A New Methodological Approach for Studying Axonal Transport:
Cytofluorometric Scanning of Nerves

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A new technique for studying axonal transport has been developed. The technique, which is based on histofluorescence techniques, enables the measurement of several different accumulated substances and parameters within a single nerve in relation to a nerve crush or local cooling. Any substance that can be made to fluoresce can be measured. The tissue is treated according to the formaldehyde-induced fluorescence method of Hillarp and Falck for visualization of monoamines, or according to the indirect immunofluorescence method. For immunofluorescence the nerve is cryostat-sectioned and various sections can be incubated with primary antisera against different antigens. After incubation and mounting the sections are placed in a cytofluorimeter (Leitz MPV II). They are passed under a measuring slit at a steady speed by a motor driven cross-table. The fluorescence intensity passing through the measuring slit is continuously registered by a recording unit with an integrator. This recorder produces a graphical nerve accumulation profile, and the area under the profile, relating to the fluorescence, is expressed in arbitrary units.

Introduction

Axonal transport of endogenous substances, such as neurotransmitter organelles and enzymes involved in synthesis and breakdown of transmitter organelles, takes place in all mammalian and invertebrate neurons. This transport, which occurs in both anterograde and retrograde directions, is essential for the function of the neuron (21,25,36). The study of axonal transport of endogenous substances has mostly taken advantage of the "stop flow" principle, which includes interruption of the axonal transport by a ligature or a crush (2,4,8,12,28,31,32,35,49), cold block (3), or application of various chemicals, e.g., microtubule destroying agents, (1,9,10,14,24,26,43) to the nerve. The interruption gives rise to an accumulation proximal and/or distal to the area of block and the accumulation can then be studied by quantitative or morphological methods.

Quantitative assays of noradrenaline (NA) content (2,12,13), of enzyme activities of dopamine β-hydroxylase (DBH) (17,22,31,39,40,42), of DOPA decarboxylase (DDC) (49), or of tyrosine hydroxylase (TH) (4,28,32,41,49) in single or pooled nerves have allowed calculations of the average rate of transport, transportable fractions, directions of transport, and events occurring during axonal transport e.g., loading of the granular stores of NA during the transport/accumulation period (13). Morphological methods, on the other hand, have included the

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formaldehyde-induced fluorescence method (FIF) of Hillarp and Falck (7,11,16) for localization of catecholamines (CA) and 5-hydroxytryptamine, immunofluorescence methods, e.g., the indirect immunofluorescence method of Coons (6) for studying various macromolecules, such as enzymes (17, 39–42), and cytochemical procedures, e.g., acetylcholinesterase (AChE) staining (cf. ref. 36).

The morphological techniques permit the study of localization and distribution of accumulated material within nerves or axons. With morphological methods it is possible to estimate relatively large variations of accumulated material on a subjective scale (e.g., strong, moderate, weak). Objective quantification of changes are, however, difficult to carry out.

The present article will describe a sensitive method, using material prepared according to morphological methods, that can give quantitative information as well. The advantages of such a method may be several but one important issue is that several substances can be measured in a single nerve. The material to be studied must be fluorescent, and thus the fluorescence intensity of the accumulated material in axons along and across the nerve is then recorded by cytofluorimetric quantitation during scanning.

Materials and Methods

Animals and Biological Models

Male Sprague-Dawley rats (180–200 g) were used. Accumulations in crushed sciatic nerves were used as a measure for axonal transport. Nerve crushes were made 15 mm below the foramen infrapiriformis using a silk suture (3 x 0) that was carefully introduced under the nerve and gently pulled against a glass rod (4 mm diameter), placed on top of and alongside the nerve, for 5 sec. (For a detailed description of the method, see ref. 35). All operations were performed under ether anesthesia. The following three types of experiments were performed:

1) Experiments concerning technical standardizations. Nerves crush-operated 12 or 18 hr before sacrifice were used.

2) Measurements of accumulation rates for NA, DBH, and TH. The nerves were crushed as described above and dissected out 0,1,3,6,9, and 12 hr after this operation for NA measurements, and 0,3,6,9, and 12 hr after this operation for DBH and TH.

