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Distinguishing intrapulmonary immune cells from intravascular immune cell populations: the intrajugular approach.

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July 23rd, 2020

Vineeta Bajaj
Science Editor,
Journal of Visualized Experiments (JoVE)

Dear Dr. Bajaj

Thank you for giving us the opportunity to be reviewed and permitted to respond to the reviewer's comments on our manuscript entitled **"Distinguishing intrapulmonary immune cells from intravascular immune cell populations: the intrajugular approach**". We have addressed all the comments from the reviewers. Due to COVID-19 related unforeseen and extenuating conditions, we have not been able to carry out additional experiments. However, we have addressed all concerns in the response to reviewers as well as the relevant portions of the manuscript. We have also incorporated all editorial directions provided on the previous iteration of the manuscript. We believe that the method we propose is reliable and lends itself to be modified for use in different contexts. As such it should be of interest of investigators well beyond pulmonology.

Overall, we believe that the comments from the reviewers has allowed us to improve our manuscript and we hope the current submission satisfies them.

With best wishes,

Shaon Sengupta
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TITLE:

Distinguishing Intrapulmonary Immune Cells from Intravascular Immune Cell Populations: the Intrajugular Approach

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KEYWORDS:

Circadian, inflammation, lung, intravascular-labeling

SUMMARY:

The aim of the current study is to describe a protocol for differentiating between intravascular and intraparenchymal immune cells in studies of lung inflammation. We use an intrajugular injection of a fluorescent tagged antibody prior to lung harvest. Further, we use an inflation-based lung digestion process to improve the yield of leukocytes from the lung.

ABSTRACT:

Circadian rhythms refer to oscillations in various biological process that occur with a 24 h period. At the molecular level, such rhythms are comprised of a web of transcriptional-translational feedback loops (TTFL) of core clock genes. Individual tissues and organ systems, including the immune system have their own clock. In the systemic circulation, various members of the $CD45^+$ population oscillate across the day; however, many of these rhythms are not identical or even similar in the tissue resident $CD45^+$ leukocyte population. When studying the role of circadian regulation of lung inflammation, $CD45^+$ within the lung may need to be investigated. However, despite optimized perfusion methods, leukocytes trapped from the circulation persist in the lungs. The goal in designing this protocol was to distinguish between intravascular and intraparenchymal leukocytes. Towards this end, mice are injected with a fluorescent tagged CD45

antibody intra-jugular shortly before lung harvest. Thereafter, the lung is digested using a customized lung digestion technique to obtain a single cell suspension. The sample is stained for the regular panel of antibodies for intraparenchymal immune cells (including another CD45 antibody). Flowcytometric analyses shows a clear elucidation of the populations. Thus, the method of labeling and defining intrapulmonary $CD45^+$ cells will be particularly important where the behavior of intrapulmonary and circulating immune cells are numerically and functionally distinct.

INTRODUCTION:

We describe here efficient and reliable methods of differentiating intravascular leukocytes from pulmonary leukocytes. Even with the best perfusion techniques, studies have revealed residual $CD45^+$ from circulation persists in the lung. This impairs the ability to distinguish between the rhythms in the circulation and the lung. This effect is further amplified in cases of lung inflammation. This is particularly relevant for the study of circadian regulation of inflammation.

Circadian rhythms refer to the diurnal oscillations in various biological processes that occur with a period of 24 h. The circadian system is an evolutionarily conserved anticipatory mechanism that confers protection on the host as it faces changes in its environment such as threat of infections. At the cellular level, the clock is organized into self-sustained transcriptional, translational feedback loops comprising the core clock genes[1]. The immune system has its own clock that impacts its response to pathogens and inflammatory insults[2,3]. As an organ exposed to the environment constantly, circadian rhythms are particularly important in the lung[4]. Various immune processes in the lung are under clock control[5-7]. However, the phase of various biological processes in the lung and the systemic circulation are not the same[8], which by extension, also suggests that the oscillations of leukocytes in the lung and the circulation may not be identical. Thus, having a method to efficiently distinguish between pulmonary and intravascular leukocytes will be critical in the circadian context.

The aim of this study was to devise a method that can differentiate between intravascular and intraparenchymal leukocytes reliably. For this, we used a labeling of intravascular leukocytes and lung digestion method. For the labeling of intravascular leukocytes, we use intrajugular injection, which targets a large blood vessel and can be reproducibly used in mice of all strains and sizes. Many other methods have used tail vein injection[9,10], which are notoriously harder to perform in Bl6 mice[11]. The intrajugular injection does necessitate use of anesthesia and is best done under direct visualization with dissecting microscope or magnifying loupes. Thus, the ease and reliability of the intrajugular injection should be weighed against the need for anesthesia and special equipment. However, given that ready availability of these equipment in most research labs, we do not view this to be a limiting factor. However, a case-by-case consideration seems prudent.

PROTOCOL:

All animal studies were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and met the stipulations of the Guide for the Care and Use of Laboratory Animals.

NOTE: The overall process may be divided into 1) intravenous CD45 labeling, 2) harvest, 3) digestion, and 4) staining and flow cytometry. These steps have been summarized in **Figure 1**.

1. Solutions/Reagent preparation

1.1. Prepare Dissociation Media by adding 5 mL of 2 mM L-glutamine, 20 mL of Fetal Bovine Serum (FBS), 1 mL of 2-mercaptoethanol, and 10 mL of Pen/Strep to 500 mL of DMEM.

NOTE: Dissociation Media is stable for up to 2 months when stored at 2-4 °C.

