Time-Lapse Imaging of Fluorescently Labeled Live Cells in the Embryonic Mammalian Forebrain

Stephen C. Noctor

Time-lapse imaging of fluorescently labeled cells in organotypic slice culture provides a unique window through which to view development of the embryonic forebrain. The live imaging approach allows investigators to directly observe and record neurogenic and gliogenic divisions in the developing forebrain, to record the proliferative behavior of embryonic precursor cells, and to track newborn cells as they migrate from the proliferative zones to their final destination. This approach allows for identification and characterization of embryonic precursor cells, permits the physiological and immunohistochemical characterization of the fluorescent cells under study, and presents an opportunity to test hypotheses about mechanisms that guide developmental processes in the forebrain. This article describes a protocol for time-lapse imaging of fluorescently labeled cells in the embryonic forebrain of organotypic slice cultures. The procedure does not require prohibitively expensive on-stage incubation systems, and can yield reliable data from embryonic slices for up to 1 wk. Tracking the movement of labeled cells in slice culture is relatively straightforward in those cases in which there are few labeled cells. Although this approach is time intensive, it provides beautifully detailed images that reveal morphological and functional data from labeled cells over time, and can yield a significant amount of data from each cell.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPE: Please see the end of this article for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Artificial cerebral spinal fluid (aCSF) (1×) <R>

Low-melting-point agarose (4%, prepared in aCSF)

Prepare in a 250-mL media storage glass bottle. Shake vigorously and microwave until the agarose goes into solution. Make sure the solution does not bubble over. Store in a 47°C water bath with the cap loosened slightly so that bubbles can come out of the agar solution. Some laboratories use lower concentrations of agarose for cutting embryonic brains.

Rats, pregnant

See Discussion for information on labeling cells.

Slice culture medium <R>

Sylgard 184 silicone elastomer kit

Equipment

Beakers (100 mL)

Culture incubator preset to 37°C

Culture plates (six-well)

Dissection microscope

Dissection pins

Fine hair brush, small

Fyrite gas analyzer

Gas supply (95% O_2/5% CO_2)

Glass cutter, diamond

Imaging setup

> *An inverted confocal microscope equipped with a long-working-distance lens is used to image fluorescently labeled cells. At desired intervals, culture plates are moved from an incubator to the inverted microscope stage. The well of interest is placed directly over the objective lens and fluorescent cells are imaged through the bottom of the plate. After images have been acquired, the plate is returned to the incubator until the next set of images is taken. Standard water-dipping lenses (used on upright microscopes equipped for electrophysiology) work very well for this system. For example, 40× water lenses have a working distance of >3 mm, a numerical aperture (NA) of up to 0.8, and produce sharp quality images. If water-dipping lenses are not available, then long-working-distance 10× or 20× air lenses can also produce quality images if the fluorescently labeled cells are sufficiently bright. For further information on the imaging setup and choosing an objective, see Discussion.*

Krazy Glue

Microsurgical tools: angled microscissors, bone elevator, fine curved forceps, fine forceps, medium scissors (4.5 in.), medium-toothed forceps (4.5 in.), scalpel blade, scoopula

Millicell cell culture inserts, 30 mm, hydrophilic PTFE (polytetrafluoroethylene), 0.4 μm (Millipore, PICM03050)

Osmometer

Parafilm

Pasteur pipettes, glass

> *Using Pasteur pipettes to transfer brains and slices minimizes contact with brain tissue and therefore minimizes potential damage from excessive handling. Pasteur pipettes can be sterilized by soaking in 70% alcohol.*

Petri dishes, plastic (35, 50, and 90 mm)

Razor blades

Sterile cabinet

Stone bubblers

Suction bulbs, rubber

Vibratome

Water bath preset to 47°C

METHOD

Preparation of Brain Dissection Dishes

1. Mix Sylgard kit solutions following the manufacturer's instructions.

2. Carefully fill the bottom half of a 50-mm plastic Petri dish with Sylgard to about one-third full. Let the Sylgard set overnight.

