Microtubules of the Kinetochore Fiber Turn Over in Metaphase but Not in Anaphase

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Abstract. In previous work we injected mitotic cells with fluorescent tubulin and photobleached them to mark domains on the spindle microtubules. We concluded that chromosomes move poleward along kinetochore fiber microtubules that remain stationary with respect to the pole while depolymerizing at the kinetochore. In those experiments, bleached zones in anaphase spindles showed some recovery of fluorescence with time. We wished to determine the nature of this recovery. Was it due to turnover of kinetochore fiber microtubules or of nonkinetochore microtubules or both? We also wished to investigate the question of turnover of kinetochore microtubules in metaphase. We microinjected cells with x-rhodamine tubulin (x-rh tubulin) and photobleached spindles in anaphase and metaphase. At various times after photobleaching, cells were detergent lysed in a cold buffer containing 80 μM calcium, conditions that led to the disassembly of almost all nonkinetochore microtubules. Quantitative analysis with a charge coupled device image sensor revealed that the bleached zones in anaphase cells showed no fluorescence recovery, suggesting that these kinetochore fiber microtubules do not turn over. Thus, the partial fluorescence recovery seen in our earlier anaphase experiments was likely due to turnover of nonkinetochore microtubules. In contrast fluorescence in metaphase cells recovered to ~70% the control level within 7 min suggesting that many, but perhaps not all, kinetochore fiber microtubules of metaphase cells do turn over. Analysis of the movements of metaphase bleached zones suggested that a slow poleward translocation of kinetochore microtubules occurred. However, within the variation of the data (0.12 $\pm$ 0.24 μm/min), it could not be determined whether the apparent movement was real or artifactual.

PHOTOBLEACHING studies of cells microinjected with fluorescent tubulin have shown that microtubule assembly and disassembly in metaphase cells occurs rapidly (2, 7, 13, 18, 21, 23). The mechanism by which this turnover takes place is presumably by the steady-state depolymerization and repolymerization of microtubules, but the precise sites of tubulin loss and addition remain controversial. The treadmilling model of mitosis (11) predicts loss of subunits at the pole (minus end) and addition at the kinetochore (plus end). In cultured mammalian and amphibian cells, Wadsworth and Salmon (23) did not observe treadmilling of microtubules toward the pole. However, Hamaguchi et al. (7) reported possible treadmilling in spindles of sand dollar embryos. Light microscopic photobleaching studies of living mitotic cells generally do not permit unambiguous discrimination of the assembly dynamics of kinetochore and nonkinetochore microtubules. Cassimeris et al. (2) tried to decrease the proportion of nonkinetochore microtubules by performing photobleaching studies at room temperature in mammalian cells at metaphase. However, no quantitative estimate of the ratio of kinetochore fiber microtubules to nonkinetochore microtubules was provided.

In a another approach Mitchison et al. (15) injected biotinylated tubulin into metaphase cells, fixed the cells at short times thereafter and prepared them for electron microscopic immunocytochemistry. From their results they argued that the kinetochore fiber microtubules in metaphase cells treadmill, polymerizing at the kinetochore and depolymerizing at the pole. These experiments, in which cells are injected with haptenized tubulin and fixed at short times for evaluation by electron microscopy have their own limitations. Only a small number of kinetochore fibers can be easily sampled. Moreover microtubules rapidly diverge from the plane of section and thus can be traced with surety only a short distance from the kinetochore.

Recently the question of how microtubule assembly and disassembly is regulated within the kinetochore fiber has taken on increased importance with evidence that microtubule disassembly alone may be sufficient to drive chromosome motion. In a population analysis performed on isolated chromosomes that had been allowed to capture microtubules, Koshland et al. (10) reported that dilution-induced disassembly could bring the minus ends of microtubules, the ends that in vivo are associated with the pole, nearer to the kinetochore.

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In anaphase the microtubules of the kinetochore fibers lose subunits and shorten while simultaneously, elsewhere in the cell, other microtubules lengthen (4, 12). In previous studies we provided evidence that most disassembly of kinetochore fiber microtubules in anaphase occurs at the kinetochore (5, 6). In those studies we injected fluorescent derivatives of tubulin into early mitotic tissue culture cells and used photobleaching to mark domains within the spindle microtubules just after anaphase onset. We found that the photobleached domains could be followed to very late anaphase, well after cytokinesis had begun. Thus it was clear that, in anaphase, the vast majority of kinetochore fiber microtubules remained stationary with respect to the pole. However, during the course of these experiments, some partial recovery of fluorescence within the bleached zones was detected. Because of this partial recovery we were unable to eliminate the possibility that a small minority of the kinetochore fiber microtubules did translocate toward the pole in anaphase. Alternatively the partial recovery might be due to turnover of kinetochore or of nonkinetochore microtubules. We sought to resolve these questions by analyzing exclusively the fluorescence recovery of kinetochore fiber microtubules. For comparison we also investigated the behavior of kinetochore fiber microtubules of cells in metaphase.

To accomplish our goals we developed a method of performing photobleaching experiments in a manner that allows us to specifically examine microtubule turnover within the kinetochore fiber at the light microscopic level. We took advantage of the greater stability of kinetochore fiber microtubules to cold and calcium (1, 15, 17). We photobleached cells and, at various times, detergent lysed them into a cold buffer containing calcium. In this way, in our final images, the contribution of nonkinetochore microtubules to the fluorescence in the bleached zones was eliminated. The results of these experiments permit us to discriminate the behavior of these two classes of spindle fibers. They indicate that kinetochore fiber microtubules of anaphase cells do not turn over detectably while kinetochore fiber microtubules of metaphase cells show considerable turnover.

Materials and Methods

Cell Culture and Microinjection

LLC-PK cells were grown in DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS (HyClone Laboratories, Logan, UT), 20 mM Hepes, and antibiotics. Cells were cultured on glass coverslips containing a pattern marked with a diamond scribe and microinjected in prophase or prometaphase with x-rhodamine (x-rh)[1] tubulin. The derivatized tubulin was prepared and characterized as previously described (6, 20). The preparations used in these experiments had a dye-to-protein ratio of 0.5:1.0 and were loaded into the micropipet at a concentration of 8 mg/ml. Microinjections were estimated to be 5% the cell volume. Based on a tubulin concentration in the cell of 3 mg/ml (8), the estimated final dye-to-protein concentration of tubulin in the injected cells was <0.1:1.0. After microinjection, the medium over the cells was replaced with fresh medium from which phenol red was omitted. The medium was overlaid with mineral oil, and cells were then returned to the incubator until they had reached the appropriate stage of mitosis.

