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Transposon mediated integration of plasmid DNA into the subventricular zone of neonatal mice to generate novel models of glioblastoma
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Abstract:	An urgent need exists to test the contribution of new genes to the pathogenesis and progression of human glioblastomas (GBM), the most common primary brain tumor in adults with dismal prognosis. New potential therapies are rapidly emerging from the bench and require systematic testing in experimental models which closely reproduce the salient features of the human disease. Herein we describe in detail a method to induce new models of GBM with transposon-mediated integration of plasmid DNA into cells of the subventricular zone of neonatal mice. We present a simple way to clone new transposons amenable for genomic integration using the Sleeping Beauty transposon system and illustrate how to monitor plasmid uptake and disease progression using bioluminescence, histology and immuno-histochemistry. We also describe a method to create new primary GBM cell lines. Ideally, this report will allow further dissemination of the Sleeping Beauty transposon system among brain tumor researchers, leading to an in depth understanding of GBM pathogenesis and progression and to the timely design and testing of effective therapies for patients.
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If this article needs to be filmed by a certain date to due to author/equipment/lab availability, please indicate the date below and explain in your cover letter.	

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To the editors of the Journal of Visualized Experiments:

Dear Editor,

Please consider for publication our manuscript entitled: "Transposon mediated integration of plasmid DNA into the subventricular zone of neonatal mice to generate novel models of glioblastomas".

We have prepared the technical report submitted hereby to instruct a wide number of researchers about an easy, rapid and versatile method of creating new models of glioblastomas in mice using the Sleeping Beauty transposase system by injecting plasmid DNA into the lateral ventricles of neonatal mice. We demonstrate that tumors generated with this model reproduce the salient features of the human disease and are amenable to test new therapeutic approaches for GBM. Ultimately our hope is that many more researchers will use this method to accelerate discovery in the field of glioblastoma, knowledge which will be rapidly translated to the clinic to improve patient outcome.

The procedure to inject plasmid DNA into the ventricles of neo-natal mice is delicate and requires a good understanding of the different steps involved and an accurate perception of the spatial coordinates required for a successful injection. The format of your visual journal is ideally suited to convey such knowledge for reproducible outcomes.

We have prepared this report at the invitation of your editor Nandita Singh.

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Acquisition of data:	Anda-Alexandra Calinescu, Carl Koschmann, Felipe Núñez and Brad Kolb
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Thank you so much for your kind consideration,
Sincerely,

Maria G. Castro
Professor

Title:

Transposon mediated integration of plasmid DNA into the subventricular zone of neonatal mice to generate novel models of glioblastoma

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Glioblastoma models, Sleeping Beauty transposase, subventricular zone, neonatal mice, cloning of novel transposons, genomic integration, GBM histology, GBM neurospheres.

Short Abstract:
Here we describe an efficient and versatile protocol to induce, monitor and analyze novel glioblastomas (GBM) using transposon DNA injected into the ventricles of neonatal mice. Cells of the subventricular zone, which take up the plasmid, transform, proliferate and generate tumors with histo-pathological characteristics of human GBM.

Long Abstract:
An urgent need exists to test the contribution of new genes to the pathogenesis and progression of human glioblastomas (GBM), the most common primary brain tumor in adults with dismal prognosis. New potential therapies are rapidly emerging from the bench and require systematic testing in experimental models which closely reproduce the salient features of the human disease. Herein we describe in detail a method to induce new models of GBM with transposon-mediated integration of plasmid DNA into cells of the subventricular zone of neonatal mice. We present a simple way to clone new transposons amenable for genomic integration using the Sleeping Beauty transposon system and illustrate how to monitor plasmid uptake and disease progression using bioluminescence, histology and immuno-histochemistry. We also describe a method to create new primary GBM cell lines. Ideally, this report will allow further dissemination of the Sleeping Beauty transposon system among brain tumor researchers, leading to an in depth understanding of GBM pathogenesis and progression and to the timely design and testing of effective therapies for patients.

Introduction:
Glioblastoma multiforme (GBM) is the most common (60%) primary brain tumor in adults, with a median survival of 15-21 months when treated with surgery, radiation therapy, and chemotherapy[1]. Novel therapies for GBM are imperative. Experimental therapies require testing in animal models which adequately reproduce the salient features of the human disease. Strategies to induce GBM in rodents include chemical mutagenesis with alkylating agents, germline or somatic genetic alterations, or transplantation using glioma cell lines[2]. The most commonly used models employ the implantation of glioma cell lines, either orthotopically, into the brain or subcutaneously using syngeneic cells in animals with identical genotype; or xenogeneic cells, most commonly human GBM cell lines, implanted in immune compromised mice[3]. Xenografts offer many advantages for the study of intracranial tumors: convenience of reproducibility, standardized growth rates, time of death and tumor localization.

However, these models have limitations due to the artificial, invasive surgical approach used for implantations and limited ability to accurately reproduce histological features characteristic of human GBM (WHO grade IV): pseudo-pallisading necrosis, nuclear atypias, diffuse invasion, micro-vascular proliferation and the formation of glomeruloid vascular abnormalities[4-7]. Induction of GBM by altering the genome of somatic cells with oncogenic DNA, either with viral vectors[8-12], or with transposon-mediated integration[13], reproduces more closely the etiology of human disease and recapitulates histo-pathological features of human GBM.

Historically, tumors of the CNS have been classified based on the perceived cell of origin, which it was observed, would be a predictive factor for survival[14]. GBMs are classified into primary and secondary. Emerging evidence today points to the highly heterogeneous nature of primary glioblastoma[15]. Secondary GBM (15%), the result of malignant transformation of low grade astrocytomas (WHO grade I) and anaplasic astrocytomas (WHO grade II), are associated with earlier onset of the disease, better prognosis and a "proneural" pattern of gene expression, whereas primary GBM (85%) show a late onset, poor prognosis and glial (classical), neural or mesenchymal expression patterns. Whether these patterns of gene expression correlate with the actual cell of origin of the tumor is still being actively investigated. Accumulating data shows that the combination of genetic mutations associated with GBMs are predictive for survival. For example, loss of heterozygocity (LOH) of chromosomes 1p/19q, IDH1 mutations, PDGFRα amplifications, are associated with secondary GBMs, proneural expression pattern and better prognosis, whereas EGFR overexpression, Notch and Sonic hedgehog pathway activation, Nf1 and PTEN loss and mutations of p53 are correlated with neural, classical, or mesenchymal primary GBM and worse prognosis[16,17]. The advent of large scale sequencing projects and the accumulation of numerous patient specimens available for testing brings a wealth of new information with respect to genetic mutations and pathways implicated in GBM pathogenesis and progression and the possibility of individualized medicine, where therapies can be specifically tailored to the genetic abnormalities of the patient. Ultimately, to assess the predictive value of these mutations and pathways in a systematic way, and to test possible treatments in each case, requires animal models of GBM with pre-determined genetic alterations. Transposon mediated integration of genomic DNA offers a feasible approach.

The Sleeping Beauty transposon system, member of the Tc1/*mariner* class of transposons, was "awakened" (constructed) in a multi-step process of site specific mutagenesis from a salmonoid transposase gene, which became dormant more than 10 million years ago[18,19]. In essence, DNA transposons flanked by specific sequences (inverted repeats/direct repeats: IR/DR) can be integrated into the genome in a "cut and paste" manner by means of the activity of the Sleeping Beauty transposase. The transposase recognizes the ends of the IR sites, excises the transposon and integrates it randomly into another DNA site between the bases T and A, bases which are duplicated at each end of the transposon during transposition (**Figure 1a**). The Sleeping Beauty transposase is comprised of three domains, a transposon binding domain, a nuclear localization sequence and a catalytic domain. Four transposase molecules are

required to bring the two ends of the transposon together and allow for transposition, however, if too many molecules of transposase are present, they can dimerize and tetramerize to inhibit the transposition reaction[20]. An efficient transposition reaction requires an optimal ratio of transposase to transposons. The DNA encoding the transposase can be delivered on the same plasmid with the transposon (in cis) or on a different plasmid (in trans). To ensure the optimal ratio between the transposase and the transposons, a promoter with adequate activity can be chosen for the expression of the transposase (for the "cis" model) or the ratio of the plasmids in the injection solution can be optimized (for the "trans" model). The Sleeping Beauty transposon system can be used successfully for functional genomics, insertional mutagenesis, transgenesis and somatic gene therapy[21]. Being a synthetic construct re-engineered to a functional molecule from a dormant salmonoid variant, the Sleeping Beauty transposase does not bind to other transposons in humans or other mammals[20]. Since its discovery, molecular engineering has enhanced the transposition efficacy of the SB transposon system through changes in the IR sequences and addition of TATA dinucleotides flanking the transposon, resulting in the pT2 transposons. These transposons have optimized binding of the SB to the IR site and increased efficacy of excision. The SB transposase also underwent significant improvement; the transposase used in experiments presented herein is the SB100X, a hyperactive transposase generated by a DNA shuffling strategy followed by a large-scale genetic screen in mammalian cells[22].

In this report we present a rapid, versatile and reproducible method to induce intrinsic GBM in mice with non-viral, transposon-mediated integration of plasmid DNA into cells of the sub-ventricular zone of neonatal mice[23]. We present a simple way to create transposons with novel genes amenable for genomic integration using the Sleeping Beauty transposase and demonstrate how to monitor plasmid uptake and disease progression using bioluminescence. We also characterize histological and immuno-histochemical features of the GBM reproduced with this model. In addition, we present a quick method to generate primary GBM cell lines from these tumors. The Sleeping Beauty model, in which tumors are induced from cells original to the animal, allows the functional assessment of the role of candidate GBM genes in the induction and progression of tumors. This system is also well suited for the testing of novel GBM treatments, including immune therapies in immuno-competent mice, without the need of invasive inflammatory surgical procedures, which may alter the local microenvironment.

Protocol:

All animal protocols have been approved by the University of Michigan Committee for the Use and Care of Animals (UCUCA).

1.   Cloning of the sequence of interest into new Sleeping Beauty transposons

Note: To insert genes or inhibitory elements (as shRNA) using the Sleeping Beauty transposase system, clone the sequence of interest into the backbone of a pKT or pT2 plasmid. Direct the cloning such that the regulatory elements, gene of interest and markers remain flanked by the inverted repeats (IR/DR). An example of cloning PDGFβ

into a pKT backbone is detailed below (see also **Figure 1b**).

1.1.   Digest 5 µg of plasmid vector pKT-IRES-Katushka for 4 hours at 37 °C with 5-10 units of XbaI/XhoI restriction enzymes in 50 µl of reaction volume.

