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Visualization of Single Neurofilaments by Immunofluorescence Microscopy of Splayed Axonal Cytoskeletons

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Treatment of cultured neurons with non-ionic detergents under certain conditions causes the axonal microtubules to splay apart from each other, allowing individual microtubules to be visualized by immunofluorescence microscopy [Brown et al., 1993, J. Cell Sci. 104: 339–352]. I have investigated whether axonal neurofilaments separate from each other under similar conditions. Cultures of dissociated dorsal root ganglion (DRG) neurons from fetal rats were treated with non-ionic detergent and fixed with formaldehyde. Neurofilaments were visualized by immunofluorescence microscopy using a polyclonal antiserum specific for NF-L. Treatment of the neurons with Triton X-100 or saponin caused filamentous structures to splay apart from each other along the entire length of the axon. Quantitative analysis of fluorescence intensity along the filamentous structures indicated that many of them represent single neurofilaments and that single and bundled neurofilaments can be distinguished based on their fluorescence intensity. The extent of this splaying phenomenon was dependent on time and detergent concentration. Temporal analysis indicated that short portions of single neurofilaments initially loop out from the axonal bundle and then subsequently splay apart further along their length and adhere to the polylysine/laminin coated substrate. The maximum observed length for a single axonal neurofilament was 183 μm in neurons after only 1 day in culture, which indicates that neurofilaments can attain remarkable lengths in these young cultured neurons. The splayed axonal cytoskeleton preparation described here allows individual axonal neurofilaments to be visualized by immunofluorescence microscopy, which is not possible in conventional preparations due to the dense packing of these polymers in axons. Cell Motil. Cytoskeleton 38: 133–145, 1997.

Key words: neurofilament, cytoskeleton, axon

INTRODUCTION

Neurofilaments are space-filling cytoskeletal protein polymers that contribute to the shape and mechanical integrity of neurons and their cytoplasmic processes. The function of these cytoskeletal elements is best understood in axons where they are required for the growth of axon caliber, which is an important determinant of axonal conduction velocity [for review, see Cleveland et al., 1991]. The contribution of neurofilaments to the growth of axon caliber appears to be maximized by the highly charged and highly phosphorylated carboxy-terminal domains of the neurofilament polypeptides, which project out from the filament backbone and keep neighboring neurofilaments at ''arm's length'' [Hisanaga & Hirokawa, 1988; Price et al., 1988; Price et al., 1993].

Neurofilaments in birds and mammals are composed of three polypeptides called NF-L, NF-M, and NF-H (low, medium, and high molecular weight, respectively). These three polypeptides are known collectively

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Abbreviations: ADU (analog-to-digital units); Cy3 (indocarbocyanine); DRG (dorsal root ganglion); EGTA (ethylene glycol-bis[β-aminoethyl ether] N,N,N′,N′ tetra-acetic acid); FITC (fluorescein isothiocyanate); HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]); LRSC (lissamine rhodamine sulfonyl chloride); NF-L (low molecular weight neurofilament triplet polypeptide); NF-M (medium molecular weight neurofilament triplet polypeptide); NF-H (high molecular weight neurofilament triplet polypeptide); PBS (phosphate buffered saline); PHEM (buffer containing PIPES, HEPES, EGTA and $MgCl_2$); PIPES (piperazine-N,N′-bis[2-ethanesulphonic acid]); TBS (Tris/HCl buffered saline).

as the ''neurofilament triplet'' [for review, see Lee & Cleveland, 1996; Shaw, 1991]. Neurofilaments *in vivo* are obligate heteropolymers, composed of NF-L and either one or both of the other two triplet polypeptides [e.g. Lee et al., 1993]. Recent evidence suggests that the requirement of NF-L for filament formation is due to the inability of NF-M and NF-H to form stable assembly-competent subunits in the absence of this polypeptide [Carpenter & Ip, 1996; Cohlberg et al., 1995].

To study neurofilaments, many researchers have taken advantage of the amenability of cultured neurons to direct experimentation and manipulation. In a previous study on microtubules, I and others have shown that treatment of cultured neurons with certain non-ionic detergents can cause these cytoskeletal polymers to splay apart from each other, allowing individual axonal microtubules to be visualized by fluorescence microscopy [Brown et al., 1993]. This technique has proven to be a simple yet powerful tool for investigation of the assembly dynamics and post-translational modification of axonal microtubules along their length [Brown et al., 1993; Li & Black, 1996; Rochlin et al., 1996]. In the present paper, I show that axonal neurofilaments also splay apart from each other under these conditions. Quantitative analysis of fluorescence intensity along the length of the splayed filaments indicates that many of them represent single neurofilaments and that single and bundled neurofilaments can be distinguished based on their staining intensity. Thus the splayed axonal cytoskeleton preparation allows individual axonal neurofilaments to be resolved by fluorescence microscopy, which would normally require electron microscopy due to the dense packing of these polymers in axons. The splaying technique provides a simple method for investigating the post-translational modification and polypeptide composition of neurofilaments in axons using conventional immunofluorescence microscopy.

MATERIALS AND METHODS

Cell Culture

Primary cultures of dissociated neurons were established from the dorsal root ganglia of fetal rats. Female Sprague Dawley rats in their seventeenth day of pregnancy (E16.5) were anesthetized with carbon dioxide and killed by cervical dislocation. Embryos and dorsal root ganglia were dissected essentially as described by Kleitman et al. [1991]. The age of the embryos was confirmed using the morphological characteristics described by Long & Burlingame [1938] and Christie [1962]. Ganglia were pooled in Liebovitz's L15 medium (#11415, Gibco BRL, Grand Island, NY) and dissociated by treatment with 2.5 mg/ml trypsin (TRL3, Worthington Biochemical Corp., Freehold, NJ) in phosphate buffered saline (PBS; 138 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, pH 7.4) followed by trituration in culture medium lacking methylcellulose, essentially as described by Higgins et al. [1991]. The resulting cell suspension was diluted in culture medium lacking methylcellulose and then plated at low density onto polylysine/laminin coated glass coverslips in special 35mm plastic tissue culture dish assemblies as described by Brown et al. [1992]. The cells were allowed to settle down onto the glass under gravity and then culture medium containing methylcellulose was added.

The culture medium contained a 1:1 mixture of Ham's F-12 (#11765, Gibco) and Dulbecco's Modified Eagle's Medium (#11960, Gibco) supplemented with 0.5 mg/ml BSA (Fraction V, Calbiochem Corp., La Jolla, CA), 0.1 mg/ml bovine transferrin (Sigma Chemical Co., St. Louis, MO), 10 µg/ml bovine insulin (Sigma), 5 ng/ml sodium selenite (Sigma), 0.2 mg/ml L-glutamine (Sigma), 50 ng/ml nerve growth factor (Collaborative Biomedical Products, Bedford, MA), 0.5% hydroxypropyl methylcellulose (Methocel™ F4M PREM; The Dow Chemical Co., Midland, MI), and 10% adult rat serum. This medium was prepared in batches without methylcellulose and serum and stored frozen in 40 ml aliquots. The methylcellulose was sterilized in an autoclave and dissolved in the medium by shaking overnight at room temperature. Adult rat serum was prepared from the blood of adult male or female rats essentially as described by Hawrot and Patterson [1979]. Cultures were maintained in an incubator at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. All studies were performed between 1 and 3 days after the time of plating.

