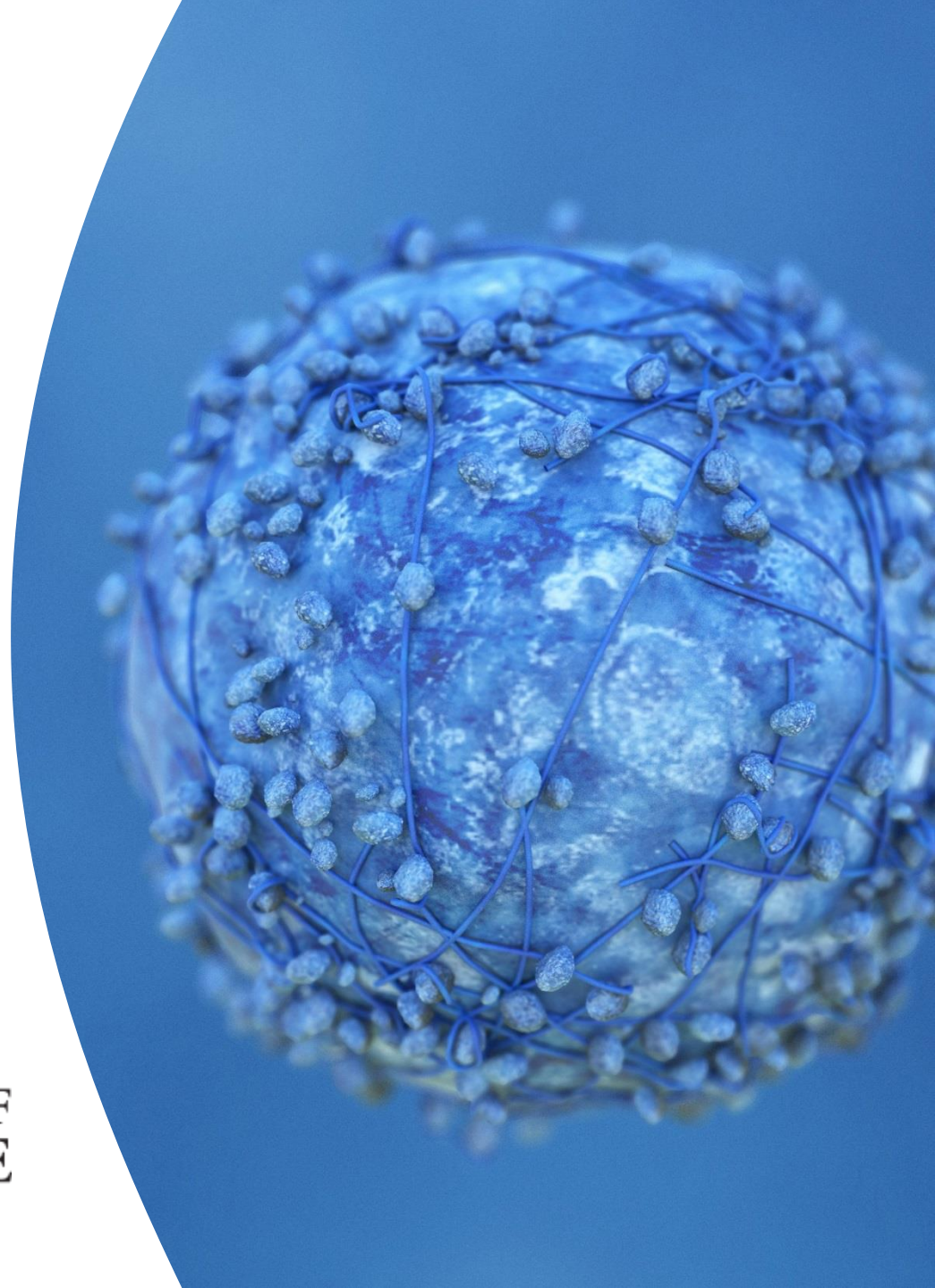
INTRODUCTION TO SINGLE CELL RNA-SEQ



CRUK CI Bioinformatics Summer School 2020

Katarzyna Kania (CRUK CI Genomics Core Facility)

24th July 2020

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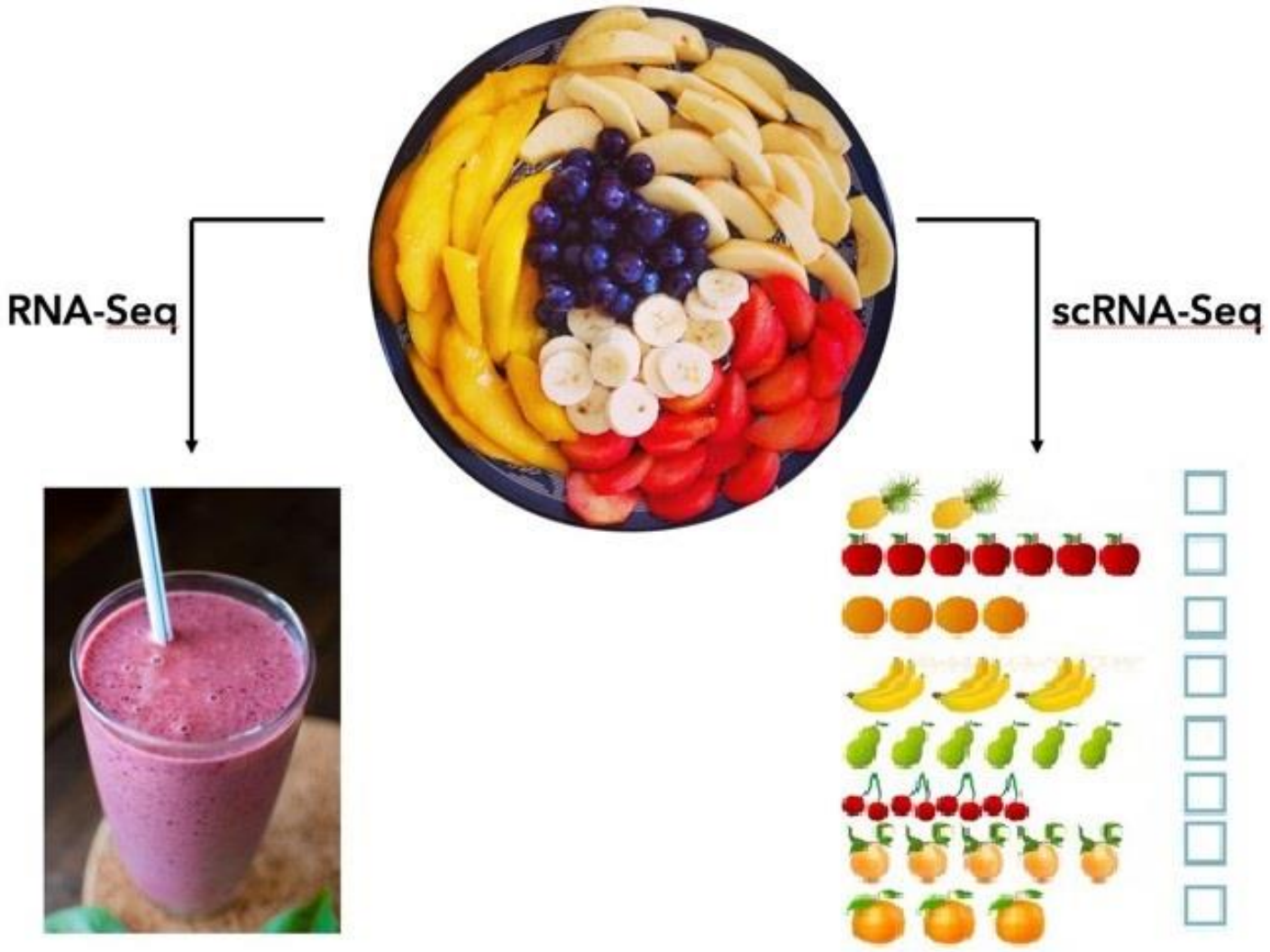
UNIVERSITY OF CAMBRIDGE

Together we will beat cancer

BULK VS SINGLE CELL RNA-SEQ

Average expression level
- Comparative transcriptomics
- Disease biomarker
- Homogenous systems



Separate populations
- Define heterogeneity
- Identify rare cell populations
- Cell population dynamics

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BULK VS SINGLE CELL RNA-SEQ

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 ~3000 cells / well
   - 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. (even GAPDH)
   - If you only looked at transcripts observed in all cells numbers drop dramatically.

Source: Sarah Boswell, Harvard Medical School, September 2020

BULK VS SINGLE CELL RNA-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

Source: Sarah Boswell, Harvard Medical School, September 2020

Disadvantages of scRNA-seq

- Dropouts and noisy data
- Lowly expressed genes might be undetected
- Samples will contain doublets
- Replicates without batch effect are unlikely
- Expensive

APPLICATIONS

nature medicine

Letter | Published: 08 June 2020

A single-cell atlas of the peripheral immune response in patients with severe COVID-19

Aaron J. Wilk, Arjun Rustagi, Nancy Q. Zhao, Jonasel Roque, Giovanny J. Martínez-Colón, Julia L. McKechnie, Geoffrey T. Ivison, Thanmayi Ranganath, Rosemary Vergara,

LETTER

https://doi.org/10.1038/s41586-018-0394-6

A single-cell atlas of the airway epithelium reveals the CFTR-rich pulmonary ionocyte

Lindsey W. Plasschaert[1,5,7], Rapolas Žilionis[2,3,7], Rayman Choo-Wing[1,5], Virginia Savova[2,6], Judith Knehr[4], Guglielmo Roma[4], Allon M. Klein[2]* & Aron B. Jaffe[1,5]*

nature

Article | Published: 20 February 2019

A single-cell molecular map of mouse gastrulation and early organogenesis

Blanca Pijuan-Sala, Jonathan A. Griffiths, Carolina Guibentif, Tom W. Hiscock, Wajid Jawaid, Fernando J. Calero-Nieto, Carla Mulas, Ximena Ibarra-Soria, Richard C. V.

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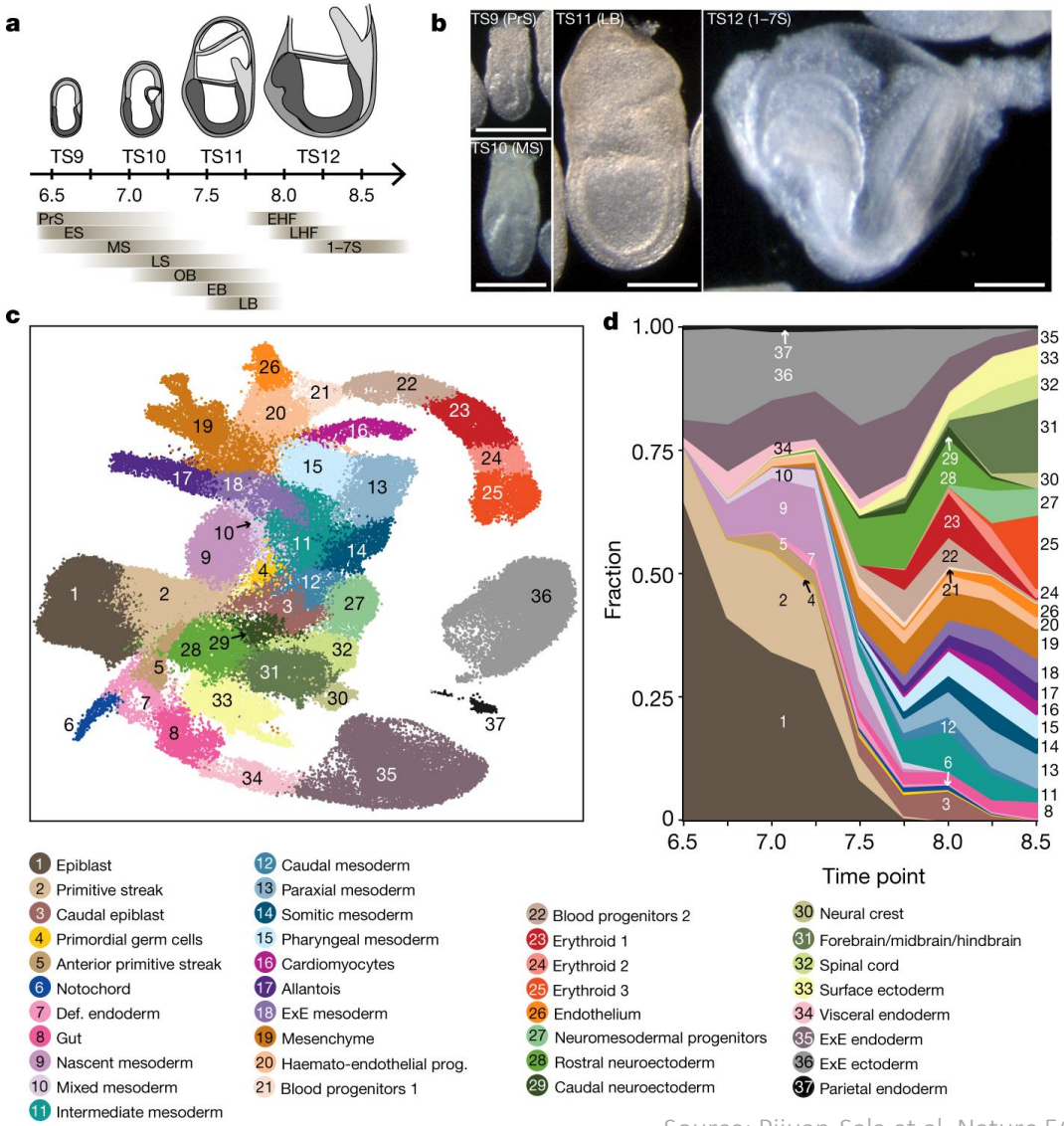
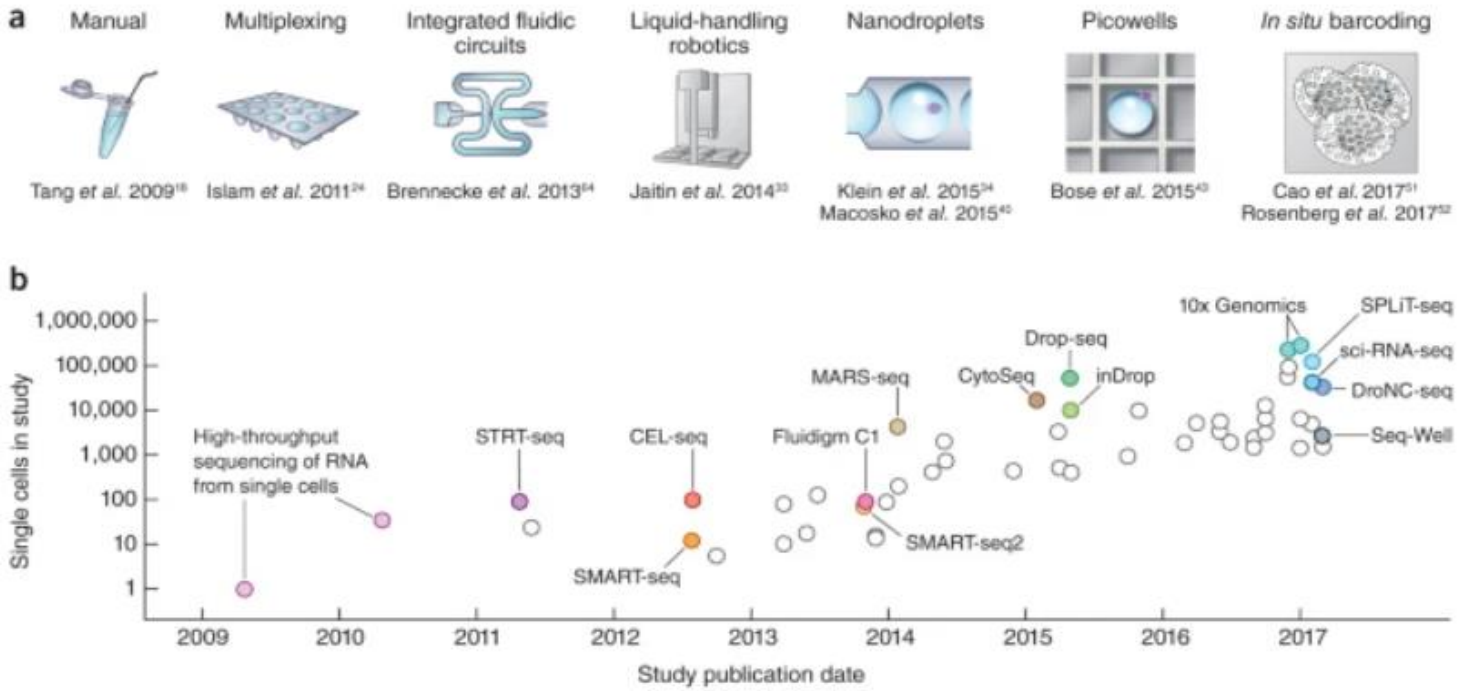


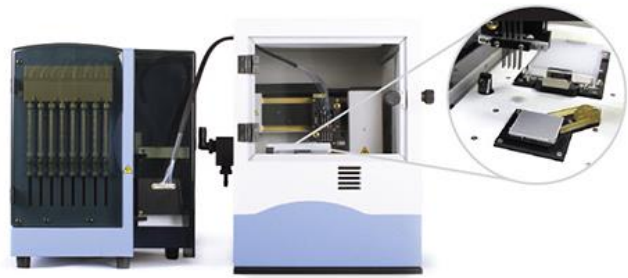
Source: Pijuan-Sala et al. Nature 566, 490–495 (2019)

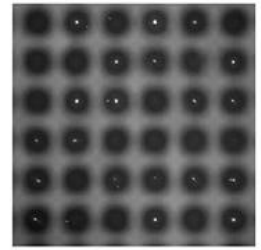
TECHNOLOGIES

Figure 1: Scaling of scRNA-seq experiments.



