Optoporation
Laser-Assisted Permeation of Vertebrate Cell Membranes

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by

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Introduction

It is often necessary during the course of biological and medical studies to introduce foreign materials into cells. Several methods have been developed to accomplish this, the three main ones being electroporation, microinjection, and optoporation. Electroporation uses an electric shock to temporarily disrupt the cell's membrane, thereby allowing materials to pass in. Microinjection is self-explanatory: the desired materials are literally injected into the cell with a fine-tipped pipet called a micromanipulator.

The final method, optoporation, temporarily opens the cell membrane in a liquid medium with the assistance of a laser [1]. One of the main advantages of optoporation is that, unlike electroporation and microinjection, it does not appear to perturb the cell membrane [2]. Exactly what causes the temporary poration is unknown, though it is almost certainly associated with a cavitation event inside the liquid. Cavitation is the creation and subsequent collapse of a bubble inside the cell medium caused when the laser beam is focused in the medium. Both the shock wave and the fluid flow over the cells caused by the creation of the bubble are two candidates likely to be the cause of temporary poration. Recent research favors the shock wave as the cause [3, 4]. Figure 1 shows the cavitation event inside the cell medium with the subsequent shock wave and fluid flow in the liquid.
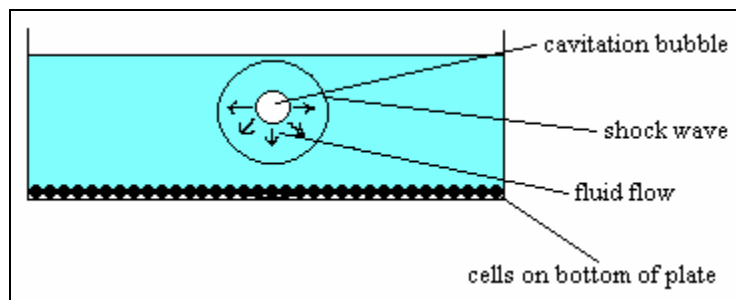

Figure 1

Since the laser energy is absorbed nonlinearly, the cavitation event will occur at the point where the laser is most tightly focused. The size of the bubble created can be found using the following equation:

$$P\left(\frac{4\pi}{3}\right)R^3 = \frac{1}{2}E \qquad (1)$$

where P is atmospheric pressure, R is the radius of the bubble, and E is the average laser energy.

Optoporation has been demonstrated using membrane-impermeable dextran-conjugated dye molecules [1]. The dye molecules, which do not cross the cell membrane unaided, have been observed inside the cells after exposure to the laser. After exposure to the laser, a circle— or "spot"—of cells is torn from the plate, a ring of cells surrounding this empty spot is successfully optoporated, and the rest appear unaffected. Figure 2 shows a bright field image of the empty spot where the laser has removed cells from the plate. Figure 3 shows the same spot with dye fluorescing in the ring of cells surrounding the empty spot. The cells that are fluorescing are the ones that were successfully optoporated.
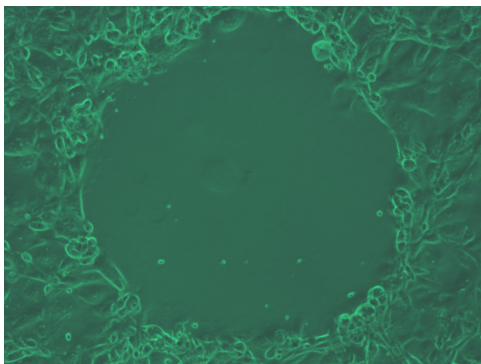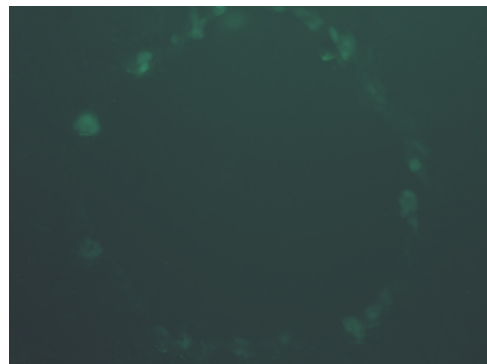

Figure 2: Cells after exposure to laser

Figure 3: Dye fluorescing within cells

Previously, optoporation was successfully carried out using dextran-conjugated molecules of up to 2000kDa in mass. (A *Dalton* is the term biochemists use to refer to an atomic mass unit.) However, attempts at optoporation using Green Fluorescent Protein (GFP) have not

been successful. In order to better understand why this is, it is important to first understand how to optimize the success of optoporation when it does work.

