Analysis of scRNA-seq using R

Annamaria Carissimo, Monika Krzak, Dario Righelli, Claudia Angelini
Istituto per le Applicazioni del Calcolo “Mauro Picone”, CNR, Naples, Italy.

Nettab+BBCC 2019 Tutorial on Analysis of scRNA-seq using R, Fisciano (SA), November 14, 2019

Outline and organization

- Introduction to **scRNA-seq**
- Overview on scRNA-seq **data analysis**
- **GitHub** training material and datasets
- **Step-by-step** data example

Claudia

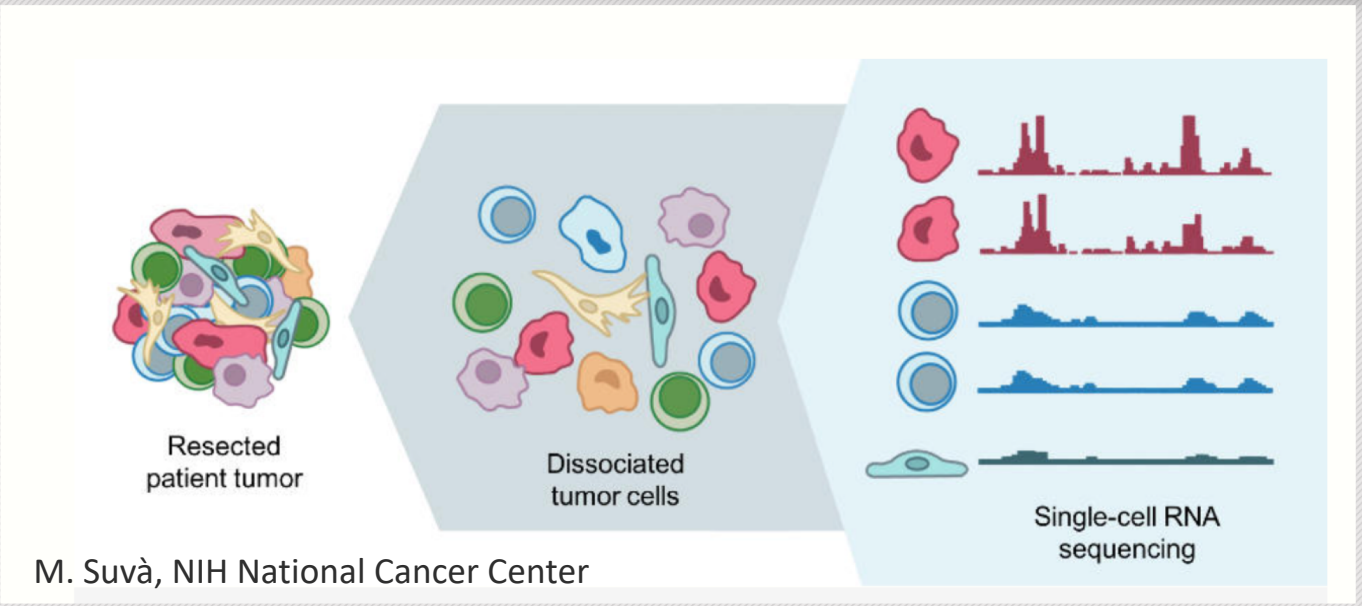
Dario, Annamaria, Monika,

- **Try yourself** (after coffee breaks …*in groups*)

- Present your **results** (afternoon)
- Let's do a joint **discussion** and **question time** (afternoon)
- Some **advanced applications**

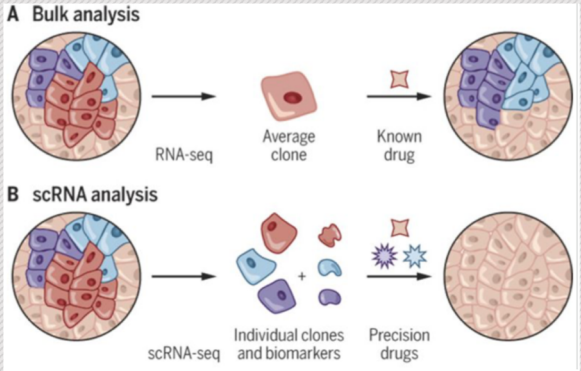
Introduction to scRNA-seq

Why scRNA-seq?



M. Suvà, NIH National Cancer Center

Shalek and Regev (2016)



scRNA-seq could play a key role in personalized medicine by facilitating characterization of cells, pathways, and genes associated with human diseases such as cancer. Sci Transl Med. 2017

What can I investigate with scRNA-seq?

Most relevant scRNA-seq **applications** include

- Study cellular **heterogeneity**
- Discover n**ovel** cell populations
- Predict **cell fate** differentiation

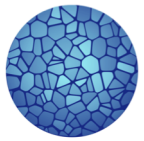
} By-cell analysis

- Detect cell-type specific **differentially** expressed genes
- Understand cell-type specific **regulatory** networks

} By-gene analysis

Other applications include

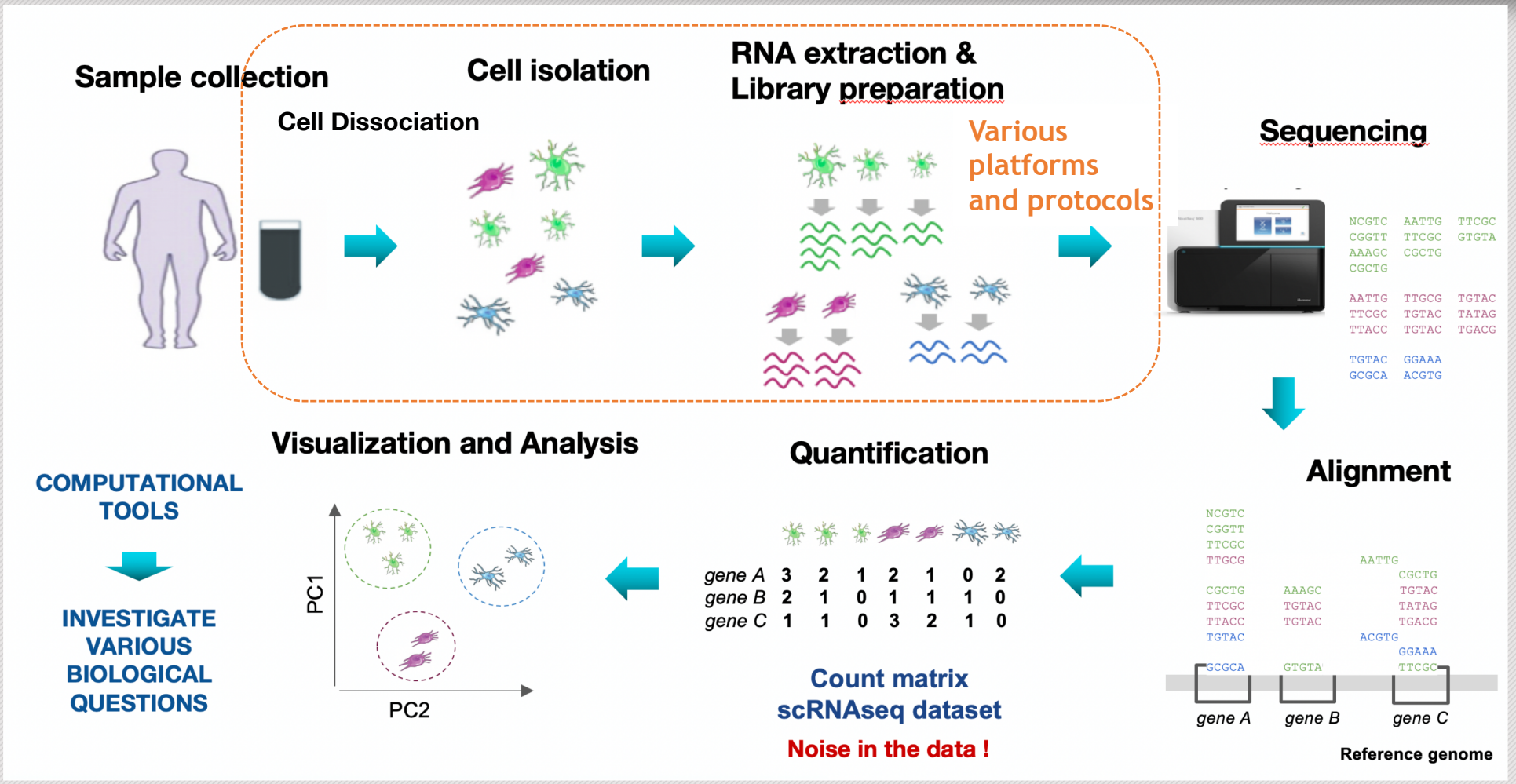
- **Cell Atlas**: Human Cell Atlas, Mouse Cell Atlas,….



HUMAN CELL ATLAS

To create comprehensive reference maps of all human cells—the fundamental units of life—as a basis for both understanding human health and diagnosing, monitoring, and treating disease.

ScRNA-seq overview



Wet experimental phase

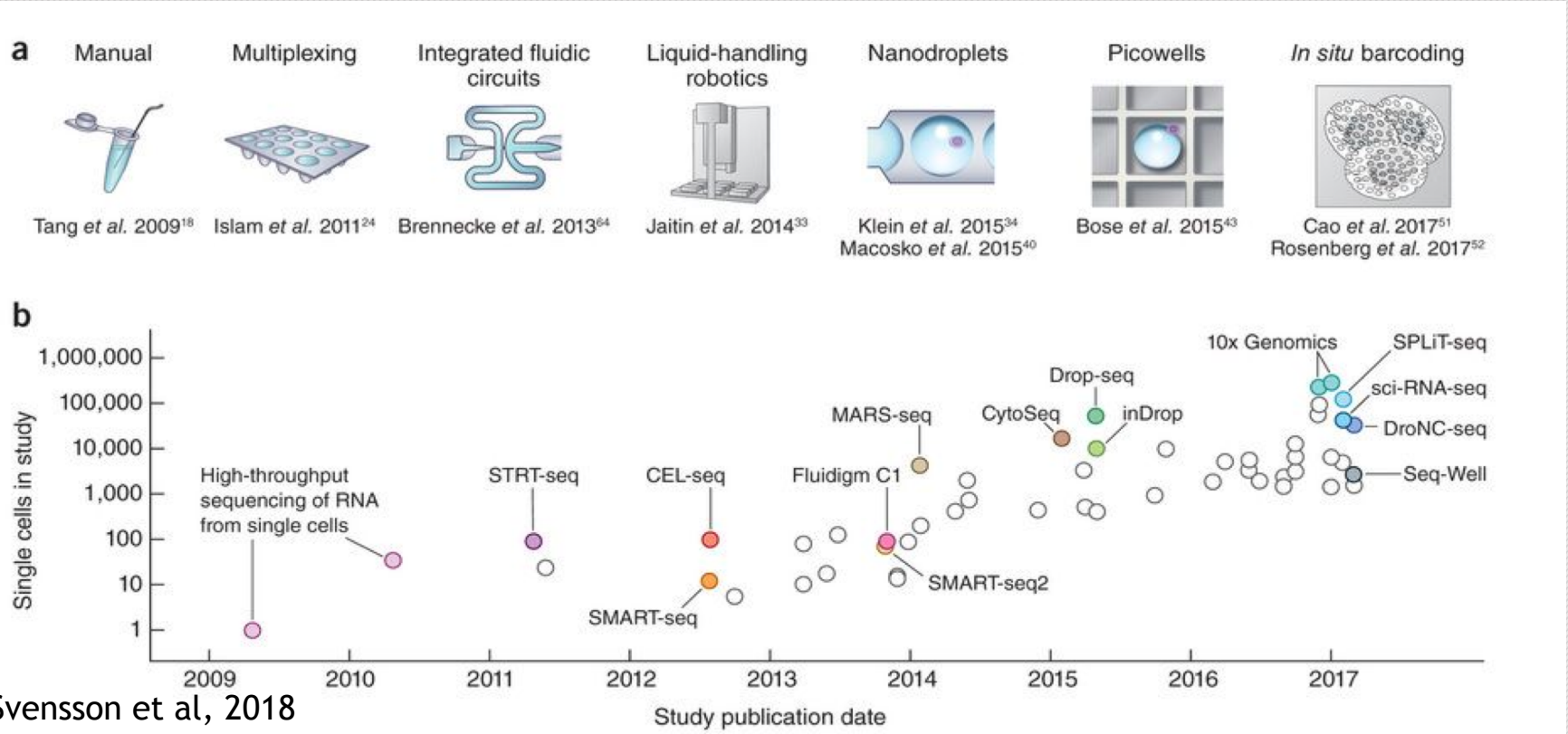
Platforms isolate cells, extract mRNA, and prepare libraries for sequencing

Protocols refer to the specific chemistries used to prepare sequencing libraries

platforms may be protocol-specific (proprietary) or allow multiple protocols (open-source)→**platform+protocols =systems**

Data Analysis phase

ScRNA-seq experimental systems



Svensson et al, 2018

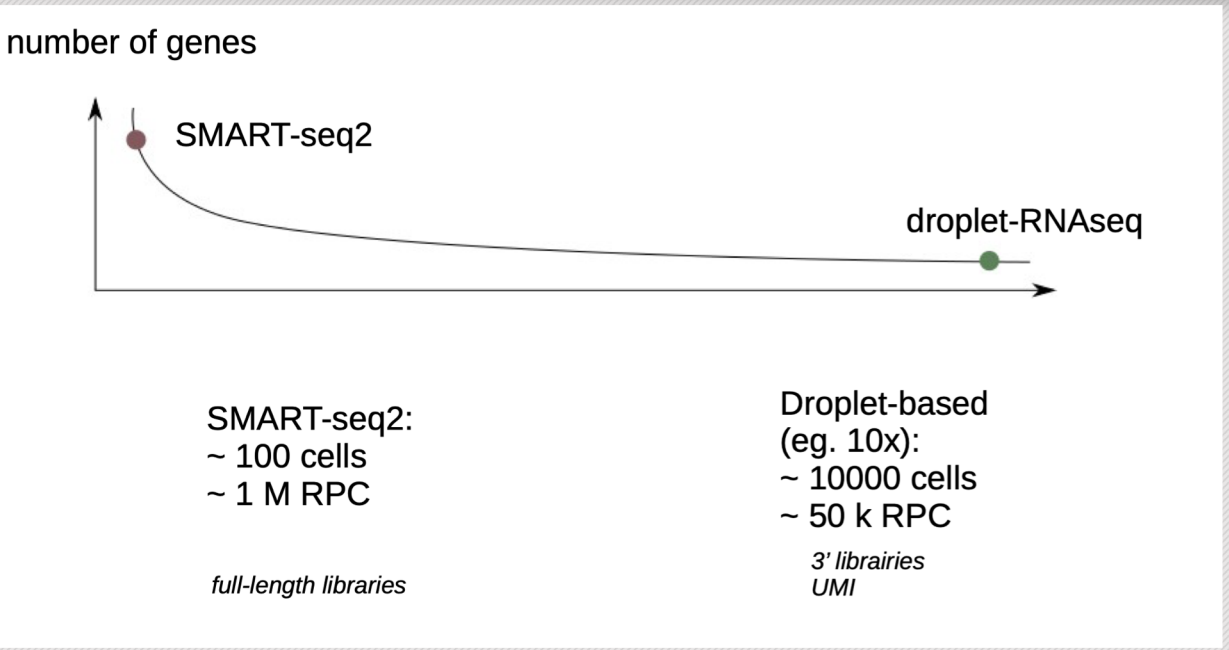
Systems differ with respect to the tecnology and chemistry used to dissociate, isolate cells, extract RNA and prepare the library.