Detergent Treatment and Fixation

Cultures were rinsed once with PBS, once with PHEM (60mM NaPIPES, 25 mM NaHEPES, 10 mM NaEGTA, 2 mM $MgCl_2$, pH 6.9), and then treated with PHEM containing 0.19M NaCl and various concentrations of Triton X-100 (Sigma, X100) or saponin (Sigma, S2149) at room temperature. Neurofilaments are known to be stable when cultured neurons are extracted under these solution conditions [Black et al., 1986]. After various periods of time, the detergent solution was removed and the cells were fixed for 30 min at room temperature in a solution of 4% (w/v) formaldehyde in PHEM containing 0.19M NaCl. The formaldehyde was

prepared fresh from paraformaldehyde on the day of use. After fixation, the dishes were rinsed with PBS and then treated with 0.1% Triton X-100 + 0.3M NaCl in PBS for 15 min (''post-fixation extraction'') to ensure complete demembranation of the cells prior to immunostaining. For experiments on microtubules, the detergent solution contained 10 μM taxol and 0.1% dimethylsulfoxide to stabilize the microtubules and the fixative contained a mixture of 0.5% glutaraldehyde and 4% formaldehyde. To eliminate autofluorescence induced by glutaraldehyde fixation, the fixed cells were treated with sodium borohydride [Brown et al., 1992].

Immunostaining

After post-fixation extraction the cultures were rinsed with tris-buffered saline (TBS; 50 mM Tris/HCl, 138mM NaCl, 2.7mM KCl, pH 7.4) and then ''blocked'' with 4% normal donkey serum (Jackson Immunoresearch, West Grove, PA) in TBS (''blocking solution''). To visualize neurofilaments the cells were stained with a rabbit polyclonal antiserum specific for NF-L (''primary antibody''; gift of Dr. Virginia Lee of the University of Pennsylvania) diluted 1:500 in blocking solution. This anti-serum was raised against a synthetic peptide identical to the 20 amino acid sequence at the extreme carboxy terminal of human NF-L [Trojanowski et al., 1989]. Unbound primary antibody was rinsed off with TBS and the dishes were blocked again prior to incubation with LRSC-conjugated donkey anti-rabbit IgG (''secondary antibody'') diluted 1:200 in blocking solution. Unbound secondary antibody was rinsed off with TBS and the dishes were mounted in TBS containing 70% (w/v) glycerol and 10 mg/ml n-propyl gallate, pH 7.0.

To visualize microtubules, the immunostaining protocol was identical except that the primary antibody was a monoclonal specific for β-tubulin (Amersham Corporation, Arlington Heights, IL) [Blose et al., 1984] diluted 1:300 in blocking solution and the secondary antibody was Cy3-conjugated donkey anti-mouse IgG diluted 1:200 in blocking solution. For quantitative analysis of fluorescence intensity, the immunostaining protocol was identical to that described above for visualizing neurofilaments except that the cells were stained with a secondary antibody consisting of FITC-conjugated goat anti-rabbit IgG diluted 1:200 in blocking solution and then a tertiary antibody consisting of LRSC-conjugated donkey anti-goat IgG diluted 1:200 in blocking solution. All conjugated antibodies were purchased from Jackson Immunoresearch and had been affinity purified and pre-adsorbed against immunoglobulins from rat and other species. Pre-adsorption against rat immunoglobulins was a precaution because of the use of adult rat serum in the culture medium. All antibody incubations were performed for 45 min at 37°C.

Microscopy and Image Acquisition

The stained cells were observed by epifluorescence microscopy using a Nikon Diaphot 300 inverted microscope (Nikon Inc., Melville, NY) equipped with a 100 Watt mercury arc lamp and fluorescein and rhodamine filter sets (HQ FITC 41001 and HQ TRITC 41002, Chroma Technology, Brattleboro, VT). Images were captured using a CH250 cooled CCD camera (Photometrics Ltd., Tucson, AZ) that contained a Kodak KAF 1400 CCD chip with an array size of 1317 × 1035 pixels and a 12-bit readout. Image acquisition was performed on an Apple Macintosh computer using a Nu200 camera controller board and the Oncor-Image (formerly BDS-Image) image processing and analysis software package (Version 2.0, Oncor Inc., Gaithersburg, MD). Images were acquired with Nikon CF 40×/1.0 NA and CF N 100×/1.4 NA Plan Apo oil-immersion objectives using the full usable area of the CCD chip with no pixel binning. Illumination of the sample was controlled using a Uniblitz® electronic shutter (Vincent Associates, Rochester, NY), which was operated from within the Oncor-Image software using a MAC 2000 Communications Interface Module (Ludl Electronic Products Ltd., Hawthorne, NY). Using the 100× objective, each pixel in the image measured 68.5 × 68.5 nm. To image axons or filaments that were too long to fit in a single field, a series of overlapping images was acquired. The magnification of the CCD images was calibrated using a stage micrometer. All images in this article are digital images that were prepared for publication in Adobe Photoshop 4.0 (Adobe Systems Inc., San Jose, CA).

Quantitative Analysis of Fluorescence Intensity

To compare the fluorescence intensity of neurofilaments in different images quantitatively it is necessary to ensure that all filaments are processed identically and that the extent of photobleaching is comparable for all images. To ensure identical processing, I only compared filaments from the same dish. To ensure comparable photobleaching throughout the dish, I visualized the primary NF-L antibody with a secondary FITC-conjugated goat anti-rabbit antibody followed by a tertiary LRSC-conjugated donkey anti-goat antibody. In this way, I was able to perform all routine inspection and focusing on the rhodamine channel without any bleaching of the fluorescein. The fluorescein channel was reserved exclusively for image acquisition and all images were acquired using the 100× objective and identical exposure times, thereby ensuring identical and minimal photobleaching.

To correct for non-uniformity in image brightness across the field of view, which is inherent in light microscopic images, an image of the background fluorescence was obtained for each image by a digital filtering. This procedure involved sequential application of a local minimum filter, a local maximum filter, and a local

average (smoothing) filter to each image. A more detailed description can be provided on request. Appropriate choice of filter dimensions enabled the filaments and/or axons in the field to be filtered out. The resulting image of the background fluorescence was used to correct the original raw image according to standard ''flat-fielding'' procedures [Brown et al., 1992]. I call this digital filtering procedure ''intrinsic flat field correction'' because it does not require the acquisition of a separate flat-field image. An important constraint on this procedure is that the objects to be analyzed must occupy a small area of the image in comparison to the background. This is not a problem for studies on neurofilaments in splayed axonal cytoskeletons but it does limit the applicability of this technique to other types of images.

