Guidos Lab: CyTOF Surface and Intracellular Staining Bootcamp Training
Last update: February 15, 2021, Tina Chen

Staining cells for CyTOF experiments, though operationally not very different than staining for flow experiments, requires much more technical precision in pipetting and aspiration. *This difference likely stems from the inherently greater stickiness of metal-tagged Abs, coupled with many more washing and aspiration steps. We have seen that inconsistent aspiration of the wash results in variable cell loss across the tubes, causing significant staining inconsistencies across tubes in the same experiment. This problem is often most evident in experiments involving staining for both surface and intracellular markers. The latter requires cells to be fixed and permeabilized after surface staining. If any fix/perm solution remains in the tube when the intracellular Ab cocktail is added, the fixative cross-links the Abs onto cells (even if they don't express the marker), and the excess Ab cannot then be removed by washing. Thus you will observe high background staining. Therefore, we highly recommend that* ***you carry out this exercise, which will help determine where you need to optimize your technique BEFORE doing important CyTOF (or Flow) experiments.*** *The idea is to perform a mock staining experiment (with no Abs) on at least 4 replicate tubes of the same cell types, and count the cells in each tube after each step to assess your losses.*

Well before beginning your experiment:

1) Prepare all necessary buffers and stock solutions, including MilliQ water. See **Guidos Lab SOP**: "***Reagents for Flow or Mass Cytometry"***.
2) Decant aliquots CyTOF staining media (CSM), calf serum (CS) and other buffers you will need (see below) into 15 ml or 50 ml tubes.
3) Ensure that you have enough Cisplatin and Iridium aliquoted, stored and ready to use.

On the day of your experiment get ready to stain as follows:

1) Prepare Cisplatin working stock: thaw 10 μl aliquot of **10 mM Cisplatin** (10,000X). Dilute 5000X into PBS (no protein) to make a 2 μM (ie., 2X) working stock. For example, add 1 uL 10 mM Cisplatin to 5 mls PBS. Store on ice until ready to use.
2) Prepare, wash, count and aliquot your cells into staining tubes as per **Guidos Lab SOP**: "***Cell Preparation for Flow or Mass Cytometry"***. Mouse spleen, thymus or lymph node cells, OR human PBMC are ideal samples to use for this exercise.
3) Pre-label FACS staining tubes: 4 replicates for each cell type to be used in the experiment.

Staining Protocol:

In the protocol, "wash, pellet, aspirate and flick" means (UNLESS otherwise specified):

1. **Wash**: add 1 ml CSM (or 5-10X staining volume), then underlay with 0.3 ml CS. *See last page for CS underlay rationale and technique*.
2. **Pellet** cells for 5' at 300 x g at 4º C (after fix/perm, pellet at **800 x g** at 4°C).
3. Carefully but **completely aspirate** supernatant, then **flick** bottom of tube 2-3 times to re-suspend pellet in residual liquid. *See last page for aspiration technique*.
4. Note: do NOT aspirate more then 5-6 tubes at a time or the pellets may dry out and compromise cell viability.

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A. Aliquot Cells into Staining Tubes

1. Aliquot 1-2x10^6 cells into FACS tubes. Use 4 replicates for each cell type for which you want to assess your technique and optimize recoveries.
2. Wash, pellet, aspirate and flick.

B. Perform Mock Staining for Surface Antigens

1. Re-suspend pellet in 50 µl of CSM, pipette up and down to mix and incubate 30' at **RT**.
2. Wash, pellet, aspirate and flick.

C. Cisplatin Viability Staining (Protein-Free PBS)

1. Wash with 2 ml of protein-free PBS to remove any residual serum or BSA – **NO CS underlay!**
2. Pellet cells, flick and re-suspend in 100 µl PBS (RT).
3. **Count cells:** Remove 5 µl and dilute with 45 µl (10X) 0.1% Trypan Blue to count manually, OR with 5 µl (2X) Invitrogen Trypan Blue to use Countess II for automated count
4. ***Record total cell number for each tube in the table at the end of this document***
5. Add 100 µl of 2 µM (2X) Cisplatin working stock to each tube**.**
6. Swirl or pipette up and down immediately to mix.
7. Incubate for 5' at RT.
8. Quench the Cisplatin: add 2 ml CSM.
9. Underlay with 300 µl CS, pellet, aspirate and flick to re-suspend.

❖ Cells should be fixed **immediately** after Cisplatin staining.

Go to Step D if you do cytokine protocol
Go to Step F if you do Surface only protocol

D. Fixation & Permeablization

Choose the Fix/Perm buffer set most appropriate for the intracellular markers in your experiment (See "Reagents for Flow & Mass Cytometry"):
1) BD Cytofix/Cytoperm (CF/CP) for cytoplasmic antigens.
2) BD Transcription Factor (TF) or E-biosciences FoxP3 (FP3) Buffer for nuclear antigens.

- *Make 1 X solutions of Fix/Perm and Perm/Wash buffers using diluent provided* from appropriate kit.
- After fixation, cell pellets will not be as visible as pellets of unfixed cells, so exercise extra care when aspirating.
- After fixation always pellet cells at **800 x *g*** to improve recovery.

1. **Carefully** aspirate media, vortex briefly to re-suspend in residual fluid.
2. Add 250 µl (CF/CP) or 1 ml (TF or FP3) of 1X Fix/Perm solution for up to 3x10^6 cells.
3. Vortex gently for 3 seconds, then incubate cells **on ice** for 30'.
4. Wash with 1 ml of 1X Perm/Wash buffer from appropriate kit.
5. Pellet cells at **800 x *g*** for 5', 4°C, aspirate and flick to re-suspend.
6. *Repeat wash*: add 1 ml of 1X Perm/Wash buffer, pellet (**800 x *g***) aspirate and flick.

