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Neuroprogenitor Cell Differentiation in Culture

Olga Miakotina and Michael E. Dailey

University of Iowa, Department of Biology, 143 Biology Building, Iowa City IA 52242 USA
(**olga-miakotina@uiowa.edu; michael-e-dailey@uiowa.edu**)

Neuroprogenitor cells differentiate into neurons, astrocytes, and oligodendrocytes. This experiment for an upper level laboratory allows students to obtain hands-on experience in sterile tissue culturing, to observe how factors stimulate neuroprogenitor cell proliferation and differentiation, to recognize neural cell types in culture, and to explore the influence of co-culturing with endothelial cells on neuroprogenitor cell fate. During this unit, students prepare substratum and cell culture medium, triturate cells, co-culture neuroprogenitors and endothelial cells, perform live imaging of differentiating cells, and immunostain cells to enable quantification of neurons and astrocytes. Results are presented in the format of a scientific manuscript.

Keywords: tissue culture, neuroprogenitors, differentiation, inquiry lab, upper level lab

Link to Supplemental Materials

http://www.ableweb.org/volumes/vol-35/v35/miakotina/supplement.htm

Introduction

Neuroprogenitor cells are capable of differentiating in cell culture to give rise to neurons, astrocytes, and oligodendrocytes (Goldman 2003). To stimulate undifferentiated neuroprogenitors to differentiate, cells are plated on an adhesive substratum and progenitor medium is substituted with differentiation medium. The neuroprogenitor cultures are started with or without endothelial cell-conditioned medium and in 4 to 5 days, cultures begin to express markers for all three types of neural lineage cells. Culturing for a total of 7 days promotes proliferation and differentiation of neural cells according to the scheme: neuroprogenitors (neurogenic) → neurons and gliogenic neuroprogenitors→oligodendrocytes and astrocytes (Goldman 2003, Yoshimura et al. 2001, Zhang 2001).

This experiment for an upper level laboratory allows students to get an opportunity to obtain hands-on experience in sterile cell culturing, to observe how factors stimulate neuroprogenitor cell proliferation and differentiation, to recognize neural cell types in culture, and to explore the influence of co-culturing on neuroprogenitor proliferation and differentiation (Nakagomi et al. 2009, Shen et al. 2004, Sun et al. 2010). Students are provided with progenitor cells in suspension, and prepared endothelial cell cultures on tissue plate inserts. During this unit, students prepare a substratum for cell attachment, make medium and filter-sterilize it, triturate cells, set up a co-culture of neuroprogenitors and endothelial cells, perform live imaging of differentiating cells, fix cells, and stain cells on coverslips using double fluorescence staining for neurons and astrocytes and DAPI nuclear counterstaining. As an outcome of the project, students take pictures of fluorescence staining, count cells of different cell types in control conditions (neuroprogenitors only) and experimental conditions (co-cultured with endothelial cells), summarize, analyze and present results in the format of a scientific manuscript. Instructional staff provide neuroprogenitor cultures, endothelial cells plated in inserts, and two or three types of media (one for culturing is made by students), and also make a backup of cell cultures in case student cultures fail. In general, the preparations include ordering of tissue culture materials, restocking supplies, starting progenitor cultures and endothelial cell cultures a few days before the lab, set up and care of fluorescence microscopes, and carrying out the experiment in parallel with students for the backup.

The co-culture of neuroprogenitor cells and endothelial cells represents a robust system with great flexibility. Primary neuroprogenitor cells keep co-cultures free of bacterial, fungal, or yeast contamination. Developing cultures undergoing both proliferation and differentiation allow students to monitor different relationships depending on a set of antibodies used: progenitors versus differentiated progeny, differentiated cell types in the presence or absence of endothelial cells, and neuronal fate compared to glial fate. We have successfully used the following antibodies: TuJ1 (for differentiated neurons), GFAP (for astrocytes), Nestin (for progenitor cells), O4 or NG-2 (for oligodendrocyte precursors) and DAPI staining (for nuclei).

In order to upgrade this laboratory to an inquiry lab, we restructured the exercise and made the following changes:

a) eliminated the time-table with listed procedures performed each week that had been closely nurtured by the instructional staff;

b) formed teams according to out-of-class time availability and added group assessments aimed to reveal problems within groups to facilitate formation of collaborative teams;

c) added quizzes with peer instruction;

d) added laboratory notebook evaluations;

e) adopted a format similar to a "flipped classroom" with prepared instructional videos explaining all tissue culture procedures, eliminating the need for demonstrations of techniques during lab time;

f) introduced performance-based assessments to verify skills on fluorescence microscope handling and sterile techniques;

g) allowed students to develop their own projects including choosing a research question and hypothesis, planning their experiment, and sharing the workload between group members; and

h) incorporated group interviews to monitor group progress (Luckie et al. 2013).

