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# The Micro-Robotic Laboratory: Optical Trapping and Scissing for the Biologist

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With the addition of tightly focused laser beams, microscopes have been turned into elaborate preparative tools that permit not only allow detailed observation of a specimen but also the capture, displacement, and microdissection of biological samples in vitro with astonishing ease and accuracy. LaserTweezers are used to capture and manipulate cells and organelles. LaserScissors are used to perform microdissections at the submicrometer level. After a short technical description of the instrumentation and its principles of operation, several examples of applications are given relevant to the field of clinical research that could only be achieved using such modern technology. For instance, LaserTweezers and LaserScissors offer a unprecedented means to study the immune response to cancer, to control the growth of nerve cells, or expand the significance of assisted reproductive technologies. It is suggested that newly developing procedures and assays using laser-assisted technologies will prove beneficial for future clinical laboratory testing. J. Clin. Lab. Anal. 11:28–38.


**Key words:** optical trapping; laser ablation; single-cell assay

## INTRODUCTION

In recent years, the light microscope has become a powerful analytical instrument. This has been particularly true with the increasing use of computer sciences for data acquisition and image processing and with the development of modern optical technologies and laser-assisted techniques such as optical trapping and optical scissing. For example, a continuous wave laser-based optical trap can be used to capture and manipulate microscopic particles in vitro (1). To minimize photodamage of the trapped particle that could result from excessive heating, wavelengths in the near infrared (IR) are preferred (e.g., 800–1,100 nm) because they are poorly absorbed by biological tissue (2). A laser beam of IR light, focused by the microscope objective lens, produces localized forces that displace the microscopic particles in suspension. The method is nondamaging and it can be applied to the manipulation of single isolated cells (3,4). The question as to whether certain parameters of the incident laser beam might induce abnormalities has been addressed with several biological systems, such as cells and organelles (5). Toward this end, the optimal range of wavelengths for the optical trapping of chromosomes has been studied using mitotic cells as a model, because such cells are extremely sensitive to any kinds of perturbations (6). Laser beam parameters have also been studied for the purpose of safely trapping human spermatozoa. Establishing the thresholds of trapping power is an important factor that contributes to the safe use of these techniques in human reproduction (7).

Other laser-based devices associated with a microscope have been introduced that complement optical trapping techniques. For example, a pulsed laser microbeam that emits in the ultraviolet (UV) or visible range of the electromagnetic spectrum can be used to form submicrometer cuts in biological material or to ablate specific cells. More recently, the value of using certain wavelengths in the IR range to achieve ablation of biological tissues was described for reproductive biology applications (8). The principles and mechanisms of laser ablation have been well described (2,9). At UV wavelengths, proteins are the primary energy absorbers and destruction of tissue bonds is due to direct absorption of the laser light. In the IR range, above 1.3 μm, ablation results from heat transfer from the water, which is the primary absorber at these wavelengths. When applied to microscopy, selective laser ablation can be confined to cellular or subcellular objects in a consistent and controllable way. Precise disruption of a specific target can be obtained within a strictly delimited region of a living cell (10). The characteristics of the laser microbeam, such as intensity, pulse duration, and wavelength, that

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are necessary to achieve the microdissection of a given particle, might vary from one specimen to another. Optimum experimental conditions have not yet been documented for each potential application. However, laser ablation used in combination with optical microscopy has already been demonstrated for a large variety of specimens and applied to various studies in physical, chemical, and biological sciences (11–13). Several review articles have been published that describe many of these investigations with biological specimens (10,14,15).

The purpose of this paper is to describe how optical trapping and optical scissing instrumentation has transformed research microscopes into modern microrobotic laboratories that, placed in the hands of biologists, offer singular opportunities for developing new clinical laboratory applications. Examples of applications are discussed in cancer research, neurosciences, and reproductive biology.

## MATERIALS AND METHODS

### Optical Trapping

Optical trapping manipulations were performed using a LaserTweezers 2000 system (Cell Robotics, Albuquerque, NM) mounted onto a Zeiss Axiovert 100 inverted microscope (Carl Zeiss, Inc., Thornwood, NY). The LaserTweezers system included an XY, automated, high-precision, variable-speed, motorized microscope stage that positions the sample on top of the lens, with respect to the stationary laser beam. Z-axis motion was controlled through a motorized focus adjuster. A three-dimensional trackball was used for control of the system. The trapping laser module was fitted with a semiconductor, 1-watt, single-mode MOPA laser diode with emission at 980 nm (SDL, San Jose, CA). The output power of the laser could be adjusted, using the LaserTweezers' electronic controller, with 1% increments from 0 to 1,000 mW. While the laser module of the LaserTweezers was firmly attached to the microscope's body, the laser beam could be precisely aligned relative to the optical path of the microscope, using a beam steering device that works with a pair of quasi-parallel mirrors optimized for IR reflection.

### Optical Scissing

Laser microdissections were performed using a LaserScissors (Cell Robotics). The laser microdissection system was attached to the epi-illumination port of the same Zeiss inverted microscope on which was simultaneously mounted the LaserTweezers. The LaserScissors was either fitted with a pulsed nitrogen laser or a nitrogen-pumped dye laser (Laser Science, Newton, MA). Using the pulsed nitrogen laser, the emission wavelength was 337 nm at 120 joule (J) maximum pulse energy. The nitrogen-pumped dye laser was used with a BBQ dye solution (4,4'''-bis[(2-butyloctyl)oxy]-1,1':4',1'':4'',1'''-quaterphenyl) (Exciton, Dayton, OH, USA) for a peak emission wavelength of 390 nm and a maximum pulse energy of 15 J. The chassis of the LaserScissors was firmly fixed onto the microscope's body. Precise alignment of the laser beam was achieved using either a pair of RM-2 reflectors or a pair of enhanced aluminum mirrors (Newport Co., Irvine, CA). Parfocality adjustment was through a defocused Galilean beam expander. The focusing and steering optics of the LaserScissors were employed to control spot size and position. A graduated variable neutral density filter (100 : 1) was used for adjusting laser beam intensity. Incisions in the zona pellucida of mammalian oocytes were performed at 337 nm. Transections of the tails of sperm cells and of extending neurites were made at 390 nm. The laser pulse duration was 3–4 nsec. The laser was triggered by a handheld push button that could be used in either single or repetitive pulse mode (≤12 Hz). The spot size (dimension of the beam waist) was $<0.5\ \mu m$.

