Video Article

Establishing Intracranial Brain Tumor Xenografts With Subsequent Analysis of Tumor Growth and Response to Therapy using Bioluminescence Imaging

Tomoko Ozawa[1], C. David James[1]

[1]Department of Neurological Surgery, University of California, San Francisco - UCSF

Correspondence to: C. David James at david.james@ucsf.edu

URL: https://www.jove.com/video/1986
DOI: doi:10.3791/1986

Keywords: Neuroscience, Issue 41, brain tumors, implantation, xenograft, athymic mice, bioluminescence imaging, therapeutic testing

Date Published: 7/13/2010

Citation: Ozawa, T., James, C.D. Establishing Intracranial Brain Tumor Xenografts With Subsequent Analysis of Tumor Growth and Response to Therapy using Bioluminescence Imaging. *J. Vis. Exp.* (41), e1986, doi:10.3791/1986 (2010).

Abstract

Transplantation models using human brain tumor cells have served an essential function in neuro-oncology research for many years. In the past, the most commonly used procedure for human tumor xenograft establishment consisted of the collection of cells from culture flasks, followed by the subcutaneous injection of the collected cells in immunocompromised mice. Whereas this approach still sees frequent use in many laboratories, there has been a significant shift in emphasis over the past decade towards orthotopic xenograft establishment, which, in the instance of brain tumors, requires tumor cell injection into appropriate neuroanatomical structures. Because intracranial xenograft establishment eliminates the ability to monitor tumor growth through direct measurement, such as by use of calipers, the shift in emphasis towards orthotopic brain tumor xenograft models has necessitated the utilization of non-invasive imaging for assessing tumor burden in host animals. Of the currently available imaging methods, bioluminescence monitoring is generally considered to offer the best combination of sensitivity, expediency, and cost. Here, we will demonstrate procedures for orthotopic brain tumor establishment, and for monitoring tumor growth and response to treatment when testing experimental therapies.

Video Link

The video component of this article can be found at https://www.jove.com/video/1986/

Protocol

1. Tumor Cell Preparation.

Cells for human brain tumor xenografts can be sourced either from tumors propagated as subcutaneous growths in athymic mice, or from cell culture. Utilization of both cell sources is discussed below, along with demonstration of a method for cell implantation.

To prepare cells from subcutaneous tumors for transfer to the intracranial compartment, excised flank tumors are placed in culture dishes, where the tissue is initially minced with a scalpel and then mechanically disrupted by repetitive pipetting to create a cell aggregate suspension[1]. The cell aggregate suspension is then passed through a 70 μM nylon mesh filter to produce a single cell suspension suitable for intracranial injection. The cell suspension is centrifuged at 1000 rpm for 10 minutes at 4°C, and the supernatant aspirated before resuspending the cell pellet in an appropriate volume of serum-free media to obtain a final working concentration (see below). For preparing established cell lines for intracranial implantation, cells are harvested by trypsinizing monolayers, or by collecting neurosphere suspension cultures, then centrifuging and resuspending the cells as indicated above [2]. The number of cells injected is variable dependent on neuroanatomical location of injection. For supratentorial injections we routinely inject 3-5 x 10^5 cells in 3 μL of serum-free media (DMEM), whereas for brainstem injections [3], as few as 5 x 10^4 cells are injected in 0.5 μL. Injecting larger volumes than recommended can result in tumor cell reflux through the needle tract, with resultant exophytic (Figure 1), rather than intracranial tumor growth. After withdrawing sample for intracranial injection, the remaining cell suspension should be placed in ice, with contents mixed frequently to maintain appropriate concentration while completing intracranial tumor establishment among the members of an injection series.

2. Tumor Cell Implantation

Note, all procedures described below have been reviewed and approved by the Institutional Animal Use and Care Committee at University of California San Francisco.

