OPTO-PORATION AND DIRECT UPTAKE OF HIS-TAT-NLS-CRE AS SINGLE CELL TRANSFECTION TECHNIQUES FOR CANCER INITIATION

A Thesis

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Masters of Science

by

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ABSTRACT

Two potential methods to excise genes in a single cell by inserting Cre Recombinase into its nucleus are described here. Optical transfection using a femtosecond laser was found to be effective at getting dyes into cells, but because of issues with spontaneous transfection and cell death, was inadequate as a method to introduce plasmids into cells. Direct delivery of His-NLS-TAT Cre with a micro-pipette was able to excise the DNA in a small number of cells without causing significant damage to them. It however was unable to reproducibly localize the location of the cells produced to less than a 600 micron diameter circle on the plate.

BIOGRAPHICAL SKETCH

Jesse graduated from the 2001 class of Buchanan High School in Clovis California with enough college units from passing fourteen Advanced Placement exams to qualify as a Junior. He went on to do his undergraduate work at California State University Fresno graduating in four years with Majors in Chemistry, Physics, and Biology and minors in Mathematics and Economics. While there he spent two summers in the National Science Foundation's Research Experiences for Undergraduates program at IBM's Almaden Research Center doing work on nanotube chemistry, and water-cored waveguides using nanoporous silicon. During these undergraduate years he was also married, divorced, and had a daughter, Makayla Elizabeth Rorabaugh.

Upon completion of this Master’s degree from Cornell University he is seeking employment among energy companies in the San Jose, and San Francisco

ACKNOWLEDGEMENTS

There were many people at Cornell, and elsewhere, I depended upon to be able to finish this research. All of the following people made important contributions to my previous and future success.

The primary person responsible for me finishing this degree is Warren Zipfel. I am not sure I have ever met a more intelligent or patient person. Never have I seen anyone more capable of mastering a huge number of highly complex scientific topics. It is stunning to watch him move flawlessly from conversations about advanced engineering topics, than move seamlessly to talk about biology. There were few if any experiments I ran while at Cornell that did not go exactly as he predicted, regardless of whether my interpretation of all the literature I could find lead me to expect different results. He also was of great assistance in ensuring my time at Cornell will assist my long term career plans: often letting me enroll in courses completely unrelated to my research; and assisting me in my transition both into biomedical engineering, and away from it.

Every member of the Zipfel/Webb group helped me along at some point in my time there. This included Rebecca Williams, Johanna Dela Cruz, Hyungsik Lim, Ina Pavlova, Valerie Anderson, Huimin Chen, Elaine Farkas, Alex Kwan, Jesse McMullen, Evan Spiegal, Paul Lee, Lisa Toth, Mark Williams, Avtar Singh, Sally Kim and Pamela Gordnier. Whenever I found myself faced with a problem I didn't know how to solve, my typical method of solving it was to ask someone on that list for help, than if they didn't know the answer continuing down the list. The background of those people is so diverse that I could count on someone in the lab having been faced with the same issue, and having many good ideas on how to solve it.

Alex Nikitin, and Claudia Fishbach-Teschl who contributed many ideas for this project and served on my committee. Katy Munson, who helped get me started with

the DNA science I needed to do transfection experiments.

Andrea Flesken-Nikitin, who assisted me in learning advanced cell culture techniques. Dave Stevenson, who helped me get the opto-perforation experiments up and running. Xianshu Cui at the Howard Hughes Medical Institute, who supplied me with the cell line I required to run the experiments presented in Chapter 2. My mother, Patricia Rorabaugh, who makes a living making documents look pretty and therefore knows far more about Microsoft Word than I ever will and helped me format this thesis.

The Tax Payers for paying for me to attend Cornell via NIH/NCI R01 CA116583 to W. Zipfel

My daughter Makayla who was demanding enough to make me get my life in order.

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Introduction:

Early cancer development is challenging to observe. In human cancers a tumor often is not found before it shows symptoms. The knowledge of early tumor development therefore must be inferred from: the genetic differences in tumor cells from the surrounding tissues; observations of risk factors leading to cancer such as certain viruses, chemicals, and inflammation[1]; or taken from one of the few cancers with easily observed precancerous states such as cervical[2, 3] and colon cancer[4,5]. The typical model of cancer development that has come out of these studies is that a gene is turned off either by mutation or epigenetic silencing[6, 7, 8], followed by another genic disruption, and possibly another until finally the cell has compiled the 2 or mutations typically required to become malignant[9].

These early steps in cancer development should be able to be modeled in a lab by causing these initial mutations in a single cell either an in vivo or in vitro model. The cell would then either develop into a tumor following the typical route that cells take; or fail to produce a typical tumor providing evidence that there is something incomplete about our understanding tumorigenesis. A negative result could possibly be even more interesting than a positive one as there are still debates over exactly which cells are capable of becoming tumors. Many hypotheses have been proposed where stem cells are the cells that initiate tumors, perhaps tumors are only produced when as a result of stem cell fusion for example[10]. Further experimentation with a negative result could provide evidence for such an alternate method of tumor formation.

In the event a tumor is produced this model could then be applied to studies on any early event in cancer development, including observations on mutations or chromosomal instability in early tumor development[11, 12, 13, 14]. For example, there is still debate as to the timing of chromosomal instability in tumor development[12]. Observations of chromosomes in tumors created with this method could provide

evidence for when it occurs. Another option for what to study could be the changes in genetic expression as the tumor develops. Microarray studies have been done in vivo in both colon[4] and cervical[3] cancer. These experiments however cannot go quite as far back into the development of the tumor as models based off of a single cell method. In all of these cases great care must be taken to ensure the results are similar to those in vivo. This can be done by growing up a tumor to the size of the ones being studied by in vivo models and demonstrating that both those produced by this model, and the natural ones, are similar both morphologically and genetically.

There are many genetic models for cancer that this methodology could be applied to. For these experiments a particularly good one would be an animal model with conditional control of the tumor suppressor genes p53 and Rb[15], such as the mouse model developed by the Laboratory of Dr. Alexander Nikitin. These two tumor suppressor genes have been shown to be involved in many human cancers. In these particular transgenic mice the tumor suppressor genes are flanked by loxP sites so that addition of the protein Cre Recombinase to the cell excises the genes. Some of the Nikitin labs more recent models also turn on a gene for Green Fluorescent Protein (GFP) when the tumor repressor genes are excised.

To turn this idea into a method for studying early cancer development it is necessary to find a way to get Cre into a single cell without damaging the cell. Two methods were investigated: (1) Optical transfection using a femtosecond laser, which is described in Chapter 1; and (2) direct delivery to the cell membrane of a Cre protein modified to cross the membrane without the requirement of pores being created in it, which is described in Chapter 2.

Chapter 1: Optical Transfection with Femtosecond Lasers:

Introduction to Optical Transfection:

In 2002 a paper by Tirlapur and Konig was published in nature[16] demonstrating a new transfection technique utilizing femtosecond laser pulses. To get plasmids containing GFP into Chinese hamster Ovarian (CHO) or rat-kangaroo kidney epithelial (PtK2) cells, the cells were placed in a solution containing plasmid and then a femtosecond laser (~100 fs pulsewidth) was focused through a high Numerical Aperture (NA) lens onto the cell membrane. Laser powers of 50-100mW applied during 16 ms durations were used. The results of these experiments were transfection efficiencies that had previously been unmatched by any previous technique:

> *Irrespective of the cell type, the transfection achieved by this technique was invariably 100%... without any detrimental effects on growth and division, and virtually no cell death or sign of apoptosis*

Though these results managed to draw significant attention to the field—they unfortunately remain the most optimistic assessment of the technique ever made. Still, several different groups have managed to achieve varying levels of success with the technique or modified versions of it.

