# Sample Processing for Single Cell Dissociation

## Reagents (Backstock Locations)

* RPMI and RPMI/10% FCS media – 9th floor cold room
* Dissociation consumables – Under Jamie’s desk, or beside the fume hood
* H, R, A enzymes – 20°C freezer in S920, second shelf
* Backup enzymes (to be aliquoted) – 4°C fridge in S920, top shelf
* RBC Lysis Buffer (ACK Buffer) – Shelf above bench where Countess II sits
* Trypan Blue – shelf to left of fume hood
* Cell counting slides – Behind Countess II Counter

## Tissue dissociation

1. Add 5 ml of serum free RPMI media into a MACS C tube (purple top)
2. Use sterile forceps and sterile scalpel to cut the tumor (<1g) into 1 mm pieces on a cell culture plate **on ice**.
3. Transfer the samples into the MACS C tube, add the three enzymes (H, R, A) to the media.
4. Place the C tube upside down on the octo dissociator and be sure the cap is clicked into place. Place the heating jacket over the MACS C tube.
5. Use the 37C\_h\_TDK\_3 program and let sit for half hour.
6. Ensure that centrifuge is set to 4°C.
7. After the dissociation is over, place a 100-micron filter on a 50ml falcon tube **on ice** and add ~5 mL of RPMI + 10% FCS to wet the filter. Pour the sample over the filter.
8. Use the back of a syringe to push the cells through the filter.
9. Add 5ml of RPMI + 10% FCS to the sample to pass the rest of the cells though the filter and to inactivate the enzymes.
10. Spin cells down at 1500 rpm for 4 minutes at 4°C, discard supernatant and resuspend in 2mL of RBC lysis buffer (ACK lysing buffer).
    1. If the sample looks too bloody, wash with RBC lysis buffer again.

**\*\*\*** (process ascites sample simultaneously)

1. Incubate at room temperature for 3 minutes, and quench with 8mL RPMI + 10% FCS.
2. Run sample through 70-micron filter into a falcon tube **on ice**.
3. Count cells

## \*\*\*Ascites processing

1. Spin cells down at 1500 rpm for 4 minutes at 4°C, remove supernatent (for DMT collaboration: store supernatant in 15ml falcon tubes and freeze down)
2. Resuspend pellet in 2mL of RBC lysis buffer (ACK lysing buffer).
3. Incubate at room temperature for 3 minutes, and quench with 8mL RPMI + 10% FCS.
4. Run sample through 70-micron filter. Rinse filter with 5ml RPMI + 10% FCS into a falcon tube **on ice**.
5. Count cells

# Cell counting

Using Countess II automated cell counter

1. Ensure all samples are on ice
2. Invert falcon tube prior to pipetting sample
3. Add 10ul of sample to 10ul of Trypan blue dye, **mix well**
4. Insert Countess cell counting slide into Countess II
5. Record the following info:
   1. Cell counts (cells/ml)
   2. Cell viability (%)
   3. Cell counts of live cells (cells/ml)
   4. Total volume of sample
6. Aliquot ~2-3 million cells per sample into a new falcon tube for antibody staining
7. Aliquot ~300,000 cells (from any sample with high cell yields) into a 1.5ml epi tube to be used for the negative and L/D controls for antibody staining (2 tubes of 300,000 cells each)
8. Place remaining cells on ice for storage

NOTE: Formula for counting on hemocytometer:

(Cells counted / 4 ) X 10,000 X Dilution factor = cells / mL

For total # of cells, multiply by total volume.

# Antibody Staining

## Reagents

* RPMI and other media – 9th floor cold room
* PBS – On shelf above cell line bench
* CD45 and Fc block – Grey box in 4°C fridge under DNA extraction bench, first shelf
* GhostRed780 dye – Blue box in 20°C freezer under pathology bench (Fresia’s bay)
* Ultracompetent beads – “Various” box in 4°C fridge under DNA extraction bench, bottom shelf
* FACS tubes – On shelf above cell line bench

Sample summary:

1. Samples (single cell sites)
2. L/D positive control
3. Negative control

Single cell samples

1. Spin single cell aliquots at 1500 rpm for 4 minutes at 4°C, remove supernatant
2. Prepare PBS with L/D dye (GhostRed780 @ 1:2000) and Fc block (Human TruStain FcX @ 1:100) master mix
   1. *1ml PBS + 0.5ul GhostRed780 – Take 500ul of mixture + 5ul TruStain FcX block*
3. Incubate samples for 10 minutes on ice in 505ul prepared master mix

NOTE: While samples are incubating, prepare RPMI + 2% FBS with antibody (CD45 @ 1:100) master mix.