1. The surgical area should be prepared by spraying all surfaces with a disinfectant, such as 2% chlorhexidine solution. After using the disinfectant, the following supplies should be placed in the surgical area:
    - Heating pad to maintain mouse body temperature
    - Two small Petri dishes; one containing 3% hydrogen peroxide, and one containing 2% chlorhexidine
    - Sterile gauze and cotton swabs

- Sterile disposable scalpels
- Autoclaved surgical stapler

2. For anesthesia an injected anesthetic should be used; typically a ketamine-xylazine mixture.
3. Once a mouse is anesthetized, the scalp is prepared by swabbing several times with a piece of sterile gauze dipped in the chlorhexidine solution. Eye ointment should be applied to maintain adequate moisture during the procedure. Using a sterile scalpel, complete a sagittal incision over the parieto-occipital bone, approximately 1cm long. The exposed skull surface is then cleaned using a cotton swab soaked in a 3% hydrogen peroxide solution. The bregma should be apparent at this point (see video).
4. The coordinates for injection of tumor cells will vary according to the desired site for tumor establishment. The following represents the procedure we use for intracerebral tumor establishment[2] in a neuroanatomical location at which many brain tumor patients experience tumor development. Other locations of interest in brain tumor research include the pons, for anatomic modeling of brainstem tumors[3], and subdural injections for modeling the location of meningeal tumors[4]. Prior to tumor cell injection, use a sterile 25 gauge sharp needle to puncture the skull at 2 mm to the right of the bregma and 1 mm anterior to the coronal suture, thereby creating an opening for the injection of tumor cells (see video). This procedure works well for both mice and rats (22 gauge needle for rats).
5. Prior to drawing cells into the syringe, mix the contents of the cell suspension by tapping with your finger. Load the syringe with the desired amount of cell suspension, being careful to avoid creating air bubbles. The outside of syringe should then be cleaned with an alcohol swab to wipe the exterior free of any adherent cells, which will help prevent extracranial tumor establishment and growth (Figure 1A). To ensure that the appropriate injection depth is achieved, use a scalpel to cut 3mm off the pointed end of a P20 pipetteman tip. This section of the tip can be fitted over the syringe and will act to limit the injection depth, and will additionally ensure that the tip of the syringe needle is 3mm from the underside of the skull. Place the syringe perpendicular to the skull and in the hole previously created, and slowly inject the cell suspension (a 3μL suspension should be injected over a 1 minute period). An inappropriate angle of syringe insertion can result in intraventricular injection of cells and subsequent spinal dissemination (Fig. 1B: Right) Upon completing injection, leave the needle in place for another minute, then slowly withdraw (see video): these steps will help reduce tumor cell reflux.
6. As an alternative to the unassisted or free-hand approach to intracranial tumor cell implantation[5], one can use a small animal stereotactic frame (panel F of schematic overview), which generally promotes more consistent injection location, but at the expense of substantial amounts of procedural time. In our experience, two surgical staff can inject as many as 60 mice/hour when using the free-hand approach, whereas maximal injection rate with a small animal stereotactic frame is approximately 15 mice/hour. Procedural expediency is an important consideration when injecting large series of mice, and helps reduce the time in which tumor cells, to be injected, are left on ice.
7. Clean the skull with 3% hydrogen peroxide and dry using a sterile dry cotton swab. Apply sterile bone wax to the hole. Using a forceps, draw the scalp together over the skull and staple to close. For optimal healing, the scalp should be stapled with the dermis of each side of the scalp against each other (underside against underside). The stapled scalp should be cleaned using Chlorhexidine solution, and buprenorphine then administered by subcutaneous injection for post-operative pain relief.
8. Monitor all mice post-operatively until they become ambulant and retain normal activity. Typically, recovery time is around 30 minutes.

