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TITLE:

Isolation of Myoepithelial Cells from Adult Murine Lacrimal and Submandibular Glands

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SUMMARY:

The lacrimal gland (LG) has two cell types expressing α-smooth muscle actin (αSMA): myoepithelial cells (MECs) and pericytes. MECs are of ectodermal origin, found in many glandular tissues, while pericytes are vascular smooth muscle cells of endodermal origin. This protocol isolates MECs and pericytes from murine LGs.

ABSTRACT:

The lacrimal gland (LG) is an exocrine tubuloacinar gland that secretes an aqueous layer of tear film. The LG epithelial tree is comprised of acinar, ductal epithelial, and myoepithelial cells (MECs). MECs express alpha smooth muscle actin (αSMA) and have a contractile function. They are found in multiple glandular organs and are of ectodermal origin. In addition, the LG contains SMA+ vascular smooth muscle cells of endodermal origin called pericytes: contractile cells that envelop the surface of vascular tubes. A new protocol allows us to isolate both MECs and pericytes from adult murine LGs and submandibular glands (SMGs). The protocol is based on the genetic labeling of MECs and pericytes using the *SMA$^{CreErt2/+}$:Rosa26-TdTomato$^{fl/fl}$* mouse strain, followed by preparation of the LG single-cell suspension for fluorescence activated cell sorting (FACS). The protocol allows for the separation of these two cell populations of different origins based on the expression of the epithelial cell adhesion molecule (EpCAM) by MECs, whereas pericytes do not express EpCAM. Isolated cells could be used for cell cultivation or gene expression analysis.

INTRODUCTION:

Myoepithelial cells (MECs) are present in many exocrine glands including lacrimal, salivary, harderian, sweat, prostate, and mammary. MECs are a unique cell type that combines an epithelial and a smooth muscle phenotype. MECs express α-smooth muscle actin (SMA) and have a contractile function[1,2]. In addition to MECs, the lacrimal gland (LG) and the submandibular gland (SMG) contains SMA+ vascular cells called pericytes, which are cells of

endodermal origin that envelop the surface of vascular tubes[3]. Although MECs and pericytes express many markers, SMA is the only marker that is not expressed in other LG and SMG cells[1,3].

Within the last 40 years, several laboratories reported assays for dissociation of different exocrine gland tissues, in which non-enzymatic and enzymatic approaches were applied. In one of the first reports published in 1980, Fritz and coauthors described a protocol to isolate feline parotid acini using sequential digestion in a collagenase/trypsin solution[4]. In 1989, Hann and coauthors adjusted this protocol for acini isolation from rat LGs using a mixture of collagenase, hyaluronidase and DNase[5]. In 1990, Cripps and colleagues published the method of non-enzymatic dissociation of lacrimal gland acini[6]. Later, in 1998, Zoukhri and coauthors returned to an enzymatic dissociation protocol for following up Ca^{2+}-imaging on LG and SMG isolated acini[7]. Within the last decade, researchers have turned their focus on isolation of stem/progenitor cells from exocrine glands. Pringle and coauthors described a protocol in 2011 for isolation of mouse SMG stem cells[8]. This method was based on isolation of stem cell-containing salispheres, which were maintained in culture. The authors claimed that proliferating cells expressing stem cell-associated markers could be isolated from these salispheres[8]. Shatos and coauthors published the protocol for progenitor cell isolation from uninjured adult rat LGs using enzymatic digestion and collecting “liberated” cells[9]. Later, in 2015, Ackermann and coauthors adjusted this procedure to isolate presumptive "murine lacrimal gland stem cells" ("mLGSCs") that could be propagated as a mono-layer culture over multiple passages[10]. However, none of the before mentioned procedures allowed for distinguishing cellular subtypes and individual populations of isolated epithelial cells. In 2016, Gromova and coauthors published a procedure for isolation of LG stem/progenitor cells from adult murine LGs using FACS[11]. However, this protocol was not intended to isolate MECs.

Recently, we have shown that we are able to isolate SMA+ cells from 3-week-old SMA-GFP mice[12]. However, at this time we have not separated different populations of SMA+ cells. Here we established a new procedure for the direct isolation of differentiated MECs and pericytes from adult LGs and SMGs.

PROTOCOL:

All animal work was conducted according to the National Institute of Health (NIH) guidelines and was approved by Institutional Animal Care and Use Committee of the Scripps Research Institute. All efforts were made to minimize the number of mice and their suffering. All experimental animals received a standard diet with free access to tap water.

NOTE: The main steps for MEC and pericyte isolation are outlined schematically in **Figure 1A-F**. All reagents and equipment used for this procedure are described in **Table 1**.

1. **Mice and labelling the SMA cells**

1.1. Use adult (2-4 months old) tamoxifen-inducible, αSMA driven reporter mice *SMA$^{CreErt2/+}$:Rosa26-TdTomato$^{fl/fl}$*.

NOTE: The *SMA^{CreErt2}* strain was kindly provided by Dr. Ivo Kalajzic[13]. *Rosa26-TdTomato$^{fl/fl}$* (*B6.Cg-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J*, also known as *Ai9*) strain (# 007909) were purchased from Jackson Laboratory (Sacramento, CA). SMA+ cells were labeled by intraperitoneal tamoxifen (TM) administration.

1.2. Preparation of tamoxifen solution

1.2.1. Prepare filtered corn oil. Use 0.22 µm vacuum filter since corn oil is viscous.

1.2.2. Transfer 1 g of TM powder from the bottle into a 50 mL tube. Add 1 mL of ethanol to the bottle, cap and shake it to rinse then add to a 50 mL tube. Repeat once more with another 1 mL of ethanol.

1.2.3. Add filtered corn oil to make 50 mL of a 20 mg/mL TM solution. Vortex the tube, wrap it in foil, and put it in a shaking water bath or shaking incubator at 45 °C.

1.2.4. It may take about 12-24 h to dissolve the TM. From time to time, remove the tube and check for any remaining crystals. Once the TM is completely dissolved, aliquot and store at -80 °C. A thawed aliquot can be reused.

1.3. To label SMA+ cells, inject mice intraperitoneally (IP) with TM on two sequential days.

1.3.1. Inject 3-4 weeks old *SMA$^{CreErt2/+}$:Rosa26-TdTomato$^{fl/f}$* any gender mice with TM at 100 µL/20 g (or 2 mg/20 g) body weight (**Figure 1A**). Mice are ready to be used for cell isolation in 2-3 days after the last TM injection. If needed, injected mice can be sacrificed at longer periods of time after TM injection.

NOTE: As controls for proper compensation during FACS, one wild type (C57Bl/6) mouse and one *SMA$^{CreErt2/+}$:Rosa26-TdTomato$^{fl/fl}$* mouse not injected with TM (with "unstained" MECs) of the same age would be required. Use the same calculations provided for 2 *SMA$^{CreErt2/+}$:Rosa26-TdTomato$^{fl/fl}$* mice. Not injected *SMA$^{CreErt2/+}$:Rosa26-TdTomato$^{fl/fl}$* will allow evaluation of the DSRed background. The C57Bl/6 mouse will serve as a negative control of unstained cells.

