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Using Organotypic Tissue Slices as Substrata for the Culture of Dissociated Cells

Daniel E. Emerling and Arthur D. Lander

1. Introduction

Dissociated cell culture has proved extremely valuable for studying the functional relationship between specific cell surface and extracellular matrix (ECM) molecules and a variety of cellular behaviors such as proliferation, differentiation, migration, and neuronal process outgrowth. When cells are cultured on substrata made from purified cell surface or ECM components, the direct actions of these molecules on cells can be examined easily. However, for some in vitro studies, it is desirable to examine the functions of such molecules in an environment that more closely approximates that which exists in vivo. Such studies might include, for example, investigations in which the endogenous distribution and heterogeneity of such molecules is important, screens for uncharacterized factors that effect a particular cell behavior, or studies examining the combinatorial effects of the many cell surface and ECM components that occur in intact tissue.

We devised an assay in which dissociated cells are plated onto living slices of tissue, much as they can be plated onto tissue culture plastic. This procedure allowed us to examine cell behaviors within the organotypic environment of a tissue substratum. Primary neurons were plated onto slices of living mouse forebrain, cocultured at 37°C, fixed, and then examined. The degree of attachment of the cells and the orientation of process outgrowth from them was found to be highly dependent upon the anatomical region of the forebrain slice onto which the cells settled when plated (**Fig. 1** and **ref. *1***). When the same cells were plated onto cryostat sections of the same tissue, neither the degree of overall attachment and outgrowth nor the region-dependent effects were as dramatic ***(1)***.

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Fig. 1. Laminar specific attachment of thalamic neurons to a living slice of embryonic mouse cerebral neocortex. Dissociated and fluorescently labeled embryonic thalamic neurons were plated onto sagittal slices of embryonic day 15 cortex and were cocultured for 3 h at 37°C. Cocultures were then rinsed to remove nonattached thalamic cells, fixed, counterstained, and visualized. **(A)** Cortical laminae of a bisbenzamide-stained slice can be seen under UV fluorescence as differences in nuclear density. Rostral is to the right, and caudal is to the left. **(B)** The same slice viewed under rhodamine optics shows the distribution of attached thalamic cells. The pial and ventricular edges of the cortex are demarcated by dotted white lines at the top and bottom of each figure. Lines are positioned in the two photos to reference the same points in the two views of the slice. Very few thalamic cells attach to the cortical plate (CP), whereas more attach to the intermediate zone (IZ), marginal zone (MZ), and ventricular zone (VZ) as well as to the culture support off the slice (seen at the edges of the photos). Density of attached cells is greatest on the intermediate zone just subjacent to the cortical plate (i.e., the subplate). Scale bar is 500 μm.

The living-slice method of coculture is amenable to the same experimental manipulations that are performed on typical dissociated cell cultures (e.g., pharmacological treatment, enzymatic digestions, antibody blockade, etc.). For example, we used this approach to investigate the functions of chondroitin sulfate in neuronal attachment and axon pathfinding. We treated slices with chondroitinase ABC to enzymatically remove endogenous chondroitin sulfate, and then compared the behaviors of primary neurons plated on treated and control slices (**Fig. 2** and **ref.** ***2***).

