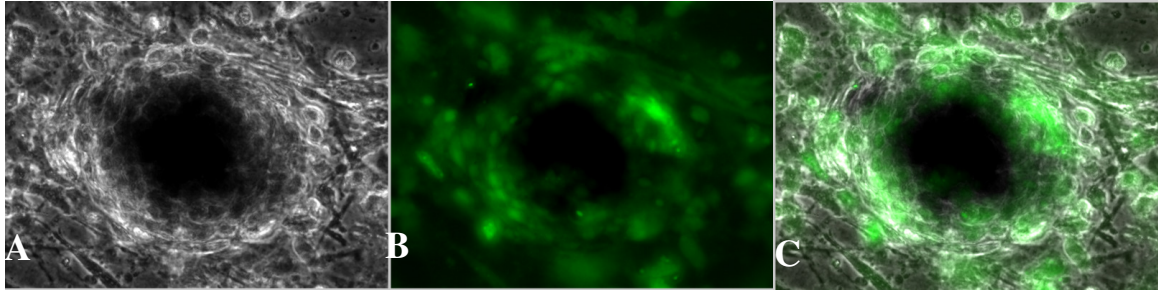
In the sample that was electroporated with the OKS-2A plasmid, no colonies were observed. However, on day 23 (10 days after last transfection), 3 colonies appeared in the sample with individual factors. One of these colonies, shown in Figure 4-21, had a large population of GFP positive cells. When imaged, all three colonies had tightly packed cells and distinct boundaries.



**Figure 4-21: Simultaneous imaging at day 23 of iPS-like colony derived from human fibroblasts after 4<sup>th</sup> electroporation at 1400 volts, 20 ms, 2 pulses. (A) phase contrast image, (B) GFP fluorescence (green), (C) merged image.**

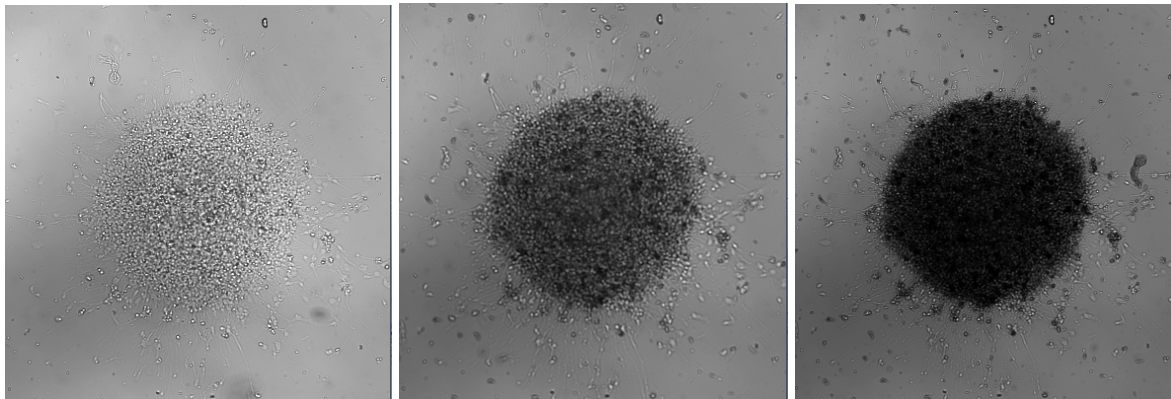
On day 36 (23 days after last transfection), two of the colonies were tested for alkaline phosphatase (AP). The test, which is the most basic test for iPS and ES colony formation, resulted in a dark blue precipitate within 10-15 minutes in the centre of each colony (Figure 4-22). It can be observed that precipitation had formed only in the colony, but not in any of the surrounding fibroblast cells.





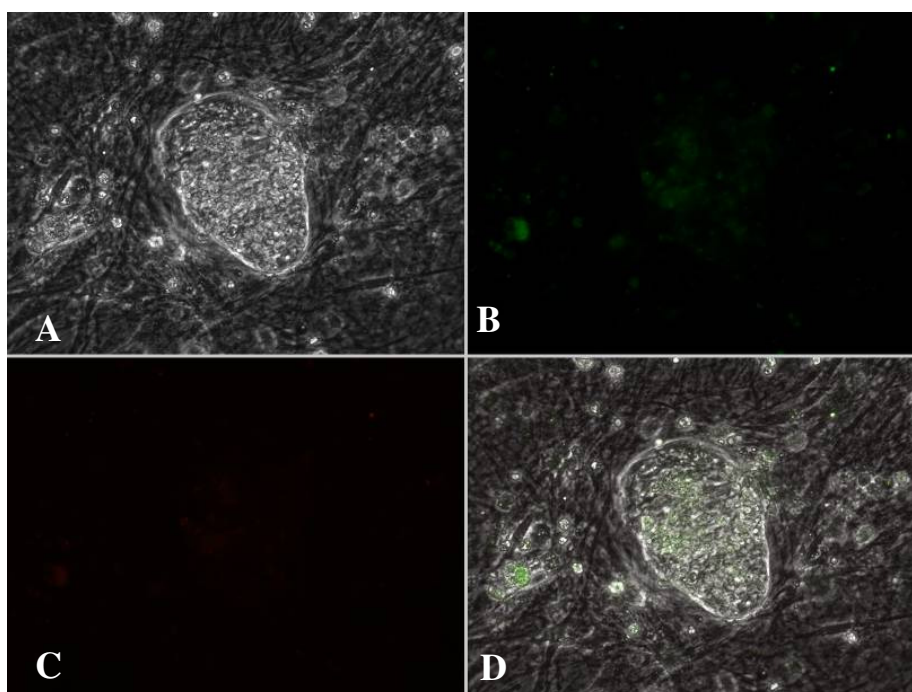
**Figure 4-22: Simultaneous imaging of a 36 day old iPS-like colony undergoing the alkaline phosphatase precipitation test. (A) phase contrast image, (B) GFP fluorescence (green), (C) merged image. 40x objective lens used.**

Figure 4-23 captures 3 frames of a time-lapse sequence of a true iPS colony (derived through retroviral transfection) during alkaline phosphatase staining. The precipitate took approximately 10 minutes to form, which stained the entire colony. In panel 2 of the figure below, a small number of cells within the colonies could be seen to have darkened from precipitation much sooner than the other cells. The time taken for precipitation to occur was similar to the above mentioned colonies derived through electroporation. The colour and level of precipitation was also consistent with the above results.



**Figure 4-23: Images of a timelapse sequence of a true iPS colony undergoing the alkaline phosphatase precipitation test. Duration of timelapse was 10 minutes. 10x objective lens used.**

The positive result of this test suggested that the 3 colonies derived through plasmid transfection could be iPS colonies, but further stringent tests were required for further verification. Positive staining by SSEA4 and TRA-1-81 are considered robust and standard indicators of reprogrammed human iPS cells ever since Yu et al. (2007) generated human iPS cells. All 3 colonies were stained simultaneously with SSEA4-tagged-Alexafluor 488 (green fluorescence) and TRA-1-81-tagged-Phycoerythrin (red fluorescence) antibodies. SSEA4 (cat. no.: 53-8843) and TRA-1-81 (cat. no.: 12-8883) were purchased from eBioscience, UK. These tests do not require cells to be fixed. After the test, none of the 3 colonies had stained positive for either of these antibodies. One example is shown in Figure 4-24.

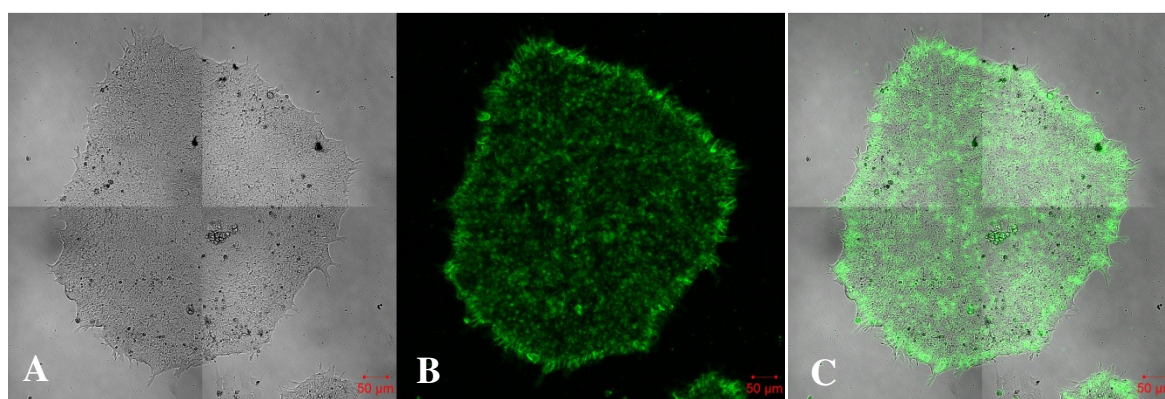


**Figure 4-24: Simultaneous imaging of an iPS-like colony stained with iPS surface marker antibodies. (A) phase contrast image, (B) SSEA4-Alexafluor 488 (green), (C) TRA-1-81-PE (red), (D) merged image. 20x objective lens used.**

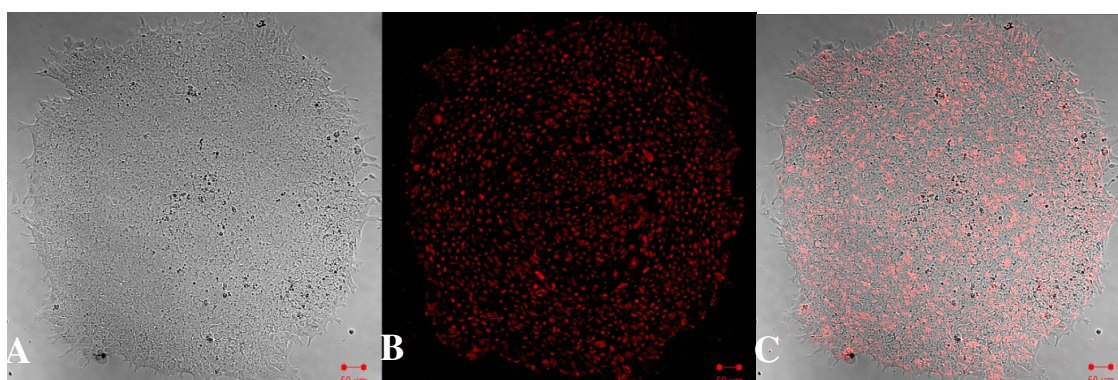
The true iPS colonies easily stained positively for SSEA4 (Figure 4-25) and TRA-1-81 (Figure 4-26). When compared to the newly formed colonies above, the true iPS colonies fluoresced

brightly. Nearly all cells in the colony stained positively for both pluripotent markers. In contrast, the newly formed colonies showed minimal, if not any, staining at all. Furthermore, the entire colony did not homogeneously stain for these markers.

In terms of morphology, both sets of colonies had well defined borders that allowed the colony to be easily distinguished from the background. The true iPS colonies did not need to be cultured on feeders as they were grown in mTesr (StemCell Technologies) and contained the necessary supplements for feeder free conditions.



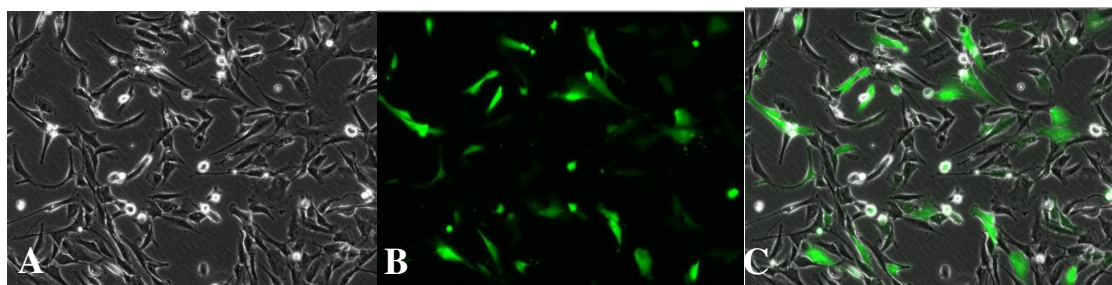
**Figure 4-25: Simultaneous imaging of a true iPS colony stained with SSEA4 antibody. (A) transmission image, (B) SSEA4-Alexafluor 488 (green), (C) merged image.**



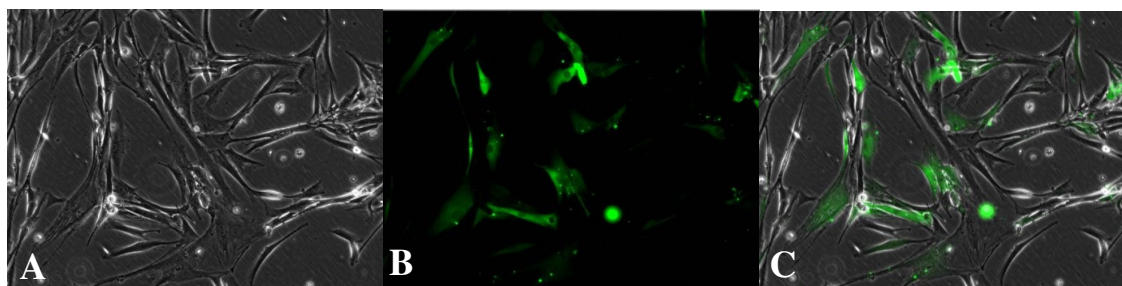
**Figure 4-26: Simultaneous imaging of a true iPS colony stained with TRA-1-81 antibody. (A) transmission image, (B) TRA-1-81 (red), (C) merged image.**



After consultation with experts from Invitrogen, the amounts of each plasmid were adjusted so that more of the regulatory factors could be transfected at the expense of GFP plasmid. The amount of regulatory factors was increased from 1  $\mu\text{g}$  to 1.2  $\mu\text{g}$  each. GFP plasmid was reduced to 0.4  $\mu\text{g}$ . HDFa cells were electroporated twice every 4 days at the same optimal electrical parameters. After day 1 and 3 (Figure 4-27 and Figure 4-28 respectively), the cells were observed to express GFP at high efficiency. This expression remained high even by day 3, which suggested that the reduction in GFP plasmid concentration did not have a significant effect on the production of GFP protein.



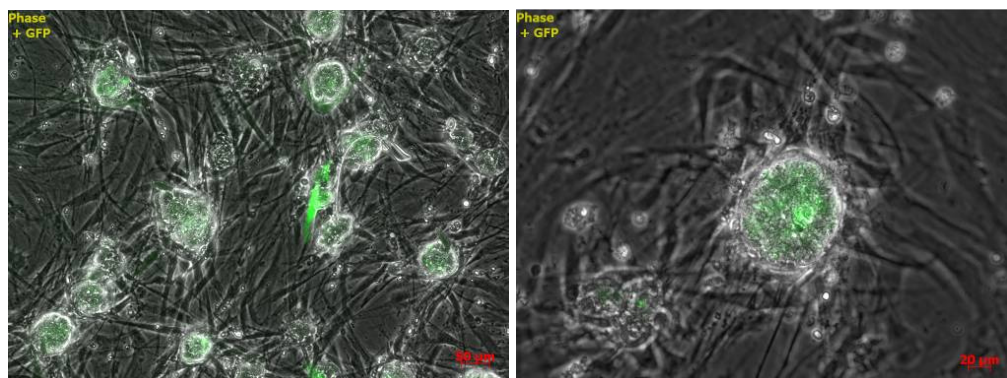
**Figure 4-27: Simultaneous imaging of human fibroblasts 1 day after electroporation at 1400 volts, 20 ms, 2 pulses with increased O,S,K plasmid. (A) phase contrast image, (B) GFP fluorescence (green), (C) merged image. 10x objective lens used.**



**Figure 4-28: Simultaneous imaging of human fibroblasts 3 days after electroporation at 1400 volts, 20 ms, 2 pulses with increased O,S,K plasmid. (A) phase contrast image, (B) GFP fluorescence (green), (C) merged image. 10x objective lens used.**

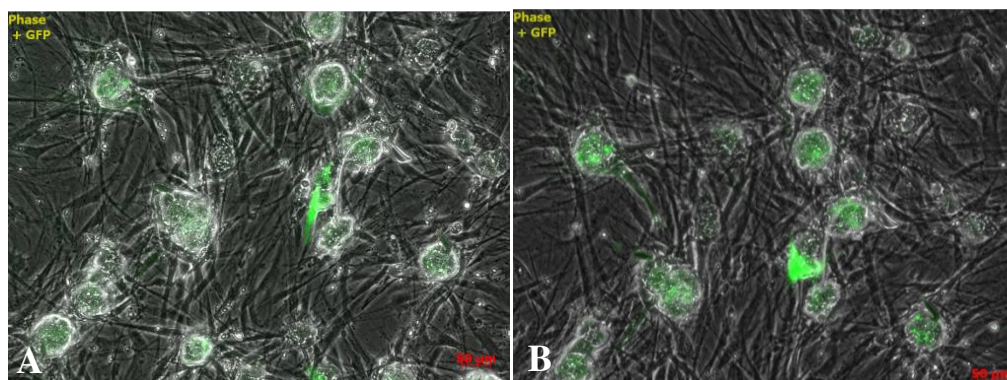


Sixteen days after the second transfection (day 20), 3 GFP expressing colonies had appeared. The cells were tightly packed within the discrete boundary of each colony. Interestingly, the nearby fibroblastic cells no longer expressed GFP. The colonies were subsequently split onto feeder cells. By day 24, more colonies appeared on feeders. In one location, approximately 11 small GFP expressing colonies had formed (Figure 4-29).



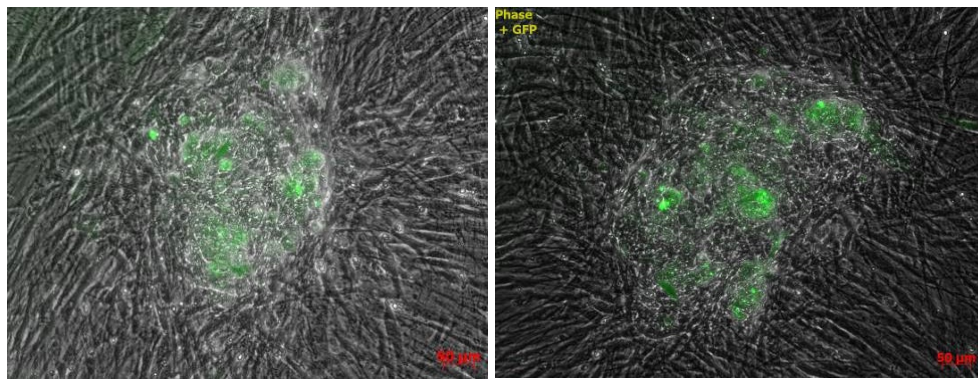
**Figure 4-29:** Simultaneous GFP fluorescence (green) and phase contrast image of iPS-like colonies 20 days after second electroporation at 1400 volts, 20 ms, 2 pulses with increased O,S,K plasmid.

After 4 more days (day 28), some of the colonies below did not appear to grow at all when comparing the colonies at different timepoints (A and B of Figure 4-30). There was no noticeable change in the expression of GFP as well.



**Figure 4-30:** Simultaneous GFP fluorescence (green) and phase contrast image of iPS-like colonies showing no significant change between (A) 20 days and (B) 24 days after 2<sup>nd</sup> electroporation at 1400 volts, 20 ms, 2 pulses with increased O,S,K plasmid.

However, in the other colonies shown below in Figure 4-31, the average diameter had increased by approximately 100 - 150  $\mu\text{m}$ . The diameter of the colonies had almost tripled over the last four days. However, the colonies no longer resembled iPS colonies as they now lacked a distinct boundary around them. Most of the colonies' peripheral cells also began to merge with the background fibroblast layer and started to share similar morphologies with them. This made the colonies difficult to find in some cases.

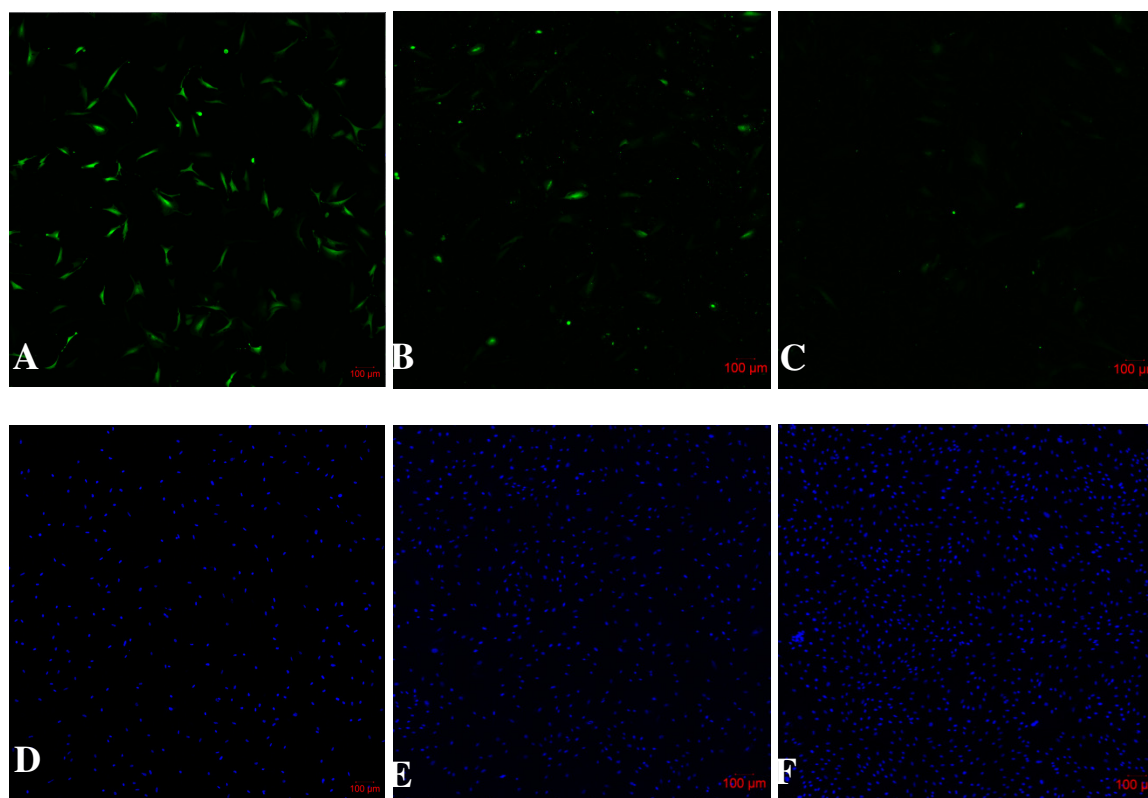


**Figure 4-31: Simultaneous GFP fluorescence (green) and phase contrast image of 6 iPS like colonies 24 days after 2<sup>nd</sup> electroporation at 1400 volts, 20 ms, 2 pulses with increased O,S,K plasmid.**

#### **4.5.4 Electroporation with STEMcircles**

Using the optimised electrical parameters as above, human fibroblasts were electroporated and subsequently lipofected with STEMcircles (StemCell Technologies) using the protocol from Jia et al. (2010). The plasmid contains a GFP reporter gene and causes the transfected cell to fluoresce green. One day after transfection, the efficiency was approximately 30%. The expression of GFP was high according to fluorescence imaging (Figure 4-32 A & D). Plating 100,000 cells/well in a 6-well dish, the resulting confluency was 30 - 40%, which allowed the cells to grow unhindered as they recovered for 4 days.

When the same batch of cells were imaged 3 days later (Figure 4-32 B & E), the expression level of GFP had dropped. This further continued to day 5 (Figure 4-32 C & F) when the sample became almost fully confluent. At this timepoint, the number of GFP expressing cells had dropped to about 10% of the original number of GFP cells from day 1.



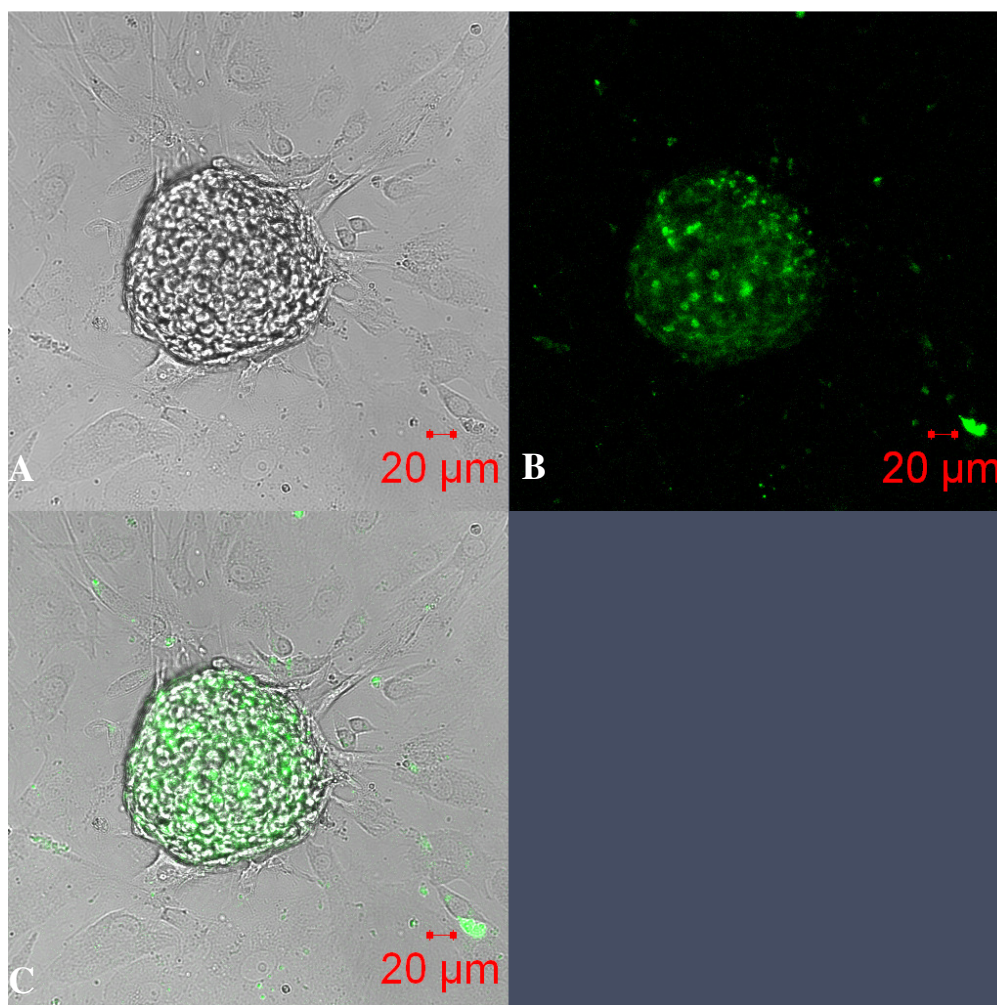
**Figure 4-32: Simultaneous imaging of human fibroblasts 1 (A&D), 3 (B&E), and 5 (C&F) days after electroporation at 1400 volts, 20 ms, 2 pulses with STEMcircles. (A – C) GFP fluorescence (green), (D - F) Hoechst nuclear staining (blue). Images are representative from 1 experiment.**

Jia et al. (2010) state that expression of the STEMcircle genes must continue for 2 weeks in order for successful iPS regeneration. Thus, the cells had to be retransfected using Lipofectamine LTX. From Figure 4-32 B, the expression of GFP already dropped significantly by day 3, so this timepoint was selected for the next transfection. This recovered the declining expression of GFP where at least 50% of the cells were now showing high expression again. After another 2 days, the cells were retransfected with Lipofectamine LTX again at the same concentrations of reagent and plasmid. The resulting efficiency was again approximately 50%. These cells were



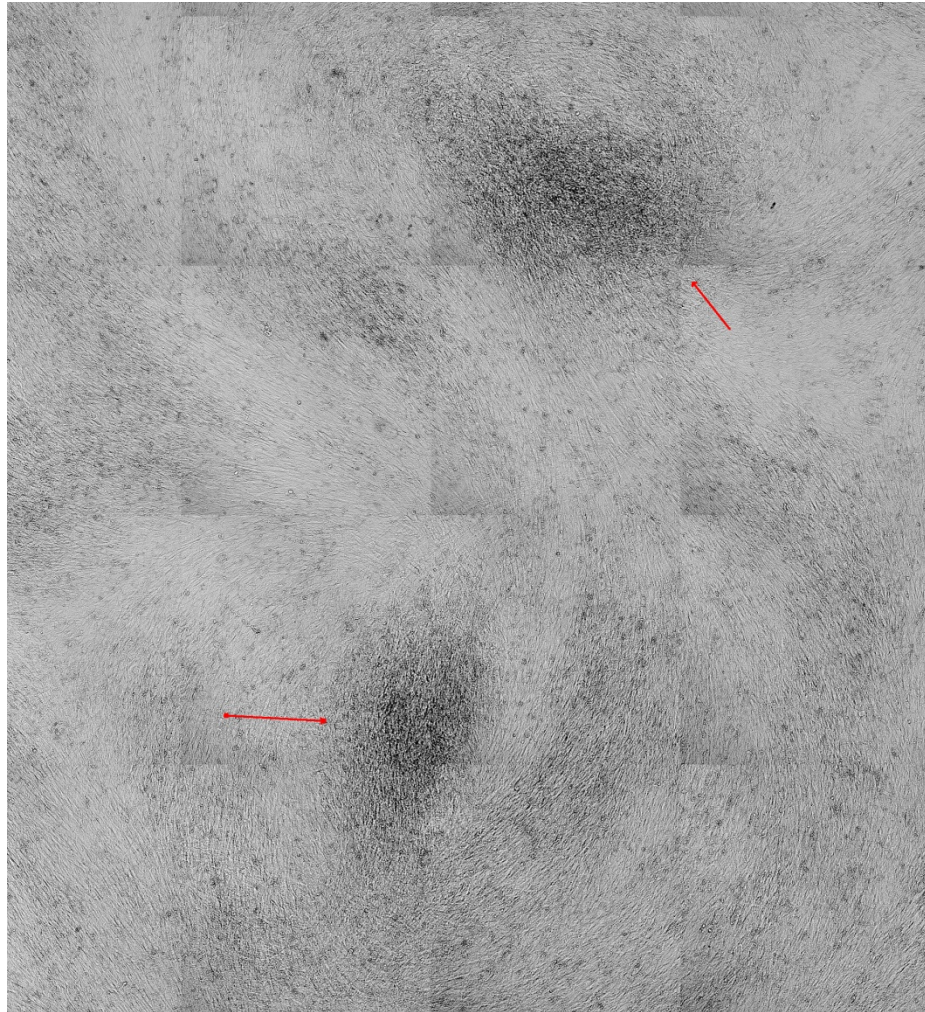
transferred onto feeders after another 2 days at a very low density so as to allow the cells to divide for 3 more weeks.

On day 28 after the first transfection, one circular colony was observed to highly express GFP, shown below. Its diameter was approximately 80  $\mu\text{m}$  and remained at that size for 2 more weeks until the end of the experiment (Figure 4-33).



**Figure 4-33: Simultaneous imaging of a 28-day old iPS-like colony derived using STEMcircles. (A) transmission image, (B) GFP fluorescence (green), (C) is merged image.**

In another independent experiment that was repeated under the same conditions, other poorly formed colonies were also observed later by day 38 (shown with red arrows in Figure 4-34). These colonies appeared dark against the feeder background layer and lacked distinct boundaries. The peripheral cells were highly fibroblastic in morphology. Even after 2 more weeks in ES cell culture medium, the colonies did not grow in size.



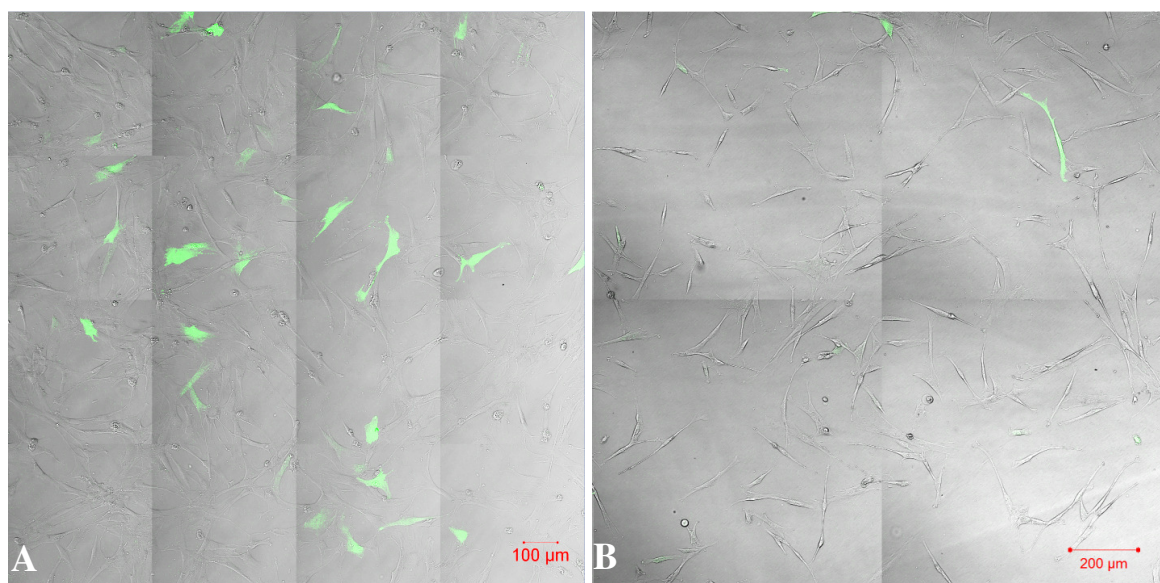
**Figure 4-34:** Transmission imaging of 2 38-day old partially reprogrammed iPS clusters (denoted by red arrows) seeded on feeders derived using STEMcircles. 10x objective lens used.



### 4.5.5 Supplementation with small molecules

Current results so far have suggested that the clusters derived through electroporation and lipofection were partially reprogrammed iPS cells. In some cases, the morphology and alkaline phosphatase staining were very similar to hESC colonies but failed to produce the correct surface markers. According to Feng et al. (2009), the supplementation of small molecules may not only assist in deriving fully reprogrammed iPS colonies, the time required could also be reduced.

After electroporation of HDFa cells with OCT4, SOX2, KLF4, and GFP (at 1400 volts, 20 ms, 2 pulses), the media was supplemented with 5'azacytidine, SAHA, or both. Before supplementation, the human fibroblast cells were healthy and had fibroblastic morphology as shown in Figure 4-35 A. The cells were seeded at 50% confluency so that growth was still permitted.



**Figure 4-35: Simultaneous imaging of human fibroblasts electroporated (A) once and (B) twice after 3 days at 1400 volts, 20 ms, 2 pulses showing GFP fluorescence (green). Image taken before supplementation of small molecules.**

After 48 hours, different batches of electroporated cells were supplemented with different combinations of small molecules and listed below. The medium was changed every second day after electroporation. In total, 3 courses of electroporation were performed at 3 day intervals. It

was expected that the number of GFP expressing cells would progressively increase as the cells were electroporated repeatedly. The batches of cells were imaged 1 day after supplementation of small molecules.

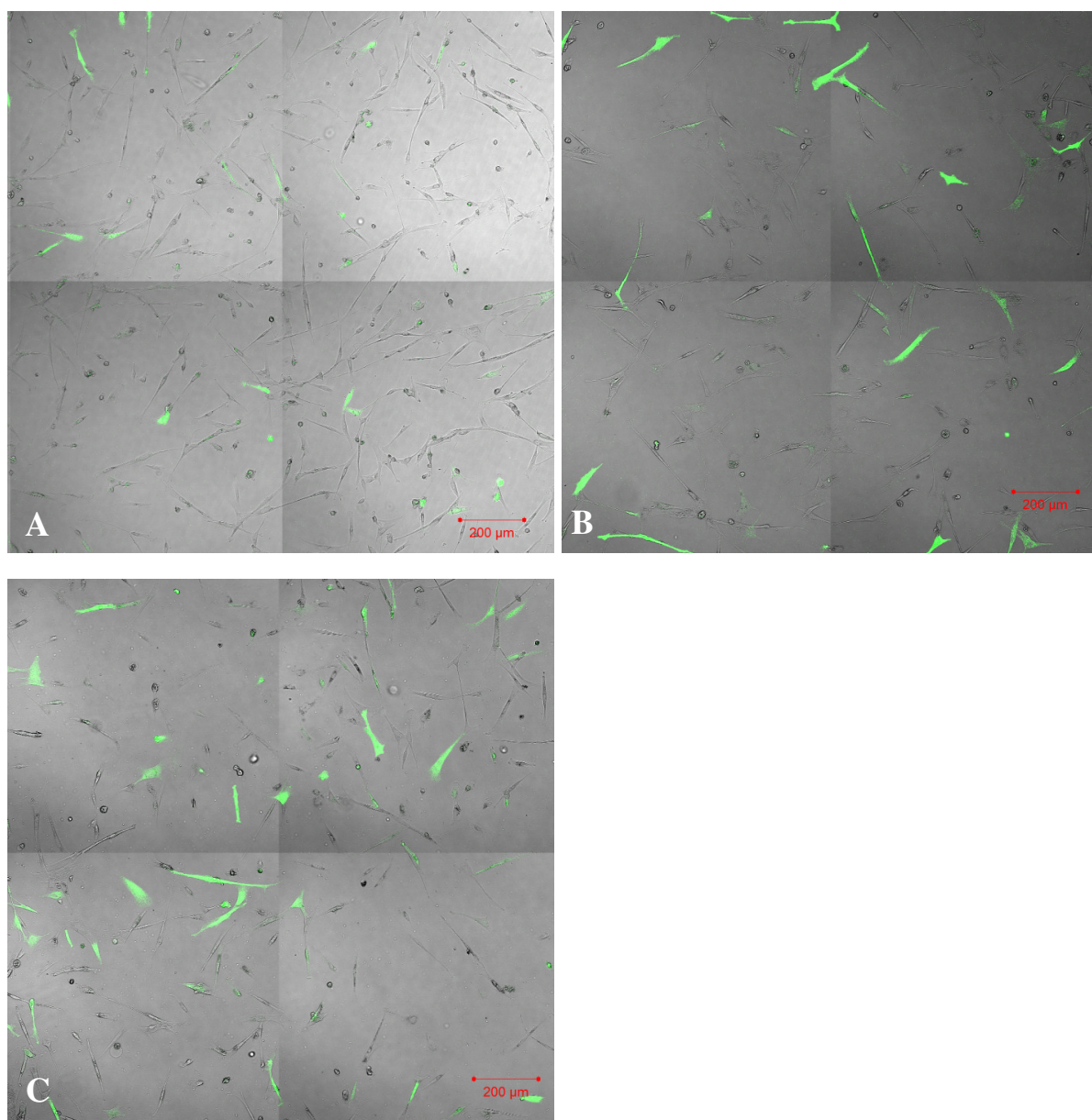
An interesting observation was that the cells grew much slower in the presence of small molecules. The division time increased dramatically. This caused problems when repeating electroporation as a large number of cells is generally required. Electroporation causes high cell death, thus, repeated electroporation is only feasible if the surviving cells can divide quickly enough to recover the total cell population again.

#### ***4.5.5.1 Control***

Figure 4-35 B depicts cells which have been electroporated for the second time without any supplementation of small molecules. It can be seen that the efficiency was very low at less than 5% despite repeated electroporation. The cells were also healthy showing that cells can be electroporated repeatedly with just 3 days of recovery.

#### ***4.5.5.2 5'AZA***

At 10  $\mu$ M of 5'AZA, the cells began to look unhealthy characterised by the rounding and detaching of cells (Figure 4-36). The next day, a large number of cells were seen to be floating suggesting that the recommended concentration of 10  $\mu$ M may have been too toxic.



**Figure 4-36: Simultaneous GFP fluorescence (green) and transmission imaging of human fibroblasts electroporated at 1400 volts, 20 ms, 2 pulses second time. Image taken 1 day after addition of (A) 10  $\mu$ M of 5'AZA (B) 10  $\mu$ M of SAHA, and (C) 5  $\mu$ M of SAHA and 10  $\mu$ M of 5'AZA.**

#### **4.5.5.3 SAHA**

At 10  $\mu$ M of SAHA, the majority of the cells were not attached and appeared round, however, the expression of GFP was much more prevalent bringing the efficiency up to approximately 50% (Figure 4-36 B). There were clearly more GFP expressing cells when SAHA was used compared to using 5'AZA or none at all. Of the living cells, the morphology was still very fibroblastic and consisted of long and spindly shapes.

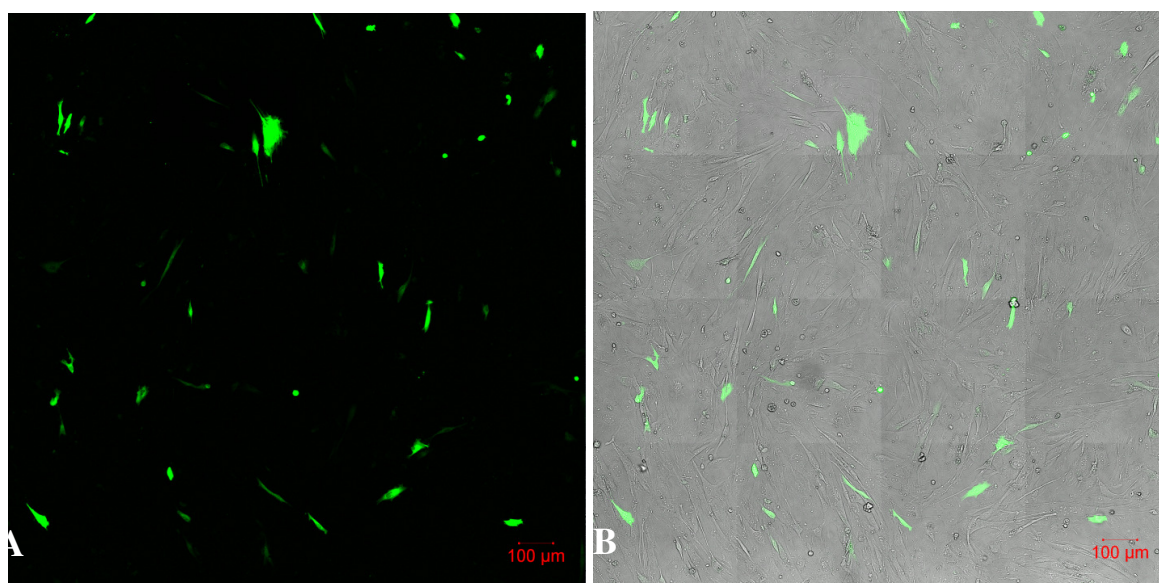


#### 4.5.5.4 SAHA + 5'AZA

When both small molecules are used simultaneously at 5  $\mu$ M and 10  $\mu$ M respectively, the most striking observation is that cell death had increased. There were more rounded cells present than when either SAHA or 5'AZA were used individually (Figure 4-36 C). However, the efficiency of GFP expression was not significantly higher compared to when 5'AZA was used alone. Electroporation was carried out for the third time and supplementation of small molecules to human fibroblast cells continued.

#### 4.5.5.5 2<sup>nd</sup> dose of SAHA & 5'AZA

There was very little improvement in the expression of GFP after the second addition of 5'AZA and SAHA+5'AZA (Figure 4-37). Also, cells continued to appear unhealthy after supplementation despite reducing the concentration of 5'AZA from 10  $\mu$ M to 5  $\mu$ M.



**Figure 4-37: Simultaneous imaging of human fibroblasts electroporated at 1400 volts, 20 ms, 2 pulses third time showing (A) GFP fluorescence (green) and (B) merged with transmission image. Image taken 1 day after addition of 5  $\mu$ M SAHA and 10  $\mu$ M of 5'AZA.**

The electroporated cells were allowed to grow in hESC medium on feeders for 2 more weeks. No iPS like colonies were observed during that time.

## **4.6 Discussion**

### **4.6.1 Reprogramming efficiency**

Compared to viral-based iPS generation, there are few papers which use plasmid-based transfection in mouse (Okita et al., 2008 ; Kaji et al., 2009 ; Gonzalez et al., 2009), human (Jia et al., 2010 ; Tayeb et al., 2010) and even equine fetal fibroblasts (Nagy et al., 2011). The efficiency is also generally low (0.0002 – 0.006%) compared to viral-based methods (up to 7%) for human cells. All publications involving plasmid-based reprogramming of human cells have used at least 4 plasmids: either a combination of O, S, K, M or O, S, LIN28 and NANOG. In my work, I used O, S, K and still managed to obtain an efficiency of 0.0001 – 0.00014% using lipofection or electroporation, which is only slightly lower than the aforementioned plasmid-based studies. In another set of experiments using the minicircle STEMcircles (O, S, LIN28, and NANOG), I obtained an efficiency of just 0.00017%, which is more than 3 times lower compared to work done by Jia et al. (2010). The reason for the large difference is likely due to the modified protocol I had to adopt. Without access to a cell sorter, it was not possible to enrich the GFP positive cell population for further transfection. The electroporators employed for Jia et al.'s (2010) and my experiments were also different (Neon, Invitrogen vs Nucleofector, Amaxa). The Nucleofector, used by Jia et al. (2010), allowed for more customisation of the electrical pulses, which may have given rise to improved transfection efficiency. Lipofection (Invitrogen) was similarly used in both studies, however.

### **4.6.2 Starting cell type**

It is widely acknowledged that the starting cell type can influence the efficiency of iPS generation (Maherali & Hochedlinger, 2008). With human keratinocytes (Aasen et al., 2008), amniotic fluid cells (Li et al., 2009), and umbilical cord matrix stem cells (Cai et al., 2010), the reprogramming efficiency can be as high as 0.4 - 1.5%. This is much higher when compared to

studies using adult fibroblasts where the efficiency is between just 0.001 - 0.1% (Takahashi et al., 2007 ; Nakagawa et al., 2008 ; Park et al., 2008). In this thesis, adult human fibroblasts were selected because of their ease of isolation and culture, hence, offering potential clinical value (compare this with neural progenitor cells, which must be harvested from fetal brains (Shi et al., 2008b)). Being associated with a clinic / hospital could also mean a large supply of available tissue from donors.

#### **4.6.3 Completion of reprogramming**

The colonies generated in this thesis stained positive for alkaline phosphatase but not TRA-1-81 or SSEA4. This result is insufficient to prove that true iPS colonies have been generated (Brambrink et al., 2008). Thus, based on morphology, the colonies are likely to have only been partially reprogrammed. The addition of chemical compounds may assist in iPS reprogramming, as stated by Feng et al. (2009) and Huangfu et al. (2008), the latter who used VPA to replace KLF4 while further boosting reprogramming efficiency. Huangfu et al. (2008) observed a 3-fold increase in reprogramming efficiency by supplementing medium with 2  $\mu$ M of 5'AZA in mouse fibroblasts retrovirally infected with O, S, and K. I also tried adding 5'AZA 1 day after transfection at 10  $\mu$ M in human fibroblasts, but no colonies were generated. At higher concentrations, 5'AZA was toxic, but according to Mikkelsen et al. (2008), 5'AZA was also shown to cause cell death when used earlier than 8 days. Thus, the allowable concentration before 5'AZA became highly toxic in my experiments may have simply been too low to cause significant DNA methylation in human cells of key genes.

SAHA has been used at 5  $\mu$ M by Huangfu et al. (2008) but only in mouse fibroblasts. In this thesis, SAHA was used at 10  $\mu$ M since I did not observe significantly more cell death compared to 5  $\mu$ M in human cells. No colonies were generated despite Huangfu et al. (2008) showing that

SAHA doubled the reprogramming efficiency in mouse fibroblasts which were retrovirally infected with O, S, K, and c-Myc. Again, this is likely because I only had access to 3 plasmids instead of 4.

#### **4.6.4 Reprogramming time**

When I used minicircles for iPS generation, colonies could be observed after 28 days. Jia et al. (2010), on the other hand, obtained colonies after just 18 days. This may have been due to the authors enriching the population of cells for further transfection. When O, S, and K were electroporated individually into human cells, I observed colonies after 23 days from the first transfection. This is 5 days shorter than Tayeb et al. (2010) who had used Fugene instead of electroporation and 4 plasmids (OCT4, SOX2, LIN28, and NANOG). Thus, electroporation may be more effective than Fugene in delivering more copies of plasmid into cells, thus, leading to higher and sustained expression of key transcription factors. This has yet to be confirmed as there are currently no publications which have compared Fugene and electroporation in great detail for the generation of iPS cells.

#### **4.7 Summary**

In summary, the optimal conditions for efficient lipofection and electroporation have been described in this chapter. The expression of OCT4, SOX2, KLF4 and GFP have subsequently been shown by immunostaining, indicating that the expression vectors are working. Colonies were derived using both methods and share similar morphological characteristics to true iPS cells. Table 4-4, below, illustrates the resultant efficiencies in generating iPS cells from mouse and human fibroblasts. In one experiment, iPS-like colonies derived from human fibroblasts using electroporation also stained for alkaline phosphatase, a universal pluripotent marker for

true iPS cells. However, they failed to stain positive for TRA-1-81 and SSEA4. This could be a result of incomplete reprogramming, which is commonly seen when c-MYC has been omitted from the iPS cocktail. Colonies from other experiments were not tested for alkaline phosphatase or iPS surface markers.

**Table 4-4: Approximate efficiencies of iPS generation for each method of transfection. Efficiency is the number of colonies divided by starting cell population. With the exception of Electroporation with OSK in human fibroblasts, experiments that yielded 0 colonies were excluded from the calculation. Electroporation of mouse fibroblasts yielded colonies in 2 experiments. In all other conditions, colonies were only generated in 1 experiment each.**

| <u>Method</u>   | <u>Cell type</u>  | <u>Plasmid</u> | <u>Starting cell population</u> | <u>Number of colonies</u> | <u>Efficiency (%)</u> | <u>AP staining</u> | <u>iPS surface markers</u> |
|-----------------|-------------------|----------------|---------------------------------|---------------------------|-----------------------|--------------------|----------------------------|
| Lipofection     | Human fibroblasts | O,S,K,G        | $9 \times 10^6$                 | 13                        | 0.00014               | Not performed      | Not performed              |
| Electroporation | Mouse fibroblasts | OSK,G          | $3 \times 10^6$                 | 3 - 8                     | 0.00018               | Not performed      | Not performed              |
|                 | Human fibroblasts | OSK,G          | $3 \times 10^6$                 | 0                         | 0                     | Not performed      | Not performed              |
|                 | Human fibroblasts | O,S,K,G        | $3 \times 10^6$                 | 3                         | 0.0001                | Positive staining  | Negative staining          |
|                 | Human fibroblasts | STEMcircles    | $0.6 \times 10^6$               | 1                         | 0.00017               | Not performed      | Not performed              |

To circumvent the low reprogramming efficiency, different combinations of small molecules were deployed in the hopes to drive the completion of reprogramming. However, after more than 2 weeks, no iPS colonies were observed.

# Chapter 5

## Optoporation of Nucleic Acids

In this chapter, optoporation was used to introduce red fluorescent protein (DsRed) and green fluorescent protein (GFP) plasmid into primary human fibroblasts. Similar to other transfection methods, optimisation is necessary and includes laser power, exposure time (also known as pixel dwell time) and culture conditions, and these factors will be discussed in this chapter. Despite having optimised these parameters using fluorescent small molecules, I was not able to consistently and reliably transfect human primary cells using optoporation. At the end of this chapter, I theorised that the reason for the low efficiency is the requirement that the plasmid must also cross the nuclear membrane, which would occur only rarely, particularly in slow-dividing fibroblasts.

### 5.1 Optimisation of Laser Parameters for Optoporation in OptiMEM

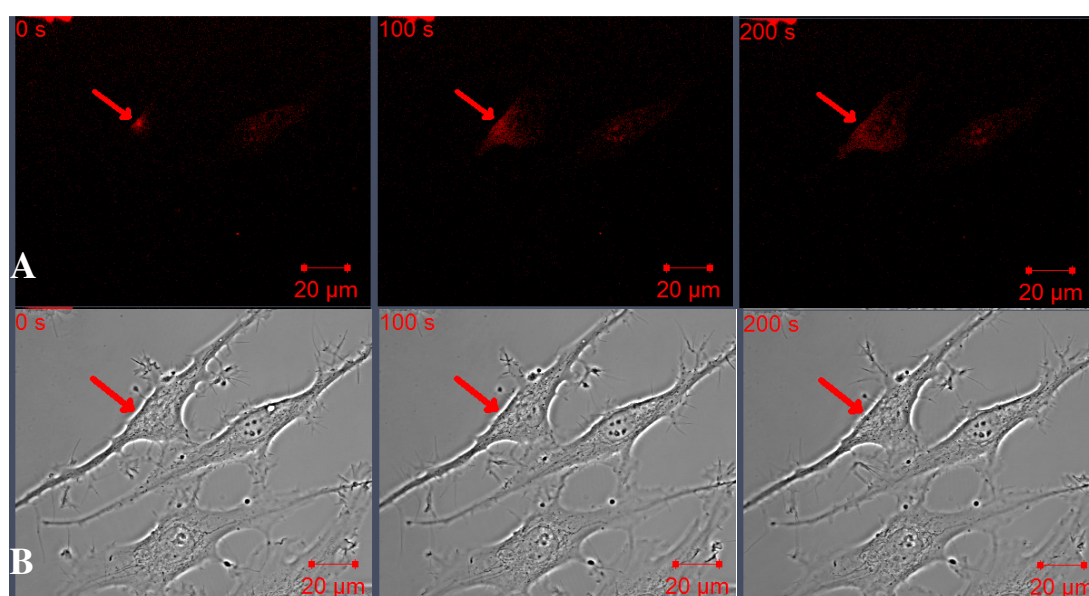
#### 5.1.1 Materials and methods

First, cells were prepared on glass dishes as described in Chapter 3.1.2. The laser parameters for optoporating human fibroblast cells (HDFa) were optimised using propidium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI) at 1 µg/ml and 20 µg/ml respectively in 600 µl of OptiMEM without serum and antibiotics. The spot scan function, which allows exposure times of the order of milliseconds, was attempted first and the laser was directed at a site on the membrane near to

the cell's nucleus. Various laser powers (10 - 200 mW) and exposure times (16 - 200 ms) were investigated. An image of each optoporated cell was taken before and after exposure. The position of the cell was also stored in memory so that the cell's viability could be reexamined later. Viability was determined based on morphology, attachment, and the positive staining of calcein-AM (green). The medium was not changed at anytime so as not to perturb the cells.

