

A PRACTICAL GUIDE FOR CORES AND THE RESEARCHERS THEY SERVE

SINGLE CELL SEQUENCING: FROM SAMPLE PREP TO ANALYSIS

October 1, 2026

Why Single Cell?

Bulk vs. scRNA-seq: A Difference of Resolution

THE SMOOTHIE IS A USEFUL ANALOGY FOR UNDERSTANDING THE DIFFERENCE

Bulk RNA



scRNA-seq



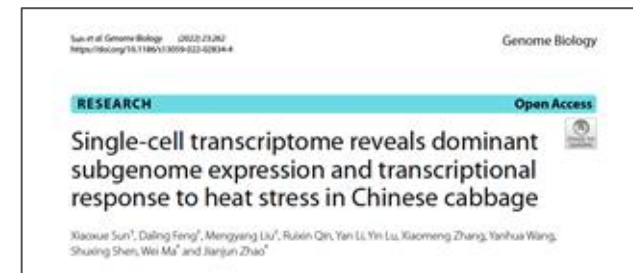
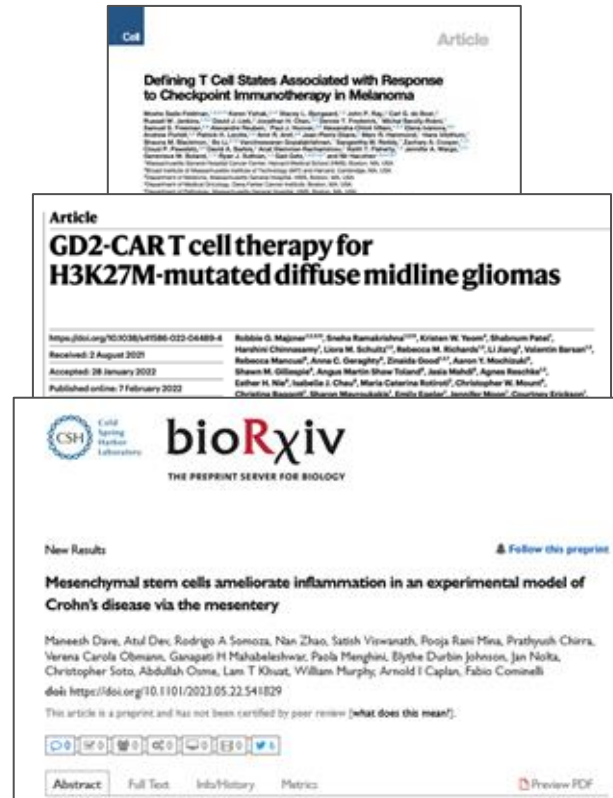
Why Perform scRNA-seq?

Characterizing tissue heterogeneity

Mapping Drug Response and Mechanism of Action

Understanding Environmental Influences on Gene Expression

Enabling Time course Experiments



Cell Atlas Efforts



**HUMAN
CELL
ATLAS**



PLANT CELL ATLAS



Tabula Muris



FLY CELL ATLAS

Zebrahub

sequencing and imaging atlas
of zebrafish development

TAHOE

A Cell Atlas of Worm

The *C. elegans* transcriptome at single cell resolution



Single Cell Platforms

Single Cell Advancements

CELLS IN WELLS, DROPLET BASED, COMBINATORIAL BARCODING

Published: 22 July 2012

Full-length mRNA-Seq from single-cell levels of RNA and individual circulating tumor cells

Daniel Ramsköld, Shujun Luo, Yu-Chieh Wang, Robin Li, Qiaolin Deng, Omid R Faridani, Gregory A Daniels, Irina Khrebukova, Jeanne F Loring, Louise C Laurent, Gary R Schoth & Rickard Sandberg

Nature Biotechnology 30, 777–782 (2012) | [Cite this article](#)

50k Accesses | 1017 Citations | 84 Altmetric | [Metrics](#)

An [Author Correction](#) to this article was published on 03 Feb 2013

Abstract

Genome-wide transcriptome analyses are routinely used to type-specific gene expression, but it has been technically challenging to profile from single cells. Here we describe a robust mRNA-Seq applicable down to single cell levels. Compared with existing improved read coverage across transcripts, which enhances transcript isoforms and identification of single-nucleotide polymorphisms, the sensitivity and quantitative accuracy of Smart-Seq for single-cell evaluation it on total RNA dilution series. We found that although from single cells have increased noise, hundreds of different genes identified using few cells per cell type. Applying Smart-Seq to melanomas, we identified distinct gene expression patterns for melanoma circulating tumor cells. Our protocol will be useful for biological problems requiring genome-wide transcriptome

> Cell. 2015 May 21;161(5):1202–1214. doi: 10.1016/j.cell.2015.05.002.

Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets

Evan Z Macosko¹, Anindita Basu², Rahul Satija³, James Nemesh⁴, Karthik Shekhar⁵, Melissa Goldman⁶, Itay Tirosh⁵, Allison R Bialas⁷, Helen Kimble⁴, Emily M Hartenstein⁸, John J Trombetta⁵, David A Weitz⁹, Joshua R Sanborn¹⁰, Steven A McCarroll¹²

Affiliations + expand

PMID: 26000488 PMCID: [PMC4481139](#) DOI: [10.1016/j.cell.2015.05.002](#)

[Free PMC article](#)

Abstract

Cells, the basic units of biological structure and function, are the focus of genomics can characterize cell identity and function, but its broad application. Here we describe Droplet-based single-cell RNA sequencing (DRS) that profiles thousands of individual cells by separating them into droplets, each with a different barcode with each cell's RNAs, and sequencing mRNA transcripts from thousands of individual cells. We analyzed transcriptomes from 44,800 transcriptionally distinct cell populations, creating a map of retinal cell classes and novel candidate cell subtypes. This method enables routine transcriptional profiling at single-cell resolution.

RESEARCH

SINGLE-CELL GENOMICS

Single-cell profiling of the developing mouse brain and spinal cord with split-pool barcoding

Alexander B. Rosenberg,^{1,*} Charles M. Roco,^{2,*} Richard A. Muscat,¹ Anna Kuchina,¹ Paul Sample,¹ Zizhen Yao,³ Lucas T. Graybuck,³ David J. Peeler,² Sumit Mukherjee,¹ Wei Chen,⁴ Suzie H. Pun,² Drew L. Sellers,^{2,5} Bosiljka Tasic,³ Georg Seelig^{1,4,6}†

To facilitate scalable profiling of single cells, we developed split-pool ligation-based transcriptome sequencing (SPLiT-seq), a single-cell RNA-seq (scRNA-seq) method that labels the cellular origin of RNA through combinatorial barcoding. SPLiT-seq is compatible with fixed cells or nuclei, allows efficient sample multiplexing, and requires no customized equipment. We used SPLiT-seq to analyze 156,049 single-nucleus transcriptomes from postnatal day 2 and 11 mouse brains and spinal cords. More than 100 cell types were identified, with gene expression patterns corresponding to cellular function, regional specificity, and stage of differentiation. Pseudotime analysis revealed transcriptional programs driving four developmental lineages, providing a snapshot of early postnatal development in the murine central nervous system. SPLiT-seq provides a path toward comprehensive single-cell transcriptomic analysis of other similarly complex multicellular systems.

Technology Choices Impact Scale

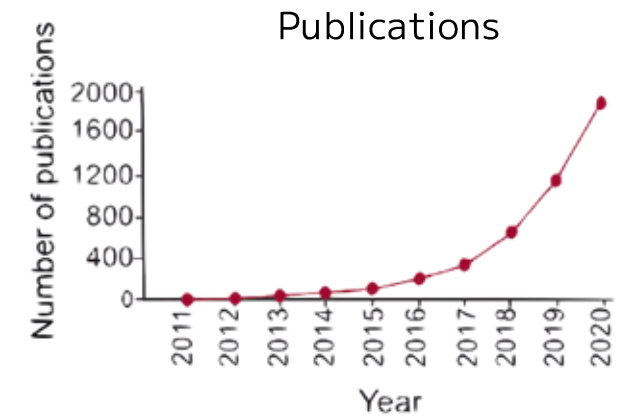
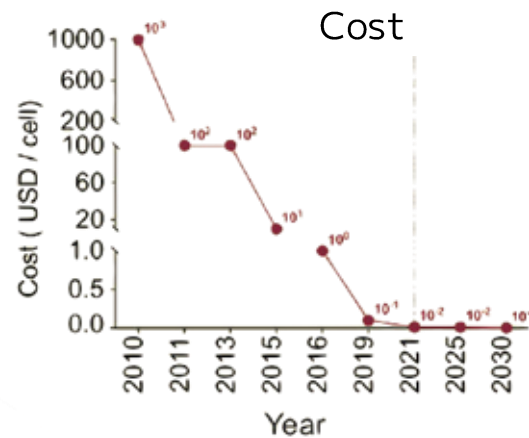
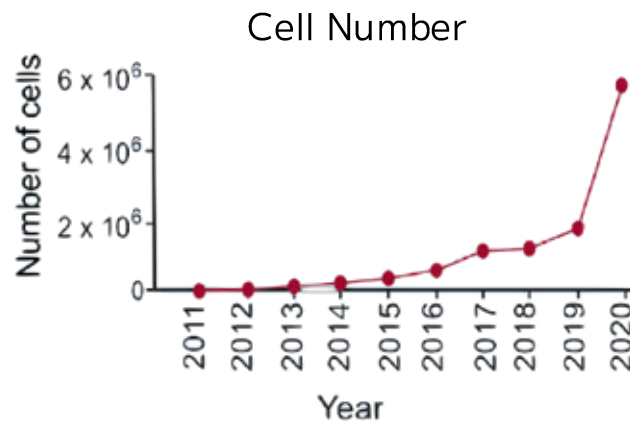
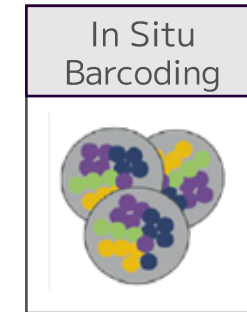
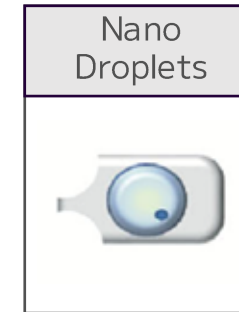
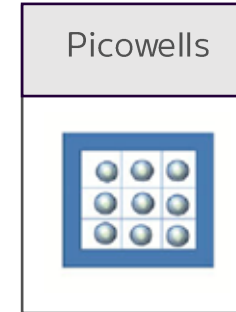
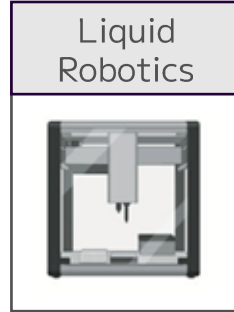
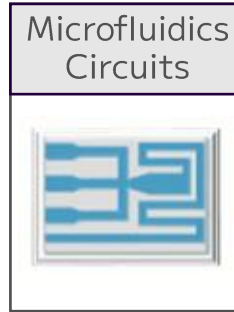
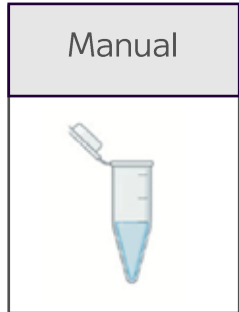


Image adapted from <https://onlinelibrary.wiley.com/doi/10.1002/ctm2.694>

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Experimental Design Considerations

Include Core in Experimental Design Discussion

Cores can provide guidance on:

- QC metrics
- Concentrations and viability
- Sample prep images, pre- and post-fixation
- Counting aliquots
- Core's bandwidth for taking on project



Experimental Design: Fixed vs Fresh Samples

	Fresh	Fixed
Clinical samples/large animal experiments	Complicated timing	Fix and store samples
Removal of time constraints	No	Yes
High throughput	Platform dependent	Yes - multiplex large number of samples
Reduced technical variation	No. Can create batch effects	Yes. Process all samples in one day