### Sample Observations

Optical manipulations of the samples using the LaserTweezers and the LaserScissors were performed in disposable coverglass chambers. A video camera mounted on the microscope's camera port provided a video display of the specimen on a color video monitor. Observation was performed using either a 40× objective lens or a large numerical aperture (>1.2) 100× oil immersion lens. Experiments were recorded from the monitor's video signal output using a standard recorder and an 8-mm tape. Single frames were stored as TIF files using a frame grabber. The contrast of the videoprints was then digitally enhanced for purpose of publication. On the videoprints, a map of the chamber as well as the X- and Y-stage coordinates, indicated in micrometers, are located at the bottom left of the image. Position of the optical trap along the Z axis is also indicated in 0.5–μm increments.

### Single Cell Sorting

For the purpose of achieving routine single cell sorting, the remote controlled precision microsyringe pump "CellSelector" (Cell Robotics) was installed onto the moving platform of the motorized microscope stage, and placed on the right hand side. The CellSelector consisted of a DC servo motor with optical encoders for driving the plunger of a microsyringe and was controlled through an analog variable output driver. The flow rate was commanded by a spring loaded potentiometer associated with a digital readout of linear plunger displacement. The motor assembly was rigidly mounted on a stand for X, Y and Z positioning. The motion along the Z axis was along an arc with the radius centered at the base plate. Control of microvolumes of fluid down to 6 picoliters at a time was achieved when using a 10 microliter precision 701 LT syringe (Hamilton Co., Reno, NV). Glass micropipettes (1.2 mm OD) were pulled using a micropipette puller from single-barrel borosilicate (type 7740) glass capil-

laries with standard wall thickness. Capillaries with an inner filament were preferred for quick filling of the micropipette. A straight micropipette holder was used to mount the pulled glass micropipette onto the luer tip of the syringe (World Precision Instruments, Sarasota, FL).

A suspension of human lymphocytes was held in a double well Lab-Tek coverglass chamber (Nunc Inc., Naperville, IL). These chambers are disposable vessels manufactured on a coverslip of about 170 micron thick (number 11/2). The coverslip window makes the chamber compatible for use with a large numerical aperture objective lens as required for optical trapping applications. First, the tip of the micropipette was placed in an area of the dish free of any cells or other contaminants. The coordinates of the micropipette tip were then stored in the memory of the electronic controller. The dish was scanned and a single cell, captured by means of optical trapping, was held stationary above the ojbective lens. Using the automated motion of the motorized stage, the tip of the micropipette was then transported back to the cell (i.e. to the objective lens). The selected cell was recovered by aspiration of the surrounding buffer. The micropipette was then rocked back, out of the dish, and a new recovery well was placed on the stage. The microsyringe was then rocked down into place and the selected cell was delivered by flushing the micropipette. Because the fine vertical adjust comes to rest on a rigid stop, readjusting the Z axis was not necessary.

### Suspension Culture of Cancer Cells and Natural Killer Cells

Lymphocytes and natural killer (NK) cells were prepared from healthy human volunteers as previously described (16). Briefly, peripheral blood lymphocytes (PBL) were prepared from heparinized peripheral blood, which was separated on Ficoll-Paque (Pharmacia, Piscataway, NJ) and depleted of monocytes by two rounds of absorption to plastic. NK cells were enriched by negative selection of $CD5^-$ $CD20^-$ PBL using PBL coated with monoclonal antibodies (mAb) directed against CD5 and CD20 (T1 and B1, Coulter Immunology, Hialeah, FL), in combination with magnetic beads coated with antibodies directed against mouse IgG (BioMag, Perceptive Diagostics, Cambridge, MA). NK cell preparations typically consisted of 89–95% $CD56^+$ NK cells and only 3–5% $CD3^+$ T cells (17). NK cells were routinely suspended in serum-free HB104 tissue culture medium (TCM) (Irvine Scientific, Santa Ana, CA), cultured overnight at 37°C under 5% $CO_2$, and used for experimentation the following day. The NK-sensitive K562 myeloid leukemia target cell line was routinely passaged in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS).

For single-cell adhesion assays, NK and K562 target cells were suspended in fresh standard solution, which consisted of the following inorganic ions [in mM]: $Na^+$ [145]; $K^+$ [5]; $Mg^{2+}$ [1]; $Ca^{2+}$ [1]; $Cl^-$ [150]; and $SO_4^{2-}$ (1). This was supplemented with 10 mM HEPES, 10 mM glucose, and 1 mg/ml human serum albumin (HSA, Sigma, St. Louis, MO). The pH was adjusted to 7.4. In previous studies (17), NK cell cytolytic activity and conjugate formation in standard solution was comparable to that observed in HB 104 medium or RPMI-1640 supplemented with 10% fetal calf serum. NK cells were exposed to 100 nM phorbol myristate acetate (Sigma) for 10 min at 37°C to activate adhesion.

### Culture of Neurons

Dissociated chick dorsal root ganglion (DRG) neurons (18) were plated in defined medium in Falcon dishes (35 mm) equipped with a poly-L-lysine-precoated glass coverslip (80 $\mu g/cm^2$). Neuron cultures used for either cell communication, neuronal pathfinding, or microdissection experiments, were maintained following already established culture methods as described in detail elsewhere (19–21).

### Mammalian Gametes

The gametes used in these studies consisted of bovine oocytes obtained from a slaughterhouse or of degenerate unfertilized human oocytes discarded from an in vitro fertilization program. Human sperm cells were collected from an healthy patient and washed in saline solution.

Ovaries of mature cows were obtained from an abattoir and placed into warm saline solution for transport. Oocytes were aspirated from visible follicles and washed in HEPES-buffered Tyrode's solution. After 24 hr of in vitro maturation culture, the cumulus cells were removed and the oocytes were loaded into 0.25-ml plastic semen straw for storage. Laser-assisted microdissections of the zona pellucida surrounding the bovine oocytes were performed in disposable double-well Lab-Tek coverglass chambers (Nunc, Naperville, IL).