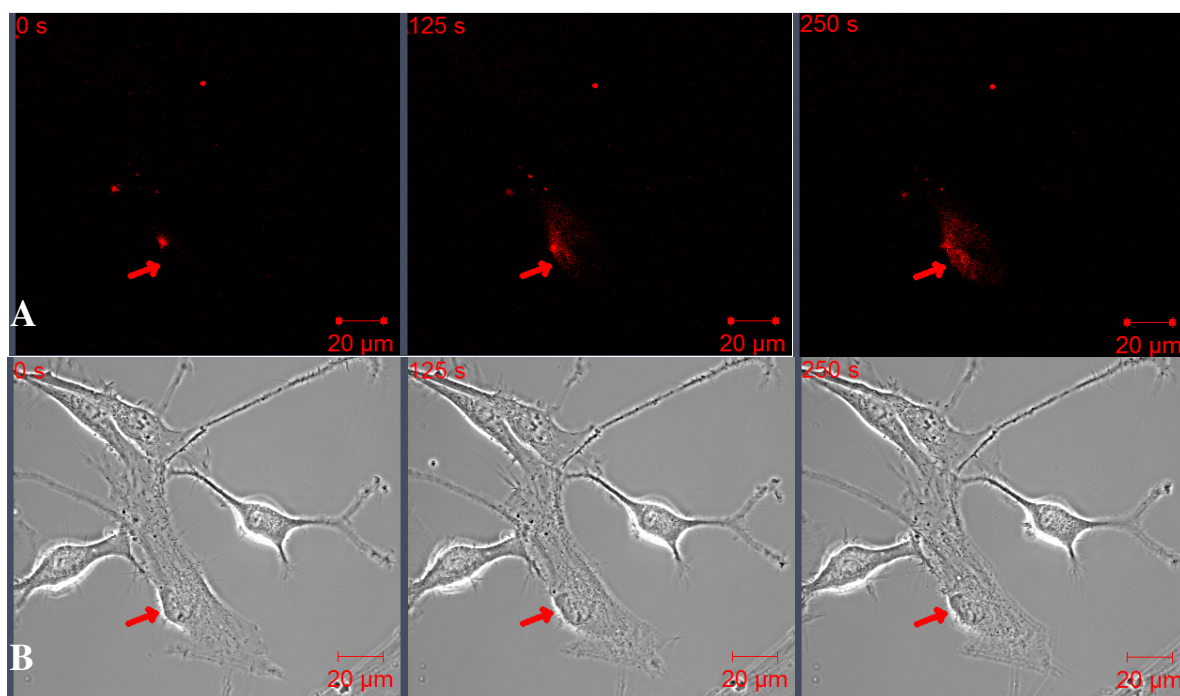
### 5.1.2 Result of optoporation with PI

The laser power was first set at 100 mW for 41 ms (see Table 5-1). All cells showed immediate and rapid uptake of PI, as shown in Figure 5-1 A. The PI, observable by red fluorescence, first entered through the site of poration and diffused into the cytoplasm of the cell. As the PI diffused further into the cell, it began to enter the nucleus. Only the dark regions, possibly the nucleic-acid-rich nucleoli, in the nucleus fluoresced brightly after 3 minutes subsequent to optoporation. Most of the PI may be binding to these nucleic-acid rich-nucleoli. A small amount of PI that was only sufficient to partially stain the nucleus had been optoporated into the cell. This was based on the observation that red fluorescence was mostly observed at the nucleoli as opposed to the entire nucleus. The original morphology of the cells was maintained even after 3 minutes.



**Figure 5-1: Simultaneous timelapse (A) propidium iodide fluorescence (red) and (B) phase contrast image of a human fibroblast cell immediately after optoporation at 100 mW and 41 ms. Site of optoporation denoted by red arrow. Images are representative of 100 cells from 1 experiment.**

To determine the upper limits of optoporation parameters, the power was increased up to 150 mW at 41 ms. The cells were continuously imaged for at least 4 minutes to observe PI uptake over time as shown in Figure 5-2 A.

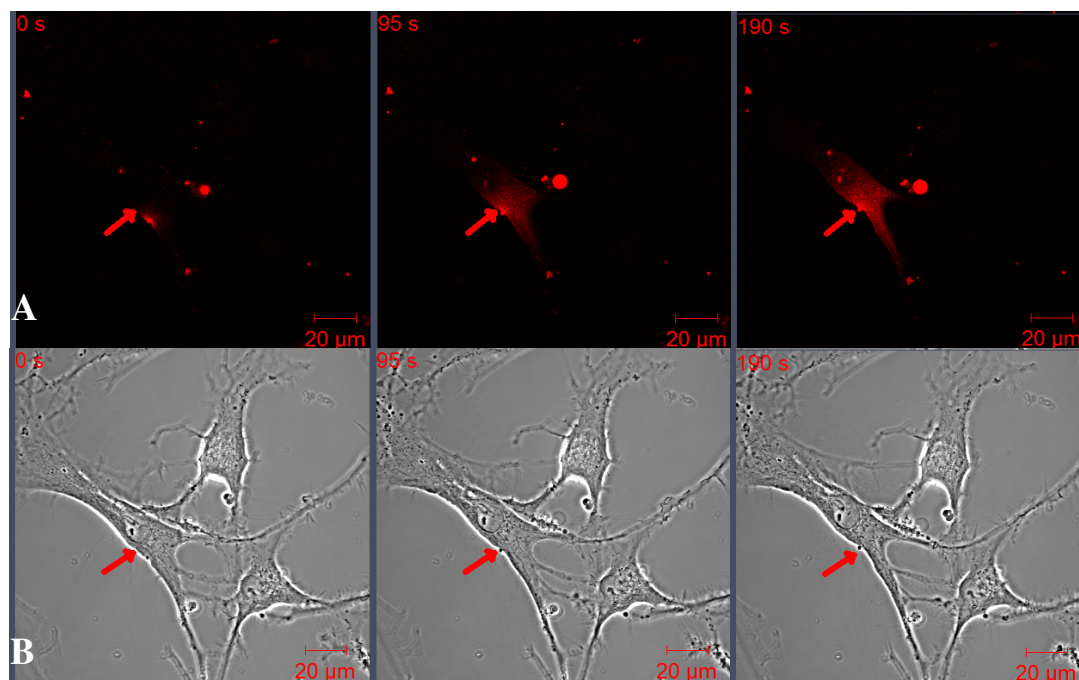


**Figure 5-2: Simultaneous timelapse (A) propidium iodide fluorescence (red) and (B) phase contrast image of a human fibroblast cell immediately after optoporation at 150 mW and 41 ms. Site of optoporation denoted by red arrow. Images are representative of 100 cells from 1 experiment.**

From Figure 5-2 and Figure 5-3, the red fluorescence was more intense suggesting that more PI had entered the cells. The PI continued to diffuse into the cells after 3 minutes. Unlike when 100 mW of power was used, the red fluorescence was still intense and clearly visible after 3 minutes subsequent to optoporation. It was observed that the cells displayed a more drastic change in morphology soon after optoporation. In the cells shown in Figure 5-2 B and Figure 5-3 B, much of the cytoplasm had leaked out causing the cells to appear deflated and shrivelled. Also, the impact of the laser had clearly perturbed the membrane significantly at the site of poration. It was assumed that these cells had died and would be unsuitable for further study. For future



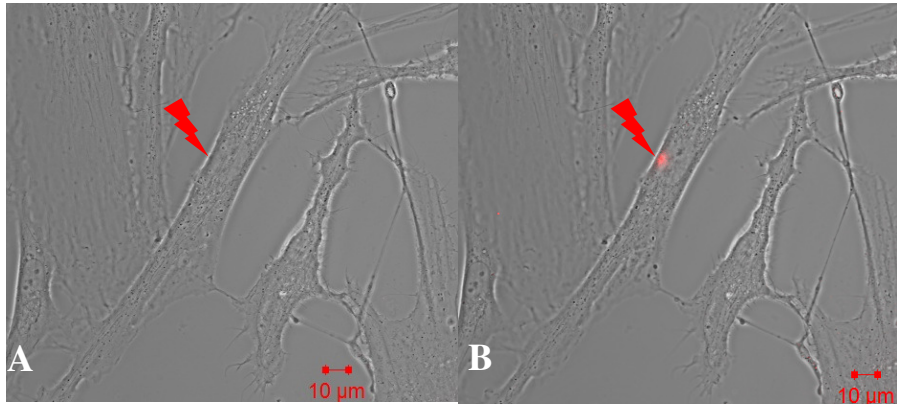
experiments, 150 mW of power at 41 ms should be avoided. Below 100 mW, the cells did not consistently show any red fluorescence or change in morphology (see Table 5-1). Thus, 100 mW was selected for further viability testing by calcein-AM staining.



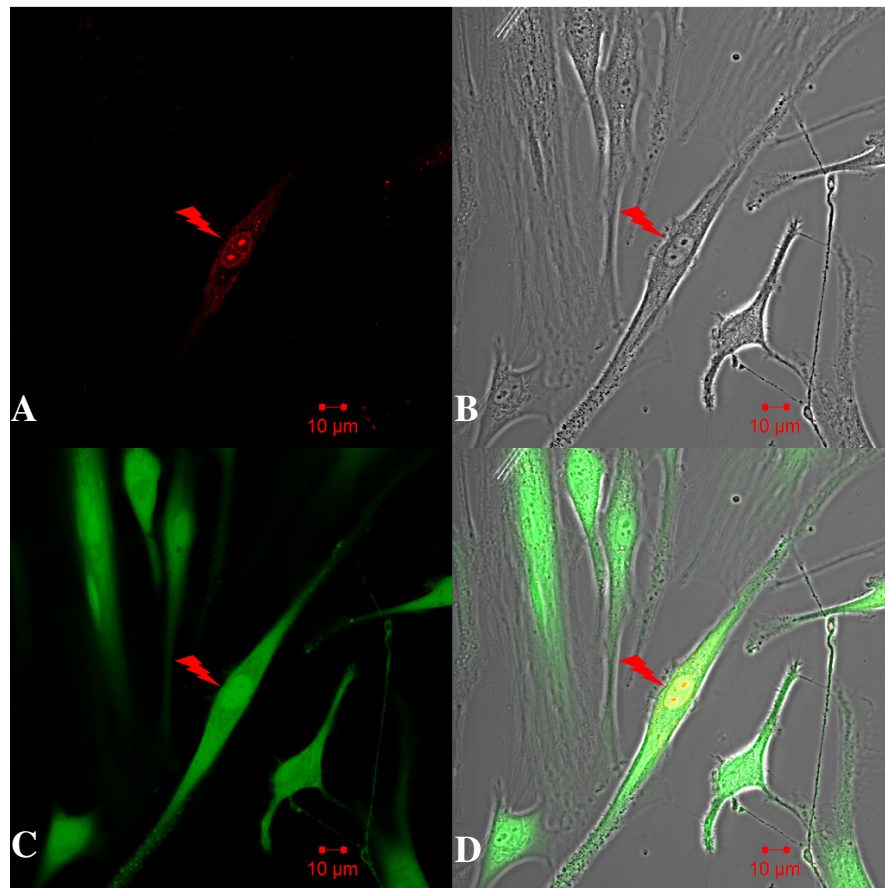
**Figure 5-3: Simultaneous timelapse (A) propidium iodide fluorescence (red) and (B) phase contrast image of another human fibroblast cell immediately after optoporation at 150 mW and 41 ms. Site of optoporation denoted by red arrow.**

### 5.1.3 Determining viability after optoporation in OptiMEM

Cells were optoporated at 100 mW for 41 ms and then tested for their viability. After optoporation with PI, the sample was allowed to recover for 90 minutes before rinsing the sample with medium containing calcein-AM. After 30 minutes of incubation, the treated samples were imaged again. Calcein –AM stains living cells and causes them to fluoresce green. For example, Figure 5-4 A & B show a cell before and after optoporation respectively at 100 mW for 41 ms. The same cell stained positive for calcein-AM 2 hours later (shown by green fluorescence in Figure 5-5 C) at the same intensity of the neighbouring cells which had not been optoporated, while retaining PI (shown by red fluorescence in Figure 5-5 A. This suggests that the optoporated cells were still viable 2 hours after PI uptake.



**Figure 5-4: Simultaneous timelapse propidium iodide fluorescence (red) and phase contrast image of a human fibroblast cell (A) before and (B) immediately after optoporation at 100 mW and 41 ms. Site of optoporation denoted by red lightning bolt.**

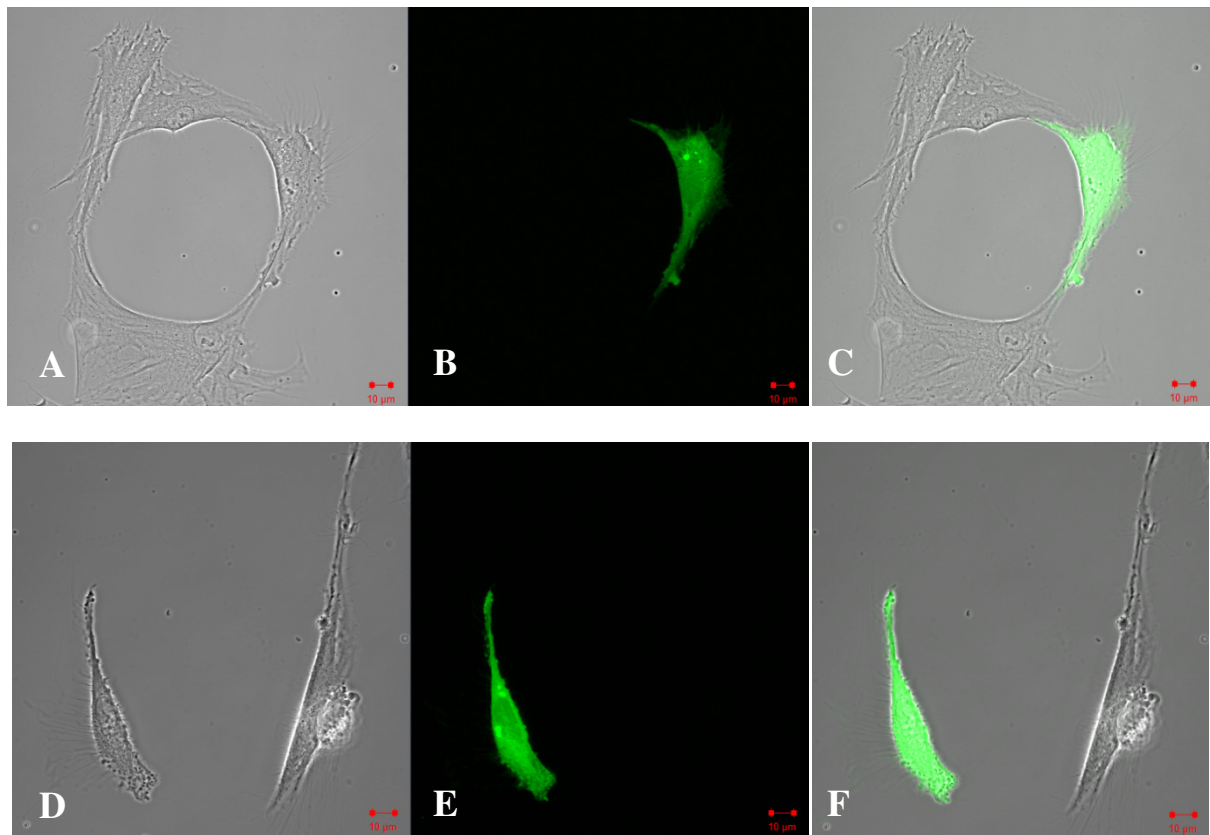


**Figure 5-5: Simultaneous imaging of the human fibroblast cell from Figure 5-4 surviving 2 hours after optoporation at 100 mW and 41 ms. (A) propidium iodide (red), (B) phase contrast image, (C) calcein-AM (green), (D) is merged image. Site of optoporation denoted by red lightning bolt**

#### 5.1.4 Optoporation with GFP plasmid in OptiMEM

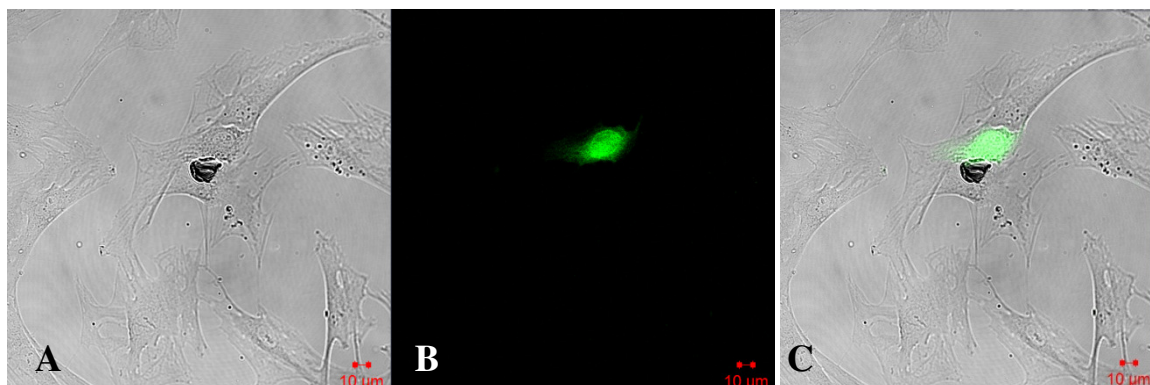
After identifying 100 mW at 41 ms as a possible optimal parameter, GFP plasmid was substituted for PI at a concentration of 20  $\mu\text{g/ml}$  in OptiMEM. After optoporation, the cells were washed twice to minimise spontaneous plasmid uptake. Surprisingly, out of 100 optoporated cells, only 5 expressed GFP protein overnight. There were no floating green fluorescent cells, which rules out the possibility that some cells had died after being transfected.

Two cells are shown in Figure 5-6 A-C and D-F. The control sample did not have any GFP positive cells, which suggested that all 5 cells in the treated sample were likely due to optoporation. The occurrence of spontaneous uptake has been reported to be as low as 1 in  $10^6$  cells (Stevenson et al., 2006) and even 0 (Uchaganova et al., 2008).



**Figure 5-6: Simultaneous imaging of 2 human fibroblast cells surviving 1 day after optoporation at 100 mW and 41 ms. (A,D) phase contrast image, (B,E) GFP fluorescence (green), (C,F) is merged image.**

The experiment was repeated once again on a fresh batch of cells to determine the reproducibility. This time, only one cell expressed GFP, shown below in Figure 5-7. The control was empty. These results are summarised in Table 5-2.



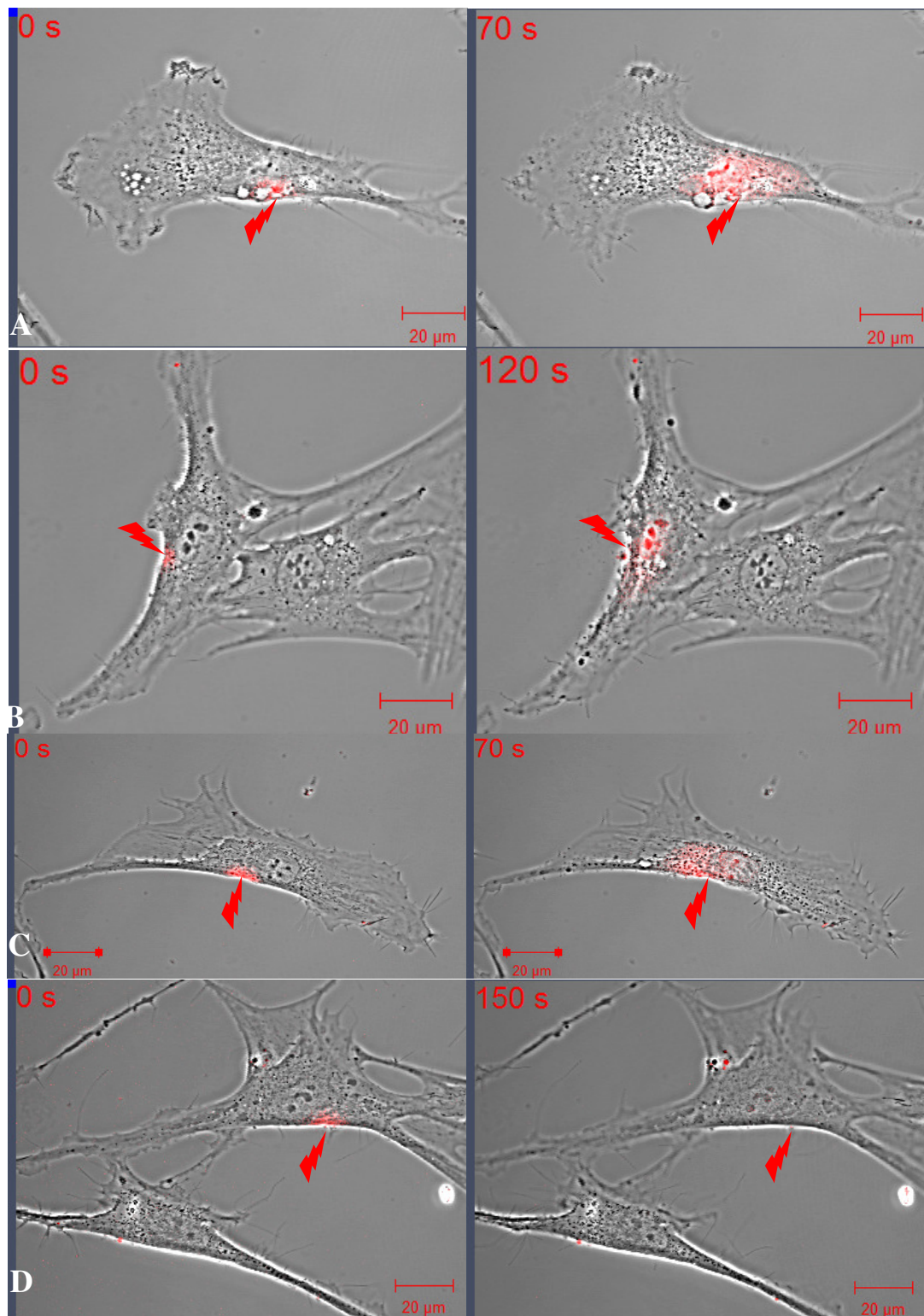
**Figure 5-7: Simultaneous imaging of a human fibroblast cell surviving 1 day after optoporation at 100 mW and 41 ms in another independent experiment. (A) phase contrast image, (B) GFP fluorescence (green), (C) is merged image.**

## **5.2 Re-Optimisation of Parameters with PI in Hank's Buffered Saline Solution (HBSS)**

A different medium, HBSS, was tried instead of OptiMEM. HBSS does not contain phenol red which may be absorbing and converting light to heat energy, hence causing damage to cells. HBSS also contains vital calcium, magnesium, and phosphate ions at normal physiological levels.

The exposure time was kept constant at 41 ms while the power was altered from 10 mW to 80 mW at regular intervals. With HBSS, optoporation could be realised at just 20 – 80 mW already, so attempting higher powers, such as 100 mW, was pointless (see Table 5-1 for a summary of parameters tested). At least 3 cells were optoporated for each power level and recorded as timelapse images shown in Figure 5-8.

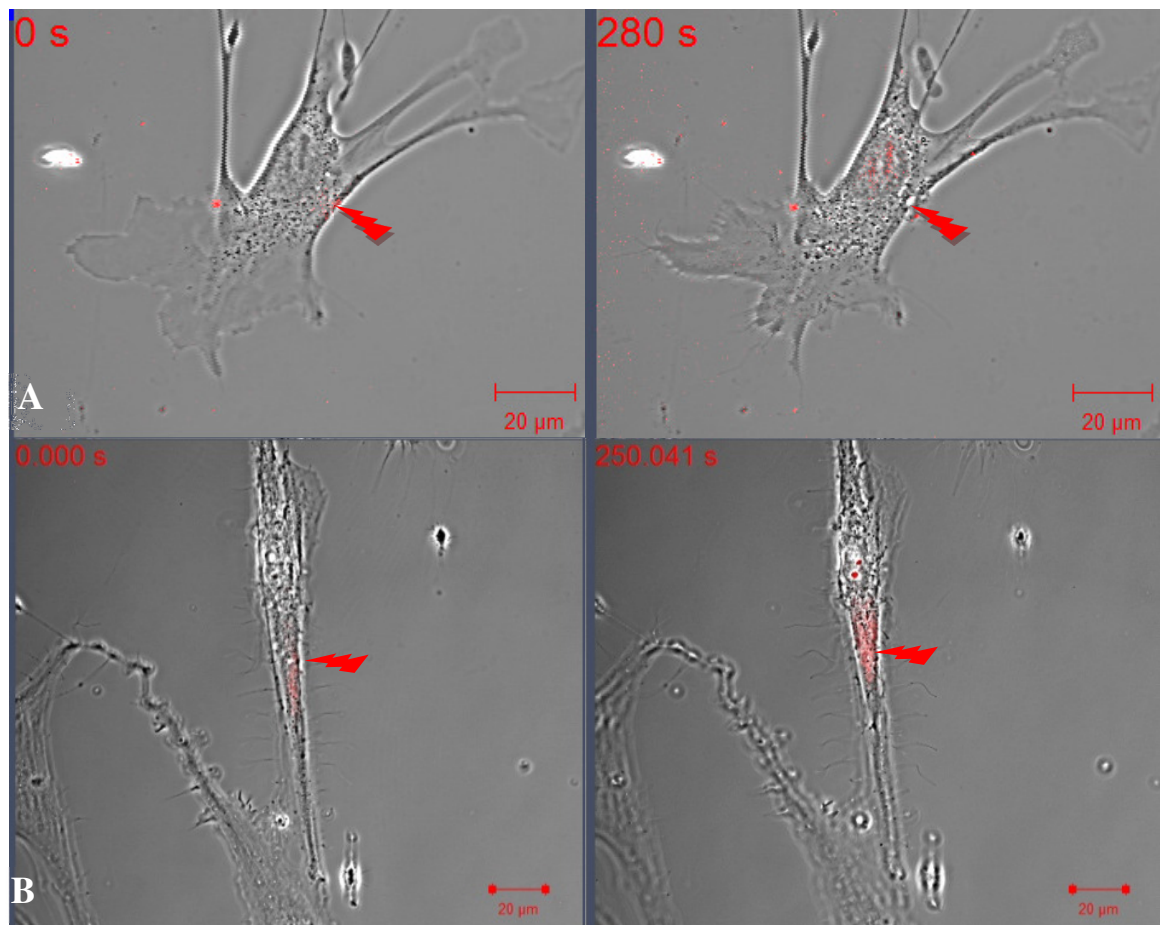




**Figure 5-8: First and last frames of propidium iodide fluorescence (red) and phase contrast timelapse imaging of 4 human fibroblast cells after optoporation at (A) 80 mW, (B) 40 mW, (C) 20 mW, (D) 10 mW at 41 ms. Site of optoporation denoted by red lightning bolt. Images are representative of 100 cells from 1 experiment.**

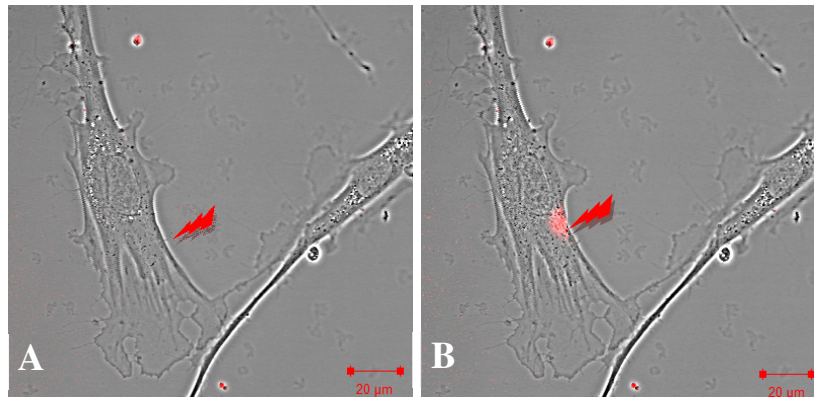
Optoporation can be realized at just 20 mW at 41 ms pixel dwell time as shown by the entry of propidium iodide in Figure 5-8 C. PI began to enter the nucleus within 30 seconds (not shown). However, the cells did not appear healthy even at this level of intensity. When the power was further lowered to 10 mW power, the PI that had entered was reproducibly no longer visible by 150 seconds (Figure 5-8 D). Timelapse imaging continued for 250 seconds (not shown) without any trace of red fluorescence.

Another parameter that could be altered is the *exposure time*. Maintaining the power between 6-12 mW, the exposure time was doubled to 82 ms to determine if labelling of the nucleus could now be observed for longer periods while still maintaining cell viability (see Table 5-1). In Figure 5-9 B, there was an immediate uptake of PI after optoporation at 12 mW and, by 250 seconds, PI could be seen to cross the nuclear membrane and into the nucleus. At 6 mW (Figure 5-9 A), PI also entered the cell but at a less rapid rate and volume. Nevertheless, red fluorescence was still observed in the nucleus, and after 280 seconds, the cell continued to appear healthy. I observed that the red fluorescence remained even after 500 seconds.

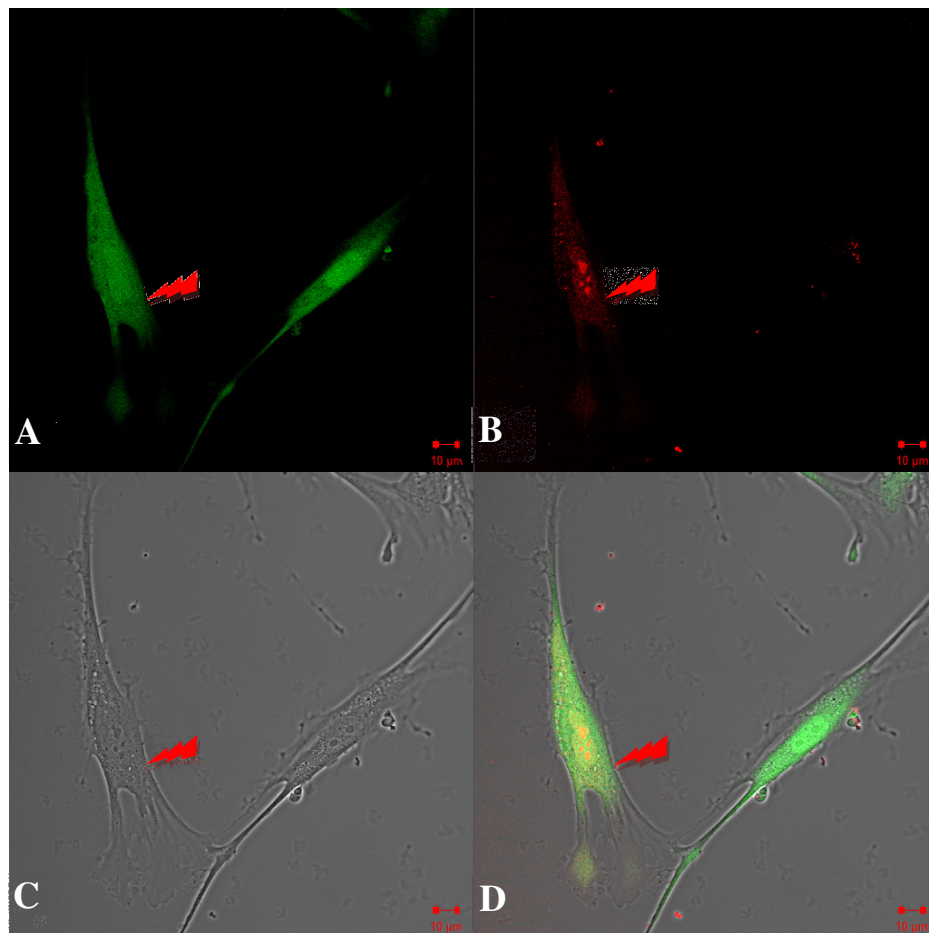


**Figure 5-9: Start and end frames of propidium iodide fluorescence (red) and phase contrast timelapse imaging of a human fibroblast cell after optoporation at (A) 6 mW and 82 ms, (B) 12 mW and 82 ms. Site of optoporation denoted by red lightning bolt.**

Using 6 mW gave more consistent results as a larger number of cells survived the procedure while displaying an uptake of PI. After optoporation, the cells were allowed to recover in normal culture medium for 90 minutes. The cells were then stained with calcein-AM and incubated for 30 minutes and then washed. Figure 5-10 shows the cell before and immediately after optoporation. From Figure 5-11, I observed that the same optoporated cell fluoresced green, was still viable, displayed minimal change in morphology, and showed uptake of PI.



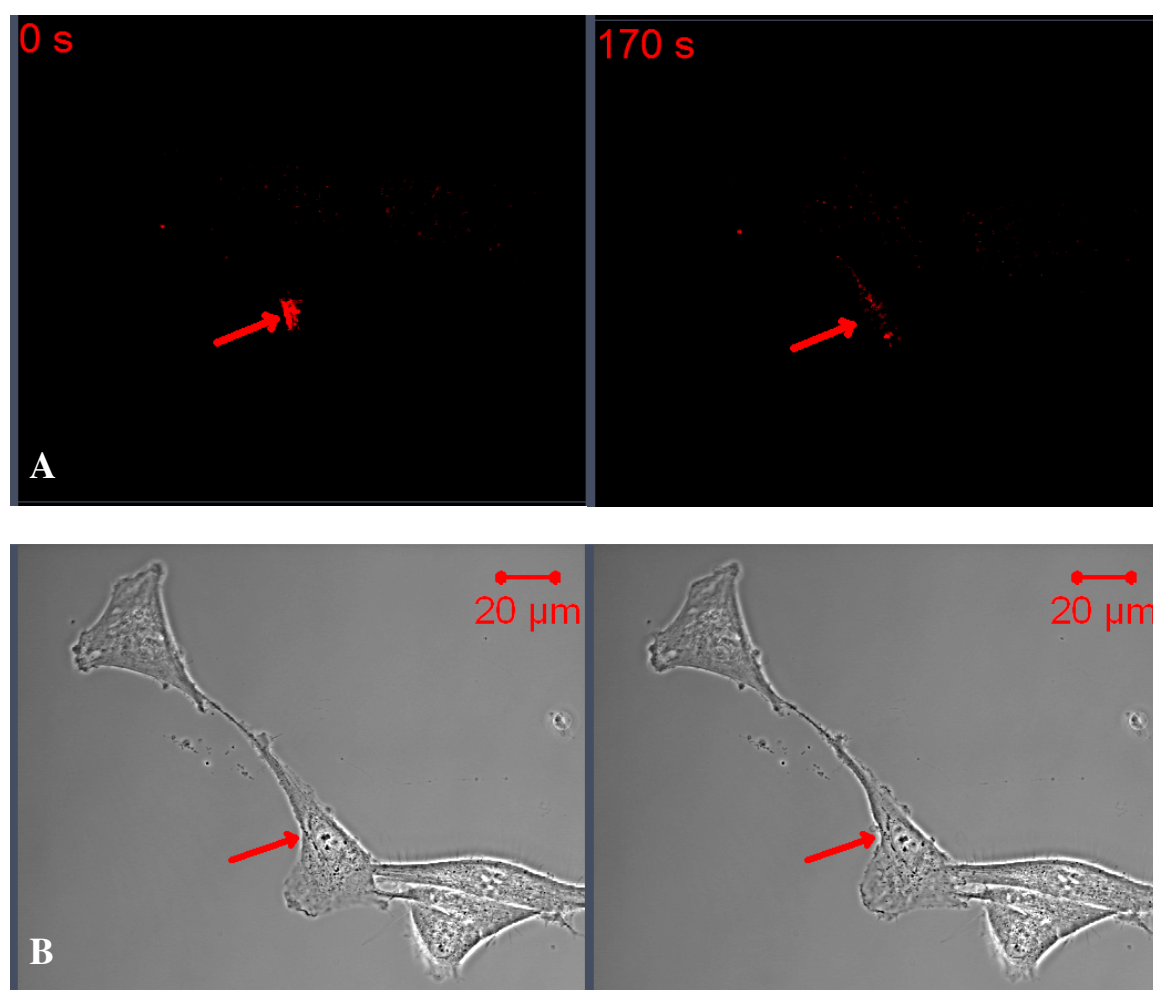
**Figure 5-10: Simultaneous timelapse propidium iodide fluorescence (red) and phase contrast image of a human fibroblast cell (A) before and (B) immediately after optoporation at 6 mW and 82 ms. Site of optoporation denoted by red lightning bolt.**



**Figure 5-11: Simultaneous imaging of the human fibroblast cell from Figure 5-10 surviving 2 hours after optoporation at 6 mW and 82 ms. (A) calcein-AM (green), (B) propidium iodide (red), (C) phase contrast image, (D) is merged image. Site of optoporation denoted by red lightning bolt.**

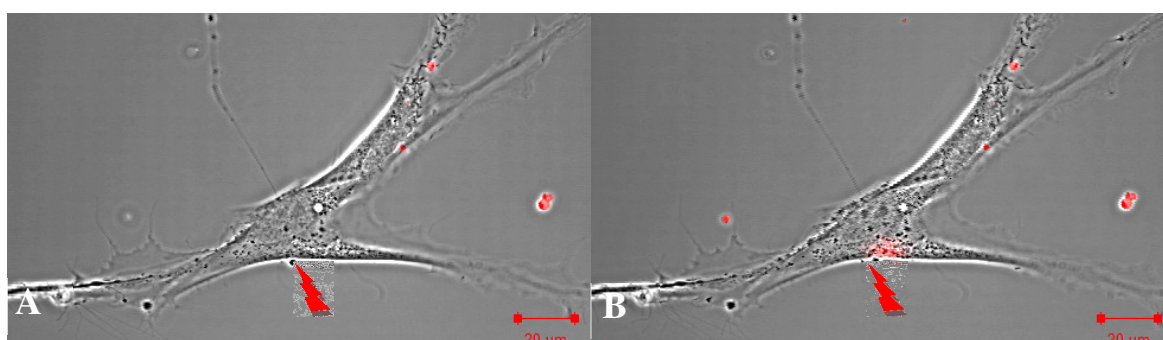


Earlier in section 5.2, when the power and exposure time was set to 20 mW and 41 ms respectively, PI stained the nucleus rapidly after just 30 seconds (Figure 5-8 C). However, the cell began to appear unhealthy. The experiment was repeated at the same power (20 mW) but the exposure time was halved from 40 to 20 ms. After 35 seconds, the nucleus fluoresced faintly (not shown) from PI and continued past 170 seconds, as shown in Figure 5-12. The cell also did not show a drastic change in size and morphology, which suggested that this was a possible optimal parameter.

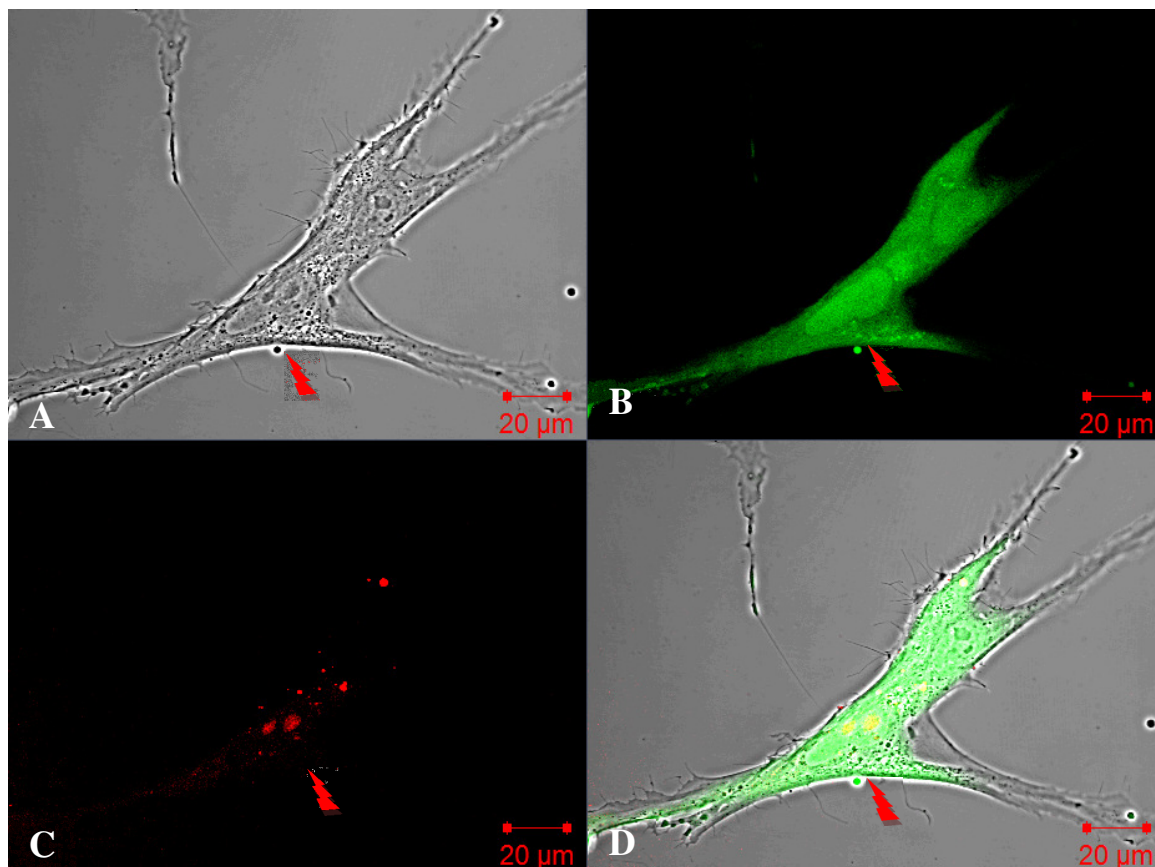


**Figure 5-12:** Two frames taken from a timelapse image showing (A) propidium iodide fluorescence (red) and (B) phase contrast of a human fibroblast cell after optoporation at 20 mW and 20 ms. Site of optoporation denoted by red lightning bolt.

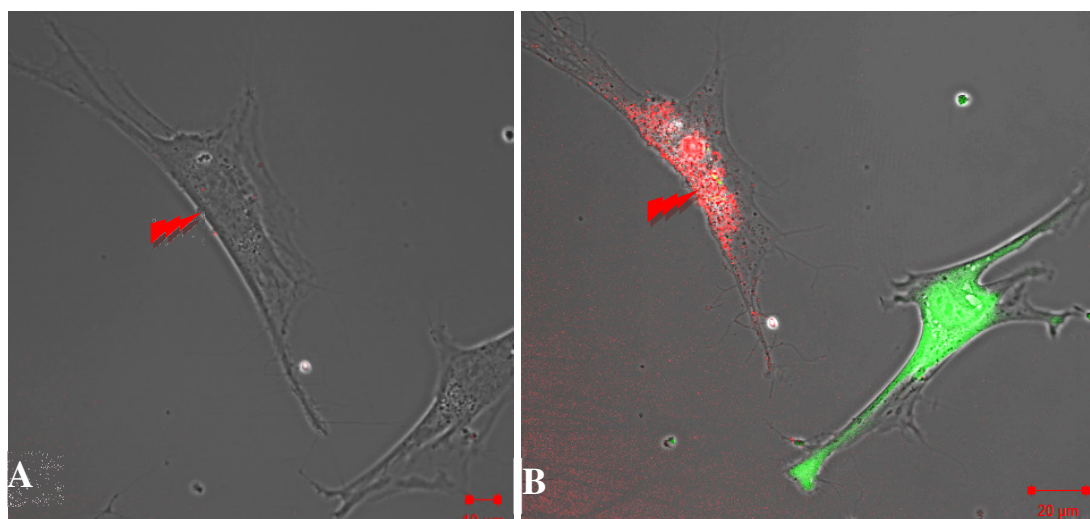
Uptake of PI could be observed within 30 seconds while maintaining cell viability based on observing the cell morphology over time. Viability was verified using calcein-AM. In about 20% of cells tested, this parameter showed clear PI uptake and nuclear staining soon after optoporation, shown in Figure 5-13. Furthermore, the same cells displayed minimal change in morphology and fluoresced green from calcein-AM, which suggested that the cells had survived (Figure 5-14 A & B). However, this parameter also caused cell death in the remaining 80% of cells tested (Figure 5-15).



**Figure 5-13: Simultaneous propidium iodide fluorescence (red) and phase contrast image of a human fibroblast cell (A) before and (B) immediately after optoporation at 20 mW and 20 ms. Site of optoporation denoted by red lightning bolt.**



**Figure 5-14:** Simultaneous imaging of the human fibroblast cell from Figure 5-13 surviving 2 hours after optoporation at 20 mW and 20 ms. (A) phase contrast image (B) calcein-AM (green), (C) propidium iodide (red), (D) is merged image. Site of optoporation denoted by red lightning bolt.



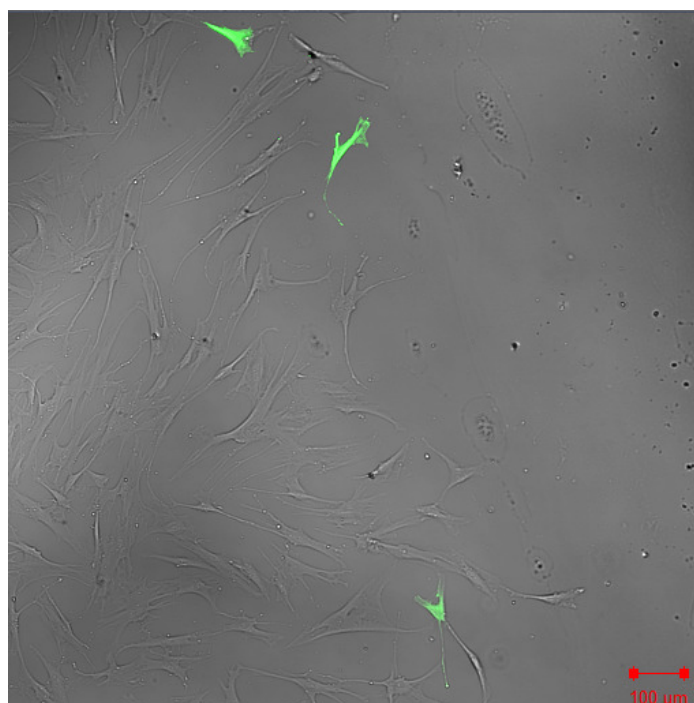
**Figure 5-15:** Simultaneous timelapse calcein-AM (green), propidium iodide fluorescence (red) and phase contrast image of a dead human fibroblast cell (A) before and (B) 2 hours after optoporation at 20 mW and 20 ms. Site of optoporation denoted by red lightning bolt.

Clearly, the contents of the treated cell in Figure 5-15 A had leaked out to the point where the cell was unable to recover. This cell did not fluoresce green after incubation in calcein-AM, unlike its neighboring untreated cell (Figure 5-15 B). Since the reproducibility of optoporation at this parameter was poor, 20 mW for 20 ms was avoided. Instead, 6 mW for 82 ms was adopted for further experiments with GFP transfection.

### 5.2.1 Optoporation with GFP plasmid in Hank's buffered saline solution

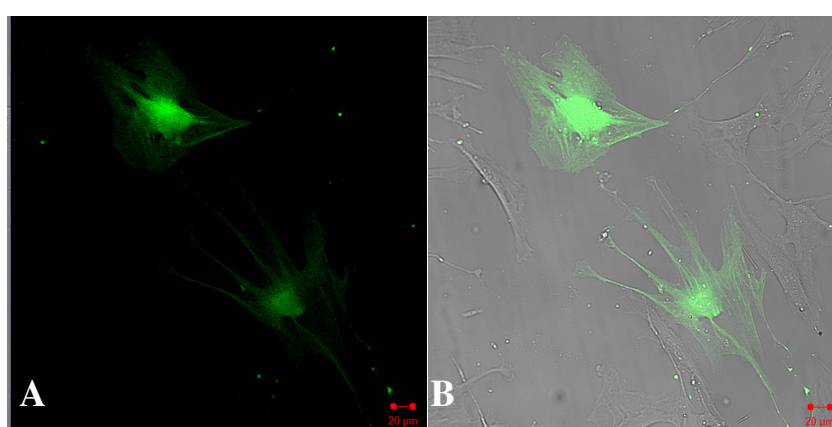
Human fibroblast cells were bathed in 250  $\mu$ l of HBSS containing 20  $\mu$ g/ml of GFP plasmid. Optoporation was carried out using the best parameter from optimisation with PI. Approximately 50 cells were optoporated using Spot Scan at 6 - 12 mW for 82 ms (see Table 5-2).

After 24 hours, 3 GFP positive cells were observed. The cells were also in close proximity of each other as can be seen in Figure 5-16. Interestingly, the control sample also contained 2 GFP positive cells (result not shown).



**Figure 5-16: Simultaneous imaging of GFP fluorescence (green) and transmission imaging of 3 GFP positive human fibroblasts 1 day after optoporation at 12 mW and 82 ms in the presence of 20  $\mu$ g/ml GFP plasmid.**

Based on the presence of GFP cells in the control sample, the suitability of using this parameter was inconclusive as the number of cells in the treated and control sample were very similar. Thus, the experiment was repeated. 15 GFP positive cells were obtained when the experiment was repeated (Figure 5-17). In this experiment, GFP plasmid was mixed thoroughly in a larger volume of 1 ml of HBSS at 20  $\mu\text{g}/\text{ml}$ . After optoporation, the cells were incubated with plasmid for 1 hour before rinsing twice with HBSS. The medium was then changed to regular culture medium.



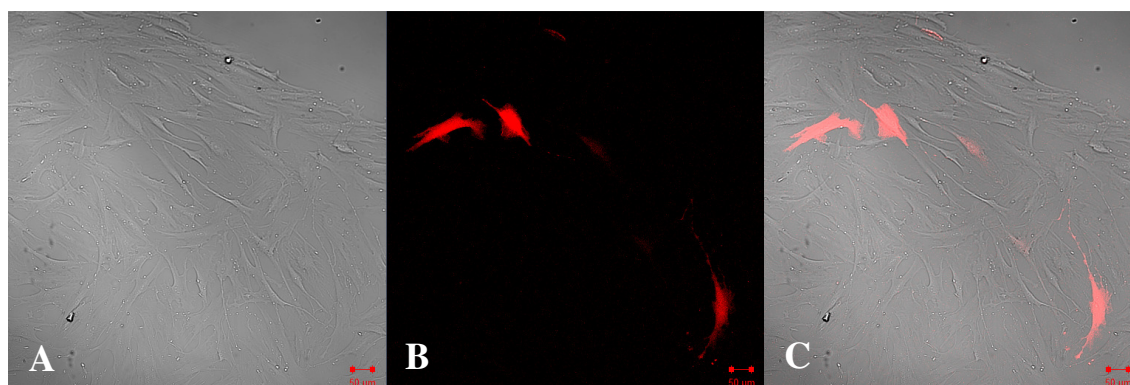
**Figure 5-17:** Simultaneous imaging of 2 GFP positive human fibroblast cells from a population of cells that were optoporated at 6 and 12 mW throughout the dish for 82 ms in 20  $\mu\text{g}/\text{ml}$  of GFP in HBSS. (A) GFP fluorescence (green), (B) is merged with transmission image.

The control sample contained 7 GFP positive cells (result not shown). Once again, the number of GFP cells located in the treated and control samples were too similar to conclude whether the transfection was due to optoporation or spontaneous plasmid uptake (see Table 5-2). As mentioned earlier, Uchaganova et al. (2008) and Stevenson et al. (2006) have reported the chances of spontaneous uptake to be nearly 0, however, they used lower concentrations of plasmid. Uchaganova et al. (2008) used as little as 0.4  $\mu\text{g}/\text{ml}$  in contrast to 20  $\mu\text{g}/\text{ml}$  used in this thesis, which may account for the number of GFP positive cells in the control sample. However, it is unclear why there were no GFP positive cells in the control sample when OptiMEM was used for the culture medium. It was also interesting to observe that all GFP positive cells in the



treated and control sample were located near or at the periphery of the dish. This is despite optoporation the dish in different areas including the centre.

To ensure that the reason for the poor efficiency was not potentially due to the GFP plasmid's unsuitability for optoporation, the experiment was repeated using DsRed plasmid at the same concentration. Zero DsRed cells were obtained in the treated sample. In contrast, there were several DsRed cells in the control sample (Figure 5-18). They were found to be close to the periphery of the dish.



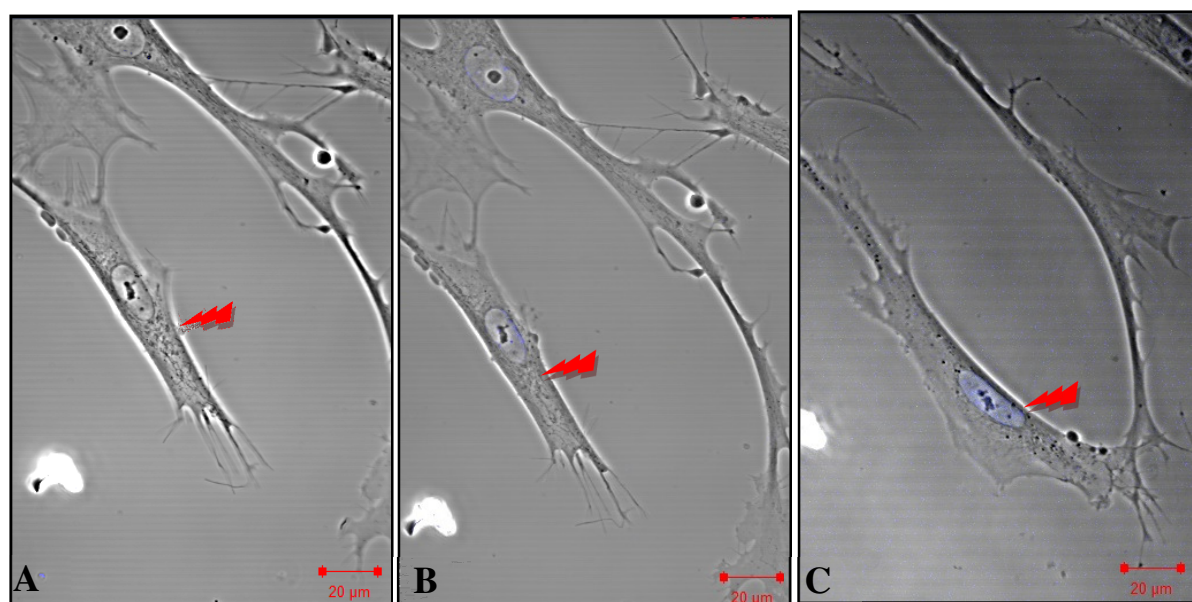
**Figure 5-18: Simultaneous imaging of several DsRed positive human fibroblast cells surviving 1 day after optoporation at 6-12 mW and 82 ms in 20 µg/ml of DsRed in HBSS. (A) transmission image, (B) DsRed fluorescence (red), (C) is merged image.**

### **5.3 Re-Optimisation of Laser Parameters for Optoporation Using DAPI**

Since PI can be extremely toxic to cells, optoporation was reoptimised using DAPI. I theorised that DAPI may be less toxic than PI, and that cells would withstand higher power levels when using DAPI, thus, allowing for a larger pore for more plasmid to enter. Laser powers of 100, 80, 70, 60, and 50 mW for 12 ms were tested in the presence of DAPI. The optoporated cells were reimaged after 18 hours. Laser powers above 50 mW showed high cell death. At 50 mW of power, 80% of the optoporated cells survived, which was the highest of all tested parameters (Figure 5-19). Since 80% was sufficiently high for my work, I retained this parameter for use

with GFP without further optimisation. It is interesting to note that this parameter is, in fact, milder than when using PI. Therefore, my initial theory that cells would be able to withstand higher power levels was wrong, and that DAPI may be more toxic than PI after all.

Apart from using lower power levels, cells were also optoporated at a point far from the nucleus. As long as the pore was located away from the nucleus, the leakage of cytoplasm caused minimal disruption to the nucleus. The cell would then have time to recover before the vital contents around the nucleus drained out of the cell and into the surrounding medium. If a pore was created close to the nucleus, cells were observed to die soon after as the pore had less time to recover before the cytoplasm immediately around the nucleus drained out. Human fibroblasts were optoporated with DAPI (4  $\mu\text{g/ml}$ ) and GFP (20  $\mu\text{g/ml}$ ) in OptiMEM. However, still no GFP expression was observed the next day.