Fluorescence intensity was analyzed along filaments in the flat-field corrected images using the segmented mask method described elsewhere [Brown et al., 1993; Brown et al., 1992]. Briefly, digital image processing techniques were used to create a mask of the neurofilament and this mask was subdivided into contiguous segments of equal length. A complementary background mask that surrounded the neurofilament mask was also created so that each segment in the neurofilament mask was flanked by two segments in the background mask. To correct for background, the mean pixel intensity was calculated for each pair of segments in the background mask and this value was subtracted from each pixel in the corresponding segment of the neurofilament mask. The total fluorescence intensity for each segment in the neurofilament mask was then calculated by summing the corrected intensities of the individual pixels in that segment. All image processing and analysis was performed using the Oncor-Image software and application programs written using the Oncor-Image programming language.

RESULTS

Characterization of Neurofilament Splaying

Previous studies on microtubule splaying in the presence of non-ionic detergent have all been performed on short-term cultures of sympathetic neurons from the superior cervical ganglia of embryonic or neonatal rats [Brown et al., 1993; Li & Black, 1996; Rochlin et al., 1996]. I have found that these cultures are less suitable for studies on neurofilaments because the neurons contain relatively few neurofilaments and their axons frequently exhibit discontinuities in neurofilament staining along their length (unpublished observations). For this reason, I have used cultured DRG neurons for the present study; these cells are known to express large amounts of neurofilament protein and their axons contain numerous neurofilaments [Sharp et al., 1982; Shaw & Weber, 1981].

Fig. 1 shows axons of cultured DRG neurons stained with NF-L antibody to visualize neurofilaments. The axon in Fig. 1A was fixed without prior exposure to detergent. The neurofilaments form a continuous overlapping array that extends along the entire length of the axon, but individual neurofilaments cannot be identified because they are too close to each other to be resolved by fluorescence microscopy. The axons in Figs. 1B-E were treated with 0.05% saponin for various times prior to fixation. In the presence of this detergent, filamentous structures initially looped out from the axonal bundle (e.g. arrowhead in B) and then subsequently splayed apart further along their length so as to expose free filament ends (e.g. arrowhead in C). The number and length of the splayed filaments increased with time and at later times some filaments became completely isolated from the axonal neurofilament bundle so that both filament ends could be identified (e.g. arrowhead in E). The lengths of the isolated filaments that I encountered in this study ranged from 1–183 μm. At higher concentrations of saponin, the rate of neurofilament splaying was too rapid for me to resolve the early stages of the process.

The extent of neurofilament splaying was also dependent on the detergent concentration. For example, Fig. 2A,C & E show the effects of treatment with 0.2%, 0.5% and 1% saponin for 5 min on the splaying of axonal neurofilaments in cultured DRG neurons. Neurofilaments splayed apart at all three concentrations but the extent of splaying (i.e. the length and number of the splayed filaments) was greater at higher detergent concentrations. The minimum saponin concentration necessary to induce neurofilament splaying was about 0.02% and no significant differences were observed with Triton X-100 (data not shown). Microtubules also splayed apart from each other in the presence of saponin, but the extent of splaying was much less. For example, Fig. 2B,D & F show the effects of treatment with 0.2%, 0.5% and 1% saponin for 5 min on the splaying of axonal microtubules in cultured DRG neurons. No splaying was observed in 0.2% saponin (Fig. 2B), and the extent of splaying at higher concentrations was less than for neurofilaments (compare C with D and E with F in Fig. 2). These observations indicate that the extent of microtubule and neurofilament splaying is dependent on detergent concentration but that the concentration required to produce splaying is different for these two types of cytoskeletal polymer.

Neurofilament splaying was observed along the entire length of the axons, though occasionally I encountered an axon that exhibited significantly more splaying distally than proximally. Fig. 3 shows a montage of a DRG neuron that was treated with 0.5% saponin for 5 min prior to fixation. This neuron was chosen because its short axon fits conveniently in a single figure, but the pattern of

