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Journal

Experimental Cell Research, 204(1)

ISSN

0014-4827

Authors

Liang, Hong
Wright, William H
Cheng, Steve
et al.

Publication Date

1993

DOI

10.1006/excr.1993.1015

EXPERIMENTAL CELL RESEARCH 204, 110–120 (1993)

Micromanipulation of Chromosomes in PTK2 Cells Using Laser Microsurgery (Optical Scalpel) in Combination with Laser-Induced Optical Force (Optical Tweezers)

HONG LIANG, WILLIAM H. WRIGHT, STEVE CHENG, WEI HE, AND MICHAEL W. BERNS[1]

Beckman Laser Institute and Medical Clinic, University of California, Irvine, Irvine, California 92715

An optical scalpel and optical tweezer have been combined to perform intracellular microsurgery and micromanipulation *in vivo*. When only laser microsurgery was performed on metaphase chromosomes, the dissected sister chromatid fragments drifted off to either the side of the spindle or completely off the spindle. At anaphase the fragments separated and the two arms generally moved to their respective daughter cells. When the chromosome arm was cut during anaphase A and B, the distal chromosome fragment separated from the rest of the chromosome and moved toward the pole, following the proximal chromosome fragment. Distal chromosome fragments laser-dissected during metaphase were held together throughout anaphase using the optical trap. Optical trapping of dissected chromosome fragments during anaphase A and B inhibited movement of the chromosome fragment to its pole. As a result, the trapped chromosome fragments were (1) incorporated into the opposite daughter cell, (2) lost in the cleavage furrow during cytokinesis, or (3) eventually incorporated into the correct daughter cell. These results indicate that optical traps are effective in holding laser-dissected chromosome fragments throughout mitosis. This new tool should be useful for studies on chromosome movement and cell genetics. © 1993 Academic Press, Inc.

INTRODUCTION

Since the development of cellular and subcellular laser microsurgery and micromanipulation, a large number of laser microbeam studies involving nuclear components, especially chromosomes, have been performed [1–24]. The first experiments showed that a focused laser beam would delete tiny chromosome segments in living salamander lung cells with cell survival for several days [1]. Later studies demonstrated that it was possible either to selectively delete the nucleolar organizer region of the chromosome [2, 3] or to remove an entire chromosome from the spindle by irradiating the kinetochore [8–10]. Further studies determined optimal laser parameters and laser dosimetry [9, 11] as well as cloning techniques [6, 10, 11] and led to the establishment of cellular sublines with deleted ribosomal genes resulting from laser irradiation of the rDNA site on the mitotic chromosome [18, 19].

The recent demonstration of optical trapping of biological cells by Ashkin *et al.* [25, 26] using a laser microbeam provides a noninvasive and nondestructive tool for the manipulation of cellular and subcellular organelles. Berns *et al.* [27] first reported the manipulation of chromosomes in mitotic PTK_2 cells *in vitro* by optical trapping. In this study, later extended by Liang *et al.* [28], an optical force applied to a late moving metaphase chromosome caused it to accelerate toward the metaphase plate. Furthermore, it was also found [28] that anaphase chromosomes could be held motionless by the trapping force, suggesting that this technique may be used as a noninvasive micromanipulator for the study of cell division and cell genetics.

We have taken the logical next step of combining the laser microbeam used for surgery with the optical trap. In this paper, we report on the microdissection and micromanipulation of chromosomes in mitotic cells by laser microsurgery (optical scalpel) in combination with laser-induced optical forces (optical tweezer) with the purpose of manipulating chromosomes in mitotic living cells. In these studies we use the optical scalpel to cut chromosomes at a preselected site, and the optical tweezer to hold and manipulate the cut chromosome fragment.

MATERIALS AND METHODS

Cell and cell culture. The cells used in this study are male rat kangaroo *Potorous tridactylis* kidney cells (PTK2). They are ideal because of their flat morphology and easily recognizable chromosomes. Cells

[1] To whom correspondence and reprint requests should be addressed at Beckman Laser Institute and Medical Clinic, 1002 Health Sciences Road East, Irvine, CA 92715. Fax: (714) 856-8413.

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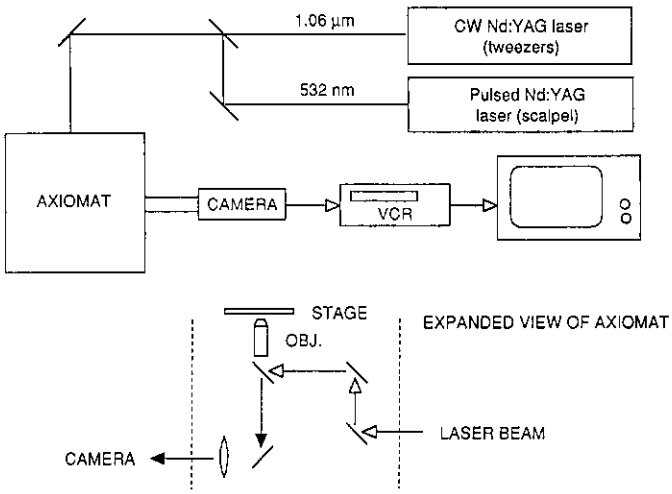

FIG. 1. Simplified instrument configuration for the combined use of a pulsed microsurgical laser beam and a single-beam gradient force optical trap.

were grown as monolayer cultures in T25 flasks in minimal essential medium (GIBCO Laboratories, Grand Island, NY) containing 10% fetal calf serum and 1% 200 m*M* glutamine. Cultures were maintained at 37°C in a 5–7.5% CO_2 incubator. Cells were subcultured once a week using a 0.125% viokase solution for digestion. Two or three days before each experiment, cells collected from the stock flasks were seeded into Rose chambers [29] by injection. Rose chambers with healthy mitotic cells were chosen for experiments.

