Steady State Dynamics of Intermediate Filament Networks

Karen L. Vikstrom,* Soo-Siang Lim,‡ Robert D. Goldman,* and Gary G. Borisy‡

*Department of Cell, Molecular, and Structural Biology, Northwestern University Medical School, Chicago, Illinois 60611; and ‡Laboratory of Molecular Biology, University of Wisconsin, Madison, Wisconsin 53706

Abstract. We have conducted experiments to examine the dynamic exchange between subunit and polymer of vimentin intermediate filaments (IF) at steady state through the use of xrhodamine-labeled vimentin in fluorescence recovery after photobleaching (FRAP) analysis. The xrhodamine–vimentin incorporated into the endogenous vimentin IF network after microinjection into fibroblasts and could be visualized with a cooled charge-coupled device (CCD) camera and digital imaging fluorescence microscopy. Bar shaped regions were bleached in the fluorescent IF network using a beam from an argon ion laser and the cells were monitored at various times after bleaching to assess recovery of fluorescence in the bleached zones. We determined that bleached vimentin fibers can recover their fluorescence over relatively short time periods. Vimentin fibers in living cells also can exhibit significant movements, but the recovery of fluorescence was not dependent upon movement of fibers. Fluorescence recovery within individual fibers did not exhibit any marked polarity and was most consistent with a steady state exchange of vimentin subunits along the lengths of IF.

INTERMEDIATE filaments (IF)[1] are generally considered to be the most stable of the cytoskeletal components. Even so, consideration of their properties supports the view that IF are dynamic. In this regard, the ability of IF networks to undergo transitory changes in response to different stimuli is well documented. For example, fibroblasts reorganize their vimentin-IF networks from a juxtanuclear "cap" to an extended cytoplasmic network during cell spreading and shape formation (Goldman et al., 1986). Dramatic organizational changes in IF networks also are seen in response to different stimuli such as adipose conversion of cultured cells (Franke et al., 1987) or mitogenic stimulation of lymphocytes (Paulin-Levasseur and Brown, 1987). In addition, IF exhibit dynamic properties during mitosis in numerous cell types (Aubin et al., 1980; Franke et al., 1982; Horwitz et al., 1981; Lane et al., 1982; Jones et al., 1985; Rosevear et al., 1990). In BHK-21 cells, these properties include the depolymerization of the IF network to form amorphous aggregates during metaphase and the eventual reformation of polymerized, cytoplasmic IF at the conclusion of mitosis (Rosevear et al., 1990). Likewise, another member of the IF protein family, the nuclear lamins, exhibits cell cycle–dependent dynamics and becomes disassembled during mitosis or meiosis (Gerace and Blobel, 1980; Dessev and Goldman, 1988). Although the molecular mechanisms governing these processes remain unclear, they can be divided into three categories: (*a*) the molecular requirements for IF assembly; (*b*) the site(s) of assembly; and (*c*) the temporal sequence of events leading to assembly.

Transfection studies have been used with great success to determine the domains of IF proteins that are essential for assembly in vivo. The effect on IF assembly of mutant IF proteins lacking portions of their nonhelical amino or carboxyl domains has been addressed using this technique (Albers and Fuchs, 1987, 1989; Gill et al., 1990; Lu and Lane, 1990; Raats et al., 1990; Wong and Cleveland, 1990). While transfection of mutated IF genes is a powerful technique for mapping the functional domains of IF proteins, its temporal resolution is low with respect to determining IF assembly mechanisms. This makes it less suitable for studying post-translationally regulated IF dynamics.

Studies of the site(s) of assembly have suggested subunit incorporation into IF either in juxtanuclear organization centers or throughout the cytoplasm. Data from transfection experiments (Albers and Fuchs, 1987; Chin and Liem, 1989; Sarria et al., 1990) and microinjection of vimentin (Vikstrom et al., 1989) support the initiation of IF assembly in the juxtanuclear region. On the other hand, studies of newly synthesized IF after keratin mRNA injection into fibroblasts (Kreis et al., 1983) as well as microinjection of keratin (Miller et al., 1991) have demonstrated that keratin assembly does not necessarily require a juxtanuclear organizing center. That vimentin and desmin assembly can also proceed without the aid of a juxtanuclear organizing center has also been supported by transfection studies (Ngai et al., 1990; Sarria et al., 1990; Raats et al., 1990). Some of the dis-

S. Lim's present address is Department of Anatomy, Indiana University Medical Center, 635 Barnhill Dr., Indianapolis, IN 46202.

K. L. Vikstrom's present address is Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461.

1. *Abbreviations used in this paper*: IF, intermediate filaments.

crepancies concerning the nature of the sites of IF assembly may have resulted from attempts to compare steady state versus non-steady state experiments. Microinjection and most transient transfections may provide the cell with a sizable pool of unpolymerized IF proteins and the events seen may not be typical of the assembly of IF in interphase cells at steady state (Ngai et al., 1990; Steinert and Liem, 1990).

To investigate the sequence of events leading to assembly, biochemical analysis has shown that some nascent vimentin molecules associate with IF during translation (Isaacs et al., 1989). This suggests that IF can polymerize cotranslationally (Isaacs et al., 1989). Pulse-chase experiments indicate that newly synthesized vimentin may also enter a soluble pool before all (Blikstad and Lazarides, 1983) or a portion of the newly synthesized vimentin (Soellner et al., 1985) incorporates into the insoluble cytoskeleton. At this point, it has not been clearly established whether after entering a soluble pool of newly synthesized protein, vimentin becomes stably incorporated into polymer or whether there is an equilibrium between soluble and polymeric forms of vimentin. Clarifying this issue through the use of analytical techniques with greater temporal and spatial resolution is central to determining the molecular mechanisms underlying the distribution and composition of IF.

We hope to resolve some of these issues by examining the dynamic properties of IF at steady state using the technique of FRAP (Petersen et al., 1986). FRAP analysis allows steady state systems to be analyzed with high temporal resolution and has proven to be extremely valuable for studying the steady state dynamics of both microtubules (for examples, see Wadsworth and Salmon, 1986; Gorbsky et al., 1988; Sammak and Borisy, 1988) and microfilaments (Kreis et al., 1982; Wang, 1985). In this report we use this technique to demonstrate that there is an equilibrium between subunit and polymer in the IF systems of living cells.

Materials and Methods

Cell Culture

3T3 cells were grown at 37°C in DME containing 10% calf serum, penicillin, and streptomycin. For microinjection, cells were plated onto etched grid coverslips (Bellco Biotechnology, Vineland, NJ) which had been cleaned with RBS-35 (Pierce Chemical Co., Rockford, IL). The coverslips were attached with a 1:1:1 mixture of Vaseline, beeswax, and lanolin to a 35-mm dish in which an 18-mm hole had been drilled (Lim et al., 1990).

Preparation of Xrhodamine-labeled Vimentin

Vimentin was purified from bovine lens and polymerized into IF as described elsewhere (Vikstrom et al., 1989, 1991). IF were collected by centrifugation at 100,000 *g* for 30 min at 4°C. The resulting pellet was solubilized in disassembly buffer (8 M urea, 5 mM sodium phosphate, pH 7.2, 0.2% 2-mercaptoethanol, 1 mM PMSF) by stirring for 30 min at room temperature. The sample was then dialyzed overnight at room temperature versus subunit buffer (5 mM sodium phosphate, pH 7.4, 0.2% 2-mercaptoethanol, 0.2 mM PMSF). The protein concentration was determined by the method of Bradford (1976) using BSA as a standard and then it was adjusted to 2.0–2.5 mg/ml. A 40:1 molar excess of 5-(and-6)-carboxy-X-rhodamine succinimidyl ester was added to this preparation (Molecular Probes, Inc., Eugene, OR). Immediately before adding to the sample, the labeling reagent was dissolved in dimethyl formamide. The final concentration of dimethyl formamide in the preparation was 10% vol/vol.

