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| Tissue dissociation**Using human tumor dissociation kit from Miltenyi****Procedure:**1. The tissue (~5 mm3) is supplied in 5 ml of Tissue Storage Solution in a 50 ml tube on ice.

Other procedures, including centroifugation, are performed at RT, unless noted otherwise.1. Put a drop of 200 µL the Enzyme Mix at the corner of a tissue culture plate, which is secured to be held at the 45° angle, and place the tissue in the drop. Cut/chop into as small pieces as possible using scissors and scalpel.

 *Remove fat, fibrous, and necrotic areas from the tissue.**Transfer (see point 3) the remaining tissue in a tube with Tissue Storage Solution to keep it until you are sure to have the required yield of cells. Mouse lung tissue is stable in this solution for up to 6 hours.**Put the leftover in a cryo vial with <1mL of Freezing Medium (kept on ice) and keep in Mr. Frosty™ Freezing Container at -80°C overnight. Then, transfer to a liquid N2 tank for storage for future research. If the tissue is already dissociated, the cells can be snap frozen in N2 or -80*°C *ethanol.* 1. Transfer the 200 µL of the chopped tissue to a 2 mL tube using a 1000-µL Gilson pipet with cut-off tip. Collect the leftovers from the plate two times with a half of the prepared Enzyme Mix (each time), transferring to the 2 mL tube. Tightly close the tube and put it in the most outside extremity (to increase rpm) of the rotator at 37°C.

 1. Incubate sample for 15 minutes at 37°C with maximum rotation, pipetting it up and down (3x) for 2 min. every 5 minutes using a 1000-µL Gilson pipet tip (without cut off). After 15 min., remove the tube and vigorously resuspend the sample using a 1000-µL Gilson pipet tip for 2 min until you see no more macroscopic chunks.

*Checkpoints:* Counting with Trypan Blue - use a 10 µL aliquot.1. Place a strainer in a 50 mL tube and pre-wet the strainer with 1 mL DMEM. Apply the cell suspension to the strainer, collecting the content by gravity.
2. Wash the strainer with 12 mL of DMEM, collecting the content by gravity.

*Before discarding the strainer, make sure that the cells of interest are not retained in the strainer. Place the strainer in a 30 mm dish, fill it in with 1 mL PBS, collect the liquid while pipetting up and down and count a cell aliquot by hemocytometer.**Checkpoints:* Counting with Trypan Blue - use a 10 µL aliquot.Immunofluorescent labeling – use a 200 µL aliquot, to which add 24 µL paraformaldehyde (upto ~4 % paraformaldehyde) for 20 min. After that, centrifuge, remove the fixative and keep the sample in PBS until permeabilization and staining.*If multiple experimental procedures are pursued in parallel, split the sample in several tubes before proceeding with the centrifugation. Use the appropriate cell number for each and appropriate tubes.*1. Centrifuge the cell suspension at 300×g for 7 min. Collect the supernatant in a separate tube.
2. Resuspend the cell pellet in the appropriate Buffer, such as PBS/0.04% BSA or for further enrichment, ACK lysis buffer.

*Any leftover can be mixed up with the Freezing Medium (on ice) and frozen.* 1. **ACK lysis**

**Based on the protocol from Lonza** [**https://bioscience.lonza.com/lonza\_bs/US/en/Culture-Media-and-Reagents/p/000000000000181463/ACK-Lysing-Buffer-(1X)**](https://bioscience.lonza.com/lonza_bs/US/en/Culture-Media-and-Reagents/p/000000000000181463/ACK-Lysing-Buffer-%281X%29)* 1. For the pellet volume around 1 mm3, use 1 mL of ACK lysis buffer. Perform the treatment in a 15 mL tube. Homogenize the pellet by pipetting 10 times with a 1000-µL cut-off tip. Keep the sample on a bench for up to 2 min.
	2. Fill the tube with 10 mL PBS/0.04% BSA. Centrifuge at 300×g for 5 minutes. Check the redness of the pellet. Remove the supernatant using a serological pipet first and then the leftovers with a 200-µL tip, while leaving the cell pellet intact.

*If the pellet is still red, repeat the process one more time.* * 1. Resuspend the cell pellet in an appropriate solution.

*Checkpoints:* Counting with Trypan Blue.Immunofluorescent labeling.***Proceed straight to* *scRNA-seq or******preliminary cell separation by magnetic beads, in order to enrich for cell of interest*** | 05/15/2019**Reagents and Checkpoints:**21.4 ml DMEM at RT (room temperature). Make the aliquot in 1.5 ml tube for the Enzyme Mix to keep at 37°C.MACS Tissue Storage Solution, 100 ml (Miltenyi #130-100-008)Miltenyi Tumor Dissociation Kit, human(Miltenyi #130-095-929)Enzyme H; Enzyme R; Enzyme AScissors, Tweezers, ScalpelStrainer (40 μm)**Enzyme Mix** Prepare in 1.5 mL tube.50 μL Enzyme H 25 µL Enzyme R6.25 μL Enzyme A1119 µL DMEM (corning)PBS/0.04% BSA10 mL:9.99 mL 1x PBS40 µL BSA 10% (Sigma, # A1595 Sigma, fridge Max lab)ACK lysing buffer (Lonza # BW10548E)*Other:* Freezing Medium:90% FBS 10% DMSO37% Formaldehyde Solution (Fisher)Mini Incubator Labroller Rotator Combo, Labnet International  |