

Introduction to Single Cell RNA Sequencing

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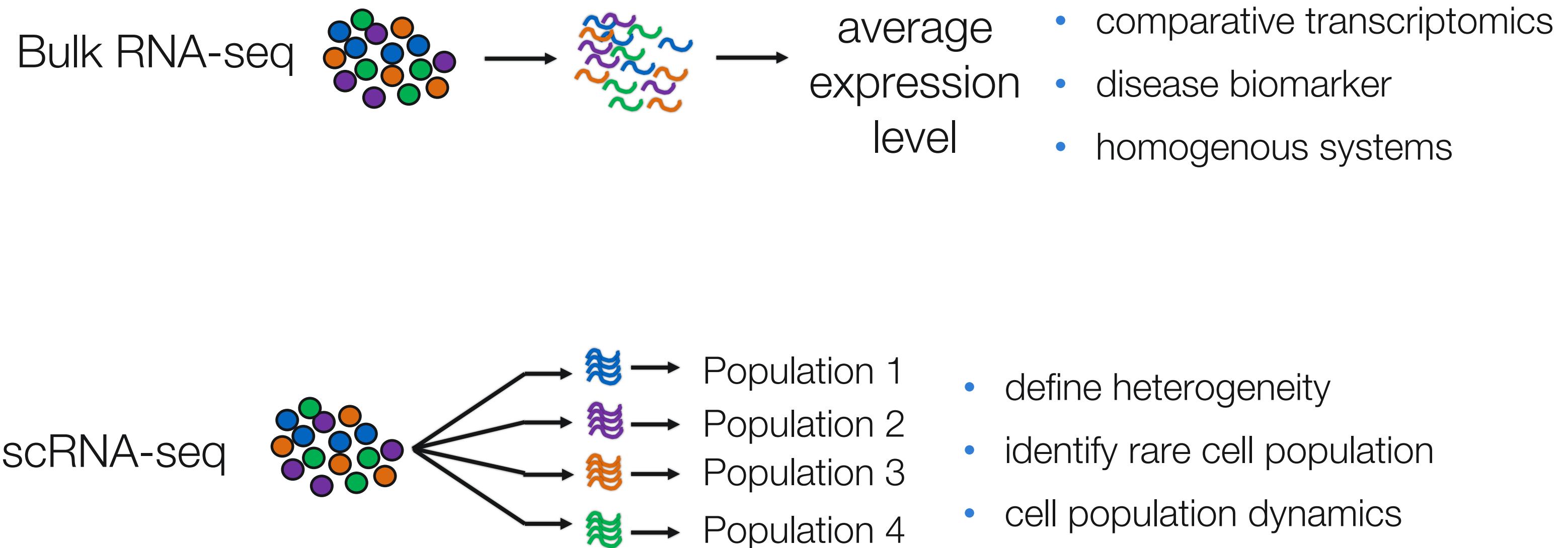
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Introduction to Single Cell RNA Sequencing

- Common applications of single cell RNA sequencing.
- Overview of single cell RNA sequencing platforms.
- Modified scRNA-seq workflows
- Sample preparation and experimental design.
- Effects of sample prep and sample type on analysis.

Bulk vs Single Cell RNA Sequencing (scRNA-seq)



Transcriptome Coverage (mRNA)

1. mRNA: TruSeq RNA-Seq (gold standard)

- ~20,000 transcripts
 - More when consider splice variants / isoforms
- Observe 80-95% of transcripts depending on sequencing depth

2. Low Input Methods

- 4000-6000 transcripts per sample
 - Limiting to transcripts observed across all samples
- Observe 20-60% of the transcriptome

3. Single Cell Methods

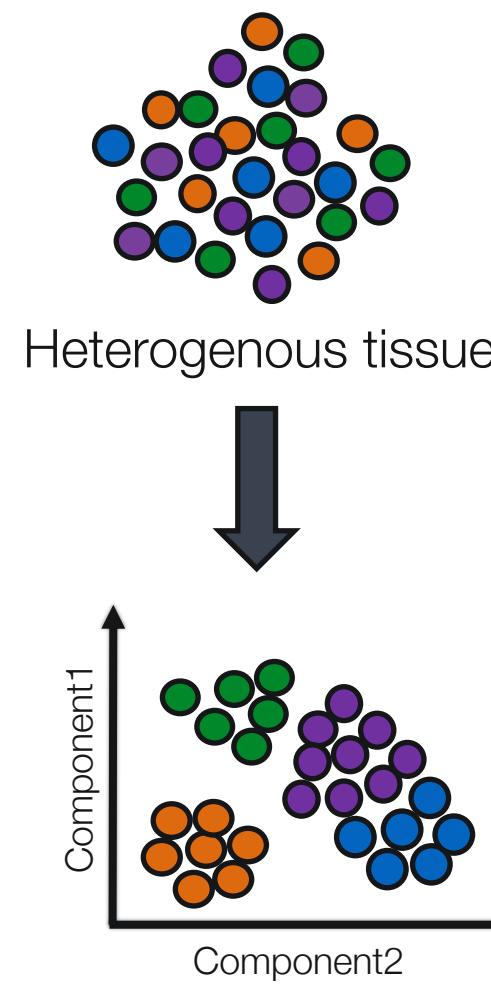
- 200 -10,000 transcripts per cell
- Observe 10-50% of the transcriptome
- Many transcripts will show up with zero counts in every cell (eg. GAPDH, ACTB).
- If you only looked at transcripts observed in all cells numbers drop dramatically.

The World Between Bulk & scRNA-seq

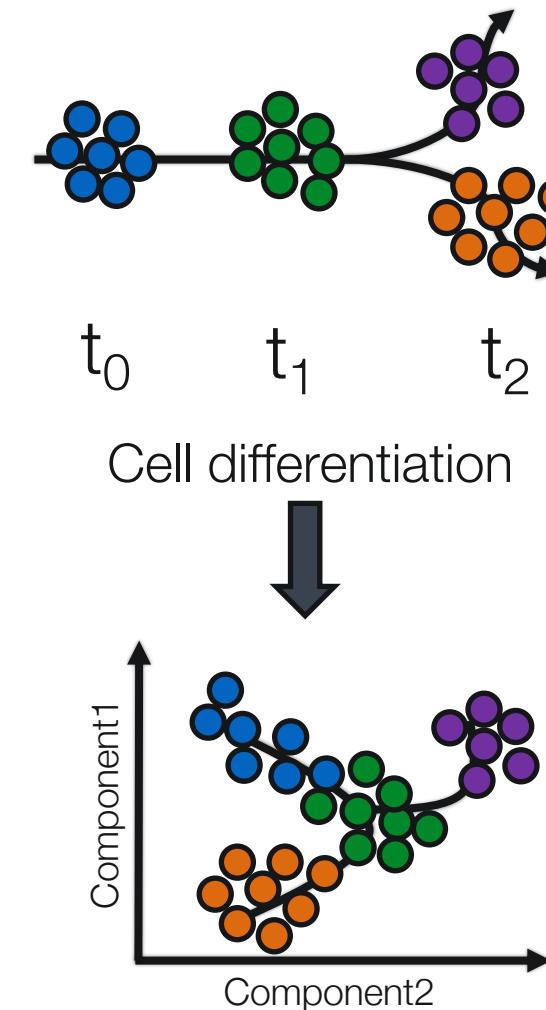
	Deep RNA-seq	Sort-seq	Low input	scRNA-seq
Transcriptome Coverage	High	High	Moderate	Low
Throughput	Moderate	Low	High	Low
Cell Subtype Information	None	Moderate	None	High
Sequencing Depth	Moderate	Moderate	Low	High
Cost per Sample	Moderate	Moderate	Low	High

Common Applications of scRNA-seq

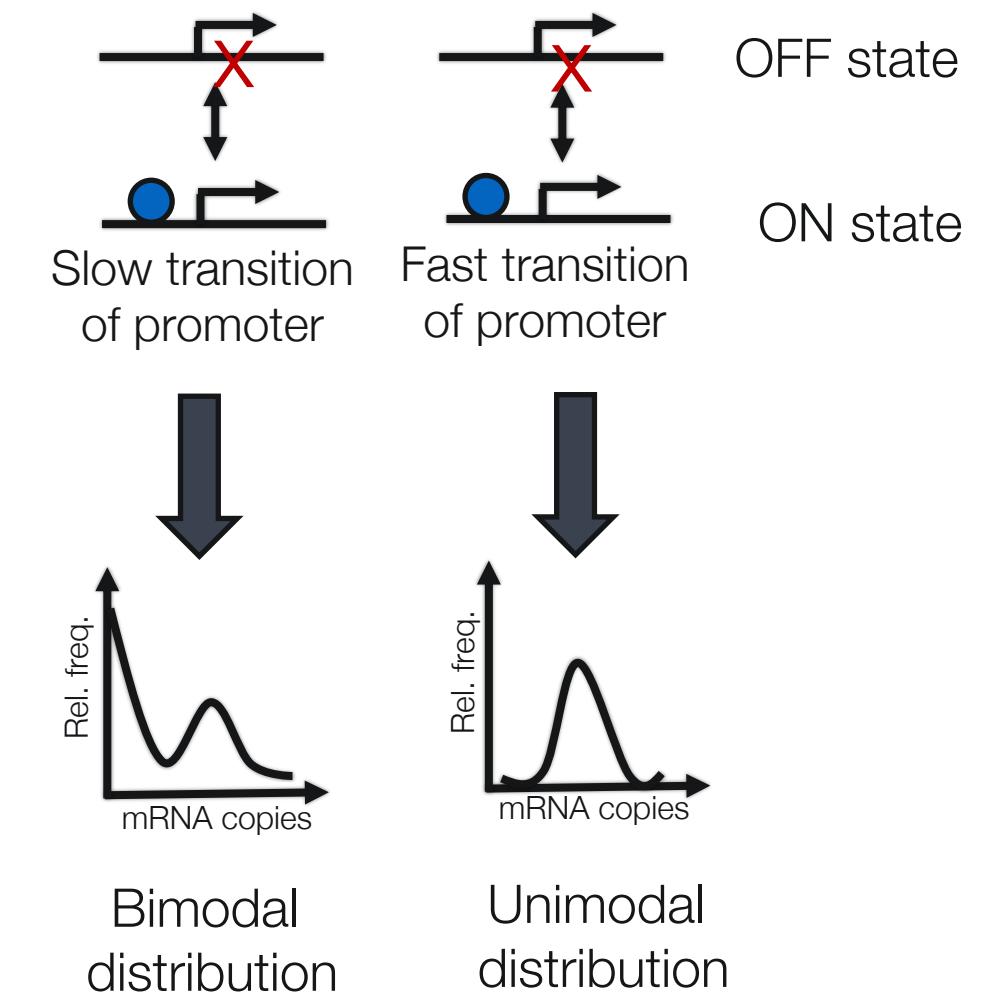
Studying heterogeneity



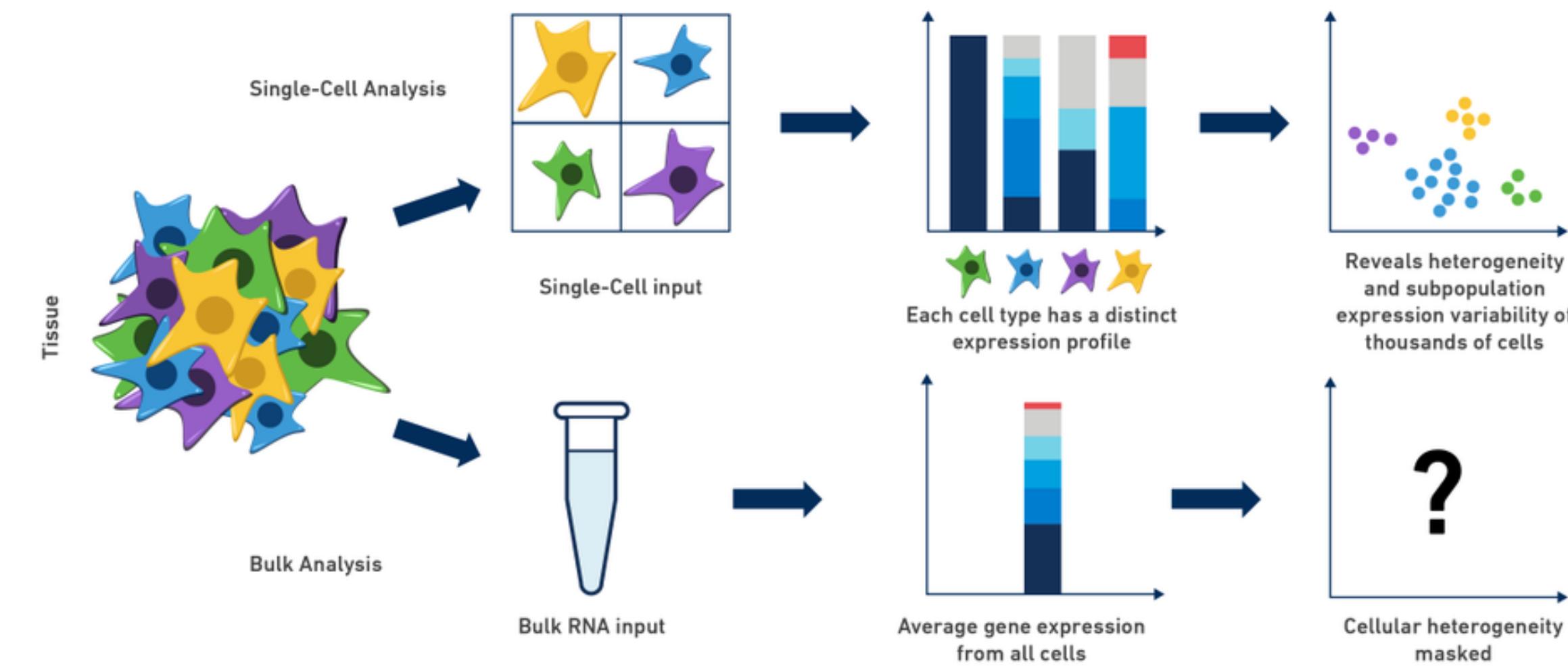
Lineage tracing study



Stochastic gene expression

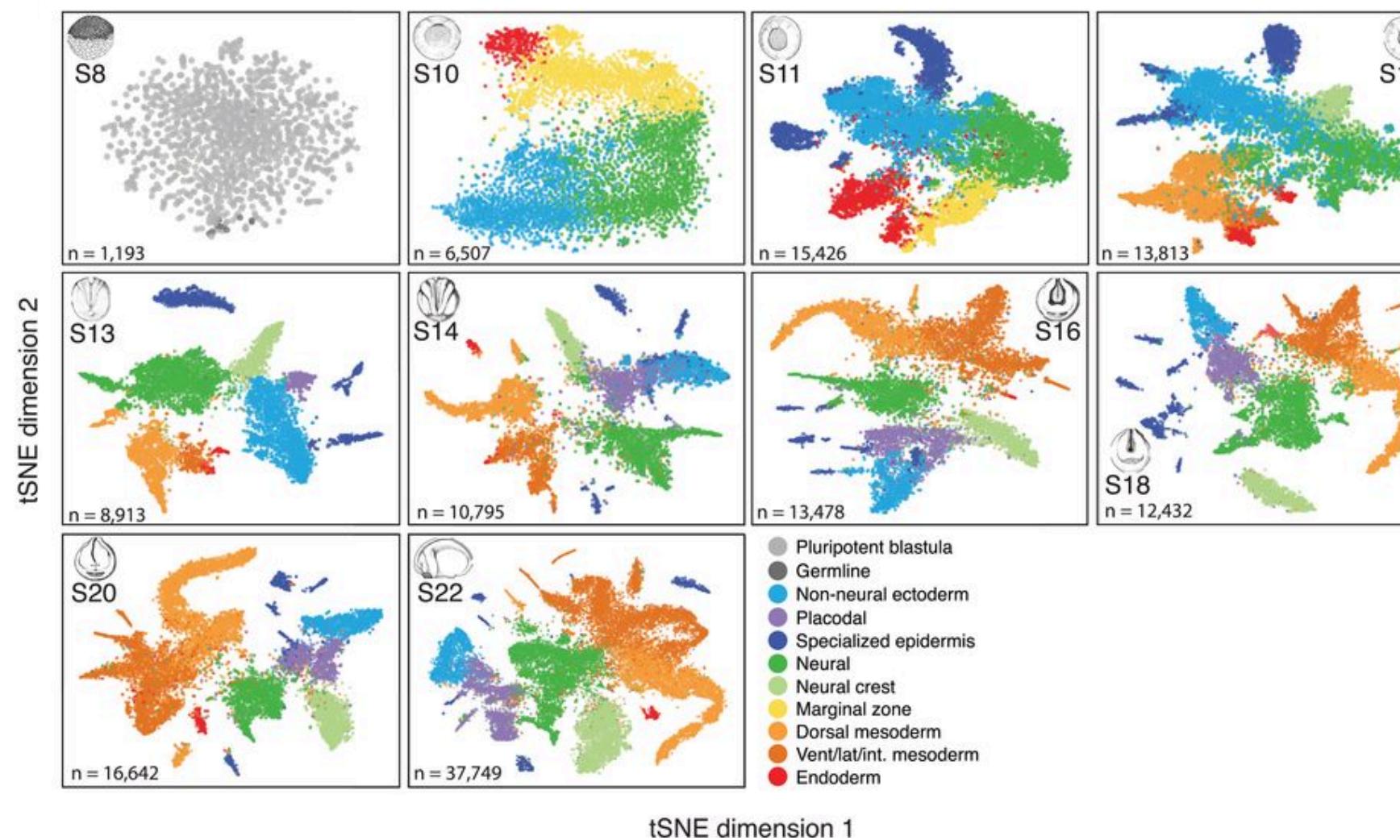


Tumor, Tissue, Organoid Heterogeneity

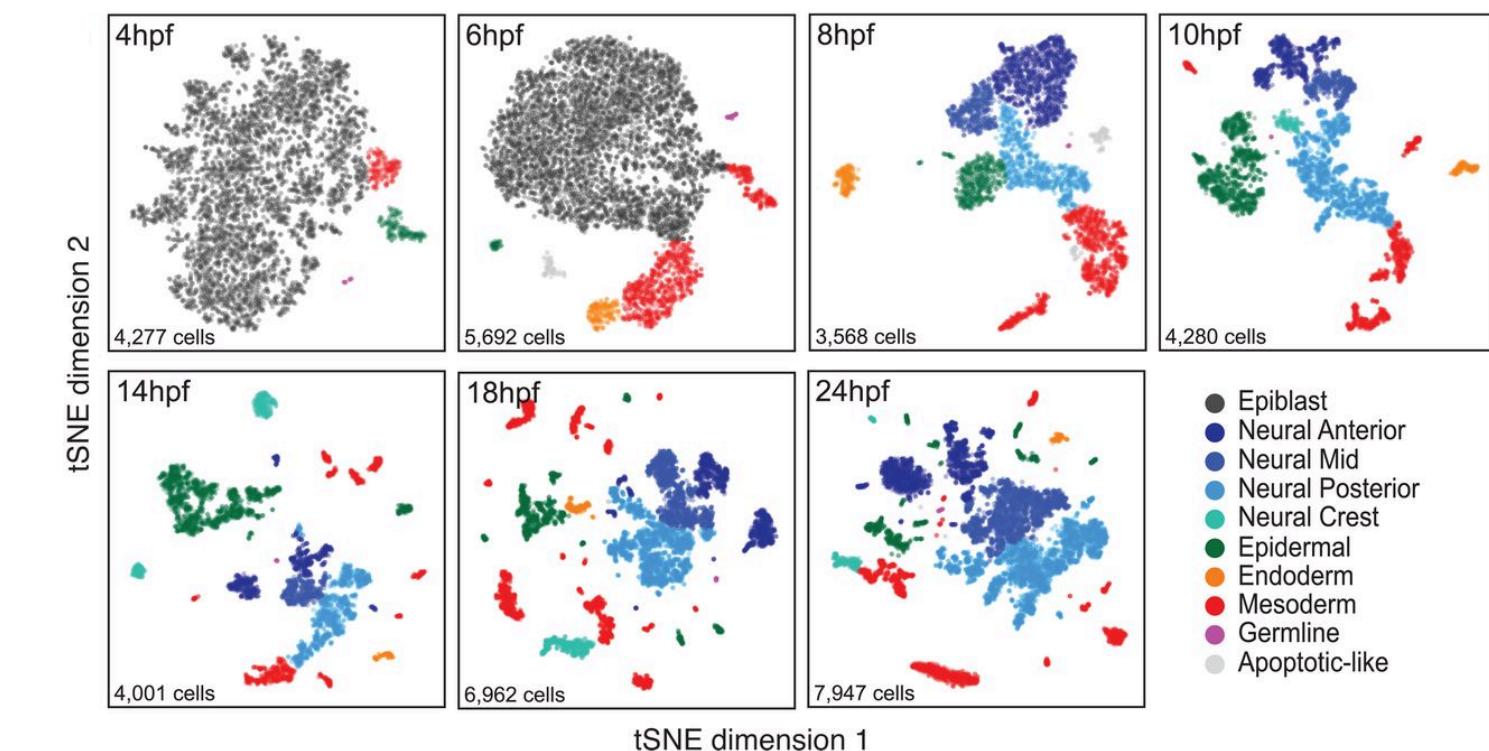


Development Lineage Tracing

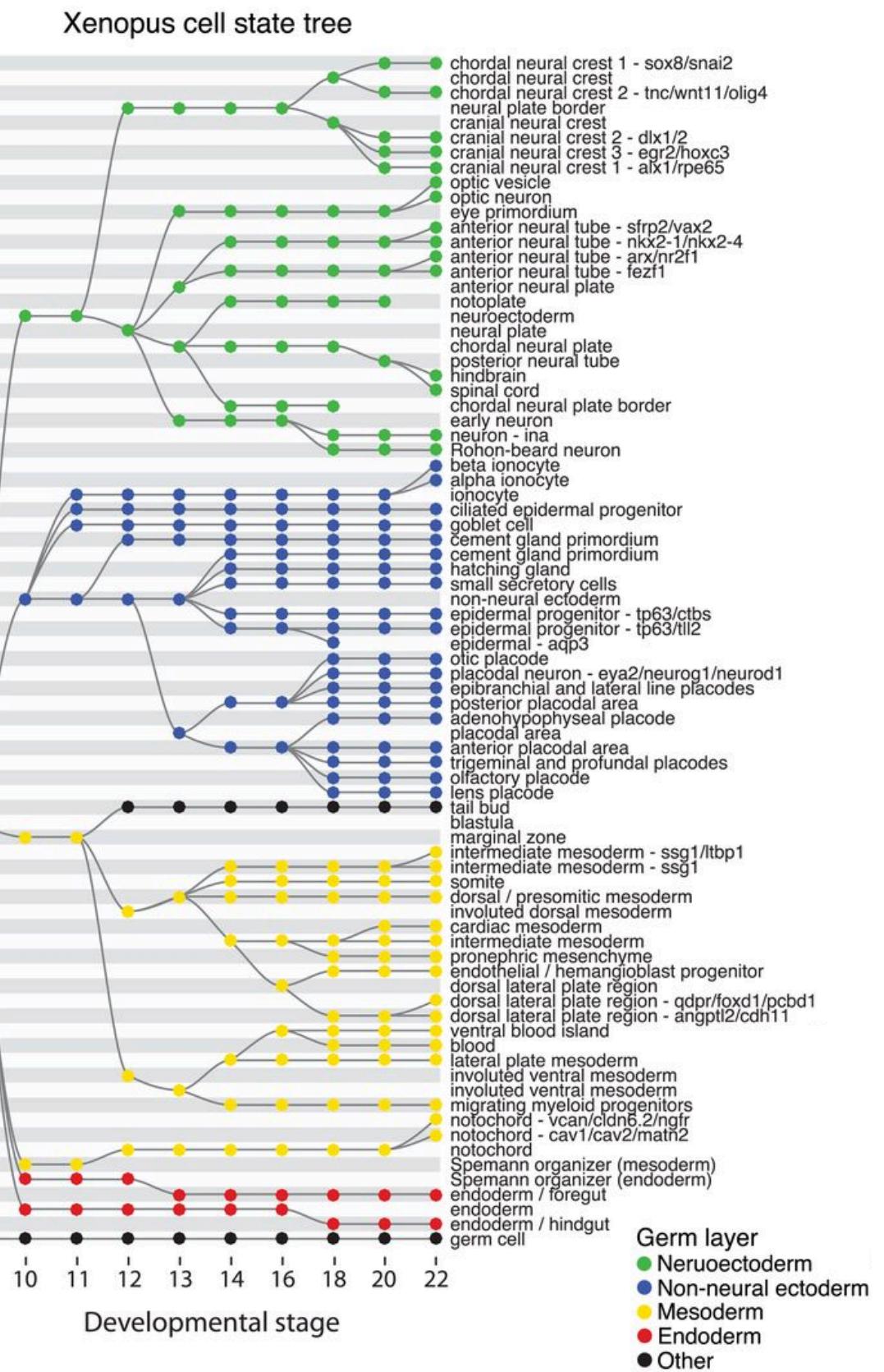
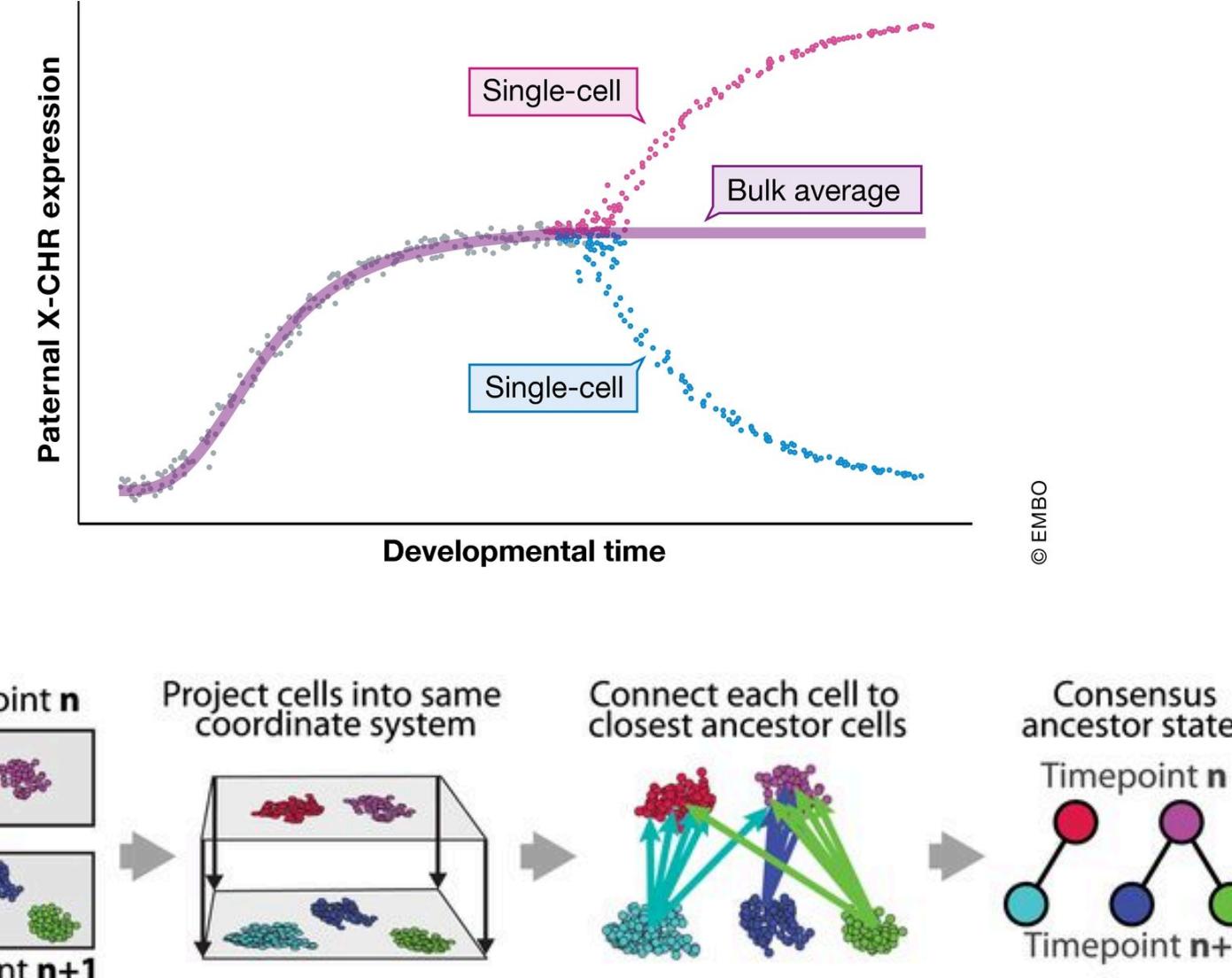
Frog



