Protocol for primary striatal cultures

Day 1:

Add poly D-lysine/laminin solution to 24-well plate. The solution contains poly D-lysine at a final concentration of 0.05 mg/ml and laminin at a final concentration of 0.005 mg/ml. Swirl the plate to ensure that the coating mix covers the entire bottom of the plate. Leave the dishes/coverslips in the 37oC/5% CO2 incubator overnight.

Day 2:

1. Wash the dishes/coverslips twice with sterile water; remove the final wash and leave them liquid-free in the incubator.
2. Make up Neuronal Growth Media with serum:
    - 911 mL BME Eagle media without L glutamine and with Earle salts
    - 50 mL bovine calf serum
    - 24 mL 1_1/2 solution (To 97.6 mL of H2O, add 1.4 mL of 2.5 M glucose solution, 0.5 mL of 0.2 M L-glutamine solution, and 0.5 mL of Pen/Strep solution)
    - 4.5 mL Stable Vitamin Mix (To 198 mL of distilled water, mix in the following by stirring: 600 mg L-proline (Sigma P-0380), 600 mg L-cysteine (Sigma C-8755), 200 mg p-aminobenzoic acid (Sigma A-9879), 80 mg vitamin B12 (Sigma V-2876), 400 mg i-inositol (meso)(Sigma I-5125), 400 mg choline chloride (Sigma C-1879), 1 g fumaric acid (Sigma F-2752), and 16 mg coenzyme A (Pharmacia 28-3001-02). Then add the following to 10 mL of distilled water: 0.4 mg d-biotin (Sigma B-4501) and 100 mg DL-6,8-thioactic acid (Sigma T-5625). Shake to resuspend. Then quickly pipette out 2 mL of the

biotin solution and add it to the 198 mL solution above. The ingredients do not dissolve completely, so stir before aliquoting into Neuronal Growth Media).

- 0.5 mL ITS (stock solution is 5 mL H2O added to Sigma product I1884)
- 5 mL Putrescine (1.6 mg/mL stock solution using Sigma product P7505)
- 5 mL Transferrin (5 mg/mL stock solution using Sigma product T2252)
- 100 uL Progesterone (1.2 mM stock solution using Sigma product P6149).

3. Make up Optimem/glucose solution (add 4 mL of 2.5 M glucose to 500 mL Optimem (Invitrogen)).

4. Make up DM/KY, sterile filter, and place on ice. Make the DM/KY slightly basic (just pinkish, approximate pH of 7.5-7.6). During the dissection process, you'll notice that the DM/KY solution next to brains in the dish turns yellow. This is from lactic acid release. Making the DM/KY slightly more basic helps better neutralize this massive lactic acid release. Composition of DM/KY solution:

   Dilute the 10x KY stock prepared below into the appropriate volume of DM.

   To make 1000ml DM, add the ingredients below to distilled water for a total volume of 1000 ml and then filter sterilize. Store at 4oC in refrigerator.

DM Ingredient	Final concentration	Stock concentration	Volume to add for 1000 mL
Na2SO4	81.8 mM	1 M	81.8 mL
K2SO4	30 mM	0.5 M	60 mL

MgCl2	5.8 mM	1 M	5.8 mL
CaCl2	0.25 mM	0.1 M	2.52 mL
HEPEs	1 mM	1 M	1 mL
Glucose	20 mM	2.5 M	8 mL
Phenol Red	0.001%	0.5%	2 mL
NaOH	0.16 mM	0.1 N	1.6 Ml

For 10x KY solution, gradually add small amounts of kynurenic acid to water containing phenol red and use the color of the phenol red to titrate the pH of the solution back up to about 7.4 as the acid dissolves. Filter the mixture of ingredients below and store at 4oC.

10X KY Ingredient	Final concentration	Stock concentration	Volume to add for 1000 mL
Kynurenic acid	10 mM		1.8925 g
Phenol Red	0.0025%	0.5%	5 mL
HEPES	5 mM	1 M	5 mL
MgCl2	100 mM	1 M	100 mL
NaOH		1 N	Add dropwise to titrate pH

5. Make up the trypsin inhibitor solution and the papain solution BUT DO NOT add papain at this point; place solutions on ice. To prepare the solutions, add 150 mg of trypsin inhibitor to 10 mL of DM/KY and pH the solution until it is again slightly basic (estimate a pH of 7.5-7.6). Add 2-3 mg of cysteine to 10 mL of DM/KY to make the papain solution and again pH to around 7.5-7.6. Leave these solutions at room temperature.

6. Pour ice-cold DM/KY solution into several culture dishes: 1 large dish for the pups and 10cm dishes for the pup heads, for the intact brains and for the dissected striatum. Place dishes on ice. Pour all solutions under the hood to keep things as sterile as possible for as long as possible.

