

# Enabling Subcellular Nanotechnology: An Applications Overview

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## Introduction

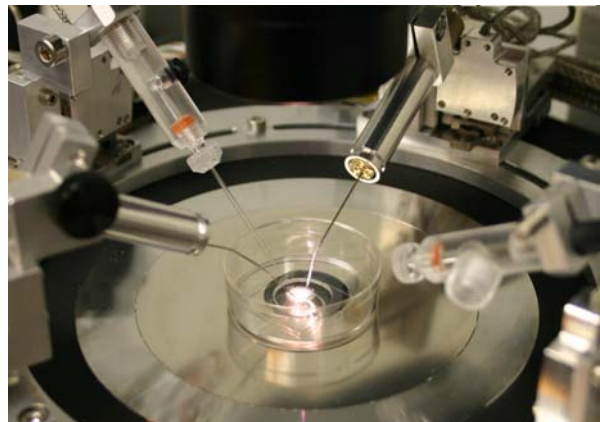
Part of the Zyvox NanoWorks® product line, the L200 Nanomanipulator System is a highly versatile nanomanipulation platform adaptable to both scanning electron and optical microscopes. The L200 possesses a much smaller form factor than existing manipulation systems (Figure 1). Its small footprint easily accommodates four independently controllable nanopositioners (Figure 2) and is upgradeable to six nanopositioners with fine positioning (5 nm) and eight nanopositioners with standard (100 nm) positioning. Each nanopositioner is capable of holding and manipulating end-effectors, such as glass capillaries for micro- or nano-injection, mechanical probes functionalized with pH-sensitive chemistry for intracellular pH measurement, and electrodes for accurate, precise electrical characterization.

With standard 100 nm precision for positioning of glass capillaries (down to 5 nm precision for positioning of mechanical probes), the L200 enables the user to carefully maneuver end-effectors near cell membranes and within the intracellular space. For non-cellular applications, such as biomaterials characterization, the L200 enables the fine manipulation of micron and sub-micron scale materials.

The L200 is a piezo-motor actuated nanomanipulation tool with four degrees of freedom – one in each orthogonal axis (X, Y, and Z) and a fourth for tilting capability.



**Figure 1** Compact L200 control cabinet, 27" H x 21" W x 26" D (left). L200 installed on a Nikon TE2000 inverted confocal microscope (right).



**Figure 2** L200 with capillary adaptors and NanoEffector® Probes.

The rail-mounted positioners can be easily re-positioned for arbitrary placement. The integrated electrical interfaces (up to 5 per positioner) make it possible to electrically stimulate samples and provide a path for electrical power to attached electromechanical devices (such as microgrippers). Inserting electrodes into electrical interfaces enables precise electrical measurements. In addition, the L200 allows for intuitive control of positioners using a single joystick or integrated software.

This application note briefly describes the following applications enabled by the L200:

- Biomaterials mechanics
- Intracellular pH sensing
- Nano-injection
- Intra- and extracellular probing
- Patch clamping
- Optical manipulation
- Cellular nanosurgery
- Nanowriting and nanoetching