Laser instrumentation The configuration of the combined pulsed laser surgical microbeam and continuous wave optical trapping microbeam is diagrammed in Fig. 1. The pulsed laser consisted of a picosecond Nd-YAG laser (Coherent Model Antares 76-YAG, Palo Alto, CA) amplified by a Continuum Model RGA 60–10 regenerative amplifier at a pulse rate of 10 Hz. The output was frequency-doubled to a wavelength of 532 nm. Ten shots were fired in rapid succession, approximately 0.5 s apart, for each chromosome irradiation. The power level at each laser pulse was approximately 70 nJ at the specimen plane. A second Nd-YAG laser (Quantronix Model 116, Smithtown, NY) operating continuous wave (CW) at a wavelength of 1060 nm in the TEM_{00} mode was used to generate the optical trap. Both lasers were directed by a series of mirrors into an inverted Zeiss Axiomat microscope and focused onto the specimen plane by a Neofluar X-100 phase-contrast objective (Zeiss), having a numerical aperture of 1.3. A dichroic reflector inside the microscope was used to reflect both the infrared optical trap beam and the green microsurgical beam into the microscope while transmitting an image to the video camera attached to one of the observation ports of the microscope. The video image was recorded by a half-inch time lapse VCR (Panasonic) and displayed on a monochrome monitor. In all the experiments, the target chromosomes were exposed to the 532-nm pulsed laser microbeam or infrared continuous laser optical trap while being continuously observed by video. All the micrographs presented in this paper were reproduced from the stored videotape image.

For each individual experiment, the laser power was carefully monitored and controlled so that it remained constant throughout the experiment. For the pulsed microsurgical beam a Scientech Model 365 was used to measure the energy before the beam entered the microscope. An attenuator (Karl Lambrecht Model k1174) located after the energy meter controlled the energy of the microsurgical beam entering the microscope. A laser power of 60 mW was used for the optical trap and was checked at the objective focal plane using a Coherent Model 210 power meter.

RESULTS

Microdissection of Chromosomes

Flat cells in prometaphase to anaphase were chosen for experimentation. A total of 22 cells, consisting of 7 at prometaphase, 5 at metaphase, 5 at anaphase A, and 5 at anaphase B were used for laser microdissection only. Large chromosomes, most likely chromosome numbers 1–4, were selected for irradiation. Microdissection of the selected chromosome was observed by projecting the chromosome image onto the TV monitor with a cross hair depicting the point of laser focus. The target area of the chromosome was then moved under the cross hair and the laser was triggered by remote control until a pale lesion was observed at the exposure site on the chromosome, as shown in Fig. 3B. The chromosome fragments were observed under phase-contrast microscopy until the cell either reached telophase or divided into two daughter cells.

Figure 2 is an example of chromosome microdissection during metaphase. After the sister chromatid fragments were cut with the laser microbeam (Fig. 2A), they drifted into the cytoplasm and remained separated from each other in the middle of the cell through the remainder of mitosis (Figs. 2B and 2C). However, by the end of cytokinesis, the chromosome fragments ended up in their respective daughter cells (Fig. 2D). The remaining centromere-containing sister chromatids of the irradiated chromosome remained attached to each other and stayed together until the onset of anaphase. At anaphase, the sister chromatids of the irradiated chromosome separated normally from one another in a manner similar to that of the nonirradiated chromosomes (Fig. 2B). The sister-chromatid fragments, however, separated from each other at the beginning of anaphase, although their rate of separation was slower than that of the nonirradiated chromosomes. In three out of five metaphase cells (not shown) the irradiated sister chromatid fragments remained in the mitotic spindle, eventually reached their corresponding poles, and were integrated into the entire set of chromosomes as the cell entered telophase. Out of 12 prometaphase and metaphase cells studied, there were two exceptions in which the irradiated sister chromatid fragments did not separate from each other. As a result, these two chromosomal fragments were incorporated into one daughter cell. Nevertheless, the behavior pattern of the majority of the chromosomal fragments generated by laser microsurgery during prometaphase and metaphase is that they eventually are incorporated into their respective daughter cells regardless of whether they remained on

