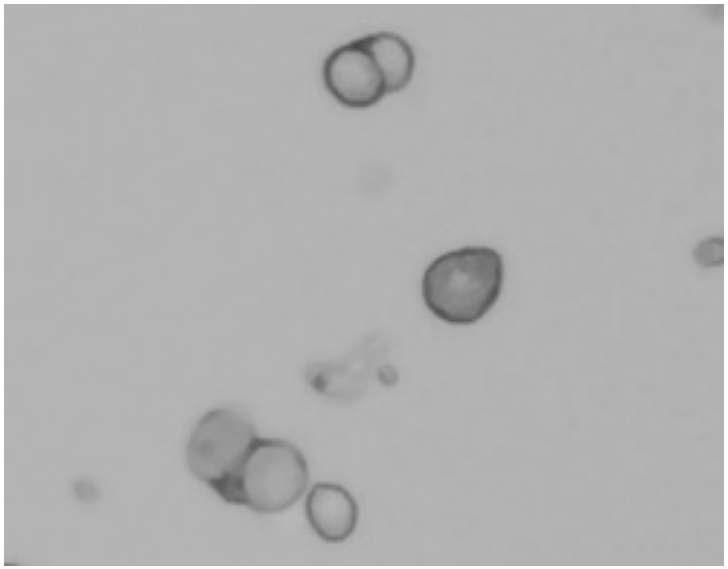
Single Cell Sample Preparation



Diana Burkart-Waco, PhD

Employed by

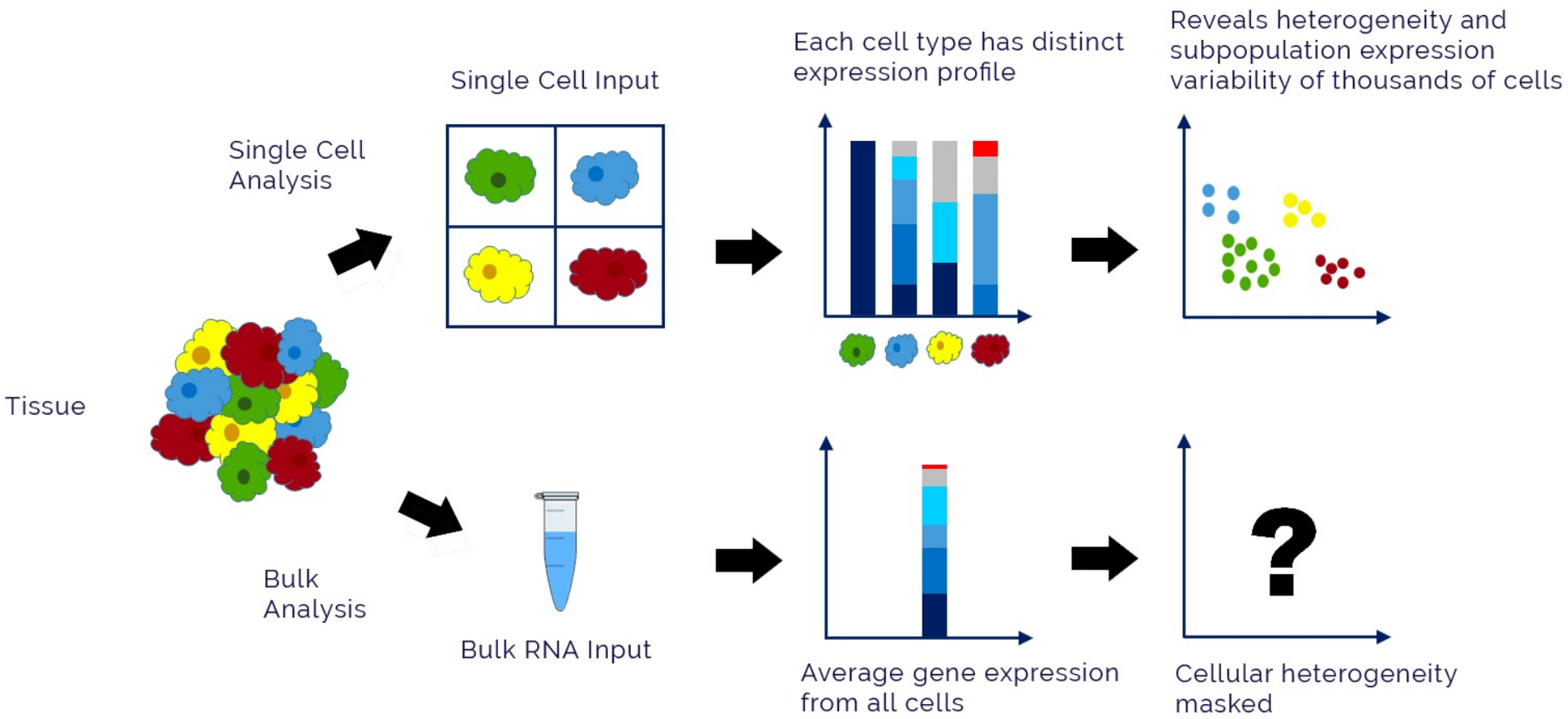
10x GENOMICS

(but views in presentation are not 100% supported by 10x)

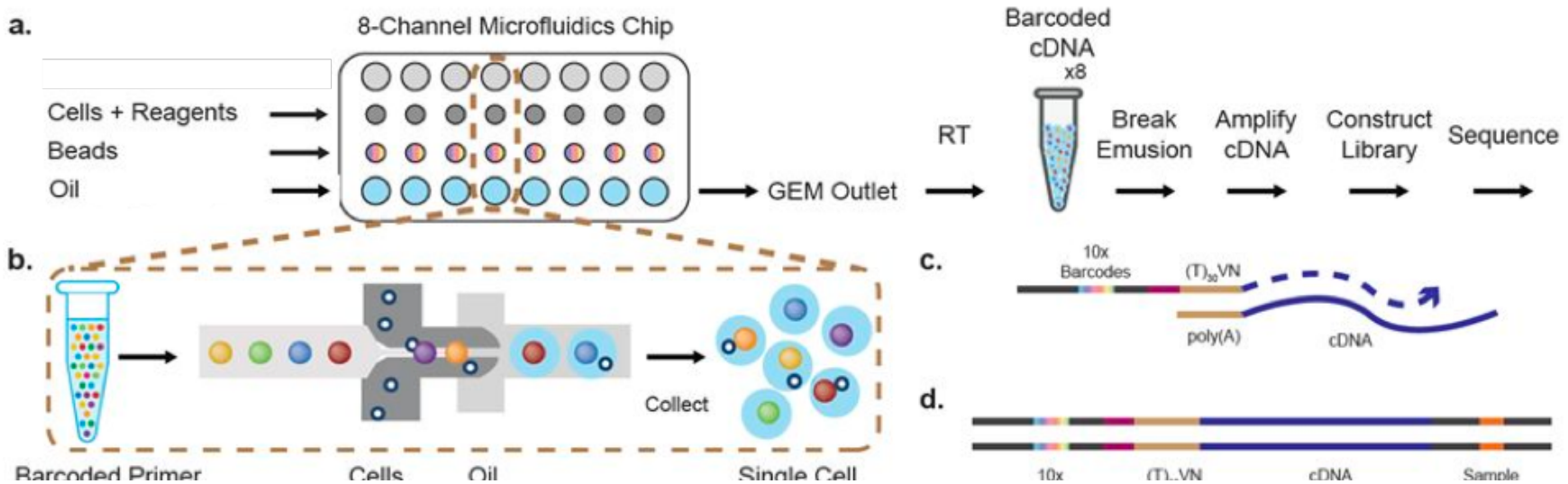
Overview

- Why single cell?
- Methods – SC isolation
  - –Single cell isolation
  - –Single nuclei
- Methods - sample QC
  - –Do I have single cells?
  - –Are they alive?
  - –Are they too big?
  - –Did I isolate the correct cells?
- Cell labeling
- Single cell studies at DNA Tech

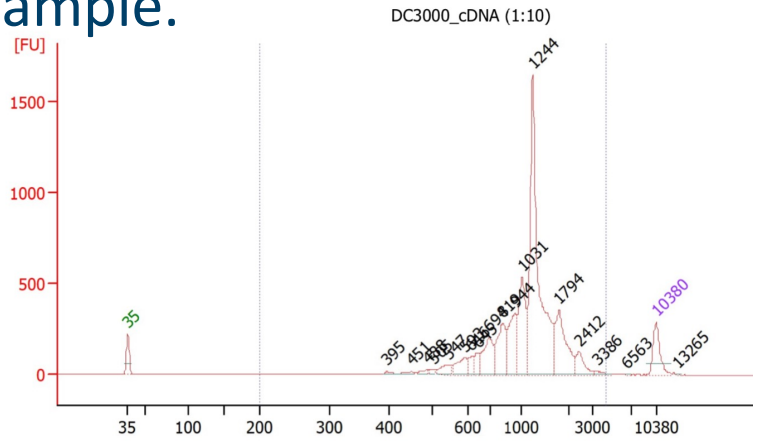
scRNA-seq


Single Cell Input
Each cell type has distinct expression profile
Reveals heterogeneity and subpopulation expression variability of thousands of cells
Single Cell Analysis
Tissue
Bulk Analysis
Bulk RNA Input
Average gene expression from all cells
Cellular heterogeneity masked
?

10X Genomics



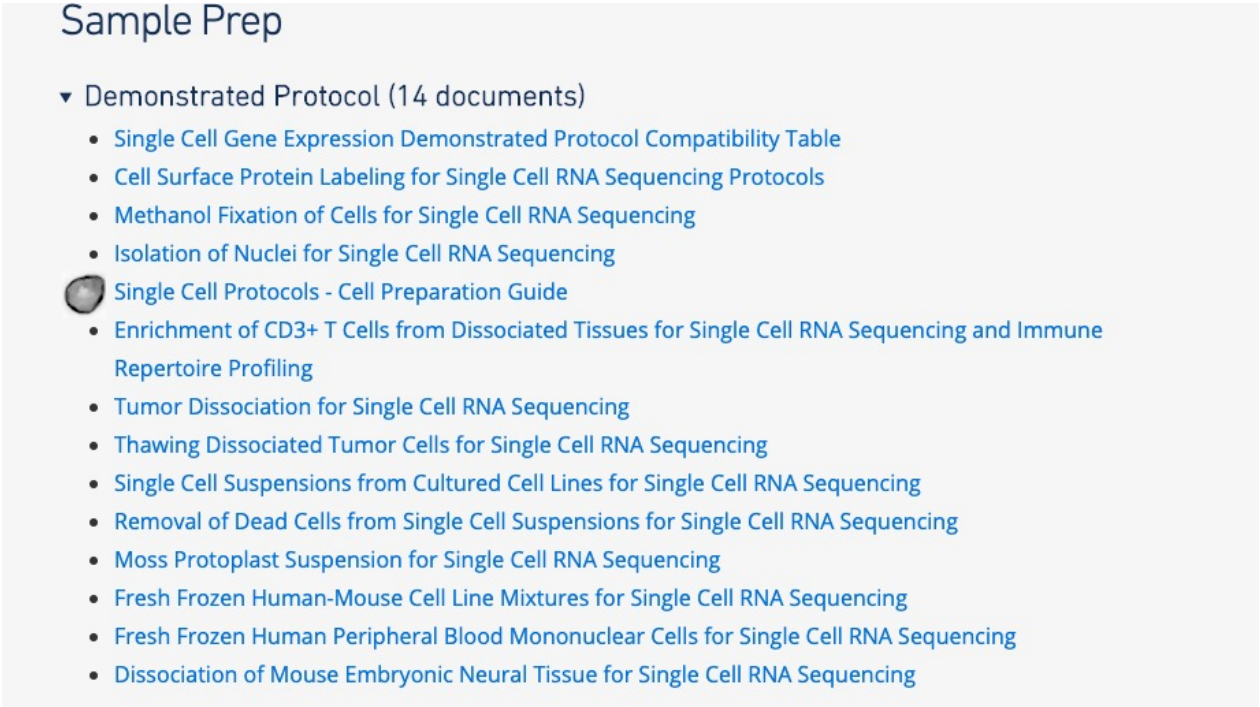
- Captures 100-10,000+ cells per sample.
- Recovers up to ~65% of cells.
- Low doublet rate.
  - (~0.8% per 1,000 cells).
- 30-40 micron size limit.



