

Protocols

Long-lived retrograde fluorescent labeling of corticospinal neurons in the living animal

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Abstract

For pathophysiological studies, it is advantageous to label specific neuronal populations in living animals. This study aimed to establish a method for stable and long-lasting fluorescent labeling of corticospinal neurons in the living animal. The two fluorescent dyes Fluoro-Red and Fluoro-Green were injected in the cervical spinal cord of anesthetized newborn rats. After a recovery period, treated rats were returned to the mother. After 24 h and 14 days, fixed brain sections revealed wide-spread fluorescence in elongated or pyramidal-shaped cell profiles in a discrete internal cortical layer, consistent with layer V pyramidal cells. Labeled neurons displayed spontaneous synaptic activity using the slice patch clamp method. These results suggest that these dyes are effective tools for pathophysiological and slice patch clamp studies focused on specific neuron groups.

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1. Introduction

A variety of retrograde axonal tracers, from horse radish peroxidase (HRP) to fluorescent molecules, such as Nuclear Yellow, fluorescent rhodamine–dextran–amine, and Fluoro-Gold, have been described [1–3,9,10,14–16]. Dong et al. [5] reported that two novel fluorescent dyes were useful for retrograde labeling of rat central nervous system, and Dijkstra et al. [4] applied these fluorescent dyes for labeling of corticospinal tract neurons. They suggested the usefulness of these dyes for acute experiments. For studies of pathophysiological mechanisms, the long-lived fluorescent staining in living animals is important. Therefore, we tested

the use of these dyes for subacute/chronic experiments, such as would be useful for the study of neurodegenerative diseases, and explored their application to *in vivo* fluorescent staining for electrophysiological studies.

The control of mammalian motor activity is a central theme of neurobiology, and the upper motor neurons of the corticospinal tract are selectively destroyed in certain neurodegenerative conditions (see review of Rowl and Shneider [13]). However, knowledge of the specific cell biology of this neuronal population is limited, and physiological studies have been limited, hampered by the difficulties of their specific identification *in vitro*. The retrograde labeling of corticospinal neurons with these tracers allows their positive identification and the study of their features. The aim of this study is to establish a method for stable and long-lasting fluorescent labeling of corticospinal neurons in the living animal, suitable for studies in slice or dissociated cell preparations.

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2. Materials and methods

Fluoro-Red and Fluoro-Green were purchased from the Meisei-Kaken (Saitama, Japan). These dyes were stored at 4 °C and used without further dilution. Their chemical compositions, according to information provided by the manufacturer (Tombow Pencil, Tokyo, Japan), were as follows: Fluoro-Red (A) water solution of 0.5% 3,6-bis(ethylamino)-9-[2-(methoxycarbonyl)phenyl]-2,7-dimethyl-xanthylium chloride and 0.5% 3,6-bis(diethylamino)-9-[2-(ethoxycarbonyl)phenyl]-xanthylium chloride, (B) dilution solution: 74% distilled water, 8% ethylene glycol, 8% glycerin, 8% D-sorbitol, and 2% thiabendazade. The proportion of concentrated dye solution (A) to diluent (B) was 39:61%. Fluoro-Green (A) water solution of 0.9% 2-[7-(dimethylamino)-2-oxo-2H-1-benzopyran-3-yl]-1,3-dimethyl-1H-benzimidazolium chloride and 0.1% 3,7-bis(diethylamino)-phenoxazin-5-ium chloride, (B) dilution solution: 70% distilled water, 8% ethylene glycol, 8% glycerin, 12% D-sorbitol and 2% thiabendazade. The proportion of concentrated dye solution (A) to diluent (B) was 50:50%. It should be noted that these formulas differ from those reported in Dong et al. [5]. Green fluorescent Nissl stain reagent (NeuroTrace) was purchased from Molecular Probes.

Fluorescence spectra of the dye solutions were recorded with an F4000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Dyes were diluted 100,000 times with distilled water for measuring the fluorescent characteristics. For Fluoro-Green, the emission spectrum was measured with excitation wavelength fixed at 450 nm, and the excitation spectrum was measured with emission at 535 nm. For Fluoro-Red, excitation and emission wavelengths of 495 and 580 nm were employed, respectively.

