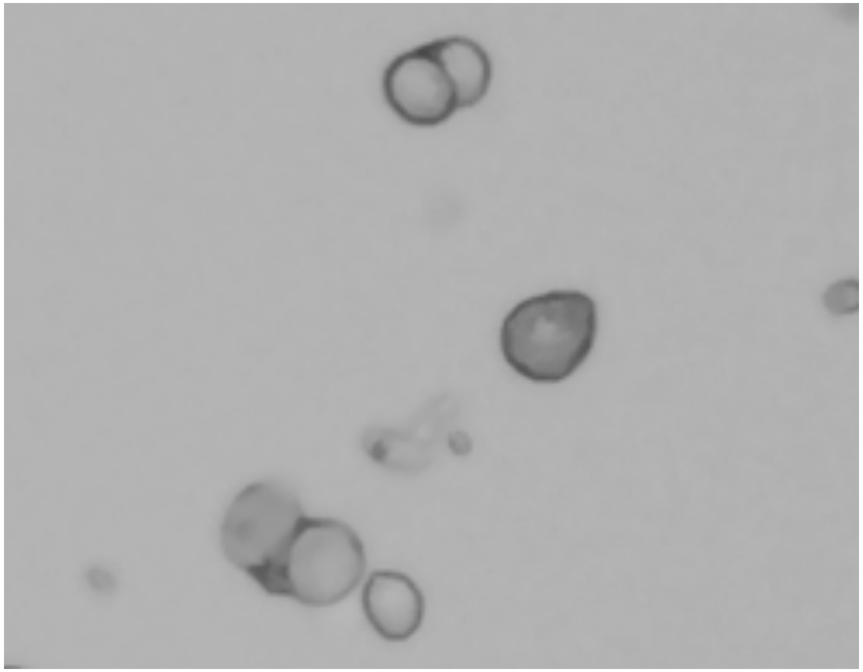
Cell preparation for 10X



Diana Burkart-Waco, PhD

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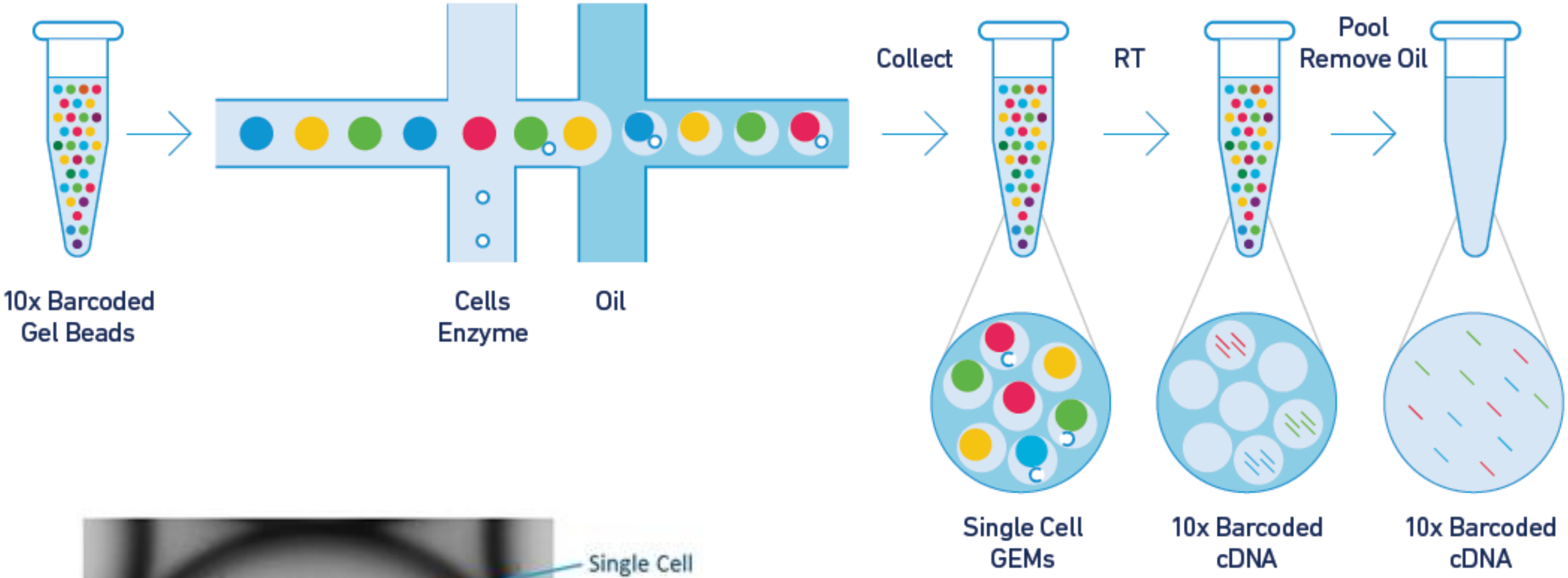
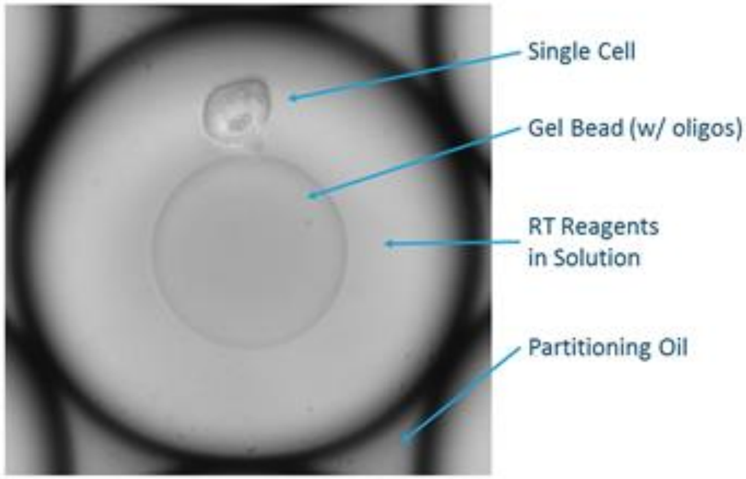
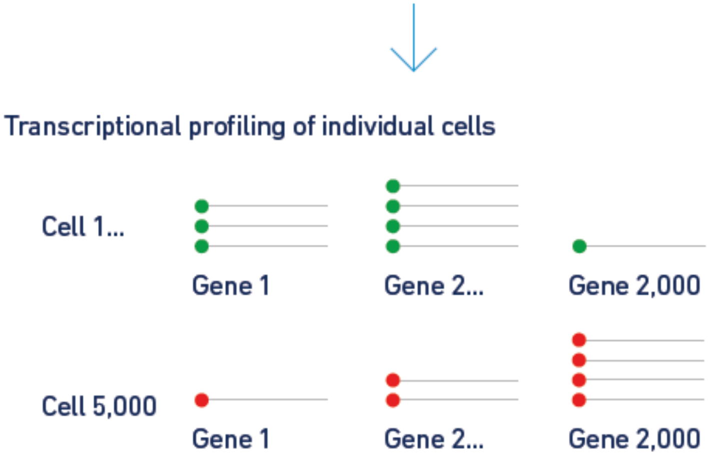
Overview

- Technology overview.
- Single cell isolation.
- Sample QC.
  - Do I have single cells?
  - Are they alive?
  - Are they too big?
  - Did I isolate the correct cells?
- How do I count them?
- Methanol Fixation.

10X technology

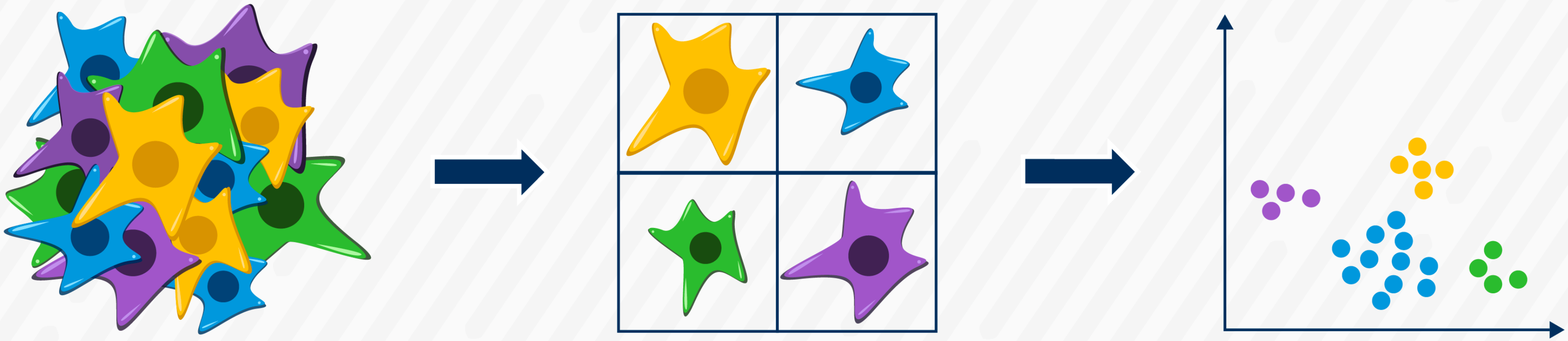






Sample preparation

- Single cell isolation is key.
- No one protocol fits all.
- Invest time sample preparation.



