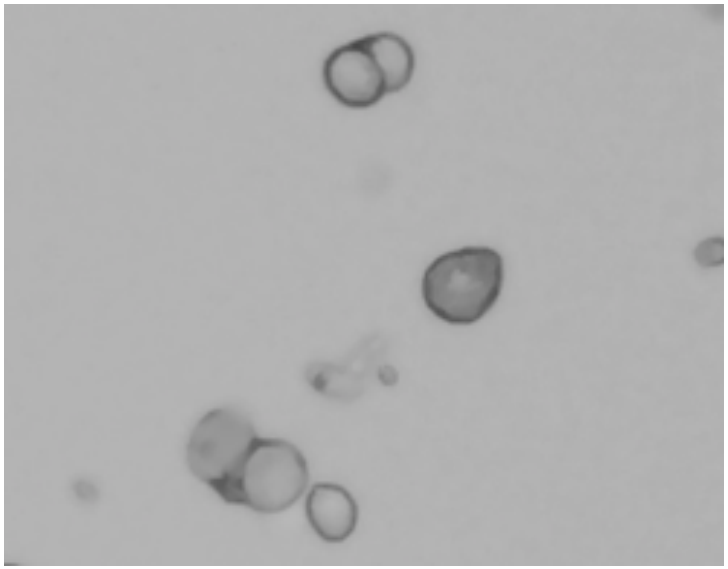
Single Cell Sample Preparation



Diana Burkart-Waco, PhD

dburkart@ucdavis.edu

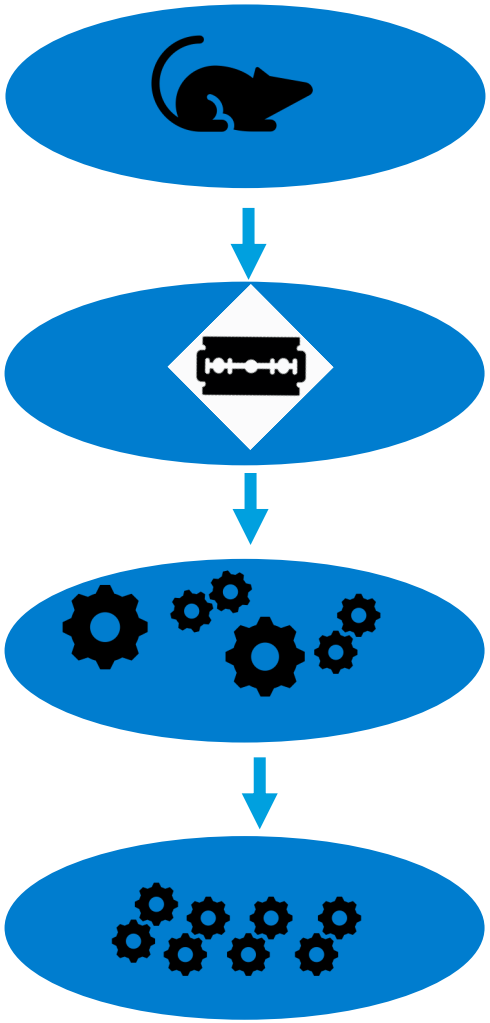
SRA at UC Davis DNATECH Core

https://dnatech.genomecenter.ucdavis.edu/

General Workflow

Tissue dissection

Fast, reliable, clean.



Mechanical mincing

Trituration, razor blade, etc.

Enzymatic breakdown

E.g. Trypsin, dispase, collagenase)

Often with agitation

Cell enrichment (optional)

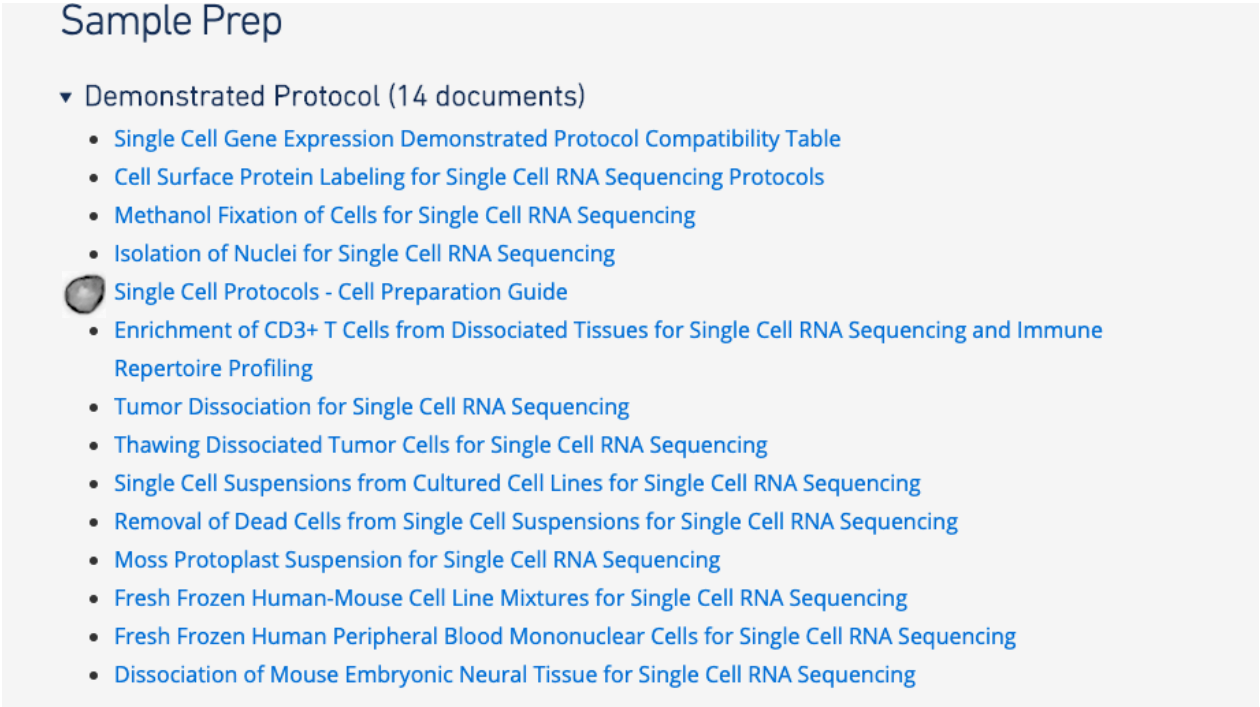
Obtain cell interest (flow)

Remove dead cells (beads, etc.)

Cell isolation

- Cell isolation guides available at:

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



Sample Prep

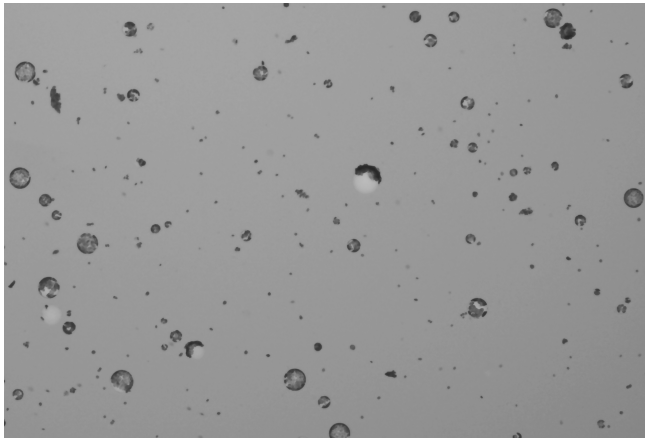
▾ Demonstrated Protocol (14 documents)

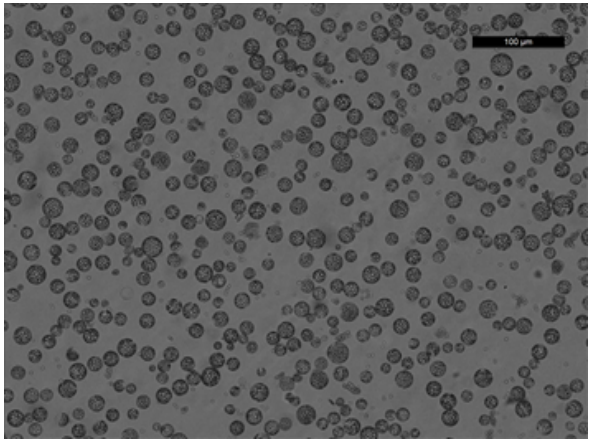
- Single Cell Gene Expression Demonstrated Protocol Compatibility Table
- Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols
- Methanol Fixation of Cells for Single Cell RNA Sequencing
- Isolation of Nuclei for Single Cell RNA Sequencing
- Single Cell Protocols - Cell Preparation Guide
- Enrichment of CD3+ T Cells from Dissociated Tissues for Single Cell RNA Sequencing and Immune Repertoire Profiling
- Tumor Dissociation for Single Cell RNA Sequencing
- Thawing Dissociated Tumor Cells for Single Cell RNA Sequencing
- 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

- Cell isolation → biggest source technical variation.
- Dissociation and preparation depends on cell type.

