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Preparation of cells from embryonic organs for single cell RNA sequencing

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Abstract

While scRNA-seq has become one of the most powerful transcriptome analysis methods, the quality of scRNA-seq data largely depends on cell preparation. Cell preparation for cultured cells and tissues requires different methods due to their inherent differences. Compared to cultured cells, tissues have more extracellular matrix and the cells are generally more adherent and thus difficult to dissociate. A challenge here is to achieve sufficient dissociation, cell counts, and viability all at the same time. This protocol will describe approaches that help achieve these goals. These include a cold dissociation technique using cryophilic proteases active at cold temperature, timing of trituration during protease digestion, as well as filtration and washing methods that optimize cell viability and retention. Materials and equipment that optimize the process will also be discussed.

Keywords

scRNA-seq; embryonic organ; embryonic tissue; cold dissociation; cryophilic protease

INTRODUCTION

RNA-seq uses deep-sequencing technologies that allow precise measurements of transcriptome levels in biological samples (Wang, Gerstein et al. 2009). Compared to bulk transcriptomes, which produce averaged datasets, a major benefit of single cell transcriptomes is the ability to resolve cell-to-cell variations (Wang and Navin 2015). Through high-resolution analysis of transcripts expressed by individual cells, scRNA-seq has allowed identification of both known and novel cell types in developing organs such as mammary gland (Bach, Pensa et al. 2017, Pal, Chen et al. 2017), pancreas (Stanescu, Yu et al. 2017), kidney (Brunskill, Park et al. 2014), lung (Treutlein, Brownfield et al. 2014), hair

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follicle (Yang, Adam et al. 2017), and other organs (Dong, Hu et al. 2018). Using scRNA-seq data, cell type-specific expression of key transcripts (e.g., cell fate determinants and causative genes for disorders) can also be discovered, which would help unravel developmental and disease processes that rely on intercellular interactions (Brucher and Jamall 2014, Montoro, Haber et al. 2018, Sekiguchi and Yamada 2018). Moreover, identification of cell type-specific transcripts could be important in future development of cell type-based targeted therapy (Zhou and Rossi 2014, Banovich, Boateng et al. 2017).

While scRNA-seq has become one of the most powerful transcriptome analysis methods, the quality of scRNA-seq data largely depends on cell preparation. Cell preparation for cultured cells and tissues require different methods due to their inherent differences. Compared to cultured cells, tissues often contain a limited number of cells, making it difficult to obtain a sufficient final concentration for successful capture. In addition, complete dissociation of all cell types in a given tissue may be difficult because cells in tissues can be surrounded by exogenous extracellular matrix and are generally more adherent to each other or because certain cell populations may be more adherent than others. A challenge here is to achieve both high yields and viability. One of the key determinants in preserving RNA integrity during dissociation is temperature. While a common dissociation protocol involves incubation with proteases at 37°C for at least 15 minutes, this induces major transcriptome changes (Adam, Potter et al. 2017). In contrast, gene expression changes induced by protease digestion are minimal when cells are dissociated with cryophilic proteases at 6°C for 15 minutes (Adam, Potter et al. 2017).

This protocol has been developed to optimize the cold dissociation method for preparation of embryonic organs for scRNA-seq. The protocol was optimized for limited samples (e.g., tiny embryonic tissues) to maximize cell recovery. The *Basic Protocol* describes in detail the steps of sample dissociation, filtration, washing, and cell counting. More specifically, these include a cold dissociation technique using cryophilic proteases active at cold temperature, timing of trituration during protease digestion, as well as filtration and washing methods that optimize cell viability and retention. Materials and equipment that are best suited for each process will also be discussed. The *Alternate Protocol* describes another cell preparation method that uses papain to dissociate cells from whole glands.

