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TITLE:

Modeling Brain Metastasis via Tail-Vein Injection of Inflammatory Breast Cancer Cells

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KEYWORDS:

brain metastasis, mouse model, tail-vein injection, inflammatory breast cancer

SUMMARY:

We describe a xenograft mouse model of breast cancer brain metastasis generated via tail-vein injection of an endogenously HER2-amplified inflammatory breast cancer cell line.

ABSTRACT:

Metastatic spread to the brain is a common and devastating manifestation of many types of cancer. In the United States alone, about 200,000 patients are diagnosed with brain metastases each year. Significant progress has been made in improving survival outcomes for patients with primary breast cancer and systemic malignancies; however, the dismal prognosis for patients with clinical brain metastases highlights the urgent need to develop novel therapeutic agents and strategies against this deadly disease. The lack of suitable experimental models has been one of the major hurdles impeding advancement of our understanding of brain metastasis biology and treatment. Herein, we describe a xenograft mouse model of brain metastasis generated via tail-vein injection of an endogenously HER2-amplified cell line derived from inflammatory breast cancer (IBC), a rare and aggressive form of breast cancer. Cells were labeled with firefly luciferase and green fluorescence protein to monitor brain metastasis, and quantified metastatic burden by bioluminescence imaging, fluorescent stereomicroscopy, and histologic evaluation. Mice

robustly and consistently develop brain metastases, allowing investigation of key mediators in the metastatic process and the development of preclinical testing of new treatment strategies.

INTRODUCTION:

Brain metastasis is a common and deadly complication of systemic malignancies. Most brain metastases originate from primary tumors of the lung, breast or skin, which collectively account for 67-80% of cases[1,2]. Estimates of the incidence of brain metastasis vary between 100,000 to 240,000 cases, and these numbers may be underestimates because autopsy is rare for patients who died of metastatic cancer[3]. Patients with brain metastases have a worse prognosis and lower overall survival relative to patients without brain metastases[4]. Current treatment options for brain metastases are largely palliative and fail to improve survival outcomes for most patients[5]. Thus, brain metastasis remains a challenge, and the need remains pressing to better understand the mechanisms of brain metastasis progression to develop more effective therapies.

The use of experimental models has provided important insights into specific mechanisms of breast cancer metastatic progression to the brain and allowed evaluation of the efficacy of various therapeutic approaches[6-16]. However, very few models can accurately and fully recapitulate the intricacies of brain metastasis development. Several experimental in vivo models have been generated via inoculation of cancer cells into mice by different routes of administration, including orthotopic, tail-vein, intracardiac, intracarotid arterial, and intracerebral injections. Each technique has advantages and disadvantages, as reviewed elsewhere[3]. None of these mouse models, however, can fully replicate the clinical progression of brain metastasis.

Brain metastases are particularly common in patients with inflammatory breast cancer (IBC), a rare but aggressive variant of primary breast cancer. IBC accounts for 1% to 4% of breast cancer cases, but it is responsible for a disproportionate 10% of breast cancer-related deaths in the United States[17,18]. IBC is known to rapidly metastasize; indeed, one-third of IBC patients have distant metastasis at the time of diagnosis[19,20]. Specific to brain metastasis, patients with IBC have a higher incidence of brain metastasis than do patients with non-IBC[21]. Recently, we demonstrated that the MDA-IBC3 cell line, derived from the malignant pleural effusion fluid of a patient with $ER^-/PR^-/HER2^+$ IBC that recapitulates IBC characteristics in mouse xenografts, has an enhanced propensity to develop brain metastases rather than lung metastases in mice when injected by tail-vein, making this cell line a good model for studying the development of brain metastasis[16].

Herein we describe the procedures to generate brain metastasis via tail-vein injection of MDA-IBC3 cells and to evaluate the metastatic burden via stereofluorescent microscopy and luciferase imaging. This method has been used to discover key mediators of breast cancer metastasis to the brain and to test the efficacy of therapeutic interventions[16,22]. The disadvantage of this technique is that it does not recapitulate all the steps in the brain metastatic process. Nevertheless, its major advantages include robustness and reproducibility, involvement of the relevant metastasis biology of intravasation, traversing the lungs and extravasation into the brain, and its relative simplicity in terms of technique.

PROTOCOL:

The method described here has been approved by the Institutional Animal Care and Use Committee (IACUC) of the MD Anderson Cancer Center and complies with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The schematic workflow, with all steps included, is presented as **Figure 1**.

1. Cell preparation

NOTE: The MDA-IBC3 ($ER^-/PR^-/HER2^+$) cell line, generated in Dr. Woodward's lab[23], was stably transduced with a luciferase–green fluorescent protein (Luc–GFP) plasmid described in detail in previous work[16,23].

1.1. Culture transduced cells in Ham's F-12 media supplemented with 10% fetal bovine serum (FBS), 1 µg/mL hydrocortisone, 5 µg/mL insulin, and 1% antibiotic-antimitotic.

1.2. Plate MDA-IBC3-Luc-GFP cells at 37 °C and 5% CO_2 in a T-75 flask until confluent. Change media every 3 days before cells are confluent enough to passage.

1.3. Harvest cells by removing media, washing each flask with 10 mL of 1x Dulbecco's (i.e., calcium- and magnesium-free) phosphate-buffered saline (DPBS), and adding 2 mL of 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA). Incubate for 3-5 min at 37 °C until cells detach.

