CyTOF Experimental Design Questions

What kinds of cells will you analyse?

- Easily made into single cell suspensions – eg. Whole blood, PBMC, lymphoid tissue?
  - ✓ CyTOF should be straight forward

- Solid tissue that requires disaggregation?
  - ✓ Will need to optimize gentle enzymatic method that preserves surface epitopes

- Expected viability and sample quality?
  - ✓ If <70% viable and/or lots of debris, RBC or degraded cells
    - pilot clean up steps: RBC lysis>Miltyeni kit for dead cell or debris removal

- Single Experiment Pilot or full Project?
  - ✓ How many groups, replicates/grp, tissue types per subject?
  - ✓ How many samples to process per experiment day?
  - ✓ How many cells to analyze per sample?

Sample Preparation Considerations

Why is Gentle Sample Preparation important?

Miltenyi Biotec

Garbage in…

…garbage out

Viability

Yield

Removal of debris and cell aggregates

Filters and strainers

Removal of dead cells and other unwanted material

Removal reagents

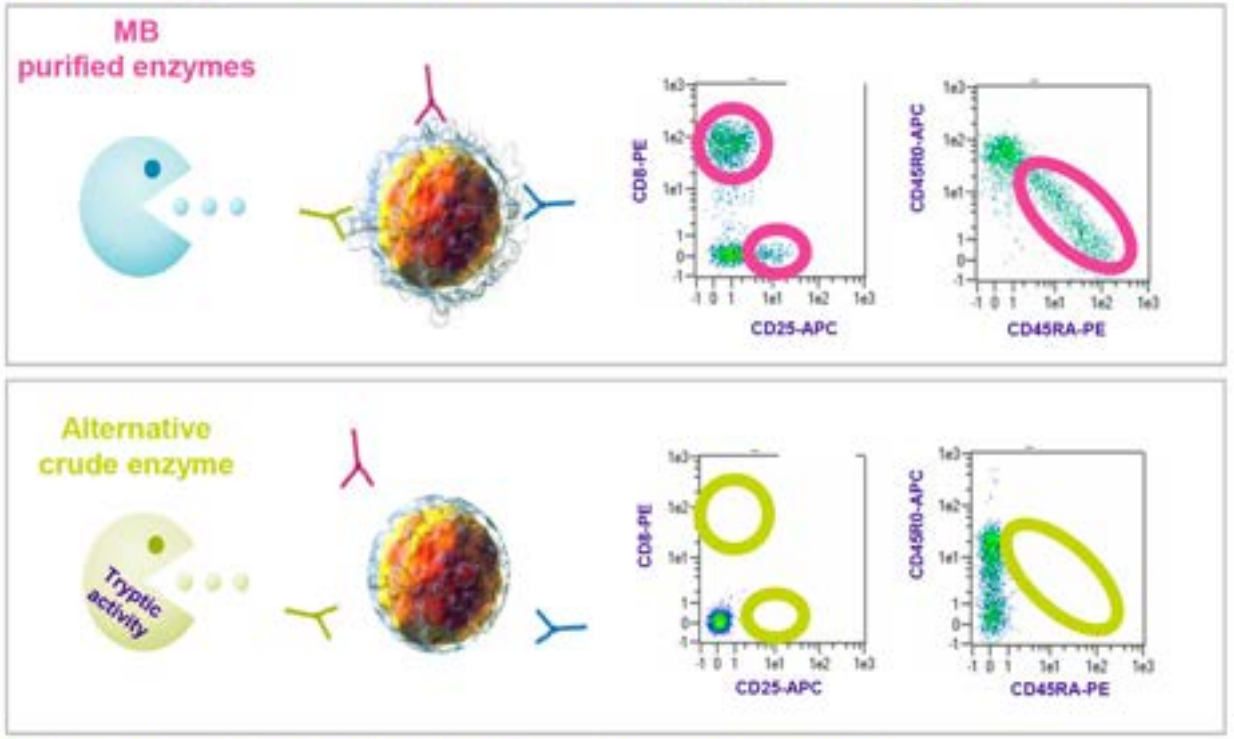
-Dead Cell Removal Kit

-Debris Removal Solution

-RBC lysis Solution

gentleMACS™ Technology – Enzyme quality is key

Miltenyi Biotec



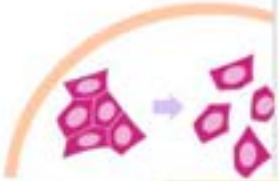
Highly purified enzymes for minimal epitope degradation and standardized results

GentleMACS™ technology: A perfect match: innovative robust instruments and optimized kits

Miltenyi Biotec

Mechanical disruption

Enzymatic digestion



Unique Dissociation Tubes:
gentleMACS™ Tubes

Easy-to-use Tissue Dissociation Kits:
e.g. Tumor Dissociation Kit

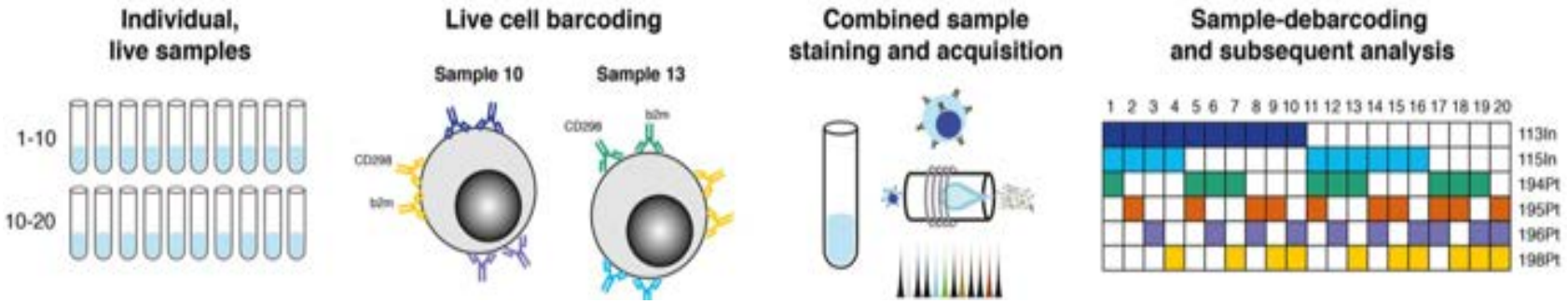
Innovative Instruments:
gentleMACS™ Dissociators

