Protocol

Brain Tumor Imaging: Live Imaging of Glioma by Two-Photon Microscopy

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Glioblastoma is a highly invasive and aggressive brain tumor that is very difficult to treat. The rat glioma cell line CNS-1 is a widely used, well-characterized model of infiltrative glioma. We have used CNS-1 to study tumors that initiate within the brain parenchyma. The CNS-1 cells were stably transfected with the yellow fluorescent protein (YFP) variant Venus to enable standard one-photon and two-photon imaging. In this protocol, we describe how to prepare a cranial window and how to inject the tumor cells into the cerebral cortex at a depth suitable for two-photon imaging. Imaging can begin 24 h after implantation of the cells. Two-photon imaging uses a long excitation wavelength (920 nm); the emission spectrum is the same for the fluorophore as for standard one-photon imaging (emission maximum = 528 nm). Two-photon microscopy permits tissue imaging to depths of 500 μm with three-dimensional (3D) high resolution and minimal photodamage to surrounding tissues. Multiple imaging sessions can be conducted over weeks in the same animal, depending on how long the cranial window remains clear and how quickly the tumor grows. Using this technique, the kinetics of tumor growth and invasion into the surrounding brain parenchyma can be measured in the same animal. This model can be used for determining the molecular and cellular players in brain tumor growth and invasion and for testing potential drug therapies to prevent brain metastasis.

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 material used in this protocol.

Reagents

Avertin
Buprenorphine
CNS-1 cells
Cyanoacrylate glue
Immunocompromised nude (nu/nu) mice
Paraformaldehyde (4%)
Phosphate-buffered saline (pH 7.4)
Prolong Gold Antifade Reagent with DAPI (Invitrogen P36931)
Saline (sterile)

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Sucrose (10% and 30%)

Tissue culture reagents (Invitrogen) (keep all solutions sterile):

- Dulbecco's phosphate-buffered saline (PBS) containing 0.5% FBS (both at room temperature and ice-cold)
- Fetal calf serum (FCS)
- RPMI medium containing 10% FBS
- RPMI medium containing 0.1 mg/mL Hygromycin B and 10% fetal bovine serum (FBS)
- Trypsin (0.25% with EDTA)

Equipment

Cell counter

Centrifuge

Cover glass (7-mm-diameter) (Fisher)

Dental cement

Drill (high-speed, with a sterile burr tip)

Epifluorescence microscope (Olympus) equipped with SPOT Pursuit digital camera and attached computer equipped with the SPOT Advanced software package (see Step 31)

To produce epifluorescence images of excised brain sections, an Olympus BX51 epifluorescence microscope, equipped with filters for yellow fluorescent protein (YFP) (emission maximum = 528 nm) and DAPI (emission maximum = 461 nm), a Spot Pursuit camera, and SPOT Advanced software, was used. ImageJ software (download from: <http://rsb.info.nih.gov/ij/download.html>) was used for analysis of fluorescence images.

Gelfoam

Hamilton syringe (low-volume) and 31-gauge needle

Heat pads

Incubator set at 37°C

Malis dissector

Microslides and coverslips

Microtome

Stainless steel bar with tapped screw holes (see Step 21)

Stereotactic frame with earbars and bite bars

Surgical instruments (sterile)

Two-photon imaging equipment (see Step 25)

For two-photon microscopy, a mode-locked, tunable Ti:sapphire laser (MaiTai; Spectra Physics) provides 920-nm excitation light. Beam scanning is accomplished by a Fluoview 3 scanner (Olympus) via an XLUMPlan FI 20×/0.95 NA objective water-immersion lens. Fluorescence passes through a 690-nm short-pass dichroic mirror and a 550DF150 emission filter (Chroma) and is detected by external (i.e., non-descanned) HC125-02 photomultiplier tubes (Hamamatsu). Our images were scanned at the slow setting with 1024 × 1024-pixel images in the x-, y-, and z-directions with a step size 3–5 μm in the z-direction.

