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Processing of human surgical samples for single-cell sequencing

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ABSTRACT

Human clinical samples pose a challenge for single-cell RNA-seq (scRNA-seq) and single-nucleus ATAC-seq (snATAC-seq) analysis, due to their heterogeneity in cell type composition and degree of degradation or necrosis. Moreover, although some tissues can be sampled by surgical resection, most biospecimens are derived from core needle biopsies or fine needle aspirates, and must be allocated to pressing needs such as pathological analysis, thereby severely limiting the number of cells available for analysis. We have developed a protocol for obtaining robust, high-quality single-cell sequencing data from human biospecimens from a variety of biopsy types, enabling greater access to clinical cohorts. A critical factor is to limit ischemic time and transport to the lab to under an hour, and to restrict subsequent processing time to under 3 hours. This protocol uses a commercial tissue disruptor for dissociation. It was optimized on diverse human lung tumor samples, including pleural effusions, but is widely applicable to normal and diseased human clinical tissues from different organ sites.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Joseph M Chan, Álvaro Quintanal-Villalonga, Vianne Gao, Yubin Xie, Viola Allaj, Ojasvi Chaudhary, Ignas Masilionis, Jacklynn Egger, Andrew Chow, Thomas Walle, Marissa Mattar, Dig VK Yarlagadda, James L. Wang, Fathema Uddin, Michael Offin, Metamia Ciampricotti, Umesh K Bhanot, W Victoria Lai, Matthew J Bott, David R Jones, Arvin Ruiz, Travis Hollmann, John T Poirier, Tal Nawy, Linas Mazutis, Triparna Sen, Dana Pe'er, Charles M Rudin. Single cell profiling reveals novel tumor and myeloid subpopulations in small cell lung cancer. *bioRxiv* 2020.12.01.406363; doi: https://doi.org/10.1101/2020.12.01.406363

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MATERIALS TEXT

Equipment

⊠gentleMACS Octo Dissociator with Heaters **Miltenyi Biotec Catalog # 130-096-427**

⊠GentleMACS C tube **Miltenyi Biotec Catalog #130-093-237**

⊠MACS SmartStrainers (70 µm) **Miltenyi Biotec Catalog #130-098-462**

⊠Corning™ Falcon™ Test Tube with 35µm Cell Strainer Snap Cap **Corning Catalog #352235**

⊠SepMate™-50 (IVD) 100 Tubes **Stemcell Technologies Catalog #85450**

Reagents

⊠RPMI 1640 [ ] L-glutamine, 25 mM HEPES **Corning Catalog #10-041-CV** (any format)

⊠DPBS (Dulbeccos Phosphate Buffered Saline) 1x [-] calcium magnesium **Corning Catalog #21-031-CV** (any format)

⊠FBS **Gemini Bio-Products Catalog #900-108** (any format)

⊠ACK Lysing Buffer (1X) (red blood cell lysis buffer) **Lonza Catalog #10-548E**

⊠Tumor Dissociation Kit human **Miltenyi Biotec Catalog #130-095-929**

⊠Human TruStain FcX™ **BioLegend Catalog #422302**

⊠PE anti-human CD45 antibody **BioLegend Catalog #368510**

⊠calcein AM **BioLegend Catalog #425201**

⊠DAPI (46-Diamidino-2-Phenylindole Dihydrochloride) **Invitrogen - Thermo Fisher Catalog #D1306**

⊠Ficoll Paque PLUS **Ge Healthcare Catalog #17144003-500 ml**

Sample collection and transport to the lab 2h

1 ⚠

Rapid transportation of the surgical sample to the lab after resection or biopsy is key to the success of this protocol. To maximize cell viability and sequencing data quality, processing of clinical samples in the lab should begin within an hour of surgical collection, and never more than two hours after.

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1.1 Surgical samples should be placed in PBS, saline (0.9% w/v sodium chloride) or RPMI media after resection, and transported to the lab on ice.

Sample preparation

2 The specimen first needs to be processed to obtain a single-cell suspension that is free of red blood cells. For **typical resections** (>100 mg), proceed to **Section 3**. For **core needle biopsies** (at least one core, typically 10-100 mg) or **fine needle aspirates** (typically 10-50 mg), proceed to **Section 4**. For **pleural effusion** samples, proceed to **Section 5**.