Sample Prep Guides

- Cell isolation guides available at:

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

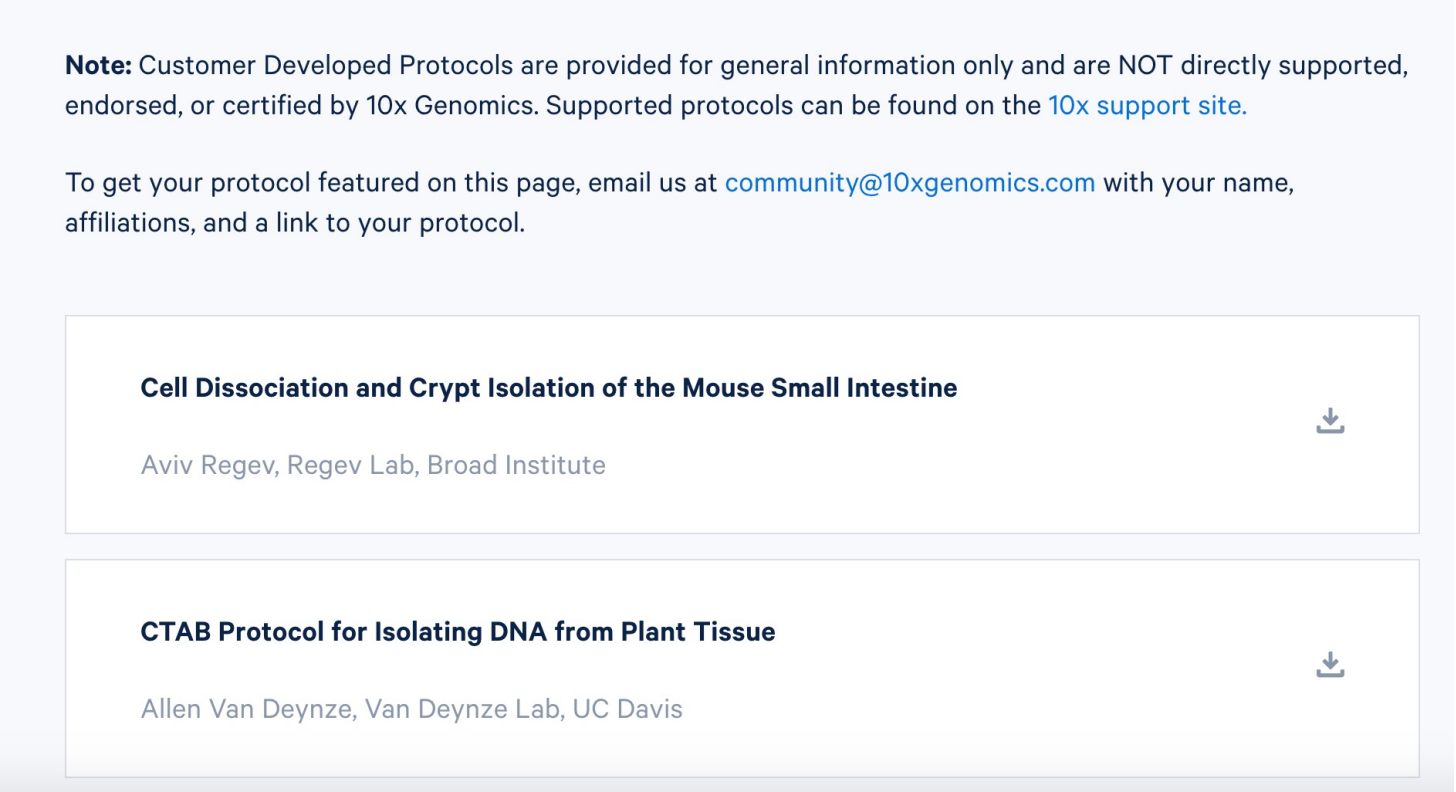


- Cell isolation → biggest source technical variation.
- Dissociation and preparation depends on cell type.

Customer Protocols

- User-developed protocols:

https://www.10xgenomics.com/resources/customer-developed-protocols



Note: Customer Developed Protocols are provided for general information only and are NOT directly supported, endorsed, or certified by 10x Genomics. Supported protocols can be found on the 10x support site.

To get your protocol featured on this page, email us at community@10xgenomics.com with your name, affiliations, and a link to your protocol.

Cell Dissociation and Crypt Isolation of the Mouse Small Intestine

Aviv Regev, Regev Lab, Broad Institute

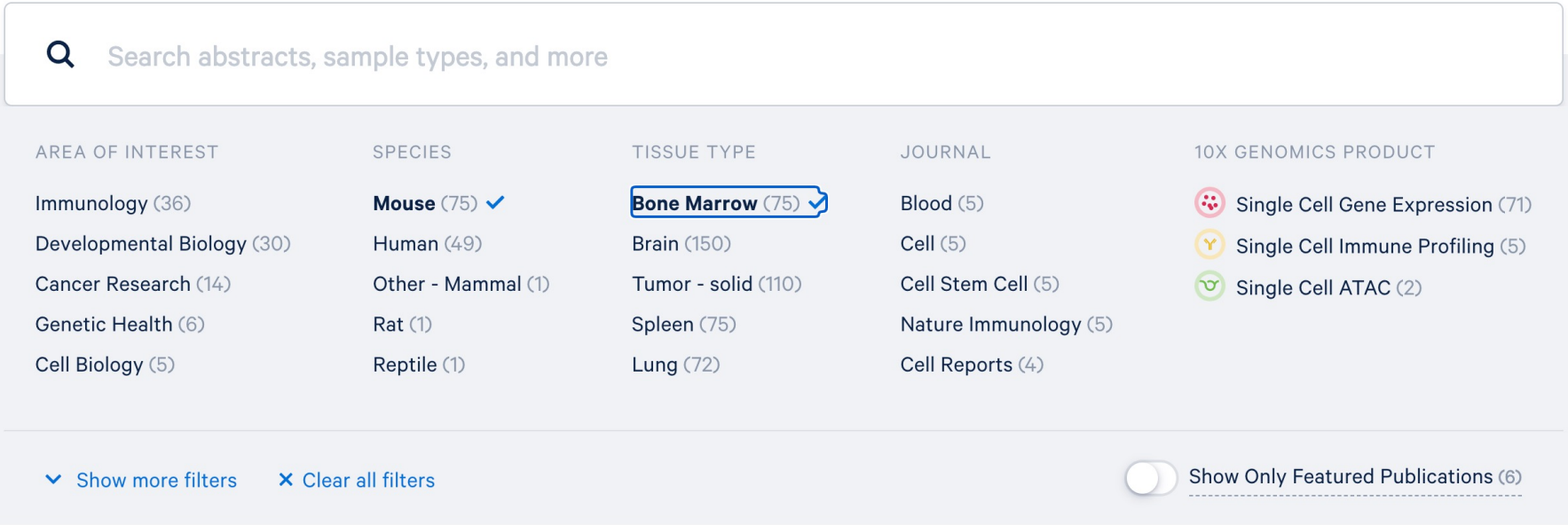
CTAB Protocol for Isolating DNA from Plant Tissue

Allen Van Deynze, Van Deynze Lab, UC Davis

Additional Resources

- Publication library

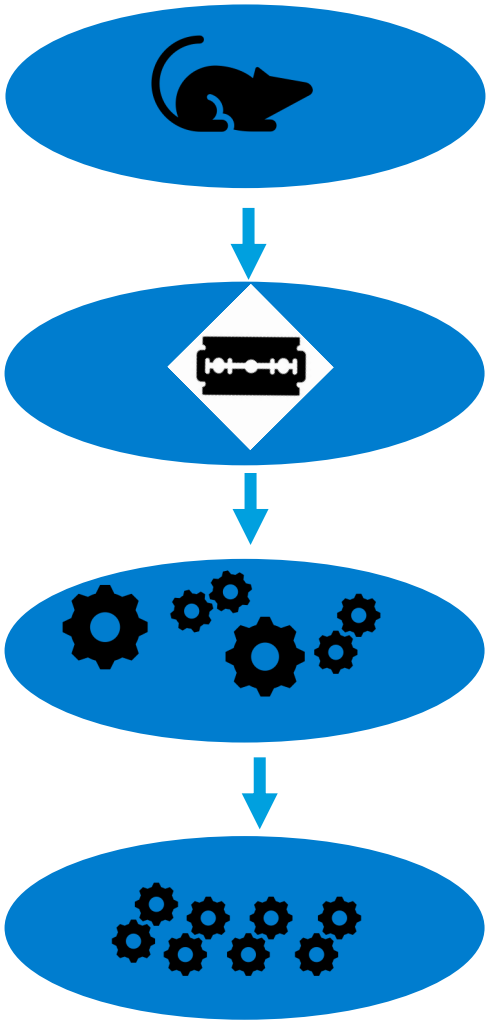
https://www.10xgenomics.com/resources/publications



General Workflow

Tissue dissection

Fast, reliable, clean.



Mechanical mincing

Trituration, razor blade, etc.

Enzymatic breakdown

E.g. Trypsin, dispase, collagenase)

Often with agitation

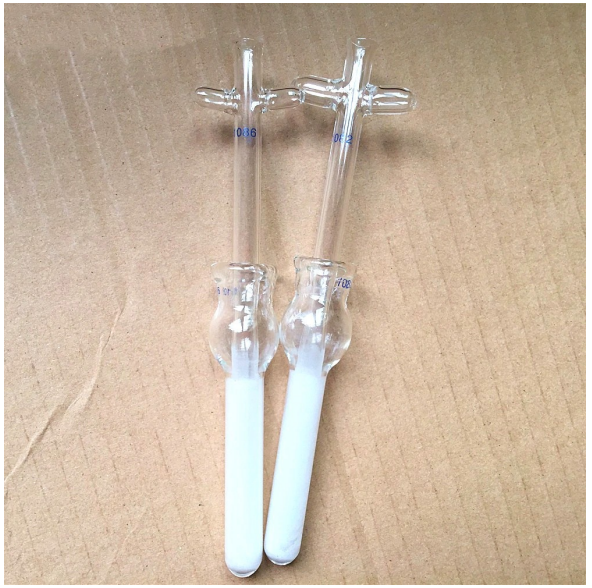
Cell enrichment (optional)

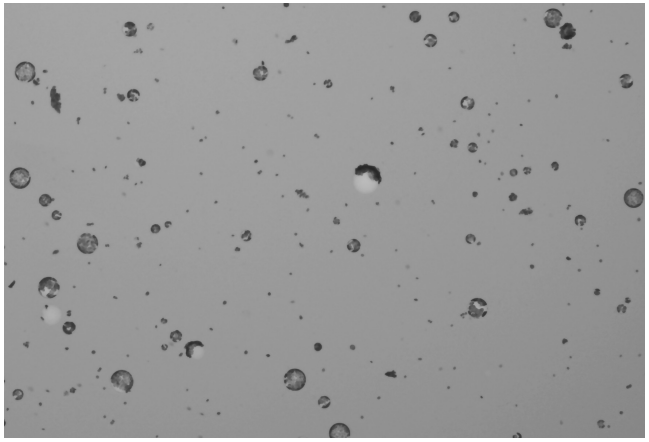
Obtain cell interest (flow)

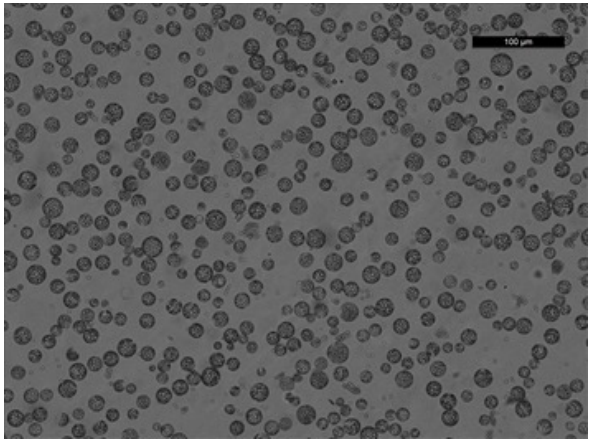
Remove dead cells (beads, etc.)

