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Human breast tissue dissociation and FACS vs Flowmi processing for scRNA-Seq

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ABSTRACT

Single cell RNA-Seq of human breast tissue requires tissue dissociation into viable single cell suspensions. Here, we describe a protocol for digestion of human breast tissue and compare two methods to obtain viable single cell suspensions using either fluorescence activated cell sorting (FACS) or several wash steps and Flowmi cell filtering.

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MATERIALS TEXT

MATERIALS:

- Collagenase Type I powder: Thermo Fisher Scientific, Catalog #17100017
- DMEM and Hams F-12 50/50: Fisher Scientific, Catalog #MT10090CV
- Deoxyribonuclease I (DNAse I): Sigma Aldrich, Catalog #D4263-5VL
- Dulbecco's Phosphate-Buffered Salt Solution 1X: Fisher Scientific, Catalog #MT21031CV
- 0.05% Trypsin-EDTA: Fisher Scientific, Catalog #MT25051CI
- HyClone™ Penicillin Streptomycin 100X Solution: Fisher Scientific, Catalog #SV30010
- Fetal Bovine Serum: Omega Scientific. Inc., Catalog #FB-12
- Invitrogen™ UltraPure™ BSA (50 mg/mL): Fisher Scientific, Catalog #AM2616
- ACK Lysing Buffer: Thermo Fisher Scientific, Catalog #A1049201
- Feather® Conventional Disposable Sterile Scalpels, Size 10 (#10): Graham-Field, Catalog #2975#10
- 1.5 mL microcentrifuge Eppendorf tubes: Fisher Scientific, Catalog #022431021
- Fisherbrand™ Colored Labeling Tape: Fisher Scientific, Catalog # 15-901-5E
- SYTOX™ Green Dead Cell Stain, for flow cytometry: Thermo Fisher Scientific, Catalog #S34860
- MULTI-seq reagents: Request from Zev Gardner's lab
- 150 x 22 mm tissue culture plates: VWR, Catalog #10062-882
- Corning™ Centrifuge Tubes with CentriStar™ Cap: Fisher Scientific, Catalog #05-526B
- Falcon™ Round-Bottom Polystyrene Test Tubes with Cell Strainer Snap Cap, 5mL: Fisher Sci, Cat # 08-771-23
- Fisherbrand™ Sterile Cell Strainers, 70 µm: Fisher Scientific, Catalog #22-363-548
- SCIENCEWARE® Flowmi™ Cell Strainers for 1000µl Pipet Tips, 40 µm, Bel-Art: VWR, Catalog # 10032-802
- ART™ Wide Bore Filtered Pipette Tips: Thermo Fisher Scientific, Catalog #2079G
- Countess™ Cell Counting Chamber Slides: Thermo Fisher Scientific, Catalog #C10228
- Trypan Blue Stain (0.4%) Thermo Fisher Scientific, Cat#T10282
- Chromium Single Cell 3' Reagent Kits v3: 10X Genomics, Catalog #CG000183

INSTRUMENTS

- Shaking incubator (set to 37°C)
- Centrifuge (cooled to 4°C)
- Tissue scale
- Vortexer
- FACSAria Fusion with 100 µm nossel or alternative fluorescence-activated cell sorter
- Countess™ Automated Cell Counter
- 10x Chromium Controller

SAFETY WARNINGS

Wear proper PPE and take precautions when handling potentially infectious patient material. Disinfect any work areas contaminated with patient tissue or blood immediately with 10% bleach.

BEFORE STARTING

Prepare the following reagents and materials:

REAGENTS

Media

DMEM + 10% FBS: DMEM/Ham's F12 + 10% FBS + 100 U/mL Penicillin + 100 µg/mL Streptomycin
DMEM + 5% FBS: DMEM/Ham's F12 + 5% FBS + 100 U/mL Penicillin + 100 µg/mL Streptomycin
Keep refrigerated or on ice.

Buffers

PBS (calcium/magnesium-free). Keep on ice.

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PBS (calcium/magnesium-free) + 1% BSA. Keep on ice.
Digestion buffer: 4 mg/mL Collagenase type I in DMEM/Ham's F12 + 5% FBS. Prepare and keep on ice until enzymatic
digestion.
ACK Red Blood Cell lysis buffer. Keep at room temperature.

DNase I Stock Solution
1 mg/mL DNase I in DPBS (with calcium and magnesium). Keep on ice until DNase I treatment.

Trypsin-EDTA
Thaw 0.05% Trypsin-EDTA. Keep at room temperature until required.

Viability dye
Thaw SYTOX Green. Keep at room temperature until required.

MULTI-seq reagents
Anchor & Co-Anchor LMOs; MULTI-seq barcodes. Refer to McGinnis *et al.*, Nat Methods, 2019 (doi: 0.1038/s41592-
019-0433-8) for detailed instructions. Keep on ice.

MATERIALS
Pre-heat shaker-incubator to 37°C.
Cool down centrifuge to 4°C.
Turn on FACS sorter (e.g. FACSAria Fusion) and attach 100 µm nossel.
Fill ice bucket(s) with ice.
Prepare reagents from 10x Single Cell 3' Reagent Kits according to manufacturer's instructions.