BASIC PROTOCOL

Preparation of cells from embryonic organs for scRNA-seq

Introductory paragraph—The goals of this protocol are to achieve a sufficient concentration of dissociated cells (~1,000 cells/μL for the 10x Genomics platform) while maintaining high viability (>90%). The protocol has been tested to work with embryonic days 11.5–14.5 salivary or lacrimal glands. Embryonic day 12 salivary glands from time-mated Hsd:ICR mice (Envigo) are used to illustrate this protocol. The protocol describes the preparation process of two samples: epithelial and mesenchymal cells. The rationale behind this separate preparation of two samples as opposed to the whole organ is to increase the resolution in gene profiling of the epithelial sample. In early embryonic organs, the mesenchyme is disproportionately large in relation to its epithelial counterpart. Separate sample preparation ensures capturing enough epithelial cells for high-quality data.

Materials

Reagents and Solutions: Dulbecco's Modified Eagle Medium (DMEM)/F12 (ThermoFisher, 11039021): 10 mL in 15 mL tube

DMEM/F12 supplemented with 5% bovine serum albumin (BSA) (BSA from Sigma-Aldrich, A8577): 1 mL in 1.5 mL tube

Dispase II (1.6 U/mL, prepared in DMEM/F12) (ThermoFisher, 17105041): 40 μ L in 1.5 mL tube

Protease mix (100 μ L in 1.5 mL tube), each containing:

- 45 μ L of Accutase (Stem Cell Technologies, 07920)
- 45 μ L of Accumax (Stem Cell Technologies, 07921)
- 1 mg of Bacillus Licheniformis protease (Creative Enzymes, NATE0633) in 10 μ L DPBS (no calcium/magnesium)

Hank's Balanced Salt Solution (HBSS), no calcium/magnesium (e.g., ThermoFisher, 14170112): 1 mL in 1.5 mL tube

Dulbecco's Phosphate-Buffered Saline (DPBS), no calcium/magnesium (e.g., ThermoFisher, 14190250), supplemented with 10% filtered fetal bovine serum (FBS): 5 mL in 15 mL tube

Filter FBS using a vacuum-driven filtration system (e.g., Millipore, SCGP00525), store 1-mL aliquots at -20°C , and thaw on the day of capture.

DPBS, no calcium/magnesium, supplemented with 1% FBS: 3 mL in 15 mL tube

Sample Material—Time-mated mice at a desired gestational stage.

Other Materials and Equipment: 35 mm dish (e.g., Corning, 353001)

Nuclease-free 15 mL tubes x 2 (Corning, 430053)

Nuclease-free 1.5 mL tubes x 2 (Eppendorf, 022431021)

Low-binding pipette tips, 20 μ L, 200 μ L and 1,000 μ L (e.g., Rainin, 17014351; 17014356; 17014361)

Flowmi cell strainer, 40 μ m x 2 (Bel-Art, H13680-0040)

Dissection microscope

Forceps and tungsten microneedles (e.g., Fine Science Tools, 11252-23; 10130-05)

Humidified 37°C incubator with 5% CO₂

Table-top centrifuge

Cell counter and live/dead cell counting dye

Protocol steps—*Step annotations*

1. Organ isolation and tissue separation. See Figure 1 for a schematic representation.
 1. Before tissue dissection, prepare all reagents and solutions in nuclease-free tubes and put them on ice.
 2. Using forceps and a dissection scope, isolate embryonic organs and collect them on a 35-mm dish containing ~40 μ L DMEM/F12. For detailed dissection methods for embryonic organs, see *Unit 19.8* (Sakai and Onodera 2008).

For embryonic day 12 salivary glands that are about 100–150 μ m in diameter, 10 to 12 glands are sufficient.
 3. Mix 40 μ L of dispase (1.6 U/mL) and 40 μ L of DMEM/F12 in a nuclease-free 1.5 mL tube.

Dispase, which cleaves collagen IV, laminin, and fibronectin at the basement membrane, separates the epithelium from the mesenchyme. To ensure optimal enzyme activity, keep dispase on ice until use, and dilute immediately before use.
 4. Submerge organs in the dispase solution on a 35-mm dish and incubate in a humidified 37°C incubator with 5% CO₂ for 10 minutes.