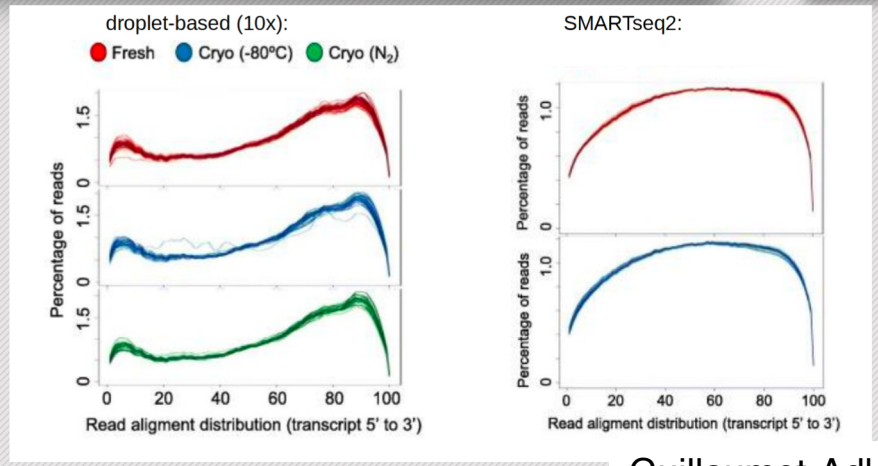
Different systems:

- Low vs high cell throughput
- Read count vs UMI
- Full lenght vs 3'/5' coverage
- Sequencing depth and accuracy
- RNA capturing efficiency
- Multiplexing
- Others….

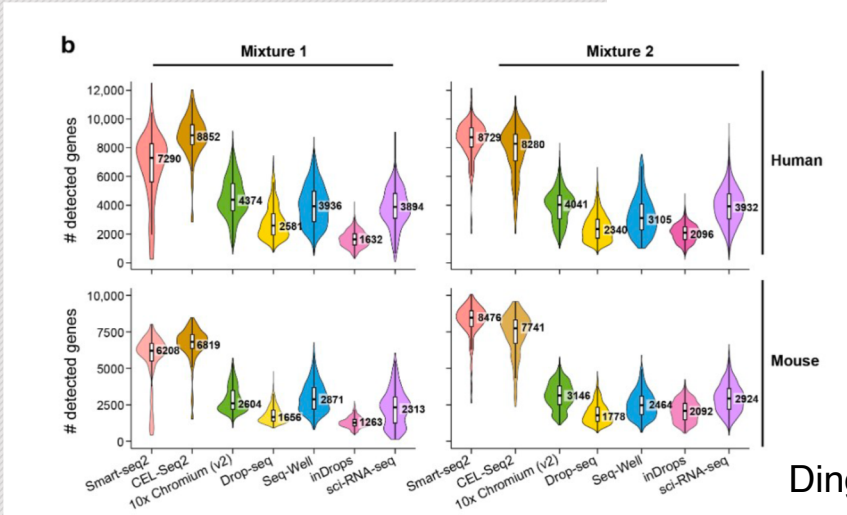
Nowadays, droplets technology allows to process up to tens of thousands of cells together.

ScRNA-seq experimental systems





Guillaumet-Adkins et al 2017

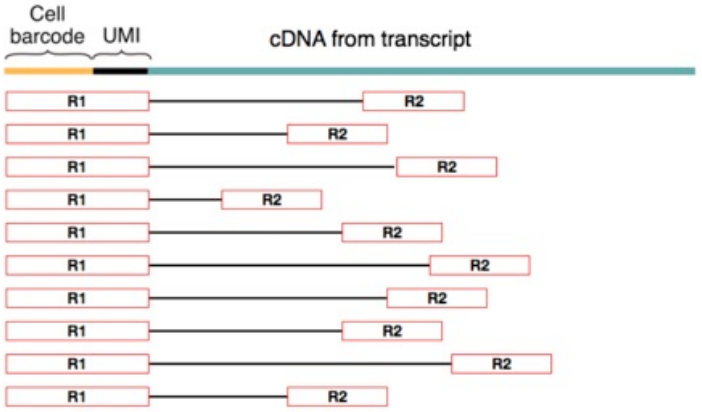


Ding et al 2019

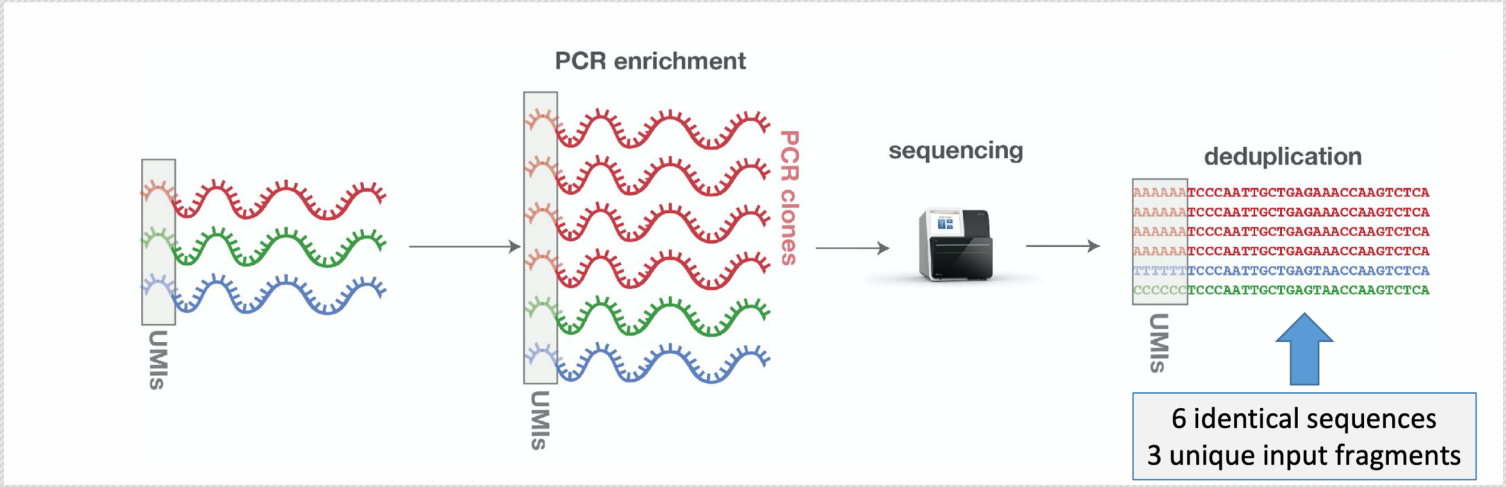
- The choice of the system has a big impact on data analysis (i.e., **counts type, noise, data size**)
- **Not all questions** can be investigated with all systems (isoform abundance requires full lenght sequencing)

Unique Molecular Identifiers (UMIs)

- PCR introduces **nonlinear** amplification bias
- **UMI**s are a way to tag each unique molecule in the sequencing library (before PCR)
- Number of possible UMIs = **4^L**, where L is the length of the UMI
- Low L or sequencing errors can cause barcode collisions
- Afterward, sum up only distinct UMIs (collapse reads)



Biased paired-end reads.



Issues in scRNA-seq systems

- Transcriptional bursting
- Dropouts
- Amplification bias
- Doublets and/or empty wells/droplets
- Dying cells, damaged or apoptosis
- Broken mebrane cells
- Ribosomal proteins
- Bias due to cell cycle, cell size, and other unwanted factors
- Strong batch effects and background noise that require to be adjusted

scRNA-seq are much more noisy than bulk RNAseq….they also are very sparse

But you have much more cells ….and hopefully good Bioinformaticians

Orchestrating Single-Cell Analysis with Bioconductor

Robert A. Amezquita, Vince J. Carey, Lindsay N. Carpp, Ludwig Geistlinger, Aaron T. L. Lun, Federico Marini, Kevin Rue-Albrecht, Davide Risso, Charlotte Soneson, Levi Waldron, Hervé Pagès, Mike Smith, Wolfgang Huber, Martin Morgan, Raphael Gottardo, Stephanie C. Hicks

doi: https://doi.org/10.1101/590562

Overview on scRNA-seq data analysis

Current best practices in single-cell RNA-seq analysis: a tutorial

Malte D Luecken, Fabian J Theis

Identifying cell populations with scRNASeq

Tallulah S. Andrews, Martin Hemberg*

Wellcome Trust Sanger Institute, Hinxton, Cambridgeshire, UK

Challenges in unsupervised clustering of single-cell RNA-seq data

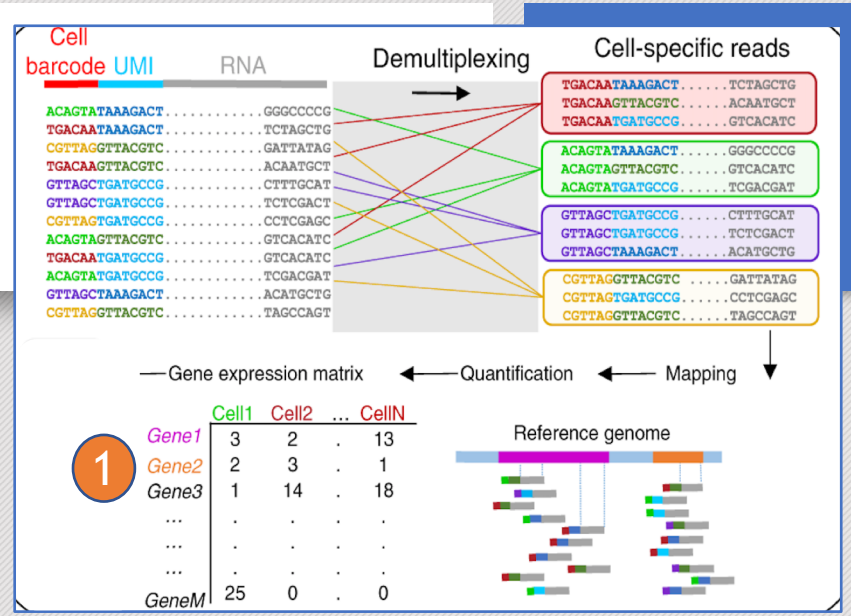
Vladimir Yu Kiselev, Tallulah S. Andrews & Martin Hemberg

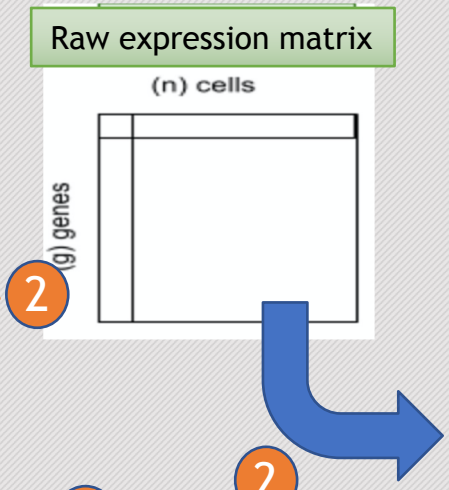
Using single-cell genomics to understand developmental processes and cell fate decisions

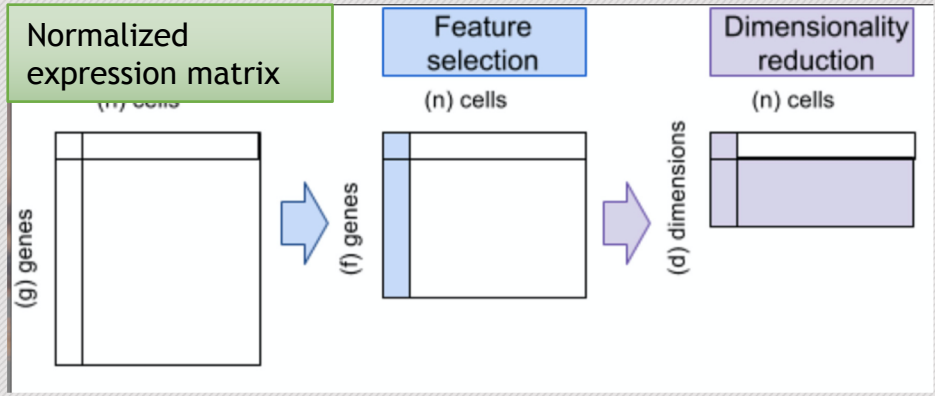
Jonathan A Griffiths[1], Antonio Scialdone[2,3,4,5] & John C Marioni[1,2,6,*]

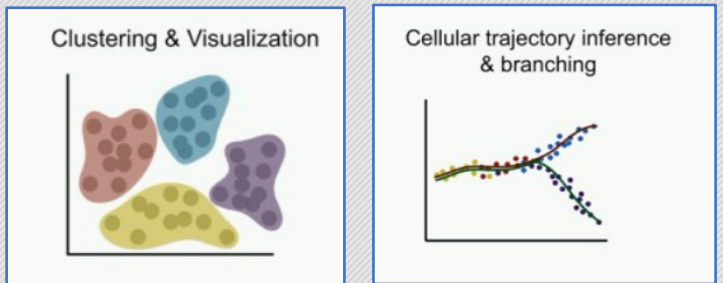
scRNA-seq data analysis overview

- **Read alignment**
- **Count matrix**
- **Quality control** and **filtering**
- **Normalization** and **Batch effect removal**
- **Feature selection** and **dimension reduction**
- **Downstream analysis** (clustering, trajectory inference, differential expression,…)
- **Interpretation** (cell type identification, marker detection, novel cell type functions, etc…)



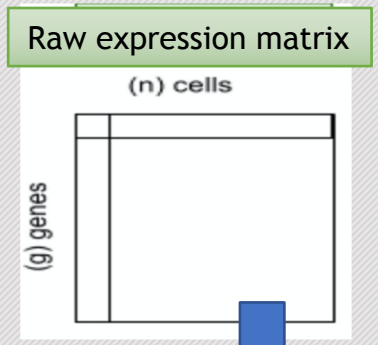


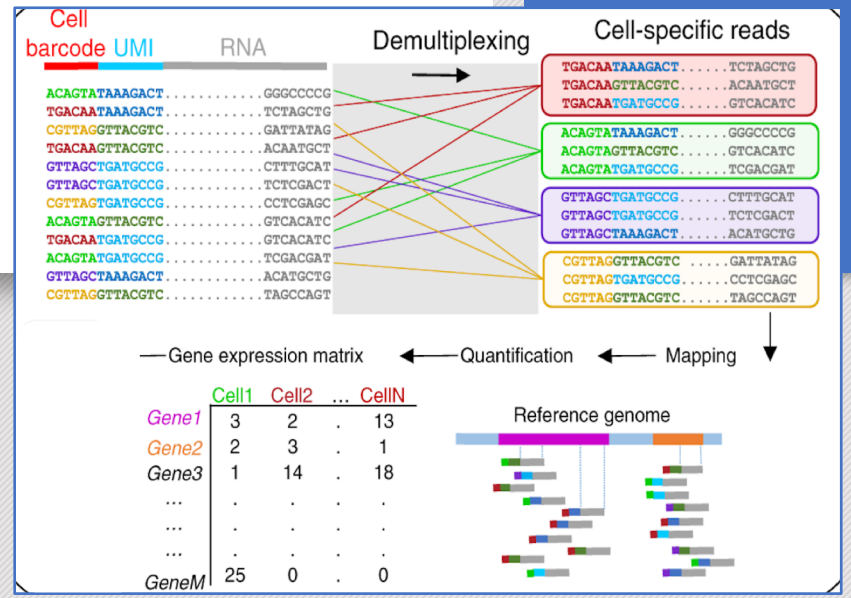


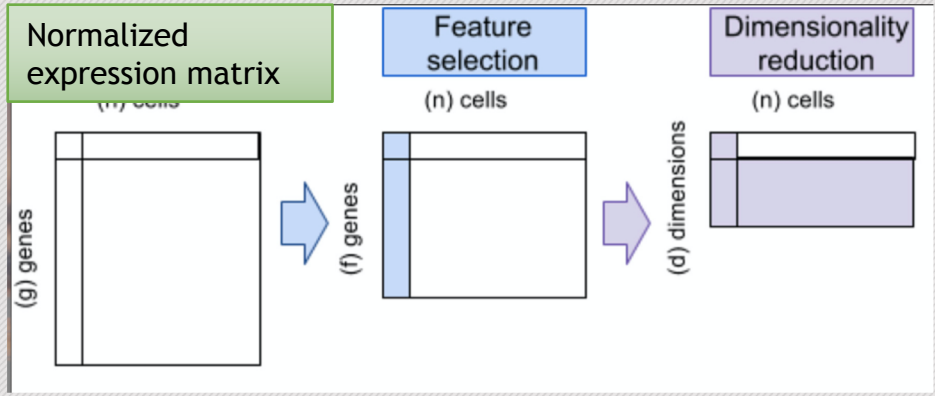


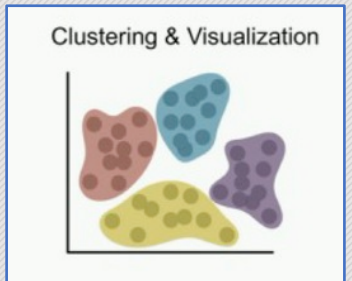
scRNA-seq data analysis overview

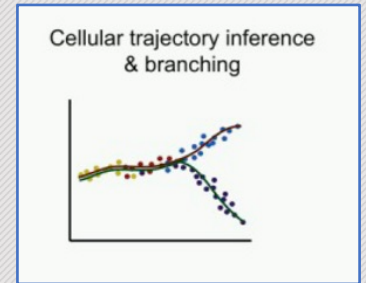
- The **pipelines** depend on the system, raw count types, downstream analysis of interests, specific problems and confounders in the dataset, dataset size, etc
- Some steps can be omitted, others can be combined or are hidden in a more comprehensive procedure
- **Visualization** and **data exploration** is fundamental





