



INTRODUCTION TO SINGLE CELL RNA-SEQ

Analysis of single cell RNA-seq data - online course

Katarzyna Kania (CRUK CI Genomics Core Facility)

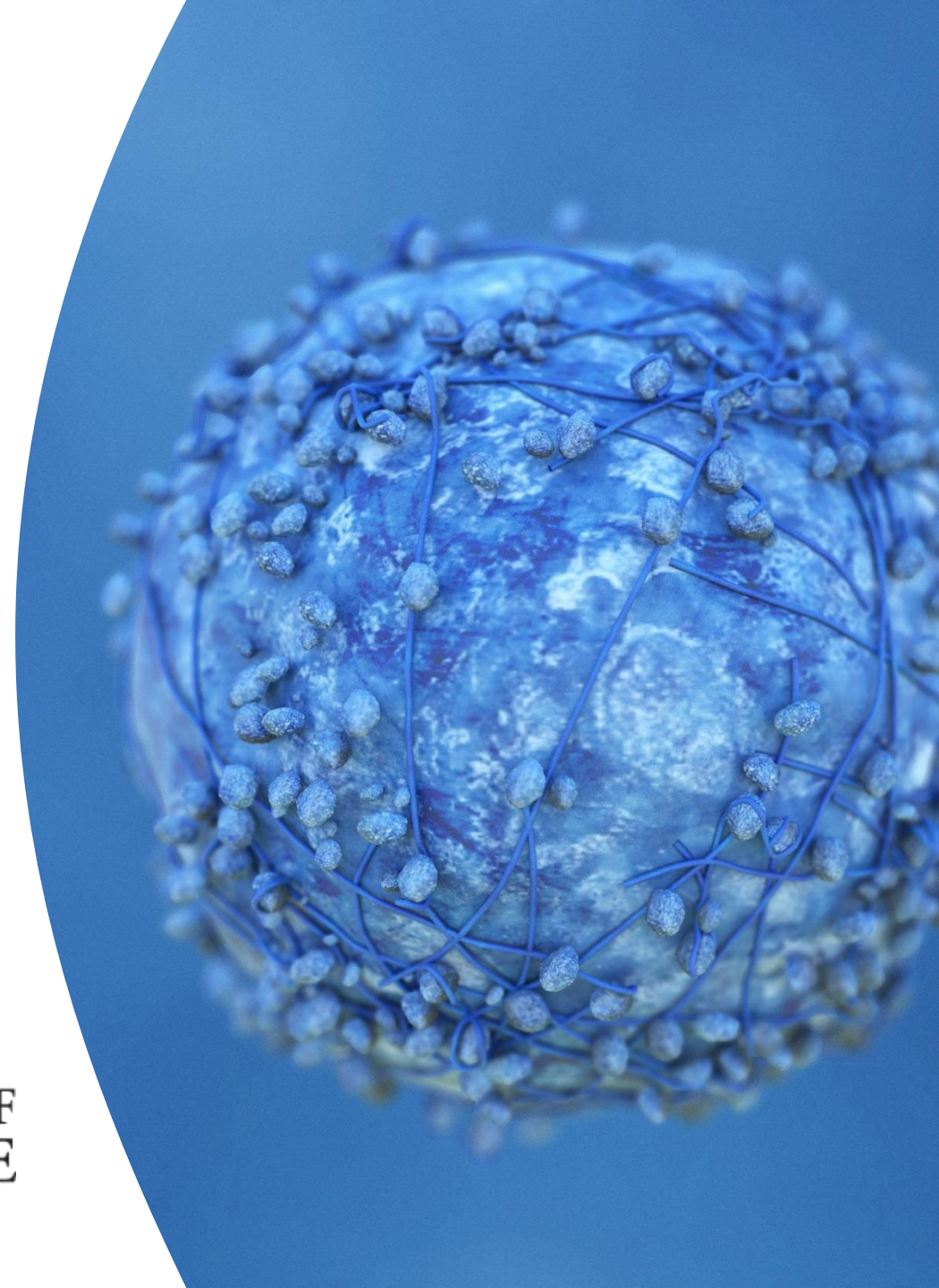
04th November 2021



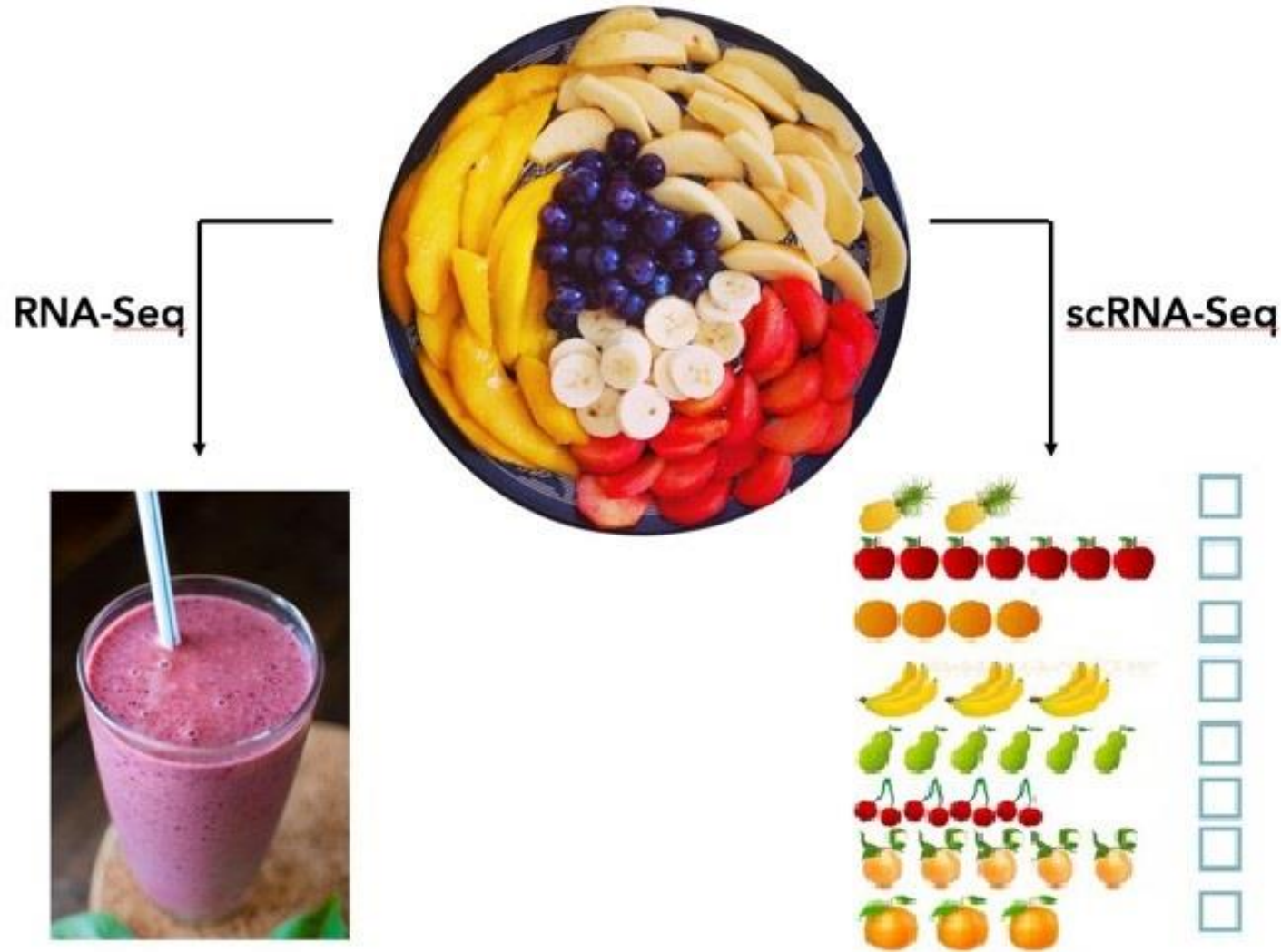
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BULK VS SINGLE CELL RNA-SEQ



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

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

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

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

Source: Sarah Boswell, Harvard Medical School, September 2020

APPLICATIONS

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. 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^{1,3,7}, Rapolas Zilionis^{2,3,7}, Rayman Choo-Wing^{1,5}, Virginia Savova^{2,6}, Judith Knehr⁴, Guglielmo Roma⁴, Allon M. Klein^{2,6} & Aron B. Jaffe^{1,5,*}

nature

Article | Published: 20 February 2019

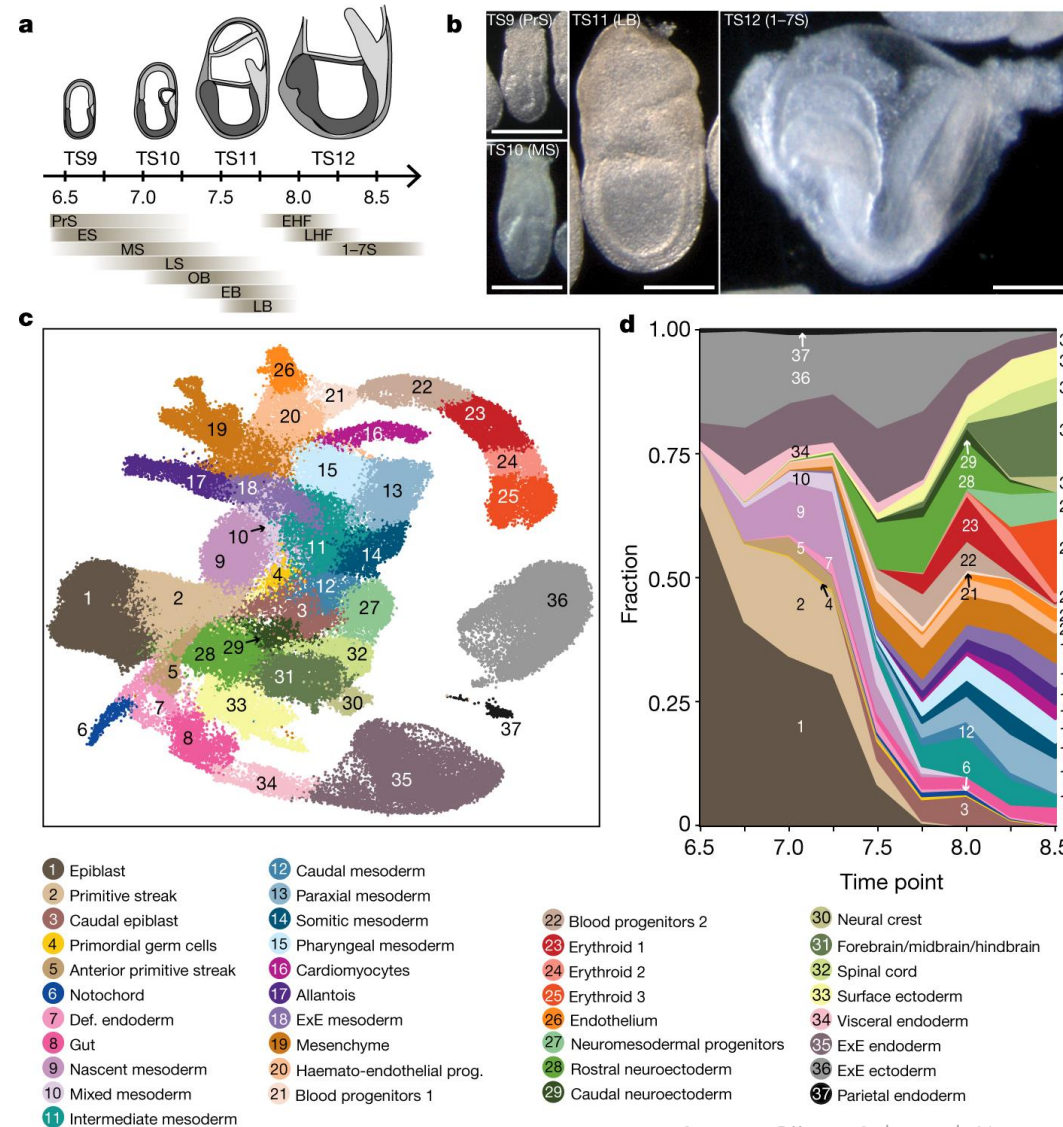
A single-cell molecular map of mouse gastrulation and early organogenesis

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.