Tissue Dissociation

- Many ways to dissociate tissue:
  - Mechanical
  - Enzymatic
  - Automated blending
  - Microfluidics devices
- Considerations:
  - Speed
  - Consistency in results
  - Good representation of all cell types





Arabidopsis protoplasts

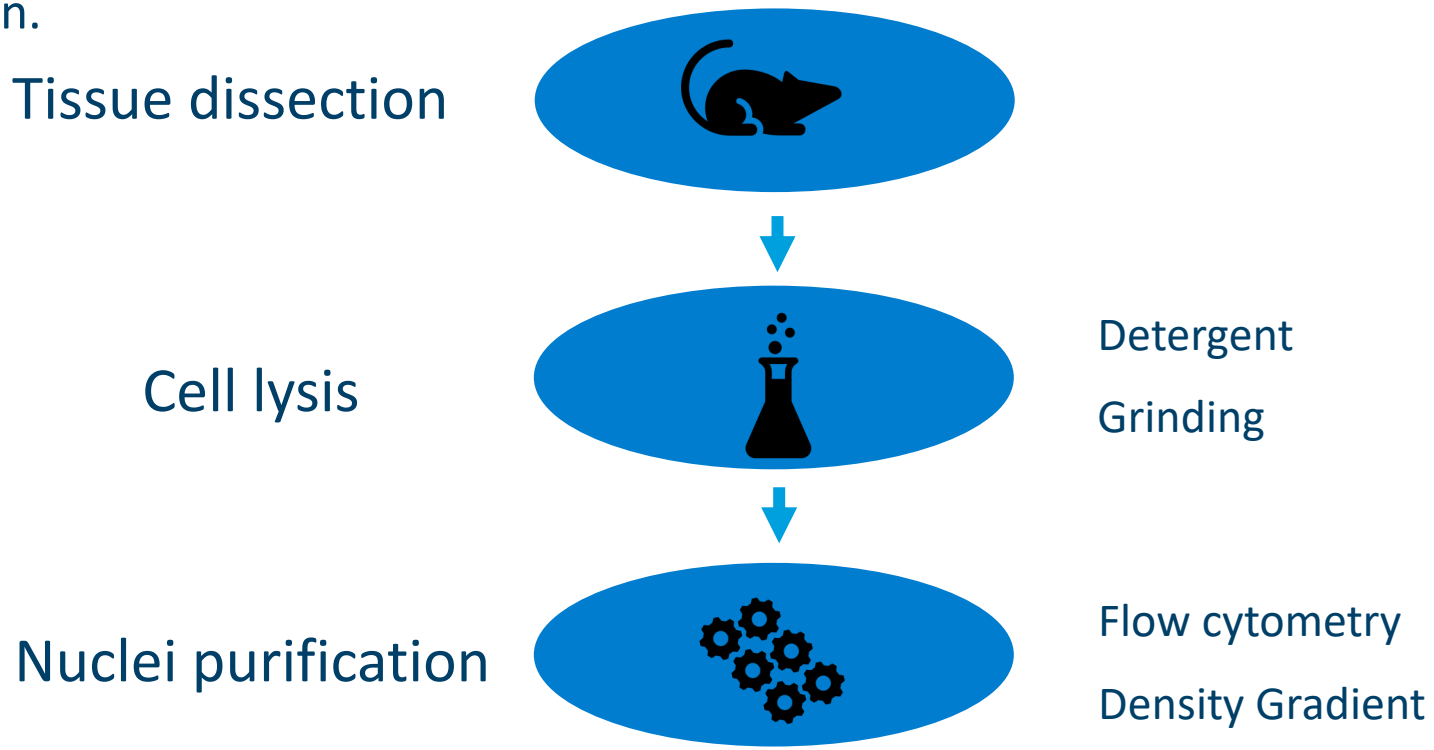
Same method, different days.

Factors Influencing Dissociation

Unfortunately, SC sample optimization is best achieved through trial and error…

Single cell vs. nuclei

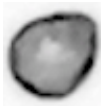
- Single cell captures more transcripts, but is a harder protocol.
- When to use nuclei:
  - Cells cannot be harvested intact or viable (e.g. adipocytes, neurons).
  - Cells are too big for capture (e.g. cardiomyocytes).
  - Tissue frozen.



Many protocols available…

- Start here:

Customer Developed Protocol

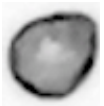


'Frankenstein' protocol for nuclei isolation from fresh and frozen tissue

Contributed by:
Luciano Martelotto, Ph.D., Melbourne, Centre for Cancer Comprehensive Cancer Centre

www.collaslab.com

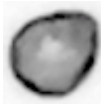
Isolation of Nuclei from Somatic Cells



1. HeLa Cells, 293T Cells, NT2 Cells

Cell preparation

- harvest cells from flasks as per standard protocol
- spin cells in 50 ml conical tube at 1500 rpm for 10 min at RT
- resuspend cells in 30 ml PBS; take a 50 l sample to determine concentration



10x Genomics®

Sample Preparation Demonstrated Protocols

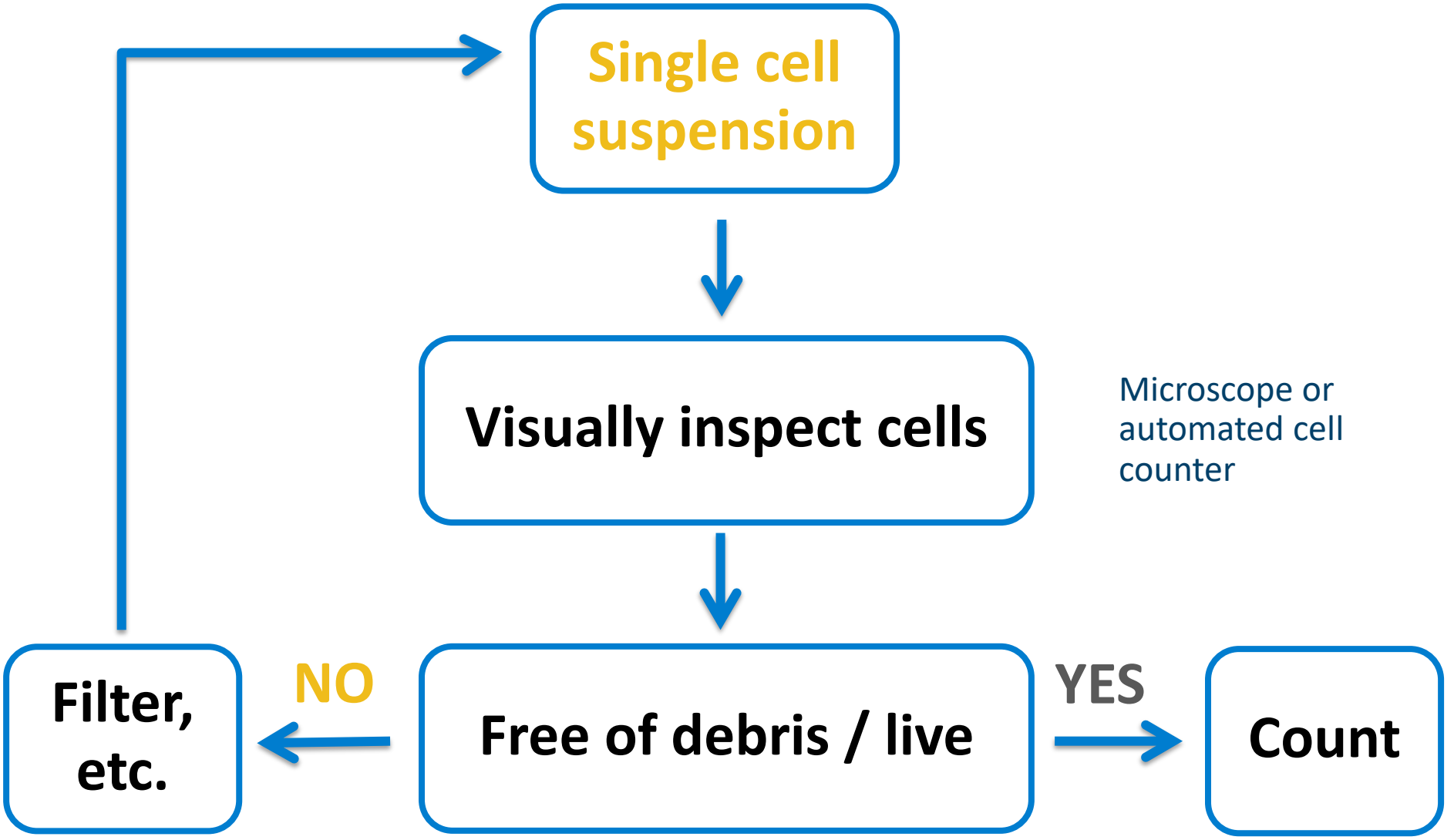
Isolation of Nuclei for Single Cell RNA Sequencing

Factors influencing success

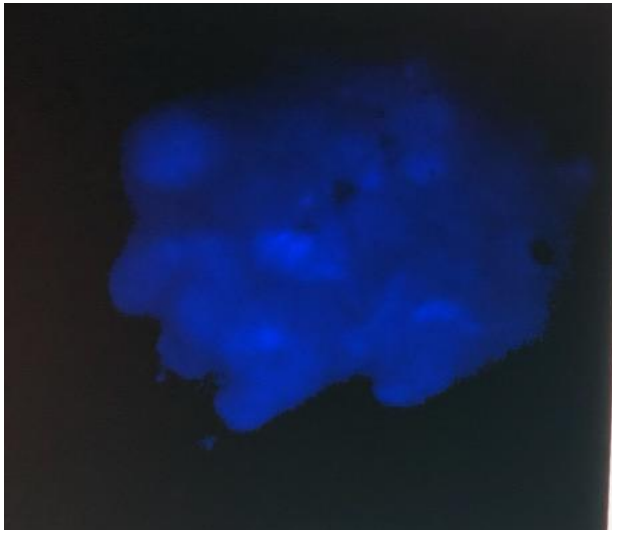
- Cell debris / dead cells
  - Clog microfluidics, free floating RNA → noise.
- Aggregates
  - No longer single cell data.
  - Clog microfluidics.
- Buffer
  - Inhibit polymerase → decrease library complexity.
- Storage conditions

