# Differential Dynamics of Neurofilament-H Protein and Neurofilament-L Protein in Neurons

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*Abstract.* Neurofilaments (NFs) are composed of triplet proteins, NF-H, NF-M, and NF-L. To understand the dynamics of NFs in vivo, we studied the dynamics of NF-H and compared them to those of NF-L, using the combination of microinjection technique and fluorescence recovery after photobleaching. In the case of NF-L protein, the bleached zone gradually restored its fluorescence intensity with a recovery half time of $\sim$35 min. On the other hand, recovery of the bleached zone of NF-H was considerably faster, taking place in $\sim$19 min. However, in both cases the bleached zone was stationary. Thus, it was suggested that NF-H is the dynamic component of the NF array and is interchangeable, but that it assembles with the other neurofilament triplet proteins in a more exchangeable way, implying that the location of NF-H is in the periphery of the core NF array mainly composed of NF-L subunits. Immunoelectron microscopy investigations of the incorporation sites of NF-H labeled with biotin compounds also revealed the lateral insertion of NF-H subunits into the preexisting NF array, taking after the pattern seen in the case of NF-L. In summary, our results demonstrate that the dynamics of the L and H subunit proteins in situ are quite different from each other, suggesting different and separated mechanisms or structural specialization underlying the behavior of the two proteins.

MONG intermediate filament proteins, the neurofilament (NF)[1] triplet proteins are quite unique in that they assume a characteristic alignment that is composed of the three subunit proteins usually referred to as NF-L, NF-M, and NF-H (Hoffman and Lasek, 1975; Liem et al., 1978; Hirokawa, 1991). In other words, NFs take on a heteropolymeric form in cells (Delacourte et al., 1980; Geisler and Weber, 1981; Ching and Liem, 1993; Lee et al., 1993). These proteins belong to the class IV intermediate filaments together with $\alpha$-internexin (Fliegner et al., 1990). Although other intermediate filaments such as vimentin and peripherin are also expressed in neuronal cells during the postmitotic stage, the neurofilament triplet proteins are major intermediate filament proteins specifically and abundantly expressed in mature neurons. Each subunit protein is different in its molecular structure and molecular weight (Hoffman and Lasek, 1975; Liem et al., 1978), which is mainly due to a long-extended COOH-terminal tail region of NF-M and NF-H. All neurofilament triplet proteins comprise three domain structures, that is to say, an $NH_2$-terminal head domain, a

coiled-coil rod domain of $\sim$310 amino acid residues (Steinert and Roop, 1988), and a COOH-terminal tail domain. The amino acid composition of the coiled-coil rod domain is highly conserved between the class I to IV intermediate filaments. Several lines of evidence support the idea that this portion plays an important role in constructing the 10-nm filament structure (Van den Heuvel et al., 1987; Albers and Fuchs, 1989; Coulombe et al., 1990; Gill et al., 1990; Lu and Lane, 1990), and mutation in the coiled-coil region results in severe disorganization of the filament assembly (Cheng et al., 1992; Chipev et al., 1992; Rothnagel et al., 1992). On the other hand, both flanking terminal domains vary with each component of the intermediate filaments including the neurofilament triplet proteins, and the variability in these regions may determine the specific functions of each member. The length of the amino acid chain of the carboxy-terminal tail domain differs greatly among the members of intermediate filaments. NF-H has an exceptionally long COOH-terminal composed of 607 amino acids.

Morphologically, the COOH-terminal of the H subunit is suggested to participate in the formation of crossbridges between NFs (Hirokawa, 1982; Hirokawa et al. 1984). The NF-H COOH-terminal includes consensus tripeptide sequences (Lys-Ser-Pro: KSP) (Geisler et al., 1987), which are the preferential sites for phosphorylation by second messenger-independent protein kinase (Leterrier et al., 1981; Tanaka et al., 1984; Hisanaga et al., 1991; Miyasaka et al., 1993), and in turn, the degree of phosphorylation is

1. *Abbreviations used in this paper:* DRG, dorsal root ganglion; FRAP, fluorescence recovery after photobleaching; MBB, maleimidobutyryl biocytin; SH, sulfhydryl.

believed to have a great effect on regulation of the interaction of NFs with other cytoskeletal proteins and conformation of NF-H (Hisanaga and Hirokawa, 1989; Shea et al., 1990). For example, phosphorylation of NF-H at the COOH-terminal has been implicated in the regulation of the interaction of NFs with MTs (Miyasaka et al., 1991). In addition, protein kinase only localized in neurons has been found, which catalyzes phosphorylation of the NF-H COOH-terminal (Wible et al., 1989).

Interestingly, NF-H is known as a protein which is expressed latest through the course of neuronal development (Shaw and Weber, 1982; Willard and Simon, 1983; Pachter and Liem, 1984; Szaro and Gainer, 1988; Szaro et al., 1989; Charnas et al., 1992). This developmentally regulated manner of expression also brings us to the idea that NF-H has special functions for establishing the more sophisticated architecture of the mature neuron, or that NF-H is under separate developmental control and itself behaves in a different manner from that seen in the case of NF-L dynamics. Although the existence of neurofilaments has been known reproducibly for over a century by Ramón y Cajal (1899) and Bielshowsky (1908) by using silver impregnation method as neurofibrils, and much has since been clarified about the biochemical nature regarding the amino acid sequence and phosphorylation of particular epitopes, their dynamics in vivo and their exact cellular functions are still open questions.

In the previous study (Okabe et al., 1993), we demonstrated that a major component of the neuronal intermediate filament triplet protein, NF-L, which is strongly suggested to construct the backbone of the neurofilament arrays in axons (Willard and Simon, 1981; Hirokawa et al., 1984), turns over within a small region of the axoplasm by the mechanism of lateral and segmental incorporation of new subunits, and the form of NFs travelling down the axon is not polymer but oligomer or monomer. Coincidentally, we estimated the recovery half time of the photobleached area to elucidate the dynamic properties of NFs in the axon, and it was determined to be ~40 min. From the aforementioned many lines of evidence concerning the biochemical and developmental aspects, it is strongly suggested that NF-H behaves in a different manner from that of NF-L. However, direct evidence that NF-H behaves in a specific manner, or that it may be transported by a different mechanism from NF-L, has not been reported yet. Also unanswered is whether NF-H subunits turn over between the soluble form and preexisting filaments once the triplet is established. In the present study, the combination of microinjection and fluorescence recovery after photobleaching procedures was applied for evaluating the dynamic behavior of NF-H, and the obtained results were compared with that of NF-L to unveil a part of the mechanism by which the specialized behavior of NF-H in the neuron is regulated and the architecture of the neuronal intermediate filament is maintained.

## Materials and Methods

### Preparation of the Neurofilaments

Neurofilaments were purified according to the methods described elsewhere (Geisler and Weber, 1981; Hisanaga and Hirokawa, 1988). Briefly, bovine spinal cords purchased from a local slaughterhouse were immediately immersed in crushed ice and within an hour meninges with blood vessels were removed with a pincette. They were then homogenized in an equal volume of PEM buffer (100 mM Pipes, 1 mM EGTA, and 1 mM MgCl₂ pH 6.6) supplemented with 1 μg/ml leupeptine, 1 mM PMSF, and 1 mM DTT. Next they were cooled down for half an hour and centrifuged at 14,000 rpm in a Beckman JA-14 fixed angle rotor (Beckman Instrs., Inc., Fullerton, CA) for 30 min. Supernatants were collected and glycerol was added to 20% of the final volume followed by centrifugation at 25,000 rpm (Beckman 45Ti) for 1 h. One cycle of polymerization and depolymerization was carried out to remove tubulins or other contaminating proteins. The crude extract obtained was applied to an anion exchange chromatographic column (DE-52; Whatmann, Maidstone, England) preequilibrated with 6 M urea buffer (6 M urea, 1 mM EDTA, 1 mM EGTA, 100 mM phosphate buffer, pH 7.5, 1 mM DTT, 0.5 mM PMSF, and 1 μg/ml leupeptine). Fractions were eluted with the same buffer of preequilibration with a linear gradient of sodium chloride ranging from 0 to 250 mM and were collected with a fraction collector. The obtained fractions were checked by SDS-gel electrophoresis to determine which fractions contained the protein of our objective. After confirming the fractions, eluents containing neurofilament protein were collected and concentrated by ultrafiltration to ~2 mg/ml. The concentrated NF solution was dialyzed against an assembly buffer P₂₀EM (20 mM Pipes, 1 mM EGTA, 1 mM MgCl₂, 1 mM EDTA, 1 mM DTT, and 0.17 M NaCl, pH 6.6) overnight to remove urea and then was used for labeling with fluorescent dyes or biotinylated probes. The part of the solution not used immediately was frozen under liquid nitrogen in the form of small aliquots and stored at −80°C.

