

INTRODUCTION TO SINGLE CELL RNA-SEQ

CRUK CI Bioinformatics Summer School 2020

Katarzyna Kania, 24th July 2020



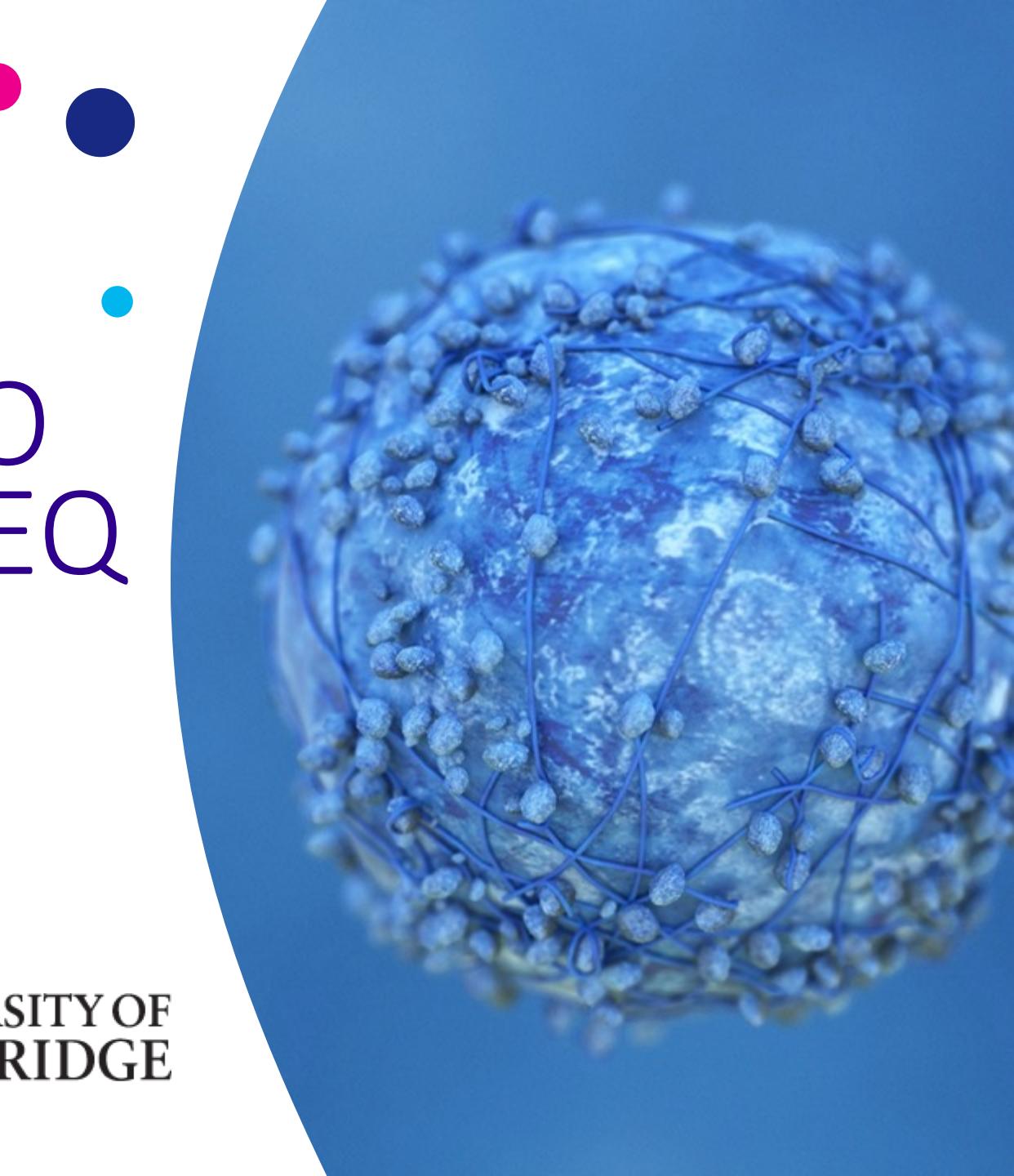
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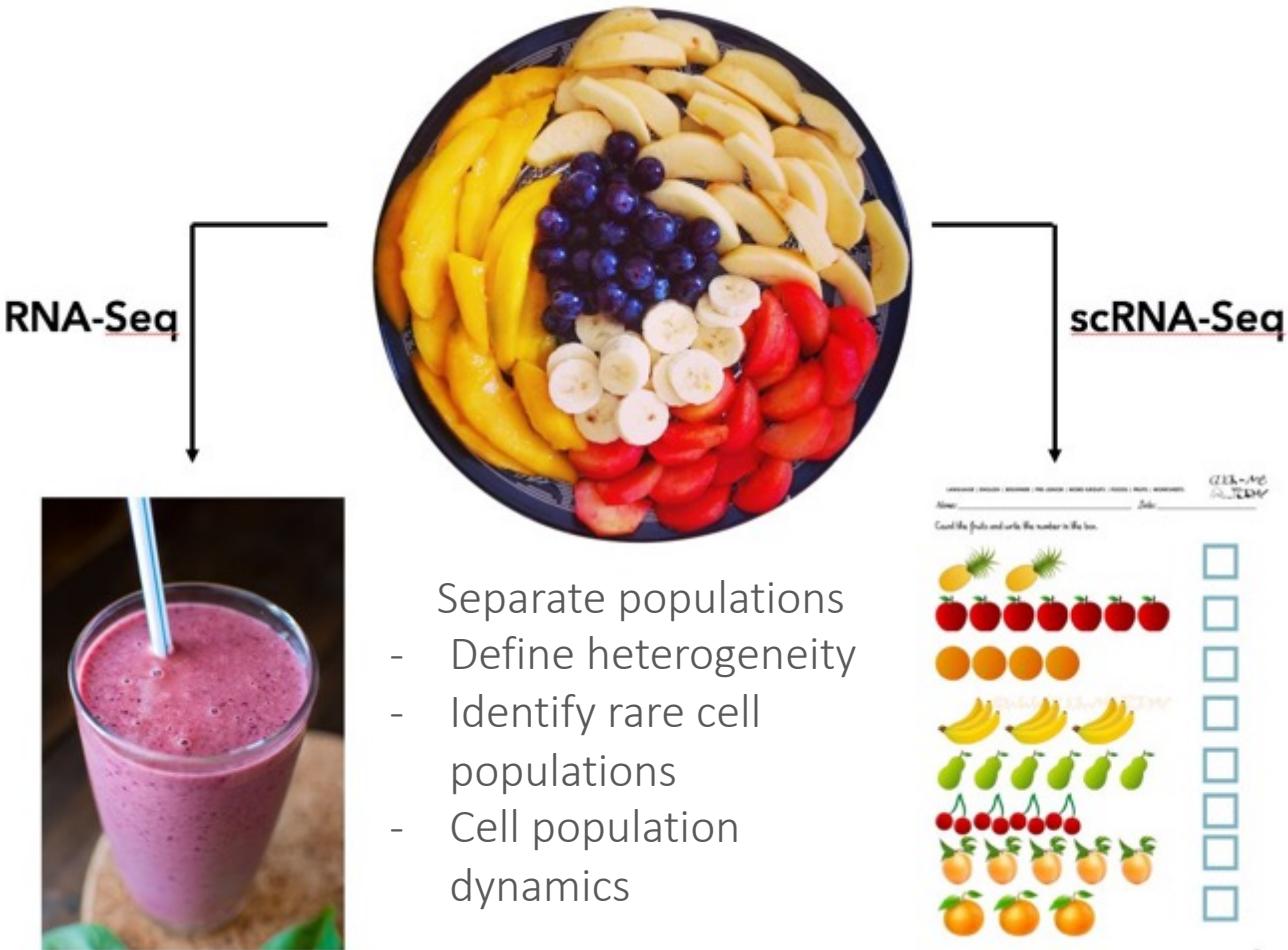
Together we will beat cancer



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BULK VS SINGLE CELL RNA-SEQ



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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.

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



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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

Article | Published: 20 February 2019

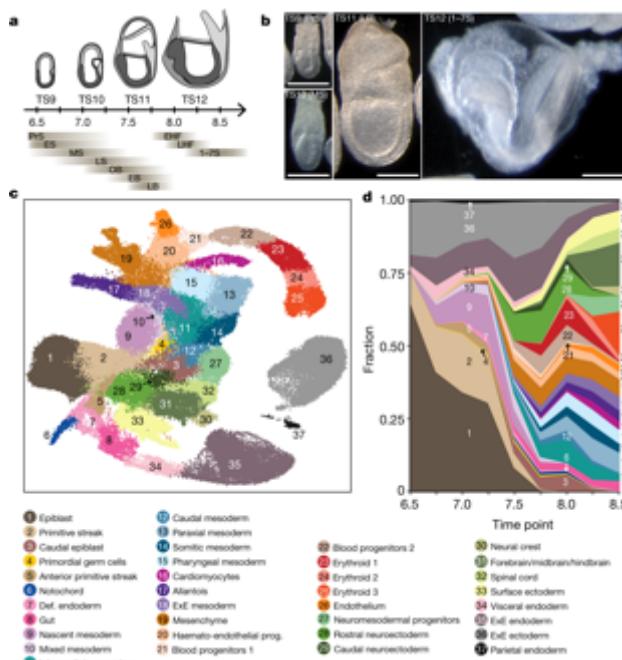
A single-cell molecular map of mouse gastrulation and early organogenesis

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. Tyser, Debbie Lee Lian Ho, Wolf Reik, Shankar Srinivas, Benjamin D. Simons, Jennifer Nichols, John C. Marioni & Berthold Göttgens

Nature 566, 490–495(2019) | Cite this article

42k Accesses | 70 Citations | 460 Altmetric | Metrics

Development lineage tracing



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

naturemedicine

Letter | Published: 08 June 2020

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

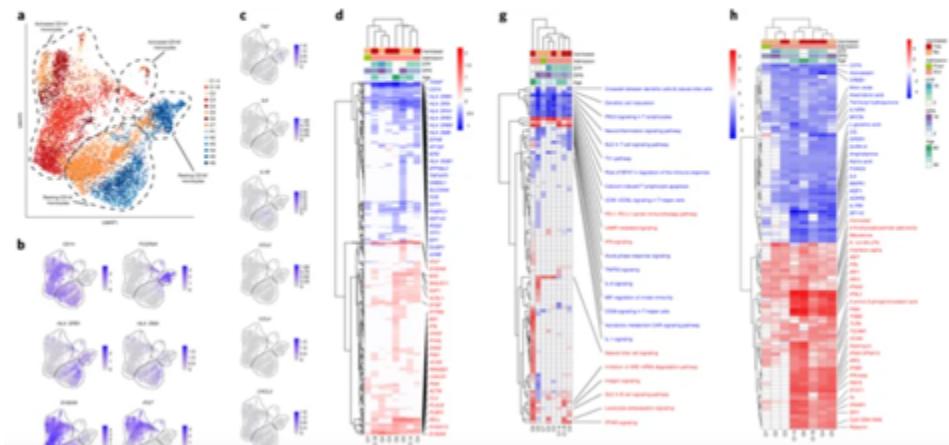
Aaron J. Wilk, Arjun Rustagi, Nancy Q. Zhao, Jonasel Roque, Giovanny J. Martinez-Colón, Julia L. McKechnie, Geoffrey T. Ivison, Thanmayi Ranganath, Rosemary Vergara, Taylor Hollis, Laura J. Simpson, Philip Grant, Aruna Subramanian, Angela J. Rogers & Catherine A. Blish

Nature Medicine 26, 1070–1076(2020) | Cite this article

35k Accesses | 8 Citations | 180 Altmetric | Metrics

Studying cellular responses

Fig. 2: Robust HLA class II downregulation and type I interferon-driven inflammatory signatures in monocytes are characteristics of SARS-CoV-2 infection.



Source: Wilk et al. Nat Med 26, 1070–1076 (2020)

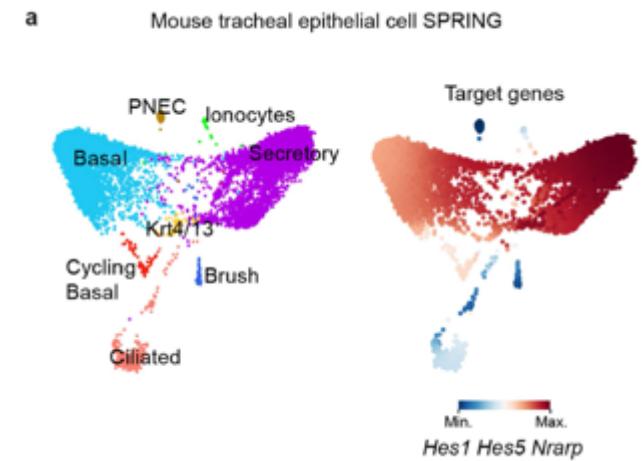
LETTER

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

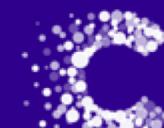
A single-cell atlas of the airway epithelium reveals the CFTR-rich pulmonary ionocyte

Lindsey W. Plaschaert^{1,5,7}, Rapolas Zillionis^{2,3,7}, Rayman Choo-Wing^{1,5}, Virginia Savova^{2,6}, Judith Knehr⁴, Guglielmo Roma⁴, Alton M. Klein^{2*} & Aron B. Jaffe^{1,5,8}

Studying heterogeneity



Source: Plaschaert et al. Nature 560, 377–381 (2018)

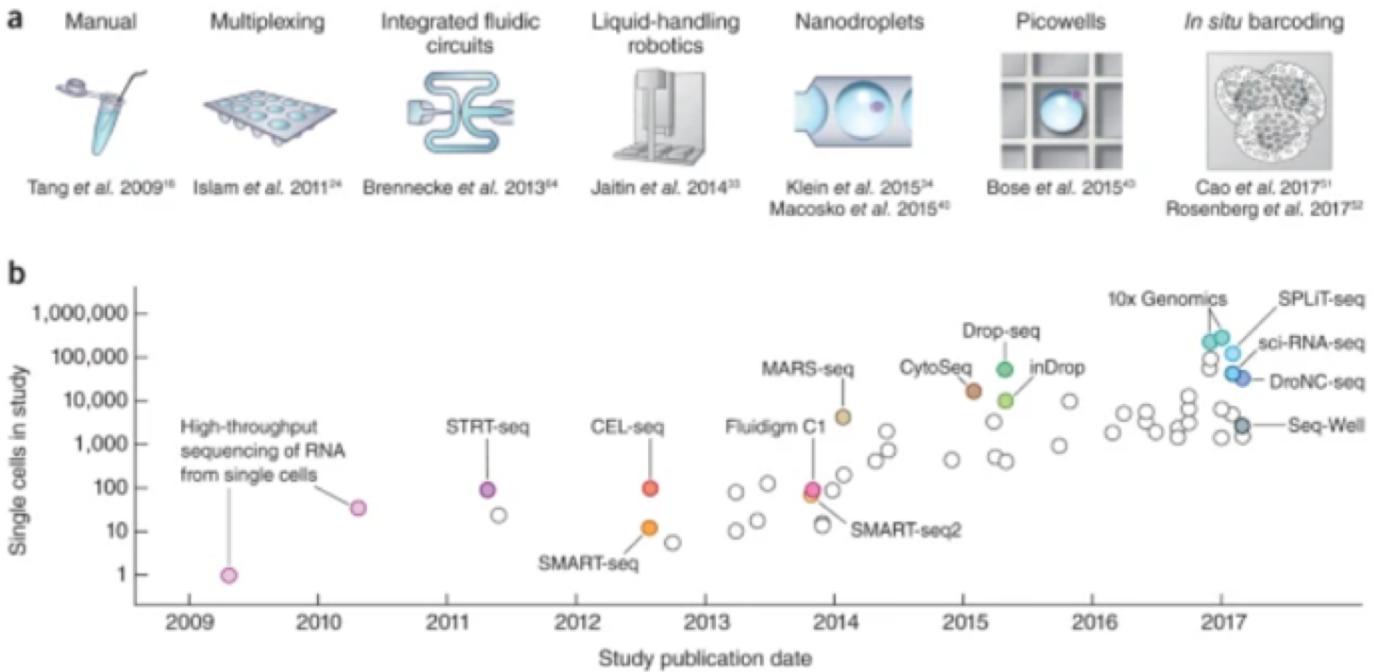


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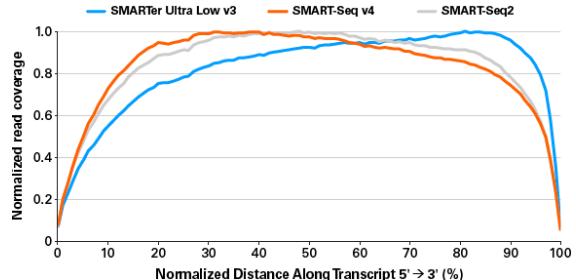
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TECHNOLOGIES

Figure 1: Scaling of scRNA-seq experiments.