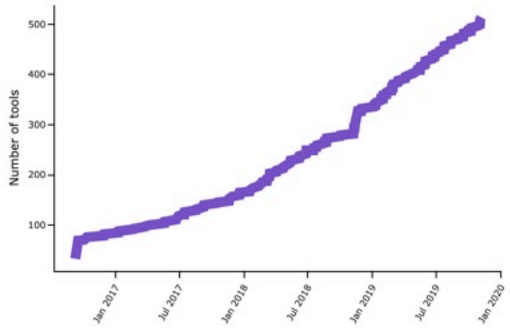




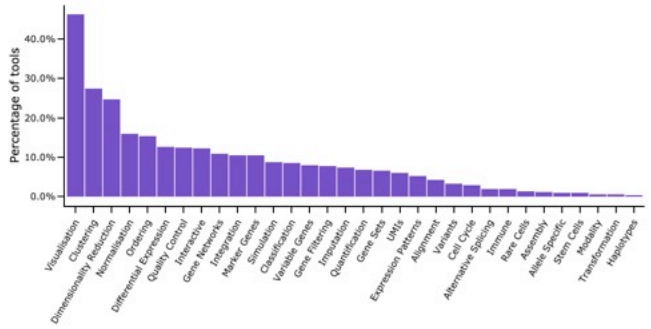


Computational challenges in scRNA-seq

- Still lack of '***golden***' standard due to relatively immaturity of the field
- High number of **tools** available (**>400**) and great variety of **programming languages**
- Very **complex pipelines** that integrate several **steps**
- The analysis depends on the **question of interest.** i.e., heterogeneous populations, rare cell identification, cell composition, trajectory inference, differential expression…
- Exploding **dataset size** due to improvement of technologies and decrease of costs
- **Heterogeneous noise** structure, extremely sparse (**zero-infated**)



We currently track **514** tools...

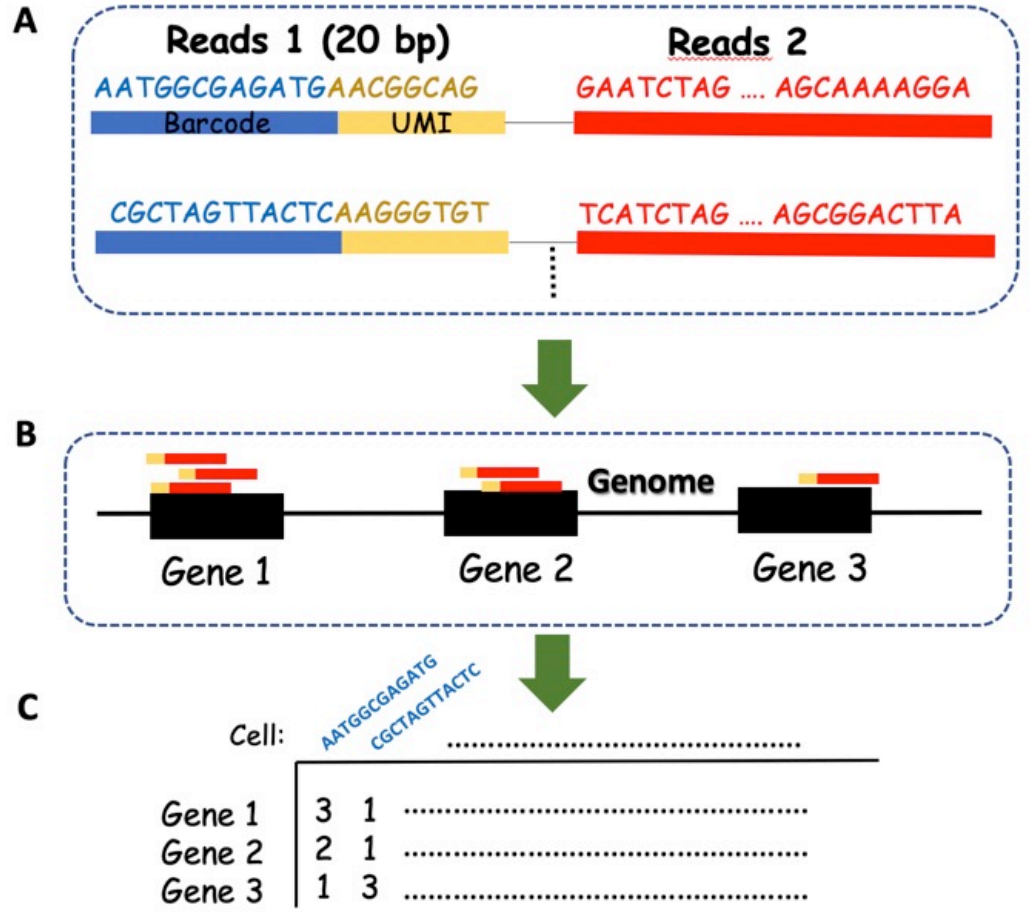


...in over **30** categories

https://www.scrna-tools.org

From sequences to raw counts

- Inspect sequence quality (**FastQC**)
- Remove reads with low phred quality at the barcodes
- Remove or trim low quality sequence (**TrimGalore, cutadapt**)
- Demultiplexing → Group reads by cell barcode
- Align reads to genome or transcriptome, using classical RNAseq aligners (**STAR**), or pseudo-aligner (**Kallisto** or **Salmon**)
- Quantify reads per transcript/gene with tools widely used for bulk (**Rsubread, RSEM, HTSeq**) or specialized tools (collapse UMIs and demultiplex)
- If using UMI → collapse UMIs to count on gene/transcripts (**Umi-tools**)



The '*count*' data: Reads counts vs UMI counts

Usually we only retain **uniquely mapped reads**, thus reads mapping on multiple areas of the genome are discarded. Multiple mapping reads can be "*rescued*"

Read counts

	cell 1	cell 2	cell 3	...	cell M
gene 1	0	0	0		0
gene 2	20	22	1		5
gene 3	90	26	10		10
...					
gene N	5	5	1		5

Regardless the count type, scRNA-seq data are **extremely sparse**, i.e., there is a high proportion of **zero read counts**.
This '***zero-inflation***' arisez for both **biological** reasons and **technical** reasons.

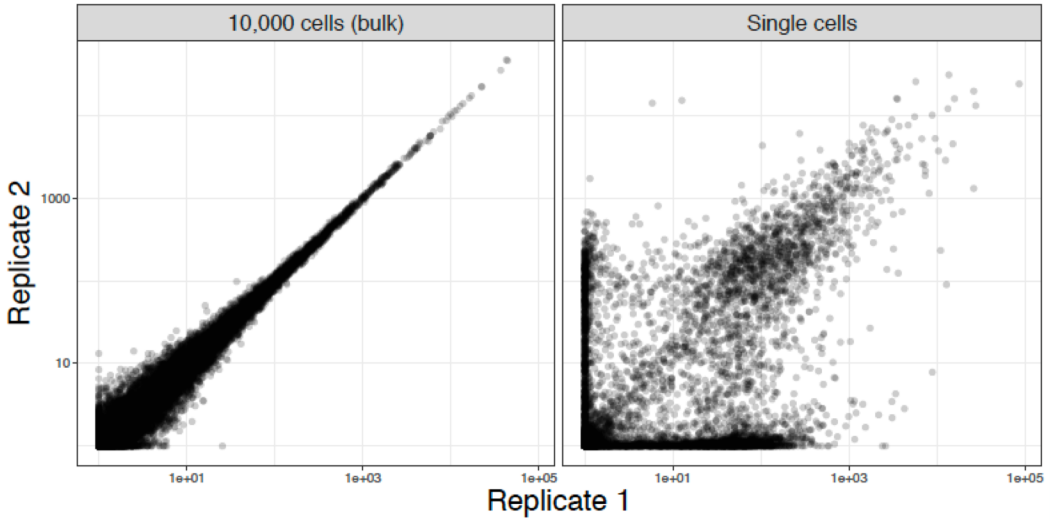
UMI counts

	cell 1	cell 2	cell 3	...	cell M
gene 1	0	0	0		0
gene 2	10	5	1		2
gene 3	27	10	3		3
...					
gene N	3	2	1		0

- Larger counts
- Include potential PCR artefacts
- Large extra Poisson variability
- Both full-length and tag based (3'-5')

- Smaller counts
- UMIs reduce extra Poisson variability
- No PCR artefacts
- Only tag based (3'-5')

scRNA-seq vs bulk RNA-seq counts



scRNA-seq are much more sparse and noise is higher….

..But there are many more cells than bulk RNA-seq samples

…Moreover, they can help facing more questions than bulk RNA-seq

Methods developed for bulk RNA-seq might not works for scRNA-seq→ novel methods are required

Quality control and filtering

Quality control at **cell level**

- Removing dying cells or with broken membrane
- Removing doublets, empty drops/wells

Quality control at **gene/transcript level**

- Removing not expressed genes/transcripts when dealing with dropouts
- Removing genes with very high expression (MALAT1, some lincRNAs, mitocondrial and ribosomal genes, actin, hemoglobin)

Some QC-metrics

- % of uniquely mapped reads
- Total counts per cells/barcode
- Number of expressed genes per cells/barcode
- Fraction of counts from mitocondrial genes per cells/barcode
- Spike-in detection – ratio, if availble

- Count per gene
- % of gene contribution to the gene profile
- Some specific gene category that do not contribute to cell variation

FILTERING
low quality cells

	cell1	cell2	cell3
gene A	18	28	3
gene B	6	140	0
gene C	180	35	0
gene D	0	0	2

FILTERING
lowly expressed genes

	cell1	cell2	cell3
gene A	18	28	3
gene B	6	140	0
gene C	180	35	0
gene D	0	0	2

Quality control and filtering

- **QC-metrics** can be computed on the raw counts matrix to filter out problematic cells or genes
- Look at the QC-metric distribution before deciding of **cutoffs**
- **It might be advisable to look for joint/combined-rules**
- Try to **visualize the impact** of QC→ plots of **PCA-components** and **QC-metrics** help to identify outlier cells.
- Always go-back at the QC-metrics after the analysis, repet the QC-filtering if needed → You may have clusters of low quality cells/doublets!

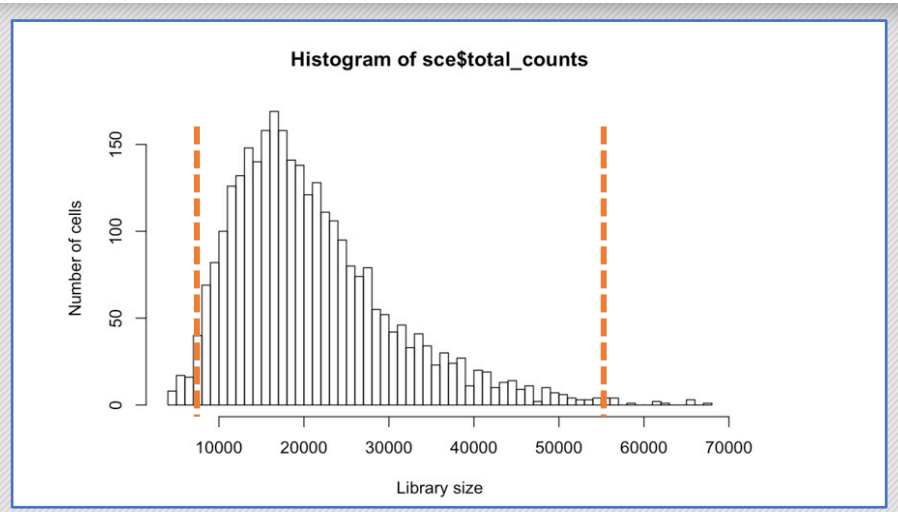
QC-metrics:

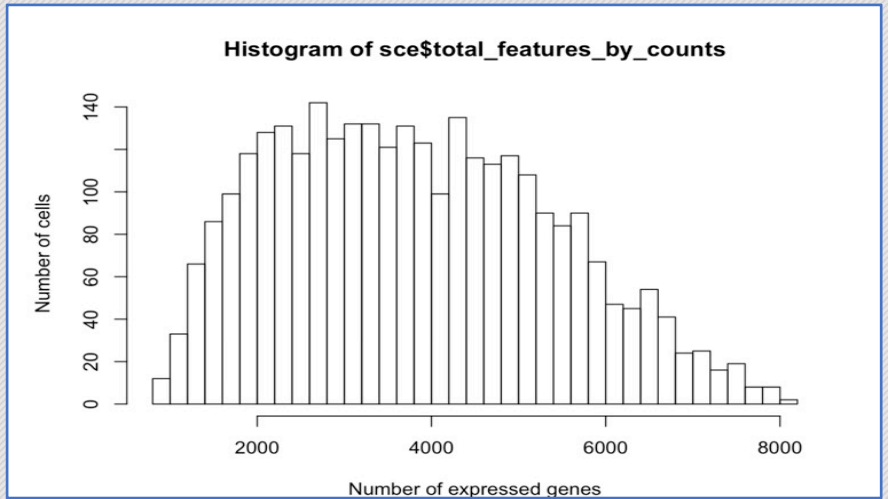
scater::calculateQCMetrics()

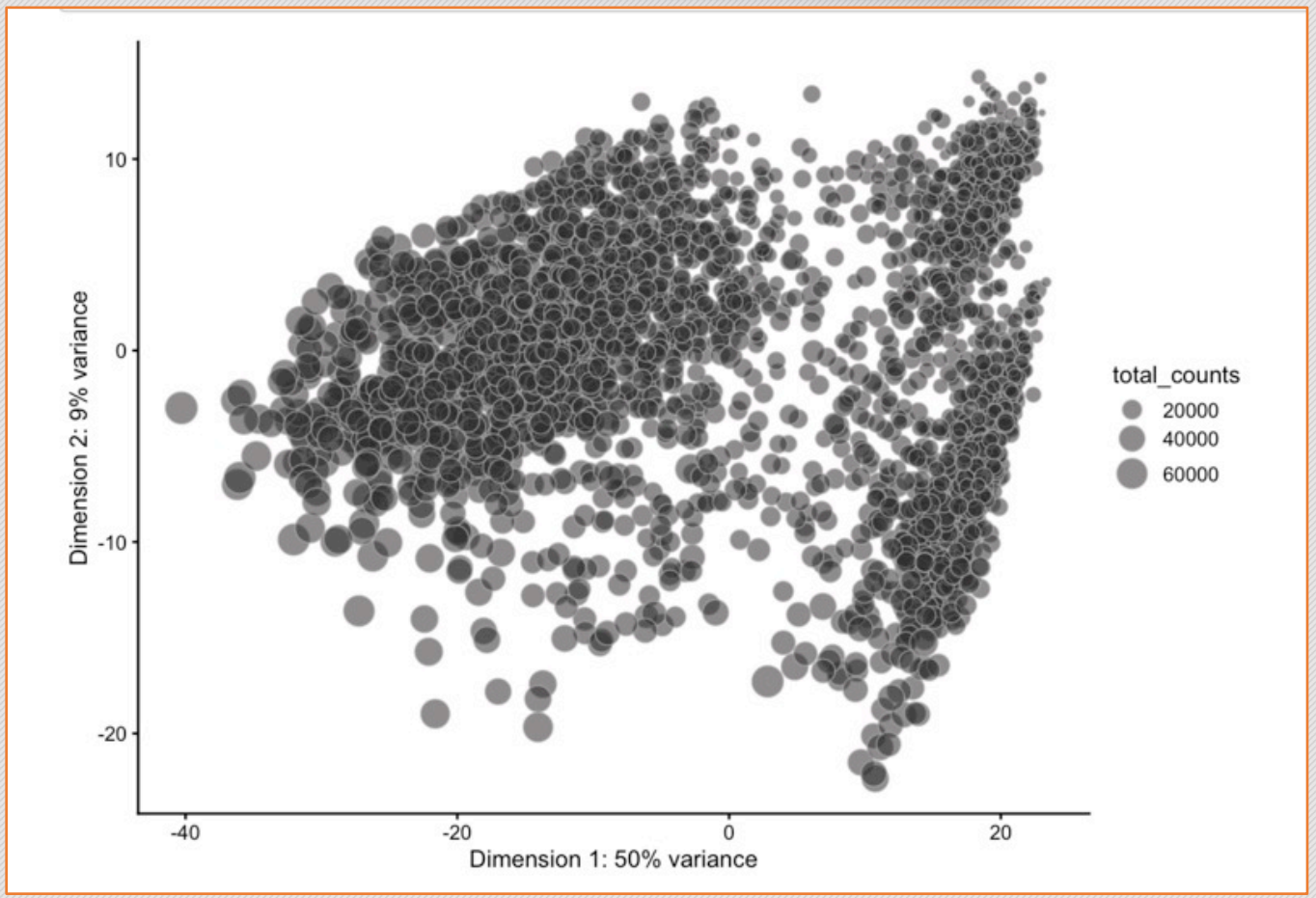
It provides:
1. Cell-level QC metrics
2. Feature level QC metrics

Scater package also provides
3. Diagnostic plots
4. Filtering
5. Basic normalization
6. Other visualization plots

Example


Histogram of sce$total_counts
Number of cells
0
50
100
150
10000
20000
30000
40000
50000
60000
70000
Library size


Histogram of sce$total_features_by_counts
Number of cells
0
20
40
60
80
100
140
2000
4000
6000
8000
Number of expressed genes


Dimension 2: 9% variance
10
0
-10
-20
-40
-20
0
20
Dimension 1: 50% variance
total_counts
20000
40000
60000

Spike-in or not?