But the most important factor is **cell / nuclei counting!**

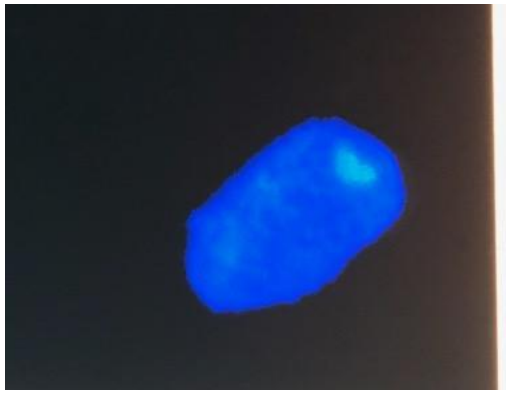
Workflow


Single cell suspension
Visually inspect cells
Microscope or automated cell counter
Free of debris / live
NO
Filter, etc.
YES
Count

Tissue aggregates



Tissue clump



Single nucleus

DAPI stain

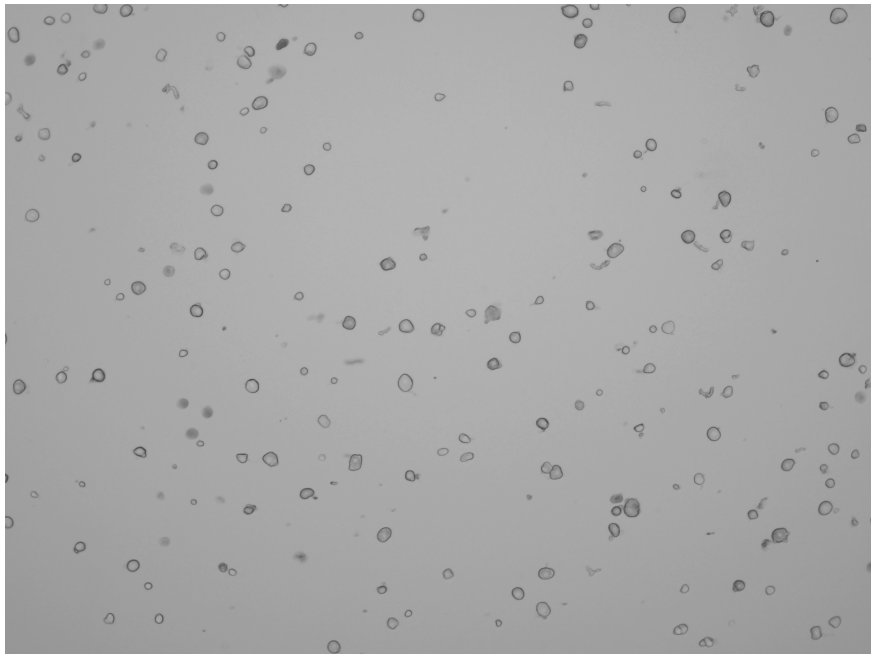
60X

No scale bar

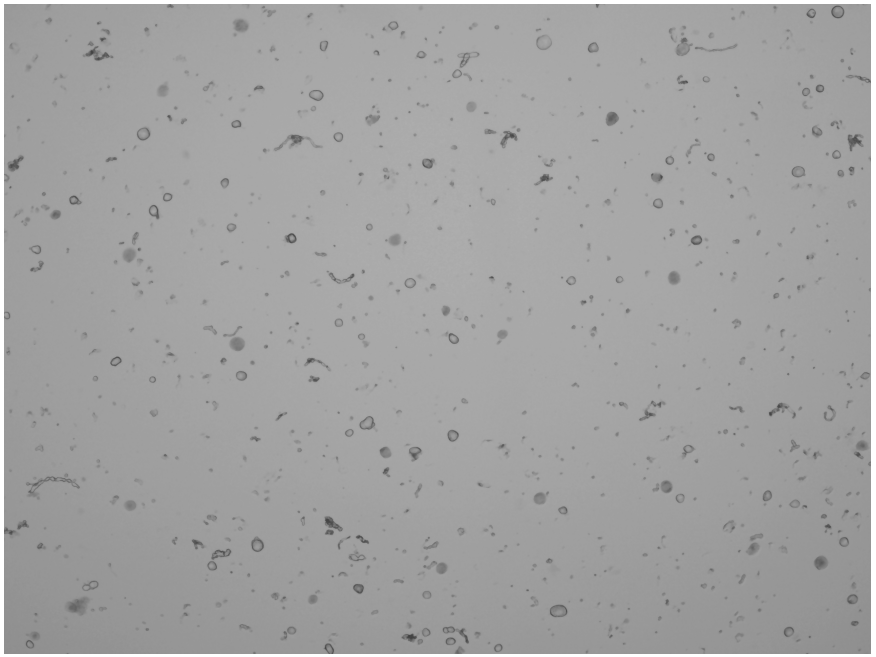
Recommended treatment: optimize tissue dissociation

Cell debris

- Mouse DRG.



Clean DRG sample

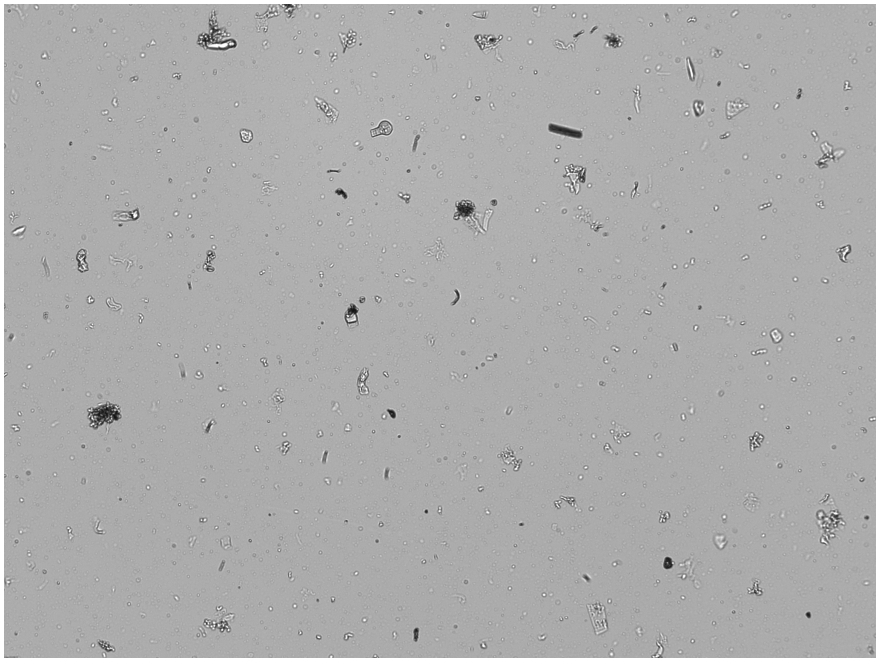


Noisy sample

Recommended treatment: filtration, centrifuge, add blocking agent.

Cell debris II

- Lettuce nuclei prep.



