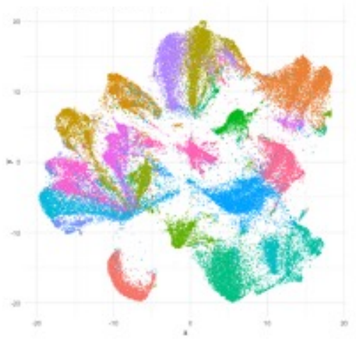
HARVARD MEDICAL SCHOOL | BLAVATNIK INSTITUTE SYSTEMS BIOLOGY

The Single Cell RNA-seq Workflow:

A practical guide to ensure experimental success



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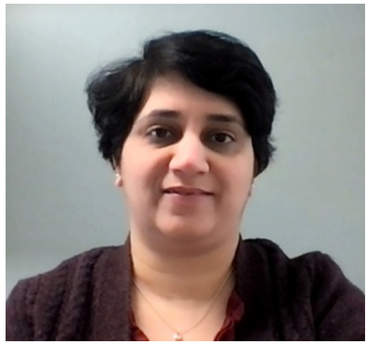
HMS's Single Cell Core: The Team and Faculty Advisors

Mandovi Chatterjee
Director



Arpita Kulkarni
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Alexa Yeagley
Research Assoc.

Dr. Ollie
scPawlice & Floof

Dr. Allon Klein

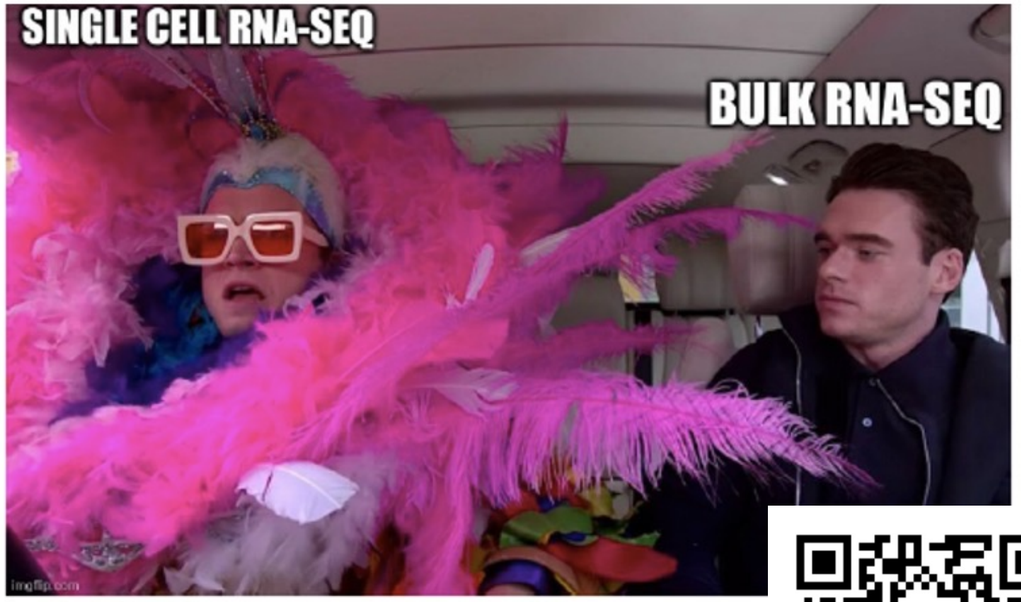
Dr. Jeff Moffitt

Dr. Chris Benoist

HMS’s Single Cell Core: our mission

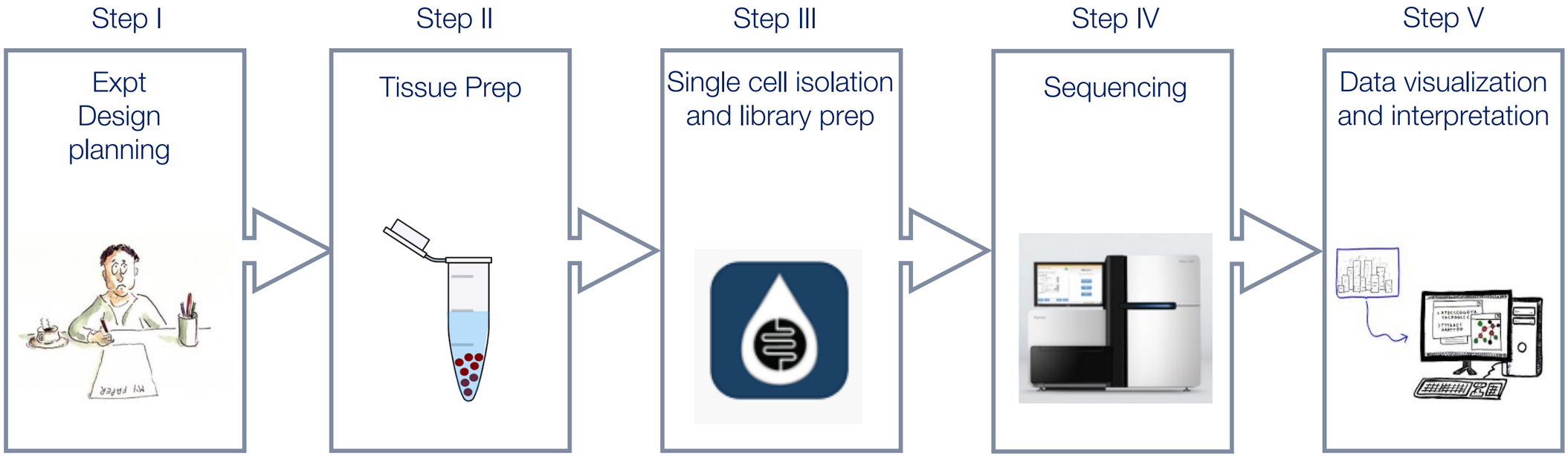
Enable discoveries by guiding in the design, execution & interpretation of single cell and spatial -omics assays

- We are one of the oldest single cell core’s on campus
- Open to all, fee-for-service core
- Worked w/ >500 PI’s and 50,000+ samples to date



Outline for today's talk (~40mins)

- A background into scRNAseq
- What good scRNAseq data looks like (defining quality metrics for goal setting)
- The scRNAseq workflow



Outline for today's talk (~40mins)

1. This is an "overview" type talk (not comprehensive given the scope and time constraints)

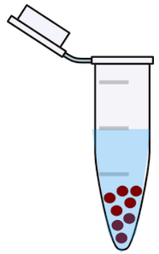
2. Geared towards beginners, to reduce barrier of entry

Step I

Expt Design planning

Step II

Tissue Prep



Step III

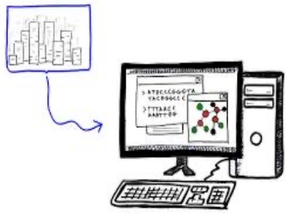
Single cell isolation and library prep

Step IV

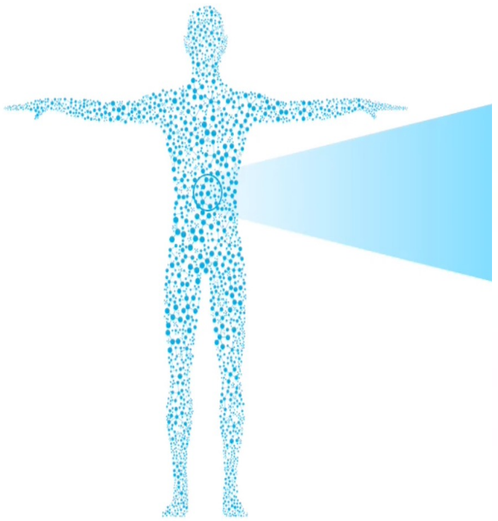
Sequencing

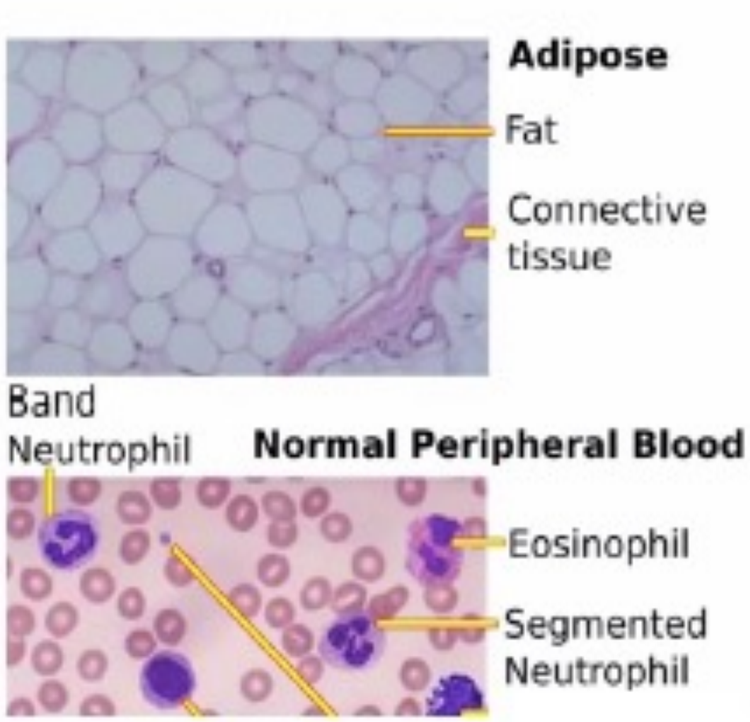
Step V

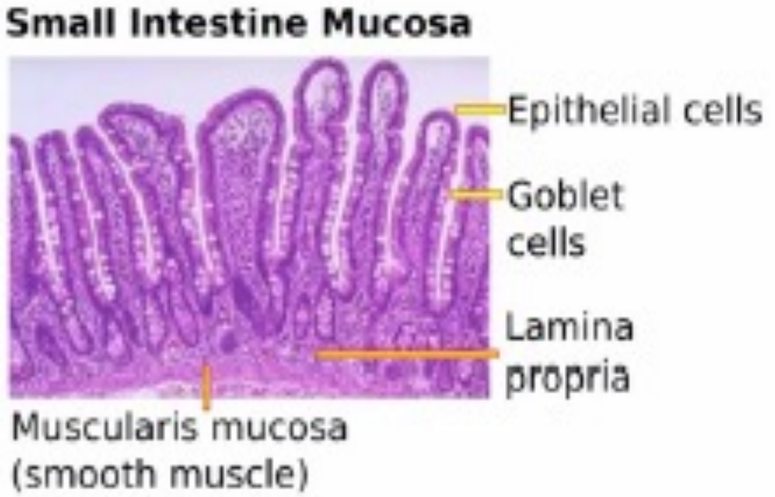
Data visualization and interpretation



We know tissues are heterogeneous







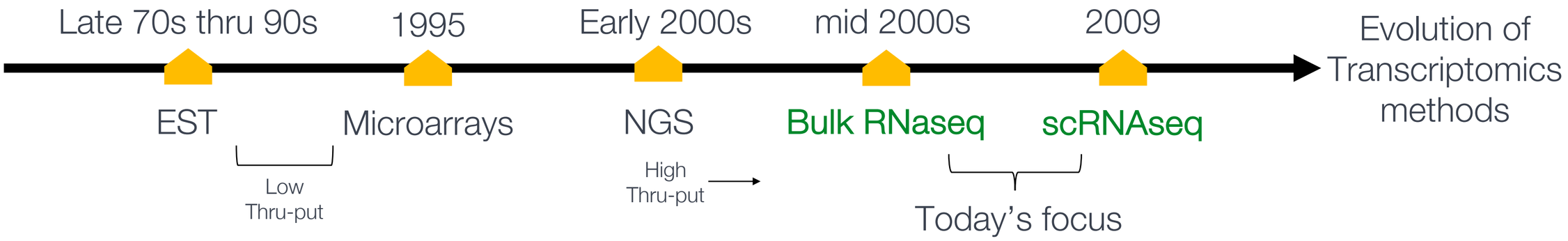
Q. What makes cells (& tissues) different from one another? How do we measure these differences?