Source: Svensson et al. *Nat Protoc* 13, 599–604 (2018)





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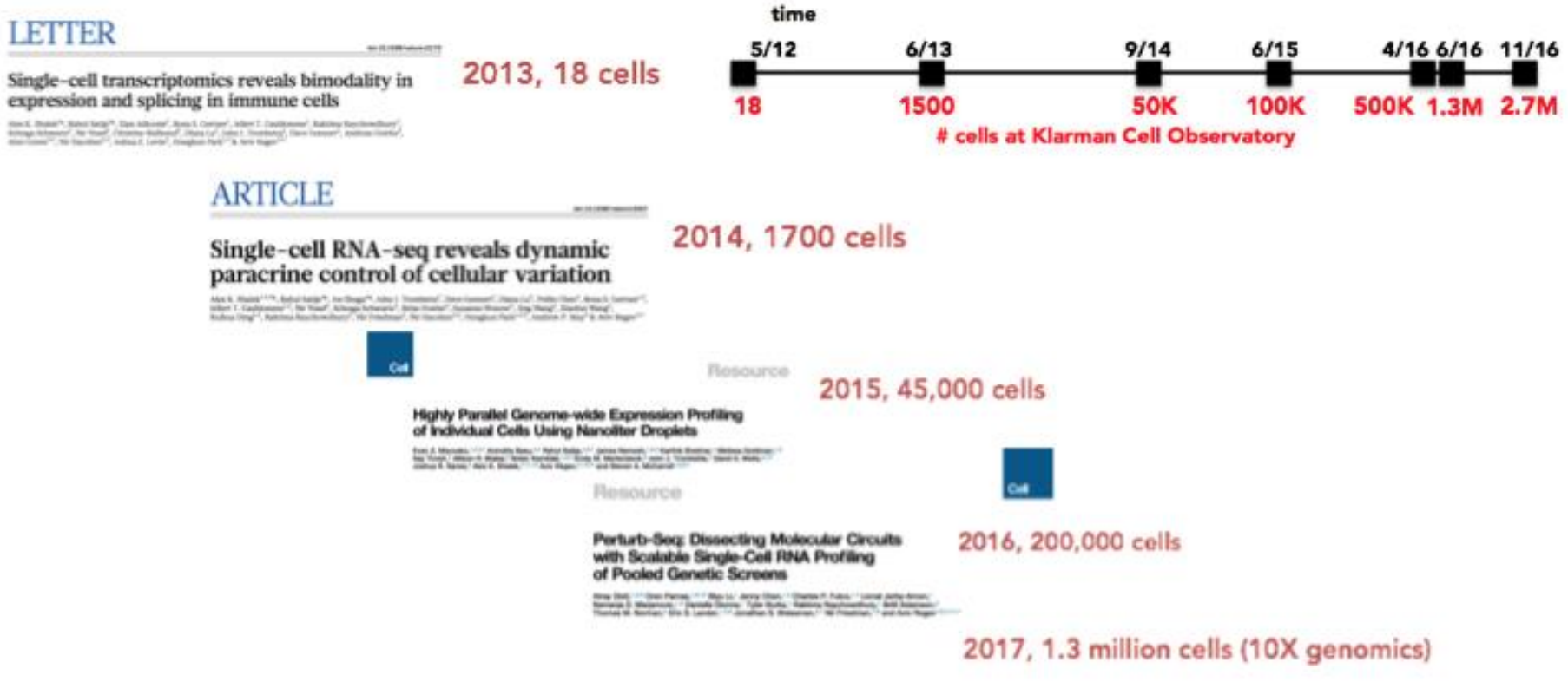
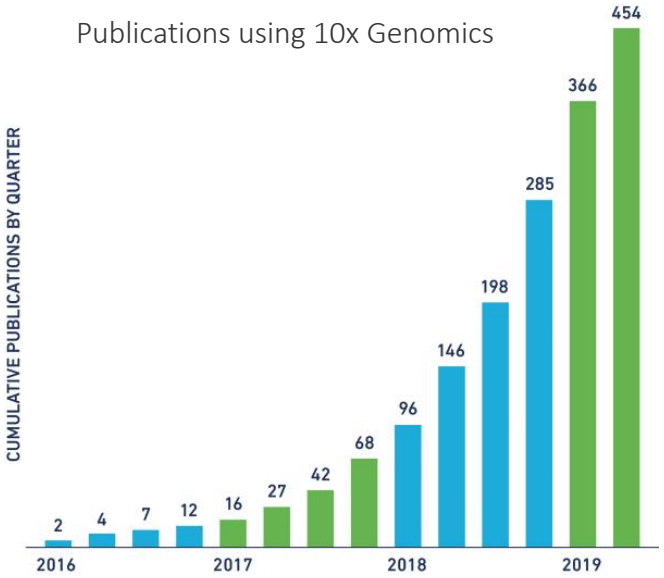
HISTORY AND PROGRESS

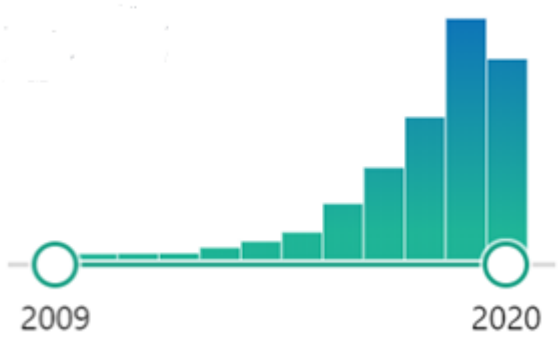


Publications using 10x Genomics



PubMed search for 'scRNA-seq'

RESULTS BY YEAR



Source: Introduction to scRNASeq, Timothy Tickle & Brian Haas, Broad Institute, 2017

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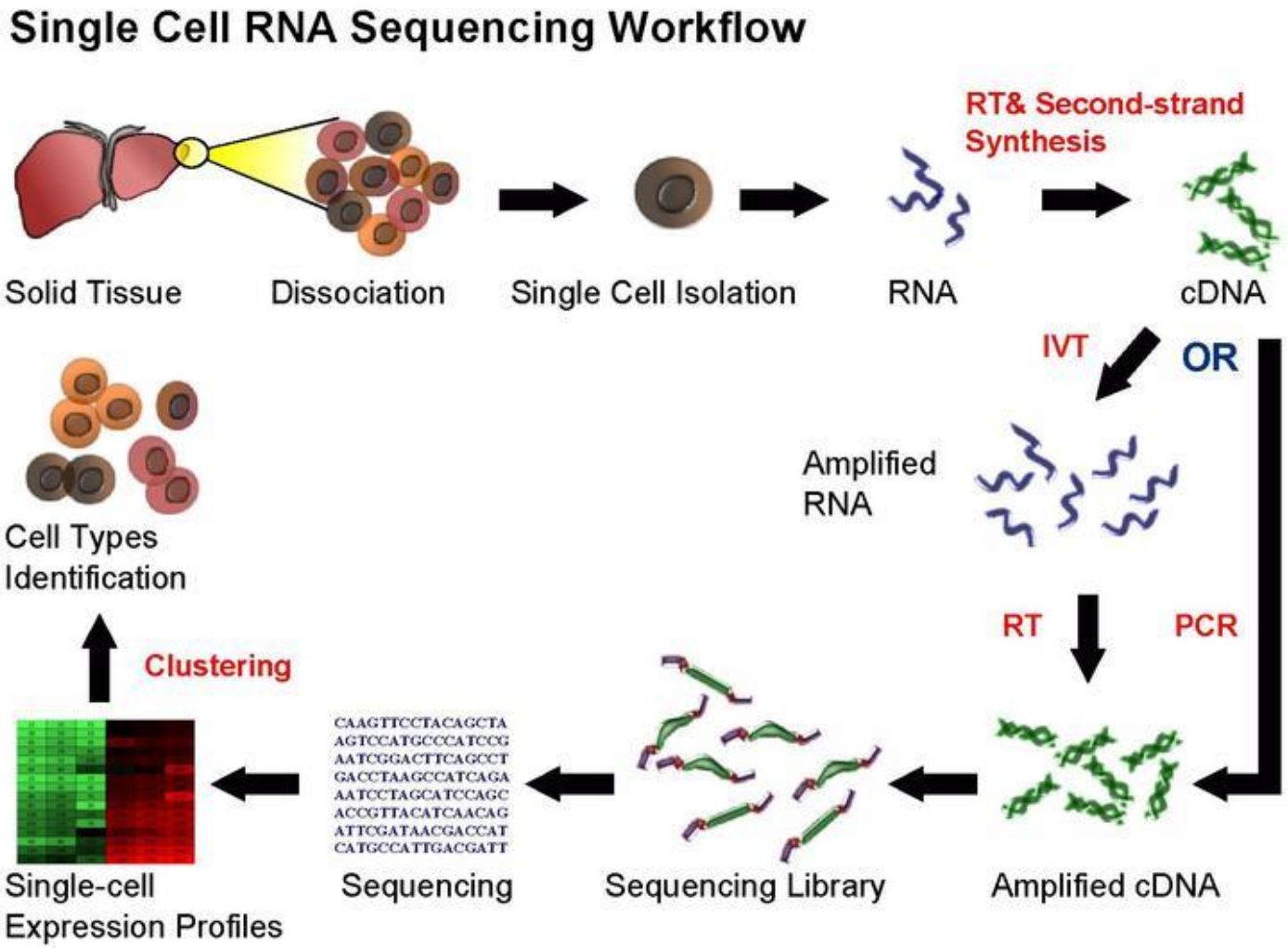
WORKFLOW

Good sample preparation is key to success!



Source: https://en.wikipedia.org/wiki/Single_cell_sequencing

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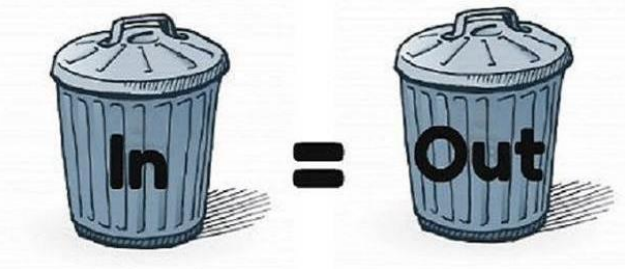
SAMPLE PREPARATION



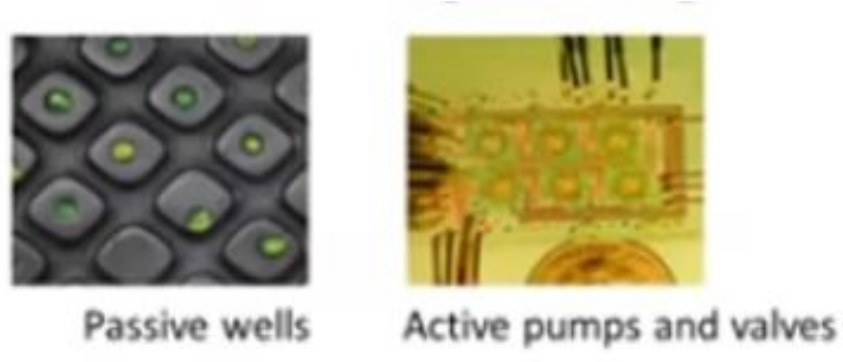
- Understand well the nature of the sample (sampling conditions, preparation, purity)
- Identify the source of technical difficulties in order to resolve them first
- Practice your sample preparation, optimise the protocol well, do not rush to the final experiment
- A well planned pilot experiment is essential for evaluating sample preparation and for understanding the required number of cells.
- You need your cells to be highly viable (>90-95%), have no clumps and no debris. Cell membrane integrity is a must!
- Free-floating RNA will make analysis more challenging
- Be cautious about FACS (especially with more fragile cells). If FACS necessary for enrichment, remember that time is crucial factor
- Count with haemocytometer or cell counter (Countess II Automated Cell Counter) – do not trust sorter counts
- Fixation and cryopreservation are not compatible with many techniques – and generally should be avoided if possible (Nuc-seq might be a solution for frozen samples)

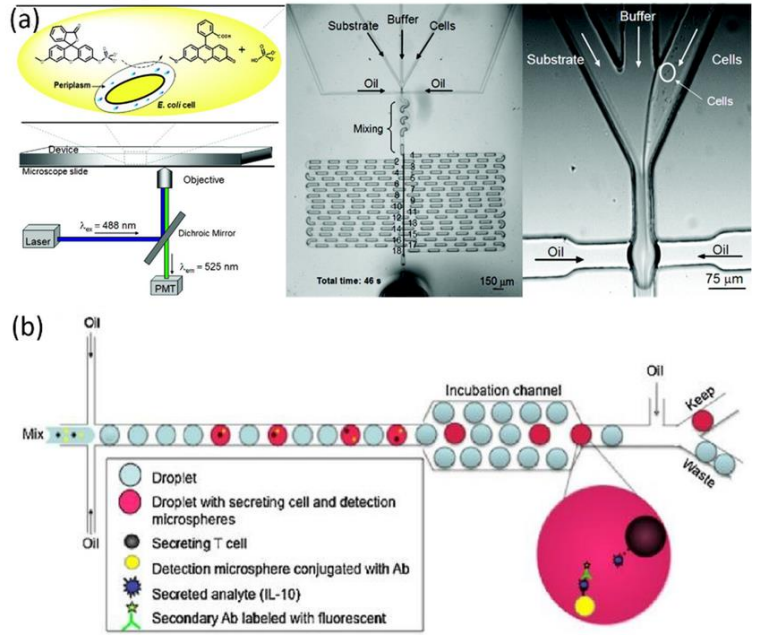
METHODS

1) Cells in wells, traps and valves (nanowell, Flow sorting, CellenOne, Fluidigm C1, SmartSeq)
   - Screen for and retrieve single cells of interest
   - Enrich for rare cells wit decided properties
   - Control the cellular microenvironment
   - Monitor and control cell-cell interactions
   - Precise/extensive manipulation of single cells

2) Droplets (Drop-seq, 10x Genomics)
   - Introduce distinct 'packets' of reagents to single cell (e.g. barcodes)
   - Perform amplification on individual cells
   - Sort large population of single cells

3) Combinatorial indexing (SCI-seq, SPLiT-seq)
   - Economic use of reagents for cell separation
   - Efficiency of handling larger population than Drop-seq
   - Maintain complexities of population without bias from droplet or well





Source: Wen et al. Molecules (2016)

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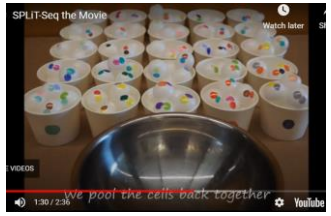
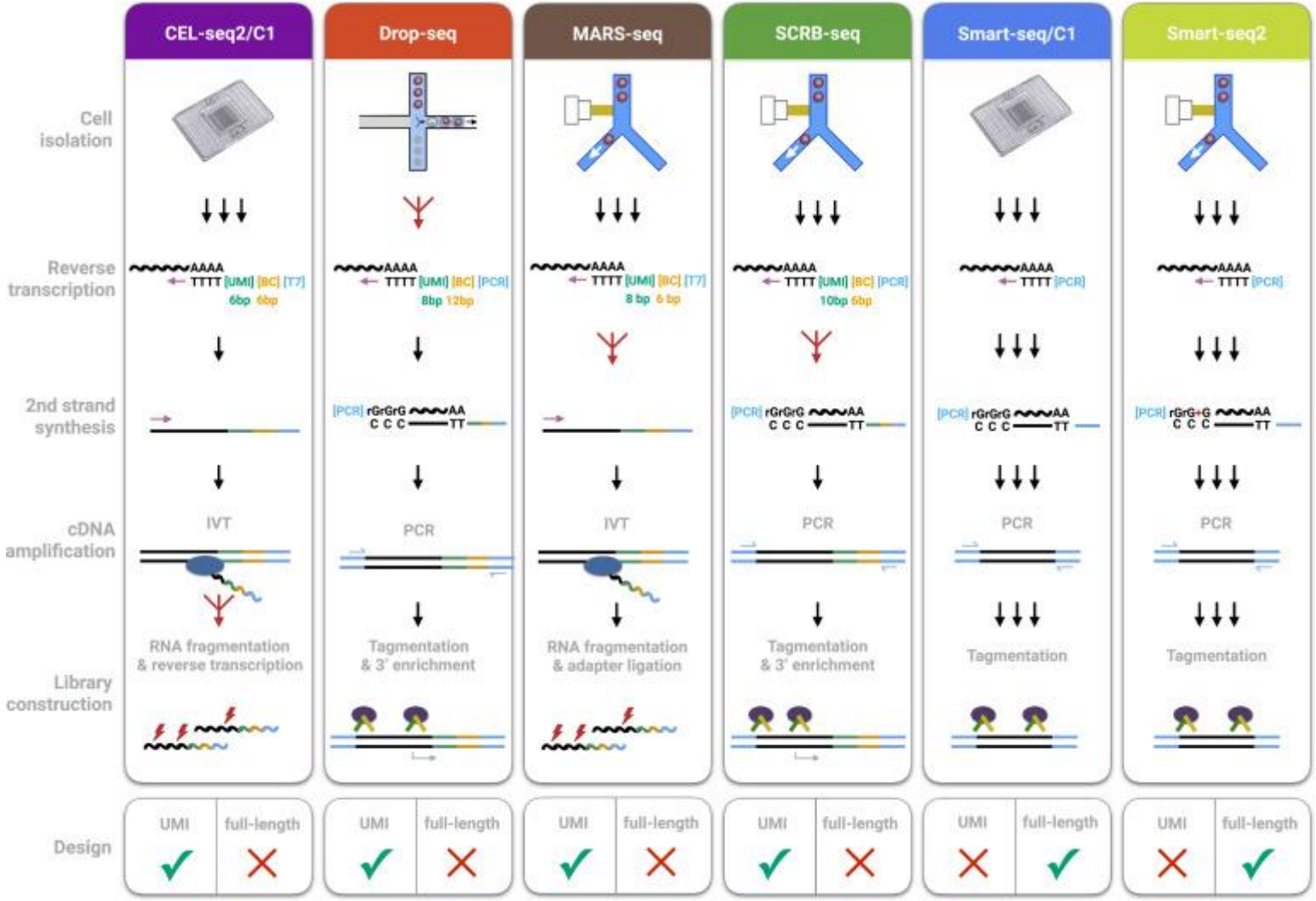


