Published in final edited form as:

Photochem Photobiol. 2009 ; 85(5): 1218–1224. doi:10.1111/j.1751-1097.2009.00581.x.

Laser Selection Significantly Affects Cell Viability Following Single-Cell Nanosurgery

Maxwell B. Zeigler and Daniel T. Chiu*

Department of Chemistry, University of Washington, Seattle, WA

Abstract

This paper compares the viability of over 700 NG108 cells after membrane disruption either with a single 3 ns pulse at 337 nm or with a 5 ms train of 110 fs pulses (80 MHz) at 770 nm. Cell viability was monitored over a period of 12 h so as to understand the effect of laser ablation-induced cell apoptosis. The use of one-photon membrane disruption with the UV-laser resulted in ~36% cell viability after 12 h while the use of two-photon ablation with the femtosecond laser resulted in a much higher viability of ~79% after 12 h, which was the same within error of the ~79% viability of cells in the control group. Changing the laser power to achieve a 90% probability of membrane disruption (PMD) from 50% PMD did not change the percentage of viable cells after 12 h, regardless of whether one- or two-photon ablation was employed. A systematic comparison between different methods of cellular ablation and their effect upon the viability of single cells has not been done before over such a long time frame. These results show the importance of laser choice when cell viability postsurgery is a concern.

INTRODUCTION

Lasers have long been used as tools for the manipulation of single cells. A laser with a Gaussian intensity profile, for example, will form a single-beam gradient trap (or optical tweezer) when focused through a high numerical aperture objective (1); optical tweezers have been used extensively for the manipulation of single cells, organelles and micro/nanoparticles. Any cellular structure that is resolvable by light microscopy can be altered by laser ablation, allowing for improved spatial and temporal resolution over previous methods like micromanipulation (*e.g.* with micropipettes or microneedles) for altering cellular compositions. Optical manipulation also offers other benefits such as: high throughput, high reproducibility and no direct physical contact with the cells, which allows for a sterile experimental environment. Laser ablation can cause disruption of the cell's cytoskeleton or organelles (2), facilitate *in vitro* fertilization by weakening of the oocyte's zona pellucida (3), ablation of plant cell walls for transfection (4), or to assist in patch clamping (5). Cellular membrane disruption also allows optical trapping of subcellular compartments for removal from the cell (6), or transient cell permeabilization for introduction of exogenous molecules into the cell (7–9). Visualization and surgery of subcellular components can be improved by the addition of photosensitizing dyes or green fluorescent protein tagging (10). Addition of material can take place via plasma-mediated or mechanical delivery to multiple

*Corresponding author: Chiu@chem.washington.edu (Daniel T. Chiu).

cells (11–13), or transient membrane disruption of a single cell (14–17). Laser surgery has also been used on a larger scale for a variety of medical procedures or lysis of entire cells for single-cell characterization (18), the study of insect embryonic development (19), or for ablating single synapses to modify neural networks in *C. elegans* (20).

In the past years, we have been working to develop and apply optical techniques for single-cell nanosurgery, in which subcellular structures either removed or introduced into a living mammalian cell in culture (6,20,21). In this procedure, a small portion of the cell membrane is disrupted; this disruption can occur via a variety of different laser–membrane interaction mechanisms that are determined primarily by the irradiance within the focal volume (22). The laser–membrane interaction can occur over a wide range of timescales from fs to cw and use a variety of lasers. The mechanism of optical disruption has been well-studied in the literature (23–25), and can occur due to photothermal interaction, photoablation or plasma-induced ablation (22). Photoablation occurs when absorption of a single photon by an electron promotes the electron from a bonding to a nonbonding orbital, resulting in dissociation of the molecule. Photoablation also results in a mechanical pressure wave radiating from the focal volume, a mechanism also known as cavitation. Plasma-induced ablation is due to a multiphoton absorption process resulting in quasi-free electrons and ionization within the focal volume (23). Like photoablation, plasma-induced ablation may also lead to the formation of a cavitation bubble. Plasma-induced ablation is also associated with plasma formation, which can minimize excess damage in nearby tissues (26). These different mechanisms of laser–cell interaction can lead to significantly different outcomes for the targeted cell. The critical factors that determine which of these mechanisms dominate in membrane disruption are the duration of the laser pulse and its irradiance. Although there is a large body of literature that discusses the relationship between pulse duration and ablative mechanism, there are few functional studies on single-cell viability postlaser ablation (21). In this paper, we compare the effect of a nanosecond laser pulse (at 337 nm) and femtosecond laser pulses (at 770 nm) on single-cell viability postmembrane disruption.