## Biomaterials Mechanics

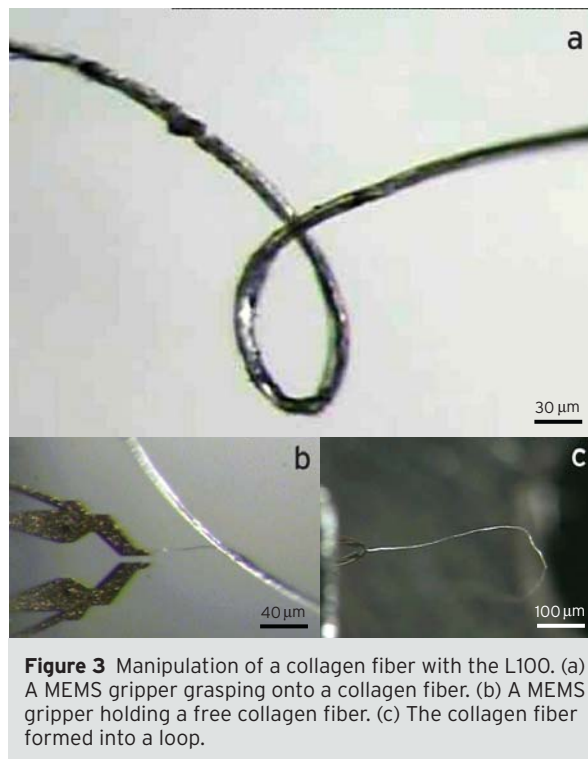
The Zyvex L200 is an enabling instrument for quantitative characterization of a wide range of mechanical characteristics of biomaterials. Some potential applications include studying the morphology, structure, and nanomechanical properties of cells. This has applications in the study of different cell types, comparison of normal cells to cancer or other pathological cells, etc. Cellular mechanics, functions of cytoskeletal and membrane proteins, and nanomechanics of actin-myosin systems can be quantitatively characterized to yield information at the molecular level.

The Zyvex L200 also has the potential to study the elastic, viscous, and plastic properties of connective tissue (such as ligament, tendon, cartilage and bone). Techniques that can be used to estimate these properties include nano-indentation, and micro-tensile and micro-fatigue testing, thus revolutionizing research in tissue regeneration, bone re-growth, and impact biomechanics. The L200 could also enable mechanical characterization of other biomaterials such as nanofibers, proteins, DNA molecules, etc.

The biomechanical characterization of collagen fibers used as scaffolding material in tissue engineering has been recently demonstrated. Current techniques for measuring the mechanical properties of collagen (involving custom built or proprietary tensile loading systems) can only handle macro-scale fibers and fibrils; they are not suitable for micron to sub-micron fibers such as collagen. The L200 provides a solution to problems that limit the science of biological material characterization. The L100 (an earlier version of the L200) has been used to grasp, pull, and twist collagen fibers for mechanical characterization (Figure 3).

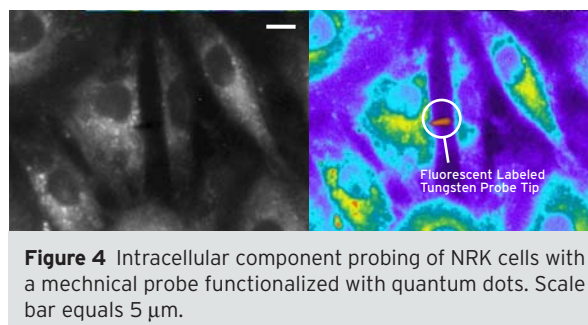
## Intracellular pH Sensing

Intracellular pH, and the pH within subcellular organelles, varies widely depending on conditions within the cell and the functions of subcellular organelles. The pH within cells and certain subcellular organelles has been measured using fluorescent dyes, such as fluorescein, whose fluorescence emission is a strong function of pH. However, localizing the dye at precise locations in cells, or within organelles, can be problematic. An alternative approach to measuring pH is to coat a nanoprobe with a pH-sensitive fluorescent dye and position the probe tip at the desired location within a cell using the Zyvex L200, followed by monitoring changes in the pH-sensitive fluorophore by fluorescence microscopy.



**Figure 3** Manipulation of a collagen fiber with the L100. (a) A MEMS gripper grasping onto a collagen fiber. (b) A MEMS gripper holding a free collagen fiber. (c) The collagen fiber formed into a loop.