Tissue Dissociation

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



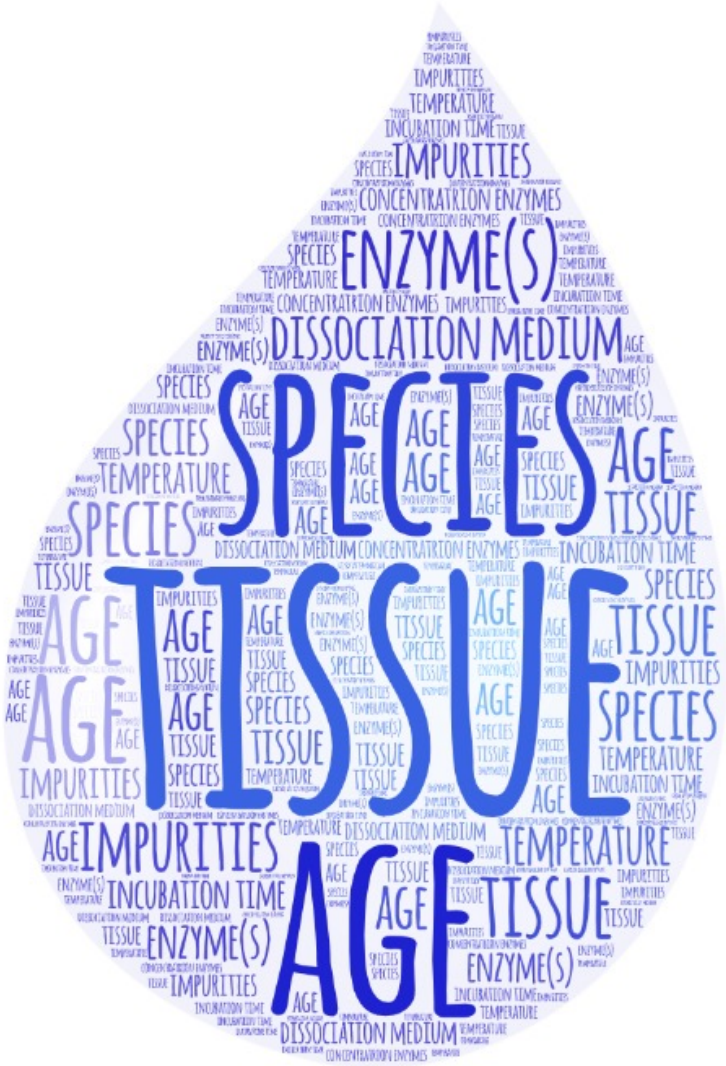




Arabidopsis protoplasts

Same method, different days.

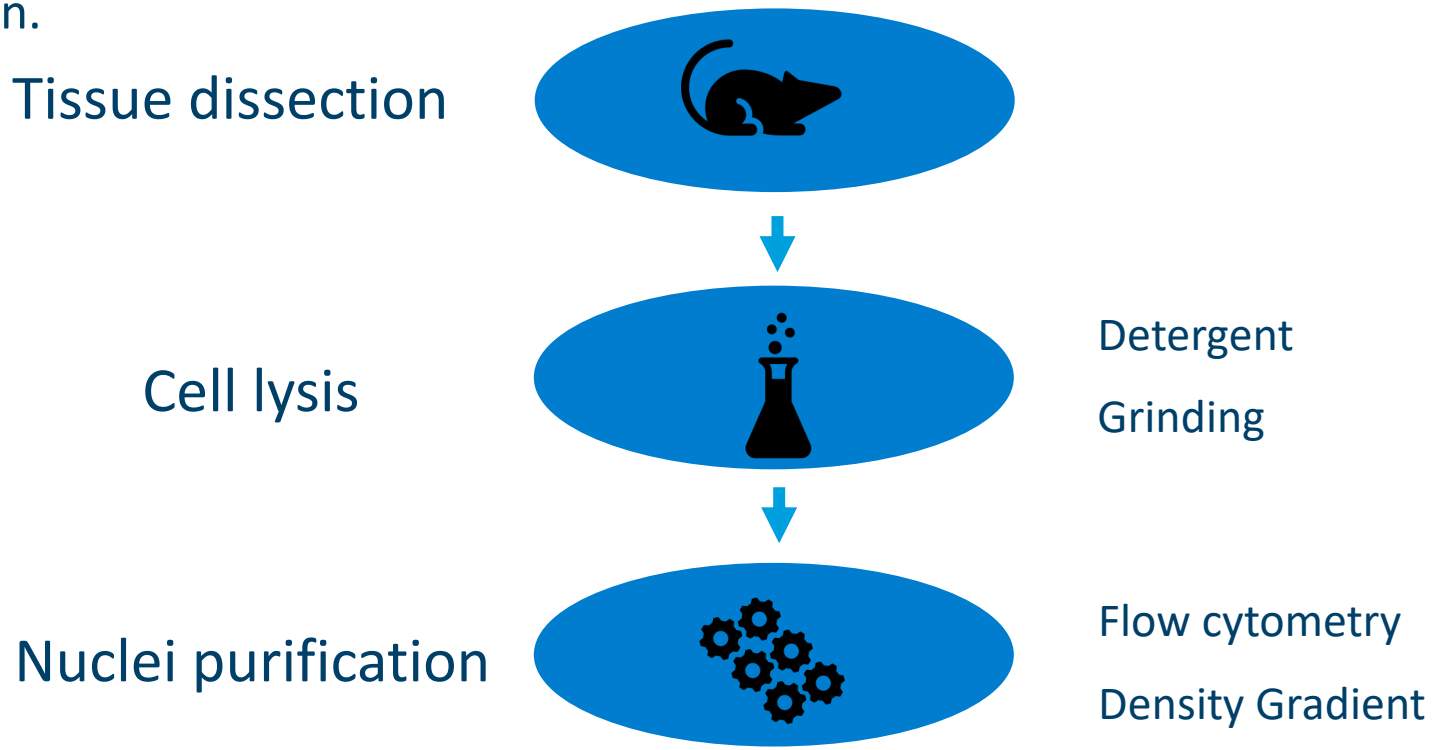
Factors Influencing Dissociation



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.



Nuclei protocols

- 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

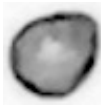
www.collaslab.com

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

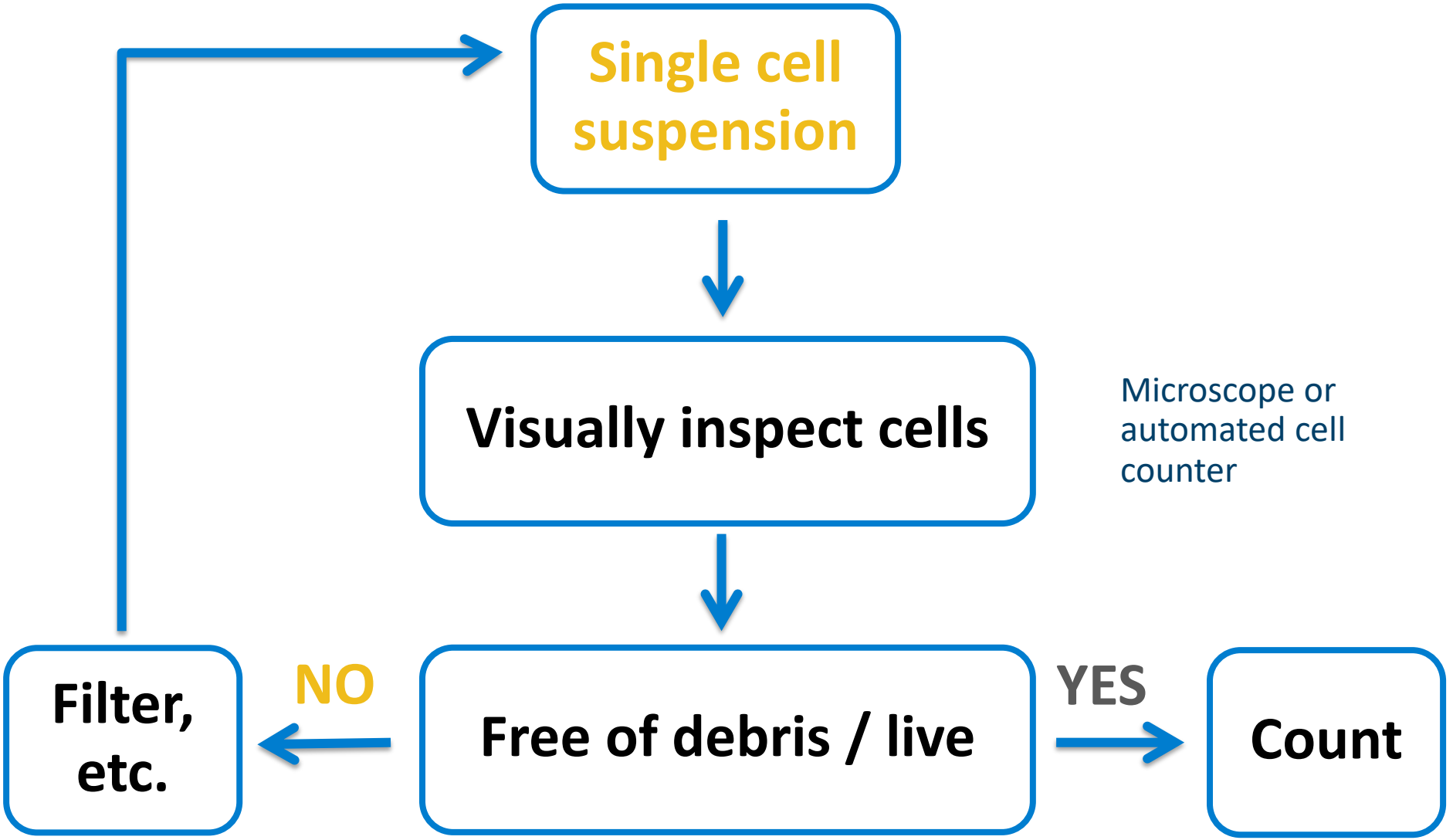
Isolation of Nuclei for Single Cell RNA Sequencing