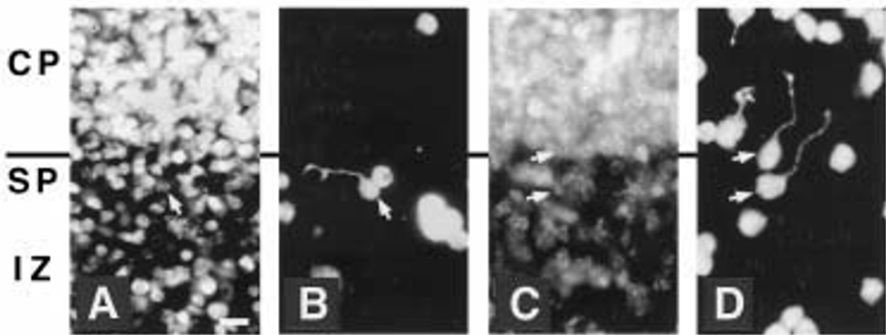

Fig. 2. Cell attachment and neurite extension on slices of embryonic mouse cerebral cortex treated with chondroitinase ABC. Cocultures of cortical slices and thalamic neurons were prepared as in **Fig. 1**, except that slice cultures were treated with carrier (complete medium), or 1 U/mL chondroitinase, for 3 h at 37°C, prior to the addition of dissociated and labeled thalamic neurons. High-magnification, fluorescence micrographs show the bisbenzamide-stained cortical plate (CP), subplate (SP), and intermediate zone (IZ) of control **(A)** and treated **(C)** tissue slices. **(B,D)** The same fields viewed under rhodamine optics reveal the attached, thalamic cells and their neurites. Arrows indicate neurite-bearing thalamic cells and their corresponding nuclei. In control cocultures **(A,B)**, cells attach well to the subplate, but poorly to the cortical plate. Neurites that extend on the subplate and intermediate zone tend to orient parallel with the cortical layers, and neurites that cross from the subplate onto the cortical plate are extremely rare. In cocultures treated with chondroitinase (C, D), cells attach well to the cortical plate, neurite outgrowth on the cortical plate is enhanced, and processes that originate on the subplate often cross onto the cortical plate. Scale bar is 10 µm.

This chapter will focus on how to cut tissue slices for use as culture substrata and how to make and fluorescently label a dissociated cell suspension from tissue. It is assumed that the investigator already has procedures to obtain the tissue(s) of interest. The first four parts of the protocol involve preparing the slices and dissociated cells for coculture and include: 1. embedding the tissue in agarose and slicing it on a vibratome, 2. mounting the tissue slices onto supports and transferring them to culture, 3. dissociating tissue into a cell suspension, and 4. labeling dissociated cells with a vital dye (**Fig. 3**). The fifth section deals with culturing, fixing, and counterstaining the cocultures, as well as how to mount them onto microscope slides for visualization.

The choice of technique for visualizing the cells on the slices (e.g., immunofluorescence, *in situ* hybridization, transgenic marker, etc.) will, of course, depend upon the experimental needs of the investigator. The method described below involves labeling the cells in suspension, before plating, with a fluorescent, fixable, cytoplasmic dye that makes it easy to discern plated cells from