1 Initial Tissue Preparation

1. Transfer breast tissue specimen to 150 x 22 mm cell culture dish. Remove medium and wash 3 times with 50-100 mL ice-cold PBS.
2. *Optional: If histological analysis is desired, separate tissue pieces and process as desired.*

2 Mechanical Digestion

1. Using forceps and scalpels, remove soft, white/yellow adipose-rich regions to enrich for epithelial/fibrous areas with a roseate appearance and rather stiff consistency.
2. Transfer tissue to a new 150 x 22 mm dish and weigh. Record tissue weight. Discard removed tissue according to biosafety guidelines for biohazardous material.
3. Fix a 150 x 22 mm dish containing breast tissue to benchtop/hood surface using tape. This will facilitate mechanical digestion by avoiding that the dish slides while cutting tissue. Mechanically digest breast tissue by mincing it using two no. 10 sharp scalpels. Chop until tissue gets to a paste-like consistency (this can take up to 1.5 hours, depending on tissue amount and stiffness).

3 Enzymatic Digestion

1. Transfer up to 5 ml of minced tissue per 50 ml conical tube and add 40 ml digestion buffer.
2. Incubate tissues in digestion buffer at 37°C in a shaker at 180-200 rpm for 6 hours.
3. Pellet digested tissue at 350 g for 5 minutes at 4°C, aspirate supernatant, and wash with 50 ml ice-cold PBS. Repeat wash and pellet cells.
4. Gently resuspend pellet in 2 ml 0.05 % Trypsin-EDTA, pipette mix and incubate at 37°C for 6 minutes. Pipette mix gently every 2 minutes using a p1000 pipette.
5. Quench Trypsin with 10 ml DMEM with 10% FBS and pellet cells by centrifuging at 350 g for 5 minutes at 4°C.
6. Resuspend pellet in 1 ml DMEM with 10 % FBS and add 100 µl DNase I stock (1 mg/mL) for a final concentration of 10 µg/ml. Incubate for 5 minutes at 37 °C.
7. Quench reaction by adding 10 ml DMEM with 10 % FBS. Resuspend cells gently and pass through a 70 µm cell strainer into a new 50 mL conical tube.

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8. Centrifuge at 350 g for 5 minutes at 4°C.

4 Red Blood Cell Lysis

1. Aspirate supernatant and wash cells with 20 mL ice-cold PBS. Centrifuge at 350 g for 5 minutes at 4°C.
2. Aspirate supernatant and gently resuspend pellet in 1 ml ACK red blood cell lysis buffer. Gently swirl cell suspension. Incubate for 30 seconds – 1 minutes at room temperature.
3. Quench by adding 10 ml ice-cold PBS (without serum or BSA) and spin down at 350 g for 5 minutes. Resuspend pellet in 1 ml DMEM + 10% FBS and transfer to 1.5ml Eppendorf tubes. Count cells with Trypan blue on a Countess automated cell counter.
4. Split cell suspension into two parts: half of the cell suspension will be subjected to FACS sorting and the other half will be washed and filtered using a 40 µm Flowmi filter. Always keep cells on ice.

5 FACS sorting

1. Pass cell suspension to be sorted through a 35 µm filter capped FACS tube.
2. Separate an aliquot with 50,000 – 100,000 cells into another FACS tube. This will be an unstained sample to set up the live/dead cell gate.
3. Prepare one 5 mL FACS tube with 1 mL DMEM + 10% FBS medium for collecting sorted cells.
4. Add SYTOX Green viability dye to the remaining sample to a final dilution of 1:1,000.
5. Sort viable single cells on FACSAria Fusion (or alternative fluorescence-activated cell sorter). Gate out debris and doublets and use unstained sample to set up live/dead cell gate. Sort all live cells (SYTOX Green negative) in the sample using 4-way purity precision setting.
6. Pellet sorted cells by centrifuging at 350 g for 5 minutes at 4°C. Resuspend cells gently using a wide bore p100 pipette in 1 mL PBS + 1% BSA. Count cells and always keep cells on ice.

6 Flowmi filtering

1. Pass cells kept for Flowmi filtering through a 35 µm filter capped FACS tube. Add 2 mL PBS + 1% BSA and pellet at 350 g for 5 minutes at 4°C.
2. Aspirate supernatant and gently resuspend pellet in 2mL PBS + 1% BSA using p1000 wide bore pipettes. Repeat centrifugation from previous step.
3. Gently resuspend cells in 1 mL PBS + 1% BSA.
4. Pass cell suspension through a 40 µm Flowmi filter attached to a wide bore p1000 pipette into a new 1.5 mL Eppendorf tube. Count cells. Always keep cells on ice.

7 *Optional:* MULTI-seq labeling and 10x Chromium GEM generation

1. If multiplexing is desired, proceed to MULTI-seq labeling of each sample according to McGinnis *et al.,* Nat Methods 2019 (doi: 0.1038/s41592-019-0433-8). MULTI-seq labeling can be done using a 1 µM final concentration of LMOs and barcodes for up to 500,000 cells.
2. After sample barcoding, pool samples and centrifuge at 350 g for 5 minutes at 4°C. Aspirate supernatant and resuspend in PBS + 1% BSA. Count cells always keep cells on ice.

8 Loading onto 10x chip for GEM generation

1. Proceed to 10x Chromium GEM generation with a targeted recovery of 10,000 cells per lane according to manufacturer's instructions.

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