

# Active Transport of Photoactivated Tubulin Molecules in Growing Axons Revealed by a New Electron Microscopic Analysis

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**Abstract.** To determine whether tubulin molecules transported in axons are polymers or oligomers, we carried out electron microscopic analysis of the movement of the tubulin molecules after photoactivation. Although previous optical microscopic analyses after photobleaching or photoactivation had suggested that most of the axonal microtubules were stationary, they were not sufficiently sensitive to allow detection of actively transported tubulin molecules which were expected to be only a small fraction of total tubulin molecules in axons. In addition, some recent studies using indirect approaches suggested active polymer transport as a mechanism for tubulin transport (Baas, P.W., F.J. Ahmad. 1993. *J. Cell Biol.* 120:1427–1437; Yu, W., V.E. Centonze, F.J. Ahmad, and P.W. Bass. 1993. *J. Cell Biol.* 122:349–359; Ahmad, F.J., and P.W. Bass. 1995. *J. Cell Sci.* 108:2761–2769). So, whether transported tubulin

molecules are polymers or not remain to be determined. To clear up this issue, we made fluorescent marks on the tubulin molecules in the axons using a photoactivation technique and performed electron microscopic immunocytochemistry using anti-fluorescein antibody. Using this new method we achieved high resolution and high sensitivity for detecting the transported tubulin molecules. In cells fixed after permeabilization, we found no translocated microtubules. In those fixed without permeabilization, in which oligomers and heterodimers in addition to polymers were preserved, we found much more label in the regions distal to the photoactivated regions than in the proximal regions. These data indicated that tubulin molecules are transported not as polymers but as heterodimers or oligomers by an active mechanism rather than by diffusion.

**S**INCE there is no protein synthesis machinery in axons, axonal and synaptic proteins must be transported down the axon following their synthesis in the cell body. It is well established that some kinds of proteins are transported with membranous organelles (fast transport) (Grafstein and Forman, 1980; Hirokawa, 1993, 1996; Brady, 1995), but the mechanism of transport of cytoskeletal or cytosolic proteins (slow transport) is not well understood (Hirokawa and Okabe, 1992; Nixon, 1992; Hirokawa, 1993).

Though previous radiolabeling studies have indicated that cytoskeletal proteins are actively transported down the axon at a rate of 0.2–10 mm/d (Black and Lasek, 1980; Lasek et al., 1984; Tashiro and Komiya, 1989), the molecules being transported had not been directly observed morphologically until now. As a result, whether the transported cytoskeletal proteins are polymers or oligomers is still problematic (Bamburg et al., 1986; Lasek, 1986; Nixon

and Logvinenko, 1986; Hollenbeck, 1989, 1990; Cleveland and Hoffman, 1991; Nixon, 1991; Reinsch et al., 1991; Hirokawa and Okabe, 1992; Baas and Ahmad, 1993; Joshi and Baas, 1993; Yu et al., 1993; Ahmad et al., 1994; Ahmad and Baas, 1995).

To investigate the microtubule dynamics and the mechanism of tubulin transport in axons, photobleaching and photoactivation studies have been performed making marks on the tubulin molecules in axons. Under optical microscopy, it was clearly shown that the marks made in these studies did not move (Lim et al., 1990; Okabe and Hirokawa, 1990, 1992, 1993; Sabry et al., 1995; Takeda et al., 1995), but fluorescent peaks traveling down the axon, which were expected to indicate the presence of actively transported tubulin molecules, could not be observed. In some specialized neurons (e.g., cultured *Xenopus* neurons), however, anterograde movement of photoactivated or photobleached marks was observed (Reinsch et al., 1991; Okabe and Hirokawa, 1992, 1993). Nonetheless, it is now considered that this polymer movement is not related to the mechanism of slow transport (Okabe and Hirokawa, 1992; Sabry et al., 1995), but reflects the passive pulling of the whole axoplasm by growth cones (Okabe and Hiro-

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kawa, 1992; Takeda et al., 1995). Recent photobleaching and photoactivation studies performed using *in vivo* systems also supported the possibility that polymers are stationary (Sabry et al., 1995; Takeda et al., 1995). Although these studies suggested that most of the axonal microtubules were stationary, the transported tubulin molecules could not be detected because of the low sensitivity of observation. Since the transported tubulin molecules are expected to be only a small fraction of the total tubulin molecules in axons (Hoffman et al., 1992), we could not determine whether they were polymers or oligomers. Furthermore, some recent studies using cultured neurons proposed movements of microtubules although they are based on indirect evidence (Ahmad et al., 1994; Ahmad and Baas, 1995; Baas and Ahmad, 1993).

