

Application of Laser Optical Tweezers in Immunology and Molecular Genetics

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Optical tweezers, based on a compact diode pumped Nd:YAG laser providing 350 mW at 1,064 nm coupled into a Zeiss IM 35 microscope, were used to sort CD4⁺ T cells into a capillary for further mechanical handling and to establish contact between single human natural killer (NK) cells and human erythroleukemia cells (K562) as targets. After contact and a lag phase of a few tens of seconds, the target cell starts to change its morphology and membrane blebbing occurs. The kinetics of the attack of the NK cell on K562 cells is not straightforward but governed by temporal oscillations in the shape of the target cell (zeosis).

In a second application, the optical tweezers are combined with a UV laser

microbeam based on a pulsed UV laser and with flow cytometry and sorting. With the pulsed laser, segments of sorted chromosome 1 of the chinese hamster karyotype (CHV 79) can be easily microdissected and subsequently collected using the optical tweezers. This allows preparation of a few hundred chromosome segments per day without mechanical contact and in an absolutely sterile way and thus may provide an interesting basic technique in any type of genome sequencing project.

Key terms: Laser microbeam, natural killer cells, microdissection, DNA libraries, Nd:YAG laser, excimer laser, single cell technique

The optical tweezers are revolutionizing micromanipulation of biological objects (1-3,5,6). Typically, conventional Nd:YAG lasers are used as a light source; they are somewhat bulky in connection with the microscope and the ultrafine handling required in that context. For miniaturization, either a laser diode with limited beam quality and 100 mW power at a wavelength of 1.330 nm can be used (24), or the conventional Nd:YAG laser can be replaced by a compact diode pumped Nd:YAG laser with a small primary beam divergence. Such a laser (DPY 301, ADLAS, Lübeck, FRG) used in the present work provides 350 mW at a wavelength of 1.064 nm. This is sufficient to transport cells on a cover slide and thus can be used, for example, in experiments of immunological interest. Due to the clonal nature of cells of the immune system, single cell techniques are required in order to obtain information on the individual behaviour of these cells. Micromanipulation by glass needles is in principle possible (36-38), but the pressure (i.e., force per contact area) exerted may be locally very high. The forces exerted by the

optical tweezers, in contrast, are distributed over a large part of the cell and thus the local pressure is much smaller. Therefore, damage to sensitive immune cells, such as natural killer (NK) cells, can be minimized.

NK cells are a poorly defined and heterogeneous group of cytotoxic immune cells (17), which are obviously complementing the function of T cells. They show, for example, defective activity in AIDS patients (12). An important function of NK cells is the elimination of tumor cells in the early tumor growth phase (10,14,28). However, they can control only small tumors less than 10⁶ cells in size (10). Therefore, an efficient interaction is required to establish contact between NK and tumor cell. Knowledge on the very early process of attack on a single cell basis may help to develop strategies to control that process by drugs. Single cell techniques for studies of immune attack using a mechanical micromanipulator have been developed (36-38) but not used with human cytolytic cells. In another type of experiment, effector and target cells are mixed, centrifugated at 500g for a few minutes,

resulting in a force of the order of 10^{-4} dyn, and plated on a thin agarose film (7,8). Such experiments with radiolabeled target cells cannot reveal the heterogeneity of the NK-cell population and the variable early kinetics of cytotoxicity. Here, the optical tweezers provide a gentle and sterile tool to study that important process. Forces of 10^{-6} dyn rather than 10^{-4} dyn are acting and represent in vivo cell contact probably better than the forces acting during centrifugation. In addition, using optical tweezers, the cells can be observed immediately after contact formation.

Micromanipulation as described above is highly suited to replace mechanical techniques. A complementary tool is the laser microbeam for processing of such materials. Unlike the optical tweezers, which have a continuous wave infrared laser of moderate power as light source, recent versions of the laser microbeam use pulsed UV lasers with pulse lengths of 3–20 ns and power densities up to 10^{13} W/cm². While in earlier versions visible light was used, now excimer pumped dye lasers, frequency multiplied Nd:YAG lasers (4,19) or diffraction limited N₂ lasers are preferred. With a laser microbeam, for example, genetic material has been introduced into animal (19,27) and plant cells (29–33). Cells of different types have been fused (34,35). It is obvious that combination of optical tweezers with a laser microbeam allows microprocessing and micromanipulation without any mechanical contact. First experiments of that type have been reported by Wiegand-Staubing et al. (35; see also 22), where mammalian cells have been fused by a laser microbeam after contact has been established with the optical tweezers. In addition, the combined use of these tools has been suggested (11,15,16) for various applications in cellular and molecular biology.

