Intracellular Nanosurgery

By Meghana Honnatti and Gareth Hughes, Zyvex Corporation

Introduction

Selective modification of intracellular chemistry has been the preferred method to study cell function. In recent decades, selective modification of intracellular morphology – deletion or knock-out of entire intracellular organelles or structures – has been gaining significant importance. Directed deletion of such sub-cellular features could prove important to better understand the structural and functional relationship between the organelles and the cell as a whole. The potential of this approach has been beautifully demonstrated in applications such as revealing the structural organization of peptidoglycan fibers of the cell wall [1] and nanosurgery of individual chromosomes [2].

Intracellular organelles could range from a few nanometers to several hundred nanometers. The ability to manipulate and operate on such small organelles depends on the availability of spatially and temporally precise and minimally invasive manipulation techniques and probes. Zyvex Corporation, a leading provider of nanotechnology solutions, now offers the L200 Nanomanipulation System designed specifically for biomedical applications.

This application note presents a brief description of intracellular nanosurgery, its significance and current techniques, and also describes how the L200 system can be used to perform directed intracellular nanosurgery, without causing any other collateral damage to the cell. As a model system for this application note, we have targeted sucrosomes – lysosomes swollen with sucrose – which range in size from 100 nm to 4 µm.

Background

What is intracellular nanosurgery and why is it important?

Cellular surgery is any technique that involves precise manipulation of cellular components without causing significant damage to the cell. The manipulation could encompass any of several operations – injection of molecules into the cell, extraction of molecules from the cell for further analysis, or knock-out of molecules or even entire organelles in the cell. In this application note, we discuss specifically the deletion of nanoscale subcellular compartments.

The ability to selectively delete cellular organelles is important to better understand the structural and functional roles of the particular organelle in the cell. For example, some studies indicate that the mitochondria form physically separate units [3], whereas some others suggest that the mitochondria form a continuous network [4]. In addition, mitochondria have been shown to exhibit morphological heterogeneity in different stages of a cell cycle as well as in different parts of the same cell [3]. Here, deletion of individual mitochondria in a cell could provide more insight into morphological characteristics of mitochondria as demonstrated by Shen *et al* [5] .

Release of the organelle's contents such as proteins, enzymes, and molecules into the intracellular space would provide a better understanding of their role in the cell as well as in the organelle. In addition, pre-packaging of various biochemicals such as proteins, nucleic acids, or even artificial liposomes into an intracellular organelle and subsequent release of the organelles' contents in a directed and timed fashion could open new doors in controlled drug delivery and therapeutic applications.

Current Nanosurgical Techniques and the Zyvex Approach.

Currently available techniques for cellular nanosurgery include near-infrared laser ablation [5-7], pulsed UV laser surgery [8], atomic force microscopy tips [9, 10], and the use of chemical agents. Laser ablation for nanosurgery involves using ultra-short laser pulses such as nanosecond, picosecond, or femtosecond pulses focused onto the target using high numerical aperture objectives to result in plasma-mediated ablation of biological materials [11]. Researchers have also used AFM tips to simultaneously image cells, as well as to perform nanosurgery by "scratching" the tip over the substrate. This method does not ablate the subcellular contents like laser-based nanosurgery.

While each of these techniques are valuable in their own right, they prove limiting to some of the applications discussed in the previous section. For example, in the case of laser ablation, minimal collateral damage to the cell occurs, yet it results in the complete ablation of not only

the subcellular structure in question, but also its contents. In addition, the force applied by the AFM tip for imaging prior to nanosurgery is known to damage certain cell types [9].

Therefore, in order to study nanoscale processes/structures and their physiological importance more effectively, there is a need for a gentle surgical technique that involves minimal invasion and damage to a given cell. Additionally, as with laser and AFM-based techniques, a targeted approach to cellular surgery enables single organelle deletion or modification as opposed to more traditional biochemical methods (which are ensemble approaches). For any cellular surgical technique, it is imperative to be able to manipulate nanoscale structures with nanometer precision.

The Zyvex L200 model provides Life Sciences researchers with the capability of positioning surgical tools, probes, electrodes or pipettes in close proximity to the cell with nanometer precision. This high resolution positioning allows researchers to probe previously-unknown regions of the cell with unprecedented ease and accuracy.

System Setup

Microscope

The L200 can be mounted onto most optical microscopes, as well as on electron microscopes. For this application, the L200 was mounted on a standard inverted optical microscope (Nikon Model TE2000 as shown in **Figure 1**). High magnification and high numerical aperture oil immersion objective lenses (60X or 100X, 1.45 NA) were used to enable visualization of intracellular organelles. Differential interference contrast microscopy aided in further enhancing the contrast of these small organelles.