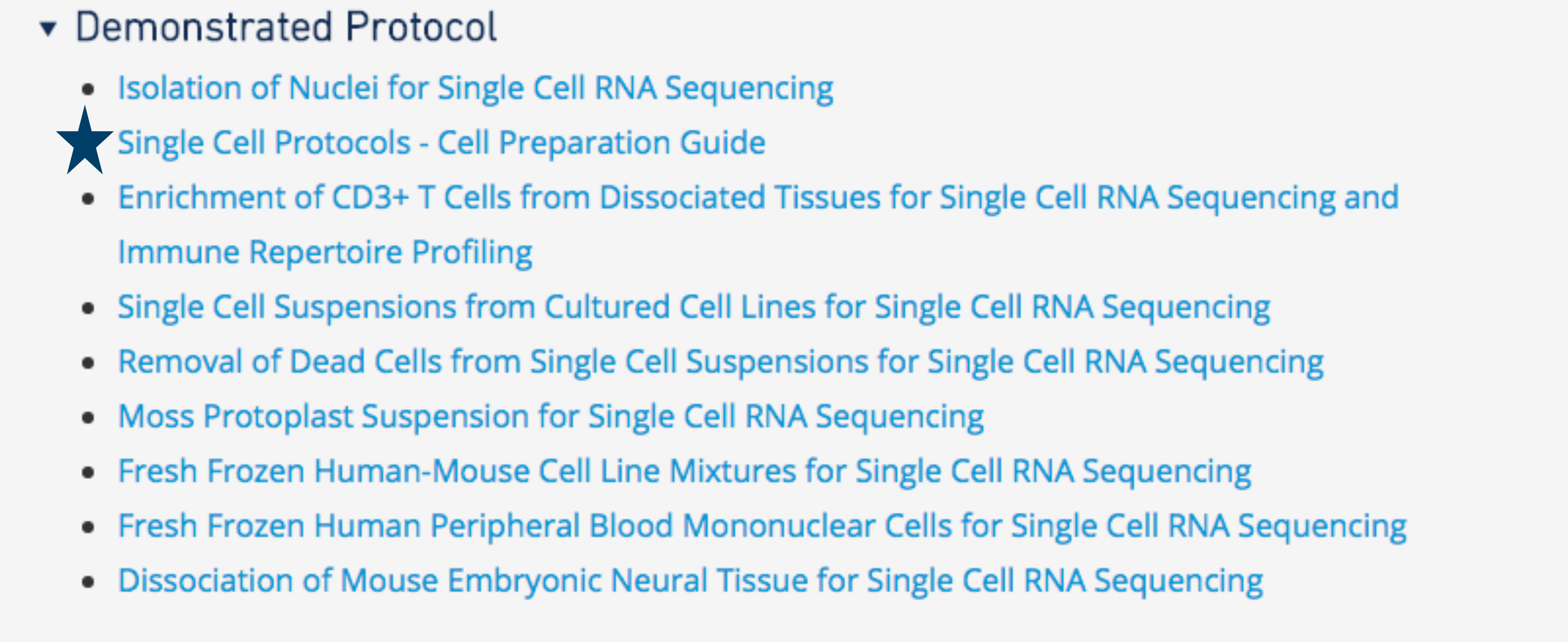
10xgenomics.com/

Cell isolation

- Cell isolation guides available at:

https://www.support.10xgenomics.com/single-cell-gene-expression/sample-prep/

  - Dissociation and preparation depends on cell type.



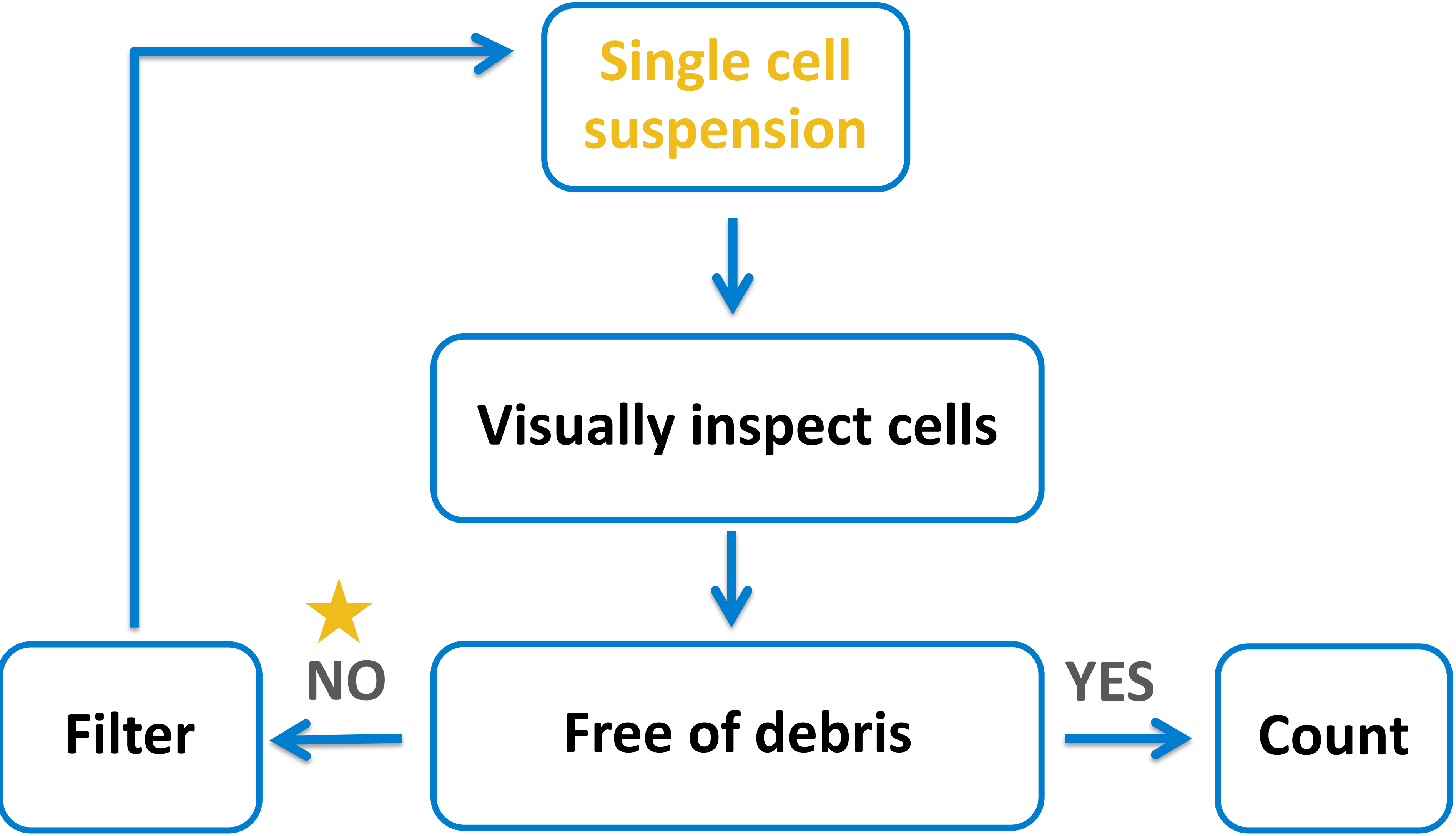
▾ Demonstrated Protocol

- Isolation of Nuclei for Single Cell RNA Sequencing
- ★ Single Cell Protocols - Cell Preparation Guide
- Enrichment of CD3+ T Cells from Dissociated Tissues for Single Cell RNA Sequencing and Immune Repertoire Profiling
- Single Cell Suspensions from Cultured Cell Lines for Single Cell RNA Sequencing
- Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing
- Moss Protoplast Suspension for Single Cell RNA Sequencing
- Fresh Frozen Human-Mouse Cell Line Mixtures for Single Cell RNA Sequencing
- Fresh Frozen Human Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing
- Dissociation of Mouse Embryonic Neural Tissue for Single Cell RNA Sequencing

Factors influencing success

- Cell debris.
- Aggregates.
- Proper concentration estimates.
- Buffer.
- Storage.

