Sample Prep and Experimental Design

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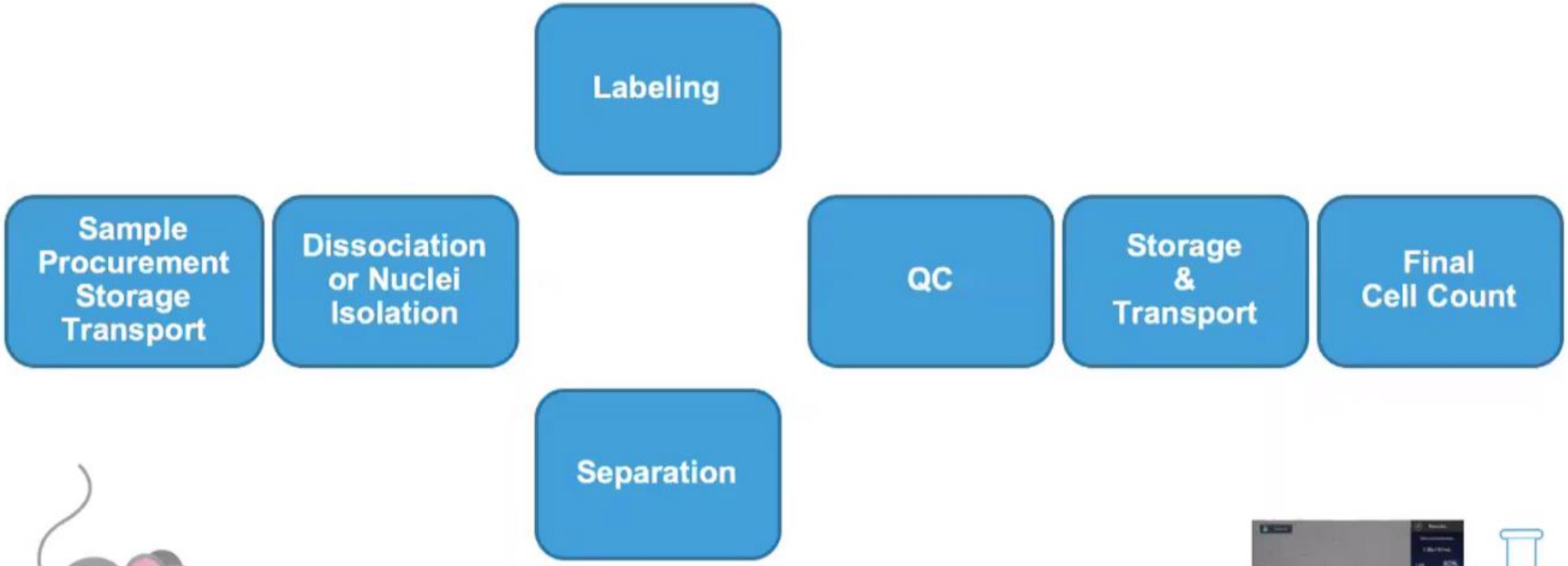
What is sample prep?

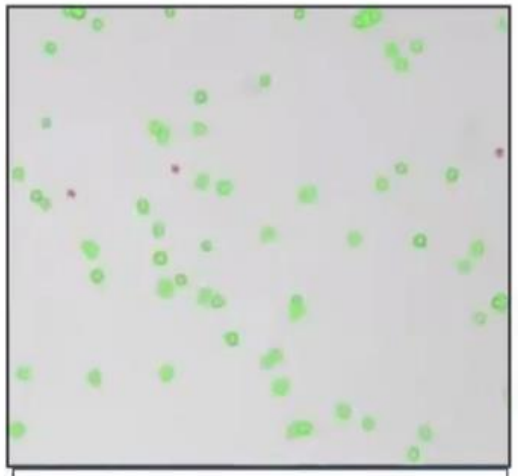
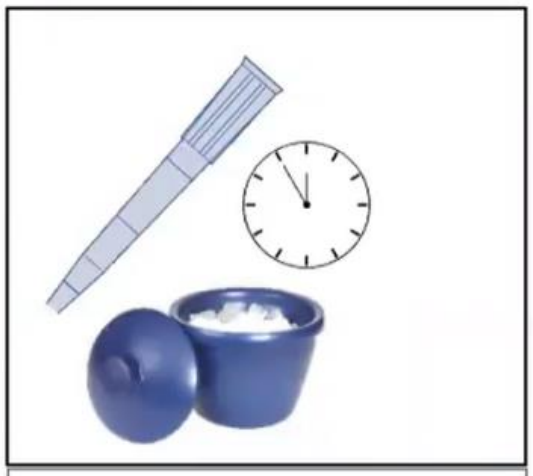