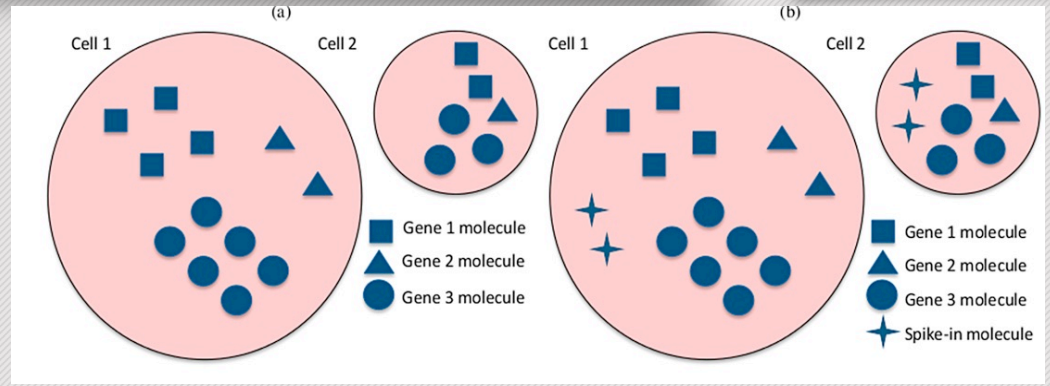
External molecules added in a known concentration to your cells.

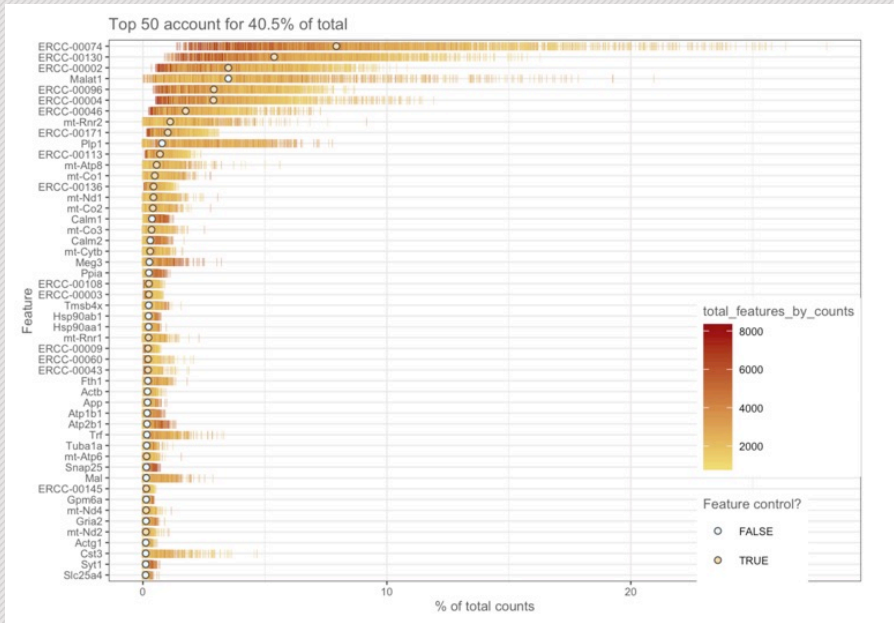
Spike-ins can be used for :

- **Quality control** (technical noise, drop-out rates/capture efficiency, amplification due to low starting amount of RNA in cells)
- **Data normalization**

Problems:

- Spike-ins behave differently to endogenous genes
- Spike-ins are not easy to dose and require **extra read depth**
- Cannot be used in **drop-seq** methods





Dropouts: Impute or Not?

scRNA-seq data are **extremely sparse** due to **dropouts**

The **zeros** arise for different reasons:

- The gene was not expressed in the cell → there are no transcripts to sequence. **It is a true zero.**
- The gene was expressed, but for some reason (trancriptional bursting or capturing efficiency) the transcripts were lost somewhere prior to sequencing. **It is a dropouts zero.**
- The gene was expressed and transcripts were captured, but the sequencing depth was not sufficient to produce any reads. **It is a dropouts zero.**

Data imputation aims to replace zero-abundance values with expected values under a drop-out model

Imputation is a difficult challenge and prone to creating false-positive results in downstream analysis.

→ In alternative, one can use implicit approaches that model counts with **zero-inflated distributions**

There are many different imputation methods available MAGIC (Dijk et al. 2017), DrImpute and scImpute (Li and Li 2017).

Normalization

Aims: Removing systematic non-biological variation (i.e., biases) and making **count distributions comparable**

Biases in scRNAseq data are due to several factors

- Low mRNA amount per cell
- Variable mRNA capture efficiency
- Variable sequencing depth
- Technical batches
- etc

Normalizing single-cell RNA sequencing data: challenges and opportunities

Catalina A Vallejos[1–4,10], Davide Risso[5,9,10], Antonio Scialdone[2,10], Sandrine Dudoit[5,6] & John C Marioni[2,7,8]

Note: normalization depends on the type of Raw counts

- **3'-tagged or UMI counts**, only cell-specific normalization is usually done (i.e., between cell normalization)
- **full-length read counts**, normalization for gene length can also be done (i.e., within and between cell normalization)

Normalization

Normalization can have a large impact on downstream results → it is critically important

- **Main factors** a global scaling/non linear to account for library size/sequencing depth
- **Batch effects** and **other confounders** (both known or unknown variation)

Main assumption: *most of the genes are non differentially expressed*

→ There is no method that is approapriate for all datasets. There is a strong dependency on the platform/protocols

→**Exploratory data analysis** (and visualization) is essential

- The **scone** package provides a wrapper to different normalization methods and **8** metrics to rank them on a given dataset

scone::metric_sample_filter()
scone::scone()

- The **scran** package computes basic and deconvolution based scaling factors

scran::quickCluster()
scran::computeSumFactors()
scran::computeSpikeFactors()
scrater::normalize()

- The **SCnorm** package
- The **sctransform** package

Scaling approaches for normalization

- **Global scaling:** It attemps scaling expression measures within each cells by a constant factor
- **Non-linear scaling:** It attemps scaling expression measures depending on the expression level

Main approaches

1. (Adapt) bulk RNA-seq **size factors**
2. Pooling and deconvolution approaches

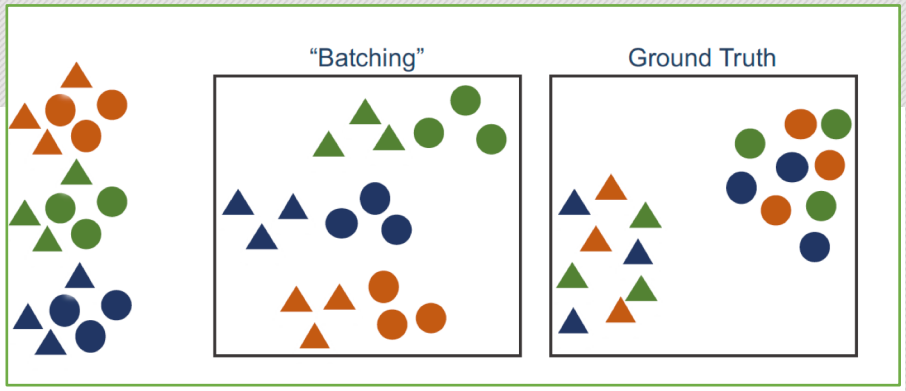
- **scran**: *Lun et al. 2016* proposed a pooling and deconvolution approach. Cells are pooled together and normalized against a global pseudoreference, then deconvolved by solving a system of linear equations

3. Regression based approaches

- **SCnorm**: *Bacher et al 2017* use two step quantile regression

Classical bulk RNA-seq normalization methods (CPM, TMM, upper quartile, etc) might not work well for scRNA-seq data since data are **zero-inflated** due to dropouts and transcriptional bursting

What is a Batch-effect ?

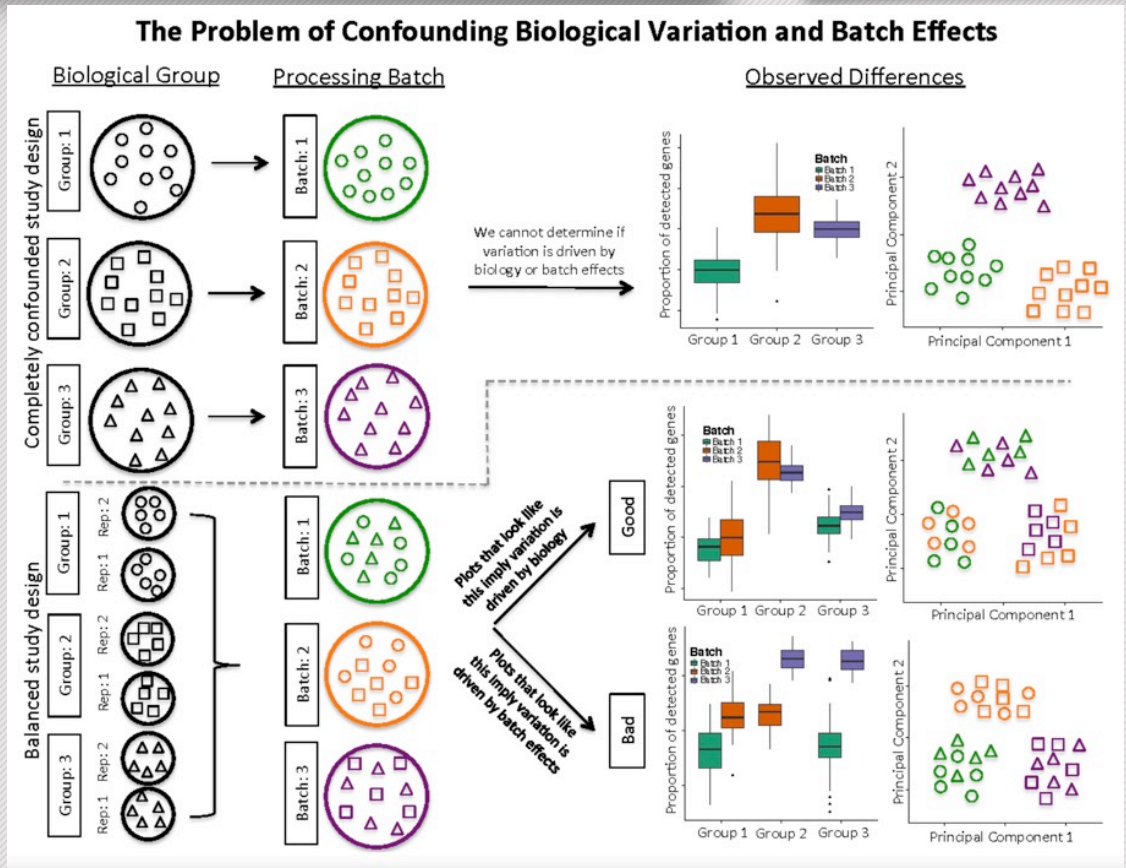


- Data may have been be generated in multiple laboratories, at different times, using different cell dissociation and handling protocols, library-preparation technologies and/or sequencing platforms.

All of these factors result in **technical batch** effects, in which the expression of genes in one batch differs systematically from that in another batch.

Such differences can **mask underlying biology** or introduce **spurious structures** in the data → to avoid misleading conclusions, they must be corrected before further analysis.

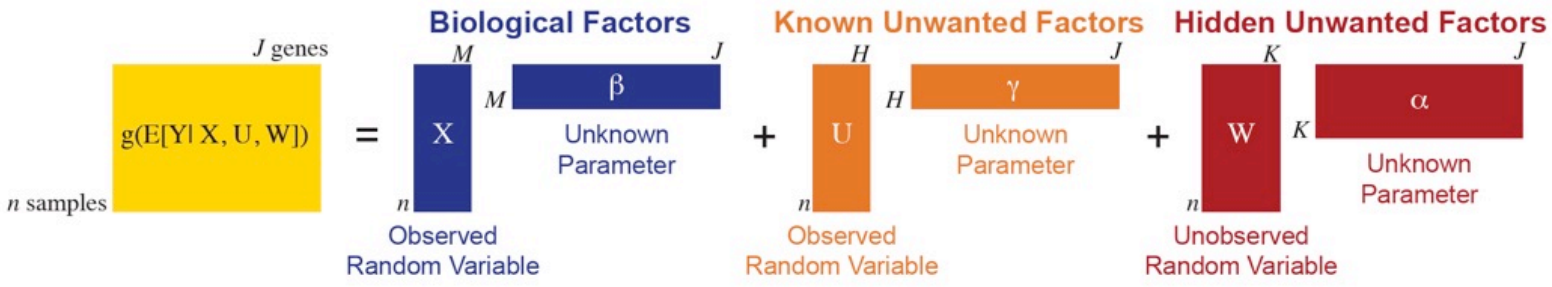
→ **We distinguish between known and unknown factors**



Note there could be **biological batches** we are not interested in (cell size, age, gender, donor …)

Batch-effect and unwanted variation

There is a growing interest for Batch removal approaches for large scale studies, cell Atlas, etc



- Regressing out unwanted covariates **removeBatchEffect**()
- **Ruvseq** or **SVAseq** approach
- **ComBat** approach

Emerging approaches … data integration using **graphlets**

- Mutual Nearest Neighbors (**MNN**) as in the *mnnCorrect* package
- Canonical correlation analysis (**CCA**) using Seurat package
- Integrative Non-negative matrix factorization (**NMF**) using LIGER package

Note: there is a **trade-off** between preseving clustering/cell heterogeneity (i.e., wanted biological) variation and removing batch effect (unwanted variation)

