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Running Head:

Complex tissue dissociation for single-cell transcriptomics

Practical considerations for complex tissue dissociation for single-cell transcriptomics

i. Abstract

Single-cell and single-nucleus RNA sequencing have revolutionised biomedical research, allowing analysis of complex tissues, identification of novel cell types, and mapping of development as well as disease states. Successful application of this technology critically relies on the dissociation of solid organs and tissues into high-quality single-cell (or nuclei) suspensions.

In this chapter, we examine several key aspects of the tissue handling workflow that need to be considered when establishing an efficient tissue processing protocol for single-cell RNA sequencing (scRNA-seq). These include tissue collection, transport and storage, as well as the choice of the dissociation conditions. We emphasise the importance of the tissue quality check and discuss the advantages (and potential limitations) of tissue cryopreservation. We provide practical tips and considerations on each of the steps of the processing workflow, and comment on how to maximise cell viability and integrity, which are critical for obtaining high-quality single-cell transcriptomic data.

ii. Key Words

tissue dissociation, cryopreservation, enzymatic digestion, single-cell suspension, tissue viability, lung tissue, single-cell transcriptomics, single-cell RNA-seq,

1. Introduction

Recent advances and widespread implementation of the single-cell technologies have transformed basic and biomedical research, allowing unprecedented insights into complex tissues[1-3], identification of previously unknown cell types[4, 5], reconstitution of developmental trajectories[6, 7] and mapping of disease states and drivers[8-12]. As we are moving into the aera of personalised medicine, single-cell RNA sequencing (scRNA-seq), single-nucleus RNA-seq (snRNA-seq) and single-cell multiomics sequencing (sc multiomics-seq) hold great promise for biomarker discovery for disease diagnosis, prognosis, and response to treatment [13, 14].

For single-cell transcriptomics, solid organs and tissues need to be dissociated to release individual cells from their extracellular matrix. When tissue complexity or storage format (e.g., fixed or snap frozen samples) preclude intact cell isolation, suspensions of single nuclei can be prepared[15, 16]. With the development of large collaborative single-cell initiatives, like Human Cell Atlas Project [2, 17, 18], numerous human tissue processing protocols compatible with the scRNA-seq workflow have been developed and benchmarked. Importantly, preparation of a high-quality single cell suspension is a key prerequisite for obtaining high-quality single-cell transcriptomics data[19]. Therefore, each parameter of the tissue processing protocol needs to be carefully tested and optimised. Independent of the exact platform used, the ideal dissociation protocol should preserve cellular composition of the original tissue, yield single-cell suspension with high viability, and maintain high RNA integrity. Moreover, some tissues are more difficult to work with then others and their processing may require extensive optimization.

When developing a tissue processing workflow for single-cell RNA-seq several steps need to be carefully considered and optimised (**Figure 1**), including:

1. Tissue collection (patient inclusion criteria, sampling method, required amount, transport logistics)
2. Tissue storage before dissociation (storage buffer, time and temperature, possibility of cryopreservation/freezing)
3. Tissue quality check and histological characterisation
4. Tissue dissociation into single-cell suspension (dissociation temperature, selection of the enzyme mix and dissociation procedure)

In the next paragraphs, we discuss the importance of each of these steps and provide practical considerations on how to maximise cell viability and integrity, which are critical for obtaining high-quality single-cell RNA-seq data. Our workflow, described in detail in section 3, was originally developed for processing human lung tissue, but similar considerations should be given for other complex tissues and organs.

Tissue collection

Patient inclusion criteria

As single cell -omics technologies remain expensive, usually relatively small samples sizes are analysed[20]. Hence, for disease studies, it is important to establish strict patient inclusion criteria for prospective tissue collection to ensure the best possible sample characterisation and matching of the control and disease groups. Selection criteria depend on the exact experimental question and need to be set up specifically for each project. We usually match our lung donors in terms of age, gender, body mass index, smoking status, and smoking history. In addition, whenever possible, we also recommend collecting patient medical history and tests results for the best possible characterization of the included samples and disease classification.