2. Solutions and buffers

NOTE: The LG is an epithelial origin gland that contains an extracellular matrix that makes dissociation of cells difficult. Therefore, using a special combination of enzymes and a multistep digestion process described below is recommended.

2.1. Dispase type II stock solution (25x)

2.1.1. Dissolve 120 mg of dispase type II powder in 2 mL of 50 mM HEPES/150 mM NaCl to prepare a 25x stock solution (final concentration of dispase should be 30 Units/mL). Units per milligram may vary depending on the number of mice and concentration of the dispase should be adjusted accordingly.

2.1.2. Prepare 200 µL aliquots and store them at -70 °C for up to 6 months or 4 °C for several days. Do not freeze/thaw the aliquot of dispase more than once to prevent enzyme degradation.

2.2. DNase type I stock solution

2.2.1. Dissolve 5 mg of DNase type I powder in a 5 mL solution of 50% glycerol, 20 mM Tris buffer (pH 7.5), and 1 mM $MgCl_2$ (stock concentration should be approximately 2000 Units/mL). Units per milligram may vary depending on number of mice and thus concentration of DNase should be adjusted accordingly.

2.2.2. Filter the stock solution using a 0.22 µm filter and a 10 mL syringe.

2.2.3. Prepare 200 µL aliquots and store them at -70 °C for up to 6 months or 4 °C for several days. Do not freeze/thaw more than once to prevent enzyme degradation.

2.3. Digestion medium

2.3.1. To 10 mL of DMEM low glucose without glutamine, add 100 µL of cell culture supplement (e.g., Glutamax, see **Table of Materials**) for a dilution of 1:100.

2.3.2. To 2 mL of DMEM low glucose with cell culture supplement, add 6 mg of Collagenase type I and mix thoroughly by pipetting (enzyme on wet ice), 160 µL of dispase stock solution (2.4 U/mL final concentration), 16 µL of DNase type I stock solution (8 U/mL final concentration), and 12 µL of 1 M $CaCl_2$ (6 mM final concentration).

NOTE: Calcium is required to increase enzymatic activity[14,15]. All calculations are provided for isolation of cells from four lacrimal glands from two adult mice. The volume of digestion medium may vary depending on the amount of tissue and number of replicates. Do not use more than 4 lacrimal glands from 2-4 months old mice per 2 mL medium.

2.4. Blocking medium I

2.4.1. To 25 mL of DMEM/F-12, add FBS (15% final concentration), 250 µL of cell culture supplement (see **Table of Materials**) for a dilution of 1:100, and 50 µL of 0.5 M EDTA pH 8.0 (1 mM final concentration).

NOTE: Of the different types of medium that were compared for this protocol DMEM/F-12 gave the best results. This medium has also been used by other researchers to isolate/culture epithelial cells[16,17].

2.5. Blocking medium II

2.5.1. To 25 mL of PBS, add 50 µL of 0.5 M EDTA pH 8.0 (1 mM final concentration).

2.6. Recovery medium

2.6.1. To 2 mL of HBSS supplemented with 5 mM $MgCl_2$, add 100 µL of DNase type I stock solution to 100 U per 2 mL final concentration. Relatively high concentrations of DNase-type I is required to reduce aggregation of epithelial cells.

2.7. Fluorescence activated cell sorting (FACS) buffer

2.7.1. To 486.5 mL of PBS, add 12.5 mL of serum (2.5% final concentration) and 1 mL of 0.5 M EDTA pH 8.0 (1 mM final concentration).

NOTE: The buffer can be stored at 4 °C for a maximum of 6 weeks.

3. Adult mouse lacrimal gland harvesting and microdissection

3.1. Anesthetize the mouse by isoflurane inhalation (adjust the isoflurane flow rate or concentration to 5% or greater) and sacrifice by cervical dislocation. Perform anesthesia and euthanasia according to institutional IACUCs recommendations.

3.2. Using fine forceps and scissors, remove skin between the eye and ear (**Figure 2A**).

3.3. To dissect a LG, gently pull LG using tweezers and at the same time scratch the connective tissue around the LG using the sharp tip of small scissors to free it (**Figure 2B**).

3.4. Avoid cutting with scissors, as the LG and parotid salivary glands are located very close to each other and must be separated prior to dissection. When LG and parotid glands are separated, cut the LG out using scissors. Place glands into a 35 mm dish with 2 mL of cold PBS (keep on ice) (**Figure 1B**).

3.5. As the LG is covered by a connective tissue capsule/envelope, trim any surrounding fat and connective tissue under a dissecting microscope and remove the LG capsule with two forceps.

3.5.1. Repeat this step for all glands.

3.6. Check a small piece of tissue under fluorescent microscope to ensure cell labeling (**Figure 1C**).

4. **Preparation of LG single-cell suspension**

4.1. Transfer all LGs into a 35 mm dish with 0.5 mL of room temperature (RT) digestion media and mince LGs using small scissors into very small pieces (approximately 0.2-1 μm^2). Normally, it takes about 3 min to mince 4 LGs (**Figure 2C**).

4.2. Transfer minced tissue into a 2 mL round bottom tube using a wide-bore pipette filter tip. Use a normal sized pipette tip with the tip cut off (**Figure 2D**).

4.3. Add up to 2 mL digestion medium and mix by inverting the tube.

4.4. Place tube in a shaking incubator (or shaking water bath), at 37 °C, 100-120 rpm for 90 min.

4.5. Every 30 min slowly pipette gland pieces 20-30 times using a 1,000 µL filter tip with the decreasing bore size (**Figure 2D**). After incubation/trituration, take a 10 µL aliquot and inspect under a microscope for clusters. If clusters persist, continue digestion.

4.6. After 90 min, pass the sample 2-3 times through an insulin syringe needle (31G) to further release cells into suspension.

NOTE: No visible lacrimal gland pieces should remain in the solution once digestion is completed (**Figure 1D**).

4.7. Transfer cell suspension to a 15 mL tube and add blocking media type I to a total of 5 mL. Invert the tube 2-3 times to mix.

4.8. Pass the cell suspension through a 70 µm cell strainer placed on a 50 mL tube. Wash the strainer with 1 mL of blocking media type I. Repeat step 4.8 again.

4.9. Centrifuge samples at 1,242 x *g* for 5 min at RT.

4.10. Aspirate the supernatant. Re-suspend cells in 2 mL of blocking medium type II using a 1 mL pipette tip and transfer the cell suspension into a 2 mL microcentrifuge tube.

4.11. Centrifuge the cells at 0.4 x *g* (24 x 1.5/2.0 mL rotor; see **Table of Materials**) for 3 min at RT.

4.12. Aspirate supernatant and re-suspend cells in 1 mL of cell detachment solution (see **Table of Materials**).

NOTE: Here, the cell detachment solution is Accutase, a marine-origin enzyme with proteolytic and collagenolytic activity that detaches/dissociates cells for analysis of cell surface markers.