Video available at: https://sites.google.com/uw.edu/splitseq

COMPARISON OF METHODS



Source: Ziegenhain et al. Mol Cell. 65 (4). (2017)

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COMPARISON OF METHODS

Method	Fluidigm C1 system (SMART-seq)	Fluidigm C1 system (mRNA Seq HT)	SMART-seq2	10X Genomics Chromium system	MARS-seq
cDNA coverage	Full-length	3′ counting	Full-length	5′/3′ counting	3′ counting
UMI	No	No	No	Yes	Yes
Amplification technology	Template switching-based PCR	Template switching-based PCR	Template switching-based PCR	Template switching-based PCR	*in vitro* transcription
Multiplexing of samples	No	Yes	No	Yes	Yes
Single cell isolation	Fluidigm C1 machine	Fluidigm C1 machine	FACS	10X Genomics Chromium single cell controller	FACS
Cell size limitations	Homogenous size of 5–10, 10–17, or 17–25 μM	Homogenous size of 5–10, 10–17, or 17–25 μM	Independent of cell size	Independent of cell size	Independent of cell size
Required cell numbers per run	≥10,000	≥10,000	No limitation	≥20,000	No limitation
Visual quality control check	Microscope examination	Microscope examination	No	No	No
Long term storage	No, must process immediately	No, must process immediately	Yes	No, must process immediately	Yes
Throughput	Limited by number of machines	Limited by number of machines	Limited by operator efficiency	Up to 8 samples per chip	Process is automated
Cost	+ + + + +	+ + +	+ + + +	+	+ +
Sample Preparation Scenario 1 (~5000 single cell)	Targeted cell No: 4992 cells	Targeted cell No: 4800 cells	Targeted cell No: 4992 cells	Targeted cell No: 5000 cells	Targeted cell No: 4992 cells
	26 rounds of 2 runs (2 C1 machines; concurrent)	3 rounds of 2 runs (2 C1 machines; concurrent)	26 rounds of 2 96-well plates	1 run	13 runs of 1 384-well plate
	~26 weeks	~3 weeks	~26 weeks	~2–3 days	~7 weeks
Sample Preparation Scenario 2 (~96 single cell)	Targeted cell No: 96 cells	Targeted cell No: Minimum 800 cell	Targeted cell No: 96 cells	Targeted cell No: Minimum 500 cells	Targeted cell No: 96 cells
	1 run (1 C1 machine)	1 run (1 C1 machine)	1 run of 96-well plates	1 run	1 run of 384-well plate
	~1 week	~1 week	~1 week	~2–3 days	~2–3 days

Source: See et al. Frontiers in immunology, 9, 2425. (2018)

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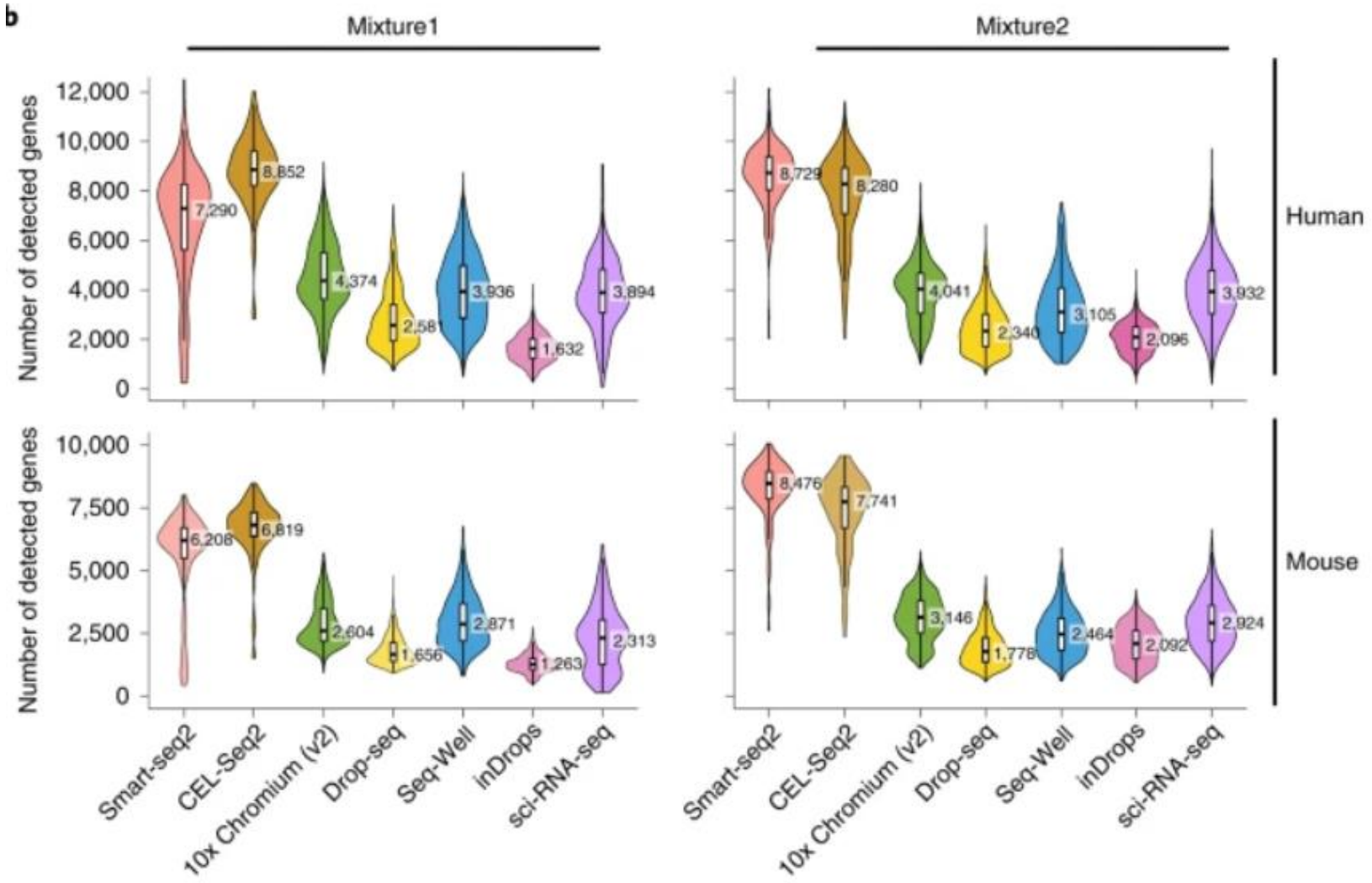
PERFORMANCE

Distribution of the number of genes in human or mouse cells in the two mixture experiments.



Source: Ding et al. *Nat Biotechnol* 38, 737–746 (2020).

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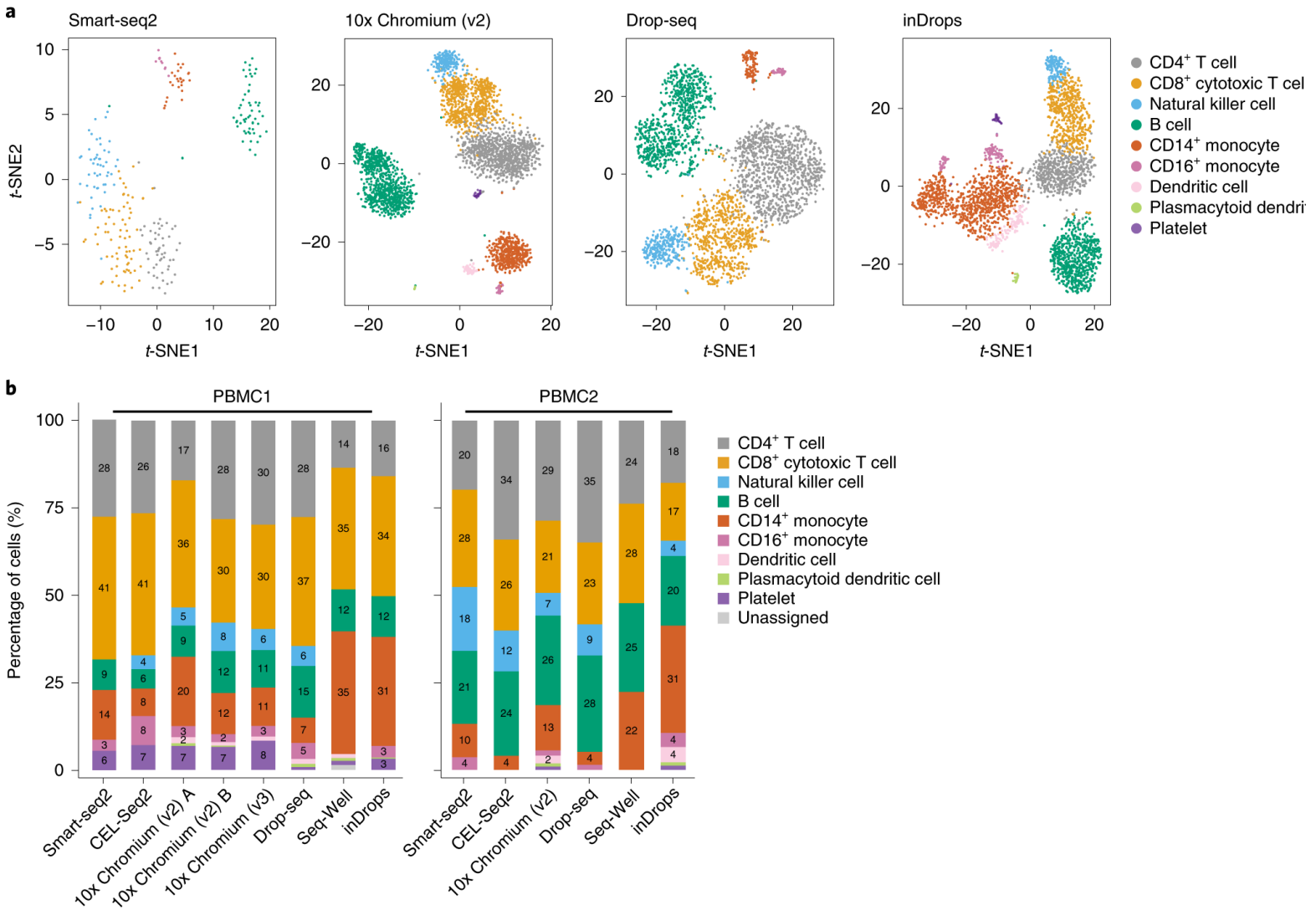
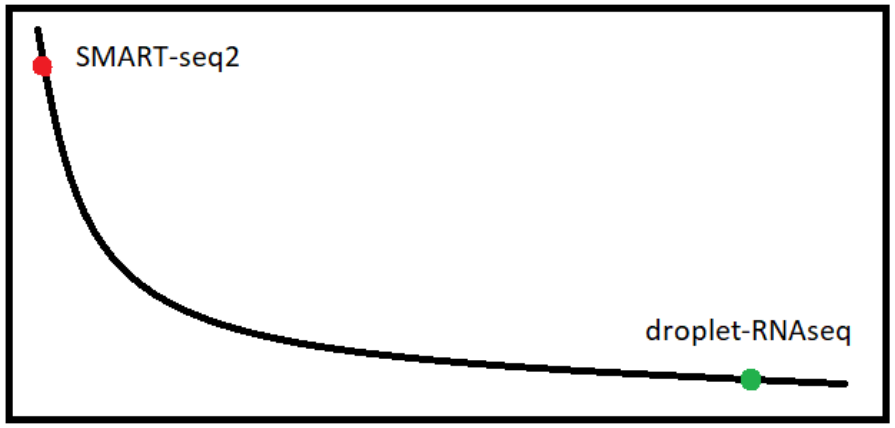
PERFORMANCE

Cell type identification and assignment in PBMCs



Source: Ding et al. *Nat Biotechnol* 38, 737–746 (2020).

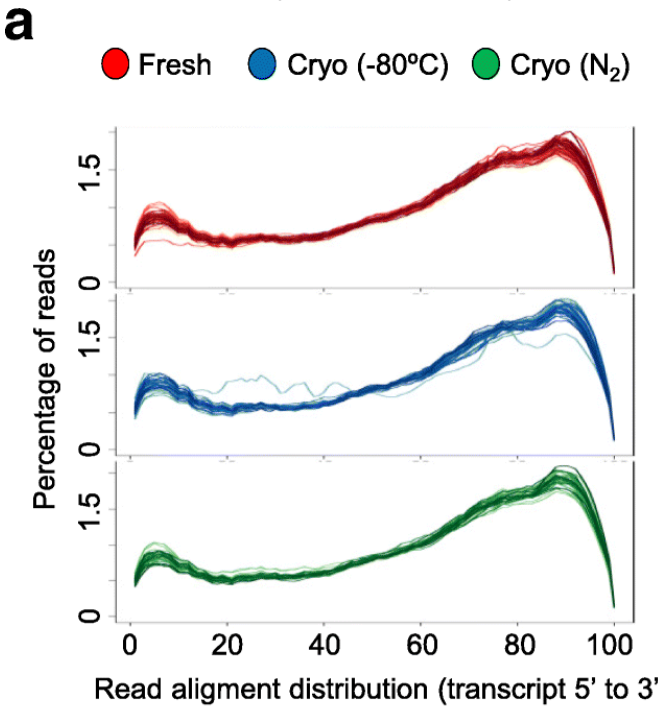
MORE CELLS OR MORE GENES?



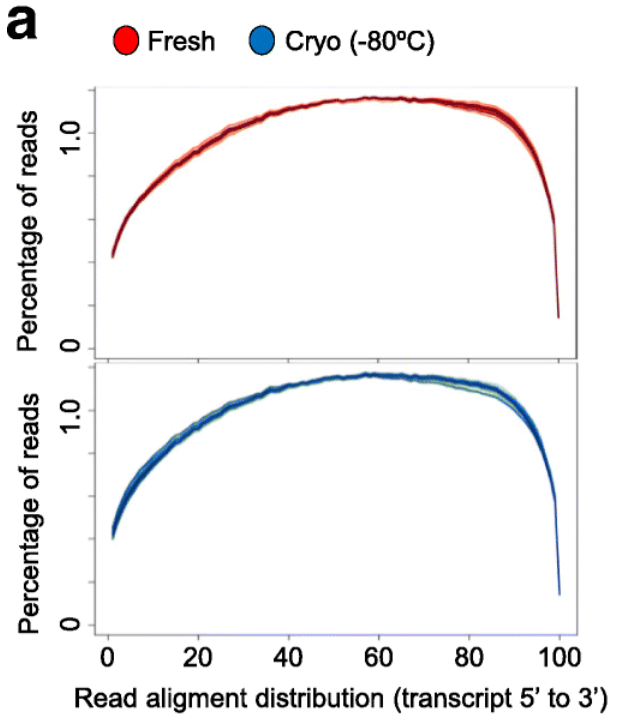
SMART-seq2
- 100 cells
- Full-length libraries
- 1M reads per cell

Droplet-RNAseq
- 10000 cells
- 50k reads per cell
- 3’/5’ bias

Droplet-RNAseq



SMART-seq2



Source: Guillaumet-Adkins, et al. Genome Biol 18, 45 (2017).

- Required number of cells increases with complexity of the sample.
- Number of reads will depend on biology of sample
- Cell-type classification of a mixed population usually requires lower read depth
- You can always re-sequence your samples.

