Video Article

Image-Guided Resection of Glioblastoma and Intracranial Implantation of Therapeutic Stem Cell-seeded Scaffolds

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Abstract

Glioblastoma (GBM), the most common and aggressive primary brain cancer, carries a life expectancy of 12-15 months. The short life expectancy is due in part to the inability of the current treatment, consisting of surgical resection followed by radiation and chemotherapy, to eliminate invasive tumor foci. Treatment of these foci may be improved with tumoricidal human mesenchymal stem cells (MSCs). MSCs exhibit potent tumor tropism and can be engineered to express therapeutic proteins that kill tumor cells. Advancements in preclinical models indicate that surgical resection induces premature MSC loss and reduces therapeutic efficacy. Efficacy of MSC treatment can be improved by seeding MSCs on a biodegradable poly(lactic acid) (PLA) scaffold. MSC delivery into the surgical resection cavity on a PLA scaffold restores cell retention, persistence, and tumor killing. To study the effects of MSC-seeded PLA implantation on GBM, an accurate preclinical model is needed. Here we provide a preclinical surgical protocol for image-guided tumor resection of GBM in immune-deficient mice followed by MSC-seeded scaffold implantation. MSCs are engineered with lentiviral constructs to constitutively express and secrete therapeutic TNFα-related apoptosis-inducing ligand (TRAIL) as well as green fluorescent protein (GFP) to allow fluorescent tracking. Similarly, the U87 tumor cells are engineered to express mCherry and firefly luciferase, providing dual fluorescent/luminescent tracking. While currently used for investigating stem cell mediated delivery of therapeutics, this protocol could be modified to investigate the impact of surgical resection on other GBM interventions.

Video Link

The video component of this article can be found at https://www.jove.com/video/57452/

Introduction

Glioblastoma (GBM) is the most common primary brain cancer in adults, with a dismal median survival of just 12-15 months[1,2,3,4,5]. Survival has not significantly improved since 2005 when the current clinical standard of maximal surgical resection followed by radiation and concomitant and adjuvant temozolomide chemotherapy was adopted[6,7]. While this treatment provides patients with a temporary relief of symptoms, standard of care treatment invariably results in recurrence as invasive cancer foci evade resection and are protected from systemic therapies by the blood-brain barrier (BBB). Strategies which target invasive tumor foci while circumventing the BBB are urgently needed to gain traction against this aggressive and debilitating disease.

Human mesenchymal stem cells (MSCs) show promise as drug delivery vehicles for GBM due to their native tumor tropism[8,9]. MSCs possess receptors for and migrate towards soluble factors that tumors secrete, including stromal cell-derived factor 1α (SDF-1α), matrix metalloproteinase-1 (MMP-1), and monocyte chemoattractant protein-1 (MCP-1) among others[10,11,12,13]. Engineering MSCs to express and secrete cytotoxic drugs allows them to be harnessed as tumor-homing drug delivery vehicles. Engineered MSCs move toward invasive tumor foci and deliver therapeutic proteins. This approach has demonstrated feasibility in a variety of preclinical GBM models[9,14]. However, the vast majority of these models do not include surgical resection despite the clinical relevance of this component. Emerging studies using new models of resection revealed that surgical tumor removal reduces the persistence of stem cells that are directly injected into the surgical cavity[15]. Loss of viability resulted in reduced efficacy, likely due to decreases in the dose and duration of drug delivered to the invasive tumor foci.

To increase stem cell viability and drug delivery, MSCs can be seeded onto scaffolds prior to implantation. In this protocol, biocompatible and resorbable electrospun nanofibrous poly(lactic acid) (PLA) is used as scaffolding for the MSCs. PLA flexes and conforms to the shape of the resection cavity upon implant, which maximizes therapeutic coverage and minimizes the distance MSCs must travel to reach tumor cells. MSCs remain on the scaffold during implantation and then migrate off the scaffold toward tumor cells after implantation[16,17]. MSCs and the cytotoxic drugs they carry then accumulate at the tumor foci. Delivery of cytotoxic drug to the tumor requires MSC viability and persistence, both of which are aided by implantation on scaffolds.