4.13. Incubate cells at 37 °C, at 100-120 rpm for 2-3 min. Over-digestion with cell detachment solution may damage cellular membranes.

4.14. Transfer cell suspension into a 50 mL tube and add up 10 mL of blocking medium type I. Centrifuge tube at 0.4 x *g* (24 x 1.5/2.0 mL rotor; see **Table of Materials**) for 5 min.

4.15. Discard supernatant and re-suspend cells in 6 mL of recovery media and incubate cells for 30 min at RT.

4.16. Check 10 µL of cell suspension under the microscope to ensure complete cell dissociation (**Figure 4**).

4.17. Count cells using a cell counter and Trypan blue. Normally, we expect 4 x 10^5-6 x 10^6 cells from four LGs (one sample).

4.18. Centrifuge cells at 0.4 x *g* (24 x 1.5/2.0 mL rotor; see **Table of Materials**) for 3 min at RT and proceed to antibody staining.

5. Antibody staining

5.1. Add up to 5 x10^5 cells to a 2 ml tube containing 400 µL of FACS buffer. Add 5 µL of Brilliant Violet 421 anti-mouse CD326 (EpCAM) and 0.5 µL of Ghost Red 780 (Viability Dye).

5.2. In parallel, prepare controls to adjust FACS compensation*:*
Negative control-1 (cells from wild type mouse)
Background control unstained cells from *SMA$^{CreErt2/+}$:Rosa26-TdTomato$^{fl/fl}$*
Cy7-780 stained cells (cells from the wild type mouse stained with the Ghost Red 780 Viability Dye)
EpCAM-Brilliant Violet 421 (cells from wild type mouse stained with the EpCAM-Brilliant Violet 421 antibody).

NOTE: For each control sample use a minimum of 1 x 10^5 cells per 400 µL of FACS buffer.

5.3. After adding each reagent mix cells thoroughly by pipetting.

5.4. Wrap tube(s) with foil and rotate tubes for 45 min at 4 °C.

5.5. Centrifuge samples at 0.4 x *g* (24 x 1.5/2.0 mL rotor; see **Table of Materials**) for 3 min at 4°C.

5.6. Re-suspend cells in 1 mL of FACS buffer. It is important to wash cells to decrease the background during compensation.

6. **Fluorescence activated cell sorting**

6.1. Transfer cell suspension into 5 mL FACS tubes and proceed with FACS analysis. Keep cells on ice.

6.2. Adjust compensation using single color controls.

6.3. Sort cells at 20 psi through a 100 µm nozzle using appropriate flow cytometer (see **Table of Materials**). Gating strategy18 is shown in **Figure 1E** and **Figure 3**.

6.4. Collect sorted cells into medium, RNA-later, FACS or lysis buffers depending on downstream procedures (**Figure 1E,F**).

REPRESENTATIVE RESULTS:

Mouse model to isolate SMA+ MECs and pericytes

The established protocol allows for the isolation of two pure populations: MECs and pericytes from LGs and SMGs (see **Table 1**). These two types of cells have a different size and appearance. Microvascular pericytes, develop around the walls of capillaries (**Figure 5A**) and have a squared shape (**Figure 5B**), while MECs surround the LG secretory acini, have long processes and occupy a relatively large area (**Figure 5A,B**). The described procedure is based on genetic cell labeling of SMA+ in the TM-inducible *SMA$^{CreErt2/+}$:Rosa26-TdTomato$^{fl/fl}$* mouse strain. Additonally, EpCAM antibodies allow the researchers to distinguish epithelial SMA^{+}:EpCAM^{+} cells of ectodermal origin (MECs) and SMA^{+}EpCAM^{-} cells of endodermal origin (pericytes).

Preparation of single-cell suspension

The LG contains a filamentous extracellular matrix that must be digested thoroughly. The provided protocol allows preparation of a single cell solution for FACS analysis and further applications. The example of dissociated cells is shown in **Figure 4**.

MEC and pericyte isolation by FACS

To distinguish MECs from pericytes, single cells were stained with antibody to EpCAM, which detects only epithelial cells. The main population of cells was determined by forward and side scatter area gating (**Figure 3A**). Doublets were excluded by plotting the forward scatter area versus width and with the side scatter area versus width (**Figure 3B**). Dead cell exclusion was done via Ghost Red 780 (Viability Dye) (**Figure 3C**). An unlabeled control (**Figure 3E**), background control and antibody control labeled with a single primary antibody were used to determine the background noise (nonspecific antibody binding) and to establish proper compensation for optimum separation between signals (**Figure 3D**). Data analyses were performed by using FlowJo software.

MECs and pericytes were gated by DsRed labeling. DsRed$^+$ dim (not shown) and DsRed$^+$ bright cells within both MEC and pericyte cell populations were detected (**Figure 4D**). The brightness of labeled cells may depend on level of SMA expression or degree of reporter activation upon TM injection[19]. Only DS Red$^+$ bright cell population were collected since only fully differentiated cells were required. The DS Red$^+$ dim cell populations require further investigation.

Downstream applications

It is well known that MECs play an important contractile function in exocrine glands. Moreover, they are very plastic cells and have features of stem cells. Therefore, isolated MECs could be used in multiple applications. For example, cells can be cultured, used for RNA isolation or transplantation (**Figure 1F**)[12,20-22].

Table 1. Modifications of the protocol for isolation of cells from the submandibular gland (SMG). The table describes major modifications required to isolate MECs and pericytes from murine SMG in comparison with the procedure for murine LG.

Figure 1. A schematic representation of the experiment. (**A**) IP injections of the *SMA$^{CreErt2/+}$:Rosa26-TdTomato$^{fl/fl}$*mice with TM. (**B**) Isolation and mincing of the LG or SMG. (C) Analysis of cell labeling using fluorescence microscope. (**D**) Multi-step enzymatic digestion to prepare a single-cell solution. It is critical to check digestion steps under a light microscope to ensure that cells are released from clusters. (**E**) Example of gating showing SMA+ bright DS Red+/EpCAM+ (MECs) and DS Red+bright/EpCAM- (pericytes). (**F**) Collected MECs and pericytes could be subjected to different downstream procedures including cell cultivation, RNA isolation and gene expression analysis and cell transplantation.

Figure 2. Critical steps of LG isolation/mincing. (**A**) Removal of skin between the eye and ear to dissect LG. Dashed yellow circle indicates LG location. (**B**) LG dissection. Yellow arrowhead points out area between lacrimal and parotid glands. (**C**) LG mincing in digestion medium using scissors with curved, blunt ends. (**D**) Transfer of minced tissue into 2 mL tube. Yellow arrowhead depicts wide bore size 1 ml tip required for transferring.

Figure 3. Identification of murine LG MECs and pericytes using FACS. (**A**) Determination of the main population of LG cells by forward and side scatter area gating. (**B**) Exclusion of doublets via forward scatter area versus width. (**C**) Dead cell exclusion via Ghost Red 780 (Viability Dye). (**D**) MEC (SMA$^+$ bright/EpCAM$^+$) and pericyte (SMA$^+$ bright/EpCAM$^-$) populations distinguished based on staining with EpCAM antibody. (**E**) Unlabeled control (cells from wild type mouse). On each plot the % of gated cells is provided.

