**Editorial comments:**

•***NOTE: Please download this version of the Microsoft word document (File name: 54929\_R1\_060616) for any subsequent changes. Please keep in mind that some editorial changes have been made prior to peer review.***                    
  
•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.

•Formatting:  
-Short abstract exceeds 50 words and must be reduced.

We have reduced the words in abstract to 50.

-Two tables are mentioned, but only one is provided. Please delete the reference to the tables in the figure legends section, as only the Table of Materials should be included with this manuscript. Please delete all references to Table 1.

Corrected.   
  
•Grammar:  
-2.2.1 – “Cut the off head”  
-3.1 – “1000P”  
-3.2 – “After discarding the soup” – do you mean discarding the supernatant? Please use “supernatant” rather than “soup”.  
-Line 352 – “be also be”

We have modified these grammar errors. Now we used professional English editing service to correct typos and grammar errors.   
  
•Additional detail is required:  
-1.6 – Are the coverslips placed in the 6-well plate for coating? Or are the wells and coverslips coated separately?

For routines monolayer culture, we used coated 6-well plates. When immunostaining is required, 18-mm coverslip was inserted to each 12-well plate prior to coating, so that cells can attach the glass surface.

-2.2.1 – How is the skin removed?

We have added an additional description to clearly explain the skin removal step.

“To immobilize the head, hold both sides of the head tightly. Cut the skin with scissors from the midline in a caudal-rostral direction.”

-2.2.2 – How is the skull opened after making the incision?

We made clear statement how we removed skull to isolate brain.

“Remove the caudal part of skull (posterior to lambda) first, and then insert the forceps between the skull and brain at the dorsal midline position. Grab the left (or right) parietal bone and carefully peel it off. Repeat this procedure for the removal of the other parietal bone.”

-2.3 note – Please clarify “Ensure that the angle of the brain fits the brain matrix”. What angle of the brain?

The following sentence was added to the revised manuscript:

“In order to obtain parallel plane of coronal sections, ensure the brain fissure is placed in the midline of the brain matrix, it is critical to avoid unnecessary variations in the sections.”

-2.4 – How long can the tissue be stored on ice without solution?

Immediately we chopped dissected SCZ region in 35 mm plastic petri dish after tissue dissection. We recommend that this process is done within 1 min. However, if longer time was required for dissecting SCZ tissue, immerse dissected tissues in a cold PBS before chopping. Additional information was added in Step 2.5 as a Note.

“**Note**: If longer time is required for dissecting SCZ tissue, immerse dissected tissues in a cold PBS before chopping”

-2.4 note - What culture condition? It does not seem as though cells are cultured here; rather they are stored prior to digestion.

Sorry for the mistake, corrected.

-3.4 – Are cultures incubated with shaking? This is not clear.

We have added the following sentence to clarify this issue:

“With hand, shake gently the 6-well culture dish to mix well the added growth factors with plated cells.”

-4.3, 4.5, 5.1, 5.3 – What volumes are used?

We have added volume information to all steps.

-6.2.2.2 – What are secondary antibodies diluted in?

We have indicated it in Step 6.5.

“…incubate with secondary antibodies conjugated to fluorescent dyes (1:500) in blocking solution for 30 min at room temperature.”

•Branding should be removed from 1.6, 4.1, Results, Discussion – Accutase

Removed.

•Results:  
-Figure 2A – Please describe the two images in the legend.

We have described the two images in the legend.

Figure 3 – Please describe the insets in the legend. From which part of the original image are they taken?

We have now described the insets in the legend.   
  
•Discussion: Please describe the other techniques that are mentioned when discussing significance and provide citations. Please discuss any modifications/troubleshooting that can be performed.

We have now described other techniques that we earlier mentioned in the discussion

“Methods for isolating aNSCs from neurogenic region of adult brain including SVZ and dentate gyrus (DG), have been established [22](#_ENREF_23). Although such protocols have promoted the isolation and cultivation of aNSCs in vitro, there are several limitations to obtain high number of cells. Many protocols utilize brain tissue chopper that may cause loss of brain tissue during chopping procedure. Another approach to isolate aNSCs from neurogenic regions uses a coronal cut through the brain with scalpel, followed with microdissection of SVZ, or cut along the longitudinal fissure with scalpel to micro-dissect the DG.”

•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.