Photobleaching

The photobleaching apparatus has been described in detail previously (5, 19, 20). Briefly, photobleaching was performed with an argon-ion laser (model 2020; Spectra-Physics Inc., Mountain View, CA) channeled through the epi-illumination port of a Zeiss IM-35 inverted microscope (Carl Zeiss Inc., Thornwood, NY). A 300-mm focal length lens and a 200-mm focal length cylindrical lens were used to produce a bar-shaped beam perpendicular to the spindle axis. The laser was operated at 500 mW with an exposure time of 100 ms. In preliminary photobleaching experiments performed on interphase cells microinjected with x-rh tubulin, we found that these exposures did not cause microtubule breakage as assayed by subsequent antitubulin immunofluorescence. When focused through a planapochromat 100×, 1.3 NA objective, these conditions produced a bleached domain 1–2 μm wide in the spindle of a typical injected cell.

Cells were photobleached either in metaphase or anaphase. For each cell, a phase contrast image and the light emitted from x-rh molecules during photobleaching were simultaneously recorded on a single frame of T-Max 400 film. Cells were then observed by phase contrast microscopy until the time of lysis. The temperature of the stage was maintained at 35–37°C by means of an air curtain incubator (Nicholson Precision Instruments Inc., Gaithersburg, MD).

Detergent Lysis

At given times after photobleaching each dish was removed from the stage of the microscope and quickly rinsed twice in 0.1 M Pipes, pH 6.95, at room temperature. The dish was then plunged into a beaker containing 100 ml of 0.1 M Pipes with 80 μM $CaCl_2$ and 1.0% Triton X-100 and left for 5 min. The lysis buffer was maintained at 0–2°C by means of an ice-water bath. After lysis the dish was rinsed twice in room temperature 0.1 M Pipes. The dish was then filled with a buffer containing 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 2 mM $MgSO_4$, pH 6.95 (PHEM) to which had been freshly added 1.0 μg/ml taxol and 0.1% *n*-propyl gallate. After rinsing and drying the exterior of the dish, the cells were returned to the stage of the microscope to record the image of the fluorescence from the injected microtubules that had not depolymerized during the cold-calcium lysis. In some experiments cells were imaged for x-rh fluorescence after fixation with 5 mM ethylene glycolbis (succinimidylsuccinate) (5) and subsequently labeled with an antitubulin antibody and a fluorescein secondary antibody. In one experiment, after imaging of the x-rh fluorescence in the lysed and unfixed cells, samples were fixed for 10 min in 1% glutaraldehyde. They were then rinsed with water, treated for 30 min with 10 mg/ml $NaBH_4$, and labeled for antitubulin immunofluorescence. Antitubulin staining was carried out to ensure that photobleaching did not disrupt kinetochore fibers.

Observation and Analysis

A cooled charge coupled device camera (model 200; Photometrics Ltd., Tucson, AZ) was used to collect fluorescent and phase contrast images and to decrease noise and background. Images were digitized to 14-bit depth and stored on a WORM drive optical disc (type 3363; IBM Corp., Danbury CT). The brightest pixels of our fluorescent images never exceeded 12 bits. Thus the camera was never operated near saturation.

The fluorescent images were analyzed in the following manner. The spindle was displayed on the video monitor with the poles aligned along the horizontal axis. Using a mouse we delineated a rectangle on the monitor surrounding the entire spindle. The fluorescent intensity of each vertical row of pixels within the rectangle was averaged. These averages were then plotted versus distance along the spindle axis. Hardcopies of the graphs displayed on the video monitor were obtained by means of a video printer (model P-61U; Mitsubishi Electric Sales America, Inc., Rancho Dominguez, CA) and enlarged xerographically two times.

The poles of the cell were defined on the graphs of the fluorescent images as follows. A baseline of background fluorescence was drawn. Next, tangents were drawn at the inflection points of the outermost descending limbs of the plot. The points where the tangents intersected the baseline were defined as the poles. In fluorescence images, the midpoint of the spindle was defined as the point equidistant between the poles. In phase images the midpoint was defined as the centroid of the chromosomes.

Because the amount of x-rh tubulin injected into cells varied somewhat, the following normalization was necessary. The minimum fluorescent intensity at the bleached zone was divided by the intensity at the corresponding point in the unbleached half spindle. The resulting values were taken as the measure of fluorescence in the bleached zones.

We also examined the distances moved by bleached zones in anaphase and metaphase cells. For each cell we first measured the distance of the bleaching beam from the midpoint of the spindle in the image taken at the

1. *Abbreviations used in this paper*: PHEM, 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 2 mM $MgSO_4$, pH 6.95; x-rh, x-rhodamine.

moment of photobleaching. We then measured the distance of the bleached zone from the midpoint in the image of the final lysed cell.

Computer-aided regression analysis was used to fit lines to the data. For fluorescence recovery in anaphase and for movement of the bleached zones in anaphase and metaphase, linear regression was chosen. Fluorescence recovery in metaphase was fit by nonlinear least squares approximation to the perturbation-relaxation equation $F_t = F_i + [F_f - F_i] \times [1 - e^{-kt}]$, where F_t is the fluorescence intensity at time t, F_i is the fluorescence intensity just after photobleaching, and F_f is the final intensity at time infinity (9, 18). The half-time for fluorescence recovery was then calculated from the coefficient k by the formula $t_{1/2} = \ln 2/k$.

Electron Microscopy

Photobleached and control cells were lysed in PHEM or in the cold, calcium buffer as described above. After lysis they were rinsed 2× in PHEM and fixed with 2% glutaraldehyde, 0.2% tannic acid in PHEM for 1 h at room temperature. The samples were then rinsed 2× in water and postfixed with 0.2% osmium tetroxide and 1.0% uranyl acetate for 30 min. The cells were dehydrated with a graded series of ethanol and embedded in Eponate 12 (Ted Pella Inc., Redding, CA). Coverslips were removed with hydrofluoric acid and gold sections were cut with a diamond knife. The sections were treated with ~7% uranyl acetate for 1 h and photographed with a Phillips 300 or JEOL 100S electron microscope.

