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Single-Cell Nanosurgery

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Abstract

This chapter explains the steps necessary to perform laser surgery upon single adherent mammalian cells, where individual organelles are extracted from the cells by optical tweezers and the cells are monitored post-surgery to check their viability. Single-cell laser nanosurgery is used in an increasing range of methodologies because it offers great flexibility. Its main advantages are (a) there is not any physical contact with the cells so they remain in a sterile environment, (b) high spatial selectivity so that single organelles can be extracted from specific areas of individual cells, (c) the method can be conducted in the cell's native media, and (d) in comparison to other techniques that target single cells, such as micromanipulators, laser nano-surgery has a comparatively high throughput.

Keywords

Laser nanosurgery; Optical tweezers; Apoptosis; Opticution; Necrosis

1 Introduction

The effects of focused laser light on living bodies, ranging from single cells to entire organisms, have been thoroughly researched (1–3). At the scale of entire organisms, lasers have been used in neuroscience applications, like the ablation of individual synapses in *Caenorhabditis elegans* (4, 5), or in developmental biology with model organisms such as *Drosophila melanogaster* (6) or zebra fish (7). Laser surgery is also particularly advantageous for single-cell or subcellular studies because mammalian cells are highly heterogeneous and segregated into biologically relevant units (8), such as organelles, which are comparable in size to the diffraction-limited focal spot of a laser.

High numerical aperture (NA) objective lenses can focus laser light down to spots on the order of hundreds of nanometers, which provide excellent spatial resolution without the need to physically manipulate the sample. When these diffraction-limited spots for spatially localized ablation are paired with a single-beam gradient optical trap, also known as optical tweezers, cell surgery can add exogenous material (9) or remove organelles from individual cells (1). Optical tweezers are not limited to biological samples and can be used to trap any object with a higher refractive index than its surroundings, assuming it is of an appropriate size and the trapping laser has sufficient power. A setup with optical tweezers and an ablation laser is amenable to a wide array of complementary biophysical laboratory techniques, such as fluorescence microscopy, and they have generated great interest for some time (10, 11).

Post-surgery, the cell may do one of three things: It may recover and remain a viable cell; it may undergo laser-induced necrosis, known as “optication”; or it may undergo apoptosis over a period of many hours. Our area of interest has been in the viability of different cell lines following cell surgery (1) and the effect of different membrane disruption techniques on cell survival (3). The method described here explains how to monitor the long-term viability of a single cell after the removal of an organelle by laser nanosurgery.

2 Materials

2.1 Polydimethylsiloxane Wells Used for Cell Surgery and Staining

1. Sylgard elastomer kit (Dow Corning, Midland, MI).
2. Photoetched glass coverslips (Bellco, Vineland, NJ).
3. Scientific oven.
4. Plasma cleaner (Harrick Plasma, Ithaca, NY).
5. Vacuum pump.
6. Vacuum chamber.

2.2 Cell Culture Equipment and Reagents

1. All cell types were grown in 75-cm² culture flasks and stored in an incubator with 5% CO₂ atmosphere at 37°C.
 - a. NG108-15 cells (ATCC, Manassas, VA) grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 8% fetal bovine serum (FBS) and 1% penicillin/streptomycin.
 - b. CHO-M1 cells (ATCC) grown in DMEM with 10% FBS and 1% penicillin/streptomycin.
 - c. ES-D3 (ATCC) grown in DMEM with 10% FBS and leukocyte inhibitory factor to prevent differentiation.
2. 0.25% trypsin-EDTA solution.
3. Polydimethylsiloxane (PDMS) wells for cell surgery.
4. Milli-Q water.

2.3 Optical Setup

1. Microscope: TE2000 microscope (Nikon, Melville, NY) with epi-illumination port, camera port, turret for optical filter cubes, bright-field light source, and condenser.
2. Objective: 100× magnification 1.3 NA S Fluor oil immersion objective (Nikon, Melville, NY).
3. Differential Interference Contrast (DIC) with de Sénarmont optics for bright-field imaging to improve contrast.

4. Video capture: CCD camera (Cohu, San Diego, CA).
5. Ablation laser: Nanosecond pulse 337-nm N₂ Ultraviolet laser (Laser Sciences, Inc., Franklin, MA).
6. Laser for optical tweezers: continuous wave (CW) Nd:YAG laser with a TEM₀₀ Gaussian beam profile (Spectra-Physics, Mountain View, CA).
7. Laser power meter (Ophir Optronics, Jerusalem, Israel).
8. Assorted neutral density (ND) filters, dichroics, lenses, optical filters, mirrors, and optomechanics (Omega Optical, Burlington, VT; Thorlabs, Newton, NJ; and Chroma Tech Corp, Rockingham, VT).