Figure 4. Confocal and differential interference contrast (DIC) images illustrating dissociated single cells from murine LG. (**A-C**) single cells isolated from two LGs of one 4 month old *SMA$^{CreErt2/+}$:Rosa26-TdTomato$^{fl/fl}$* mouse. Nuclei are stained with DAPI (blue). White arrowheads denote SMA+ (DS Red) cells: MECs or pericytes. Scale bar is 15 µm.

Figure 5. Confocal images showing difference in distribution and shape between MECs and pericytes isolated from murine LG. (**A**) Whole mount preparation of LG with labeled cells showing difference in distribution between MEC and pericytes. (**B**) The shape of MEC and pericyte is different. Pericyte is relatively small and has squired shape, whereas MEC is large and has irregular shape and several long processes. Arrowheads: MECs and pericytes.

DISCUSSION:

This manuscript described a protocol of MEC and pericyte isolation from LG and SMG. This procedure was based on genetic labeling of SMA, the only reliable biomarker of MECs and pericytes.

The urgency to develop this protocol was motivated by the almost total absence of literature highlighting the isolation of MECs from murine LGs and SMGs. Although genetic labeling was previously used, using SMA-GFP mice to isolate SMA+ cells from young three-week-old LGs[12], it did not allow the use these older mice for cell isolation due to partial loss of signal in adult mice. In addition, GFP-labeled cells give a relatively high background in FACS applications[23] and require additional compensations. In contrast, *the SMA$^{CreErt2/+}$:Rosa26-TdTomato$^{fl/fl}$* mouse line shows no or a low background and high levels of SMA labeling activation throughout mouse postnatal development, adulthood and aging. Usage of the *SMA$^{CreErt2/+}$:Rosa26-TdTomato$^{fl/fl}$* mouse is especially important for studies focused on disease progression or aging, since SMA+ cells in these mice could be labeled prior to disease development and studied later when disease/aging progresses. Another critical step of the protocol is thorough LG mincing and the following digestion. This step may reduce the cell number obtained during FACS analysis. Overall, isolation and immediate analysis of primary cells is also important due to significant changes in the transcriptional profiles of cells maintained in culture[24].

Additionally, the described protocol for MEC and pericyte isolation from murine LGs enables different downstream procedures. Protein, RNA and DNA extractions are possible from both single-cell populations, although several mice/samples need to be processed in parallel or sequentially to increase the number of cells. Taken together, the obtained results demonstrate an effective and relatively straightforward way for MEC and pericyte isolation from different murine glandular tissue.

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DISCLOSURES:

The authors declare no competing financial interests and no conflicts of any other interests.

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Figure

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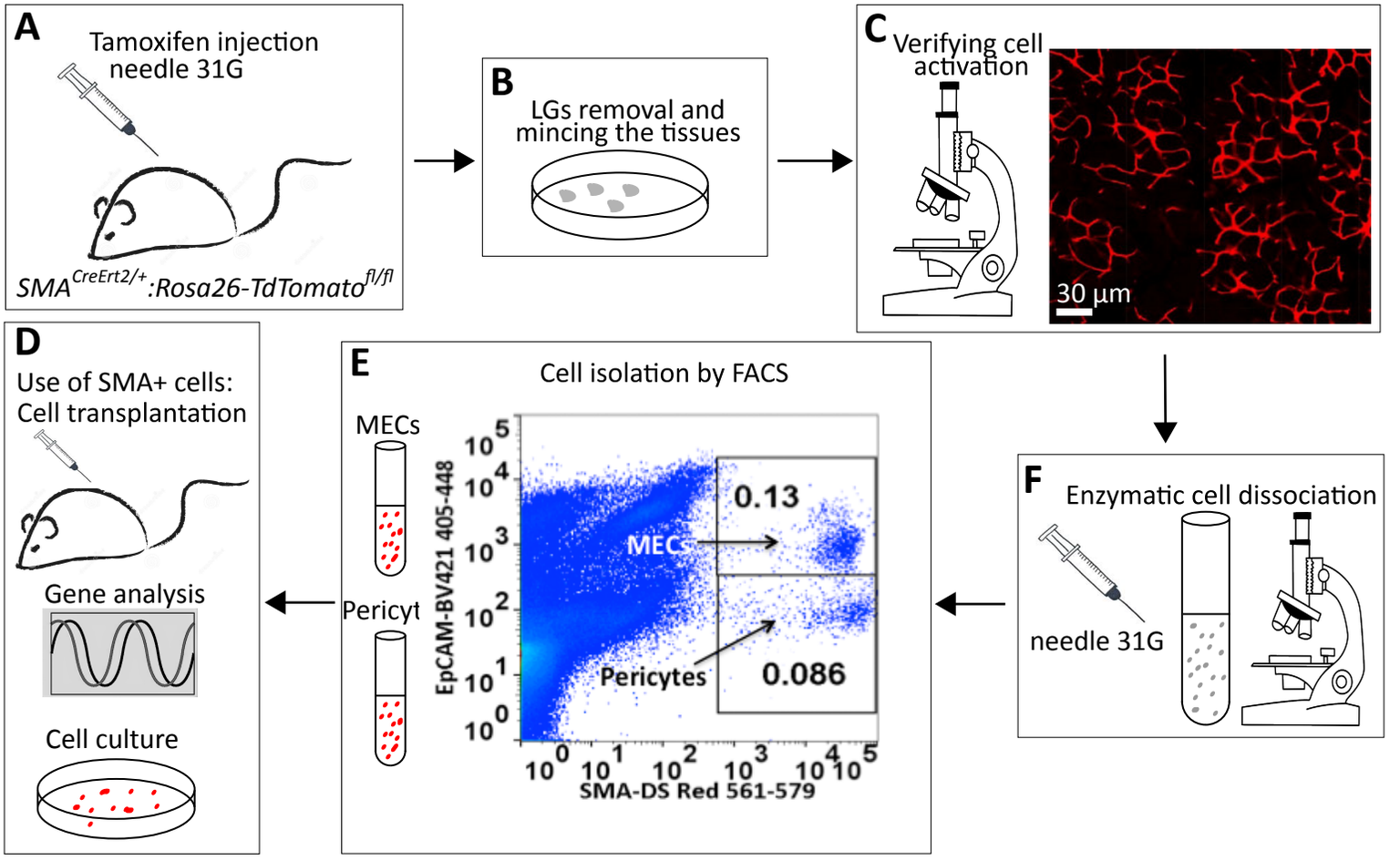