1.4. Add 5 mL of complete media to the flask to collect the cells and transfer the entire contents to a 15 mL centrifuge tube (optimized to 50 mL centrifuge tube depending on the total number of cells needed). Centrifuge at 290 x *g* for 5 min to pellet cells.

NOTE: Complete media is Ham's F-12 media supplemented with 10% fetal bovine serum (FBS), 1 µg/mL hydrocortisone, 5 µg/mL insulin, and 1% antibiotic-antimitotic.

1.5. Discard the supernatant. Then wash the cells with 10 mL of 1x DPBS. Centrifuge at 290 x *g* for 5 min to pellet the cells and discard the supernatant carefully. Resuspend the cells with 1 mL of fresh 1x DPBS and mix well by pipetting up and down.

1.6. To make single-cell suspensions, filter cells through 40 µm sterile cell strainers.

1.7. Calculate cell density by using an automated cell counter.

NOTE: To reduce errors in counting, create different cell dilutions and count them separately, and calculate the average value to determine the concentration of cells (number of cells/mL).

1.7.1. Collect 2 µL of cell suspension + 8 µL of 1x DPBS to make a 1:5 dilution sample.

1.7.2. Collect 1 µL of cell suspension + 9 µL of 1x DPBS to make a 1:10 dilution sample.

1.7.3. Add 10 µL of 0.4% trypan blue stain. Mix the sample mixture well by pipetting it up and down a few times.

1.7.4. Gently pipette 10 µL of the sample into the half-moon-shaped sample loading area of the counting chamber slides. Make sure there are no bubbles inside; wait for 30 s to allow the cells to settle in the chamber before counting.

1.8. Dilute the cells with DPBS to a density of 5 x 10^5 or 1 x 10^6 cells per 100 µL. Transfer cell suspension into 1.5 mL microcentrifuge tubes for tail-vein injection. Place cells on ice until injection to maintain viability.

2. Tail vein injection

2.1. Use 4- to 6-week-old female athymic SCID/Beige mice.

2.2. Prepare a 30 G syringe to draw 100 µL of cell suspension. Remove all air bubbles.

2.3. Place mouse in a rodent restrainer (diameter about 1 inch) to facilitate tail-vein injection and prevent injuries from movement during the injection process.

2.4. Use alcohol cotton pads (made with 70% ethanol) to gently wipe the mouse's tail 3–4 times and hold for 5–10 s to make the tail vein more clearly visible.

2.5. Insert the syringe into the tail of the mouse at an angle of 15-30˚. Slowly push the cell suspension into the tail vein.

2.6. Remove the syringe gently and use the cotton pad to hold the tail for few seconds to help stop any bleeding.

2.7. Return the mice to their cages and check them 2–4 h after injection to ensure that no adverse effects occur.

3. Evaluation of brain metastasis burden

3.1. Prepare diluted D-luciferin solution.

NOTE: Stock concentration is 47.6 mM (15.15 mg/mL), and the concentration for use is 1.515 mg/mL.

3.1.1. Add 5 mL of 1x DPBS to the D-luciferin bottle.

3.1.2. Place 2.5 mL of the solution into each of two 50 mL conical tubes.

3.1.3. Add another 5 mL of 1x DPBS to the D-luciferin bottle again to rinse it. Put 2.5 mL into each 50 mL conical tube.

3.1.4. Place another 5 mL of 1x DPBS into the bottle and rinse it again. Repeat the rinsing process twice.

3.1.5. Add 1x DPBS to each 50 mL tube to a total of 66 mL (about 33 mL per tube). There should be about 33 mL of the solution in each tube.

3.1.6. Mix the tubes well and then combine both into a sterile filtration unit (150 mL PES filter 0.45 µm).

3.1.7. Filter the solution and then aliquot the filtered solution into sterile brown 1.5 mL microtubes.

3.2. Detecting brain metastases by luciferase imaging.

3.2.1. Thaw D-luciferin by keeping on ice, and vortex before injection into the mice.

3.2.2. Clean the area of injection with alcohol cotton pads and inject 100 µL of D-luciferin per mouse intraperitoneally by using a 0.5 mL insulin syringe, one syringe for each cage.

3.2.3. After 10 min, anesthetize the mice with isoflurane (2% O_2 – 2.5% isoflurane) for 5 min using the veterinary vaporizer connected to the small animal induction chamber.

3.2.4. Turn on the in vivo imaging system. While the mice from the first cage are under anesthesia, inject the D-luciferin into the mice in the next cage.

3.2.5. Put mice into the in vivo imaging system machine and obtain ventral and dorsal images. Select an exposure time of 2 min, and in the field view choose option **E** (whole body). Press **Acquire**. Next, save the image (**Figure 2**).

NOTE: If the image shows saturation of luminescence, reduce the exposure time.

3.3. Detecting brain metastases by GFP imaging.

3.3.1. Brain tissue preparation.

3.3.1.1. About 8-10 weeks post injection of cells or when the mice are moribund due to brain metastasis burden, euthanize the mice by inhalation of an isoflurane overdose followed by cervical dislocation, in accordance with protocols approved by the IACUC.