PC: 10x Genomics

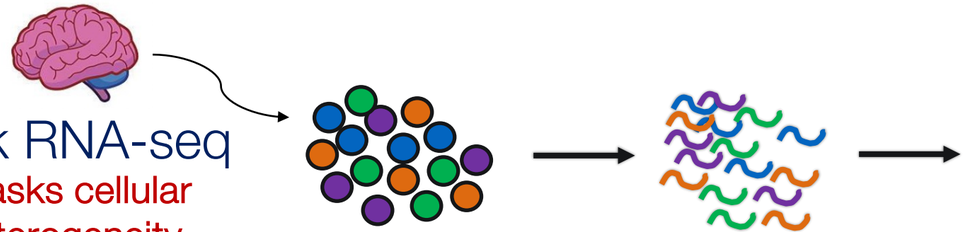
Transcriptomics: Unraveling the nature of cellular identity

DNA → RNA → Protein

Measuring (m)RNA “transcriptomics” is a reasonable & powerful proxy to unravel cellular identity



Bulk vs single cell RNA sequencing (scRNA-seq)



Bulk RNA-seq

⚠ (masks cellular heterogeneity, averaging misleading)

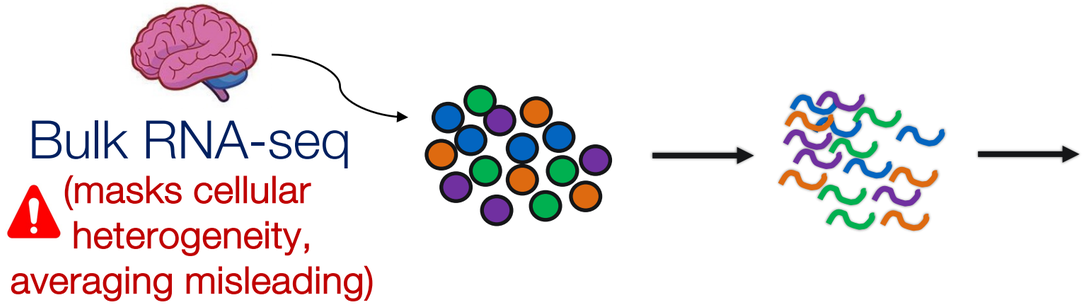
average expression level across a population

Bulk RNA-seq good for -

- comparative transcriptomics
- ID disease biomarkers
- homogenous systems

Allows identification of broad level differences in gene expression

Bulk vs single cell RNA sequencing (scRNA-seq)



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(masks cellular heterogeneity, averaging misleading)

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Bulk RNA-seq good for -

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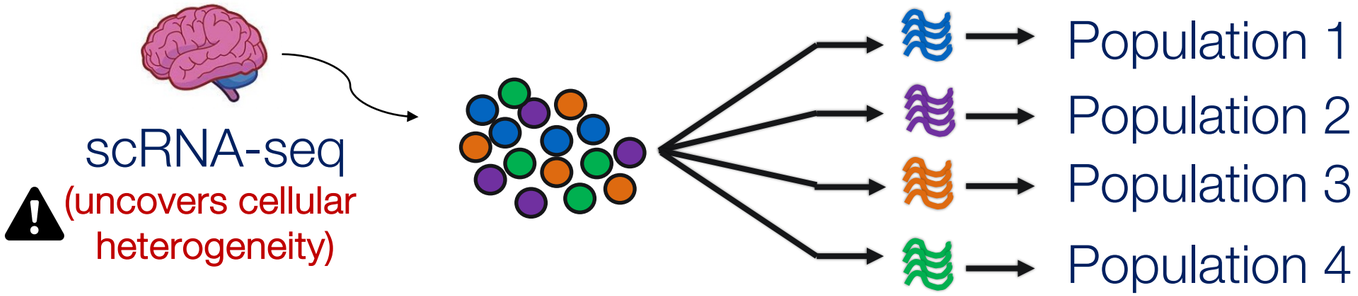


scRNA-seq
(uncovers cellular heterogeneity)

scRNA-seq good for -

- defining heterogeneity
- identify rare cell population(s)
- cell population dynamics

Allows identification of cell to cell variation in gene expression

Bulk vs scRNA-seq: a difference of resolution

smoothie

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

Bulk RNA-seq

Mix-Fruit salad

scRNA-seq

Individual components

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

Which technique to use when?

Bulk vs scRNA-seq: not always an either/or situation

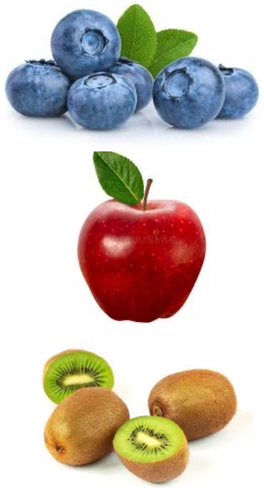
Cost effective, good quality data

smoothie

Bulk RNA-seq

Mix-Fruit salad

scRNA-seq



Individual components

Bulk RNAseq as a –

1. Complementary first step
2. For homogenous systems
3. Broad level differences

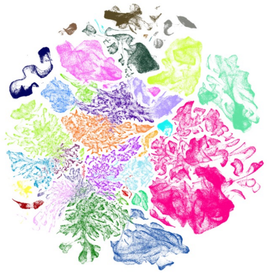
Separate populations
- Define heterogeneity
- Identify rare cell populations
- Cell population dynamics

Common applications of scRNA-seq – why use scRNAseq?

a) "cell atlas"-type studies
- Heterogeneous populations

Uncover cellular heterogeneity

e.g. Allen brain atlas, Tumor environment etc



b) "timeseries"-type studies
- Snapshots in biol. process

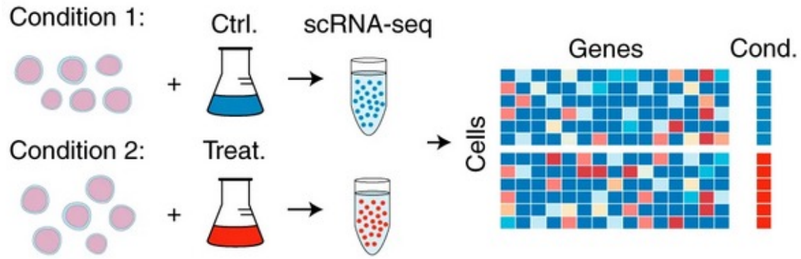
Bio. trajectories/cell fate, Dev timelines, lineage tracing

e.g. embryogenesis

c) "screening"-type studies
- Single cells as individual expt.

Uncover GEX diff on perturbation

e.g. CRISPR studies, Therapeutics discovery



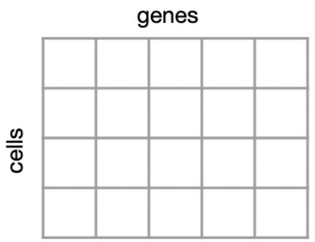
Defining metrics for good quality scRNAseq data

scRNAseq final goals -

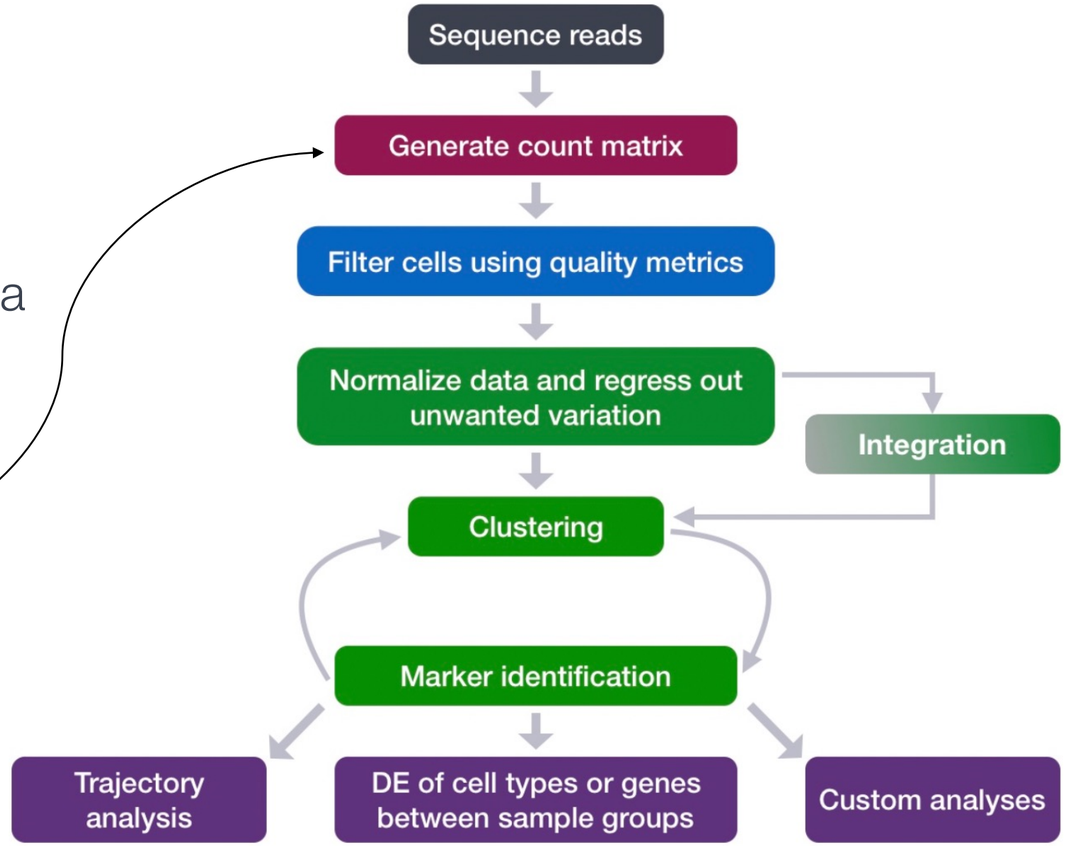
- Gain novel biological insights
- publish & advancement of scientific work

So lets imagine, you've done the expt and have the data

INPUT: cells



OUTPUT: gene expression matrix



https://hbctraining.github.io/scRNA-seq/lessons/04_SC_quality_control.html

Defining metrics for good quality scRNAseq data

Filtering: Cells have to pass this first QC to get to green/purple/yellow

Quality metrics -

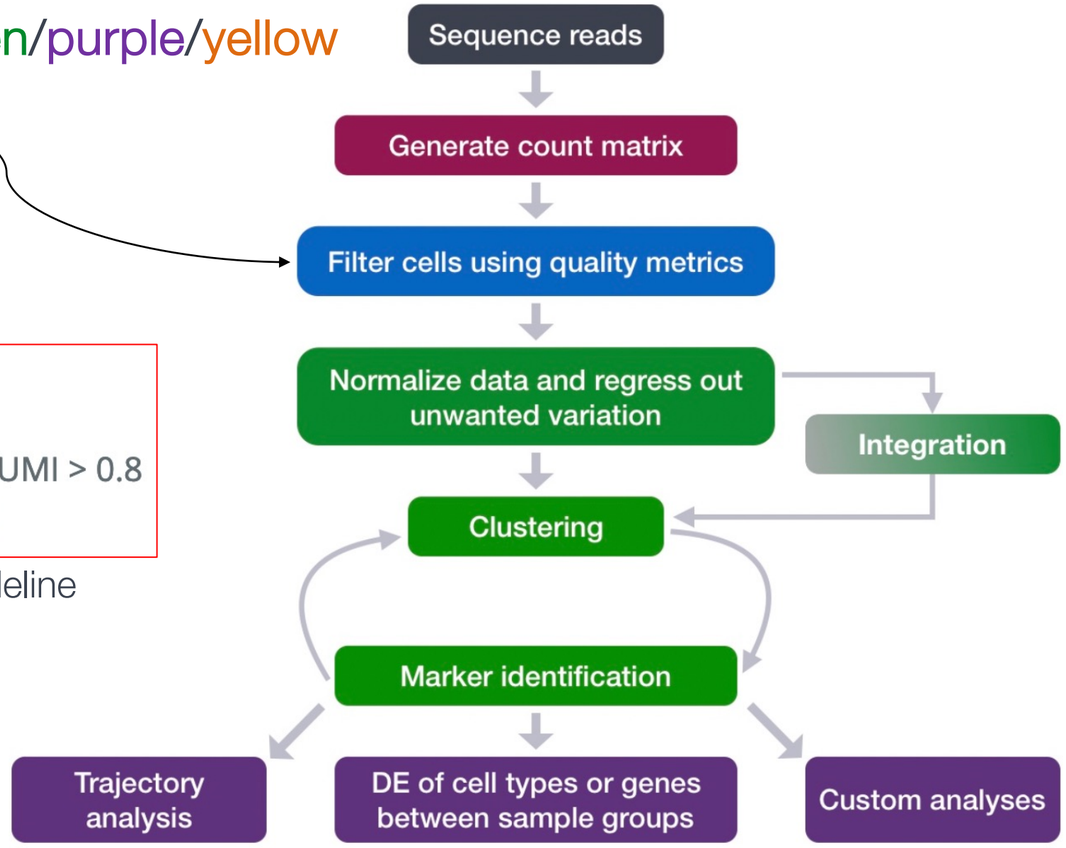
1. Cell counts
2. nUMI counts (transcripts) per cell and nGenes per cell
3. Mitochondrial counts ratio
4. Novelty or complexity score

