# Benchmarks

## Inexpensive Method for Viewing Fluorescent DiI-Labeled Cells with a Dissecting Microscope

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Fluorescent tracers and bioluminescent proteins allow researchers to label specific cells and to study their biological properties in vivo or in vitro. However, the epifluorescence compound microscopes typically used to observe labeled cells have a short working distance, and they are not suited for observing and manipulating labeled cells in vivo. Commercial epifluorescence attachments are available for dissecting microscopes, but they are quite expensive (several thousand dollars). We describe modifications to a standard dissecting microscope equipped with a fiber-optic illuminator that provide sufficient sensitivity to detect small clusters of fluorescent cells in intact living tissue and that cost less than $15. Using this approach, we have been able to greatly increase the fraction of fluorescently labeled neurons placed into primary cultures. This technique is also likely to be useful to researchers who want to transplant labeled cells in vivo.

We have previously used the relatively nontoxic fluorescent lipophilic tracer [1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate; DiI-$C_{18}$-(3)] to retrogradely label sympathetic preganglionic neurons in the spinal cord of chick embryos by making injections into the sympathetic chains (1–5,7). The preganglionic neurons were then placed into cell culture for studies of synapse formation (5), neurotransmitter receptor regulation (1,2) or axon outgrowth (8). A limitation of this approach was that it was common to also label somatic motor neurons, because their axons pass very close to the sympathetic chain. With careful dissection with tungsten needles, it was possible to separate the spinal cord into two pieces, in each of which the only labeled cells were of the desired type. However, carrying out experiments was time-consuming because it was necessary to section and observe the material left behind to verify that only the desired population had been placed into cell culture. Furthermore, the labeled neurons comprised only a small portion of the cultured cells. Our yield of labeled cells could be increased by removing unlabeled cells using fluorescence-activated cell sorting (1), but the time, effort and cost that went into cell sorting were also high. We therefore needed a method that would allow us to observe clusters of DiI-labeled neurons in the living spinal cord, so that we could remove the appropriate cell populations under visual inspection. Rather than purchasing a commercial epifluorescent filter attachment, we have developed simple and cheap modifications to a standard dissecting microscope.

We use Roscolux plastic filters that are designed for theatrical lighting as both excitation and barrier filters (Rosco Filters, Port Chester, NY, USA [available at most local theatrical distributors and from Edmund Scientific, Barrington, NJ, USA]). In keeping with the wide variety of lighting effects used in the theater, a huge number of filters are available, each with well-defined spectral characteristics. These filters come in sheets that are 40–130 μm thick and can easily be cut to the desired size and shape. Filters are made from either co-extruded polycarbonates or deep-dyed polyester; they are heat-resistant up to temperatures of 160° or 125°C, respectively (although the manufacturer's instructions warn that they should not directly contact the light source). For determining the appropriate combination of filters to use with a particular dye, a sample book with 1½- × 3¼-in pieces of 100 different filters (available for under $10) is very convenient. Once appropriate filters are identified, a typical 20- × 24-in sheet costs only $5.95.

Mercury or xenon arc lamps are typically used in fluorescence microscopy because they have a high luminous density, but a fiber-optic 150-W Model 180 Fiber-lite® Quartz Halogen Illuminator (EKE lamp; Dolan-Jenner, Lawrence, ME, USA) at its maximum setting provided sufficient illumination (40 000 ft-c) with minimal heat production. To attach the excitation filter to the light source, a circular piece of filter was placed into the bottom of a 35-mm Petri dish, which was then attached with Velcro® to the fiber-optic lens. The Velcro allowed for quick insertion and removal of the filters. On one of our dissecting microscopes, a removable ring in front of the objective lens was used to mount the barrier filter; on the other, the barrier filter was placed inside a 60-mm Petri dish and then attached to the microscope body with Velcro.

Figure 1A shows a cross section of a chick spinal cord 16 h after injection of DiI-$C_{18}$-(3) into the sympathetic chains on both sides of the embryo, viewed with white light produced by the fiber-optic illuminator with no filters in place. This section was cut from living tissue with a Beaver® Microsharp® Blade (Becton Dickinson Labware, Bedford, MA, USA). In Figure 1B, the same section is viewed with the appropriate plastic filters in place. In this preparation, both preganglionic neurons (medial-dorsal cluster, arrows) and somatic motor neurons (ventral-lateral cell cluster, arrowheads) were labeled. Neither population of labeled neurons was visible with unfiltered white light (Figure 1A). Thus, these filters allowed the visualization of labeled cells, so that the isolation of preganglionic or motor neurons could be done more easily. Our previous experiments (3) demonstrated that DiI-labeled neurons survive well when placed into culture (Figure 1D); spinal cords that had been viewed through the plastic filters prior to dissection gave rise to the expected number of viable, fluorescently labeled neurons, so there is likely to be little or no excess cell death associated with the procedure.

