This protocol is used for the dissociation of human primary lung cancer resections to a single-cell suspension compatible with droplet-based single-cell RNA-Seq technology.

For the Human Tumor Atlas Pilot Project (HTAPP) it has been successfully applied to non-small cell adenocarcinoma from which it was able to capture a diversity of cell types, including malignant (epithelial) and non malignant cells such as mesenchymal, endothelial, myeloid, and lymphoid cells. In some instances, this protocol can be combined with a protocol for CD45-cell depletion, which is used for fast depletion of immune cells for enrichment of malignant and stromal cells.

Description of this protocol and guidance for testing and selecting methods for processing other tumor and sample types can be found in Slyper et al.

GUIDELINES

- Keep sample on ice and use cold reagents unless noted otherwise.
- Report information as indicated in the protocol.

MATERIALS
We use this protocol and it’s working.

Created: Jun 09, 2020

Last Modified: Aug 24, 2020

PROTOCOL integer ID: 37960

MATERIALS

- ACK Lysing Buffer Thermo Fisher Scientific Catalog #A1049201
- PBS, pH 7.4 Thermo Fisher Scientific Catalog #10010049
- Pronase from Streptomyces griseus Sigma Aldrich Catalog #10165921001
- Dispase II (neutral protease grade II) Sigma Aldrich Catalog #4942078001
- Collagenase A from Clostridium histolyticum Sigma Aldrich Catalog #10103578001
- Collagenase Type 4 100MG Thermo Fisher Scientific Catalog #NC9836075
- Elastase from Porcine Pancreas Thermo Fisher Scientific Catalog #NC9301601
- DNase I from bovine pancreas Sigma Aldrich Catalog #11284932001
- Trypan Blue solution 0.4% Sigma Aldrich Catalog #T8154-20ML
- Noyes Spring Scissors - Tungsten Carbide Fine Science Tools Catalog #15514-12
- Flex-Tube® 1.5 mL PCR clean colorless Eppendorf Catalog #022364120
- Tips RT-LTS-A-10µL-/F/L-960/10 Rainin Catalog #30389226
- Tips RT-LTS-A-200µL-/F/L-960/10 Rainin Catalog #30389240
- Tips RT-LTS-A-1000µL-/F-768/8 Rainin Catalog #30389212
- Falcon® 100 mm TC-treated Cell Culture Dish 20/Pack 200/Case Sterile Corning Catalog #353003
- Falcon® 15 mL High Clarity PP Centrifuge Tube Conical Bottom with Dome Seal Screw Cap Sterile 50/Rack 500/Case Corning Catalog #352097
- Falcon® 5 mL Round Bottom Polystyrene Test Tube with Cell Strainer Snap Cap 25/Pack 500/Case Corning Catalog #352235
- Pipet-Lite LTS Pipette L-1000XLS Rainin Catalog #17014382
- Pipet-Lite LTS Pipette L-200XLS Rainin Catalog #17014391
- Pipet-Lite LTS Pipette L-20XLS Rainin Catalog #17014392
<table>
<thead>
<tr>
<th>Item</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifuge 5430 R refrigerated with Rotor FA-45-30-11 incl. rotor lid keypad 120 V/50 – 60 Hz (US)</td>
<td>Eppendorf</td>
<td>022620601</td>
</tr>
<tr>
<td>Shake n Stack™ Hybridization Ovens</td>
<td>Thermo Fisher</td>
<td>6241</td>
</tr>
<tr>
<td>Aspen Surgical™ Bard-Parker™ Protected Disposable Scalpel</td>
<td>Fisher</td>
<td>02-688-78</td>
</tr>
<tr>
<td>HBSS no calcium no magnesium</td>
<td>Thermo Fisher</td>
<td>14170112</td>
</tr>
<tr>
<td>MACS SmartStrainers (70 µm)</td>
<td>Miltenyi</td>
<td>130-098-462</td>
</tr>
<tr>
<td>MACS SmartStrainers (100 µm)</td>
<td>Miltenyi</td>
<td>130-098-463</td>
</tr>
<tr>
<td>Eppendorf Tubes™ 5.0 mL</td>
<td>Fisher</td>
<td>14-282-305</td>
</tr>
<tr>
<td>MACS SmartStrainers (30 µm)</td>
<td>Miltenyi</td>
<td>130-098-458</td>
</tr>
<tr>
<td>UltraPure™ BSA (50 mg/mL)</td>
<td>Ambion</td>
<td>AM2616</td>
</tr>
<tr>
<td>NanoEnTek Disposable Hemocytometer</td>
<td>Westnet</td>
<td>C-CHIP</td>
</tr>
</tbody>
</table>