The human oocytes had been recovered by follicle puncture using transvaginal ultrasound. At 6 hr following recovery, each oocyte was inseminated with $1 \times 10^5$ sperm in a total volume of 1 ml culture medium. After 18 hr of incubation, the oocytes were freed of their surrounding cumulus cells and observed for the presence of pronuclei. Oocytes without pronuclei were again inseminated and incubated another 18 hr. Oocytes not having pronuclei at 18 hr after this second insemination were considered degenerate. These oocytes were placed into HEPES-buffered human tubal fluid medium containing 0.4% human serum albumin (Irvine Scientific, Irvine, CA) for storage.

## RESULTS AND DISCUSSIONS

### Optical Trapping: Brief Technical Background

The noninvasive in vitro manipulation of microscopic particles using optical trapping has been well documented during the past few years (14,22–24). In practice, the near-IR

laser beam, when focused through a large numerical aperture objective lens, converges to form an optical trap at the focal point for the laser. The lateral trapping force moves a particle in suspension toward the center of the beam. The longitudinal trapping force moves it in the direction of the focal point. The optical trap levitates the particle and holds it in position. Positioning the particle relative to the rest of the sample is then achieved by moving the microscope stage. As no physical contact occurs during manipulation, there is no risk of damage to the particle or of sticking to a micropipette. Many research laboratories are now applying this technique for various applications in both biological and nonbiological fields of research. The use of lasers offers strong advantages over alternate methods such as those that use mechanical micromanipulators fitted with microtools and micropipettes. For example, capturing, selecting, and handling single cells in a culture vessel is very easy to perform using the LaserTweezers. There is no unwanted movement such as occurs when a particle is expelled from a micropipette. Positioning and trapping of the particle occurs under direct visualization, ensuring separation of only the desired particle.

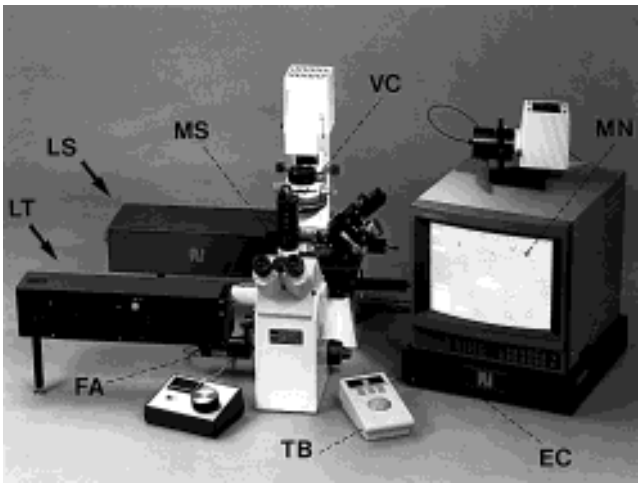


**Fig. 1.** The microrobotic laboratory. The LaserTweezers (LT) and the LaserScissors (LS) mounted on an inverted microscope. Sample positioning is using a high-precision motorized X-Y stage (MS) and focus adjust (FA). A video camera (VC) and monitor (MN) facilitate sample observation. Complete system operation is through an electronic controller (EC), using a multifunction trackball (TB) and an on-screen command menu overlay.

## Photothermal Ablation: Brief Technical Background

Many laser microdissection procedures are based on the principle of photothermal ablation, which results from phase changes of liquid and solid components. In this application, a different region of the electromagnetic spectrum is selected than for the formation of an optical trap. A variety of wavelengths of various intensity can be used, ranging from the UV through the visible spectrum and to the IR light. Although it depends on the wavelength favored, photothermal ablation occurs when heat is produced as the laser light is absorbed by the target specimen. As the pulsed laser beam is focused through the microscope objective lens, it converges to form an optical scalpel at the focal point for the laser. Positioning the sample relative to the cutting laser beam is achieved by moving the microscope stage. The ablation phenomenon in the specimen is strongly influenced by the target substrate and by the eventual presence of chromophores that absorb the laser light. In the UV, such as at 337 or 390 nm, cellular structures and proteins govern the absorption coefficient. Pigmented tissues, melanin, and hemoglobin absorb strongly in the visible range. In the IR light, the ablation phenomenon results from the strong absorption of the water molecules, starting at about 1,200 nm, and exhibiting a peak at around 1,480 and 2,900 nm.

## Single-Cell Assays

Modern laser technologies such as optical tweezers and optical scalpels have transformed the standard inverted microscope from a purely analytical tool into an elaborate revolutionary microrobotic laboratory (Fig. 1). This tool not only permits the detailed examination of a sample such as single isolated particles, but also allows performance of unprecedented studies and manipulations at the microscopic level. The fundamental advantage of these techniques is that they offer the investigator the possibility to select, capture, manipulate, dissect, and sort a specimen on a single-cell basis. This is of relevance for various scientific fields. Detection capabilities down to the molecular level, for instance, are demonstrated with capillary liquid chromatography (HPLC) and electrophoresis (HPCE), or in molecular biology (PCR, FISH). These methods allow researchers to focus their investigations on the single particles or molecules isolated from a specimen and to perform analysis with great accuracy.

In Figure 2, the purification of a single human lymphocyte is demonstrated in a dry chamber. The dry recovery chamber permits an appreciation of the small volume in which the cell is recovered. A small volume of liquid (typically 300–500 picoliters) was necessary to deliver the cell. To break the surface tension and detach the droplet from the micropipette, it was essential to establish contact with the bottom of the chamber. Cell recovery could also be performed in a micro well pre-filed with culture medium, using a sterile Terasaki format plate for example. The three-axis positioning stand of the CellSelector was essential in facilitating the routine and rapid positioning of a micropipette in a microwell. The tilt handle allowed the microsyringe pump to rock back and forth so that the second chamber could be placed on the microscope while the micropipette returned to the identical Z position. This feature allowed repositioning of the tip of the micropipette repeatedly with great accuracy, eliminating the risk of pipette tip breakage. Unquestionably, the association of LaserTweezers for the selection and capture of single cells,

