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Technical Note

Neuronal Tracing with DiI: Decalcification, Cryosectioning, and Photoconversion for Light and Electron Microscopic Analysis[1]

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The molecule 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) is a fluorescent dye which diffuses within cell membranes. The properties of DiI diffusion and fluorescence are maintained in aldehyde-fixed tissue, thereby allowing selective neuronal tracing post mortem. We describe three modifications of this tracing method. First, while DiL diffuses along neuronal membranes the tissue can be decalcified in EDTA at 37°C. Tracing in decalcified tissue extends the possible application of the DiI technique to the investigation of neuronal tissue enclosed in bony structures. Second, we describe a protocol that allows sectioning of DiI-injected tissue on a cryostat with minimal subsequent spread of DiI in dried sections. Third, we demonstrate that DiI label of fluorescent neurons in cryosections as well as Vibratome sections can be photo-oxidated and converted to a stable diaminobenzidine reaction product. The photoconverted DiI label is electron dense and allows analysis of labeled cell bodies and processes at the electron microscopic level. DiI does not stay confined to the surface cell membrane in fixed tissue but reaches internal organelles, presumably via membranes of the endoplasmic reticulum, and concentrates in microsomal structures adjacent to mitochondria. Photoconversion of DiI label is compatible with gold immunocytochemistry. Long-term incubation and subsequent photoconversion of post-mortem DiI-labeled neurons provides remarkable tissue preservation at the ultrastructural level. (*J Histochem Cytochem 38:725-733, 1990*)

KEY WORDS: Cryosectioning; Decalcification; Fluorescence microscopy; Neuronal tracing technique; Fluorescent carbocyanine tracers (DiI); Intramembranous diffusion; Diaminobenzidine (DAB) photooxidation; Fixed slice; Electron microscopy; Bird (chicken).

Introduction

The carbocyanine compounds DiI and DiO not only label neurons in vivo and in vitro (8,9) but are also suitable for post-mortem neuronal tracing in aldehyde-fixed tissue (7). This technique is particularly useful for the study of embryonic circuits (7,8,12,18) and has been successfully applied to human neuronal tissue (4). Neuronal structures that are enclosed in bony tissue must be either dissected or decalcified before sectioning. We tested several decalcification methods for their compatibility with DiI labeling. Here, we report that DiI-injected tissue can be decalcified with EDTA, but other decalcifying agents (e.g., formic acid) are not compatible with DiI tracing.

In general, the use of a vibratome rather than a cryostat is recommended for sectioning of fixed tissue injected post mortem with DiI (7). In tissue fixed with paraformaldehyde, label spreads within minutes after cryosectioning (9), and drying of tissue is believed to produce severe degeneration of DiI label (7). The present study describes a fixation protocol that preserves DiI label in cryosections dried for several months (and in coverslipped cryosections for hours to weeks) without significant spread of label into adjacent tissue.

Fluorescent label in tissue sections can be transformed to an insoluble diaminobenzidine reaction product by photo-oxidation (11,12,14). The present study shows that DiI-labeled cryosections, as well as Vibratome sections, can be photoconverted and describes the ultrastructural distribution of photoconverted DiI label and its compatibility with immunogold labeling in tissue that has been incubated in fixative for up to 2 years. The distribution of DAB-photoconverted DiI label in cell bodies at the electron microscopic level provides new insights into the abilities and limitations of DiI diffusion in fixed tissue.

Materials and Methods

The dyes DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) and DiO (3,3'-dioctadecyloxacarbocyanine perchlorate) were obtained from Molecular Probes (Eugene, OR). Post-hatch chickens and chicken embryos (White Leghorn; H+N International, Redmond, WA) were used for investigation. The National Research Council's guide for care and use of laboratory animals was followed. Most dye injections were made into the

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otocyst (n = 41), auditory brainstem nuclei (n = 32), and the paratympanic organ (n = 30). Other injections were made into the vestibular, facial, trigeminal, and the glossopharyngeal ganglia and peripheral portions of these cranial nerves. DiO was tested for cryosectioning but not for decalcification and photo-oxidation. The following protocols and comments are based on a total of 124 injections of DiI or DiO into neuronal tissue.

DiI or DiO Injection. Older chicken embryos (>12 days of incubation) and post-hatch birds were anesthetized with sodium pentobarbital (Nembutal; 50 mg/kg body weight). All animals were perfused or immersion-fixed (younger embryos) with 1.3–2.0% paraformaldehyde and 0.5–1.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). DiI and DiO were applied as a crystal, or they were dissolved by sonication in dimethylformamide (final concentration 0.5–1.0%). In most cases we used micropipettes (tip diameter 25–70 μm), and pressure-injected 1–5 μl of DiI solution with a Picospritzer (General Valve Corporation; East Hanover, NJ). The injected tissue was stored for periods of 2 weeks to 2 years at room temperature or at 37°C, usually in the same fixative used for the perfusion of the animal.

EDTA Decalcification. Perfused or immersion-fixed chicken heads were injected with DiI and placed in a mixture of 1 part fixative to 9 parts EDTA. We used EDTA prepared according to the protocol of Brain (2): 100 g ethylenediaminetetraacetate disodium (EDTA) salt were dissolved in 600 ml of water, and 280 ml of 1 N sodium hydroxide solution were added (final pH 7.4); this solution was diluted to a final volume of 1000 ml. The tissue was stored in the decalcification solution at 37°C. The volume ratio of specimen and decalcification solution was in the range of 1:50. After 2–4 months, the tissue was washed in three or four changes of fixative (1.3–2.0% paraformaldehyde and 0.5% glutaraldehyde) over a period of 12 hr before transfer to a 30% sucrose solution (see below).