2.4 Cell Viability Assay

1. 2-μM ethidium homodimer.
2. 200-nM MitoTracker Red (Invitrogen, Grand Island, NY).
3. Annexin V Alexa Fluor 488 (Invitrogen, Grand Island, NY).
4. 488-nm CW sapphire laser (Coherent, Santa Clara, CA).

3 Methods

3.1 Making PDMS Wells

1. Mix the Sylgard elastomer kit base and catalyst thoroughly in a 10:1 ratio in a disposable cup; 100 g of base and 10 g of catalyst are enough to make ~12 wells.
2. Place the uncured silicone mixture in the vacuum chamber and pump air out of the chamber to remove air bubbles.
3. Pour the PDMS into a large petri dish. We have found that placing evenly spaced glass vials with a diameter slightly smaller than the “1” photoetched coverslips makes suitable molds to shape the PDMS as it cures. To ensure that the vials do not move through the PDMS before it hardens, keep the vials in place using a petri dish lid with holes drilled such that the vials go through the holes and thus are held in place by the holes in the lid.
4. Bake at 60°C for at least 1 h to cure.
5. Pull out the glass vials from the PDMS, remove the PDMS from the petri dish, and cut out the individual wells.
6. Expose PDMS and photoetched glass coverslips to oxygen plasma for 60 s.
7. Seal the PDMS to the coverslip by bringing both together; apply light pressure to expunge any air pockets between the glass and PDMS.

3.2 Membrane Disruption

1. Split cells into a PDMS well 24 h before surgery using the cell's native media. The cells should be split at a low density to aid in identification during surgery

and subsequent observation (see Note 1). Just prior to surgery, supplement the media in the PDMS well with ~25% Milli-Q water to induce cell swelling and help organelle extraction.

2. For the pulsed N₂ laser to disrupt the membrane, use a beam expander or lens pair to collimate and expand the beam to fill the back aperture of the microscope objective. This will produce a submicron focal spot size. We would recommend mounting at least one lens base on a rail to facilitate alignment of the focal spot in the axial direction.
3. Each laser pulse needs ~800 nJ of energy per pulse when measured before the objective. Our particular laser has a 10% pulse-to-pulse variability in pulse energy at 2 Hz at a 3-ns duration, and the objective has a roughly 30% transmission efficiency. This power is sufficient to disrupt the plasma membrane of an NG-108 cell with a single pulse to allow organelle extraction 50% of the time. Greater laser powers could lead to excessive cell damage and large cavitation bubbles; lower powers would have no visible effect. We modulate the pulsed laser's power using a neutral density filter and select the laser pulses using a shutter controlled by a transistor–transistor logic (TTL) switch.
4. Laser pulses should target the cell's plasma membrane to maximize membrane disruption and minimize cell damage (see Note 2). Focusing on the coverslip leads to ablation of the coverslip and immediate cell lysis; focusing on the cell cytoplasm creates large cavitation bubbles and excessive cell damage. Regardless of where the laser focus is directed, morphological changes will be evident for all cells following surgery.
5. If post-surgery cell viability is important and the cell line is particularly susceptible to death after surgery, a femtosecond near-IR pulse laser, instead of a nanosecond UV pulse laser, may improve cell viability. The mechanism of membrane disruption greatly affects cell viability after surgery (3). Membrane disruption by multiphoton absorption from a femtosecond laser produces a smaller focal volume, smaller cavitation bubbles, and other effects that help cell viability (2). The drawbacks of using a femtosecond laser for surgery are added cost and complexity.

3.3 Optical Trap Construction

This section provides brief guidance on the construction of a simple, robust, single-beam, gradient-force, three-dimensional optical trap with commercially available components. If more detail or explanation of optical alignment is needed, more comprehensive guides are available (12). With additional modification, trap position sensing (13), vortex-shaped optical traps (14), multiple traps (15, 16), force measurements (17, 18), and a myriad of other techniques are possible (19) but are beyond the scope of this chapter.

1. An inverted microscope is a stable and flexible platform for optical trapping. The back epifluorescence port is convenient for directing the trapping beam into the objective. Most research-grade inverted microscopes also come with a turret where multiple beam splitter cubes may be installed, which makes it convenient

to switch between cell surgery and epifluorescence imaging modes. It is possible to build a platform for optical trapping without a microscope, but this requires a high degree of expertise in microscopy.