Example

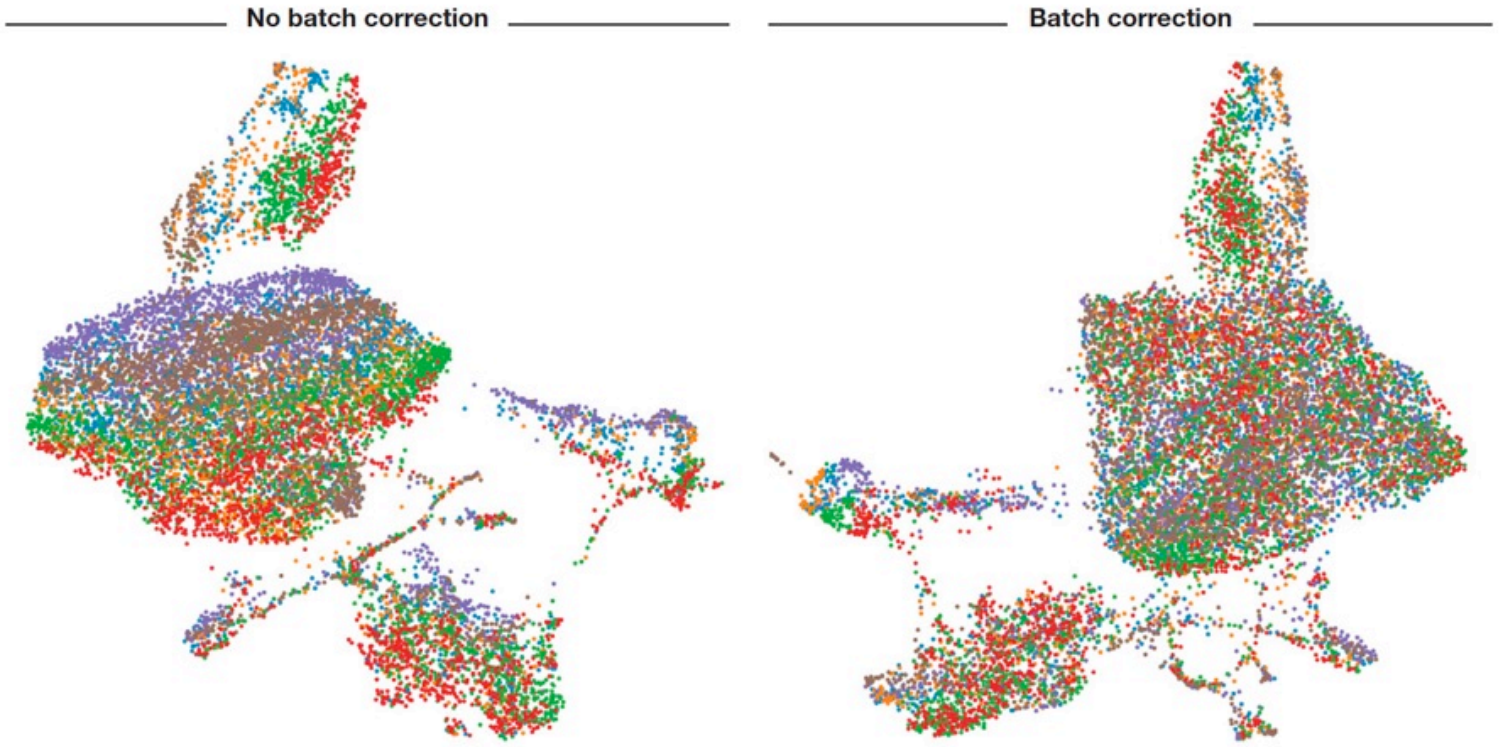
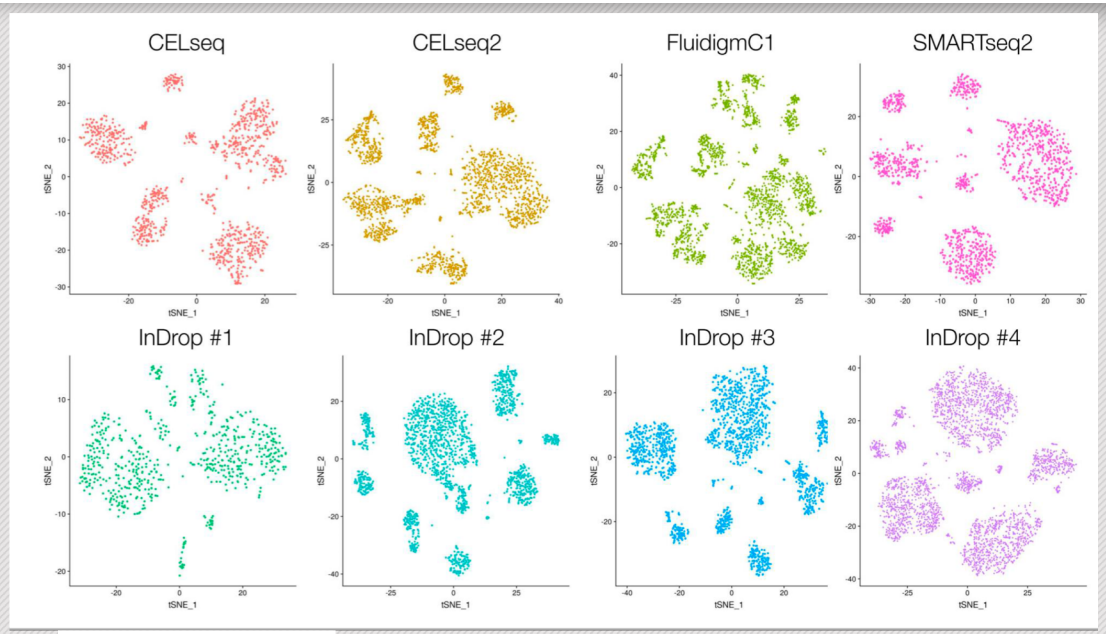


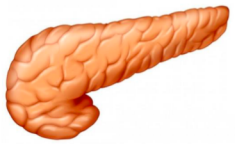
Figure 3. UMAP visualization before and after batch correction.

Cells are coloured by sample of origin. Separation of batches is clearly visible before batch correction and less visible afterwards. Batch correction was performed using ComBat on mouse intestinal epithelium data from Haber *et al* (2017).

Building Cell Atlas: Dataset integration



Human pancreas

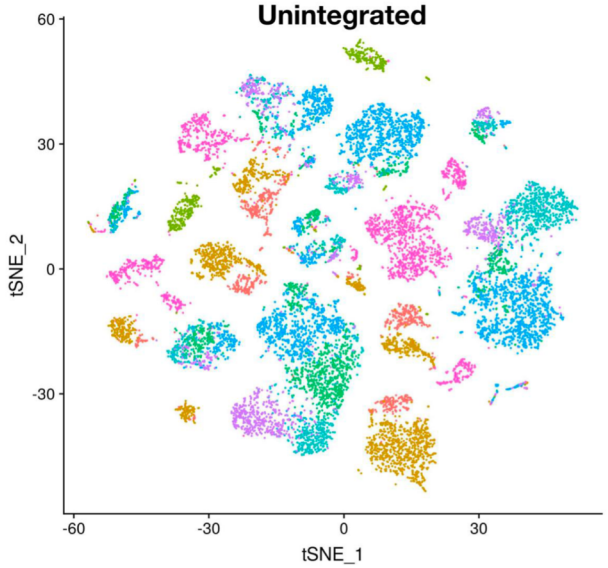


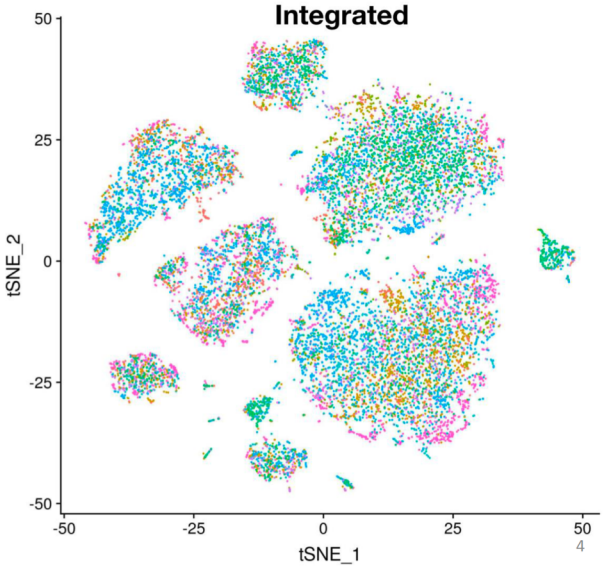
Baron et al. 2016, *Cell Syst.*
Lawlor et al. 2017, *Genome Res.*
Grun et al. 2016, *Cell Stem Cell*
Muraro et al. 2016, *Cell Syst.*

8 maps of the human pancreas

Cluster by batches (i.e sequencing system)

Cluster by cell types





Some R tools for quality control and normalization

- **scater:** quality control, dimension reduction, visualization (Bioconductor)
- **scran:** normalization, doublet detection, batch effect correction, **D**etection of HGV, dimension reduction, clustering (Bioconductor)
- **SCnorm:** normalization (Bioconductor)
- **scone:** normalization, batch effect correction, quality filtering (Bioconductor)
- **Seurat:** general purpose tool, including normalization and batch removal (CRAN)

Others

- **scruff:** UMI tools, quality control (Bioconductor)
- **Cellity:** quality control(Bioconductor)
- **simpleSingleCell:** quality control, normalization (Bioconductor)
- **sctransform:** normalization (CRAN)
- **DropletUtils:** removal of empty droplets (Bioconductor)

Feature selection and Dimension reduction

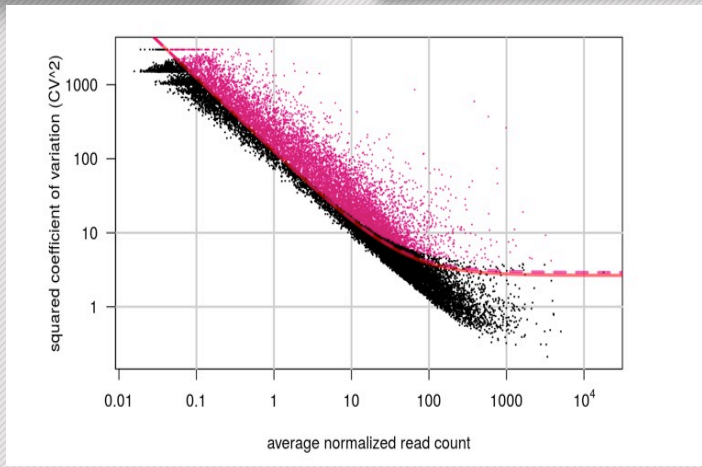
ScRNA-seq are **high dimensional** data → dimensionality has to be reduced before proceeding into the **analysis for reducing** both **technical noise** and **computational burden**

- **Feature selection** is aimed at selecting a subset of the most '*biologically associated*' genes, say for example the **HGV** (Highly variable genes)
- **Dimension reduction** consists of either linear or non linear embeddings that project data into a lower dimensional space that keep most of the biological signal (i.e., cell heterogeneity)

The main assumption is that only a portion of genes will show a response to the biological condition of interest, e.g. differences in cell-type, drivers of differentiation and so on…

Feature selection: Highly Variable Genes (HVG)

- **Assumption:** large differences in expression across cells are due to biological differences between the cells rather than technical noise.
- Due to the nature of count data, there is a positive relationship between the **mean expression** of a gene and **the variance** in the read counts across cells.
- **HGV** consists in **identifying genes that exhibit high cell-to-cell variation in the dataset**



*[Brennecke et al., 2013] implemented in **M3Drop** package*

There are several others similar ideas

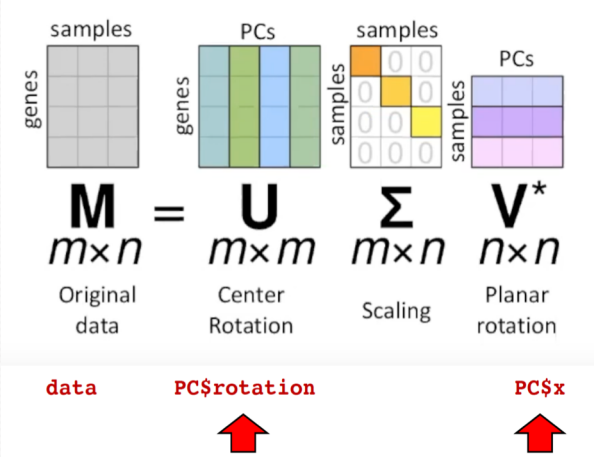
1. *normalize data*
2. *calculate the mean and the square coefficient of variation (CV^2) for each gene.* $CV^2 = \frac{\delta^2}{\mu^2}$
3. *Fits a quadratic model (gamma generalized linear model) to the relationship between mean expression and the CV^2*
4. *Use a chi-square test to find genes significantly above the curve*

Dimensionality Reduction for scRNA-seq

Principal component analysis (PCA):

It is a well known linear and interpretable method based on SVD

<u>What is the goal of dimension reduction?</u>
Reduce dimensionality for downstream analysis or for visualizing the data → The number of components to retains depends on the aim



PC1 and PC2 often correlate with cell heterogeneity, however they might also correlate with batches such as sequencing depth, cell cycle and others → PCs can be also used to visualize and remove batches

When PC components correlate with cells heterogeneity, **PCs loadings** can be used to identify gene contribution to cell populations

- There are plenty of other methods both based on ***matrix factorization*** such as ICA, NMF, or on ***graph-theory*** (tSNE, UMAP, Isomap, Diffusion maps)
- Moreover, there are sc-specific approaches, such as zero inflated factor analysis (**ZIFA**) or zero-inflated negative binomial wanted variation extraction (**ZINBWaVE**)

Dimensionality Reduction: Other Approaches

tSNE (*t-distributed Stochastic Neighbor Embedding algorithm*)

- It is a non linear approach aimed at preserving local distances (i.e., distance between nearest neighbor cells) → **local embedding,** it does not preserve a global data structure
- It is an **iterative** and **stochastic** method that depends on an inizialization seed and several tuning parameters (i.e., perplexity, number of iterations, many others)
- Note, for large datasets use a novel more efficient implementation based on FFT

UMAP (*Uniform Manifold Approximation and Projection*)

- It is a non linear approach based on **topological structures**
- It is **both local** and **global embeddings**
- Not completely stochastic
- As tSNE, it has many tuning parameters (minimum distance, number of neighbors)
- It is quite fast

tSNE and **UMAP** are implemented in
- Seurat3
- Scater
- Monocole3

Etc

UMAP is relatively novel approach that is becoming extremely popular

Some R tools for quality control, normalization and dimension reduction

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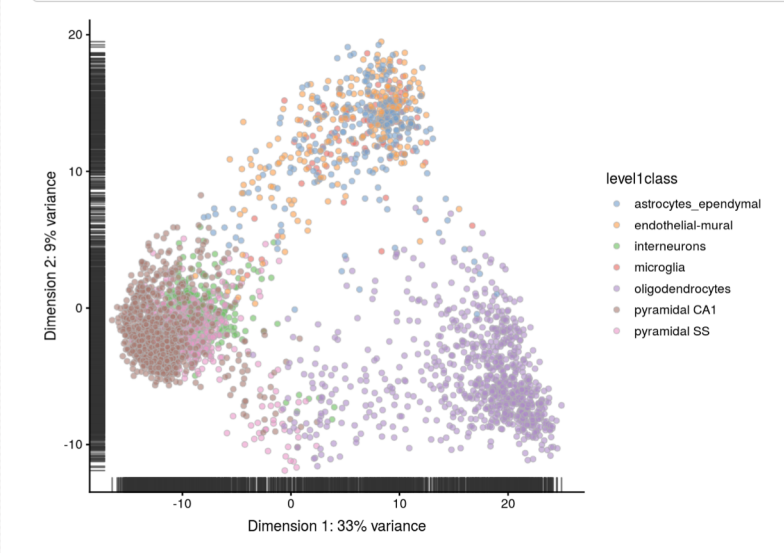
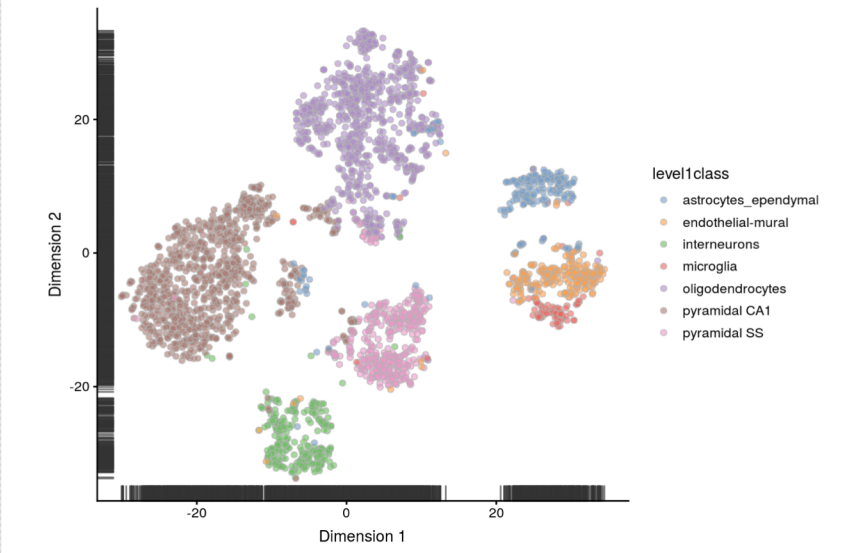
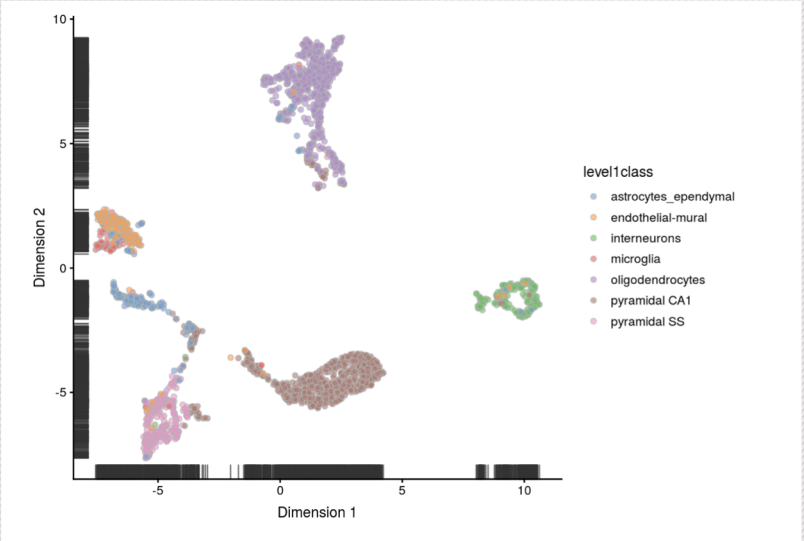
- **ZINB-WaVE**: dimension reduction (Bioconductor)
- **sctransform:** normalization (CRAN)
- **DropletUtils:** removal of empty droplets (Bioconductor)