In addition, we tested decalcification with formic acid–sodium citrate (10). DiI injections were performed either before or after decalcification. Chicken heads were placed for 10 days in three or four changes of 45% formic acid and 20% sodium citrate. The end point of decalcification was determined with 5% ammonium hydroxide and 5% ammonium oxalate. The specimens were washed for 4 hr in tap water and stored in fixative, or were injected with DiI before transfer to the fixative.

Cryosectioning. Before cryosectioning, the tissue was placed in a 30% sucrose buffer (pH 7.4) for cryoprotection. It remained in this solution for 12 hr or until it sank to the bottom of the container. After trimming, the tissue was covered with embedding medium (Tissue-Tek OCT compound; Miles, Elkhart, IN) and frozen on dry ice. Sections of 20–40 μm were cut on a cryostat and collected directly on gelatin-coated slides (0.5% gelatin). One series of sections was coverslipped with Glycergel (DAKO Corp; Santa Barbara, CA) or other water-based mounting media (e.g., Citifluor, Plano, W. Plannet GmbH, Marburg, FRG; Gel/Mount, Biomedia Corp, Foster City, CA). Another series of sections was dried on the slide and viewed directly without a coverslip. To view DiI-labeled sections, we used epifluorescence with the rhodamine filter set (Zeiss, BP 546, FT 580, LP 590), for DiO the FITC filter set (Zeiss, BP 450-490, FT 510, LP 520). Fluorescent DiI and DiO label was documented using TMAX 400 film (Kodak; Rochester, NY) and exposure times ranging from 1 sec–2 min.

DiI Photoconversion. Fluorescent DiI label was photoconverted using diaminobenzidine (DAB; Sigma, St Louis, MO). To enhance penetration of DAB, some sections were pre-incubated for 15–60 min in 1.5–2.0 mg/ml DAB–Tris-HCl buffer (pH 7.6) at 4°C in the dark. Slides with cryosections that were not coverslipped were placed on the stage of a Zeiss fluorescence microscope and a drop of cold DAB (2 mg/ml in 50 mM Tris-HCl buffer, pH 7.6) was placed on the section or part of the section. Because DAB is a carcinogen, appropriate precautions were taken for protection. An area 500–1500 μm in diameter was illuminated through ×16 or ×10 objectives, using the rhodamine filter set (Zeiss, BP 546, FT 580, LP 590) and a 50-W HBO lamp. Some sections were photoconverted on a Leitz Aristoplan microscope equipped with a 100-W mercury lamp. The incubation solution was replaced by a new (cold) drop of DAB solution about every 15 min and the microscope was refocused; fluorescence illumination was maintained for 30–120 min. In some cases, this procedure was repeated for several sections on the same slide. The sections were then rinsed in distilled water, counterstained with thionin, dehydrated, and coverslipped in DPX mountant (Gallard and Schlesinger; Carle Place, NY). Nomarski optics were used to view and document DAB-converted DiI label.

Electron Microscopy. Two 20-day-old chick embryos were perfused with 0.1 M phosphate buffer (PB, pH 7.4), followed by 2% paraformaldehyde and 1% glutaraldehyde. They were decapitated at a level just caudal to the nucleus magnocellularis and received a small injection of DiI (0.5% in dimethylformamide) into the crossed dorsal cochlear tract, using pressure injection. The heads of the animals were kept in PB containing 0.8% paraformaldehyde and 0.4% glutaraldehyde at room temperature in the dark for 2 years, and then the brains were dissected from the fixed heads, blocked, and sectioned on a Vibratome at 50 μm. For electron microscopic analysis, we tested only Vibratome-sectioned tissue, but not cryosections. Free-floating sections were pre-incubated for 30–90 min in a 1.5 mg/ml DAB solution in 50 mM Tris-HCl buffer (pH 7.6) at 4°C in the dark; some sections were stored in 0.1 M PB at 4°C in the dark for up to 4 weeks before pre-incubation and photoconversion. The pre-incubated sections were mounted on glass slides, covered with a fresh 1.5 mg/ml DAB solution, and illuminated (photoconverted) for 1 hr on either a Zeiss or Leitz fluorescence microscope, using the same protocol described above for the photoconversion of DiI in cryosections.

After two washes in Tris buffer and one wash in PBS, sections were osmicated for 30 min in 1% OsO_4 in PBS followed by three washes in PBS (10 min each). They were dehydrated through a graded ethanol series and embedded via propylene oxide in Poly/Bed 812 (Polysciences; Warrington, PA). Thin sections of 70–90 nm were cut and mounted on copper grids. Sections were viewed in a Philips 420 transmission electron microscope, either unstained or conventionally stained with uranyl acetate and lead citrate.

Immunogold Labeling of DiI-photoconverted Tissue. Sections from the same brain, injected with DiI and incubated for 2 years (described above), were used for immunocytochemistry. Vibratome sections of 50 μm were kept for 1 month in phosphate buffer (PB) at 4°C before photoconversion. After photoconversion, the sections were washed in four changes of PB and incubated free-floating in 3% normal goat serum for 1 hr, followed by an antiserum against gamma-aminobutyric acid (Incstar Corp; Stillwater, MN), diluted 1:3000 in 0.5 M Tris buffer (pH 7.6), for 36 hr at room temperature on a shaker table. After three washes, first in Tris buffer, then in PB, the sections were incubated with secondary antibody (AuroProbe EM, GAR G10; Janssen Biotech NV, Lammerdies, Belgium) at a dilution of 1:33 for 24 hr. The sections were washed again, embedded, and thin-sectioned for electron microscopy as described above.

