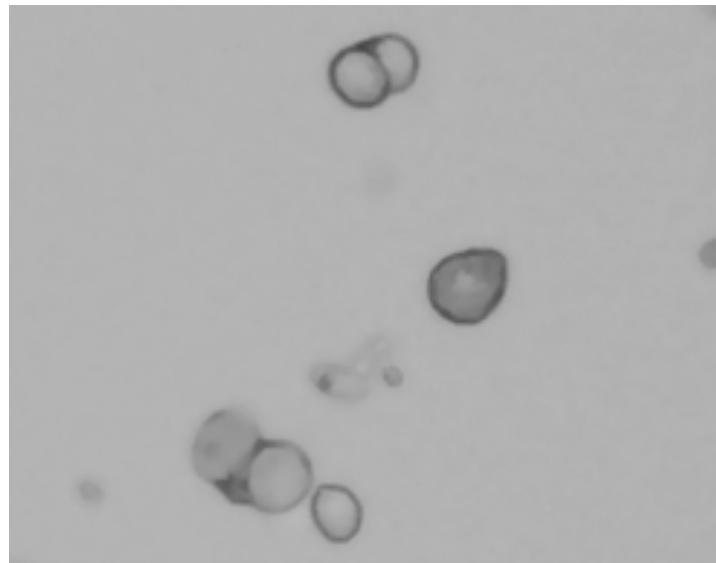


# Single Cell Sample Preparation



Diana Burkart-Waco, PhD

[dburkart@ucdavis.edu](mailto:dburkart@ucdavis.edu)

SRA at UC Davis DNATECH Core

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

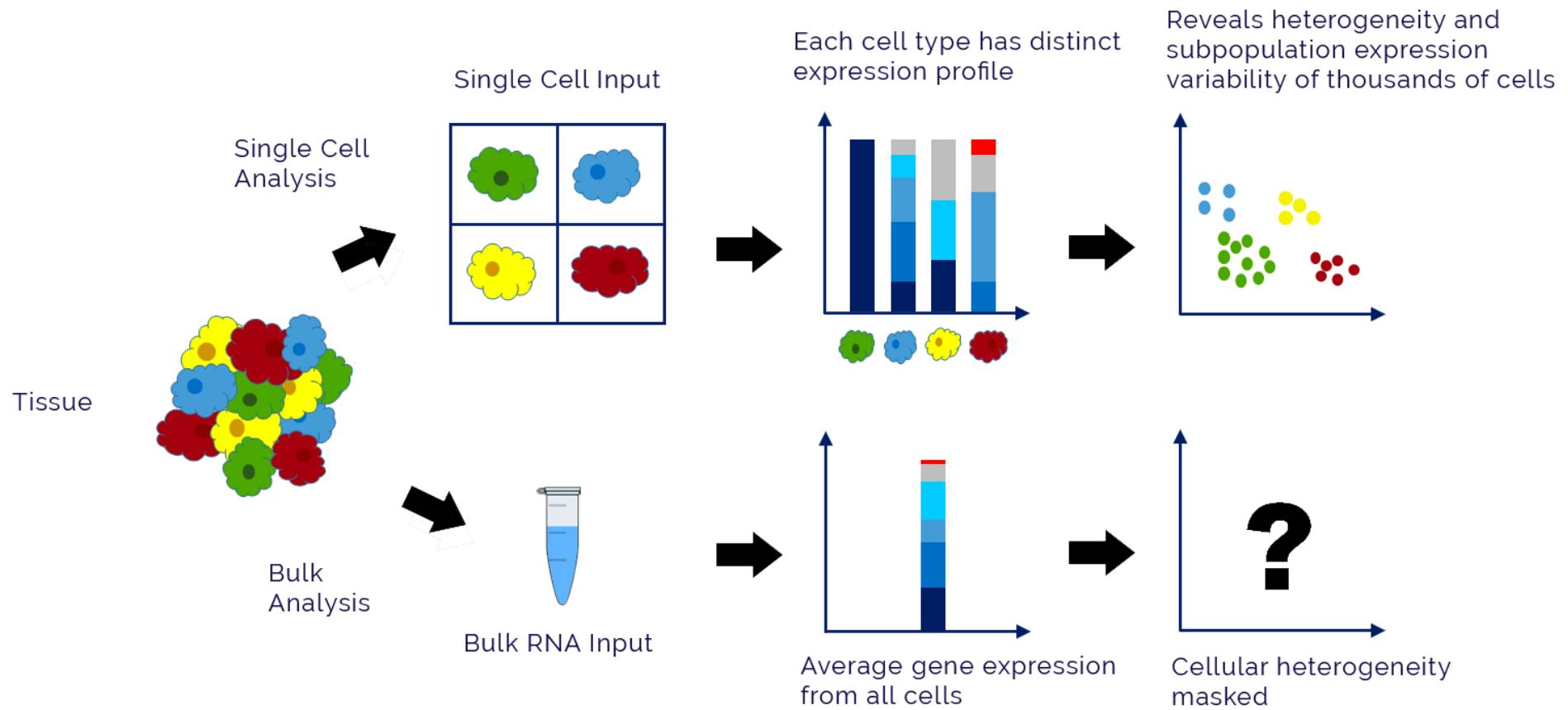
# Overview



## 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



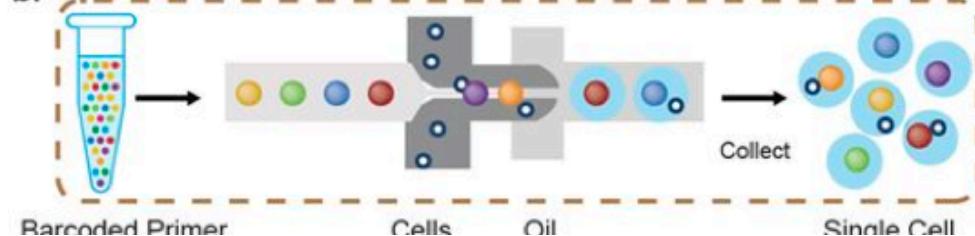
# 10X Genomics

DNA  
TECH

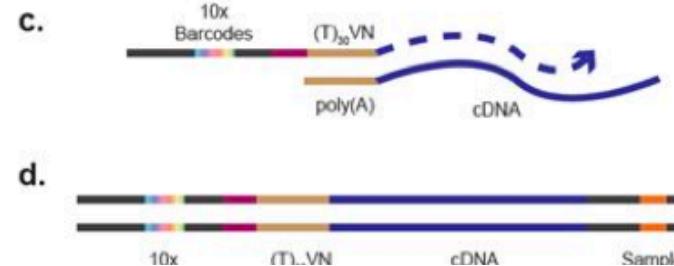
a.



b.



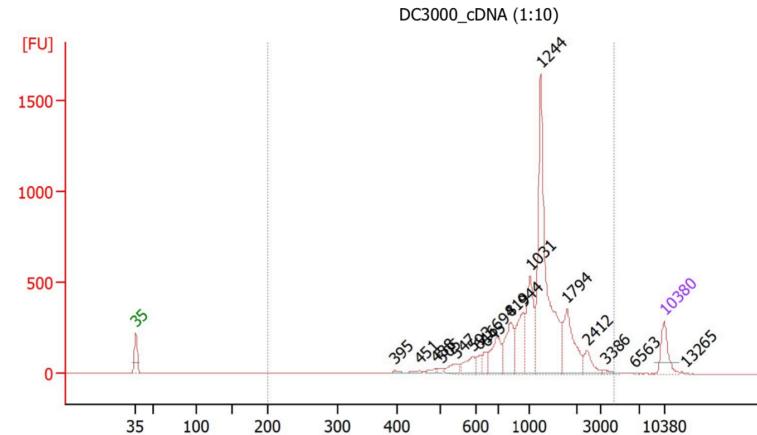
c.



d.



- Captures 100-10,000+ cells per sample.
- Recovers up to ~65% of cells.
- Low doublet rate.  
– (~0.9% per 1,000 cells).
- 40 micron size limit.
- Expensive!



# Alternative technologies



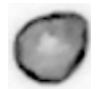
- Homemade drop-seq.
- Qiagen UPX.
- SPLIT-seq.
- Dolomite Nadia System.
- Fluidigm.
- And the list goes on...

Need both **single cells** and to maintain **sample composition**.