Dispase preserves tissue cohesion and integrity of the epithelium and most of the mesenchyme (Figure 2). Dispase treatment of glands at 37°C for 10 minutes should have negligible effects on RNA integrity of separated tissues.
 5. Inactivate dispase by adding 80 μ L of cold DMEM/F12 supplemented with 5% BSA to the organs submerged in the dispase solution.
 6. Using a pipette, transfer glands to a new 35 mm dish containing 80 μ L of DMEM/F12 supplemented with 5% BSA.
 7. Mechanically separate the epithelium and the mesenchyme using tungsten microneedles.
 8. Using a pipette, transfer the epithelial and mesenchymal tissues separately to 35-mm dishes, each containing 80 μ L of HBSS.

From here, two samples—epithelial and mesenchymal samples—are prepared separately in different dishes/tubes. HBSS is used to rinse off DMEM/F12 and BSA before cell dissociation. While HBSS can be substituted with DPBS, HBSS is generally better for cell health.
2. Cell dissociation with protease

1. Put the epithelial and mesenchymal tissues in separate 1.5 mL Eppendorf LoBind tubes, each containing 80 μ L of the protease mix.
2. Use a low bind 200 μ L pipette tip to gently pipette up and down for 2 minutes for each sample.

Mixing by pipetting up and down every 4–5 seconds for a total of 2 minutes only at the beginning of digestion (as opposed to 2–3 times throughout the digestion period) reduces cell loss and facilitates protease digestion.

3. Incubate the sample-containing tubes on ice for 15 minutes.

This is a good time to take a 5-minute break. Approximately 5 minutes before the completion of incubation, have cell strainers, 15 mL tubes, and DPBS supplemented with 10% FBS ready.

3. Cell filtration and wash

- (1) With a low bind 1,000 μ L pipette tip, add 920 μ L of DPBS (no calcium/magnesium) supplemented with 10% FBS to each 1.5 mL tube. Gently mix by pipetting up and down 4–5 times.
- (2) After mixing, take all the solution (with the same 1,000 μ L pipette tip used for mixing) and filter through the Flowmi 40- μ m strainer into a Corning 15 mL tube.

Cell suspensions of the two samples are placed into two separate 15 mL tubes. A video on filtering samples with the Flowmi strainer can be watched at: <https://www.belart.com/flowmi-cell-strainers-for-1000-l-pipette-tips.html>

- (4) Add 500 μ L of DPBS (no calcium/magnesium) supplemented with 10% filtered FBS to each 15 mL tube containing cell suspension.

A high concentration (10%) of FBS facilitates inactivation of residual proteases.

- (5) Centrifuge at 112 g for 7 minutes.
- (6) Remove supernatant carefully so as not to disturb cell pellets, leaving ~60–70 μ L. Use the very bottom line that marks 100 μ L on the Corning tube as a guide.
- (7) Add 1 mL of DPBS (no calcium/magnesium) supplemented with 1% filtered FBS to each 15 mL tube.

Up to 10% FBS has been shown to have no downstream deleterious effects by 10x Genomics; however, FBS concentration is reduced here since 1% FBS is enough to maintain cell health of embryonic salivary glands. This media—DPBS supplemented with 1% FBS—serves as a final buffer.

For more details on final buffers, see 10x Genomics cell preparation guide: https://assets.ctfassets.net/an68im79xiti/56DIUZE8VOWc8sSG42KQis/35cbcf6dcd4b0c0196263ee93815b0ae/CG000053_CellPrepGuide_RevC.pdf

- (8) Centrifuge at 112 g for 4 minutes. Carefully remove supernatant, leaving ~40–50 μ L.
- (9) With a 20 μ L pipette, resuspend the cell pellets by gently pipetting up and down several times. Transfer cell suspension to a 1.5 ml Eppendorf LoBind tube.
- (10) Count cells. If the cell concentration is excessively high, dilute with DPBS (no calcium/magnesium) supplemented with 1% filtered FBS and recount cells.

ALTERNATE PROTOCOL

Alternative preparation of cells from embryonic organs for scRNA-seq

Introductory paragraph—This alternate protocol describes single cell dissociation of whole glands using the Worthington papain dissociation system. Embryonic days 12 to 14 submandibular salivary glands from time-mated Hsd:ICR mice have been used to develop this protocol. This alternate protocol is appropriate if the cell type of interest is present in sufficiently high numbers.