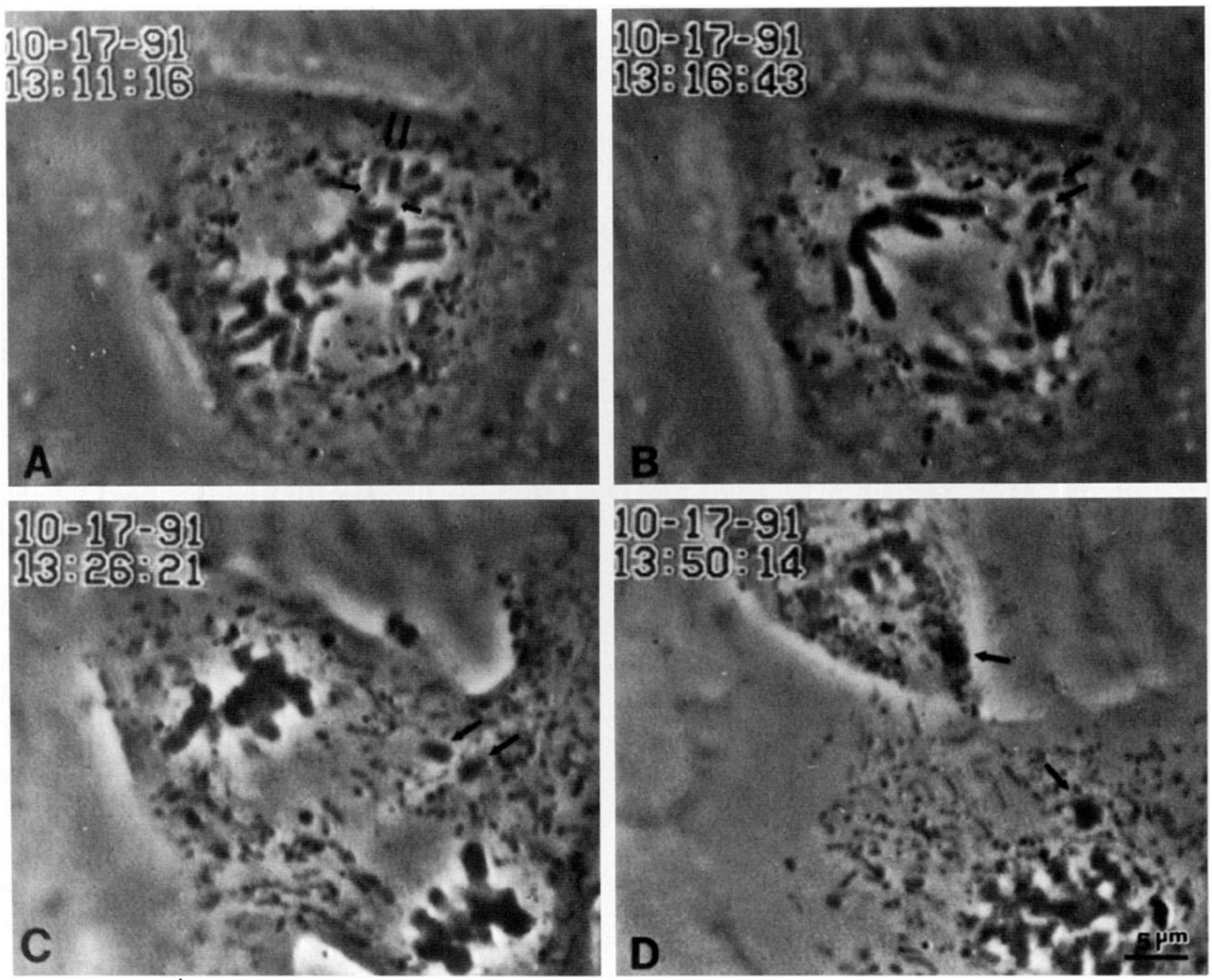

FIG. 2. Laser microsurgery of a metaphase chromosome. (A) A few seconds after laser microsurgery. Small arrows indicate the irradiation site. (B) The two dissected sister chromatid fragments (indicated by arrows) drifted into the cytoplasm during anaphase. (C) The chromosome fragments remained in the interzone cytoplasm while the cell completed anaphase. (D) Eventually, the two chromosome fragments were separated into their corresponding daughter cells.

the mitotic spindle or drifted into the cytoplasm after laser irradiation.

For a chromosome that was dissected during anaphase A or B, the chromosomal fragment separated from the remainder of the daughter chromatid in all cases. An example of chromosome dissection during anaphase B is shown in Fig. 3. Note that the fragment followed the daughter chromatid towards the pole (Fig. 3C). This was true regardless of the size of the laser-dissected fragment. There was no difference in the behavior of the chromosomal fragment cut during anaphase A or B. As a result of these experiments, the majority of the chromosomal fragments produced in prometaphase to anaphase were incorporated into their corresponding daughter cells.

Optical Trapping of Chromosomal Fragments after Laser Microsurgery

A total of 23 cells, consisting of 7 at metaphase, 8 at anaphase A, and 8 at anaphase B were used to study the effect of optical trapping on chromosomal fragments produced as a result of laser microsurgery. We first dissected a fragment from a chromosome arm using the pulsed laser microbeam, as described in the last section. Next the dissected chromosome fragment was trapped by the IR laser microbeam. Each cell was observed continuously during microsurgery and trapping. Of the 23 cells studied, all survived and completed mitosis. The experiment was terminated at the end of mitosis.

The behavior of the dissected sister chromatid frag-

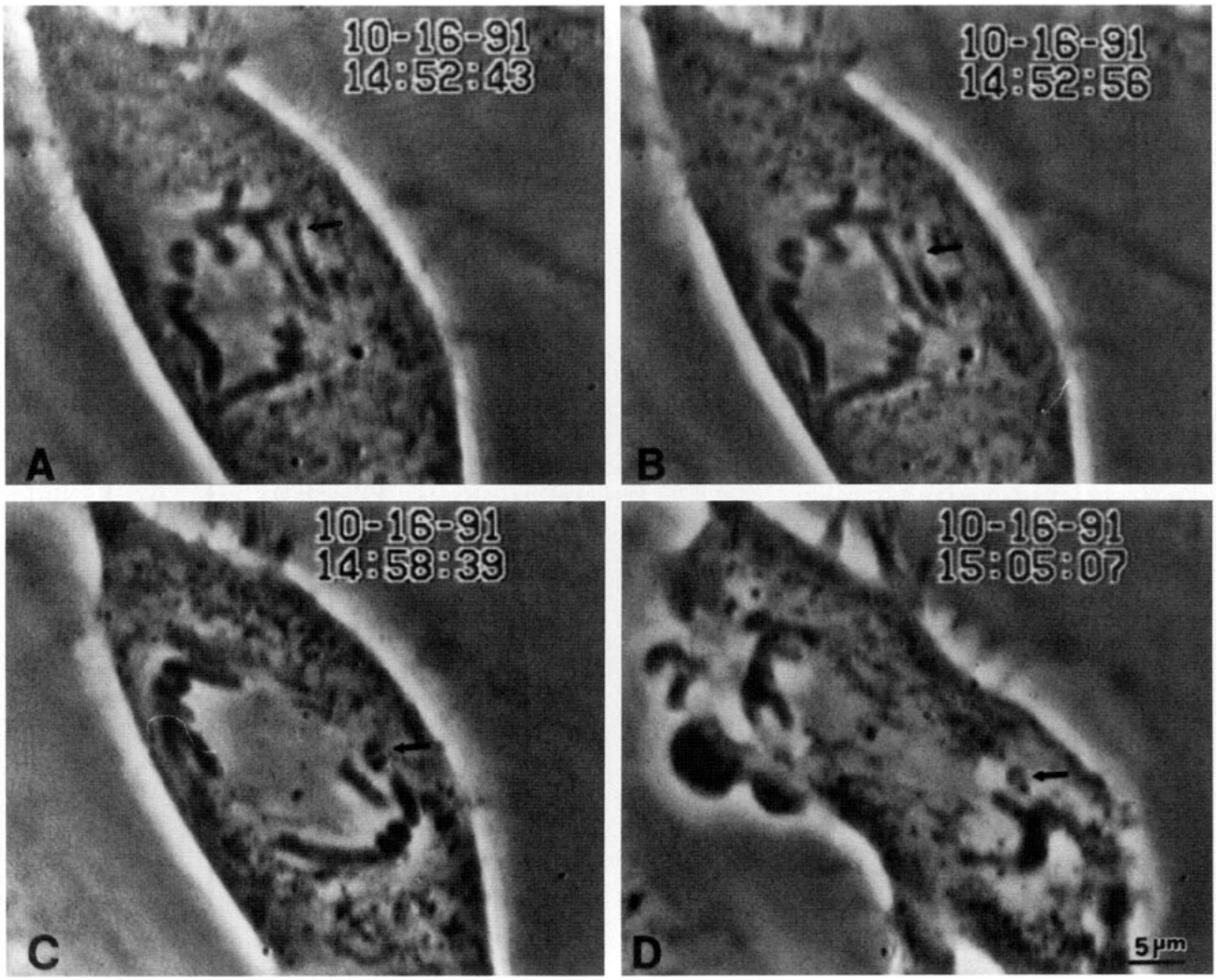

FIG. 3. Laser microsurgery of a chromosome during anaphase B. (A) The selected chromosome (indicated by arrow) prior to laser microsurgery. (B) The chromosome immediately after pulsed laser microirradiation. Note that a pale lesion appeared in the irradiation area (indicated by arrow). (C) This chromosomal fragment (indicated by the arrow) followed the daughter chromatid toward the pole as the spindle elongated. (D) The chromosome fragment is at the pole as the cell enters telophase.

ments changed when the optical trap force was applied. After laser microsurgery of one metaphase chromosome, the trapped sister chromatid fragments separated but remained close to each other throughout anaphase, as shown in Fig. 4. The trap was switched off at the end of anaphase (Fig. 4D), where we observed that the trapped sister chromatid had moved back together with no observable separation between them. Approximately 6 min later (Fig. 4E), the chromatid fragments had fused together into one entity. As a result of optical trapping, the fused chromosomal fragment was still located in the interzone at the end of telophase and eventually remained in one of the daughter cells (Fig. 4F). In one cell, the fused chromosome fragment was incorporated into a micronucleus when the fragment was located far from the other chromosomes. In two other cells, the dissected chromatid fragments were lost in the cleavage furrow during cytokinesis.