3) Cholchicine/lumi-cholchicine experiments. The mitosis inhibitor colchicine (COL; Sigma Chemical Co., St. Louis, MO) was used as test substance and for preparation of its virtually inactive isomer lumi-colchicine (lumi-COL; for detailed description, see ref. 48). Under ether anesthesia the sciatic nerves were injected subperineurally with 3–5 μl of a solution (0.1 M) of COL, lumi-COL, or the vehicle (10% ethanol in saline) about 5 mm distal to the foramen infrapiriformis, using a 32 gauge needle and a microsyringe. All injections were made under a dissection microscope. Six hours after injection, the nerves were crush-operated 10 mm distal to the site of injection, and the nerves were dissected 18 hr later. After dissection, the nerves were treated for visualization of NA according to the FIF method (see below).

Fluorescence Microscopic Methods

Noradrenaline. The FIF method of Hillarp–Falck was used (7,11,16). Dissected nerves were placed on small stainless steel plates, with a groove for the nerve to keep it straight, rapidly frozen in liquid propane and freeze-dried for 3 days. After freeze-drying, the nerves were reacted with paraformaldehyde vapor at 80°C for 1 hr and then embedded in paraffin in vacuo and sectioned in 10 μm sections.

Dopamine β-hydroxylase and tyrosine hydroxylase. The indirect immunofluorescence method of Coons was used (6). The animals were perfusion fixed intracardially with ice-cold 4% buffered paraformaldehyde, pH 7.4. After dissection the nerves were postfixed for 4 hr and rinsed for at least 12 hr in phosphate buffer, pH 7.4, with 5% sucrose for cryoprotection. The nerves were sectioned at 10 μ in a cryostat, placed on gelatin-coated slides, and incubated with specific antisera (produced in rabbits) (20) against DBH or TH in the first step (+4°C for 12–18 hr) and with swine anti-rabbit immunoglobulin (lg) labeled with fluorescein isothiocyanate (FITC) (Dakopatts, Copenhagen, Denmark) for 1 hr at 37°C in the second step. Preincubation with normal swine serum (1:10, 1 hr at 37°C) was performed to reduce nonspecific staining. After careful rinsing in phosphate buffered saline (PBS), the sections were mounted in PBS–glycerin (1:1), pH 7.3.

Cytofluorimetric Scanning

The cytofluorimetric scanning device (Figure 1) consisted of a fluorescence microscope with incident and transmitted illumination (Orthoplan, Leitz) equipped with a cytofluorimeter (Leitz MPV2). The cytofluorimetric system, using the incident light pathway, consisted of 1) a high pressure mercury lamp (Osram HBO 200) operated on

![Figure 1. Representation of the technical setup for the scanning equipment.](https://example.com/figure1.png)
Data Analysis

In the scanning graphs the site of the crush as well as the accumulation area were easily identified, and the basal level of fluorescence was determined (Figure 2). The integrator registered total fluorescence along the nerve. Therefore the integrated value (arbitrary units) under the accumulation curve corresponded to basal area plus accumulation area (T in Figure 2). The nerve basal area was then calculated for a similar length of nonaccumulated nerve, and subtracted from the total accumulation area. (Total accumulated area (T) minus basal area = accumulated fluorescence in arbitrary units, as noted in Figure 2). The figure obtained represented the net accumulation in the nerve, expressed in arbitrary units.

Variations between Sections

This was tested for NA fluorescence. Nerves crushed 12 hr before the rats were killed were chosen for presentation (below), but such tests were performed for every nerve assayed by cytofluorimetric scanning. Fifteen sections per nerve of five individual nerves were analyzed. In Figure 3 the scanning curves for 15 sections of one such nerve are shown; each curve has its individual profile, but the general form of the curves is the same. The fluorescence (arbitrary units) for the accumulation areas are given in each curve, and the scatter is between 18 and 22, mean ± SEM = 20.2 ± 0.35. The superimposed curves give an accumulation profile characteristic for NA accumulations in 12 hr crushed rat sciatic nerves.