3.3.1.2. After euthanasia with isoflurane followed by cervical dislocation, spray the mice with a 70% ethanol solution and remove the fur from the head area and ears with scissors. Next, make

a cut in the cervical area of the neck (being careful to not decapitate the mouse). Cut the skull and pull it out. Next, carefully remove the brain.

3.3.1.3. Place the collected brains into tissue cassettes.

3.3.1.4. Transfer the cassettes to a container with cold 1x DPBS.

NOTE: Brain samples should not be kept in 1x DPBS for more than 1 h. If several brain samples are to be collected at the same time, euthanize only a few mice at a time (10 per round).

3.3.2. Stereomicroscopic imaging.

3.3.2.1. Transfer the whole brain onto a 100 cm tissue dish lid.

3.3.2.2. Turn on the stereomicroscope and the UV light, at position 3 with lens 0.5x, to allow visualization of the whole brain as shown in **Figure 3A** (red square).

3.3.2.3. Press **Live view** (**Figure 3A**, green square).

3.3.2.4. Focus the view while the brain is in the ventral position.

3.3.2.5. Select **GFP** (if cells are GFP-labeled) or **TXRED** (if cells are RFP-labeled) (**Figure 3A**, yellow square) in the software, select the proper filter on the microscope, and take a picture.

NOTE: Keep the SOLA light source setting at about 30%; if the GFP is too bright, reduce until the proper signal is observed.

3.3.2.6. Without moving the sample, turn on the bright light and select BF (**Figure 3A**, yellow square). Take a picture.

3.3.2.7. Repeat the previous steps with the brain in the dorsal position.

NOTE: Depending on the size, the same GFP positive brain metastasis lesion can appear in both ventral and dorsal positions. In such a case, avoid duplicate quantification of the same lesion. Save the images with a TIFF extension (which keeps all the information in the picture file).

3.3.2.8. Repeat steps for all samples.

3.3.2.9. Convert the TIFF images into JPEG so that the files can be opened with any computer that does not have the associated software installed (**File | Import/Export | convert files**).

3.3.2.10. To merge the fluorescent image with the bright field image, open both images and go to **File | Merge channels**. Select the proper components, green for GFP and brightfield for BF, and press **Ok**. Save the merged image.

3.3.2.11. To quantify brain tumor area, use the fluorescent image. Select **Measure | Manual Measurement | Area**. Select automatic selection (**Figure 3B**, green square), and move the arrow to the GFP-positive area and click to automatically create a selection. Click again to confirm the selection and then all measurements will show (**Figure 3B**, red square). If more than one GFP-positive area is present, repeat the procedure.

3.3.3. After brain images are obtained, proceed to routine tissue fixation protocols using 10% neutral buffered formalin.

3.3.3.1. Once the brains are fixed, proceed to standard histological sectioning followed by hematoxylin and eosin staining to confirm the presence of brain metastasis and immunohistochemical staining to detect selected markers.

NOTE: Immunocytochemistry was performed at the Pathology Core Laboratory at UT MD Anderson Cancer Center that has standardized protocol for immunohistochemical staining of known markers.

REPRESENTATIVE RESULTS:

With the rationale that labeled cells facilitate monitoring and visualization of brain metastasis in preclinical mouse models, we tagged MDA-IBC3 cells with Luc and with GFP to monitor brain metastases and quantify the metastatic burden by using bioluminescence imaging and fluorescent stereomicroscopy. Injection of the labeled MDA-IBC3 cells into the tail veins of immunocompromised SCID/Beige mice resulted in high percentages of mice developing brain metastasis (i.e., 66.7% to 100 %)[16,24]. Brain metastatic lesions could be detected as early as 8 weeks after injection by luciferase imaging **(Figure 2)** or stereofluorescent microscopy (**Figure 4).** GFP imaging allows us to detect, count, and calculate the area of each metastatic lesion. After imaging, portions of the brain metastases are formalin-fixed and processed for hematoxylin and eosin staining to validate the presence of brain metastasis lesions (**Figure 5A**) and for immunohistochemical staining to detect specific protein markers (**Figure 5B,C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic workflow for generating brain metastasis via tail-vein injection.

Figure 2: D-luciferin images of mice in dorsal and ventral positions.

Figure 3: Screenshots of imaging software used for stereomicroscopy. (**A**) Steps showing how to obtain images. The green square at left shows the live view button; the red square at right shows the position of the microscope for the nosepiece lens and zoom position; and the yellow square highlights the filter selection. (**B**) Screenshot of the steps involved in measuring tumor burden. The green square at upper left shows the auto selection mode for area calculation; the red square beneath it shows the area values and other measurements after the brain metastasis lesion was selected.

Figure 4: Stereoscopic images of mouse brains with metastases from tail-vein injection of the MDA-IBC3 cell line. The brightfield picture at left is merged with the GFP image (middle) and then merged (right). Scale bar = 100 µm.

Figure 5: Slides showing stained images of MDA-IBC3-derived brain metastases in mice. (**A**) Hematoxylin and eosin stains provide histologic confirmation of brain metastasis. Immunostaining of the MDA-IBC3-derived brain metastatic tumors show positive staining for HER2 (**B**) and E-cadherin (**C**). Scale bar = 100 µm.

