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Lung Tumor Cell Recruitment Assay

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TITLE:
Lung Tumor Cell Recruitment Assay

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SHORT ABSTRACT:
This manuscript describes a method to quantify tumor cell accumulation in the lungs in an animal model of tumor metastasis.

LONG ABSTRACT:
To investigate the molecular mechanisms governing tumor metastasis, various assays using the mouse as a model animal have been proposed. Here, we demonstrate a simple assay to

evaluate tumor cell extravasation or micrometastasis. In this assay, tumor cells were injected through the tail vein, and after a short period, the lungs were dissected and digested to count the accumulated labeled tumor cells. This assay skips the initial step of primary tumor invasion into the blood vessel and facilitates the study of events in the distant organ where tumor metastasis occurs. The number of cells injected into the blood vessel can be optimized to observe a limited number of metastases. It has been reported that stromal cells in the distant organ contribute to metastasis. Thus, this assay could be a useful tool to explore potential therapeutic drugs or devices for prevention of tumor metastasis.

INTRODUCTION:

Tumor metastasis accounts for high mortality in cancer-related diseases. From the viewpoint of investigations at the molecular level, metastasis can be divided into multiple steps: tumor initiation at the primary tumor site, primary tumor growth and invasion into surrounding tissues, intravasation, circulation through blood vessels, extravasation at distant organs, and tumor re-growth. Each step involves different sets of molecules/signals[1].

Studies to date have been concerned with events occurring in distant organs before the tumor cells start circulating in the blood, which is also known as the pre-metastatic phase[2-6]. Our mouse model studies revealed that the pre-metastatic phase facilitates lung metastasis by creating long-lasting and low-level inflammatory responses in the lungs. The pre-metastatic phase is characterized by hyperpermeability of the microvasculature, recruitment of bone marrow derived cells (BMDC), and upregulation of cytokine/chemokine-like molecules including S100A8 and SAA3[3,4]. It has been reported that lysyl oxidase modifies the extracellular matrix in the lungs to recruit BMDCs[7]. Another report has revealed the importance of Angpt2, MMP3, and MMP10 in the pre-metastatic phase[8]. In other words, the intricate interactions between primary tumors, bone marrow, and remote organs need to be understood and properly modulated (for instance, by using drugs that target proteins involved in these interactions) to prevent future metastasis[9]. To regulate the pre-metastatic phase, it should first be visualized and evaluated for diagnostic or therapeutic interventions. Here, we report a lung tumor cell recruitment assay that enables the study of the pre-metastatic phase in the lungs.

Tumor cells directly injected into the blood vessel of the mouse are similar to circulating tumor cells in cancer patients, although there may be differences between cultured cells and circulating tumor cells. Circulating tumor cells themselves undergo some characteristic changes when they enter circulation from the primary tumor. Nevertheless, cultured cells can form tumors in immunodeficient mice when they are injected in the tail vein. Additionally, in the mouse model system, fluorescence labeled tumor cells can be used that allow tracking of these cells in the body. The behavior of circulating tumor cells is critical as they determine the ultimate consequences in cancer patients[10]. Blood is not a good place for survival of tumor cells[2]. They need to escape from attack by the host immune system and need anti-apoptotic signals to prevent apoptosis due to a lack of physical support. Tumor cells with higher abilities of tumor initiation at metastatic sites generate more tumor nodules. If the tumor cells show specific organ tropism, metastasis should be observed in those particular organs. In this assay, the initial steps of the metastatic cascade (primary tumor growth and invasion) are not included,

and so the focus is on the interaction between circulating tumor cells and the stromal cells (endothelial cells, epithelial cells, and blood cells). In conventional tumor cell injection assays, researchers have to wait until tumor nodules grow to a tangible size to detect the metastasis. In this case, the exact number of tumor cells in the blood vessel cannot be quantified. Additionally, this process includes a tumor regrowth phase, indicating that other factors in the tumor cells may affect the outcome.