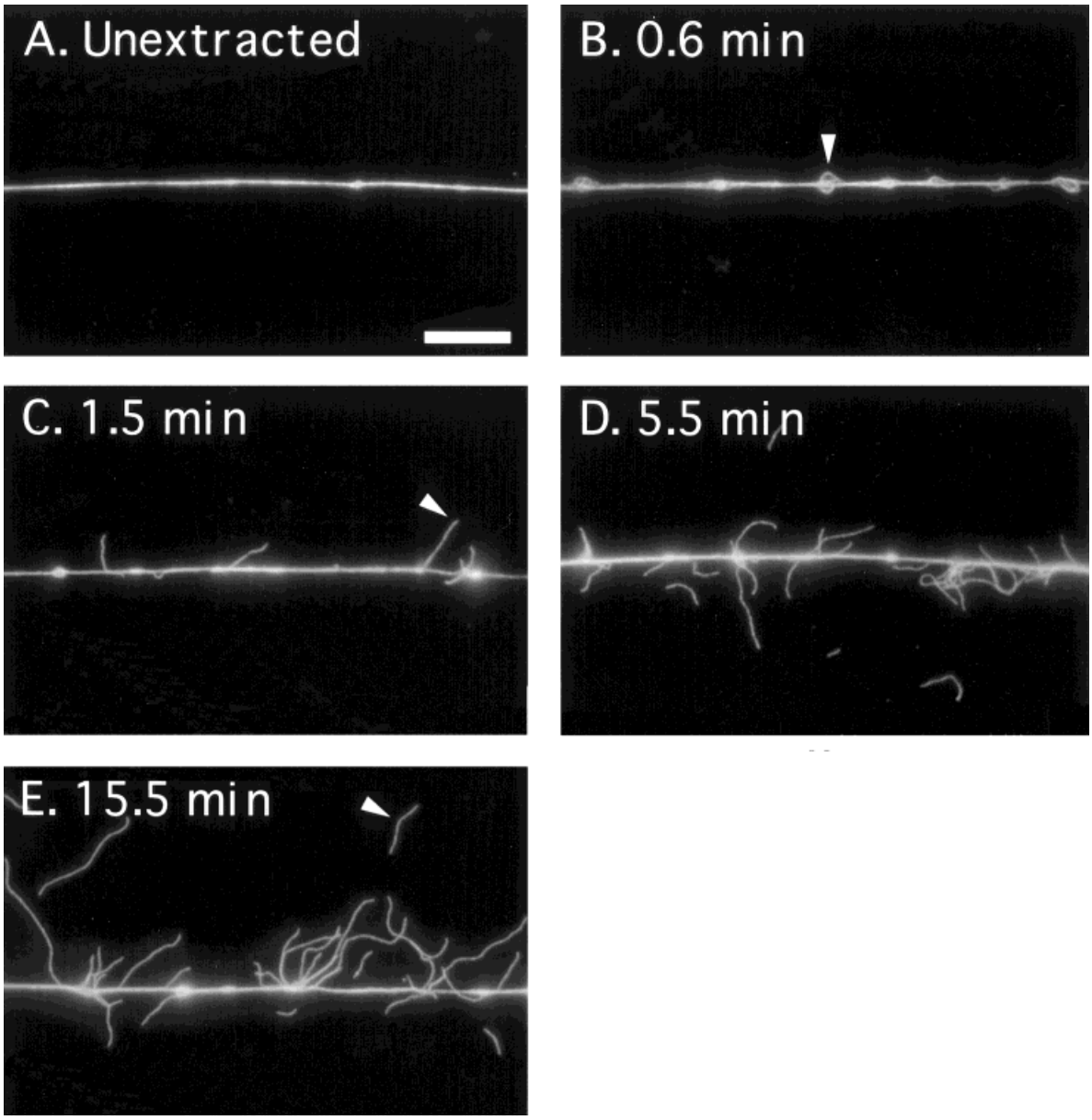


Fig. 1. Time course of neurofilament splaying. Axons fixed without prior saponin treatment (**A**) or after treatment with 0.05% saponin for times ranging from 0.6–15.5 minutes (**B**-**E**) and then processed for immunofluorescence microscopy to visualize the neurofilaments. In unextracted axons the neurofilaments are bundled together and cannot be resolved (A). In the presence of detergent, the neurofilaments initially loop out from the axonal bundle (e.g. **arrowhead** in B) and then subsequently splay apart further along their length so as to expose free filament ends (e.g. **arrowhead** in C). The number of splayed filaments increases with time and an increasing number of filaments separate from the axon so that both ends are visible (e.g. **arrowhead** in E). Scale bar = 10 μm.

splaying seen here is typical of neurons with much longer axons. During the course of this study I have observed cultures ranging in age from 1–3 days after plating and axons ranging from 225–2300 μm in length and I have observed no correlation between these variables and the extent of splaying.

Most filaments in the splayed cytoskeletons were static, which suggests that they adhered to the polylysine/laminin coated glass substrate after they splayed apart from the axonal neurofilament bundle. However, in some experiments portions of some of the splayed filaments were observed to jiggle rapidly and continuously by Brownian motion, which indicates that these particular filaments were not firmly attached to the substrate along their entire length. The rate of jiggling was too rapid to visualize using the slow-scan cooled CCD camera. Often the mobile regions were interrupted or flanked by regions that were static, suggesting that the filaments were

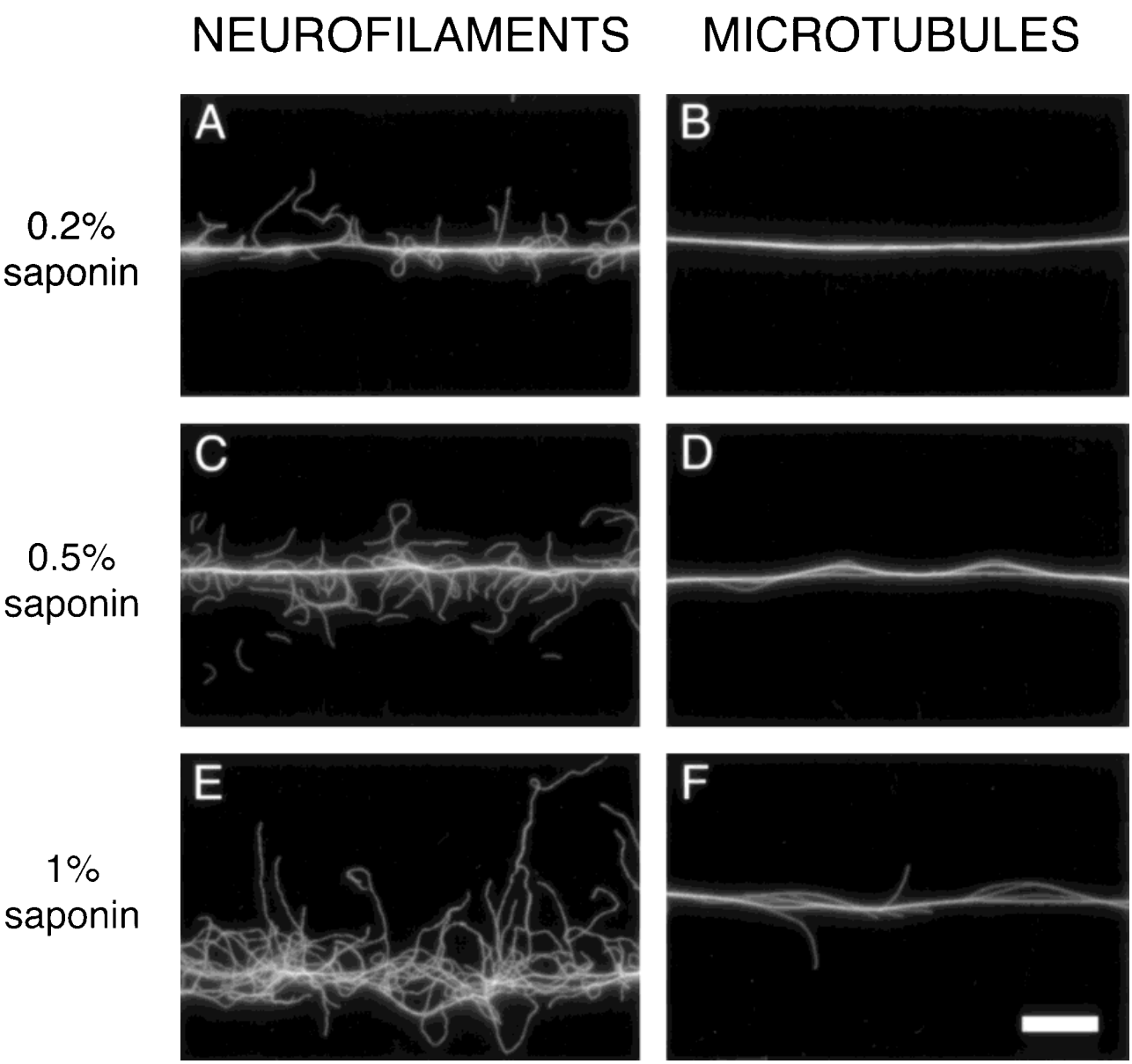
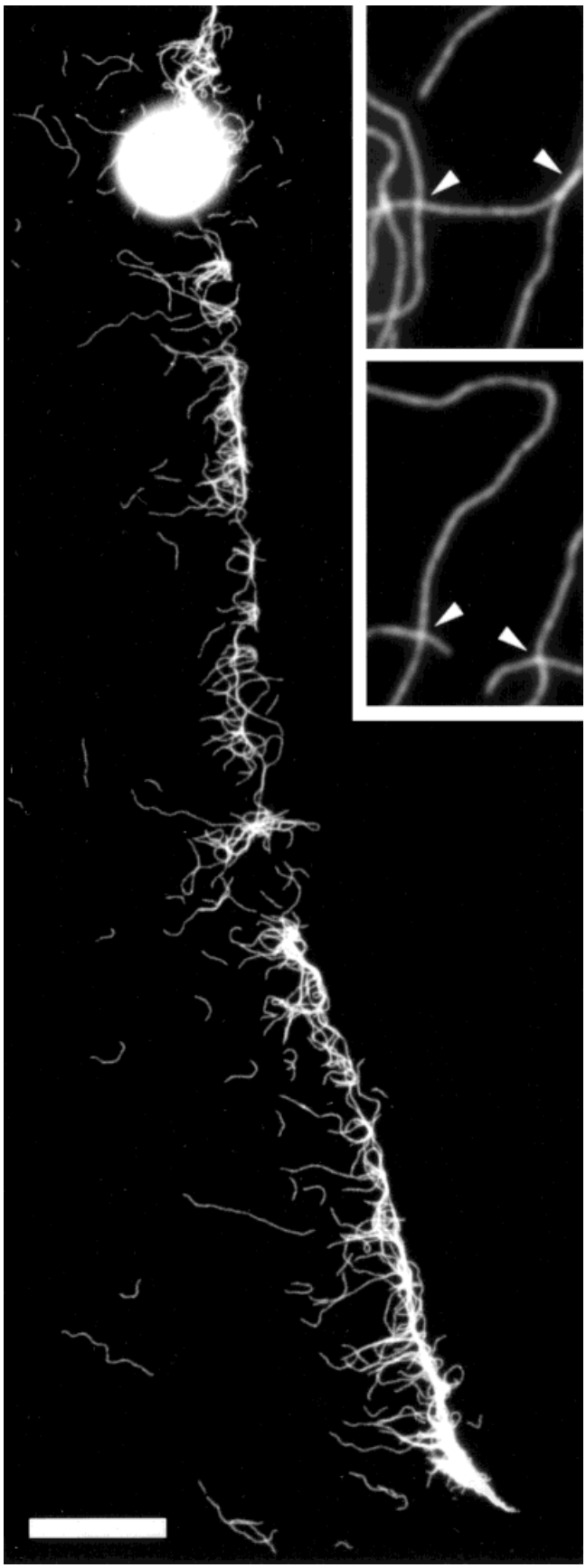