Optical trapping of chromosome fragments created at anaphase was also performed. Figure 5 shows a chromosomal fragment at the beginning of anaphase held by the optical trap immediately after laser microsurgery. While the trap was on, this fragment was held motionless at the midplate. The chromosomal fragment remained in the cleavage furrow at cytokinesis. Figure 6 shows another example of a dissected chromosomal fragment at the start of anaphase and subsequently held with the optical trap. This fragment also remained motionless at the midplate, but was incorporated into a daughter cell rather than being lost in the cleavage

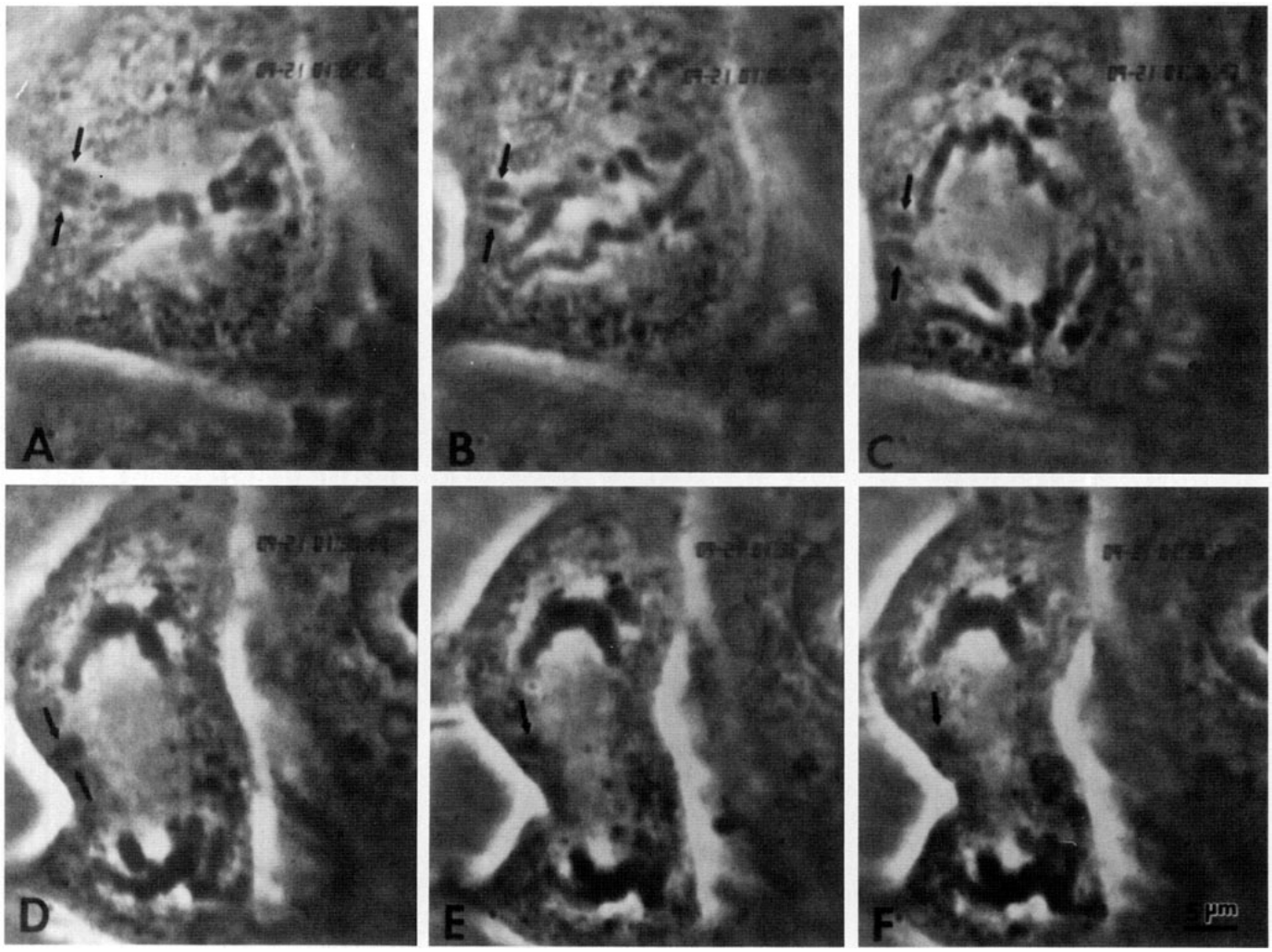

FIG. 4. Optical trapping of metaphase sister chromatid fragments after laser microsurgery. (A) The cell immediately after laser microsurgery. The dissected sister-chromatid fragments are indicated by arrows. (B) The sister chromatid fragments separated slightly from each other as the cell entered anaphase. (C) The sister chromatid fragments were kept motionless by the optical trap as the cell entered anaphase B. (D) The sister chromatid fragments (indicated by arrows) have moved together as the cell completes anaphase. The optical trap was switched off at this point. (E) The sister chromatid fragments have fused together to become one fragment. (F) The fused chromosomal fragment was incorporated into the upper daughter cell.

furrow. Instead of being incorporated into their appropriate daughter cell as in the controls, the optically trapped dissected chromosomal fragments were often incorporated into the opposite daughter cell. Figure 7 shows a tiny chromosomal fragment that was cut from a chromosome of the lower chromosome set during anaphase B, and subsequently held by the optical trap until telophase. The chromosomal fragment was finally incorporated into the upper daughter cell. Figure 8 is another example of a tiny chromosomal fragment after laser surgery of one anaphase B chromosome. The optical trap held this fragment effectively at the midplate. As a result, this fragment was eventually lost in the cleavage furrow. Of eight anaphase B cells studied, there were four cells in which the dissected chromosomal fragment was incorporated into its appropriate daughter cell, similar to the control cells. In summary, after being laser microdissected at anaphase A or B, trapped chromosome fragments were either (1) incorporated into the opposite daughter cell (five cases), (2) lost in the cleavage furrow during cytokinesis (four cases), or (3) eventually incorporated in the appropriate daughter cell (seven cases).