3. Bioluminescence Monitoring of Tumor Growth

1. Background. Bioluminescence imaging (BLI) is based on the oxidation of luciferin [d-(-)-2-(60-hydroxy-20-benzothiazolyl)-thiazone-4-carboxylic acid] in the presence of oxygen and adenosine triphosphate (ATP). This reaction is catalyzed by the enzyme luciferase, which converts chemical energy into photons with resultant emission of light. Human cells can be modified to express luciferase (see below), with only luciferase-expressing cells capable of emitting light in the presence of the luciferin substrate. There is minimal background luminescence from animals hosts treated with luciferin, such that there is a very favorable signal-to-noise ratio for detecting luminescence emissions from luciferase-modified tumor cells, allowing highly sensitive monitoring of tumor growth and response to therapy in vivo. Moreover, luciferase and its substrate, luciferin, have been shown to be non-toxic to mammalian cells, and we have observed no functional differences between cells expressing luciferase relative to corresponding unmodified cells. Luciferin readily crosses cell membranes and the blood-brain barrier after intraperitoneal (i.p.) or intravenous (i.v.) injection in mice, thereby allowing imaging of every compartment that contains luciferase-modified cells. The level of photon emission and the spectrum of emitted light from luciferase-modified cells is adequate to penetrate tissues of small research animals, such as mice and rats, and can be detected externally with low-light imaging cameras. The noninvasive nature of bioluminescence imaging allows repeated monitoring of tumor growth and response to therapy in individual animal subjects.
2. Implanted cell sources should be stably modified for firefly luciferase expression. Such cell sources can be purchased, or produced in individual laboratories using lentivirus that have been constructed for constitutive expression of luciferase. We strongly recommend the use of cells modified with luciferase encoding lentivirus, rather than plasmid, to ensure stable cellular expression of luciferase in vivo, which is necessary for quantitative luminescence imaging to provide accurate indication of changes in tumor burden for individual animal subjects that are repeatedly imaged during the course of an experiment.
3. BLI monitoring. We recommend conducting quantitative bioluminescence imaging (qBLI) 1x-2x weekly, beginning one week following tumor cell injection. Our qBLI is conducted using the IVIS Lumina imaging station (Caliper Life Sciences), and our results indicate similar data are obtained in using the IVIS Lumina, the IVIS 150, or the IVIS 200 imaging station. In preparation for imaging, mice are simultaneously anesthetized with Ketamine/Xylazine and administered luciferin (D-Luciferin potassium salt, 150 mg/kg, Caliper Life Sciences) via intraperitoneal injection, with mice imaged 12 minutes after injection. The pharmacokinetics of luciferin indicate that the time between administration of luciferin and determination of cell luminescence should be between 10-15 minutes post injection of luciferin, in order to obtain maximum luminescence emission and greatest sensitivity of detection. The length of time selected within the 10-15 minute time interval should be maintained as constant among the animals that are being imaged. It is extremely important to maintain consistency in length of time between injection of luciferin and obtaining BLI readings. Regions of interest encompassing the intracranial area of signal are defined using Living Image software (Figure 2), and the total photons/s/sr/cm2 (photons per second per steradian per square cm) are recorded (see video).
4. Data analysis. Whereas tumor growth and therapy response are monitored in individual animals, we highly recommend treatment groups of at least 8 for increasing the statistical certainty of conclusions regarding tumor response, or lack thereof, to therapy. With regard to presenting qBLI results, luminescence readings are converted to normalized values by dividing each mouse s luminescence, obtained during and subsequent to completion of therapeutic regimen, with its corresponding maximal pretreatment luminescence reading[6,7]. For

survival analysis, the Kaplan-Meier estimator[8] is used, and from which survival curves are generated, and median survival values determined. Differences between survival curves are compared using a log-rank test[9].

5. Retrieval of brain for subsequent analysis of treated and untreated tumor. Upon animal euthanasia, the brain of the mouse should be quickly excised (see video), and either placed in formalin for subsequent analysis of tumor morphology and immunohistochemistry, or should be mounted in OCT for specimen freezing.