Fig. 2. Dependence of neurofilament and microtubule splaying on detergent concentration. Axons fixed after treatment with 0.2% (**A,B**), 0.5% (**C,D**), and 1% (**E,F**) saponin for 5 min and then processed for immunofluorescence microscopy to visualize neurofilaments (A,C,E) or microtubules (B,D,F). The extent of splaying increases with increasing saponin concentration for both types of polymer. Scale bar = 10 μm.

attached or ''tethered'' to the substrate at these sites. The reason that jiggling filaments were observed in some experiments and not others is not clear, though it is likely that it reflects differences in the adhesiveness of the substrate or in the efficacy of the fixation.

Analysis of Fluorescence Intensity Along The Length of Individual Splayed Filaments

In principle, the filamentous structures that splay apart from each other in these preparations could represent single neurofilaments or bundles of two or more neurofilaments. If the latter were true then one would expect the fluorescence intensity of the filamentous structures to differ depending on the number of neurofilaments that they contained. In addition, one would expect to observe variations in fluorescence intensity along the length of the filamentous structures due to staggered overlap of the bundled neurofilaments because it is highly unlikely that all the neurofilaments in a bundle would be exactly the same length and be arranged in precise register.



Contrary to these predictions, close visual inspection of the splayed cytoskeletons strongly suggests that most of the splayed filaments do not represent neurofilament bundles. For example, the inset in Fig. 3 shows two regions of a splayed axonal cytoskeleton at higher magnification. Most filaments that have splayed apart from the axonal neurofilament bundle appear to stain uniformly along their length and with comparable intensity to each other. Departures from the uniform intensity of these filaments can generally be attributed to the cross-over or co-alignment of two or more filaments (for example, see arrowheads in Fig. 3 inset). These observations suggest that many of the filaments in the splayed cytoskeletons actually represent single neurofilaments and that it is possible to distinguish single and bundled filaments based on their fluorescence intensity.

To test these proposals quantitatively, I have analyzed the fluorescence intensity along the length of splayed filaments. To examine the uniformity of staining along a single filament, I analyzed the longest filament that I encountered in these studies. Fig. 4A shows an image of this filament and Fig. 4B shows the result of a quantitative analysis of fluorescence intensity along its length, performed using the segmented mask method (see Materials and Methods). The total length of this filament is ambiguous because it overlaps with another filament towards its right end, but it measures 183 μm from its left end to the point of overlap. The average fluorescence intensity was 18.0 ± 1.8 ADU/nm (analog-to-digital units per nanometer filament length; min. = 14.2, max. = 21.0) and was relatively constant along the length of the filament over a distance of more than 160 μm (it was not possible to analyze the entire 183 μm length because the constrained distance transformation employed in the segmentation procedure was unable to cope with the sharp hairpin bend at the left end of this filament).

Most splayed filaments in these preparations traced independent paths but occasionally two or more filaments were observed to co-align (‘‘pair up’’) along their length (for example, see Fig. 3 inset). Though this pairing could reflect an interaction between the filaments, it is important to note that the resolution of the light microscope is

Fig. 3. Splayed axonal cytoskeleton at low magnification. Neuron fixed after treatment with 0.5% saponin for 5 min and then processed for immunofluorescence microscopy to visualize the neurofilaments. Neurofilaments splay apart along the entire axon. Note the numerous isolated filaments which have separated completely from the axonal neurofilament bundle so that both ends are visible. **Inset** shows splayed filaments at higher magnification. The filaments have an apparent thickness of about 250–300 nm, which corresponds to the diffraction-limited resolution of the light microscope for the wavelengths of light used in these experiments. **Arrowheads** point to examples of the cross-over or pairing of two filaments. Scale bar = 25 μm for the low magnification image; 5 μm for the high magnification inset.

