Neuronal Mapping: A Photooxidation Reaction Makes Lucifer Yellow Useful for Electron Microscopy

Abstract. *Irradiating Lucifer yellow–filled neurons with intense blue light in the presence of 3,3'-diaminobenzidine produces an electron-opaque osmiophilic polymer within the injected cells. This technique is valuable when cobalt or horseradish peroxidase injections are difficult or when a second intracellular marker is needed to demonstrate neuronal contacts.*

The development of intracellular markers for light and electron microscopy has facilitated our understanding of neuronal structures and interactions (*1–10*). Dyes compatible with both forms of microscopy enable one to determine the complete branching pattern of a physiologically identified neuron at the light level and its interactions with other neurons at the ultrastructural level. Cobalt injection followed by either sulfide precipitation (*1*) or reaction with diaminobenzidine (*2*) has been used to such ends, but the technique disrupts ultrastructure (*1, 2*) and variable success is obtained when injection is attempted in some organisms such as the leech (*3*). Another metal-based dye, Procion brown, has been used for light and electron microscopy (*4*), but its low sensitivity at the light level and the requirement for extremely short osmium fixation times have made it unpopular. Horseradish peroxidase (HRP) has probably been the most successfully applied intracellular marker compatible with both forms of microscopy because it diffuses widely within injected cells and its reaction products are intensely opaque to light and electrons (*5–7*). Injecting this relatively large molecule, however, with either pressure (*5*) or depolarizing current (*6*) is often more difficult than injecting smaller dyes because HRP blocks microelectrode tips more readily.

The fluorescent markers, Procion yellow (*8*) and Lucifer yellow (*9*), which are easily injected and more sensitive at the level of light microscopy, have also been used to visualize processes of identified neurons with electron microscopy (*10, 11*). Because the dyes are not electron-opaque, it has been necessary to photograph thin sections or adjacent thick sections with fluorescence optics for reference before examining thin sections with electron optics. In addition to the inconvenience and difficulty of performing these correlations, osmium tetroxide quenches the fluorescence of these dyes, thereby requiring an osmium substitute (*11*) or shorter fixation times, which may result in poorer membrane preservation (*10*).

I now report a technique for directly visualizing Lucifer yellow–injected neurons with the electron microscope. When such cells are irradiated with intense blue light in the presence of diaminobenzidine, an electron-opaque osmiophilic polymer is formed within the cells. The advantages of this technique are that (i) Lucifer yellow is highly visible even in unfixed whole mounts, it is easy to inject, and it diffuses rapidly within an injected cell (*9*); (ii) injected cells appear as electron-opaque profiles; (iii) the technique is compatible with osmium fixation; and (iv) it can be used in conjunction with other intracellular markers such as HRP to facilitate studies of neuronal interactions at the ultrastructural level.

Neuronal somata of the leech *Hirudo medicinalis* and the medial giant fiber in the abdominal nerve cord of the crayfish *Orconectes virilis* were injected with Lucifer yellow. The cells to be stained were impaled with microelectrodes containing Lucifer yellow CH (5 percent weight to volume in H_2O). Dye was injected by applying brief pulses of pressure to the back of the electrode as described for HRP injection (*5*) or iontophoretically with constant hyperpolarizing current in the range of 5 to 10 nA. Injection was stopped when the cells appeared yellow under white light. At this point the preparation could be removed from the recording chamber and its filled cells could be examined with fluorescence optics to verify cell identity and assess fill quantity. While on the microscope stage, the normal Ringer solution bathing the preparation was replaced with a solution of 1.5 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) per milliliter of normal Ringer solution readjusted to *p*H 7.5 (*12*). After 10 to 20 minutes of diffusion of DAB through the tissue, the preparation was illuminated with intense blue light from the microscope source for 30 minutes or until the Lucifer yellow fluorescence faded below visibility (*13*). After irradiation, the preparation was inspected with white light to verify the presence of the reddish-brown reaction product (*14, 15*) within the injected cells. The tissue was then washed with two changes of normal Ringer solution, fixed in 2.5 percent glutaraldehyde in 0.1*M* cacodylate buffer (*p*H 7.5) for 1 hour (*16*), fixed in 1 percent OsO_4 in cacodylate buffer for 3 hours, embedded in Epon or Spurr's plastic, and sectioned for electron microscopy.

