Permeating Vertebrate Cell Membranes Through the Use of Optoporation

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Physics from the College of William and Mary in Virginia,

by

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Abstract:

We worked towards attempting cellular "optoporation" with Xenopus kidney cells. Our eventual goal is to use lasers to permeate the cell membrane for the purpose of inserting exogenous molecules. Thus far research has focused primarily on growing and testing cells to find the maximum amount of (laser) energy they can withstand without being removed from the plates as well as determining the effect this energy has on the cells. This is being done to prepare for actual attempts at optoporation with Enhanced Green Fluorescent Protein. Progress was made on determining a relationship between power and plate adherence as well as the kill rates of cells affected by the laser.

Background:

"Optoporation" is the term given to the process of using light within a liquid medium full of cells with the intention of permeating their cellular membrane [1]. A laser is used to focus this light onto the liquid medium, which subsequently creates bubbles through cavitation events. The resultant shock wave and/or fluid flow is theoretically capable of instantaneously porating the cell membrane and allowing exogenous molecules into cells [1]. However, optoporation is not a widely established method and is still in its infant stages.

"Electroporation" is commonly used with bacteria for the purpose of inserting plasmid DNA into cells in order to modify their genetic systems [2]. Despite its relative success with bacteria, it is much harder to cause this type of vector poration in vertebrate cells [2]. The most success with non-bacterial subjects has been with the process of

"transfection," in which the DNA for insertion is incorporated into a viral vector [3]. However, viral transfer has low efficiency and potentially serious side effects [3].

Published results have shown a successful transfection rate of ~40% through optoporation, as opposed to a success rate of only ~10% through the more widely used methods of lipofection (lipid-mediated transfection) and electroporation [4]. In addition, optoporation can theoretically be used on specific cells and hard-to-reach areas because of the flexibility of permeating cells with an external stimulus: a laser beam [1].

Purpose:

The aim of laser optoporation work is to create a more efficient means of genetically altering cells. Previous work at William and Mary has indicated that very short bursts of laser energy in the area immediately above a monolayer culture of *Xenopus* (frog) kidney cells can disrupt the cells without killing them. However, it has been shown that an excess of energy causes the cells to be "blown off" (removed from the adhesive surface of) the plate. It is unsure whether the shock wave from the cavitation events or the field flow from the liquid is creating this effect. As a result, the current work is directed at determining whether sufficient bursts of energy can force plasmids containing specific DNA fragments across the cell membrane without lysing the cells. In order to test if these exogenous molecules are able to cross the cell membrane EGFP (Enhanced Green Fluorescent Protein)-containing plasmids can be inserted. This test is relatively simple to analyze because—if the plasmid is successfully inserted into the cell—the cell will begin

to make green fluorescent proteins and glow in a distinct manner when illuminated by fluorescent light.

Thus far this research has focused primarily on growing and testing cells to find the maximum amount of (laser) energy they can withstand without being "blown off" the plates. This relationship is important because it will ensure that the cells are being hit with the most power they can withstand without having any effect—essential for attempts at disrupting their membranes enough for plasmids to enter. In the end, this characterization will be essential for actual attempts at optoporation with EGFPs.

The effect of the laser on those cells that were and were not blown off was also tested. It is presumed that those cells that were not blown off but were close to the laser provide a good opportunity for optoporation as they might have been able to withstand lysis caused by the shockwave and fluid flow from the laser while still having their membrane temporarily permeated. The health of these "peripheral" cells (that are adjacent to the channels of cells that were blown off) is important to determine as they might provide the best opportunity for successfully transfected cells through optoporation. Those cells that were blown off might also be candidates for successful optoporation. Any cells that survived the laser blast that detached them from the plates would likely have received the most power allowable and therefore would have the best chance for temporarily permeated membranes that could allow plasmids into the cell. If the viability of both the blown off and peripheral cells is determined it would provide important information about the likely location of transfected cells after attempting optoporation.

Methods:

The *Xenopus* kidney cells are cultured in Nunc^{TM} Nunclon^{TM} flasks using *Xenopus* A6 media (for detailed recipe see: http://dept.kent.edu/projects/cell/MEDIA.HTM). They are transferred to 35mm Nunc Nunclon culture dishes for use with the laser apparatus. Nunc flasks and dishes are used due to the *Xenopus* cells' adherence to its surface. Cells are grown until they near confluence on the bottom of each dish. Various amounts of media have been put in the culture dishes due to its effect on the experiment (see **Results**).