It is important to minimize the dissociation and processing time as much as possible, to improve cell viability and quality of sequencing data.

Surgical specimen dissociation 1h

3 This standard protocol should be used when tissue is not limiting.

3.1 Add **250 µl of Enzyme H, 110 µl of Enzyme R, and 40 µl µL of Enzyme A** from **HumanTumor Dissociation Kit** (Miltenyi) into a **GentleMACS C Tube** (Miltenyi) with **7.5 µl** of medium (RPMI without antibiotics or FBS).

3.2 Aspirate the liquid in which the tumor has been submerged and transfer the tumor to a petri dish. Chop into ~5 mm^3 pieces using a new sharp razor, and transfer the tumor pieces to the **GentleMACS C Tube** containing the enzyme mix.

3.3 Place the **GentleMACS C Tube** in the **GentleMACS Octo Dissociator with Heaters** (Miltenyi), and run program **37C_H_TDK3** for **00:15:00** . After this time, check the solution to ensure complete dissociation (no tissue chunks visible to the naked eye). If the sample is not completely dissociated, continue running the program for rounds of **00:10:00** until completely dissociated. Do not continue any longer than necessary, as extended incubation can damage fragile cells and reduce RNA quality.

3.4 Apply the cell suspension to a **70 µm MACS SmartStrainer** (Miltenyi) placed in a 50 mL conical tube.

3.5 Wash the **70 µm MACS SmartStrainer** gently with **20 mL** to **25 mL** of **PBS 2.5% FBS** buffer. Remove strainer.

3.6 Spin **800 x g, Room temperature , 00:03:00** and aspire supernatant carefully so as not to disturb the pellet.

3.7 Gently resuspend cell pellet in **1 mL** of **Red Blood Cell Lysis Solution (ACK buffer)** (Lonza) by pipetting up and down 2-3 times. Incubate for 2-3 minutes.

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RBC lysis should be included even if blood is not visually evident in the sample.

3.8 Add **20 mL** to **25 mL** **PBS 2.5% FBS** to dilute the ACK buffer, centrifuge again at **800 x g, Room temperature , 00:03:00** , and aspirate supernatant carefully so as not to disturb the pellet.

At this point, the pellet should be whitish and not red. If it still looks red, repeat steps 3.7 and 3.8.

3.9 Resuspend cells in **3 mL** to **4 mL** of **PBS 2.5% FBS** and transfer to a **Blue cap 35 µm filter FACS tube** (Corning) by pipetting onto the cap filter. Proceed to **Section 6**.

Small specimen processing

4 Smaller samples are more effectively dissociated in reduced volumes for shorter periods, and extra care needs to be taken to avoid cell loss at each step.

4.1 Add **85 µl** **of Enzyme H,** **40 µl** **of Enzyme R, and** **15 µl** **µL of Enzyme A** from **HumanTumor Dissociation Kit** (Miltenyi) into a **GentleMACS C Tube** (Miltenyi) with **2.5 µl** of medium (RPMI without antibiotics or FBS).

4.2 Aspirate the liquid in which the tumor has been submerged and transfer the tumor pieces to the **GentleMACS C Tube** containing the enzyme mix.

If the cores are disaggregated, centrifugation at **800 x g, Room temperature , 00:02:00** is recommended to collect all tumor pieces at the bottom of the tube before liquid aspiration and transferring to the **GentleMACS C Tube** containing the enzyme mix.

4.3 Place the **GentleMACS C Tube** in the **GentleMACS Octo Dissociator with Heaters** (Miltenyi), and run program **37C_H_TDK3** for **00:15:00** . After this time, check the solution to ensure complete dissociation (no tissue chunks visible to the naked eye). If the sample is not completely dissociated, continue running the program for rounds of **00:05:00** until completely dissociated. Do not continue any longer than necessary, as extended incubation can damage fragile cells and reduce RNA quality. 20m

4.4 Apply the cell suspension to a **Falcon™ Test Tube with 35 µm Cell Strainer Snap Cap** (Corning) though the cell strainer cap. To improve cell recovery, add **1.5 mL** of **PBS 2.5% FBS** to the **GentleMACS C Tube** and wash the tube with it. Then, add it to the same **Falcon™ Test Tube with 35 µm Cell Strainer Snap Cap** (Corning) though the cell strainer cap.