Most of the students had introductory neurobiology course work preceding this laboratory course. The detailed outline of the inquiry-based laboratory schedule from Neurobiology, Spring 2013 is shown in Appendix A.

Even in the previous format of a classical type of laboratory course, students gave great evaluations for this unit, and instructors were impressed with the high quality of final lab reports.

Student Outline

Included here are laboratory materials from Dr. Michael Dailey's Neurobiology Laboratory (002:186) from the spring semester of 2012 in a classical lab format. In the 3-week long cell culture unit, students performed culturing of neuroprogenitor cells with or without endothelial cells on substratum, and compared the number of differentiated neural cells (neurons, astrocytes and oligodendrocytes) in control conditions (with neuroprogenitors alone) and in co-culture.

Materials Distributed to Students (Spring 2012) *(See Supplement - Student Instructions)*

1. Neuroprogenitor cell culture techniques - Week 1 of the Unit.
2. Preparation of coverslips (Handout #1).
3. Working with contamination (from *At the Bench: A Laboratory Navigator* Kathy Banker, Cold Spring Harbor Lab Press, 1998) - not reproduced here.
4. Gary Banker and Kimberly Goslin. Types of nerve cell cultures, their advantages and limitations (from Banker and Goslin, Culturing Nerve Cells, Second Edition, MIT Press, 1998) - not reproduced here.
5. Starting co-cultures of endothelial and neuroprogenitor cells (Handout #2).
6. Recipes for media (Handout #3).
7. GFAP and TuJ1 immunocytochemical (ICC) staining of cell cultures on coverslips - Week 2 of the Unit.
8. Summary of O4 immunostaining procedure - Week 2 of the Unit.
9. How to differentiate between different types of neural cells in culture.
10. Guidelines for Neurobiology lab report on cultured neuroprogenitor cells.

Materials

List of Materials and Equipment for Cell Culturing

Live Materials and Media

- Neuroprogenitors, NP (BrainBits, NNSph Neuroprogenitor Neurosphere tissue is provided live, unseparated, fresh from E18 rat cortex/hippocampus including subventricular zone), one vial for four groups
- NEUROBASAL™ Medium (#21103-049, Invitrogen)- two 500 mL bottles for four groups
- B-27 Supplement Minus Vitamin A (50X), liquid (#12587-010, Invitrogen/Gibco) - one or two 10 mL vials for four groups
- Human recombinant bFGF (#13256-029, Invitrogen) - one 10-50 μg vial for four groups
- Human EGF (#13247-051, Invitrogen; #PHG0314 GIBCO) - one 10-50 μg vial for four groups
- GlutaMAX, 100X (#35050, Invitrogen) - one vial for four groups
- Gentamicin sulfate, 50 mg/mL (CellGro) - one bottle for four groups
- Penicillin / Streptomycin (Invitrogen) - 100 mL bottle for four groups
- Horse serum (Equine Serum, #SH30074.03, HyClone Laboratories) - 500 mL bottle for four groups
- Endothelial cells, C166 - yolk-sac derived endothelial cells (ATCC, CRL-2581)
- DMEM (GIBCO, with Glucose, 4.5 g/L, + L-Glutamate, - Sodium pyruvate) - one 500 mL bottle
- 100 mm cell culture plates - one sleeve (20 plates)
- Uncoated low adhesion plastic 6 well plates, 24/pack (Costar, #3471) - one pack for four groups
- Papain (Worthington, LS003119) - 100 mg per classroom
- Hibernate E-Ca (BrainBits) - 5 or 10 mL per classroom.
- 15 mL sterile polystyrene (PS) or PET centrifuge tube (Corning #430055 or Sarstedt #62.553.002) - from four to six packs per four groups
- 50 mL sterile polystyrene (PS) or PET centrifuge tube (Corning #430304) - four to six packs per four groups
- Medium sterile forceps to handle inserts-- one per group
- Steriflip (Millipore) - up to six units
- Stericup (Millipore), 150 mL or 250 mL - from six to eight units
- Water bath, 37°C
- Hemacytometer (VWR #15170-079 or Fisher #0267110)
- Trypan blue (Sigma #T8154)
- Inverted phase contrast microscope (Nikon TMS) - minimum two per classroom
- UltraPure Distilled water (18 Mohm, #10977, Invitrogen/Gibco) - one 500 mL bottle per classroom
- Fire polished borosilicate pasteur pipettes in a holder, sterile - up to six pipettes per group. To polish, pasteur pipettes are run through a flame for short time to narrow the opening and smoothen sharp edges
- Pasteur pipettes in a holder, sterile - up to ten pipettes per group
- Micropipettors and sterile tips - one set of P1000, P200, and P20 pipettors and one box of corresponding tips per group
- Pipet aids - one per group
- Tweezers (Dummond #5, 11 cm, World Precision Instruments, #14098) - one per group
- Borate buffer (2.38 g boric acid and 1.28 g borax in 500 mL of pure water)
- 10 mL or 20 mL syringes - two or three per group
- Syringe-tip filter units (0.2 μm) - two or three per group
- Glass coverslips, treated, 18 mm round (two coverslips per well) - see "Coverslips" protocol - 8 or 12 per group
- Glass petri dishes to sterilize coverslips - one per group
- 95% ethanol
- Poly-D-Lysine hydrobromide (#P-6407-5 mg, Sigma) - from two to four bottles
- 0.25 % Trypsin/EDTA - one 100 mL bottle
- 6 well plates (#35-3046, Falcon) - one or two per group
- Tissue transwells, 0.4 μm, 24 mm clear, for 6-well plates (#3412, Corning) - from two to six wells per group
- Table-top swinging bucket centrifuge - one per room
- Shaking incubator at 30°C - one per room
- Cell culture CO_2 incubator - one per room
- Cell culture enclosures, a tabletop simplified version of cell culture hood with a UV light and a timer, without air flow - one per group. (See Supplement - Tissue Culture Enclosure Photograph.)