Experimental Technique

Optical Setup: The laser we used for this experiment is the 2nd Harmonic (554nm) of a Q-switched Nd:YAG laser. The full width-half maximum (FWHM) of the pulses were 2ns. Initially, the average power at 10Hz was 2mW, meaning that each pulse had an energy of 200μJ. Later, an average power of 4mW at 10Hz was used, meaning that each pulse had an energy of 400μJ. The Q-switch was controlled manually in order to produce single shots. The laser beam was focused up through a microscope objective, through the plastic base of a cell plate resting on a stage, and through the medium in which the cells are growing (Figure 4). The height of the stage apparatus could be set to the desired level using a z-direction micrometer. The horizontal position of the stage could also be adjusted.
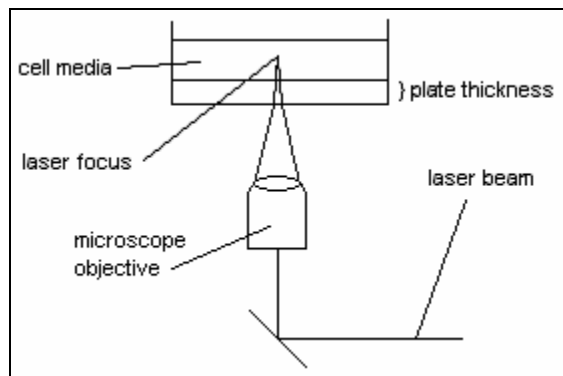

Figure 4: Laser focus

The actual height of the cavitation event above the surface of the plate was then determined by multiplying the distance the focus has moved according to the stage micrometer by 1.3, the index of refraction for the medium. (Figure 5)

For example, assume that the beam is focused at the surface of the plate when the micrometer reads 6.0mm. Moving the stage by 0.3mm (so that it read 6.6mm) means that the

laser is actually focused at 6.69mm because of the index of refraction. In other words, the index of refraction in the medium is 1.3, so when the stage micrometer says that the focus has moved up 0.3mm, it has actually moved up 0.39mm.
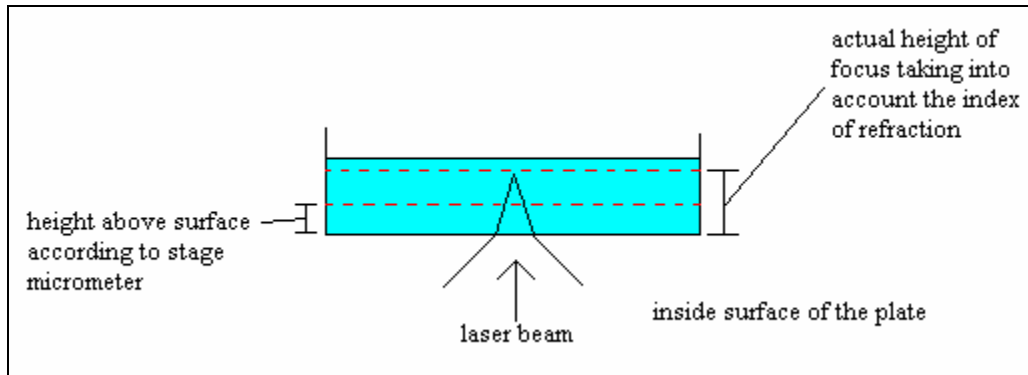

Figure 5

Characterizing Cavitation: Pinpointing the cavitation event is synonymous with pinpointing the height of the beam focus. Because cavitation takes place within the cell medium, we first determined the height at which the laser was focused at the surface of the cell plate.

First, we placed an empty plate on the stage and moved it vertically, so that the range over which the laser cracks the plastic was audible. After recording the range over which the popping occurred, we made burn marks in the plastic with a single laser shot at different heights. We made one burn mark where the popping stopped closer to the surface of the plate. Then we made several more marks at intervals of 0.05mm from that point upwards.

The procedure was carried out on four plates to ensure that the results were repeatable. The plates were analyzed with an inverted microscope, which we focused on the surface of the plate before looking for burn marks. The spot with the tightest burn mark at the surface indicated the height of the surface, since we knew the height at which the spot was made. The pictures below were all taken at the surface of each of the four plates. The first time we used this

method, it clearly indicated that the laser was focused on the top surface of the plate when the micrometer stage read 6.3mm.

