An Introduction to scRNA-seq

GSND 5340Q, BMDA

W. Evan Johnson, Ph.D.
Professor, Division of Infectious Disease
Director, Center for Data Science
Rutgers University – New Jersey Medical School

2024-06-09

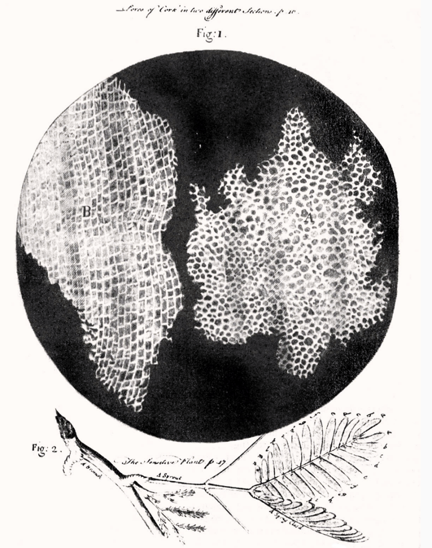
Installing and using the SCTK

```r
install.packages("devtools")
devtools::install_github("wevanjohnson/singleCellTK")
library(singleCellTK)
singleCellTK()

### Example: open downstream_analysis/
### features_combined.txt and meta_data.txt
```

Cells are Important

- Fundamental unit of life
- Autonomous and unique
- Interactive
- Dynamic - change over time
- Evolution occurs on the cellular level



Robert Hooke's drawing of cork cells, 1665

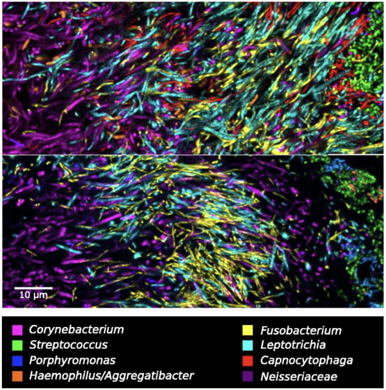
Cells are Diverse

Type	Prokaryotes	Eukaryotes
Typical size	$\sim$ 1-5 μm	$\sim$ 10-100 μm
DNA form	Circular	Linear
DNA location	Cytoplasm	Nucleus
DNA amount	$\sim$ .3-16 fg	$\sim$3-300,000 fg
RNA amount	$\sim$ 5-26,000 fg	$\sim$ 1,000-350,000 fg

Landenmark HKE, Forgan DH, Cockell CS (2015). An Estimate of the Total DNA in the Biosphere. PLoS Biol 13(6): e1002168. https://doi.org/10.1371/journal.pbio.1002168

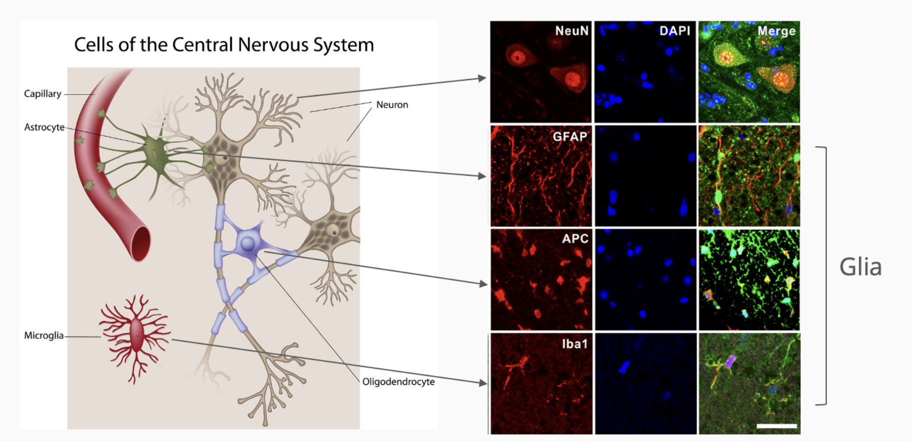
Cells are Diverse: Microbial Ecology

- Environments on Earth support microbial life
- Microbes usually work together in a balance
- Imbalances can disrupt the function of overall ecology
- Which specific microbes out of millions cause a particular effect?