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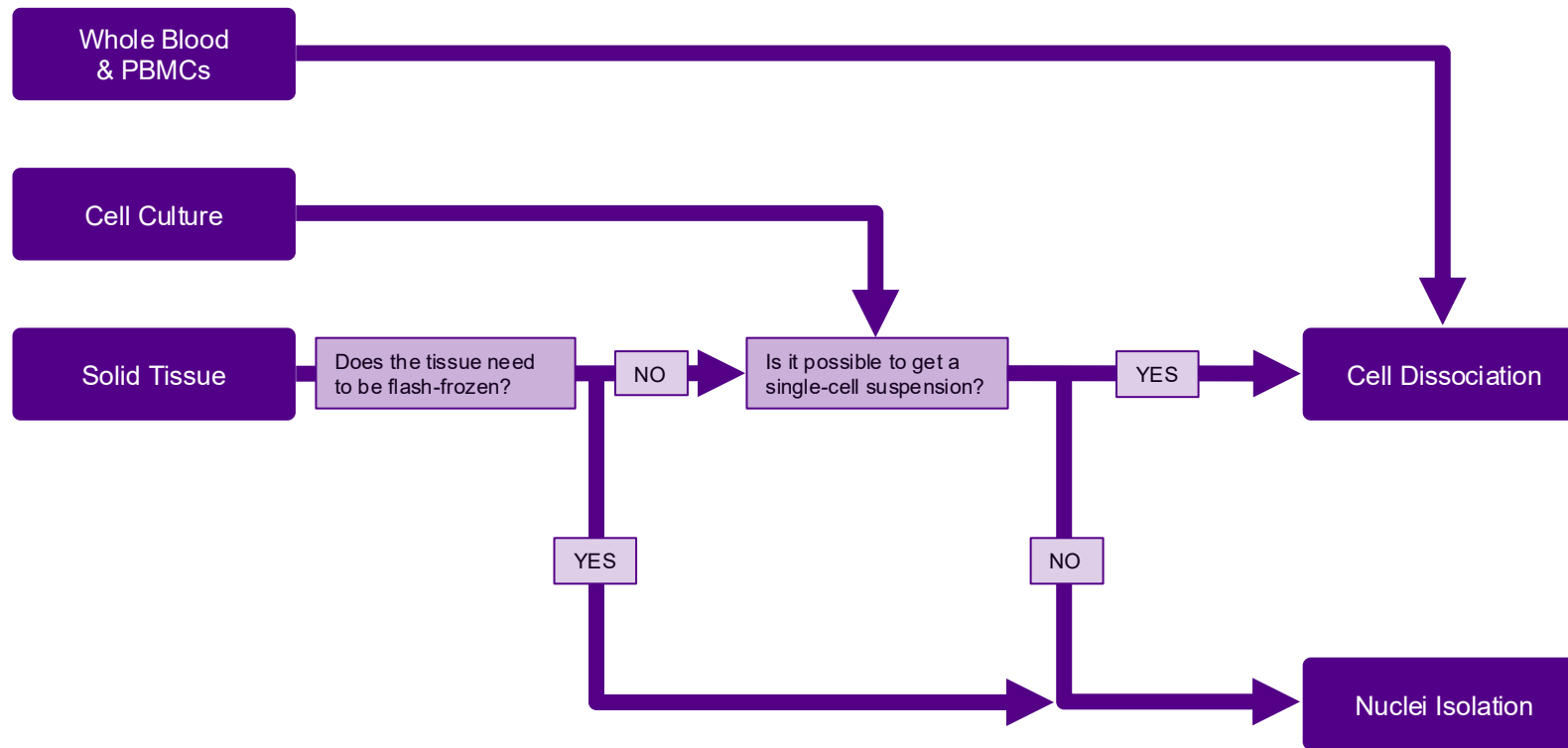
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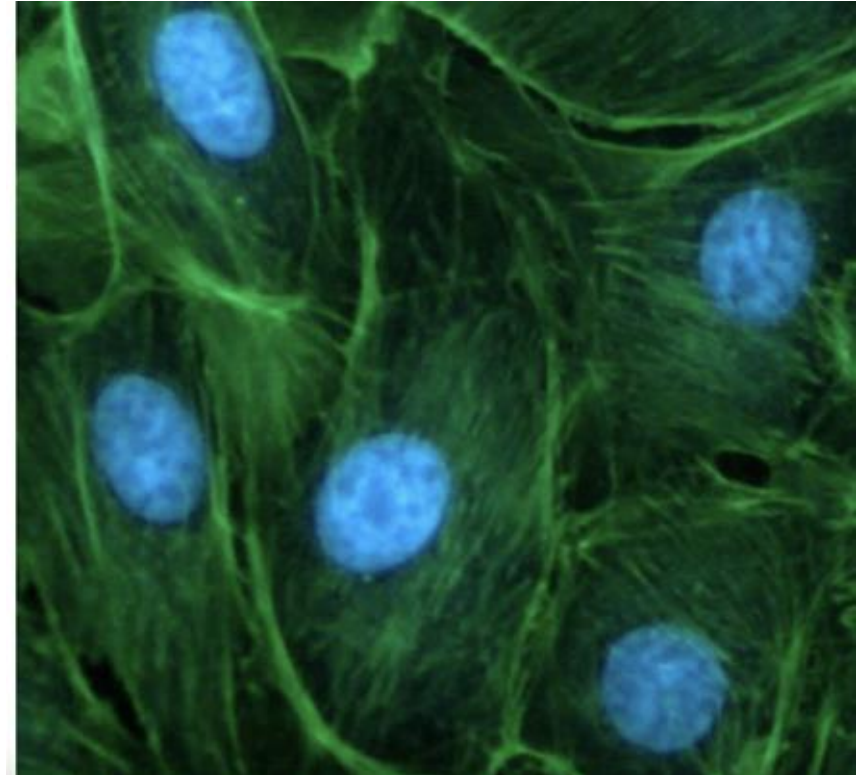
Experimental Design: Cells or Nuclei?



Experimental Design: Reasons to Use Nuclei

Bovine Pulmonary Arterial Cells

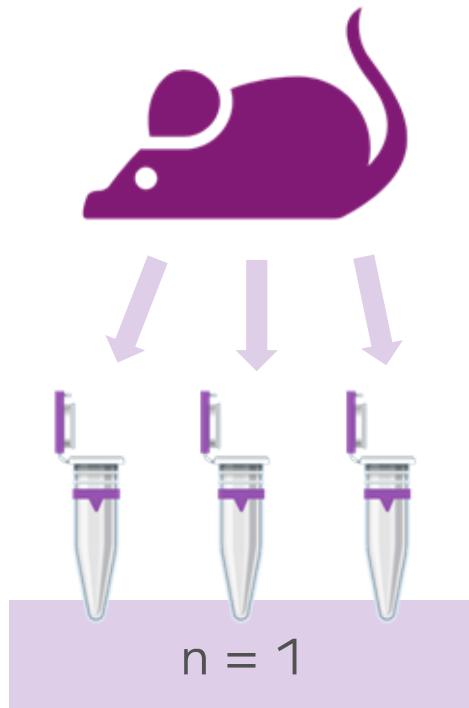
- Dissociation is difficult
- Viability is low
- When you have flash-frozen tissues



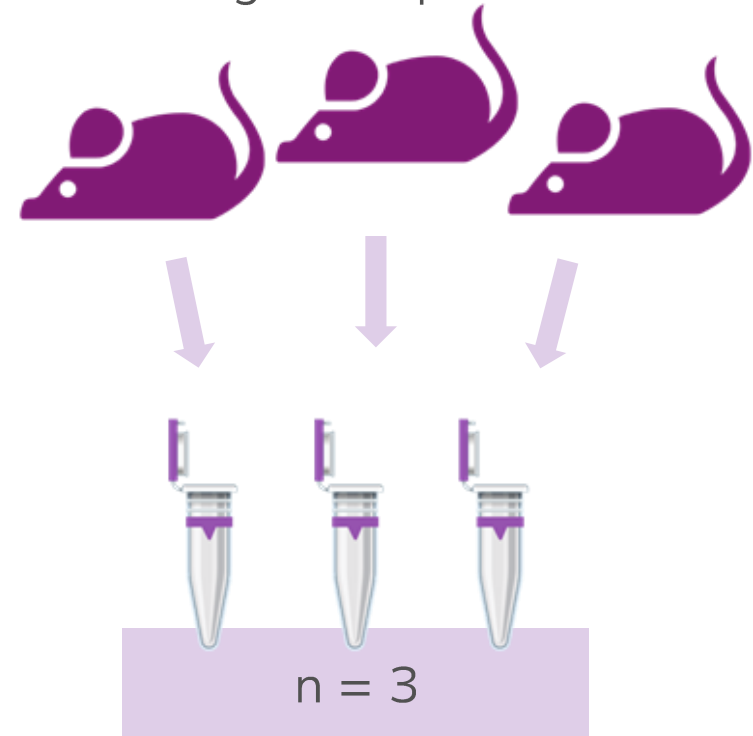
<https://www.microscopyu.com/gallery-images/bovine-pulmonary-artery-cell-nuclei-3>

Experimental Design: Biological vs. Technical Replicates

Technical Replicates

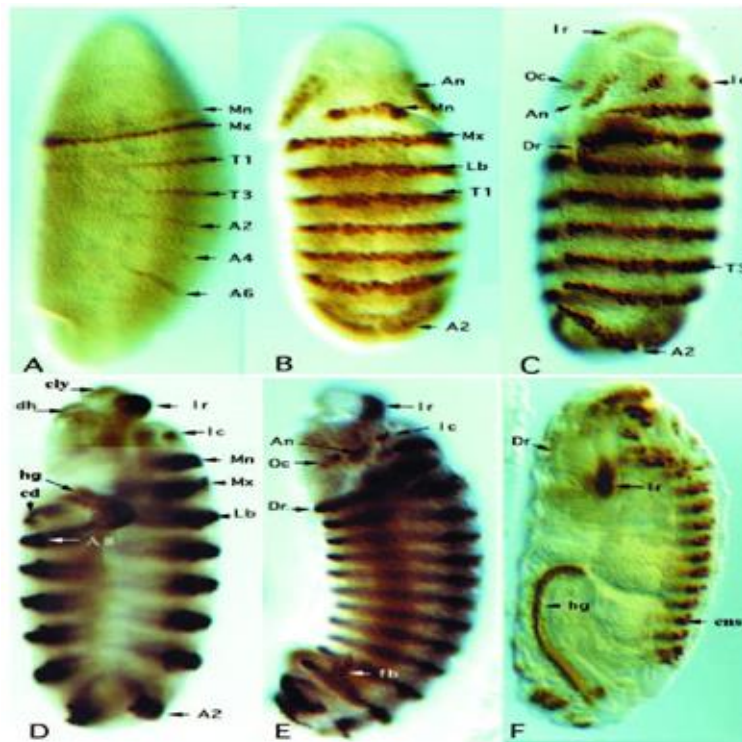


Biological Replicates



Experimental Design: Working with Low-input, Precious Samples

Consider pooling samples from small tissues, embryonic, or unique organisms



https://www.researchgate.net/publication/14256499_Structure_of_the_insect_head_as_revealed_by_the_EN_protein_pattern_in_developing_embryos



https://en.wikipedia.org/wiki/BALB/c#/media/File:Lightmatter_lab_mice.jpg

Variables of Sample Preparation



Cell
morphology



Cell
viability



Time



Temperature

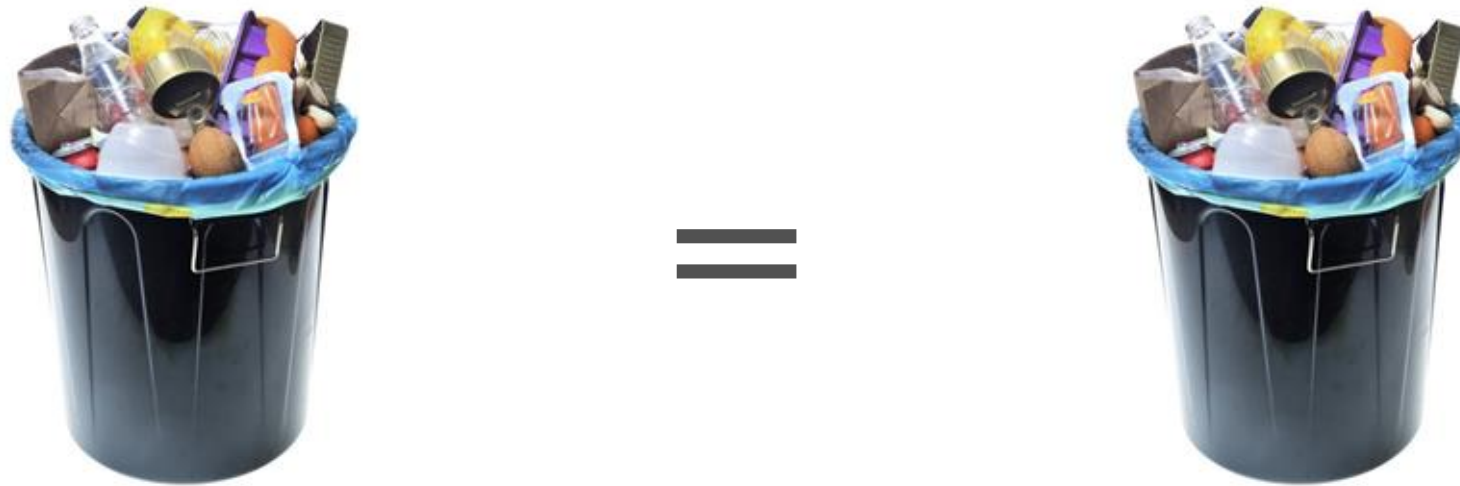


Aggregation
and Debris



Cell
counting

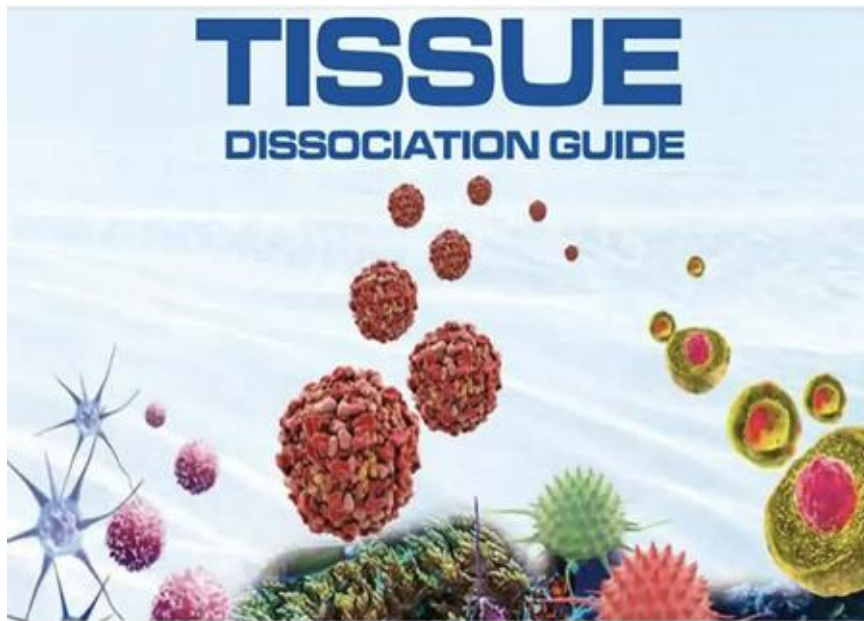
Poor Quality Input Leads to Poor Quality Output



Enzymatic Tissue Dissociation

Your Core can be an excellent resource (Equipment, Knowledge-base)

Every tissue dissociation protocol is a specific recipe, protocol, and method



<https://www.technologynetworks.com/cell-science/how-to-guides/tissue-dissociation-guide-334918>