Plate 1   Plate 2   Plate 3   Plate 4

6.3mm →   surface of plate
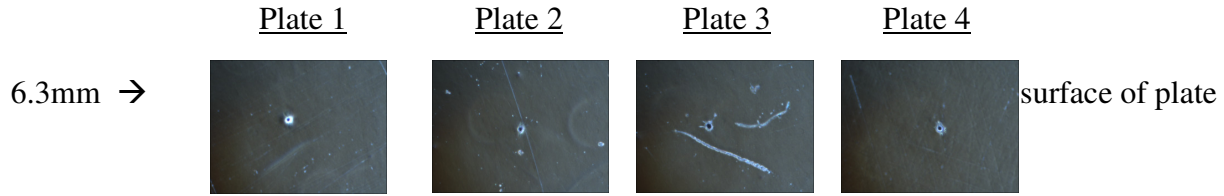
Unfortunately, the micrometer reading at the surface varied over weeks, perhaps due to the fact that the stage apparatus sags. The height of the plate is not consistent over long periods of time but is constant for long enough to be measured accurately using the above procedure. Therefore, we checked the height of the focus before each experiment. Furthermore, at least one spot per experiment was reserved to make a burn mark at the surface to ensure that the beam focus fell where it was thought to according to the above procedure.

From this point on, distances will be given relative to the top surface of the plate. For example, 0mm will mean that the laser beam is focused on the surface of the plate, whereas 0.50mm will mean that the beam is focused inside the cell medium 0.50mm above the surface of the plate.

Calculating Rayleigh Range: The Rayleigh range is the depth over which a laser remains in focus. For a TEM_{00} Gaussian beam, the Rayleigh range is defined by the following equation, where Z_R is the Rayleigh range, $2\omega_0$ is the beam diameter, and λ is the wavelength:

$$Z_R = \frac{\pi\omega_0^2}{\lambda} \hspace{3cm} (2)$$

We found the beam diameter empirically by measuring the diameter of a burn mark made by the laser focused on the surface of a cell plate. We then measured the burn mark diameter using an inverted microscope and stage micrometer (Figures 6, 7). Both pictures below were taken at 100x magnification.
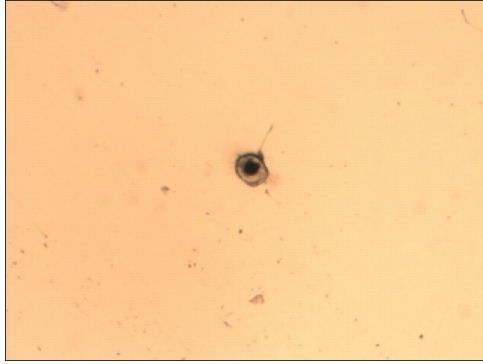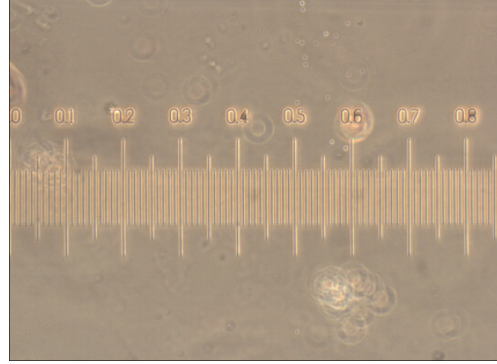
Figure 6: Burn mark at surface

Figure 7: Stage Micrometer

The diameter of the spot was measured with the stage micrometer to be 50μm, and the Rayleigh range was subsequently calculated (using Equation 2) to be 50μm. Inside the media, this is multiplied by the index of refraction to give 65μm, which means that cavitation occurs within ±30μm of the focus.

Preparation of the Cells: *Xenopus* kidney cells (A6) were grown to confluence in the bottom of 35mm NUNC culture plates. Immediately before the cells were brought to the laser, the old cell medium was decanted and replaced with the proper concentration of dextran-conjugated Fluorescein Isothiocyanate (FITC-Dextran) in cell medium. In this case, we used 4000Da FITC-Dextran, so the old medium was replaced with 0.048mL of the dye and 2.952mL of medium, for a total of 3mL in each plate. After the dishes were prepared, they were wrapped in foil to protect them from sunlight (which may photobleach the dye) and transported to the laser.