In 2005, a paper by Dave Stevenson et al published in Optics Express[17] made an attempt at further investigating and developing the method. They used the technique on a total of 4000 CHO cells and widely varied the laser power and pulse time used. This paper found that very different conditions produced the highest transfection rate (~160mW average power, 60 ms optoporation time), and that the highest transfection rate they could get was about half of what the previous nature paper had shown (50% +/-10%). While both papers showed impressive transfection efficiencies it should be noted that there is reason to expect this value was significantly inflated. The procedure followed to determine efficiency was to focus the laser on several cells, wait 48 hours, than count the number of fluorescent cells. They completely ignored the simple fact

that the cells could have divided. In the worse case scenario the cells could have been dividing once every twelve hours. If that was the case, a single cell that had been transfected could result in 16 fluorescent cells. This would reduce the efficiency from 50% down all the way to 3%. That is of course a worst case estimate, and the true value should be expected to lie somewhere between the two values. The Tirlapur paper never mentions any procedure to eliminate the problem of cell division so it is quite possible they also over-estimated their transfection efficiencies because of this.

In February 2008 the most recent, and plausible, estimation of the efficiency of the technique was published in by Baumgart et al[18] This paper side-stepped the question of cell division by focusing mostly on three questions: what percentage of cells uptake membrane impermeable dye, what percentage of cells survive, and how much volume is taken up when a laser is focused on their membrane. They showed using patch-clamps attached to cells while the laser was focused, as well as watching fluorescence as a dye enters, on it that about 40% of the cell volume was exchanged with the media. This is a substantial volume, and unsurprisingly it causes the deaths of many cells subjected to it. The viability and Dye experiments showed that the ideal laser power to use was 40ms and 80mW, where the viability 90% was and the efficiency was 70%. While the efficiency depends on the physical properties of the membrane and laser, the viability could depend strongly on the biology of the cell. It is reasonable to expect that an experiment on only one cell type might not be representative of the technique in all cell types, this could be the explanation for some of my differing results.

One of the big questions being addressed in the literature is whether femtosecond laser transfection can deliver genes to cells while producing little to no cell damage. A paper published in early 2008 tried to address this question in zebra-fish embryos[19]. If femtosecond laser pulses can be focused on cells in early stages of

embryo growth without causing alterations in their development than perhaps it can become widely used. This paper supported this idea by showing that femtosecond laser pulses have no effect on the hatching rates or morphology of zebrafish embryos at any time during the first week of development.

There is also literature trying to alter the laser beam, or use different laser types to improve the technique. Using a laser with pulses on the order of a few hundred femtoseconds does have advantages, but it is not clear that this is the ideal laser system for the application. It is difficult to automate this method of transfection. One of the reasons for this is the laser must be focused to within a couple microns of the cell membrane. Two methods have been reported to try to remove this problem, the use of a continuous wave Violet Diode laser[20], and a Bessel beam from a femtosecond laser, rather than a Gaussian one[21]. While neither of these methods reported higher transfection efficiencies than the traditional beam, it was possible to be significantly out of focus and still get dye or plasmids into the cell. This decreases the issues involved with creating an automated system that can transfect a large number of cells. Neither of these papers reported the creation of such a device, and it is not clear that such a device would be more effective than the simpler method of electroporation, however if an application requiring such a device was found than this would be a significant step towards it. Another, potentially more interesting, method is the use of sub-20 femtosecond lasers[22]. Transfection efficiencies for CHO cells were reported to be 90%, and the method successfully was used to transfect stem cells which can be a challenge to transfect under other conditions. To avoid the overstatement of efficiencies found in earlier papers this one tracked the individual cells to be sure how many times each cell had divided. This makes its results more credible than those previous papers produced. Perhaps this method will prove capable of providing the ultra-high efficiencies femtosecond laser transfection was originally reported to be

capable of producing but that remains to be clearly seen.

Another line of research that has been undertaken is to use a femtosecond laser as a tool in genetic immunization[23]. A paper published in 2007[24] attempted to do this by focusing a femtosecond laser with a lower NA lens (0.45) ~400 microns into the skin of a mouse in an area where a plasmid for Hepatitis B surface antigen had been injected. The paper convincingly demonstrated that using the femtosecond laser increased the immune response of the mouse too the antigen. However it is unclear that their experiment operated on the same physical principal as other papers published in the field. They were using significantly less power than used by other groups (30 mW) for a significantly longer period of time (2-3minutes). Also, and even more surprisingly, the paper reported transfection of cells in culture using 15-30mW, and 2 ms pulse train durations. This is less than half the power used by any other published paper, and an eighth of the optoporation time. These results are a good example of the more difficult aspects of working with laser based transfection methods. There is little to no agreement as to the ideal laser conditions for transfection.

The mechanisms leading to these effects have also been the subject of research. Focusing a laser on a cell leads to chemical, thermal, and mechanical effects. The primary ones leading to pore formation in the cell membrane are the creation of plasma, and the production of a long-lasting bubble that results from heating and tissue dislocation into volatile fragments.[25]

The primary goal in performing the experiments presented here was to determine whether or not optical methods of transfection were appropriate for use in experiments on cancer initiation. Because there is so little agreement on the conditions required to transfect cells by optoporation (Table 1) it was first necessary to determine what conditions work on a particular cell type to work with than primary cells. Than,

if results were encouraging enough, this method could be applied to cancer studies.

Table 1. Conditions reported as optimal by four papers using the same laser type

	Laser Power	Focus Time	Plasmid Concentration	N.A. Of lens
Stevenson(2006)	~170mW	~60ms	12ug/mL	0.85
Baumgart(2008)	80mW	40ms	50ug/mL	0.8
Tirlapur(2002)	50-100mW	16ms	0.1ug/mL	“high”
Zeira(2007)	15-30mW	2ms	1ug/mL	0.45

Experiments were run analogous to both types found in the literature. In some of the experiments the ability of the laser to cause a cell to uptake dye was measured. In others the ability to uptake and express a plasmid was measured. In different experiments different parameters were altered to try to optimize their values, these parameters include: the length of time the laser was focused on the membrane, the power of the laser, the numerical aperture of the lens used, and the concentration of the plasmid in the media.

Methods for Optical Transfection*:*

Experimental setup:

The Experimental setup used was a standard two-photon imaging system and is shown in figure 1. The beam from a titanium sapphire laser is directed through a pockel cell to control its power. Steering mirrors then direct the beam into a biorad scanbox and through an objective. The sample is imaged in transmission mode by placing a photodiode under the sample to create a laser scanned transmission image. In experiments using dyes, a two-photon image of the sample was simultaneously taken.

Computer programs were developed to control the shutter, and pockel cell while transfecting cells. To park the laser beam on a cell, a signal is sent telling the laser beam to stop at a particularly location. The program then opens the shutter at the same time as a voltage is sent to the pockel cell to set the laser power. To ensure the

correct pulse shape the photodiode was attached to an oscilloscope and the reading from the oscilloscope was observed when a laser pulse was created. This showed the shutter can reproducibly open and shut as quickly as 20ms while the pockel cell can go down to 2 us or less. It also found that there was a delay between when the computer sent the shutter a signal, and when the shutter opened. This caused the pockel cell to set the power at a different time from the shutter. Delay times added into the software ensured square waveforms of the correct length for any park time greater than 20ms. To create a pulse less than 20 ms the pockel cell had to be used rather than the shutter. The pockel cell could never quite create a beam of zero power however. This lead to a time after the pulse should have been completed where ~5mW of laser power was still focused on the cell as shown in Figure 2. Since no groups have reported transfection of a cell with less than 15 mW of power it is reasonable to assume that this extra laser power did not lead to a different biological effect than a pulse than a square wave profile.

