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Single Cell Isolation from Human Skin Biopsies

johannng¹, Maksim Plikus²¹University of Michigan; ²University of California, Irvine

1 Works for me

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ABSTRACT

This protocol describes necessary reagents and step-by-step procedures for processing normal human skin (from skin punch biopsy) for single-cell isolation to be used for scRNA-seq experiments.

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Samples

- 1 Determine beforehand whether skin epidermis and dermis will be sent for sequencing separately or batched (and if so at what proportion; e.g. all:all or 1:1)

Determine number of cells targeted (1,000 to 10,000)

- We generally target 10,000 to increase diversity at the expense of depth
- For skin samples, actual number of recovered cells is generally ~3,000-5,000

General workflow

- 2 **Day 0:** Receive skin samples, transfer to Dispase, and incubate overnight at 4C
Day +1: Create single-cell suspensions and submit to core for further processing

Procedure details

3 Day 0, Dispase treatment of skin samples

Materials/reagents:

1 aliquot/sample of 0.4% Dispase (Life Technologies 17105-041; located at 4C) in HBSS (Gibco 14025-092)

Collagenase II (Life Technologies 17101-015): 4C

Collagenase V (Sigma C9263-1G): -20C

Plain media (IMDM or RPMI) without FBS: 4C

0.25% Trypsin-EDTA (Gibco): 4C

Dispase treatment:

Transfer each skin specimen to labeled **Dispase** aliquot and place at 4C overnight (ideally ~18h or less)

- If samples are received early in the day and scRNA-seq appointment is available, it is acceptable to process day 0 after 1h RT
- Request appointment for scRNA-seq the next day (if not already done when the biopsy was scheduled)

Other chores for Day 0:

- Check stocks of Dispase to ensure it is available for next setup
- If needed make more 3-5 mL aliquots of 0.4% Dispase in HBSS (for 40 mL, add 160 mg Dispase under sterile conditions and store in 3-5 mL aliquots at 4C)

Day 0 prep for next-day immediate scRNA-seq (can also do morning of day 1):

- Make two aliquots of 0.2% Collagenase II + 0.2% Collagenase V in plain medium
- In the hood, add 20 mg of Collagenase II and 20 mg of Collagenase V to 10 mL plain media, mix, divide into two 15 mL conicals

- May also make ahead of time to keep at -20C
 - Measure out two aliquots of 0.25% trypsin-EDTA x2 5 mL aliquots in 15 mL conicals
 - Store the above at 4C overnight
 - Note: DNase must be added next morning

4 Day +1, Processing Dispace samples for scRNA-seq

Materials/reagents:

Biopsies incubated in 0.4% Dispace in HBSS overnight at 4C (or 1h at RT)
 Collagenase aliquots and Trypsin-EDTA aliquots (see above)
 DNase I (Thermo Scientific 90083 – says 2500 U/uL but is actually ~5000 U/uL): -20C
 FBS (Atlanta Biologicals S11550): -20C in aliquots
 Complete media (e.g. IMDM +10% FBS +P/S, complete KSM, complete RPMI + GlutaMAX, etc.)

Supplies:

MACSMix
 Forceps x2
 10 cm plates x3
 Razor (or scalpel) blades x2
 50 mL tubes x2, labeled
 70 nm nylon mesh strainers x2
 1cc or 3cc syringes x2
 0.5 mL Eppendorf tube x1 per sample labeled for scRNA-seq

Protocol for cell isolation from skin biopsy for scRNA-seq:

- Warm complete media, FBS, aliquots x2 each of Collagenase and Trypsin-EDTA
- To each 5mL aliquot of Collagenase or Trypsin, add 10U/mL DNase solution and invert to mix
- Calculate quantity based on U on slip (~11uL for 50,000U in 5mL)

■ ***Specimen dissociation:***

- Clean MACSMix and place in 37C incubator
- Pour 70% EtOH into 10 cm plate to wash blades; lean blades on edge of second plate to dry
- Swirl Dispace and pour biopsy into 10 cm plate
- With two pairs of forceps, peel epidermis off of dermis

■ ***Processing of epidermis and dermis:***

- Transfer epidermis to Trypsin-EDTA + DNase and rock at highest (top) MACSMix setting at 37C for 1h
- Set timer for 1h to quench Trypsin-EDTA with FBS
- Transfer dermis to new 10 cm plate with drop of Dispace to avoid drying
- Using an ethanol-sterilized razor (dried with Kimwipe) and forceps, mince dermis into 1-2 mm cubes
- Transfer pieces Collagenase + DNase; pipet some out to rinse mincing dish and add back to tube