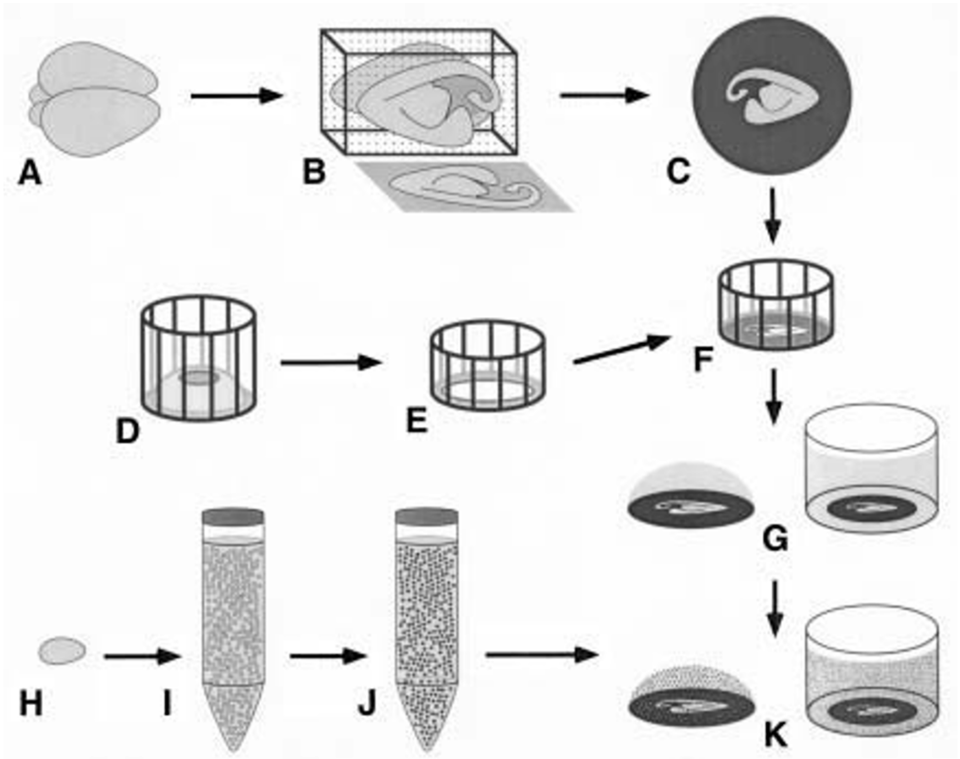

Fig. 3. Steps to making cocultures of dissociated cells plated onto living tissue slices. The tissue to be sliced is dissected **(A)**, embedded in agarose and sliced on a vibratome **(B)**, and then placed onto nitrocellulose disks for support **(C)**. Disks with mounted slices are transferred from the vibratome using a holder made from the cap of a round-bottom tube (**D**, *see* **Subheading 2.2.3.**). A hole is punched in the top of the cap, leaving a rim, and the cap is shortened **(E)** so that the disk can be placed in the bottom of the cap, forming a well **(F)**. After transfer from the vibratome, disks are removed from the holder (*see* **Subheadings 3.2.4.** and **3.2.5.** for details), and placed either on parafilm under a bubble of medium or in tissue culture wells **(G)**. To obtain a cell suspension, the tissue is dissected **(H)**, dissociated **(I)**, and labeled with a fluorescent vital dye **(J)**. Cells are then allowed to settle onto the slice cultures **(K)**.

those in the slice. Cells can be similarly labeled with membrane-intercalating vital dyes such as DiI *(3)*. Bisbenzamide, a blue-fluorescent, nuclear stain, is useful for making visible the overall anatomy of cultured slices. Protocols for immunostaining such slices can be found elsewhere *(1,2)*.

We optimized this method for making living slices of perinatal mouse forebrain to use in acute (<24 h) cocultures with dissociated primary neurons. The sectioning protocol should be applicable for use with most tissues, but may

require some modifications depending upon the properties of the particular tissue used. Similarly, the dissociation protocol was optimized for neural tissue and may require modification for other tissues or for particular experimental requirements.

We found that, after 1 d in culture, the tissue slices may begin to flatten. This process alters anatomical structure and borders. The particular questions and needs of the investigator will determine whether longer culture periods are needed and if such in vitro alterations could affect the interpretation of data. Regardless of the culture period, investigators should take care to ensure that particular results are not because of artifacts such as folds, tears, or other incongruities in the tissue slice. A wise approach is to examine all fixed, mounted slices for such defects prior to examining the behaviors of the labeled dissociated cells plated on them.

2. Materials

All solutions should be sterile (except paraformaldehyde). Unless noted, all chemicals are from Sigma, St. Louis, MO. All media and balanced salt solutions are from Gibco, Gaithersburg, MD. Procedures should be performed aseptically and, when possible, in a laminar flow hood.

2.1. Embedding and Slicing Tissue

1. Phosphate Buffered Saline (PBS): 137 m*M* NaCl, 2.68 m*M* KCl, 7.83 m*M* Na_2HPO_4, 1.47 m*M* KH_2PO_4.
2. PBS/glucose: PBS supplemented with 0.45% glucose and 1 m*M* sodium pyruvate (from 100X stock, Gibco). Filter sterilize.
3. Low melting point (LMP) agarose solution: 2% LMP agarose (Gibco-BRL) in PBS. Autoclave to dissolve agarose and to sterilize. When cool, but still liquid (<50°C), supplement to 0.3 % final glucose with sterile 30% glucose stock. Stable at 4°C in sealed bottle at least 6 mo.
4. Vibratome tray (Technical Products International, Inc., St. Louis, MO) and teflon coated vibratome blades (Ted Pella).
5. Tissue adhesive, such as Locktite™ (Ted Pella) or any comparable glue may work.