   > *Be careful not to create bubbles when pouring Sylgard into the dish; the solution should be translucent.*

3. Glue the 50-mm Petri dish with Sylgard into the bottom half of a 90-mm Petri dish.

4. When the glue is dry, fill the area between the 50-mm dish and the inner portion of the 90-mm dish with aCSF.

5. Place the brain dissection dish in a −20°C freezer.

Preparation for Surgery and Sectioning

6. Prepare for surgery.

 i. Set out tools to retrieve embryos:
- medium scissors (4.5 in.)
- medium-toothed forceps (4.5 in.) to open the abdomen
- fine curved forceps for removing embryos from uterine horn
- scoopula for transferring embryos
- several 50-mm Petri dishes filled with ice-chilled aCSF

 ii. Set out tools to remove brains:
- two fine forceps
- angled microscissors
- bone elevator
- glass Pasteur pipettes with rubber suction bulbs

  Cut the small end of a pipette off with a diamond cutter. Place a rubber suction bulb over the cut end of the pipette. Put two Pasteur pipettes in the sterile cabinet for later use.

 iii. Retrieve a brain dissection dish with frozen aCSF from the −20°C freezer (from Step 5). Place it on the stage of the dissection microscope.

 iv. Fill the center of the brain dissection dish with ice-chilled aCSF.

 v. Insert four dissection pins in the Sylgard.

7. Prepare the vibratome.

 i. Place the bottom half of a 35-mm Petri dish in each of two 100-mL glass beakers. Fill the beakers with aCSF. Place a stone bubbler on the bottom of the beaker, outside the Petri dish. Bubble the solution with 95% O_2/5% CO_2.

  Embryonic slices collected from the vibratome will be kept in these beakers until sectioning is complete.

 ii. Pour aCSF into two additional 100-mL glass beakers. Place on ice.

  These beakers will be used to fill the vibratome cutting chamber when sectioning begins.

 iii. Place ice into the cooling chamber that surrounds the cutting chamber of the vibratome (unless using a self-cooling vibratome).

 iv. Prepare blades for the vibratome.

 v. Set out tools for slice handling:
- two glass pipettes and rubber bulbs for transferring slices
- one fine-hair brush for manipulating slices
- pair of angled microscissors for cutting meninges to free slices from brain if necessary
- scalpel blade to trim agar

8. In a sterile culture cabinet, fill two 50-mm Petri dishes with slice culture medium. Cover the dishes and place them in an incubator preset to 37°C and 5% CO_2.

  The temperature and CO_2 level in the incubator should be checked regularly and calibrated using an accurate thermometer and a Fyrite CO_2 gas analyzer.

9. Prepare the slice culture plates.

   i. In a sterile culture cabinet, fill each well of a six-well culture plate with 1.5 mL of culture medium.

   ii. Place a Millicell insert in each well.

   iii. Fill the inside of each insert with ~2 mL culture medium (i.e., enough so the tip of a Pasteur pipette will be completely submerged in the medium when transferring slices).

   iv. Place the six-well plate in the incubator so that the pH of the culture media equilibrates in the 5% CO_2 incubator atmosphere.

Embryo Removal

10. Before starting surgery, make sure the pregnant animal is fully anesthetized and completely insensitive to pain following IACUC (Institutional Animal Care and Use Committee)-approved protocols.

11. Cut open the abdomen by making an incision in the skin, followed by an incision through the abdominal muscles to expose the uterine horns.

12. Remove the uterine horns one at a time.

   This can be performed very gently using fingers, being especially careful not to damage the embryos.

13. Once exposed, gently grasp the far end of one uterine horn with small curved forceps. Make an incision through the uterine wall using medium-sized scissors to expose the amniotic sac of one embryo.

14. Cut through the amniotic sac. Gently remove the embryo. Cut the umbilical cord. Transfer the embryo to a small Petri dish containing ice-chilled aCSF.

15. Kill the embryo by decapitation. Use the scoopula to transfer the head to the brain dissection dish on the stage of the dissection microscope.

Brain Removal and Embedding

16. Pin the embryonic head to the dissection dish with the nose facing away from you.

17. Starting at the base of the head, reflect the skin laterally using fine forceps.

   This can be performed very quickly with the forceps; scissors are not needed.

18. Remove the brain by one of the two following methods.

   For young embryos

   i. Make a lateral incision over the cerebellum, being careful not to harm the cerebral cortex.

   ii. Use fine angled microscissors to make a central cut through the skull above the longitudinal fissure, being careful not to damage the cortex with the tips of the scissors.

   iii. Reflect the skull and remove the brain from the cranial cavity with the bone elevator.