Results

Injections of DiI into neuronal tissue yielded consistent labeling of neuronal processes. The appropriate time to allow for sufficient diffusion of DiI along the neuronal membranes depended on the length of the pathway to be labeled. A distance of 1 mm took about 2 weeks of diffusion time, 5–10 mm about 4 months. Some of our tissue was stored at 37°C; this treatment did not speed up the diffusion time by more than 10–20% compared with storage at room temperature. Incubation of injected tissue for prolonged periods (up to 2 years) had no adverse effects on the labeling characteristics. In young embryos, we sometimes observed transfer of DiI to secondary neurons and/or glial cells, possibly via tight junctions

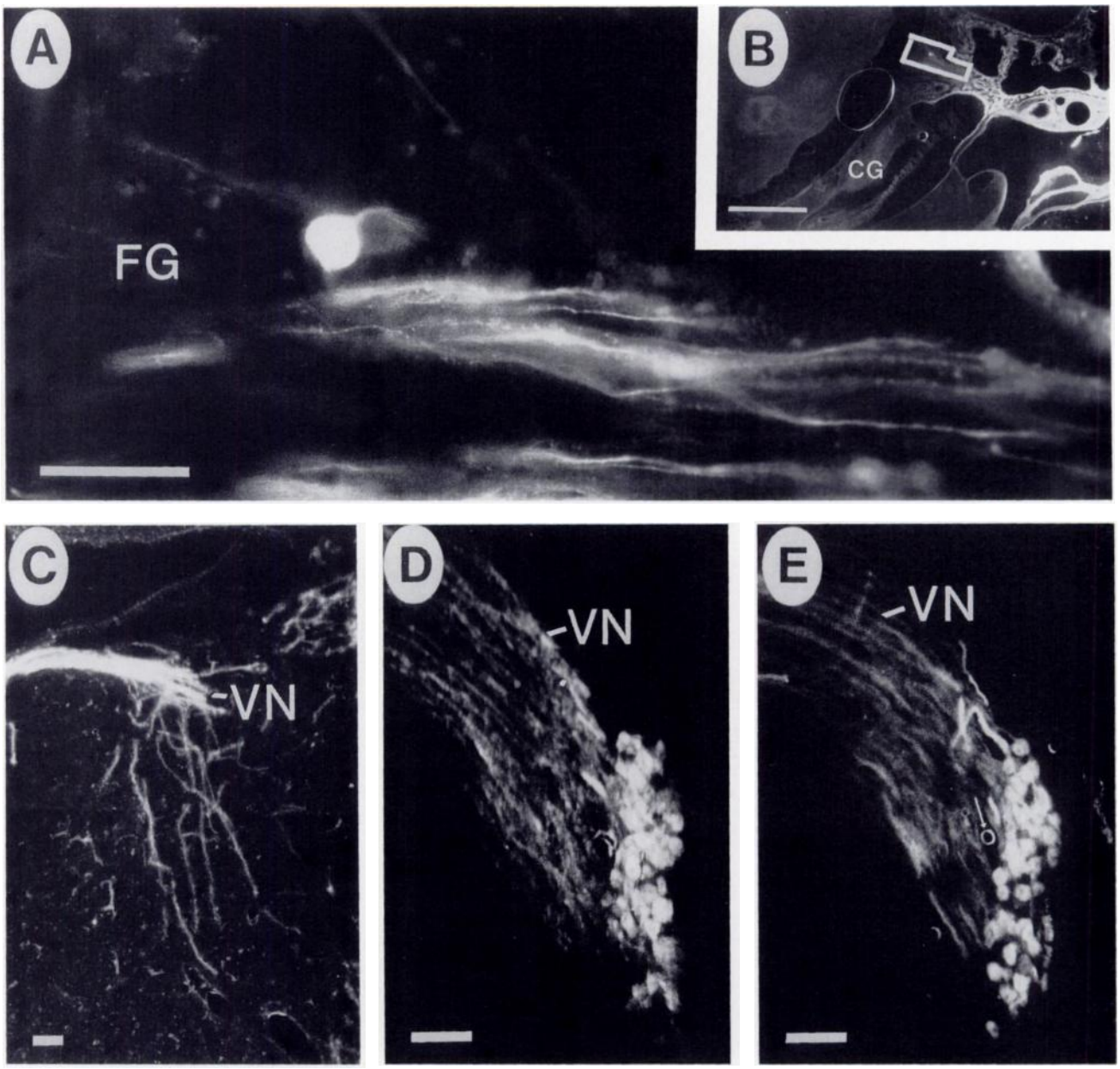

Figure 1. (**A**) Cryosection of tissue from a one-day-old chicken head that was aldehyde-fixed, injected with DiI, and decalcified with EDTA. The dried section was coveslipped with Glycergel. Note one labeled ganglion cell in the proximal facial ganglion (FG) and several labeled fibers in the facial nerve. The position of this region is shown in **B**. (**B**) The injection site in the middle ear is visible on the right side. The area shown at higher magnification in **A** is boxed. CG, cochlear ganglion. Cryosections of DiI-labeled vestibular nerve fibers in the brainstem (**C**) and the vestibular ganglion (**D, E**) of a 17-day-old chicken embryo. Cryosections were dried and left without coverslip (**C, D**) or were coverslipped with Gel/Mount (**E**). Sections **C** and **D** were photographed 1 week after cryosectioning. DiI stayed confined to the labeled neurons in uncovered sections (**C, D**), but optical resolution was superior in coverslipped sections (**E**, photographed 2 hours after cryosectioning). Arrow, air bubble; VN, vestibular nerve. Bars: **A, C–E** = 100 μm; **B** = 1 mm.

(cf. 5) as has been noted in the chick's developing visual system (7,8). In older embryos and hatchling chicks, we never saw labeling across synaptic links. The occurrence and degree of transsynaptic DiI diffusion may depend on the lipid composition of membranes, which is species and age dependent (B. Fritzsch, personal communication).