The conjugation reaction was initiated with soluble vimentin to avoid the sampling error inherent in calculating the protein concentration of polymerized IF at high concentrations. Therefore, 0.17 M sodium chloride was added to the reaction mixture to induce filament formation during the labeling reaction (Zackroff and Goldman, 1979). After incubating for 60 min at room temperature the protein was collected by centrifugation at 100,000 *g* for 30 min at 4°C. The sample was then subjected to two cycles of in vitro disassembly/reassembly to ensure that only polymerization-competent protein was used for the microinjection experiments.

To begin the first cycle of disassembly/reassembly the pellet of xrhodamine-labeled IF was solubilized in disassembly buffer and passed over a Sephadex G-25 column which had been equilibrated in subunit buffer. Sodium chloride was added to a final concentration of 0.17 M and the preparation was dialyzed overnight at room temperature versus PBS containing 0.2% 2-mercaptoethanol and 0.2 mM PMSF (Zackroff and Goldman, 1979). The in vitro disassembly/reassembly conditions included both gel filtration chromatography over Sephadex G-25 and dialysis to completely remove unconjugated xrhodamine from the preparation. After a second cycle of disassembly/reassembly the xrhodamine vimentin sample was frozen dropwise in liquid N_2 and stored at −70°C. A typical vimentin preparation contained 0.5 mole xrhodamine per mole of vimentin, calculated using an extinction coefficient of $5.3 \times 10^4\ M^{-}\ cm^{-}$ for xrhodamine in 5 mM sodium phosphate and a molecular weight of 55,000 for vimentin.

Microinjection

Xrhodamine-labeled vimentin was prepared for microinjection as described for biotinylated vimentin (Vikstrom et al., 1989, 1991). Volumes were adjusted at all steps to yield final samples with concentrations of 2.0–3.0 mg/ml xrhodamine-vimentin in 5 mM sodium phosphate, pH 8.5, 0.05% 2-mercaptoethanol. Microinjections were performed on an inverted microscope (Nikon Inc., Melville, NY) with the aid of a micromanipulator (The Leitz Co., Overland Park, KS) (Lim et al., 1989). After microinjection, the cells were returned to the incubator for 5–13 h to allow full incorporation of xrhodamine-labeled vimentin into the endogenous vimentin networks. At this time, the culture medium was changed to phenol red-free DME containing 10% calf serum and antibiotics.

Photobleaching

The photobleaching apparatus was set up essentially as described by Lim et al. (1989) following the general procedure of Petersen et al. (1986). Microinjected cells were located on the etched grid and the image of the fluorescent IF network was focused using a SIT TV camera. Since excessive illumination of fluorescent cytoskeletal components such as microtubules can be deleterious (Vigers et al., 1988), neutral density filters were placed in front of the mercury arc lamp to attenuate the light and to minimize photodamage to the cells. Cells were bleached with a bar of green light (514 nm) by 200 ms of irradiation from an argon ion laser operating at 200 mW. These conditions result in laser exposures that are eightfold less than those reported to damage microtubules in vitro (Lim et al., 1990). Fluorescence and phase contrast images of the cells were taken before and after photobleaching using a cooled charge-coupled device (CCD) camera (Photometrics Ltd., Tucson, AZ) and the resulting digitized images were stored on a computer disk and archived on an optical disk using a WORM drive (Model 3363, IBM Corp., Danbury, CT) (Lim et al., 1990).

Image Analysis

Images were first flat-fielded using the Photometrics system and further analysis was carried out with the Image-1 system (Universal Imaging Corp., West Chester, PA). To adjust for sample fading during image acquisition the brightness of the prebleach image of each cell was measured and this value was used to normalize the brightness level of subsequent images of that cell. Digitized pixel values were read along each fiber being measured. The maximum pixel value across the width of the fiber was recorded both in the bleached and unbleached regions of the individual xrhodamine-vimentin-containing fibers. The average pixel intensity of the background fluorescence of the coverslip was subtracted from these values and the resulting relative fluorescence intensities were plotted.

Immunofluorescence

Cells were rinsed rapidly with PHEM (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.95) at 37°C, and then lysed for 30 s in 0.15% Triton X-100 in PHEM (Schliwa and van Blerkom, 1981). After lysis the cells were fixed for 15 min at room temperature in 5 mM EGS (ethylene glycol bis [succinic acid *N*-hydroxy succinimide ester]) (Sigma Chemical Co., St. Louis, MO) in PHEM (Gorbsky et al., 1988). After fixation the coverslips were rinsed with PBS and stored overnight at 4°C. Fixed cells

on coverslips were absorbed with 1% BSA in PBS for 30 min at 37°C and then incubated with a rabbit polyclonal antibody directed against vimentin (Yang et al., 1985) for 30 min at 37°C. After their incubation, the coverslips were washed with PBS (4–5 changes over 15 min) and then incubated with a fluorescein-labeled goat anti-rabbit antibody (Kirkegard & Parry Labs, Inc., Gaithersburg, MD) for 30 min at 37°C. The coverslips were washed as described above and then examined using the CCD camera and narrow band fluorescein and rhodamine filters.

Results

Xrhodamine–Vimentin Forms IF in vitro

Twice-cycled xrhodamine vimentin IF (see Materials and Methods) were examined by negative stain EM and only samples which contained smooth walled 10-nm diameter IF (Fig. 1 *a*) were frozen and stored at −70°C for use in subsequent experiments. When examined by SDS-PAGE, the resulting preparation contained one major polypeptide as detected by Coomassie-blue staining, which was fluorescent when unstained gels were examined under UV illumination (Fig. 1 *b*).

Microinjected Xrhodamine–Vimentin Is Incorporated into the Vimentin-IF Network of 3T3 Cells

For xrhodamine–vimentin to be useful in photobleaching studies, it must be incorporated into the endogenous vimentin IF network after microinjection into cultured cells. In addition, the fluorescent vimentin networks must not be damaged under the conditions used to photobleach a bar in this network. To determine if we had achieved these conditions, xrhodamine–vimentin was microinjected into 3T3 cells growing on "locator" coverslips and their positions were noted. Injected cells were incubated for 5–13 h and then examined by fluorescence microscopy using the cooled CCD camera. A bar was bleached in the fluorescent IF network and the cell was lysed and fixed within 2 min of photobleaching (see Materials and Methods). The fixed preparation was processed for indirect immunofluorescence with an antibody directed against vimentin and a fluorescein-conjugated second antibody. In fixed-stained cells, xrhodamine–vimentin colocalized with endogenous vimentin. Furthermore, endogenous vimentin appeared intact in regions where the rhodamine fluorescence had been bleached (compare Fig. 2, *A* and *B*, and *C* and *D*). In addition, over the course of these experiments we did not observe any dissolution of the fluorescent IF network such as had been described for photo damaged fluorescent microtubule networks (Vigers et al., 1988). These data indicate that rhodamine–vimentin is incorporated into the cytoplasmic IF network and that photobleaching does not cause obvious breaks or damage in the vimentin fibers. We refer to these structures as fibers since the limit of resolution for the light microscope does not allow individual IF to be distinguished from small bundles of IF.