In single-cell nanosurgery, we first optically trap—either with optical tweezer (21) or with polarization-shaped optical vortex trap (6)—the sub-cellular organelle of interest, and then ablate a small opening ($\sim 1 \mu\text{m}$) in the membrane, hence, we can remove the organelle from the cell. In this paper, we focus on the effect of membrane disruption on cell viability, because our previous study established that cell viability in this procedure is not affected by organelle removal or irradiation by optical tweezers, but due to the disruption of the plasma membrane (21). In the previous study, cellular damage was shown to lead quickly to either recovery or necrosis, and that cells would go through a period where they exhibited morphological stress responses like membrane blebbing before recovering. This prior study, however, only monitored cell viability postsurgery over a short timescale (4 h), while cell apoptosis has been known to occur over a longer timescale (27,28). In the current study, we have followed the effect of membrane disruption on single cells over a period of 12 h using a combination of live/dead stain, apoptosis assay and morphological observations, and compare the effect of one-photon (nanosecond UV pulse) and two-photon (femtosecond near IR pulses) membrane disruption on cell viability and apoptosis. In total, we have followed over 700 cells and determined that a significant fraction of cells undergo apoptosis and that two-photon membrane disruption offers the best result in enhancing cell viability.

MATERIALS AND METHODS

Cell culture

NG108-15 cells (ATCC) were cultured in 75 cm² culture flasks in an incubator kept at 37°C with 5% atmospheric CO₂. Cells were grown in Dulbecco's modified Eagle's medium (Gibco #11965) with 8% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Gibco). Cells were split into home-made poly-(dimethylsiloxane) (PDMS) wells bonded to a photoetched glass coverslip (Bellco Glass) by bringing the pieces together after oxidation in oxygen plasma for 1 min. Cells were split into the PDMS wells 24 h before surgery and the surgery was conducted in the cell's native media. The entire surgery was conducted and all images were taken of cells that had been split into the PDMS wells. Cells were split at a low density to aid in identification during surgery and subsequent observation.

Cell surgery

Two home-built optical setups were used for the cell surgery procedures. The schematic representation shown in Fig. 1 is a combination of the two optical setups which were on separate tables. The schematics were combined to economize figure space. The one-photon setup can be described as follows: a pulsed 337 nm N₂ laser with a 3 ns pulse width (Laser Sciences, Inc.) was spatially filtered using an aspheric lens pair and a 100 μ m pinhole to improve beam profile. Pulse duration is reported as the full-width at half-maximum intensity. The intensity of the pulsed laser was modulated using a neutral density filter. The beam was passed into a TE300 microscope (Nikon Instruments) with a 100 $\times$ 1.3 NA Super Fluor objective (Nikon Instruments) with improved UV transmission. Laser power was 800 nJ per pulse before the objective with 10% pulse-to-pulse variability and ~30% objective transmission at 337 nm. The focal spot size of the laser was determined by measuring the area of ablation of a thin layer of PMMA spin-coated onto a glass coverslip. Spot size for the UV laser was ~1.3 μ m in diameter. The power level was chosen by finding the minimum pulse power necessary to disrupt the NG108 cellular membrane 50% of the time.

For the two-photon surgery, a Ti:Sapphire tuned to 770 nm with a 110 fs pulse width and 80 MHz pulse frequency (Newport Tsunami) was directed through a neutral density filter (Thorlabs, Inc.) and a 5 ms mechanical shutter (Uniblitz) with a home-built controller. The beam was passed into a TE2000 inverted microscope (Nikon Instruments) with a 100 $\times$ 1.3 NA Super Fluor objective. Laser power was 0.150 W before the objective and the spot size was ~1.3 μ m in diameter, measured by the same technique described above. The focal spot size of the Ti:Sapphire laser was increased to more closely resemble the spot size of the UV laser. The primary descriptive characteristics of the two lasers are shown in the table inset in Fig. 1.