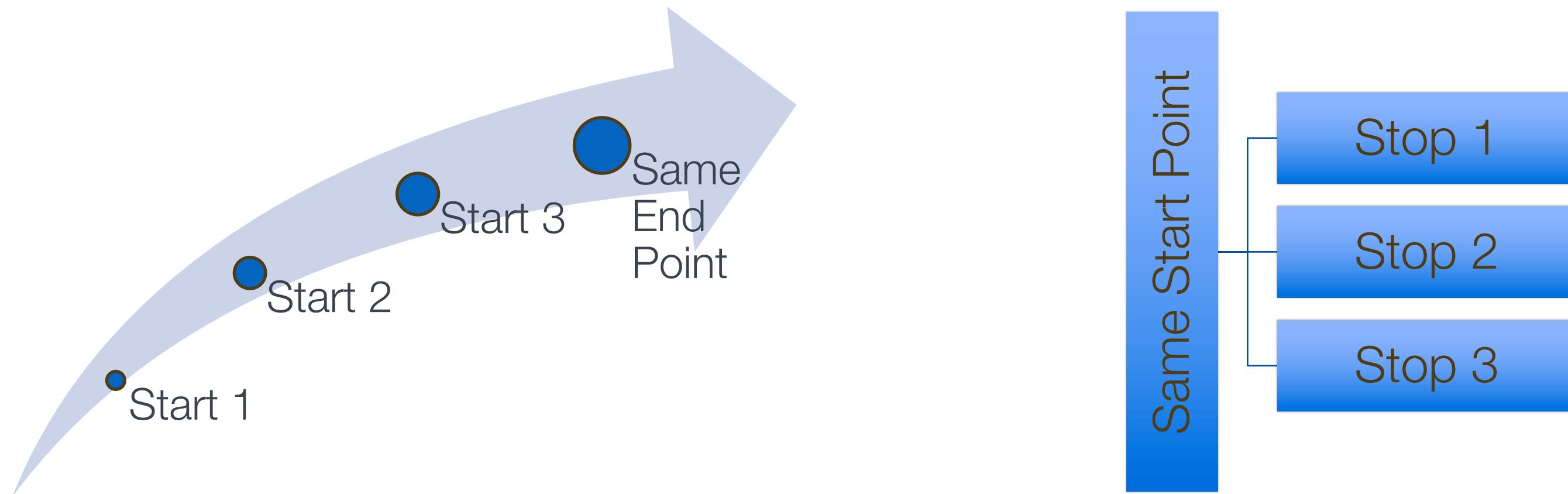
Zebrafish



Development Lineage Tracing

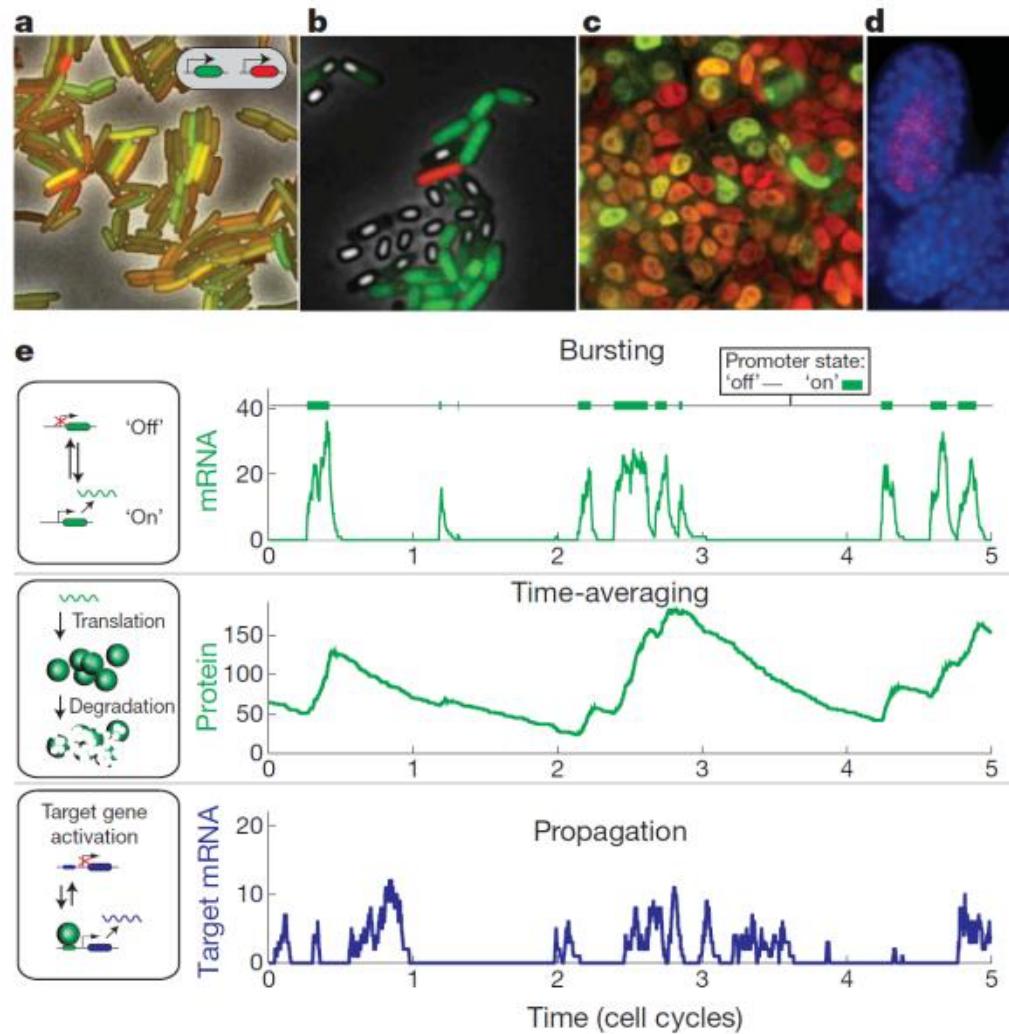


Time Course or Development Experiment



- Collect all samples and prep libraries together in one batch.
- Biological duplicates (at minimum)

Stochastic Gene Expression

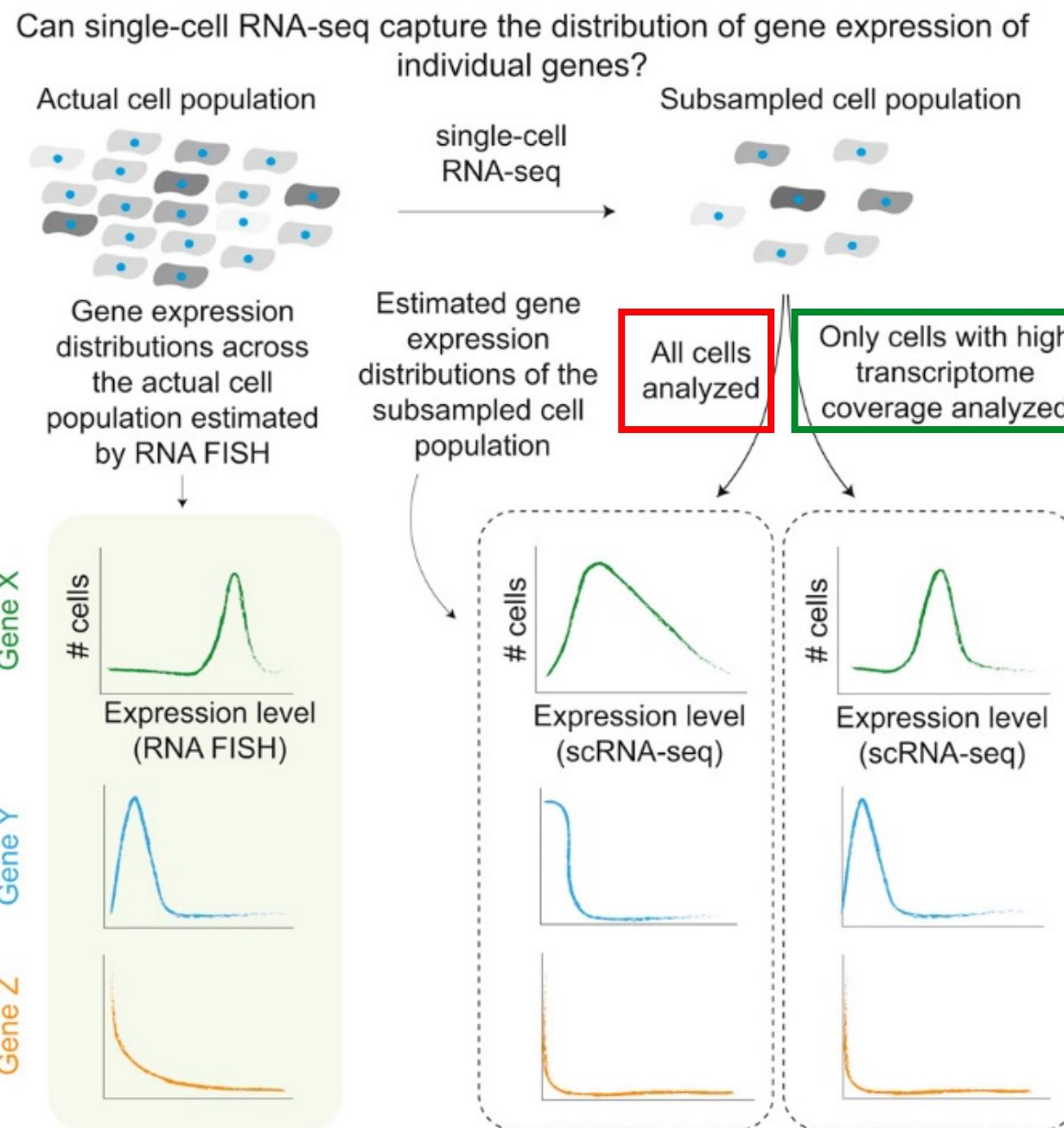


- Gene expression is heterogeneous and “bursty”.
- Genes fluctuate between “On” and “Off” promoter states.
- Stochastic expression of one gene can propagate to generate more stochasticity in downstream genes.

Eldar & Elowitz; Functional roles for noise in genetic circuits, Nature 2010 (doi: 10.1038/nature09326)

B Hwang et al. Single-cell RNA sequencing technologies and bioinformatics pipelines, EMM 07 Aug 2018 (doi: 10.1038/s12276-018-0071-8)

Stochastic Gene Expression



- Low mRNA capture efficiency of scRNA-seq makes it difficult to draw definitive conclusions about expression at the single-cell level.
- Number of cells and depth of sequencing critical for understanding rare gene expression phenotypes.

More Cells or More Sequencing Reads?

- Required number of cells increases with complexity of the sample.
- As the number of genes involved in the biology decrease then the coverage requirements increase (more reads).
- Cell-type classification of a mixed population usually requires lower read depth and can be sequenced at 10,000-50,000 reads per cell.
- Suggest starting with 25,000-55,000 reads per cell. Can always re-sequence your samples.

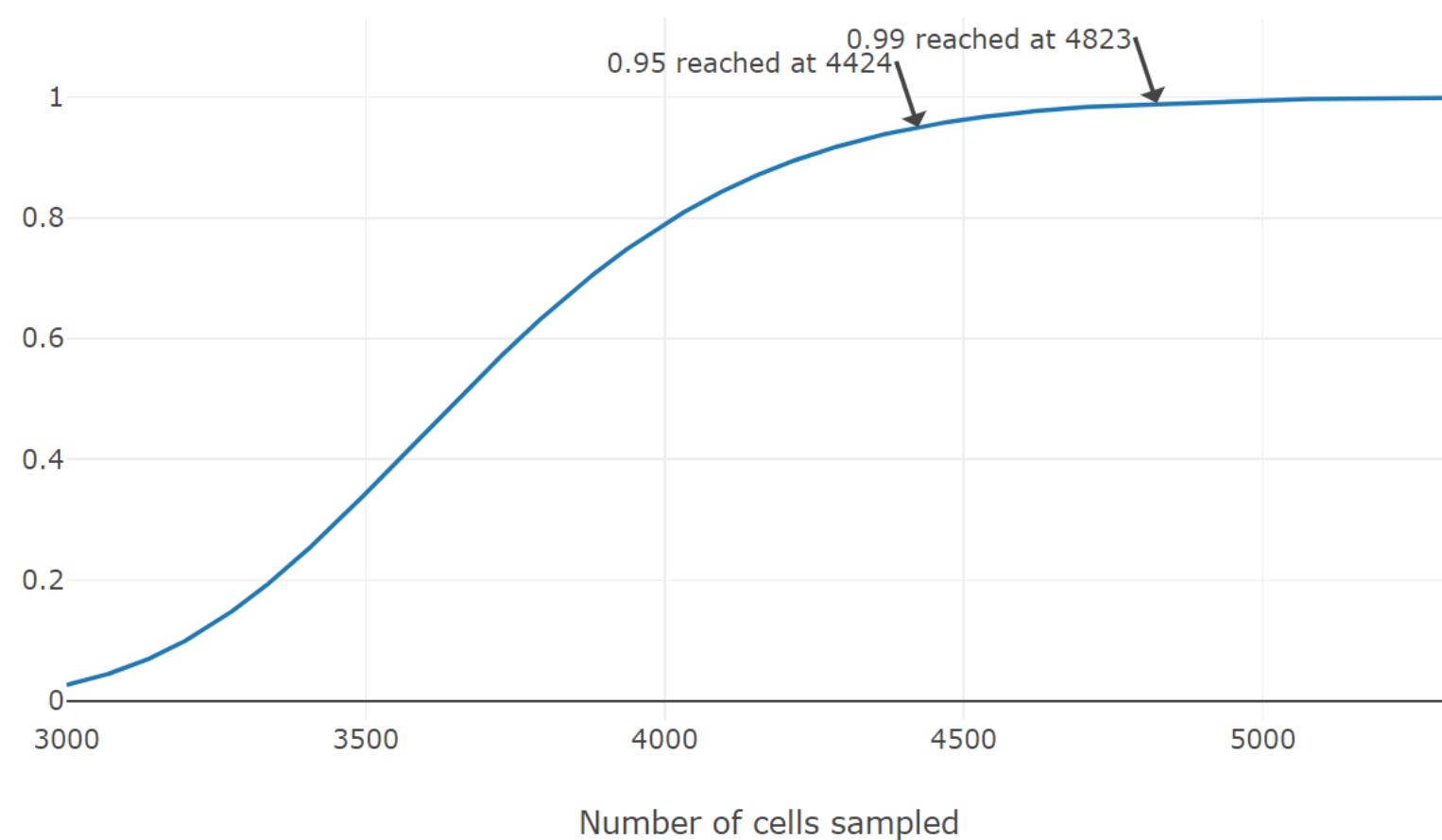
<https://satijalab.org/howmanycells>

Assumed number of cell types
6

Minimum fraction (of rarest cell type)
.01

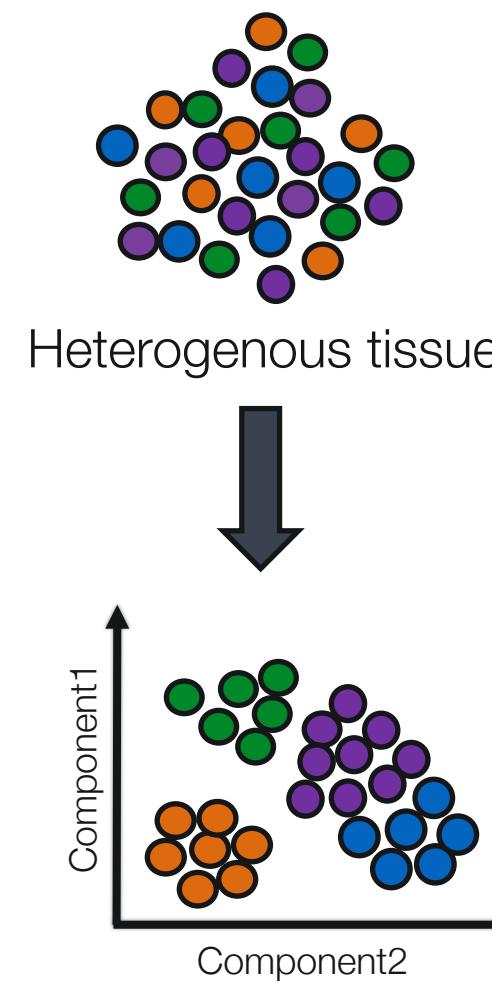
Minimum desired cells per type
30

Probability of seeing at least 30 cells from each cluster

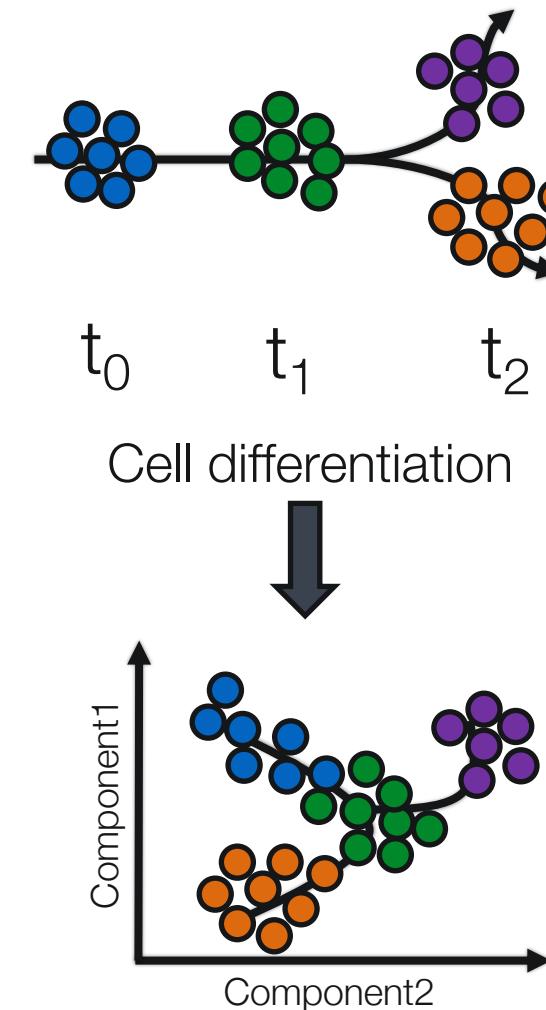


Common Applications of scRNA-seq

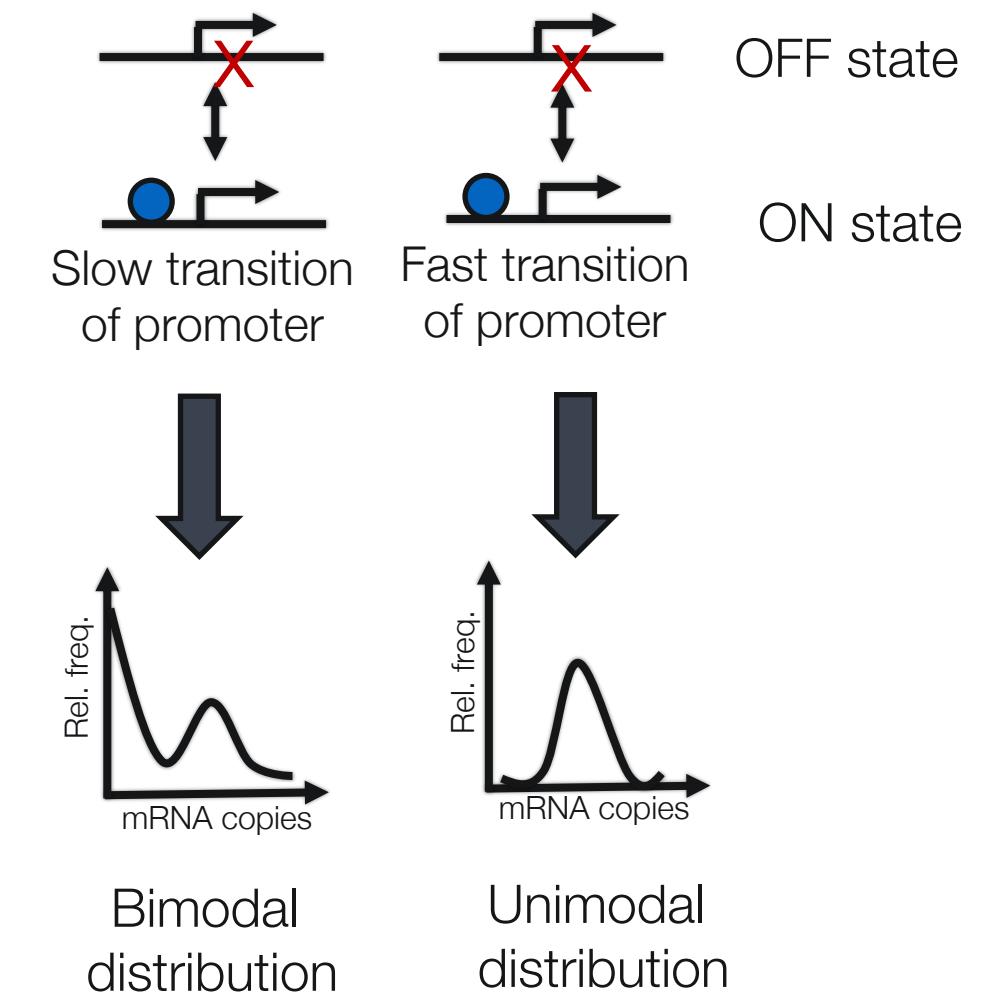
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Lineage tracing study



Stochastic gene expression



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Comparison of Single Cell Methods

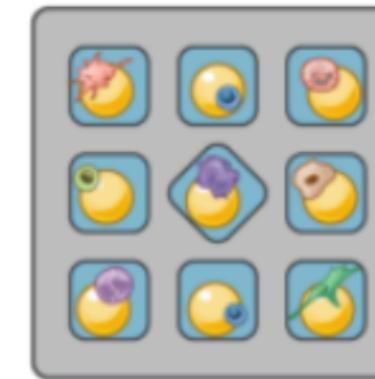
CELL-seq
MARS-seq
SMART-seq
SCRB-seq



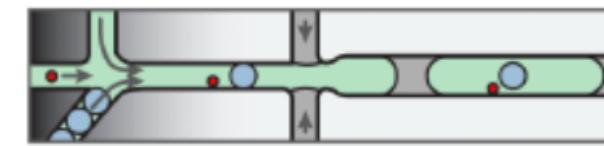
Chromium (10x)



