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Title: Protocol for Single-Cell Dissociation, RNA Sequencing, and Isolation of Myoepithelial Cells and Fibroblasts from the Aged Lacrimal Gland

Authors and Affiliations:

Anastasiia Ivanova^{*}, Vanessa Delcroix^{*}, Vina Dang, Anastasiya Kuznetsova, Neda Rashidi, Sicong Wong, Katja A. Lamia, and Helen P. Makarenkova

Department of Molecular and Cellular Biology, Scripps Research Institute

^{*}These authors contributed equally

Corresponding Authors:

Helen P. Makarenkova hmakarenk@scripps.edu

Email Addresses for All Authors:

Anastasiia Ivanova	aivanova@scripps.edu
Vanessa Delcroix	vdelcroix@scripps.edu
Vina Dang	VinaD@scripps.edu
Anastasiya Kuznetsova	akuznetsova@scripps.edu
Neda Rashidi	nrashidi@scripps.edu
Sicong Wong	siwang@scripps.edu
Katja Lamia	klamia@scripps.edu
Helen P. Makarenkova	hmakarenk@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? **yes**

If **Yes**, can you record movies/images using your own microscope camera?

No

If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit.

If your microscope does not have a camera port, the scope kit will be attached to one of the eyepieces and **you will have to perform the procedure using one eye.**

Zeiss Stemi CV6 and Leica M205FA (fluorescent microscope)

SCOPE shots: 2.4.2, 2.4.3, 2.5.1

Videographer: Please film the above-mentioned shots using the scope kit

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: 25

Number of Shots: 56 (3 Scope)

Introduction

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

Authors: Please note that **the questions will not appear on screen. Please answer in *stand-alone full sentence with sufficient context.***

Answers are slightly edited to match the length in accordance with the journal guidelines

INTRODUCTION:

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

- 1.1. **Helen Makarenkova:** Our lab studies how age-related changes affect different cell types in the lacrimal and salivary glands, using single-cell analysis and flow cytometry.
 - 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. **Helen Makarenkova:** Standard dissociation methods poorly recover fragile epithelial cells from aged lacrimal and salivary glands; this optimized protocol improves viability and enables reliable single-cell analysis and cell sorting.
 - 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. **Vina Dang:** This protocol is designed to isolate viable single cells from lacrimal glands for molecular characterization of epithelial, stromal, and immune populations.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~**Describe the most important consideration or challenge when performing this protocol.**~~

- 1.4. **Vina Dang:** The main challenge is minimizing mechanical and enzymatic stress during dissociation of fragile aged tissue while maintaining high cell viability for single-cell RNA sequencing.

- 1.4.1. 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?~~

- 1.5. **Vina Dang:** Following this procedure, isolated cells can be used for single-cell RNA sequencing, bulk RNA sequencing, and molecular profiling of specific cell populations. Sorted cells can also be cultured for downstream functional assays.
 - 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 Institutional Animal Care and Use Committee (IACUC) at The Scripps Research Institute

Protocol

2. Lacrimal Gland Tissue Isolation from *Pdgfra*^{EGFP} Transgenic Mouse

Demonstrator: Vina Dang

2.1. To begin, position the euthanised mouse supine on the dissection table [1].

2.1.1. WIDE: Talent positioning the euthanised mouse supine on the dissection table.
TXT: Anesthesia: Isoflurane; Euthanasia: Cervical dislocation; *Pdgfra*^{EGFP} transgenic mouse

2.2. Using fine forceps and scissors, incise the skin between the eye and the ear [1]. Gently retract the skin to expose the lacrimal gland [2].

2.2.1. Talent making an incision in the skin between the eye and the ear using fine scissors while holding the skin with fine forceps.

2.2.2. Talent retracting the skin to expose the lacrimal gland.

2.3. Using dull-tipped forceps, lift the lacrimal gland slightly [1]. Carefully separate the surrounding connective tissue by gently teasing it away in a circular motion [2]. Identify the visible boundaries of the lacrimal gland and separate it from adjacent tissues, including the parotid gland [3].