### Labeling of the Neurofilaments with Fluorescent or Biotinylated Probes

For the purpose of labeling with fluorescent agents or biotin compounds, neurofilament protein stored in the form of frozen small aliquots, which took on a polymerized form, was used after dissolving in 6 M urea buffer to alter the state of the protein into monomers. Because labeling of the NF protein with sulfhydryl-specific maleimide was successfully achieved previously (Angelides et al., 1989; Okabe et al., 1993), we used 5'-iodo-acetoamide fluorescein (Molecular Probes Inc., Eugene, OR) and maleimidobutyryl biocytin (MBB) (Molecular Probes, Inc.) dissolved in DMSO to concentrations of 50 and 25 mg/ml, respectively. For NF-L, sequence analysis has shown only one cysteine residue at position 321 (Lewis and Cowan, 1985), meaning that the reaction between protein sulfhydryl (SH) and the agents would be specific and stoichiometric (Okabe et al., 1993), and therefore labeling of NF-L is achieved at the stoichiometry of 1:1. On the other hand, NF-H has four cysteine residues within its primary structure (Julien et al., 1988; Chin and Liem, 1990). In the case of NF-H, to the best of our knowledge, there have been no reports about labeling with sulfhydryl-specific maleimide, and we were able to carry out labeling with these agents successfully.

The reactions were carried out for 2 h at room temperature or 6 h at 4°C, and were stopped by adding DTT to a final concentration of 1 mM. The reactants were applied to a desalting column (gel filtration column) PD-10 (Pharmacia LKB, Uppsala, Sweden) and eluted with 1 ml of 6 M urea buffer three times. The obtained solution containing labeled protein was determined by the assay of Bradford (1976), and fractions containing more than 2 mg of the protein were dialyzed against the assembly buffer to eliminate excess fluorophore and urea as mentioned before. The part of the solution not used immediately was frozen under liquid nitrogen in the form of small aliquots and stored at −80°C.

For usage, the assembled protein was centrifuged, solubilized in 6 M urea buffer to attain a disassembled monomeric state, and dialyzed against 10,000 vol of injection buffer (5 mM Hepes, 1 mM DTT, 1 mM EDTA, and 1 mM EGTA, pH 8.5) for more than 6 h. This NF protein was then used for microinjection.

### Cell Culture

Dorsal root ganglion (DRG) sensory neurons were used for cell culture. They were cultured according to the method previously described (Okabe and Hirokawa, 1990). Briefly, young adult mice (C57 BL) ranging from 8 to 12 weeks old were killed to obtain neurons for culture. The dissected DRGs were dissociated by immersing them in 0.25% collagenase (Wako, Co., Osaka, Japan) followed by 0.25% trypsin (Difco, Detroit, MI) solution, then plated onto a coverslip which was attached to the bottom of a 35-mm dish in which a 12-mm hole had been drilled, and grown in MEM supplemented with horse serum (GIBCO BRL, Gaithersburg, MD) and fetal calf serum (Mitsubishi Kasei Co., Tokyo, Japan). Nerve growth factor

(Takara, Kyoto, Japan) was added to a concentration of ~50 ng/mL. After that, they were incubated at 37°C for 12 h for the photobleaching experiment and 48 h for immunoelectron microscopic examination.

Bovine fibroblast cell line BSC1 maintained in our laboratory was used for the study. The cells stored in liquid nitrogen were melted quickly in a warm bath and resuspended in DMEM containing 10% fetal calf serum (GIBCO BRL, Gaithersburg, MD) after removal of the cryoprotectant, DMSO. The cells were allowed to grow and were used for the experiment after 8 h.

## Microinjection Technique and Fluorescence Recovery After Photobleaching

These procedures were carried out as reported previously (Okabe and Hirokawa, 1990; Vikstrom et al., 1992; Okabe et al., 1993). A detailed description of the optical alignment for the fluorescence recovery after photobleaching (FRAP) method can be obtained from several reports (Okabe and Hirokawa, 1990; Tanaka and Kirschner, 1991; Okabe et al., 1993). Microinjection of the labeled NF subunit for the photobleaching experimental run was performed with DRG neurons 12 h after plating onto D-poly-lysine-coated coverslips when the neurons had no neurites. It was recommended that the injection of the tagged proteins is most appropriate when the cells are without neurites or with only minor sprouting, as incorporation of the injected proteins into the cytoskeletal arrays of neurites will be achieved most efficiently (Okabe et al., 1993). After allowing the cells to grow freely in an incubator for a further 12–24 h, photobleaching as described previously was carried out. By doing so, the influence of the pressure-directed incorporation of neurofilaments on the measurement of the cytoskeletal dynamics can be mitigated. The results after the photobleaching procedure were recorded with Argus-100 (Hamamatsu Photonics, Co., Hamamatsu, Japan) under low-light level microscopy at various times to assess the recovery of fluorescence at the bleached zone. The obtained data were analyzed by Argus-100 to quantify the rate of fluorescence recovery and to estimate the value of the recovery half time.

## Detergent Extraction of DRG Cell

Detergent extraction was carried out by a procedure similar to that described by Okabe et al. (1993). However, we adopted more stringent conditions, where 0.5% Triton X-100 in PHEM (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl$_2$, pH 6.6) buffer was used for 5 min.

## Immunoelectron Microscopy and Immunofluorescence

Microinjection of NF-H labeled with maleimidobutyryl biocytin was performed to visualize the incorporation of the injected proteins into the intrinsic neurofilament network of a neuron. In this case, in contrast to the photobleaching fluorescence recovery experiment, plated DRG cells were allowed to grow for ~48 h, because it is hardly possible to evaluate the dynamic turnover of the NF-H within the preexisting axonal neurofilament arrays unless mature neurites have developed. 1–24 h after microinjection the cells were processed for immunoelectron microscopical investigation. Prior to fixation, the dishes were washed with warm PBS or PHEM buffer, and permeabilized with 0.3% Triton X-100 supplemented with 1 mM GTP, 10 μM Taxol, and 10% glycerol dissolved in PHEM buffer. They were fixed with 0.1% glutaraldehyde and 2% paraformaldehyde buffered by PBS for half an hour. Then the cells were treated with 5% skimmed milk solution to block nonspecific reaction with antibodies. The first antibody, rabbit anti-biotin antibody (Enzo Biochem. Inc., New York), was incubated with cells at room temperature for 3 h, followed by overnight incubation with the second antibody, anti-rabbit antibody IgG fraction conjugated with 10-nm colloidal gold particles (Amersham International Plc., Buckingham-shire, UK). After washing the culture dishes, rhodamine-conjugated third antibody was used to visualize the cells which had been injected with MBB-labeled NF proteins. After identifying the cells incorporating the NF-H protein labeled with MBB, they were postfixed with 1% glutaraldehyde containing 0.1% tannic acid in 100 mM phosphate buffer. Then they were immersed in 1% osmium tetraoxide in PBS for 10 min, followed by extensive washing with distilled water. After that they were stained with 1% uranyl acetate, dehydrated with a series of graded concentrations of ethanol, and embedded in Epon (Polyscience Inc., Warrington, PA). The Epon blocks were processed for ultrathin sections, and these sections were observed under an electron microscope (1200 Ex; JEOL) after staining with 1% uranyl acetate and 1% lead citrate.

Immunofluorescence studies of the plated cells for the detection of intrin-

sic NF-H expression at the time corresponding to both microinjection (~12 h after plating) and the photobleaching run (~24 h after plating) were also done. A detailed description of the immunostaining procedure can be found in the previous report (Okabe et al., 1993). Anti-neurofilament H (Biomakor, Rehovot, Israel) was used at a dilution of 1:100 and was incubated with the cells for 1 h. After washing the dishes with PBS, the second antibody was reacted for 30 min and the cells were then observed under a Zeiss Axiophot microscope.

For immunostaining of vimentin filament in BSC1 injected with fluorescein-labeled NF-L and NF-H, we followed the procedures of Okabe et al. (1993).