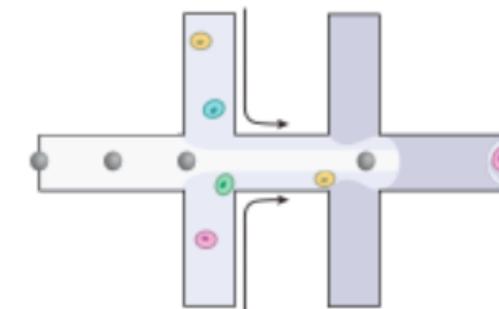
Seq-Well



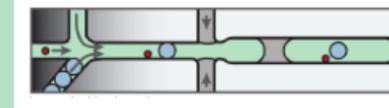
inDrops



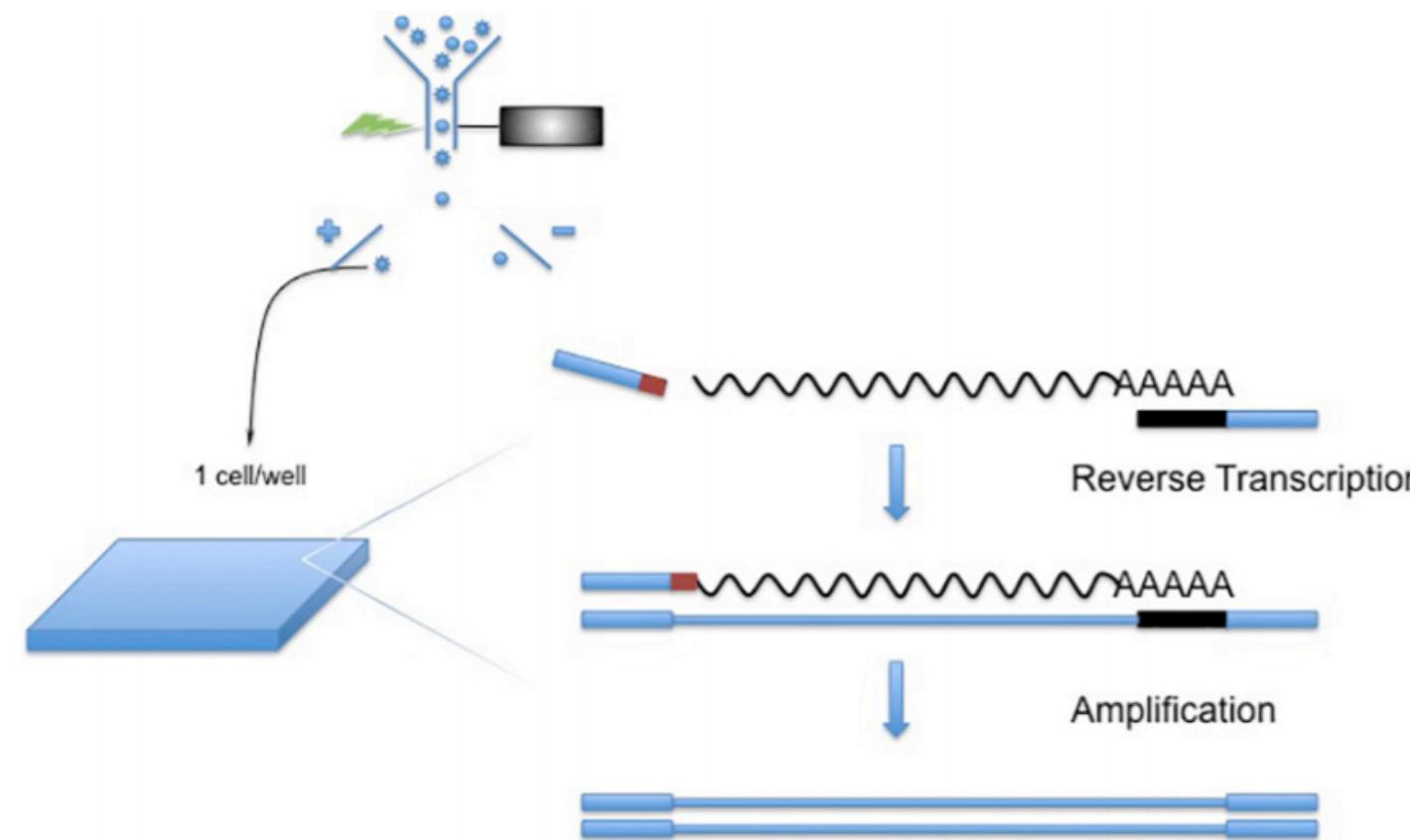
Drop-seq



Comparison of Single Cell Methods

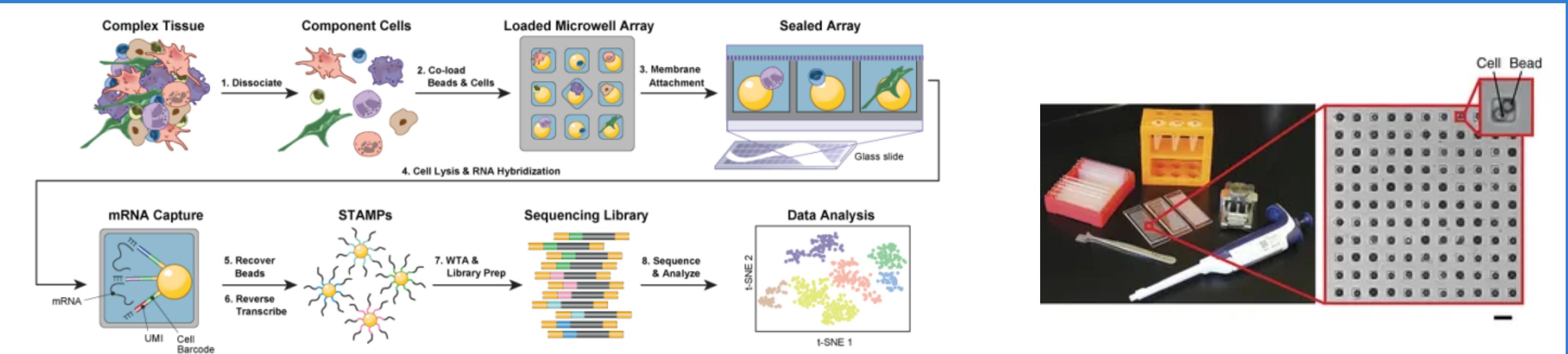
	inDrops	10x Genomics	Drop-seq	Seq-well (Honeycomb)	SMART-seq
Cell capture efficiency	~70-80%	~50-70%	~10%	~80%	~80%
Time to capture 10k cells	~30min	10min	1-2 hours	5-10min	--
Encapsulation type	Droplet 	Droplet 	Droplet 	Nanolitre well 	Plate-based 
Library prep	CEL-seq Linear amplification by IVT	SMART-seq Exponential PCR based amplification	SMART-seq Exponential PCR based amplification	SMART-seq Exponential PCR based amplification	SMART-seq Exponential PCR based amplification
Commercial	Yes	Yes	--	Yes (Summer 2020)	Yes
Cost (~\$ per cell)	~0.06	~0.2	~0.06	~0.15	1
Strengths	<ul style="list-style-type: none"> Good cell capture Cost-effective Real-time monitoring Customizable 	<ul style="list-style-type: none"> Good cell capture Fast and easy to run Parallel sample collection High gene / cell counts 	<ul style="list-style-type: none"> Cost-effective Customizable 	<ul style="list-style-type: none"> Good cell capture Cost-effective Real-time monitoring Customizable 	<ul style="list-style-type: none"> Good cell capture Good mRNA capture Full-length transcript No UMI
Weaknesses	Difficult to run	Expensive	Difficult to run & low cell capture efficiency	Available Soon	Expensive

Full Length Transcripts: SMART-seq



- Sort cells of interest into single well.
- Only single cell method that gives full transcript information.
- Currently best option for low cell number samples. (100's – 1,000's)

Seq-Well: Honeycomb Biotechnologies

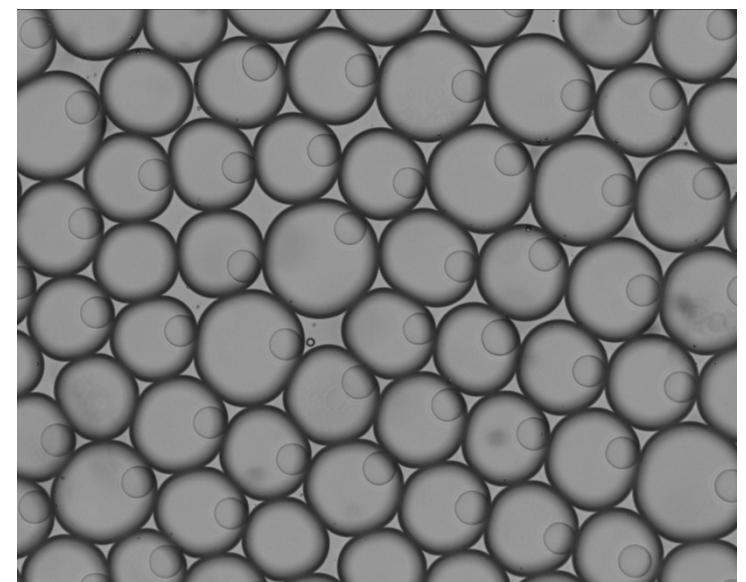


- Up to 1ml of sample is loaded into nanowells (100's – 1,000's of cells)
- Samples settle by gravity into wells.

TM Gierahn et al., Seq-Well principle: low-cost RNA sequencing at single cell and high throughput. Nat Methods. 2017 Apr;14(4):395-398. doi: 10.1038/nmeth.4179

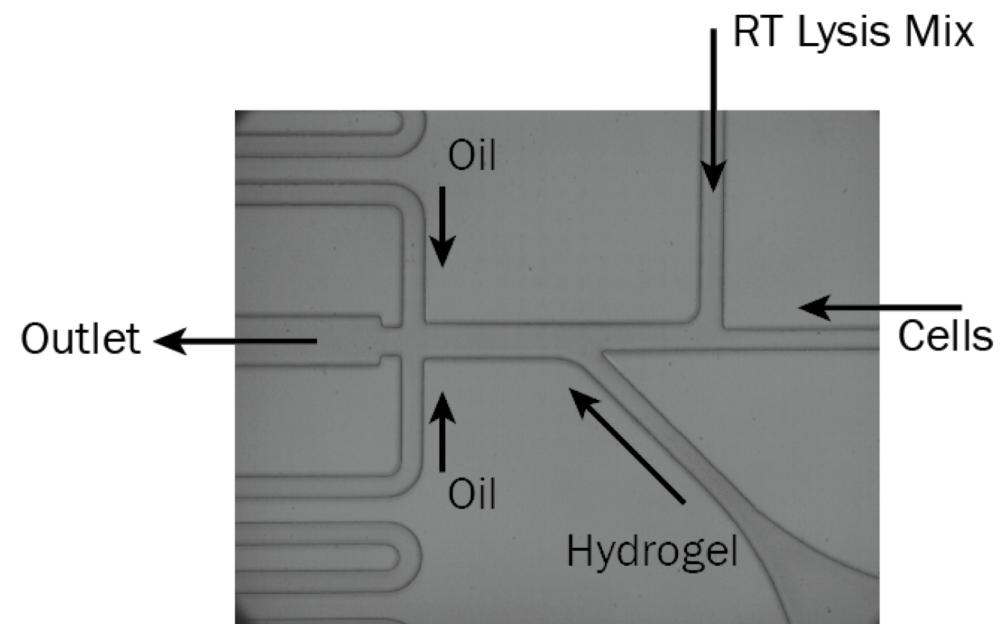
Droplet scRNA-seq

- Droplet methods give single cell information, BUT require high cell numbers to achieve best results. (>10,000 cells)
- Capture 50-80% of the input cells depending on the platform used.
- Extreme 3'-bias in data. Can not look at splicing / isoforms.



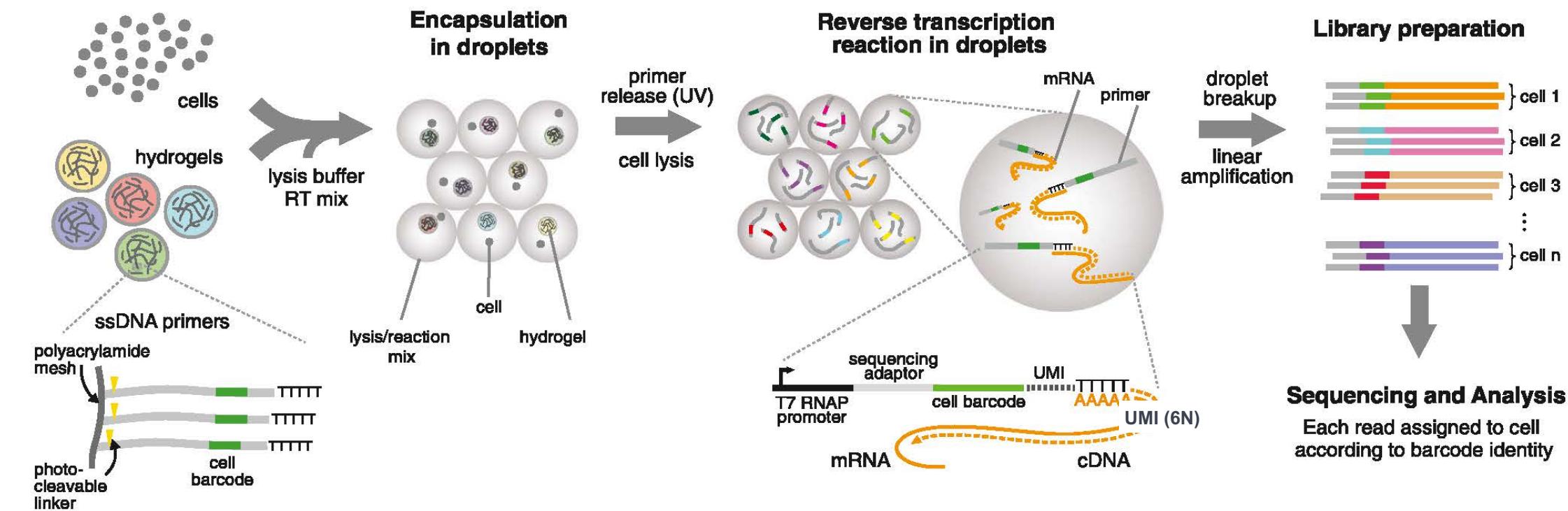
inDrops Method Overview

- Single cell suspension injected at density of ~80,000 cells / ml



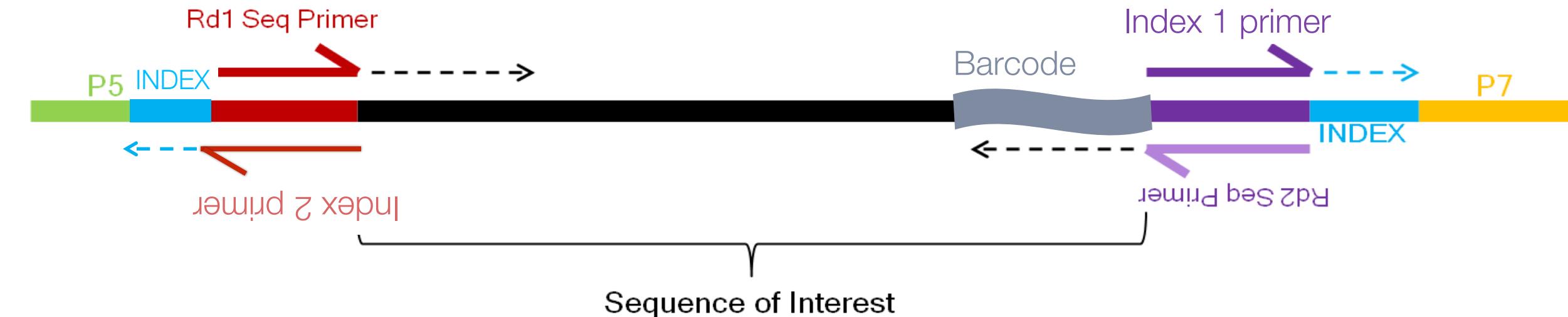
- Matching the speed of bead injection with the speed of droplet generation it is possible to set conditions in which nearly every droplet would be loaded.

inDrops Method Overview



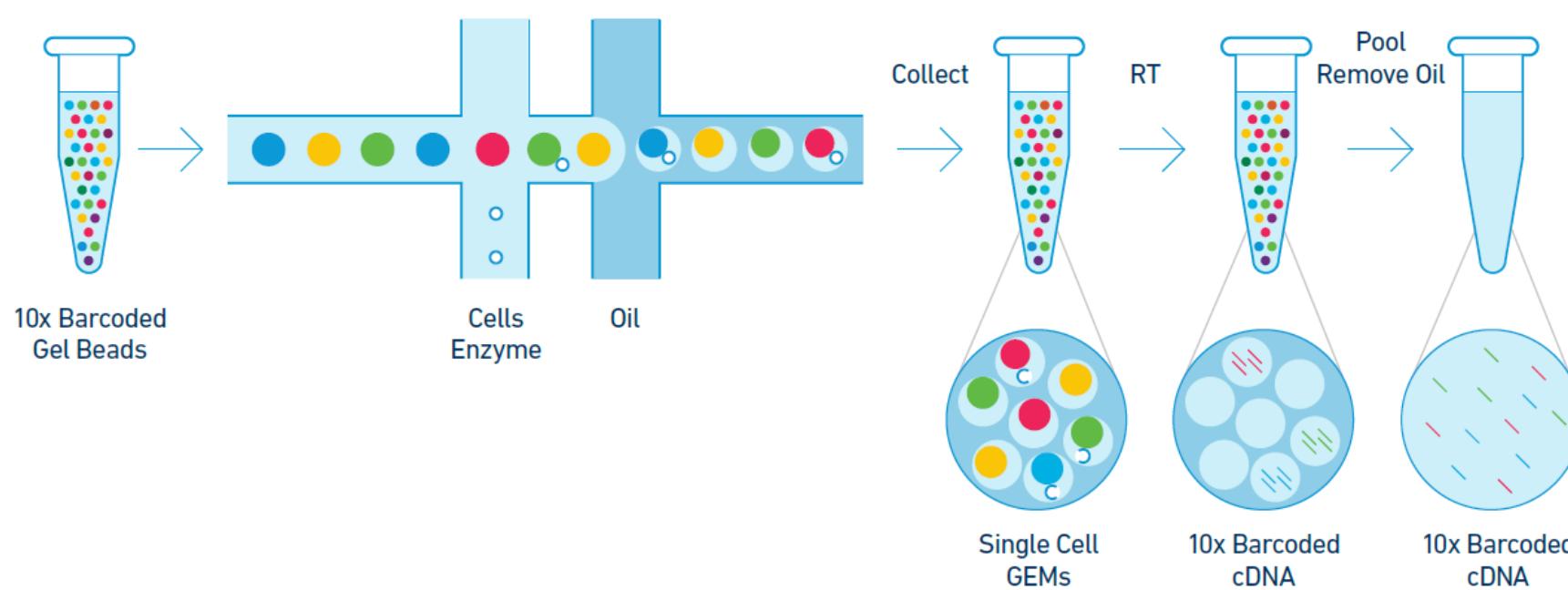
- Lysis and reverse transcription occurs in the beads
- Samples are frozen after RT as RNA:DNA hybrid in gel
- Library prep is based on CEL-seq method

scRNA-seq Library Structure (inDrops)

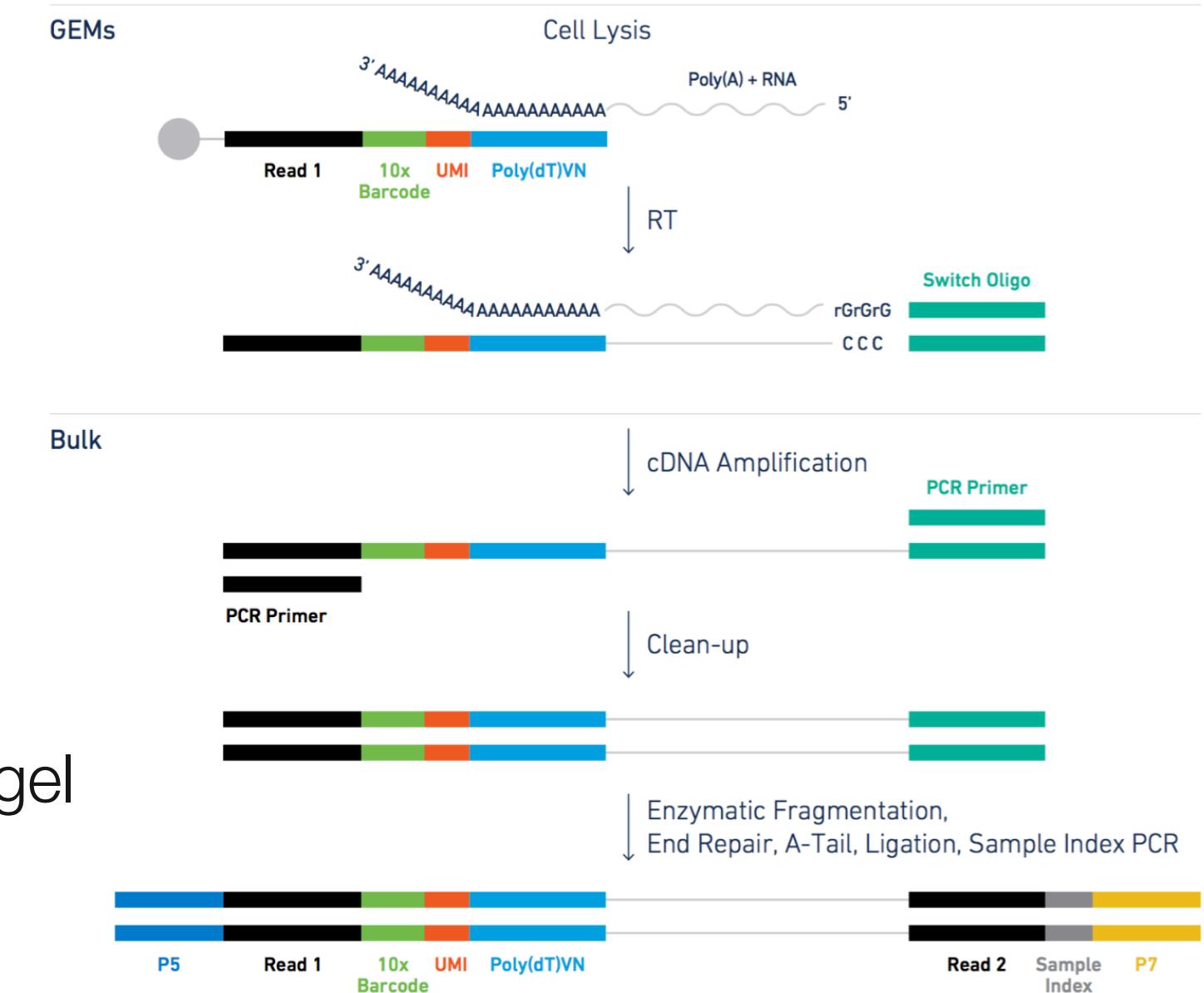


Sequencing Read	Description	Number of Cycles	Notes
Read1	Insert (Transcript)	61	Can read longer into transcript if desired
I7 index	Single Cell Barcode	8	Reads first half of barcode
I5 index	Library Index	8	Distinguish multiplexed samples
Read 2	Single Cell Barcode & UMI	14	Reading longer will read into PolyA tail.

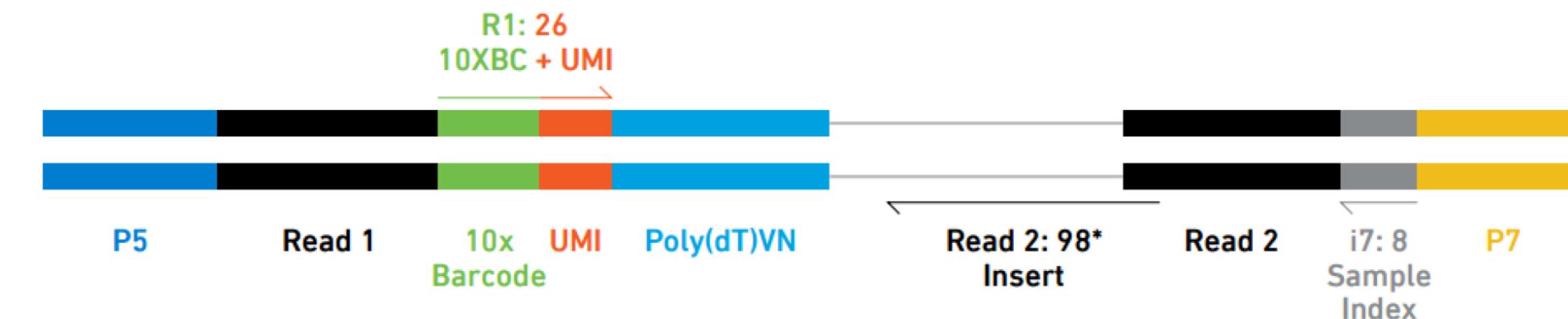
10x Genomics Method Overview



- Lysis and reverse transcription occurs in the beads
- Samples are frozen after RT as RNA:DNA hybrid in gel
- Library prep is similar to SMART-seq method
- GEM = Gel Beads-in-emulsion



10x Genomics Method Overview



Sequencing Read	Description	Recommended Number of Cycles	Notes
Read 1	10x Barcode Read (Cell) and Randomer Read (UMI)	26 cycles	It cannot be shorter than 26 bp If longer than 26 bp it will be ignored by Cell Ranger
i7 Index	Sample Index Read	8 cycles	If longer than 8 bp it will need to be trimmed during base calling
i5 Index	N/A	0 cycles	N/A
Read 2	Insert Read (Transcript)	98 cycles	Can be adjusted*

*User controlled trade-off between read length and mapping rate

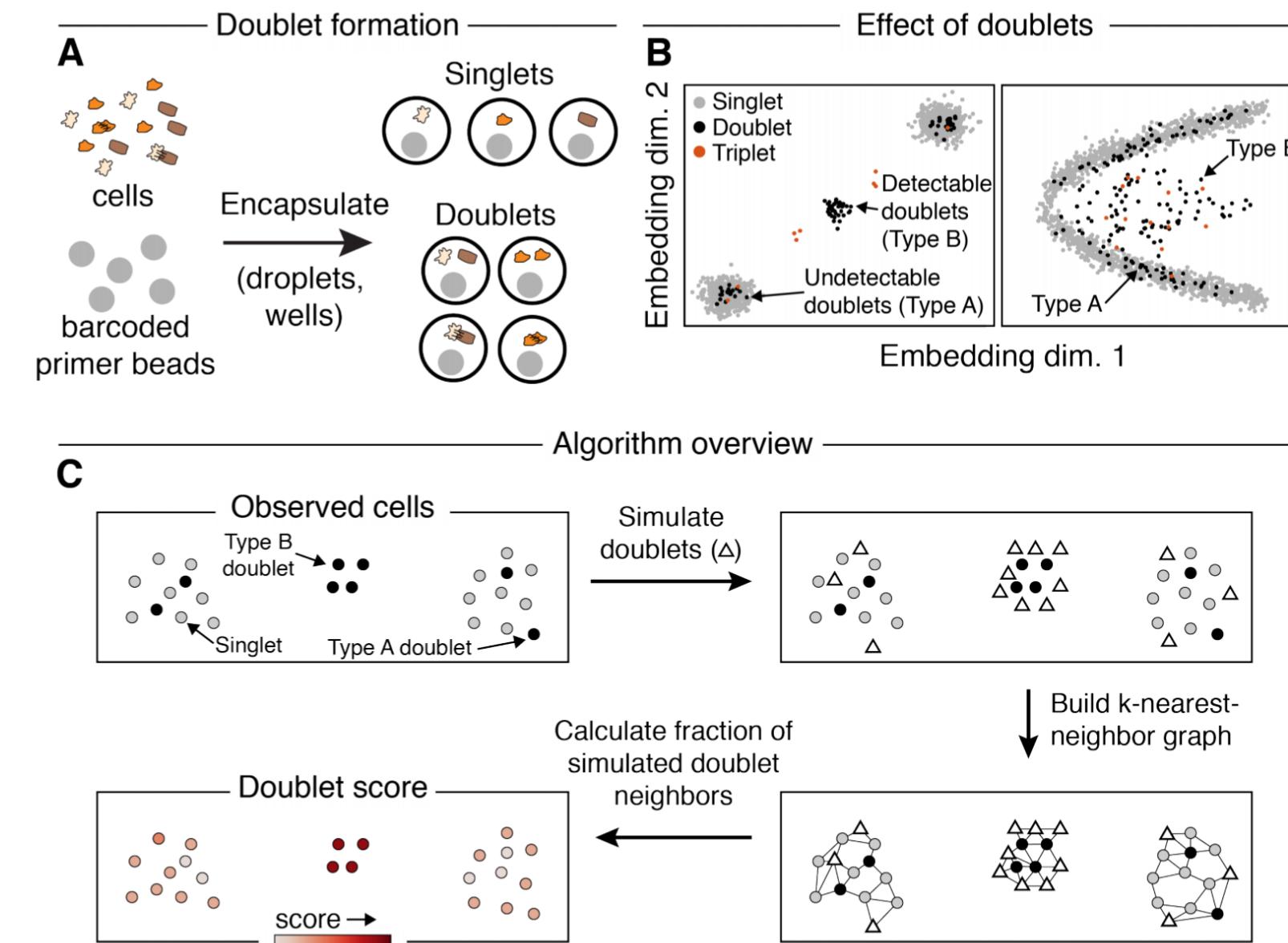
Doublets / Cell Density

- Rate of doublets depends on the cell density and the flow rate used for encapsulation.

