December 7, 2017

Alisha DSouza, Ph.D.  
Review Editor  
[JoVE](http://www.jove.com/)

Dear Dr. Dsouza,

Thank you very much for reviewing our manuscript entitled “Immunophenotyping of orthotopic homografts (syngeneic) of murine primary KPC pancreatic ductal adenocarcinoma by flow cytometry” by An *et al*, and the suggested revisions. I sincerely appreciate the time and the thorough review by the three reviewers. We are in complete agreement with reviewers’ critiques. We have now completed the revision of the manuscript per editor/reviewers’ suggestions and are submitting the final version for publication. We have also addressed the reviewers’ comments item by item, as shown in **Red bold font** as below. In addition, we have carefully edited the text and hope that it is now acceptable for publication.

Please note that I have attached a clean version along with the one with tracked change for reference. I have also attached original Figure 1C Tiff file photo in case the problem with resolution.

Please do not hesitate to contact me if you have any question regarding this submission. Thank you again, and I look forward to hearing from you.

Sincerely,

Sincerely,

Henry Q.X. Li, Ph.D.

Vice President, Translational Oncology, Crown Bioscience, Inc.

#6 W. Beiijng Rd., Taicang, Jiangsu, China

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Dear Dr. Li,

Your manuscript, JoVE57460 Immunophenotyping of orthotopic homografts (syngeneic) of murine primary KPC pancreatic ductal adenocarcinoma by flow cytometry, has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits. After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually.

Your revision is due by **Dec 19, 2017**.

To submit a revision, go to the [JoVE submission site](http://www.editorialmanager.com/jove) and log in as an author. You will find your submission under the heading "Submission Needing Revision".

Best,  
  
Alisha DSouza, Ph.D.

Review Editor

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**Editorial comments:**

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. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. **Yes, we have done so thoroughly per the suggestion.**

2. Unfortunately, there are a few sections of the manuscript that show overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 76-81, 309-316, 374-391. **Thanks for pointing this out. Some of the mentioned work will also appear in a proposed chapter of a book which has yet to be published, although submitted. It is likely that the editor for that book is still waiting for other chapter contributors to put them together. Most likely it will be published after this current manuscript. In any event, we have now revised the mentioned sections as per the suggestion. Meanwhile, we will also revise our other manuscript (chapter) for the same rationale, so both final versions will be sufficiently different.**

3. Figure 1: Please provide scale bars for panel C. **We added “40x10” in the figure legend.**

4. 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. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. **Removed as suggested.**

5. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. **Removed as per suggestions.**

6. Step 1 of the protocol can be removed entirely. Please ensure all of this information is in the Materials Table instead. **Removed as per suggestions.**

7. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. **Revised per suggestions.**

8. What type of mouse is used? **We added C57BL/6 mice in the text and Figure 1 legend.**

9. How is the tumor surgically removed? We need more details on the surgical procedure.

**We now added the details per suggestions.**

10. Please mention how proper anesthetization is confirmed.

**We now added the confirmation per suggestions.**

11. 4.1.4: Inoculate the tumor how?

**Now, we added the description.**

12. Please specify all surgical tools used and when.

**Now, we added more descriptions per suggestion.**

13. Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency. **Added.**

14. Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered. **Added.**

15. What is the gentleMACS program? Please use generic terms. **Added description.**

16. Please highlight 2.75 pages or less 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. **Done**

17. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. **Done.**

18. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol **(described)**

b) Any modifications and troubleshooting of the technique **(done)**

c) Any limitations of the technique **(added)**

d) The significance with respect to existing methods **(yes)**

e) Any future applications of the technique **(done)**

**Reviewers' comments:**  
Reviewer #1: Manuscript Summary: The manuscript by An, et al. describes the method of immunophenotyping of a orthotopic pancreatic adenocarcinoma. The paper appears to have all the necessary details to successfully reproduce the experiment

Major Concerns:

Minor Concerns: Since identifying and quantifying the various immune cells is a key part of the manuscript, more details in the Figure legend and the Results section for Figure 3 would greatly enhance the manuscript. **Added more description per suggestions.**

Reviewer #2: Manuscript Summary:

The manuscript describes the method for setting up a mouse model for orthotopic homograft of pancreatic tumor and flow cytometry-based phenotyping of immune cells in that tumor.  
The authors have extensive previous experience with patient tumor-derived xenograft mouse models. Now they are applying their expertise to the orthotopic homograft tumor mouse model.  
Although immunotherapy made an important breakthrough in treating cancer patients, majority of patients are still not getting clinical benefits. There is an increasing awareness that the more "physiological" pre-clinical tumor models will provide new insights into the improvement of cancer immunotherapy. Therefore, the aim and scope of this paper is timely. The manuscript is well written and is easy to follow. The level of technical details provided in this manuscript with video demonstration is appropriate and will help others implement this model.

Major Concerns:

The quality of results section needs to be improved.

