HTAPP_Dissociation of human primary colorectal cancer resection specimens into single cell suspension for single cell RNA-seq V.2

Version 2
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ABSTRACT

This protocol describes the dissociation of primary human colorectal cancer (CRC) resection specimens into single cell suspensions for single cell RNA-seq applications. It was successfully used for droplet-based single cell RNA-seq of more than 60 patient specimens using the 10x Genomics platform, including 21 HTAPP CRC cohort samples. It can also be used upstream of other single cell RNA-seq approaches such as plate-based SS2 single cell RNA-seq protocols.

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MATERIALS TEXT

MATERIALS

RPMI 1640 Medium **Thermo Fisher Scientific Catalog #11875093**

Scalpel **VWR International Catalog #89176-380**

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

Cell strainer Falcon 70um **VWR International Catalog #21008-952**

Human Serum **Sigma Aldrich Catalog #H3667-100ml**

ACK Lysis buffer **Thermo Fisher Scientific Catalog # A1049201**

BSA **Cell Signaling Technology Catalog #9998S**

Falcon® 5 mL Round Bottom Polystyrene Test Tube with Cell Strainer Snap Cap **Corning Catalog #352235**

Zombie Violet™ Fixable Viability Kit **BioLegend Catalog # 423113**

PBS pH 7.4 **Gibco - Thermo Fischer Catalog #10010-023**

Trypan Blue Stain (0.4%) for use with the Countess™ Automated Cell Counter **Thermo Fisher Scientific Catalog #T10282**

Sample Description and Allocation

1 Report sample information.

Sample ID:

Time specimen out:

Time start tissue triage:

Other assays tissue is allocated to:

2 Sample allocation:

Transfer resection specimen into a Petri dish kept on ice. Take a picture of the specimen alongside a ruler. Remove fat, necrotic and fibrous areas. Wash off residual blood and stool using cold RPMI as needed. Dissect tissue using a scalpel in order to allocate tissue to assays as required. Weigh specimen allocated for scRNAseq using a precision scale. Work quickly. In order to weigh the tissue, carefully tap tissue onto a kimwipe to remove excess liquid and transfer onto a petri dish sitting in a precision scale. Move tissue back into petri dish on ice as quickly as possible and proceed to the

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next step immediately. If delays occur, make sure to cover the specimen with RPMI.

4 °C wet ice

Picture of sample:

Description of sample appearance:

Weight of specimen allocated for scRNAseq:

Tissue Dissociation

3 Mechanical dissociation: Mince tissue allocated for scRNASeq into small pieces (~1 mm^3) using a scalpel. Work quickly and keep the specimen in the petri dish on ice during this procedure. Mechanical dissociaton is critical to ensure efficient enzymatic digestion.

4 °C wet ice **00:05:00**

4 Enzymatic digestion: Transfer dissociated tissue into 1 ml eppendorf tubes, each containing 1 ml of enzymatic digestion mix (Miltenyi, human Tumor Dissociation kit) containing:

Enzyme H	100 ul
Enzyme R	50 ul
Enzyme A	12.5 ul
RPMI 1640	2.35 ml

Enzymatic digestion mix (according to Miltenyi, Human Tumor Dissociation kit)

Use 1 ml of digestion mix per 50 mg of tissue. Invert tubes to resuspend the tissue. Transfer eppendorf tubes to a rotation shaker set to 37˚C and 550 rpm and incubate shaking for 20 min.

37 °C rotation shaker, 550 rpm **00:20:00**

5 70 um filter step: Transfer digestion mix including any remaining tissue pieces onto a 70um cell strainer sitting on a 50 ml falcon tube on ice. Cut off the end of pipette tip and use low retention tips in order to prevent the tissue from sticking to the pipette tip. Flush the eppendorf tube once with RPMI containing 2% human serum. Use plunger of a 1 ml syringe in order to carefully squeeze the tissue pieces through the filter. Flush with RPMI containing 2% human serum as needed until the suspension has passed through the filter.

4 °C wet ice

6 Centrifugation: Centrifuge cell suspension at 500 g for 7 min in 4˚C pre-cooled centrifuge.
Note: If the cells fail to pellet after this spin, repeat this step.

4 °C **500 x g** **00:07:00**

Red Blood Cell removal

7 Carefully transfer supernatant into a 50 ml falcon labeled 'Supernatant' kept on ice. Try to remove as much liquid as possible without disturbing the pellet (usually keeping about 100-500 ul on the pellet). The supernatant will be discarded unless the pellet got disturbed in which case step 6 needs to be repeated with the supernatant tube.