Know what you are working with

Cells Tested	Species	Cell Source	Total RNA (pg/cell)	Cell Size (µm)
PBMC	Human	Extracted from blood	~0.75	~5-10
E18 neuron	Mouse	Brain tissue	~2-3	~9
Jurkat	Human	Suspension	5.5	~12
Raji	Human	Suspension	7.3	~12
293T	Human	Adherent	14.2	~18
3T3	Mouse	Adherent	16.1	~18
HCC1954	Human	Adherent	15.7	~18
HCC38	Human	Adherent	21.6	~30

Quality is critical

Goal is to minimize

Clean	Healthy	Gentle
		
• Aggregates/clumps • Subcellular debris • Free-floating RNA/DNA	• RNA leakage • RNA degradation	• Physical decomposition

Handle with care

Minimize handling, both pipetting and centrifugation

Metric	Control	Wide Bore (Rough)	Narrow Bore (Rough)	Vortex 5s
Number of Cells	1,118	846	1,012	983
Reads per Cell	50,000	50,000	50,000	50,000
Valid Barcodes	95.40%	95.50%	95.30%	95.50%
Fraction Reads in Cells	79.40%	72.80%	54.00%	63.10%
Reads Mapped Confidently to Transcriptome	70.50%	71.40%	71.80%	71.00%
Valid UMIs	99.40%	99.40%	99.40%	99.40%
Median Genes per Cell	3,137	3,180	2,833	2,934

Sample procurement, storage, transport

CNV

Cultured cells, PBMCs
*Fresh and cryopreserved
*10x protocols work as-is

Fresh tissue
*Minimal optimization

Snap-frozen tissue
*Minimal optimization

Fixation?
*Not tested but likely (ethanol or methanol)

ATAC

Cultured cells, PBMC
*Fresh and cryopreserved
*10x protocols work as-is

Fresh tissue
*Optimization

Snap-frozen tissue
*Optimization

Fixation?
*Not tested

GEX

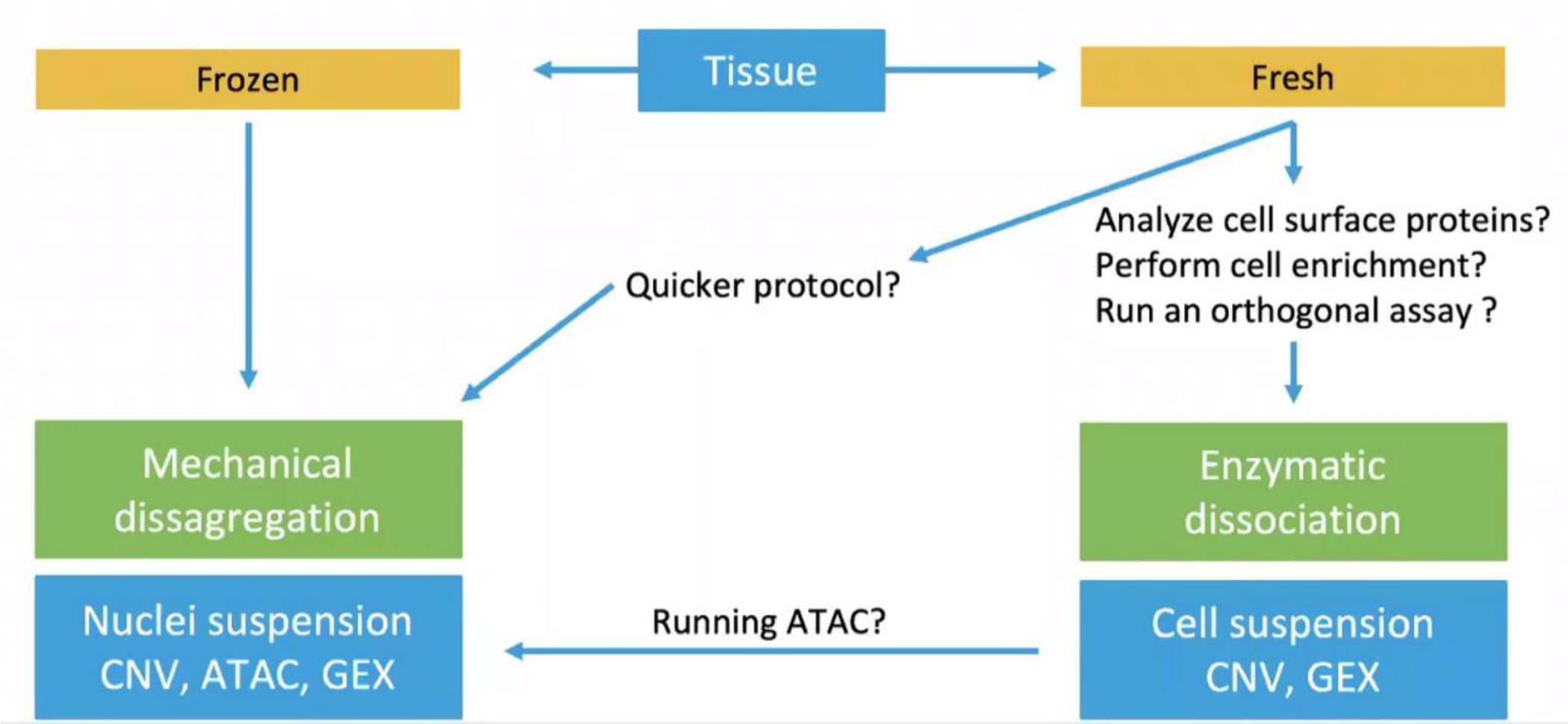
Cultured cells, PBMC
*Fresh and cryopreserved
*10x protocols work as-is