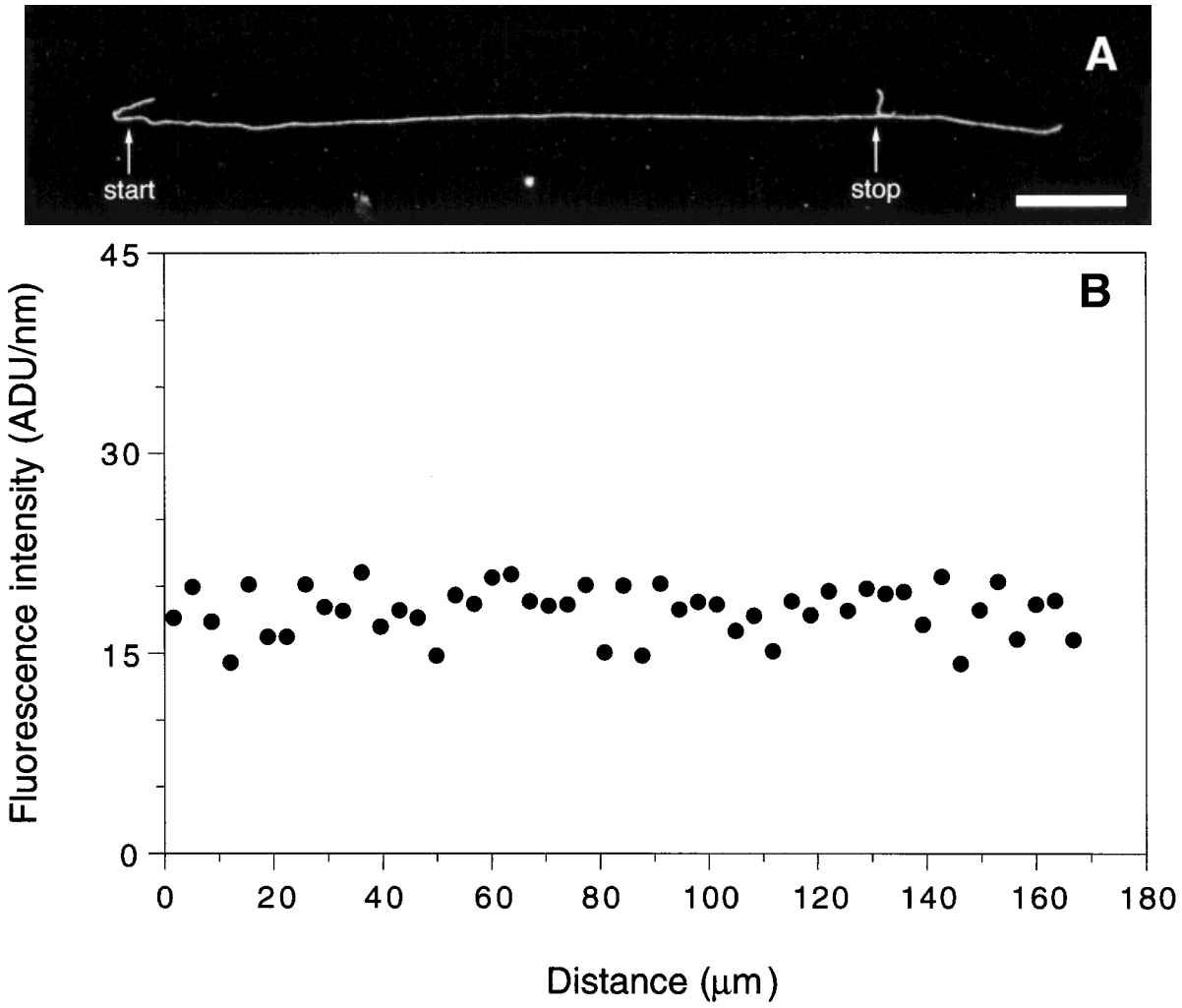


Fig. 4. Analysis of fluorescence intensity along the length of a single filament. **A** shows an image of a long filament which forms a hairpin bend at one end and appears to overlap with a second filament at the other. The filament measures 183 μm from the left-most end to the point of overlap. The **arrows** indicate the region of this filament that was analyzed by the segmented mask method, which measured 169 μm. **B** shows a graph of fluorescence intensity versus distance along the filament. Each point in the graph represents the fluorescence intensity (corrected for background) for one segment in the mask (segment length = 3.4 μm). The units of fluorescence intensity are analog-to-digital units per nanometer of filament length (ADU/nm). The fluorescence intensity is relatively constant along the length of the filament. Scale bar = 25 μm.

diffraction-limited, which means that the two filaments could actually be several hundred nanometers apart and not be resolved. Situations where the co-alignment of three or more filaments could be identified unambiguously were very rare, so I confined my analyses to examples of the pairing of two filaments. To compare the staining intensity of paired and unpaired filaments, I photographed splayed axonal cytoskeletons using a procedure designed to ensure identical processing and minimal photobleaching, and then analyzed the fluorescence intensity along the length of the filaments using the segmented mask method (see Materials and Methods). Filaments were rejected if they contained sharp bends (which cause segmentation artifacts in the segmented mask procedure) or could not be traced unambiguously for at least 10 μm.

Fig. 5 shows three examples of filament pairing from a total of eleven examples that I have analyzed. The segmented masks that were used to quantify fluorescence intensity along the filaments are shown in yellow (Fig. 5A, D & G). Each point in the graphs represents the fluorescence intensity for a single segment in the mask. Comparison of the data for the unpaired (Fig. 5 B, E & H) and paired (Fig. 5 C, F & I) filaments shows that the intensities within each group are similar but that the intensities of the paired filaments are about twice that of the unpaired filaments.

Fig. 6 shows two histograms which summarize the quantitative data for all eleven paired and unpaired filaments, three of which are shown in Fig. 5. If the unpaired filaments represent single neurofilaments then the average intensity of the paired filaments should be twice that of the unpaired filaments. In contrast, no such simple relationship would be expected if the unpaired filaments represented bundles of two or more filaments because it is unlikely that all the bundles would contain exactly the same number of neurofilaments. Statistical comparison of the paired and unpaired filaments in Fig. 6 shows that the variances are not significantly different ($p > .05$, F-test) but that the means are significantly different ($p < 0.001$, t-test). However, multiplication of the fluorescence intensities for the unpaired filaments by a factor of two results in a predicted mean that is not significantly different from the observed mean for the paired filaments ($p > 0.9$, t-test). These observations support the hypothesis that the unpaired filaments in the splayed preparations represent single neurofilaments and they demonstrate that single and bundled filaments can be distinguished based on their staining intensity.

DISCUSSION

I have shown that treatment of cultured neurons with non-ionic detergent causes the axonal neurofilaments to separate from each other in a manner similar to that described previously for axonal microtubules [Brown et al., 1993], allowing individual axonal neurofilaments to be visualized by immunofluorescence microscopy. Brown et al. [1993] called this phenomenon ''fraying'' (fray vb. 1. (trans.) a: to wear (as an edge of cloth) by or as if by rubbing. b: to separate the threads at the edge of: Merriam-Webster, 1993), but more recently Rochlin et al. [1996] have referred to it as ''splaying'' (splay vb. 1. (trans.) to cause to spread outward: Merriam-Webster, 1993). I have decided to adopt the term splaying because I believe that it is a better description of this phenomenon; the term fraying may be taken to imply that the polymers themselves are disrupted.