For decalcification of tissue injected with DiI, we tested EDTA as well as several formic acid/sodium citrate protocols. Formic acid caused an unspecific spread of DiI, even if the tissue was decalcified first, washed for several days, and subsequently injected with DiI. With EDTA, on the other hand, decalcification could be performed while DiI diffused along the neuronal membranes, and at 37°C (Figures 1A and 1B). Occasional changes of EDTA solution may have accelerated the decalcification process, but were not es-

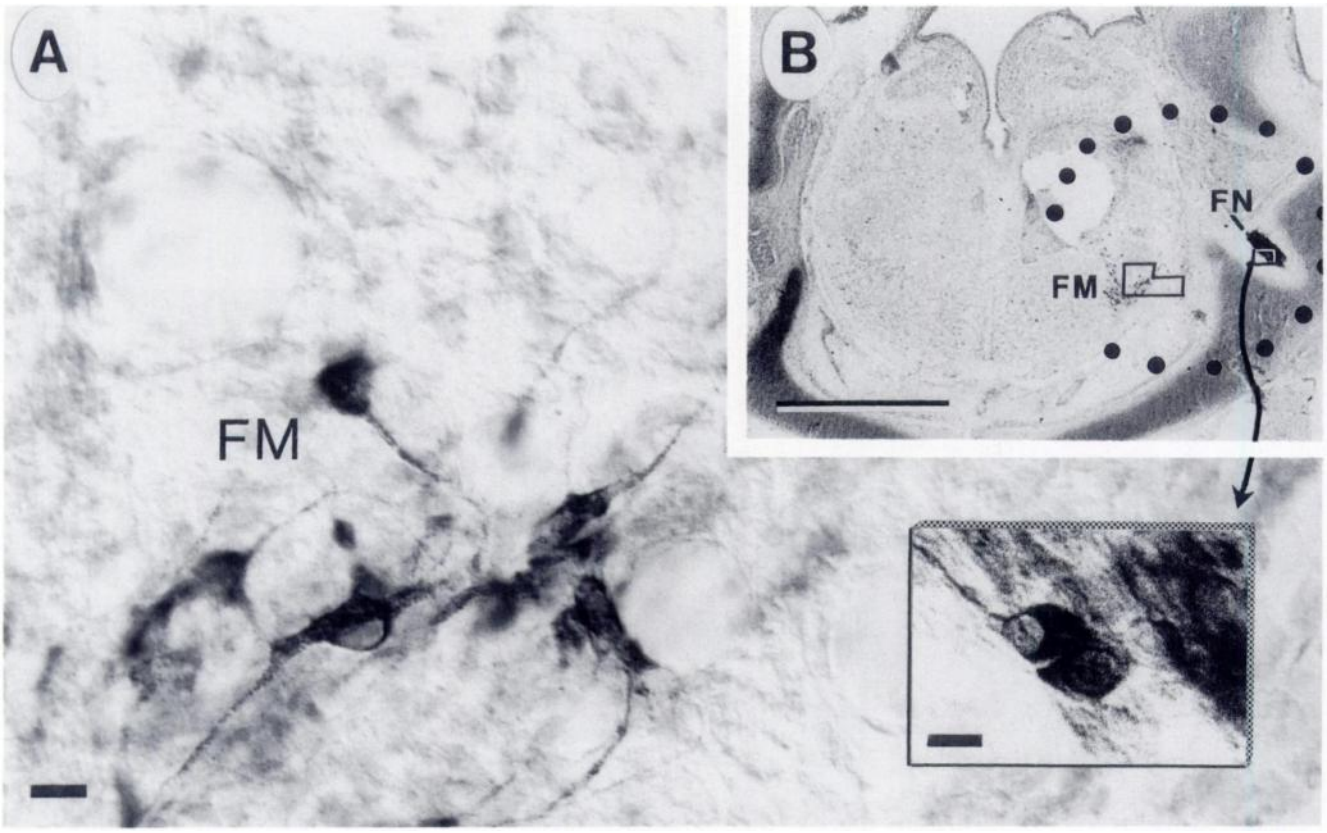

Figure 2. Section through the facial motor nucleus (FM) of a 9-day-old chicken embryo after fixation and injection of DiI into the facial nerve. The tissue was stored for 8 months before cryosectioning at 35 μm. DiI label was photooxidated in the presence of diaminobenzidine (DAB), using a 50-W HBO lamp and a ×10 objective. The section was lightly counterstained with thionin, dehydrated, coverslipped, and photographed with Nomarski optics. **(A)** Labeled neurons in the facial motor nucleus (FM). Background staining is due to thionin counterstain. The DAB label spares the cell nuclei. **(Inset)** Two labeled ganglion cells of the facial nerve. The positions of labeled neurons are shown in **B**. **(B)** The region indicated by the dotted circle was photo-oxidated. The position of the higher magnified region shown in **A** is boxed. FN, facial nerve. Bars: **A, inset** = 10 μm; **B** = 1 mm.

sential for tissue (about 1 cm in diameter) that was kept in solution for 8 weeks at 37°C. Because the tissue needed to be incubated for several weeks to obtain sufficient diffusion of DiI, we did not determine the shortest time period necessary for adequate decalcification. The time course of DiI diffusion in EDTA-fixative appeared to be similar to that in fixative without EDTA.

The DiI label was visible with both the rhodamine filter (bright red signal on dark red background and the FITC filter (yellow signal on dark green background), whereas the DiO label could be seen only when excited through the FITC filter (bright green signal on dark green background). The concentrations of fixatives appeared to be crucial for the level of background as well as for keeping DiI within the neuronal membranes. In dried sections, a 2% paraformaldehyde and 0.5% glutaraldehyde solution provided a light nonspecific background fluorescence which facilitated the differentiation of normal histological structure (Figure 1C) (18). Background fluorescence decreased when sections were coverslipped. Because of limited optical resolution, sections that were not coverslipped were viewed only at lower magnification (×10–×25 objectives).