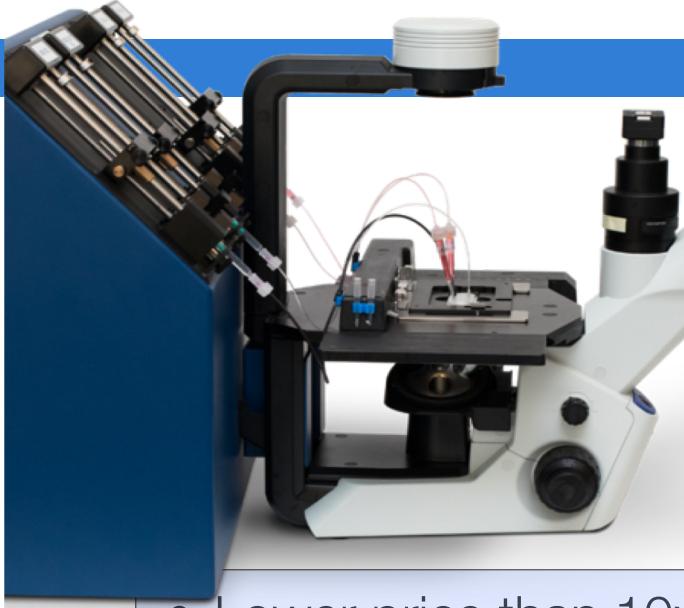
Multiplet Rate (%)	# of Cells Loaded	# of Cells Recovered
~0.4%	~870	~500
~0.8%	~1700	~1000
~1.6%	~3500	~2000
~2.3%	~5300	~3000
~3.1%	~7000	~4000
~3.9%	~8700	~5000
~4.6%	~10500	~6000
~5.4%	~12200	~7000
~6.1%	~14000	~8000
~6.9%	~15700	~9000
~7.6%	~17400	~10000

- “Sweet spot” for loading a 10x is recovery of 5000-6000 cells.

Scrublet: Computational Identification of Doublets



inDrops vs. 10x Genomics



inDrops

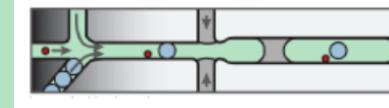
- Lower price than 10x
- Sample cost depends on number of cells and libraries prepped.
- Ability to collect sample backups at reduced cost per sample.
- Samples run 1 at a time for ~20 minutes per 6000 cell sample
- Observe ~1/2 the genes/cell of 10x
- Customizable



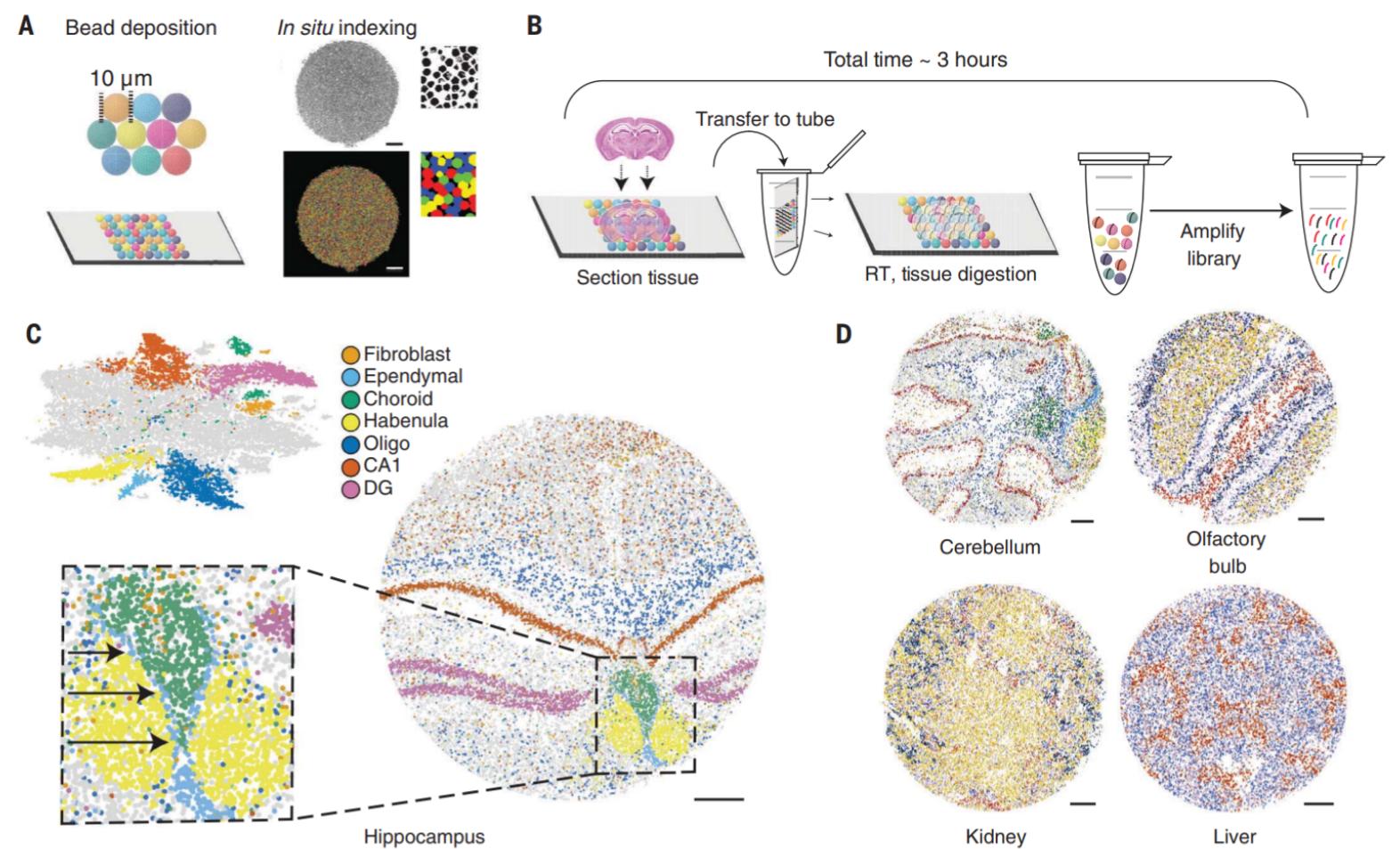
10x Genomics

- Higher price, reduced with hashing.
- Sample cost is per sample, number of cells not part of cost.
- Backup samples come at full sample cost.
- Up to 8 samples run in parallel in 10 minutes
- Observe 2x genes/cell than inDrops
- Out-of-the-Box

Comparison of Single Cell Methods

	inDrops	10x	Drop-seq	Seq-well	SMART-seq
Cell capture efficiency	~70-80%	~50-65%	~10%	~80%	~80%
Time to capture 10k cells	~30min	10min	1-2 hours	5-10min	--
Encapsulation type	Droplet 	Droplet 	Droplet 	Nanolitre well 	Plate-based 
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Weaknesses	Difficult to run	Expensive	Difficult to run & low cell capture efficiency	Still new!	Expensive

On the Horizon: Spatial Transcriptomics



- All methods are in their infancy.
- Require fresh-frozen tissue sections.
- Kharchenko lab working on setting up Slide-seq (10 micron resolution)
- Slide-seq recovers 100's genes/cell
- 10x Visium (50-100 micron resolution)

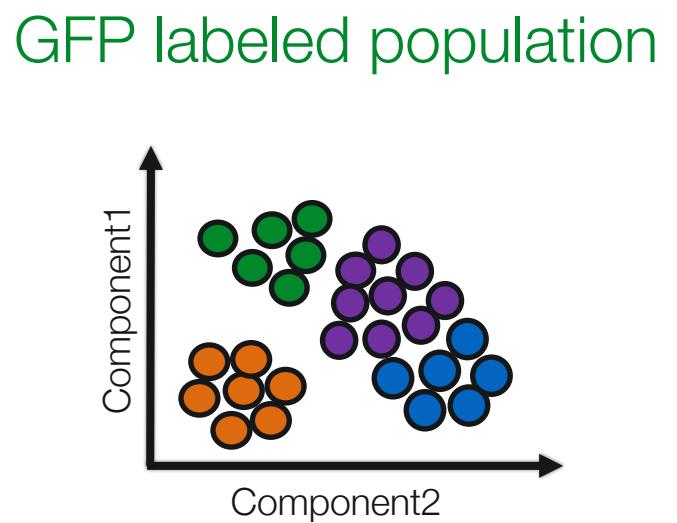
Rodrigues et al, Slide-seq: A scalable technology for measuring genome-wide expression at high spatial resolution.
Science. 2019 Mar 29;363(6434):1463-1467.

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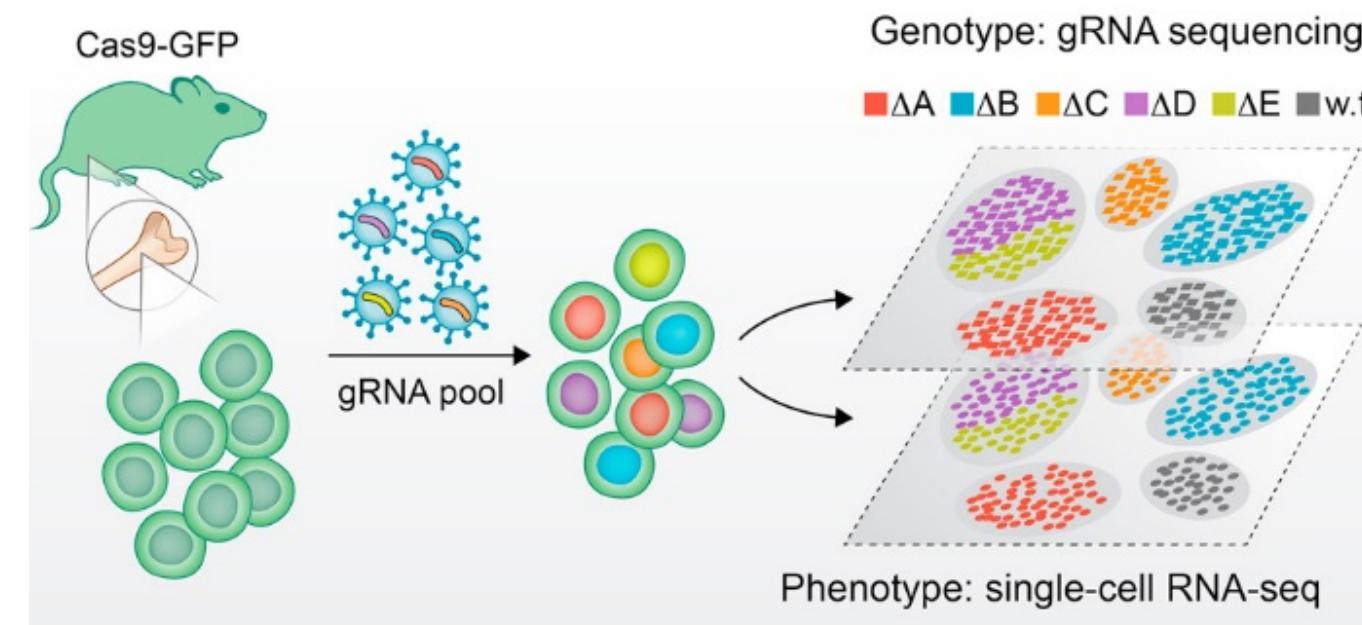
Transcript Specific Library Prep

- Identify cells in pooled CRISPR screens
- Identify barcoded cells
- V(D)J immune cell profiling
- Identify labeled (GFP, mCherry) cells.
- Enrich for genes of particular interest for your experiment.



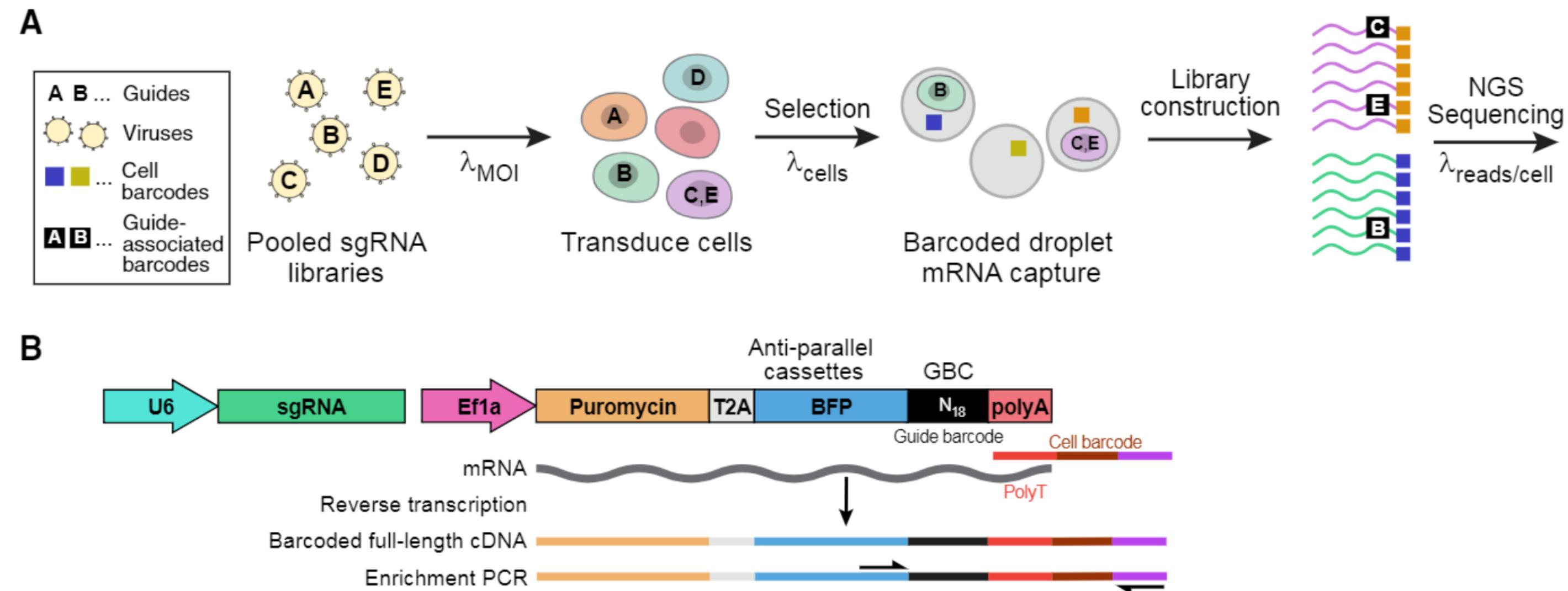
Transcript Specific Library Prep

- CRISPR pool vector backbone must contain a transcribed poly-adenylated unique guide index (UGI), which can include a fluorescent marker



- scRNA-seq library to phenotype cellular transcriptome (NextSeq/HiSeq)
- gRNA-targeted library to ensure proper cell identification (MiSeq)

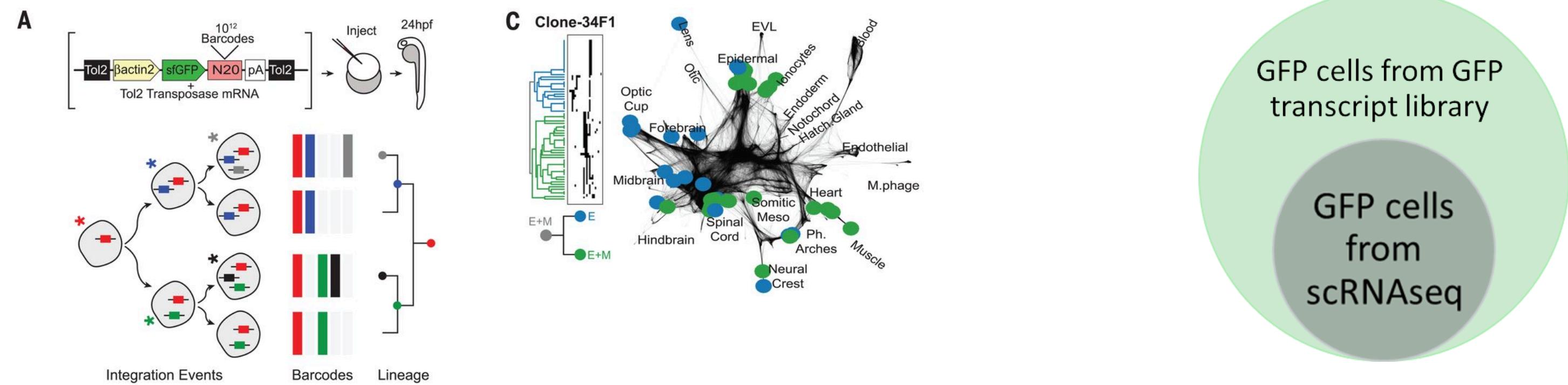
Transcript Specific Library Prep



Transcript Specific Library Prep

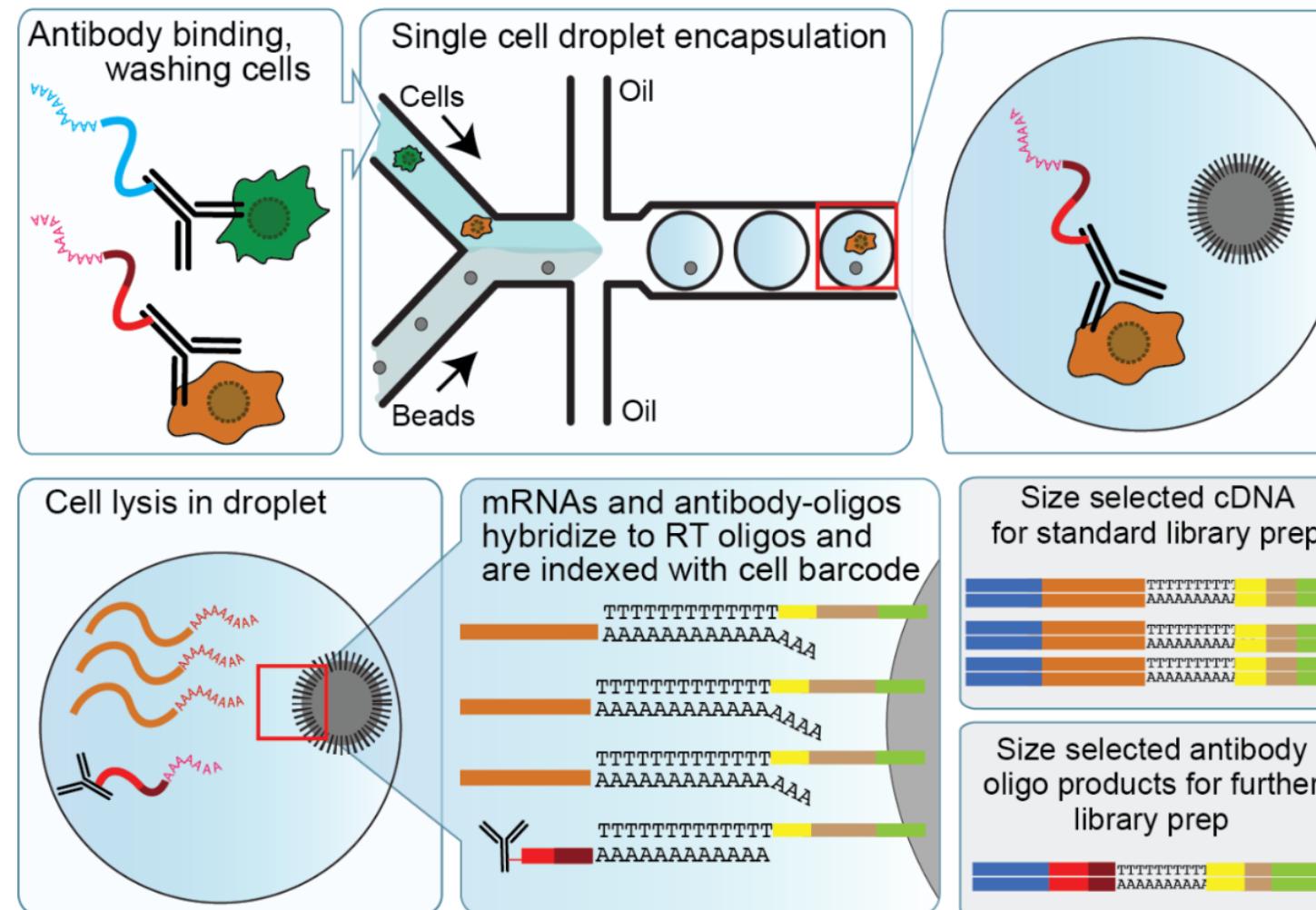
- Make standard library
 - NextSeq/HiSeq/NovaSeq sequencing to identify cell barcodes in sample
- Make transcript specific library with aliquot of partially prepped library
 - MiSeq to identify cells with transcripts of interest or spike back into full transcriptome sample
- Match barcodes identified in both sequencing runs

Transcript Specific Library Prep



- Transcript of interest often not observed all cells it was expressed in.
 - Specifically amplify desired transcript to identify more cells of interest.
 - Recover double the number of GFP cells with the addition of a transcript specific library

CITE-seq / Cell Hashing

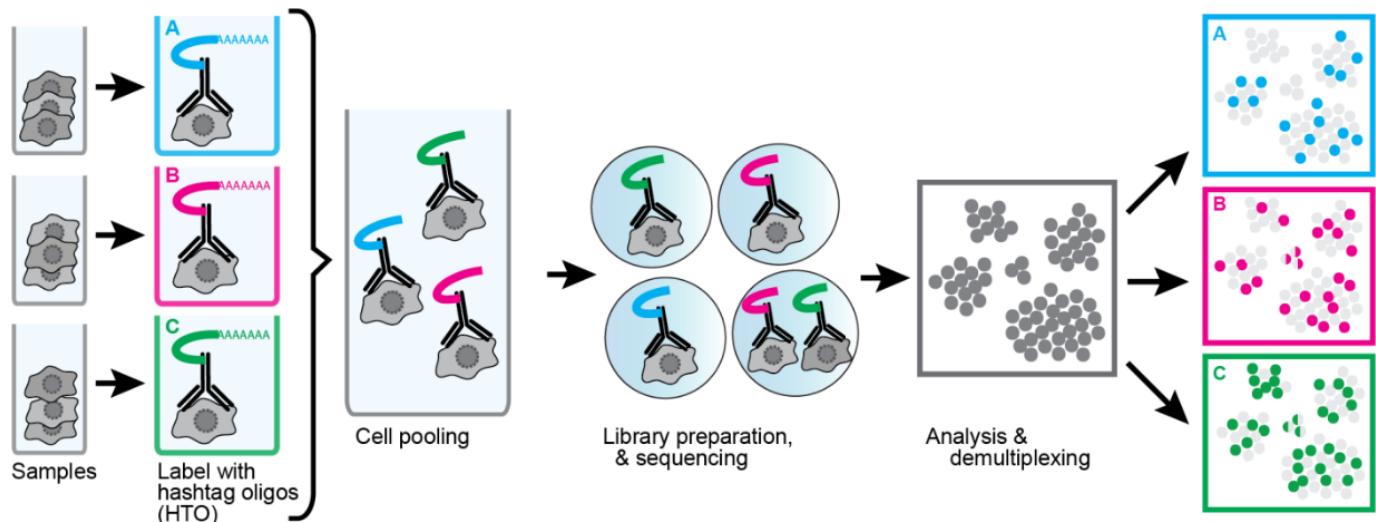


- Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-seq)
- CITE-seq uses DNA-barcoded antibodies to convert detection of proteins into a quantitative, sequenceable readout.

Cell Hashing / CITE-seq

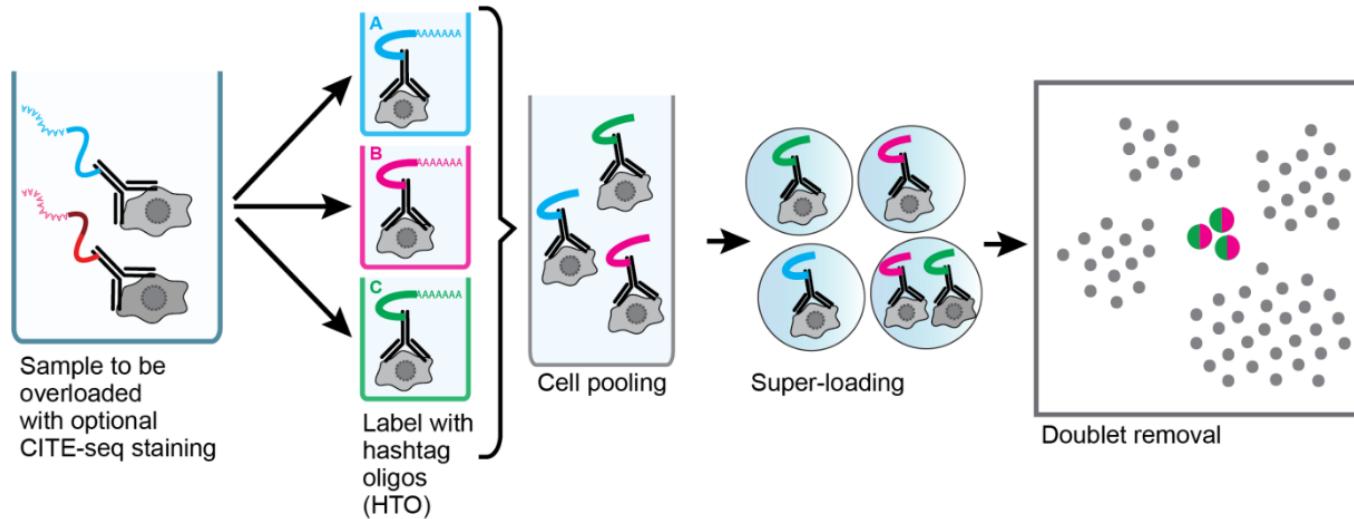


Sample multiplexing schematic:

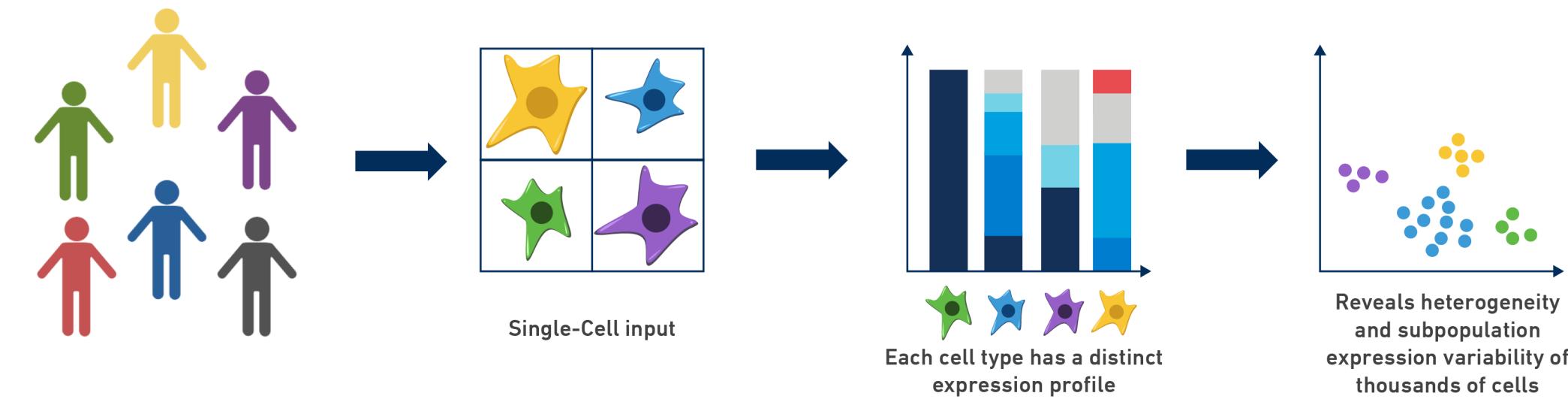


- Cell Hashing is the same idea as CITE-seq just using ubiquitously expressed surface proteins.
- Allows for multiplexing samples into a single encapsulation.
- By sequencing tags alongside the cellular transcriptome, we can assign each cell to its sample of origin, and robustly identify doublets originating from multiple samples.