To clear up this issue, we made fluorescent marks on the tubulin molecules in axons using a photoactivation technique and performed electron microscopic immunocytochemistry using anti-fluorescein antibody obtaining high sensitivity and resolution simultaneously. In cells permeabilized and fixed after an appropriate interval following photoactivation to allow clear observation of microtubules, we found no labeled microtubules out of the photoactivated regions. This indicated that there are no moving microtubule polymers in axons. In those fixed without permeabilization, in which oligomers and heterodimers in addition to polymers were preserved, we found many labels out of the photoactivated regions. These labels out of the photoactivated regions indicated the presence of moving tubulin molecules from the photoactivated regions. In addition, the labels were more abundant in the regions distal to the photoactivated regions than in the proximal ones. This means that we observed not only simple diffusion but also the active anterograde transport of tubulin molecules. These data indicated that tubulin molecules are transported not as polymers but as heterodimers or oligomers by an active mechanism rather than by diffusion.

## Materials and Methods

### Preparation of Caged Fluorescein-labeled Tubulin

Phosphocellulose-purified hog brain tubulin was labeled with *bis*-caged carboxyfluorescein (caged fluorescein) (Mitchison, 1989; Okabe and Hirokawa, 1992; Umeyama et al., 1993) (Biscaged-Fluorescein Sulfo OSu; Dojindo Laboratories, Kumamoto, Japan). After the labeling reaction, the preparation was subjected to two cycles of polymerization and depolymerization selecting the assembly competent molecules. Labeled tubulin preparations after photoactivation were analyzed by SDS gel electrophoresis.

### Antibody Preparation

Antiserum against fluorescein was produced using fluorescein-labeled keyhole limpet hemocyanin for the antigen. Antibody against fluorescein was affinity purified by CNBr sepharose affinity column (Pharmacia LKB Biotechnology, Uppsala, Sweden) coupled with fluorescein-conjugated BSA.

### Cell Culture, Microinjection, and Photoactivation

Dorsal root ganglion (DRG)<sup>1</sup> neurons were isolated from adult mice and plated onto laminin-coated coverslips (Okabe and Hirokawa, 1992). For

1. *Abbreviation used in this paper:* DRG, dorsal root ganglion.

identification of photoactivated axons after embedding in resin, we used CELLocate microgrid coverslips (Eppendorf, Hamburg, Germany). The meshes etched on these coverslips were transferred to the resin block and we could easily and precisely identify the photoactivated axons after embedding. Neurons without any process or with only minor sproutings were microinjected with bis-caged carboxyfluorescein-labeled tubulin 3–8 h after plating and further incubated for 10–20 h. To identify microinjected neurons, cells were coinjected with caged fluorescein-labeled tubulin and rhodamine-BSA as previously described (Okabe and Hirokawa, 1992).

Photoactivation was performed as previously described (Mitchison, 1989; Okabe and Hirokawa, 1992; Umeyama et al., 1993).

When we microinjected the neurons with fluorescein-labeled BSA, we used the neurons 15–20 h after plating. About 2 h after injection, we fixed the cells and stained for electron microscopic observation.

### Preparation of Fluorescein-labeled BSA

5 mg/ml BSA in PEM (100 mM Pipes, 1 mM EGTA, and 1 mM MgCl<sub>2</sub>, pH 6.6) was reacted with 2.5 mg/ml 5(6)carboxyfluorescein succinimidyl ester for 30 min at 37°C. The reaction was stopped by adding K-glutamate and free dye was separated by gel filtration using a Sephadex G-25 column. Fluorescein-labeled BSA-containing fractions were collected and dialyzed against K-glutamate buffer (50 mM K-glutamate, 100 mM KCl, and 1 mM MgCl<sub>2</sub>, pH 6.8) and used for microinjection (Salmon et al., 1984; Popov and Poo, 1992; Okabe and Hirokawa, 1991).