2. The objective lens is probably the most critical element of the optical trapping setup. For stable trapping, a high NA objective lens is necessary to generate the tight focus (20), such as the Nikon S Fluor 1.3 NA 100× oil immersion objective lens.
3. CW lasers emitting in the NIR, like the Nd:YAG laser, are used for optical trapping because they produce sufficient power at a wavelength that is relatively transparent to cells. Wavelengths shorter than ~700 nm are more readily absorbed by cells and should be avoided if possible because the high intensity in the focal volume results in excessive photodamage. Also, the laser should have an $M^2 < 1.1$ and emit the TEM₀₀ mode to produce a light gradient steep enough to form a stable optical trap. For the sake of safety, a shutter should be installed at the point immediately after the beam exits the laser (see Note 3).
4. Place a dichroic mirror in the microscope's laser filter cube so that it will reflect IR and UV light but allow visible light to pass through. After alignment of the IR laser, an emission filter used should also block back-reflected IR and UV light from the optical tweezers and the ablation laser (see Note 4).
5. The power of the trapping beam should be minimized, but still be great enough to stably trap the organelle of interest. Most Nd:YAG lasers emit significantly more power than is necessary. The power is best modulated by using a $\lambda/2$ wave plate installed in a rotating mount followed by a polarizing beam splitter and a beam block that absorbs the excess laser power. With this configuration, the laser's power can be attenuated by rotating the wave plate. Controlling the laser power by external means instead of adjusting the drive current of the laser improves the laser power stability.
6. After the power attenuator, insert a pair of lenses into the beam path that expand the beam to fill or slightly over fill the back aperture of the objective. Beam expanders are commercially available for this purpose.
7. The most convenient way to steer the optical trap is by using a combination lens pair and a mirror to create a 4f system. Mount the lens nearest the microscope on an x, y, z movable lens mount and arrange the lens pair so that each lens is the same distance from the central focal spot as its focal length. The lenses closest to the objective can be used to fine-tune the alignment of the optical tweezers. The movement of the final lens along the optical axis can be used to adjust the height of the optical trap above the objective. This adjustment will ensure that the plane of the optical trap and the image are parfocalized (see Note 5). The mirror is installed in a kinematic mirror mount at the conjugate focal plane of the objective and can be used to steer the optical trap (Fig. 1).

3.4 Organelle Trapping

1. Organelle trapping is performed using the optical tweezers described in the previous section. For our setup, a power of ~200 mW at the nosepiece of the microscope generates a sufficient gradient force to trap individual organelles. The power transmitted through the microscope's objective will vary depending on the % transmission through the objective and whether or not the trapping beam is larger than the back aperture of the objective (13).
2. Organelles near the spot of membrane disruption can be extracted from the cell after steering of the trapping beam (see Note 6). Adding Milli-Q water to the media improves the ease of organelle extraction without significantly reducing cell viability, but a small amount of cytoplasm is still often extracted with the targeted organelle (Fig. 2). The extracted organelles are often lysosomes or mitochondria. These organelles can be differentiated with the appropriate fluorescent stain.
3. We have observed that the high fluence of the IR laser leads to fast photobleaching of organelle stains and possible photodamage to the organelle. If the extracted organelle is intended for further analysis, such as single mitochondria capillary electrophoresis (21), photobleaching and photodamage to the organelle can be reduced by using an optical vortex trap (14) instead of the Gaussian optical trap described above.

3.5 Cell Viability Monitoring

1. After surgery, incubate the cells at regular intervals in pre-warmed media for 10 min containing the viability reagents (see Subheading 2.4). The particular dyes are chosen to determine cell viability because (a) they can all be excited with a single 488-nm laser, (b) their fluorescence is spatially and spectrally distinct so they are easily resolved, and (c) in addition to cell necrosis, they also suggest apoptosis prior to the morphological signs of apoptosis, which may take many hours to become apparent. Ethidium homodimer is a membrane-impermeable dye that will brightly stain the cell nucleus if the membrane loses integrity. MitoTracker Red is a mitochondrial stain that will have a significant decrease in brightness if the mitochondria lose the ability to maintain a proton gradient across their membrane. Annexin V Alexa Fluor 488 is a membrane-impermeable antibody that binds to phosphatidyl-serine, a phospholipid that can be found on the external leaflet of a cell's lipid bilayer only during apoptosis.
2. After 10 min, remove the cells from the incubator and wash with fresh pre-warmed media. Cell viability can be determined by observing the fluorescent stains and cell morphology. Some morphological indicators that a cell is dying, such as membrane blebs, a granular appearance in their cytoplasm, more spherical shape, and loss of adherence to the coverslip, can be viewed under bright-field microscopy.
3. Cell apoptosis can be differentiated with bright-field DIC and epifluorescence illumination (Fig. 3). The viable cell (a1, a2) has a normal morphology under