2.2. Mounting Slices onto Supports for Culture

We chose to mount tissue slices onto black nitrocellulose disks because this material provided the rigidity we needed for our experimental manipulations. Black was chosen to minimizes reflection and scattering of light during examination by fluorescence microscopy. To ensure that slices attach well to the support, the disks are pretreated with the lectin concanavalin A, however, other proteins (e.g., laminin, fibronectin, etc.) may work as well or better for a particular tissue. For this treatment and for coculturing, disks can be placed into 12- or 24-well flat bottom tissue culture plates. Alternatively, disks can be

placed onto Parafilm™ (*see* **Subheading 2.2.1.**). Holders (*see* **Subheading 2.2.3.**) are made to transfer disks with mounted tissue slices out of the vibratome tray without damaging the slices.

The preparation and use of nitrocellulose disks as supports is somewhat laborious (*see* below and **Subheading 3.2.**), and they are not suitable for transmitted light microscopy. As an alternative, slices can be mounted onto commercial culture well inserts. Several suppliers (Millipore, Bedford, MA, Costar, Cambridge, MA, and Nunc, Wilsbaden-Biebrich, Germany) manufacture these inserts with growth surfaces made from a variety of translucent or transparent polymer materials (e.g., polycarbonate, cellulose, etc.) and treated with a variety of ECM molecules (e.g., collagen, laminin, etc.). Characteristics such as autofluorescence, transparency, rigidity, and porosity vary greatly among the different membranes and, based on the particular experimental needs, should be taken into account when making a choice of insert to use.

1. Parafilm™ dishes: When pipeted onto nitrocellulose disks that are on Parafilm™ (American National Can), small volumes (≈100 µL) of solutions remain in a bubble over the disk because of the hydrophobic properties of the Parafilm™. Cut a circle of Parafilm™ (≈10–12 cm diameter), remove backing, and place down in a 150-mm plastic Petri dish. Using both hands, hold the sheet flat and, with the back edge of a forceps or other blunt metal object, scratch several short lines, perpendicular to the edges of the Parafilm™, around its perimeter. This will score the Parafilm™ into the plastic of the dish. Sterilize under ultraviolet (UV) light (>1 h).
2. Nitrocellulose disks: Sterilize the cutting edge of a cork borer (12 mm diameter) with flame and/or ethanol. Place a sheet of sterile, black nitrocellulose (0.45 µm; Sartorius, Bohemia, NY) into a plastic Petri dish and press the borer into it to cut out disks. Transfer the cut disks with a sterile forceps to another Petri dish for storage.
3. Holders for nitrocellulose disks: A holder is made from the cap of a 6-mL round bottom tube (Falcon, #2063). The cap is inverted and used as a well, on the bottom of which the tissue-bearing nitrocellulose disks will be placed. Take a plastic cap and place it onto the cutting end of an 11-mm cork borer, as if to cap the end of the cork borer as a tube. The indent at the top of the cap should just fit into the hole of the borer. Using a large rubber stopper as a backing, punch a hole out of the cap. This should leave the top of the cap with just a rim. Discard the rest of the cap top that was cut out. Using a scalpel or razor blade, cut the length of the cap in half, discarding the half that contains indents on the inner side of the cap and saving the half that has the rim (**Fig. 3E**). Sterilize by storing in ethanol.
4. Concanavalin A: 1.3 mg/mL in DMEM (or other medium or balanced salt solution). Filter sterilize and store aliquots at –20°C.
5. Complete medium: DMEM (glutamine free, 4.5 g/L glucose) supplemented with 10 µg/mL transferrin, 5 mg/mL crystalline grade bovine serum albumin (BSA;

ICN Biochemicals), 20 n*M* progesterone, 30 nM sodium selenite, 100 µ*M* putrescine, 10 µg/mL bovine insulin, 1 m*M* sodium pyruvate, 50 I.U./mL penicillin, 50 µg/mL streptomycin, and 25 m*M* HEPES (pH 7.2). Filter sterilize. Store at 4°C. Use preferably within 24 h. Other basal medium and supplements may be more appropriate for the particular tissue and dissociated cells used.