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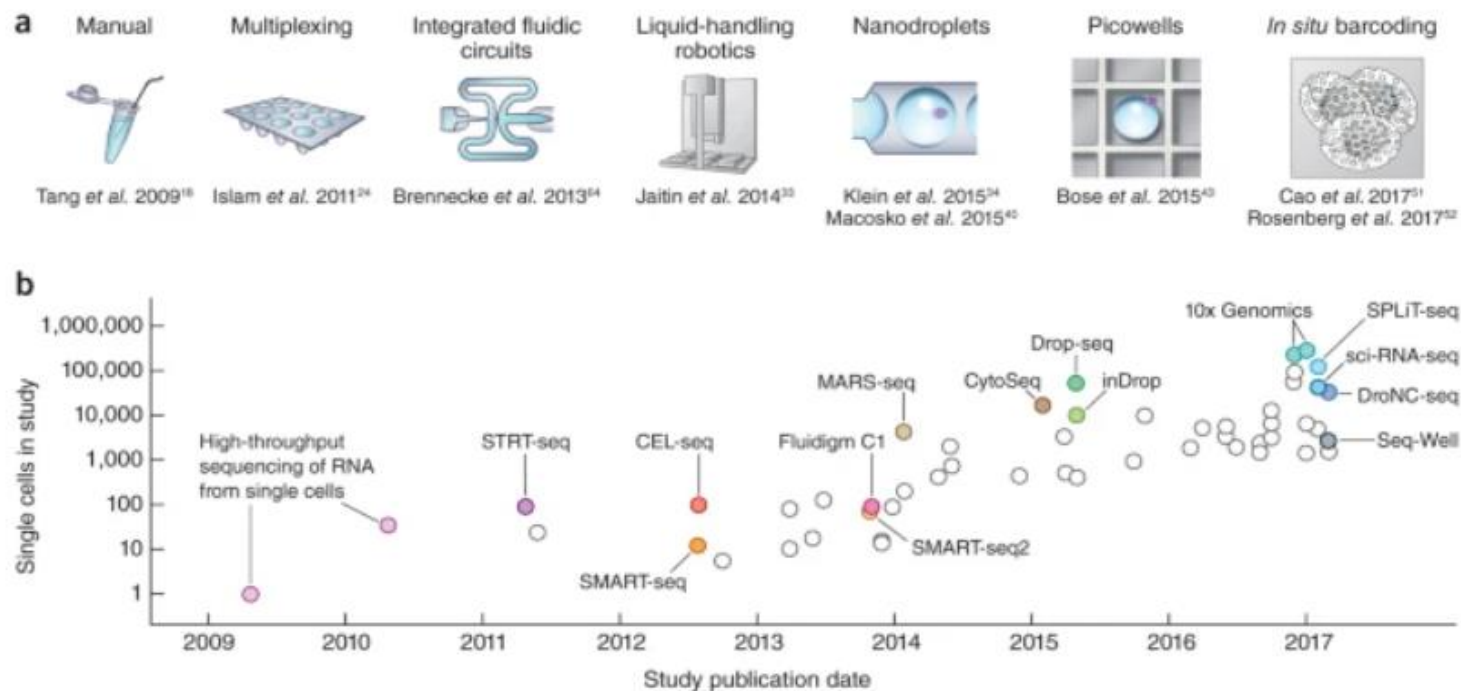
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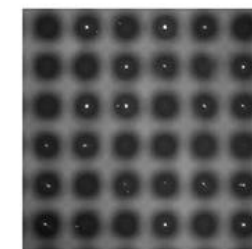
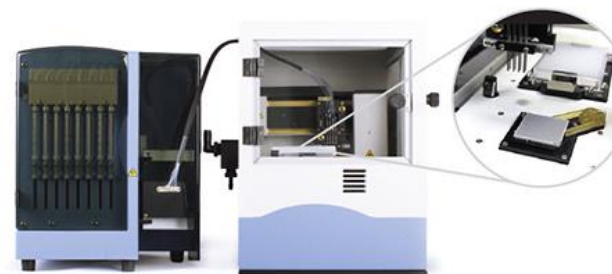
Source: Pijuan-Sala et al. Nature 566, 490–495 (2019)

TECHNOLOGIES

Figure 1: Scaling of scRNA-seq experiments.



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



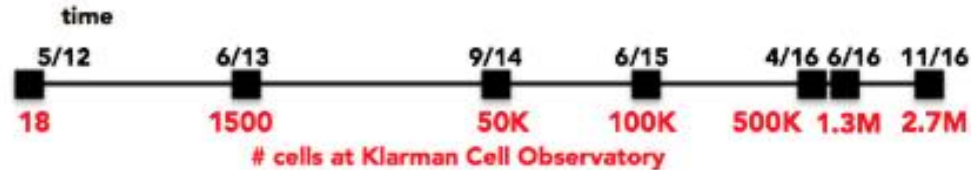
HISTORY AND PROGRESS

LETTER

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

David S. Shulman¹, Robert Kelly², Dan Adelman³, Ryan S. Campbell⁴, Robert T. Campbell⁵, Anthony Richardson⁶, Anthony Richardson⁷, Ray Yip⁸, Alexander B. Heger⁹, John C. Lutz¹⁰, Timothy J. Staudt¹¹, David G. Klarmann¹², Anthony Richardson¹³, Anthony Richardson¹⁴, Anthony Richardson¹⁵, Anthony Richardson¹⁶, Anthony Richardson¹⁷, Anthony Richardson¹⁸, Anthony Richardson¹⁹, Anthony Richardson²⁰

2013, 18 cells



ARTICLE

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

David S. Shulman¹, Robert Kelly², Dan Adelman³, Ryan S. Campbell⁴, Robert T. Campbell⁵, Anthony Richardson⁶, Anthony Richardson⁷, Ray Yip⁸, Alexander B. Heger⁹, John C. Lutz¹⁰, Timothy J. Staudt¹¹, David G. Klarmann¹², Anthony Richardson¹³, Anthony Richardson¹⁴, Anthony Richardson¹⁵, Anthony Richardson¹⁶, Anthony Richardson¹⁷, Anthony Richardson¹⁸, Anthony Richardson¹⁹, Anthony Richardson²⁰

2014, 1700 cells



Resource

2015, 45,000 cells

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

David S. Shulman¹, Robert Kelly², Dan Adelman³, Ryan S. Campbell⁴, Robert T. Campbell⁵, Anthony Richardson⁶, Anthony Richardson⁷, Ray Yip⁸, Alexander B. Heger⁹, John C. Lutz¹⁰, Timothy J. Staudt¹¹, David G. Klarmann¹², Anthony Richardson¹³, Anthony Richardson¹⁴, Anthony Richardson¹⁵, Anthony Richardson¹⁶, Anthony Richardson¹⁷, Anthony Richardson¹⁸, Anthony Richardson¹⁹, Anthony Richardson²⁰

Resource



Perturb-Seq: Dissecting Molecular Circuits with Scalable Single-Cell RNA Profiling of Pooled Genetic Screens

David S. Shulman¹, Robert Kelly², Dan Adelman³, Ryan S. Campbell⁴, Robert T. Campbell⁵, Anthony Richardson⁶, Anthony Richardson⁷, Ray Yip⁸, Alexander B. Heger⁹, John C. Lutz¹⁰, Timothy J. Staudt¹¹, David G. Klarmann¹², Anthony Richardson¹³, Anthony Richardson¹⁴, Anthony Richardson¹⁵, Anthony Richardson¹⁶, Anthony Richardson¹⁷, Anthony Richardson¹⁸, Anthony Richardson¹⁹, Anthony Richardson²⁰

2016, 200,000 cells

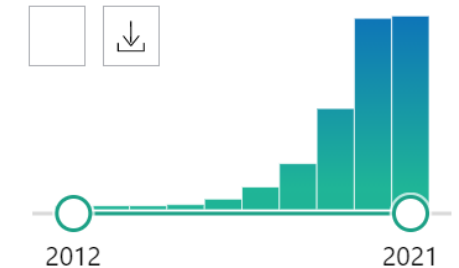
2017, 1.3 million cells (10X genomics)

Publications using 10x Genomics



PubMed search for 'scRNA-seq'

RESULTS BY YEAR



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



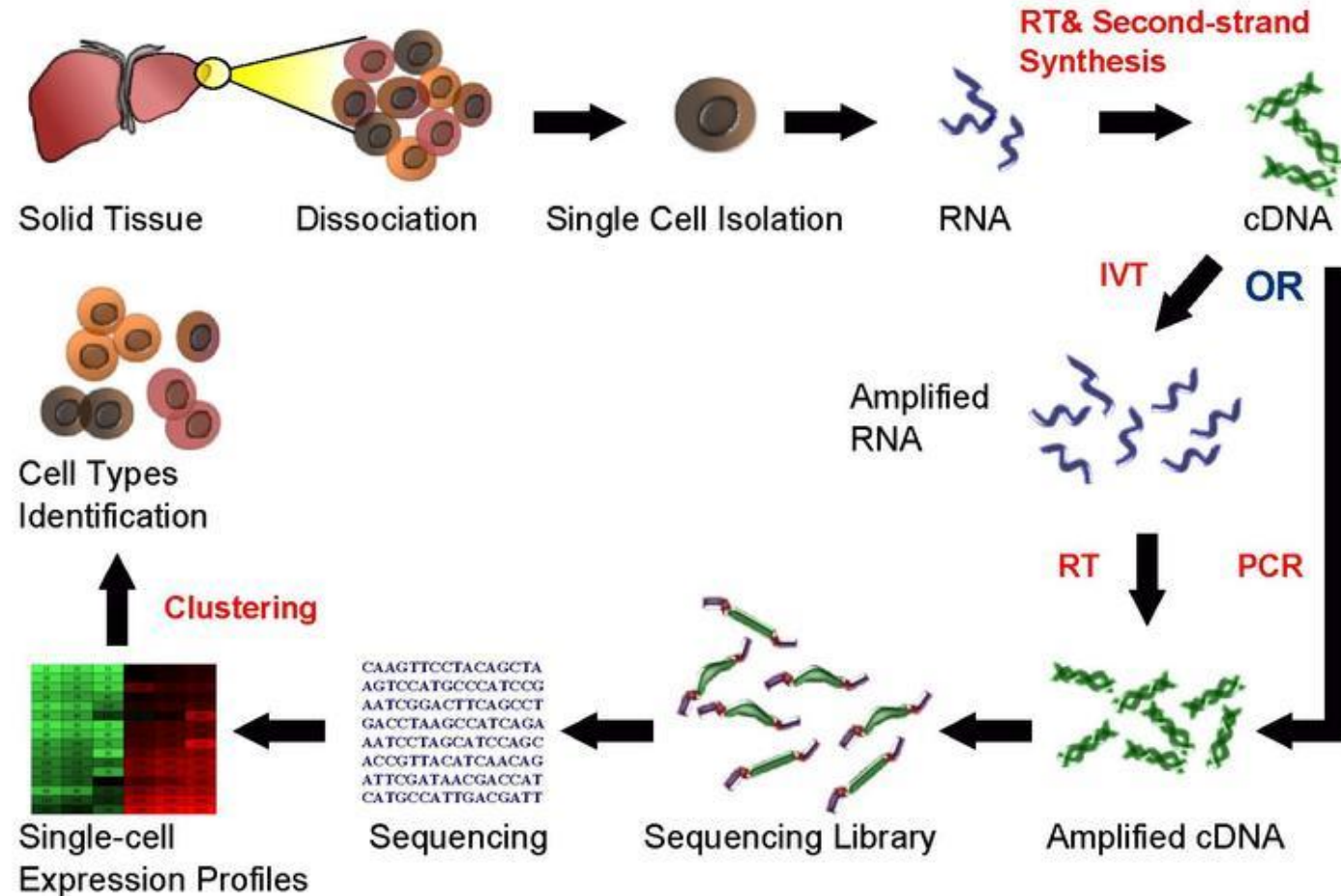
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WORKFLOW

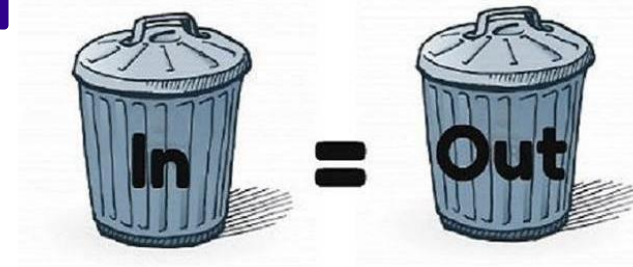
Single Cell RNA Sequencing Workflow



Good sample preparation is key to success!



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)



TISSUE PRESERVATION

Research | [Open Access](#) | Published: 02 June 2020

Systematic assessment of tissue dissociation and storage biases in single-cell and single-nucleus RNA-seq workflows

[Elena Denisenko](#), [Belinda B. Guo](#), [Matthew Jones](#), [Rui Hou](#), [Leanne de Kock](#), [Timo Lassmann](#), [Daniel Poppe](#), [Olivier Clément](#), [Rebecca K. Simmons](#), [Ryan Lister](#) & [Alistair R. R. Forrest](#) 

[Genome Biology](#) **21**, Article number: 130 (2020) | [Cite this article](#)

14k Accesses | 39 Citations | 40 Altmetric | [Metrics](#)

[Immune Netw.](#) 2020 Aug; 20(4): e34.

Published online 2020 Jul 15. doi: [10.4110/in.2020.20.e34](#)

PMCID: PMC7458795

PMID: [32895621](#)


Effects of Cryopreservation and Thawing on Single-Cell Transcriptomes of Human T Cells

[Jeong Seok Lee](#),^{1,†} [Kijong Yi](#),^{1,†} [Young Seok Ju](#),^{1,2} and [Eui-Cheol Shin](#)^{1,2}

► [Author information](#) ► [Article notes](#) ► [Copyright and License information](#) [Disclaimer](#)

Article | [Open Access](#) | Published: 23 July 2019

DMSO cryopreservation is the method of choice to preserve cells for droplet-based single-cell RNA sequencing

[Christian T. Wohnhaas](#), [Germán G. Leparo](#), [Francesc Fernandez-Albert](#), [David Kind](#), [Florian Gantner](#), [Coralie Viollet](#), [Tobias Hildebrandt](#) & [Patrick Baum](#) 

[Scientific Reports](#) **9**, Article number: 10699 (2019) | [Cite this article](#)

14k Accesses | 20 Citations | 49 Altmetric | [Metrics](#)

Research | [Open Access](#) | Published: 10 May 2021

Cryopreservation of human cancers conserves tumour heterogeneity for single-cell multi-omics analysis

[Sunny Z. Wu](#), [Daniel L. Roden](#), [...] [Alexander Swarbrick](#) 

[Genome Medicine](#) **13**, Article number: 81 (2021) | [Cite this article](#)

1757 Accesses | 34 Altmetric | [Metrics](#)

[Genome Biol.](#) 2020; 21: 1.

