Sulzer lab

Postnatal ventral midbrain dopamine neuronal culture protocols

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Acknowledgments

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Thanks also to the technicians in our lab who advanced these methods, particularly Viviana Davila, Johanna Bogulavsky, Gerald Behr, Anthonia Hananiya, Kester Phillips, Candace Makeda Moore, Carl St. Remy, Megan Antonelli, from our lab, and Geetha Rajendran of Steve Rayport's lab.

Thanks to Christian Bjerggaard, who visited our lab to learn these techniques in April 2004, and revised and added to an earlier addition of this booklet.

Introduction

This manual is designed to instruct in a detailed manner the steps our group uses to produce postnatally-derived ventral midbrain cultures. The approach used here has been used in numerous studies from our and Stephen Rayport's labs (Sulzer and Rayport, 1990; Rayport et al., 1992; Sulzer et al., 1993; Cubells et al., 1994; Rayport and Sulzer, 1995; Pothos et al., 1996; Przedborski et al., 1996; Sulzer et al., 1996; Fon et al., 1997; Mena et al., 1997a; Mena et al., 1997b; Burke et al., 1998; Pothos et al., 1998; Sulzer et al., 1998; Kholodilov et al., 1999; Pothos et al., 2000; Sulzer et al., 2000). In addition, these cultures have been used in studies from Louis Trudeau's lab (Bourque and Trudeau, 2000; Michel and Trudeau, 2000; Congar et al., 2002), Susan Amara's lab (Prasad and Amara, 2001; Ingram et al., 2002) and Mark Cookson's groups (Petrucelli et al., 2002).

Previously, dissociated culture studies of dopamine neurons use embryonically-derived cultures that produce a fraction of dopamine neurons of 1% or less. The postnatally derived cultures developed by Stephen Rayport and myself when I was a postdoc in his lab, have several advantages, the most striking being that the fraction of dopamine neurons is between 20-70%. The fraction is chiefly depending upon the area dissected.

Other advantages are that the neurons are more mature than embryonically-derived neurons. Not only do many more display tyrosine hydroxylase (TH), but the dopamine uptake transporter appears to be more highly expressed in the postnatally-derived neurons. Under optimal conditions, these cultures have survived for as long as 6 months, although most survive about 2 months. We suspect that the death is mostly due to increased osmolarity of the medium, and could probably be controlled

by weekly partial exchange with conditioned media, testing the osmolarity of the medium over time with an osmometer and compensating with the addition of water, using a dish lid that allows air transfer but not evaporation. The latter approach has recently been introduced by Steve Potter, but has not been attempted to date for the postnatal dopamine neurons.

The techniques shown here have also been adapted by our group to produce postnatal cultures of other brain areas, such as the dorsal striatum. However, we suspect that the dopamine neurons are particularly challenging to culture, mostly because dopamine easily oxidizes to produce fairly potent neurotoxins. An odd choice for a neurotransmitter; perhaps we will eventually discover why the property of auto-oxidation to a reactive substance provides a selective advantage. Alternatively, maybe catecholamines were chiefly useful as skin and fur pigment, and simply adapted to produce additional neurotransmitters!

We've also added the protocol for Chromaffin Cell cultures.

We have trained numerous visitors to the lab in these techniques. The following is a lab manual intended to help both visitors and those who cannot visit the lab.

Overview of techniques

First, we outline how we prepare the glass coverslip petri dishes in our lab. While we have used various approaches, including multiwell plates, to good effect, we generally culture on glass coverslips that are glued to petri dishes, so that the neurons produce small, dense cultures. This appears to keep them healthier, and to require no feeding with new media. The glass allows optimal microscopy with DIC/Nomarski imaging and fluorescent imaging. The central glass well allows all of the neurons to be approached with electrodes. We also make neuronal cultures on free-floating coverslips, so they can be removed from the dish for microscopy later. An alternate approach we also outline is the use of Aclar coverslips, which are well adapted for processing for electron microscopy.

We have found it necessary to culture these neurons on glia monolayers. We have also attempted the "Banker" technique, in which glia are grown close by on an inverted coverslip. However, to date, the dopamine neurons die within 3 days unless they actually touch the glia. Thus, we outline the techniques to produce glia monolayers followed by a protocol how to dissociate the neurons and plate them onto the glia.

We use similar techniques for culturing of other types of neurons (hippocampal, cortical, striatal, etc.).

Dish / coverslip preparations

Aclar dishes for electron microscope preparations

1. Cut Aclar sheets into squares ~ 1.5 cm and drop into Nanopure dd H_2O for 5 min
2. Swirl the squares in a beaker (if they don't all immediately float to the top)
3. Rinse twice in Nanopure dd H_2O for 5 min
4. Expose to 70% ethanol in Nanopure dd H_2O for 30 min
5. Rinse twice in Nanopure dd H2O for 5 min
6. Expose to 1M HCl in Nanopure dd H_2O for 30 min
7. Rinse again several times in water
8. Maintain in 70% ethanol, covered
9. Sylgard the squares to the dishes after the holes are prepared:
    a. Wearing gloves, prepare about 5 ml of Sylgard in a large weighing boat by combining 10 parts resin with 1 part catalyst (by weight) and stirring extensively with flame-sealed Pasteur pipette. Pour contents into new weighing boat to avoid use of resin without catalyst.
    b. Dip an empty 15 ml Falcon tube or glass scintillation vial upside-down into the Sylgard, using it as a *rubber stamp* to make a circle of Sylgard around the hole, on the outside of the dish.
    c. Place the Aclar sheets on the circle of Sylgard, covering the hole.
    d. Allow Sylgard to spread (about 15 min).
    e. To cure the Sylgard, place dishes, upside-down, at 37°C overnight, or at room temperature for 2 to 3 days (cured Sylgard is no longer sticky to the touch).
    d. Store dishes in zip lock bags: initial and date.
10. Coat with PDL:

a. Add 200 μL of the 40 μg/ml PDL solution to each dish. Expose for 2-4 H at RT in a culture hood without UV light.

b. Rinse 3 times for 5 min each in tissue culture water.

c. Remove water and expose to UV for 1 H to sterilize.

11. Note that these dishes must be coated with laminin before use, just as we do with glass coverslips *(see glia dissection protocol)*

    (Note: Left over sylgard from dish preparation can be used to make sylgard circles.)