# 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

# Cell isolation

- Cell isolation guides available at:

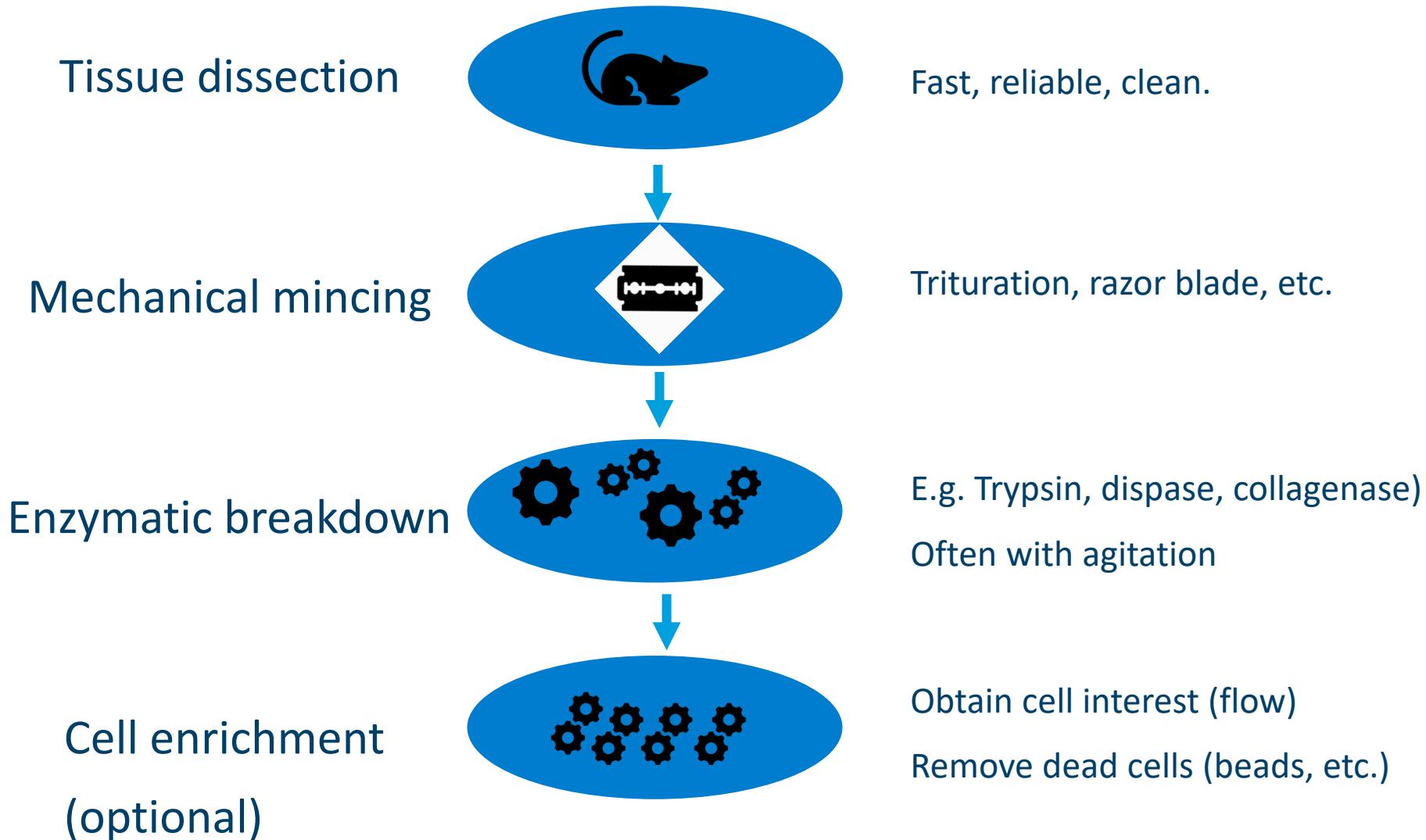
<https://www.support.10xgenomics.com/single-cell-gene-expression/sample-prep/>

## 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

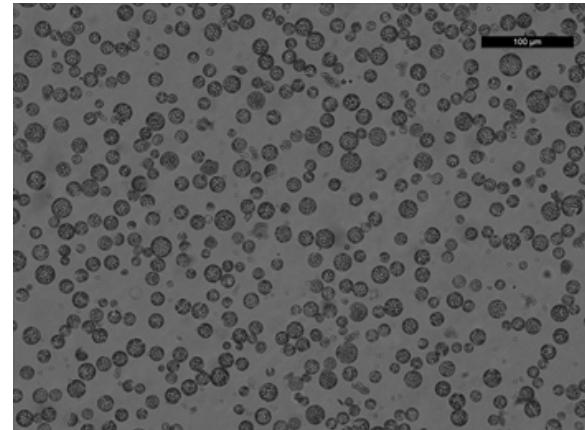
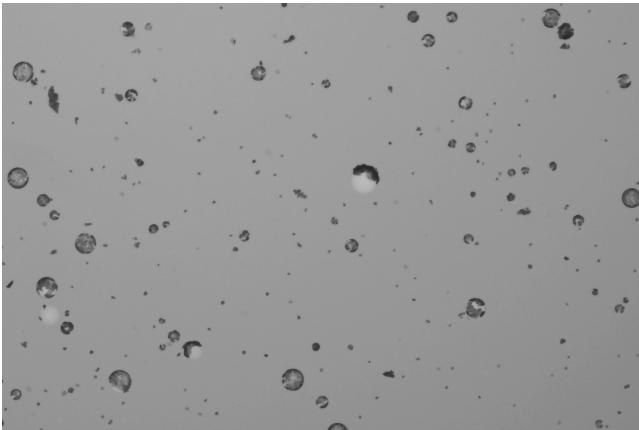
- Cell isolation → biggest source technical variation.
- Dissociation and preparation depends on cell type.

# General Workflow



# Tissue Dissociation

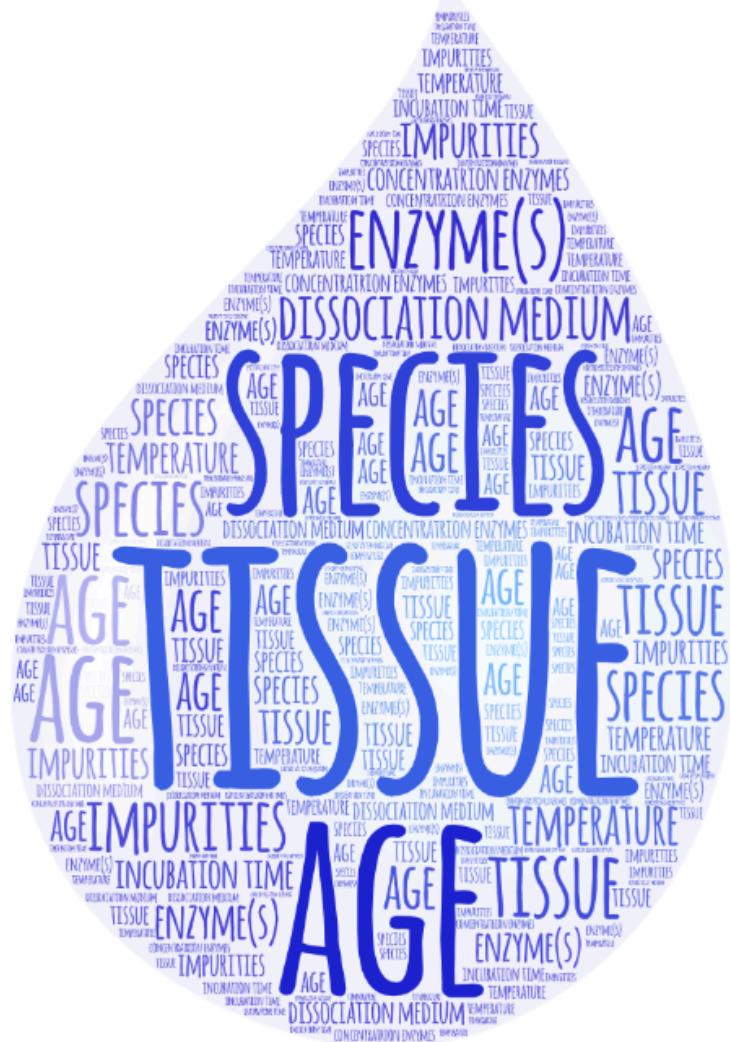
- Many ways to dissociate tissue:
  - Mechanical
  - Enzymatic
  - Automated blending
  - Microfluidics devices
- Considerations:
  - Speed
  - Consistency in results
  - Good representation of all cell types



Arabidopsis  
protoplasts

Same method,  
different days.

# Factors Influencing Dissociation

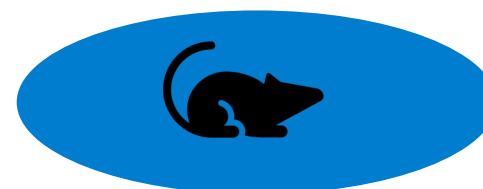


Unfortunately, SC sample optimization is best achieved through trial and error...

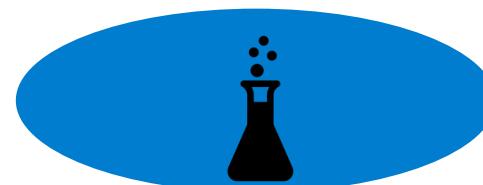
# Single cell vs. nuclei

- 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.