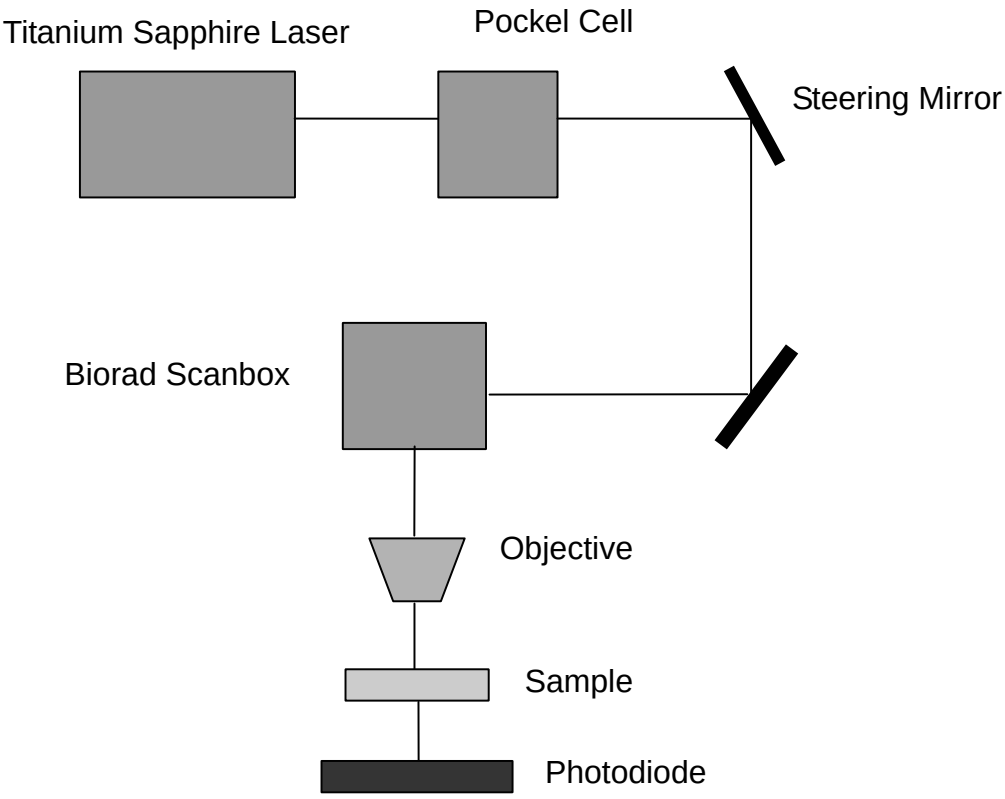


Figure 1. The experimental setup used for optical transfection methods.

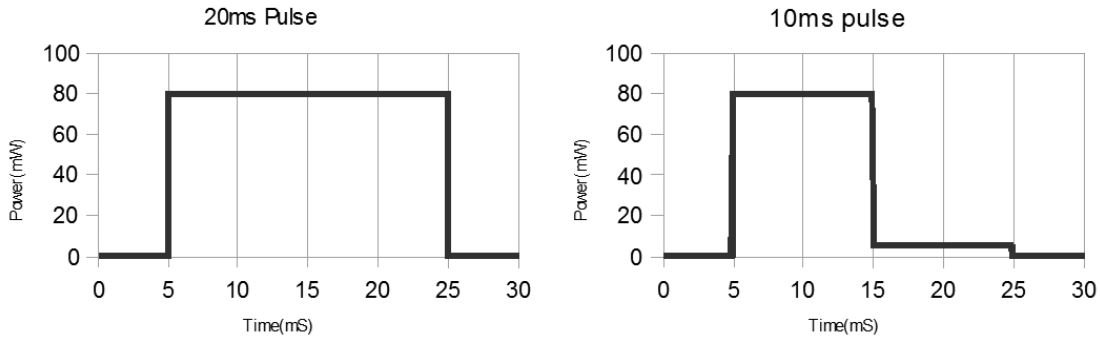


Figure 2. Typical pulse shape of an 80mW pulse greater than, or less than 20ms. The pulse shape is square greater than 20ms, but when a pulse length, t<20ms, is used there is a tail 20-t ms in length where a 5mW output is still present. This is because the pockel cell was not able to block all incoming light as effectively as the shutter.

The system was optimized to the point where transfecting a cell only required moving the microscope stage to the correct location, and pressing three keys on the computer. This allowed for the transfection per cell to be reduced to around twenty seconds. For most laser transfection experiments 25 cells were targeted on a single dish. The total incubator-to-incubator time for the experiment was approximately 15 minutes.

Dye experiments:

To ensure that the laser was in fact producing pores in the cell membrane, experiments were done to get dye into a single cell. Ethidium Bromide proved to be a successful choice of dye. 3T3 cells were first grown up on glass bottomed petri dishes. The media used was DMEM (Gibco 11965) containing 10% Bovine Calf Serum. Media was removed, and 5 mM of dye in Optimem low serum media (Invitrogen 31985**)** was added to the well. A cover slip was placed over the well, and a drop of water for the immersion fluid was placed over the cover slip. The system was set up to have the desired power, and pulse length. Typically 25 cells on a plate had the laser focused on them at the given power and optoporation time.

The dye uptake experiment was repeated on more than 1000 cells covering a wide range of laser focus times and powers. Cells were imaged using a 20X 0.95 NA

lens. Both transmission images from the photodiode, which showed clearly where the cell was and whether a pore had been created, and a two-photon image, which showed whether dye had in fact entered the cell were simultaneously collected.

Plasmid experiments:

These experiments were designed to find the optimal laser parameters to transfect cells. Cells were grown in a glass bottomed petri dish as before. The cells were washed twice in Phosphate-Buffered Saline (PBS) before filling the well with PIRES-eGFP plasmid in serum-free Optimem media. This media has HEPES buffer which is enough to keep the cells viable for the fifteen minutes they are typically kept out of the incubator. In an experiment the laser was focused on 25 cells on a plate for a laser power ranging from 30 – 225 mW, and pulse length of 2 – 200 ms. The cells were then washed twice in media and returned to the incubator. Fluorescent cells, if they were seen, usually became visible within 24 hours, and always within 48 hours.

To try to reduce problems of spontaneous uptake in plasmids two additional sets of experiments on CHO cells were run. The first was to determine if there was a plasmid concentration where plasmid would not be spontaneously taken up by cells. For concentrations of pIRES-eGFP ranging from 0.1μg/mL to 50μg/mL one plate of cells had plasmid added but did not have a laser focused on it, while another had the laser focused on 25 cells while in media containing the plasmid.

Since the uptake of plasmid by cells is proportional to the number of cells on a dish, it was expected that lowering the number of cells on a dish would remove the issue of spontaneous transfection. This would also make it possible to know for sure the cells being observed were the cells that the laser was focused on. Another way of doing this would have been the use of gridded plates, but the grids on these were found to be difficult to image on the two-photon system. Adding 1μL of media containing cells to the center of a plate than letting them settle onto the plate for

twenty minutes created a circle of cells that could be used for this purpose. Now it was possible to be sure the cells being focused on with the laser were the ones subsequently observed; so the experiments where spontaneous transfection were observed were repeated on these plates.

The experiments performed on CHO cells that had resulted in spontaneous transfection were repeated in 3T3 cells at a variety of plasmid concentrations and numerical apertures. This was to try and identify whether spontaneous plasmid uptake was only a problem with certain cell lines.

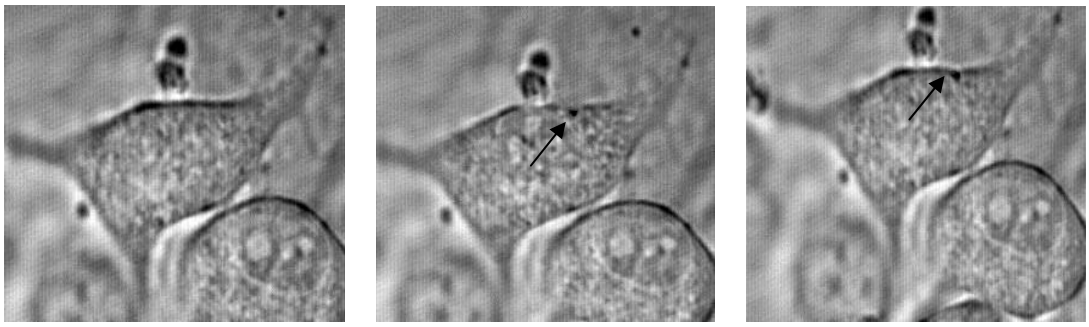
Results:

The first evidence that the experimental system was working as intended came from visual inspection of cells before and after they had the laser focused on them. One of three possible outcomes was observed. Either: no visible change was seen, a small dark spot was seen on the cell that typically disappeared anywhere from a couple seconds to a couple minutes later, or the cell was seen to increase in size likely as a result of cytoplasm spilling out through the hole that had been created. All three of these situations were also observed in the literature[12]; examples of each of these outcomes are shown in Figure 3.