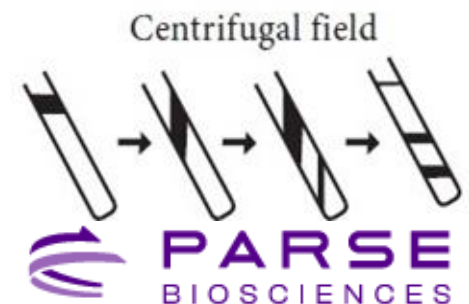
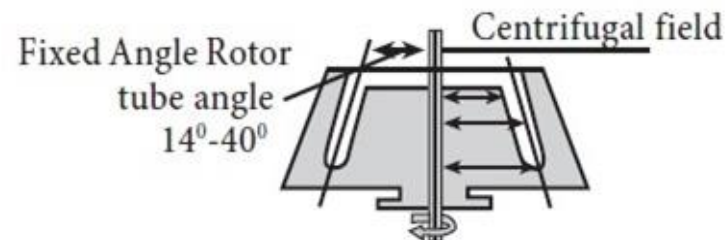
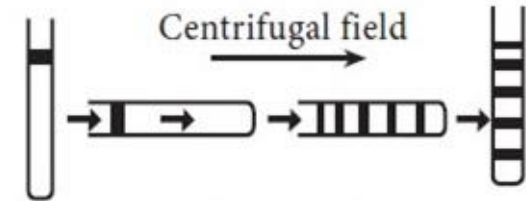
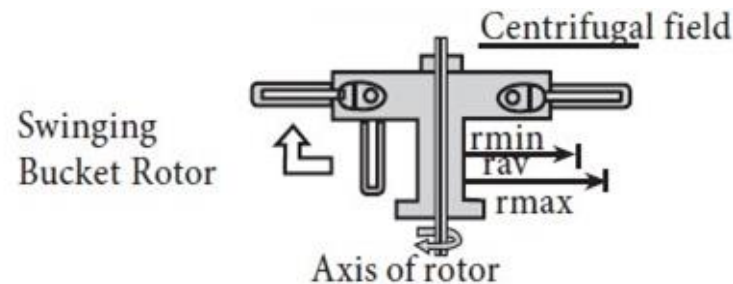
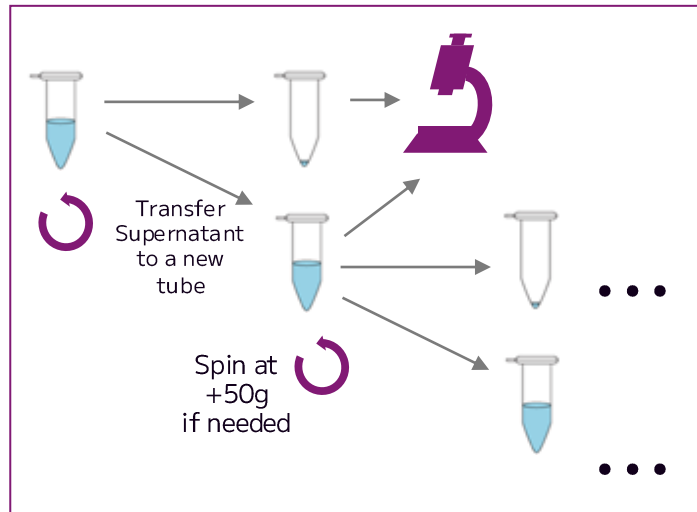


<https://www.miltenyibiotec.com/US-en/products/gentlemacs-octo-dissociator-with-heaters.html> and <https://s2genomics.com/singulator-platform/>

Optimize Centrifugation Speed and Time

- Optimize spin speeds to maximize cell retention: 200-500g, 4°C, 5-10 min
- Smaller cells or nuclei may require higher speeds than larger cells
- Save supernatant and check with hemocytometer
- Use your optimized spin speed throughout workflow

Swinging bucket rotor, 4°C
NOT a fixed angle rotor



Cell Viability is Essential

Desired sample viability >70%, Ideally >90%

- Maintain samples on ice
- Handle and pipette gently
- Proceed into fixation workflow as quickly as possible

Morphology visualization and viability are best calculated with a fluorescent dye

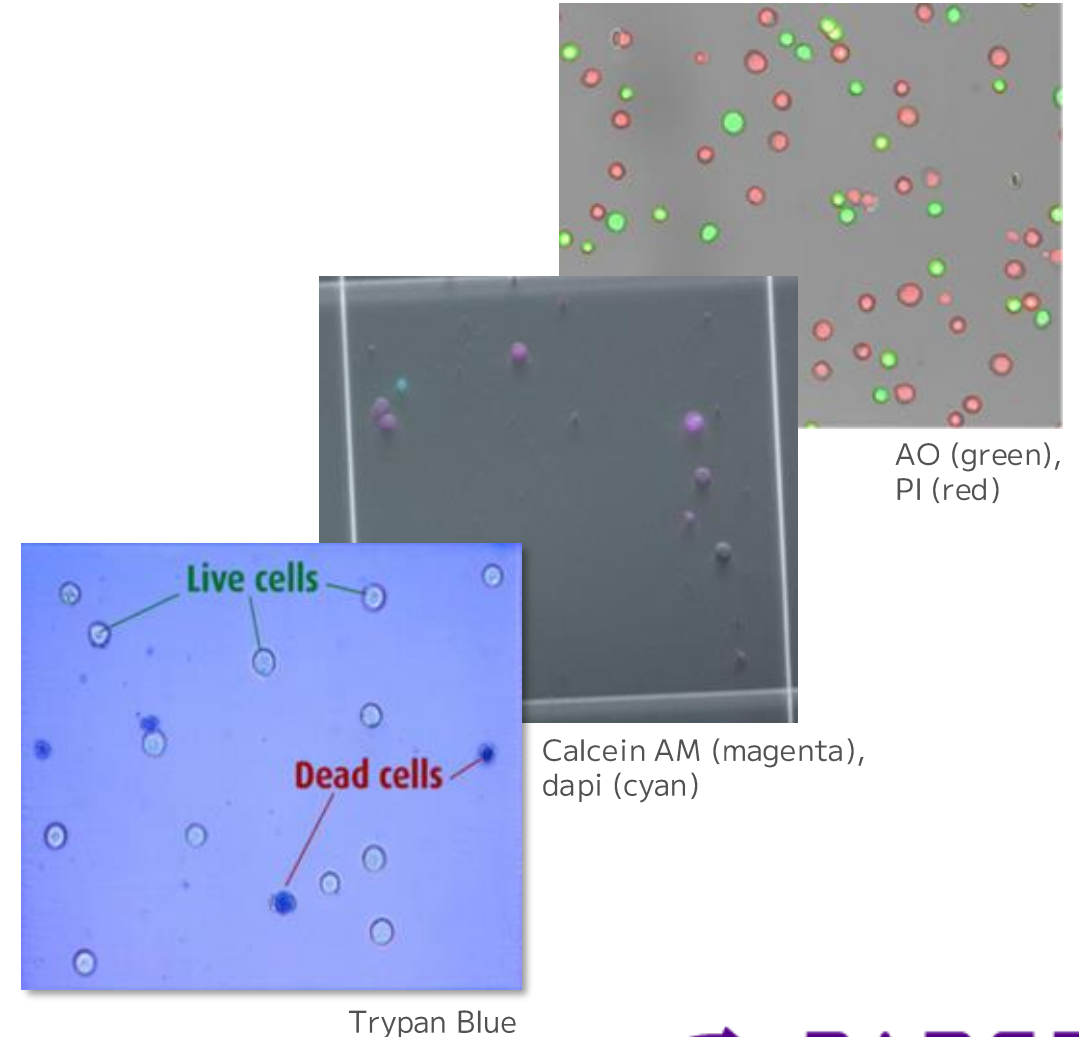
- AOPI, Calcein AM, eFluor780, Sytox, etc.

Commercial dead cell removal kits

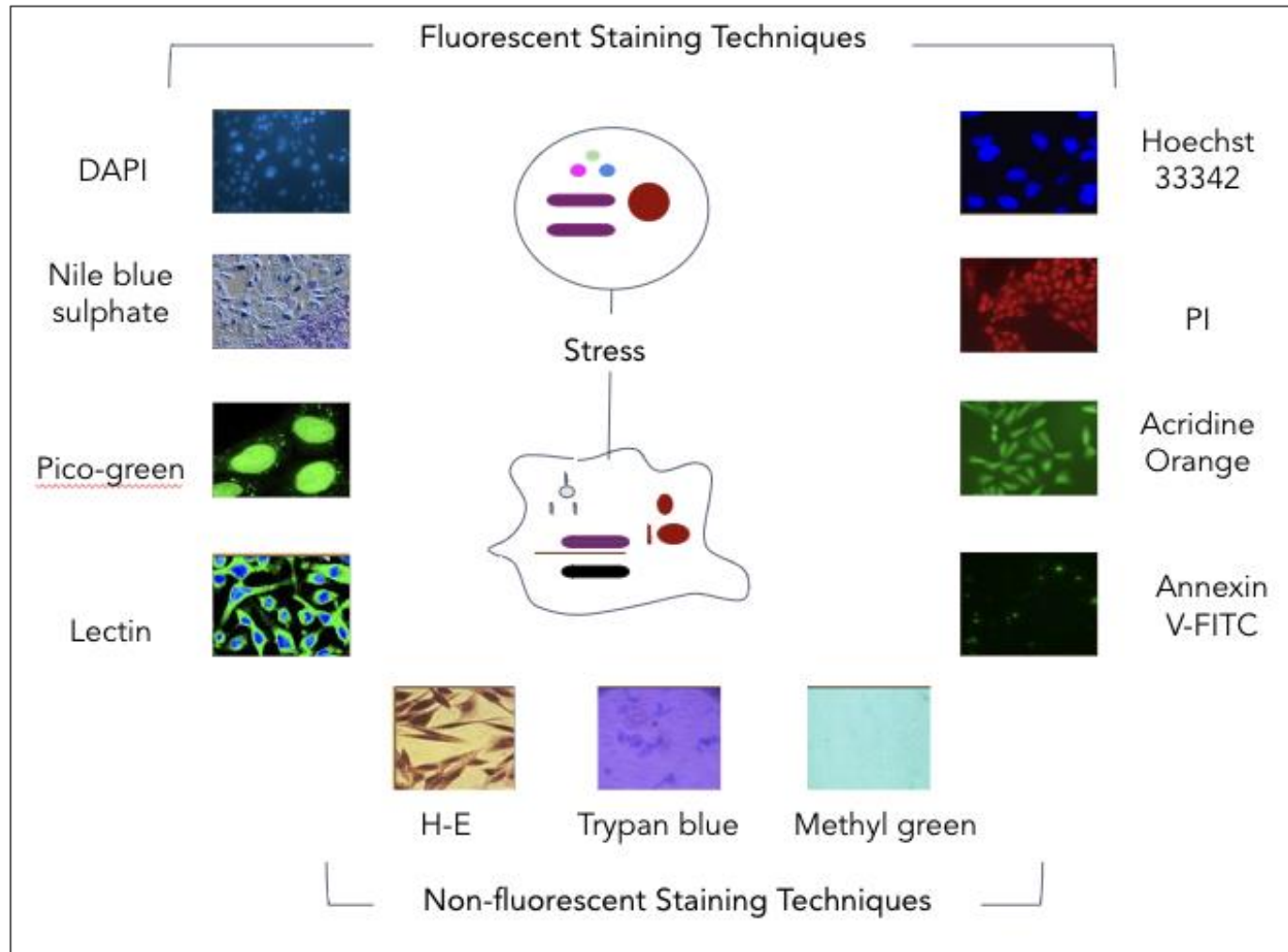
- StemCell EasySep Dead Cell Removal – PN 17899
- Miltenyi Dead Cell Removal – PN 130-090-101

FACS using viability markers

Sytox, DAPI, Calcein AM, AO/PI, 7AAD



Tools for Assessing Cell Membrane Integrity

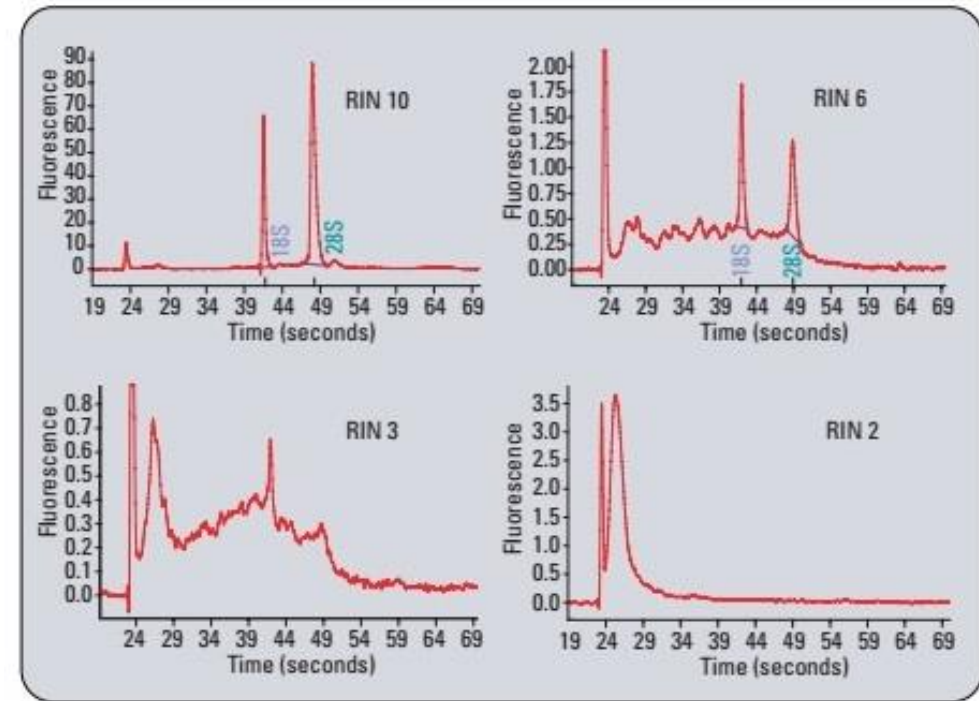


Poor cell membrane integrity can be directly related to poor scRNA-seq results

Image adapted from Cell-death assessment by fluorescent and nonfluorescent cytosolic and nuclear staining techniques <https://doi.org/10.1111/jmi.12133>

RNA Integrity Number Quality Check

- Core Instruments may be needed
- RIN score runs from 1-10
- Can be performed on cells or tissue
- Higher RIN score = better data
 - Denotes good quality RNA
 - Will yield good quality data!