The present protocols for animal experiments were approved by the University of Chicago Institutional Animal Care and Use Committee. Retrograde fluorescent tracers were injected into the cervical spinal cord of newborn rat pups (postnatal day 1). Newborn rats (Sprague–Dawley purchased from Charles River Laboratories, Wilmington, MA; both sexes, less than 24-h old) born to mothers were weighed and then anesthetized with pentobarbital (15–30 mg/kg, i.p.). Rats were fixed under surgical microscope and sterilized from head to back with 70% ethanol. Bupivacaine (0.5%) was injected subcutaneously at the cervical operative site prior to skin incision. Surface hair was removed, and skin and muscle just above the cervical 1 (C1) vertebra were cut. A laminectomy was performed at C1 level, allowing visualization of the upper cervical spinal cord. Tracer (approximately 0.2 µl per injection) was injected uni- or bilaterally, 0.5 mm lateral to the midline, at 1 mm depth, by use of a glass capillary tube drawn to a tapered tip of approximately 50 µm diameter. Pressure injection was conducted by an oral tube and pipette holder (Sigma). After injection, the injection glass capillary was held at the injection site for 30 s and then removed. The wound was closed by nylon suture (#5). After surgery,

Ringer's solution (0.1–0.2 ml) was injected (i.p.) for fluid and electrolyte balance, and animals were kept on heating pad at 37 °C for at least 6 h. Rats were fed skim milk manually for every 2–4 h after surgery. After a recovery period of at least 9 h, treated rats were cleaned with phosphate-buffered saline (PBS) and then returned to the mother.

For brain sectioning, isoflurane-anesthetized rats were sacrificed after periods of 24 h and 14 days following surgery. Phosphate-buffered paraformaldehyde (4% in saline) was perfused via heart injection to replace the blood and fix the brain. After in situ fixation, brain was removed and washed with phosphate-buffered sucrose (30%) for overnight at 4 °C and then washed with PBS for at least 6 h. After washing, brain was frozen with Tissue-Tek O.C.T. compound (Sakura Finetek USA, Torrance, CA, USA). Frozen tissues were sectioned of thickness at 8–10 µm using a cryo-microtome. In some experiments, 25-µm sections were stained with green fluorescent Nissl reagent according to the manufacturer's protocol. The fluorescent microscopic filters used in this study were Chroma High Q filters 41001 and 41002b (Rockingham, VT, USA).

Electrophysiological responses of labeled cells were determined by whole-cell patch clamp methods applied to acute brain slices. Animals were deeply anesthetized with isoflurane and rapidly decapitated. Brains were removed and quickly transferred to ice-cold artificial cerebrospinal fluid (aCSF) consisting of (in mM): NaCl 125, KCl 2.5, MgCl₂ 1, CaCl₂ 2.5, Glucose 20, NaH₂PO₄ 1, and NaHCO₃ 25, bubbled continuously with 95% O₂, 5% CO₂. After removal of the cerebellum and olfactory bulbs, the brain was glued to the chuck on its caudal end with cyanoacrylate. After mounting, the chuck was rapidly loaded into the slicing chamber of a Vibratome (Campden Instruments) filled with ice-cold oxygenated aCSF. Coronal sections of 350- to 400-µm thickness were cut. The sections were allowed to recover for at least 1 h at 37 °C in oxygenated aCSF before moving to the recording chamber. The recording chamber was continuously perfused with oxygenated aCSF at a rate of approximately 2 ml/min. Recordings were conducted at room temperature. The slice recording upright fixed stage microscope was equipped with a 10 × objective for gross positioning of the electrodes and the microperfusion system. A 40 × 0.75 n.a. water-immersion objective and infrared illumination enabled visualization of neurons as deep as 200 µm in the slice. Cells were visualized with a CCD camera and video monitor. The slice recording microscope was also equipped with epifluorescence illumination. For visualization of fluorescently labeled corticospinal neurons, the tissue was exposed to 480 nm light, and emission at 535 nm was visualized. A cell of interest was oriented in a specific location in the field and depth of the cell in the tissue. The optics were then switched to infrared DIC for visualization and placement of recording electrode. Recording electrodes (2–7 MΩ) contained (in mM) K-gluconate 154, KCl 1, EGTA 1, HEPES 10, glucose 10, ATP 5, and

GTP 0.1. For recording well-defined AMPA receptor-mediated mEPSCs, tetrodotoxin-, bicuculline-, and 2-amino-5-phosphonopentanoic acid-containing aCSF were used.