The laser beam being used is the 2nd harmonic of a Q-Switched neodymium: yttrium-aluminum-garnet (ND:YAG) laser with various optical density filters to achieve the desired energy. The energy density of the laser varied between .16-.85 GW/cm^2 (the specific value for each run is labeled in **Results**). The size of the beam at the focal point was ~.50 microns. The confocal parameter was ~500 microns. The frequency of the laser was set at 7 hertz for the first 3 experiments (10-1-04 to 10-22-04) and 10 hertz for the experiments thereafter. The pulse width was about 2 ns. The laser is focused with a microscope lens (focal length= 25 mm.) into the culture dish, which rests upon a platform. The platform is adjustable on the micrometer scale in all three dimensions. This gives precise control of how high the laser focuses within the culture dish and allows for a large number of cells to be tested at once by horizontally displacing the dish in relation to the laser. A power detector is used to measure the average power of the incident laser energy. A camera that is attached to a computer displays the output of the laser at the focal point and is used to determine crucial positions such as the bottom of the

cultures dish and the top of the media. These points can be determined due to the nature of the reflection that can be seen on the computer screen.

Cavitation events occurring at the focal point of the laser provide the means for cellular poration. Bubbles that develop from the energy the laser imparts upon the media create a shock wave as well as bulk fluid flow within the liquid [5]. It is not clear which of these mechanisms porates the cell (and blows them off the dish)—although published results have given credit to the emergence of these bubbles [1,6].

The laser travels through the bottom of the culture dish and a layer of media before it focuses at a set distance above the cells. Therefore, due to the varying index of refractions of these substances the focus of the laser will be vertically adjusted according to Snell's law: $N_1 \sin(q_1) = N_2 \sin(q_2)$. The index of refraction of the media is close to that of water (~1.3) while that of plastic is a little higher (~1.4). Therefore, the laser focuses about 1.3x higher than what is measured by the vertical adjustment micrometer. This has been factored into the heights recorded in the results section.

When cells are blown off they form "channels" due to the horizontal displacement that is used to test multiple cells at a constant height. These channels become wider when more cells are blown off. About 6 channels could be created (to measure 6 various focal point heights) on each culture dish. To measure these channels a microscope (at 100x magnification) was used with the width of the channels being measured as a fraction of the microscope diameter (~2.2mm). These channel widths were then graphed versus the

height of the focal point from the bottom of the plate (factoring in the index of refraction the focal point is at 1.3 times the altimeter height). Various heights were used in order to determine an "ideal" height at which cells were no longer blown off the plate.
Once this "ideal" height at which cells were no longer blown off was determined for various power densities they were graphed to determine if any relationship existed. Any sort of relationship provides important characterization about the maximum amount of power the cells could withstand without being detached from the plate.

In order to determine the health of blown off and peripheral cells the following method was used. First, cells were allowed to grow to confluence at the bottom of plates. Before they were shot with the laser, the media was emptied and 4mL of fresh media was put in each plate. After the cells were done being tested with the laser (and multiple channels due to blown off cells exist on each plate) the media in each plate was decanted into a new plate. 4mL of fresh media was then placed back in the original plate and decanted into a second new plate. Finally, 4mL of fresh media was placed back in the original plate. Therefore, each original plate contained fresh media with a negligible amount of blown off cells still floating in the media. The plates with the first and second decant of media contained those cells that were blown off during the experiment. This provided a means for determining if the peripheral cells in the original plate grew back into the open space created by the channels soon after the laser experiment (~24 hrs. later). Fig. 9 displays a before and after picture of peripheral cells over a ~24 hour period. Decanting the original media from these plates removed all blown off cells that might have settled into these channels in place of peripheral cells. The two plates that contained the

decanted media could then be tested in the same timeframe to determine if the blown off cells survived the shockwave and fluid flow that detached them from the plate. This procedure was done with the plates displayed in Fig. 7.