Laser Procedure: The plates were placed on the stage apparatus and, using single laser pulses, we created sixteen spots in a grid pattern at the desired heights, once again using the micrometers to control the position of the plate (Figure 8). While the pulses nominally had energies of 200μJ and 400μJ respectively, the pulse energy actually varied slightly from shot to shot. After the first experiment, four shots were taken at each height to ensure that results were repeatable. The heights at which shots were taken were chosen in increments (usually of 0.05mm) starting with

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the inside surface of the plate. This meant that the four spots indicated by the box in Figure 8 were always reserved for surface shots.
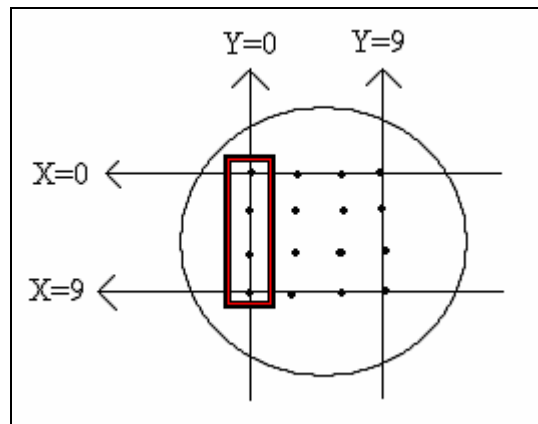

Figure 8: Overhead view of a dish on stage apparatus

Post-laser Procedure: Once the laser procedure was complete, the dye and media mixture was decanted off the cells and the plate was washed, first with 2mL of fresh media and then with 1mL of fresh media twice. We put fresh media back into the plates and used the inverted microscope to take two pictures of each spot: one in bright field and one with the fluorescence lamp on to see if any dye got into the cells. We also recorded the radius of the circle of cells removed from the plate at each spot. (Figure 9)
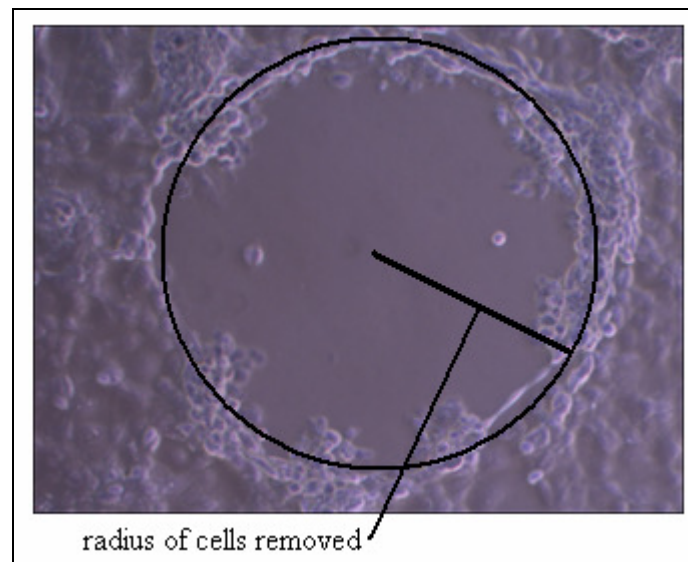

Figure 9

Results

The relative stage position is the height of the focus above the plate recorded from the z-direction micrometer. For Experiment One, the laser did not remove any cells from the plate above the relative stage position of 0.56mm, but cavitation could still clearly be heard. Since the medium is 3mm high inside the plate, this is because cavitation occurred too far away to have an effect. The average laser energy used in Experiment One was 200μJ/pulse. According to Equation 1, this corresponds to a bubble radius of 0.62mm.