Tissue sampling

In addition to patient inclusion criteria, the choice of tissue collection method needs to be considered depending on availability, feasibility, required amount and suitability to the research question. Tissue can be obtained via different sampling methods (e.g, from surgical resections or biopsy extraction, from life or deceased donors). The choice of starting material, location of the collected tissue piece within the organ and the sampling method are important parameters as they may yield different cellular representation in scRNA-seq analysis.

Tissue transport and storage (before dissociation)

The tissue piece is typically collected in transport medium from the surgery theatre and sent on ice to the processing laboratory via an organised transport route (e.g., courier). The transport medium should allow an optimized storage of fresh tissue and maintain high viability. There must be sufficient buffering capacity and osmolarity. A variety of organ and tissue transport solutions have been developed[21] and can be tested when optimising a new workflow. For human tissue transport, country-specific human tissue regulations apply and require special packaging, labelling, and tracking of the material. Typically, a double sealed container system is required. During tissue procurement and transport, care should be taken to prevent contamination, cross-contamination between samples, mislabelling and deterioration of the tissue. To obtain the highest possible tissue and cell viability, it is essential to keep the overall time from tissue collection to final processing as short as possible. Hence, the logistics of tissue collection, transport and processing needs to be well organised and optimised.

Standard tissue storage formats, like formalin-fixed paraffin-embedded (FFPE) or flash-frozen do not yield viable cell suspensions and are therefore not compatible with scRNA-seq analysis (can be used for snRNA-seq ans sc multiomics). Hence, most single-cell transcriptomic studies to date have relied on direct processing of fresh tissue received from the hospital. Processing of fresh tissue is attractive, as it does not introduce any further tissue

treatments steps apart dissociation, minimising additional biases[19, 22]. However, fresh tissue processing poses several challenges, especially in clinical settings, including access to specialised equipment and specialist facilities where the material needs to be dissociated immediately and further processed to avoid sample degradation[22]. This restricts complex experimental designs, does not allow histological evaluation before processing, and may introduce technical biases related to processing of fresh samples in multiple batches [22, 23]. Alternative protocols include cold storage of the tissue [24], addition of various fixatives and stabilisers to dissociated cells or minced tissues [25-29] or cryopreservation [30-32].

Tissue storage

In order to allow sufficient time to assess the quality of the collected tissue and to design and organize the follow up processing, clinical fresh specimens may be stored by cryopreservation. Cryopreservation entails controlled freezing of the small pieces of tissue in the presence of a cryopreserving agent (e.g., DMSO), protecting tissue and cell viability [30]. Alternatively, collected samples can also be cryopreserved as dissociated single-cell suspensions for subsequent scRNA-seq analysis[29]. The main advantage of cryopreservation is that it in enables a longer-term storage of the viable tissue. Hence, it allows thorough sample quality check before further processing, permitting exclusion of low-quality samples[30]. In addition, separating location and time of tissue collection from downstream scRNA-seq analysis facilitates complex designs and collaboration between multiple laboratories. Prior to cryopreservation, tissues can be macroscopically separated in areas of interest (eg. airways from lung parenchyma, or different parts of tumors, distal, central) allowing to expand and define the collection of viable tissue. However, small biases in population proportions might occur in cryopreserved samples [32, 33]. Hence, consistency across samples for a given tissue and experiment is recommended.