In the present study, I have found that both neurofilaments and microtubules splay apart from each other in the presence of either saponin or Triton X-100. The minimum concentration of saponin required to produce splaying was 0.2% for microtubules and 0.02% for neurofilaments. The observation that microtubules splay apart in the presence of saponin is contrary to the observations of Brown et al. [1993], who reported that microtubules splay apart from each other in the presence of Triton X-100 but not in the presence of saponin. This discrepancy could reflect differences in cell type and/or culture conditions, but it is more likely due to the presence or absence of salt in the extraction solution. The extraction solution used in the present study contained 0.19M NaCl whereas the extraction solution of Brown et al. [1993] did not; salt is known to enhance the extent of microtubule splaying in the presence of Triton X-100 [Brown et al., 1993] and therefore it seems reasonable to suggest that this might also be the case for saponin.

Visual inspection of the splayed filaments and quantitative analysis of fluorescence intensity along their length both suggest that the majority of the filaments in the splayed cytoskeletons are single neurofilaments and that single and bundled neurofilaments can be distinguished based on their staining intensity. The proposal that single neurofilaments splay apart from the axonal cytoskeleton in the presence of non-ionic detergent is supported by the observations of Letourneau using electron microscopy [1982]. In this study, cultured chick sensory neurons were allowed to extend axons across electron microscopy grids and then treated for 5 min with a microtubule-stabilizing solution containing 0.4% Triton X-100. The axons crossing the electron microscopy grids were then examined as whole-mount preparations by electron microscopy. Splaying of the axonal microtubules was not apparent in this study, but numerous individual neurofilaments were observed to loop out from the detergent-extracted axonal cytoskeletons in a manner very similar to that described in the present work. As in the present study, most of the splayed neurofilaments were single filaments and showed little tendency to bundle together.

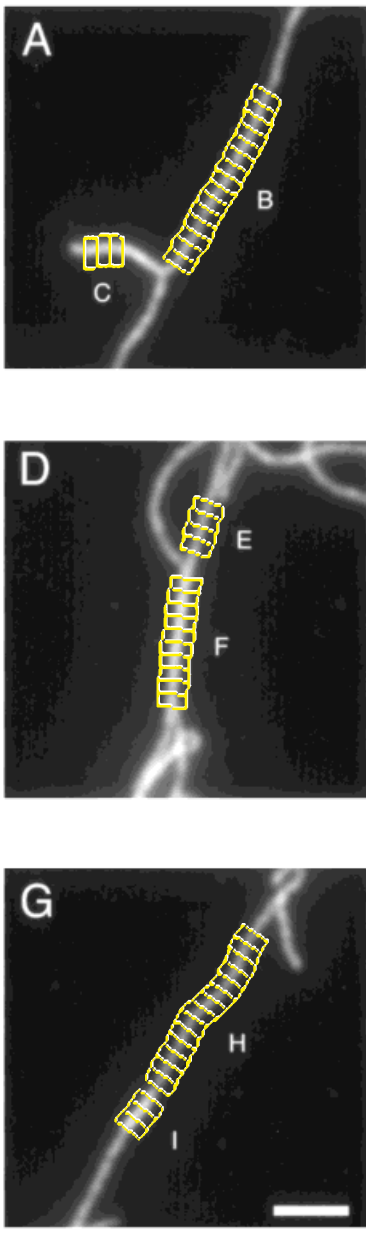
One striking feature of the splayed axonal cytoskeletons is that many of the neurofilaments can be traced for many tens of micrometers, indicating that these polymers can attain relatively long lengths in axons. In most cases it was not possible to measure the total length of these filaments because one or both of the filament ends was obscured. However, filaments that had separated completely from the axonal neurofilament bundle could often be traced unambiguously and these filaments ranged from 1 μm to 183 μm in length. In comparison, serial reconstruction of axons by electron microscopy has shown that the average length of microtubules in cultured embryonic rat DRG neurons is 108 μm [Bray & Bunge, 1981]. Thus the present study indicates that at least some axonal neurofilaments can attain lengths comparable to microtubules in these axons. This is consistent with the measurements of Burton & Wentz [1992] on neurofilaments in bullfrog olfactory neurons. Using serial reconstruction of axons by electron microscopy, these authors estimated that the average length of the neurofilaments was 118 μm.

The dependence of microtubule and neurofilament splaying on detergent concentration suggests that this

phenomenon involves partial or complete dissolution of the axonal plasma membrane. The lateral movement of the polymers apart from each other in these detergent-treated axons most likely reflects diffusion in response to the thermal forces of Brownian motion. The difference in the detergent concentration required to cause splaying of neurofilaments and microtubules may reflect specific features of the interaction or organization of these polymers in axons, but I suspect that it is principally due to differences in their length and flexibility. Neurofilaments, which are narrower and more flexible than microtubules, may jiggle their way apart in a partially demembranated axon more readily. By contrast, microtubules are wider and more rigid [Felgner et al., 1996] and thus may require more extensive demembranation in order to spread apart.

Ultrastructural studies on neurofilaments in axons have led to the impression that these polymers are extensively cross-linked by their side-arm projections. This perspective has been reinforced by quick-freeze deep-etch electron microscopy of large neurofilament-rich axons, in which these polymers are packed very densely [Hirokawa, 1982; Schnapp & Reese, 1982; Tsukita et al., 1982]. However, several lines of evidence suggest that the side-arm projections that appear to interconnect neurofilaments actually represent weak or transient associations and that their principal function may be to hold adjacent polymers at arm's length, thereby maximizing their space-filling properties, rather than to link them together. For example, statistical analysis of neurofilament organization in axonal cross-sections has demonstrated that these polymers adopt a random distribution in regions where they are not packed densely, indicating that they do not have a strong affinity for each other [Price et al., 1993; Price et al., 1988]. Furthermore, studies on squid axons have shown that neurofilaments appear to move apart freely after axoplasm is separated from the axonal plasma membrane by extrusion [Brown & Lasek, 1993; Brown & Lasek, 1995]. Thus the tendency of neurofilaments to splay apart from each other in detergent-treated axons of cultured neurons is consistent with a growing body of evidence indicating that these cytoskeletal polymers are not extensively cross-linked to each other in axons.

In conclusion, I have demonstrated that individual neurofilaments can be visualized in splayed axonal cytoskeletons using conventional immunofluorescence microscopy. This technique provides a simple method that should prove useful to researchers that are interested in studying the polypeptide composition or post-translational modification of axonal neurofilaments along their length. Presently I am using the splaying technique to investigate variations in the phosphorylation state of neurofilament proteins along the length of single neurofilaments.



2 COLOR

Fig. 5A,D,G.