<u>Experiment One</u>:

relative stage position (mm)	height adjusted for index of refraction (mm)	radius of removed cells (mm)
0.08	0.104	0.19
0.11	0.143	no spot
0.14	0.182	0.21
0.17	0.221	0.275
0.2	0.26	no spot
0.23	0.299	0.215
0.26	0.338	0.2
0.29	0.377	0.225
0.32	0.416	no spot
0.35	0.455	0.31
0.38	0.494	0.35
0.41	0.533	0.35
0.44	0.572	0.335
0.47	0.611	0.25
0.5	0.65	0.35
0.53	0.689	0.35
0.56	0.728	0

Plotting the radius of removed cells versus the height of the laser focus (adjusted for the index of refraction) results in the following graph, where the expected bubble radius is indicated by the vertical line:
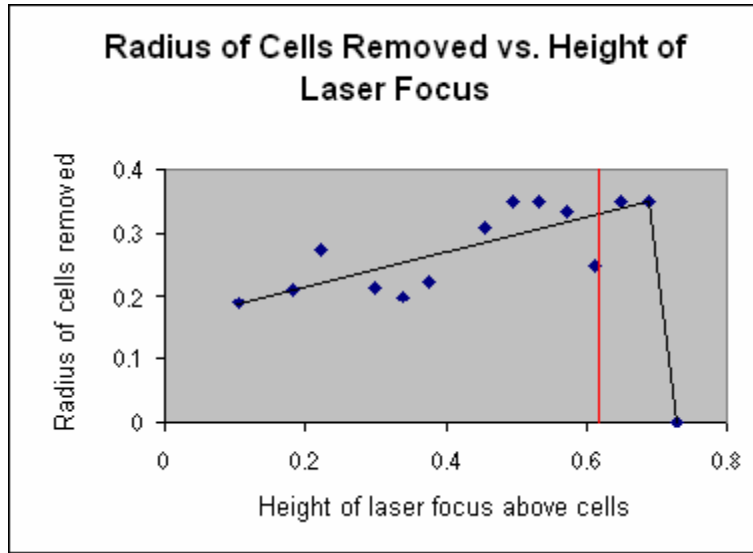
Graph 1: Experiment One

For Experiment Two, as in Experiment One, an average laser energy of 200μJ/pulse was used. Four shots were taken at each height, so the recorded radius of removed cells is the average radius for those four shots. Once again, the number of cells removed from the plate dropped to zero suddenly (though cavitation was still heard), this time at the relative stage height of 0.5mm above the cells.

Experiment Two:

relative stage position (mm)	height adjusted for index of refraction (mm)	average radius of removed cells (mm)
0	0	0.155
0.1	0.13	0.234
0.2	0.26	0.2335
0.3	0.39	0.284
0.4	0.52	0.2665
0.5	0.65	0

Plotting the radius of removed cells versus the height of the laser focus above them (adjusted for the index of refraction) results in the following graph where the expected bubble radius is indicated by the vertical line:

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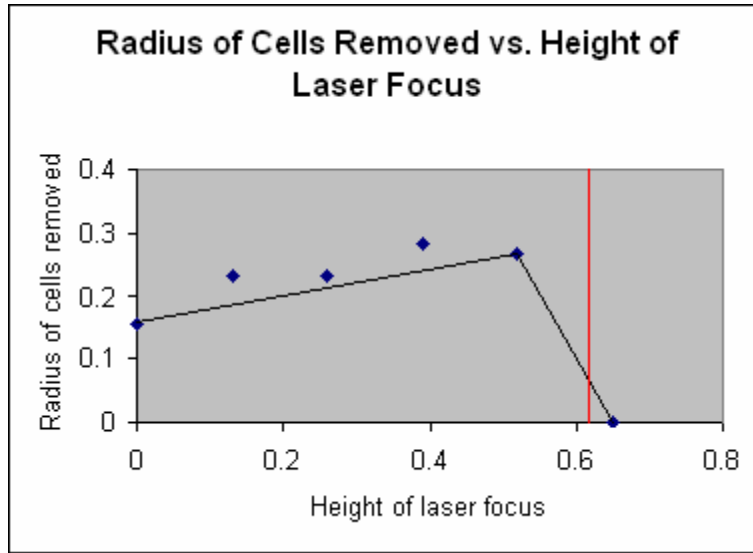
Graph 2: Experiment Two

For Experiment Three, we once again took four shots at each height, so the recorded

radius of removed cells is the average radius for those four shots. This time the average laser

energy we used was400μJ/pulse. According to equation 1, this corresponds to a cavitation

bubble radius of 0.78mm. As in Experiments One and Two, the radius of removed cells dropped

off suddenly, this time at the relative stage height of 0.6mm.