1.2. Prepare Fluorescence Activated Cell Sorting (FACS) Buffer by adding 10 mL of FBS and 500 mg of sodium azide to 500 mL of PBS without magnesium or calcium.

NOTE: With the addition of sodium azide, FACS Buffer can be stored at 2-4 °C for months.

1.3. On the day of sample collection, add the DNase and Liberase solutions to Dissociation Media at a 1:100 dilution (i.e., add 10 µL of DNase and Liberase for every 1 mL of Dissociation Media).

NOTE: For each mouse, digesting the whole lung requires 10 mL/mouse and half of the lung requires 5 mL/mouse.

2. Intravenous CD45 labeling

2.1. For this experiment, use adult C57Bl6 mice aged 8-12 weeks old.

2.2. Anesthetize the mice with agents of choice. A combination of xylazine and ketamine were used for this purpose, but other agents are acceptable as well. The aim is to get moderate to deep level of anesthesia that lasts about 5 -10 minutes.

NOTE: The xylazine and ketamine anesthesia mixture is administered intraperitoneally. Use 10-15 mg/kg of xylazine and 120-150 mg/kg of ketamine.

2.3. Once the pedal reflex is negative, position the animal on its back and tape down its limbs gently to keep the head as central as possible.

2.4. Expose the jugular vein and pectoral muscle by lifting the skin with forceps and snipping with sharp surgical scissors.

2.5. Inject 200 µL of anti-CD45 antibody (flow cytometry grade antibody; diluted 1:300 in PBS) into the jugular vein using a 28 G needle. Wait 2-4 minutes so the antibody can circulate throughout the vasculature.

NOTE: Entering through the pectoral muscle into the jugular vein from a shallow angle prevents considerable bleeding from occurring

2.6. Thereafter euthanize the animal by exposure to CO_2 for 10 minutes. Proceed to lung perfusion and harvest lungs and other tissues.

NOTE: Any other method of humane euthanasia that adheres to the AVMA guidelines for the euthanasia of animals is also acceptable.

3. Dissection/Harvest (Figure 1)

3.1. Position the animal on its back on a flat board and pin the paws down, keeping the head central.

3.2. Spray the body with 70% ethanol. Open the thoracic cavity with forceps and scissors to expose the lung, heart, and trachea.

3.3. Perfuse the lung by making a small incision in the left ventricle of the heart and injecting 10 mL of cold PBS through the right ventricle.

3.4. Snip an opening in the trachea and insert an intravenous cannula. Once the cannula is in, pass a suture string about 6-8 cm long underneath the trachea and tie it twice to the cannula.

3.5. Tuck the surgical string into the cannula and attach a syringe containing Dissociation Media (5 mL for half a lung or a 10 mL for a whole lung) as depicted in **Figure 1B**.

3.6. Gently cut away the lung from the rest of the body and place the syringe with the lung attached into a 50 mL conical tube.

4. Digestion to single cell suspension

4.1. Incubate the lung at 37 °C for 30-40 minutes, instilling 1 mL (for half the lung) or 2 mL (for the whole lung) of Dissociation Media every 5 minutes.

4.2. Once all the media is instilled, remove the syringe and cannula, and place the 50 mL conical tube into a shaking water bath at 180 rpm for the remainder of the incubation for better yield.

NOTE: Alternatively, the tubes may be manually shaken every 5 minutes.

4.3. Add 10 mL of PBS and shake vigorously for 1 minute to stop the reaction.

4.4. Pass the solution through a cell strainer (70 µm) into a new 50 mL conical tube. Use a 5 mL syringe rubber stopper to pass any clumps of tissue through the strainer. Add PBS so that the

final volume is 30 mL.

4.5. Centrifuge the samples for 10 minutes at 1,200 x *g* and 4 °C. Discard the supernatant without disturbing the pellet. Pipet out any remaining solution.

4.6. Add 1 mL of Red Blood Cell (RBC) Lysis Buffer and mix with the cell pellet by pipetting.

4.7. Incubate at room temperature for 60-90 s. Add PBS so that the final volume is 30 mL to stop the reaction.

NOTE: Incubation time depends on the amount of blood in the cell pellet; the redder the cell pellet, the longer the incubation time.

4.8. Centrifuge the samples for 10 minutes at 1,200 x *g* and 4 °C. Discard the supernatant without disturbing the pellet. Pipet out any remaining solution.

4.9. Add 1 mL of FACs buffer to the cell pellet and mix by pipetting.

5. Staining cells for flow cytometry

5.1. Use a cell counter to determine the total number of cells in the cell suspension

5.2. Transfer the cell suspension into each labeled FACS tube so that there are 3 x 10^6 cells total per sample.

5.3. Add Fc Block (diluted 1:100) and incubate for 15 minutes on ice.

5.4. Centrifuge the tubes for 5 minutes at 1,200 x *g* and 4 °C. Discard the supernatant without disturbing the pellet.

5.5. Stain the samples with a specified antibody mixture and incubate for 20 minutes on ice protected from light (i.e., by covering with aluminum foil). Mix by racking tubes against the tube holder halfway through incubation.

5.6. Add 1 mL of FACS buffer to wash and mix by pipetting.

5.7. Transfer the cell suspension into another FACS tube by pipetting the suspension slowly through a 35 µm strainer.

5.8. Centrifuge the tubes for 5 minutes at 1,200 x *g* and 4 °C. Discard the supernatant without disturbing the pellet.

5.9. Add 150 µL of FACS buffer and mix by pipetting.

5.10. Add 10 µL of DAPI (1:100) to each tube immediately prior to running the samples.

NOTE: The samples are now ready to be run on the flow cytometer.

