Procion Brown: An Intracellular Dye for Light and Electron Microscopy

Abstract. *Procion brown has been used to inject lamprey giant interneurons. The dye is opaque to both light and electrons, and imparts a pink color to the neuron in situ during iontophoresis. It has the advantage over cobaltous chloride that postinjection treatment with ammonium sulfide is eliminated and the integrity of the surrounding neuropil is ensured.*

Alterations in the functional importance of central pathways may be reflected by morphological changes of synapses on neurons. Experiments designed to examine this possibility require careful morphology after physiological study of the synapses. Conventional microelectrode techniques may be used to study the physiology of the neurons and the electron microscope (EM) used to examine the ultrastructure of synapses on those neurons only if the neuronal processes can be located in the sectioned material. Thus, a technique is needed for identifying all of the processes belonging to one neuron to be examined in the microscope.

Several techniques have been described for labeling central neurons for later identification in the EM. One involves intracellular injection of cobaltous chloride (*1*). The metal is precipitated as a sulfide by immersion of the injected tissue in a solution of ammonium sulfide. The resulting precipitate is electron-opaque and can be easily located by its dense appearance in the EM. The disadvantage of cobalt as an intracellular marker is that the ammonium sulfide may produce distortion of the ultrastructure. Furthermore, cobalt tends to diffuse out of an injected cell unless the tissue is immersed in ammonium sulfide soon after the injection. This precludes the injection and subsequent study of cells which may be serially connected. In another method the dye Procion yellow is used (*2*, *3*). Fluorescence micrographs are made from thick sections and compared with electron micrographs of ultrathin sections for similarities in outlines of cellular processes. The major disadvantage of Procion yellow is that it is not electron-opaque and one must rely on a comparison of fluorescence micrographs with the electron micrographs for identification of the injected processes. On the other hand, the Procion dye does not appear to diffuse out of the cell, and injected material is often left several hours to maximize the extent of diffusion down processes before fixation.

Other Procion dyes now available incorporate metals in their structure, which makes them visible in the EM. The dye used in the experiments reported here is Procion brown (MX5-BR). Each molecule of this dye contains one atom of chromium (*4*). Another dye, Procion rubine (MX-B), contains copper in its molecular structure and may be a possible substitute for Procion brown. The dye is introduced into the cell from a microelectrode, much in the same manner as described by Stretton and Kravitz (*5*) for Procion yellow. Although it does not fluoresce, Procion brown has a slight pinkish color and is readily identified in the light microscope. In the EM the dye appears as a dense precipitate with no obvious structure. It is the density of the cytoplasm of the injected cell which distinguishes it from the cell cytoplasm of the surrounding neuropil. The visibility of the dye in the light microscope is used for comparison of cellular profiles in the EM according to the technique described by Purves and McMahan (*2*).

In continuing studies of synaptic mechanisms in the central nervous system this technique was applied to large cells in the spinal cord of the sea lamprey (*Petromyzon marinus*). The spinal cord in larvae and young adults is small and lacks an obvious blood supply. It can be removed from the animal and placed in an artificial physiological solution, where cells remain viable for several hours, as indicated by their ability to maintain resting potentials and conduct action potentials. Microelectrode placement for recording of electrical events and injection of dye can be done visually because the cells can be seen in the living tissue by transillumination of the spinal cord.

The giant interneurons described by Rovainen (*6*) were studied by this combined physiological-anatomical technique. Lamprey spinal cord contains several giant interneurons bilaterally located in the caudal one-third of the cord. After these cells are visually located, a microelectrode (tip diameter, 1 to 2 μm) filled with a solution of Procion brown in distilled water

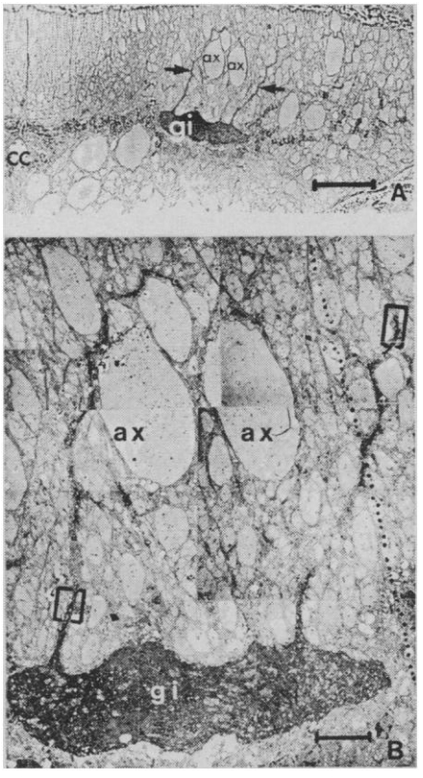

Fig. 1. (A) Light micrograph of a 5-μm plastic cross section of one-half of a larval lamprey spinal cord showing a part of the injected giant interneuron (*gi*) with two dorsally projecting processes (arrows); (*ax*) two of the several giant axons visible; (*cc*) central canal; scale bar, 50 μm. (B) Montage made from several electron micrographs from a thin section cut from the remounted 5-μm section shown in (A); (*gi*) giant interneuron; and (*ax*) giant axons; scale bar, 10 μm.