Tissue dissection



Cell lysis



Nuclei purification



Detergent  
Grinding

Flow cytometry  
Density Gradient

# 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](http://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  $\mu$ l sample to determine concentration



10x Genomics®  
**Sample Preparation  
Demonstrated Protocols**  
Isolation of Nuclei for Single Cell RNA  
Sequencing

# Overview



- Why single cell?
- Methods – SC isolation
  - Single cell isolation
  - Single nuclei



## Methods - sample QC

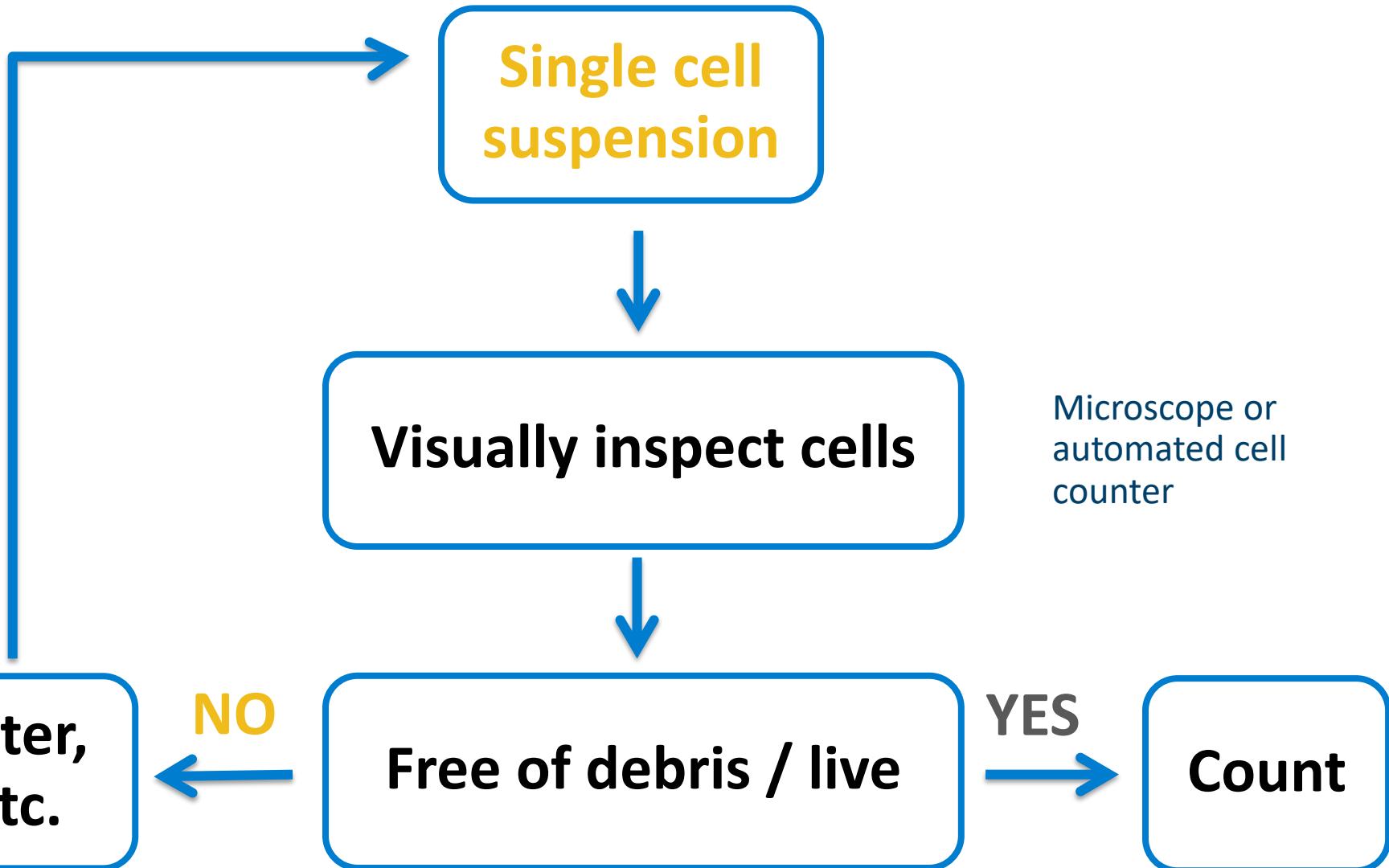
- Do I have single cells?
  - Are they alive?
  - Are they too big?
  - Did I isolate the correct cells?
- Cell labeling
  - Single cell studies at DNA Tech

# Factors influencing success

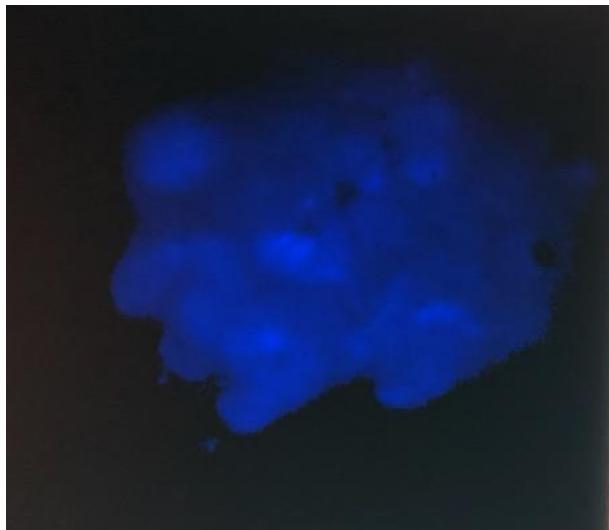
- Cell debris / dead cells
  - Clog microfluidics, free floating RNA → noise.
- Aggregates
  - No longer single cell data.
  - Clog microfluidics.
- Buffer
  - Inhibit polymerase → decrease library complexity.
- Storage conditions

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

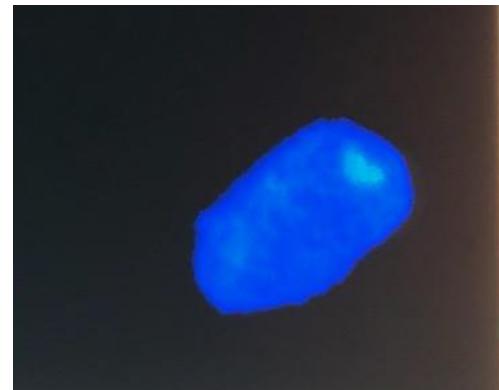
# Workflow



# Tissue aggregates



Tissue clump



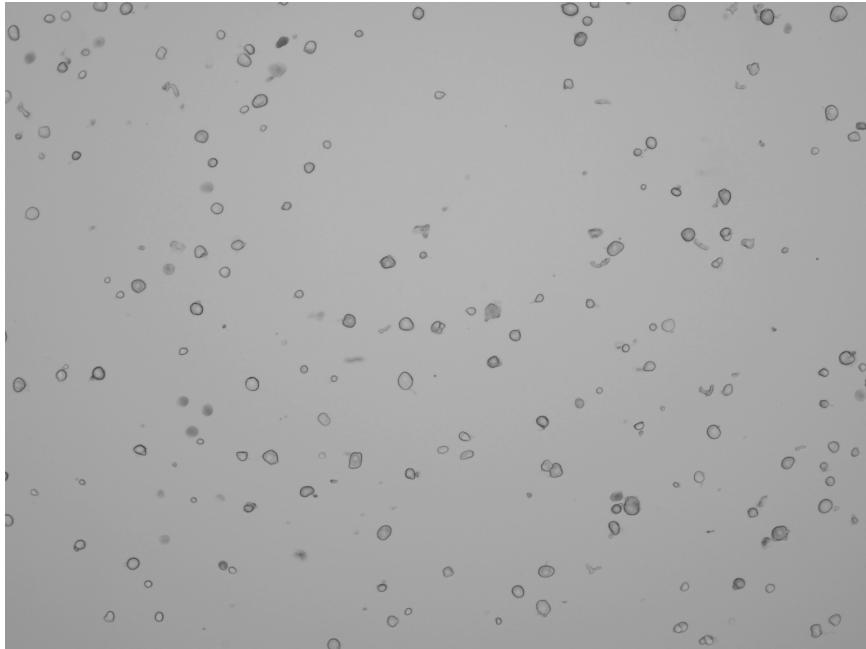
Single nucleus

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.