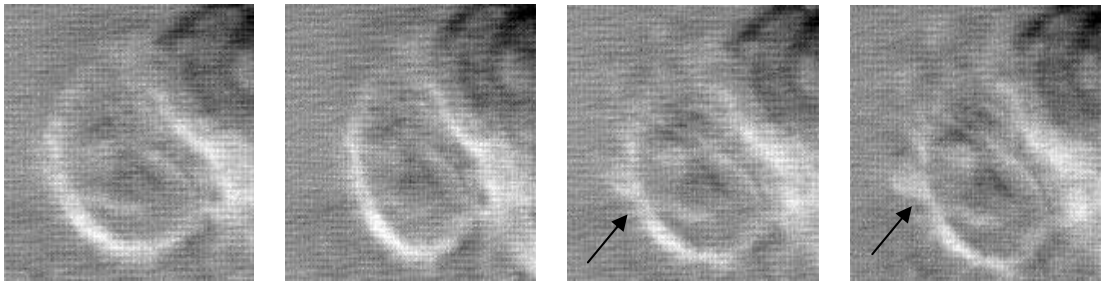
Next, the dye uptake experiments were run to determine whether or not the laser allowed surrounding media into the cells. An example image of some cells before and after treatment with the laser is given in Figure 4. These results are typical of when a dark spot, likely a pore, was observed on the surface of the surface of the cell. The nucleus is seen to light up within seconds after the laser is focused. Typically a front of dye crossing the nucleus can be seen to move from the side of the cell where the laser was focused across the nucleus until all of it is uniformly stained. When no dark spot was observed on the cell – dye would not enter the cell. Because of the high concentration of dye used, some cells spontaneously uptake the dye. These cells were

simply avoided when running the experiment. This did not create a serious experimental problem since it was quite clear from the images taken that dye was entering the cell through the pore created by the laser within seconds of the cell being optoporated. Spontaneous uptake was a slower and more diffuse process.

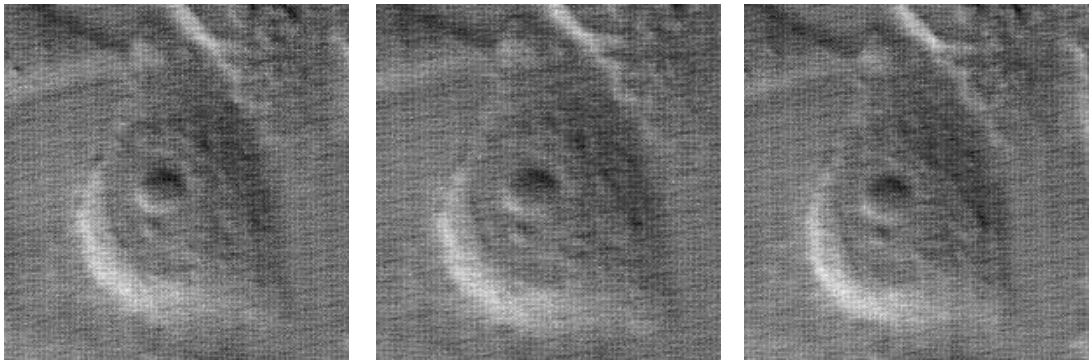
This information, showed in table 2, was then entered into the graph shown in figure 5. Efficiencies of uptake higher than 90% were found when using high laser powers, but as would be expected they dropped off dramatically at lower focus times and lower powers. Most of the experiments were run on what were considered likely values for future experiments, in particular 40ms/80mW and 16ms/80mW reported as the ideal laser parameters by Baumgart et al and Tirlapur and Konig respectively. Conditions similar to those reported by the other two papers cited in Table 1 were only tested on one plate as initial results did not seem to justify further experimentation.



A. *Before* *Immediately After* *25 Seconds After*



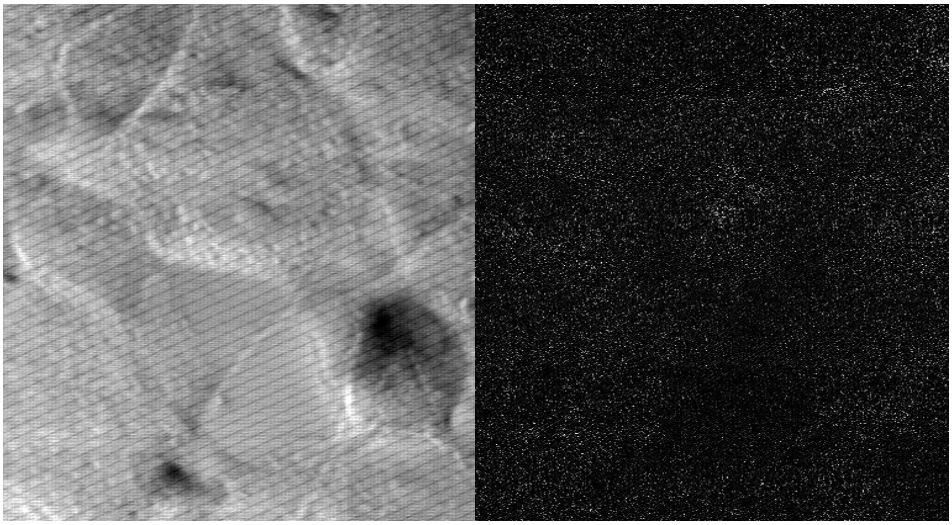
B. *Before* *Immediately After* *25 Seconds After* *1 Minute After*



C. Before Immediately After 35 Seconds After

Figure 3: The three morphological outcomes seen from focusing a laser on the membrane. (A) Small black dot formed where the laser was focused. (B) Blebbing of the cell where the laser was focused. (C) No visible change in the cell.

Before



After

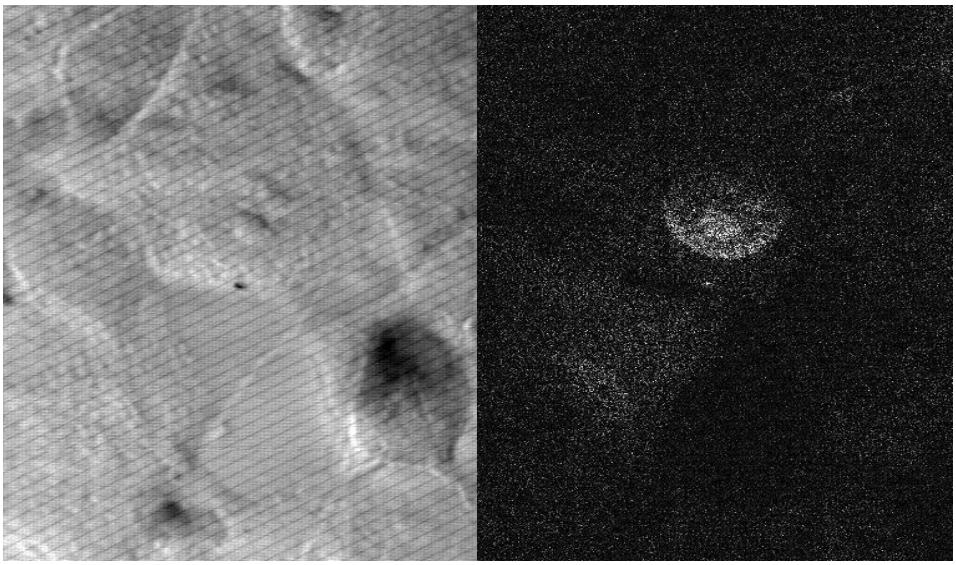


Figure 4: The result of an experiment where the laser is focused upon a cell in the presence of ethidium bromide. The image on the left is a transmission image – to the right is a two-photon image showing the dye. Within seconds, the nucleus of the cell is stained from the dye, particularly on the bottom side where the dye is entering. In this case dye can also be seen entering the cell on the bottom left. This sometimes happens when the laser is focused close to the edge of a cell. Although unusual, on occasion up to three cells can uptake the dye at once.