In this procedure, lentiviral vectors are used to induce stable expression of fluorescent (*in vitro* tracking) and bioluminescent (*in vivo* tracking) markers in both cancer and stem cell lines. The human GBM line U87 is infected with mCherry and firefly luciferase (U87 mCh-Fl), and the non-therapeutic MSCs with GFP and renilla luciferase (MSC GFP-Rluc). The therapeutic variant of MSCs express TNFα-related apoptosis inducing

ligand (MSC-TRAIL). TRAIL, a constitutively-secreted protein, binds to nearby death receptors on cancer cells and initiates caspase-mediated apoptosis[18].

Here, we provide a protocol for preclinical image-guided GBM surgical resection and implantation of MSC-seeded scaffolds. In brief, nude mice are given a craniotomy followed three days later by stereotactic orthotopic injection of U87 mCh-Fl to establish the primary tumor. The engrafted tumor grows for a period of approximately one week. PLA scaffolds are seeded with MSCs 48 h prior to resection surgery. The tumor is then resected under fluorescent guidance, and the MSC-loaded scaffold is implanted into the resection cavity. Tumor burden and mouse survival are then tracked post-operatively with bioluminescence imaging (BLI). A timeline of these procedures is provided below (**Figure 1**).

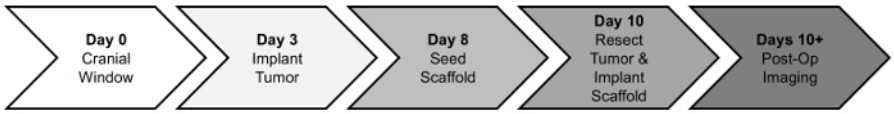

Figure 1: Timeline of procedures. Mice initially receive a cranial window (Day 0). After a recovery period of three days, tumors are implanted (Day 3) and grow for approximately one week. Scaffolds are seeded with MSCs (Day 8) two days in advance of the tumor resection and implantation procedure (Day 10). Tumor progression and therapeutic efficacy are evaluated via post-operative imaging thereafter (Day 10+).
Please click here to view a larger version of this figure.

Protocol

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of The University of North Carolina at Chapel Hill.

NOTE: This procedure is performed on mice with an open craniotomy, as a modified version of the protocol by Mostany and Portera-Cailliau[19] wherein the bone tissue that is surgically removed is discarded, leaving the brain accessible for establishment and eventual resection of GBM tumors. Following craniotomy, tumors are established via stereotactic implantation as described in Ozawa and James[20]. We implant 1 x 10^5 U87 mCh-Fl cells at the following stereotactic coordinates (in mm from bregma): (2.5, 0, -0.5).

1. Cell Culture and Scaffold Preparation

NOTE: Scaffolds should be prepared 48 h prior to implantation in mice. The following volumes are provided on a per-scaffold basis. Multiply quantities as needed for additional scaffolds.

1. Cut PLA scaffolds into resection cavity-sized (approximately 2 mm x 2 mm) pieces. Scaffolds can be cut by hand using scissors or using a hole punch for repeatability.
2. Sterilize by immersing in 70% ethanol for 15 min followed by immersing in PBS. Place scaffold in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin while preparing cells for seeding.
3. Lift cultured MSCs using 0.05% trypsin (3-5 mL for a T75 flask). Incubate at 37 °C for 5 min. Ensure that the cells have lifted, then add 7-10 mL DMEM to the flask to inactivate trypsin.
4. Transfer flask contents to a 15-mL centrifuge tube. Count cells using a hemocytometer. Pellet 5 x 10^5 MSCs for each scaffold via centrifugation at 100 x g for 5 min.
5. Aspirate off supernatant and resuspend MSCs in 5 µL DMEM per 5 x 10^5 cells.
6. Prepare the scaffolds for seeding by patting them dry.
    1. Remove a scaffold from DMEM immersion with forceps and temporarily place it on the lid of the 6-well plate. Lift the scaffold off the lid, leaving behind a droplet of excess DMEM.
    2. Place the scaffold back on the lid again in a new, dry location. Repeat 3-5 times, then place the partially dried scaffold in a new 6-well plate for seeding. Repeat for each scaffold.
    NOTE: A partially dried scaffold provides optimal cell seeding results. If the scaffold is too wet, cells will slide off the scaffold and adhere to the well plate below. If the scaffold is too dry, the droplet will not spread over the entire scaffold, resulting in poor initial cell distribution.
7. Using a pipette, gently mix the vial of stem cells to homogenize the suspension as some cells may have settled to the bottom.
8. Slowly pipette 2.5 µL freshly mixed MSC suspension directly on the scaffold, creating a small droplet over the top of the scaffold.
9. Add 300 µL DMEM to edges of each well. This will prevent rapid evaporation of the cell droplet. Incubate at 37 °C for 30 min, allowing the cells to attach to the scaffold.
10. With forceps, gently flip the scaffolds over in the well plate. Seed 2.5 µL freshly mixed MSC suspension (this results in a total of 5 x 10^5 cells seeded per scaffold). Incubate at 37 °C for 30 min, allowing the cells to attach to the scaffold.
11. Cover the scaffolds in DMEM by adding 2 mL to each well of the 6-well plate. Gently lift the scaffolds to allow media to flow underneath them. Incubate at 37 °C in this state for 48 h prior to implantation surgery.
12. Check the scaffolds after 24 h and add/change media if excess evaporation or discolored media due to pH imbalance is observed.