## Negative Staining and Immunogold Labeling of Reconstructed NFs

Neurofilament proteins labeled with the aforementioned two tagging agents were assembled in assembly buffer as described above the examine their ability to polymerize after chemical modification. NF-L, NF-H, and their mixture at molar ratios of 2:1 and 4:1 were examined by negative staining according to Tokutake et al. (1984). Immunogold labeling of the NFs was carried out essentially according to Balin et al. (1991), but with several minor modifications. NFs reassembled from native NF-L and chemically modified NF-H at the molar ratio of 4:1 were fixed with 2% paraformaldehyde supplemented with 0.1% glutaraldehyde for 30 min, and after being dispersed on carbon, formvar-coated grids, they were washed with droplets of PBS for several times. To prevent nonspecific reaction of the antibody, the whole grid was blocked with 5% skimmed milk before incubation with the first antibody. In the case of single labeling of NF-H, the first antibody was mouse anti-NF-H (Biomakor) at a dilution of 1:100, and for double-labeling rabbit anti-NF-L (Chemicon International Inc., Temecula, CA) at a dilution of 1:40 was also added. After incubating with the first antibody for 2 h, the grids were washed with TBS. Following thorough washing, the grids were incubated with secondary antibodies diluted to 1:20 in TBS for 1 h. The secondary antibodies were either anti-rabbit or anti-mouse antibody conjugated with 10 and 5 nm gold, respectively. After incubation, the grids were washed and postfixed with fixative (2% paraformaldehyde and 0.1% glutaraldehyde), washed again, and then stained with 4% methanolic uranyl acetate. For control for each immunogold labeling, the following procedures were also performed: (a) incubation of NFs without a primary antibody, (b) incubation of NFs with antibodies specific for vimentin intermediate filament (Amersham International Plc.).

## Results

### Characterization of the Neurofilament Used for Microinjection

To assess the state of the labeling by 5′-iodoacetoamide fluorescein, we carried out electrophoresis of the labeled protein both before and after passing through the gel filtration column. Before the procedure, the lane of SDS–gel electrophoresis under ultraviolet illumination revealed unbound-dye at almost the same level as the CBB dye-front (data not shown). The labeled protein could also be recognized at a level of mobility corresponding to those of NF-L and NF-H. Then, after passing through the gel filtration column, the unconjugated dye was first completely eliminated from the preparative sample to be used for microinjection (Fig. 1, lanes 1 and 4). This had to be done for two reasons, both of which would likely affect the monitoring accuracy of the dynamics of the cytoskeletal proteins. First, unbound dye generates more free radicals than the state where no unbound dye exists. Free radicals are noxious to cells under investigation (Vigers et al., 1988). Second, injected unbound dye probably hastens the apparent recovery time of fluorescence, as it is assumed to behave like a molecule that can easily move into the photobleached region by simple diffusion. On observation of the dye-stained gel, there was no upward or downward shift of the bands in relative mobility, implying
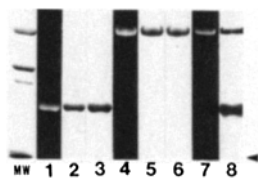
*Figure 1.* Characterization of fluorescein- or biotin-labeled neurofilament polypeptides L and H. SDS–polyacrylamide gel analysis of the preparation of fluorescein- and biotin-labeled NFs. Fluorescein-labeled NF-L and H are visualized by UV illuminator (lanes *1* and *4*) or by dye (CBB) staining (lanes *2* and *5*). Biotin-labeled NFs are stained with CBB (lanes *3* and *6*). In both cases, only a single band is detectable. Mixture of native NF-L and fluorescently labeled NF-H at the molar ratio NF-L/NF-H = 4 is visualized by UV illuminator (lane *7*) and by CBB staining (lane *8*). Note that only NF-H is visible in lane *7,* and the NF-L band in lane *8* is much thicker than that of NF-H. Arrowhead indicates dyefront, where no single band can be observed, indicating complete elimination of the unbound dye. M$_r$: molecular weight standards, from top to bottom, 200, 116, 92, and 31 kD.



*Figure 2.* Electron microscopical observation of the negatively stained neurofilaments. (*A*) Fluorescein- or (*B*) biotin-labeled NF-L formed smooth-walled filaments with a diameter of 10 nm. However, NF-H per se did not form a smooth 10-nm filament but a stubby thread-like structure (*C,* fluorescein-labeled NF-H; *D,* biotin-labeled NF-H). Bar, 100 nm.

that the overall profile of NFs after chemical modification is very similar to unlabeled proteins. Furthermore, no other contaminating or degrading proteins could be seen as exclusive bands on the gel.

It is essential to evaluate the ability of the neurofilament protein to polymerize while it is labeled with various kinds of agents which react with proteins at SH-group sites, before utilizing them as a tool for investigating the dynamics of the proteins. Although cysteine residues in the rod domain of the neurofilament triplet protein have been suggested to be involved in the formation of cross-linking between filaments, cleavage of these bonds by treatment with mercaptoethanol did not destroy or bring them into a fragile state (Carden and Eagles, 1983). However, cysteine residues where fluorescein or biotin compounds chemically bind are considered to be important for modulating assembly properties of NF-H, especially those located at the conserved region of the head (Chin et al., 1990; Gill et al., 1990; Wong and Cleveland, 1990) and rod domains. Taking both these factors into consideration, evaluation of the assembly competence of the chemically modified NF subunits at their cysteine residues is indispensable, because our experiment is based mainly on the assumption that these chemically modified NF subunits behave the same as those in native form. We therefore performed the following two experimental runs to address this issue: in vivo and in vitro reassembly studies together with immunogold labeling.

In vitro reassembly of NFs was performed by dialyzing the monomer NF solution dissolved in urea buffer against assembly buffer at 37°C for 12 h. After that, the solution containing NFs was dispersed onto grids and incubated in 4% uranyl acetate solution for 2 min to negatively stain the filaments. In this portion of the study, three pats of the NF solution with different proportions of subunit content were prepared. The first sample contained only the NF-L subunit at a concentration of ∼100 μg/ml, which was labeled with maleimidobutyryl biocytin or 5′-iodoacetoamide fluorescein. They formed bona fide smooth-surfaced long filaments (Fig. 2, *A* and *B*) with a diameter of ∼10 nm (typical 10-nm filament; Balin et al., 1991), and were indistinguishable from unlabeled NF-L proteins, suggesting that the ability of the NF-L subunit to assemble after labeling with tagging agents was not disturbed.

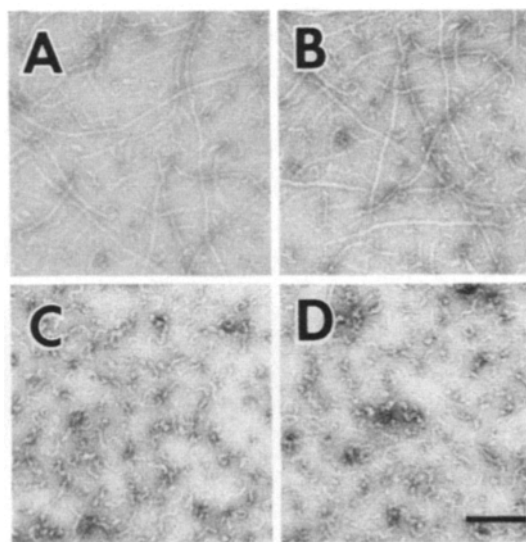Examining the ability of the NF-H protein to assemble it-

self into the filamentous structure is not as easy as in the case of NF-L, in the point that it can not form smooth-surfaced 10-nm filaments without existence of NF-L. Although NF-H proper can form so-called component-specific filaments as reported by Tokutake et al. (1984), it is necessary to incubate it with native NF-L and to evaluate the appearance of filaments reconstituted from both proteins. So, in the second part of the experiment, we used the solution containing both NF-L and NF-H at a molar ratio of 4:1. In this case, the NF-L protein was not labeled with modifying agents such as 5′-iodoacetoamide fluorescein or maleimidobutyryl biocytin, but was used in its native form. Therefore, by observing the degree of the apparently fine filamentous structures that these proteins could form, it could be determined whether the injected NF-H that was labeled with either fluorophore or biotin compound maintained its capability to coassemble with endogenous NFs. As shown in Fig. 3, *A* and *B*, both fluorophore- and biotin-labeled NF-H protein can form 10-nm filament structures without the short thread-like structures composed solely of NF-H which are not able to associate with NFs.