Mark Welch, et al. 2016. "Biogeography of a Human Oral Microbiome at the Micron Scale." Proceedings of the National Academy of Sciences of the United States of America 113 (6): E791–800.

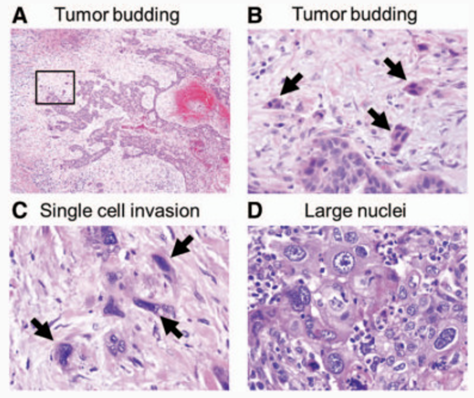
Cells are Diverse: Human Brain



The vast majority of cells (10x-50x) in your brain are glia, not neurons

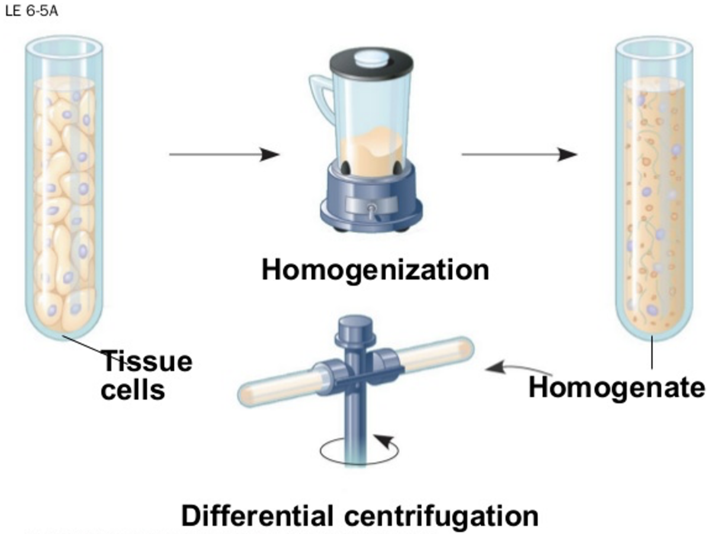
Cells are Diverse: Tumors

- Tumors are seeded by single mutated cells
- Founder cells divide and further mutate
- Large tumors undergo angiogenesis
- Selectively kill cancer cells: cure the cancer
- But which cells to target?



Kadota, Kyuichi, et al. 2014. "Comprehensive Pathological Analyses in Lung Squamous Cell Carcinoma: Single Cell Invasion, Nuclear Diameter, and Tumor Budding Are Independent Prognostic Factors for Worse Outcomes." Journal of Thoracic Oncology: Official Publication of the International Association for the Study of Lung Cancer 9 (8): 1126–39.

The Forest: Tissue Homogenate



The Trees: Cells

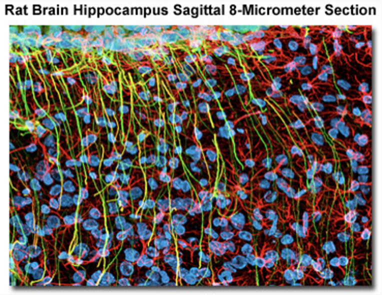
- What cell types are in a sample?
- What are their proportions?
- How does their transcription differ?
- Which/how do specific cells respond to stimulus?
- How do cells develop over time?
- What is the level of mosaicism in tissues

Single Cell Sequencing Workflow

1. Dissociation of tissue, isolation of cells
2. FACS sorting (optional)
3. Nucleic acid extraction and processing
4. Sequencing library prep + sequencing
5. Analysis

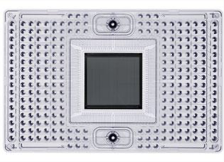
Dissociation of Tissue

- Cells in complex tissue are highly intermingled
- Separate cells without destroying or breaking membranes
- Complex cellular morphology (e.g. neurons) makes dissociation challenging
- Can isolate nuclei instead:
  - Contain DNA/some RNA
  - Much more input material needed



