The manuscript has been modified to include line numbers and minor formatting changes. The updated manuscript **57585\_R0.docx** is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink to download the .docx file. **Please download the .docx file and use this updated version for future revisions.** The file is also attached.  
  
You will find Editorial comments and Peer-Review comments listed below. Please read this entire email before making edits to your manuscript.  
NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.   
  
**Changes recommended by the JoVE Scientific Review Editor:**  
  
• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.  
  
• Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.  
  
• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.  
  
• **Protocol Detail:** Please add more details to the following protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure that all additional details in the protocol section are written in the imperative tense, as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.).

All the points mentioned below has been taken care of except 12.

1. 2.1: What are Enzyme A, B, D?  
   2) 3.1: What kind of tumor? What is the mouse strain, age, sex, weight? What size of the tumor? Are these subcutaneous tumors? Are they xenograpfts? How are they dissected? Is the animal anesthetized/euthanized? Mention the method. What is the size of tumor?  
   3) 3.2: What is the recommended size of pieces?  
   4) 3.3: Mention dissociator speed, duration and temperature in generic terms.  
   5) 3.4: What is the rotation speed?  
   6) 3.5: Mention dissociator speed, duration and temperature in generic terms.  
   7) 4.1: Mention all antibody concentrations.  
   8) 4.3: Unclear how the washing steps are performed.  
   9) 4.5: It sounds like there may be a missing centrifugation step. Mention all antibody concentrations.  
   10) 4.5: check double numbering  
   11) 4.6: Mention centrifuge speed and duration.  
   12) 5.1 to 5.4: Unclear what is done here. If this is to be filmed more specific details are needed, e.g. button clicks and menu selections.

These steps involve using flow cytometry instrument, normally only done by a trained personnel. The steps cannot be precisely wriiten as it depends on what instrument is being used and what is the associated program with it.  
13) Section 5: Step numbers are repeated.  
14) 6.1, 6.2: Penstrep %?  
15) 6.2: How are cells counted?  
16) 6.4: Mention e.g. of cytokines you tested and their concentrations.  
17) 7.4: When and how are the cells counted?  
18) 7.6: Mention step numbers where this is described.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE’s instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.  
  
• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight 2.75 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE’s instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.  
  
• **Results:** Please mention the statistical tests performed and report sample sizes.  
  
• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.  
  
• **Figures:**:  
1) Please provide each figure (if multiple panels are present per figure, keep them within 1 file) as an individual PDF, TIFF, JPEG or PNG file.  
  
• **Figure/Table Legends:**:  
1) Please expand the legends to adequately describe the figures/tables. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.  
2) Define \*\*, \*\*\*, \*\*\*\*.  
  
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• **Table of Materials:**Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as all enzymes, media, FACS buffer, dissociator, antibodies and their concentrations,cytokines and neutralizing antibody, etc.  
  
• Please define all abbreviations at first use.  
  
• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.  
  
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**Comments from Peer-Reviewers:**   
  
**Reviewer #1:**   
Manuscript Summary:  
The manuscript "Isolation of myeloid derived suppressor cells from mouse tumor and determining their migration potential in vitro" describes the experimental procedure to isolate MDSCs from mouse solid tumors by flow cytometry sorting and perform an in vitro MDSC migration assay. Its introduction clearly indicates the importance of MDSC studies in cancer field. The protocol is generally well-written and easily understandable. However, some detailed explanation may be needed in some parts of the protocol and figures.  
  
Major Concerns:  
Protocol 2.1:  
The author does not explain what Enzyme D, R and A are and their functions in tumor tissue dissociation. There is no material information too.

Response: We have edited the text to avoid using commercially available reagents. The exact composition of Enzymes D, R and A are not known.

How long can the dissociation buffer be stored? It needs to be clarified since enzymes are easily degraded.

Response:The dissociation buffers need to be made fresh..

It would be better to add the method for murine ovarian tumor injection, such as the number of cells for implantation and tumor growth rates.

Response: Details added to text.

There is no detailed information about how and when tumors necropsied and processed.  
Protocol 2.4:

Response: Details added to text.

The protocol says that the staining buffer with antibodies can be stored for long term. But antibody can be degraded and we generally avoid to keep the antibody dilution for long time. It needs to be clarified. It also does not indicate the concentration of each antibody.

Response: The staining buffer is stable for up to 1 week if stored appropriately. This is useful only if staining experiments are done on a regular basis. We have edited the text to avoid such confusion.  
  
Minor Concerns:  
Protocol 3.7: What is the volume of RPMI medium for resuspension?

Response: We used 25ml. We have added the information to the text.

Protocol 3.12: It would be better to indicate the approximate number of single cells in 1gm of tumor tissue for antibody dilution and flow cytometry sorting analysis.