- nUMI > 500
- nGene > 250
- log10GenesPerUMI > 0.8
- mitoRatio < 0.2

General guideline

- no metric in isolation

- setting thresholds is an iterative process (starting from lenient to stringent)



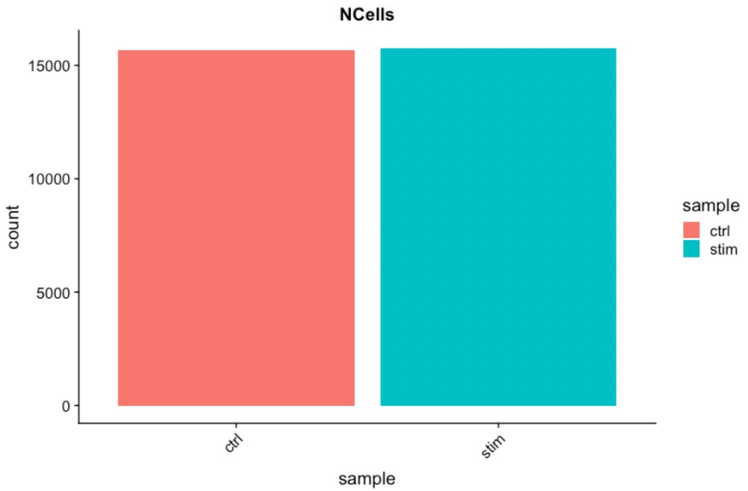
Fame and fortune await

Defining metrics for good quality scRNAseq data

Assessing quality metrics –

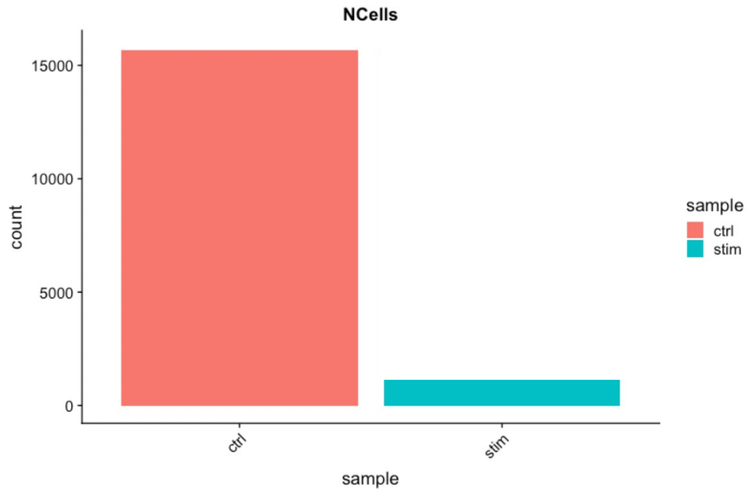
1. Cell counts (how many unique cellular barcodes detected, *ideally* same as #cells you started with, but 60-80% cell recovery is good)

✓



E.g. You start w/ 20K cells, and recover 15K cell barcodes – 75% recovery!

✗



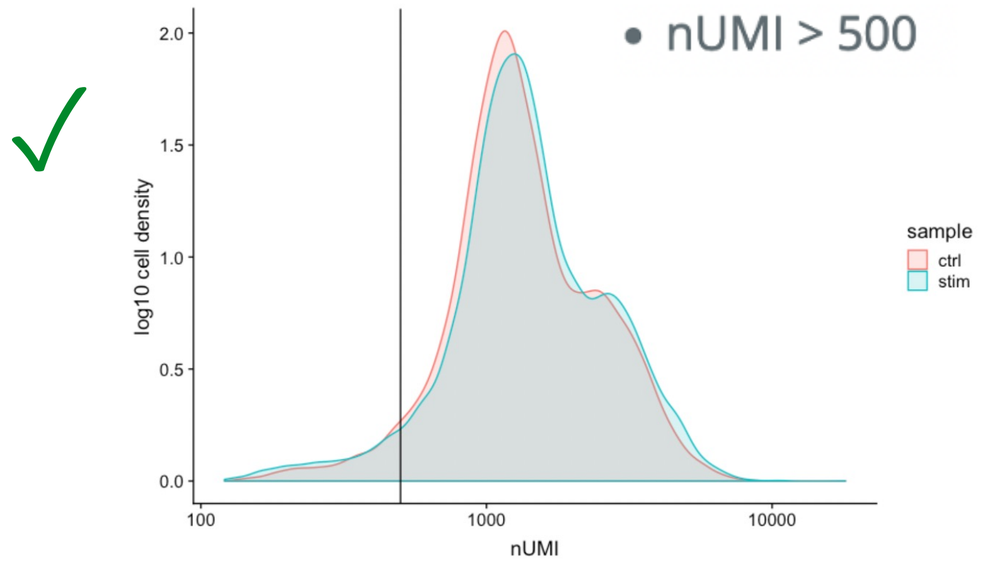
E.g. 1. You start w/ 10K cells, and recover 15K cell barcodes
2. You start w/ 10K and recover 1K
Was cell count wrong? Is it an issue w/ barcodes?
Likely that junk “cells” present

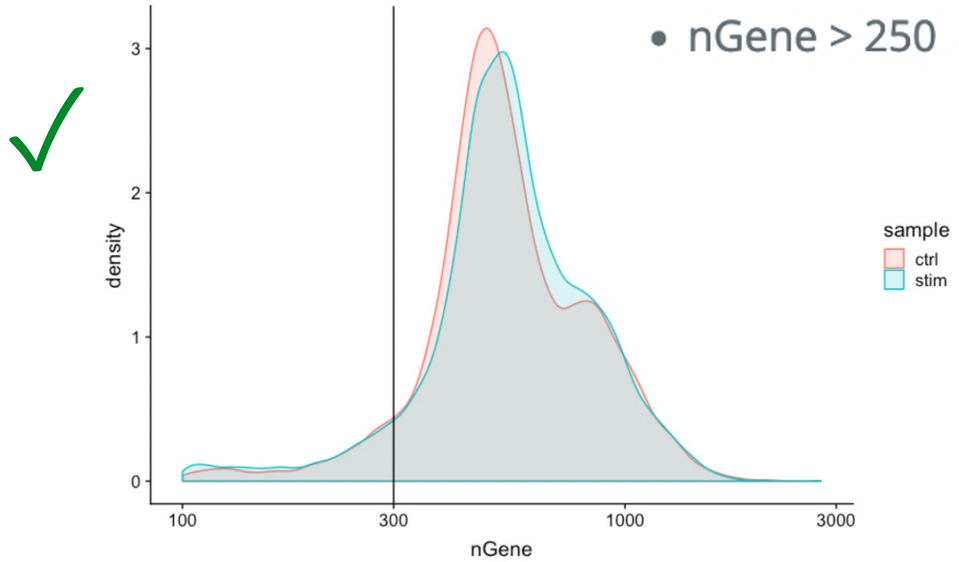
https://hbctraining.github.io/scRNA-seq/lessons/04_SC_quality_control.html

Defining metrics for good quality scRNAseq data

Assessing quality metrics –

2. UMI (transcripts) per cell and genes per cell





UMIs <500 is bad, between 500-1000/cell is usable, but 1000+ is great!

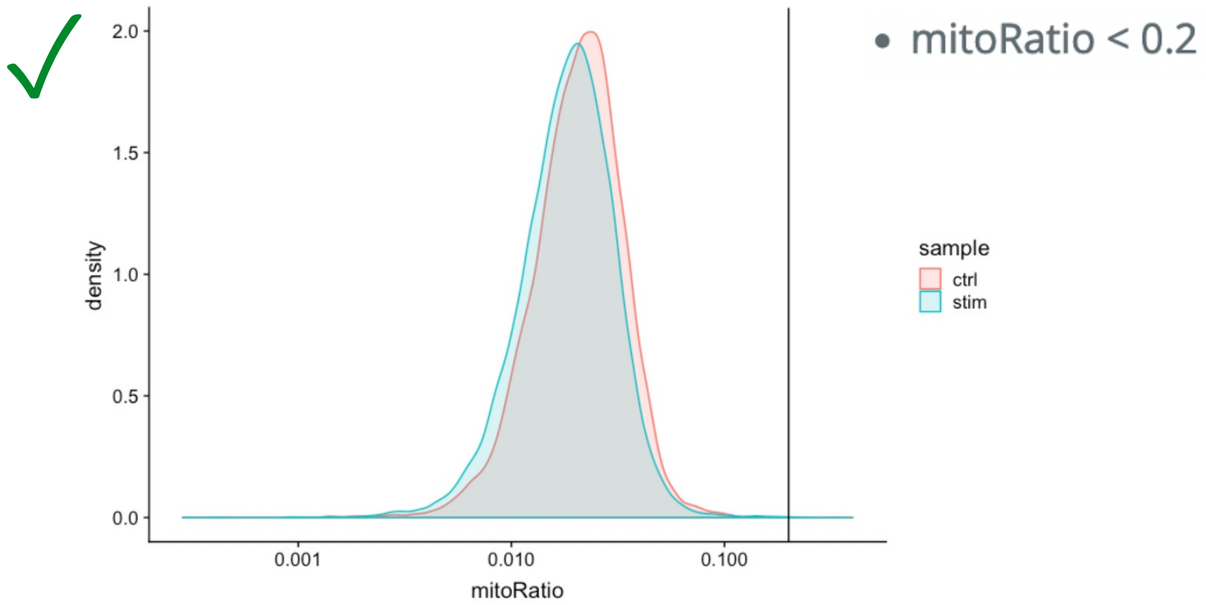
V low or high UMIs and genes per cell can indicate technical artifacts during prep, or poor quality cells

https://hbctraining.github.io/scRNA-seq/lessons/04_SC_quality_control.html

Defining metrics for good quality scRNAseq data

Assessing quality metrics –

3. Mitochondrial counts ratio (unless expected, high counts indicative of unhealthy, dying or lysed cells)



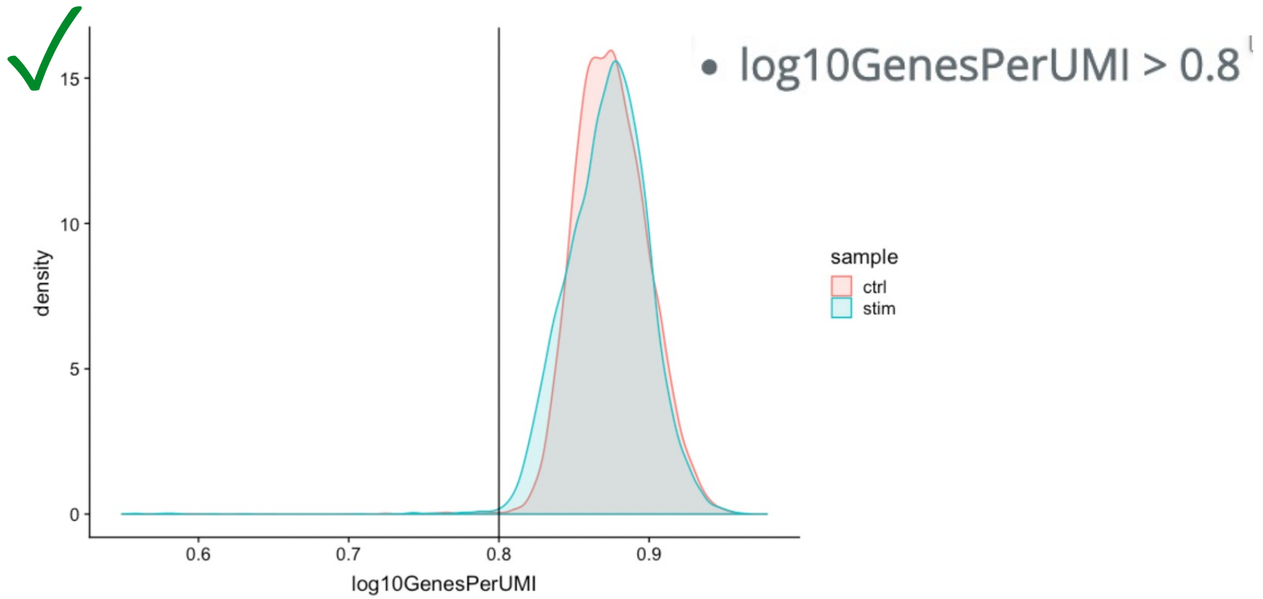
Mitochondrial read counts >0.2, when sample prep bad

https://hbctraining.github.io/scRNA-seq/lessons/04_SC_quality_control.html

Defining metrics for good quality scRNAseq data

Assessing quality metrics –

4. Novelty or complexity score (more genes detected per UMI, more complex the data, in terms of RNA species)



(low scores indicative of no seq saturation, contamination (if multimodal peaks present), and low complexity cells at sample prep)