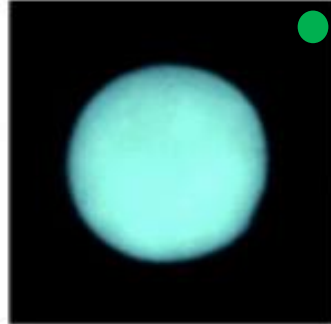


<https://blog.genohub.com>

Nuclei Integrity QC

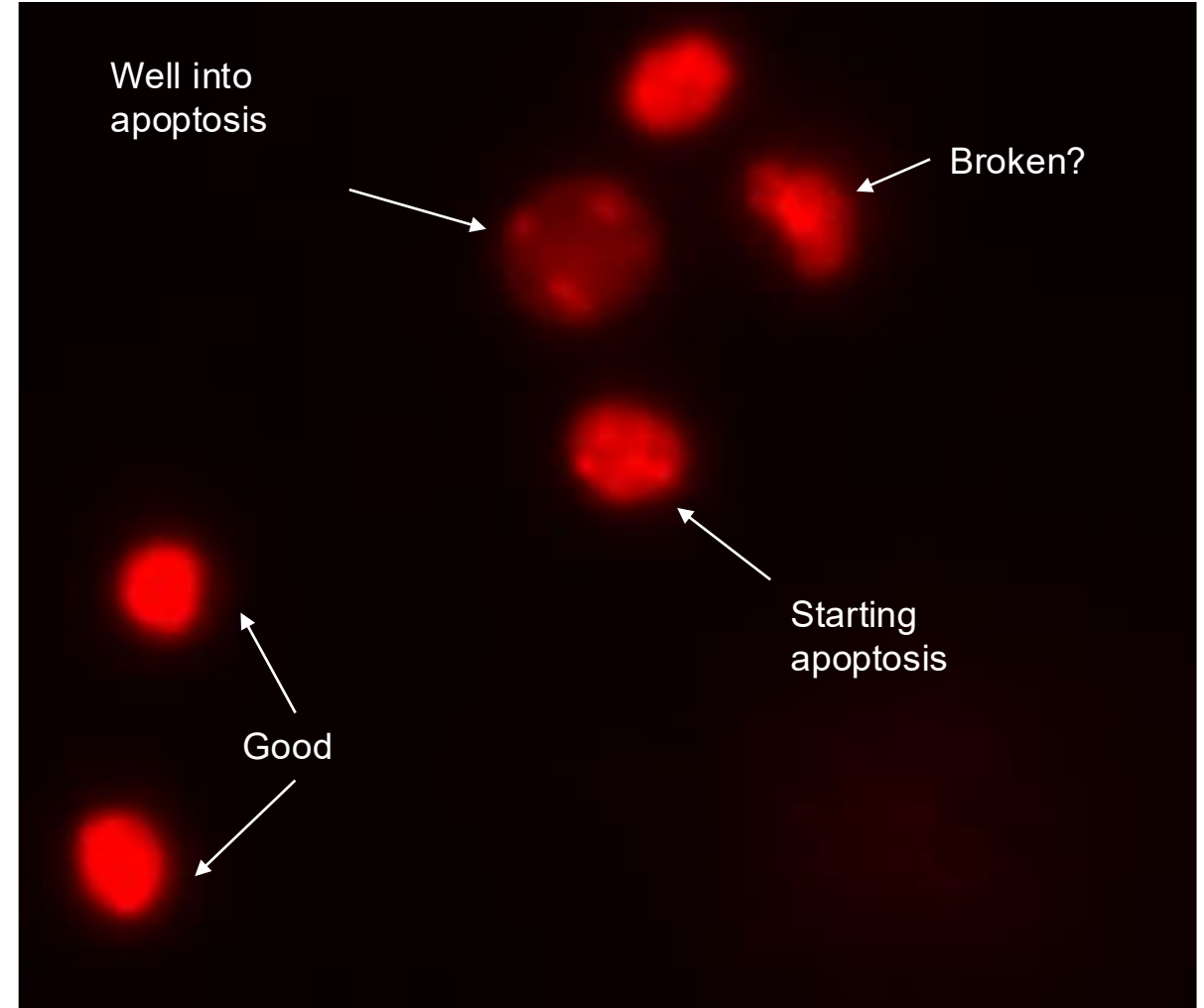
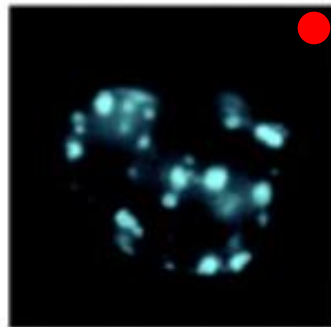
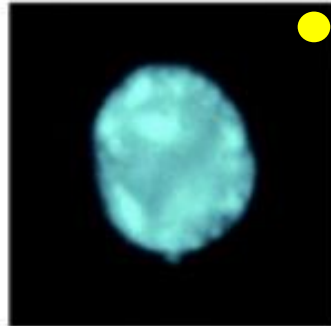
Happy nuclei

- Round
- Smooth borders
- Uniform staining



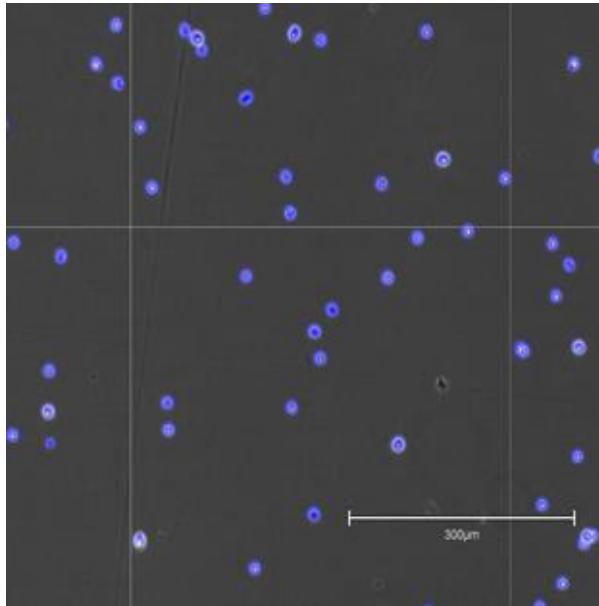
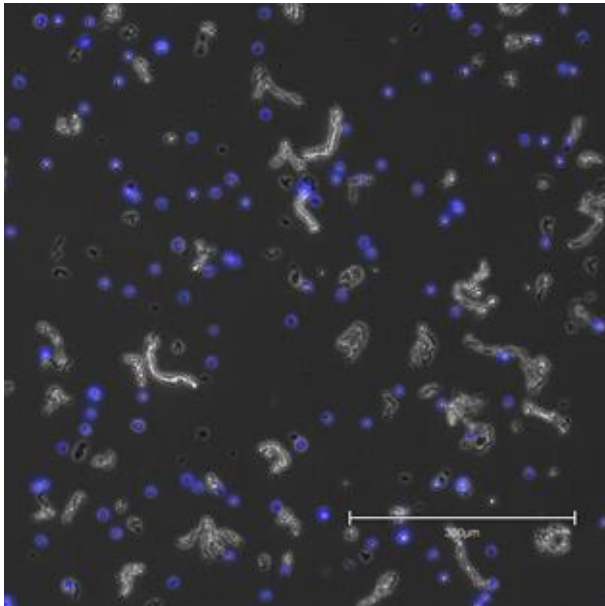
Unhappy Nuclei

- Blebbing
- Irregular shape
- Chromatin condensation
- Leaky nucleic acids

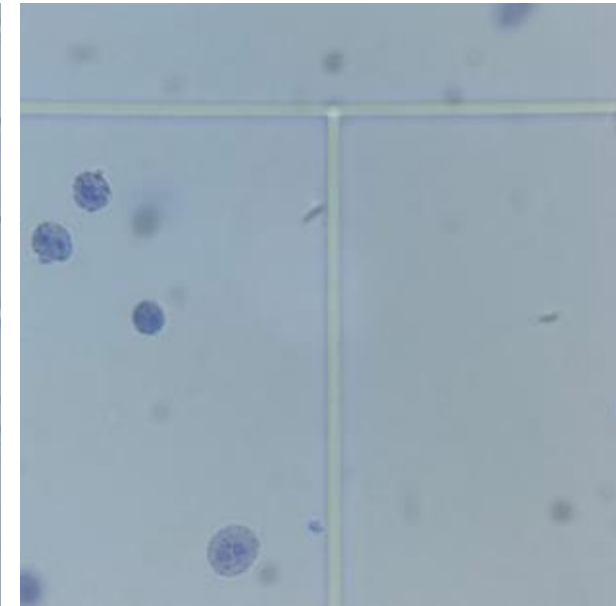
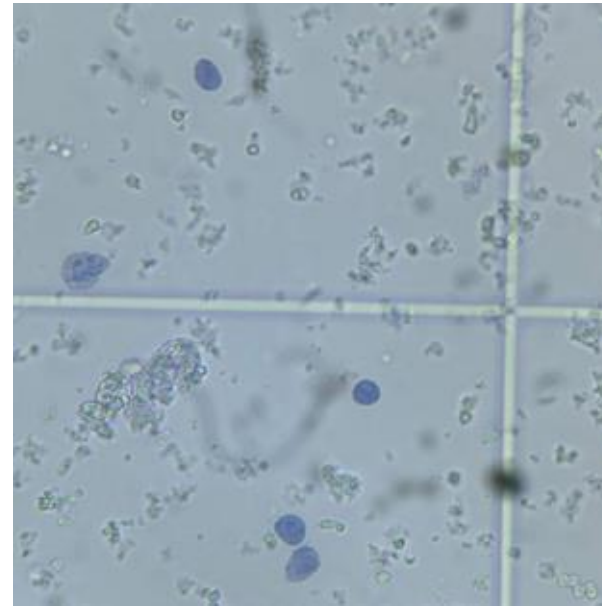


Nuclei Debris Cleanup

- Debris removal can help prevent clumping
- Nuclei prep from mouse brain tissue
- Multiple methods for nuclei cleanup:
 - Gradient centrifugation (optiprep/iodixanol, sucrose)
 - Anti-Nucleus Beads



- Sigma-Aldrich OPTIPREP PN# D1556
- dapi stained

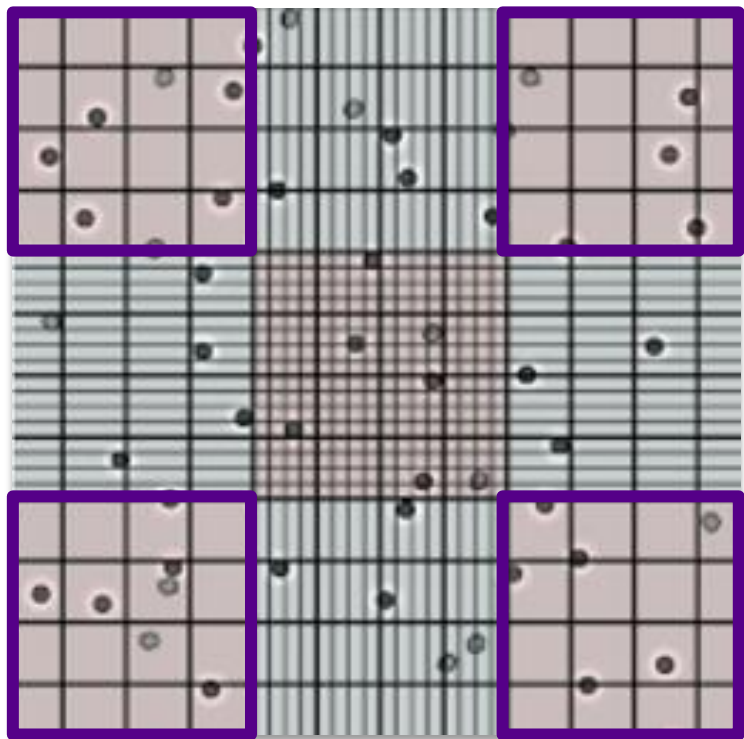


- Miltenyi Anti-Nucleus MicroBeads PN# 130-132-997
- trypan stained

How to get Accurate Cell Counts

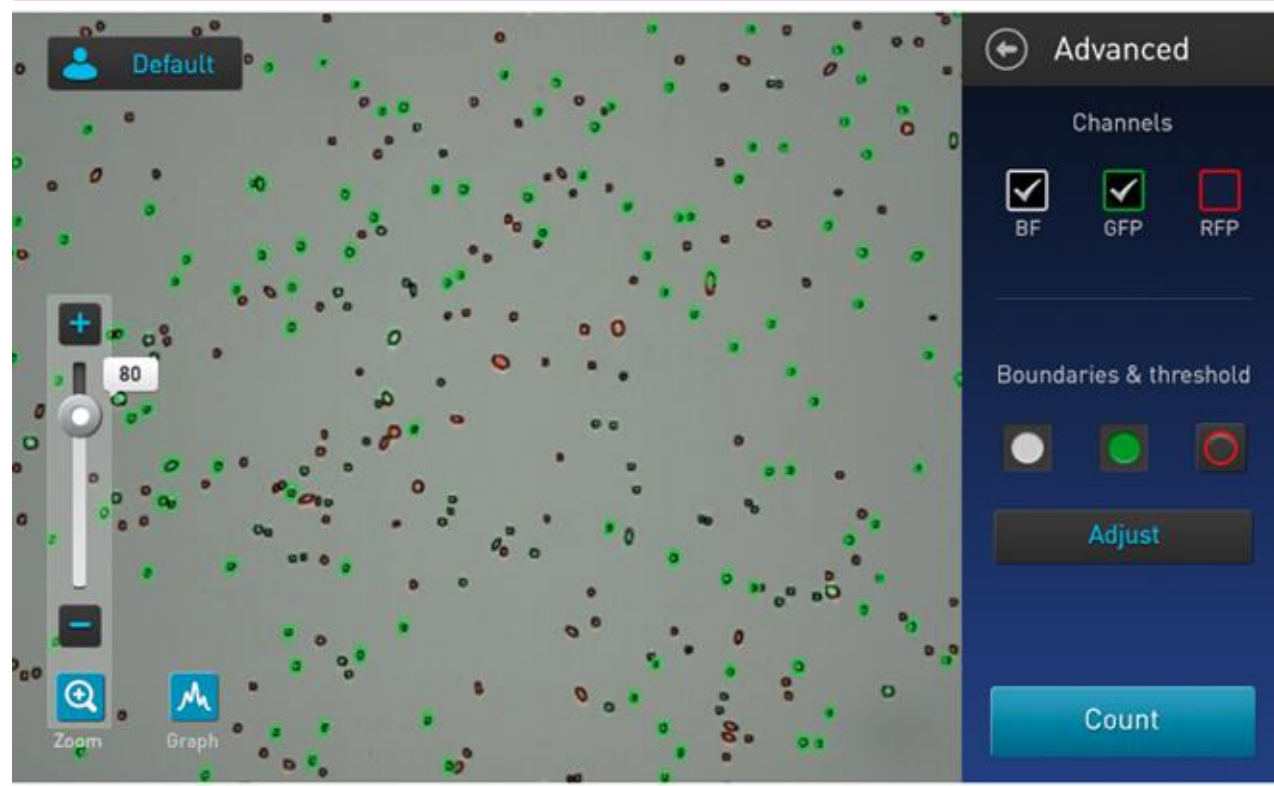
Cell count accuracy is key to success!

- Count all 4 quadrants of each of the 2 grids
- Ensure cell/nuclei are within concentration range
- Only use the viable cell count for input
- After fix you are counting "dead cells"



Ensuring Precision with an Automated Cell Counter:

- Follow manufacturer's instructions to calibrate.
- Validate automated cell counter accuracy by comparing results with a hemocytometer.