   For older embryos

   Use this method for removing brains from older embryos (E18 rats and older) and postnatal animals when the skull is harder (Fig. 1).

   i. Use angled microscissors to cut from the rear through the foramen magnum and along a central line above the longitudinal fissure to a point anterior of the bregma. Be careful not to damage the cerebral cortex with the tip of the scissors.

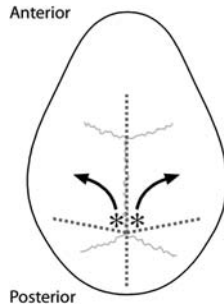   ii. Make cuts starting from λ at the midline and progressing laterally.

FIGURE 1. Removing embryonic brains from the cranial cavity. Illustration of the placement of three incisions (dotted lines) for the removal of older embryonic rat brains (E18). The first incision starts at the foramen magnum and continues anteriorly above the midline longitudinal fissure following the dotted line. Be careful not to damage the cerebral cortex with the tip of the scissors. Next, make lateral cuts starting at the midline from λ (posterior sutures shown in gray) and progressing laterally (along the dotted lines). The corners of the skull tissue (asterisks) are grasped with fine forceps and the skull is gently peeled away in an anterolateral direction (arrows).

   iii. Grasp the corners of the skull tissue and gently peel the skull away in an anterolateral direction.

   iv. Gently remove the brain from the cranial cavity with the bone elevator.

19. Embed the brains in agar.

    This procedure improves agar embedding.

   i. Pour 4% agarose into two 35-mm Petri dishes.

   ii. Using a Pasteur pipette, immediately transfer the embryonic brains from the brain dissection dish to the first agarose-filled Petri dish.

    Late embryonic brains can be too large to fit in the pipette but can be transferred using a scoopula.

   iii. Gently swirl the brain around in the agarose to dilute any aCSF that was transferred along with the brain.

   iv. Transfer the brain to the second agarose-filled Petri dish.

    You may need to use a fresh Pasteur pipette, so be sure to have a second one available.

   v. Quickly orient the brain appropriately for coronal sectioning (or other orientation as needed).

   vi. Place the Petri dish on ice. Cover it with the dish lid and cover with ice. Be sure that the agarose solution does not touch the inside of the Petri dish lid.

    The agarose will harden in several minutes, but the brains can remain on ice while several more embryonic brains are quickly removed and embedded. Postnatal and adult brain tissue is much more sensitive to anaerobic conditions and must be sectioned immediately.

Vibratome Sectioning

If the laboratory has sufficient space, vibratome sectioning can be performed in a sterile cabinet, but this is not necessary.

20. Once the brains have been embedded and the agar has hardened, use a scalpel blade to cut a block of agar containing the brain.

21. Cut the block in a pyramidal shape such that the base of the block is wider than the top.
    This increases the stability of the brain during vibratome sectioning.

22. Gently dab the bottom of the agar block with a laboratory tissue to remove excess moisture.

23. Place one or two drops of glue on the vibratome cutting block. Place the agar block on the glue.

    For coronal sectioning, the olfactory bulbs should be facing up, the base of the brain oriented toward the blade, and the cerebral cortex facing away from the blade.

24. Immediately pour ice-chilled aCSF (from Step 7.ii) in the cutting chamber to completely cover the brain.

    If there is sufficient room in the cutting chamber, include a stone bubbler to provide 95% O_2/5% CO_2 for the brain and slices.

25. Section the agar-embedded brains coronally at ∼375–400 μm.

    Slices can be cut thinner on the vibratome at later stages of embryonic development. However, ∼400 μm is thin enough for the tissue to receive nutrients from the culture medium, while keeping a larger portion of tissue intact so that cellular connections and associations remain. Brains obtained during early stages of cortical development (e.g., E12 rat) must be cut thinner because the cortical wall can be <100-μm thick and the cortical tissue will not be stable when plated on the culture well insert.

    See Troubleshooting.

26. Transfer each slice using a Pasteur pipette to a beaker containing room temperature aCSF aerated with a stone bubbler (from Step 7.i).

    It might be necessary to trim slices down with a scalpel blade so they can be transferred with a Pasteur pipette. Some of the slices might not remain embedded in the agar, but this is fine.

Plating Slices in Culture Wells

27. Retrieve the 50-mm Petri dishes containing media from the incubator (from Step 8). Place them next to the beaker containing the slices.

