Analysis of single-cell RNA-seq data (I)

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ENAR 2021 short course
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Course outline

- **8-9:15: Intro and data preprocessing.**
- 9:15-9:45: Lab: preprocessing and visualization.
- 10-11:15: Normalization, batch effect, imputation, DE, simulator.
- 11:15-12: Lab: Normalization, batch effect, imputation, DE, simulator
- 12-1: lunch break
- 1-2: Clustering and pseudotime construction
- 2-2:30: Lab: Clustering and pseudotime construction
- 2:45–3:30: Supervised cell typing & related single cell data sources
- 3:30-4: Lab: supervised cell typing.
- 4:15-5: scRNA-seq in cancer

Other useful resources

- https://github.com/theislab/single-cell-tutorial/
- https://scrnaseq-course.cog.sanger.ac.uk/website/index.html
- https://broadinstitute.github.io/2019_scWorkshop/

Outline for this session

- **Background**
  - Scientific motivation
  - Technology
  - UMI
- **Pre-processing:** Alignment, QC, GE quantification
- **Data visualization**: tSNE, UMAP

Background

- Most of the biological experiments are performed on “bulk” samples, which contains a large number of cells (millions).
- The “bulk” data measure the average signals (gene expression, TF binding, methylation, etc.) of many cells.
- The bulk measurement ignores the inter-cellular heterogeneities:
  - Different cell types.
  - Variation among the same cell type.

Single cell biology

- The study of individual cells.
- The cells are isolated from multi-cellular organism.
- Experiment is performed for each cell individually.
- Provides more detailed, higher resolution information.
- High-throughput experiments on single cell is possible.

Single cell sequencing

- Different types of sequencing at the single-cell level:
  - DNA-seq
  - ATAC-seq, ChIP-seq
  - BS-seq
  - RNA-seq
  - Multi-omics
- Very active research field in the past few years.

Basic experimental procedure

- Isolation of single cell. Techniques include
  - Laser-capture microdissection (LCM)
  - Fluorescence-activated cell sorting (FACS)
  - Microfluidics
- Open the cell and obtain DNA/mRNA/etc.
- PCR amplification to get enough materials.
- Perform sequencing.

Single cell RNA-seq (scRNA-seq)

- The most active in the single cell field.
- Scientific goals:
  - Composition of different cell types in complex tissues.
  - New/rare cell type discovery.
  - Gene expression, alternative splicing, allele specific expression at the level of individual cells.
  - Transcriptional dynamics (pseudotime construction).
  - Above can be investigated and compared spatially, temporally, or under different biological condition.

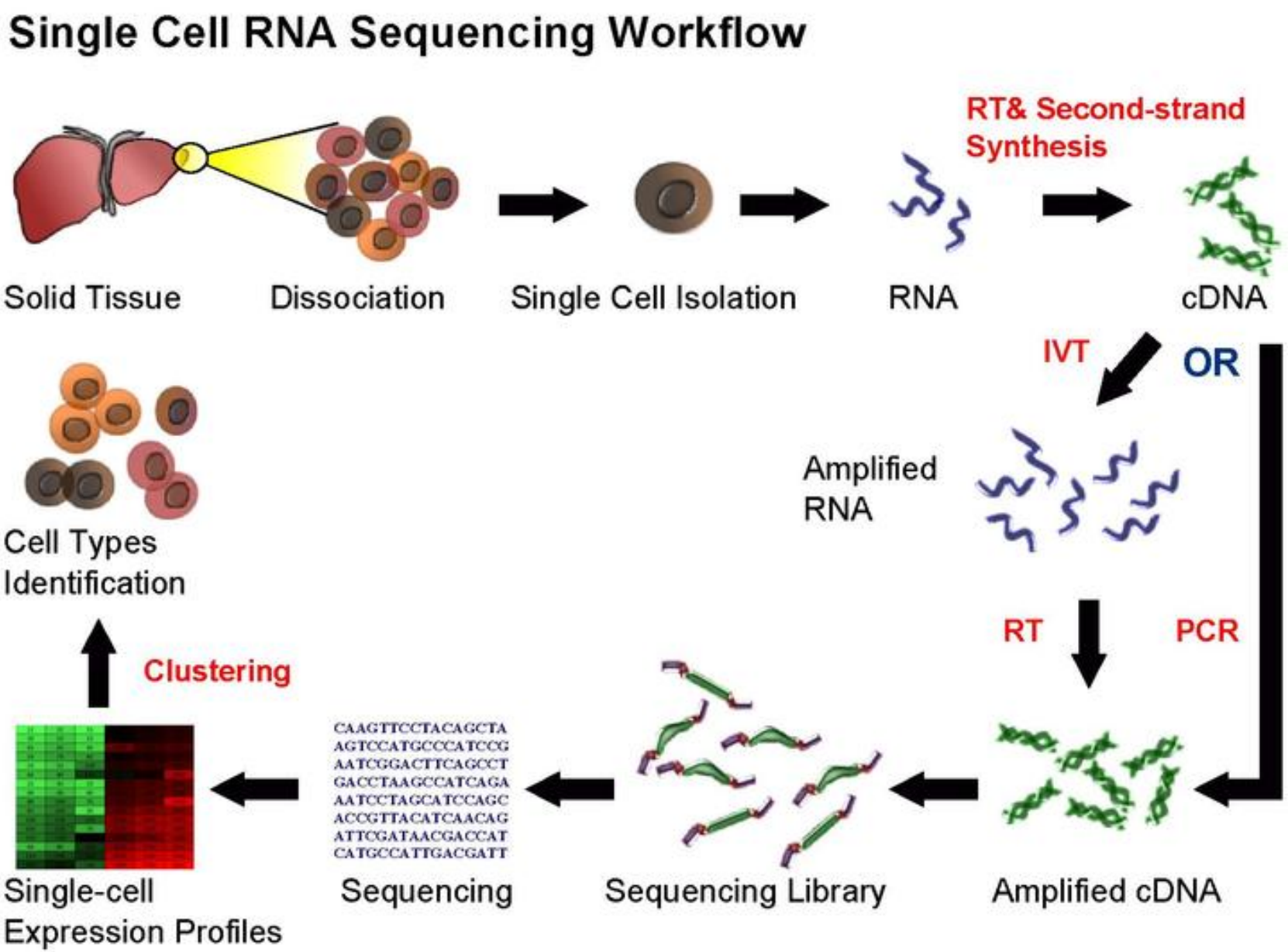


Figure source: Wikipedia

Technologies by cell capturing method

- **Plate-based methods**: Smart-Seq/Smart-Seq2, CEL-seq:
  - Sort cells into the wells on a multi-well plate.
  - Lower throughput (in terms of number of cells).
  - High sequencing depth
  - Can be combined with FACS for cell sorting.
  - Better at detecting low expression genes
  - Good for isoform analysis, allele specific expression