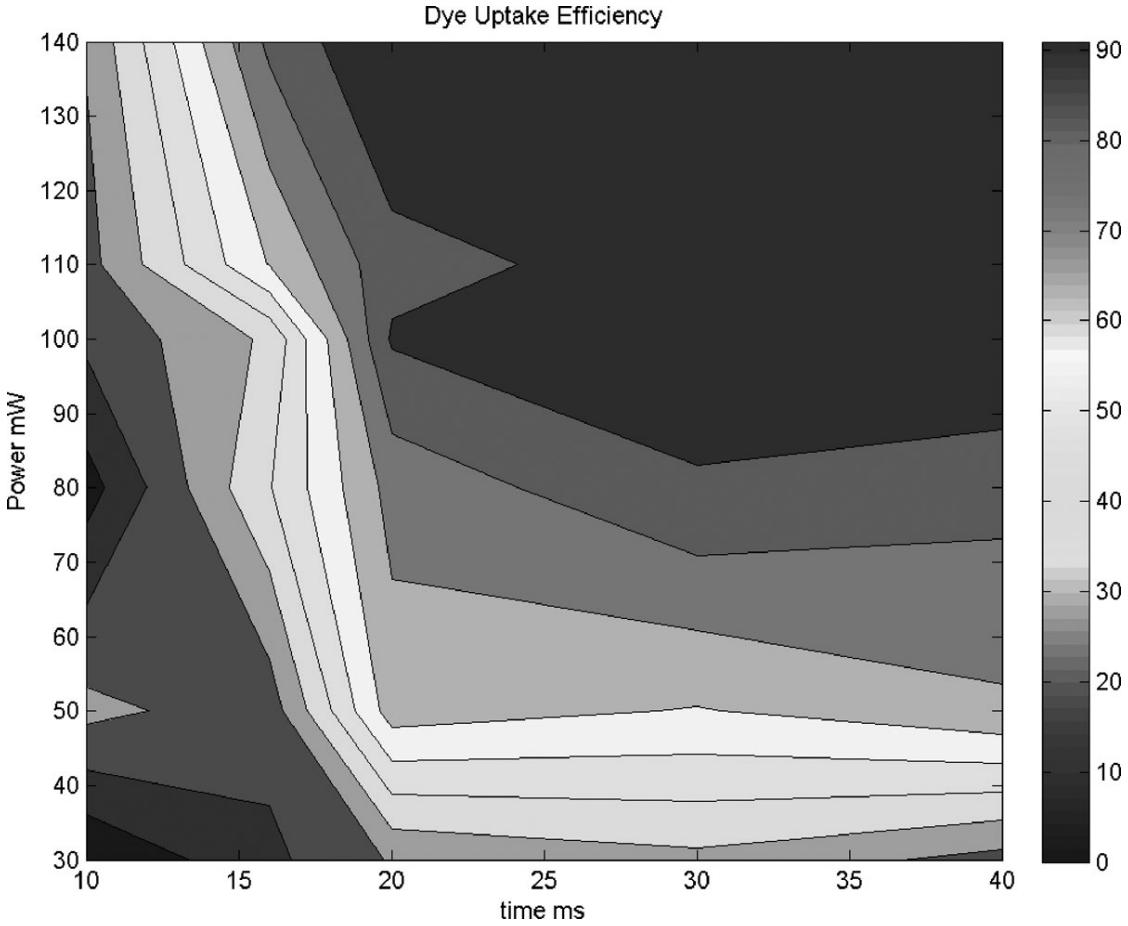


Figure 5. Dye uptake efficiency for ethidium bromide experiments. The efficiencies came from the number of cells that took up dye when the laser was focused on them, divided by the number of cells the laser was focused on.

Table 2. The raw data used to make Figure 5. Data is sparse in regions less likely to be of biological importance, more data was taken for laser powers expected to be useful. Since 25 cells were optoporated on a plate, it is possible to give a range for parameters used on two or more plates, and standard deviation for those with three or more plates. While the error is high, it is not so high as to invalidate general trends in the graph.

Park Time(ms)	Laser Power(mW)	% dye entered	# of Cells hit	# dye entered	Range(in %)	Standard Deviation(in %)
100	15	32	25	8		
30	30	34	50	17	28	
10	30	0	25	0		
16	30	16	25	4		
20	30	28	25	7		
40	30	24	25	6		
60	30	72	25	18		
100	30	92	25	23		
10	50	30	50	15	20	
16	50	22	50	11	4	
20	50	68	75	51	12	7
30	50	63	125	79	44	18
40	50	71	75	53	32	16
60	50	76	25	19		
30	80	90	50	45	12	
20	80	76	75	57	8	4
10	80	5	75	4	8	5
16	80	45	75	34	8	5
40	80	85	75	64	16	9
60	80	92	25	23		
10	100	20	25	5		
16	100	38	100	38	36	17
20	100	92	25	23		
30	100	96	25	24		
40	100	100	25	25		
10	110	24	25	6		
16	110	64	25	16		
20	110	88	25	22		
30	110	88	25	22		
40	110	92	25	23		
10	140	28	25	7		
16	140	84	25	21		
20	140	100	25	25		
100	140	100	50	50	0	

After the encouraging results for dye experiments it was hoped that similar graphs could be produced for the ideal conditions to get cells to uptake plasmid. Experiments were therefore set up to do just this. Twelve plates of cells had 25 cells each transfected with the laser over a variety of conditions shown in table 3. Also a plasmid with no laser control, and a no action control was taken. Four other plates had one cell each transfected and they, unfortunately, should be interpreted as additional negative controls given the results.

Table 3. Experiments on CHO cells to determine the correct laser parameters for laser transfection. If there were any cells transfected by the laser they are hidden by spontaneously uptake the plasmid.

Laser Power(mW)	Park Time(ms)	# of Cells Hit with Laser	Fluorescent cells 48 hours later
180	125	25	12
180	80	25	13
180	50	25	31
180	20	25	12
120	125	25	46
120	80	25	70
120	50	25	10
120	20	25	38
60	125	25	8
60	80	25	46
60	50	25	20
60	20	25	76
Plasmid only	Control	0	12
No Action	Control	0	0
60	50	1	71
60	50	1	32
60	50	1	10
60	50	1	6

Clearly the cells are spontaneously taking up plasmid. This is most dramatically seen in the negative control which has fluorescent cells. The cells in the two sample groups look identical, as shown in figure 6, and cannot be sorted by morphology to determine which way the plasmid entered cells. A series of experiments were performed to attempt to remove this problem. As shown in table 1, plasmid concentrations used by other groups varied wildly. The experiment shown in table 3 used a rather high plasmid concentration of 10μg/ml; successively lower plasmid concentrations were used to see if there was a concentration where cells treated with the laser would uptake plasmid but not cells in the negative controls. The results of these experiments are shown in Table 4.