Results

Lysis in Cold and Calcium

Lysis of cells in a microtubule stabilizing buffer such as PHEM resulted in the preservation of microtubules within and outside the kinetochore fibers. In contrast, when cells were lysed in cold Pipes buffer containing 80 μM calcium, kinetochore fiber microtubules were retained but other classes of microtubules were depolymerized in both anaphase and metaphase cells (Fig. 1).

Ultrastructure of Photobleached Cells

We examined the ultrastructural integrity of spindle microtubules in injected cells photobleached under our standard conditions. Confirming our immunofluorescence control data in this and previous publications (5, 6), we did not detect any structural damage to microtubules. Microtubules in photobleached regions appeared identical to those in unbleached areas in the opposite half-spindle (Fig. 2).

Fluorescence Recovery

A total of 17 anaphase cells and 23 metaphase cells were analyzed for fluorescence recovery after photobleaching. In spindles of cells lysed a few seconds after photobleaching, a well-defined bleached zone was evident in the kinetochore fiber microtubules that resisted depolymerization in the cold and calcium-containing buffer (Fig. 3). In cells that were photobleached in early anaphase and allowed to progress for some time, clear bleached zones persisted without apparent recovery (Fig. 4). Consistent with our earlier studies (5, 6) bleached zones placed just ahead of the advancing chromosomes were overrun and could not be detected in the final images of the lysed cells (data not shown). In contrast to the

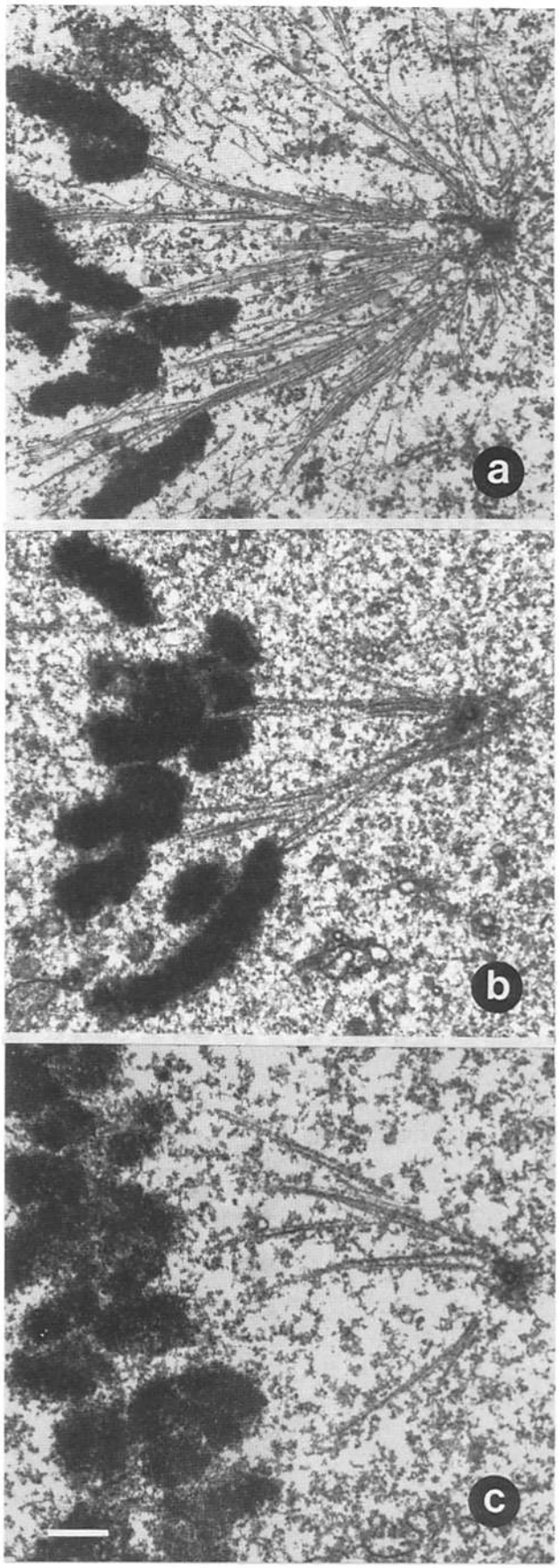

Figure 1. Ultrastructural comparison of cells lysed in PHEM and cold-calcium buffers. Midanaphase cell lysed in PHEM to preserve both kinetochore fiber and nonkinetochore microtubules (*a*). Midanaphase (*b*) and metaphase (*c*) cells lysed in cold Pipes buffer containing 80 μM calcium to depolymerize nonkinetochore microtubules. Bar, 1.0 μm.