Results

Accumulation of NA

The accumulation of NA increased with time proximal and distal to a crush, as indicated in Figures 4 and 5. Proximal accumulations increased gradually with time, while distal accumulations appeared to reach a maximum at 6 hr and then remained unchanged, as described earlier (8,12). The time course for NA accumulation 0–12 hr after crushing is presented in Figure 6. At least three nerves per each time period following crushing were measured and 11–28 sections were

Figure 3. Scanning profiles from a rat sciatic nerve crushed 12 hr prior to being treated for visualization of noradrenaline (NA) according to the FIIF method. Fifteen individual nerve sections and a superposed profile of them are shown, demonstrating the variation of accumulation profiles within one nerve. Figures in the graphs represent the net accumulation expressed in arbitrary units. The mean value was 22.2 ± 0.35.

A mean value ± SEM was calculated for each substance in the nerve; it represents at least 5 sections/substance studied.

Figure 2. Schematic of the basic principles of the scanning technique. At a constant speed (40 µ/sec) the nerve section is passed under the measuring slit. The fluorescence intensity is continuously recorded, as indicated in the lower part of the figure. The nerve basal area (calculated for a length of nerve similar to the length of the total accumulation area) is then subtracted from the area T, giving a net accumulation, expressed in arbitrary units.
Figure 4. Fluorescence micrographs of accumulation of NA in rat sciatic nerve proximal and distal to a crush performed 3–12 hr earlier: (a) 3 hr; (b) 6 hr; (c) 9 hr; and (d) 12 hr. The proximal part of the nerves are at the top of each plate. The site of the crushing is indicated (>). Original magnification ×63. Bar = 10 μm.
scanned per nerve. This time course is strikingly similar to the accumulation curve obtained by biochemical assays (12,13).

It is interesting to note that the scanning technique also registered a "supralinear" increase in NA content after 9 hr as had been observed in a previous biochemical study (13). An explanation for this finding lies in the increased loading of the NA vesicles during accumulation.

Accumulation of DβH-like Immunoreactivity

Strongly fluorescent DβH-like immunoreactive material accumulated both proximal and distal to a crush as early as 3 hr after the crush. The proximal accumulations thereafter increased linearly up to 12 hr, while the distal accumulation appeared unchanged after 6 hr. The increase on the proximal side during the first 6 hr was more rapid than later (Figure 6). This is in agreement with other investigations where the enzymatic activity of DβH was studied in crushed sciatic nerve of guinea pig (39,40) or rat (22). A striking similarity between the accumulations of DβH and of NA fluorescence was observed up to 9 hr after crushing. This observation is in agreement with the co-compartmentalization of NA and DβH within the same organelle during fast axonal transport in the adrenergic axons. After 9 hr, the NA accumulation increased more rapidly than the DβH-accumulation, as mentioned above. This deviation between the accumulations of the two substances seen after 9 hr is in all likelihood explained by the estimated increase in granular content of NA due to local synthesis in the axons of NA, which is then incorporated into granules during the accumulation period (13). DβH, on the other hand, can only increase by delivery of material by axonal transport, since virtually all neuronal synthesis of protein takes place in the cell bodies (cf. ref. 33).

Accumulation of TH-like Immunoreactivity

As seen from Figure 6, TH-like immunoreactivity accumulated less rapidly (Figure 7) than did DβH-like material, but much faster than was expected for a purely soluble enzyme (cf. ref. 49). This result differed from data from dog splenic nerve (32) where no accumulation of TH enzyme activity was found, in contrast to the marked accumulation of DβH enzyme activity (31). However, data from rat (40) and rabbit sciatic nerve (4) demonstrate that TH activity accumulates almost as rapidly as DβH activity proximal to a crush. This may suggest that in the intact animal TH is not an entirely soluble enzyme, but may be reversibly organelle affiliated (cf. ref. 4).