Published online 2019 Dec 31. doi: [10.1186/s13059-019-1906-x](#)

PMCID: PMC6937944

PMID: [31892341](#)

scRNA-seq assessment of the human lung, spleen, and esophagus tissue stability after cold preservation

[E. Madisson](#),^{#1,2} [A. Wilbrey-Clark](#),^{#1} [R. J. Miragaia](#),¹ [K. Saeb-Parsy](#),³ [K. T. Mahbubani](#),³ [N. Georgakopoulos](#),³ [P. Harding](#),¹ [K. Polanski](#),¹ [N. Huang](#),¹ [K. Nowicki-Osuch](#),⁴ [R. C. Fitzgerald](#),⁴ [K. W. Loudon](#),⁵ [J. R. Ferdinand](#),⁵ [M. R. Clatworthy](#),⁵ [A. Tsingene](#),¹ [S. van Dongen](#),¹ [M. Dabrowska](#),¹ [M. Patel](#),¹ [M. J. T. Stubbington](#),^{1,6} [S. A. Teichmann](#),¹ [O. Stegle](#),² and [K. B. Meyer](#)^{#1}

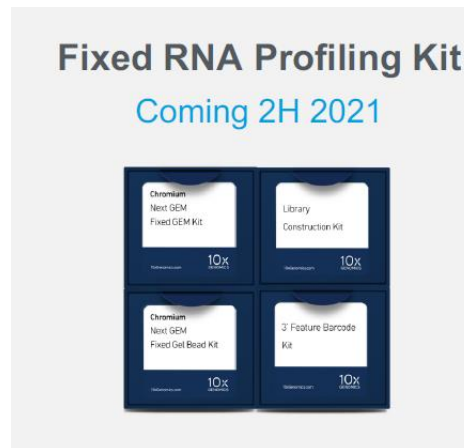
Research article | [Open Access](#) | Published: 09 November 2020

Single-cell transcriptome conservation in a comparative analysis of fresh and cryopreserved human skin tissue: pilot in localized scleroderma

[Emily Mirizio](#), [Tracy Tabib](#), [Xinjun Wang](#), [Wei Chen](#), [Christopher Liu](#), [Robert Lafyatis](#), [Heidi Jacobse](#) & [Kathryn S. Torok](#) 

[Arthritis Research & Therapy](#) **22**, Article number: 263 (2020) | [Cite this article](#)

2256 Accesses | 4 Citations | 4 Altmetric | [Metrics](#)



METHODS

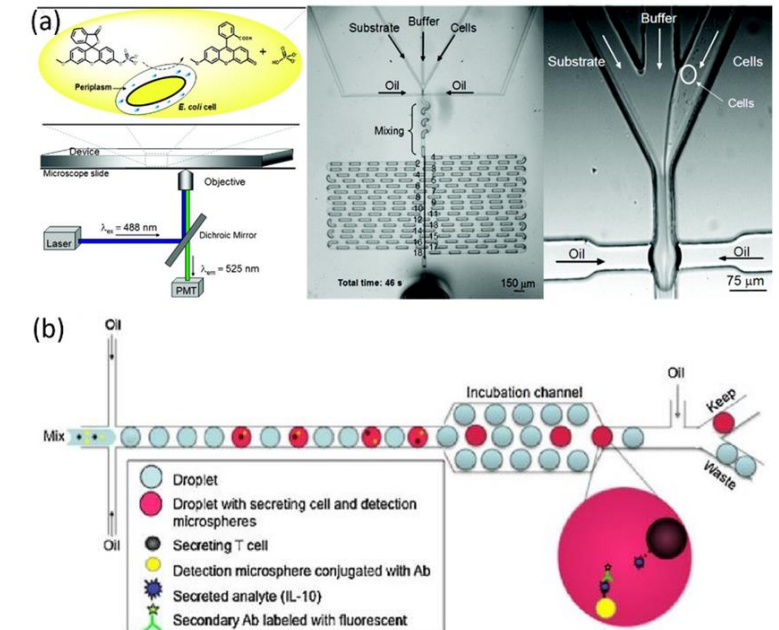
- 1) Cells in wells, traps and valves (nanowell, Flow sorting, CellenOne, Fluidigm C1, SmartSeq)
 - Screen for and retrieve single cells of interest
 - Enrich for rare cells with decided properties
 - Control the cellular microenvironment
 - Monitor and control cell-cell interactions
 - Precise/extensive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



Passive wells

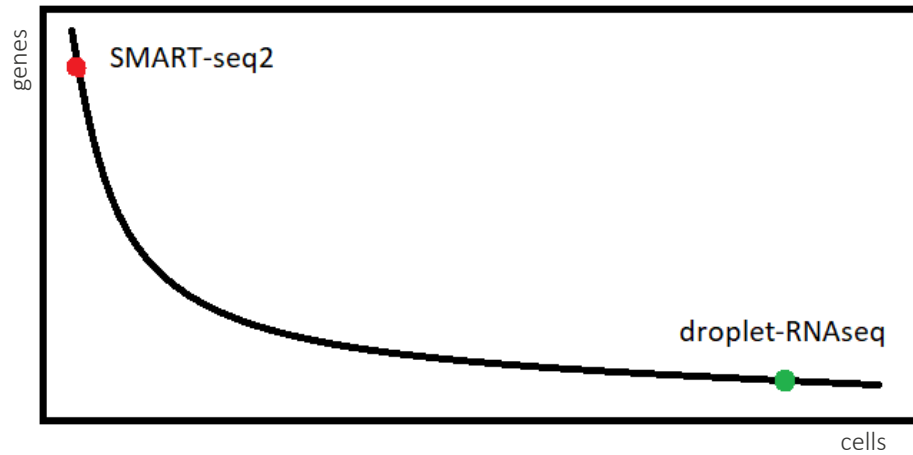


Active pumps and valves



Source: Wen et al. Molecules (2016)

MORE CELLS OR MORE GENES?

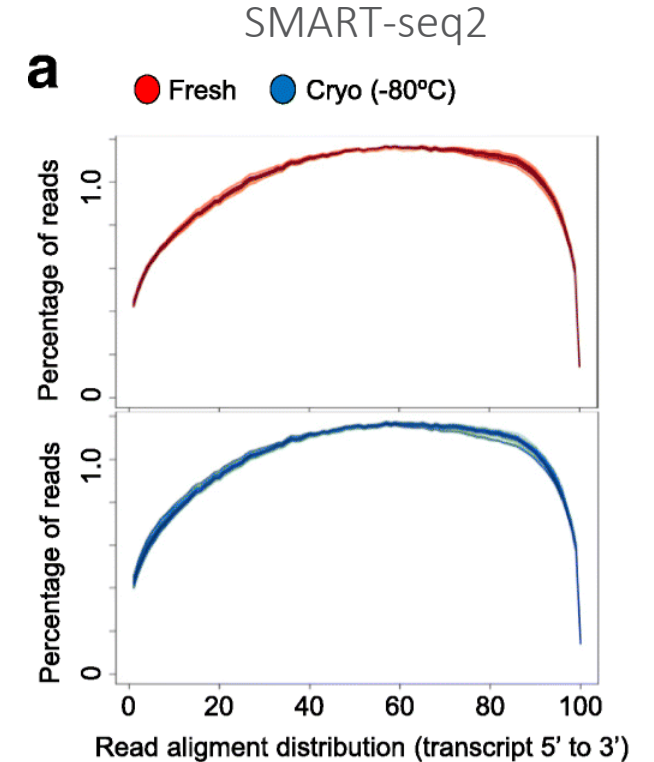
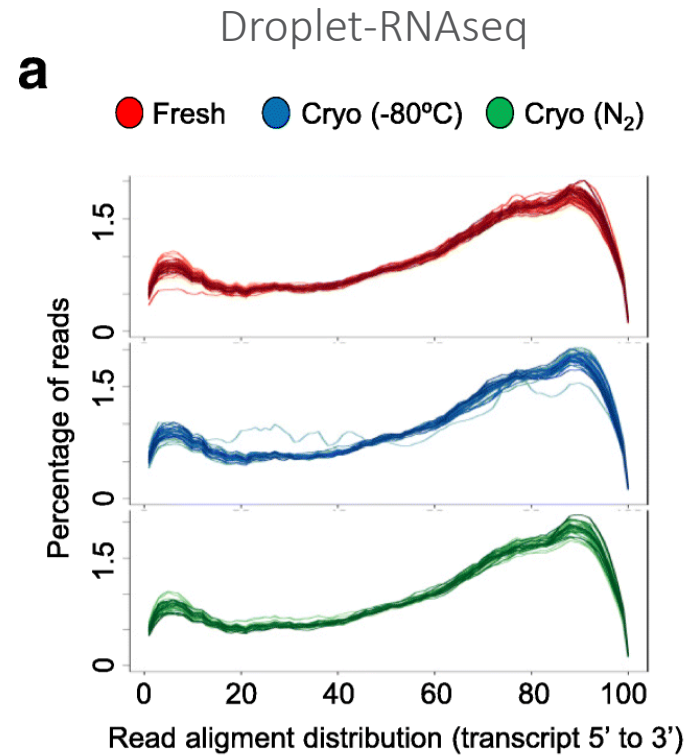


SMART-seq2

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

Droplet-RNAseq

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



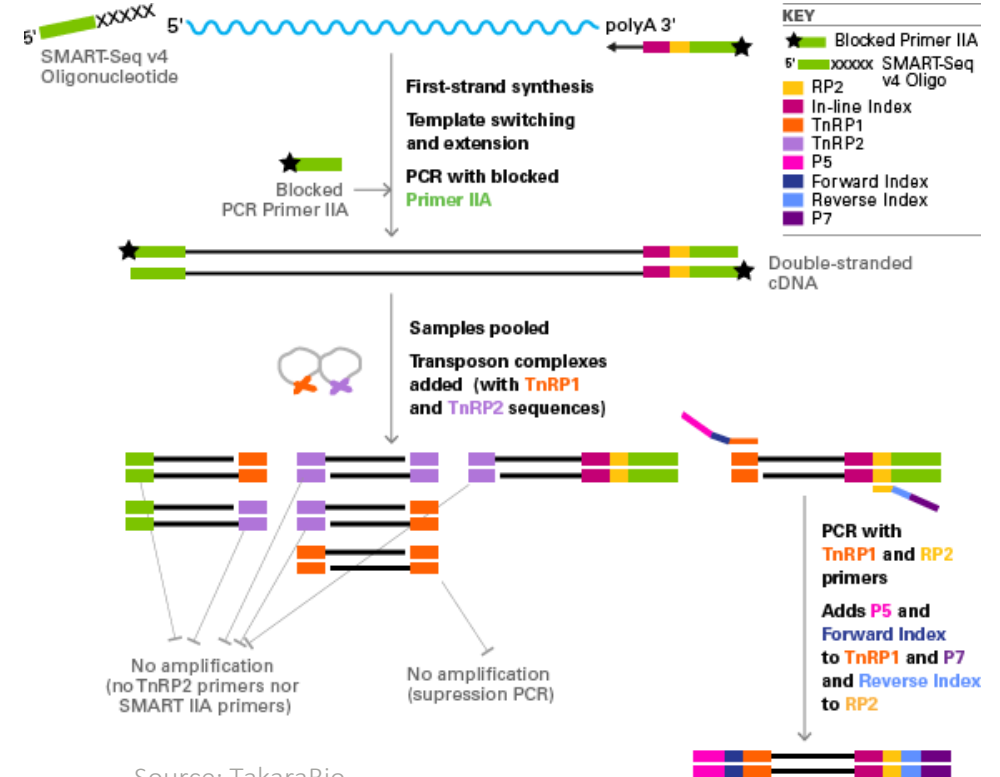
Source: [Guillaumet-Adkins, et al. Genome Biol 18, 45 \(2017\).](#)

- 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.

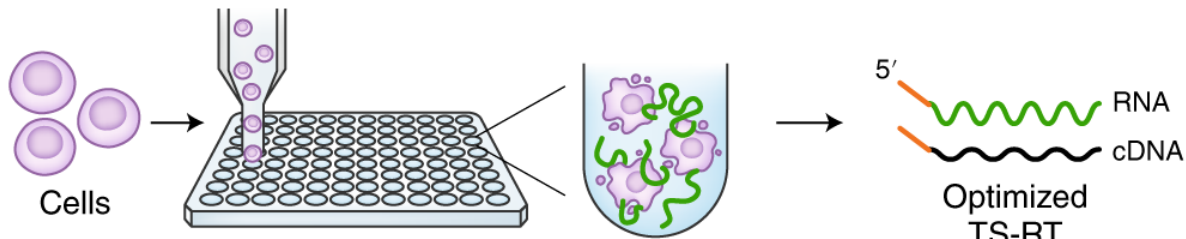
SMART-SEQ2/3/4 OVERVIEW

Developed for single cell but can be 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

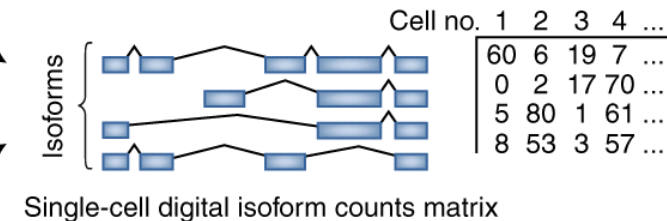


5' tagged reads

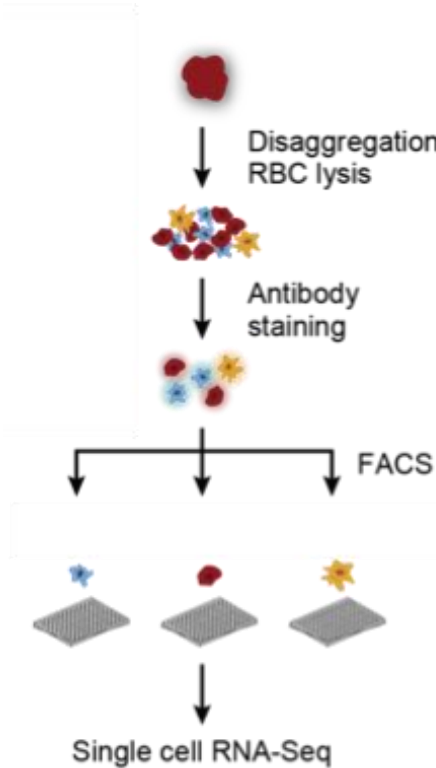
Digital count of transcripts
5' isoform structure