Microwell plates

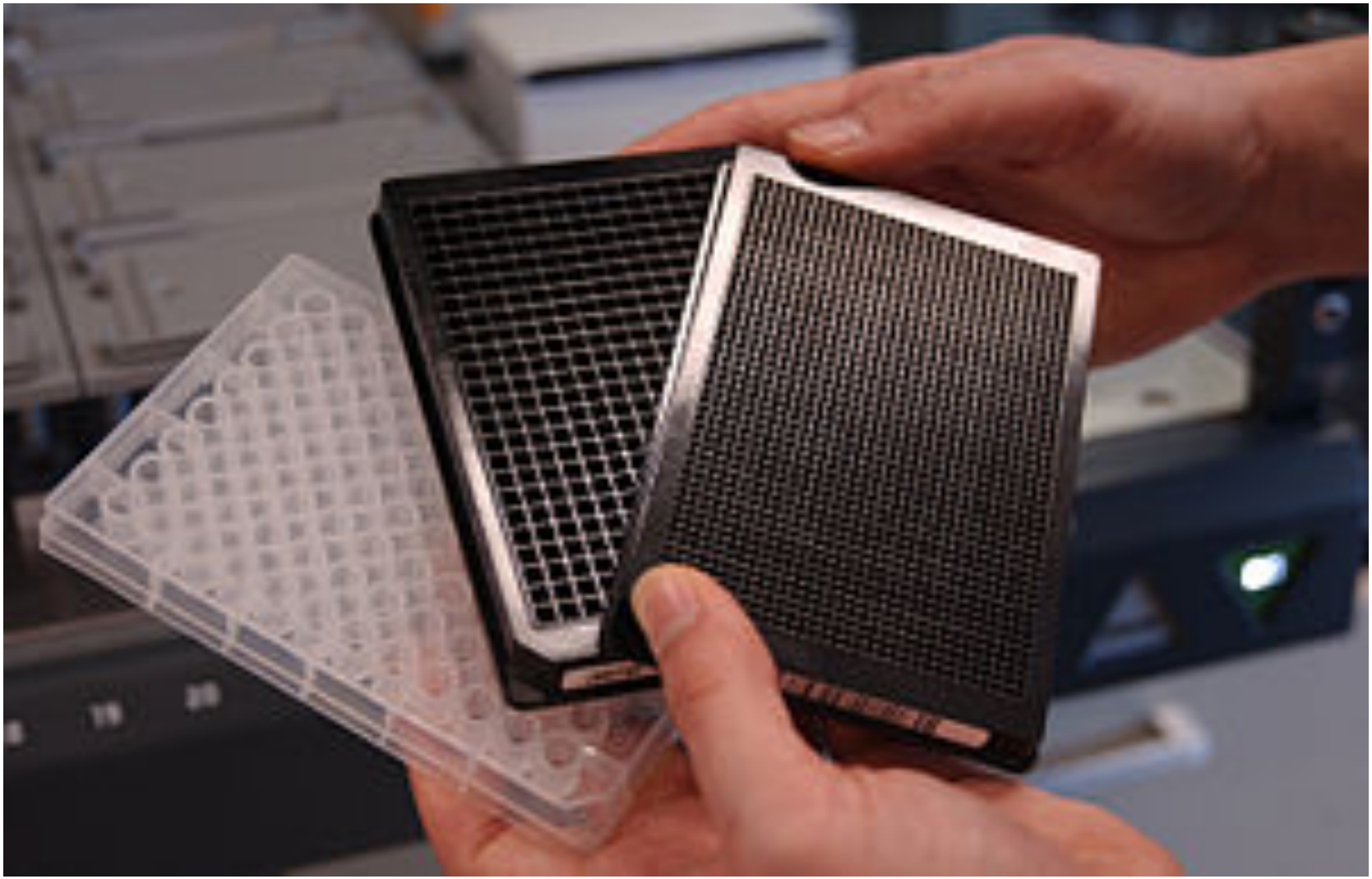
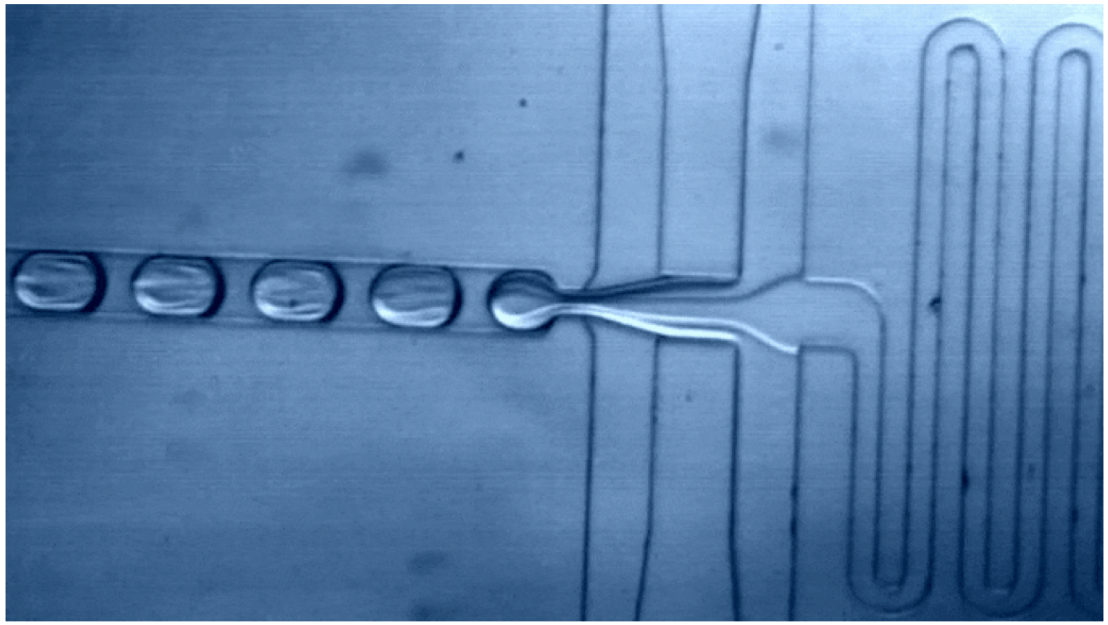
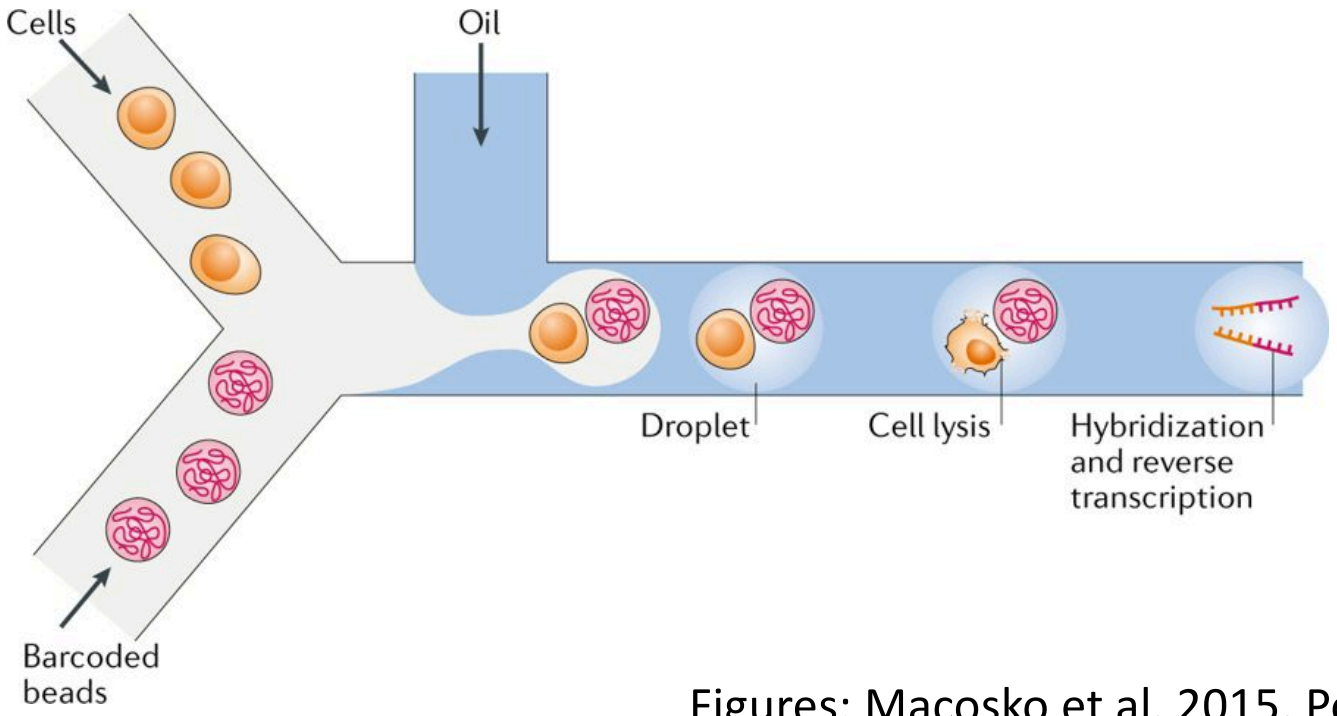