Glass coverslip preparation

Need: Coverslips
Beaker, 200 ml
95% EtOH
Forceps
Poly-D-Lysine, MW 70,000-150,000
Water, "nanopure" or tissue culture grade
Glass dish

1. Drop 100 coverslips (15 x 15 mm) one-at-a-time into a 200 ml beaker containing 100 ml of 95% EtOH. (Leave approx. 1 minute at least)

2. Prepare a solution of poly-d-lysine in "nanopure" or tissue culture grade water. The final concentration should be 40 μg/ml and 250mL are adequate for 100 coverslips. Place the poly-D-lysine solution in a glass dish of enough area to give 3/4"/2 cm height to the poly-D lysine solution. (the walls of the dish should be at least 1.5"/4 cm, the area no less than 40"/100 cm square)

3. Throw (pour) away excess EtOH. Place ethanol soaked cover slips on to a paper or glass container.

4. Individually flame dry the coverslips (one pass through flame); use forceps.

5. After putting the slip through the flame, allow it to cool in the air in forceps for approximately 10 seconds while moving back and forth (cracking or sizzling sounds mean it did not cool enough).

6. Put the coverslip into the poly-D-lysine solution. Repeat for all slips.

7. Let sit for 1 hour, then pour off the poly-D-lysine, then let slips dry (overnight in the hood with the blower on).

8. Prepare dishes with 10 mm holes punched with a bench punch for wells where neurons and glia will grow.
   Sylgard the coverslips to the dishes after the holes are prepared.

   a. Wearing gloves, prepare about 5 ml of Sylgard in a large weighing boat by combining 10 parts resin with 1 part catalyst (by weight) and stirring extensively with flame-sealed Pasteur pipette. Pour contents into new weighing boat to avoid use of resin without catalyst.

   b. Dip an empty 15 ml Falcon tube or glass scintillation vial upside-down into the Sylgard, using it as a *rubber stamp* to make a circle of Sylgard around the hole. Use caution when applying the Sylguard as too much will result in excess Sylgard covering the culturing surface of the coverslip whereas inadequate Sylgard will result in a leaky well.

   c. Place the coverslips on the circle of Sylgard, covering the hole.

   d. Press down on coverslip gently using forceps to eliminate bubbles.

   e. Allow Sylgard to spread (about 15 min).

   f. To cure the Sylgard, place dishes, upside-down, at 37°C overnight, or at room temperature for 2 to 3 days (cured Sylgard is no longer sticky to the touch). Once the dishes are cured they can be stored in zip-lock bags at 4° C until needed.

Similar dishes are now available commercially.

9. Note that these glass coverslips must be coated with laminin before use *(see glia dissection protocol)*

(Note 1: Left over sylgard from dish preparation can be used to make sylgard circles.)
(Note 2: if these coverslips ever become unavailable, the Bellco #2 photoetched coverslips have been recommended)

The TechTip Preparation

These are used for triturating cells

1. Obtain a box of 1000ul pipette tips (unfiltered). Wear gloves.

2. Flame all tips in order to seal off hole (we recently started using tips without sealing the hole, making additional holes with needles as described below).

3. Using a needle, poke a hole as close to the tip as possible through one side and out the other. This will result in two holes. The purpose of piercing right through both sides is to prevent cells getting caught in the pipette. Rather, they will have an outlet hole. Make 3 types of tips, with Large (21G 1½), Medium (22G 1½) and Small (26G 1½) size holes.

4. Autoclave as usual

Sylgard Circles

For use during dissection as a platform to do the cutting:

1. Weigh out the Elastomer Base and the Elastomer Curing Agent. Mix in the ratio 10:1
2. Mix well and pour into 12 micro well plate, about 3-4 mm in height.
3. Cure in oven at 60°C for 30 minutes or at room temperature for 5-7 days.
4. Cut circles out of wells using razor and individually wrap each circle in aluminum foil, autoclave to sterilize.

(Note: Left over sylgard from dish preparation can be used to make sylgard circles.)

Things to Prepare During the Week before Plating Glia or Neurons

Three days before culturing

1. Make sure that there is an ample supply of glass dishes, Sylgard circles, Techtips and both types of plastic pipette tips (for 1000uL and 200uL).
2. Figure out the number of dishes and types of neurons required. Calculate the number of pups needed. Pups needed vary by dissection technique, but we have found 1 rat cortex is enough material to easily plate ~110-130 dished, and one mouse VM enough to plate 2-3 dishes.
3. Clean and sterilize dissection tools (This should be done right after each dissection!)
    a. First wipe clean with 70% ethanol.
    b. Wipe again with 100% ethanol to speed drying process.
    c. Let dry!
    d. Leave in the oven at 200°C for at least 1 hour.
4. Clean and sterilize the plastic rings and magnetic stir-bars:
    a. Wipe off any debris from rings and stir-bars.
    b. Let soak in 1N HCL for 1 hour.
    c. Rinse at least 20 times with de-ionized water and let dry.

d. Place rings into 100 ml beakers and individually distribute the stir-bars into small tubes (the 1.5 ml snap-top tubes are fine) and autoclave.

5. Once-a-month, autoclave the flask and glass tube (NOT THE RUBBER STOPPER) that is used for bubbling the carbogen through water for papain dissociation.

On the day before culturing

1. Prepare appropriate media as needed (SF1C, M10C-G, PBS).

2. **For neuronal culturing**, take glial dishes and wash them twice with 2ml of cold MEM. Replace MEM with 2 ml of SF1C. Alternatively, neurobasal medium A/B27 can also be used (Table 16.9). While A/B27 is easier to make, we have had slightly better success with SF1C in certain toxicity assays (unpublished observations).

3. Place sterile ring in each dish to the side of the glass coverslip well, using forceps with sterile tips. (see dish and media prep. for neurons). For free-floating coverslips, place the ring in the middle of a coverslip.

4. Leave dishes with SF1C in the incubator overnight. This allows the media to condition over the glial cells. Alternatively, use preconditioned SF1C media on the day of the culture.

Glia preparation on coverslip petri dishes

Laminin coating

1. Before the dissection/digestion time, dilute 1 aliquot of laminin (140ul of 0.5mg/ml laminin = 70 μg) in 7 ml sterile MEM. Final concentration 10ug/ml.

2. Add at least 100 μl 10 μg/mL laminin per round well (enough to cover the surface) for minimum 1 hour at room t (can be done O/N at +4'C) before plating cells and leave in flow bench. Aspirate off laminin and wash each well with MEM. Place into 37° C incubator to warm dishes before plating the cells. 1 aliquot is enough for about 50-60 dishes.