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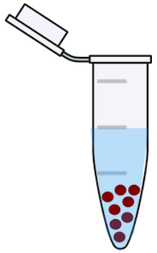
Steps in a scRNAseq work flow

Step I

Expt Design planning

Step II

Tissue Prep



Step III

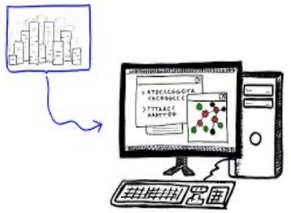
Single cell isolation and library prep

Step IV

Sequencing

Step V

Data visualization and interpretation



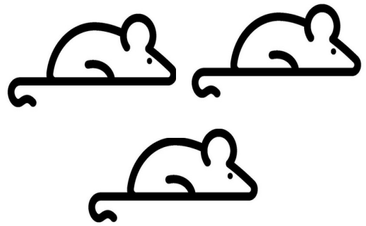
Goal: Put a well-thought-out, holistic plan in place, because single cell expts require clarity from the get-go

Experimental design considerations: taking stock

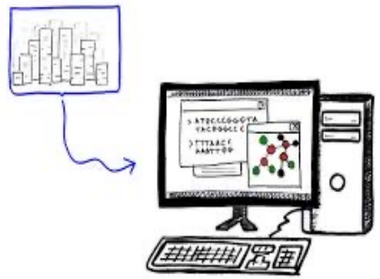
End goal:
Hypothesis testing
Publication
Grant-writing
New study or conti

Resources:
Funding/budget
Time
Effort/Team

Sample type:
Cells vs nuclei
Fresh/frozen/fixed
Abundance
Access

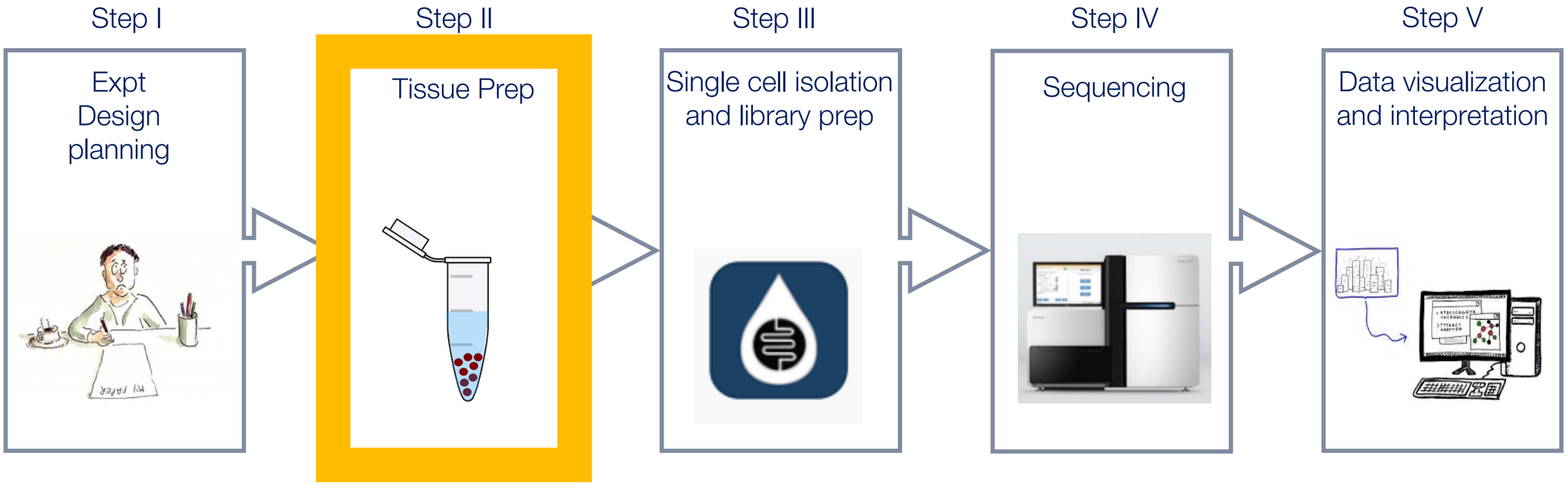


Scale:
Technical &
biological replicates
Comparative groups
Platform choice
#cells barcoded



Bioinformatics & analyses capabilities:
Cloud storage
Computing power
Bioinformaticians
Analysis pipelines
Sequencing costs

Steps in a scRNAseq work flow



Goal: Get high quality, viable, single cell suspension from sample, assess prep and perform QC

Sample prep protocol varies by cell type and logistics

- Enzyme-based dissociation- trypsin, collagenase, liberase, accutase.
- Gentle washes.
- Dead cell removal kit, filtering out the debris.
- Density gradient (Ficoll, Optiprep)

Solid tissue

Adherent cell culture

Dissociation

Suspended cell culture

Liquid tissue

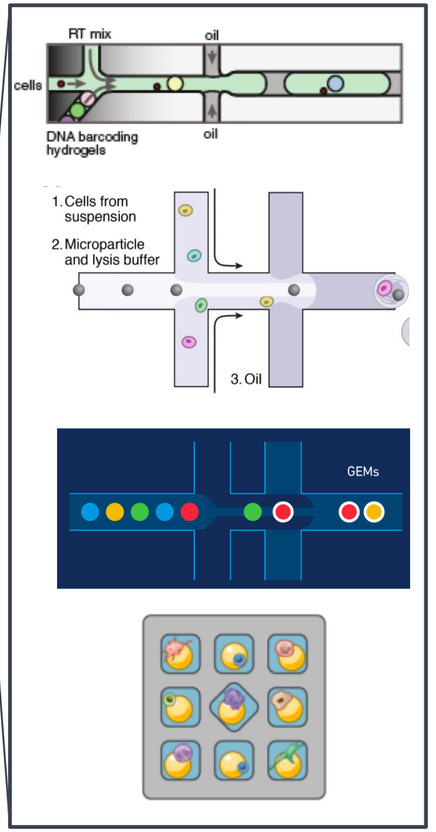
Enrichment

- Magnetic bead selection (MACS)
- FAC sorting

Quality check

- Cell concentration
- Cell Viability (Trypan Blue)

Note: the final sample buffer should be devoid of heparin, Ca^{2+}, Mg^{2+} and EDTA



Dissociation: making a single cell suspension

1. Mechanical methods – cutting, shearing, laser dissections, FACS

Centrifugation

Vortexing

Pipette mixing

2. Enzymatic dissociation

3. Combination

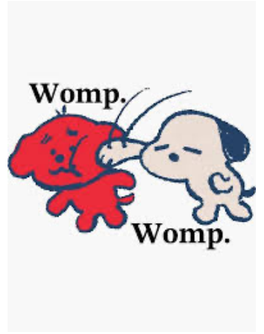
4. Automated

Enzymes (trypsin, collagenase liberase, accutase)

Dissociator

No universal protocol

Requires optimization of protocol for every tissue/cell type



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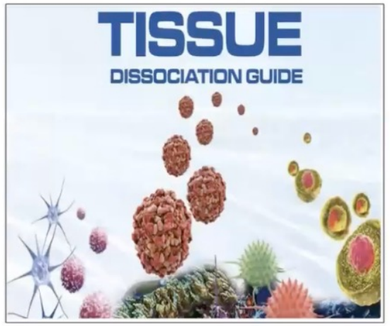
Dissociator

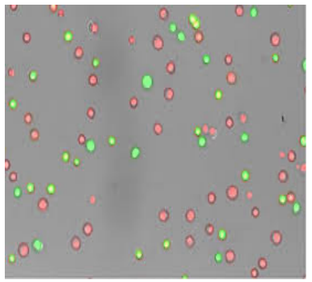
How to find a protocol-

- Publications/Literature
- Technology websites
- Customer support
- Online resources
- Talk to experts
- Use ready-to-use dissociators
- Trial-n-error



https://www.technologynetworks.com/cell-science/how-to-guides/tissue-dissociation-guide-334918

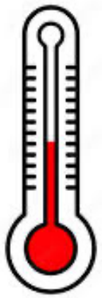
Factors affecting sample preparation (& data quality): top 5



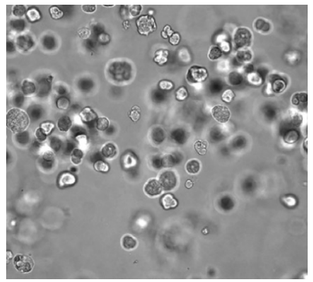
Cell Viability

Cell Count

Time



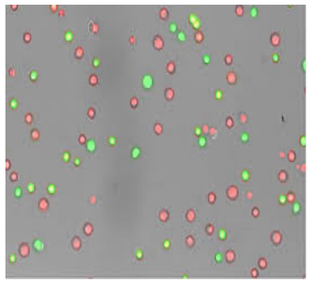
Temp



Quality

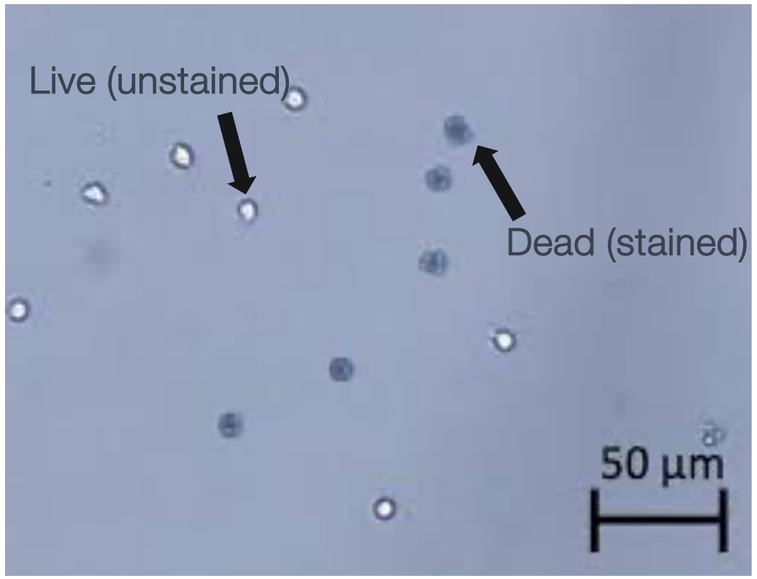
Factors affecting sample preparation: cell viability

1

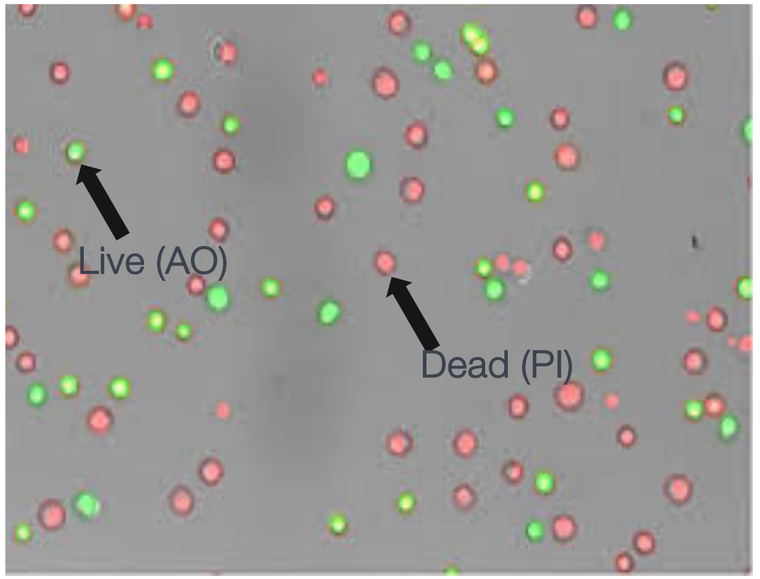


Cell Viability
(high >70%)