Tissue quality check before downstream analysis

As mentioned above, tissue cryopreservation enables the pathological evaluation of the sample before downstream processing, ensuring that only high-quality samples are processed and sequenced. For this, representative tissue slides (i.e. adjacent to the tissue piece that is cryopreserved) can be stained with haematoxylin and eosin (H&E) and analysed by experienced pathologists. If needed, specific informative markers can be used in immunohistochemistry analysis for further evaluation (e.g. cytokeratins for certain tumors). For diseased samples, histopathological evaluation allows better patient characterisation based on disease phenotypes (e.g., for lung tissue: presence of emphysema, fibrosis, alveolar thickening, extent and type of immune infiltration, tumor cell content, necrosis, etc.). For non-diseased (control) samples, it confirms the absence of disease phenotypes. Accurate selection of control and diseased samples based on both patient medical history and histological tissue characterisation will result in more meaningful data. For example, the presence of tumor cells or extensive inflammation in control samples increase the experimental noise and may mask disease-associated gene expression signatures. Hence, tissue quality check is critical, as it enables exclusion of low-quality samples, which is important for the discovery of disease-relevant changes. Additionally, having access to representative tissue slides of the cryopreserved tissue allows the validation and investigation of the genes identified by scRNA-seq analysis in a spatial approach and at a protein level.

Tissue dissociation into single-cell suspension

Dissociation of an organ or tissue into a single-cell suspension often requires a lengthy and complex protocol. Several parameters of the dissociation protocol need to be considered and optimised, including dissociation temperature, time, as well as the choice of the enzyme mix and dissociation device.

Digestion temperature: Dissociation temperature is an important parameter that needs to be tested and optimised when developing a new tissue processing workflow, as the use of warm

or cold dissociation may alter gene expression profiles. Tissue dissociation is commonly conducted using proteolytic enzymes that require incubation at 37 °C for optimal activity. Digestion at 37 °C may however introduce gene expression artifacts in specific cell populations (e.g., induction of heat shock proteins and immediate-early response genes) that correlate with the digestion time since mammalian transcriptional machinery is also active at this temperature[33, 34]. Hence, alternative protocols have been developed based on cold-adapted proteases that show high activity at low temperatures, where mammalian transcription is minimised[35, 36]. Additionally, computational approaches can be used to remove dissociation-affected cells from single-cell data sets in silico [34].

Dissociation enzymes: For tissue digestion several enzymes are typically used in various combinations and concentrations (e.g., trypsin, collagenase, liberase or dispase), often aided with mechanical agitation or dissociation. The choice of the enzymatic mix typically depends on the starting material and needs to be carefully optimised. Notably, when optimising a new workflow for fresh (or cryopreserved) tissue, different enzymatic combinations should be tested, as they may lead to different representations of cellular populations [37].

(Lung) Tissue processing workflow for scRNA-seq

Our complete tissue processing workflow, originally developed for human lung and described in detail in **section 3**, consist of four main steps: 1) Tissue collection 2) Tissue cryopreservation 3) Tissue quality check and 4) Tissue dissociation (**Figures 1 and 2**). First, tissue is collected in transport medium from the surgery theatre and sent to the processing laboratory. After initial washing, representative tissue pieces are collected for FFPE for subsequent histological analysis. The remaining material is cut into small pieces (approx. 4x4 mm) and cryopreserved for long-term storage (**Figure 1 top row**). The FFPE slides are stained by H&E and evaluated by an experienced pathologist. This tissue quality check is a critical step that allows characterisation and classification of the samples, and most

importantly, the exclusion of low-quality material. After histopathological evaluation, the cryopreserved tissue is thawed, washed and dissociated into homogenous single-cell suspension using a combination of mechanical and enzymatic processing. Finally, dissociated single-cell suspension with high-viability can be subjected to a scRNA-seq workflow (not covered in detail in this chapter).

2. Materials and Equipment

Required buffers, media, solutions, and specialist equipment are provided separately for the different steps of the workflow.