6. Sterile filter paper or gauze. Bleached (white), filter paper from a sealed pack should be suitable.

2.3. Dissociating Tissue into a Cell Suspension

1. Balanced Salt Solution (BSS): calcium–magnesium-free, Hank's Balanced Salt Solution. This can be supplemented with 10 m*M* HEPES, pH 7.2, if good pH control is needed (BSS will become somewhat basic in room air).
2. Trypsin stock: 5 mg/mL of trypsin from bovine pancreas in BSS. Sterile filter. Store as aliquots at –20°C.
3. DNase stock solution: 1 mg/mL DNase I (grade II, Boehringer Mannheim, Germany) in DMEM or other basal medium. Sterile filter. Store as aliquots at –20°C.
4. Trypsin inhibitor: 5 mg/mL trypsin inhibitor (Type I-S) from soybean in DMEM or other basal medium. Sterile filter. Store as aliquots at –20°C.
5. 9" Pasteur pipets with cotton plug. Autoclave to sterilize.
6. 4% BSA: 4% bovine serum albumin (crystalline grade; ICN Biochemicals) in BSS; bring to pH ≈ 7.3 with 1 M NaOH. Sterile filter. Store as 2.0–2.5 mL aliquots at –20°C.

2.4. Labeling Dissociated Cells

Dye stock solution: Use Cell Tracker™ (Molecular Probes, Eugene, OR) dyes, such as CMTMR (rhodamine derivative) or CMFDA (fluorescein derivative). Make 10 m*M* stock solution in DMSO and store as aliquots at –20°C.

2.5. Coculture, Fixation, Counterstaining, and Mounting

1. Paraformaldehyde: 4% paraformaldehyde (J. T. Baker, Phillipsburg, NJ) in 0.1 *M* sodium phosphate buffer (pH 7.5) or PBS. Paraformaldehyde is toxic, so perform this procedure in a fume hood. Add paraformaldehyde to water (one half of the final solution volume), stirring at 65°C. Add 5 *M* NaOH dropwise until paraformaldehyde goes into solution. Bring to the final volume with a 2X stock of buffer (0.2 M sodium phosphate or 2X PBS). Filter to remove particulate. For paraformaldehyde/sucrose, make fix with 0.12 *M* final sucrose. Add sucrose with the 2X buffer. Good for 2 wk at 4°C.
2. Bisbenzamide (Hoechst 33258): 1 mg/mL stock solution (100X) in water, then filter and store at 4°C. For use, make fresh by diluting in PBS to 10 µg/mL.
3. Mounting solution: Saturated sucrose solution containing 0.1% sodium azide (Fluka AG). Store at room temperature. Other aqueous mounting media may work as well.
4. Glass microscope slides, 22 × 22 mm coverslips, super glue, and clear nail polish.