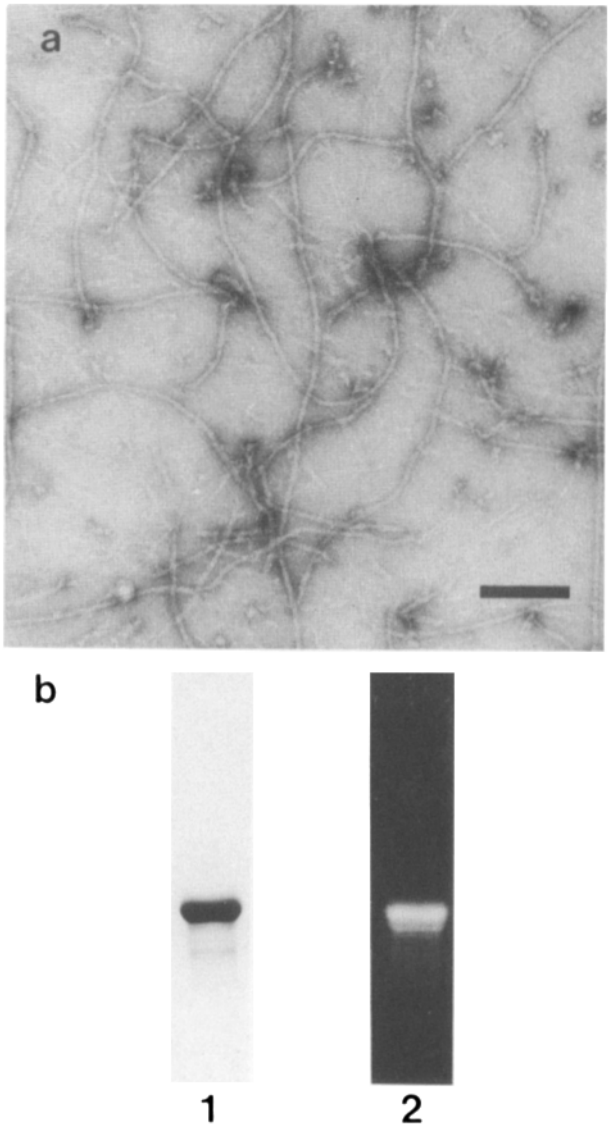

Figure 1. (*a*) Negative stain electron micrograph of twice cycled xrhodamine-labeled vimentin IF. Xrhodamine–vimentin IF were applied to carbon- and parlodion-coated electron microscope grids and negatively stained with 1% uranyl acetate. (*b*) SDS-PAGE was performed on 7.5% gels using the method of Laemmlli (1970). Unstained gels were examined under UV illumination to make certain that fluorescent vimentin was present and to detect any contaminating, unconjugated xrhodamine running in the dye front. Although the pyronin Y indicator dye fluoresced pink in the dye front (not shown), no red fluorescence was detected in the dye front of this sample which indicated that all of the unconjugated xrhodamine had been removed. (lane *1*) xrhodamine–vimentin stained with Coomassie blue and (lane 2) direct fluorescence of the xrhodamine–vimentin in an unstained gel. Bar, 200 nm.

Photobleached Vimentin Networks Recover Their Fluorescence

The ability of bleached zones to recover their fluorescence was assessed. After microinjection with xrhodamine–vimentin, 3T3 cells were allowed to incorporate the injected protein into their endogenous vimentin IF networks. Flat, peripheral regions of microinjected cells were selected and reference images were taken and stored on a computer. A bar was then bleached in the vimentin IF network and images of the cell were taken at 1–10 min intervals to monitor recovery in the bleached zone (Fig. 3). In some cases fluorescent fibers were seen crossing the bleached zone within 5 min, and by 13–14 min after photobleaching many of the bleached fibers had recovered a significant proportion of their fluorescence (Fig. 3 *C*).

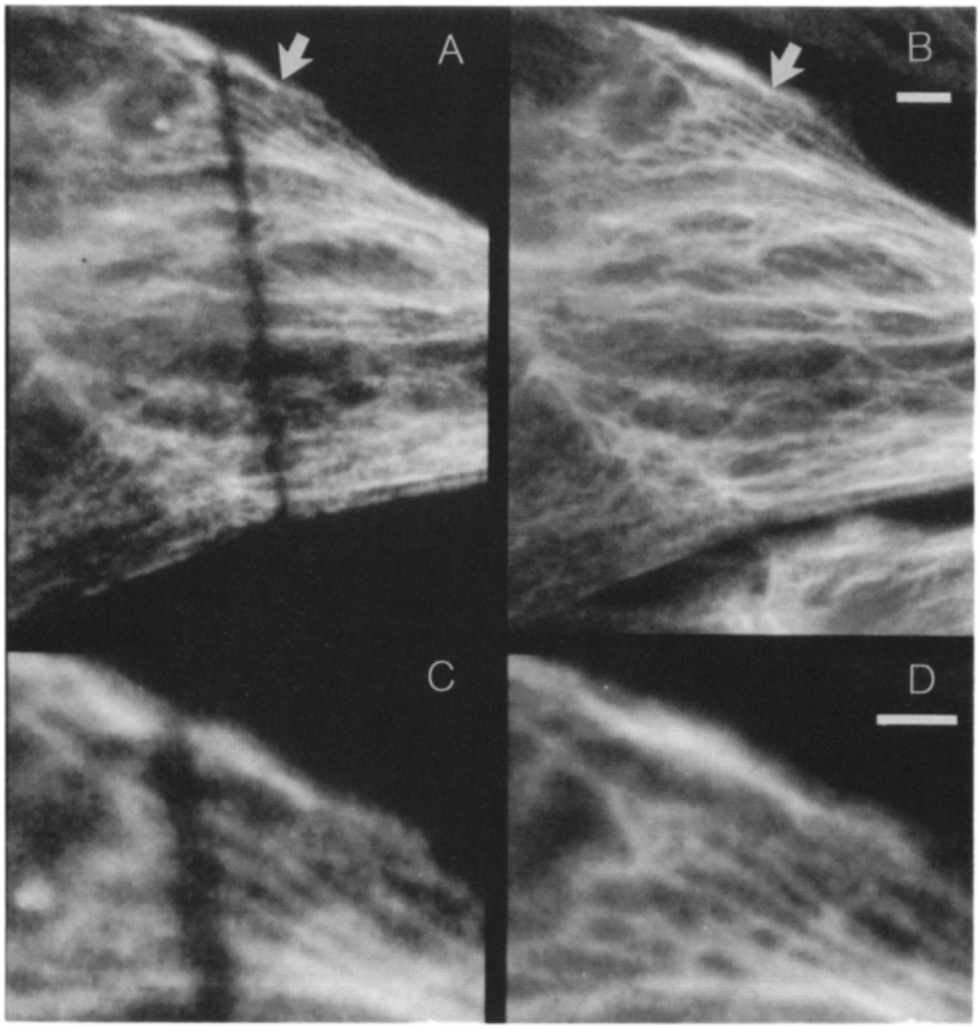

Figure 2. A 3T3 cell microinjected with xrhodamine-vimentin and photobleached 8 h later. The cell was lysed and fixed 2 min after photobleaching. (*A*) Direct fluorescence of the xrhodamine-vimentin pattern. (*B*) Indirect immunofluorescence using an antibody against vimentin and a fluorescein-conjugated secondary antibody to show continuity of the vimentin fibers running through the bleached zone. (*C* and *D*) Higher magnification of the region indicated in *A* and *B*. Bar, 5 μm.