Figure

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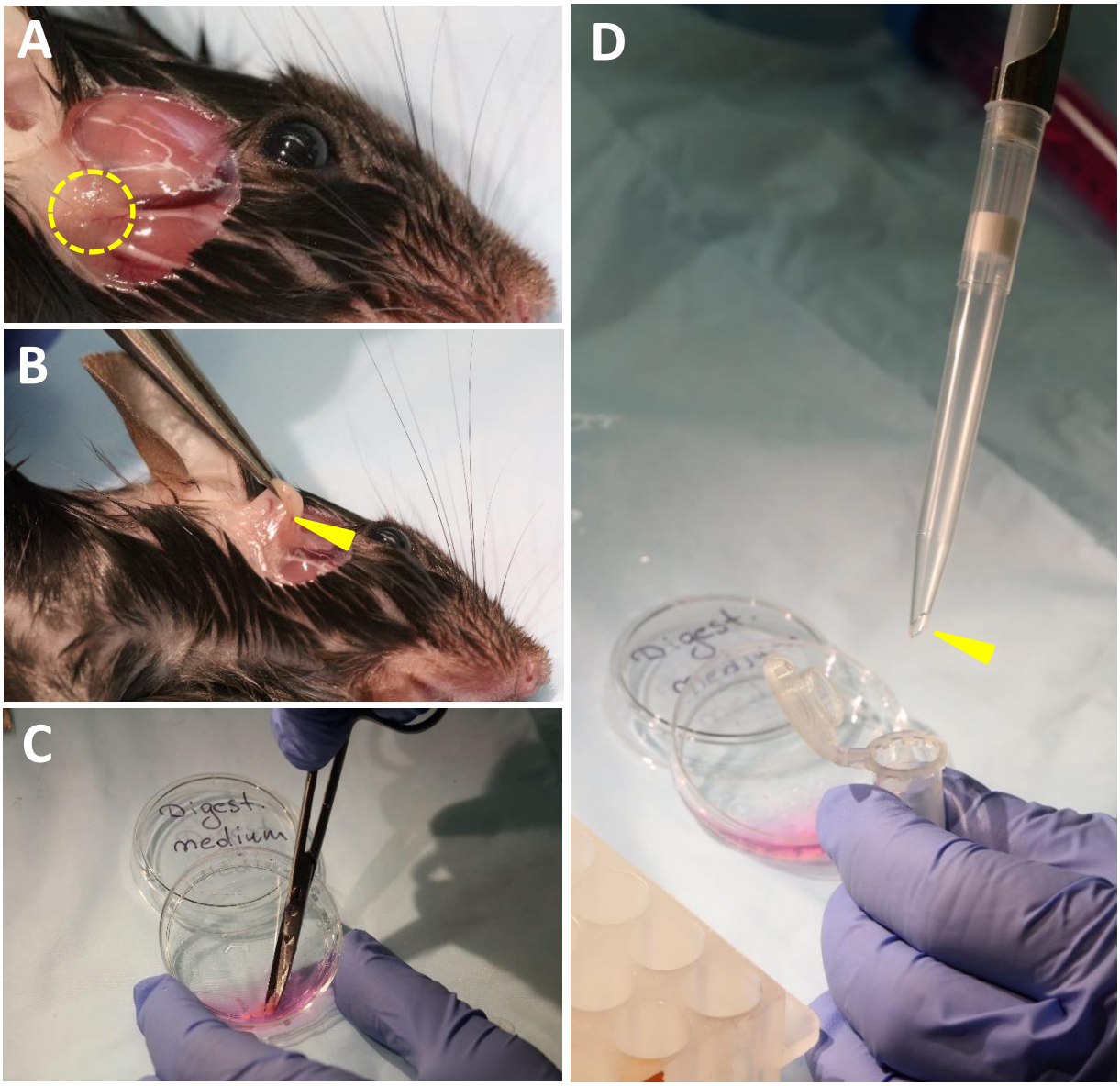

Figure

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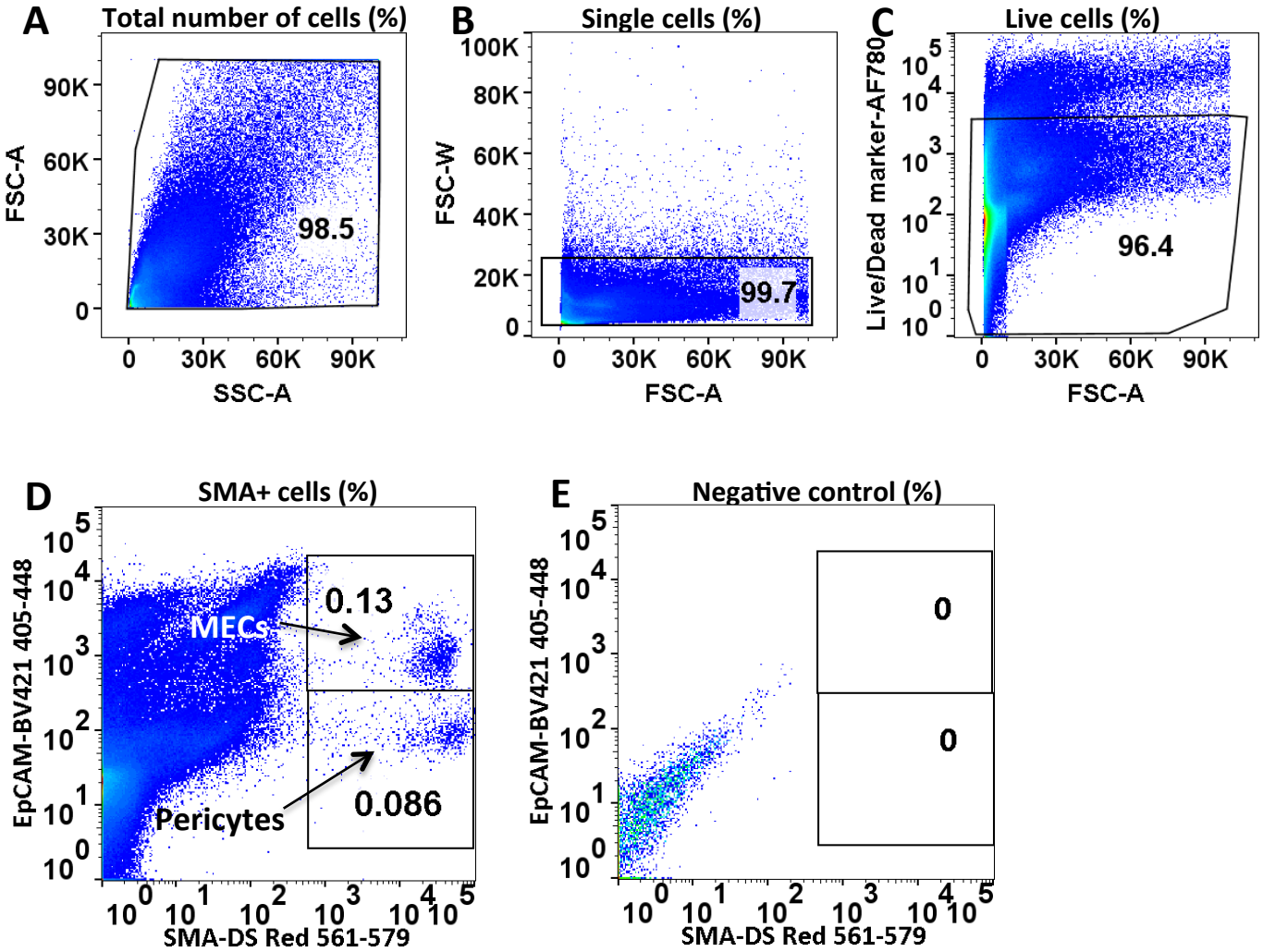