2.3.1. Talent lifting the lacrimal gland slightly using dull-tipped forceps.

2.3.2. Talent teasing away surrounding connective tissue around the gland in a circular motion with the forceps.

2.3.3. Shot showing the lacrimal gland boundaries and separating it from adjacent tissues including the parotid gland.

2.4. Now, transfer the lacrimal gland to a 35-millimeter Petri dish containing 2 milliliters of cold PBS on ice [1]. Under a dissecting microscope, remove the remaining fat [2] and carefully peel off the connective tissue capsule [3].

2.4.1. Talent placing the isolated lacrimal gland into a 35 millimeter Petri dish containing cold PBS on ice. **Authors: This need not be a scope shot. This can be a normal shot**

2.4.2. SCOPE: View of the lacrimal gland as the remaining fat is removed under the dissecting microscope. **Videographer: Please film the SCOPE shots using the**

scope kit

- 2.4.3. SCOPE: Talent peeling off the connective tissue capsule from the gland using two forceps.
- 2.5. Excise a small fragment from the lacrimal gland tissue [1]. Examine the fragment under a fluorescence microscope [2]. Verify EGFP fluorescence in the tissue fragment to confirm labeling of PDGFR α -expressing cells before proceeding to enzymatic digestion [3-TXT].
 - 2.5.1. SCOPE: cutting a small fragment from the lacrimal gland tissue.
 - 2.5.2. Talent positioning the sample on the fluorescence microscope stage.
 - 2.5.3. SCREEN: Fluorescent signal visible in the examined tissue fragment. **TXT: PDGFR α : Platelet-Derived Growth Factor Receptor Alpha**

Authors: This shot requires a green signal to be seen. The videographer can film the computer screen when you obtain an image. Or you can also provide us with a representative image for this shot

3. Fragmentation of Harvested Lacrimal Gland Tissue

- 3.1. Next, place a sterile 35-millimeter Petri dish on ice [1]. Add 800 to 1000 microliters of cold PBS to the center of the dish [2] and transfer one lacrimal gland into the PBS [3].
 - 3.1.1. Talent placing a sterile 35 millimeter Petri dish on ice.
 - 3.1.2. Talent pipetting 800 to 1000 microliters of cold 1 times PBS into the center of the dish.
 - 3.1.3. Talent transferring one lacrimal gland into the PBS.
- 3.2. Ensure the tissue remains submerged in PBS [1]. Using two sterile scalpels, finely mince the lacrimal gland into small fragments until it reaches a soft, paste-like consistency [2].
 - 3.2.1. Close shot showing tissue remaining submerged in PBS.
 - 3.2.2. Talent mincing the lacrimal gland into small fragments using two sterile scalpels.
- 3.3. Using a pipette, collect the tissue fragments to one side of the dish [1]. Aspirate the residual PBS [2] and wash the fragments with 1 milliliter of fresh cold PBS [3].

- 3.3.1. Talent gathering minced tissue fragments to one side of the Petri dish using a pipette.
- 3.3.2. Talent aspirating the remaining PBS from the dish.
- 3.3.3. Talent adding 1 milliliter of fresh cold PBS to wash the tissue fragments.