Working in the ultraviolet allows very precise and efficient micromachining of biological material. Thus, chromosomes have been microcloned after microdissection with a UV laser microbeam (11,21). This is of importance for genome sequencing, since it allows preparation of large amounts of chromosome segments for subsequent microcloning and thus reduces erroneous results, when, for example, the polymerase chain reaction or microcloning into plasmids or phage vectors is employed. The use of a laser microbeam alone has highly speeded up the preparation of chromosome segments (20), and it is possible to prepare about 100 segments per day. The limiting step at present is collecting the chromosome segments after microdissection. Here we speed up that process further by combined use of microbeam and optical tweezers on flow-sorted chromosomes.

MATERIALS AND METHODS

Optical Tweezers and Microbeam

The optical tweezers used consist of a cw diode pumped Nd:YAG-laser (ADLAS, DPY 301, Lübeck, FRG) which is directed through the fluorescence illumination path into a microscope (Zeiss, IM 35,

Oberkochen, FRG) (Fig. 1). The specifications of the cw Nd:YAG laser are as follows: wavelength, 1,064 nm; beam diameter, 1.0 mm; output power, adjustable from 0 to 350 mW; and beam divergence of <1.8 mrad. The Nd:YAG beam is expanded by a telescope system and reflected by a dielectric mirror (coating for 337 nm and 1,064 nm, Laser Optik, Garbsen, FRG) and focussed into a microscope through a Zeiss Ultrafluar 100/1.25 Glyc. objective. Objects are translated by fixing them in the focus of the Nd:YAG laser and moving the motor driven XY stage of the microscope.

A N₂-laser (VSL-337ND, Laser Science Inc., Cambridge, MA), coupled into the microscope as described for the Nd:YAG laser, was used as light source for the microbeam to microdissect chromosomes. The N₂-laser emits at 337 nm and has a pulse duration of 3 ns and a maximum pulse energy of 270 μ J at a repetition rate of 20 Hz.

To observe the specimen during irradiation, a TV-camera, monitor, and a video system (Hamamatsu, photonic microscope system C 1966-20) were used. Figure 2 shows the setup of optical tweezers and microbeam.

Flow Cytometry and Sorting

Flow cytometric measurements and sorting were carried out with the HEIFAS equipment (Heidelberg Flow Analyzer and Sorter). This system is described in detail elsewhere (25). The UV lines (351 nm and 364 nm at 800 mW) of an argon ion laser (Spectra Physics, Darmstadt, FRG, 171-19) were utilized for fluorescence excitation. For DNA analysis alone a 386 nm cutoff filter (KV 386, Schott) was used to separate the scattered UV light from the fluorescence emission. The HEIFAS instrument was connected to a personal computer (COM-PAC, DESKPRO 386/16). Data acquisition, real time display, chromosome analysis, and graphical documentation has been realized in the "C" language. The interface between HEIFAS flow cytometer and personal computer is the DT-2828 board (Data Translation) (13).

Petri Dishes

The Petriperm dishes used (Heraeus, Hanau, FRG) have a diameter of 5 cm. The bottom consists of a thin hydrophobic PTFE film to reduce adhesion forces between cells and the bottom of the dish. Both lasers can be easily focussed through the PTFE film without significant scattering losses and without damaging the film.