This method depends on coating probes with fluorescent dyes. A gold-coated tungsten probe was incubated with an eleven-carbon alkane thiol terminated at one end with biotin. Via binding of the thiol to the gold-coated probe, a self-assembled monolayer displaying biotin was generated. The probe was then incubated with fluorescent streptavidin, which binds tightly to the biotin, labeling the probe with the fluorescent dye on the streptavidin. This same technique can be used to attach a pH-sensitive fluorophore to the probe and, upon inserting the probe into cells using the nanomanipulator, measurement of pH in the immediate



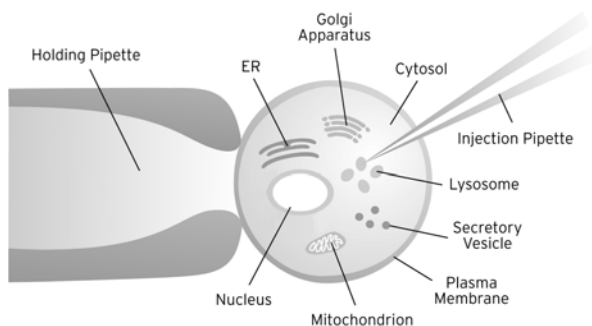
**Figure 4** Intracellular component probing of NRK cells with a mechanical probe functionalized with quantum dots. Scale bar equals 5 μm.

environment of the probe becomes possible. **Figure 4** demonstrates the insertion of a fluorescent probe into a normal rat kidney (NRK) cell.

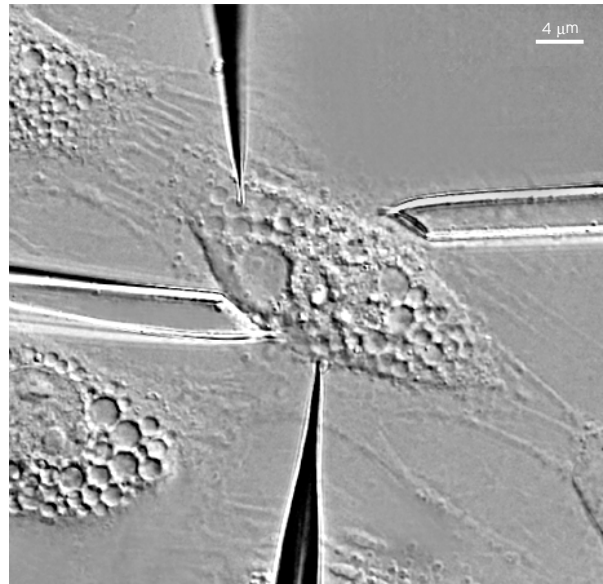
## Nanoinjection

Cell microinjection techniques are generally of two basic types, depending on whether the target cells adhere to the bottom of a culture dish or whether the cells are non-adherent and float freely in the medium. With adherent cells, the objective is usually to inject DNA or protein into either the nucleus or the cytoplasm while the cell is spread out on the bottom of the dish. The injection procedure is tedious for large numbers of cells and the procedure frequently damages and kills the cells, depending on the skill of the operator. One factor contributing to cell death is that most microinjection instruments do not have very fine control over needle movement and the cell membrane is torn during injection. The Zyvex L200 has very fine positional control, which increases the efficiency of the injection process by reducing cell death.

Microinjection with non-adherent cells involves a holder pipette that applies gentle suction to the cell, holding it in place, so that the injection needle does not move the cell. This situation is typical in veterinary cloning laboratories where material is injected into non-adherent oocytes or embryos during cloning procedures; it also occurs in certain types of human *in vitro* fertilization where a sperm is directly injected into the oocyte. Fine control over needle movement, possible with the Zyvex L200, reduces the probability of cell damage. Nanoinjection into intracellular organelles is depicted in **Figure 5**.



**Figure 5** Microinjection Process Illustration.

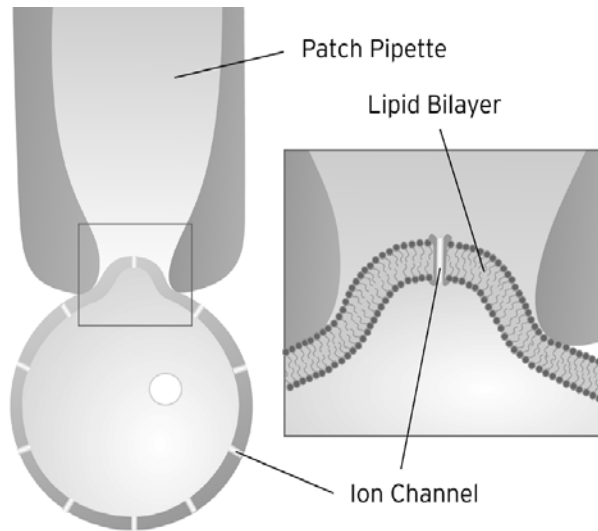


**Figure 6** Intracellular component probing of an NRK cell, with 2 mechanical probes and 2 micropipettes using a 4 manipulator L200 setup.