Results:

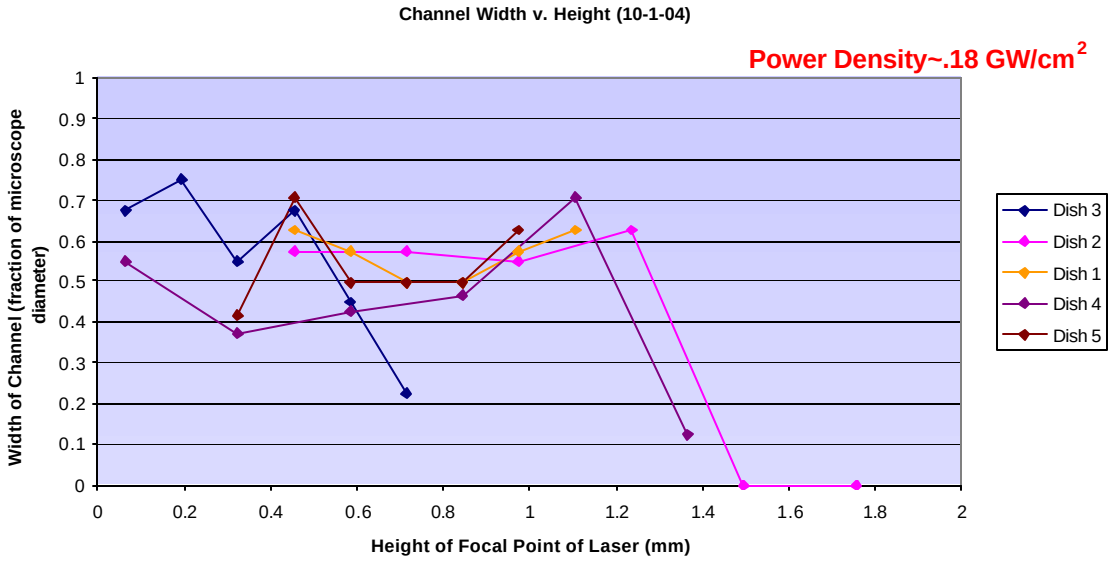

Figure 1: The first cells that were tested were grown in 2mL of media in each of the 5 culture dishes. The power of the laser was measured at ~.18 GW/cm^2.

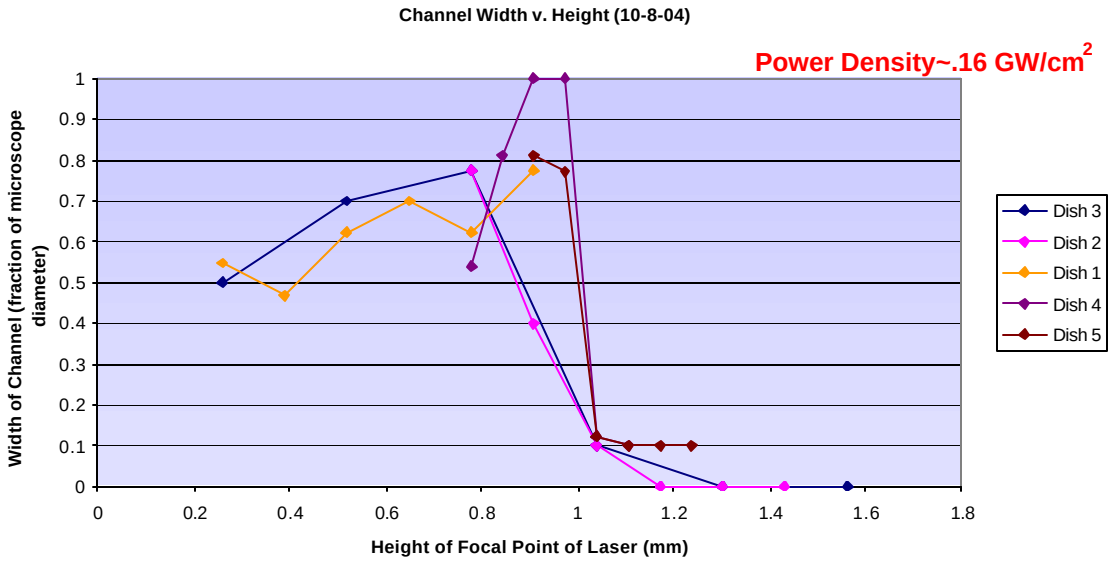

Figure 2: The next set was also grown in 2mL of media in each of the 5 culture dishes. The power of the laser was measured at ~.16 GW/cm^2.