Counting labeled tumor cells under a fluorescence stereomicroscope is a simple process. The stereomicroscope provides a wide field of view so that the whole lung can be scanned. Flow cytometry is also a useful tool that instantly gives an accurate number of tumor cells in the tissue. Counting fluorescent cells in the tissue under a confocal microscope is also possible and displays the most accurate pictures of metastatic tumor cells, but it is time-consuming and may give biased results if only a selected area is counted. The ultimate goal of this assay is to observe how easily tumor cells accumulate in the lungs. As reported previously[3,4], if the lungs are in pre-metastatic phase due to inflammatory signaling from the primary tumor, the injected tumor cells are prone to accumulate in the lungs, thus implying higher chances of lung metastasis. Other conditions (rheumatoid arthritis, asthma, *etc.*), and their treatment with anti-inflammatory agents (such as anti-TNFα) may attenuate lung metastasis. Thus, we conclude that this assay is a powerful tool to assess the possibility of lung metastasis.

PROTOCOL:

All procedures performed with mice were approved by the Animal Research Committee of Tokyo Women's Medical University.

1. Tail Vein Injection of Lewis Lung Carcinoma (LLC) Cells

1.1. Maintain LLC cells in DMEM supplemented with 10% FCS, in a humidified 5% CO_2 incubator at 37 °C. Cells should contain a fluorescent protein expression system (*e.g.,* GFP).

1.2. Remove cells from culture dish by using a non-enzymatic cell dissociation reagent. Follow manufacturer's protocol.

1.3. Collect cells in a 15 mL tube, and centrifuge at 400 x g for 3 min. Resuspend the cells in 5 mL of PBS by pipetting, and centrifuge the tube (400 x g, 3 min). Then, remove the supernatant.

1.3.1. Repeat this washing step one more time.

1.4. After the second wash, pass the cells through a cell strainer (40 µm pore size), and resuspend in PBS to give a final cell density of 1 x 10^6 cells/mL.

1.5. Inject 0.1 mL of cell suspension (1 x 10^5 cells, 29G needle) into each C57BL/6 mouse (8 – 10 week old males) via the tail vein.

2. Isolation of the Lungs and Counting the Cells Under a Fluorescence Stereomicroscope

2.1. Euthanize the mice by CO_2 inhalation.

2.2. Remove the skin on the ventral surface of the chest with scissors. Then, cut the ribs and the diaphragm to expose the thoracic cavity. Flush PBS (100 cmH_2O, total 15 mL) through the right ventricle with a winged infusion 25G needle and tubing.

2.3. Cut out the heart and the thymus. Grip the trachea with forceps, and pull up, dissecting the connective tissues around trachea with scissors. Dissect out the lungs and wash them in PBS.

2.4. Detach each lobe and observe it under a fluorescence stereomicroscope.

3. Lung Digestion

3.1. Dissolve 10 mg of collagenase, 10 mg of dispase, and 10 µg of DNase in 10 mL of serum-free DMEM. Filter through a 0.22 µm syringe filter to prepare the enzymatic digestion solution.

3.2. Dice the isolated caudal lobe with scissors. Place the lung pieces in a 10 mL syringe, followed by the plunger.

3.3. Aspirate the digestion solution (5 mL) into the syringe by pulling back on the plunger. Operate the plunger up and down until the lung sinks into the solution. Then, place the lung and digestion buffer in a 50 mL tube.

3.4. Shake the tube for 15 min at 37 °C on a reciprocal shaker (150 rpm). Lungs may become tattered during this incubation.

3.5. Pipette up and down until lung cells are completely dispersed. Shake the tube for additional 30 min at 37 °C on a reciprocal shaker (150 rpm).

3.6. Pass the lung cells through a 40 µm cell strainer. Spin down the cells at 400 x g for 3 min.

4. Flow Cytometry of Lung Cells

4.1. Dissolve 100 mg of BSA in 10 mL of PBS and filter through a 0.22 µm syringe filter to prepare PBS-1% BSA.

4.2. Remove supernatant of the sample obtained at step 3.6 using a pipette and add 2 mL of red blood cell lysis solution. Then, spin down the cells at 400 x g for 3 min. Discard the supernatant.

4.3. Resuspend the cells in 5 mL of PBS-1% BSA by pipetting, and centrifuge the tube (400 x g, 3 min). Discard the supernatant.