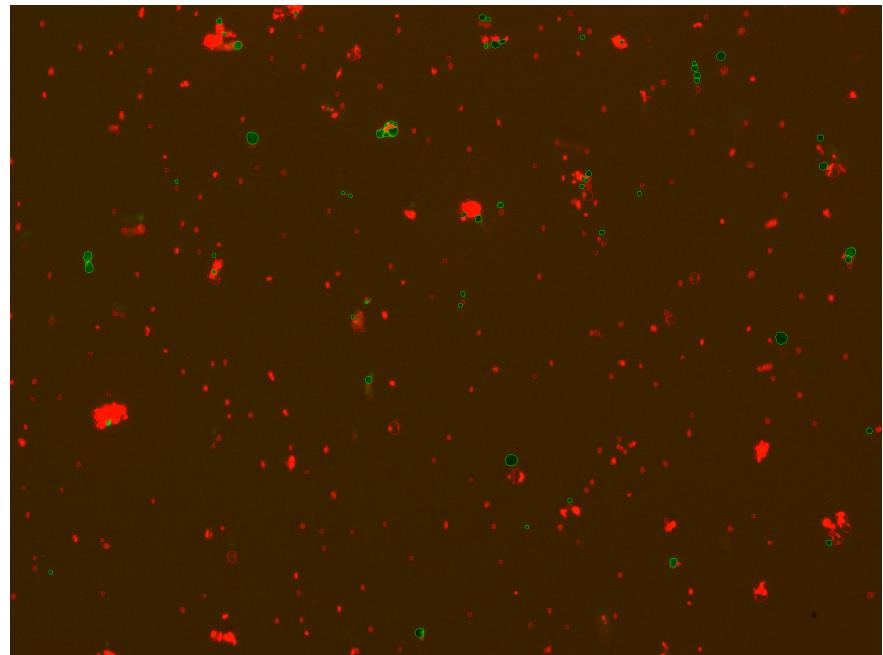
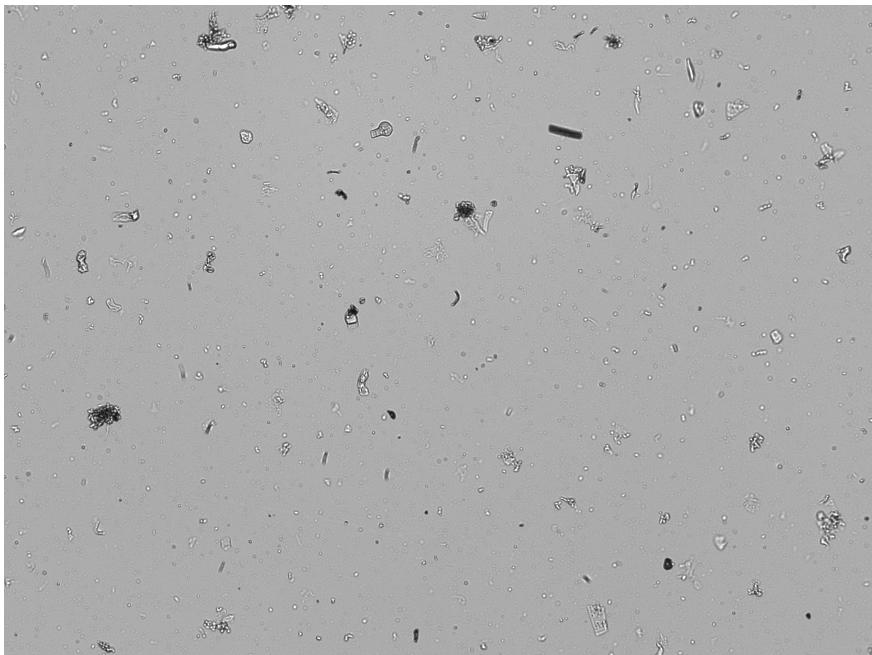
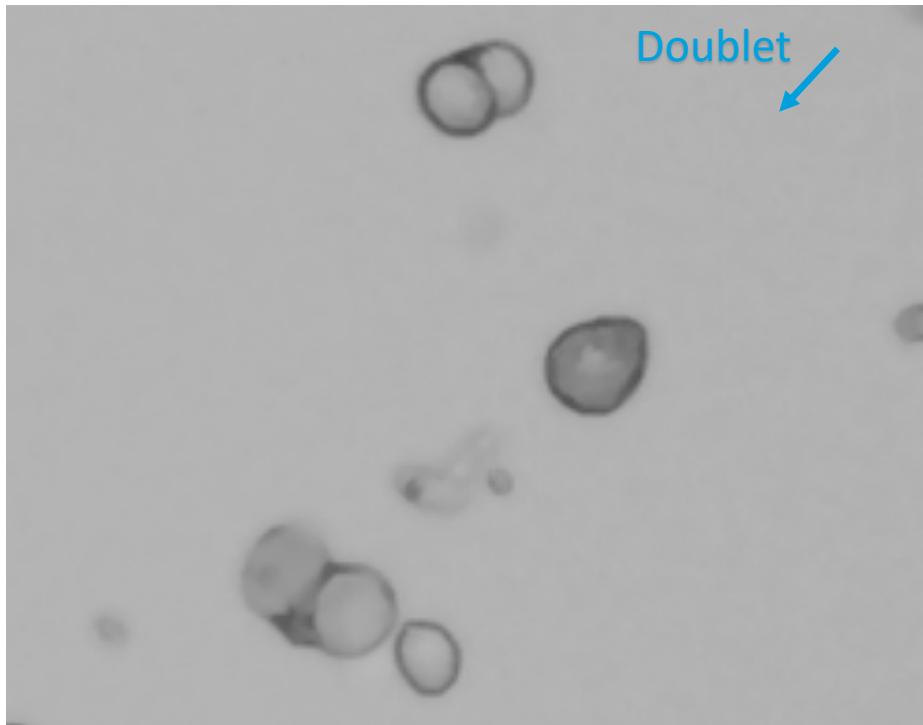


Photo credit: Mohan Prem Anand Marimuthu

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

# Doublets

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

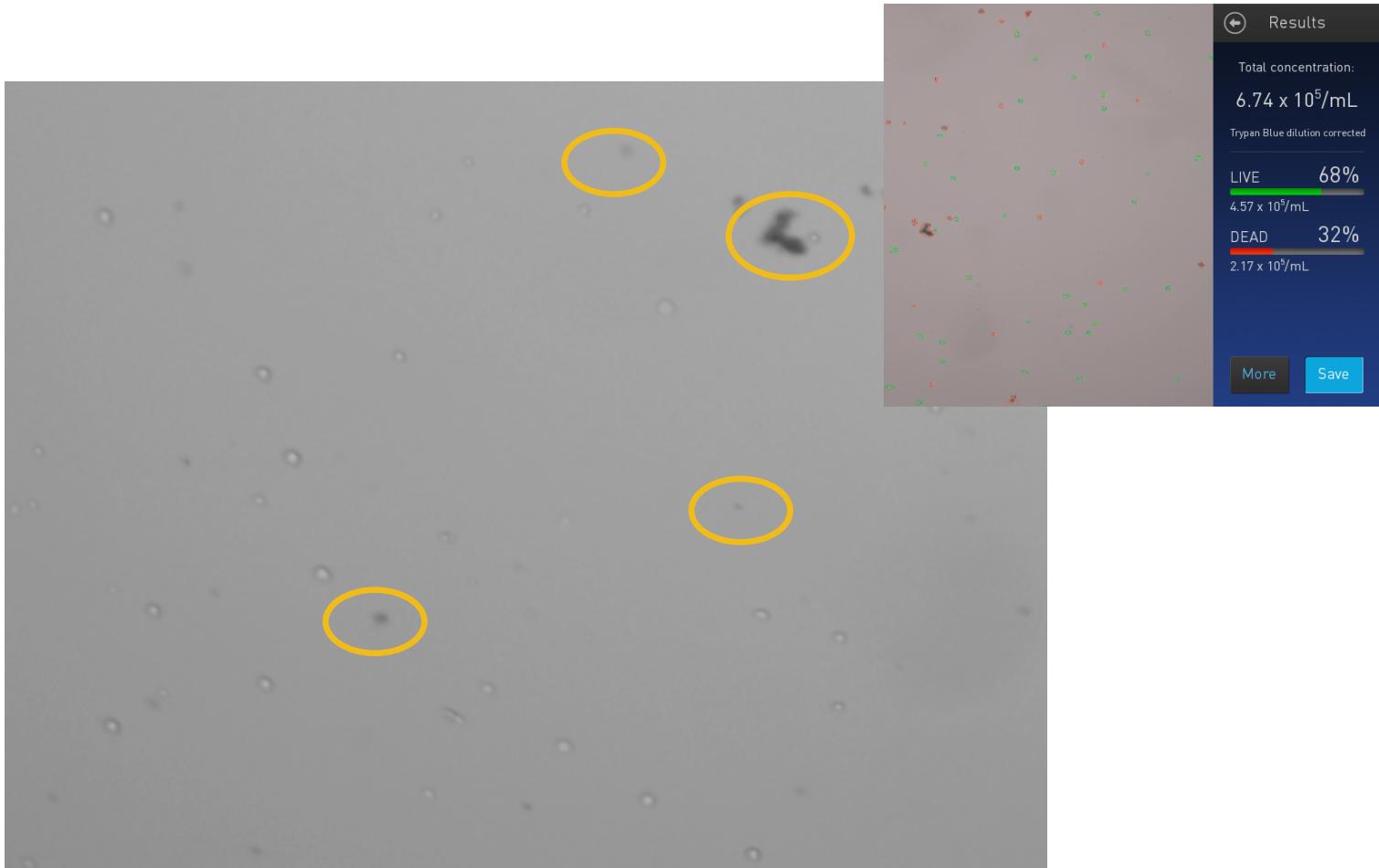


Bad for any  
single cell  
experiment.



Recommended treatment: filtration, change [blocking agent]

# Dead cells (debris?)



Recommended treatment: dead cell removal.

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

# Nuclei QC

- Automated cell counters suboptimal, but possible with fluorescence.

- Countess II FL, Devonix CellDrop.