28. Transfer slices from the beaker to the Petri dishes using a Pasteur pipette.

29. Place the Petri dishes containing the slices into a sterile cabinet.

30. Working in the sterile cabinet, transfer the slices from the Petri dishes to the slice culture inserts in the six-well culture plates (from Step 9).

    Proper orientation of the slice on the insert can increase the maximum time possible for time-lapse imaging. Investigators who focus on neuronal migration might not need to concern themselves with this issue. Ideal placement of slices on inserts for long time-lapse imaging experiments should take into account the curvature of the ventricular surface of the embryonic brain. This curvature is retained in coronal sections after vibratome sectioning (see Fig. 2A,B). The ventricular zone surface slope is most apparent in anterior and posterior coronal sections, but is present in all coronal sections, and the process of cutting live unfixed embryonic tissue can exaggerate the slope.

    i. Depending on experimental conditions, place three or four small slices in each well.

       When depositing slices, do not expel media from the pipette. Instead, touch the pipette to the surface of the media in the well and allow the slices to gently settle down onto the insert.
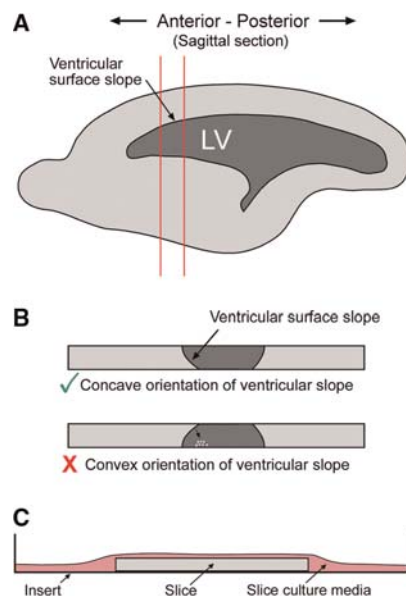
       See Troubleshooting.

FIGURE 2. The slope of the ventricle, and how this can impact time-lapse imaging of the embryonic forebrain. (A) A drawing of an E18 embryonic brain shown in a sagittal section. The lateral ventricle (LV, dark gray) is curved. Coronal sections cut through the brain will retain the sloped surface of the ventricle. The two red lines approximate the thickness of a coronal slice. (B) Coronal slices can be placed on the insert with the ventricular slope in a concave (upper panel) or convex (lower panel) orientation. When slices are placed in the convex orientation, the ventricular slope falls down and cells crumble onto the surface of the insert, making it more difficult to identify the surface of the ventricle. (C) Illustration of the appropriate level of culture media (red) for organotypic slice cultures. The level should be adjusted so that it just barely covers the top of the slice. When looking down on the slice, one should see a deflection in the media just above the slice.

ii. To ensure proper orientation of the slice, place each slice on an upright microscope equipped with a water-dipping lens and fitted with a slice chamber stage perfused with aerated aCSF.

Placing the slice on the culture insert in a concave orientation will allow the researcher to more easily identify the ventricular surface for a longer period of time and will prevent ventricular zone cells from crumbling into the space of the former ventricle. Time-lapse experiments that visualize ventricular surface mitoses or precursor cells that maintain contact with the ventricular surface will benefit the most from concave placement of the slice (see Fig. 2B).

iii. Visualize the slice to determine the orientation of the ventricular surface. Retrieve the slice with a Pasteur pipette. Place on the slice culture insert with the desired orientation.

iv. Adjust the position of the slices on the insert using the pipette tip to gently push against the agar or the base of the brain.

31. Remove excess media so that the slice is just covered with media (see Fig. 2C).

When viewed from above a slight deflection of light on the media should be visible where it covers the slice.

32. Place the six-well culture plate in the incubator. After 30 min, check the level of the media.

It often is necessary to remove more media from inside the cell insert to achieve an appropriate level. Do not move the slices after this point; they will remain on the inserts in the culture plates for the duration of the imaging experiment.

33. For experiments that last >3 d, exchange one-half of the media in the well with fresh media that has been prewarmed and equilibrated to the incubator atmosphere.

Data Acquisition and Processing

See Figure 3.

34. Retrieve the culture plate from the incubator and place it on the stage.

35. If using a water-dipping lens, place a drop of water on the lens. Place the culture well of interest on the water drop (see arrow in Fig. 3C).