The higher the viability, the better (minimum 70-75%, ideally >90%)



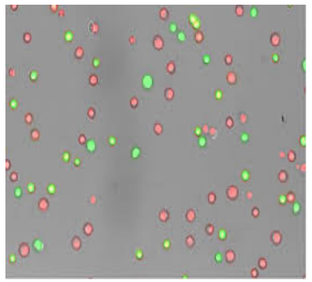
Trypan Blue (dead)



Acridine orange (live)/
Propidium iodide (dead)

Factors affecting sample preparation: cell viability

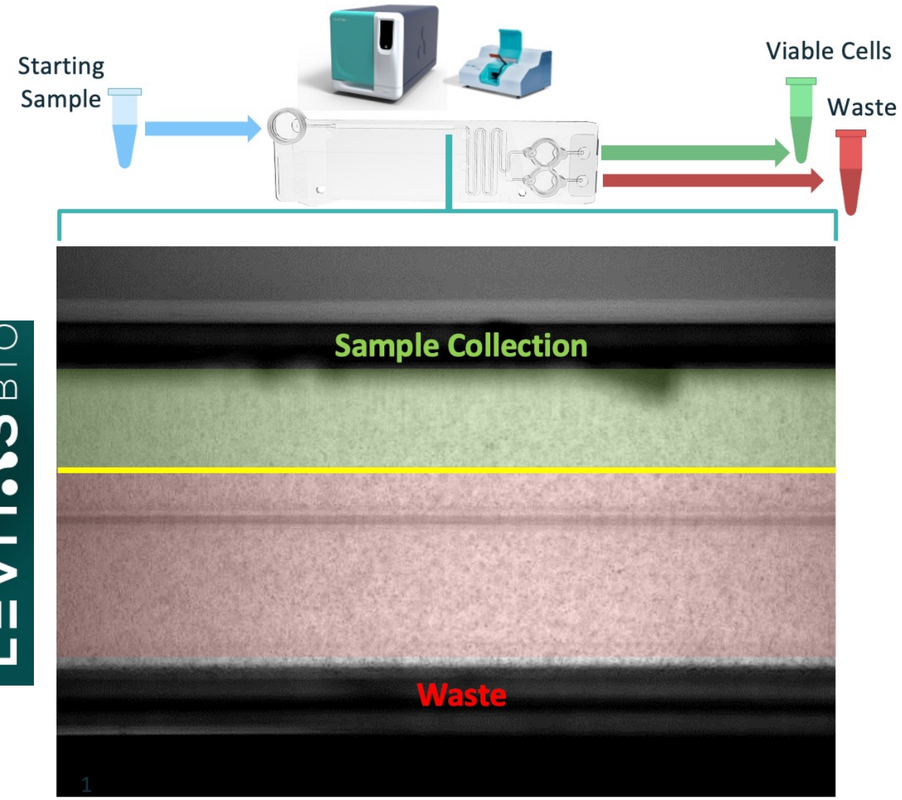
1



Cell Viability
(high >70%)

Dead cell removal,
Enrichment for live cells

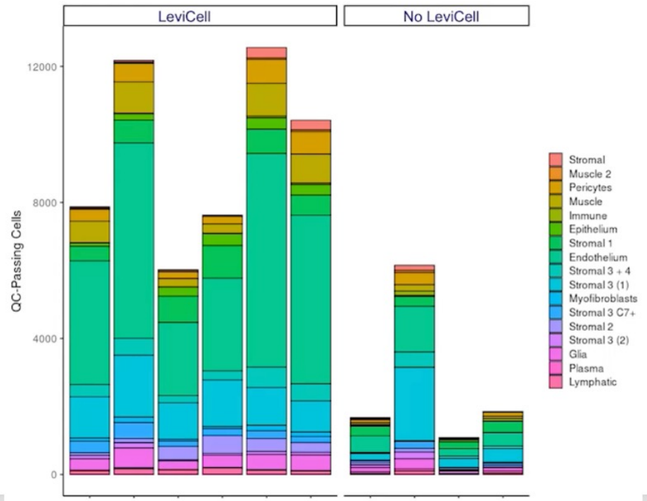
LeviCell gently enriches for viable cells improves SC data



Up to 5-fold increase in cell recoveries post-sequencing

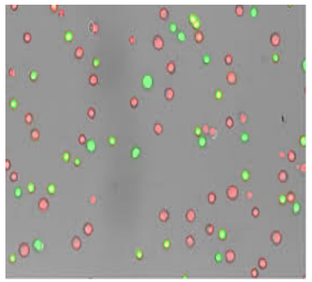
Dr. Agne Antanaviciute

"With particularly bad-quality samples….the platform [LeviCell] becomes really applicable because it can rescue projects that would otherwise fail."



Factors affecting sample preparation: cell viability

1



Cell Viability
(high >70%)

Dead cell removal,
Enrichment for live cells

Dead cell removal and any sort of enrichment comes at a cost!

1. How many dead cells are you removing?
2. What does this mean for the biology you are studying?
3. Are you recording this metadata?

Dead cells may increase the free-floating RNA in your prep
(check supernatant for RNA using e.g. Ribogreen Kit)

Low viability
Ambient RNA

Filter parameters:

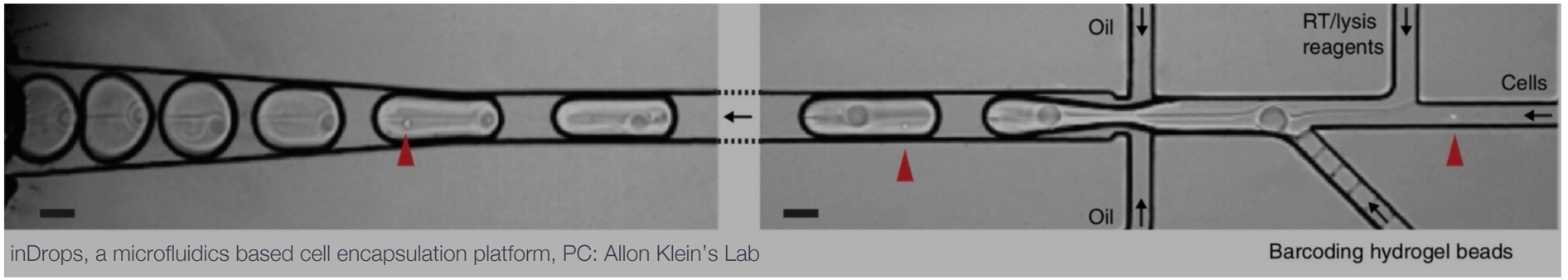
- nUMI > 500
- nGene > 250
- log10GenesPerUMI > 0.8
- mitoRatio < 0.2

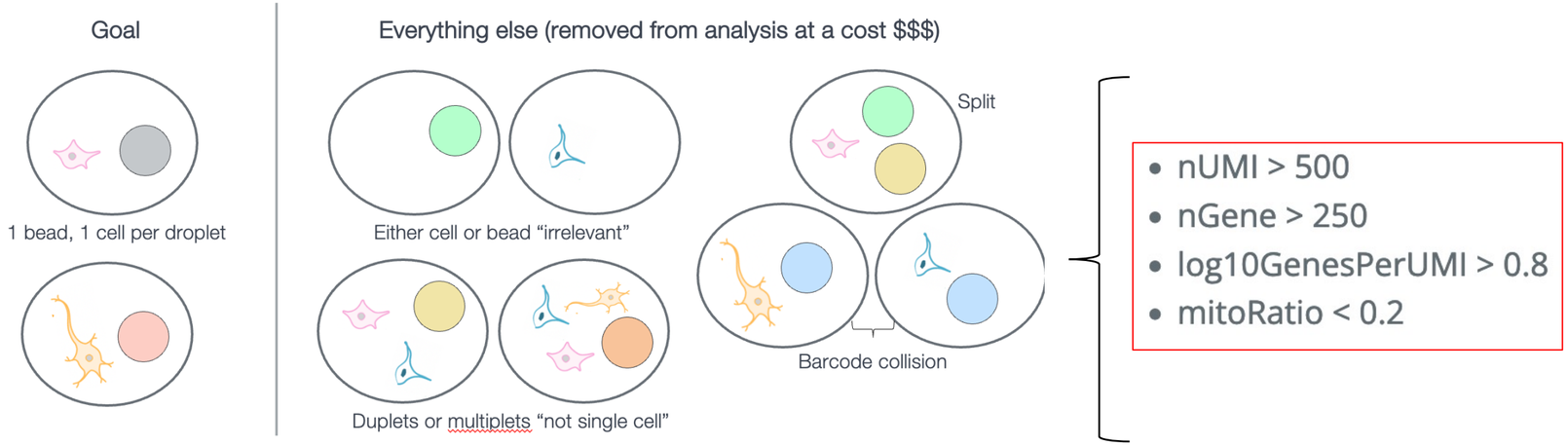
Factors affecting sample preparation: cell count

2

Cell Count
(accurate)

Single cell encapsulation follows Poisson's distribution (& tightly controlled math)

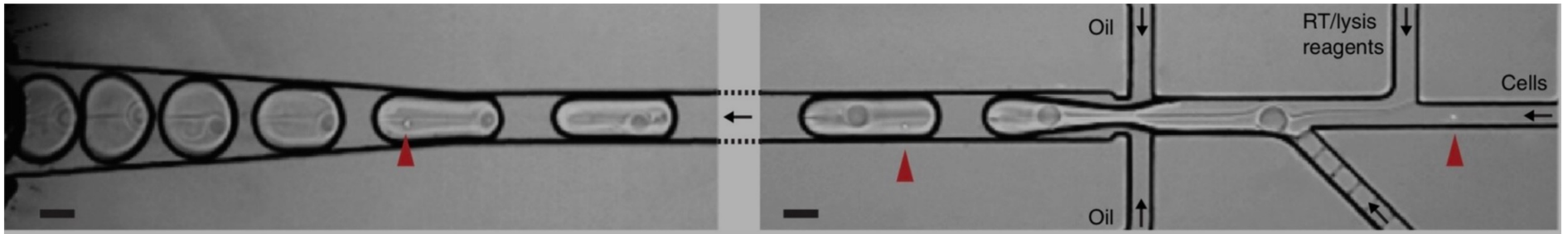




Factors affecting sample preparation: cell count

2

Single cell encapsulation follows Poisson's distribution

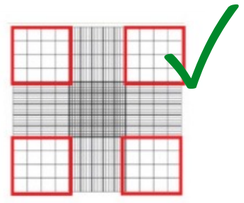


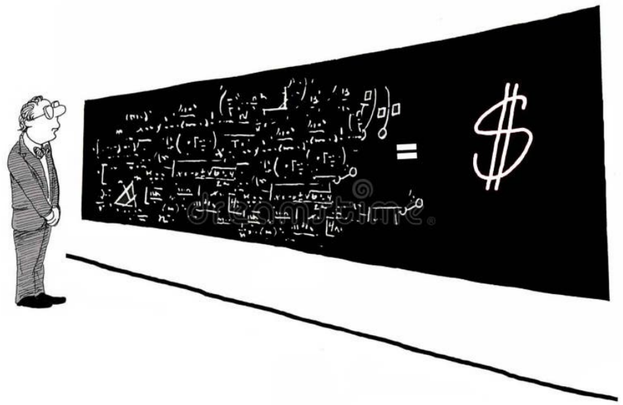
inDrops, a microfluidics based cell encapsulation platform, PC: Allon Klein's Lab

Cell Count (accurate)

Manual count at optimization

- Calibrate count by manual counting (hemocytometer)
- Cell sorters can overestimate by as much as 5-50%





Factors affecting sample preparation: time and temp

3

Time
(short)

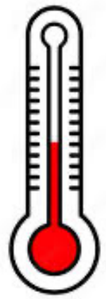
Less is more!