Cryosectioning and drying of uncoverslipped sections generally posed no problems. In a few cases we noted a spread of label within a few hours after sectioning; in these cases, subsequent coverslipping did not prevent loss of signal. Similar problems were reported for cryosectioned tissue that was fixed with paraformaldehyde only (9), but not in tissue fixed with 0.25–1.0% glutaraldehyde (16,18). Because we used a combination of paraformaldehyde and glutaraldehyde for fixation, spread of dye after sectioning may be related to different cross-linking properties of aldehyde fixatives; glutaraldehyde in low concentrations may help to keep the DiI molecule within the neuronal membranes (S. E. Fraser, personal communication); it may also reduce the speed of DiI diffusion (15). In most cases, we stored uncovered sections for several months at room temperature or at 4°C without significant spread or decrease of label.

In coverslipped cryosections, DiI tended to leak from the membranes at a faster rate than in sections left without a coverslip. None of the three aqueous mounting media tested was able to prevent some "running" of DiI. Sections coverslipped with Gel/Mount often developed many small air bubbles (Figure 1E). Usually, DiI stayed confined to the membranes for several hours before it started to run, and this delay provided sufficient time to analyze and document labeled sections. Because of the tendency of DiI to leak from

the membranes in coverslipped sections, we collected alternate cryosections on two different sets of slides and coverslipped only one set (Figures 1D and 1E).

For photoconversion of fluorescent DiI label, we used a protocol similar to ones described previously (3,11,12,14). DiI label in cryosections as well as Vibratome sections was photoconverted to a diaminobenzidine (DAB) reaction product. At the light microscopic level, weakly fluorescent fibers did not seem to photoconvert, but strongly fluorescent fibers and cell bodies accumulated DAB label and rendered satisfying morphological detail after photoconversion (Figures 2A and 2B). Labeled neurons showed granular reaction product in the cytoplasm of the cell body and in the proximal processes; the cell nucleus was spared (Figure 2A). In the peripheral fiber bundle close to the injection site, abundant DAB reaction product obscured labeling of individual nerve fibers; the labeling pattern of ganglion cell bodies (Figure 2A, inset) was similar to that of motoneurons in the brainstem.

At the electron microscopic level, DiI-photoconverted label was readily observed in unstained thin sections (Figures 3A, 3B, and 4A), but label was virtually impossible to detect in sections stained with uranyl acetate and lead citrate because the counterstain darkened membranes regardless of whether they were labeled or unlabeled (data not shown). In the case demonstrated in Figures 3 and 4, a 20-day-old chick embryo was perfusion-fixed and then received a small injection of DiI into the crossed dorsal cochlear tract. The DiI label was photoconverted 2 years after injection of DiI. The tracer labeled the processes and cell bodies of a few dozen neurons in the nucleus magnocellularis and the medial vestibular nucleus on both sides of the brainstem. Considering the fact that the fixed tissue had been stored for 2 years at room temperature, the ultrastructure appeared remarkably well preserved. For example, myelin sheaths, mitochondrial cisternae, and the vesicles of synaptic terminals were clearly visible (Figures 3C and 4B).

The labeling pattern in cell bodies differed between the two cell types observed. In nucleus magnocellularis neurons, heavy label was observed in lysosome-like structures adjacent to or indented into mitochondria; the endoplasmic reticulum was devoid of label (Figure 3A). In neurons of the medial vestibular nucleus, the endoplasmic reticulum was also heavily labeled (Figure 3B). Labeled myelinated fibers could be clearly distinguished from unlabeled myelinated fibers because the myelin was darker (Figures 3B and 3C). Occasionally, labeled myelin was observed immediately juxtaposed to unlabeled myelin (Figure 3C), indicating that DiI may not diffuse from labeled myelinated axons to immediately adjacent myelin of unlabeled axons. "Powdery" electron-dense particles were frequent at sites with disruption of myelin (Figure 3C), presumably owing to long-term incubation of fixed tissue. "Powdery" particles were observed in labeled as well as unlabeled myelinated fibers and obviously do not represent photoconverted DiI label. DiI-photoconverted sections labeled with immunogold show that DiI label can be clearly distinguished from the uniform gold particles (Figure 3D).

In labeled cell bodies, the heaviest label was found in structures resembling lysosomes (Figures 4A and 4B). These particles typically showed a ring-like structure with an unlabeled core, an electron-dense outer layer of 20–50 nm thickness, and a total diameter of 100–300 nm (Figure 4B). They were spherical rather than tubular. Similar lysosome-like structures were present in unlabeled neurons, but they were less frequent, considerably smaller, and not as electron dense as in labeled neurons (data not shown). Labeled "lysosomal" bodies were frequently associated with mitochondria (Figures 4A and 4B), but were absent in the endoplasmic reticulum. Some mitochondria in labeled cells appeared darker than those in unlabeled cells (Figures 3A, 4A, and 4B), but mitochondrial membranes were not consistently labeled in all neurons. In some presumably DiI-labeled presynaptic elements we observed electron-dense "lysosomal" bodies (Figure 4A); the membranes and synaptic vesicles of these terminals did not differ from those in unlabeled terminals.

DiI-photoconverted label was not observed in the surface membranes (Figures 3A, 3B, and 4A–4C), but the nuclear envelope appeared to be labeled in a few neurons (Figure 3B). In cells with label of the endoplasmic reticulum, the lamellae sometimes were labeled heterogeneously (Figure 4C); label was particularly heavy in the endoplasmic reticulum in peripheral portions of the cell and at sites where the reticulum was contiguous with the surface membrane (Figure 4C). Diffusion of DiI appeared to be largely restricted to membranes in the cytoplasm between the surface membrane and the nuclear cell membrane. The nuclear chromatin remained virtually unlabeled, and DiI did not appear to cross the surface membrane and spread to neighboring neurons (Figures 3A and 4A–4C). The differential labeling patterns of the two types of retrogradely DiI-labeled neurons are shown schematically in Figure 5.