Figure source: wikipedia

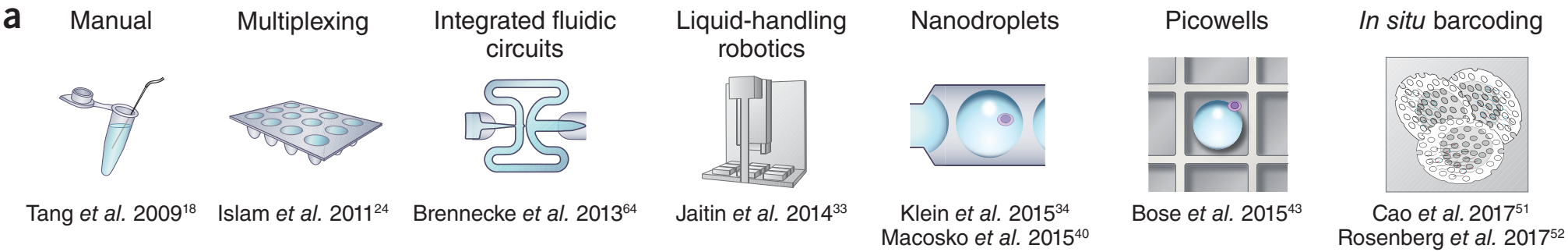
- **Droplet-based methods:** Drop-seq, inDrop, 10x genomics
  - Put each cell in a nanoliter droplet with a bead.
  - Each droplet is a reactor for PCR.
  - Each bead has a unique barcode, so all beads can be pooled and sequenced together.
  - Much higher throughput in terms of number of cells.
  - Lower sequencing depth.
  - Good for identifying cell subpopulations.

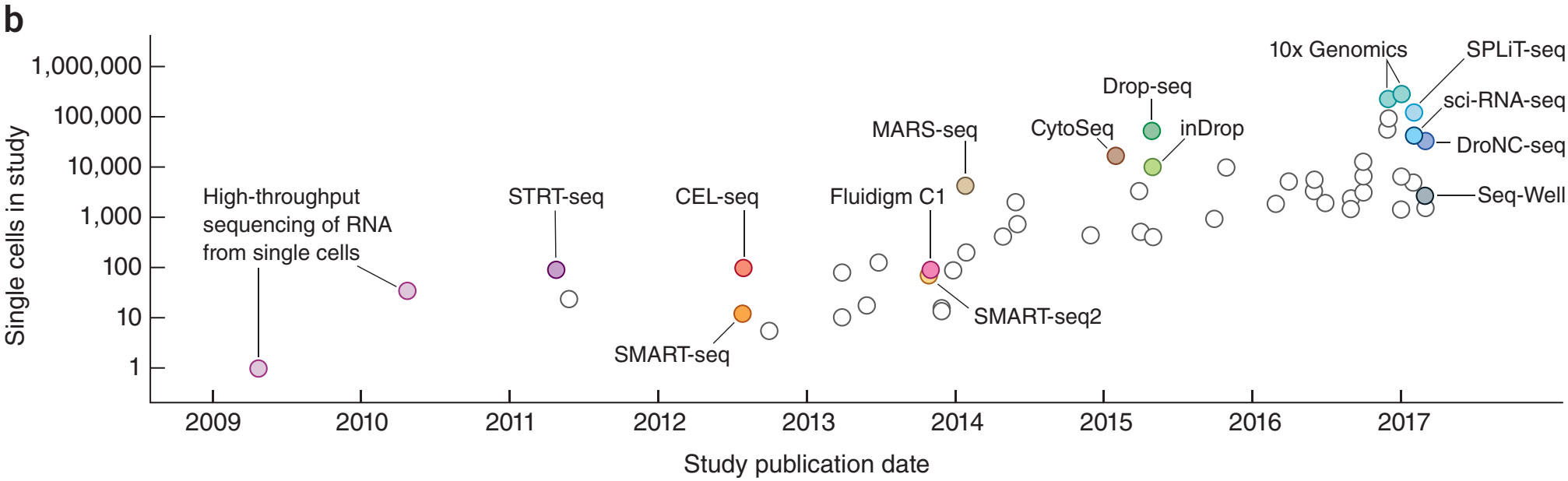




Figures: Macosko et al. 2015, Potter SS. 2018

Technologies over the years





Svensson et al. (2017) Nature Protocols

Unique molecule identifier (UMI)

NM 2014

Quantitative single-cell RNA-seq with unique molecular identifiers

Saiful Islam[1], Amit Zeisel[1], Simon Joost[2], Gioele La Manno[1], Pawel Zajac[1], Maria Kasper[2], Peter Lönnerberg[1] & Sten Linnarsson[1]