Internal reads

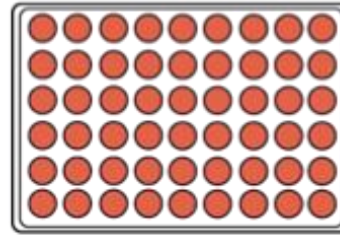
Full isoform reconstruction



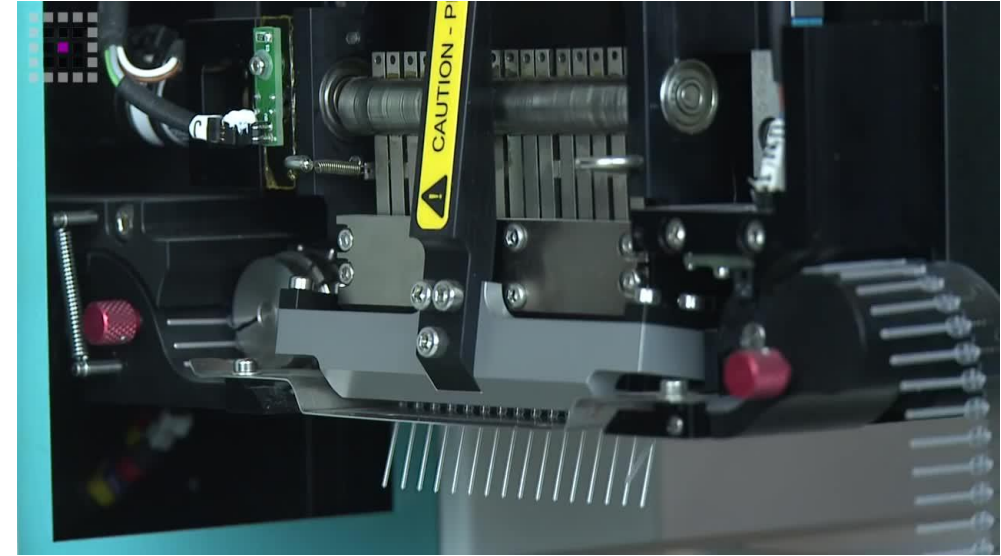
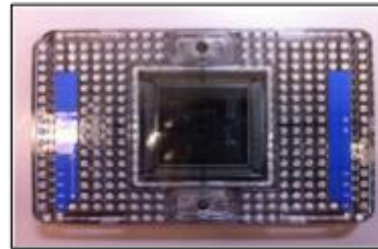
SMART-SEQ2/3/4 + MOSQUITO LV



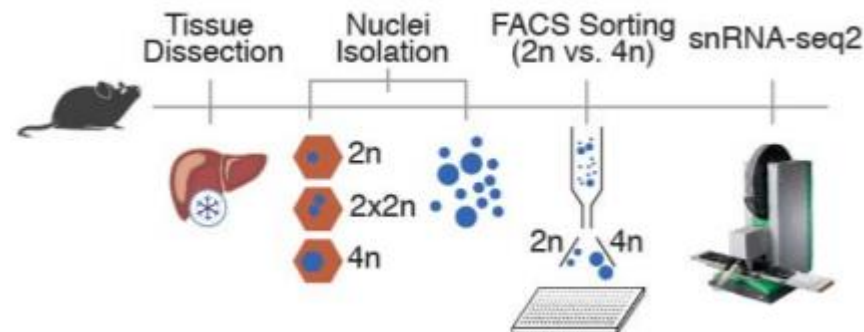
FACS sorting on 96/384-well plates



Fluidigm C1-autoprep system

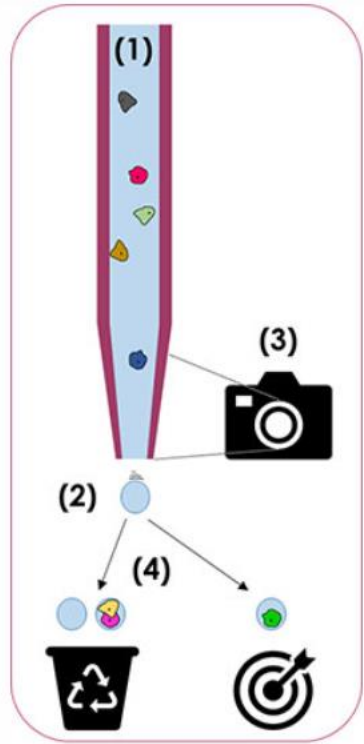


Source: SPT Labtech



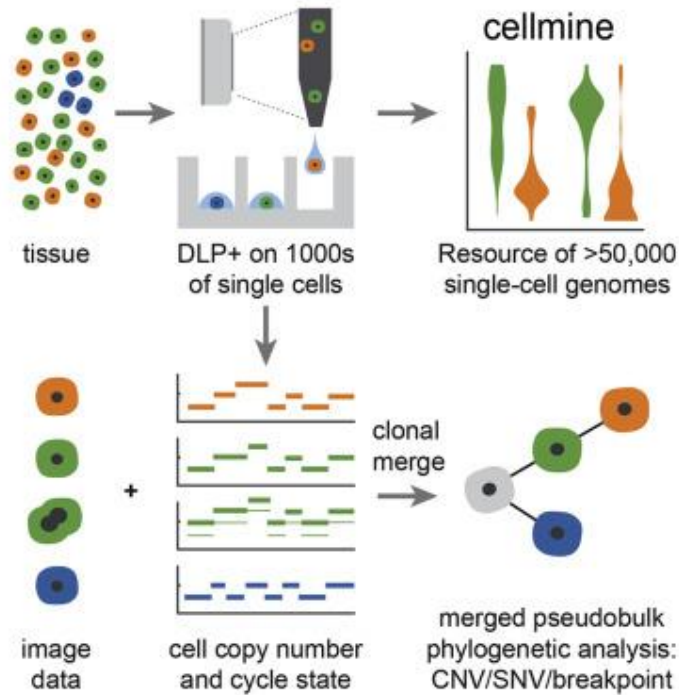
- Mosquito LV makes assay miniaturisation simple, leading to significant savings on precious reagents and time.
- Mosquito LV offers highly accurate and precise multichannel pipetting from 25 nL to 1.2 μ L.
- SmartSeq2 cost reduced from \$12 to \$4 per cell

CELLENONE

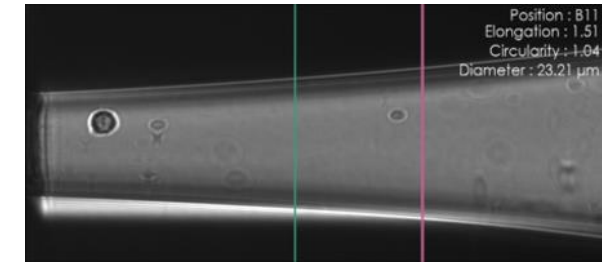
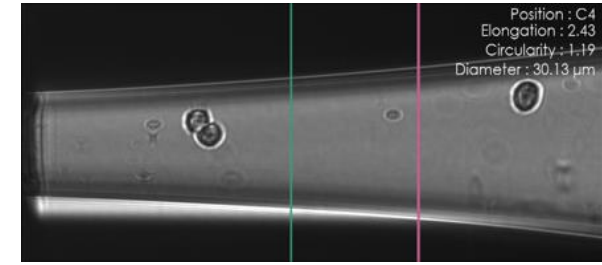


Source: Cellenion

- 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 cell are dispensed into recycling tube



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

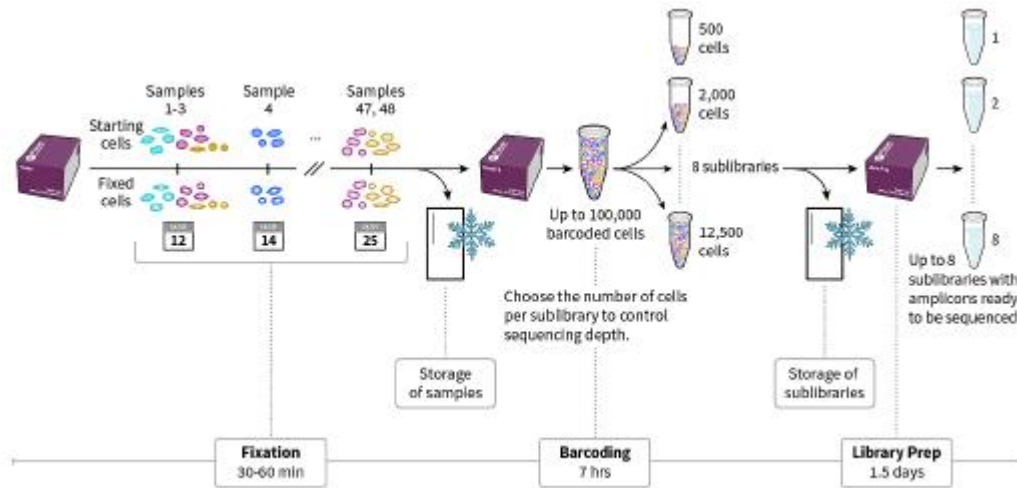


Source: Cellenion

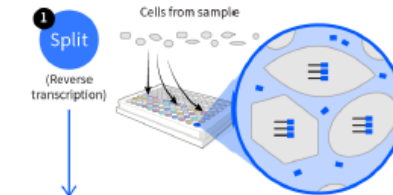
SPLIT-SEQ OVERVIEW (PARSE BIOSCIENCES)



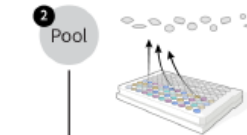
Video available at: <https://sites.google.com/uw.edu/splitseq>



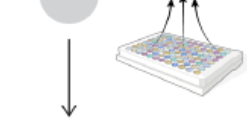
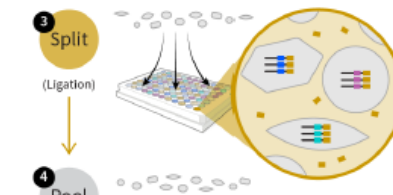
In the first round of barcoding, fixed cell samples are distributed into 48 wells, and cDNA is generated with an in-cell reverse transcription (RT) reaction using well-specific barcoded primers.



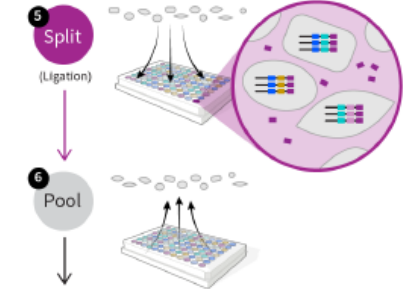
Cells from each well are pooled back together.



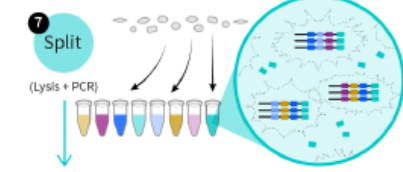
Cells are then distributed into 96 wells, and an in-cell ligation reaction appends a second well-specific barcode to the cDNA.



The third-round barcode is appended with another round of in-cell ligation.



After three rounds of barcoding, the cells are pooled and split into 8 distinct populations we term sublibraries. The user can choose the number of cells in each sublibrary to control the depth of sequencing. Cells will not be pooled again after this step. After this final split cells are lysed and the barcoded cDNA is isolated. A fourth sublibrary-specific barcode is introduced by PCR to each cDNA molecule.



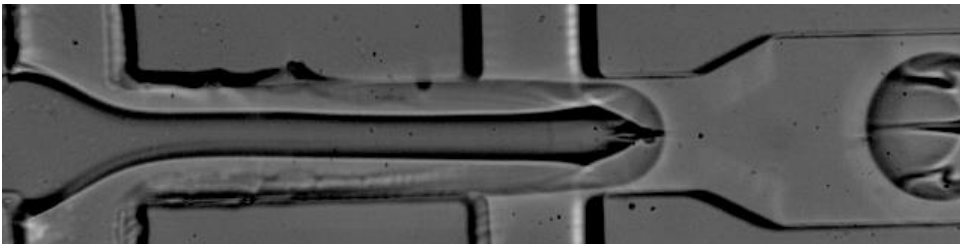
After sequencing, each single cell transcriptome is assembled by combining reads containing the same four-barcode combination.



- Time flexibility – single experiment for samples collected on different dates (up to 6months storage)
- No instrument required for experiment. Computational pipeline available
- Up to 48 samples / 100k cells in total – kit has to be used at once
- Retail price of \$9,800 per kit (+fixation kits)
- Doublet rate of 0.27% per 1000 cells (3.4% per library)
- No 3'/5' bias – random hexamers method
- Median genes detection of about 12,000 genes
- Works with any species, any sizes of cells/nuclei & results in lower background noise

DROP-SEQ OVERVIEW

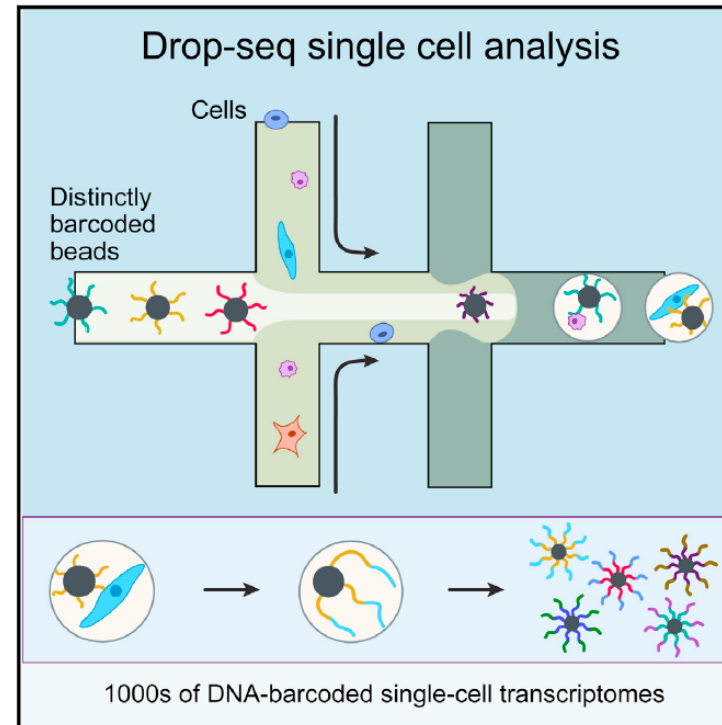
- Moved throughput from hundreds to thousands.
- Droplet-based processing using microfluidics
- Nanoliter scale aqueous drops in oil.
- 3' End
- Bead based (STAMPs).
- Single-cell transcriptomes attached to microparticles.
- Cell barcodes use split-pool synthesis.
- Uses UMI (Unique Molecular Identifier)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



Cell

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

Graphical Abstract



Resource

Authors

Evan Z. Macosko, Anindita Basu, ...,
Aviv Regev, Steven A. McCarroll

Correspondence

emacosko@genetics.med.harvard.edu
(E.Z.M.),
mccarroll@genetics.med.harvard.edu
(S.A.M.)