- Stains

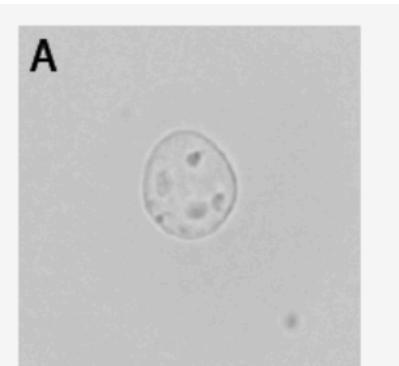
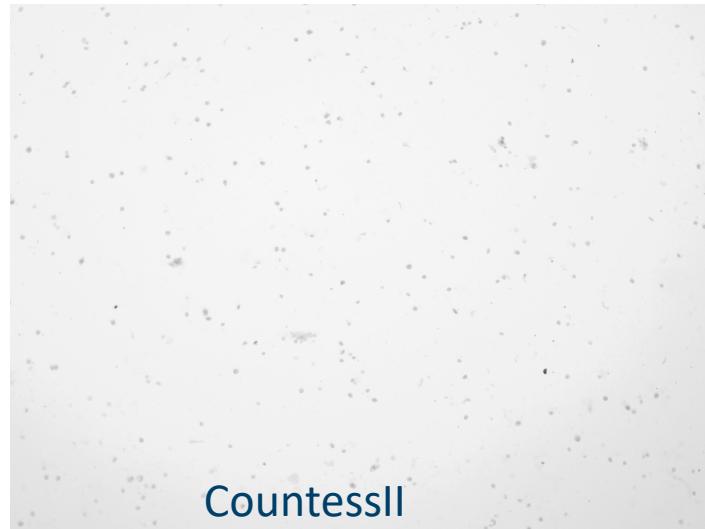
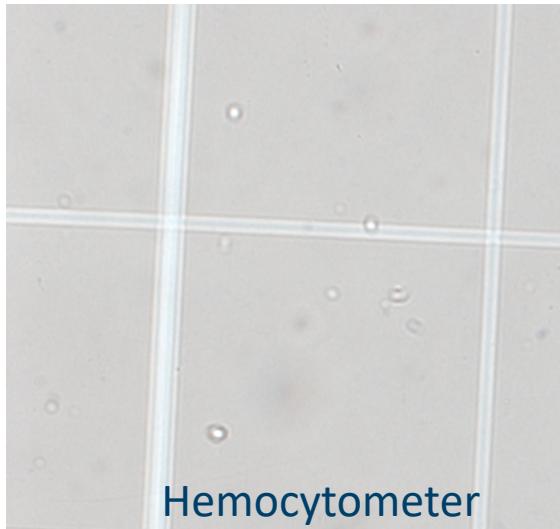
- Trypan blue → 100% dead (not great).

- Ethidium homodimer stain (good for excluding debris).

- DAPI, PI other options

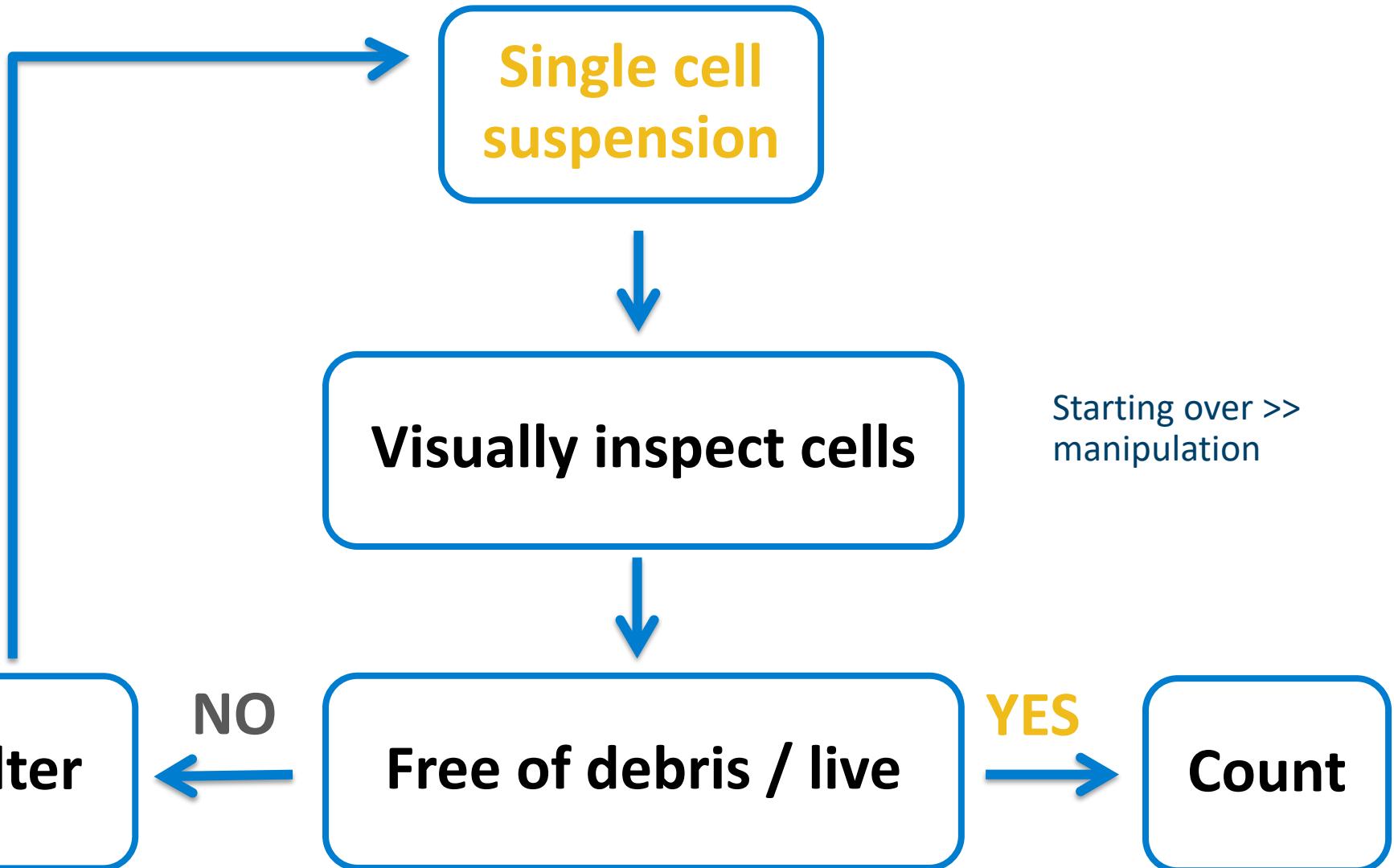


60X: DAPI



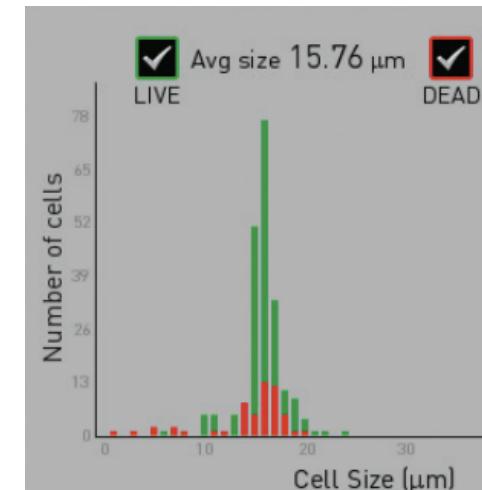
Credit: 10X

# Workflow



# Automated counting

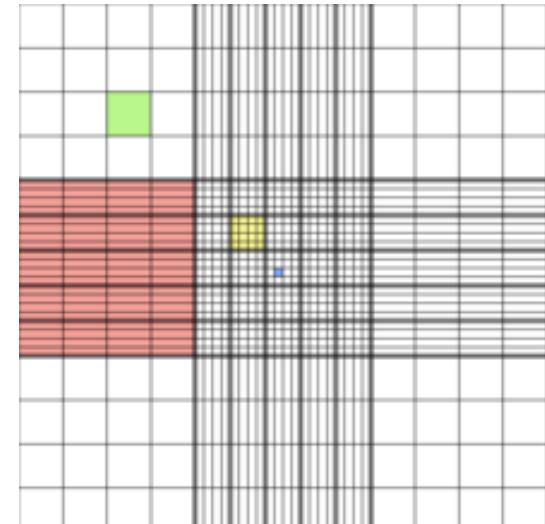
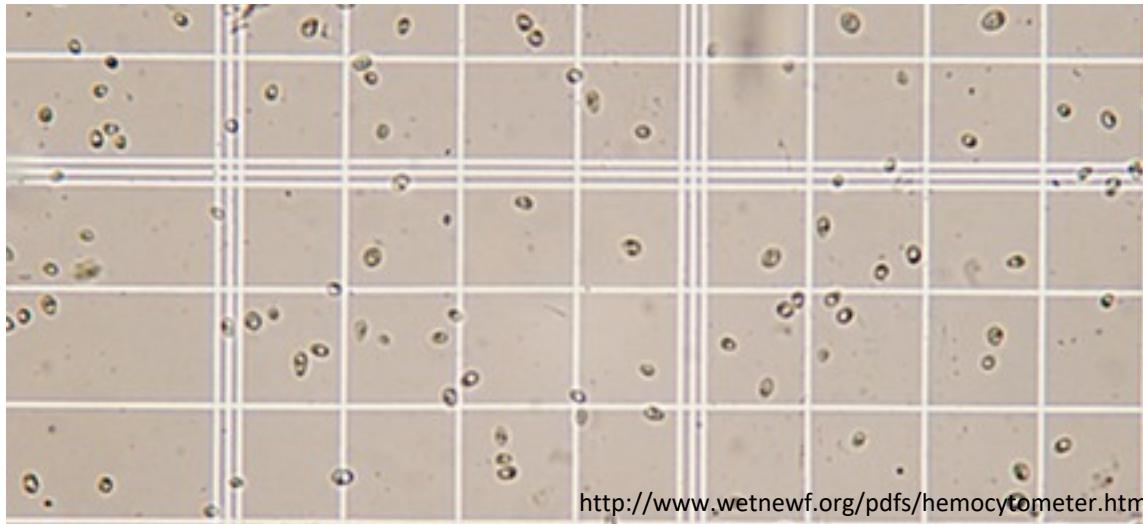
- Countess II.



