SUPPLEMENTARY MATERIAL 1

Chick Forebrain Neuron Isolation (version 1.5, updated 7/24/12)

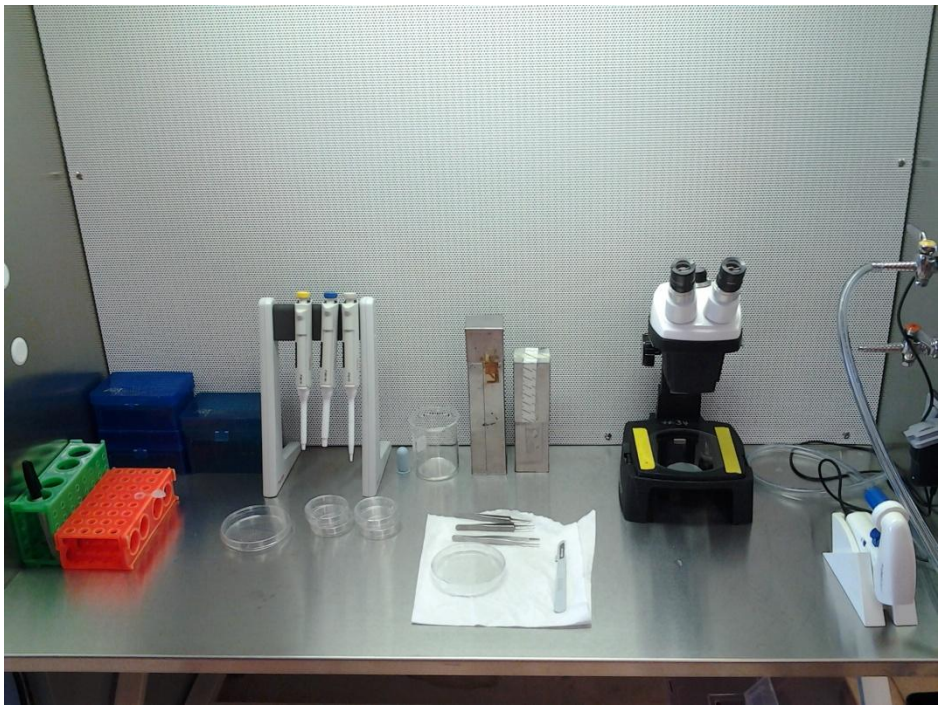
Note: Everything that goes into the ethanol soaked paper towel before being placed into the hood.

The dissection implements should be repeatedly soaked or rinsed with ethanol and wiped with a clean paper towel to keep contamination carry over from step to step to a minimum.

Keep the dissection cold as you progress by using a 100mm dish packed with ice or a flat cold pack from the freezer as a dissection base.

Supplies Needed:

- Ice bucket with ice
- Trash bag for egg waste
- 2x100mm & 5x60mm dishes
- Normal culture hood supplies
- ~100 mls HBSS Ca/Mg(-) and ~10 mls HBSS Ca/Mg(+)
- 2x70um filters for embryo rinsing
- Trypan blue
- 10 & 25ml serological pipets
- 1 dissection pack
    - 2 fine forceps, 1 large forceps, disposable scalpel
- Hemacytometer and cell counter
- 25 mls warmed media for 1x48 well plate (poly-L-lysine coated)

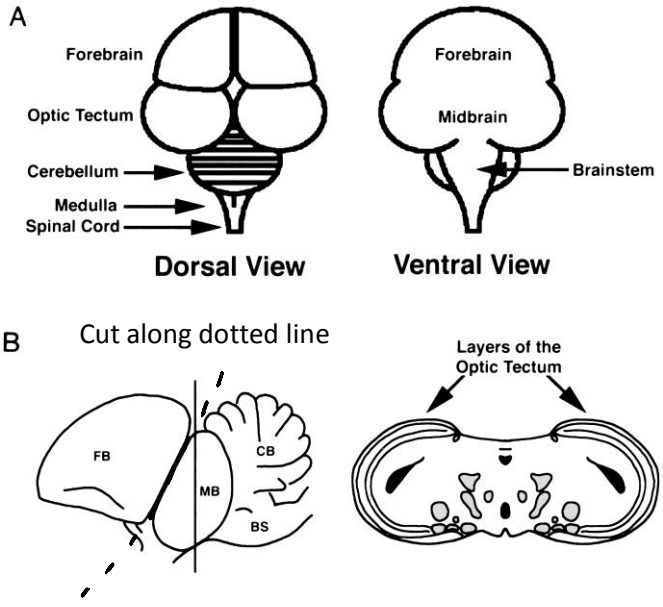


1. At least ½ hr before needed, turn on water bath, cooled centrifuge and hood (if the blower is not already on)
2. Place a tube with at least 25 mls of media into the water bath
    a. A full tube will take ~20 minutes to equilibrate when the water bath is up to temp