COL/lumi-COL Experiments

As an example of the possible applications of this scanning technique for studying localization and amounts of accumulated fluorescence along a nerve, the COL/lumi-COL experiment was performed. It has been demonstrated earlier with the use of the two mitotic inhibitors COL and vinblastine that local injections of these substances into the rat sciatic nerve arrest axonal transport of NA (9,10,24,26). Local injection with the vehicle saline had no such effect. Since COL binds to cellular membranes other than microtubules this COL experiment does not constitute evidence for the involvement of microtubules in axonal transport of NA-storing organelles. The isomer to COL, lumi-COL, has many of the membrane-binding characteristics of the "mother" substance COL, but a much lower affinity to tubulin (43,48). This isomer has been used earlier to investigate whether axonal transport in motor nerves is dependent on microtubules. It was found that the anterograde transport of acetylcholine, AChE, and choline-acetyltransferase (ChAT) were inhibited by COL, but not by lumi-COL (14,24). The COL/lumi-COL test was now applied to the adrenergic system. Figure 8 shows two scannings, one from a COL-treated nerve and one from a nerve treated with...
lumi-COL (0.1 M in 3–5 μl). A set of microphotographs from the same nerves as in Figure 8 are shown in Figure 9 for comparison. The drugs were injected 8 hr before a crush 9–10 mm more distal, and dissected out 18 hr later. In this experiment COL caused NA accumulations over a 4 mm length of the nerve in the area of injection. A small accumulation was present just above the crush in this nerve section. The isomer, lumi-COL, had no transport inhibitory effect in the area of injection, and consequently a large accumulation of NA fluorescence was present proximal to the crush. This is consistent with previous findings (9,10) that COL, which depolymerizes microtubules, interferes with the axonal transport of NA granules along the nerve. Lumi-COL, however, which does not bind to tubulin, has no detectable effect on axonal transport. This supports the view that microtubules are essential for axonal transport of NA granules but the exact mechanism is so far unknown (1,9,10).

Discussion

The present scanning technique is based on principles for cytofluorimetric assay of tissue monoamines (15,29,30) and cytofluorimetric scanning of sympathetically innervated tissues (29,44,45). These principles have now been adapted to a scanning device that makes it possible to measure an accumulation profile along a nerve. When using this method to study axonal transport phenomena in nerve certain technical details are of great importance.

Methods for Preparation of Sections

The procedure quantitates the fluorescence intensity along a nerve section and great care must be taken in the preparation of tissues to obtain standardized conditions and to avoid artifacts and disturbing factors.

**Straight nerve sections.** Nerve sections must be straight along the entire length to be measured, since the scanning device, which only operates in X or Y axis, cannot “follow” a nerve section that is curved. For the FIF method the technique employed was as follows: The nerve was frozen on a steel plate with a groove for the nerve to keep it straight during freezing and freeze-drying. This methodological approach was successful; no freezing artifacts were noted and the specimens were relatively easy to handle during the following steps. For immunohistochemistry, the fixed nerve was frozen on similar steel plates prior to sectioning. This procedure, which gave straight nerves, greatly improved the quality of the sections.

**Perpendicular crushes.** A perfectly perpendicular crush prevents interference between proximal and distal accumulations during scanning. Such crushes can be obtained by careful operation and dissection procedures. A small deviation, only to the point where it is still possible to recognize the crush area in the graph, may, however, be acceptable.

**Artifacts in the nerve section.** Sections with artifacts, such as dust or hair, cracks or foldings, may represent a problem. Hair and air borne dust are autofluorescent and will be...
Figure 9. Fluorescence micrographs from the nerves in Figure 8 demonstrating the distribution of NA in a colchicine (a, c) and in a lumi-colchicine (b, d) injected nerve. In the colchicine-treated nerve accumulations of NA are present in the injection area (a), while only a small amount was present proximal to the crush (c). In the lumi-colchicine-treated nerve there was no accumulation in the injection area (b) but much transported NA had accumulated proximal to the crush (d). Site of crushes are indicated by arrows. Original magnification ×63. Bar = 10 μm.
TH, 12 h accumulation

a

no pre-incub.