1.2.   Digest the mPDGFβ cDNA of 5 µg of the pGEM-T- mPDGFβ plasmid for 4 hours at 37 °C with 5-10 units of NcoI/SacI restriction enzymes.

1.3.   Precipitate the total DNA by adding 2.5 volumes of 100% ethanol, and 1/10 volume of 3 M Na Acetate, pH 5.8 and incubate overnight at -20 °C.

1.4.   Centrifuge the samples at 13,800 x g for 15 min.

1.5.   Wash the DNA pellet with 500 µl of 70% ethanol, centrifuge at 13,800 x g for 10 min, repeat once and re-suspend in 19 µl of sterile DNase free water.

1.6.   Follow the manufacturer's instructions in the Quick Blunting Kit to blunt the ends of both vector (pKT-IRES-Katushka) and insert (mPDGFβ).

1.7.   Load the blunted vector and insert on a 1.2% ethidium bromide stained agarose gel and run at 80 V for 40 min. Visualize the bands, excise them with a clean razor-blade and gel purify the DNA using a gel purifying kit according to manufacturer's protocol.

1.8.   Ligate the insert and vector DNA purified in step 1.7 by adding them in a tube at a 4:1 molar ratio (insert:vector). Into this tube, pipette 1 µl of T4 ligase and 2 µl of T4 ligase buffer (containing ATP), and adjust the volume to 20 µl with sterile water. Incubate the ligation reaction for 18 h at 16 °C.

1.9.   Transform chemically competent DH5α bacterial cells with the ligated products.

1.9.1. Thaw the competent cells on ice.

1.9.2. Add the entire volume of the ligation reaction to 50 µl of competent cells, gently shake the tube and incubate the mix on ice for 30 minutes. Heat-shock the bacteria for 45 s at 42 °C and return immediately to ice; incubate on ice for another 2 min.

1.9.3. Add 950 µl of Luria Broth to the culture and incubate with shaking at 37 °C for 1 h. Centrifuge bacteria at 2400 x g for 3 min and re-suspend the pellet in 200 µl of LB.

1.9.4. Spread the transformed bacteria onto a Petri dish coated with LB-agarose and the corresponding antibiotic. Incubate the plate overnight at 37 °C.

1.9.5. Finally, collect 5-10 bacterial colonies and culture overnight at 37 °C in 5 ml of LB media with antibiotic.

1.9.6. Purify plasmid DNA using a plasmid DNA mini kit according to manufacturer's instructions. Perform a diagnostic restriction digest to verify the proper incorporation of the insert into the vector and submit positive clones for DNA sequencing to ensure the right orientation of the insert.

1.10   Select the correct colonies and scale up the DNA production using a high quality, endotoxin free plasmid preparation kit.

1.11   Elute the DNA in sterile water or 1 mM Tris-HCl. Store DNA at -20 °C until ready to inject into neonates.

2   Intra-ventricular neonatal injections

2.1.   Three to four weeks prior to experiment, set up a mouse breeding cage with one male and one female mouse, and a plastic colored igloo. This provides an enriched environment and aids the mating process.

2.2.   When pregnancy is confirmed, remove the male to a new cage. Eighteen days after mating, monitor cage daily for delivery. Perform intraventricular injections on postnatal day 1 (P1).

2.3.   Preparation of the injection solution

Note: The injection solution is made by mixing the plasmid DNA with the transfection reagent to create the particles for transfection. Below is an example on the preparation of an injection solution using a plasmid encoding the Sleeping Beauty transposase and luciferase: pT2/SB100x-Luc and two transposon plasmids: pT/CAGGS-NRASV12 and pT/CMV-SV40-LgT, at a ratio of 1:2:2. All the plasmids have a concentration of 2 µg/µl. Important details on the preparation of the injection solution are presented in the Discussion.

2.3.1. Prepare the DNA solution by adding: 4 µg (2 µl) of pT2/SB100x-Luc, 8 µg (4 µl) of pT/CAGGS-NRASV12 and 8 µg (4 µl) of pT/CMV-SV40-LgT and 10 µl of 10% glucose to a final volume of 20 µl at a concentration of 1 µg /ml DNA and 5% glucose.

2.3.2. Prepare the PEI solution by adding 2.8 µl of *in vivo* jet PEI, 7.2 µl of sterile water, and 10 µl of 10% glucose to a final concentration of 5% glucose.

2.3.3. Add the PEI solution to the DNA solution, mix and vortex and let sit at room temperature (RT) for 20 min. The solution is now ready for injection. After one hour at RT, keep the solution on ice.

2.4.   Fit a 10 µl clean syringe equipped with a 30 gauge hypodermic needle with 12.5° bevel into the micropump (**Figure 2 #1**).

2.5.   To verify proper functioning of the injection system, use the automatic injector

(**Figure 2 #1**) to withdraw 10 μl of water into the syringe and then empty the syringe completely

2.6.   Cool the neonatal stereotaxic stage (**Figure 2 #3**) with a slurry of dry ice and alcohol to a temperature of 2-8°C.

2.7.   Anesthetize a pup by placing it on ice for 2 minutes.

2.8.   Fill the syringe with the DNA/PEI solution.

2.9.   Immobilize the pup in the stereotaxic frame by placing its head between the gauze covered ear bars (**Figure 2b).** Ensure that the dorsal side of the skull is horizontal, parallel to the surface of the frame and that the cranial sutures are clearly visible. Wipe the head with 70% ethanol.

2.10.   Lower the needle of the syringe and adjust stereotaxic coordinates using dials of the stereotaxic frame until the needle touches the bregma (the midline intersection of the parietal and occipital bones) (**Figure 2b**).

2.11.   Lift the needle and adjust stereotaxic coordinates to 0.8 mm lateral and 1.5 mm rostral to the bregma (**Figure 2b**).

2.12.   Lower the needle until it touches and slightly dimples the skin. Measure the coordinates. Lower the needle another 1.5 mm. The needle will pierce the skin and skull, and pass through the cortex to enter the lateral ventricle (**Figure 2c**).

2.13.   Using the automatic injector, inject 0.75 μl of the DNA/PEI solution at a rate of 0.5 μl/min

2.14.   Keep the pup on the frame for another minute to allow for the solution to disperse into the ventricles. In the meantime, anesthetize another pup, on ice for 2 min in preparation for the next injection.

2.15.   Gently and slowly lift the needle, and place the pup under a heating lamp. Monitor breathing and activity. If necessary, provide gentle stimulation of the limbs until first breathing ensues.

2.16.   Return the pup to its mother after it has warmed up, has reached a rosy color, is breathing regularly and is active, normally 5-7 minutes. If more than one pup is injected at a time, keep all pups together until all the injections are done. If injections take longer than 30 min, return half of the pups after the first 30 min and the rest at the end of the procedure.

2.17.   Monitor that the dam is responsive and nurturing to her pups. If necessary, add a surrogate mom to the cage.

2.18.   Ensure that the interval between placing the pup on ice and placing it under the warming lamp is less than 10 minutes. The quicker the procedure the better the survival. Usually, the time it takes to anesthetize and inject a pup is 4 minutes.

3.   Bioluminescence monitoring of tumor formation and progression

3.1.   Monitoring plasmid uptake after injection

3.1.1. 24-72 hours after injection, remove all pups from the mother's cage and place in a 6 well clean tissue culture dish, one pup per well.

3.1.2. Prepare a 30 mg/ml solution of luciferin dissolved in normal saline or phosphate buffer. Prepare this solution in advance and keep in small aliquots at -20 °C.

3.1.3. Using a 1 ml syringe equipped with a 30 gauge hypodermic needle inject 30 µl of luciferin subcutaneously between the shoulder-blades of the pup. Ensure that the needle does not pierce any organs, by pinching the skin and lifting it slightly before inserting the needle.

3.1.4. Image immediately, using an *in vivo* bioluminescence imaging system. For the IVIS Spectrum, use the following settings for acquisitions: automatic exposure, large binning, and aperture f=1.

3.2.   Monitoring tumor formation and progression

Note: Animals will start forming macroscopic tumors detectable by bioluminescence and histology within 2½-6 weeks, depending on the oncogenic plasmids injected.

3.2.1. Using a 1 ml syringe equipped with a 26 gauge needle inject 100 µl of a 30 mg/ml luciferin solution intra-peritoneally.

3.2.2. Place the animal in the anesthesia chamber. Inject up to five animals at the same time.

3.2.3. Wait 5 minutes then start the oxygen/isoflurane flow (1.5-2.5% isoflurane) to anesthetize the animals. After 3-4 minutes, remove the animals from the anesthesia chamber, start the oxygen /isoflurane flow in the bioluminescence chamber. Place the animals in the respective slots equipped with glass nose cones to allow for anesthesia and unobstructed passage of light.

3.2.4. Typically, acquire a series of 6 images, at 2 min intervals with an automatic exposure time, median binning and an open aperture of f=1.

3.2.5. Set the region of interest for all the animals imaged, typically an oval over the head region, and measure the luminescence intensity using the calibrated units photons/s/cm^2/sr.

4.   Histological and immuno-histochemical analysis of new GBMs

Note: When the tumors have reached the experimental time-point desired, animals can be sacrificed with brains perfused, fixed and analyzed following standard protocols and procedures. A brief description of these methods is presented below.

4.1.   Perfusion and Fixation

4.1.1.   Anesthetize the mouse with an intra-peritoneal injection of 100 mg/kg of ketamine and 10 mg/kg of xylazine.

4.1.2. Check the pedal reflex by pinching the foot of the mouse. When this reflex is abolished, indicating deep anesthesia, immobilize the mouse on a dissection board with pins, open the abdominal cavity, dissect the diaphragm and open the thoracic cavity to visualize the heart.

4.1.3. Insert a blunt 20 gauge cannula into the left ventricle of the heart. Connect the cannula with plastic tubing passing through a peristaltic pump to a container with oxygenated and heparinized Tyrode's solution (0.8% NaCl, 0.0264% $CaCl_2$ $2H_2O$, 0.005% NaH_2PO_4, 0.1% glucose, 0.1%$NaHCO_3$, 0.02% KCl). Start the flow of Tyrode's solution by adjusting the speed of the peristaltic pump to ensure a slow and constant flow, approximately one drop per second or less if the animals are smaller.

4.1.4. Make a small incision into the right atrium of the heart to permit the drain of blood and Tyrode's.

4.1.5. Monitor perfusion efficiency by observing blanching of internal organs (most obvious in the liver and kidney) as they become devoid of blood.