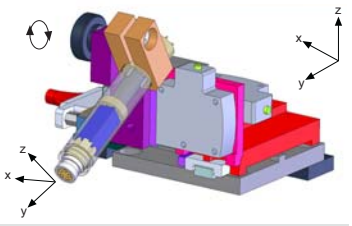

Figure 1a (left) L200 installed on a Nikon Model TE2000 inverted microscope.

Figure 1b (right) Drawing of coarse and fine positioners with axes depiction.

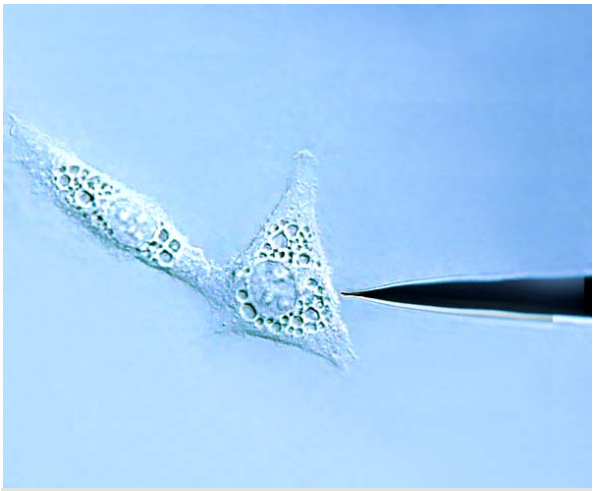

Figure 2 DIC image of a cell with sucrosomes.

Cells

For this application note, we used normal rat kidney (NRK) cells seeded onto a glass-bottomed culture dish. For experiments where biomolecule delivery is demonstrated, NRK cells were incubated overnight with medium containing 30mM sucrose. Sucrose gets internalized into the lysosomes. The indigestible solute can not be cleaved by lysosomal hydrolases and causes the lysosomes to swell, referred to as sucrosomes, as shown in **Figure 2**. We used sucrosomes as a model system to demonstrate nanosurgical techniques. To demonstrate loading of intracellular organelles with biomolecules, we included dextran tagged with the fluorophore TRITC in the culture medium (2 mg/mL). This step can be viewed as an emulation of loading intracellular organelles with specific biomolecules.

Mechanical Probes

Successful mechanical probing of intracellular organelles necessitates surgical tools of similar, if not smaller, sizes. A small diameter mechanical probe is essential for impaling micron-sized sucrosomes. Besides, minimal deformation of cells during manipulations is essential to prevent undesirable mechanical stresses and deformation to the cellular structure. Zyvex, a provider of nanotechnology solutions for applications such as this, offers a wide range of end-effectors as part of our NanoEffector® Tools product

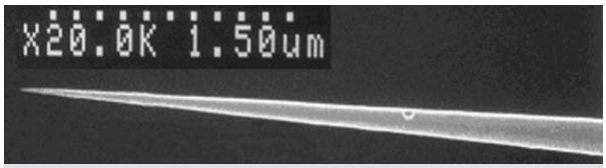

Figure 3 SEM image of a tungsten probe.

line. We used ultrasharp tungsten probes with a 50 - 150 nm tip diameter (see **Figure 3**), for this application. The probes were cleaned with acetone to get rid of any organic residues, followed by rinses in isopropyl alcohol and methanol, and air-dried before use. Cleaned probes were inserted into an adaptor that inserts into one of the holes of the fine positioner (see **Figure 4**). The adaptor was bent to a conformation that allows the adaptor and probe to go over the lip of the cell culture dish.

Manipulation

For this application, a desirable cell and the sucrosome to be deleted were first selected. Once the sucrosome was selected, the approach angle of the probe to the sucrosome was decided, a fine positioner was selected, and then the capillary adaptor with the tungsten probe was inserted into the respective fine positioner. To enable better visualization, the probe was bent (as shown in Figure 4) and inserted in a near horizontal orientation. A horizontal approach angle reduced the flare introduced due to the out-of-focus light of the shank.

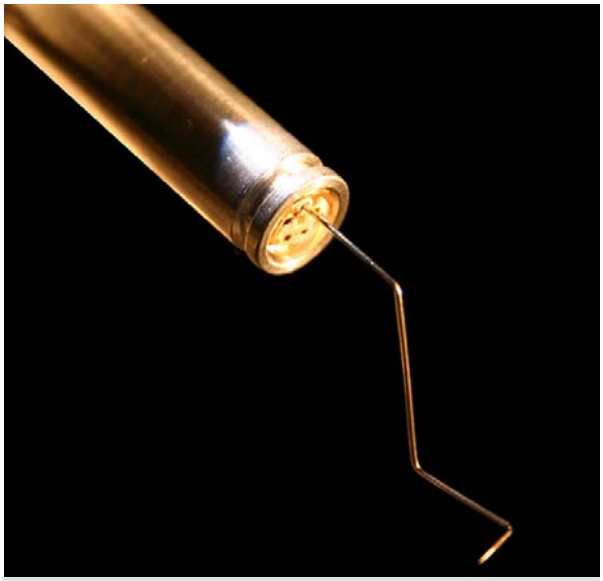

Figure 4 Bent probe inserted into a fine positioner through the bent capillary adaptor.