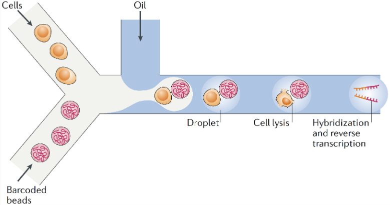
Cell Isolation Techniques

Microfluidics

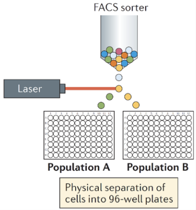


Fluidigm C1
Integrated Fluidic Circuit (IFC)

Droplet Based



Fluorescence Activated Cell Sorting (FACS)

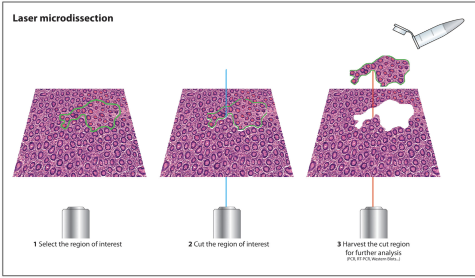


Potter, S. S. 2018.
Papalexi, E. Satija, R. 2018.

Some technologies use all three!

Laser Capture Microdissection

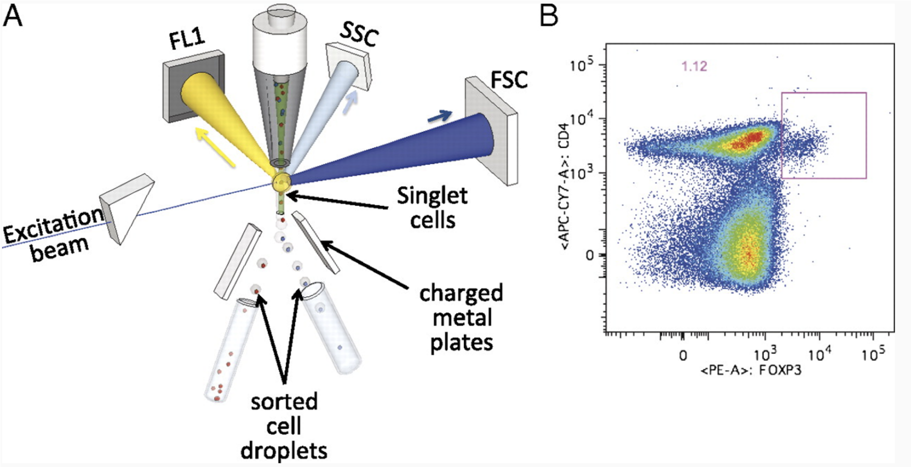
- Technique for isolating groups of cells in situ
- Low throughput, requires expensive equipment
- Laser causes damage to tissue and degrades RNA
- Not generally suitable for single cell sequencing



Fluorescence-Activated Cell Sorting (FACS)

- Cells with known surface markers are tagged with fluorescent antibodies
- Tagged cells excited by lasers during flow cytometry
- Excited and non-excited cells separated and collected
- Cell type specific populations can be sequenced and studied

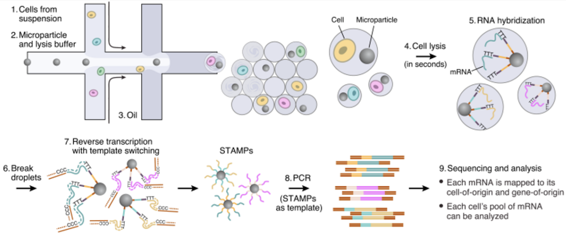
Fluorescence-Activated Cell Sorting (FACS)

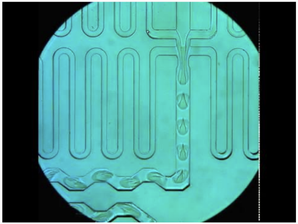


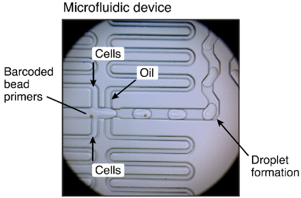
Jaye, David L., Robert A. Bray, Howard M. Gebel, Wayne A. C. Harris, and Edmund K. Waller. 2012. "Translational Applications of Flow Cytometry in Clinical Practice." Journal of Immunology 188 (10): 4715–19.