Chamber for Cell Selection

The chamber for microsorting of single cells was specifically developed for this purpose. It consists of three silicon wells (Wacker-Chemie GmbH, München, FRG), stuck on a 50 mm Petriperm dish. The wells are connected with each other by narrow channels (Fig. 3). One of the wells contained a glass capillary. The well containing the capillary and the well in the middle was filled with *Chromosomenmedium B* (Biochrom, Berlin, FRG). The cell suspension was carefully syringed into

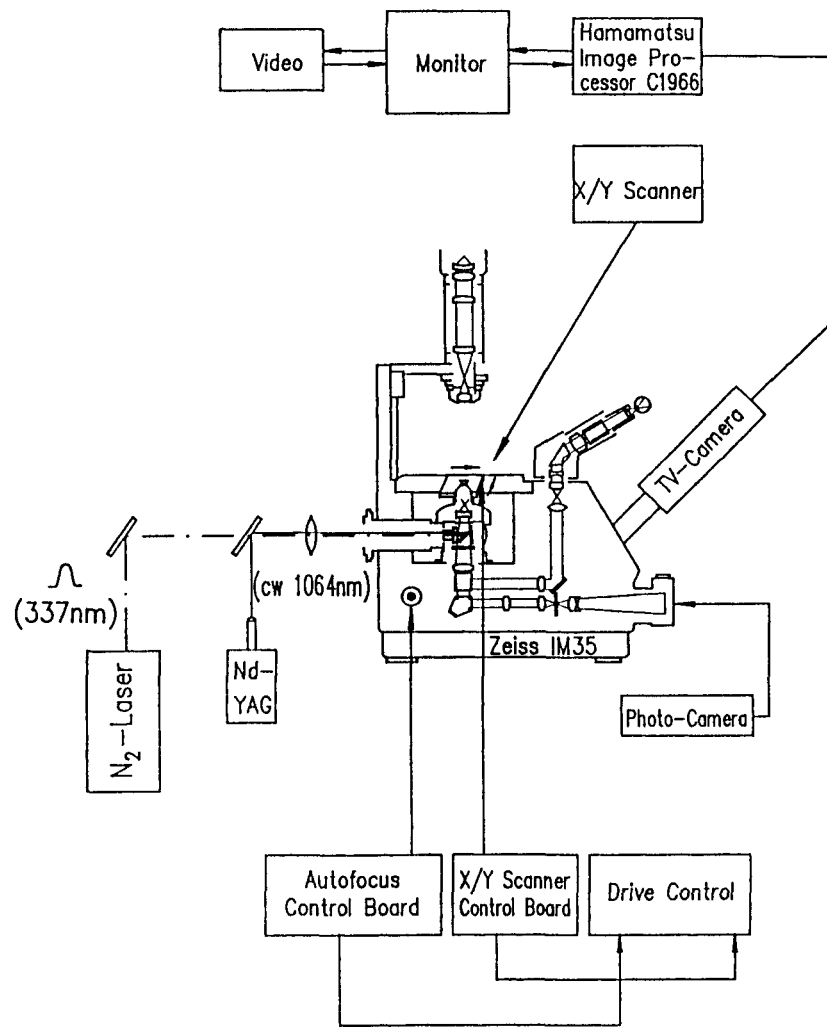


FIG. 1. Schematic diagram of the optical tweezers and UV-laser microbeam. The basic components are a cw-diode pumped Nd-YAG laser (ADLAS) of 1,064 nm wavelength as optical tweezers; a nitro-

gen-laser (LSI) of 337 nm wavelength at 3 nm pulse duration as UV-microbeam; the inverted microscope (IM 35, Zeiss); AVEC image processing system (Hamamatsu) and video system for documentation.

the third well. For trapping of sedimented cells it is useful to minimize forces due to the adhesion between cells and bottom.

Target Cells and NK-Cells

The continuous erythroleukemia cell line K562 was cultured at 37°C, 5% CO₂ and humidified air in RPMI 1640 (Nordvac, Sweden) supplemented with 10% heat-inactivated fetal calf-serum (Boehringer, Mannheim, FRG), 1% glutamine, and antibiotics. NK cells were obtained from the mononuclear fraction of human blood after Ficoll (Pharmacia, Freiburg, FRG) gradient centrifugation of 5 ml fresh human blood. The mononuclear fraction was washed twice with phosphate-buffered saline, PBS (Serva, Heidelberg, FRG).