4. Enzymatic Digestion of the Tissue Fragments and Primary Washes

- 4.1. Now, transfer the washed tissue fragments into a 12-well plate containing 2 milliliters per well of pre-warmed digestion medium [1-TXT].
 - 4.1.1. Talent transferring the washed tissue fragments into a well of a 12 well plate containing digestion medium. **TXT: Digestion medium contains: Collagenase IV (125 U/mL); Dispase II (1.25 U/mL); DNase I (20 U/mL)**
- 4.2. Place the plate in a shaking water bath and incubate at 37 degrees Celsius and 60 revolutions per minute for 30 minutes [1].
 - 4.2.1. Talent placing the 12 well plate into a shaking water bath for incubation.
- 4.3. Using a wide bore 1 milliliter pipette tip, gently triturate the tissue 15 times [1]. Return the plate to the shaking water bath and incubate for an additional 30 minutes [2].
 - 4.3.1. Talent gently triturating the tissue suspension in the well using a wide bore 1 milliliter pipette tip.
 - 4.3.2. Talent placing the plate back into the shaking water bath.
- 4.4. Next, using a regular 1 milliliter pipette tip, gently triturate the sample 10 times [1] and incubate again for 30 minutes [2].
 - 4.4.1. Talent gently triturating the sample in the well using a regular 1 milliliter pipette tip.
 - 4.4.2. Talent placing the 12 well plate back into the shaking water bath.
- 4.5. Then, gently triturate the sample 10 times [1] and transfer the cell suspension into 2 milliliter Dolphin microcentrifuge tubes [2].
 - 4.5.1. Talent gently triturating the sample in the well using a pipette.

- 4.5.2. Talent transferring the cell suspension into 2 milliliter Dolphin microcentrifuge tubes.
- 4.6. Add 1 milliliter of cold blocking medium 1 or BM-1 to each well of the plate to collect the remaining digestion medium containing residual cells [1] and place the plate on ice until the next step [2].
 - 4.6.1. Talent adding 1 milliliter of cold BM1 into each well of the plate.
 - 4.6.2. Talent placing the plate on ice.
- 4.7. Centrifuge the tubes containing the cell suspension at 300 g for 5 minutes at 4 degrees Celsius [1].
 - 4.7.1. Talent placing the Dolphin microcentrifuge tubes containing the cell suspension into a centrifuge.
- 4.8. Discard the supernatant from each tube [1]. Transfer the BM1 from each well of the plate containing residual cells into the corresponding tube and gently resuspend the cell pellets [2].
 - 4.8.1. Talent aspirating and discarding the supernatant from the Dolphin microcentrifuge tube.
 - 4.8.2. Talent transferring BM1 from a well of the plate into the corresponding Dolphin microcentrifuge tube and mixing by pipetting.
- 4.9. Next, add 600 microliters of cold BM1 to each well to rinse the remaining cells [1] and transfer the wash into the corresponding tube to combine with the cell suspension [2].
 - 4.9.1. Talent adding 600 microliters of cold BM1 into a well of the plate.
 - 4.9.2. Talent transferring the wash from the well into the corresponding Dolphin microcentrifuge tube. **NOTE: 4.9.2 and 4.10.1 may have been shot together**
- 4.10. Gently resuspend the cell suspension [1]. Centrifuge the tubes again at 300 g for 5 minutes at 4 degrees Celsius [2] and discard the supernatant [3].
 - 4.10.1. Talent gently resuspending the cell suspension in the Dolphin microcentrifuge tube using a pipette.
 - 4.10.2. Talent placing the Dolphin microcentrifuge tubes into the centrifuge.
 - 4.10.3. Talent aspirating and discarding the supernatant from the tube.