**Figure 5-19: Simultaneous DAPI fluorescence (blue) and phase contrast imaging of 3 human fibroblasts surviving 1 day after optoporation at 50 mW at 12 ms in 20  $\mu\text{g/ml}$  of GFP plasmid in OptiMEM. Site of optoporation denoted by red lightning bolt. (A) before optoporation, (B) immediately after optoporation, (C) overnight. Images are representative of 100 cells from 1 experiment.**

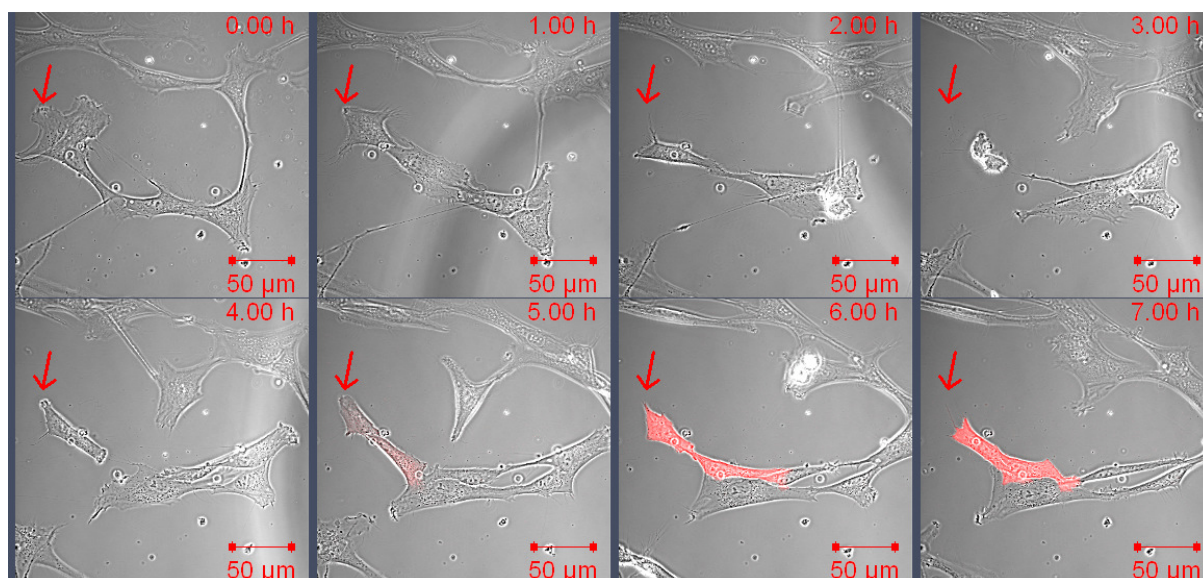


## 5.4 Optoporation with Nupherin-Neuron

Transfection with GFP and DsRed has not been consistent. A possible reason is that the size of the pore excludes the entry of plasmid DNA, which is 2 orders of magnitude larger than small molecules such as DAPI and PI. Another possible reason is that the plasmid had not entered the nucleus through the nuclear membrane, which may be required for expression.

Two possible remedies are to optoporate the nuclear membrane as well or to use cationic peptides, such as Nupherin-Neuron (Biomol) and PLUS reagent (Invitrogen) to facilitate the delivery of DNA into the nucleus. The latter technique has been shown to be especially beneficial in raising the efficiency (Praveen et al., 2011) of transfection in cell lines, and may also be advantageous in slow dividing fibroblast cells where nuclear envelope breakdowns are rarer due to lengthy doubling times.

To test this theory, cells were optoporated with DsRed plasmid complexed with the cationic peptide Nupherin-Neuron (NN). Shockwave optoporation was used at 180mW for 1  $\mu$ s and to ensure that pore size was large enough to allow plasmid through; please see section 7.1.5 for explanation of the technique. Only 1 out of 22 cells, shown in Figure 5-20, fluoresced red the following day. This particular fibroblast was optoporated before it was about to divide. Before cell division, it was possible that the plasmid had entered the plasma membrane by optoporation but remained in the cytoplasm. It was only during cell division that the plasmid was able to migrate into the nucleus through the broken down nuclear membrane. Because expression of DsRed only occurred in the case of Figure 5-20, the use of cationic peptides may not be beneficial in primary cell lines. However, it does suggest that nuclear membrane breakdown may be necessary for expression of optoporated plasmids.



**Figure 5-20: Timelapse imaging of a dividing human fibroblast immediately after shockwave optoporation at 180 mW for 1  $\mu$ s (site of optoporation shown with red arrow). Cell division occurred 3 hours after optoporation. Expression of DsRed occurred 2 hours after cell division.**

## 5.5 Discussion

Successful optical transfection has been achieved in a number of papers (Mohanty et al., 2003 ; Satoh et al., 2005 ; Paterson et al., 2005 ; Tirlapur & Konig, 2002 ; Stevenson et al., 2006 ; Zeira et al., 2007 ; Baumgart et al., 2008 ; Uchuganova et al., 2008 ; Praveen et al., 2011) in a variety of cell types. The latter 7 publications utilised a Ti:Sapphire laser similar to the one used in this thesis. Stevenson et al. (2006) and Baumgart et al. (2008) both used a laser power of 80 mW and an exposure time of 40 ms, while Tirlapur & Konig (2002) tried 50 – 100 mW for 16 ms. In contrast, Zeira et al. (2007) used low power (30 mW) but with an exposure time as long as 2 – 3 minutes. The reason for this unusual set of parameters is likely due to the fact that optoporation was performed *in vivo* at a depth of 400  $\mu$ m through the skin. After optimisation with PI, I selected 100 mW for 41 ms as suitable laser parameters (see section 5.1.2 and 5.1.3), which is not dissimilar from that used by Stevenson et al. (2006) and Baumgart et al. (2008). Likewise, my parameters after optimisation with DAPI was similar to that used by Tirlapur and Konig (2002) (50 mW for 12 ms from section 5.3). In section 5.4, with shockwave optoporation, higher

powers would be needed, thus, a laser power of 120 mW was used to transfect a cell with DsRed plasmid (Figure 5-20). From my observations, only a short exposure time of the order of microseconds was needed to permeabilise cells in this way. It is difficult to compare these values to the aforementioned literature since they involved direct irradiation of the membrane instead. Although shockwave optoporation has been used in 3 other studies (Yamaguchi et al., 2008 ; Souhayer et al., 2002 ; Clark et al., 2006), it should be noted that the latter 2 used a different laser, the Nd:YAG. These publications do not list sufficient information to be able to calculate mean powers and exposure times used and, therefore, cannot be reliably compared with the results in this thesis. Yamaguchi et al. (2008), however, did use a Ti:Sapphire laser but with a much lower repetition rate of 125 Hz, compared to 80 MHz as configured in this thesis. This meant that Yamaguchi et al.'s (2008) average laser power was several orders of magnitude lower at 1.25  $\mu$ W. Although this power has been successfully reported for the entry of nanoparticles, it may not be suitable for introducing larger-sized plasmid DNA into cells, which has not been attempted by the authors.

The reported efficiencies of transfection with a plasmid encoding for a fluorescent protein are 40-60% (Stevenson et al., 2006 ; Praveen et al., 2011), 70% (Baumgart et al., 2008), and 70-80% (Uchuganova et al., 2008) in either cell lines or stem cells. Since transfection requires the plasmid to enter the nucleus (ie. during nuclear breakdown and cell division), it is understandable that high transfection efficiencies can be achieved in rapidly dividing cells. Praveen et al. (2011) further used Nupherin-Neuron, a nuclear localization signal which assists in bringing the plasmid into the nucleus. In my work, human fibroblasts appear to divide much less frequently, therefore, the chances of the plasmid entering the nucleus before possible degradation is lower, which could result in the extremely low efficiencies observed. When Nupherin-Neuron was used here in fibroblasts, only 1 dividing cell was transfected, which suggests that transfection was due to cell division and nuclear membrane breakdown, and not because of the

nuclear localization signal. This may also mean that Nupherin Neuron is not suitable in primary cells.

## **5.6 Summary**

In summary, optoporation was optimised for human fibroblast cells using DAPI and PI in two different culture media: Serum-free OptiMEM and HBSS. The absence of serum was preferred as there was a risk of DNase denaturing the plasmid. Table 5-1 summarises some of the parameters that were attempted. As described earlier, cells that experienced the most promising parameters were tested with calcein-AM for their survivability. Table 5-2 shows the number of GFP and DsRed cells obtained by optoporation using these optimised parameters from Table 5-1.

Fibroblasts could be optoporated with DAPI and PI at a high efficiency with the cells remaining viable. However, when the same laser parameters were used for GFP and DsRed plasmid, almost no cells were transfected. Furthermore, it was not possible to obtain consistent transfection efficiencies. Although the plasmid may have been able to enter into the cytoplasm through the transient pore created by optoporation, it is likely that it was still impeded by the nuclear membrane. Optoporating the nuclear membrane has not been attempted here. Instead, the plasmid was conjugated with Nupherin-Neuron, a nuclear localization sequence, and optoporated as described by Praveen et al. (2011). Despite implementing this modification, DsRed expressing cells were still not observed. Since Praveen et al.'s (2011) experiments with CHO cells were successful, it is possible that Nupherin-Neuron may not be suitable in primary cells.

**Table 5-1: Comparison of laser parameters and different media for optoporation of DAPI and PI. Each condition was performed on 100 cells from 1 experiment. 'YES' in capitals denotes conditions which gave best results based on fluorescent molecule uptake and cell survivability.**

| <b>Fluorescent molecule</b> | <b>Medium</b> | <b>Power (mW)</b> | <b>Exposure time (ms)</b> | <b>Uptake of florescent molecule</b> | <b>Survival shown by calcein-AM</b>   |
|-----------------------------|---------------|-------------------|---------------------------|--------------------------------------|---------------------------------------|
| <b>PI</b>                   | OptiMEM       | 100               | 41                        | YES                                  | YES                                   |
|                             |               | 150               | 41                        | Yes                                  | N/A, but drastic change in morphology |
|                             | HBSS          | 20-80             | 41                        | Yes                                  | N/A – cells appeared unhealthy        |
|                             |               | 10                | 41                        | No                                   | N/A since no uptake of PI             |
|                             |               | 6-12              | 82                        | YES                                  | YES                                   |
|                             |               | 20                | 20                        | Yes                                  | Not consistent                        |
| <b>DAPI</b>                 | OptiMEM       | 50                | 12                        | YES                                  | YES                                   |
|                             |               | 60-80             | 12                        | Yes                                  | N/A, cells died                       |

**Table 5-2: Summarised results of GFP and DsRed transfection by optoporation. Each condition shows the result of an experiment where there were transfected cells. Experiments with no transfected cells were ignored. Except for when Nupherin Neuron was used, 100 cells were optoporated each time.**

| <b>Plasmid</b> | <b>Medium</b>  | <b>Power<br/>(mW)</b> | <b>Exposure<br/>time (ms)</b> | <b>Number of<br/>cells<br/>optoporated</b> | <b>No. of<br/>transfected<br/>cells in<br/>treated<br/>sample</b> | <b>No. of<br/>transfected<br/>cells in<br/>control<br/>sample</b> |
|----------------|----------------|-----------------------|-------------------------------|--------------------------------------------|-------------------------------------------------------------------|-------------------------------------------------------------------|
| <b>GFP</b>     | OptiMEM        | 100                   | 41                            | 100                                        | 5                                                                 | 0                                                                 |
| <b>GFP</b>     | HBSS           | 6-12                  | 82                            | 100                                        | 3                                                                 | 2                                                                 |
| <b>GFP</b>     | HBSS           | 6-12                  | 82                            | 100                                        | 15                                                                | 7                                                                 |
| <b>DsRed</b>   | OptiMEM        | 6-12                  | 82                            | 100                                        | 0                                                                 | 5                                                                 |
| <b>DsRed</b>   | OptiMEM<br>+NN | 120                   | 1.58 $\mu$ s                  | 22                                         | 1                                                                 | 0                                                                 |

# Chapter 6

## Optoporation with Small Molecules

The optoporation of nucleic acids in chapter 5 resulted in a low efficiency and required overnight incubation before the production of fluorescent protein could be observed. I therefore, tried optoporating fluorescent small molecules (DAPI and PI) which immediately stained the nucleus after entry into the cell. I proceeded to measure the kinetics of small molecule entry.

Using the optimised parameters for DAPI and PI optoporation, I also showed that optoporation could be used to substitute inhibitors of the histone demethylase JMJD3. The remainder of this chapter will discuss two methods to over-express JMJD3 in HeLa cells and the optoporation of two cell-impermeable compounds (8-Hydroxyquinoline, or 8HQ ( $M_r = 145.16$ ), and FMF1293) to inhibit demethylation H3K27 by JMJD3.

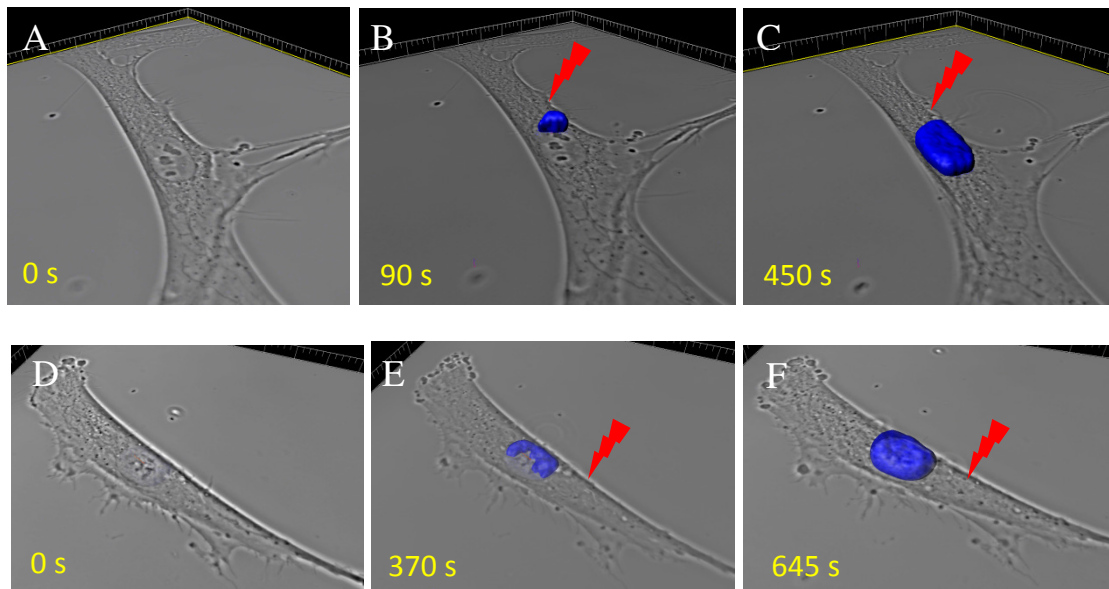
### 6.1 Single Cell Optoporation with DAPI

Human fibroblasts were prepared on glass dishes and optoporated as described in Chapter 3.1.2. Optoporation was performed in antibiotics-free medium containing DAPI (Sigma-Aldrich) at a dilution of 1:1000 resulting in a final concentration of 20  $\mu\text{g/ml}$ . The laser power and exposure time was set to 120 mW and 0.79  $\mu\text{s}$  respectively. This laser setting was borrowed from optoporation with BSA (see chapter 7). Since DAPI is smaller than BSA, and the cells survived the optoporation procedure, I reasoned that this laser setting could be



similarly used for introducing DAPI into living cells as well. Further optimisation was not necessary since the survivability was sufficiently high already (approximately 70%) in order to monitor the kinetics of the DAPI fluorescence signal as it entered the cell.

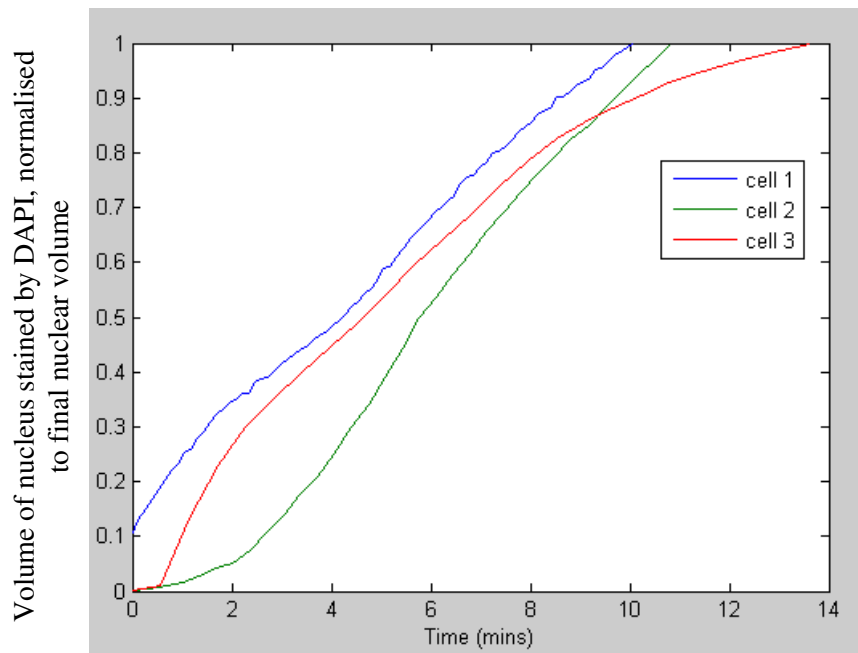
A 3D timelapse image was recorded before and after the procedure for at least 10 minutes. The 4D imaging data for the blue fluorescence channel was imported into Imaris (Bitplane AG, Switzerland) for analysis and volumetric rendering as shown in Figure 6-1. Imaris generates a voxel for every blue pixel (from the DAPI fluorescence) that is over a certain threshold on each plane. The volume is automatically calculated and normalised to the final volume in the dataset, which is assumed to be the full volume of the nucleus. It can be seen that DAPI entered the nucleus from the side which was closest to the site of optoporation. Eventually, the entire nucleus was filled within 10 - 15 minutes.



**Figure 6-1: Timelapse imaging of DAPI nuclear uptake (blue) in (A-C) cell 1 and (D-F) cell 3. Red lightning bolt indicates location of optoporation. Images are representative of 3 cells from 1 experiment.**

The progression of nuclear staining by DAPI was normalised to each of the 3 nucleus' final volume and then plotted against time for each cell (Figure 6-2) in MATLAB. Normalisation was necessary since the size of the nuclei for every cell would vary. From the graph, it can be seen that the rate of DAPI entering the cell and, thus, staining the nucleus (gradient) was

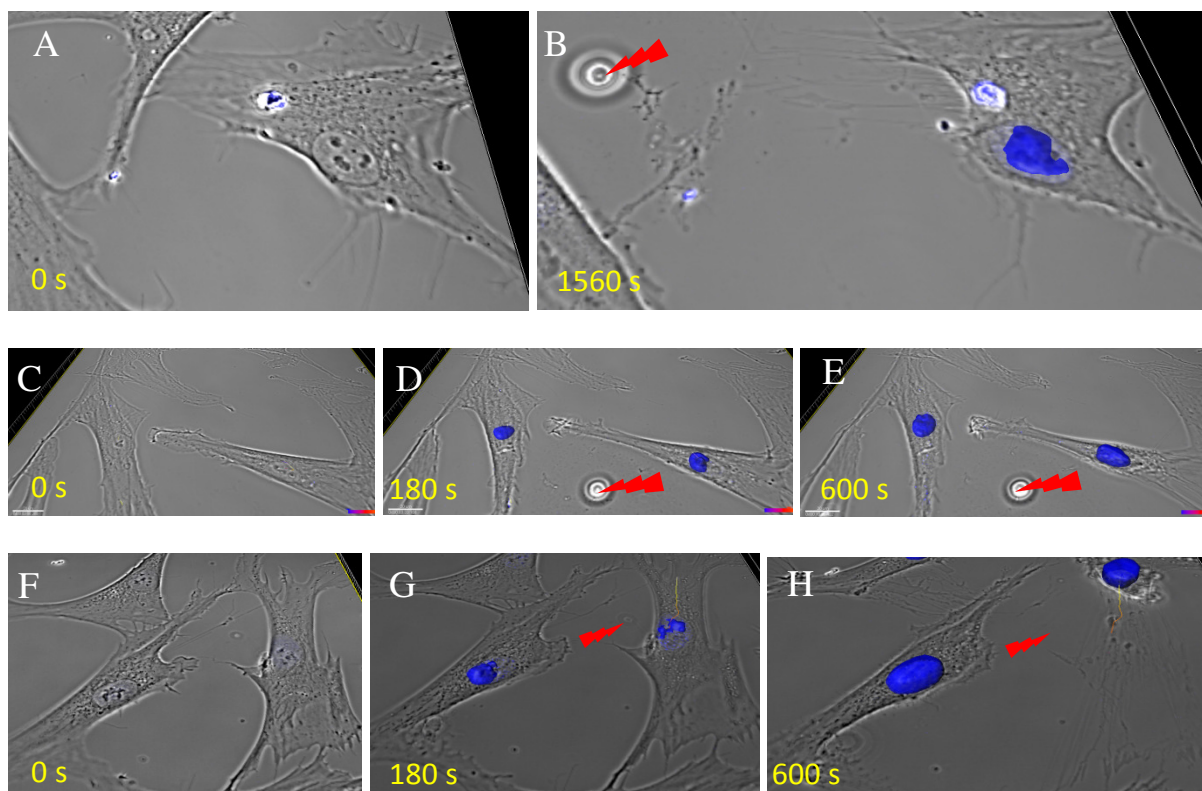
similar between all cells. In cell 3, the rate also tapered off as the increase in DAPI staining seemed to saturate.



**Figure 6-2:** Graph showing nuclear volume of 3 cells stained by DAPI normalised to final nuclear volume vs time (mins) after single cell optoporation

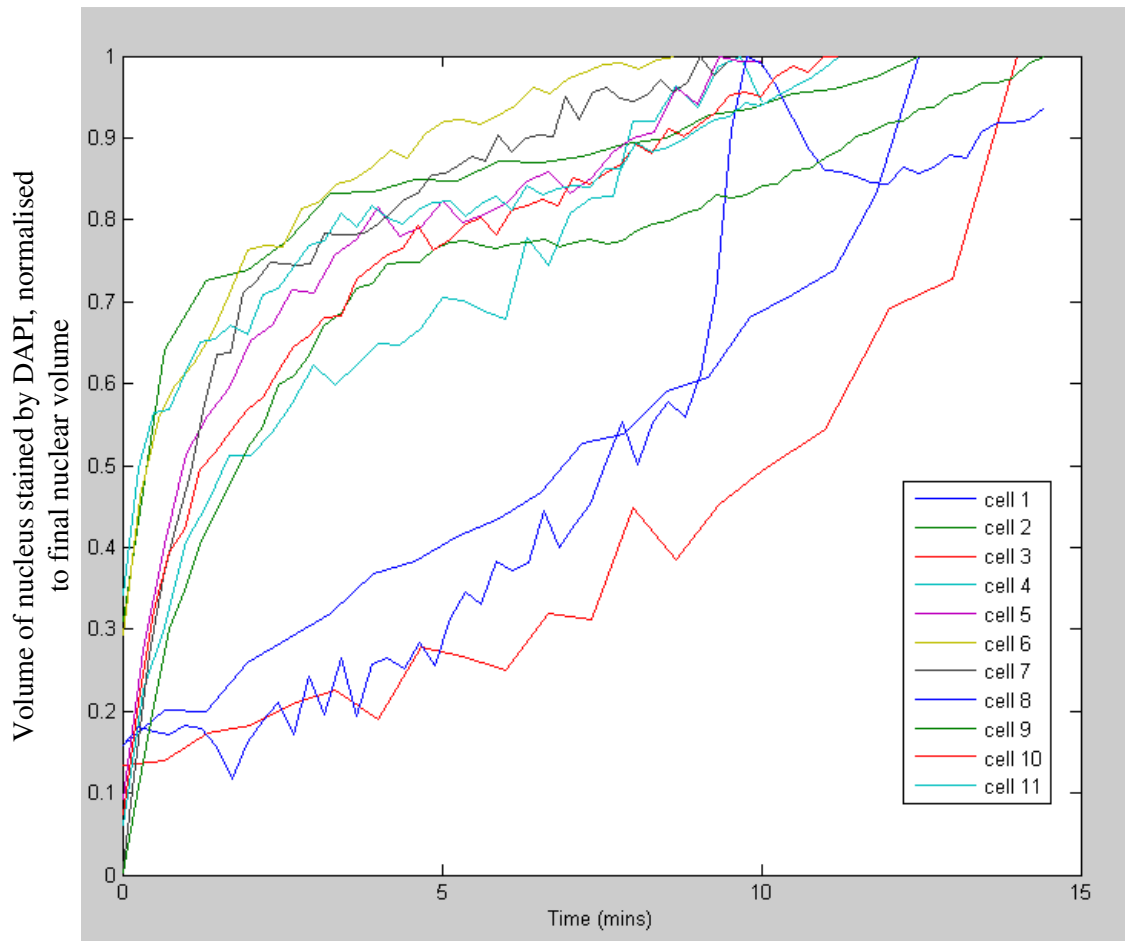
## 6.2 Shockwave Optoporation with DAPI

Shockwave optoporation involves targeting the substrate that the cells are adhered to with the laser at high power. Yamaguchi et al. (2008) and Soughayer et al. (2002) have used this technique where the laser was aimed approximately 30 – 40  $\mu\text{m}$  and 20  $\mu\text{m}$  away from the cell membrane respectively. In this thesis, the laser was focused at about 20  $\mu\text{m}$  away from the cells and below the interface between glass and medium. The rationale of using this technique is to explore the possibility of increasing the size and/or number of the pore(s), thus, allowing more DAPI to enter. The laser was emitted for approximately 50  $\mu\text{s}$  at 50 - 80 mW. In several instances, a single shot was able to optoporate at least 2 cells simultaneously. The uptake of DAPI in these cells was recorded as a z-stack so that the blue fluorescence channel could be surface rendered, as shown in Figure 6-3.



**Figure 6-3: Timelapse imaging of DAPI nuclear uptake (blue) in (A & B) cell 3, (C-E) cell 4 (left cell) and cell 5 (right cell) and (F-H) cell 8 (right cell) and cell 9 (left cell). Red lightning bolt indicates location of optoporation. Images are representative of 11 cells from 1 experiment.**

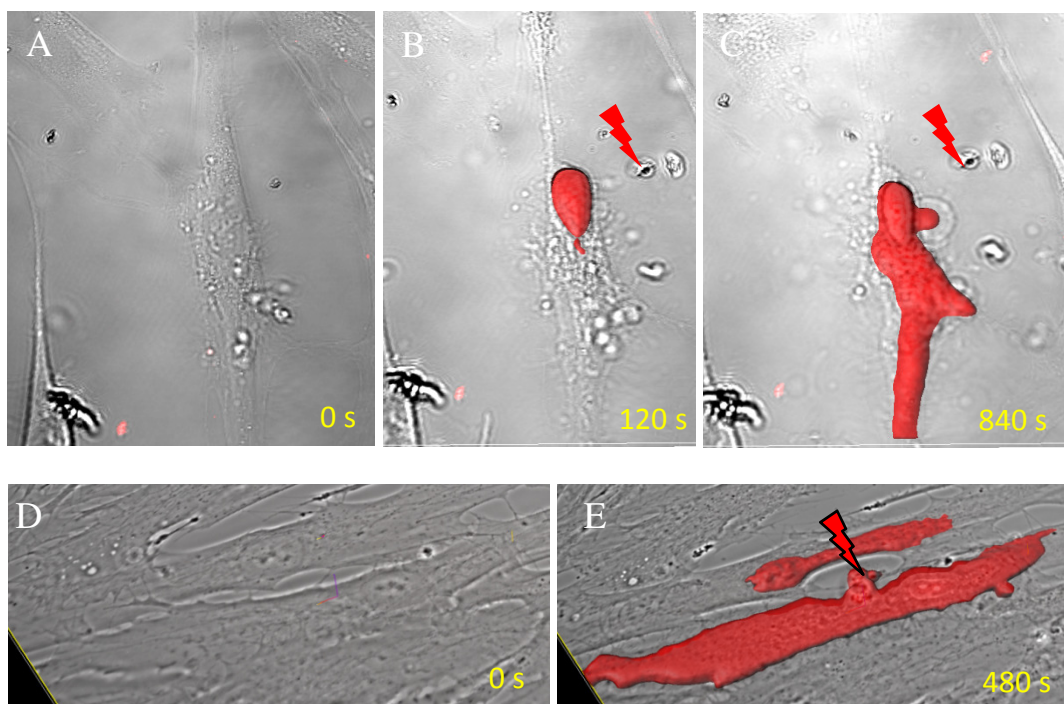
Interestingly, when the uptake of DAPI was plotted versus time for all cells, the population seemed to fall into two types of groups. The more common group displayed a curve which had an initial rapid increase in DAPI uptake but eventually displayed saturation towards the end of the timelapse course. Cells 4 and 5 in Figure 6-3 C-E and cell 9 of Figure 6-3 F-H were some of the cells that displayed this trend. Based on morphology, these cells appeared to remain viable after optoporation as they did not experience a drastic change in morphology throughout the entire procedure. On the other hand, cell 3 in Figure 6-3 A & B and cell 8 in Figure 6-3 F-H rapidly shrivelled and did not recover their original morphology. These cells were likely not viable after optoporation. When the uptake of DAPI was plotted, their trend was entirely different from the viable cells. The rate of DAPI uptake was initially gradual but increased rapidly and tended towards infinity by about 12 - 15 minutes.



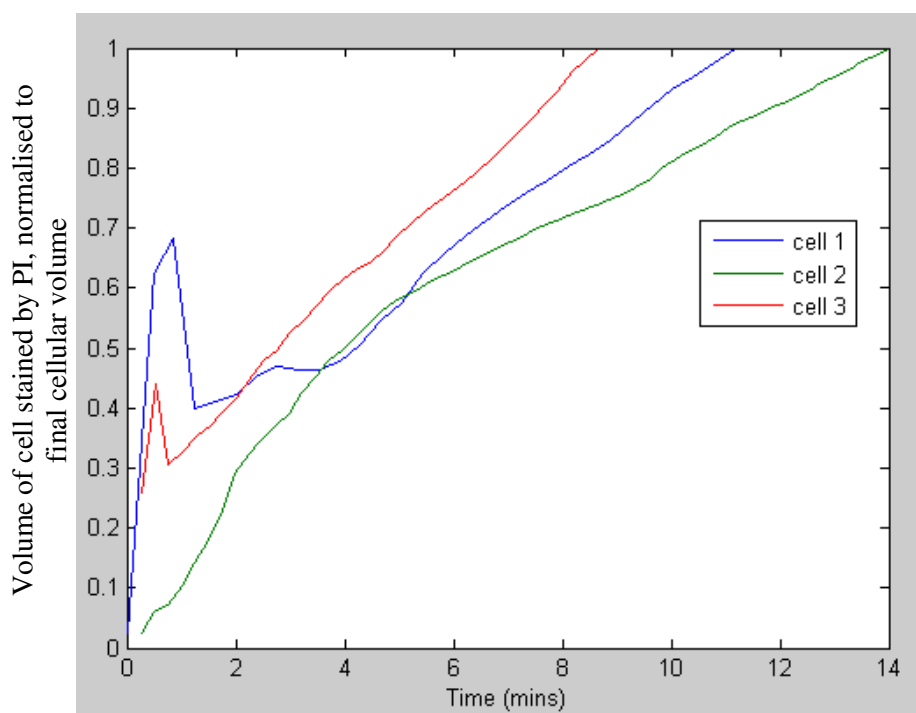
**Figure 6-4: Graph showing nuclear volume of 11 cells stained by DAPI, normalised to final nuclear volume vs time (mins) after shockwave optoporation**

### 6.3 Shockwave Optoporation with Propidium Iodide

The experiment was repeated with propidium iodide (Invitrogen) diluted 1:1000 to a final concentration of 1  $\mu\text{g}/\text{ml}$  with laser parameters of 70 - 90 mW for 1.58  $\mu\text{s}$ . When the cells were imaged in 4D for PI uptake, they appeared to fall into three categories. Cells 1 and 3 in Figure 6-5 A-C and D & E respectively show ruptured cell membranes with contents of the cytoplasm leaking out. PI filled the entire cell and this was consistent with the plot of PI uptake versus time. The trend of these cells was a continuous increase in PI uptake without saturation.



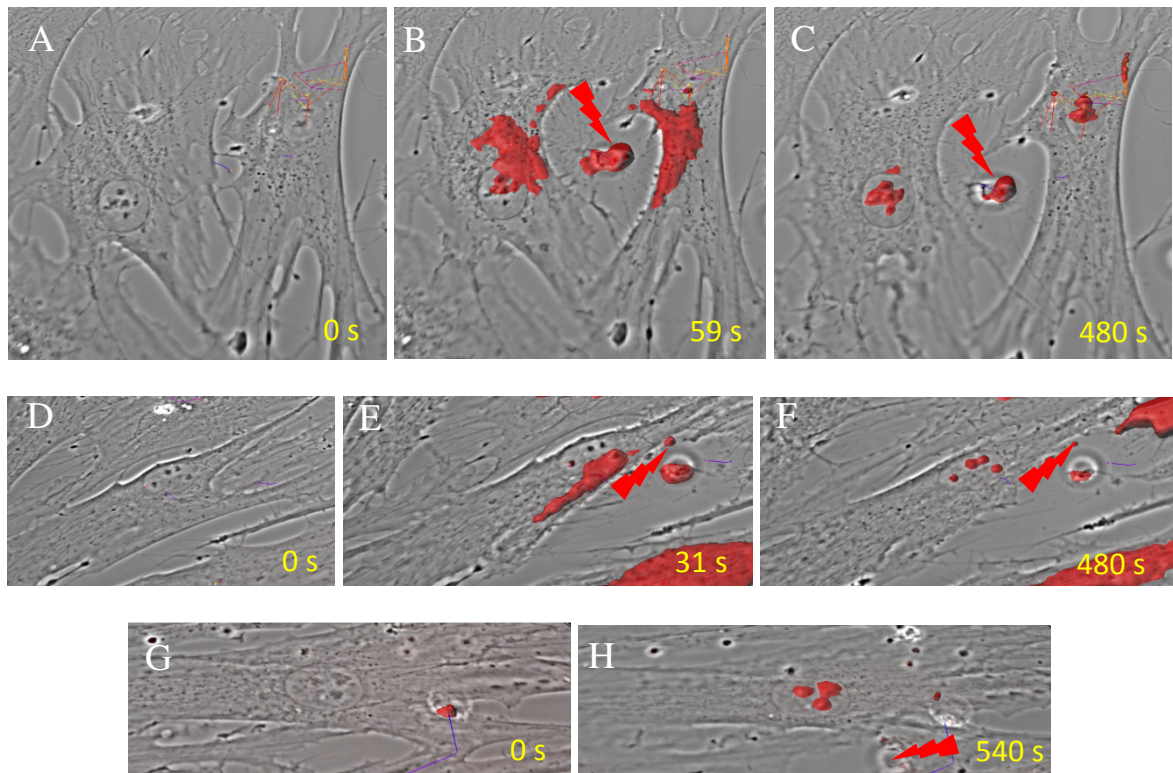
**Figure 6-5: Timelapse imaging of PI nuclear uptake (red) in (A-C) cell 1 and (D & E) cell 3 (bottom). Red lightning bolt indicates location of optoporation. Images are representative of 3 cells from 1 experiment.**



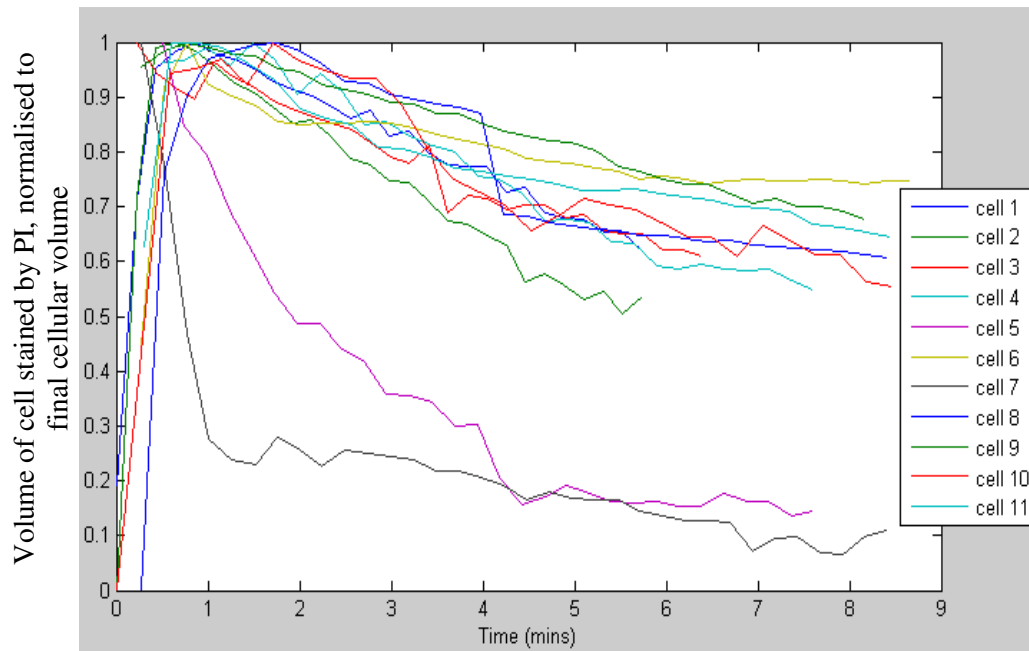
**Figure 6-6: Graph showing volume of cell stained by PI, normalised to final cellular volume for 3 cells versus time (mins) after shockwave optoporation**



During the experiment, some cells displayed a different trend of PI uptake. After the initial rapid entry of PI, these cells were able to recover and reseal their membranes, thus, preventing more PI from entering. From the timelapse images in Figure 6-7, PI migrated from the cytoplasm near the entry site to the nucleus and flooded it. Interestingly, from then on, the fluorescence from PI started to diminish after reaching a peak at about 1 minute, shown in Figure 6-8. The drop in PI fluorescence appeared to reach a plateau around the 8 minute mark. In the case of cells 5 and 7, however, the decrease stabilised by about 4 and even 1 minute respectively. By this time, PI could only be found in the dark nuclear spots of the optoporated cells as shown in Figure 6-7 C, F, and H. The confinement of PI to the small and dark nuclear regions was consistent with the downward trend shown in Figure 6-8.



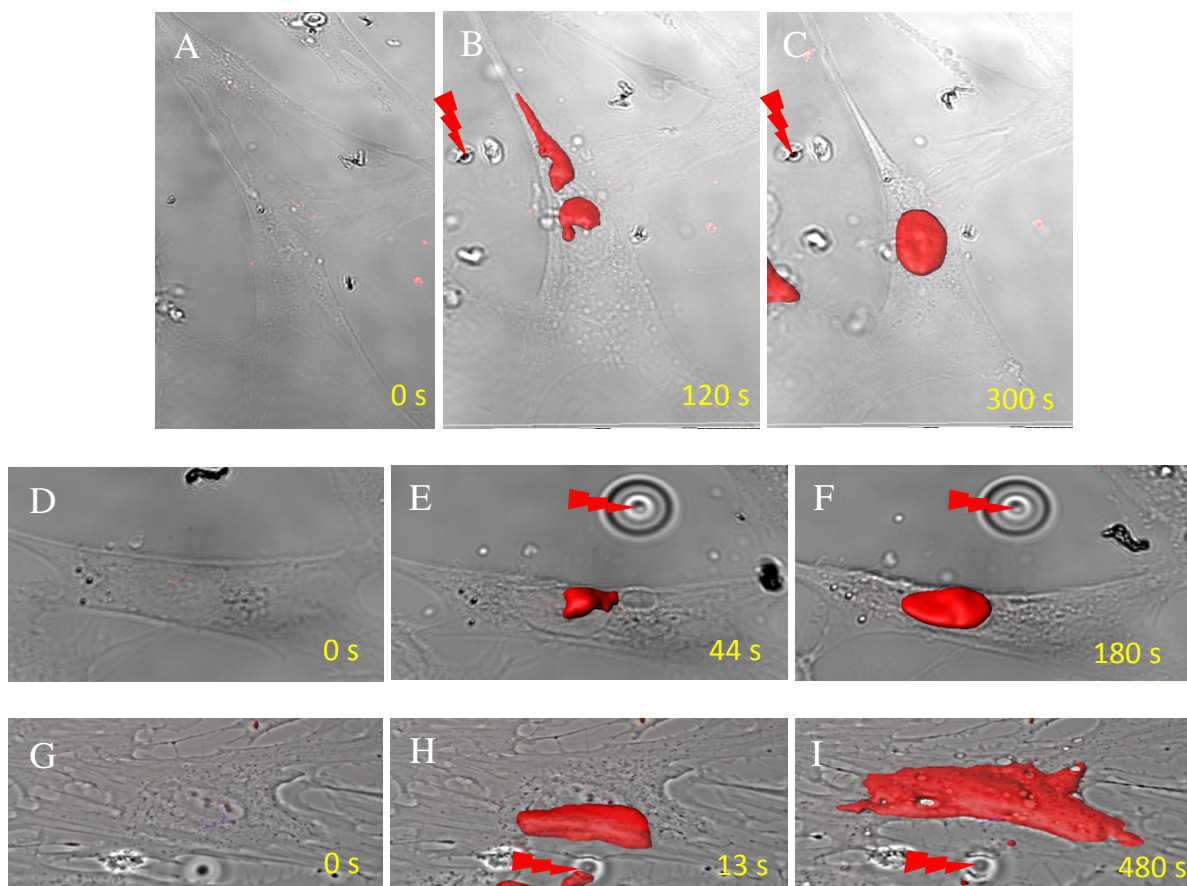
**Figure 6-7: Timelapse imaging of PI nuclear uptake (red) in (A-C) cell 4 (left cell) and 5 (right cell), (D-F) cell 7, (G & H) cell 10. Red lightning bolt indicates location of optoporation. Images are representative of 11 cells from 1 experiment.**



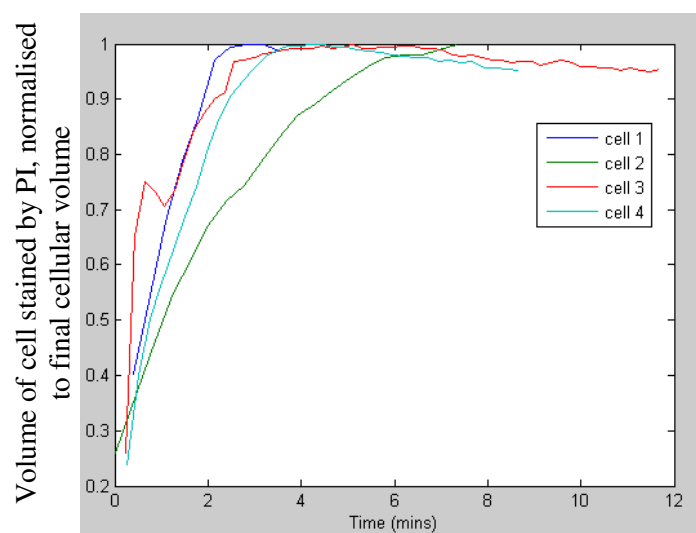
**Figure 6-8: Graph showing volume of cell stained by PI, normalised to final cellular volume for 11 cells versus time (mins) after shockwave optoporation**

Cells also fell into a third category of PI uptake kinetics. After optoporation, the uptake of PI was rapid and once the membrane resealed itself, the cell appeared viable. Further entry of PI was prevented and the amount of PI entering the cell therefore stabilised which is consistent with the plot. In these cells, PI entered the cell and localised into the nucleus and remained there, as shown in Figure 6-9 A-C and D-F.





**Figure 6-9: Timelapse imaging of PI nuclear uptake (red) in (A-C) cell 1, (D-F) cell 2, (G-I) cell 3. Red lightning bolt indicates location of optoporation. Images are representative of 4 cells from 1 experiment.**



**Figure 6-10: Graph showing volume of cell stained by PI, normalised to final cellular volume for 4 cells versus time (mins) after shockwave optoporation**

## **6.4 Optoporation of Demethylase Inhibitors for Drug Development**

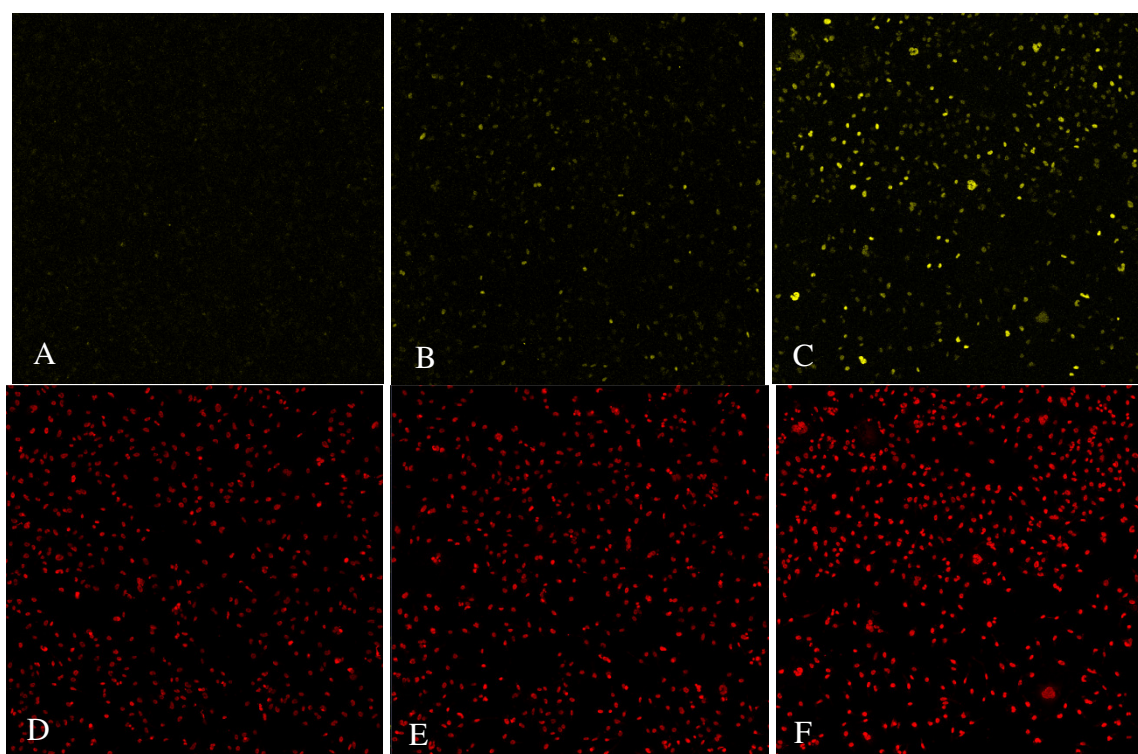
In sections 6.1 - 6.3, I showed that optoporation could be used to bring in fluorescent small molecules such as DAPI and PI into living human fibroblasts. The function of these dyes is to fluoresce intensely after intercalating with DNA. Section 6.4 will expand on this by using optoporation to introduce inhibitors of the histone demethylase JMJD3. First, this section will discuss two methods to over-express JMJD3 in HeLa cells. Then, this section goes on to discuss the optoporation of two membrane-impermeable compounds (8-Hydroxyquinoline, or 8HQ, and FMF1293) into the cells to inhibit the demethylation of H3K27 by JMJD3. I chose to optoporate cells directly on the membrane at 120 mW for 0.79  $\mu$ s as outlined in section 6.1. Since cells had to be cultured at high density for transfection, the use of shockwave optoporation was unsuitable.

For immunostaining, a primary antibody for H3K27me3 (rabbit, IgG, Millipore) was used with a secondary antibody (Alexafluor 568 goat anti-rabbit IgG, Invitrogen).

### **6.4.1 Overexpression of JMJD3 by BacMam virus**

HeLa cells were seeded onto glass bottom dishes at 60% confluency and transfected with a 10% mixture of BacMam (Invitrogen) virus encoded with JMJD3 fused with enhanced yellow fluorescent protein (eYFP), provided by GlaxoSmithKline. The cells were incubated until maximum expression of eYFP could be observed by fluorescence imaging. A 27-hour timecourse study revealed that expression of eYFP increased and peaked at 18 hours post infection. By 15 - 18 hours, the advantage of the BacMam system could be seen as nearly all cells had been transfected giving high transfection efficiency (Figure 6-11C). However, when the H3K27me3 marks were analysed (Figure 6-11D – F), the resultant staining and methylation marks did not decrease over time as expected. It is not exactly known why this is the case, however, one reason could be due to the inability of the BacMam virus to cause over-expression of JMJD3 in HeLa cells to cause global demethylation. On the other hand,

transient transfection with Fugene HD (see section 6.4.2) allowed cells to produce sufficient JMJD3 protein to induce global demethylation.

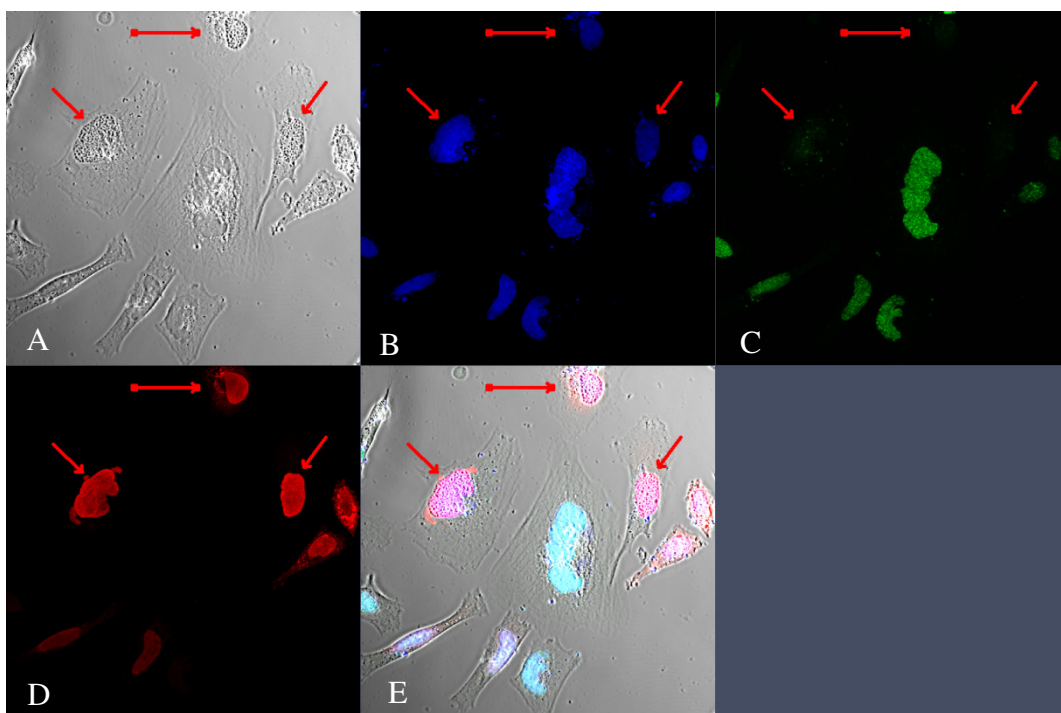


**Figure 6-11:** Expression of (A – C) eYFP (yellow) and the (D – F) resulting H3K27me3 stain (red) at (A & D) 0 hours (control), (B & E) 10 hours, (C & F) 18 hours after infection with JMJD3 BacMam virus. Images are representative from 3 experiments.

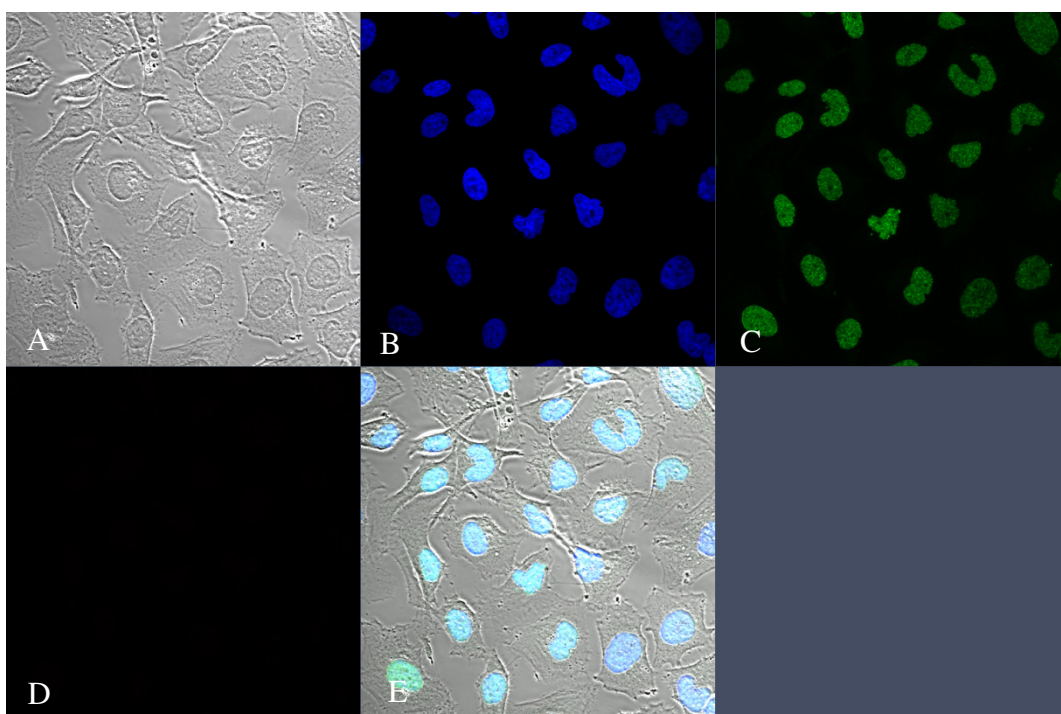
#### **6.4.2 Overexpression of JMJD3 by Fugene HD**

I used Fugene HD (Roche Diagnostics) as an alternative to transfect HeLa cells. This produced very low transfection efficiency (approximately 5 - 15%). As Figure 6-12 C & D show, cells that had been transfected (red) with JMJD3 displayed a significant reduction in H3K27me3 (green) after just 18 hours. Cells in the control sample showed no obvious decrease (Figure 6-13).

For immunostaining, a primary antibody for H3K27me3 (rabbit, IgG, Millipore) was used with a secondary antibody (Alexafluor 488 goat anti-rabbit IgG, Invitrogen). The secondary antibody for the FLAG-tag was Alexafluor 594 goat anti-mouse IgG (Invitrogen).