Dissection

Need:

Rat pups, P1-P3	Ketamine (100mg/mL)
Dissecting scope & light	Laminin, 70 μg aliquot
3 cc Syringe	Dissecting instruments
Aluminum foil pouch for bodies	Ice chips in bucket
Aluminum foil square for heads	Chilled, sterile PBS
70% EtOH	Scissors
18 gauge needles	sterile disposable filters
sterile microstir bars	
Petri dish [Falcon 100x15] w/ Sylgard square stuck on	

3. Obtain one or two P1-P3 rats.

4. Prepare the papain dissociation solution. Each vial should hold tissue from a maximum of 2 animals though it's better to digest 1 rat cortex per vial.

5. Sterilize the top of each Nunc specimen vial (25 ml) with ethanol. Punch two holes in the top with an 18 gauge needle, using a separate needle to bore holes. In one hole place a new needle with a 0.22 μm filter attached. Leave the other hole empty for ventilation.

6. Add an HCl-cleaned, autoclaved micro-stir bar to the vial. Fill each tube with 5-10 ml of freshly made papain solution (sterile filtered with 0.22 μm filter). Place in temperature-controlled 37°C water bath with magnetic stirrers.

7. Arrange dissection tools, microscope, dissection light, a bunch of transfer pipettes, sterile Petri dish with Sylgard circle, beaker, and 70% ethanol in a 50 ml tube in the sterile hood.

8. Prepare two buckets of ice to chill the PBS and for the pups.

9. Cut aluminum foil squares (about 15 x 15 cm).

10. Perfuse dissociation tube (papain tube) with a steady flow of humidified carbogen (95% oxygen, 5% CO_2) delivered through the filter/needle (flow rate adjusted to about 1 bubble per second).

11. When the papain has reached a pH of 7.2 - 7.4 in about ½ - 1 h (indicated by a red color; purple indicates that the papain solution is too alkaline and orange that it is too acidic), anesthetize pups and begin dissection.

12. Anesthetize pups with an intraperitoneal injection of ketamine (0.01 and 0.05 ml of 100 mg/ml for mouse and rat pups, respectively).

13. When pups begin to show sedation, put them on ice till hypothermic.

14. Rinse aluminum foil square, scissors and head with 70% EtOH.

15. Decapitate pup, allowing head to fall onto aluminum foil square and move to hood. Using (toothed) forceps hold head by the eyes (using left hand if you are right handed). Using the other hand and a curved or angled forceps, pinch the scalp just behind the eyes and pull back, tearing it off. Next, use a micro-scissor to cut around the circumference of the skull, and gently peel the skull off with forceps, being careful in case any tissue still connects it to the rest of the skull. Gently remove brain (using a small scoop shaped scalpel) and place into Petri dish with one SYLGARD® circle stuck onto the bottom and filled with ice cold sterile PBS (use enough PBS such that the brain lying on the SYLGARD® circle is submerged).

16. Remove the olfactory lobes and brain stem (with V-cut). Hemi-sect brain and then peel off meninges. Shell out cortex, taking care to remove the hippocampus. Remove any torn areas to avoid DNA release. Cut into 1 mm^3 chunks.

Digestion

17. Place chunks in papain as with neuronal prep, *without kynurenate* (1 cortex/vial). Chunks will be digested enough to triturate during the initial 5-10 minutes. We judge by the appearance of the chunks (like cotton candy) when they are ready.

Trituration and plating

18. Warm up 45 ml sterile M10C-G for rinsing and trituration.

19. From this point forward, everything should be done in a sterile laminar hood with sterile technique.

20. Remove chunks from enzyme vial using transfer pipette and place in 15 ml conical test tube.

21. Rinse chunks 3 times using 2 ml M10C-G, allowing them to settle each time and discard supernatant.

22. Using 1 ml warm M10CG triturate up to a maximum of 10 times (3-5 times should be sufficient) using a regular 1ml pipette tip.

23. Remove supernatant to a separate tube, and replace the volume with 1 ml fresh M10C-G.

24. Repeat trituration using the tech tips with smaller holes each time until the chunks have all fallen apart.

25. Pellet the glial cells at 1000rpm for 5 minutes.

26. Resuspend pellet in 10 ml M10C-G, and count the cells using hemocytometer.

27. Dilute to plating density of 1,000,000-1,500,000 cells/ml.

28. Remove laminin from wells and plate about 80,000 cells per well. Plate by dripping the cell suspension from about 10cm above the dish to help minimize number of surviving neurons in glial prep.

29. Tap the sides of the trays (not for island cultures) in order to evenly distribute cell suspension.

30. Allow cells to settle for 1.5-2 hours in incubator. For island preps allow 2-5 hours.

31. Chill M10C-G in meantime.

Rinsing and feeding

32. For monolayers: squirt 2 ml of cold MEM into each well, moving the pipette around in a circular motion to wash each well evenly.

33. Aspirate off MEM and repeat wash.

34. For island cultures: One dish at a time, lift the cover and tilt the dish at a 45° angle with the hood countertop. Using a transfer pipette very gently, drip 2 ml of cold MEM onto a spot on the dish just above the well so that it slides down the incline and over the well.

35. Aspirate off the MEM.

36. Feed with 2.0 ml of cold M10C-G.

37. Add FDU to the dishes when cells are ~70% confluent (about 3-5 days). If neurons are still present at this point, remove the medium and cold-feed with 2.0ml M10C-G before adding FDU.

38. Astrocytes dishes should be at least one week old and at most 4 weeks old at the time the neurons are plated. Feed with cold M10C-G at one month if not already used.

Neuronal preparation on coverslips with Glia monolayer

Preparation

Need: Magnetic Stirrer w/ 37°C water bath
Carbogen, which is a mixture of 5% CO_2 and 95% O_2
Papain solution
18 Gauge needles
Micro stir bars (Fisher #14-511-68)
0.22 μm syringe filters
Dissociation vials, Nunc specimen vials
Dissecting instruments:
- 2 fine forceps and tooth forceps
- fine scissors
- larger scissors
- weighing scoop
- scalpel with size 11 blade
- hemostat

70% EtOH in a 50 ml tube
Sterile slide rings
Cold, sterile PBS
Sterile, conditioned prewarmed SF1C

1. Please see sheet on **Things to Prepare during the Week before Plating Glia and Neurons.**

2. First, turn on the dissociation water bath and let warm to 37°C.

3. Make the papain dissociation solution (see *Media* section). Each vial can hold tissue from 1 to maximum 10 mice preps.