3. Results

For *in vivo* retrograde labeling of corticospinal neurons, we employed the novel fluorescent tracers Fluoro-Red and Fluoro-Green. These dyes were reported to have advantages of rapid retrograde transport, lack of diffusion away from the injection site, and lack of trans-synaptic leakage [5].

First, we tested the appropriate excitation and emission wavelength of these dyes by scanning fluorescence spectrophotometry. The wavelength patterns were shown in Fig. 1. The excitation maximum wavelength of Fluoro-Green was found as 447 nm and its emission maximum as 501 nm, respectively. Those of Fluoro-Red were also determined as 536 nm for excitation, and 586 nm for emission, respectively. These results suggest the compatibility of these dyes in double-staining experiments.

We sought to establish methods for stable and long-lasting retrograde fluorescent labeling of corticospinal neurons in the living animal, especially newborn pups. The fluorescent tracers were injected into the cervical spinal cords of newborn rat pups, followed by surgical wound closure and resuscitation of pups and survival for periods of various lengths prior to sacrifice. At least a 9-h period was required for recovery from surgery and anesthetic effects. During this period, the operated rats were maintained by warming, a single intraperitoneal fluid resuscitation, and

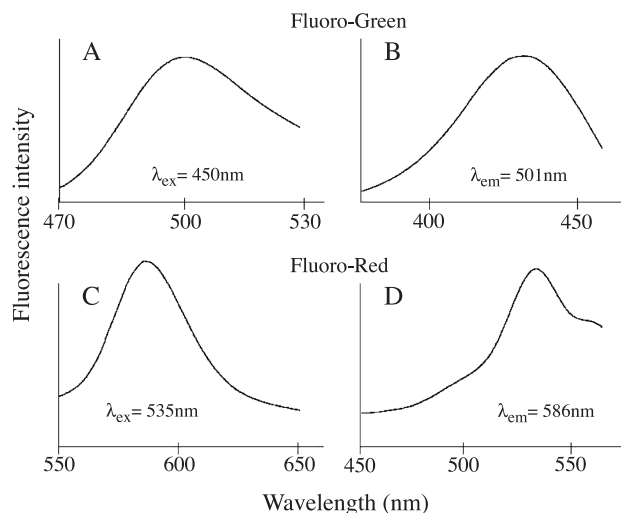


Fig. 1. Fluorescence spectra of Fluoro-Green and Fluoro-Red. (A) Fluoro-Green emission spectrum, maximum value at 501 nm (excitation wavelength, 450 nm). (B) Fluoro-Green excitation spectrum, maximum value at 447 nm (emission wavelength, 501 nm). (C) Fluoro-Red emission spectrum, maximum value at 586 nm (excitation wavelength, 535 nm). (D) Fluoro-Red excitation spectrum, maximum value at 536 nm (emission wavelength, 586 nm).

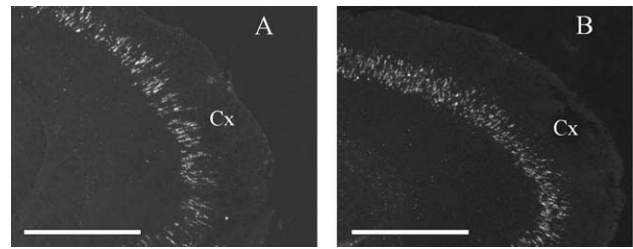


Fig. 2. Fluoro-Red (A) and Fluoro-green (B) staining (24 h after injection) pattern of neocortex. Coronal section, with dorsal side up. Cx: neocortex. Scale bar: 200 μ m.

attempts at feeding by pipette. Despite these efforts, some rat pups failed to recover from the initial surgery. After recovery, injected rats were returned to the cages of their mothers. Commonly after neonatal surgery on the rat, the pup is attacked by the mother rat upon return, presumably because of blood odor. However, we found that maternal neglect or aggression toward the postoperative pups was avoided by careful washing. Mother rats readily accepted and nurtured the injected pups. The pups that were successfully resuscitated after surgery apparently developed normally. After a 24-h recovery interval, they were seen to be growing, moving about, and suckling normally along with unoperated littermates. Mother rats held and fed newborn rats without distinction between operated and unoperated rats. From 24 h following surgery onward, there were no apparent differences in behavior between treated and untreated rats. Operated rats grew at rates similar to control littermates.