DISCUSSION

The application of optical tweezers after laser surgery of chromosomes could be used to (1) prevent the separa-

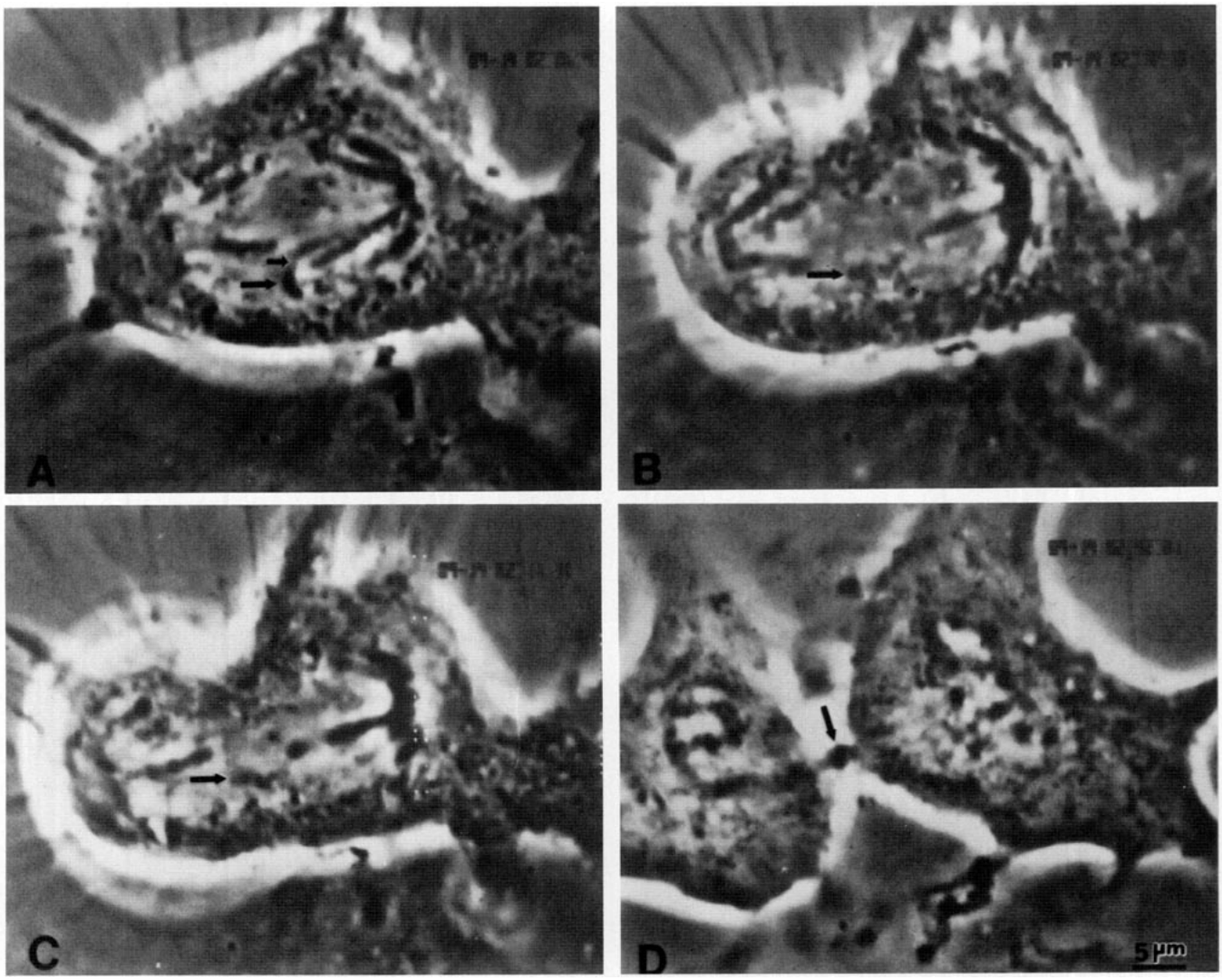

FIG. 5. Optical trapping of one chromosomal fragment after laser microsurgery at anaphase A. (A) The dissected chromosomal fragment (indicated by the longer arrow) was cut off from one chromosome attached to the right pole. Small arrow indicates the cut site. (B, C) The dissected chromosomal fragment was held at midplate by the optical trap. (D) Finally, the fragment was lost in the cleavage furrow.

tion of metaphase sister chromatid fragments and keep them close to each other, and (2) hold metaphase or anaphase chromosomal fragments motionless. The behavior of optically trapped chromosomal fragments is distinct from that of laser-dissected chromosome fragments that are not exposed to the optical trap. Our experimental results indicate that the use of the optical scalpel (laser microsurgery), in combination with optical tweezers (optical trap), can be used to micromanipulate chromosomes in living cells.

Laser microbeam surgery of metaphase chromosomes demonstrates that the sister chromatid fragments that were cut from the chromosome also separated from one another at the onset of anaphase, even though they did not contain kinetochores and their orientation was random. In their studies of laser microirradiation of kinetochores in PTK cells, Brenner and Berns [12, 13] demonstrated that if both kinetochores on one chromosome are irradiated in metaphase, the chromosome drifts off the metaphase plate, but the two chromatids remain attached to each other. However, when the nonirradiated chromosomes underwent the initial chromatid separation immediately prior to the beginning of anaphase movements, the irradiated chromatids also separated from each other. In subsequent studies it was further demonstrated that when the kinetochore of a single chromatid is irradiated at mitotic prometaphase or metaphase, the chromatids of the irradiated chromosome remain attached to one another until anaphase, at which time the damaged chro-