Sample super-loading schematic:

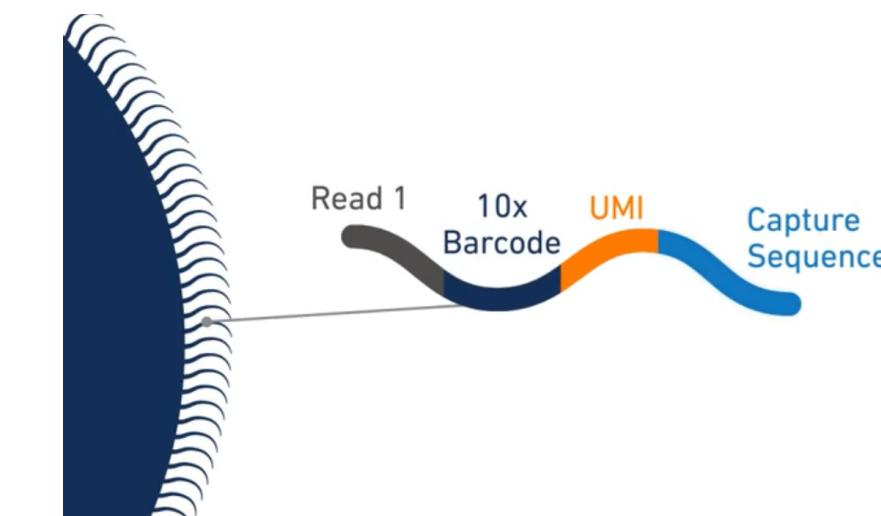
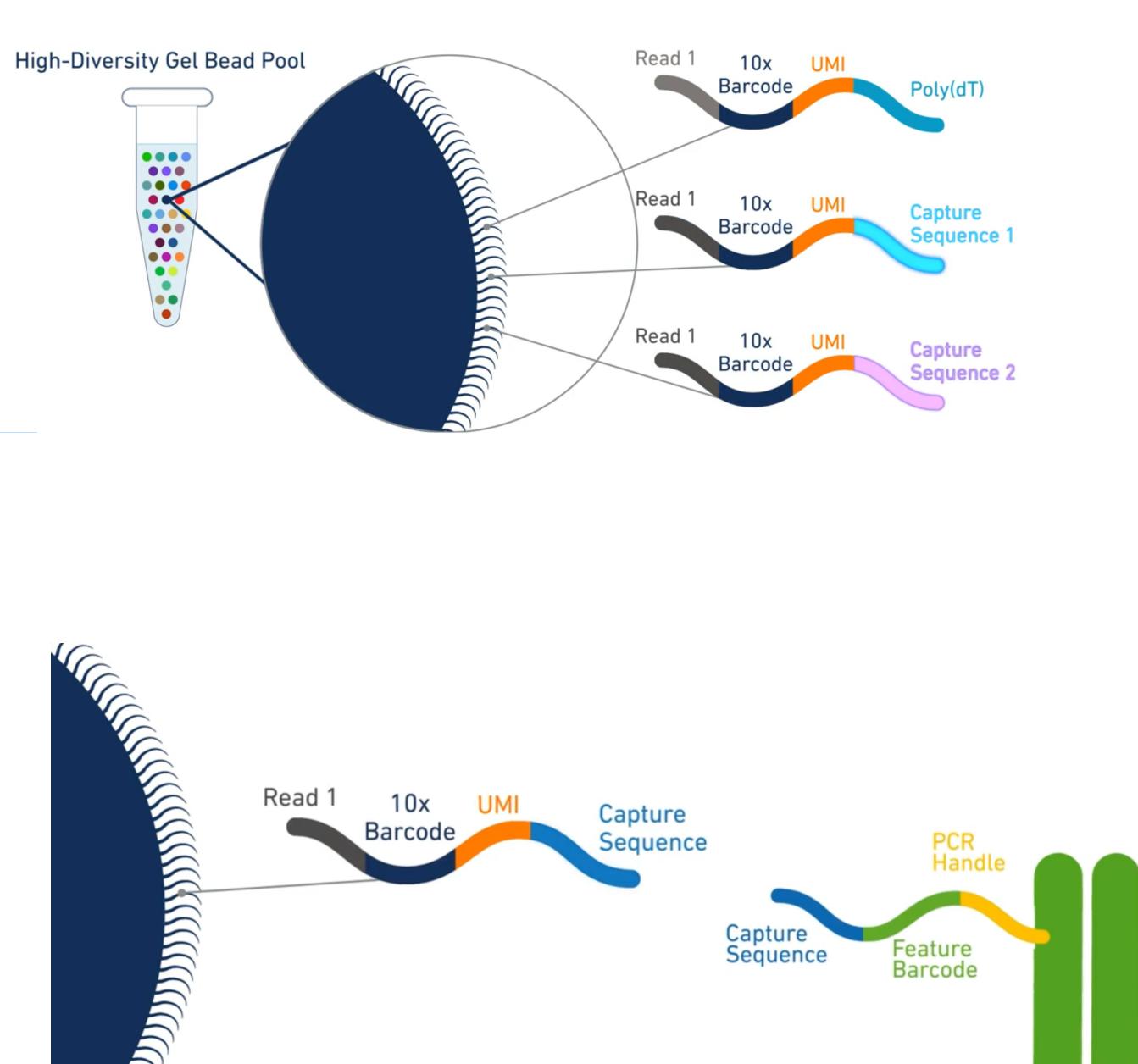


Label-Free Multiplexing of Patient Samples



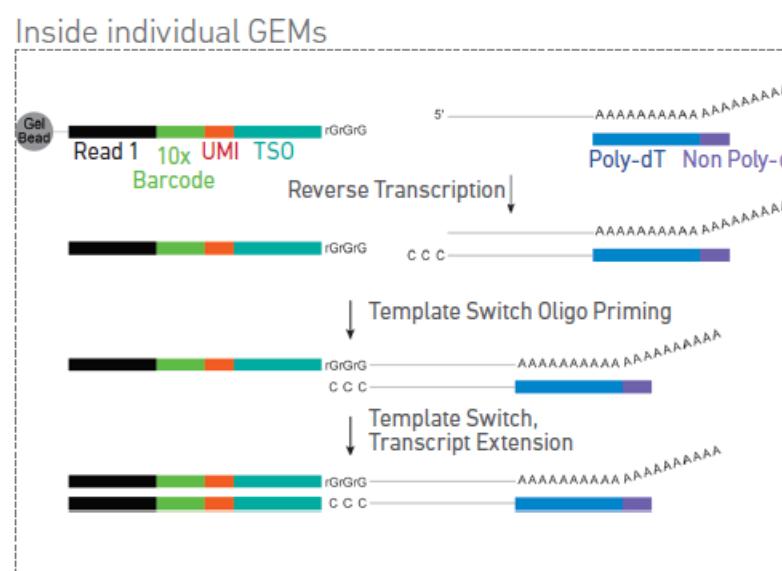
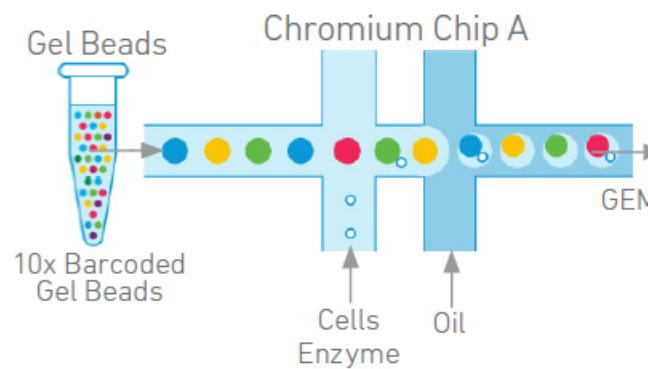
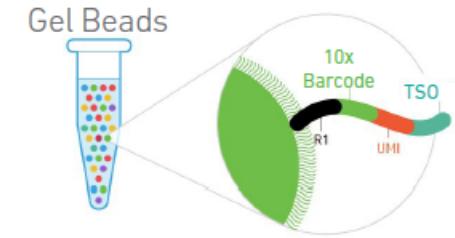
- Genetic differences between patient samples are inferred directly from scRNA-seq data to demultiplex samples.
- Will not be able to link patient data back unless have data on patient SNPs.
- Allows for super-loading and doublet identification.

10x Capture Sequence / Feature Barcode



- V3 10x kits offer specific capture sequences you can engineer into your system.
- CITE-seq, CRISPR pools, etc.

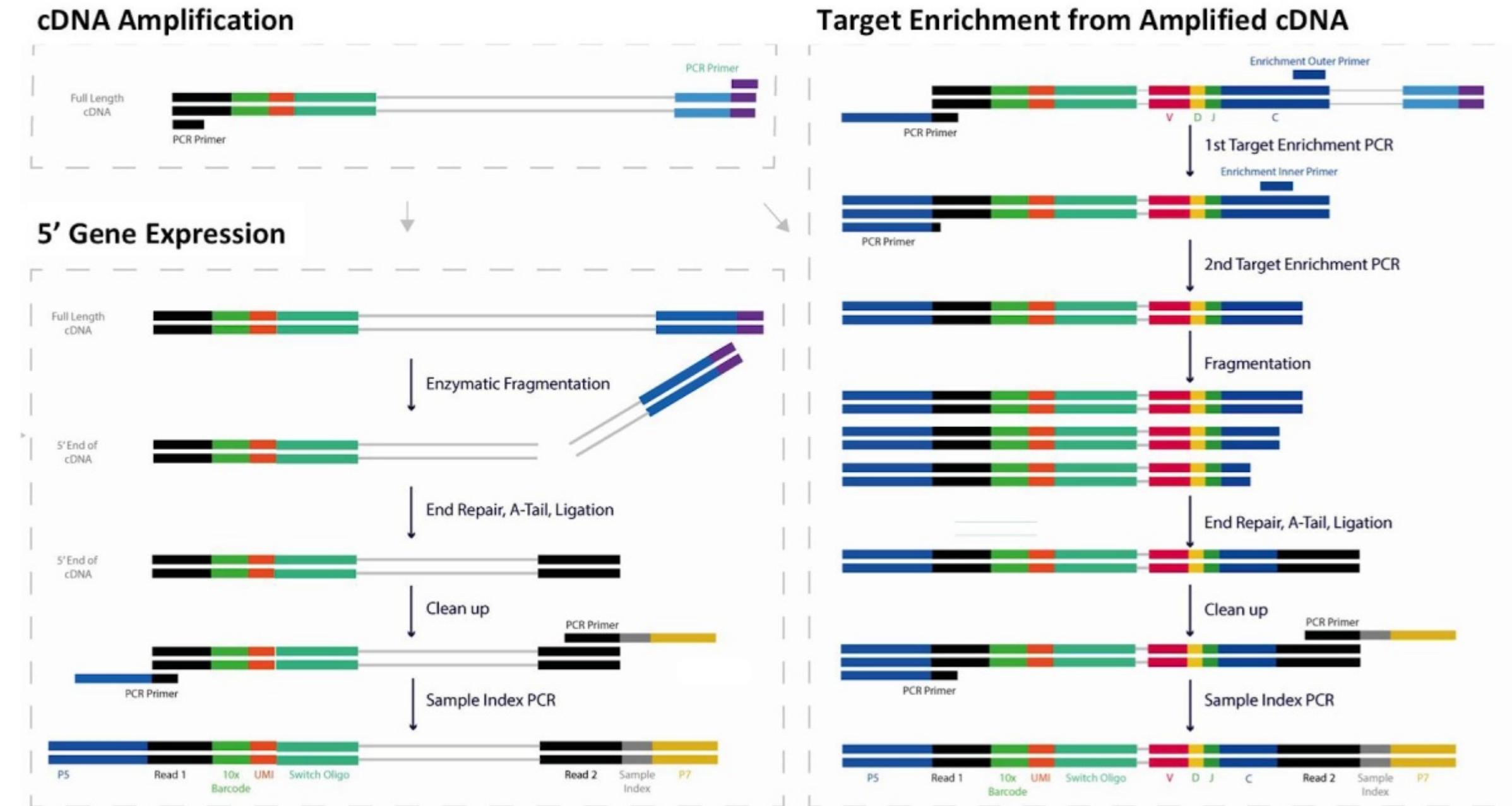
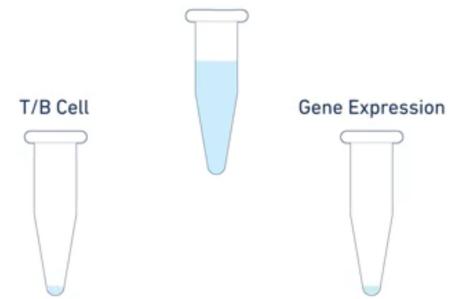
10x V(D)J Immune Profiling & 5' gene expression



- 3' gene expression assay the polydT sequence attached to gel bead.
- 5' assay the polydT is supplied as an RT primer.
- 5' VDJ kit: profiles full length (5' UTR to constant region) paired T-cell receptor (TCR), or B-cell immunoglobulin (Ig) transcripts.
- If both T and B cells are expected to be present in the cell population, TCR and Ig transcripts can be enriched in separate reactions from the same amplified cDNA material.

10x V(D)J Immune Profiling

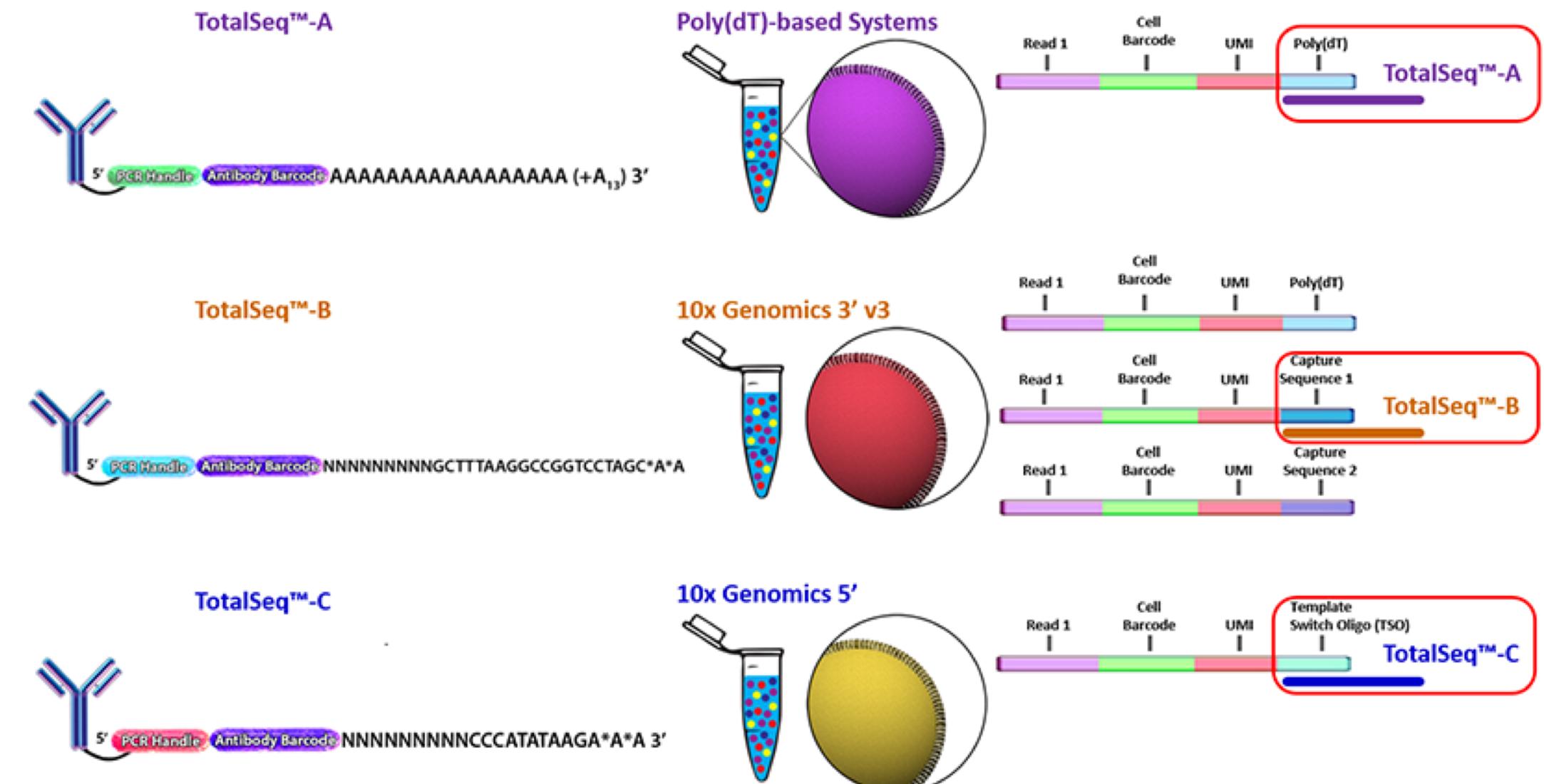
From the same 10x Barcoded cDNA



TotalSeq



- TotalSeq – A: captures using a polyA Tail; 10x 3' transcriptome V2 or V3
- TotalSeq – B: captures using sequence 1; 10x 3' transcriptome V3
- TotalSeq – C: captures using sequence TSO; 10x 5' transcriptome V3



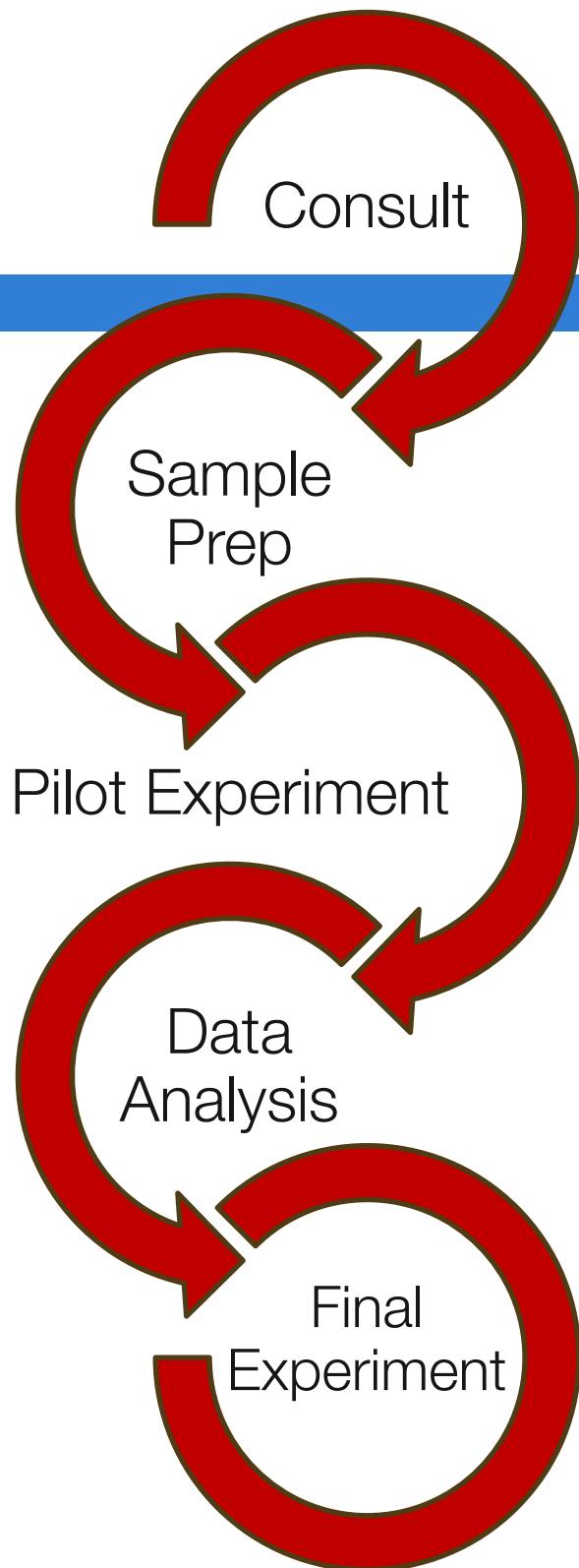
<https://kb.10xgenomics.com/hc/en-us/articles/360019665352-What-is-the-difference-between-TotalSeq-A-B-and-C->
<https://www.biologgend.com/en-us/totalseq>

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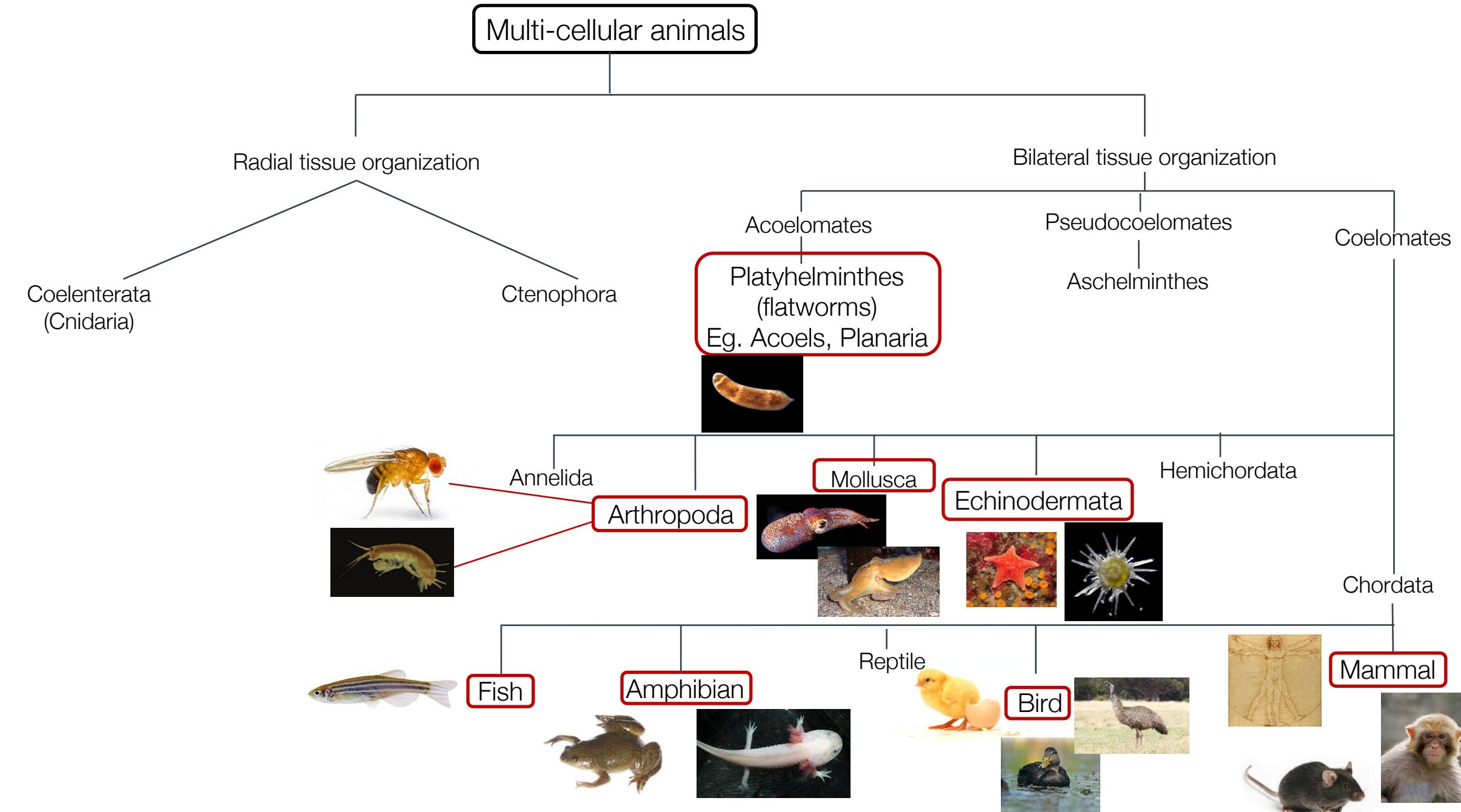
- Common applications of single cell RNA sequencing.
- Overview of single cell RNA sequencing platforms.
- Modified scRNA-seq workflows
- Sample preparation and experimental design.
- Effects of sample prep and sample type on analysis.

Single Cell Core Workflow

- Good sample prep is the key to success.
- A well planned pilot experiment is essential for evaluating sample preparation and for understanding the required number of cells.
- Do not rush to the final experiment.