METHOD

Preparation of Cells for Injection

1. Maintain CNS-1 cells in RPMI medium containing 0.1 mg/mL Hygromycin B and 10% fetal bovine serum.
2. When cells reach 70%–80% confluence, trypsinize them with 0.25% trypsin/EDTA for 3 min at 37°C.

3. Stop the trypsin reaction by adding RPMI containing 10% FBS.
4. Centrifuge the cells at 200g for 5 min at 4°C.
5. Resuspend the cells in 5 mL of Dulbecco's PBS containing 0.5% FBS.
6. Centrifuge the cells at 200g for 5 min at 4°C.
7. Resuspend in 1 mL of ice-cold Dulbecco's PBS containing 0.5% FCS.
8. Count the cells and adjust volume to yield a concentration of 10^7 cells/mL.
9. Place cells on ice until injection.

Preparation of a Cranial Window

The duration of the entire surgical procedure (Steps 10–21), including anesthesia and glue-drying time, is ~1 h.

10. Pretreat immunocompromised nude (nu/nu) mice (2–6 months of age) with 0.5 mg/kg buprenorphine subcutaneously, then anesthetize with 200 mg/kg Avertin (intraperitoneally) and place on a heat pad; stabilize the head in a stereotactic frame using a bite bar and earbars.
11. Make a longitudinal incision of the skin between the occiput and the forehead.
12. Remove a disk of skin from the top of the skull, and scrape off the underlying periosteum to the temporal crests.
13. Draw a 6-mm circle over the frontal and parietal regions of the skull.
14. Using a sterile high-speed drill with a burr tip, make a groove on the margin of the drawn circle.
15. Use repetitive gentle drilling to deepen the groove until the bone flap becomes loose.
16. Using a Malis dissector, separate the bone flap from the underlying dura mater and remove the bone. Place Gelfoam on the cut edge if needed.
17. Rinse the dura mater continuously with sterile saline.

Continuous rinsing minimizes window dehydration and heat buildup that can damage the preparation.

Tumor Cell Injection into the Brain

18. Using a special low-volume Hamilton syringe, aseptically inject a 1- μ L volume containing 1×10^4 cells into the center of the craniotomy.

For this injection, the needle is placed 1.5 mm below the surface and then retracted back to 1.0 mm to create a small space for the cells. Over a period of 5 min, the cells are slowly injected into the frontal cortex of the brain as the needle is gradually retracted.

19. Dip the 7-mm round cover glass into sterile saline, place over the craniotomy, and secure to the surrounding bone with cyanoacrylate glue.
20. Build up a shallow well with glue around the edge to hold water for imaging using the water-immersion lens (see Fig. 1).
21. Mount a stainless steel bar with tapped screw holes onto the front of the skull using dental cement for head stabilization during imaging.
22. Maintain the animals until ready for imaging.

See Troubleshooting.

Two-Photon Imaging

Imaging can begin as early as 1 d after injection of the CNS-1 cells.

23. Anesthetize the mouse with 200 mg/kg Avertin intraperitoneally. Place the anesthetized mouse on a heat pad.
24. To minimize motion artifacts, stabilize the head by attaching the head bar to a frame.

FIGURE 1. Chronic cranial window for imaging brain tumors. The diagram shows a cranial window in cross section. A craniotomy is performed and the dura (gray) is broken in a small peripheral area to inject brain cancer cells (green) into the cerebral cortex. The injection pipette is withdrawn, the craniotomy is filled with sterile saline, and a small coverslip is secured in place using dental cement to allow chronic optical access to the brain.

25. Use the parameters provided for two-photon imaging. Take low-magnitude (1× digital zoom) images to reveal the organization of the tumor mass. Use the patterns of blood vessels as landmarks for alignment purposes between imaging sessions. Take higher resolution (5×–10× digital zoom) images to reveal the morphology and dynamics of individual cells within the tumor and at the tumor margins. See Figure 2 for maximum intensity projections of two-photon images of CNS-1 Venus cells.

Imaging sessions can be repeated periodically until the window becomes cloudy or the tumor becomes too large.