### Immunoelectron Microscopy

To observe individual microtubules clearly, we permeabilized the cells before fixation as described previously (Baas and Black, 1990) with slight modifications after an appropriate interval after photoactivation. The photoactivated samples were permeabilized for 5 min with 1% Triton X-100 in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 2 mM MgCl<sub>2</sub>, pH 6.9) containing 10 μM taxol and 0.2 M NaCl and fixed for 30 min by adding an equal volume of 1% glutaraldehyde in PHEM. After fixation, the samples were washed with PBS (pH 7.2) and treated with 50 mM glycine in PBS for 30 min followed by incubation with blocking solution containing 5% skimmed milk in PBS. Then, we incubated the cells with the anti-fluorescein antibody which had been newly raised for this study. Antiserum was used at a dilution of 1:500 in PBS with 0.5% skimmed milk. After overnight incubation at 4°C, cells were washed with PBS for 1 h and incubated at 37°C with 5-nm gold-conjugated anti-rabbit antibody (Amersham Life Science, Buckinghamshire, England, RPN 420) for 5 h at a dilution of 1:10 in PBS. After washing, we fixed the cells again and processed for silver enhancement (Amersham Life Science, RPN 491) followed by osmification, block staining, dehydration, and embedding. The block was serially sectioned and examined. Since most of the silver granules but not the core gold particles dissolved in the osmium tetroxide solution, the size of the labels was varied.

To observe not only microtubules but also tubulin oligomers and heterodimers, we fixed some photoactivated neurons without the preceding permeabilization. (In some cases, as controls, cells were photoactivated after fixation without the preceding permeabilization.) To examine whether soluble proteins are really preserved during the fixation procedure employed here, fluorescein-labeled BSA-injected neurons were also processed according to the following fixation procedure. We fixed these neurons 1 or 20 min after photoactivation with 0.1% glutaraldehyde, 2% paraformaldehyde in PEM. Neurons injected with fluorescein-labeled BSA were also fixed with the same fixative and processed as follows. We treated the neurons with 1% Triton X-100 in PBS for 30 min. After a 30-min incubation with 50 mM glycine in PBS and treatment with 5% skimmed milk in PBS for 30 min, we incubated the samples overnight with affinity-purified anti-fluorescein antibody in BSA-PBS (0.5% BSA, and 0.1% gelatin in PBS) containing 0.5% skimmed milk at 4°C. After complete washing with BSA-PBS containing 0.5% skimmed milk, cells were incubated overnight at 4°C with 1.4-nm gold-conjugated anti-rabbit Fab' fragment (Nanoprobes, Inc., Stony Brook, NY Nanogold™-anti rabbit) diluted 500 times in BSA-PBS containing 1% skimmed milk, 500 mM NaCl and 0.05% Tween 20. Complete wash with BSA-PBS containing skimmed milk, NaCl and Tween 20 was followed by fixation and silver enhancement in developer (30% gum arabic, 0.85% hydroquinone, and 0.11% silver lactate in citrate buffer) (Hayat, 1989). Cells were then block stained, dehydrated, and embedded without osmification for serial sectioning and observation.

## Analysis of the Distribution of Labels Out of the Photoactivated Regions

We counted the number of gold labels in the regions proximal or distal to the activated segments on serial sections of the cells fixed 1 min after photoactivation. The quantification was performed by workers who were not concerned with this experiment. Then the numbers of labels in the two regions were compared.

We performed binomial tests to reject the null hypothesis that labels appeared in equal possibility in the two regions.

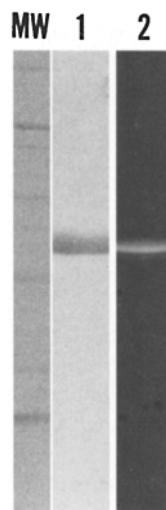
## Results

### Characterization of Caged Fluorescein-labeled Tubulin

Phosphocellulose-purified hog brain tubulin was labeled with bis-caged carboxyfluorescein (Mitchison, 1989; Okabe and Hirokawa, 1992; Umeyama et al., 1993). Bis-caged carboxyfluorescein is a nonfluorescent derivative of carboxyfluorescein, changes into carboxyfluorescein and becomes fluorescent by ultraviolet (UV) illumination (photoactivation). After the labeling reaction, the preparation was subjected to two cycles of polymerization and depolymerization selecting the assembly competent molecules. Labeled tubulin preparations after photoactivation were analyzed by SDS gel electrophoresis (Fig. 1). The sample was purified to a high degree and under ultraviolet light a distinct single band was observed, suggesting that substantially all of the fluorophore were covalently bound to tubulin molecules.