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



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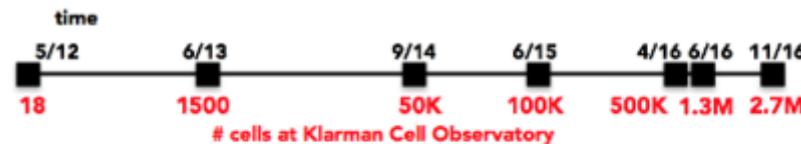
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HISTORY AND PROGRESS

LETTER

Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells

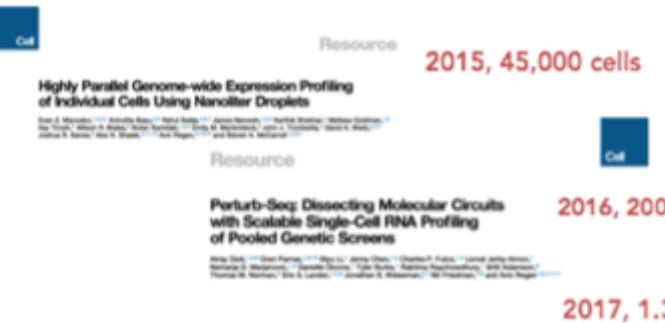
2013, 18 cells



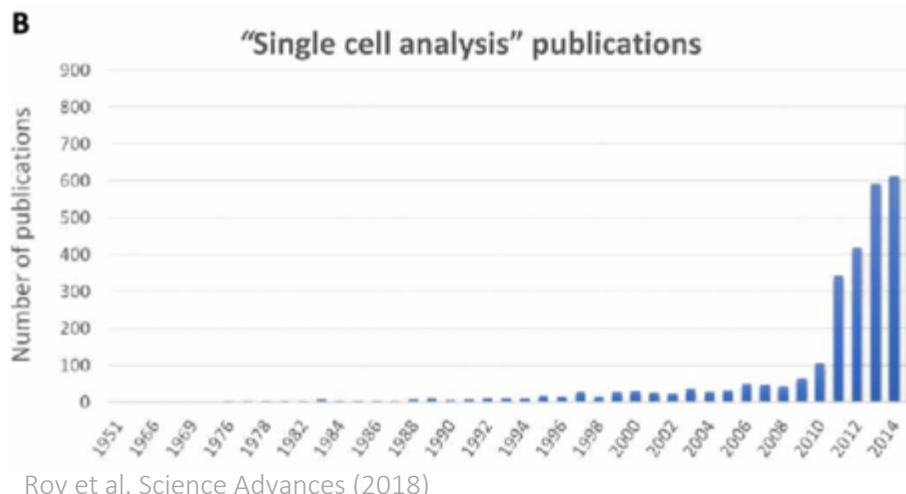
ARTICLE

Single-cell RNA-seq reveals dynamic paracrine control of cellular variation

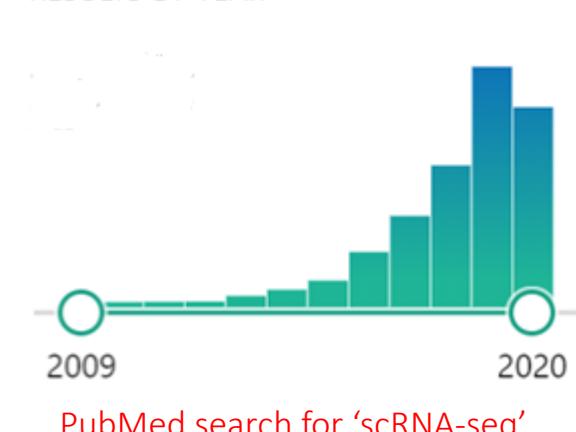
2014, 1700 cells



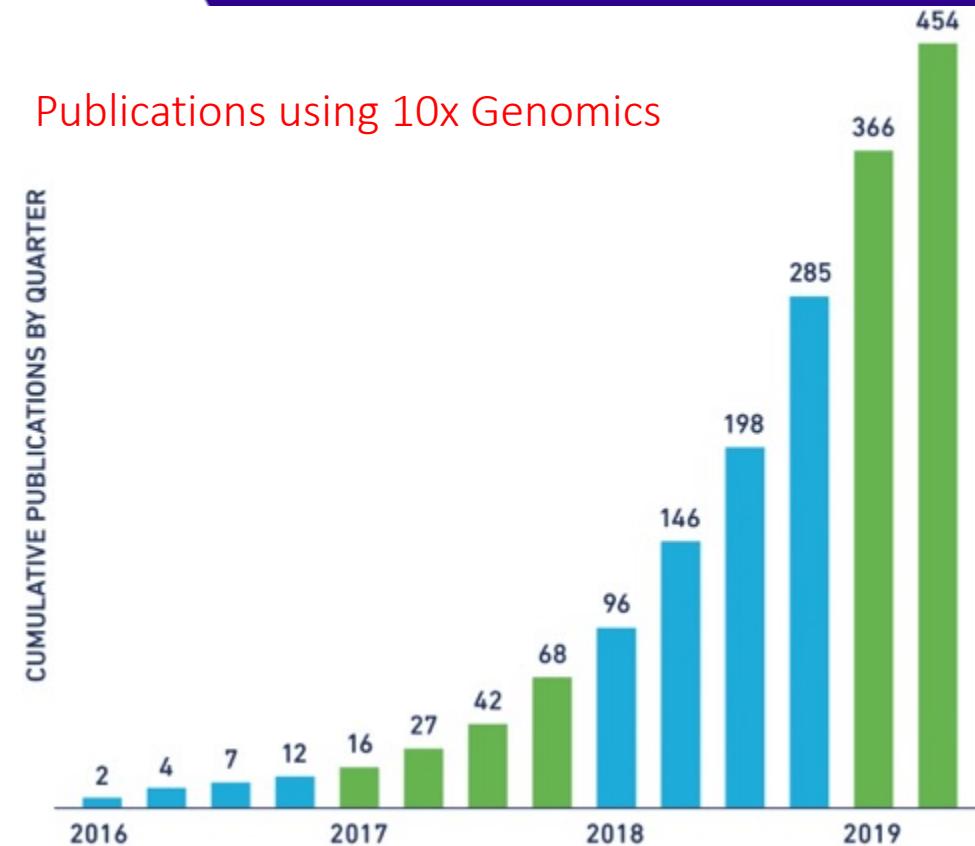
Source: Introduction to scRNASeq, Timothy Tickle & Brian Haas, Broad Institute, 2017



RESULTS BY YEAR



Publications using 10x Genomics



Source: <https://www.sec.gov/Archives/edgar/data/1770787/000119312519224368/d737378ds1.htm>



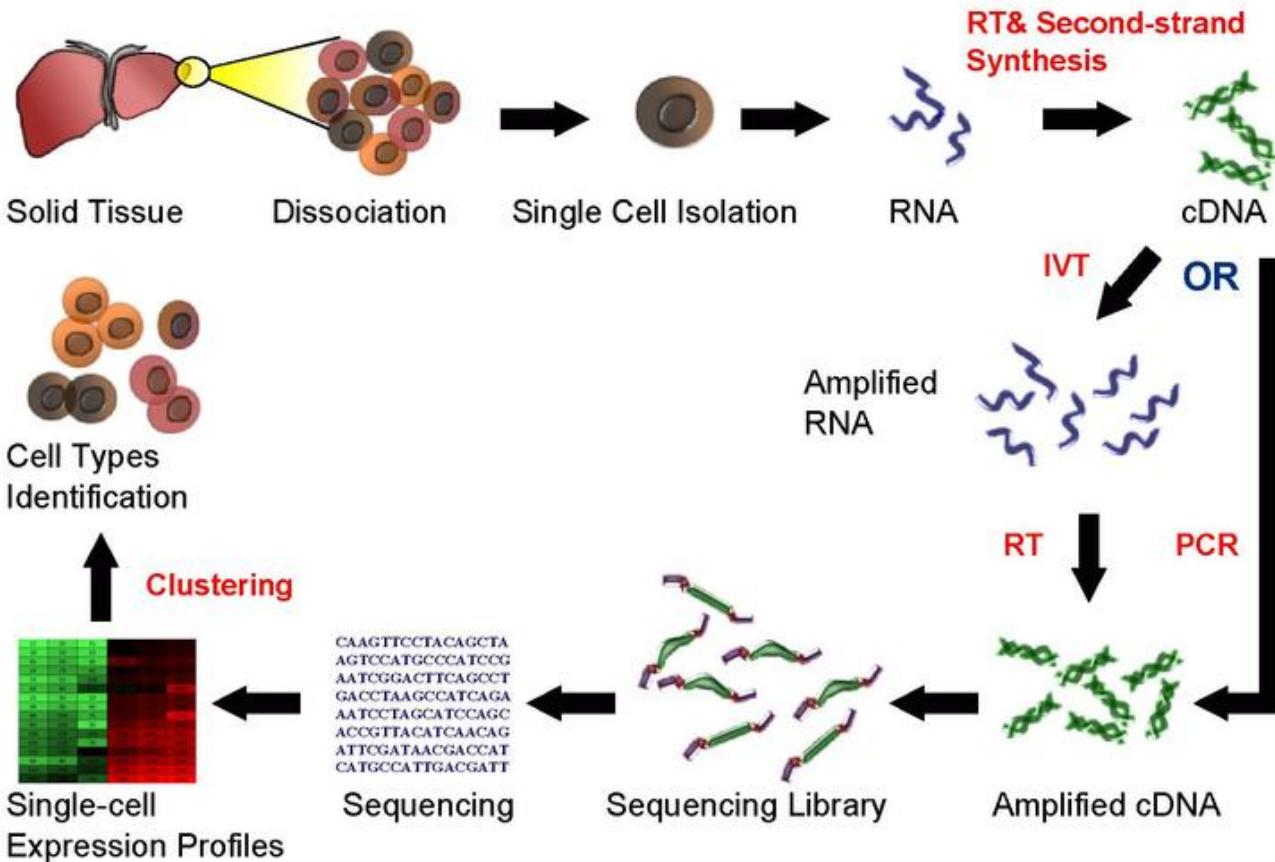
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WORKFLOW



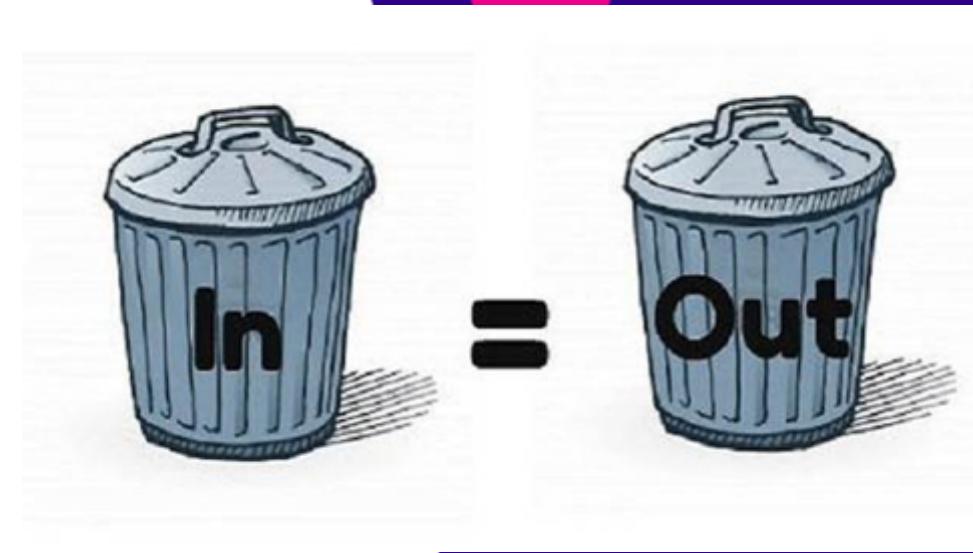
Single Cell RNA Sequencing Workflow



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

SAMPLE PREPARATION

- Understand well the nature of the sample (sampling conditions, preparation, purity)
- Identify the source of technical effects to help resolving them
- 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!
 - Gentle treatment, short sample preparation, low temperature.
Protocols will vary for different cell type
 - Usual buffer: PBS with 0.1-1% BSA (no EDTA, Mg+, Ca+, Tween)
- 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)



TECHNOLOGIES

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 with decided properties
- Control the cellular microenvironment
- Monitor and control cell-cell interactions
- Precise/extensive manipulation of single cells



2) Droplets (Drop-seq, 10x Genomics)

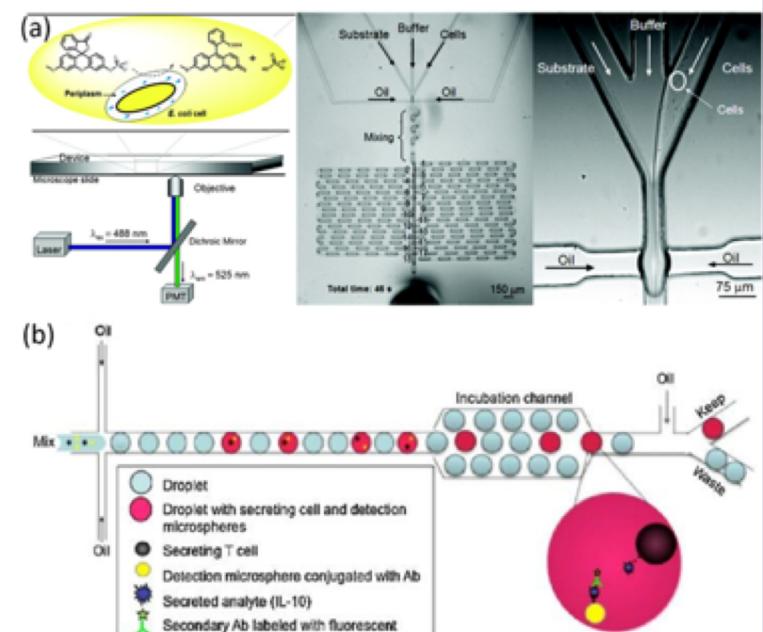
- Introduce distinct 'packets' of reagents to single cell (e.g. barcodes)
- Perform amplification on individual cells
- Sort large population of single cells

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

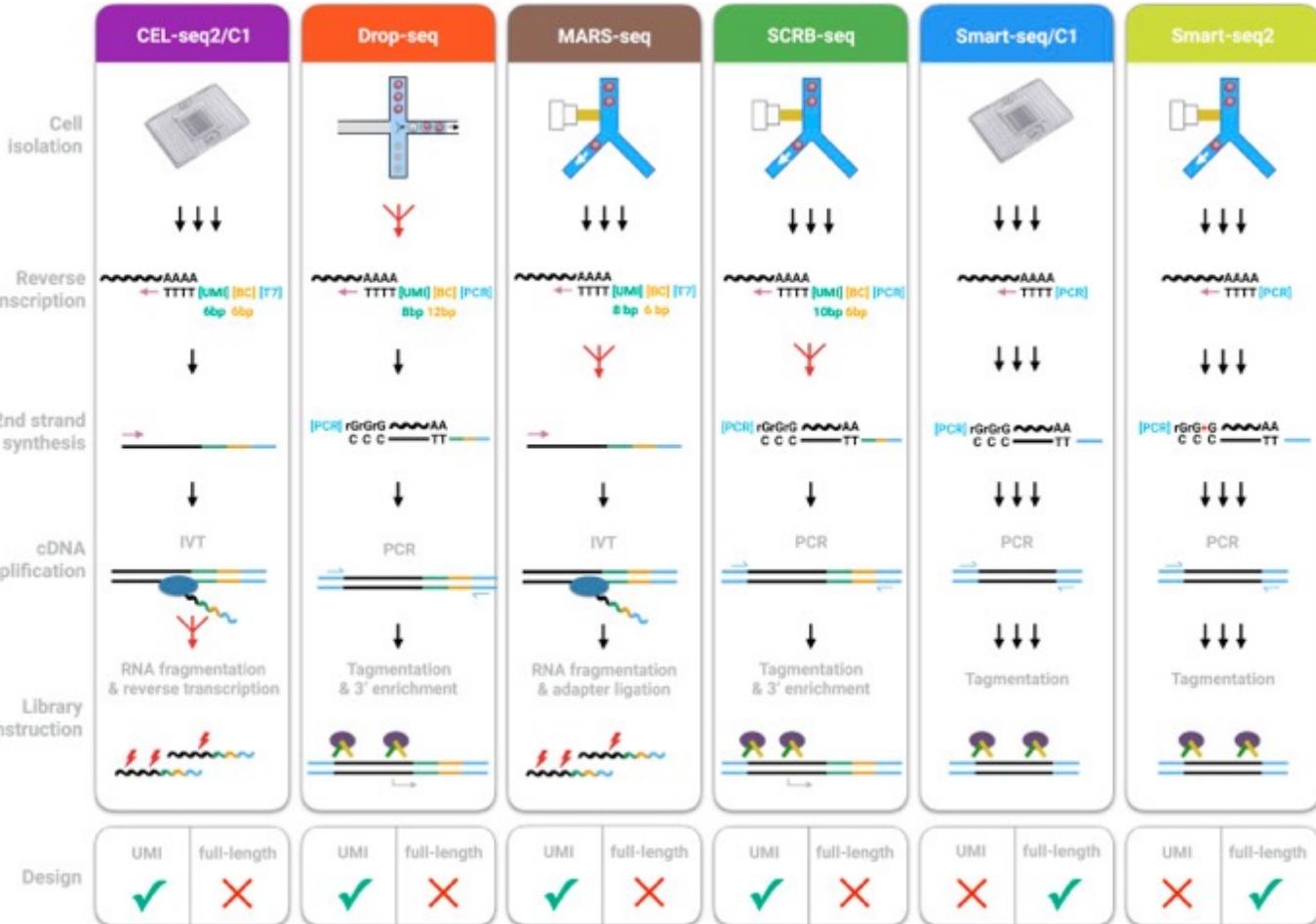


Source: <https://sites.google.com/uw.edu/splitseq>



Source: Wen et al. Molecules (2016)