- 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

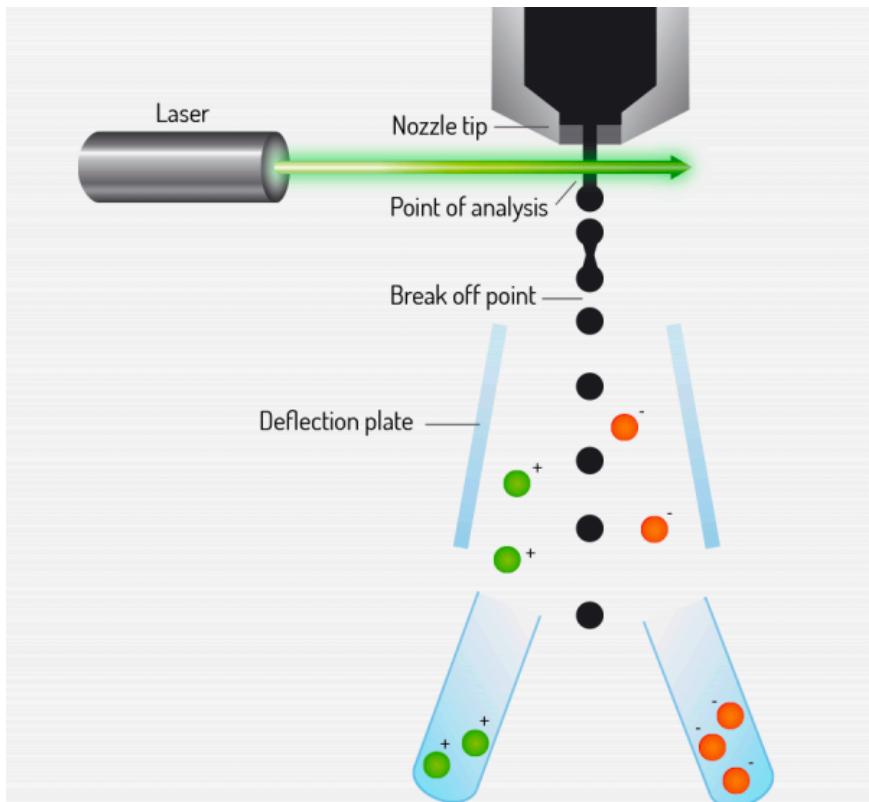
- Hemocytometer.



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

# Cell sorting

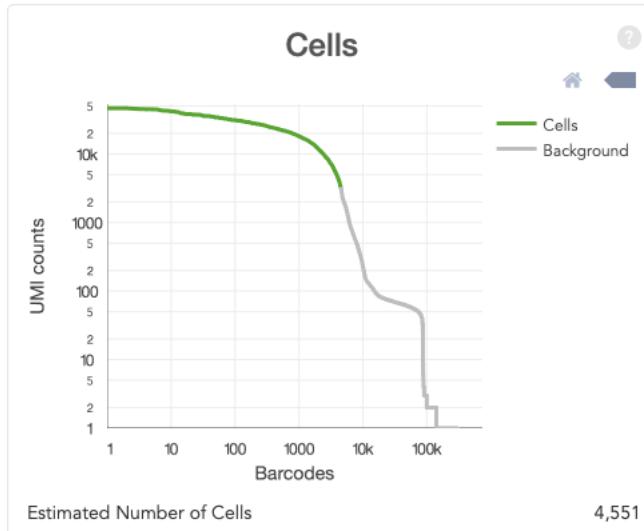
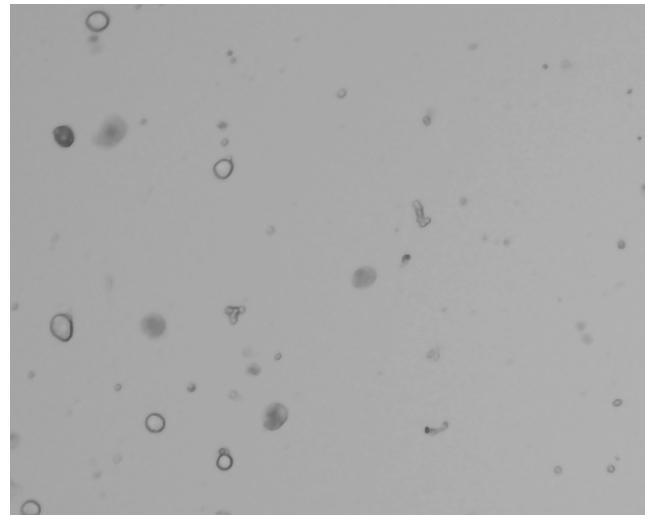
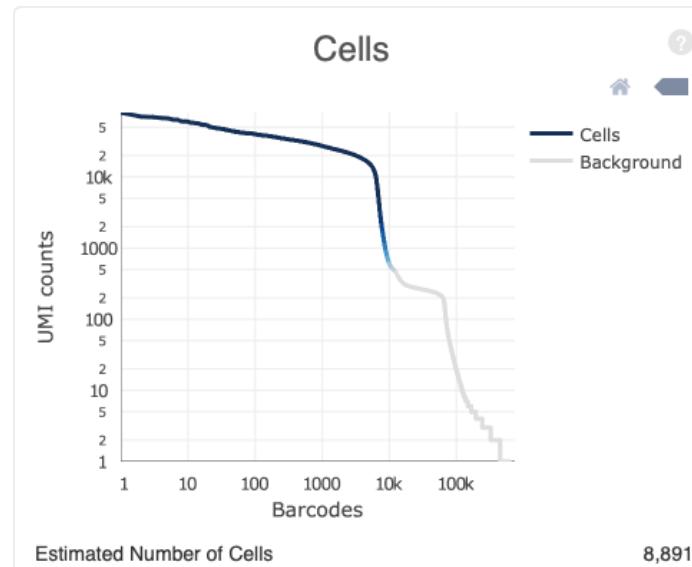
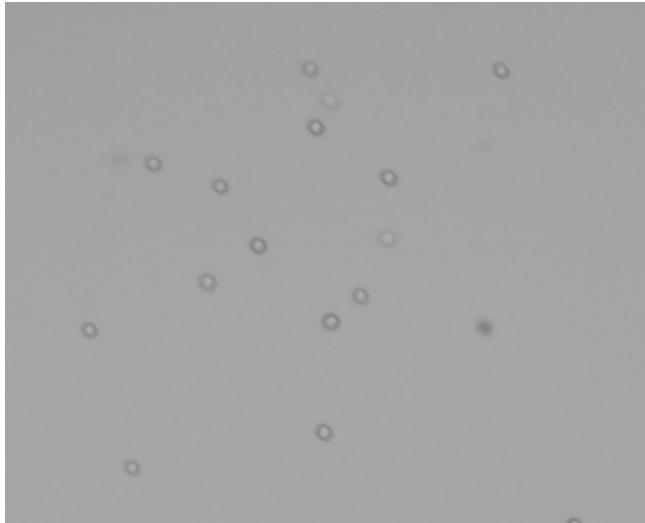
- FACS