Labelling of CD4⁺-Cells and NK-Cells

After separation the mononuclear cells were resuspended in PBS to a concentration of approximately

2·10⁶ cells/ml. One hundred microliters of cell suspension were pipetted into a plastic tube (Greiner GmbH, Frickenhausen, FRG). In the case of NK cell labelling, 200 µl PBS and 20 µl of anti-NK monoclonal antibody (Dianova, Hamburg, FRG) were added, gently mixed, and incubated for 40 min at +4°C. After washing twice with PBS, 20 µl of dichlorotriazinyl amino fluorescein (DTAF)-labelled anti-mouse immunoglobulin solution (F[ab]₂ goat antimouse IgG (H+L)-DTAF diluted 1/50 in PBS) (Dianova, Hamburg, FRG) was added to the tube, gently mixed, and incubated for 40 min at +4°C. After incubation the cell suspension was washed twice with PBS and resuspended in *Chromosomenmedium B*. NK cells were mixed with target cells in petri dishes. CD4⁺-cell labelling was carried out in the same way; instead of using an anti-NK monoclonal antibody cells were labelled with anti-CD4 monoclonal antibody (Dianova, Hamburg, FRG).

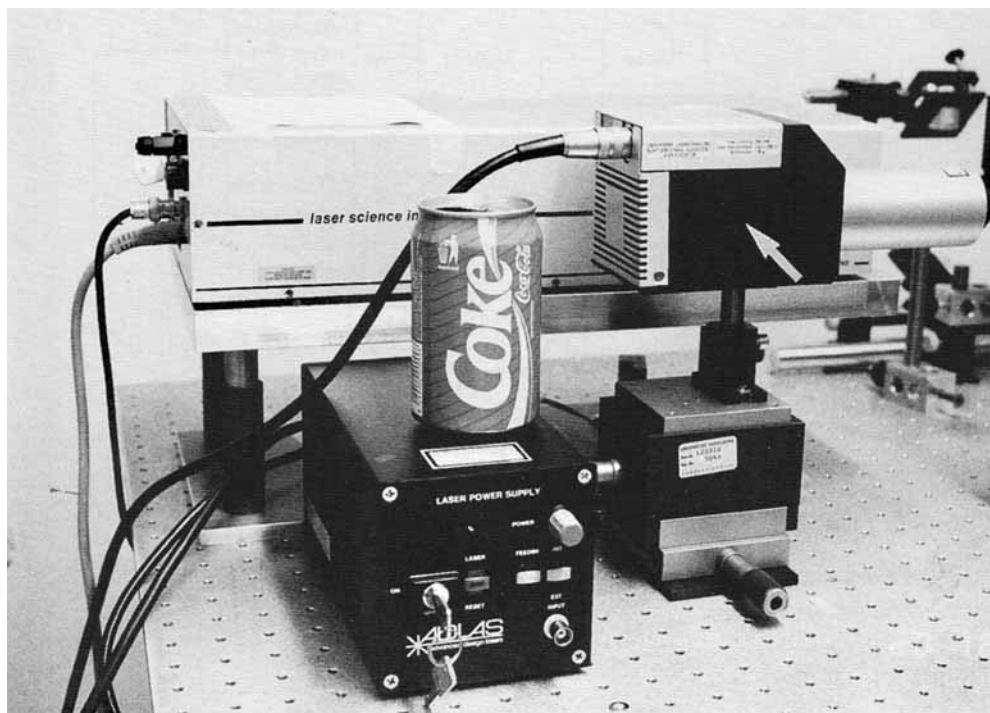


FIG. 2. The setup of the lasers for the optical tweezers and microbeam. **Front:** Nd-YAG-laser (arrow). **Background:** N₂-laser.

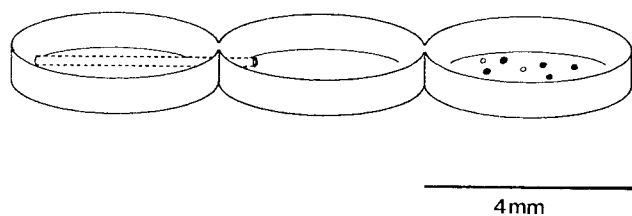


FIG. 3. Chamber for microsorting (see text).

Human Mononuclear Cells

Human mononuclear cells were obtained by Ficoll gradient centrifugation of 5 ml fresh human blood. The Ficoll was obtained from Pharmacia (Freiburg, FRG).