Drop-seq

- Microfluidics used to pair cells and barcodes/reagents into separate oil droplets
- Concentrations carefully controlled to get 1:1 cell/barcode matches in each oil droplet with high statistical confidence

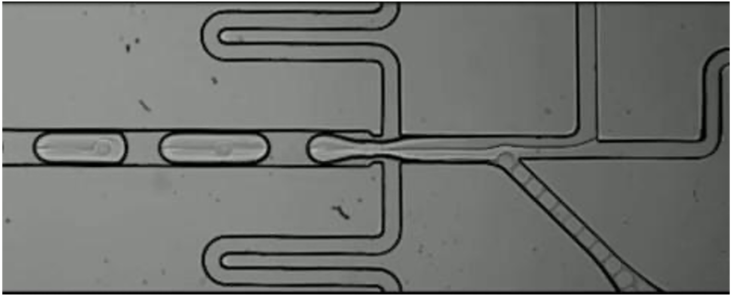


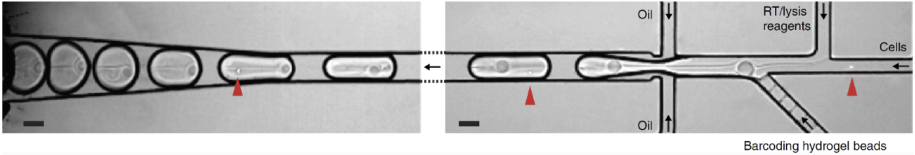




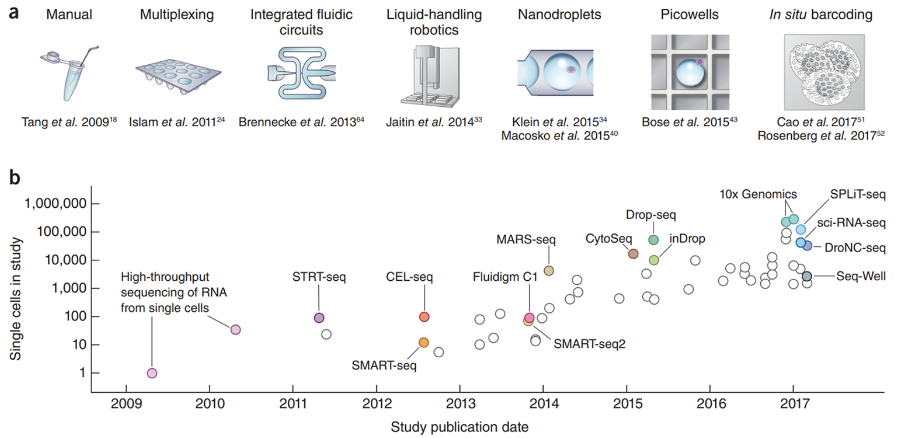
inDrops

Essentially the same strategy Drop-Seq, but uses hydrogel beads





A decade of single cell RNA-seq



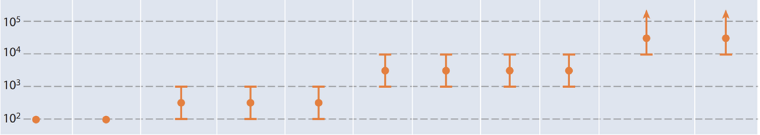
[Svensson et al. 2018. DOI:10.1038/nprot.2017.149](https://doi.org/10.1038/nprot.2017.149)

Slide by Lior Pachter and Matt Thomson

A decade of single cell RNA-seq

	SMART-seq2	CEL-seq2	STRT-seq	Quartz-seq2	MARS-seq	Drop-seq	inDrop	Chromium	Seq-Well	sci-RNA-seq	SPLiT-seq
Single-cell isolation	FACS, microfluidics	FACS, microfluidics	FACS, microfluidics, nanowells	FACS	FACS	Droplet	Droplet	Droplet	Nanowells	Not needed	Not needed
Second strand synthesis	TSO	RNase H and DNA pol I	TSO	PolyA tailing and primer ligation	RNase H and DNA pol I	TSO	RNase H and DNA pol I	TSO	TSO	RNase H and DNA pol I	TSO
Full-length cDNA synthesis?	Yes	No	Yes	Yes	No	Yes	No	Yes	Yes	No	Yes
Barcode addition	Library PCR with barcoded primers	Barcoded RT primers	Barcoded TSOs	Barcoded RT primers	Barcoded RT primers	Barcoded RT primers	Barcoded RT primers	Barcoded RT primers	Barcoded RT primers	Barcoded RT primers and library PCR with barcoded primers	Ligation of barcoded RT primers
Pooling before library?	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Library amplification	PCR	In vitro transcription	PCR	PCR	In vitro transcription	PCR	In vitro transcription	PCR	PCR	PCR	PCR
Gene coverage	Full-length	3′	5′	3′	3′	3′	3′	3′	3′	3′	3′