Workflow



Single cell suspension

Visually inspect cells

Free of debris

★ NO

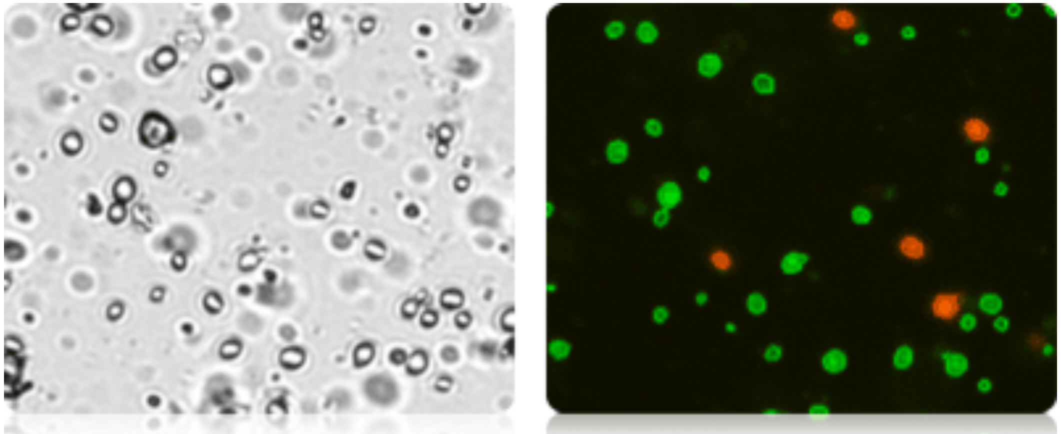
Filter

YES

Count

Debris

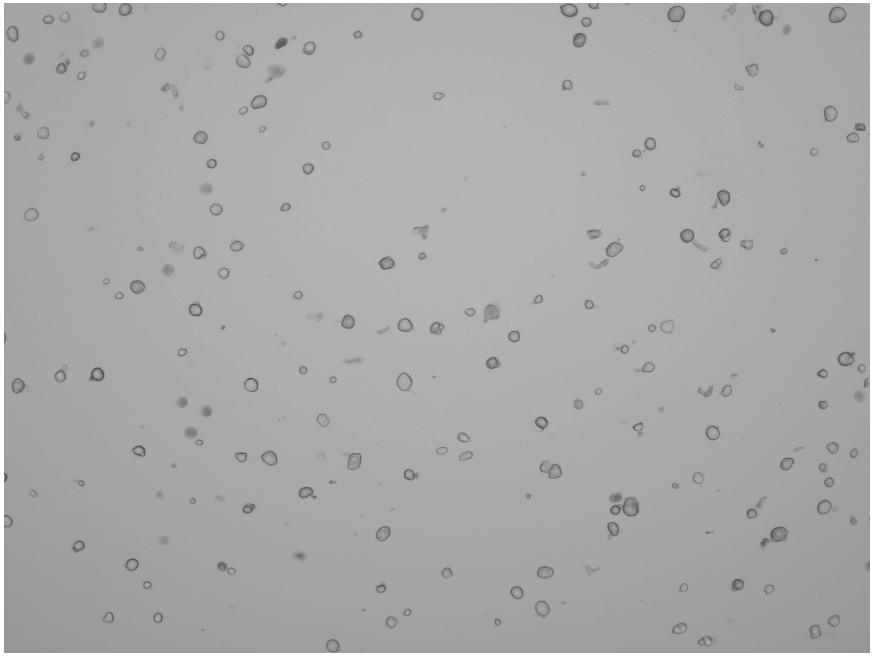
- Organic matter left over from dead cells.
- Impacts targeted cell recovery.
  - Free RNA → noise.
  - Hard to obtain cell counts.
  - Clog microfluidics



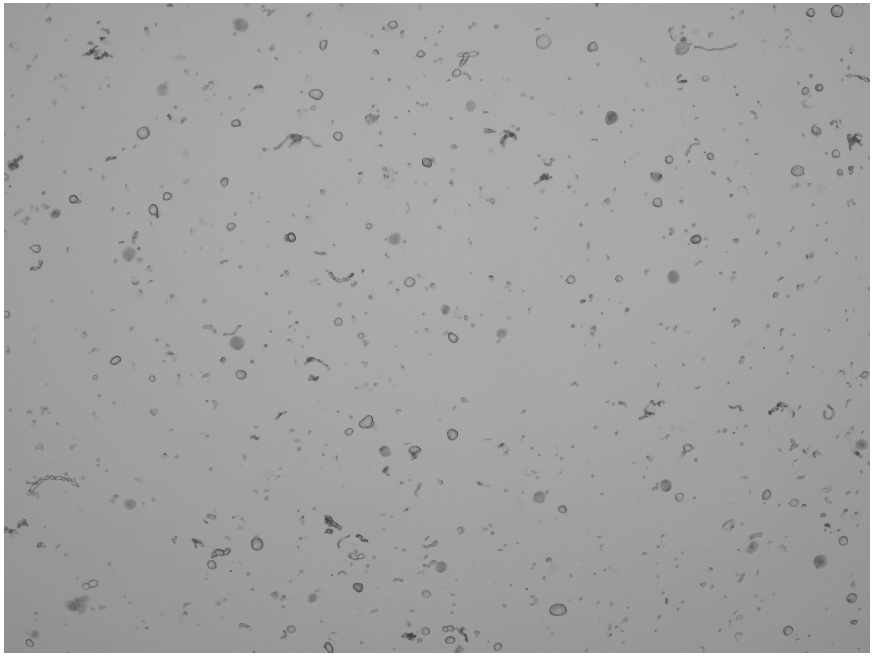
http://www.nexcelom.com/Cellometer-Vision-CBA/

Cell debris example I

Mouse



Clean DRG sample



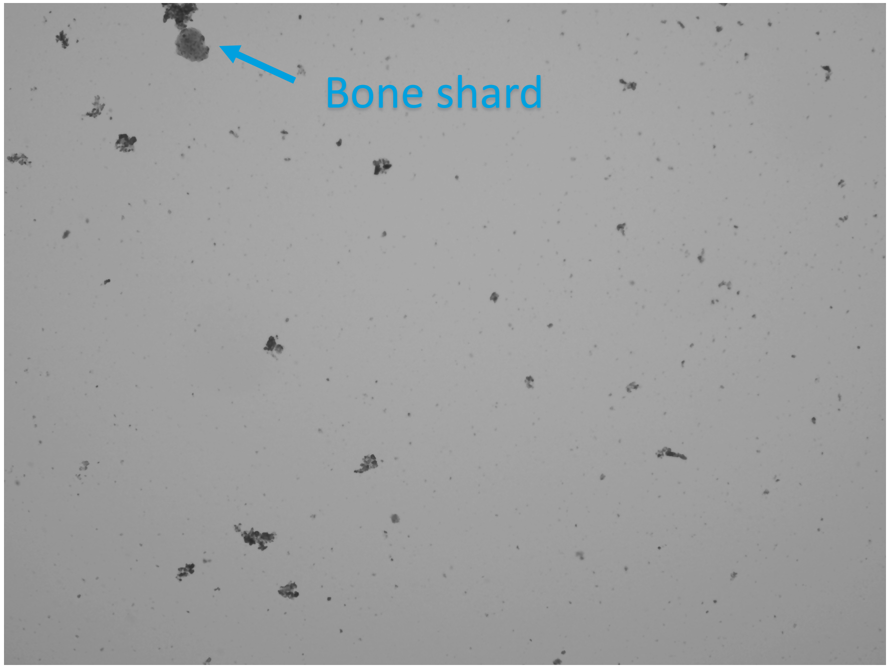
Noisy sample

Recommended treatment: filtration, centrifuge, add blocking agent.

Cell debris example II

Human

Clean human sample

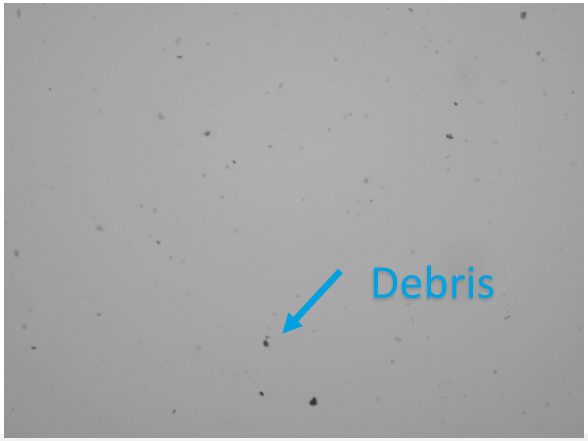


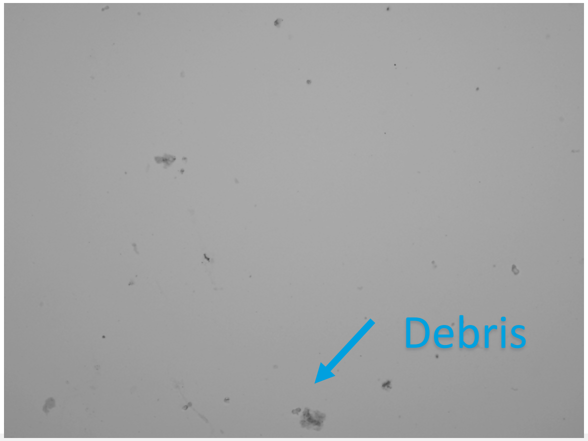
All debris

Recommended treatment: redo isolation.