Tissue collection and transport:

1. Transport buffer (CO_2-i +++): CO_2-independent medium as base medium (Thermo Fisher Scientific), 1 % BSA, 1 % penicillin/ streptomycin, 1 % amphotericin B
2. 50 ml canonical (Falcon) tubes
3. Resealable plastic bag
4. Sterile forceps
5. Transport box filled with ice

Tissue washing and cryopreservation:

1. Plastic Petri dishes
2. 15 ml and 50 ml canonical (Falcon) tubes
3. Cryogenic tubes, 2.0 ml
4. Syringes (2.5 ml and 5 ml)
5. Syringe needles (0.5x25 mm)
6. 10 % neutral buffered formalin (Sigma)

7. Tissue wash buffer (HBSS++++): Hanks balanced salt solution (HBSS) without Ca2+, Mg2+ and phenol red as base buffer (Fisher Scientific), 2 mM EDTA, 1 % BSA, 1 % penicillin/streptomycin, 1 % amphotericin B
8. Cryopreservation medium for lung tumor and parenchyma: Dulbecco's Modified Eagle Medium (DMEM), high glucose, GlutaMAX™ as base medium (Thermo Fisher Scientific), 20 % Fetal Bovine Serum (FBS), 10 % Dimethylsulfoxide (DMSO)
9. Optional: Cryopreservation medium for airways and vessels (or dissociated single-cell suspensions): Cryostor (Sigma, C2874)
10. Sterile forceps and dissection scissors
11. Balance
12. Rocking table (at 4 ℃)
13. Two large ice boxes filled with ice
14. Cell freezing containers (e.g., MrFrosty from Thermo Fisher Scientific)
15. -80 ℃ freezer
16. Liquid nitrogen dewar and freezing boxes

Tissue dissociation:

1. Gentle MACS™ C tubes (Miltenyi Biotec)
2. Falcon cell strainers 40 µm, 70 µm, 100 µm
3. Human tumor dissociation kit (Miltenyi Biotec, 130-095-929) (see **Note 1**)
4. ROCK inhibitor, Y-27632, 2HCl (e.g., Adooq Bioscience) (see **Note 2**)
5. 100 µg/ml DNase I (e.g., ProSpec-Tany TechnoGene)
6. 20 % Fetal Bovine Serum (FBS), heat-inactivated (Gibco)
7. ACK lysis buffer (Thermo Fisher Scientific)
8. Trypan Blue solution 0.4 %, sterile
9. Sterile scalpels and forceps

10. Gentle MACS dissociator (Miltenyi Biotec) (see **Note 3**)
11. MACSmix™ tube rotator (Miltenyi Biotec)
12. Hemocytometer Counting Chamber (e.g., Neubauer Improved)
13. Water bath set to 37 ºC
14. Balance

    Optional:
15. FACS analyser/sorter
16. SyTOX blue viable dye

3. Methods

Tissue collection, transport, and initial processing:

1. Prepare 50 ml conical tubes filled with around 30 ml of CO_2-independent medium (CO_2-i+++) and store them at 4ºC until use (see **Note 4**).
2. Collect lung tissue piece from the surgery, place it inside a 50 ml conical tube containing CO_2-independent medium+++, carefully label the tube, put it in inside a plastic bag, seal tightly and keep on ice during transport in a sealed container. Transfer to the sterile tissue processing area as quickly as possible (see **Note 5**).
3. Under a class 2 cell culture hood disinfect forceps and cutting tools with 70 % ethanol, put them on a Petri dish and wait until ethanol has completely evaporated before starting the dissection procedure.
4. Place the tissue piece on a Petri dish, weigh it and record the tissue mass.
5. Wash the tissue extensively, first with ice-cold PBS and afterwards with ice-cold HBSS++++ (see **Note 6**).
6. (Optional): Carefully remove the pleura using surgical scissors (see **Note 7**).
7. For histological analysis of the tissue, slice representative pieces of the lung tissue/tumor and put in into a 15 ml conical tube containing 10 % neutral buffered

formalin for fixing. Rock the tube at 4 ºC for 24 h. Next day, discard the formalin (requires special waste), wash with PBS, and transfer the tissue to a 15 ml conical tube containing 70 % ethanol. Rock for an additional 24 h at 4 ºC and keep at 4 ºC in 70 % ethanol until paraffin embedding (see **Note 8**). For next steps on histological processing, refer to the tissue quality check section below.