DISCUSSION:

The protocol includes several critical steps. Cells should be kept on ice for no longer than 1 hour to maintain viability. Alcohol cotton pads should be used to wipe the tails of the mice before injection, with care taken to not wipe too hard or too often to avoid damaging the tail skin. Ensure that no air bubbles are present in the cell suspension, to prevent mice from dying from blood vessel emboli. Maintain the angle of injection at 45˚ or less to avoid piercing the blood vessel in the tails and insert at least 1/3 of the needle into the tail-vein to ensure successful injection of all cells. The total volume of injected cells can be adjusted according to the weight of the mice, but the total number of cells should be kept as similar as possible. In this protocol, the SCID / Beige mice were all 4 to 6 weeks old and weighed between 15 and 20 g. For mice that weigh less than 15 g, the injection volume could be adjusted to less than 100 µL; otherwise, 100 µL is injected. After its removal from the skull, the whole brain must be kept in 1x DPBS for no longer than 1 hour before imaging by stereoscopic microscopy to prevent signal reduction and tissue degeneration. For immunofluorescence imaging, brain tissues should not be placed in formalin, because it generates endogenous autofluorescence that hinders the acquisition of high-quality images. We have noted that luciferase imaging does not always reveal brain metastases, especially very small lesions; however, GFP imaging by stereomicroscopy can visualize all lesions. Moreover, GFP imaging can show more than 1 lesion, whereas luciferase imaging does not.

The proposed procedures can be modified slightly according to user preferences. First, the number of injected cells and the duration of metastasis formation could be adjusted for different studies. Second, the tail vein of the mice could be visualized more clearly by using warm water to dilate the vein or UV light to illuminate the veins. Finally, the size of the needle in the syringe can be either 30 G or 28 G.

The advantages and limitations of existing mouse models of brain metastasis has been reviewed elsewhere[3] .The brain metastasis model we described here does have its limitations and strengths. One limitation is that it does not recapitulate all the steps of the brain metastatic process and does not allow interrogation of the initial stages of the metastatic process, i.e., the dissemination of primary breast cancer cells into the circulation. Also, this model cannot be used to study the interactions between tumor cells and the host immune microenvironment during the process of brain metastasis or to evaluate immunotherapeutic applications. However, this model has several advantages over other brain metastasis models. First, unlike spontaneous models in which only a small fraction of the mice develop brain metastases at variable intervals, our model offers the advantage of consistently leading to metastasis to the brain, typically in

more than 70% of mice. Second, tail-vein injection allows the dissemination of cells primarily to the lung with subsequent spread to the brain, whereas inoculation via the intracarotid artery allows cells to disseminate directly to the brain; intracardiac injections allow systemic distribution of the cancer cells to the brain as well as to extracranial sites such as the lung and bone. Thus, our model recapitulates the brain metastatic colonization step better than the commonly used intracardiac or intracarotid injection models because the cells traverse the lung capillary beds and survive in the circulation before generating brain lesions. Finally, injection of breast cancer cells via the tail vein is technically less challenging than intracarotid or intracardiac injection.

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DISCLOSURES:

The authors declare no conflicts of interest.