4.1.6. When the solution draining from the heart is clear (3-5 minutes), switch the solutions from Tyrode's to 4% paraformaldehyde for fixation (4% PFA, pH 7.34 in phosphate buffered saline).

4.1.7. Monitor good fixation of the tissues by the increased rigidity of the neck and tail.

4.1.8. Decapitate the mouse and carefully dissect the brain from the skull.

4.1.8.1.   Pull the fur which covers the skull rostrally, towards the nose, and grab it firmly to stabilize the head, dorsal side up.

4.1.8.2.   Using a sharp scalpel, cut downwards along the edge of the orbit, to

transect the orbital muscles and the underlying zygomatic arches.

4.1.8.3. Turn head ventral side up and remove attached neck muscles using a rongeur. Crush the first vertebra and remove it from the brainstem.

4.1.8.4. Visualize the cochlea, grab the overlying temporal bone with the rongeur and create an opening between the temporal and occipital bone. Peel off the occipital bone from the surface of the cerebellum.

4.1.8.5. Crush the bone overlying the nose with the rongeur. Carefully remove the remaining frontal and parietal bones from the surface of the brain being mindful not to damage the surface of the brain. Pay particular attention in the orbital area.

4.1.8.6. Turn the head ventral side up. The brain will slide down from the remainder of the skull, connected now solely through the cranial nerves. Using small scissors cut the olfactory, optic and trigeminal nerves. This will release the brain.

4.1.9. Place brain in 4% PFA overnight at 4 °C for post-fixation.

4.1.10. For histological analysis and hematoxylin-eosin staining, transfer the brains to phosphate buffer for 24 h and then proceed to paraffin embedding.

4.1.11. For immuno-histological analysis, transfer brains into a 30% sucrose solution in phosphate buffered saline for 24-48 h (until the brains sink to the bottom of the tube)

4.1.12. Section brains in half through the middle of the tumor region, place the cut side down into a freezing mold, embed into cryo-embedding media (OCT compound), and flash-freeze in a slurry of dry-ice ethanol, dry-ice isopentane or into liquid nitrogen. Keep frozen blocks at -80 °C until sectioning and staining.

4.2. Hematoxylin-Eosin staining of paraffin embedded brains for histo-pathological analysis of intrinsic GBMs

4.2.1. Section paraffin embedded brains at 4 µm thickness, drop in a 42 °C water bath and mount onto glass slides.

4.2.2. De-paraffinize slides by placing them for 10 min in a 60 °C oven and then transferring them at 5 min intervals through a series of three slide containers filled with xylene.

4.2.3. Hydrate the slides through a series of ethanol baths: 100% ethanol for 2 min, repeat once, 95% ethanol for 2 min, repeat once, 70% ethanol for 2 min and then transfer slides to water.

4.2.4. Stain the slides by dipping them in a slide container filled with Harris Hematoxylin

for 2 min.

4.2.5. Rinse in tap water until water is clear.

4.2.6. Dip slides twice into bluing solution (0.1% Sodium Bicarbonate).

4.2.7. Let slides stand in tap water for 2 min, then transfer to 80% ethanol for 2 min.

4.2.8. Stain the slides by dipping them in a container filled with Eosin for 5 min.

4.2.9. Dehydrate slides in a series of ethanol baths (80% ethanol 3-4 dips, 95% ethanol 2 min, repeat once, 100% ethanol 2 min, repeat once).

4.2.10.   Clear with 3 consecutive baths of xylene, 3 min each.

4.2.11.   Coverslip with a xylene-based mounting medium and let dry on a flat surface at room temperature until ready for imaging. Store at room temperature.

4.3.   Immuno-histochemistry of cryo-preserved embedded brains for molecular and cellular characterization of de novo induced GBMs

4.3.1. Retrieve frozen blocks from the -80 °C storage, place into cryostat and allow temperatures to equilibrate for 30 min.

4.3.2. Section 12-14 µm thick sections and mount 2-3 sections onto positively charged glass slides. Cut sections serially, by collecting adjacent sections on different slides. This allows the staining with different antibodies on adjacent tissue sections within the tumor.

4.3.3. Dry slides in a desiccator for at least 1 h after sectioning to allow for good adherence of the tissue.

4.3.4. Create a hydrophobic barrier (with a hydrophobic marker) at each end of the slide to allow for liquid to cover the sections and to stay on the slide during the washes. Cover the sections with a solution of Phosphate Buffered Saline with 0.1% Triton-X (PBS 0.1% Tx) and gently shake for 5 min on a horizontal shaker to permeabilize the tissue. Repeat twice.

4.3.5. Block nonspecific binding with a solution of 10% normal goat serum (or serum from the animal in which the secondary antibody was raised) for 30 min with gentle shaking

4.3.6. Prepare primary antibody solution in 2% normal goat serum in PBS 0.1% Tx and pipette the solution over the sections. Place slides in a covered humid chamber in the dark and keep at room temperature overnight.

4.3.7. The next day, wash sections three times for 5 min with PBS 0.1% Tx and incubate in fluorescent secondary antibody diluted in 2% normal goat serum PBS 0.1% Tx for one hour.

4.3.8. Wash sections three times for 5 min with PBS 0.1% Tx, counterstain with a nuclear stain (e.g., DAPI) for 2 min, wash once more, clear in water and coverslip with a water based mounting medium that will preserve the fluorescence. Place slides on a flat surface and let them cure for at least 24 h, in the dark, until ready for imaging. Keep at 4 C thereafter.

5.   Generation of primary tumor cell lines with specific genetic alterations.

5.1.   To generate cell lines from Sleeping Beauty tumor-bearing mice select a mouse with confirmed large tumor (bioluminescence 10^7-10^8 photons/s/cm^2/sr).

5.2.   Euthanize the mouse with an overdose of isoflurane anesthetic, decapitate the animal and carefully dissect the brain from the skull (as described in section 4). Place the brain in a clean Petri dish.

5.3.   Using a razor blade, make coronal cuts anterior and posterior to the tumor. The location of the tumor is usually recognizable due to the transparency of the brain.

5.4.   Dissect the tumor, usually 5-7 mm in diameter with fine forceps and place into a 1.5 ml tube filled with 300 µl Neural Stem Cell Media (NSC Media: DMEM/F12 with B-27 supplement, N2 supplement, and Normocin, supplemented with human recombinant EGF and bFGF at a concentration of 20 ng/ml each).

5.5.   Gently homogenize tumor with a plastic pestle, which fits to the walls of the tube.

5.6.   Add 1 ml of enzyme-free tissue dissociation solution and incubate at 37 °C for 5 minutes.

5.7.   Pass cell suspension through a 70 µm cell strainer, wash the strainer with 10 ml of NSC media, centrifuge at 300 x g for 4 minutes, decant supernatant and re-suspend pellet into 7 ml of NSC media.

5.8.   Plate onto a T25 culture flask and culture at 37 °C in a tissue culture incubator with and atmosphere of 95% air and 5% CO_2. After 3 days, some cells have died, some adhered to the bottom of the culture dish, and some are forming neurospheres, freely floating in the media.

5.9.   Remove these suspended cells and re-plate onto a T75 tissue culture flask.

5.10.   After another three days of culture, collect neurospheres, dissociate as described in step 5.6, strain through cell strainer and count cells. Freeze aliquots of cells in Fetal Bovine Serum 10% DMSO and passage cells at a density of one million cells in 14 ml of

NSC supplemented with EGF and FGF for a T75 flask. The growth rate will depend on the oncogenes used to generate tumors. Adapt cell culture conditions to prevent overgrowth and maintain cells at relatively high density.

Representative Results:
To characterize histo-pathological features of SB-induced glioblastomas, C57/ BL6 neonatal mice were injected at P1 with a plasmid encoding luciferase (pT2/SB100x-Luc) in combination with plasmids encoding transposons with oncogenic DNA, i.e., NRAS (pT/CAGGS-NRASV12) and SV40 LgT (pT/CMVSV40-LgT) (**Figure 3c**) or a plasmid encoding a short hairpin p53 with PDGFβ and a GFP reporter (pT2shp53/GFP4/mPDGFβ) in combination with NRAS (**Figure 3d**). Animals were monitored for bioluminescence the day after injection (**Figure 2a**) and periodically until reaching the moribund stage when they were euthanized. Moribund stage is defined as the clinical stage when the animal becomes symptomatic showing impaired mobility, hunched posture, scruffy fur and weight loss. Sometimes, animals develop seizures or abnormal patterns of movement, like walking in circles and sudden jumping. At the end of the experiment animals were anesthetized. The brains were perfused, embedded in paraffin, and processed for hematoxylin and eosin staining. Data show tumors displaying the hallmarks of human GBM (WHO grade IV) with hemorrhages (**Figure 3c**), pseudo-pallisading necrosis (**Figure 3e**) and perivascular and diffuse invasion into the brain parenchyma (**Figure 3g, f**). The formation of pseudopallisades is preceded by the rupture of large glomeruloid vessels with leaky endothelia (**Figure 3i**) which result in regions of hemorrhage with massive infiltration of mononuclear cells(**Figure 3h**). Atypical mitoses (**Figure 3j**) and gigantic multinucleated tumor cells (**Figure 3k**) are also pathognomonic of human GBM.

Glioblastomas generated using the Sleeping Beauty transposase system can be monitored throughout the progression of tumor growth with bioluminescence, if the plasmids injected encode luciferase. An example experiment is shown in **Figure 4a**. Animals usually succumb of tumor burden when luminescence reaches an intensity of 10^7-10^9 photons/s/cm^2/sr. The median survival of animals is predictably dependent on the combinations of oncogenic transposons injected into the neonatal brain, as illustrated in the survival curves presented in **Figure 4b**. Note that the most aggressive tumors are induced with NRAS and SV40 LgT antigen (median survival of 30 days), whereas the median survival of animals with GBM induced with shp53 NRAS and PDGF is 62.5 days and of animals injected with shp53 and NRAS 83 days.
De novo formed tumors can be characterized immuno-histochemically by their expression of molecules characteristic of glial tumors. **Figure 5** shows a nascent tumor (22 dpi, bioluminescence 2x10^5 photons/s/cm^2/sr), induced with shp53 and NRAS. The injected shp53 plasmid encodes for green fluorescent protein (GFP) to allow the identification of transfected cells and their progeny (pT2/shp53/GFP). GFP+ tumor cells express the neural stem cell marker nestin and some GFP+ cells also express glial fibrillary acidic protein (GFAP). **Figure 6** illustrates a tumor from a moribund animal at 56 dpi, tumor which was also induced with shp53/GFP and NRAS. Numerous GFAP+ astrocytes are surrounding the tumor. GFP+ tumor cells express nestin, but not GFAP.