8. Cut the remaining tissue into small pieces (approx. 4x4 mm) using surgical scissors (see **Note 9**).
9. Proceed with tissue cryopreservation (next section) or if processing fresh tissue, continue with tissue dissociation from step 3 (section after next) (see **Note 10**).

Tissue cryopreservation: (optional)

1. Transfer the small lung tissue pieces into a 2.0 ml cryotubes so that it each tube is filled no more than the 500 µl mark (equals approx. 10-15 pieces per tube).
2. Add 1-1.4 ml of ice-cold cryopreserving medium to fill the tubes, close the tubes, and flip them to distribute the medium within the tissue pieces. Make sure there are no tissue pieces stuck to the lid after flipping the tube.
3. Incubate the tubes on ice until tissue sinks in the tube (maximum15 min). This enables the cryopreserving medium to penetrate the tissue pieces.
4. Place the tubes in the freezing containers (e.g., Mr. Frosty) and bring them to a -80 °C freezer. In the following days, transfer the tubes containing cryopreserved tissue to a storage box pre-cooled at -80ºC and place it in the liquid nitrogen dewar for long-term storage (see **Note 11**).

Tissue quality check:

1. Embed the fixed tissue pieces in paraffin blocks. Cut 4 µm slides using a microtome and transfer to adhesive glass slides. Perform H&E and immunohistochemistry (IHC) staining, if needed, for subsequent histological analysis (see **Note 12**).
2. H&E/IHC-stained tissue slides should be evaluated by an experienced pathologist to 1) confirm and score disease phenotypes (for diseased samples) and 2) confirm the absence of disease phenotypes in control samples (see **Note 13**).

Tissue dissociation:

1. Thaw cryopreserved lung tissue pieces for 2 min in a water bath set to 37 °C (skip this step if fresh tissue is used) (see **Note 14**).
2. Quickly transfer all tissue pieces by flipping the thawed tubes onto a cell strainer (use forceps if same pieces are left in the tube) and wash them twice with HBSS++++ (skip this step if fresh tissue is used).
3. Transfer the tissue to a new Petri dish tube and weigh the tissue pieces on a balance. It is important to determine the exact weight of the used tissue to adjust the amount of enzyme and MACS C dissociation tubes that will be needed (see **Note 15**).
4. Under a class 2 cell culture hood, using scalpels or surgical scissors and forceps mince the tissue thoroughly into smaller pieces before transferring to MACS C tubes for mechanical and enzymatic dissociation
5. Transfer the minced tissue to a MACS C tube containing 4.5 ml of CO_2-i+++, add the enzyme mix from the human tissue dissociation kit, consisting of 200 µl enzyme H, 100 µl enzyme R, 50 µl enzyme A, 10 µM ROCK inhibitor and 100 µl DNaseI (see **Note 16**)
6. Close the MACS C tubes tightly and place them into the MACS dissociator for mechanical dissociation (see **Note 17**). Following the recommended program for lung tissue in the following order:

[Insert Table 1 here]

Dissociation step	Dissociator program	Incubation
1	h_tumor_01	15 min at 37 °C on MACSmix rotator
2	h_tumor_01	15 min at 37 °C on a MACSmix rotator
3	h_tumor_02	15 min at 37 °C on a MACSmix rotator
4	h_tumor_02	Transfer cells to class 2 cell culture hood