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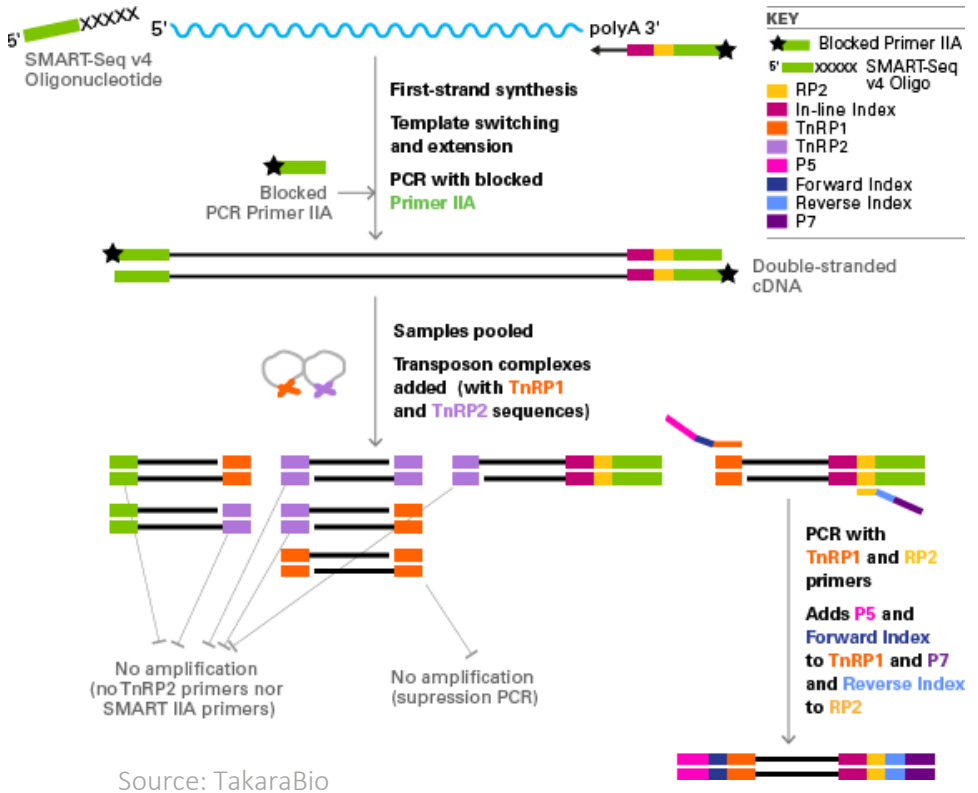
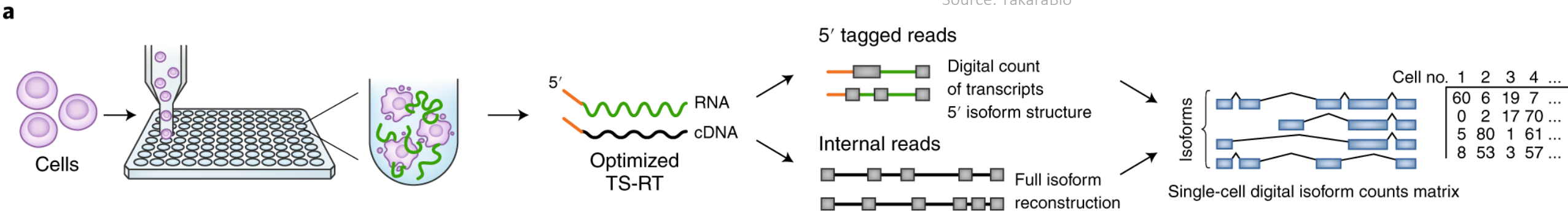
SMART-SEQ2/3/4 OVERVIEW

Developed for single cell but can performed using total RNA.

- Selects for poly-A tail.
- Full transcript assay.
- Uses template switching for 5' end capture.
- Standard illumina sequencing.
- Plate-based solution so labour intensive, slow and costly (~$12/cell)

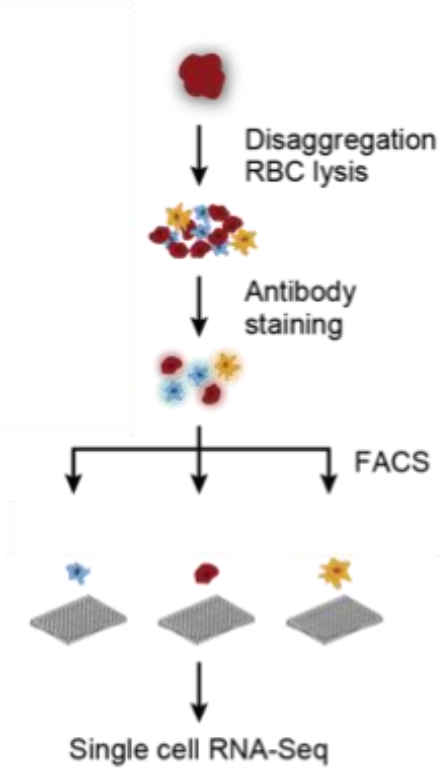


Source: TakaraBio

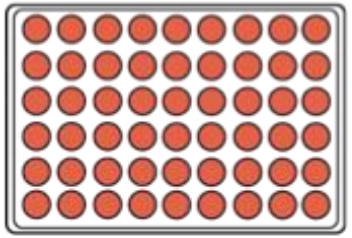


Source: Macosko, Nat Biotechnol 38 (2020).

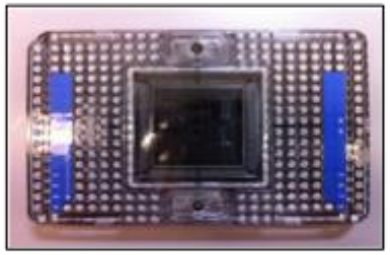
SMART-SEQ2/3/4 + MOSQUITO LV

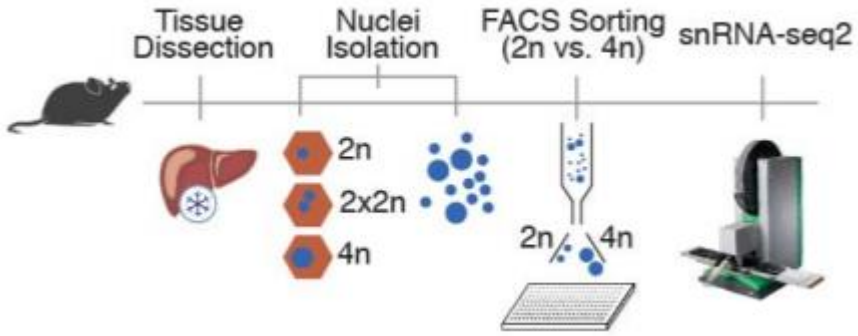


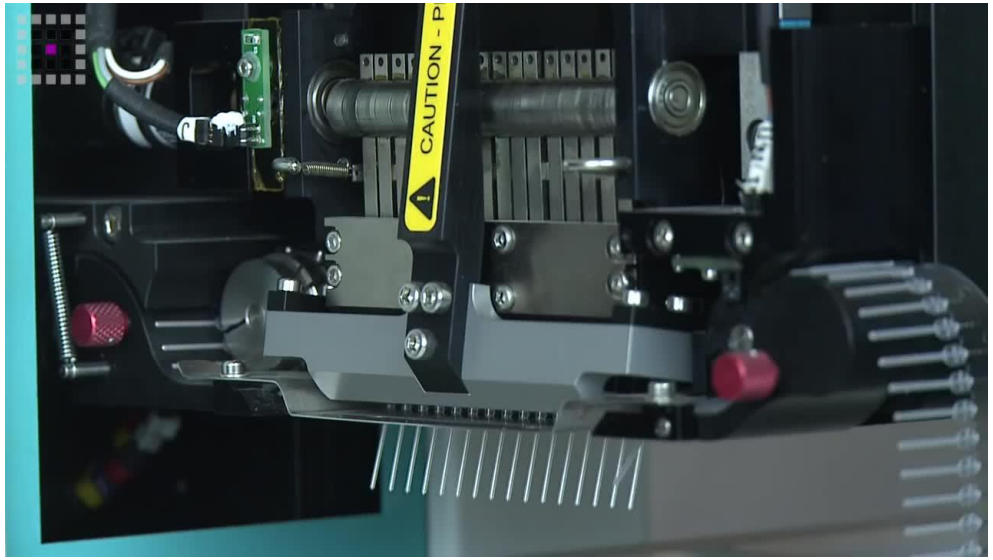
FACS sorting on 96/384-well plates



Fluidigm C1-autoprep system







Source: SPT Labtech

- Mosquito LV makes assay miniaturisation simple, leading to significant savings on precious reagents and time.
- Mosquito LV offers highly accurate and precise multichannel pipetting from 25 nL to 1.2 µL.
- SmartSeq2 cost reduced from $12 to $4 per cell

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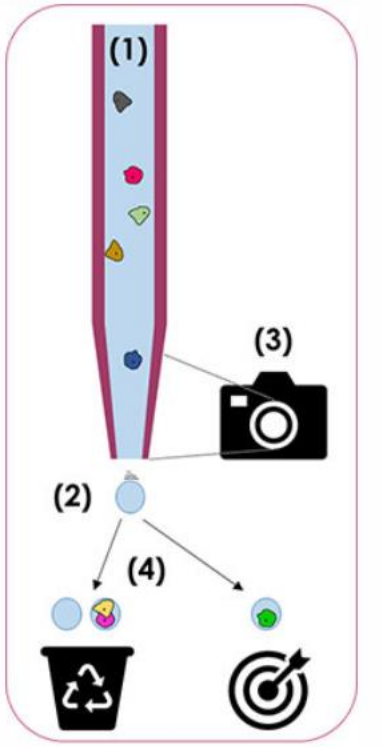
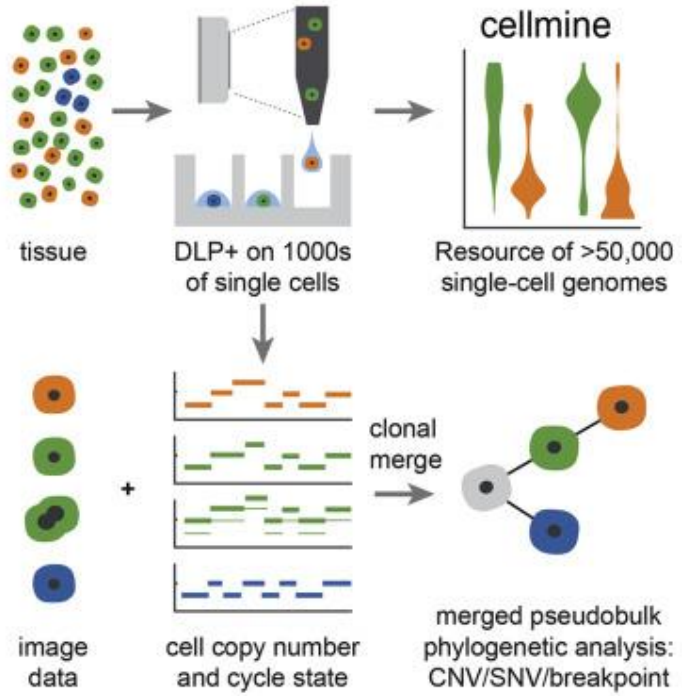
Richter et al. bioRxiv 2020.07.11.193458

CELLENONE

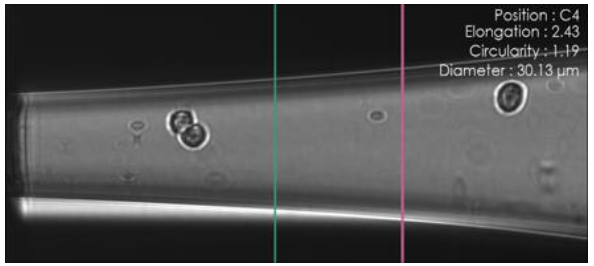


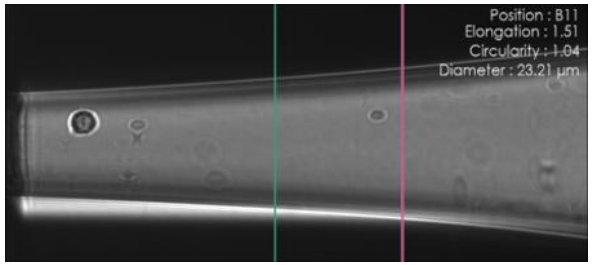
Source: Cellenion

1) Cell suspension is aspirated into a glass capillary
2) Generation of drops on demand, in air
3) Thanks to automated imagining, cellenONE tracks cells and determines if upcoming drops will contain or not a single cell
4) Drops containing single cells are dispensed into selected targets, drops without cells or with more than one cells are dispensed into recycling tube



Source: Laks et al. *Cell*. 179(5):1207-1221.e22. (2019)





Source: Cellenion

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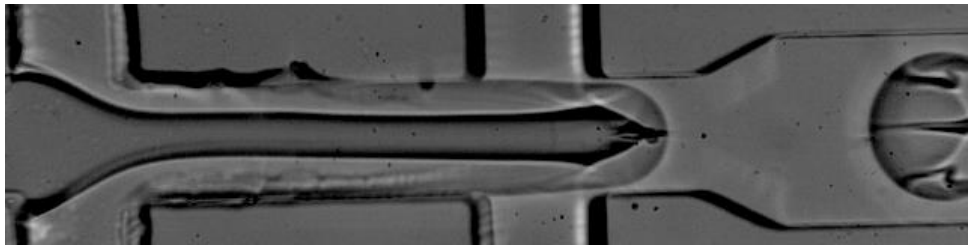
DROP-SEQ OVERVIEW

- Moved throughput from hundreds to thousands.
- Droplet-based processing using microfluidics
- Nanoliter scale aqueous drops in oil.
- 3' End
- Bead based (STAMPs).
- Single-cell transcriptomes attached to microparticles.
- Cell barcodes use split-pool synthesis.
- Uses UMI (Unique Molecular Identifier)
- Chance to have two cells within one droplet



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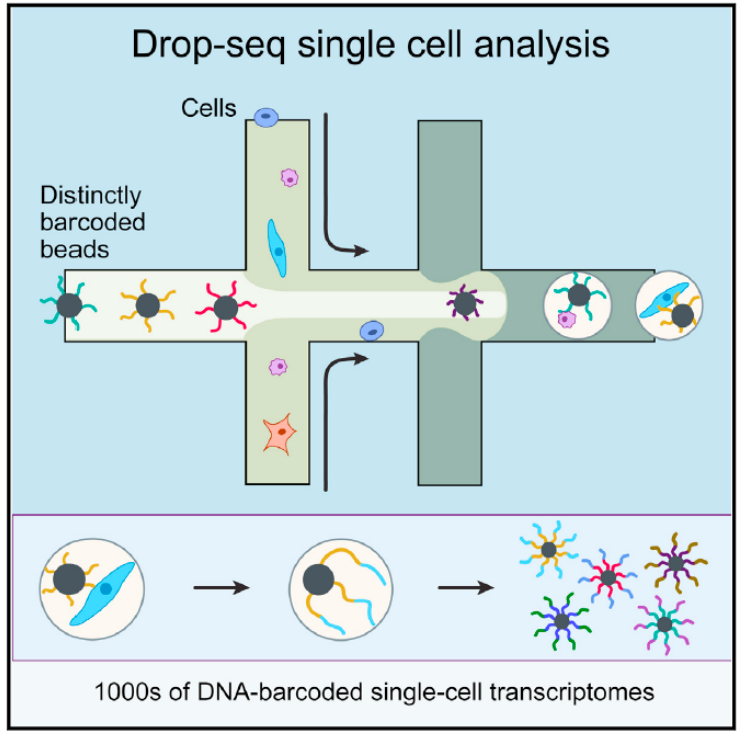
Resource

Cell

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

Graphical Abstract



Authors

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Correspondence

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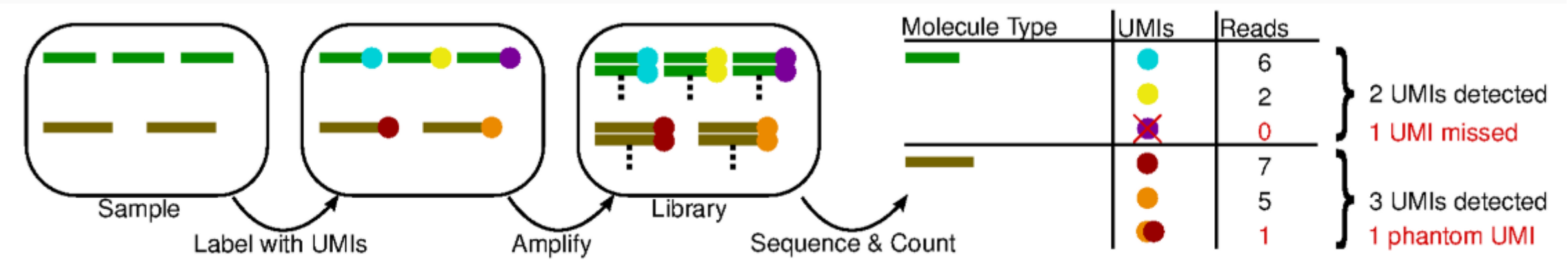
In Brief

Capturing single cells along with sets of uniquely barcoded primer beads together in tiny droplets enables large-scale, highly parallel single-cell transcriptomics. Applying this analysis to cells in mouse retinal tissue revealed transcriptionally distinct cell populations along with molecular markers of each type.