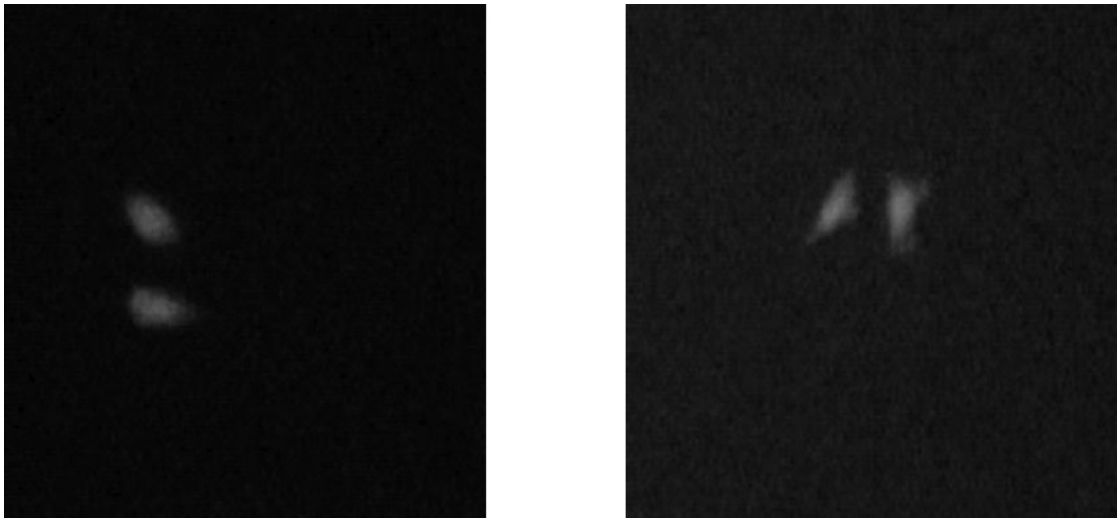


Figure 6. Example fluorescent cells from a plate where no laser was used (left panel), and a plate where the laser was focused on several cells (right panel). No consistent morphological differences can be seen between the two groups.

Table 4. The results of experiments where plasmid concentration was successively lowered to try to determine a concentration where the cells would uptake plasmid only when the laser was focused on them. Concentrations range from the lowest used in the literature, to the highest. No concentration was found with the desired effects.

Plasmid Concentration	Presence of Fluorescent cells when laser is used	Presence of Fluorescent cells when no laser is used
50ug/mL	Yes	Yes
10ug/ml	Yes	Yes
5ug/mL	Yes	Yes
1ug/mL	No	No
.1ug/mL	No	No

An example of a plate where cells were grown in a small circle at the center of the plate is shown in Figure 7. Experiments with this method where 25 cells were transfected on some plates while others were left as controls were run. The results of the experiments were that the total number of fluorescent cells in the dishes where the laser was used was about the same as those in the control. The total number of fluorescent cells dramatically decreased but the trend was the same, the laser did not significantly change the number of fluorescent cells found on a dish. The results of a typical experiment using this technique are given in table 5.

Table 5. Results of a typical experiment where the only cells on the plate were in a small circle in the center. Negative controls and experimental plates are still statistical equivalent. If there is successful laser transfection, it is of very low efficiency

	Laser Power(mW)	Plasmid Concentration (ug/mL)	Park Time (ms)	# of Cells Hit with Laser	Fluorescent cells 48 hours later
No Action Control 1	NA	0	NA	0	0
No Action Control 2	NA	0	NA	0	0
- Control 1	NA	5	NA	0	2
- Control 2	NA	5	NA	0	3
- Control 3	NA	5	NA	0	0
- Control 4	NA	5	NA	0	1
Plate 1	80	5	16	25	4
Plate 2	80	5	80	25	2

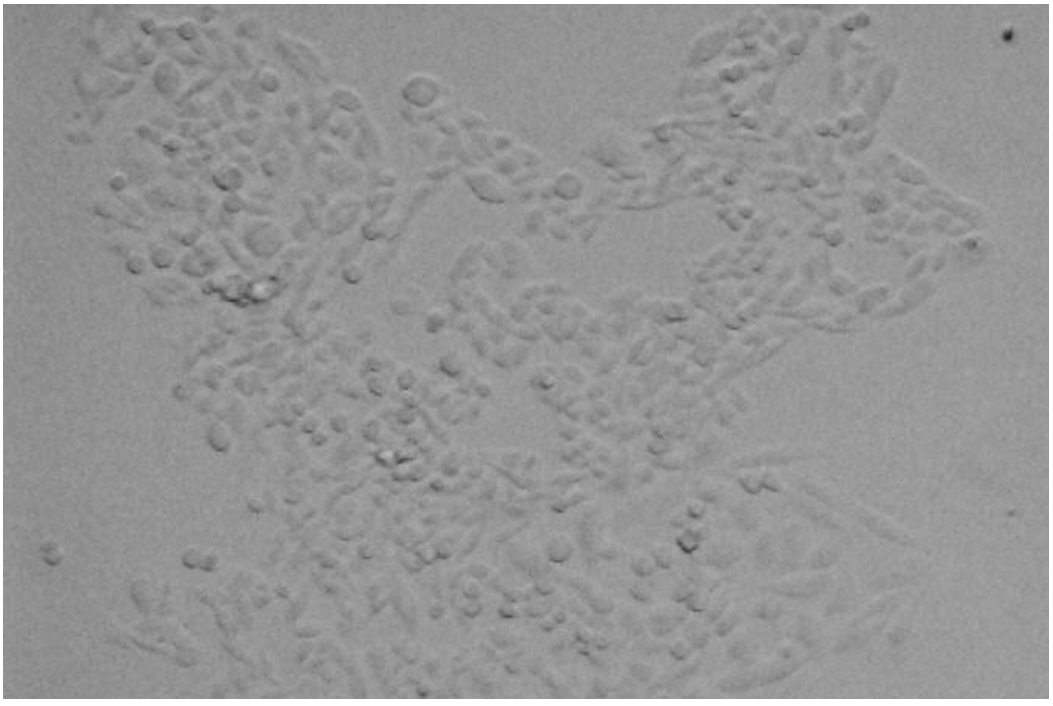


Figure 7. An example of the type of group of cells created in the center of a dish when a small volume of media with cells is allowed to sit until cells adhere. There are few cells far away from where this image was taken.

Table 6. The results of experiments on 3T3 cells where the concentration of plasmid was varied across the spectrum of published values. No Fluorescent cells were produced.

Laser Power(mW)	Plasmid Concentration (ug/mL)	Park Time(ms)	# of Cells Hit with Laser	Fluorescent cells 48 hours later
- Control	0.1		0	0
80	0.1	16	25	0
- Control	1		0	0
80	1	16	25	0
- Control	10		0	0
80	10	16	25	0
- Control	50		0	0
80	50	16	25	0

Table 7. The results of experiments where the numerical aperture of the lens focusing the laser on the cell membrane was changed across the range of published values. No fluorescent cells were produced under any of these conditions.

N.A. Of Lens	Laser Power(mW)	Park Time(ms)	# of Cells Hit with Laser	Fluorescent cells 48 hours later
Control			0	0
0.5	80	16	25	0
Control			0	0
0.7	80	16	25	0
Control			0	0
0.95	80	16	25	0
Control			0	0
1.15	80	16	25	0

Since it was clear this method was not working with CHO cells, essentially identical experiments were performed with 3T3 cells. Unlike in CHO cells spontaneous transfection was not an issue in 3T3 cells. However on no occasions were any fluorescent cells produced in any of the experiments involving 3T3 cells. Results of these experiments are given in Tables 6, and 7. Table 6 presents an attempt to get the method to work by changing the concentration of the plasmid in the media across the spectrum of published values. Table 7 attempts to get the method to work by changing the numerical aperture of the objective across the range of published values.

Conclusions:

The results of experiments involving focusing the laser upon more than 2000 cells were presented. The most consistent interpretation of this data is that the laser is causing the death of cells, but that has not been conclusively shown. Given the high efficiency in which the laser creates pores in the membranes of cells it is reasonable to expect that if this technique were to be used on a cell line that was tough enough to survive the treatment it would result in extremely high transfection efficiency, similar to that reported in the literature. It does not seem that this method is suitable for the cancer experiments it was hoped that it could be of use for. It is not able to consistently transfect cells without causing significant cell damage, at least with the laser and cell types used. Perhaps further developments in the field, probably through the application of different lasers, will improve the technique to the point that it can be as useful as the initial report in the literature suggested it could be.