Number of cells per assay



Chen et al. 2018. DOI:10.1146/annurev-biodatasci-080917-013452

Nucleic Acid Extraction + Processing

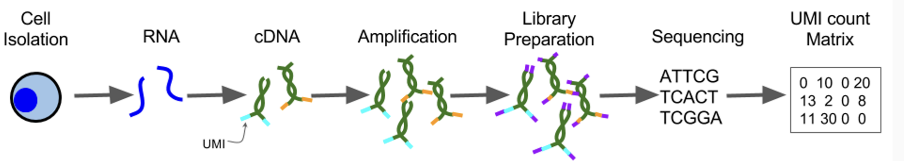
femto to picograms of input material

Each cell is:

- Assigned a unique DNA barcode
- Optionally treated with UMIs
- Amplified by one of:
  - Reverse transcriptase (RNA)
  - Multiple displacement amplification (DNA)
  - In vitro transcription (RNA)

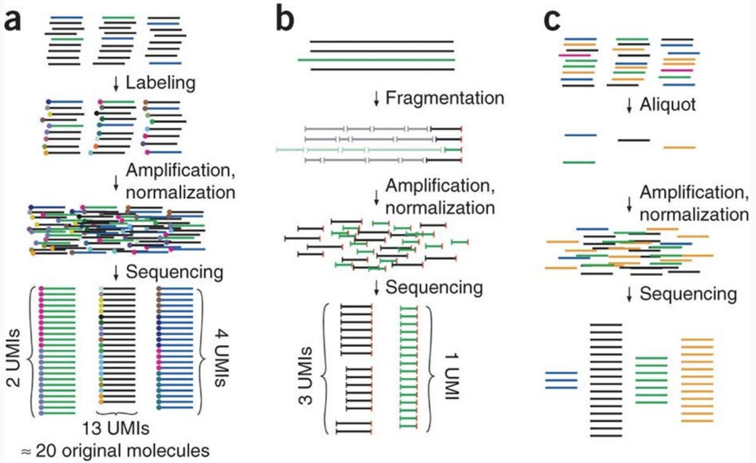
Unique Molecular Identifiers (UMIs)

- Low input material may cause amplification bias
- UMIs are sequences that correspond to one fragment
- Sequenced reads with the same UMI are from the same fragment
- Unique sequences collapsed/deduplicated for counting



Unique Molecular Identifiers (UMIs)

Strategies for counting individual molecules



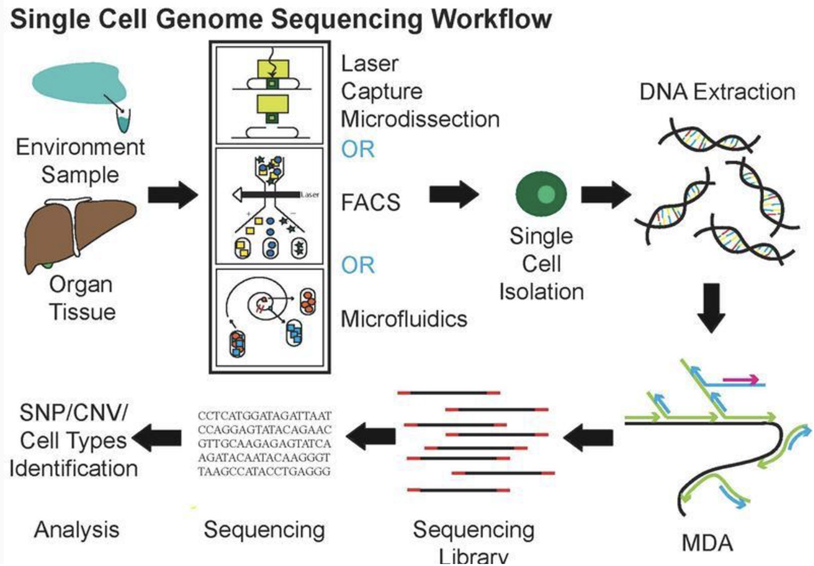
Kivioja et al. 2011. "Counting Absolute Numbers of Molecules Using Unique Molecular Identifiers." Nature Methods 9 (1): 72–74.