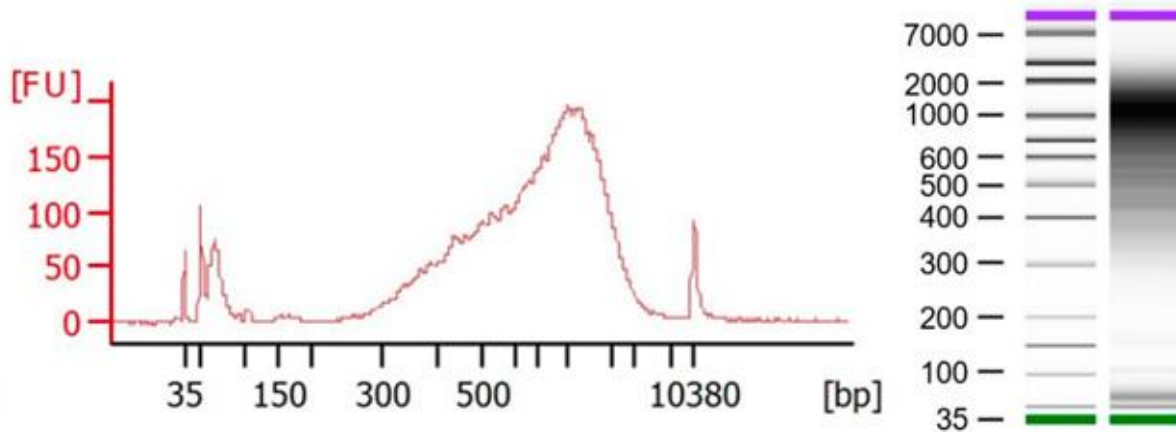
Assay QC Metrics

Work with the Core on Their Procedures for
Generating Vital Assay QCs

cDNA Amplification QC Traces

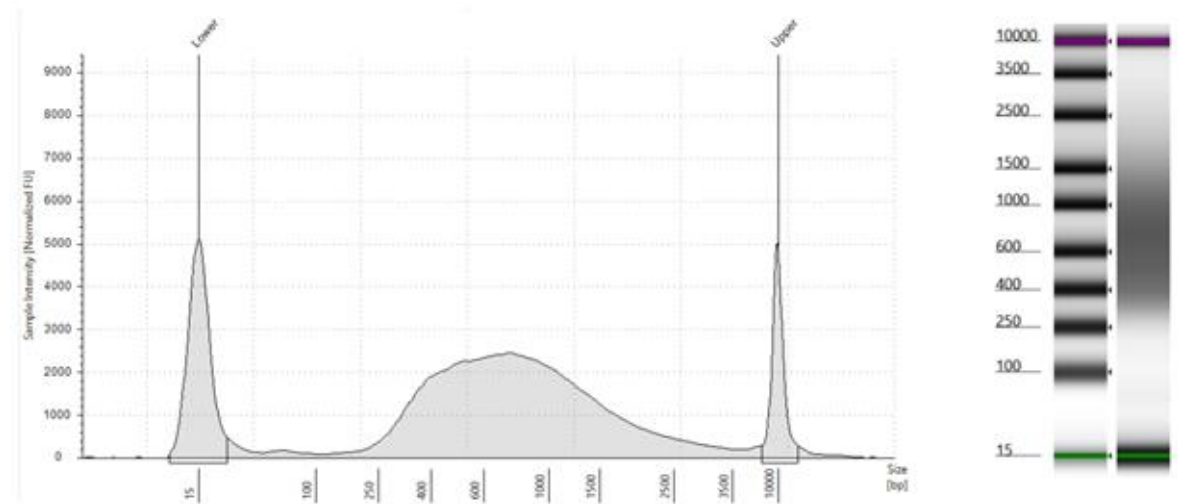
BioAnalyzer

Yield	Mean Peak
> 0.5 ng/uL	~1200bp



TapeStation

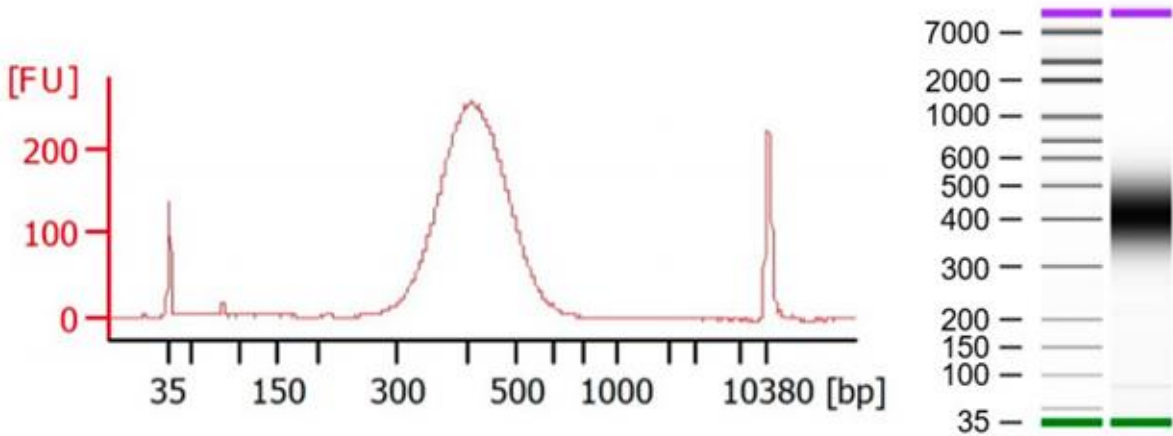
Yield	Mean Peak
> 0.5 ng/uL	~500-800bp



Final Library QC Evaluation

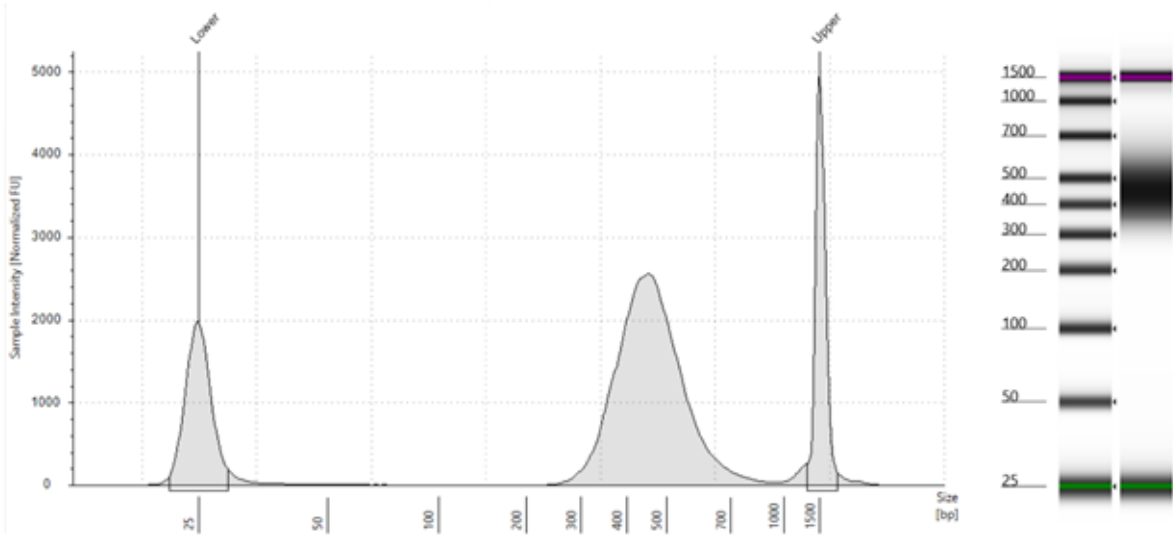
BioAnalyzer Trace

Yield	Mean Peak
> 5.0 ng/uL	~400-500bp



TapeStation Trace

Yield	Mean Peak
> 5.0 ng/uL	~400-500bp



Utilizing Your Core for Assay QC Metrics

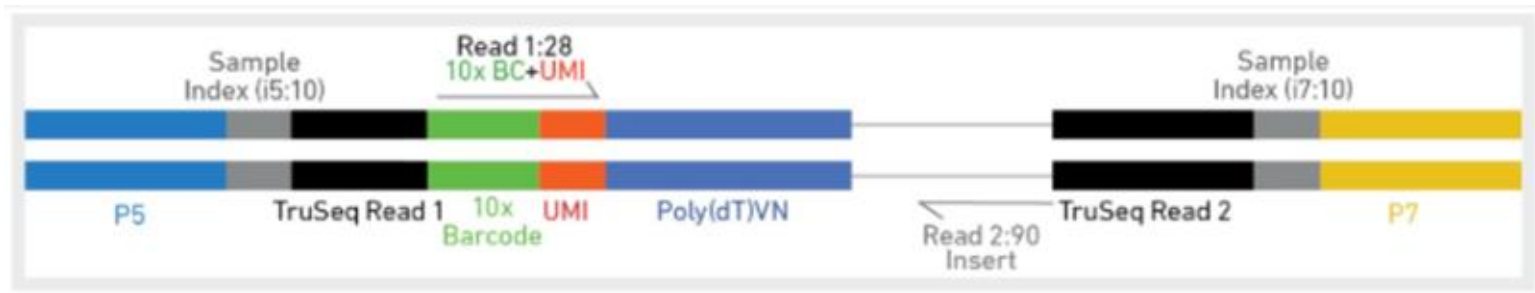
- Most researchers might not have needed equipment
- Reach out to cores to determine how to deliver samples for QC
 - μ L volumes, tube type
- Highlight assay type/specific needs
 - High Sensitivity? D1000/5000?



Sequencing

Working with the Core to Sequence your Single Cell Project

What to Communicate to the Core When Sequencing Your Project



- Number of reads per cell you are targeting
- Phi-X spike-in percentage
- Unique Dual Indices to demultiplex your run
- Read structure of library
- Number of cells in each library

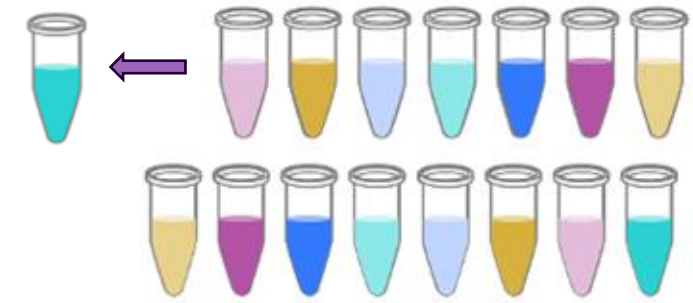
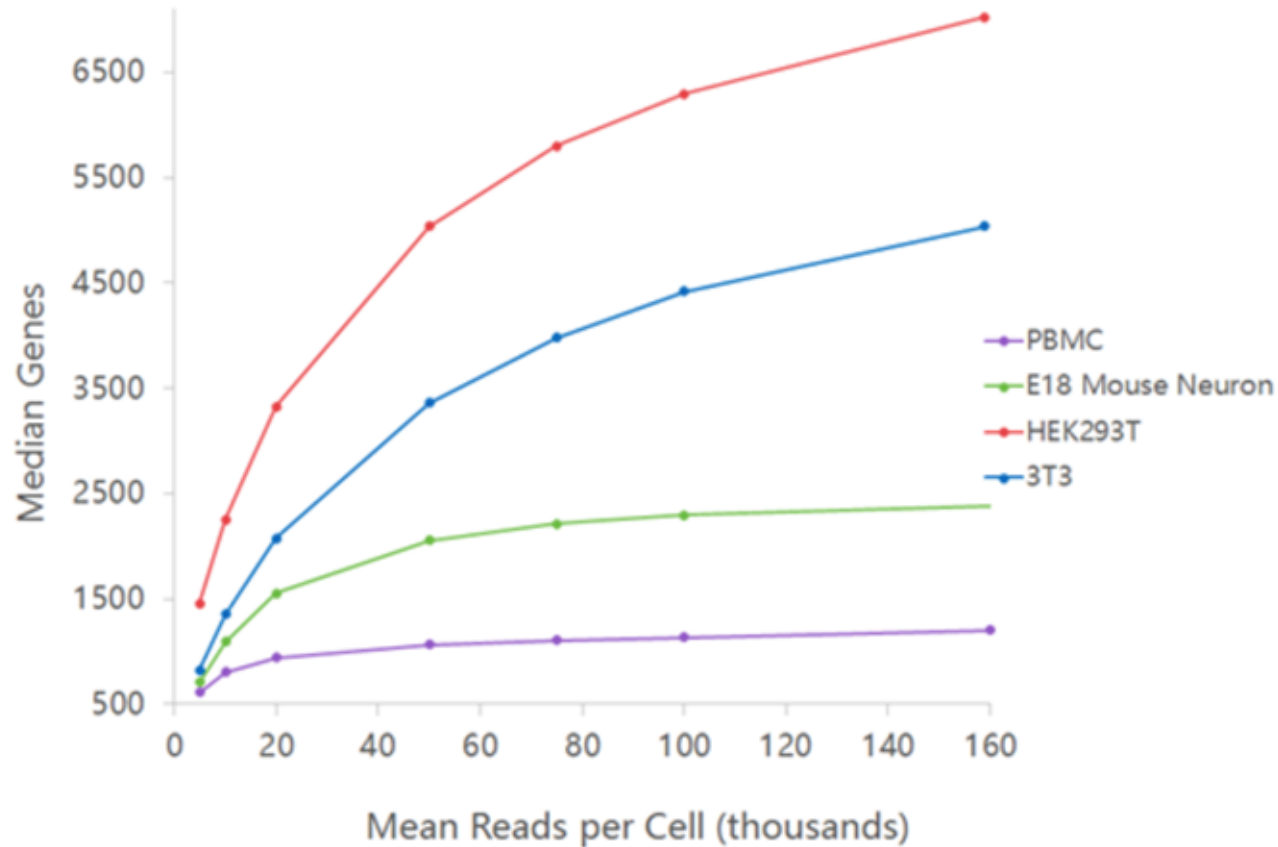
Flow Cell Considerations

SEEK GUIDANCE FROM YOUR SEQUENCING PROVIDER/CORE WHEN SEQUENCING YOUR PROJECT

Parse Kit	Samples Per Run (Cells)	Number of Cells Per Run at 20K Reads/Cell										
		MiSeq	NextSeq 550		NextSeq 2000		NovaSeq 6000			NovaSeq X		
		V3	Mid	High	P2	P3	S2	S4	S4 x2	1.5B	10B	25B
WT Mini	1 – 12 (10K)	1k	5k	10k	10k	-	-	-	-	-	-	-
WT	1 – 48 (100K)	-	-	20k	20k	50k	100k	-	-	80k	-	-
WT Mega	1 – 96 (1M)	-	-	-	-	-	200k	500k	1M	80k	500k	1M