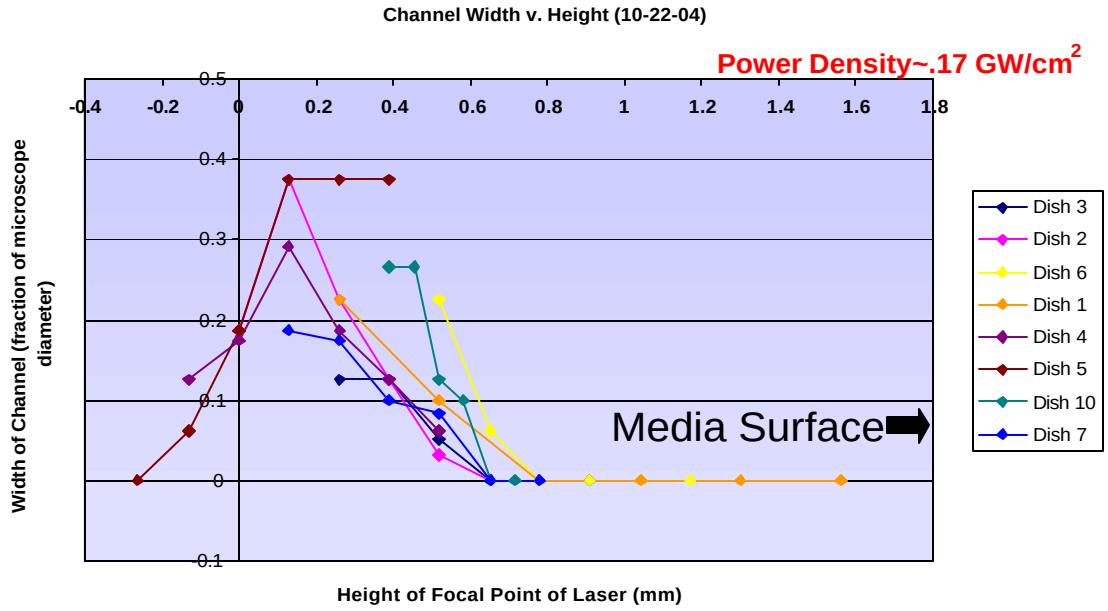

Figure 3: This set was grown in 4mL of media in each of the 8 culture dishes. The power density of the laser was measured at ~.17 GW/cm^2.

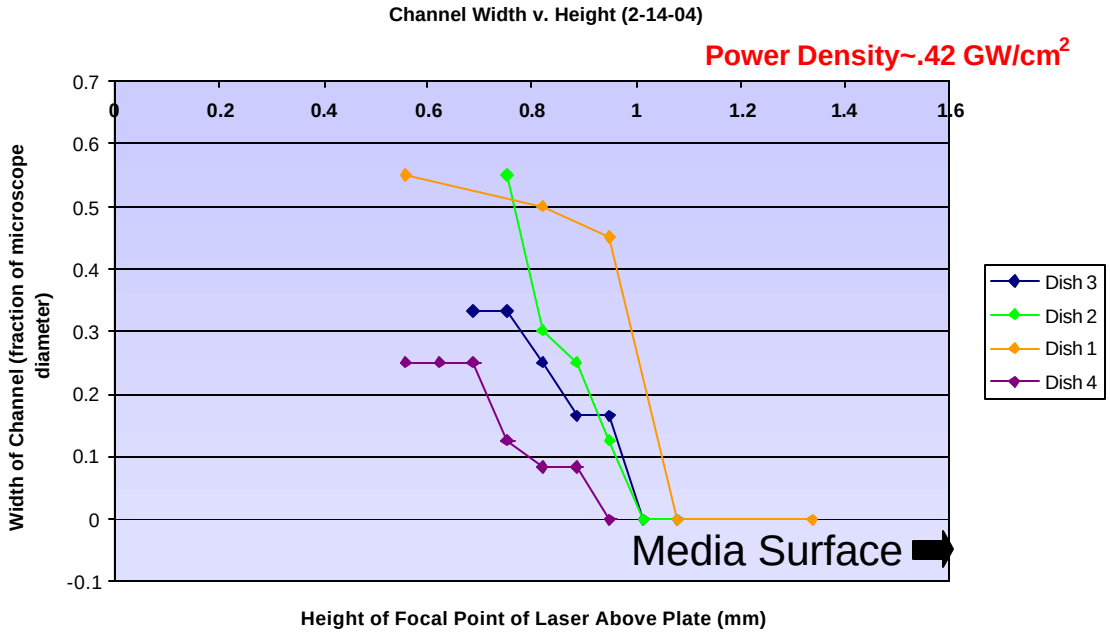

Figure 4: This next set was grown in 4mL of media in each of the 4 culture dishes. The power density of the laser was measured at ~.42 GW/cm^2.