After periods of 24 h and 14 days following surgery, operated rat pups were sacrificed and the brains were removed. Then, brain was frozen and sectioned in the coronal plane in slices of 10- μ m thickness. Fluoro-Red fluorescence was widely observed in elongated or pyramidal-shaped cell profiles in a discrete internal layer, consistent with layer V pyramidal cells. Fluorescence was found in a widespread distribution in the lateral neocortex (Fig. 2A). Fluoro-Green injection produced fluorescence in a similar distribution (Fig. 2B). This localization of labeled cells is identical to that described for corticospinal neurons of the pyramidal tract as demonstrated in previous pathohistochemical studies [12].

In labeled cells, these dyes produced a punctate or globular fluorescence pattern rather than a diffuse fluorescence distribution (see Fig. 3). To clarify the cellular localization of fluorescence, we applied a fluorescent Nissl stain to brain sections from Fluoro-Red-treated animals, and utilized fluorescence microscopy to visualize the resulting staining (Fig. 4A). The green fluorescent Nissl stain produced a pattern of labeling consistent with a cytoplasmic distribution, sparing nuclei. Fluoro-Red punctate overlay the Nissl-labeled areas. This pattern indicated a cytosolic localization of Fluoro-Red fluorescence, without identifiable nuclear or surface staining of cells, and with no visible sign of trans-synaptic spread of fluorophore to surrounding cells.

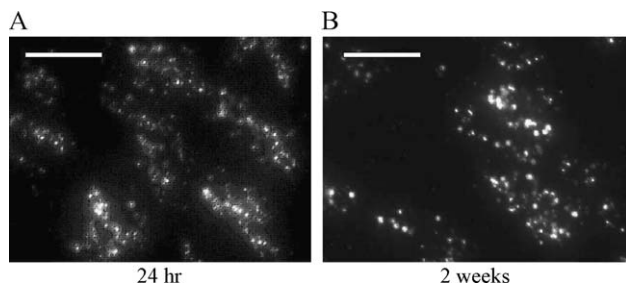


Fig. 3. Fluoro-Red staining in neocortex slices after 24 h (A) and 2 weeks (B) survival following cervical spinal cord injection. Scale bar: 20 μ m.

In some experiments, we injected Fluoro-Red to right side of C1 spinal cord and Fluoro-Green to the left side. Fig. 4B showed the fluorescent localization of right cerebral cortex of 2 weeks after treated mice. Each fluorescent dye injected unilaterally was observed in both sides of cerebral cortex. No cells contained both dyes. These results suggested that corticospinal neurons in each brain hemisphere projected bilaterally to the cervical spinal cord in the neonates, but individual corticospinal neurons each apparently projected to only one side of the spinal cord, either ipsilaterally or contralaterally.

Fluorescent labeling was long-lived. There were no apparent differences in the pattern or intensity of fluorescence in brain sections of rats sacrificed after 24 h or 14 days of postoperative survival. Punctate fluorescence remained confined to a single discrete cortical layer after

2 weeks, without signs of further spread to other cells (Fig. 3).

To test the viability and responsiveness of labeled cells, we undertook pilot patch clamp electrophysiological studies of labeled neurons in acute cortical slices, taken from brains harvested 2 weeks after neonatal Fluoro-Green injection. Fluorescently labeled cells were easily identified within the 350- μ m thick acute slices (Fig. 5). Spontaneous excitatory synaptic activity could be detected with excitatory postsynaptic potentials of 5–20 pA amplitude at a holding potential of -70 mV (Fig. 6). These results indicated that the labeled neurons retained physiological functions at least for 2 weeks after labeling.

4. Discussion

Corticospinal neurons, controlling spinal motor activities, are of central neurobiological importance. In addition, these neurons, like spinal motor neurons, selectively degenerate in amyotrophic lateral sclerosis. Nevertheless, few physiological or pathophysiological studies have examined corticospinal motor neurons, in part due to the difficulties in specifically identifying them in physiological preparations. Therefore, a new experimental system for long-term labeling of these neurons was needed. Fluoro-Red and Fluoro-Green were developed as pink and green fluorescent marker inks for commercial purposes. Their application to biological preparations was first reported by Dong et al [5], who