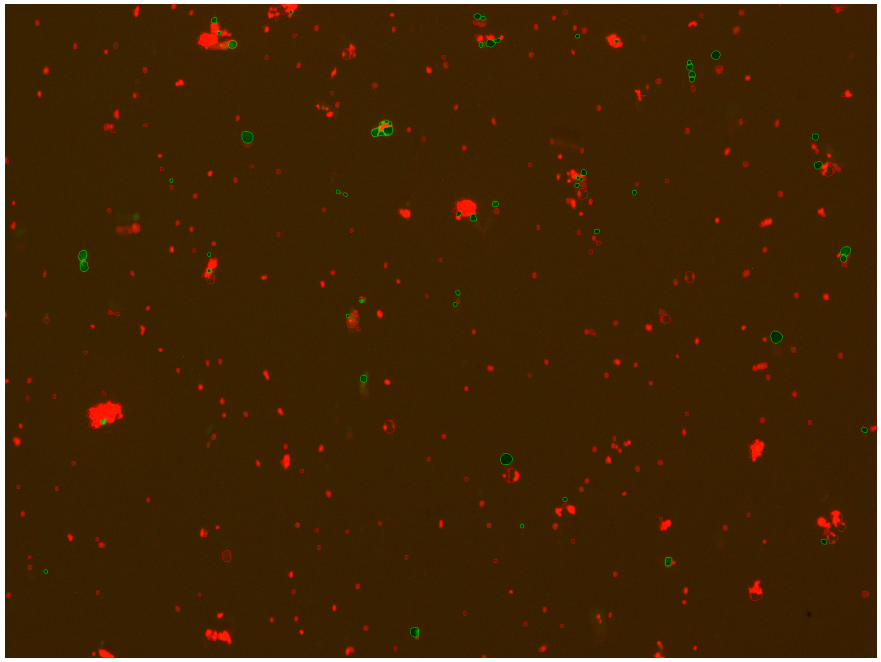
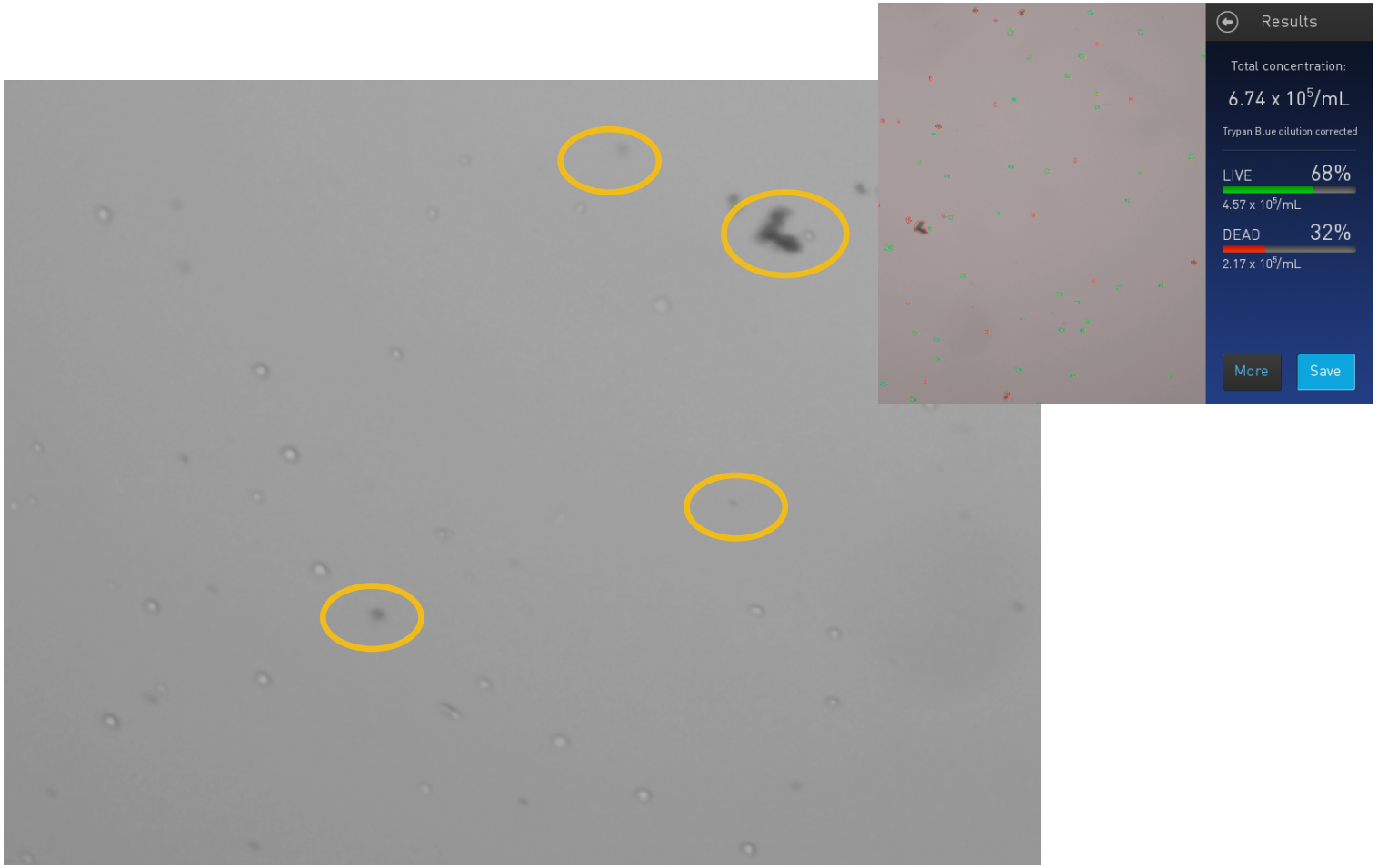


Photo credit: Mohan Prem Anand Marimuthu

Recommended treatment: modify dissociation times / detergent concentrations, change density gradient.

Dead cells (+debris?)

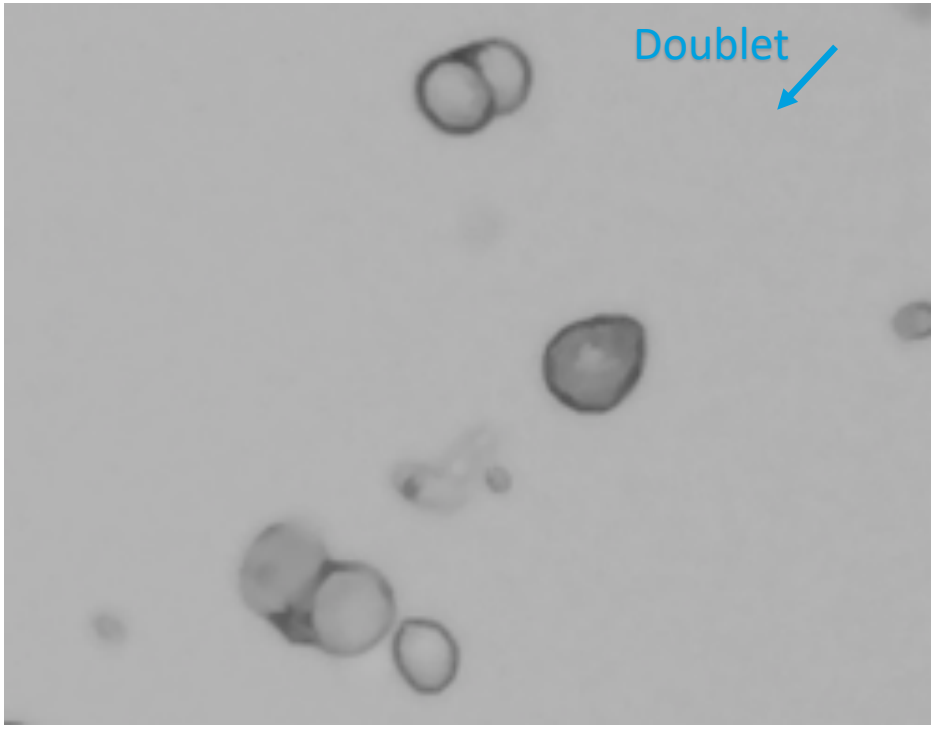


Recommended treatment: dead cell removal.

https://www.miltenyibiotec.com/US-en/products/dead-cell-removal-kit.html

Doublets

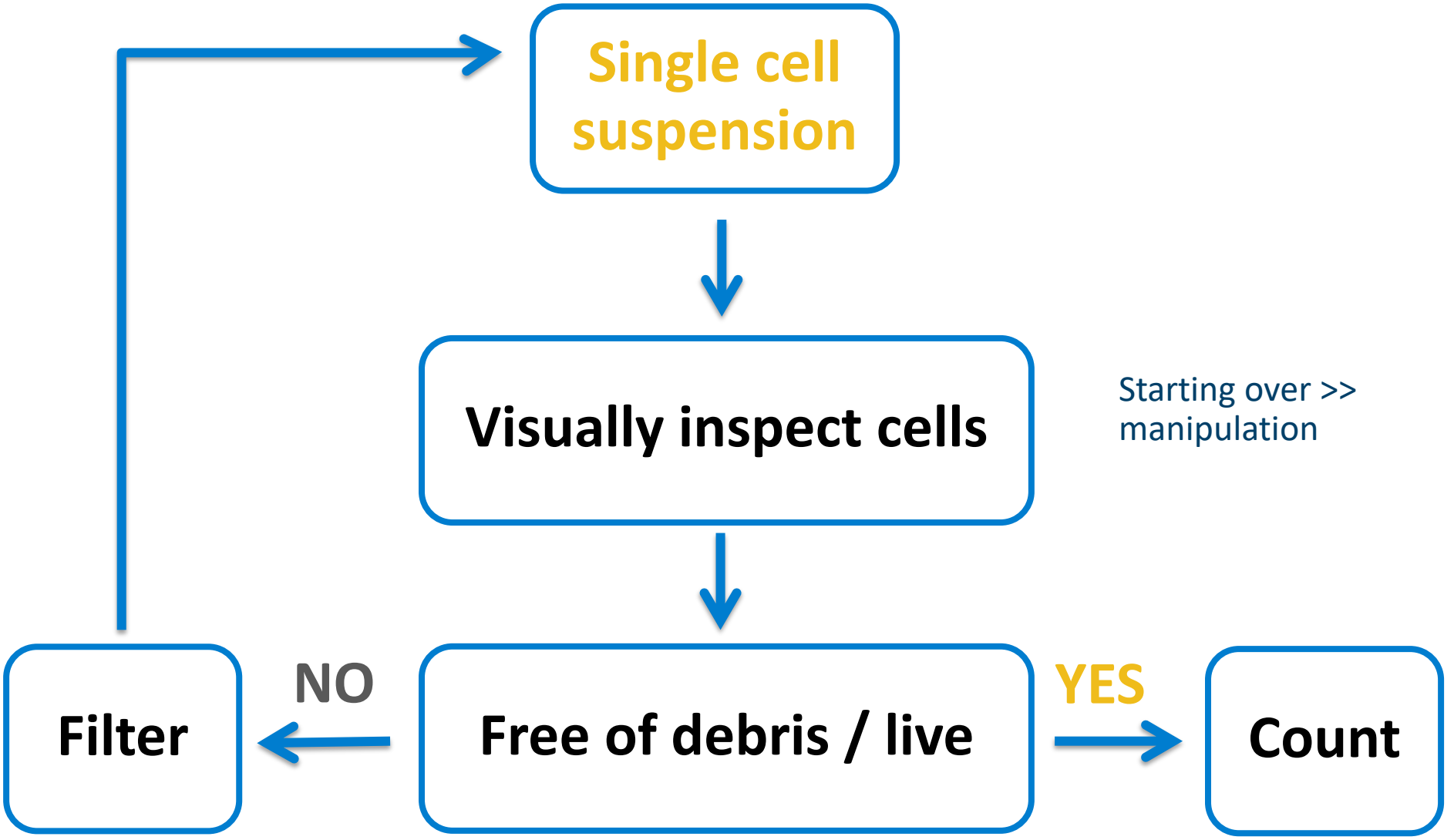
- Non-single cell clumps.
- Integrated into droplets and cannot* distinguish from single cells.