4.3.1. Repeat this washing step one more time.

4.4. Suspend cells in 1 mL of PBS-1% BSA and pass through a new 40 µm cell strainer.

4.5. Analyze the cells with a flow cytometer to count tumor cells (using filter setup FL1 for GFP-LLC).

REPRESENTATIVE RESULTS:

The lungs present many GFP-LLC cells 2 h after injection (**Figure 1A**). It should be noted that the fluorescent spots also detected in the red filter should be excluded from the cell number count. The vast majority of LLC cells disappear from the lungs 24 h after injection (**Figure 1C**).

To confirm the number of the GFP-LLC in the lungs, one of the lobes can be used for flow cytometric analysis (**Figure 2**).

Figure Legends:

Figure 1: Detection of GFP-LLC cells under a fluorescence stereo microscope. (A) Lungs are isolated 2 h (A: filter set GFPLP, B: filter set ET/CY3) or 24 h (C: filter set GFPLP, D: filter set ET/CY3) after injection of 3 x 10^4 GFP-LLC cells. In (C), a GFP-LLC cell is marked by a yellow arrow. The spots marked by blue arrows are not GFP-LLC cells. Scale bar indicates 750 µm.

Figure 2: Flow cytometry analysis of lung cells. (A) and (B); Flow cytometric analysis of GFP-LLC cells used for tail vein injection. To exclude dead cells the cell suspension buffer contained propidium iodide (1 µg/mL). (A) shows FSC *vs.* SSC plot, and cells in the region of (C) are developed in (B) to show FL1 *vs.* FL3 plot. Cells in the region (D) are considered to be GFP-LLC cells. (E) and (F); Dot plots of caudal lobe cells isolated 24 h after the tail vein injection. The GFP-LLC cells are observed in region (D).

DISCUSSION:

Pre-treatment of mice with antibodies, drugs, a high-fat diet or tumor-conditioned medium prior to tumor cell injection is possible. The most difficult step in this assay is the tail vein injection. Incomplete injection results in inconclusive data. The tail vein in C57BL/6 is particularly difficult to identify, resulting in failed injections. Placing the mice on a heating pad (37 °C) helps dilate the tail vein so that injection becomes easier.

This assay uses a large number of (1 x 10^4 - 1 x 10^5) tumor cells, which is much larger than that observed in patient blood. A one-time injection of such a large number of the cells may bring about unwanted responses from the host animals. In the fluorescence stereo microscopy counting, tumor cells deep inside the tissue are hard to detect. In flow cytometry analysis, lungs are completely digested to count the tumor cells in the lungs. This process causes loss of valuable information about tumor cell location (inside or outside of the blood vessel). On the contrary, detection of tumor cells under a confocal fluorescence microscope allows the distinguishing tumor of cells in the blood vessel from the others[10].

Fluorescence stereo microscopy is a very simple method. Using flow cytometry to count the tumor cells has no bias compared with microscope scanning. It is possible to scan entire lung sections under a confocal fluorescence microscope; however, the assay will then be laborious. Other types of tumor cells or stromal cells may be mixed with the injected tumor cells. Co-injection of cancer-associated cells enhances the possibility of metastasis. The tumor metastasis rate heavily depends on the type of the tumor cell and the animal used.

As mentioned above, the most critical step in this protocol is the tail vein injection. An accurate number of cells needs to be injected with no leakage, but is technically difficult. Carefully observing the handling by skilled researchers may be of great help.

A series of mouse model studies have reported that 20% of the injected tumor cells underwent extravasation, 3% of them formed micrometastases, and only 0.02% developed into tangible metastatic nodules[2]. In case of GFP-LLC cell injection (3 x 10^4 cells), 150-300 GFP-LLC cells were detected by flow cytometry analysis in the caudal lobe isolated 2 h after injection. The total number of GFP-LLC in the lungs is assumed to be 600-1,200, indicating that 2-4% of the injected cells remained alive. This ratio decreases in a time-dependent manner[3,11]. The other cells are assumed to undergo apoptosis due to lack of physical support or nutrition, or due to exclusion by the immune system. In our recent data, 24 h after the injection of GFP-LLC (6 x 10^4 cells), the number of GFP-LLC in the lungs was found to be about 20. These results may be affected by the mouse strain, tumor cell, and other factors. For instance, it has been reported that the number of the tumor cells in lungs was elevated in the pre-metastatic phase[12], or in gene modified mice[13, 14].

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None

DISCLOSURES:
The authors have nothing to disclose.