### Photoactivated Microtubules Are Stationary in Growing Axons

We microinjected cultured mouse DRG cells with bis-caged carboxyfluorescein-labeled tubulin. Neurons without any process or with only minor sproutings ( $<50\ \mu\text{m}$ ) were microinjected 3–8 h after plating and further incubated for 10–20 h. During this incubation, the axons started to extend. We photoactivated axons longer than 100  $\mu\text{m}$ . When small axonal segments of injected cells ( $\sim 5\ \mu\text{m}$  wide) were illuminated with 365-nm UV light, carboxyfluorescein-labeled tubulin molecules were generated only in the small regions. Photoactivation was performed within



**Figure 1.** Characterization of bis-caged carboxyfluorescein-labeled tubulin. SDS-PAGE of the bis-caged fluorescein-labeled tubulin after photoactivation revealed a distinct single band at the level of 55 kD (lane 1). Under ultraviolet illumination, only a single band corresponding to that of Coomassie brilliant blue-stained gel was visualized (lane 2). Molecular mass standards (from top to bottom) are 200, 116, 92, 66, 42, and 31 kD.

50  $\mu\text{m}$  from the cell bodies. To analyze the movement of the generated fluorescent tubulin molecules electron microscopically, we stained the cells using polyclonal anti-fluorescein antibody newly raised for this study and gold-conjugated secondary antibody.

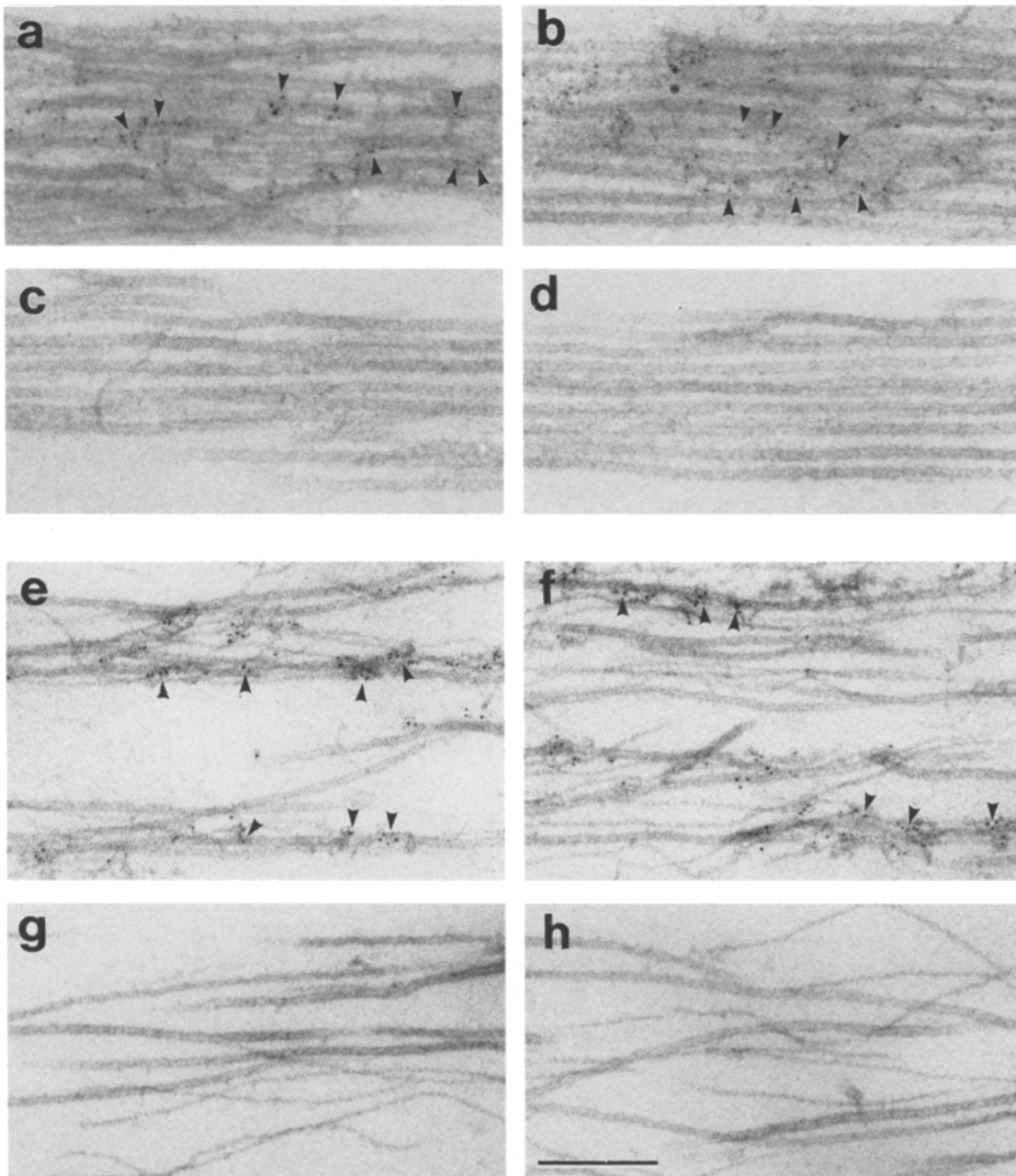
First, to determine whether microtubules traveling down the axons are present or not, we permeabilized, fixed, and stained the cells after an appropriate interval after photoactivation. By permeabilization in the presence of taxol, a microtubule-stabilizing agent, we could clearly identify individual microtubules in the axon. Fig. 2 shows two axons which were processed 1 h (Fig. 2, *a–d*) and 10 min (Fig. 2, *e–h*) after the photoactivation. Although ultrathin serial sectioning allowed us to observe microtubules with many labels in the photoactivated small regions (Fig. 2, *a, b, e, and f*), we could find no such microtubules in the proximal regions (Fig. 2, *d and h*) or in the distal regions (Fig. 2, *c and g*). This showed that no microtubule polymer moved from the activated regions. Since the axon shown in Fig. 2, *a–d* extended more than 50  $\mu\text{m}$  during the incubation after photoactivation (observation by DIC microscopy) and that shown in Fig. 2, *e–h* was also extending at a rate of 60  $\mu\text{m}/\text{h}$  (observation by DIC microscopy for 30 min just before the photoactivation), if the transported tubulin molecules were polymers, we would have been able to detect them. This result indicated that tubulin molecules are transported not as polymers but as oligomers or heterodimers.