COMPARISON OF TECHNOLOGIES



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

Table 1 Brief overview of scRNA-seq approaches

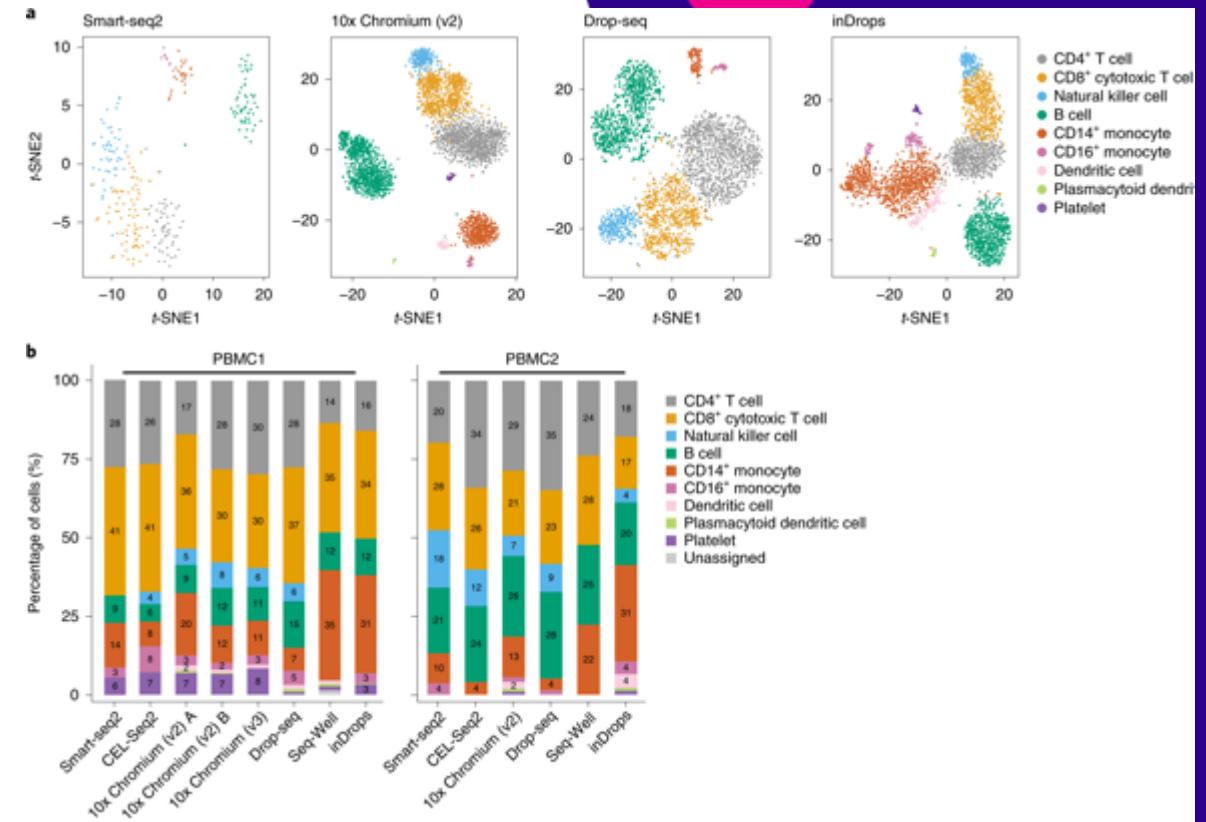
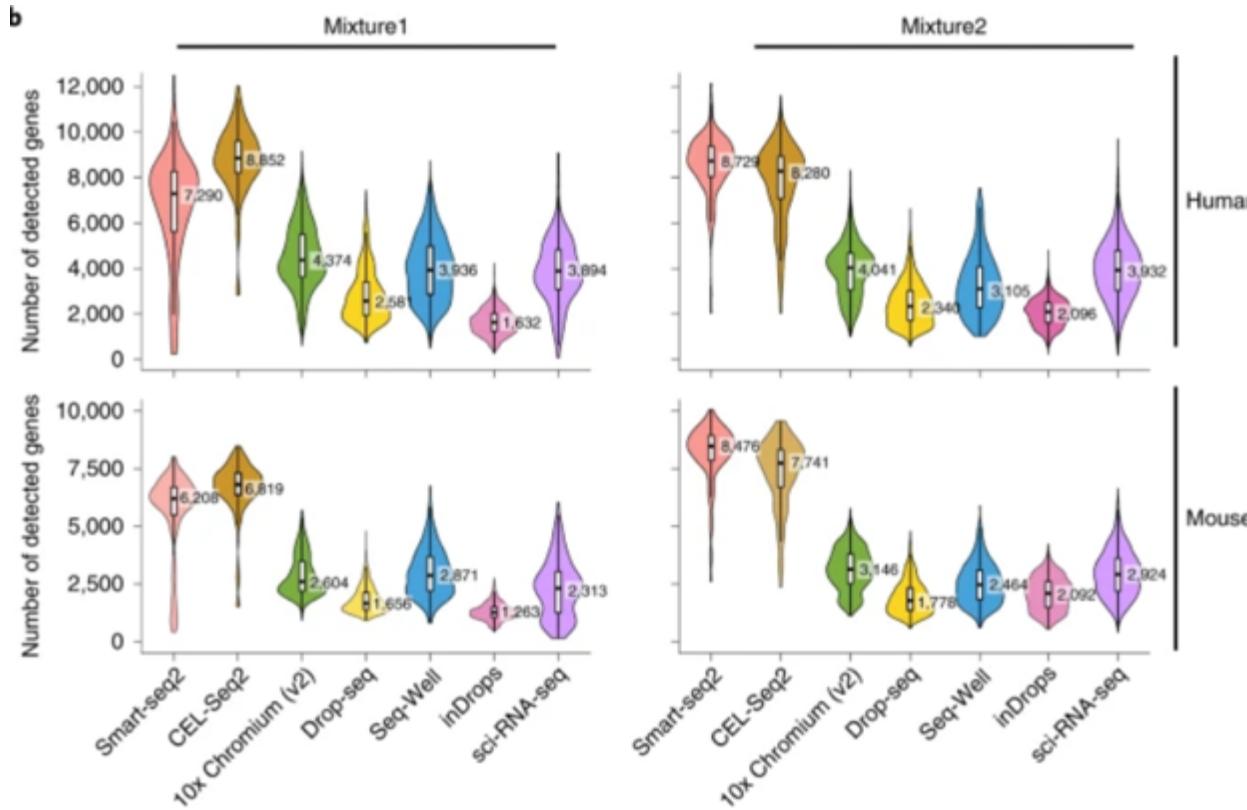
Protocol example	C1 (SMARTer)	Smart-seq2	MATQ-seq	MARS-seq	CEL-seq	Drop-seq	InDrop	Chromium	SEQ-well	SPLIT-seq
Transcript data	Full length	Full length	Full length	3'-end counting						
Platform	Microfluidics	Plate-based	Plate-based	Plate-based	Plate-based	Droplet	Droplet	Droplet	Nanolwell array	Plate-based
Throughput (number of cells)	$10^2\text{--}10^3$	$10^2\text{--}10^3$	$10^2\text{--}10^3$	$10^2\text{--}10^3$	$10^2\text{--}10^3$	$10^3\text{--}10^4$	$10^3\text{--}10^4$	$10^3\text{--}10^4$	$10^3\text{--}10^4$	$10^3\text{--}10^5$
Typical read depth (per cell)	10^6	10^6	10^6	$10^4\text{--}10^5$	$10^4\text{--}10^5$	$10^4\text{--}10^5$	$10^4\text{--}10^5$	$10^4\text{--}10^5$	$10^4\text{--}10^5$	10^4
Reaction volume	Nanoliter	Microliter	Microliter	Microliter	Nanoliter	Nanoliter	Nanoliter	Nanoliter	Nanoliter	Microliter
Reference	[63]	[57]	[39]	[10]	[64]	[45]	[46]	[47]	[101]	[38]

Source: Haque et al. Genome Med 9, 75 (2017)

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	<i>in vitro</i> transcription
Multiplexing of samples	No	No	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	$\geq 10,000$	$\geq 10,000$	No limitation	$\geq 20,000$	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) ~26 weeks	3 rounds of 2 runs (2 C1 machines; concurrent) ~3 weeks	26 rounds of 2 96-well plates	1 run	13 runs of 1 384-well plate
Sample Preparation Scenario 2 (~96 single cell)	Targeted cell No: 96 cells 1 run (1 C1 machine) ~1 week	Targeted cell No: Minimum 800 cell 1 run (1 C1 machine) ~1 week	Targeted cell No: Minimum 500 cells 1 run of 96-well plates ~1 week	~2–3 days	~7 weeks Targeted cell No: 96 cells 1 run of 384-well plate ~2–3 days

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

PERFORMANCE



Distribution of the number of genes in human (top) or mouse (bottom) cells in the two Mixture experiments.

Cell type identification and assignment in PBMCs

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

MORE CELLS OR MORE GENES?

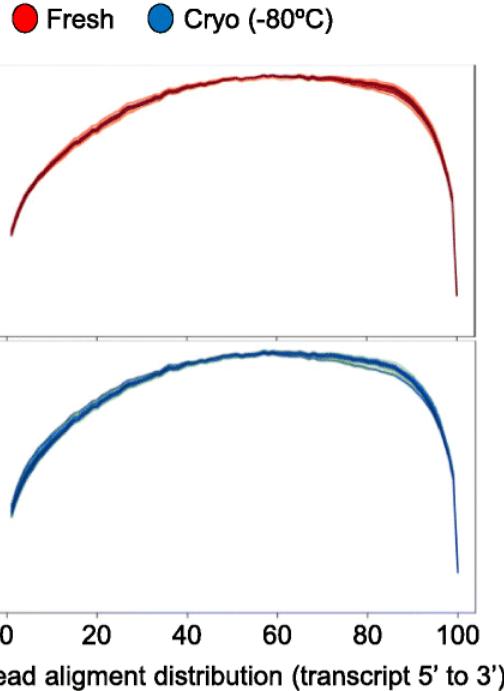
SMART-seq2

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

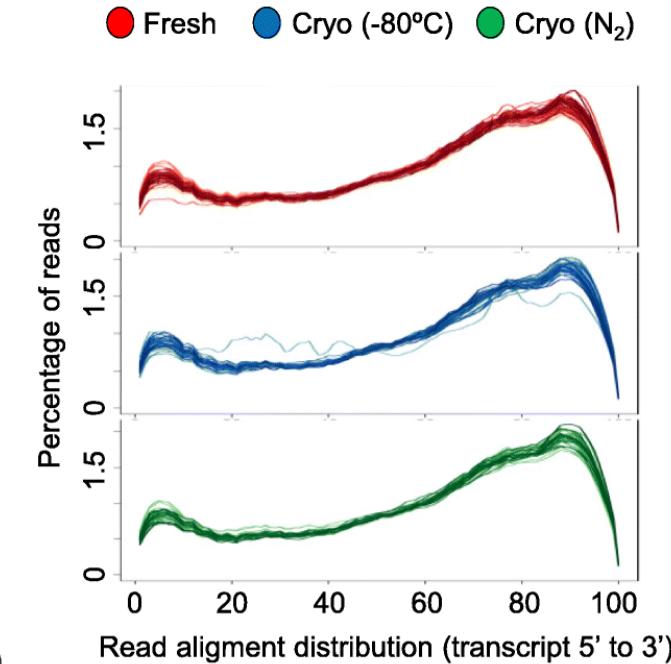
Droplet-RNAseq

- 10000 cells
- 50k reads per cell
- 3'/5' bias

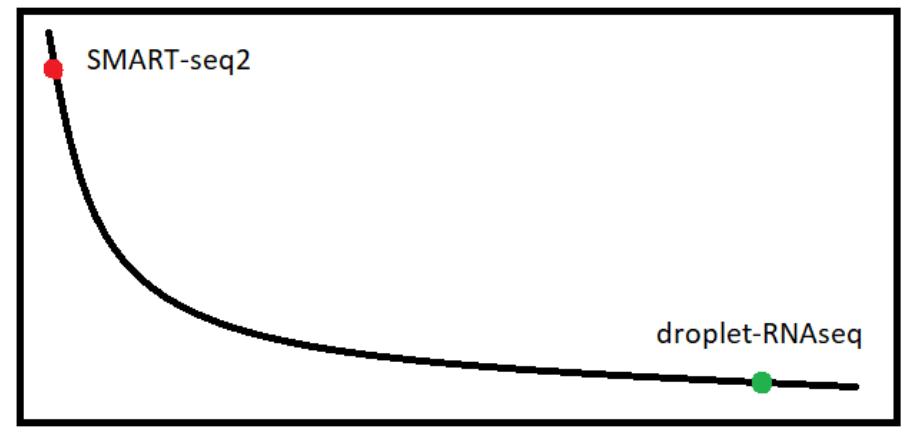
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Source: Guillaumet-Adkins, et al. Genome Biol 18, 45 (2017).



- 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.



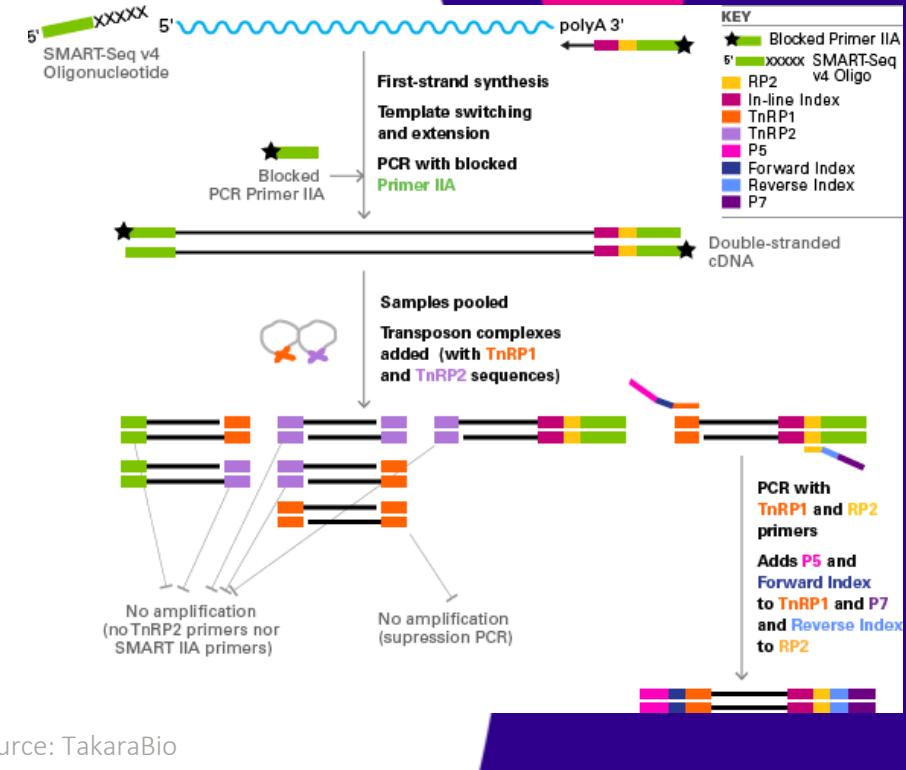
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SMART-SEQ2/3/4 OVERVIEW

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)



a



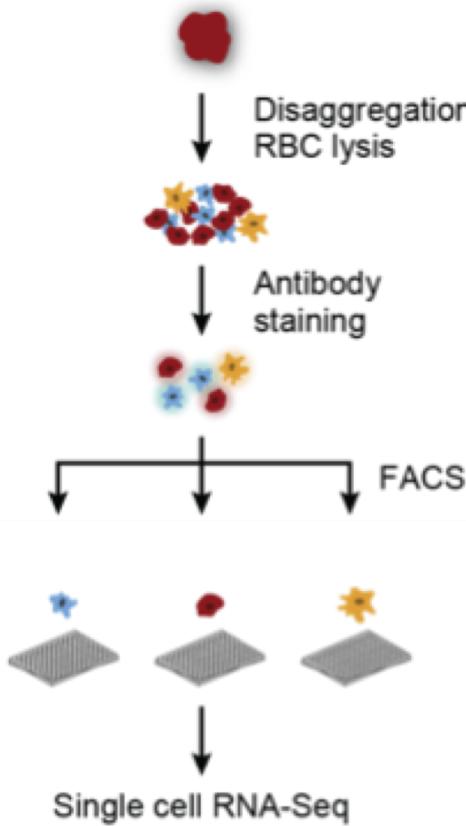
Source: Macosko, Nat Biotechnol 38 (2020).