UMI

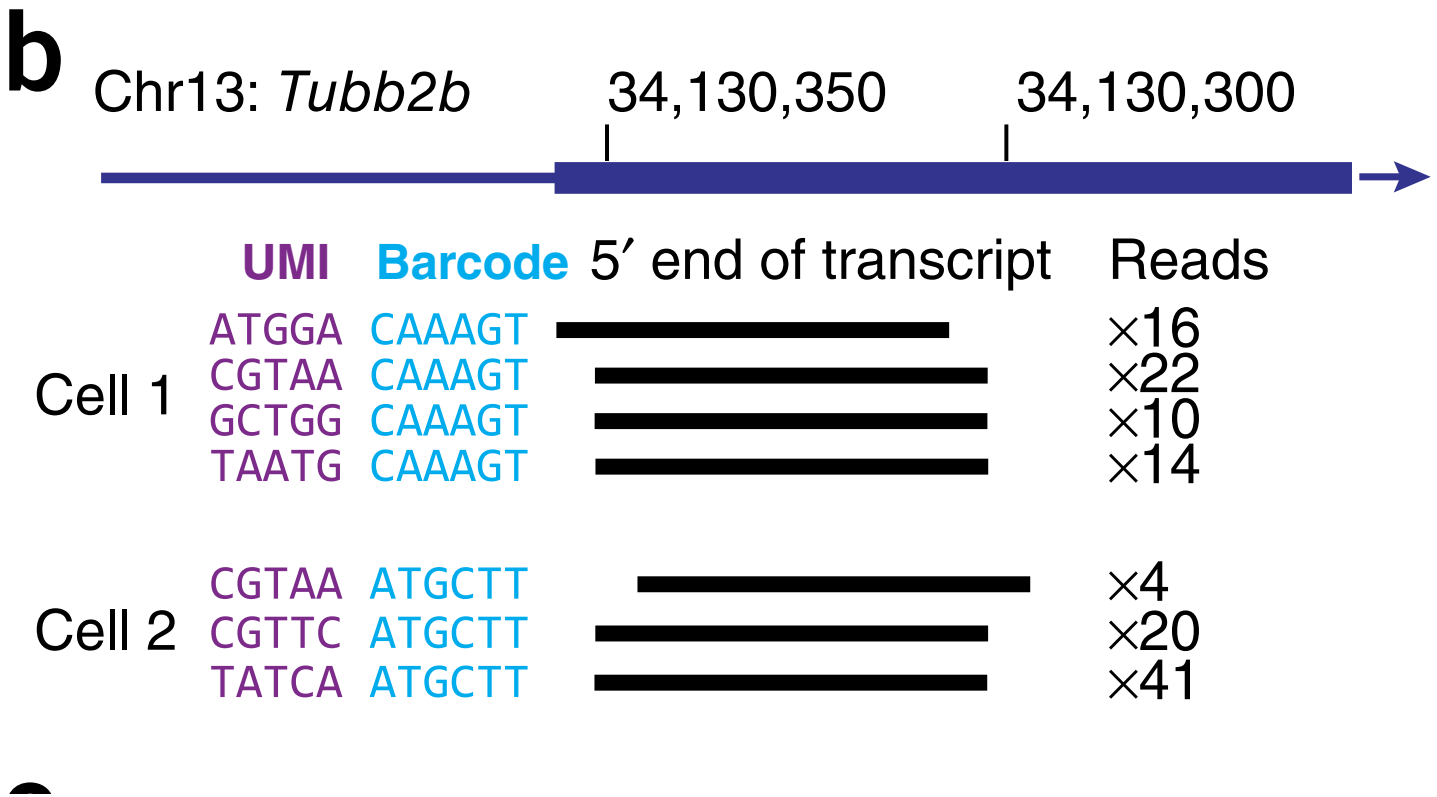
- PCR introduces nonlinear amplification bias
  - Factors influencing PCR: sequence content, chromatin structure, etc.
- UMIs are short sequence tag added to each unique mRNA molecular before PCR, for reducing PCR bias.
- Number of possible UMIs = 4^L, where L is the length of the UMI

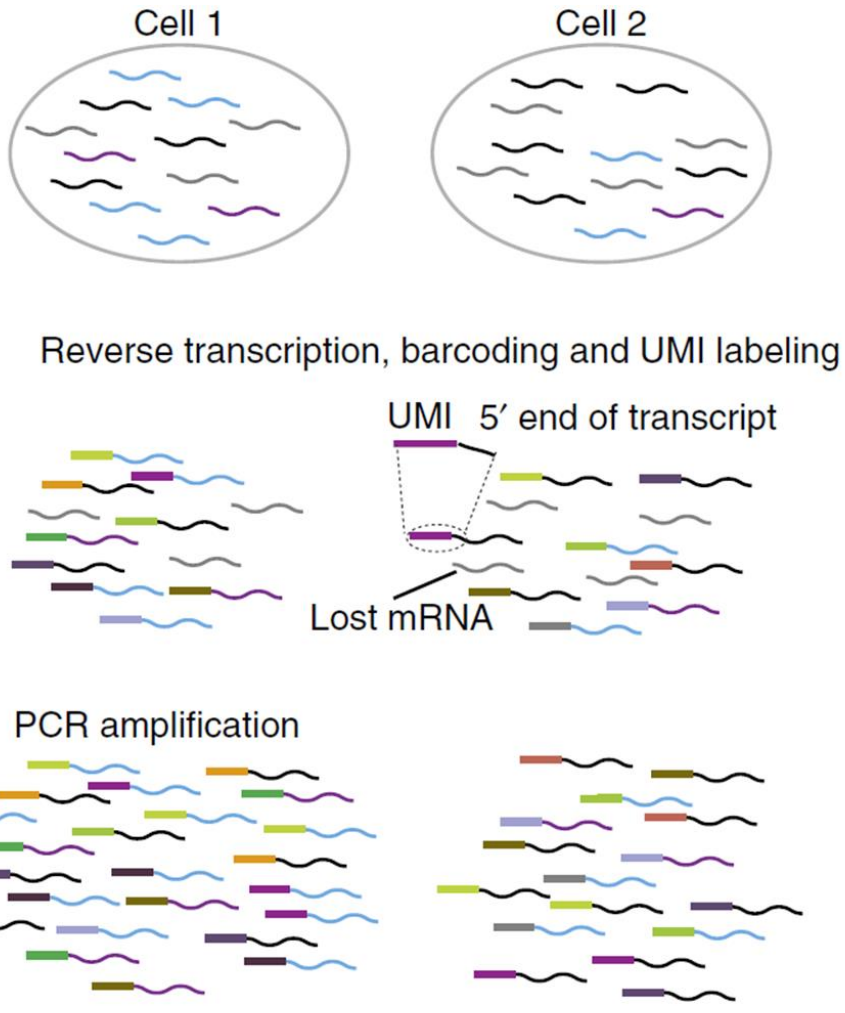
Multiplexing

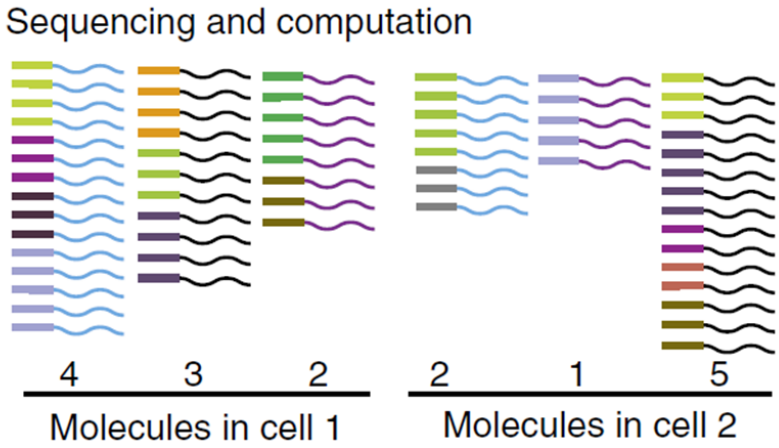
- Technology to pool many cells together for each sequencing lane.
- Each cell is uniquely marked by a **barcode** (short sequence tag).
- A combination of barcode and UMI can quantify unique transcripts in each cell.

UMI + barcode

- Assumption: number of identical mRNA is small (say, <100) for most genes.
- Use 5-bp UMI (can mark 1024 molecules)
  - When a transcript has, say, 20 mRNA molecules, the probability of two molecule having the same UMI is small.
- Use 6-bp barcode to identify cells (up to 4096 cells).
- Use molecule counts instead of read counts as gene expression measurements.