Sequencing Library Prep and Sequencing

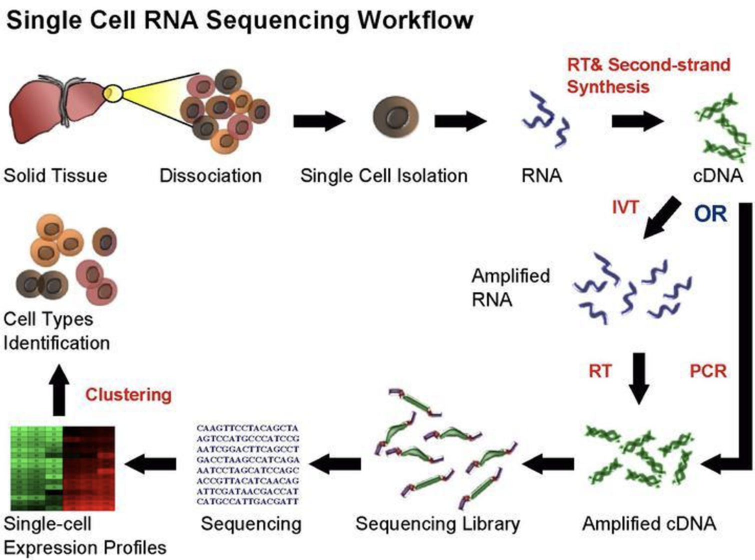
- Previous protocols typically include sequencing primers
- Sequencing depth = (# of cells) x (required depth):
  - RNA - 50k paired end reads / cell for cell type classification
  - RNA - .25M-1M paired reads / cell for transcriptome coverage
  - DNA - 30-100x per cell
- e.g. 1000 cell scRNA-Seq = 250M-1B reads per sample!
- Sequences in one PE fastq file are entirely barcodes
- Read length > 50bp for annotated genome
- Single cell sequencing is still *very expensive*

Rizzetto, et al. 2017. "Impact of Sequencing Depth and Read Length on Single Cell RNA Sequencing Data of T Cells." Scientific Reports 7 (1): 12781.

scDNA-Seq Workflow

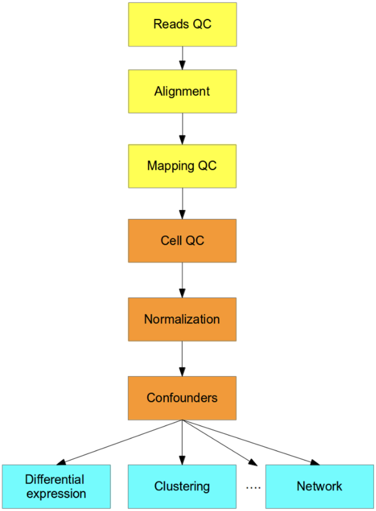


scRNA-seq workflow



Analysis Overview

1. Sequence QC
    1. Demultiplex
    2. UMI Collapsing
2. Alignment
3. Quantification
4. Normalization
5. DE, Clustering, etc



scruff R/Bioconductor package

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scruff

platforms all | rank unknown | posts 0 | in Bioc < 6 months
build ok | updated < 1 month

DOI: 10.18129/B9.bioc.scruff

Single Cell RNA-Seq UMI Filtering Facilitator (scruff)

Bioconductor version: Release (3.8)

A pipeline which processes single cell RNA-seq (scRNA-seq) reads from CEL-seq and CEL-seq2 protocols. Demultiplex scRNA-seq FASTQ files, align reads to reference genome using Rsubread, and generate UMI filtered count matrix. Also provide visualizations of read alignments and pre- and post-alignment QC metrics.

Author: Zhe Wang [aut, cre], Junming Hu [aut], Joshua Campbell [aut]

Maintainer: Zhe Wang <zhe at bu.edu>

Citation (from within R, enter `citation("scruff")`):

Wang Z, Hu J, Campbell J (2019). *scruff: Single Cell RNA-Seq UMI Filtering Facilitator (scruff)*. R package version 1.0.3.