Safety Equipment

- Protective gloves of various sizes
- Lab coats for each student
- 70% ethanol spray bottle for cleaning - one per group
- Large kimwipes for cleaning - one per group
- Bleach for decontamination

Materials and Equipment for Immunostaining

- DAPI (Invitrogen) - one vial
- Formaldehyde - one 500 mL bottle per classroom
- Cytoseal (VWR) - one 100 mL bottle per classroom
- Horse serum - one 500 mL bottle per classroom
- 6 well plates (#35-3046, Falcon) - two per group
- Plastic transfer pipets
- Glass slides
- Triton X-100 - one 500 mL bottle per classroom
- Phosphate buffered saline (PBS)
- Parafilm - two or three rolls per classroom
- Small kimwipes - one per group
- 150 mm plastic dishes for humidified chambers - one per group
- Round filters for humidified chambers - one pack per classroom

- Rectangular glass staining containers - one per group
- Rotary shaker - on or two per classroom
- Fluorescence microscope with digital video camera

Antibodies

- Anti-glial fibrillary acidic protein,GFAP (#G9269-1ML, Sigma) - one vial
- Neuronal Class III β-Tubulin (MMS-435P -TUJ1, Covance) - one vial
- Anti-O4 Mouse IgM, clone 81, (Millipore) -50 µg
- Anti-NG2 Chondroitin Sulfate Proteoglycan, rabbit polyclonal (Millipore) - one vial
- Alexa Fluor 488, goat anti-mouse IgG (Invitrogen) - one vial
- Alexa Fluor 568, goat anti-mouse IgG (Invitrogen) - one vial
- Alexa Fluor 568, goat anti-mouse IgM (Invitrogen) - one vial

Notes for the Instructor

The prerequisite for this lab should be knowledge of neural cell types, fluorescence microscopy with image capturing, and immunocytochemistry. These skills are taught during the first 4 weeks of the course. This upper level laboratory required at least two times more out-of-class time than an announced number of semester hours (4 hours of class time and 8+ hours of out-of-class work). Intensive training of students with different culture techniques, detailed instructions, and individual assessments of tissue culture skills allow students to perform outside bench work without supervision. When we posted detailed instructional videos of culturing techniques, students were able to work completely independently, and instructors contributed via different type of assessments and project discussions.

This tissue culture unit uses commercially available primary cells isolated directly from embryonic tissue, neuroprogenitors, lowering the risk of bacterial, fungal or yeast contamination of these cultures (contamination actually happened only in one well one time over 4 years). Culturing endothelial cell lines is more prone to contamination, but should not be an issue if it is performed by instructional staff. Materials and detailed protocols for preparing progenitor cell culture can be found in Appendix B. Students performed their tissue culture work mostly in table top enclosures (one per group), which are a closed containment containing light (including fluorescence and UV), and a timer for light. A laminar flow tissue culture hood is available for students as well. Safety tip: Students should never work under the UV lights ON. Safety issues are addressed in safety training courses which students completed at the beginning of this course (Biological safety - Basic, Chemical safety, PPE awareness, Formalin users). Personal protective equipment (gloves and lab coats) are used for the entire cell culture unit.