b

pre-incub. NSS

← proximally

Figure 10. Scanning graphs from a nerve treated for visualization of TH immunoreactivity 12 hr after crush operation both with (b) and without (a) preincubation with normal swine serum (NSS) to reduce nonspecific staining. The figure demonstrates the reduction of fluorescence intensity (~30%) caused by depression of nonspecific fluorescence in monocytes by preincubation with NSS. The remaining fluorescence is due solely to TH immunoreactivity.

registered in the same way as specific fluorescence. However, with the MPV-2 equipment used, the investigator can continuously control the nerve section by eye and make note of any artifacts disturbing the measurements.

Another problem may be "artifacts" of biological origin, e.g., erythrocytes, mast cells, or monocytes, invading the crush area. Erythrocytes in the section originate from blood vessels within the epineurium that have been injured during the crushing procedure. Erythrocytes may have a yellowish fluorescence, particularly in sections prepared according to the FIF method, but sometimes also in nerves perfusion fixed for immunocytochemistry. However, carefully performed nerve crushes reduced this artifact and less than 1% of the nerves in the study had to be excluded for this reason. Mast cells in rats contain high amounts of serotonin (5-HT), and their fluorescence may seriously disturb the measurements. When present, however, mast cells are usually located in the connective tissue sheet in the periphery of the nerve and hence, interfere very little with the registrations in the present study. A further improvement was introduced in the latter part of this study by stripping the connective tissue sheet in conjunction with the dissection procedure. Stripping is, however, not suitable for nerves longer than 5–7 mm because of the trauma involved, which may cause derangements between the nerve fasicles, disorganizing the crush area. Monocytes are often fluorescent in sections prepared for immunofluorescent techniques, and localize near the crush area. Preincubations of the sections with normal swine serum (NSS), or bovine serum albumin prior to the first incubation with the specific antisera, efficiently reduced the fluorescence of the monocytes to levels that did not interfere with measurements of the accumulated substance. Figure 10 shows an example of sections from a nerve accumulated for 12 hr and prepared for demonstration of TH-like material. This nerve was heavily infiltrated with monocytes between the accumulated axons. Preincubation with NSS (1:10, 1 hr at 37°C) virtually abolished the nonspecific fluorescence of the monocytes and thereby reduced the registered fluorescence value of the accumulation area by approximately 30%. Thus, artifacts in the nerve sections are possible to avoid or to reduce to an acceptable level. However, in general some sections of each studied nerve must be excluded from measurements.

Scanning speed and size of the measuring slit. All scanning must be performed at the same speed. Even a small deviation in scanning speed will result in false estimations of accumulated amounts. In our system, careful investigations of the time needed for the measuring slit to move a certain distance (10 mm) were regularly performed and no deviation was observed. To minimize mistakes the scanning device was preset at the lowest speed to avoid adjustment errors. The size of the measuring slit was chosen to fit the size across our model nerve, which was approximately 1000 μm. The width of the slit was 100 μm, which was found to give an optimal signal-to-noise ratio.

Fading of fluorescence. In quantitative studies the fading of the fluorescence is generally a problem. This problem can be circumvented if the time of fading is constant. In this system, the scanning speed and the intensity of the exciting light are constant and the area under the measuring slit is always illuminated for the same length of time, i.e., the time needed to scan the distance from the advancing periphery of the visual field to the measuring slit. By mounting the tissue sections in high pH (8.6) solution, the intensity of FITC fluorescence may be increased (37); however, this was not done in this study. The rate of fading is reported to be decreased if the sections are mounted in media containing paraphenylene diamine (28a). This will be tested in forthcoming experiments.