2. Fluorescence-guided Resection and Scaffold Implantation

NOTE: Sterilize all tools prior to initializing surgery. Administer prophylactic analgesia as outlined in your institutional IACUC protocol.

1. Place the stereotaxic frame on the stage of a fluorescence dissecting stereomicroscope.

2. Anesthetize the mouse under inhaled isoflurane in an induction chamber. Once anesthetized, secure the mouse in the stereotaxic frame with continuous inhaled anesthesia supply via an isoflurane nose cone adapter. Maintain body temperature with a heating pad and/or probe.
   NOTE: 4-5% isoflurane is suitable for induction and 2-3% is suitable for maintenance, but this must be adapted and monitored for each mouse.
3. Apply ophthalmic ointment to the eyes to prevent drying of the cornea. Sterilize the incision site of the scalp with a series of three alcohol and three betadine wipes.
4. Perform toe pinch reflex test on each limb and confirm negative response to ensure proper anesthetization. Ensure a steady respiratory rate at approximately 55-65 breaths/min.
5. Using forceps, pinch and gently lift the scalp. Make a midline linear rostral-caudal incision with surgical scissors. Irrigate the incision site with PBS and remove subdermal fat with a cotton tip applicator in a circular brushing motion. Arrange the skin such that the previously-established cranial window is fully visible.
6. Gently puncture the dura just interior to the borders of the cranial window using an 18-G needle. Repeat until the incision fully traces the interior of the window.
7. Remove the dura by peeling it away using fine forceps, revealing the underlying parenchyma and tumor.
8. Locate the U87 mCh-Fl tumor by turning room lights off and turning the stereomicroscope fluorescence-mode on. Prepare for resection by loading a 1-200 µL pipette tip into the end of the tubing of a vacuum pump.
9. Turn the vacuum pump on. Resect the tumor by gently aspirating fluorescent tissue until no signal remains. Turn the vacuum pump off and discard the pipette tip. Turn the fluorescence off and the room lights back on.
   NOTE: To control bleeding, irrigate with cold PBS and apply steady pressure with a cotton tip applicator. In more severe cases, the resection cavity can also be temporarily packed with a hemostatic agent as long as it is removed after the bleeding has stopped followed by another PBS irrigation.
10. Immediately prior to implantation, slowly dip an MSC-seeded PLA scaffold in PBS to remove unwanted media and associated components. Implant the scaffold into the resection cavity. If needed, add 1 µL fibrinogen (67-106 mg/mL) followed by 1 µL thrombin (400-625 U/mL) to secure the scaffold in place.
11. With the dura removed and the bone flap from the cranial window already discarded, seal the wound simply by closing the skin and applying surgical glue. Remove the animal from inhaled anesthetic and allow to recover on a heated surface.
12. Once ambulatory, return mouse to cage. Administer analgesic on IACUC-approved schedule. Repeat procedure for each mouse.