REPRESENTATIVE RESULTS:

Using this technique, the total cell count of the naïve dissociated lungs (only the left lobes were used for the representative data) was between 27.3 x 10^6 to 71.1 x 10^6 cells/mL. After gating on size and gating out doublets and dead cells (gating scheme in **Figure 2**), the leukocyte counts ranged from 6.9 x 10^6 to 13.5 x 10^6 cells/mL. Circulating leukocytes that remain trapped even after perfusion to clear the lungs constituted approximately 4% to 13% of the live cells in the experiment. While all leukocytes in the dissociated lung stain for CD45 on the PE-Cy7, only the circulating leukocytes are double stained with the CD45 antibody on both fluorophores, PE-Cy7 and Pac blue. Despite standardizing the process of perfusion, we found that about 24% to 70% of the total leukocytes in the dissociated lung belonged to the circulating leukocyte pool. The mean leukocyte counts without accounting for the intravascular labeling was 10.2 x 10^6 cells/mL, while the corrected value was 4.93 x 10^6 cells/mL (**Figure 3A**). Therefore, failing to differentiate residual intravascular leukocytes that persist after perfusion results in a significant overestimate the number of pulmonary leukocytes. For this experiment, animals were 8-12 weeks of age and weighed 25-30 g at the time of dissection. Dose adjustments may need to be made for extremes of ages and weight. Other useful controls include using an intravascular sample to demonstrate good labeling of intravascular leukocytes and a sample from an unlabeled tissue (such as a lymph node) to exclude the possibility to diffuse labeling of all tissues by transmigration of labeled leukocytes from the blood.

Since research questions often involve circadian sampling, we further broke down the data by time of day (for the animal) at which the lung harvest was performed. By circadian convention ZT0 refers to the time when lights turn on. We used dawn (ZT23: onset of rest phase) and dusk (ZT11; onset of active phase) as the two time points. We note that the proportion of the intraparenchymal and the residual intravascular leukocytes varied by the time of day at dissection (**Figure 3B**). In this experiment, the mice were maintained in reverse light-dark (LD) cycles using light-controlled circadian boxes, so that ZT11 and ZT23 mice were in reverse phases at the same conventional time and could be harvested simultaneously. Thus, lungs from both groups were labeled, harvested and digested at the same time of the day and therefore these differences in **Figure 3** cannot be attributed to differences in processing.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of lung inflation and digestion.

(**A**) Overall experimental design starting from anesthetizing of the mice to the antibody staining step. (**B**) Image of the cannula insertion step of the lung harvest.

Figure 2: Gating strategy: Gating strategy used for differentiating intravascular leukocytes from leukocytes that truly resident in the lungs.

Figure 3: Comparison of intraparenchymal and intravascular leukocytes.

(A) Leukocyte counts (in 10^6 cell/mL) using traditional digestion and labeling compared with the counts corrected for residual intravascular leukocytes that were not cleared out of the lungs. $P<0.0001$ by student's t-test. Data represented as mean±SEM.
(B) Leukocyte numbers and percentage from lungs harvested at dawn (ZT23) or dusk (ZT11). (right panel) 2-way ANOVA, $p >0.05$ for both time of harvest and compartment labeled, but $p<0.05$ for interaction. Data represented as mean±SEM.

DISCUSSION:

Careful studies of lung inflammation and pulmonary immune responses are crucial to the understanding of many disease conditions. Flow cytometry is routinely used to enumerate and ascribe functional relevance to pulmonary leukocytes. The function of leukocytes depends at least partly on where they are found. Although there is accumulating evidence to support that even after perfect perfusion protocols, many intravascular leukocytes persist in the lungs, most studies do not differentiate between intrapulmonary and intravascular leukocytes. Further, the residual intravascular leukocytes that remain post-perfusion in the lung are likely to be randomly distributed across the samples depending on the preparation, such that predicting how it affects the results is impossible. This hampers the generalizability of the results and is a threat to the rigor and reproducibility of those studies involving flowcytometry[12].

We have described here a method to determine the residual leukocytes from the vasculature and to differentiate them from the truly intrapulmonary leukocytes. Although such methods have been used in the past[9,13,14], we have refined the labeling, as well as digestion, to make it the most efficient preparation. Other reports describe using the retro-orbital or tail vein injections for the intravascular labeling[10]. Retro-orbital injections inflict more pain on the animal and thus require a higher level of regulatory scrutiny. Although the tail vein injection does not need anesthesia, it is technically more challenging[11] especially in C57bl6 animals. This intrajugular approach overcomes both these disadvantages by making the procedure relatively painless and easy to perform under microscopy. Although we have described the procedure here for young adult mice, given the larger size of the intrajugular, this protocol may be used for smaller animals[15]. The ease of the procedure makes the labeling consistent across animals and thus would add to the rigor and reproducibility of the results.

The critical steps in this protocol include the reproducibility of the intravascular injection and the lung harvest. Some additional considerations that are needed for intrajugular injection are need for anesthesia and magnifying equipment (magnifying loupes or dissecting microscope). Both these steps require practice to master and may add to variability if not well mastered. With intrajugular injection, a faulty technique may lead to excessive bleeding and precipitate death. Thus, there is an emphasis on equipment to aid visualization. In the case of lung harvest, poor technique may result in rupture of the trachea. While it may still proceed with other methods of lung digestion (such as using a scalpel to chop the lung, etc.), in our experience it yields much lower cell counts and tends to increase variability between samples.

Another important concern emerging from any additional steps is that it increases the total processing time and may reduce the viability the samples, and therefore the quality of the data.

