# Sample Collection

NACT/PDS processing steps are the same only differences is in labeling and funding

Immediate upon notification from Michelle of a new case:

* Check flow/IGO, if available, no further correspondence needed
* If not available, but the case is rare/important:
  + Email flow core staff for waitlist –
    - include date of submission, time range (1:30-4:30pm), 2 hour time slot, type out iLab submission form details
      * Mark Kweens ([kweensm@mskcc.org](mailto:kweensm@mskcc.org))
      * Fang Fang ([fangf@mskcc.org](mailto:fangf@mskcc.org))
      * Joana Da Silva Leite ([dasilvaj@mskcc.org](mailto:dasilvaj@mskcc.org))
    - Check every day for cancellations even without response from above
    - If no go within ~24h before case
  + Register for IGO’s waitlist in their iLab page
    - If really important case Jamie will email IGO manager Andrea Farina ([farinaa@mskcc.org](mailto:farinaa@mskcc.org)) indicating we have a 10X 5’ run, [#] samples to submit, can he accommodate w the other user?
    - Both waitlist and Andrea will respond by email if yes
    - If no response within ~24h of the case then answer is probably no

Getting off the waitlist actions:

|  |  |  |  |
| --- | --- | --- | --- |
| **Flow** | **IGO** | **Lab tech action** |  |
| Yes | Yes | As planned |  |
| Yes | No | Cancel flow within 24h of case | Full booking fee is charged if not canceled in time |
| No | Yes | Cancel IGO within 3h of case | Pay a fee (unsure, Jamie has never not canceled in time) |
| No | No | Storage only |  |

* Update “Patient OR Collection Record” (will move to new central SPECTRUM folder) all pt info copied from Michelle’s “pt tracker”
* Updates surgery time, type, flow/IGO, IRB consents, and any notes (reasons for no flow/IGO, identity of person collecting if not Jamie)

~48h prior to case start

* Check Michelle pt tracker for updated study ID
* Check HIS for start time, OR location and room #
* Update Jamie’s spread sheet w this info
* Prep OR collection forms (PBMC, tissue)
* Prep tubes for bloods (when applicable)
* Ensure OR kit is stocked
  + Forceps (under Jamie’s desk)
  + Scalpel (second drawer under the microdissection bench)
  + Gauze (shelf between culture hoods)
  + Collection forms (above)
  + Pen
* Check covid status 24h before

Emails to OR staff and PPBC (if applicable) sent out around this time Michelle to expand this

GOING TO THE OR

* Bring collection/OR kit, box of ice w tubes of media, scrubs
* MAIN
  + Take B elevators to floor 6
  + Out of elevators turn right and go through automatic door,
  + 1st door on left after automatic door – wait for when someone opens the door
  + grab scrub cap and booties from shelf (on the right), change in locker room

JOSIE

* Enter building on 61st and York entrance
* Jamie: add OR room floors
* Elevator to floor 13 for locker rooms
* Out of elevator turn left, enter doors, obtain scrubs from machines on the right
* Change in locker room
* Ensure hands are washed and face mask is on
* Enter OR
  + Double check pt info on electronic screen to ensure you’re in the right surgery
* Step to the side while staff prep – **avoid touching sterile items (anything covered in a blue sheet) and staff who have scrubbed in**
* When appropriate, let fellow know you’re there to collection tissue specimens for research
  + If lsc case, also give the collection marking sheet
  + Fellows usually come into the case a little earlier and are at the computer or standing around
  + Do not approach if fellow is working directly on the pt in the OR

## Blood Collection from OR

1. Call OR room (MAIN x5924, JOSIE x125 7070, request OR room) half an hour before case begins (defined as “Rm Set-Up St” status in greaseboard – indicates case OR staff are in room) to inform Anesthesiologist/cRNA that you will be needing to collect blood prior to surgery, let nurses know that you need blood collected before they tuck the patient’s hands
2. Arrive to the OR 15 mins after the OR update indicates “Patient in Room” (for Josie leave lab as soon as status is “Rm Set-Up St”- **if it’s first case just be in the OR by 7:30am**)
3. Wait until pt is intubated (when pt has a tube inserted in her mouth), let staff know total volume of blood needed
   1. When anesthesiology team start prepping pt’s arms – approach with tubes
   2. If above does not start prior to RNs start of prepping pt (steriziling pt’s abdomen/vagina w orange fluid) – approach with tubes
4. Either staff will fill the tubes directly or will give you a syringe and you will need to inject the tubes yourself
5. Invert tubes several times – gently
6. Ensure tubes are each labeled with pt label
   1. Labels are on the nurse’s bench in the OR or ask a circulating nurse (a nurse that is not scrubbed in) if can’t find yourself
7. After blood is collected:
   1. **CPT**
      1. Complete IMF form with time of blood collection and name of staff member who did the blood draw (Anesthesiologist/CRNA name is on the electronic pt info screen
      2. Before 3:30pm same day – submit to Immune Monitoring Facility (15th floor Zuckerman)
   2. **Streck** –
      1. Before drop off make sure Michelle has released the CIS order
      2. Once order is released, submit to Lab Medicine (Floor B2 on 64th Street between 1st and 2nd Ave)

## During Time Out

1. Surgeon/OR staff will call “time out”
2. Make sure you stop what you’re doing and pay attention
3. Surgeon will review case and pt info and anything important for the surgery
   1. Double check name, MRN
   2. Listen for details that could be relevant to your tissue collection

## Tissue Collection from OR

1. Start prepping collection station
   1. Grab bench cover (white absorbant sheet with blue edges) from metal cabinet
   2. Find a surface area to work on and cover w the above
   3. Lay out supplies needed for collection
2. Collect 3-7 sites and ascites
   1. Sometimes the surgeon will prompt you to tell them what sites you want, sometimes will just provide tumor samples as they go. This preference is a case by case thing
3. Take images of sample size (using ruler), if possible – use cell phone
   1. Upload pictures to folder in shared drive
4. Remind the surgeon and fellow to submit a fresh sample to pathology from corresponding collected sites
5. Complete **SPECTRUM Intraoperative Collection Form** (page 1)
6. Record the following details on the back of the SPECTRUM Intraoperative Collection Form:
   1. Surgery scheduled time
   2. Surgery start time
   3. Blood collection time
   4. # sites collected
   5. Processing time (start/end)
   6. Cell count time (start/end)
   7. Antibody staining time (start/end)
   8. Flow sort time (start/end)
   9. Time cells submitted to IGO
   10. Labels and storage time (start/end)
7. If the case is a Laparoscopic Biopsy, have the surgeon or fellow complete **SPECTRUM Intraoperative Collection Form** (page 2) in the OR.
8. Process samples for single cell dissociation

NOTE: Ensure samples are kept **on ice** at all times

# Sample Processing

## Reagents

* RPMI and other 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