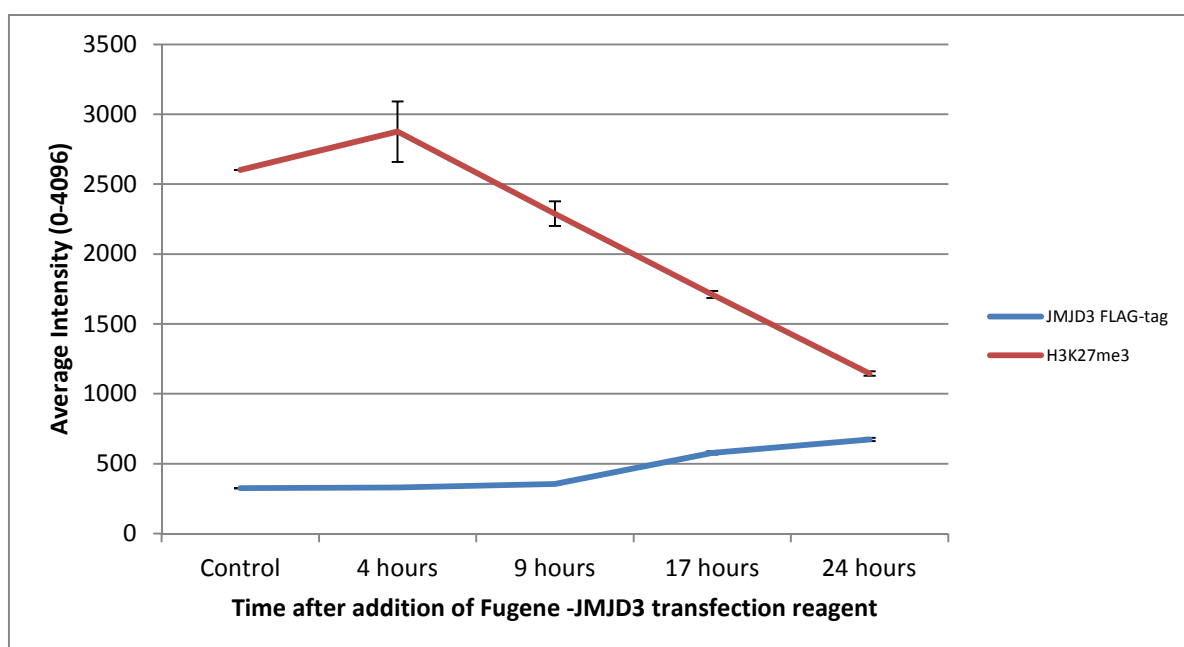


**Figure 6-12: JMJD3 FLAG-tag transfected sample using Fugene HD. (A) phase contrast image, (B) DAPI nuclear stain, (C) H3K27me3, (D) JMJD3 FLAG-tag, (E) merged image. Red arrows indicate transfected cells undergoing demethylation. Images are representative from 1 experiment.**



**Figure 6-13: Control sample without transfection of JMJD3. (A) phase contrast image, (B) DAPI nuclear stain, (C) H3K27me3, (D) JMJD3 FLAG-tag, (E) merged image. Images are representative from 1 experiment.**

Both the intensities of JMJD3 and H3K27me3 in the control and transfected samples were analysed at different timepoints using an adherent cell counter (BD Pathway, BD Biosciences). From Figure 6-14, JMJD3 expression (blue) could be observed to increase 9 hours after transfection. As expected, there was a reduction in H3K27me3 (red) beginning after 4 hours and continued through to the end of the experiment. The final intensity had reduced by approximately 2-fold. It is interesting to note that H3K27me3 appears to decrease before JMJD3 expression. One possibility for this anomaly is that the transfection reagent itself may have caused an early decrease in global demethylation at 4 hours. Such a control (with Fugene, but without JMJD3 plasmid) was not conducted so this cannot be confirmed. Another possibility is that the quality of the secondary antibody was poor, thus, the staining at 4 hours may have simply been weak despite the presence of JMJD3 protein. This seems reasonable since the fluorescence of the secondary antibody against JMJD3 was still relatively low even at 24 hours, where it should have been highest.



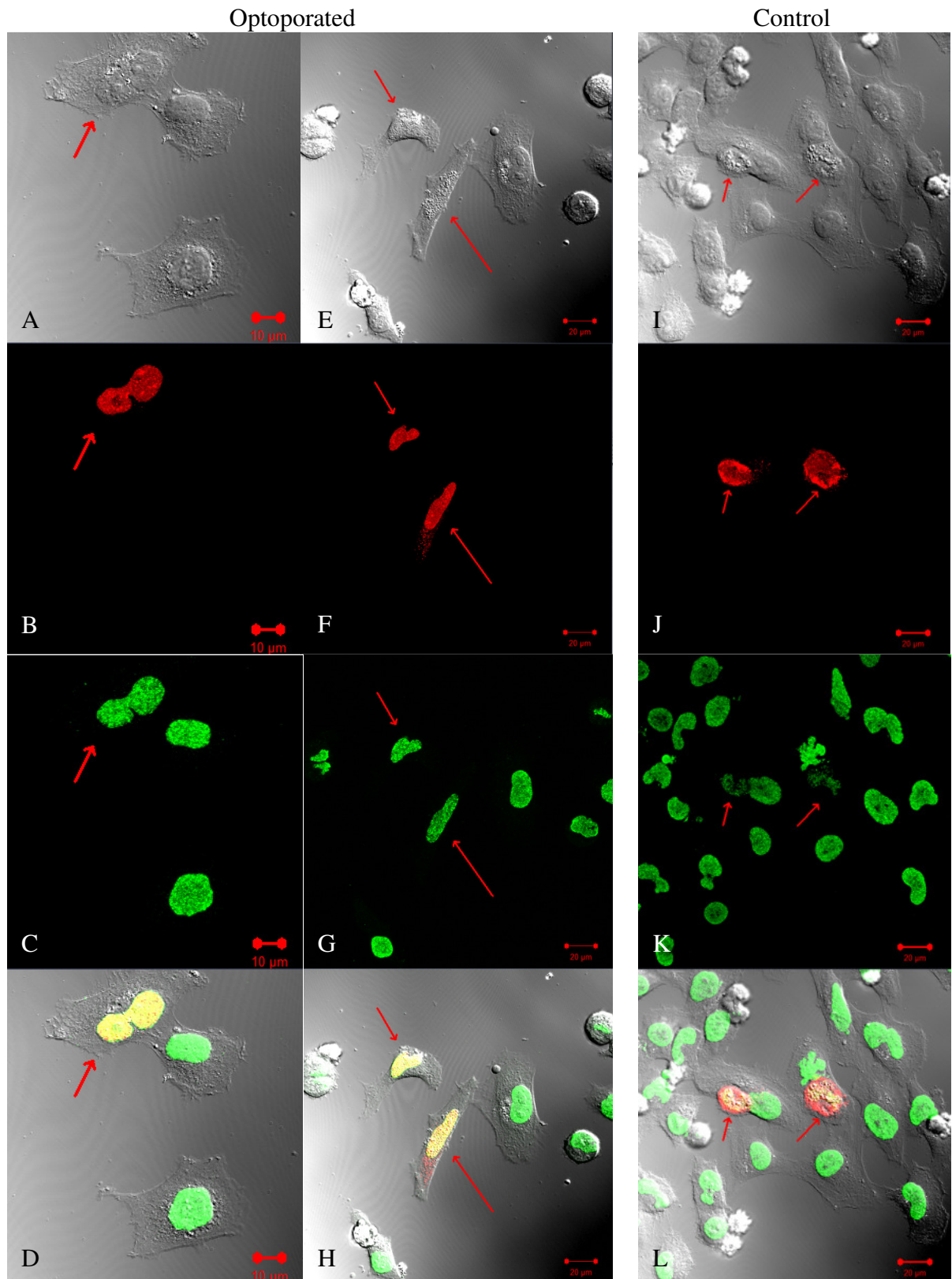
**Figure 6-14:** Graph showing decrease in H3K27me3 and increase in JMJD3 expression over 24 hours in HeLa cells. Data is representative of about 600 – 1200 cells from 1 experiment.

### 6.4.3 Optoporation of 8-hydroxyquinoline (8HQ)

Six hours after transfection, the medium was changed to OptiMEM (Invitrogen) supplemented with 8HQ at 20  $\mu$ M. 8HQ was first reported to inhibit JMJD2 with an  $IC_{50}$  of 86.5  $\mu$ M by King et al. (2010) and has been proposed to possibly have an effect on JMJD3 as well. This compound, according to King et al. (2010), has a suitable structure for which other selective inhibitors can be derived from through modification. Approximately 100 - 150 HeLa cells were optoporated with 8HQ in 1 hour at 120 mW for 0.79  $\mu$ s. Imaging and optoporation were performed at the same wavelength of 700 nm instead of the usual 800 nm because the transmission detector is optimal at lower wavelengths and generated a clearer image. To be able to return to the optoporated cells for later viewing, the positions of each cell were stored. The optoporated cells were chosen at random as there was no way the transfected cells could in any way be identified until they had been fixed and stained with antibodies. After optoporation, the medium was changed back to antibiotics-free culture medium and the cells were incubated for a further 6 hours before fixing and staining.

As shown in Figure 6-15 C & G, the JMJD3 transfected cells (red) that underwent optoporation in the presence of 8HQ showed positive staining of H3K27me3 (green). The intensity of the staining was visually similar to the neighbouring untransfected cells. In contrast, although the transfected cells in the control sample (Figure 6-15 J) had been cultured in the presence of 8HQ, they were not optoporated and, therefore, did not stain as intensely with H3K27me3 (Figure 6-15 K).



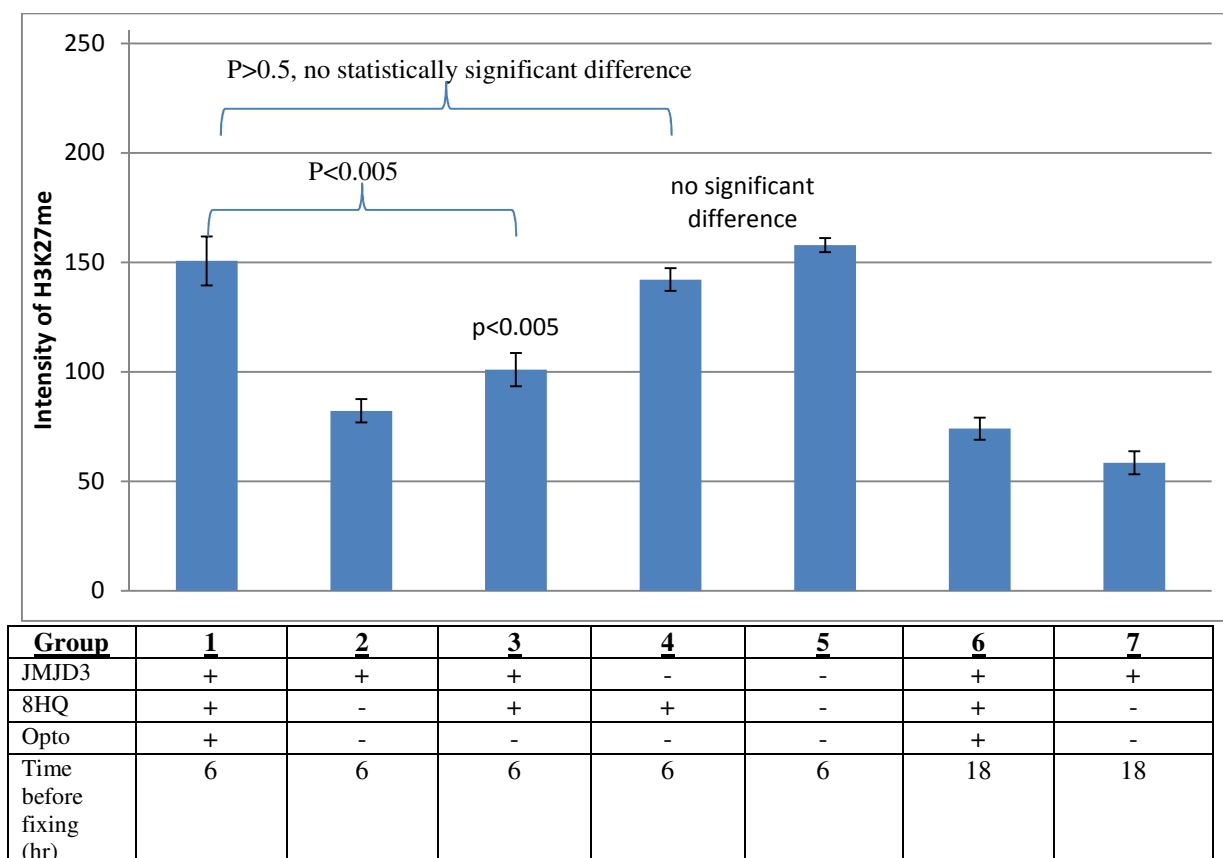


**Figure 6-15: Simultaneous imaging of 3 sets of HeLa cells transfected with JMJD3. (A-H) cells optoporated with 8HQ 6 hours after transfection with JMJD3. Cells were fixed and incubated with antibodies a further 6 hours later. (I-L) control samples cultured in presence of 8HQ but not optoporated. (A, E, I) are phase contrast images. (B, F, J) and (C, G, K) are fluorescence images of JMJD3-FLAG (red) and H3K27me3 (green) respectively. (D, H, L) merged images. Cells of interest are shown with red arrows. Images are representative from 3 experiments.**



Because the transfection of JMJD3 by Eugene was low (less than 10%), the probability of optoporation a cell expressing JMJD3 without prior knowledge of whether it had been transfected or not was also low (n=11). Cells which happened to be optoporated as well as stained positively for the FLAG tag of JMJD3 had their H3K27me3 intensities measured and plotted (Figure 6-16). The graph shows that there is a statistical significance due to optoporation with the drug (compare Group 1 vs. Group 2 and 3). On the other hand, there was no significant difference between the treated group and control cells (Group 4 and 5) which experienced no transfection or drug treatment at all. This suggested that optoporation of 8HQ had inhibited the demethylation caused by the overexpression of JMJD3.

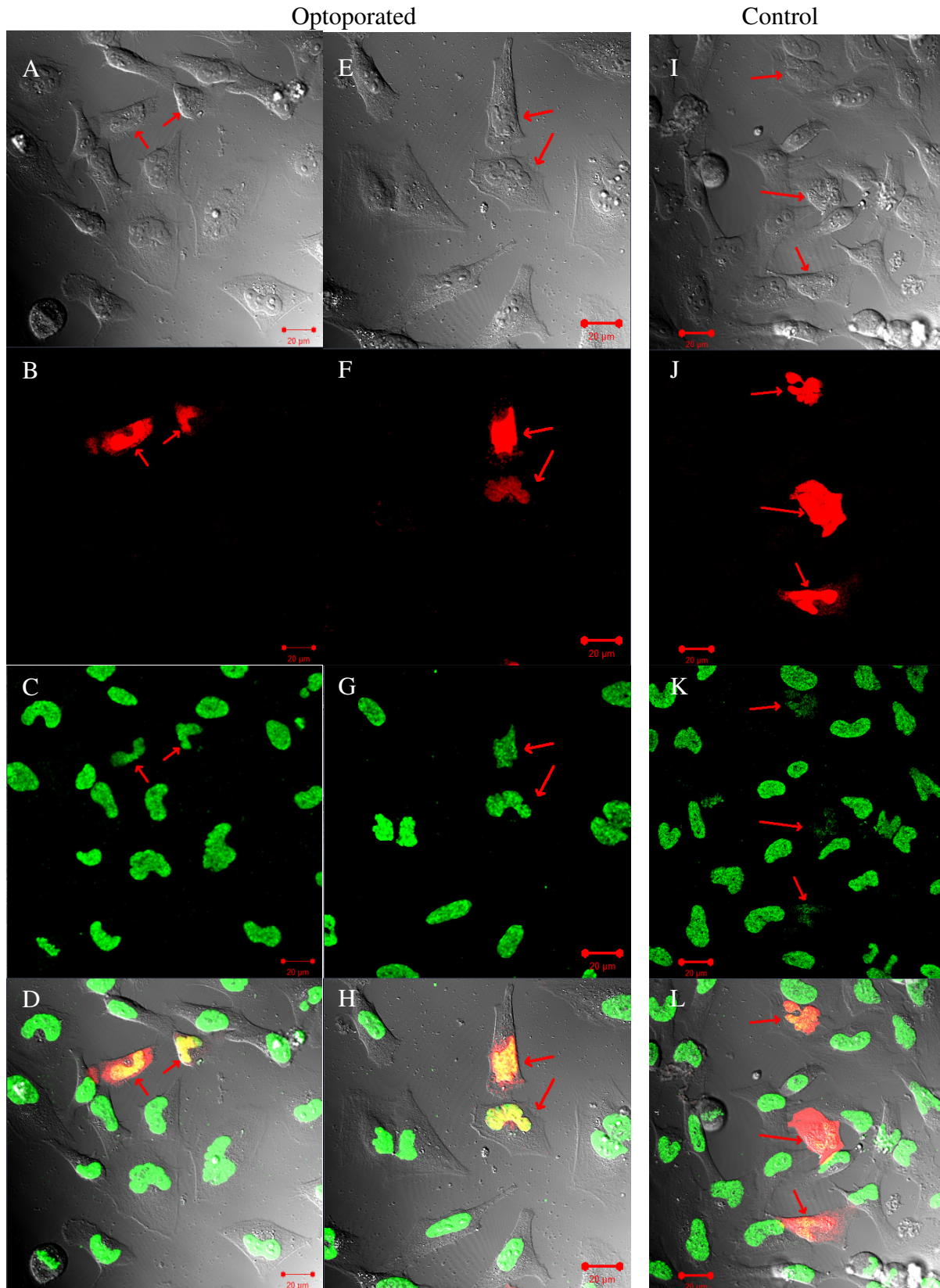
Interestingly, if I had allowed the cells to incubate with the drug for as much as 18 hours after optoporation (24 hours after transfection), there was no significant inhibition of demethylation (compare Group 6 vs. Group 7). The intensity of H3K27me3 staining in the 18-hour treated and control samples were similar.



**Figure 6-16:** Graph showing change in intensity of H3K27me3 staining after treatment with 8HQ. A region of interest was drawn around the nuclei of optoporated cells and the average intensity measured. Data from Group 1 taken from 11 cells from 3 independent experiments. Other Groups represented by >15 cells. Standard error bars are shown. Statistical significance was calculated using Mann-Whitney test.

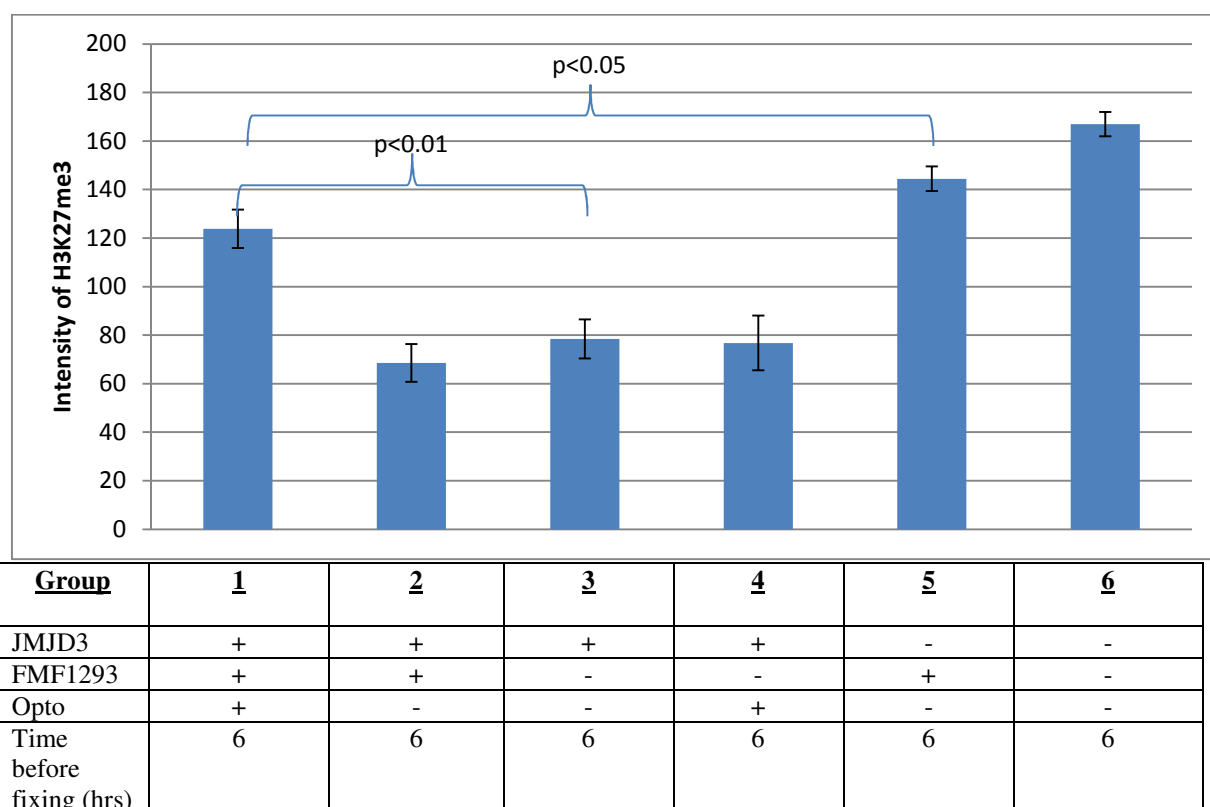
#### 6.4.4 Optoporation of FMF1293

Cells were also optoporated in the presence of FMF1293 using the same laser parameters. This compound has been known to be less active in cells compared to 8HQ even at 100  $\mu$ M. The purpose of this experiment was to determine if optoporation could inhibit demethylation at just 20  $\mu$ M. As expected, from Figure 6-17 J, the control cells which overexpressed JMJD3 (labelled with red arrows) did not stain strongly for H3K27me3 (Figure 6-17 K). On the other hand, the optoporated cells in Figure 6-17 C & G showed more intense staining.



**Figure 6-17: Simultaneous imaging of 3 sets of HeLa cells transfected with JMJD3. (A-H) cells optoporated with FMF1293 6 hours after transfection with JMJD3. Cells were fixed and incubated with antibodies a further 6 hours later. (I-L) control samples cultured in presence of FMF1293 but not optoporated. (A, E, I) are phase contrast images. (B, F, J) and (C, G, K) are fluorescence images of JMJD3-FLAG (red) and H3K27me3 (green) respectively. (D, H, L) merged images. Cells of interest are shown with red arrows. Images are representative from 1 experiment.**

By visual inspection of Figure 6-17 C & G, the optoporated cells did not stain as intensely as neighbouring untransfected cells. This was confirmed when the intensities were plotted as shown in Figure 6-18. Although Group 1 was significantly different from transfected cells without drug treatment (Group 2 - 4), it was also significantly different from the JMJD3-groups (Group 5 & 6) as well. Thus, FMF1293 was unable to *fully* inhibit demethylation with optoporation.



**Figure 6-18:** Graph showing change in intensity of H3K27me3 staining after treatment with FMF1293. A region of interest was drawn around the nuclei of optoporated cells and the average intensity measured. Data from Group 1 taken from 6 cells in 1 experiment. Other Groups represented by >13 cells. Standard error bars are shown. Statistical significance was calculated using Mann-Whitney test.

## 6.5 Discussion

Several fluorescent dyes have successfully been reported to be optoporated into cells. These are propidium iodide (Lei et al., 2008, Marchington et al., 2010, Antkowiak et al., 2010), Lucifer yellow (Peng et al., 2007), Glycine conjugated with Texas Red (Soughayer et al., 2000), merocyanine 540 (Mohanty et al., 2003), and cascade blue acetyl azide (Stracke et al.,

2005). Only in the case of Soughayer et al. (2000) was shockwave optoporation used and this was carried out with a Nd:YAG laser with a 5 ns pulse at an unknown mean laser power. Here, using a Ti:Sapphire laser, propidium iodide was successfully optoporated into human fibroblasts using 70 – 90 mW for 1.58  $\mu$ s. So far, the kinetics of propidium iodide and DAPI fluorescence as they enter living and dying cells has not been quantified. In living cells, I observed that the increase in fluorescence is rapid within the first 1-2 minutes before it levels off. This is likely due to the cell healing as it recovers from the wound. The rate of dye entry progressively decreases. This trend has also been shown by Peng et al. (2007) but with Lucifer yellow, which has a smaller molecular weight than propidium iodide, but larger than DAPI. In their paper, the increase in fluorescence is much more gradual than that shown by PI and DAPI in this chapter. This is likely due to the fact that both DAPI and PI exhibit an increase in fluorescence emission after binding to nucleic acids, whereas Lucifer Yellow does not. The kinetics of DAPI and PI fluorescence in irrecoverable cells have not been quantified by Peng et al. (2007) or in any other publication. Here, however, DAPI and PI have been shown to exhibit an approximate linear increase in signal. There is no plateauing of signal intensity likely because the pore does not seal. Peng et al. (2007) have described changes in morphology for dying cells, which are consistent with the results here. For example, Peng et al. (2007) and Stracke et al. (2005) report that cells can appear to “explode” (such as in Figure 6-5 A- C) or “curl up from the bottom of the cell culture dish” (such as in Figure 6-3 A - B & F - H). A vapour bubble has also been reported to appear following optoporation, however, this could not be observed on the imaging system here since it is not equipped with a CCD camera.

Instead of using shockwave optoporation, Lei et al. (2008) and Marchington et al. (2010) focused the laser to a spot on the membrane. The former authors used a power of 20 mW for 100 ms. On the other hand, Marchington et al. (2010) employed a different strategy by using

2 to 3 doses of 90 mW of power for 4 ms. Since the exposure time was less in the latter study, the laser power would have had to be increased to account for this. However, with multiple laser pulses, it is likely that Marchington et al. (2010) would not have needed to increase the power above 90 mW. In this thesis, when the laser was focused onto the cell membrane, I needed a laser power of 120 mW for 0.79  $\mu$ s in order to introduce DAPI into cells. Since the exposure time is several orders of magnitude lower than the two aforementioned studies, the laser power had to be increased. Such short laser pulses were only possible using bleach mode (see section 3.2.3), which has the advantage that the laser can be targeted onto multiple cells in one scan. This increases the throughput of optoporation. Clearly, all the laser settings from the two aforementioned studies and this thesis are not the same and cannot be compared easily. For example, Marchington et al. (2010) optoporated each cell in suspension with multiple doses whereas only 1 dose was used in this thesis. Additionally, Lei et al. (2008) optoporated neurons whereas this thesis involved fibroblasts instead. If cells are assumed to behave in the same way under the influence of a laser, then this shows that short exposure times are necessary if high power is to be used. Likewise, if the exposure time is lengthy (as in the case of Lei et al. (2008)), then the laser power must be reduced to avoid irreparable cell damage.

The use of optoporation to introduce inhibitors of demethylation has not been attempted prior to this thesis as a search into scientific literature has revealed. The  $IC_{50}$  for 8HQ and FMF1293 against JMJD3 have also not been published. However, King et al. (2010) determined the  $IC_{50}$  of 8HQ against JMJD2A to be 86.5  $\mu$ M and demonstrated that there was nearly 100% inhibition of the enzyme at approximately 400  $\mu$ M. If this concentration were to be similar in JMJD3, then optoporation has been shown to significantly reduce this. Here, I demonstrate that almost 100% inhibition of JMJD3 can be achieved at just 20  $\mu$ M with optoporation. A lower concentration is advantageous especially when compounds show high

toxicity or poor specificity. Thus, in some cases, optoporation may actually be necessary for testing otherwise impermeable compounds for their efficacy at less than toxic concentrations.

King et al. (2010) obtained results from a minimum of 400 cells for each concentration. This was not possible in this thesis due to the low transfection efficiency of JMJD3 and the speed at which optoporation could be carried out. With an efficiency of just 5 - 10%, approximately 5 – 10 JMJD3 positive cells could successfully be optoporated in 1 hour. As the transfection efficiency increases, the reliability of this technique should improve.

Despite the low efficiency, testing of chemical compounds using optoporation is actually quicker. After transfection, King et al. (2010) incubated cells with 8HQ for 24 hours before analysing the cells by immunofluorescence. In contrast, I had to only incubate cells with 8HQ for just 6 hours and this was sufficient to show 100% inhibition of JMJD3. Thus, with optoporation, I have been able to test compounds within 1 day, compared to King et al. (2010) who required 18 hours longer. Shorter incubation times have not been tried yet and may potentially lead to even quicker experiments and higher throughput.

The amount that is delivered into cells by optoporation is generally unpredictable because of the variability of the size of the pore. Future work may include radiolabelling as a means to measure the fraction of the drug that enters the cell. For example, the chemical compound could be radiolabelled with Carbon-14 and optoporated into a large number of cells. Any excess radiolabelled compound could be rinsed off and the plate or dish read by a scintillation counter to measure the number of decay events. This can be correlated to the number of radiolabelled compounds in the sample and then normalised to the number of optoporated cells to determine the amount introduced into each cell. A limitation of this technique is that one would have to account for the possibility of compounds sticking to the membrane or being engulfed by the cell, which would give a false positive. However, this could be



accounted for with an optoporation-free control. A second method could be to optoporate the compound radiolabelled with tritium ( $^3\text{H}$ ) instead, followed by immersing the sample in photographic emulsion. This technique, which uses  $^3\text{H}$  due to its quick energy dissipation and, therefore, improved spatial resolution, has been explained by Wilson and Goulding (1986). The authors state that as the tritium decays, it reduces the silver halide in the emulsion to metallic silver grains, which can be observed. As each grain originated from one decay event, the number of grains can be counted to determine the amount of compound present. This technique has the further advantage that one can observe in which cellular compartment the compound is located.

## 6.6 Summary

In this chapter, the uptake of small molecule fluorescent probes in living and dead fibroblasts was quantified after optoporation. When fibroblasts were optoporated directly on the membrane using DAPI, the rate of uptake was consistent between the cells tested. These cells were shown to be viable. When the fibroblasts were optoporated using shock waves, there were 2 distinct kinetics of uptake. Cells that displayed a rapid increase in DAPI but saturation within 5 minutes could be expected to remain viable after optoporation. In non-viable cells, the volume of DAPI entering the cell continued to increase even after 10 minutes while the overall size of the cell shrivelled.

Likewise, the kinetics of propidium iodide was also measured. Non-viable cells showed no plateauing of PI uptake even after 10 minutes. With viable cells, there existed 2 distinct kinetics of uptake. In one group of cells, the uptake of PI saturated within 2 - 6 minutes. In another group, the uptake of PI increased rapidly but the volume quickly diminished after 1 minute and continued to decrease before saturating to a new baseline after 6 - 7 minutes.

Accordingly, it may be possible to determine a cell's fate after optoporation, by quantitating the uptake of fluorescent probes.

This chapter also presented work in introducing two chemical compounds used for inhibiting the demethylation of H3K27 by JMJD3. These drugs (8HQ and FMF1293) are impermeable unless used at high concentrations, which can be toxic. Optoporation temporarily permeated the cell membrane to allow entry of these compounds into the cell where they later freely diffused into the nucleus as observed by DAPI and PI in section 6.1 - 6.3.

I observed that 8HQ was able to inhibit the action of JMJD3 and allow restoration of the H3K27 trimethyl marks. FMF1293 is known to be inactive in cells at below 100  $\mu$ M. Optoporation was also able to allow FMF1293 into cells at an even lower concentration, however, it was not as effective as 8HQ in inhibiting JMJD3. I theorised that higher concentrations (but still below 100  $\mu$ M) may be required.

It was interesting to note that the timing of optoporation after transfection is important. Generally, if optoporation with the drug compound occurs too early, the drug may not remain in the cell long enough when it is needed, particularly when expression of JMJD3 begins to increase. If optoporation occurs too late, demethylation may have already exceeded the drug's ability to inhibit JMJD3 any further thereby preventing methyltransferases from recovering the methyl marks.

The duration of the experiment is also important. If the cells are fixed too early, there would not be sufficient time for JMJD3 to demethylate H3K27. Thus, the treated and control sample would appear almost identical and I would not be able to identify whether the drug had worked or not. If the cells are fixed too late (ie. after 18 hours as can be seen in Figure 6-16), the drug may have been metabolised or transported out. Once this happens, the demethylation

of H3K27 would proceed further. As Figure 6-16 shows, the trimethyl marks were never restored (compare Group 5 & 6).

The work described in this chapter presents the technique of optoporation as a proof of principle for screening novel chemical compounds (in much less time compared to other protocols in literature) by selectively introducing them into cells. Optoporation creates a transient pore in the membrane of a cell, which allows a small amount of the compound to enter the cell, but as shown in this chapter, was sufficient to inhibit JMJD3 and restore methyl marks.

# Chapter 7

## Optoporation of Proteins into Fibroblasts

This chapter presents my work in introducing the proteins bovine serum albumin (BSA), phalloidin, and anti-actin antibody into primary fibroblast cells using optoporation. Each protein is discussed in its own subchapter containing specific materials and methods, results and discussion.

### 7.1 Optoporation of BSA-FITC

To optimise the optoporation settings for proteins, I chose BSA (labelled with FITC) as it was larger than phalloidin and approximately the same molecular weight (approximately 66 kDa) as the actin antibody. Also, BSA has not been known to be toxic in cells. I tried 4 strategies in introducing BSA into cells:

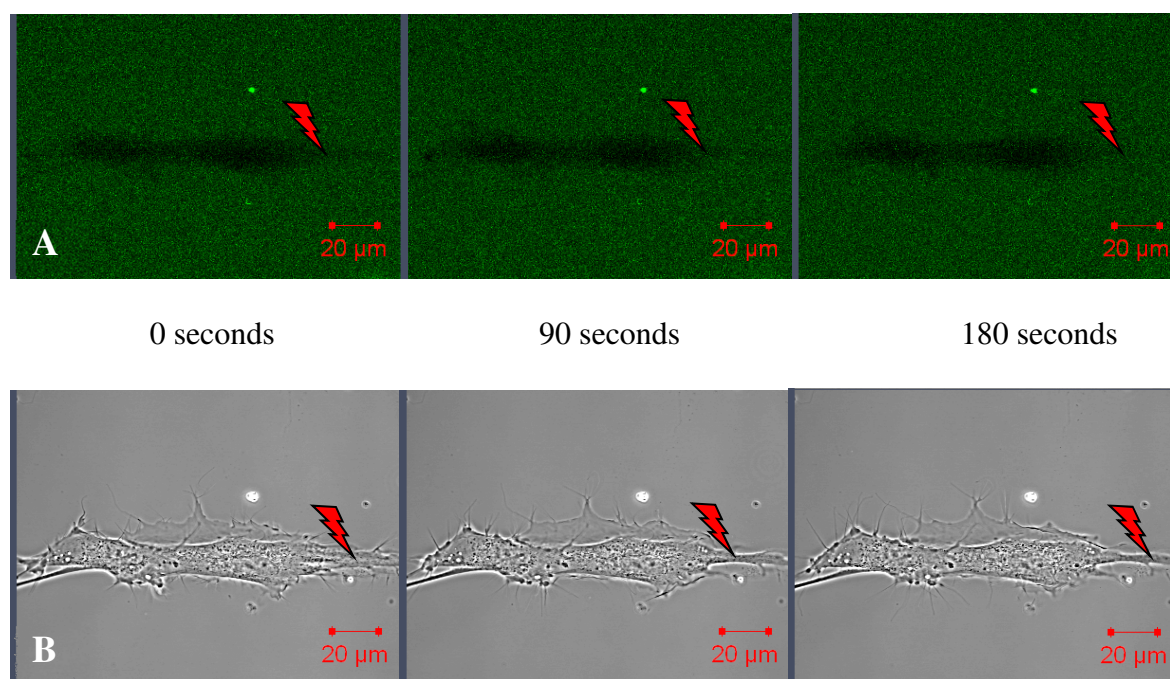
- Low power and short exposure time on the cell membrane
- High power and long exposure time on the cell membrane
- High power and long exposure time on cell membrane with high potassium content in OptiMEM
- Shockwave optoporation

### 7.1.1 Materials and methods

Bovine serum albumin protein (Thermo-Scientific) was conjugated with a FITC fluorophore and passed through a MicroSpin G25 column (GE Healthcare, UK). The eluent was diluted to a concentration of 0.1 mg/ml. BSA-FITC was used at a working concentration of 20  $\mu\text{g/ml}$  in 600  $\mu\text{l}$  of OptiMEM. Optoporation was conducted at 37 degrees Celsius and 5%  $\text{CO}_2$  in a humidified chamber. Human fibroblasts were first seeded on glass dishes as described in chapter 3.1.2.

### 7.1.2 Low power and short exposure time on cell membrane

During optimisation of parameters, human fibroblasts were first optoporated at 50 mW of power for 12 ms based on the optoporation of DAPI into HDFa cells with 80% efficiency. At this power level, no BSA was observed to enter any of the treated cells even after 180 seconds (Figure 7-1 A). The cells appeared to shrink slightly before recovering within 90 seconds. After this, the morphology of the cell was stable (Figure 7-1 B).

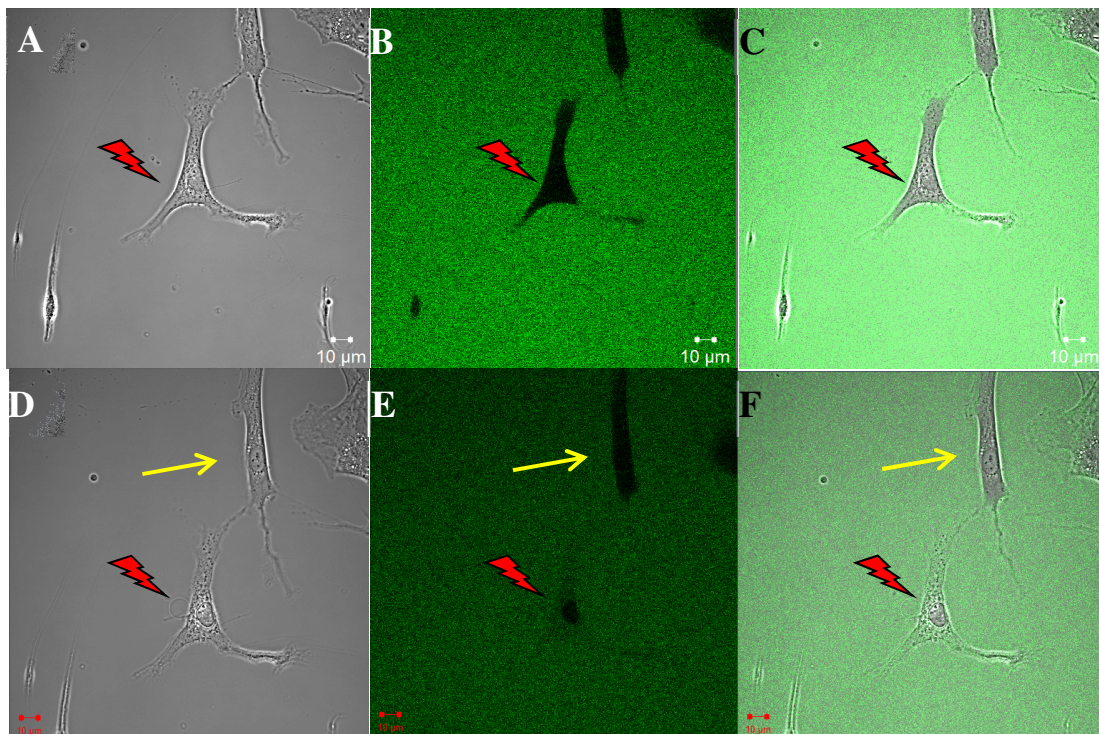


**Figure 7-1 : Simultaneous timelapse imaging of a human fibroblast cell optoporated at low power in the presence of BSA. Each slice was recorded at 0, 90, and 180 seconds. (A) BSA fluorescence (green), (B) corresponding phase contrast image. Images are representative of 20 cells from 1 experiment.**

### 7.1.3 High power and long exposure time on cell membrane

The power and exposure time were increased to 20 - 100 mW for 82 ms to allow more BSA to enter (see Table 7-1). I theorised that the pore would need to remain open longer. Pores that sealed quickly worked for DAPI since only a small amount of DAPI was needed to stain the nucleus intensely. However, this would not be sufficient for BSA-FITC because the fluorophore generally shows no increase in signal after entering the cell. A larger amount of BSA-FITC had to enter and diffuse throughout the entire cell volume just to be detected by fluorescence imaging.

Below, in Figure 7-2, a healthy cell is shown before and after optoporation surrounded by medium containing BSA protein (shown in green).



**Figure 7-2:** Simultaneous imaging of a human fibroblast before (A-C) and 30 minutes after (D-F) optoporation with BSA-FITC. (A & D) phase contrast image, (B & E) FITC fluorescence (green), (C & F) is the merged image. Site of optoporation is shown by red lightning bolt. Yellow arrow denotes an unoptoporated cell. Images are representative of 20 cells from 1 experiment.

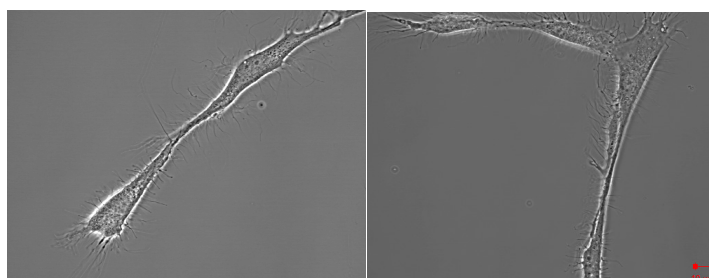
Thirty minutes after optoporation, in Figure 7-2 D-F, BSA had actually entered the cell. The cell, apart from the nucleus, eventually filled with the green protein as shown in Figure 7-2 E. From

the phase contrast image in Figure 7-2 D, it can be seen that a bubble had developed near the pore on the membrane, known as blebbing. Furthermore, the cell's membrane appeared to have collapsed, unlike the cell optoporated with DAPI in Figure 6-1. In contrast, the unoptoporated cell, labelled with a yellow arrow in Figure 7-2, remained dark, which shows that BSA did not enter without optoporation. Thus, by increasing the power and the duration of the pore opening, the cytoplasm had been allowed to leak out causing the cell to collapse and die.

#### 7.1.4 Low power and long exposure time on cell membrane with supplementation of K<sup>+</sup> ions

As the cytoplasm leaked out, vital ions that are needed for the cell to survive were likely to have been lost. Potassium was identified as one of the most important and abundant ions in cells. Thus, to prevent a drastic outward flow of potassium ions, the surrounding medium was supplemented with potassium carbonate to a final concentration of 140 mM so that both of the extra and intra cellular concentrations were similar.

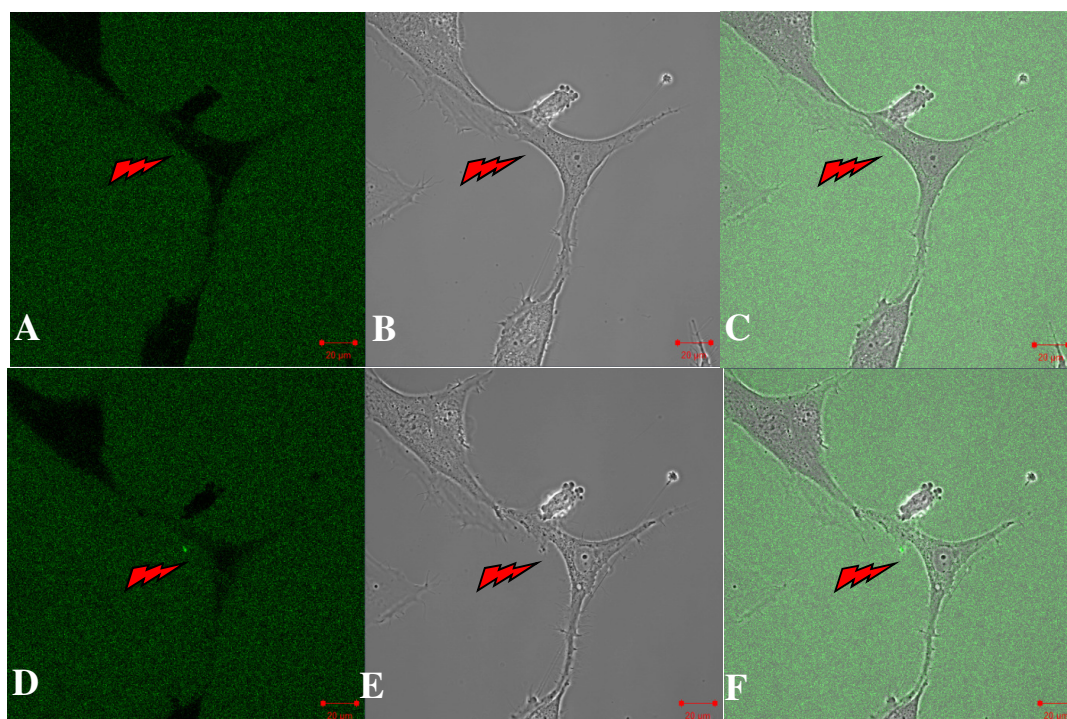
As expected, the higher external concentration of carbonate and potassium caused the majority of cells to shrink due to water leaving the cell through osmosis. Figure 7-3 below shows two examples of cells that have shrivelled. The cells' attachment proteins are easily discernible in this state.



**Figure 7-3: Examples of shrivelled fibroblast cells cultured in high potassium medium**

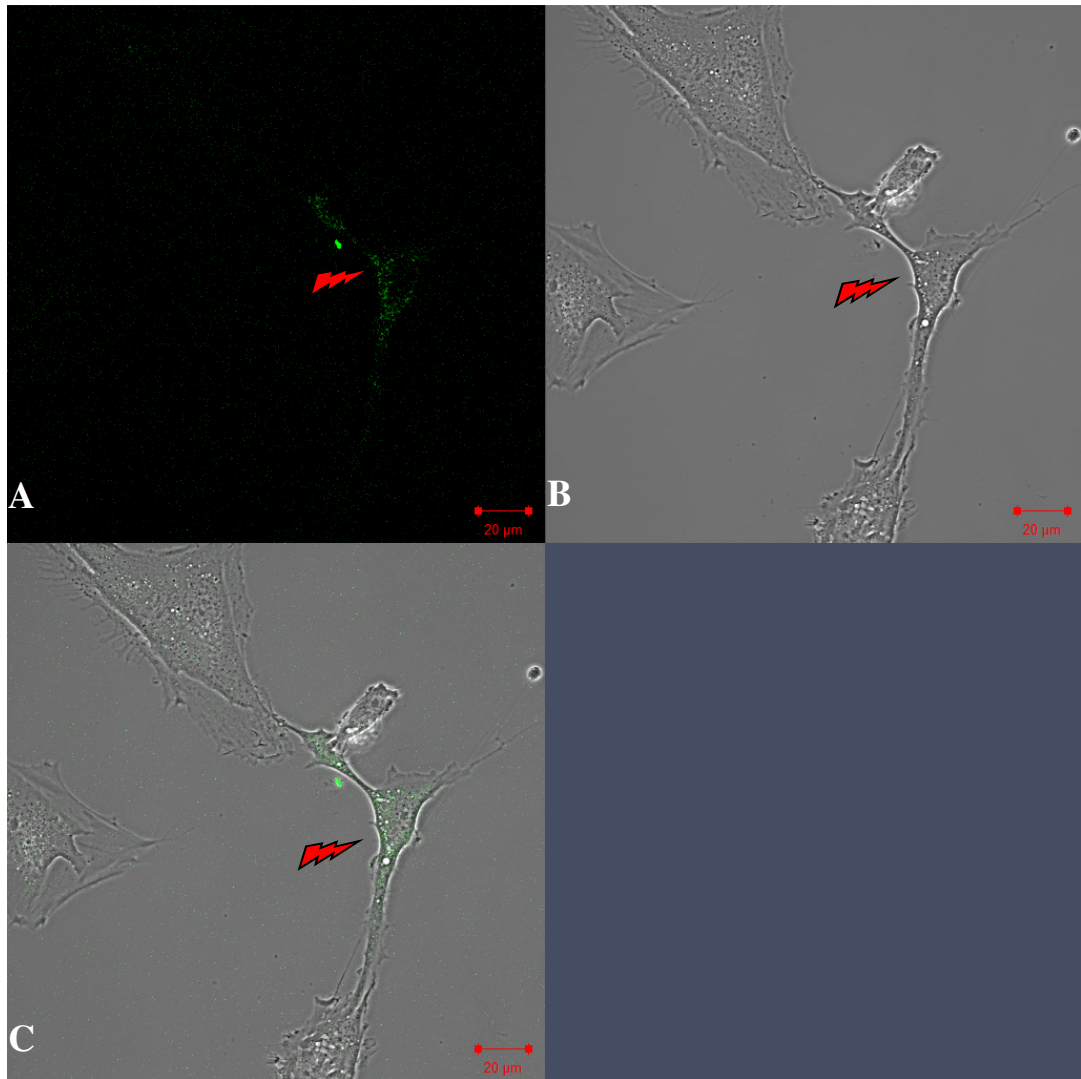


A small number of cells still appeared viable (Figure 7-4). Cells were optoporated at 18 mW (not shown) and at 36 mW for 82 ms (see Table 7-1). Almost the entire cell was filled with BSA apart from the nucleus (Figure 7-4 B). Some vesicles had also appeared inside the cell, which usually suggests that the cell was under stress.



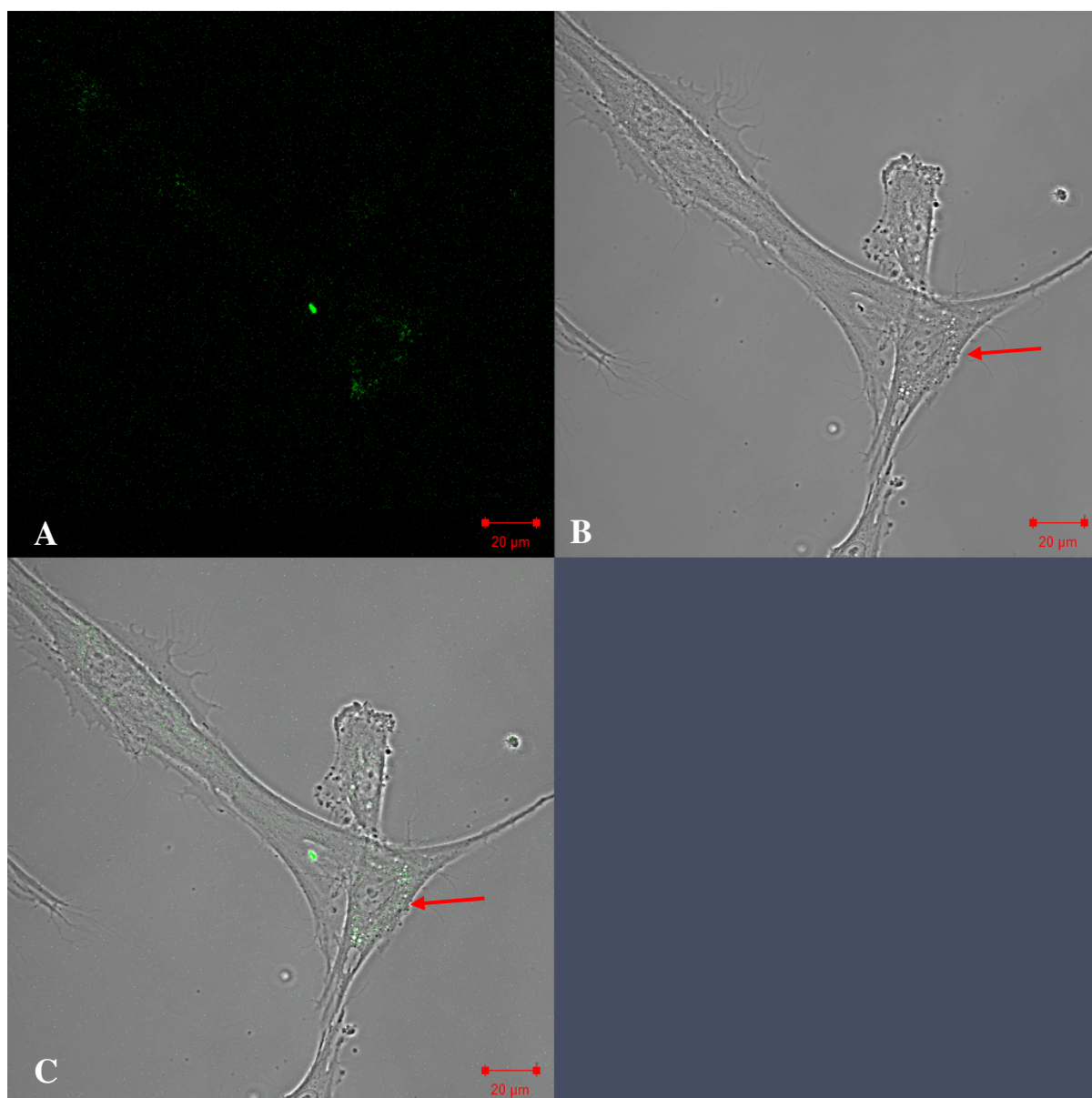
**Figure 7-4:** Simultaneous imaging of a human fibroblast before (A-C) and 30 minutes after (D-F) optoporation with BSA-FITC at 36 mW for 82 ms. (A & D) FITC fluorescence (green), (B & E) phase contrast image, (C & F) is the merged image. Site of optoporation is shown by red lightning bolt. Images are representative of 20 cells from 3 experiments.

After 2 hours and having washed the BSA away, the cell retained more BSA and had a stronger green fluorescence (Figure 7-5 A) when using 36 mW as opposed to using just 18 mW. This showed that a larger amount of BSA had entered the treated cell when higher power was used. Despite using higher laser powers, the cell seemed able to recover. The cell membrane had reformed, however, much of the cell's contents were missing and the cell had shrunk significantly (Figure 7-5 B). The vesicles were still present in the cytoplasm.



**Figure 7-5: Simultaneous imaging of (A) fluorescein fluorescence (green) and (B) phase contrast of a human fibroblast cell 2 hours after optoporation at 36 mW at 82 ms. (C) is the merged image. Site of optoporation is shown by red lightning bolt.**

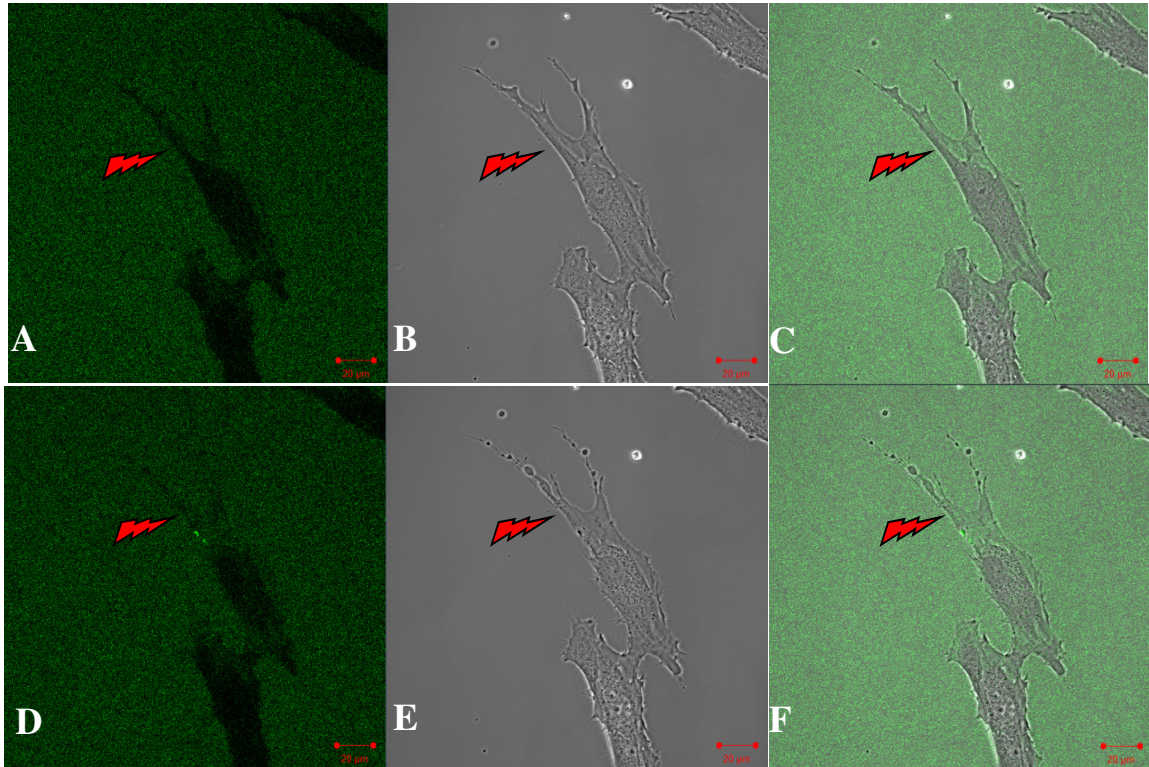
The following day, the same cell appeared much healthier and larger, thus, recovery had taken more than 12 hours to complete (shown with red arrow in Figure 7-6). During the recovery, neighbouring unoptoporated cells had further attached to the treated cell (Figure 7-6 B).