3. Methods

3.1. Embedding and Slicing Tissue

1. Autoclave or ethanol flame the vibratome tray to sterilize it and then insert it in the vibratome.
2. Melt the LMP agarose solution, then cool to and keep at 37°C in a water bath.
3. Dissect tissue for slicing and store on ice in PBS/glucose.
4. Fill a 35-mm plastic Petri dish or similar container with the agarose and place the dissected tissue in the dish (*see* **Note 1**), being careful not to transfer too much liquid with it (*see* **Note 2**). Place the dish on ice to speed the hardening of the agarose.
5. Cut a block (sides ≈1.5 cm in length) containing the tissue out of the agarose in the dish using a sterile scalpel or razor blade. In another dish, you can further cut the block so that the tissue is oriented in the desired plane of sectioning and is at the correct height for the blade when the block is placed in the vibratome tray (*see* **Note 3**).
6. Place a drop of adhesive on the dry tray surface and immediately place the agarose block on the glue in the proper orientation. Once the glue has set (*see* **Note 4**), fill the tray with ice-cold PBS/glucose so that the block is submerged and surround the tray with an ice bath.
7. Insert a sterile vibratome blade in the holder on the vibratome (*see* **Note 5**).
8. Section the tissue into slices 150–200µm thick (*see* **Note 6**). The optimum vibratome settings for speed and vibration of the blade will depend upon the size and consistency of the particular tissue and should be empirically determined (*see* **Note 7**). Once cut, allow the slice to settle to the bottom of the tray and separate it from any agarose (*see* **Note 8**).

3.2. Mounting Slices onto Supports for Culture

1. Treat the nitrocellulose disks with the concanavalin A solution for 4–18 h at 4°C (*see* **Note 9**) in a culture well plate or on Parafilm™ in a humidified atmosphere. Rinse well in PBS before use.
2. Place a disk in the vibratome tray and position it near the slice on the bottom of the tray. Carefully manipulate the tissue slice onto the disk (*see* **Note 10**). Multiple slices can be placed on the disk, if they can fit.
3. Take a sterile disk holder (*see* **Subheading 2.2.3.**) and, with forceps, hold it on the tray bottom next to the disk (*see* **Note 11**). With another forceps in the other hand, grab the edge of the disk with mounted slice(s) and place it into the holder so that the disk forms the bottom of a well (**Fig. 3F** and *see* **Note 12**).
4. Lift the holder and disk out of the tray so that the well they form remains filled with the PBS/glucose (*see* **Note 13**). Place the holder onto the sterile filter paper or gauze so that the buffer is drawn down, through the nitrocellulose, by capillary action, and rapidly lift the holder off the paper just before the slice gets dry (*see* **Note 14**).
5. Using forceps, remove the disk from the holder and transfer it to parafilm or a culture well plate. Add complete medium before the tissue dries out (≈100 µL for

disks on parafilm). Slices can be stored for short periods (≈1.5 h) in medium on ice until all slices collected. Culture in a humidified atmosphere at 37°C (*see* **Note 15**).

3.3. Dissociating Tissue into a Cell Suspension

1. Dissect tissue for dissociation and place in 1 mL BSS in a 15-mL polypropylene, conical tube (*see* Note 16).
2. Add 45 μL of the trypsin stock and 100 μL DNase (final concentrations of 0.2 mg/mL and 0.09 mg/mL, respectively). Incubate for ≈10 min at 37°C with occasional, gentle mixing (*see* **Note 17**).
3. Add another 250 μL DNase, 300 μL of complete medium, and 100 μL of the trypsin inhibitor. These additions raise the final DNase concentration to 0.2 mg/mL and the inhibitor to 0.3 mg/mL. Incubate for another 2–5 min at 37°C.
4. Make a trituration pipet by flame-polishing the tip of a Pasteur pipet so that it tapers to a hole ≈0.2–0.7 mm in diameter (*see* **Note 18**). To prevent loss of cells on the glass, coat the inside of the pipet with 4% BSA or serum just before use.
5. Gently triturate the tissue by drawing and ejecting the entire tissue suspension in and out of the pipet (*see* **Note 19**). To avoid introducing bubbles into the suspension, eject it from the pipet along the wall of the tube at a height above the final liquid level. Repeat until the most of the tissue has dissociated (about 5X, *see* **Note 20**).
6. Add ice cold BSS to bring the final volume to 10–12 mL, mix, and allow the undissociated tissue to settle by gravity, while keeping the tube on ice (5–10 min). Transfer the cell suspension to a new tube and discard the settled chunks.
7. If cells are to be labeled, go to **Subheading 3.4.1.** If cells are to be plated directly, go to **Subheading 3.4.4.**