4. Sterilize the top of each Nunc specimen vial with EtOH. Make two holes in the top with an 18 gauge needle, using a separate needle to bore holes. In one hole place a *new* needle with a 0.22 μm filter attached. Leave the other hole empty for ventilation.

5. Add an HCl-cleaned, autoclaved micro-stir bar to the vial. Fill each tube with 2-10 ml of freshly made papain solution (0.22 μm filtered). Place in temperature-controlled 37ºC water bath on top of magnetic sturrer.

6. Arrange dissection tools, microscope, dissection light, a bunch of transfer pipettes, sterile Petri dish with sylgard circle, beaker, and 70% ethanol in a 50 ml tube in the sterile hood.

7. Get 2 buckets of ice to chill the PBS and to anesthetize the pups.

8. Rip aluminum foil squares (about 15 cm^2).

9. Fill “insulin” syringe with 100 mg/ml ketamine solution.

10. Obtain as many P1-P3 rat or mouse pups as needed (animals which are one to three days old).

11. Superfuse dissociation tube (papain tube) with a steady flow of humidified carbogen delivered through the filter/needle (Flow rate adjusted to about 1 bubble per second).

12. When the papain has reached a pH of 7.2 - 7.4 in about 30-45 minutes (indicated by the red color, not too purple, not too orange), anesthetization and dissection of pups can begin.

Dissection

13. Anesthetize pups with an intraperitoneal injection of ketamine (0.01 ml and 0.05 ml of 100 mg/ml ketamine for mouse and rat pups, respectively).

14. When pups begin to show sedation, put them into ice till hypothermic.

15. Rinse aluminum foil square, scissors and head with 70% EtOH.

16. Decapitate pup, allowing head to fall onto aluminum foil square and move to hood.

17. Gently remove brain into ice cold PBS-filled Petri dish as described for glia. With anterior of brain facing left on the SYLGARD® circle (dissection will be described for right handed people) use a fine forceps in the left hand to pin the two hemispheres of the brain to the SYLGARD®. Remove any remaining meninges.

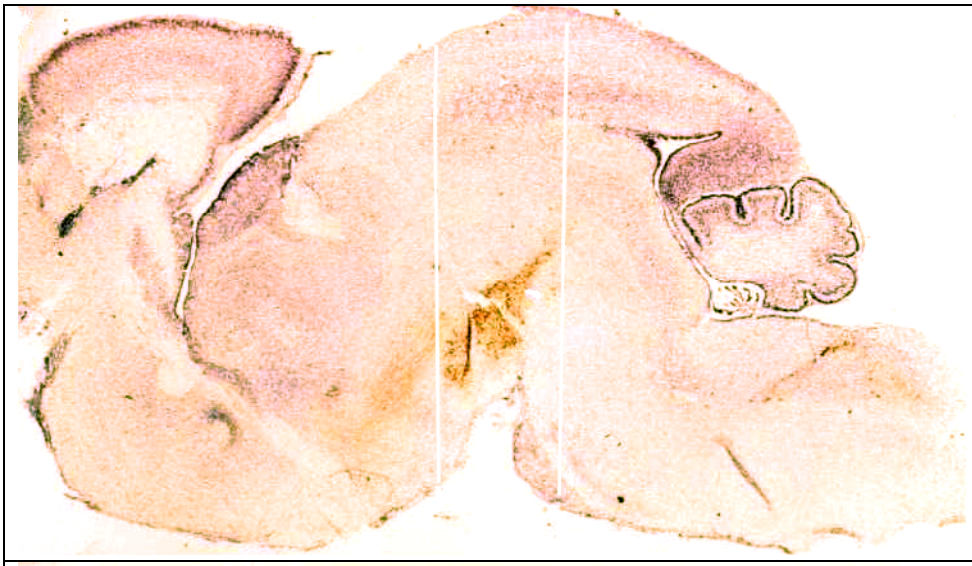
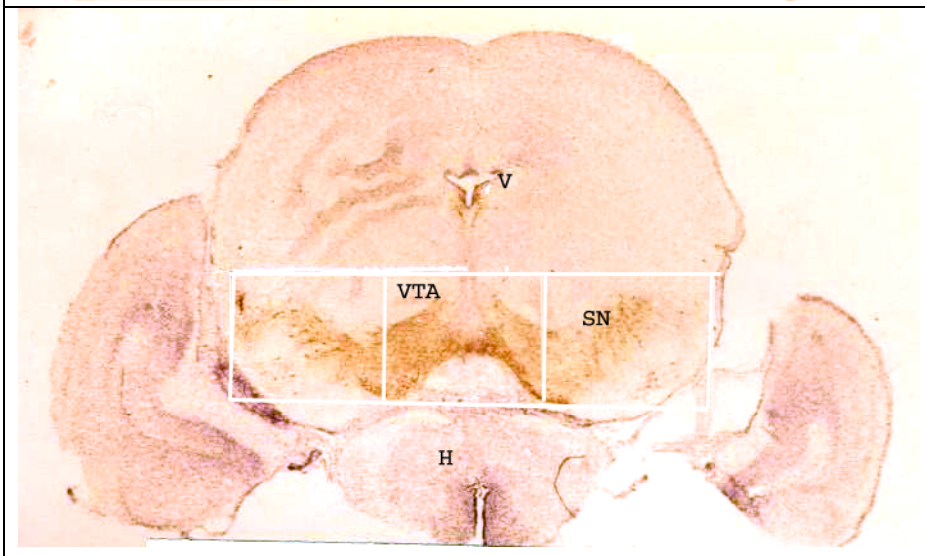
18. Place the brain, dorsal side down, anterior facing left onto the SYLGARD®. Using forceps, pin the brain to the SYLGARD® to immobilize. Make the initial cut through the entire brain caudal to the midbrain flexure (right line, Fig 1 below), and the second cut rostral to the flexure, including the caudal aspect of the hypothalamus. Lay the resulting slice flat on the SYLGARD® with the ventral side facing right, and the dorsal side to the left and the caudal aspect up. Remove any remaining cortex. Place the forceps through the aqueduct and push into the SYLGARD® to immobilize the slice. Cut the ventral edge of the slice along the top of the hypothalamus as indicated by the line in Figure below. Next cut approximately halfway between the new ventral edge of the slice and the aqueduct. Cut into 1 mm^3 segments and place all three segments into the vial with papain solution for ventral midbrain cultures. For SN cultures only use the two outer segments and for VTA only use the inner segment. Use both SN and VTA for combined VM cultures.