A great advantage when using this technique to induce GBMs is the ability to generate novel GBM cell lines with unique genetic alterations by means of the transposons injected. In addition, custom cell lines can be generated using transgenic animals with specific genetic makeup. These cell lines are instrumental in asking many mechanistic questions using biochemical assays. They are ideally suited for cytotoxicity studies with novel chemotherapeutic agents. **Figure 7** illustrates a neurosphere from a Sleeping Beauty tumor induced with shp53, PDGF and NRAS, showing expression of GFP, which is encoded on one of the injected plasmids. Note that expression is not equally intense in all the cells, indicating the heterogeneous nature of these primary GBM cells.

Figure 1. a) Schematic representation illustrating the "cut and paste" mechanism used by the Sleeping Beauty transposase to integrate transposons into the host chromosomal DNA.
A donor transposon plasmid is depicted with the gene of interest flanked by the inverted repeats/direct repeats (IR/DR; red arrow boxes) sequences. The SB transposase (green) binds to the IR/DR, excises the transposon and reintegrates it in between random TA dinucleotide base pairs on the host chromosomal DNA.
b) Example of cloning a gene of interest (mPDGFβ**) into the backbone of a SB vector (**pKT-IRES-Katushka). The SB vector contains two IR/DR repeat sequences (red) and a gene expression cassette which includes promoter and enhancer sequences, a multiple cloning site (MCS; blue), internal ribosomal entry sites (IRES), a fluorescent reporter marker (Katushka: yellow), and a polyadenilation site (polyA). The oncogene of interest (mPDGFβ; orange) is contained in a cloning vector pGEMT. The oncogene, flanked by specific restriction sites (NcoI/SacI) is released by enzymatic digestion, and blunted by the blunting enzyme. The vector is linearized and blunted as well. Finally, the mPDGFβ oncogene is inserted into the donor vector by means of a blunt-end ligation reaction to generate the new plasmid transposon vector: pKT-mPDGFβ-IRES -Katushka.

Figure 2. Experimental Setup and guides for intra ventricular injections in neonatal mice
(a) A stereotaxic frame (2) with micrometer dials is fitted with an automatic injector holding a 10 µl syringe (4). Inside the U frame, a neonatal adaptor frame is securely fastened (3). The control panel of the automatic injector (1) allows for precise selection of syringe, volume and rate of flow.
(b) Photograph of a neonatal mouse (P1) with a needle inserted at the coordinates required for injections into the lateral ventricle: 1.5 mm ventral and 0.8 mm lateral to the bregma.
(c) Illustration of a coronal section through the brain of a neonatal mouse (P1) highlighting the relative dimensions and position of the ventricles.

Figure 3. Tumors induced with the Sleeping Beauty transposon system show the histological hallmarks of human GBM
(a) Bioluminescence image of a neonatal pup 24 h after intraventricular injection with plasmids encoding NRAS, SV40 LgT. **(b)** Bioluminescence image of an adult animal (50 dpi) with a large tumor induced with shp53 NRAS and PDGF. **(c)** Hematoxilin and eosin

stain of a coronal section from a brain of a moribund mouse with a tumor induced with NRAS and LgT (28 dpi). **(d)** Coronal section from a brain of a moribund mouse with a tumor induces with shp53 NRAS and PDGF. **(e)** Pseudopalisading necrosis, histological hallmark of human GBM is observed in *de novo* generated tumors. Arrow points to cells arranged in palisades migrating away from the central area of necrosis (N) **(f)** and **(g)** *de novo* generated tumors are highly invasive, showing invasion along blood vessels **(g)** and diffuse **(f)** into the normal brain parenchyma**. (h)** Region of hemorrhage with massive invasion of mononuclear cells (arrow), the initial stage of an area of pseudopalisading necrosis. **(i)** Large glomeruloid vessel with leaky endothelium (arrow) at the origin of diffusing hemorrhage inside a tumor induced with NRAS and SV40 –LgT. **(j)** Atypical mitosis in a tumor cells (arrowhead). **(k)** Gigantic tumor cell with multiple large nuclei (arrowhead).

Figure 4. Tumor-bearing animals can be monitored with bioluminescence throughout the duration of disease progression. The median survival of animals is predicted by the combination of oncogenic DNA injected. (a) Example of bioluminescent traces from a cohort of 7 C57/BL6 mice. Note that mice succumb about a week after bioluminescence reaches 10^8 photons/s/cm2/sr. **(b)** Sample survival curves comparing median survival of animals with Sleeping Beauty tumors generated with different plasmid combinations. Median survival of tumors induced with NRAS and SV40 LgT is 30 dpi whereas of animals with tumors induced with shp53 NRAS and PDGF or shp53 and NRAS is 62.5 dpi or 83 dpi respectively. dpi: days post injection.

Figure 5. Nascent macroscopic GBM (22 dpi) induced with shp53 and NRAS show expression of nestin and GFAP in GFP+ tumor cells (confocal micrographs). Panel **(a)** shows a nascent tumor expressing GFP. Panel **(b)** represents the same field showing nestin expression. Panel **(c)** is an overlay of **(a)** and **(b)** illustrating co-localization of nestin and GFP**. (d)** GFP expression in tumor cells. **(e)** GFAP expression in some tumor cells and in cells surrounding the nascent tumor. **(f)** Overlay projection of **(d)** and **(e)** illustrating some tumor cells (white) co-expressing GFAP and GFP. Scale bars in (a) and (d) represent 75 µm.

Figure 6. GBM induced with shp53, NRAS and PDGF from a moribund animal (56 dpi) showing GFP and nestin expression in tumor cells and abundant staining for the mature glial marker GFAP surrounding the tumor. Panel **(a)** represents Nissl stain of a coronal section through the brain of a moribund animal with a tumor (intense blue staining from increased cellularity) induced with the Sleeping Beauty transposon system using shp53, PDGFB and NRAS. Panel **(b),** a coronal adjacent section to the one illustrated in panel (a) stained with the nuclear stain DAPI, showing expression of GFP in tumor cells. Panel **(c)** represents a confocal micrograph of the tumor border showing GFP expression in the tumor, identified by the high nuclear density with DAPI. Panel **(d)** illustrates the same field of view as in **(a),** showing intense GFAP expression in cells adjacent to the tumor. Panel **(e)** represents the overlay of panels (c) and (d). Panel **(f)** is a confocal micrograph of the tumor border, showing GFP expression in tumor cells. Panel **(g)** represents the same field of view as

in **(f)**, showing expression of nestin in tumor cells. Panel **(h)** is the overlay projection of panels (f) and (g). Scale bar in (c) represents 150 µm and in (f) represents 75 µm.

Figure 7. Neurospheres from a Sleeping Beauty tumor induced with shp53, PDGF and NRAS after 5 days in culture, passage 2. The cells are expressing GFP encoded on the shp53 PDGF plasmid (pT2shp53/GFP4/mPDGFβ).
Panel **(a)** is a brightfield micrograph of a neurosphere. Panel **(b)** represents an epi-fluorescent micrograph of the same view as in (a) showing expression of GFP in the neurosphere cells. Panel **(c)** represents the overlay of panels (a) and (b).

Discussion:
In this article, we detail a versatile and reproducible method for generating new models of GBM using SB transposase- mediated integration of oncogenic plasmid DNA into cells surrounding the subventricular zone of neonatal mice. We present a protocol to generate transposon plasmids with new genes of interest, illustrate how to monitor the progression of the tumors in live animals, and how to characterize histo-pathological and immuno-histochemical features of these tumors.

As our lab (**Figure 3**) and others[13] have shown, this model reliably creates tumors with the salient characteristics of human GBM including (1) pseudo-palisading necrosis, (2) vascular proliferation, (3) nuclear atypia, (4) abnormal mitoses and (5) perivascular and diffuse invasion. The use of neonatal mice is optimal from a logistical standpoint, providing a relatively easy technical procedure with minimal equipment and sedation/recovery time, allowing for rapid generation of large sample sizes of tumors with specific genetic alterations. These tumors cause animals to succumb to tumor burden with a predictable median survival dependent on the genetic alterations induced. Finally, the SB model has been used as a therapeutic intervention screen for treatment response[24].

There are several important factors to consider when preparing the solutions used for the neonatal injections. DNA solutions need to be sterile, endotoxin free and concentrated (2-7 µg/µl) to allow for minimal volume of injections. The optimal ratio of nitrogen residues in the polyethyleneimine (PEI) to the phosphate residues in the DNA (N/P ratio) is 7. This ensures the formation of positively charged particle complexes which will bind anionic moieties on cell surfaces and will be endocytosed. Once in the cytoplasm, osmotic influx will cause the particles to burst and release the encapsulated plasmid DNA. The *in vivo* jet-PEI solution has a concentration of 150 mM expressed as nitrogen residues. Given that one µg DNA has 3 nmol of anionic phosphate, the amount of transfection reagent necessary can be calculated with the formula:

µl of *in vivo* jet-PEI = [(µg DNA x 3) x N/P ratio]/150.

For example, to prepare 40 µl of 0.5 µg/µl DNA solution with an N/P ratio of 7, 20 µg of DNA are needed. The volume of PEI required will be 20*3*7/150=2.8 µl.

Up to five different plasmids can be injected simultaneously. Experiments presented

herein deliver the Sleeping Beauty transposase in trans on a plasmid that also encodes luciferase (SBLuc). As mentioned in the introduction, the ratio of transposase molecules to transposons is important to ensure optimal transposition. The optimal ratio (empirically established) of the SB100x plasmid to the transposon DNA plasmid is 1:4. Hence, if one uses two different transposon plasmids, T1 and T2 for example, the ratio of SBLuc:T1:T2 will be 1:2:2; or for a total of 20 µg DNA in a 40 µl solution 4 µg of SBLuc will be mixed with 8 µg of T1 and 8 µg of T2. For three different transposon plasmids the ratio will be SBLuc:T1:T2:T3 = 1:1.33:1.33:1.33 and for four transposons: 1:1:1:1:1.

It is useful to verify the uptake of the plasmid DNA by bioluminescence 24-72 h after injection. As the animals grow, the increased optical density of the fur, skin, skull and brain, prevents monitoring of the tumor progression until the tumor has reached a threshold size, roughly 1-2 mm^3 for darkly pigmented C57BL6 mice. Better transmission of the light is possible in these mice if their fur is shaved, or when using mice with white or no fur.