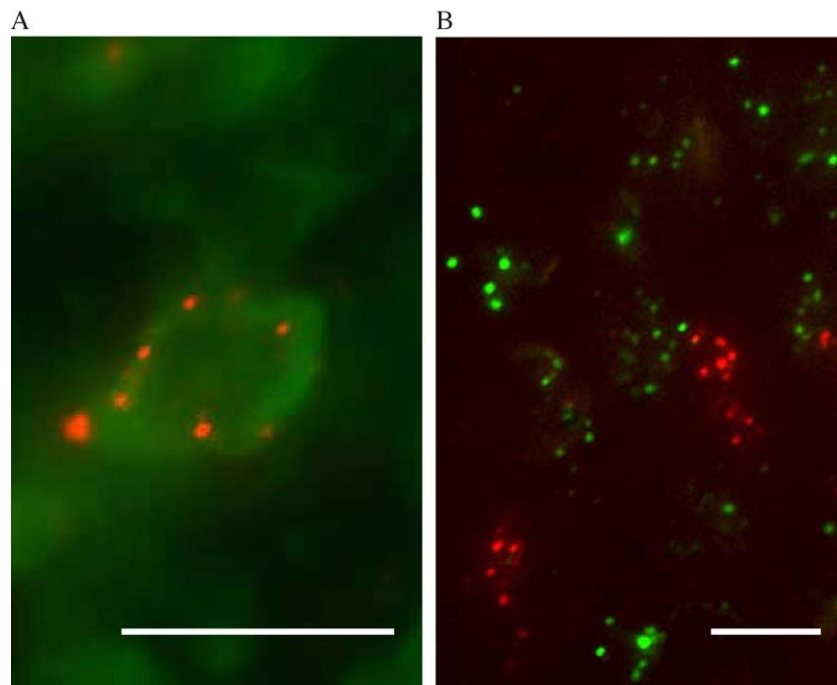


Fig. 4. (A) Intracellular localization of Fluoro-Red fluorescence (2 weeks after injection). Cells were stained with fluorescent Nissl reagent (green), marking cytoplasmic areas, and Fluoro-Red. Scale bar: 20 μ m. (B) Neocortex (right) slice from a rat pup injected with Fluoro-Green (left spinal cord) and Fluoro-Red (right spinal cord), after 2 weeks survival interval. Scale bar: 20 μ m.

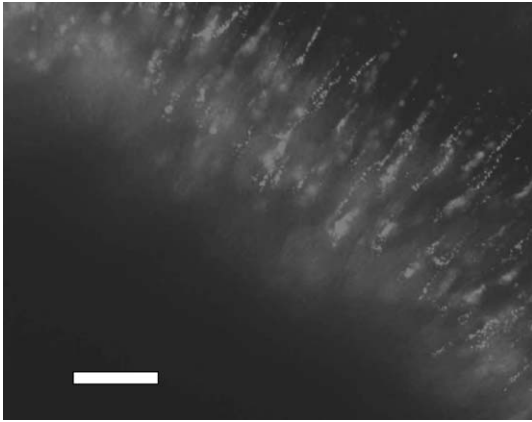


Fig. 5. Fluoro-Green fluorescent found in the rat neocortical acute slice (350- μ m-thick) after 2 weeks dye injection. Scale bar: 20 μ m.

reported several advantages of these dyes including little diffusion at injection sites, lack of leakage from labeled cells, strong fluorescence, lack of toxicity, and low cost. Subsequently, Dijkstra et al. [4] reported the application of Fluoro-Red for retrograde labeling of corticospinal neurons in acute experiments, with sacrifice of animals 20 h after tracer injection, and short-term (20 h) culture of dissociated neurons from labeled cortices. These studies showed the usefulness of these novel fluorescent dyes for acute studies. However, their applicability for longer-term *in vivo* labeling of neurons without toxicity remained uncertain. In the present study, we tested the application of Fluoro-Red or Fluoro-Green in long-term fluorescent labeling of a specific region of the rat brain.

The fluorescent characteristics of Fluoro-Green and Fluoro-Red (Fig. 1) show strong and well-separated peaks of excitation and emission wavelengths, indicating that these dyes may be useful for double-labeling experiments. In addition, the excitation and emission wavelengths of Fluoro-Green and Fluoro-Red were close to those of FITC and rhodamine, respectively, allowing use of widely available commercial fluorescent filters for their detection.