19. Set the magnetic stirrer on low. Chunks should swirl around in the vial gently.

20. Digest for approx. 7-20 min or until chunks have fallen apart and look something like cotton candy. Periodically examine the tubes to see if segments are sticking to the stir bars; if so, tap vials to loosen tissue.

Figure indicating dissection an TH immunolabel in mouse sections

These sections of neonatal mice were cryosectioned and immunolabeled for tyrosine hydroxylase by Serge Przedborski.

	This sagittal section is taken through the midline of the brain. The two "knife cuts" above the midbrain flexure indicate how I produce the coronal section below (of course that dissection is of the brain, not a crysection!). Note the brown label just above the midbrain flexure indicating the midbrain dopamine neurons
	This shows the above slice flipped on the rostral side. The ventricle is indicated by V, and the hypothalamus by H. Typically, I call the outer two boxes resulting from the "knife cuts" the "substantia nigra", and the medial third "ventral tegmental area", with the combined three sections the "ventral midbrain". If it is important to differentiate SN and VTA, I will take the most lateral SN, the most medial VTA, and discard the remainder.

Special Instructions for Mouse Neurons

21. Some types of mouse tails (for ex, VMATs) need to be saved for genotyping. Prepare a numbered 1.5ml tube for each mouse. After the mouse has been decapitated, remove the tail using a fresh, sterile razor blade and place tail in numbered tube. Put all tubes on ice during the dissection. After dissection, store tubes in -80°C freezer. Alternatively, number the pups with the marker on their back, cut the tails and genotype the pups before the cultures, and no later than 1-day old. This way the pups with the same genotype can be cultured together rather than individually.

Dish and Media Preparation

22. On day before dissection, take glia dishes and wash them twice with 2ml cold MEM. Replace MEM with 2 ml of SF1C. Leave dishes with SF1C in the incubator overnight. Alternatively, do this step on the day of the culture, using preconditioned media.

23. After dissection, place tubes containing SF1C for rinsing and triturating in the incubator until ready to triturate so that the pH stays between 7.2 and 7.4, and the media is warm. The media must be at the correct pH (indicated by the red color) or the neurons may die.

Trituration

24. From this point forward, everything should be done in a sterile laminar hood with sterile technique.

25. Remove chunks from enzyme vial using transfer pipette and place in 15 ml conical test tube. **IMPORTANT NOTE: Take great care to prevent bubble formation because bubbles are harmful to neurons. If bubbles occur, aspirate them off.**

26. Rinse chunks 2-3 times using about 2 ml SF1C (warmed to 37'C). **After adding SF1C for each wash, flick the tube so that the cells swirl around and shake off the papain**. Allow tissue to settle each time and discard as much of the supernatant as possible without exposing cells to air.

27. Triturate in 2 ml of room temperature SF1C with large-bore tech-tips a couple of times (when done properly the medium becomes slightly opalescent with dissociated cells).

28. Repeat the trituration process up to three times with a medium-, then a small-bore tech-tip. The chunks should now be completely dissociated into individual cells.

29. Centrifuge cells at 1000rpm for 5 minutes. Resuspend the pellet in 0.2-0.5 ml (for mouse cultures) or 1-2 ml (for rat cultures) of media, mix and count (see below). Dilute cell suspension until 50-100 μl of media contains 80,000 cells. Add the appropriate volume to the well (into the slide ring) of the SF1C-containing dishes so that each well contains 80,000 cells (use a circular motion to spread the cells around). Again, the rings should ensure that the neurons settle into the well and do not float away or get washed away when moving the dishes.

Attachment

30. After one and a half hours, cells should have attached to the surface of the well. Remove rings from each well using forceps, which have sterile pipette tips at the end.

31. Add 100 μl GDNF (200 ng/ml) to each culture for a final concentration of 10 ng/ml GDNF (see *media and solutions* section how to prepare GDNF).

Next Day - Mitotic Inhibition

32. The next day inhibit the growth of non-neural cells with 5-fluorodeoxyuridine (FDU). Dilute 1000X stock 1:10 by adding 200 μl stock to 1.8 ml MEM. Add 20 μl diluted FDU solution to each dish (2ml media).

33. Media should be changed only when necessary (change of color) – aspirate half the media and add fresh preconditioned (pre-warmed at 37'C). Try to avoid this step if possible as neuronal cells are very sensitive to the media exchange.

34. Disturb cultures as little as possible, avoiding multiple observations (especially in the first 2 days after culturing).

35. Process cells as needed for the individual experiment (ideally, between 5 days to 2 weeks old).

Note: If necessary after fixation/immunostaining, the coverslips can be taken off the Petri dish (cut between the glass and Sylgard with a scalpel – *careful not to break the glass!!!*) and mounted on a glass slide. Alternatively, cells can be cultured on free-floating coverslips, which can be later removed from dishes for the experiments.

Microglial Culture

Prepare astrocyte cultures in tissue culture flasks (225 cm^2 for rat and 75 cm^2 for mice cultures)

Dissection

Need:

Rat or Mouse pups, P1-P3	Ketamine (100mg/mL)
3 cc Syringe	Dissecting instruments
Aluminum foil pouch for bodies	Ice chips in bucket
Aluminum foil square for heads	Chilled, sterile PBS
70% EtOH	Scissors
18 gauge needles	sterile disposable filters
sterile microstir bars	Dissecting scope & light

Petri dish [Falcon 100x15] w/ Sylgard circle stuck on

1. Obtain one or two rat or five mouse pups P1-P3.

2. Anesthetize pups with an intraperitoneal injection of ketamine.

3. When pups begin to show sedation, put them on ice till hypothermic.

4. Rinse aluminum foil square, scissors and head with 70% EtOH.

5. Decapitate pup, allowing head to fall onto aluminum foil square and move to hood. Gently remove brain into ice cold PBS filled Petri dish (with one Sylgard circle – should be covered with cold PBS!).

6. Remove the olfactory lobes and brain stem (with v-cut). Hemi-sect brain and then peel off meninges. Shell out cortex. Remove any torn areas to avoid DNA release. Cut into 1 mm^3 chunks.