Materials

Reagents and Solutions: Sterile Earle's Balanced Salt solutions (EBSS) with calcium, magnesium, bicarbonate and phenol red, 100 mL bottle (Worthington Corporation, LK003150)

Papain containing L-cysteine and EDTA, (vial 1) yields a solution at 20 units of papain per mL in 1 mM L-cysteine with 0.5 mM EDTA

Ovomucoid protease inhibitor with bovine serum albumin. One vial per package, 32 mL upon reconstitution

1. Mix 5mL of papain (20 U/mL) and 5ml of EBSS solution
2. Mix 35mL of EBSS solution with the Ovomucoid protease inhibitor with bovine serum (10 mg) albumin
3. Equilibrate at 37°C for 30 minutes

HBSS, Gibco (14175–095 500 mL)

Bovine serum albumin (Sigma, A8577–50ml)

Sample Material

Timed-pregnant mice at a desired gestational stage

Other Materials and Equipment: 50 mm Glass Bottom dishes (P50G-1.5–14-F)

Nuclease-free 1.5 mL tubes x 1 (Eppendorf, 022431021)

Low-binding pipette tips, 20 μ L, 200 μ L and 1,000 μ L (e.g., Rainin, 17014351; 17014356; 17014361)

pluriSelect pluriStrainer 20 μ m (43–50020-01), 1x

BD Falcon FACS tube , 5ml Polystyrene round-bottom tube (352235)

Dissection microscope

Forceps and tungsten microneedles (e.g., Fine Science Tools, 11252–23; 10130–05)

Humidified 37°C incubator with 5% CO₂

Table-top centrifuge

Cell counter and live/dead cell counting dye

Protocol:

1. Isolate embryonic glands.
 1. Before tissue dissection, equilibrate reconstituted papain and albumin solutions in the incubator at 37°C for 30 minutes.

The Earle's balanced salt solution contains a pH sensitive indicator dye.
 2. Using forceps and a dissection scope, isolate embryonic organs and collect them in a 50-mm dish containing ~ 200 μ L DMEM/F12 supplemented with vitamin C and transferrin.

For embryonic day 12 submandibular salivary glands that are about 150 μ m in diameter, 7 glands are sufficient.
2. Remove DMEM/F12x and transfer glands into 1.5 mL Eppendorf tube containing 500 μ L of papain. Incubate in a humidified 37°C incubator with 5% CO₂ for 30–40 minutes. Gently triturate the papain solution containing the embryonic glands every 10 minutes.

Tissue is dissociated by incubation with activated papain at 37°C. Trituration is a crucial procedure during incubation.
3. Inactivate papain by adding (1:1) 500 μ L of ovomucoid inhibitor at 10 mg of albumin per mL to the 1.5ml Eppendorf tube containing 500 μ L of papain.
4. Using a low-bind 200 μ L pipette, gently pipette up and down with the albumin for 2 minutes to inactivate papain.
5. Cell filtration and wash

1. Centrifuge the 1.5 mL Eppendorf tube containing the papain and ovomucoid protease inhibitor at 300 g for 5 minutes.
2. Gently remove the supernatant, leaving the pelleted cells.
3. Add 1,000 μ L of HBSS (no calcium/magnesium) supplemented with 1% filtered BSA in HBSS to the 1.5 ml Eppendorf tube containing the pelleted cells and gently mix.
4. After mixing, take all the solution (with the same 1,000 μ L pipette tip used for mixing) and filter through the 20- μ m strainer into a BD Flacon FACS (352235) 5 mL tube.

Depending on the size of the cells in the organ of interest, the use of a 20 μ L filter may exclude large cells.

5. Centrifuge at 300 g for 5 minutes.
6. Remove supernatant carefully so as not to disturb cell pellets, leaving ~50 μ L.
7. Add 1 mL of 1% filtered BSA in HBSS to each 15 mL tube.
8. Count cells. If the cell concentration is excessively high, dilute with HBSS (no calcium/magnesium) supplemented with 0.04% filtered BSA and recount cells.