Figure

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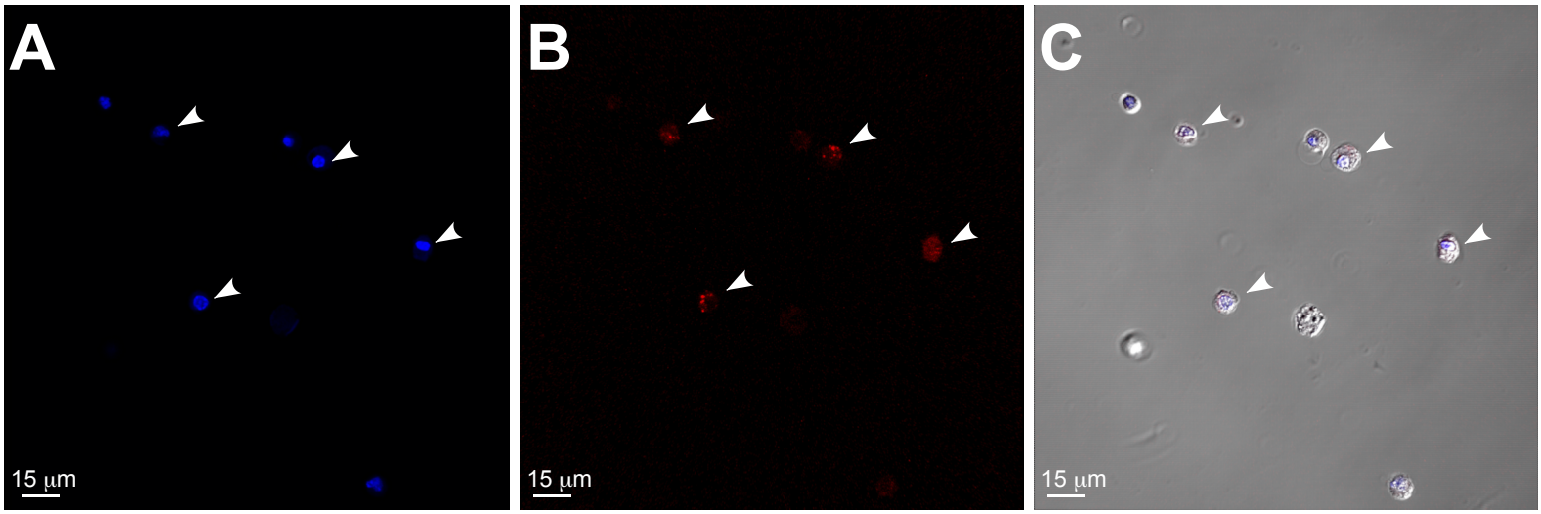

Figure

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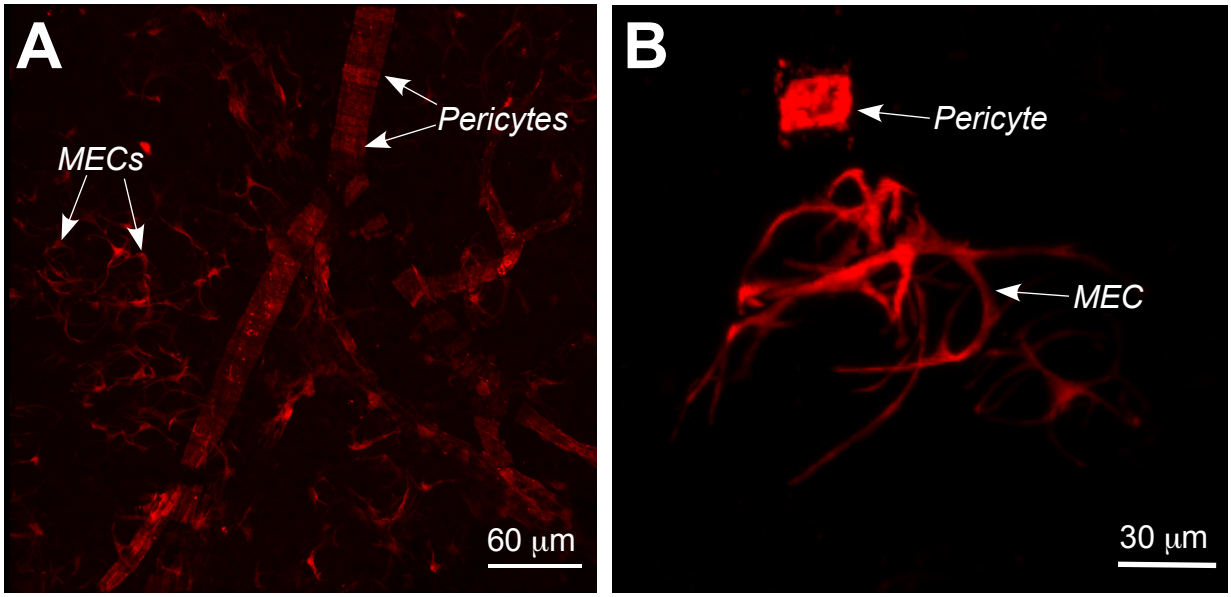

Parameter
Number of mice per sample
Number of glands per sample
Dissection glands
Concentration of collagenase per sample
Approximate cell number after enzymatic dissociation
Recovery step (see the section “Adult Mouse Lacrimal Gland Single-cell Dissociation”, point 15)
Volume of FACS buffer during antibody staining

Lacrimal gland	**Submandibular gland**
2	1
4	2
Separate from parotid gland	Separate from sublingual gland
6 mg/2 mL	9 mg/2 mL
$4x10^5$-$6x10^5$	$9x10^5$-$1.5x10^6$
Re-suspend cells in 6 ml of recovery media	Re-suspend cells in 12 mL of recovery media
400 µL	2 tubes by 400 µL; it is better to split the cells into two or three tubes that each tube has not more than $6x10^5$

Table of Materials

Click here to access/download;Table of Materials;table_JoVE_Materials 021319 corrected.xlsx