3. Post-Operative Imaging

1. Using the 28-G insulin syringe, inject mice with D-luciferin (150 mg/kg intraperitoneal).
2. Wait 10 min. This will allow the luciferin to circulate throughout the body and react with the luciferase-expressing cancer cells in the brain to obtain peak expression.
3. Under isoflurane anesthesia (as mentioned previously), image mice in a bioluminescence imaging (BLI) system to visualize the tumors. Vary exposure times as needed (seconds to minutes) depending on tumor size.
4. Repeat imaging procedure as needed to determine tumor growth kinetics. Continue to monitor animal health.
   NOTE: The therapeutic MSCs will constitutively express TRAIL, killing cancer cells and suppressing overall tumor growth. Imaging every 2-5 days provides sufficient frequency for this purpose.
5. When predetermined endpoints outlined in the IACUC protocol have been met, sacrifice mice by transcardial perfusion and collect tissues for analysis.

Representative Results

U87 tumor cells were engineered to express mCherry and firefly luciferase (U87 mCh-Fl) prior to injection, and to allow for image-guided resection and bioluminescence tracking (**Figure 2A-C**). Stem cells were similarly engineered with diagnostic GFP-Rluc (MSC-GFP) or therapeutic GFP-TRIAL (MSC-TRAIL) and seeded onto the PLA scaffolds to confirm attachment and proliferation (**Figure 2D-F**).

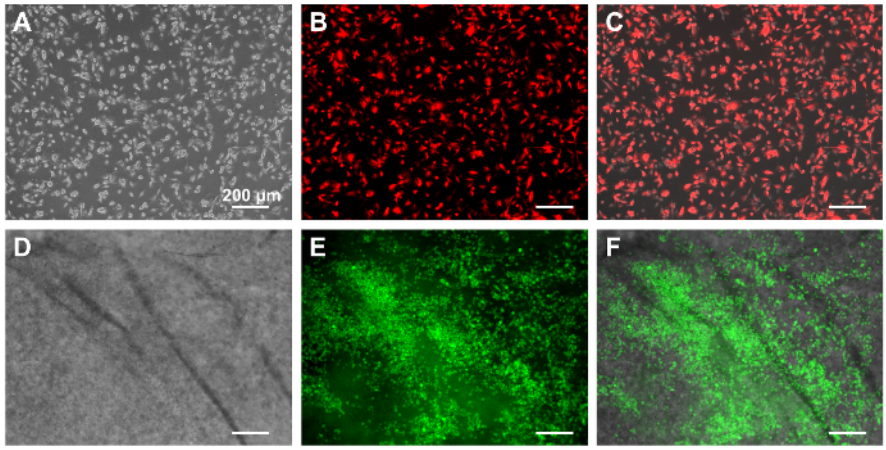

Figure 2: Representative images of fluorescent cancer cells and MSCs seeded on PLA. A-C) Phase, fluorescent, and combined images, respectively, show U87 cancer cells engineered with lentiviral constructs to express mCh-FI markers. **D-F**) Corresponding images of stem cells engineered to express GFP-RI or therapeutic GFP-TRAIL seeded onto the PLA scaffolding material. Scale bars = 200 µm. Please click here to view a larger version of this figure.

Intraoperative images are provided below to highlight portions of the surgical procedure for tumor resection and scaffold implantation (**Figure 3**). The animal is first anesthetized and aligned in the stereotaxic instrument (**Figure 3A**). The skin is opened, and the dura is peeled back. The tumor is resected (**Figure 3B**), and when examined under white light appears to be fully removed. However, fluorescent images of the tumor before (**Figure 3C**) and after (**Figure 3D**) resection indicate while the majority of the tumor was removed, a residual amount remains. This phenomenon resembles clinical cases wherein surgeons are unable to remove tumor cells that quickly migrate away from the primary mass and into inoperable areas in the brain. Migratory therapeutic MSCs move toward residual tumor foci that remain following surgical resection. To deliver MSCs into the resection cavity, the MSCs are first seeded onto PLA, and then the scaffold construct is placed in the resection cavity. Presence of the MSCs on the scaffold are confirmed post-implant by fluorescently imaging GFP signal (**Figure 3E**). *Ex vivo* whole brain images show scaffold dimensions relative to the resection cavity (**Figure 3F-H**). The scaffold appears significantly larger when laid flat, but can be molded into the shape of the resection cavity during implantation. By coating the entire cavity, the distance therapeutic MSCs have to migrate to reach tumor foci is minimized. After surgery, BLI is used to track tumor growth over time and determine scaffold-delivered MSC-TRAIL efficacy.