COMMENTARY

Background Information

One of the key determinants in preserving RNA integrity during dissociation is temperature. Cells and tissues have been commonly dissociated at 37°C for at least 15 minutes in preparation for scRNA-seq capture. Protease digestion at this high temperature for this long induces unwanted changes in transcriptome expression, with the changes increasing further as incubation time lengthens (Adam, Potter et al. 2017). In contrast, gene expression changes are minimal when cells are dissociated with cryophilic proteases for 15 minutes at 6°C (Adam, Potter et al. 2017). The first protocol has been developed to optimize the cold dissociation method for preparation of limited tissues such as early embryonic organs.

Critical Parameters

Unsuccessful capture can result from compromised cell/RNA quality, components inhibiting the reverse transcription reaction, and/or technical errors. Ensuring cell health by efficient preparation under optimal conditions is essential. Minimizing the time between final cell counting and 10x Genomics capture is just as important as efficient preparation. As cells likely start to become unhealthy and/or form aggregates over time, samples should be ideally captured within 30 minutes from the time of counting. To minimize RNase contamination, the use of RNase-free reagents and materials is also essential. The final buffer should not contain components known to inhibit the reverse transcription reaction. For instance, more than 0.1 mM EDTA or 3 mM magnesium are known to inhibit the reverse transcription

reaction. Since both Accutase and Accumax contain 0.5 mM EDTA, washing of cells is a critical step to prevent inhibition of subsequent reverse transcription. For an additional list of components that can have downstream effects, see the 10x Genomics article at: <https://kb.10xgenomics.com/hc/en-us/articles/115001937123-What-buffers-can-be-used-for-washing-and-cell-resuspension->

Understanding Results

At the time of cell counts, cells should be separate from one another by microscopy, with a minimal, if any, amount of aggregates (Figure 3a). While any established cell counting methods can work, an automated fluorescence cell counter is a preferred method over a hemocytometer for efficiency. Figure 3b shows dissociated cells stained with acridine orange and propidium iodide and imaged with the Cellometer Auto 2000 Cell Viability Counter (Nexcelom).

After single cell captures and cDNA synthesis, the yield and size distribution of the second strand cDNA provide a preliminary indication of the quality of capture. After sequencing, the Cell Ranger analysis pipeline will generate a number of statistics that also serve as indications of how the project is performing. High-quality data should contain sufficient numbers of reads, captured cells, genes per cell, and unique molecular identifiers. Data validation can be performed using different techniques such as bulk RNA-seq and qPCR.

Time Considerations

For the first protocol, the whole process of preparing two samples from the time of harvest to cell count should take approximately 2.5 hours (Figure 1).

1. Harvesting of embryos and tissue microdissection: 50 minutes
2. Enzymatic and mechanical tissue separation: 40–50 minutes
3. Protease digestion: 20 minutes
4. Cell filtration and washes: 20 minutes
5. Cell count, and if necessary, dilution to achieve optimal concentration: 10 minutes

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Significance Statement

Preparation of small tissues such as early embryonic organs for single cell RNA sequencing (scRNA-seq) comes with challenges of 1) gently dissociating adherent cells into singlets, 2) minimizing cell loss, and 3) maintaining cell viability. Temperature during protease digestion is of particular importance. While 37°C is the optimal temperature for many enzymes, keeping cells at a cooler temperature helps preserve RNA integrity. This protocol introduces a simplified and modified protocol of a cold dissociation technique previously described for a neonatal organ. The protocol has been optimized for limited amounts of samples (e.g., tiny embryonic tissue) to maximize cell recovery.

Figure 1. Overview of the cell preparation workflow.
Schematics illustrating the procedures for the preparation of cells for scRNA-seq.

Figure 2. Embryonic salivary gland after dispase treatment.
Separated embryonic day 12 submandibular mesenchyme (left) and epithelium (right).

Figure 3. Dissociated cells visualized in a cell counter.

(A) Brightfield image of dissociated cells; (B) Live cells are stained green with acridine orange, whereas dead cells, though not visible here due to the very high viability of this sample, will be stained red.