- Minimal handling
- Gentle protocol
- Reduce/arrest metabolic activity of cells
- Not induce extra stress response in cells (high mito)

- nUMI > 500
- nGene > 250
- log10GenesPerUMI > 0.8
- mitoRatio < 0.2

Simple protocol, minimal steps
1-3h

4



Temp.
(cold, 4C)

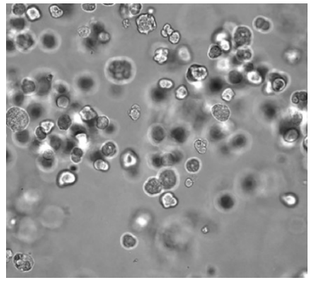
- RT accelerates cell death, clumping, results in “ambient RNA” or degraded RNA
- Ambient RNA creates noisy, unusable data, at high costs, and wasted $

- nUMI > 500
- nGene > 250
- log10GenesPerUMI > 0.8
- mitoRatio < 0.2

On ice,
RNAse-free

Factors affecting sample preparation: quality

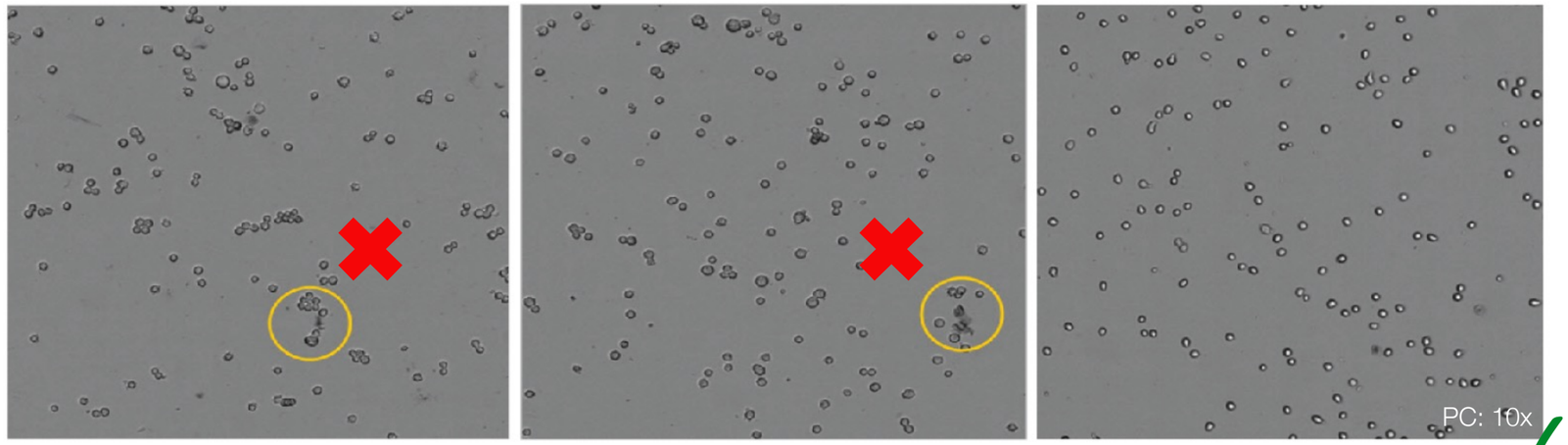
5



Quality
(no clumps/debris)

Use micron-filters,
Gentle pipette-mixing
or centrifugation (<400-500g, 4C),
Use Dnase,
Be quick

Your expt is not single-cell if there are clumps! (removed at filtering)



Cell aggregates

< 10% doublets ✓

Single cell platforms do not distinguish between live or dead cells, debris or clumps and will encapsulate everything, making your data noisy/unusable

- nUMI > 500
- nGene > 250
- log10GenesPerUMI > 0.8
- mitoRatio < 0.2

Common causes of a bad prep: too many!

- Long prep times (>3-4h)
- Harsh dissociation conditions or harsh handling
- Too many dead cells
- Debris
- Using wrong buffer/media
- Cell/Nuclear membrane damage

PRACTICE PRACTICE PRACTICE!

This is why the actual “scRNA-seq run day” should not be the 1st time you attempt the protocol

Wide variety of input samples compatible for scRNAseq-

decouple sample collection from sample processing

✓ Cryopreserved samples

- Success dependent on cell type
- Beware of data bias: some cells more prone to death upon thaw

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

Christian T. Wohnhaas, Germán G. Leparc, Francesc Fernandez-Albert, David Kind, Florian Gantner, Coralie Viollet, Tobias Hildebrandt & Patrick Baum

Cryopreservation with BAMBANKER™

✓ Snap-Frozen samples

- Segue into next slide
- Offers greater flexibility

'Frankenstein' protocol for nuclei isolation from fresh and frozen tissue for snRNAseq

Luciano Martelotto[1]

✓ Fixed samples

- Opens up a treasure trove!
- Offers greater flexibility

HONEYCOMB

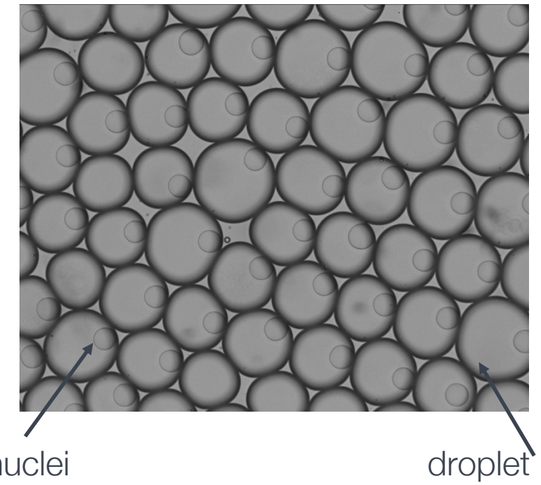
FixNCut: A Practical Guide to Sample Preservation by Reversible Fixation for Single Cell Assays

Method | Open access | Published: 08 April 2021

ACME dissociation: a versatile cell fixation-dissociation method for single-cell transcriptomics

single nuclei RNA-seq (snRNAseq): data comparable to scRNAseq

- Removes transcriptional noise from dead/dying cells
- snRNAseq most often used for



✓ difficult to isolate/dissociate samples e.g. neuronal samples

✓ low viability samples e.g. good for flash frozen clinical samples

✓ tissues problematic for sc-processing e.g. adipose tissue, where fat inhibits RT enz. or pancreatic tissue (high in RNAses)

✓ Cell types hard to get from single cell preparations or cells too big to encapsulate on microfluidic platforms

✓ ATAC (to study the epigenome)/Multiome studies (to study the epigenome along w/ transcriptome)

Same QC metrics to evaluate sample prep

PC: Grindberg et al., 2013

Garbage in, Garbage out, every single time

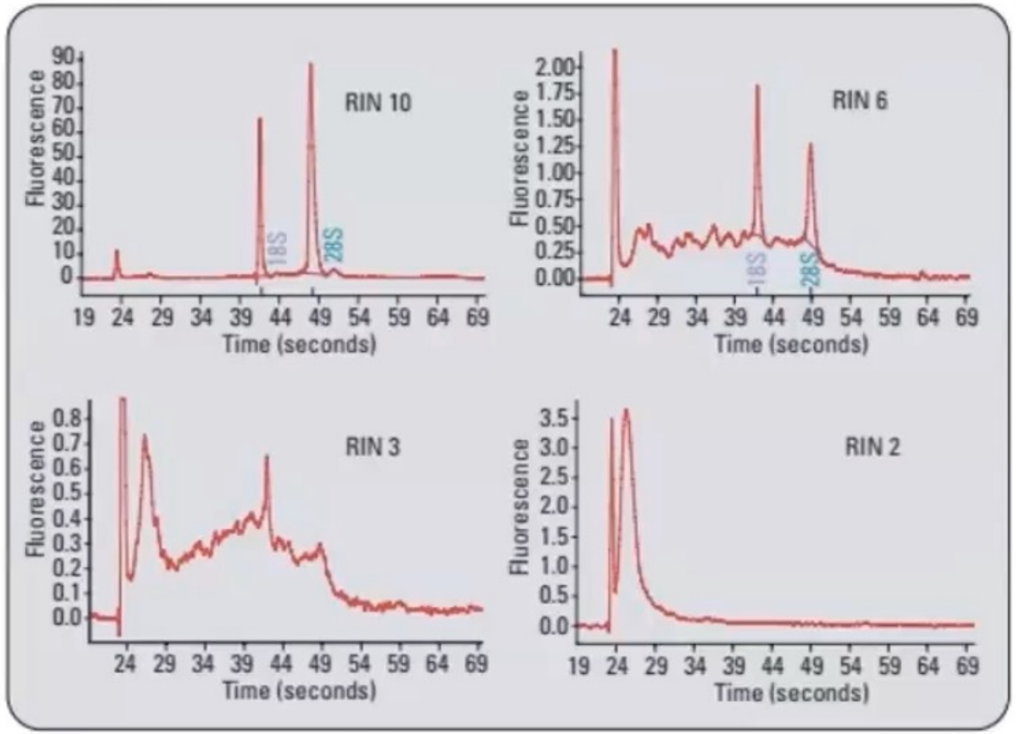
Poor quality input (cells) contributes to poor quality output (data) in scRNAseq!

The RIN score: RNA integrity as final check at optimization

RIN stands for “**R**NA **I**ntegrity **N**umber”,
i.e. how degraded is the RNA in your sample(s)

RIN 7-10 (Proceed), RIN < 3 (no go)

Informative for:
Assessing prep quality
Predicting data quality



PC: blog.genohub.com

Do a (small scale) pilot experiment

pilot is key to success (closes gaps between theory & practice)

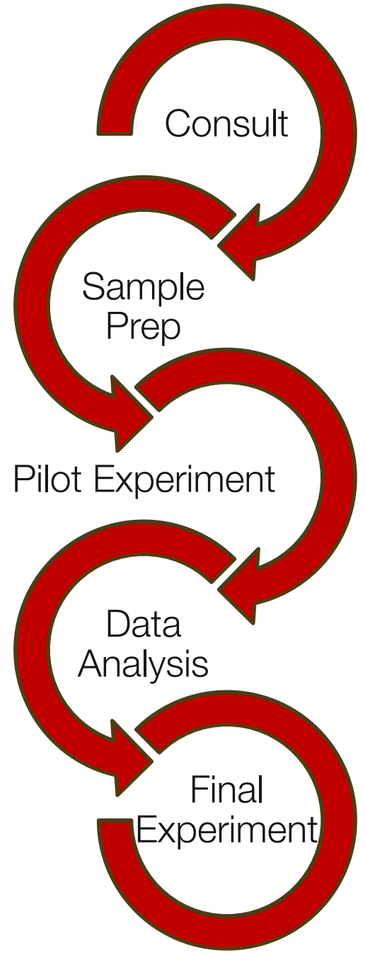
- Do not rush to the final experiment
- A well-planned pilot experiment is essential for

✓ tweaking or redefining bio. objectives

✓ providing rational expt design/optimal approach for research Q

✓ evaluating sample preparation

✓ figuring our the required number of cells needed statistically to answer your biological question



A pilot will warn you about the sources of technical noise (before you've spent big money)

"Technical Noise": When non-biological, technical factors cause changes in the data produced by the expt. leading to wrong conclusions

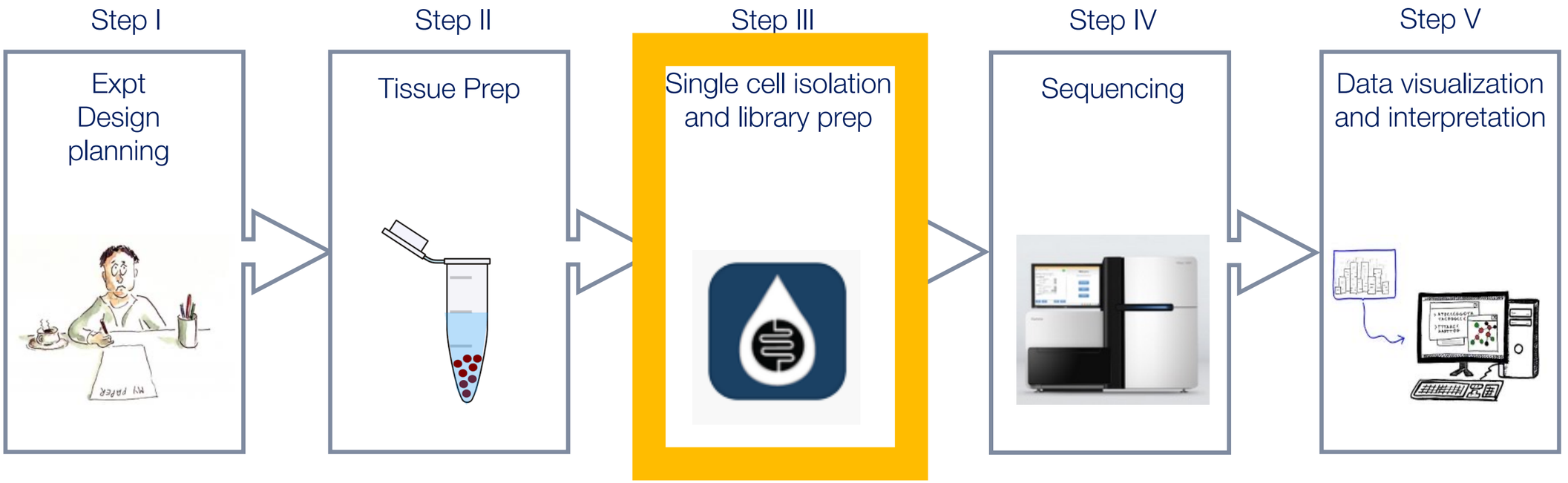
Single cell expts prone to technical noise