SAFETY WARNINGS

- Follow general lab safety and institutional guidelines for working with human samples and sharps.
BEFORE START INSTRUCTIONS

- Set centrifuge to 4°C.
- Set hybridization oven with rotator to 37°C.
- Label one 50 mL conical tubes as “Supernatant 1” and one 15 mL conical tube as “Supernatant 2” and keep on ice. These tubes will be used to collect supernatant before and after red blood cell removal, respectively, and prevent accidental loss of cells. If needed, cells can be recovered from the supernatants by centrifugation using settings from the protocol.
- Store ACK Lysing Buffer at 4°C or cool down on ice.
- Prepare PBS with 0.4% BSA, keep on ice. This solution can be prepared in advance and stored at 4°C for several weeks.
- Prepare dissociation mix immediately before use as described in the table below and keep on ice. The suggested amount is 3 mL per 25-200 mm³ resection, and may be adjusted depending on the size of the resection. Record in the table the volumes used.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock concentration (mg/mL)</th>
<th>Final concentration (µg/mL)</th>
<th>Volume for 3 mL Mix (µL)</th>
<th>Volume Prepared (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>-</td>
<td>-</td>
<td>2692</td>
<td></td>
</tr>
<tr>
<td>Collagenase A</td>
<td>150</td>
<td>1500</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Collagenase IV</td>
<td>100</td>
<td>100</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>DNAse I</td>
<td>10</td>
<td>100</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Dispase II</td>
<td>10</td>
<td>100</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Elastase</td>
<td>1</td>
<td>9.2</td>
<td>27.6</td>
<td></td>
</tr>
<tr>
<td>Pronase</td>
<td>20</td>
<td>1250</td>
<td>187.5</td>
<td></td>
</tr>
</tbody>
</table>

Sample Description and Allocation

1. Report sample processing information.

4 °C Wet Ice

Report sample processing information.
Transfer sample to a Petri dish with cold PBS (or HBSS without phenol red) kept on ice to better visualize its composition. Take a picture of the resection alongside a ruler and annotate its different regions. Tumors are typically stiff and light-colored, often with dark spots in lung cancer. Necrotic regions tend to be soft and crumbly.

Transfer the resection to a Petri dish with cold HBSS kept on ice and dissect the tumor from non-tumor tissue using a scalpel. Describe the decisions taken to dissect the sample and document them with an annotated picture.

If required, divide the tumor using a scalpel and allocate pieces to different assays following each assay’s requirements. Recommended dimensions to obtain enough cells for 10x Genomics Single-Cell RNA-seq system are 3x3x3 mm or larger. Describe the decisions taken to allocate the sample, including the dimensions of each piece, and document the allocation process with an annotated picture.
Describe Sample Allocation:

Dimensions of Tissue Piece Allocated (mm):

Insert Annotated Picture(s) Documenting Sample Allocation:

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**Tissue Dissociation**

5 If the dimensions of the tumor piece are greater than 3x3x3 mm, cut into smaller fragments using safety scalpels.

6 Transfer the tumor piece or fragments to a 5 mL Eppendorf tube containing 3 mL cold dissociation mix. Report time at which dissociation starts.