7. Pipet the dissociated mix up and down using a 10 ml serological pipet to support dissociation, aiming for a smooth mixture without clumps. Avoid bubbles.
8. Stop the dissociation process by adding 2 ml of 20 % FBS per MACS C tube (see **Note 18**).
9. To remove any leftover aggregates, tissue pieces and undissolved tissue matrix, pass the suspension through cell strainers of descending mesh size. Start with 100 µm, then transfer the flowthrough to a 70 µm cell strainer and collect into a 50 ml conical tube.
10. To maximise cell recovery, wash the tubes and strainers with 10 ml HBSS++++, pass through the strainer and combine with the cell suspension.
11. Centrifuge the cell suspension at 4 °C, 300 g for 8 min.
12. Resuspend cell pellet in 4 ml ACK lysis buffer and incubate for 4 min at room temperature (see **Note 19**).
13. Add 20 ml HBSS++++ and pass the cell suspension through a 40 µm cell strainer.
14. Centrifuge the cell suspension at 4 °C, 1.400 rpm (around 300 g) for 8 min and resuspend the cell pellet in 500 µl HBSS++++.

15. Take an aliquot of the cell suspension and determine cell number and viability. For example, when using trypan blue, mix the aliquot with trypan blue solution in a 1:1 ratio, load into a counting chamber (e.g., Improved Neubauer Chamber) for cell counting and to determine cell viability (see **Note 20**).
16. As a last step we recommend calculating the number of cells obtained per tissue weight by dividing the counted cell number by the initial mass of tissue used for dissociation (see **Note 21**).
17. The cells can be further processed by fluorescence-activated cell (FACS) sorting to enrich specific populations or cryopreserved as single-cell suspension before proceeding with the scRNA-seq workflow (if processed from fresh tissue).

4. Notes

Note 1: Upon arrival, the kit should be stored at 4°C. After reconstitution (following manufacturer's instructions), the enzymes should be aliquoted and stored at -20°C

Note 2: Y-27632 is a selective, cell-permeable inhibitor of Rho-associated kinase (ROCK). It is used to increase the survival of human stem cells after dissociation into single cells. We usually prepare a 10mM stock in DMSO, and store in small aliquots at -20°C for 6 months to 1 year. Once thawed, the aliquot should be stored at 4°C and used within 2 weeks.

Note 3: There are newer MACS dissociator models available with eight cartridges and integrated heaters (e.g., gentleMACS™ Octo Dissociator with heaters; 130-096-427, Miltenyi Biotec), which enable performing the dissociation procedure in one device. When using gentleMACS™ Octo, MACSmix™ tube rotator is not required.

Note 4: We typically obtain 0.5-10 g of human lung tissue from the surgery, but the protocol can be upscaled if needed for larger pieces (use multiple 50 ml Conical tubes or larger containers for transport).

Note 5: Care should be taken to carefully label the tubes, especially if multiple samples are being collected (e.g., tumor, parenchyma) to maintain traceability and avoid sample swap. To obtain the highest possible tissue and cell viability, the overall time from tissue collection to final processing should be as short as possible. We therefore usually process the tissue with several people in parallel. It is helpful to bring all the necessary materials to the class 2 hood area in advance of the tissue arrival and prepare and label all the required tubes and petri dishes. We usually also prepare at least two large ice boxes to maintain all the buffers ice-cold during tissue processing.

Note 6: The washing step is required to remove as much blood as possible from the tissue. Larger tissue pieces may be cut into smaller parts if washing is not efficient. Using a syringe

with a needle, infuse and wash the tissue piece gently, by "inflating it" with ice-cold PBS while holding it with the forceps. Repeat this step at least 4-5 times using ice-cold HBSS++++.

Note 7: The removal of the pleura is optional and depends on whether this part is of interest for analysis. The easiest way to remove the pleura, is to lift the tissue with the forceps and cut out the pleura with scissors as close as possible and parallel to the surface.