Factors influencing success

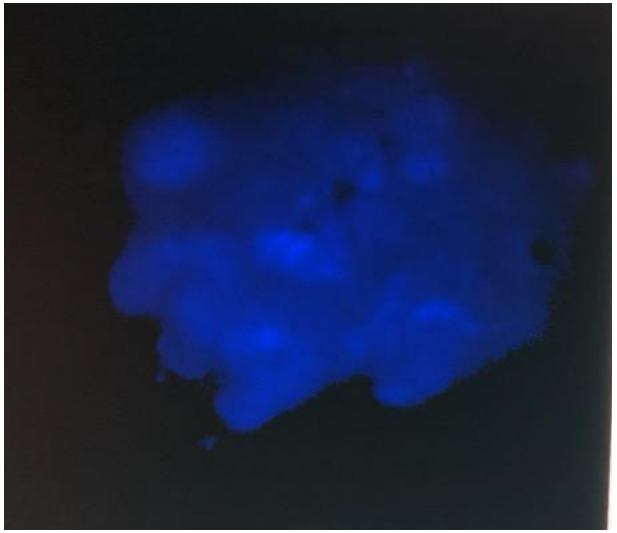
- Cell debris / dead cells
  - Clog microfluidics, free floating RNA → noise.
- Aggregates
  - No longer single cell data.
  - Clog microfluidics.
- Buffer
  - Inhibit polymerase → decrease library complexity.
- Storage conditions

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

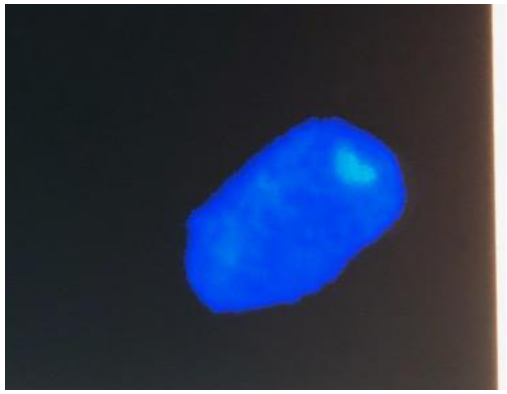
Workflow


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

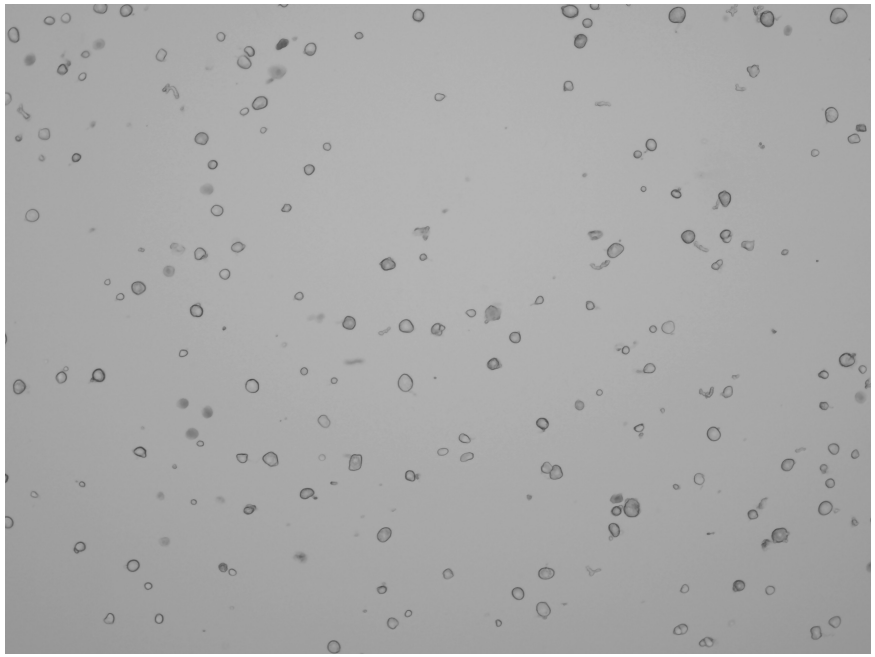
60X

No scale bar

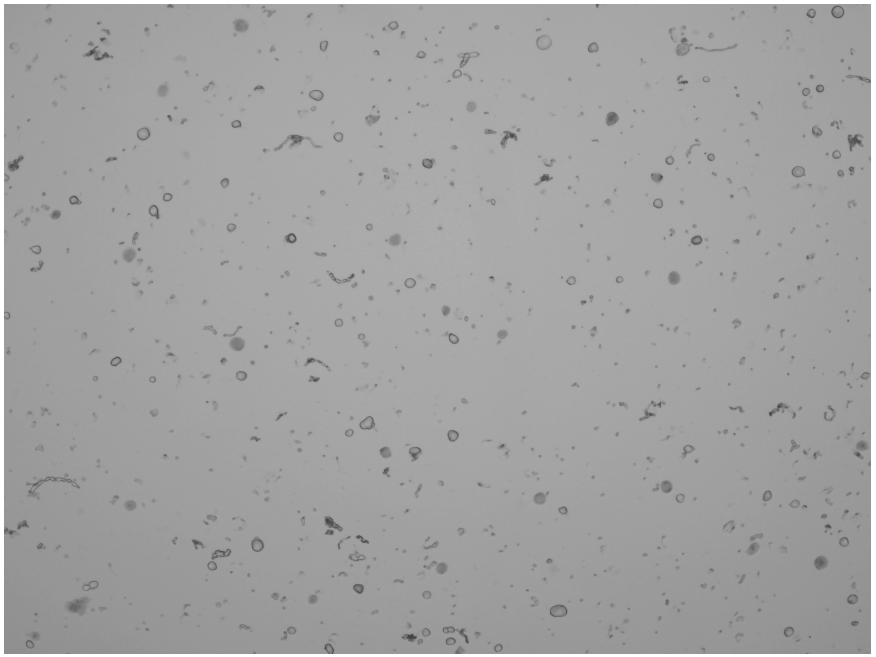
Recommended treatment: optimize tissue dissociation

Cell debris

- Mouse DRG.



Clean DRG sample

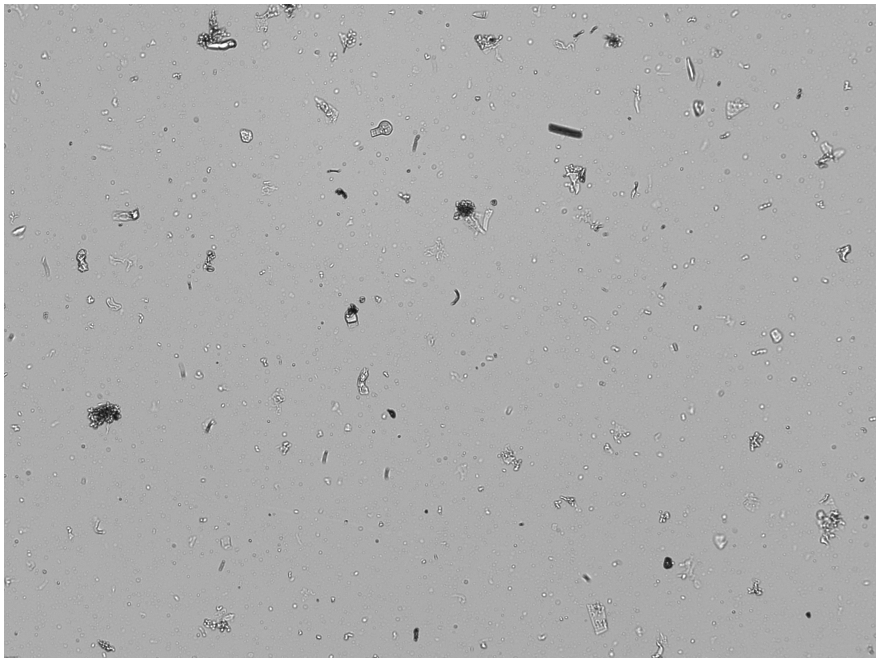


Noisy sample

Recommended treatment: filtration (If debris big), centrifuge, add blocking agent.

Cell debris II

- Lettuce nuclei prep.



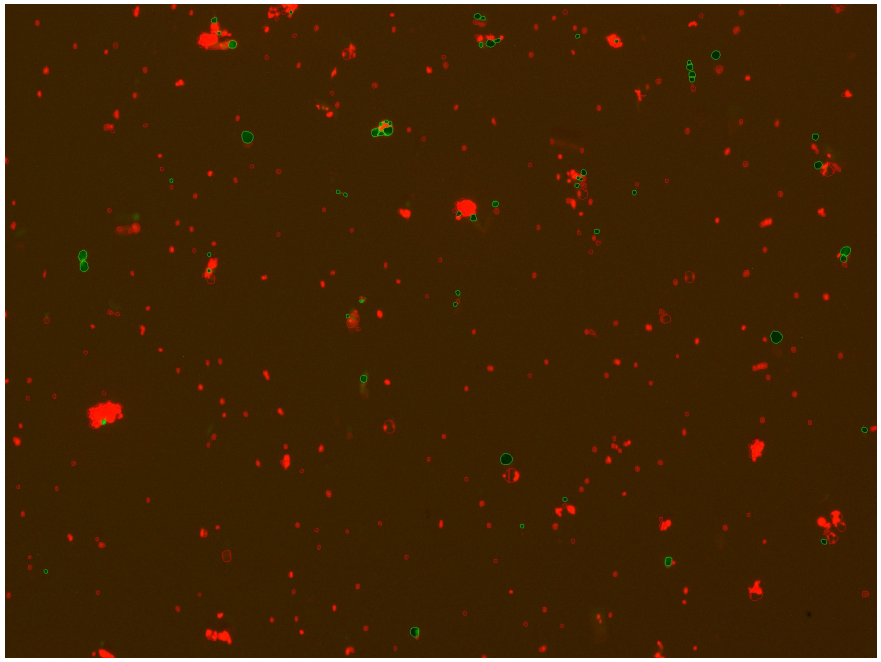
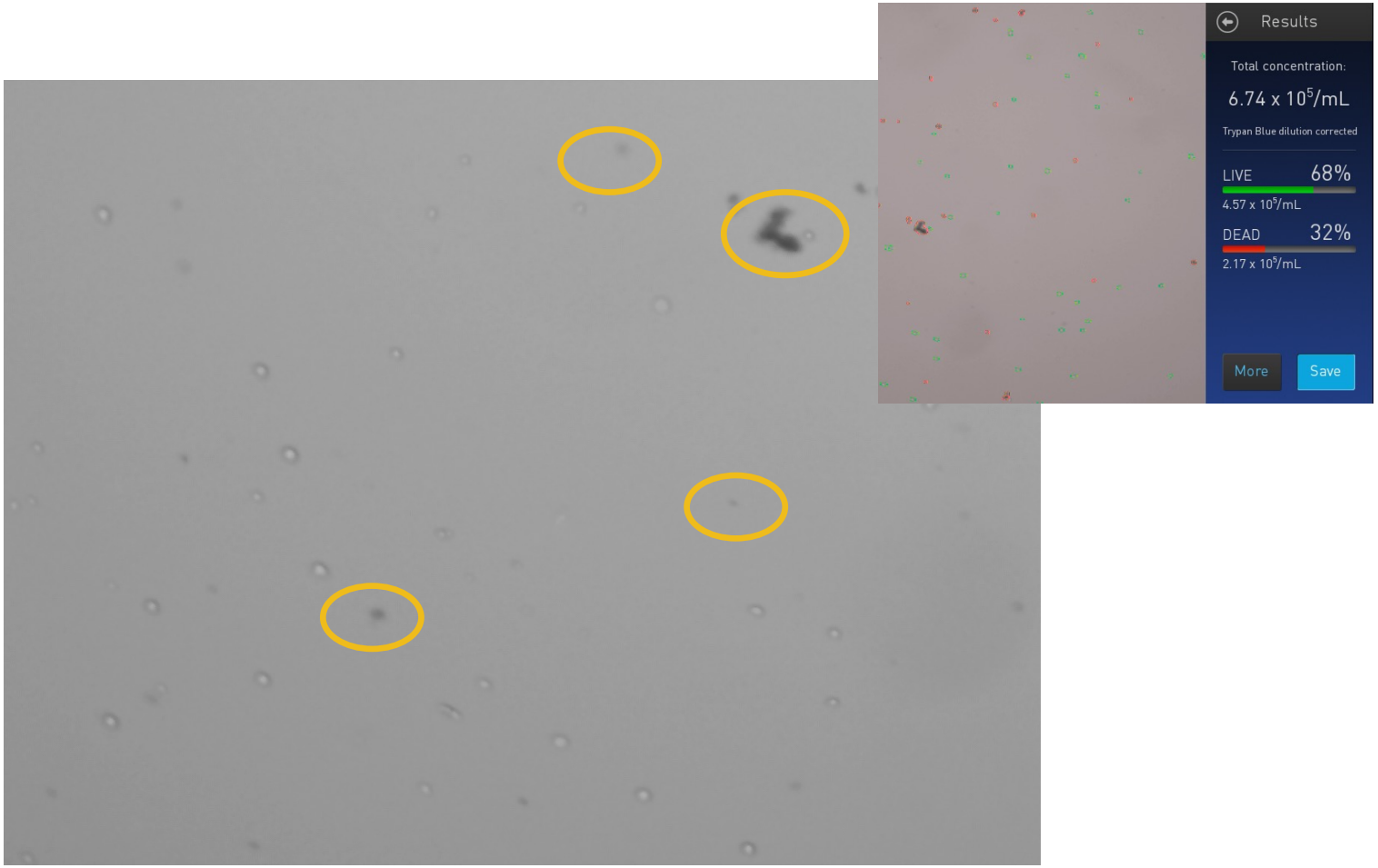


