

Submission ID #: 70452

Scriptwriter Name: Sri Lakshmi Priya

Project Page Link: <https://review.jove.com/account/file-uploader?src=21487908>

Title: Optimized Preparation of Whole Murine Tumor-Bearing Lung Tissue for Flow Cytometry and Single-Cell RNA-Sequencing

Authors and Affiliations:

Sicong Wang, Anastasiia Ivanova, Helen P. Makarenkova, Katja A. Lamia

Department of Molecular and Cellular Biology, The Scripps Research Institute

Corresponding Authors:

Katja A. Lamia (klamia@scripps.edu)

Email Addresses for All Authors:

Sicong Wang	(siwang@scripps.edu)
Anastasiia Ivanova	(aivanova@scripps.edu)
Helen P. Makarenkova	(hmakarenk@scripps.edu)
Katja A. Lamia	(klamia@scripps.edu)

Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar?

No

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage?

No

3. Filming location: Will the filming need to take place in multiple locations?

No

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials.

No

Current Protocol Length

Number of Steps: 23

Number of Shots: 42

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Sicong Wang**: Our research investigates how circadian clocks control biological processes and how their disruption promotes tumorigenesis.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Katja Lamia**: To overcome limited subpopulation analysis, we developed this protocol to sequence all cell types within tumor-bearing lungs together.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Sicong Wang**: This protocol allows researchers to study the heterogeneity in various murine lungs models, including tumor-bearing whole lungs.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.4. **Sicong Wang**: The protocol also enables downstream analyses such as cell-cell communication mapping to further characterize the tumor microenvironment.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Sicong Wang**: Future studies could investigate how circadian rhythm disruption promotes tumorigenesis through cell-cell communication and microenvironment remodeling.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Scripps Research Institutional Animal Care and Use Committee at The Scripps Research Institute

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Preparation of Buffers

Demonstrator: Sicong Wang

- 2.1. To begin, gather the required reagents [1]. Prepare Dulbecco's Modified Eagle Medium or DMEM (*D-M-E-M*) and F 12 (*F-12*) medium by adding 2% volume per volume heat inactivated fetal bovine serum or FBS (*F-B-S*) to the medium [2].
 - 2.1.1. WIDE: Talent walking into the room with a tray containing required reagents.
 - 2.1.2. Talent adding heat-inactivated FBS to the DMEM and F-12 medium.
- 2.2. Prepare the cell wash buffer by adding heat inactivated FBS to calcium and magnesium free phosphate buffered saline or PBS (*P-B-S*) [1].
 - 2.2.1. Talent adding heat-inactivated FBS to Ca^+/Mg^+ free PBS.
- 2.3. Pre-chill calcium and magnesium free PBS to use as the perfusion buffer [1].
 - 2.3.1. Talent placing the Ca^+/Mg^+ free PBS in the cold room.
- 2.4. Prepare the enzyme stock solutions and digestion medium [1] by adding the solutions [2] and mixing thoroughly [3].
 - 2.4.1. Talent holding a bottle and pipette.
 - 2.4.2. TEXT ON PLAIN BACKGROUND:
Enzyme stock solutions:
Collagenase IV stock: 10 mg (1600 U/mg) in 1 mL DMEM/F-12
Dispase II stock: 42 mg (1.19 U/mg) in 1 mL DMEM/F-12
DNase I stock: 5 mg in 1 mL DMEM/F-12

Digestion medium:
Collagenase IV stock solution - 250 μl
Dispase II stock solution - 200 μl
DNase I stock solution - 100 μl
DMEM/F12 medium - 5 mL
 - 2.4.3. Talent holding the bottle and manually swirling it in a circular motion to mix the solution thoroughly.
- 2.5. Prepare the resuspension buffer by adding 1 microliter of 0.5 molar ethylenediaminetetraacetic acid or EDTA (*E-D-T-A*) [1] and 1 milliliter of heat-

inactivated FBS to the bottle containing 50 milliliters of calcium and magnesium-free PBS [2].