Cell Culture and Arresting of Mitotic Cells

Chinese hamster cells (CHV 79) were cultivated in tissue flasks (Falcon, 3024F) with minimal essential medium (Eagle, Cat. No. 10-101-26) supplemented with 20% fetal calf serum (Biochrom, Berlin, FRG) and 2.2 g NaHCO₃. Cells were grown as monolayer cultures for 3 h at 37°C in the presence of colcemid (Ciba Geigy, Cat. No. 27645, Basel, Switzerland; stock solution: 25 mg colcemid/250 ml PBS; working solution: 200 ml of stock solution/20 ml growth medium). This high colcemid dose and prolonged time of colcemid inhibition guaranteed a high metaphase yield (mitotic index = 95%) (26).

Isolation of Metaphase Chromosomes and Staining

After colcemid arrest of metaphase cells, the entire cell suspension was centrifuged at 50g for 10 min. The pellet was resuspended in 5 ml of hypotonic solution (0.075 M KCl) for 5 min at room temperature. After centrifugation the pellet was resuspended in 1 ml of 1% acetic acid in hexylene glycol buffer at pH 3.2. The cell suspension was syringed through a needle No. 20 (No. 20 Luer-Konus, Erosa, FRG) five to ten times to release the chromosomes (18).

HEXYLENE GLYCOL BUFFER

25 mM tris-HCl buffer (pH 7.5), 0.75M Hexandiol-(1.6) (Merck, No. 8043308), 0.5 mM CaCl₂ × 2H₂O, containing 1% acetic acid.

Staining Solution

Stock solution: 10 mg 4.6-diamidino-2-phenyl-indol/100 ml Tris buffer. Working solution: 100 μl of the DAPI stock solution were added to 1 ml of chromosome suspension.

RESULTS

As a first application, a T cell, identified with a fluorescence labelled anti-CD4 antibody as a T helper cell, is sorted into a micro-capillary (see Fig. 3) for further mechanical handling. The inner diameter of the capillary is approximately 80 μm, its wall is 20 μm thick.