Digestion

7. Place chunks in papain as with neuronal prep, *without kynurenate* (1 rat cortex/vial, or 5 mouse cortexes/vial). Chunks will be digested enough to triturate during the initial 7 to 10 minutes. We judge by the appearance of the chunks (like cotton candy) when they are ready.

Trituration and plating

1. Warm up sterile M10C-G to 37'C for rinsing and trituration.

2. Remove chunks from enzyme vial using transfer pipette and place in 15 ml conical test tube.

3. Rinse chunks 3 times using 2 ml warm M10C-G, allowing them to settle each time and discard supernatant.

4. Using 1 ml warm M10CG triturate 5-10 times using the just regular pipette tip.

5. Repeat trituration using the tech-tip with smaller size hole, until the chunks have all fallen apart.

6. Pellet the glial cells at 1000rpm for 5 minutes.

7. Resuspend pellet in 15 ml M10C-G.

8. Transfer cell suspension to tissue culture flask. Use big T-225 flask for rat culture and T-75 flask for mouse culture. The yield from two rat brains is ideal for one

9. T-225 flask. And the yield from 5 mouse brains is ideal for one T-75 flask.

10. Allow cells to settle for at least 2 hours, better leave them overnight in incubator.

Rinsing and feeding

11. For astrocyte monolayer remove M10CG from flask and squirt ~ 20 ml of cold MEM onto the base of upright tissue culture flask. Gently allow the media to swirl around the bottom of the flask to remove unattached cells.

12. Aspirate off MEM and feed with cold M10C-G (30ml per T-225 flask, or 15ml per T-75 flask), and leave ~2 weeks in the incubator.

DO NOT ADD FDU TO FLASKS!!!!

Microglia isolation

Once the cell layers are stable and you start seeing rounded microglial cells on top of the layers (usually 10-14 d after the cortical culture is plated) and floating in the medium. You should harvest the floating microglia at this time.

12. Shake the flask with a back-and-forth motion several times to help lift the microglia off. Shake additional ~10 min placing the flask on a shaker at moderate speed.

13. Collect the fluid in two 15 ml conical tube and centrifuge at 3,000rpm for 5 min. Note that the speed is higher than for regular cells centrifugation, which is normally 1,000rpm.

14. Remove supernate and GENTLY resuspend the cell pellet in 1 ml warm conditioned M10CG with regular 1ml pipette tip.

15. Count cells on hematocytometer to determine appropriate volume for cells density @ 60,000 cells/well on neuronal cultures or 150, 000 cells/well 96 well plates for biochemical assays.

16. When plating on neuronal cultures place plastic ring around the well and plate cells in the ring. Remove ring after 2 hr.

****NOTE- PLEASE BE SURE TO REMOVE NEURONAL MEDIA (SFIC) AND REPLACE WITH 2-3 DAY ASTROGLIAL CONDITIONED MEDIA BEFORE PLATING MICROGLIA.**

TO CONDITION MEDIA:

- have T-225 flask with astrocytes monolayer prepared at least 10 days prior;
- replace media in the flask with freshly made 200ml of M10CG media. Incubate for 1-2 days. Collect conditioned media and filter to sterilize.

Note: to condition neuronal media, add 200ml of SF1C media to the flask with astrocytes and incubate overnight to 2 days. Collect conditioned media and filter-sterilize.

Media and solutions

M10C-G (for glia)

200 ml *(can be made the day before)*

Component	Amount
1. MEM	180 ml
2. Calf serum fresh, *not* heat-inactivated	20 ml
3. Glucose 45%	1.5 ml
4. Pen-Strep	0.24 ml
5. Insulin (25 mg/ml)	40 μl, dissolve in 20mM HCl
6. Glutamine (200 mM)	0.5 ml

1. Use a bent neck tissue culture flask to measure and mix solutions.
2. Filter using a 0.22 μm filter unit, label, and store at 4°C till needed.

3. Please note that L-Glutamine stock (200mM) is for most common culture media recipes is 100x (final concentration in media 2mM). We use less – final concentration 0.5mM.

SF1C (for neurons)

200 mL *(can be made the day before)*

Ingredient	*Amount*	*Notes*
1. BSA	0.50 g	fraction V. 0.25% final conc.
2. MEM liquid	94.0 ml	Sigma
3. DME liquid	80.0 ml	Sigma
4. F-12 liquid	20.0 ml	Sigma
5. Glucose 45% liquid	1.5 ml	Sigma solution
6. Glutamine 200 mM	0.50 ml	Aliquotted Sigma solution
7. DiPorzio Conc.[1]	2.0 ml	see below
8. Calf Serum	2.0 ml	Gibco, heat-inactivated. 1% final conc
9. Liquid Catalase	0.10 ml	Sigma solution
10. Kynurenic acid 0.5M	200 μl	0.5 M kynurenic acid is 946 mg in 10 mL. Dissolve in 1N NaOH
11. HCl 5N	50 μl	

1. Combine ingredients in a bent necked flask.

2. Shake till BSA goes into solution.

3. Filter using a 0.22 μm filter unit, label and refrigerate.

4. Note – neurons can be cultured in serum-free media. Make sure to condition the serum-free SF1C over astrocytes before using this media on neurons.

Laminin

Need Laminin 70 μg aliquot (enough for 50-70 dishes). Stock solution – mouse Laminin, 0.5mg/ml, from Chemicon, Sterile, cat # CC095. One 140ul-aliquot = 70ug.
15 ml centrifuge tube
7 ml cold sterile MEM

1. Dilute 1 aliquot laminin (70 μg) in 7 ml MEM. Final concentration 10ug/ml.

2. Add at least 100 μl per round well, enough to cover the surface, 1 hour at room t in the hood before plating cells (can be done overnight at +4'C).

Glia Derived Neurotrophic Factor (GDNF)

Human Recombinant GDNF – Chemicon, cat # GF030, 10ug.

1. Add 4.8ml of H_20 (tissue culture grade, Sigma # W3500) to 10ug of GNDF

2. Aliquot (76.9μl per tube – enough for 8 cell dishes) and freeze at -20'C

3. Add 725μl SF1C media to a tube with 77-ul aliquot and add 100μl of this solution to each cell dish (per 2ml media). This procedure results in a final concentration of 10ng/ml media.