Several limitations need to be considered when using this model. First, the genetic material introduced with the injected plasmids can be integrated randomly in the host genome in different locations and number of copies. This may lead to variability between tumor cells and between tumors. Second, the DNA introduced is inserted primarily into intronic TA repeat sequences DNA[25], however, the possibility of interference with coding host genomic DNA remains. Third, intraventricular injections may cause a small but persistent rate of hydrocephalus in young mice, which becomes clinically visible and symptomatic in the 3rd-4th week of life. Some strains of mice are more prone to hydrocephalus than others (i.e., C57/BL6 more than FVB). To minimize the risk of inducing hydrocephalus, it is recommended to inject as small a volume as possible (less than 1 µl in C57/BL6 mice), to ensure that the needles are sharp, the solutions have low salinity and to provide growing pups with enriched food, in order to allow for timely ossification of the skull bones. Finally, late-stage tumors often develop necrosis, which has to be considered when monitoring mice by bioluminescence. A lower reading may indicate an advanced tumor with large areas of necrosis and not necessarily a resolution of the tumor as a consequence of treatment. Ultimately, histological analysis remains the gold standard to assess tumor progression.

The advent of tumor whole genome sequencing in recent years has opened the door to exploration of the role of recurrently mutated genes in GBM. The Sleeping Beauty model is optimal for validating potential oncogenes or tumor suppressors. As in the example given in this paper with PDGFβ ligand, cloning the mouse cDNA sequence of a candidate oncogene into an established SB plasmid backbone will lead to constitutive expression within transfected cells. Evaluation of the role of tumor suppressors can be effectively performed by cloning into the backbone of the SB plasmid with candidate sequences, (available for example in the RNAi codex database[26]), to create robust expression of second-generation miR short-hairpins.

A current debate in the field of GBM research is related to the identity of the cell-of-origin. It has recently been shown that in mice, tumors induced by down-regulating p53 and Nf1 in neural stem cells, originate from oligodendrocyte precursor cells[27]. Using the Sleeping Beauty transposon model, similar studies can be designed to test whether other genetic alterations preferentially target other populations of stem cells/precursor cells to generate GBMs. Knowledge from such studies will greatly enhance our understanding of GBM etiology and provide avenues for novel therapeutic approaches.

Disclosures:
The authors have nothing to disclose.

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Figure 2

a

1

2

4

3

b

needle

1.5 mm
rostral

0.8mm
lateral

bregma

c

1.5 mm
deep
(ventral)

lateral
ventricle
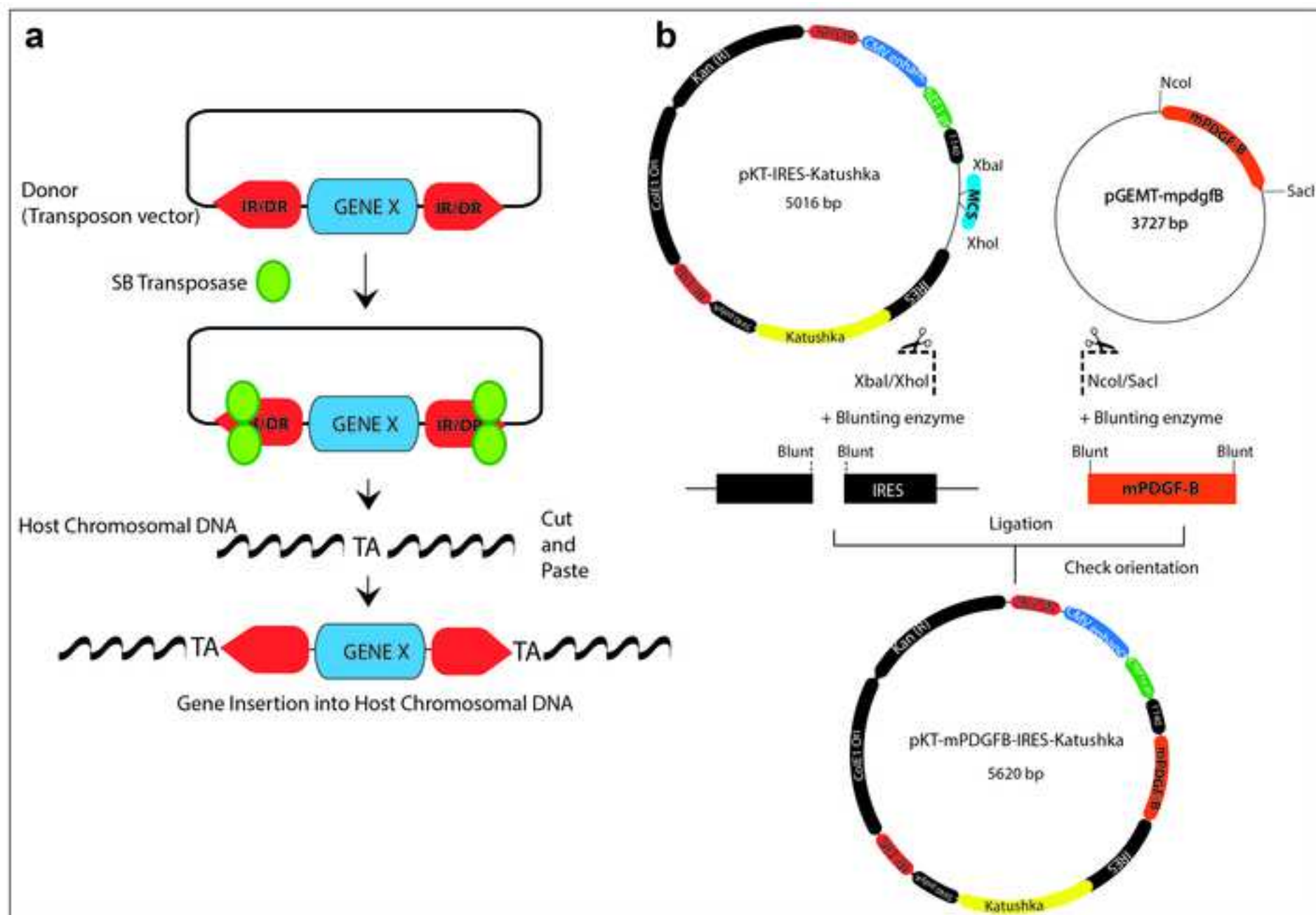
3rd
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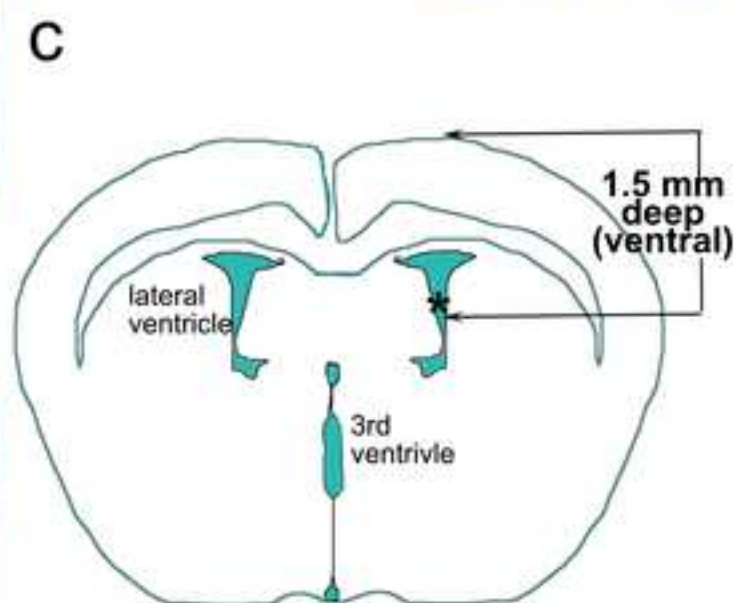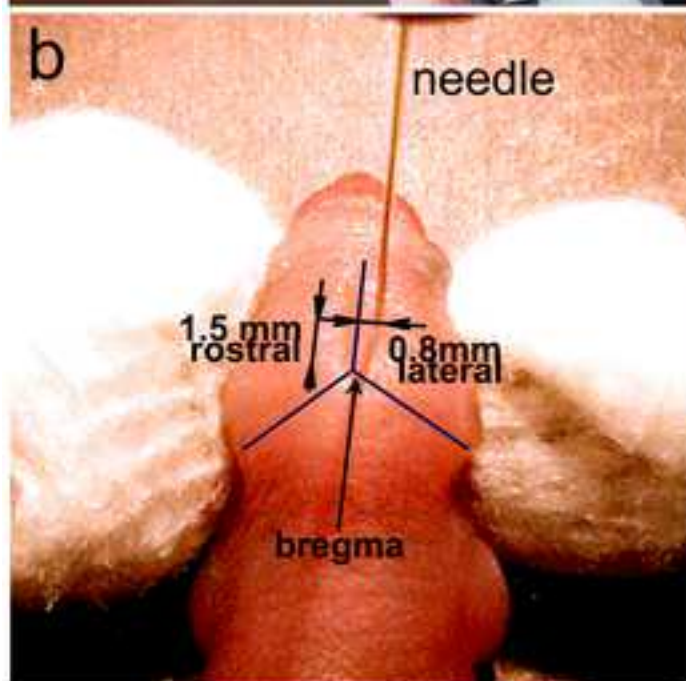
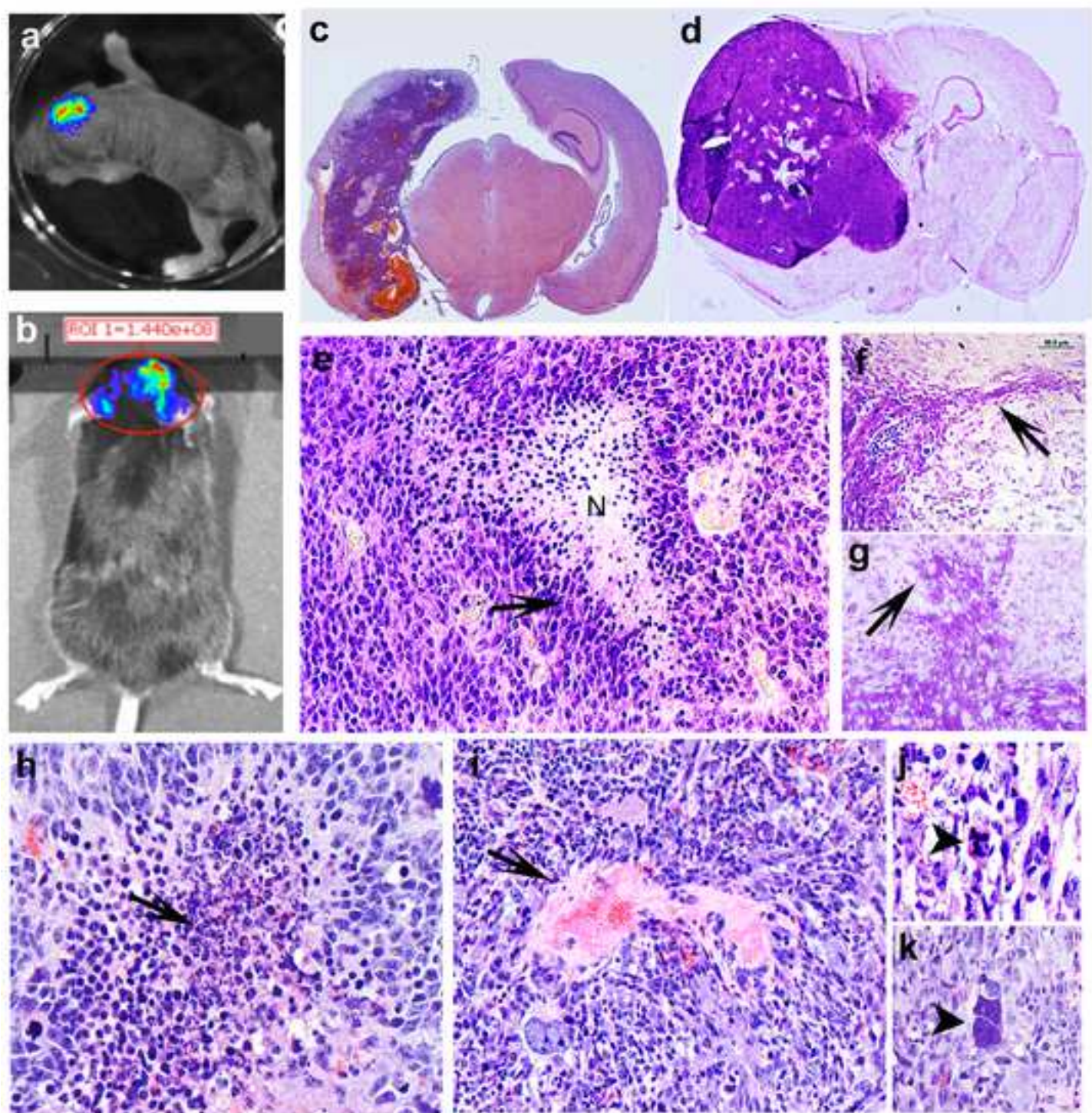Figure 3
Click here to download high resolution image