Visualization: PCA, tSNE, UMAP







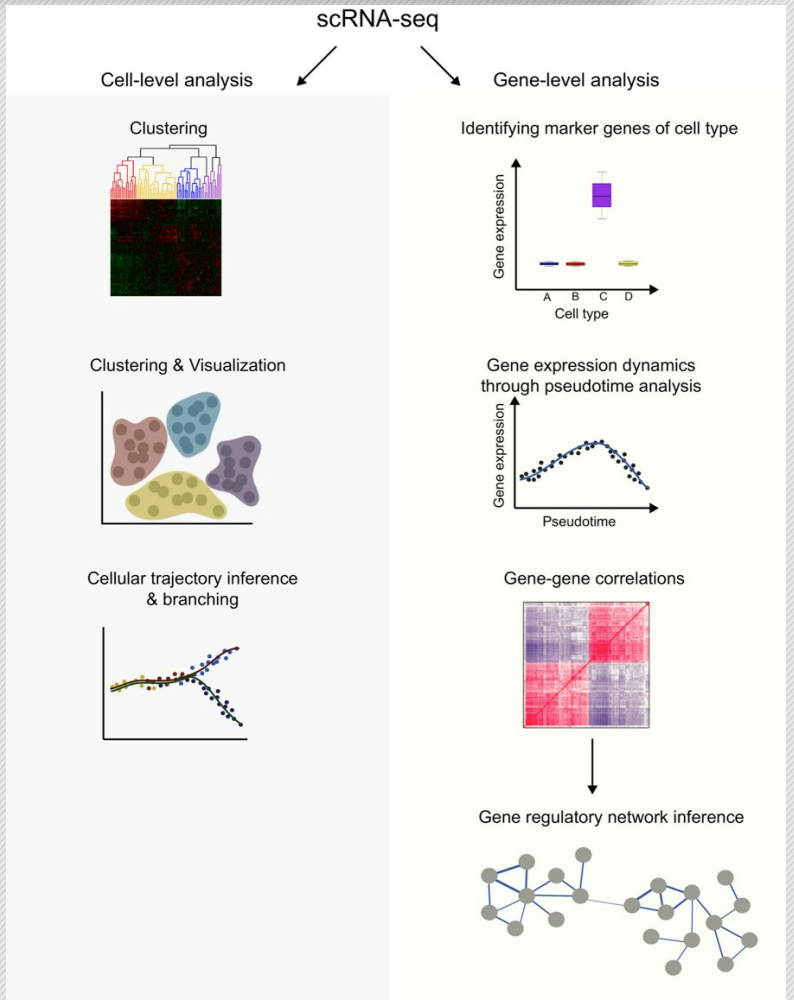
PCA **tSNE** **UMAP**

Other visualization methods such as **Diffusion Maps** might be more appropriate for cell differentiation.
Diffusion map is a non linear method for dimension reduction where the distance between the points are measured as probability from going from one to another.

Introduction to downstream analysis

- Clustering
- Cluster annotation
- Compositional analysis
- Trajectory inference and branching
- Cell marker identification
- Differential expression
- Gene regulatory networks
- Others… continuosly emerging

Each question would require a specific course



Clustering

Clustering is one of the most popular scRNA-seq applications, since it allows to identify subpopulations of cells

The most popular clustering approaches are:

- **Hierarchical clustering**: build and cut the dendrogram based on pairwise distance matrix
- **K-means clustering**: iterative approach that reassign cells to the nearest cluster center
- **Density based clustering**: clusters are defines as areas of higher density (DBSCAN)
- **Mixture model clustering**: each cluster is modelled as a mixture, followed by frequentist or Bayesian inference
- **Graph-based clustering**: Cells are first organized in a network using a KNN approach, then cluster are identified as network modules (i.e, regions of highly connected nodes)

Challenges in scRNA-seq

- What is the number of clusters?
- What about dropouts and other scRNA-seq technical issues ?
- Scalability: in scRNA-Seq experiments the number of cells could be millions, tools developed for single-cell data don't scale well.

Clustering scRNA-seq

Plenty of methods that can be used in different modes and with differently pre-processed data

Table 1 | **Clustering methods for scRNA-seq**

Name	Year	Method type	Strengths	Limitations
scanpy[4]	2018	PCA + graph-based	Very scalable	May not be accurate for small data sets
Seurat (latest)[3]	2016			
PhenoGraph[32]	2015			
SC3[22]	2017	PCA + *k*-means	High accuracy through consensus, provides estimation of *k*	High complexity, not scalable
SIMLR[24]	2017	Data-driven dimensionality reduction + *k*-means	Concurrent training of the distance metric improves sensitivity in noisy data sets	Adjusting the distance metric to make cells fit the clusters may artificially inflate quality measures
CIDR[25]	2017	PCA + hierarchical	Implicitly imputes dropouts when calculating distances	
GiniClust[75]	2016	DBSCAN	Sensitive to rare cell types	Not effective for the detection of large clusters
pcaReduce[27]	2016	PCA + *k*-means + hierarchical	Provides hierarchy of solutions	Very stochastic, does not provide a stable result
Tasic et al.[28]	2016	PCA + hierarchical	Cross validation used to perform fuzzy clustering	High complexity, no software package available
TSCAN[41]	2016	PCA + Gaussian mixture model	Combines clustering and pseudotime analysis	Assumes clusters follow multivariate normal distribution
mpath[45]	2016	Hierarchical	Combines clustering and pseudotime analysis	Uses empirically defined thresholds and a priori knowledge
BackSPIN[26]	2015	Biclustering (hierarchical)	Multiple rounds of feature selection improve clustering resolution	Tends to over-partition the data
RaceID[23], RaceID2[115], RaceID3	2015	*k*-Means	Detects rare cell types, provides estimation of *k*	Performs poorly when there are no rare cell types
SINCERA[5]	2015	Hierarchical	Method is intuitively easy to understand	Simple hierarchical clustering is used, may not be appropriate for very noisy data
SNN-Cliq[80]	2015	Graph-based	Provides estimation of *k*	High complexity, not scalable

DBSCAN, density-based spatial clustering of applications with noise; PCA, principal component analysis; scRNA-seq, single-cell RNA sequencing.

Identifying cell populations with scRNASeq

Tallulah S. Andrews, Martin Hemberg*

Wellcome Trust Sanger Institute, Hinxton, Cambridgeshire, UK

Challenges in unsupervised clustering of single-cell RNA-seq data

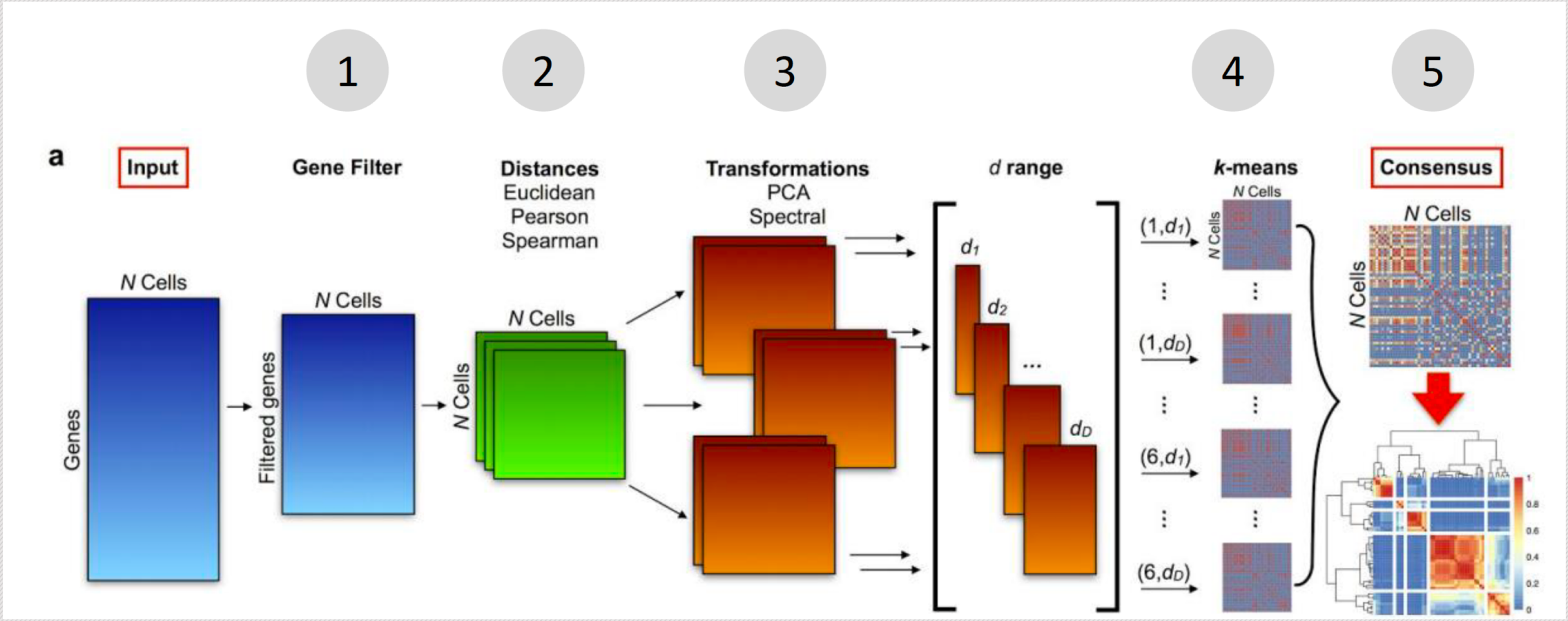
Vladimir Yu Kiselev, Tallulah S. Andrews & Martin Hemberg

Benchmark and parameter sensitivity analysis of scRNAseq clustering methods.

Monika Krzak[1*], Yordan Raykov[2], Alexis Boukouvalas[3], Luisa Cutillo[4], Claudia Angelini[1]

- There is still a significant space for improvments
- Scalability is becoming a serious issue for several methods

SC3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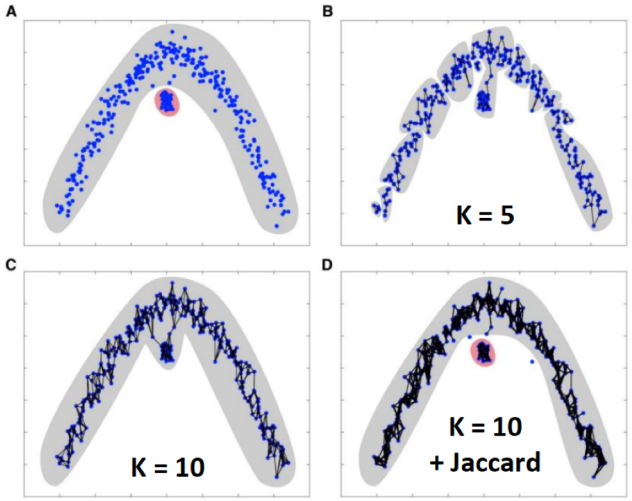
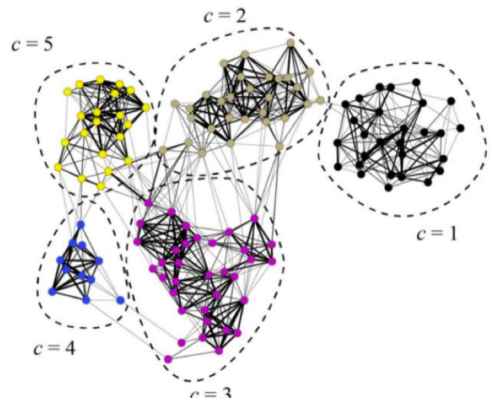
k-means
Consensus
N Cells
Genes
Filtered genes
d_1
d_2
d_D
$(1,d_1)$
$(1,d_D)$
$(6,d_1)$
$(6,d_D)$

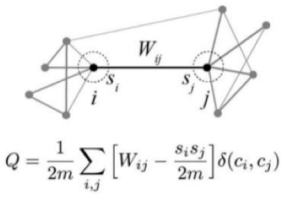
Seurat Clustering

1. Reduce dimensionality using PCA
2. Construct KNN (*k-nearest neighbor*) graph based on the Euclidean distance in PCA space using some of the first components
3. Refine the edge weights between any two cells based on the shared overlap in their local neighborhoods (Jaccard distance, i.e. how many shared edges).
4. Cluster cells by optimizing for modularity (*Louvain algorithm*)

Note that it does not require to explicitly choose the number of clusters







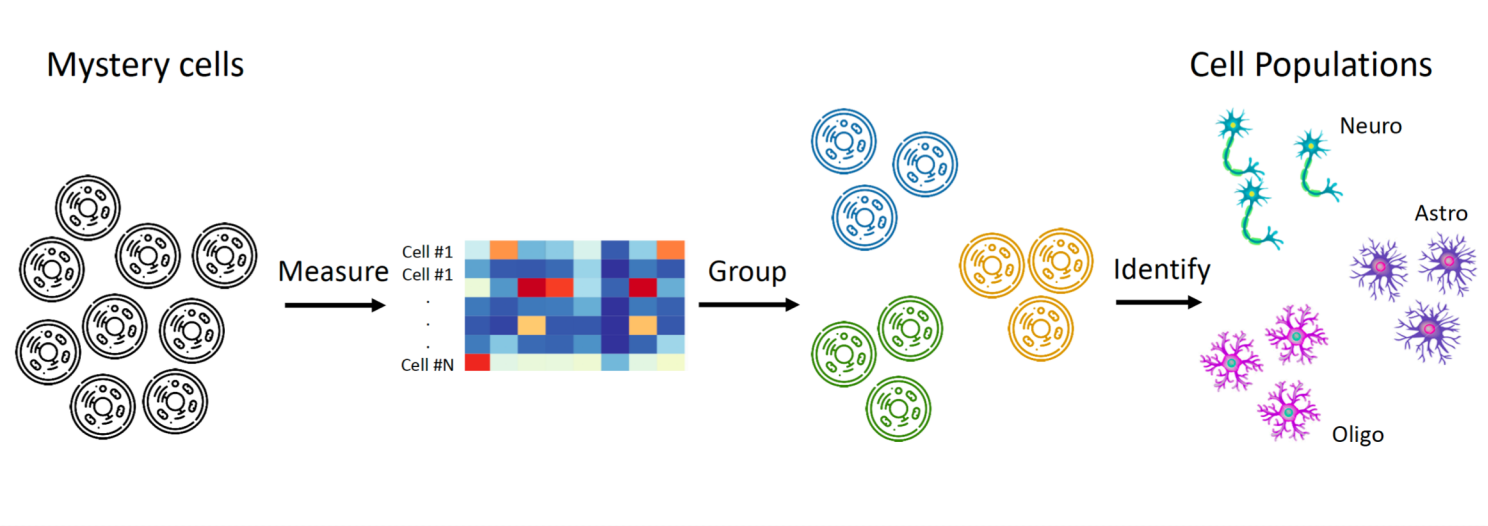
Clustering assessment

Clustering algorithms always return a partion → It is important to check quality and robustness of the partition