### Immunoelectron Microscopic Observation of Cytoplasmic Soluble Protein

Next, as we wanted to preserve unassembled tubulin molecules for observation of actively transported tubulin molecules directly, we first fixed the cells with 2% paraformaldehyde and 0.1% glutaraldehyde in PEM and then permeabilized them with 1% Triton X-100 in PBS. To confirm that soluble protein molecules in axons were preserved during the procedure adopted, we first injected fluorescein-labeled BSA into the neurons and observed. Fig. 3 *a* shows a living axon observed using a fluorescence microscope before fixation. Fig. 3 *b* shows the same axon after fixation and permeabilization. The axon was brightly fluorescent after permeabilization after fixation. This showed that a large part of the fluorescein-labeled BSA was preserved. In addition, we stained the cells with affinity-purified anti-fluorescein antibody and 1.4-nm gold-conjugated second antibody for electron microscopic observation. Fig. 3 *c* shows an axon of a neuron injected with fluorescein-labeled BSA. In Fig. 3 *d* we show an example of an axon of an uninjected neuron as a control. The presence of many labels in Fig. 3 *c* clearly showed that the immunoelectron microscopic procedure employed here really preserved soluble cytoplasmic molecules in axons.

### Actively Transported Tubulin Molecules in Axons Are Visualized by Electron Microscopy

Next, since we wanted to observe the actively transported tubulin molecules directly, we fixed, permeabilized, and stained the cells which were injected with caged fluorescein-labeled tubulin. This process allowed us to observe not only microtubules but also tubulin oligomers and heterodimers. Fig. 4 shows electron micrographs of three axons