5-Fluorodeoxyuridine (FDU)-solution

Make 1000X stock

Ingredient	*Amount*	*Final Conc.*
Uridine	247 mg	16.5 mg/ml
5-FDU	100 mg (bottle)	6.7 mg/ml
Tissue Culture Water	15 ml	

1. Make a little over 15 ml of 16.5 mg/ml uridine.

2. Add 15 ml uridine solution to 100 mg bottle of FDU to make 6.7 mg/ml solution FDU.

3. Divide FDU stock into 200μl aliquots and freeze at -20'C.

Adding FDU solution to cultures:

4. Add 1.8 ml of MEM into the 200 ul aliquot of FDU stock. Add 20 ul of diluted FDU solution to each dish (20ul per 2ml media).

Calf Serum

Use Heat Inactivated calf serum for neurons (SF1C), and Not-Heat Inactivated calf serum for glia (M10C-G).

Heat Inactivation (note: serum can be purchased heat inactivated):

1. Thaw serum in the refrigerator overnight.

2. Next morning, finish thawing in room temperature water bath.

3. Put serum on counter, raise bath temperature to exactly 56°C. Then place serum in bath for 30 min exactly, mixing every 10 min by turning bottle.

Papain Solution for GLIA

- prepare ON the day of culturing

10 ml solution:

Ingredient	Amount	Final Conc.
1. Cysteine Water	7.87 mL	1 mM cysteine
2. Papain	varies	20units/ml
3. H&B conc.	2 mL	
4. 0.5% Phenol red	20 µl	0.001%
5. Carbogen	95% O_2 + 5% CO_2	

Papain Solution for NEURONS

- *prepare ON the day of culturing*

20 ml solution:

Ingredient	Amount	Final Conc.
1. Cysteine Water	15.75 mL	1 mM cysteine
2. Papain	varies	20 units/ml
3. H&B conc.	4 mL	
4. HCL 5N	15 µl	
5. 0.5% Phenol red	40 µl	0.001%
6. Kynurenate 0.5M	20 µl	0.5 mM
7. Carbogen	95% O_2 + 5% CO_2	

Notes:

Dissolve papain in cysteine water first by mixing well. Then add remaining ingredients. Otherwise, papain will not dissolve and will be removed by filtering.

Make the Papain solution just before dissection, sterilize by filtering through 0.22u-syringe filter into NUNC dissociation vial, and effuse with Carbogen at least 30 minutes before you are ready to dissect (judge by the change of color, from pink-red to red-orange).

Check the H&B concentrate before adding to make sure there is no crystal formation in solution. If there is, and you don't have time to make more, put solution in warm water bath for approximately 5 minutes or until crystals disappear.

The Papain solution should be a light magenta color before effusion with Carbogen. Check periodically to make sure the magenta color is converted into a light salmon color (optimal pH for dissociation).

For **Neurons**, **YOU MUST ADD** Kynurenate (kynurenic acid) and depending on the color, if it's too purple then add small quantities (<2ul) of 5N HCL at a time.

Do not add kynurenate or HCL to the papain solution for glia!

Use syringe filter to **STERILIZE** the Papain solution and put 2-10 ml of solution in each Nunc dissociation vial. Each vial could now hold a maximum of 7-10 animals' VM tissue. For Glia culture, you should only put 1 rat cortex in each tube with 10ml due to the excessive amount of tissue.
Always make sure to label the vials with the **type** of cells and the **time** after the tissue put into the vials.

The dissociation process should range from 7 to 20-25 minutes at the most. Keep an eye on the cells periodically. Do not let the stir bar spin too quickly and adjust the stirring to slow down the dissociation process, if necessary.

Diporzio concentrate

Single substance stocks to make up the DiPorzio Conc. Stock (see below)

Need:			**Combine:**		**Aliquot:**			
Additive	*Solvent*	*Tube*	*Amount*	*ml*	*ml/tube*	*Conc*	*Amount*	*#Aliq.*
Insulin	20mM HCl [(1)]	plastic	250 mg / bottle	10	1	25 mg/ml	25 mg	10
Transferrin	Hank's		500 mg / bottle	5	1	100 mg/ml	100 mg	5
SOD	Hank's		70 mg / bottle	14	1	5 mg/ml	5 mg	14
Putrescine	Hank's		50 mg	2.5	0.12	20 mg/ml	2.4 mg	21
Na_2SeO_3	Hank's		0.104 mg [(2)]	10	0.5	10.4 μg/ml	5.2 μg	20
T3	10mM NaOH		2 mg	10	0.1	0.2 mg/ml	20 μg	100
Progesterone	100% EtOH	glass	12.5 mg	10	0.05	1.25 mg/ml	62.5 μg	1
Cortisol	100% EtOH	glass	20 mg	10	0.02	2.0 mg/ml	40 μg	1

Notes:
SOD=superoxide dismutase, T3=3,3',5-triiodo-L-thyronine (Na salt) or Liothyronine; Stock solutions should be kept frozen at -80°C.

1. 20mM HCl = 41.5μl conc HCl (12N)/ 25ml water

2. Na_2SeO_3 Make 1mg/ml stock and add 104 μl to 10 ml.

3. Keep stocks at -80°C

Combine the following to make up the DiPorzio Conc. Stock (100X) – 10ml

Additive	Amount (ml)	Final concentration in stock	Final concentration in SF1C buffer		ml for two batches
1. Progesterone	0.05	6.25 μg/ml	62.5 ng/ml	200 nM	0.1
2. Cortisol	0.02	4 μg/ml	40 ng/ml	125 nM	0.04
3. Hank's BSS	6.21				12.42

4. Insulin	1	2.5 mg/ml	25 μg/ml		2
5. Na2Seo3	0.5	0.52 μg/ml	5.2 ng/ml	30 nM	1
6. T3	0.1	2 μg/ml	20 ng/ml	30 nM	0.2
7. SOD	1	0.5 mg/ml	5 μg/ml		2
8. Putrescine	0.12	0.24 mg/ml	2.4 μg/ml	15 μM	0.24
9. Transferrin	1	10 mg/ml	100 μg/ml		2

Use a 15 ml polypropylene test tube (translucent) and perform quasi-sterility.

1. Add the progesterone and the cortisol.
   Use an aspirator pipet and vacuum to speed evaporation of EtOH:
   Use a 5ml pipet broken in half and place half-way down into the tube being very careful not to aspirate EtOH liquid. Hold pipet securely in place with Kimwipes and then bring the tip down to the 500μl mark located on the side of the tube.