Take care not to drip water over sensitive parts of the microscope, and that water does not drip down the side of the lens to enter the lens turret. Do not use salt solutions (e.g., PBS [phosphate-buffered saline] or aCSF) on inverted water lenses because this increases the difficulty of cleanup.

36. Locate and image a region of interest.

i. If a small population of cells has been labeled (e.g., using a retrovirus), scan through the slices using epifluorescence to find target cells of interest.

ii. If a large population of cells has been labeled (e.g., using electroporation), scan through the slices to find a section that includes the population of interest.

See Troubleshooting.

Assembling Images into a Time-Lapse Sequence

If optical planes that do not contain cellular data are included in the z-stack when making a projection, the "empty" data averaged into the projection results in a dimmer image of the target cell. Excluding such empty optical planes produces projection images that better reveal the morphology of target cells. Most confocal microscope software programs include a basic feature to allow this operation. Also, even if the six-well culture plate is placed on the microscope stage in the same position to obtain images for each time point, some movement of the slice invariably occurs. It is therefore necessary to properly register each image relative to other images that were taken during the time-lapse experiment to visualize true cellular movements.

37. Save the z-stack images taken at each time point as flattened projection images.

38. Prepare the z-stack projection in such a way as to maximize brightness of the target cell (e.g., by excluding optical planes that do not contain the cell of interest when making the projection).
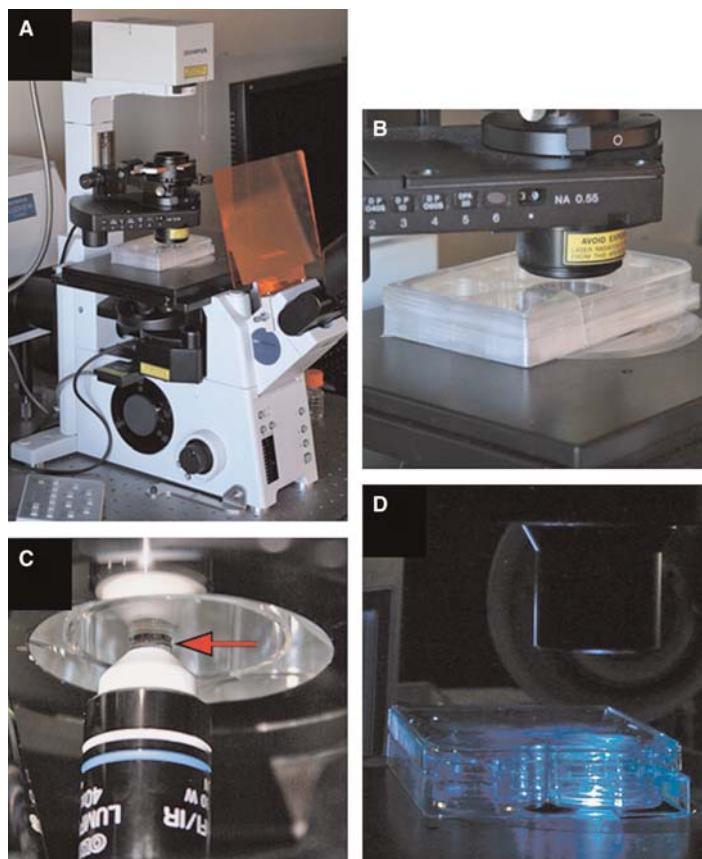
FIGURE 3. Photographs of time-lapse imaging using a six-well culture plate and an inverted confocal microscope. (*A*) Placement of the culture plate on the stage of an inverted confocal microscope. (*B*) The condenser can be kept in its proper position, which will be necessary for collection of transmitted light images. The edges of the six-well culture plate should be carefully wrapped with a thin strip of Parafilm each time it is removed from the incubator to reduce the likelihood of contamination. (*C*) A water-immersion lens beneath the stage of an inverted microscope. A drop of water is placed on the lens (red arrow) and the culture plate is placed on the stage in contact with the water. (*D*) Operation of the laser scanning confocal microscope using the 488 line to image eGFP-labeled cells in the cultured slices.

39. Register individual time-point images with one another using a software program such as Adobe Photoshop.

    i. Open the first image (e.g., $t = 0$) in Photoshop. Save the file as a Photoshop "psd" file type that has layers.