E. Perform Mock Staining for Intracellular Antigens

1. Re-suspend pellet in 50 µl of 1X Perm/Wash buffer.
2. Incubate for 30'at **RT.**
3. Wash, pellet (800 x g), aspirate and flick to re-suspend.

F. Iridium Labeling of Cellular DNA

1. Dilute Iridium stock 1000X (100 μM) in PBS/Sap+ 1.6% FA to achieve 100 nM (1X) working solution.
2. Add 1 ml of 100 nM (1X) Iridium in PBS/Sap containing 1.6% FA.
3. Vortex briefly to ensure all cells are re-suspended.
4. **Count cells:** Remove 10 μl and dilute with 10 μl with 10 μl (2X) Invitrogen Trypan Blue to use Countess II for automated count.
5. ***Record total cell number for each tube.***
6. Incubate 1h at RT **OR** overnight or up to 72h at 4°C.
7. Storage:

   Do NOT wash BEFORE storing cells as it can result in significant cell loss.

   A. If you need to store stained samples >72h up to 1 week before running on the CyTOF, pellet (600 x *g*), after Iridium staining, aspirate and add 50 ul PBS/FA 1.6% solution and store at 4°C.
   B. For storage longer than 1week freeze the stained samples -80°C in 50 ul of 10% DMSO + 90% FBS.

G. Final Washes (Immediately Before Acquisition)

1. About 1 hr before running cells on CyTOF, remove Iridium solution by pelleting 800xg for 5 min at RT.
2. Aspirate, flick to re-suspend and wash with 2 ml CSM. NO CS UNDERLAY from this point forward.
3. Aspirate, flick and re-suspend in 1 ml PBS, Remove ~30 μl and count
4. ***Count cells: Record total cell number for each tube***
5. Add 1 ml more of PBS. NO CS UNDERLAY.
6. Pellet (800 x g) and completely aspirate PBS, place tubes with dry cell pellets on ice.
7. Bring cell pellets, the cell count/tube, and your panel information to the CyTOF facility to assess sample quality.
8. Ask the Operator to add 0.5 ml of 0.1X EQ bead solution and run your Ir-stained cells + EQ beads. Ask them to collect 50,000 events per tube.

- ***Expected cell recovery:*** You should expect to recover at least 50% of the number of cells you started with in each tube, although this number may be lower for cell types that are very sticky or otherwise difficult to deal with.
- ***Acceptable Precision:*** If your technique is highly precise, the variance in recovery between your replicate tubes should not be greater than 15-20%. If it is greater, you need to improve your technique before proceeding to do an actual CyTOF staining experiment.
- ***Sample Quality:*** When you analyze the FCS data files, the Ir-stained cells ($DNA1^+$ $EQ140Ce^-$ events should be >90% of the total events in the sample for all tubes. If lower, you lost too many cells during the procedure.

Calf Serum Underlay Technique for Cell Washing:

Pelleting cells through the CS underlay:

- makes a more compact cell pellet
- gets rid of some dead cells and cellular debris
- makes the supernatant easier to aspirate without loss of cells
- better separates unbound antibody from the cell pellet
- leaves less residual fluid behind that can further dilute the antibody solution.

1. Place a glass pasteur pipette into the tube
2. For a wash volume of 0.5ml in a standard 4.5 ml FACS tube:
    a. Add 0.3 ml of CS into the top opening of the Pasteur pipette (use more CS for larger wash volumes in larger tubes)
3. Slowly remove the pipette (move up and down to dislodge air bubbles) to allow the denser CS to form a layer under the SM containing the cells.

Aspiration Technique for Cell Washing:

Residual fluid can further dilute the Ab cocktail and adversely affect your staining results. This aspiration technique minimizes this possibility.

1. Use a paster pipette attached to a vacuum flask with vacuum turned on.
2. Place the pipette tip at the top of the fluid to be aspirated and move it slowly down as the fluid level decreases. Do NOT stick the pipette tip way below the surface of the fluid and aspirate from below – this can leave a lot of residual fluid on the side of the tube that will slowly run down and dilute your cell pellet.
3. If desired, place pipetman tip (for a P200) on the tip of the Pasteur pipette – the aspiration will be slower but more controlled.
4. Ensure that you "back-light" the tube as you aspirate so that the cell pellet is visible (assuming sufficient cells were in the tube!) at all times.
5. When the tip and fluid level reach a few mm above the pellet – slowly tip the tube so that the residual fluid runs into the tip, rather than moving the tip closer to the cell pellet.
6. After removing the pipette from the tube, gently flick the tube 3X with your finger to dislodge the cells into the residual fluid, rather than leaving them as a tight pellet.

Samples Notes:

- Miltenyi magnetic bead enriched cell populations can be acquired on Helios instruments.
- Consult with the Instrument manager for acquiring Nano-particles.
- The upper limit for particle size is 100 μm in diameter, which is the diameter of the nebulizer internal capillary. The largest cell type Fluidigm has tested is 25 μm in diameter. The lower limit is defined not by size but by labeled epitope density. All particles are ionized in the plasma and expand like a gas to ~2 mm diameter at the plasma/cones interface. Therefore, particle detection is dependent entirely upon signal, not size (adapted from Fluidigm FAQs).

Cell Count

STEP	Tube 1	Tube 2	Tube 3	Tube 4
A1				
C3/4				
F4/5				
G4				