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Vineeta Bajaj, Ph.D.
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Dear Dr. Bajaj,

We are incredibly grateful to you and the reviewers for your careful consideration of our manuscript by Geisler *et al.* entitled “Modeling brain metastasis through intracranial injection and magnetic resonance imaging”. This manuscript was submitted for your consideration as an original methods article for publication in the *Journal of Visualized Experiments* methods collection “Modeling solid tumor metastasis *in vivo*”. While the reviewers had favorable comments, they did make several suggestions for revision, which we have addressed as detailed below. Changes to the manuscript are denoted through red text for easy visualization.

Reviewer #1:

The protocol provided here can be used for all tumor models that metastasize to the brain (eg. melanoma and lung). Why only limit to the text to breast cancer since similar clinical outcomes are observed across multiple tumor types that metastasize to the brain? In this case the authors could also provide references describing the intracranial approach for different cell tumor models.

Thank you for this comment. We agree there is wide applicability to this method and have altered our title, abstract and introduction appropriately with additional references.

Could the authors briefly describe and/or add references describing the advantages of using MRI over other modalities?

We have added text in the introduction regarding the advantages of MRI. We have also expanded the limitation section of the discussion regarding use of MRI.

What is the smallest detectable tumor size by MRI? A small discussion about the imaging resolution would be helpful.

We are able to observe a 0.5 mm diameter tumor routinely with a minimum limit around 0.3 mm. This is now discussed (page 12).

The protocol described highly depends on a specific set of equipment. Would be informative to provide additional references of complementary "hands-on" intracranial injection methods.

Discussion of complementary methodology has now been added (page 12).

Furthermore, MRI may be cost prohibitive to some researchers. The authors should suggest alternative imaging/monitoring methods.

We agree this method could be cost prohibitive, and under “Limitations” we provide alternative methods, including bioluminescent imaging (page 12).

Figure 1 is underwhelming. Please add details about injection references (e.g. vessels) and stereotactic coordinates in the schematic. Also include a schematic about head/teeth placement on the mouse bar together with the body placement.

Thank you for pointing this out. We agree that Figure 1 required extensive labeling to be informational, and have modified appropriately. We hope the reviewer agrees that having a visual reference of this complex equipment is useful. To address the reviewers concern regarding injection references, injection coordinates and head/teeth/body placement, we have greatly modified Figure 2. Figure 2A now details tooth placement in the mouse bar. Figure 2B now details head placement. Figure 2C (original Figure 2) now includes additional labeling, including injection references (Bregma) and stereotactic coordinates.

It is important to have intermittent breaks during drilling to avoid over-heating the tissue. Recommended - to have sterile saline solution for regular rinsing. How should one handle bleeding when it occurs?

We completely agree with the reviewers the amount of heat created by the drill can cause thermal injury to the surrounding tissues (*e.g.* thermal necrosis). We apologize for not pointing this out. We have now added a statement to reflect that dropping sterile saline at the site of drilling can be used to negate this effect (Step 1.5.5). We have also added a note on how to handle bleeding if it occurs (Step 1.5.2).

In step 1.6.2, it is recommended to always re-suspend/mix cells before putting into syringe to avoid clump formation. The cell number injected will be consistent when regularly refreshing cell slurry in the injection syringe.

We thank the reviewer for this point. We have amended the protocol accordingly (Step 1.6.2).

What is the rationale behind the times proposed for the cell injection steps?

The prolonged times during the injection procedure are important to reduce pressure changes in the brain and the unintended seeding of cells along the needle tract. We have added this rationale to the protocol (Steps 1.6.6, 1.6.7, 1.6.8, 1.6.9).

Sections 3.2 and 3.3 are not very informative. Could the authors use this space to discuss missing points such as tumor growth dynamics with resolution of MRI, about maximum tumor volume size, common neurological symptoms from this injection site, statistical considerations (power, tumor collection….)

We thank the reviewer for this suggestion and have removed sections 3.2 and 3.3. Text regarding tumor growth dynamics, max tumor volume and common neurological symptoms have been added to the discussion on page 12. We have chosen not to add in statistical considerations as this will be very specific to individual experimental paradigms.

Minor Concerns:

In the intro, the authors mention that only the intracranial inoculation model specifically forms brain metastasis. However, the intracarotid method does also provide cells exclusively to the brain. Furthermore, brain-tropic cell lines can be injected intracardiacally.

Thank you for catching our missed discussion of these two points. We have added text commenting on the intracarotid method and the brain tropic lines in the introduction.

It is recommended to add a PBS wash/spin step after 1.1.4

Thank you. This has been added as Step 1.1.5

In 1.1.5, what would be an acceptable range of injectable volume (1 up to 5uL)?

We have clarified this step (now 1.1.6) to reflect an appropriate range of volume.

All injection and surgical tools must be autoclaved

We have amended step 1.3.1 to reflect that all tools should be cleaned and sterilized prior to the procedure.

Step 1.5.1 is not necessary

We respectfully disagree. Post-surgical infection is a complication that through this important step is easily avoided.

Reviewer #2:

Line 61-62 I do not agree that tail vein and intracardiac injections are easier to perform technically. In my experience these methods often lead to tumour burden at other metastatic sites due to the characteristics of the cell line injected more than the actual technique.

Thank you for pointing this out. We have altered the text appropriately (now lines 77-82).

Line 72 Don't other injection methods that use brain trophic cell lines also allow for the study of these points? Is it possible that a limitation could arise from surgical intervention when monitoring the mechanisms within the brain?

Yes, we agree. Other injection methods using brain tropic cells similarly can address research points 1-3 in the intro (now lines 95-99). To clarify, we have added discussion regarding the limitation of brain tropic cells (lines 88-90) as well as how inflammation resulting from the intracranial injection itself can be problematic (lines 518-525).

Line 166 How is this anesthetic plane confirmed at this step? What is the appropriate anesthetic plane?

Thank you. We have updated 1.4.2 to include anesthetic monitoring criteria.

Line 190 Might be useful to have a figure demonstrating the mouse skull stabilization in their contraption. It seems to be an important step to ensure correct placement of the injection and to avoid skull movement and damage to the brain.

Thank you for this comment. We have modified Figure 2 to demonstrate skull stabilization utilizing ear bars as well as the utilization of the mouth bar for nose-cone anesthetic administration.

Line 271 Where should the intraperitoneal injection be? Is there an ideal area on the mouse to avoid hitting other organs or a particular side of the mouse that should be used?

The injection is standard. We have included a reference to a JoVE article that demonstrates this procedure in step 2.1.1.

Line 319 It is also important that the same window/leveling be used between each contouring session and between users. How is this achieved with ImageJ?

We absolutely agree that image windowing should remain consistent between imaging sessions. The MRI settings embedded in the DICOM file are maintained in ImageJ and are not adjusted. We have added a note in the text (step 3.1.2) to make sure brightness/contrast is not altered in ImageJ so to maintain consistency between sessions.

Line 328 Is it useful to also know the total brain volume to accurately assess the tumour burden? Are they averaging the total volume of all tumours or the total volume of each tumour?

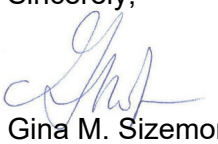
We calculate the tumor volume for each animal then average the groups. We do not quantify total brain volume as a comparison measure; however, this could be accomplished using the same measurement technique if desired.

Figure 1 Is it possible to upload a higher quality image so when zoomed it is still clear?

We have submitted a higher quality image so that the resolution is acceptable with digital zoom.

Once again, we are thankful for the opportunity to include our work at *JoVE.* We look forward to continue working with you and the journal staff.

Sincerely,



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