Comment is ignored        
                                                  
•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 citing directly from PubMed. In these cases, please manually include DOIs in reference information.

We have included DOIs except for 5 references (#3, #18, and #19) because there are no DOI.   
                                             
•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:*  
Understanding of neural stem cells (NSC) behaviors in adult brain is critical for therapeutic development for various neuro-psychiatric disorders as well as for promotion of normal brain functions such as memory. Thus in vitro culture systems for adult NSCs (aNSCs) offer an important platform for those researches. aNSC culture methods have been previously demonstrated in many studies, but we cannot say that the aNSC culture is a commonly utilized technique up to date, mainly due to fastidious culture methods and lack of clarity in their protocol descriptions. The paper of Kim et al. describes the detailed methods for isolation, expansion, passaging, differentiation of aNSCs, specifically from subcallosal zone (SCZ), which has recently been shown as another neurogenic brain region, responding to injury at posterior brain regions. Considering that aNSCs in subcallosal zone (SCZ) are much less abundant than the other neurogenic brain regions such as SVZ and hippocampal SGZ, the protocol for successful SCZ-aNSC culturing described in this paper seems to be an advance in aNSC culture methods. Thus this article provides a description of a technique that will be of interest to researchers, especially who have been experiencing difficulties in aNSC cultures. Only several minor comments are made as follows:  
  
*Major Concerns:*  
N/A  
  
*Minor Concerns:*  
1. Page 2, line 4, 'white matter' can be changed into 'corpus callosum (white matter)'

We have changed.

2. An English editing service may be required, due to many awkward and unclear descriptions, the followings are the examples:  
- P. 3, 1.2) 'For brain wash'  
- P. 3 1.3) 'dissociation brain'  
- P.3 1.4) 'growth medium by containing'  
- P.3, 1.6) 'For the cell split'  
- P.4, line 5, 'affect affects'  
- P4, 3.1) 'below 5 times'  (<5 times)  
- P.4 3.3) 'non-coating'  'uncoated', 1ml suspended cells 1ml of suspended cells, make final 2ml growth medium  make final volume to 2ml by adding growth medium

Thank you for the comments, and we utilized an English editor service to correct grammatical errors.

3. The following issues can be discussed at least in discussion section:  
-How long (or How many passages) can the SCZ-aNSC be expanded in vitro?

We can culture SCZ-aNSCs over 10 passages. We have added this point in the discussion section.

“SCZ-aNSCs were passaged for extended periods, <5 passages in a neurospheres format and> 10 passages as a monolayer, this consistent with previous that suggests monolayer culture system maintains NSCs in vitro in long-term cultures.”

- What is differentiation propensity of the SCZ-aNSCs in culture? (% of neurons, astrocytes, oligodendrocytes)

We published a study including this data. We have mentioned this point briefly in discussion.

“Extended passaging affects to multipotency and neuronal differentiation with increased chromosome aberration. Therefore, to avoid extended passaging effect, it is recommended to use early passage (< passage 5) cells for proliferation and differentiation analysis. Although the potential for self-renewal of SCZ-aNSCs is similar to SVZ-aNSCs, less neuronal differentiation was shown in SCZ-aNSCs”

- Description about the aNSC property changes during in vitro expansions (passages).

We have mentioned.

“Extended passaging affects to multipotency and neuronal differentiation with increased chromosome aberration. Therefore, to avoid extended passaging effect, it is recommended to use early passage (< passage 5) cells for proliferation and differentiation analysis. Although the potential for self-renewal of SCZ-aNSCs is similar to SVZ-aNSCs, less neuronal differentiation was shown in SCZ-aNSCs”

4. Fig. 1A: It is more comprehensive to indicate other brain regions (Lateral ventricle, corpus callosum, hippocampus…) alongside SCZ highlighted in red.

We have modified Fig 1A and included other brain regions (Lateral ventricle, hippocampus).

*Additional Comments to Authors:*  
N/A

**Reviewer #2:**  
*Manuscript Summary:*  
This manuscript demonstrates a simple technique to isolate adult neural stem cells (NSCs) from subcallosal zone (SCZ). Since SCZ has recently been focused as a novel stem cells containing region, isolation, expansion, and analyzing detailed differentiation properties of these SCZ derived NSCs are important for potential therapeutic approach such as traumatic brain injury. The authors tried two well-established NSCs culture system, sphere culture, and monolayer culture, to show that isolated SCZ-NSCs possess ability to differentiate into three major types of neural cells.  
Since protocol is simple and don't need any specialized instruments or fancy dissection tools, scientists who are interested in isolating these NSCs would easily try to follow this method. However, except for the utilization of brain matrix to separate SCZ, most of author's provided methods in here are more or less the copy of well-established systems that are used in these research fields. Detailed comparison of NSC's character of SCZ-NSCs with SVZ or SGZ-derived NSCs would help increasing the value of current studies.