In Brief

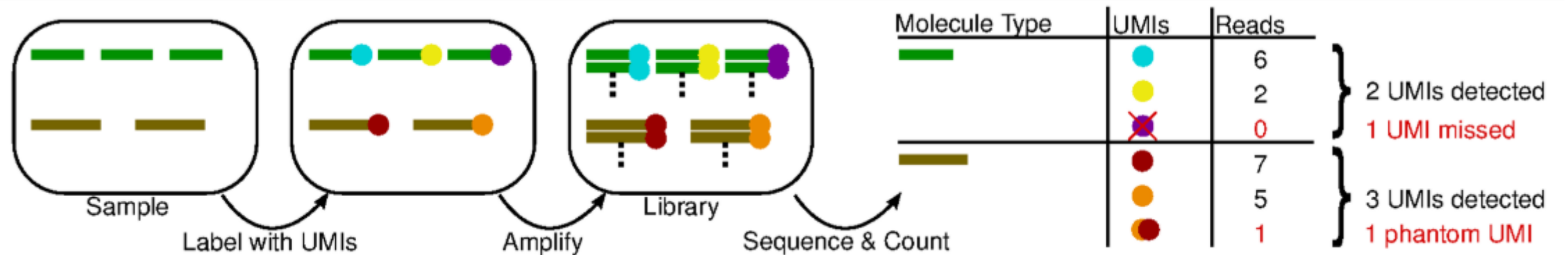
Capturing single cells along with sets of uniquely barcoded primer beads together in tiny droplets enables large-scale, highly parallel single-cell transcriptomics. Applying this analysis to cells in mouse retinal tissue revealed transcriptionally distinct cell populations along with molecular markers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

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.

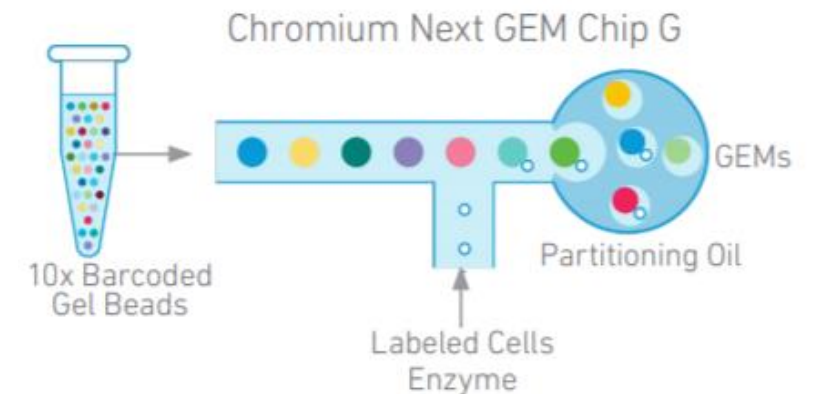
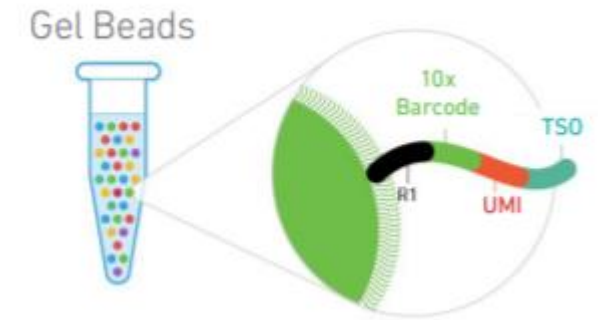
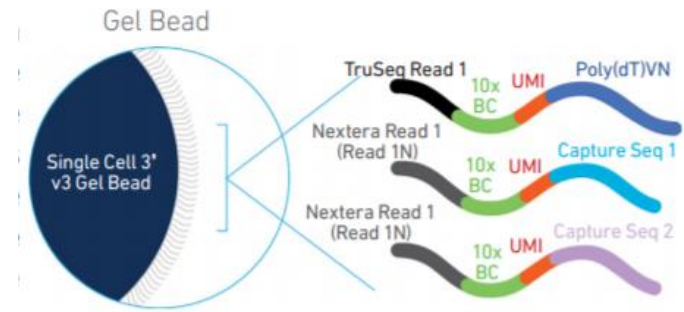
When performing variant analyses, these falsely overrepresented fragments can result in incorrect calls and thus wrong diagnostic findings



Source: Pflug et al. Bioinformatics (2018)

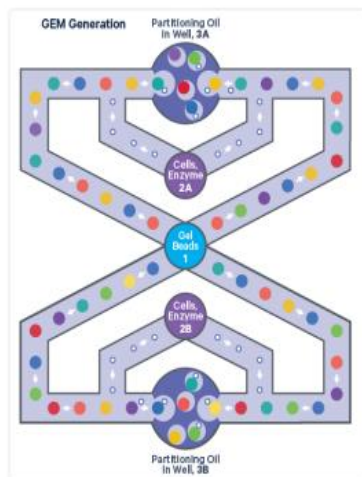
10X GENOMICS OVERVIEW

- 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

10X GENOMICS OVERVIEW

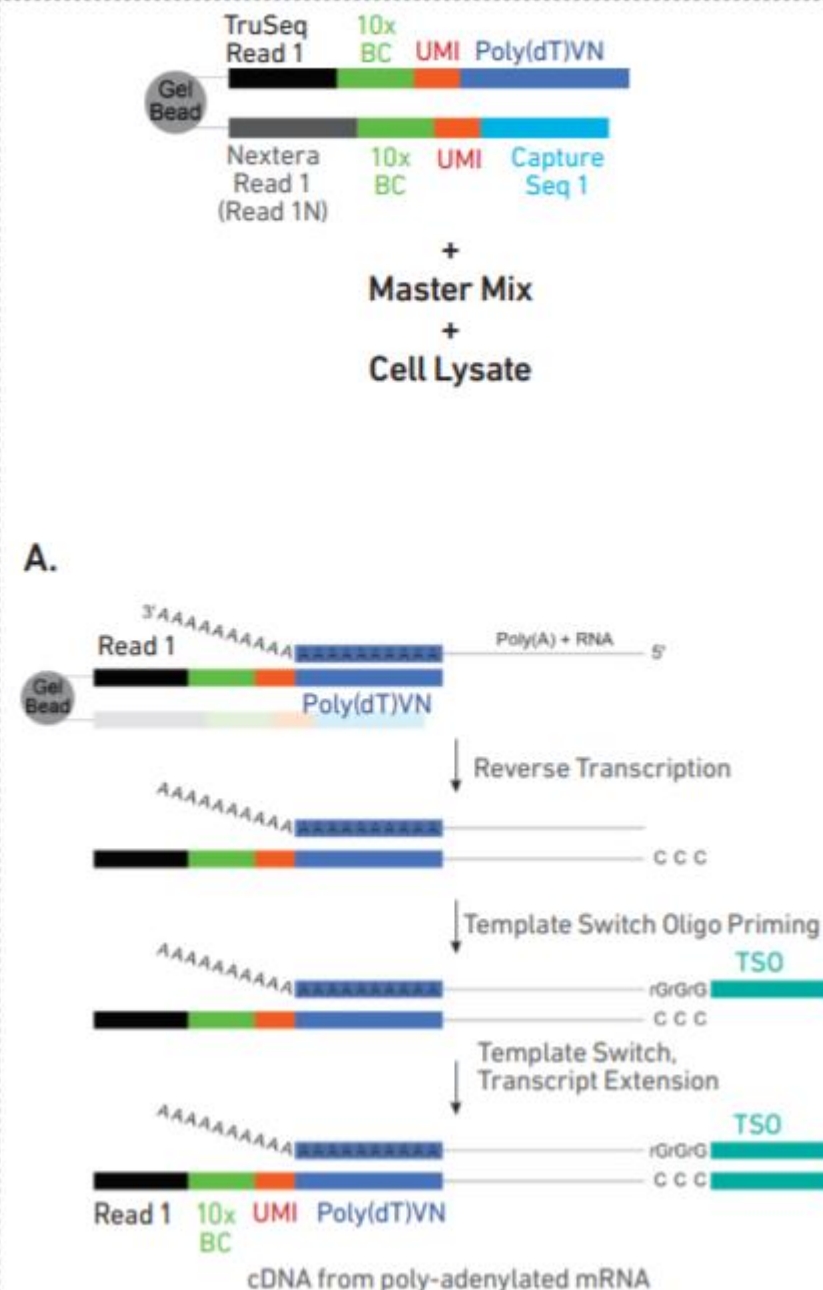


Chromium X



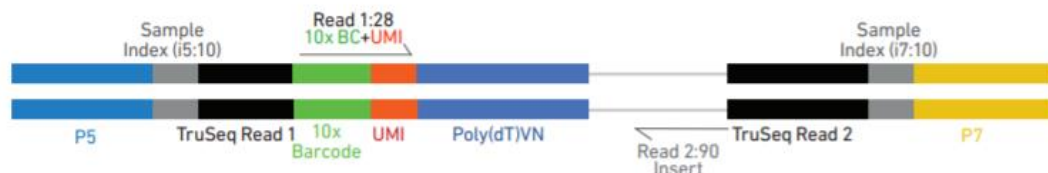
Making 1 million cell experiments routine

Inside individual GEMs

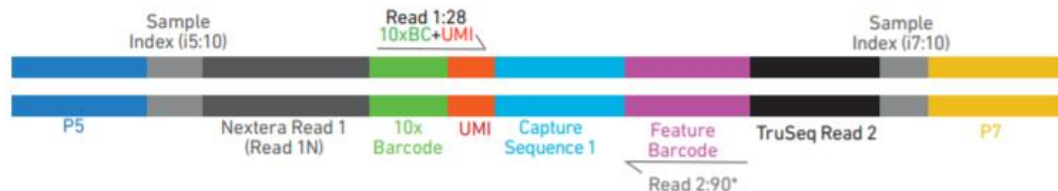


10X GENOMICS LIBRARIES

Chromium Single Cell 3' Gene Expression Dual Index Library



Chromium Single Cell 3' Cell Surface Protein Dual Index Library



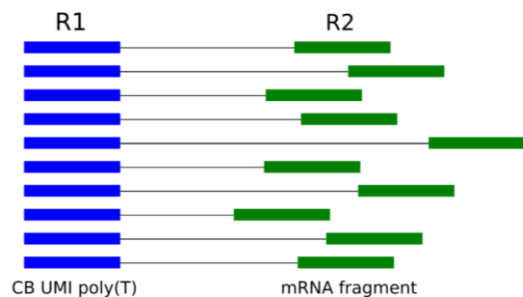
Chromium Single Cell V(D)J Dual Index Library



Chromium Single Cell 5' Gene Expression Dual Index Library



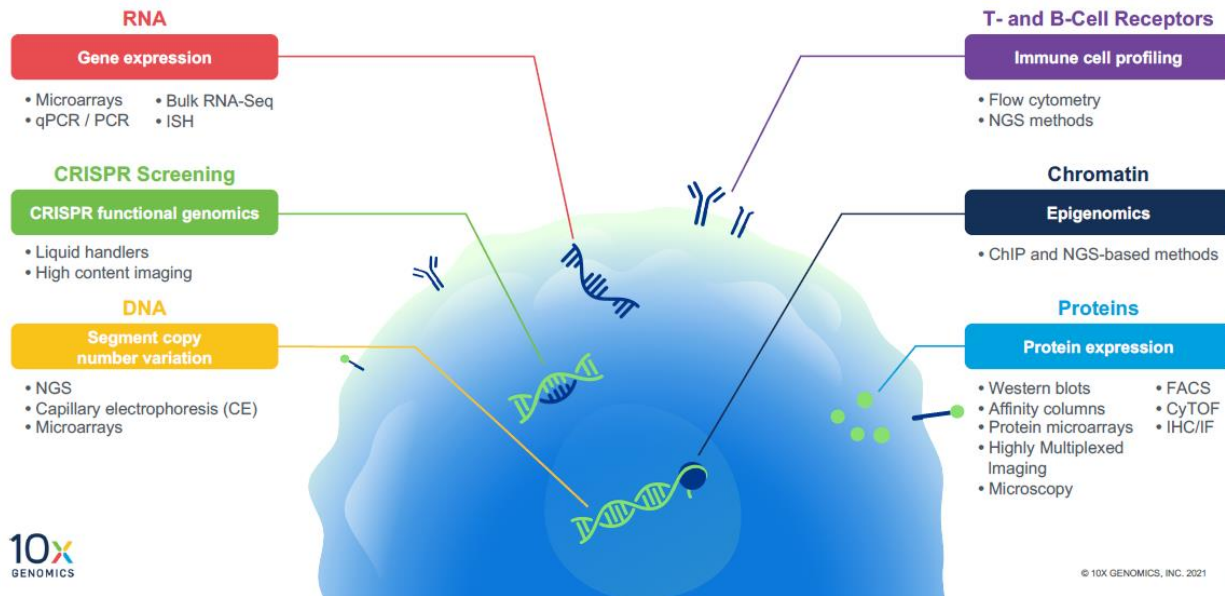
Source: 10x Genomics



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

MULTIOMICS AGE

Replacing the Legacy Toolkit Across Biology

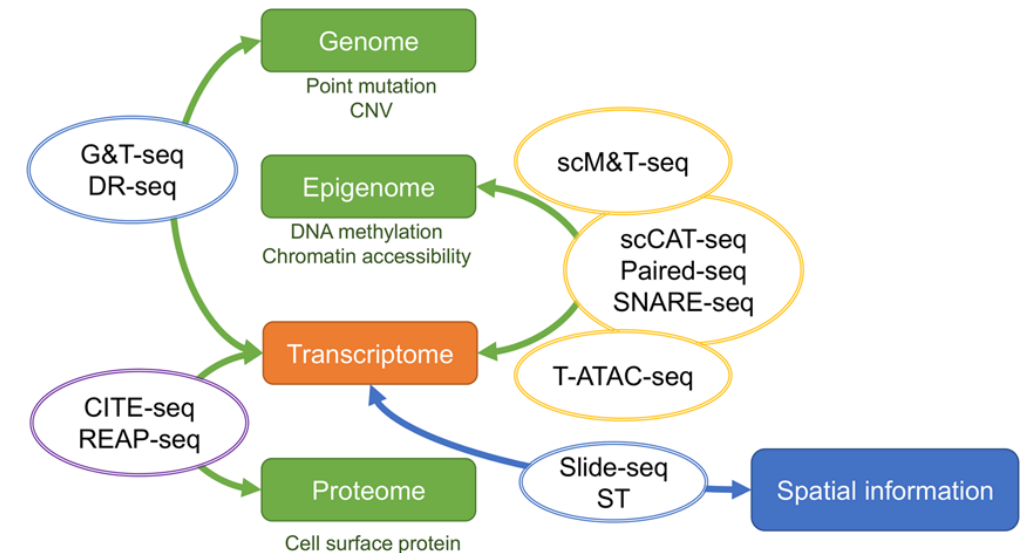


TECHNOLOGY FEATURE | 19 July 2021 | Correction 21 July 2021

Single-cell analysis enters the multiomics age

A rapidly growing collection of software tools is helping researchers to analyse multiple huge ‘-omics’ data sets.