3.4. Labeling Dissociated Cells

1. Make fresh labeling medium by diluting the dye stock in warm complete medium to a final concentration of 1–25 μM (*see* **Note 21**). Centrifuge the labeling medium to remove undissolved dye and filter sterilize before use.
2. Centrifuge the cell suspension (100–300*g*, 10 min, 4°C) and gently resuspend in 0.5–2 mL labeling medium. Incubate at 37°C for 30–50 min with occasional mixing to resuspend settled cells (*see* **Note 22**).
3. Add cold BSS to bring final volume to 10–12 mL and mix.
4. Below the cell suspension, underlay a 2–2.5 mL cushion of cold, 4% BSA. Place the tip of a Pasteur pipet, filled with BSA solution, at the bottom of the tube and slowly eject the solution so as not to disturb the cushion as it forms (*see* **Note 23**). Centrifuge (150–300*g*, 10 min, 4°C).
5. Resuspend the pellet in complete medium and dilute the cell suspension to the appropriate density for plating (*see* **Note 24**).

3.5. Coculture, Fixation, Counterstaining, and Mounting

1. Carefully aspirate or pipet the medium off the slices and add the cell suspension. Alternatively, you can add the cells in a small volume directly to the medium

already on the slices or after removing a portion of it (*see* **Note 25**). Coculture in a humidified atmosphere at 37°C (*see* **Note 15**).

2. To fix cocultures, slowly pipet warm paraformaldehyde/sucrose under the medium of the cocultures. Alternatively, you can transfer disks directly to warm paraformaldehyde (*see* **Note 26**). Fix at room temperature for 15–30 min. Stop the fix by replacing it with PBS (*see* **Note 27**). Fixed cocultures can be stored at 4°C in PBS with 0.02% sodium azide.
3. To fluorescently counterstain the nuclei of cells in the slice, add bisbenzamide (10 µg/mL final in PBS) to live or fixed cocultures at room temperature and incubate for at least 10 min (*see* **Note 28**).
4. To mount cultures on microscope slides, a "stage" is built up on the slide to hold the coverslip over the coculture without crushing it. Use superglue to affix two 22 × 22 mm coverslips on a slide at least 15 mm apart but so that a third coverslip can bridge across them when placed over the gap. Place a disk on the slide in the space between the two stages and fill the area with mounting medium (*see* **Note 29**). Overlay the third coverslip and aspirate off any excess solution. Seal the edges with nail polish and let dry. Fixed and mounted cocultures can be stored at 4°C.

4. Notes

1. The tissue may sink to the bottom of the dish. To prevent this, pour the agarose in two layers. Pour a bottom layer, and when it begins gelling, but is not yet solidified, pour a top layer and add the tissue. It should sink to the top of the solidifying bottom layer.
2. Any space created by liquid trapped between the tissue surface and the hardened agarose can cause uneven slicing. To prevent this, thoroughly coat the tissue by moving it around in molten agarose before it hardens. Avoid making bubbles.
3. To minimize the deflection of the block each time the blade enters it, make cuts to taper the side of the block that the blade will enter. When placed in the tray, the taper of the block should point toward the blade like an arrow.
4. If the glue does not hold well, there may have been too much liquid on the agarose. Aspirate excess liquid before placing the block on the glue. Dry glue on the tray can be removed with a razor blade.
5. Oil placed on the blades by the manufacturer can be removed with ethanol. If flame-sterilizing the blades, minimize exposure to heat since this can dull and distort the blade edge.
6. At this thickness, slices of embryonic and postnatal mouse forebrain were sturdy enough to be manipulated and not tear, but were thin enough so that flattening and distortions of the slice surface were minimal during culture. Some tissues may allow thinner, or require thicker, sectioning.
7. For embryonic and postnatal mouse forebrain, a relatively slow speed and the maximum vibration provided the best settings to begin cutting the tissue. After most of the slice had been cut, however, the vibration intensity was lowered to minimize damage to the delicate, sectioned tissue. In general, the softer the tissue, the slower the speed and the greater the vibration needed.