7. Put all the dissection tools you will be working with (several pairs of forceps, a chemical spatula, one large and one small pair of scissors, and anything else you will need) into an alcohol bath to sterilize. If infections have been a problem, consider flame sterilizing the dissection tools and leaving them on the edge of a surface so that the parts that touch the rat embryos don't touch any unsterilized surface. While you are doing your dissections below, be sure to place your dissection tools (when not in use) in a way that maintains their relative sterility. One way to do this is to have the top cover of a 15 cm dish and place the forceps/scissors/spatula on the cover such that the end of the instruments that will be touching the rat brains is hanging off the edge of the dish cover.

8. Obtain pregnant rat

Dissection of striatum:

1. Sacrifice the rat.

2. After the rat fails to move spontaneously or in response to pain (touch the eye and look for a reflex), puncture each lung with a needle. Clean the belly of the rat with alcohol and then incise along the abdomen and remove the uterus. Place the pups into the large culture dish on ice. Try hard to prevent the pups from touching the outside of the mother or other unsterile surfaces. The better you can get the pups straight into the large culture dish, the better.

3. Remove the heads of the pups and place in a 10cm dish on ice.

4. The rest of the protocol is done on ice, but under a dissection microscope. For each head, remove the skin and place the two prongs of one set of forceps into and through the eyes of the head (the head should be positioned so you are looking down on the top of the head). This pins the head down and allows you to use the other set of forceps to cut into the head. Use the other forceps (in a closed position) to puncture the part of the skull that is exactly midline where all the sutures meet. Then carefully run your forceps towards the eyes and then towards the back of the head to open up a midline cut in the skull (don't dig down too far or you'll scrape brain). Once you have a big enough opening, remove your other pair of forceps from their position through the eyes and peel the skull back using each forceps, pulling in opposite directions; this kind of counter-traction is most effective in breaking open the skull. Once enough of the brain is exposed, take the chemical spatula, dig underneath the brain, and scoop the brain out. Place the brain into a new 10 cm dish with DM/KY on ice.

5. Repeat the process, but BE SURE that the dish which contains your newly dissected brains gets swirled every once in a while. This prevents the local buildup of lactic acid around each brain, which decreases viability of your neurons.

6. Once you have removed all the brains, you are ready to dissect the striatum. Orient yourself so that the brain is facing forward (the olfactory bulbs are at the top of your view and you are looking down at the top of the brain, rather than looking down on the brainstem). For each hemisphere, use your forceps to dissect longitudinally (sagittal incision) down the hemisphere. There should be equal amount of cortex to the left and to the right of your cut. Don't dissect too deep. Your goal is to just expose the structures underneath the superficial cortex. When you've split open the surface of the cortex, you should eventually be able to see the fine capillary network that makes up the choroid plexus of the lateral ventricles (you

may need to very gently use the blunt aspect of both forceps to open up the longitudinal cut you've made to see the plexus. Once you've located the plexus, you want to push your forceps down into the incision you made such that you are splitting the cortices exactly along the plane where the choroid plexus is. This will leave you with a lateral half of the cortical hemisphere. Once you have isolated this lateral half of the cortical hemisphere, place it so that the side facing you was previously buried in the brain and the side facing the dish is the lateral surface of the cortex. From there, you should be able to faintly see a semi-circle like structure. The cortex will appear to slightly indent (like it has folded over something else) on the side closest to the top of the brain (i.e. not the side that was previously buried). We will call this side the lateral surface. The indentation of the cortex near the lateral surface creates a semi-circle structure. The striatum is the brain material that is medial to this semi-circle indentation. You may see the hippocampus posteriorly (it looks like a banana – a curvy line inside a banana-peel). Be sure to dissect away the hippocampus. Cut along the semi-circle line and throw out the cortex and hippocampus that came off. You should now be left with a semi-circle piece of tissue that is MAINLY striatum. However, the cortex actually coats the piece of tissue you have ON THE BACK SIDE (i.e. the side facing the dish). You need to be sure to dissect this remaining cortex off the striatum. Do this by placing the semi-circle piece of tissue such that the medial aspect of the semi-circle (the part of the semi-circle that was oriented towards the bottom of the culture dish when the brain was intact) is again facing down towards the bottom of the dish. This now means that the lateral edge of the semi-circle is on top and closest to your eyes. You should be able to discern a faint tissue plane in looking at the tissue from this angle. The lateral side of that tissue plane is the cortex that is wrapped around the back of the striatum. Use your forceps to dissect down through this tissue plane to remove the cortex that has coated the striatum. Take your piece of freshly dissected striatum and place it in a new 10 cm dish with DM/KY on ice.