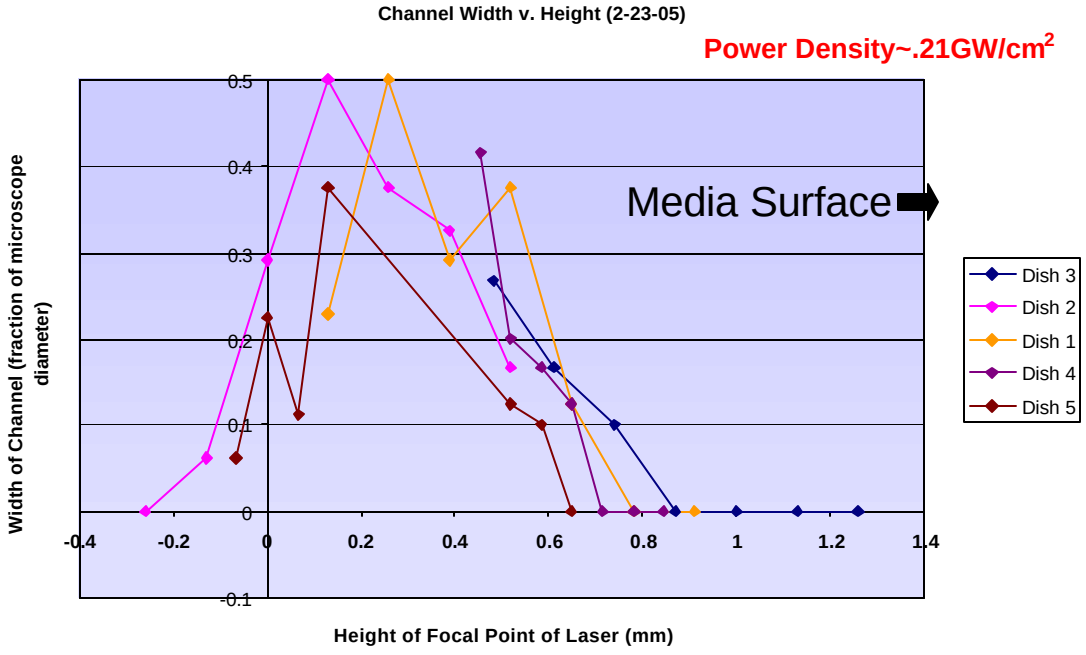

Figure 5: This set was grown in 4mL of media in each of the 5 culture dishes. The power density of the laser was measured at ~.21 GW/cm^2.