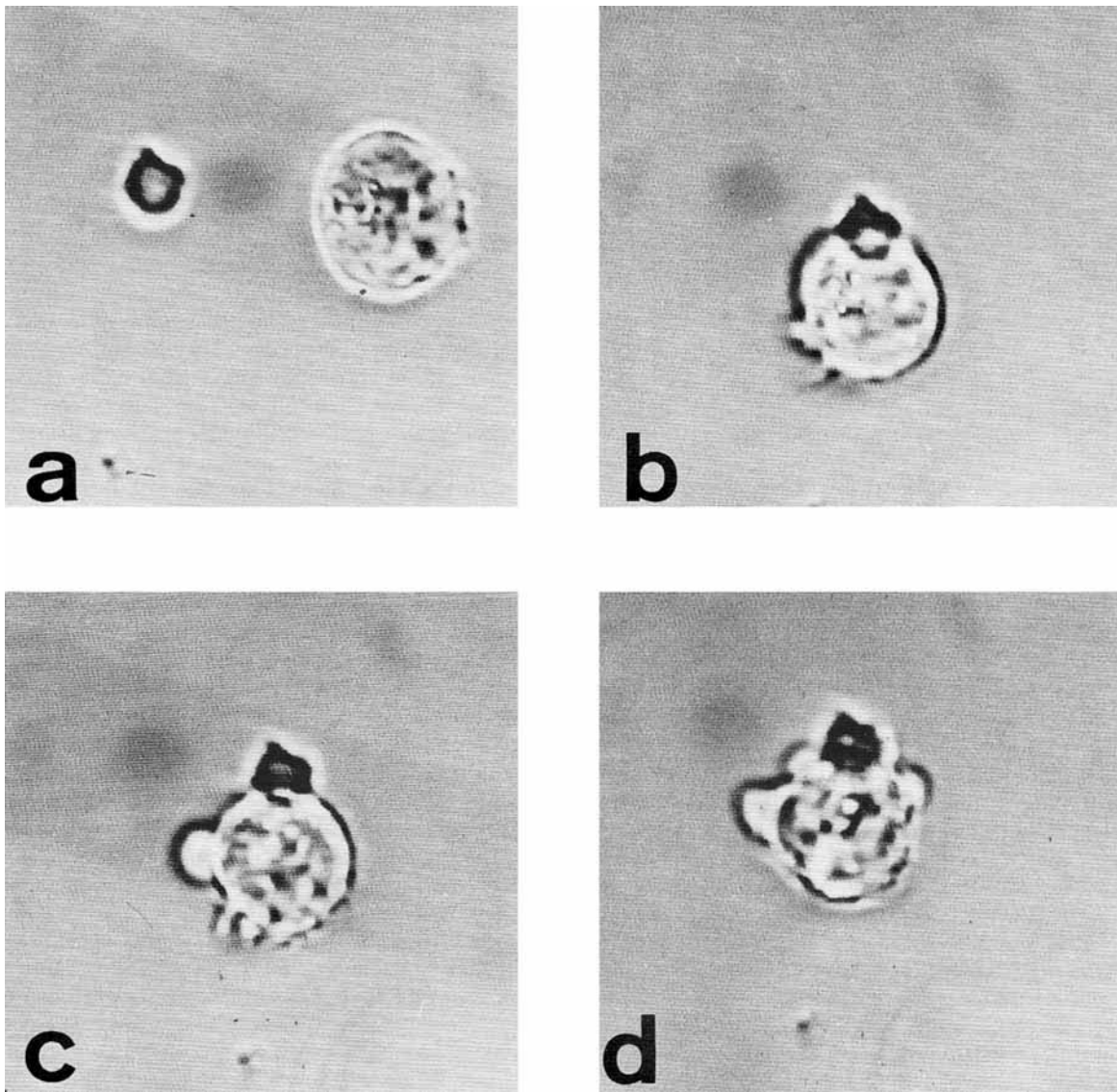


FIG. 4. Attack of a natural killer cell against a erythro leukemia cell K562. The contact between the cells was induced by the optical tweezers. K562 target (right) and NK cell (left) a) before contact, b) immediately after contact, and c) 160 s and d) 350 s later.

After the cell has been moved with the optical tweezers close to the end of the capillary, it has to be lifted against gravity. This is achieved by moving the focus of the Nd:YAG laser. When the distance between trapped cell and the bottom of the dish is about 20 μm , it can easily be translated into the capillary. This procedure takes less than 5 min. Afterwards the whole capillary can be easily handled mechanically; for example, it can be transported with a mechanical tweezers. In total, the laser has been used as a kind of simple micro-cell-sorter.

A second application for *immunology* is shown in Figure 4a–d. It shows a target cell and a effector cell identified using a fluorescent antibody binding typi-

cally to NK cells (Dianova, Hamburg, FRG). It is trapped in the laser beam and the microscope stage is moved, and thus contact is established between effector and target cell (Fig. 4b). The changes in morphology and the membrane blebbing is clearly visible.

The apparent area of the target cell after the attack of a NK cell is shown in Figure 5. Swelling and shrinking of the target cell can be measured immediately after contact with the killer cell. The apparent area of the cell increases by 15% within 20 s. A tentative explanation for the observed temporal oscillation may be that repair mechanisms in the target cell attempt to counteract the NK attack.

The experiments described in Figures 4 and 5 were

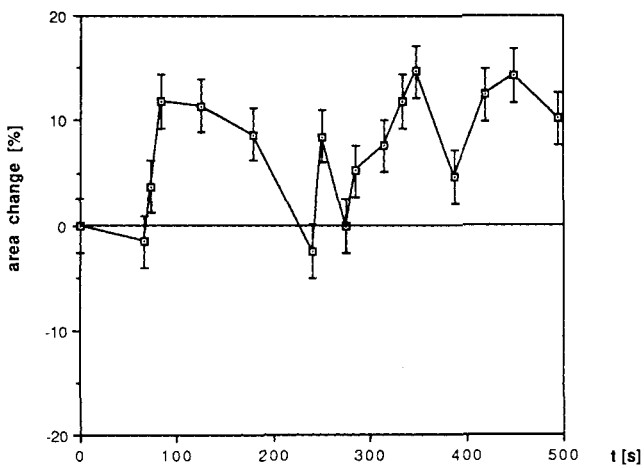


FIG. 5. Apparent temporal area changes of a target cell during the attack of a killer cell.

reproduced several times. In all cases, either the oscillatory kinetics was observed, albeit with varying time scales, or no effect on the target cell at all was observed; i.e., the lag phase was longer than the observation time. These differences may be caused just by different viability of effector or target cells or by not yet understood differences in handling of the cells during preparation of the experiment. They may, however, also reflect the clonal heterogeneity of NK cells themselves.