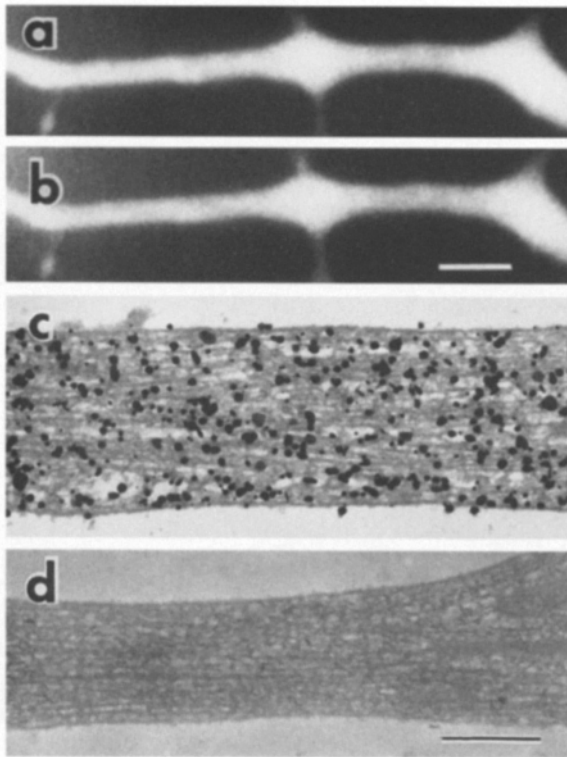
**Figure 2.** Behavior of photoactivated microtubules in extending axons. After incubation following photoactivation of the small regions of the axons, cells were permeabilized, fixed, and stained by anti-fluorescein antibody. (*a-d*) An axon processed 1 h after photoactivation; (*e-h*) another axon processed 10 min after photoactivation; (*a, b, e, and f*) photoactivated region. Microtubules decorated with gold particles (*arrowheads*) were observed. *c, d, g, and h* show examples of the regions out of the photoactivated regions. (*c and g*) Regions  $\sim 4 \mu\text{m}$  and  $8 \mu\text{m}$  distal to the photoactivated region, respectively. (*d and h*) Regions  $\sim 4 \mu\text{m}$  proximal to the photoactivated regions. Out of the photoactivated regions, no microtubules with labels were observed. Bar, 200 nm.

which were processed for 1 min (Fig. 4, *a-f*) and 20 min (Fig. 4, *g-i*) after photoactivation. In all cases, we could find many labels which indicated the presence of carboxy-fluorescein-labeled tubulin molecules out of the photoactivated regions.

To evaluate our procedure and to check whether these labels out of the photoactivated regions really represented the moving tubulin molecules in the axons or not, we illuminated small axonal regions of previously fixed cells

with UV, stained, and observed. In these axons, we could scarcely find labels out of the photoactivated regions (Fig. 4 *j*).

In addition, we analyzed the distribution of the labels out of the photoactivated regions. We counted the number of gold labels in the symmetrical regions proximal and distal to the activated segments in serial sections. Labels were more abundant in the distal than in the proximal regions (Table I). We performed binomial tests to reject the null



**Figure 3.** Axons of neurons microinjected with fluorescein-labeled BSA. (a) An axon of a living neuron injected with fluorescein-labeled BSA. (b) The same axon after permeabilization after fixation. (c) Electron microscopic photograph of another axon stained with anti-fluorescein antibody after permeabilization after fixation. Many labels were observed. (d) An electron micrograph of an axon of another neuron which was not microinjected. This cell was processed just as the cell in c. Labels were scarcely observed. Bars: (b) 5  $\mu\text{m}$ ; (d) 500 nm.

hypothesis that labels appeared in equal possibility on both regions.

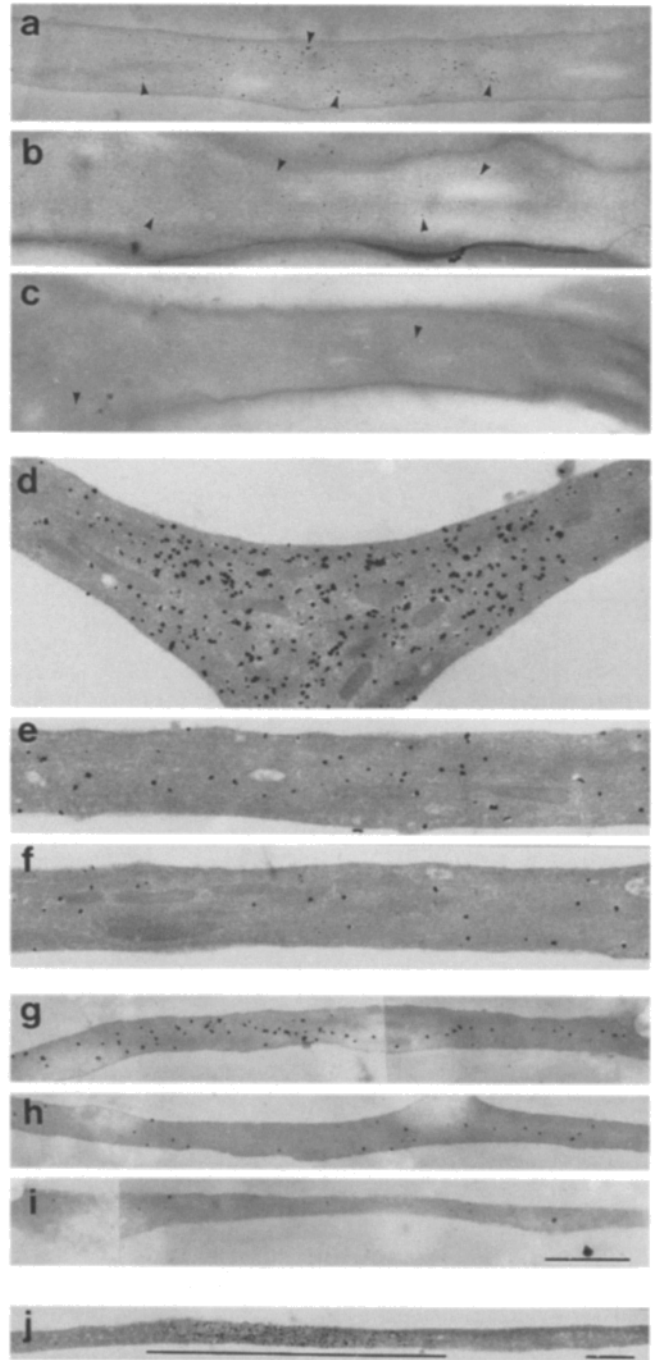
This suggests that we observed not only simple diffusion but also active anterograde transport of tubulin heterodimers or oligomers.