Fresh tissue
*Significant optimization

Snap-frozen tissue
*Nuclei (no cell surface proteins)
*Technically challenging
*Significant optimization

Fixation?
*Methanol (but we prefer cryopreservation)

How to choose a dissociation method



Separation

Separate intact cells and nuclei from

- Aggregates/clumps
- Debris
- Free-floating mRNA
- Dead cells
- Enrichment/depletion

Challenges with separation

- Samples are fragile
- Physical stress
- Buffers
- Time
- Yield

Want the minimum handling necessary. Maintain sample integrity.

There are many separation methods

Method	Thorough c'fuge (e.g. 3x in PBS + 0.04% BSA)	Gentle c'fuge (e.g. 1x in media)	Magnetic beads	Density Gradient	FACS
10x Protocol Example	PBMC (CG000039)	Cell Prep Guide (CG000053)	Dead cell removal (CG000093)	Nuclei isolation (CG000124)	Customer Developed Protocol (Martelotto)
Can be used with	Abundant	Limited	Abundant	Abundant	Limited
Benefit	Clean	Gentle	Specific, easily accessible, scalable	Removes Debris	Versatile, quick
Challenges	Lossy, can be harsh	Less thorough	Lossy	Lossy, harsh	Expensive, can be harsh

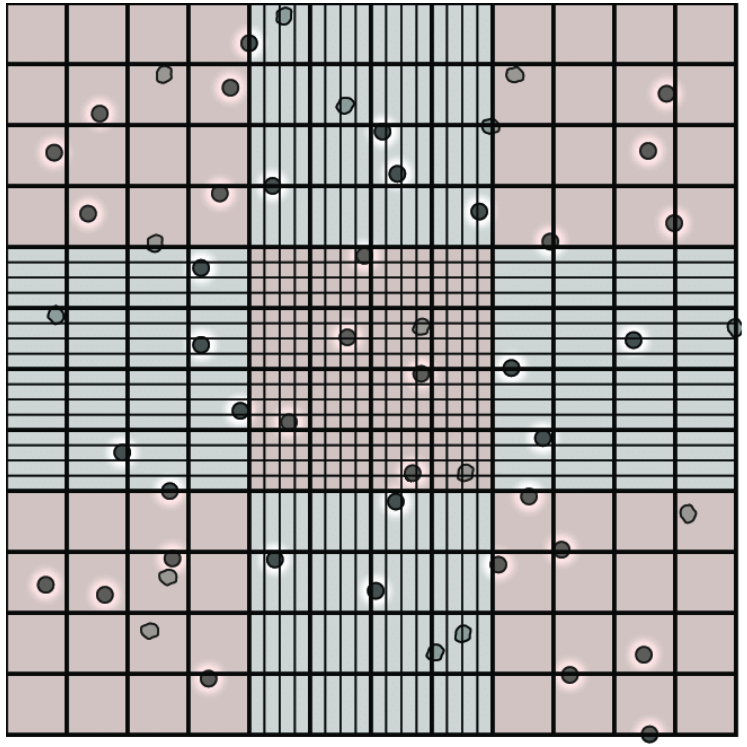
Washing / straining

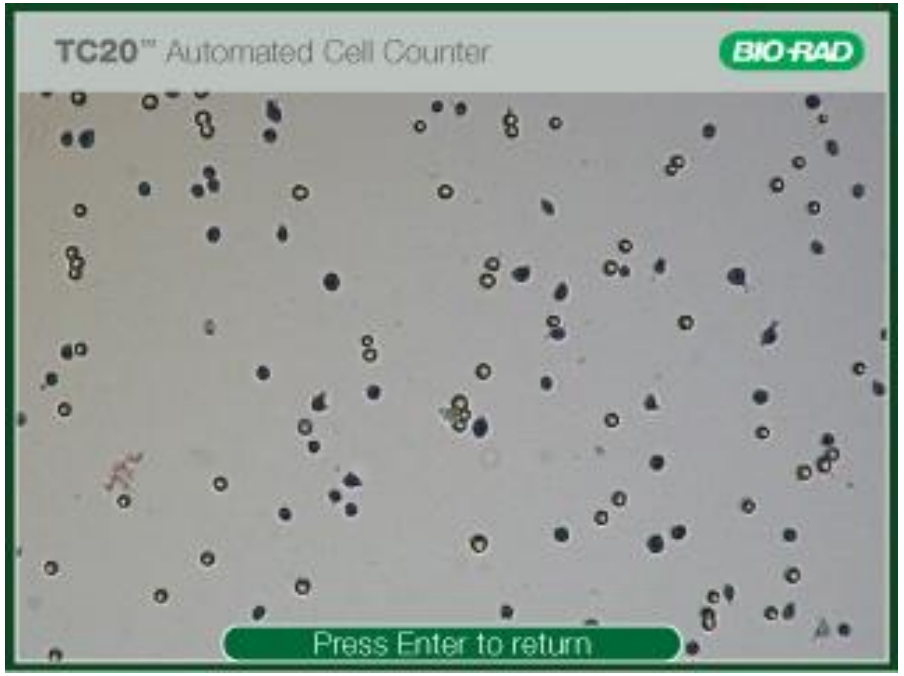
- 3 washes in PBS + 0.04% BSA before loading
- Use a relatively large volume for washes relative to cell number
  - Concentrated cells can clump
- Large cell aggregates or debris can increase the risk of clogs

  => Use a cell strainer, 30 to 40 µm

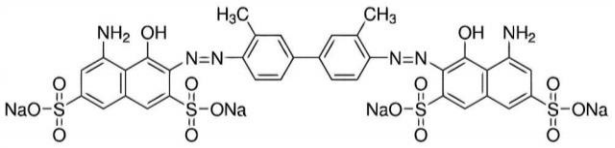
  BUT – this will result in sample loss, volume loss, and possible cell loss