4. Representative Results

In the example shown in Figure 3A, mice receiving intracranial tumor cell injection were monitored for intracranial luminescence until successive mean luminescence values indicated progressive tumor growth, and at which time therapy was initiated (gray arrow beginning at day 34: erlotinib administered daily at 150 mg/kg until required euthanasia). Luminescence values for each mouse are set to a normalized value of 1 at time of initiating therapy, with subsequent luminescence readings for each mouse normalized to its final pretreatment imaging value. As an example, a mouse with a final pre-treatment luminescence reading of 2.0 x 10^7 photons/sec at day 34, whose luminescence had increased to 6.0 x 10^7 photons/sec at day 38, would have a day 38 normalized luminescence value of 3.0. Mean normalized bioluminescence and corresponding standard error for control and treatment groups are plotted for each imaging time point. In this example, a significant difference in mean normalized luminescence is apparent at the first imaging time point subsequent to the initiation of therapy (day 38), with the difference in mean group luminescence showing further increase at subsequent time points. In most instances, anti-tumor activity of therapy, as indicated by qBLI, is accompanied by a corresponding significant difference in survival (i.e., $p < 0.05$), as is the case here (Figure 3B). Panels 3C and 3D show adjacent hematoxylin & eosin and anti-EGFR stained sections of mouse brain obtained at time of euthanasia, following placement of resected brain in formalin and subsequent paraffin-embedding for sectioning.

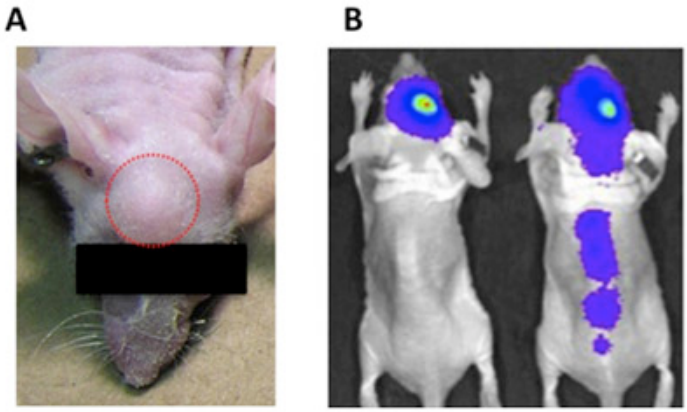

Figure 1. Indications of intracranial injection errors. A) Exophytic (extracranial) tumor growth (red circle) can be caused by too large an injection volume, residual cell suspension attached to the syringe, or from withdrawing the syringe too quickly after injecting the tumor cells. B) Injecting tumor cells into the ventricles can cause spinal dissemination of tumor (mouse to the right), in contrast to properly injected tumor cells, the signal for which stays localized to the injection site (mouse to the left).

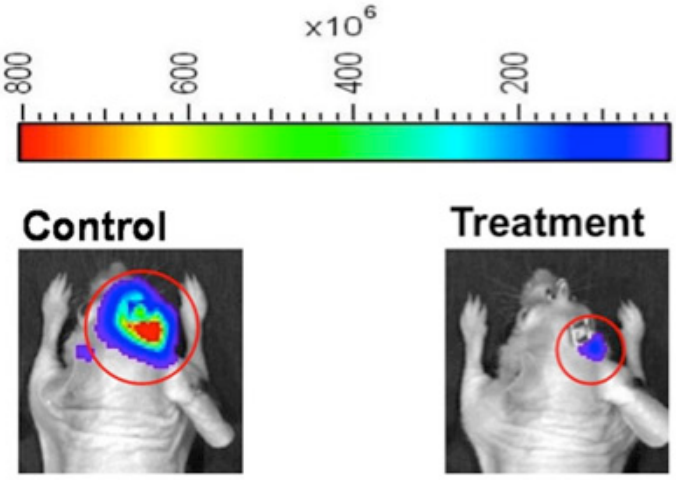

Figure 2. Heat map image representations of bioluminescence intensity for representative mice from control (left) and treatment (right) groups of a therapy response experiment. The Living Image software can be set to define regions of interest (red circles), or instrument operators can define regions of interest manually. For using images such as these for figure construction, we recommend that the instrument operator shows heat map images using the same bioluminescence heat map range (upper portion of figure), to provide visual representation of extent of bioluminescence difference between animal subjects.

