<u>Backfilling reticulospinal neurons</u> (Jon Clarke, UCL)

You will need:

3-5 day old larvae
fish anaesthetic
fluorescent dextrans (3000 or 10000 MW lysinated rhodamine dextran from Molecular Probes)
tungsten micropins made from 0.5mm diameter wire
fine forceps
2 Sylgard-bottomed petri-dishes and micropins for dissection (don't throw these dishes away!!!)
1.5% low melt agarose
water bath at approx 40º C
glass microscope slides and coverslips
glass pasteur pipette
4% paraformaldehyde containing 4% sucrose ("sweet fix")

1. Clean a couple of Sylgard-covered Petri-dishes with distilled water or clean tank water.

2. Prepare dextran mixture. You need a small pile of dextran crystals on a Sylgard-coated Petri-dish. Add to the crystals a tiny amount of distilled water (I usually do this by picking up a drop with fine forceps but you could be scientific and use a micropipette) so that the crystals dissolve and make a <u>thick, sticky</u> solution. Or dry down from a 10mg/100ul solution.

3. Catch your fish.

4. Anaesthetise fish in 0.03% MS222 (aka MESAB or Tricaine). This takes a few minutes.

5. Load up a tungsten micropin with sticky dextran. Your dextran will probably have solidified by now but just add another <u>tiny</u> drop of distilled water to edge of dextran sludge and pick up a thick drop onto the micropin. Put this dextran-laden pin carefully aside.

6. This is the tricky bit. Using a glass pasteur pipette, transfer 1 larvae in as small a volume of medium as possible (single drop if you can) into warm (NOT HOT) agarose. Quickly suck larvae plus agarose back up the pipette and transfer a small drop of agarose containing the larvae onto a glass slide. Using your other micropin quickly orient larvae so that it lies on its side and as close to the surface of the agarose drop as possible (too deep and it makes the dye application difficult).

7. Allow 2 or 3 minutes for agarose to set firm. Test this by gently prodding it with forceps.

8. This is the other tricky bit. Using a clean sharp tungsten micropin make an incision through the spinal cord of the larvae. Insert the tip of the micropin between notochord and ventral spinal cord and flick pin dorsally to cut spinal cord. Use quick movements of the pin to cut through the tissue and aim to make a complete transection of the spinal cord. Do this at about the level of the 5th somite (or more caudally if you wish).

9. Quickly thrust the dextran-laden micropin into the incision you have just made and hold it there for a few seconds. The dextran will dissolve off the pin and into the hole in the fish. Some of the dextran will diffuse into the cut axons of the spinal cord and this is how the reticulospinal axons become labelled. You can pick up more gooey dextran and repeat application onto the spinal cord if you are worried you didn't get enough in first time, but don't go mad as excess background fluorescence will spoil the prep. This procedure will also label many neurons and axons in the spinal cord caudal to the transection. NB. Speed is important here – the damages axons will quickly seal over and then no dye will be able to get into them.

10. Leave for about 2 minutes before adding fresh embryo medium to the top of the agarose. Now carefully carve the larvae out of the agarose block and then suck this up in a pipette and transfer to a Petri dish containing embryo medium or clean tank water for recovery.

11. Repeat this for several larvae.

12. Leave for 30 mins or longer (good time for a break or lunch). You can check the quality of the labeling at this stage using a fluorescence dissecting stereomicroscope, the fluorescent cells should be easily visible in the living embryo viewed dorsally.

13. Re-anaesthetise and fix fish in 4% paraformaldehyde with 4% sucrose for 1 hour or more (you can store them in this in fridge until dissection).

14. Dissect out the midbrain, hindbrain and anterior spinal cord as a single chunk of tissue. Pin the fish through its tail and any convenient ventral head structure into a Sylgard dish containing PBS. Use micropins and fine forceps to remove overlying ectoderm and as many melanocytes as you can without damaging brain, and then seperate brain from underlying mesoderm. This is a bit fiddly but the brain is surprisingly robust. Also save the tail if you want to look at any spinal neurons you have filled.

15. Place dissected brain, ventral side up on a microscope slide (pick up the tiny bit of tissue either

in a pasteur or carefully between tips of forceps). Make 4 small pillows of silicone grease to support a 22x22 coverslip and gently lower a coverslip onto the brain and silicone grease pillows. Do not crush the brain. Add a small volume of 70% glycerol from the side of the coverslip and this will be sucked across the specimen by capillary action. Mop up excess glycerol so it doesn't flow onto microscopes.

16. Analyse specimen under a fluorescence microscope or confocal. Store specimens in fridge.

Alternatives

Try labeling spinal motor neurons by poking the dextran into the segmental muscle blocks. Place the dextran into the ventral half of the muscle blocks to avoid damaging spinal cord.

You can also use a pressure injection system, such as Picospritzer, to inject the fluorescent dextran solution into the spinal cord.