**Figure 7-6: Simultaneous imaging of (A) FITC fluorescence (green) and (B) phase contrast of the human fibroblast cell from Figure 7-5 1 day after optoporation at 36 mW at 82 ms. (C) is the merged image. Optoporated cell is shown by red arrow.**

The experiment was repeated with even higher powers of 100 mW for 82 ms (Figure 7-7 A-C), the result was similar to when 36 mW of power was used. However, the cell shrunk as BSA quickly entered (Figure 7-7 D-F).

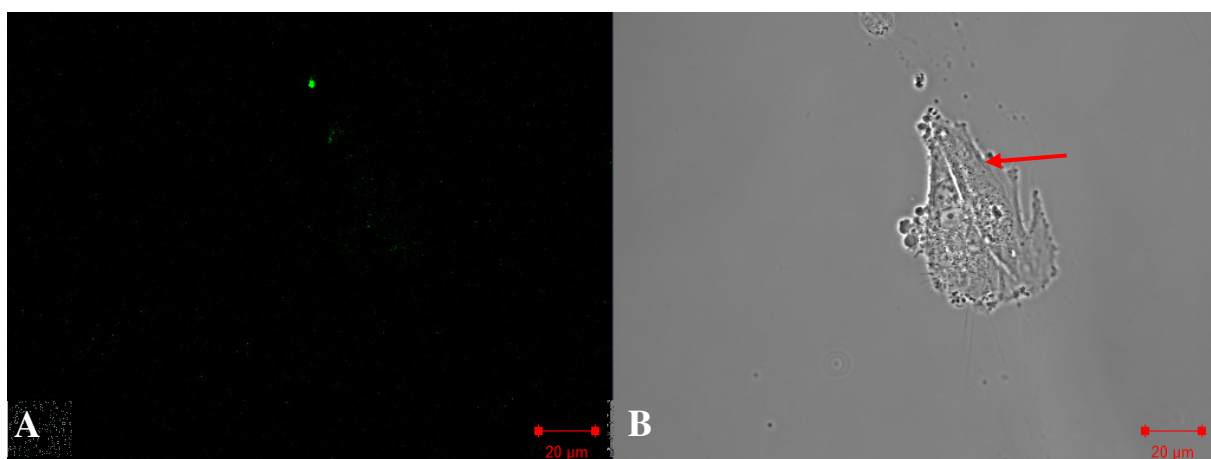




**Figure 7-7:** Simultaneous imaging of a human fibroblast before (A-C) and 30 minutes after (D-F) optoporation with BSA-FITC. (A & D) FITC fluorescence (green), (B & E) phase contrast image, (C & F) is the merged image. Site of optoporation is shown by red lightning bolt. Images are representative of 20 cells from 3 experiments.

After 2 hours, the cell continued to shrink dramatically. Overnight, the treated cell (shown with a red arrow in Figure 7-8) did not return to its original size. Not only had it shrivelled, it lacked the characteristic fibroblast morphology that the cell exhibited before optoporation in Figure 7-7 B.

Thus, although it was possible to introduce BSA into cells at 100 mW of power, the cells did not appear healthy for future cultivation. At 36 mW, the cells still exhibited bright green fluorescence from the relatively large amount of BSA that had entered. In contrast, these cells were able to recover well overnight. It is important to note that the success rate of optoporation and cell recovery was very low at 5%. Nevertheless, this efficiency was reproducible in 3 independent experiments consisting of 25 cells each.

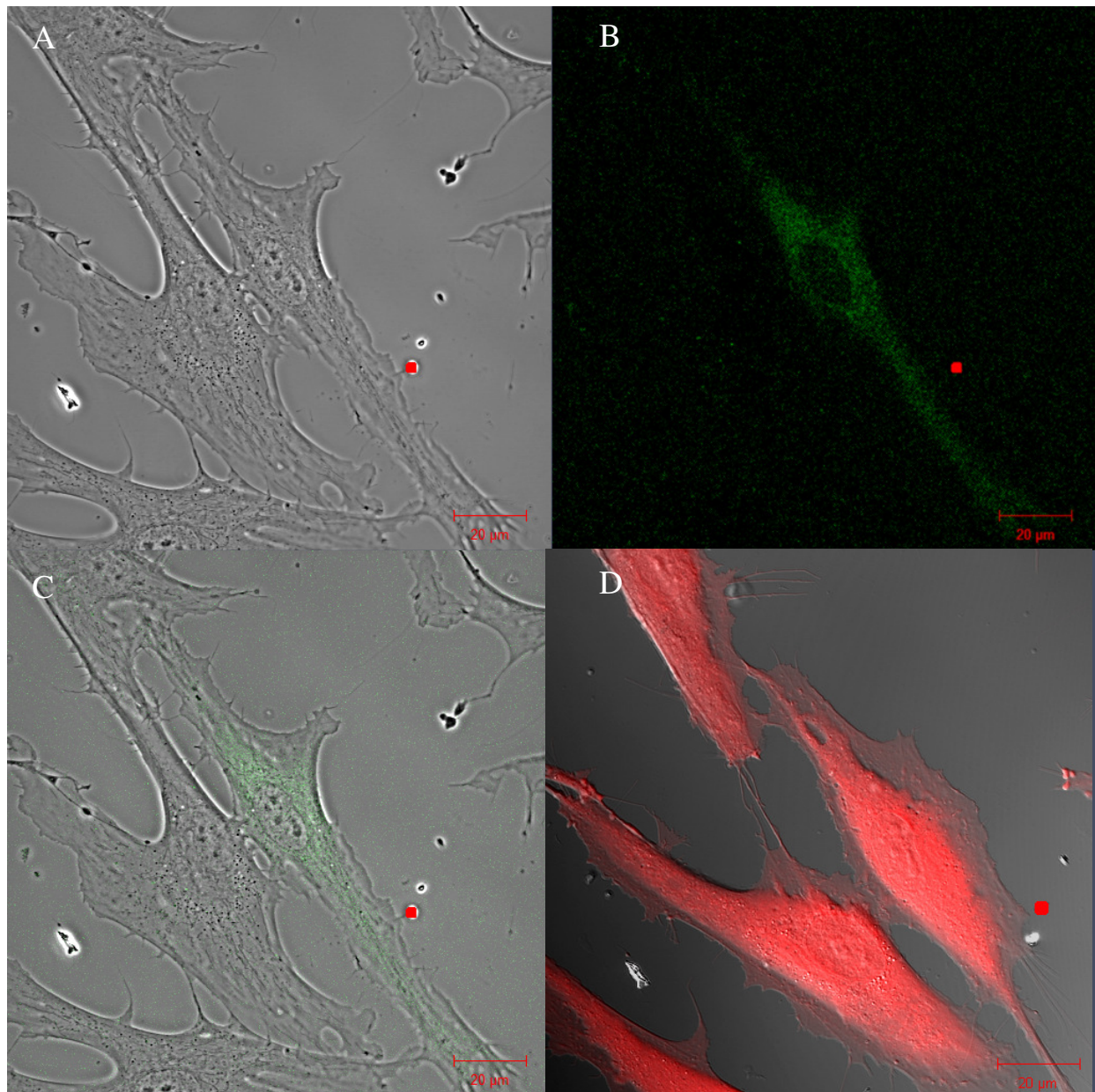


**Figure 7-8: Simultaneous imaging of (A) fluorescein fluorescence (green) and (B) phase contrast of a human fibroblast cell 1 day after optoporation at 100 mW at 80 ms. Optoporated cell is shown by red arrow.**

### 7.1.5 Shockwave optoporation in OptiMEM

The final technique employed was to induce a shockwave by irradiating the glass substrate at a site within 10  $\mu\text{m}$  of the cell. The change to the morphology of the cell was much more drastic but most cells were still able to recover. In fact, 8 out of 13 optoporated cells recovered after BSA uptake.

Cells were observed using phase contrast when focusing the image. The wavelength of the tunable laser was then set to 800 nm with power and exposure time of 180 mW and 1  $\mu\text{s}$  respectively (see Table 7-1). As shown by the green fluorescence in Figure 7-9 B & C, uptake of BSA could be realised. Because shockwave optoporation utilises more laser power and induces drastic morphology changes than single cell optoporation, viability was confirmed by calcein-AM red staining after 1 hour (Figure 7-9 D). The optoporated cell stained with red fluorescence at a similar intensity to its neighbouring unoptoporated cell, which suggested that it had recovered.



**Figure 7-9: Simultaneous imaging of a human fibroblast undergoing optoporation with BSA-FITC. (A) phase contrast, (B) BSA-FITC (green), (C) merged image of (A) and (B), (D) the same cell stained with calcein-AM red (red) at 1 hour post-optoporation. Site of optoporation is shown with red dot. Images are representative of 20 cells from 1 experiment.**

When comparing the 4 strategies, the shockwave optoporation method was the most optimal in that the efficiency was the highest (8 out of 13 recoverable cells). The next best method was to use a medium with high potassium content where only 5 out of 100 cells survived. Thus, the shockwave optoporation method will be adopted for optoporating phalloidin and the actin antibody.



A summary of parameters that were tried for protein optoporation is shown in Table 7-1.

**Table 7-1: Summary of parameters tested for optoporation of proteins. Each condition was tested on 20 cells from 1 experiment, except when high K<sup>+</sup> was used where 3 experiments were carried out. Survivability and BSA uptake were determined and compared between conditions by visual examination based on set imaging parameters.**

| <b><u>Laser power</u></b> | <b><u>Exposure time</u></b> | <b><u>Medium</u></b>             | <b><u>Number of cells optoporated</u></b> | <b><u>Efficiency (%)</u></b> | <b><u>Comments</u></b>                            |
|---------------------------|-----------------------------|----------------------------------|-------------------------------------------|------------------------------|---------------------------------------------------|
| 50 mW                     | 12 ms                       | OptiMEM                          | 20                                        | 0                            | No BSA uptake                                     |
| 20 - 100 mW               | 82 ms                       | OptiMEM                          | 20                                        | 0                            | BSA uptake, but cells shrivelled                  |
| 18 mW                     | 82 ms                       | OptiMEM with high K <sup>+</sup> | 20 x 3 experiments                        | 0                            | BSA could not be observed after 2 hours           |
| 36 mW                     |                             |                                  | 20 x 3 experiments                        | 5                            | Good BSA uptake. Cells appeared stressed          |
| 100 mW                    |                             |                                  | 20 x 3 experiments                        | 0                            | Poor cell viability. Drastic change in morphology |
| 180 mW                    | 1 $\mu$ s                   | Shockwave in OptiMEM             | 20                                        | 62                           | Good BSA uptake and higher viability              |



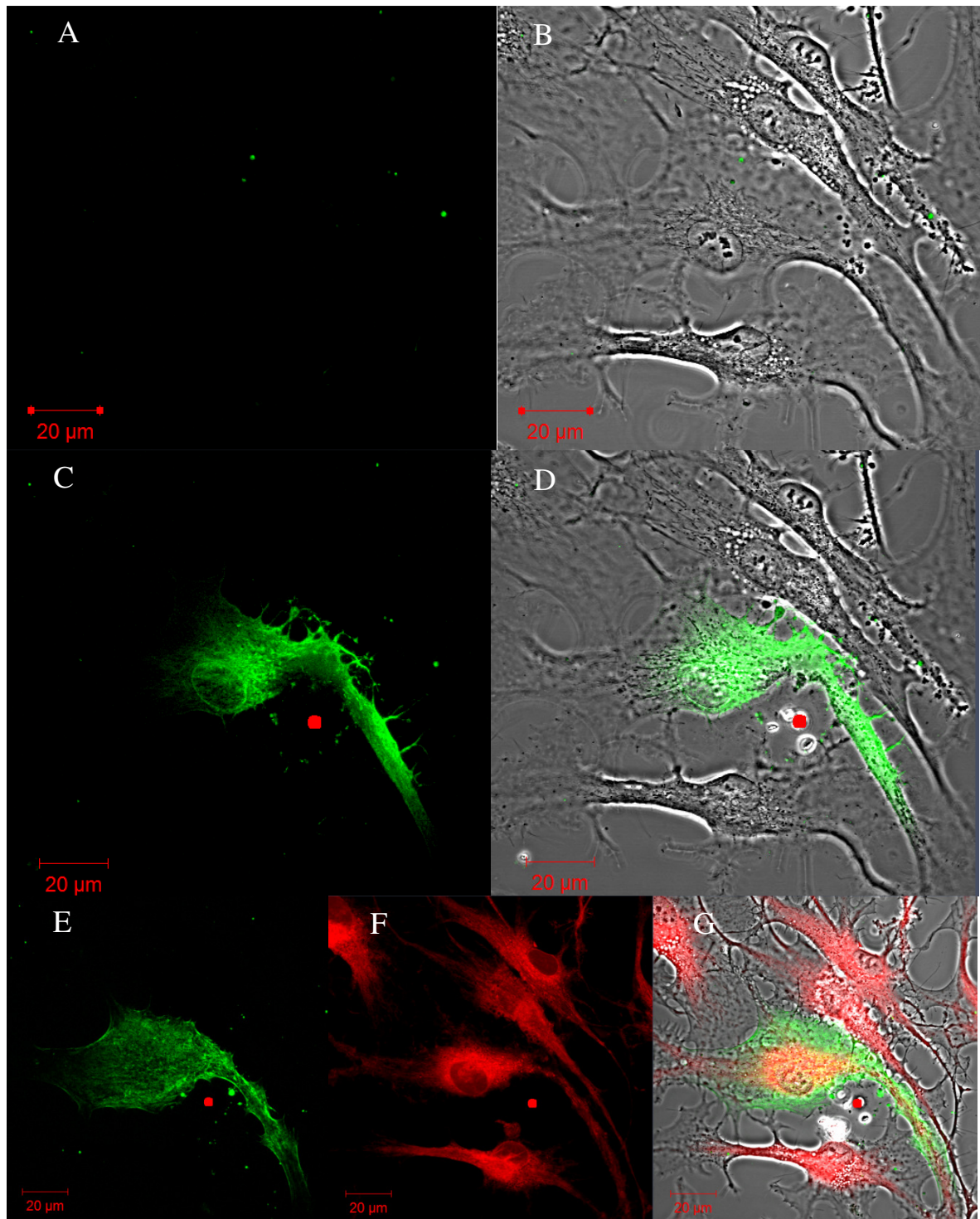
## **7.2 Optoporation of Phalloidin-FITC**

### **7.2.1 Materials and methods**

Phalloidin (Sigma Aldrich), a peptide with a molecular weight of 1.2 kDa, is impermeable to living cells but, when fixed, binds to F-actin identically to actin-specific antibodies (Wulff et al., 1979). It was tagged with FITC and used for shockwave optoporation at a concentration of 20 - 40  $\mu\text{g/ml}$  in antibiotics-free culture medium. The laser was emitted at 160 -180 mW of power for 1  $\mu\text{s}$  at a location on the glass coverslip at 5 - 10  $\mu\text{m}$  away from the cell membrane. The cell was observed for several minutes and its position was stored in memory for later recall. One hour after optoporation, the cells were stained with calcein-AM red (Invitrogen) to determine their viability. The medium was not changed so as to not perturb the cells.

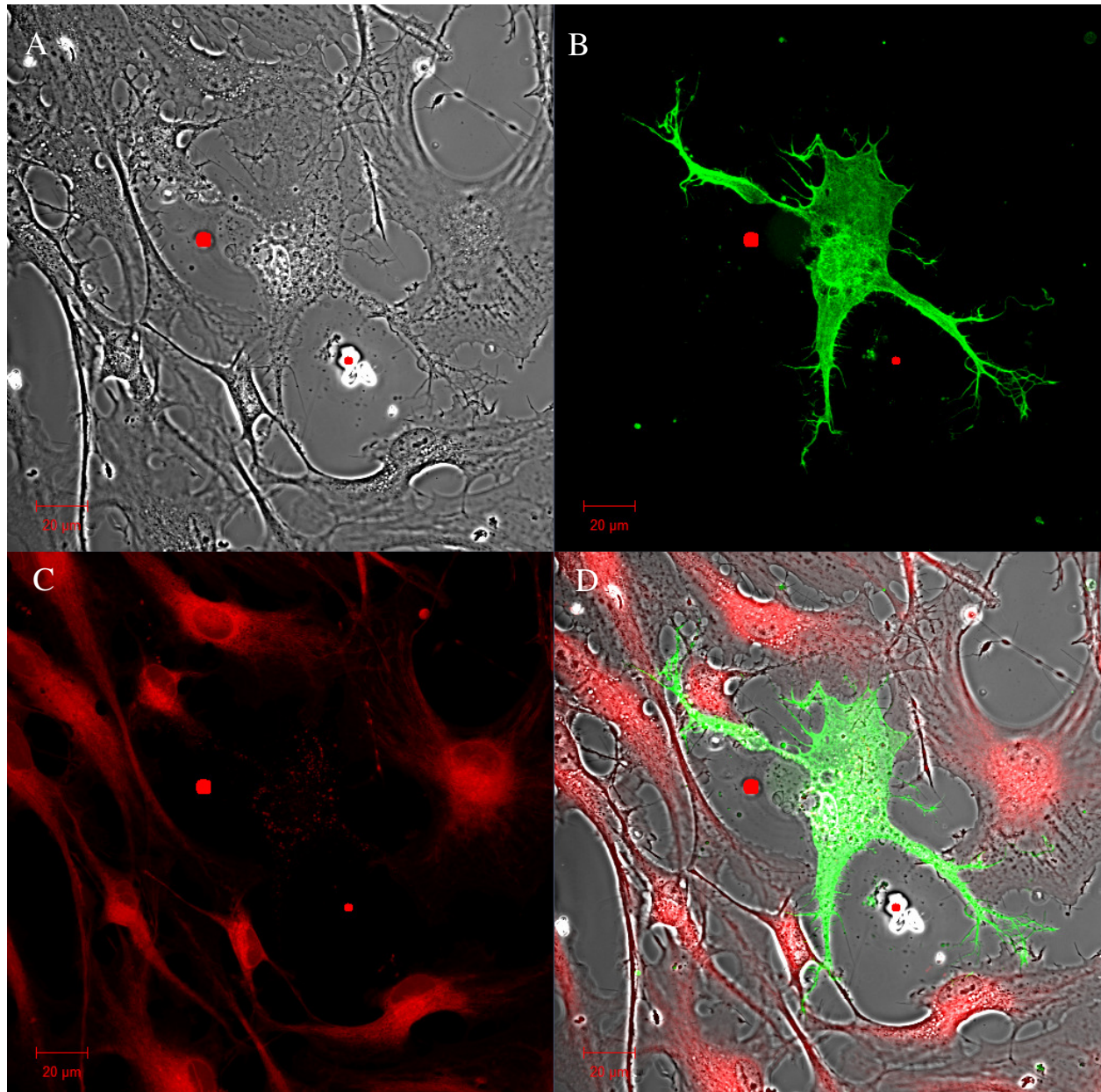
### **7.2.2 Result of optoporation**

Immediately after the procedure, the area of the cell closest to the laser target began fluorescing green due to the entry of phalloidin-FITC (Figure 7-10 C). Only a small amount entered before the cell's membrane resealed and effectively prevented more phalloidin from entering. After 1 hour, the cells were incubated with calcein-AM red to determine the cell's viability and reimaged. In the cells that took a small amount of phalloidin before resealing, these cells showed red fluorescence of similar intensity to its neighbours, which suggested that they had survived the procedure (Figure 7-10 F).



**Figure 7-10: Simultaneous imaging of a human fibroblast (A & B) before, (C & D) after, and (E-G) 1-hour after optoporation. (A, C, E) show fluorescence from phalloidin-FITC (green). (F) shows fluorescence from calcein red-orange AM (red). (B, D, G) are merged images. Red dot indicates location of optoporation. Images are representative of 20 cells from 1 experiment.**

Of those cells where the entire cell was labelled bright green (Figure 7-11 B), these cells did not recover from the shockwave and did not fluoresce red from the calcein-AM red (Figure 7-11 C). This was in contrast to the unaffected cells nearby which continued to fluoresce red.



**Figure 7-11: Simultaneous imaging of a dead human fibroblast 1-hour after optoporation. (A) phase contrast image, (B & C) show fluorescence from phalloidin-FITC (green) and calcein AM red-orange(red) respectively. (D) is the merged image. Red dot indicates location of optoporation. Images are representative of 20 cells from 1 experiment.**

### **7.3 Optoporation of Antibodies**

Human fibroblasts were also optoporated in the presence of a synthetic actin binding antibody sAB-27, which is known to be toxic in that it bundles actin filaments (Rizk et al., 2009). The authors thus proposed that this antibody has applications in cancer therapeutics.

In this thesis, sAB-27 was used to determine if an antibody with an actual function could be optoporated into cells. Since sAB-27 was known to induce actin bundling, its effects could be observed almost immediately. This is in contrast to BSA, which is a protein but not an antibody and therefore, is unlikely to show an immediate effect in cells.

#### **7.3.1 Materials and methods**

The antibody sAB-27 (approximately 66 kDa) was conjugated with CF 633 using a Mix-n-Stain CF dye antibody labelling kit (Biotium, USA) and used at a working concentration of 20 µg/ml in antibiotics-free culture medium. sAB-27 was optoporated either on its own or together with phalloidin-FITC (20 µg/ml) and DAPI (4 µg/ml) in order to compare actin staining and to mark cells that were optoporated respectively.

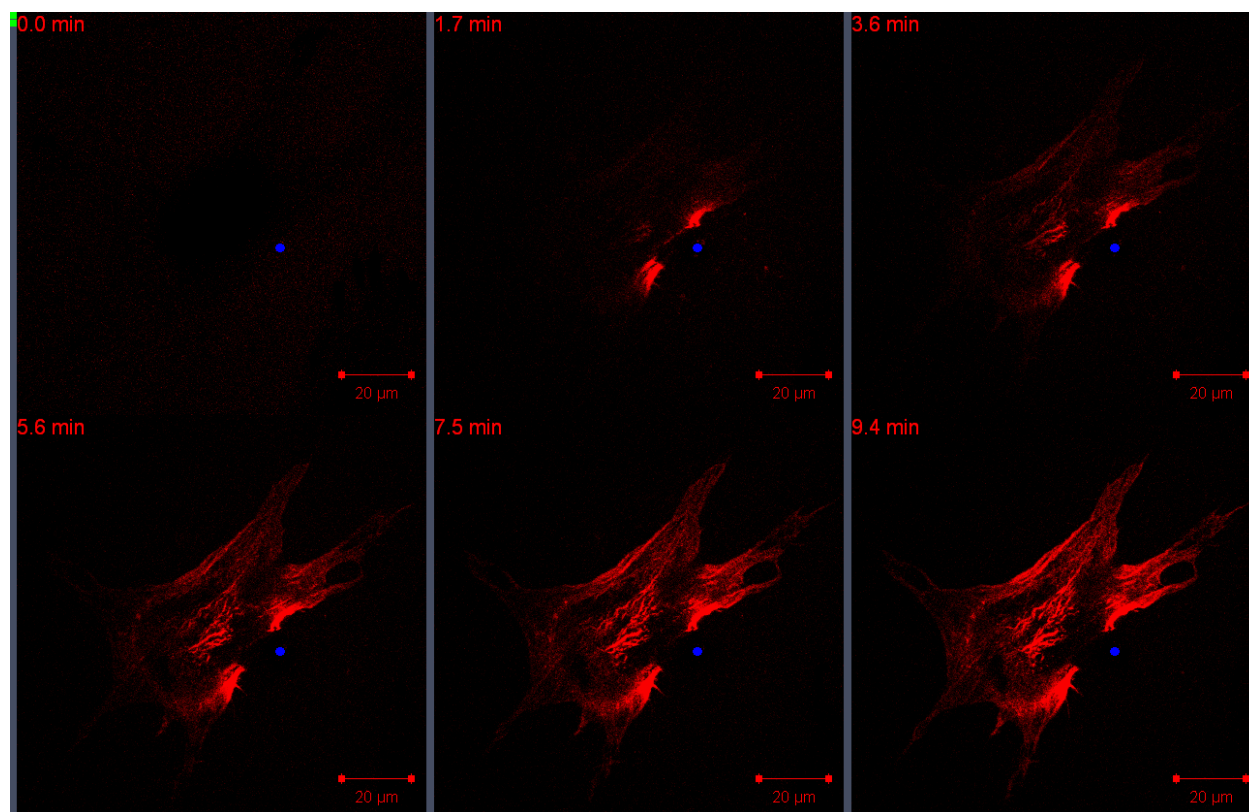
Cells were optoporated at a distance of 5 - 10 µm away from the membrane of cells with laser powers of 180 mW for 1 µs at 800 nm. After optoporation, cells were imaged using the 405 nm, 488 nm, and 633 nm laser to visualise all three fluorescent stains. To test for viability, cells were stained with calcein-AM red (Invitrogen, UK), which was excited using a 561 nm laser.

#### **7.3.2 Result of optoporation**

When sAB-27 was optoporated on its own into living human fibroblasts, the antibody was observed to first stain the actin filaments near to the site of impact, as shown in Figure 7-12

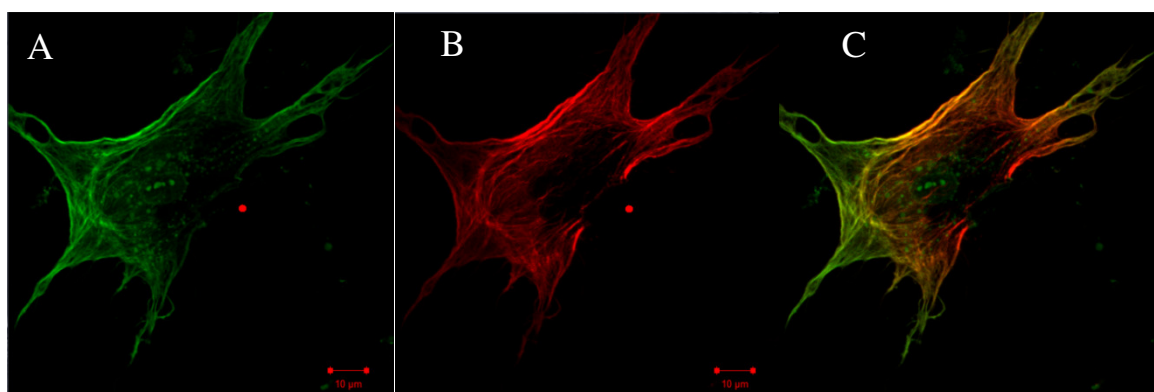


(@1.7 min). Excess antibody continued to stain the remaining actin structures throughout the cell. Figure 7-12 was acquired as a 3D timelapse image with a time resolution of 10 seconds with an axial resolution of 0.75  $\mu\text{m}$ . The timelapse continued for 20 minutes in total (not shown).



**Figure 7-12: Timelapse imaging of a human fibroblast before and after shockwave optoporation in presence of sAB-27 actin antibody (red). Site of shockwave labelled with blue dot. Images are representative of 20 cells from 1 experiment.**

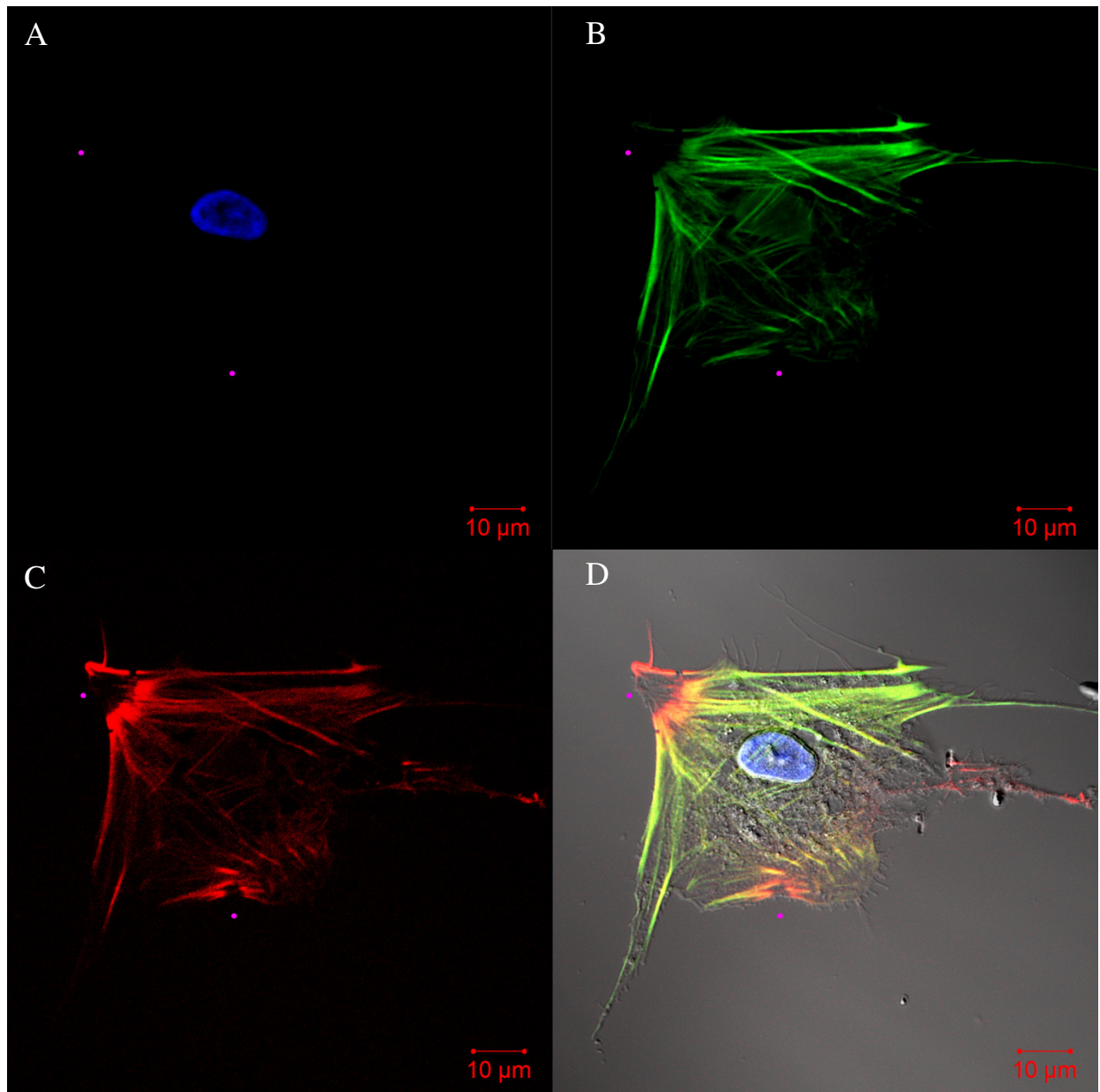
I rinsed the excess sAB-27 antibody off and incubated the sample with phalloidin-FITC for 5 minutes. When reimaged, the cells also fluoresced green from the phalloidin as shown in Figure 7-13 A. When the staining patterns of phalloidin and sAB-27 (Figure 7-13 B) were compared, both showed colocalisation and appeared similar, which suggested that the antibody was indeed targeting actin.



**Figure 7-13: Simultaneous imaging of human fibroblast after addition of phalloidin-FITC to optoporated cell. (A) Phalloidin-FITC (green), (B) sAB-27-CF633 (red), (C) merged image. Site of optoporation shown with red dot. Images are representative of 20 cells from 1 experiment.**

The result in Figure 7-13 was repeatable and shows that antibodies can be introduced into cells without being impeded by the size of the pore caused by shockwave optoporation. Furthermore, I repeated the experiment by optoporating sAB-27 *and* phalloidin at the same time (Figure 7-14). Similarly, the staining patterns between the two colocalised and allowed the actin filaments to be observed.

Although it was possible to permeate the membrane to allow entry of the antibody, the optoporated cells died within 1 hour. However, optoporation of BSA-FITC using identical experimental conditions (discussed in section 7.1.5, green fluorescence in Figure 7-9 B & C) showed that the procedure of optoporation itself was not toxic to cells, as evidenced by the positive calcein-AM red staining (Figure 7-9 D). As mentioned, 8 out of 13 BSA-optoporated cells stained positive for viability compared to zero sAB-27-optoporated cells. Thus, it can be deduced that cell death was caused by sAB-27 and is likely due to its actin bundling characteristics.



**Figure 7-14: Simultaneous imaging of a human fibroblast optoporated in the presence of (A) DAPI (blue), (B) phalloidin-FITC (green), and (C) sAB-27 conjugated with CF 633 (red). (D) is the merged image. Site of optoporation shown with pink dots. Images are representative of 20 cells from 1 experiment.**



## 7.4 Discussion

Shockwave optoporation was able to bring phalloidin, BSA, and sAB-27 into cells. Similar work has been shown where dextran (3, 40 and 500 kDa) and protein kinase C (80kDa) were optoporated into cells (Clark et al., 2006 ; Souhayer et al., 2000 ; Stracke et al., 2005). The first two groups used shockwave optoporation by positioning the laser at 28  $\mu\text{m}$  and 31 - 40  $\mu\text{m}$  respectively from cells, and this allowed loading of more than 1 cell with each shot. Souhayer et al. (2000) even showed that amounts too low to detect by fluorescence detection could still be optoporated at even 75  $\mu\text{m}$  away. The work in this thesis shows that optoporation of proteins could be achieved if the laser was positioned just 10  $\mu\text{m}$  away from cells. Beyond this distance, the cells experienced no uptake of protein or signs of perturbation. This could be due to different power levels and lasers (Clark et al. (2006) and Souhayer et al. (2000) used an Nd:YAG laser instead of Ti:Sapphire). Clark et al. (2006) also introduced dextran by firing several low-powered shots at the cell membrane, however, one exposure was sufficient to introduce phalloidin (similar sized to dextran) into cells.

The introduction of BSA into living cells has been shown through microinjection by Valster et al. (1997) and Turner (2004). The former showed no toxicity and recovered from the wound after 10 minutes. This was based on visualisation of the cell's morphology. Similarly, in Figure 7-9, human fibroblasts were shown to have recovered and remained alive 1 hour after optoporation with BSA judged by presence of calcein-AM fluorescence. BSA-FITC was also observed to be localised to the cytoplasm and absent from the nucleus, which is consistent with findings from Turner (2004).

Rizk et al. (2009) state that sAB-27 had a drastic effect on cell viability due to its actin-bundling properties. Likewise, when this antibody was optoporated into cells, I found that no cells could survive. The staining pattern of sAB-27 (Figure 7-12 - Figure 7-14) is similar to Cleary's (1995) work, which showed that actin staining is absent near the site of injection/optoporation.

However, the observed staining pattern differed from Rizk et al. (2009). In this thesis, the optoporated antibody stained the entire actin cytoskeleton and co-localised with phalloidin. In contrast, Rizk et al. (2009), showed that the antibody altered the arrangement of the actin fibres so that they appeared short and arranged in thick bundles. The reason for this difference could be that Rizk et al. (2009) allowed the antibody to incubate for as long as 2 hours, whereas it was only observed for 10 minutes here. Thus, more time may be needed for the antibody to function. In support of this, it took 2 hours to notice any severing effect of actin filaments when Scheer et al. (1984) microinjected Fragmin into cells.

## 7.5 Summary

This chapter shows proof of principle that proteins such as BSA, phalloidin, and antibodies can be introduced into living cells using optoporation. Because of their size, these proteins would normally be impermeable on their own unless the cells were fixed and permeated. Optoporation allows the introduction of proteins without having to fix the cells. Thus, the effect of certain proteins can be continuously monitored *in vitro* in living cells. Two methods were used to bring sufficient protein into cells so they can be observed. First, the extracellular concentration of potassium was increased so as to minimise the amount of potassium that would leak out of cells after optoporation. This allowed cells to be optoporated at higher powers and thus, create pores which would be opened longer. Second, the laser was directed at the glass itself below the cell's focal plane at high power (180 mW for 1  $\mu$ s). This created a shockwave which significantly perturbed the cell but still within repairable range. The resultant pore was large, and recovery took long enough to allow sufficient phalloidin and anti-actin antibody to enter the cell and clearly stain the actin.

# Chapter 8

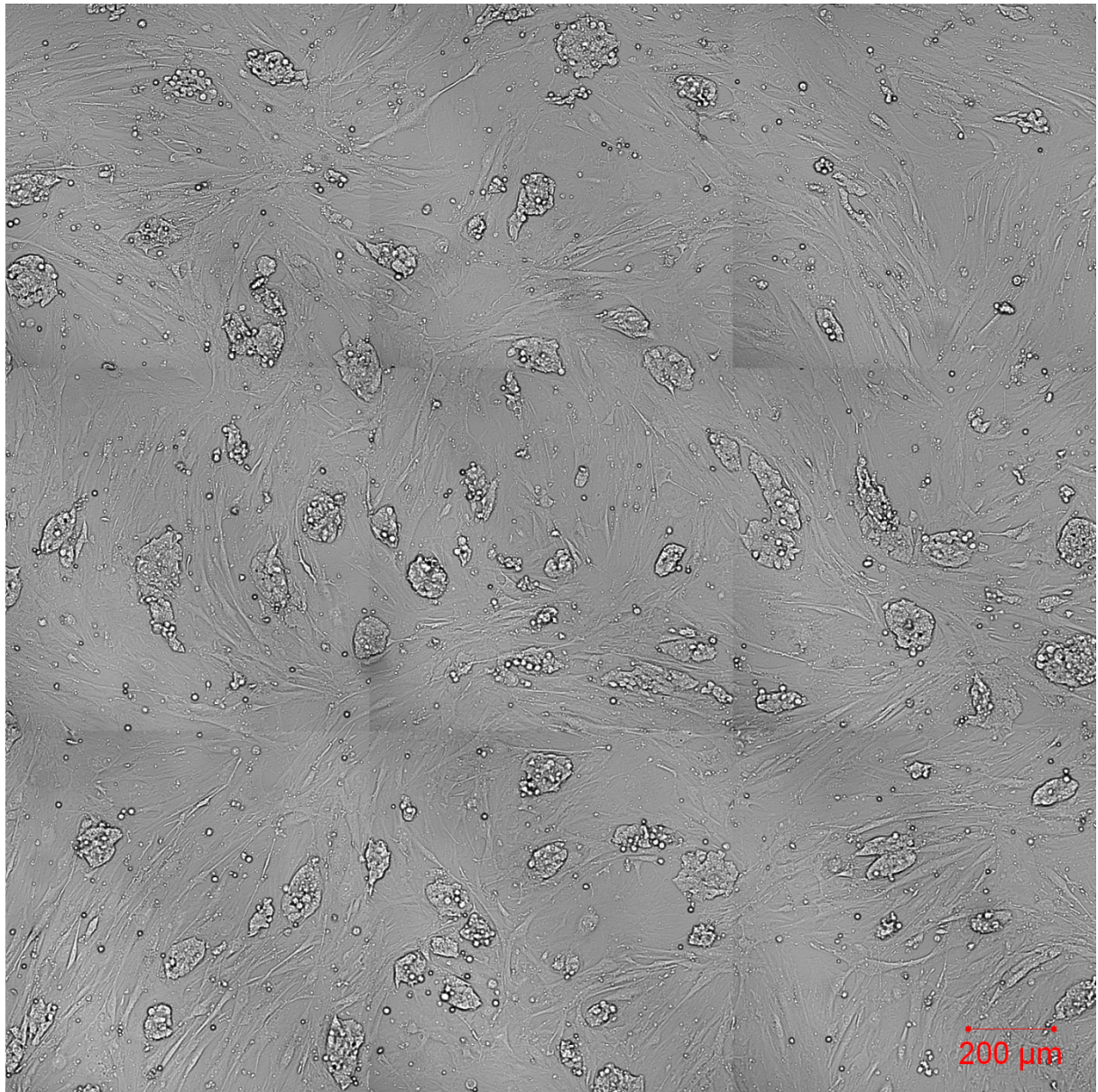
## Automated Colony Recognition Program

### 8.1 Automation of Colony Recognition

Chapter 5 described how optoporation might be used to transfect cells with nucleic acids. A specific application for this technique is for introducing iPS transcription factors (OCT4, SOX2, KLF4) and GFP plasmid into cells. This chapter describes an algorithm to automatically identify iPS-like colonies based on morphology, and to report their positions within the experimental setting. The approach could also be modified for future experiments to monitor changes in cell morphology after the addition of small molecule compounds.

The identification of stem cell colonies is time consuming, laborious and prone to errors (Barber et al., 2001, Dahle et al., 2004, Bottigli et al., 2007). ES and iPS cells grow in colonies, which are characteristically compact and circular as opposed to being loosely packed, as observed with many other cell types, e.g. fibroblasts. It has been shown that these colonies can be cultured on gelatin coated plastic dishes, but this requires the use of expensive conditioned medium. Colonies can also be cultured on a confluent layer of mitotically inactivated mouse feeder cells. However, this introduces another cell type with its own morphology into the culture system. In addition to the colonies, the feeder layer can also be observed in the images acquired with a microscope (such as that shown in Figure 8-1). Furthermore, this image suffers from poor

contrast and high background texture resulting in difficulties in segmenting the colonies for identification.



**Figure 8-1: Tiled phase image of ES colonies cultured on a mouse feeder layer**

The current standard in identifying true stem cell colonies is through destructive antibody staining. It would be more desirable if such tests were non-invasive and quick. Various methods of segmenting and recognising colonies have been implemented such as using standard image processing techniques (Barber et al., 2001, Dahle et al., 2004), neural networks (Bottigli et al.,

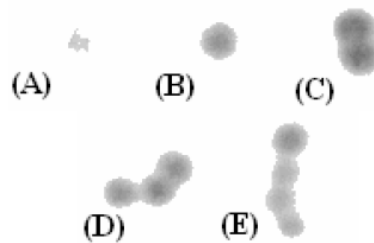
2007, Masala et al., 2007), machine vision (Narkilahti et al., 2007), and texture segmentation using wavelets (Mangoubi et al., 2007). In this thesis, I also attempted to use wavelet analysis to identify colonies based on texture (results not shown) but to no avail. It was not possible to identify key frequencies in the colonies' texture that could be used to separate the colonies from the background feeder layer. Instead, I resorted to using a series of image processing techniques combined with conditional statements or a fuzzy logic system to identify and count colonies on feeders.

### **8.1.1 Current techniques to identify colonies**

The aim of the study by Barber et al. (2001) was to develop algorithms to identify colonies from different cell types quickly so that a large number of culture flasks can be screened. The watershed algorithm was reported as being computationally intensive and slow (Barber et al., 2001). Therefore, the group relied on standard image processing algorithms to find human colonies, which were discrete and separated. However, for colonies which were overlapping or lacked clear boundaries, Barber et al. (2001) relied on the compact Hough transform. Images of colonies were acquired using a CCD (charge-coupled device) camera and underwent contrast enhancement, thresholding, edge detection, hole filling, and filtering based on size. The remaining objects were colonies which were labelled with a white dot.

Dahle et al. (2004) expanded on Barber et al.'s (2001) study by using a flat bed scanner in order to scan more than one culture at the same time. To count merged colonies, the algorithm analysed the elongation and the number of inflexions around the object's shape. These numbers were used to classify how many colonies had merged in a particular object based on prior observations. The software was able to classify and color-code objects containing up to 10 colonies.

Neural networks are another alternative to identifying and classifying colonies. Bottigli et al. (2007) and Masala et al. (2007) first used 'region growing' to segment cells instead of standard image processing algorithms in the previous mentioned studies. 'Region growing' begins by selecting a seed pixel as part of a region. Nearby pixels were added to this region only if they were above a certain threshold. When no more pixels were selected, a new seed pixel was selected and the process was repeated until all pixels had been accounted for. Each region was then presented to a Sanger neural network which identified shape properties such as the area and perimeter. Using these inputs, each object was classified into 5 categories, depicted in Figure 8-2. Class A is a false colony, while B-E are clusters ranging from 1 -4 colonies.



**Figure 8-2: Examples of 5 classes of joined clusters to be recognised by neural network: (A) false colony composed of fragments, (B-E) shows 1, 2, 3, and 4 joined colonies. (Adapted from Bottigli, U. M. C., Fiori, P. L., Golosio, B., Marras, A., Masala, G. L., and Oliva, P. (2007). "A New Automatic System of Cell Colony Counting." International Journal of Mathematical and Computer Sciences 3(4): 5).**

'Region growing' was also compared to 3 other methods : watershed-convolve, watershed-median, and a Laplace-based filter. 'Region growing' and watershed-convolve gave the most similar readings to the manual count because they retained more detail than the other two methods. While watershed-convolve eliminated some of the false colonies, Masala et al.'s (2007) method used neural networks to do this instead. It is not clear why this was preferred but computational time could be one factor. In the previous mentioned studies, the researchers had to stain the colonies with crystal violet in 95% ethanol. For embryonic stem cell colonies, this is undesirable due to their sensitivity. It would be preferable to identify colonies in their natural state without using extra probes and stains which may compromise the pluripotency or health of

the stem cell colonies. Using a Cell IQ system (Chip-Man Technologies), Narkilahti et al. (2007) extracted images of the background feeder layer, differentiated and undifferentiated colonies from 2 cell lines at different passages as sample images. These were then entered into the Cell IQ system, which extracted features (not listed) for teaching the system's classifier. Using this system, the researchers calculated the percentage area of the colony which was composed of undifferentiated cells.

Analysing texture by wavelet decomposition could be another way of identifying colonies. This has been applied to segmenting and separating out textures within the same images (Arivazhagan et al., 2003), classifying textures (Hiremath et al., 2006a), image retrieval based on texture (Wang et al., 2008), and even identifying animal fibres (Zhang et al., 2010).

Wavelet decomposition involves convolving a signal with a mother wavelet. This is analogous to separating a signal into its low and high frequencies by performing both a low-pass and high-pass filter on the signal respectively. On a 2D image, a low- and high- pass filter can also be applied in different sequences (Figure 8-3 A). A second decomposition can be performed on the result of the first low-pass subimage to decompose the frequencies further (Figure 8-3 B). Decomposing the signal or image with a low- and high-pass filter allows the calculation of wavelet coefficients (Lu et al., 1997). The frequencies that most contributed to the signal or image will have higher coefficients based on the subimages obtained through the high-pass filter.



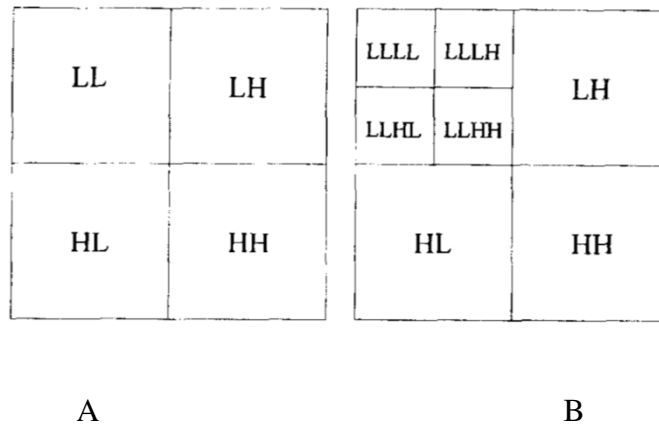


Figure 8-3: (A) Single level wavelet decomposition, (B) two level wavelet decomposition. Reprinted from Pattern Recognition, 30/5, Lu C., Chung P., Chen C., Unsupervised texture segmentation via wavelet transform, 729-742, Copyright (1997), with permission from Elsevier.

Undifferentiated colonies have cells which are tightly compacted giving a homogenous texture (Figure 8-4 a & c). Differentiated colonies, on the other hand, have a heterogeneous texture due to the cells lacking compactness. The boundaries of differentiated colonies are also not distinct and sharp, as shown in Figure 8-4 b & d.

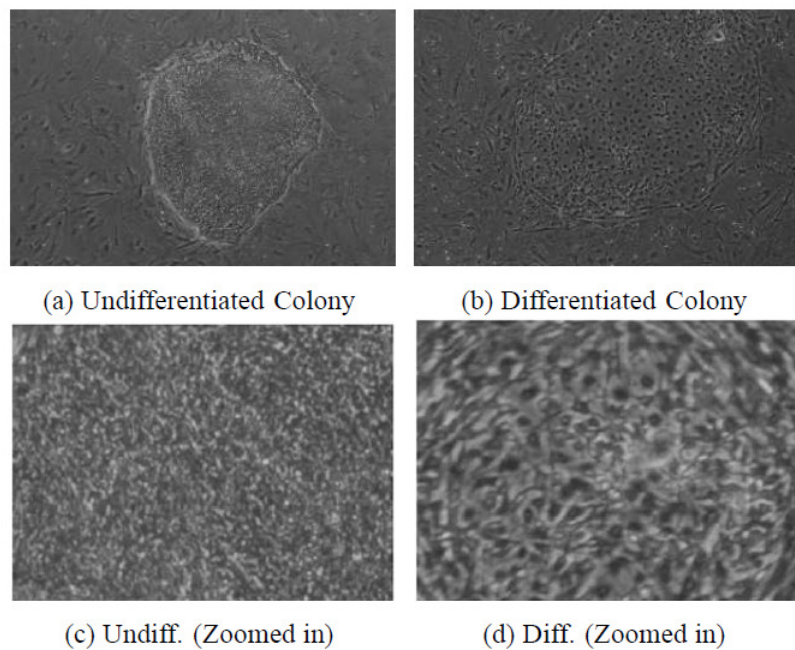


Figure 8-4: Brightfield images highlighting the different textures of an (a & c) – undifferentiated colony, and (b & d) differentiated colony. Adapted from Mangoubi et al., Non-invasive image based support vector machine classification of human embryonic stem cells, Biomedical Imaging: From Nano to Macro, 2007. ISBI 2007. 4th IEEE International Symposium on. © 2011 IEEE.

Mangoubi et al. (2007) also analysed linescans from undifferentiated and differentiated colonies. It appeared that there were more fluctuations and high frequencies in the undifferentiated case (Figure 8-5 A) than in the differentiated colony (Figure 8-5 B). This suggested that texture could be used to distinguish undifferentiated and differentiated colonies from each other.

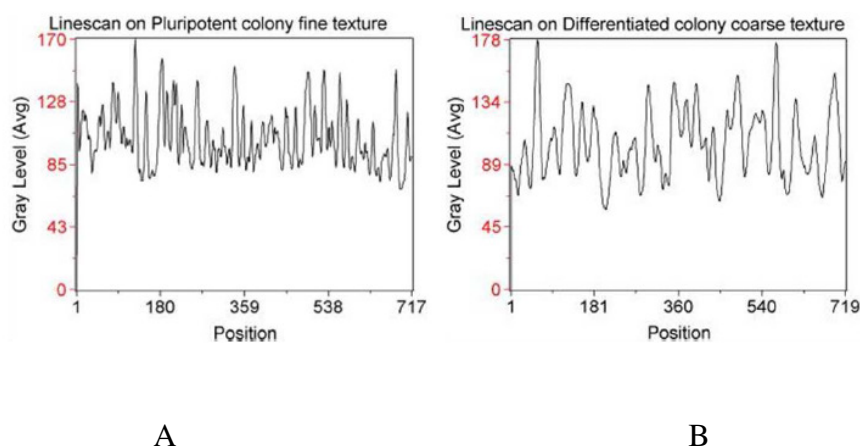


Figure 8-5: Line scans of (A) undifferentiated colonies, (B) differentiated colonies. Adapted from Mangoubi et al., Non-invasive image based support vector machine classification of human embryonic stem cells, Biomedical Imaging: From Nano to Macro, 2007. ISBI 2007. 4th IEEE International Symposium on. © 2011 IEEE.

## 8.2 Design of Colony Recognition Program

The method employed consisted of a series of image processing steps to minimise the effects of noise and to segment the colonies into objects. The flowchart shown in Figure 8-6 details the key steps in segmenting the colonies. As can be seen, the shape properties of each of these objects were then derived and analysed by either conditional *if-then* statements (section 8.3) or a fuzzy logic system (section 8.4).