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Figure 1

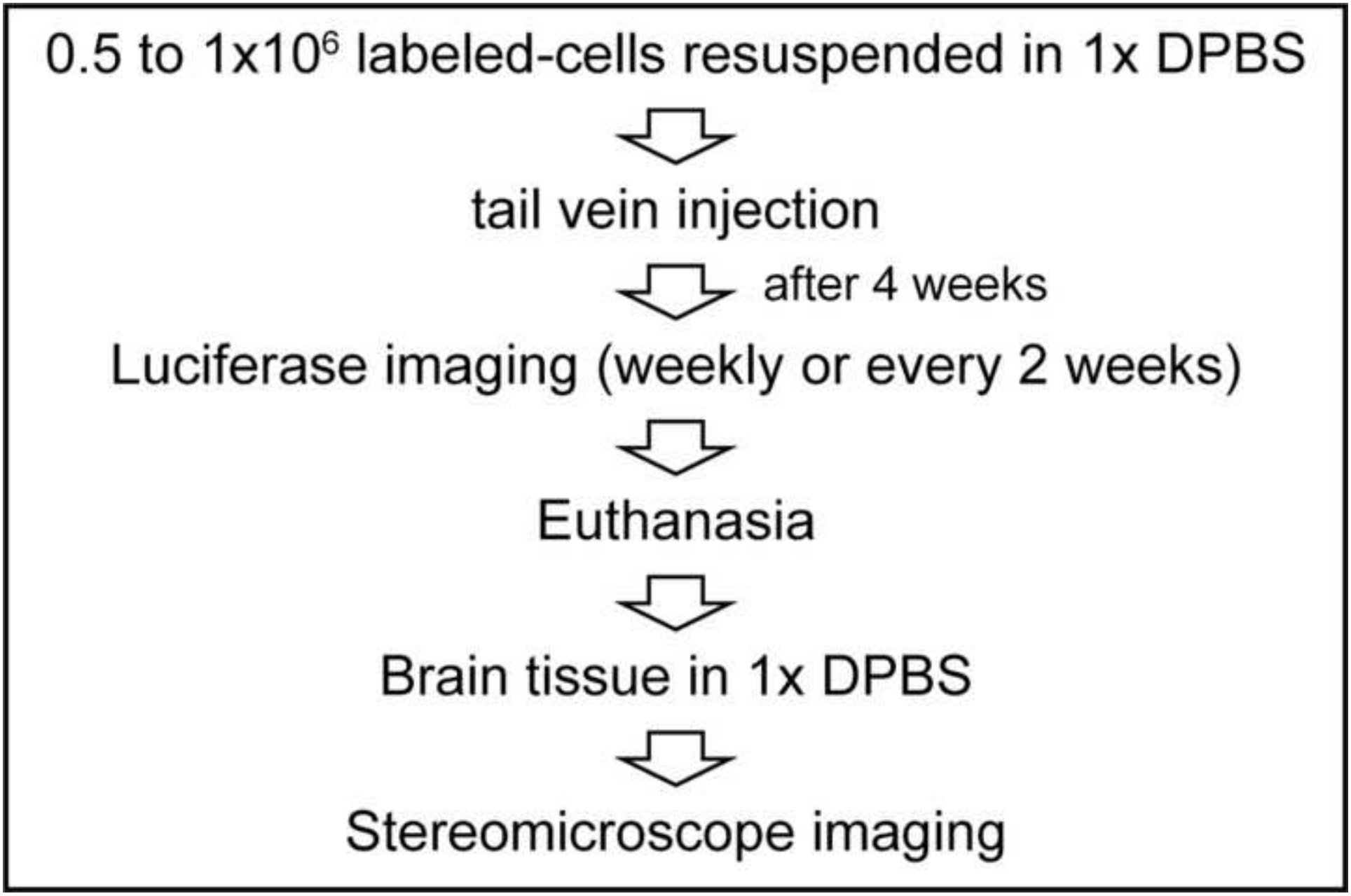