5' tagged reads

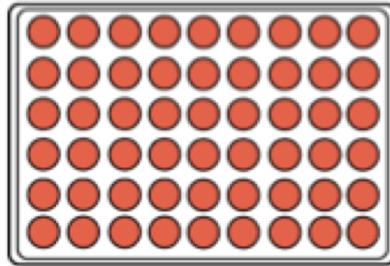


Cell no. 1 2 3 4 ...

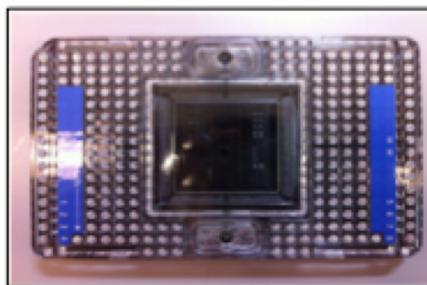
SMART-SEQ2/3/4 + MOSQUITO



FACS sorting on 96/384-well plates



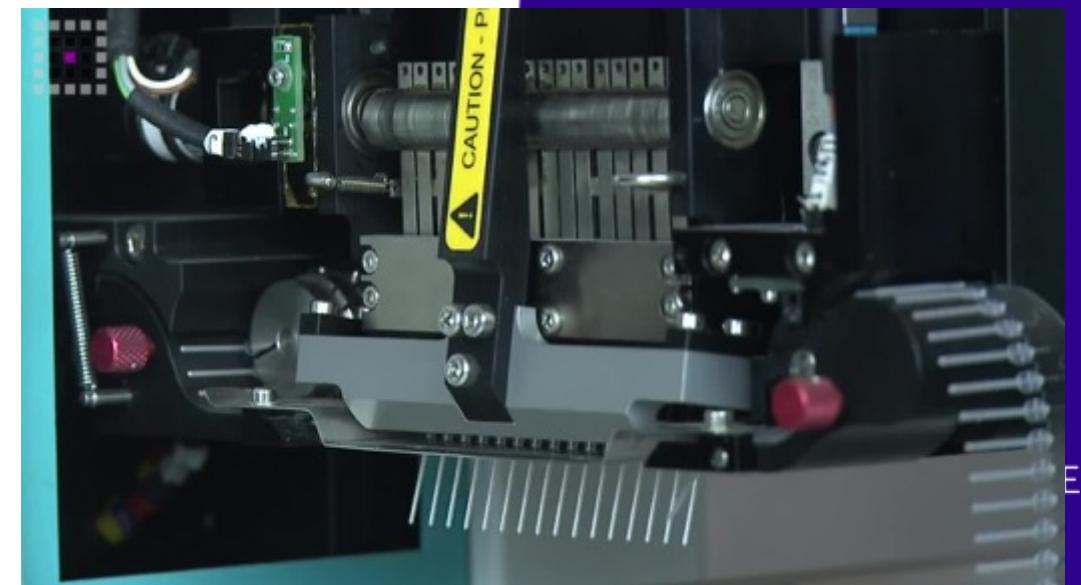
Fluidigm C1-autoprep system



- 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



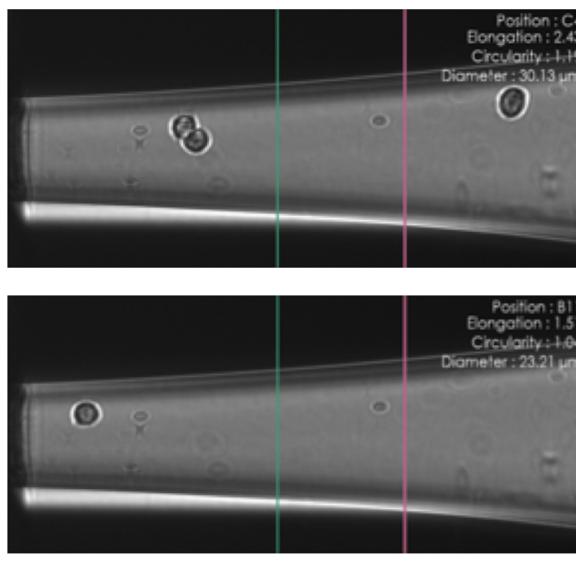
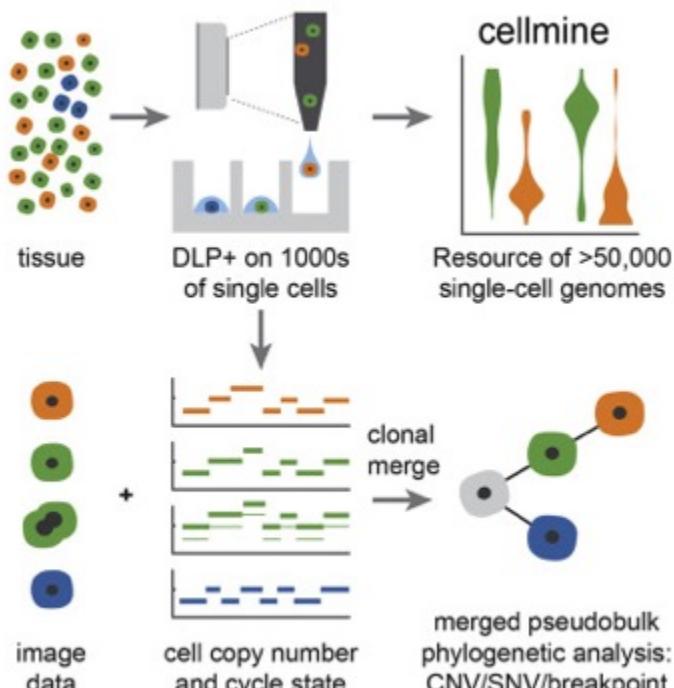
Source: SPT Labtech



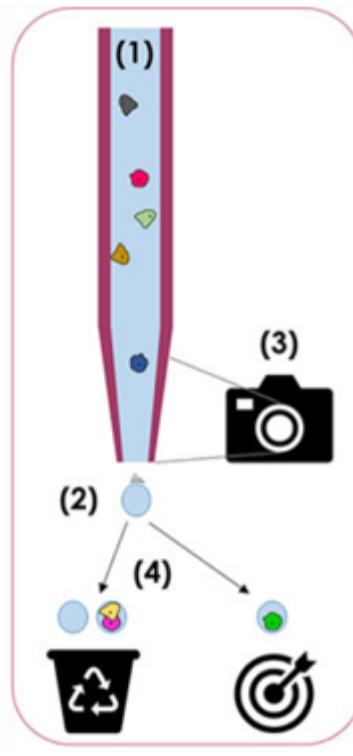
Source: SPT Labtech

CELLENONE

- 1) Cell suspension is aspirated into a glass capillary
- 2) Generation of drops on demand, in air
- 3) Thanks to automated imaging, 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: Cellenion



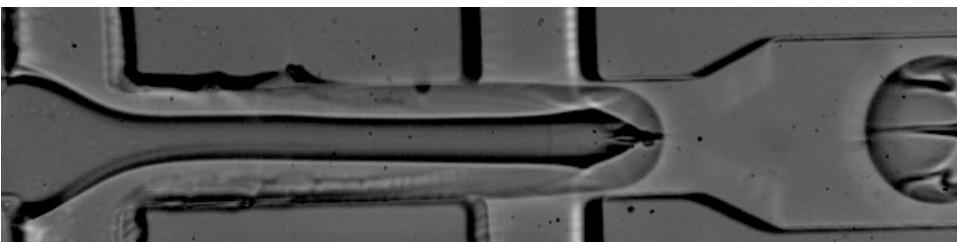
Source: Cellenion



Source: Cellenion

DROP-SEQ OVERVIEW

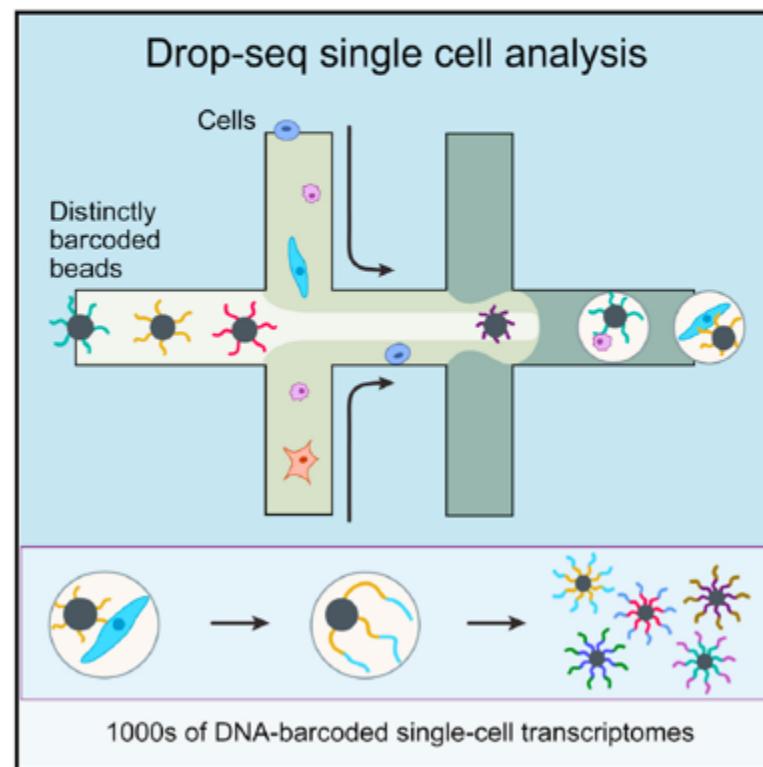
- Moved throughput from hundreds to thousands.
- Droplet-based processing using microfluidics
- Nanoliter scale aqueous drops in oil.
- 3' End
- Bead based (STAMPs).
- Single-cell transcriptomes attached to microparticles.
- Cell barcodes use split-pool synthesis.
- Uses UMI (Unique Molecular Identifier)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



Cell

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

Graphical Abstract



Authors

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(S.A.M.)

In Brief

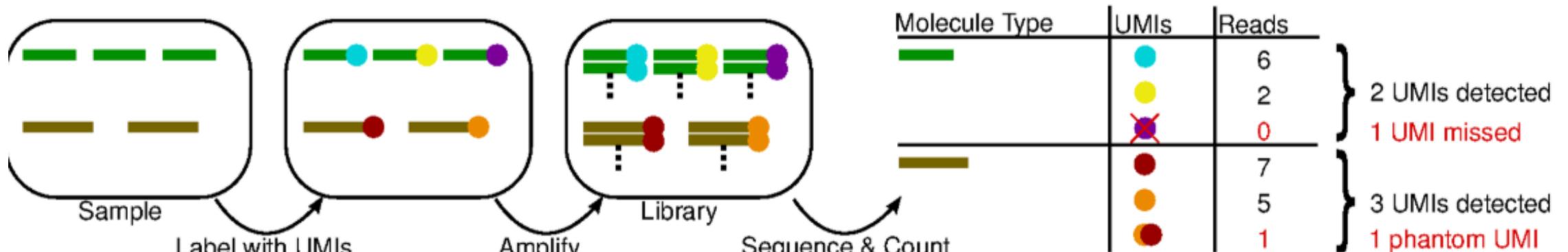
Capturing single cells along with sets of uniquely barcoded primer beads together in tiny droplets enables large-scale, highly parallel single-cell transcriptomics. Applying this analysis to cells in mouse retinal tissue revealed transcriptionally distinct cell populations along with molecular markers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

Resource

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 duplicates. 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)

The relevant steps of library preparation when the UMI method is used. The sample initially contains three copies of molecule graphic and two copies of graphic, which are made unique by labelling with UMIs (graphic). Each of those molecules is expanded into a molecular family during amplification, and a random selection of molecules from these families is sequenced. Counting unique UMIs then counts unique molecules, unless UMIs have read-count zero (graphic) or phantom UMIs are produced (graphic).

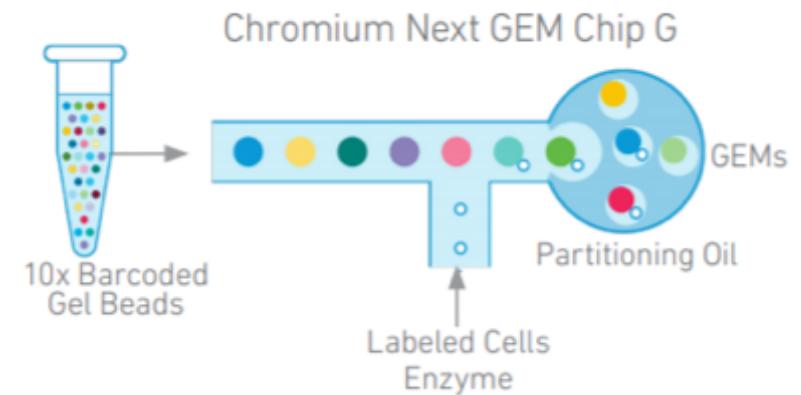
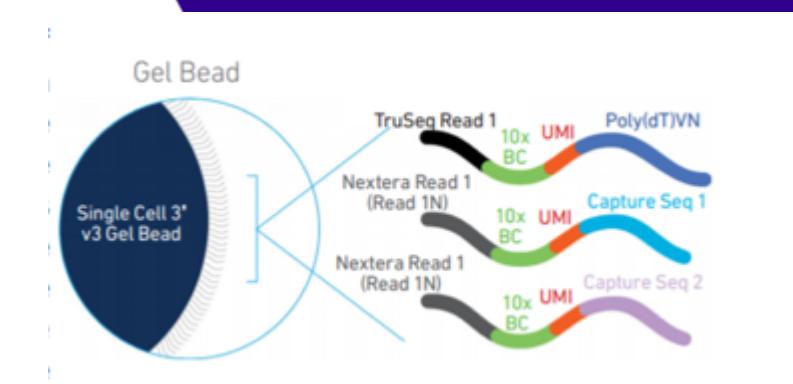


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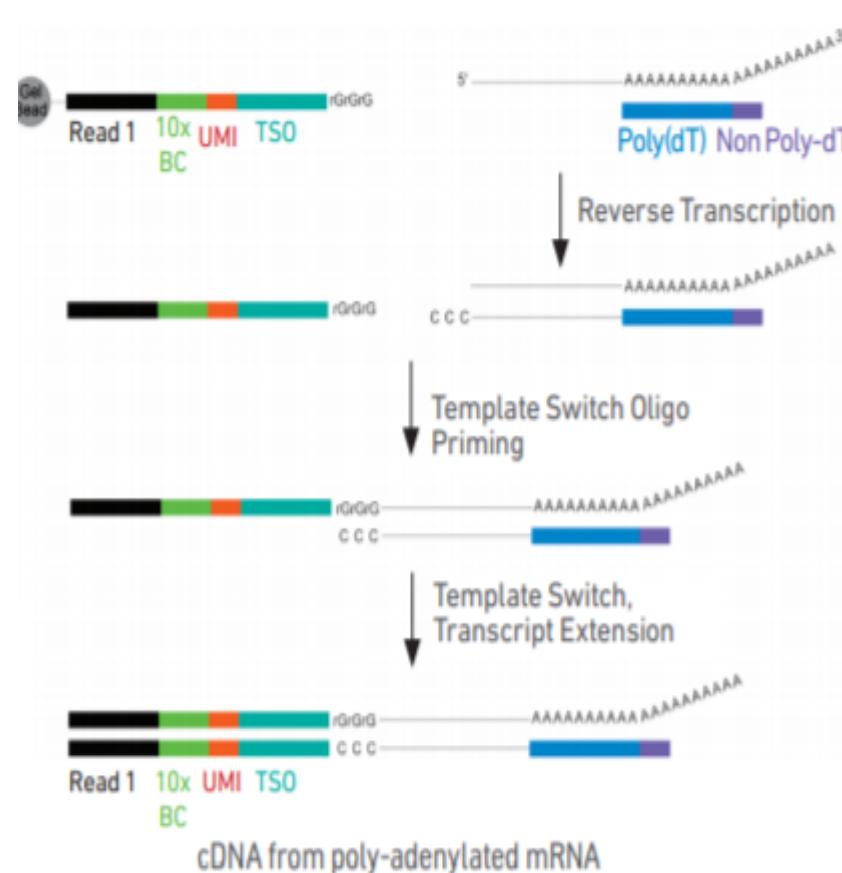
10X GENOMICS OVERVIEW