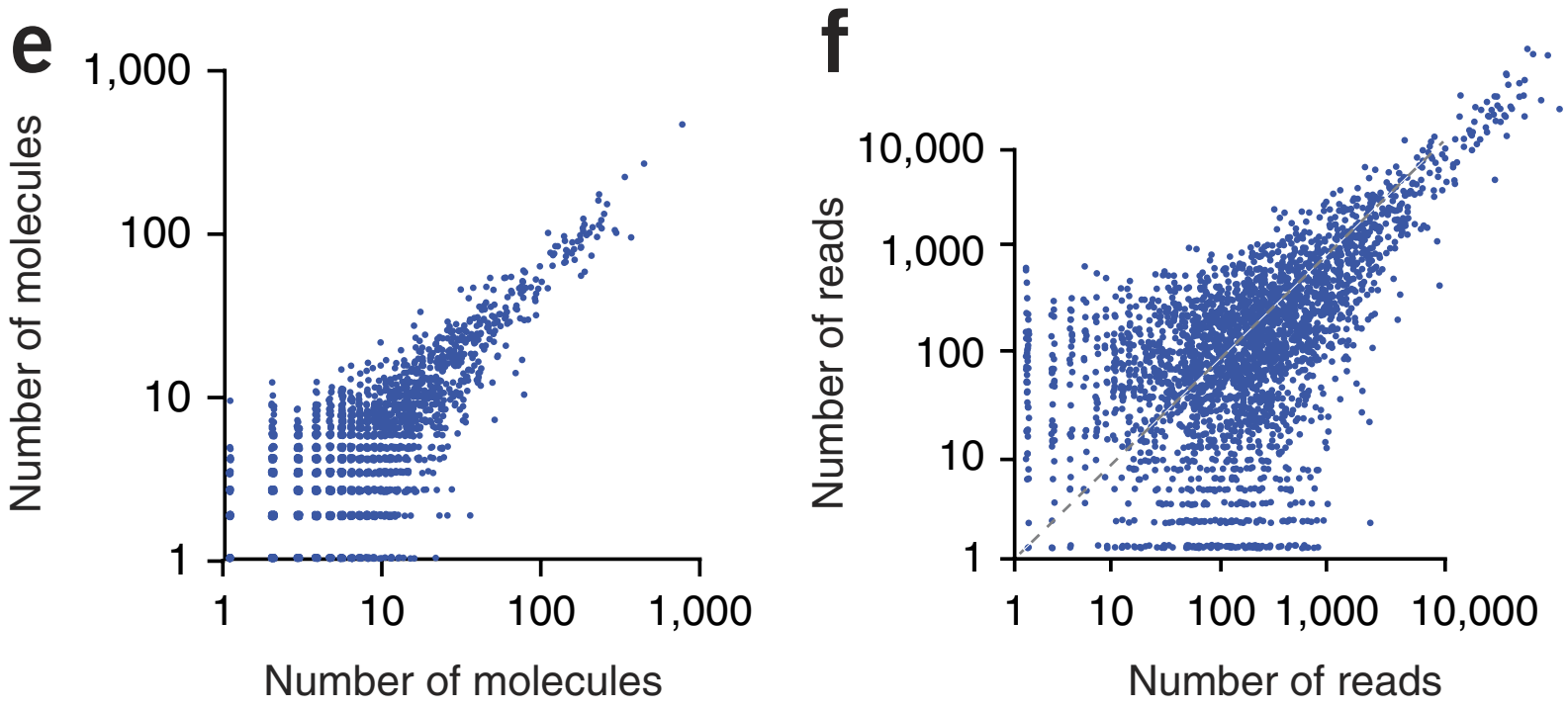

b
Chr13: Tubb2b
34,130,350
34,130,300
UMI
Barcode
5′ end of transcript
Reads
Cell 1
ATGGA CAAAGT
CGTAA CAAAGT
GCTGG CAAAGT
TAATG CAAAGT
×16
×22
×10
×14
Cell 2
CGTAA ATGCTT
CGTTC ATGCTT
TATCA ATGCTT
×4
×20
×41





Saiful Islam ••• Sten Linnarsson

UMI provides better measurements and reproducibility



Single nucleus RNA-seq (snRNA-seq)

- Profile gene expressions in nucleus, instead of the whole cell
  - Transcripts can be in cytoplasm and nucleus
- Useful when the cells are difficult to isolate
  - Frozen tissues
  - Highly connected cells such as neurons
- Analysis methods are similar.

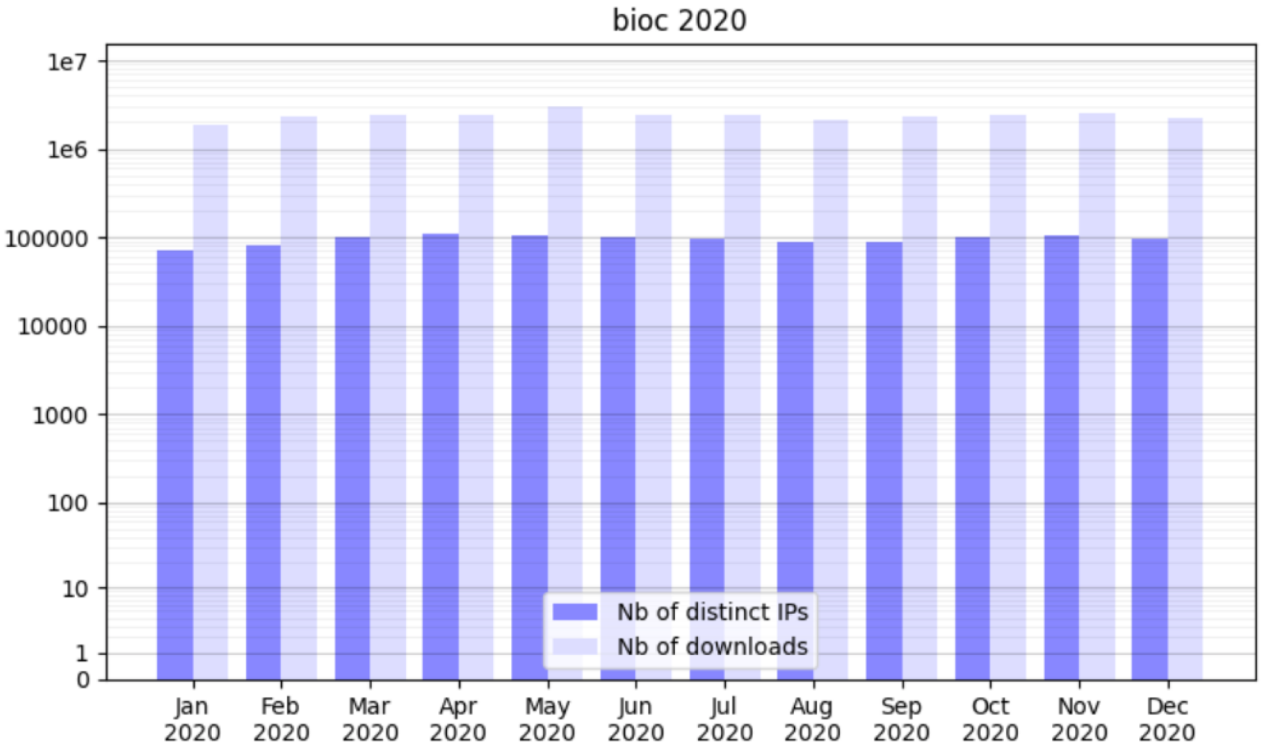
Multi-omics single cell assays

- CITE-seq (**C**ellular **I**ndexing of **T**ranscriptomes and **E**pitopes by **Seq**uencing)
  - Jointly profile transcriptome and proteome.
- scNMT-seq (single-cell **N**ucleosome, **M**ethylation and **T**ranscription sequencing)
  - Jointly profile chromatin accessibility, DNA methylation, and transcription

Brief introduction to Bioconductor

- A collection of R packages
- The *de facto* language for genomic data analysis.

2020



Month	Nb of distinct IPs	Nb of downloads
Jan/2020	71347	1863031
Feb/2020	82959	2327549
Mar/2020	100156	2437796
Apr/2020	109245	2445530
May/2020	107201	3059277
Jun/2020	99529	2406886
Jul/2020	96776	2409421
Aug/2020	86995	2119491
Sep/2020	90269	2280596
Oct/2020	100693	2427302
Nov/2020	103036	2572492
Dec/2020	95193	2225497
2020	**816065**	**28574868**

bioc_2020_stats.tab

Functionalities

- "*Bioconductor provides tools for the analysis and comprehension of high-throughput genomic data. Bioconductor uses the R statistical programming language, and is open source and open development.*"
- Provides close to 2000 packages for:
  - microarrays.
  - second generation sequencing.
  - other high-throughput assays.
  - annotation.
- Most of the packages are contributed.