## Discussion

### *A New Method for the Detection of Molecular Translocation In Vivo Using Electron Microscopic Analysis*

To observe the molecular transport of various molecules in the cell, making marks on the molecules in some restricted regions is necessary. Many photobleaching and photoactivation studies have been performed to make marks on tubulin molecules to analyze the mechanism of its transport in axons. However, although these approaches are powerful, they couldn't detect the moving population of

**Figure 4.** Electron micrographs of transported tubulin molecules in the axon. (a–i) After appropriate incubation after photoactivation of small segments of the axons, cells were fixed without the pre-



ceding permeabilization and stained by anti-fluorescein antibody. Many labels were observed out of the photoactivated regions. (a–c and d–f) Two axons processed 1 min after photoactivation. (a) Photoactivated region. Many labels were observed. (b and c) Regions  $\sim 20 \mu\text{m}$  distal (b) and proximal (c) to the photoactivated segment, respectively. (d) Photoactivated region. (e and f) Regions immediately distal (e) and proximal (f) to the photoactivated region. (g–i) Another axon processed 20 min after photoactivation. (g) Photoactivated region. (h and i) Regions 10  $\mu\text{m}$  distal (h) and proximal (i) to the photoactivated region. In a–c some of the labels are indicated by arrowheads. (j) An axon photoactivated after fixation. The photoactivated region is underlined. In a–i we found many labels out of the photoactivated regions, while the labeling density is higher in the distal regions than in the proximal regions. Bar, 1  $\mu\text{m}$ .

**Table I. The Distribution of Labels Out of the Photoactivated Regions**

	Counted region			Proximal	Distal	P
	Counted	activated	counted			
Cell 1	-4μ-	-4μ-		29	66	<.01
	Counted	activated	counted			
	-5μ-	-5μ-	-5μ-			
Cell 2	-3μ-	-3μ-		220	327	<.01
	Counted	activated	counted			
	-10μ-	-5μ-	-10μ-			
Cell 3	-8μ-	-8μ-		95	173	<.01
	Counted	activated	counted			
	-17μ-	-5μ-	-17μ-			

We counted the number of gold labels in the regions proximal or distal to the photoactivated regions on serial sections in three independent cells fixed 1 min after photoactivation. Labels were more abundant in the distal than in the proximal regions (Binomial test).

tubulin molecules because of the insufficiency of signal to noise ratio (Lim et al., 1990; Okabe and Hirokawa, 1990, 1992; Sabry et al., 1995; Takeda et al., 1995). It was impossible to detect the moving molecules if they were <10% of the total tubulin molecules in axons (Sabry et al., 1995). In addition, low resolution of the observation using optical microscopy didn't allow us to observe individual microtubules because they are densely packed in axons.

To clear up these problems, we developed a new procedure for electron microscopic analysis. In previous photoactivation and photobleaching studies, the marks made on axonal tubulin molecules were observed by optical microscope as fluorescent or nonfluorescent regions, respectively. In this study, we combined the photoactivation technique and immunoelectron microscopy to obtain a high signal to noise ratio and high resolution simultaneously. We stained the photoactivated sample with anti-fluorescein antibody newly raised for this study and gold-labeled second antibody to detect the fluorescein molecules generated in the photoactivated region electron microscopically. This procedure allowed us to observe a single microtubule and a small population of transported tubulin molecules in axons. This method would be useful in many other fields of cell biology to observe the behavior of photoactivated molecules at the electron microscopic level.