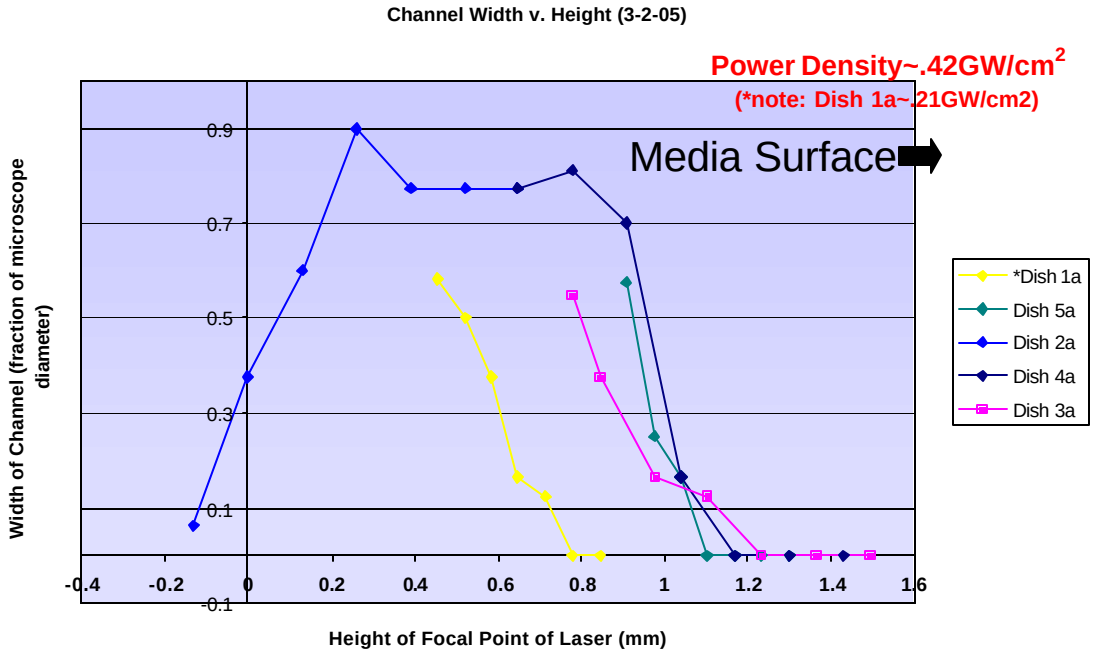

Figure 6: This set was grown in 4mL of media in each of the 4 culture dishes. The power density of the laser was measured at ~.42 GW/cm^2.

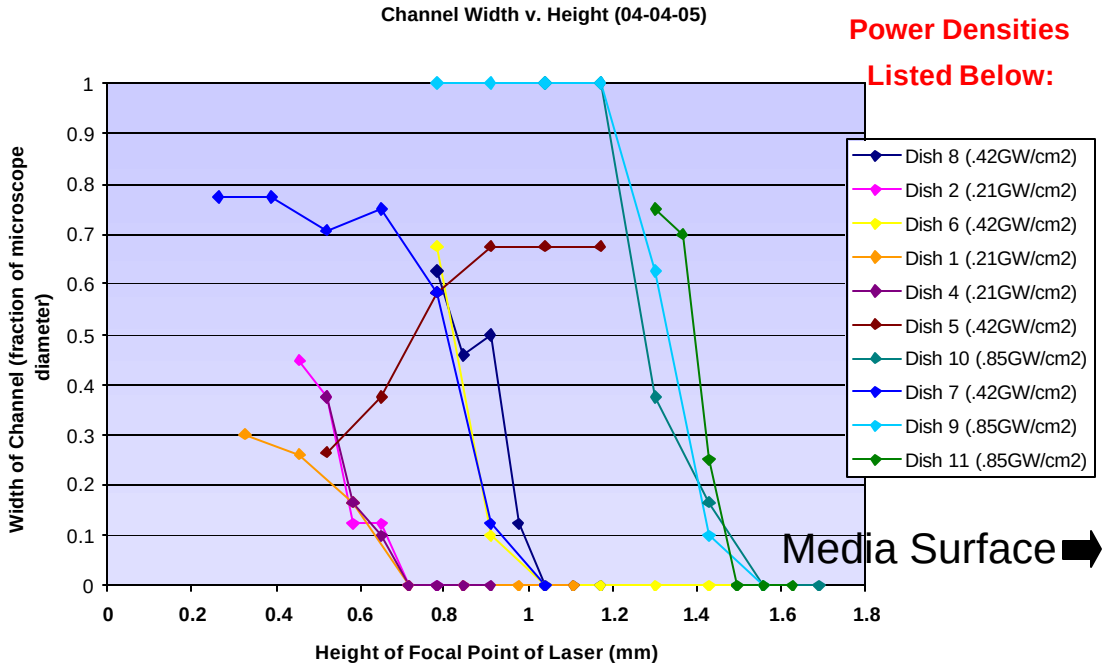

Figure 7: This set was grown in 4mL of media in each of the 11 culture dishes. The power density of the laser was between ~.21 GW/cm^2 to ~.85 GW/cm^2.