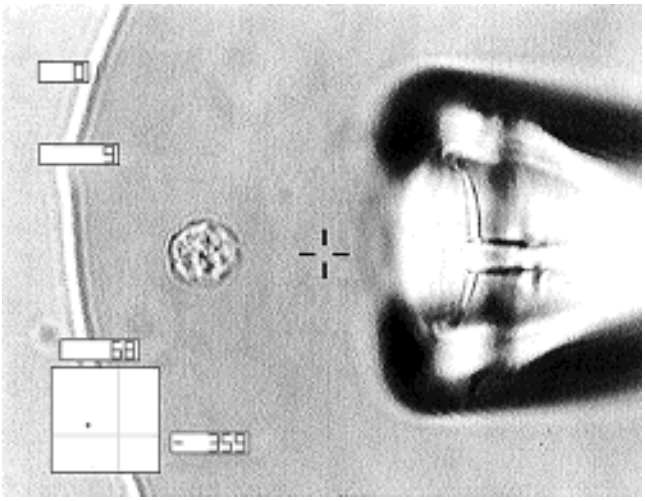

**Fig. 2.** Single cell purification. The sorting of a single human lymphocyte was made possible by the combined use of the LaserTweezers and CellSelector. The tip of the micropipette (*right hand side*) and the circular edge of the recovery droplet (*left*) are clearly visible.

precision microsyringe pumps for the control over minute volume of fluid and miniature culture chambers for the recovery of rare cells will be decisive in the development of numerous single-cell assays. Routine single cell sorting should expand the scope of clinical diagnostics when tracking cancer cells or establishing the genetic fingerprints of various mutations.

## Cancer Research

The LaserTweezers proved to be a powerful tool for the manipulation of human cells in suspension. In particular, this tool allowed development of radically new experimental procedures that would otherwise be impossible or extremely difficult to achieve using other methods and techniques, for example, studying the interactions that might occur at the membranous level between different types of cells in culture. With the optical trap, individual cells can be easily selected and spatially arranged relative to each other. Because there is no need to mechanically establish a contact with the cells (e.g., with a micropipette), this procedure can be performed within a closed culture dish in order to maintain sterile conditions of culture. This point is important not only because it indicates that the safe handling and manipulation of a precious or rare cell culture is now possible, but also because it emphasizes optimized working conditions and protection level for the operator (e.g., when working with pathogenic organisms). Furthermore, since the procedure is simple to perform and nondestructive for the cells, an investigator can repeat the procedure quite routinely as necessary to collect sound scientific data based on a large number of observations and to establish reliable statistical information. In comparison, mechanical micromanipulators are not so user friendly, often require delicate micropipette alignment procedures and result, for the nonspecialist, in difficult to manage and time consuming manipulations.

Figure 3 shows an example of cell-to-cell interaction that was promoted using the LaserTweezers. NK cells collected and purified from a healthy volunteer were mixed in vitro with an established cell line (K562 myeloid leukemia). First, using the optical trap to select, displace, and position the cells in the suspension cell culture, a large number of human NK cells were gathered and placed in the vicinity of a cancer cell. Then, one at a time, the NK cells were brought into contact with the target cancer cell. Thus, within a short period of time, many contacts were promoted between the two types of cells. This approach to the study of the cellular mechanisms that lead to the destruction of cancer cells is unique in that it is the only one that permits an investigator to control and to monitor live, and in real time, the interaction of the NK cells with a specific target cell. Using optical trapping for this application is extremely advantageous over any other method considering the versatility and precision that this technique allows in the manipulation of living cells. Some NK cells bound easily and almost instantaneously to a cancer cell, however, a large number of NK cells were not capable of establishing any binding with the target cancer cell even if the two cells were maintained in close apposition in the trap for an extended period of time (up to 5 min). While attempting to promote cellular interactions, a given NK cell was presented from many angles to the target; for example by rolling it over the surface of the cancer cell (not unlike two gears meshing on each other). Thus, this procedure allowed testing of the potential adhesion properties of the NK cell from many angles all around its surface. Failure of NK cells to bind to a target cell was clearly not due to inappropriate polarity of NK cell orientation with respect to the cancer target cell. In reciprocal experiments, heteroge-

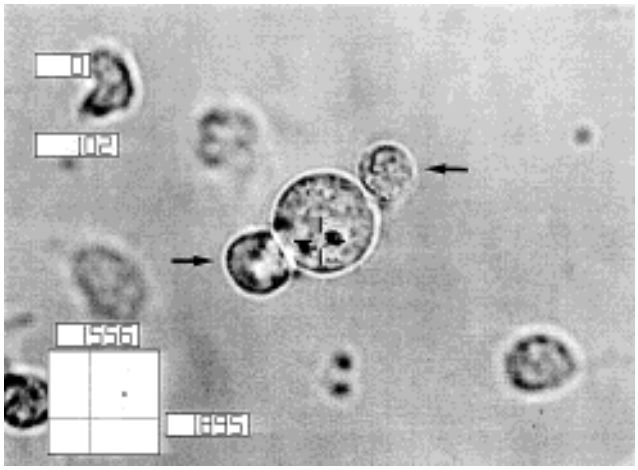

**Fig. 3.** Cell-to-cell interaction. Natural killer (NK) cells *(arrows)* are brought into contact with a cancer cell, using the LaserTweezers. Although many NK cells do not bind to the cancer cell, some exhibit a particularly strong affinity for it and lead to the rapid formation of a conjugate. This might permit the distinction of a population of NK cells with marked adhesive properties.

neity in the ability of different cancer target cells to be bound by a single NK cell was also discerned. Some of the cancer cells may have lacked membrane structures capable of triggering or supporting the NK cell adhesion response or, alternatively, may have inactivated the NK cell adhesion response. Optical trapping represents a unique and convenient approach by which to identify, isolate, and clone such adhesion-resistant cancer cells under sterile conditions for more detailed experimental analysis. Experiments of this type may prove a powerful approach by which to investigate biological mechanisms underlying NK resistance of some cancer cells and NK-sensitivity of others. Using a similar technical approach, scientists would have a powerful tool to investigate various drugs and compounds for their potential in enhancing the adhesion and killing properties of the NK cells. This approach would further permit a detailed molecular analysis of interactions between NK and target cells to promote a better understanding of the cellular mechanisms of the immune response to cancer.

Studying the cellular interactions at the single-cell level does not have application solely in cancer analysis but is also of great interest for many other areas of clinical research. The LaserTweezers' optical trap has tremendous potential for the understanding of the invasion mechanisms of infectious diseases, for example in the case of human immunodeficiency virus (HIV). Several in vitro experiments suggest that the formation of syncytia between infected and healthy T cells, as well as the presence of soluble gp120 in the blood and lymph are two important phenomena responsible for the depletion of T cells in acquired immunodeficiency syndrome (AIDS) patients [for an overview of the immune response to HIV infection, see Kuby (25)]. The development of a vaccine will probably first require precise evaluation and quantification of the interactions that occur at the cellular level and that result in the depletion of uninfected cells. It is also likely that the ability to safely manipulate specific cells in vitro will be beneficial to the testing of drugs intended to minimize or eliminate these events. LaserTweezers should therefore offer a unique opportunity for several experiments intended to investigate the mechanisms that lead to the decline of $CD4^+$ T cells.