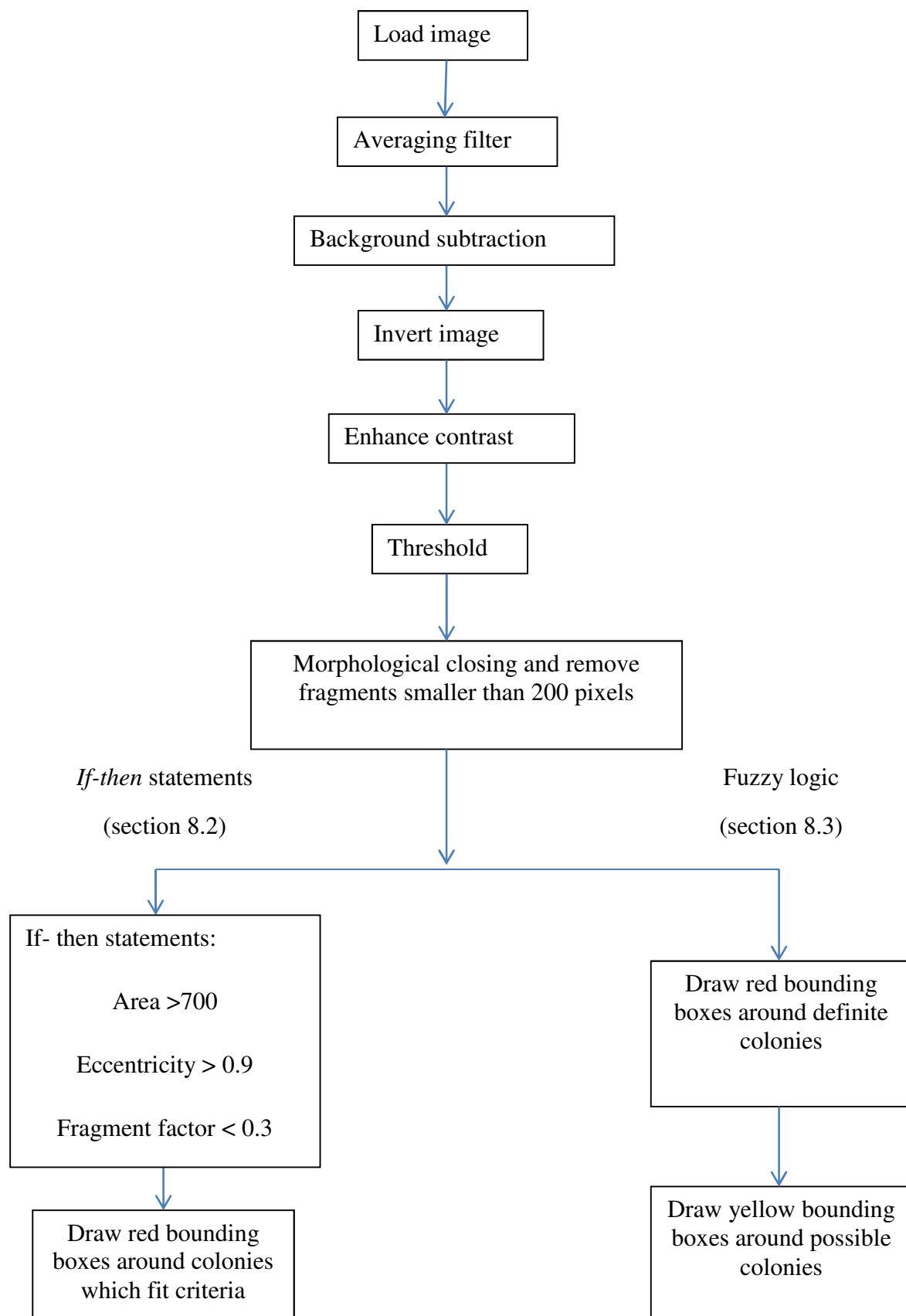
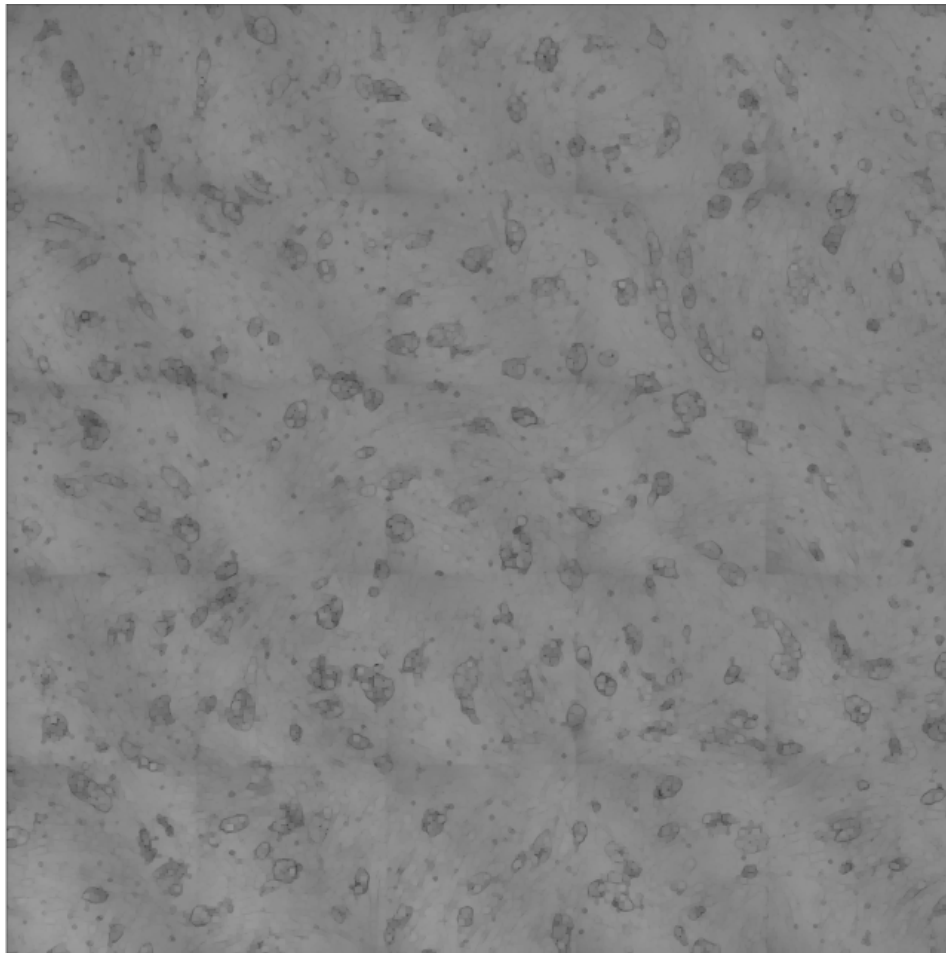
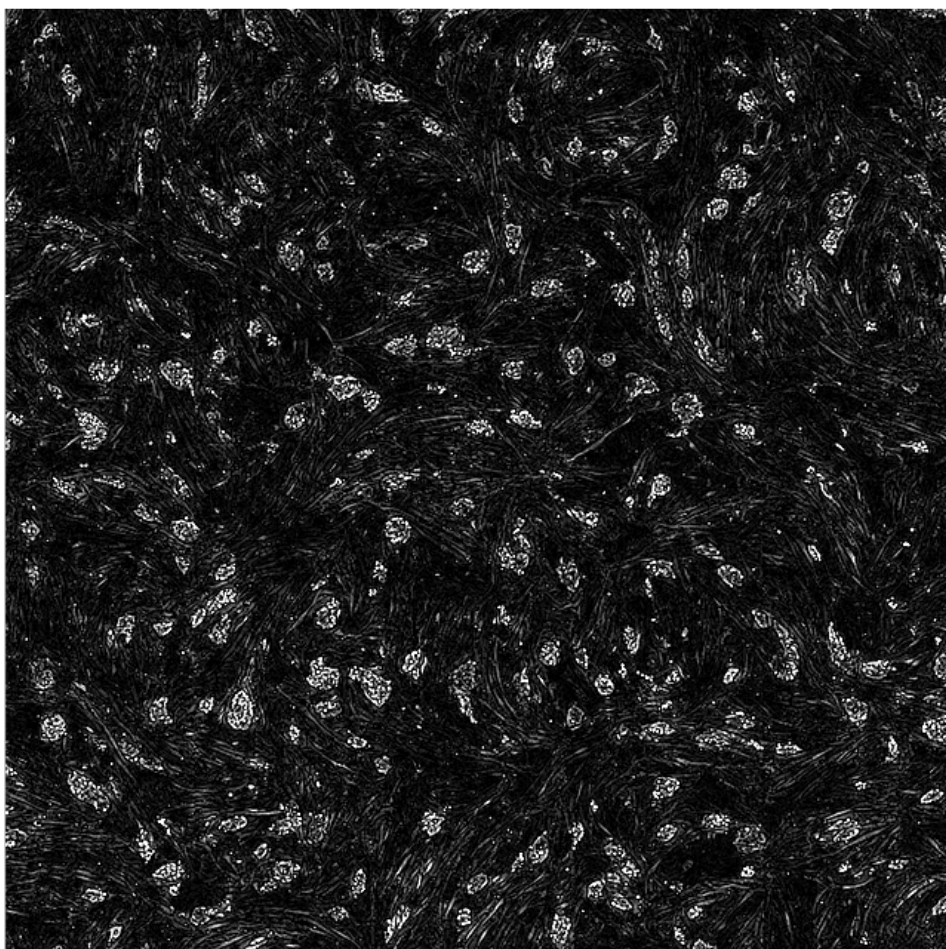


Figure 8-6: Flowchart showing colony recognition algorithm using standard *if-then* statements and Fuzzy logic control

The primary goal was to generate sufficient contrast so as to separate the foreground colonies from the background feeder layer. As shown in Figure 8-1, the colonies appeared similar to the foreground. It was necessary, therefore, to subtract the background from Figure 8-1 through background subtraction. A morphological opened version of Figure 8-1 (shown in Figure 8-7) was used for subtraction. Since the colonies in Figure 8-7 were darker due to having smaller pixel values, there was less overall reduction in their intensity after subtraction in the resultant image. On the other hand, this made the background feeder layer much darker. The resulting image, after a further inversion and contrast enhancement step, is shown in Figure 8-8.

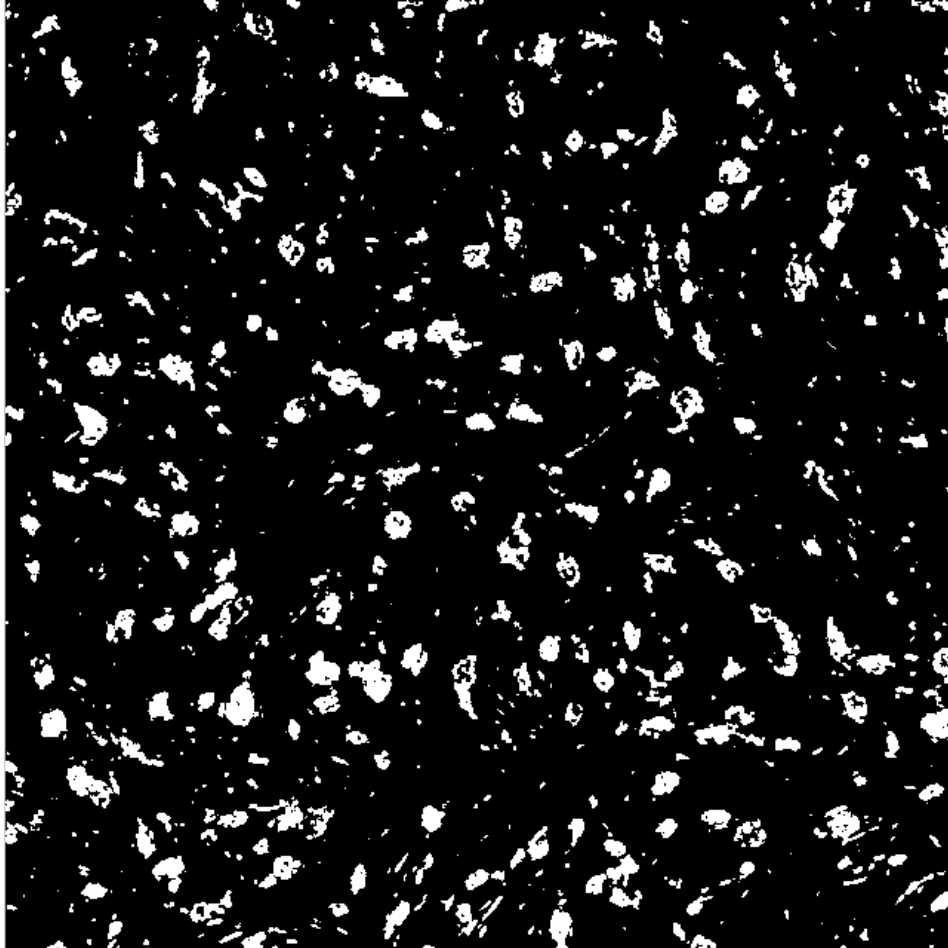


**Figure 8-7:** The background was extracted by removing the cells within the colonies using morphological opening



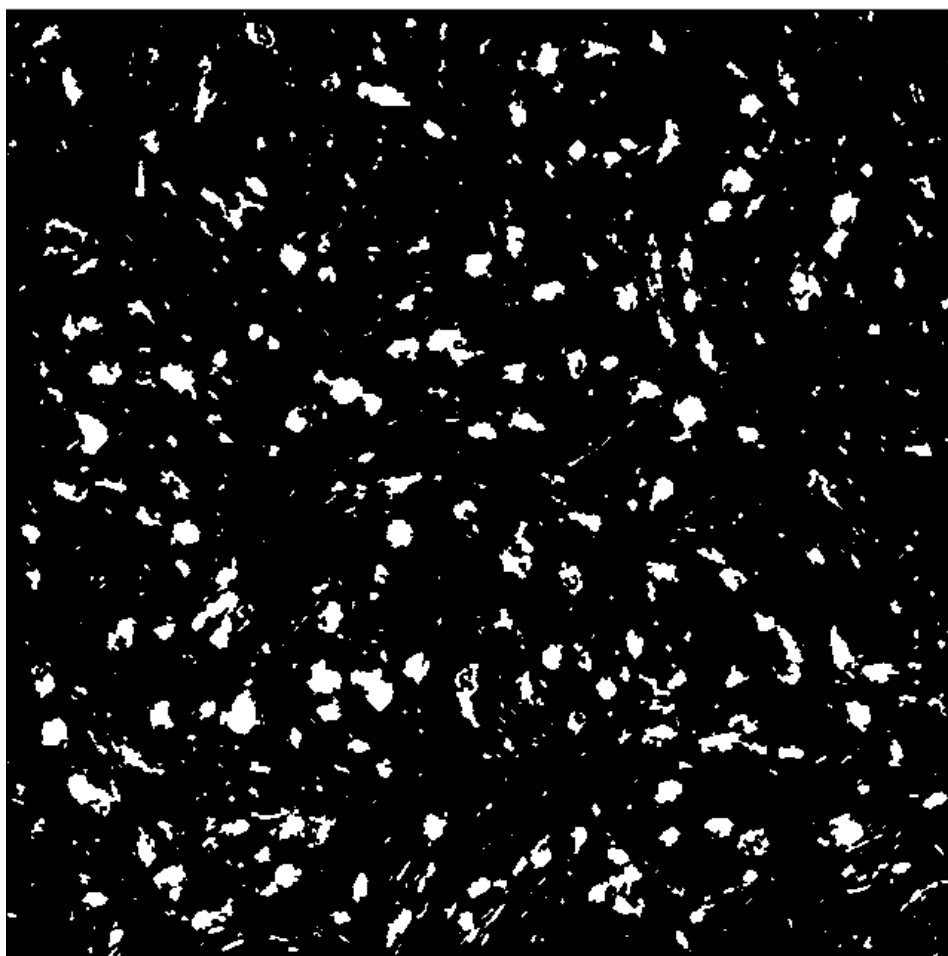
**Figure 8-8: Contrast enhancement allowed for improved visualization of colonies**

Thresholding attempts to remove the background altogether by forcing all background pixels less than 64 pixels to black. This threshold value was identified empirically. Figure 8-9 shows a typical result.



**Figure 8-9: Thresholding the contrast enhanced image with a value of 64**

Thresholding also resulted in holes within the colonies. Applying a morphological closing operation filled these holes, as shown in Figure 8-10. A final image processing step was to remove any objects that were in contact with the border as they represented incomplete shapes. The analysis of these may result in false positives. The shape properties of the resultant objects were then analysed using one of the following two methods.



**Figure 8-10: Morphological closing operation to fill holes in colonies**



### 8.3 Method 1 – *If-then* Statements

The properties of each object in the image were measured and classified according to the following shape properties as listed in Table 8-1. The justification of using these properties will now be discussed. Area has been used as a criterion by Narkilahti et al. (2007) in selecting colonies. Classifying based on area is also important because, from my experience, the cells in the centre of overgrown colonies tend to receive less nutrients, resulting in unhealthy colonies. Mangoubi et al. (2007) also list border circularity and texture homogeneity when identifying colonies. In this thesis, these will be adopted and termed as eccentricity and fragment factor respectively.

**Table 8-1: List of shape properties for classifying objects as colonies with *if-then* statements**

| Shape Property  | Description                                                                     | Condition    |
|-----------------|---------------------------------------------------------------------------------|--------------|
| Area            | total number of pixels the object occupies                                      | > 700 pixels |
| Eccentricity    | the extent to which the object is not circular (0-1)                            | > 0.9        |
| Fragment factor | the percentage of the object which does not contribute to its convex hull (0-1) | < 0.3        |

Each object was checked as to whether its properties fulfilled all of three criteria using the values I empirically determined (in Table 8-1) to identify as many colonies as possible. In general, if the object did not satisfy all three criteria, the object was unlikely a colony and was deleted.

#### 8.3.1 Justification of conditional statements

When the objects were not checked for their fragment factor, several objects which displayed fragmentation remained in the image, shown in Figure 8-11 & Figure 8-12. This means that the circularity and area alone were not sufficient to identify possible true colonies. An object which is highly inhomogeneous (not solid) may still be circular if it has matching overall widths and lengths.

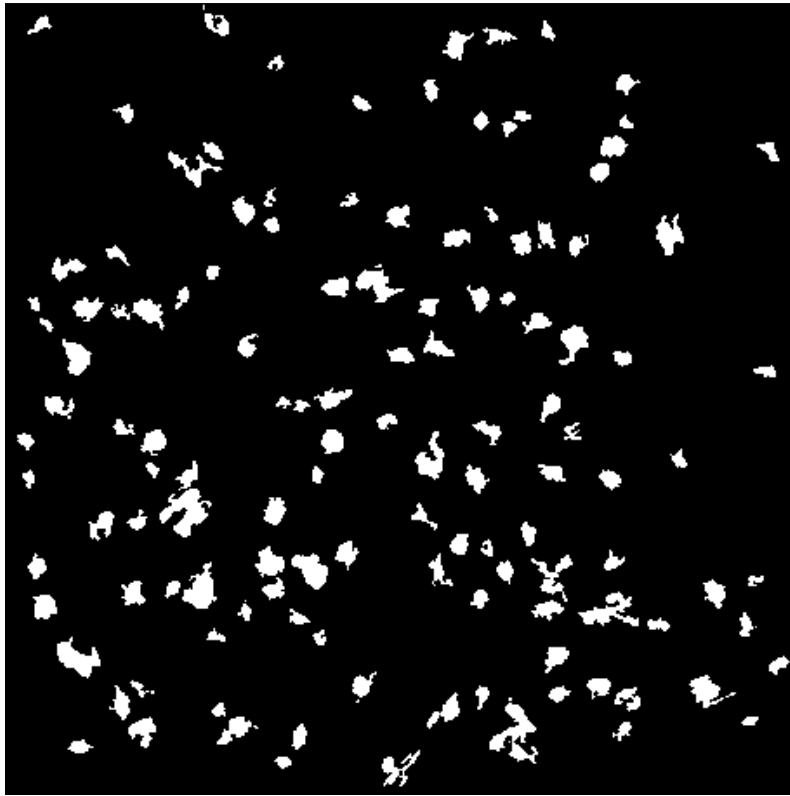


Figure 8-11: Routine repeated with only 2 criteria: area and eccentricity

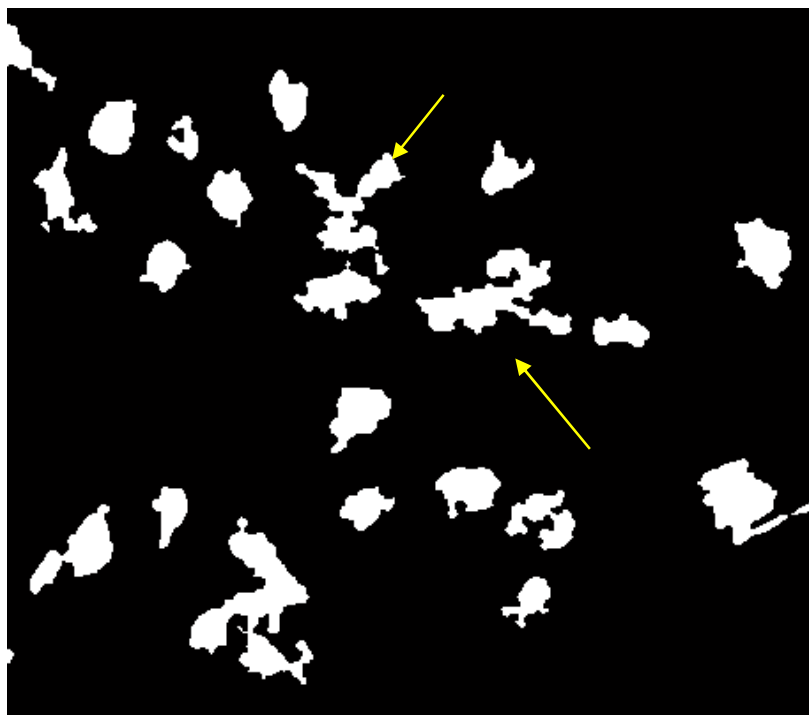
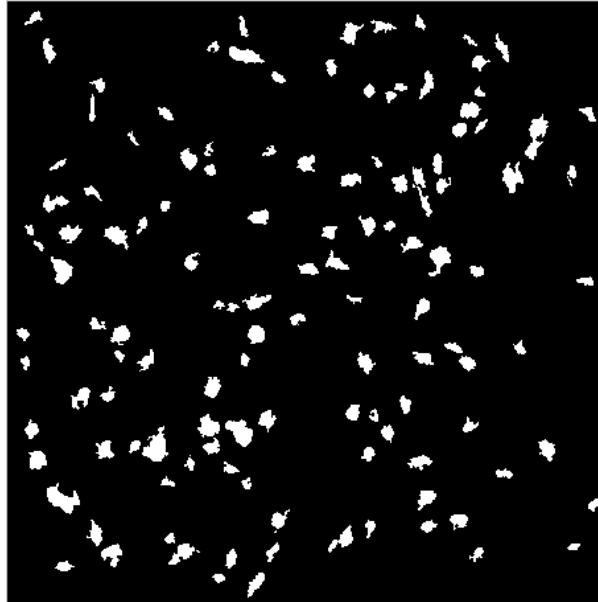
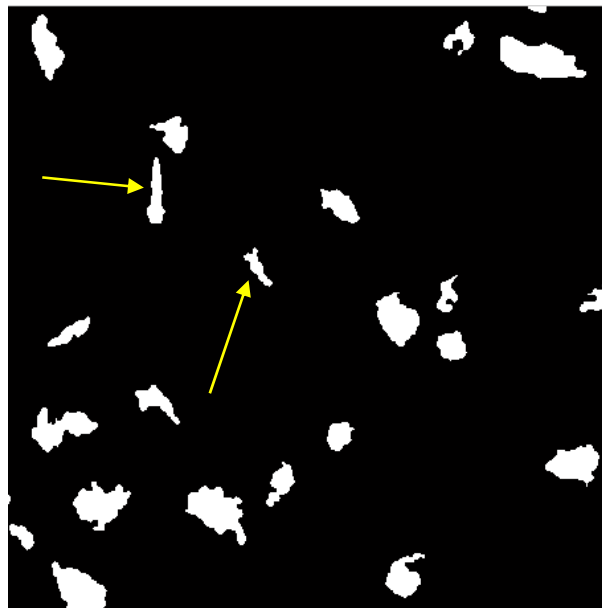


Figure 8-12: Close up showing objects with low solidity and homogeneity (yellow arrows)

When the eccentricity criteria was omitted, the resultant image contained objects which were highly elliptical and narrow as shown in Figure 8-13. Figure 8-14 shows a closeup of these narrow objects which are sufficiently large and solid, but were not circular or wide enough to be developed colonies.



**Figure 8-13: Routine repeated with only 2 criteria: area and fragment factor**



**Figure 8-14: Close up showing objects which are narrow and elliptical (yellow arrows)**

In summary, method 1 was able to identify and locate most GFP positive colonies. However, as seen in Figure 8-16, many GFP positive colonies were missed and therefore not marked with a red box. This may be due to some of the image processing steps, such as Gaussian blurring, which remove definition from the colonies' borders.

### **8.3.2 Evaluation of conditional *if-then* statements**

For comparison, the identified colonies have been marked on the GFP channel of the same set of colonies as shown in Figure 8-16. On closer inspection of Figure 8-10, it can be seen that several individual round colonies (marked with yellow arrows in Figure 8-16) have been segmented as a single shape, thus, presenting a false shape for analysis. The image processing steps used in method 1 have not been able to properly segment the colonies into individual objects.

Despite expressing GFP, other colonies were not identified as their morphology was uncharacteristically elliptical and/or fragmented suggesting that these colonies could have been in a gradual state of differentiation while producing small amounts of GFP. Such colonies would have been less vital in practice and were, therefore, not marked.

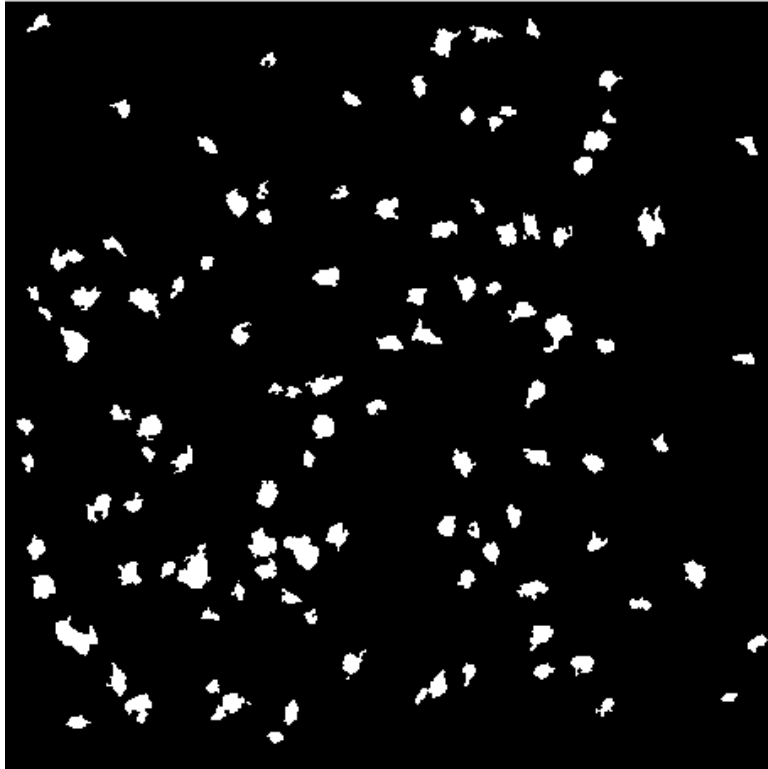


Figure 8-15: The remaining objects after comparing their area, eccentricity, and solidity

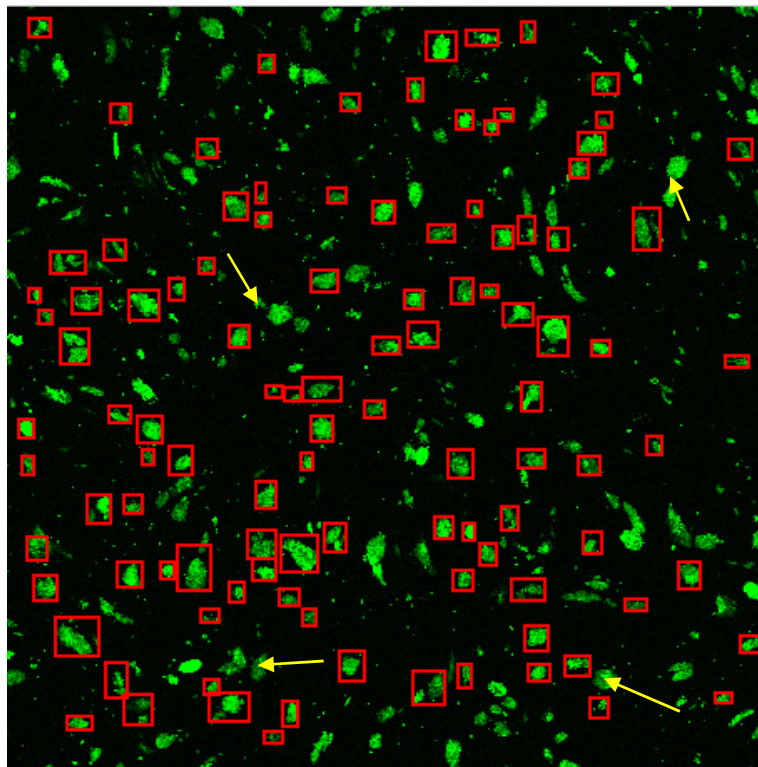
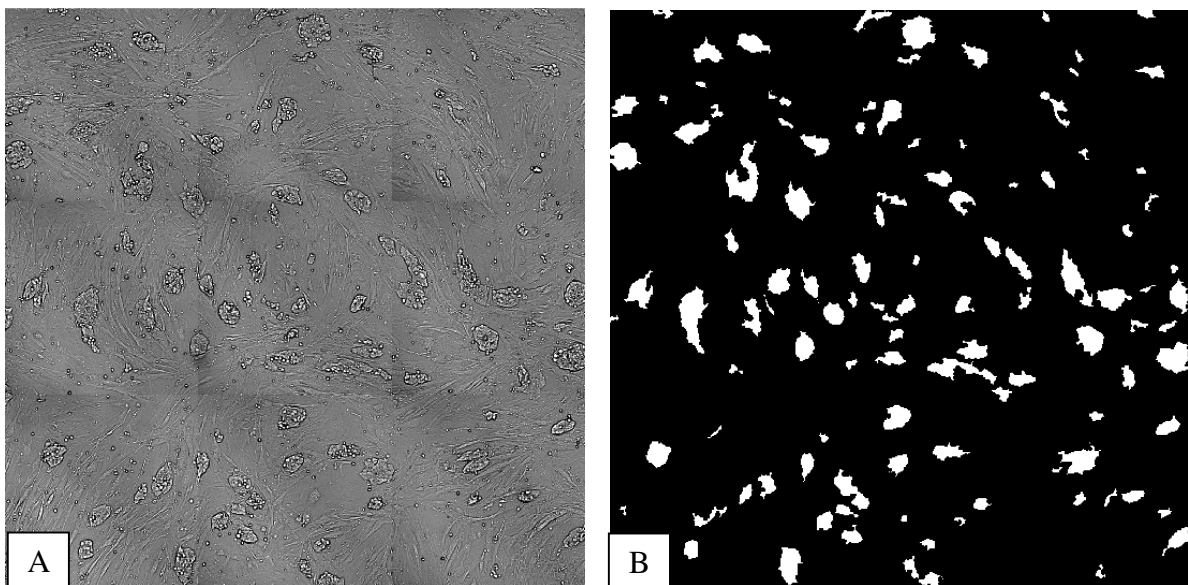


Figure 8-16: Fluorescence image showing GFP positive colonies. Identified colonies shown in red bounding box. Yellow arrows show unidentified colonies

## 8.4 Method 2 – Shape Classification with Fuzzy Logic

In method 2, background subtraction and Gaussian blurring were omitted so as to retain the contrast of the colonies against the feeder layer. Typically, a Gaussian or averaging filter would be applied to remove noise. However, in this case, this had the detrimental effect of softening the edges, and made subsequent segmentation difficult. Instead, the image contrast was enhanced, which made the edges more pronounced (compare Figure 8-1 and Figure 8-17 A).

This improved the object segmentation once thresholding, inverting, morphological closing, and removal of small fragments had taken place (Figure 8-17 B). For the last step, a size of 200 pixels was chosen as it effectively reduced the largest number of unwanted objects while retaining the colonies. This had the further advantage of lowering the overall processing time by half.



**Figure 8-17: (A) enhancing contrast, (B) after removing fragments of less than 200 pixels in size**

From the segmented objects, the following shape properties were extracted and calculated in Table 8-2. Partiality refers to the missing area which prevents the colony from having a smooth

shape equivalent to its convex area. It is calculated by subtracting the actual area of the colony from the convex area. The convex area is the smallest convex polygon that can contain all the pixels of a region. The fragment factor is a ratio of the missing area from the actual size of the colony. It is a measure of the compactness of the colony. The properties for each colony are analysed using a Fuzzy Logic System described in the next section.

**Table 8-2: List of shape properties for classifying objects as colonies with fuzzy logic system**

| <b>Property</b> | <b>Description</b>                                               |
|-----------------|------------------------------------------------------------------|
| Area            | total number of pixels occupied by colony                        |
| Partiality      | convex area minus the actual area                                |
| Fragment factor | $(\text{convex area} - \text{actual area}) / \text{actual area}$ |
| Circularity     | the opposite of eccentricity                                     |

#### **8.4.1 Fuzzy logic system**

Once the 4 inputs (area, partiality, fragment factor, and eccentricity) were calculated for each colony, they were entered into a fuzzy logic system (Figure 8-18). This produced an output, called ‘colony’, which is a score from 0 to 1. It defines how ‘true’ each colony is likely to be based on the 4 inputs.



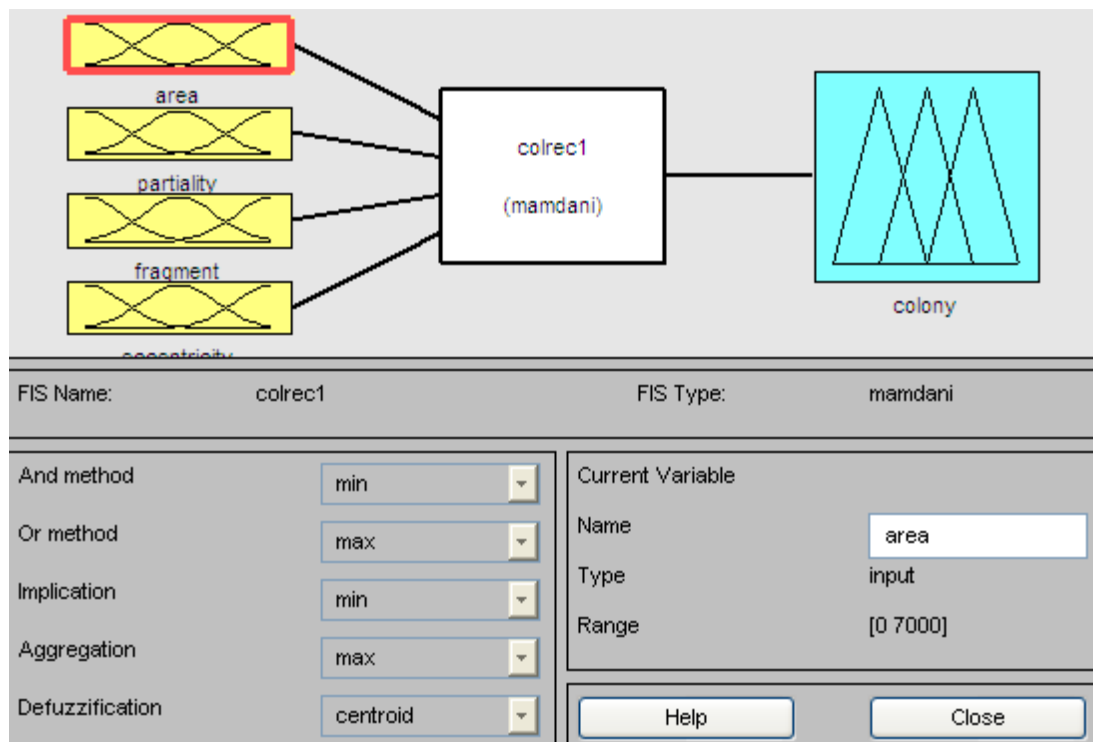


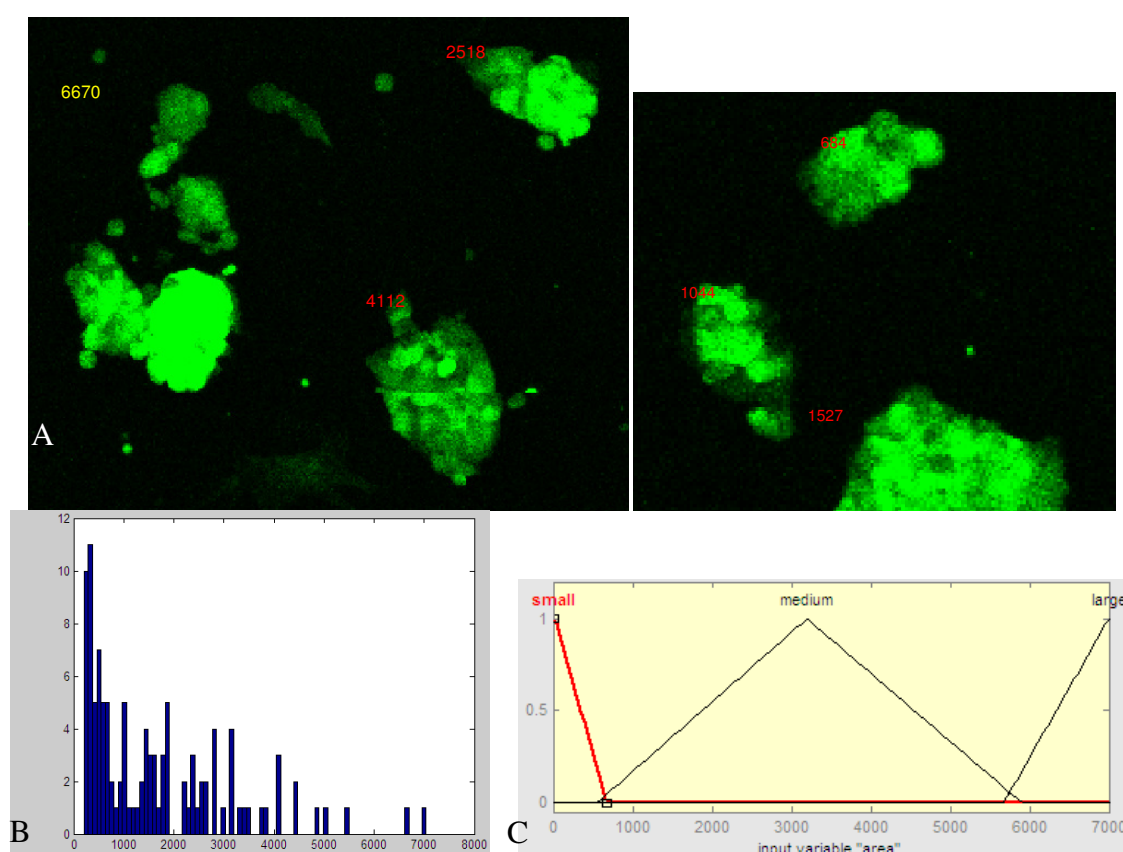
Figure 8-18: The Fuzzy Inference System (FIS) editor allows visualisation of inputs and output(s)

#### 8.4.1.1 Membership functions

Membership functions accept shape properties as inputs and translate them into a weighted degree of association. They are used to compute a final overall output value based on a set of rules. Each membership function has overlapping regions to give the system some flexibility. For example, assume that a rule had been designed so that any colony smaller than 700 pixels should be considered ‘small’. If a colony of 701 pixels is present, it would be immediately and unreasonably classified as ‘large’. From experience, 700 and 701 sized colonies are similar enough to be classified as the same size. Fuzzy logic allows such considerations to be taken into account. The following subsections describe how each variable’s membership function is designed.

### 8.4.1.2 Area

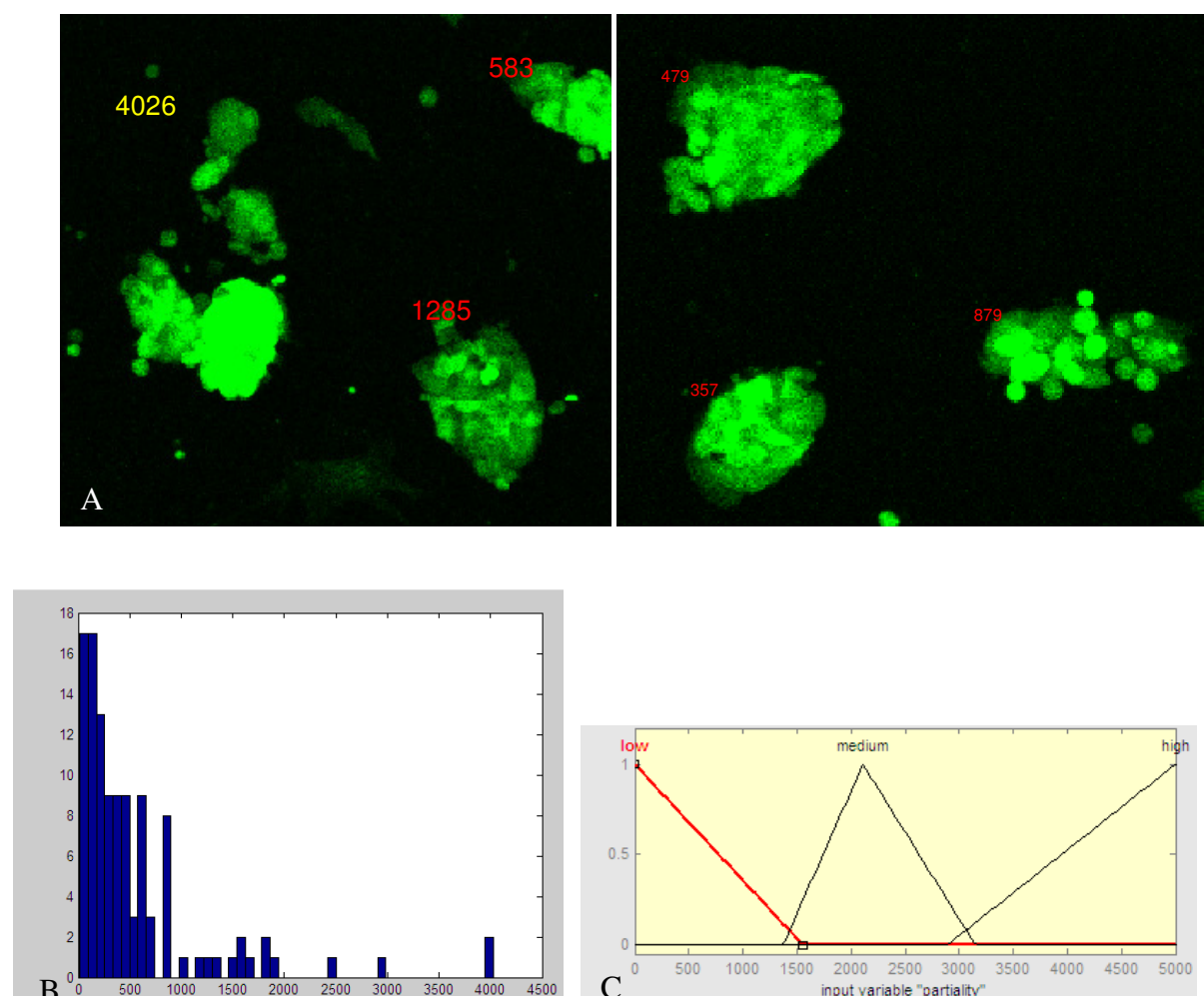
A histogram of colony area is shown in Figure 8-19 B. It can be seen that colonies were of various sizes, with the smallest recognizable colony being about 684 pixels in area while some of the largest as much as 4,000 pixels and above. Colonies such as the leftmost one in Figure 8-19 A were very large ie. 6,670 pixels in area. Such colonies can be observed as outliers in the histogram (Figure 8-19 B). The histogram also shows that, apart from the outliers, there was a spread of sizes between 200 and 5,500 pixels. It was observed that colonies of around 200-500 pixels in size were just fragments and were unlikely to be considered a colony yet. Thus, the membership function for Area was designed to classify objects only between 700 to 6,000 pixels in size as candidate colonies (Figure 8-19 C).



**Figure 8-19:** (A) GFP fluorescence images of colonies with their area (pixels), (B) histogram of frequency vs area of colony, (C) Membership function of area input

### 8.4.1.3 Partiality

There were a large number of colonies which had just 800 pixels or less of area missing from its convex area. This is shown in the histogram (Figure 8-20 B) with some examples shown in Figure 8-20 A. Other colonies had more than 1,200 pixels missing but, from experience, could still be considered as a stem cell colony. The colony on the left of Figure 8-20 A, however, was missing more than 4,000 pixels and therefore should be excluded. The membership function (Figure 8-20 C) was therefore designed to include colonies with less than 1,500 pixels missing as ‘whole’. Colonies missing an area as large as 3,000 pixels and above were deemed as porous and therefore, excluded.



**Figure 8-20: (A) GFP fluorescence images of colonies with their partiality (missing area when compared to convex area in pixels), (B) histogram of frequency vs partiality of colony, (C) Membership function of partiality input**

#### 8.4.1.4 Fragment factor

The lower the fragment factor, the more compact the colony. This is one of the most important characteristics of a true colony. According to the histogram Figure 8-21 B, the fragment factors spread from under 0.1 to about 0.55, however, the trend was skewed to the left so that there appeared to be more compact colonies with fragment factors of 0.3 and below. Examples of such colonies are shown in Figure 8-21 A, and it is reasonable to assume upon examination that these were true instances of colonies. The colony on the right of Figure 8-21 A, however, depicts a fragmented colony with a factor almost as high as 0.5. Thus, the membership function (Figure 8-21 C) was designed to disfavor fragment factors of 0.4 and above.

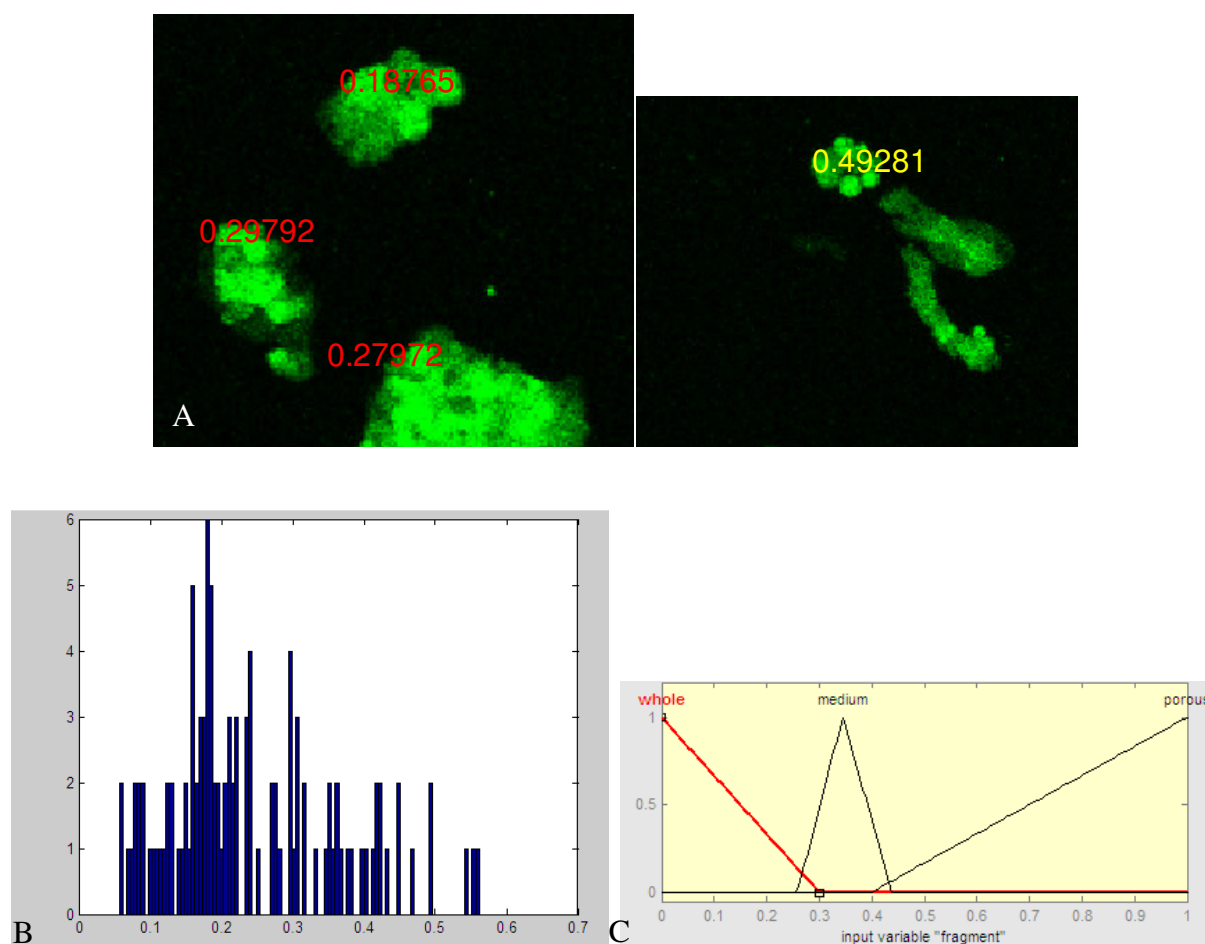
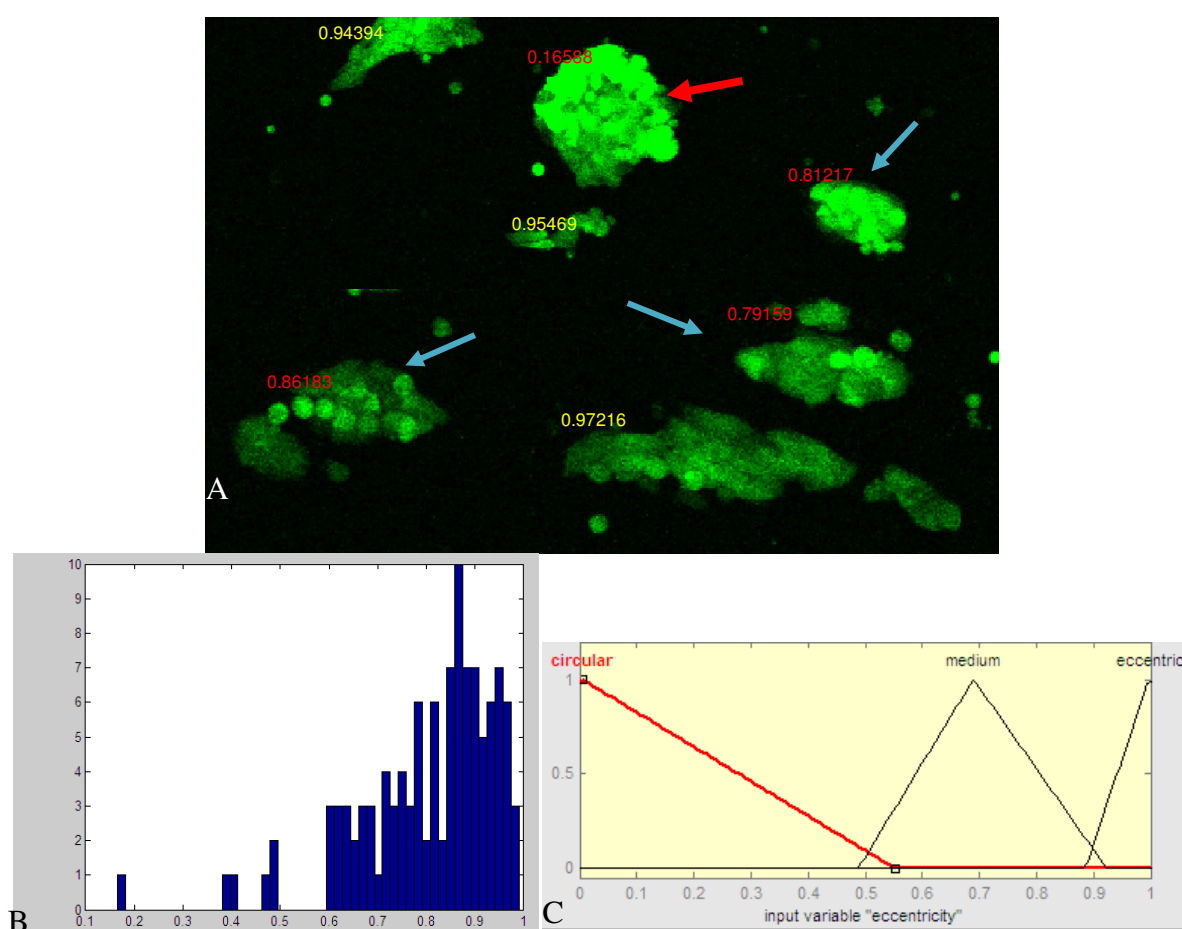


Figure 8-21: (A) GFP fluorescence images of colonies with their fragment factors, (B) histogram of frequency vs fragment factor of colony, (C) Membership function of fragment factor input

### 8.4.1.5 Eccentricity

The eccentricity (opposite of circularity) has values from 0 to 1.0, with 0 describing a perfectly circular true colony. The eccentricity was the most difficult membership function to design because the majority of colonies (including those which would be considered true) were mostly grouped between 0.6 and 1.0 with very little statistical distinction between circular and eccentric objects. A small number of colonies had values less than 0.5 (one example shown in Figure 8-22 A with red arrow), which can be clearly classified as a stem cell colony. Based on experience and visual examination, colonies that could also be considered true had values even as high as about 0.8 (shown with blue arrows), while those that were eventually weighted as eccentric were approximately between 0.9-1.0.



**Figure 8-22:** (A) GFP fluorescence images of colonies with their eccentricity values, (B) histogram of frequency vs eccentricity of colony, (C) Membership function of eccentricity input

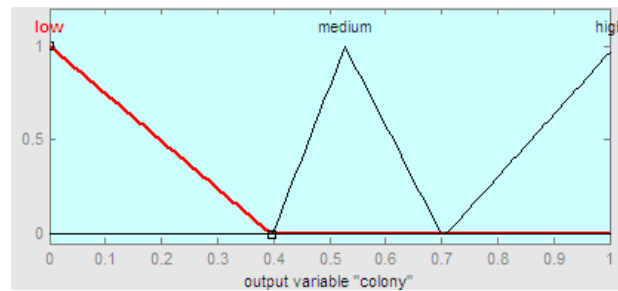


Figure 8-23: Membership function output of 'colony' (ie. score of colony likeness)

## 8.4.2 Rules

For each colony, the fuzzy logic system obtained the degree of membership (between 0 and 1) for each input based on the above membership functions. These were then compared to each of the following 7 rules. A rule was true if all conditions for that statement were met. Each rule can be represented in an easy-to-understand 'if-then statement' shown in Figure 8-24.

1. If (partiality is high) and (fragment is porous) and (eccentricity is eccentric) then (colony is low) (1)  
 2. If (area is medium) and (partiality is low) and (fragment is whole) and (eccentricity is circular) then (colony is high) (1)  
 3. If (area is medium) and (partiality is medium) and (fragment is medium) and (eccentricity is medium) then (colony is medium) (1)  
 4. If (area is medium) and (partiality is medium) and (fragment is porous) and (eccentricity is eccentric) then (colony is medium) (1)  
 5. If (area is large) and (partiality is high) and (fragment is medium) and (eccentricity is medium) then (colony is medium) (1)  
 6. If (area is small) then (colony is low) (1)  
 7. If (area is medium) and (partiality is low) and (fragment is whole) and (eccentricity is medium) then (colony is high) (1)

| If                               | and                           | and                               | and                                     | Then                          |
|----------------------------------|-------------------------------|-----------------------------------|-----------------------------------------|-------------------------------|
| area is                          | partiality is                 | fragment is                       | eccentricity is                         | colony is                     |
| small<br>medium<br>large<br>none | low<br>medium<br>high<br>none | whole<br>medium<br>porous<br>none | circular<br>medium<br>eccentric<br>none | low<br>medium<br>high<br>none |
| <input type="checkbox"/> not     | <input type="checkbox"/> not  | <input type="checkbox"/> not      | <input type="checkbox"/> not            | <input type="checkbox"/> not  |

Connection: ☐ or ☒ and

Weight: 1

Buttons: Delete rule, Add rule, Change rule, <<, >>

Figure 8-24: Rule editor showing 7 rules used in distinguishing true and false colonies

The seven rules are better explained here:

- Rule 1:* This defines the scenario in which a colony is highly false: all 4 inputs are highly suboptimal. A 'low' score is given.
- Rule 2:* This describes a colony where all 4 inputs are close to optimal. The colony is likely to be a true iPS or ES colony and is appropriately given a 'high' score.
- Rule 3:* A colony which falls under this rule has all 4 inputs as average. A 'medium' score is given.
- Rule 4:* If the colony is of the medium size and partiality, but suffers from low solidity and circularity, the colony is given some tolerance and a 'medium' score.
- Rule 5:* Some large colonies may actually be mistaken for 2 or more colonies in very close vicinity, thus giving an erroneous large area. Such instances should be eliminated as they may have already come into contact leading to reduced health and pluripotency. For single colonies which are truly large, their fragment factor and circularity must then be optimal to be given a 'medium' score.