- 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 is available and user friendly



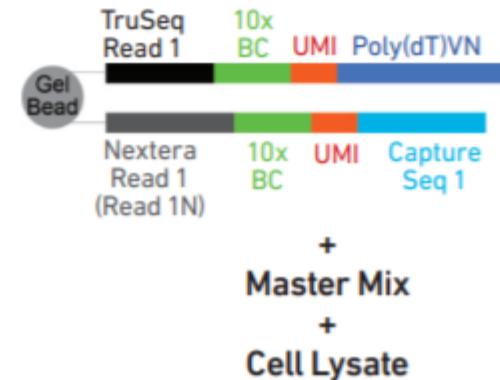
Source: 10x Genomics

10X GENOMICS OVERVIEW

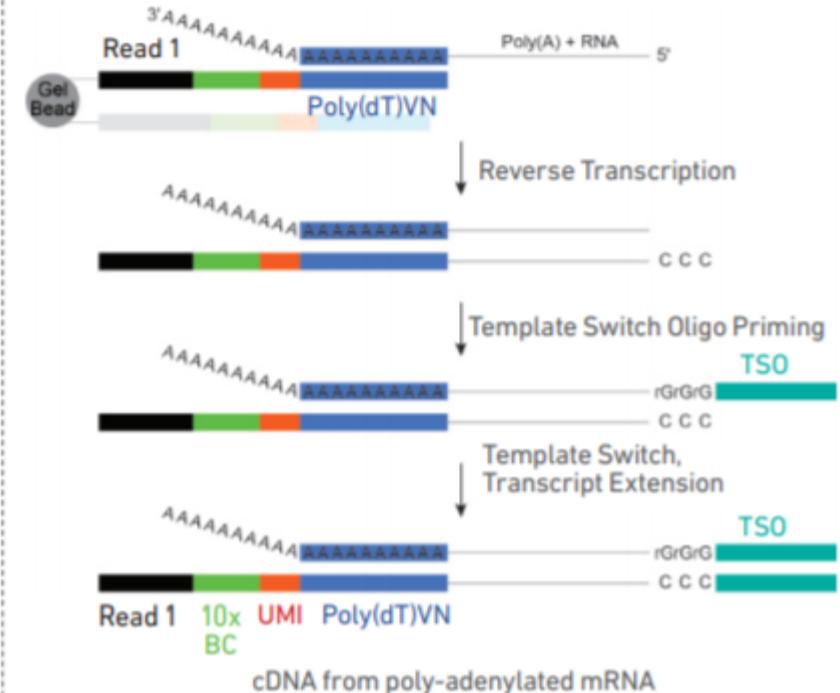


Source: 10x Genomics

Inside individual GEMs

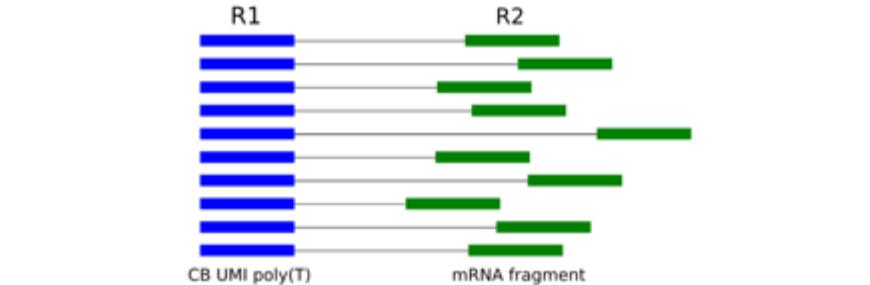
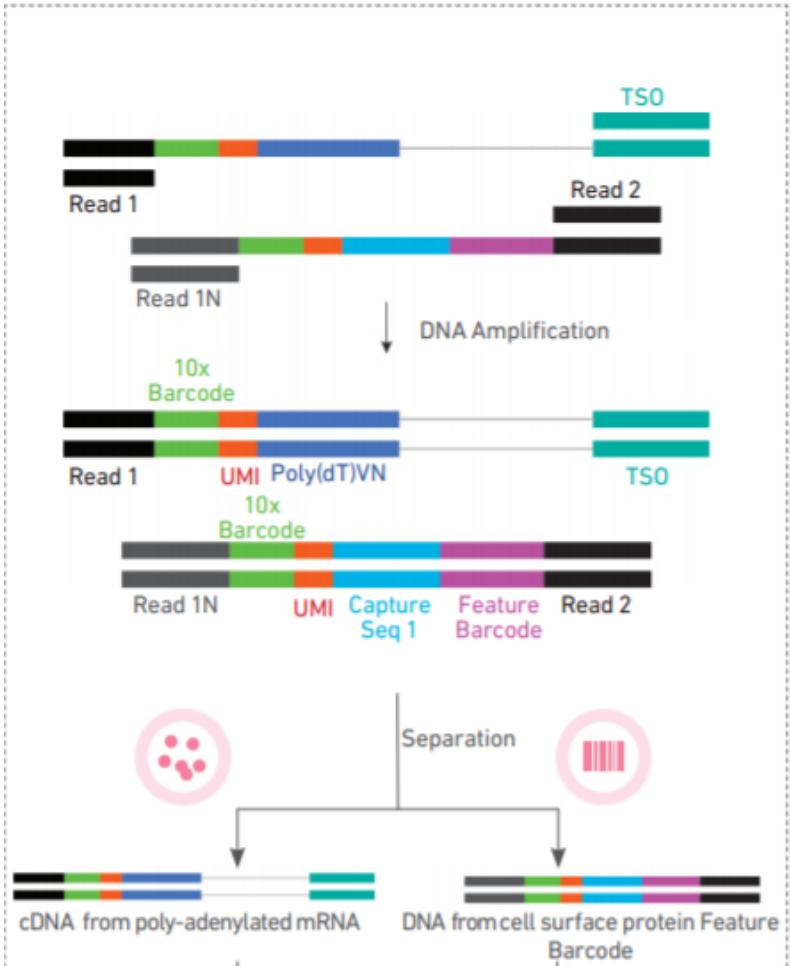


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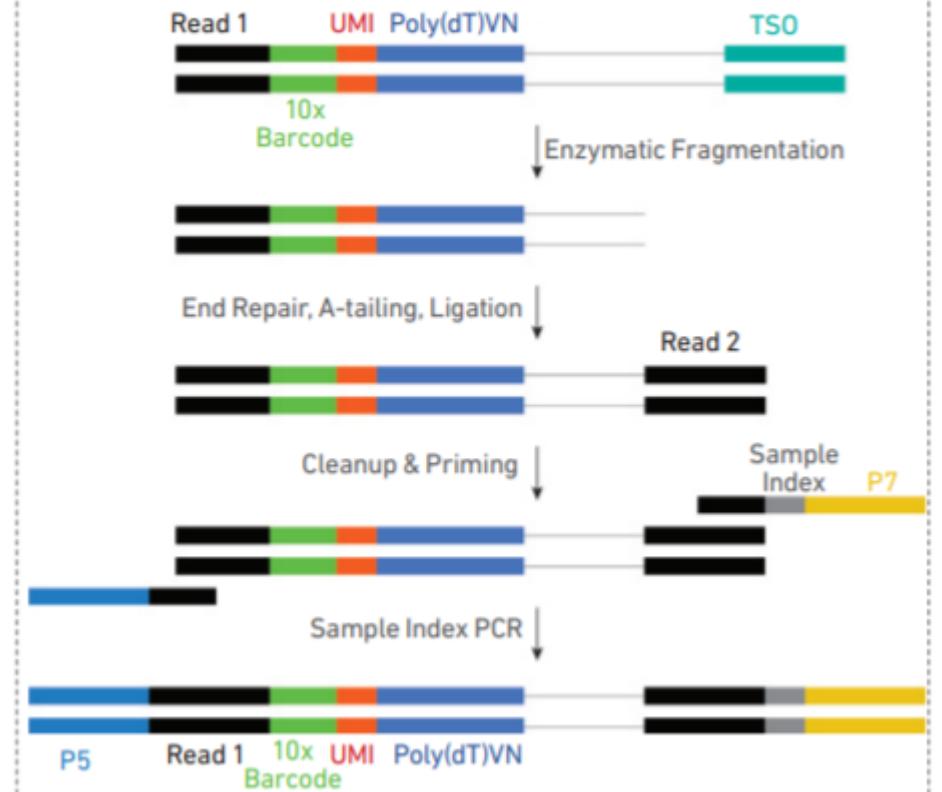
10X GENOMICS OVERVIEW

Pooled cDNA amplification



In 3' libraries, actual coverage vary according to the level of duplication of a given cDNA.

Pooled amplified cDNA processed in bulk



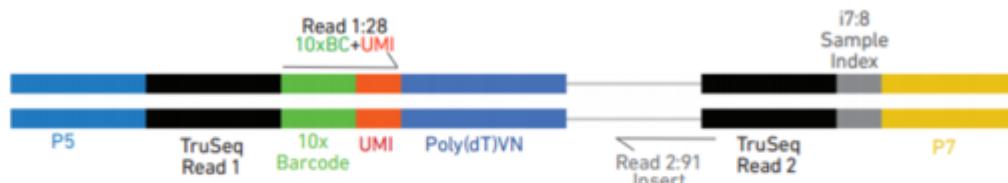
Source: 10x Genomics



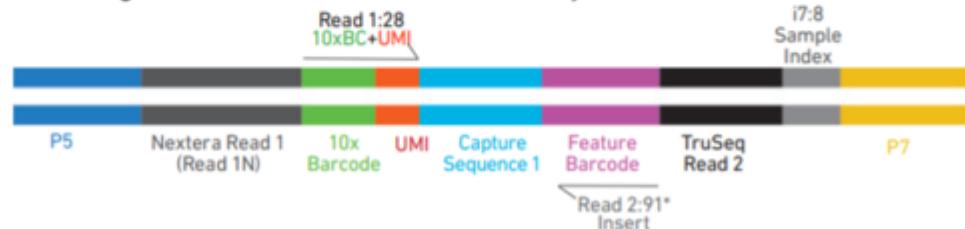
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10X GENOMICS LIBRARIES

Chromium Single Cell 3' Gene Expression Library



Chromium Single Cell 3' Cell Surface Protein Library



*Minimum required Read 2 length for Cell Surface Protein libraries is 25 bp

Source: 10x Genomics

Chromium Single Cell V(D)J Enriched Library



Chromium Single Cell 5' Gene Expression Library



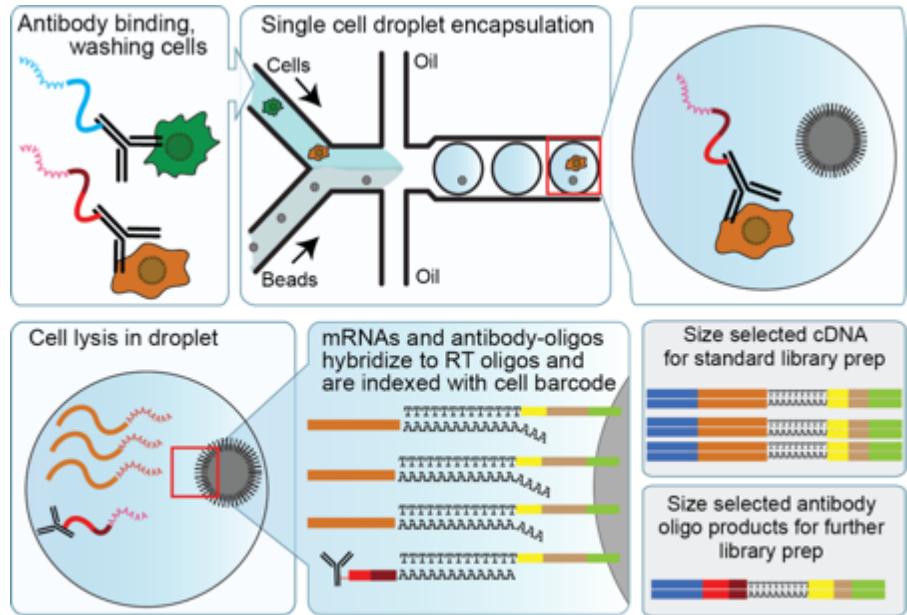
Sequencing Read	Description	Number of cycles
Read1	10x Barcode Read (Cell) + Randomer Read (UMI)	28bp
i7 index	Sample index read	8bp (soon 10bp)
i5 index	Sample index read	0 (soon 10bp)
Read2	Insert Read (Transcript)	91bp (soon 90bp)



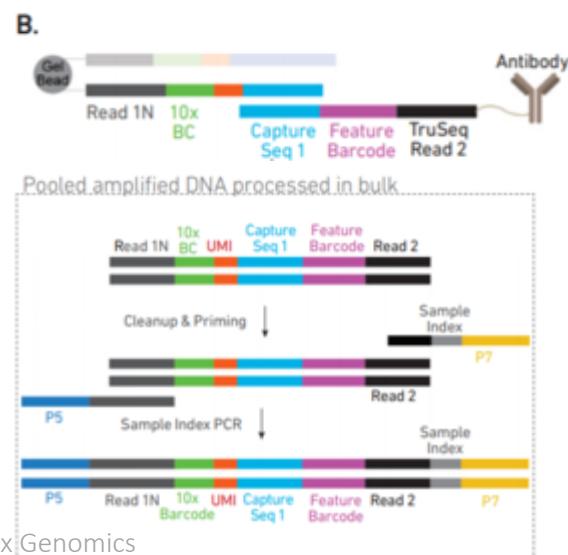
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CITE-SEQ: CELLULAR INDEXING OF TRANSCRIPTOMES AND EPITOPE BY SEQUENCING

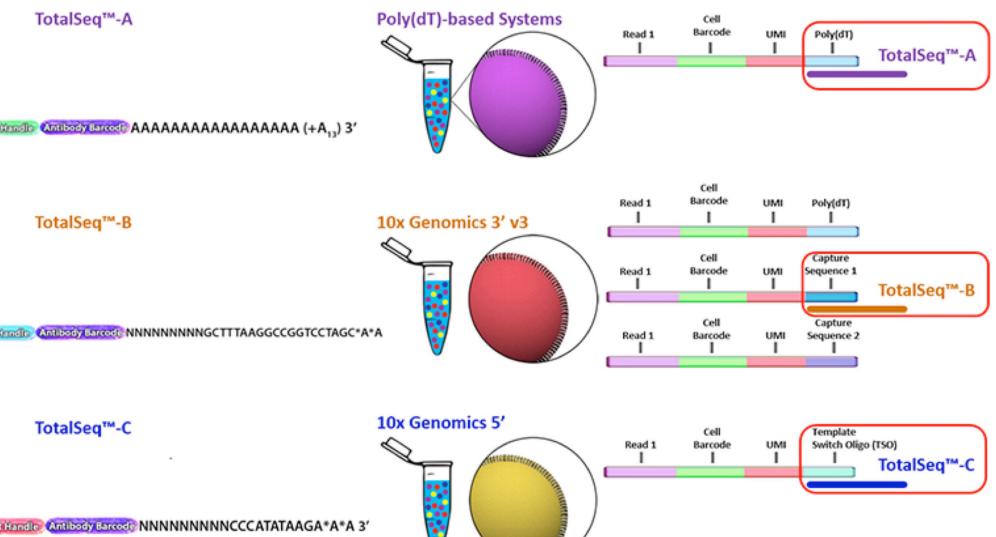


Source: cite-seq.com

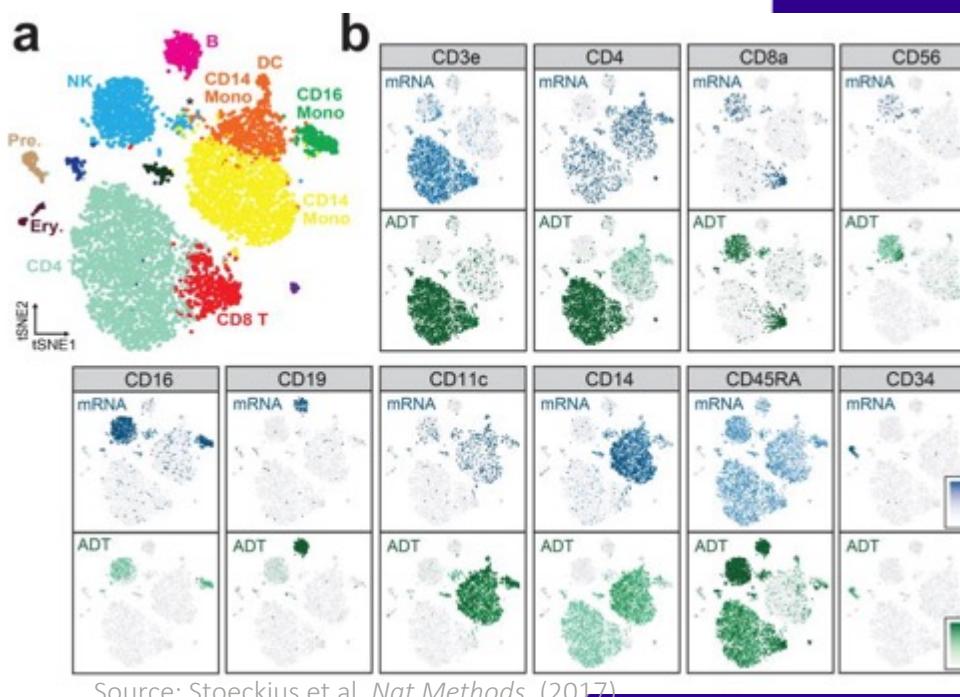


CITE-seq uses DNA-barcoded antibodies to convert detection of proteins into a quantitative, sequenceable readout.