Installation

To install this package, start R (version "3.5") and enter:

Documentation »

Bioconductor

- Package vignettes and manuals.
- Workflows for learning and use.
- Course and conference material.
- Videos.
- Community resources and tutorials.

R / CRAN packages and documentation

Support »

Please read the posting guide. Post questions about Bioconductor to one of the following locations:

- Support site - for questions about Bioconductor packages
- Bioc-devel mailing list - for package developers

Sequence QC

One sample is 100s or 1000s of cells

- i.e. ~1,000 fastq files per sample
- May or may not be already demultiplexed by core

UMI-Tools - open source UMI software

Normal fastq processing and QC:

- Adapter and quality trimming
- fastqc, multiqc

Alignment and Quantification

STAR+htseq-count, kallisto, salmon, CellRanger

Each sample has a different # of cells

Each cell has the same number of measurements (e.g. genes) = (# of samples) x (# of cells) x (# of genes)

Sparse: most will be zero!

Amarel Submission Script for CellRanger

```
#!/bin/bash
#SBATCH --partition=main
#SBATCH --job-name=cellranger_bam
#SBATCH --array=0-3,5,6
#SBATCH --cpus-per-task=30
#SBATCH --mem=100G
#SBATCH --time=04:00:00

# path to fastq files
FASTQPATH=/scratch/$USER/tmp/awsbucket/fastqs/

# get sample to process
INDEX=$(($SLURM_ARRAY_TASK_ID))
INPUT=($(ls -d $FASTQPATH*R1_001.fastq.gz))
FASTQ=($(echo ${INPUT[$INDEX]##*/} | cut -d_ -f1-1))

# Path to cellranger
crpath=/projects/f_wj183_1/apps/cellranger-8.0.1/
```

Amarel Submission Script for CellRanger

```
# path to reference library
refpath=/projects/f_wj183_1/reflib/2024_cellranger/refdata-gex-GRCh38-2024-
cd $FASTQPATH

# load python
module load python/3.8.2

$crpath/./cellranger count --id=$FASTQ
   --create-bam=true \  # true or false, necessary
   --sample=$FASTQ \ # prefix of files to align
   --fastqs=$FASTQPATH \
   --localcores=30 \
   --localmem=100 \
   --chemistry=SC3Pv2 \ # optional
   --transcriptome=$refpath
```

The Counts Matrix

- Counts matrix contains either:
  - Read counts or
  - UMI counts if used
- Each cell has:
  - Total number of counts (col. sum, “library size”)
  - Number of non-zero genes
- Each gene has:
  - number of non-zero cells
  - Non-zero mean/variance
- Matrix is sparse: many zeros
- Zeros may be:
  - Cell lacks gene
  - A “drop-out”: gene present but was missed by qPCR

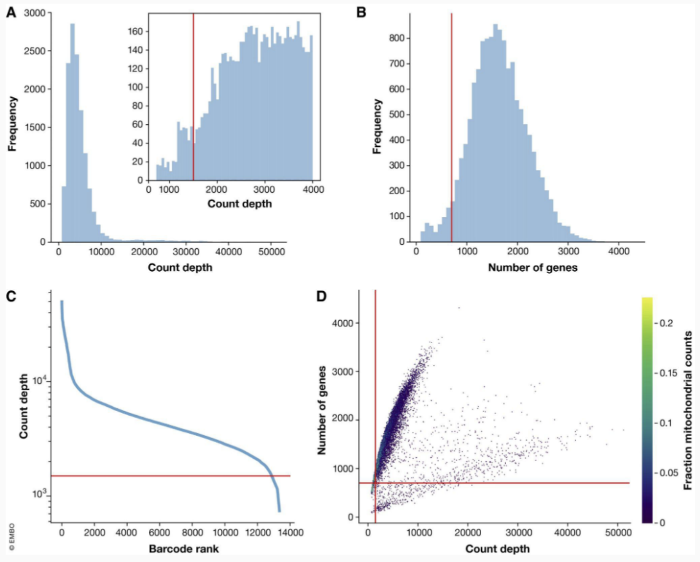
The Counts Matrix

	cell1	cell2	cell3	cell4	cell5	cell6	...	cellM
gene1	93	25	0	0	3335	0		82
gene2	5	2	0	3	1252	0		12
gene3	0	0	0	0	0	0		0
gene4	98	21	1	1	5318	0		75
gene5	0	0	513	0	0	325		135
gene6	0	0	113	0	1	497		255
gene7	3	0	0	0	6	0		0
...								
geneN	68	52	0	2	4313			63