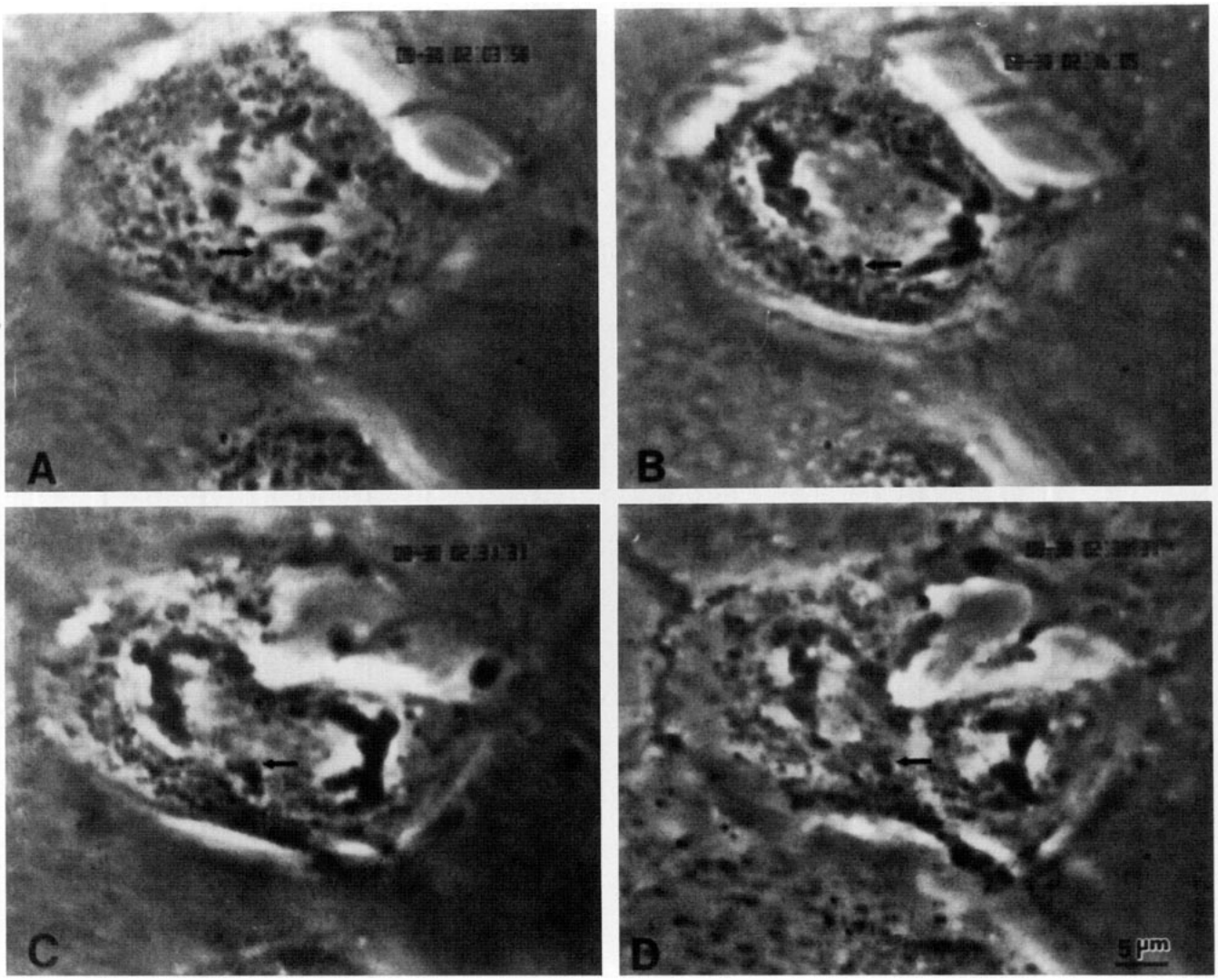

FIG. 6. Optical trapping of one chromosomal fragment after laser microsurgery at anaphase A. (A) The dissected chromosomal fragment (indicated by the arrow) from a chromosome attached to the right pole was trapped. (B, C) The chromosomal fragment was kept motionless from anaphase to telophase. (D) The chromosomal fragment drifted to the left daughter cell during cytokinesis.

matid did not move toward the opposite pole but remained parallel to, and slightly separated from, the undamaged chromatid, resulting in nondisjunction of the irradiated chromosome [16]. Our experimental results are in accordance with these earlier studies and demonstrate again that the initial event of chromatid separation does not rely upon microtubule-mediated forces at the kinetochore.

Nevertheless, it should be pointed out that once the dissected sister chromatid fragments separated from one another, they would either move to their corresponding poles if they remained within the spindle, or they would become incorporated into their corresponding daughter cells when they drifted into the cytoplasm. Our observation that chromosome fragments dissected during anaphase A or B moved toward the pole at the same rate as the proximal fragment with an intact kinetochore suggests that the distal fragment: (a) is still attached to the remaining chromosome, (b) is attached to polar microtubules, or (c) appears to be moving due to spindle elongation. Additional EM studies are required to resolve this issue.

Optical trapping of dissected chromosomal fragments had two significant results. First, the dissected sister chromatid fragments during metaphase were kept close to each other by the trapping force throughout anaphase. As a result, there was no separation of these chromatid fragments. We observed in some cases (e.g., Fig. 4) that as a result of the trapping process, the two dissected sister chromatids fused together. One possibil-