Although the above observations seem to be enough to prove the assembly competence of conjugated NF-H, we also carried out immunogold labeling of the reconstituted filaments to provide additional evidence that chemically modified NF-H can behave in a similar manner to the native molecule. In the case of fluorescently labeled NF-H coincubated with native NF-L, the filaments were decorated with both 10- and 5-nm gold on their smooth surface (Fig. 3 *C*). Similar image was also obtained in the case of biotin labeled NF-H coincubated with native NF-L (Fig. 3 *D*). Distribution of the 5-nm gold particles, which depicts localization of chemically modified NF-H, did not show regular intervals on the filamentous structure, being compatible with the results of the experiment by using the quick freeze deep etch
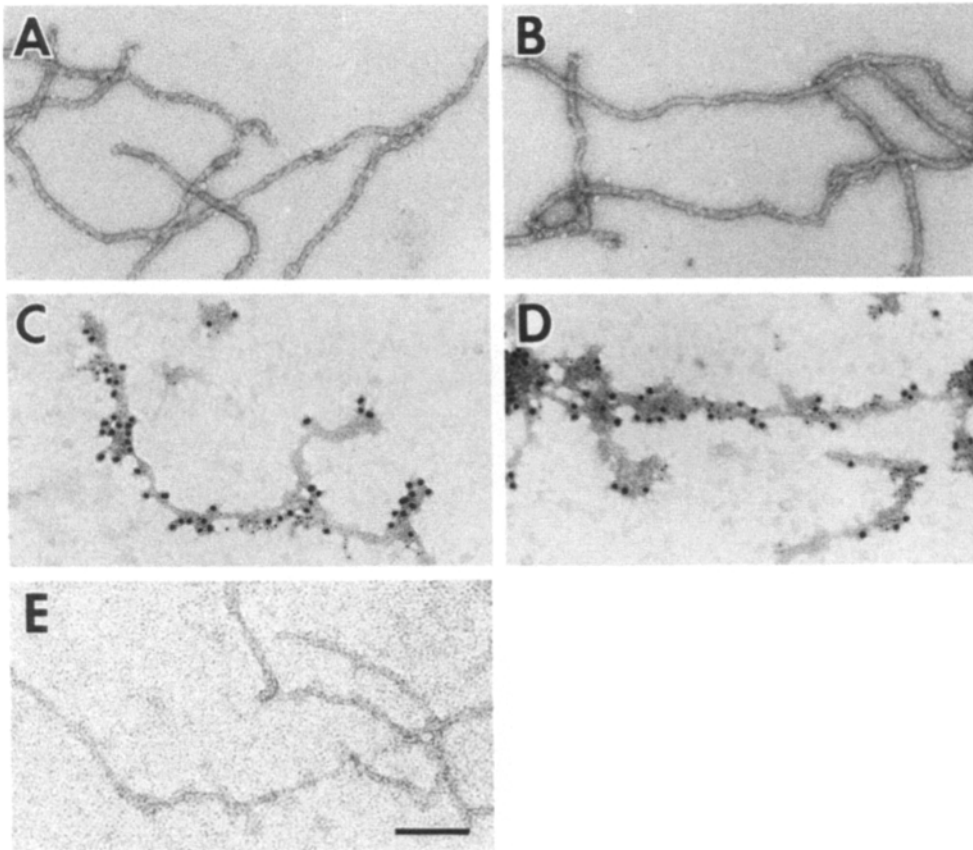
*Figure 3.* Electron microscopical observation of the negatively stained and immunogold labeled NFs. NFs reconstructed from native NF-L and (*A*) fluorescein- or (*B*) biotin-labeled NF-H at a molar ration of 4:1 was negatively stained with 4% methanolic uranyl acetate. In both pictures there are few short thread-like filaments. Immunogold labeling of the reconstituted neurofilaments from the same content as described in *A* and *B* shows double-labeling of the same filaments with 5- and 10-nm gold, where 5-nm gold particles depict localization of tagged H subunits and 10-nm gold particles decorate the native L subunit (*C*, fluorescein-labeled NF-H; *D*, biotin-labeled NF-H). Control study with incubation of anti-vimentin antibody as a first antibody shows few gold particles on the NFs (*E*). Bar, 100 nm.

method (Hirokawa et al., 1984). The width of the filaments varied along their length because of antibody decoration on their surface. These decorations with immunogold could be specific with respect to the control study, where first antibody was absent (data not shown) or anti-vimentin antibody was incubated (Fig. 3 *E*). In the control study, we could not find gold particles decorating the surface of the reassembled NFs. Although the negative staining pattern seen in the case of NFs processed for immunogold labeling did not resemble that before processing (Fig. 3, *A* and *B*), this was probably due to the immunogold processing itself, and a similar view was presented in a previous study (Balin et al., 1991).

Finally, the solution containing only NF-H (∼100 µg/ml) was also dialyzed against the assembly buffer to confirm the capability of the chemically modified H subunit to form component- or subunit-specific filaments. Although the H subunit has been reported as not forming homopolymers (Zackroff et al., 1982), it has been suggested that NF-M and NF-H proteins assemble themselves, respectively, to make component-specific filaments (Tokutake et al., 1984). As expected from its known in vitro assembly properties, NF-H made very short thread-like filaments of a slightly smaller diameter (less than 10 nm; Fig. 2, *C* and *D*), as also reported by Gardner et al. (1984).

The in vivo experiment of microinjecting labeled NFs into bovine fibroblast BSC1 (Fig. 4, *A* and *C*) revealed that the injected NF proteins followed almost the same intracellular distribution pattern as the intrinsic intermediate filament protein of BSC1 cells visualized by anti-vimentin antibody (Fig. 4, *B* and *D*), suggesting that they coassemble with the endogenous vimentin filament network. This is consistent

with the reports that describe the coassembly of Class III and IV intermediate filaments (Chin and Liem, 1989; Monterio and Cleveland, 1989; Soifer et al., 1991). From these two sets of experiments concerning the biochemical and biological properties of tagged neurofilament proteins, it is indicated that the labeled subunit protein retains its ability to form the 10-nm filamentous structure.

## Immunofluorescent Detection of NF-H Protein in DRG before Microinjection

We used DRG cells of large diameter of investigate the dynamic behavior of NF protein, because it has been reported that cells with a small diameter do not express or express only scanty amounts of NF protein (Duce and Keen, 1977). In addition, we have also confirmed before carrying out microinjection and FRAP that the dissociated neurons definitely express endogenous NF-H. At 10 and 30 h after plating the cells, they expressed NF-H as revealed by immunofluorescence (Fig. 5). A DRG cell with a long extending axon visualized by immunofluorescence (Fig. 5, *arrow*) was used for analysis by FRAP. As a criterion for the identification of DRG axon used for the FRAP experiment on the plating dish, we chose a long extended neurite not branched at the proximal portion, corresponding to the peripheral ramus in living animals.

## Detergent Extraction of DRG Cells

We performed detergent extraction of DRG cells that were injected with either rhodamine-labeled BSA or fluorescein-labeled NF-H. DRG cells without neurites or with minor
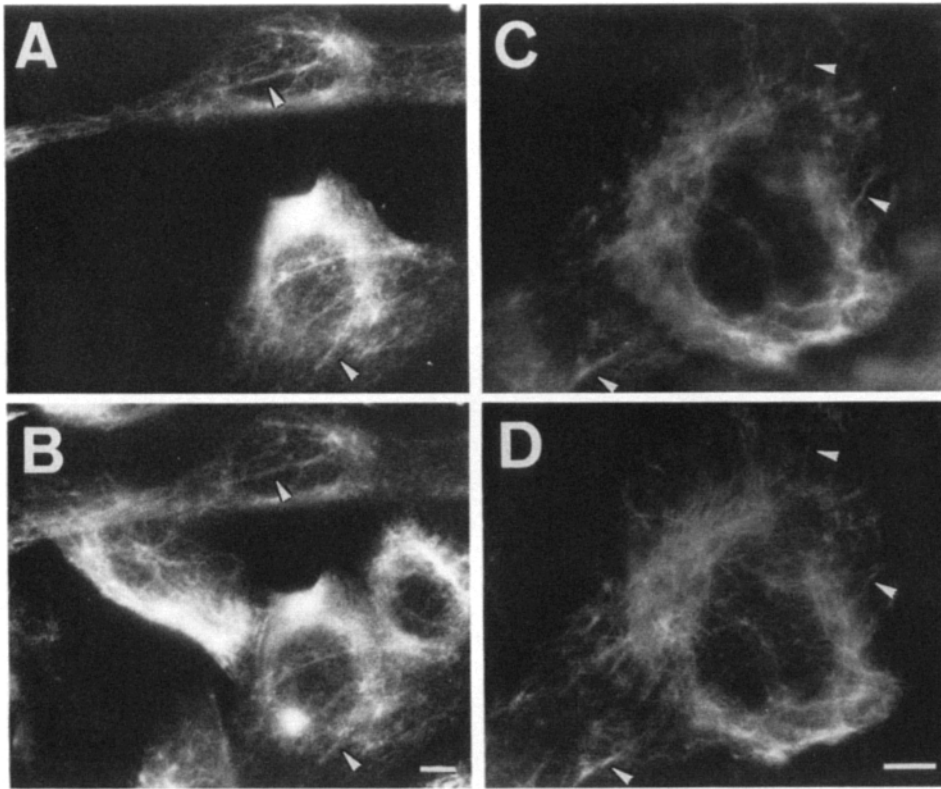
*Figure 4.* A fibroblast BSC1 injected with fluorescently labeled NF-H (*A* and *B*) or NF-L (*C* and *D*), fixed 16 h after microinjection. In both cases, cells were processed for indirect immunofluorescence for anti-vimentin antibodies and rhodamine labeled second antibodies. Note that the filament organizations of both sets of panels are almost identical to each other (*arrowheads*). Bar, 10 μm.

processes were injected with a mixture of these two proteins. They were permitted to grow freely in an incubator for ~24 h until the neurites had grown to a length of 200–300 μm. At that time, if the injected proteins tagged with fluorogenic probes had actually been incorporated into the preexisting intermediate filament network, they could no longer be totally lost from the neurites after detergent extraction. That is to say, incorporated NF subunits would be resistant to detergent extraction, and this would be reflected by the fact that the intensity of the fluorescence of the neurites after extraction would not be so different from that before extraction.