Dialing in Sequencing

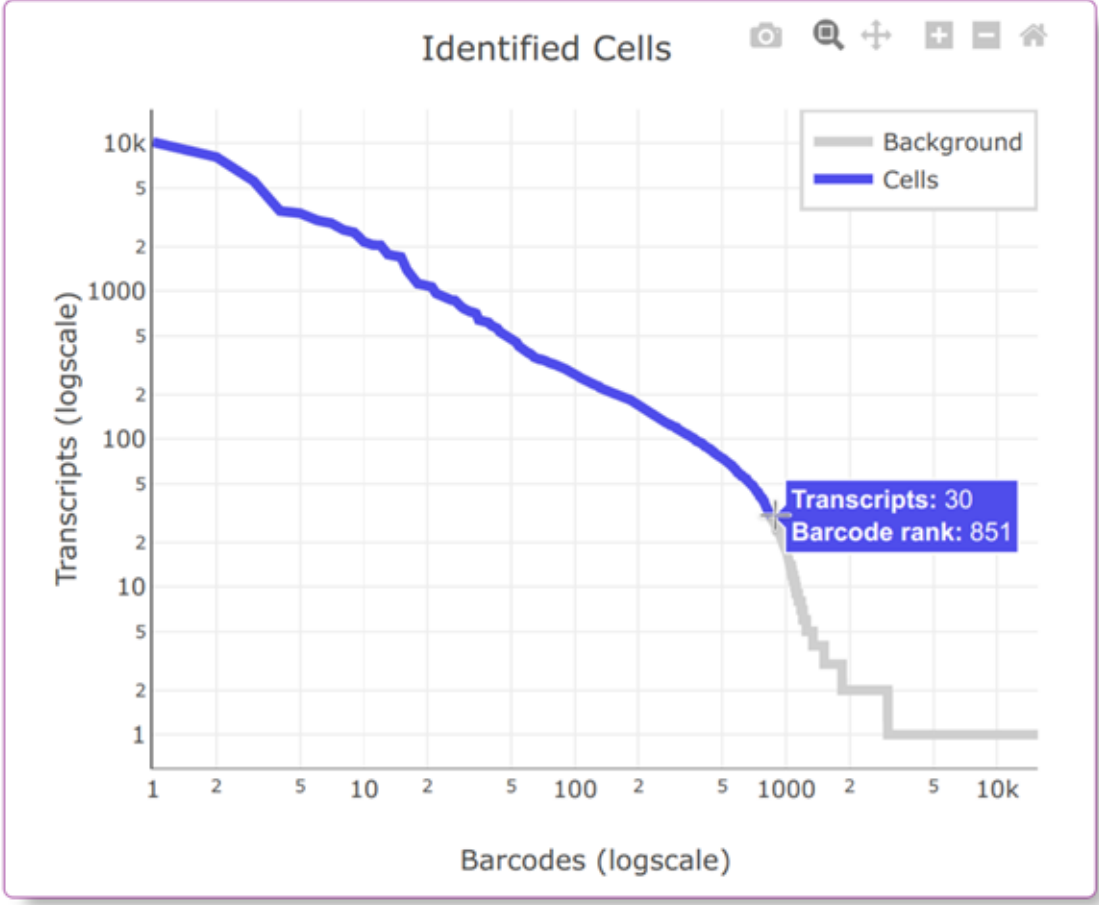
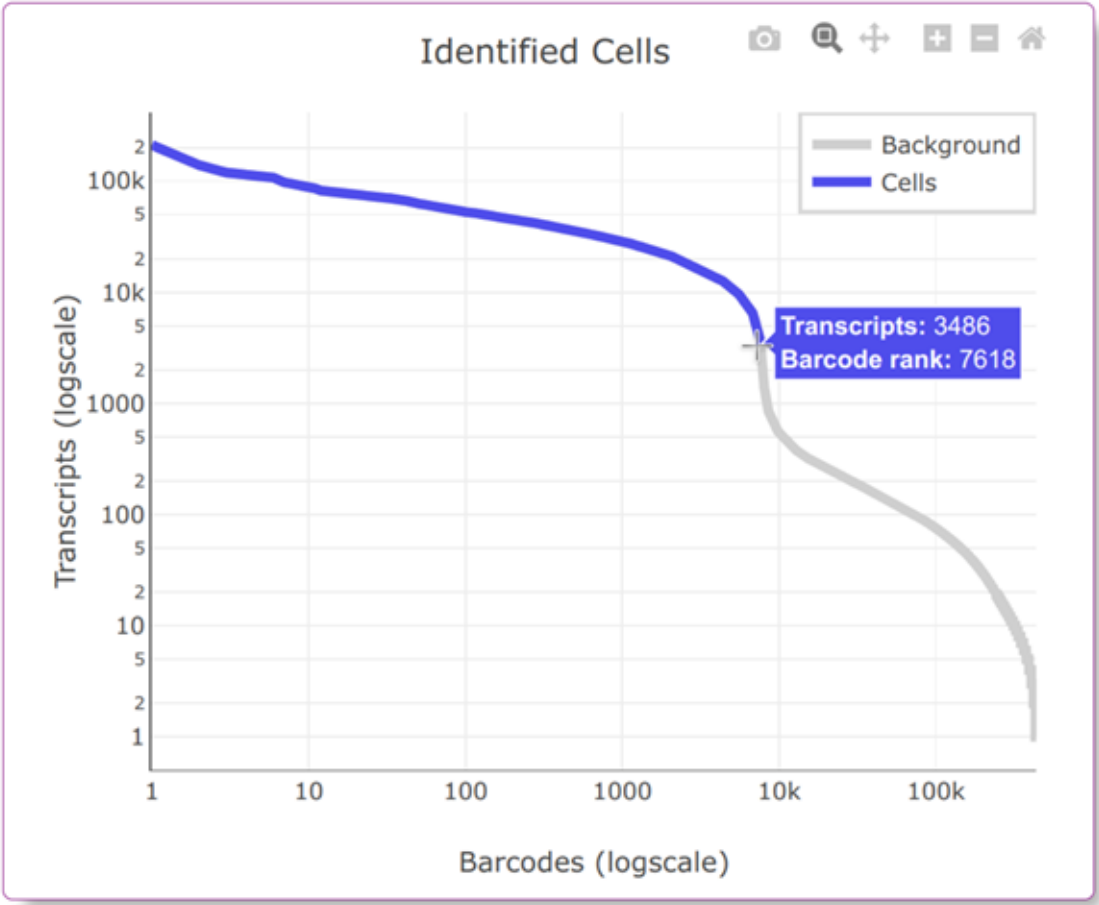


- Sequencing depth
- RNA amount by cell type
- Sample quantity metric

<https://kb.10xgenomics.com/hc/en-us/articles/115005062366-What-is-sequencing-saturation->

Post-Sequencing QC Metrics

Barcode Rank Plot: Cells vs. Ambient RNA



Cell cutoff threshold with distinct inflection point in curve

An excess of dead and damaged cells creates a smoothness to the curve

Experimental QC Metrics

Cell totals by sample

Gene/Transcript level

Sequencing saturation

Q30 Score

Transcriptome map fraction

STATISTIC	SAMPLE 1	SAMPLE 2	SAMPLE 3
number_of_cells	3313	3043	2877
GRCm39_median_tscp_per_cell	15493	17817	17257
GRCm39_median_genes_per_cell	4096	4387	4318
mean-reads_per_cell	59505	68179	65290
sequencing_saturation	0.3694	0.3691	0.3691
valid_barcode_fraction	0.8243	0.8243	0.8243
bc1_Q30	0.8652	0.8652	0.8653
bc2_Q30	0.8947	0.8947	0.8947
bc3_Q30	0.8866	0.8866	0.8866
cDNA_Q30	0.9159	0.9159	0.9159
transcriptome_map_fraction	0.7969	0.7969	0.7969

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sequencing_saturation	0.3694	0.3691	0.3691
valid_barcode_fraction	0.8243	0.8243	0.8243
bc1_Q30	0.8652	0.8652	0.8653
bc2_Q30	0.8947	0.8947	0.8947
bc3_Q30	0.8866	0.8866	0.8866
cDNA_Q30	0.9159	0.9159	0.9159
transcriptome_map_fraction	0.7969	0.7969	0.7969

Experimental QC Metrics

Cell totals by sample

Gene/Transcript level

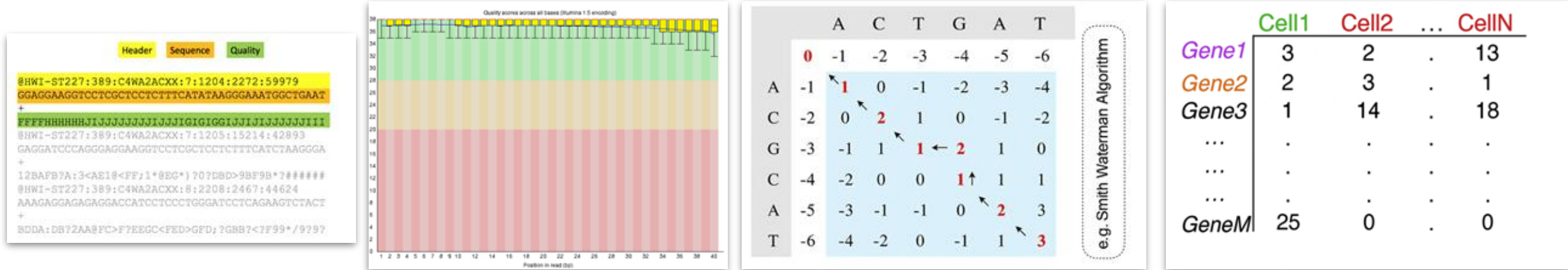
Sequencing saturation

Q30 Score

Transcriptome map fraction

STATISTIC	SAMPLE 1	SAMPLE 2	SAMPLE 3
number_of_cells	3313	3043	2877
GRCm39_median_tscp_per_cell	15493	17817	17257
GRCm39_median_genes_per_cell	4096	4387	4318
mean-reads_per_cell	59505	68179	65290
sequencing_saturation	0.3694	0.3691	0.3691
valid_barcode_fraction	0.8243	0.8243	0.8243
bc1_Q30	0.8652	0.8652	0.8653
bc2_Q30	0.8947	0.8947	0.8947
bc3_Q30	0.8866	0.8866	0.8866
cDNA_Q30	0.9159	0.9159	0.9159
transcriptome_map_fraction	0.7969	0.7969	0.7969

CONVERTING FASTQ FILES INTO COUNT MATRICES



What are Count Matrices?

FASTQ file processing pipelines produce count matrices which we can analyze to extract **insights about your samples**:

- Number of counts for every gene in each cell
- Usually provided with three files
 - Matrix
 - Cell metadata
 - Gene list

	Cell1	Cell2	...	CellN
Gene1	3	2	.	13
Gene2	2	3	.	1
Gene3	1	14	.	18
...	.	.	.	.
...	.	.	.	.
...	.	.	.	.
GeneM	25	0	.	0

Data Visualization and Interpretation

A WEALTH OF BIOINFORMATICS TOOLS ARE AVAILABLE



Split-Pipe



Trailmaker™



Satija Lab

<https://github.com/satijalab/seurat>



F. Alexander Wolf, Philipp Angerer, Fabian J. Theis

<https://github.com/scverse/scanpy>



Yosef Lab

<https://scvi-tools.org/>

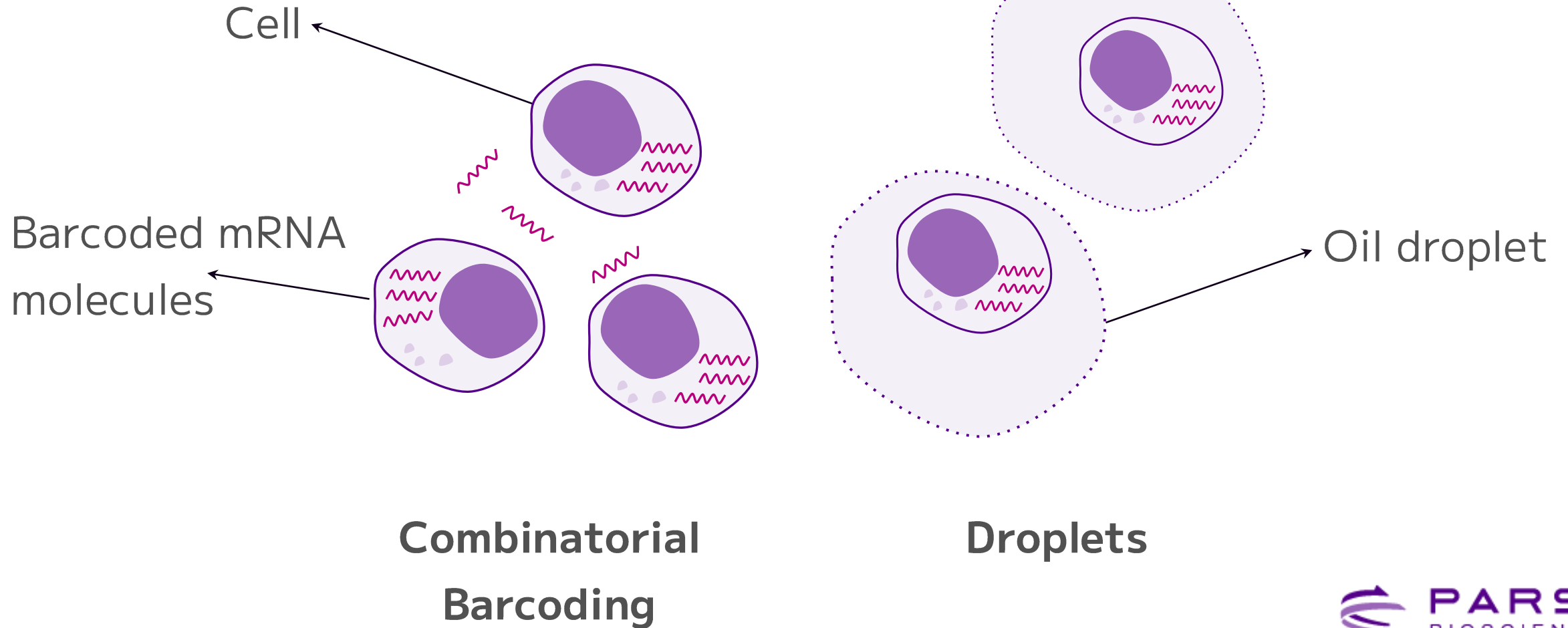


Trapnell Lab

<https://cole-trapnell-lab.github.io/monocle3/>

scRNA-seq Quality Control

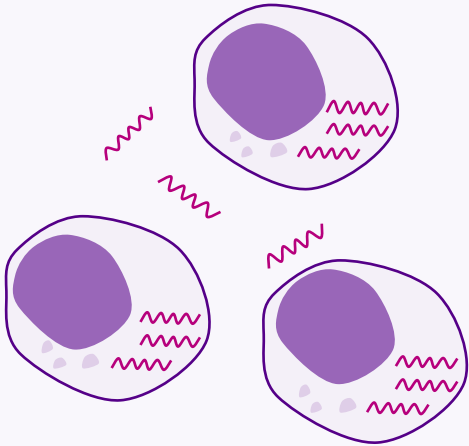
WHAT DO WE EXPECT IN AN IDEAL SCENARIO?