5. Accutase and DNase I Treatment

- 5.1. Add 1 milliliter of Accutase to the cell pellets [1] and gently resuspend the cells [2]. Incubate the tube at 37 degrees Celsius and 60 revolutions per minute for 5 to 10 minutes [3]. Using a 1 milliliter pipette tip, gently triturate the sample 10 times [4].
 - 5.1.1. Talent adding 1 milliliter of Accutase to the Dolphin microcentrifuge tube containing the cell pellet.
 - 5.1.2. Talent gently resuspending the cells in the tube using a pipette.
 - 5.1.3. Talent placing the tube into a shaking water bath for incubation.
 - 5.1.4. Talent gently triturating the cell suspension in the tube using a 1 milliliter pipette tip.
- 5.2. Then, add 1 milliliter of cold BM2 to the tube to terminate the enzymatic reaction by diluting the Accutase [1]. Centrifuge the tube at 300 g for 5 minutes at 4 degrees Celsius [2] and discard the supernatant [3].
 - 5.2.1. Talent adding 1 milliliter of cold BM2 into the tube containing the cell suspension.
 - 5.2.2. Talent placing the tube into the centrifuge.
 - 5.2.3. Talent aspirating and discarding the supernatant from the tube.
- 5.3. Add DNase 1 containing medium to the tube to resuspend the cells and fill the tube to the top [1-TXT]. Incubate the tube for 10 minutes at room temperature and gently resuspend once after 5 minutes [2].
 - 5.3.1. Talent adding the Deoxyribonuclease treatment medium into the tube and gently resuspending the cells. **TXT: DNase I: 200 U/mL**
 - 5.3.2. Talent gently resuspending the cell suspension in the tube during incubation.
- 5.4. Meanwhile, place a 70-micrometer cell strainer over a 15-milliliter conical tube [1] and pre wet the strainer with 1 milliliter of BM3 [2].
 - 5.4.1. Talent placing a 70 micrometer cell strainer on top of a 15 milliliter conical tube.
 - 5.4.2. Talent adding 1 milliliter of BM3 onto the cell strainer to pre wet it.

- 5.5. After incubation, transfer the cell suspension onto the strainer [1]. Add 1 milliliter of BM3 into the original collection tube to rinse it [2] and transfer the rinse onto the same strainer [3].
 - 5.5.1. Talent pipetting the cell suspension onto the 70 micrometer cell strainer.
 - 5.5.2. Talent adding 1 milliliter of BM3 into the original collection tube.
 - 5.5.3. Talent transferring the rinse from the tube onto the cell strainer.

- 5.6. Wash the strainer with an additional 5 milliliters of BM3 [1].
 - 5.6.1. Talent adding 5 milliliters of BM3 onto the cell strainer to wash it.

- 5.7. Finally, determine cell concentration and viability of the filtered cell suspension using Trypan Blue stain and an automated cell counter [1]. Proceed with downstream applications such as single-cell RNA sequencing for gene expression profiling or flow cytometry-based cell sorting of fluorescently labeled cell populations [2].
 - 5.7.1. Talent loading the Trypan Blue stained cell suspension into a counting chamber.
 - 5.7.2. ~~Talent labeling a tube with clear solution as "For RNA Seq".~~ **Author's NOTE: We were not able to film 5.7.2 and 5.7.3 as described in the script. We filmed an alternate shot for 5.7.3**
 - 5.7.3. ~~Talent placing the sample in a flow cytometer or working at the flow cytometer station.~~ Talent resuspending the sample in 1 ml FACS buffer.

Results

6. Results

6.1. UMAP analysis of single-cell RNA sequencing data identified well-represented epithelial and stromal populations in the lacrimal gland, including myoepithelial cells and fibroblasts [1], as well as diverse immune cell populations [2].

6.1.1. LAB MEDIA: Figure 3AB.

6.1.2. LAB MEDIA: Figure 3C

6.2. Aged lacrimal glands contained abundant plasma and activated B cells expressing *Ighm* (I-G-H-M) [1] and *Ighd* (I-G-H-D) [2].

6.2.1. LAB MEDIA: Figure 3D. *Video editor: Highlight the regions with blue shade.*

6.2.2. LAB MEDIA: Figure 3E. *Video editor: Highlight the regions with blue shade.*

6.3. Sequential flow cytometry gating enabled clear separation of EpCAM-positive, GFP-positive myoepithelial cells from EpCAM-negative, GFP-positive mural cells [1].

6.3.1. LAB MEDIA: Figure 4C. *Video editor: Highlight the plot on the extreme right and focus on MEC quadrant.*

6.4. GFP-positive fibroblasts expressing PDGFR alpha were EpCAM negative, allowing discrimination from epithelial cells [1].

6.4.1. LAB MEDIA: Figure 4D. *Video editor: Highlight the plot on the extreme right and focus on 11% quadrant.*

6.5. In *PdgfraEGFP* (P-D-G-F-R-alpha-E-G-F-P) mice, all GFP-positive cells also expressed collagen 1, confirming fibroblast identity [1].