4.5 Spin **800 x g, Room temperature , 00:02:00** and aspire supernatant carefully so as not to

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disturb the pellet.

4.6 Gently resuspend cell pellet in **0.2 mL** of **Red Blood Cell Lysis Solution (ACK buffer)** (Lonza) by pipetting up and down 2-3 times. Incubate for 2-3 minutes.

RBC lysis should be included even if blood is not visually evident in the sample.

4.7 Add **2 mL** to **3 mL** **PBS 2.5% FBS** to dilute the ACK buffer, centrifuge again at **800 x g, Room temperature , 00:02:00** , and aspire supernatant carefully so as not to disturb the pellet. Proceed to **Section 6**.

At this point, the pellet should be whitish and not red. If it still looks red, repeat steps 4.6 and 4.7.

Pleural effusion processing

5 Pleural effusions are cell suspensions that can be effectively concentrated from much larger volumes using density gradient centrifugation.

5.1 To concentrate the cells, divide the fluid into conical tubes and centrifuge at **500 x g, Room temperature , 00:10:00** . Carefully remove supernatant and resuspend the pellets into **10 mL** to **40 mL** of **PBS 2.5% FBS**.

The resuspension volume depends on the size of the pellets obtained. Typically, **10 mL** of **PBS 2.5% FBS** are used per **250 mL** of initial fluid volume.

5.2 Prepare **SepMate™-50 (IVD)** tubes (STEMCELL Technologies) by pipetting ~ **15 mL** of **Ficoll-Paque PLUS** (GE Healthcare) directly and gently into tube's separating hole until the fluid level is just above the dividing plastic. One **SepMate™-50 (IVD)** tube will be needed per **20 mL** of concentrated cell suspension in **PBS 2.5% FBS** .

5.3 Carefully layer **20 mL** concentrated cell suspension in **PBS 2.5% FBS** onto the **Ficoll-Paque PLUS** in the **SepMate™-50 (IVD)** tube (drop by drop).

When layering the sample, try to minimize mixing of the **Ficoll-Paque PLUS** solution and the concentrated cell suspension.

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5.4 Centrifuge at **1200 x g, Room temperature , 00:10:00 , Accelerator and break off** .

5.5 Pipette off **15 mL** of upper fluid layer from each **SepMate™-50 (IVD)** tube into a Falcon tube. For the remaining ~ **5 mL** above the dividing plastic, try to pipette up and down gently to loosen and collect the residue sitting above the dividing plastic.

5.6 Centrifuge at **800 x g, Room temperature , 00:03:00** and discard supernatant.

5.7 Gently resuspend cell pellet in **1 mL** of **Red Blood Cell Lysis Solution (ACK buffer)** (Lonza) by pipetting up and down 2-3 times. Incubate for 2-3 minutes.

RBC lysis should be included even if blood is not visually evident in the sample.

5.8 Add **20 mL** to **25 mL** **PBS 2.5% FBS** to dilute the ACK buffer, centrifuge again at **800 x g, Room temperature , 00:03:00** , and aspire supernatant carefully so as not to disturb the pellet. Proceed to **Section 6**.

At this point, the pellet should be whitish and not red. If it still looks red, repeat steps 5.7 and 5.8.

Sample staining 25m

6

Staining will allow dead cells to be selectively removed based on calcein AM and DAPI stains. Additionally, CD45 staining will allow live cells to be further sorted by CD45 marker status to separate immune (CD45+) from non-immune, tumor-enriched (CD45-) cell fractions.

The goal of CD45 staining and sorting is to enrich tumor cells in samples for which these make up a relatively small fraction of the total tumor mass (e.g. lung adenocarcinoma). CD45 staining does not need to be performed if tumor cell enrichment is not required; however, sorting for live (DAPI-, Calcein+) cells is always required to fulfill the high viability requirements of library preparation and sequencing steps.

6.1 Centrifuge the FACS tube **800 x g, Room temperature , 00:03:00** and aspire supernatant carefully so as not to disturb the pellet.