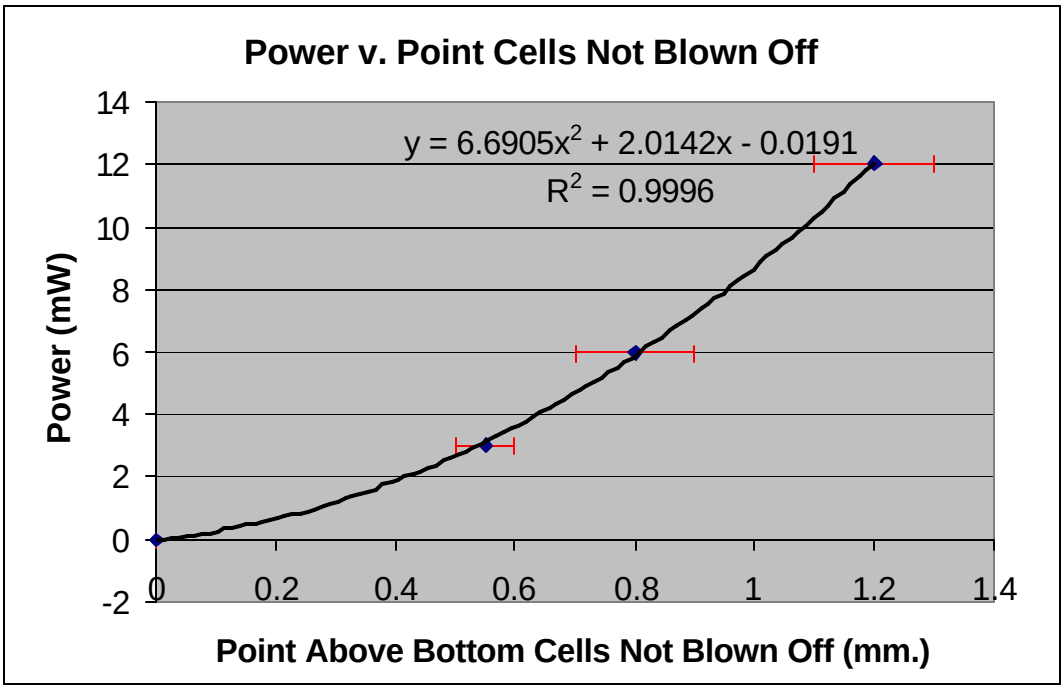

Figure 8: This graph displays the laser power at which cells were no longer blown off. The relationship between these quantities is best modeled by a polynomial form.

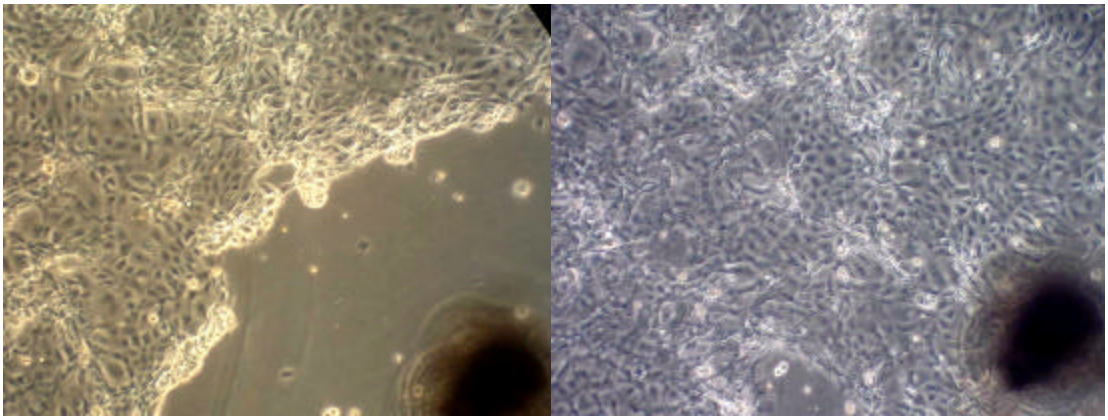

Figure 9: These pictures are before and after shots of a plate of cells shot at ~.84 GW/cm^2. The first picture was taken immediately after the channel of blown off cells was created by the laser. The second was taken ~24 hours later after fresh media was added.

Plate #	Blown Off Cells Counted in Decants	Estimated # Cells Blown Off	Blown Off Cells Surviving
1	**113**	**402**	**~28%**
2	**127**	**509**	**~25%**
3	**383**	**509**	**~75%**
4	**308**	**905**	**~34%**
5	**324**	**509**	**~64%**

Table 1: This table shows an estimate of the number of blown off cells that survived the laser shock. After a single laser shot was focused over the plates the number of cells blown off was estimated by measuring the area of the spot size and multiplying by the cell density of confluent cells (estimated to be ~800 cells/mm^2). Next, the media in the initial plate was then decanted into a second plate. After, the media was replaced in the initial plate and then decanted into a third plate. Living cells from the second and third plates were then counted after an incubation period of ~24 hours to find an approximation of the number of surviving cells. These numbers were divided to give an estimate of the number of blown off cells that remained viable.