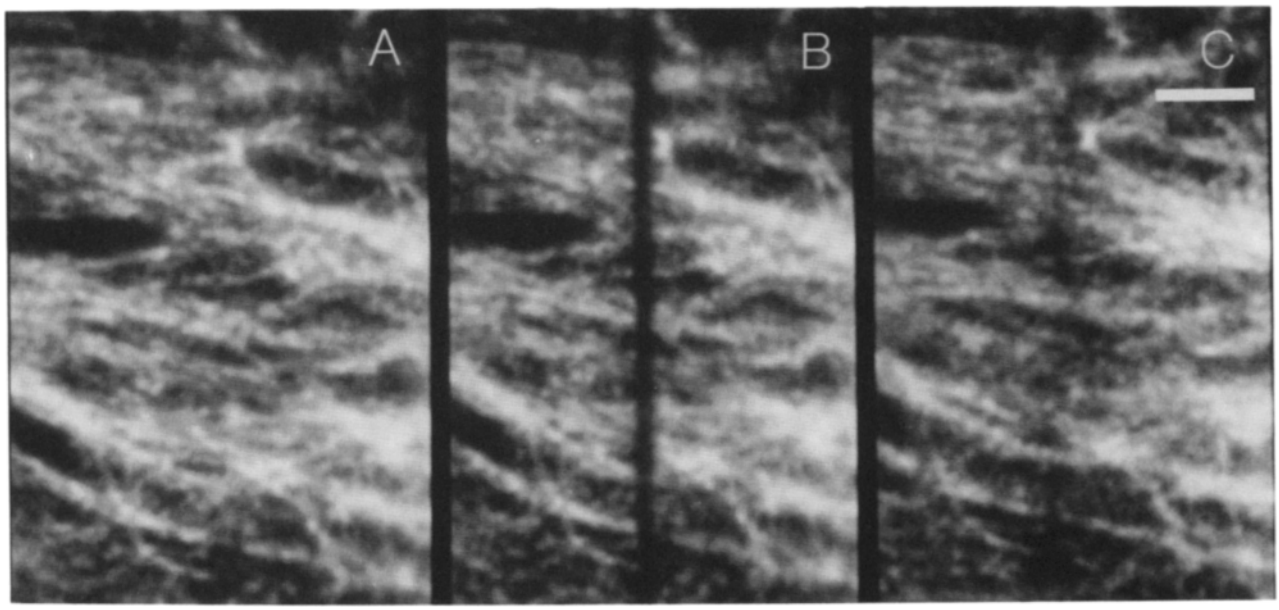

Figure 3. Recovery of fluorescence in xrhodamine-vimentin networks in living cells after photobleaching. A 3T3 cell was microinjected with xrhodamine-vimentin and 13 h later was photobleached with a 200 ms pulse from a 200 mW argon ion laser beam. (*A*) Cell before photobleaching; (*B*) image of the cell after 0.5 min; and (*C*) 13.5 min after photobleaching. Bar, 5 μm.

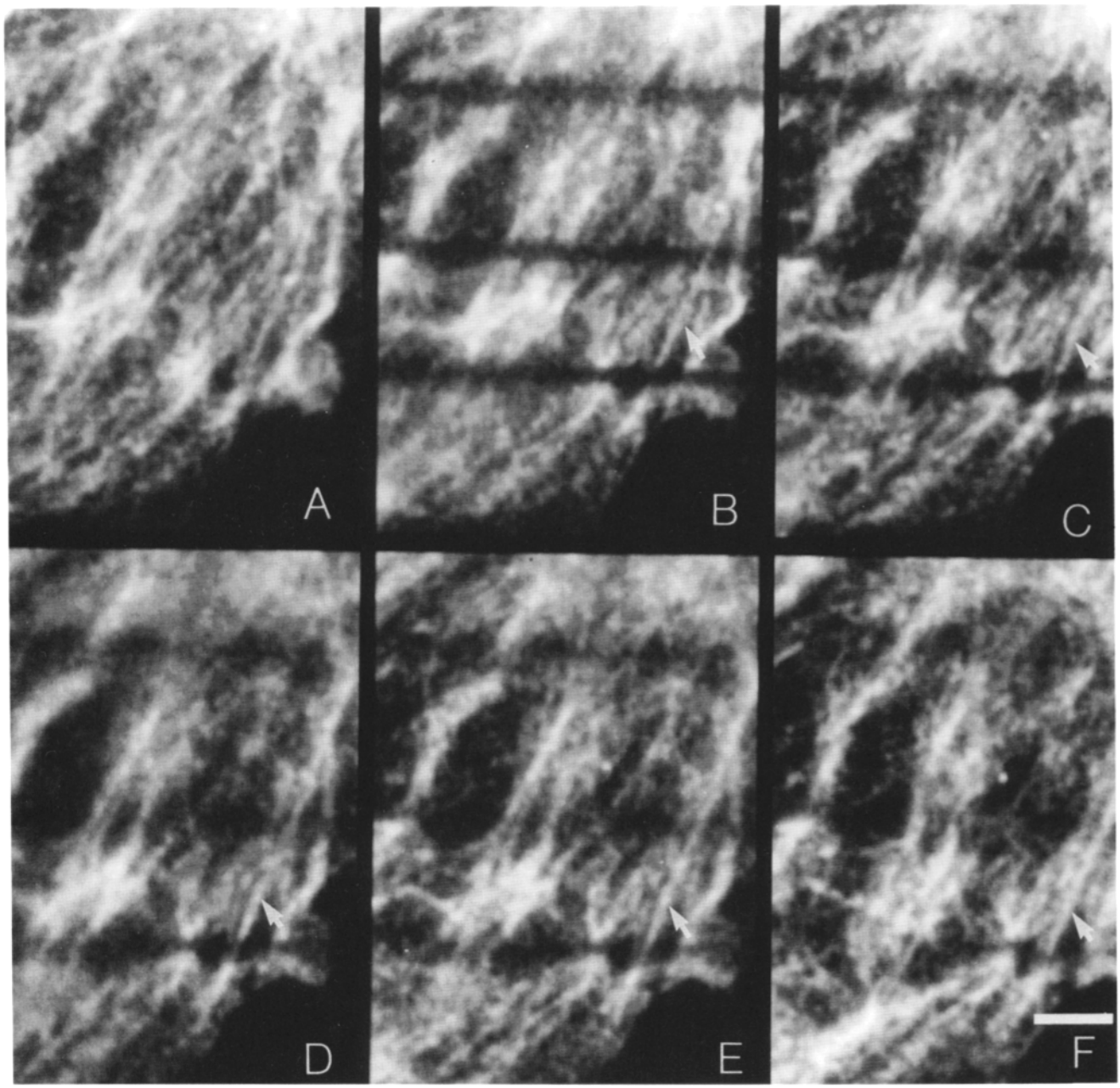

Figure 4. Recovery of fluorescence in a 3T3 cell with multiple photobleached bars. 8 h after the cell was microinjected with xrhodamine–vimentin, three bars were bleached in the fluorescent vimentin network over a 30-s period. The cell was then monitored at time intervals for 40 min; images of the living cell were taken at the times indicated. (*A*) Xrhodamine–vimentin network in the living cell before photobleaching, and (*B*) 0.5, (*C*) 6.5, (*D*) 12.5, (*E*) 34.5, and (*F*) 39.5 min after photobleaching of the third bar. Bar, 5 μm.