Students monitor differentiation of cell cultures mostly outside of class time using inverted phase contrast or Hoffman modulation contrast microscopy.

Fixation and staining of cell cultures using three antibodies would be very challenging if not practiced a few weeks before. Possible errors could include a lack of adequate labeling of coverslips and slides, missed steps, especially if staining is split between group members, wrong solutions used for incubation, incorrect mounting of coverslips, and broken coverslips (at least 4 coverslips per condition are used). Instructional staff usually have prepared fixed cultures on coverslips as a backup to cover these mistakes and to assure that students have accomplished staining of all conditions.

Microscopy and capturing images of stained cultures are done by students outside the scheduled class time. They practice cell counting with the instructor to recognize dead and live cells, as described in the student handout "How to differentiate between different types of neural cells in culture", and then proceed to actual counts on their own. The class results from Spring 2012 are shown in the online supplement-*Cell Culture Results*).

Time-Table and Steps

1. Instructors: Set up progenitor cell cultures (tissue dissociation) a few days before the start of the Unit. Takes 3 hours to complete.
2. Instructors: Start endothelial cell cultures (the procedure takes 1 hour, should be done 1 week before use).
3. Instructors: Plate inserts with endothelial cells for overnight culturing (takes 1 hour, performed one day before use).
4. Students: prepare substratum on coverslips, make neuronal differentiation medium for co-cultures, and plate progenitors on the coverslips in the bottom chamber of transwells, add inserts with endothelial cells - takes 3 hour class period, incubation lasts for 1-3 days in progenitor medium.
5. Students: optional - discard inserts and change medium in cultures to neuron differentiation medium or astrocyte differentiation medium and incubate for additional 4-7 days (takes 30 minutes).
6. Students: monitor cell differentiation by taking pictures over the 7-day incubation: Day 0, Day 3 or 4, Day 6 or 7 (takes 1 hour for each time point).
7. Students: fix and stain cells with TuJ1 (for neurons) and GFAP (for astrocytes) antibody as double staining, or for NG2 or O4 antibody (oligodendrocyte progenitors). Each staining takes 4 hours.
8. Students: take pictures of stained cultures by digital fluorescence microscopy; count neurons, astrocytes, and oligodendrocytes in digital images; analyze results - takes 4 hours.

9. Students: write a lab report in the format of a scientific paper. Optional: student poster presentations or slide shows.

Assessment

1. Students work in groups of three. All four groups of students in Spring 2012 were able to accomplish all steps of the laboratory and successfully quantified cells in captured images of stained cultures.
2. Lab reports showed a good understanding of the research question and required steps of the procedures. They showed that students understood the limitations in their results and applied original methods to obtain quantitative data in their projects.
3. Students in Spring 2012 evaluated this lab in an anonymous survey on a scale of 0-5 for interest and usefulness. Results were the following:

   Interest: 4.9±0.9 (n=7)
   Usefulness: 5.0±0.6 (n=7)

Here are some comments about this exercise which our students added in their evaluations:

"Favorite lab section of the course, I felt that the addition of the endothelial exposure was also positive addition and should be continued"

"Loved it! I wish we spent more time on these or did cultured 2 rounds of cells using different variables. It also would have been cool to use more types of stains with different specificity of binding or found a stain that stained neurons a color other than red, green, or blue and then used all the stains we used in the same culture so we could see astrocytes, oligo, and neurons in one coverslip"

"This was an awesome few weeks of lab. All of the techniques were really useful and it was a great way to integrate all that we learned thus far. Definitely, my favorite experiment. The lab report was also useful and good preparation for a research life. I feel like the entire experiment was very well organized and worthy enough for honors!"

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About the Authors

Dr. Olga Miakotina is a Lab Coordinator for the Department of Biology at the University of Iowa. She received her MS and PhD degrees in Biochemistry at the Moscow State University (Russia). Olga joined the Biology Department in 2008. She teaches Human Biology (BIOL:1140), Animal Behavior (BIOL:3244), Developmental Biology lab (BIOL:3736) and Neurobiology lab (BIOL:3656).