3. Fill a bucket with ice (if one is not already in the lab), and place two tubes of HBSS on ice. One tube should be Ca/Mg containing (+) HBSS, the other should lack (-) Ca/Mg.
4. Tape a small trash bag into a larger one to collect egg waste
5. Check the vacuum traps to make sure they will not be overfilled by your procedure
6. Put on gloves, and wipe down everything in the hood and the hood working surface with ethanol soaked paper towel
7. If an egg is not waiting for you in your hood, obtain one containing a chick embryo (this is determined by candling with a powerful flashlight) and wipe it down very well with ethanol before placing it in the hood.
    a. The egg shell is one of the biggest potential source of contaminating microbes, so pay particular attention to washing the outside very well with an ethanol soaked paper towel
8. Place 2 x 100 mm dishes and 5 x 60 mm dishes into the hood
9. Add approximately 4 mls of ice cold Ca/Mg (-) HBSS into each of the 60mm dishes
10. In one of the 100mm dishes, place 2 fine forceps and 1 blunt forceps from an autoclaved dissection tools pack, as well as a new disposable scalpel. Add 70% ethanol to this dish at a level that covers the working ends of these implements.
11. Place the other 100 mm dish on an ethanol soaked paper towel in the middle of your work space
12. Crack open the egg on the side of the empty 100mm dish as you would if you were cooking with it. With some practice, you should be able to let the yolk drain out of the egg into the 100mm dish while retaining the embryo inside the shell. The goal is to keep yolk contamination (and its associated microbiota) of the embryo to a minimum
13. Using the blunt forceps, pinch the embryo at the base of the neck and remove the head into the first 60mm dish. **Place this dish and all successive dishes you are working with on top of a covered 100mm dish packed flat with ice to keep the dissection as cold as possible.**
14. Throw the 100mm dish containing the yolk, egg shell and embryo body into the egg waste trash bag. Thoroughly wipe up any mess from the egg with an ethanol towel.
15. Place one of the 70 um filters into a 50 ml tube. Use the blunt forceps to place the head in the filter, and use a 25 ml serological pipet to rinse the head well with 1 pipet full of cold Ca/Mg negative HBSS, being careful not to aspirate fluid into the pipet cotton plug. Flip the head over

halfway through dispensing to clean the entire surface. Make sure the rinsing fluid does not overflow the receiving tube.

16. Use the blunt forceps to move the head into a new 60mm dish. Bring the dissection scope to the front of the hood (keeping it 6+ inches inside of the hood), and carefully place the 60mm dish containing the head on the stage
17. Focus the scope on the head using the adjustments.
18. Looking through the dissecting scope, use the beak as a point of reference. Hold the head or neck gently with the blunt forceps, remove the midbrain, cerebellum and the rest of the hindbrain with the scalpel by cutting along the fissure separating it from the forebrain and front of the head (see A and B in the figure below). The beak and eyes will still be attached to the forebrain (see C below; your chick won't be this color though!).
19. Leaving the eyes attached for reference points, and keeping a close watch under the dissecting scope, use the fine forceps (pinch them together to use as a probe) to gently push into the space between each telencephali hemisphere and the eye. Don't remove the eye, but do open up more of a space between the brain and eye. Do the same for the other hemisphere. It should now be possible to roll the telencephali out of the head, flipping the front of the brain back and over into an upside down position. Move the telencephali to a new 60mm dish using the blunt forceps.



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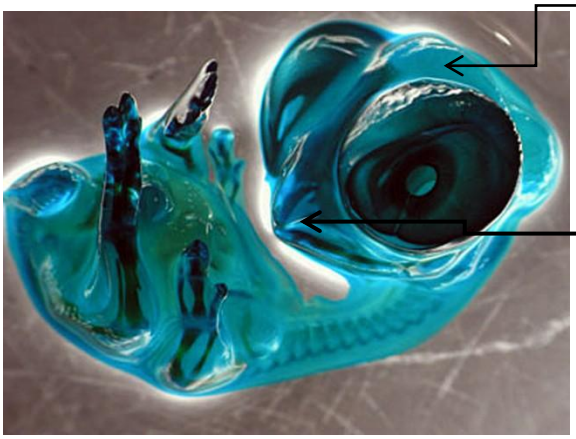

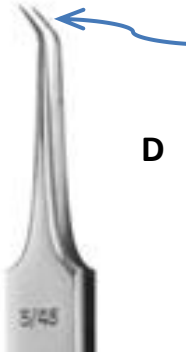

Flat angled end of the forceps.
Pinch/scrape meninges with this side when removing