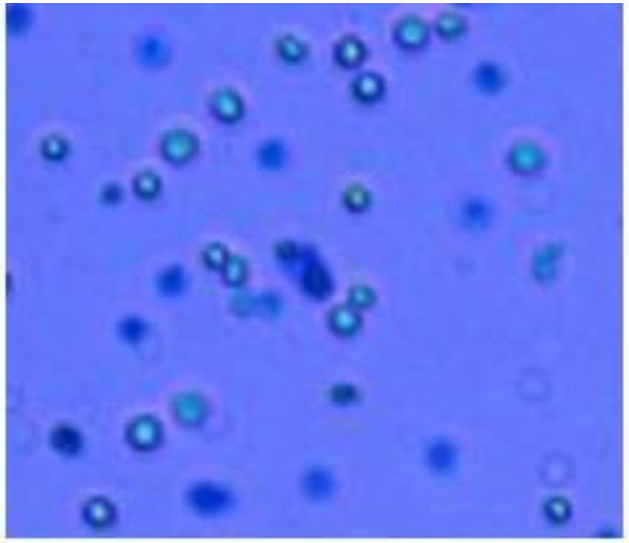
Counting and viability assessment

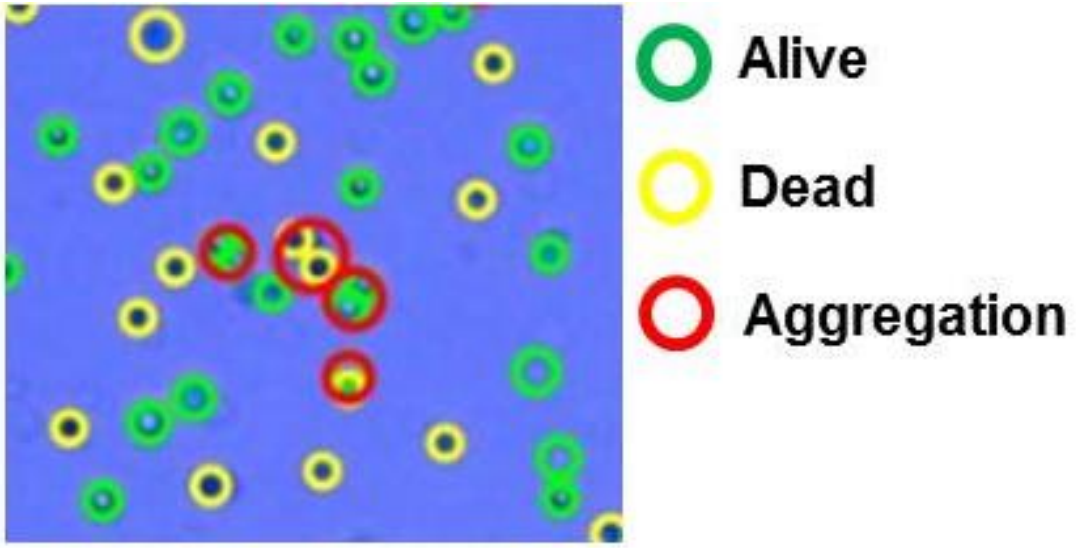




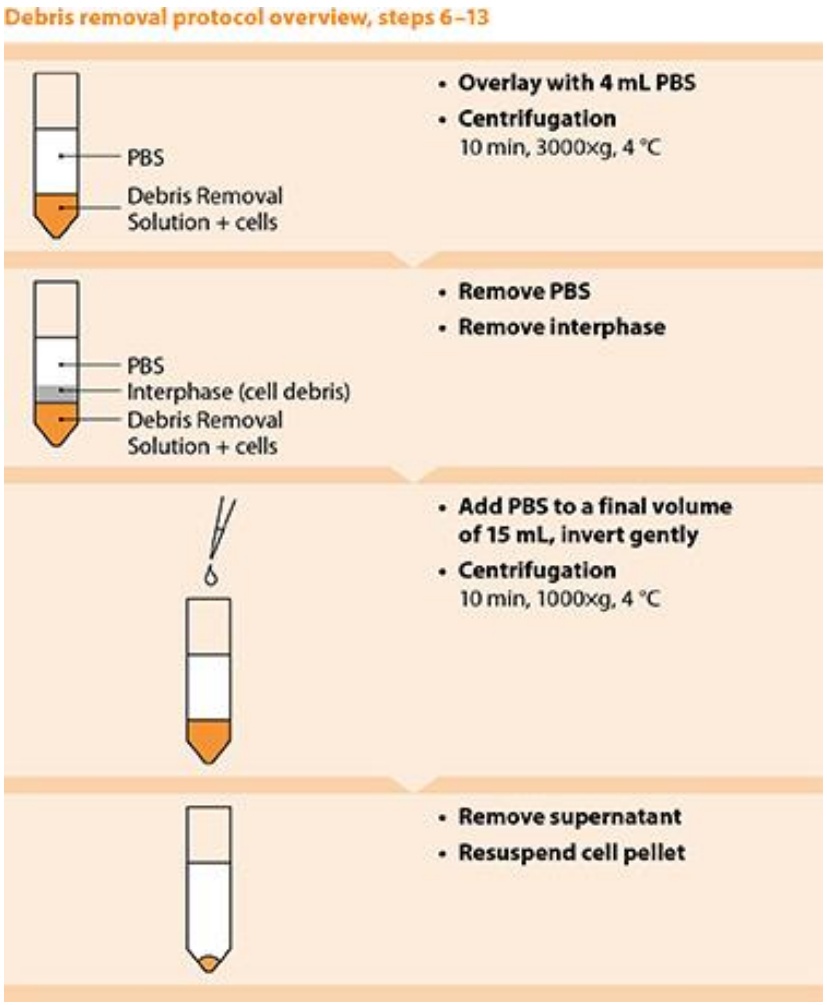
Counting and viability assessment – live/dead