## Intra and Extracellular Probing

The 5 nm positioning accuracy of the Zyvex L200 opens up exciting new opportunities in intracellular stimulation and probing; just a few are discussed in this section. One application is to probe subcellular domains of the plasma membrane. For example, one could compare the membrane potentials (in different plasma membrane domains) of live neurons during the firing process. One could also measure the potentials across the membrane of subcellular organelles. With probe tips of less than 1 micron diameter, it is possible to insert a probe into an organelle of a living cell while keeping another probe in the cytoplasm. Thus, the real-time membrane potential across various subcellular membranes under varying conditions can also be recorded. One example of intracellular probing is demonstrated in **Figure 6** where two probes were inserted into an NRK cell.

By inserting electrodes into a cell, and measuring the impedance between the electrodes at various locations in the cell, one could construct an impedance map of the intracellular space – a technique known as “electrical impedance tomography.” Since different biomolecules have different electrical properties, a map of the intracellular space would provide information about the distribution of these molecules inside the cell. This knowledge provides



**Figure 7** Patch Clamping Process Illustration

new insights into biological processes at the single-cell level. One could insert a functionalized probe into a cell and extract proteins from specific subcellular compartments for further detection or even detect certain proteins within the cell by optical or electrochemical methods. With this new advance in single-cell proteomics, one will now be able to study biological responses evident only at the single-cell level, but which have, for decades – for lack of better technology – been studied at the bulk level.

## Patch Clamping

The L200's ability for sub-micron precision in manipulation using multiple probes (while still having a small foot-print) opens up new vistas in electrophysiology. Not the least among them is the possibility of simultaneously patch clamping multiple locations on neuronal processes such as dendrites and axons. This will enable the study of electrical and biophysical properties, such as the local distribution and modulation of ion channels in dendrites (Figure 7). With its multifunctional capability, the L200 makes it possible to monitor the propagation of synaptic and action potentials down the axon within the dendritic tree. Combining patch clamping with either atomic force microscopy (AFM), scanning probe microscopy (SPM) or

scanning ion conductance microscopy (SICM) brings up the possibility of simultaneously obtaining both topographical and electrophysiological information from the same cell. This information greatly aids the study of the distribution and effects of various ion channels in dendritic processes. Imaging and controlled application of reagents and biomolecules and controlled drug delivery are now feasible.

In addition, the Zyvex L200 could also enable the investigation of mechano-sensitive ion-channels in cochlear hair cells or smooth muscle cells of the jejunum. When combined with patch clamping, the mechano-transduction of signals and sensitivity of mechano-sensitive ion channels to membrane strain and tension can be studied.

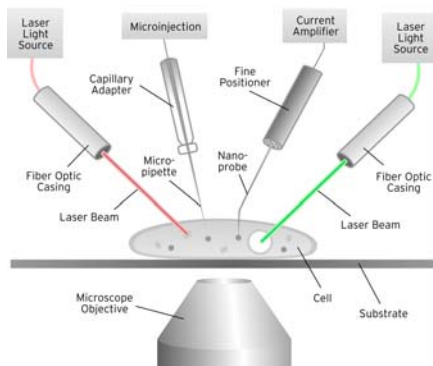
## Optical Manipulation

Optical tweezers use the energy of light to trap and manipulate microscopic particles without mechanical contact. The ability to remotely trap, manipulate and track particles has wide-ranging applications in different disciplines of science. A few vigorously pursued biological applications in recent years include studies of nanoscale mechanics of biological motors, inter-particle interactions, and protein folding and unfolding.