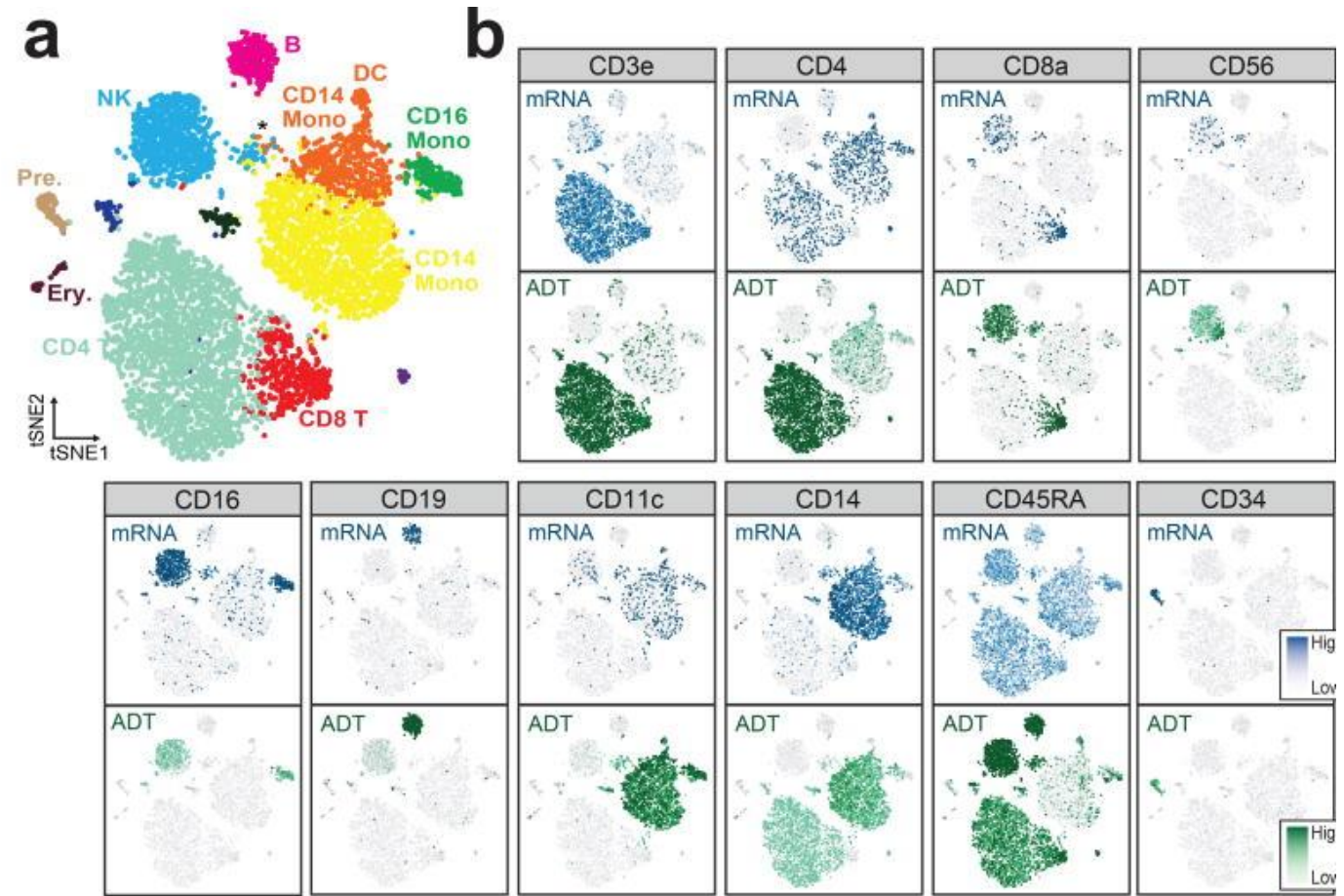
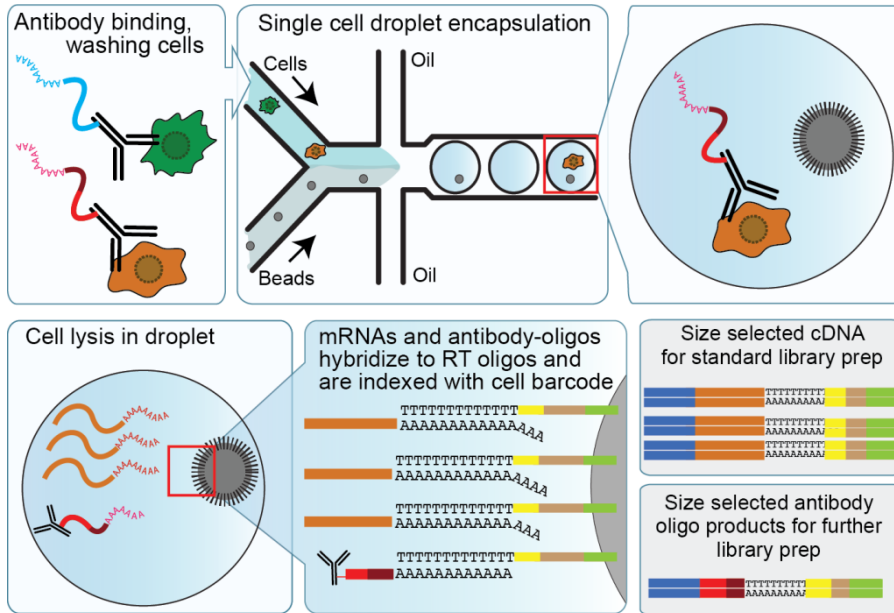
Jeffrey M. Perkel



Kashima Y et al. Exp Mol Med 52, 1419–1427 (2020)

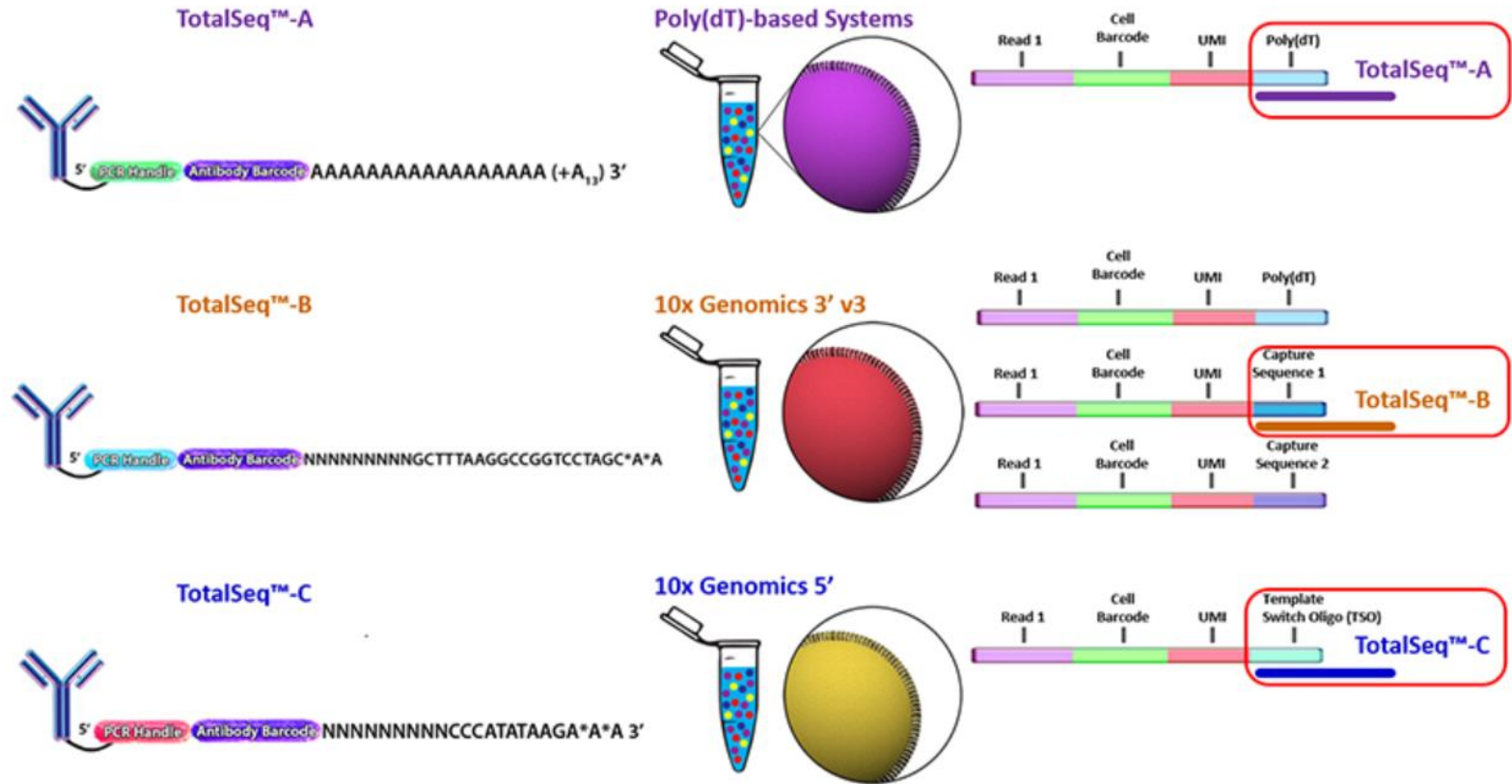
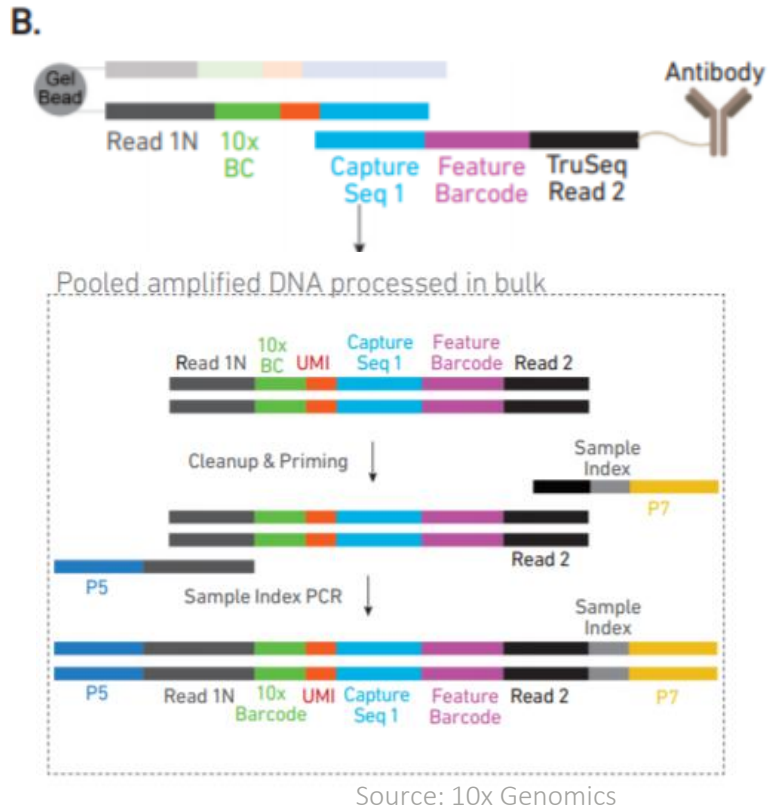
CITE-SEQ

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

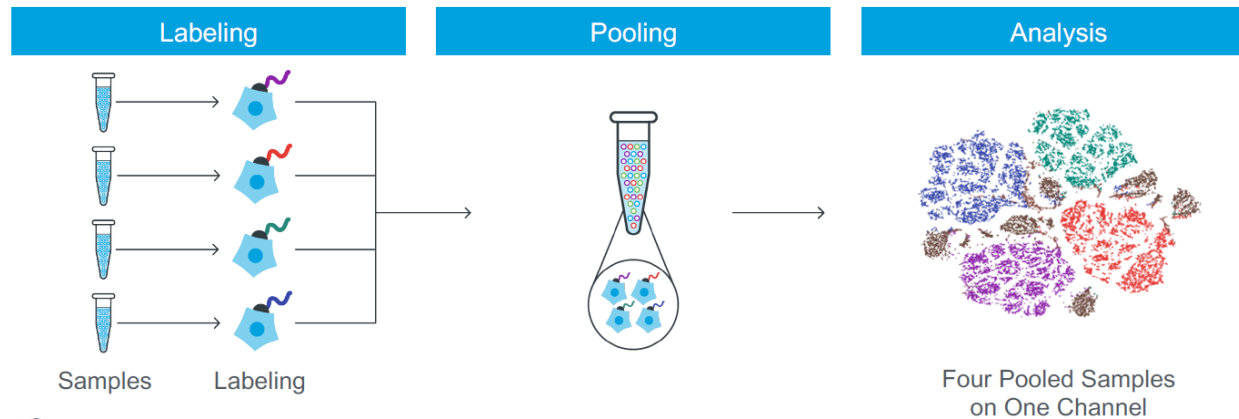
CITE-SEQ



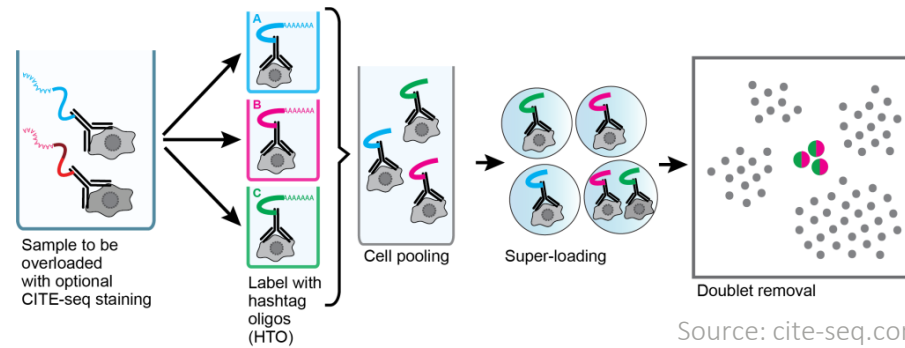
Source: BioLegend

CELL HASHING

Reduces cost of running multiple samples by adding hashtag oligos and pooling into single channel of 10x chip (10x CellPlex or TotalSeq antibodies)