Relations between Fluorescence Intensity and Amounts of Accumulated Material

It is well established that the intensity of the FIF-induced NA fluorescence is proportional to the concentration of NA in tissues. Even with the high NA concentrations in nerve terminals (varicosities of the rat iris), cytofluorimetric measurements gave results very similar to biochemical assays, and a
linear relationship between NA fluorescence and NA concentrations was established (29,30,43,44). Using the present method, the NA accumulation curves from rat sciatic nerve, assayed biochemically (12), and the cytofluorimetric scanning curve was nearly identical. The accumulation curves for FITC-induced immunofluorescence of DβH and TH were similar to those obtained by biochemical assays of enzyme activities (22,48). Previously several investigators have performed cytofluorimetric quantitations, using FITC-labeled antibodies with success (18,19,27,46), and thus, immunofluorescence can be quantified also.

There have been other attempts to quantify accumulations of immunoreactive DβH in sections of the sciatic nerve (see e.g., refs. 38 and 40). In those studies the areas of accumulation of FITC fluorescence were cut out from micrographs and weighed. The scanning technique described in this article measures not only the areas of accumulation, but can also register variations in intensity of fluorescence in various parts of the accumulated axons, e.g., higher near the crush and decreasing with distance proximally or distally. (If the nerve is scanned transversally instead of longitudinally, recordings demonstrating the accumulation pattern in individual axons or groups of axons can be made.) It cannot be claimed that the cytofluorimetrically registered fluorescence is directly proportional to the number of immunoreactive molecules present in the nerve, as is the case for NA fluorescence. However, the differences in FITC fluorescence in various parts of the axons gives information on the relative concentration of immunoreactive molecules. A detailed study comparing scanning data with radioimmunoassays of the macromolecule to be studied is needed before the exact relationship between FITC fluorescence and content of immunoreactive material in accumulated nerves can be established.

Nevertheless, when compared with data from biochemical studies on DβH activity in rat sciatic nerve (22), the proximal accumulation curves, based on scanning data of DβH immunofluorescence, were very similar. The enzyme activity of a macromolecule does not always, however, parallel the number of protein molecules, as shown beautifully for the retrograde accumulations of DβH in guinea pig sciatic nerve (41). The DβH accumulating distally (in transit somatopetally) had a lower enzyme activity per immunoreactive unit of DβH than the proximally accumulated DβH, in transit from the soma. This indicates that the DβH that is transported in the retrograde direction is partly inactive (41). Immunofluorescence studies recognize the DβH molecule, whether it is enzymatically active or not. Therefore, the present scanning technique will prove useful for quantitations of not only FIT-induced fluorescence, but also of immunofluorescence of substances undergoing intraaxonal transport.

Limitations and Advantages

At present the method is not sensitive enough to measure normal levels of fluorescent material in a reliable manner. Therefore estimations of “transportable fractions” of axonally transported material (e.g., the clearance of material 5–10 mm distal to a single crush, or in the middle part of a nerve segment, isolated by two simultaneously applied crushes) (cf. ref. 48) cannot be performed as yet. Thus, the absolute rates of transport of a substance cannot be determined, but the average rates of axonal transport can be estimated from the slope of the accumulation curve. This limitation can, however, be circumvented by using a cold-block technique instead of ligation to block transport (3). Material accumulated against the cold-block region can, after rewarming, resume axonal transport, and a peak of fluorescent material will move along the nerve and can be registered by the cytofluorimetric scanning procedure.

The advantages with the method may be many.

1) Many different substances can be studied in the same nerve, if consecutive sections are treated with various antisera. Also monoamine fluorescence can be studied in tissues perfusion-fixed for immunofluorescence, if the composition of the fixative is suitable (see ref. 37). This allows the direct comparison of the effects of a particular experimental treatment on various substances in the same animal and nerve.

2) The number of animals used can be decreased, since each nerve can be used for studying many various substances.

3) Quantitative information can be obtained from sections where the morphology can also be studied and documented photographically.

4) Morphometric studies can be performed and the distribution of fluorescence material within single axons or axon bundles can be documented on curves and plots.

5) It might also be possible to use the method to study axonal transport in in vitro systems, e.g., tissue culture of neurons where the single individual neuron can be studied.

6) The retrograde (and anterograde) transport of fluorescent tracers in various systems can be analyzed.

Literature Cited


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