Bad for any single cell experiment.

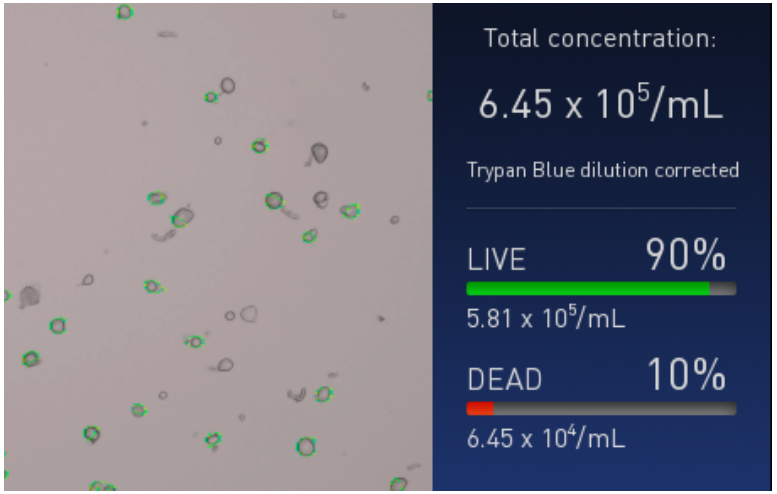
Recommended treatment: filtration, change [blocking agent]

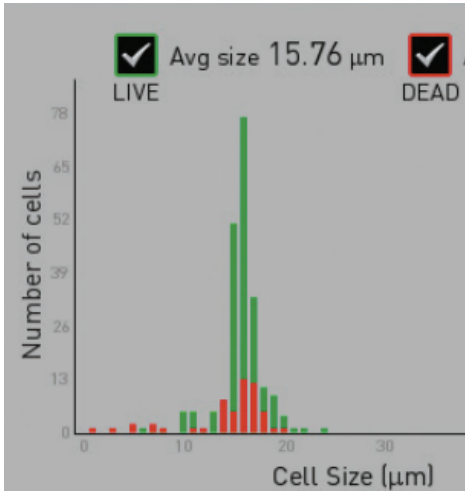
Workflow


Single cell suspension
Visually inspect cells
Starting over >> manipulation
Free of debris / live
YES
Count
NO
Filter

Automated counting

- Countess II.

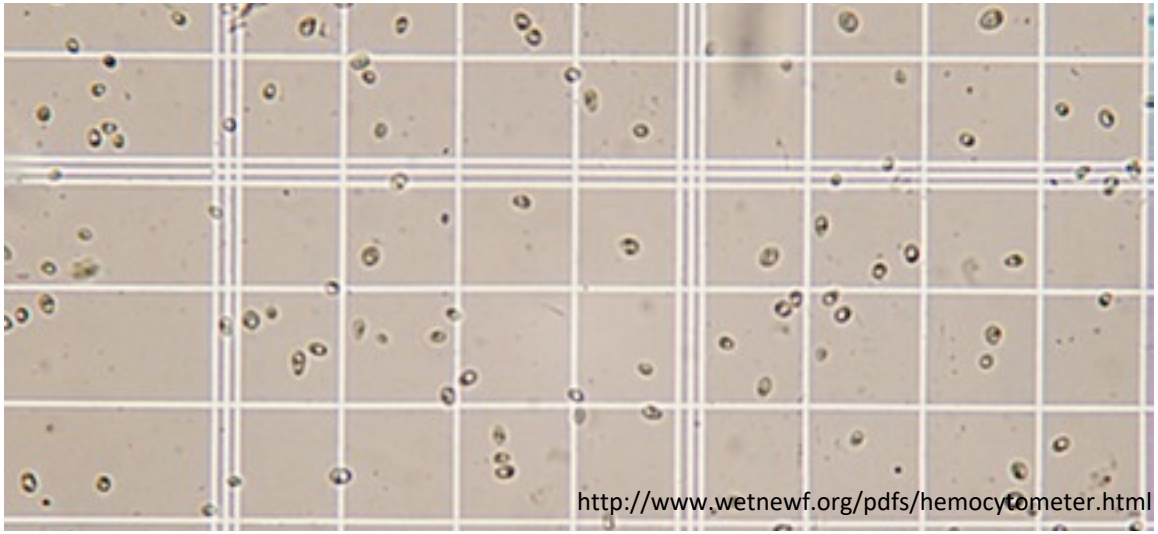


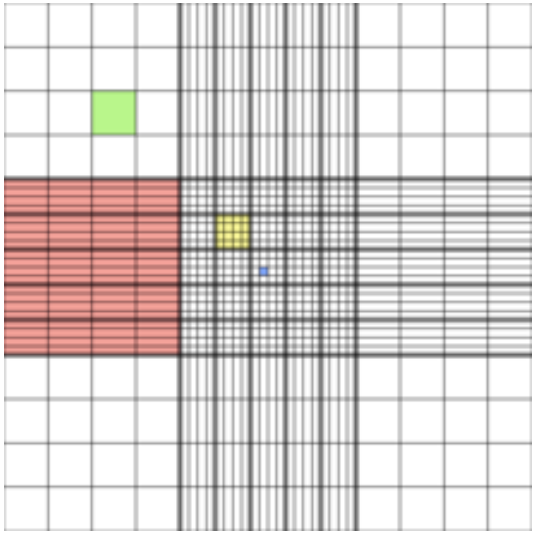


- Pros (+):
  - Fast.
  - Live/dead cell counts.
  - Cell size estimates.
- Cons (-):
  - Cell size limits (4-30um).
  - Doesn't do well with odd cell shapes, debris, nuclei.

Manual counting

- Hemocytometer.



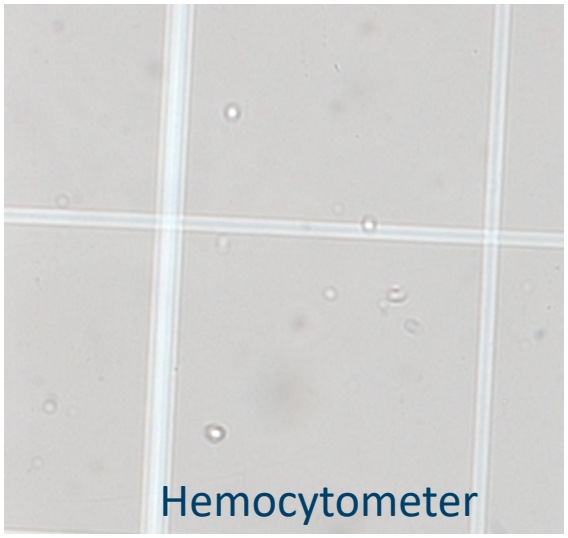


- Pros (+):
  - Reliable cell counts.
  - Count small cells.
  - Visualize cells vs. debris.
- Cons (-):
  - Slow.

Nuclei QC

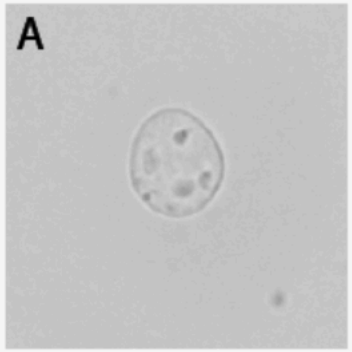
- Use fluorescence.
  - Countess II FL, Devonix CellDrop, Luna FL.
- Stains
  - Trypan blue → 100% dead (not great).
  - Ethidium homodimer stain (good for excluding debris).
  - DAPI, PI other options