By labeling intravascular leukocytes before the digestion, we ensure that the viability of the prepared sample is not adversely affected in the protocol. Further, using the inflation technique to distribute the digestive enzymes throughout the lung, we ensure that the digestion is uniform and yields reliably more cells than other methods to dissociate the lung tissue[7]. Few additional concerns that may be considered are common to this labeling method independent of the route of administration of the intravascular antibody. This includes possible competitive binding between the two CD45 antibodies. This may be partially mitigated by using different clones of the CD45 antibody, although care must be taken to ensure that the two antibodies used have very similar binding affinities. The other aspect to consider is if the time from injection to harvesting (here, 2-4 minutes) is enough for labeling the majority of the intravascular leukocytes. Given that the intravascular labeling precedes the overall CD45 labeling, and at a heart rate of 300-700 beats per minute in mice[16], it is very likely that there is complete labeling of the intravascular CD45 compartment. This method was adapted from others who have used very similar concentrations and times form administration to harvest[9,10,13]. Finally, another issue to consider is the possible transmigration of the resulting leukocytes, technically in either direction in the time from the injection to the harvest. This may also vary by the underlying experimental condition. Although, not used here, one way to overcome this at least partially would be to use other standard methods of euthanasia that reduce the time from injection to harvest, such as cervical dislocation.

This work revolves around the elucidation of circadian regulation of lung injury, repair and regeneration. Although the entire body is under circadian regulation, the phases of such regulation vary by organ and cell type. Thus, the phase of the rhythms of leukocyte or their functional subtypes may be different based on the organ or site of circulation. Not differentiating these two populations may in fact result in false negative results in the analyses of various leukocyte sub-populations (if the circulating and pulmonary populations are in opposite phase, the averages may not be vastly different). Thus, differentiating between circulating leukocytes that remain in the lung even after perfusion from those that have already migrated into the lung and are actively participating in the inflammatory cascade is essential to discovering the biology of circadian regulation. The protocol described here helps this differentiation. While we have done this in the lung, this method lends itself to being adapted for other organs as well. However, adding these steps does lengthen the time to harvest and may not be suitable for all circadian experiments. This would thus entail a customized consideration on where differentiating between the intravascular and intraparenchymal leukocytes is most relevant.

In conclusion, we have described in detail a method of distinguishing residual intravascular from intrapulmonary leukocytes and inflation-based lung digestion. These techniques will enhance the yield of single cell suspension from the lung and improve the rigor and reproducibility of flowcytometry studies. Finally, this is of particular relevance for those studying circadian rhythms.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1(A)

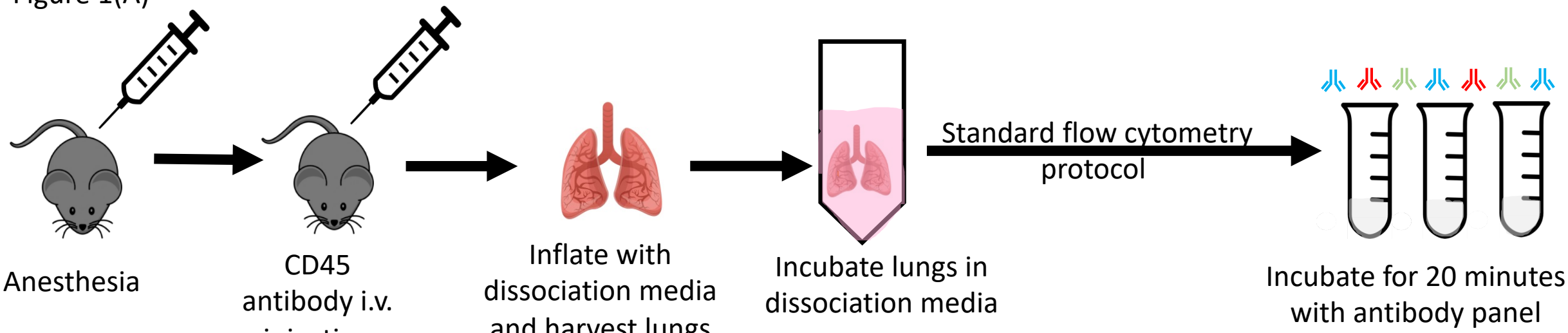


Figure 1 (B)