One important issue regarding this simple approach for visualizing fluorescently labeled material is its sensitivity. Figure 1 (C and D) demonstrates that single cells can be successfully imaged. In these experiments, we labeled E7 chick dorsal root ganglia neurons with DiI during dissociation as described by Honig and Hume (3) and then placed them into low-density cultures. Figure 1C shows the cultured neurons as they appear through the dissecting scope with the appropriate filters in place. Figure 1D shows the same field viewed with a Model IM35 Inverted Epifluorescent Microscope (Carl Zeiss, Thorn-

wood, NY, USA). Each of the small fluorescent dots visible in Figure 1C corresponds to a single cell or a very small clump of cells when viewed at higher resolution in Figure 1D.

Our choice of the appropriate filters depended on the absorption spectrum of DiI and the human visual system's color processing. Under white incident light, DiI appears red, but clusters of DiI-labeled cells are very difficult to identify, because the amount of energy transmitted by the fluorescence is less than background lighting and decreases with the square of the magnification (6). DiI absorbs maximally at 500 and 550 nm and emits maximally at 565 nm (information provided by Molecular Probes, Eugene, OR, USA). Thus, a green plastic filter (Primary Green No. 91) placed in front of our light source as the excitation filter and a light red filter (Fire No. 19) placed in front of the objective lens as a barrier filter proved to be a good combination. This barrier filter did not completely block the transmission of green light, so a dim green background was reflected by the unlabeled tissue, and DiI-labeled cells appeared orange-yellow. Using Medium Red No. 27 as a barrier filter reduced the amount of transmitted green light but did not improve sensitivity because it also reduced intensity of the fluorescence. The green background was an advantage because it allowed us to see our dissecting instruments during tissue preparation and also improved color contrast.

In addition to DiI, we have examined DiO [3,3′-dioctadecyloxa-carbocyanine perchlorate; DiO-$C_{18}$-(3)], lucifer yellow and green fluorescent protein (GFP)-labeled material. These fluorochromes absorb blue light (428–490 nm) and emit yellow or green