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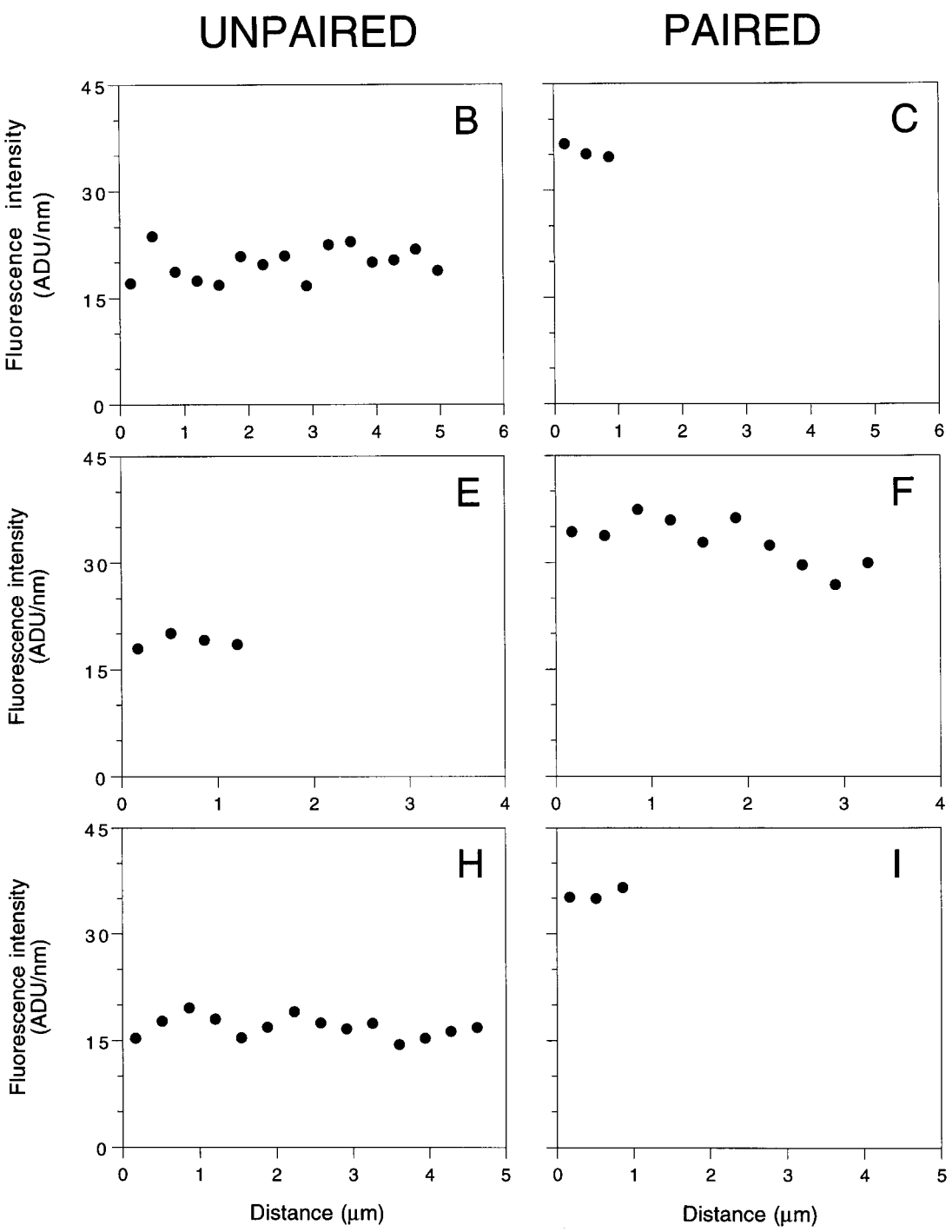

Fig. 5. Analysis of fluorescence intensity along paired and unpaired filaments. This figure shows three examples in which two filaments were observed to pair up. **A, D** and **G** show images of the paired and unpaired neurofilaments and the segmented masks (yellow) that were used for the analyses. For simplicity, the background masks which were used to compute the background fluorescent intensity are not shown. Graphs of fluorescence intensity versus distance along the filament are shown for one of the two unpaired filaments in each image (**B,E,H**) and for each paired filament (**C,F,I**). Each point on the graphs represents the total fluorescence intensity (corrected for background) for one segment in the mask (segment length = 0.34 µm). The units of fluorescence intensity are analog-to-digital units per nanometer of filament length (ADU/nm). Distance is measured from top to bottom for masks that are orientated vertically in the images and from right to left for masks that are orientated horizontally. The mean fluorescence intensities were 19.9 ± 2.3 (n = 15) in B, 18.9 ± 0.9 (n = 4) in E, and 16.9 ± 1.4 (n = 14) in H for the unpaired filaments and 35.4 ± 1.0 (n = 3) in C, 32.9 ± 3.3 (n = 10) in F, and 35.5 ± 0.9 (n = 3) in I, for the paired filaments. Scale bar = 2 µm.

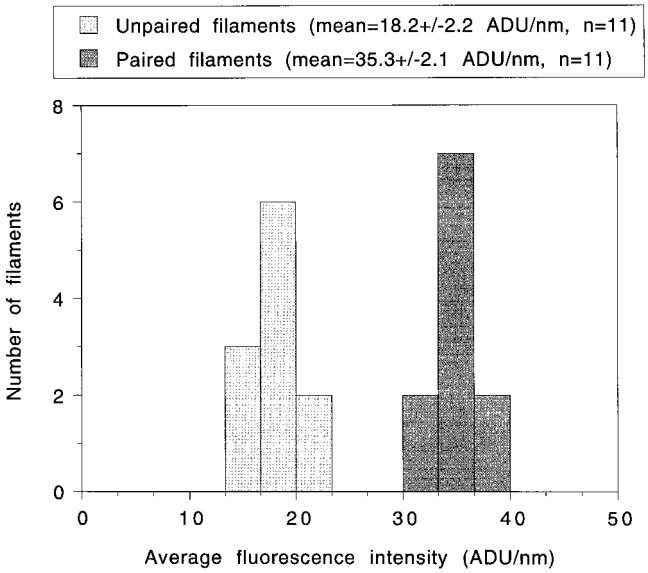

Fig. 6. Histogram summarizing the quantitative analyses of 11 unpaired and paired filaments, including the three examples shown in Fig. 5. The number of segments in the masks ranged from 4–33 for the unpaired filaments (mean = 16) and from 3–22 for the paired filaments (mean = 9). The fluorescence intensities for the unpaired and paired filaments ranged from 16.0–22.7 ADU/nm and 32.9–39.2 ADU/nm respectively. The variances for the two groups are not significantly different ($p > 0.5$, F-test) but the means are significantly different ($p < 0.001$, t-test). Multiplication of the intensities of the unpaired filaments by a factor of two yielded a mean fluorescence intensity of 36.3 ± 4.4 ADU/nm, which is not significantly different from the actual intensity measurement for the paired filaments ($p > 0.9$, t-test).

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