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



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

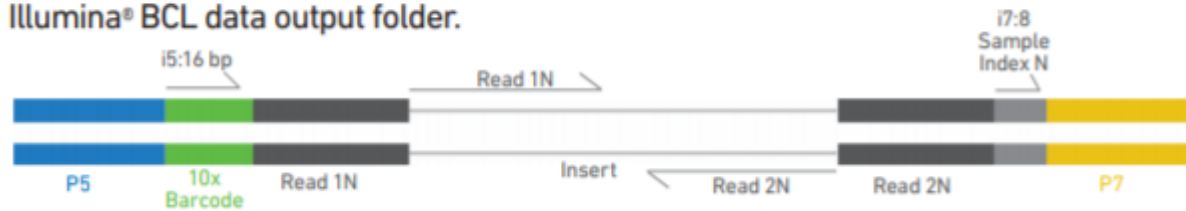
Christopher S. McGinnis, David M. Patterson, Juliane Winkler, Daniel N. Conrad, Marco Y. Hein, Vasudha Srivastava, Jennifer L. Hu, Lyndsay M. Murrow, Jonathan S. Weissman, Zena Werb, Eric D. Chow & Zev J. Gartner

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

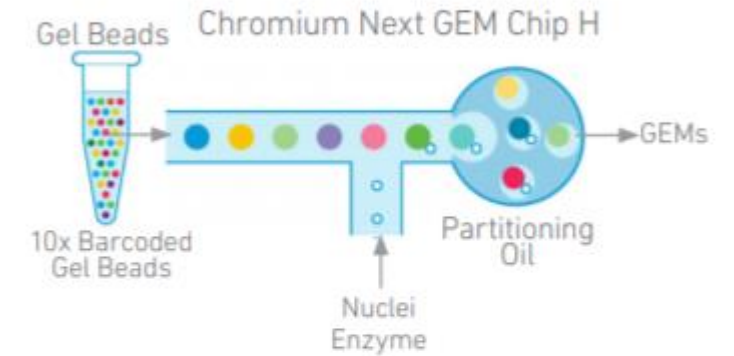
15k Accesses | 27 Citations | 85 Altmetric | Metrics

10X ATAC

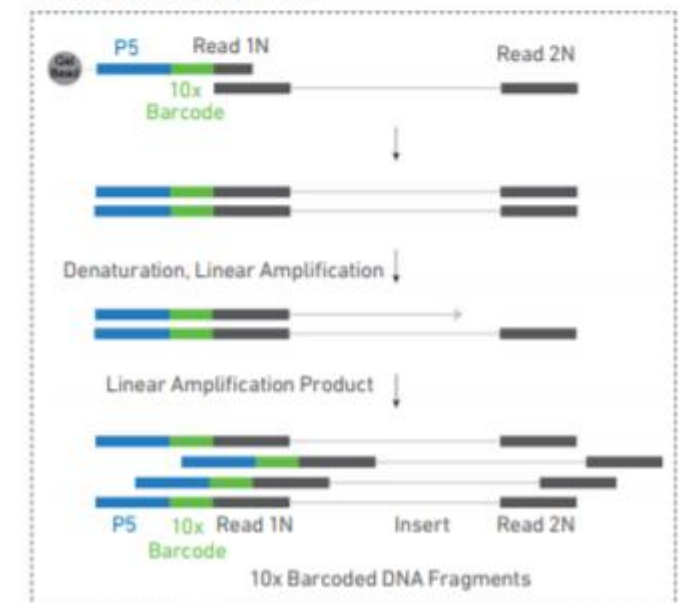
Chromium Single Cell ATAC libraries comprise double stranded DNA fragments which begin with P5 and end with P7. Sequencing these libraries produces a standard Illumina® BCL data output folder.



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



Inside Individual GEMs

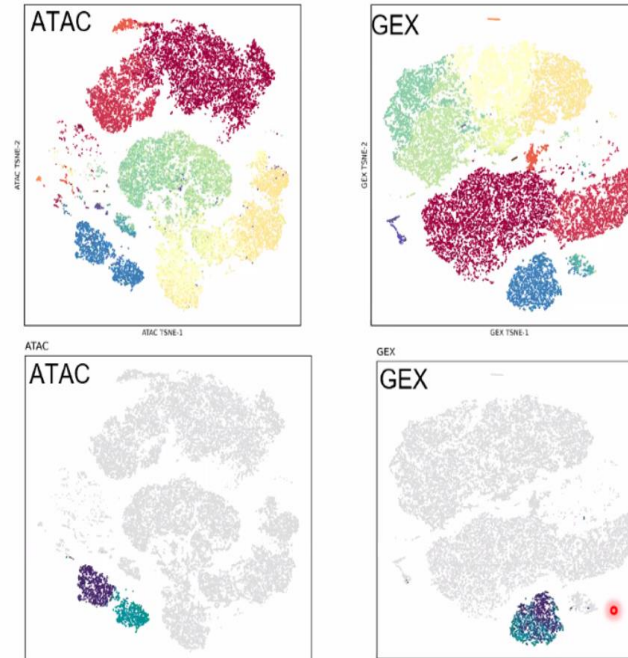
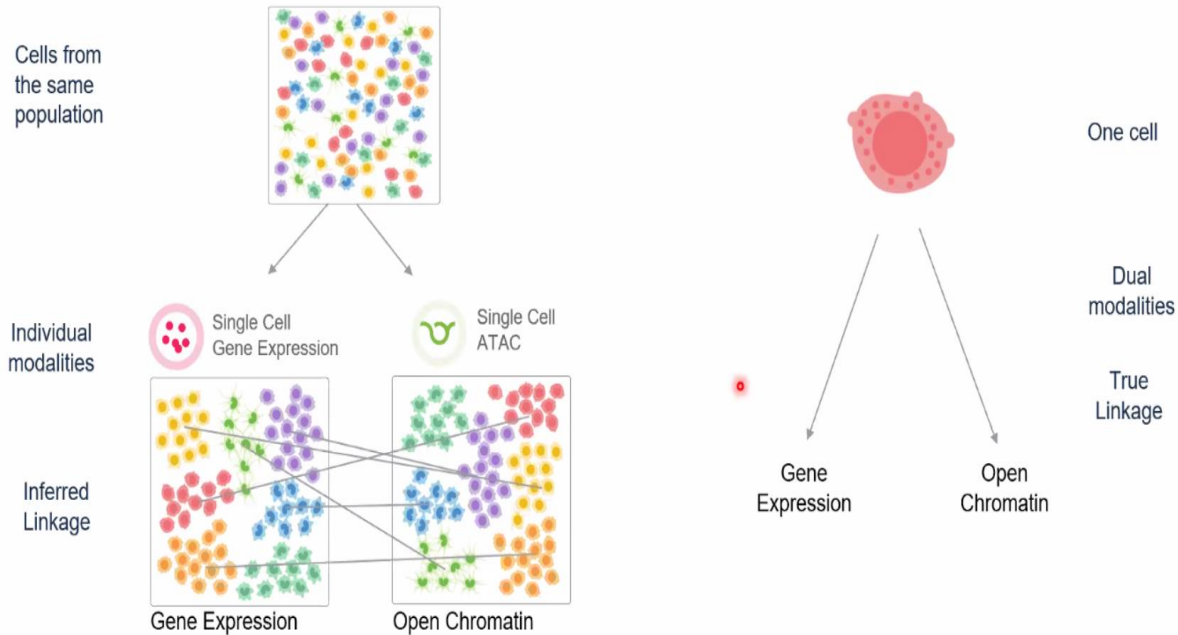


Source: 10x Genomics

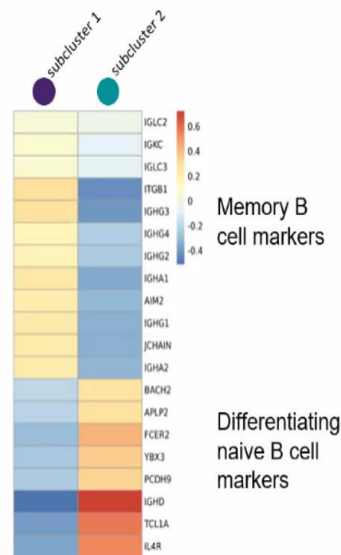
10X MULTIOME (RNA+ATAC)

Profiling Different Modalities To Gain Deeper Insights

Dive Deep Where It Matters



Top differentially expressed markers

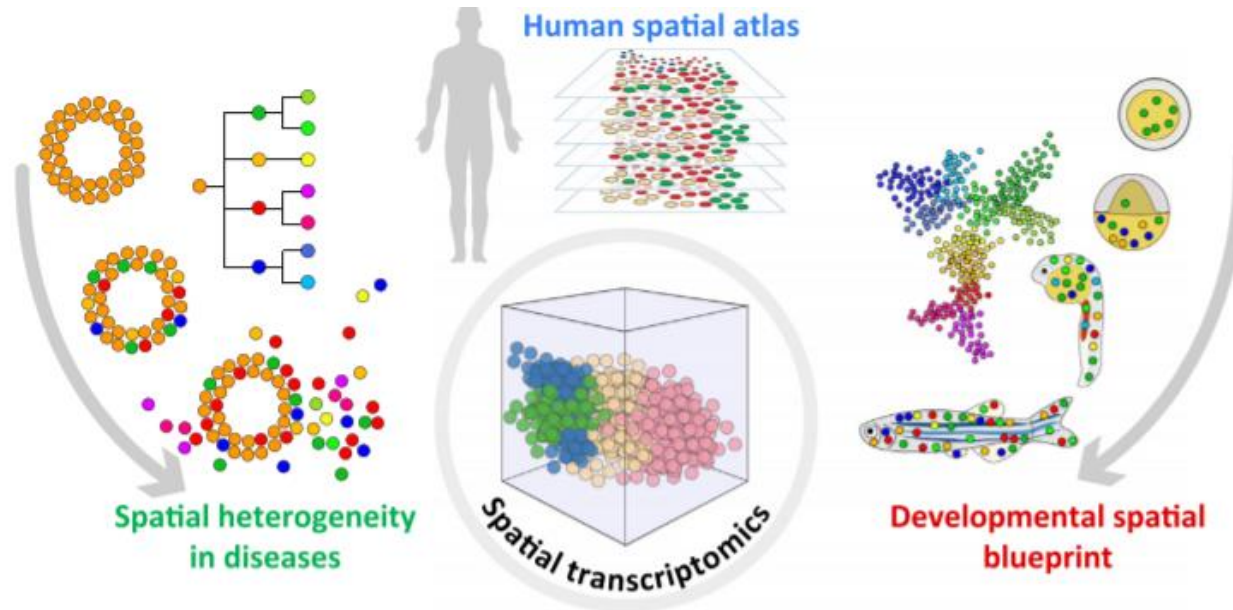


subcluster 1 → Memory B cells

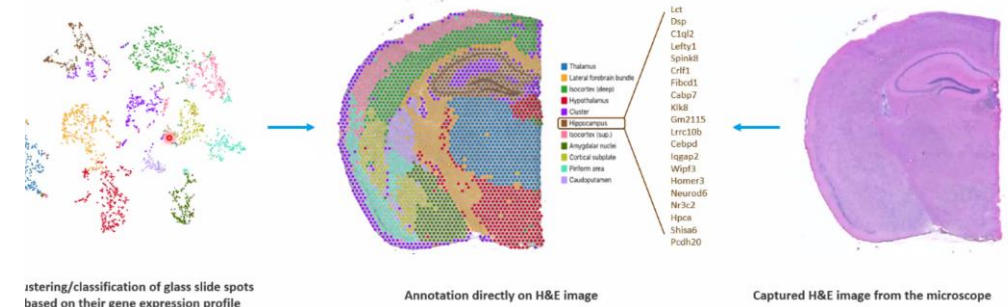
subcluster 2 → Differentiating Naive B cells

Source: 10x Genomics

SPATIAL TRANSCRIPTOMICS

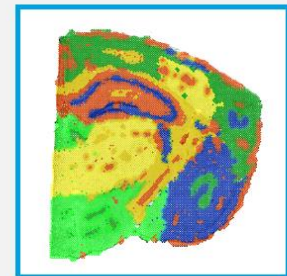


55ul spots -> 1 and 10 cells captured per spot



Source: 10x Genomics

2022



Visium HD
Resolution 1,500x

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.