Name of Material	Company
Biosafety Cabinet	SterilCard Baker
10 ml Disposable serological pipets	VWR
10 mL Disposable serological pipets	VWR
15 mL High-clarity polypropylene conical tubes	Falcon
25 mL Disposable serological pipets	VWR
5 mL FACS round-bottom tubes	Fisher Scientific, Falcon
50 mL High-clarity polypropylene conical tubes	Falcon
Antibiotic-antimycotic	Invitrogen
Appropriate filter and non-filter tips	Any available
BD Insulin Syringes	Becton Dickinson
BD Syringes 10 mL	Becton Dickinson
Brilliant Violet 421 anti mouse CD326 (EpCAM)	Biolegend
$CaCl_2$ 1M solution	BioVision
Cell culture dishes 35 mm	Corning
Collagenase Type I	Wortington
Corn oil	Any avaliable
Corning cell strainer size 70 µm	Sigma-Aldrich
Digital Stirrer PC-410D	Corning
Dispase II	Sigma-Aldrich
Dissecting scissors, curved blunt	McKesson Argent
DNase I	Akron Biotech, catalog number
Dulbecco's Modified Eagle's Medium – low glucose (DMEM)	Sigma-Aldrich
Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12)	Millipore
Easypet 3 pipette controller	Eppendorf
Ethanol	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Fisher Vortex Genie 2	Fisher Scientific
FlowJo version 10	Any available
Fluorescence binocular microscope Axioplan2	Carl Zeiss

Ghost Red 780 Viability Dye	Tonbo Biosciences
GlutaMAX Supplement	ThermoFisher Scientific, Gibco
Glycerol 99%	Sigma-Aldrich
Hand tally counter	Heathrow Scientific
Hank's Balanced Salt Solution (HBSS)	Sigma Millipore
Hank's Balanced Salt Solution (HBSS)	ThermoFisher Scientific
Hausser Bright-Line Phase Hemocytometer	Fisher Scientific
HEPES 1M solution	ThermoFisher Scientific, Gibco
HyClone Fetal Bovine Serum (FBS)	Fisher Scientific
Hydrochloric Acid (HCl), 5N Volumetric Solution	JT Baker
Innova 4230 Refrigerated Benchtop Incubator	New Brunswick Scientific
Iris scissors	Aurora Surgical
Isoflurane Inhalation Anesthetic	Southern Anesthesia Surgical (SAS)
$MgCl_2$ 1M solution	Sigma-Aldrich
Microcentrifuge tubes 1.5 mL	ThermoFisher Scientific
Microsoft Power Point	Any available
NaCl powder	Sigma-Aldrich
Nalgene 25 mm Syringe Filters	Fisher Scientific
Pen Strep	Gibco
pH 510 series Benchtop Meter	Oakton
Phosphate buffered saline (PBS)	ThermoFisher Scientific
Pure Ethanol 200 Proof	Pharmco-Aaper
Red blood cell lysis buffer 10x	BioVision
Roto-torque Heavy Duty Rotator	Cole Parmer
Safe-lock round bottom Eppendorf tubes 2 mL	Eppendorf Biopur
Scissors	Office Depot
Sorting flow cytometer MoFlo Astrios EQ	Beckman Coulter
Sorvall Legend Micro 17R Microcentrifuge	Thermo Scientific
Sorvall RT7 Plus Benchtop Refrigerated Centrifuge	Thermo Scientific
Stemi SV6 stereo dissecting microscope	Carl Zeiss
Tamoxifen	Millipore Sigma

Trizma base powder	Sigma-Aldrich
Trypan blue solution	Millipore Sigma
Two Dumont tweezers #5	World Precision Instruments
Upright microscope	Any available
Vacuum filtration systems, standard line	VWR
Variable volume micropipettes	Any available

Catalog Number	Comments/ Description
19669.1	Class II type A/B3
89130-910	Manufactured from polystyrene and are supplied sterile
89130-908	Manufactured from polystyrene and are supplied sterile
352196	
89130-900	Manufactured from polystyrene and are supplied sterile
14-959-11A	
352070	
15240-062	
Any available	
328468	with BD Ultra-Fine needle ½ mL 8 mm 31G
309604	Sterile
118225	Monoclonal Antibody (G8.8)
B1010	sterile
430165	Non-pyrogenic, sterile
LS004194	
Any avaliable	From grocery store
CLS431751-50EA	
Item# UX-84302-50	
D4693-1G	
487350	Metzenbaum 5-1/2 Inch surgical grade stainless steel non-sterile finger ring handle
AK37778-0050	
D5546-500ML	with 1000mg/L glucose and sodium bicarbonate, without L-glutamine
DF-042-B	without HEPES, L-glutamine
4430000018	with 2 membrane filters 0.45 µm, 0.1 – 100 mL
E7023-500ML	
E6758	
12-812	
Any available	
ID# 094207	

13-0865-T100	
35050061	
G-5516	
HEA6594	
H6648-500ML	Modified, with sodium bicarbonate, without calcium chloride, magnesium sulphate, phenol red.
14025092	With calcium, magnesium, no phenol red.
02-671-51B	02-671-51B
15630-080	Dilute 1/10 in ddH20
SH3007002E	
5618-03	To adjust Tris buffer pH
SKU#:	Shaker; 37 °C, 5% CO_2 in air
AS12-021	Pointed tips, delicate, curved, 9 cm, ring handle
PIR001325-EA	
63069-100ML	
3451	Clear, graduated, sterile
Any available	
S-3014	
724-2020	
15140-122	
SKU: BZA630092	
10010023	pH 7.4
111000200	
5831-100	
MPN: 7637-01	
22600044	PCR inhibitor, pyrogen and RNAse-free
375667	
B25982	With Summit 6.3 software
75002441	All centrifugation performed at RT
ID# 21550	RTH-750 Rotor. All centrifugation performed at RT
455054SV6	With transmitted light base
T5648-1G	

T1503	
T8154	
500342	11 cm, Straight, 0.1 x 0.06 mm tips
Any available	With transmitted light base
10040-436	
Any available	

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Author(s): Tatiana Zgrianova, Liana Basova, Helen P. Makarenkova

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We are grateful to the editor and all reviewers for their constructive comments. We have addressed each comment and made significant changes in the manuscript.

Editorial comments:
Changes to be made by the author(s):
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.
Grammar and typos have been corrected

2. Please revise lines 43-44, 60-62, 260-262, 264-266 to avoid previously published text.
This issue has been addressed

3. Keywords: Please provide at least 6 keywords or phrases.
Keywords have been added.

4. Please abbreviate liters to L to avoid confusion.
Corrected.
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Commercial language has been removed

6. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
The ethic statement has been included.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.
Corrected.
8. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Corrected.

9. Please revise the protocol to contain only action items that direct the reader to do something (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." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly, and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.
Corrected.

10. 1.1: Please specify the type of filter that is used to filter the corn oil.
The filter has been specified

11. Line 104: Please specify the type, age and gender of mice.
The gender of mice hsas been specified.

12. Line 166: Please specify the concentration of isoflurane and mention how proper anesthetization is confirmed.
It has been done.

13. Line 180: Please define the size of very small pieces.
Specified.

14. Line 229: Please use centrifugal force (x g) for centrifuge speeds.
Corrected.

15. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.
Corrected.

16. After you have made all the recommended changes to your protocol (listed above), please highlight 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. Please do not highlight sections other than Protocol.
Done

17. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.
Done.

18. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
This has been done.