2 kinds of technical noise -

- from experimental designs and handling (e.g. different personnel, reagent lots, PCR amp cycles, equipment, protocols etc) -> "Batch effect correction"
- from sequencing (e.g. library prep, GC content, amp bias etc) -> "Normalization"

- nUMI > 500
- nGene > 250
- log10GenesPerUMI > 0.8
- mitoRatio < 0.2

Steps in a scRNAseq work flow



Goal: Capture and isolate single cells on a suitable platform, & prep libraries

Scale impacts technology choice ↔ Technology choice impacts scale

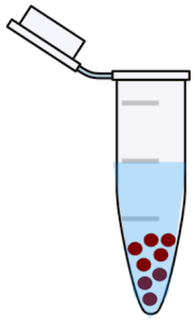
Low Through-put approaches

High Through-put approaches

1st Gen

2nd Gen

3rd Gen



Manual

Plate/Wells

Liquid handlers

Microfluidics/Droplet

1-few cells | 100-1000s cells | 1000s-100K+ cells | 1M cells

Minimal equipment
Cost effective
Scale prohibitive

Sophisticated equipment
Cost prohibitive
Scale effective

Scale impacts technology choice ↔ Technology choice impacts scale

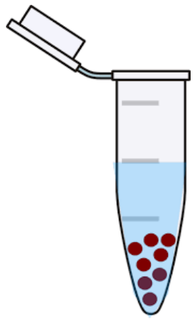
Low Through-put approaches

High Through-put approaches

1st Gen

2nd Gen

3rd Gen



Manual

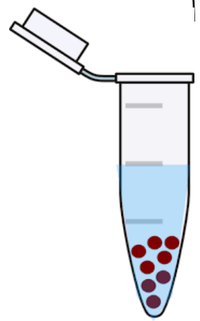
BD

Plate/Wells

Liquid handlers

Microfluidics/Droplet

10x GENOMICS



Equipment free

Parse BIOSCIENCES

Fluent BioSciences

1-few cells → 100-1000s cells → 1000s-100K+ cells → 1M cells

Minimal equipment
Cost effective
Scale prohibitive

Today's science demands scale

Minimal equipment
Cost effective
Scale effective

Scale impacts technology choice ↔ Technology choice impacts scale

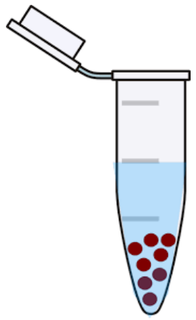
Low Through-put approaches

High Through-put approaches

1st Gen

2nd Gen

3rd Gen



Manual

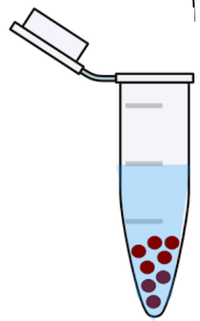
BD

Plate/Wells

Liquid handlers

Microfluidics/Droplet

10x GENOMICS



Equipment free

Parse BIOSCIENCES

Fluent BioSciences

1-few cells → 100-1000s cells → 1000s-100K+ cells → 1M cells

Minimal equipment
Cost effective
Scale prohibitive

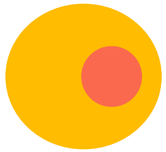
Ideal method does not exist

- nUMI > 500
- nGene > 250
- log10GenesPerUMI > 0.8
- mitoRatio < 0.2

Minimal equipment
Cost effective
Scale effective

Structure and scRNA-seq library preparation (just an illustrative example)

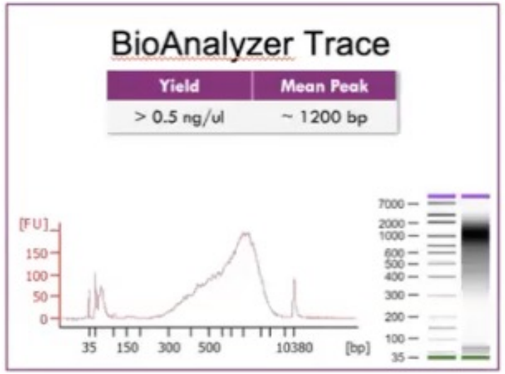
1. Isolate single cell and lyse



2. mRNA capture by PolyA tail

3. Convert mRNA into cDNA using RT and amplify cDNA, QC

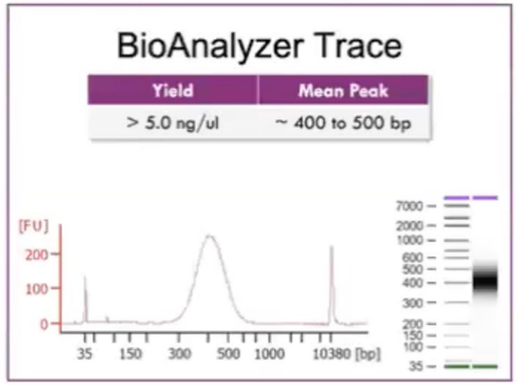
Amplified cDNA



- Quantify
- Size
- contam

4. Fragment cDNA, add adaptors for Illumina sequencing

Index-PCR Library

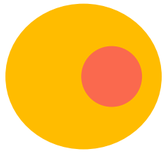


- Quantify
- Size
- contam

5. PCR amplify library, QC, pool and sequence

Structure and scRNA-seq library preparation (just an illustrative example)

1. Isolate single cell and lyse



2. mRNA capture by PolyA tail

3. Convert mRNA into cDNA using RT and amplify cDNA, QC

4. Fragment cDNA, add adaptors for Illumina sequencing

5. PCR amplify library, QC, pool and sequence

Library structure is sc-platform, kit & sequencing platform dependent

Lib prep is the biggest source of batch effect

- nUMI > 500
- nGene > 250
- log10GenesPerUMI > 0.8
- mitoRatio < 0.2

- When possible, prep all libs together
- Prep control and treatment together, per run, so you can normalize

Parallel, multimodal assays to add layered info to scRNAseq data

Multiple libraries from same sample for multimodal sc-analysis:

- scRNAseq (transcriptome) + scATACseq (epigenome)
- scRNAseq (transcriptome) + CITEseq/Hashing/VDJ (surface proteins)
- scRNAseq (transcriptome) + DNA (genome)

More informative data at same or lower cost!
But expt has to be designed as such at the beginning

Mix-n-match

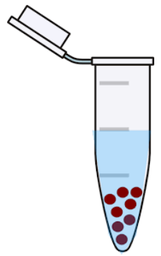
Steps in a scRNAseq work flow

Step I

Expt Design planning

Step II

Tissue Prep



Step III

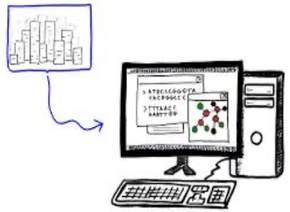
Single cell isolation and library prep

Step IV

Sequencing

Step V

Data visualization and interpretation



Goal: sequence your libraries on the appropriate platform

Step IV: Sequencing platforms for scRNAseq

(current) common compatible single cell sequencing platforms – NextSeq and NovaSeq

More output
Simple benchtop
Affordable & low cost
Fast data turnaround

Advantages	Power of high-throughput sequencing with the simplicity and affordability of a benchtop system	Unprecedented output and throughput
Ideal for	Mid- to high-throughput sequencing applications and average scale single-cell sequencing studies, such as studies to profile cell function in both development and disease.	Extensive screening studies, such as pharmaceutical screens and cell atlas studies.

PC: Illumina

Step IV: how much should one sequence?

Sequencing depth dependent on sample type and experimental objective

Table 8: Recommended reads for different single-cell sequencing applications

Method	Recommended no. of reads[a]
3′ gene expression	15K–50K reads per cell
5′ gene expression ✓	50K reads per cell
Antibody sequencing	100 reads per antibody/cell
scATAC-Seq	50K reads per nuclei
5' TCR/BCR	5K reads per cell
Takara SMARTer	1M–2M reads per cell (> 300,000 reads per cell)

The recommended number of reads is based upon manufacturer recommendations

Reference: 10x sequencing guideline

Step IV: choosing a platform

Example: You have 4 samples & you have barcoded 10K cells each for 5'GEX = 40K barcoded cells
40K x 50,000 reads/cell = 1 Billion total reads needed

NovaSeq 6000 System

Flow Cell Type	SP ✓	S1	S2	S4
Single-end Reads	650–800 M	1.3–1.6 B	3.3 B–4.1 B	8-10 B
Paired-end Reads	1.3–1.6 B ✓	2.6–3.2 B	6.6–8.2 B	16–20 B

Shallow seq

- nUMI > 500
- nGene > 250
- log10GenesPerUMI > 0.8
- mitoRatio < 0.2




Step IV: choosing a platform

Example: You have 4 samples & you have barcoded 10K cells each for 5'GEX = 40K barcoded cells
40K x 50,000 reads/cell = 1 Billion total reads needed

1 slice of bread does not need an entire jar of peanut butter!
Similarly, you don't need sequencing-overkill on your sample

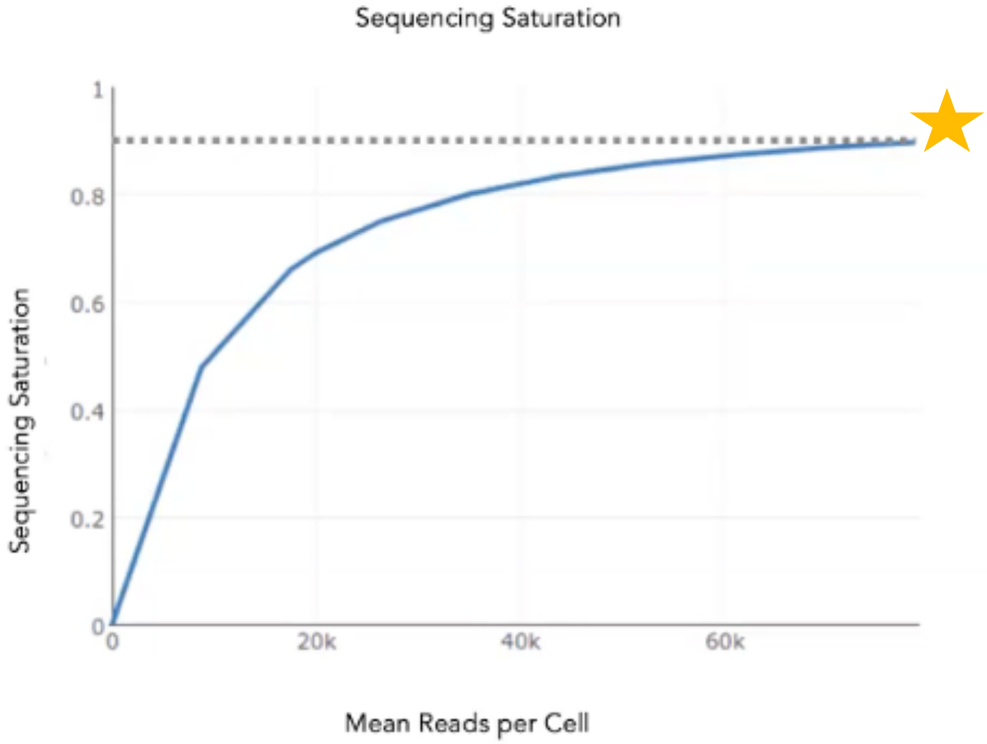
Step IV: dialing in on sequencing saturation

How to know what's sequencing over-kill?