UMI – UNIQUE MOLECULAR IDENTIFIERS

After PCR enrichment, without UMIs, one can not distinguish if multiple copies of a fragment are caused by PCR clones or if they are real biological duplicated.
By using UMIs, PCR clones can be found by searching for non-unique fragment-UMI combinations, which can only be explained by PCR clones.
When performing variant analyses, these falsely overrepresented fragments can result in incorrect calls and thus wrong diagnostic findings



Source: Pflug et al. Bioinformatics (2018)

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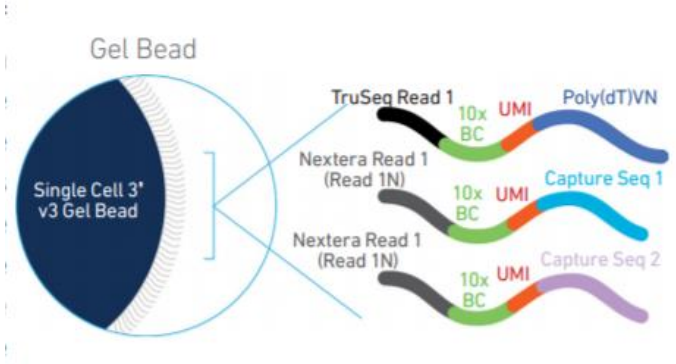
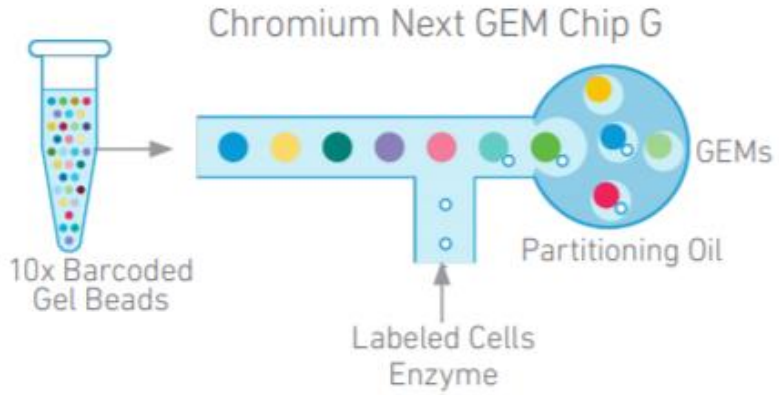
10X GENOMICS OVERVIEW

- Droplet-based similar to Drop-Seq, 3’ or 5’ mRNA
- In contrast to Drop-seq, where solid beads are used for RNA capture, 10X uses soft hydrogels containing oligos. These enable “single Poisson loading” leading to capture of >60% of input cells.
- Standardized instrumentation and reagents (unhackable so no customisation or control)
- Very easy to use and less processing time
- More high-throughput scaling - 8 samples can be processed simultaneously with up to 10000 cells captured per sample
- The doublet rate increases with number of cells loaded
- CellRanger and CellLoupe software are available and user friendly





Source: 10x Genomics

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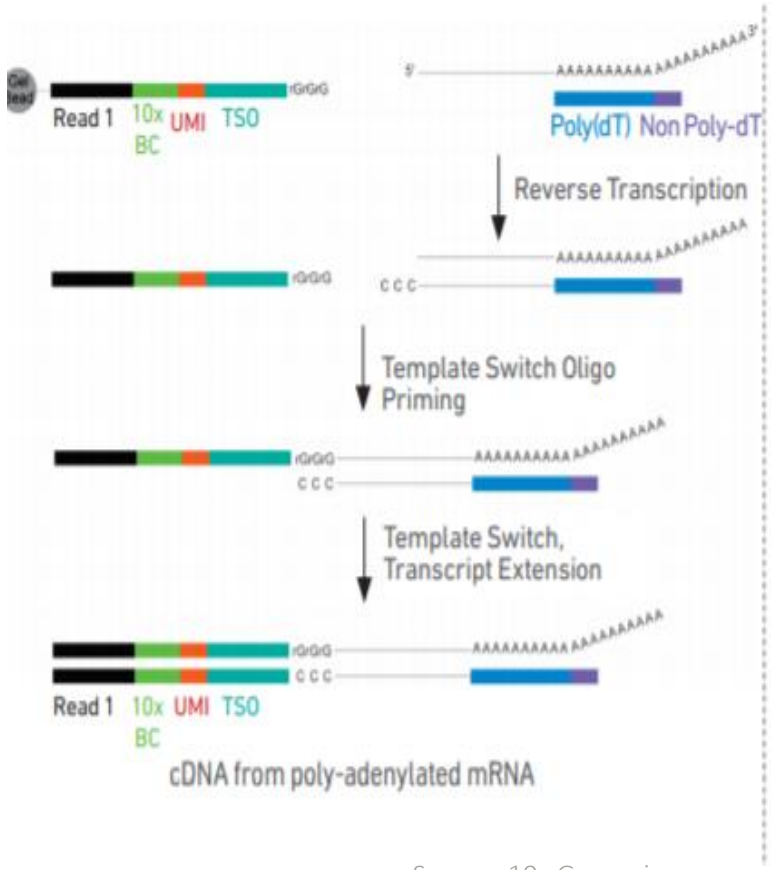
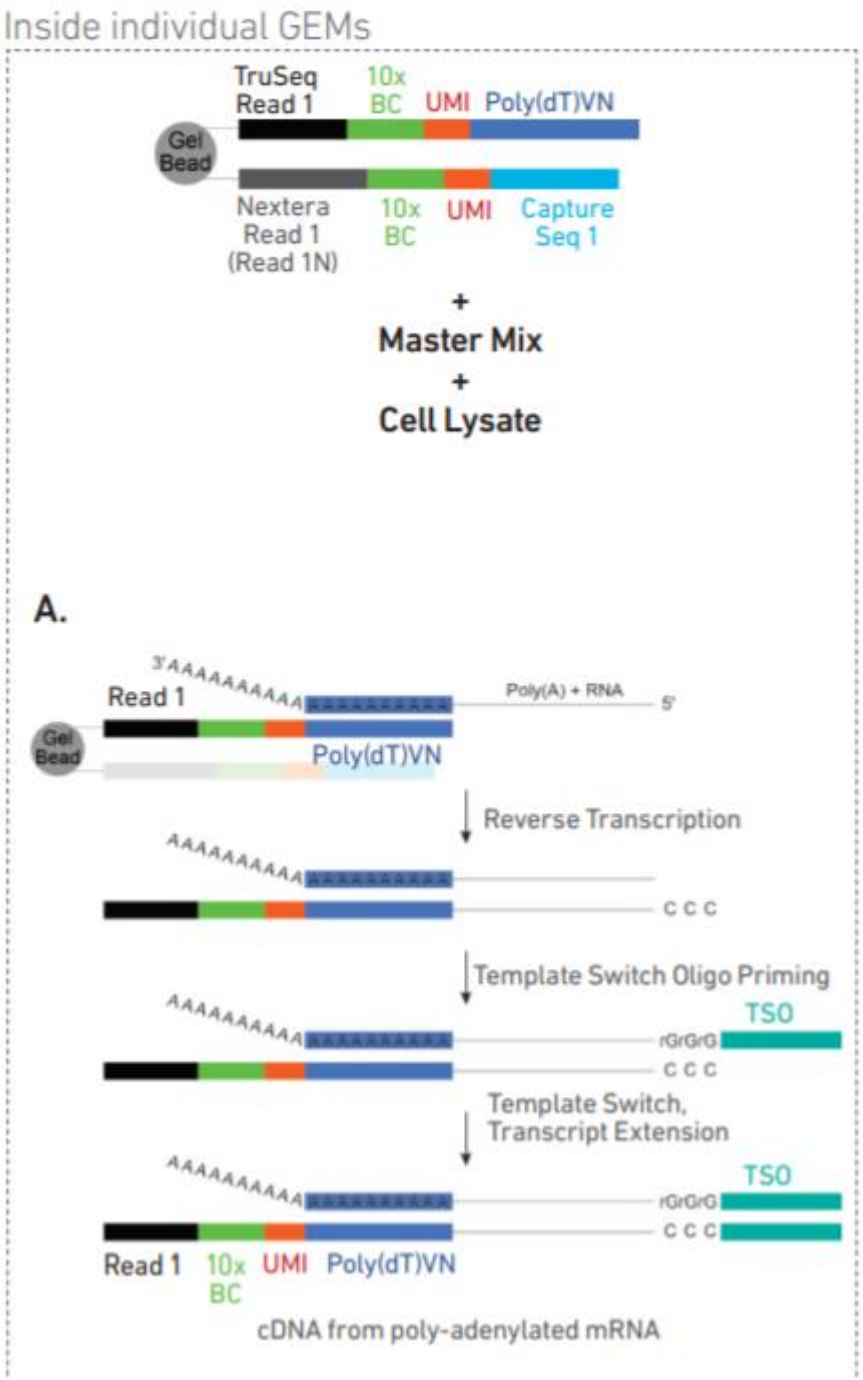
10X GENOMICS OVERVIEW





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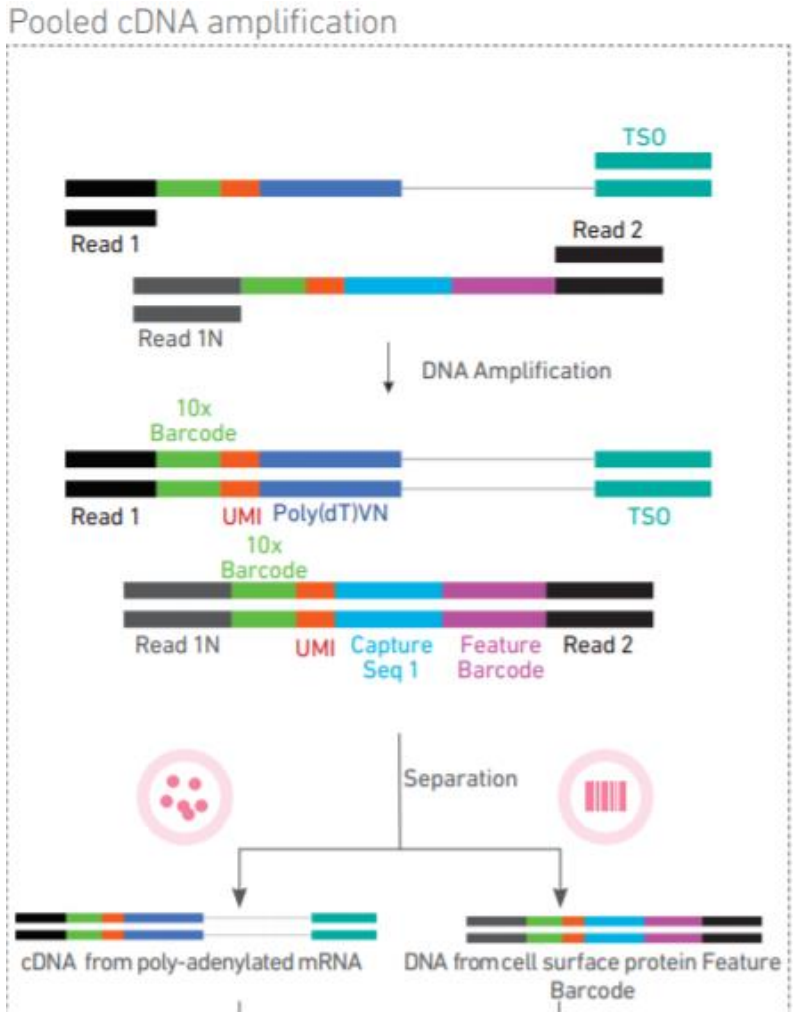
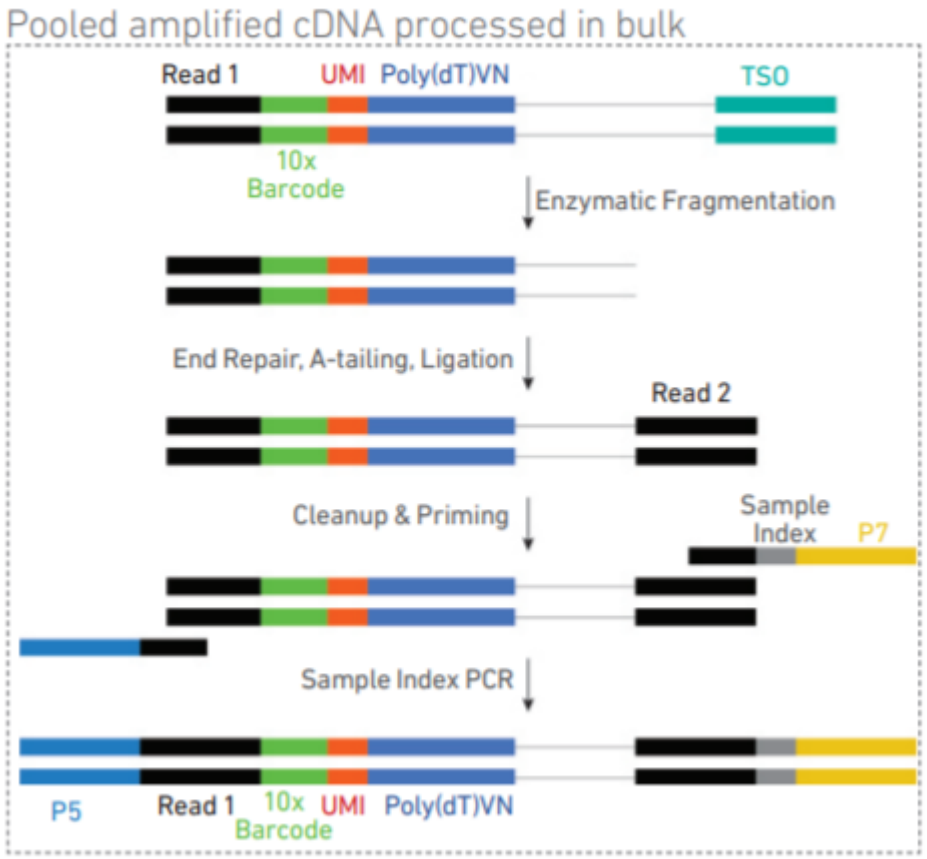
Source: 10x Genomics

10X GENOMICS OVERVIEW





Source: 10x Genomics

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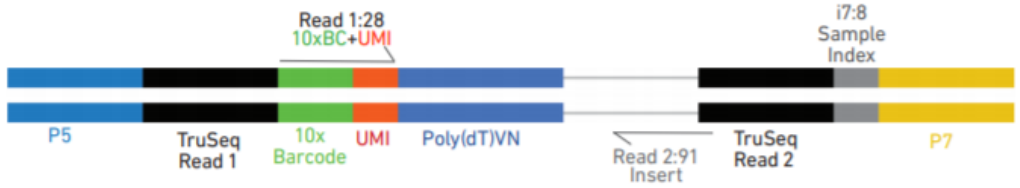
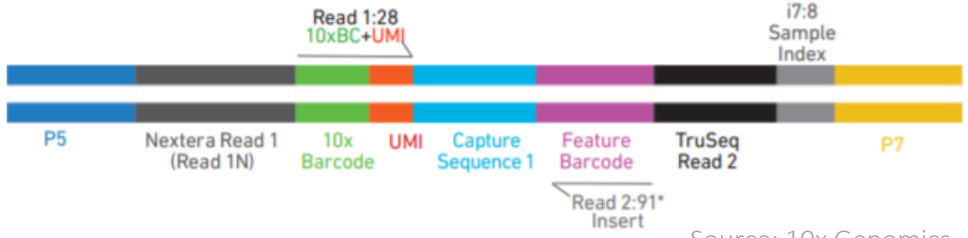
10X GENOMICS LIBRARIES

Chromium Single Cell 3' Gene Expression Library



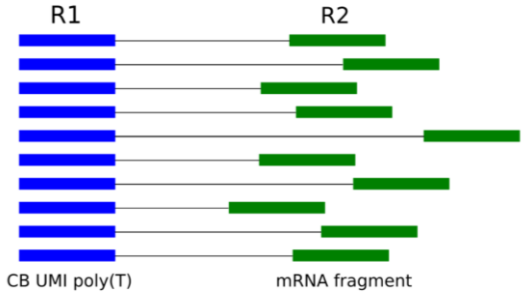
Chromium Single Cell V(D)J Enriched Library

Chromium Single Cell 3' Cell Surface Protein Library



Chromium Single Cell 5' Gene Expression Library

Source: 10x Genomics



Sequencing Read	Description	Number of cycles
Read1	10x Barcode Read (Cell) + Randomer Read (UMI)	28bp
i7 index	Sample index read	8bp (soon 10bp)
i5 index	Sample index read	0 (soon 10bp)
Read2	Insert Read (Transcript)	91bp (soon 90bp)