## Neurosciences

Using the optical trap of the LaserTweezers, it is possible to select and very accurately position nerve cells in culture. In Figure 4, several living nerve cells were spatially arranged relative to each other. Placing cells in a precise pattern favors the formation of specific interconnections that is a prerequisite for the building of distinct neuronal circuits.

Microdissection of neuronal processes using the LaserScissors is a singularly attractive method that permits not only control of the growth of extending nerve cells, but the establishment of a reliable assay system for the study of cell response to injury as well. Figure 5 shows the transection

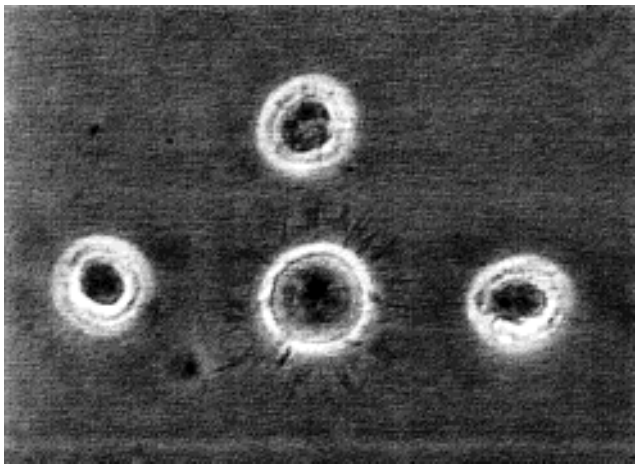

**Fig. 4.** Cell communications. Four nerve cells are accurately positioned relative to each other using the LaserTweezers. Spacial arrangement of nerve cells in a precise pattern is important to study the formation of neuronal interconnections and to build specific neuronal circuits.

of a mammalian neurite. Cuts as small as 1 μm in width can be made on the neuronal processes. It should be emphasized that the cells survive after such manipulation. The LaserScissors offers an alternative to the use of a mechanical micromanipulator and eliminates the cumbersome alignment steps required when using microdissection tools. In addition, it allows for the use of enclosed culture dishes for optimal sterile environment.

Remarkable advances in neurosciences have recently been made possible with the availability of commercial, table top, optical tweezers, and optical scissors. At the Department of Anatomy and Neurobiology of the Colorado State University, scientists are using LaserTweezers and LaserScissors to control, with a rare precision, the growth of single neurons in

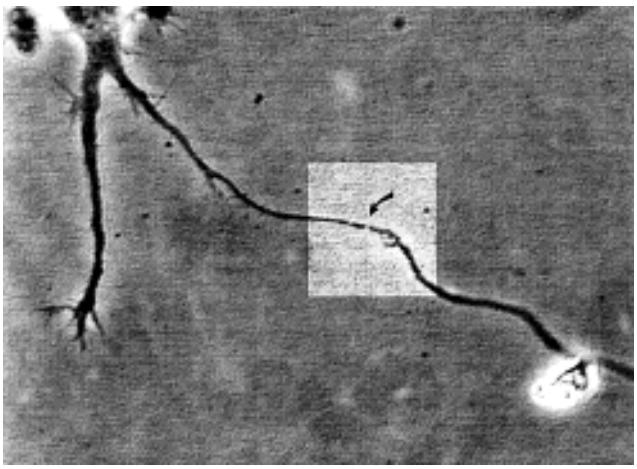

**Fig. 5.** Transection of mammalian neurite. The microdissection of mammalian neurons using the LaserScissors, is shown here with the pyramidal neurons from the hippocampus of developing rats. *Arrow*, submicrometer cut in a neuronal process.

vitro. The objective of these experiments is to better understand the mechanisms that lead to the formation of functional neuronal circuits during the development of the nervous system. Using the LaserTweezers, it is possible to reproduce in the laboratory the conditions that influence the spatial navigation of elongating neurons (Fig. 6). Growth cones, located at the tip of elongating processes, are critical for the process of elongation. It has been suggested that the presence of specific environmental information acts as a cue to guide the advancing growth cones during their elongation concurrent to the embryonic phases of development. Microscopic guideposts, such as plastic or glass microspheres, can be used to provide similar information to the elongating neurons in vitro. Positioned using the optical trap in precise locations in the vicinity of the growth cones, the pattern of guideposts controls direction of neuronal growth. Accurate positioning of the microspheres around the cells is a difficult process. The use of a mechanical micromanipulator is not recommended for this application, as the microtools themselves, or the waves they produce while in motion, are constant obstacles to the fine positioning of several beads close to each other. On the other hand, the precise manipulation of the microspheres is considerably simplified using the beam of light. The ability that LaserTweezers provided to design precise experimental conditions was key to examining guidepost-mediated neuronal pathfinding (26). It was demonstrated that model guideposts, composed of a single molecular species, are sufficient to change the navigation and the behavior of advancing growth cones well beyond the time of contact. Directional guidance information was shown to be dependent upon the type of molecular species associated with the guideposts. A sustained increase in growth cone velocity was obtained using laminin-coated guideposts. Decrease in growth cone velocity was observed with fibronectin-coated guideposts (20).