Source: 10x Genomics



Source: BioLegend



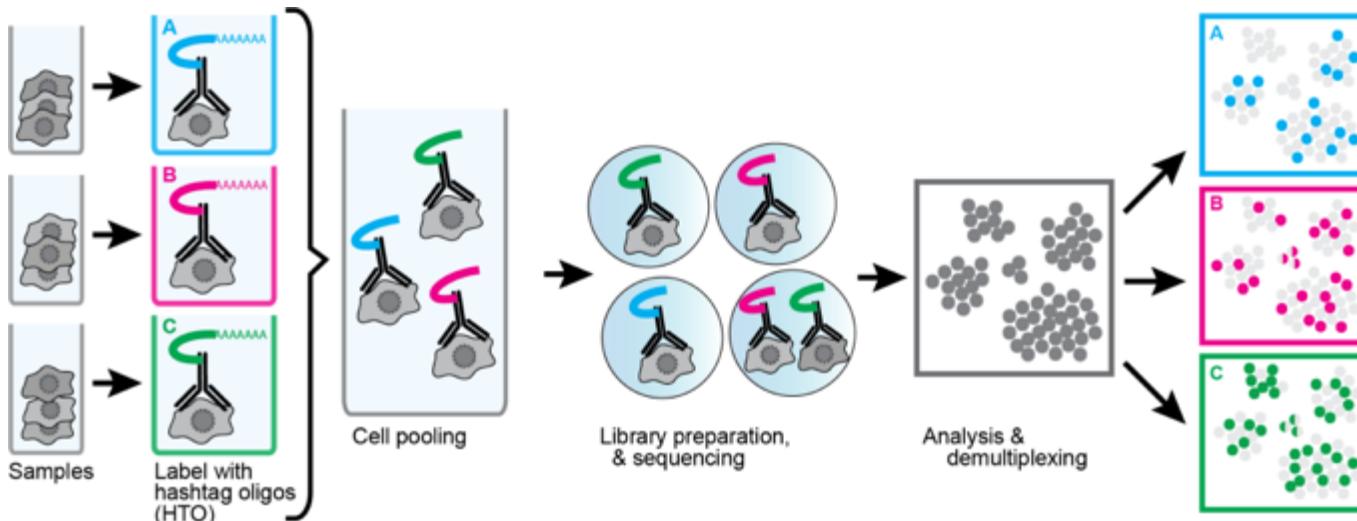
Source: Stoeckius et al. *Nat Methods.* (2017)

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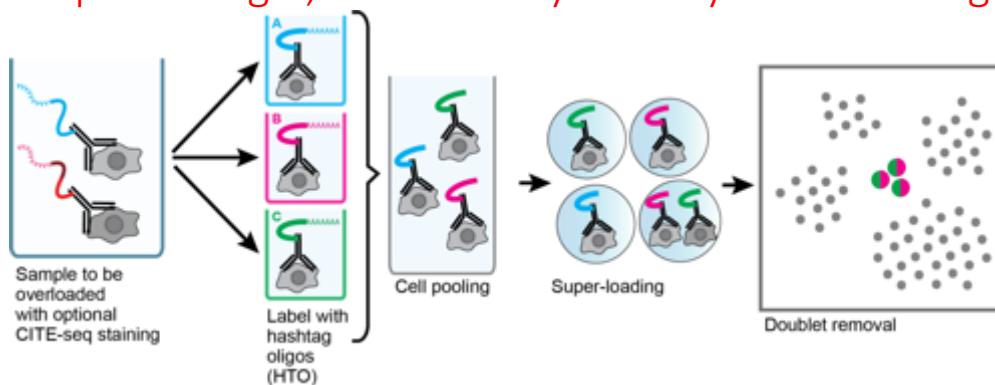
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CELL HASHING

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

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

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

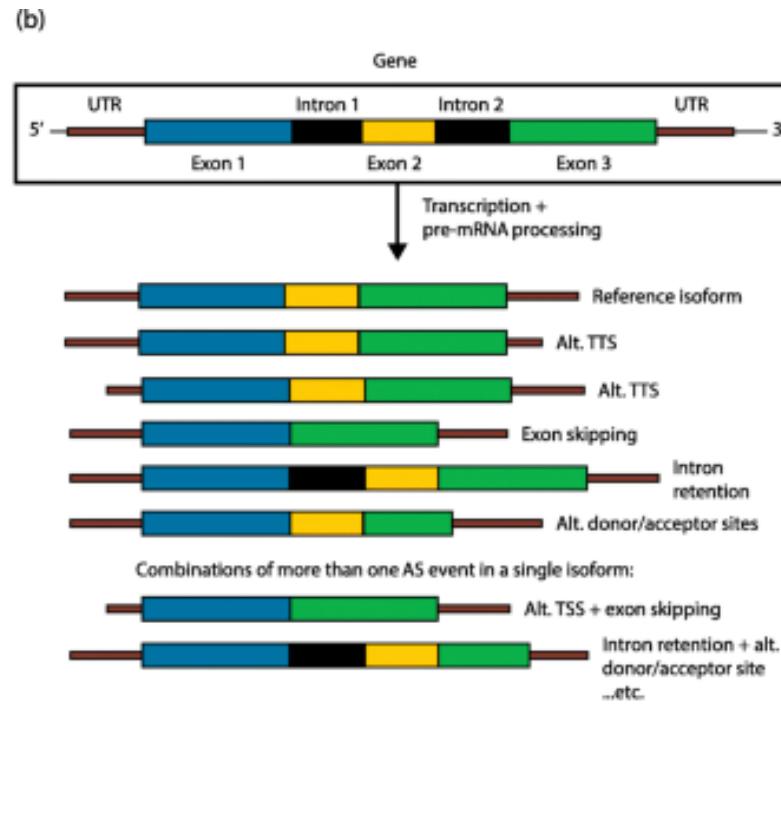
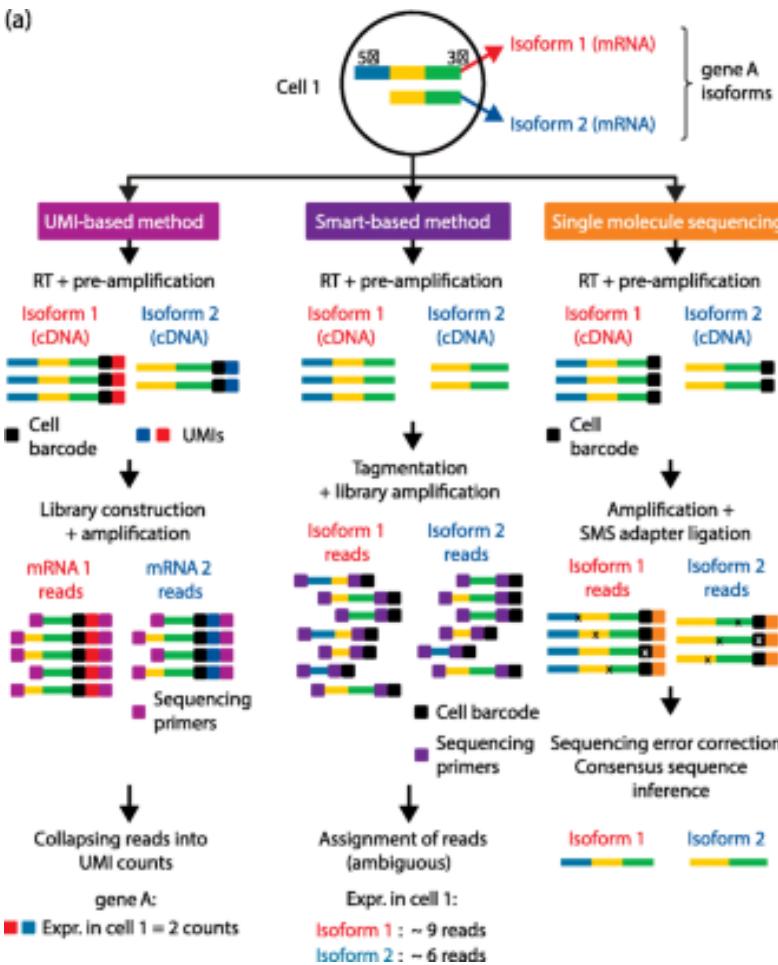
Jun Xu^a, Caitlin Falconer^b, Quan Nguyen^b, Joanna Crawford^b, Brett D. McKinon^{b,e}, Sally Mortlock^b, Alice Pébay^{f,g,h,i}, Alex W. Hewitt^{f,g,h,i}, Anne Senabouth^d, Nathan Palpant^{a,b}, Han Chiu^b, Stacey Andersen^{a,b}, Grant W. Montgomery^{a,b}, Joseph Powell^{c,d}, Lachlan Coin^{a,b,*}



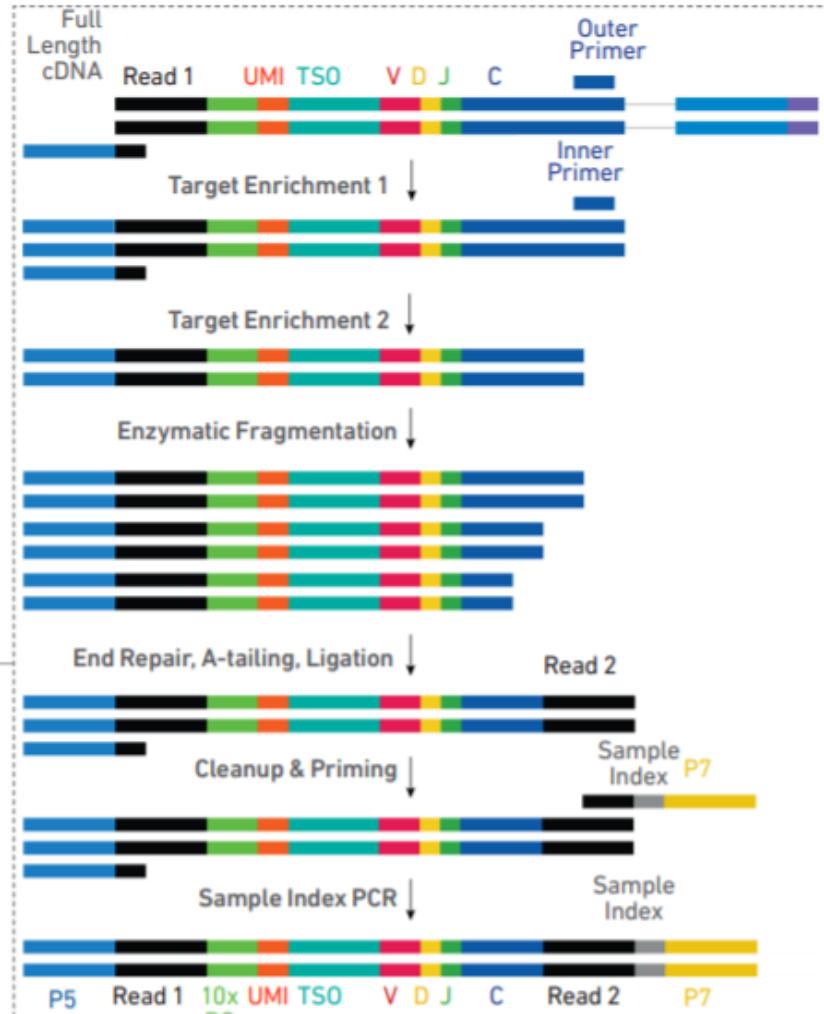
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ISOFORMS



Pooled amplified cDNA processed in bulk



Source: 10x Genomics

Source: Arzalluz-Luque, Á., Conesa, A. *Genome Biol* 19, 110 (2018).

10x 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.



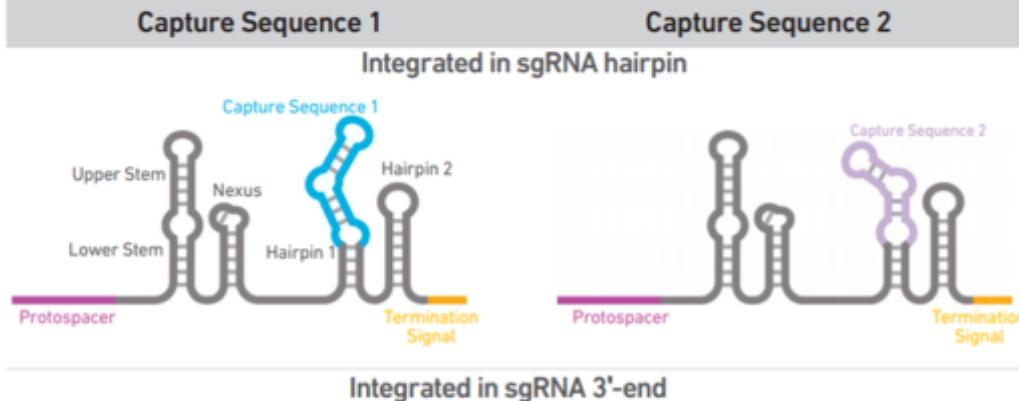
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Source: 10x Genomics

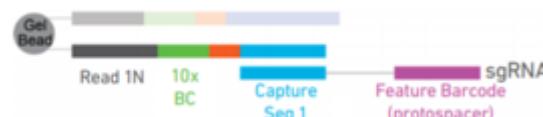
CRISPR SCREENING

Capture Sequence Integration in sgRNA



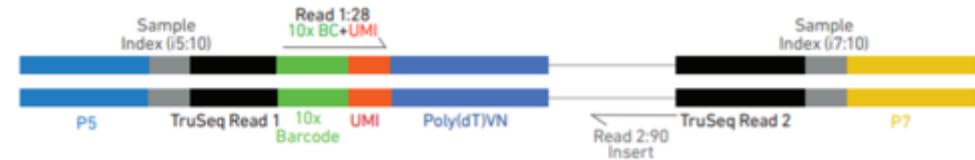
Direct Capture of sgRNA

Only Capture Sequence 1 is illustrated in the example

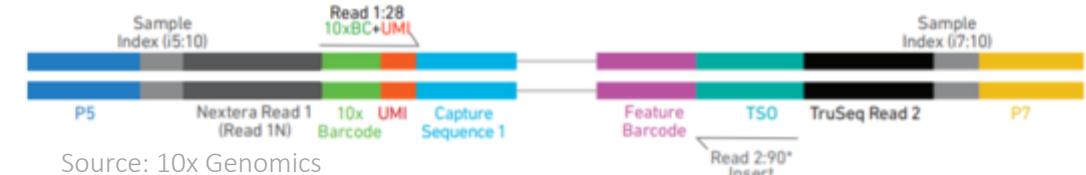


Source: 10x Genomics

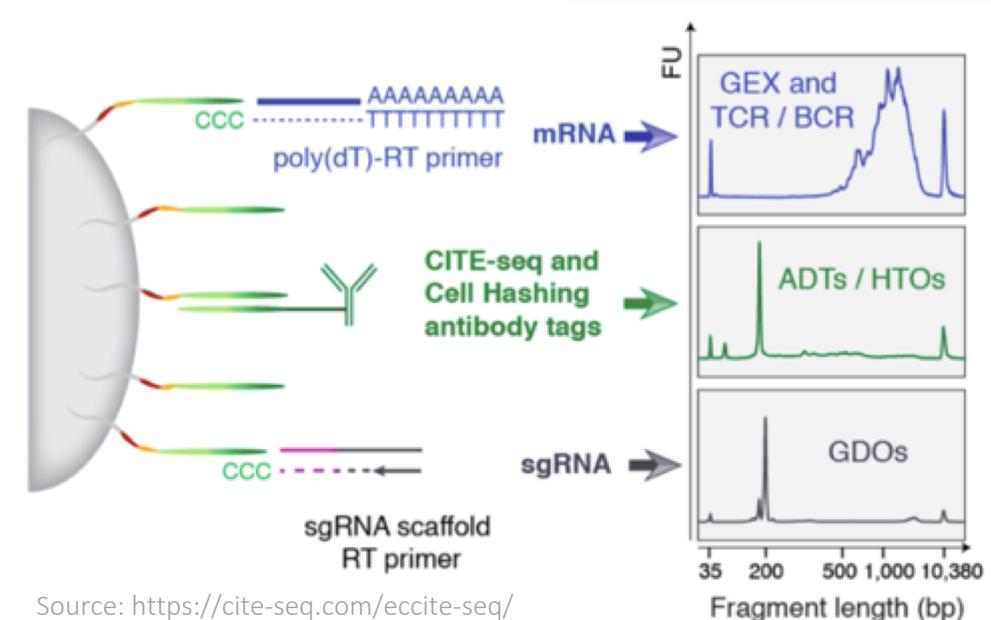
Chromium Single Cell 3' Gene Expression Dual Index Library



