April 30, 2013

Dr. Michelle Kinahan, Ph.D.

Science Editor

Journal of Visualized Experiments

Dear Dr. Kinahan,

# Thank you very much for your recent letter regarding our manuscript JoVE50716R1 'Functional interrogation of adult hypothalamic neurogenesis with focal radiological inhibition'. We have re-arranged the figures as requested by the reviewers, and addressed all comments below, with our responses in bold font.

# Sincerely,

# Daniel Lee, Ph.D.

# Seth Blackshaw, Ph.D.

Editorial comments:  
  
\* All of your previous revisions have been incorporated in to the most recent version of the manuscript. Please download this version of the Microsoft word document from the "file inventory" to use for any subsequent changes. 

**This has been done.**

\* Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

**This has been done.**

\* You vary between using OCT and O.C.T. Please make this consistent throughout the manuscript.

**This has been corrected. Both OCT and O.C.T. have been replaced with the non-commercial nomenclature of “freezing medium”.**

\* Please remove commercial language from step 2.17. (Superfrost Plus slides ) 

**This has been removed, and replaced with electrostatically charged microscope slides.**

Reviewers' comments:  
  
Reviewer #1:   
  
*Manuscript Summary:*   
This (apparently revised) manuscript is a useful description of Computer tomography-guided focal irradiation (CFIR) of defined regions in the mouse brain. Specifically, the authors focus on the hypothalamic median eminence, for which they have recently demonstrated increased production of new neurons in response to a high-fat diet. Such and related experiments often require local suppression of neurogenesis and irradiation is often a method of choice. Problem is that in most cases large areas of the brain become exposed to radiation, thus bringing into question the relevance of the response. This problem becomes particularly acute for the studies of hypothalamic neurogenesis, where neighboring pituitary may become exposed to radiation and change the hormonal status of the entire organism. The authors present a detailed description of a small animal radiation research platform that they have developed (there are similar devices available commercially and their use may also benefit from the detailed description of the procedure in the current manuscript). The procedure is described clearly and fully, starting with preparing mice for the experiments and ending with the tests for the radiation effects, such as gamma-H2AX histone immunocytochemistry. The importance of the manuscript is in clear description of a method that has value beyond the studies of the hypothalamic neurogenesis per se and may be adjusted for irradiating other subregions of the rodent brain. The manuscript is written clearly (line 152 - should "control" be "controlled"?) and the illustrations are relevant to the material. **(line 152 has now been corrected).** Overall, this is a useful methods paper which should help researchers in the field. My only request would be to provide a better discussion of how this protocol can be adjusted for other models of CT-guided irradiation setups, because that will increase the range of researchers interested in the protocol; at this point the use of this technique is essentially limited to the authors' research groups with an access to a unique device. 

**We thank the Reviewer for his/her positive evaluation. To address the Reviewer’s request, we have now discussed more extensively how this CFIR protocol can be used to provide conceptual advances in other research areas. Specifically, we discuss how CFIR could be used to evaluate the function of progenitor populations that have been suggested in other circumventricular organs such as the area postrema (Bauer et al., 2005; Hourai and Miyata, 2013), subfornical organ (Bennett et al., 2009; Hourai and Miyata, 2013), and the pituitary (Gleiberman et al., 2008). We also discuss how using this technique could address longstanding questions of the role of neurogenesis in maintaining birdsong, which have been hampered by the ability to specifically inhibit neurogenesis in specific brain regions relevant to birdsong. It is our hope that publicizing this methodology that will allow researchers outside of our immediate research focus to extend this technique to provide conceptual advances in their own field.**

Reviewer #2:   
  
*Major Concerns:*  
This is an innovative well-written manuscript.

**We thank the Reviewer for his/her positive evaluation.**

The following issues should be addressed.  
1. It is unclear why female mice and relatively young mice are being used. Does this not work with older and male mice?