Cell viability

For the time-sequenced cell viability assay, ethidium homodimer (Invitrogen) and calcein AM (Invitrogen) were used to assay cell viability over the course of 12 h. The cells were stained with 4 μ M calcein AM and 2 μ M ethidium homodimer every hour for 12 h. After each assay, the cells were rinsed and returned to their native media.

Cell viability and apoptosis was monitored by a combination of fluorogenic dyes ethidium homodimer, MitoTracker Red and the fluorescently labeled antibody Annexin V Alexa488 (Invitrogen). Cells were stained 11.25 h after surgery with 200 nM MitoTracker Red and 2 μ M ethidium homodimer and incubated for 30 min; they were rinsed and then stained with Annexin V Alexa488 for 15 min. Cells were viewed using epifluorescent illumination in a TE300 inverted microscope, a 20 $\times$ 0.4 NA Plan objective (Nikon) and epi-illuminated by a 488 nm sapphire laser (Coherent). Cells were imaged using a CCD camera (Cohu 4912). Color images were taken using differential interference contrast microscopy (DIC) with de Sénarmont compensator and a color SLR CCD camera (Olympus E-300).

RESULTS AND DISCUSSION

Choice of cell line

We have shown in our previous study that cell viability postsurgery is widely variable depending on the cell line (21). In this prior work, we used a single 3 ns pulse at 337 nm for membrane disruption and a 1064 nm YAG laser for optical trapping and removal of subcellular organelles. Of the three cell lines we studied (CHO: Chinese Hamster Ovary cells; NG108: a neuroblastoma fused with glioma cell line; ESD3: murine embryonic stem cells), we observed their 4-h postsurgery viability varied from ~80% (CHO) to ~30% (NG108) and ~10% (ESD3). The largest effect on cell viability was shown to be due to membrane disruption by the UV laser. To form a useful comparison between the one-photon disruption with 337 nm nanosecond laser and the two-photon disruption with 770 nm femtosecond laser, we decided the NG108 cells would offer the most information: if two-photon offers better cell viability than the one-photon process, we might not see the improvement with CHO cells because of their already high viability at 80%; on the other hand, if two-photon offers less cell viability than one-photon disruption, then we might not see the decrease in cell viability with ESD3 cells because of their already low viability of 10%.

Membrane disruption with nanosecond UV laser leads to apoptosis

As mentioned above, previous characterizations of cell viability did not encompass a sufficient duration to observe surgery-induced apoptosis. Figure 2 shows apoptosis induced in NG108 cells after single-pulse membrane disruption with the nanosecond UV laser. In this study, we compared cell viability over 12 h between cells that have undergone surgery and those that have not (control). All cells were stained with ethidium homodimer (ethidium) and calcein AM and then washed before counting the number of live and dead cells. Ethidium and calcein AM are common live/dead stains. When a cell is no longer viable, its membrane becomes permeable to large molecules such as ethidium, which intercalates with the cell's DNA and exhibits strongly orange fluorescence. Calcein AM is a membrane permeable substrate that becomes fluorescent after it is cleaved by a cell's esterases, thus causing the cytoplasm of viable cells to fluoresce green. Therefore, cells were determined to be viable if they show green fluorescence in the cytoplasm and dead if they exhibit orange fluorescence in the nucleus. Figure 2a shows a flowchart that summarizes our procedure, in which we used ethidium and calcein AM to check the viability of both control cells and cells that underwent surgery every hour for 12 h.