19. Figure 1C: Please include a space between the number and its unit of the scale bar (30 µm).
Corrected.

20. Figure 2: Please describe panel A in the figure legend.
Corrected.

21. Figure 4: The uploaded Figure 4 only has three panels while its figure legend indicates six panels. Please revise to be consistent.

The figure legend and figure have been corrected.

22. Figures 4 and 5: Please revise the unit of the scale bars so that letters do not overlap.
Corrected

23. Table 2: Please abbreviate liters to L to avoid confusion.
Corrected.

24. Table of Materials: Please do not number the Table of Materials. Please sort the items in alphabetical order according to the name of material/equipment.
Corrected.

25. Discussion: Please discuss critical steps and any limitations of the technique.
Important steps and limitations have been highlighted.

26. References: Please do not abbreviate journal titles.
This Endnote bibliography template was provided by JOVE.

Reviewers' comments:

Reviewer #1:
In this manuscript, the authors describe a detailed protocol for isolating myoepithelial cells and pericytes from lacrimal and submandibular glands. They used aSMA-CreErt2; Rosa-tdTomato mouse line to genetically label both of these cell populations and further used antibody against Epcam to separate myoepithelial cells from pericytes using FAC sorting. This is a useful protocol for not only FAC sort SMA-positive cells in these glands but also, can used as a guideline to harvest single cells from other organs. I have suggestions for minor corrections which are outlined below.
We thank the reviewer for her/his kind comments. We have taken the reviewer's concerns seriously and addressed them in the revised manuscript.

Minor Concerns:

1) Figure 4: Panel is not complete or there may be typo in numbering. Also labeling of the images will be helpful.
Corrected.

2) In Figure 5 A, expression of SMA in the blood vessel seems to be present all over the vasculature. If it is because of the high exposure during imaging, then background could be reduced using 30 sec TrueBlack staining or reducing the exposure time for a clearer image.
The image in Figure 5A is not a staining, all red cells are genetically labeled SMA+ cells. In the lacrimal gland pericytes present only over the thicker vasculature. This image was specifically taken at the place with larger number of thicker blood vessels to visualize pericytes.

3) Table2 -For SMG cell isolation, does the concentration of Ab for staining remain same with the increase in volume of the Facs buffer? Does the cell concentration also kept same for staining? Description for the differences in the protocol for SMG can be more elaborate with possible explanation for the need to modify.
Table 2 is now table 1. We mentioned that up to 5 x10^5 cells should be diluted in 400 µL of FACS buffer. If the number of isolated SMG cells is higher, we recommend splitting cells equally into two tubes with a 400 µL of FACS buffer in each tube. The concentration of antibodies for SMG cells in the same volume of FACS buffer remains the same. We added this information to Table 1.

4) Table 2- recovery step reference point number has typo.
Table 2 is now Table 1. Corrected.

5) Also, it is important to provide Flowjo analysis for quantification of these populations which are sorted. It will be an important piece of information to proceed for any downstream application. If possible, an estimate % of live cells being sorted per lacrimal gland/SMG will be helpful to determine the efficiency of this protocol.
This analysis has been shown in Figure 3. In each plot there is an estimated % of cell. We showed that the number of live cells is 96.4%. We updated the legend for Figure 3 showing the % of gated cells for each plot.

Reviewer #2:
Manuscript Summary:
A well written article with a detailed but briefly protocol about isolation of MEC of adult murine lacrimal glands.
Thank you for this comment!

Major Concerns:
no major concerns

Minor Concerns:

in the title and the abstract you wrote about submandibular gland isolation but in this work you just uses adult lacrimal glands. I would recommend deleting submandibular glands from the title and the manuscript completely even if the protocol is working with these glands too.
We would like to keep submandibular gland in the protocol since this kind of the protocol would be useful for scientists who study submandibular gland.

Just point out briefly why it is of interest to isolate epithelial cells of glands, in my opinion "downstream procedures" is a little bit to superficial or inaccurate, please quote actual work.
We discuss it in a new version of the manuscript.

Page 4 107-110 I don't get the point, please rewrite.
Done

Page 5 165 - Page 7 240 why is the text underlaid with Yellow?
The editors require highlighting text that is going to be included into video demonstration.

Page 6 187 what is trituration?
We replaced it with pipetting

Page 8 283 - 303 Yellow underlaid text see above.
Addressed.

Figure 2 A maybe you should use a higher magnification because i can see nothing within the yellow circle.
Done.

Figure 3 legend it should be SMA+ bright/EpCAM negative for pericytes or? you wrote EpCam+
Corrected.

Figure 4 You wrote about 5 images A-F but i can see only 3 images A-C, please submit all the images mentioned in the text or correct the text of the legend of figure 4
Corrected

Reviewer #3:
Manuscript Summary:
The manuscript details a method for the separation of lacrimal gland (LG) myoepithelial cells (MEC) from pericytes. The methods are very well described and could be useful for other investigators in the field. However, since most of the data and visual descriptions are centered on the LG, the authors are encouraged to remove the submandibular glands (SMG) from the title and simply state that the protocol can be adapted for isolating MEC and pericytes from the SMG as described in Table 1.
Thank you!

Below are a few comments/corrections for the authors to address.
-Lane 77, reference 12 is not the right citation.
The reference is correct. In this publication, we isolated and cultured MECs from the SMA-GFP transgenic mice PBMID 29967327.

-Lanes 84 and 85, remove "the" before Figure 1A-F and before reagents.
Corrected.
-Lane 90, list city and state for the Jackson Laboratory.
Corrected.

-Lane 99, add TM after makes a 20mg/ml…
Corrected
-Lane 104, add body before weight (i.e., 2mg/20g body weight).
Corrected.

-Lane 109, should read: the same age would be required.
Corrected.

-Lanes 120 and 128, please explain why the units per milligram may vary. Is it from batch to batch?
It varies from batch to batch.

-Lane 126, Glycerol should be a lower-case g.
Corrected.
-Lane 143, can the authors provide a mean for adjusting digestion volumes based on number of glands.
Provided in the note.

-Lane 150, mediums should be media.
Corrected.

-Lane 161, can the authors explain the use of goat serum.
We used goat serum because it is cheaper. “Goat” is removed. Any serum can be used.

-Lane 166, the method of anesthesia and euthanasia should be left to be done according to institutional IACUCs recommendations.
Added.

-Lane 179, is the digestion media pre-warmed or is it at room temperature? Please pick one.
Corrected: Room temperature.

-Lane 181, please specify if the 3-minute mincing is for 1 LG.
Corrected: Normally, it takes about 3 min to mince 4 LGs.

-Lane 182, filter tip should probably read pipette tip.
Corrected: wide-bore pipette filter tip.

-Lane 184, please list the precise volume of digestion media instead of up to 2ml.
We added 2 ml of digesting media

-Lane 195, Repeat the entire step: please define the step number.
Corrected.

-Lane 248, in addition to surrounding LG secretory acini, MECs can also be found associated with ducts.
It is correct.

-Lane 283, the protocol should read a protocol.
Corrected.

-Lane 361, since the words pericyte and MEC are listed next to the arrows in the Figure 2, there is no need to add this to the legend.
Corrected