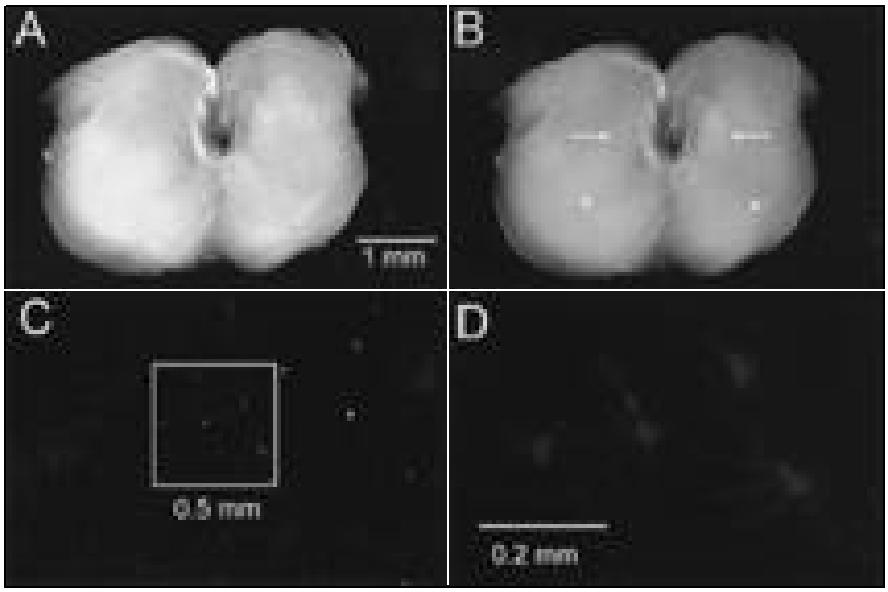


**Figure 1. DiI-labeled neurons can be seen using a plastic filter set and halogen light source.** (A and B) Cross section of the thoracic portion of an E7 chick spinal cord after pressure injection of DiI-$C_{18}$-(3) (2.5 mg/mL in 100% ethanol) into both sympathetic chains and a 16-h incubation in Tyrode's solution bubbled with 95% $O_2$ and 5% $CO_2$ as in Reference 3. (A) The section viewed with unfiltered white light illumination. Labeled neurons are not apparent. (B) The same section viewed with appropriate plastic filters. Clusters of DiI-labeled sympathetic preganglionic neurons (arrows) and somatic motor neurons (arrowheads) are easily distinguished. (C and D) Dissociated E7 chick dorsal root ganglia neurons labeled with DiI. (C) A region of the culture dish viewed through a dissecting microscope equipped with the appropriate plastic filters. (D) The region of the culture dish within the white box in Image C viewed at higher power with the Inverted Epifluorescent Microscope. Images A, B and C were taken with a three-chip color charge-coupled device (CCD) camera (Model DEL-750; Optronics Engineering, Goleta, CA, USA) mounted onto a dissecting microscope (a Model M5 Wild Heerbrugg [Heerbrugg, Switzerland] was used for Images A and B, and a Model SZ40 [Olympus America, Melville, NY, USA] was used in Image C). Image D was taken with a cooled CCD-300-RC Camera (Dage-MTI, Michigan City, IN, USA) mounted on the Inverted Epifluorescent Microscope. All images were captured using a Model LG-3 Imaging Board and Image software (Scion, Frederick, MD, USA) and modified for publication using Adobe® Photoshop® software (San Jose, CA, USA).

light (510–535 nm) (information provided by Molecular Probes and CLONTECH Laboratories, Palo Alto, CA, USA). We were able to observe DiO or lucifer yellow fluorescence using Night Blue No. 74 or Brilliant Blue No. 69 as the excitation filter and Medium Straw No. 14 or Deep Straw No. 15 as the barrier filter. DiO and lucifer yellow appeared orange and yellow, respectively, against a background of blue-green light, which improved color contrast. However, the fluorescence of cells labeled with these molecules was not as vivid as the fluorescence of cells labeled with DiI. This may be due to the halogen lamp's decreased radiance at shorter wavelengths (information provided by Dolan-Jenner). We were not successful in finding a plastic filter set that allowed us to observe GFP fluorescence on our dissecting microscope when either S65T or wild-type GFP was expressed in zebrafish embryos or frog tadpoles, even though GFP expression was readily observed when the same specimens were viewed on the Inverted Epifluorescent Microscope with the appropriate filter sets. We suspect that the considerable overlap between the absorption and emission spectra of GFP might explain our difficulty in detecting its fluorescence. If so, appropriate narrow bandpass interference filters may be sufficient to detect GFP fluorescence at a somewhat greater cost ($80–$200 each) than the plastic filters we have tested.

Although this technique lacks the sensitivity of commercial epifluorescence microscopes with interference filters, its simplicity and cost-effectiveness make it a valuable tool. Besides identifying labeled cells prior to culturing them, it will likely be useful to those who use DiI as a marker for transplanted cells (7) and fate mapping (9). Finally, this technique is affordable for educational demonstrations on the uses of fluorescent tracers and the principles of fluorescence microscopy.

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## Improved Measurement of Calcium Mobilization by Flow Cytometry

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Calcium mobilization is a common way to assess eukaryotic cellular activation. Resting cells typically maintain low concentrations of intracellular calcium ions (100 nM) as compared to the extracellular (1 mM). Intracellular calcium concentrations are often increased by extracellular signals such as an agonist binding to a receptor. Experimentally, these changes in calcium concentrations can be visualized as fivefold to tenfold increases in fluorescence of fluorescent indicator dyes, such as fluo-3, or as a shift in the emission intensity altering the emission ratio of such indicators as fura-2 or indo-1. Depending on the agonist and the signaling pathway, the mobilization of calcium can be fast and transient, imposing time constraints on its measurement.

Protocols for flow cytometric analysis exist that permit the analysis of agonist-induced calcium mobilization, indicating a signaling event (4,8). However, because the sample tube must be continuously pressurized while on the flow cytometer, data acquisition must be interrupted by removal of the sample tube from the instrument to add the agonist (2). This introduces a gap of approximately 10–20 s in data recording while the agonist is added, the sample mixed and the tube returned to the sample port, and then data acquisition is resumed. Thus, it is possible to miss fast transients that might occur during this time. Alternatively, extensive or costly modifications may be made to the cytometer to circumvent this limitation (3,5). To solve this problem, we have devised a simple and inexpensive method that permits continuous data acquisition while the agonist is added.

As illustrated in Figure 1, the technique uses a small, Teflon®-coated magnetic stir bar coated with the agonist and held above the suspension of cells in the tube as baseline data are acquired. The small magnet inside is kept in place by two larger magnetic stir bars held outside of the tube. After an arbitrary length of time acquiring back-