60X: DAPI



Hemocytometer

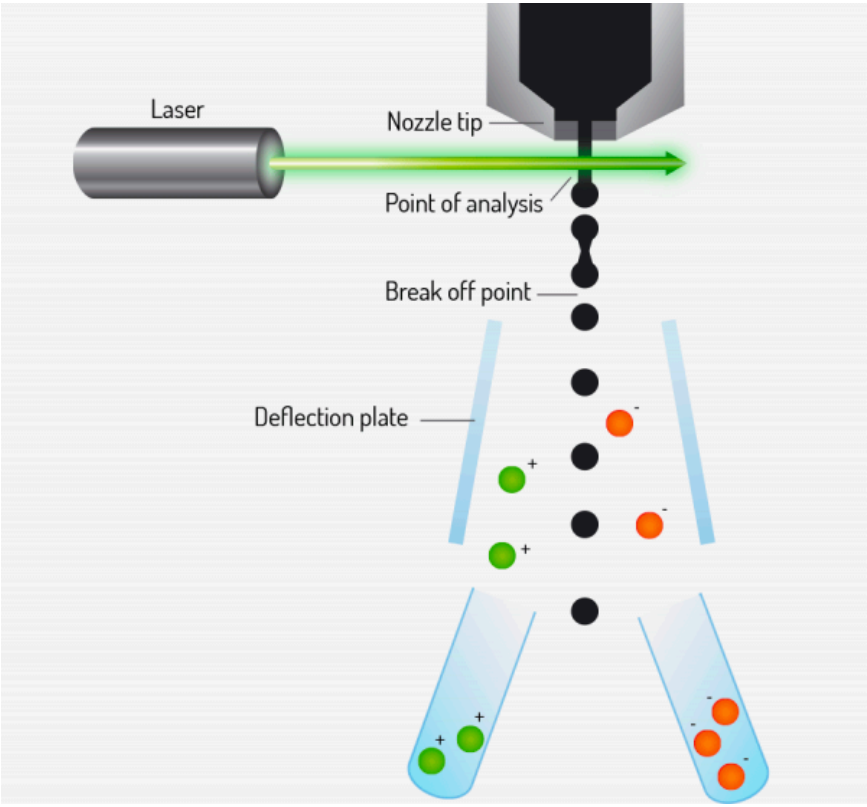
CountessII



Credit: 10X

Cell sorting

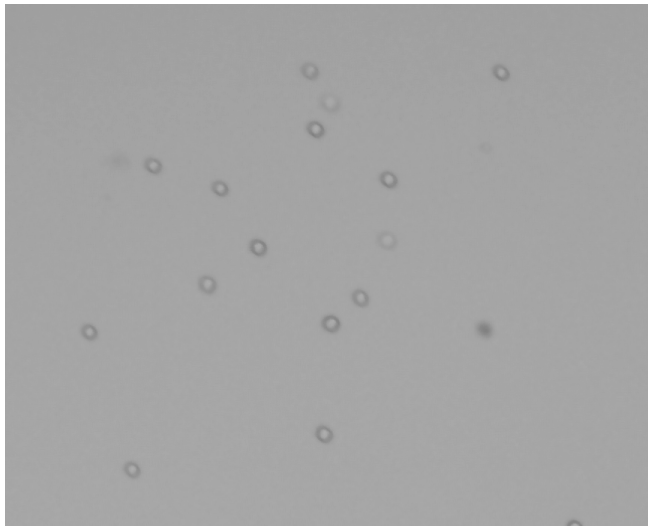
- FACS

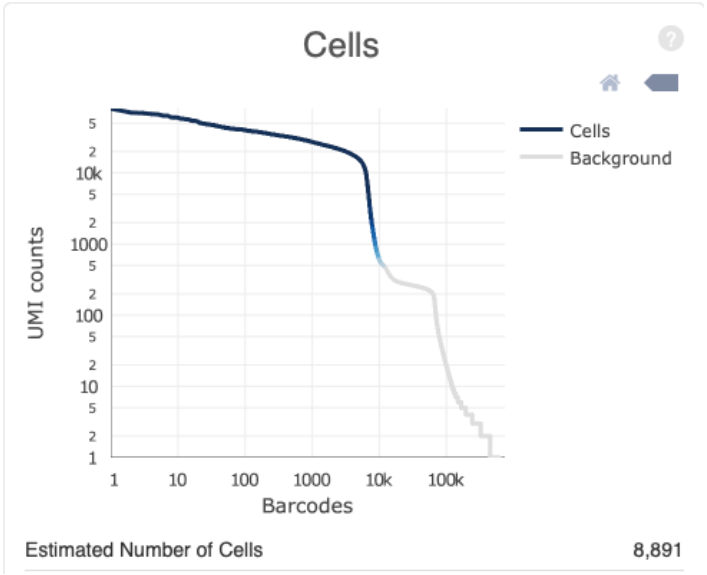


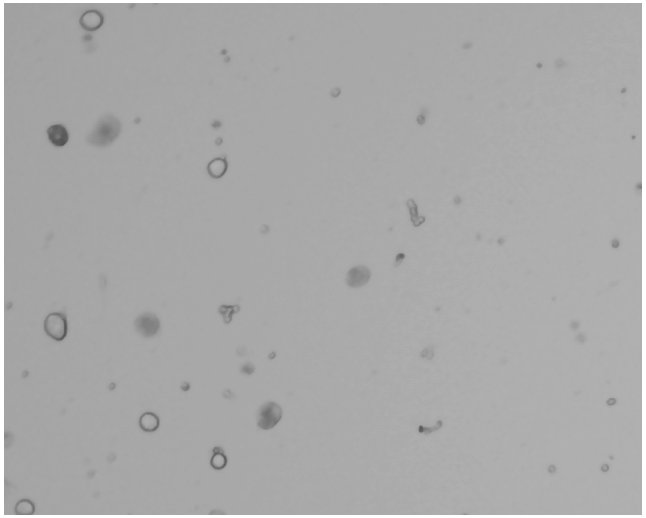
https://flowcytometry-embl.de/cell-sorting/

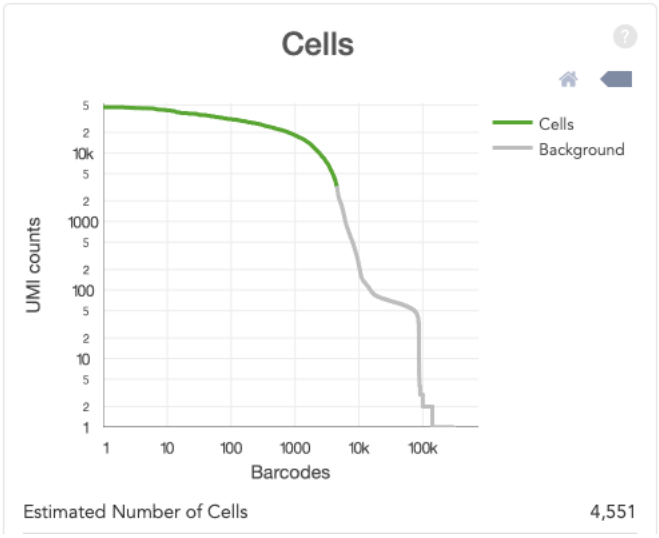
- Pros (+):
  - Sort live / dead.
  - Enrich cells of interest.
  - Determine whether correct cells isolated (qPCR works too).
- Cons (-):
  - Overestimates cell counts (~2x).

The good…

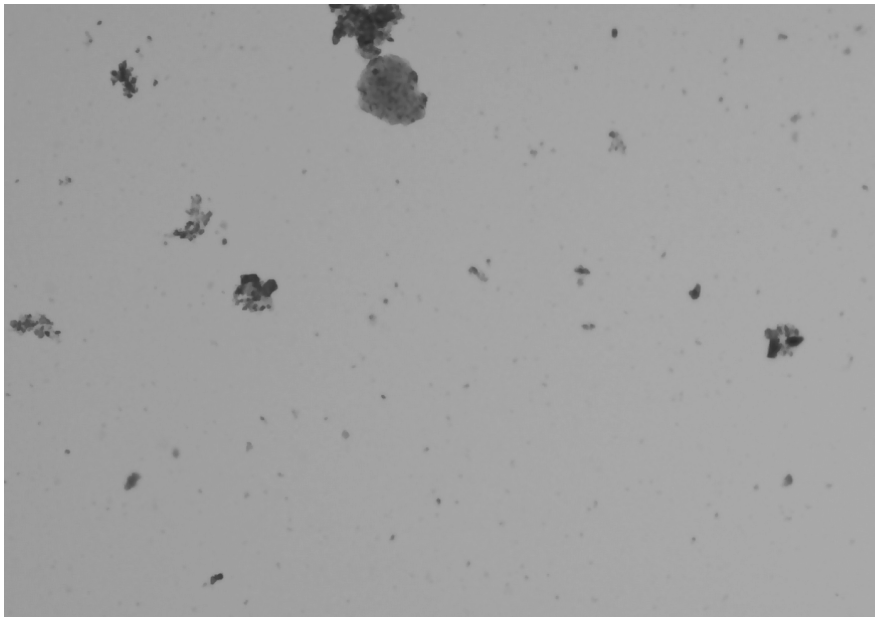


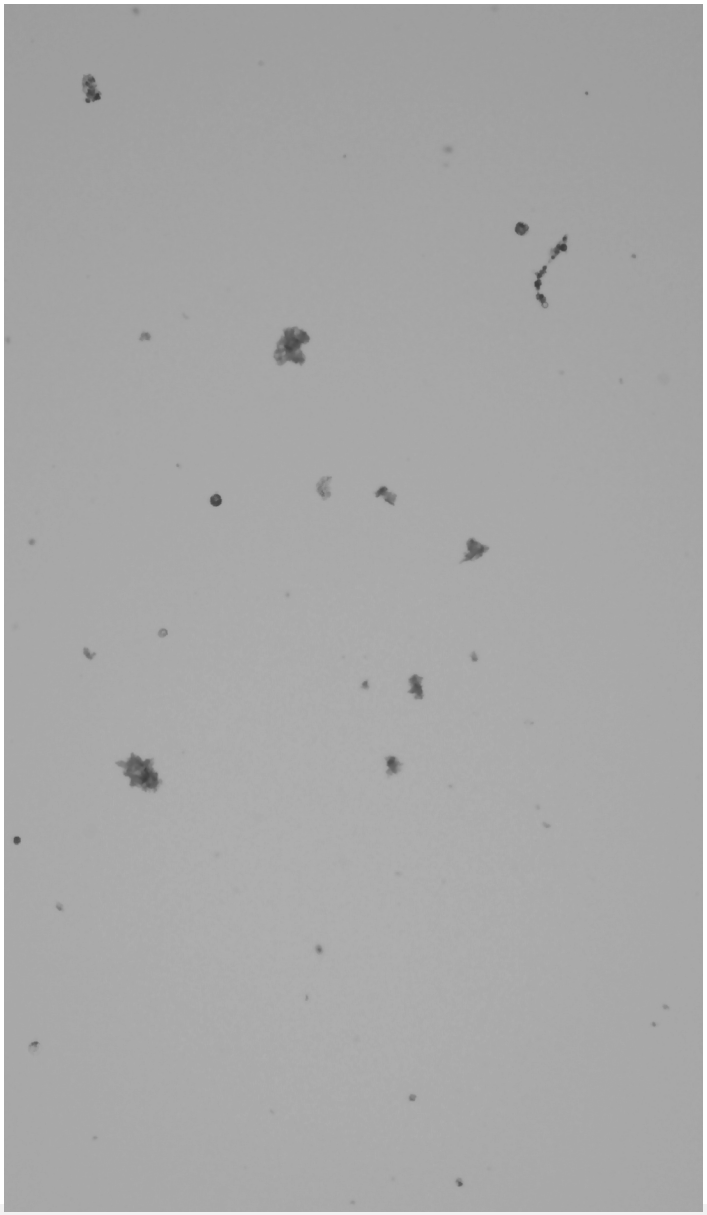

Cells
Cells
Background
UMI counts
5
2
10k
5
2
1000
5
2
100
5
2
10
5
2
1
1
10
100
1000
10k
100k
Barcodes
Estimated Number of Cells
8,891