Figure 2b shows our results, in which the difference in survival rates between hours 1 and 12 of the two groups (control *versus* postsurgery cells) is clear. More than 50 cells were observed in both the control group and the UV laser group for a total of over 100 cells. Between surgery and hour 1, 39% of the NG108 cells survived after their membranes had been disrupted compared with 95% survival in the control group. We attribute cell death between 0 and 1 h mostly to cell necrosis. Between hours 1 and 12, of the 39% postsurgery cells that survived after hour 1, only 39% of the remaining cells survived at hour 12. In contrast, of the 95% control cells that are alive after hour 1, 80% of the remaining cells are still viable at hour 12. Overall, 16% of the UV-laser-treated group was viable after 12 h compared with 76% in the control. The right-hand side of Fig. 2b summarizes the percent of cells that died between hours 1 and 12, which we attribute to apoptosis. The ratio of cells that died from hours 1 to 12 between laser disrupted cells to control cells is ~3:1. This result suggests that laser ablation of cell membrane affects not only short-term cell viability, but also can lead to apoptotic cell death later.

Morphological effects of cell surgery

In addition to fluorescent live/dead stains, the morphology of cells also provides valuable information in determining necrotic and apoptotic cells. Nonviable cells often develop large membrane blebs, lose adherence to the surface, develop granular appearance of cytoplasm, have condensation of the nucleus, and conform to a more spherical shape. In Fig. 3, one cell was viewed for 30 min (a1–a8) after it underwent surgery (a1); here, the cell increased in granularity (a2–a8) and formed a large membrane bleb and died (a5–a8). Another cell was viewed after surgery for a period of 12 h (b1–b12); this cell formed blebs (b3–b5), then recovered (b6–b11), but ultimately underwent apoptosis (b12). It is important to note that although the cell appeared healthy and recovered after 10 min, cell apoptosis was verified visually after 12 h.

Apoptosis assay

Given the presence of apoptosis, in our comparison between one-photon and two-photon membrane disruption, we chose to monitor the cells after 12 h using three assays: MitoTracker Red, ethidium and the fluorescent antibody conjugate Annexin V Alexa488. MitoTracker Red was used to monitor mitochondrial membrane potential; it is strongly fluorescent in the membranes of mitochondria that are able to maintain a potential gradient and fluorescence decreases sharply as mitochondria lose function and are unable to maintain a potential gradient. Annexin V Alexa488 (AVA488) is a membrane impermeable antibody conjugated to a fluorophore (Alexa488) that binds to phosphatidylserine (PS). AVA488 can be used to detect apoptosis because PS is localized to the inner leaflet of the lipid bilayer of a healthy cell but becomes present on the outer leaflet and allows AVA488 to bind to it as the cell undergoes apoptosis. It is quite beneficial to use AVA488 combined with MitoTracker Red because the two in conjunction can be used to visualize early stages of apoptosis before more obvious morphological changes become visible.

Figure 4 shows the appearance of a healthy (a1–a2) and apoptotic (b1–b2) cell as visualized with these three assays. Compared with the healthy cell in a2, the apoptotic cell in b2 shows increased granularity in the cytoplasm, decreased MitoTracker Red fluorescence and visible

fluorescence from both AVA488 and ethidium. In all subsequent experiments using these three assays, the viability of each cell was only counted once at the end (~ 12 h) of the experiment, which was done to maximize cell viability by limiting cell exposure to cytotoxic membrane stains and repeated rinsing.

Viability of cells after one- and two-photon membrane disruption

We disrupted the membranes of over 600 cells and then monitored their viability after 12 h to better understand the effect of membrane disruption using one-photon *versus* two-photon ablation. NG108 cells were treated with two different lasers, each at two different powers. For each laser, we chose two power levels, one of which corresponds to a 50% probability of membrane disruption (PMD), and another that corresponds to a 90% PMD. Over 100 cells were monitored for each laser treatment and each power level alongside over 200 cells whose membranes were left intact for a total of over 600 cells. To determine whether the membrane was disrupted, we used single laser pulses (one-photon surgery) or pulse trains (two-photon surgery) and visually verified the formation of transient membrane holes.