- Visualization
- Average silhouette
- Modularity Score
- Bootstrapping approaches
- Adjusted Rand Index
- **Biological interpretation**

- Check also **quality metrics** in your clusters, you might have clusterized doublets, or other technical issues
- Be aware… a cluster does not necessarity corresponf to a cell type

Cluster annotation - Cell identity



Making sense of the data

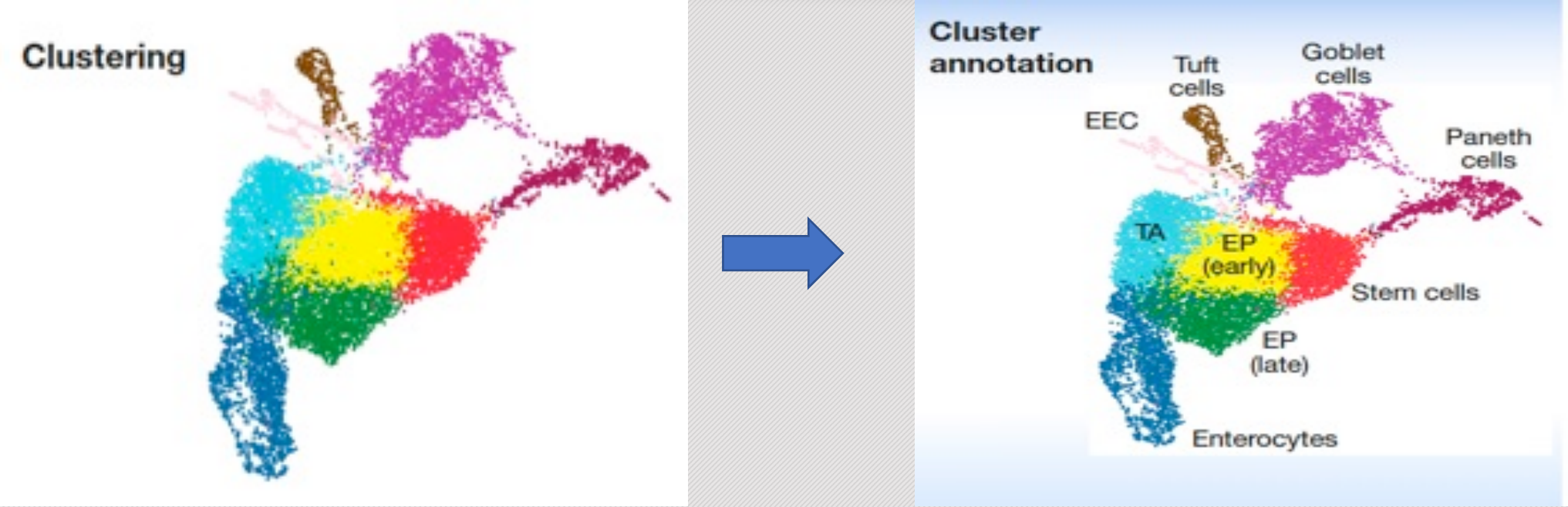
- Samples are heterogeneous, either in cell composition and abundance
- Important in particular for **immunology** and **cancer research**
- Discovering novel cell types

A comparison of automatic cell identification methods for single-cell RNA sequencing data

Tamim Abdelaal[1,2†], Lieke Michielsen[1,2†], Davy Cats[3], Dylan Hoogduin[3], Hailiang Mei[3], Marcel J. T. Reinders[1,2] and Ahmed Mahfouz[1,2*]

- **Manual approaches**: Use cell surface markers or other gene markers to identify known cell population
- Databases with cell type gene signatures (**PanglaoDB, CellMarker**, **Tabula Muris, Cell Atlas**..)
- **Automatic approaches**: Classification/mapping based approaches (**scmapm SingleR**, ..)
- Tumor cells are much more challenging
- Did you identify novel cell sub-population?..... is it a true functionally characterized populations?

Cluster annotation - Cell identity



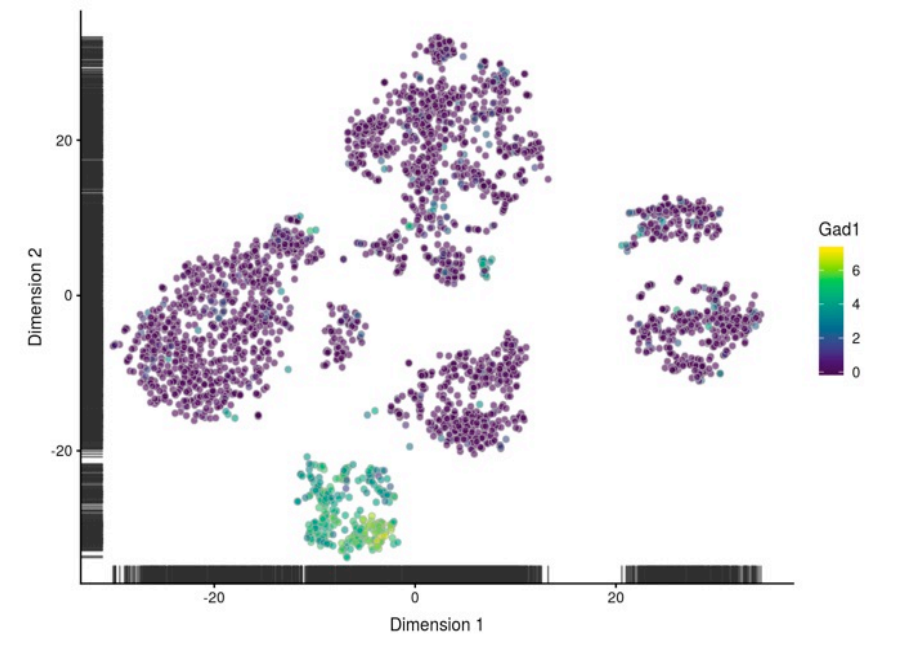
Note

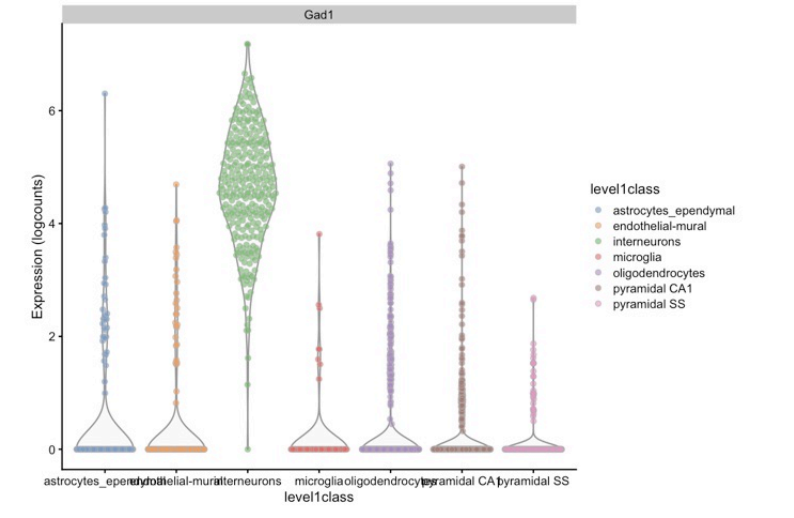
- It is not always clear what constitutes a '*cell type*' (i.e, at what resolution types are different)
- Cells of the same cell type in different states may be detected in separate clusters

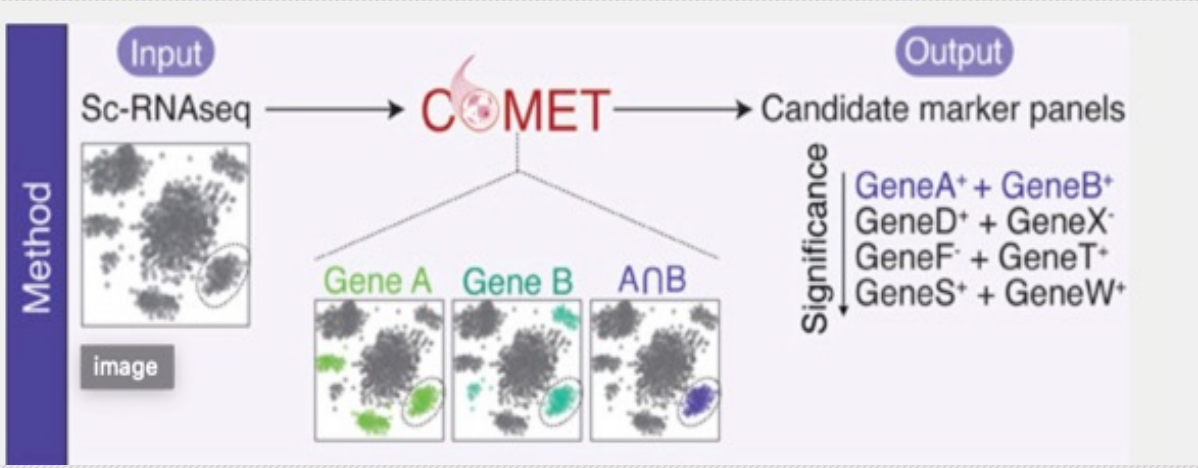
→ it is better to use the term "cell identities" rather than "cell types"

The iteration of clustering, cluster annotation, re- or subclustering and re-annotation can be time-consuming

Example







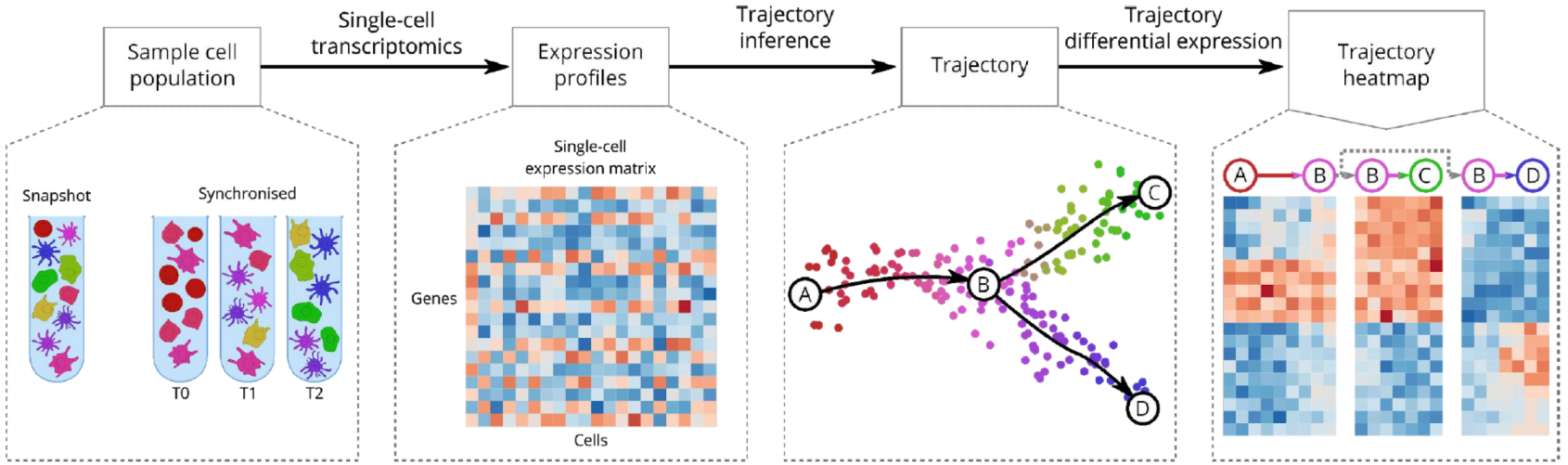
Combinatorial prediction of marker panels from single-cell transcriptomic data

Conor Delaney, Alexandra Schnell, Louis V Cammarata, Aaron Yao-Smith, Aviv Regev, Vijay K Kuchroo, Meromit Singer

Compositional Analysis

- Clusters can be analyzed in terms of its **compositional** structure.
- Compositional data analysis revolves around the **proportions of cells** that fall into each cell-identity cluster and their **change** in response to disease/treatment.
- Investigating **compositional changes** in scRNA-seq data requires sufficient cell numbers to robustly assess cell-identity cluster proportions, and sufficient sample numbers to evaluate expected background variation in cell-identity cluster compositions
- Statistical tests over changes in the proportion of a cell identity cluster between samples are **dependent** on one another → emerging methods come from **mass cytometry** and **microbiome** literature

Trajectory inference



REVIEW

Computational methods for trajectory inference from single-cell transcriptomics

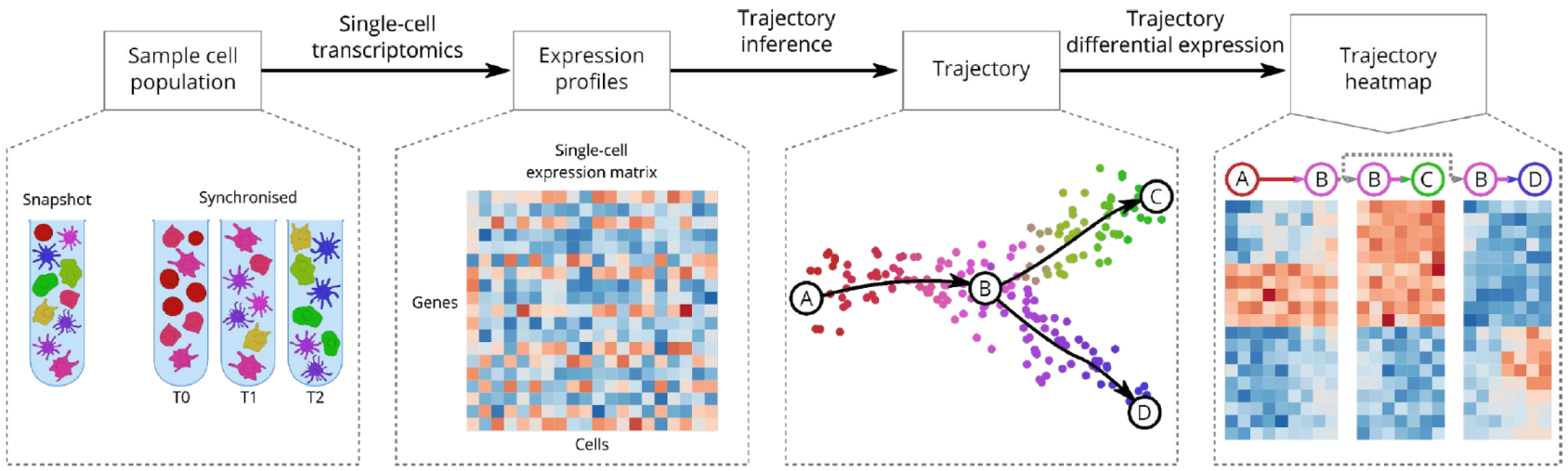
Robrecht Cannoodt[1,2,3,4], *Wouter Saelens*[1,2] *and Yvan Saeys*[1,2]

Using single-cell genomics to understand developmental processes and cell fate decisions

Jonathan A Griffiths[1], Antonio Scialdone[2,3,4,5] & John C Marioni[1,2,6,*]