Dr. Michael Dailey is Associate Professor of Biology at the University of Iowa. He received his PhD in Biology and Biomedical Sciences (Neural Science) from Washington University (St. Louis) and did postdoctoral work in Molecular and Cellular Physiology at Stanford University Medical School. His research utilizes time-lapse confocal and multiphoton imaging to study glial cell development and function. He has been teaching upper level cell biology and neurobiology lab courses at the University of Iowa for 15 years.

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APPENDIX A

Assessments and Points for Neurobiology Lab (2013)

This is a summary of the topics, skills, assessment methods and grading as proposed for the inquiry laboratory format.

Weeks - Topic	*Skills*	*Assessments*	*Time, points*
Week 1: **Gross Examination and Dissection of Sheep Brain**	a) Organization of the brain, comparison of human and sheep brain b) Cranial nerves (type, location, functions) c) Anatomical terminology, planes d) Lab notebook	a) The handout on "Comparison of Sheep and Human Brains"	10 points, in Wk 2
		b) Sheep brain + Cranial nerves - Quiz	50 points, Wk 2
		c) Cranial nerve report	50 points, Wk 4
		d) Start lab notebook (sample, description online)	Wk 1, check on Wk 2
		e) Team builder - preliminary questionnaire	Finish by Wk 2
Week 2: **Team formation** • Sheep brain + Cranial nerves - Quiz • Check on lab notebook (each student) • Handout on "Comparison of Sheep and Human Brains"	a) Teamwork=science skill: why essential for research, trouble-shooting	a) Several exercises on team formation (in class)	
Week 3: **Classic stains, Fluorescence Microscopy and Imaging** • 1 hour of work with each group (all three students) to use fluoroscopes	a) Describe and compare different staining techniques. b) Prepare stained slides using classic stains. c) Use a transmitted light microscope to view samples. d) Identify structures in brain sections and relate to gross anatomical structures. e) Distinguish between neurons and neuroglia in histochemically stained brain tissues. f) Identify neuronal cell body, axon, dendrites, synaptic structures in Golgi stained brain sections. g) Fluorescence microscope: parts, function h) Using fluorescence microscope - 2 different per group i) Taking images using fluorescence microscope and image processing (cont. next page)	a) "Neurohistology-I Worksheet"	20 points by Wk 4
		b) Present results of classic staining and BrainStaining (with scale) - each students capture images of their own slides, group presentation of the best work of each student	10 points for presentation, in class
		c) Performance-based assessment- fluoroscopes (each student) - be ready	25 points, Wk 4
		d) Questions at the end of lab outline	10 points, Wk 4

Week 3: continued	j) Improvement of images, multi-color images k) Basic steps of staining l) Pipettor use m) Dilutions n) Coverslipping		
Week 4: **Antibodies and Immuno-fluorescent labeling of Glia** • Cranial nerve report • PBA on fluoroscopes (each student) • Questions at the end of lab outline • Neurohistology-I Worksheet • Overview of sterile technique (videos)	a) Antibody staining (flowchart with purpose of each step) b) Fluorescent immunostaining (direct, indirect, advantages, pitfalls) c) Staining tissue d) Images: capturing and interpretation	a) Quiz - pipettor use, dilutions, ab staining steps (individual and group answers)	20 points, Wk 4, in class
		b) Present results of fluorescence staining (with scale) - each student captures images of their own slide and presents with explanation (deduction of what cell type stained)	10 points for presentation, Wk 4 in class
		c) Check notebook -1	12 points, Wk 4 in class
		d) Team assessment-1 (CATME)	10 points, by Wk 5
Week 5: **Sterile Technique and Neuroprogenitor cell culture techniques.** • Overview of cell culture experiment - coculture of neuroprogenitors and endothelial cells • Demonstration of techniques • Have a list of materials and protocols available online • Optional field trip - confocal scope	a) Sterile techniques b) Tissue culture experiments (cell types, conditions, critical parameters), advantages, disadvantages c) Cell culture techniques (substratum preparations, medium prep, trituration, centrifugation, precautions) d) Scientific method (asking question, generating hypothesis, predictions) e) Scientific method (designing experiments, developing timeline, conducting experiments)	a) Quiz on sterile techniques with peer instructions (individual and group assessment)	15 points, Wk 5 in class
		b) Write up: Research question, hypothesis, prediction, methods, time line for research project - from a group or from each student	25 points, Wk 6
		c) Group - interview (brief presentation of project in progress)	25 points, by Wk 7