Discussion:

It can be seen from these graphs the effect that the media height has on the channel width. When 2mL were in each culture dish (Figs. 1 and 2) the results were fairly inconsistent. It is believed that this is because the focal point of the laser was approaching the top of the media and this affected the consistency of the cavitation events and consequently the channel width. Fig. 2 appears to fall off consistently around an altimeter height of 1.2-1.3 mm. However, this seems to be due to the nearby proximity of the top of the fluid and not because the focal point was at an ideal height above the cells for them not to be blown off.

Fig. 3 was done with 4mL in each culture dish in order to eliminate the effect of the focal point nearing the top of the media. This run was quite consistent. As can be seen the width of the channel reached 0 (where no cells are blown off) at similar values for each dish. In fact, 4 of the 8 dishes reached this point at about .65mm while the slope of dish 4 indicates that it would have neared this point too had it been measured at that height. Therefore, it is clear that adding more media to each culture dish had a positive effect on the reliability of the experiment. It also seems that at a laser power of ~.17 GW/cm^2 cells were no longer blown off at a height of about .65-.8mm.

This led to further tests to find the point at which no cells were blown off at various powers. Power densities of ~.21 GW/cm^2, ~.42 GW/cm^2 and ~.84 GW/cm^2 were used in Figs. 4-7. Figs. 5 and 7 show that at ~.21 GW/cm^2 cells are no longer blown off at ~.7-.8 mm above the plate surface. Figs. 4, 6 and 7 display no cells being blown off at a laser focal point of ~1-1.1 mm. from the top surface of the plate at ~.42 GW/cm^2. Fig. 7 also displays the focal point at which cells are no longer blown off to be ~1.5-1.6 mm. at ~.84 GW/cm^2.

Using the focal point values at which cells were no longer blown off and graphing them in comparison to the power density of the laser provided a relationship shown in Fig. 8. As can be seen, this graph is best represented by a polynomical curve (assuming the point [0,0] represents where no cells are blown off when there is no laser shock). Therefore, in this range the power density is proportional to the square of the amount of cells being

blown off (with an R^2 value of .9996 indicating a good fit). Considering the relative consistency of the focal point where cells are no longer blown off for each power (as shown by Figs. 4-7 and the related error bars on Fig. 8) this relationship appears to be fairly accurate.

Analyzing the plates tested in Fig. 7 also provided notable information about the effect of the laser on the peripheral and blown off cells. The channels that formed in the original plates all filled in with peripheral cells within ~24 hours after the cells were shot (see Fig. 9). This was true for separate plates tested at all three powers (~.21 GW/cm^2, ~.42 GW/cm^2 and ~.84 GW/cm^2). This indicates that the peripheral cells were healthy even after notable amounts of adjacent shells were blown off the plate.

In addition, many blown off cells that were decanted into fresh media reattached onto the surface of the new plates—a clear sign of life. This was true for both the first and second decants into the new plates at all three power levels. These results are shown in Table 1. While the percentage of the blown off cells that survived was fairly variable a clear percentage (25-75%) survived in each test. This variability is due to the different powers and time of laser exposure that were done on each plate. Therefore, a substantial number of cells that received enough laser power (and the resultant shock wave and fluid flow) to blow them off the plates remained viable. These—as well as the peripheral cells—could very well provide the best chance for successfully transfected cells in attempted optoporation as they receive the most power without cell death.

Future Work:

The final goal of this research is to insert exogenous molecules into *Xenopus* kidney cells by optoporating their cell membranes. The characterization done to this point has given a successful relationship between the power and the amount of cells that are blown off the plates as well as an idea of which cells would have the best possibility for optoporation. Therefore, the next step is to attempt optoporation using Enhanced Green Fluorescent Protein to determine if this procedure can successfully permeate the cell membrane and allow exogenous molecules to enter the cell.

In addition, it will be important to determine if it is the shock wave or the fluid flow that is blowing off the cells from the culture dishes. This might be achieved by placing some kind of barrier between the focal point of the laser and the cells in order to try and stop the fluid flow and consequently isolate the shock wave. It might also be possible to create a shock wave without any fluid flow by focusing the laser on the plastic below the cells. Another possibility is trying to get an idea of the relative pressure incident on the cells from each of these processes by using a microphone. This information could be particularly important if optoporation is successful as it will help determine if it is the shock wave or fluid flow that permeates the cell membranes.

Acknowledgements:

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