2.5.1. Talent adding EDTA to the bottle containing Ca^+/Mg^+ free PBS.

2.5.2. Talent adding heat inactivated FBS to the bottle containing Ca^+/Mg^+ free PBS.

3. Collection of Murine Lung Tissue

3.1. After placing the euthanized mouse on its back on a dissection pad, pin the feet [1-TXT]. Spray the mouse liberally with 70% ethanol [2].

3.1.1. Talent pinning the feet of the mouse positioning on its back on the dissection pad. **TXT: Euthanasia: CO_2 inhalation**

3.1.2. Talent spraying the mouse with 70% ethanol.

3.2. Using disinfected surgical instruments, make a 2 to 3 centimeter wide midline incision through the skin and muscles of the lower abdomen [1]. Carefully open the thorax without cutting the lungs or the heart [2].

3.2.1. Talent making a midline incision through the lower abdomen.

3.2.2. Talent opening the thorax.

3.3. Attach a safety multifly 21 gauge needle to a 50 milliliter syringe [1]. Aspirate 30 milliliters of perfusion buffer into the syringe [2].

3.3.1. Talent attaching the safety multifly 21 G needle to the 50 mL syringe.

3.3.2. Talent aspirating perfusion buffer into the syringe.

3.4. Insert the needle into the left ventricle [1]. Using the sharp, straight scissors, make a small incision in the right atrium [2].

3.4.1. Talent inserting the needle into the left ventricle.

3.4.2. Talent making a small incision in the right atrium with the sharp, straight scissors.

3.5. Perfuse the whole body with 15 milliliters of perfusion buffer [1] until the liver is whitened [2].

3.5.1. Talent perfusing the whole body with the perfusion buffer.

3.5.2. CU: Shot of the liver whitening after perfusion.

3.6. Remove the lungs from the thorax [1]. Using scissors, cut away the excess tissue attached to the lungs [2]. Place the lungs in 10 milliliters of pre cooled calcium and magnesium free PBS to wash away excess blood, and maintain the sample on ice between each step throughout the experiment [3].

3.6.1. Talent removing the lungs from the thorax.

3.6.2. Talent trimming away excess tissue attached to the lungs.

3.6.3. Talent placing the lungs into pre-cooled Ca^+/Mg^+ free PBS.

- 3.7. Transfer the lungs into a 1.5 milliliter tube containing 500 microliters of ice-cold DMEM and F 12 medium [1].

- 3.7.1. Talent transferring the lungs into a 1.5 mL tube containing ice-cold medium.

4. Preparation and Assessment of Lung Single-cell Suspension

- 4.1. Using an extra-narrow straight scissors, cut the lung tissue into small pieces of 1 to 2 cubic millimeters for 2 to 3 minutes [1].

- 4.1.1. Talent cutting the lung tissue into small pieces with the extra-narrow straight scissors.

- 4.2. Transfer the minced lung tissue to a 6 centimeter dish containing 5 milliliters of digestion medium [1].

- 4.2.1. Talent transferring the minced lung tissue into the 6 centimeter dish with digestion medium.

- 4.3. Place the 6 centimeter dish on a thermostatic shaker set to 50 revolutions per minute for 25 minutes at 37 degrees Celsius [1]. Using a 1 milliliter pipette, mix the suspension every 8 minutes [2].

- 4.3.1. Talent placing the 6 cm dish on the thermostatic shaker.

- 4.3.2. Talent mixing the suspension in the dish with a 1 mL pipette.

- 4.4. Wet the 100 micrometer mesh strainer with 1 milliliter of DMEM and F 12 medium [1] and place it in a 10 centimeter dish [2].

- 4.4.1. Talent wetting the mesh strainer with medium.

- 4.4.2. Talent placing the mesh strainer in the 10 cm dish.

- 4.5. Transfer the digested suspension into the strainer [1]. Using the rubber end of a sterile syringe plunger, push the tissue through the strainer [2].

- 4.5.1. Talent transferring the digested suspension into the strainer.