Figure 4

Sample bioluminescence recording from a cohort of mice with Sleeping Beauty tumors

Sample survival curves from mice with Sleeping Beauty tumors generated with different plasmid combinations

Figure 5
Click here to download high resolution image
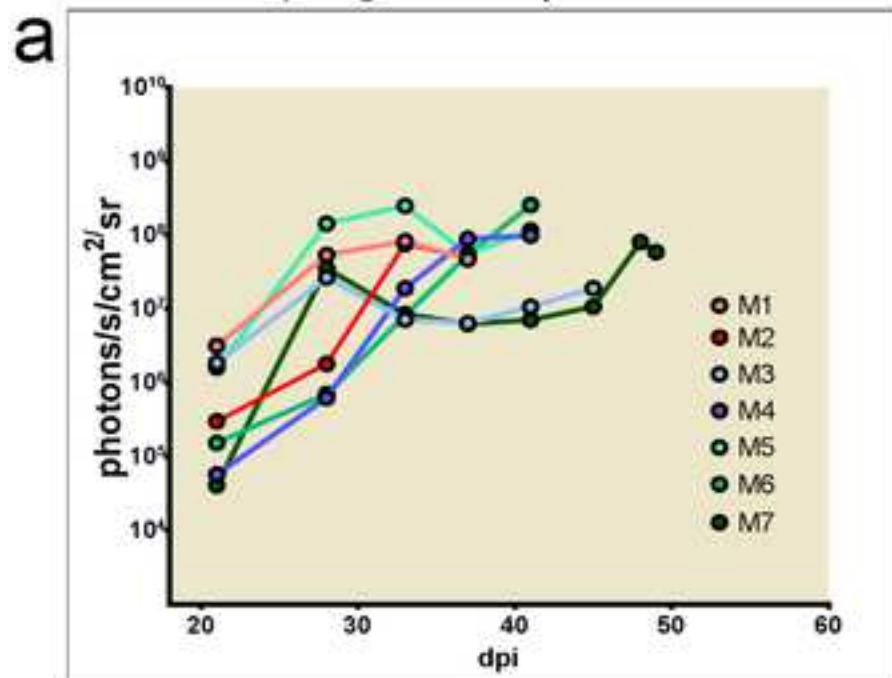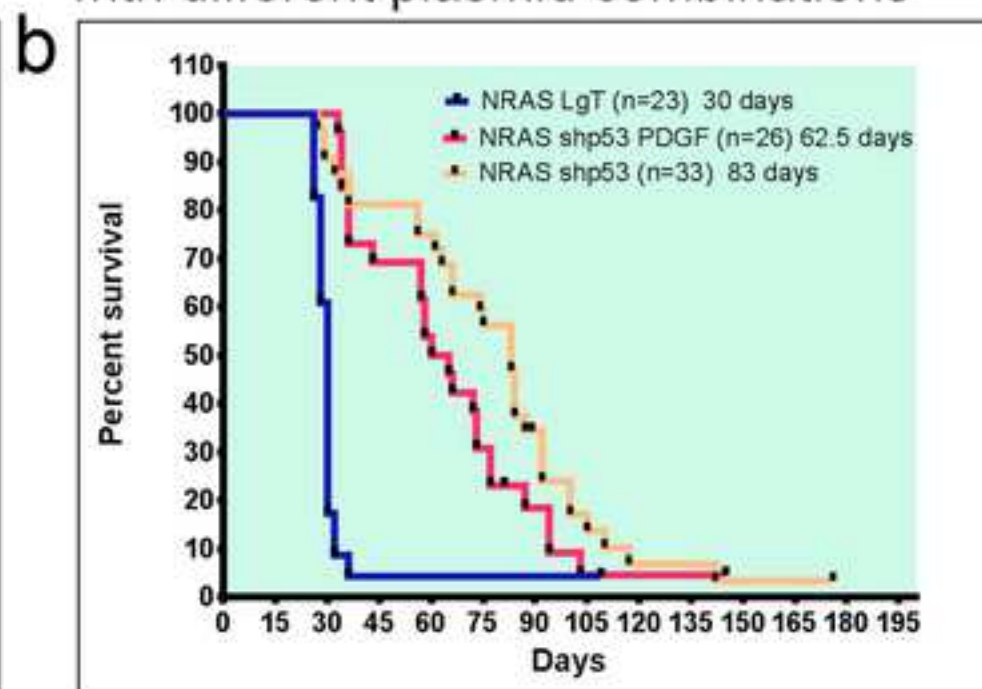
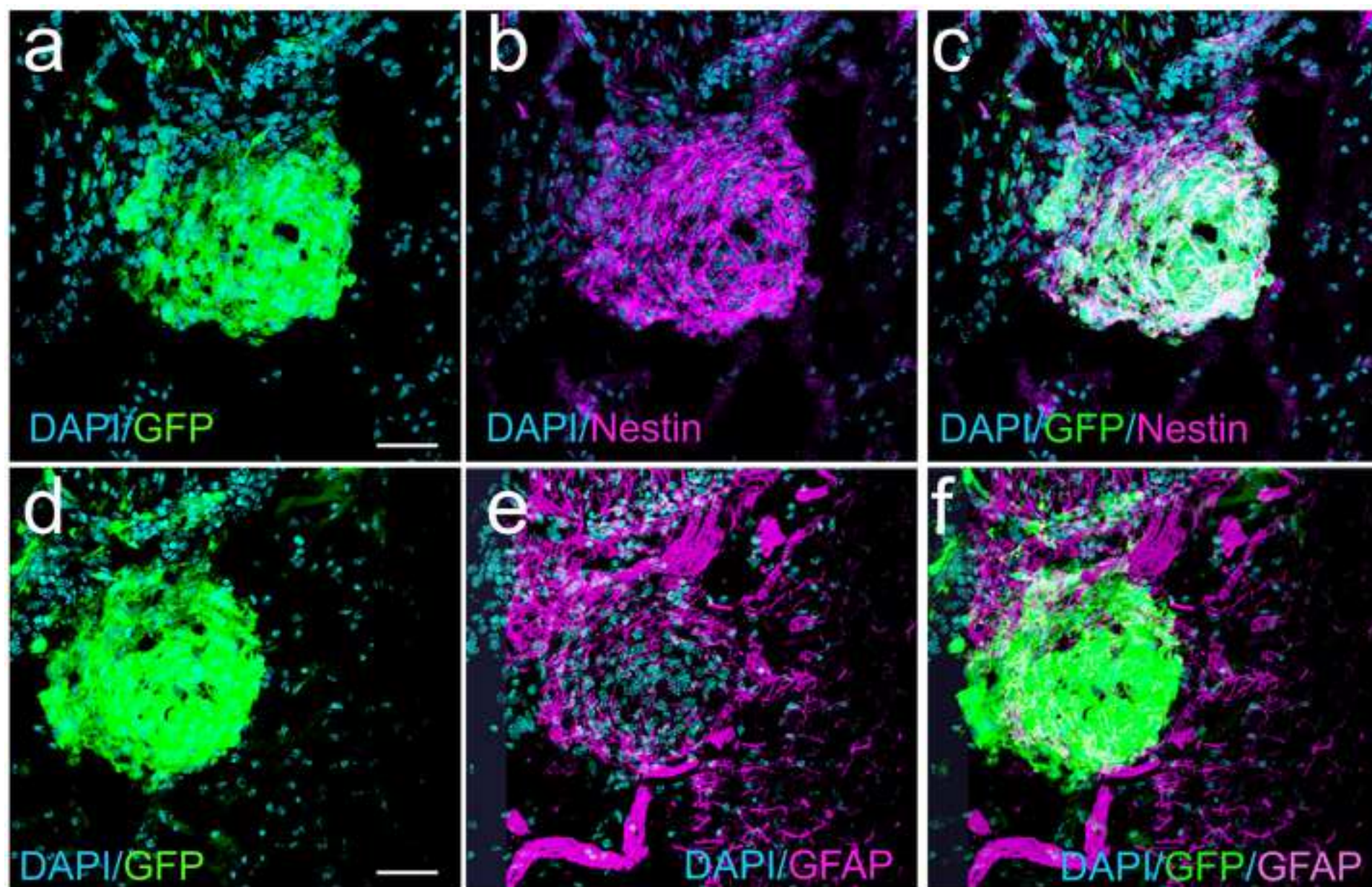Figure 6
Click here to download high resolution image