6.2 Add **100 µl** of staining cocktail per ~1 million cells and vortex briefly to mix.

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Staining cocktail preparation (per **100 µl**) for **live and CD45+/- cell sorting**:

- **91.5 µl** **PBS 2.5% FBS**
- **5 µl** of **human TruStain FcX**
- **3 µl** of **PE anti-human CD45 antibody**
- **0.5 µl** of **1 Milimolar (mM)** **calcein AM**

Staining cocktail preparation (per **100 µl**) for **live cell sorting**:

- **100 µl** **PBS 2.5% FBS**
- **0.5 µl** of **1 Milimolar (mM)** **calcein AM**

6.3 Incubate cells with staining cocktail for **00:15:00** on ice or at **4 °C** .

6.4 Wash by adding **2 mL** of **PBS 2.5% FBS** and centrifuging at **800 x g, Room temperature , 00:03:00** . Aspirate supernatant carefully and repeat once, for a total of two washes.

Sample sorting 30m

7 Cell sorting can be performed on any standard FACS instrument.

7.1 Resuspend cells in **100 µl** **RPMI 2.5% FBS** per million cells with **DAPI** (final concentration of 0.1-1 µg/mL), and place on ice.

7.2 Prepare Eppendorf tubes containing **50 µl** of **RPMI 2.5% FBS** that will serve as sort collection tubes.

7.3 Set sorting gates to capture the cell populations of interest. If tumor cell enrichment is required and was performed in **Step 3**, set the following gates (example below) and record cell fractions in your notes:
- Live immune cells: DAPI-, FITC+ (Calcein+), PE+
- Live non-immune (tumor-enriched) cells: DAPI-, FITC+ (Calcein+), PE-

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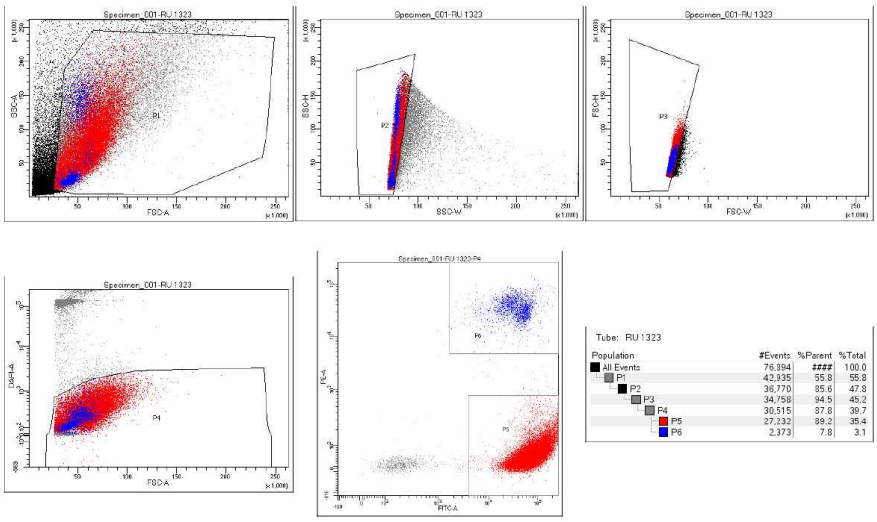

Sorting strategy to isolate live immune and non-immune (tumor-enriched) cells separately.

Alternatively, if tumor cell enrichment is not required and CD45 staining was not performed, set the following gates:
- Live cells: DAPI-, FITC+ (Calcein+)

For smaller samples, using more permissive (lower purity) sorting conditions improves cell recovery, ensuring enough cells for successful sequencing library preparation.

7.4 If CD45 staining and sorting was performed, tumor cell enrichment can be performed by mixing immune (CD45+) and non-immune (tumor-enriched, CD45-) cell fractions back together at the desired ratio, according to your research interests.

7.5 Concentrate sample by centrifuging **800 x g, 4°C, 00:03:00** in an oscillating rotor centrifuge, very carefully aspire supernatant, and resuspend in **RPMI 2.5% FBS** to 700 cells/µL.

The use of a swinging bucket centrifuge is ideal for maximizing cell recovery.

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