Single Cell Core Sample Repertoire

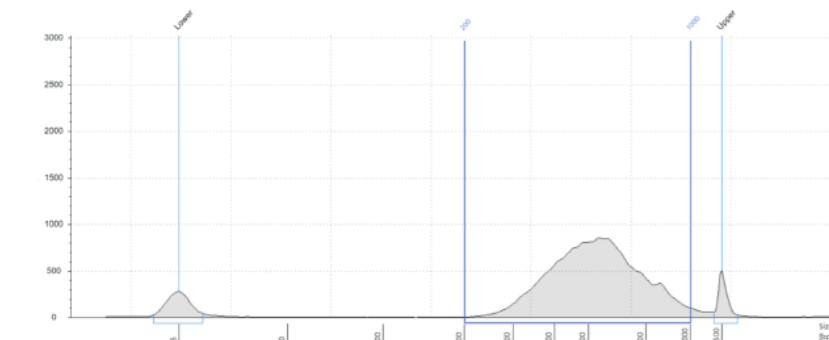


Single Cell Core Sample Repertoire

Primary cell/ tissue:

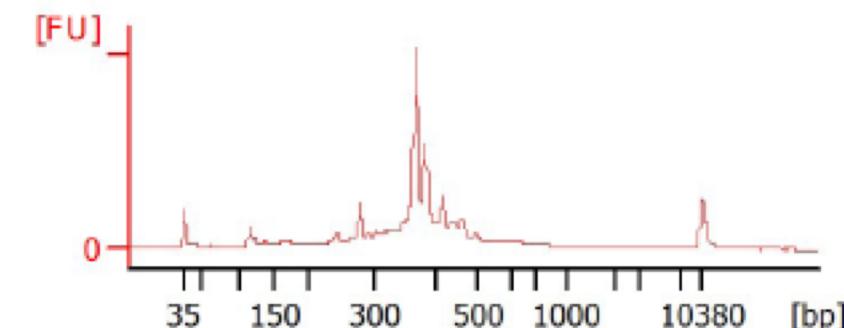
- Whole embryo
- Blood (immune cells)
- Bone marrow (hematopoietic stem cells)
- Embryonic stem cells
- Gut, Lung, Stomach, Colon (epithelium)
- Adipose tissue (non-adipocytes)
- Hepatocytes
- Brain and spinal cord (neurons, microglia, astrocytes)
- Thymus
- Various patient samples

Standard Library



Fly / Arthropod Library

S1- 1.1 PC



Cells/ tissues grown in vitro:

- Differentiated cells from iPSCs
- Organoids

Single nuclear samples:

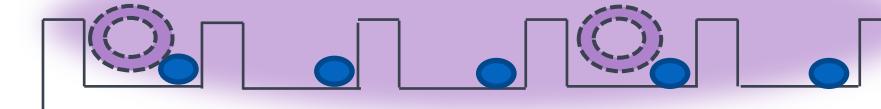
- Brain and Kidney

Key to Success: Sample Preparation

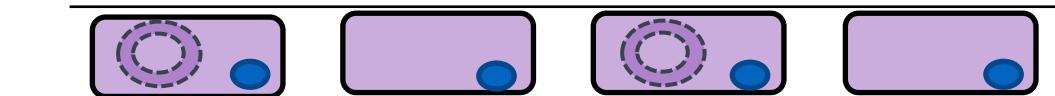
- High cell viability (>90-95% preferred)
- Minimal free-floating RNA
- Single cell suspension
- Dissociation protocol is cell type dependent
- Primary samples are much more difficult
- Cryopreservation or Nuc-seq are optional for some samples

Key to Success: Sample Preparation

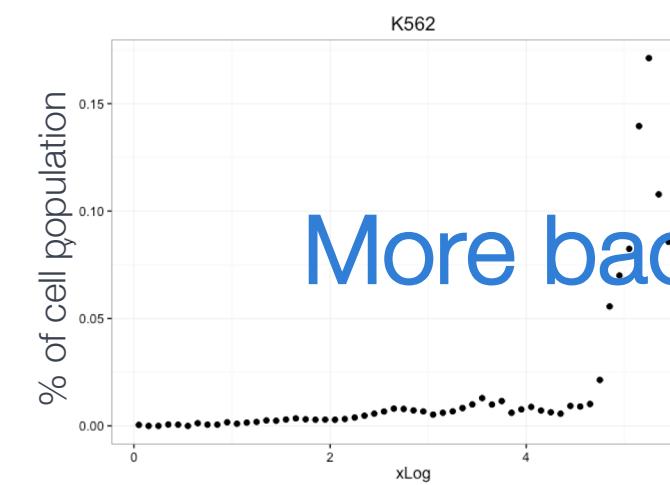
- Good single cell suspension. No clumps and minimal debris.
- More than 90% viability is ideal.
- Cell membrane integrity is required until they are encapsulated.



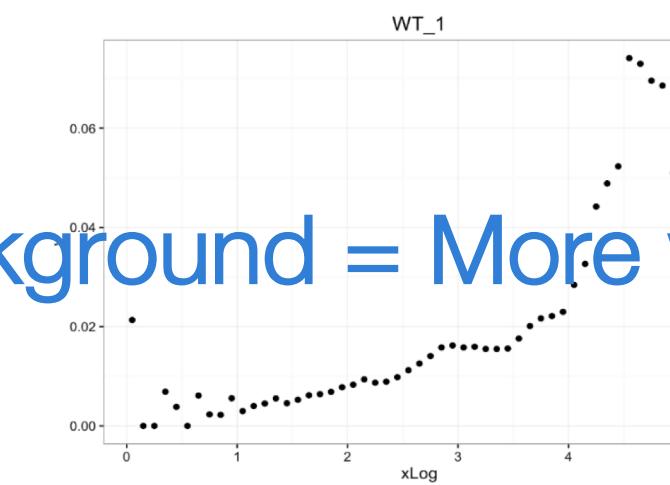
Nanowell-based barcoding platform



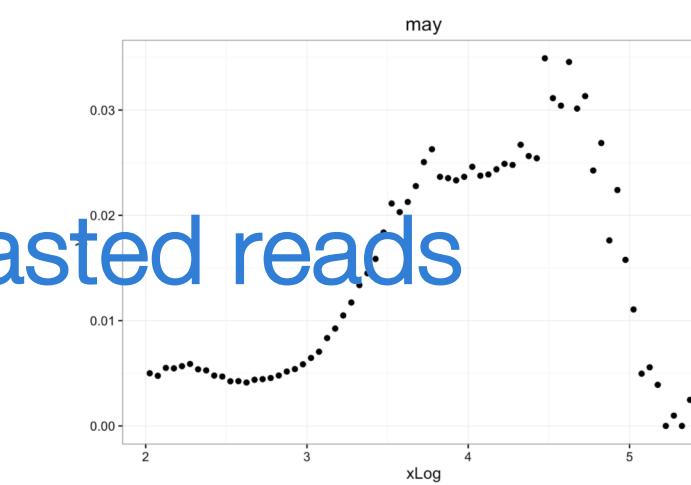
Droplet-based barcoding platform



Ideal data
Reads/ cell barcode

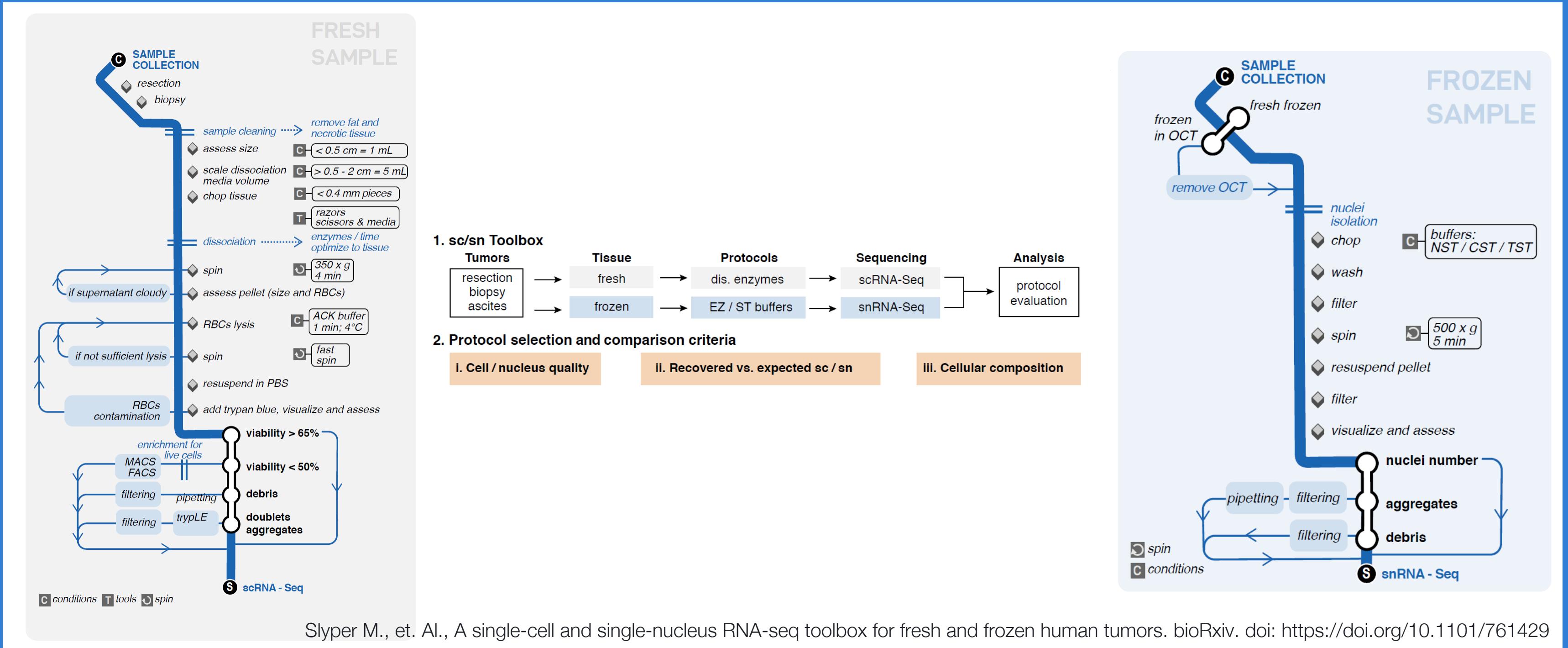


Reads/ cell barcode



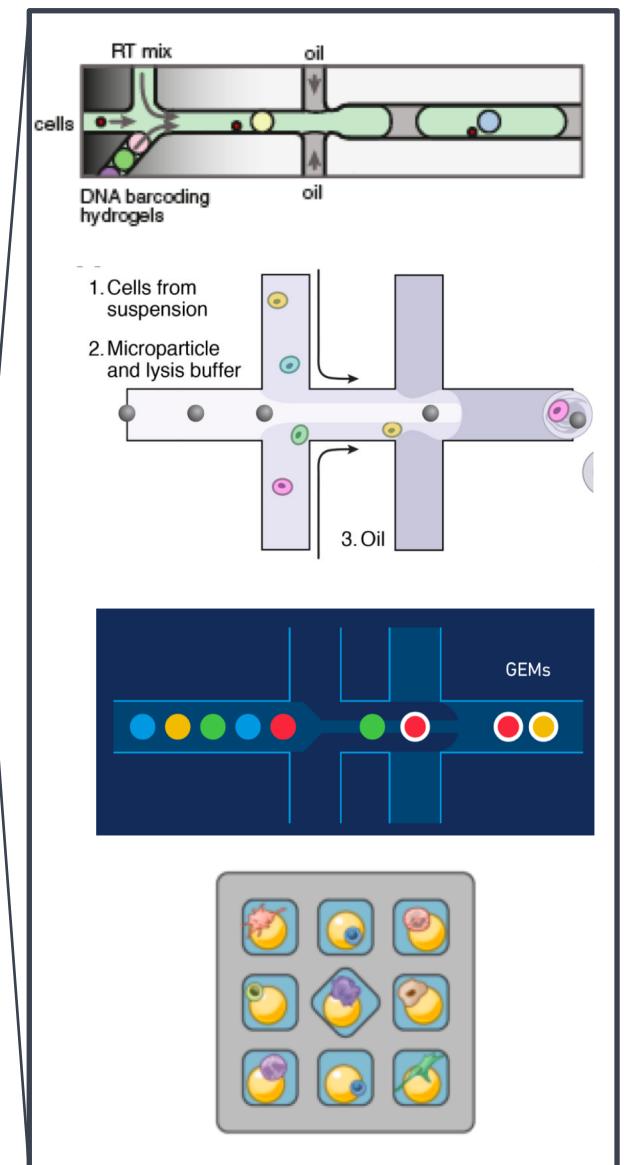
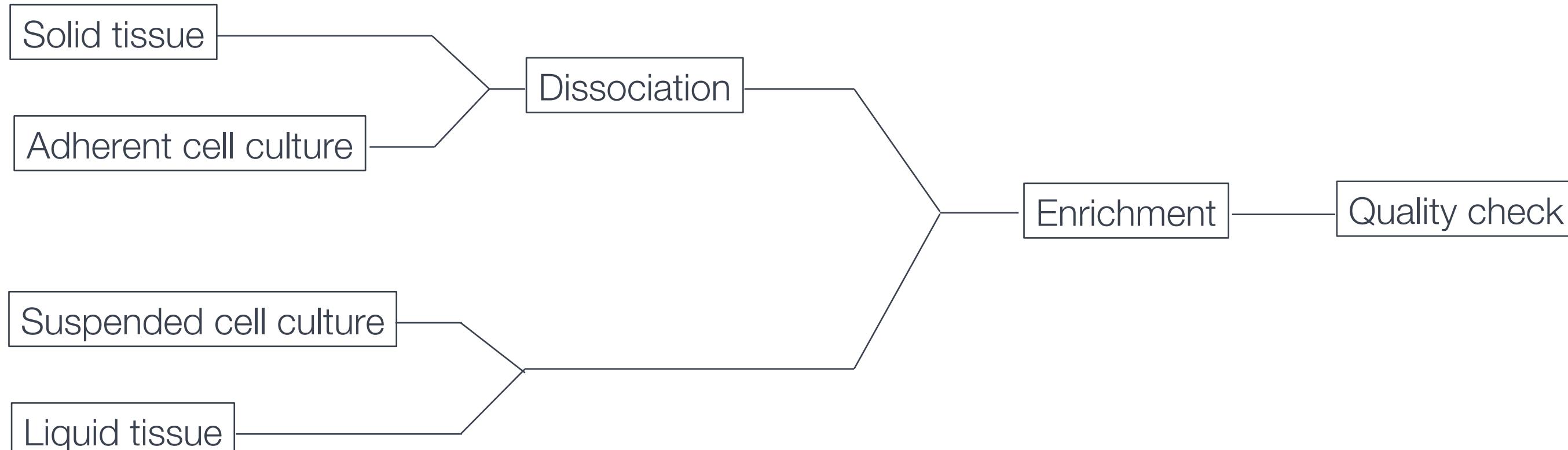
Free-floating RNA
Reads/ cell barcode

Sample Preparation Varies by Cell-Type



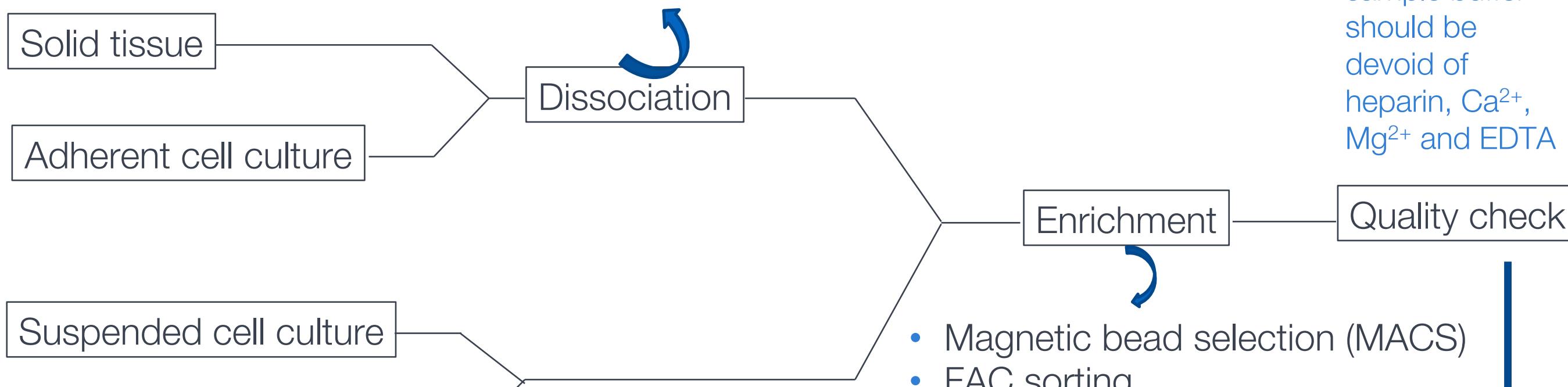
Key to Success: Sample Preparation

- What is your sample of interest? And how would you obtain that?
 - Which population in a tissue should be examined?
 - Does it require some sort of enrichment?



Sample preparation protocol varies by cell-type

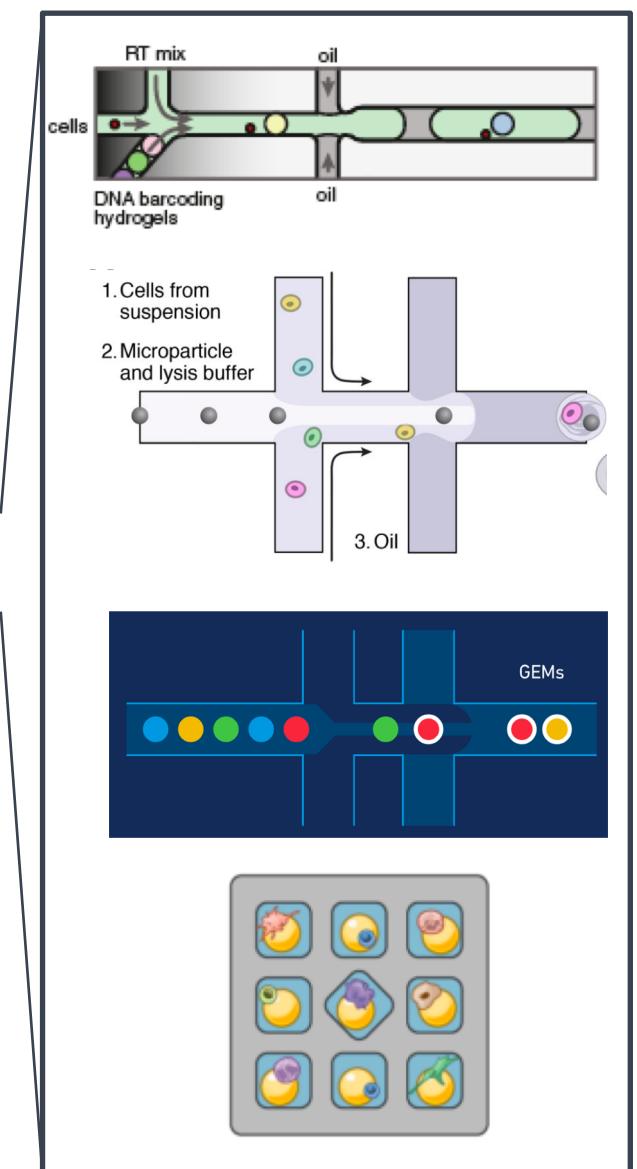
- Enzyme-based dissociation- trypsin, collagenase, liberase, accutase.
- Gentle washes.
- Dead cell removal kit, filtering out the debris.
- Density gradient (Ficoll, Optiprep)



Note: the final sample buffer should be devoid of heparin, Ca^{2+} , Mg^{2+} and EDTA

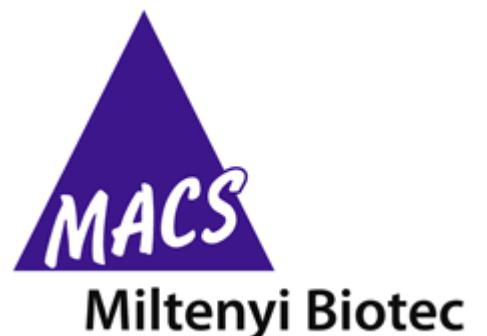
- Magnetic bead selection (MACS)
- FAC sorting

- Cell concentration
- Cell Viability (Trypan Blue)



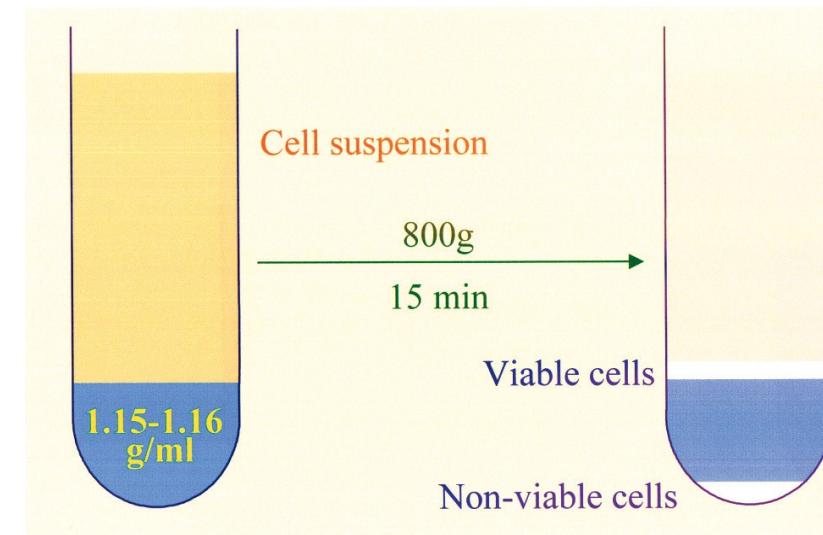
Sample Preparation: increasing cell viability

- Mild dissociation reagent (TrypLE, StemPro, Accutase, Liberase)
- Shorten dissociation time
- Reduce dissociation temperature
- Adding a DNase step can help reduce clumping of dead cells.
- Using ROCK inhibitor/ apoptosis inhibitor (esp. epithelial cells)
- Avoid cell pelleting
- Avoid FACS sorting on more fragile cell types
- Try magnetic activated cell sorting (MACS)



Sample Preparation: single cell suspension

- Use appropriate cell strainer to get rid off clumps.
- Use metabolically inert, non-toxic density gradient media to fractionate cells.
 - We routinely use 15% Opti-prep to keep cells in single cell suspension while loading sample, but the concentration might vary from cell to cell.



- Make sure final buffer does not contain calcium, EDTA, or heparin (inhibit RT).

Enrichment Methods: pros & cons

FACS (Fluorescence activated cell sorting)

Pros:

- Enrichment is robust.
- Yields good single cell suspension.
- Live/dead sorting by DNA stains, eg. DAPI.

Cons:

- Uses high pressure to sort the cells.
- Can introduce bias in the experiment.
- Long sample prep protocol.

MACS (Magnetic activated cell sorting)

Pros:

- Gentle on cells and faster protocol
- Greater number of cells can be processed.
- Not limited by FACS sorter availability.

Cons:

- Number of available surface marker-conjugated to magnet is limited.
- Enrichment is not precise. Not applicable for rare population.

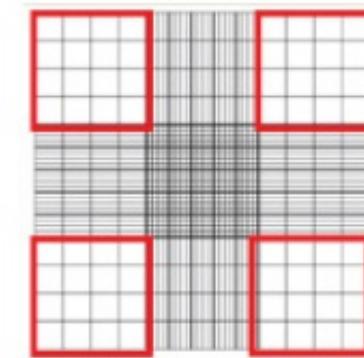
Enrichment Methods: cell staining

- Standard staining used for FACS
- DAPI staining to remove dead cells in sorting
- Live cell sorting: 7-AAD from Biolegend and Live 488 from Thermo
- Biotium's NucSpot 470 for nuclear staining; requires cell permeation

Can always test if your dye of interest will inhibit RT in a mock tube based reaction.

Sample Preparation: cell numbers

- Droplet methods have a 10,000-25,000 cell minimum
 - need ~50-100 cells with a unique transcriptome to identify a population cluster
 - 100-1000 cells per ul = 100,000-1,000,000 cells per ml
- Count cells by hemocytometer – do not trust sorter counts
 - counts from the sorter are often ½ of actual cell counts
- Try negative selection to remove unwanted cells
- Sort on a broader marker to increase cell numbers
- For unavoidably low density samples
 - spike the sample with cells with distinct expression profile



Sample Preparation: buffers

- Suggest final sample prepared in:
 - PBS with 0.1-1% BSA
 - Defined media without calcium, magnesium, or EDTA
- 2% FBS in defined media without calcium or EDTA.
- Make sure buffer does not contain calcium, magnesium, EDTA, or heparin (inhibit RT).

Sample Preparation: viability checks

- Check viability of sample over time
 - If viability decreases over a short period of time this will be reflected in transcriptional data.
 - Will see high mitochondrial read counts.
- Check single cell suspension supernatant for the presence of free floating RNA (Ribogreen)
 - Creates background noise in all samples and complicates analysis.
- Number of trypan positive cells × number of wasted sequencing reads
 - If 30% of your cells are dead at the time of encapsulation then at most you will be able to use 70% of your sequencing data.

Sample Preparation: dead cell removal

- FACS out dead cells
 - Will have all associated complications of FACS.
- Miltenyi dead cell removal kit
 - Magnetic beads used to remove dead cells & debris.

Food For Thought

- How many dead cells are you removing?
- What does this mean for the biology you are studying?
- Record your sample prep metadata!!!

Sample Preparation: cryopreservation

- Several papers have come out using various cryopreservation techniques on samples (PBMC's or cell lines).
- Success of cryopreservation is dependent on the sample type.
- Have seen this worked well on blood and immune cells.
- Key is the viability of the cells upon rehydration.
- Consider Nuc-seq as an option from cryopreserved cells.