- 4.5.2. Talent pushing the tissue through the strainer with the rubber end of the sterile syringe plunger.

- 4.6. Place a pre-wetted 70 micrometer mesh strainer over a 50 milliliter centrifuge tube [1].

- 4.6.1. Talent placing the 70 μ m mesh strainer over the 50 mL centrifuge tube.

- 4.7. Filter the cell suspension through the strainer [1]. Rinse the strainer with 5 milliliters of DMEM and F 12 medium [2].

- 4.7.1. Talent filtering the cell suspension through the strainer.

- 4.7.2. Talent rinsing the strainer with medium.

- 4.8. Centrifuge the filtered cell suspension at 400 times relative centrifugal force for 10 minutes at 4 degrees Celsius [1]. After removing the supernatant, resuspend the cell pellet in 1 milliliter of red blood cell lysing buffer for 2 minutes at room temperature [2].
 - 4.8.1. Talent placing the filtered cell suspension in the centrifuge.
 - 4.8.2. Talent resuspending the cell pellet in red blood cell lysing buffer.
- 4.9. Add 14 milliliters of cell wash buffer to the suspension [1]. After centrifuging as previously demonstrated, discard the supernatant [2].
 - 4.9.1. Talent adding cell wash buffer to the suspension.
 - 4.9.2. Talent discarding the supernatant.
- 4.10. Once the pellet appears clear, resuspend the cells in 20 milliliters of resuspension buffer [1].
 - 4.10.1. Talent resuspending the cells in resuspension buffer.
- 4.11. Mix 10 microliters of the single-cell suspension with an equal volume of trypan blue [1]. Add 10 microliters of the mixture to the cell counting chamber slide [2]. Check live cell viability, cell number, and single-cell status using a cell counter [3].
 - 4.11.1. Talent mixing the single-cell suspension with trypan blue.
 - 4.11.2. Talent loading the mixture onto the cell counting chamber slide.
 - 4.11.3. Talent placing the slide into the cell counter and starting the measurement.

Results

5. Results

- 5.1. Cell viability was below 80% before optimization [1] and increased to above 85% after optimization [2].
 - 5.1.1. LAB MEDIA: Table 1. *Video editor: Highlight the values in the row "Percentage of live cells" under Repeat 1 and Repeat 2 in "Before optimization".*
 - 5.1.2. LAB MEDIA: Table 1. *Video editor: Highlight the values in the row "Percentage of live cells" under Repeat 1 and Repeat 2 in "After optimization".*
- 5.2. Cell Ranger quality assessment identified 23,488 estimated cells in sample 1 and 21,436 in sample 2 [1].
 - 5.2.1. LAB MEDIA: Table 2. *Video editor: Highlight the values in the row "Estimated Number of Cells" under Sample 1 and Sample 2.*
- 5.3. The barcode rank plot indicated minimal ambient ribonucleic acid contamination [1].
 - 5.3.1. LAB MEDIA: Figure 2A, B. *Video editor: Emphasize the grey line labelled as "Background".*
- 5.4. Principal component analysis and uniform manifold approximation and projection revealed well-defined clusters with all major lung cell types represented [1].
 - 5.4.1. LAB MEDIA: Figure 3A. *Video editor: Highlight all the clusters in the UMAP plot.*
- 5.5. Dot plots of representative marker genes showed that canonical biomarkers were highly expressed in their corresponding cell types [1].
 - 5.5.1. LAB MEDIA: Figure 3B. *Video editor: Highlight all circles in blue shades.*
- 5.6. TdTomato positive cells were enriched in epithelial-related clusters [1].
 - 5.6.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the blue cluster labelled as 7 and AT2 cells.*
- 5.7. By flow cytometry [1], TdTomato positive and epithelial cell adhesion molecule positive cells were successfully isolated [2].
 - 5.7.1. LAB MEDIA: Figure 1. *Video editor: Show the plots with FSC-A on the X-axis and TdTomato-A on the Y-axis, and FSC-A on the X-axis and EpCAM-A on the Y-axis.*
 - 5.7.2. LAB MEDIA: Figure 1. *Video editor: Show the same plots used in 5.7.1 and highlight the gated region labeled epcam+ in the EpCAM-A plot and the rectangular region labeled Ep+ Td+ in the TdTomato-A plot.*