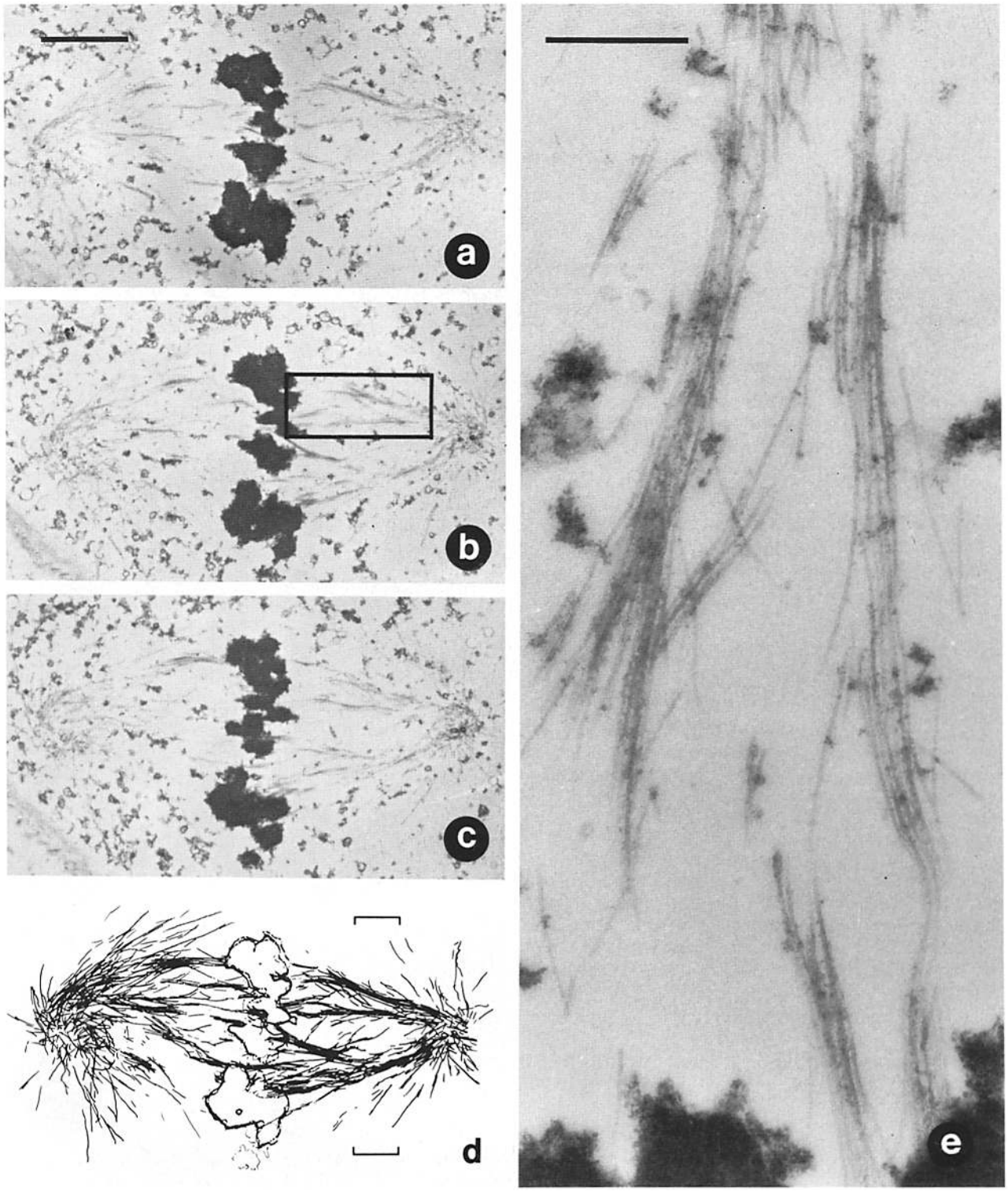

Figure 2. Ultrastructural analysis of spindle microtubules after photobleaching. A metaphase cell was photobleached across one half-spindle, lysed in PHEM 12 s later, and prepared for electron microscopy. Analysis of serial sections (*a–c*) revealed no apparent effect on the microtubules. A reconstruction of the three sections (*d*) showing the location of the bleaching beam (*brackets*) reveals that bundles of microtubules run uninterrupted from the pole to the chromosomes. Detail of the microtubules that were photobleached is shown in *e*, which is an enlargement of the boxed region of *b*. No damage to the microtubules can be detected. Bars: (*a–c*) 5 μm; (*e*) 1 μm.

situation in anaphase, metaphase cells exhibited considerable recovery with time (Fig. 5).

Quantitation of the fluorescence recovery within the bleached zones confirmed these general observations. Anaphase and metaphase cells lysed within 20 s after photobleaching showed kinetochore fibers that were bleached to an average of ~35% the level of fluorescence in the unbleached half spindle. Fluorescence in kinetochore fibers of