Alles, J. et al. Cell fixation and preservation for droplet-based single-cell transcriptomics. *BMC Biol.* 2017 (doi: 10.1186/s12915-017-0383-5)
Guillaumet-Adkins, A. et al. Single-cell transcriptome conservation in cryopreserved cells and tissues. *Genome Biol.* 2017 (doi: 10.1186/s13059-017-1171-9)
Habib, N. et al. Massively parallel single-nucleus RNA-seq with DroNc-seq. *Nat. Methods* 2017 (doi: 10.1038/nmeth.4407)

Sample Preparation: cryopreservation

- The quality of the tissue at the time of freezing is a major factor in the quality of data downstream.
- Single Cell Core has seen best results with cells frozen in their standard growth media supplemented with 5% DMSO.
 - Primary cells observed to have 20% cell death upon thawing.
- If freezing tissue for use later, you may want to consider cryopreserving 50 mg tissue chunks in BAM Banker cryopreservative. (Core has not tested yet)

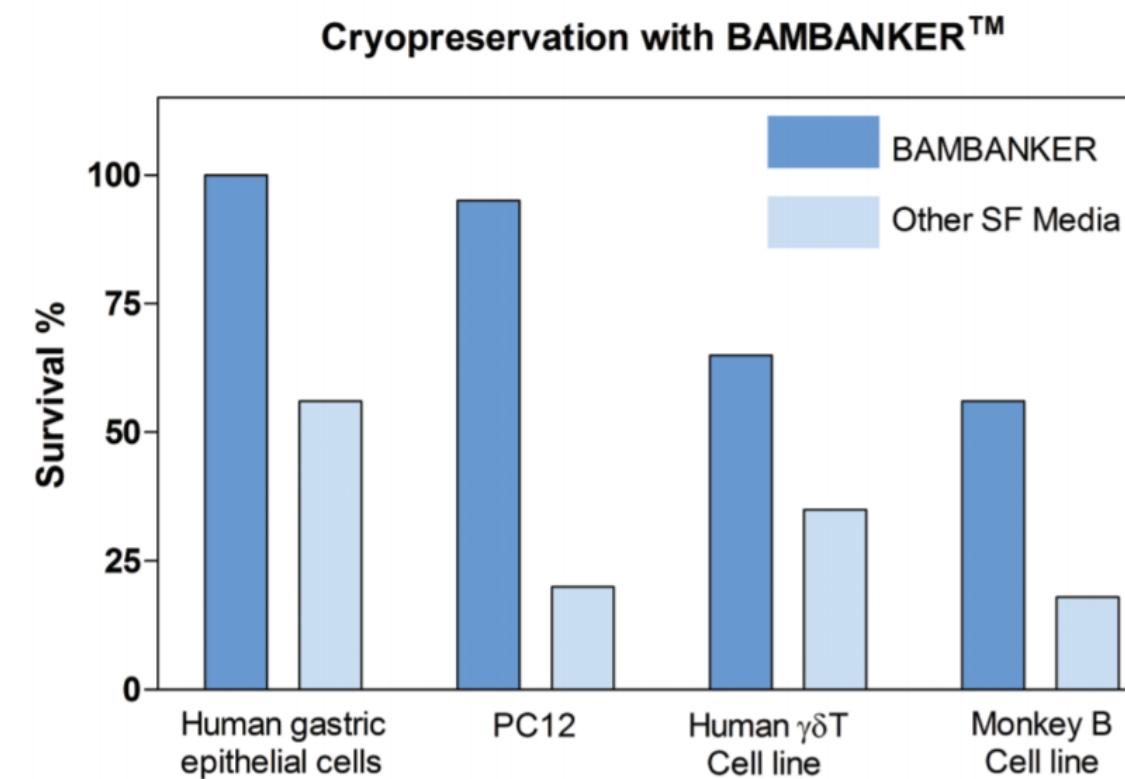
<https://www.protocols.io/view/isolation-of-nuclei-from-frozen-tissue-for-atac-se-6t8herw/guidelines>

Corces MR, et al. An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues. Nat Methods. 2017 Oct;14(10):959–962.

Sample Preparation: cryopreservation

BAMBANKER cell freezing media has been tested with numerous cell types and shown to deliver excellent cell recovery rates.

Cryopreservation tests with human gastric epithelial cells, PC12 (rat-derived pheochromocytoma) cell line, activated human $\gamma\delta$ T Cells and Monkey B Cell lines show improved cell survival using BAMBANKER cell freezing media when compared to another serum-free media.



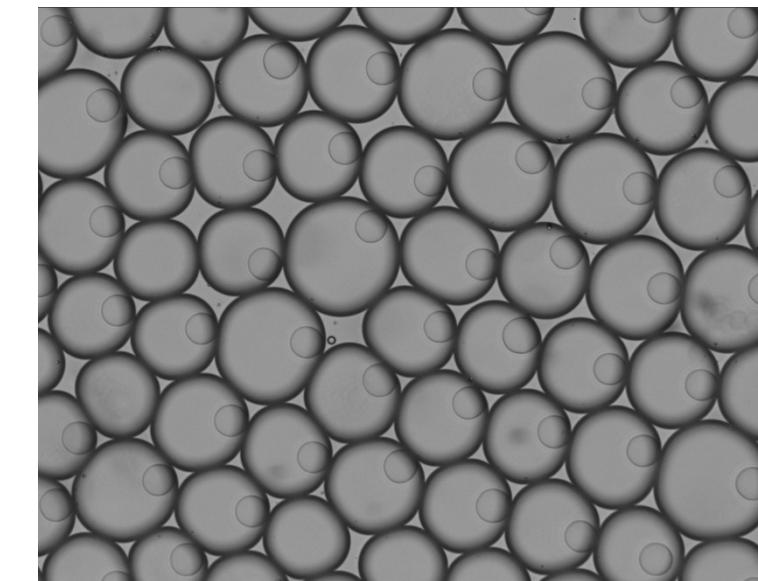
Also tested with Daudi (human B cell line), P3U1 (mouse myeloma cell line), K5625 (human leukaemia cell line), OKT4 (mouse hybridoma), activated lymphocytes derived from human peripheral blood and activated lymphocytes from mouse spleen.

BAMBANKER cell freezing media is available in two pack sizes to suit different usage patterns. For high volume users storing large batches of cells we recommend the 120ml pack size. Alternatively the pack of 5 smaller 20ml vials is ideal for individual use. To minimise cross contamination of cultures or culture facilities, separate vials could also be dedicated for use with specific cell lines or culture areas.

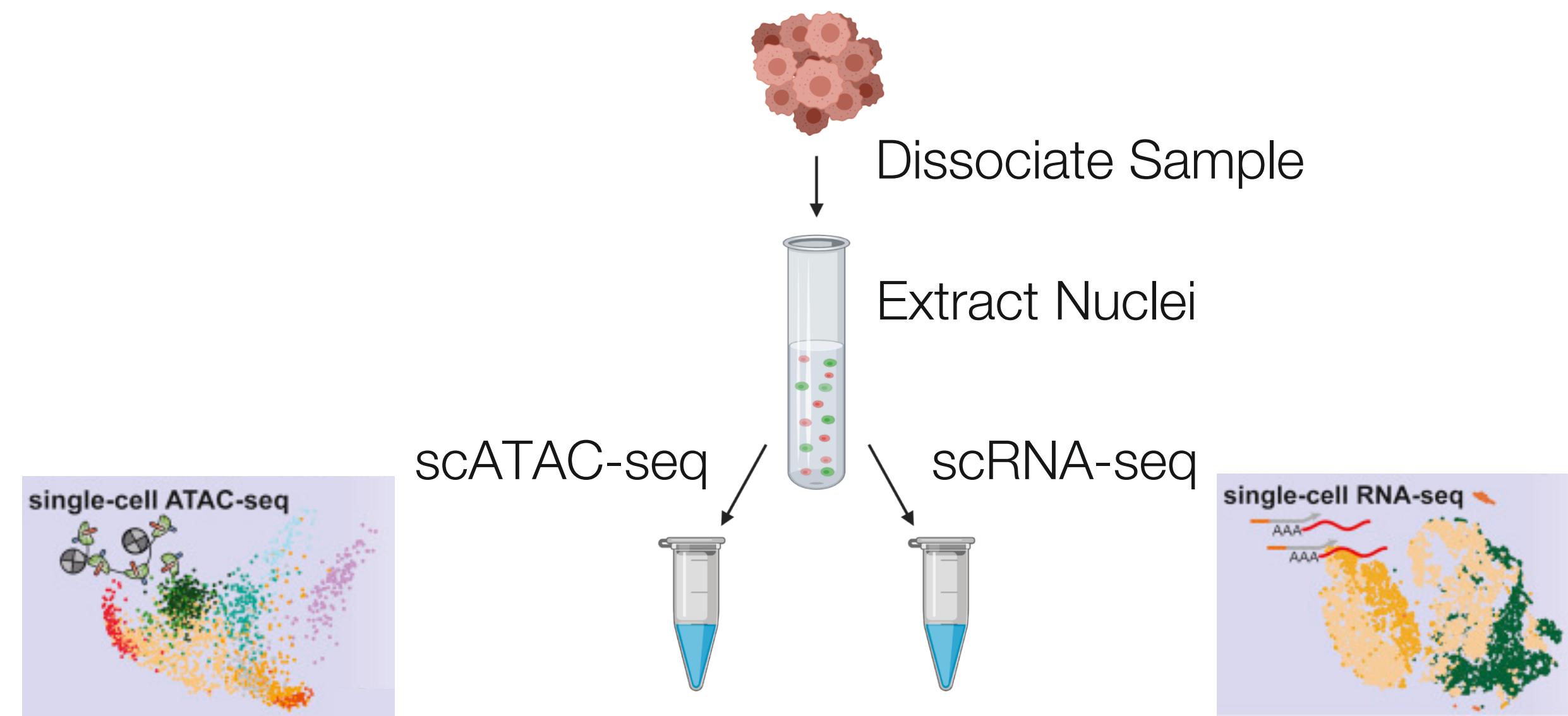
- Ready-to-use
- Freeze & store at -80 C
- Quality assured*

Sample Preparation: nuclear RNA sequencing

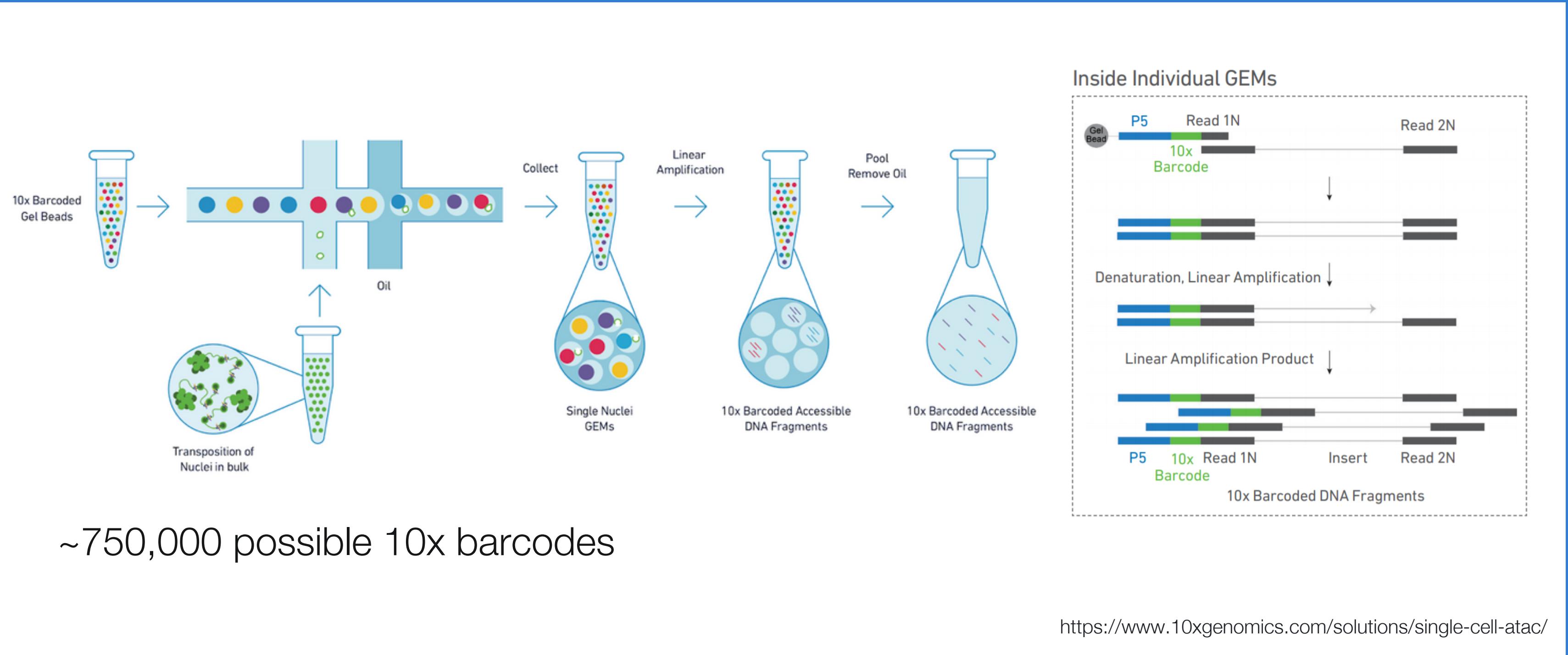
- Extract nuclei from sample of interest.
- Removes transcriptional noise from dead/dying cells.
- Most often used for neuronal samples.
- Good for flash frozen clinical samples.
- Several studies have shown nuclear transcripts represent a considerable portion of the whole cell transcripts.
- Analysis is more difficult due to presence of introns and non-coding RNA.



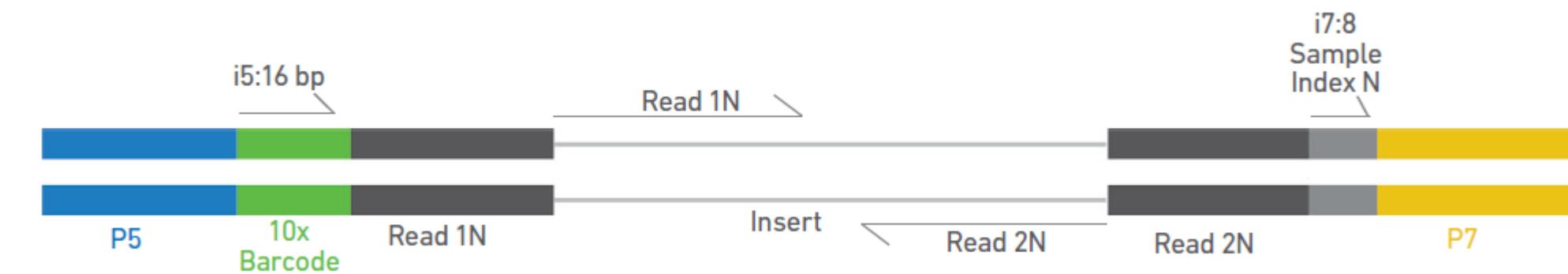
Nuclear Preparation



10x Genomics scATAC-seq Method Overview



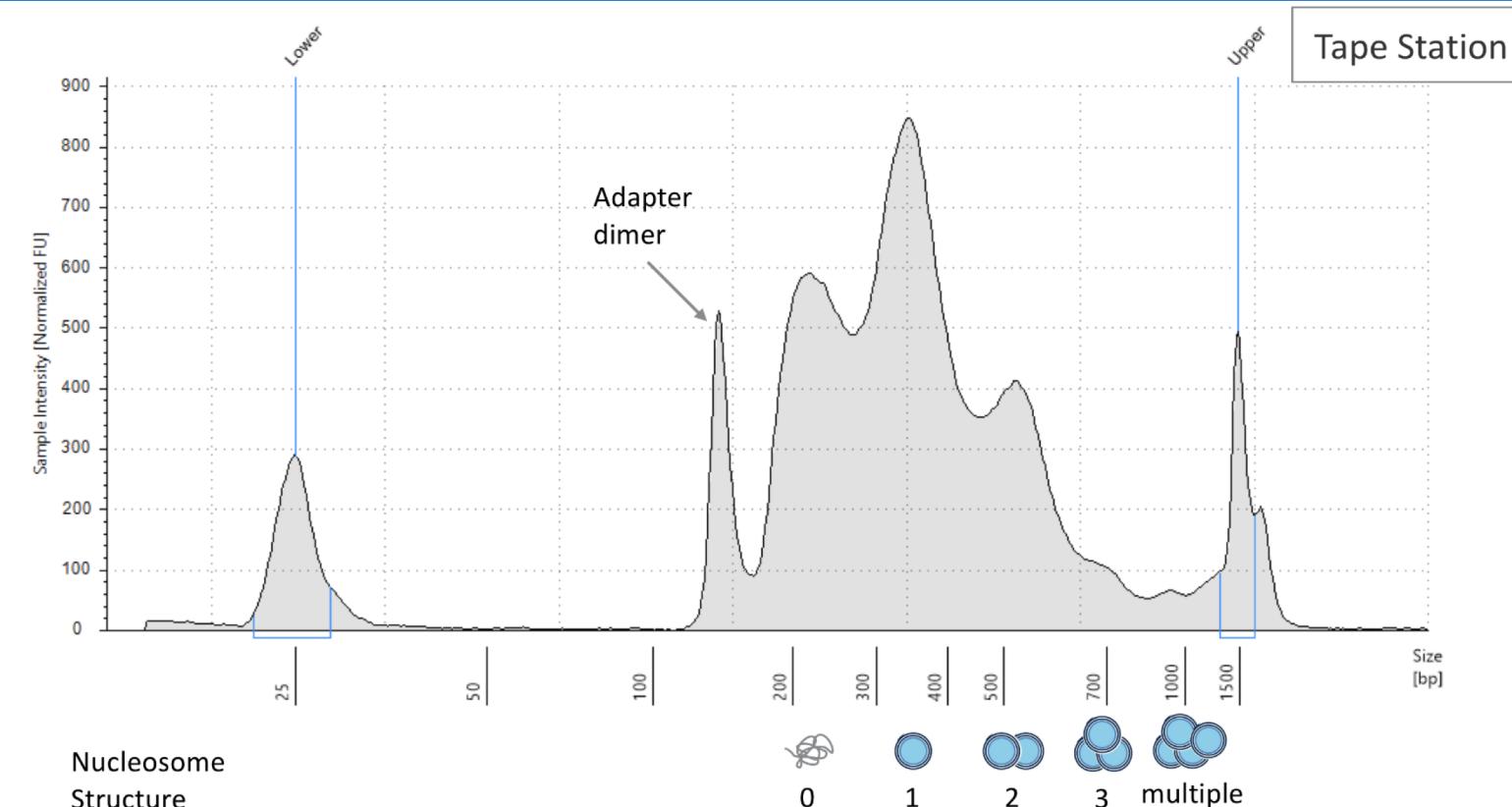
10x Genomics scATAC-seq Method Overview



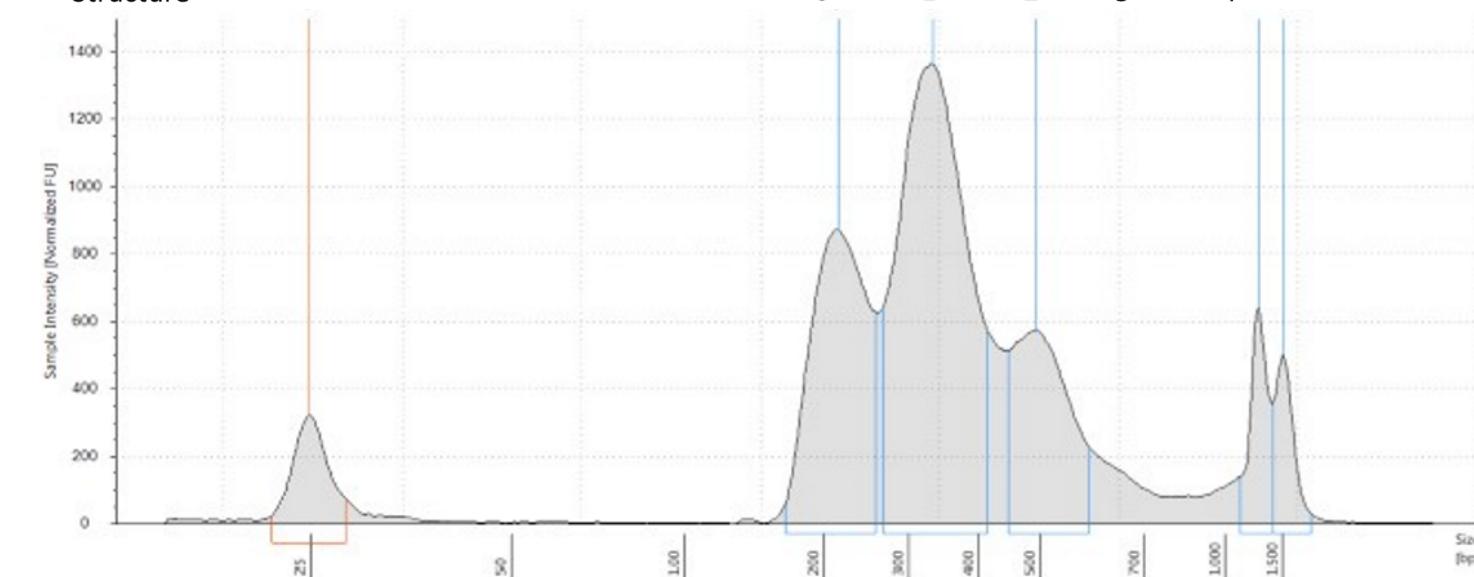
Sequencing Read	Description	Number of Cycles	Notes
Read1	Insert (genomic)	50	Can read longer if desired
I7 index	Sample Index	8	Sample Index
I5 index	Nuclei barcode	16	Nuclei barcode
Read 2	Insert (genomic)	50	Can read longer if desired

10x Genomics scATAC-seq Method Overview

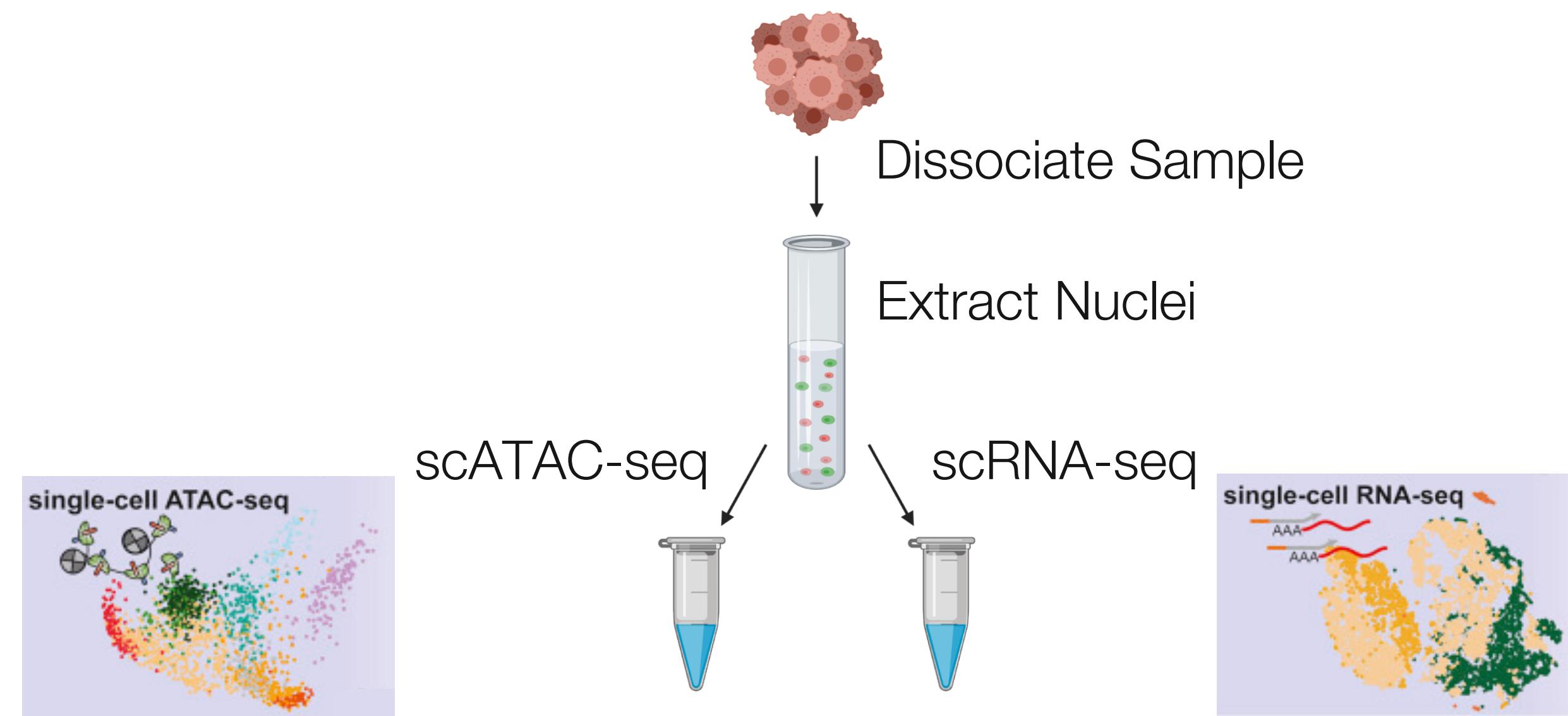
- 10x Example



- Single Cell Core

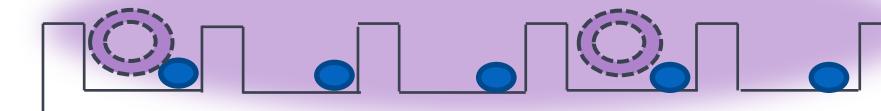


Nuclear Preparation

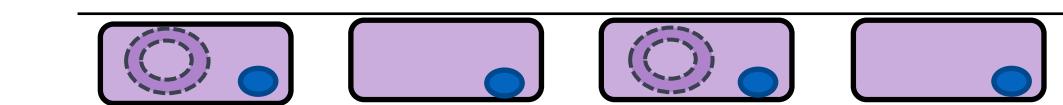


Sample Preparation: nuclear RNA sequencing

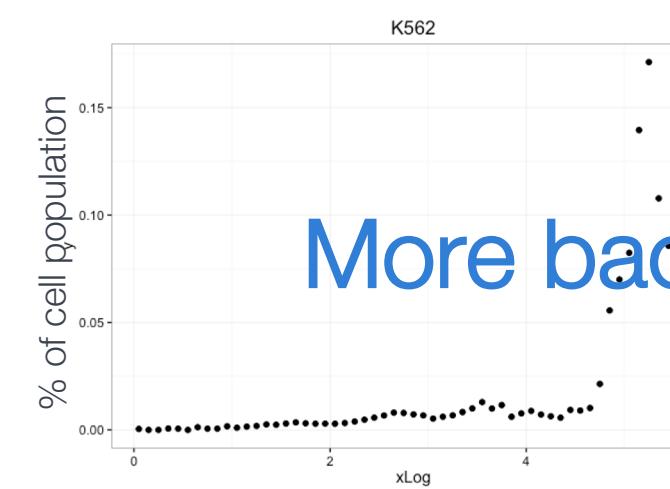
- Good single nuclei suspension. No clumps and minimal debris.
- Nuclear membrane integrity is required until cells are encapsulated.
- May need to DNase treat nuclear prep.