Postimaging Analysis

26. After the last imaging session, perfuse the mouse intracardially with PBS (pH 7.4) followed by 4% paraformaldehyde for fixation.
27. Remove the brain from the skull and postfix overnight in 4% paraformaldehyde.
28. Cryoprotect in 10% sucrose for 24 h, followed by 30% sucrose for 24 h.
29. Cut 50-μm frozen sections from the area of the injection.
30. Mount the sections on microslides, counterstain with Prolong Gold Antifade with DAPI, and coverslip.

FIGURE 2. Imaging gliomas in vivo. (A) Two-photon image of CNS-1 Venus-expressing cells forming a tumor in the brain imaged under a cranial window. The image was taken 5 d after intracranial cell injection and window implantation and is 800 μm across. (B) Higher-resolution image showing individual CNS-1 cells in the brain. Notice that some cells have many small processes that invade the brain (arrow), whereas other cells wrap around blood vessels and have a doughnut-like shape (arrowhead). Scale bars, 100 μm (A); 20 μm (B).

31. Examine sections under epifluorescence with appropriate filters for Venus and DAPI. Take photomicrographs of the tumor with a SPOT Pursuit digital camera in conjunction with the SPOT Advanced software package.
32. Analyze the size and spread of the tumor and the number of fluorescent foci per section using NIH Image J software and compare with the results obtained with two-photon microscopy.

TROUBLESHOOTING

Problem (Step 22): Rapid tumor growth is observed.

Solution: As CNS-1 tumors enlarge, brain edema and neurological damage may lead to neurological impairments such as seizures. Animals should be closely monitored. Kill animals that display neurological impairment. Inject fewer cells if impairment occurs too rapidly.

Problem (Step 22): A cloudy cranial window results in poor imaging.

Solution: Inflammation and bleeding may result in a cloudy window that is unsuitable for imaging. Maintain sterility and minimize damage to the brain and brain dura during preparation of the cranial window. Observe the window daily. Cloudy windows may clear up on their own in a day or two. If the damage is too severe, the window will not clear up, and the animal should be killed.

DISCUSSION

Despite progress in treating primary tumors in the periphery, tumors that initiate in the central nervous system (CNS) or that preferentially migrate from peripheral primary or secondary metastatic sites into the brain remain refractory to treatment (King et al. 2005; Lin et al. 2004; Weil et al. 2005). Neurons and the supporting glial cells of the brain create a distinct microenvironment for primary brain tumors, and metastatic tumor cells must adapt to this unique environment (Weil et al. 2005). Furthermore, to enter the brain, tumor cells and therapeutics must cross the blood–brain barrier. Animal models that recapitulate all aspects of brain tumor pathogenesis are critical to improve our understanding of CNS tumors and to develop more effective therapies that inhibit their development.

Glioblastoma is an invasive brain tumor with a high mortality rate, even with treatment (King et al. 2005). The rat CNS-1 cell line is a widely used, well-characterized model of infiltrative glioma grown in nude mice. CNS-1 cells are implanted in nude mice because these mice are deficient in T lymphocytes and unable to mount an acquired response to the tumor, although their innate response is normal. Cranial windows provide long-term optical accessibility to the brain for high-resolution two-photon microscopy, creating a 3D view of the region in which CNS-1 cells are growing and spreading. With clear, intact cranial windows, tumors can be imaged many times over weeks in the same animal until the tumor grows large enough to induce neurological impairment. The injection technique controls the placement of tumor cells, reducing animal-to-animal variability caused by microenvironmental differences. By incorporating other fluorophores, tumor cell interactions with neurons, glial cells, and blood vessels can be studied and manipulated. This model is not useful for understanding the process of brain metastasis, which requires tumor cells to cross the blood–brain barrier. Also, although two-photon microscopy permits imaging to a depth of 500 μm (deeper than standard one-photon imaging), many tumors grow in regions of the brain that are not optically accessible, and therefore are not amenable to the high-resolution advantages of two-photon microscopy. With this model, experiments manipulating the surrounding environment and the tumor cells will elicit a greater understanding of the molecular pathways critical to glioma growth and spread, leading to more effective therapeutics directed against this aggressive brain tumor.

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