Summary - debris

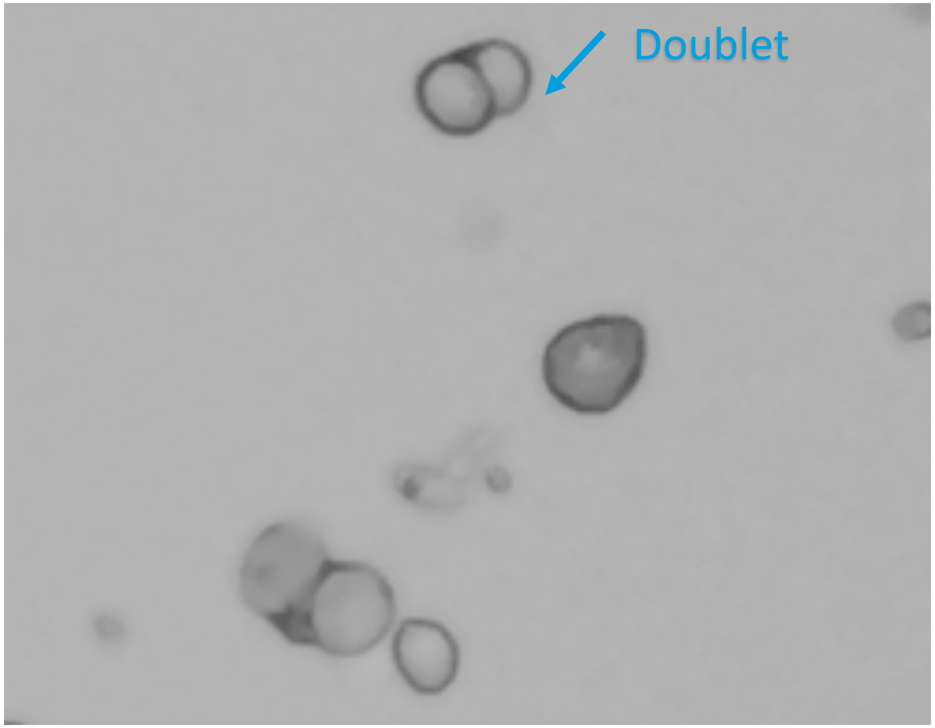
- Work on gentle dissociation cells.
  - Ex) Trituration with P-1000 first then P-200.
- Filtration (ex, 40 micron strainer).
- Density gradient centrifugation.
  - Ex) ficoll or sucrose gradient.
- Detergents.
- Redo cell isolation.





Doublets

- Non-single cell clumps.
- Integrated into droplets and cannot* distinguish from single cells.

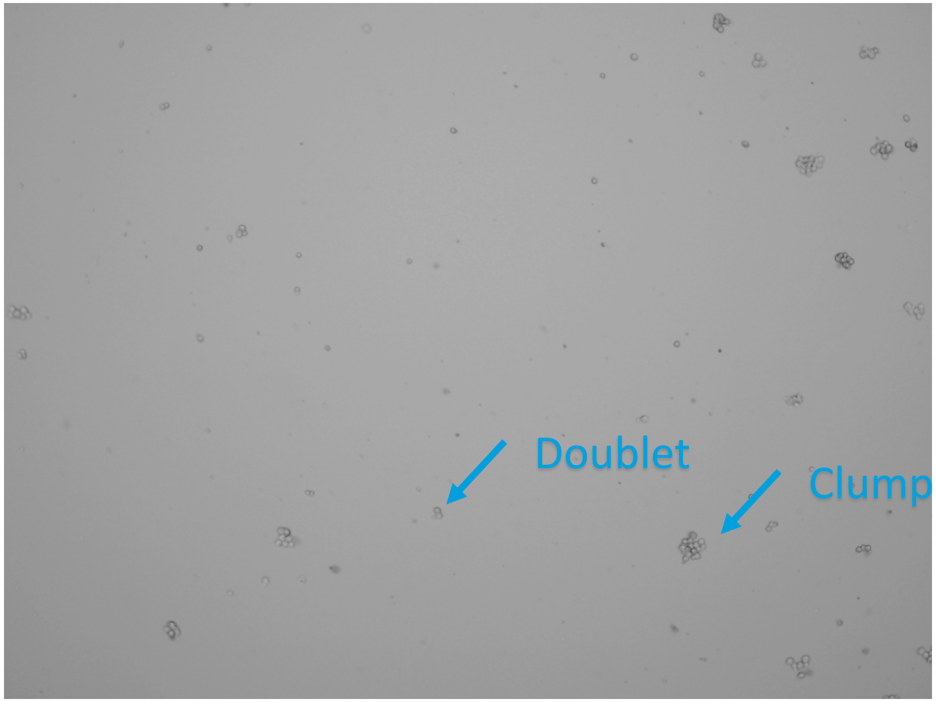


Bad for any single cell experiment.

*Call some doublets in tagged cells (multi-seq or ‘Feature Barcodes’)

Doublets example I

Good sample

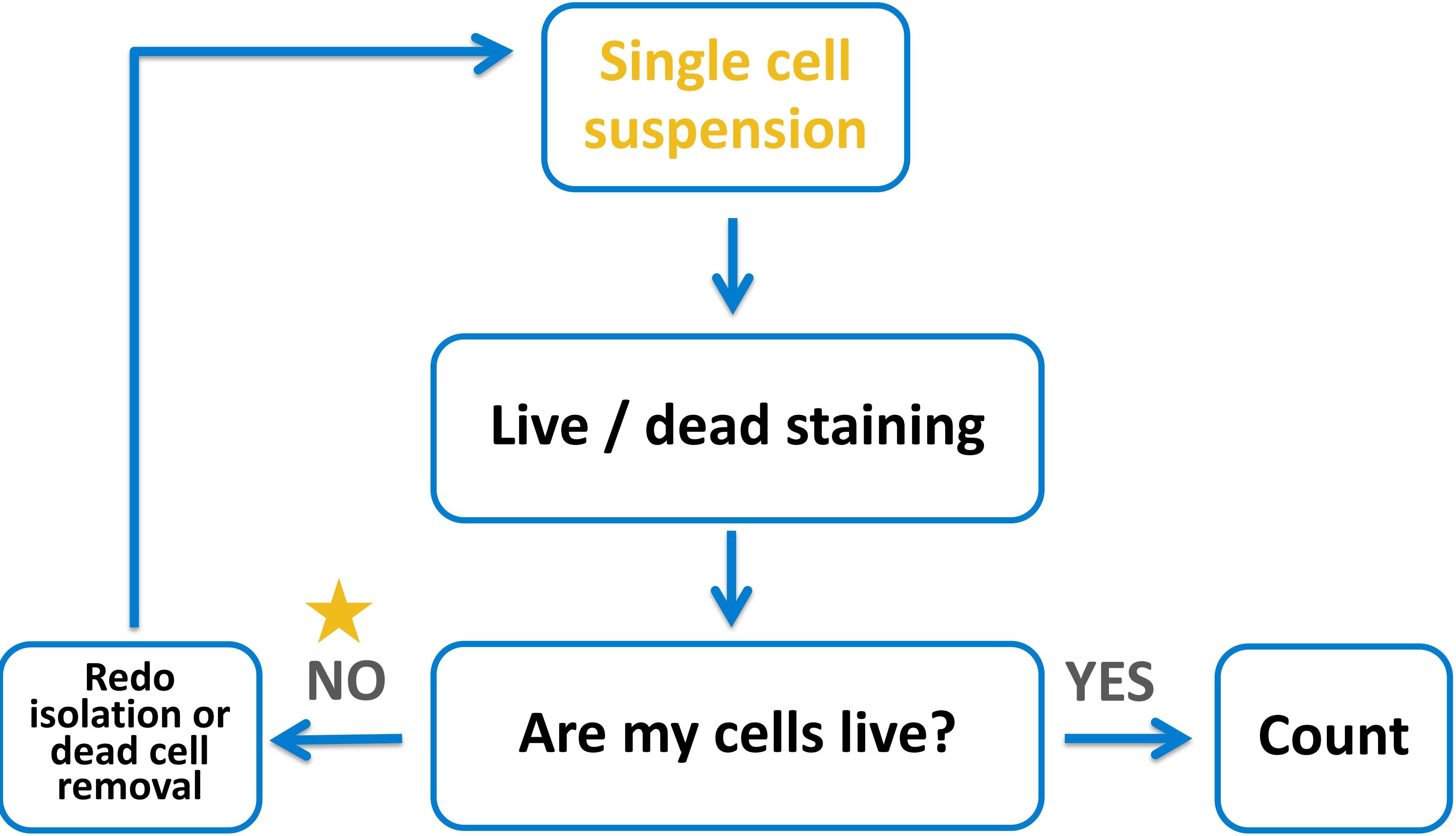


Many multiplets

Summary - doublets

- Did you fully dissociate your tissue?
- Cells can sit too long → clumps.
  - Depends on cell type.
  - Know the temperature your cells are happy at.
    - Ex) some bovine cells like to be warm; neurons RT, etc.
- Add blocking agent.
  - BSA or FBS (make sure conc. compatible with 10X)

Dead cells



Single cell suspension

Live / dead staining

Are my cells live?