In the experiment presented here, two differently labeled proteins, NF-H protein and a non-cytoskeletal protein, BSA,

were injected into a DRG cell. Before detergent extraction, as viewed by rhodamine and fluorescein band-pass filters, the neurites showed relatively strong intensity of fluorescence with a homogeneous pattern (Fig. 6, *A* and *C*). The fluorescence intensity of the axon (indicating the presence of NF-H), although slightly decreased, was not so greatly changed after detergent extraction (Fig. 6 *B*), while the signal intensity of rhodamine was almost lost (Fig. 6 *D*). However, small spotty intensity in the cell body was still observed after detergent extraction in the case of rhodamine BSA (data not shown). It is suggested that the remaining intensity does
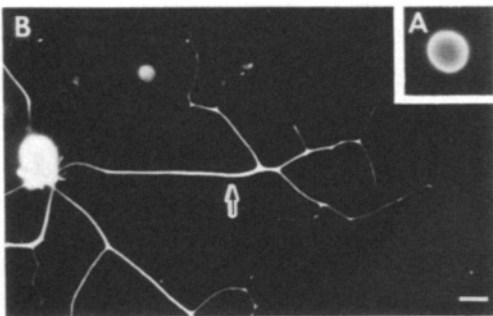


*Figure 5.* Immunofluorescence micrographs of DRG cell stained with anti–NF-H antibody. About 30 h after plating the cell (*B*), a long, extended neurite was clearly visualized. Arrow indicates the site where photobleaching was carried out. 12 h after plating the cell, there were no identifiable processes (*A*). Microinjection of fluorescently labeled NF-H into cells was performed as shown in *A*. Bar, 10 μm.
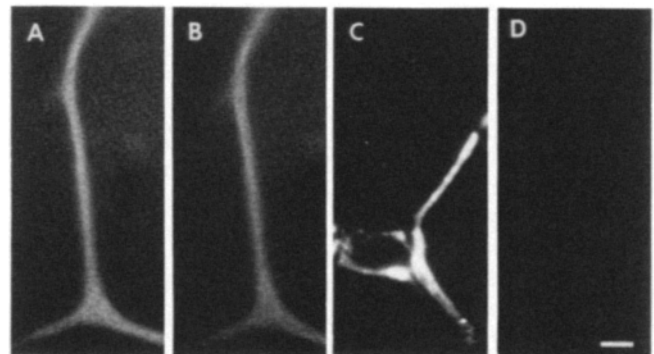


*Figure 6.* Detergent extraction of the axon of DRG cell injected with fluorescently labeled NF-H (*A* and *B*) and with rhodamine-labeled BSA (*C* and *D*). Before extraction, the processes could be visualized by low-light level microscopy (*A* and *C*). After detergent extraction, the signal from the rhodamine-BSA–injected cell was lost (*D*), and the signal intensity from the fluorescein–NF-H–injected cell was also slightly decreased compared to that of preextraction. Bar, 10 μm.

**G**

100

intensity

0

0 length (micrometer) 30

0 min

**H**

100

intensity

0

0 length (micrometer) 30

30 min

**I**

100

intensity
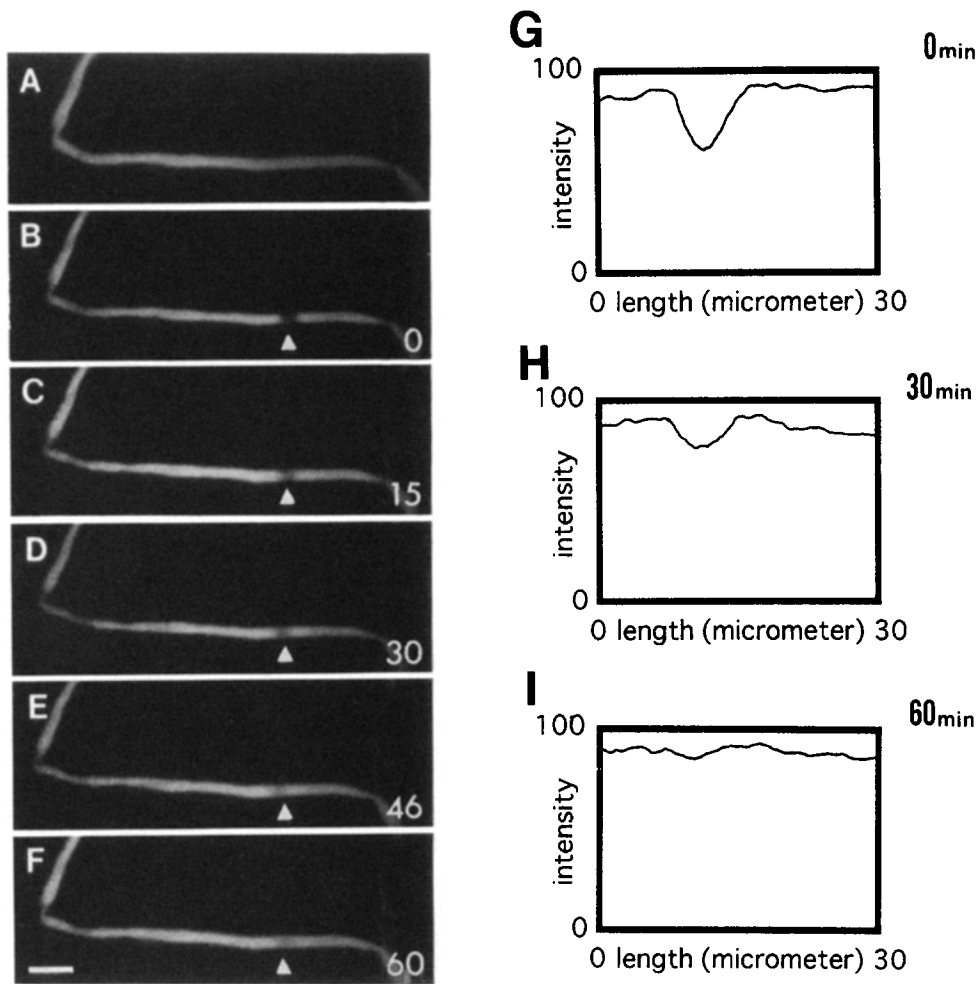
0

0 length (micrometer) 30

60 min

*Figure 7.* Photobleaching fluorescence recovery of DRG axon injected with fluorescently labeled NF-H. Fluorescent images of photobleached NFs were recorded intermittently. Rapid recovery of the bleached zone was observed. Almost complete recovery was achieved 30 min after photobleaching as shown in *H*. Quantitative measurement of signal intensity and location showed no antero- or retrograde movement of the bleached zone (*G–I*). Arrowheads (*A–F*) indicate the bleached zone. Elapsed time in min is indicated in the right bottom corner of each panel. Bar, 10 μm.

not represent the physiological incorporation of the injected BSA into the preexisting NF network, but rather that it is being processed in vesicles such as lysosomes.

## Fluorescence Recovery after Photobleaching (FRAP) of DRG Cells

Although several arguments have been raised concerning the reliability of the photobleach fluorescence recovery method, it has proved to be useful for assessing the local turnover of the NF protein (Okabe and Hirokawa, 1993). A major disadvantage of the method used here was the possible photoablation or photodamage of the cytoskeletal components labeled with fluorogenic probes or of the adjacent protein structures mainly due to generation of free radicals by the application of the argon laser beam (Vigers et al., 1988). However, we have also demonstrated that the level of energy used for photobleaching determines whether photodamage to the intracellular components occurs or not (Okabe et al., 1993). The present experiment was performed under the same energy level conditions as those that did not disturb the proteins in the previous study (Okabe et al., 1993).