6.5.1. LAB MEDIA: Figure 4E. *Video editor: Highlight the green cells that overlap with the red collagen I staining signal.*

6.6. Flow cytometry analysis showed that 99.6% of GFP-positive cells corresponded to PDGFR alpha-immuno-stained cells [1].

6.6.1. LAB MEDIA: Figure 4F. *Video editor: Highlight the value "99.6%" associated with the GFP-positive cell gate.*

7. Lacrimal
Pronunciation link: <https://www.merriam-webster.com/dictionary/lacrimal>
IPA: /'læk.rə.məl/
Phonetic Spelling: lak·ruh·muhl
8. Isolation
Pronunciation link: <https://www.merriam-webster.com/dictionary/isolation>
IPA: /,aɪ.sə'leɪ.ʃən/
Phonetic Spelling: eye·suh·lay·shuhn
9. Transgenic
Pronunciation link: <https://www.merriam-webster.com/dictionary/transgenic>
IPA: /trænz'dʒɛn.ɪk/
Phonetic Spelling: tranz·jen·ik
10. Euthanised
Pronunciation link: <https://www.merriam-webster.com/dictionary/euthanize>
IPA: /'ju:.θə'naɪz/
Phonetic Spelling: yoo·thuh·nyze
11. Supine
Pronunciation link: <https://www.merriam-webster.com/dictionary/supine>
IPA: /'su:.paɪn/
Phonetic Spelling: soo·pyne
12. Isoflurane
Pronunciation link: <https://www.merriam-webster.com/dictionary/isoflurane>
IPA: /,aɪ.sou'flʊr.ɛn/
Phonetic Spelling: eye·soh·floor·ayn
13. Cervical
Pronunciation link: <https://www.merriam-webster.com/dictionary/cervical>
IPA: /'sɜ:.vɪ.kəl/
Phonetic Spelling: ser·vih·kuhl
14. Dislocation
Pronunciation link: <https://www.merriam-webster.com/dictionary/dislocation>
IPA: /,dɪs.loʊ'keɪ.ʃən/
Phonetic Spelling: dis·loh·kay·shuhn
15. Retract
Pronunciation link: <https://www.merriam-webster.com/dictionary/retract>
IPA: /rɪ'trækt/
Phonetic Spelling: ree·trakt
16. Connective
Pronunciation link: <https://www.merriam-webster.com/dictionary/connective>
IPA: /kə'nek.tɪv/
Phonetic Spelling: kuh·nek·tiv
17. Parotid
Pronunciation link: <https://www.merriam-webster.com/dictionary/parotid>
IPA: /pə'rɑ:.tɪd/
Phonetic Spelling: puh·raa·tid

- 18. Capsule**
Pronunciation link: <https://www.merriam-webster.com/dictionary/capsule>
IPA: /'kæp.sju:l/
Phonetic Spelling: kap·syool
- 19. Fluorescence**
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
IPA: /flʊ'res.əns/
Phonetic Spelling: floor·es·uhns
- 20. Platelet-derived**
Pronunciation link: <https://www.merriam-webster.com/dictionary/platelet>
IPA: /'plɛt.lət/
Phonetic Spelling: playt·luht
- 21. Fragmentation**
Pronunciation link: <https://www.merriam-webster.com/dictionary/fragmentation>
IPA: /,fræg.mən'teɪ.ʃən/
Phonetic Spelling: frag·muhn·tay·shuhn
- 22. Submerged**
Pronunciation link: <https://www.merriam-webster.com/dictionary/submerge>
IPA: /səb'mɜːdʒ/
Phonetic Spelling: sub·merj
- 23. Scalpel**
Pronunciation link: <https://www.merriam-webster.com/dictionary/scalpel>
IPA: /'skæl.pəl/
Phonetic Spelling: skal·puhl
- 24. Enzymatic**
Pronunciation link: <https://www.merriam-webster.com/dictionary/enzymatic>
IPA: /,ɛn.zai'mæt.ɪk/
Phonetic Spelling: en·zy·mat·ik
- 25. Collagenase**
Pronunciation link: <https://www.merriam-webster.com/dictionary/collagenase>
IPA: /kə'lædʒ.ə.neɪs/
Phonetic Spelling: kuh·laj·uh·nays
- 26. Dispare**
Pronunciation link: <https://www.howtopronounce.com/dispare>
IPA: /'dɪs.pɛɪs/
Phonetic Spelling: dis·pays
- 27. Triturate**
Pronunciation link: <https://www.merriam-webster.com/dictionary/triturate>
IPA: /'trɪtʃ.ə.reɪt/
Phonetic Spelling: trɪch·uh·rayt
- 28. Microcentrifuge**
Pronunciation link: <https://www.merriam-webster.com/dictionary/microcentrifuge>
IPA: /,maɪ.kroʊ'sɛn.trə'fjuːdʒ/
Phonetic Spelling: my·kroh·sen·truh·fyooj