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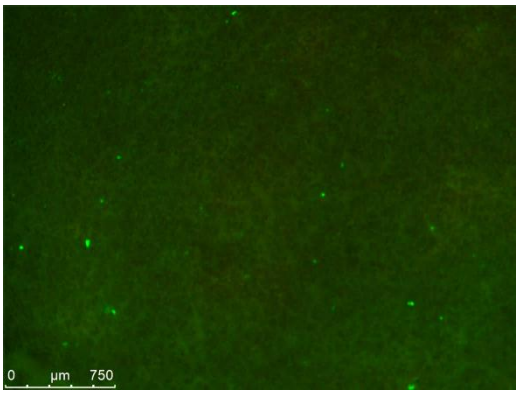
Figure 1

A



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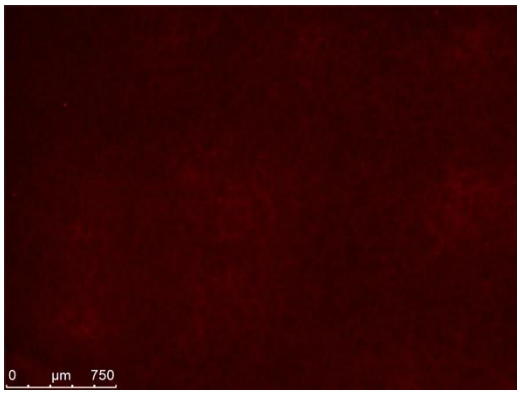

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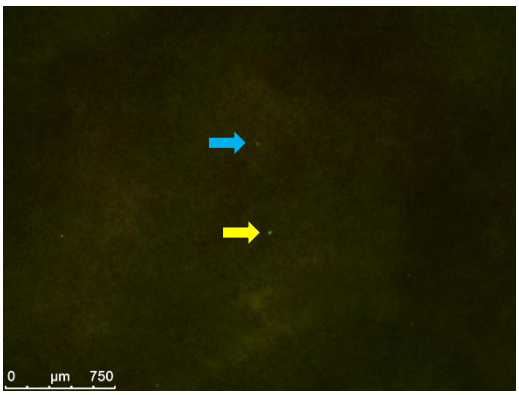

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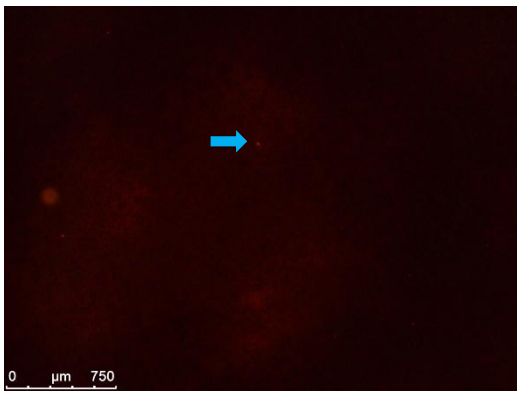

Figure 2

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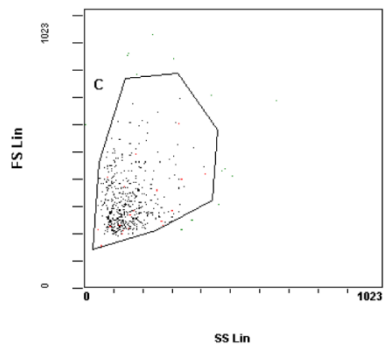

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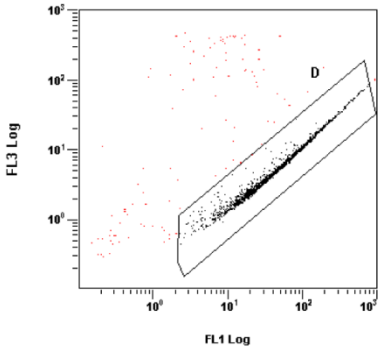

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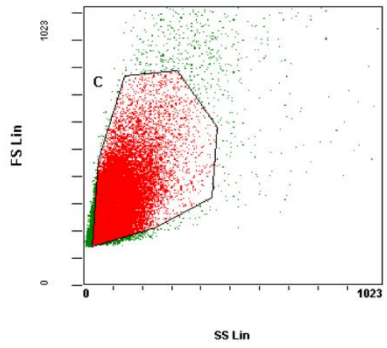