Response: We have added the information to the text.

Protocol 5.4: It needs to indicate the approximate number of MDSCs in 1gm of tumor tissue after sorting. It gives ideas to set up MDSC migration assay.

Response: It is difficult to determine the number of MDSCs as it depends on the tumor type.

Figure 1: It would be better to show all steps of sorting strategy. It does not show the plots of total events and negative controls. It needs to be clear that the gates of CD45, CD11b and GR1 positive cells is reasonable. It also needs to show the percentages or numbers of MDSCs by graphs.  
Response: We have added the gatings to the figure.

**Reviewer #2:**  
This manuscript is interesting. Data analysis and results are also perfect. In this manuscript, the authors have discussed the methodology to isolate MDSCs from mouse ovarian tumor. The same  
method can be utilized for isolating MDSCs or other immune cells from any solid normal tissue or solid tumor using cell specific markers.  
Although the manuscript is well written, few minor revisions needs to be done before publication.  
  
1. Did you treat the cells with Mitomycin -C to check the migration.

Response: the cells were not treated with Mitomycin-C  
2. Please also stain the migrated cell and quantify by manual method or by absorbance based method.

Response: The cells were quantified my flow cytometry based method.  
  
  
**Reviewer #3:**   
In this manuscript (JoVE57585), Sarkar describes a protocol to isolate myeloid-derived suppressor cells from tumors and assess their migration capacity. As noted in the introduction, similar methods have been reported for isolation of other types of immune cells, including for MDSCs. Although migration assay provides a slightly different perspective, the value of this section is weakened by the lack of some important steps that would verify the robustness of the method.  
The manuscript needs editing for clarity and the proper use of scientific language/terminology. There are some knowledge gaps that should be corrected in the introduction.  
Throughout the manuscripts the term MDSCs is spelled out as myeloid derived suppressor cells. It should be corrected as myeloid-derived suppressor cells.

Response: We have edited the text.

In Figure 1, it would benefit the readers to show granulocytic and monocytic MDSCs on the same flow plot (likely needed to show additional plots) and re-label axes with the antibody (not channel).

Response: We have modified the figure to accommodate the changes suggested.

Line 22: Although immunological mechanism that associate with different disease states might remain elusive, the importance of immune system in cancer is known and appreciated. It is unclear what the other diseases are.

Response: We have added a few examples of other non-cancer diseases where immune system plays a role.

Line 26: For precision it should be emphasized MDSCs negatively influence "anti-tumor" or "pro-inflammatory" immune response.

Response: We have edited the text accordingly.

Line 38-44: There are no citations in this section.

Line 48: Unlike macrophages and dendritic cells, monocytes are immature cells. Could you please cite the respective articles reporting MDSC differentiation into these cell types?

Response : We have added the reference.

Line 51: COX2 is not a secretory molecule.  
Response : We have corrected that.

Line 65: "Commonly CD14-CD11b+ cells are characterized as MDSCs" This statement is wrong. Monocytic MDSCs are positive for both markers, while granulocytic MDSCs express CD15 instead of CD14.

Response: We apologize for the error. We have corrected it in our revision.

Step 2.3) Addition of EDTA could be important to ensure single cell suspensions.

Response: We have not added additional EDTA in the solution.

Although CD11b, Gr1 are commonly used to analyze MDSCs as a bulk population, there is an ongoing debate about how to discriminate between MDSCs and neutrophils with this staining profile. It would be best if the author could show the Ly6C and Ly6G staining pattern of the sorted cells to address the issue of MDSC subsets.

Response : We have added the flow gating but the experiment has been done with Gr1+CD11b+ cells.

Line 169: It is important to emphasize the approximate cell density appropriate for cell sorting.

Response : We have added the information.

Line 194: Can the author please comment on the yield of sorting process? Despite increased frequencies in tumors, 0.3-0.5 10^6 sorted MDSCs seems to be a difficult number to reach.

Response : We have used intraperitoneal tumors for the purpose. In this case we get enough tumor to perform the experiment. About 25% of all viable cells are MDSCs in this tumor type. For other tumors cells from multiple tumors can be pooled together if genetic background of tumors and host mice remain the same, for a general idea about the nature of MDSCs in those tumor type.

Did the author confirm the viability of MDSCs immediately after post-sorting and 6 hours later? Immature myeloid cells have very short half-life and unless exposed to stimulants, they start dying within hours. The lack of transmigration in untreated or anti-TNFa containing groups could simply be accumulation of more dead cells in the upper chamber.

Response : We appreciate the criticism. The cell viability was determined while counting cells post sorting and before plating into inserts for migration assay.

6 hours post migration cells were stained with cell viability dye for flow cytometry. We have added that information to the protocol.