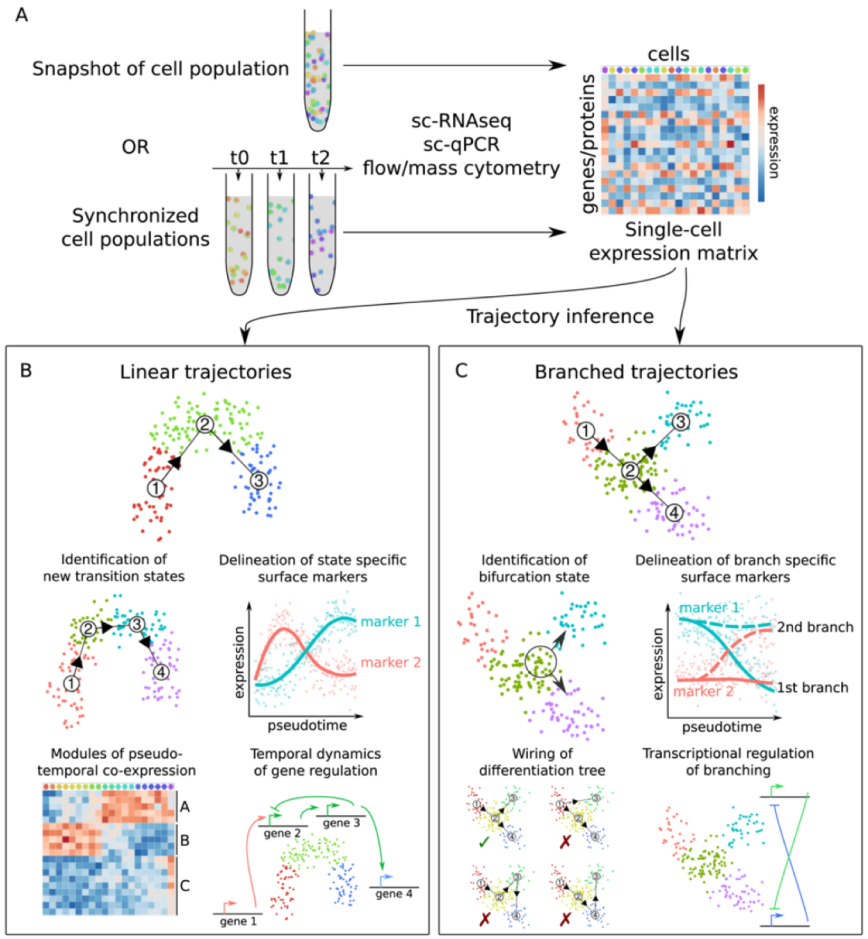
- Cellular diversity cannot be fully captured using a **discrete system** such as clustering.
- **Biological processes** driving **cell development** are **continuous processes.**
- Individual cells will differentiate in an **unsyncrhonized** manner and can go toward **different fates**
- To capture **transitions between cell identities**, branching differentiation processes we need expression **dynamic models**

Trajectory inference

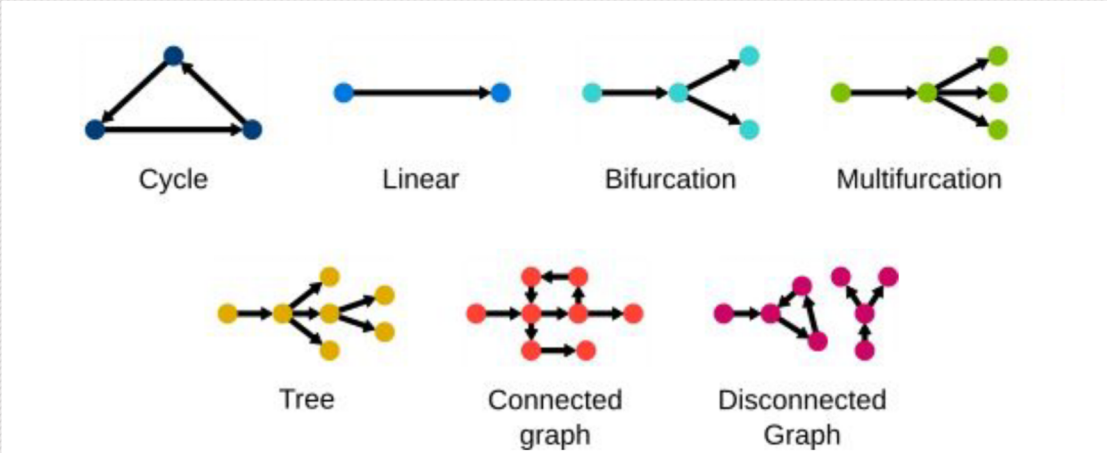


- Trajectory inference methods interpret scRNA-seq data as a **snapshot** of a **continuous process.**
- The process is reconstructed by finding **paths through cellular space that minimize transcriptional changes** between neighbouring cells
- The ordering of cells along these paths is described by a **pseudotime** variable.

Pseudotime and Branching



Trajectory types



Pseudotime is an ordered abstract unit of progress

It has been introduced in the original Monocle paper

The single-cell transcriptional landscape of mammalian organogenesis

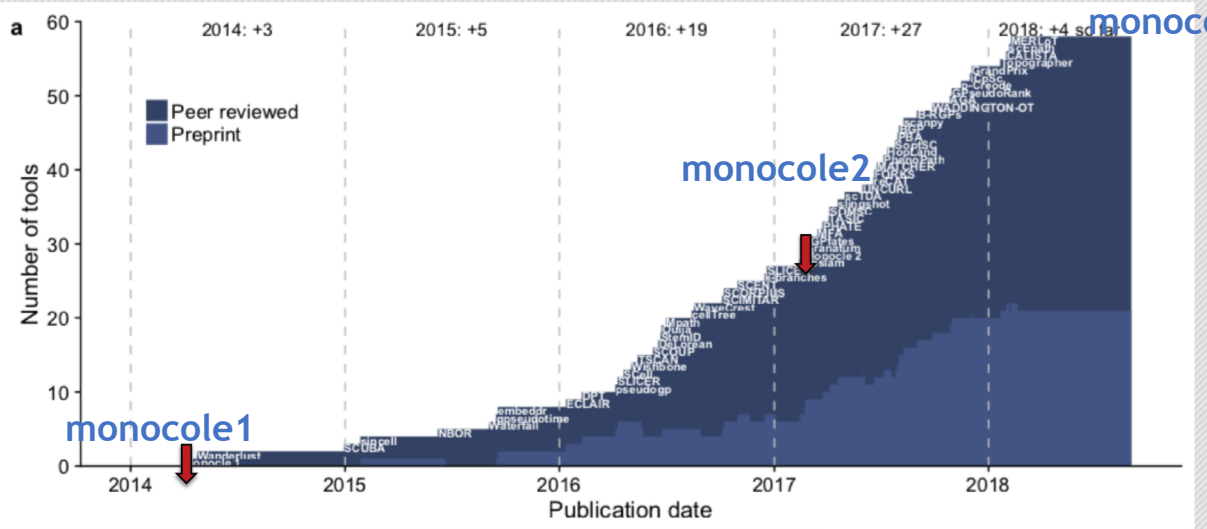
Junyue Cao, Malte Spielmann, Xiaojie Qiu, Xingfan Huang, Daniel M. Ibrahim, Andrew J. Hill, Fan Zhang, Stefan Mundlos, Lena Christiansen, Frank J. Steemers, Cole Trapnell, and Jay Shendure

Nature 2019

Trajectory inference with scRNA-seq

Any dataset can be forced into a trajectory even without any biological meaning

- Are you sure to have a developmental trajectory?
- Which assumptions can be done on the type of trajectory (ie., branchings)?
- Where is the starting point? And end point? Do you know a bit of the biology behind?
- Many methods available (minumim spanning tree, reverse graph embeddings, Gaussian processes, latent variables, etc)



- Monocole1; Monocole 2; Monocole 3
- PAGA
- RaceID
- Scorpius
- Slingshot
- TSCAN
- Cell Router

Many others…

They differs in accuracy, scalability, stability, usability

A comparison of single-cell trajectory inference methods

Wouter Saelens, Robrecht Cannoodt, Helena Todorov & Yvan Saeys

Nature Biotechnology **37**, 547–554 (2019) | Download Citation

Differential Expression

Comparison of cell types (often within a single sample) to find **"marker genes"** or cell-type specific **pathways**

However cell types is usually unknown

- **Step 1**: Get the cell populations using clusteting approaches
- **Step 2**: Compare expression levels between populations

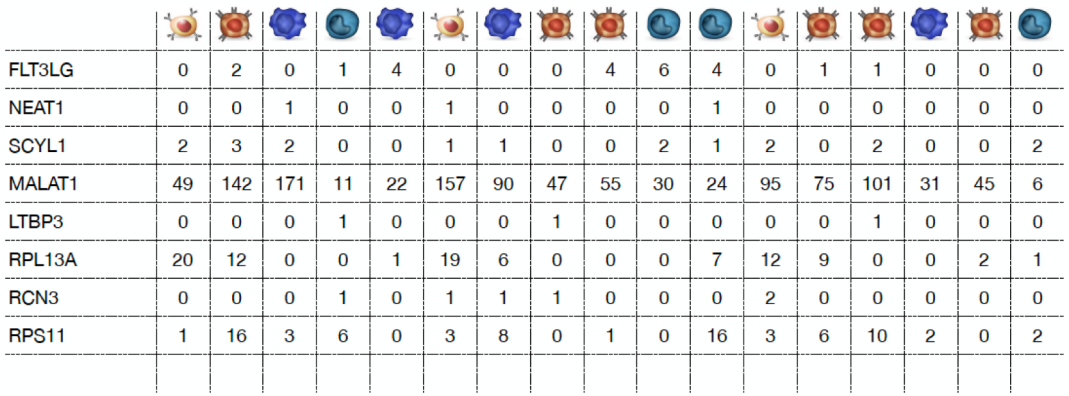
Bulk RNA-Seq methods:

- edgeR, DESeq2, etc

→ scRNAseq data are much more sparse, with higher variability, many more cells than the typical number samples of bulk RNA-seq

Single-cell methods:

- Single Cell Differential Expression (**SCDE**)
- Model-based Analysis of Single-cell Transcriptomics (**MAST**)
- Many others



FLT3LG	0	2	0	1	4	0	0	0	4	6	4	0	1	1	0	0	0
NEAT1	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0
SCYL1	2	3	2	0	0	1	1	0	0	2	1	2	0	2	0	0	2
MALAT1	49	142	171	11	22	157	90	47	55	30	24	95	75	101	31	45	6
LTBP3	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0
RPL13A	20	12	0	0	1	19	6	0	0	0	7	12	9	0	0	2	1
RCN3	0	0	0	1	0	1	1	1	0	0	0	2	0	0	0	0	0
RPS11	1	16	3	6	0	3	8	0	1	0	16	3	6	10	2	0	2

For a given cluster, are we interested in "*marker genes*" that are:

- DE compared to all cells outside of the cluster
- DE compared to at least one other cluster
- DE compared to each of the other clusters
- DE compared to "most" of the other clusters

`scran::findMarkers`

Gene Regulatory Networks

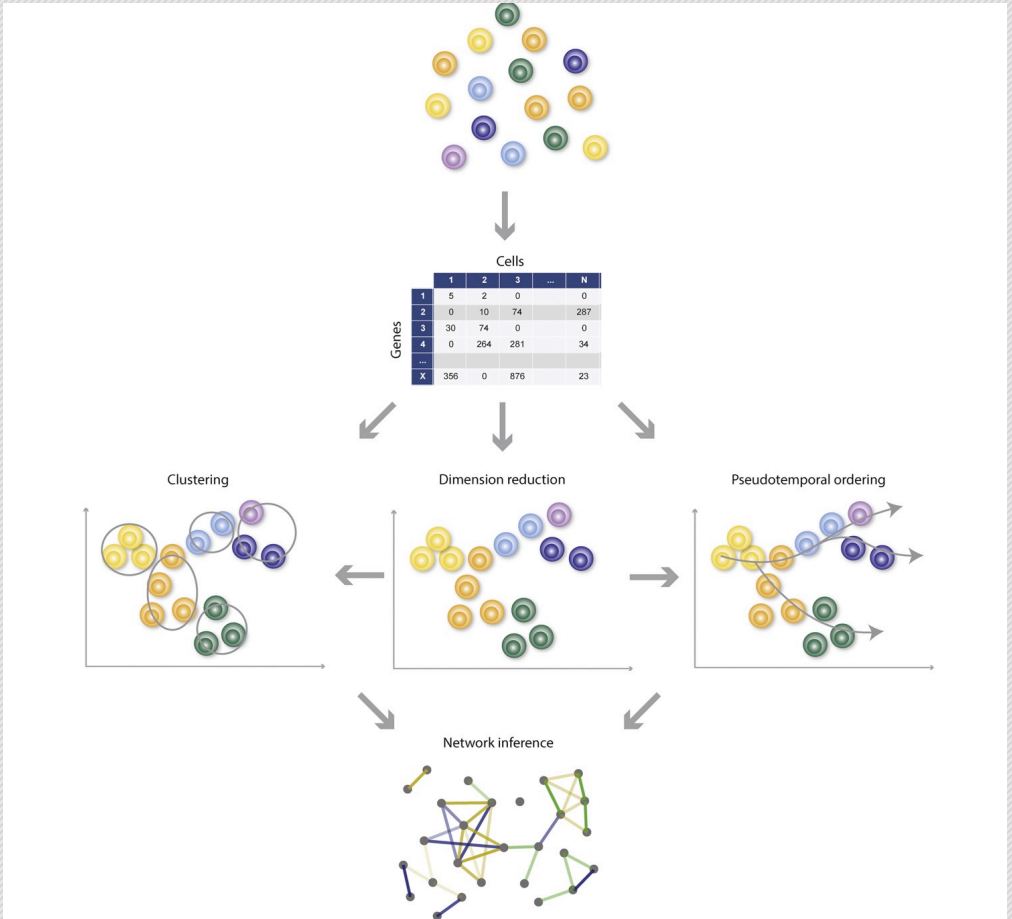
Comparison of cell types (often within a single sample) to find cell-type specific **GRN**

- Commonalities and differences among cell populations
- Regulatory dynamic during development

Benchmarking algorithms for gene regulatory network inference from single-cell transcriptomic data

Aditya Pratapa[1], Amogh P. Jalihal[2], Jeffrey N. Law[2], Aditya Bharadwaj[1], and T. M. Murali[1]

[1]Department of Computer Science, Virginia Tech, Blacksburg, USA 24060
[2]Genetics, Bioinformatics, and Computational Biology Ph.D. Program, Virginia Tech, Blacksburg, USA 24060



Some R tools for Downstream analysis

- SC3: Clustering (Bioconductor)
- Seurat: QC and pre-processing, detection of HGV, dimension reduction, clustering, DE (CRAN)
- Monocole3: Clustering, differential expression, trajectories, identification of marker genes (Bioconductor and CRAN)

Other applications

- Slingshot : trajectories (Bioconductor)
- MAST: DE (Bioconductor)
- SCDE: DE (Bioconductor)
- SCENIC : gene regulatory networks (GitHub)
- SCODE : gene regulatory networks (GitHub)
- ISEE: Shiny-based graphical user interface for exploring data (Bioconductor)

More tools at

- https://www.scrna-tools.org
- https://github.com/seandavi/awesome-single-cell

Other applications

ScRNA-seq applications are greatly increasing to study different aspects of biology

- Identification of novel and rare cell populations
- Alternative Splicing
- Allelic specific expression
- Single Cell Atlas … organisms, conditions
- Spatial transcriptomics and in-situ transcriptomics
- …
- Multiomics single cell integration

Challenges and emerging directions in single-cell analysis

Guo-Cheng Yuan, Long Cai, Michael Elowitz, Tariq Enver, Guoping Fan, Guoji Guo, Rafael Irizarry, Peter Kharchenko, Junhyong Kim, Stuart Orkin, John Quackenbush, Assieh Saadatpour, Timm Schroeder, Ramesh Shivdasani & Itay Tirosh

A practical guide to single-cell RNA-sequencing for biomedical research and clinical applications

Ashraful Haque, Jessica Engel, Sarah A. Teichmann & Tapio Lönnberg

Integrative single-cell analysis

Tim Stuart & Rahul Satija

Nature Reviews Genetics **20**, 257–272(2019) | Cite this article

… And then, **How to interpret result?** What is the impact for developmental cell biology, immunology, cancer biology?

Conclusions

- **scRNA-seq** is a novel and rapidally evolving technology that allows to investigate cell heterogeneity and development at an unprecedenent level of resolution and throughtput
- **scRNA-seq** data are by far **noisier** and **sparser** than bulk RNA-seq data, → novel computational methods are required
- While their preprocessing (i-e. Data cleaning, normalization, etc) is quite general, the downstream analysis strongly depends on the biological question of interest
- More than **400 methods** have been developed→ **no 'golden standard'**
- scRNA-seq **data size** is rapidally increasing→ novel challenge to **scalability** of methods in terms of **memory** and **run time**
- **Novel applications** of scRNA-seq are continuolsy proposed

Thank you for the attention

WE ARE RECRUITING

We are seeking motivated undergraduate/PhD/PostDoc students to start in the **winter/spring of 2020**.

If you are interested, please, contact me (send your CV) at **claudia.angelini@cnr.it**