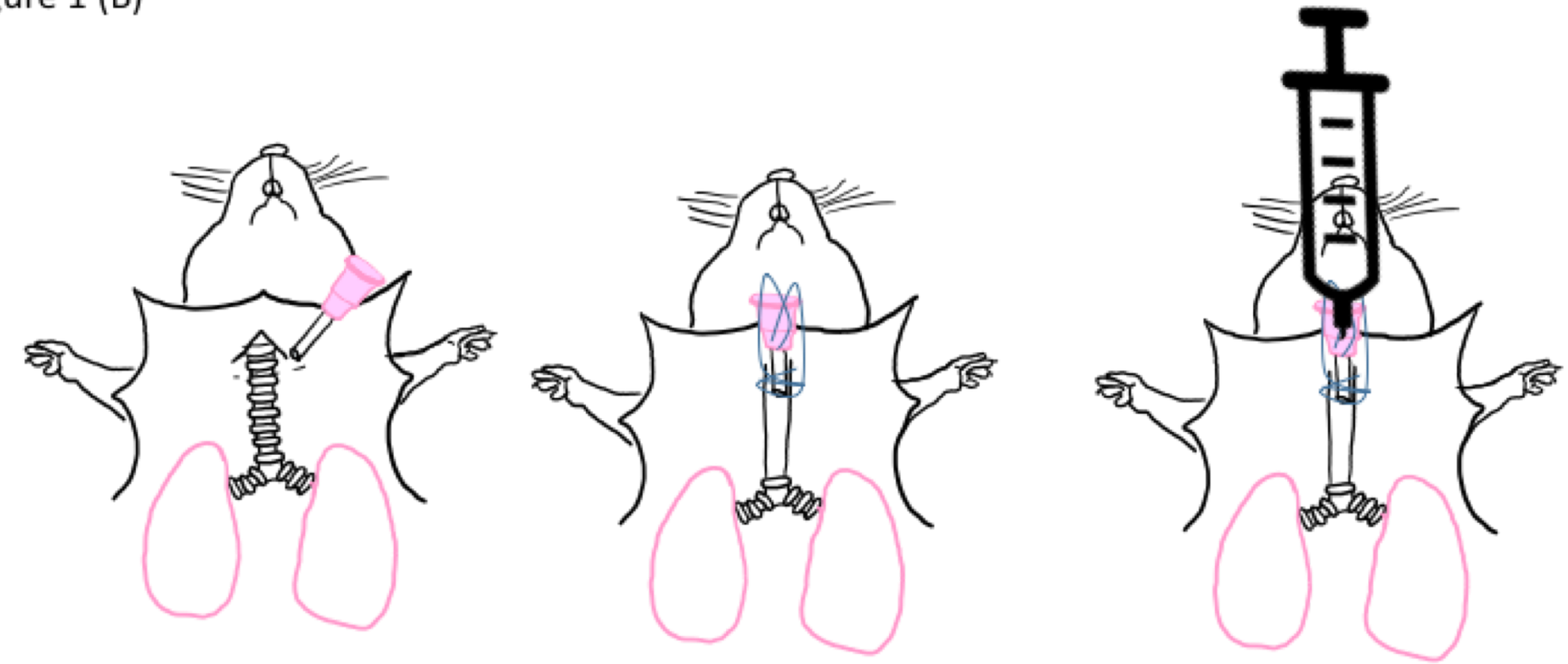


Figure 2

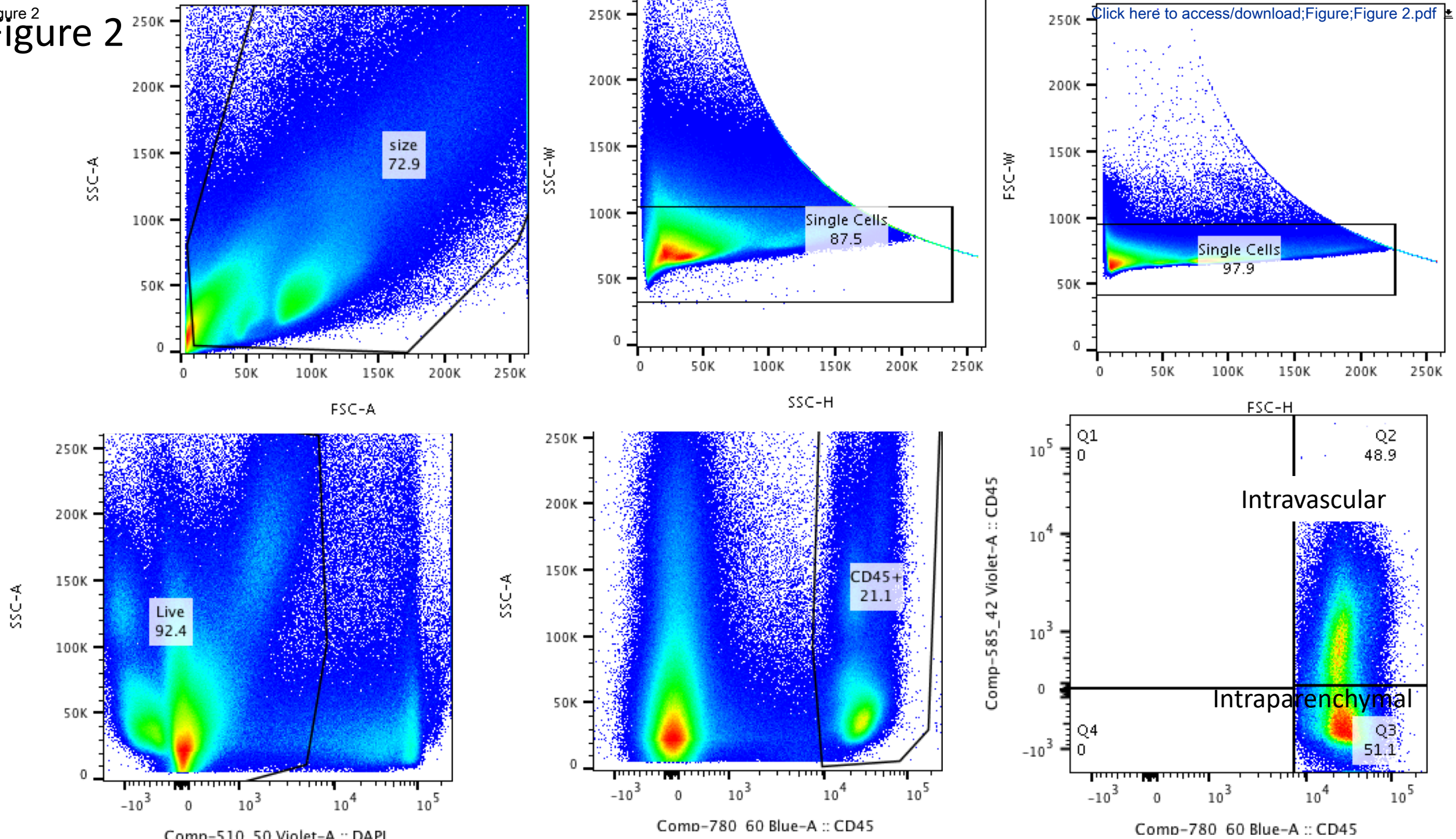
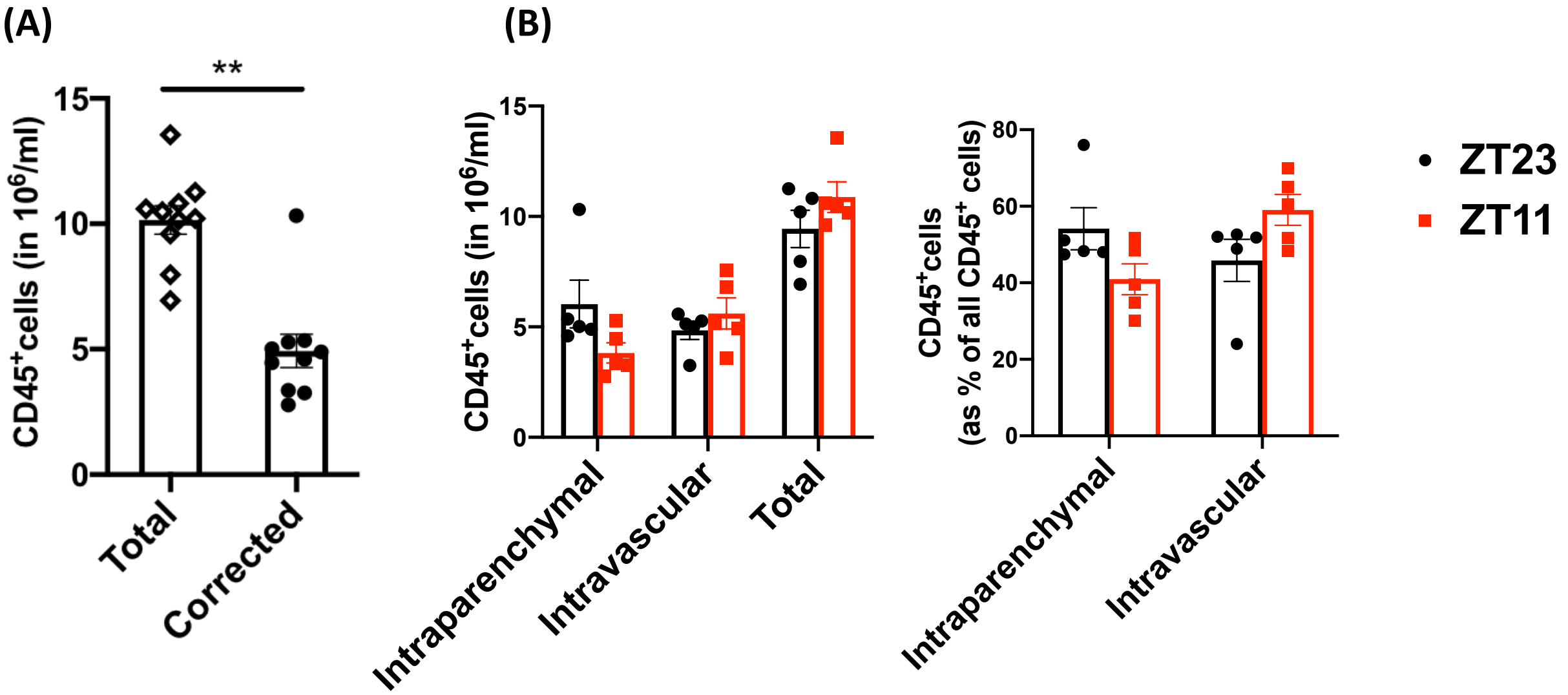


Figure 3



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Boekel Scientific Medium Water Bath	Boekel Grant Scientific	290200	
10 mL BD Syringes with BD Luer-Lok Tip	BD Biosciences	309604	
5 mL BD Syringes with BD Luer-Lok Tip	BD Biosciences	309646	
Anti-CD45- Pac Blue	Biolegend	103114	
Anti-CD45- Pe/Cy7	Biolegend	103114	
Cell strainer 70 µm Nylon	Fisher	352350	
Corning Conical-Bottom Centrifuge Tube 50 mL	Avantor	21008-714	
Corning Falcon Test Tube with Cell Strainer Snap Cap	EMSCO	10004637	
Dissection Microscope	Olympus	SZX-SDO2	
DMEM, high glucose	Life Technologies	11965084	
Dnase	Roche	10104159001	
DPBS without Ca^{++} & Mg^{++}		14190136	
Fc Block	Biolegend	101320	
HyClone Fetal Bovine Serum	GE Healthcare	SH30071.03	
L-Glutamine (200 mM)	Life Technologies	25030-081	
Liberase Research Grade	Sigma	5401127001	
Penicillin-Streptomycin (10,000 U/mL)	Life Technologies	15140-122	
Precision Shaking Water Bath	Thermo Fisher	TSSWB15	
Red Blood Cell Lysing Buffer	Sigma	R7757	
Suture Silk 4-0	Roboz	SUT-15-2	

Antibodies	Clone	Company	Flurophore	Catalog number
CD45	30-F11	Biolegend	Pac Blue, Pe/Cy7	103114
FcBlock	93	Biolegend	N/A	101320

We thank all the reviewers and editorial board for their comments and questions. These have helped up improve the quality of the submission. Our point-by-point response is as below.