       Save the file with a unique name and the "psd" file extension so that the original data are not overwritten.

    ii. Open the second image (e.g., $t = 5$ min). Drag it onto the $t = 0$ file as a new layer.

    iii. Repeat Step 39.ii for the projection images for each subsequent time point (i.e., $t = 0$ as the bottom layer; the $t = 5$ min image situated above $t = 0$ in the layers panel of Photoshop; $t = 10$ min above the $t = 5$ min image, etc.).

40. Orient the time-point images in each layer so that they are properly registered with one another.

    The investigator will have to choose appropriate registration points for the region under study. The cortical striatal junction can often serve as one registration point. In some cases, certain cell types such as radial glial cells can serve as registration points. For example, the ventricular and the pial contacting processes of radial glial cells do not move laterally and can therefore be used as registration points if they are present in the slice. When tracking the movements of cells migrating freely in the slice, one can use the transmitted light images that have been taken at each time
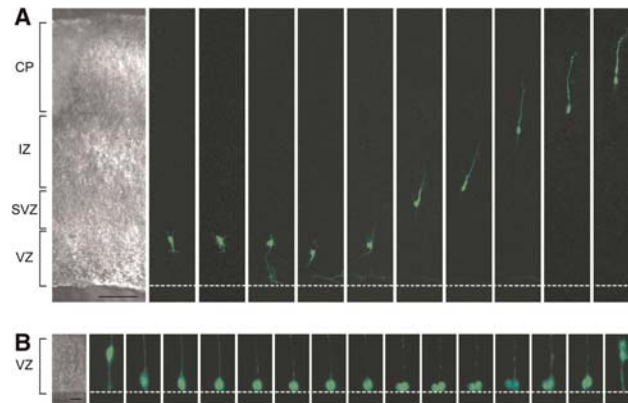
FIGURE 4. Time-lapse imaging of retrovirally labeled cells in the embryonic cerebral cortex using long or short time-lapse intervals. Fluorescently labeled cells can be imaged a limited number of times. Longer intervals between imaging allow cell movements to be tracked over a longer period (*A*), whereas shorter intervals between imaging allow the behavior of a given cell to be recorded with greater temporal resolution (*B*). (*A*) Time-lapse sequence recorded >96 h showing the migration of a postmitotic neuron in the embryonic forebrain from the proliferative zone to the cortical plate. The panel on the left is a transmitted light image of the cultured slice taken during time-lapse imaging. (Adapted, with permission, from Noctor et al. 2004.) (*B*) Time-lapse sequence recorded >8 h showing the division of a radial glial cell in the embryonic forebrain. The panel on the left is a transmitted light image taken at the start of the experiment. During the G-2 phase, the radial glial cell body drops to the surface of the ventricle (dotted white line). The cell undergoes division at the ventricle, and the two nascent daughter cells move away from the ventricle. Scale bars, 100 µm (*A*); 10 µm (*B*). (Adapted, with permission, from Noctor et al. 2008.)

> point to place the position of the cell in the slice at one time point in relation to its position at a previous time point.

i. Turn off all layers except the bottom two ($t = 0$ and $t = 5$) by clicking the eye-shaped icon to the left of each layer in the layers window panel.

ii. Reduce the opacity of the $t = 5$ layer, allowing the $t = 0$ layer beneath to be seen.

> *This option is also found in the layers window panel. The default level of opacity for each layer is 100%.*

iii. Rotate and/or move the $t = 5$ layer in the x–y plane so that the $t = 0$ and $t = 5$ layers are perfectly registered with one another.

iv. Return the $t = 5$ opacity to 100%.

v. Turn on the layer $t = 10$ visibility by clicking the eye icon.

vi. Reduce the layer $t = 10$ opacity. Register the $t = 10$ layer with the $t = 5$ layer (as described in Step 40.iii).

vii. Repeat Steps 40.iv–40.vi for each successive layer in the file until finished.

41. Adjust the gain and contrast for each layer so that each layer is similar to all other layers. Adjusting contrast/brightness allows the reader to focus on cellular movements rather than being distracted by changes in the level of light for each frame in the time lapse.

42. After each image in the layered Photoshop file has been properly oriented, save the separate layers as individual tiff or jpeg image files.

> *These files can be assembled into a time-lapse sequence, or saved as a QuickTime video with simple user-friendly programs such as Apple's iPhoto. More sophisticated imaging programs can also be useful because they provide more control over frame speed. For examples of time-lapse sequences, see Figure 4. For examples of time-lapse QuickTime movies, see Noctor et al. (2004, 2008).*

TROUBLESHOOTING

Problem (Step 25): Vibratome slices remain attached to the brain by the pial mater.
Solution: Cut the attachment with a pair of microscissors to free the slice.