Firstly, we performed photobleaching of a DRG cell injected with fluorescein labeled NF-H (Fig. 7). The argon laser beam was applied to the small area of neurites for 1/15 s. Then the bleached area was monitored under low-light level microscopy to follow the recovery of fluorescence in-

tensity. The bleached area did not move either antero- or retrogradely (Fig. 7, *arrowheads*). Almost complete recovery was observed 46 min after photobleaching the axon (Fig. 7 *E*), and after 60 min the fluorescence reached the same state as that of prebleaching. There was no outstanding deformity of the axon throughout the course of fluorescence recovery. The bleached zone restored its fluorescence intensity relatively rapidly, but the site of bleaching also did not move in terms of quantitative measurements of the intensity profile (Fig. 7, *G–I*).

Secondly, to compare the local turnover rate of NF-H with that of NF-L, the same experiment was also carried out with NF-L (Fig. 8, *left column*). In the previous study, the recovery half time of NF-L was nearly 40 min (Okabe et al., 1993). The slope of the recovery curve in the first hour after photobleaching also clearly demonstrates the difference in the rate of turnover between NF-L and NF-H (Fig. 9, *A* and *B*). We calculated the recovery half time in the same manner as in the previous study as follows; we quantified the intensity recovery of the bleached zone by using the data analysis mode of Argus-100, and drew a graph with the elapsed time on the abscissa and the relative intensity of fluorescence on the ordinate. Then a line was drawn from the point of 50% on the ordinate, which was parallel to the abscissa, to cross the line of the intensity recovery line. From the crossing point, a vertical line was drawn to the abscissa, and we read the value on the abscissa as the recovery half time. In the
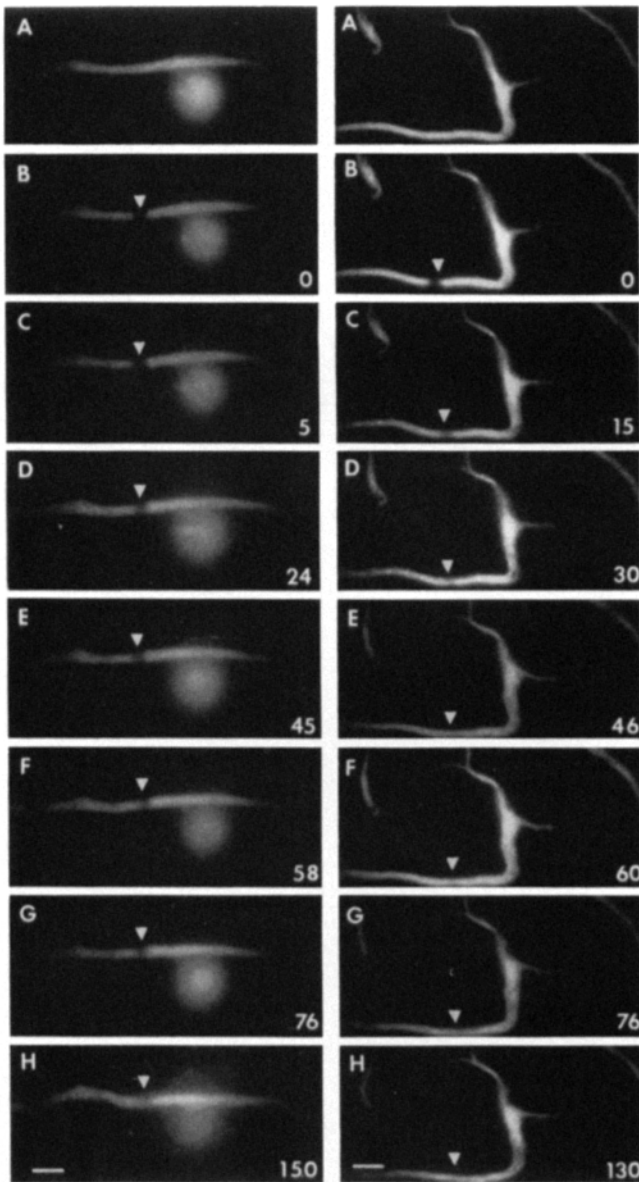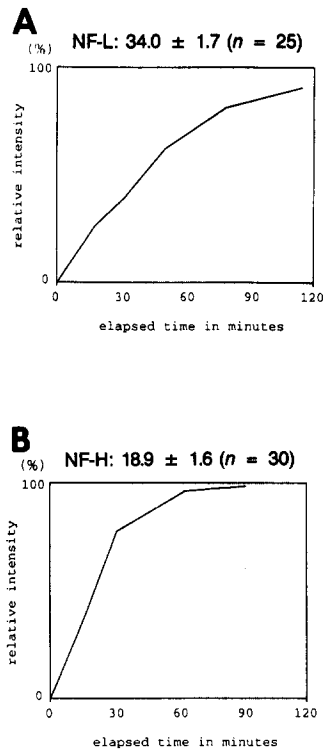
Figure 9. Graphical presentation of the photobleaching fluorescence recovery of both NF-L and NF-H. Fluorescence intensity of the bleached area was plotted against time for the axons shown in Fig. 8. Recovery half times calculated from these graphs are shown on the top of the graphs. For NF-L the value was 34 min, and for NF-H it was ~19 min.

Figure 8. Comparison of photobleaching in DRG axons injected with fluorescently labeled NF-L (left column) and NF-H (right column). Recovery of fluorescence is considerably slower in the case of NF-L. On the contrary, fast recovery was observed in the case of NF-H. Arrowheads indicate the zones where photobleaching run was done. Elapsed time in minutes is indicated in the right bottom corner of each picture. Bar, 10 $\mu$m.

same way, other intensity measurement points of NF-L and H, respectively, were determined. The estimated recovery time for each subunit protein was averaged and the overall recovery half time was calculated. For NF-L, the recovery half time was $34.0 \pm 1.7$ min ($n = 25$), and for NF-H the value was $18.9 \pm 1.6$ min ($n = 30$). Comparison of the data of the respective subunit proteins clearly demonstrated that each behaves in a different manner or is controlled under separate mechanisms.

Thirdly, we have carried out an experiment by injecting a mixture of native NF-L and fluorescently labeled NF-H at a molar ratio of 4:1. In the experiments stated above, we injected only NF-H or NF-L protein into DRG cells. However,

the possibility exists that greatly increased amounts of a single subunit in a DRG cell may interfere with the milieu intérieur or physiological behavior of NF subunits, especially NF-H, because the amount of NF-H incorporated into the NF architecture is at least less than that of NF-L. A large excess of NF-H, which is not incorporated into the preexisting array of neurofilaments, may exist in a form of free monomer and may easily replace the NF-H already taken into the filaments. This phenomenon may hasten the turnover rate of NF-H in the cell to be faster than that observed in the cell of normal conditions. Therefore, to eliminate this possibility, we carried out this experiment as a third part.