Discussion

The significance of DiI and DiO for neuronal tracing has been discovered only recently. Honig and Hume (8,9) described the advantages of these compounds for selective labeling of nerve cells in vivo and in vitro. Subsequently, Godement and co-workers (7) reported that labeling properties of DiI and DiO were preserved in tissue that had been fixed with aldehydes.

Tracing with DiI

The unique properties of the carbocyanine dyes make these compounds valuable and convenient neuronal tracers for a variety of reasons. They are applicable to dead tissue and therefore can be used when access to the area of interest is impossible or difficult in the living animal (e.g., embryos), when experimental handling of living specimens is limited for other reasons, e.g., human tissue, extinct species (provided that soft tissue is preserved), or when the survival of the animal for the period necessary for marker transport is not possible (7). Tracing with carbocyanines is sensitive and reliable. It is a safe (non-hazardous) technique and the hands-on procedures are simple and short. Two colors with different illumination properties are available, red (DiI) and green (DiO), and recently a fluorescent counterstain has been described for DiI (12,13). As demonstrated in the present work, DiI labeling can be combined with decalcification. Tissue fixed with paraformaldehyde needs to be sectioned with a Vibratome; tissue fixed with paraformaldehyde *and* glutaraldehyde can be either cryosectioned or Vibratome-sectioned. Fluorescent DiI label can be photoconverted to a stable DAB product that allows dehydration and viewing of labeled neu-

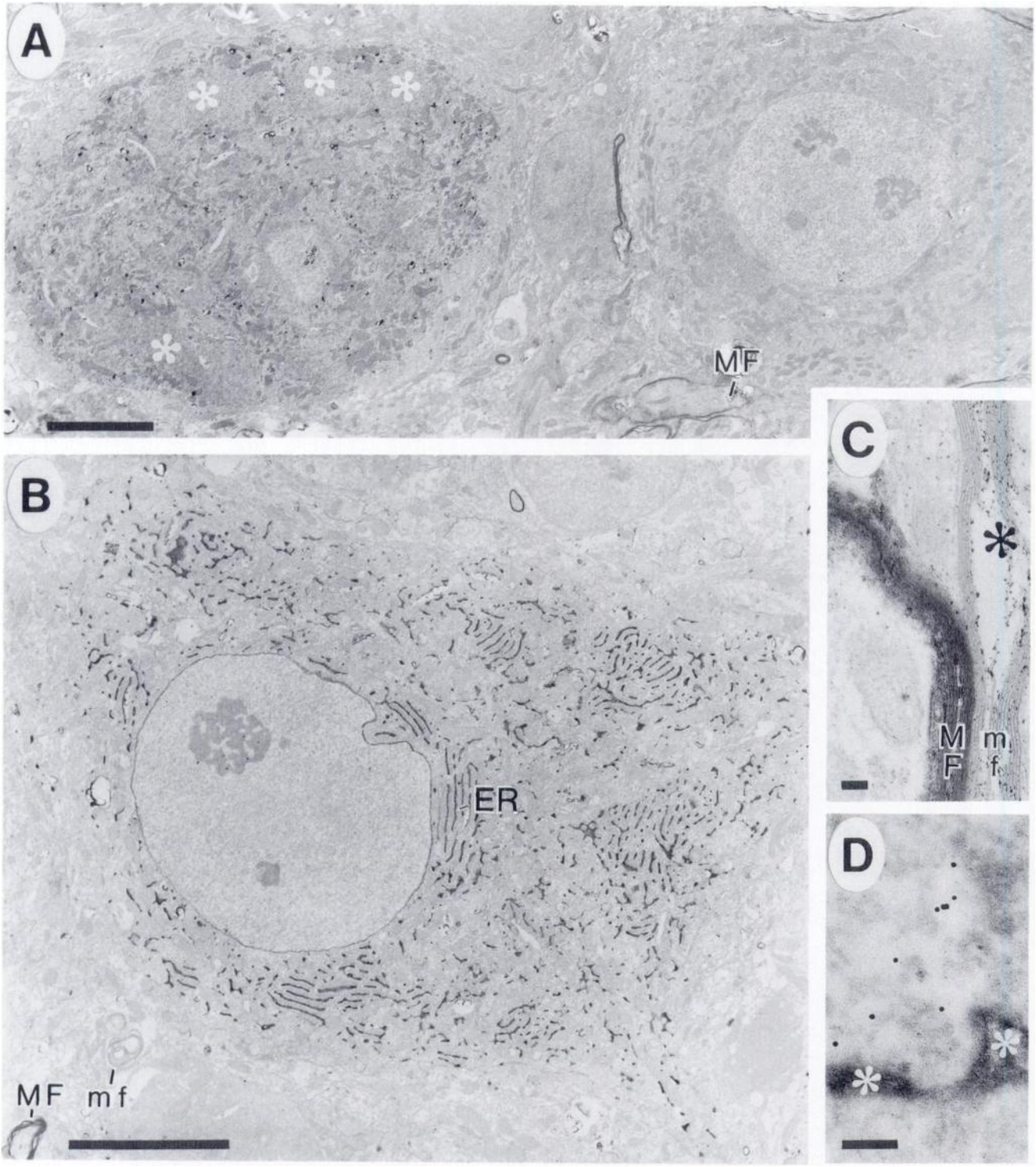

Figure 3. (**A**) Electron micrograph of two neurons in the nucleus magnocellularis of a 20-day-old chicken embryo. DiI label was photoconverted 2 years after injection of DiI into fixed tissue. The tissue was osmicated; thin sections were not counterstained. The neuron on the left is retrogradely labeled; the neuron on the right is unlabeled. Note a bundle of labeled myelinated fibers (MF). Electron-dense profiles (lysosome-like bodies; see text) are distributed throughout most of the cytoplasm, but spare the endoplasmic reticulum (white asterisks). (**B**) Electron micrograph of a retrogradely DiI-labeled neuron in the medial vestibular nucleus. Note heavy labeling of the endoplasmic reticulum (ER). Note also labeled (MF) and unlabeled (mf) myelinated fibers. (**C**) Myelin sheaths at higher magnification. Note dark, labeled myelin sheaths (MF) immediately adjacent to unlabeled myelin sheaths (mf). Precipitations of "powdery" particles in sites of deteriorated myelin (asterisk) are artifacts. (**D**) Immunogold-labeled structure (presumably a terminal or pre-terminal process immunoreactive to gamma-aminobutyric acid) adjacent to DiI-label (asterisks) in a photoconverted neuron. Bars: **A, B** = 5 μm; **C, D** = 0.1 μm.