Problem (Step 30.i): The quality of the embryonic slices cut on the vibratome is not sufficient.

Solution: It is possible the brain is not embedded properly in the agar. Try adjusting the speed and amplitude of the vibratome blade. Also, blades can become dull over time. The blade should be changed after cutting one or two brains.

Problem (Step 36): There is bacterial or fungal contamination of the organotypic cultures detected by growth in the culture well.

Solution: The closed six-well culture plate and the inclusion of antibiotics in the culture media will generally keep the slices free of most bacterial contamination for the duration of time-lapse imaging. However, spores can enter the culture plate and quickly ruin experiments. Consider the following:

1. Keep experimental rooms free of food, particularly bread.
2. Wear gloves when handling the culture plates, and wrap the plate with a thin strip of Parafilm each time it is removed from the incubator (see Fig. 3B). This must be performed carefully to avoid jostling the slices in the plate. Be sure to remove the strip of Parafilm before replacing the six-well culture plate in the incubator so that the slices will be exposed to the appropriate gases.

Problem (Step 36): There is bleaching of fluorescently labeled cells.

Solution: To minimize excessive exposure to energy, carefully balance laser intensity, laser scanning speed, Kalman averaging, and image size to achieve the best quality image and the least exposure to higher laser intensities. The laser power should be set manually to avoid unnecessary overexposure and oversaturation of the image. In addition, use defined regions of interest to further reduce exposure.

Problem (Step 36): Cells appear to drift in the x–y plane in a z-stack when images are acquired with a water-immersion lens.

Solution: Use a lens heater to reduce heat drain from the culture plate into the water drop/lens that can produce real or apparent drift of the cells/tissue in each image of the z-stack.

DISCUSSION

Successful time-lapse imaging of fluorescently labeled cells in organotypic slice cultures depends on several important factors, including the labeling method itself and the preparation of slices from the embryonic forebrain.

Labeling Embryonic Cells

There are two basic approaches one can use for time-lapse experiments: Label a few cells to obtain information about the behavior of single cells over time, or label a large number of cells to obtain information about the behavior of a large population of cells over time. Both methods provide important detailed information about developmental processes in the embryonic forebrain. Each procedure has pros and cons; investigators must choose an appropriate cell-labeling technique to suit their goals. Labeling multiple embryonic brain cells in utero (e.g., by electroporation; see Saito and Nakatsuji 2001) yields important data about large populations, but the technique produces dense fields of similarly labeled cells, making it difficult to evaluate the morphology, migration, or proliferation of a given cell. Researchers may also take advantage of transgenic animals that express fluorescent reporter proteins in the developing forebrain under the control of cell type-specific promoters. Transgenic animals carry the advantages of label specificity and of not requiring surgeries or procedures to label cells. However, depending on the cell type under study, transgenics could also produce fluorescent labeling in dense fields of similarly labeled cells. On the other hand, low-titer viral injections (e.g., Noctor et al. 2001) label fewer cells, making it possible to obtain reliable data on the morphology and behavior of

single cells. However, this approach requires multiple experiments to achieve a sufficient number of data points for the results to be significant.

Imaging Setup

Slice cultures from embryonic cortex are maintained in six-well culture plates throughout the duration of the experiment. Multiple slices can be plated in each well; a single six-well plate can hold 15 to 20 slices. Data can be collected from multiple slices in one imaging session, decreasing the need for replicating experiments. At desired intervals, culture plates are moved from an incubator to the inverted microscope stage and then returned to the incubator after images have been acquired, where they remain until the next set of images is taken. This approach allows the researcher to image labeled cells in embryonic cortical tissue on a confocal microscope without an onstage incubation system. Furthermore, it allows other investigators to use the setup between imaging sessions. Investigators who plan to gather a large amount of time-lapse data might find it helpful to have the incubator next to or near the microscope to minimize the distance that the culture plate must be transported.