Figure 2

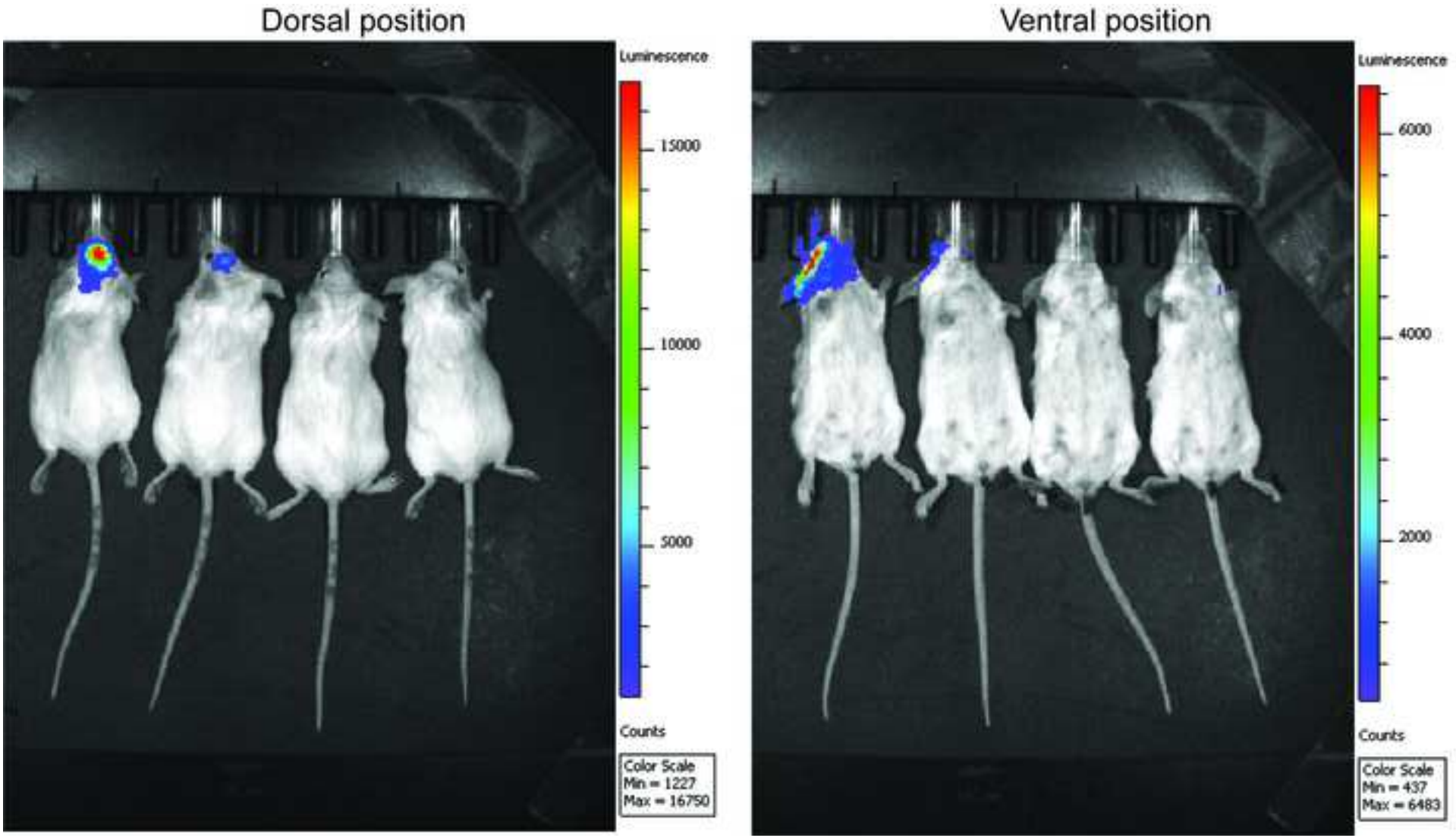
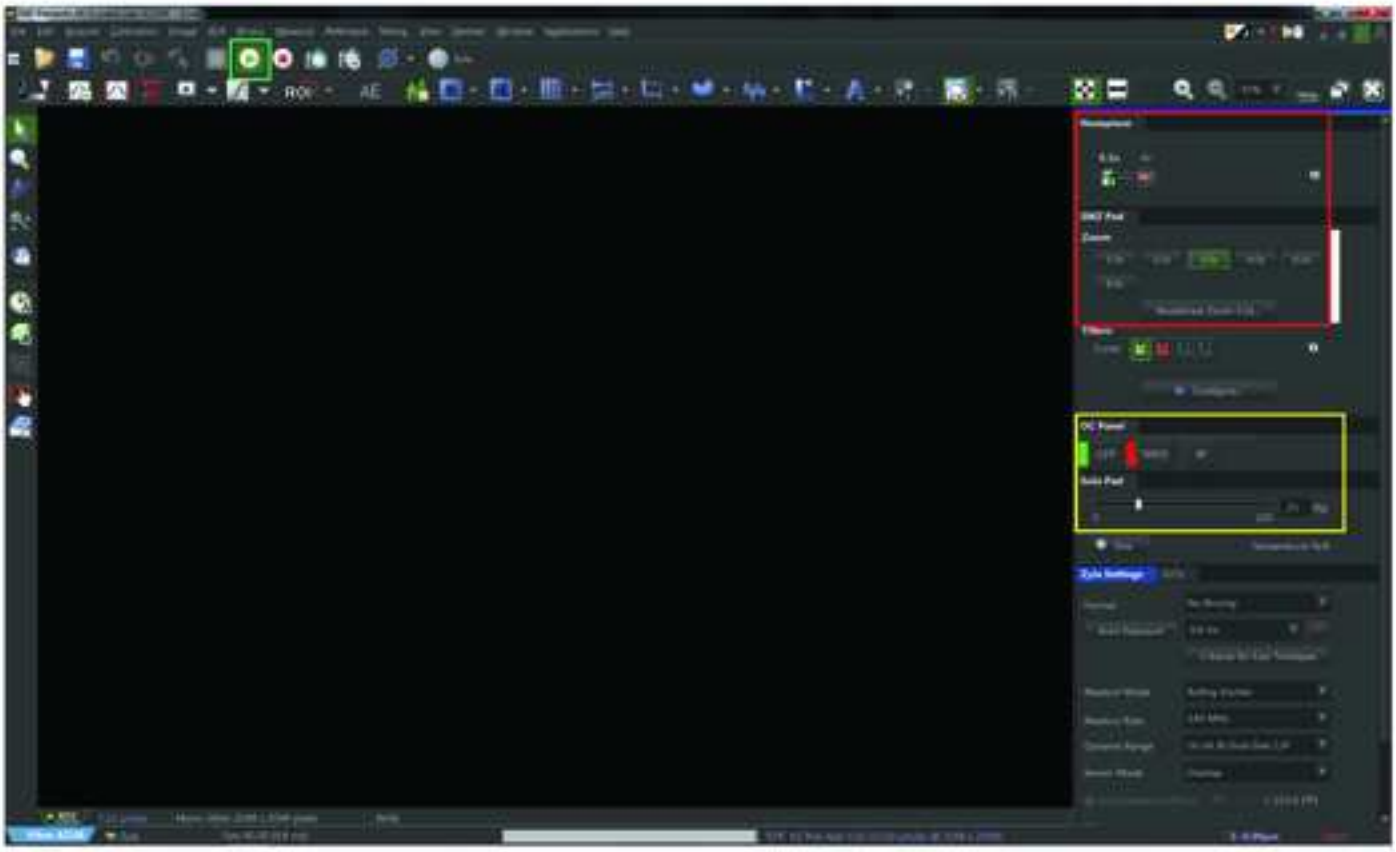