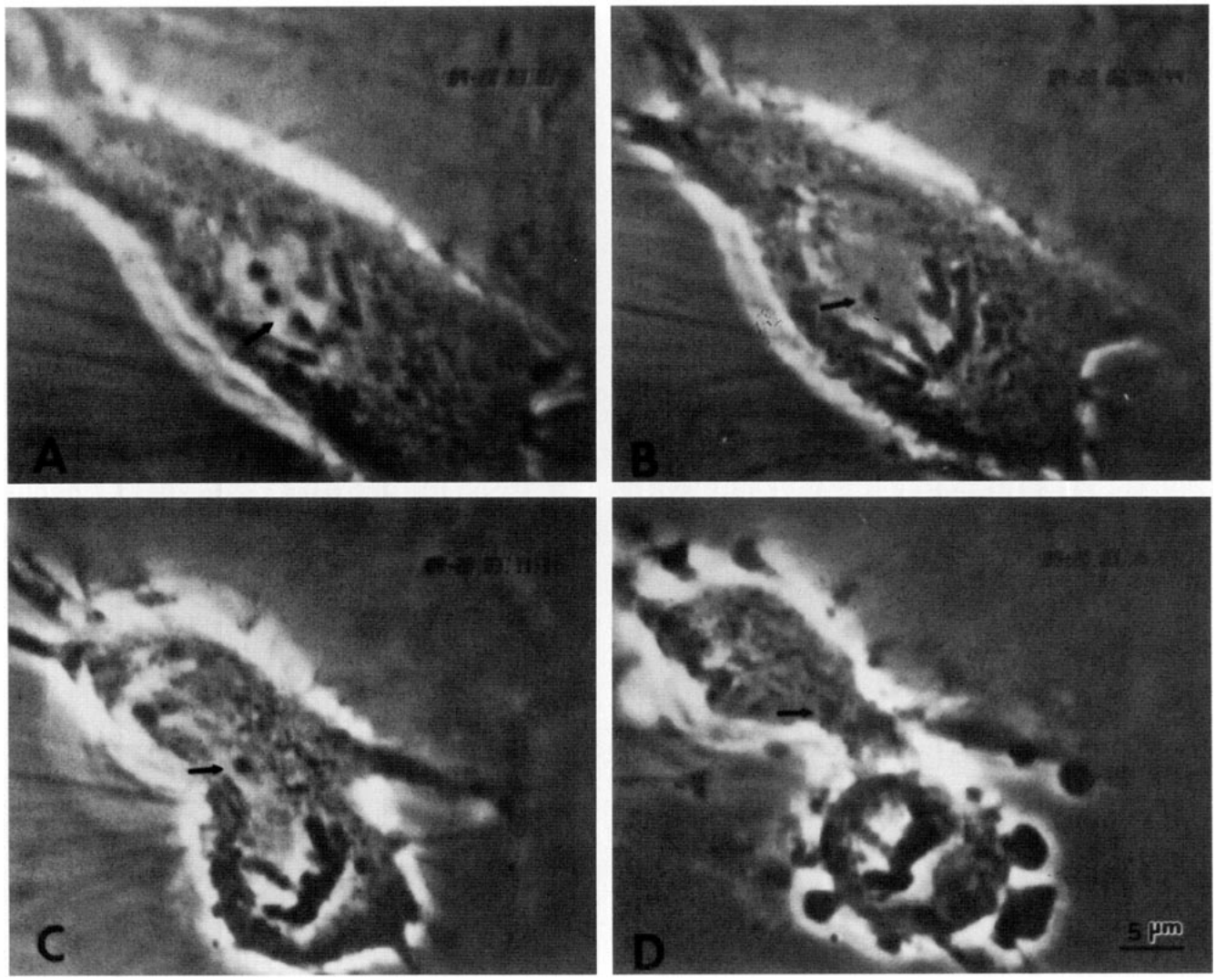

FIG. 7. Optical trapping of one anaphase B chromosomal fragment after laser microsurgery. (A) The dissected chromosomal fragment cut from a chromosome attached to the lower pole was held by the trap. Arrow indicates the cut site. (B, C) The chromosomal fragment was kept motionless as cell went through mitosis. Note that chromosomes at the upper pole are out of focus. (D) The chromosome fragment drifted to the upper daughter cell during cytokinesis.

ity is that the two chromatid fragments were not fused together and did not separate after the trap was turned off. We note from the experiments where only microdissection was performed (e.g., Fig. 2) that over the course of approximately 15 min (Fig. 2A–2C) the two chromatid fragments increased their separation to ~5 μm. From Fig. 4, the time that the chromatid arms are together is approximately 8 min (Fig. 4D–4F). (The trap was turned off at Min 30 and the last plate is at Min 38). In addition, we observed this cell out to Min 45 and saw that the chromatid fragments were still fused together. If the chromatid fragments had not fused together, we would have expected to see some separation, perhaps in the range of a few micrometers, as a result of diffusion. Since we did not observe a displacement of this magnitude, we believe that the two chromatid fragments fused together. The fusion may have resulted from the thermal denaturation of the DNA due to the absorption of a small amount of the trapping beam or from some unknown photochemical process. Second, the dissected chromosomal fragments during anaphase could also be held by the optical trap and kept motionless throughout anaphase while the irradiated proximal kinetochore-containing chromosomal fragments and nonirradiated chromosomes separated and moved toward the poles normally. The results of this study are consistent with our earlier study [28] in which the optical trap was able to hold anaphase chromosomes motionless.