bright field and displays bright-orange fluorescence under epifluorescent illumination from the MitoTracker stain. The cell undergoing apoptosis (b1, b2) has a more granular appearance under bright field. Under epifluorescence, it has decreased emission intensity from MitoTracker but a brightly fluorescent nucleus from the ethidium homodimer and a green fluorescent plasma membrane as Annexin V Alexa 488 binds to the lipid bilayer.

4 Notes

1. Cells are known to migrate over time, a property dependent on the cell line. If long-term observation is necessary, be sure to either split the cells at a lower density or take images regularly to track cell movement. Photoetched glass coverslips also help to track individual cells.
2. When performing cell surgery, watch the surgery with a CCD camera. Do not use the microscope's oculars. IR light can leak through the microscope's oculars so place a light blocker over them when appropriate to prevent accidental exposure.
3. Proper laser safety is crucial and should include the appropriate laser goggles, beam blocks, shut-off keys, IR laser display cards, and so on. Safety measures are especially important in cell laser surgery because both the trapping and ablative lasers are outside the visible range and scattered light from either laser may be intense enough to cause eye damage.
4. Back reflection from optical tweezers can be intense enough to damage the camera so make sure its power is minimized during alignment and that the proper IR emission filter is in place.
5. The distance from the focal spot where an object is trapped by the optical tweezers can change with the object's size and composition. Some tweaking of the trap's focal spot may be necessary when trapping different samples to keep the objects in the same plane as the imaging plane.
6. If the emission filter blocks 100% of the back-reflected IR and UV light, then the trapping beam and ablation laser are invisible to the camera. Organelle extraction is easier if you find a method for tracking the position of the trapping beam and the ablative pulsed laser so that they do not get lost between experiments or are steered outside the field of view of the camera. There are many ways to track the beams depending on the application: It can be as complex as building a trapping positioning system or as simple as marking the laser beam focal points on the monitor with a dry-erase marker.

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Fig. 1.

Schematic of optical setup. The outputs of a pulsed N_2 laser (337 nm; 3-ns pulse width) and a CW Nd:YAG laser (1,064 nm) were aligned collinearly and sent into the microscope. The two beams were directed into the objective (NA = 1.3) by a dichroic mirror and focused onto the cell by the objective lens. The microscope was equipped with Nomarski optics, but the components are omitted in the schematic for clarity. The pulsed N_2 laser ($\sim 0.7 \mu\text{J}$) was used to ablate a small hole ($\sim 1\text{--}3 \mu\text{m}$) in the cell membrane, and the YAG-trapping laser (200 mW) was employed to extract a subcellular organelle (*arrow* pointing away from focal volume). To track individual cells post-surgery, we cultured cells in PDMS wells formed by sealing PDMS to photoetched coverslips (*upper left inset*). *HWP* half-wave plate, *BS* polarizing beam splitter, *BB* beam blocker, *ND* neutral density filter, *M* mirror, *L* lens. Reprinted with permission from (1)

Fig. 2.

Organelle extraction in successful surgeries. An NG108-15 cell (**a**) before, (**b**) during, and (**c**) after surgery; the *arrows* point to the organelle that was extracted. A similar sequence showing a CHO cell (**d**) before, (**e**) during, and (**f**) after surgery. A small cavitation bubble is visible in (**e**); (**f**) shows the organelle (*arrow*) that was completely removed from the cell. An ES-D3 stem cell (**g**) before, (**h**) during, and (**i**) after surgery. Scale bars = 10 μm . Reprinted with permission from ref. 1

Fig. 3. Apoptosis assay. A healthy cell is observed using (a1) DIC microscopy and then with (a2) epifluorescence microscopy. Another cell undergoes apoptosis, as observed using (b1) DIC microscopy and (b2) epifluorescence microscopy. In panel (a2), the intense fluorescence from MitoTracker Red indicates that the mitochondria are able to maintain a potential gradient. In panel (b2), the cell is undergoing apoptosis so the MitoTracker Red fluorescence is decreased. In addition, ethidium has penetrated the cell's membrane and intercalated with the cell's DNA and Annexin V Alexa 488 has bound to the cell's membrane. Reprinted with permission from ref. 3