The one way this technique may prove to be of some use in these cancer experiments is through its ability to kill cells. The other technique to get cre recombinase into the cell presented here has a tendency of getting protein into too many cells. This method could potentially be used to kill off all but one cell that has taken up the protein thereby making the method a truly single cell method.

Chapter 2: *Direct Uptake of His-TAT-NLS-Cre*

Introduction:

Even unmodified Cre Recombinase is capable of crossing a cell membrane[26], with further modification of the protein this feature can be augmented to make for a particularly effective method of delivery. The addition of a TAT group to the end of a Cre protein is one common way this can be done; this group dramatically increases the probability that the protein will cross a membrane. However simply getting the protein into the cell does not ensure activity, to be able to excise DNA the protein must enter the nucleus. To increase the probability that the protein enters the nucleus, a Nuclear Localization Signal (NLS) can be added along with the TAT group[27, 28, 29]. This results in a protein that can enter into, and excise DNA between loxP sites, in up to 80% of cells on a dish; without the requirement of any more complex vectors such as viruses[29].

While this method has not to my knowledge been used to genetically alter cells one at a time, it holds great potential for this. All that is required for a single cell method is the ability to deliver a protein such as His-NLS-TAT-Cre to the cell membrane of one cell. The protein should then take over from that point and excise the DNA between two loxP sites. When compared to currently used methods to genetically modify a single cell this is among most gentle. Other methods typically require some sort of pores be produced in the membrane such as in single cell electroporation or microinjection; while this one does not physically damage the membrane.

To ensure the technique is working a reporter cell line is required. An ideal cell line for this application is one which turns on a fluorescent protein whenever an active Cre protein cuts out loxP sites in the nucleus. There are several such cell lines, but the

one used here was tex.loxp.eg. This is a T cell line with some history of use in published work with these proteins[29]. The cells are not adherent, but they do settle down on the surface and stay there unless significant agitation of the dish breaks them off of the surface.

There exist dozens of possible methods to deliver His-NLS-TAT-Cre to the surface of a single cell. The method chosen for these experiments was the use of a patch pipette. Of the ways to attempt to deliver the protein to small numbers of cells this one is among the simplest, and uses commonly available laboratory equipment.

The initial question asked is whether or not the protein can be delivered to a small area, without either sticking to the inside of pipette tips or evenly dispersing around the plate. If the proteins are taken up slowly enough by cells than it would be expected that they would diffuse far across the plate before entering into cells; if they are taken up quickly than cells would be localized to a small region near the grid square where the protein was released.

Next it was desired to know whether the protein could be delivered to a single cell while using this method. To attempt to do this, micro-pipettes full of protein were held up to cells for varying lengths of time with varying concentrations of protein this time without putting pressure on the back of the pipette.

Materials and Methods:

These experiments centered on the use of micro-pipettes. These pipettes had tip sizes between 1-5μm which was enough smaller than the diameter of a cell to hope to be able to deliver the protein to just one. The tips were pulled on a Narishige PC-10 puller; the 1 μm tips were pulled with the settings: temperature 1 = 65; temperature 2 = 55; top slider setting, 4.5; bottom slider setting, 10. The settings for the 5μm tips were the same except for the second temperature setting was reduced to 55.

Tex.loxp.eg cells were obtained from Xianshu Cui at the Howard Hughes

Medical Institute. They were cultured in RPMI 1640 containing 10% FBS, 100 I.U. penicillin and 100 μg/ml streptomycin and passaged twice a week. MaTek tissue culture plates with a 7 mm diameter coverslip containing 600 μm grid spacings were used for experiments so the same location on the dish could be found again the day after experiments were run. Cells were grown up to around 30% confluence at the start of experiments, the use of higher cell confluences was found to cause problems since the cells sometimes lift up off the dish in large numbers when at high densities.

The His-NLS-TAT Cre was produced and purified by the Cornell University Protein Facility. It is stored at a concentration of 0.45-0.7 mg/mL in a -80°C freezer as small pellets in 50 mL tubes. This concentration was as high as the Protein Facility members could get it up to before it came out of solution. The buffer it is in consists of: 20mM Tris pH 8.0, 500mM NaCl, 140mM Imidizole, and 5% Glycerol.

To position the tip next to cells a Luigs & Newmann SM-5 Micromanipulator was used. A 100μL syringe was attached to the back of the pipette which allowed for the pressure to be increased when necessary. The location tip could be controlled well enough to consistently bring it within a couple microns of a cell membrane. The grid square where that cell could be found could also be recorded for future use.

To run the proof of principle experiments, cells were grown up on gridded plates as previously described. Micro-pipette tips were then held in the middle of a grid square and a small volume, ~3μL, of protein was forced out of the tip by increasing the pressure with the syringe. The number of the grid where the protein was produced was recorded; and the cells were returned to the incubator. No special precautions to prevent contamination were taken other than the use of antibiotics; the plates were exposed to untreated laboratory air.

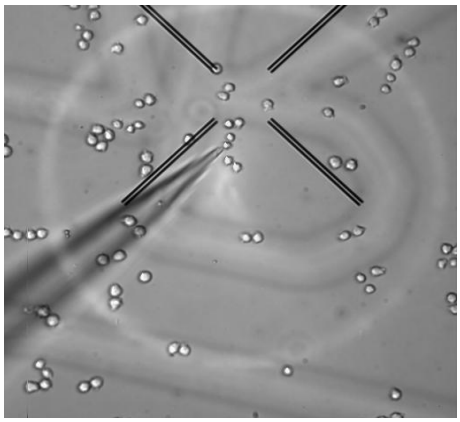


Figure 8. An example of a micro-pipette full of protein being held up to a single cell.

To attempt to get the protein into a smaller number of cells, tips containing 20μL of protein were brought up to a single cell in a group of six cells(shown in figure 8.) and held there for varying lengths of time(10min, 5min, 2min, <20sec). There is a reasonable chance that disrupting the plate too soon after the tip was removed would disperse the protein, increasing the distance away from the tip that fluorescent cells are found. Therefore in some samples the dish was not disturbed for two minutes after the tip was removed. In most experiments a concentration of 0.45mg/mL was used, but in one set of experiments the concentration of the protein was reduced to 0.13mg/mL. At the conclusion of experiment the cells were returned to the incubator. The number of fluorescent cells found on the plate 18 hours after the experiments were run was counted. After every forth experiment a positive control plate was run to ensure that the protein was still active and not sticking to the pipettes. The procedure for this consisted in filling a pipette with 20μL than injecting the whole volume from a micro-pipette into the plate of cells.

Results:

Of the six replicates of the proof of principle experiment, all had fluorescent cells. Only one had a reasonably small number of cells, 12, the rest had greater than fifty, with one having greater than five hundred. Cells were not randomly disbursed

around the plate, and in all cases were concentrated near the grid square where they were released. The farthest distance fluorescent cells were found was three grid spaces away, or 1800 about microns. Since the coverslip on the bottom of the dish was 7000 microns in diameter this represents a small percentage of the plate. An example of what is seen can be seen in figure 8. The huge variation in the number of cells found is unsurprising since the flow out of the tips was crudely controlled and monitored with a syringe.