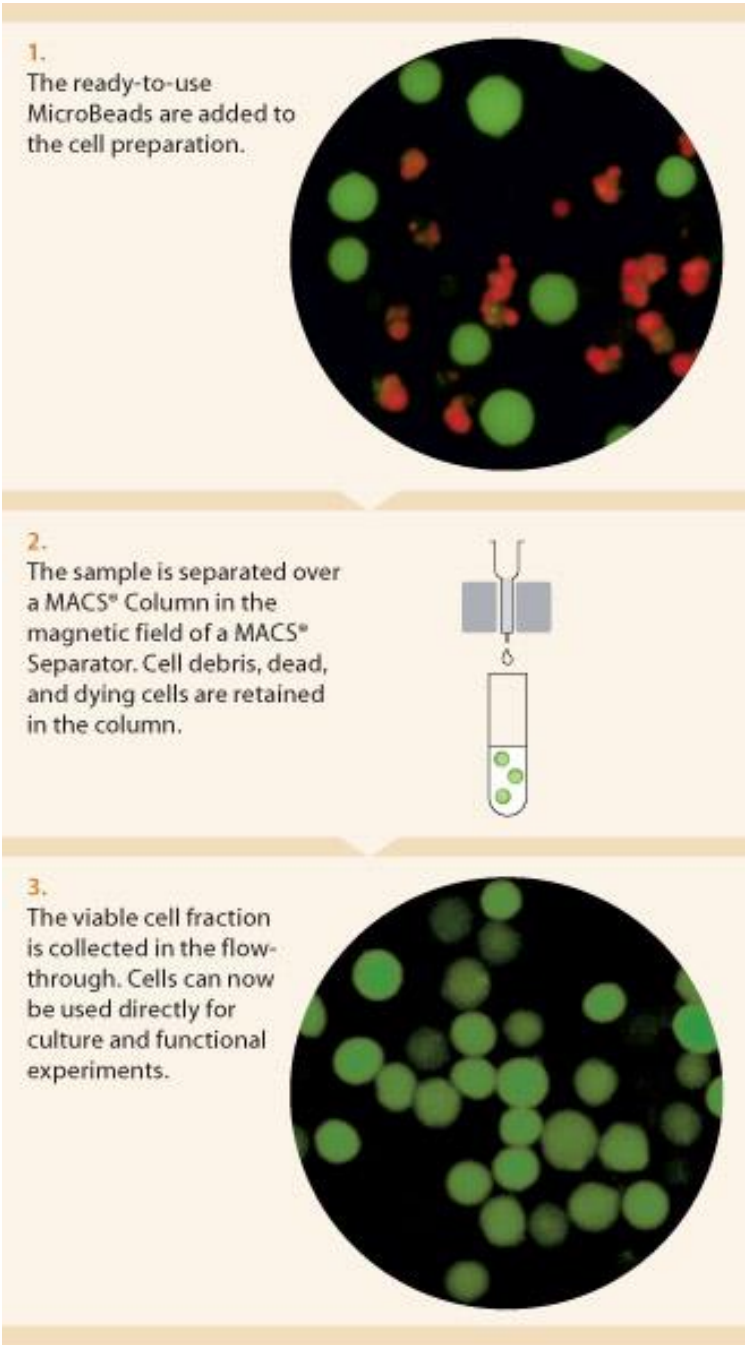




Dead cell and debris removal



Source:
Miltenyi



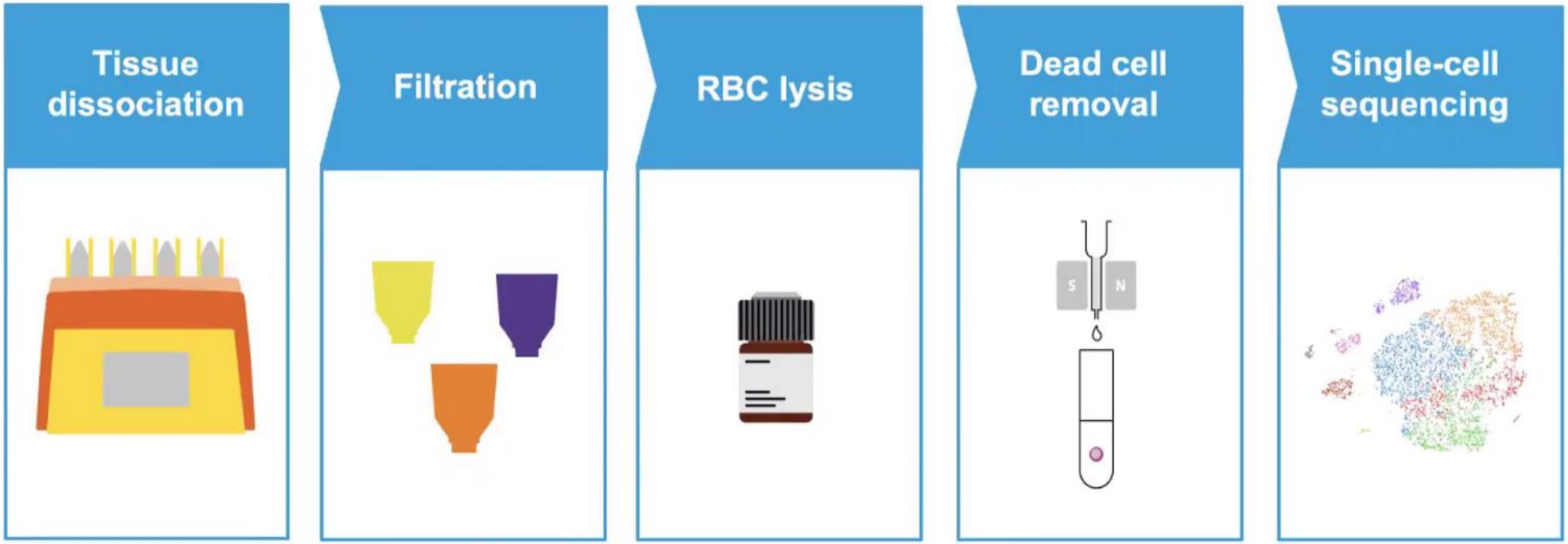
Storage after preparation?