   *Tip: The suggested amount of 3 mL works well on 25-200 mm³ resections, but may be reduced or increased for smaller or larger resections, respectively.*

7 Mince the tissue with spring scissors into <0.5 mm fragments for approximately 3 min.

   *Tip: If spring scissors are not available, scalpels can be used instead. In this case, the sample should be minced in a Petri dish within 1 mL dissociation mix before transferring the resulting tissue fragment suspension to a 5 mL Eppendorf tube containing an additional 2 mL of dissociation mix.*

8 Incubate for 10 minutes at 37°C, with rotation at approximately 14 rpm.

   *Tip: Wrap the lid of the Eppendorf tube with Parafilm to prevent any leakage, and use lab tape to secure tubes on the rotator.*
9 Continue dissociation by pipetting up and down 20-30 times using a pipette with 1 mL tip. 
*Tip: Do not put the tube on ice at this step as repeated, drastic changes in temperature may be detrimental to cell viability. Pipette down pushing against the wall of the tube for optimal dissociation. If fragments are too large for pipetting, continue mincing with spring scissors before pipetting.*

10 Incubate for another 10 minutes at 37˚C, with rotation at approximately 14 rpm.
*Tip: Wrap the lid of the Eppendorf tube with Parafilm to prevent any leakage, and use lab tape to secure tubes on the rotator.*

11 Continue dissociation by pipetting up and down 20-30 times using a pipette with 1 mL tip. No or only very small fragments should be visible by eye and the solution should appear cloudy. 
*Tip: Pipette down pushing against the wall of the tube for optimal dissociation. If fragments are too large for pipetting, continue mincing with spring scissors before pipetting.*

12 Filter through a 70 µm cell strainer set on a 15 mL conical tube kept on ice to get rid of fragments. Wash strainer with 10 mL cold HBSS. 
*Tip: If the mixture appears to have a lot of undigested tissue pieces or mucus, which may clog the 70 µm strainer, use a 100 µm strainer instead.*

13 Centrifuge at 400 g for 5 minutes in 4˚C pre-cooled centrifuge. This long spin helps to get rid of fat more efficiently than subsequent short spins.

14 Report pellet color (e.g., red, pink, white) in the first row of the table at Step 20.

15 Carefully transfer supernatant to the 50 mL “Supernatant 1” tube kept on ice, making sure to remove any fat and to not disturb the pellet. 
*Tip: If any fat is visible, aspirate and discard it before transferring the remaining of the
16. Resuspend pellet in 300-500 µL cold ACK Lysing Buffer to lyse red blood cells. If cells are in a 15 mL tube, transfer them to a 1.7 mL Eppendorf tube kept on ice. 

*Tip: The volume of ACK should be adjusted to the size and color of the pellet and may be increased up to 1 mL if the pellet is large or extremely bloody.*

17. Incubate for 1 minute on ice.

18. Mix in a volume of PBS equal to twice the volume ACK Lysing Buffer. Proceed quickly to the next step.

*Tip: If more than 500 µL ACK Lysing Buffer was used, mix in the largest volume of PBS that can safely fit in a 1.7 mL Eppendorf tube (500 µL - 1 mL) and proceed quickly to the next step.*

19. Centrifuge for 8 sec at 4°C using short spin setting, with centrifugal force ramping up to (but not exceeding) 11,000 g. Proceed quickly to the next step.

*Tip: Do not spin for a longer duration or at a higher centrifugal force, as this would result in cell death.*

20. If the pellet is pink or red, revealing a significant portion of red blood cells, repeat steps 15-19. For each round of ACK Lysing Buffer treatment, report the ACK Lysing Buffer volume, treatment duration, and pellet color in the table below.

*Tip: Avoid exceeding three rounds of 1 min ACK Lysing Buffer treatment, as this may result in important loss of cell viability.*

<table>
<thead>
<tr>
<th>Repeat</th>
<th>ACK Lysing Buffer Volume (µL)</th>
<th>ACK Lysing Buffer Treatment Duration (min)</th>
<th>Pellet Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
21 Carefully transfer supernatant to the 15 mL “Supernatant 2” tube kept on ice without disturbing the pellet.