Bioconductor installation

- Use **`BiocManager::install()`**.
- Basic installation: installing default (core) packages:

```
if (!requireNamespace("BiocManager"))
    install.packages("BiocManager")
BiocManager::install()
```

- Installing a specific package:

```
BiocManager::install("limma")
```

Data processing

- Preprocessing
  - QC
  - Alignment
  - Expression quantification
- Normalization
- Batch effect correction
- Imputation

scRNA-seq data preprocessing

- QC:
  - FastQC is a popular tool for checking a single sample.
  - MultiQC: create a single report with interactive plots for multiple QC reports.
- Read trimming:
  - cutadapt (with a wrapper Trim Galore!)
- Bioconductor package “scater” provides useful and easy-to-use functions for QC and data visualization.

Alignment and quantification

- Alignment
  - Bulk RNA-seq alignment software (Tophat, STAR, HISAT, etc.) can be used.
  - Some commercial software, such as CellRanger for 10x genomics data.
- Quantification (to obtain count matrix from aligned reads)
  - Most alignment software provide such functionality.

Some data characteristics

- Data is very sparse (many zeros), especially for Drop-seq data.
- Number of transcripts detected is much lower compared to bulk RNA-seq under the same sequencing depth.

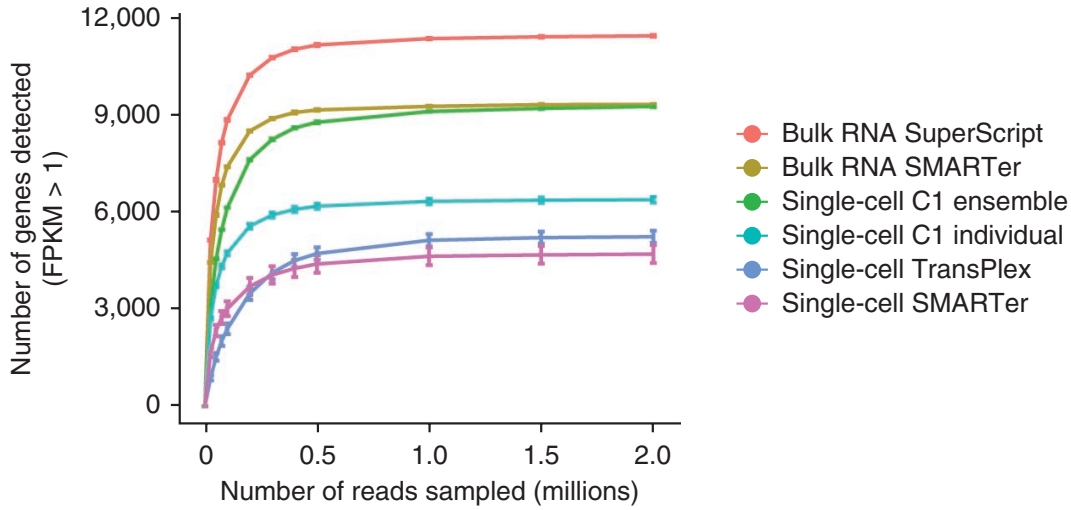
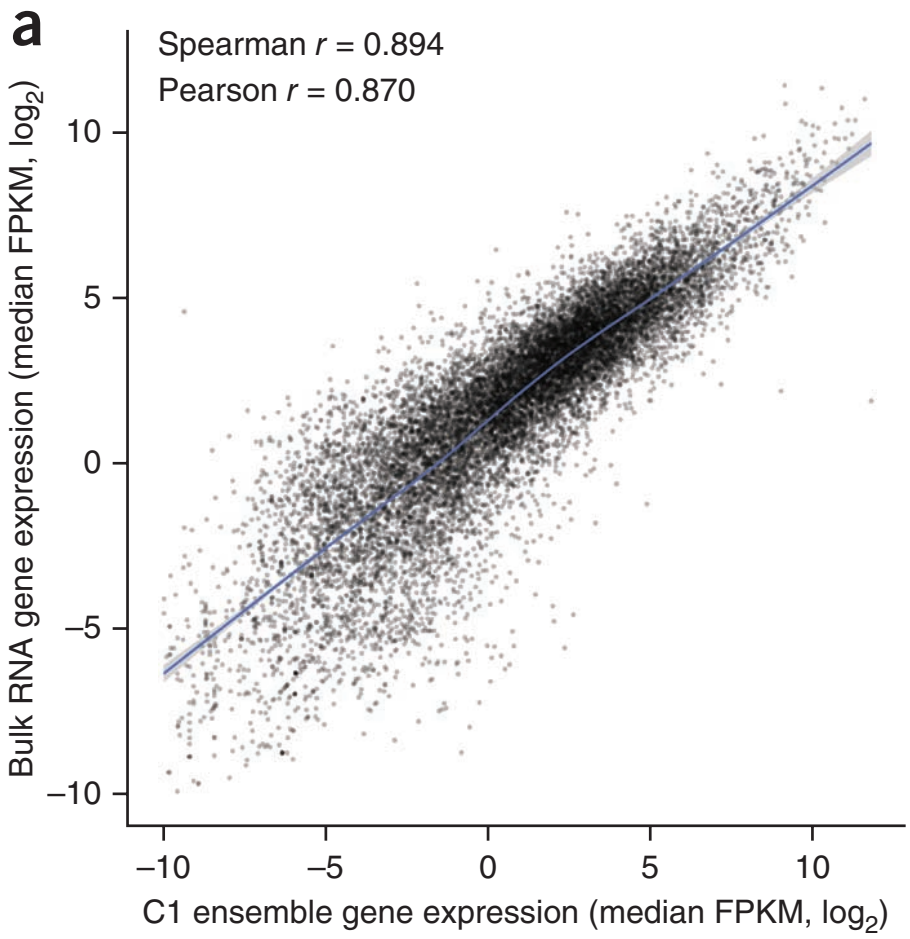


Figure 5 | Saturation curves for the different sample preparation methods. Each point on the curve was generated by randomly selecting a number of raw reads from each sample library and then using the same alignment pipeline to call genes with mean FPKM >1. Each point represents four replicate subsamplings. Error bars, standard error.