- Cryopreservation (DMSO)
  - No quantifiable difference between fresh and cryopreserved scRNA-seq samples
- Methanol fixation

**NOTE: both approaches have shown a potential bias in cell-type composition, and it is strongly recommended to thoroughly evaluate preservation methods for new cell types that have not been tested.

- iCell8 and Rhapsody microwells – freeze plate after single cell sorting

Example: tumor processing workflow



Summary of key sample prep lessons

- Treat cells gently and keep them happy
  - Try gentle(r) lysis conditions
  - Reduce washing steps*
  - Use a swinging bucket centrifuge
  - Keep cells in media + FBS instead of PBS**
- Work quickly
  - Consider sorting
  - Minimize handling steps
- *Consider the benefits and drawbacks of different techniques*

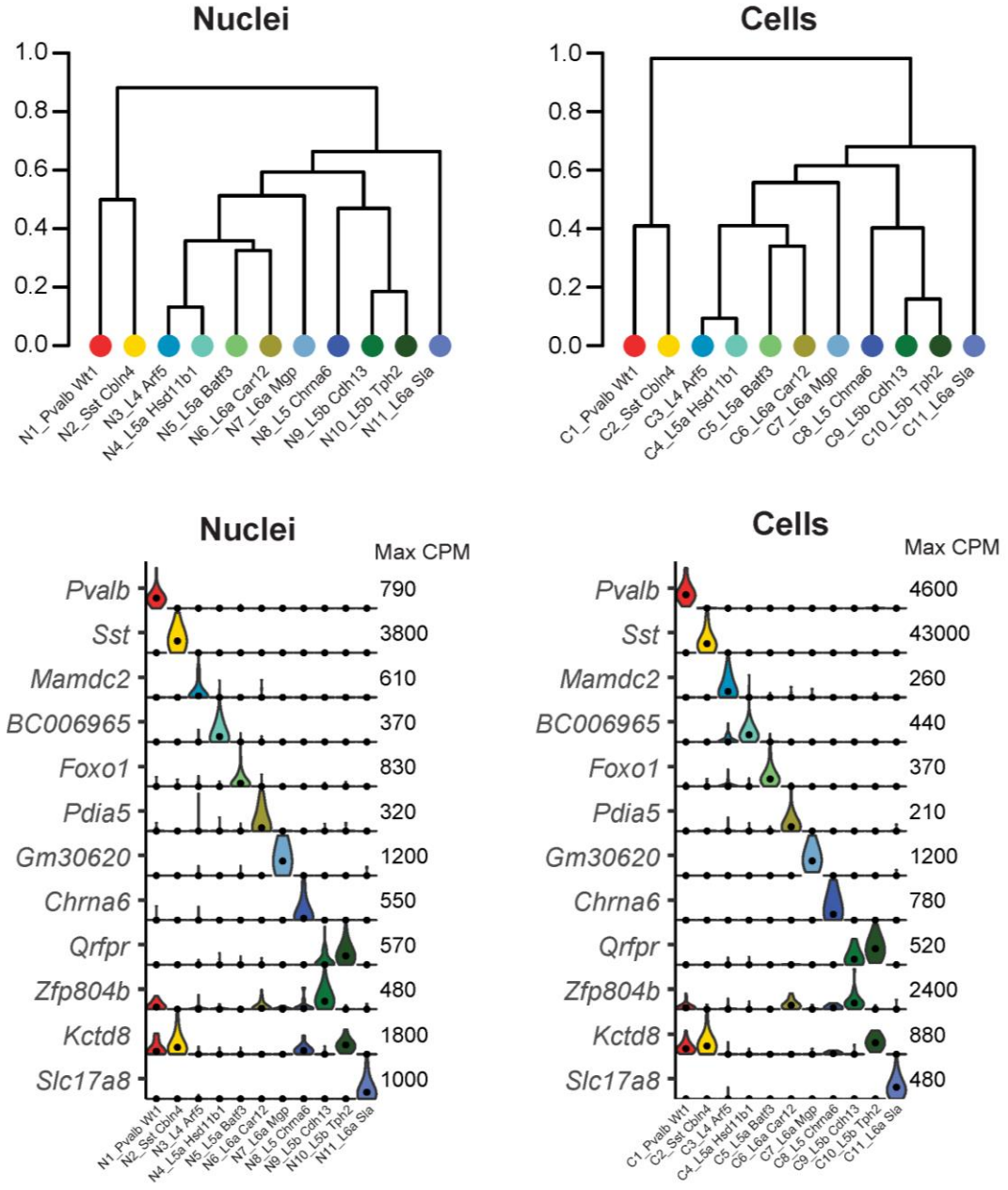
Nuclei vs whole cell

What if cells are not an option?

- Neurons
- Snap-frozen
- FFPE*

snRNA-seq is nearly identical to scRNA-seq

- Fewer unique transcripts
- More variability in per-nucleus content



Bakken et al PLOS ONE 2018

How many samples? How many cells?