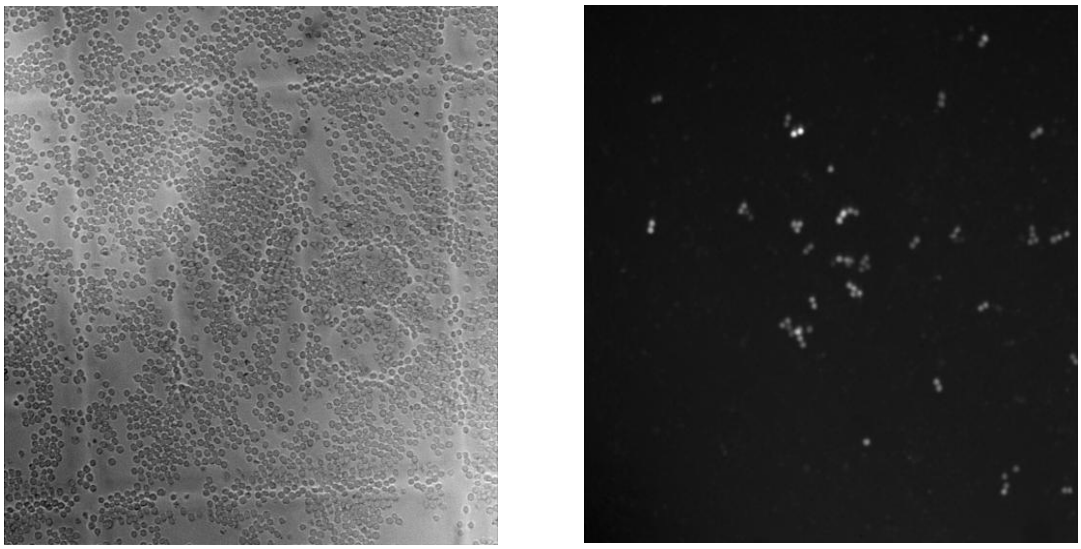


Figure 9. An example of what is seen when large amounts of protein are released from the center of the grid shown. Cells are found in a high concentration in the center and slowly lower in numbers until around three grid spaces away when none of the cells are fluorescent. This plate had a below average number of cells for this type of experiment, but was by no means the plate with the lowest number.

The results of initial experiments with releasing protein were considered encouraging enough to continue with the experiments where the pipette was held still near a group of cells. The results of these experiments over a wide range of variables are summed up in table 8. A total of 36 replicates are presented in this figure. When a positive control came up negative the results were thrown out and not reported. This did not happen with any experiments run using a procedure similar to those in Table 8, but did happen in some experiments where the protein was mixed with dye to try to better observe the flow of liquid.

Table 8. Results of experiments where tips were held up to cells without the application of pressure to force a high flow rate of protein out of the tip.

Time Tip Held	Wait Time	Tip Size	Protein Concentration	Number of Fluorescent Cells on Each Plate
10min	0Min	5µ	0.53mg/ml	50+, 50+, 50+, 50+
5min	0Min	5µ	0.53mg/ml	1, 12, 20, 50+, 50+, 50+, 50+, 50+
5min	2min	5µ	0.53mg/ml	0, 0, 2, 18, 19, 24, 29, 50+
5min	2min	1µ	0.53mg/ml	0, 0, 0, 2
5min	2min	5µ	0.13mg/ml	0, 0, 0, 0
1min	2min	5µ	0.53mg/	0, 0, 0, 0
<20seconds	2min	5µ	0.53mg/ml	0, 0, 0, 7

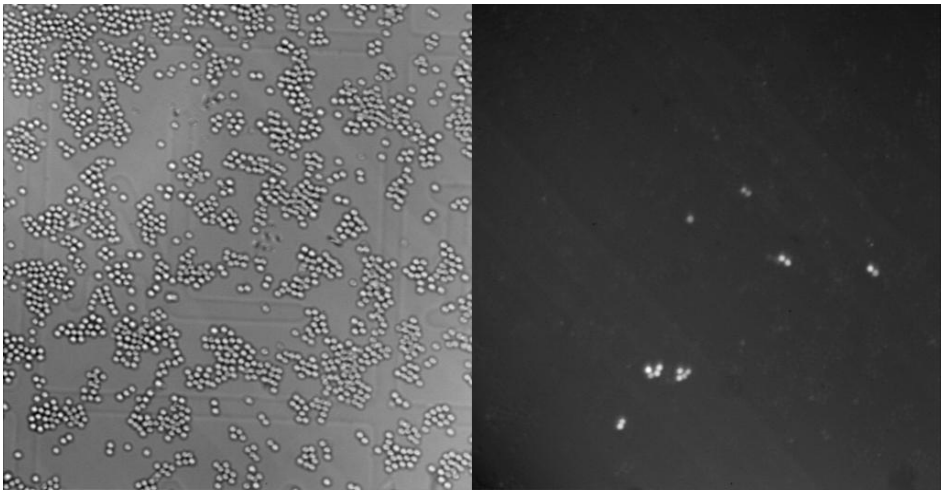


Figure 10. Image of a group of cells produced with this method, two cells were outside of the region shown, and the rest were within the same grid square where the tip was held. It is reasonable to assume that as few as 8 cells were initially altered from the protein since this is the number of groups of cells produced.

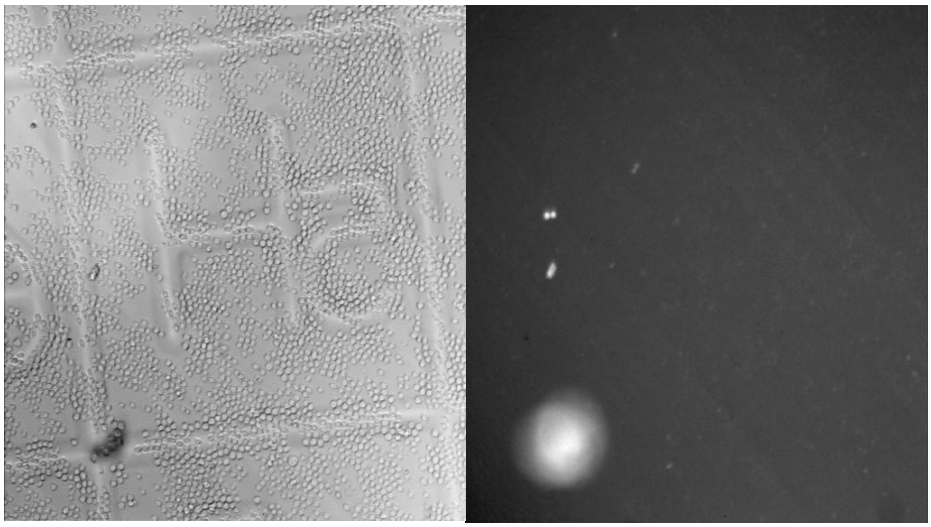


Figure 11. Image of a group of cells produced with this method. In this case the hope of a single cell method is nearly achieved, 2-4 fluorescent cells being on the plate 18 hours later. All of the cells are within 300 microns of where the protein was released. It would be reasonable to assume, although certainly not proven, that all of the fluorescent cells on this plate came from a single cell that has divided.

When the tip was held up to the cell for five minutes 14 out of 16 plates had fluorescent cells in the grid square where the cell was released. There was still a rather random distribution for the number of fluorescent cells found. This is not unexpected given that small variations in the amount of protein released and number of nearby cells willing to uptake it. Still, with this parameter the method is successful as a way to excise DNA between loxP sites in a small number of cells, without physically damaging the membrane. By lowering either the concentration of DNA, or the amount of time the tip is held next to cells to less than five minutes than the technique approaches closer to a single cell method, however with the caveat that most of the dishes produced will have no fluorescent cells at all. Example images of some plates that were produced with this method are given in Figures 9, and 10.

Conclusions:

This method can excise DNA in a very small number of cells in a reasonably reproducible manner and without excessive stress on the cells. It however does have issues getting the number of fluorescent cells produced, and the location of such cells to be consistent. If an experiment only requires the genetic alteration of a single cell, within a defined 600 micron circle, and it is not important which cell gets altered than this method can produce that. It will however require many replicates since some plates will have the protein get into too many cells and in some it will enter no cells. Another way around this problem is to kill off all but one excised cell using a specific method such as laser ablation.

This method may however be a reasonable tradeoff to make for the advantages this method has over using traditional transfection techniques, which can lead to significant damage of the cell. Further experimentation with other delivery methods for this protein may result in a way to better localize the protein. To be successful any method used will need to bind the protein strongly enough that it cannot simply

diffuse away into the media, but has to wait until it comes in contact with a cell membrane to unbind. Trapping the protein inside a shell of a more hydrophobic material, and attaching it to a Nickel surface are both plausible ways of doing this.

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