Neuroanatomical Tracing of Neuronal Projections with Fluoro-Gold

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1. Introduction

The study of neuronal connectivity requires the ability to trace axons from the neuronal cell body to its axon terminal (anterograde tracing) and from the terminal back to the soma (retrograde tracing). Such neuroanatomical tracing is frequently used to identify neurons on the basis of their pre- or post-synaptic connections. Neuroanatomical tracing has become particularly important in the field of nervous system regeneration and repair, allowing investigators to follow the axon projections of newly born, transplanted, or axotomized neurons in lesioned or neurodegenerative environments. To allow further study of neurons identified and labeled in this way, it is particularly important that tracers are compatible with other tissue processing such as immunocytochemistry.

Fluoro-Gold (FG; Fluorochrome Inc., Denver, CO, USA) is a fluorescent retrograde marker, commonly used for neuronal labeling and neuroanatomical tracing. Like other retrograde markers, FG is taken up by axon terminals, or through injured axons, and retrogradely transported to neuronal cell bodies, thus specifically labeling neurons that project to the region of application. The main advantages of FG, as compared to other retrograde markers, are (1) its ability to be visualized without additional processing; (2) its extensive labeling of distal dendrites; and (3) its stability for relatively long periods of time after application and under a variety of fixation and histochemical conditions.

FG is taken up by intact axon terminals and injured axons, but not by intact axons at nonterminal sites ("axons of passage") (1). Intact axonal uptake occurs via endocytosis at nerve terminals (2). Following retrograde transport, FG labels neuronal soma and dendrites. In the soma, FG is associated with vesicles.
in the cytoplasm, the plasma membrane, and the nucleolus (1). It does not
diffuse from labeled neurons, and it is not transported trans-synaptically (1).

Because of its stability, FG may be applied to axon terminals or cut nerves
by a relatively wide variety of methods. FG may be administered directly to the
target region via pressure injection (typically <1 μL); FG can be iontophoresed
(+5 to +10 μA/10 min) from small-tipped pipets that can be used to record
physiological activity from cells before labeling (3); and FG may be placed in
the target region in crystalline form. FG also may be applied indirectly (and
nonspecifically) to axon terminals by intraperitoneal (IP) or intravenous (IV)
injection (4). FG widely labels axon terminals in the peripheral nervous system
following IP or IV injections. However, it does not cross the blood-brain barrier
(BBB), and therefore does not label neurons that project to areas within the
CNS protected by the BBB (1,4).

A significant limitation of FG as a marker of specific projection neurons is
its tendency to diffuse from the site of injection in neural or muscle tissue. This
diffusion can lead to nonspecific labeling of neurons that project to regions
neighboring the intended target region (5).

FG is delivered to the cell body by fast axonal transport (6). The time
required for retrograde labeling depends on distance and on degree of dendritic
filling desired. FG labeling of cell bodies has been detected as soon as 2 h after
injection (7). Typically, minimum survival time for axonal and cytoplasmic
labeling is 1–2 d. Longer survival periods result in more complete labeling
of high-order neurites, up to hundreds of microns beyond the soma (8). FG
labeling is stable for weeks following injection, and persists up to 2 mo in
some reports (1).

FG is functional at a wide range of concentrations (1–10% w/v). Lower
concentrations (2–4%) are recommended for most procedures. Higher concen-
trations and larger injection volumes cause more intense retrograde labeling,
but also induce more necrotic damage at the injection site (1). FG labeling in
the neuronal cell body is non-toxic at recommended concentrations.

FG has been reported to locally label non-neuronal cells in vivo. Almost all
cells at the injection site become labeled. Rinamin et al. (9) found phagocytic
microglia and other macrophages containing FG following degeneration of
retrogradely labeled axotomized neurons, presumably due to ingestion of
dying FG-labeled neurons. Similarly, Streit et al. (10) reported FG-containing
glia following toxic injury to motor neurons, but not in control (nontoxic)
conditions. Injection of large volumes of FG into the lateral ventricle in rats
labels periventricular astrocytes and ependymal cells (1).

FG can be visualized directly by fluorescence microscopy using an ultraviolet
filter (excitation, 323 nm; emission, 408 nm). Color varies slightly with changes
in pH from gold (neutral and basic pH) to blue (acidic pH) (1). The ability to detect FG without additional histochemical processing allows for identification and dissection of FG-labeled regions under UV light (11). Visualization by fluorescence microscopy is limited by FGs tendency to photobleach, although its photostability far exceeds commonly used chromophores such as AMCA or Cy-3. Ju and Han (12) suggest that photobleaching is largely due to water in tissue sections, and may be avoided or minimized by the use of alternative mounting media.

Several methods have been developed to convert FG’s fluorescence into more stable, electron-dense products for electron microscopy (EM). FG can be converted directly to an electron-dense diaminobenzidine (DAB) reaction product by photooxidation to allow for EM visualization (13). The development of antisera against FG (14) has also allowed FG to be detected with DAB or immunogold-silver (8). In these protocols, FG is detected not only in lysosomes, but also dispersed throughout the cytoplasm and distal dendrites, two to three branch points beyond the soma (8). FG is reported to be stable in a variety of fixatives, or in the absence of fixation, and is not affected by most standard immunohistochemical procedures.