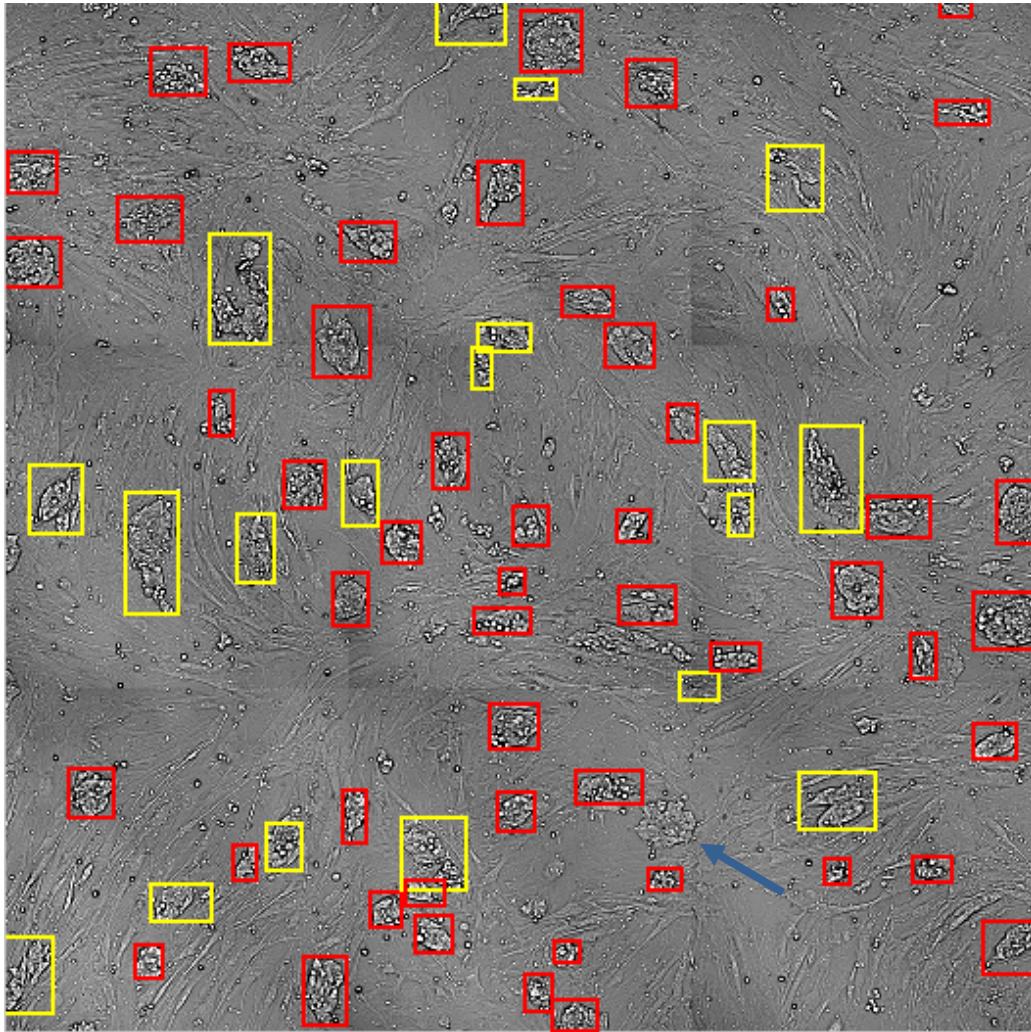


*Rule 6:* Any colony which is too small is immediately given a ‘low’ score. This is to rule out any colonies which need more time to develop before they can be confirmed as true based on image analysis.

*Rule 7:* Finally, for optimum colonies which only have a medium amount of circularity, some tolerance is provided for this as well, in which case a ‘high’ score is given. From experience, the image processing may sometimes distort the shape of colonies causing them to appear more oblong than they really were. This artifact was sometimes sufficient to cause a ‘true’ colony to be classified as false. Thus, this rule is to deal with such rare instances.

### **8.4.3 Classification of colonies**

The fuzzy logic system scored each colony with a value between 0 and 1 (1 being absolutely certain the colony was true). Any colony with a score higher than 0.7 (empirically chosen) was deemed to have a *high possibility* of being true. These are highlighted with a red bounding box (shown in Figure 8-25). Any colony with a score between 0.47 and 0.69 (also empirically chosen) was *only likely* to be true to some extent. These colonies failed more rules which would have led them to be classified as stem cell colonies. They were boxed with a yellow bounding box so as to give users the chance to manually confirm them before deciding. Colonies with a score lower than 0.47 were confidently deemed not ‘stem-like’ in morphology and were therefore not boxed.



**Figure 8-25: Contrast adjusted phase image showing discovered colonies which are highly confident to be true (boxed in red), and less likely to be true but where manual verification is still recommended (boxed in yellow). Blue arrow denotes a colony that was missed by the algorithm.**

The colonies that were used in this example had been genetically modified to fluoresce green (GFP fluorescence) prior to imaging. Thus, to test the algorithm, the bounding boxes were superimposed onto the GFP fluorescence image (Figure 8-26). According to Figure 8-26, it can be seen that the algorithm found colonies instead of cells belonging to the background feeder layer with the exception of one instance shown with a blue arrow marked '1'. Also, the algorithm had successfully accounted for the majority of the colonies. All of the colonies shown with a red bounding box appeared to be true and consisted of individual colonies. Some of the colonies in yellow (eight, specifically) were also stem cell colonies and will need the help of the

user to manually confirm this. However, there were three colonies that had been boxed in yellow but suffered from being too oblong and/or large (shown with blue arrows marked '2'). Also, two colonies had been mistaken for one, also boxed in yellow. These rare instances were caused by colonies which were only slightly similar to true colonies but were still sufficiently different that they will need to be verified by the user. Only 1 out of 60 true colonies was missed by the algorithm (shown with blue arrow marked '3'). This was acceptable as it represented only a small fraction (<2%) of the much larger total number of colonies. This anomaly was attributable to this particular colony's poor edge contrast in the phase image (refer to blue arrow in Figure 8-25). To summarise, 51 out of 60 (85%) true colonies were correctly identified. Of the 9 remaining colonies, 8 were identified as likely colonies and needed manual verification (13.3% of total). Apart from these eight colonies, eleven were falsely boxed in yellow as requiring manual verification when in fact they should have been disregarded. Only one true colony out of 60 (1.6%) was not recognized and accounted for (false negative). The algorithm was tested on 5 images containing a large population of colonies (>70 colonies) and the results are summarised in Table 8-3.

The sensitivity can be calculated by:

$$N_{tp}/(N_{tp} + N_{fn}) \times 100$$

where  $N_{tp}$  is the number of correct colonies that the algorithm finds, and  $N_{fn}$  is the number of true colonies that the algorithm misses or mistakenly believes to be false.

In Table 8-3, the algorithm achieved a sensitivity of 95.0 - 98.5% on five different images. This suggests that the majority of true colonies were found, with a small proportion of false negatives.

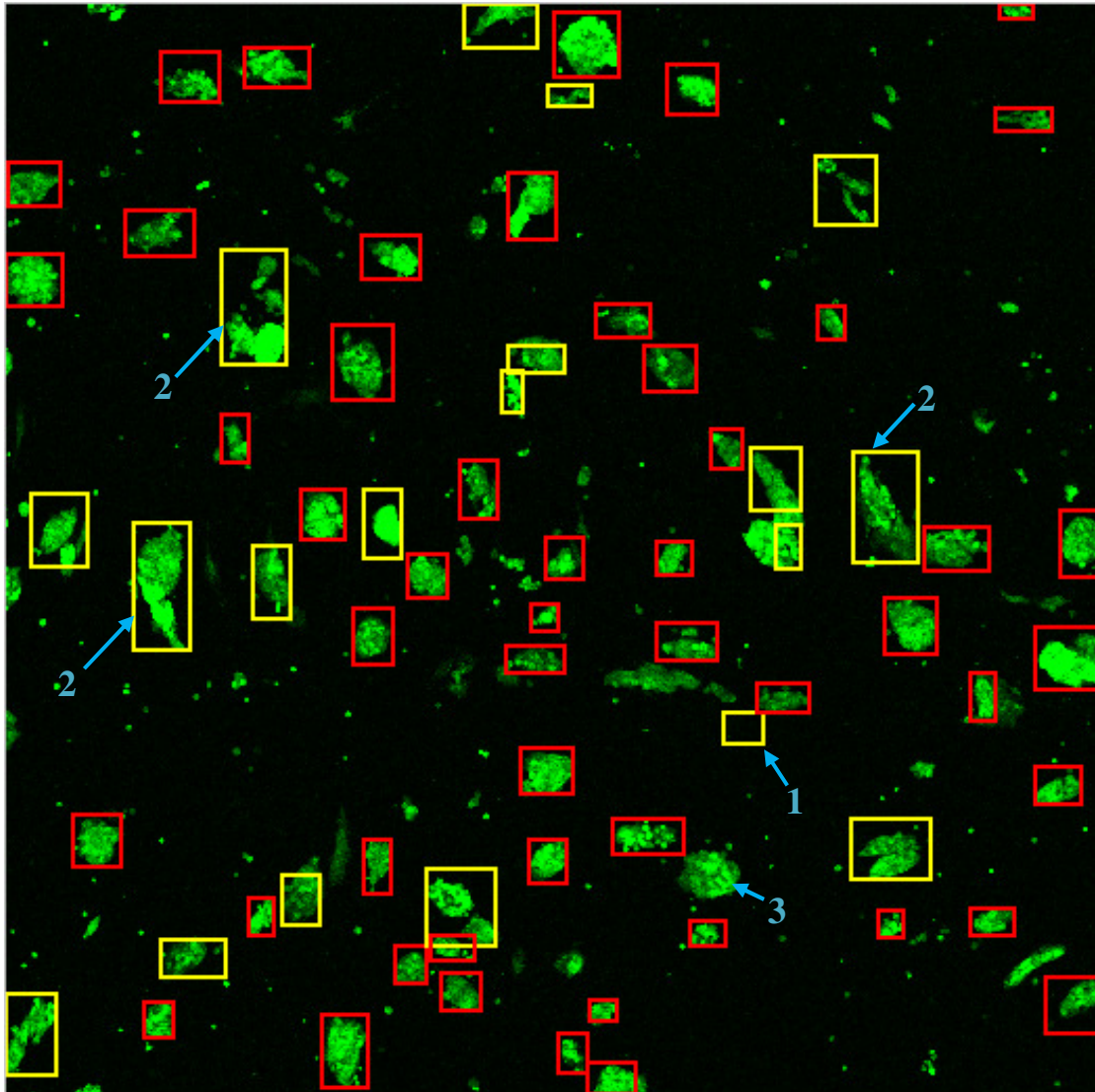


Figure 8-26: The algorithm repeated but with the results displayed on the GFP fluorescence image of the same colonies for validation purposes. Colonies marked with red and yellow boxes were identified by the algorithm as ‘true’ and ‘likely true’ respectively. Cells of interest are marked with blue arrows. (1 & 2) false positive, (3) missed positive colony

**Table 8-3: Assessment of sensitivity of fuzzy logic system on five different images consisting of >100 colonies**

| <b>Image</b> | <b>Confirmed<br/>true colonies<br/>(Marked in<br/>Red)</b> | <b>Possible true<br/>colonies<br/>(Marked in<br/>Yellow)</b> | <b>False<br/>positives<br/>(Marked<br/>in Red)</b> | <b>False<br/>possible<br/>positives<br/>(Marked<br/>in Yellow)</b> | <b>Unidentified<br/>true<br/>colonies</b> | <b>Sensitivity<br/>(%)</b> |
|--------------|------------------------------------------------------------|--------------------------------------------------------------|----------------------------------------------------|--------------------------------------------------------------------|-------------------------------------------|----------------------------|
| 1            | 43                                                         | 33                                                           | 0                                                  | 6                                                                  | 2                                         | 97.2                       |
| 2            | 66                                                         | 27                                                           | 1                                                  | 6                                                                  | 3                                         | 97.2                       |
| 3            | 53                                                         | 32                                                           | 2                                                  | 5                                                                  | 2                                         | 97.5                       |
| 4            | 48                                                         | 22                                                           | 2                                                  | 2                                                                  | 3                                         | 95.0                       |
| 5            | 51                                                         | 19                                                           | 1                                                  | 5                                                                  | 1                                         | 98.5                       |

## 8.5 Comparing Performances between Method 1 and Method 2

The proposed algorithm (method 2) was compared with method 1. Two distinct differences emerged:

1. Method 1 subtracted the original image from a morphologically opened version of itself to remove the background layer. Method 2 thresholded the inverted edges of the colonies which were brighter than the background layer. The image was then dilated to fill holes.
2. Method 1 used a single *if-then* statement, which rejected any object that displayed any of the following characteristics:
  - Area smaller than 700
  - Fragment factor greater than 0.3
  - Eccentricity greater than 0.9

Method 2 used a fuzzy logic system to evaluate the above mentioned characteristics of each segmented object

The performance of each method can be compared and described using four criteria and is summarised in Table 8-4.

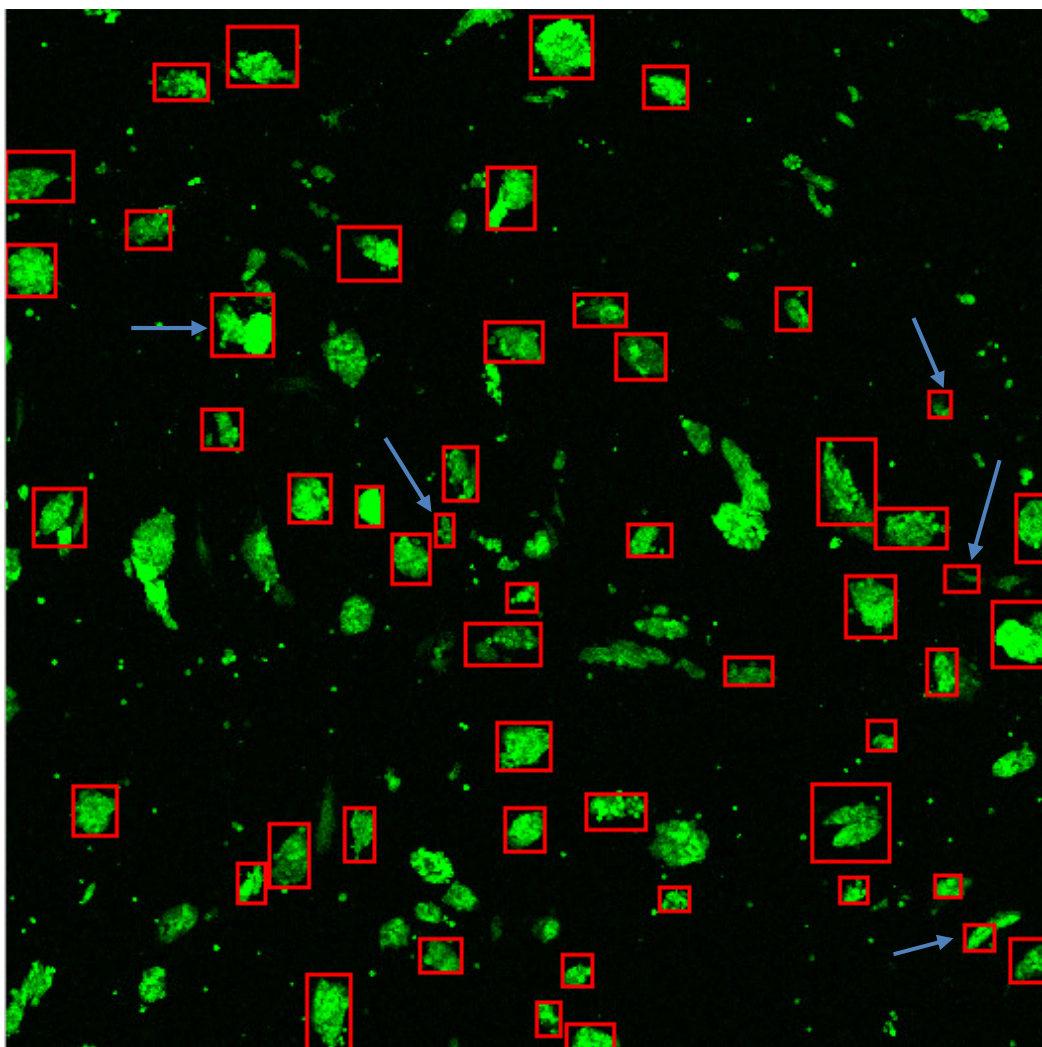
**Table 8-4: Comparison between method 1 and 2**

| <b>Criteria</b>           | <b>Method 1<br/>(‘if-then’ statements)</b>       | <b>Method 2<br/>(Fuzzy Logic System)</b>                      |
|---------------------------|--------------------------------------------------|---------------------------------------------------------------|
| Stringency                | More stringent – single <i>if-then</i> statement | Less stringent – more tolerant to possible colonies           |
| Yield                     | Lower due to higher stringency                   | Higher due to highlighting both certain and possible colonies |
| Number of false negatives | Fewer (5 colonies)                               | More (11 colonies)                                            |
| Computational time        | Longer (11.5 seconds)                            | Shorter (7.5 seconds)                                         |

### 8.5.1 Stringency

Method 1 evaluated colonies more stringently as there was only a single ‘*if-then* statement’. Colonies were labelled as either ‘true’ or ‘false’. The result in Figure 8-27 only contained colonies which the algorithm was highly certain were true. Partially matching colonies were completely excluded. This algorithm was able to find 45 out of 60 (75%) true colonies (boxed in red). Because this algorithm did not highlight partially matching colonies, the remaining 15 true colonies had been disregarded. Method 2 gave more tolerance to colonies that did not meet all three characteristics. These were marked in yellow boxes and allowed the operator to manually verify these extra colonies.





**Figure 8-27: Method 1 repeated with fewer colonies boxed in red on the GFP fluorescence image. False positives are marked with blue arrows**

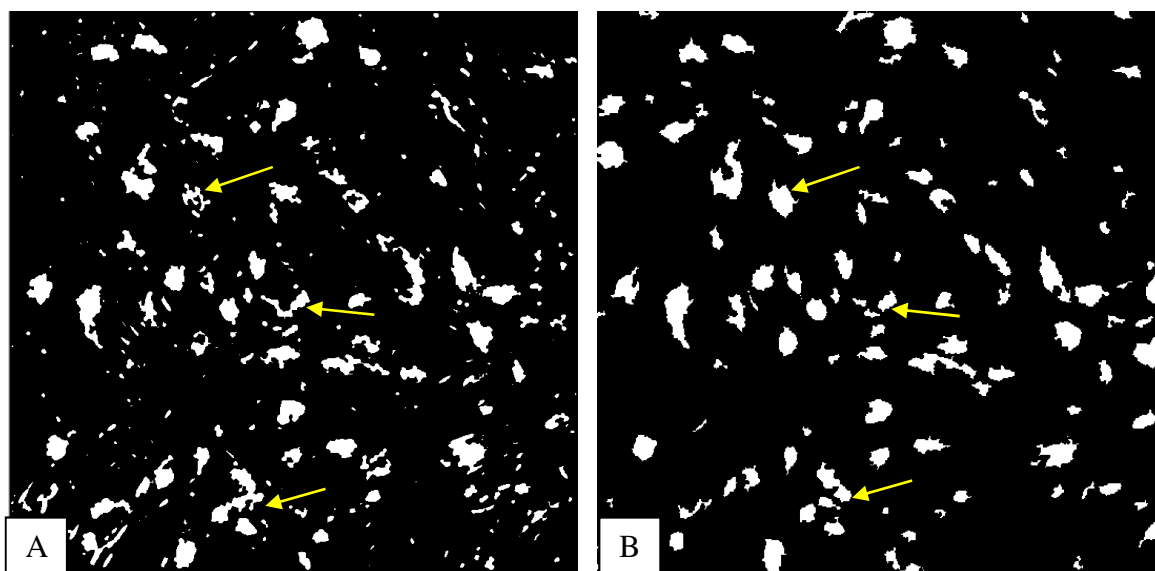
### 8.5.2 Yield (number of true positives)

Because method 2 marked ‘highly certain’ and ‘partially certain’ colonies in red and yellow boxes respectively, more colonies were identified, thus, increasing the yield. Method 1 recognised fewer colonies than method 2 (75% versus 85% or 98% with manual verification).

The higher yield was due to the improved image processing techniques used in method 2, which segmented the colonies more accurately. Compared to method 1 (Figure 8-28 A) on the other hand, the set of colonies (marked with yellow arrows) were incorrectly identified as single



objects. With method 2, however, the same set of colonies was better separated into individual objects and, therefore, resulted in more accurate shape property analysis.



**Figure 8-28:** Comparison of colony segmentation by (A) method 1 and (B) method 2 . Yellow arrows represent colonies that have been segmented properly using method 2 but not with method 1.

### 8.5.3 Number of false negatives

Method 1 found fewer false negatives (5 compared to 11 in Method 2), shown with blue arrows in Figure 8-27. In a large batch of colonies such as in this example, the number of false negatives may only account for approximately 10% of all the true colonies.

### 8.5.4 Computational time

Method 1 also took 4 seconds longer than method 2, which may be significant when a large number of images are analysed. The images shown here had an area of approximately  $9\text{mm}^2$ . A 35mm dish has an area of approximately  $960\text{ mm}^2$ , almost 100 times more. Thus, method 1 could take as much as 400 seconds longer than method 2.

## 8.6 Discussion

The automated colony finding algorithm proposed here implements image processing techniques and fuzzy logic control for the purpose of identifying and sorting colonies into classes respectively. Other publications displaying similar work exist (Mangoubi et al., 2007 ; Narkilahti et al., 2007 ; Bottigli et al., 2007 ; Masala et al., 2007). For example, Mangoubi et al. (2007) have used a neural network approach in classifying 4 types of colonies as opposed to 3 in my algorithm. In order to classify colonies, Mangoubi et al. (2007) had to locate the colonies manually and propose them to a neural network. In this thesis, the algorithm here can be given large tile-scanned images consisting of an undefined number of colonies. The throughput and speed is improved since it can identify and determine the positions of all the colonies that it finds simultaneously.

The aforementioned publications have not stated the throughput of their algorithms, however, an expert in stem cell culture took 5 - 10 minutes to identify most of the colonies in a tile-scanned image (see Figure 8-1). My algorithm only needed 11.5 seconds for the same image and found more colonies than the expert.

Mangoubi et al. (2007) state that the accuracy of their algorithm is 96.7% at best when distinguishing between distinctly undifferentiated from differentiated colonies. Narkilahti et al. (2007) used the ChipIQ's capabilities to monitor hESC growth. The system quantified the proportion of undifferentiated cells using machine vision and compared it against FACS; the results were 86% vs 81% respectively. Bottigli et al. (2007) and Masala et al. (2007) both claim that their algorithms reach accuracies between 91 - 94% and can classify up to 4 and 7 connected colonies respectively. In contrast, my algorithm can achieve a sensitivity between 95.0 - 98.5% based on test images consisting of approximately 500 colonies altogether.

The criteria of my algorithm are different from those of Masala et al. (2007) and Bottigli et al. (2007). The latter 2 publications involve counting the number of *bacterial* colonies, which are generally round, and thus, do not exhibit the irregular shapes of ES / iPS colonies. Furthermore, the bacterial colonies were cultured on clear plastic making it much easier to segment using image processing. My algorithm had to deal with a background monolayer of cells. The goal of the algorithms developed by Masala et al. (2007) and Bottigli et al. (2007) is to count the number of colonies regardless of whether they are in contact with each other. ES / iPS colonies, on the other hand, may suffer from reduced health and pluripotency if they are grown too close together. Therefore, the algorithm developed in this thesis not only counts healthy colonies, but excludes those which are connected.

## 8.7 Summary

This chapter presented a novel approach to identify stem cell colonies on a monolayer of feeder cells. Once the colonies were segmented, they were sorted into whether they represented true colonies based on their shape properties. The two original methods of classification compared were an ‘*if-then* statement’ and a fuzzy logic system. I analysed some of the key strengths. The advantage of the latter method was that it identified both true and possible colonies, which would give users the option of selecting only true colonies where stringency was important, or selecting both true *and* possible colonies if yield was essential. My results showed that the fuzzy logic system had high sensitivity of 95 - 98.5% and was able to identify large numbers of true and possible colonies quickly.

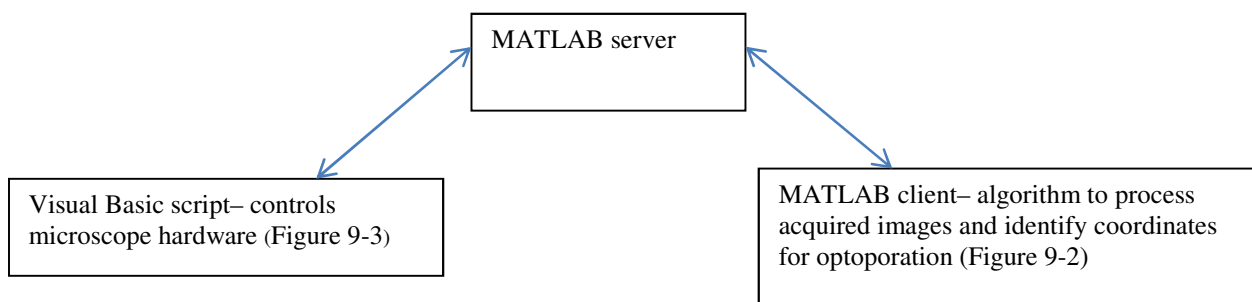
## Chapter 9

# Optoporation with Automated Transfection Routine

Optoporation is conventionally performed manually by either moving the microscope stage relative to a stationary laser beam, or by directing the laser to different areas around a fixed stage. Both methods are time consuming and labour intensive as they require a human operator to identify and target the cells, consequently limiting the yield of optoporated cells. Hence, the development of an automated optoporation system using image processing to identify cells would be beneficial. A possible solution is presented and discussed in this chapter.

## 9.1 Design of Automated Transfection Routine

An automated transfection routine was written in a combination of Visual Basic (VB) (Figure 9-3) and MATLAB (Figure 9-2). As shown in Figure 9-1, both programs shared variables through a MATLAB server. Visual Basic exported data into this server, which can then be imported into the MATLAB client's workspace. The latter program was responsible for image processing and deciding where along the cell membrane the laser emission should be focused, also known as 'parking' the laser beam. The VB script file received the coordinates from the MATLAB program and controlled the microscope stage, laser, and the overall optoporation procedure.



**Figure 9-1: Flowchart showing how Visual Basic and MATLAB exchange data through the MATLAB server**

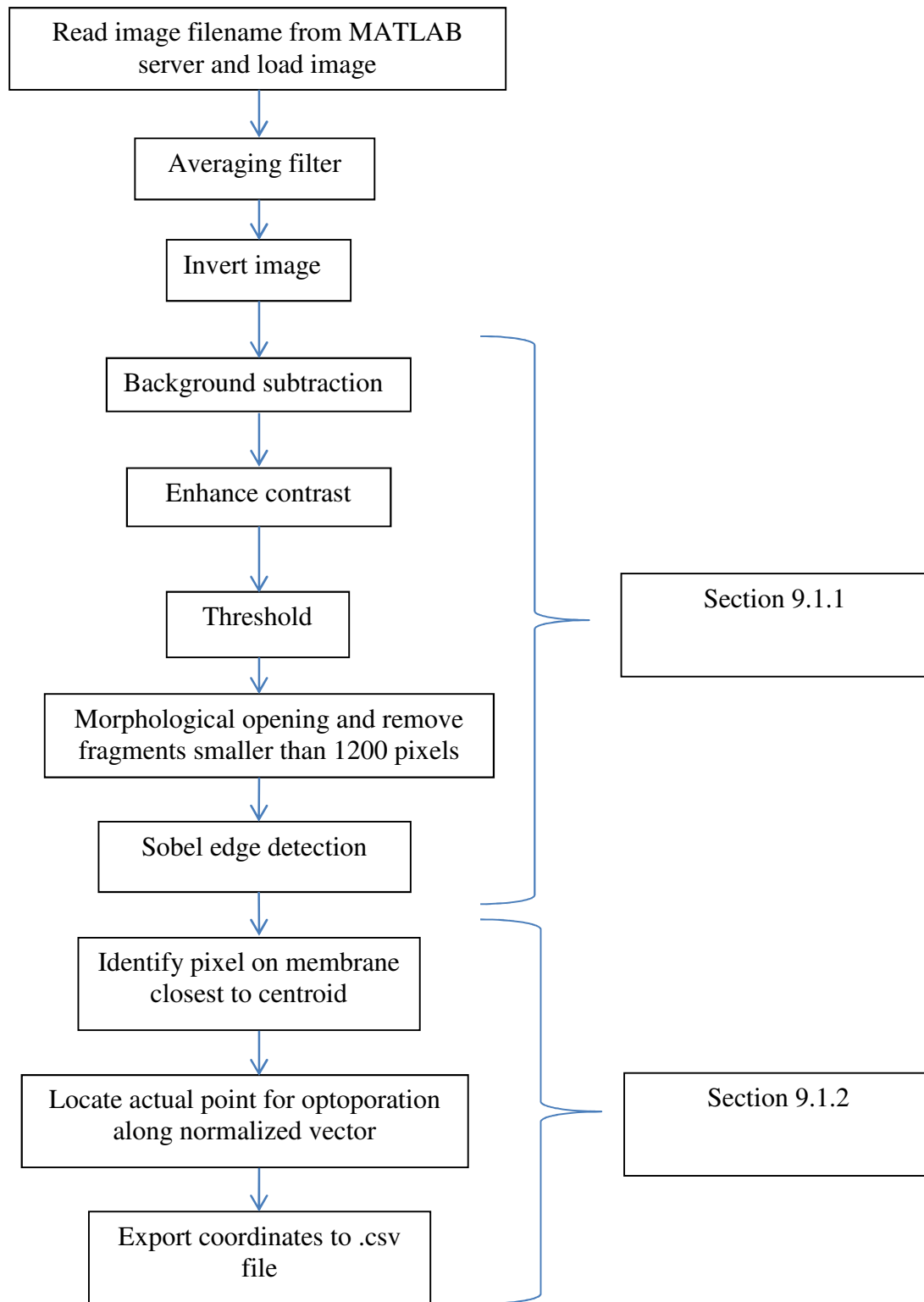
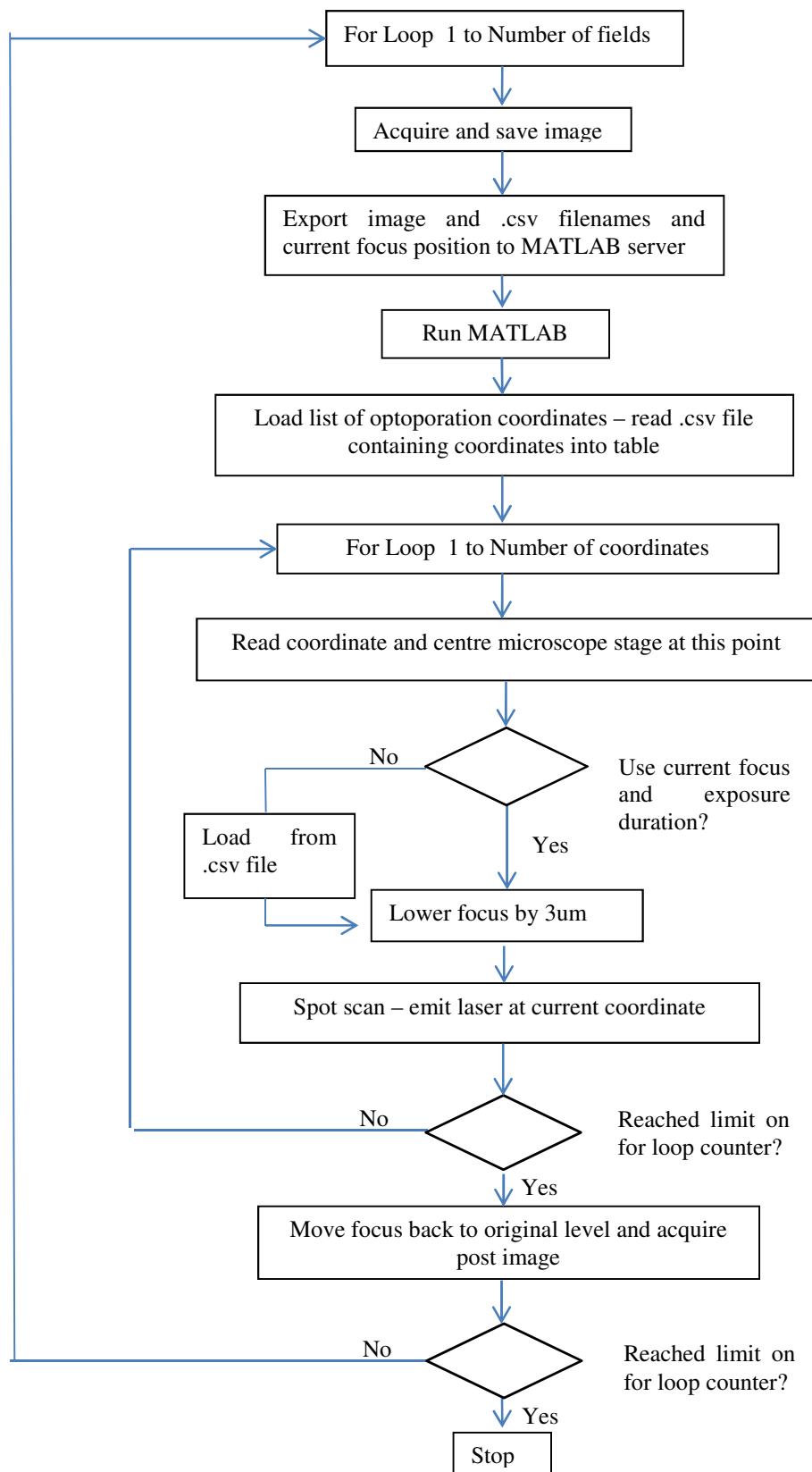


Figure 9-2: Flowchart showing MATLAB algorithm for locating cells and identifying coordinates for parking laser

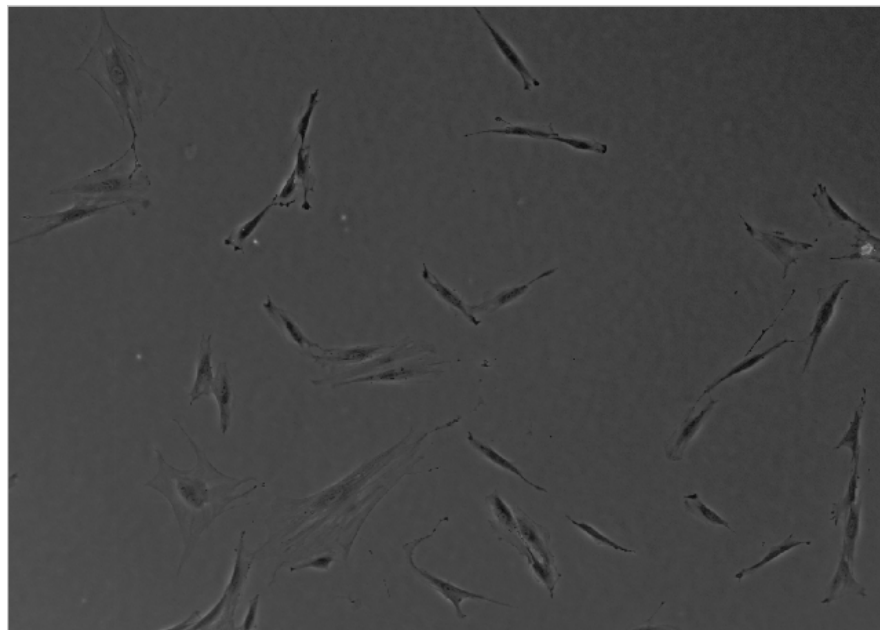


**Figure 9-3: Flowchart showing Visual Basic script to control microscope hardware**



### 9.1.1 Image segmentation

An image of a cell monolayer was first taken with a 20x or 40x phase objective (Figure 9-4) using the microscope control software ZEN 2009. The cells were seeded so that they were not in contact with each other, which allowed the algorithm to resolve each cell individually. Using this objective with phase contrast, the cells appeared darker than the background as indicated in Figure 9-4.



**Figure 9-4: Monolayer of human fibroblasts imaged with a phase objective**

After inverting the image and enhancing the contrast, the resultant image was thresholded using an empirically determined threshold value to produce a binarised image, Figure 9-5. This procedure segmented the foreground into individual objects. Each object corresponded to one cell. However, some small fragments still remained. A morphological opening step was applied to remove fragments which were smaller than 1200 pixels (Figure 9-6). With the cells segmented, the next subchapter will describe the approach in determining the coordinates for optoporation.



Figure 9-5: Thresholding the grayscale image into a two-tone image



Figure 9-6: Elimination of objects smaller than 1200 pixels using morphological opening. In this image, each identified object is labelled with a different intensity of grey.

### 9.1.2 Determination of coordinates for optoporation

This subchapter describes and compares two techniques for determining the coordinates for optoporation each cell:

1. Directly on membrane
2. Fixed distance away from membrane

#### 9.1.2.1 *Directly on membrane*

A Sobel edge detector detected points on the perimeter of each cell. The point on the membrane that was closest to the centroid was selected for optoporation. The pseudocode for this procedure is shown below.

*For i: 1 to number\_of\_points\_on\_perimeter*

*Calculate distance between point(i) and centroid*

*If distance < shortest\_distance then*

*shortest\_distance = distance*

*End if*

*End loop*

When cells were optoporated using the above method, there was frequent cell death due to the proximity of the laser to vital cellular components such as the nucleus. When the algorithm was changed to the *farthest* point from the centroid, I nevertheless continued to observe frequent cell death. This was because cells are thinner at its farthest edges, and therefore generated less contrast. Consequently, the accuracy of the segmentation was poorer and produced incorrect

optoporation coordinates, as shown in Figure 9-7. The inaccurate segmentation has resulted in failed edge detection, causing the laser to be ‘parked’ destructively within the cell body instead of at the membrane (Figure 9-7 B).

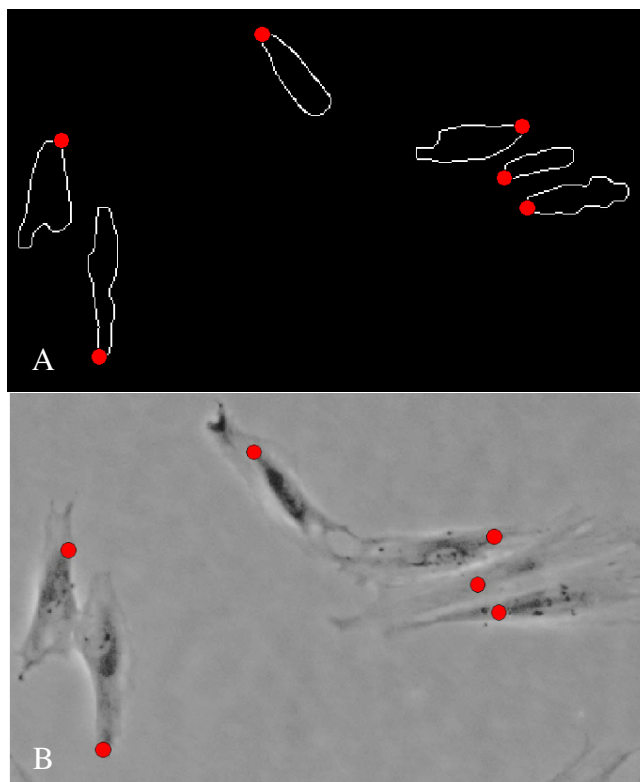
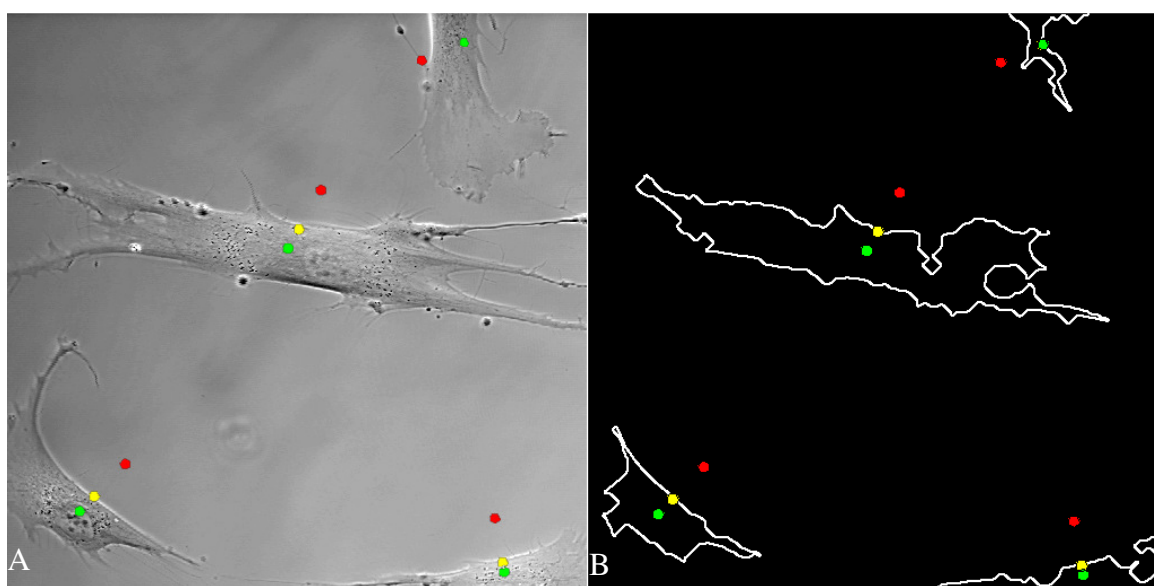


Figure 9-7: Furthest pixel from the centroid shown by a red dot overlaid (A) after edge detection, (B) and on the original raw image itself

#### ***9.1.2.2 Fixed distance away from membrane***

This section suggests and implements a possible solution to the inaccurate segmentation described in section 9.1.2.1. Specifically, the glass substrate was optoporated at a fixed distance away from the cell at high power. This appeared to induce a shockwave which permeated the edge of the cell closest to the site of impact. The advantage of this method was that segmentation was allowed to be less precise. The shockwave permeated cells that were within a certain range, rather than only at a fixed spot. Furthermore, two or more cells can be optoporated at the same time to increase yield.

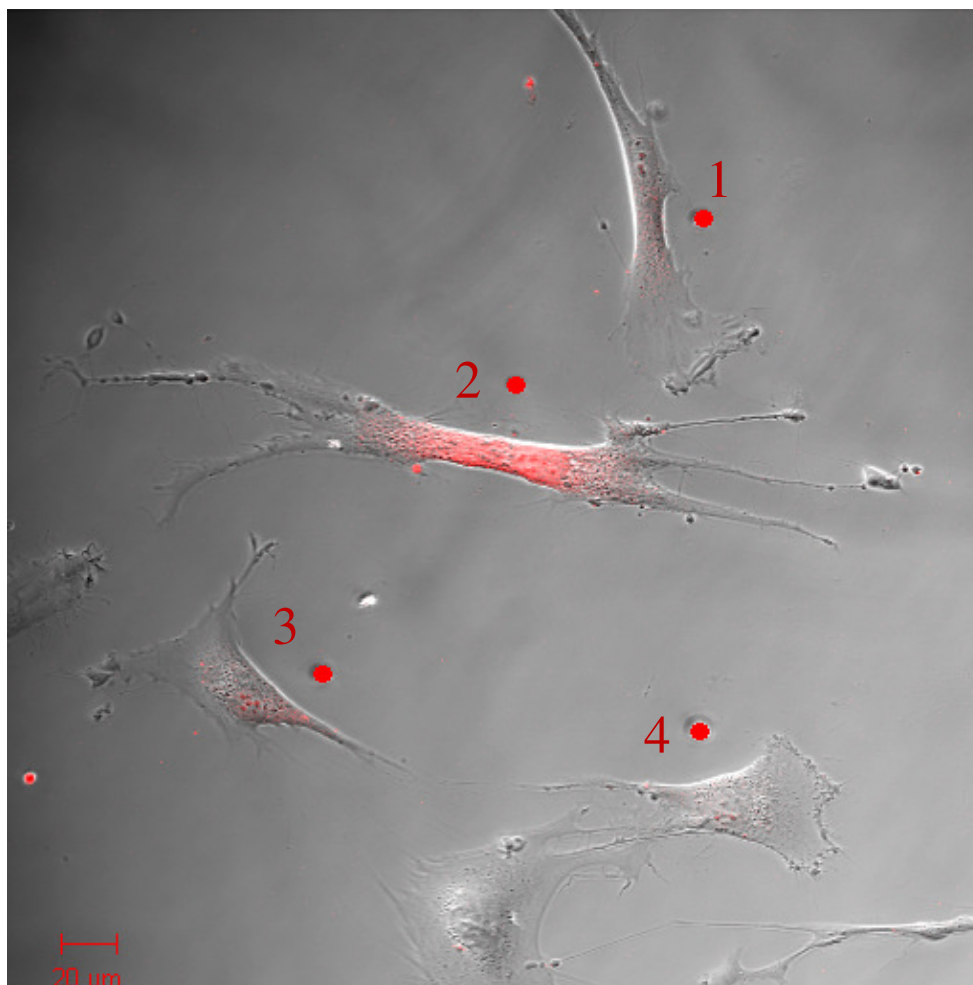
The location of the shockwave was decided by first calculating the normalised vector between the centroid and its closest coordinate on the perimeter, shown as the green and yellow dots in Figure 9-8 B respectively. The origin of this vector was set at the latter of these two points and multiplied by a scaling factor of 40 pixels (empirically determined). The laser was irradiated at the terminal point of this vector (red dots in Figure 9-8 B).



**Figure 9-8:** (A) Phase contrast image of human fibroblasts, (B) edge detection of segmented cells with centroid, coordinate on membrane closest to centroid, and actual point of optoporation (marked with green, yellow and red dots respectively)

## 9.2 Results

Figure 9-9 displays the result when the cells from Figure 9-8 A were optoporated away from the membrane at the locations shown by red dots in Figure 9-8 B. All four cells appeared to show uptake of PI within seconds after irradiation. The membranes of cells 1, 3, and 4 appeared to have recovered and were intact within 5 minutes after optoporation. Cell number 2 showed the highest uptake of PI shown by the intense red fluorescent signal. Expectedly, its morphology also narrowed significantly, which suggested that the cell was the most permeated.



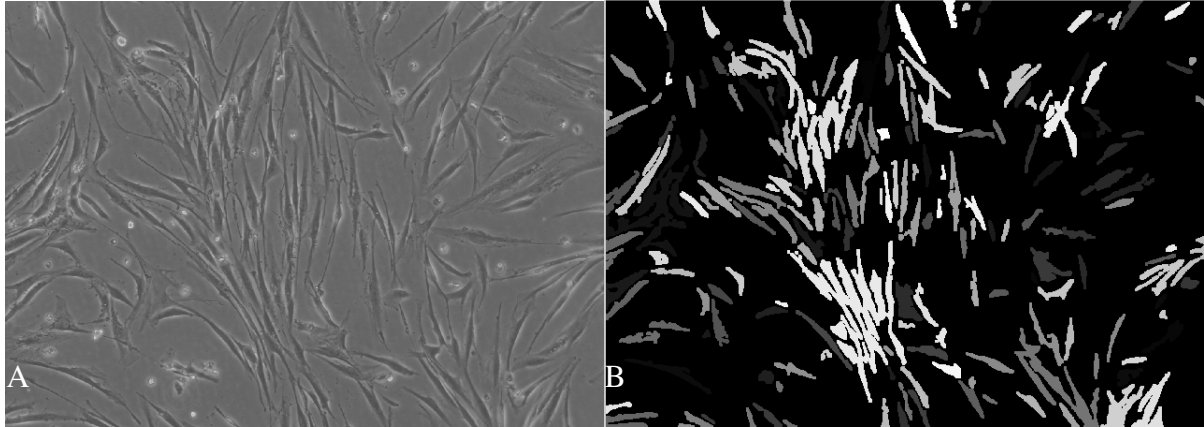
**Figure 9-9 : Phase contrast and fluorescence imaging of PI (red) uptake in human fibroblasts after shockwave optoporation (beam parked at locations shown by red circles)**

### 9.3 Evaluation of Script Robustness

It should be noted that cells need to be plated at a single cell density and there should be no contact between them, such as in Figure 9-4. Here, cells were seeded at  $1 \times 10^5$  cells per 35 mm dish and imaged the next day. This is vital because cells cannot be individually segmented when cells are seeded at a high density or allowed to grow to confluency. Figure 9-10 A shows a layer of cells which are far too confluent.

In Figure 9-10 A, the algorithm was unable to distinguish and segment individual cells especially those in the clusters (Figure 9-10 B). These clusters were resolved as single objects during edge detection even though each cluster consisted of approximately 10 cells as shown in Figure 9-11

A. Figure 9-11 B is a closeup of such a region of high confluency in Figure 9-11 A where segmentation had failed.



**Figure 9-10: Gray scale image of high density culture. (A) original, (B) segmented objects. Each object is represented by a shade of grey.**



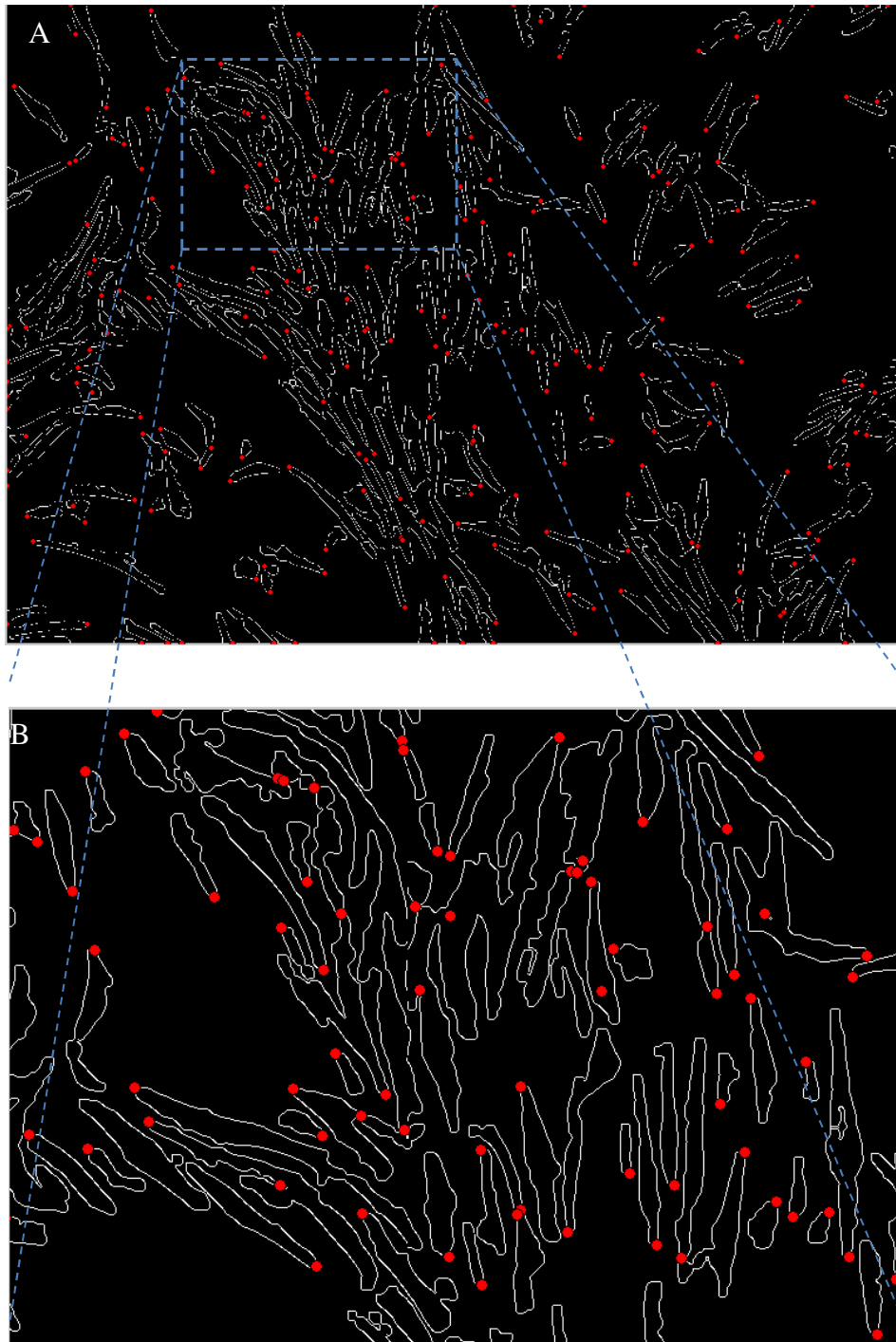


Figure 9-11: (A) Edge detection of clumps of cells, (B) close up of (A) marked by blue dashed rectangle

## 9.4 Validation of Script Usefulness

With manual optoporation, approximately 100 cells could reasonably be targeted in 1 hour. When the algorithm was used, 4 cells could be optoporated in just 30 seconds. Theoretically, this

would correspond to 480 cells in 1 hour assuming that there were always 4 cells in each field of view. This is a significant improvement of nearly 5 times faster. I note, however, that the algorithm failed after optoprating 3 fields of cells due to the loss of imaging focus. The reason for this is due to variations in the flatness of the dish as it was not possible to assume that the glass-bottom dish was totally level. One solution that I attempted was to model the surface of the glass as a plane using 3 points in order to interpolate the focal plane at different areas of the dish. Despite this, the dish still exhibited both global and local unevenness, and the algorithm continued to fail after approximately 3 fields. Another solution was to optoprate at five different levels with the assumption that the focal plane would coincide with one of these levels. This was highly unsuccessful because the step-size between each level was too coarse. Furthermore, using more levels made the algorithm time consuming. One possible method which has yet to be tried here would be to generate a Bessel beam (Tsampoula et al., 2007), which could be implemented in future projects based on the simple addition of an axicon lens as described by the aforementioned publication. The Bessel beam exhibits two-photon excitation along the entire focal length of the beam, thus, negating the need to focus the beam along the z-axis.