Thank you for the comments. It is true that NSCs culture system is well established. However, the novelty of our method is the micro-dissection of SCZ out of a complex brain structure and study the behavior of cells of SCZ in vitro, as this reviewer commented. In addition, we also proposed the efficient way to expand SCZ-aNSCs by neurosphere formation (for isolation of aNSCs) and subsequent monolayer culture (to maintain and expand aNSCs). We have compared these cells to SVZ-aNSCs and recently published in Stem cells (Kim et al., 2016).

*Major Concerns:*  
Is there any difference for the differentiation potential and proliferation speed of SCZ-derived NSCs, compared to others? (Beside authors declared in page 5 line 186.) How about general proliferation speed in monolayer cultures? When SCZ-NSCs are differentiated, what are the percentages of cells generating each type of cells (neurons, astrocytes, and oligodendrocytes).

We have added a description in the discussion section. We examined and demonstrated the characteristics of SCZ-aNSCs including proliferation and differentiation in recent Stem Cell journal (Kim et al., 2016).

“SCZ-aNSCs were passaged for extended periods, <5 passages in a neurospheres format and (> 10 passages as a monolayer), this consistent with previous that suggests monolayer culture system maintains NSCs in vitro in long-term cultures. However, the proliferating speed decreased, and the portion of dying cells increased over 5 passages. Extended passaging affects to multi-potency and neuronal differentiation owing with increased chromosome aberration. Therefore, to avoid extended passaging effect, it is recommended to use early passage (< passage 5) cells for proliferation and differentiation analysis. Although the potential for self-renewal of SCZ-aNSCs is similar to SVZ-aNSCs, less neuronal differentiation was shown in SCZ-aNSCs.”

*Minor Concerns:*  
1. Protocol has to be arranged along the time series from beginning.  
It would be good to demonstrate from 'coating procedures'. Since coating is important step, and coating material is sometimes very toxic to cells. I wonder more detailed protocol for this section.

In this protocol, coating step is required for maintenance of aNSCs with passage 1 after primary neurosphere culture is done. We have modified and added more detail protocol in coating section in Step 1.6, 1.6.1, and 1.6.2.

“**Preparation of coating plate and coverslip:**

Prepare poly-L-ornithine (PLO; 0.01%) and Laminin (10 µg/ml dissolved in DH2O).

1.6.1) To coat 6-well plates for maintenance of SCZ-aNSCs as monolayer or 18-mm coverslips for immunostaining, incubate them with PLO overnight at 4 °C, followed 3 times washing with DH2O. Allow plates and coverslips to dry after the last washing.

1.6.2) Next, incubate the plates with Laminin overnight at 4 °C, followed 3 times washing with DH2O.

**Caution**: Do not dry Laminin, which affects cell attachment.

**Note:** Coating solutions can be re-used 3 times.”

2. There are several grammar mistakes (eg. page 1 line 35, 'recognized as novel a neuroblast-' should be 'recognized as a novel neuroblast-') and appropriate expression (eg. page 4 line 169, What does the soup means?).

We have corrected grammatical errors according to your comments. The soup is the supernatant. For better understanding, we re-stated the word from soup to the supernatant.

3. It appears that author's English expression make it difficult to imagine their experimental procedure especially for page 3 and 4. English correction by a native-speaker would help understanding these things. Definitely movie will help a lot.

Thank you for the comment, and we have rephrased our paragraphs in page 3 and 4. In addition, we have used English editor service to overcome grammatical mistakes. As you stated, a video will definitely help to follow up with our procedures.

4. I found some incorrect information. (eg. page 2 line 80. Not many current protocols use whole brain to isolate NSCs. Even the paper they referenced, Walker et.al., they dissected out subventricular zone and dentate gyrus to isolate adult NSCs.

Sorry for the mistake, and we have revised and erased incorrect information from the revised manuscript.