Week 6: Students' cell culture projects • Write up: Research question, hypothesis, prediction, methods, time line for research project - from a group • Group - interview for entire week	a) Communication skills (writing, oral PowerPoint presentation, delivering in interview) b) Experimental design and conduction of independent project	a) Check notebook-2	12 points, Wk 6 in class
		b) PBA - cell culture techniques	25 points , Week 6 in class
		c) First draft of final paper, (introduction, materials, methods) - written by every member of group and reviewed by every member of group - submitted	25 points, Week 7, with feedback next week (Wednesday - submit to group members, Thursday - feedback from group members, Friday - to instructors)
Week 7: Students' cell culture projects • First draft for feedback on Wednesday		a) Second interview for project in progress (results of staining), short presentation, problems, preliminary conclusions (30 min/ group) b) Team assessment-2 (CATME)	25 points, Week 8 10 points, by Week 8
Week 8: Students' cell culture projects • Second interview for entire week 8 • Team assessment-2 (CATME)		a) Second draft of final paper, Week 8 - (introduction, materials, methods, some results, some conclusions, abstract) - written by every member of group and reviewed by every member of group	25 points, Week 8, with feedback next week (Wednesday - submit to group members, Thursday - feedback from group members, Friday - to instructors)
Week 9: Students' cell culture projects • Feedback on Draft 2			
Week 10: Students' cell culture projects • Final paper	Final grade is a combination of personal assessments (lab notebook, team exercises, PBA-1 and 2, participation in presentations and interviews-1 and 2, quizzes, worksheets, drafts), and group assessments (presentations, write-ups)		

Appendix B

Instructional Protocols for Progenitor Culture and Endothelial Cell Culture

Preparation of Progenitors Culture

Materials: Information on specific brands is found in the Materials section:

1. Neuroprogenitors - NNSph Neuroprogenitor Neurosphere tissue (BrainBits).
2. Hibernate E - Ca (BrainBits), warmed to room temperature.
3. Papain.
4. Progenitor growth medium, warmed to room temperature.
5. Sterile 15 mL centrifuge tubes.
6. Sterile 50 mL centrifuge tubes.
7. 5 mL disposable pipettes with pipette aid.
8. Sterile fire-polished borosilicate pasteur pipettes (to polish pipettes pass them through a flame a few times to decrease tip diameter and smoothen edges).
9. Sterile pasteur pipettes.
10. Pipettors.
11. Uncoated low adhesion plastic, 6-well plates.
12. 0.4 % Trypan Blue/PBS: dissolve 0.04 g Trypan Blue in 1X PBS, not sterile, store at room temperature.
13. Hemacytometer.
14. Phase contrast microscope.
15. Table-top swinging bucket centrifuge.
16. Shaking incubator at 30^{o}C.
17. Water bath, 37^{o}C.
18. Steriflip and Stericup units.
19. 95% ethanol.
20. Concentrated nitric acid.
21. Sodium bicarbonate (baking soda from grocery store).
22. 18 mm coverslips (treated, autoclaved - see below).
23. Glass petri dishes.

Performed Under a Tissue Culture Hood: Takes 3 Hours

Water bath, 37^{o}C
Shaking incubator at 30^{o}C - setting 26 in 149 BB
Progenitor growth medium, room temperature

- Prepare papain solution: reconstitute 20 mg of papain (very light powder, do not wear gloves) in 2.0 mL of 'Hibernate E - Ca' in a sterile 50 mL tube (10 mg/mL solution), incubate at 37^{o}C for 10 minutes, vortex, filter sterilize using Steriflip (0.22 μm pore size);
- Dilute 400 μL of 10 mg/mL papain with 1.6 mL of 'Hibernate E - Ca' (without B27) medium to have 2 mg/mL papain solution, use a sterile 15 mL tube;
- Aseptically open a vial with brain tissue (Neuroprogenitors), remove most of tissue medium (Hibernate E/B27)using 5 mL pipette and save it in a new sterile 15 mL tube;