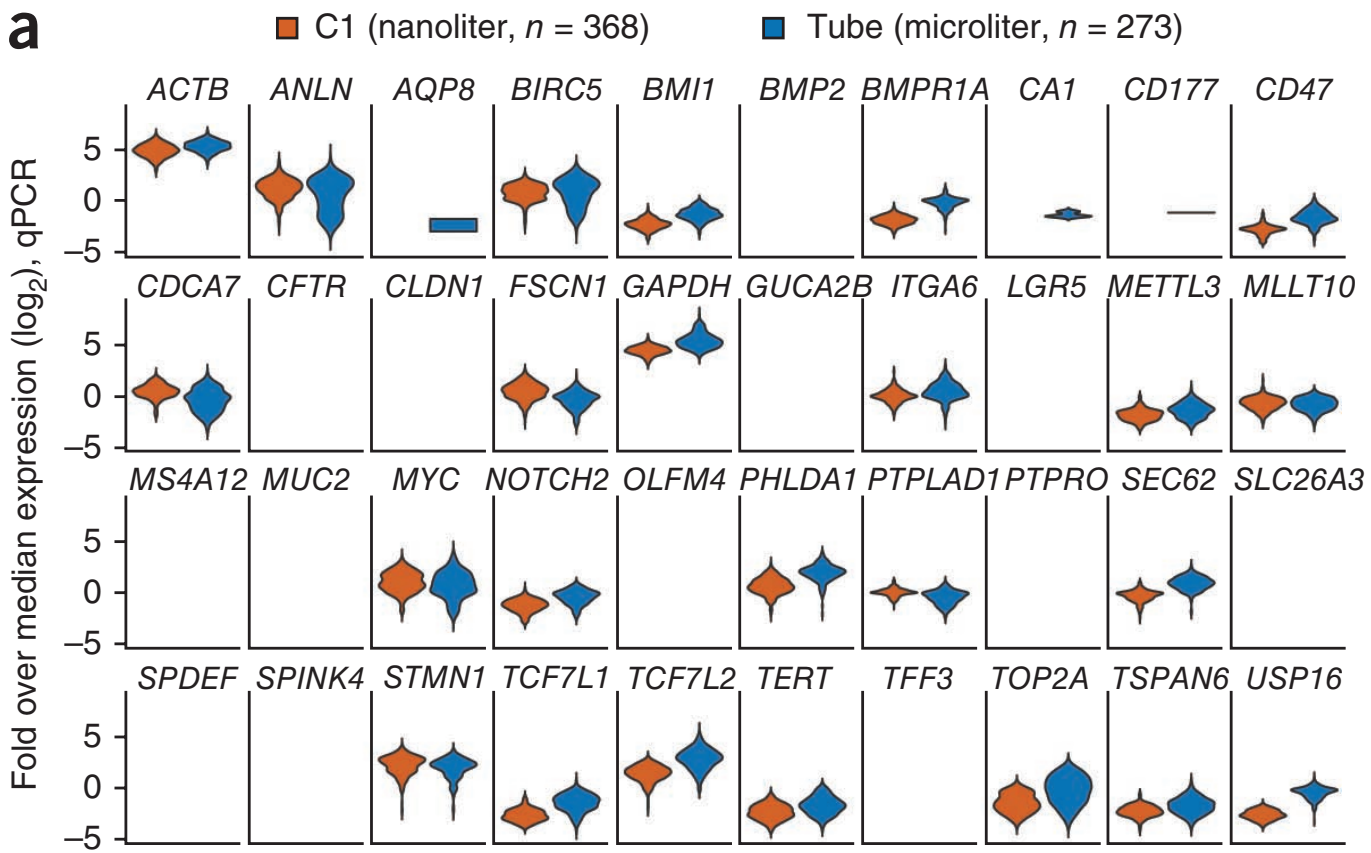
Wu et al. 2013 Nature Method

- Bulk and aggregated single cell expressions have good correlation.



Wu et al. 2013 Nature Method

- Expression levels for a gene cross cells sometimes show bimodal distribution.



Wu et al. 2013 Nature Method

scRNA-seq data after processing

- A matrix of read counts: rows are genes and columns are cells

	AACGGTACCTTCGC_1	AGAGAAACGCCCTT_1	AGGCAGGACGAATC_1
ENSG00000228463	0	0	0
ENSG00000230021	0	0	0
ENSG00000237491	0	0	0
ENSG00000177757	0	0	0
ENSG00000225880	0	0	0
	ATACCTTGCCGATA_1	ATAGGCTGGCTTCC_1	
ENSG00000228463	0	0	
ENSG00000230021	0	0	
ENSG00000237491	0	0	
ENSG00000177757	0	0	
ENSG00000225880	0	0	

A few useful R packages

- SingleCellExperiment:
  - Bioconductor package. Defines "SingleCellExperiment" class for storing single cell data: expression matrix, gene and cell information, etc.
- Visualization tools:
  - tSNE
  - UMAP

SingleCellExperiment

- Installation:

```
BiocManager::install("SingleCellExperiment")
```

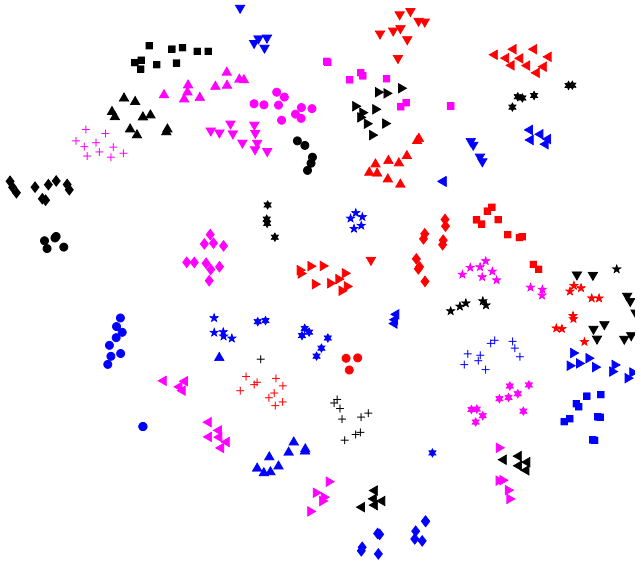
- Create SingleCellExperiment object:

```
sce <- SingleCellExperiment(list(counts=counts),
    colData=DataFrame(label=celllabels),
    rowData=DataFrame(genenames=genenames),
    metadata=list(study="GSE111111")
)
```

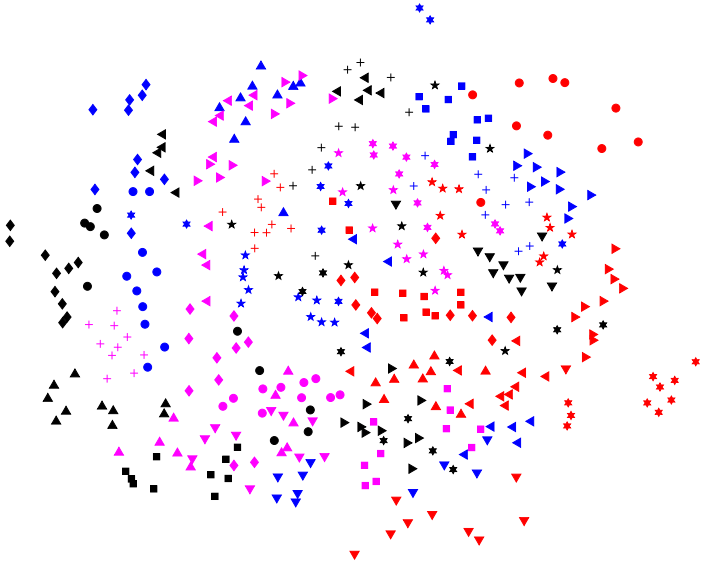
- Functions to access contents of the object: `counts`, `rowData`, `colData`, etc.

t-SNE: a useful visualization tool

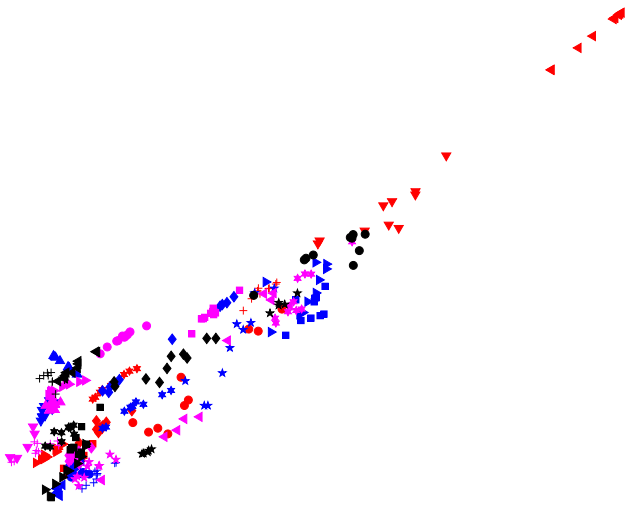
- t-SNE (t-distributed stochastic neighbor embedding): visualize high-dimensional data on 2-/3-D map.
- When project high-dimensional data into lower dimensional space, preserve the distances among data points.
  - Try to make the pairwise distances of points similar in high and low dimension.
- Has “tsne” and “Rtsne” package on CRAN.