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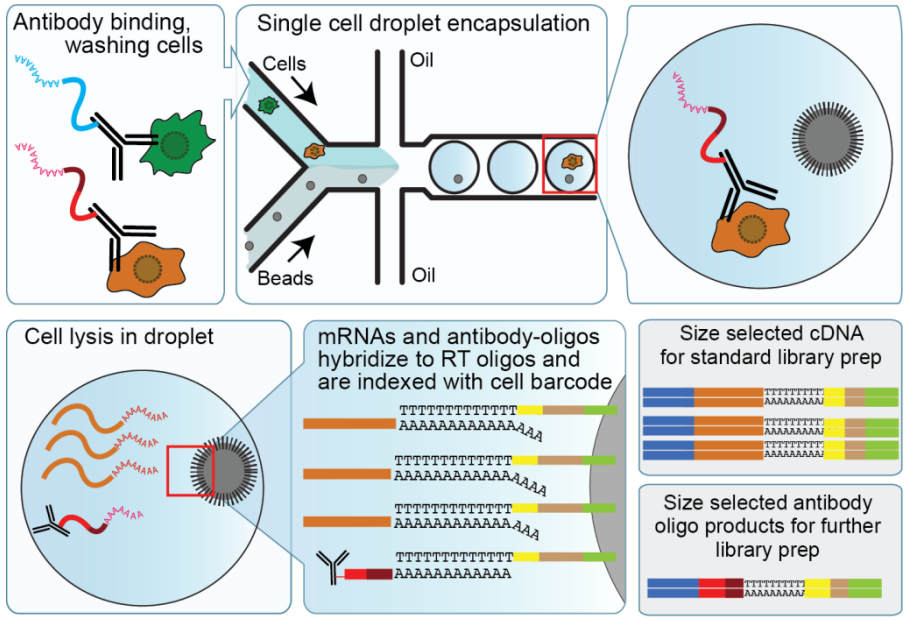
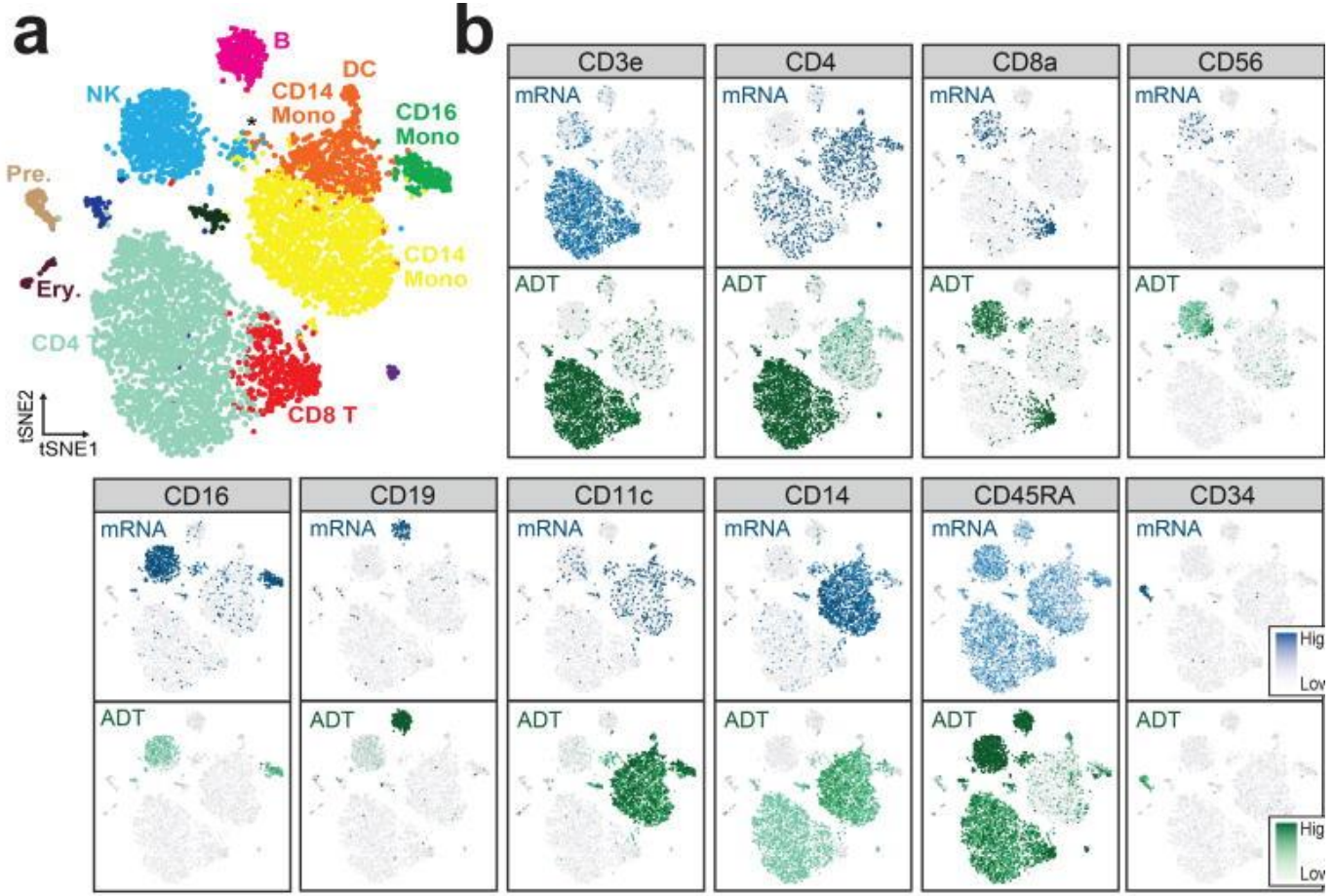
CITE-SEQ

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



Source: cite-seq.com



Source: Stoeckius et al. *Nat Methods*. (2017)

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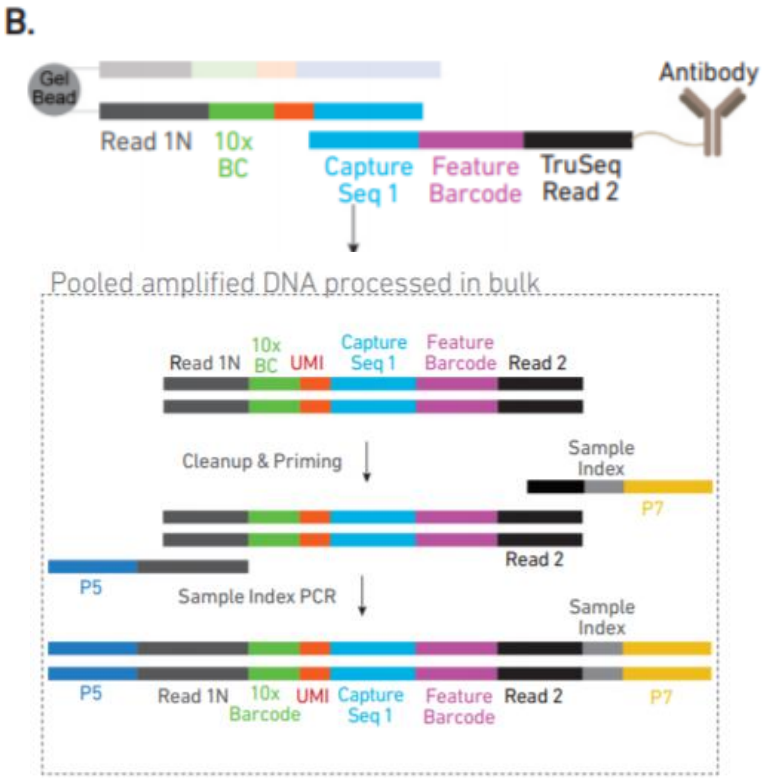
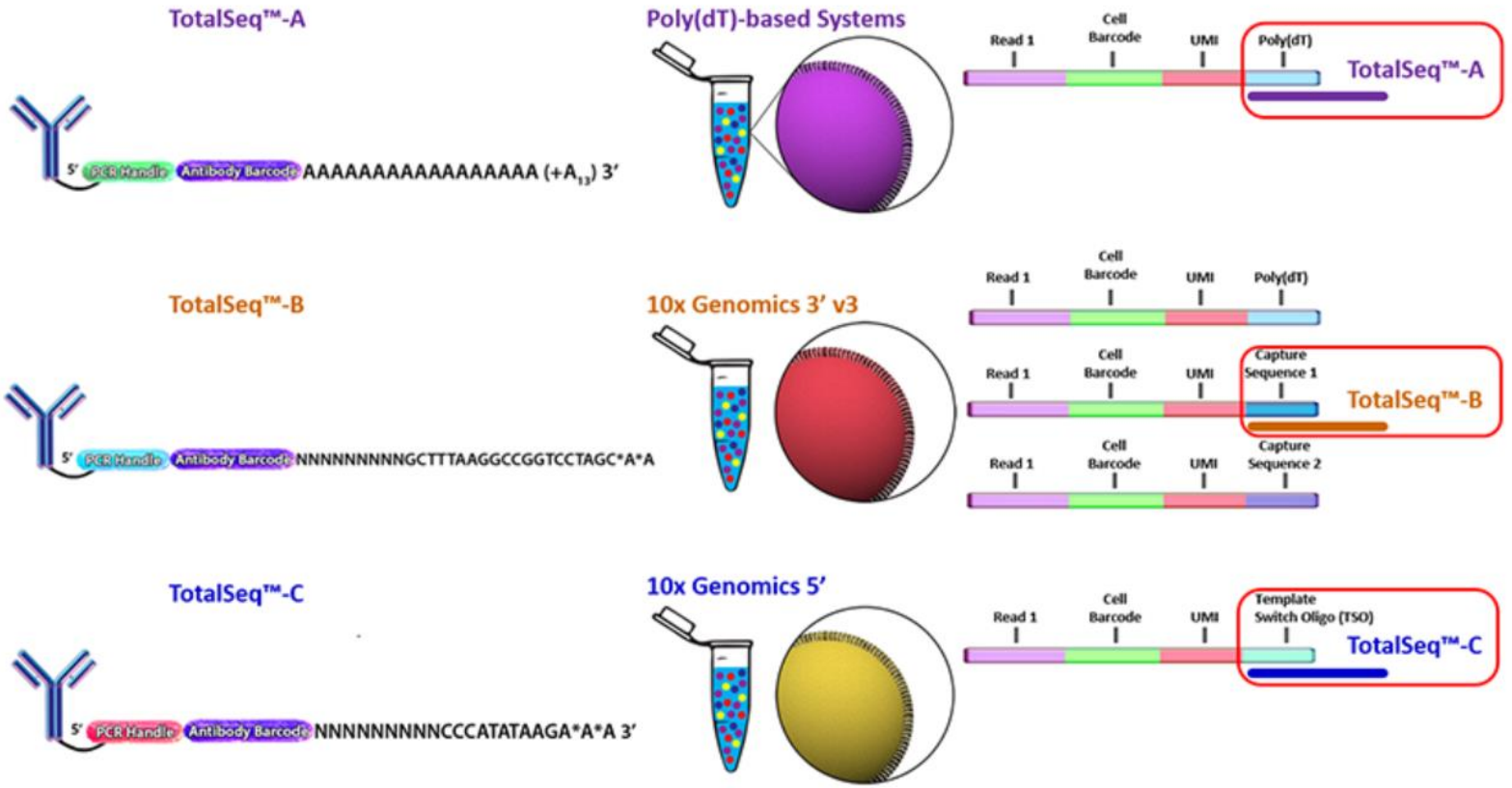
CITE-SEQ



Source: 10x Genomics



Source: BioLegend

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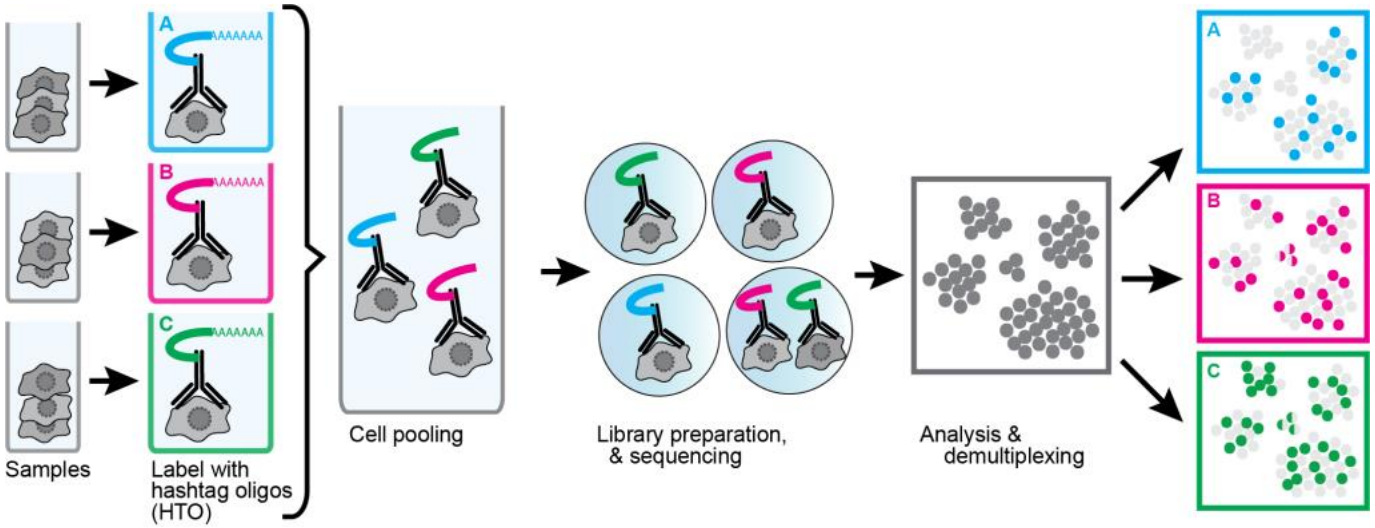
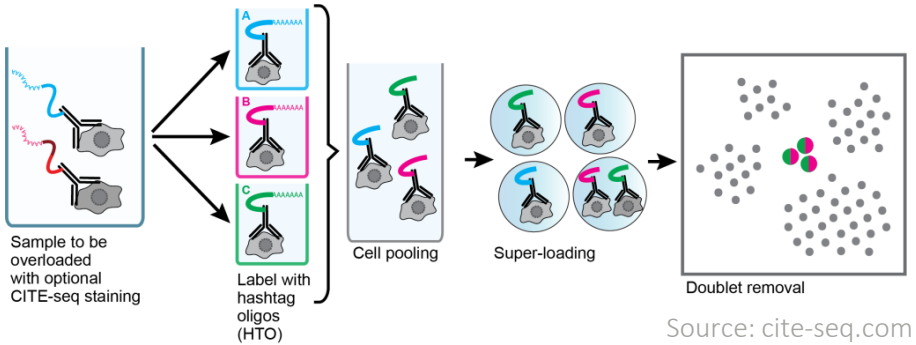
CELL HASHING

Reduces cost of running multiple samples by adding hashtag oligos and pooling into single channel of 10x chip



Allows overloading as 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



Source: cite-seq.com

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Genotype-free demultiplexing of pooled single-cell RNA-Seq

Jun Xu[a], Caitlin Falconer[b], Quan Nguyen[b], Joanna Crawford[b], Brett D. McKinnon[b,e], Sally Mortlock[b], Alice Pébay[f,g,h,i], Alex W. Hewitt[f,g,h,i], Anne Senabouth[d], Nathan Palpant[a,b], Han Chiu[b], Stacey Andersen[a,b], Grant W. Montgomery[a,b], Joseph Powell[c,d], Lachlan Coin[a,b,*]

nature methods

Article | Published: 17 June 2019

MULTI-seq: sample multiplexing for single-cell RNA sequencing using lipid-tagged indices

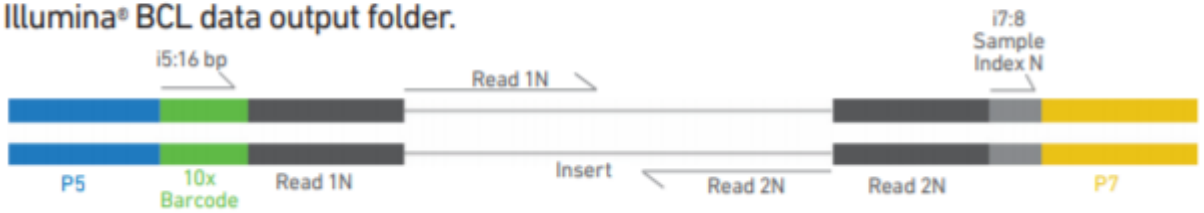
Christopher S. McGinnis, David M. Patterson, Juliane Winkler, Daniel N. Conrad, Marco Y. Hein, Vasudha Srivastava, Jennifer L. Hu, Lyndsay M. Murrow, Jonathan S. Weissman, Zena Werb, Eric D. Chow & Zev J. Gartner

Nature Methods **16**, 619–626(2019) | Cite this article

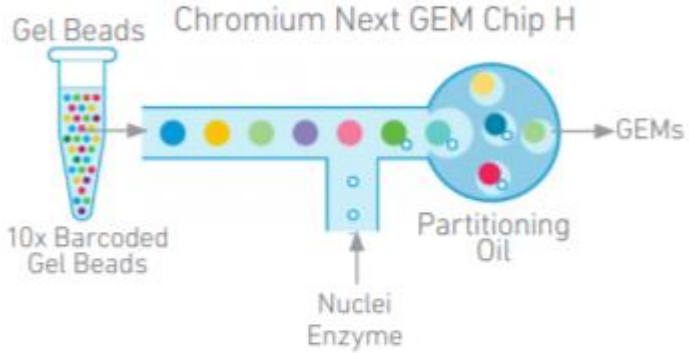
15k Accesses | **27** Citations | **85** Altmetric | Metrics

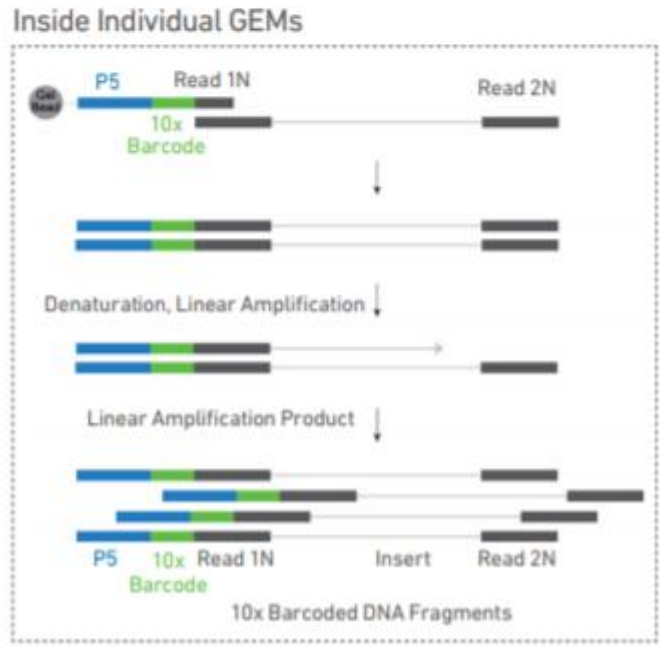
SINGLE CELL ATAC-SEQ

Chromium Single Cell ATAC libraries comprise double stranded DNA fragments which begin with P5 and end with P7. Sequencing these libraries produces a standard Illumina® BCL data output folder.