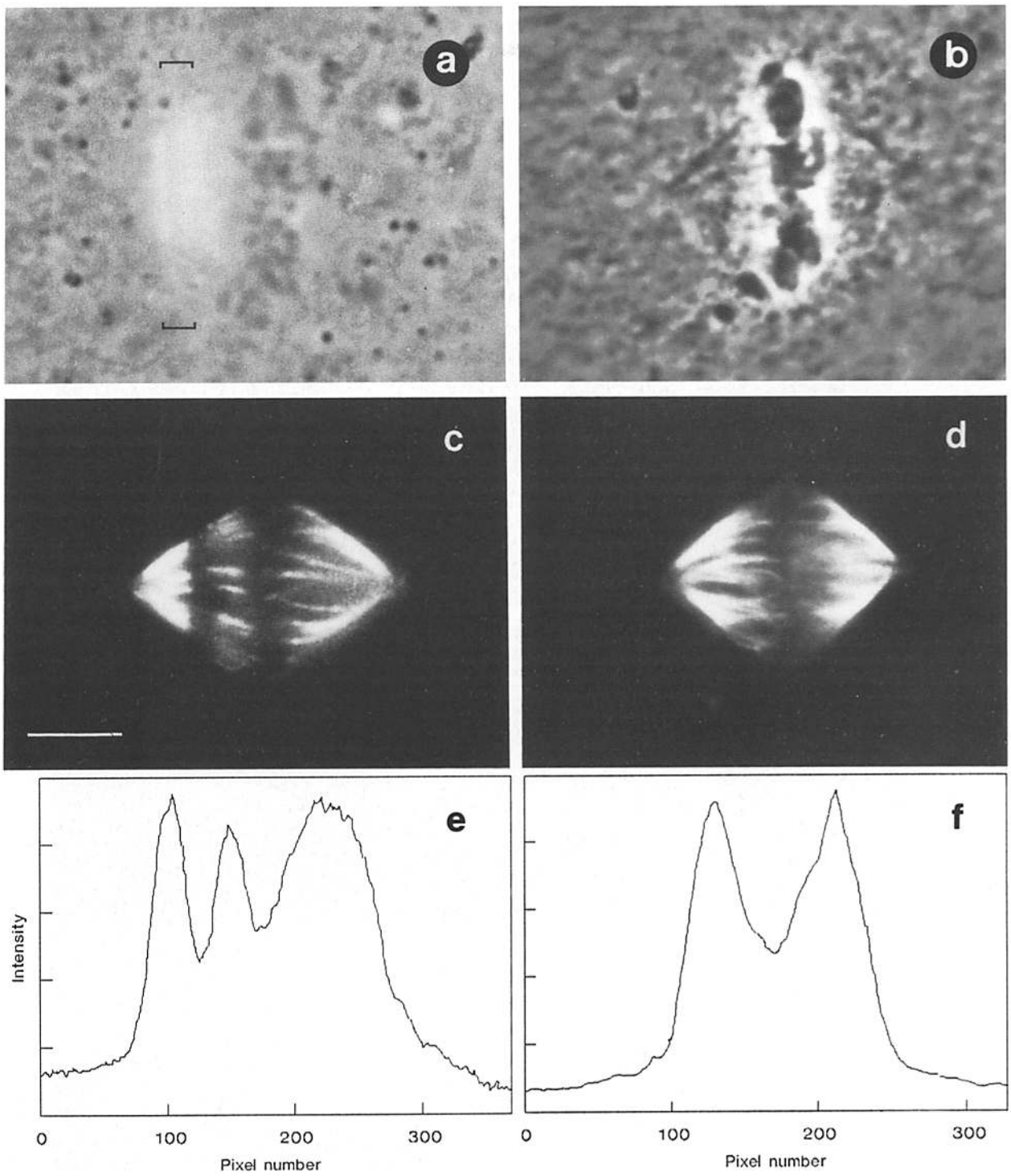

Figure 3. Anaphase cell lysed shortly after photobleaching. Cell was photobleached just after anaphase onset (*a*). Brackets denote bleaching beam which appears as a light band in the phase micrograph. The cell was lysed in cold-calcium buffer 10 s later. The cell was imaged in phase contrast (*b*) and x-rh fluorescence (*c*) and subsequently labeled for antitubulin immunofluorescence (*d*). Quantitation of the fluorescence across the spindle was performed for the x-rh fluorescence (*e*) and for the antitubulin immunofluorescence (*f*). The photobleached zone appears in the x-rh graph as a large trough in the left half-spindle. The antitubulin graph shows that the distribution of microtubules is symmetrical. Note that the average fluorescent intensity of the x-rh image of the unbleached half-spindle peaks about halfway between the chromosomes and the pole suggesting that microtubules are most numerous in that region. This result is consistent with the electron microscopic counts of microtubules in cold-stable kinetochore fibers of PtK1 cells reported by Reider (16). As was consistently found, the length of the spindle in the antitubulin image appears somewhat shorter in comparison to the x-rh image due to the failure of antibodies to penetrate fully into the region of the pericentriolar material at the poles. Bar, 5 μm.

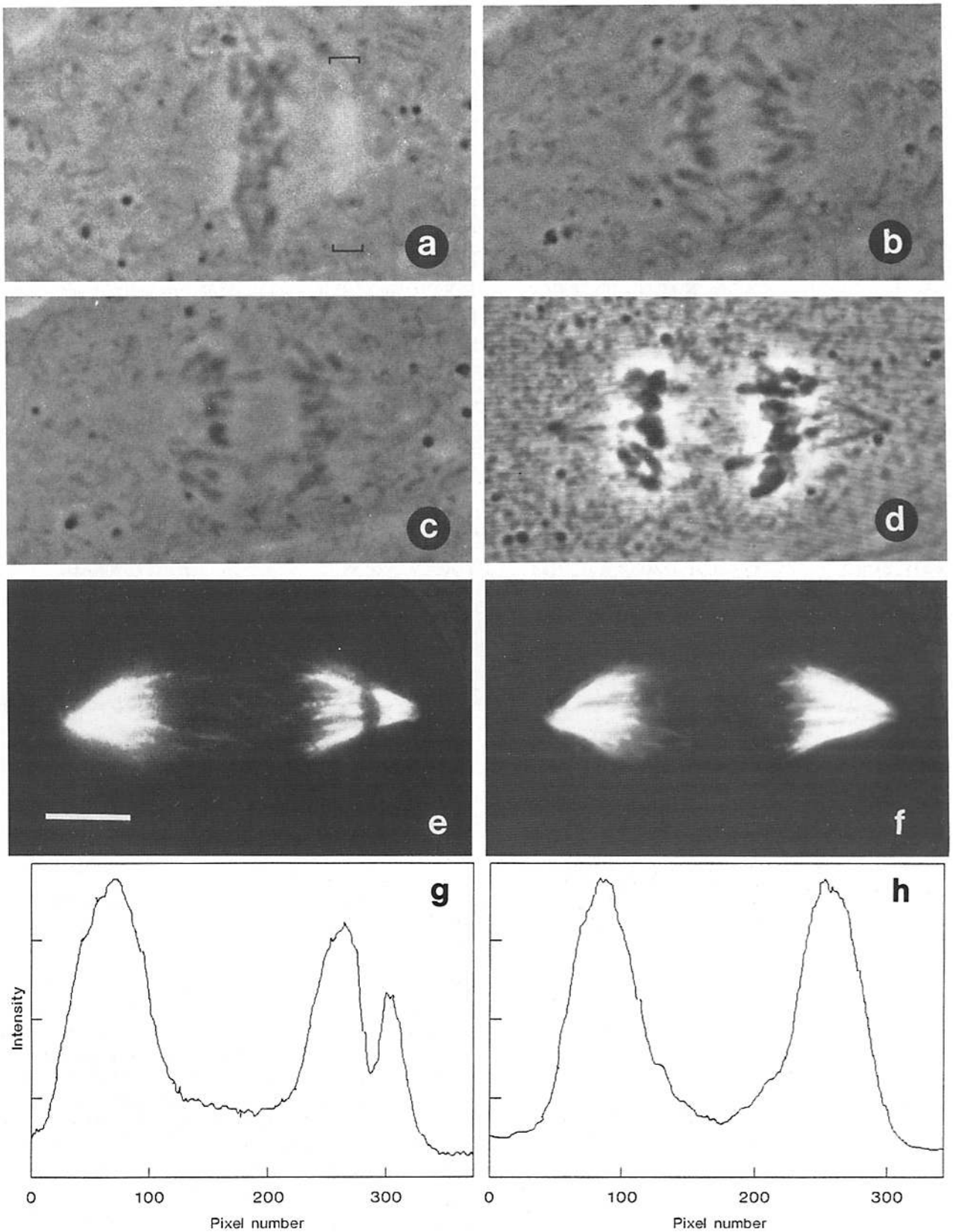

Figure 4. Anaphase cell allowed to continue for some time after photobleaching. Cell was photobleached just after anaphase onset in the right half spindle (*brackets*) (*a*). The cell was photographed with phase contrast while live at 90 (*b*) and 120 s (*c*) after photobleaching. At 150 s after photobleaching the cell was lysed in cold-calcium buffer and imaged in phase contrast (*d*) and x-rh fluorescence (*e*). The cell was then labeled for antitubulin immunofluorescence (*f*). Quantitation of the x-rh fluorescence (*g*) reveals that the bleached zone in the anaphase kinetochore fibers has persisted without recovery. The antitubulin graph (*h*) shows that the microtubules are distributed symmetrically. Bar, 5 μm.