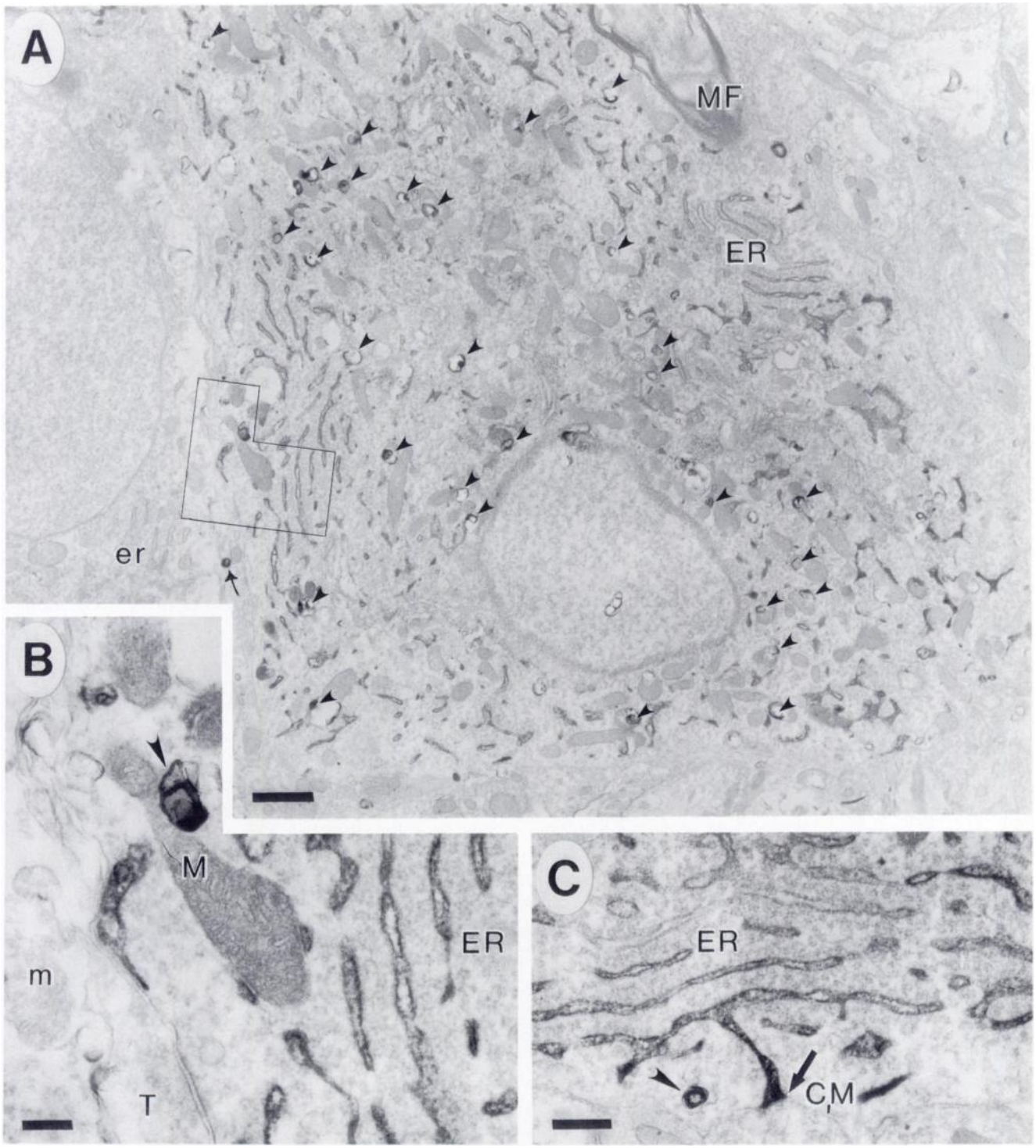

Figure 4. Electron micrographs of a DiI-labeled neuron adjacent to the nucleus magnocellularis of a 20-day-old chicken embryo. After perfusion-fixation, DiI was injected into the crossed dorsal cochlear tract and the tissue was kept in fixative for 2 years before photoconversion. The tissue was osmicated; thin sections were not counterstained. (**A**) Labeled cell body with prominently labeled lysosomal structures (arrowheads) and endoplasmic reticulum (ER). Compare with endoplasmic reticulum (er) in the adjacent unlabeled cell (left). The cell nucleus is unlabeled; the nuclear and surface membranes are inconspicuous. One labeled lysosomal structure (long arrow) is present in the terminal also shown in **B** (boxed). (**B**) Detail of the DiI-labeled cell demonstrating electron-dense endoplasmic reticulum (ER) and dark mitochondria (M) as well as an unlabeled mitochondrion (m) in the adjacent cell. Arrowhead, lysosomal structure. The cell membrane of the labeled cell is contacted by a terminal (T); note the symmetric synaptic profile. (**C**) Electron micrograph of a section through the same neuron at a different level. The cell membrane appears unlabeled, but the endoplasmic reticulum (ER) is intensively electron dense at sites of close apposition (arrow) between the ER and the cell membrane (CM). Label of peripheral ER is heavier than that of central ER. Arrowhead, lysosome-like structure. Bars: **A** = 1 μm; **B** = 0.2 μm; **C** = 0.5 μm.