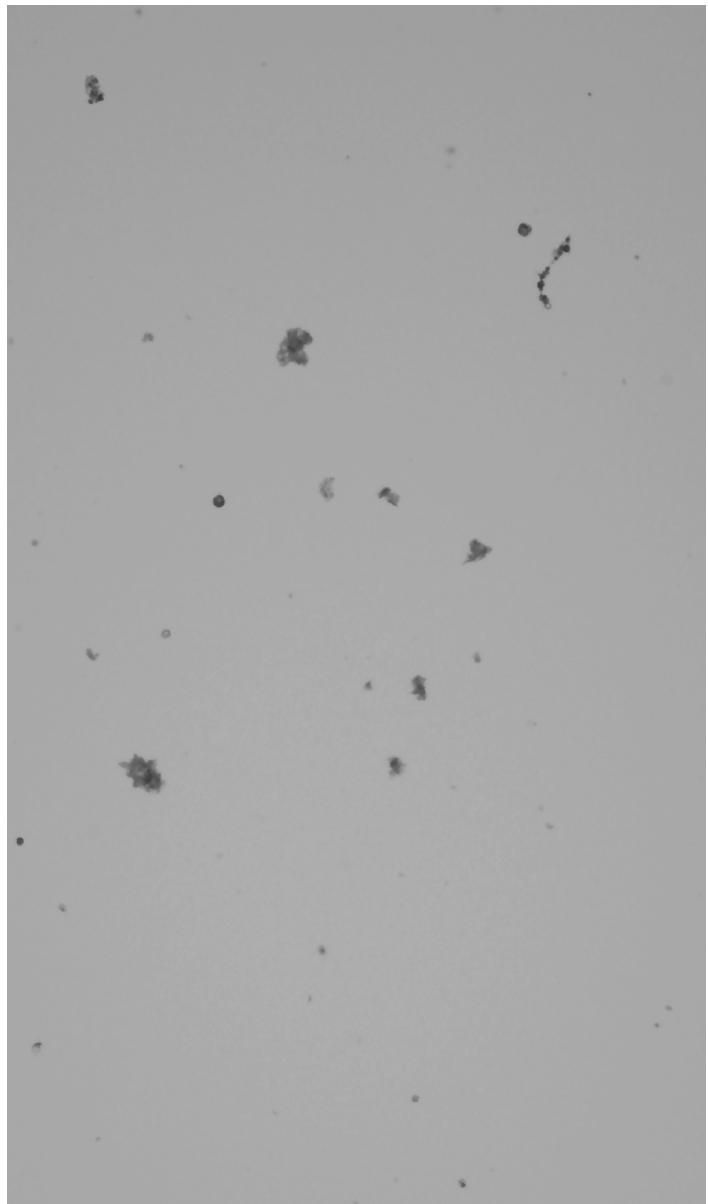
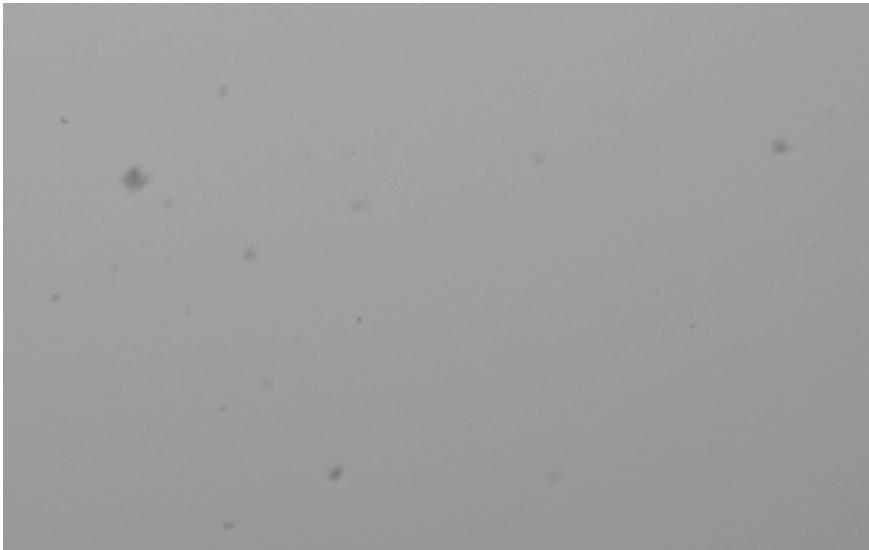
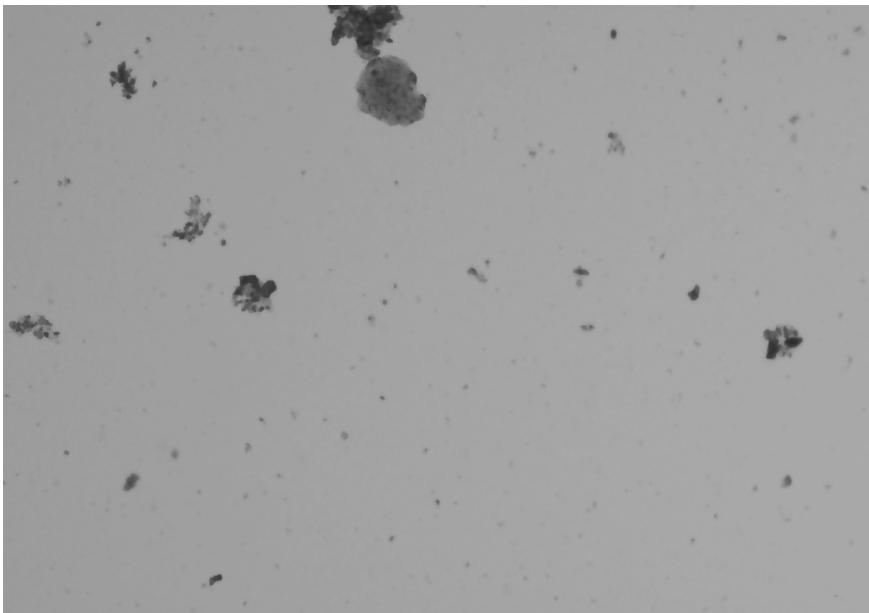
- Pros (+):
  - Sort live / dead.
  - Enrich cells of interest.
  - Determine whether correct cells isolated (qPCR works too).
- Cons (-):
  - Overestimates cell counts (~2x).

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

# The good...

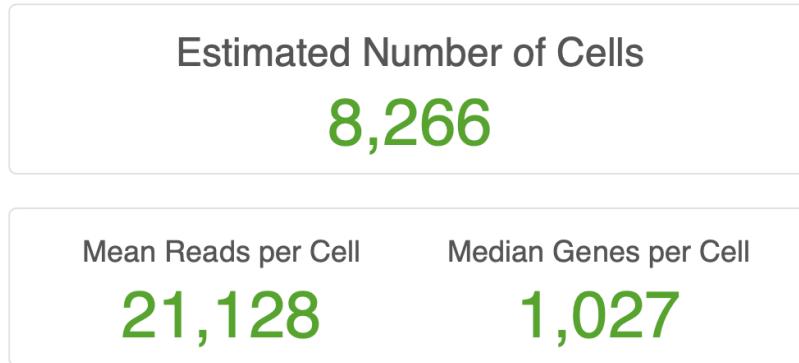
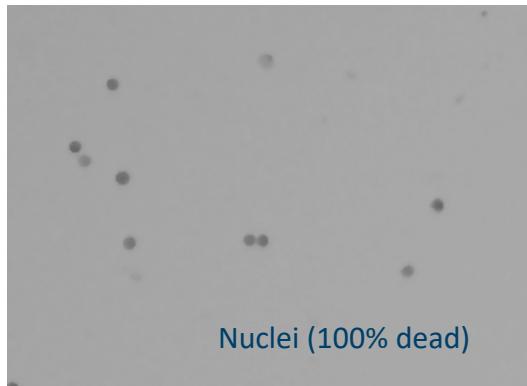


# The bad...

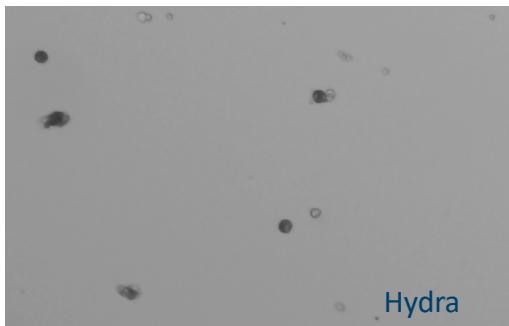


# Challenges Cell Recovery

- 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.



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



Hydra



Human bone

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..

# 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?

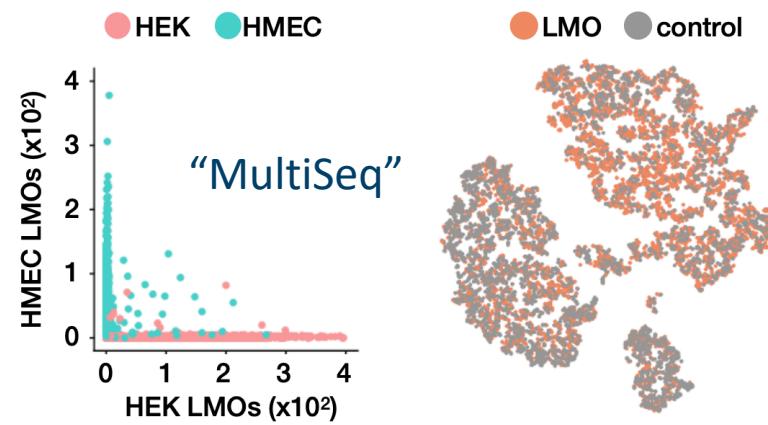
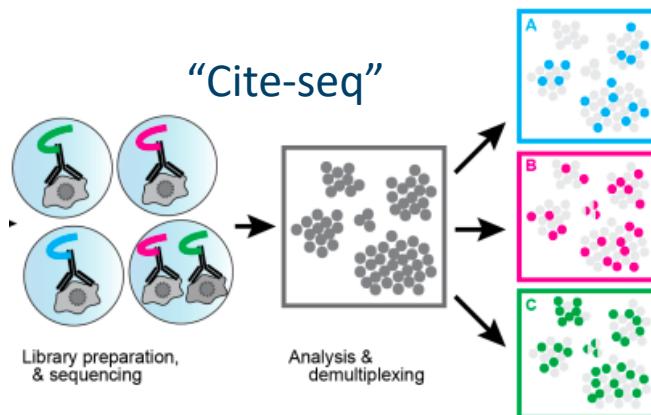


## Cell labeling

- Single cell studies at DNA Tech

# Cell Hashing

- Label cells (either oligo-tagged antibodies) or oligos.
- Allows for multiplexing and super-loading scRNA-seq platforms – cost saving and minimize tech variation.
- Many options available:
  - MULTI-seq (C. McGinnis / Z Gartner @ UCSF)
  - Biolegend TotalSeq
  - BD Single-Cell Multiplexing Kit
  - 10X CellPlex (coming later this year)

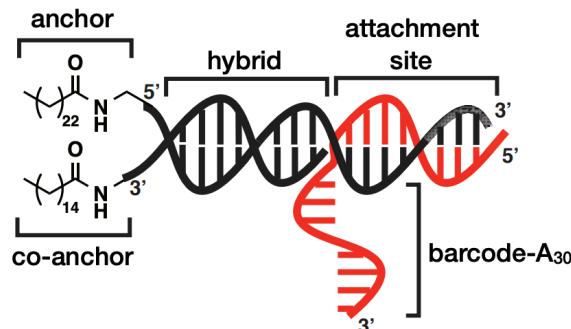


# MultiSeq Tag Design

- Oligo targeted to plasma membrane with 5' lignoceric acid amide.
  - Co-anchor increases stability and specificity.
- 8bp barcode conjugated to 3' Poly-A capture sequence.