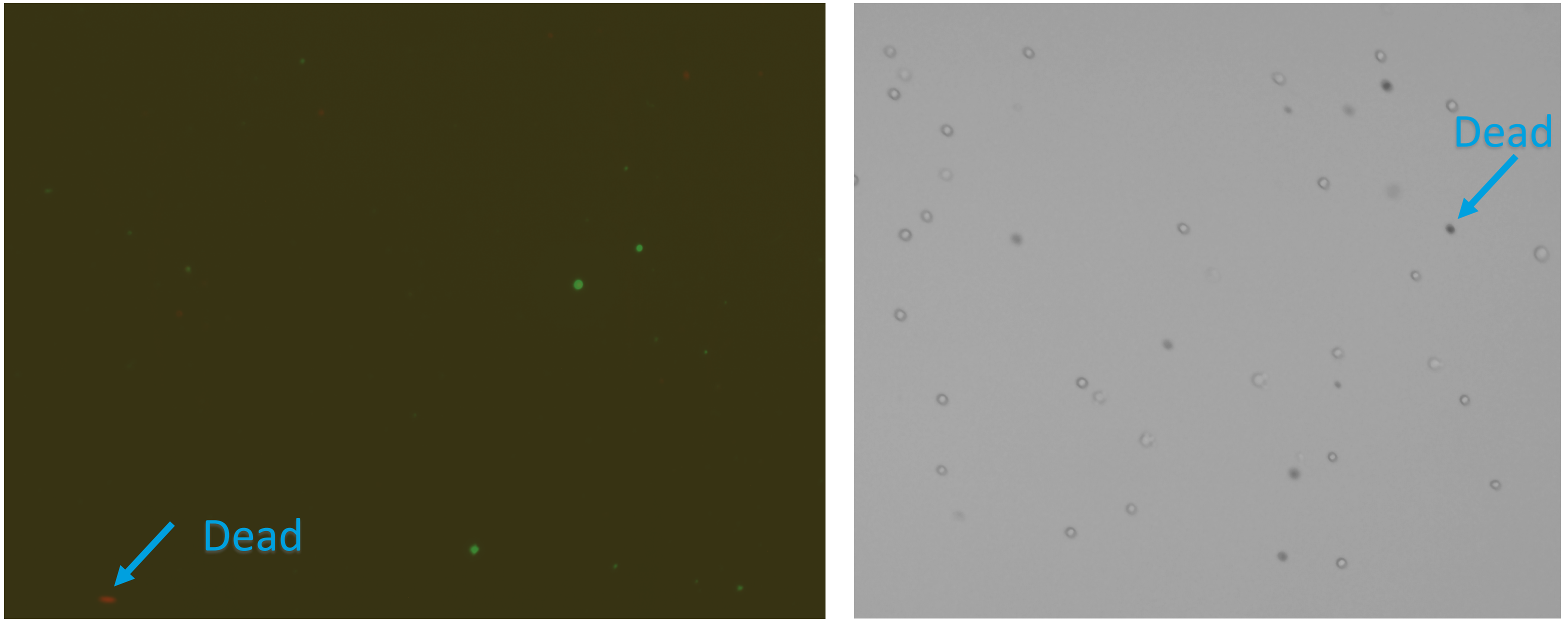
YES

Count

NO ★

Redo isolation or dead cell removal

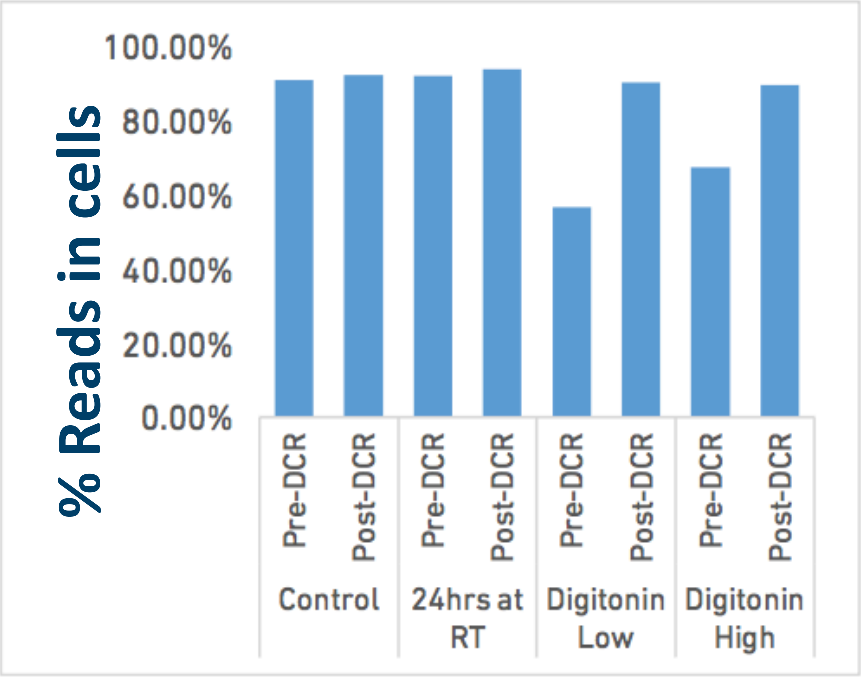
Assessing cell death

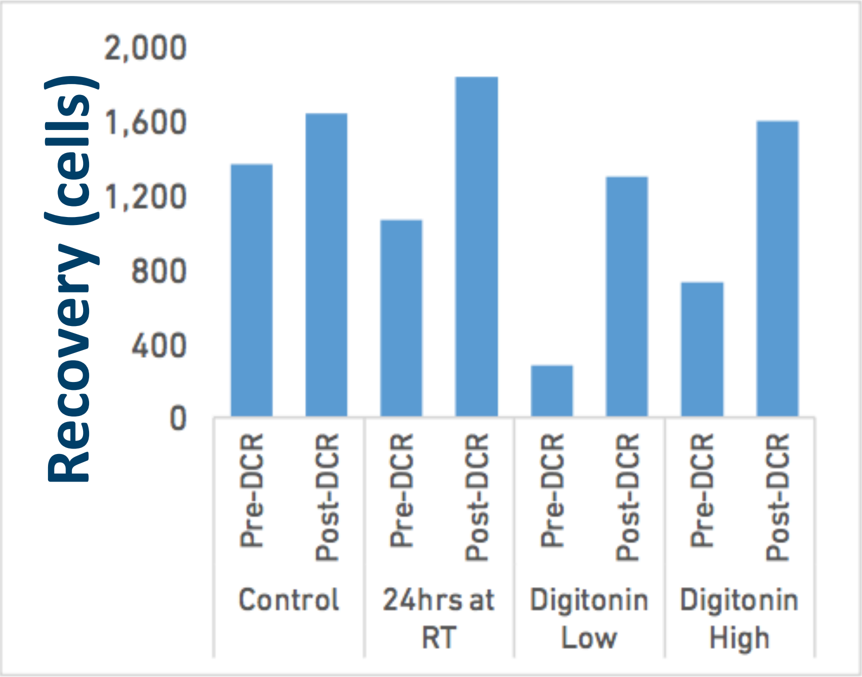


- Use cell-impermeant dye or fluorescent labels.
  - Automated cell counter okay, but sometimes better by hand.
    - Very small cells may stain ‘dead’ automated cell counter.
- Cells die during isolation, but also during (improper) cryopreservation.
  - Count before and after cryopreservation.

Do I need a clean suspension?

- Digitonin (mimics dead cells) treated cell suspension.
- DCR – **D**ead **C**ell **R**emoval





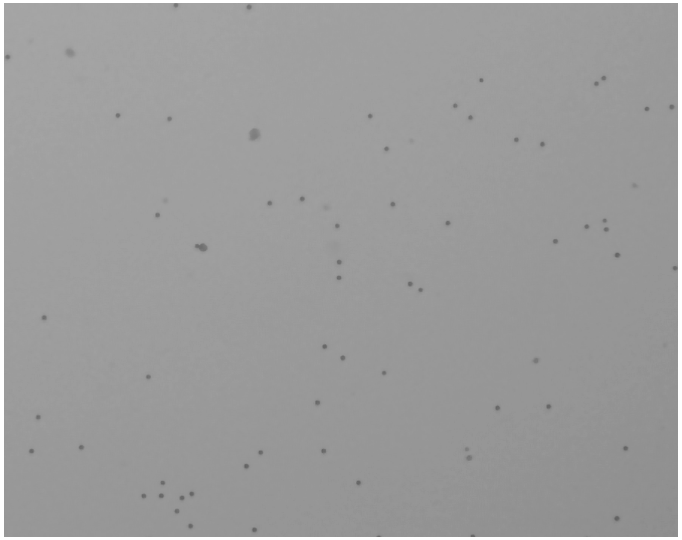
↑ Reads in cells.

↑ Targeted recovery.