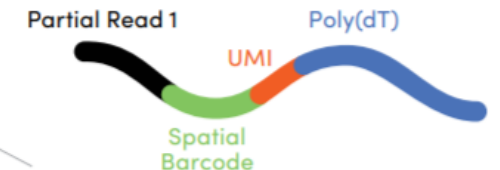
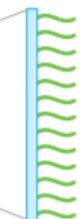
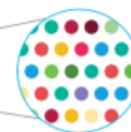
Source: Liao et al. Trends in Biotechnology. (2020)

Trends in Biotechnology

Visium Spatial
Gene Expression Slide

Capture Area with
5000 Barcoded Spots

Visium Gene Expression
Barcoded Spots



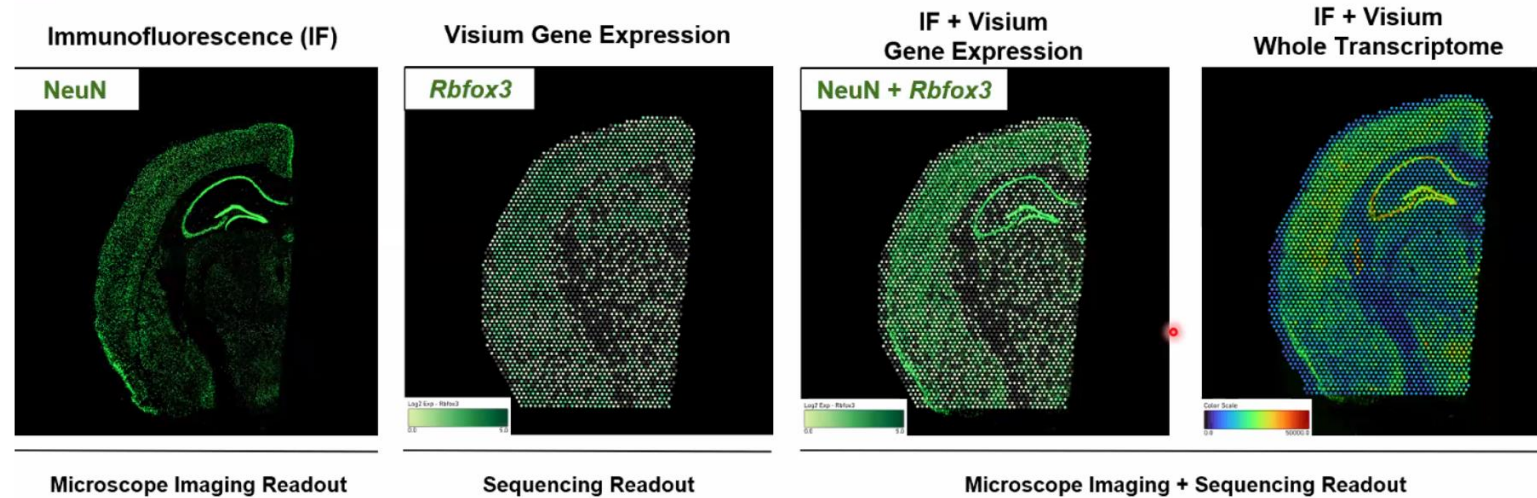
Source: 10x Genomics



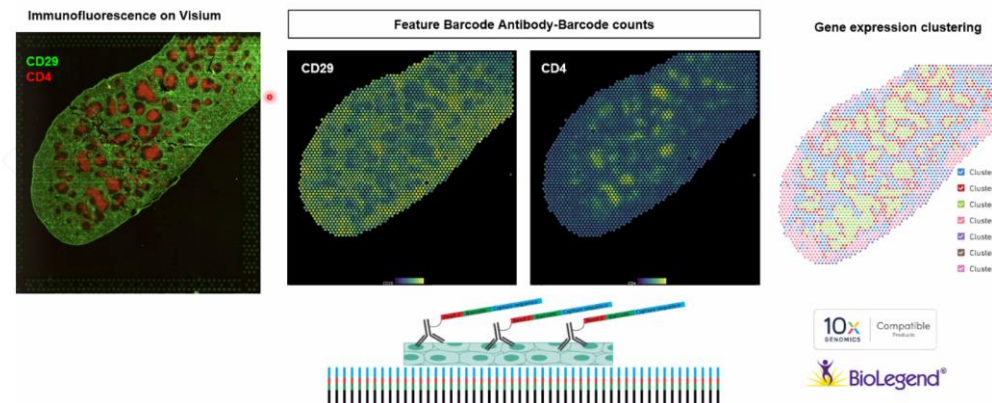
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SPATIAL TRANSCRIPTOMICS

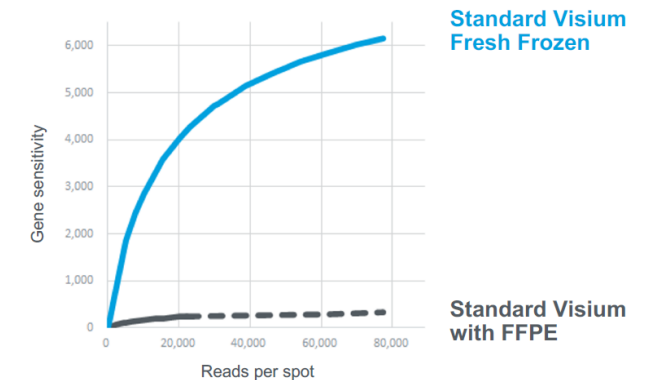
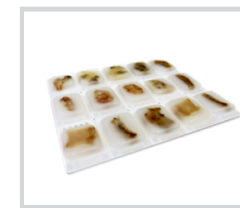


Feature Barcode Correlates with Immunofluorescence



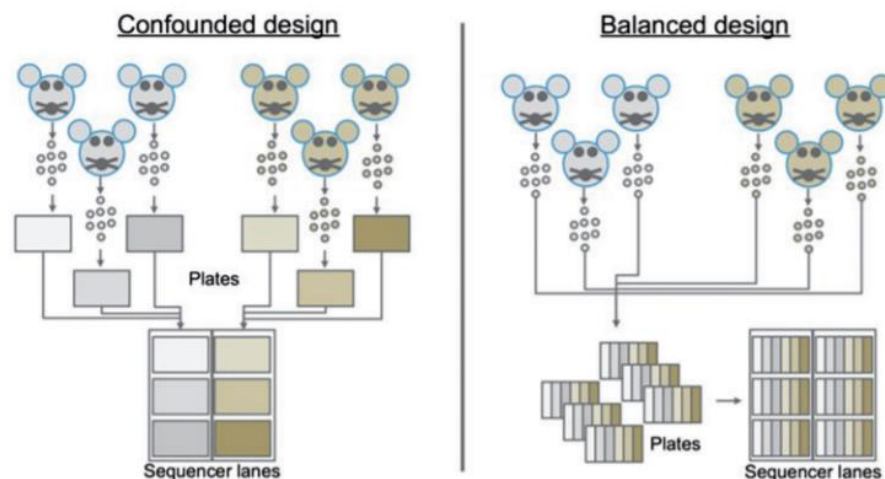
The Challenge of FFPE Samples

FFPE tissue preservation degrades RNA



Source: 10x Genomics

EXPERIMENTAL DESIGN



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

I. Tissue Procurement



Source:

- Primary human
- Model organism
- Cell culture

Key considerations:

- Biological variation
- Sampling/handling variation
- Duration of sourcing

Study design:

- Biological replicates
- Technical replicates
- Cell number calculation
- Workflow optimization

II. Tissue Dissociation



Method:

- Mechanical mincing
- Enzymatic digestion
- Automated blending
- Microfluidics devices

Key considerations:

- Experimental consistency
- Shortest duration
- Highest cell/nucleus quality
- Representation of all cell types

Quality control:

- FACS analysis
- qPCR for marker genes
- Imaging of cell integrity
- RNA quality (RIN)

III. Cell Enrichment (optional)



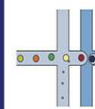
Method:

- Differential centrifugation, sedimentation, filtration
- Antibody labeling for positive/negative selection
- Flow cytometry or bead-based enrichment
- Dead cell removal

Key considerations:

- Additional handling
- Longer duration
- Loss of RNA quality
- Transcriptome changes

IV. Single Cell RNAseq Platform



Method:

- Droplet-based
- Tube-based after FACS
- Microwell-based
- Microfluidics-enabled

Key considerations:

- Cell throughput and handling time
- Gene coverage and cell type detection
- Whole transcript versus 3' end counting
- Imaging capability for doublet detection

V. Library Sequencing



Method:

- Illumina NGS
- Compatible with cDNA library

Sequencing depth considerations:

- 3' end counting: low depth ~50K RPC
- Whole transcript: high depth ~1M RPC
- Alternative splicing: ~20-30M RPC
- Iterative optimization for biological system

VI. Computational Analysis



Key considerations:

- Separation of batch and condition
- Technical vs. biological variation

Sample Batch correction approaches:

- Cell Hashing
- Demuxlet
- Canonical correlation analysis (CCA)
- MAST

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 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:

- 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).
- Haque et al. A practical guide to single-cell RNA-sequencing for biomedical research and clinical applications. *Genome Med* 9, 75 (2017).
- Hwang et al. Single-cell RNA sequencing technologies and bioinformatics pipelines. *Exp Mol Med* 50(8):96. (2018).
- Baran-Gale et al. Experimental design for single-cell RNA sequencing. *Briefings in Functional Genomics*, Volume 17, Issue 4, Pages 233–239 (2018).
- Kashima, Y., Sakamoto, Y., Kaneko, K. et al. Single-cell sequencing techniques from individual to multiomics analyses. *Exp Mol Med* 52, 1419–1427 (2020).
- Liao et al. Uncovering an Organ’s Molecular Architecture at Single-Cell Resolution by Spatially Resolved Transcriptomics. *Trends in Biotechnology*. (2020).
- Laks et al. Clonal Decomposition and DNA Replication States Defined by Scaled Single-Cell Genome Sequencing. *Cell*. 179(5):1207-1221.e22. (2019).
- Macosko et al. Single-cell RNA sequencing at isoform resolution. *Nat Biotechnol* 38, 697–698 (2020).
- McGinnis et al. MULTI-seq: sample multiplexing for single-cell RNA sequencing using lipid-tagged indices. *Nat Methods* 16, 619–626 (2019).
- Nguyen et al. Experimental Considerations for Single-Cell RNA Sequencing Approaches. *Front Cell Dev Biol* 6:108. (2018).
- Pijuan-Sala et al. A single-cell molecular map of mouse gastrulation and early organogenesis. *Nature* 566, 490–495 (2019).
- Plasschaert et al. A single-cell atlas of the airway epithelium reveals the CFTR-rich pulmonary ionocyte. *Nature* 560, 377–381 (2018).
- Richter, M.L et al. Single-nucleus RNA-seq2 reveals functional crosstalk between liver zonation and ploidy. *Nat Commun* 12, 4264 (2021).
- See et al. A Single-Cell Sequencing Guide for Immunologists. *Frontiers in immunology*, 9, 2425. (2018).
- 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).



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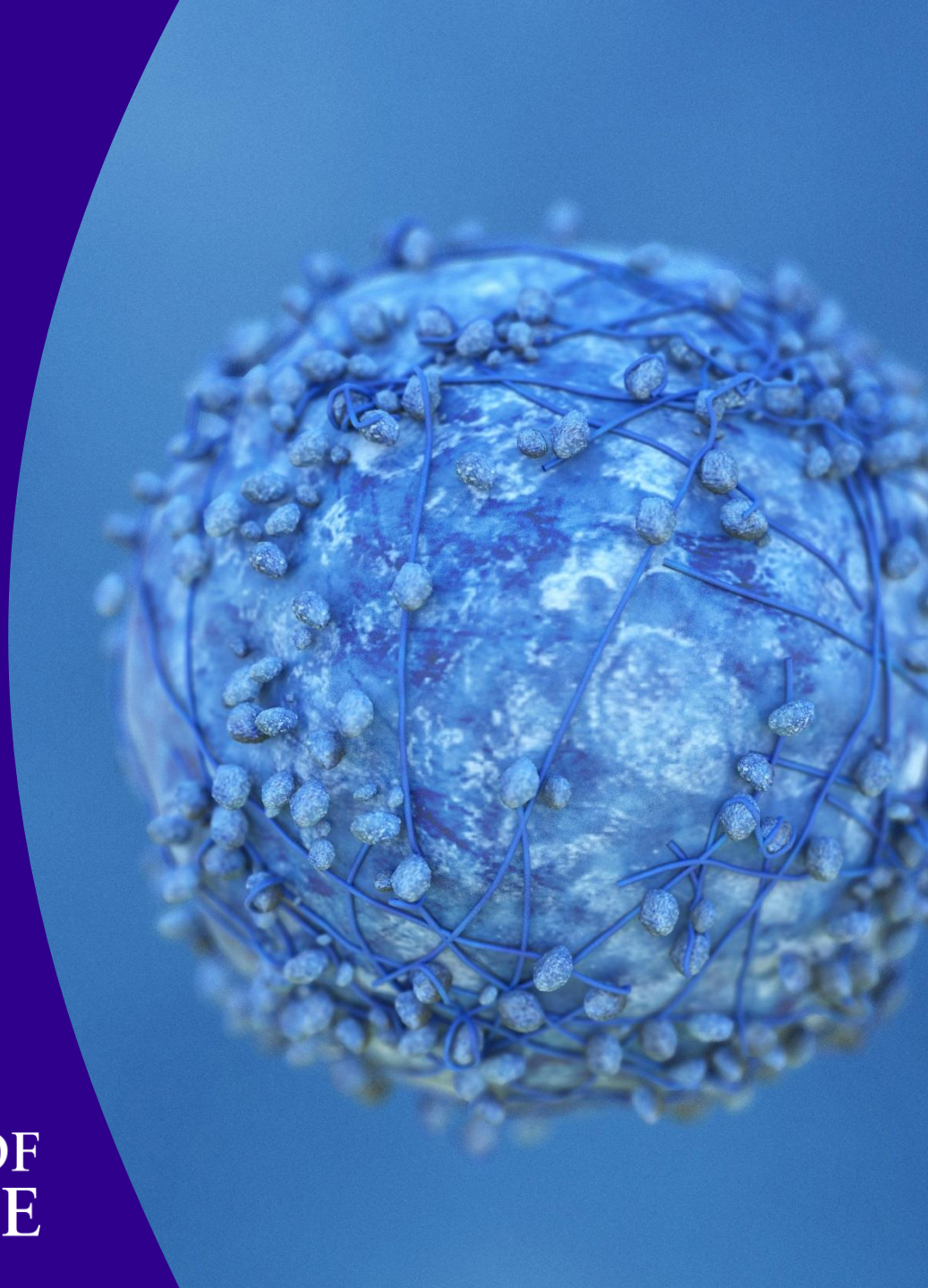
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USEFUL RESOURCES:

- 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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