For the N₂ laser the energy required was 800 nJ and 1.0 μ J for 50% and 90% PMD, respectively; in this paper, we used a single pulse to ablate the membrane. For the fs laser, we used a 5 ms train of pulses at 80 MHz and at powers of 150 mW and 190 mW for 50% and 90% PMD, respectively. If a hole was not visible after one pulse or pulse train, the cell was excluded from further observation. Again, the focal spot size for both lasers was ~ 1.3 μ m in diameter, and all powers were measured before the objective. The viability of each cell was determined by observing cell morphology concurrently with the three assays shown in Fig. 4. Figure 5a is a flow chart that summarizes the procedure we used in this experiment.

Figure 5b is a histogram of cell survival that shows our results. There is a large difference in cell viability between the control and UV-ablated cells after 12 h, consistent with our previous findings (21). The control group remained 79% viable after 12 h, while 36% of the cells disrupted by a UV pulse at both 50% and 90% PMD were viable after 12 h. The cells disrupted by the femtosecond pulse train were 79% viable in both the 50% PMD and 90% PMD groups, which is identical, within error, to the viability rate of the control group. For both the one-photon and two-photon laser treatments, ~25% more energy was required to increase the probability of membrane disruption from 50% to 90%, but in each case we have shown that the increase in energy used for ablation had no effect on cell viability. Additionally, the amount of total energy used to disrupt the cell's membrane is higher by a factor of ~400 in the case of the two-photon membrane disruption mechanism compared with the one-photon membrane disruption. This shows that the amount of energy used for cell surgery is less important than the mechanism of ablation.

CONCLUSION

The experiments presented here constitute a functional study of the end effect of laser ablation on cell viability. Our biological results agree well with the physical studies on the mechanism of one-photon and two-photon ablation reported in the literature, which shows one-photon ablation usually results in the formation of larger cavitation bubbles that lead to

more extensive damage than two-photon ablation. From the results of our study, we can draw three conclusions: (1) Although most cell death occurs immediately (within 1 h) after laser surgery, a significant portion of cells die because of laser-surgery-induced apoptosis, (2) two-photon ablation offers significant enhancements in cell viability compared with the one-photon ablation and should be used whenever possible, and (3) the difference in energy required to increase PMD to 90% from 50% is not large enough to affect cell viability, thus the amount of energy used to disrupt a cell's membrane is not as important as the mechanism of ablation. The drawback of two-photon ablation is the significant increase in cost of instrumentation, as femtosecond lasers currently are about an order of magnitude more expensive than nanosecond pulsed lasers. Nevertheless, this added cost might be worthwhile for applications that require high cell viability postlaser surgery.

Acknowledgments

We gratefully acknowledge support of this work by the National Institutes of Health (EB 005197).

References

1. Neuman KC, Block SM. Optical Trapping Rev Sci Instrum. 2004; 75:2787–2809. [PubMed: 16878180]
2. Heisterkamp A, Maxwell IZ, Mazur E. Pulse energy dependence of subcellular dissection by femtosecond laser pulses. Opt Express. 2005; 13:3690–3696. [PubMed: 16035172]
3. Blake DA, Forsberg AS, Johansson BR, Wikland M. Laser zona pellucida thinning—An alternative approach to assisted hatching. Hum Reprod. 2001; 16:1959–1964. [PubMed: 11527905]
4. Tirlapur UK, König K. Femtosecond near-infrared laser pulses as a versatile non-invasive tool for intra-tissue nanoprocessing in plants without compromising viability. Plant J. 2002; 31:365–374. [PubMed: 12164815]
5. Henriksen H, Taylor AR, Brownlee C, Assmann SM. Laser microsurgery of higher plant cell walls permits patch-clamp access. Plant Physiol. 1996; 110:1063–1068. [PubMed: 11541144]
6. Jeffries GDM, Edgar JS, Zhao Y, Shelby JP, Fong C, Chiu DT. Using polarization-shaped optical vortex traps for single-cell nanosurgery. Nano Lett. 2007; 7:415–420. [PubMed: 17298009]
7. Kohli V, Acker JP, Elezzabi AY. Reversible permeabilization using high-intensity femtosecond laser pulses: Applications to biopreservation. Biotechnol Bioeng. 2005; 92:889–899. [PubMed: 16189821]
8. Mehier-Humbert S, Guy RH. Physical methods for gene transfer: Improving the kinetics of gene delivery into cells. Adv Drug Deliv Rev. 2005; 57:733–753. [PubMed: 15757758]
9. Stevenson D, Agate B, Tsampoula X, Fischer P, Brown CTA, Sibbett W, Riches A, Gunn-Moore F, Dholakia K. Femtosecond optical transfection of cells: Viability and efficiency. Opt Express. 2006; 14:7125–7133. [PubMed: 19529083]
10. Khodjakov A, Cole RW, Rieder CL. A synergy of technologies: Combining laser microsurgery with green fluorescent protein tagging. Cell Motil Cytoskeleton. 1997; 38:311–317. [PubMed: 9415373]
11. Krasieva TB, Chapman CF, LaMorte VJ, Venugopalan V, Berns MW, Tromberg BJ. Cell permeabilization and molecular transport by laser microirradiation. SPIE Proc. 1998; 3260:38–44.
12. Palumbo G, Caruso M, Crescenzi E, Tecce MF, Roberti G, Colasanti A. Targeted gene transfer in eukaryotic cells by dye-assisted laser optoporation. J Photochem Photobiol B: Biol. 1996; 36:41–46.
13. Soughayer JS, Krasieva T, Jacobson SC, Ramsey JM, Tromberg BJ, Allbritton NL. Characterization of cellular optoporation with distance. Anal Chem. 2000; 72:1342–1347. [PubMed: 10740880]