Cells
Cells
Background
UMI counts
5
2
10k
5
2
1000
5
2
100
5
2
10
5
2
1
1
10
100
1000
10k
100k
Barcodes
Estimated Number of Cells
4,551

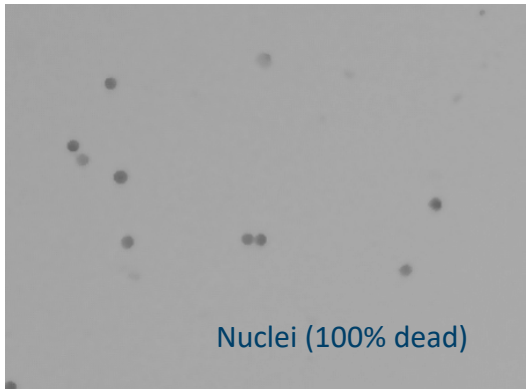
The bad…





Challenges Cell Recovery

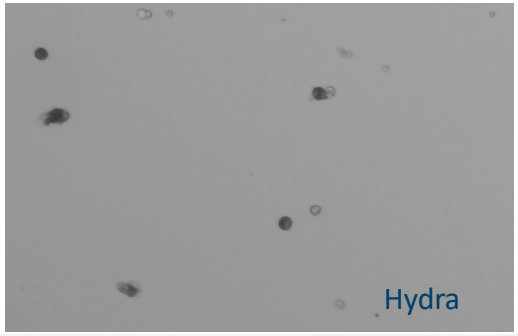
- 10X is optimized for model cell types – human PBMCs, etc.
  - Round, easy to count, size well below size of microfluidics.
  - These yield consistent results. 10K target. 8K-10K recovered.

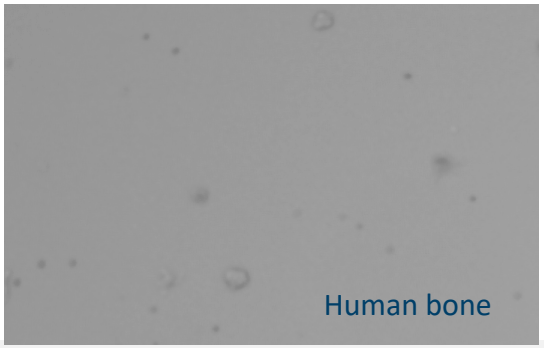


Estimated Number of Cells	
8,266	

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

- But most experiments outside of culture don't look like this.



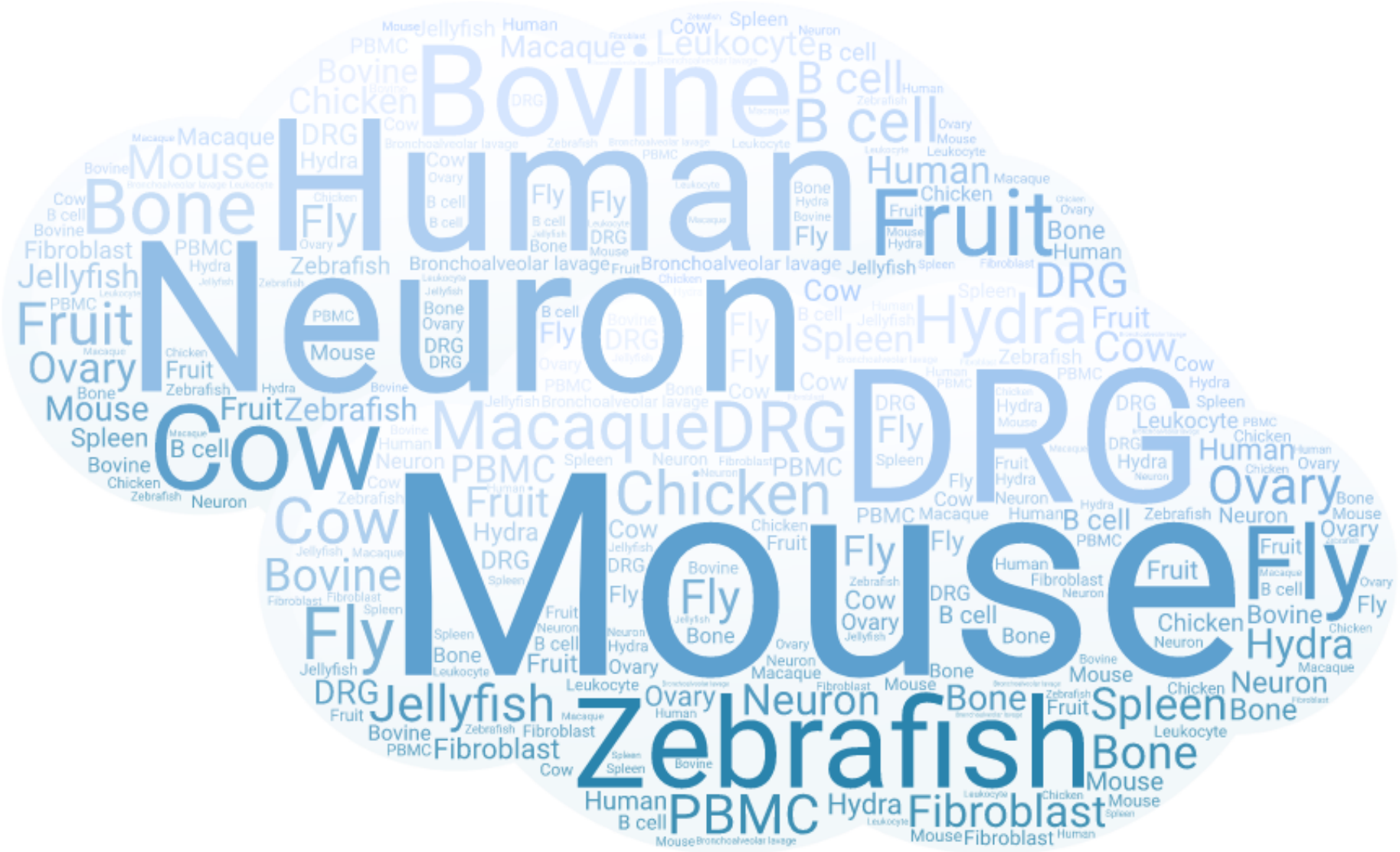


Variable size, shape, and viability

How maximize output?