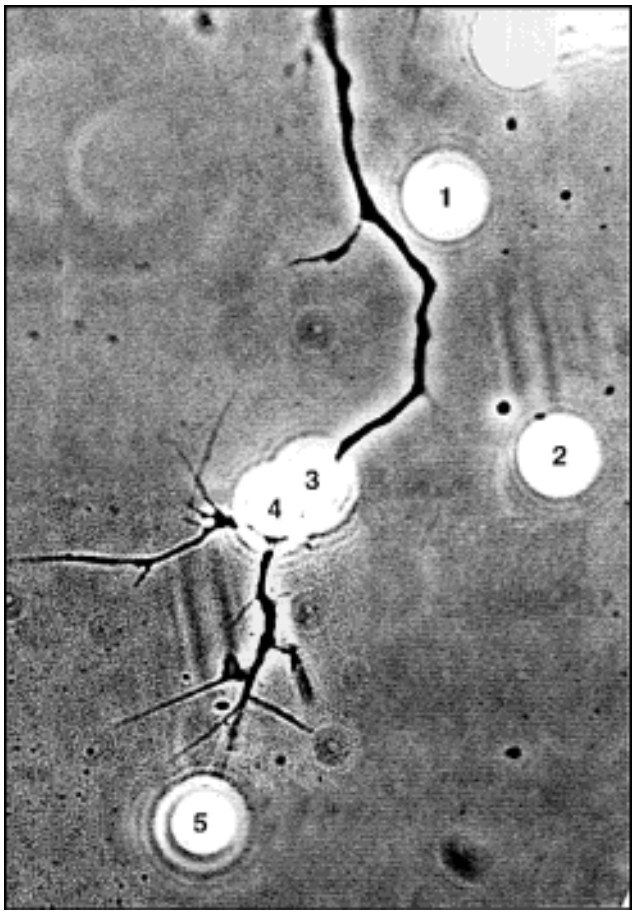


**Fig. 6.** Positioning of signaling molecules. Microspheres coated with laminin, were placed in specific locations around a neuronal growth cone of a chick dorsal root ganglion cell. Contacts are successively established by the progression of growth toward and beyond the beads, while earlier contacts are sequentially terminated. Consequently, the pattern defined by the microscopic guide posts governs the neuronal pathfinding. [From Kuhn et al. (20).]

With the introduction of these modern techniques and with the development of such new procedures, neuroscientists now have access to an unprecedented means to manage the distribution and the growth of nerve cells in vitro. Specific cell communications can be promoted between a selection of nerve cells by precisely controlling their spatial arrangement. Existing communication links can be interrupted by the laser-assisted microdissection of specific neuronal processes. Finally, elaborate neuronal pathfinding can be guided using a predefined distribution of microscopic guideposts. It is suggested that such procedures will offer opportunities for the in vitro construction of "neuronal connectors", including both nerve cells and glial cells, that might then be used for spinal cord splicing.

## Reproductive Biology

During the past two decades, tremendous advances have been reported in the field of reproductive medicine. Several techniques are available to assist infertile couples to achieve pregnancy. Worldwide success rates vary, but reports show that the live birth rates are approximately 15–20% per treatment cycle. This relatively low success rate places both emotional and financial constraints on many patients and calls for procedure improvements. Recent developments in assisted reproductive technologies (ART) include gamete and embryo micromanipulations. Improvements in oocyte fertilization rates were made (primarily in cases of severe male factor infertility) by assisting the entry of the sperm into the oocyte, while improvements in embryo implantation rates were achieved by facilitating the hatching process of the developing embryo. Typically, gamete and embryo manipulations are operated with the aid of precision mechanical micromanipulators fitted with fine microtools such as glass or metal microneedles, microscalpels, and micropipettes. However, these devices are difficult to use, time consuming to prepare and expose gametes and young embryos to questionable sterile conditions. The use of lasers in assisted reproduction biol-

ogy, however, offers singular advantages over mechanical techniques and conventional methods. A wide range of laser-assisted manipulations have already been demonstrated, principally in research laboratories, but also in a few clinical settings. Optical trapping has proven to offer a unique and very powerful means to select, capture, and manipulate a sperm cell in three dimensions and has also allowed measurement of the relative force generated by the swimming cells (7,27). Partial dissection of the zona pellucida has been achieved using laser beams of various wavelengths, from the UV to the IR range of the electromagnetic spectrum (28,29). By combining two laser beams, one for performing microdissection of the zona pellucida and one for generating an optical trap, it has become possible to easily perform elaborate in vitro fertilization procedures (30). For example, the subzonal insertion of a selected sperm cell can be accomplished with the optical trap through a trench or tunnel previously drilled in the zona with the dissecting laser beam (31). Another very promising method is the in vitro laser-assisted hatching of the preimplantation embryo. This method consists of preparing several cuts in the zona pellucida using a laser beam before transferring the embryo into the uterus. It offers the advantage of not exposing the embryo to an acidic solution or to a glass micropipette as currently done with alternative procedures. Laser-assisted hatching has already proved successful in promoting human pregnancies (32).

With the LaserTweezers and the LaserScissors, laser-based technologies are made user-friendly, affordable, and thus accessible to a wide range of laboratories. The time has gone when solely the few research laboratories equipped with complex and expensive laser systems could establish new protocols and evaluate the value of lasers in reproduction biology. Presently, it is not necessary to be a laser expert to have access to the most promising technique in gamete and embryo manipulation. Figure 7 shows the severing of the tail from a human sperm cell. The tail has been cut in a few seconds, by using the focused UV laser beam of the LaserScissors. The procedure is reliable and very simple. The head of the sperm cell can then be trapped and manipulated using the LaserTweezers. Lower laser intensities are preferred when an operator wishes not to completely separate the head of the sperm but to only break its tail. Breaking the sperm cell tail is of interest for applications such as the intracytoplasmic injection of a sperm into an oocyte (33), in which case motile sperm must be immobilized before injection into the oocyte.

Figure 8 shows the laser-assisted dissection of the zona pellucida of a bovine oocyte. Multiple cuts were performed for demonstration purposes in order to illustrate two kinds of applications. First, a single cut all the way through the zona makes it easy for the sperm cells to access the egg and is necessary for the subzonal insemination of a sperm cell using the optical trap (30). Second, several incisions in the zona pellucida facilitates the hatching process of the embryo. For this particular application, only partial dissection of the zona

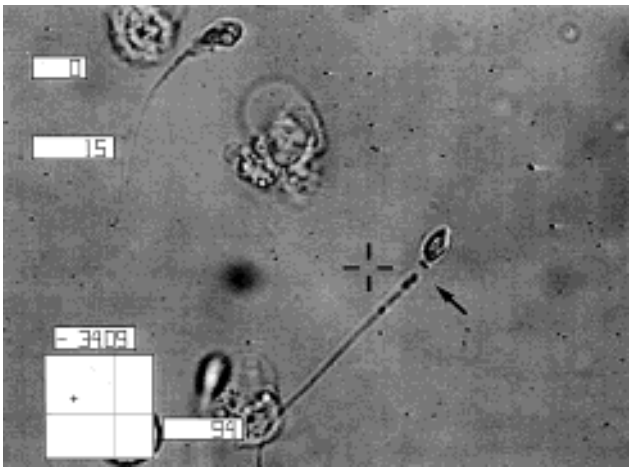

**Fig. 7.** Sperm tail removal. The tail has been easily cut (arrow) and separated from the head of a human sperm using the LaserScissors.