* 1. *500ul RPMI/2% FCS + 5ul CD45 antibody*

1. Post-incubation, spin sample down (1500 rpm for 4 minutes at 4°C), remove supernatant
2. Incubate for 30 minutes on ice in RPMI/2%FBS with antibody master mix

NOTE: While samples are incubating:

1. Prepare controls (see below “Flow controls”).
2. Prepare enough 1.5ml eppendorf tubes (no need to label) – 2 tubes per site to be sorted (CD45+/CD45-), 1 extra tube per site to collect any extra CD45- cells (3 tubes/site in total)
   1. Wash the walls of each epi tube with RPMI + 2% FCS prior to filling tube with 200ul RPMI + 2% FCS (this is to ensure that when the cells are being sorted into each tube, they do not stick to the walls of the dry tubes)
3. Place tubes on ice and bring to flow sorting session
4. Spin sample down (1500 rpm for 4 minutes at 4°C), remove supernatant
5. Resuspend sample in 500ul RPMI + 2% FCS and use the same pipette tip to transfer sample
6. Transfer sample to blue cap FACS tubes
   1. Label FACS tubes with corresponding “spectrum ID-sample site abbreviation” (eg. 001-RA)

Flow controls (Live/Dead positive control)

1. Spin the live/dead aliquot down in the centrifuge on the cell line bench at 1500rpm for 4 minutes at 4°C (can be done concurrently with the negative control)
2. Remove the supernatant and resuspend the cell pellet in 500ul RPMI + 2% FCS and 0.5ul of GhostRed780
3. Transfer sample to blue cap FACS tubes

Flow controls (Negative control)

1. Spin the aliquot down in the centrifuge on the cell line bench at 1500rpm for 4 minutes at 4°C (can be done concurrently with the positive control)
2. Remove the supernatant and resuspend the cell pellet in 500ul RPMI + 2% FCS
3. Transfer sample to blue cap FACS tube and keep on ice

Flow controls (CD45 positive control)

1. **Vortex ultra-competent beads thoroughly**
2. Add 2 drops of ultra-competent beads to tube and stain with 2ul of CD45 antibody
3. Incubate on ice for 15 minutes
4. Wash with 500ul PBS (Spin at 1500 rpm for 4 minutes at 4°C)
5. Resuspend in 100ul PBS, transfer to blue cap FACS tube and keep on ice

Bring prepared samples to Flow Cytometry (RRL 9th floor for ARIA 5 and 6, or Zuckerman 16th floor for ARIA 1 and 3) to have samples sorted into CD45+ and CD45- populations. Sort for maximum amount of cells for each population (minimum 150,000 cells, if possible). Each epi tube will hold approximately 300,000 cells. Request that flow summary and all fcs files are emailed post-sort.

While samples are being sorted, prepare IGO iLabs submission form (10X Genomics 3’ Gene Expression) and REX (Sample Submission form). Target 5,000 cells per sample, if possible. REX Sample IDs should be in the format “OV#SiteAbbreviation\_CD45P/N” (eg. 001RA\_CD45P).

1. After samples are sorted into their respective CD45+ and CD45- tubes, bring samples **on ice** to IGO for 10X scRNA seq submission
2. Ensure that cell counts are sufficient for scRNA sequencing (check with IGO tech)
3. Bring cryovials, labels, freezing foam container and freezing media to IGO and have them store any remaining CD45- samples
4. If there are extra aliquots of CD45- populations from sort, please see 3. from Sample Storage below.

# Sample labelling and storage

## Reagents

* Freezing media (FCS + 10% DMSO) - 4°C fridge under the Countess II Counter
* FCS - 20°C freezer under the plate reader bench (behind fume hood)
* DMSO – in shelf left of fume hood

Sample storage

1. Spin down remaining sample (1500 rpm for 4 minutes at 4°C) and store approximately 3-5million cells, if possible, in 1ml of freezing media (FCS + 10% DMSO) per cryovial.
2. Label and place cryovials into a freezing foam container in the -80°C freezer
3. If there are any extra CD45- sorted populations, spin down, remove supernatant and resuspend in freezing media as above for storage. Place cryovials into a freezing foam container in the -80°C freezer. Ensure cryovials are labelled accordingly below.
4. After 24 hours, move cryovials into respective storage boxes and update the inventory spreadsheet.

Sample labels

SPECTRUM-OV-001

RUQ CD45- SCS

01/01/2019 JL

SPECTRUM-OV-001

RUQ SCS

01/01/2019 JL