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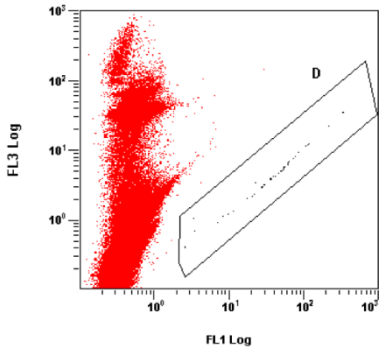

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Bovine serum albumin	Sigma	A7030	
DPBS	Gibco	14190-144	no calcium, no magnesium
Deoxyribonuclease I	Sigma	DN-25	trace amount
RBC lysis buffer	Sigma	R7757	
Cell strainer	BD	352340	40 micrometer mesh
Fluorescence labeling kit	Sigma	MIN26-KIT	
DMEM	Gibco	11965-092	
0.22 μm syringe filter	sartorius	17597K	
O.C.T. compound	Sakura Finetek	4583	
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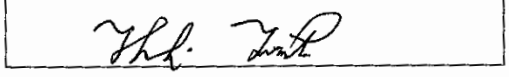
Signature:	 Date: 1/15/2015

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3rd May 2018

Dr. Jaydev Upponi, Ph.D.
Science Editor
JoVE

Dear Dr. Upponi,

Thank you for your e-mail of 16th April 2015 regarding our manuscript entitled "JoVE53172R4" We have corrected all the points raised by the reviewers. The followings are the point-by-point responses to the reviewer's comments. The changed, or added sentences in the text are highlighted. In addition, I would like to note that the revised manuscript contains direct cell counting using a fluorescent stereo microscope. This process is very simple and is worth being included in the manuscript.

Sincerely,

Takeshi Tomita
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Editorial comments:

1) Grammar:
-The manuscript would benefit from copy editing for numerous grammatical errors, preferably by a native speaker. In particular, please make sure all grammar in the long abstract is correct.
-Figure 2 legend: please clarify/correct grammar for "red fluorescence possibly does their remnants."

[Answer1] Thank you for your comments. I sent the manuscript a proofreading service.

2) Highlighting – Under half a page is highlighted for filming (as we cannot film mouse euthanasia). At a minimum, the tail injection from section 1 should also be filmed, as it is the most critical step in the protocol.

[Answer2] I would like to film from step 2.1) (except for euthanization) to step 4.4).

3) Additional detail is required:
-1.3 – Please provide stepwise detail on how the tail injection is performed, given this is a critical step. What steps are taken to minimize leakage or incomplete injection?
-2.2 – How is euthanasia confirmed? This information is required for ethical reasons.
-3.4 – Please provide stepwise detail or a citation for flow cytometry.
-4.1 – Are the lungs still inside the carcass when they are injected with OCT compound? If so, how are the lungs removed?

[Answer3] -Unfortunately, we cannot provide tips for the tail vein injection. Our hands are not as good as those of experienced technicians. We only did repeat practice to improve our technique.
-For euthanasia, we follow the guideline provided by NIH OACU. "The CO_2 gas flow is maintained for a minimum of 1 minute after observation of each mouse for lack of respiration and faded eye color."
-We use FC-500 flow cytometer. Its detailed procedure is unique to the model so that it cannot be applied to the other types of flow cytometer. If this does not matter, I will provide the detail.
-My answer is yes. The lungs are still inside when they receive OCT or PFA/PBS injection. This part is removed in the revised version.

4) Discussion: A citation for the information the second sentence of the last paragraph is necessary.

[Answer4] I added the reference.

5) Unnecessary branding in 3.4 - FC-500.

[Answer5] I removed it.

6) 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.

[Answer6] The format is maintained.

7) Please take this opportunity to proofread thoroughly your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript, and any errors in your submitted revision may be present in the published version.

[Answer7] Thank you. As abovementioned, the manuscript is checked by a proofreading service.

8) 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.

[Answer8] Our data in this manuscript are original and never published before.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This manuscript describes a quantification method to study the tumor metastasis with intravenous injection of PKH26-labed tumor cells. In general, this manuscript is well written and presented in a logical fashion. The authors elaborated the advantages to studying the tumor metastasis with intravenous injection of tumor cells. However, the limitation of this method should also be discussed.