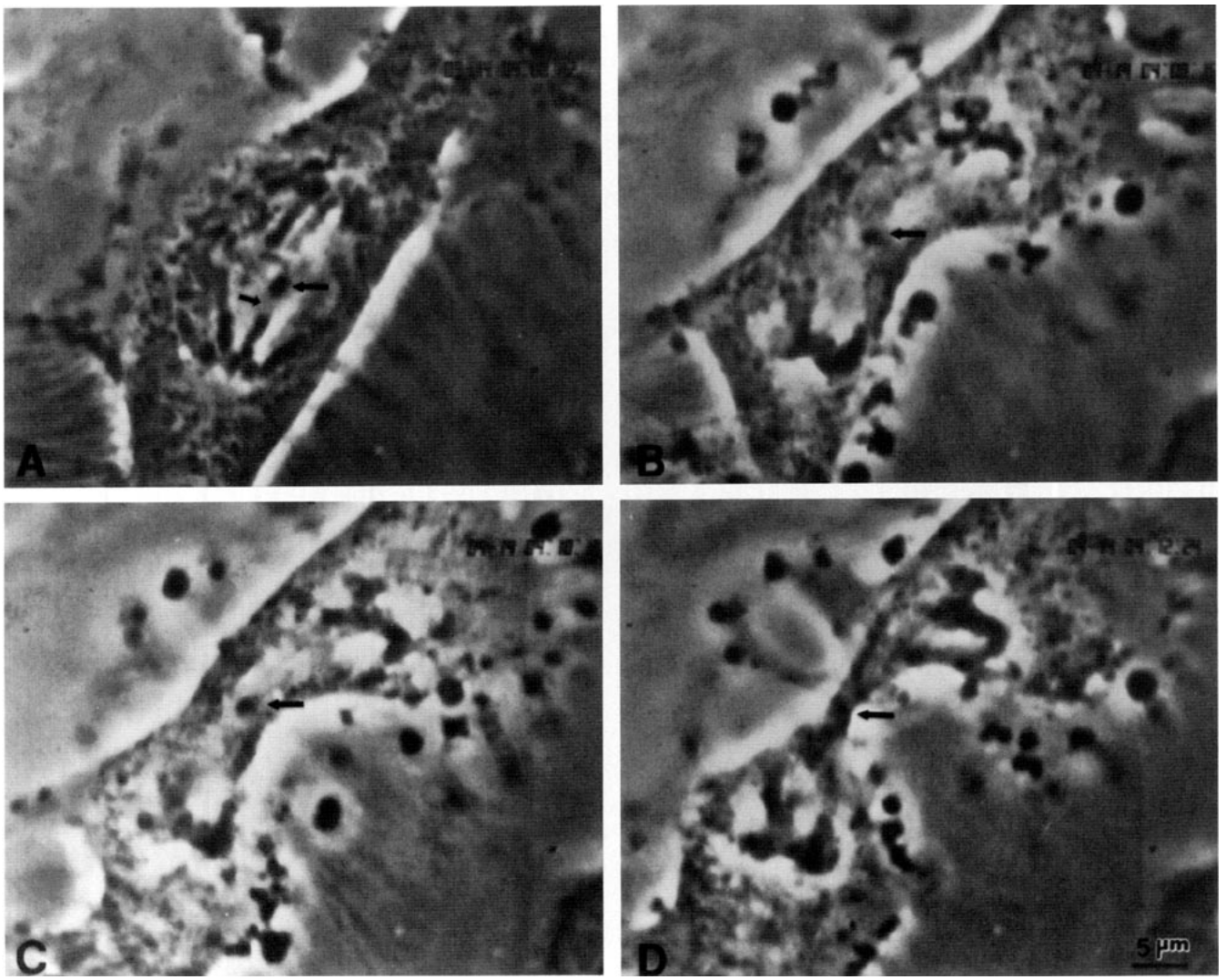

FIG. 8. Optical trapping of one anaphase B chromosomal fragment after laser microsurgery. (A) Immediately after laser microsurgery. Small arrow indicates the cut site. (B) The chromosomal fragment was kept motionless from anaphase to telophase using the optical trap. (C, D) The chromosomal fragment was finally lost in the cleavage furrow.

However, in this study, we were unable to move the dissected chromosomal fragment with the optical trap even though we hypothesized that a chromosomal fragment without a kinetochore and microtubule attachment should be easier to manipulate. This means that the forces necessary to move these chromosomal fragments are much higher than anticipated. It appears unlikely that the dissected chromosome fragment is attached to the mitotic spindle given that the poleward movement of chromosomes is retarded only by viscous drag forces [31] and that the point of force application on the chromosome occurs at the kinetochore. Instead, the most likely explanation is that a combination of high viscosity in the mitotic spindle combined with a low trapping efficiency for the chromosomal fragment are the reasons why it is not possible to move the fragment except at very low speeds. For example, a rough estimate for the force required to move a large chromosome is $\sim 10^{-8}$ dyn at a velocity of 1 μm/min and a spindle viscosity of 1 poise (1 dyn-s/cm^2) [32]. It is reasonable to assume that the dissected fragment requires an order of magnitude less force, or 10^{-9} dyn. However, due to the design of our motorized stage that incorporates a stepper motor that moves in increments of 0.5 μm, it is difficult to get smooth motion at speeds less than 10 μm/s. Taking this speed as a lower limit, and using Stokes Law to describe the drag force, the estimated drag force on the fragment now becomes 6×10^{-7} dyn or 600 times larger! For an optical trap, the force is given by the relation $F = qn_1P/c$, where q is an efficiency parameter, n_1 is

the refractive index of the mitotic spindle, P is the laser power in Watts, and c is the speed of light in free space. In the absence of a specific value for the refractive index of the mitotic spindle in PTK_2 cells, we take n_1 to be that of water (1.33). The efficiency parameter q is dependent on the optical characteristics of the particle. For the case of large spherical particles in the limit of geometrical optics and tightly focused laser beams, q can be quite large, as high as 0.3 for a sphere with a relative refractive index of 1.2 when trapping in the direction transverse to the laser beam propagation [33]. However, the efficiency is expected to decrease as the size of the particle decreases. No calculations have been described to date for particles on the order of 1 μm diameter; however, one reported measurement [34] indicates that q is $\sim$0.03 for a micrometer-sized sphere. For a chromosome of irregular shape and composition, it is reasonable to expect that q will be much lower, perhaps by an order of magnitude. Taking q for the chromosomal fragment to be 0.003, and a laser power of 60 mW, the calculated trapping force exerted on the fragment is 8.1×10^{-8} dyn. This is almost an order of magnitude less than the calculated drag force. To improve the situation, we must move the fragment at a slower rate, improve the trapping efficiency, or increase the power. In the case of the metaphase chromosome fragments trapped in the cytoplasm, mechanical interference from the cytoskeletal matrix may also be significant.

An alternative to motorized microscope stages based on stepper motors is the piezoelectric translator (PZT). These devices create movement when a voltage is placed across a material that is piezoelectric. Displacements on the order of nanometers are realizable; the speed can be controlled by the change in voltage per unit time applied to the PZT. Attachment of small "handles" (polystyrene spheres) to the end of mitotic chromosomes analogous to the *in vitro* work done by Chu *et al.* [35] could be an effective method of applying sufficient forces to the chromosomes by increasing the "effective" q of the chromosome. Increasing the power of the trapping beam is limited to the damage threshold of the cell specimen. Previous work [28] employed trapping power levels in the range of 60–200 mW at a wavelength of 1.06 μm to hold chromosomes. We have observed morphological changes to PTK_2 cells at power levels >200 mW (unpublished data). Using a wavelength that causes less damage, such as 700 nm, may increase the trapping force by a factor of $\sim$2 [30]. Alternately, applying two optical traps to the same chromosome at different locations might be successful. This would minimize the deleterious effects of high laser power at one focal point while permitting the application of higher forces to the chromosome. In addition, measurement of the key parameters, such as the trapping efficiency, spindle viscosity, and spindle refractive index will help pinpoint the magnitude of the trapping and drag forces.

Despite the inability to actually move laser-generated chromosome fragments, our experimental results are the first report demonstrating that the optical tweezer can be used to manipulate chromosomes in combination with laser scissors in living cells. Future studies will employ the tunable titanium–sapphire laser at wavelengths that are even less absorbed than the 1060-nm YAG laser [30] as well as multiple optical traps in the same field to increase the force applied to the chromosome fragments. In addition, the microscope stage will be modified to accept the piezoelectric translators to permit movement at slower speeds. The combination of the optical scissors with the optical tweezers provides a new tool that undoubtedly will be useful for studies on chromosome movement and cell genetics.

This work was supported, in part, by grants from NIH (RR01192), Office of Naval Research (N00014-91-C-0134), Department of Energy (DE-F03-91ER 61227), and the Beckman Laser Institute Endowment.

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Received July 1, 1992
Revised version received September 23, 1992