Tissue Dissociation Kits

Unique Dissociation Tubes:
gentleMACS™ M and C Tubes

gentleMACS™ Octo Dissociator
& gentleMACS™ Dissociator

Barcoding for Multi-plexed Staining and Acquisition



A: Live cell barcoding scheme for CyTOF analysis: Schematic from Hartmann *et al* 2018.

Should I Barcode?

- How many experiments will you do?
- How many samples per experiment will you acquire?
  - ✓ Barcoding is not usually worth it for Pilots – a few experiments, <6 tubes per experiment
  - ✓ Barcoding is highly recommended for study with multiple experiments, >6 tubes per experiment

Barcoding Advantages

- Higher through-put for staining & acquisition on Helios
- Better doublet discrimination
- Less technical variation and cell loss during multiplexed staining

Barcoding Disadvantages

- Longer sample prep times
- Adds reagent cost
- Can be challenging for experiments with multiple cell/tissue types that may not barcode with the same efficiency

How Should I Barcode?

Live Cell Barcoding (Any Metal)

- Most common method: Uses Multiple tags of the same Ab to a broadly expressed surface marker (eg., CD45, b2M, CD298)

- Advantages:
  - ✓ Done prior to fixation so avoids loss of fixation-sensitive epitopes
  - ✓ Flexible: can customize 4c2=6, 5c3=10, 6c3=20 or higher multiplexing as needed
  - ✓ Can use any metal that can be tagged using MaxPar (lathenides) or MCP9 (Cadmium) kits
- Disadvantage:
  - ✓ Reduces # of metal tags available for markers of interest
  - ✓ Can be challenging to find the ideal surface marker

Metal Tag Barcoding (Usually Pd)

- Most common method: Fluidigm Cell-ID 20-Plex Barocding kit that uses 6c3 Pd isotopes>20 codes

- Advantages:
  - ✓ Easy to use kit comes with all buffers plus 3 sets of 20 pre-aliquoted microtubes each with one BC
  - ✓ Pd tags not usually used for Ab tagging
- Disadvantages:
  - ✓ Expensive!
  - ✓ Requires that cells be fixed & permeablized prior to staining
    - Can reduces or eliminate staining of some surface markers>Need to prospectively test if your surface markers are impacted by F/P
    - To avoid this problem, barcode AFTER staining and then pool for multiplexed acquisition
      - This will greatly reduce acquisition time and cost
      - It will also allow better doublet discrimination

How Many Cells Can I (or Should I) Acquire?

Number of Events to Collect for Flow or Mass Cytometry

Adapted from: https://utahflowcytometry.wordpress.com/2013/07/26/how-many-cells-should-i-count/

Technical issues: flow cytometry and rare event analysis.

Hedley BD, Keeney M.Int J Lab Hematol. 2013 Jun;35(3):344-50. doi: 10.1111/ijlh.12068.PMID: 23590661

We are often asked:

How many events/sample should I collect in my flow or mass cytometry experiments?

Here is the answer!

The total number of cells you should collect is determined by:

1) **the frequency of the rarest population** you want to measure ***and***
2) **the precision**, represented as the coefficient of variation (CV), with which you want to measure it.

- The precision of your measurement is calculated using the Poisson distribution based on the number of events you count in your subset of interest.
- The essential feature of Poisson distributions is that if N events are observed in your target subset, then the standard deviation (SD) associated with that count is square root of N.
- The coefficient of variation (CV) is then given by: CV(%) = 100/sqrt N.
- Thus, as you count more cells in your target subset, the CV will decrease, indicating higher precision.

For a CV of (%)	***2.5***	***5***	***10***	***20***
Events Counted (N)	***1,600***	***400***	***100***	***25***
Subset Frequency (%)				
10	16,000	4,000	1,000	250
1	***160,000***	***40,000***	10,000	2,500
0.1	1,600,000	***400,000***	***100,000***	25,000
0.01	16,000,000	4,000,000	1,000,000	***250,000***

For most applications CVs in the 2-5% range are considered ideal:

- For a CV of 5%, you need to count 400 cells of your population of interest.
- For a 1% population, you will need to count 40,000 total events to achieve a CV of 5%.
- For a 0.1% population, you will need to count 400,000 total events to achieve a CV of 5%.
- Note that if your sample has many dead cells and/or doublets you need to consider the **% of total cells** collected represented by your target, **not the % of live singlets**.
- Lower precision (10-20%) is generally acceptable for rare event analysis when it is not feasible to collect enough events to achieve higher precision

Helios Acquisition Rate:

- 300-500 cells/sec

For cells @500,000/ml:

- Can collect ~100,000 cells in 10’

Subset Freq. (%)	Events (N)	CV
10	10,500	1.0
1	1,050	3.1
0.1	105	9.8

Other throughput considerations:

- Need to wash ~10’ btwn each tube
- For barcoded tube, can acquire 2-3 million events per day (4-5h run time)