is necessary. It is interesting to note, however, that different approaches have been applied to the dissection of the zona pellucida. These approaches differ in the range of wavelengths used and the method of sample irradiation [for a review, see Tadir et al. (34)]. Several reports in animal research describe the use of UV or visible wavelengths. The advantage of using these wavelengths is that the laser beam can be delivered to the specimen directly though the objective lens of the microscope and the laser-assisted dissection is a *noncontact* mode. However, it is difficult to assess how much scattered light could irradiate the embryo. Consequently, when using UV light, there is legitimate concern about the potential absorption of light energy by DNA material. Radiations that have no mutagen effect, such as those in the IR range, may be more appropriate for the dissection of the zona in clinical applica-

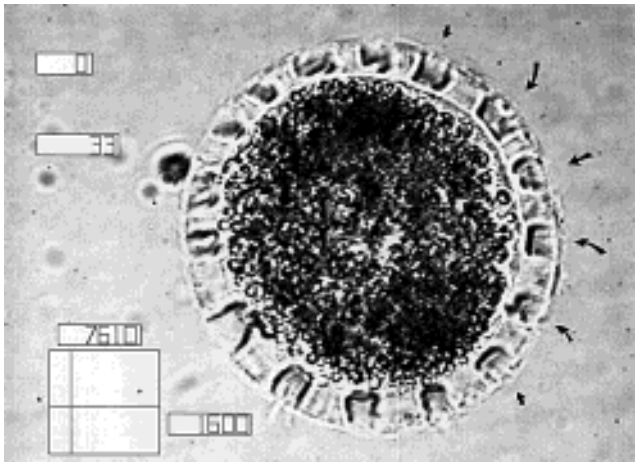

**Fig. 8.** Zona dissection. Partial zona dissections of a bovine oocyte were achieved using the LaserScissors *(arrows)*. Laser-assisted zona drilling finds application for the subzonal insemination of a sperm cell or for assisting hatching of the embryo.

tions. To date, many healthy babies have been delivered that were conceived with the aid of laser-assisted technologies. An overwhelming number of these procedures were conducted using an IR laser source at 2,900 nm (28). At this wavelength, the absorption of light by water is such that the laser beam cannot be delivered to the sample in a noncontact mode through the objective lens. For optimal transmission, the light needs to be guided to the sample using an optical fiber. When using the optical fiber approach, a mechanical micromanipulator is needed to position the fiber at the target and so this inconvenience remains. At Cell Robotics, we are currently investigating and developing an alternate method that will allow the noncontact mode, laser-assisted dissection of the zona pellucida to be performed in the IR range of the spectrum. This approach is particularly attractive for clinical applications because it offers the convenience of the noncontact method, while drastically minimizing any potential mutagen effect on the embryo. Preliminary experiments, using oocytes of murine origin, have shown that the precise noncontact dissection of the zona can be achieved at 1,480 nm, based on the absorption properties of water (8). A LaserScissors operating at 1,480 nm will provide research and clinical laboratories with probably the most appropriate tool for the assisted dissection of the zona pellucida. It is expected that physicians in fertility clinics will singularly benefit from laser-assisted technologies because they greatly facilitate current procedures and techniques in reproduction biology, are more efficient and more user-friendly, offer better control over sterility during specimen manipulations and should result in important time and cost savings. Furthermore, the laser beam itself can also be used as a diagnostic tool. Using varying laser intensities, it becomes possible to precisely assess the quality of the zona pellucida and to measure its hardness. Appropriate treatment can then be selected for each patient and each embryo, and pregnancy results studied retrospectively. The LaserTweezers and the LaserScissors are intended to make in vitro fertilization procedures more successful and more accessible to various clinical laboratories for the purpose of significantly improving fertility rates.

Finally, laser-assisted technologies have great potential for the routine genetic analysis of the preimplantation embryo [for further information regarding current preimplantation genetic analysis procedures, see Verlinsky and Cieslak (35)]. After cutting the tunnel in the zona pellucida using the microdissecting laser beam, the LaserTweezers is used for capturing the polar body from within the perivitelline space, making it available for further DNA testing after PCR amplification (Fig. 9). It is suggested that a similar approach could be used for the capture and recovery of individual blastomeres from precompaction embryos for genetic analysis.

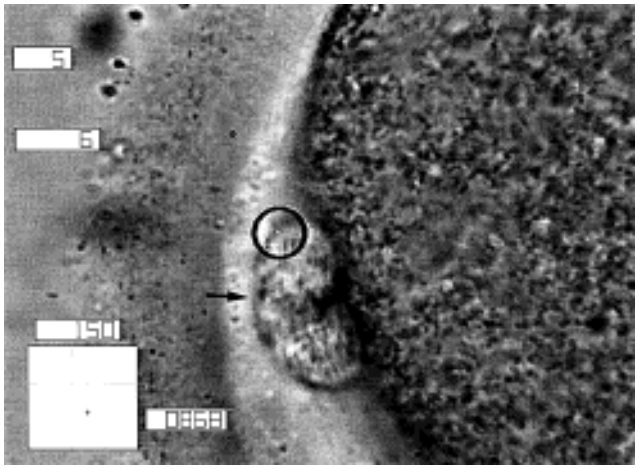

**Fig. 9.** Preimplantation genetic analysis. The polar body *(arrow)* can be captured from within the perivitelline space and recovered, as shown here with a degenerate human oocyte.

## Computer Controlled Workstation

Initially, technologies presented for optical trapping and laser microdissection applications were mainly the result of efforts established in highly specialized laser research laboratories. Consequently, instruments were not available to the widest variety of scientists and physicians. Primary prototypes were cumbersome and difficult to use. Large lasers resulted in prohibitive development costs and these systems required the presence of well trained personnel. Often, research programs were focused on studying the optical trapping and/or ablative phenomena themselves rather than to develop elaborate and new biological applications. Laser micromanipulations were then a science per se. With the introduction of more user friendly systems, optical trapping and scissing have found their way to a larger number of laboratories and are now considered for routine clinical diagnosis applications. The microrobotic laboratory depicted in Figure 1 constitutes the first attempt to offer these revolutionary techniques to a wide audience of scientists from various fields. With the microrobotic laboratory, optical trapping manipulations, single cell sorting and laser microdissections have now reached the level of well established techniques and are used in an exponentially growing number of applications.