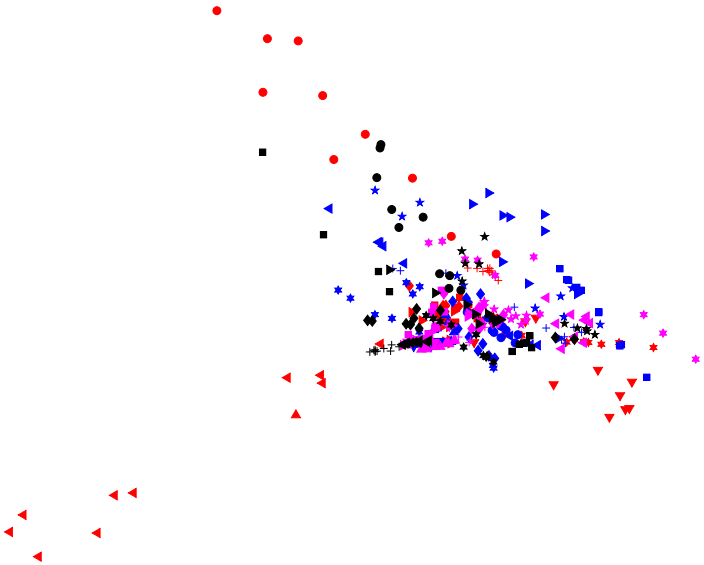
(a) Visualization by t-SNE.



(b) Visualization by Sammon mapping.



(c) Visualization by Isomap.



(d) Visualization by LLE.

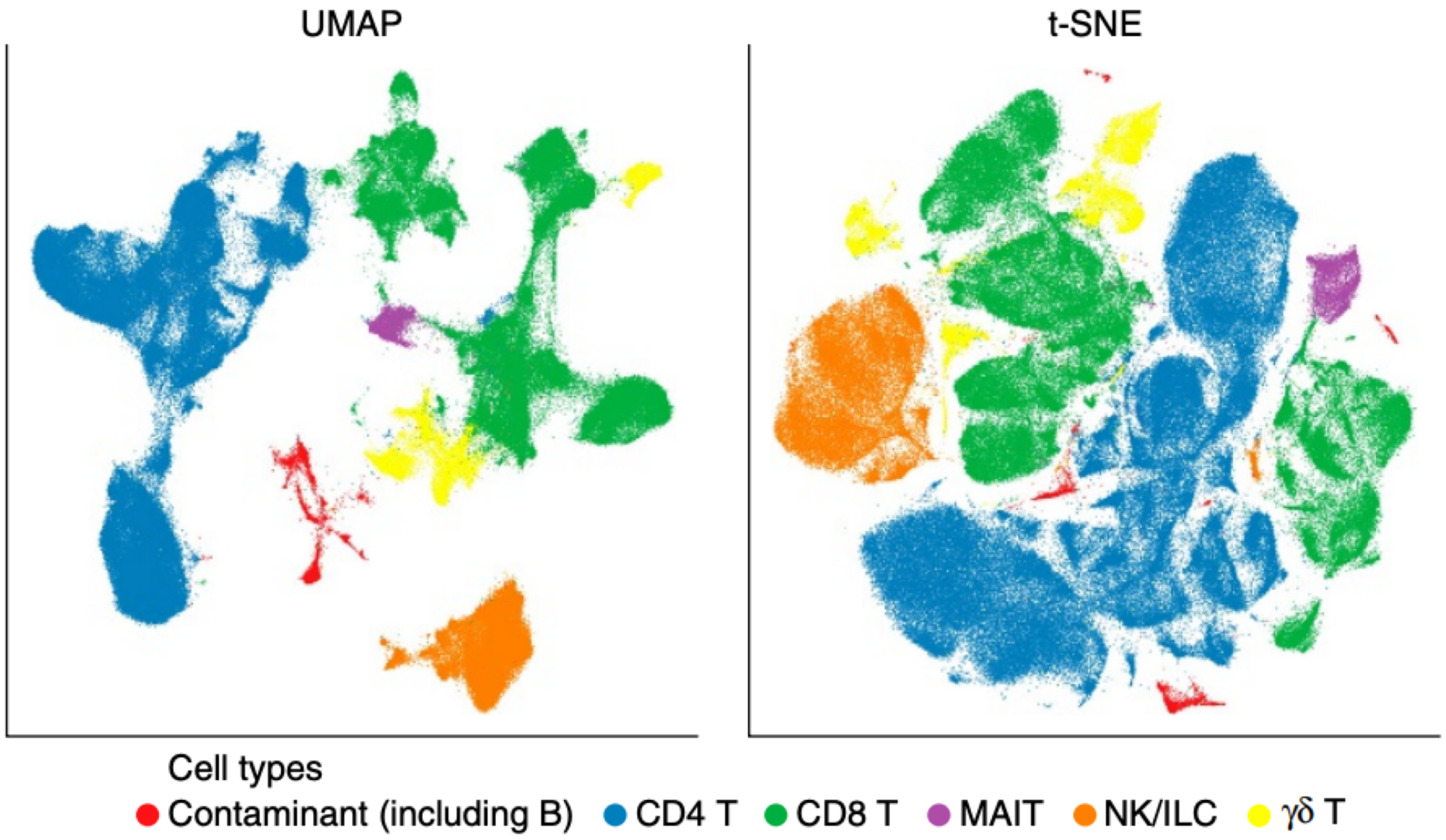
Example code for t-SNE

```
library(Rtsne)
tsne_model_1 = Rtsne(datamatrix,
                     check_duplicates=FALSE, pca=TRUE,
                     perplexity=30, theta=0.5, dims=3)
tsne_out = as.data.frame(tsne_model_1$Y)

plot(tsne_out$V1, tsne_out$V2,
    pch = 19, cex = 0.4, col = mycolor)
legend("bottomleft", col = mycolor,
       legend = uniqCT, pch = 19,
       cex = 0.5, bty = "n")
```

UMAP: a newer (and better?) visualization tool

- UMAP (uniform manifold approximation and projection): a recently developed dimension reduction tool
- "*Comparing the performance of UMAP with five other tools, we find that UMAP provides the fastest run times, highest reproducibility and the most meaningful organization of cell clusters.* " ---- Betcht et al. 2018 Nat Biotech
- "*UMAP, which is based on theories in Riemannian geometry and algebraic topology, has been developed, and soon demonstrated arguably better performance than t-SNE due to its higher efficiency and better preservation of continuum.*" ---- Mu et al. 2018 GBP
- Has "umap" package on CRAN.



Betcht et al. 2018 Nat Biotech

Example code for UMAP

```
library(umap)
sim_umap <- umap(datamatrix)
sim_umap2 <- sim_umap$layout
colnames(sim_umap2) <- c("UMAP1", "UMAP2")

plot(sim_umap2[,1], sim_umap2[,2],
    pch = 19, cex = 0.4, col = mycolor)
legend("bottomleft", col = mycolor,
       legend = uniqCT, pch = 19,
       cex = 0.5, bty = "n")
```