D

20. Use the fine forceps in pairs to remove the shiny clear layer on top of the telencephali if still present (the embryonic skin and skull), as well as the meninges. Careful removal of the meninges is key to a neuron pure culture. “Never a trace of red” is the mantra here (except for the very fine vasculature within the brain tissue itself). Hold the telencephali down gently with one pair of fine forceps with using the flat angled end (D; see above) of the other pair to gently scrape and tug away at the meninges. Try to remove them as a sheet of tissue if possible. Sometimes it is possible to pull the sheet of meninges up out of the dish with the forceps and the weight of the brain and surface tension of the water will allow clean separation of the two. Wipe meninges on edge of 35mm dish to remove from forceps if necessary. Try to keep each hemisphere intact during meninges removal.
21. Use the fine forceps to move the telencephali to a new 70um filter in a 50ml tube and rinse gently with a 25ml pipet full of cold Ca/Mg (-) HBSS. If you can’t pick the hemispheres up with the fine forceps, use the 1000 ul pipet set at ~200 ul with just a small amount of pressure on the plunger and aspirate up the telencephali with just enough HBSS to move to the filter. Avoid aspirating up any meninges.

22. Move the telencephali to a new 60mm dish after rinsing.
23. Using the scalpel and/or both sets of fine forceps, mince the telencephali into small pieces to facilitate pipet trituration.
24. Pipet the brain pieces and HBSS into a 15ml tube with the 1000 ul pipet.
25. Use a flame narrowed 9" Pasteur pipet (in the tall metal can) with rubber bulb to triturate the brain as many times as it takes to homogenize the tissue (30-40+ times most likely). If this isn't done sufficiently, you will have large clumps of cells when you count, which is not good. Do just a little volume at a time to avoid aspirating the homogenate into the filter plug at the top of the pipet.
    a. Keep a close eye on the fluid and bubbles in the pipet to make sure it doesn't hit the plug. If you're getting close and can't force any more fluid out of the pipet with the bulb, keep the bulb squeezed and remove it from the pipet to release the fluid back into the tube.
26. Add 2x volumes (compared to what is in the homogenate tube) of Ca/Mg containing (+) HBSS to the homogenate
27. Let any unhomogenized tissue settle to the bottom of the undisturbed tube for 2-3 minutes. With a 1000ul pipetor, gently pipet the supernatant to a new tube and centrifuge for 2 minutes.
    a. The centrifuge should be precooled to 4 degrees C
28. Spin the tube (with a balancing tube) at 300 g for 3 minutes.
    a. An asterisk (*) next to the speed indicates gravities (g)
29. While the tube is spinning, remove the warm media tube from the water bath, dry with a paper towel, wipe well with an alcohol towel, and then place in the hood. Also, put the hemacytometer, cover glass and your Trypan blue tube near the hood on the center bench.
30. Remove the tube from the centrifuge, wipe it with an alcohol towel, place back into the hood, and gently aspirate off the supernatant with the vacuum and a short Pasteur pipet.
    a. Leave behind a little bit (250ul - 500ul or so) of HBSS if necessary to avoid aspirating the cell pellet
31. Resuspend the cell pellet with 5 mls of media using the serological pipetor set on slow and a 10 ml pipet
    a. If you have less than 2 full telencephali, from your dissection, use less media for resuspension to ensure a sufficient concentration of cells.
32. Gently shake a small microfuge tube out of the bottle in the hood

33. Making sure the cells have been well resuspended, use a 100 ul pipetor to take up 20 ul of cells from near the middle of the 15 ml tube. Keep the shaft of the pipetor clean.
34. Add the 20 ul of cells to the small microfuge tube
35. Take the microfuge tube out of the hood and use a 100 ul pipetor from the center bench to add 20 ul of Trypan blue, for a total volume of 40 ul
36. Triturate gently with the pipetor to mix the cells and Trypan blue, and pipet up 20 ul of the mixture.
37. Slowly add the mixture to the groove of the hemacytometer until blue dye covers the grid area on one side
38. View the entire hemacytometer with the 10x lens to ensure you have an even distribution of cells before counting. Count the Trypan blue negative cells per the cell counting protocol
    a. If you have clumps of more than 3-4 cells on your hemacytometer, you will need to go back into your cell containing tube and retriturate the cells with another flamed pipet. Repeat the counting as necessary.
39. Determine # of cells per ml in your tube
40. Dilute the cells as appropriate by adding the correct volume of cells from your tube to fresh media in a 15 or 50 ml tube
    a. An entire 48 well plate will need 9.6 ml of media
    b. Always make up 10-20% extra cell mixture for plating if possible
41. Add 200 ul per well of diluted cells to each well of your poly-L-lysine coated 48 well plate
    a. The plates are not coated from the manufacturer; we do this ourselves, so do not use new plates that have not been coated.
42. View your wells briefly under the microscope to ensure there are cells present
43. For L-15 media (non-CO_2 dependent media), place cells in humidified 37 degrees C incubator
44. Clean up the hood and other equipment/materials you used during the dissection
    a. Wipe *everything* in the hood with an ethanol towel when you are finished
    b. Add ~100-200 ml of 10% bleach or 70% ethanol to the empty waste tip beaker, swirl gently, aspirate with the vacuum hose.