5. There are several mistakes in concentration or units. Obviously, these information are important, thus author should be again point by point confirmation.  
(page 5 line 179, 200 ml should be 200 ul? )

We are sorry for the error. Concentrations and units were correctly stated in the revised manuscript.

6. Table1. Coating materials, PLO, comments, 'Working concentration: 0.1%'. Is this correct? Should be 0.01%.

We have edited accordingly.

*Additional Comments to Authors:*  
N/A

**Reviewer #3:**  
*Manuscript Summary:*  
Jun et al, present a method to isolate and culture subcallosal zone NSCs. This paper is in general well written and of interest to the scientific community. We have only minor concerns that should be addressed.  
\*On line 65, the sentence should be changed from "The number and size of the cells in the neurosphere…" to "The number of cells and size of the neurosphere…"

We have changed it according to your comments.

\*The structure and wording of the paragraph starting on line 88 and continuing to line 92 should be edited since it is confusing (i.e. "Neurospheres could be formed from SCZ-aNSCs in the culture system and maintained continuously as neurospheres or monolayers")

We have edited.

“After SCZ-aNSCs form neurospheres, they also can be maintained as neurospheres or monolayers for expansion”

\*Step 1.3 on line 105 should clarify what is the meaning of "prepare the surgical tools".

We have included “autoclaved surgical tools” in this section.

\*Step 2.2.2 on line 132 should add a sentence clarifying the skull must be removed to extract the brain.

We have added a description of brain removal from the skull.

“Remove the caudal part of skull (posterior to lambda) first, and then insert the forceps between the skull and brain at the dorsal midline position. Grab the left (or right) parietal bone and carefully peel it off. Repeat this procedure for the removal of the other parietal bone.“

\*The note under step 2.4 on line 152 should have a reference if published.

We cited two references. And to culture SCZ-aNSCs, we modified the culture method of SCZ-aNSCs compared to other protocols to get a high yield of aNSCs *in vitro*.

\*Step 3.2 on line 169 mentions the discarding of the "soup" when it should be supernatant.

Corrected

\*Step 3.3 on line 172 should clarify if there is a specific number of cells that should be plated as stated on Figure 1.

We did not count the number of cells prior to plating SCZ-aNSCs. Instead, all single cells that were obtained from digested SCZ brain tissue were plated.

\*Step 4.1 on line 182 should add that the neurospheres are transferred into a conical tube before the incubation on Accutase.

Yes, we have added this in Step 4.1.

“Gather neurospheres and transfer to a new 15 ml conical tube.”

\*The note under step 4.1 on line 185 should clarify if the number of neurospheres quantified is per well or total.

We have added “per well”

\*A note needs to be added to Step 5.3 on line 215 indicating that BrdU must be added to live cells before fixation if BrdU staining is of interest.

We have added note that explain BrdU treatment.

“**Note**: BrdU can be incorporated into newly synthesized DNA of proliferating cells. Therefore, if desired, BrdU (10 g/ml) can be added to live cells before fixation of cells.”

\*Step 6.2.1 should clarify the units of BrdU (i.e. ug/ml).

We have given the units of BrdU in note of Step 5.3.

“**Note**: BrdU can be incorporated into newly synthesized DNA of proliferating cells. Therefore, if desired, BrdU (10 g/ml) can be added to live cells before fixation of cells.”

\*The duration of BrdU incubation should be clarified. The duration of the incubation is defined as overnight on line 265 while on line 232 it's defined as 2 hours.

Thank you for the comment. We have clarified the duration of BrdU incubation time. Two hours incubation is right. We have edited that in manuscript. And we have added this information in the note of Step 5.3.

“**Note**: BrdU can be incorporated into newly synthesized DNA of proliferating cells. Therefore, if desired, BrdU (10 g/ml) can be added to live cells before fixation of cells.”

\*On line 318, the sentence discussing cell death occurring due to non-NSCs should be referenced if published.

We have added references.

\*On line 337, the sentence discussing the lack of nutrients reaching the core of neurospheres should be referenced if published.

We have added references.

\*On line 339, the sentence discussing multiple passages of NSCs should mention there's a decline in multipotency and/or neuronal differentiation after multiple passages if this is the case as it is for adult SVZ NSCs.

We have added accordingly.  
  
*Major Concerns:*  
N/A  
  
*Minor Concerns:*  
N/A  
*Additional Comments to Authors:*  
N/A