Stationary Fibers Recover Their Fluorescence

In some cases fluorescent fibers moved during the recovery period such that the remnants of the bleached bar did not remain straight. To clearly distinguish between fluorescence recovery because of subunit exchange or because of movements of fibers we carried out two experiments. First, we bleached multiple parallel bars in the fluorescent network to provide additional landmarks in assessing the movement of fibers. In addition, we looked for fluorescence recovery in fibers which remained stationary during the time we monitored the cell. When stationary fibers were monitored after photobleaching, fluorescence recovery was observed. In addition, the position of the bleached bars in the cells remained the same. In the example shown in Fig. 4, the bleached zones were clearly apparent 0.5 min after photobleaching. By 6.5 min, the bleached zones no longer had distinct margins and fluorescent fibers could be seen crossing them (Fig. 4). Many bleached fibers that remained stationary (e.g., see the fiber indicated by arrows in Fig. 4, *B–F*) regained fluorescence during the time that the cell was observed with no apparent translocation of the bleached zones along the fibers.

Fluorescence Recovery Exhibits No Detectable Polarity

To further assess the nature of fluorescence recovery in pho-

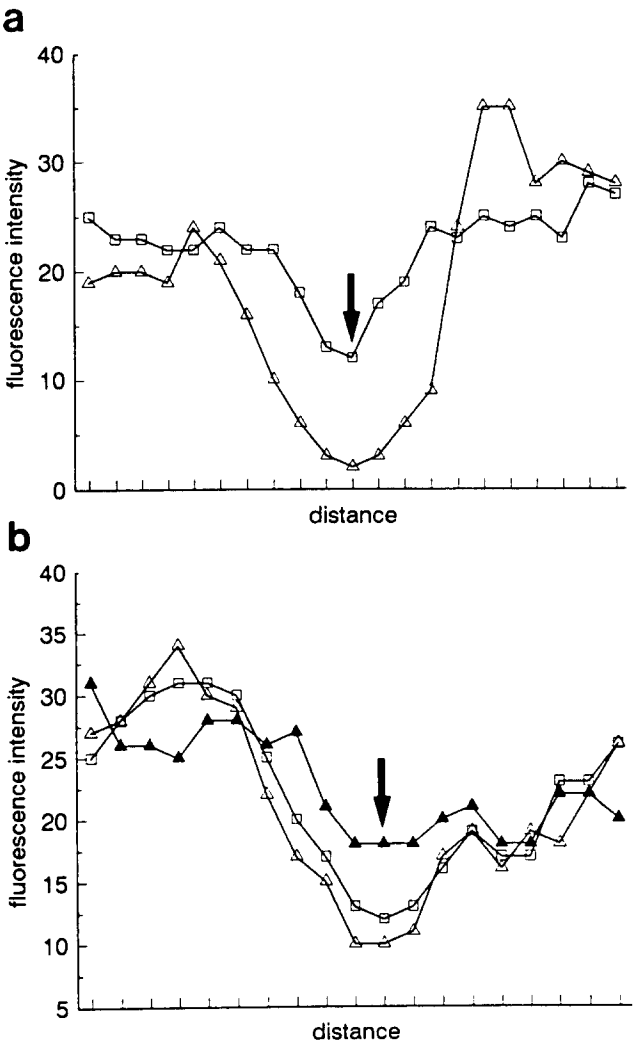

Figure 5. Fluorescence intensity measurements along photobleached fibers during the initial stages of recovery. The arrow marks the center of the bleached zones. The times after photobleaching are indicated. (*a*) 0.5 (△), min, 13.5 (□) min, (*b*) 0.5 (△), 2.5 (□) and 12.5 (▲) min.

tobleached vimentin fibers, we undertook measurements of the fluorescence intensity during recovery. To clearly distinguish the position of the bleached zone we restricted our measurements to the initial stages of recovery. The intensity of fluorescence along a fiber was measured in images of that fiber taken within 15 min of photobleaching. Images of cells at several times during the initial stages of recovery were adjusted to compensate for sample fading during image acquisition and the relative fluorescence intensity was measured along individual fibers in 10 different cells. The measurements of fluorescence intensity were plotted graphically and examined for any trends in the initial stages of recovery. The fluorescence intensities along bleached fibers increased with time after photobleaching, but we did not detect preferential recovery from either side of the bleached zone (Fig. 5). These data indicate that the recovery of fluorescence intensity exhibits no apparent polarity.

To confirm this observation, we then monitored fluorescence recovery in fibers that had been bleached longitudinally. In this manner we were able to produce an effectively wider bleached zone without changing the photobleaching parameters. When longitudinally bleached fibers were monitored over time, fluorescence returned to the fibers throughout their length (Fig. 6). As in the previous experiment there was no apparent polarity to the recovery.

Discussion

Microinjection of labeled IF proteins has been used to determine the location of sites where subunits incorporate into IF (Vikstrom et al., 1989; Mittal et al., 1989). In this study we combine microinjection with FRAP to determine exchange of vimentin subunits in vivo under conditions which approximate steady state. We report that vimentin IF are dynamic in steady state situations, and therefore, they appear to exchange subunits with a pool of depolymerized or disassembled vimentin.

Xrhodamine-Vimentin Functions as a Probe for Examining the Vimentin Networks of Living Cells

Central to using fluorescently labeled IF proteins as probes for IF dynamics is the premise that once microinjected into living cells they will function as a tracer for the behavior of the entire IF network. Therefore, care must be taken to ensure that the fluorescent protein subunits can become incorporated into IF and that once incorporated, they behave normally. The primary in vitro functional assay for fluorescent IF protein is its ability to polymerize into IF (Ip and Fellows, 1990). We have found that vimentin conjugated with 0.5 mole xrhodamine per mole protein retains its ability to assemble into morphologically normal IF in vitro. In addition, the in vivo pattern of xrhodamine vimentin seen after microinjection appears to reflect the entire IF network (see Fig. 2). The excellent correspondence between xrhodamine-vimentin and the total IF network seen 5–10 h after microinjection argues against the possibility that the microinjected protein only polymerizes into new IF. More vigorous proof of the incorporation of xrhodamine-vimentin into IF in vivo would require the localization of xrhodamine subunits at the electron microscope level. Using immuno-EM techniques, biotinylated vimentin has been localized to IF in microinjected cells (Vikstrom et al., 1991). However, similar localization of xrhodamine-vimentin would require probes that are not yet available, such as anti-rhodamine antibodies.

Fluorescence Recovery in Bleached Zones Indicates Subunit Exchange

Xrhodamine does not recover its fluorescence after bleaching (Jacobson et al., 1983), so recovery of fluorescence in the bleached zone is because of the incorporation of unbleached vimentin into that region. There is evidence for a small soluble pool of tetrameric vimentin subunits in living cells (Soellner et al., 1985) and the unbleached xrhodamine-vimentin molecules could be provided by this pool. In this case, diffusion of soluble vimentin tetramers into the bleached zone should occur within seconds. However, in most cells recovery was seen over a period of 30–40 min. Therefore, it seems unlikely that fluorescence recover is rate limited by diffusion of unbleached subunits from the soluble pool. Consequently, the rate of fluorescence recovery may