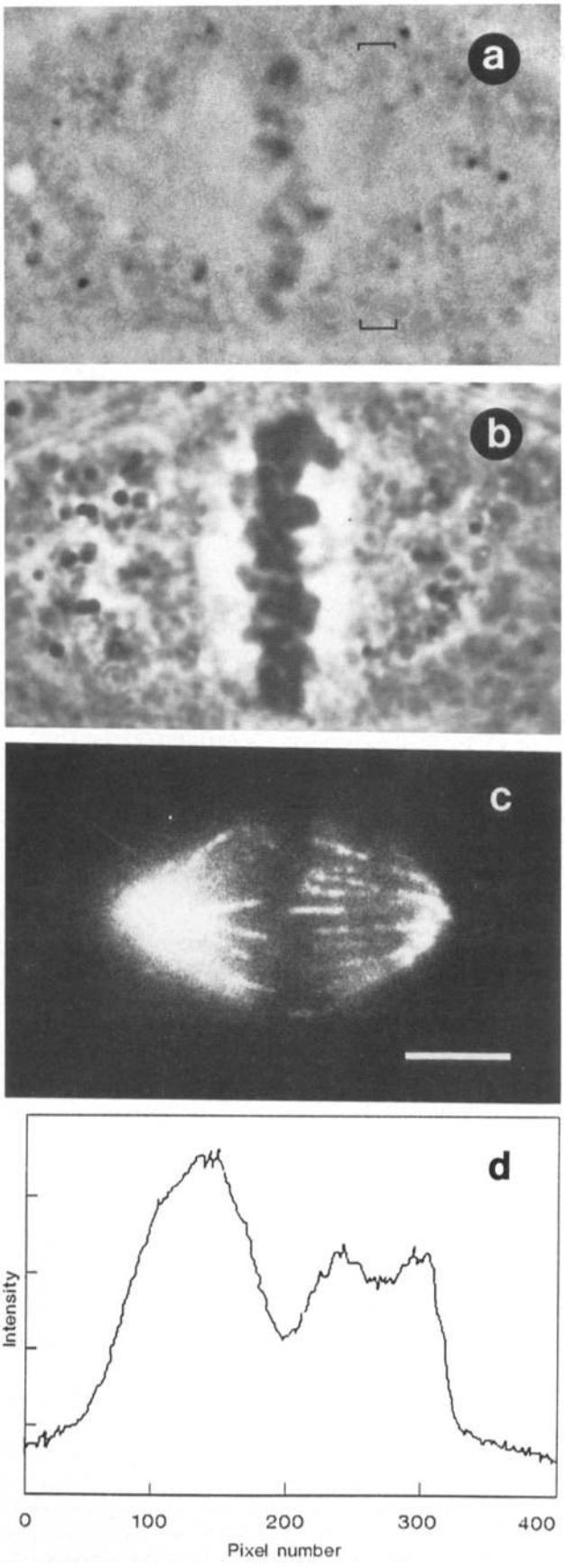

Figure 5. Metaphase cell allowed to continue for some time after photobleaching. The metaphase cell was photobleached at the brackets (*a*). Because this cell had received only a small injection of x-rh tubulin, the x-rh molecules during bleaching were of insufficient intensity to be revealed over the phase contrast image. The cell was observed for 235 s when it was lysed in cold-calcium buffer and imaged in phase contrast (*b*) and x-rh fluorescence (*c*). Quantitation of the fluorescence (*d*) indicated that considerable recovery had taken place in the bleached zone. Bar, 5 μm.

anaphase cells did not recover (Fig. 6). In contrast, metaphase spindles recovered to an average of ~70% the fluorescence in the unbleached half spindle, with an approximate half-time for recovery of 70 s.

One metaphase cell lysed at 13 s showed an unusually high level of fluorescence (77%) in the bleached zone. We speculate that due to experimental error, this cell was not photobleached to the same extent as the others. (The fluorescence in the bleach zone of this cell was nearly six standard deviations from the mean fluorescence intensity of the other metaphase cells lysed before 17 s. In calculating the approximate half-time for recovery it was excluded based on Chauvenet's criterion for the rejection of outliers [3].) As also seen in Fig. 6, four cells at later times departed significantly from the plotted recovery curve. (These points could not be excluded based on Chauvenet's criterion.) Elimination of these four points did not significantly affect the half-time for recovery, reducing it from a calculated value of 71 to 69 s. We also tested whether the metaphase data could be accommodated within a model of no recovery, i.e., whether the plot of percentage fluorescence versus time could be fit to a straight line with slope = 0. Based on statistical tests for comparison of regression models (16), we found that the metaphase data was significantly better fit to a third order polynomial, indicating recovery, than to any straight line with slope = 0 ($P < 0.01$).

Movement of Bleached Zones

The degree of spindle shrinkage during the lysis procedure was determined by examining cells lysed <20 s after photobleaching. For each such cell we compared the distance of the bleached zone from the spindle midpoint in the micrograph of the living cell taken at the time of photobleaching to the distance of the bleached zone from the spindle midpoint in the images of the lysed cell. It was found that lysis caused the bleached zone to midpoint distance to decrease an average of 1.7 ± 0.6 μm (mean ± SD, $n = 14$). To correct for the shrinkage during lysis 1.7 μm was added to the bleached zone-to-midpoint distances measured in the images of lysed cells.

As shown in Fig. 7, bleached zones in anaphase cells showed clear movement outward from the spindle midpoint at a mean rate of 1.2 μm/min with a 95% confidence interval of 0.9–1.5 μm/min. Previously, we imaged living cells in which bleached zones in anaphase spindles accompanied the near pole during pole–pole separation (6). The mean rate of pole movement away from the spindle midpoint recorded in that study was only 0.6 μm/min. The reason for the disparity with the rate obtained here is likely that cells in the earlier study were generally followed over longer times in anaphase, and pole movement slows considerably in late anaphase. Thus the total mean rate reported in our earlier study was less. The outward movement of the bleached zone detected