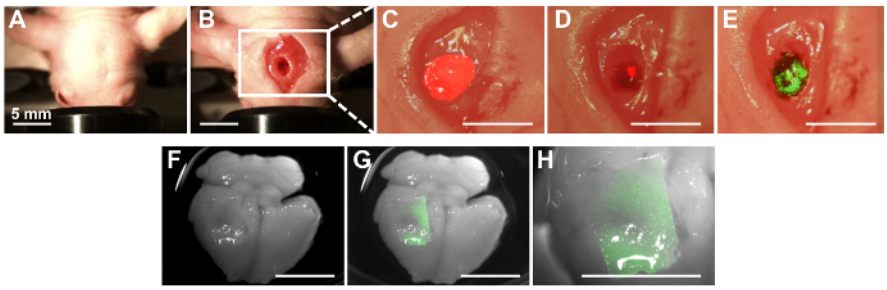

Figure 3: Representative intraoperative and postoperative images. A) Intraoperative series showing mouse before incision. **B**) Incised mouse with cranial window revealing tumor mass. **C**) Bright field and fluorescent overlay image showing location of tumor. **D**) Post-surgical resection cavity with remnant tumor foci. **E**) Implanted PLA scaffold seeded with MSC-TRAIL. **F**) Post-mortem brain tissue with scaffold overlaid. **G**) Fluorescent overlay highlighting GFP-TRAIL cells. **H)** Magnified version of G. Scale bars = 5 mm. Please click here to view a larger version of this figure.

Discussion

Surgery can typically be completed within 30 min per mouse, given that the following points are taken into consideration to maximize precision and avoid time-consuming pitfalls. First, ensure the mouse is properly positioned in the stereotaxic instrument prior to starting the procedure. Unwanted head movements will limit surgical accuracy of the craniotomy, location of the tumor implantation, and degree of tumor resection. Prior to resection, fully remove the portion of the dura covering the tumor. The tough, fibrous dura hardens as it dehydrates during aspiration. If incompletely removed, the hardened dura may limit aspirator tip mobility and prevent complete tumor resection. During resection, blood vessels are often inadvertently resected along with the tumor, causing significant bleeding. Excess blood diminishes the intensity of the fluorescent signal and obscures the tumor. Maintaining tumor visibility during resection ensures proper extent of resection, and limits the removal of healthy tissue and excess bleeding. For smaller bleeds, irrigate the damaged vessel with cold saline and apply gentle pressure with a cotton tip applicator. If bleeding continues, pack a hemostatic agent into the cavity for 2-3 min and then remove with forceps. Irrigate with PBS afterwards to restore physiological pH prior to implanting the cell-seeded scaffold. When resection is completed and bleeding has stopped, the final pitfall is inadequate skin wound closure. This is most often caused by excess moisture (PBS or blood) that prevents the surgical glue from bonding the incised skin. Avoid this by gently drying the skin with a cotton tip applicator prior to applying the glue. It is also possible to accidentally glue the skin directly to the skull if the wound gap is not first held in a closed position with forceps prior to gluing.

With these considerations in mind, the above protocol produces consistent and reliable GBM surgical resections that mimic the standard of care for accurate *in vivo* testing of emerging cell and small molecule therapies. Still, several components can be modified to serve particular experimental needs. For instance, tumor properties such as size, location, and extent of invasion can be adjusted by changing the initial number and depth of injected cells, time between tumor establishment and resection, or by switching the tumor cell line itself. Furthermore, this study is performed in athymic nude mice whose compromised immune system tolerates human cells. Immune competent mice may be more appropriate for studies using mouse cells.

Compared to a closed system (*i.e.*, the bone or a coverslip is put back into place), the open craniotomy performed in this protocol reduces intracranial pressure (ICP). Since increased ICP is responsible for the onset of symptoms including seizures, mice will experience an artificial prolongation of survival in this model. While the impact is minimized due to the delay applying to both control and treatment groups, future models could re-close the bone flap to restore ICP and determine the role that it plays in cell therapy for GBM.

Disclosures

Drs. Sheets, Bagó, and Hingtgen have equity in Falcon Therapeutics who has licensed aspects of the stem cell and scaffold technology from UNC Chapel Hill.