The probe was then located in the field of view of the microscope and brought into focus with the cell. It was positioned as close as possible to the target sucrosome using either the coarse mode at its lowest speed setting or the fine mode. The probe was then inserted very slowly (in fine mode) past the cell membrane into the sucrosome. The probe was typically held in the sucrosome for approximately one second before withdrawing it slowly. As the probe was withdrawn, one could observe the sucrosome collapse and release its contents into the intracellular space (see **Figures 5 and 6**).

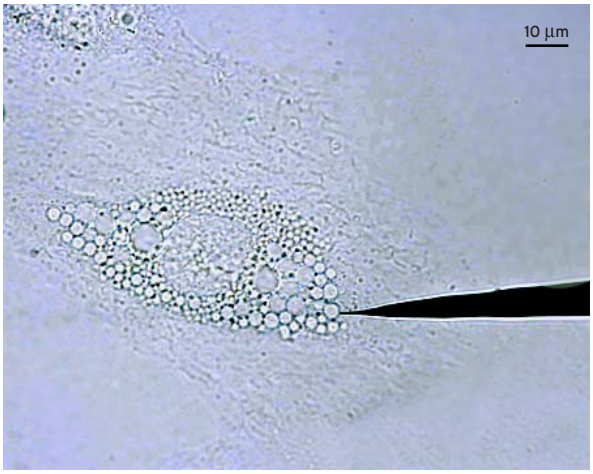

Figure 5a (top) A 3 µm sucrosome in a NRK cell before being penetrated by a sharp tungsten probe.

Figure 5b (bottom) The same cell two minutes after surgery.

In addition, sucrosomes were loaded with TRITC/dextran and released in a controlled manner by impaling the sucrosomes with tungsten probes. This is evident in the DIC and fluorescence images (as shown in **Figure 6**) where the target sucrosome was observed to disappear in DIC images and its fluorescence diminished. There was also some residual fluorescence near the site of the deleted sucrosome (see **Figure 6F**). We speculate this was due to sucrosomes that were out of the plane of the "popped" sucrosome. **Figure 7** shows a sequence of images depicting the structural changes in the sucrosome as it collapses. In addition, the procedure was gentle enough on the cells to enable them to retain their viability for at least 10 minutes post surgery (see **Figures 6F and 7F**).

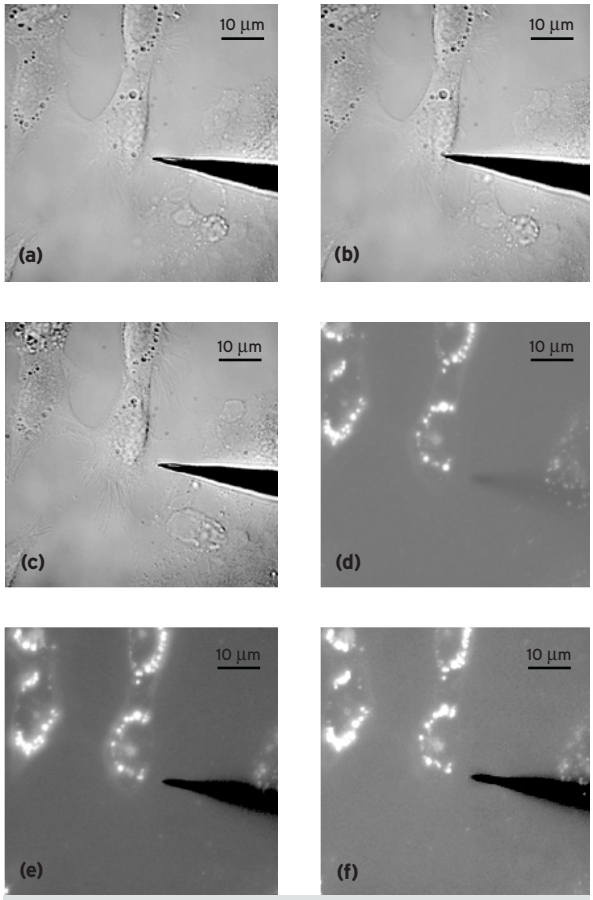

Figure 6 Sequence of images showing a sucrosome being burst in DIC **(a,b,c)** and fluorescence **(d,e,f)** microscopy. **(a)** Prior to being inserted into the sucrosome. **(b)** Probe inserted into sucrosome t=0. **(c)** t=7 min. **(d)** Before the probe was inserted into the sucrosome. **(e)** t=4 min. **(f)** t=11 min.