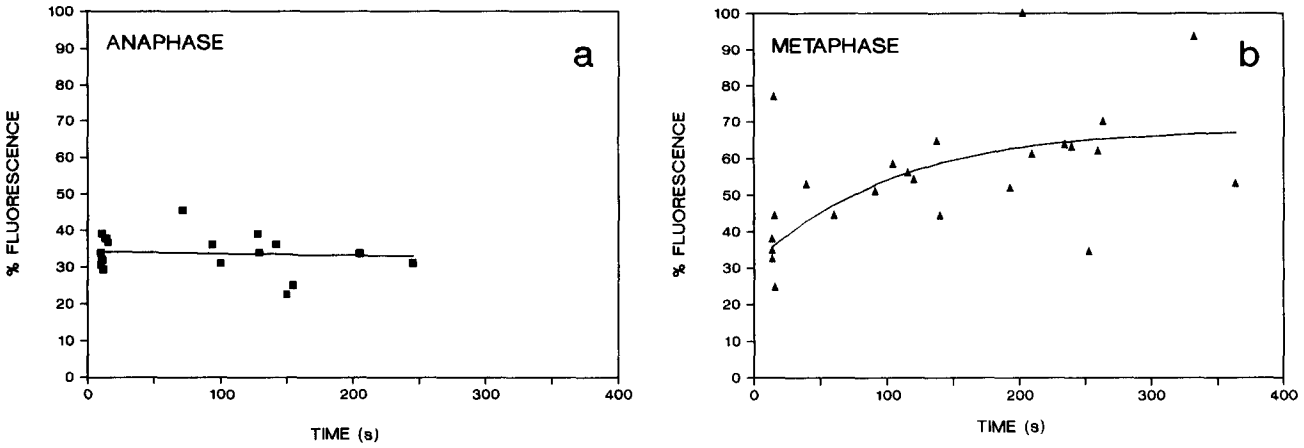

Figure 6. Fluorescence recovery in kinetochore fibers of anaphase cells (*a*) and metaphase cells (*b*). Fluorescence intensity just after photobleaching in both anaphase and metaphase cells was ~35%. (One metaphase cell lysed shortly after photobleaching showed an abnormally high fluorescence intensity in the bleached zone. That cell was excluded for plotting the recovery curve.) No recovery of fluorescence in the bleached zones of anaphase cells was detected. In metaphase a nonlinear least squares fit yielded a recovery half-time of 70 s and a recovery extent of ~70% over the course of the experiment.

in our current experiments is again consistent with the interpretation that the kinetochore fibers accompany the near pole in its anaphase B movement.

For metaphase cells, the data indicated a mean rate of bleached zone movement of 0.12 μm/min suggesting a slow poleward translocation of the kinetochore fibers. However, due to variation in the data, we were unable to determine whether this apparent motion was real since the 95% confidence interval on the data included rates of from 0.12 μm/min toward the cell equator to 0.36 μm/min toward the pole.

Discussion

Interpretations of fluorescence recovery in spindles in previous photobleaching studies (5, 6, 7, 13, 18, 21, 23) have been complicated by the presence of different classes of microtubules. In an effort to decrease the contribution to fluorescence recovery due to nonkinetochore microtubules, Cassimeris et al. (2) recently performed photobleaching studies on mitotic Ptk1 cells at room temperature. However, no estimation was given for the degree of reduction in the proportion of nonkinetochore microtubules. We sought to determine unambiguously whether microtubule turnover occurs within kinetochore fibers in anaphase and in metaphase. We took advantage of the fact that kinetochore fiber microtubules have been found to be notably more resistant to microtubule depolymerizing agents such as cold and calcium (1, 15, 17). We developed a detergent lysis procedure that retained kinetochore fibers under conditions where astral and interzonal microtubules were largely depolymerized, as assayed by antitubulin immunofluorescence. We confirmed with electron microscopy that the method effectively removed nonkinetochore fiber microtubules but left the kinetochore fibers in both anaphase and methaphase cells. While we can not be certain that every microtubule originally present within the kinetochore fiber was preserved, sizable bundles of microtubules extending from the poles to the chromosomes were seen by both fluorescence and electron microscopy under our extraction procedure.

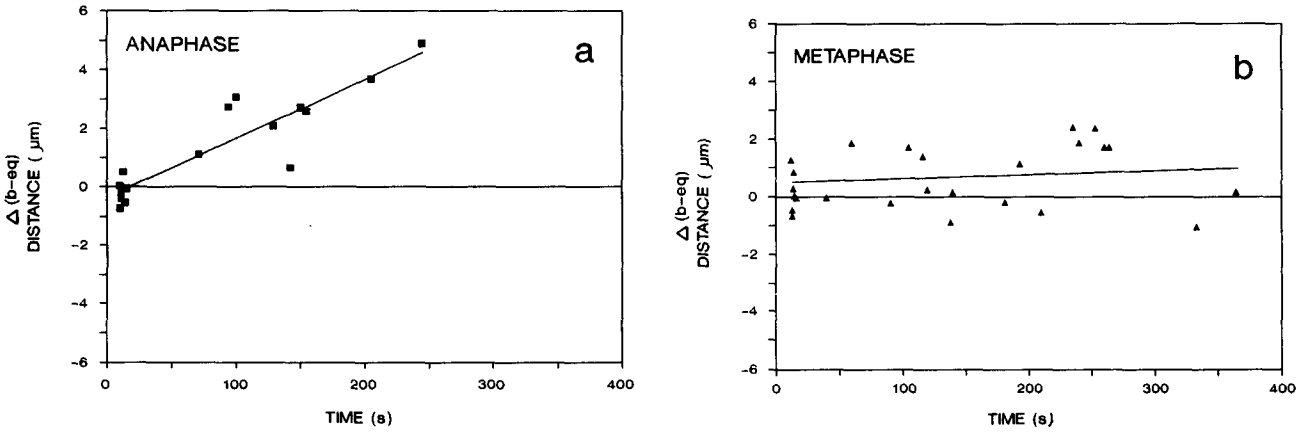

Figure 7. Movement of bleached zones in anaphase cells (*a*) and metaphase cells (*b*). As expected bleached zones moved away from the spindle equator (*b-eq*) during anaphase at a rate of 1.2 μm/min. This result is consistent with the movement of kinetochore fibers accompanying the pole during anaphase B. Linear regression of the data from metaphase cells gave a positive slope of 0.12 μm/min; however, given the spread in the data, this value is not significantly different from zero.

Recently Vigers et al. (22) have cautioned that photobleaching and live fluorescent observation of fluorescent microtubules leads to their dissolution. We recognized this potential problem and took measures to perform our studies under illumination conditions that did not noticeably affect microtubule behavior. To avoid possible photodamage, cells were injected such that the in vivo dye-to-protein ratio was <0.1:1.0. Cells were not imaged while live. They were brought to proper focus by phase contrast microscopy and were subjected to only one or two flashes of 0.1-s duration of green light from a 100-W mercury arc lamp that was attenuated with a 1.0 OD neutral density filter. The short flashes were used only to ensure that a cell identified by phase microscopy had indeed been microinjected. It should be noted that Vigers et al. (22) showed that x-rh–labeled microtubules at a dye-to-protein ratio of 0.5:1.0 and treated with taxol, remained visible by DIC microscopy after 120 s of full intensity mercury arc illumination.