Sequencing Read	Description	Number of cycles
Read1	Insert Sequence 1N	50bp
i7 index	Sample index read	8bp
i5 index	10x Barcode Read (Cell)	16bp
Read2	Insert Sequence 2N (opposite end)	50bp





Source: 10x Genomics

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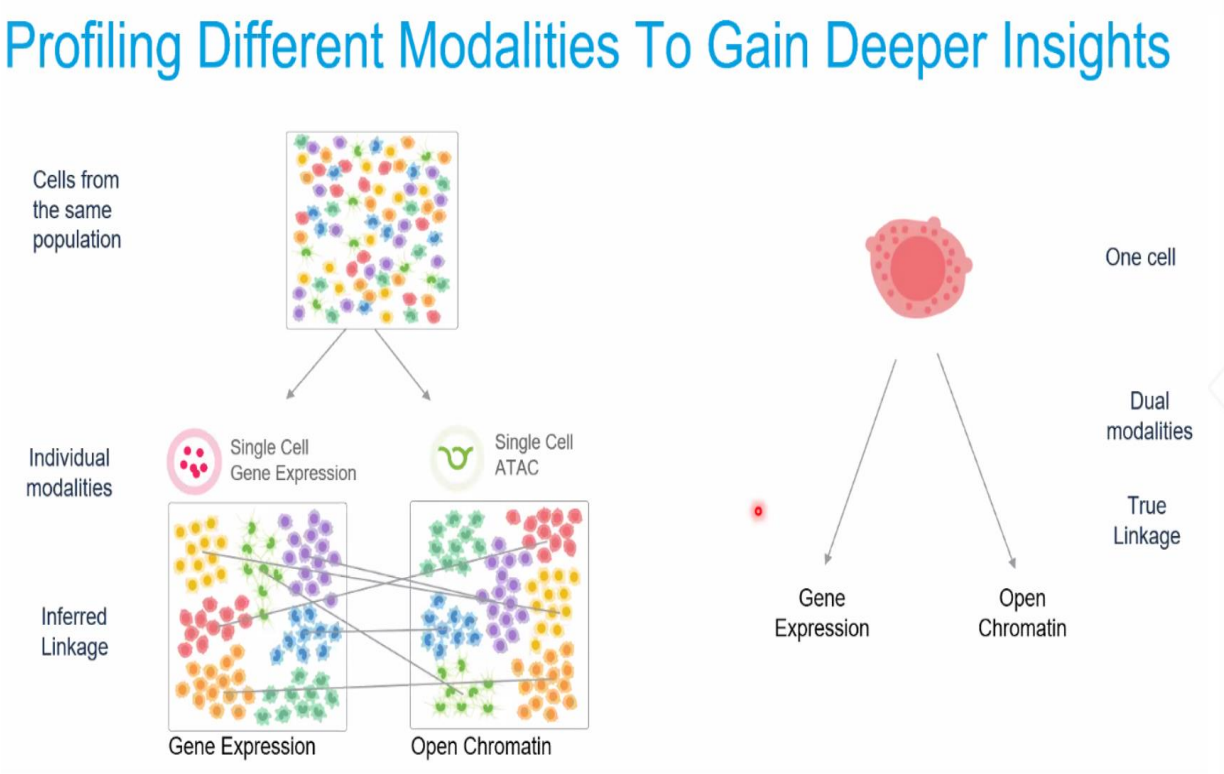
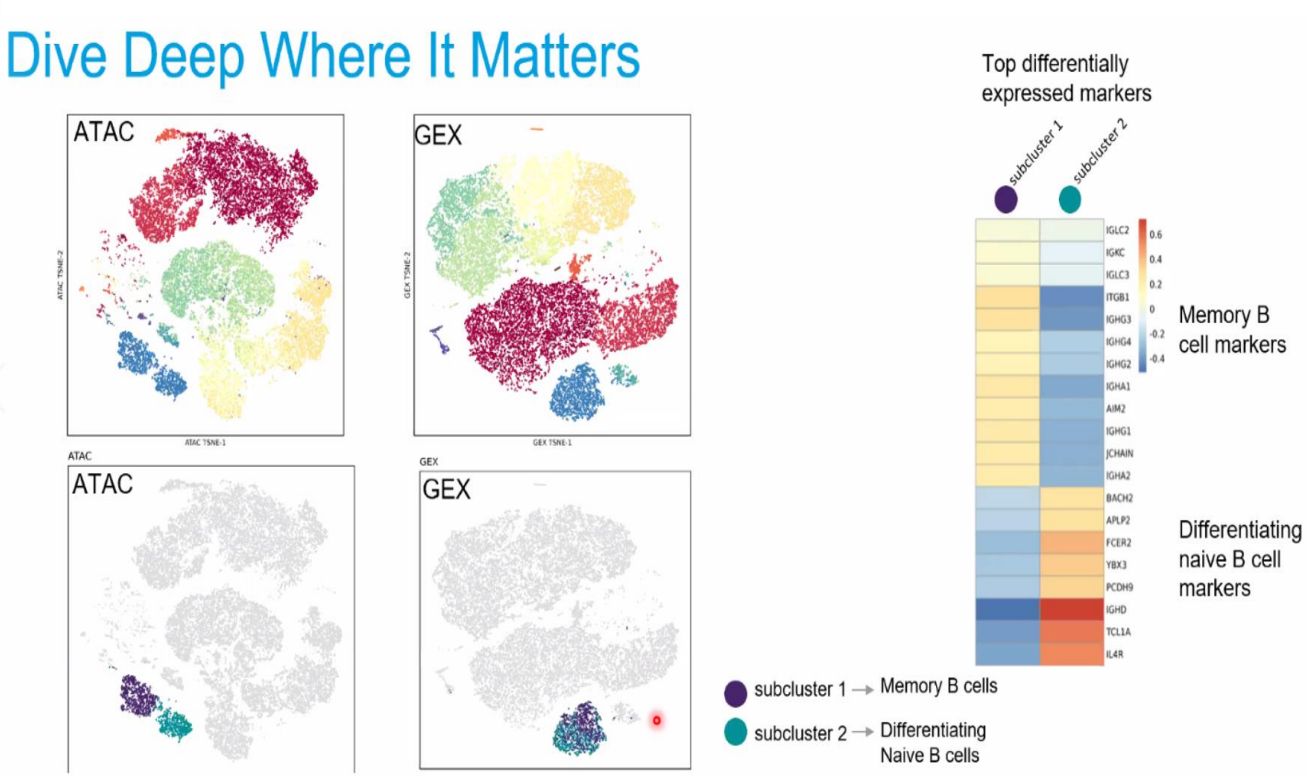
ATAC-SEQ + RNA-SEQ





Source: 10x Genomics

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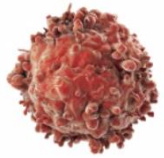
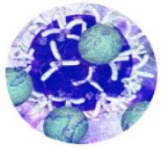
TARGETED PANELS

Accelerate research in 4 major areas



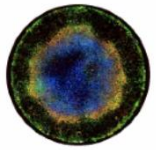
Human Pan-Cancer
1,253 genes

- **33 cancer types**, key biomarkers, pathways, and cellular processes
- Profile tumor microenvironment and heterogeneity, and tumor immune status in a wide variety of tumors



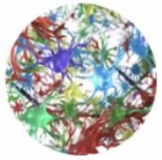
Human Immunology
1,056 genes

- Covers **innate and adaptive immunity**, **inflammation** and **immuno-oncology**
- Comprehensively profile the immune response in cells and tissues



Human Gene Signature
1,142 genes

- **Disease and drug targets,** including kinases, GPCRs, cell cycle/checkpoint
- Analyze the **activation or inhibition** of important signaling pathways, and discover mechanism of action of small molecules



Human Neuroscience
1,186 genes

- Covers **neural development, neurogenesis, neurodegenerative diseases** and **neuro-oncology**
- Characterize changes in gene expression in brain injury and disease

Increased experimental efficiency

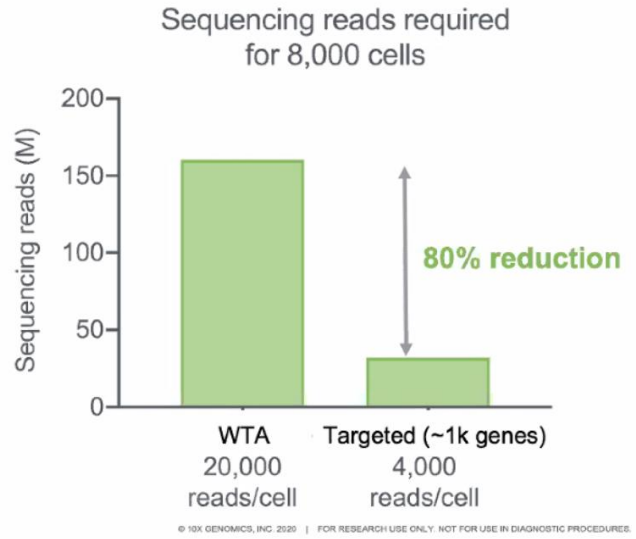
Reduced sequencing cost

WTA and targeted gene expression from the same cells

Core assay compatibility

Content and customization

10x GENOMICS



Source: 10x Genomics

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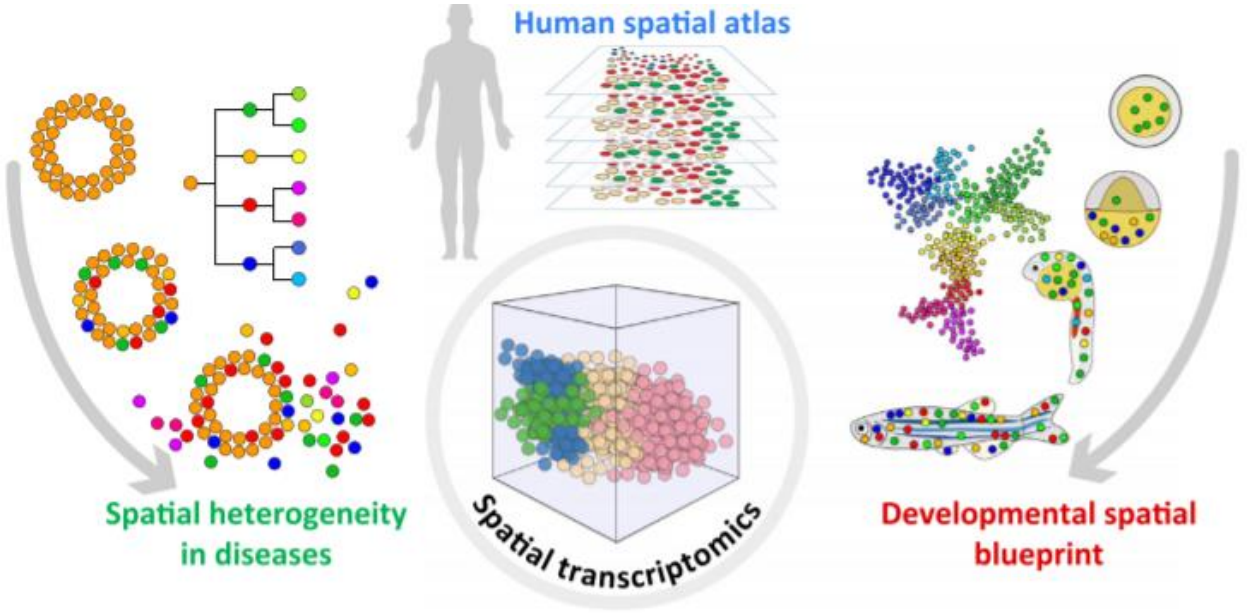
SPATIAL TRANSCRIPTOMICS

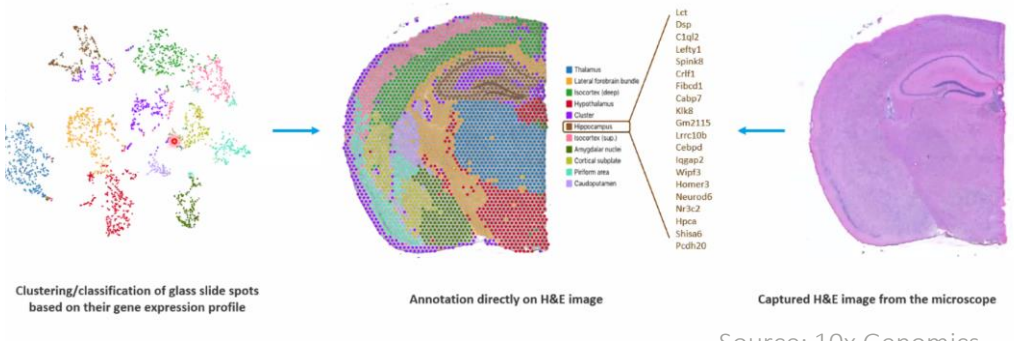


Trends in Biotechnology

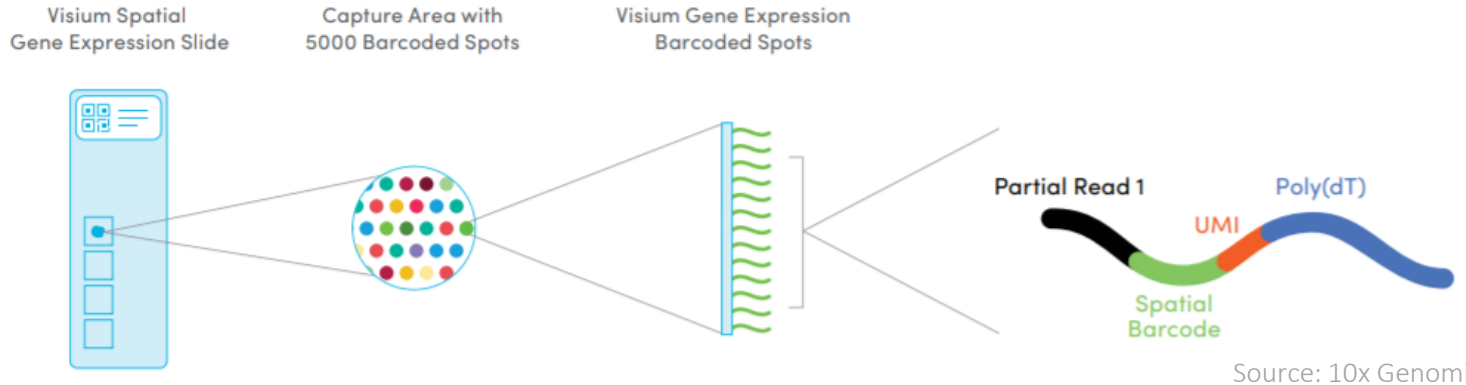
Figure 3. Applications for Spatially Resolved Transcriptomics. Three primary kinds of hot issues can be resolved by spatially resolved transcriptomics: left, discovering spatial heterogeneity of diseases; middle, establishing spatial transcriptome atlases for the human body; and right, delineating an embryonic developmental and spatial blueprint.

Source: Liao et al. Trends in Biotechnology. (2020)

55ul spots -> 1 and 10 cells captured per spot



Source: 10x Genomics



Source: 10x Genomics

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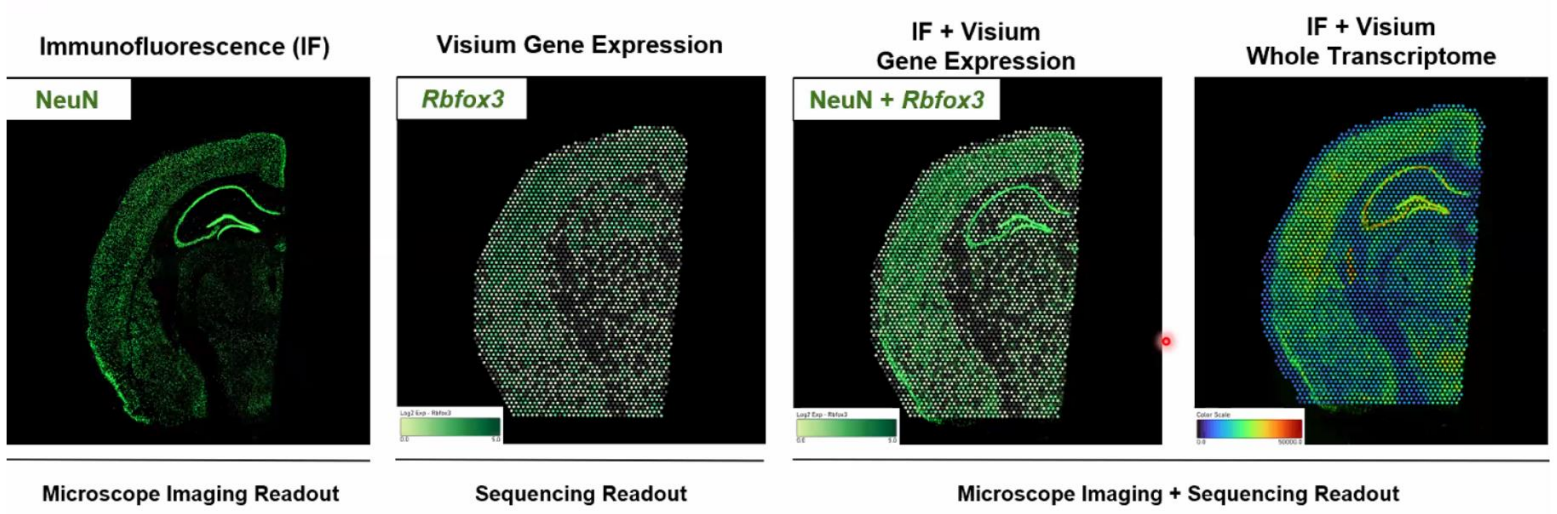
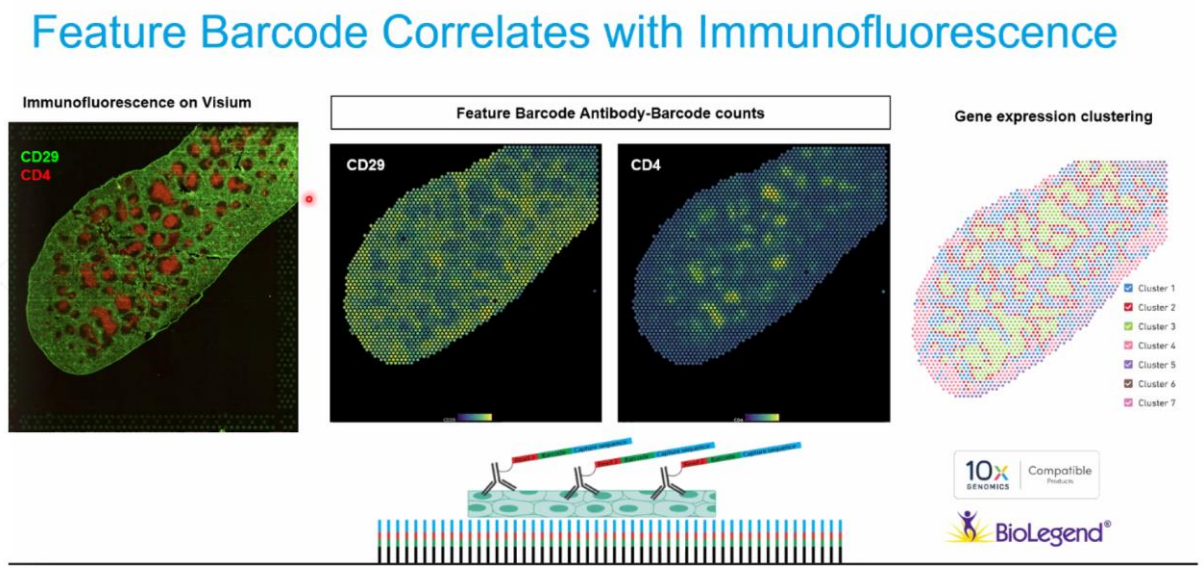
SPATIAL TRANSCRIPTOMICS