Typical optical manipulation setups consist of a laser beam focused through a high numerical aperture microscope objective onto the specimen of interest. The “trapped” particle can now be easily manipulated and its mechanical properties studied. Incorporating a laser through an objective, however, has several disadvantages: drastic modifications of the original setup are necessitated; we can typically use only a single beam through the laser and, hence, obtain a single trap; reconfiguration or recalibration of the system is typically cumbersome. Holographic and beam-splitting methods produce multiple traps, but add complexity to the optical setup.

To overcome some of these limitations, and to enable non-contact manipulation of sub-micron sized particles, we are developing optical trapping functionality as an add-on option for the Zyvex L200. The basic idea is to direct a fiber optic laser and focusing optics through the L200's



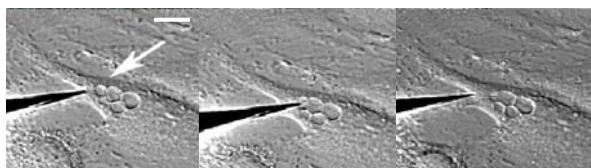


**Figure 8** Illustration of optical tweezers with one L200 positioner. This illustration also highlights the multifunctional nature of the L200 where each of the positioners is used for a different application.

nanopositioner head to trap the particle of interest in the specimen plane (**Figure 8**).

Shining lasers from above the sample means that no modification of benchtop optics is necessary to add this feature; the optical trapping system essentially functions as a “plug-and-play” module. Other inherent advantages include the ability to run multiple lasers through a single optical fiber to enable multiple beam optical manipulation and trapping. Also, the trapped particles will now move with the optical fiber (not by moving the specimen or the objective). This decoupling of the optical trapping module from the microscope objective will allow for better viewing of the sample.

Some advantages resulting from combining fiber optical trapping with the Zyvex L200 Nanomanipulator system are the obtainable precise positioning capability (down to 5 nm) and the possibility of combining optical trapping with the other modalities enabled by the L200. For example, one could now simultaneously trap a subcellular organelle with the fiber optic through one positioner, monitor its electrochemical properties with an electrode attached to the end of a second positioner, and simultaneously monitor its mechanical properties using a probe attached to a third



**Figure 9** Inserting a probe into a sucrosome of an NRK cell. Scale bar equals 10  $\mu$ m.

positioner. Other applications include stretching DNA molecules, localized photochemistry, studying the kinetics of cell motility, understanding molecular motors, intracellular surgery, and dissection of sub-micron intracellular structures, etc.

## Cellular Nanosurgery

On the micro scale, sharp probes are routinely used to excise cells from a tissue or cell group growing on a soft membrane this is accomplished by cutting a pattern around the cells, releasing them for harvesting. The probe could also be a “cookie cutter” to cut out pre-defined patterns.

On the nanoscale, a probe could be used as a “nano-scalpel” to cut subcellular organelles within living cells. Nuclei or lysosomes are reasonable test systems because of their size. For example, it should be readily possible to observe the consequences of cutting a lysosomal or nuclear membrane (**Figure 9**). For instance, the contents of individual lysosomes can be systematically released and the effects monitored in real time.

If small enough needles are available, removing subcellular organelles by applying suction on the needle is possible. In the reverse, injecting fluid or particles directly into subcellular organelles is also possible.

## Nanowriting, Nanoetching, and Other Applications

Because of the L200’s accurate positioning capabilities, nanowriting or dip pen lithography proves a very simple task. This allows controlled deposition of DNA or proteins at defined locations on a surface, and printing of very high density DNA microarrays.

An intuitive spin-off from nanowriting is nanoetching which is realizable through Zyvex Corporation’s nanoprobes and nanoeffectors. This opens up a wide range of biomedical applications ranging from cutting or dissecting biomolecules, to creating nanochannels in substrates by laying down etchant solutions.

In combination with other microscopy techniques such as AFM, SPM, and SICM, the Zyvex L200 makes controlled imaging and manipulation of single proteins possible allowing the investigation of several molecular interactions.