Note 8: If the tissue piece is heterogenous, we recommend taking representative pieces for FFPE from different parts and compartments, respectively, for a more representative characterisation and classification of the sample (e.g., presence of disease phenotypes in different lung compartments, like airways, vessels, or alveoli). Inclusion of this step allows collection of a sample for H&E/IHC staining and pathological evaluation (for more detailed discussion of the utility of that step, refer to **Note 13**). In addition, as the FFPE samples are collected from the same piece of tissue that is subjected to the scRNA-seq, they can be used for subsequent validation of the transcriptomic results (e.g., by immunofluorescence (IF) or IHC). Alternative tissue embedding methods (e.g., OCT) compatible with H&E, IF and IHC staining can be used to collect tissue for evaluation and subsequent validation. For FFPE samples, it is important not to extend the incubation time in the formalin solution beyond 24 h, as this may lead to over fixation. Once washed and transferred to ethanol, the tissue pieces can be stored at 4°C for several days before embedding.

Note 9: If an initial pre-enrichment of different tissue compartments or parts is desired (e.g., for the lung: distal versus proximal, tumor versus adjacent parenchyma, or large, airways versus alveolar parenchyma), the material can be separated prior cutting to small pieces via manual macroscopic dissection and the different fractions cryopreserved separately. We typically first cut the tissue into several smaller pieces and process one piece at the time (with several people in parallel). The lung tissue should be cut into small pieces between 9 mm^2

and 16 mm^2. The uncut lung should be kept in cold HBSS++++ (on ice) while processing the rest.

Note 10: Tissue cryopreservation permits thorough histological characterization of the collected tissue before time-consuming and expensive downstream dissociation and scRNA-seq. Additionally, it also enables more complex experimental design, as after thawing, samples from multiple donors can be processed and dissociated in parallel, technical biases related to multiple batch processing of fresh samples are avoided. However, small biases in the proportions of different cell populations may occur in cryopreserved samples [27, 33], therefore, careful consideration of the experimental design and consistency in the processing and storage across samples for a given tissue, samples and study is strongly recommended.

Note 11: We recommend the use of cell freezing containers (e.g., Mr. Frosty) to ensure a gradual temperature decrease (1°C/min). For short-term storage (up to 2 weeks), the samples can be stored at -80 ℃, but for longer storage the samples should be transferred to liquid nitrogen dewar or a -150 ℃ freezer to maintain their viability. When transferring, the frozen vials should be transported on dry ice since thawing of the samples will lead to decreased cell viability.

Note 12: Many research institutions offer tissue embedding and staining services. Alternatively, paraffin embedding, and H&E staining can also be outsourced (e.g., we successfully used service from Morphisto (Morphisto GmbH, Frankfurt, Germany) in the past). When cutting the slides by yourself, we recommend using adhesive glass slides (e.g., Superfrost Plus, Thermo Fisher). Subsequent drying of the sections O/N in a 40 °C oven removes excess water and enhances adhesion.

Note 13: Careful histological evaluation of the collected tissue is critical, as it enables exclusion of low-quality samples. From our experience with the collection of human lung

tissue from control donors with preserved lung function (as assessed by spirometry), only about 35-50 % of samples showed normal histological pattern expected for a control lung[30]. Other samples showed presence of moderate to extensive emphysema or fibrosis, alveolar wall thickening, as well as immune infiltration and other alterations. Although the implementation of the strict tissue-quality step results in a significant dropout of samples before sequencing, it results in a controlled setup, where the control and disease groups are divided not only based on clinical parameters, but also on histopathology of the same tissue piece which is used for downstream analysis.

Note 14: Transport the tubes containing cryopreserved tissue on dry ice after their retrieval from the liquid nitrogen dewar. The use of the water bath at 37 ℃ enables a fast thawing of the tissue samples, which is important to maintain high cell viability. When thawing multiple tubes, use a floating tube rack to hold the tubes immersed in the water bath.

Note 15: The dissociation kit manufacturer recommends using max. 1 g tissue per MACS C tube. If using 2 g of tissue, it is recommended to double the amount of enzyme mix, ROCK inhibitor and DNase I, since manufacturers protocol indications are for 1 g of tissue.