## **9.5 Discussion**

Cappelleri et al. (2010) state that the throughput for manual optoporation is a mere 20 cells / hour, but this depends on practice and skill. In my experience, a throughput of more than 60 cells / hour can be achieved. The automated algorithm presented here can optoprate 4 cells automatically in 30 seconds, which equates to about 8 cells / minute or 480 cells / hour resulting in an 8 to 24-fold increase compared to manual optoporation. Obviously, the throughput increases if there are more cells per field and the computational time for analyzing images could be lowered by using a more powerful PC. Cappelleri et al. (2010) have similarly designed a MATLAB program to automate optoporation (see section 2.1.5.1 for review) and is the most

similar image-based algorithm proposed than any other publication. Cappelleri et al. (2010) also assume that 4 – 6 cells will be in the field of view using a 40x objective on a system equipped with a Ti:Sapphire laser. However, their algorithm has yet to be tested on an actual working microscope capable of optoporation and, therefore, the yield of 2,250 cells / hour is only theoretical and perhaps optimistic. This does not take into account the time required for moving the stage, acquiring an image from a PMT detector, and storing images. Also, this level of throughput assumes that images are acquired using a CCD, which takes a frame in one instant. Not all microscopy systems, including the one used in this thesis, have CCD cameras but may only be equipped with a PMT so that a laser must scan the sample. This can take several seconds to complete. Furthermore, Cappelleri et al.'s (2010) algorithm does not take into account the level of flatness of the dish, thus, that it would be impossible to optoporate that many cells unless a lengthy autofocus routine was added. Thus, Cappelleri et al.'s (2010) yield needs further evaluation.

Another technique to automate optoporation was described by Marchington et al. (2010). Using microfluidics, cells were streamed in suspension into the beampath of the laser. Throughputs of 1 cell / sec or 3,600 cells / hour have been described, but only 150 cells per batch have ever been tried. This technique does not rely on image processing or other computationally intensive algorithms and is, therefore, fast. The efficiency of optoporation, however, was only 42%, again, due to focusing. In practice, one would need experience in designing, constructing and integrating microfluidic channels and pumps to an imaging system. In contrast, the technique offered in this thesis works with standard glass-bottom culture dishes and could even be extended to glass-bottom multi-well plates.

Wang et al. (2007) have devised an image-based automated microinjection system that can process 15 embryos per minute, which is nearly double the speed of my algorithm. However, Wang et al.'s (2007) system utilises two micromanipulators that work in parallel to obtain these

higher yields. In contrast, my algorithm is only equipped with 1 laser, therefore, both systems are comparable if this is taken into account. Another difference is that, in Wang et al.'s (2007) system, the 1.2 mm sized embryos, are held in place at regularly spaced locations using suction force. This reduces the search time drastically. On the other hand, in this thesis, somatic cells of just 30  $\mu\text{m}$  in diameter need to be located from random locations. Such cells are too small to be fixed in place using suction force. Both systems are similar in that they use a combination of thresholding and morphological closing to locate cells and determine the most suitable site for optoporation / microinjection. Also, cells in both systems are brought to the centre of the field of view for targeting. Wang et al. (2007) report a survivability of 96 – 100%. With careful optimization of laser parameters, my proposed technique has also shown that it can achieve similar cell viabilities.

This section compared the proposed automated algorithm with 2 other optoporation systems, 1 of which has not been tested on an actual optoporation system. Because of the similarities between optoporation and microinjection, I have also compared my automated optoporation system with an equivalent automated microinjection system.

## **9.6 Summary**

This chapter has presented an algorithm to automatically identify cells and subsequently, to identify suitable sites of optoporation for each cell. The algorithm communicates with a Visual Basic script to control the microscope stage and laser. The automated stage found on the Zeiss LSM710 was adequately precise to centre the stage at each coordinate.

It was generally not possible to consistently optoporate directly on the membrane especially when cells were thin and exhibited low contrast and, therefore, delivered poor segmentation results. This problem was overcome by optoporating the glass substrate, which caused a

shockwave to nearby cells (manual shockwave optoporation was shown by Soughayer et al., 2000). To my knowledge, my work is the first to automate optoporation by shockwave using a Ti:Sapphire pulsed laser.

The use of shockwave optoporation presents a limitation in that cells need to be sparsely seeded. At high densities, cells tend to be incorrectly segmented as one object. The Watershed segmentation algorithm may segregate highly dense cell cultures but is time consuming and may require manual marking of seeding positions.

The tight femtolitre focal volume of the femtosecond pulsed laser presents a disadvantage since precise focus is especially crucial for optoporation to work. Two methods (modeling the glass surface as a plane and optoporating at multiple levels) were tried to circumvent this issue, however, these were unsuccessful. Nevertheless, within about 3 fields, the algorithm optoporated cells 5 times faster than when performed manually.

# Chapter 10

## Conclusions

### 10.1 Summary of Achievements

The primary aim of this research was to demonstrate the use of laser transfection, also known as optoporation, to deliver small molecules, proteins, and plasmids into human primary cell lines for the purposes of drug testing and generation of iPS cells. I also looked into the feasibility of automating this technique by using image processing techniques. A summary of each chapter's main findings is highlighted below. Detailed discussion comparing the results with literature can be found in the 'Discussion' sections of each Results chapter.

In chapter 2, some of the existing scientific literature regarding optoporation were reviewed. I noted that experimental conditions were cell-specific and had to be optimised. These included laser type and power, exposure time, choice of medium, objective lenses, and optoporation technique. Optoporation could be realised either by 'parking' the laser beam directly on the membrane or on the glass substrate itself at high power to induce a shockwave. This shockwave, when positioned at about 10  $\mu\text{m}$  away from the cell membrane, was sufficient to permeate it. At higher cell densities, the shockwave was also able to permeate more than one cell, thus, increasing the yield. Much of the research in this thesis relies on this technique. Other attempts to increase the yield from optoporation have been focused on directing or automatically positioning cells under the laser beam using microfluidic channels, and the use of image segmentation. To my knowledge, this thesis is the first publication that shows the latter method

in use with a femtosecond laser coupled to an inverted multiphoton microscope (described in chapter 3). Using this experimental setup, it was possible to both optoporate and construct 4D timelapse datasets of transfected cells.

In chapters 4 and 5, I compared the efficiencies of generating iPS cells between traditional transfection methods, such as electroporation and lipofection, with the more advanced technique, optoporation. Using electroporation and lipofection, partially reprogrammed iPS colonies that stained positive for alkaline phosphatase were generated albeit at very low efficiencies ( $<0.00018\%$ ). However, these colonies did not propagate nor stain for iPS surface markers. The efficiency of plasmid-based generation of human iPS cells is higher at between  $0.0002 - 0.006\%$  (Tayeb et al. 2010 ; Jia et al., 2010). Repeated transfections in these studies were similarly necessary to maintain expression for long periods of time. In terms of reprogramming time, colonies were obtained 5 days before Tayeb et al. (2010) despite using 3 genes instead of 4. This is surprising since the colonies that were generated by Nakagawa et al. (2008) using only O, K, S took 18 days longer to form than Yu et al. (2007) who used O, S, NANOG, and LIN28.

In chapter 5, I proceeded to reprogram somatic cells into iPS cells by optoporating OCT4, SOX2, KLF4, and GFP (as a reporter gene) into human fibroblasts. Since the reported reprogramming efficiency of plasmid-based methods is already low, transfection efficiency of optoporation itself would need to be high. Based on my results, it was nearly impossible to transfect human fibroblasts reliably and consistently even with just GFP or DsRed plasmid. Based on the observation of one successfully transfected cell after it underwent cell division, I reasoned that plasmid DNA had encountered a barrier – the nuclear membrane. Therefore, even though optoporation may have allowed the plasmid to enter the cell through the plasma membrane, it however did not facilitate entry into the nucleus. This finding is in line with the published result by Wilke et al. (1996), Uchugonova et al. (2008) and Praveen et al. (2011) who used cell lines, which divide more often and, therefore, experience nuclear membrane breakdown



regularly. The fact that electroporation can transfect both primary cells and cell lines, on the other hand, may mean that it not only perturbs the membrane, but it may also permeabilise the nuclear membrane, thereby allowing the plasmid DNA to enter the nucleus and be expressed. As far as I am aware, there is no study yet, confirming this.

In chapter 6, I demonstrated a novel application of optoporation by introducing impermeable small molecules that included fluorescent DNA intercalating dyes (DAPI and PI) and potent histone demethylase inhibitors, with the latter having important applications in drug development. The chemical compounds 8HQ and FMF1293 were optoporated into vital HeLa cells to inhibit H3K27 demethylation by the demethylase JMJD3. These drugs are impermeable unless used at high concentrations, which may result in toxicity and also an off-target effect. This is the first time that optoporation had been used to introduce these potent compounds into cells at lower concentrations of just 20  $\mu$ M. Both 8HQ and FMF1293 were able to inhibit demethylation in JMJD3-overexpressing cells where optoporation had taken place. In contrast, without using optoporation, concentrations as high as 86  $\mu$ M (King et al., 2010) for 8HQ were required just to elicit a 50% response. Prior to this experiment, FMF1293 has not been shown to successfully inhibit JMJD3. Indeed in this thesis, FMF1293 had shown an inhibitory effect on JMJD3 albeit at a lower degree than 8HQ.

The limitation of using optoporation for this application, however, is the short temporal window of the order of seconds/minutes given to the compounds to enter the cell before the pore reseals. In viable optoporated cells, the pore has been observed to seal in 10 minutes or less and this has been confirmed by Kurata et al. (1986), Mohanty et al. (2003) and Peng et al. (2007). After the pore seals, there is virtually no further entry of the drug. Metabolism or exocytosis may, over time, remove any existing drug out of the cell, thus allowing demethylation to continue. It may be because of this reason that the drug was less effective after an interval of 18 hours between optoporation to the time of fixing. If the experiment time was shortened so that cells were fixed

just 6 hours after optoporation, they actually displayed significantly different trimethyl marks compared to the JMJD3-overexpressing control samples not optoporated with drugs. Thus, my thesis had demonstrated that optoporation had markedly increased the permeability of the drugs.

Biological molecules can be metabolised or exocytosed over time. Thus, if a small dosage of a low potent drug is optoporated into a cell, the drug may be metabolised even before it has the chance to function. This problem may be overcome if drugs are designed to delay metabolism/exocytosis so as to sustain efficacy. To do this, it would be useful to study the rate of metabolism of compounds and proteins after they entered the cells. One way is to use optoporation to introduce a finite quantity of a compound into cells. After the pore reseals, spontaneous uptake of more impermeable compound into cells is minimal and will, therefore, allow more accurate metabolic rate measurements to be taken of the starting quantity of drug. Radiolabelling of a chemical compound or protein to measure its amount in cells was discussed in section 6.5.

Chapter 7 extended the work with small molecules by showing that proteins such as BSA, phalloidin and novel synthetic antibodies can also be introduced into living cells by optoporation. Cells optoporated with the actin antibody quickly died. Rizk et al. (2009) confirms that the antibody is toxic and has been known to bundle actin filaments together, which may disrupt membrane healing. Although optoporation of a toxic actin-antibody had been demonstrated, it may also be possible to deliver other antibodies into living cells to act as a biological probe to study a particular biological function. Proteins are normally impermeable to cell membranes due to their size and negative charge. Attempts have been made to overcome this by fusing proteins with cell permeating peptides such as Trans-Activator of Transcription (Tat) from the human immunodeficiency virus (HIV-1) (Caron et al., 2001, Frankel et al., 1998, Chen et al., 1995), histone proteins (Kaouass et al., 2006) and arginine rich peptides (Futaki et al., 2001). Recently, Naturally Supercharged Human Proteins (NSHPs) have been shown to be

efficient due to their inherent positive charge (Cronican et al., 2010 & 2011), but these were demonstrated to have an affinity for extracellular matrices, thus, rendering them useless in tissues. Optoporation, however, has the advantage in that it can selectively introduce proteins into cells regardless of charge and into tissue without the need for fusion with NSHPs. It is important to stress that cells would traditionally need to be fixed and permeated, whereas this is not so with optoporation. Hence, this offers the possibility of introducing antibodies as reporter systems into living single cells to track phenotypical changes as well as to monitor cell connections and signalling between cells over time. Rizk et al. (2009) were able to deliver proteins selectively into cells, however, this still required conjugation of the protein to a neuropeptide. Furthermore, the conjugated protein could only be internalised in cells which expressed certain receptors. My thesis, however, has shown that both toxic and non-toxic proteins as large as 66 kDa could be selectively optoporated into cells.

A method for automatically identifying iPS and ES-like colonies was presented in chapter 8. Fuzzy logic control was used to perform rationale analytics based on the shape, size, and eccentricity of the segmented colonies. Using this method, the algorithm was able to identify 'highly-certain' and 'partially-certain' colonies, while discarding colonies which showed signs of differentiation. The algorithm has the further advantage of being able to identify stem cell colonies non-invasively and rapidly. When tested on several tile-scanned images, the sensitivity was excellent at 95.0 - 98.5% with only a few false negatives. Other automated image-based systems show accuracies of 96.7% (Mangoubi et al., 2007) and 91- 94% (Bottigli et al., 2007 ; Masala et al., 2007). The computation times were not reported, however. As mentioned in section 8.6, my algorithm needed only 11.5 seconds to search a 5 x 5 tile-scanned image and found more colonies than when done manually by an expert, who took 5 – 10 minutes.

As noted in this thesis, optoporation, unlike electroporation, is highly manual and time-consuming since cells are permeated individually. In chapter 9, I demonstrated the use of image

processing techniques to automatically locate cells and identify suitable sites for irradiation. Two strategies were employed: the first involved optoporation directly on the membrane, and the second targeting on the glass substrate (shockwave). The shockwave method gave optimal results and was more tolerant towards sub-accurate segmentation caused by out-of-focus imaging. Furthermore, because two-photon lasers excite only a femtolitre volume, they must be precisely focused, hence this poses as the main limiting factor to increasing optoporation throughput (Tsampoula et al., 2007 ; Stevenson et al., 2010). In addition, due to the limitations of present manufacturing tolerances, the glass-bottom dish was not perfectly flat and level, thus giving rise to out-of-focus problems. To overcome this, I tried modelling the surface of the dish as a plane using 3 points in order to interpolate the focal plane. However, the dish still exhibited both global and local unevenness. Within a small limited area of about 3 fields, the algorithm was able to successfully and automatically locate and optoporate cells with propidium iodide. Current publications refer to the use of a manual ‘point-and-click’ interface (Cizmar et al., 2008) or targeting in a fixed grid-like manner (Clark et al., 2006) to optoporate cells quickly. As far as I know, this thesis is the first publication that demonstrated a system using image processing to define the exact locations of cells, and thus, the beam position of a pulsed femtosecond laser for transfection of primary cells.

## **10.2 Suggestions for Future Work**

### **10.2.1 Improving yield of optoporated demethylase inhibitors**

The use of optoporation as a quick method to screen drugs in development for efficacy is very promising. Although the results presented here are derived from small sample sizes, this thesis lays the groundwork as a proof-of-principle for future development. In order to use optoporation on a routine basis, one would need to be able to transfect plasmids to overexpress demethylases at significantly higher efficiency. This would augment the chances of optoporating a cell which

had earlier been treated to undergo global demethylation. Currently, only about 10 out of 100 optoporated cells provided usable data. A solution would be to use the BacMam system described in chapter 6, which resulted in nearly 95% efficiency, but did not show signs of global demethylation in cells. The BacMam system has been shown to work in another study (Gao et al., 2007) also with a transfection efficiency of nearly 90%. It is not clear why the BacMam system was unable to induce global demethylation in this thesis. The system may require more expertise or optimisation. Alternatively, it may be possible to design a plasmid that encodes for JMJD3 but also fused with a fluorescent protein to be able to identify transfected cells. Even at low transfection efficiencies, having the fluorescent protein would be advantageous as only those cells which express it would be optoporated.

### **10.2.2 Quantifying amount of drug optoporated into cells**

My research has shown that a certain amount of DAPI, PI, 8HQ, and FMF1293 consistently entered the cell after optoporation. However, it has not been possible to accurately quantify them. Despite the use of fluorescently-tagged compounds, constant imaging may induce photobleaching and, consequently, underestimate the actual amount. One can see that there is a variation in the inhibition of JMJD3 using 8HQ and FMF1293. Therefore, the dosage in each cell by optoporation may be variable. Radiolabelling the compounds may allow quantification of the actual amount of drug that enters each cell. This technique has been described in more detail in section 6.5.

### **10.2.3 Improving reliability of automated optoporation system**

The automated optoporation system did not provide good yield because of the need to constantly re-focus the image. Future work may include the use of a Bessel beam (Tsampoula et al., 2007),

where the power is uniformly distributed along the length of the beam, thereby overcoming the aforementioned problem. The addition of the Bessel beam appears to be compatible with my system since Tsampoula et al. (2007) used a similar femtosecond pulsed laser. However, it will still be necessary to achieve accurate focus in order to acquire an image for segmentation. The algorithm proposed in this thesis could be integrated with a laser autofocus routine. This technique relies on the detection of laser reflection, where the peak reflection signal corresponds to reflection from the glass coverslip. This can be used as a reference point, to which a fixed offset can be added to locate the actual focal plane of the cells.

#### **10.2.4 Increasing the accuracy of the colony recognition program**

Although the sensitivity of this technique has been 95.0 - 98.5%, there are still some false positives. I theorise that the measurement of more shape properties may further increase the accuracy. When present in samples under the microscope, dust particles and other foreign contaminants could also be identified as false positives. Because they usually appear as overly bright solid objects, they could be eliminated by analysing their variance in pixel intensity, which would be much lower than in highly textured colonies.

#### **10.2.5 Future experiments**

As shown in this thesis, optoporation delivered FMF1293 into cells where it would have otherwise not been able to perform its function as a histone demethylase inhibitor. This drug, now demonstrated to have a particular effect, will be chemically modified to increase its permeability. Other drugs with similar structures or scaffolds may exist, but thought to have little or no effect in cells due to their poor permeability. Optoporation, as a quick screening method,

may be able to rescue such previously abandoned drugs so that they can be developed further at lower costs and within a shorter time frame.

Optoporation can introduce compounds into 3D matrices of cells and tissues. The two-photon NIR laser used in this thesis already allows for deep scanning through tissue slices because of the absence of NIR absorbers (Zipfel et al., 2003 ; Helmchen & Denk, 2005 ; Christensen & Nedergaard, 2011). It has been shown that two-photon femtosecond lasers can penetrate the tissue by as much as several hundreds of microns and, in some cases, up to 2 mm (Stevenson et al., 2010). By extension, using a NIR laser for optoporation could also permit similar screening experiments, such as those performed in chapter 6, to be done in 3D matrices of cells or even thick tissue slices such as liver samples.

Already, Uchuganova et al. (2008b) have shown that individual pancreatic stem cells can be selectively destroyed by laser treatment. By extension, embryonic and induced pluripotent stem cells could also be optoporated to deliver plasmids, proteins, or chemical compounds in order to induce differentiation into a particular lineage. Such stem cells can be differentiated in the form of 3D structures, termed embryoid bodies (Martin & Evans, 1975). Bratt-Leal et al. (2009) have recognised that only the exterior of the embryoid bodies come into contact with differentiation cues. Optoporation with a NIR laser could be used to deliver such signals into cells deep inside the structure. More recently, stem cells are also being cultured and differentiated in matrices made from collagen (Hwang et al., 2007), nanofibrous poly-lactide (Xie et al., 2011), and dextran-based hydrogels (Ferreira et al., 2007) to mimic actual physiological conditions more closely. Similarly, the long penetration depth of a NIR laser could allow for optoporation of cells embedded inside these scaffolds.

*In vivo* optoporation is possible with a NIR laser coupled with a high-resolution confocal microscope. Zeira et al. (2007) have already demonstrated that they can safely introduce plasmid DNA into the dermis of mice. Furthermore, Satoh et al. (2005) have optoporated GFP plasmid



into the central nervous system of mice. Hence, it may also be possible to use optoporation to perform *in vivo* drug testing. This is presently done *in vitro* on a cell monolayer, which does not mimic actual physiological conditions.

Optoporation also has applications in the study of developmental biology. Kohli et al. (2007) optoporated zebrafish blastomere cells with GFP plasmid. These embryos later went on to successfully develop into functional organisms. Thus, optoporation can be used to generate transgenic animals in less time compared to more traditional techniques, such as microinjection.

Optoporation can be used to study the functionality of gap junctions in connection-forming cells such as tenocytes and osteocytes. Normally, when such cells are chemically or mechanically permeated, their gap junctions are activated to allow small molecules to spread to other cells (Cherian et al., 2005 ; Waggett et al., 2006). Optoporation can be used to permeate a small number of cells in a more controlled manner. The same type of experiments can also be carried out in other cell types such as neurons, which display a wide range of complex connections. Optoporation could be used to introduce molecules into the cell body of a neuron and to determine the speed at which these small molecules are transported down axons and into other cells, similar to the method employed by Barrett et al. (2006). This could help answer basic biological questions as to whether the connections that are formed in neurons and even tenocytes and osteocytes are actually functional.

Finally, optoporation is not limited to the introduction of small and macromolecules into cells. The precision of the laser can be utilised to selectively destroy or deliberately damage, for example, neuron cells (Steinmeyer et al., 2010) to simulate injuries such as spinal cord severing. It has been shown that damaged axons can repair themselves and connections can be re-established (Fawcett & Asher, 1999 ; Horner & Gage, 2000). High resolution timelapse microscopy can be used to understand the effects of apoptosis or cell repair. Such studies have not been done with tenocytes. Furthermore, optoporation in conjunction with timelapse

microscopy may be of help to identify the timeline required for repair and also how it can be further enhanced. In densely packed cells, one could also observe how chemical signals from damaged cells affect the rest of the population.

In conclusion, optoporation clearly cannot compete with traditional transfection methods such as electroporation and lipofection which are able to deliver plasmid DNA into large numbers of cells more quickly. The procedure is also highly manual and time consuming. Having said that, optoporation does, however, have its advantages and niche applications. Its unique strength lies in its ability to selectively deliver small molecules and proteins into specific single cells based on morphology or expression of fluorescence, as this thesis has demonstrated.

# References

- Aasen, T., A. Raya, et al. (2008). "Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes." Nat Biotechnol **26**(11): 1276-1284.
- Arivazhagan, S. L. G. (2003). "Texture segmentation using wavelet transform." Pattern Recognition Letters **24**: 3197-3203.
- Atkinson, H. and R. Chalmers (2010). "Delivering the goods: viral and non-viral gene therapy systems and the inherent limits on cargo DNA and internal sequences." Genetica **138**(5): 485-498.
- Au, P. (2008). "Bone marrow–derived mesenchymal stem cells facilitate engineering of long-lasting functional vasculature." Blood **111**(9): 4551.
- Bannister, A. J., P. Zegerman, et al. (2001). "Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain." Nature **410**(6824): 120-124.
- Barber, P. R., B. Vojnovic, et al. (2001). "Automated counting of mammalian cell colonies." Phys Med Biol **46**(1): 63-76.
- Barrett, L. E., J. Y. Sul, et al. (2006). "Region-directed phototransfection reveals the functional significance of a dendritically synthesized transcription factor." Nat Methods **3**(6): 455-460.
- Barski, A., S. Cuddapah, et al. (2007). "High-resolution profiling of histone methylations in the human genome." Cell **129**(4): 823-837.
- Baumgart, J., W. Bintig, et al. (2008). "Quantified femtosecond laser based opto-perforation of living GFSHR-17 and MTH53 a cells." Opt Express **16**(5): 3021-3031.
- Bennett, B. D., T. L. Jetton, et al. (1996). "Quantitative subcellular imaging of glucose metabolism within intact pancreatic islets." J Biol Chem **271**(7): 3647-3651.
- Bestor, T. H. (2000). "The DNA methyltransferases of mammals." Hum Mol Genet **9**(16): 2395-2402.
- Boerboom, R. A., K. N. Krahn, et al. (2007). "High resolution imaging of collagen organisation and synthesis using a versatile collagen specific probe." J Struct Biol **159**(3): 392-399.
- Bottigli, U. M. C., Fiori, P. L., Golosio, B., Marras, A., Masala, G. L., and Oliva, P. (2007). "A New Automatic System of Cell Colony Counting." International Journal of Mathematical and Computer Sciences **3**(4): 5.

- Bradley, A., M. Evans, et al. (1984). "Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines." Nature **309**(5965): 255-256.
- Brambrink, T., R. Foreman, et al. (2008). "Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells." Cell Stem Cell **2**(2): 151-159.
- Bratt-Leal, A. M., R. L. Carpenedo, et al. (2009). "Engineering the embryoid body microenvironment to direct embryonic stem cell differentiation." Biotechnol Prog **25**(1): 43-51.
- Brezenoff, H. E. and D. J. Jenden (1969). "Modification of arterial blood pressure in rats following microinjection of drugs into the posterior hypothalamus." Int J Neuropharmacol **8**(6): 593-600.
- Brown, E. B., E. S. Wu, et al. (1999). "Measurement of molecular diffusion in solution by multiphoton fluorescence photobleaching recovery." Biophys J **77**(5): 2837-2849.
- Brown, C. T. A., D. J. Stevenson, et al. (2008). "Enhanced operation of femtosecond lasers and applications in cell transfection." Journal of Biophotonics **1**(3): 183-199.
- Buehler, C., K. H. Kim, et al. (1999). "Innovations in two-photon deep tissue microscopy." IEEE Eng Med Biol Mag **18**(5): 23-30.
- Burkel, B. M., G. von Dassow, et al. (2007). "Versatile fluorescent probes for actin filaments based on the actin-binding domain of utrophin." Cell Motil Cytoskeleton **64**(11): 822-832.
- Buttery, L. D., S. Bourne, et al. (2001). "Differentiation of osteoblasts and in vitro bone formation from murine embryonic stem cells." Tissue Eng **7**(1): 89-99.
- Cai, J., W. Li, et al. (2010). "Generation of human induced pluripotent stem cells from umbilical cord matrix and amniotic membrane mesenchymal cells." J Biol Chem **285**(15): 11227-11234.
- Campagnola, P. J., A. C. Millard, et al. (2002). "Three-dimensional high-resolution second-harmonic generation imaging of endogenous structural proteins in biological tissues." Biophys J **82**(1 Pt 1): 493-508.
- Capecchi, M. R. (1980). "High efficiency transformation by direct microinjection of DNA into cultured mammalian cells." Cell **22**(2 Pt 2): 479-488.
- Cappelleri, D. J., A. Halasz, et al. (2010). "Towards A Fully Automated High-Throughput Phototransfection System." JALA Charlottesville Va **15**(4): 329-341.
- Caron, N. J., Y. Torrente, et al. (2001). "Intracellular delivery of a Tat-eGFP fusion protein into muscle cells." Mol Ther **3**(3): 310-318.
- Chakravarty, P., W. Qian, et al. (2010). "Delivery of molecules into cells using carbon nanoparticles activated by femtosecond laser pulses." Nat Nanotechnol **5**(8): 607-611.

- Chambers, I., J. Silva, et al. (2007). "Nanog safeguards pluripotency and mediates germline development." Nature **450**(7173): 1230-1234.
- Chen, L. L., A. D. Frankel, et al. (1995). "Increased cellular uptake of the human immunodeficiency virus-1 Tat protein after modification with biotin." Anal Biochem **227**(1): 168-175.
- Cherian, P. P., A. J. Siller-Jackson, et al. (2005). "Mechanical strain opens connexin 43 hemichannels in osteocytes: a novel mechanism for the release of prostaglandin." Mol Biol Cell **16**(7): 3100-3106.
- Christensen, D. J. and M. Nedergaard (2011). "Two-photon in vivo imaging of cells." Pediatr Nephrol.
- Cizmar, T., V. Kollarova, et al. (2008). "Generation of multiple Bessel beams for a biophotonics workstation." Opt Express **16**(18): 14024-14035.
- Clark, I. B., E. G. Hanania, et al. (2006). "Optoinjection for efficient targeted delivery of a broad range of compounds and macromolecules into diverse cell types." J Biomed Opt **11**(1): 014034.
- Cleary, A. L. (1995). "F-actin redistributions at the division site in living *Tradescantia* stomatal complexes as revealed by microinjection of rhodamine-phalloidin." Protoplasma **185**: 152-165.
- Cooney, C. A., A. A. Dave, et al. (2002). "Maternal methyl supplements in mice affect epigenetic variation and DNA methylation of offspring." J Nutr **132**(8 Suppl): 2393S-2400S.
- Couture, J. F., E. Collazo, et al. (2007). "Specificity and mechanism of JMJD2A, a trimethyllysine-specific histone demethylase." Nat Struct Mol Biol **14**(8): 689-695.
- Cox, G., E. Kable, et al. (2003). "3-dimensional imaging of collagen using second harmonic generation." J Struct Biol **141**(1): 53-62.
- Cronican, J. J., D. B. Thompson, et al. (2010). "Potent delivery of functional proteins into Mammalian cells in vitro and in vivo using a supercharged protein." ACS Chem Biol **5**(8): 747-752.
- Cronican, J. J., K. T. Beier, et al. (2011). "A Class of Human Proteins that Deliver Functional Proteins into Mammalian Cells In Vitro and In Vivo." Chem Biol **18**(7): 833-838.
- Dahle, J., M. Kakar, et al. (2004). "Automated counting of mammalian cell colonies by means of a flat bed scanner and image processing." Cytometry A **60**(2): 182-188.
- Davidson, S., H. Truong, et al. (2010). "A microinjection technique for targeting regions of embryonic and neonatal mouse brain in vivo." Brain Res **1307**: 43-52.
- Denk, W., J. H. Strickler, et al. (1990). "Two-photon laser scanning fluorescence microscopy." Science **248**(4951): 73-76.

- Dhalluin, C., J. E. Carlson, et al. (1999). "Structure and ligand of a histone acetyltransferase bromodomain." Nature **399**(6735): 491-496.
- Eminli, S., A. Foudi, et al. (2009). "Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells." Nat Genet **41**(9): 968-976.
- Esteban, M. A., T. Wang, et al. (2010). "Vitamin C enhances the generation of mouse and human induced pluripotent stem cells." Cell Stem Cell **6**(1): 71-79.
- Esteller, M. (2005). "Aberrant DNA methylation as a cancer-inducing mechanism." Annu Rev Pharmacol Toxicol **45**: 629-656.
- Fawcett, J. W. and R. A. Asher (1999). "The glial scar and central nervous system repair." Brain Res Bull **49**(6): 377-391.
- Feinberg, A. P. and B. Vogelstein (1983). "Hypomethylation distinguishes genes of some human cancers from their normal counterparts." Nature **301**(5895): 89-92.
- Felsenfeld, G. and M. Groudine (2003). "Controlling the double helix." Nature **421**(6921): 448-453.
- Feng, B., J. H. Ng, et al. (2009). "Molecules that promote or enhance reprogramming of somatic cells to induced pluripotent stem cells." Cell Stem Cell **4**(4): 301-312.
- Ferreira, L. S., S. Gerecht, et al. (2007). "Bioactive hydrogel scaffolds for controllable vascular differentiation of human embryonic stem cells." Biomaterials **28**(17): 2706-2717.
- Fish, E. W., D. Shahrokh, et al. (2004). "Epigenetic programming of stress responses through variations in maternal care." Ann N Y Acad Sci **1036**: 167-180.
- Fraichard, A. (1995). "In vitro differentiation of embryonic stem cells into glial cells and functional neurons." Journal of cell science **108**(10): 3181.
- Frankel, A. D. and C. O. Pabo (1988). "Cellular uptake of the tat protein from human immunodeficiency virus." Cell **55**(6): 1189-1193.
- Franken, P. A. (1961). "Generation of optical harmonics." Physical Review Letters **7**(4): 118.
- Futaki, S., T. Suzuki, et al. (2001). "Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery." J Biol Chem **276**(8): 5836-5840.
- Gerger, A., R. Hofmann-Wellenhof, et al. (2008). "In vivo confocal laser scanning microscopy of melanocytic skin tumours: diagnostic applicability using unselected tumour images." Br J Dermatol **158**(2): 329-333.
- Giorgetti, A., N. Montserrat, et al. (2009). "Generation of induced pluripotent stem cells from human cord blood using OCT4 and SOX2." Cell Stem Cell **5**(4): 353-357.

- Glover, D. J., D. L. Leyton, et al. (2010). "The efficiency of nuclear plasmid DNA delivery is a critical determinant of transgene expression at the single cell level." J Gene Med **12**(1): 77-85.
- Gonzalez, F., M. Barragan Monasterio, et al. (2009). "Generation of mouse-induced pluripotent stem cells by transient expression of a single nonviral polycistronic vector." Proc Natl Acad Sci U S A **106**(22): 8918-8922.
- Göppert-Mayer, M. (1931). "Über elementarakte mit zwei quantensprüngen." Annalen der Physik **401**(3): 21
- Gordon, J. W., G. A. Scangos, et al. (1980). "Genetic transformation of mouse embryos by microinjection of purified DNA." Proc Natl Acad Sci U S A **77**(12): 7380-7384.
- Graessmann, M. and A. Graessman (1976). ""Early" simian-virus-40-specific RNA contains information for tumor antigen formation and chromatin replication." Proc Natl Acad Sci U S A **73**(2): 366-370.
- Hayden, E. C. (2011). "Stem cells: The growing pains of pluripotency." Nature **473**(7347): 272-274.
- Hebbes, T. R., A. W. Thorne, et al. (1988). "A direct link between core histone acetylation and transcriptionally active chromatin." EMBO J **7**(5): 1395-1402.
- Helmchen, F. and W. Denk (2005). "Deep tissue two-photon microscopy." Nat Methods **2**(12): 932-940.
- Henderson, J. K., J. S. Draper, et al. (2002). "Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens." Stem Cells **20**(4): 329-337.
- Hockemeyer, D., F. Soldner, et al. (2009). "Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases." Nat Biotechnol **27**(9): 851-857.
- Holliday, R. (1994). "Epigenetics: an overview." Dev Genet **15**(6): 453-457.
- Horner, P. J. and F. H. Gage (2000). "Regenerating the damaged central nervous system." Nature **407**(6807): 963-970.
- Huang, P., Z. He, et al. (2011). "Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors." Nature.
- Huangfu, D., R. Maehr, et al. (2008). "Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds." Nat Biotechnol **26**(7): 795-797.
- Huangfu, D., K. Osafune, et al. (2008). "Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2." Nat Biotechnol **26**(11): 1269-1275.
- Huff, T. B., L. Tong, et al. (2007). "Hyperthermic effects of gold nanorods on tumor cells." Nanomedicine (Lond) **2**(1): 125-132.



- Hwang, N. S., S. Varghese, et al. (2007). "Cartilage tissue engineering: Directed differentiation of embryonic stem cells in three-dimensional hydrogel culture." Methods Mol Biol **407**: 351-373.
- Jia, F., K. D. Wilson, et al. (2010). "A nonviral minicircle vector for deriving human iPS cells." Nat Methods **7**(3): 197-199.
- Jiang, X., J. Zhong, et al. (2011). "Two-photon fluorescence and second-harmonic generation imaging of collagen in human tissue based on multiphoton microscopy." Scanning **33**(1): 53-56.
- Kaji, K., K. Norrby, et al. (2009). "Virus-free induction of pluripotency and subsequent excision of reprogramming factors." Nature **458**(7239): 771-775.
- Kaouass, M., R. Beaulieu, et al. (2006). "Histonefection: Novel and potent non-viral gene delivery." J Control Release **113**(3): 245-254.
- Kohli, V., V. Robles, et al. (2007). "An alternative method for delivering exogenous material into developing zebrafish embryos." Biotechnol Bioeng **98**(6): 1230-1241.
- Kim, D., C. H. Kim, et al. (2009). "Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins." Cell Stem Cell **4**(6): 472-476.
- Kim, K., A. Doi, et al. (2010). "Epigenetic memory in induced pluripotent stem cells." Nature **467**(7313): 285-290.
- King, O. N., X. S. Li, et al. (2010). "Quantitative high-throughput screening identifies 8-hydroxyquinolines as cell-active histone demethylase inhibitors." PLoS One **5**(11): e15535.
- Koller, M. R., E. G. Hanania, et al. (2004). "High-throughput laser-mediated in situ cell purification with high purity and yield." Cytometry A **61**(2): 153-161.
- Kornberg, R. D. (1974). "Chromatin structure: a repeating unit of histones and DNA." Science **184**(139): 868-871.
- Kornberg, R. D. and Y. Lorch (1999). "Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome." Cell **98**(3): 285-294.
- Kouzarides, T. (2002). "Histone methylation in transcriptional control." Curr Opin Genet Dev **12**(2): 198-209.
- Kouzarides, T. (2007). "Chromatin modifications and their function." Cell **128**(4): 693-705.
- Kurata, S. I. (1986). "The laser method for efficient introduction of foreign DNA into cultured cells\* 1." Experimental cell research **162**(2): 372.
- Lee, J., J. R. Thompson, et al. (2008). "Distinct binding modes specify the recognition of methylated histones H3K4 and H4K20 by JMJD2A-tudor." Nat Struct Mol Biol **15**(1): 109-111.
- Lei, M., H. Xu, et al. (2008). "Femtosecond laser-assisted microinjection into living neurons." J Neurosci Methods **174**(2): 215-218.

- Li, C., J. Zhou, et al. (2009). "Pluripotency can be rapidly and efficiently induced in human amniotic fluid-derived cells." Hum Mol Genet **18**(22): 4340-4349.
- Lister, R., M. Pelizzola, et al. (2011). "Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells." Nature **471**(7336): 68-73.
- Lowry, W. E., L. Richter, et al. (2008). "Generation of human induced pluripotent stem cells from dermal fibroblasts." Proc Natl Acad Sci U S A **105**(8): 2883-2888.
- Lu, C. S. (1997). "Unsupervised texture segmentation via wavelet transform." Pattern recognition **30**(5): 729.
- Lu, V. B., D. J. Williams, et al. (2009). "Intranuclear microinjection of DNA into dissociated adult mammalian neurons." J Vis Exp(34).
- Luger, K., A. W. Mader, et al. (1997). "Crystal structure of the nucleosome core particle at 2.8 Å resolution." Nature **389**(6648): 251-260.
- Maherali, N., R. Sridharan, et al. (2007). "Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution." Cell Stem Cell **1**(1): 55-70.
- Maherali, N. and K. Hochedlinger (2008). "Guidelines and techniques for the generation of induced pluripotent stem cells." Cell Stem Cell **3**(6): 595-605.
- Maherali, N., T. Ahfeldt, et al. (2008). "A high-efficiency system for the generation and study of human induced pluripotent stem cells." Cell Stem Cell **3**(3): 340-345.
- Maiti, S., J. B. Shear, et al. (1997). "Measuring serotonin distribution in live cells with three-photon excitation." Science **275**(5299): 530-532.
- Mali, P., B. K. Chou, et al. (2010). "Butyrate greatly enhances derivation of human induced pluripotent stem cells by promoting epigenetic remodeling and the expression of pluripotency-associated genes." Stem Cells **28**(4): 713-720.
- Mali, P., Z. Ye, et al. (2008). "Improved efficiency and pace of generating induced pluripotent stem cells from human adult and fetal fibroblasts." Stem Cells **26**(8): 1998-2005.
- Marchington, R. F., Y. Arita, et al. (2010). "Optical injection of mammalian cells using a microfluidic platform." Biomed Opt Express **1**(2): 527-536.
- Martin, C. and Y. Zhang (2005). "The diverse functions of histone lysine methylation." Nat Rev Mol Cell Biol **6**(11): 838-849.
- Martin, G. R. and M. J. Evans (1975). "Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies in vitro." Proc Natl Acad Sci U S A **72**(4): 1441-1445.
- Masala, G. L., Brunetti, U. B., Carpinelli, M., Diaz, N., Fiori, P. L., Golosio, B., Oliva, P., and Stegel, G. (2007). "Automatic cell colony counting by region-growing approach." Il Nuovo cimento: 1.

- Masters, B. R. (1998). "Optical biopsy of in vivo human skin: Multi-photon excitation microscopy." Lasers in medical science **13**(3): 196.
- Matsuzaki, M. (2011). "Simultaneous two-photon activation of presynaptic cells and calcium imaging in postsynaptic dendritic spines." Neural Systems and Circuits **1**(1): 2.
- Meissner, A., M. Wernig, et al. (2007). "Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells." Nat Biotechnol **25**(10): 1177-1181.
- Mercer, W. E., C. Avignolo, et al. (1984). "Role of the p53 protein in cell proliferation as studied by microinjection of monoclonal antibodies." Mol Cell Biol **4**(2): 276-281.
- Mikkelsen, T. S., J. Hanna, et al. (2008). "Dissecting direct reprogramming through integrative genomic analysis." Nature **454**(7200): 49-55.
- Mitsui, K., Y. Tokuzawa, et al. (2003). "The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells." Cell **113**(5): 631-642.
- Mohanty, S. K., M. Sharma, et al. (2003). "Laser-assisted microinjection into targeted animal cells." Biotechnol Lett **25**(11): 895-899.
- Nagy, K., H. K. Sung, et al. (2011). "Induced pluripotent stem cell lines derived from equine fibroblasts." Stem Cell Rev **7**(3): 693-702.
- Nakagawa, M., M. Koyanagi, et al. (2008). "Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts." Nat Biotechnol **26**(1): 101-106.
- Nakanishi, M., T. S. Hamazaki, et al. (2007). "Pancreatic tissue formation from murine embryonic stem cells in vitro." Differentiation **75**(1): 1-11.
- Narkilahti, S., K. Rajala, et al. (2007). "Monitoring and analysis of dynamic growth of human embryonic stem cells: comparison of automated instrumentation and conventional culturing methods." Biomed Eng Online **6**: 11.
- Ng, S. S., K. L. Kavanagh, et al. (2007). "Crystal structures of histone demethylase JMJD2A reveal basis for substrate specificity." Nature **448**(7149): 87-91.
- Nuckolls, G. H., L. H. Romer, et al. (1992). "Microinjection of antibodies against talin inhibits the spreading and migration of fibroblasts." J Cell Sci **102** ( Pt 4): 753-762.
- O'Connor, M. D., M. D. Kardel, et al. (2008). "Alkaline phosphatase-positive colony formation is a sensitive, specific, and quantitative indicator of undifferentiated human embryonic stem cells." Stem Cells **26**(5): 1109-1116.
- Okita, K., T. Ichisaka, et al. (2007). "Generation of germline-competent induced pluripotent stem cells." Nature **448**(7151): 313-317.
- Okita, K., M. Nakagawa, et al. (2008). "Generation of mouse induced pluripotent stem cells without viral vectors." Science **322**(5903): 949-953.
- Overney, G. (2006). "Phase Contrast Illumination [online]." Retrieved 24-5-11, from <http://www.microscopy-uk.org.uk/mag/artmar06/go-phase.html>.

- Palumbo, G., M. Caruso, et al. (1996). "Targeted gene transfer in eucaryotic cells by dye-assisted laser optoporation." J Photochem Photobiol B **36**(1): 41-46.
- Park, I. H., R. Zhao, et al. (2008). "Reprogramming of human somatic cells to pluripotency with defined factors." Nature **451**(7175): 141-146.
- Patel, D. V. and C. N. McGhee (2009). "In vivo confocal microscopy of human corneal nerves in health, in ocular and systemic disease, and following corneal surgery: a review." Br J Ophthalmol **93**(7): 853-860.
- Paterson, L., B. Agate, et al. (2005). "Photoporation and cell transfection using a violet diode laser." Opt Express **13**(2): 595-600.
- Patterson, J. E., A. Lagutchev, et al. (2005). "Ultrafast dynamics of shock compression of molecular monolayers." Physical Review Letters **94**(1): 015501.
- Peng, C., R. E. Palazzo, et al. (2007). "Laser intensity dependence of femtosecond near-infrared optoinjection." Phys Rev E Stat Nonlin Soft Matter Phys **75**(4 Pt 1): 041903.
- Piston, D. W. (1999). "Imaging living cells and tissues by two-photon excitation microscopy." Trends Cell Biol **9**(2): 66-69.
- Plews, J. R., J. Li, et al. (2010). "Activation of pluripotency genes in human fibroblast cells by a novel mRNA based approach." PLoS One **5**(12): e14397.
- Ploeger, L. S., H. F. Dullens, et al. (2008). "Fluorescent stains for quantification of DNA by confocal laser scanning microscopy in 3-D." Biotech Histochem **83**(2): 63-69.
- Polo, J. M., S. Liu, et al. (2010). "Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells." Nat Biotechnol **28**(8): 848-855.
- Praveen, B. B., D. J. Stevenson, et al. (2011). "Enhancement and optimization of plasmid expression in femtosecond optical transfection." J Biophotonics.
- Ra, H., W. Piyawattanametha, et al. (2008). "Three-dimensional in vivo imaging by a handheld dual-axes confocal microscope." Opt Express **16**(10): 7224-7232.
- Rajadhyaksha, M., M. Grossman, et al. (1995). "In vivo confocal scanning laser microscopy of human skin: melanin provides strong contrast." J Invest Dermatol **104**(6): 946-952.
- Rajadhyaksha, M., S. Gonzalez, et al. (1999). "In vivo confocal scanning laser microscopy of human skin II: advances in instrumentation and comparison with histology." J Invest Dermatol **113**(3): 293-303.
- Rea, S., F. Eisenhaber, et al. (2000). "Regulation of chromatin structure by site-specific histone H3 methyltransferases." Nature **406**(6796): 593-599.
- Rice, J. C. and C. D. Allis (2001). "Histone methylation versus histone acetylation: new insights into epigenetic regulation." Curr Opin Cell Biol **13**(3): 263-273.
- Richon, V. M., S. Emiliani, et al. (1998). "A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases." Proc Natl Acad Sci U S A **95**(6): 3003-3007.

- Rizk, S. S., A. Luchniak, et al. (2009). "An engineered substance P variant for receptor-mediated delivery of synthetic antibodies into tumor cells." Proc Natl Acad Sci U S A **106**(27): 11011-11015.
- Rocheleau, J. V. and D. W. Piston (2003). "Two-photon excitation microscopy for the study of living cells and tissues." Curr Protoc Cell Biol **Chapter 4**: Unit 4 11.
- Satoh, Y., Y. Kanda, et al. (2005). "Targeted DNA transfection into the mouse central nervous system using laser-induced stress waves." J Biomed Opt **10**(6): 060501.
- Scheer, U., H. Hinssen, et al. (1984). "Microinjection of actin-binding proteins and actin antibodies demonstrates involvement of nuclear actin in transcription of lampbrush chromosomes." Cell **39**(1): 111-122.
- Scheper, W. and S. Copray (2009). "The molecular mechanism of induced pluripotency: a two-stage switch." Stem Cell Rev **5**(3): 204-223.
- Schneckenburger, H., A. Hendinger, et al. (2002). "Laser-assisted optoporation of single cells." J Biomed Opt **7**(3): 410-416.
- Shi, Y., C. Desponts, et al. (2008a). "Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds." Cell Stem Cell **3**(5): 568-574.
- Shi, Y., J. T. Do, et al. (2008b). "A combined chemical and genetic approach for the generation of induced pluripotent stem cells." Cell Stem Cell **2**(6): 525-528.
- Shirahata, Y., N. Ohkohchi, et al. (2001). "New technique for gene transfection using laser irradiation." J Investig Med **49**(2): 184-190.
- Shivashankar, S. (2006). "Texture Classification using Wavelet Packet Decomposition." ICGST International Journal on Graphics, Vision and Image Processing **6**(2): 4.
- Shotton, D. and N. White (1989). "Confocal scanning microscopy: three-dimensional biological imaging." Trends Biochem Sci **14**(11): 435-439.
- Sisan, D. R., R. Arevalo, et al. (2006). "Spatially resolved fluorescence correlation spectroscopy using a spinning disk confocal microscope." Biophys J **91**(11): 4241-4252.
- So, P., H. Kim, et al. (1998). "Two-Photon deep tissue ex vivo imaging of mouse dermal and subcutaneous structures." Opt Express **3**(9): 339-350.
- So, P. T., C. Y. Dong, et al. (2000). "Two-photon excitation fluorescence microscopy." Annu Rev Biomed Eng **2**: 399-429.
- Soughayer, J. S., T. Krasieva, et al. (2000). "Characterization of cellular optoporation with distance." Anal Chem **72**(6): 1342-1347.
- Squirrel, J. M., D. L. Wokosin, et al. (1999). "Long-term two-photon fluorescence imaging of mammalian embryos without compromising viability." Nat Biotechnol **17**(8): 763-767.

- Squirrell, J. M., R. D. Schramm, et al. (2003). "Imaging mitochondrial organization in living primate oocytes and embryos using multiphoton microscopy." Microsc Microanal **9**(3): 190-201.
- Stadtfield, M., M. Nagaya, et al. (2008). "Induced pluripotent stem cells generated without viral integration." Science **322**(5903): 945-949.
- Steinmeyer, J. D., C. L. Gilleland, et al. (2010). "Construction of a femtosecond laser microsurgery system." Nat Protoc **5**(3): 395-407.
- Stephens, D. J. and V. J. Allan (2003). "Light microscopy techniques for live cell imaging." Science **300**(5616): 82-86.
- Stevenson, D., B. Agate, et al. (2006). "Femtosecond optical transfection of cells: viability and efficiency." Opt Express **14**(16): 7125-7133.
- Stevenson, D. J., F. J. Gunn-Moore, et al. (2010). "Single cell optical transfection." J R Soc Interface **7**(47): 863-871.
- Stracke, F., I. Rieman, et al. (2005). "Optical nanoinjection of macromolecules into vital cells." J Photochem Photobiol B **81**(3): 136-142.
- Strahl, B. D. and C. D. Allis (2000). "The language of covalent histone modifications." Nature **403**(6765): 41-45.
- Tachibana, M., J. Ueda, et al. (2005). "Histone methyltransferases G9a and GLP form heteromeric complexes and are both crucial for methylation of euchromatin at H3-K9." Genes Dev **19**(7): 815-826.
- Takahashi, K. and S. Yamanaka (2006). "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors." Cell **126**(4): 663-676.
- Takahashi, K., K. Tanabe, et al. (2007). "Induction of pluripotent stem cells from adult human fibroblasts by defined factors." Cell **131**(5): 861-872.
- Talbot, C. B., J. McGinty, et al. (2008). "High speed unsupervised fluorescence lifetime imaging confocal multiwell plate reader for high content analysis." J Biophotonics **1**(6): 514-521.
- Tao, W., J. Wilkinson, et al. (1987). "Direct gene transfer into human cultured cells facilitated by laser micropuncture of the cell membrane." Proc Natl Acad Sci U S A **84**(12): 4180-4184.
- Tayeb, K. (2010). "Generation of human induced pluripotent stem cells by simple transient transfection of plasmid DNA encoding reprogramming factors." BMC developmental biology **10**(1): 81.
- Thomson, J. A. (1998). "Embryonic stem cell lines derived from human blastocysts." Science **282**(5391): 1145.
- Tirlapur, U. K. and K. Konig (2002). "Targeted transfection by femtosecond laser." Nature **418**(6895): 290-291.

- Tirlapur, U. K., W. J. Mulholland, et al. (2006). "Femtosecond two-photon high-resolution 3D imaging, spatial-volume rendering and microspectral characterization of immunolocalized MHC-II and mLangerin/CD207 antigens in the mouse epidermis." Microsc Res Tech **69**(10): 767-775.
- Tsampoula, X. (2007). "Femtosecond cellular transfection using a nondiffracting light beam." Applied physics letters **91**: 053902.
- Tsukakoshi, M. (1984). "A novel method of DNA transfection by laser microbeam cell surgery." Applied physics. B, Lasers and optics **35**(3): 135.
- Turner, J. G. (2004). Human topoisomerase II alpha nuclear export is mediated by two Crm-1 dependent nuclear export signal, Thesis and Dissertations. Paper 1276, University of South Florida.
- Uchugonova, A., K. Konig, et al. (2008a). "Targeted transfection of stem cells with sub-20 femtosecond laser pulses." Opt Express **16**(13): 9357-9364.
- Uchugonova, A., A. Iseman, et al. (2008b). "Optical knock out of stem cells with extremely ultrashort femtosecond laser pulses." J Biophotonics **1**(6): 463-469.
- Valster, A. H., E. S. Pierson, et al. (1997). "Probing the Plant Actin Cytoskeleton during Cytokinesis and Interphase by Profilin Microinjection." Plant Cell **9**(10): 1815-1824.
- Waggett, A. D., M. Benjamin, et al. (2006). "Connexin 32 and 43 gap junctions differentially modulate tenocyte response to cyclic mechanical load." Eur J Cell Biol **85**(11): 1145-1154.
- Wang, W., X. Liu, et al. (2007). "A fully automated robotic system for microinjection of zebrafish embryos." PLoS One **2**(9): e862.
- Wang, K.-A., H.-H. Lin, et al. (2008). "Implementation of an image retrieval system using wavelet decomposition and gradient variation." W. Trans. on Comp. **7**(6): 724-734.
- Warren, L., P. D. Manos, et al. (2010). "Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA." Cell Stem Cell **7**(5): 618-630.
- Welstead, G. G., P. Schorderet, et al. (2008). "The reprogramming language of pluripotency." Curr Opin Genet Dev **18**(2): 123-129.
- Wernig, M., C. J. Lengner, et al. (2008). "A drug-inducible transgenic system for direct programming of multiple somatic cell types." Nat Biotechnol **26**(8): 916-924.
- Wilke, M., E. Fortunati, et al. (1996). "Efficacy of a peptide-based gene delivery system depends on mitotic activity." Gene Ther **3**(12): 1133-1142.
- Williams, R. M., W. R. Zipfel, et al. (2005). "Interpreting second-harmonic generation images of collagen I fibrils." Biophys J **88**(2): 1377-1386.



- Wilson, K. and K. Goulding (1986). A Biologist's Guide to Principles and Techniques of Practical Biochemistry. London, Hodder and Stoughton.
- Xie, C., J. Hu, et al. (2011). "Three-dimensional growth of iPS cell-derived smooth muscle cells on nanofibrous scaffolds." Biomaterials **32**(19): 4369-4375.
- Yakubov, E., G. Rechavi, et al. (2010). "Reprogramming of human fibroblasts to pluripotent stem cells using mRNA of four transcription factors." Biochem Biophys Res Commun **394**(1): 189-193.
- Yamaguchi, A. (2008). "Nanoparticle injection to single animal cells using femtosecond laser-induced impulsive force." Applied physics. A, Materials science & processing **93**(1): 39.
- Yao, C. P., Z. X. Zhang, et al. (2008). "Laser-based gene transfection and gene therapy." IEEE Trans Nanobioscience **7**(2): 111-119.
- Yoshida, M., M. Kijima, et al. (1990). "Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A." J Biol Chem **265**(28): 17174-17179.
- Yu, J., M. A. Vodyanik, et al. (2007). "Induced pluripotent stem cell lines derived from human somatic cells." Science **318**(5858): 1917-1920.
- Zeira, E., A. Manevitch, et al. (2003). "Femtosecond infrared laser-an efficient and safe in vivo gene delivery system for prolonged expression." Mol Ther **8**(2): 342-350.
- Zeira, E., A. Manevitch, et al. (2007). "Femtosecond laser: a new intradermal DNA delivery method for efficient, long-term gene expression and genetic immunization." FASEB J **21**(13): 3522-3533.
- Zhang, J. "Identification of Animal Fibers with Wavelet Texture Analysis." Lecture notes in engineering and computer science **2183**.
- Zhang, Y. and L. C. Yu (2008). "Microinjection as a tool of mechanical delivery." Curr Opin Biotechnol **19**(5): 506-510.
- Zhao, T., Z. N. Zhang, et al. (2011). "Immunogenicity of induced pluripotent stem cells." Nature **474**(7350): 212-215.
- Zhou, W. and C. R. Freed (2009). "Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells." Stem Cells **27**(11): 2667-2674.
- Zipfel, W. R., R. M. Williams, et al. (2003). "Nonlinear magic: multiphoton microscopy in the biosciences." Nat Biotechnol **21**(11): 1369-1377.