7. Again, every once in a while, be sure to swirl both the dish that has the brains in it and the dish that has just the striatum in it to help prevent local buildup of lactic acid.
8. Repeat the process on the other cortical hemisphere. When you have just 1-2 brains left to dissect, take a break and add papain to the papain solution. You should be adding 100 units of papain to the solution. Place both the papain solution and the trypsin inhibitor solution in the 37oC water bath. Be sure the water bath is actually at 37 degrees!
9. Finish your dissections.
10. Sterile filter the papain and trypsin inhibitor solutions. Leave both solutions out at room temperature.
11. Transfer the striatal tissue to a 15ml conical tube taking as little DM/KY as possible. Once the tissue has settled remove the extra DM/KY solution.

Papain treatment:

1. Add 10ml of the papain solution to the dissected tissue and incubate at 37oC for 15min, mixing every 5min.
2. Remove the enzyme solution.

Trypsin Inhibitor treatment:

1. Add 5 ml of trypsin inhibitor, mix the tissue, and incubate for 10 min at ROOM TEMPERATURE.

2. After 10 min, remove the trypsin inhibitor and replace with a fresh 5 ml aliquot. Wait another 10 min at room temperature.

3. Remove trypsin inhibitor solution and wash with 10 mL of Optimem / glucose; the Optimem/glucose should be at room temperature. Remove the Optimem/glucose solution.

Trituration:

1. Add 5 mL of Optimem/glucose. Triturate gently with a 5 ml pipette until the solution turns cloudy. The trituration should start off extremely slow. You have to be patient during these steps of the protocol since your yield and neuronal health will go up substantially if you take your time and triturate slowly. Also, during your first few rounds of trituration, you should avoid pipetting the brain/Optimem solution directly into the bottom of the conical tube. Instead, as you pipette out, you should be simultaneously lifting your pipette higher and higher out of the conical tube. The tip of the pipette should still stay under the Optimem solution, but it shouldn't be too far under the surface of the solution. Triturate until the solution becomes somewhat cloudy (but not too cloudy!). If you triturate up and down with dissociated neurons (which make the solution cloudy), you'll cause sheering stress and kill them. So the goal is to free up enough neurons each round, but stop the trituration as soon as there are a reasonable number of neurons free in solution.

2. Allow the brain material to settle (at room temperature) and then take the cloudy supernatant and transfer it to a 50 mL conical tube that is also left at room temperature. Add 5 mL of new Optimem/glucose to the 15 mL conical tube and repeat trituration.

3. Keep repeating steps 1 and 2. As you increase the number of times you triturate, you will eventually have to triturate more aggressively (faster and pipetting against the wall of the

conical tube). You should only become more aggressive when gentler methods fail to turn the solution cloudy and you still have significant brain material left.

4. I typically triturate 10 times for striatum collected from 10-15 brains.

5. Allow everything to settle in the 50 mL conical tube and then take a pipette and suck up the random debris/DNA/etc. that has accumulated at the bottom of the tube. Be careful not to contaminate the solution with any unsterile parts of the pipette (e.g. if you are using a P1000 to suck up the left-over bits in the 50 mL conical tube, the sterile tip may not reach all the way to the bottom of the conical tube).

Plating cells:

1. Mix the cell suspension in the 50 mL conical tube and transfer a 10ul aliquot to a tube that contains 10 ul of DM/KY and 10 ul of Trypan blue. Mix thoroughly, add to the cytometer, and count the number of cells in the 16 box squares in the 2 opposite corners of the field. Average the 2 counts or recount if the 2 numbers are different by more than 10%. Multiply the average by 30, 000 to get the number of cells per ml.

2. Dilute the cells with Optimem/glucose solution to a final count of 0.5-0.6 million per mL and plate 1 mL per 24 well plate.

3. As you are plating, be sure to add the neurons to the center of the well (don’t squirt the cell suspension solution onto the side of the well). Also, after adding the cell suspension solution to all the wells in a row, swirl the plate to make sure the neurons are evenly distributed and swirl your dispensing container to make sure the neurons don’t settle.

4. Once you have fully plated the neurons, swirl the plate one last time and then don't move the plate at all. Just leave the plate in the hood (at room temperature) for about an hour. This helps ensure that the neurons settle evenly across the whole well. We've previously had problems with the neurons settling preferentially in the center of the well or the periphery of the well.

5. After an hour, place the plates in the 37 degree incubator and leave for another hour.

6. Check the plates under the microscope to ensure that the neurons have adhered to the well surface (tap the plate and observe for movements of the neurons under the scope).

7. Assuming the neurons have attached, replace the Optimem/glucose with pre-warmed Neuronal Growth Media (that includes serum).