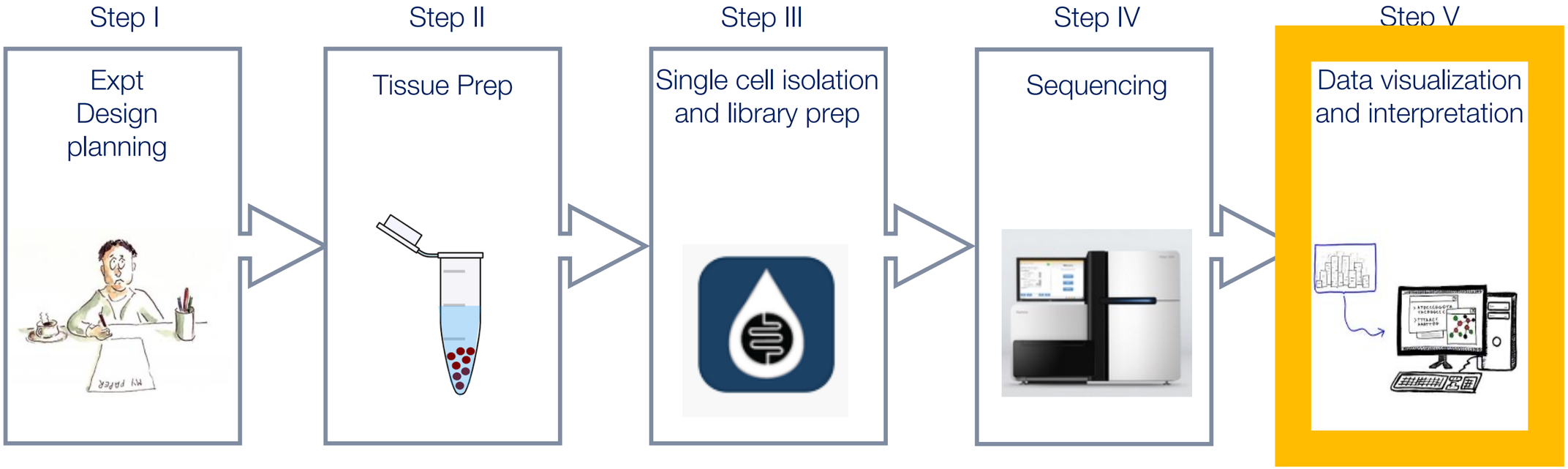
★ Seq saturation = $\frac{\text{\# of unique mRNA detected}}{\text{\# of total reads}}$

- Differs by RNA amount per cell type (cell type dependent)
- Depends on sample metrics – how many cells barcoded, what is rarest cell population of interest?
- Rarer the cell type (or transcript), more sequencing needed

You can always re-seq your libraries!

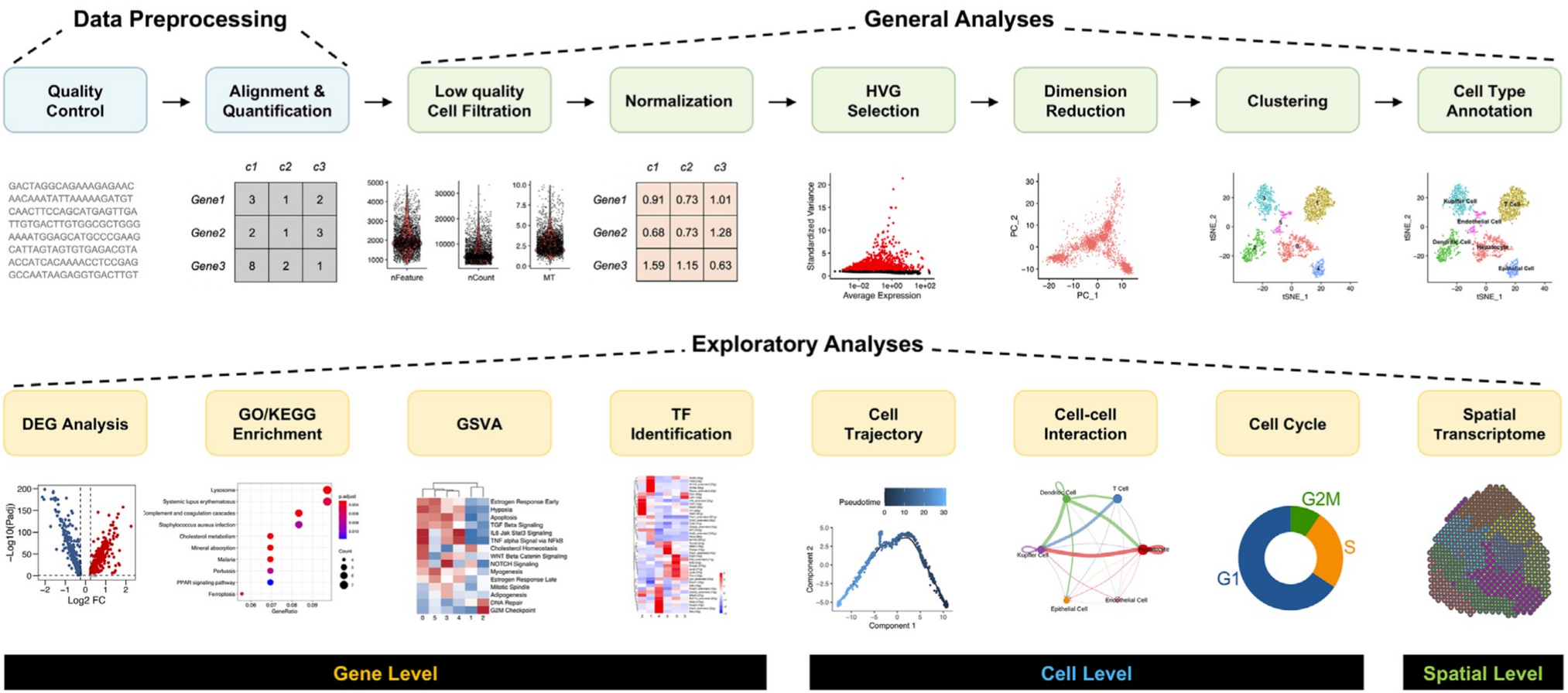


Steps in a scRNAseq work flow



Goal: analysis, interpretation and visualization of data!

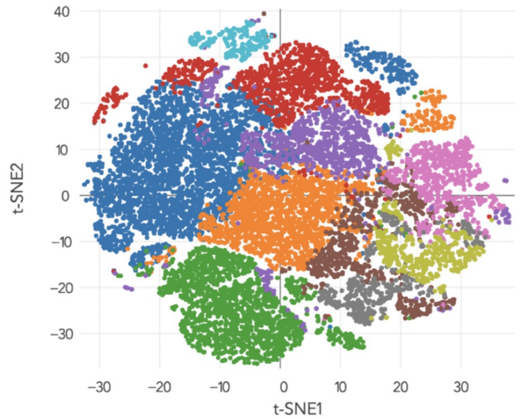
Key analysis steps in scRNAseq analysis



Kharchenko lab., 2021

Pipelines for data visualization and interpretation

A wealth of bioinformatic tools are available for scRNA-seq analysis:



Publication-worthy figs

Interpretation

Pipelines for data visualization and interpretation

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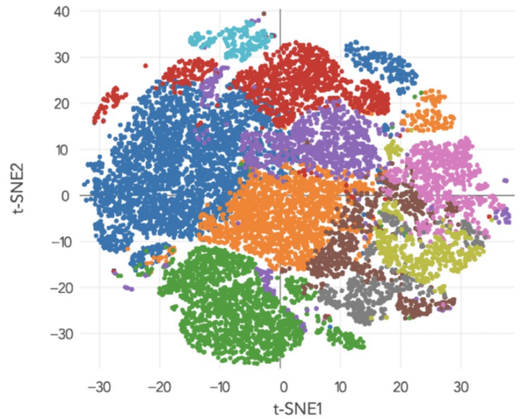


Publication-worthy figs

Interpretation

Leading pipelines for scRNAseq analysis-

- **Seurat** (Satija Lab, 2015): written in the programming language R
- **Scanpy** (Theis Lab, 2017): written in the programming language Python

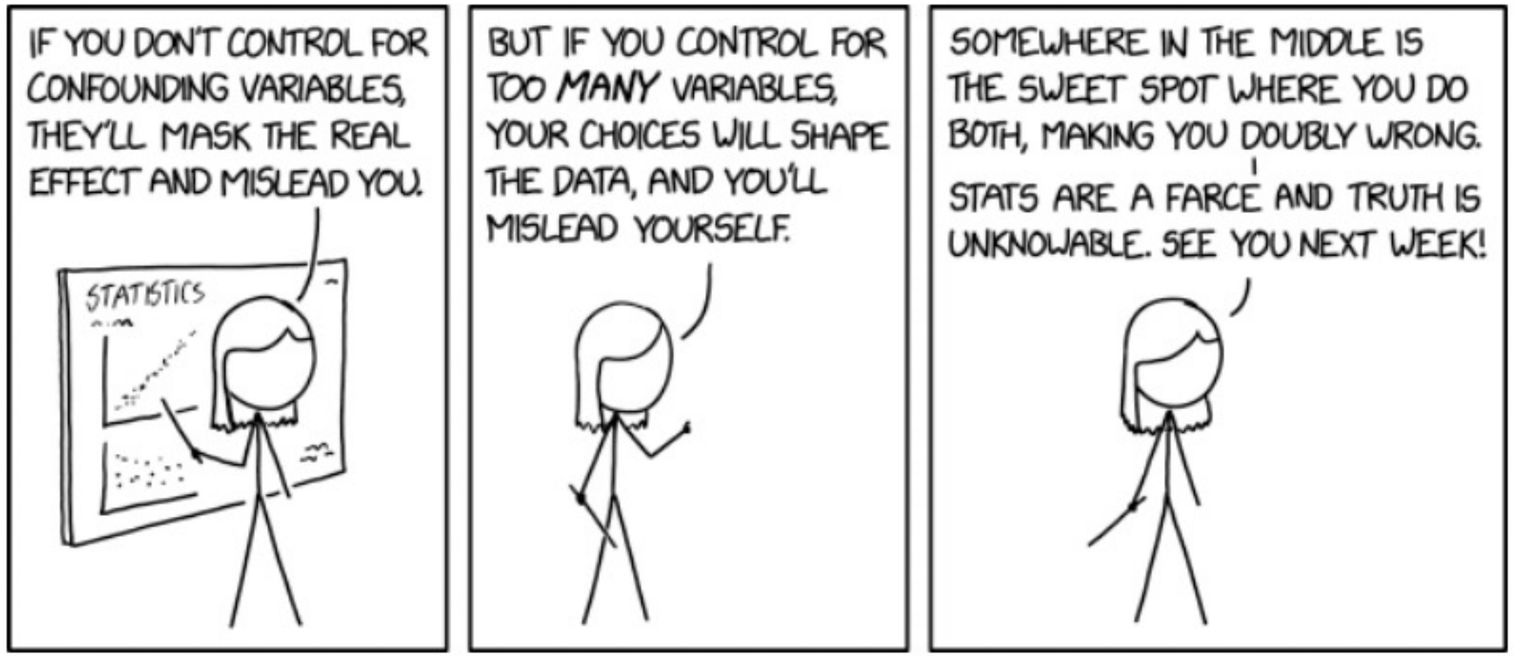
More tools do not translate to more understanding of biology

SEURAT

scanpy

scvi-tools

Monocle 3



Choice boils down to the user's programming preference

Implicit assumption: choice among packages and versions has little to no impact on the interpretation of results **(proven wrong)**

Key takeaways!

Summary –

- (I know it seems daunting) You can do this!
- Plan your experiments, put in effort into your sample prep. It pays off at data analysis

  Just remember: **Garbage in, Garbage out**
- Pay attention to doing informed, responsible, transparent and reproducible analyses
- Talk to experts – this is a fast evolving field (technology, methodology and computationally)

Thank you! Got Questions?

Get (stay) in touch!

For consultations, trainings and questions:

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Citation and use:
hbctraining/scRNA-seq_online: scRNA-seq Lessons from HCBC (first release). Zenodo. https://doi.org/10.5281/zenodo.5826256