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References

1. Adamson, C. et al. Glioblastoma multiforme: a review of where we have been and where we are going. *Expert opinion on investigational drugs.* **18** (8), 1061-83 (2009).
2. Asthagiri, A. R., Pouratian, N., Sherman, J., Ahmed, G., & Shaffrey, M. E. Advances in Brain Tumor Surgery. *Neurologic Clinics.* **25** (4), 975-1003 (2007).
3. Affronti, M. et al. Overall survival of newly diagnosed glioblastoma patients receiving carmustine wafers followed by radiation and concurrent temozolomide plus rotational multiagent chemotherapy. *Cancer.* **115** (15), 3501-3511 (2009).
4. Erpolat, O., Akmansu, M., Goksel, F., Bora, H., Yaman, E., & Büyükberber, S. Outcome of newly diagnosed glioblastoma patients treated by radiotherapy plus concomitant and adjuvant temozolomide: a long-term analysis. *Tumori.* **95** (2), 191-7 (2009).
5. Minniti, G. et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma in elderly patients. *Journal of Neuro-Oncology.* **88** (1), 97-103 (2008).
6. Stupp, R. et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *The New England Journal of Medicine.* **352** (10), 987-96 (2005).
7. Delgado-López, P., & Corrales-García, E. Survival in glioblastoma: a review on the impact of treatment modalities. *Clinical and Translational Oncology.* **18** (11), 1062-1071 (2016).
8. Wu, X. et al. In vivo tracking of superparamagnetic iron oxide nanoparticle-labeled mesenchymal stem cell tropism to malignant gliomas using magnetic resonance imaging. Laboratory investigation. *Journal of Neurosurgery.* **108** (2), 320-9 (2008).
9. Nakamizo, A. et al. Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Research.* **65** (8), 3307-18 (2005).
10. Xu, F., Shi, J., Yu, B., Ni, W., Wu, X., & Gu, Z. Chemokines mediate mesenchymal stem cell migration toward gliomas in vitro. *Oncology Reports.* **23** (6), 1561-7 (2010).
11. Park, S. et al. CXCR4-transfected human umbilical cord blood-derived mesenchymal stem cells exhibit enhanced migratory capacity toward gliomas. *International Journal of Oncology.* **38** (1), 97-103 (2011).
12. Ho, I. et al. Matrix Metalloproteinase 1 Is Necessary for the Migration of Human Bone Marrow-Derived Mesenchymal Stem Cells Toward Human Glioma. *STEM CELLS.* **27** (6), 1366-1375 (2009).
13. Bexell, D., Svensson, A., & Bengzon, J. Stem cell-based therapy for malignant glioma. *Cancer Treatment Reviews.* **39** (4) (2012).
14. Nouri, F., Wang, X., & Hatefi, A. Genetically engineered theranostic mesenchymal stem cells for the evaluation of the anticancer efficacy of enzyme/prodrug systems. *Journal of Controlled Release.* **200**: 179-187 (2015).
15. Kauer, T., Figueiredo, J.-L., Hingtgen, S., & Shah, K. Encapsulated therapeutic stem cells implanted in the tumor resection cavity induce cell death in gliomas. *Nature Neuroscience.* **15** (2), 197-204 (2011).
16. Bagó, J., Pegna, G., Okolie, O., & Hingtgen, S. Fibrin matrices enhance the transplant and efficacy of cytotoxic stem cell therapy for post-surgical cancer. *Biomaterials.* **84**, 42-53 (2016).
17. Bagó, J., Pegna, G., Okolie, O., Mohiti-Asli, M., Loboa, E., & Hingtgen, S. Electrospun nanofibrous scaffolds increase the efficacy of stem cell-mediated therapy of surgically resected glioblastoma. *Biomaterials.* **90**, 116-125 (2016).
18. Loebinger, M., Eddaoudi, A., Davies, D., & Janes, S. Mesenchymal Stem Cell Delivery of TRAIL Can Eliminate Metastatic Cancer. *Cancer Research.* **69** (10), 4134-4142 (2009).
19. Mostany, R., Portera-Cailliau, C. A Craniotomy Surgery Procedure for Chronic Brain Imaging. *J. Vis. Exp.* (12), e680 (2008).
20. 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 (2010).