Chromium Single Cell 3' CRISPR Screening Dual Index Library



Source: 10x Genomics

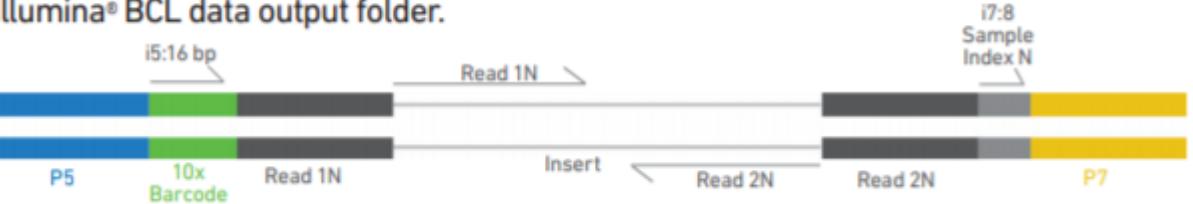


Source: <https://cite-seq.com/eccite-seq/>

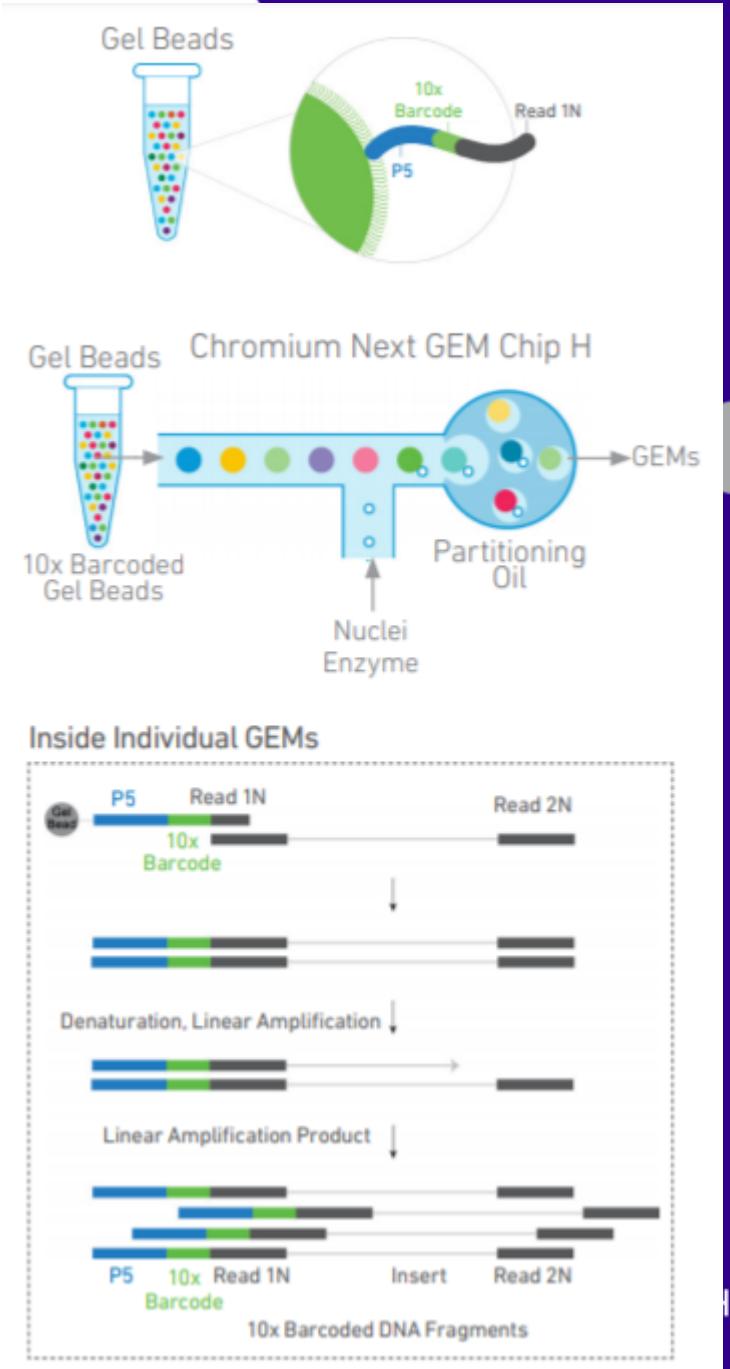
Source: 10x Genomics

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

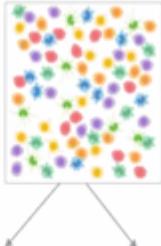


Source: 10x Genomics

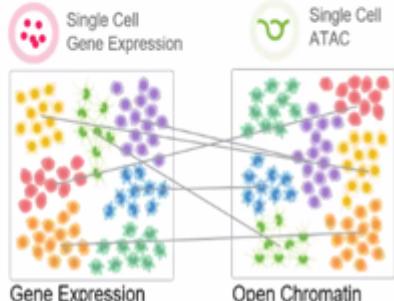
ATAC-SEQ + RNA-SEQ

Profiling Different Modalities To Gain Deeper Insights

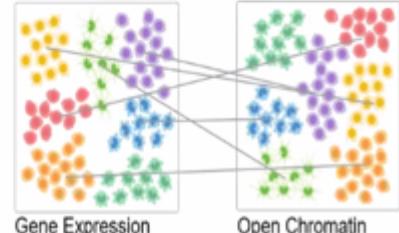
Cells from
the same
population



Individual
modalities



Inferred
Linkage



Source: 10x Genomics

Dive Deep Where It Matters

One cell

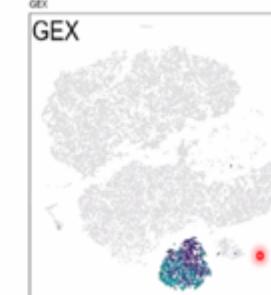
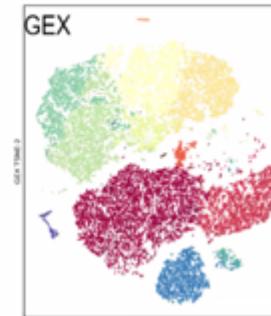
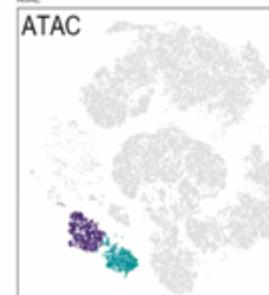
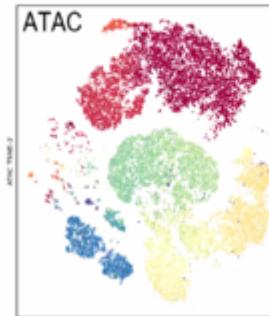
Dual
modalities

True
Linkage



Gene
Expression

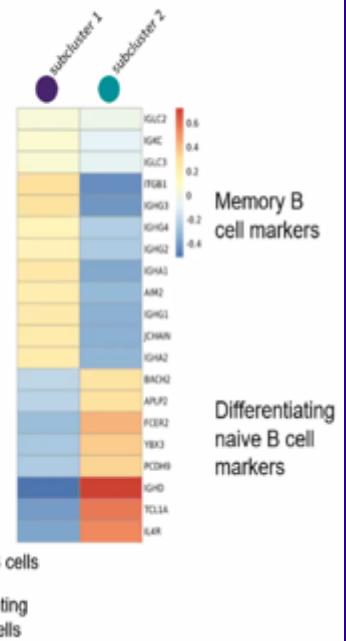
Open
Chromatin



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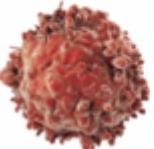
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Top differentially
expressed markers



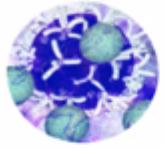
TARGETED PANELS

Accelerate research in 4 major areas



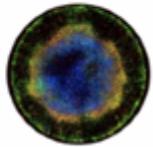
Human Pan-Cancer
1,253 genes

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



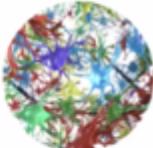
Human Immunology
1,056 genes

- 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

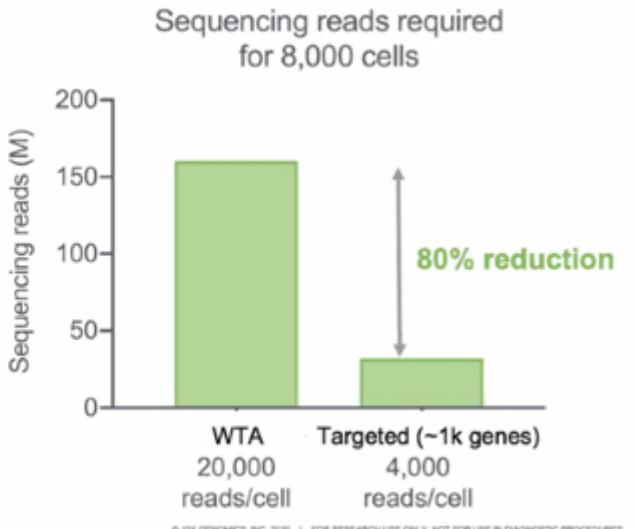
Increased experimental efficiency

Reduced sequencing cost

WTA and targeted gene expression from the same cells

Core assay compatibility

Content and customization



SPATIAL TRANSCRIPTOMICS

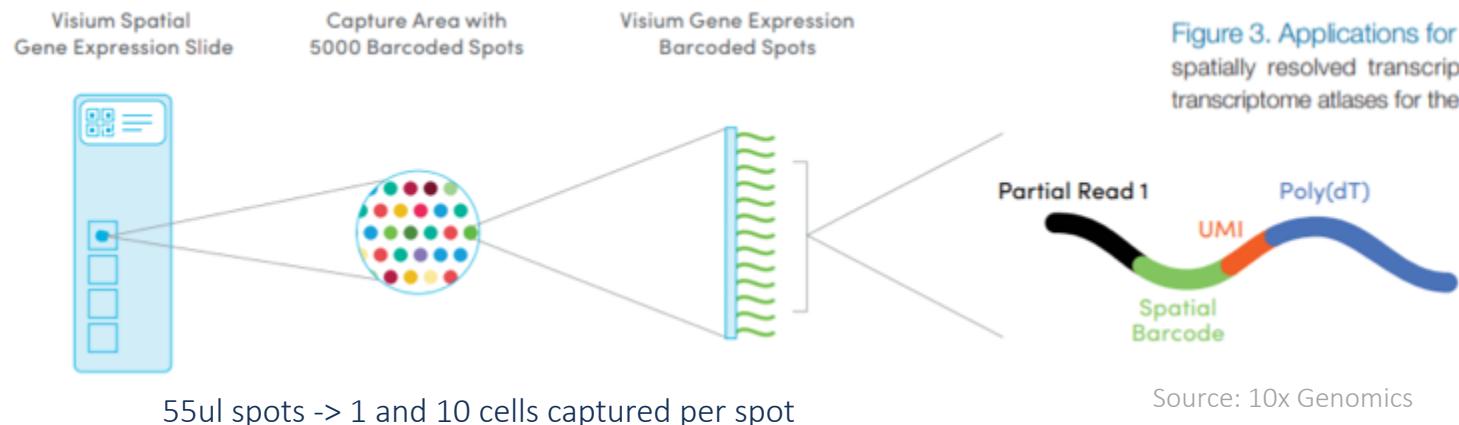
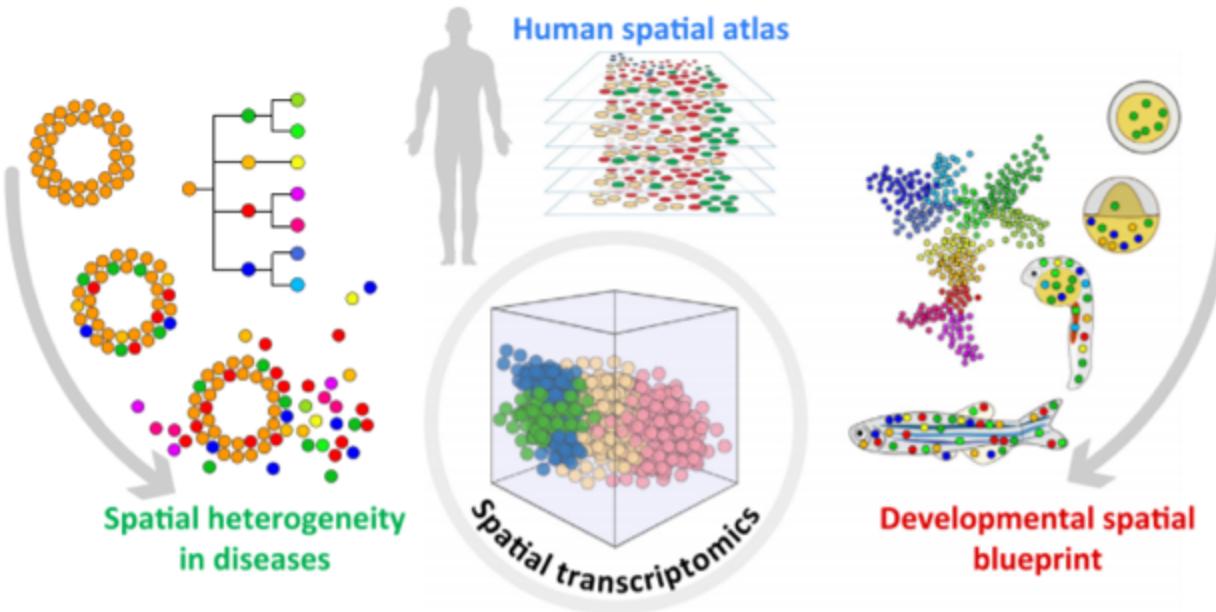
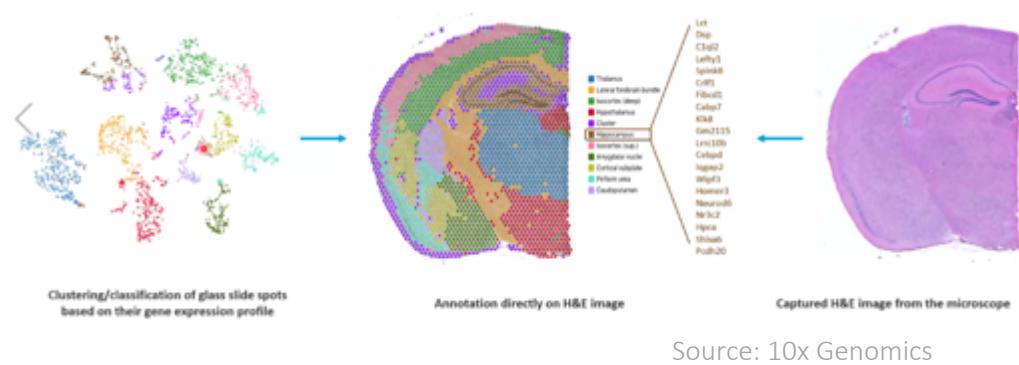


Figure 3. Applications for Spatially Resolved Transcriptomics. Three primary kinds of hot issues can be resolved by spatially resolved transcriptomics: left, discovering spatial heterogeneity of diseases; middle, establishing spatial transcriptome atlases for the human body; and right, delineating an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