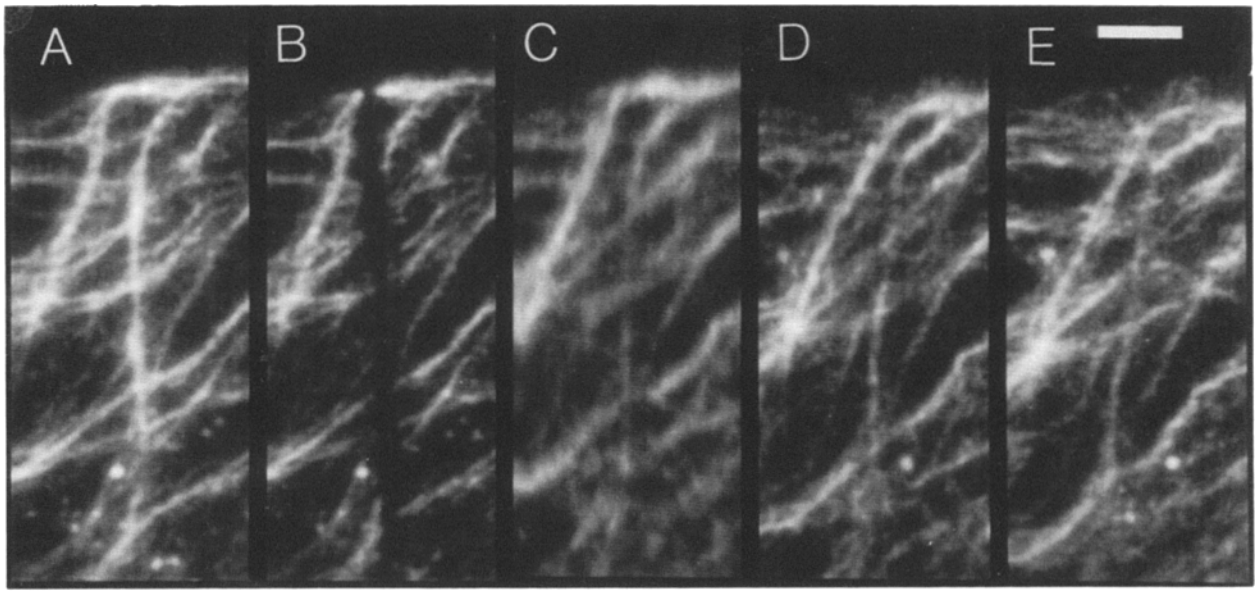

Figure 6. Recovery of fluorescence in a 3T3 cell with a longitudinal bleach zone. 12 h after the cell was microinjected with xrhodamine–vimentin, the aster beam was oriented longitudinal to a prominent fiber. The fiber was photobleached and the cell was then monitored at 15-min intervals for >45 min; images of the living cell were taken at the times indicated. (*A*) Xrhodamine–vimentin network in the living cell before photobleaching, and after (*B*) 0.5, (*C*) 15, (*D*) 30, and (*E*) 45 min after photobleaching. Bar, 5 μm.

depend on the ability of unbleached subunits to incorporate into the bleached regions of IF. This in turn may depend on the availability of assembly sites in these IF.

If bleached xrhodamine–vimentin subunits must first dissociate from IF to create an assembly site, fluorescence recovery would be limited by the rate of subunit dissociation. Angelides et al. (1989) have demonstrated an equilibrium between the subunit and polymeric forms of NF-L in vitro and have suggested that the dissociation of subunit from polymer is the kinetically slowest step in this equilibrium. This suggests that the recovery of fluorescence in photobleached vimentin fibers could be the result of such an equilibrium between soluble and polymeric vimentin. It should be noted that the possibility that vimentin can exchange between polymer and a subunit pool was originally suggested by Soellner et al. (1985).

Possible Mechanisms of Fluorescence Recovery

We do not detect any polarity in the recovery of fluorescence in bleached vimentin fibers nor do we see migration of the bleached zones towards the cell periphery. The former possibility would be expected if the incorporation of subunits into steady state IF only occurred in the juxtanuclear region, as was suggested by our previous microinjection experiments (Vikstrom et al., 1989). Furthermore, migration of the bleached zones towards the cell periphery would occur if IF exhibited treadmilling, as would be expected from the model of vectorial assembly. This model suggests that the nucleation of IF occurs at the nuclear surface and assembly progresses towards the cell surface (Georgatos and Blobel, 1987).

Based on these observations and the probability that IF are apolar structures (Geisler et al., 1985; Steven, 1990) it seems likely that vimentin-IF at steady state exchange subunits along their length (Fig. 7). This hypothesis is consistent with biophysical analyses of IF structure. For example, STEM analysis of the mass per unit length of individual IF indicates polymorphisms in the number of protein chains per cross-sectional area (Steven et al., 1982; Engel et al., 1985) and nuclear magnetic resonance (NMR) studies show that IF are highly flexible, loosely packed polymers (Mack et al., 1988). Taken together, these data suggest that IF are more like a partially disordered braid, rather than a crystalline lattice, therefore subunits would be expected to dissociate from polymer throughout the filament. The exchange of subunits along the length of IF also is supported by the microinjection of biotinylated keratin which is incorporated into tonofilaments throughout the cytoplasm, possibly along the lengths of filaments (Miller et al., 1991). In addition, transfection experiments by Ngai et al. (Ngai et al., 1990) demonstrate that newly synthesized vimentin subunits may incorporate into IF along their length. These studies are limited to determining the incorporation of subunits into IF, while our FRAP data suggest that the disassociation of vimentin subunits also may occur along the lengths of IF. A qualification should be noted, as FRAP analyses are of insufficient resolution to distinguish between events occurring within individual IF or small bundles of IF. The vimentin fibers detected by light microscopy may actually contain numerous IF of undetermined length. Therefore, multiple "ends" of individual IF could exist throughout the length of the fluorescent fibers and consequently an exchange mechanism limited to the ends of IF cannot be ruled out. However, if the biophysical picture of IF as a disordered braid is correct, subunit vacancies and ends exist within single IF, thus obviating a sharp distinction between exchange at ends and along the length of IF.

Subunit Exchange as a Mechanism to Alter the Composition of an IF Network

The exchange of subunits between a polymer and a soluble

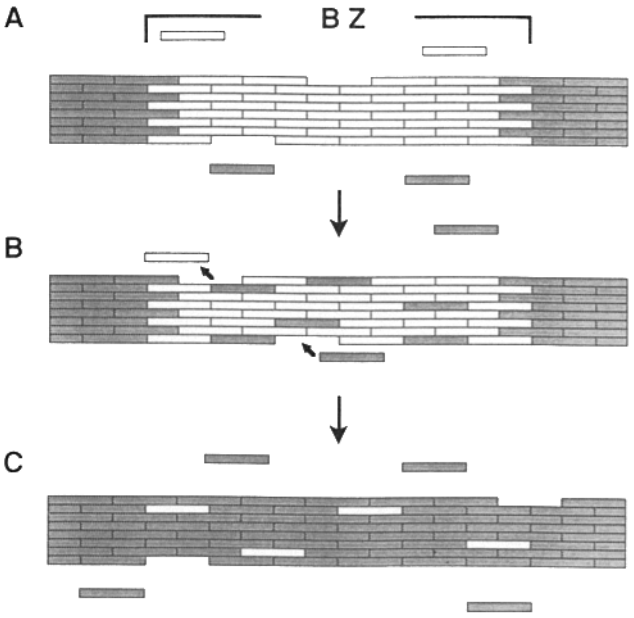

Figure 7. A model for fluorescence recovery in photobleached IF. Previous work by other investigators suggests that IF subunits in living cells may be tetrameric (Soellner et al., 1985). Therefore, in constructing this model we assumed that the exchangeable unit is a vimentin tetramer (*rectangular boxes*). In addition, we have drawn the substructure of the normally cylindrical polymer in a flattened configuration to depict eight tetramers comprising the cross-sectional diameter of the wall. This would result in the 32 protein chains thought to comprise the core structure of the filament (Steven et al. 1982; Engel et al., 1985). (*A*) Immediately after photobleaching, the portion of the polymer found in the bleached zone (*BZ*) contains bleached subunits (▭) while the regions of the polymer adjacent to the *BZ* contain unbleached subunits (▨) as is shown in the diagram. (*B*) Fluorescence recovery in photobleached IF is due to unbleached xrhodamine-labeled vimentin subunits incorporating into polymer in the *BZ*. No polarity is detected in this recovery. Instead, fluorescence returned to the vimentin-IF throughout the *BZ*. This is consistent with the exchange of vimentin subunits along the length of IF. We propose that bleached subunits dissociate from the wall of the IF and that this dissociation creates sites for the incorporation of new subunits into the polymer. Many of these subunits could be unbleached, accounting for the fluorescence recovery. (*C*) With increasing time after photobleaching, the polymer in the *BZ* will contain more unbleached xrhodamine–vimentin subunits.