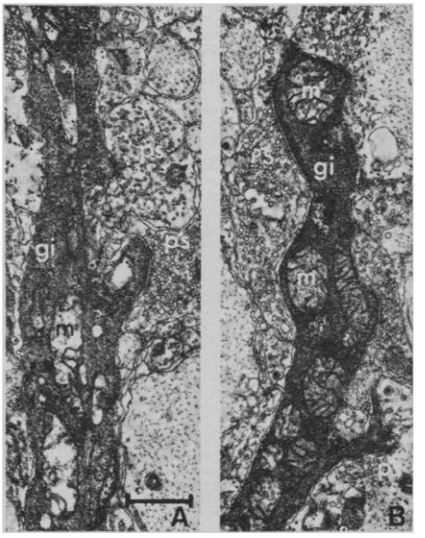

Fig. 2. (A) and (B) Higher magnification electron micrographs of the portion of the injected processes (*gi*) shown in the left- and right-hand boxes, respectively, of Fig. 1B; *m*, mitochondria; *ps*, presynaptic profiles; scale bar, 1 μm.

(4 percent, weight to volume) is advanced until it penetrates the cell. Resting membrane potentials of the order of 60 to 70 mv (inside negative) are normally recorded. The cell is injected by passing hyperpolarizing pulses (approximately 10^{-8} ampere) 500 msec in duration at a frequency of 1 per second until many of the fine processes of the cell can be observed. The time for injection is variable (10 to 30 minutes) and depends mainly on the inside diameter of the electrode tip. To date, 12 lamprey neurons, including both giant interneurons and dorsal cells, have been injected by this method. All cells successfully penetrated, as evidenced by a maintained resting membrane potential, were adequately injected for EM. After injection the tissue containing the cell is removed and prepared for sectioning by using a modified method described by Smith *et al.* (*7*). The tissue is fixed in a 2.5 percent glutaraldehyde solution in 0.05*M* cacodylate buffer (*p*H 7.4) and 1 percent sucrose for ½ hour, and postfixed in 1 percent osmium tetroxide in 0.05*M* cacodylate buffer and 2 percent sucrose for 1 to 2 minutes. Cell membrane definition is not compromised by this short osmium fixation time and the tissue is quite transparent; the injected cell and its processes are visualized much like neurons impregnated by the Golgi method. In one whole mount a process 2 to 5 μm in diameter was followed 250 μm from the cell. The tissue is dehydrated in a series of alcohol solutions of increasing concentration. A 50 percent mixture of Epon 812 and Araldite is used for infiltration and embedding of the tissue. After the plastic is cured, 5-μm sections are cut on an LKB Ultratome III and mounted on clean glass slides until the cell is completely sectioned through.

Figure 1A is a light micrograph of an unstained 5-μm plastic cross section of one-half of a larval lamprey spinal cord. After identification of the injected cell with the light microscope, thin sections for EM are cut from the same thick section according to the method described by Campbell and Hermans (*8*). Figure 1B is a montage made from several electron micrographs (taken on a JEOL 100B microscope) of one of the thin sections cut from the section shown in Fig. 1A. The increased density of the giant interneuron (*gi*) and the dorsally projecting processes distinguishes them from the surrounding tissue. A direct correlation of the injected processes can be made between the light and electron micrographs so that there can be no error as to the cellular profiles containing the dye. To test the possibility that the cellular staining was an artifact or a consequence of osmium fixation, one cell was processed for EM without the osmium fixation. In the EM the density of the injected cell cytoplasm appeared no different from that of the tissue treated with short osmium fixation times. If anything, the contrast between injected cell cytoplasm and the surrounding neuropil appears to decrease if long osmium fixation times (15 minutes) are used.

Higher magnifications of portions of the injected processes shown in the left- and right-hand boxes of Fig. 1B are shown in Fig. 2, A and B. The cytoplasm of the injected processes (*gi*) is denser than the surrounding tissue and the dye appears to be contained within the plasmalemma. Mitochondria appear somewhat swollen in Fig. 2A, although the mitochondrial membrane is intact since no dye appears within. Mitochondria in Fig. 2B appear more normal, possibly because this area is farther from the injection site. Presynaptic profiles (*ps*) are numerous and appear to make normal contact with the injected processes. The presynaptic and postsynaptic membrane thickenings can be seen and do not appear to be altered by the technique.

Procion brown combines the advantages of Procion yellow and cobalt for light and electron microscopy without incorporating their disadvantages. The dye is visible in the light microscope, remains in the cell after injection, and requires no further reaction to make it visible in the EM. Injected processes are identified by using combined light and electron microscopy applied to the same section. In preparations such as the lamprey, where both presynaptic and postsynaptic cellular elements are large enough for microelectrode penetration, this technique can be used to identify them in the EM, and a study can be made of the synaptic contact about which some of the physiology is known.

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Neuron Geometry and Circuitry via the Electron Microscope: Intracellular Staining with Osmiophilic Polymer

Abstract. *Intracellularly injected cobalt catalyzes the generation of an osmium-binding polymer from 3,3'-diaminobenzidine in neurons of* Aplysia californica. *Excellent structural preservation of nervous tissue is coupled with easy recognition of fine branches of the injected neurons in the electron microscope. This permits detailed structural analysis of synaptology.*

Morphological analysis of cell geometry and of neural networks is increasingly important in neurophysiological, genetic, developmental, and behavioral studies in the neurosciences (*1–5*). Recently introduced techniques of intracellular injection of diffusible fluorescent dyes (*2*) and metal ions (*3*) to display neuron geometry are now widely used in investigations of neural circuitry and the relations of neuronal form with function (*4*). However, dye and metal ion injection techniques have been used with good results only at the light microscope level; attempts to adapt them to use with the electron microscope have produced unsatisfactory results largely because of the compromises necessary in tissue fixation to preserve the intracellular marker. The use of Procion yellow dye to mark leech ganglion cells (*5*) in thick sections for light microscopy, which were subsequently sectioned for electron microscopy allowed only 3 minutes fixation in OsO_4. Longer fixation, necessary to preserve neuronal membranes, destroyed the fluorescent properties of the dye. Similarly, the use of cobalt precipitated as a black sulfide for an electron