Editorial comments:

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. **[Done]**
2) Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points **[Done]**

3) Please provide an email address for each author.
    - Yasmine Issah:
    - Amruta Naik: NAIKA@email.chop.edu
    - Soon Yew Tang: tangsoon@pennmedicine.upenn.edu
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    - Shaon Sengupta: SenguptaS@email.chop.edu
4) Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol. **Done. Word count is 216.**
5) Please reword lines 164-168 as it matches with previously published literature. **[Done.]**
6) Please expand the Introduction to include all of the following:
    a. A clear statement of the overall goal of this method
    b. The rationale behind the development and/or use of this technique
    c. The advantages over alternative techniques with applicable references to previous studies
    d. A description of the context of the technique in the wider body of literature
    e. Information to help readers to determine whether the method is appropriate for their application

Response: We have updated and expanded the introduction to include the same.

7) Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.). **[Done]**
8) Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” **[Done]**
9) The Protocol should contain only action items that direct the reader to do something. **[Done]**
10) The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step **[Done]**
11) Please ensure you answer the “how” question, i.e., how is the step performed? **[Done]**

12) 1.1 Age, strain, sex of mice used? Concentration of xylazine and ketamine used? **[Done]**
13) There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight in yellow 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.
14) Please do not highlight anesthesia and euthanasia steps**. Done.**
15) Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]." **Not applicable.**
16) Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. **Done.**
17) As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
    a. Critical steps within the protocol
    b. Any modifications and troubleshooting of the technique
    c. Any limitations of the technique
    d. The significance with respect to existing methods
    e. Any future applications of the technique
    Response: We have expanded the same to include these aspects highlighted here.
18) Please do not abbreviate the journal titles in the references section. **[Done]**

Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials and sort the table in alphabetical order. **[Done]**

Reviewer #1:
Manuscript Summary:
The authors propose a new variation of a commonly used method to differentiate between circulating and tissue-resident leukocytes. This is a common problem for immunologists trying to differentiate leukocyte populations, and the protocol here described will prove useful to differentiate circulating and parenchymal populations. The authors provide sufficient proof that the method is working, and since it is a variation of a well-documented method, the usefulness of their approach can be trusted. I think the method will be useful in the field.

Major Concerns:
None

Minor Concerns:
* The method may benefit of including a figure showing the IV injection route the authors chose, like what they already show for the lung inflation method in Fig1B. Since the video will show it, I do not think it's absolutely necessary, so authors should decide.

Response: We thank the reviewer for this idea. We will re-evaluate after the video has been shot and if at that time, this additional figure adds to the narrative, we will definitely include the same.

*** Figure 1** legend does not comment on the different panels. Authors should either avoid using separate panels in the figure or comment on those panels in the legend.

Response: We have clarified the panel specific information to the legends.

* In Fig. 2 the Y axis legends of some of the plots are overlapping the adjacent plots. Authors should be careful with figure placement.

Response: We thank the reviewer for drawing our attention to this. We have adjusted the figure to ensure that the figure placement is appropriate and without overlap.

* In Fig. 2 also there is no indication as to which are the intravascular and parenchymal populations in the last panel. Authors should note this on the plots for the sake of clarity.

Response: We have done the same.

* It is unclear to this reviewer why the authors plot cells per 10^6 per ml (in Fig. 3) instead of absolute counts per gram of processed tissue, which would allow to better compare between individuals. In this reviewer's humble opinion, the method would benefit if it would account for the original amount of processed tissue. In any case, it is up to the authors to reflect on this possibility if they so wish.

Response: We thank the reviewer for this intriguing suggestion. Unfortunately, we did not weigh the lungs of these animals, which would be technically challenging given our method of digestion. We do believe that this would be very relevant in case a wide range of animals (Weights and ages) were being compared. Our comparison was rather tight, the animals were all 8-12 weeks of age and weighed between 25-30 gms, as such we believe that this normalization would have yielded comparable results and have chosen to represent the data as such. Further, we take the same tissue, either the left half of the lung or the whole lung, for this experiment. Though the weight of tissue might vary amongst animals, after counting the total number of cells coming from each animals' processed lung tissue, the same number of cells are taken from each sample, thus achieving a level of normalization across all samples processed.

* Also, for Fig. 3, no statistics were shown on the data. I agree this is not the main point of a methods paper but if the authors choose so, statistics may help readers confirm the differences between the mixed (iv + parenchymal) and parenchymal-only populations.

Response: We thank the reviewer for this comment and have added the statistics same.

* Finally, in the table of materials there are different listed materials that were never mentioned in the method (such as L-glutamine). Authors should either add them to the corresponding section of the method or remove them from the materials table.

Response: We have included the composition of the dissociation media in a section called "Solution/Reagent Preparation" that lists the materials used in the media preparation.

* As a suggestion, readers may find interesting a brief discussion by the authors of the possible confounding factor of inflammation-derived vascular leakiness in the lungs, and whether reducing the time from injection of the IV-marking antibody and the perfusion would help in such cases. That includes choosing the method for euthanasia (as CO2 is known to affect the pulmonary endothelium).

Response: We thank the reviewer for this comment. We have addressed this concern in the discussion section. Indeed, the vascular leakiness may be context dependent and the method for euthanasia should be guided by experiment specific considerations.