Major Concerns:
1.How long does the signal of PKH26 last? Is it possible to detect the PKH26 labeled tumor cells 14 days (or even longer) after the intravenous injection of tumor cells?

[Answer#1-1] Thank you for your comments. In our experience, the lifetime of PKH26 label is at least more than a week. Since the PKH26 labeling is diluted in the dividing cells, we consider that tracking PKH26 labeled cells in vivo is impossible. Additionally, labeled cell is a good target for the resident macrophages. Macrophages retain the fluorescence after the intake. Thus, PKH26 labeled cells may be still detected even14 days after the injection, but the signal does not guarantee the presence of initially labeled cells.

2. Page 2, starting on row 82: The circulating tumor cells must be different from the primary tumor cells because some changes have happened before the intravasation (e.g. EMT process, etc). So, the tumor cells directly injected into the blood vessel should have some differences from the naturally found circulating tumor cells. This point is not discussed.

[Answer#1-2] Thank you for your informative comment. We added sentences to describe the difference between circulating tumor cells and artificially injected tumor cells.

Minor Concerns:
1. Page 2, starting on row 70: Many previous reports have studied the pre-metastatic phase using the method of intravenous injection of tumor cells. In the premetastatic stage, Angpt2, MMP3, and MMP10 are up-regulated in the lung which leads to the increased permeability of pulmonary vasculatures and extravasation of circulating tumor cells. This was not explicitly evaluated in the present study which is an unfortunate omission.

[Answer#1-3] Thank you for your comment. I added a sentence with the reference in the text. I believe that the manuscript is improved.

2. Page 2, starting on row 85: The author mentioned that "Behaviors of circulating tumor cells are very important; they determine the ultimate consequences of cancer patients." "Blood is not a good place for the tumor cells to survive." The references support these points should be provided.

[Answer#1-4] Thank you. The references were added in that part.

3. Page 3, row 94: As mentioned above, the phrase "is same as" is inappropriately used.

[Answer#1-5] Thank you for your comment. The tone of the sentence was changed.

4. Page 3, row 121: The relevance of the concentration of intravenously injected tumor cells needs to be shown. What is the reason for choosing the concentration that used in this experiment?

[Answer#1-6] Thank you for your comment. In the standard protocol, 1×10^4-1×10^5 cells are used for the injection. This number is much higher than naturally occurring CTC. However, given the detection limit of this assay system, at least 1×10^4 cells are necessary as input (injection into the tail vein). CTC appears to metastasize more efficiently than the cultured cells.

5. Page 3, row 133: The process of perfusion is not optional but necessary because blood cells would influence the results of following studies especially the fluorescence assay.

[Answer#1-7] Thank you for your suggestion. The perfusion process is incorporated in the main protocol.

6. Page 4, row 141: Is it sufficient to separate the lung tissues into single cells through the methods described in 2.4) and 2.5)? It would be better to cut the lung tissues into pieces with scissors before put it in the syringe.

[Answer#1-8] Thank you for your comment. I changed the protocol.

7. Page 6, row 254: The author mentioned that "This ratio decreases in a time dependent manner". When would the tumor cells begin to regrowth? How the ratio changes in the lung needs further discussion.

[Answer#1-9] Thank you for your comment. The apparent tumor cell regrowth is not observed in 48 hours after the injection. Five days after the injection, micro-size colony formations were seen in the case of LLC inoculation. In the ref 13 paper, it is reported that the tumor microenvironment in the lungs influences the ratio.

Reviewer #2:
Manuscript Summary:
The manuscript "Tumor cell lung recruitment assay" by Tomita et al describes a tail vein injection-based experimental approach to evaluate specific steps of the metastatic cascade.

Major Concerns:
A general point of objection is the statement in abstract, lines 80 and 83 of the introduction, and discussion that injected tumor cells into the bloodstream would be equivalent to circulating tumor cells (CTCs). This is not correct as CTC are a selected sub-population of all cancer cells, often with specific features (EMT, stemnes, ...). I would strongly suggest amending these statements.

[Answer#2-1] Thank you for your informative comments. Another reviewer gave us the almost same comment. In the revised manuscript, this part is amended.