Note 16: The choice of the best dissociation conditions needs to be considered carefully and each parameter experimentally optimised. This includes choice of the enzyme mix, dissociation temperature, time, and dissociation device. For the dissociation of the lung parenchyma and lung tumors, we obtained very good results using tumor dissociation kit from Miltenyi Biotech and a gentle MACS dissociator. However, the efficiency of the dissociation largely depends on the type of tissue used, sampling method and requires a careful optimisation of the digestion parameters. Refer to Miltenyi website for guidance on available dissociation kits. Alternatively, digestion enzymes can be purchased separately and mixed in different composition during optimisation steps. In this case, we would recommend using high purity enzymes to avoid unspecific enzymatic activities. After dissociation, we

recommend running a flow cytometry analysis to analyse distribution of different cell populations and their viability. Ideally, the dissociation protocol will lead to an efficient release of all cell types (including rare and fragile cells), while preserving their integrity and variability.

Note 17: Different models of the MACS dissociators are available. Gentle MACS™ dissociator allows processing two samples in parallel and has no automated heating. Hence, the samples need to be transferred between the MACSmix™ tube rotator in the cell culture incubator and the bench-top dissociator. This can be time consuming and not ideal when processing multiple samples, as it may compromise cell viability. There are newer models available with eight cartridges and integrated heaters (e.g., gentleMACS™ Octo Dissociator with heaters; 130-096-427, Miltenyi Biotec), which enable performing the entire procedure in one device, making the dissociation process more efficient, easier, and faster.

Note 18: Addition of FBS inactivates the enzyme mix. Try to avoid foaming when pipetting up and down, since it may lead to decreased yield of single cells after the dissociation procedure. We use heat inactivated FBS to destroy complement that may lead to cell lysis by antibody binding.

Note 19: This step is optional and may not be needed if the sample has low red blood cell content (< 20 %). It is recommended if the tissue looks “bloody”, because if the erythrocytes are not removed, they will add to the total number of loaded cells, increasing the amount of sequencing reads needed for coverage of cells of interest.

Note 20: Trypan blue will stain dead cells dark blue, while leaving live cells unstained. It is compatible with any manual hemocytometer counting chamber and can be also used with many automated cell counters, however other dyes can be used. In addition, cell viability of the total suspension, as well as of specific fractions (e.g., epithelial or immune), can also be

evaluated by flow cytometry using viability dyes (e.g. SyTOX staining). The correct cell count is key as it ensures appropriate cell loading and droplet formation in the subsequent scRNA-seq workflow.

The viability of the cell suspension should be as high as possible, ideally above 90 %, but at least 75 %. We typically achieve 85-90 % viability for cryopreserved tumors and 78-94 % for cryopreserved parenchyma [30]. We successfully dissociated cryopreserved lung tissue from smokers, as well as COPD donors with various stages of COPD, maintaining viability above 80 %, indicating that this protocol can be used to profile both normal and COPD tissue. If higher viability is required, enrichment of viable cells by FACS can be performed. This step leads to the removal of debris, damaged and dead cells, as well as cell doublets and aggregates.

Note 21: This step is not strictly required, but it helps to estimate how well the dissociation protocol worked. It is also very helpful when comparing different dissociation conditions and enzyme mixes. The yield varies depending on the type of the sample (e.g., lung tumors are usually easier to dissociate than parenchyma) ,location of the lung the sample was taken from (high content of vessels or airways may decrease the yield), as well as the donor group (e.g. fibrotic lungs are harder to dissociate than normal lungs).

Figure legends:

Figure 1 Schematic representation of the main key steps of tissue processing workflow that need to be considered for single cell transcriptomics, including tissue collection and transport, tissue storage and tissue dissociation.

Figure 2 Schematic representation of the detailed steps of the tissue processing workflow described in section 3 (for details refer to section 3).

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