Cell Quality Control

- Consider metrics jointly and threshold by outliers in distribution
- Most common cell QC metrics are:
  - Count depth
  - Number of genes
  - Fraction of mitochondrial genes

Cell Quality Control



Cell QC metrics should be considered together

Example 1:

Cell with high % of MT genes may represent:

- Cell subtypes highly engaged in respiratory processes (muscle, fat, etc.)
- Broken cells where the cytoplasmic mRNA has escaped, leaving only the MT RNA
- Cell with low count depth, few genes, and high % of MT genes may be a better marker of true “broken” cell events

Cell QC metrics should be considered together

Example 2:

Cell with abnormally high count depth and large amount of genes may represent:

- Doublets (droplets or events containing multiple cells)
- Larger cells (cell size can correlate with counts)
- Newly developed tools specifically handle doublets and attempt to resolve these cases (Scrublet, Doublet Finder, etc)

Cell QC metrics should be considered together

Example 3:

Cell with low counts and low amount of genes may represent:

- "Empty" droplets or events
- Quiescent cell populations

Additional QC Checks

- Remove genes that are only expressed in a few cells
  - This naturally sets a limit on the size of cell clusters you can recover
  - i.e. Removing genes expressed in fewer than 20 cells will make it difficult to detect clusters smaller than this
- Account for ambient gene expression (contaminating mRNA from lysed cells prior to library construction)

Filtering Cells and Genes

Many measurements

- e.g. 30k genes x 1ks of cells

Some cells are uninformative, e.g.:

- Very few reads, few genes detected
- Two cells sequenced together (i.e. doublets)

Some genes are uninformative:

- Low # reads, low variance across all cells
- Too few cells express gene (e.g. $<$ 10 of 10,000 cells nonzero)

Must filter genes *and* cells to reduce noise

Filtering the Counts Matrix

Cells might also be filtered:

- Very few or zero counts (cell4)
  - Empty well?
  - Ambient expression?
  - Quiescent cell?
- Very many counts (cell5)
  - Possible "doublet" of same cell type
- Inconsistent expression pattern (cellM)
  - Possible "doublet" of different cell types

Doublet: two cells with same cell barcode

Filtering the Counts Matrix

	cell1	cell2	cell3	**cell4**	*cell5*	cell6	...	cellM
gene1	93	25	0	**0**	*3335*	0		82
gene2	5	2	0	**3**	*1252*	0		12
gene3	0	0	0	**0**	*0*	0		0
gene4	98	21	1	**1**	*5318*	0		75
gene5	0	0	513	**0**	*0*	325		135
gene6	0	0	113	**0**	*1*	497		255
gene7	3	0	0	**0**	*6*	0		0
...								

Session info

```r
sessionInfo()
```

```
## R version 4.4.0 (2024-04-24)
## Platform: aarch64-apple-darwin20
## Running under: macOS Sonoma 14.2.1
##
## Matrix products: default
## BLAS:   /Library/Frameworks/R.framework/Versions/4.4-arm64/Resources/lib/libRblas.0.dylib
## LAPACK: /Library/Frameworks/R.framework/Versions/4.4-arm64/Resources/lib/libRlapack.dylib;  LAPACK version 3
##
## locale:
## [1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8
##
## time zone: America/Denver
## tzcode source: internal
##
## attached base packages:
## [1] stats     graphics  grDevices utils     datasets  methods   base
##
## loaded via a namespace (and not attached):
##  [1] compiler_4.4.0    fastmap_1.2.0     cli_3.6.2         tools_4.4.0
##  [5] htmltools_0.5.8.1 rstudioapi_0.16.0 yaml_2.3.8        rmarkdown_2.27
##  [9] knitr_1.47        xfun_0.44         digest_0.6.35     rlang_1.1.4
## [13] evaluate_0.23
```