4 °C Wet Ice

22 Resuspend in 50 µL cold PBS with 0.4% BSA.

4 °C Wet Ice

### Quality Control

23 Mix 5 µL of single-cell suspension with 5 µL Trypan blue and load on hemocytometer.

24 Count and report the number of viable single cells, dead single cells, cell doublets or clumps, and whether debris are present, then calculate additional quality control metrics below. Take picture if possible.

<table>
<thead>
<tr>
<th>Initial Quality Control</th>
<th>Quality Control after Optional Debris and Cell Clumps Removal (Step 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Viable Single Cells Counted</td>
<td></td>
</tr>
<tr>
<td>Number of Dead Single Cells Counted</td>
<td></td>
</tr>
<tr>
<td>Number of Cell Clumps or Doublets Counted</td>
<td></td>
</tr>
<tr>
<td>Concentration of Viable Single Cells (cells/µL)</td>
<td></td>
</tr>
<tr>
<td>Concentration of Dead Single Cells (cells/µL)</td>
<td></td>
</tr>
<tr>
<td>Concentration of Cell Clumps or Doublets (doublets/µL)</td>
<td></td>
</tr>
<tr>
<td>Volume of Single Cell Suspension (µL)</td>
<td></td>
</tr>
<tr>
<td>Total Number of Viable Single Cells</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>--</td>
</tr>
<tr>
<td>Proportion of Single Cells that are Viable (%)</td>
<td></td>
</tr>
<tr>
<td>Proportion of Cell Clumps or Doublets (%)</td>
<td></td>
</tr>
<tr>
<td>Description of debris (if any)</td>
<td></td>
</tr>
</tbody>
</table>

**Note**

Insert Picture for Initial Quality Control:

Insert Picture for Final Quality Control (if additional cleanup was performed as described in Step 25):

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### [Optional] Debris and Cell Clumps Removal

**25**

If the quantity of debris or cell clumps is too high to load on 10x Genomics Single-Cell RNA-seq system and the number of cells is at least double from what is required to load, strain the sample as described below.

*Tip: Samples should typically have less than 5% cell clumps. If enough cells (e.g., at least four times the number of cells to be loaded), strain only half of the sample and keep the other half as a backup.*

**25.1**

Resuspend in 500 µL cold PBS with 0.4% BSA.

![4 °C Wet Ice]

**25.2**

Filter through 35 µm FACS tube cell strainer.

*Tip: Alternately, 30 µm MACS SmartStrainer can be used to minimize cell loss.*

![4 °C Wet Ice]

**25.3**

Wash filter with an additional 500 µL of cold PBS with 0.4% BSA to recover as many cells as possible.

![4 °C Wet Ice]
25.4 Transfer to Eppendorf tube.

25.5 Centrifuge for 8 sec at 4°C using short spin setting, with centrifugal force ramping up to (but not exceeding) 11,000 g. Proceed quickly to the next step.

Tip: Do not spin for a longer duration or at a higher centrifugal force, as this would result in cell death.

25.6 Carefully transfer supernatant to the 15 mL “Supernatant 2” tube kept on ice without disturbing the pellet.

25.7 Resuspend in 50 µL cold PBS with 0.4% BSA.

25.8 Repeat steps 23-24.

Loading on 10x Genomics Single-Cell RNA-seq system

26 If necessary, adjust the concentration before proceeding to loading on 10x Genomics Single-Cell RNA-seq system, following 10x Genomics recommendations.

Tip: 8,000-10,000 live cells are typically loaded per channel. Optimal cell recovery is achieved for concentrations between 800 and 1,200 cells/µL but deviations from that range are acceptable (see 10x Technical Note on this topic). Furthermore, it is recommended that viability be higher than 60% and the proportion of cell clumps lower than 5%.

27 Report the information listed below about loading on 10x Genomics Single-Cell RNA-seq system, including the number and concentration of cells per channel.
# Note

**Time of Loading:**

**Person Loading:**

**Single-Cell RNA-seq Kit Used:**

**Concentration of Viable Cells Loaded (cells/µL):**

**Number of Cells Loaded per Channel:**

**Number of Channels Loaded:**