August 3, 2016

Journal of Visualized Experimentation

RE: A Model for Perineural Invasion in Head and Neck Squamous Cell Carcinoma

Dear Editors:

Thank you for reviewing our manuscript and providing us with the opportunity to revise and resubmit it for publication in the *Journal of Visualized Expermentation*. As requested, we have submitted two copies of the revised manuscript, one with all changes highlighted and the other a clean version of the revised paper (but with the protocol to be narrated highlighted in yellow).

We have taken into consideration all of the reviewers’ recommendations and have responded to them in the revised manuscript as well as in this letter. The exception to this statement is putting scale bars and inlayed panel identifiers in our pictures which we have discussed working on together to complete.

Thank you,

Phillip Huyett and Steve Kim

•Formatting:

* Please make sure the font in the discussion matches the rest of the manuscript.
  + This was fixed- all text is in Times New Roman.
* Length exceeds 3 pg. 2.75 pg or less must be highlighted for filming.
  + I cut out everything between the yellow highlights and get less than 2 pages.
* •Grammar: -Please copyedit the manuscript for grammatical errors. Such editing is required prior to acceptance, and some errors are noted below:
* -Latin phrases (ie in vitro and in vivo) should be in italics.
  + These were adjusted as instructed.
* -Line 119 – “1 to 36.It is not”
  + Changed to 1 to 40 as discussed by one of the reviewers below.
* -2.6 – “verterbral”
  + Fixed to vertebral
* -Line 153 – “deep to the spinal cord”
  + I’m not sure what the grammatical error is here.
* -3.2.1 – “is engaged the plate”
  + I’m not sure what the grammatical error is here.
* -4.5 – “fixe”
  + I’m not sure what the grammatical error is here.
* -“37°” should be “37 °C” throughout the manuscript
  + These were all adjusted.
* -Line 414 – “is crush”
  + changed to “in crush”
* •Additional detail is required:  -2.1 – How is euthanasia performed here?
  + We were previously instructed to remove the details regarding euthanasia, however I have added back the means by which we sacrifice the mice.
* -5.1 – How is the culture plate originally set up? Please describe briefly or provide a citation for initial growth/plating conditions.
  + This was added to 5.1.
* -5.2 – What do the dyes stain? That is, what cellular targets are stained?
  + As recommended below as well, this was added to 5.2
* -Please provide a step at the end to describe quantification. This does not need to be highlighted for filming, but should appear in the protocol rather than the results section.
  + We were previously advised to remove the quantification section from the protocol and to place in the Representative results section.
* •Results: -Please add scale bars to all microscopy images.
* -Please add panel labels to all figures so that it is clear which image is which.
  + As discussed, I was hoping that the journal could assist me with this.
* •Discussion: Please include citations to reference other similar protocols in the section on significance.
  + These references were added.
* If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.
* They are original pictures
* JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when Safiyaciting directly from PubMed. In these cases, please manually include DOIs in reference information.
* IMPORTANT: Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

* NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.

**Reviewers' comments:**

**Reviewer #1:** *Manuscript Summary:* This is a review of A Model for Perineural Invasion in Head and Neck Squamous Cell Carcinoma, Manuscript number JoVE55043R1. Description: This is a paper describing a model system for perineural invasion for of head and neck squamous cell carcinoma using dorsal route ganglion harvested from nude mice. The model allows the dorsal route ganglion to sprout neurites that grow in a radial fashion and then cancer cells are added and can be tracked for growth along neurites. Review: This is a well done, well written, and detailed article describing dorsal route ganglions as a model for perineural invasion. The technique is clearly explained, as are the benefits and limitations. The authors should be congratulated for bringing this technique forward for examination of perineural invasion in head and neck squamous cell carcinoma.  *Major Concerns:* N/A  *Minor Concerns:*

* The author should explain what the semisolid matrix is within the text in addition to the table.
  1. The semisolid matrix is Matrigel- we were not allowed to use the trade name outside of the Table or Reagents. With that said, we have explained in the introduction what Matrigel is specifically made of.
* The author should explain that primary cultures could also be used in addition to squamous cell carcinoma cell lines.
  1. This was added to 5.1
* Summary: This is an excellent description of a model for perineural invasion that may be useful for future experiments.  *Additional Comments to Authors:* N/A
  1. Thank you.