Consider sample heterogeneity and subpopulation frequency

General rule of thumb:

- Heterogenous populations require MORE cells
  - Here, a larger number of cells will provide greater statistical power
  - Heterogeneity can be reduced through experimental design (sorting, transgenics)
- Identification of rare cells requires MORE cells

Power calculations + group size estimates

- If other single-cell data is available (flow- or mass-cytometric data)
- Computational tool accessible at https://satijalab.org/howmanycells

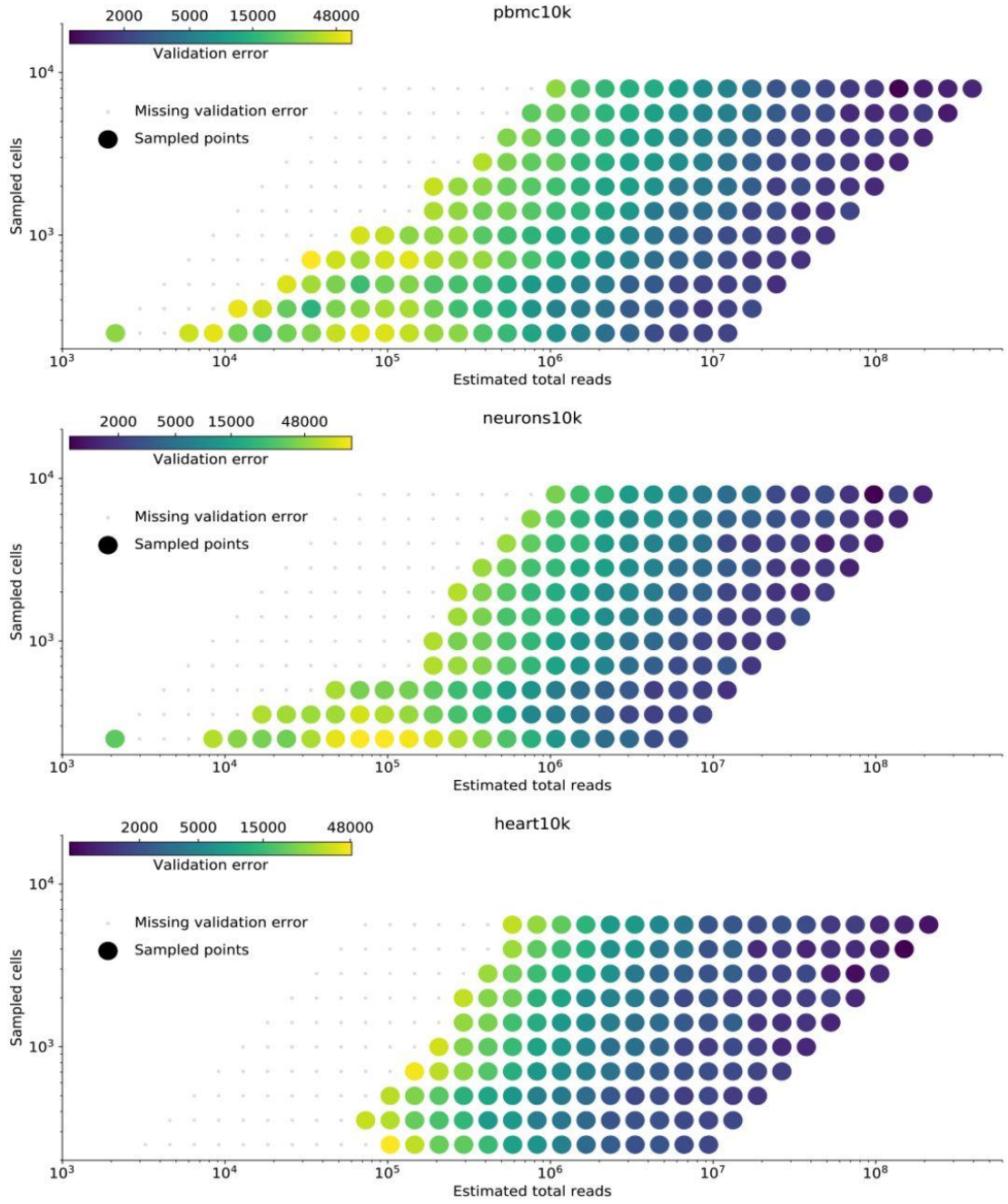
Pilot experiment recommended before large-scale data production

How deep?

For 3’ counting approaches:

<15k reads/cell, doubling reads reduces error by 30-40%

>15k reads/cell, doubling reads or cells has little effect (1-3%)



Svensson et al BioRxiv 2019

How deep?

- BUT… not all cells will have the same depth of sequencing