A second generation of more elaborate instruments are now available that feature computer control to make completing optical trapping, laser microdissection and routine single cell sorting faster and easier. As shown in Figure 10, an integrated system based on a high speed computer includes a user-friendly control panel featuring detailed virtual tools to direct stage movement and operate all modules. The software interface now directs simultaneous laser beams, selective fluorescence illumination, the precision microsyringe pump and focus adjust. The large computer memory allows a variety of commands for the stage such as fine and coarse stepping functions and point features for the storing of preferred positions than can later be revisited. Video microscopy allows to perform experiments and tests to be performed at the single cell

level entirely at the computer. A video board permits the capture of either single frame images or video clips and digitally saves them while the assay is in progress. This feature makes obsolete the need for a video tape recorder. Stored data may then be used in charts, graphs, reports or publications. Since the microrobotic laboratory has turned into a complete computer workstation, custom-designed software or image analysis software are also being designed to fit specific clinical applications, such as for example for use in cancer research, reproduction biology and in vitro fertilization.

## CONCLUSION

Laser-assisted micromanipulations have already been described in various fields of the life science arena, such as in biology and biophysics, and should also find many applications in microchemistry, micromachining, and forensics. First, optical trapping systems and optical scissing apparatus were described as expensive and complex prototypes that only a few high-tech research laboratories could afford to develop. With the recent availability of modern and user friendly commercial instrumentation, laser tweezers and laser scissors have found their way to a much larger number of scientific institutions. Also, an analysis of the literature shows a recent and dramatic increase in the number of scientific reports describing the use of such technology. Nevertheless, because the use of lasers in the clinical field is a relatively recent event, many applications remain to be explored in this domain. Routine clinical diagnosis, for example, in the case of the early detection of cancer, should benefit from the versatility provided by optical trapping for the sorting of single cells with absolute purity for further genetic screening. The study of cellular interactions between NK cells and target cancer cells may lead to a better understanding of the immune response to cancer and to the development of more efficient methods of treatment. In the prenatal care area, it is likely that the capture and isolation of fetal cells from maternal blood will play a very important role in genetic diagnosis (36), (37). For this application, the LaserTweezers is a particularly attractive tool because it gives the operator a gentle means of collecting a rare cell with a high degree of reliability. The future development of new laboratory assays, using modern laser-assisted technologies, can be predicted in reproductive biology. Genetic screening of the preimplantation embryo is one example as is sorting single sperm cells for DNA typing and for forensic investigations. Sorting single microorganisms, such as bacteria and yeasts, is appealing to both research and industrial interests (biomedical, pharmaceutical, chemical, and agronomic fields). For example, the ability to manipulate with precision and ease a single specific microorganism is a tremendous advantage in separating and cloning a rare genetic variant from among an heterogeneous population of bacteria. Using this approach, it should be possible to isolate new molecular markers and to better manage the mass production of biomolecules of medical interest.

With the present evolution of the light microscope into a versatile and elaborate microrobotic laboratory, newly developing modes of assays and of clinical testing are likely to develop rapidly. In the modern biological laboratory,

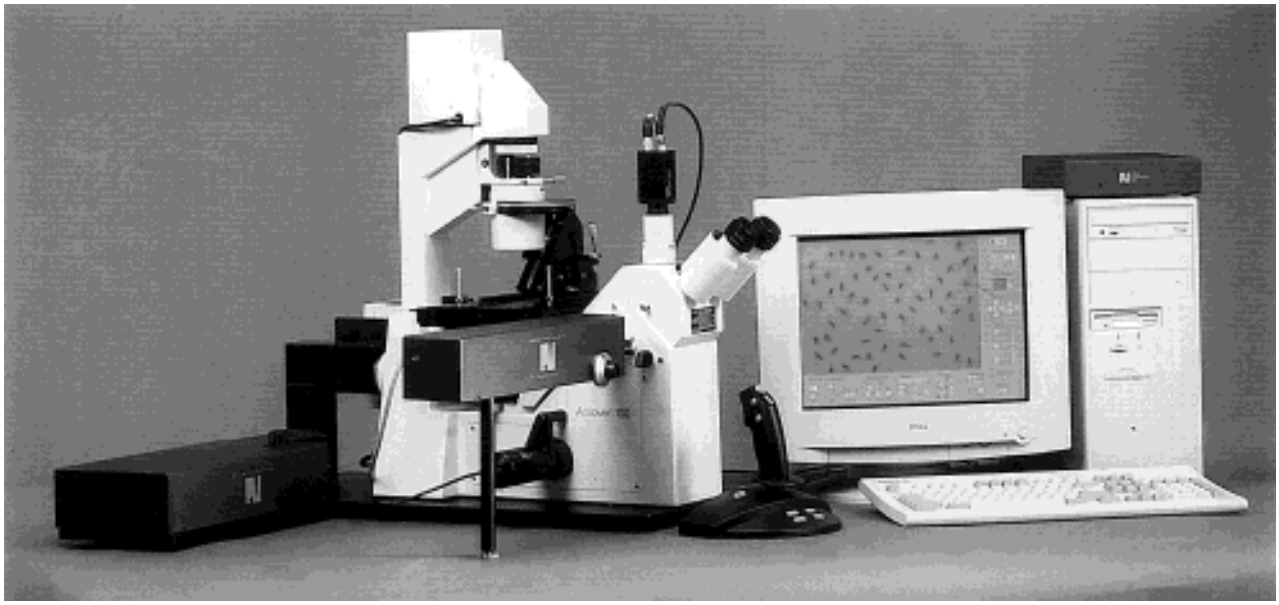

**Fig. 10.** Computer workstation. A computer controlled microrobotic laboratory includes LaserTweezers, LaserScissors, CellSelector, smart motorized stage, motorized focus adjust, video microscopy and elaborate software. The user's interface is designed for ease of use, accuracy and speed. The system can be mounted on inverted microscopes from any of the large manufacturers (e.g., Leica, Nikon, Olympus or Zeiss).

LaserTweezers and LaserScissors will soon constitute the prevailing tools for scientific investigations at the single-cell level.

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