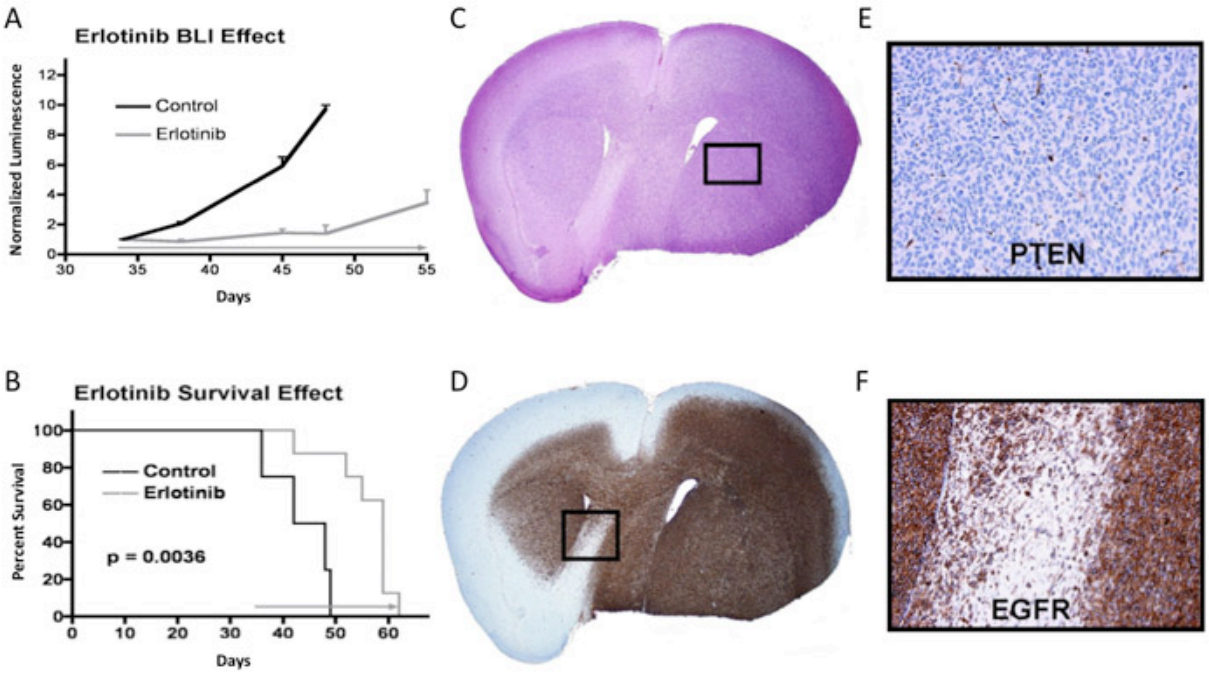

Figure 3. Bioluminescence, survival, and tumor tissue analysis from an experiment in which therapeutic response is evident. A) Plot of mean bioluminescence readings for control and treatment group mice, with standard error indicated for each imaging point. B) Survival plot for same mice; p-value determined through use of log-rank test [7]. C) H&E stained section of mouse brain with tumor. D) EGFR stained section. E and F) Magnifications of indicated areas from panels C and D, respectively, with panel E showing negative staining for tumor suppressor protein PTEN.