a

b
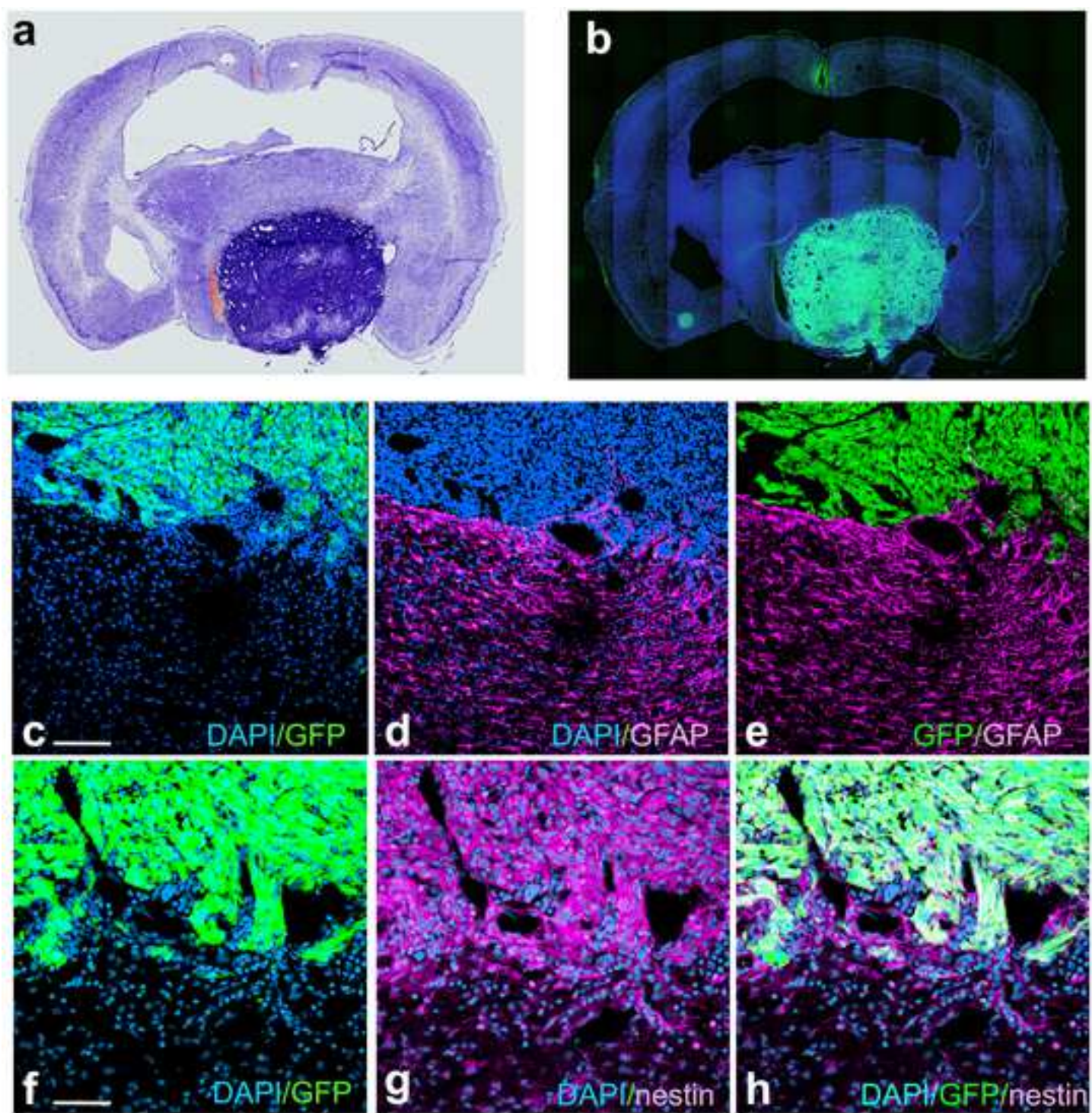
c DAPI/GFP

d DAPI/GFAP

e GFP/GFAP

f DAPI/GFP

g DAPI/nestin

h DAPI/GFP/nestin

Figure 7

Excel Spreadsheet- Table of Materials/Equipment
Click here to download Excel Spreadsheet- Table of Materials/Equipment: Sleeping Beauty JoVE_Materials - revised.xls
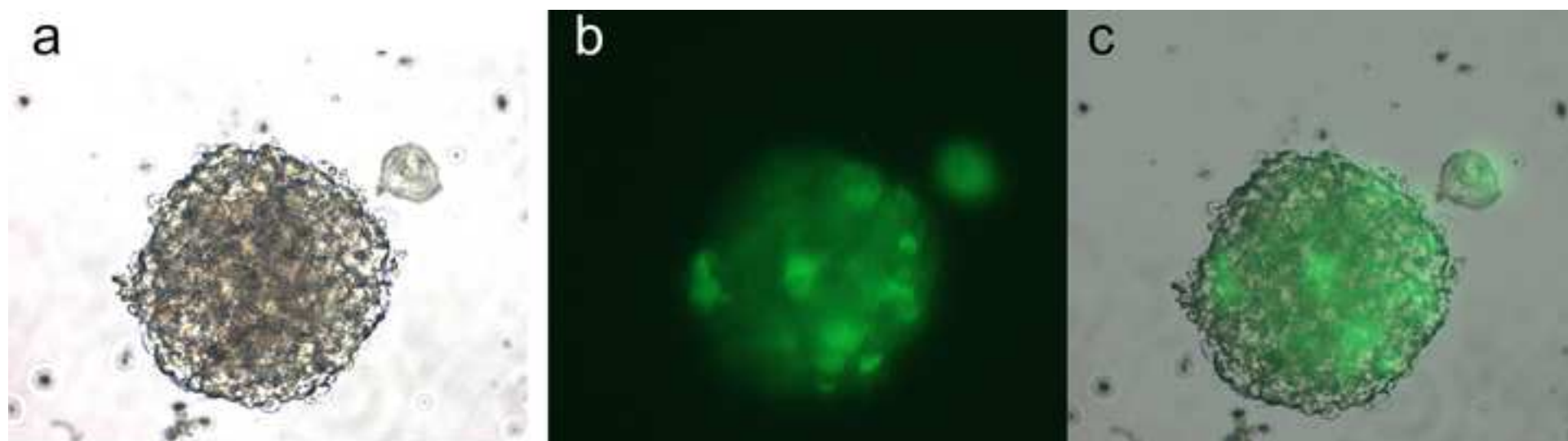
Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Stoelting's Lab Standard with Rat and Mouse Adaptors	Stoelting	51670	Other companies produce similar frames, any of them with small mouse adaptors are suitable
Quintessential Stereotaxic Injector (QSI)	Stoelting	53311	Injections can be made without it, ,but the automatic injector allows for increased reproducibility and convenience
10 µL 700 series hand fitted MICROLITER syringe	Hamilton	Model 701	This syringe model will deliver from 0.5 to 500 ul of solution reliably. Other syrnges (fro example 5 ul, Model 75) may also be used
30 gauge gauge hypodermic needle (1.25", 15° bevel)	Hamilton	S/O# 197462	This needle is ideal to pierce the skin and the skull of a neonatal mouse without the need of other invasive procedures. If it gets dull (doesn't easily enter the skin), it needs to be replaced.
in vivo-jetPEI	Polyplus Transfection	201-10G	Aliquot in small volumes and keep at -20oC
D-Luciferin, Potassium Salt	Goldbio.com	LuckK-1g	Other sources of firefly lucifierase are just as adequate
Hematoxylin Solution, Harris Modified	Sigma Aldrich	HHS128-4L	
Tissue-Tek O.C.T. Compound	Electron Microscopy Sciences	62550-01	for embedding brains for cryosectioning and immunohistovhemistry
Advanced DMEM/F-12, no glutamine	Life Technologies	12634-010	for the culture of neurospheres
B-27¨ Supplement (50X), serum free	Life Technologies	17504-044	serum free supplement for the culture of neurospheres
N2 Supplement (100x)	Life Technologies	17502-048	serum free supplement for the culture of neurospheres
Normocyn	InvivoGen	ant-nr-1	anti-mycoplasma agent for the culture of neurospheres
Recombinant Human EGF	PEPROTECH	AF-100-15	growth factor supplement for the culture of neurospheres
Recombinant Human FGF-basic	PEPROTECH	100-18B	growth factor supplement for the culture of neurospheres
HyClone HyQTase Cell Detachment Reagent	Thermo Scientific	SV3003001	for the dissociation of neurospheres
Kimble-Chase Kontes Pellet Pestle	Fisher Scientific	K749510-0590	for the dissociation of freshly dissected tumors
Falcon Cell Strainers mesh size 70um	Fisher Scientific	08-771-2	for generating single cell suspensions of dissociated neurospheres
Xylenes Histological grade	Fisher Scientific	C8H10	
Protocol Harris Hematoxylin Mercury free (acidified)	Fisher Scientific	245-678	
Protocol Eosyn Y Solution (intensified)	Fisher Scientific	314-631	

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Title of Article: Transposon mediated integration of plasmid DNA into the SVZ

Author(s): AA Calinescu, F Nuñez, C Koschmann, bad Kolb, PR Löwenstein

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CORRESPONDING AUTHOR:

Name: _Dr Maria G. Castro_

Department: _Neurosurgery, Cell and Developmental Biology_

Institution: _University of Michigan_

Article Title: _Transposon mediated integration of plasmid DNA into the SV8..._

Signature: _[signature]_   Date: _6-20-14_

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July 14, 2014

Dr. Nam Nguyen, Ph.D.
Science editor
JOVE
1Alewife Center Suite 200
Cambridge, MA 02140

Sent via email

Dear Dr. Nguyen,

Submitted hereby is the revised manuscript JoVE52443 'Transposon mediated integration of plasmid DNA into the subventricular zone of neonatal mice to generate novel models of glioblastoma'. We have accepted all your editorial changes and completed your recommended changes, as presented in detail below,

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have carefully checked the manuscript for spelling and grammatical errors.

2. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source.* **Volume** (Issue), FirstPage – LastPage, doi: DOI (YEAR).] For more than 6 authors, list only the first author then *et al.* Please include issue numbers for all references.

We have corrected the reference style to match your requirements using the Endnote JOVE style.

3. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

We have removed the trademark and registered symbols from the Table of Equipment and Materials and from the body of the manuscript.

We have provided email addresses for all the authors.

We have re-written the short abstract, limited to 50 words, to conform to your requirements.

We have corrected the reference style to match your requirements using the Endnote JOVE style.

We have revised the protocol section to make each steps an actionable imperative. We have largely re-written section 1 to comply with the specifications.
We have left a rather lengthy note at the beginning of section 2 in which we detail important factors which need to be considered when preparing the plasmid mixture to inject in mice. We consider that this is the best placement of this information in order to convey to the researchers pertinent information prior to proceeding further into executing the procedure.

We have simplified the protocol to conform to this requirement. We added more steps to minimize the number of actions per step.

We have made changes accordingly.

reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

We have removed all trademark and registered symbols and all commercial language from the manuscript and the table of Materials and Reagents.

11. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

We have converted centrifuge speeds to centrifugal forces (g)

12. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

We have re-written section 1 and added many more steps to section 4 to detail the "how" regarding the procedures described. We hope to have thusly conformed to your requirements.

13. What are the agarose gel conditions in step 1.2.7? What is the percentage of agarose used in the gel?

We added that information in the protocol.

14. Please add additional details on how to perform step 1.2.8.

We expanded this step of the protocol to provide better detail of the procedure.

15. What are the concentrations and ratios of the components of the injection solution in step 2.2.1?

We provided that information again in step 2.2.1

16. What is the concentration of xylenes used in step 4.2.2?

For histological use, xylenes are used as is. They are commercially available as a mixture of o, m, p isomers of xylenes, which is not diluted. We added the information about the reagent and supplier to the revised Materials Excel Spread Sheet.

17. What is the procedure to stain the slides with Harris hematoxylin in step 4.2.4? How much stain to use, etc.? Eosin in step 4.2.8?

We elaborated on the method to explain that to stain the slide it has to be dipped in a solution with hematoxylin or eosin respectively. The staining solutions are purchased from the supplier already diluted to the proper concentration. We provided the information for the reagents and supplier in the revised Materials Excel Spread Sheet. Staining solutions for histology need to be standardized and need to mature prior to use. Purchasing ready-made staining solutions

from established suppliers, is a practice common in clinical settings, ensures reproducible results.

18. Please specify how to euthanize the mouse in step 5.1.

We added this information to the protocol.

19. Please specify how to dissect the brain in step 5.1 (surgical approach, useful landmarks, etc.)

We added several steps to describe this procedure in detail, in section 4. (4.1.8 through 4.1.14).

20. There is 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, *i.e.,* the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We limited the highlighted steps to sections 2 (Intraventricular neonatal injections) and 3 (Bioluminescence monitoring of tumor formation). We feel that, as written, these sections provide a logical narrative with good flow.

21. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences).

See answer to question 20.

22. Please discuss all figures in the Representative Results. However for figures showing the experimental set-up, please reference them in the Protocol.

We have referenced Fig. 1 and 2 in the protocol. The other figures are discussed in the representative results section.

23. The Figure Legends should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

The required figure legends now conform to your requirements as specified above.

24. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

We have included a scale bar for images in Figure 5 and 6 and defined the scale in the Figure legend. Corrected Figures are uploaded with this letter and revised manuscript

25. Please move all URLs to the Table of Materials and Equipment.

We removed the URL from the body of the text.

Thank you for your help and consideration,

Yours sincerely,

Maria G. Castro, PhD

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July 21, 2014

Dr. Nguyen
JOVE
Via Internet

RE: **Manuscript Number JoVE52443R1**

Dear Dr. Nguyen,

We have revised the manuscript JoVE52443R1 'Transposon mediated integration of plasmid DNA into the subventricular zone of neonatal mice to generate novel models of glioblastoma' according to your requests. We have uploaded to the JOVE website the revised word document, tracking the implemented changes and also uploaded a separate document, represented by this letter, in which we address each editorial request. The details of the changes are listed below:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. There are numerous copy-editing errors throughout and the ones I caught were fixed.

> We have proofread the manuscript and checked for spelling and grammar issues.

*2. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. **Volume** (Issue), FirstPage – LastPage, doi: DOI (YEAR).] Please include issue numbers for all references.*
Please note that the Endnote Style guide on the website is not up to date and lacks the issue numbers.

> We have **manually** revised the bibliography to include the issue numbers for all references.

*3. Please ensure that **all text** in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. The Protocol should contain only action items that direct the reader to do something.*

We believe we have accomplished this task.

Please move the discussion about the protocol to the Discussion.
You can move the discussion about the preparation of the injection/transfection solution to the Discussion, where critical steps are discussed. Please change the text to complete sentences. Similarly, the first note in step 5, etc.

We have moved the discussion about the preparation of the injection solution to the Discussion. Similarly, the first note in step 5.

4. Please avoid numbered lists and bullet points.

We removed the bullets from the note at the beginning of Section 2.

5. In multiple steps, you are presenting redundant protocols. You are stating generally what to do with numbered steps, and then giving detail instructions on how to perform the steps with a specific example. This makes for a very disjointed protocol section. Please eliminate redundancy by removing the general directions and leaving the detailed instructions for specific examples. You can speak generally about the critical steps, modifications, troubleshooting and limitations of the protocol in the Discussion.

We have made changes in the protocol accordingly.

6. Please state the concentration of isoflurane used.

Isoflurane is administered in a gas mixture with oxygen in a standardized mouse anesthesia XGI-8 gas anesthesia system. The concentration of isoflurane used to anesthetize mice is usually between 1.5-2.5%. We added this information to the manuscript.

7. Please state the temperature of all incubation steps. Is there an incubation in step 5.8?

We have added specifics of the tissue culture incubator to the protocol.

8. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of

the Protocol. Currently, you have just under 10 pages of protocol steps with 3.2 pages highlighted. Please reduce the highlighting to 2.75 pages.

We have reduced the highlighted portion of the protocol to 2.75 pages. We feel that the highlighted steps form a cohesive story, easily amenable to the visual format of your journal.

9. *Please use μm (mu with an m) to specify micrometer instead of just μ (mu).*

We have made the changes accordingly.

10. *Please discuss all figures in the Representative Results. Figure 7 is not discussed.*

We have added discussion of Figure 7 to the Representative Results.

Thanking you in advance for your consideration.

Sincerely yours,

Maria G. Castro, PhD
Professor

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September 10, 2014

Dr. Nguyen
JOVE
Via Internet

RE: **Manuscript Number JoVE52443R1**

Dear Dr. Nguyen,

We have revised the manuscript JoVE52443R1 'Transposon mediated integration of plasmid DNA into the subventricular zone of neonatal mice to generate novel models of glioblastoma' according to your suggestions as well as the reviewers' comments. We have uploaded to the JOVE website the revised word document, tracking the implemented changes and also uploaded a separate document, represented by this letter. Changes to the manuscript and replies to the reviewers' comments are detailed below:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have proofread the manuscript and checked for spelling and grammar issues. We believe the manuscript in its present state is free of copy edits and ready for publication.

2. What are the reactions conditions for the digestion in step 1.2?

We have modified the manuscript to specify the reaction conditions for the digestion (4 hours at 37°C) in step 1.2.

3. In step 1.5, how much 70% ethanol should be used? What are the centrifugation conditions thereafter?

We have changed the step 1.5 of the manuscript to clarify the uncertainties:

Comments from reviewer #1.

We thank reviewer #1 for his positive feedback and appreciation of the manuscript. We also believe that this manuscript will disseminate the technical knowledge needed to use the Sleeping Beauty transposon model to generate glioblastomas in mice and will help accelerate discoveries and novel therapeutic approaches to treat this devastating disease.

1/3

Comments from reviewer #2.

With regard to the major concerns of reviewer #2 related to "safety issues facing this approach for gene therapy" we reply that this manuscript presents a method for generating GBM in mice which is by no means a gene therapeutic approach. Hence the safety concerns do not apply.

We have addressed limitations related to the integration of the transposons in variable number and locations, as well as the possibility of interference with coding host genomic DNA in our discussion section (lines 748-753). We feel that an extensive elaboration on the topic is beyond the scope of this manuscript.

Regarding the concern that the "SB transposase can induce the remobilization of transposons already sited in the recipient genome" we refer the reviewer to the article cited in our manuscript at position 20: "Hackett, P.B., S.C. Ekker, D.A. Largaespada, and R.S. McIvor. 2005. Sleeping beauty transposon-mediated gene therapy for prolonged expression. *Advances in genetics* 54:189-232.", as well as to the references within. The Sleeping Beauty transposase is a synthetic transposase constructed from the dormant salmonoid transposase of the TC1/mariner class and does not bind to other transposons in humans or other mammalian cells. We have added this statement to the body of the manuscript, lines 149-151.

Regarding the minor concerns of reviewer #2 we have addressed them as follows:

Line 230: "digest" should be replace by "digestion"
As "restriction digest" is a commonly used and accepted term to identify this procedure, we have kept the original version (see also: http://en.wikipedia.org/wiki/Restriction_digest)

Protocol section 2.3: the injection solution has to be described in this section. The authors should move lines 714 to 728 from the discussion to section 2.3.
Originally the preparation of the injection solution was presented in the methods. Following repeated discussions with our editorial reviewer and due to formatting restrictions of the journal we have moved this part to the discussion section to comply with the editorial review specific and repeated requests.

Same for the plasmids description, lines 730 to746, that fits better in section 2. The plasmids used in the report and their features have to be mentioned also in the table of material and equipment.
The same explanation (see above) goes for this comment as well.

Line 281: "in on ice" should be replaced by "on ice" or "in ice"
We have made the suggested correction.

Line 286: "Ensure that the head is level" remove "is"
We have re-written the sentence to avoid any confusion about the positioning of the head of the pup in the stereotaxic frame. The sentence (lines 288-290) now reads: "Ensure that the dorsal side of the skull is horizontal, parallel to the surface of the frame and that the cranial sutures are clearly visible."

Section 3.2: step 3.2.2 should be switched with 3.2.1
The injection is performed prior to placing the animal in the anesthesia chamber. The sequence of steps is correct as stated.

The authors need to describe clearly the experiments represented in figure 5 and 6. The reader cannot understand why the tumor cells are GFP positive.

The injected shp53 plasmid encodes for green fluorescent protein to allow the identification of transfected cells and their progeny (pT2/shp53/GFP). We have added this sentence to the results section to clarify our statements (lines 591-593 and 596). We thank the reviewer for pointing out this oversight.

Thanking you in advance for your consideration.

Sincerely yours,

Maria G. Castro, PhD
Professor