Pronunciation guides:

1. Dulbecco's
 Pronunciation link: <https://www.merriam-webster.com/dictionary/Dulbecco>
 IPA: /duːlˈbɛkoʊ/
 Phonetic Spelling: dool·beh·koh

2. Fetal
Pronunciation link: <https://www.merriam-webster.com/dictionary/fetal>
IPA: /'fi:təl/
Phonetic Spelling: fee·tuhl
3. Bovine
Pronunciation link: <https://www.merriam-webster.com/dictionary/bovine>
IPA: /'bɒʊˌvaɪn/
Phonetic Spelling: boh·vyn
4. Phosphate
Pronunciation link: <https://www.merriam-webster.com/dictionary/phosphate>
IPA: /'fɑ:sˌfeɪt/
Phonetic Spelling: faas·fayt
5. Collagenase
Pronunciation link: <https://www.merriam-webster.com/dictionary/collagenase>
IPA: /kə'lædʒəˌneɪs/
Phonetic Spelling: kuh·laj·uh·nays
6. Dispase
Pronunciation link: <https://www.howtopronounce.com/dispase>
IPA: /dɪ'speɪs/
Phonetic Spelling: dih·spays
7. Ethylenediaminetetraacetic
Pronunciation link: <https://www.howtopronounce.com/ethylenediaminetetraacetic>
IPA: /ˌɛθɪliːnˌdaɪˌæmɪnˌtetrəˌsiːtɪk/
Phonetic Spelling: eth·uh·leen·dy·am·uh·neen·teh·truh·see·tik
8. Euthanized
Pronunciation link: <https://www.merriam-webster.com/dictionary/euthanize>
IPA: /'ju:θəˌnaɪz/
Phonetic Spelling: yoo·thuh·nyz
9. Thorax
Pronunciation link: <https://www.merriam-webster.com/dictionary/thorax>
IPA: /'θɔ:rˌæks/
Phonetic Spelling: thor·aks
10. Ventricle
Pronunciation link: <https://www.merriam-webster.com/dictionary/ventricle>

IPA: /'vɛntrɪkəl/
Phonetic Spelling: ven·truh·kuhl

11. Atrium

Pronunciation link: <https://www.merriam-webster.com/dictionary/atrium>
IPA: /'ɛtrɪəm/
Phonetic Spelling: ay·tree·uhm

12. Perfusion

Pronunciation link: <https://www.merriam-webster.com/dictionary/perfusion>
IPA: /pər'fjuːʒən/
Phonetic Spelling: per·fyoo·zhuhn

13. Centrifuge

Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /'sɛntrɪˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj

14. Supernatant

Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /ˌsuːpər'nextənt/
Phonetic Spelling: soo·per·nay·tuht

15. Trypan

Pronunciation link: <https://www.howtopronounce.com/trypan>
IPA: /'traɪpæn/
Phonetic Spelling: try·pan

16. Ribonucleic

Pronunciation link: <https://www.merriam-webster.com/dictionary/ribonucleic>
IPA: /ˌraɪboʊnuː'kliːɪk/
Phonetic Spelling: ry·boh·noo·klee·ik

17. Cytometry

Pronunciation link: <https://www.merriam-webster.com/dictionary/cytometry>
IPA: /saɪ'tɑːmətri/
Phonetic Spelling: sy·taam·uh·tree

18. Epithelial

Pronunciation link: <https://www.merriam-webster.com/dictionary/epithelial>
IPA: /ˌɛpə'θiːliəl/
Phonetic Spelling: eh·puh·thee·lee·uhl

19. Adhesion

Pronunciation link: <https://www.merriam-webster.com/dictionary/adhesion>

IPA: /əd'hi:ʒən/

Phonetic Spelling: ad·hee·zhuh