Photo credit: Mohan Prem Anand Marimuthu

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

Dead cells (+debris?)

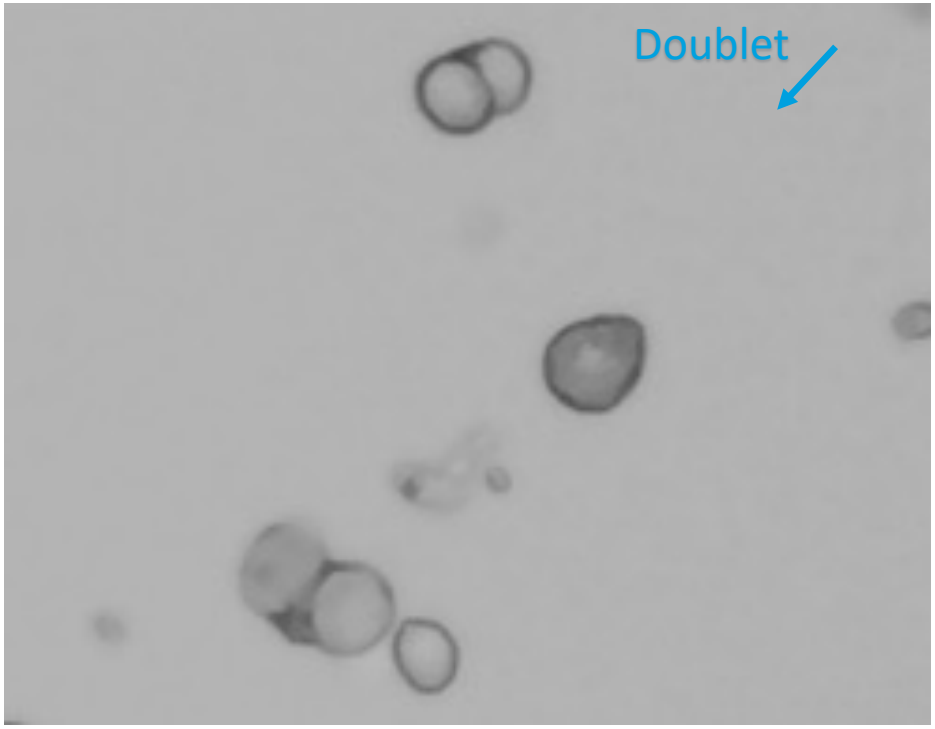


Recommended treatment: dead cell removal.

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

Doublets

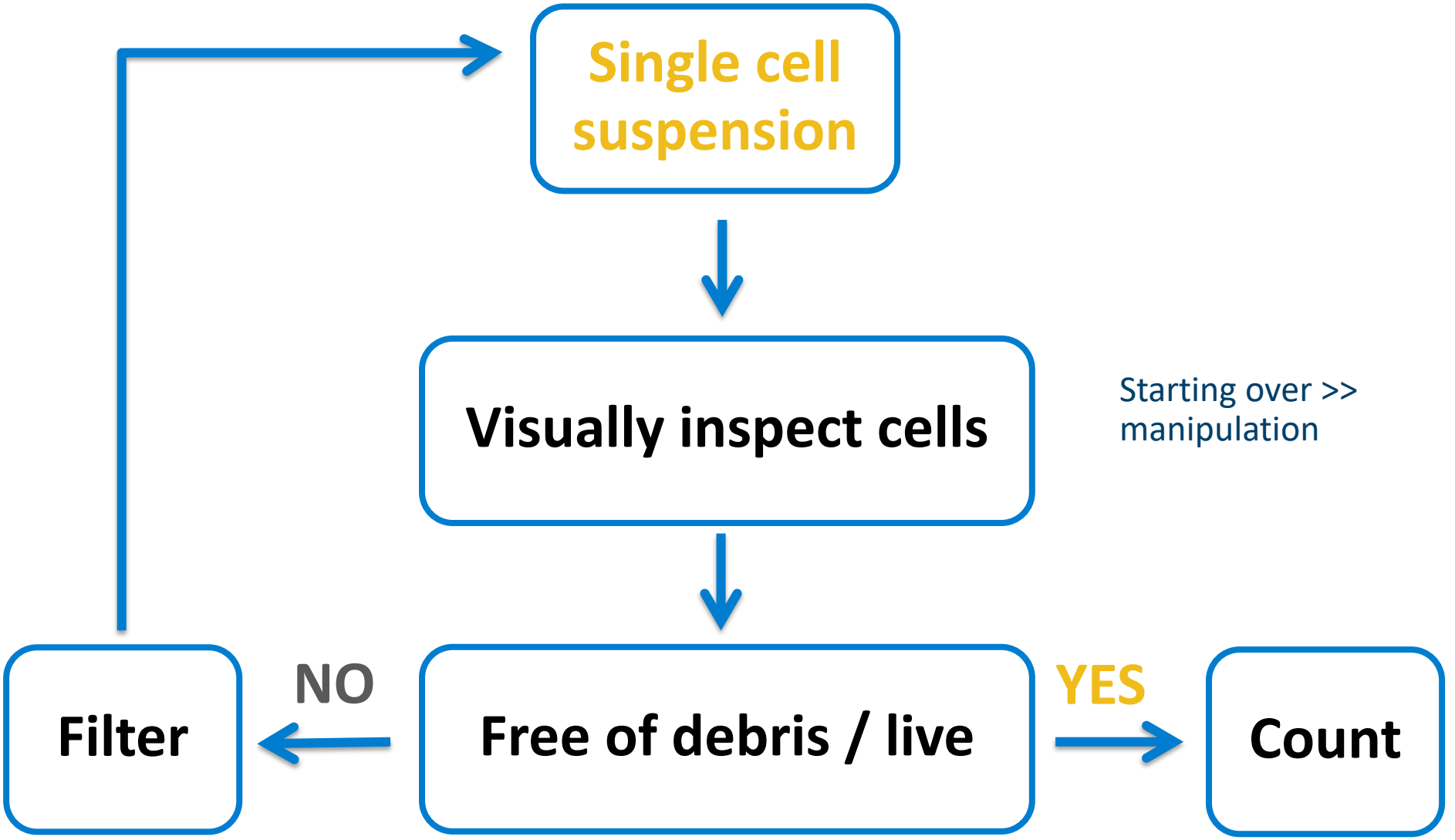
- Non-single cell clumps.
- Integrated into droplets and cannot* distinguish from single cells.



Bad for any single cell experiment.

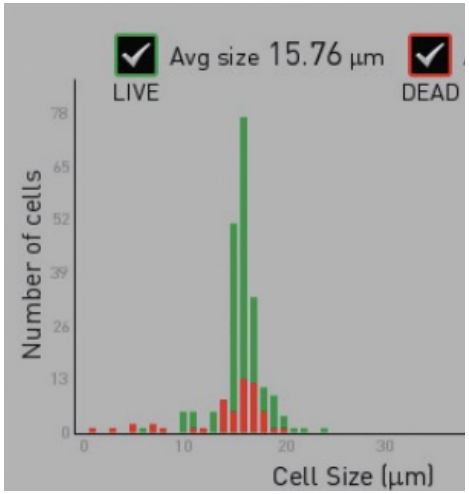
Recommended treatment: filtration, change [blocking agent]

Workflow


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

Automated counting

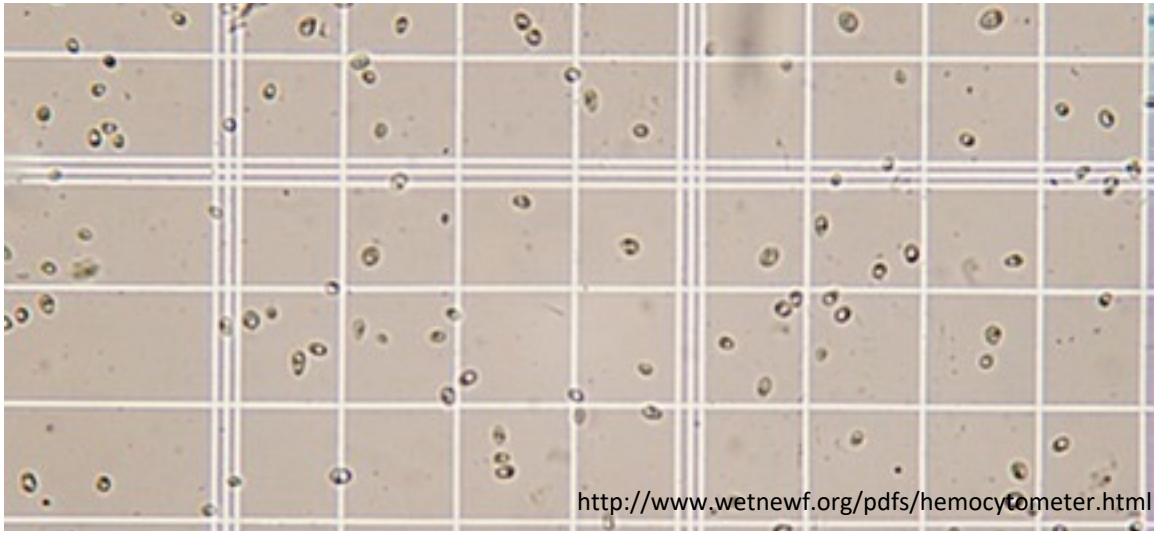
- Countess II.