8. In most cases, the agarose should easily separate from the slice. If the agarose is still attached, separate it from the slice using a pairs of forceps in each hand. Cutting the agarose with a scalpel blade may help. Although the slice can be mounted onto its support with the agarose attached, be aware that the agarose may detach after time in the culture.
9. Culture inserts can also be coated.
10. This may be done with a forceps or by lifting the slice with a flat spatula. To ensure attachment, use a forceps to lightly press the slice onto the disk in an unimportant region of the tissue. Slices can be similarly placed into inserts held under the fluid level.
11. Holders float in water. The rim of the holder should be on the bottom.
12. The slice should always remain under the surface of the liquid. Use a forceps to lightly press the disk down so that it is flush with the rim of the holder.
13. Surface tension at the air/liquid interface can damage the tissue or remove it from the disk if the disk is simply pulled from the PBS/glucose after mounting the slice.
14. This step removes most of the PBS. It also further pushes the slice onto the disk by the force of the capillary action. Alternatively, the holder and disk can be placed on parafilm and the PBS removed by careful pipeting. Inserts can be treated similarly.
15. Complete medium with DMEM is buffered for an 8% CO_2 atmosphere, but other medium may be different.
16. Large tissue chunks should be cut into smaller pieces. Volumes and additions can be scaled up for large amounts of tissue.
17. The optimal protease concentration and length of incubation may vary for different tissue types.
18. Rotate the pipet within the flame to get an even taper. The optimal bore size will depend upon the tissue type. Too small of a bore will kill cells, whereas a hole too large will not effectively dissociate the tissue. We routinely used bore sizes of 0.2–0.4 mm for perinatal mouse nervous tissue. Instead of flame-polished glass, a disposable pipet tip (e.g., 1 mL blue tips) may work as well.
19. If the entire suspension does not fit into the pipet, divide the suspension among more than one tube into volumes that fit the trituration pipet , and triturate each individually.
20. Repeatedly triturating cells that are already dissociated will lower cell viability. If further dissociation is necessary, allow the undissociated tissue chunks to settle, transfer the dissociated cell suspension to another tube, add more medium or BSS to the chunks, and continue to triturate them. Pool all fractions when done. A longer protease digestion (**Subheading 3.3.2.**) may promote easier dissociation during the trituration step, but can also lower cell viability.
21. The optimum concentration for CellTracker™ dyes will depend upon the cell type and length of culture and should be empirically determined. The optimal labeling concentration should not be exceeded because of the toxic effects of DMSO and high dye concentrations. We used 14 μm CMTMR for labeling primary embryonic neurons.

22. If stringy precipitate is seen floating, it may be DNA released from dead cells. Remove it by adding DNase (*see* **Subheading 2.3.3.** for stock solution) to 0.1–0.2 mg/mL final after tripling the volume with complete medium to dilute the dye. Continue the incubation for 5–15 min, until precipitate dissolves.
23. 4% BSA is more dense than BSS. The cushion efficiently separates the cells from dye, enzymes, and debris left from the labeling and dissociation.
24. We routinely plated $1–2 \times 10^6$ cells/disk in 90 µL on parafilm. This gives 1000–2000 cells/mm^2 in the middle portion of the disk. At the edges, densities are lower due to the shape of the medium bubble on the disk.
25. This step can be done immediately after the slices are cut or after a period of slice preculture and/or treatment. We routinely plated dissociated cells 4–5 h after slicing.
26. Transfer of cocultures through an air/liquid interface will remove cells that are not well attached to the slice.
27. After fixation, the cocultures can better withstand manipulation through an air/liquid interface.
28. If immunostaining is performed, bisbenzamide can be added after this procedure, before mounting the slides. Rinsing with PBS after the bisbenzamide may slightly lower the background fluorescence, however, the stain is effective when slices are mounted directly from the dye solution.
29. Cocultures grown in well inserts can also be mounted this way by cutting the membrane out of the insert with a scalpel blade and mounting the membrane like a disk.

Acknowledgments

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