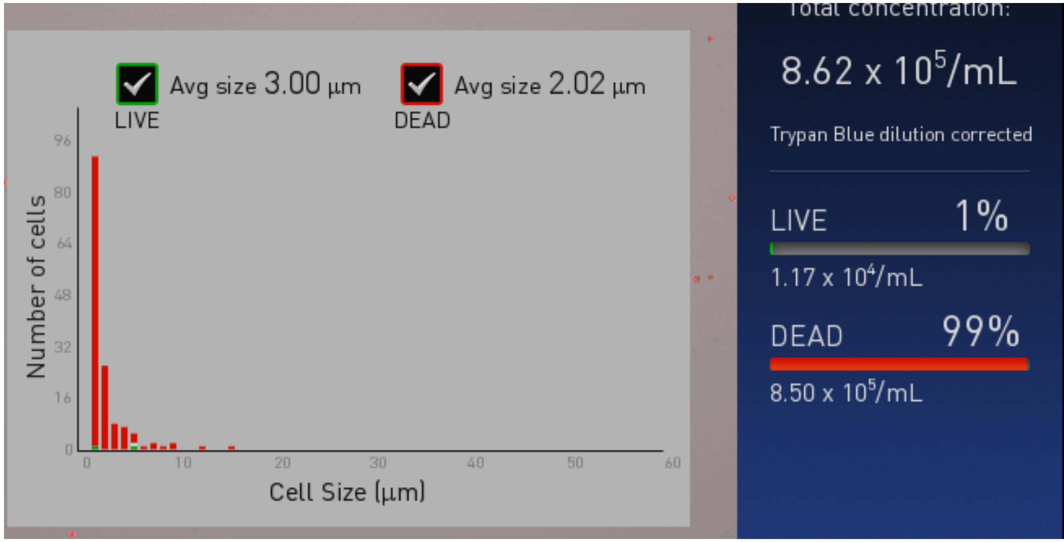
★ CG000130_10x_Technical Note_DeadCell Removal_RevA.docx

Cell Size and did I isolate the correct cells?

- Microfluidics have cell size limit
  - 40 microns or less.
  - Clogs, etc.
- Nuclei isolation great alternative to large cells.
- Know your cell size.
  - Do you have RBC contamination (‘empty’ cells).



Small dark cells



☑ LIVE Avg size 3.00 µm

☑ DEAD Avg size 2.02 µm

Total concentration:

8.62×10^5/mL

Trypan Blue dilution corrected

LIVE 1%

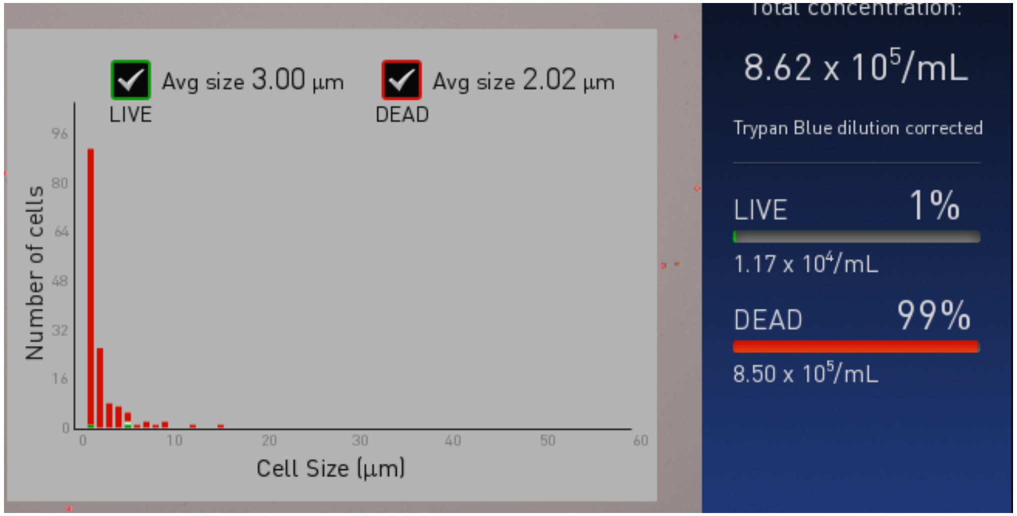
1.17×10^4/mL

DEAD 99%

8.50×10^5/mL

Histogram cell size

Cell size – example I



Total concentration:

8.62 x 10^5/mL

Trypan Blue dilution corrected

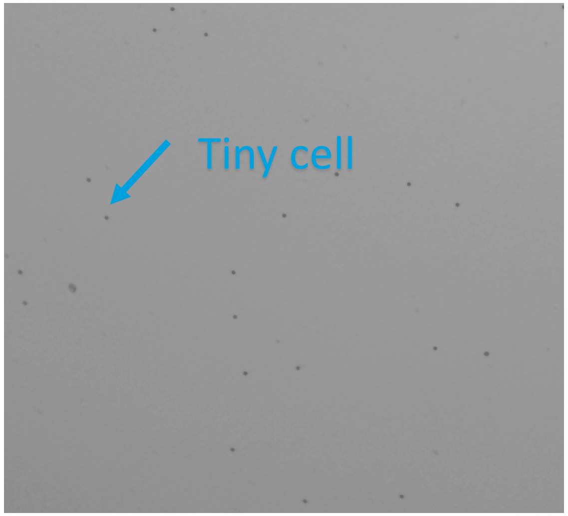
LIVE 1%

1.17 x 10^4/mL

DEAD 99%

8.50 x 10^5/mL

- Expect cells to be from 2 micron to 20 micron.
- Average size 3um.



Two different isolations from same tissue.

Left – small and uniform.

Right – variable sizes.

Overview

- Technology overview.
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- Sample QC.
    - Do I have single cells?
    - Are they alive?
    - Are they too big?
    - Did I isolate the correct cells?
- How do I count them?
- Methanol Fixation.

Cell counting – automated

- Countess II (done by DNA Tech).

Total concentration:

6.45 x 10^5/mL

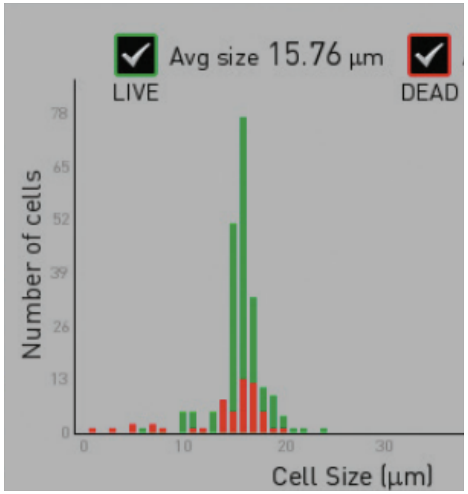
Trypan Blue dilution corrected

LIVE 90%

5.81 x 10^5/mL

DEAD 10%

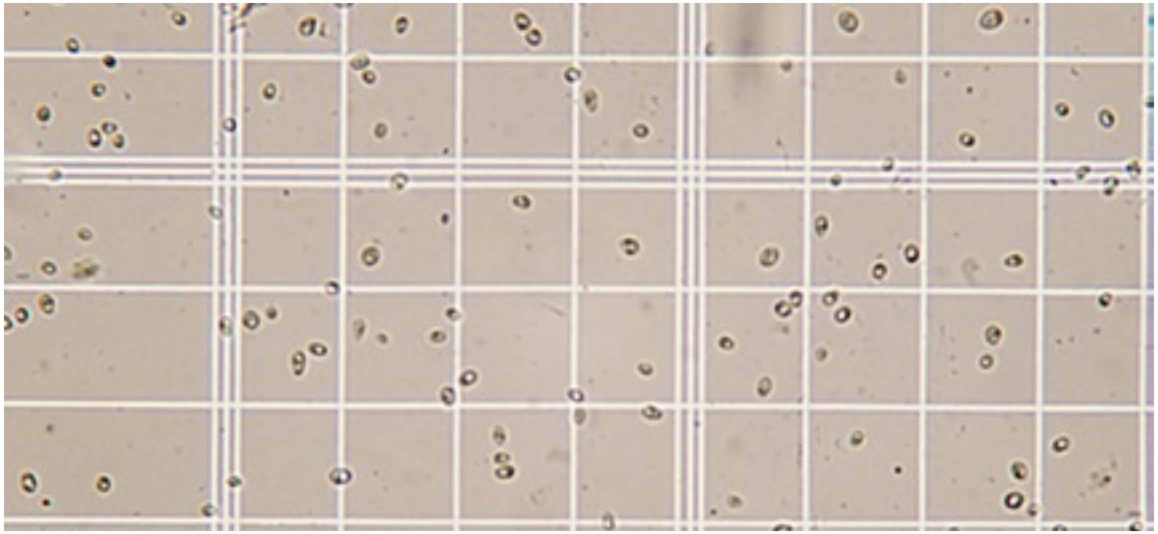
6.45 x 10^4/mL

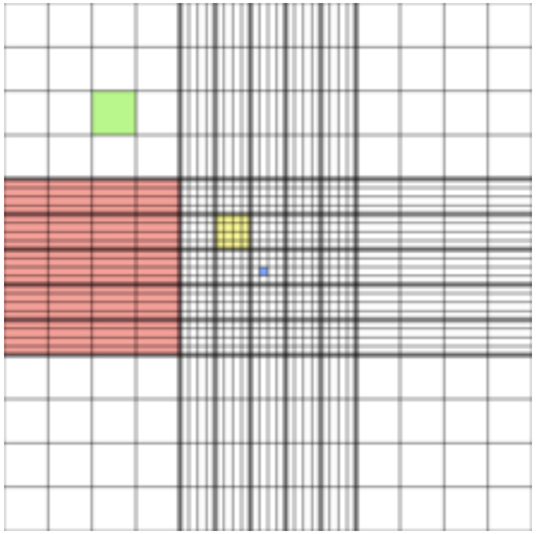


- Pros (+):
  - Fast.
  - Live/dead cell counts.
  - Cell size estimates.
- Cons (-):
  - Cell size limits (4-60um).
  - Performance poor for odd shapes.
  - Cell type limitations (DRG…)

Cell counting – manual

- Hemocytometer (customer-supplied counts).