In determining the stoichiometry of NF-H and NF-L being injected into DRG, we referred to several reports concerning estimated stoichiometry of NF subunits (Chiu et al., 1980; Mori and Kurokawa, 1980; Schecket et al., 1980; Brown et al., 1981; Moon et al., 1981; Scott et al., 1985). At the same time, we performed a reconstruction experiment as described above in another section. At the molar ratio of NF-L/NF-H = 2, there were short stubby filaments along with long smooth-surfaced filaments typical of 10-nm filaments (data not shown). However, by increasing the ratio to NF-L/NF-H = 4, almost all the fields examined showed only typical 10-nm filaments. Combining both the information from the literature and our experimental data, we determined the NF-L/NF-H ratio for microinjection as described above. As shown in Fig. 10 A, the bleached area in the axon apparently recovered its fluorescent intensity faster than in the case of NF-L, but was quite similar to that from the injection of NF-H only. We performed the same experimental run with a mixture of NF-L/NF-H = 4 to confirm that the obtained data were reproducible ($n = 12$). In these cases, overall intensity along the neurite was relatively weaker than that in the case of NF-H only, since the relative amount of fluorescently labeled NF-H being injected became smaller. How-
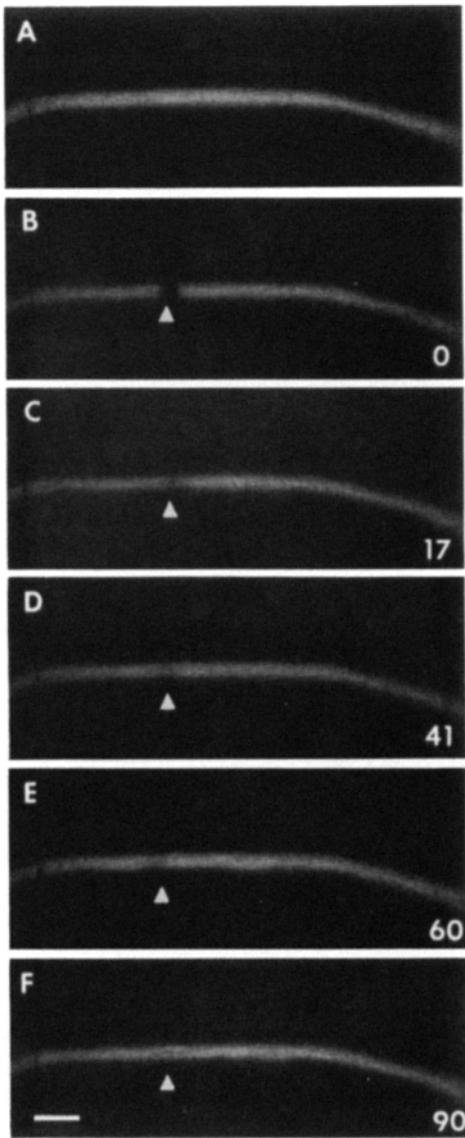
Figure 10. Photobleaching experiment of DRG cells coinjected with native NF-L and fluorescently labeled NF-H at a molar ratio of 4:1. The overall course of the fluorescence recovery mimics that of DRG neurons injected with only NF-H. Elapsed time in minutes is indicated in the right bottom corner of each picture. Bar, 10 $\mu$m.
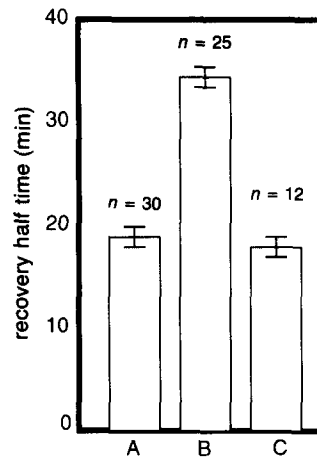


Figure 11. The recovery half time for each set of FRAP experiment. The recovery half time on abscissa and each set of the experiment on ordinate. (A) Only NF-H. $T_{1/2} = 18.9 \pm 1.6$ min (mean $\pm$ SEM). (B) Only NF-L. $T_{1/2} = 34.0 \pm 1.7$ min. (C) Coinjection of NF-L and NF-H at molar ration of 4:1. $T_{1/2} = 18.0 \pm 1.7$ min. The number presented at the top of each column indicates the number of the cells on which FRAP measurements were carried out. Bars indicate the standard error of the mean.

ever, the fluorescent intensity was above the limit of sensitivity of the SIT-camera, and the estimated recovery halftime was $18.0 \pm 1.7$ min (mean $\pm$ SEM), a value near that of injecting NF-H only. The recovery half time for each set of experiment (NF-H, NF-L, and NF-L+NF-H) is summarized graphically in Fig. 11.

## Immunoelectron Microscopical Analysis of NF-H Incorporation Sites in the Axoplasm

To address the question of how NF-H subunits in soluble form assemble with preexisting arrays of NFs, we carried out immunoelectron microscopical analysis by microinjecting biotin-labeled NF-H, which could be clearly detected by using anti-biotin antibody and colloidal gold conjugated sec-

ond antibody. DRG cells 48 h after plating were used for this procedure, because at this point the cells had almost fully extended axons, a suitable condition for our purpose. After injecting the tagged NF-H, the cells were fixed at 1, 3, 13, and 21 h. The conditions and timing of fixation were described previously (Okabe et al., 1993). We were able to process the cells without causing any kind of damage or abnormal appearance possibly resulting from the injection. 1 h after microinjection, colloidal gold particles were scattered randomly along the NF arrays $\sim$100 $\mu$m from the cell body in the axoplasm (Fig. 12 A). In addition, they appeared to have a tendency to be abundant in the region where NFs and MTs form crossbridges, suggesting the participation of the long COOH-terminal of the NF-H protein in forming these structures. There were no signs of aggregation formed by the injected NF-H. At the same time, localization of the injected NF-H in the cell body was also observed. The NFs in the cell body were densely labeled with colloidal gold. They did not show any kind of dense nucleate or have other abnormal appearances (data not shown).

3 h after the microinjection, the gold particles were observed on the neurofilament in the axon more frequently than 1 h after (Fig. 12 B). However, the tendency of lateral incorporation of the molecule into the preexisting filaments was still observed, suggesting random insertion of the injected NF-H subunit protein. No direct evidence for bulk replacement of the NF-H subunit protein at the proximal portion of the axoplasm was seen. After 13 h, the density of the insertion visualized by the gold particles had become greater (Fig. 12 C). 21 h after microinjection, more than 90% of the neurofilaments were labeled with colloidal gold (Fig. 12 D), and there was no distinct difference in the patterns of the incorporation of the biotin-labeled subunits between NF-H and NF-L. In the case of NF-L as reported by Okabe et al. (1993), the incorporation of the molecule was almost homogeneous, showing no specific pattern of tendency. In the case of NF-H, as periodical arrangement within NFs according to antibody decoration has been reported (Willard, 1981), periodical labeling of incorporated NF-H can be expected, but such obvious tendency could not be observed in our experiment.
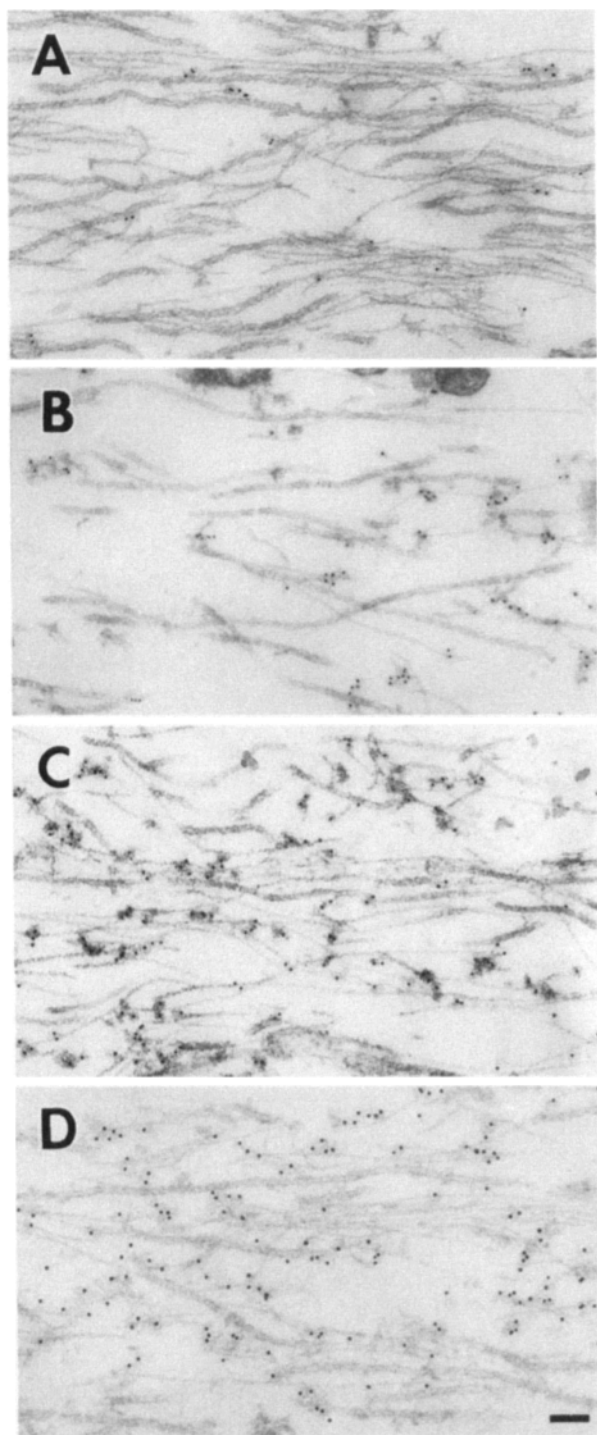
*Figure 12.* Immunoelectron microscopy of DRG axons injected with biotin-labeled NF-H. (*A*) Axons of DRG cells permeabilized and fixed about 1 h after microinjection. The processed area is ∼200 μm from the cell body. A few gold particles are associated with NFs. (*B*) Region of the axon ∼200 μm from the cell body, fixed 3 h after microinjection. Gold particles increased in number but do not form distinct segments. (*C*) 13 h after microinjection. (*D*) 21 h after microinjection.