Discussion

Orthotopic (intracranial) brain tumor xenograft establishment provides an appropriate microenvironment[10] for modeling CNS cancer to be tested for therapeutic response. This type of modeling additionally provides information regarding therapeutic access to brain and brain tumor, which is critically important to determining whether an experimental agent should be advanced to clinical trial evaluation in patients. Because the amount of intracranial xenograft tumor can not be directly measured, such as by calipers, longitudinal monitoring of intracranial tumor growth and response to therapy requires non-invasive imaging, with our experience indicating bioluminescence imaging as the most practical approach for experiments whose primary objective is assessing extent of tumor response to therapy. When the results of bioluminescence imaging are combined with animal subject survival analysis, the two data sets provide a powerful and reliable approach for evaluating experimental therapeutic efficacy.

Finally, it is critically important that intracranial brain tumor xenografts are harvested from euthanized animal subjects in order to assess morphologic and molecular effects of therapy, and for this we prefer whole brain resection at time of euthanasia, with preservation of resected brain for subsequent analysis.

Whereas the preceding presentation has been made specific to brain tumor research, the concepts are certainly generalizeable to other human cancers that are amenable to orthotopic modeling in rodents.

Disclosures

No conflicts of interest declared.

Acknowledgements

NINDS grants NS49720 and NS65819, and NCI grant CA97257

References

1. Giannini, C., Sarkaria, J.N., Saito, A., Uhm, J.H., Galanis, E., Carlson, B.L., Schroeder, M.A. & James, C.D. Patient Tumor *EGFR* and *PDGFRA* Gene Amplifications Retained in an Invasive Intracranial Xenograft Model of GBM. Neuro-Oncol. 7, 164-176 (2005).
2. Ozawa, T., Wang, J., Hu, L.J., Bollen, A.W., Lamborn, K.R. & Deen, D.F. Growth of human glioblastomas as xenografts in the brains of athymic rats. In Vivo 1,55-60 (2002).
3. Hashizume, R., Ozawa, T., Dinca, E.B., Banerjee, A., Prados, M.D., James, C.D. & Gupta, N. A human brainstem glioma xenograft model enabled for bioluminescence imaging. J Neurooncol. (2009). Epub ahead of print.

4. Baia, G.S., Dinca, E.B., Ozawa, T., Kimura, E.T., McDermott, M.W., James, C.D., VandenBerg, S.R. & Lal, A. An orthotopic skull base model of malignant meningioma. Brain Pathol. 2,172-179 (2007).
5. Ozawa, T. & James, C.D. Human Brain Tumor Cell and Tumor Tissue Transplantation Models, in *CNS Cancer: Models, Markers, Prognostic Factors, Targets, and Therapeutic Approaches* (Van Meir E, ed.), pp. 147-162, Humana Press-Springer, New York, NY, (2009).
6. Sarkaria, J.N., Yang, L., Grogan, P.T., Kitange, G.J., Carlson, B.L., Schroeder, M.A., Galanis, E., Giannini, C., Wu, W., Dinca, E.B. & James C.D. Identification of Molecular Characteristics Correlated with Glioblastoma Sensitivity to EGFR Kinase Inhibition Through Use of an Intracranial Xenograft Test Panel. Mol. Cancer Ther. 6,1167-1174 (2007).
7. Dinca, E.B., Sarkaria, J.N., Schroeder, M.A., Carlson, B.L., Voicu, R., Gupta, N., Berger, M.S. & James, C.D. Bioluminescence Monitoring of Intracranial Glioblastoma Xenograft Response to Primary and Salvage Temozolomide Therapy. J. Neurosurg. 107,610-616 (2007).
8. Kaplan, E.L. & Meier, P. Non-parametric estimation from incomplete observations. J. Am. Stat. Assoc. 53,457 481 (1958).
9. Peto, R. & Peto, J. Asymptotically efficient rank invariant procedures. J. R. Stat. Soc. Ser. A. Stat. Soc. 135,185 207 (1972).
10. Camphausen, K., Purow, B., Sproull, M., Scott, T., Ozawa, T., Deen, D.F. & Tofilon, P.J. Influence of in vivo growth on human glioma cell line gene expression: convergent profiles under orthotopic conditions. Proc. Natl. Acad. Sci. U. S. A. 102,8287-92 (2005).