### **Tubulin Molecules Transported Down the Axon Are Not Polymers but Oligomers or Heterodimers**

In previous photobleaching and photoactivation studies, the nonfluorescent or fluorescent marks made on axonal tubulin molecules were stationary. This only means that transported tubulin molecules were a small fraction of the total tubulin molecules in axons and whether the undetected transported tubulin molecules are polymers or not was left undetermined.

When we observed the extending axons fixed after permeabilization using immunoelectron microscopy, we found no labeled microtubules out of the photoactivated regions (Fig. 2). This indicated that there are no moving microtubules in axons. Because there should be transported tubu-

lin molecules in extending axons, the transported tubulin molecules must be oligomers or heterodimers.

On the other hand, when we observed the axons fixed without permeabilization, we could find many labels out of the photoactivated regions. First, the data from neurons injected with fluorescent BSA indicated that our procedure really preserved soluble proteins such as tubulin heterodimers or small oligomers. Furthermore, to check whether these labels really represented the moving tubulin molecules in the axons or not, we illuminated small axonal regions of previously fixed cells with UV, stained, and observed. In these axons, we could scarcely find labels out of the photoactivated regions (Fig. 4*j*). This shows that those labels observed out of the photoactivated regions (Fig. 4, *b, c, e, f, h, and i*) were not a result of the leakage of the UV which generated carboxyfluorescein molecules out of the photoactivated regions, nor simple background of antibody staining. Thus, the labels out of the photoactivated regions indicated the movement of the fluorescein molecules from the photoactivated regions. Since substantially all the fluorochrome introduced into the cells were covalently bound to tubulin molecules (see Fig. 1), labels out of the photoactivated regions must be derived from tubulin molecules conjugated with carboxyfluorescein which were generated in the photoactivated regions. They were concluded to represent the moving tubulin molecules in the axons. This was also supported by the fact that labels were associated to microtubules in the cells permeabilized before fixation (Fig. 2, *a, b, e, and f*). These labels indicated the presence of the moving tubulin molecules from the photoactivated regions. Labels were more abundant in the distal than in the proximal regions (Table I). If the labels out of the photoactivated regions had been the result of simple diffusion of tubulin molecules, we would have observed as many labels in the proximal as in the distal regions. Thus, we observed not only simple diffusion but also active transport of tubulin heterodimers or oligomers. At least some part of labels in the regions distal to the photoactivated regions represented actively anterogradely transported tubulin molecules. On the other hand, whether labels found in the proximal regions indicated simple diffusion or retrograde transport of tubulin molecules remains to be determined (Glass and Griffin, 1994).

To find clear peaks in the distribution of the labels out of the photoactivated regions was difficult partially because of the branching of the axons of DRG neurons. But we think this difficulty suggests that the transported molecules are in dynamic equilibrium with some stationary population of tubulin in axons (dynamic microtubules, for example) (Stromka and Ochs, 1981). Nevertheless, since we found many labels in the regions 25–30 μm distal to the photoactivated regions of those cells which were fixed only 1 min after the photoactivation, the lower limit of the rate of tubulin transport was estimated to be 0.4 μm/s (i.e., 35-mm/d). Although this estimate of the rate is based upon only one time point and is preliminary, the speed is much greater than the value obtained from radiolabeling studies (0.2–2 mm/d) (Black and Lasek, 1980; Lasek et al., 1984; Tashiro and Komiya, 1989), but we think the low speed from radiolabeling studies may be an average speed resulted from long term observations. In other words, if transported tubulin molecules are in dynamic equilibrium with

preexisting stationary polymers, long term observations of radiolabeling studies may lead to the underestimation of the velocity of tubulin molecules in the real moving phase. This idea was also suggested by previous studies (Okabe and Hirokawa, 1988, 1990). In addition, we think that the high speed of transport of tubulin heterodimers or oligomers suggests the possibility that some kinds of KIFs (kinesin superfamily proteins) (Hirokawa, 1996) play crucial roles in the transport.

Now we have clearly shown that transported tubulin molecules in axons are not polymers but oligomers or heterodimers. But how they are transported? What is the motor? These problems remain to be solved.

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