As an example for the use of optical tweezers in *molecular genetics*, we combine it with a UV laser microbeam. Such a UV-laser microbeam has already improved the accuracy and speed in the microdissection of chromosomes. In practice, it has turned out that the collection of chromosome segments after laser microdissection is still too slow and can result in failure of experiments (9) due to hydrolysis of the DNA in the metaphase preparates. As a remedy to that problem, two changes have been introduced in the original protocols for laser microdissection: The optical tweezers are used instead of glass needles and the chromosomes are prepared by chromosome sorting which provides unfixed sorted chromosomes. The latter avoids sticking of the fixed metaphase chromosomes to the object slides. Therefore, chinese hamster chromosomes (CHV 79, DKFZ Heidelberg) were sorted onto thin cover slides (76 mm \times 26 mm \times 0.2 mm) (4,000 chromosomes 1/glass).

The purity of sorted chromosomes was 91%, as counted by fluorescence microscopy (23).

A drop of suspension (HEPES buffer) is placed on the cover slide with sorted chinese hamster chromosomes. With the UV laser microbeam the chromosome which slightly adheres to the glass, can be cut into segments. Then the chromosome segment adhering to the glass is detached from the bottom by light pressure and starts to move freely in the suspension. The chromosome can

now be trapped and moved similarly as described above for the T helper cell. Figure 6 shows that procedure.

DISCUSSION

Though the experiments described here have been designed primarily to show some first applications of the optical tweezers, they reveal the immense potential this tool may gain for immunology, as well as, when combined with the UV laser microbeam, for molecular genetics and particularly for genome projects. Thus, in addition to providing interesting information on the early kinetics of NK attack on tumor cells, much more information can be obtained, since the individual effects of NK subtypes on tumor cells have not yet been studied. To test this, NK cells have to be characterized with all presently available NK markers (CD16, CD56, CD57) and a variety of different target cells has to be used. It may well be that specific NK subtypes are particularly efficient in lysing particular tumors; thus, such experiments may give valuable hints for the development of cancer therapies based on stimulation of those NK subtypes. This will doubtless remain a tedious task, but with the optical tweezers it appears feasible, since it minimizes the micromanipulation work itself and thus allows the experimenter to concentrate on the cell biology of the process. No other experimental technique presently available provides the required amount of information in such a simple way as optical tweezers. Beyond studies on NK cells, single cell techniques are generally important in many cell biological applications, and particularly in immune research, because of the clonal nature of many cells of immunological interest.

In addition, a combination of the optical tweezers and flow cytometric analysis and electronic sorting was applied in order to obtain highly enriched individual chromosomes (in this case chromosome 1 of chinese hamster karyotype with a purity of 91%). As the objects are located closely together, segments of chromosomes can easily be microdissected with a UV laser microbeam and subsequently collected using the optical tweezers.

The time-consuming and aggressive preparation of metaphase spreads (fixation with methanol:acetic acid (3:1); 2–5 days air drying of slides; staining by Giemsa-Trypsin-procedure) and the tedious microscopic identification of individual chromosomes in these metaphases can be avoided by pre-enrichment using flow sorting (gain in speed: several days). Furthermore, the collection of microdissected segments of chromosomes prepared by sorting represents gentle treatment of chromosomal DNA, while removal of fixed and air-dried metaphase chromosomes can cause mechanical and/or chemical damage.