Choosing an Objective

For excitation of fluorescent molecules to take place, light must travel through the bottom of the plastic plate and through the well insert to reach the labeled cells within the slice. Although the plastic and the insert material are sufficiently translucent to allow imaging of bright cells, long-working-distance lenses nevertheless are required for obtaining quality images. Standard water-dipping lenses work well. Using water lenses on an inverted microscope has the disadvantage that the water will evaporate over time. Oil lenses do not have this problem, but generally do not work for this method because of their short working distance. Fortunately, images are obtained within a matter of minutes and water evaporation should not be a major concern.

Time-Lapse Imaging

When imaging enhanced green fluorescent protein (eGFP)-labeled cells over several days, one can obtain a limited number of laser-scanning confocal images from a given cell, perhaps on the order of 80–100 z-stacks at a maximum (this number is greater if using a multiphoton system). There are therefore several approaches to the time-lapse experiment. A shorter experiment with short time-lapse intervals yields high temporal resolution details about cellular migration/proliferation. On the other hand, a longer experiment using longer time-lapse intervals reveals more details about migratory trajectories. A combination approach is also possible, using high temporal resolution to capture proliferative events followed by low temporal resolution to capture cellular migration. In general, when using slices prepared from embryonic slices in which the level of proliferation is high and in which there is substantial cellular migration, it might be necessary to check the cells every 2 h using epifluorescence (even when not capturing data) to update maps and keep track of the cell's position in the slice. Finally, the investigator should take advantage of imaging program options, such as user-defined regions of interest. This will restrict laser exposure to the cells of interest and reduce unwanted excitation of tissue not pertinent to the experiment.

During long-term time-lapse movies, the shape and appearance of the slice can change as a result of slice growth (produced by proliferation and migration), and from the tendency of slices to flatten a bit over time in culture. To ensure the same cells can be found for imaging at subsequent time points, it is helpful to draw a simple map depicting the shape of the slice and position of each target cell within the slice. Also, take a low-power (10×) fluorescence image of the cell using rapid scanning speed and no averaging, and a transmitted light image at 10× using a different laser line. For example, if imaging eGFP-labeled cells, use the 650-nm laser line to take the transmitted light images to avoid unnecessary overexcitation of the eGFP cells. The 10× eGFP and transmitted laser images can be superimposed to create a record of cell position in the slice for each time point. The drawings and eGFP/transmitted

light images will make it easier to locate the cells at later time points and will also help track the position of labeled cells in the slice.

When imaging samples in which only a few labeled cells are found in each slice, it may be necessary to scan through several dozen slices to encounter enough cells of interest (e.g., neurons, ventricular zone and subventricular zone precursor cells). Once the target cells are identified, acquire a z-stack that includes all visible processes of the labeled cells. This must be repeated for each slice that contains target cells of interest. Thus, images will be obtained from multiple slices throughout the experiment. Although this makes for some work, it increases the data yield.

RECIPES

Slice Culture Medium

66 mL Basal medium Eagle
25 mL Hanks' balanced salt solution
5 mL fetal bovine serum
1 mL N-2 supplement (100×)
1 mL penicillin–streptomycin–glutamine (100×)
2 mL D-(+)-glucose solution (33%) (Sigma-Aldrich)

Purchase components other than glucose from GIBCO/Invitrogen. Prepare slice culture medium fresh for each experiment. Filter through a 0.22-µm filter and store at 37°C.

aCSF (1×)

100 mL aCSF stock solution A (10×) <R>
100 mL aCSF stock solution B (10×) <R>
4.5 g glucose
600 mL H_2O

Adjust the final volume to 1 L with H_2O. Mix well and bubble with 95% O_2/5% CO_2. Check an aliquot of the 1× aCSF after making each new batch of stock solutions to confirm that the aCSF has a pH of 7.4 and an osmolarity of 310 mOsm when bubbled at room temperature with 95% O_2/5% CO_2.

ACKNOWLEDGMENTS

REFERENCES

Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstein AR. 2001. Neurons derived from radial glial cells establish radial units in neocortex. *Nature* **409:** 714–720.

Noctor SC, Martínez-Cerdeño V, Ivic L, Kriegstein AR. 2004. Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat Neurosci* **7:** 136–144.

Noctor SC, Martínez-Cerdeño V, Kriegstein AR. 2008. Distinct behaviors of neural stem and progenitor cells underlie cortical neurogenesis. *J Comp Neurol* **508:** 28–44.

Saito T, Nakatsuji N. 2001. Efficient gene transfer into the embryonic mouse brain using in vivo electroporation. *Dev Biol* **240:** 237–246.