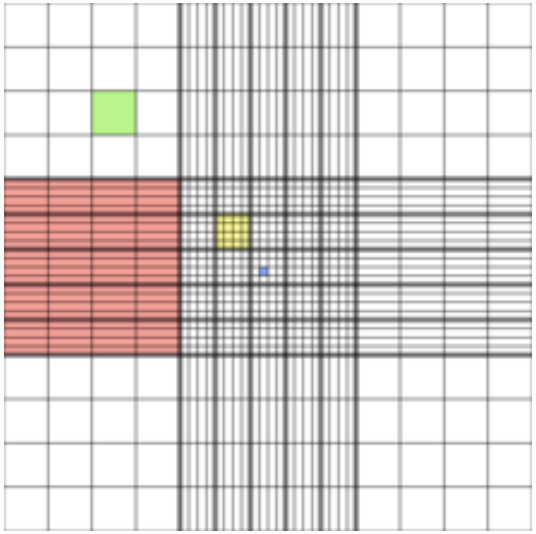


- Pros (+):
  - Fast.
  - Live/dead cell counts.
  - Cell size estimates.
- Cons (-):
  - Cell size limits (4-30um).
  - Doesn't do well with odd cell shapes, debris, nuclei.

Manual counting

- Hemocytometer.

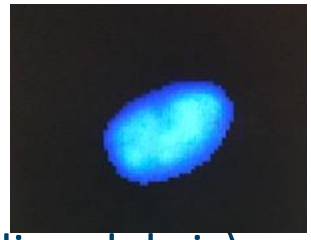




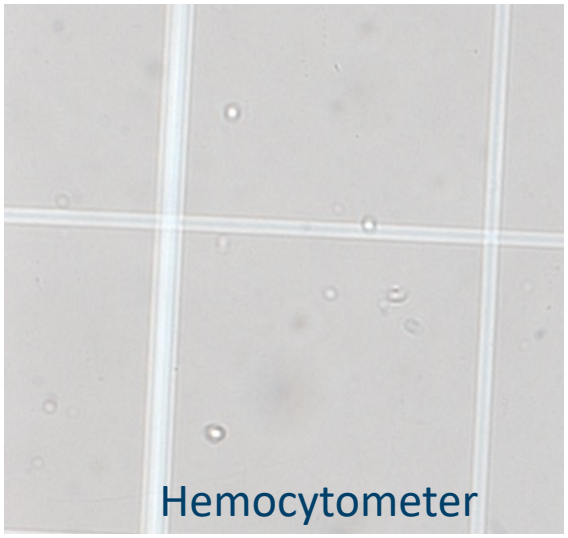
- Pros (+):
  - Reliable cell counts.
  - Count small cells.
  - Visualize cells vs. debris.
- Cons (-):
  - Slow.

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, AO/PI other options

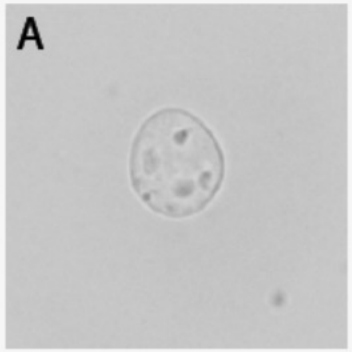


60X: DAPI



Hemocytometer

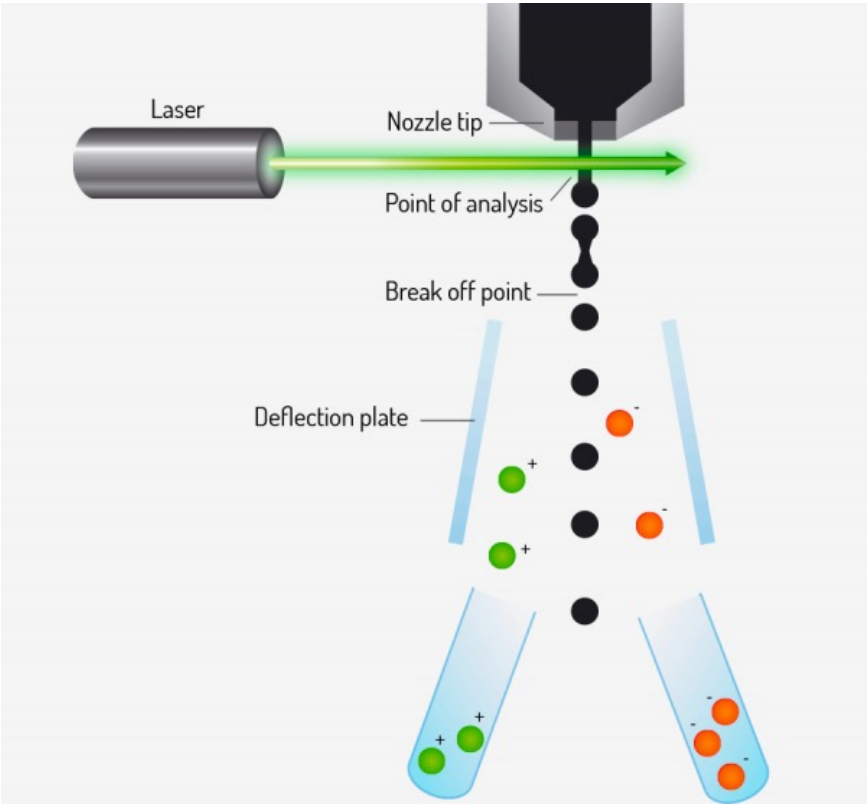
CountessII



Credit: 10X

Cell sorting

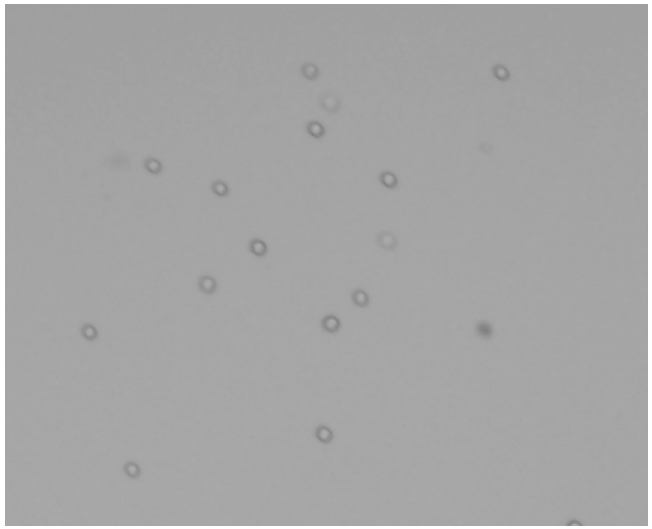
- FACS

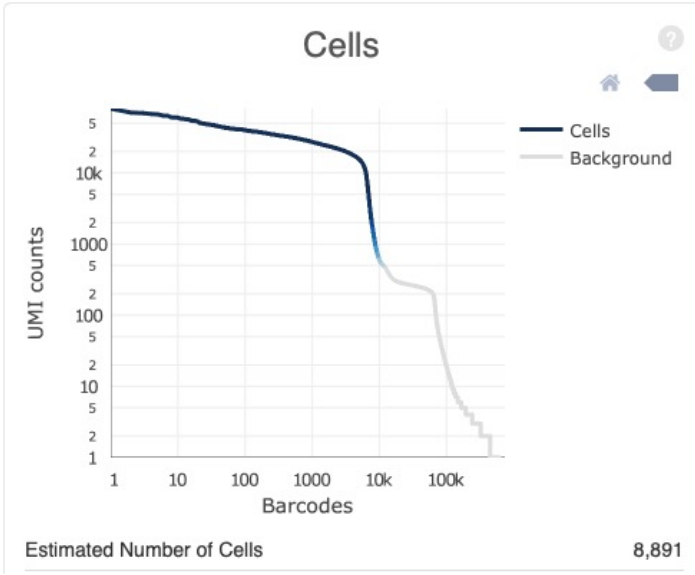


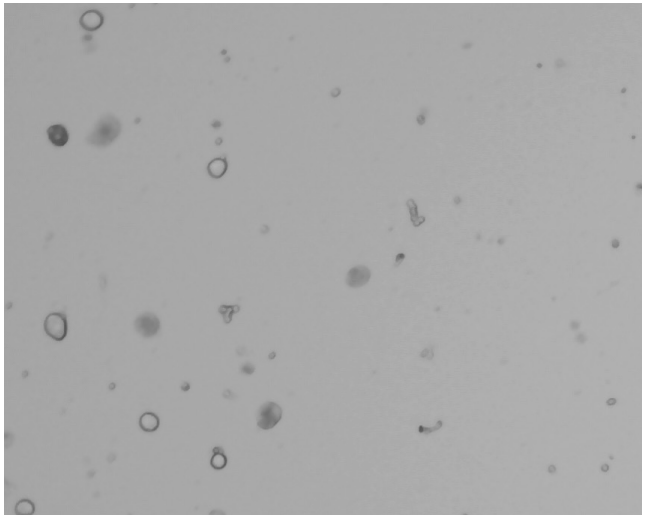
https://flowcytometry-embl.de/cell-sorting/

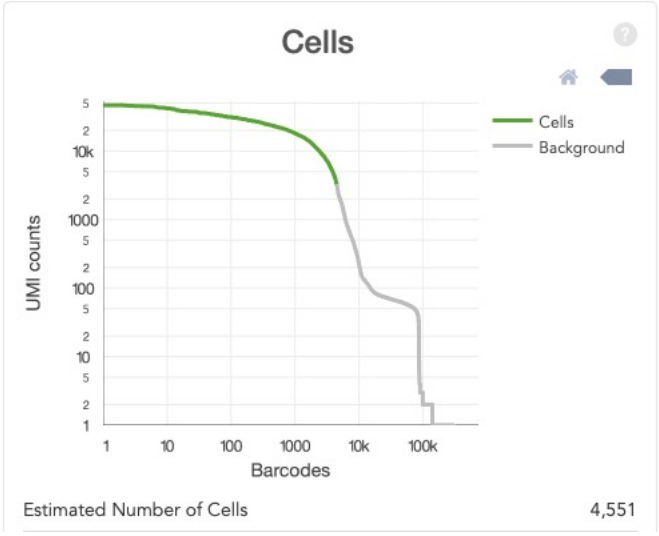
- Pros (+):
  - Sort live / dead.
  - Enrich cells of interest.
  - Determine whether correct cells isolated (qPCR works too).
- Cons (-):
  - Not accurate for cell counts.