Figure 3

Click here to access/download;Figure;Figure 3.tif

A



B

Figure 4

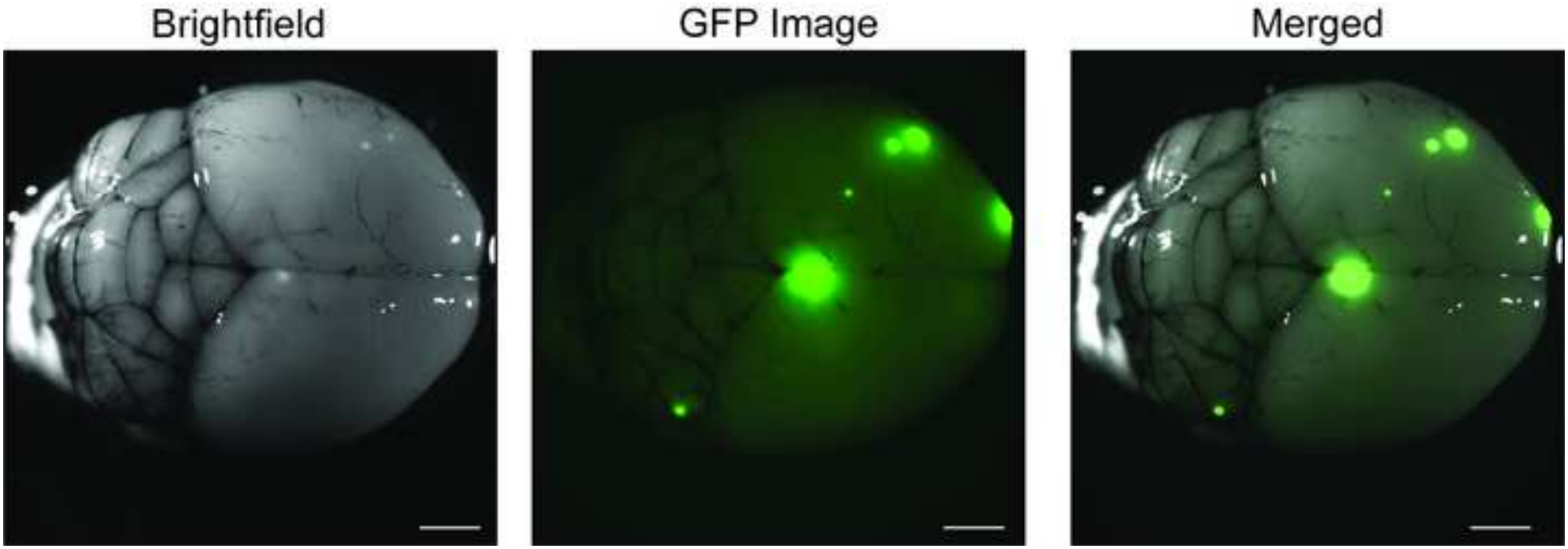
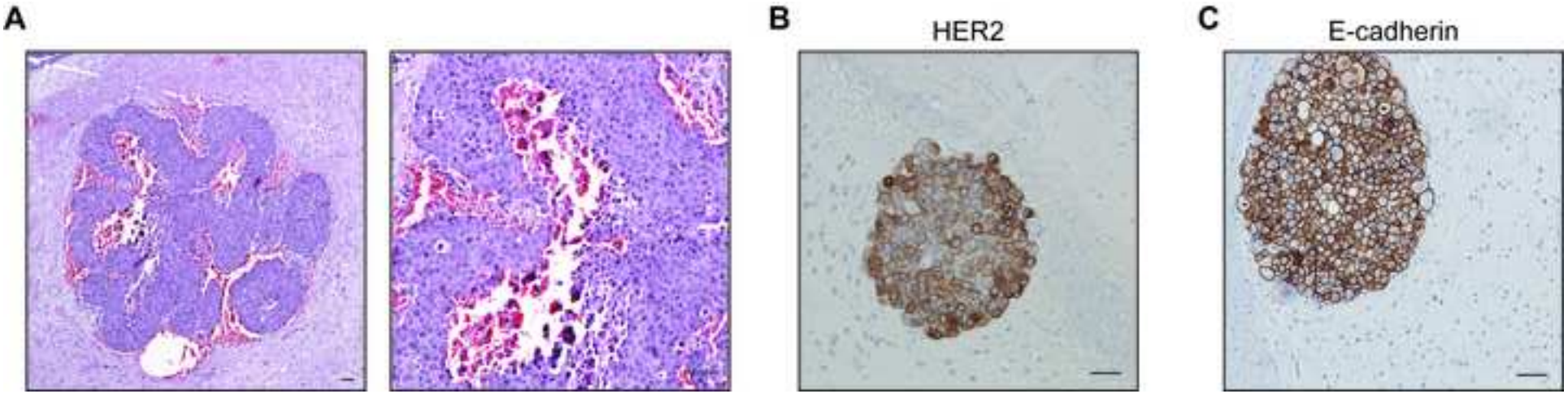


Figure 5



Name of Material/ Equipment	Company
Cell Culture	
1000 µL pipette tip filtered	Genesee Scientific
10 mL Serological Pipets	Genesee Scientific
Antibiotic-antimycotic	Thermo Fisher Scientific
Centrifuge tubes 15 mL bulk	Genesee Scientific
Corning 500 mL Hams F-12 Medium [+] L-glutamine	GIBICO Inc. USA
Countess II Automated Cell Counter (Invitrogen)	Thermo Fisher Scientific
1x DPBS	Thermo Fisher Scientific
Eppendorf centufuge 5810R	Eppendorf
Fetal bovine serum (FBS)	GIBICO Inc. USA
Fisherbrand Sterile Cell Strainers (40 µm)	Thermo Fisher Scientific
Hydrocortisone	Sigma-Aldrich
Insulin	Thermo Fisher Scientific
Invitrogen Countess Cell Counting Chamber Slides	Thermo Fisher Scientific
MDA-IBC3 cell lines	MD Anderson Cancer Center
microtubes clear sterile 1.7 mL	Genesee Scientific
Olympus 10 µL Reach Barrier Tip, Low Binding, Racked, Sterile	Genesee Scientific
TC Treated Flasks (T75), 250mL, Vent	Genesee Scientific
Trypan Blue Stain (0.4%) for use with the Countess Automated Cell Counter	Thermo Fisher Scientific
Trypsin-EDTA (0.25%), phenol red	Thermo Fisher Scientific
Tail vein injection	
C.B-17/IcrHsd-Prkdc scid Lyst bg-J - SCID/Beige	Envigo
BD Insulin Syringe with the BD Ultra-Fine Needle 0.5mL 30Gx1/2" (12.7mm)	BD
Plas Labs Broome-Style Rodent Restrainers	Plas Labs 551BSRR
Volu SolSupplier Diversity Partner Ethanol 95% SDA (190 Proof)	Thermo Fisher Scientific
Imaging	
BD Lo-Dose U-100 Insulin Syringes	BD
Disposable PES Filter Units 0.45 µm	Fisherbrand
D-Luciferin	Biosynth