14. Tao W, Joyce W, Stanbridge EJ, Berns MW. Direct gene transfer into human cultured cells facilitated by laser micropuncture of the cell membrane. *PNAS*. 1987; 84:4180–4184. [PubMed: 3473500]
15. Tirlapur UK, König K. Targeted transfection by femtosecond laser. *Nature*. 2002; 418:290–291. [PubMed: 12124612]
16. Paterson L, Agate B, Comrie M, Ferguson R, Lake TK, Morris JE, Carruthers AE, Brown CTA, Sibbett W, Bryant PE, Gunn-Moore F, Riches AC, Dholakia K. Photoporation and cell transfection using a violet diode laser. *Opt Express*. 2005; 13:595–600. [PubMed: 19488389]
17. Schneckenburger H, Hendinger A, Sailer R, Strauss WSL, Schmitt M. Laser-assisted optoporation of single cells. *J Biomed Opt*. 2002; 7:410–416. [PubMed: 12175291]
18. Quinto-Su PA, Lai HH, Yoon HH, Sims CE, Allbritton NL, Venugopalan V. Examination of laser microbeam cell lysis in a PDMS microfluidic channel using time-resolved imaging. *Lab Chip*. 2008; 8:408–414. [PubMed: 18305858]
19. Supatto W, Débarre D, Moulia B, Brouzés E, Martin JM, Farge E, Beaurepaire E. In vivo modulation of morphogenetic movements in *Drosophila* embryos with femtosecond laser pulses. *PNAS*. 2004; 102:1047–1052.
20. Allen PB, Sgro AE, Chao DL, Doecker BE, Edgar JS, Shen K, Chiu DT. Single-synapse ablation and long-term imaging in live *C. elegans*. *J Neurosci Methods*. 2008; 173:20–26. [PubMed: 18579213]
21. Shelby JP, Edgar JS, Chiu DT. Monitoring cell survival after extraction of a single subcellular organelle using optical trapping and pulsed-nitrogen laser ablation. *Photochem Photobiol*. 2005; 81:994–1001. [PubMed: 15850426]
22. Niemz MH. *Laser-Tissue Interactions: Fundamentals and Applications* Springer, Heidelberg, Germany. 2004:45–150.
23. Vogel A, Noack J, Hüttman G, Paltauf G. Mechanisms of femtosecond laser nanosurgery of cells and tissues. *Appl Phys B*. 2005; 81:1015–1047.
24. Vogel A, Noack J, Nahen K, Theisen D, Busch S, Parlitz U, Hammer DX, Noojin GD, Rockwell BA, Birngruber R. Energy balance of optical breakdown in water at nanosecond to femtosecond time scales. *Appl Phys B*. 1999; 68:271–280.
25. Venugopalan V, Guerra A III, Nahen K, Vogel A. Role of laser-induced plasma formation in pulsed cellular microsurgery and micromanipulation. *Phys Rev Lett*. 2002; 88:078103. [PubMed: 11863944]
26. Hammer DX, Jansen ED, Frenz M, Noojin GD, Thomas RJ, Noack J, Vogel A, Rockwell BA, Welch AJ. Shielding properties of laser-induced breakdown in water for pulse durations from 5 ns to 125 fs. *Appl Optics*. 1997; 36:5630–5640.
27. Jessel R, Haertel S, Socaciu C, Tykhonova S, Diehl HA. Kinetics of apoptotic markers in exogenously induced apoptosis of EL4 cells. *J Cell Mol Med*. 2002; 6:82–92. [PubMed: 12003671]
28. Lizard G, Fournel S, Genestier L, Dhedin N, Chaput C, Flacher M, Mutin M, Panaye G, Revillard JP. Kinetics of plasma membrane and mitochondrial alterations in cells undergoing apoptosis. *Cytometry*. 1995; 21:275–283. [PubMed: 8582250]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1. Schematic representation depicting the optical setup used for one-photon and two-photon membrane disruption and subsequent cell observations. For one-photon surgery, a 100 μ m pinhole was used to spatially filter laser pulses and to improve beam profile. For two-photon cell surgery, a second lens pair was added to adjust the size of the beam and to match the focal spot size to the spot size of the one-photon laser. The one-photon and two-photon treatments were done on different optical tables, but the two laser schematics have been combined to economize space. L = lens, LP = lens pair, D = dichroic, M = mirror, ND = neutral density filter, P = pinhole, S = shutter. Inset: table listing the primary characteristics of consideration between the two lasers.