The good…

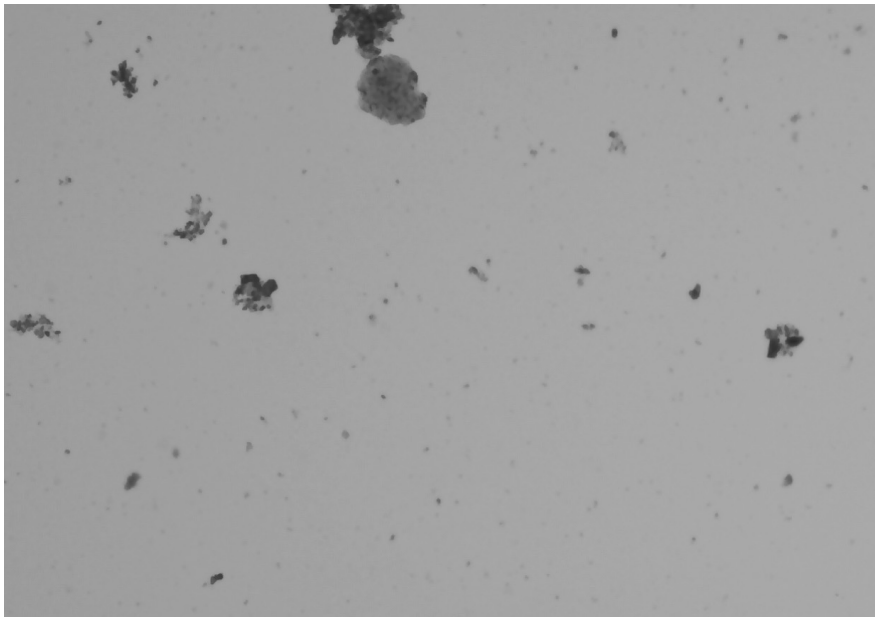


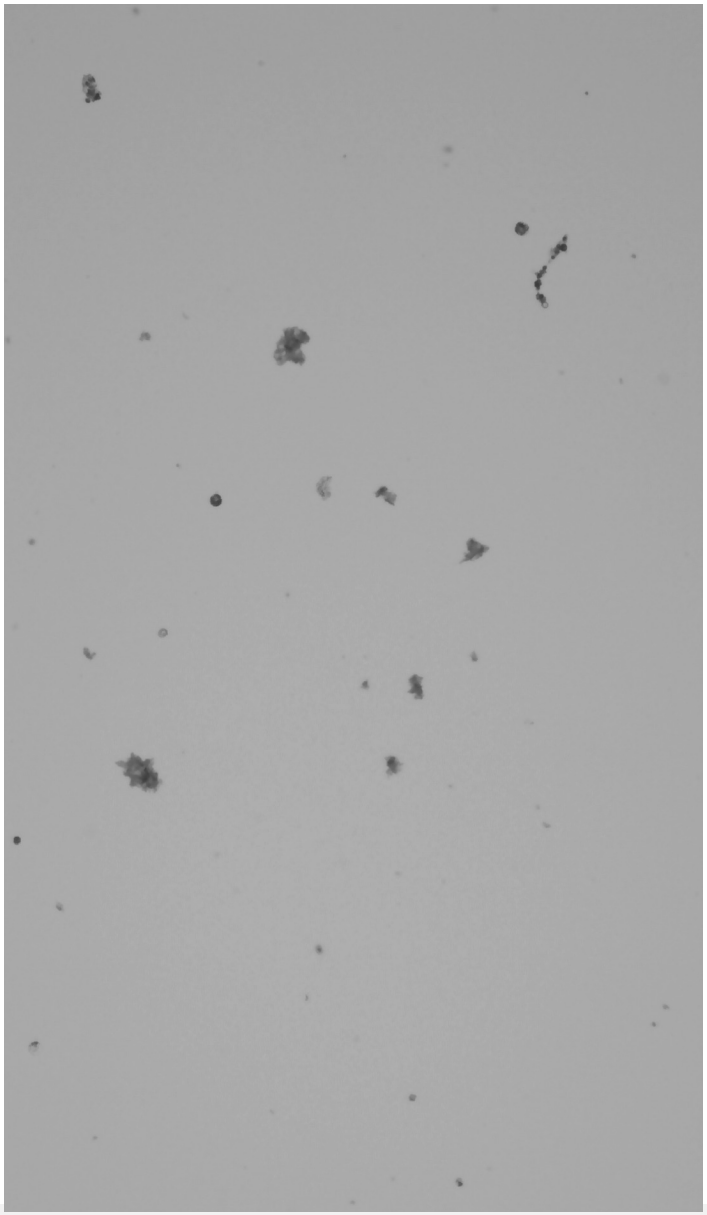

Cells
Cells
Background
UMI counts
5
2
10k
5
2
1000
5
2
100
5
2
10
5
2
1
1
10
100
1000
10k
100k
Barcodes
Estimated Number of Cells
8,891




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

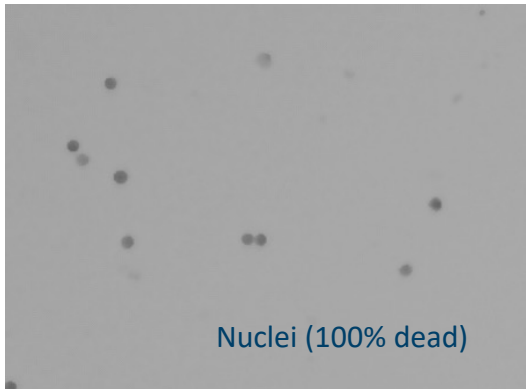
The bad…





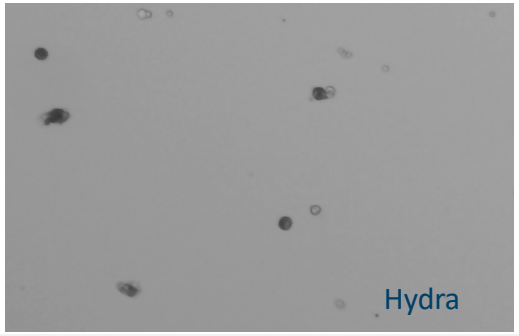
Challenges Cell Recovery

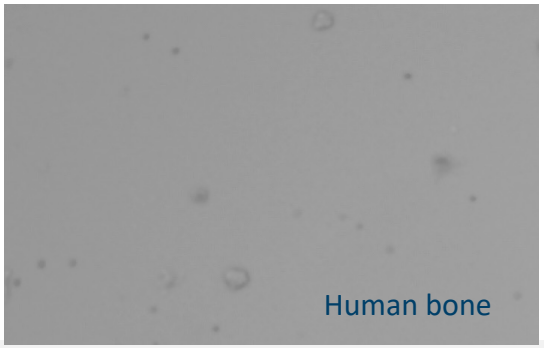
- Some samples easy - human PBMCs, etc.
  - Round, easy to count, size well below size of microfluidics.
  - These yield consistent results. 10K target. 8K-10K recovered.



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.



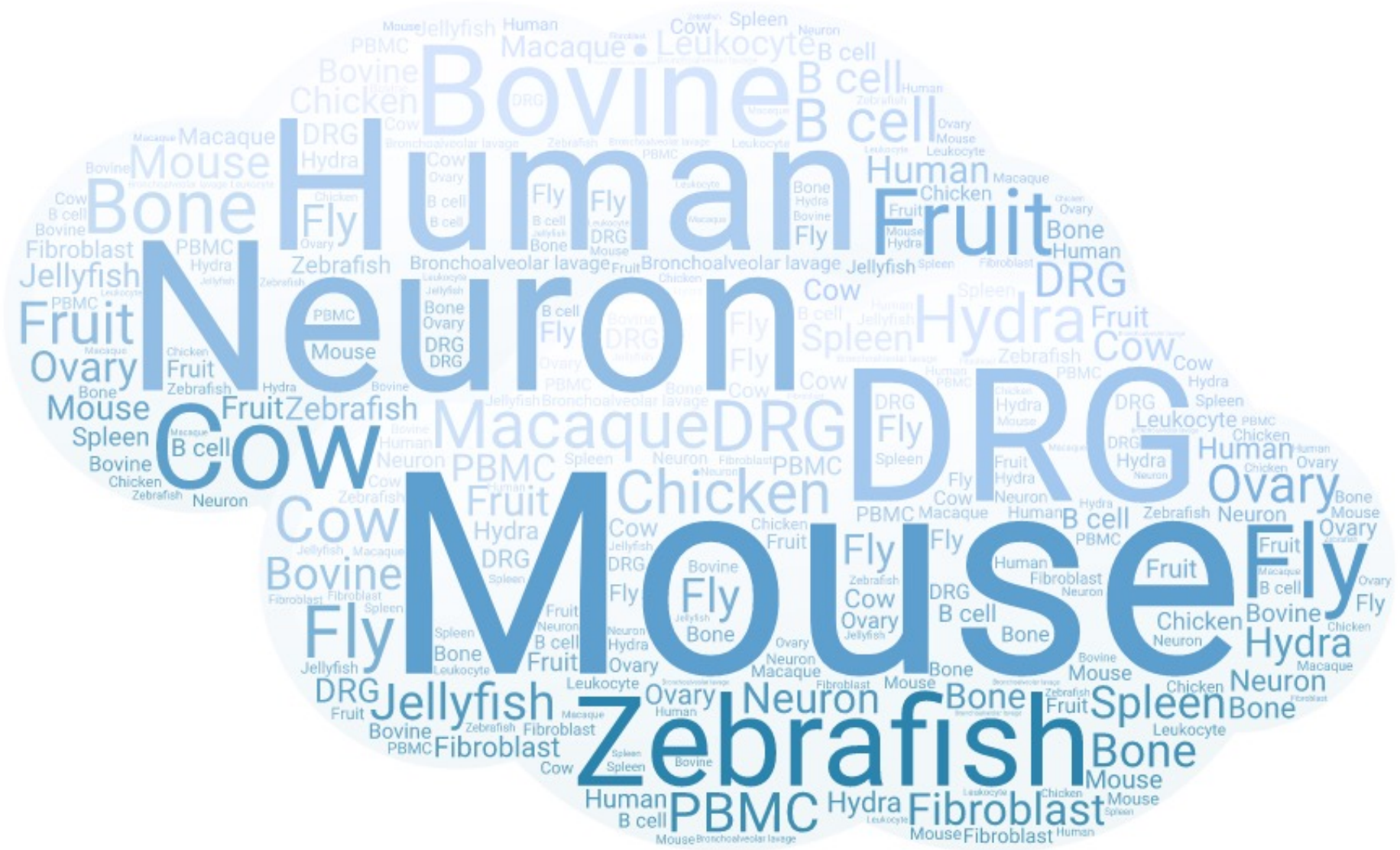


Variable size, shape, and viability

How maximize output?