Reviewer #2:

Manuscript Summary:

The authors' manuscript is well written and describes a method of labelling intravascular CD45-positive leukocytes to differentiate this population from intraparenchymal leukocytes when collected from mouse lungs. The central problem that the authors correctly identify as a significant issue, is that analysis of leukocytes collected from the lungs by FLOW cytometry is confounded by variable presence of both intravascular and intraparenchymal leukocyte populations. To address this issue and differentiate these biologically different populations, intravascular injection of anti-CD45 antibody has been used to label intravascular leukocytes. In this manuscript, the authors improve on that technique using intrajugular injection of anti-CD45 antibody prior to tissue collection and digestion followed by labeling the liberated immune cells using a panel of immune cell-specific antibodies. The total leukocyte population can thus be quantified while the intravascular and intraparenchymal leukocytes may be differentiated and analyzed independently.

The overall significance of the manuscript and the method that it describes will be of helpful to investigators who study lung immune cell biology. Despite those points, the authors do not adequately demonstrate the validity of their approach.

Major Concerns:

The major concern is that it is not clear that intravascular (IV) injection of anti-CD45 antibody labels the majority of intravascular leukocytes or that this approach is specific for intravascular leukocytes. For this reason, it remains unclear if a significant number of intravascular leukocytes are incorrectly classified as intraparenchymal (if IV antibody labeling is insufficient) or if a significant number of parenchymal leukocytes are incorrectly labeled as intravascular (if IV antibody injection results in labeling of intraparenchymal leukocyte labeling and is therefore not specific). To validate the efficacy of this approach, this outstanding question should be addressed either using immunofluorescence imaging of the intravascular and parenchymal immune cells following IV injection of anti-CD45 antibody and demonstrating the percent of intravascular leukocytes that are labeled by the IV injected antibody. It is also important to show that the IV injected antibody does not label the intraparenchymal leukocytes. The authors could also address this concern by referencing prior experiments where the validity of the IV antibody labeling approach was demonstrated.

Response: We thank the reviewer for this comment. Our work is an adaptation of previous protocols descried in this field. The level of intravascular labeling has been validated in those

experiments. (Anderson et al Nat Protocols, 2014; Gibbings et al, Methods in Molecular Biology 2015). We have however taken this opportunity to clarify the same in the results section.

Minor Concerns:
What is the approximate age and weight range of the mice that the authors have investigated using this approach?

Response: The animals were 8-12 weeks of age and weighed 25-30 gms. We have added this to the results section.

What are the components of the dissociation media used in preparation of the single cell suspension? The reagents appear to be listed in the unlabeled table that lists materials and equipment; however, a clear explanation of this would be helpful to investigators hoping to follow this approach. A good way to do this might be to have subheadings in the table.
Response: We have included the composition of the dissociation media in a section called "Solution/Reagent Preparation" that lists the materials used in the media preparation.

Please add additional details to the figure legends so that the diagrams and data may be interpreted independently from the text. This is true for all figures. Figure 3 appears to have 2 figure legends (?).
Response: We have clarified the same. Figure 3 contains a 3(A) and a 3(B). The legends have been updated as such.

Reviewer #3:
Manuscript Summary:
The paper provides a detailed protocol for distinguishing the intravascular and intraparenchymal leukocytes in the lung. This method can be used to investigate the immune responses of the lung, especially in the scope of the circadian rhythm since the two kinds of cells may present distinct rhythmic behavior.

Minor Concerns:
1 It would be better if change the labeling for the last two graphs in figure 2 to PE-Cy7 and Pacific blue. The authors seem to use the same clone F-311 for both CD45, could that cause any competing for staining, and would that better to use different clones for the two CD45, for example, clone I3/2.3?

Response: We thank the reviewer for this important point. We have included this in our discussion. We believe that both methods have trade-offs. While with the same clone there is risk of competitive binding (or lack thereof), with different clones the affinities may not be identical. We have used the same clone since by design the intravascular compartment gets to bind first, reducing the likelihood that the two antibodies are actually competing in real time. Further, in some cases of labeling specific immune population, only one good clone may be available. Some of these issues have been addressed in the literature a priori, so we focused on the intrajugular mode of delivery and our method of lung digestion.

2 It would be clearer if change the x axis to intraparenchymal and intravascular instead of lung and intravascular for figure 3B.

Response: We have changed the labels as recommended.

3 It would be useful to put the formula of the dissociation media and which buffer contains L-glutamine and penicillin-streptomycin.
Response: Done. We have included the composition of the dissociation media in a section called “Solution/Reagent Preparation” that lists the materials used in the media preparation.

Reviewer #4:
Manuscript Summary:
In this paper, Issah et al. describe a protocol to distinguish intrapulmonary immune cells from intravascular immune cells. The method based on intravascular injection of fluorescently labeled anti-CD45 antibody is simple and easy to perform to get clear results. However, there are some uncertainties shown below.

Major Concerns:
One concern is that the whole procedure might take longer time than conventional methods, which could be disadvantage for the studies that time matters. In this sense, one may argue that this method may not be the best choice for the analysis of circadian rhythm oscillation of immune cell trafficking. It seems that the method described in this paper can be useful for the other applications.

Response: Thank you for this important consideration. We have included this aspect in our discussion.

Minor Concerns:
1. In steps 1.3 and 1.4, why did the authors inject anti-CD45 antibody through the jagular vein but not through the tail vein? Is that only because of the technical difficulties? What happens if the antibody is administered through the tail vein?

Response: We thank the reviewer for this opportunity to clarify. We found the intrajugular injection to be technically reliable and easy to learn, especially for bl6 mice. The tail vein injection method has been used in the literature elsewhere, and works similarly when it can be done adequately.

2. In step 1.4, the authors waited for 2 to 4 min before euthanizing the animals by CO2 exposure for 10 min. One concern is that some cells might transmigrate during the waiting process. If so, they might be considered as intravascular immune cells even though they actually located outside the blood vessels.

Response: We agree completely and have included this concern in the discussion section of the manuscript.