Quality Control Metrics for Filtering

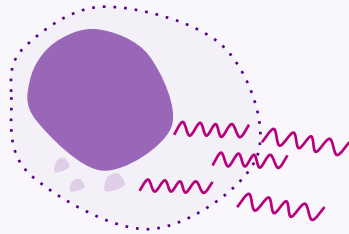
KEY CONSIDERATIONS FOR PROCESSING SINGLE CELL TRANSCRIPTOME DATA

01



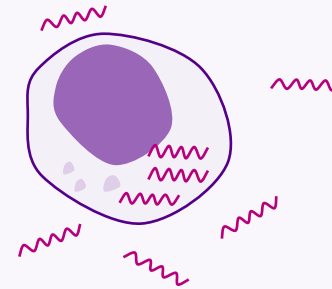
Background

02



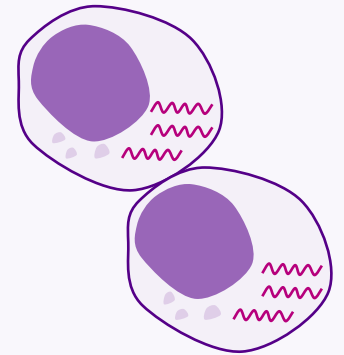
**Dead or
Dying Cells**

03



**Outliers and
Ambient RNA**

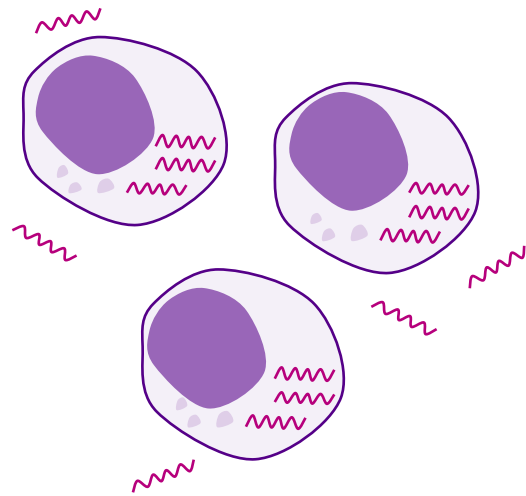
04



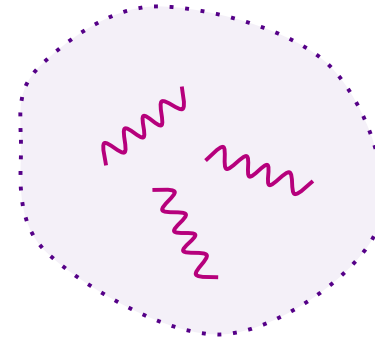
Doublets

Background and Empty Droplets

WHAT TO LOOK OUT FOR WHEN PROCESSING SINGLE CELL DATA



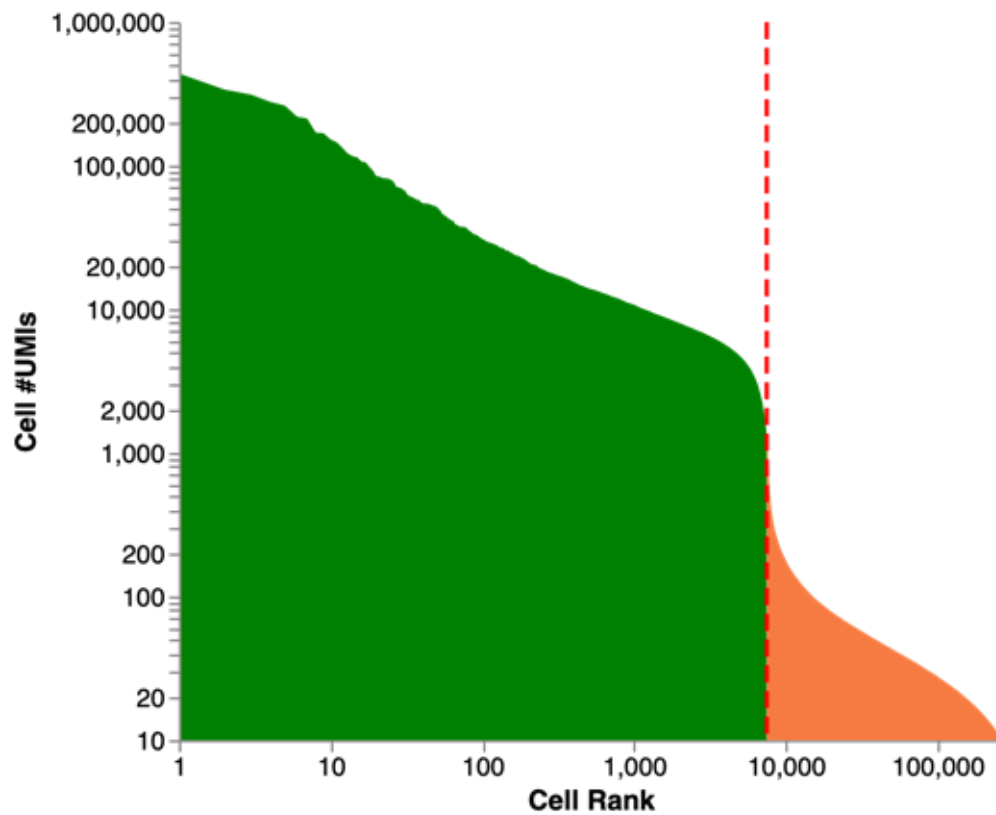
**Combinatorial
Barcoding**



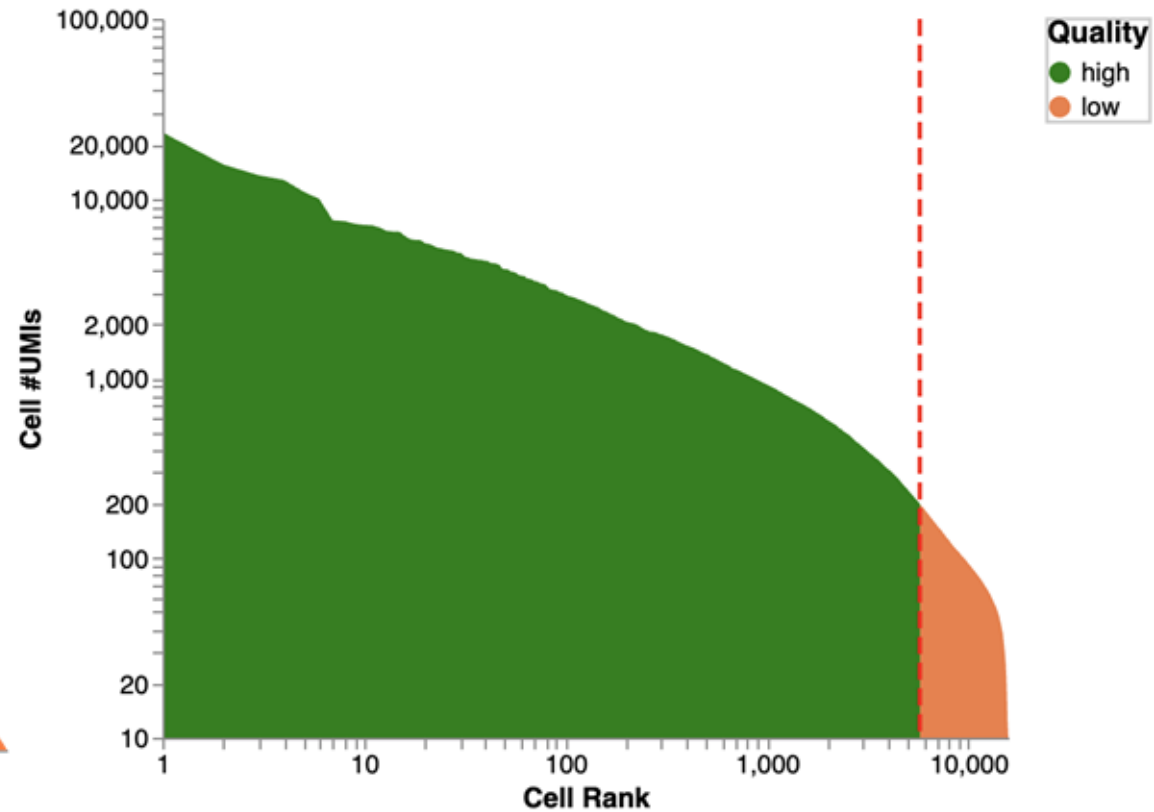
Droplets

Comparing Knee Plots

GARBAGE IN, GARBAGE OUT



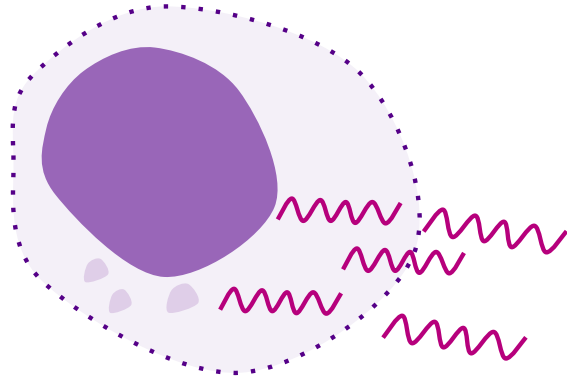
Clearly defined knee separating cells from background



Poor sample quality (debris and/or damaged cells) affecting curve

Dead or Dying Cells

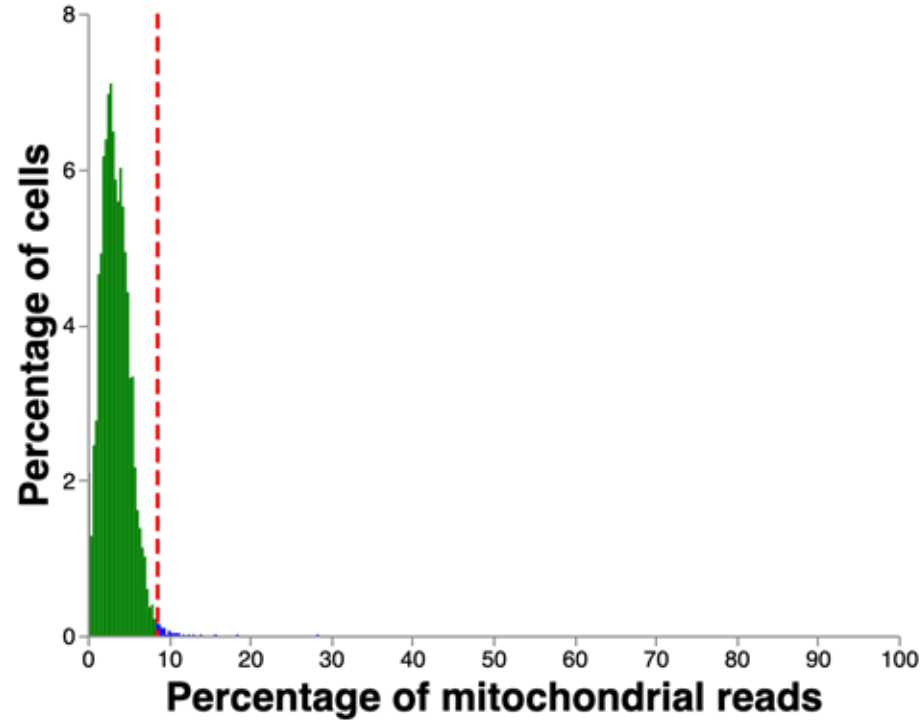
USING THE PERCENTAGE OF MITOCHONDRIAL GENES TO REMOVE DEAD CELLS



Proportion of mitochondrial genes increases as stressed cells release cytoplasmic transcripts while mitochondrial transcripts remain in the cell

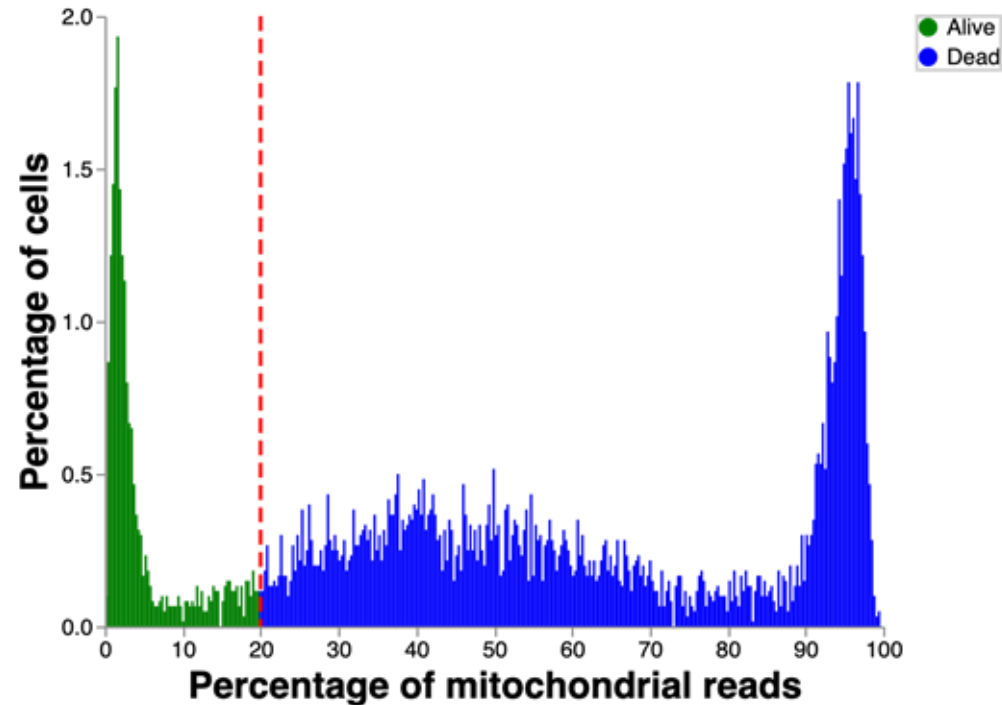
Dead or Dying Cells: Comparing Samples

A REAL WORLD EXAMPLE



"Good" sample

Sample with majority of cells containing a low fraction of mitochondrial reads

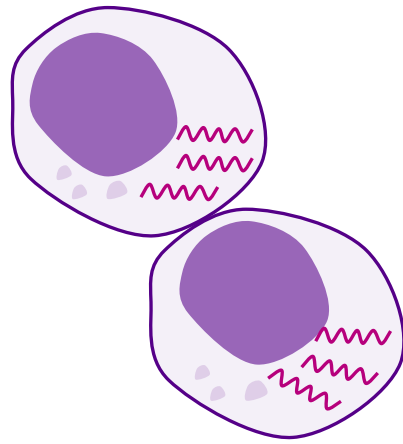


"Bad" sample or biology?