pool adds a new dimension to the dynamic properties of IF. Subunit exchange along the lengths of IF would provide multiple sites at which to modify the existing network. The work of Albers and Fuchs (1987, 1989) has shown that the entire keratin-IF network of cultured cells can be disrupted by the synthesis of assembly incompetent keratin. A similar effect is seen after transfection of mutant neurofilament genes into fibroblasts (Gill et al., 1990; Wong and Cleveland, 1990). The catastrophic disruption of the filament systems seen after the transient transfection of mutant IF proteins (Albers and Fuchs, 1987; Gill et al., 1990; Wong and Cleveland, 1990) is difficult to explain if the mutant protein only acted on the ends of IF or in a localized area of the network. However, the replacement of endogenous IF protein with mutant protein by subunit exchange throughout the IF network could result in the disruption seen in these studies.

In addition, subunit exchange may provide a mechanism to alter the protein composition of an IF system without depolymerizing the existing network. During development, the transition from vimentin to different IF proteins seen in differentiating muscle cells, glial cells and neurons may occur through a process of subunit exchange (Bennett et al., 1979; Bignami et al., 1982; Cochard and Paulin, 1984; Tapscott et al., 1981; Tokuyasu et al., 1984). It is tempting to speculate that in these instances vimentin-IF provide the initial "IF framework" into which different IF proteins exchange. It should be emphasized that at present the significance of these changes in IF composition remains unclear. However, the two-way exchange of vimentin between polymer and a cytoplasmic pool suggests another level at which IF organization is regulated; the size and composition of the subunit pool.

The authors would like to thank the reviewer who suggested the radial bleach experiment. Your comments were helpful and greatly appreciated. We would also like to thank Alice Rushforth and Rock Pulak for their unique contribution to this project.

This work has been supported by National Institutes of Health (NIH) grants GM 36806 to R. D. Goldman and GM 25062 to G. G. Borisy and by a Markey Foundation fellowship award to K. L. Vikstrom. This work was in partial fulfillment of the degree of Doctor of Philosophy in the Department of Cell, Molecular, and Structural Biology, Northwestern University Medical School for K. L. Vikstrom.

Received for publication 12 February 1992 and in revised form 1 April 1992.

References

Albers, K., and E. Fuchs. 1987. The expression of mutant epidermal keratin cDNAs transfected in simple epithelial and squamous cell carcinoma lines. *J. Cell Biol.* 105:791–806.

Albers, K., and E. Fuchs. 1989. Expression of mutant keratin cDNAs in epithelial cells reveals possible mechanisms for initiation and assembly of intermediate filaments. *J. Cell Biol.* 108:1477–1493.

Angelides, K. J., K. E. Smith, and M. Takeda. 1989. Assembly and exchange of intermediate filament proteins of neurons: neurofilaments are dynamic structures. *J. Cell Biol.* 108:1495–1506.

Aubin, J. E., M. Osborn, W. W. Franke, and K. Weber. 1980. Intermediate filaments of the vimentin-type and the cytokeratin-type are distributed differently during mitosis. *Exp. Cell Res.* 129:149–165.

Bennett, G. S., S. A. Fellini, Y. Toyama, and H. Holtzer. 1979. Redistribution of intermediate filament subunits during skeletal myogenesis and maturation in vitro. *J. Cell Biol.* 82:577–584.

Bignami, A., T. Raju, and D. Dahl. 1982. Localization of vimentin, the nonspecific intermediate filament protein, in embryonal glia and in early differentiating neurons. *Dev. Biol.* 91:286–295.

Blikstad, I., and E. Lazarides. 1983. Vimentin filaments are assembled from a soluble precursor in avian erythroid cells. *J. Cell Biol.* 96:1803–1808.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

Chin, S. S. M., and R. K. H. Liem. 1989. Expression of rat neurofilament proteins NF-L and NF-M in transfected non-neuronal cells. *Eur. J. Cell Biol.* 50:475–490.

Cochard, P., and D. Paulin. 1984. Initial expression of neurofilaments and vimentin in the central and peripheral nervous system of the mouse embryo *in vivo*. *J. Neurosci.* 4:2080–2094.

Dessev, G., and R. Goldman. 1988. Meiotic breakdown of nuclear envelope in oocytes of *Spisula solidissima* involves phosphorylation and release of nuclear lamin. *Dev. Biol.* 130:543–550.

Engel, A., R. Eichner, and U. Aebi. 1985. Polymorphism of reconstituted human epidermal keratin filaments: determination of their mass-per-unit-length and width by scanning transmission electron microscopy. *J. Ultrastruct. Res.* 90:323–335.

Franke, W., E. Schmid, C. Grund, and B. Geiger. 1982. Intermediate filament proteins in nonfilamentous structures: transient disintegration and inclusion of subunit proteins in granular aggregates. *Cell.* 30:103–113.

Franke, W. W., M. Hergt, and C. Grund. 1987. Rearrangement of the vimentin cytoskeleton during adipose conversion: formation of an intermediate filament cage around lipid globules. *Cell.* 49:131–141.

Geisler, N., E. Kaufmann, and K. Weber. 1985. Antiparallel orientation of the

two double-stranded coiled-coils in the tetrameric protofilamentous unit of intermediate filaments. *J. Mol. Biol.* 182:173–177.

Georgatos, S. D., and G. Blobel. 1987. Two distinct sites for vimentin along the plasma membrane and the nuclear envelope: a basis for vectorial assembly of intermediate filaments. *J. Cell Biol.* 105:105–116.

Gerace, L., and G. Blobel. 1980. The nuclear lamina is depolymerized during mitosis. *Cell.* 19:277–287.

Gill, S. R., P. C. Wong, M. J. Monteiro, and D. W. Cleveland. 1990. Assembly properties of dominant and recessive mutations in the small mouse neurofilament (NF-L) subunit. *J. Cell Biol.* 111:2005–2019.

Goldman, R. D., A. E. Goldman, K. J. Green, J. C. R. Jones, S. M. Jones, and H. Y. Yang. 1986. Intermediate filament networks: organization and possible functions of a diverse group of cytoskeletal elements. *J. Cell Sci. Suppl.* 5:69–97.

Gorbsky, G. J., P. J. Sammak, and G. G. Borisy. 1988. Microtubule dynamics and chromosome motion visualized in living anaphase cells. *J. Cell Biol.* 106:1185–1192.

Horwitz, B., H. Kupfer, Z. Eshhar, and B. Geiger. 1981. Reorganization of arrays of prekeratin filaments during mitosis. *Exp. Cell Res.* 134:281–290.