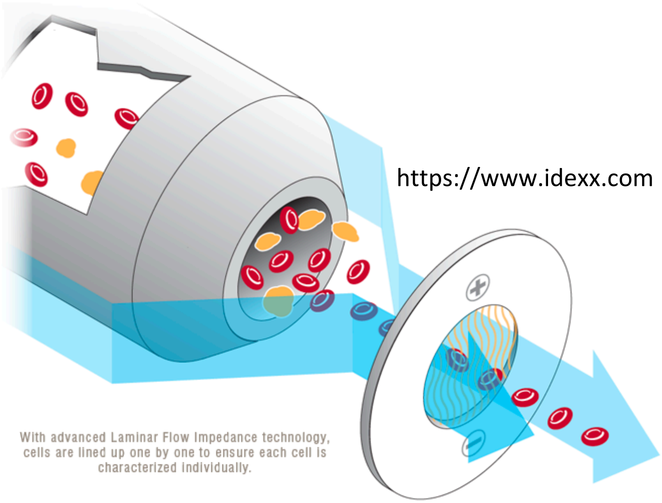


http://www.wetnewf.org/pdfs/hemocytometer.html

- Pros (+):
  - Reliable cell counts.
- Cons (-):
  - Slow?

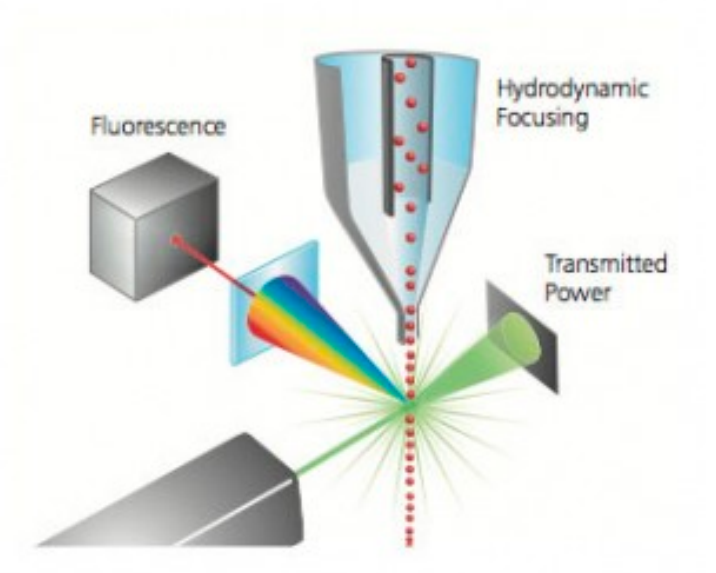
Cell counting – automated

- Flow



https://www.idexx.com

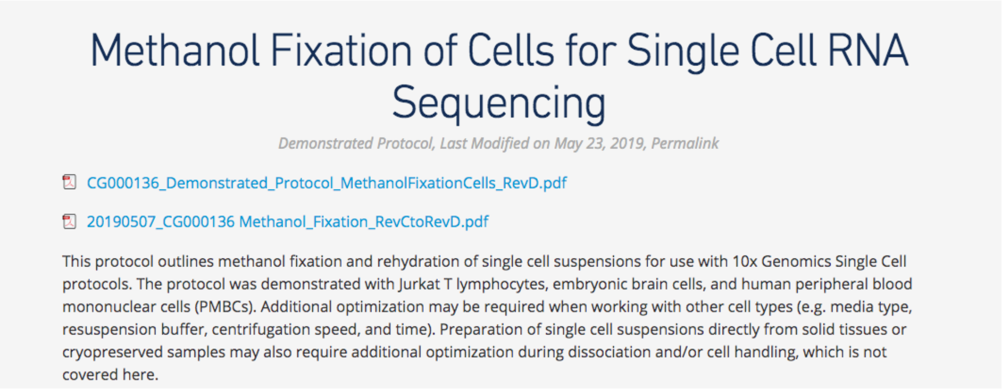
With advanced Laminar Flow Impedance technology, cells are lined up one by one to ensure each cell is characterized individually.



- Pros (+):
  - Sort based on characteristics (fluorescence, etc).
  - Characterize cells.
- Cons (-):
  - Cannot provide absolute counts.
  - Tends to overestimate cells.
  - Concentrate after.

Methanol fixation

- Methanol permeabilises cells and dehydrates proteins.
- Kills infectious agents, while maintaining cell structure.



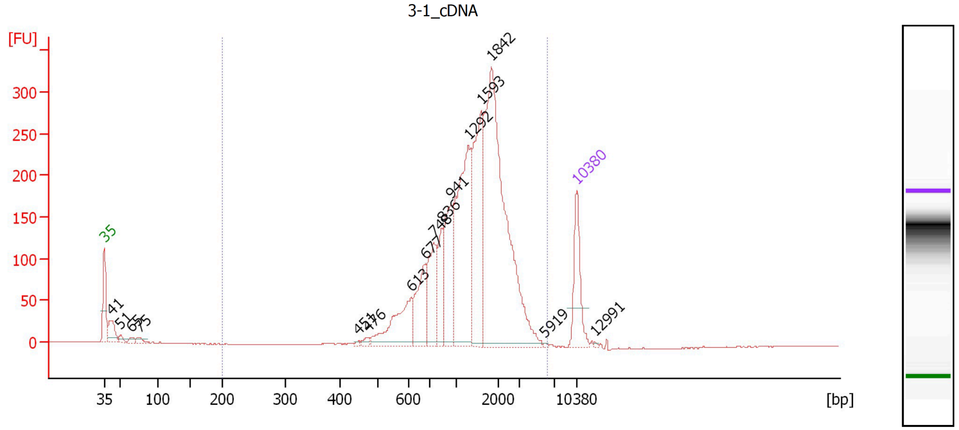
Methanol Fixation of Cells for Single Cell RNA Sequencing

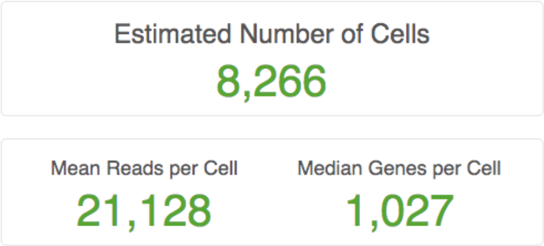
Demonstrated Protocol, Last Modified on May 23, 2019, Permalink

CG000136_Demonstrated_Protocol_MethanolFixationCells_RevD.pdf

20190507_CG000136 Methanol_Fixation_RevCtoRevD.pdf

This protocol outlines methanol fixation and rehydration of single cell suspensions for use with 10x Genomics Single Cell protocols. The protocol was demonstrated with Jurkat T lymphocytes, embryonic brain cells, and human peripheral blood mononuclear cells (PMBCs). Additional optimization may be required when working with other cell types (e.g. media type, resuspension buffer, centrifugation speed, and time). Preparation of single cell suspensions directly from solid tissues or cryopreserved samples may also require additional optimization during dissociation and/or cell handling, which is not covered here.

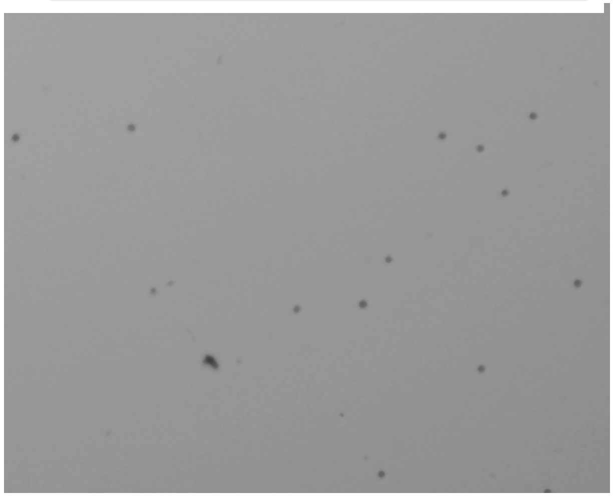




Estimated Number of Cells

8,266

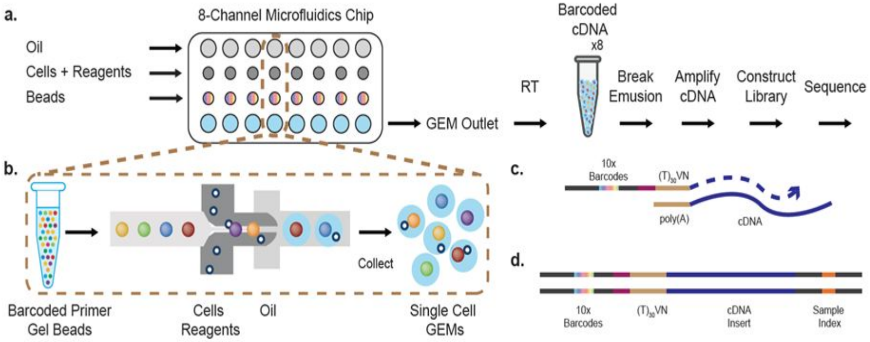
Mean Reads per Cell	Median Genes per Cell
21,128	1,027