- Transfer brain tissue to 2 mL of 2 mg/mL papain using 5 mL pipette, seal the tube with parafilm and shake for 10 minutes at 30°C for enzymatic dissociation;
- Remove papain solution from the tube using 5 mL pipette. Transfer tissue to 1 mL of original Hibernate E/B27 medium with as little papain as possible;
- Triturate tissue using fire-polished borosilicate pasteur pipettes (for higher viability): 10 times total using 2 different pasteur pipettes, avoid creating bubbles; add remaining saved Hibernate E/B27 medium;
- Let the tube with dissociated tissue sit for 1 minute to settle undispersed pieces by gravity, then transfer the supernatant to a new 15 mL tube and spin it at 1,100 rpm (200 X G) for 1 minute at room temperature;
- Discard the supernatant. Add 4 mL of Progenitor medium (provided by BrainBits) to the cell pellet, flick the tube to resuspend cells and count cells using 0.4 % Trypan Blue/PBS: dilute 20 µL of cell suspension with 20 µL of trypan blue solution, mix well and place 10 µL of the mixture on the hemacytometer. Count cells (phase bright) in four or five fields and calculate a cell density: Mean cells/field X 2 X 10^4 (cells/mL).
- Dilute cells with Progenitor growth medium (see recipe) to have 120,000 cells/mL in one or two 50 mL tube(s);
- Plate 240,000 cells (350,000 cells/well - maximum) in 2 mL Progenitor growth medium in a 6-well low attachment plate. Incubate at 37°C, 5% CO_2, 9-20% oxygen. After 2 days or longer progenitors are present. Add equal volume of fresh medium in 3 or 4 days. Later on, change one half of medium every 3 or 4 days: you may lose cells during medium change. Tilt the plate to let cells settle at the bottom and slowly remove medium from the top well corner. Add fresh medium. Progenitor culture is good for 2 or 3 weeks.

Treatment of Glass Coverslips

Takes at least two days.

- Treat 18 mm coverslips (round, glass) in concentrated nitric acid overnight to etch the glass surface.
- The next day, carefully pipet out HNO_3 into a 2 L jar with water, then neutralize water with sodium bicarbonate, and wash coverslips in a container with plenty of water till the pH is neutral,then rinse them with 95% ethanol, dry on napkins under tissue culture hood, place in glass petri dishes and autoclave them.

Neuroprogenitors and Endothelial Cells Co-Culture

Materials:

1. Endothelial cells, C166- yolk-sac derived endothelial cells.
2. Medium for C166: DMEM (Gibco, #11965 with high Glucose, 4.5 g/L, + L-Glutamate, Sodium pyruvate), 5% FBS, 1% Penicillin / Streptomycin.
3. 0.25 % Trypsin/EDTA.
4. Tissue transwell/insert, 0.4 µm, 24-mm for 6-well plate.
5. Six-well tissue culture plate.
6. Treated 18 mm glass coverslips as described above coated with poly-D-lysine (PDL), 100 mg/mL.
7. Progenitor medium components: Neurobasal, EGF, FGF, B27, Glutamax, Gentamicin.
8. Neuronal medium components: Neurobasal, Glutamax, B27, Penicillin / Streptomycin.
9. 100-mm tissue culture dishes.

Procedure:

1. Establish C166 cultures (up to one week before the lab):
    - Make media: DMEM+5% FBS + 1% Penicillin / Streptomycin for C166, warm up.
    - Thaw frozen stock of C166 quickly at 37°C.
    - Pour 10 mL of warmed medium in one 100-mm tissue culture plate, keep it warm at 37°C incubator.
    - Transfer cells into plate, swirl, and place a dish in the incubator at 37°C, 5% CO_2.
    - Next day change medium, split culture if needed.
    - Incubate C166 culture to 70-90% confluence.
    - Split cells to generate from six to eight 100-mm dishes.

2. Order Neuroprogenitors, set up Neuroprogenitor culture (one to -four days before experiment).
3. Prepare 6-well plates with coverslips treated with PDL of 0.1 mg/mL (one day before experiment). Incubate with PDL at room temperature for several hours or overnight. Wash wells with coverslips with sterile water twice. Keep wells with Progenitor medium at 37^{o}C overnight.
4. Plate endothelial cells in inserts (transwells) one day before the experiment:

   Note:1X100-mm dish of C166 contains from 2.7 to 6.0x10^{6} cells depending on confluence.

   Rinse endothelial cell culture (C166) with 1 mL of trypsin, then trypsinize endothelial cells with 2.5 mL of trypsin per 100-mm dish for 4-6 minutes at 37^{o}C, neutralize using endothelial growth medium (DMEM, 5% FBS, 1% Penicillin / Streptomycin, 5-7 mL/dish), hit the dish lightly on the bench, triturate cells to detach them from the dish and collect cells in 15 mL tube. Spin down cells at 1,000 rpm X 5 min. Remove most of medium, add 1 mL of medium for endothelial cells, triturate with 1 mL tip, 10 times. Add 3 more mL of medium, triturate with pipet and count cells: use 20 μL of cell suspension and 20 μL of trypan blue (0.4% in PBS), count cells in three or five fields of hemacytometer under phase-contrast microscope (10X), calculate a number of cells per mL (average number of cells per field X 2 (dilution factor) X 10,000). Dilute cells to have 2.5x10^{5} cells per mL.