We developed and applied an experimental protocol for retrograde labeling of corticospinal neurons in newborn rats based on that of Dijkstra et al. [4]. However, in contrast to their reported protocol, we maintained injected pups for longer-term survival for periods of 2 weeks or more. Since newborn rats require maternal care until weaning at about 3 weeks, this required returning pups to their mothers after surgery, which was accomplished with careful postoperative washing. This protocol produced successful long-term retrograde labeling of corticospinal neurons. Retrograde transport of these dyes from injected site to cerebral cortex required approximately 20 h in the newborn rats. Both Fluoro-Red and Fluoro-Green produced strong fluorescent labeling, distributed in similar patterns. The fluorescence signal was located in layer V of the cerebral cortex, in a wide distribution over the dorsal and lateral cortical surfaces. This distribution, extending beyond the primary sensory and

motor cortical areas, matches the distribution of the full extent of corticospinal projections neurons mapped with sensitive detection techniques when horse radish peroxidase (HRP) was injected in the cervical cord [12]. Some studies utilizing spinal injections of HRP have described more limited distributions of labeled neurons [7,8]. These discrepancies have been attributed to more caudal spinal injections, known to produce more limited areas of cortical labeling, and to less sensitive HRP detection techniques [12]. Thus, the present results support the high sensitivity of Fluoro-Red as a retrograde tracer. The resulting fluorescence was highly resistant to fading or photo-bleaching with no visible sign of trans-synaptic spread of fluorophore to surrounding cells. The fluorescence remained strong 2 weeks after injection. These results indicate that these dyes are not excreted from the cells, allowing corticospinal neuron identification in subacute or chronic experiments.

Fluoro-Red and Fluoro-Green were also useful tools for double staining. In this study, we injected these dyes to the opposite sides of the C1 spinal cord. Fluorescent punctate of both Fluoro-Red and Fluoro-Green were observed in either side of the cerebral cortex. Fluoro-Red and Fluoro-Green stained cells were easily recognized in same microscopic field. No single neuron contained both fluorophores. These results indicate that in the neonatal rat brain, individual corticospinal neuron axons project unilaterally to either ipsilateral or contralateral sides of the cervical spinal cord.

In the adult rat, the corticospinal tract is nearly completely crossed [8], although a small ipsilateral ventral spinal cord projection has been described (see review of Terashima [17]). During postnatal development, uncrossed corticospinal fibers extend caudally into thoracic and lumbar levels, later disappearing from levels below the upper thoracic cord [17]. Some uncrossed tracts persist in higher levels of the adult rat spinal cord [6,11,18]. Our results strongly indicate that at least, early in development in the rat corticospinal tract, there is strong ipsilateral contralateral projection of corticospinal neurons to the cervical level. Interestingly, neonatal hemispherectomy induces increased ipsilateral

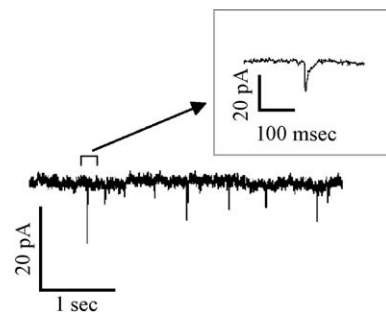


Fig. 6. Patch-slice electrophysiology recording in an identified corticospinal neuron stained with Fluoro-Green, in an acute cortical brain slice obtained 2 weeks after cervical spinal cord dye injection. Whole-cell voltage clamp recording in tetrodotoxin-, bicuculline-, and 2-amino-5-phosphonopentanoic acid-containing solution revealed well-defined AMPA receptor-mediated mEPSCs.

projections of corticospinal neurons in the surviving adult rats, with this plasticity possibly accounting for the functional recovery [8]. Whether the numerous uncrossed fibers identified in the present study are transient ipsilateral corticospinal projections that later retract, but in neonatal brain injury can provide a basis for functional recovery, or instead are only those of the normal ipsilateral ventral corticospinal tract of the adult, remains a question for further experiments, for which Fluoro-Red and Fluoro-Green will be useful tools.

Neuronal survival and physiological viability after 2 weeks of labeling with Fluoro-Green were confirmed with patch clamp electrophysiological studies in acute cortical slices. Labeled corticospinal neurons were easily visualized in thick (350 μm) brain slices suitable for the patch-slice technique. They could be distinguished from surrounding unlabeled layer V neurons and neurons of other cortical layers, allowing application of patch pipettes to specifically identified corticospinal neurons. Afferent excitatory activity and postsynaptic responsiveness of labeled neurons suggested functional viability of labeled cells after 2 weeks. After recordings, cell contents could be aspirated into the patch pipette, confirming cell identification by observation of entry of the fluorescence into the patch pipette. These experiments suggest that these dyes are economical and highly effective tools for slice patch clamp studies focused on specific neuron groups that can be retrogradely labeled via a projecting tract.

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