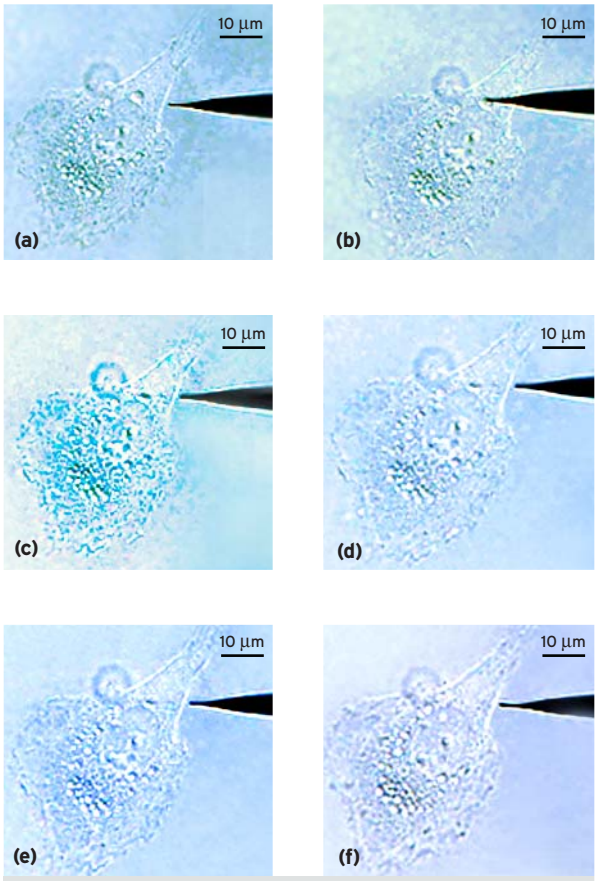

Figure 7 Bursting a sucrosome: sequence of images showing structural changes in a 2.5 µm sucrosome **(a)** after probe insertion **(b)** until it completely disappears **(f)**.

Conclusion

Intracellular nanosurgery is an important technique that facilitates knowledge about the roles of intracellular organelles. Success in this area of research requires the ability to selectively abrogate individual organelles with high precision. We have demonstrated here the possibility to perform nanosurgery on individual cellular organelles smaller than 2 mm in size. Additionally, the procedure was minimally invasive and caused no visible collateral damage to the cells. Future applications will include extraction of molecules from the cell for further analysis and nanoinjection. Thus, with the sub-5 nm motion capabilities of the L200 coupled with ultrasharp probes, researchers can now build the path to new discoveries of cellular structure and function.

Acknowledgements

The authors thank Professor Rockford Draper and Carole Mikoryak of the University of Texas at Dallas, Richardson, Texas for helpful discussions and for generously providing cells throughout this project.

References

1. Firtel, M., G. Henderson, and I. Sokolov, *Nanosurgery: observation of peptidoglycan strands in lactobacillus helveticus cell walls.* Ultramicroscopy, 2004. **101**(2-4): p. 105-9.
2. Yanik, M.F., et al., eds. *Neurosurgery: Functional regeneration after laser axotomy.* Nature. Vol. **432**. 2004.
3. Collins, T.J., et al., *Mitochondria are morphologically and functionally heterogeneous Within Cells.* Embo J, 2002. **21**(7): p. 1616-27.
4. Rizzuto, R., et al., *Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca2+ responses.* Science, 1998. **280**(5370): p. 1763-6.
5. Shen, N., et al., *Ablation of cytoskeletal filaments and mitochondria in live cells using a femtosecond laser nanoscissor.* Mechanics and Chemistry of Biosytems, 2005. **2**(1): p. 17-25.
6. Watanabe, W., et al., *Femtosecond laser disruption of subcellular organelles in a living cell.* Opt Express, 2004. **12**(18): p. 4203-4213.
7. Botvinick, E.L., et al., *Controlled ablation of microtubules using a picosecond laser.* Biophys J, 2004. **87**(6): p. 4203-12.
8. Colombelli, J., E.G. Reynaud, and E.H. Stelzer, *Subcellular nanosurgery with a pulsed subnanosecond UV-A laser.* Medical Laser Application, 2005. **17**: p. 217-222.
9. Parpura, V., P.G. Haydon, and E. Henderson, *Three-dimensional imaging of living neurons and glia with the atomic force microscope.* J Cell Sci, 1993. **104 (Pt 2)**: p. 427-32.
10. Han, S., et al., *Gene expression using an ultrathin needle enabling accurate displacement and low invasiveness.* Biochem Biophys Res Commun, 2005. **332**(3): p. 633-9.
11. Heisterkamp, A., et al., *Pulse energy dependence of subcellular dissection by femtosecond laser pulses.* Opt Express, 2005. **13**(10): p. 3690-6.