4 °C

8 Resuspend pellet in 4 ml of ACK lysis buffer and lyse red blood cells for 2 min on ice.

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4 °C wet ice 00:02:00

9 Stop ACK lysis by adding 26 ml of cold RPMI containing 2% human serum.
4 °C wet ice

10 Centrifugation: Centrifuge cell suspension at 500 g for 7 min in 4˚C pre-cooled centrifuge.
Note: If the cells fail to pellet after this spin, repeat this step.
4 °C 500 x g 00:07:00

Final cleanup

11 Carefully transfer supernantant into a 50 ml falcon labeled 'Supernatant' kept on ice. Try to remove as much liquid as possible without disturbing the pellet (usually keeping about 100-500 ul on the pellet). The supernatant will be discarded unless the pellet got disturbed in which case step 10 needs to be repeated with the supernatant tube.
4 °C

12 Resuspend cell pellet in cold 10x loading buffer (PBS containing 0.04% m/v BSA). 10x loading buffer should be freshly prepared on the day of the experiment. However, sterile filtered 4% m/v BSA in PBS stock solutions (2 g BSA in 50 ml PBS final volume) can be stored at 4˚C and diluted 1:100 at the day of the experiment. Adjust the volume to the size of the cell pellet, start with 500 ul and increase the volume if the cell suspension is too dense to filter. Use low retention tips.
4 °C wet ice

13 Filter the cell suspension through a blue cap FACS filter top. If cell suspension is not passing through the filter, dilute the cell suspension and collect the cell suspension from the bottom of the filter using a pipette. Transfer the filtered cell suspension into an 1.5 ml eppendorf tube.
4 °C wet ice

14 Centrifugation: Centrifuge cell suspension at 500 g for 2 min in 4˚C pre-cooled centrifuge. Use a swing out rotor in order to pellet cells at the bottom of the eppendorf tube.
4 °C 500 x g 00:02:00

15 Carefully transfer supernantant into a 1.5 ml eppendorf tube labeled 'Supernatant' kept on ice. Try to remove as much liquid as possible without disturbing the pellet (usually keeping about 10-20 ul on the pellet). The supernatant will be discarded unless the pellet got disturbed in which case step 14 needs to be repeated with the supernatant tube.
4 °C

16 Resuspend the pellet in cold 10x loading buffer (PBS containing 0.04% m/w BSA). Judge by the size of the pellet how much volume to use, typically around 50-500 ul.
4 °C wet ice

Quality control

17 Mix 1 ul of single cell suspension with 9 ul of trypan blue and count live and dead cells using a hemocytometer. Report cell number and viability.
Note: If cell viability is low or lots of debris is visible, go back to step 13 for an additional filter step. Viability should not be below 60%.

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Cell number:

Cell viability:

Other observations (e.g. debris, cell clumps):

10x loading

18 Adjust cell concentration to 1E6 cells/ml (counting both live and dead cells) by diluting cell suspension in cold 10x loading buffer (PBS containing 0.04% BSA) and immediately proceed to 10x loading.
Note: If more cells than needed are available, only a part of the cell suspension can be diluted for 10x loading and the remaining cell suspension can be used for other purposes.

19 Report the time at which the sample is loaded onto the 10x machine, the person loading, and the concentration and number of cells loaded.

Time of Loading on 10x machine:

Person Loading:

10x kit Used:

Numer of Cells Loaded per Channel:

Number of Channels loaded:

Optional: Post loading QC

20 *Note*: For additional quality assessment, any left over cell suspension after 10x loading can be used for flow analysis. The flow staining panel should be adjusted based on the focus of the study and available cytometer. The staining procedure and panel described below can be used to assess the general cellular composition and is compatible with SONY SH800 cell sorters if sorting of specific cell populations is desired.

21 Add 1 ml of cold PBS to remaining cell suspension to wash out any remaining BSA and centrifuge cell suspension at 500 g for 2 min in 4˚C pre-cooled centrifuge. Use a swing out rotor in order to pellet cells at the bottom of the eppendorf tube.
4 °C **500 x g** **00:02:00**

22 Live/Dead staining: Carefully remove as much liquid as possible without disturbing the pellet (usually keeping about 10-20 ul on the pellet) and resuspend the cell pellet in Zombie Violet Fixable Viability dye (Biolegend 423113, stock solution prepared in DMSO as recommended by the manufactorer) diluted 1:100 in cold PBS and incubate
4 °C **00:10:00**

23 Add 1 ml of cold PBS containing 0.5% BSA and 2mM EDTA to cell suspension and centrifuge cell suspension at 500 g for 2 min in 4˚C pre-cooled centrifuge. Use a swing out rotor in order to pellet cells at the bottom of the eppendorf tube.
4 °C **500 x g** **00:02:00**

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24 Antibody staining: Carefully remove as much liquid as possible without disturbing the pellet (usually keeping about 10-20 ul on the pellet), resuspend the cell pellet in 100 ul of the following antibody master mix and incubate

4 °C **00:20:00**

Human TruStain FcX™ Fc Receptor Blocking Solution, Biolegend 422301	5 ul
APC anti-human CD326 (EPCAM), Biolegend 324207	1 ul
PE-Cy7 anti-human CD235a, Biolegend 349112	1 ul
AF700 anti-human CD45, Biolegend 304023	1 ul
FITC anti-human CD2, Biolegend 300203	1 ul
FITC anti-human CD3, Biolegend 302805	1 ul
FITC anti-human CD19, Biolegend 302206	1 ul
FITC anti-human CD20, Biolegend 302304	1 ul
FITC anti-human CD56 Biolegend 318304	1 ul
PE anti-human CD66b Antibody, Biolegend 305105	1 ul
PBS containing 0.5% BSA and 2 mM EDTA	86 ul

Antibody master mix

25 Add 1 ml of cold PBS containing 0.5% BSA and 2mM EDTA to cell suspension and centrifuge cell suspension at 500 g for 2 min in 4˚C pre-cooled centrifuge. Use a swing out rotor in order to pellet cells at the bottom of the eppendorf tube.

4 °C **500 x g** **00:02:00**

26 Measure on flow cytometer.

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