Similarly, the combined use of optical tweezers with UV laser microbeam will allow preparation of a few hundred chromosome segments of the size of 20 megabases per day. Thus it appears feasible to prepare chromosome segment specific libraries with approxi-

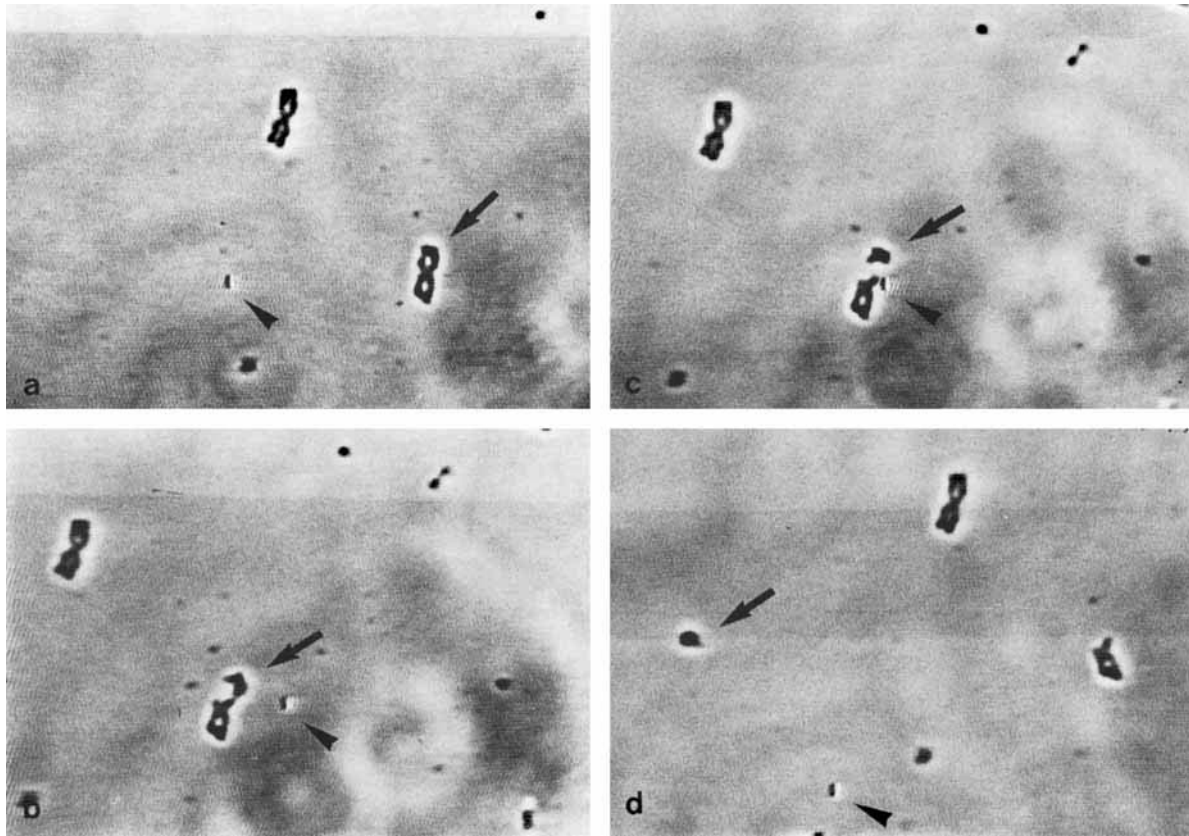


FIG. 6. The use of optical tweezers and UV-laser microbeam in microdissection and microcloning; a) a single sorted Chinese hamster chromosome before treatment. The cursor shows the position of the UV beam (triangle). b,c: Telomere cut off from chromosome 1. d: Chromosome segment transferred from the right to left by moving the

whole microscope slide and fixing the segment in the Nd:YAG beam (arrow). The position of the chromosome segment corresponds to the Nd:YAG beam position. In this experiment, the power of the Nd:YAG laser was approximately 100 mW. The chromosome segments collected in this way can be directly used for microcloning experiments.

mately 1,000 clones; i.e., the idea of preparing plasmid or phage libraries which statistically cover a whole chromosome segment is no longer unrealistic.

Finally, the use of a simple switch-on switch-off laser, such as the diode pumped Nd:YAG and the N_2 laser, will make this interesting tool available not only for laser specialists, but also for biologists who wish to concentrate on their biological problem and thus need reliable and simple-to-use equipment.

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