29. Residual
 Pronunciation link: <https://www.merriam-webster.com/dictionary/residual>
 IPA: /rɪˈzɪdʒ.u.əl/
 Phonetic Spelling: rih·zij·oo·uhl
30. Accutase
 Pronunciation link: <https://www.howtopronounce.com/accutase>
 IPA: /ˈæk.jʊ.teɪz/
 Phonetic Spelling: ak·yuh·tayz
31. Deoxyribonuclease
 Pronunciation link: <https://www.merriam-webster.com/dictionary/deoxyribonuclease>
 IPA: /di.ɑ:k.si.raɪ.boʊˈnu:.kli:.eɪs/
 Phonetic Spelling: dee·ok·see·rye·boh·noo·klee·ays
32. Micrometer
 Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>
 IPA: /maɪˈkra:.mə.tər/
 Phonetic Spelling: my·krom·uh·ter
33. Viability
 Pronunciation link: <https://www.merriam-webster.com/dictionary/viability>
 IPA: /ˌvaɪ.əˈbɪl.ə.ti/
 Phonetic Spelling: vy·uh·bil·uh·tee
34. Cytometry
 Pronunciation link: <https://www.merriam-webster.com/dictionary/cytometry>
 IPA: /saɪˈtɑ:.mə.tri/
 Phonetic Spelling: sy·tom·uh·tree
35. Sequencing
 Pronunciation link: <https://www.merriam-webster.com/dictionary/sequencing>
 IPA: /ˈsi:.kwən.sɪŋ/
 Phonetic Spelling: see·kwuhn·sing
36. Profiling
 Pronunciation link: <https://www.merriam-webster.com/dictionary/profiling>
 IPA: /ˈproʊ.fai.lɪŋ/
 Phonetic Spelling: proh·fy·ling
37. Epithelial
 Pronunciation link: <https://www.merriam-webster.com/dictionary/epithelial>
 IPA: /ˌɛp.ɪˈθi:.li.əl/
 Phonetic Spelling: ep·ih·thee·lee·uhl
38. Stromal
 Pronunciation link: <https://www.merriam-webster.com/dictionary/stromal>
 IPA: /ˈstroʊ.məl/
 Phonetic Spelling: stroh·muhl
39. Myoepithelial
 Pronunciation link: <https://www.merriam-webster.com/dictionary/myoepithelial>
 IPA: /ˌmaɪ.oʊ.ɛp.ɪˈθi:.li.əl/
 Phonetic Spelling: my·oh·ep·ih·thee·lee·uhl

40. Fibroblasts

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

IPA: /'faɪ.broʊˌblæst/

Phonetic Spelling: fy·broh·blast

41. Immuno-stained

Pronunciation link: No confirmed link found

IPA: /,ɪm.jə.noʊˈsteɪnd/

Phonetic Spelling: im·yuh·noh·staynd