

General

The general idea is to selectively reveal the morphology of a neuron that you are recording from intracellularly. I'm going with the assumption that nowadays that means using fluorescent dyes, so I won't bother to describe older, non-fluorescent methods. There are a couple of general considerations that will determine what your best choice of stain should be:

- **Direct tracers vs. secondary staining.** Obviously, it would be nice not to have to go through a long staining protocol once your specimen is fixed. However, if you are planning to double stain, e.g. with an immunohistochemical protocol, indirect methods give you free choice of fluorophore to maximize signal separation.
- **Fluorescence properties.** Two properties are important. First, the spectral properties of a fluorophore (the wavelength ranges for excitation and emission) are important for imaging (make sure you have the matching filters, lasers, etc. in your microscope) and for signal separation (if you use more than one stain). Second, different fluorophores have different quantum yields. You want your fluorophore to be as bright as possible, but you also have to consider other advantages/disadvantages.
- **Electrode quality.** Different dyes have different properties as electrolytes. Because this document is about iontophoretic injections, all dyes discussed here are charged and can be moved out of the electrode by passing current. However, no dye is as good an electrolyte as the salt solution you are usually using for intracellular recordings (like KCl, KAc, or K_2SO_4). This leads to higher electrode resistances and, potentially, electrode clogging.
- **Fixability.** If you want live stains (e.g., for 2photon imaging), this is not of concern. For everything else, you want a dye that easily and reliably fixes in standard fixatives (e.g., 4% Paraformaldehyde).

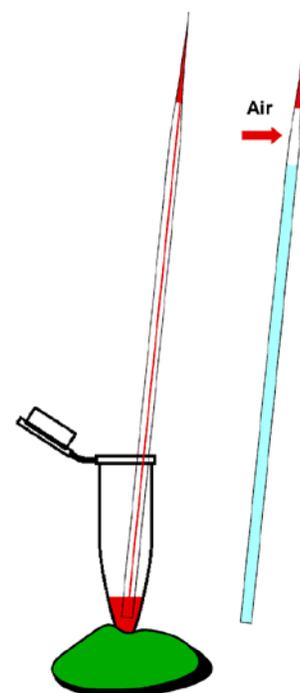
There are plenty of other dyes and stains around, but the ones listed below are the ones that to my knowledge are the most useful in the STG.

Intracellular iontophoretic staining of STG neurons

Electrodes and iontophoresis

The idea is that you fill an electrode with dye, record intracellularly from a neuron, and pass the dye into the neuron by applying current. If the current you apply has the same polarity as the charge on the dye molecules, the dye molecules should get pushed out of the electrode. All of the dyes described below are negatively charged at normal pH, so iontophoresis works pretty much the same for all of them.

Filling the electrode. Transfer a small volume of your dye into an Eppendorf tube, and stand it up by making a small base out of clay. Pull your recording electrodes. Of course, the lower the electrode resistance, the better the dye will pass. However, you don't want to compromise your recording quality too much. I recommend using the electrodes you usually use for current injections (e.g., in 2 electrode voltage clamp). The most important thing is to use capillary glass WITH filament to allow the dye to travel by capillary action and to provide electrical contact (see below). Place an electrode upside-down into the tube and wait a couple of minutes for the tip to fill (again, this won't work without filament in the glass). Contrary to the ancient wisdom of classic neuroanatomy, it is not actually necessary to fill the shanks of the electrodes with an electrolyte. In my experience, a bendy enough silver wire in your electrode holder will touch the wet filament in the electrode sufficiently to provide electrical contact. If this doesn't work for you, you can always fill the shank of the electrode with whatever is appropriate. However, make sure to leave an air bubble to prevent the dye in the tip from diluting too much.



Passing the dye. There are different ways to do this that depend on the quality of your electrode and the robustness of the cell you want to fill. Alexa hydrazides, dextrans, and Lucifer yellow are anions, so you should inject negative current. Only for neurobiotin you need positive current. If there are not too many other ions around, the amount of dye leaving the electrode is roughly proportional to the amount of current you are injecting. In theory, this means that the more current, the better. However, many neurons aren't particularly happy about large current amplitudes. In addition, dye-filled electrodes have a tendency to clog and not allow huge amounts of current, anyway. Practically, you will probably inject 5-20nA current (hyperpolarizing for

Intracellular iontophoretic staining of STG neurons

Alexa hydrazides, dextrans, and Lucifer yellow, depolarizing for neurobiotin). If you can get away with it, you can just inject constant current for 10-30 minutes. However, most cells and electrodes are happier when you use square pulses. I usually use 50%-75% duty cycle at 0.5-1Hz. If necessary, you can also give a depolarizing pulse every couple of cycles. I also often set up the injection protocol so it would pause for a couple of seconds every couple of minutes. This makes it much easier to check if your recording is still good. Finally, there is the fast and somewhat brutal option to rapidly expel dye from the electrode by turning up the capacitance compensation and letting it ring. Similarly, you can hold the clear button in the minus direction. Mostly, STG neurons are actually sturdy enough to be able to handle this, and you often get a complete fill within a few minutes. With direct tracers, you can actually watch the amount of staining.