Source: 10x Genomics

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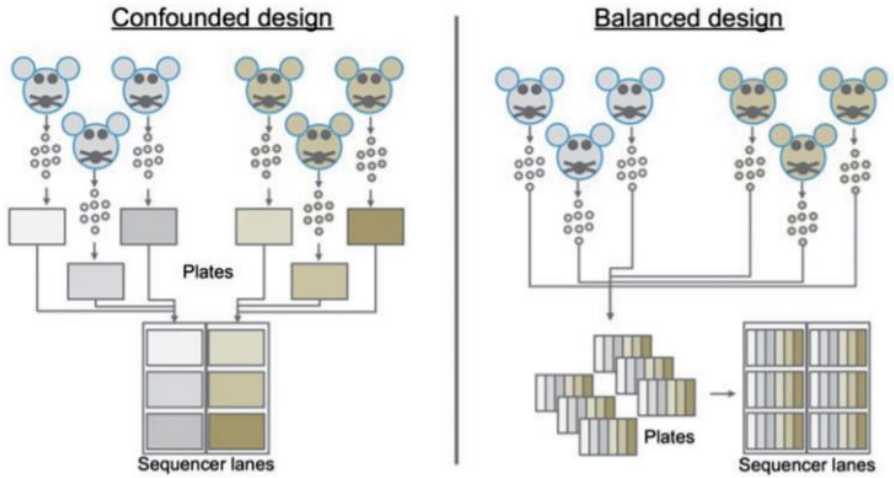
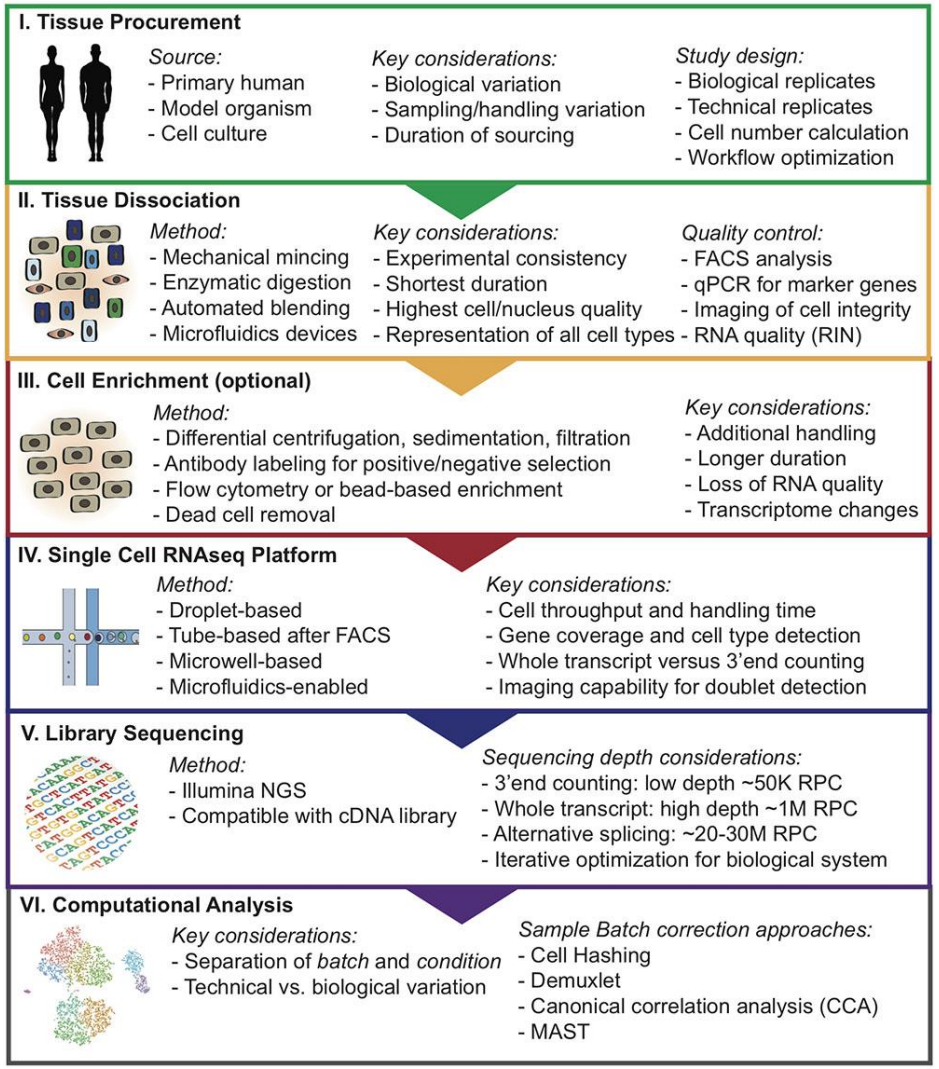
EXPERIMENTAL DESIGN



Source: Baran-Gale et al. Brief Func Genomics. 17 (4):233–239. (2018)



Source: Nguyen QH et al. *Front Cell Dev Biol* 6:108. (2018)

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WHAT PLATFORM SHOULD I USE?

Choose protocol based on:

- Throughput (number of cells per reaction)
- Sample of origin
- Cost / Labour / Time limitations
- Gene body coverage: 5'/ 3' biased or full-length?
- UMI vs no-UMI
- Sequencing depth per cell

Examples:

- If you sample is fairly homogeneous – bulk RNAseq
- If your sample is limited in cell number – plate-based method
- If you want re-annotate the transcriptome and discover new isoforms – full-length coverage (SMART-seq2)
- If you are looking to classify all cell types in a diverse tissue - high throughput
- If you have only archival human samples – nuclei isolation

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LITERATURE:

- https://hbctraining.github.io/scRNA-seq/slides/Single_Cell_2_27_20.pdf.
- https://www.slideshare.net/TimothyTickle/introduction-to-singlecell-rnaseq
- Arzalluz-Luque et al. A. Single-cell RNAseq for the study of isoforms—how is that possible?. Genome Biol 19, 110 (2018).
- Ding et al. Systematic comparison of single-cell and single-nucleus RNA-sequencing methods. Nat Biotechnol 38, 737–746 (2020).
- Guillaumet-Adkins et al. Single-cell transcriptome conservation in cryopreserved cells and tissues. Genome Biol 18, 45 (2017).
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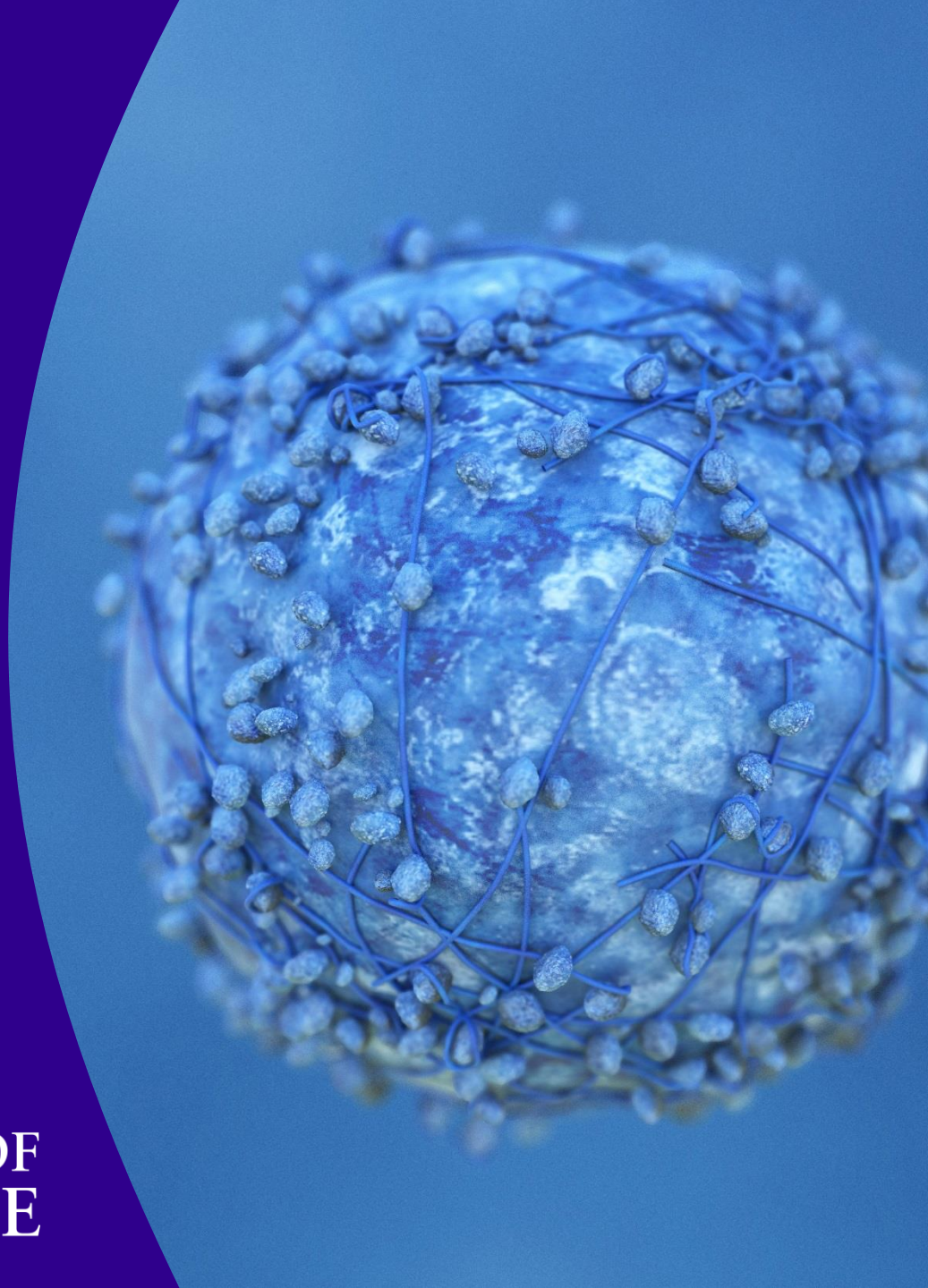
- Stoeckius et al. Simultaneous epitope and transcriptome measurement in single cells. Nat Methods. (2017).
- Svensson et al. Power analysis of single-cell RNA-sequencing experiments. Nat Methods 14, 381–387 (2017).
- Svensson et al. Exponential scaling of single-cell RNA-seq in the past decade. Nat Protoc 13, 599–604 (2018).
- Wen et al. Development of Droplet Microfluidics Enabling High-Throughput Single-Cell Analysis. Molecules. 21. (2016).
- Wilk et al. A single-cell atlas of the peripheral immune response in patients with severe COVID-19. Nat Med 26, 1070–1076 (2020).
- Xu et al. Genotype-free demultiplexing of pooled single-cell RNA-seq. Genome Biol 20, 290 (2019).
- Ziegenhain et al. Comparative Analysis of Single-Cell RNA Sequencing Methods. Mol Cell. 65(4):631-643.e4. (2017).

USEFUL RESOURCES:

- Haque et al. A practical guide to single-cell RNA-sequencing for biomedical research and clinical applications. *Genome Med*. 2017;9(1):75.
- Single cell course by Hemberg Lab, Wellcome Sanger Institute (http://hemberg-lab.github.io/scRNA.seq.course/index.html)
- Tabula Muris (https://tabula-muris.ds.czbiohub.org/)
- Human Cell Atlas (https://www.humancellatlas.org/)
- 10x Genomics demonstrated protocols for sample preparation (https://support.10xgenomics.com/single-cell-gene-expression/sample-prep)
- Worthington Tissue Dissociation Guide
- (http://www.worthington-biochem.com/tissuedissociation/default.html)
- Broad Institute Single Cell Portal (https://singlecell.broadinstitute.org/single_cell)
- List of software packages for single cell data analysis (https://github.com/seandavi/awesome-single-cell)
- SPLIT-seq (https://sites.google.com/uw.edu/splitseq)
- CITE-seq (https://cite-seq.com/)
- Biolegend TotalSeq (https://www.biolegend.com/en-us/totalseq)



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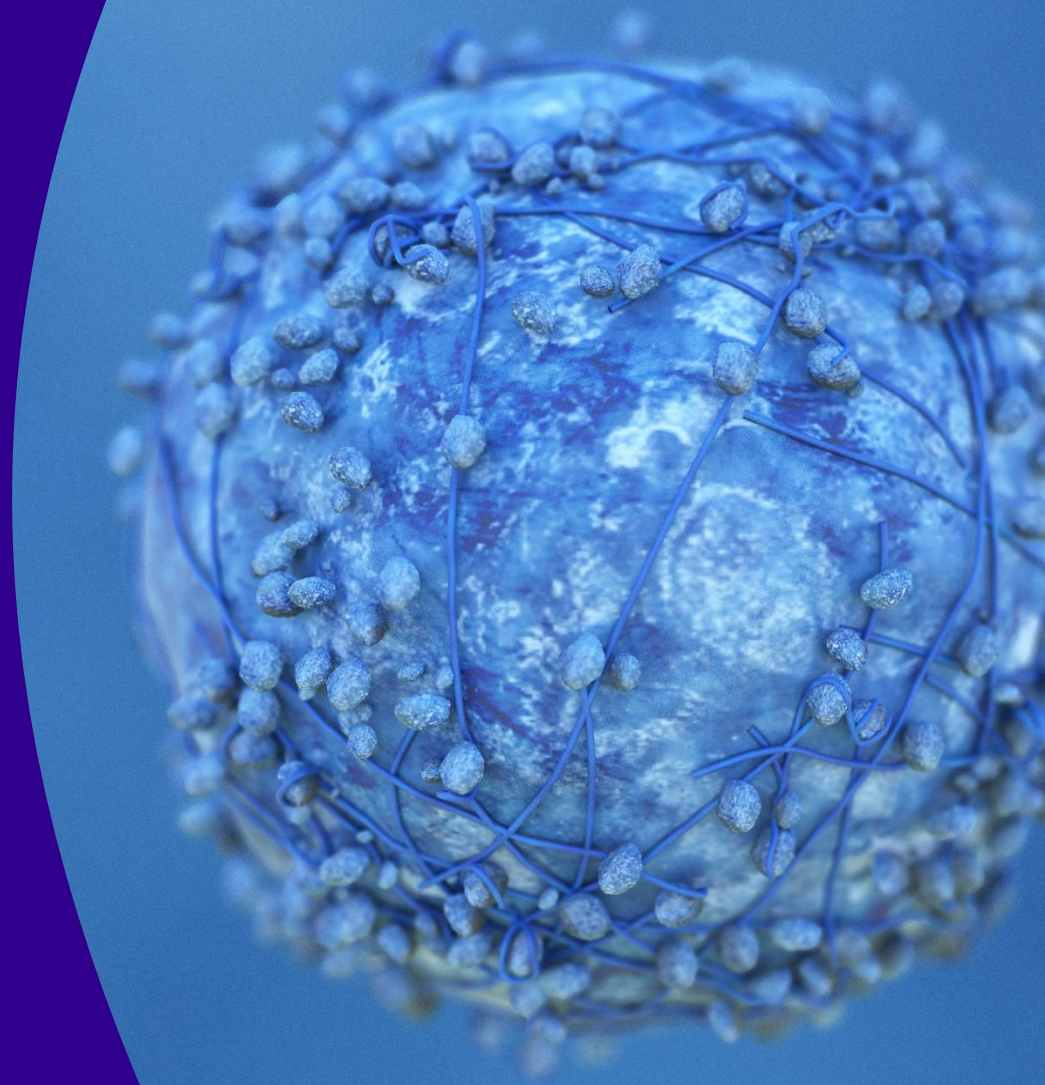
THANK YOU FOR YOUR ATTENTION!



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