Ip, W., and M. E. Fellows. 1990. Fluorescent measurement of desmin intermediate filament assembly. *Anal. Biochem.* 185:10–16.

Isaacs, W. B., R. K. Cook, J. C. Van Atta, C. M. Redmond, and A. B. Fulton. 1989. Assembly of vimentin in cultured cells varies with cell type. *J. Biol. Chem.* 264:17953–17960.

Jacobson, K., E. Elson, D. Koppel, and W. Webb. 1983. International workshop on the application of fluorescence photobleaching techniques to problems in cell biology. *Fed. Proc.* 42:72–79.

Jones, J. C. R., A. E. Goldman, H. Y. Yang, and R. D. Goldman. 1985. The organizational fate of intermediate filament networks in two epithelial cell types during mitosis. *J. Cell Biol.* 100:93–102.

Kreis, T. E., B. Geiger, and J. Schlessinger. 1982. Mobility of microinjected rhodamine actin within living chicken gizzard cells determined by fluorescence photobleaching recovery. *Cell.* 29:835–845.

Kreis, T. E., B. Geiger, E. Schmid, J. L. Jorcano, and W. W. Franke. 1983. De novo synthesis and specific assembly of keratin filaments in nonepithelial cells after microinjection of mRNA for epidermal keratin. *Cell.* 32:1125–1137.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lane, E. B., Goodman, S. L., and L. K. Trejdosiewicz. 1982. Disruption of the keratin filament network during epithelial cell division. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:1365–1372.

Lim, S. S., P. J. Sammak, and G. G. Borisy. 1989. Progressive and spatially differentiated stability of microtubules in developing neuronal cells. *J. Cell Biol.* 109:253–263.

Lim, S. S., K. J. Edsen, P. C. Letourneau, and G. G. Borisy. 1990. A test of microtubule translocation during neurite elongation. *J. Cell Biol.* 111: 123–130.

Lu, X., and E. B. Lane. 1990. Retrovirus-mediated transgenic keratin expression in cultured fibroblasts: specific domain functions in keratin stabilization and filament formation. *Cell.* 62:681–696.

Mack, J. W., D. A. Torchia, and P. M. Steinert. 1988. Solid-state NMR studies of the dynamics and structure of mouse keratin intermediate filaments. *Biochemistry.* 27:5418–5426.

Miller, R. K., K. Vikstrom, and R. D. Goldman. 1991. Keratin incorporation into intermediate filaments is a rapid process. *J. Cell Biol.* 113:843–855.

Mittal, B., J. M. Sanger, and J. W. Sanger. 1989. Visualization of intermediate filaments in living cells using fluorescently labeled desmin. *Cell Motil. Cytoskeleton.* 12:127–138.

Ngai, J., T. R. Coleman, and E. Lazarides. 1990. Localization of newly synthesized vimentin subunits reveals a novel mechanism of intermediate filament assembly. *Cell.* 60:415–427.

Paulin-Levasseur, M., and D. L. Brown. 1987. Vimentin dynamics during the mitogenic stimulation of mouse splenic lymphocytes. *Cell Motil. Cytoskeleton.* 8:227–237.

Petersen, N. O., S. Felde, and E. L. Elson. 1986. Measurement of lateral diffusion by fluorescence photobleaching recovery. Handbook of Experimental Immunology. 4th edition. D. M. Weir, editor. Blackwell Scientific Publications Ltd., Oxford. 1:24.1–24.22.

Raats, J. M. H., F. R. Pieper, W. T. M. Vree Egberts, K. N. Verrijp, F. C. S. Ramaekers, and H. Bloemendal. 1990. Assembly of amino-terminally deleted desmin in vimentin-free cells. *J. Cell Biol.* 111:1971–1985.

Rosevear, E. R., M. McReynolds, and R. D. Goldman. 1990. Dynamic properties of intermediate filaments: disassembly and reassembly during mitosis in baby hamster kidney cells. *Cell Motil. Cytoskeleton.* 17:150–166.

Sammak, P. J., and G. G. Borisy. 1988. Detection of single fluorescent microtubules and methods for determining their dynamics in living cells. *Cell Motil. Cytoskeleton.* 10:237–245.

Sarria, A. J., S. K. Nordeen, and R. M. Evans. 1990. Regulated expression of vimentin cDNA in cells in the presence and absence of a preexisting vimentin filament network. *J. Cell Biol.* 111:553–565.

Schliwa, M., and J. van Blerkom. 1981. Structural interaction of cytoskeletal components. *J. Cell Biol.* 90:222–235.

Soellner, P., R. A. Quinlan, and W. W. Franke. 1985. Identification of a distinct soluble subunit of an intermediate filament protein: tetrameric vimentin from living cells. *Proc. Natl. Acad. Sci. USA.* 82:7929–7933.

Steinert, P. M., and R. K. Liem. 1990. Intermediate filament dynamics. *Cell.* 60:521–523.

Steven, A. C., J. Wall, J. Hainfeld, and P. M. Steinert. 1982. Structure of fibroblastic intermediate filaments: analysis by scanning tunneling electron microscopy. *Proc. Natl. Acad. Sci. USA.* 79:3101–3105.

Steven, A. C. 1990. Intermediate filament structure: diversity, polymorphism, and analogy to myosin. *In* Cellular and Molecular Biology of Intermediate Filaments. R. D. Goldman and P. M. Steinert, editors. Plenum Press, New York. 233–263.

Tapscott, S. J., G. S. Bennett, and H. Holtzer. 1981. Neural precursor cells in the chick neural tube express neurofilament proteins. *Nature (Lond.).* 292:836–838.

Tokuyasu, K. T., P. A. Maher, and S. J. Singer. 1984. Distribution of vimentin and desmin in developing chick myotubes in vivo. I. Immunofluorescence study. *J. Cell Biol.* 98:1961–1972.

Vigers, G. P. A., M. Coue, and J.R. McIntosh. 1988. Fluorescent microtubules break up under illumination. *J. Cell Biol.* 107:1011–1024.

Vikstrom, K. L., G. G. Borisy, and R. D. Goldman. 1989. Dynamic aspects of intermediate filament networks in BHK-21 cells. *Proc. Natl. Acad. Sci. USA.* 86:549–553.

Vikstrom, K. L., R. K. Miller, and R. D. Goldman. 1991. Methods for analyzing the dynamic properties of intermediate filaments. *Methods Enzymol.* 96:506–525.

Wadsworth, P., and E. D. Salmon. 1986. Analysis of treadmilling model during metaphase of mitosis using fluorescence redistribution after photobleaching. *J. Cell Biol.* 102:1032–1038.

Wang, Y.-L. 1985. Exchange of actin subunits at the leading edge of living fibroblasts: possible role of treadmilling. *J. Cell Biol.* 101:597–602.

Wong, P. C., and D. W. Cleveland. 1990. Characterization of dominant and recessive assembly-defective mutations in mouse neurofilament NF-M. *J. Cell Biol.* 111:1987–2003.

Yang, H.-Y., N. Lieska, A. E. Goldman, and R. D. Goldman. 1985. A 300,000 molecular weight intermediate filament-associated protein in Baby Hamster Kidney (BHK-21) cells. *J. Cell Biol.* 100:620–631.

Zackroff, R. V., and R. D. Goldman. 1979. *In vitro* assembly of intermediate filaments from Baby Hamster Kidney (BHK-21) cells. *Proc. Natl. Acad. Sci. USA.* 76:6226–6230.