Figure 2.

(a) A flow chart summarizing the procedure we used to monitor cell viability over 12 h with live/dead stains (ethidium and calcein AM). (b) A histogram showing the viability of NG108 control cells and cells that underwent surgery. Each bar gives the number of cells alive at the given time. The data points labeled “% Apoptotic Cells” shows the percentage of cells that were viable after the first hour, but were not viable at hour 12, and thus are presumed to have undergone apoptosis. Cells that were exposed to a UV pulse were much more likely to undergo apoptosis (*i.e.* died between hours 1 and 12) than the control group. The error bars represent the 95% confidence intervals. Over 50 cells were observed in both the control and the laser-treated group for a total of over 100 cells.

Figure 3.

Monitoring of morphological changes after membrane disruption with a single nanosecond UV-laser pulse. Panels a1–a8 follow an NG108 cell as it was ablated (a1), formed a large bleb and died (a3–a8). Panels b1–b12 follow an NG108 cell as it was ablated (b1), formed blebs (b3–b5), and recovered (b6–b11) before the cell ultimately underwent apoptosis (b12). Arrows in panels a1 and b1 show sites of UV irradiation. A cavitation bubble is visible in panel a1. Cells were kept at 37°C in an integrated incubator on the microscope for long-term observation.

Figure 4.

Apoptosis assay. Panels a1 and a2 are images of the same healthy cell observed using DIC in a1 and then with epi-fluorescence in a2. Panels b1 and b2 are images of a second cell undergoing apoptosis, also observed using DIC (b1) and epi-fluorescence (b2). In panel a2, the intense fluorescence from MitoTracker Red signifies that the mitochondria are able to maintain a potential gradient. In panel b2, the cell is undergoing apoptosis: MitoTracker Red fluorescence is decreased, ethidium has penetrated the cell's membrane and intercalated with the cell's DNA, and Annexin V Alexa488 has bound to the cell's membrane.

Figure 5.

(a) A flow chart summarizing the procedure we used to monitor cell viability 12 h postsurgery using MitoTracker Red, ethidium, and Annexin V Alexa488. (b) Histogram showing overall cell viability after 12 h for 50% and 90% probability of membrane disruption (PMD). The error bars represent 95% confidence interval. At least 200 cells were observed for each of the one-photon, two-photon and control sets for a total of over 600 cells.