   Add C166 cells to transwells: 5x10^{5} in 2 mL of medium to the insert, have 2.5 mL of endothelial cell medium in the bottom chamber. Incubate in endothelial growth medium overnight.
5. Neuroprogenitors (NPCs) in conditioned medium of endothelial cells (C166):

   - Spin down NPCs from several wells, carefully remove medium and add 1 mL of Progenitor medium. Triturate neurospheres using 4 different borosilicate polished pasteur pipets (perform 20 strokes total). Add more medium to have from 200,000 to 300,000 cells per mL according to the original cell counting of cells in the progenitor culture. Alternatively, collect cells from several wells and triturate, adding more fresh medium.
   - Remove medium from wells with coverslips treated with PDL and add 1.5 mL of fresh Progenitor medium.
   - Add from 200,000 to 300,000 of NPCs on the bottom of the transwell in 1 mL of medium, cover with insert containing endothelial cells; change medium in insert with endothelial cells to Progenitor medium - 1.5 mL; incubate for 3 days; discard inserts and change medium to neuronal differentiation medium in the bottom chamber (2 mL) and incubate for three or four more days (total incubation for 7 days).
   - Remove and fix coverslips - Day 7.

6. Take pictures at 0 day, 1, 3, 5 and 7 days in culture.
7. Stain some cultures on coverslips with GFAP and TuJ1 (double staining for astrocytes and neurons, respectively), and stain other cultures with NG2 or O4 (for oligodendrocytes); DAPI is added at the end of each staining option as a nuclear stain. Count a percentage of astrocytes and neurons in some cultures and oligodendrocytes in other cultures from a total number of live cells.

Table B1. Progenitor growth medium.

Components	Final concentration	Stock concentration	Amount for 20 mL	Amount for 50 mL	Amount for 100 mL
NEUROBASAL™ Medium			19.466 mL	48.665 mL	97.33 mL
B-27 Supplement Minus Vitamin A	2%	50X	1:50 0.4 mL	1:50 1.0 mL	1:50 2.0 mL
bFGF	20 ng/mL	100 µg/mL	1:500 40 µl	1:500 100 µl	1:500 200 µl
EGF	20 ng/mL	100 µg/mL	1:500 40 µl	1:500 100 µl	1:500 200 µl
Glutamax	0.5 mM	100X, 200 mM	1:400 50 µl	1:400 125 µl	1:400 250 µl
Gentamicin	10 µg/mL	50 mg/mL	1:5000 4 µl	1:5000 10 µl	1:5000 20 µl

- bFGF - use 40 ng/mL after 1 medium change for prolonged culturing; EGF - use 40 ng/mL after 1 medium change for prolonged culturing; Filter-sterilized and 0.2 µm filter pore size.

Table B2. Differentiation medium for neurons.

Components	Final concentration	Stock concentration	Amount for 10 mL	Amount for 20 mL	Amount for 40 mL
NEUROBASAL™ Medium			9.725 mL	19.45 mL	38.9 mL
B-27 Supplement Minus Vitamin A	2%	50X	1:50 0.2 mL	1:50 0.4 mL	1:50 0.8 mL
Glutamax	0.5 mM	100X, 200 mM	1:400 25 µl	1:400 50 µl	1:400 100 µl
Penicillin/ Streptomycin	50 U/mL Pen 50 µg/mL Strep	10,000 U/mL Pen 10,000 µg/mL Strep	1:200 50 µl	1:200 100 µl	1:200 200 µl

- Filter-sterilize, 0.2 µm filter pore size

Table B3. Differentiation medium for astrocytes.

Components	Final concentration	Stock concentration	Amount for 10 mL	Amount for 20 mL	Amount for 40 mL
NEUROBASAL™ Medium			8.8 mL	17.6 mL	35.2 mL
Horse Serum	10%	100%	1:10 1 mL	1:10 2 mL	1:10 4 mL
Glutamax	3 mM	100X, 200 mM	1:67 150 µl	1:67 300 µl	1:67 600 µl
Penicillin/ Streptomycin	50 U/mL Pen 50 µg/mL Strep	10,000 U/mL Pen 10,000 µg/mL Strep	1:200 50 µl	1:200 100 µl	1:200 200 µl

- Filter-sterilized, 0.2 µm filter pore size.