Surprisingly-and disappointingly regarding the scope of the journal-the method section is highly inaccurate and/or misses essential information. A few of these problems are highlighted below:
Are all steps (without specific information) performed with room temperature material and at room temperature?
Line 119: concentration of PKH26?

[Answer#2-2] The protocol provided by the manufacturer recommends that the concentration of PKH26 be 4×10^{-6} M for staining 2×10^{7} cells. All procedures, including PKH26 labeling, are performed at room temperature unless otherwise stated.

Line 121: which wash steps are performed?

[Answer#2-3] After the labeling reaction with PKH26, cells were washed with PBS (10 ml) three times.

Line 144-151; steps2.5 and 2.7: confusing as the buffer seems to be in the plunger (2.5) and then in the tube (2.7).

[Answer#2-4] Thank you for your comment. In step 3.3), the lungs were pushed back to a 50 ml tube. I rewrote this part to avoid reader's confusion.

line 158, step 3.2: composition of red blood cell lysis buffer? How long are samples treated with it? Temperature?

[Answer#2-5] I suppose the composition of the buffer is as following; NH_4Cl, 8.26g/L H_2O, $NaHCO_3$, 1.19g/L H_2O, and EDTA2Na, 0.0378g/L H_2O. However, the manufacturer did not disclose the exact composition of the buffer. The red blood cell lysis buffer requires no incubation time after mixing with the sample. The operator can proceed to the next step immediately.

Line 165: step 3.4: how many cells are analyzed? Authors need to include gating strategy as a figure

[Answer#2-6] Thank you for your comment. I rewrote this part.

Line 171, step 4.1: please show histology of 4%PFA/PBS soaked lung; how is the pressure of H_2O controlled?

[Answer#2-7] Thank you for your comment. This part is removed in the revised manuscript. Please see a paper "Am J Physiol Lung Cell Mol Physiol 306: L10-L22, 2014". The pressure of H_2O (100-136 cm H_2O for the blood vessel perfusion, 20-25 cm

H_2O for the lung fixation) is controlled by utilizing hydrostatic pressure. A tank with PBS or saline is placed on a stand, the height of which from the operation table is set to be 100-136 cm for the blood vessel perfusion or 20-25 cm for the lung fixation. The tank outlet and a needle are connected with tubing.

Line 181, step 4.4: which microscope was used in this study?

[Answer#2-8] We used Olympus BX51. This part is removed in the revised manuscript. Instead, the new version contains fluorescence stereo microscopy using Leica M205FA.

Line 189: gating conditions: again, needs more detailed gating information: was it lung cells, singlets, positive?

[Answer#2-9] Thank you for your comment. We revised the gating conditions. Doublet removal was not introduced in the flow cytometry analysis.

Line 194: Why are there PKH26-positive signals in non-tumor-bearing mice? The increase (from 0.2-0.3% to 0.4-0.6% (line 195)) is tiny. This needs to be represented in a table or figure with statistical analysis.

[Answer#2-10] Thank you for your comment. The reason why there is PKH26-positive signal in non-tumor-bearing mice is that large number of tumor cells (1×10^5) are injected. The increase earned in the pre-metastatic phase is tiny but it is similar to the values previously reported (references 4, 10, and 12).

Line 198: how can the authors conclude that these are tumor cell remnants? Could it be dormant tumor cells, non-proliferating tumor cells...?

[Answer#2-11] Thank you for your informative comment. As you pointed out, they might be dormant tumor cells, non-proliferating cells, or even resident macrophages digesting labeled cells.

Line 210 see above, type of microscopy
Line 213: is it expected that cells become 2-color positive despite being labelled with (only) PKH26 originally?

[Answer#2-12] In this case, GFP-labeled cells were stained with PKH26 so that the resulting cells show two different colors. The PKH26 label is diluted in the cell regrowth process. On the other hand, GFP fluorescence is maintained because GFP is inherited in the descendent.

Line 222: tail vein injections are not difficult to experienced animal technicians

[Answer#2-13] Thank you for your comment. The tail vein injection is very difficult for the beginners, but there is no way other than doing practice to be a skilled operator. Unfortunately, we are not able to describe tips for the tail vein injection.

Minor Concerns:
Line 220/221: check grammar of sentence
Line 228: spelling form-from
Line 249-251: this is not mentioned in results section-why?

[Answer#2-14] Thank you. All points are corrected in the revised manuscript.