Figure 1 illustrates the appearance in whole mount of four Lucifer yellow–injected leech neurons before and after formation of the DAB reaction product. The somata and major neurites of a pair of mechanosensory neurons (T cells) (*17*) and a pair of motor neurons (AE cells) (*18*) appear brightly fluorescent even in

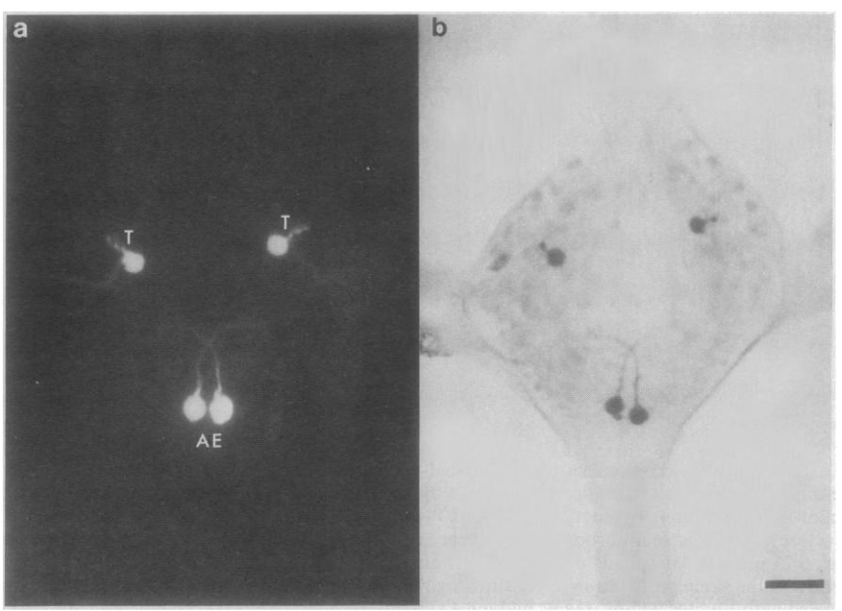

Fig. 1. (a) Light micrograph of a pair of touch sensory neurons (*T*) and a pair of annulus erector motor neurons (*AE*) within a leech midbody ganglion. The cells were injected with Lucifer yellow and photographed with fluorescence optics. (b) The same ganglion photographed with bright-field optics after the light-driven Lucifer yellow–DAB reaction. Scale bar, 100 μm.

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an unfixed and uncleared ganglion (Fig. 1a). After irradiation with intense blue light in the presence of DAB, the reddish-brown reaction product is visible within the injected cells when viewed with white light (Fig. 1b). In whole mount, cells stained by the Lucifer yellow–DAB reaction resemble cells lightly stained by the HRP-DAB reaction in that somata and large processes are visible while smaller processes are not.

In thick (1 μm) plastic sections, however, the Lucifer yellow–DAB reaction product is dense enough to allow light microscopic localization of small marked processes (Fig. 2a). In practice it is also possible to locate not only the axon and primary neurite, but also other less densely stained processes in the neuropil (not visible here) by their yellow-to-orange appearance, especially when contrasted against toluidine blue counterstaining.

Ultrathin sections through processes labeled with the reaction product (Fig. 2b) demonstrate that the product is electron-opaque. Although this process is densely labeled, the reaction product does not obscure synaptic vesicles (SV) or presynaptic densities associated with membrane. Because of the proximity of this region to the site of injection, the soma, the concentration of Lucifer yellow was high. More distal processes, which would be expected to contain lower concentrations of Lucifer yellow, contained correspondingly less reaction product as judged by either light or electron microscopy. This observation sug-