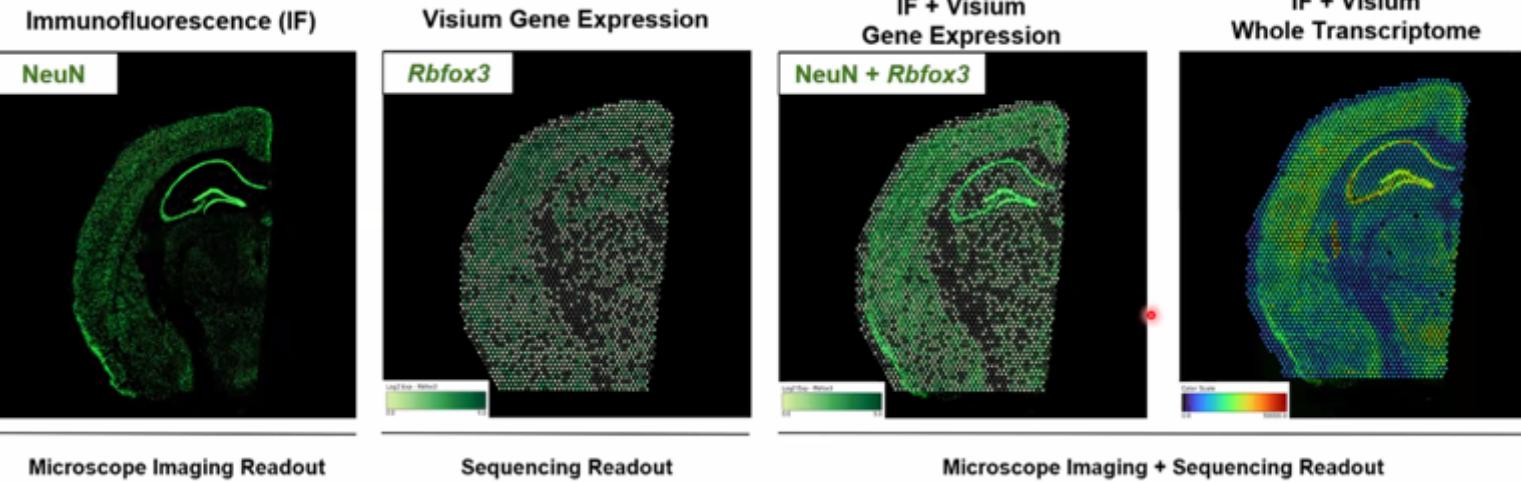
Source: Liao et al. Trends in Biotechnology. (2020)



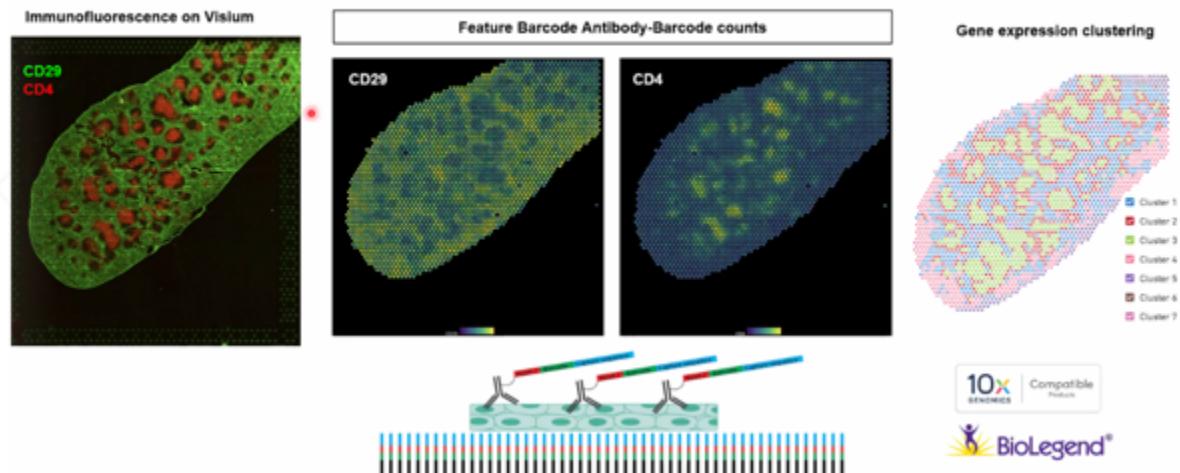
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SPATIAL TRANSCRIPTOMICS



Feature Barcode Correlates with Immunofluorescence

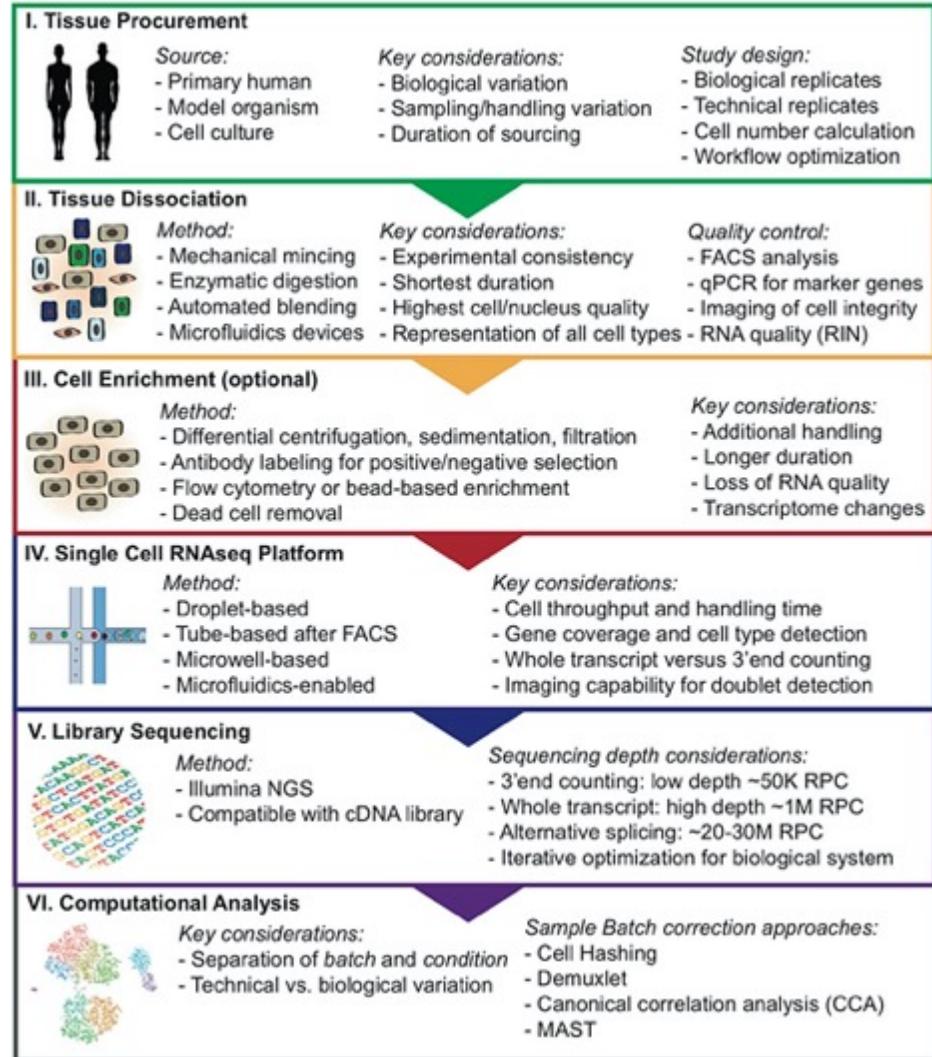


Source: 10x Genomics

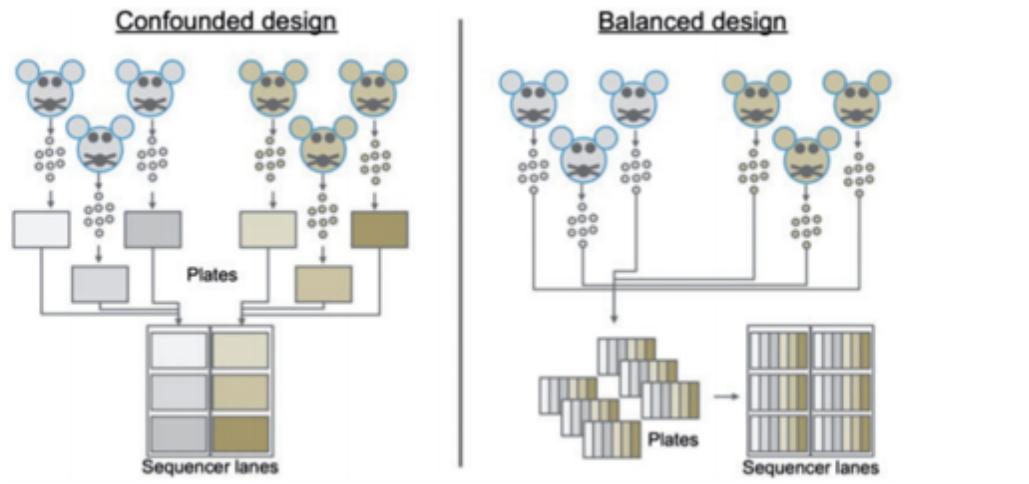


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EXPERIMENTAL DESIGN



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



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

WHAT PLATFORM SHOULD I USE?

Choose protocol based on:

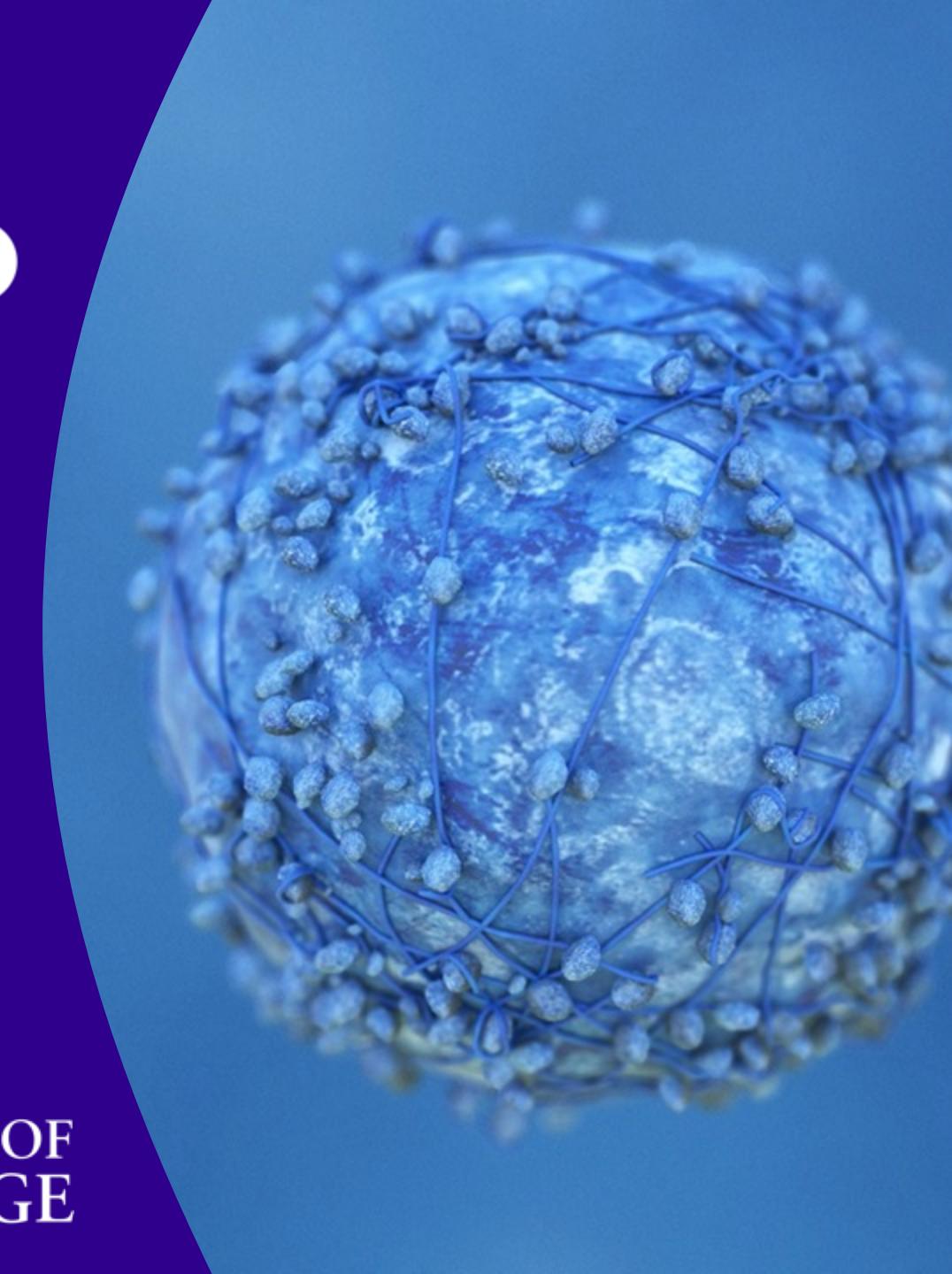
- Throughput (number of cells per reaction)
- Sample of origin
- Cost / Labour / Time limitations
- Gene body coverage: 5' / 3' biased or full-length?
- UMI vs no-UMI
- Sequencing depth per cell

Examples:

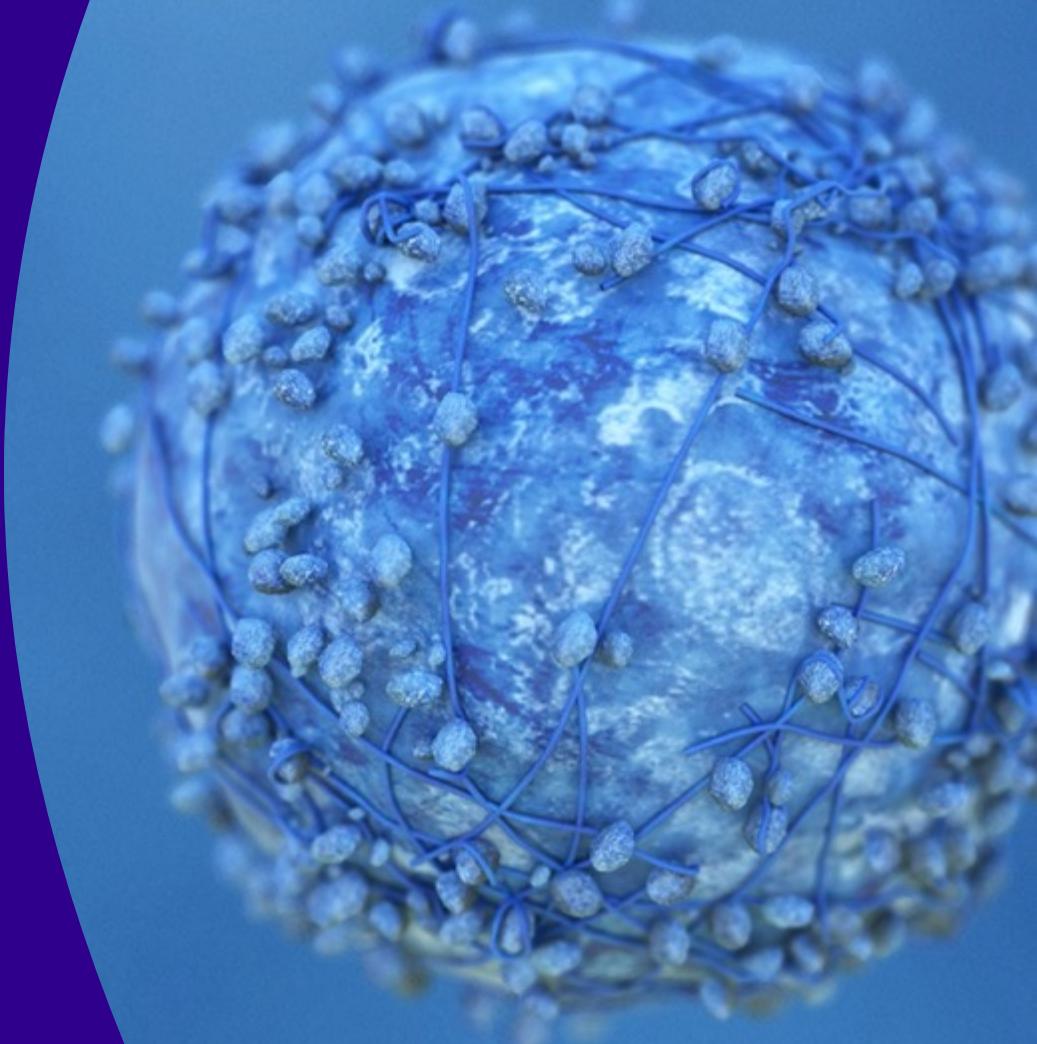
- If your sample is fairly homogeneous – bulk RNAseq
- If your sample is limited in cell number – plate-based method
- If you want re-annotate the transcriptome and discover new isoforms – full-length coverage (SMART-seq2)
- If you are looking to classify all cell types in a diverse tissue - high throughput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

LITERATURE:

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- Stoeckius M, Hafemeister C, Stephenson W, et al. Simultaneous epitope and transcriptome measurement in single cells. *Nat Methods*. (2017)
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THANK YOU FOR YOUR ATTENTION!



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