- Rock at highest MACSMix setting at 37C for 1.5h-2h
- While samples are rocking, submit samples to sequencing core
- When epidermis has incubated 1h, quench with 5 mL warm FBS
 - *Can process immediately or return to 37C rocker until dermis is ready*
- 5min before dermis is done, strain epidermis solution through 70 um mesh into labeled 50 mL conical
- Use back of syringe plunger to shred epidermis against nylon mesh, then rinse mesh with 2 mL IMDM
- Repeat several times; leftover tissue on mesh is okay
- Process dermis in the same way into the same 50mL conical, shredding and rinsing several times
- Spin down samples at 1800 RPM for 6 minutes at 4C (RT okay)

■ **scRNA-seq sample preparation:**

- For each, aspirate liquid and re-suspend in 500 uL (small pellet) to 1mL (larger pellet) of IMDM + 10% FBS
- Pipet to mix, then transfer 12 uL resuspended cells and 12 uL Trypan blue to Eppendorf tube and mix
- Add 10 uL to each side of hemocytometer and count live and dead cells
 - *Live cells exclude blue dye (clear/shiny); blue cells are dead*
 - *If <25% of cells are dead, omit live/dead column – calculate cells per uL*
- Count # cells per 9 center squares = x (20x = # of cells per uL)
- If >25% of cells are dead, consider live/dead column (see below) with caveat that this will increase handling/incubation times and likely increase cell death and bias recovery toward hardier cell types
- Prepare cooler with wet ice
- If combining cells 1:1, calculate the number of uL for 20,000 epidermal cells and 20,000 dermal cells and combine in an Eppendorf tube
 - *Bring volume up with media such that you have 40 ul (seeking concentration of 1000 cells/ul)*
 - *If sample is too dilute or concentrated, processing time will be increased at the core*
- Typically, sequencing core prefers 1000 cells/uL but can accommodate some variability, Err on the side of more concentrated – typically core prefers to avoid spinning the samples
 - *If sufficient cells, can do 40,000 and 40,000 in 80 uL, as core prefers to have plenty of cells*
 - *If insufficient cells, try to give at least 20,000 in 20 uL and warn core*
 - *Bring an aliquot of spare media for core to dilute samples*
- Label samples on lid with sample ID from submission
- Transport samples and spare media to sequencing core on wet ice
- At the core, staff will count cells/check viability while you wait to ensure you want to proceed
 - *Auto-count will show populations of larger cells (live) and smaller cells (“dead”)*
 - *Note: smaller “dead” cells are most likely lymphocytes in our samples; Trypan blue exclusion is MUCH more reliable – do not be concerned*

5 Optional procedure: live/dead column use protocol

Reagents/supplies:

MACS Dead Cell Removal Kit 1x Binding Buffer (prepared from stock; bottom of 4C, tin foil)
MACS Dead Cell Removal Kit Microbeads (Miltenyi 130-090-101; stored at 4C)
MACS magnet(s) and stand
LS columns (or MS columns)
Conical rack for collecting flow-through

Protocol for live/dead column:

- Transfer MACS binding buffer to RT
- Spin down sample at 1800 RPM for 6 minutes and aspirate off media
- Resuspend cells in 100 uL MACS microbeads (or 100 uL per 10M cells if >10M cells)
- Incubate at RT 15 minutes
- Clean MACS magnet and stand and set up in hood

- For LS columns, only need 1 per sample
- For MS columns, prepare only 1 per sample but may need second if first becomes clogged

■ ***Prepare MACS columns during incubation:***

- Load column(s) into magnet with waste tube below (throw column plungers away)
- Rinse with binding buffer (LS: 3 mL, MS: 0.5 mL) by gravity filtration
- Remove waste tube and place fresh, labeled collection tube beneath

■ ***After incubation, process each sample independently:***

- Dilute sample in appropriate volume binding buffer (LS: 3 mL, MS: 0.5 mL) and mix gently
- Load sample onto column and allow to pass through by gravity filtration
- Rinse column 4x with appropriate volume binding buffer

- Spin down sample at 1800 RPM for 6 minutes at RT
- Re-suspend pellet in appropriate volume of IMDM + 10% FBS (eg 1mL per 1M cells)
- Count with hemocytometer and return to protocol above