2. Add the subsequent aliquots in order.

3. After the addition of the insulin which makes the solution cloudy, add **20 μl of 1M NaOH** to neutralize the pH. Solution should go from yellow to pink and immediately clear.
   ALSO, after the addition of transferrin, immediately add 20μl of 1N NaOH, to neutralize the pH and prevent the formation of precipitates.

4. Draw up 10 ml into a serological pipet and divide into 5 aliquots, 2ml each (one batch). Store at -20°C.

H&B Concentrate

(100 ml of 5X)

Ingredient	M.W.	Powder/50 ml H2O	Conc. (M)	Combine (ml)	Final Conc. (mM)
NaCl	58.44	11.70 g	4	14.5	116
KCl	74.56	3.728 g	1	2.7	5.4
$NaHCO_3$	84.01	4.2 g	1	13	26
$NaH_2PO_2*H_2O$	137.99	6.90 g	1	1	2
$MgSO_4$	120.38	6.02 g	1	0.5	1
EDTA	292	Sigma 5%	0.17	1.46	0.5
Glucose	180	Sigma 45%	2.5	5	25
Tissue culture H_2O				61.84	

1. Combine amounts from stock solutions.
2. Filter sterilize (0.22 μm), divide into 4 ml aliquots and freeze at -20'C.
3. Add aliquot to cysteine water after adding papain.

Cysteine Water

157.5 ml of 1.25X
Make stock of 0.5 M $CaCl_2$ and stock of 0.02 M cysteine in water.
Filter sterilize into bottle and keep cold at +4'C.
Combine 146.9ml water, 10 ml 0.02M cysteine stock and 0.6 ml of 0.5 M $CaCl_2$.

		Components				
Ingredient	M.W.	powder (mg)	H_2O (ml)	Stock Conc. (mM)	Combine (ml)	Final Conc. (mM)
$CaCl_2$	111	555	10	500	0.60	1.9
Cysteine	121.2	24	10	19.8	10	1.25
TC water					146.9	

Catalog numbers

SIGMA CATALOG #'S/size

A4503	BSA (albumin)	50 g
C2505	Cortisol	20 mg
C3155	Catalase(liquid)	50 mg
C7352	L-Cysteine(base)	25 g
D5546	DME	500 ml
F0503	5-fluorodeoxyuridine	
G8769	Glucose, 45% w/v	100 ml
G2150	L-Glutamine, 200 mM	20 ml
I5500	Insulin	250 mg
K3375	Kynurenic Acid	5 g
M2279	MEM	1000 ml
N4888	Nutrient Mixture F-12 (Ham's)	500 ml
D1408	PBS 10x	500 ml
P0781	Pen/Strep	20 ml
P0290	Phenol red	100 ml
P0899	Poly-D-Lysine,MW70,000-150,000	10 mg
P0130	Progesterone	5 g
P7505	Putrescine	50 mg
S1382	Na_2SeO_3	10 g
S2515	Superoxide dismutase (SOD)	70 mg/300,000 units
T2752	T_3	2 mg
T1428	Transferrin	500 mg X2
W3500	Water, Tissue Culture Grade	1000ml
U3003	Uridine	

OTHER CATALOG #'S

10501-10	Aclar film	Ted Pella, Inc.
CC095	Laminin (mouse)	Chemicon
12-565-206	Dissociation vial	Nunc (Fisher)
6705-R12	Microscope slide rings	Thomas Scientific (phone 800-345-2100)
PAP	Papain	Worthington
14170-120	Hank's Balanced Salt Solution	
with Phenol	Red	Gibco
26140-087	Calf Serum	Gibco
16140-063	Calf Serum, heat inactivated	Gibco
184	Sylgard	Dow Corning Corporation (phone 989-496-6000)
14-511-68	Micro stir bars	Fischer
351006	Petri dish 50x9mm	Falcon
63-3111	Cover slips #2	Carolina Biological Supply

GF030	GDNF	Chemicon
12-565-224	NUNC Bioassay trays	Fisher
Portable Light Duty Puncher N0 XX		Roper Whitney

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Primary Adrenal Chromaffin Cell Cultures

For rat-derived cultures, adrenal glands from 7- to 12-day-old Sprague Dawley rats are dissected in ice-cold Hanks Balanced Salt Solution (HBSS).

The capsule and cortex of adrenal glands are removed and the remaining medullae cut in half.
After several washes with HBSS, the tissue is incubated with Ca2+/Mg2+-free **collagenase IA** solution in 10ml HBSS (250-350U/ml, Worthington) for about 30-45 min at 37°C with stirring.
The digested tissue is rinsed with HBSS and triturated gently in a solution containing 1% heat-inactivated fetal bovine serum and 0.02% DNase I.
Dissociated cells are centrifuged at 1000 g for 3 min to form a pellet and then resuspended in a culture medium comprised of DMEM, 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml of streptomycin, and 2mM Glutamine.
The cell suspension is plated onto poly-D-lysine- and laminin-coated glass wells in 50 mm dishes (cells from 5 rat 10day-old pups onto16 dishes) and, after 2 hr, the dishes are flooded with the culture medium (3ml per dish). Cells are maintained in a 5% CO2 incubator at 37°C.

All measurements are conducted between 1 and 7 post-plating days.

Suggestions for plating density for rat and mouse CC cultures:

3 10-day-old rat pups – 8 dishes
5 10-day-old rat pups – 16 dishes

2 adult mice - 12 dishes
10 10-day-old mouse pups - 12 dishes. Add FDU to the dishes.

CC Media 200ml:

2ml L-Glutamine
240ul Pen-Strep
20ml Fetal Bovine Serum, heat-inactivated
180ml DMEM

For 10ml trituration solution:

10ml HBSS
30ul DNase stock (final concentration 0.02%)
100ul Fetal Bovine Serum, heat-inactivated

Catalog #'s:

L-Glutamine – 200mM, Sigma # G2150
Pen-Strep - Sigma # P0781
Fetal Bovine Serum, heat-inactivated - Gibco # 16140-063
DMEM - Sigma # D5546

HBSS – Gibco # 14175-095
Collagenase Type I – Worthington # CLS-1
Dnase I – Worthington # 2006

Preparation of Dnase I stock solution:

Reconstitute with HBSS to a concentration of 2,000 U/ml
(for example: a vial with 20 mg and 3,364 U/mg is reconstituted with 33.64 ml HBSS).
Store as 500ul aliquots at -80'C.