**This CFIR protocol works with older mice, as well as male mice. In our previous work using this technique, we performed this protocol in 8 week old male mice. In Ford *et al.,* 2011, we highlight using arc targeting to focally irradiate the hippocampal subgranular zone in that publication. This manuscript goes into greater description on how to irradiate a limited focal region, and works on all ages in principle. Furthermore, as we have shown from our previous work, CFIR works on both sexes (Lee *et al.,* 2012; Ford *et al.,* 2011).**

2. The anesthesia might modulate the effects of irradiation. That should be discussed.

**This problem is not well studied. The radiation response of tissues probably does change in response to anesthesia due to temperature and oxygenation effects. An over-response has been shown in animal tumor models. For clinical purposes where patients are treated under anesthesia the response is assumed to be minimal. The key here, though, is that we are using large localized doses for ablative purposes. If there is an over response due to anesthesia it would not impact the experiment.**

3. Recent evidence suggests that DNA double strand brakes occur even if mice explore a novel environment. Therefore, using this method to determine the selectivity of radiation might often not be possible.

**There are other methods such as immunohistochemistry against 53BP1, but similar issues exist there. The number of breaks after irradiation is much higher than the background level referred to here. The purpose of the present experiment is to visualize the location of the irradiated area which is readily accomplished.**

4. It seems important to include the regular diet in the context of irradiation as well to determine if the effects seen are only seen when mice are on a HFD.

**This is now included alongside the HFD data (see revised Figure 6B). Indeed, we have only seen these effects on weight and metabolism with HFD-fed mice or with mouse models of metabolic disorders.**

Reviewer #3:   
This is a nice study demonstrating the focal irradiation of hypothalamus neurogenic zones under CT guidance. This approach has important implications on investigations of other neurogenic regions as well.

**We thank the Reviewer for his/her interest and positive evaluation.**

*Major Concerns:*  
1.For the protocol, draw it out with squares representing each steps in sequence. This is better than text.

**In keeping with the suggested guidelines of the JOVE instructions to authors, this has been kept as text. Similarly, all figures have been kept as layered psd file format so that the JOVE editorial staff can re-arrange the subfigures into square panels during the video editing process. If the editor would like us to change the protocol into squares, please do let us know. We thank the reviewer for this constructive suggestion.**

2. Make a "TROUBLE shooting" PANEL for all potential problems during this experiment. Check Nature protocol papers.

**This has now been included. We list the most common problems that we encountered initially, or that we envision others would encounter while performing this protocol. There are, of course, a number of issues that may arise related to hardware and software issues that are specific to whatever radiological research platform the end-user selects. In those cases, we would suggest they refer to the technical manuals provided at the installation of those devices.**

3. Figure 1,2 can be put together. Figure 6 is important, but it is too few data to stand alone as one figure. The author may integrate this into other figures, or include example pictures at the 3rd Ventricular zone with BrdU labeling.

**Figure 6 has now been integrated with Figure 7. This has been updated in the text as well. However, in keeping with editorial suggestions on the JOVE website, Figure 1 and 2 has been kept separate.**

4. Please discuss the previous failures or mistargeting effects. As shown in Figure 5B, the bottom region of the brain (optic tract??) also demonstrated irradiation effects. Will this harm the vision of the animal, for example?

**At the doses used here, no frank radiation injury is expected to well-differentiated normal tissue. The dose to the optic nerve for example would not visually impair the mouse. Furthermore, the irradiated zone includes very little of the optic nerve, and ongoing rates of cell proliferation in adult optic nerve are very small in any case.**

**In addition, it is the region at the very apex of these radiological beams that demonstrate substantial inhibition of cell proliferation. In our previous study (Lee *et al.*, Int J Dev Neuro, 2012), where we target the median eminence (apex), we find no defect in neurogenesis in the adjacent arcuate nucleus, even though lower levels of gamma H2AX immunostaining are also detected there immediately following irradiation.**

*Minor Concerns:*  
Spelling errors.  
line 186: 5.5-10 week should be "weeks" ? (see line 203, 205) – **this has been changed.**  
line 196: the, ventrobasal should be "the ventrobasal" - **this has been changed.**