Nanowell-based barcoding platform

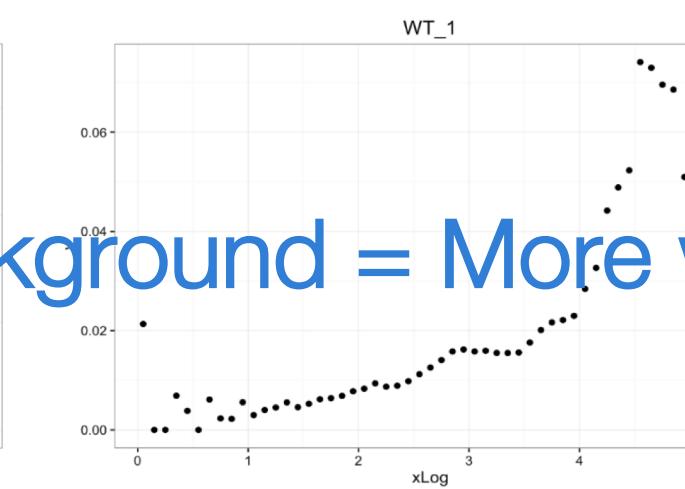


Droplet-based barcoding platform



More background = More wasted reads

Ideal data



Ideal data

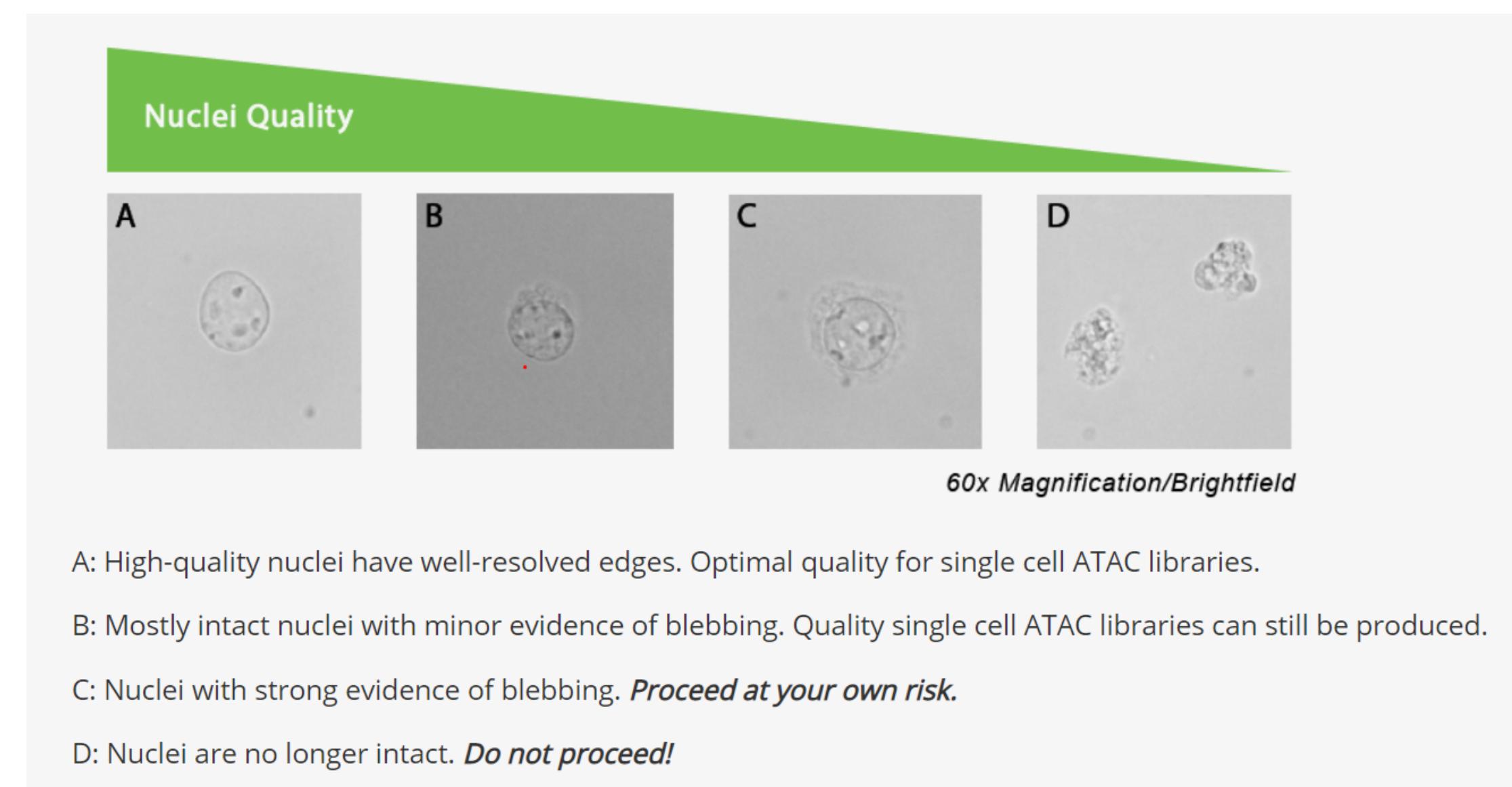


Free-floating RNA ~~DNA~~

scATAC-seq Sample Preparation

- Test lysis time
- Assess level of free floating DNA
 - DNase treat to remove background DNA
 - Only for primary cells
- Visualize nuclei to asses clumping & debris
 - Use BSA up to 2% to minimize clumping
- Count nuclei – needs to be accurate

Sample Preparation: nuclear RNA sequencing



Best practices to obtain high quality sample

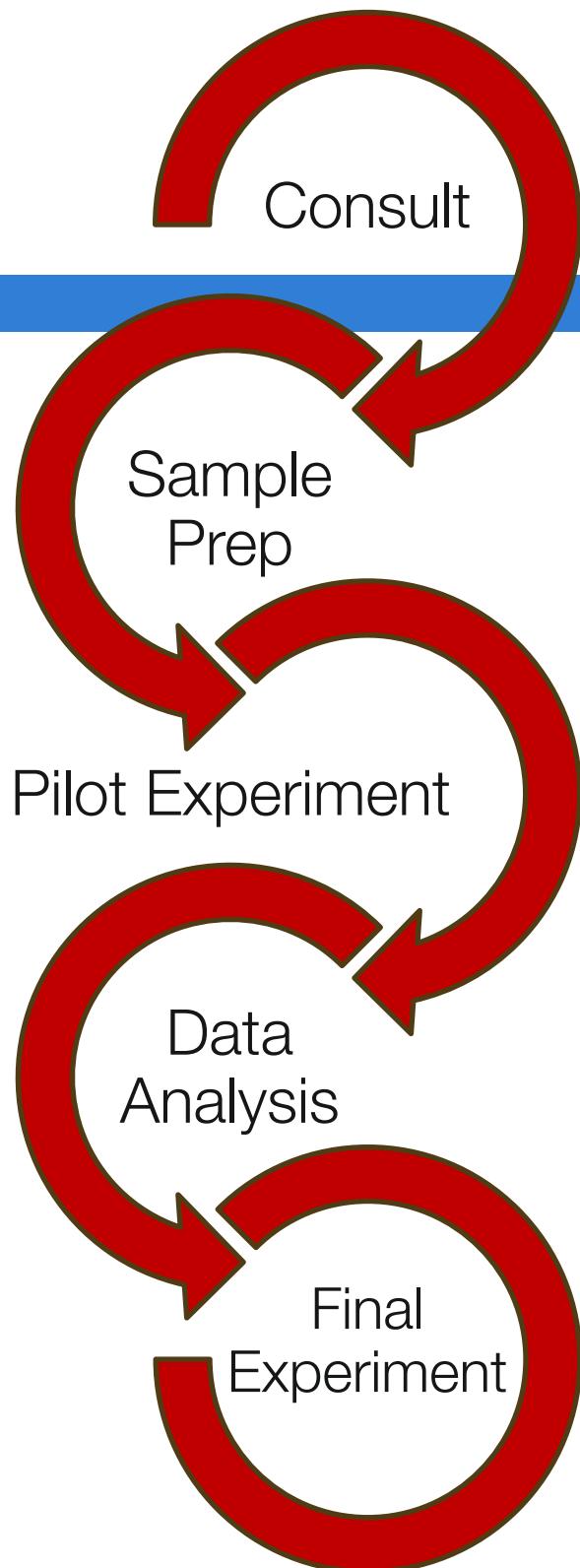
- Optimize a dissociation protocol that is best-suited for your cell type of interest.
- Short sample prep time.
- Maintaining low temperature.
- Gentle treatment
 - gentle lysis condition (low temp, short time)
 - short FACS time, slow sorting, bigger nozzle (in certain cases)
 - Gentle centrifugation (300-500xg) and resuspension
 - Removing debris by filter or density medium
- Include BSA (up to 1%) or FBS (up to 2%) in final buffer.

Resources for scRNA-seq Sample Prep

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

Single Cell Core Workflow

- Good sample prep is the key to success.
- A well planned pilot experiment is essential for evaluating sample preparation and for understanding the required number of cells.
- Do not rush to the final experiment.



Best Practices for Experimental Design

- Include biological replicates.
- Perform drug/treatment/model vs control on the same day.
- Randomize the order of samples run on different days.
- Use same sex littermates as controls in mouse experiments.
- Our experience is that library prep is the largest source of batch effect.
 - Collect all your samples in one study together then prep as one large library group.

Best Practices for Experimental Design

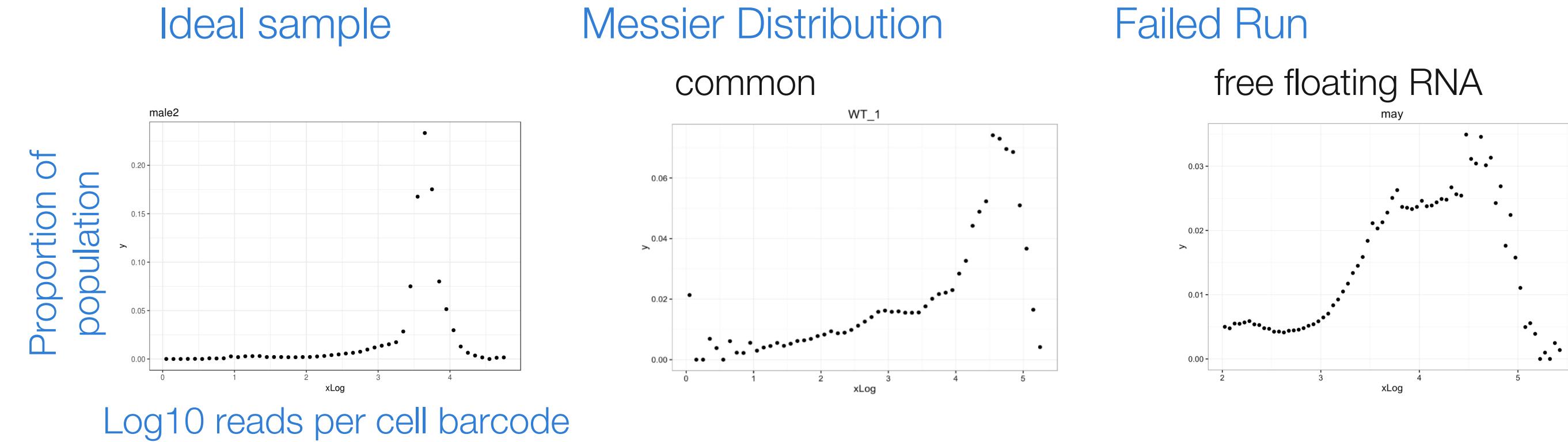
Pilot Study Experimental Design

- Control vs diseased animal
- Each sample requires pooling several animals.
 - Control and diseased littermates pooled for a single sample.
- Both control and diseased samples run on same day.
- The entire experiment is repeated on a second day running samples.
 - Load samples in opposite order.
- Libraries from the four single cell samples are prepared as one batch.
- Sequencing and analysis performed
 - Check that sample prep was of good quality.
 - Determine that the desired information can be obtained from the experiment.

Introduction to Single Cell RNA Sequencing

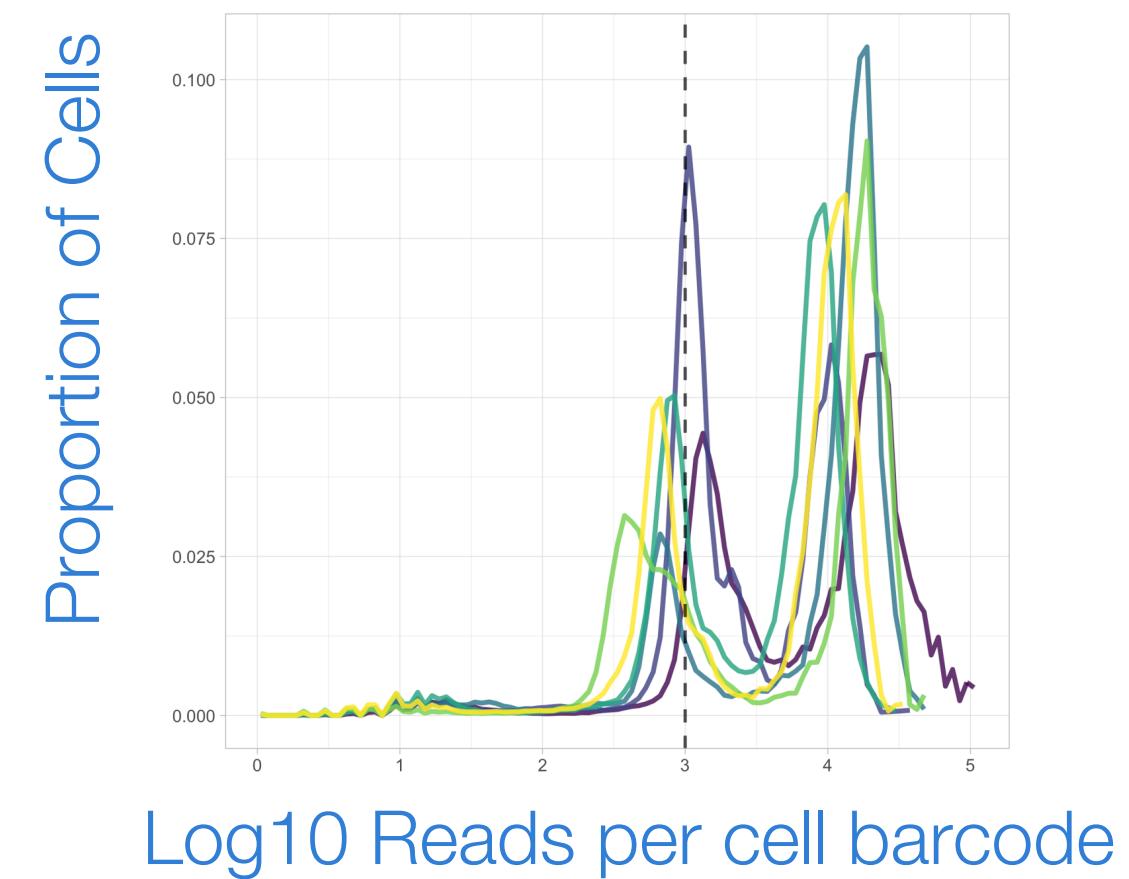
- Common applications of single cell RNA sequencing.
- Overview of single cell RNA sequencing platforms.
- Modified scRNA-seq workflows
- Sample preparation and experimental design.
- Effects of sample prep and sample type on analysis.

How Sample Prep Effects Data



- Cut off usually remove any cell with < 10,000 / 20,000 barcodes per cell
- It is normal for single cell RNA-seq data to contain a large number of low complexity barcodes.
- Exact threshold will depend on sample

How Sample Type can Effect Data



- Bimodal peak is due to sample type. (infiltrating immune cells in tumor)
- Lower peak can get filtered out in analysis.
- May want to analyze each peak separately.

Data Analysis: Quality Control (QC) metrics

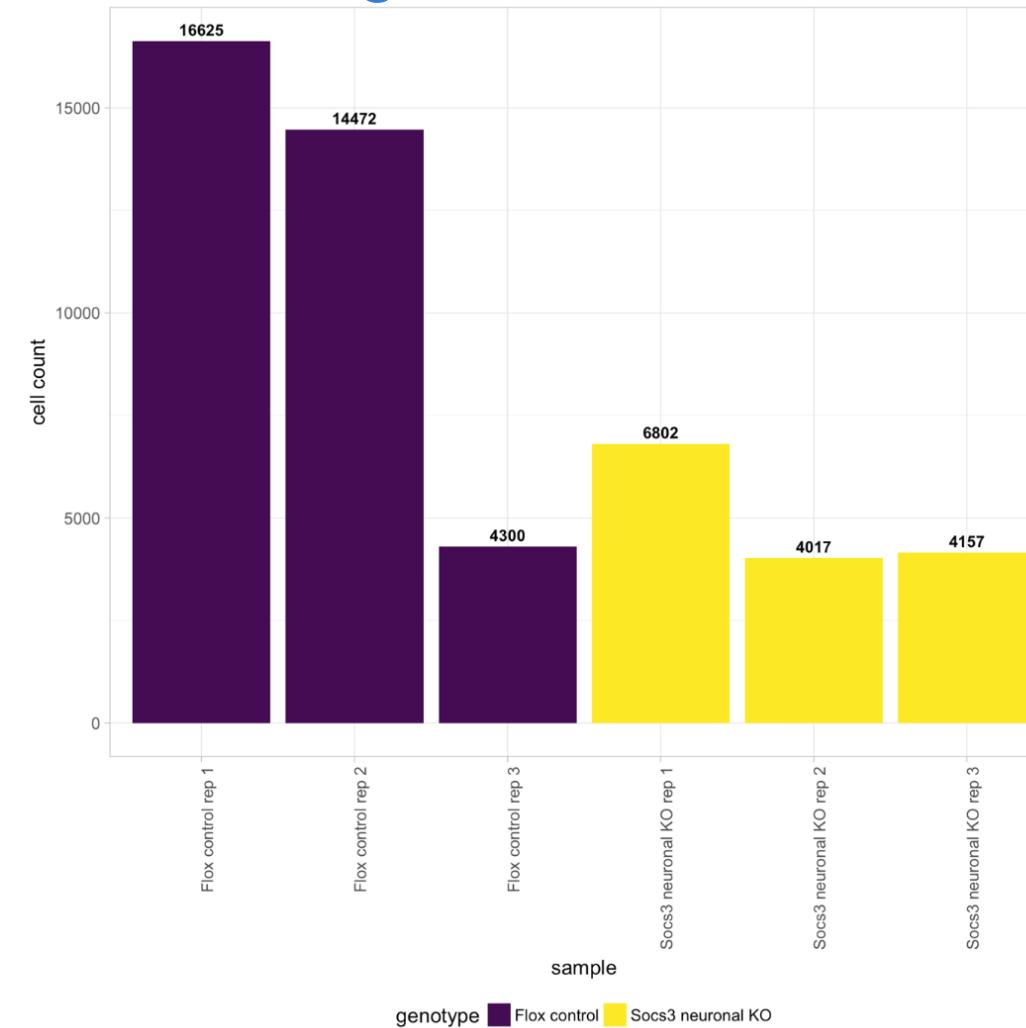
- Reads per cell: How many reads assigned to a given cell barcode
- UMI per cell: “Novelty” score looks for greater diversity genes per UMI
- Genes detected: Genes with a non-zero count measurement per cell
- Mitochondrial counts ratio: Biomarker for cellular stress

Filter parameters

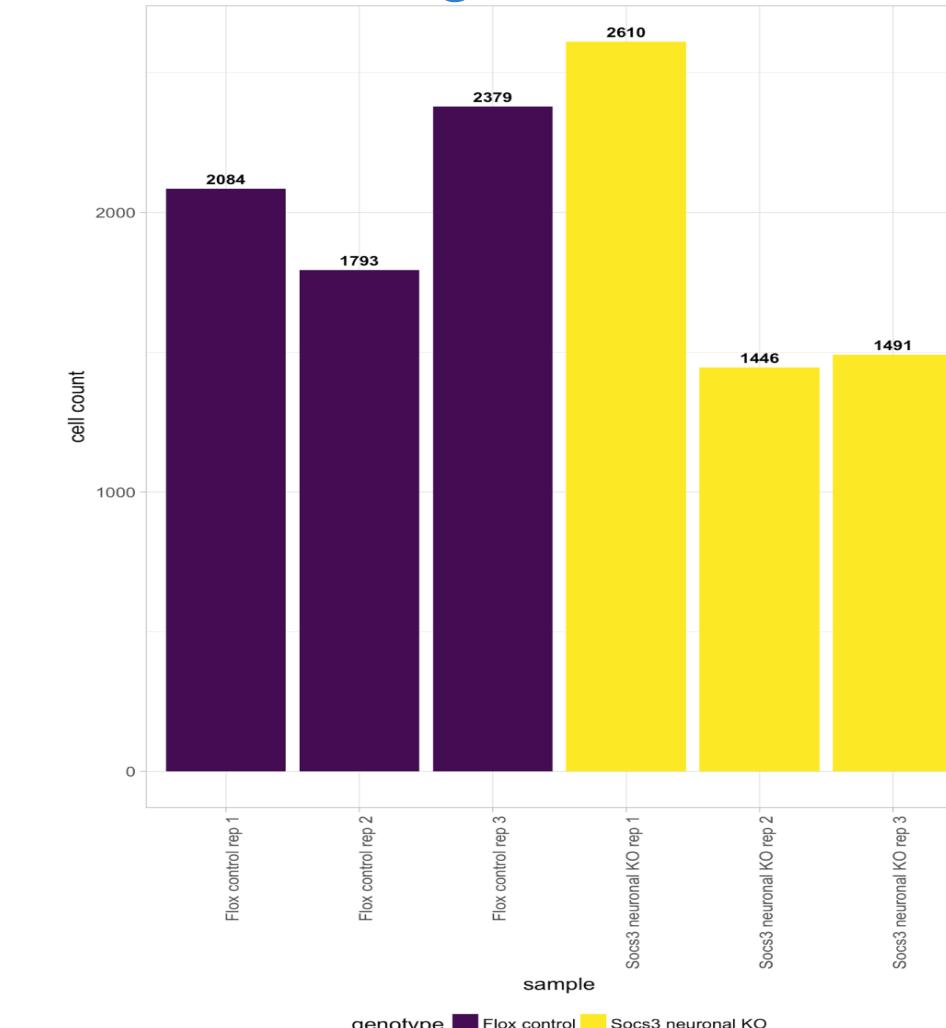
- `>= 500` UMI counts per cell
- `>= 500` genes per cell
- `<= 5000` genes per cell
- `<= 0.1` relative mitochondrial abundance
- `>= 0.8` novelty score

Data Analysis: filtering & correction

Pre-Filtering

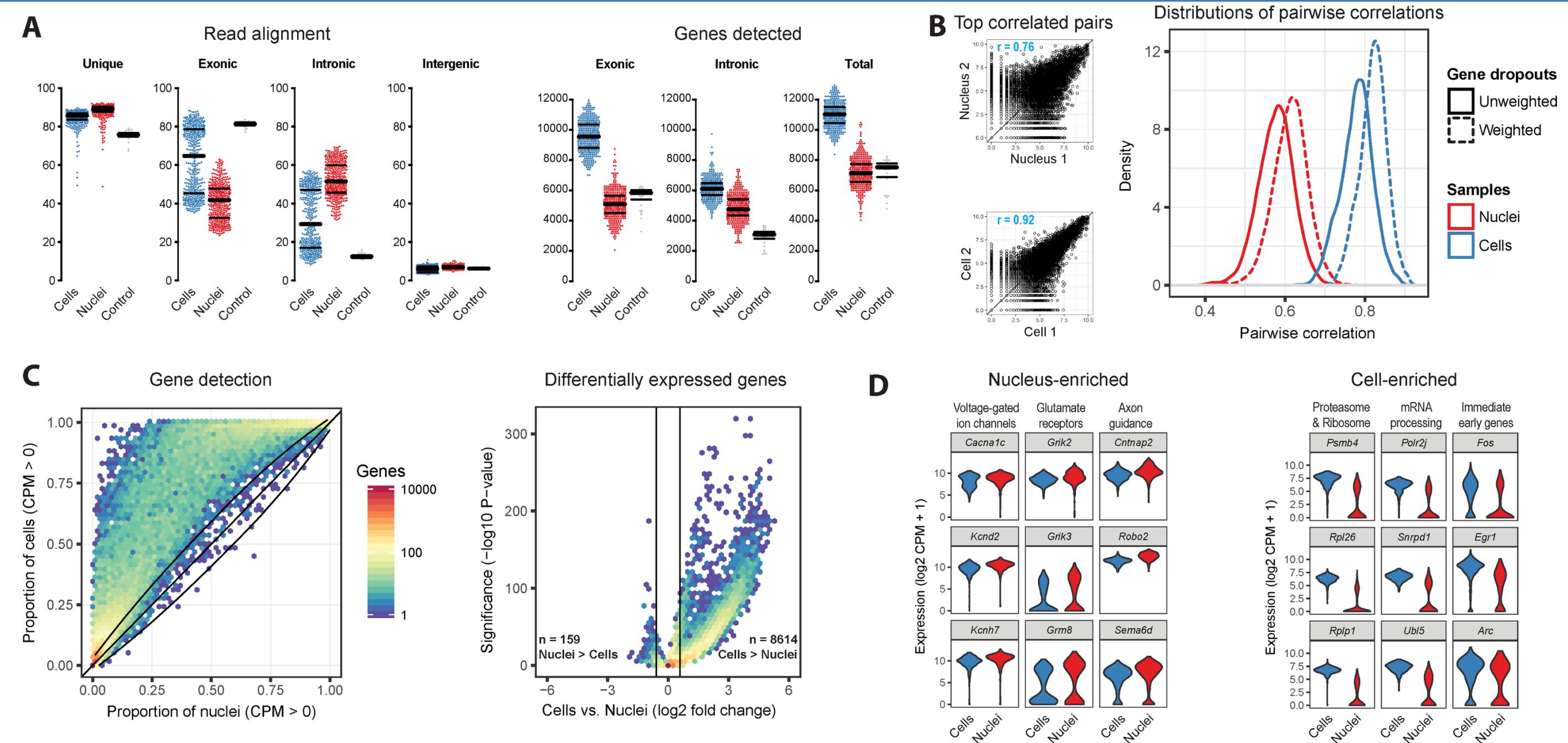


Post-Filtering



Libraries were of 3,000 cells. Post-filtering retains 50-80% of cells

Data Analysis: single cell vs. single nuclei



Data Analysis: single cell vs. single nuclei

- Nuclei are more stable to expression changes from dissociation.
- Data from nuclei detects fewer genes per cell.
- Nuclei data has much higher percentage of intronic reads.
- Single nuclei sequencing seems to recapitulate the cell type classifications observed from single cell data.

Final thoughts on scRNA-seq

- Practice your sample prep protocol. KEY to SUCCESS
- Start with a pilot sample set to ensure your protocol is working.
- Do not make your scRNA-seq run day the first day you run through the whole protocol.
- Be sure sequencing core understands the specific sequencing parameters needed for your scRNA-seq library.

qPCR

Precise quantitation is key to good clustering / sequencing