Experiment Three:

relative stage height (mm)	height adjusted for index of refraction (mm)	average radius of removed cells (mm)
0	0	0.2
0.1	0.13	0.2915
0.2	0.26	0.294
0.3	0.39	0.3115
0.4	0.52	0.2935
0.45	0.585	0.25
0.55	0.715	0.2585
0.6	0.78	0

Plotting the radius of removed cells versus the height of the laser focus above them

(adjusted for the index of refraction) results in the following graph where the expected bubble

radius is indicated by the vertical line:
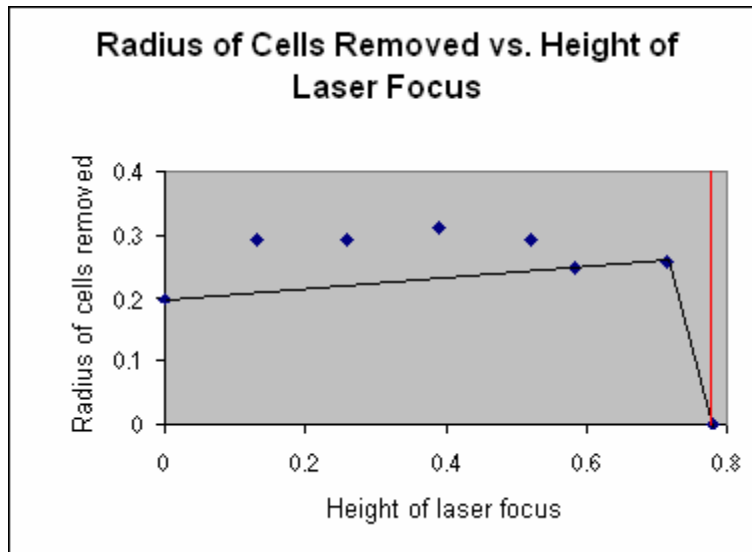
Graph 3: Experiment Three

Several shots taken during the above experiments fell inside the plastic of the plate. In other words, the beam was focused below the surface of the plate. In each of these cases, no cells were removed, nor did any poration occur. Figures 10 and 11 show an example of such a spot.
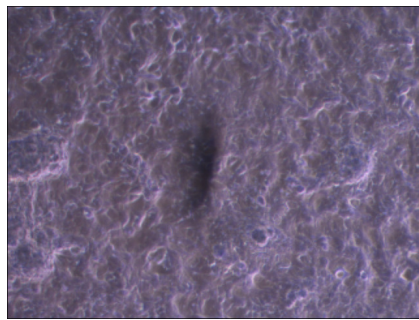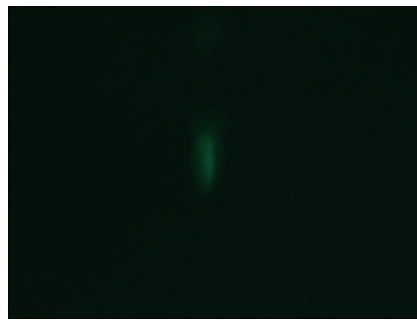

| Figure 10 | Figure 11 |

Discussion

Figures 10 and 11 show that when the beam was focused inside the plastic, no cells were removed and no poration occurred. No cavitation occurs when the beam is focused inside the plastic. This means that, while a shock wave is sent out as a result of the beam causing the plastic to crack, no fluid flows over the cells. This strongly suggests that the fluid flow, not the shock wave, is responsible both for the removal of cells from the plate and for the poration of the

cells. Further supporting fluid flow as the cause of cell removal and poration is the fact that, beyond the point where the beam is focused above the cells at a height comparable to the radius of the cavitation bubble, the effect drops off very rapidly. In Experiments One and Two, the bubble size was 0.62mm and the effect dropped off at heights (adjusted for the index of refraction) of 0.728mm and 0.65mm respectively. Keeping in mind the fact that the Rayleigh range (also adjusted for the index of refraction) is 0.065mm, it seems reasonable to say that the effect drops off at height comparable to the bubble radius. For Experiment Three, when the bubble size was 0.78mm, the effect dropped off at a height (adjusted for the index of refraction) of 0.78mm. If the shock wave were responsible, the effect would not drop off as quickly.

Conclusion

When cavitation occurs in the cell medium, it sends out a shock wave and displaces some of the cell medium, causing fluid to be pushed out over the cells. A circle of cells is removed, and a ring of cells surrounding that circle are porated. We know they were porated because of the presence of 4kDa FITC-Dextran dye in the cells after exposure to the laser. The results included in this report support the fluid flow over the cells, produced by the expanding bubble, as the cause of both the removal and poration of the cells.

References

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