Processing. After you remove the dye electrode, I would usually let the prep sit for another 0.5-1h to allow diffusion and/or transport of the dye. Fix in 4% paraformaldehyde at room temperature for 0.5 – 2h. 30 min is usually enough for dyes in the STG, but if you do other processing later (e.g. immunostaining), you should fix longer. Clearing and mounting procedures are described elsewhere.

Alexa Fluor® Hydrazides

Alexa Fluor® Hydrazides (life technologies) have some advantages over more traditional options, and they come in different colors. I recommend Alexa Fluor® 568 Hydrazide, for microinjection. It has similar spectral properties as TRITC or other rhodamine derivatives. It comes as a 200mM KCl solution and can be used straight up. Alexa dyes are named after confocal laser lines and different colors behave differently. In my hands, Alexa633 worked fine too, but Alexa488 had a tendency to precipitate and clog the electrode. Fill electrode shanks with 3M KCl if needed.

Advantages

- Very bright fluorophore.
- Dark purple non-fluorescent color (Alexa Fluor® 568), easy to see if your cell is nicely filled.
- Fixes very well.
- Survives even elaborate histochemical procedures (immuno, *in situ* hybridization)
- Because of its high quantum yield, it is fantastic for photoablations with a green laser!

Disadvantages

- Slightly toxic (not good for live imaging).
- Travels passively and slowly (usually not a problem for stains just in the STG).

Intracellular iontophoretic staining of STG neurons

Lucifer Yellow

LY is the most traditional direct tracer of the bunch. It doesn't have great fluorescent properties, but it travels well and can be used as an indirect tracer. Life technologies sells a rabbit antibody against LY that works very well. Use at 5% in 1M LiCl. Fill electrode shanks with 2M LiCl if needed.

Advantages

- Travels very well (also good for backfills!)
- As indirect tracer, free options for fluorophores.

Disadvantages

- As direct tracer, weak fluorophore and such a broad emission spectrum that it cannot easily be separated from other signals.
- Needs 405nm laser or special fluorescence cube for imaging or dye kills
- For really good signal, needs a long secondary staining protocol (primary & secondary antibody).
- As secondary tracer: antibody only comes as rabbit-IgG. Therefore, for double labels it can only be paired with non-rabbit antibodies.

Conjugated Dextrans

Dextrans come in all kinds of carbon chain length and conjugated with all kinds of different fluorophores. I have not used Alexa conjugated ones myself, so I recommend Tetramethylrhodamine, 3000 MW, Anionic, Lysine Fixable. 3000MW means it's the smallest molecule (other options are 10,000 and 30,000). It is designed to be fixable in paraformaldehyde by lysine-crosslinking. This works very well in insect nervous systems and other animals, but for some reason not in crustaceans (at least not in marine ones). No idea why. *Therefore, dextrans are only good for live imaging!* Use at 5% in distilled water. The net molecular charge depends on capping of unreacted amines and is apparently not that consistent. Therefore, sometimes it actually passes better with depolarizing pulses.

Intracellular iontophoretic staining of STG neurons

Advantages

- Travels very well (is apparently actively transported).
- Completely non-toxic (good for long-term live imaging).

Disadvantages

- Doesn't fix.
- Not that bright. Independent of fluorophore, the ratio of conjugated and unconjugated sugar molecules is pretty low.
- Pretty sticky, i.e. clogs easily and yields high electrode resistances.

Neurobiotin Tracer

Neurobiotin is a biocytin derivative designed for intracellular staining (more soluble, more charged). It is a colorless substance that can be visualized with a fluorescently labeled streptavidine (biocytin-streptavidin is the strongest known covalent bond). Use as 5% in 1M KCl. After fixing, treat like an immunoprep (except that it doesn't need a primary antibody). Stain with 1:600 Streptavidin (coupled to whatever fluorophore you like). If used in conjunction with immunostaining, add the Streptavidin to the secondary antibody step.

Advantages

- Non-toxic.
- Travels well (good for backfills).
- Free choice of fluorophore.
- In double labeling, can be used in conjunction with any antibodies.

Disadvantages

- Passes through gap junctions. Unless dye-coupling is the actual objective, you can't wait too long before fixing.
- Crustacean tissue has some intrinsic epitope that binds streptavidine. Chances are that you will get a lot more background staining than with other dye.