## Discussion

### Turnover of the NF-H Subunit in the Sensory Nerve Axon

FRAP has been suggested to provide us with two sets of information, one about the ability of subunits to assemble or disassemble locally, and the other about the manner in which cytoskeletal proteins are transported (Hirokawa and Okabe, 1992; Hirokawa, 1993). In the present study, we have obtained data which indicate that the local turnover of NF-H is significantly faster than that of NF-L. The recovery half-time of fluorescence intensity was only ∼19 min in the case of NF-H, whereas that for NF-L was ∼35 min. The restoration of the bleached zone was rapidly achieved in DRG microinjected with NF-H without moving in either antero- or retrograde direction. With reference to previous studies dealing with cytoskeletal dynamics (Okabe and Hirokawa, 1990, 1993; Okabe et al., 1993), it is reasonable to conclude from our experiment that NF-H is a dynamic component of NF arrays turning over rapidly, and it is strongly suggested that it does not move by polymer sliding mechanism as reported previously (Lasek, 1986), but possibly in a form of small oligomers. Also answered by our study is the question of whether or not the NF-H subunit exchanges between soluble and filamentous pools once the triplet is assembled. The conclusion is compatible with earlier studies formulating that the basic unit of NFs transported by SCa slow axonal transport is in the oligomeric state (Nixon, 1992; Okabe et al., 1993).

By immunoelectron microscopy, incorporation of biotin-labeled NF-H was quite sparse in the preexisting NF arrays in the axoplasm, but one day later the density of the incorporation sites had increased. We actually expected some periodicity in the case of NF-H, but in our experimental system a prominent difference between NF-L and NF-H in their pattern of NF subunit incorporation was not observed. This may have resulted partially from the resolution of techniques employed in the present study. The time course of NF-H incorporation into the preexisting NFs, taking after that of NF-L, supports the idea that both NF subunits are transported by the same slow axonal transport called SCa. In addition, the fact that colloidal gold decoration was more concentrated in the neighborhood of MTs also suggests the function of the H subunit in the formation of crossbridges between MTs and NFs (Hirokawa, 1982; Miyasaka et al., 1993).

From another point of view, our result from the reconstruction experiment using native NF-L and fluorescently labeled NF-H at a molar ratio of 4:1 might be supportive data for determining how and by what ratio the neurofilament is constructed in the axoplasm. Several reports on the estimated molar ratio of neurofilament polypeptides vary widely from ∼2:2:1 for L/M/H (Moon et al., 1981) to 9:2:1 (Mori and Kurokawa, 1980) with various intermediate values (Chiu et al., 1980; Schecket et al., 1980; Brown et al., 1981; Scott et al., 1985; Mulligan et al., 1991), but the exact ratio has not yet been determined. There are obviously several difficulties in determining the exact ratio, but delineating them is beyond the scope of the present study. Although by our study using immunoelectron microscopy, the value of the molar ratio cannot be predicted because of inherent technical limitations, the reconstitution experiment by varying the

NF-L/NF-H ratio suggests that the amount of NF-H assembled into neurofilaments is less than that of NF-L as reported by several studies (Mori and Kurokawa, 1980) despite the absence of NF-M in the reconstitution experiment.

Although the recovery half time of NF-H was revealed to be considerably faster than that of NF-L, one important question still remains unsolved. It is whether or not the local turnover of the protein is directly related to the velocity of slow axonal transport. In the case of tubulin and actin, they are believed to be transported at different rates (Tashiro and Komiya, 1989), and the turnover rates of the two proteins are also different (Okabe and Hirokawa, 1990). Fast transported tubulin turned over slower than slowly transported actin and the explanation given was that the distinct velocities of slow axonal transport are generated by differences in the exchange rates of these two cytoskeletal proteins. However, each neurofilament polypeptide is considered to be transported by the same transport rate denoted as SCa. For this reason, at least in the case of neurofilament polypeptides, we should be prudent in accepting the generalization that there is a relationship between the rate of transport and the rate of assembly/disassembly, which are reflected in the recovery half time after photobleaching.

Then how can we explain the differential turnover rates of NF-H and NF-L? First of all, we should eliminate the possibility that a large excess of NF-H in the cell after the injection may interfere with the physiological turnover of NF-H in the neuron. Concerning this problem we carried out an experiment of coinjection of NF-L and labeled NF-H into the same cell. In this case, the estimated recovery half time was quite similar to that of injecting NF-H only, implying that the difference in turnover rate between NF-L and NF-H does not result from the experimental design adopted here. Regarding other factors possibly responsible for such a difference, it is impossible to make a precise determination from our results, but speculation on the basis of the literature can be made. Two factors considered to affect the turn over rate of the NF-H are: (a) the enzymatic modification of NFs, and (b) heterogeneity in the grade of phosphorylation. Factor (a) refers to the possibility that enzymatic modification specific to the NF-H protein occurs after its synthesis. Such modifications achieved by specific proteases or posttranslational modifications including glycosylation might affect the degradation rates or assembly/disassembly rates of NFs. However, concrete information is still limited. On the other hand, phosphorylation of the COOH-terminal of the H subunit is relatively well documented (see review, Nixon and Sihag, 1991). The COOH-terminal of the H subunit is highly phosphorylated in the axon, but soon after its synthesis in the cell body it is not phosphorylated to the extent observed in the axon. However, in the present experimental system, the injected sample may contain variously phosphorylated NF-H, so that its biochemical nature of heterogeneity was not sufficient to explain the result that the behavior of NF-H being faster than NF-L truly resulted from differences in the protein nature per se, not from differences in the state of phosphorylation. In other words, the injected NF-H could not represent the in vivo environment of the cell body.

To eliminate factor (b), the possibility that the grade of phosphorylation of NFs in the cell body has an effect on the travelling of NF polypeptides down the axon, and in turn results in alteration of the local turnover, we took two mea-

sures to verify our experimental results in vivo. One was the analysis concerning the local turnover of NF-H about one day after introducing fluorescently labeled protein into the cell body, taking into consideration the fact that one day is sufficient for the injected NF-H to be modified in the cell and transported down the axon to the growing tip (in this case the photobleaching process was carried out at 200–300 μm of the axon), as the velocity of slow axonal transport by which NFs are transported is reported to be ∼1–2 mm per day (for review, Nixon, 1992).

The other measure was the performing of a preliminary study using dephosphorylated NF-H. We though the treatment with alkaline phosphatase might be necessary to achieve homogeneity of NF-H in phosphate content, and also, if the in vivo phosphorylation state of NF-H in perikarya has a pronounced effect on the turnover rate by possibly altering its affinity to some, as yet unidentified carrier protein, a dephosphorylation procedure would be pertinent to reproduce the condition in vivo. Our experiment revealed that the recovery half time estimated in the case of injecting dephosphorylated NF-H was almost equal to that of the native one (data not shown), suggesting that the inherent nature of the protein and/or phosphorylation taking place after it is transported into the axon in situ contributes to the differential behavior of the two NF subunit proteins. The results obtained have led us to believe that inherent properties of the NF polypeptides themselves possibly determine their dynamic properties.

## Turnover Rate and Its Implications on the Structure of IFs

We have speculated that the differences in turnover rate between the H and L subunits result mainly from the location where each component associates with the NFs per se. It is quite natural to suppose from the data presented here and the results of Mulligan et al. (1991) that NF-H is associated with the core NF array at the periphery and in a looser manner. Several lines of evidence on the structure of the NF array in the axon and hypotheses concerning the functional and morphological roles of NF-H have been proposed, and some of them are able to account for, or point to clues as to why the turnover of the H subunit is faster than that of the L subunit. Antibody decoration studies have also revealed the location of the H subunit in neurofilaments (Willard and Simon, 1981; Sharp et al., 1982; Hirokawa et al., 1984). Whereas the L subunit makes up the central domain of the neurofilaments, the H subunit associates with them peripherally because of their long, extended COOH-terminal, and seems to be major component of crossbridges between neurofilaments in situ (Hirokawa et al., 1984; Hisanaga and Hirokawa, 1988). This structural property makes NF-H more dissociative than the L subunit. Furthermore, dissociation of H subunits from NF arrays may be regulated by phosphorylation (Hisanaga and Hirokawa, 1989). Further detailed structural analyses are expected to determine how each subunit forms 10-nm neurofilaments.

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