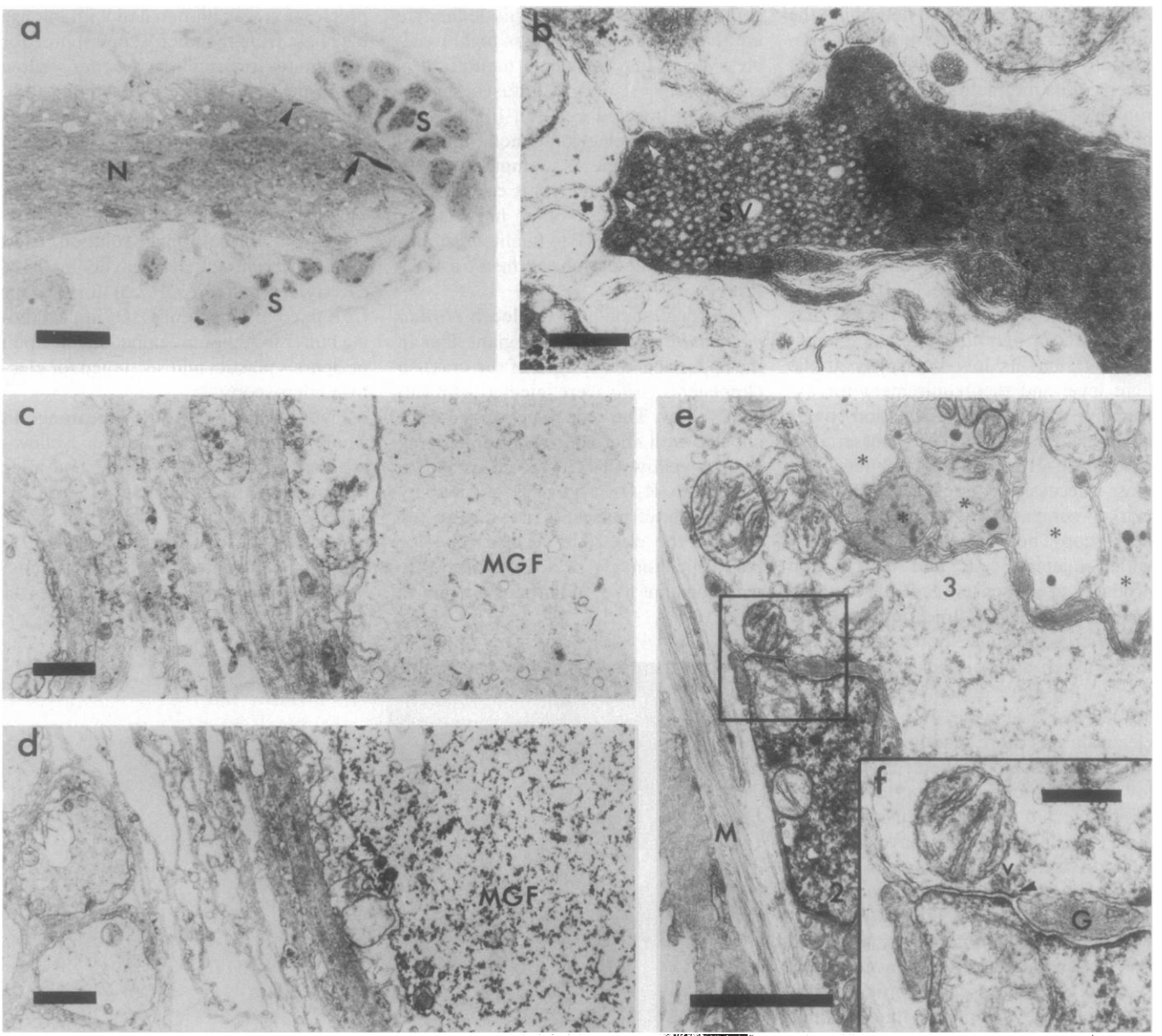

Fig. 2. (a) Light micrograph of a transverse 1-μm section through the third segmental ganglion of a leech. Neuronal somata (*S*) surround the neuropil (*N*). Visible within the neuropil are the axon (arrowhead) and major neurite (arrow) of a heart interneuron (HN) labeled by the Lucifer yellow–DAB reaction product. Scale bar, 50 μm. (b) Electron micrograph of the labeled cell from (a) in the region of the major neurite. The reaction product is seen throughout the process, although synaptic vesicles (*SV*) and presynaptic densities (arrowheads) remain visible. Scale bar, 0.25 μm. (c) An unfilled crayfish medial giant fiber (*MGF*) irradiated in the presence of DAB. (d) The contralateral MGF filled with Lucifer yellow in the same preparation as (c) showing the presence of reaction product. Scale bars for (c) and (d), 1 μm. (e) Heart interneuron HN(3) (*3*), labeled with Lucifer yellow–DAB, making a synapse (box) onto HN(2) (*2*), labeled with HRP-DAB, near the dorsal margin (*M*) of the neuropil in the third segmental ganglion of a leech. The flocculent to granular staining of the labeled processes distinguishes them from unlabeled processes (asterisks). Scale bar, 1 μm. (f) A magnified view of the boxed area in (e). Synaptic vesicles (*V*), a presynaptic density (arrowhead), together with the observation that the glial cell (*G*) has been excluded from between the two interneurons only in the area of this contact, indicate that this is a synapse. Scale bar, 0.25 μm.

gests a relationship between Lucifer yellow concentration and product density. Indeed, the amount of dye injected critically affected the amount of product, and in general it was necessary to inject enough dye to color the site of injection yellow in order to adequately label the cell's finer processes for electron microscopy.