**Reviewer #2:** *Manuscript Summary:* This manuscript describes the protocol and methods used to perform dorsal root ganglion cell culture in the presence of tumor cells from squamous cell carcinoma of the head and neck. This in vitro model is utilized to study perineural invasion - a common phenomenon in head and neck squamous cell carcinoma. The manuscript is well written and the requisite laboratory steps are clearly described. Minor issues that should be addressed before publication include the following:

1. Head neck cancer is innervated by nerve fibers originating from trigeminal ganglia. If the described method is applicable to trigeminal ganglia culture, steps to harvest trigeminal ganglia should be included.
   1. This is a very interesting idea, however, we do not have a protocol for extracting trigeminal ganglia from mice. We did briefly attempt to observe perineural invasion of the facial nerve of living mice but the results were variable- certainly not a protocol ready for publication.
2. DRGs from different segments exhibit differences in size and neurochemical expression. The authors should describe or compare differences in perineural invasion between DRGs collected from different body regions.
   1. Another excellent point that we realized early on. There are at least structural/size differences in DRG obtained from the sacral vs cervical spine. We dealt with the issue of DRG size variability by randomly assigning cell type and treatment condition with each mouse. With large numbers of duplicates in each experiment our assumption is that variability is evenly distributed. This is discussed in the first paragraph in “Representative Results.”

3. Protocol text: 1.2. line 109: what is the "semisolid matrix"? Please clarify. 1.3. line 119: up to 40 DRGs will be yield. Why only label up to 36 DRGs? Space is needed after "36". 2.1. Explain the rationale for choosing athymic nude mice.  "lower portion of the spine" is vague. Do the authors mean sacral region? Please specify.

* The semisolid matrix is described in the introduction. We were previously advised to not use the tradename in the manuscript except in the Reagents table.
* We changed the sentence describing how many plates are labelled as above.
* There was no specific reason for selecting nude mice. were used initially because our lab had prior experience working with them.
* Changed sentence describing lower portion of the spine to specify the sacral spine

 5. Specify the "fluorescent cell stains" used. 6.6. There is a typo in line 299.

* CellTracker (ThermoFisher) enters the cell freely then reacts with glutathione transferase to remain intracellularly and is passed onto daughter cells. This was added to the manuscript.

4) Critical steps within the protocol: Aseptic experimental procedures to prevent contamination in the DRG/cancer cell culture should be emphasized.

* Changed 2.2 to reflect this. In all honestly, we do disinfect the dissection surface and autoclave instruments but the dissection and plating of DRG are not done under a sterile hood and we have never had problems with contamination.

*Major Concerns:* N/A  *Minor Concerns:* N/A  *Additional Comments to Authors:* N/A

**Reviewer #3:** *Manuscript Summary:* This manuscript provides an adaptation of a previously published JOVE method by a different group for studying pancreatic cancer.

*Major Concerns:* The major concern is that given the description of this assay as an assay for perineural invasion, demonstration that the tumor cells are indeed moving along the neurites is important. It was difficult to determine at the low magnifications given whether the cells were moving along the surface of the droplet or invading into the droplet, much less whether that was along neurites. Although in figure 6 it seems that physical contact is made between the invading tumor cells and the DRG neurites, it is unclear whether they were attracted to the neurites or invaded along the neurites. If they are simply responding chemotactically to the neurites, that is a different type of response and perhaps should not be described as perineural invasion. Ideally there should be staining of the DRG in a separate color followed by confocal microscopy in order to demonstrate true perineural invasion.

* This is a legitimate concern that we and other investigators using similar techniques have looked into. Within our own experience we have innumerable micrographs depicting the cells tracking along the neurites as well as time lapse footage of the cell movement (which can be included in the video for JoVE). It is true that this does not prove that perineural invasion is occurring or represented but it is very clearly a non-random event. We do run a control wherein we plate cells at the same time as the DRG (i.e. before the neurites sprout) and find that there is no movement of the cells on or into the assays suggesting that the neurites are required to track along. This to us implies more or neural tracking/perineural invasion rather than neurotropism.

*Minor*

*Concerns:* Figure 2, 3, 4,5 and 6 individual images or image pairs should have labels. The legend needs to explain that the second pair images in Figure 4 is (presumably a later time point?).  *Additional Comments to Authors:* N/A

* As above, we will work together to fix these images.