Isoflurane	Patterson Veterinary
IVIS 200	PerkinElmer
Plastic Containers with Lids	Fisherbrand
Tissue Cassettes	Thermo Scientific
Webcol Alcohol Prep	Covidien
Stereomicroscope Imaging	
Stereomicroscope AZ100	Nikon
Formalin 10%	Fisher Chemical
TC treated dishes 100x20 mm	Genesee Scientific

Catalog Number	Comments/Description
23430	
12-112	
15240062	1%
28103	
MT10080CV	
AMQAX1000	
21-031-CV	
16000044	10%
22-363-547	
H0888	1 µg/mL
12585014	5 µg/mL
C10228	
	Generated by Dr. Woodward's lab[23]
24282S	
23-401C	
25-209	
T10282	
25200114	
SCID/beige mice	
328466	
01-288-32A	Order fromThermo Fisher Scientific
50420872	70 % used
329461	
FB12566501	filter system to sterilize the D-luciferin
L8220-1g	stock concentration = 47.6 mM (15.15 mg/mL); use concentration = 1.515 mg/mL

NDC-14043-704-06	Liquid anesthetic for use in anesthetic vaporizer
	machine for luciferase imaging, up to 5 mice imaging at the same time, with anesthesia machine
02-544-127	
1000957	
6818	
model AZ-STGE	software NIS-ELEMENT
SF100-4	
25202	

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This protocol describes preparation of a mouse model of breast cancer brain metastases. The protocol appears relatively straightforward to follow.

Major Concerns:
none

Minor Concerns:
none

***Response**:* We greatly appreciate the positive comments, thank you very much.

Reviewer #2:
Manuscript Summary:
The manuscript describes a cell line model of inflammatory breast cancer to develop brain metastasis. This cell line is HER2+ve, which shows high propensity for breast cancer brain metastasis. They used tail vein route to develop brain metastasis. Which is quite easy and will help scientific community for testing novel drug combinations.

Minor Concerns:
Although they clearly mentioned that 70% SCID mice develop brain metastatic tumors, they did not mention %age of lung and other metastasis.

Response: Thank you for your suggestion. Following tail-vein injection of MDA-IBC3, the percentage of mice that developed lung metastases was about 20%[1] while those that developed bone metastasis was 0%.

In the literature, there are some orthotopic xenograft as well as syngeneic models have been reported, it would give strength if they include these models in the discussion.

Response: Thank you for your suggestion. The advantages and limitations of existing orthotopic xenograft and syngeneic mouse models of brain metastasis has been previously reviewed[2]. In the discussion section of this manuscript, we have described the limitations and strengths of our model in relation to existing models.

1 Debeb, B. G. *et al.* miR-141-Mediated Regulation of Brain Metastasis From Breast Cancer. *J Natl Cancer Inst.* **108** (8), (2016).
2 Lowery, F. J. Yu, D. Brain metastasis: Unique challenges and open opportunities. *Biochim Biophys Acta Rev Cancer.* **1867** (1), 49-57, (2017).

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.

Done

2. Please revise the following lines to avoid previously published work: 288-291

Revised

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., (line 93) “Culture transduced cells in…” instead of “transduced cells are cultured in…”.

Corrected

4. Line 98: Please consider adding the part about the mice being ready for injection as a note to this step, since the rest of the protocol deals with the cultured cells.

Corrected

5. Line 103: Complete medium composition?

Added as a NOTE

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. E.g. “Countess II FL Automated Cell Counter (Invitrogen) “, “Eppendorf tube”, “IVIS 200 machine”, “SOLA light” etc.

Done

7. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.) e.g., Line 111

Revised

8. Lines 145, 146: Consider if these steps could be combined by adding “Repeat the rinsing process twice/ 2x” to the previous step. Also specify if the DPBS used for rinsing is added to the 50 mL tube (similar to line 144).

Done

9. Line 154: “Procedure” should be removed as the entire content is a protocol. Start directly with line 155 after the heading.

Done

10. Line 159: Please add more details about the anesthetization (e.g. chamber used, the time required, etc.)

Done

11. Line 174: Please specify the method followed.

Method of euthanasia specified.

12. Line 216-220: Please provide references to the standard procedures followed. Provide details on the immunohistochemistry procedures.

We have added as a NOTE that we used the immunocytochemistry core lab at MD Anderson that has standardized protocol for immunohistochemical staining of known markers.

13. Please highlight up to 3 pages 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Highlighted in green

14. Please remove “&” from the references, as per the JoVE format: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).]
Corrected
15. Figure 5 has been mislabeled. Figure 5A is a part of Figure 4, while Figure 5B, C have been labeled as A,B. Please check and correct.
Corrected
16. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.
Done.