In order to demonstrate at the electron microscopic level that reaction product accumulates only in the cell injected with Lucifer yellow, one of the medial giant fibers (MGF's) in a crayfish abdominal nerve cord was injected with Lucifer yellow and the whole preparation irradiated in the presence of DAB. The contralateral MGF served as a control. Transverse thin sections were made of the connectives, and the MGF's could be identified by their size and position. The results of such an experiment demonstrate that the unfilled MGF and other unfilled axons appear normal while the filled MGF develops granular patches of reaction product throughout the cytoplasm (Fig. 2, c and d). Previous experiments by Miller and Selverston (*19*) with the crayfish lateral giant fibers and by myself (*20*) with the leech giant S axon demonstrated that, although irradiation of cells filled with Lucifer yellow could cause dissolution of cytoplasmic structures, no electron-opaque reaction product was produced within the cells unless DAB was present during the irradiation. Thus, the formation of the product reported here requires the simultaneous presence of Lucifer yellow, illumination at the proper wavelength, and DAB.

The compatibility of Lucifer yellow with HRP at the light microscope level has previously been demonstrated in studies to detect sites of putative contact between pairs of neurons in the leech (*21*). To demonstrate the compatibility of the HRP-DAB and Lucifer yellow–DAB labeling techniques at the electron microscope level and to demonstrate the advantages of combining the techniques, I used them to find a recently inferred synapse between two leech heart interneurons (*22*).

The leech heartbeat rhythm is generated in the segmental ganglia by inhibitory interactions among a group of interneurons known as HN cells (*23*). Most of these interactions can be accounted for by direct synaptic connections identified physiologically. The inputs to some heart interneurons such as the HN(2) cells, however, are difficult to demonstrate physiologically because it is not possible to detect discrete postsynaptic potentials in these cells. Peterson and Calabrese (*22*) have presented indirect physiological evidence suggesting that heart interneurons in the third ganglion [HN(3) cells] could be presynaptic to HN(2) cells.

Since Peterson and Calabrese inferred that the third ganglion was a likely location for this synapse, my strategy was to inject an HN(2) soma with HRP, allow the enzyme to diffuse down the axon to the third ganglion over 2 days in culture medium (*24*), and then to inject the ipsilateral HN(3) soma with Lucifer yellow. The Lucifer yellow–DAB product was developed before fixation, and the HRP-DAB product was developed after aldehyde fixation (*5*).

Figure 2 (e and f) illustrates a synaptic contact from the Lucifer yellow–DAB labeled HN(3) cell to the HRP-DAB labeled HN(2) cell. The identity of the processes was established first by observing their origins in thick sections with the light microscope and then by following their progress through serial thin sections with the electron microscope. This method of identification was used because product density is an unreliable characteristic of each marker even though the HRP-labeled processes were generally more densely stained than the Lucifer yellow–labeled processes, as is the case in this section.

Although it would have been possible to use HRP to label both cells, Lucifer yellow was used as one of the markers because it was easier to inject HN cells with Lucifer yellow than with HRP. Furthermore, the differential density of staining aided in the process of tracking labeled processes through many serial sections.

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12. It is important to adjust the *p*H of the reaction medium after the addition of DAB because the diffusion of DAB into cells is impeded by the positive charge it acquires in acidic solution. Potassium cyanide (1 mg/ml) added to the reaction medium reduced background staining in uninjected neurons, probably by inhibiting oxidases that can oxidize DAB.
13. The Zeiss microscope was equipped with a 200W mercury lamp whose beam was filtered by a BG12 exciter filter and a 430 nm interference filter. Alternatively, a light source similar to that employed by Miller and Selverston was used (*19*). The use of the interference filter reduced background staining dramatically by passing only wavelengths at the Lucifer yellow absorption maximum, thereby preventing reactions involving endogenous chromophores with other absorption maxima. Swirling the bath during illumination facilitated DAB access to the injected cell and probably also carried heat away from the tissue.
14. The reaction product produced here and the HRP-DAB reaction product have similar physical and chemical properties. Both are soluble in 0.1*N* HCl or acetone; such solutions exhibit a broad absorption band between 350 and 600 nm with a maximum at 440 nm. These observations, together with the ability of ascorbate, an electron donor, to block the photoreaction in Lucifer yellow–DAB solutions, suggest that the product in this reaction, like that in the HRP reaction, is formed by the oxidation of DAB to an insoluble polymer.
15. Three other compounds used as proton donors for HRP in anatomical studies were inferior to DAB as reactants for the Lucifer yellow photoreaction. Benzidine [W. Straus, *J. Histochem. Cytochem.* **12**, 462 (1964)] and tetramethylbenzidine [M.-M. Mesulam, *ibid.* **26**, 106 (1978)] do not readily penetrate unfixed tissues although they are polymerized by light and Lucifer yellow in solution. *p*-Phenylenediamine with or without pyrocatechol (*7*) was too photolabile to be used in this reaction.
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