5' ----- PCR handle ----- barcode ----- AAAAAAAAAAA ----- 3'

- Once in droplets, PolyA mimics endogenous transcripts and hybridizes to dT oligos in beads.



“MultiSeq” (C. McGinnis)

Barcode → tag suspension  
AAs → capture transcripts

# 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?
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- Cell labeling



Single cell studies at DNA Tech

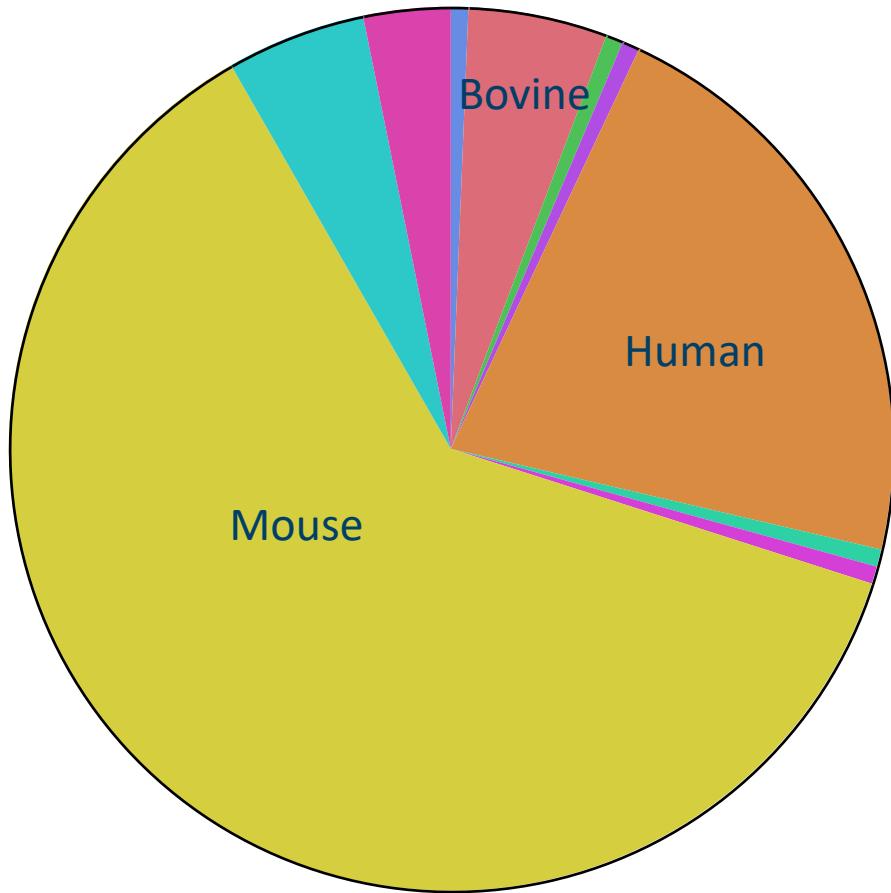
# 10X Single Cell Studies - DNATECH



# 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

# SCIENTIFIC REPORTS



OPEN

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

Received: 15 October 2018

Accepted: 27 February 2019

Published online: 19 March 2019

Kaitryn E. Ronning<sup>1</sup>, Sarah J. Karlen<sup>2</sup>, Eric B. Miller<sup>1</sup> & Marie E. Burns<sup>1,2,3</sup>

# 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**

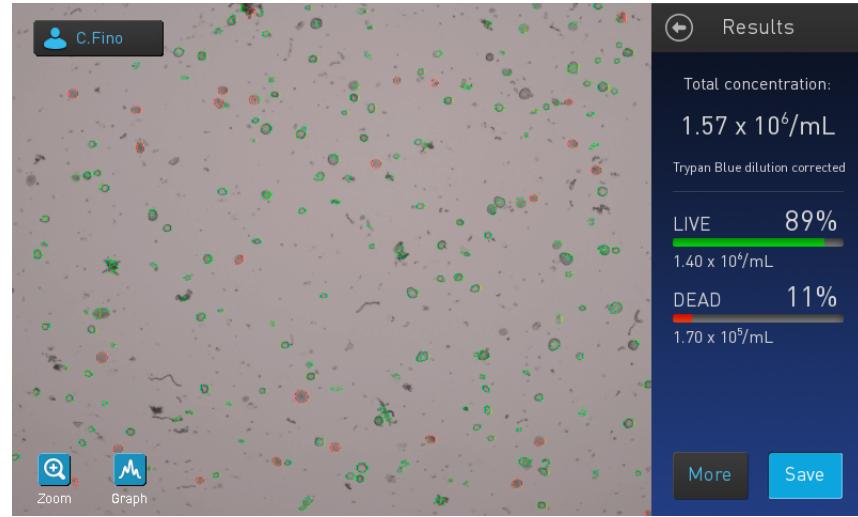
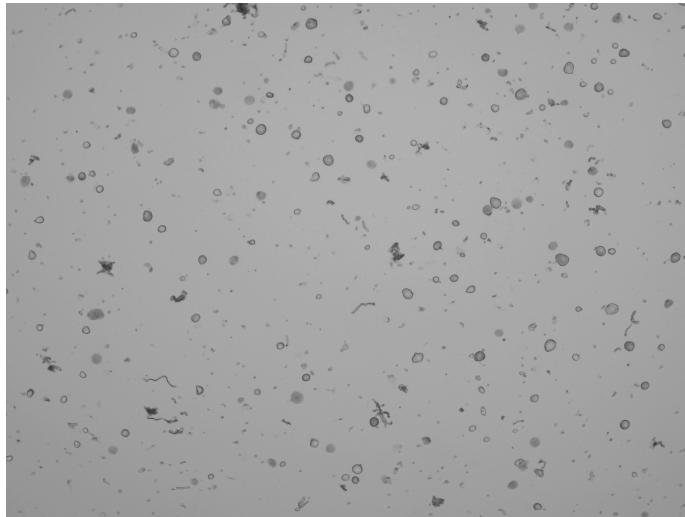
**Carrie Finno**

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](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/>

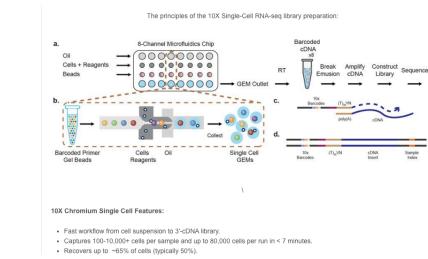
## Single Cell Expression Profiling & Genomics (10X Genomics, Fluidigm & Plate-based scRNA-Seq)

### 10X Genomics Single Cell Sequencing

Please schedule any 10X single cell experiment at least a week in advance. We highly advise a consultation prior to experiment scheduling.

Update March 2019: MULTI-seq reagents promise to enable the labeling and pooling of up to 96 cell suspension samples. Reagents are in stock. Please inquire!

The 10X Genomics Single Cell system is the **single-cell expression profiling** platform enabling the analysis of large cell numbers at the highest capture efficiency (of up to 65%). The technology allows for high-throughput single cell transcriptomics of many different cell types as well as **single-nuclei expression profiling**. The flexible workflow encapsulates 500 to 20,000 cells or nuclei per library together with micro-beads into nano-droplets. Each bead is loaded with adapters containing one of 750,000 different barcodes for the single cell RNA-seq library prep. In contrast to other protocols (e.g. Drop-Seq) the 10X controller is capable of loading "all" droplets with micro-beads, enabling single-Poisson distribution loading and thus high capture efficiencies (in contrast to double-Poisson loading of other protocols). The single-cell encapsulating process is significantly faster compared to *inDrop* or *Drop-Seq*. Up to eight samples can be processed per batch within minutes. The resulting data can be analyzed with the free *Cell Ranger* and *Loupe Cell Browser* software. In addition the **Bioinformatics Core** has developed a custom single-cell data analysis pipeline for 10X data.



# 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).

# Acknowledgements

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