1) Fig. 1A showed vehicle vs Gemcitabine. Gemcitabine was not effective. Is there any reason to believe showing gemcitabine data is helpful to understand the model? **We agree with the reviewer that the Gemcitabine treatment may not necessarily be needed to demonstrate the procedure of this model. It is just that this model happened to be insensitive to gemcitabine as the SOC, which was observed in our early model validation experiment. In the video portion of manuscript, we will not include the treatment portion.**

2) The H & E staining for SubQ Tumor in Fig. 1C needs to be replaced with the one with higher resolution. Scale needs to be provided as well.

**Replaced as suggested.**

3) Fig. 2 is not necessary.

**Figure 2 intends to show that tissue dissociator plays a critical role in successful TIL analysis.**

4) It is unclear why PD-1+ cells were excluded from T cell analysis in the Lymphocytes panel in Fig.3 A. **We revised. There is no PD-1 involved in this study.**

5) For tumor cells in Fig. 3A, are PD-L1+ cells and Ki-67+ cells mutually exclusive?

**We revised. PD-L1 and KI-67 were not involved in this study.**

6) For Lymphoid gating in Fig. 3B, CD11b staining for CD19- population was mistakenly shown.

**This is for extra parent gates for further T cell analysis. We gated CD19- cells from CD45+ (total leukocytes) which identifies all non-B cells (myeloid cells plus T cells), followed by gating for CD11b- cells to remove CD11b+ myeloid cells from CD19- cells, in another word, we tried to remove as much as non-T cells. In the end, we gated the CD3+ T cells from the remaining cell population.**

7) For Fig. 3C, authors should show data in counts in addition to %. They should also show statistics. The graph should also show individual data points or mention the number of mice analyzed. It is unclear how many times experiments were repeated.

**Done. We have edited the plots to bar graph plus dot plots to represent each individual animal.**

Minor Concerns:

1) Authors should clearly convey the benefit of the orthotopic homograft (e.g., allow to synchronize timing of tumor growth) in Introduction or Discussion. **Added now in the Introduction.**

2) Line 125: Current most up-to-date FlowJo version is v10.4. Is there any reason to use v10.0?

**We have yet to upgrade the software. We do not believe our current version (10.0) will not affect the analysis for this study.**

3) Line 145-146: It is unclear if you mean any of three is OK. **Now removed.**

4) Line 163-167: numbers are not consecutive. They should be #27-31 rather than #17-21. **Now removed.**

5) Line 166: Supposed to be "Sterile PBS"? **now removed.**

6) Line 172: Please specify "the local IACUC". Is this study approved by the Crown Bioscience Inc. IACUC? **Now specified.**

7) Line 195-197: How did you measure the tumor volume? **Using caliper, now added the description.**

8) Line 255-257: How frequently did you observe this to occur? **We believe that this is more model specific. For this particular model, we do not have sufficient data to know the frequency.**

9) Line 289: section 6.1.1 instead of 5.1.1. **done**

10) Material table was truncated. It also misses information for company and catalog number. The table needs to be complete.

**Corrected.**  
  
Reviewer #3: Manuscript Summary:

Using the immunophenotyping of murine orthotopic PDAC homograft, authors aims at profiling the tumor immuno-microenvironment. This is a well-organized paper with protocol in details. This protocol would be applicable for likely a majority of tumor models used. My opinion is " accepted with minor revision".

Major Concerns:

Digestion with enzymes can be performed. This will allow for a more thorough single cell suspension. However, it should be noted that such digestion protocols can affect surface antigen expression, so caution should be taken in interpreting these results. This can be verified using enzyme treated or un-treated samples.

Minor Concerns:

1. Page 7, line 199, after" containing 20 mL PBS", "Chill media or buffer to 4 °C prior to euthanization" is recommended; **now revised per suggestions.**

2. Page 7, change the sequence of step 3 and 5 on "3.2. Donor tumor fragment preparation"; **Changed now.**

3. Page 7, add "Only intact pieces were chosen" to step 6; **Revised per suggestion.**

4. Page 7, line 203, what is "transport medium"? Please give the detail. **Changed to PBS.**

5. Page 8, line 233, the sentence is recommended to use "Once anesthetized, the mice were fixed on an experiment board in the right lateral position." **Revised per suggestions.**

6. Page 8, line 240, please add "homoeostasis was achieved by compression" to the end of item e; **revised.**

7. Page 8, line 242, please add "If neither bleeding nor tumor tissue leakage occurred" to the ahead of "After finishing tumor implantation"; **revised per suggestions.**

8. Page 9, line 299, PBS should be cold; **revised.**

9. Page 10, line 314, add "4 °C" after "300 x g"; **done.**

10. Page 10, line 320, replace " count the cell number" with "count viable cells using trypan blue" ; **revised.**

11. Page 10, line 320, add "Include the correct isotype control antibodies to ensure staining is specific." to the end of item 2; **Revised.**

12. Figure 1, C, left photo (H&E) is unclear, ple

**Now changed**