We have used Fluoro-Gold to label both callosal projection neurons and cortico-thalamic projection neurons in vivo in adult and neonatal mouse neocortex (15–20) and projection neurons in the song networks of the avian forebrain (21). The following protocol outlines our methods for labeling corticothalamic neurons in adult mice, and can be adapted to retrogradely label different populations of projection neurons.

2. Materials

1. Fluoro-Gold Fluorochrome Inc., Denver, CO, USA [(303) 832-1212].
2. Fluoro-Gold solution. Make a 2% (w/v) solution of Fluoro-Gold in double distilled water (see Note 1).
3. Nanoject Variable (Drummond Scientific Company). The Nanoject Variable is a digitally controlled oocyte pressure injector that is especially useful for injecting nanoliter volumes of solutions extracellularly into the brain, via small diameter pulled glass micropipets.

3. Method

In this protocol, we inject Fluoro-Gold into the thalamus using a posterior approach, inserting the glass micropipet at a 30° angle from the vertical axis in order to avoid injury to more anterior cortex, which was important for the specific experiments we were conducting.

1. Anesthetize and prepare the mouse for surgery in compliance with institutional and NIH guidelines.
2. Make a midline incision in the scalp from 2 mm posterior to the interaural line to 1 mm posterior to the eyes. Expose the lambda cranial suture and regions up to 5 mm lateral to it.

3. Mount the mouse in a stereotactic frame. It is critical that the skull is mounted in a stable horizontal position. The orientation of the skull can be confirmed by measuring the height of the lambda and bregma sutures and confirming that they are equal.

4. Make a small craniotomy with a scalpel (a drill may be used for animals with thicker skulls, such as rats) using the following coordinates: medial boundary, 0.5 mm lateral to midline; lateral boundary, 2 mm lateral to midline; posterior boundary, 1 mm anterior to lambda; and anterior boundary, 2 mm anterior to lambda. One should be careful to avoid damaging vasculature in order to minimize bleeding. It is not necessary to replace the removed bone fragment after surgery with such small craniotomies (see Note 2).

5. Make three injections into the thalamus. Insert pulled glass micropipet at a 30° angle from the vertical axis at the following coordinates:

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<th>3</th>
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<tbody>
<tr>
<td>Anterior to lambda</td>
<td>1.5 mm</td>
<td>1.5 mm</td>
<td>1.5 mm</td>
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<tr>
<td>Lateral to midline</td>
<td>0.8 mm</td>
<td>1.2 mm</td>
<td>1.6 mm</td>
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<td>Depth</td>
<td>3.6 mm</td>
<td>3.7 mm</td>
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These coordinates place the pipet tip in the following locations, targeting the thalamic nuclei VLc, VPLo, Area X, and vLo, which receive projections from motor cortex:

Anterior to lambda 3.24 mm
Posterior to bregma 0.97 mm
Lateral to midline 0.9, 1.2, 1.6 mm
Depth 3.04 mm

6. Inject 32 nL of FG solution and wait 1 min. Retract the glass micropipet 100 μm, inject another 32 nL and wait an additional minute. Retract the pipet slowly. Waiting for the FG to diffuse and withdrawing the pipet slowly minimizes the amount of FG that potentially effluxes along the micropipet’s path. Repeat at each injection site (see Note 3).

7. Suture the incision area and allow the mouse to recover. We allow 7 d for retrograde transport of FG to neuronal cell bodies in adult animals (see Note 4).

8. Transcardially perfuse with 10 units/mL heparin/PBS solution followed by 4% paraformaldehyde in PBS solution. Postfix brains overnight in 4% paraformaldehyde at 4°C. Section brains at 40 μm using a vibrating microtome (Vibratome); mount sections on gelatin-coated slides using Fluoromount (BDH); and examine by fluorescence microscopy under a filter with a 350–380 nm excitation spectrum.
4. Notes

1. FG is soluble in saline solution as well, but it precipitates out of PBS, forming a suspension. FG solution stored in the dark at 4°C is stable in solution for 6 mo. FG crystals are stable for years when protected from light and stored at 4°C.
2. Cranial bleeding can usually be stopped using gel-foam absorbable gelatin sponges (Harvard Scientific, cat. no. 59-9863).
3. The inner diameter of the glass micropipettes should be as small as possible; an outer diameter of 40 μm is sufficient. It is also possible to inject Fluoro-Gold using a Hamilton syringe. However, the large bore of the metal needle is likely to injure a much larger region of the brain, and is likely to result in additional efflux along the injection path. In addition, it is difficult to accurately inject the small volumes required to target single areas of the rodent brain using a syringe and needle.
4. FG may transport in shorter periods of time. Allowing longer times for transport can result in brighter nuclear labeling and increased labeling of dendrites.

References