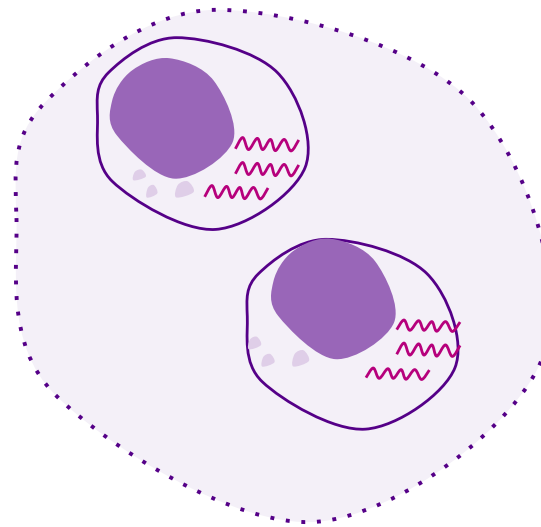
Sample containing more cells with a higher fraction of mitochondrial reads

Doublets

WHAT TO LOOK OUT FOR WHEN PROCESSING SINGLE CELL DATA



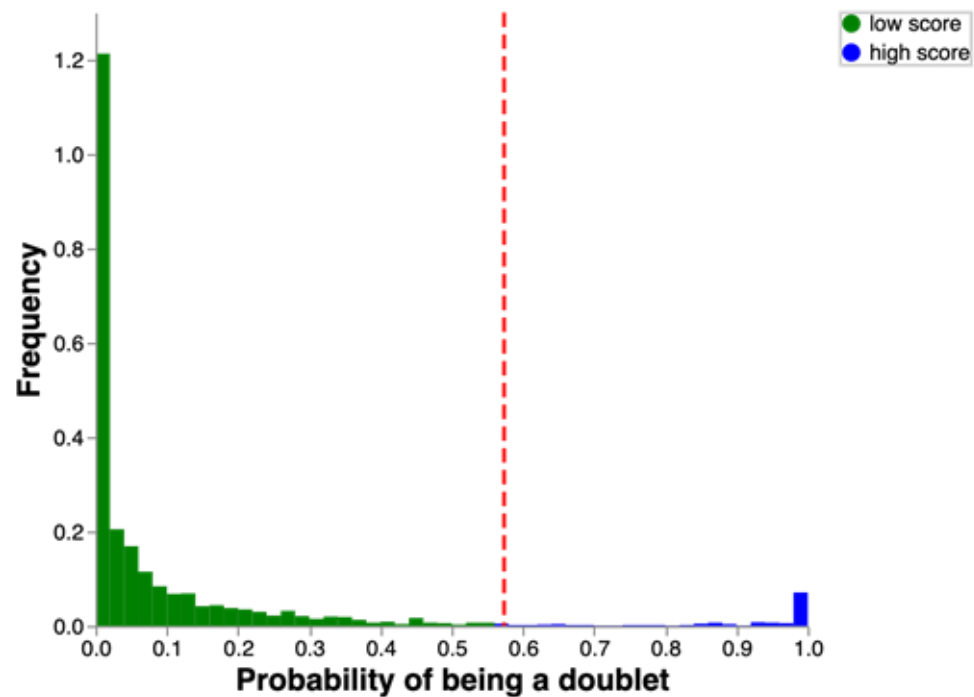
**Combinatorial
Barcoding**



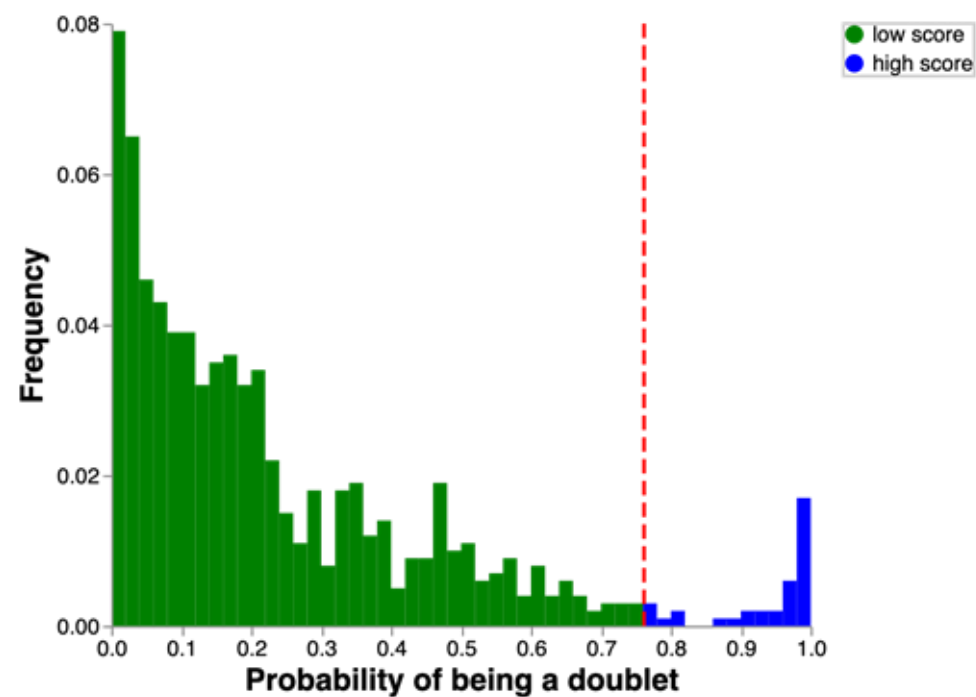
Droplets

Doublets

COMPARING TWO SAMPLES



"Good" sample

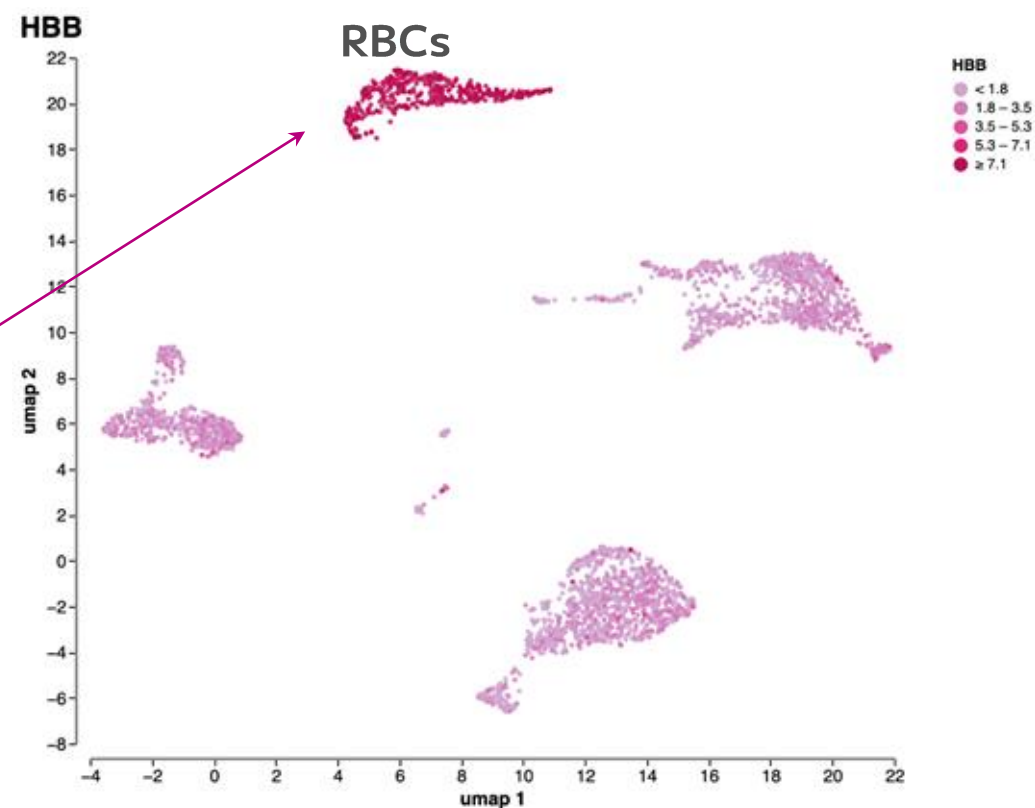
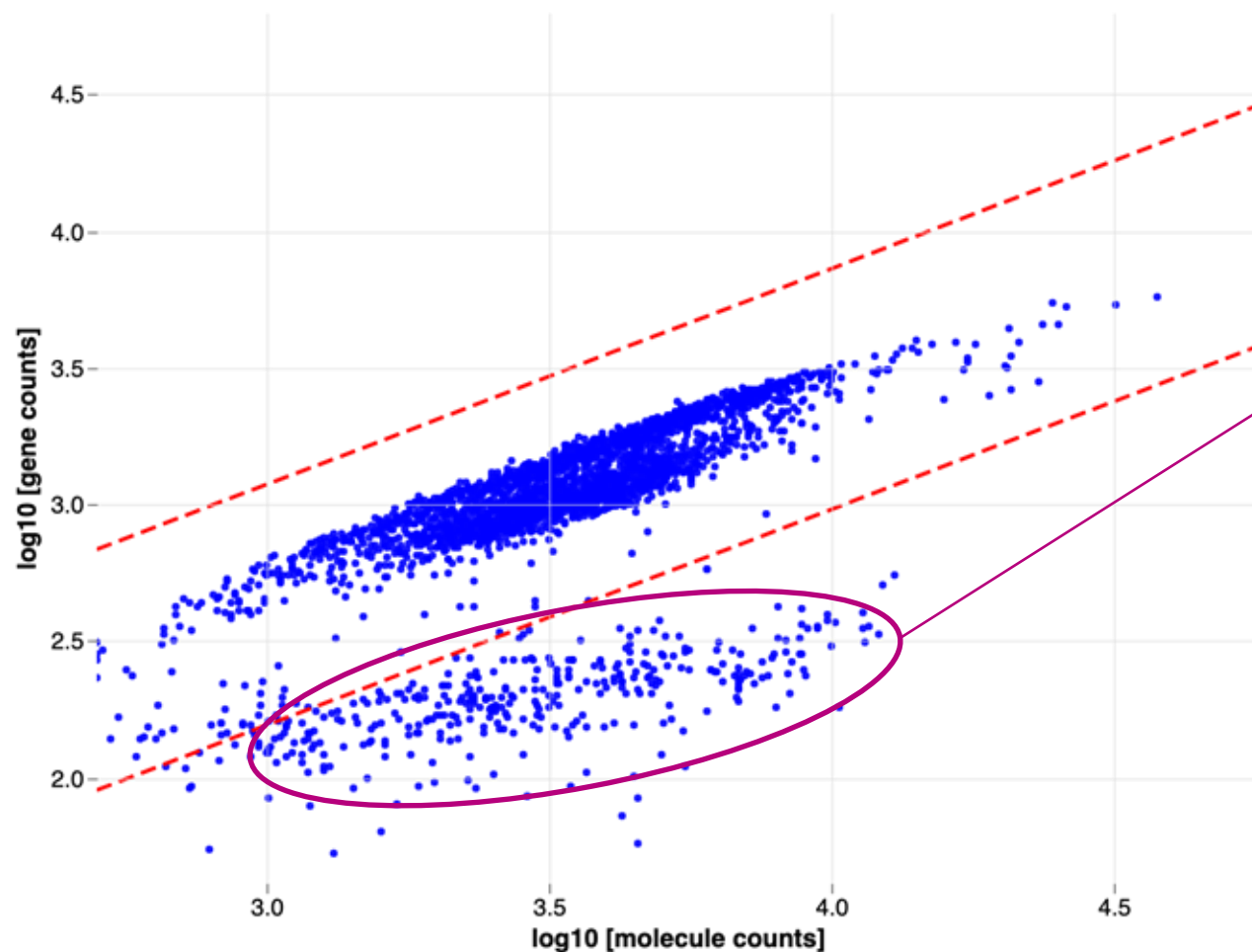


"Bad" sample

Not enough cells to accurately
compute doublets scores

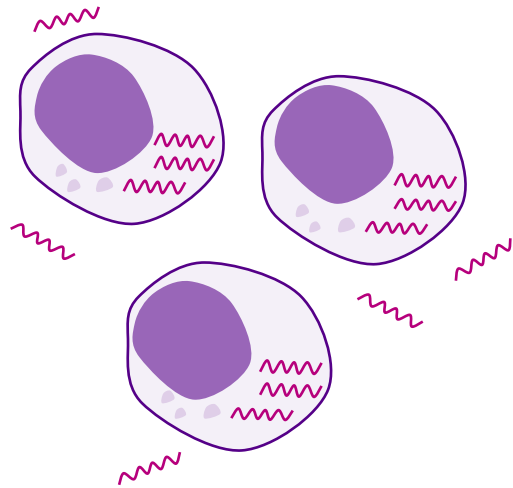
Contaminant or Low-quality Cells

SOME VISUALIZATIONS CAN AID WITH REMOVAL OF CONTAMINANT OR LOW-QUALITY CELLS

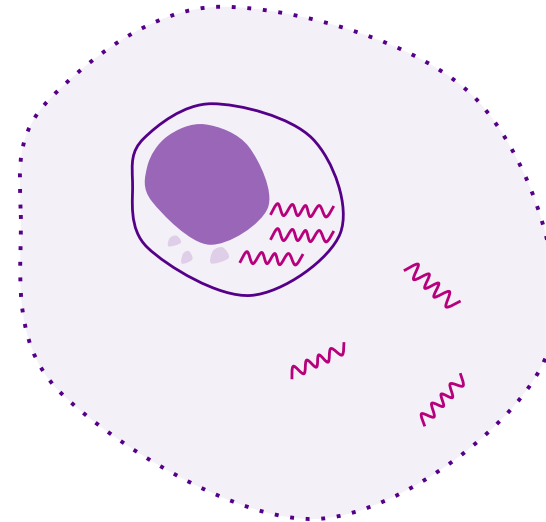


Ambient RNA

WHAT TO LOOK OUT FOR WHEN PROCESSING SINGLE CELL DATA



**Combinatorial
Barcoding**



Droplets

Key Takeaways

- 01** Planning is vital (and may include your Core!)
- 02** Taking time to generate quality samples leads to quality data
- 03** Partner with your Resource Facilities, the most experienced hands on campus

Thank you