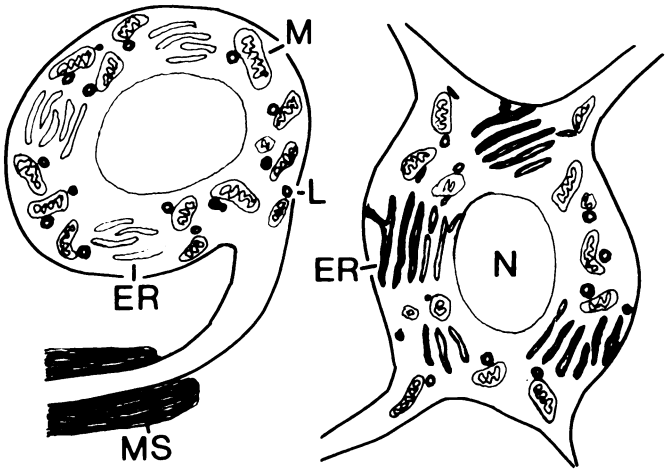

Figure 5. Schematic drawing of two neurons retrogradely labeled with DiI in fixed tissue and photoconverted with DAB. Left, a cell in the nucleus magnocellularis; right, a cell in the medial vestibular nucleus. Four different structures are labeled: lysosomes (L), often adjacent to mitochondria (M), are heavily and consistently labeled; some mitochondria appear to be lightly labeled; endoplasmic reticulum (ER) is labeled heavily near the cell surface, weaker near the cell nucleus (N); myelin sheaths (MS) are heavily labeled. The axonal and cell surface membranes appear unlabeled.

rons under brightfield illumination (12), as well as analysis of DiI label at the electron microscopic level (Figures 3 and 4). Retrogradely labeled cell bodies and myelinated fibers can be readily detected, but labeled unmyelinated fibers and terminals can also be identified at the electron microscopic level. When storage of tissue is compatible with subsequent immunostaining, DiI tracing in fixed tissue can be combined with immunocytochemistry by using fluorescent immunolabels (16,17) or, after photoconversion, by using immunomarkers for electron microscopic analysis (Figure 3D).

There also are disadvantages inherent to the DiI method. The diffusion time is relatively long, and the length of the neural pathways is a limiting factor (about 5–10 mm). The inability to dehydrate fluorescent sections in alcohol (unless photoconverted) may be inconvenient, and the fluorescence is at risk to leak from the membranes, to dissolve and eventually fade. In addition, with standard laboratory equipment, the photoconversion of larger numbers of sections is time consuming. The possibility that DiI may diffuse from labeled nerve fibers via the myelin sheaths of oligodendrocytes to other unlabeled nerve fibers (rendering false-positive label) deserves further attention. Nevertheless, the unique properties of DiI and DiO make these dyes valuable neuronal tracers at the light and electron microscopic level, particularly for the study of the short-range connectivity in embryonic and ossified tissues.

Labeling Mechanism

Diffusion of carbocyanine dyes provides a Golgi-like label of cell membranes, including axonal and dendritic membranes as well as growth cones (7,8). The mechanism of neuronal DiI and DiO labeling in fixed tissue is believed to be relatively simple: "Any labeling that is observed should occur solely by lateral diffusion in the plane of the plasma membranes" (7). Our study demonstrates that DiI can reach certain membranous structures inside the cell body even in aldehyde-fixed neuronal tissue. After a diffusion time of several months and photoconversion of DiI fluorescence, granular DAB reaction product appears in the cytoplasm of the cell body and dendritic processes (Figure 2A). At the electron microscopic level, DAB-photoconverted DiI label is abundant in lysosome-like structures and in the endoplasmic reticulum (Figure 4). Surprisingly, DiI label is not found in the surface membrane of fixed neurons photoconverted 2 years after injection of DiI.

How does DiI reach internal membranous organelles in the fixed cell? The hydrocarbon chain of the DiI molecule presumably inserts into the cell membrane and the molecule diffuses laterally within the fluid membrane (1,5). The diffusion coefficient of DiI is in the same range as that of phospholipid molecules constituting the membrane, but DiI "flip-flops" between the outer and inner layer of the membrane bilayer more frequently than phospholipids, and it rapidly diffuses through tight junctions (5). Fixation of a cell membrane with glutaraldehyde reduces the diffusion coefficient of DiI but does not affect the extent of lateral diffusion (15).

The lack of photoconverted DiI in the plasma membrane may be consistent with the notion that DiI reaches the cell body via lateral diffusion (5,7,15). DiI may have a higher affinity for certain membranes, and, over time, may reach a heterogeneous distribution owing to different affinities to membranes of different composition. The apposition of portions of the endoplasmic reticulum with the surface membrane (Figures 4B and 4C), may explain DiI's ability to label internal membranes of the cell body; DiI may reach endoplasmic extensions in contact with mitochondria via the endoplasmic reticular membranes (6,19). Interestingly, DiI label is abundant at the presumptive sites of contact between endoplasmic reticular and mitochondrial membranes, which may account for the accumulation of label in lysosome-like bodies. A continuous membrane connection between the endoplasmic reticulum and the mitochondria has been postulated for the transfer of phospholipids from the endoplasmic reticulum to mitochondria (19). Apparently, the DiI molecule has great affinity for the site at which phospholipids normally become inserted into the mitochondrial membranes (19). Diffusion may be an important mechanism for the "transport" of phospholipids as well as DiI molecules from the endoplasmic reticulum to the mitochondria.

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