To directly test whether our photobleaching protocol resulted in damage to kinetochore fiber microtubules we performed two sets of controls. For about half the cells used in the study we fixed the cells and prepared them for antitubulin immunofluorescence. Secondly, we fixed and embedded eight photobleached cells and prepared them for transmission electron microscopy. In all the electron microscopic samples and in all save one of the immunofluorescence samples we detected no effect of photobleaching on the structural integrity of the kinetochore fiber microtubules. Indeed, without reference to the x-rh image of the lysed cell or to the image taken at the time of photobleaching it was impossible to discern which half-spindle had been photobleached. The one exception was a cell whose immunofluorescent antitubulin pattern indicated a possibility of damage to the fibers. However, examination of the image taken at the time of photobleaching showed an extremely bright bar where the laser had excited the injected x-rh tubulin: This bar was considerably more intense than that of any other sample, suggesting that the cell in question received an abnormally large injection of x-rh tubulin, and hence was unusually subject to photodamage. That cell was not used in the analysis reported here.

To examine and measure the extent of fluorescent recovery in our bleached zones we used a slow scan, cooled charge coupled device that allowed us to collect and store images in digital form. This camera also allowed us to perform pixel-by-pixel reduction of background for increased accuracy. Of various methods for quantifying fluorescence in the bleached zones, we chose to measure the intensity of fluorescence at the midpoint of the bleached zone, normalizing to the intensity of a corresponding point in the unbleached half spindle. This method was simple, reproducible for different cells allowed to recover for similar lengths of time, and was relatively insensitive to the exact plane of focus in which the final images were taken. We found that changing plane of focus within the spindle altered the fluorescent intensities by at most ~10%.

Our method of assessing recovery was to plot the fluorescence present in the bleached zones of cells lysed at various times after photobleaching in a cold-calcium buffer to deplete the nonkinetochore microtubules. In anaphase little or no new assembly of fluorescent microtubules was detected. With the important assumption that the extraction does not also remove significant numbers of kinetochore fiber microtubules, we can conclude that the kinetochore fiber microtubules in anaphase cells do not turn over appreciably. These results suggest that the limited fluorescence recovery previously noted by us in living anaphase cells (6) is likely due to the turnover of their nonkinetochore microtubules. Our photobleaching studies have been thus far restricted to the region between the chromosomes and the poles. The nonkinetochore microtubules there may be identified as polar or astral microtubules. They presumably turn over by the mechanism of tempered dynamic instability (14, 15), undergoing successive rounds of depolymerization and repolymerization at their plus ends.

In contrast to the situation during anaphase, significant recovery was detected in kinetochore fibers bleached during metaphase. On average kinetochore fibers recovered to ~70% with an approximate half-time of 70 s. The analysis of these data was complicated by several factors. Some points lie well away from the plotted recovery curve, showing somewhat greater than average recovery. These cells may not have been as efficiently bleached as the typical cell. On the other hand, these cells may represent biological phenomena due to hidden variables. For example, unlike anaphase no true landmark exists signifying the start of metaphase. A particular cell photobleached at metaphase may have progressed more in the mitotic cycle than another similarly treated. The dynamic properties of kinetochore fiber microtubules in metaphase cells may change as the cells approach anaphase onset.

Why did recovery not proceed to 100%? A trivial explanation is that our method of analysis underestimated the true degree of recovery. However, our findings do parallel the level of recovery reported by Wadsworth and Salmon (23) who analyzed living metaphase cells that had been injected with fluorescein tubulin and photobleached. These authors noted as we did that recovery was only partial, but speculated that the rapid recovery might represent nonkinetochore microtubules whereas the kinetochore fiber microtubules might turn over more slowly. More recently the photobleaching study of Cassimeris et al. (2) in metaphase PtK1 cells generated a half-time for recovery of 77 s and an extent of recovery of 72%, figures very similar to our own.

One possible explanation for the partial recovery seen in metaphase kinetochore fibers is the following. Extrapolation of our plots of fluorescence recovery indicates that at time 0, the moment of photobleaching, the level of fluorescence is reduced to 30–35%. With time this level rises to ~70% and apparently levels off. Thus, approximately half the fluorescence reduced by the laser recovered. Rieder (17) has reported that within PtK1 cells treated with cold to disassemble nonkinetochore microtubules, approximately half the microtubules in the kinetochore fibers were continuous from the pole to the chromosome, the rest having one or both ends free in the fiber. It is interesting to speculate that the recovery of fluorescence within the kinetochore fibers of LLC-PK observed in our experiments might be due to rapid turnover of one subclass of microtubules within the kinetochore fiber. In metaphase, some microtubules within the kinetochore fibers might be dynamically unstable. They could lose contact with the kinetochore and depolymerize, to be replaced by new ones nucleated either at the poles or along the length of the kinetochore fiber.

In addition to acting as indicators of turnover, the bleached zones also served as positional markers on the kinetochore fiber microtubules. Using these markers, we measured, for cells in anaphase and in metaphase, the movement of kinetochore fiber microtubules with reference to the spindle midpoint. The midpoint proved a convenient reference since it was easily determined from the position of the chromosomes in the phase image taken at the time of photobleaching. In contrast, the positions of the mitotic poles could only be approximated from the phase micrographs of the living cells. In anaphase, bleached zones moved outward from the spindle midpoint. This result is consistent with our earlier photobleaching study where live cells were imaged (6). Those experiments indicated that kinetochore fibers accompany the near pole during pole–pole separation. In the present experiments regression analysis of the metaphase data were consistent with a slight outward movement of the bleached zone, but variation in the data did not permit us to conclude whether the detected motion was real.

In conclusion we have developed methods for specifically visualizing kinetochore fibers with the light microscope after microinjection of x-rh tubulin and photobleaching. Kinetochore fiber microtubules, defined as those resistant to cold-calcium extraction, in anaphase cells do not turn over while a significant percentage of those in metaphase do. Kinetochore fibers move outward as the poles separate in anaphase. In metaphase, our data are consistent with stationary kinetochore fiber microtubules that turn over by dynamic instability or by a slow poleward flux of microtubule subunits.

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