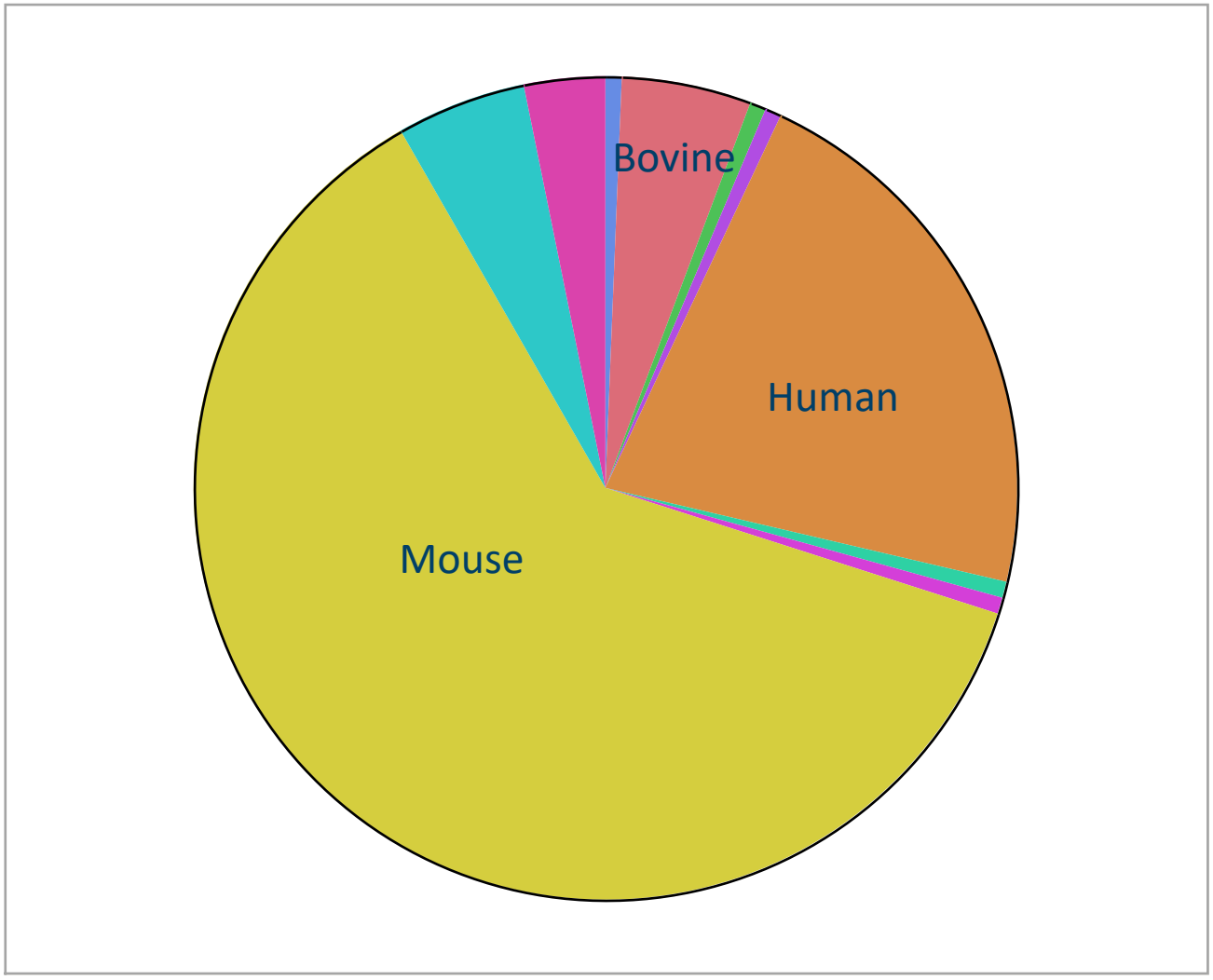
- Carefully craft experimental design and sample prep .
- QC cells before real sample set-up.
- Concentration: aim for the median.
  - **700-1,200 cells per µl optimal**.
  - Too high → dilute.
  - Too low → tough one (concentration impacts yield, pooling replicates suboptimal).
  - Count in replicates (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

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**

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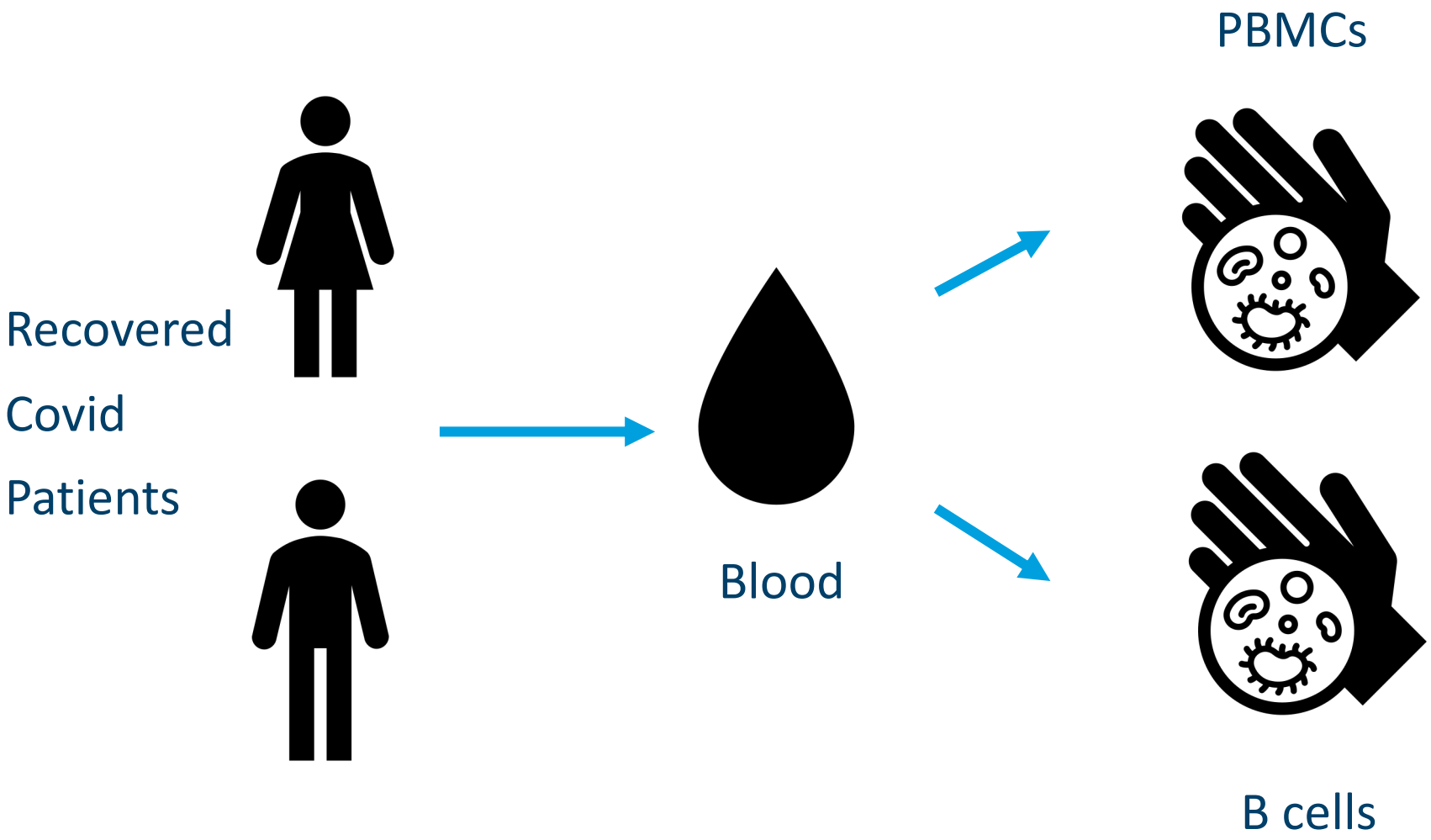
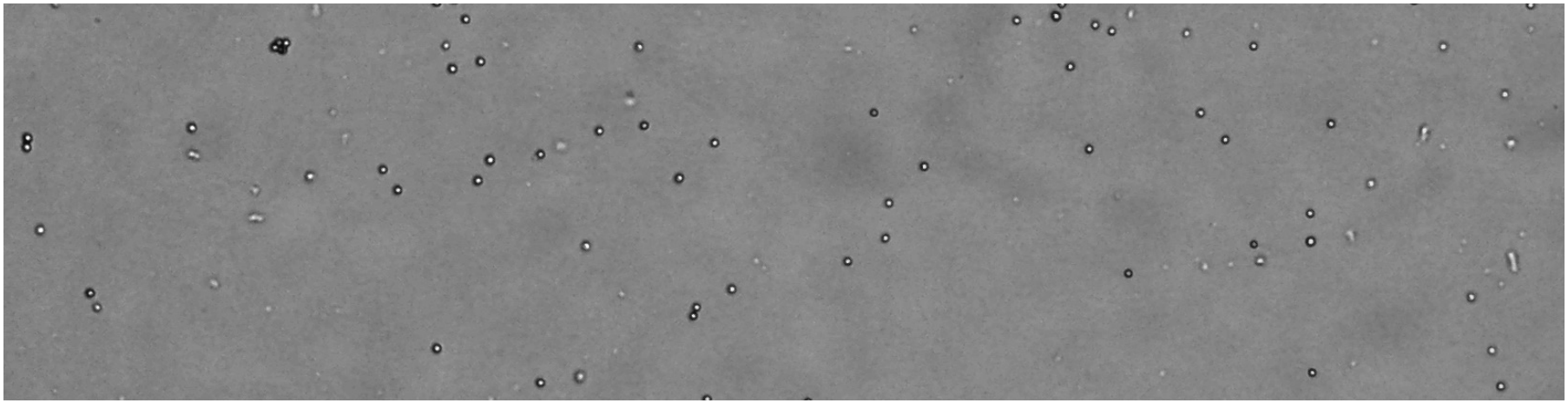
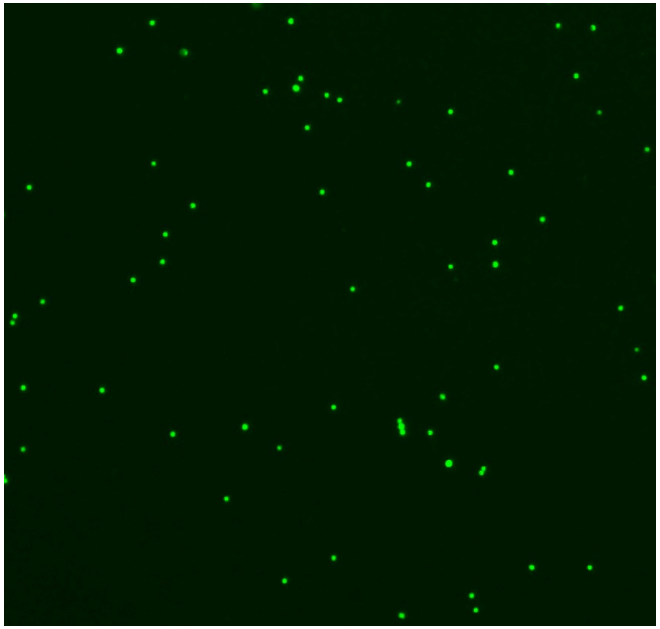
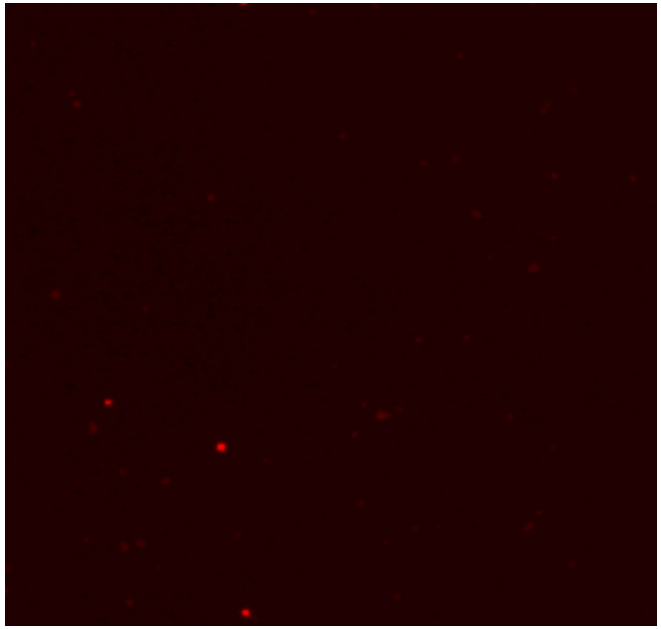
Course dataset



Input Material







Final Libraries

- Bead capture with TSO sequence → 5’ bias transcripts.
- 2 libraries from each cell type (GEX and V(D)J).

Chromium Single Cell V(D)J Enriched Library

Chromium Single Cell 5' Gene Expression Library

Important resources

- 10X Genomics
  - https://support.10xgenomics.com/single-cell-gene-expression
  - support@10xgenomics.com
  - 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/

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- UC Davis DNA Technology Core team.
- UC Davis Bioinformatics Core team.
- 10X Genomics.
  - Adam Bemis (Field Applications Scientist)
  - Paul Scott (Sales Executive)
  - Nicole Abreu (Technology Advisor)