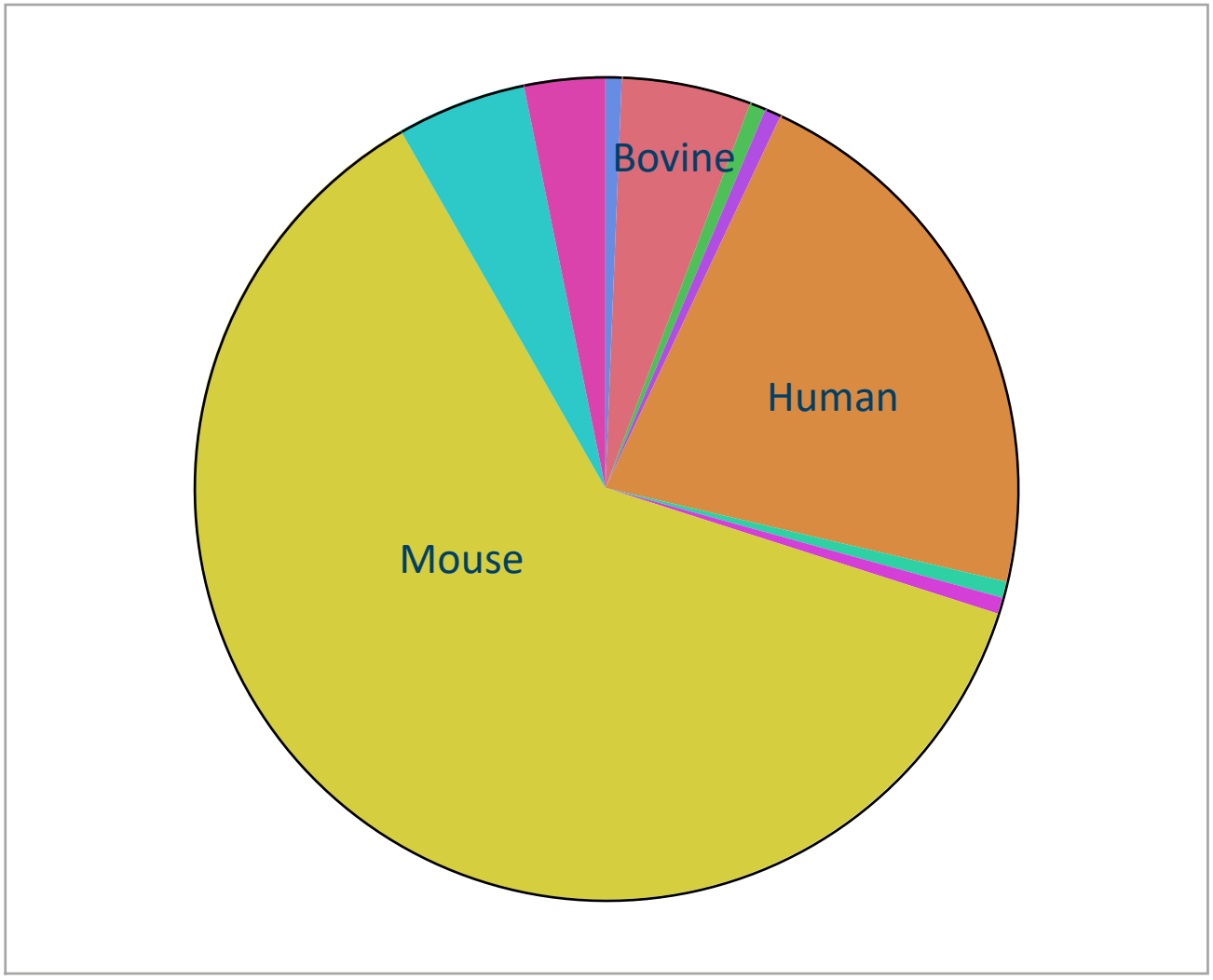
- Carefully craft experimental design and sample prep .
- QC cells before real sample set-up.
- Concentration: aim for the median.
  - **700-1,200 cells per µl optimal**.
  - Too high → dilute.
  - Too low → tough one (concentration impacts yield, pooling replicates suboptimal).
  - Count in replicates (at least n=2).
- Viability: **70% minimum**.
  - Nuclei and methanol fixed cells (0%).
- Treat cells gently.
  - Wide bore pipette tips, keep cells at preferred temp, work quickly.

10X Single Cell Studies - DNATECH



Assay summary (organism)

- 78 projects since July 2017 (primarily 3’ GEX).



Sample distribution by organism

- Aurelia aurita
- Bovine
- Chicken
- Fruit fly
- Human
- Human+Mouse
- Hydra
- Mouse
- Rhesus macaque
- Zebrafish

Retinal degeneration

SCIENTIFIC REPORTS

OPEN

Received: 15 October 2018
Accepted: 27 February 2019
Published online: 19 March 2019

Molecular profiling of resident and infiltrating mononuclear phagocytes during rapid adult retinal degeneration using single-cell RNA sequencing

Kaitryn E. Ronning[1], Sarah J. Karlen[2], Eric B. Miller[1] & Marie E. Burns[1,2,3]

Vitamin E deficiency - DRG Neurons

CellPress

Sneak Peek

A PREVIEW OF PAPERS UNDER REVIEW

Single-Cell RNA-Seq Reveals Profound Alterations in Mechanosensitive Dorsal Root Ganglion Neurons with Vitamin E Deficiency

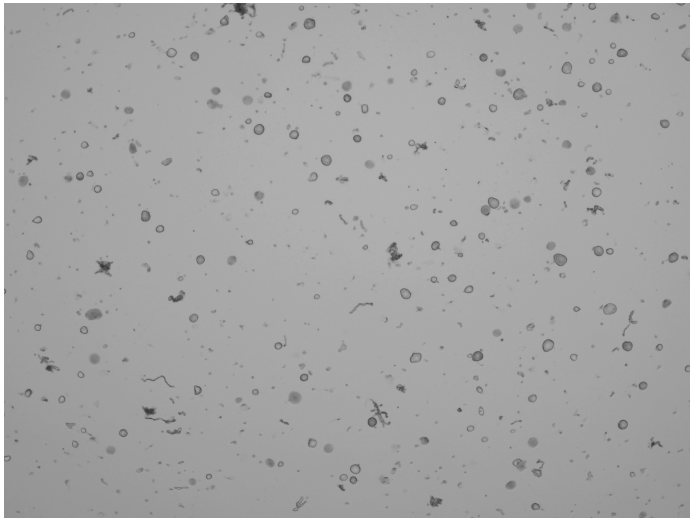
iScience

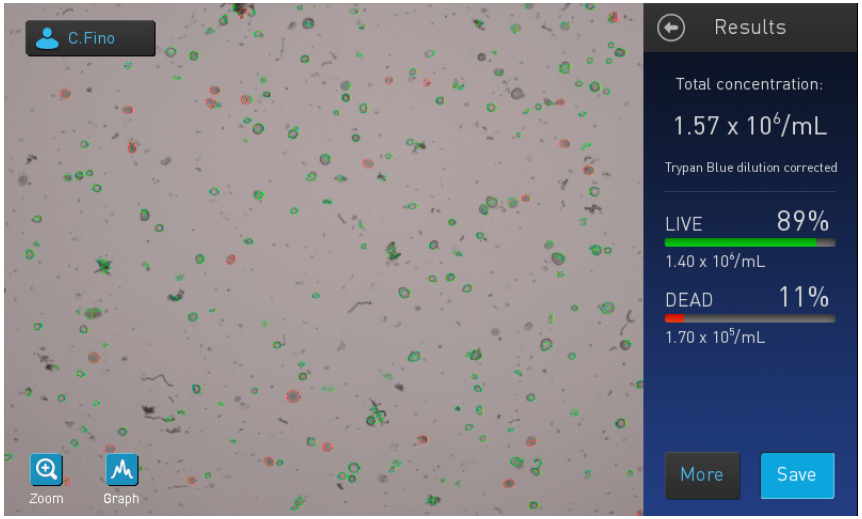
50 Pages • Posted: 5 Aug 2019 • Sneak Peek Status: **Review Complete**

Carrie Finno
University of California, Davis, School of Veterinary Medicine, Department of Population Health and Reproduction

Janel Peterson
University of California, Davis, School of Veterinary Medicine, Department of Population Health and Reproduction

‘564’ – challenges and solutions

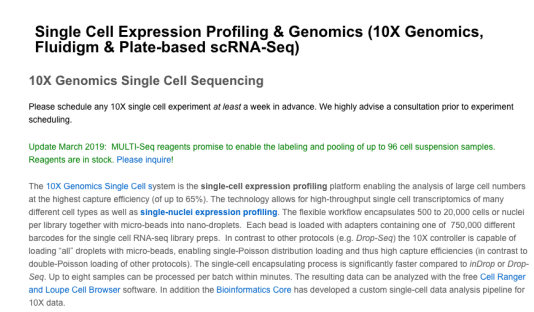


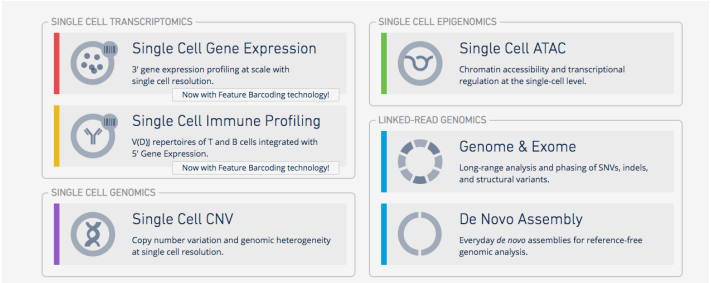


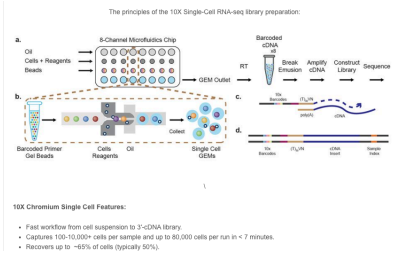
- DRG fragile, difficult to count, odd sizes (clog 10X chip?).
- Count manually → better counts.
- Accepted some degree of induced technical variation
  - Prepped samples over three days.
  - Improved sample quality and reproducibility, introduced prep day variation.
    - As Matt mentioned mitigate with experimental design.

Important resources

- 10X Genomics
  - https://support.10xgenomics.com/single-cell-gene-expression
  - Paul Scott, Sales Executive (paul.scott@10xgenomics.com)
- 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/
- UC Davis Bioinformatics Core
  - https://bioinformatics.ucdavis.edu/







Cost 10X Reagents

Product Code	Product	List Price	Discount
1000120	Chromium Next GEM Chip G Single Cell Kit, 48 rxns	USD 1,480.00	
1000121	Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead Kit v3.1, 16 rxns	USD 21,600.00	
		Quote Created Date	3/10/2020

- Chips are single use (n=1 to n=8 samples) → $247 EACH
- Reagents (n=1) → $1,350 EACH.
- Total (n=1, reagents only): $1,597.
  - This doesn't include you or your cores labor!
- Sequencing? Bioinformatics?
  - Depends on many factors.
  - We charge $1,000 for 350M reads (~10K cells with 35K reads per cell).

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