Cells stain 100% dead and can be stored for weeks

Important metrics – 10X

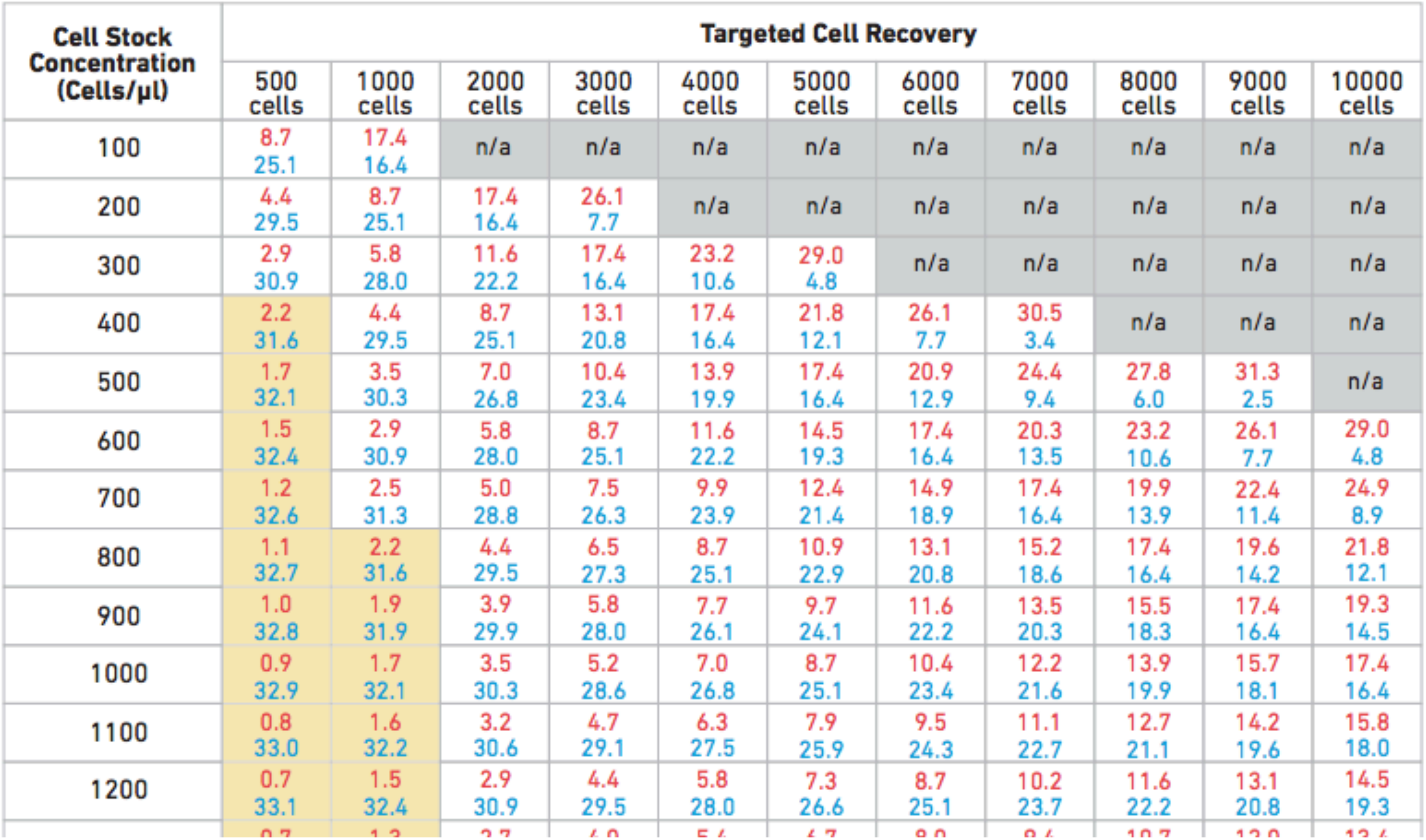
- Concentration: 100-2,000 cells per µl.
  - –**700-1,200 cells per µl**.
  - –Count in replicates!
  - –We require cell counts prior to delivery.
- Viability: **70% minimum**.
  - –Nuclei and methanol fixed cells.
- Sample buffer.
- Cell size and shape..
- Treat cells gently.
  - –Wide bore pipette tips, keep cells on ice, etc.



Credit-10X Genomics

Chip loading

- Very flexible → **cell concentration** and **recovery.**



Cell Stock Concentration (Cells/µl)	Targeted Cell Recovery										
	500 cells	1000 cells	2000 cells	3000 cells	4000 cells	5000 cells	6000 cells	7000 cells	8000 cells	9000 cells	10000 cells
100	8.7 25.1	17.4 16.4	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
200	4.4 29.5	8.7 25.1	17.4 16.4	26.1 7.7	n/a	n/a	n/a	n/a	n/a	n/a	n/a
300	2.9 30.9	5.8 28.0	11.6 22.2	17.4 16.4	23.2 10.6	29.0 4.8	n/a	n/a	n/a	n/a	n/a
400	2.2 31.6	4.4 29.5	8.7 25.1	13.1 20.8	17.4 16.4	21.8 12.1	26.1 7.7	30.5 3.4	n/a	n/a	n/a
500	1.7 32.1	3.5 30.3	7.0 26.8	10.4 23.4	13.9 19.9	17.4 16.4	20.9 12.9	24.4 9.4	27.8 6.0	31.3 2.5	n/a
600	1.5 32.4	2.9 30.9	5.8 28.0	8.7 25.1	11.6 22.2	14.5 19.3	17.4 16.4	20.3 13.5	23.2 10.6	26.1 7.7	29.0 4.8
700	1.2 32.6	2.5 31.3	5.0 28.8	7.5 26.3	9.9 23.9	12.4 21.4	14.9 18.9	17.4 16.4	19.9 13.9	22.4 11.4	24.9 8.9
800	1.1 32.7	2.2 31.6	4.4 29.5	6.5 27.3	8.7 25.1	10.9 22.9	13.1 20.8	15.2 18.6	17.4 16.4	19.6 14.2	21.8 12.1
900	1.0 32.8	1.9 31.9	3.9 29.9	5.8 28.0	7.7 26.1	9.7 24.1	11.6 22.2	13.5 20.3	15.5 18.3	17.4 16.4	19.3 14.5
1000	0.9 32.9	1.7 32.1	3.5 30.3	5.2 28.6	7.0 26.8	8.7 25.1	10.4 23.4	12.2 21.6	13.9 19.9	15.7 18.1	17.4 16.4
1100	0.8 33.0	1.6 32.2	3.2 30.6	4.7 29.1	6.3 27.5	7.9 25.9	9.5 24.3	11.1 22.7	12.7 21.1	14.2 19.6	15.8 18.0
1200	0.7 33.1	1.5 32.4	2.9 30.9	4.4 29.5	5.8 28.0	7.3 26.6	8.7 25.1	10.2 23.7	11.6 22.2	13.1 20.8	14.5 19.3

Cost – 2019 10X Single Cell

DNA TECH

UC RATES

Library prep	Total Cost
1 sample	$2,072
2 samples	$3,909
3 samples	$5,746

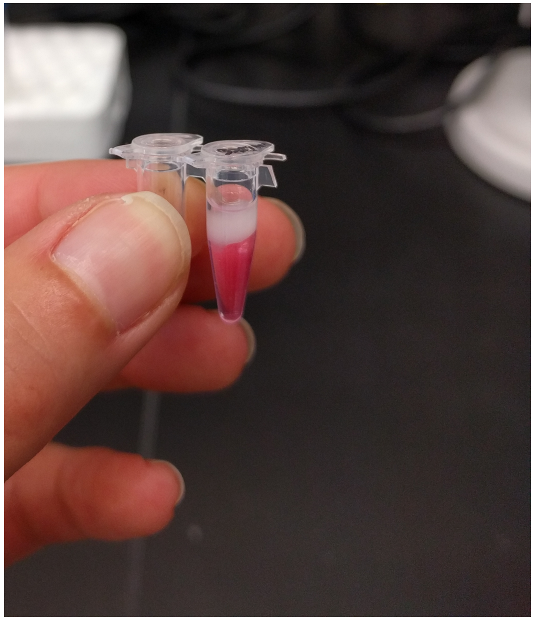
Per prep day	Cost
Labor, cell QC, chip	$235
Labor, reagents, instrument use	$1,837

Important resources

- 10X Genomics
  - https://support.10xgenomics.com/single-cell-gene-expression
- UC Davis Flow Cytometry
  - http://www.ucdmc.ucdavis.edu/pathology/research/research_labs/flow_cytometry/index.html
  - Bridget McLaughlin (Technical Director)
- UC Davis DNA Technology Core
  - http://dnatech.genomecenter.ucdavis.edu/single-cell-analyses/

Acknowledgements

- UC Davis DNA Technology Core.
- UC Davis Bioinformatics Core.
- 10X Genomics.
  - Nicole Rapicavoli (Lead Field Applications Scientist).



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