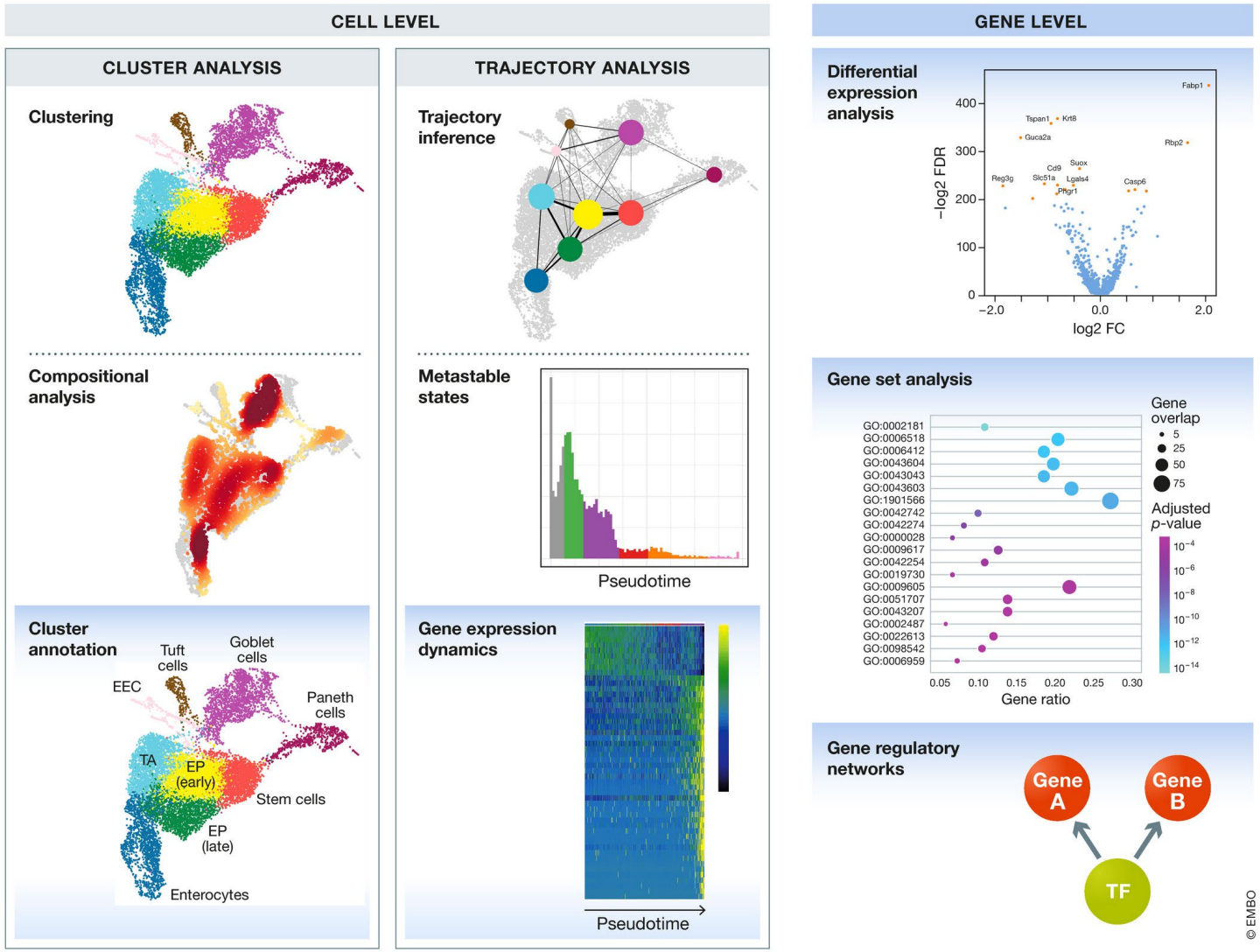
Differential expression (DE) analysis

Ahmed Mahfouz
Department of Human Genetics, Leiden University Medical Center
Pattern Recognition and Bioinformatics, TU Delft

mahfouzlab.org
@ahmedElkoussy

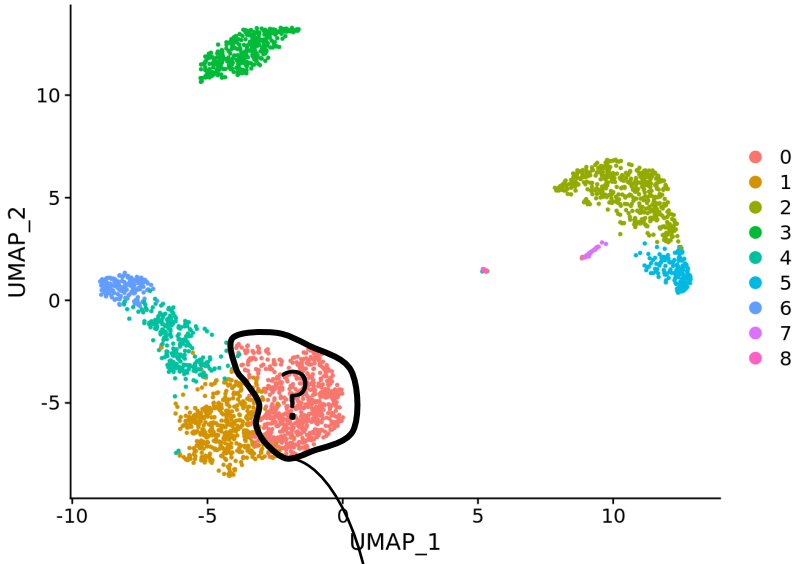
Downstream analysis of scRNA-seq data



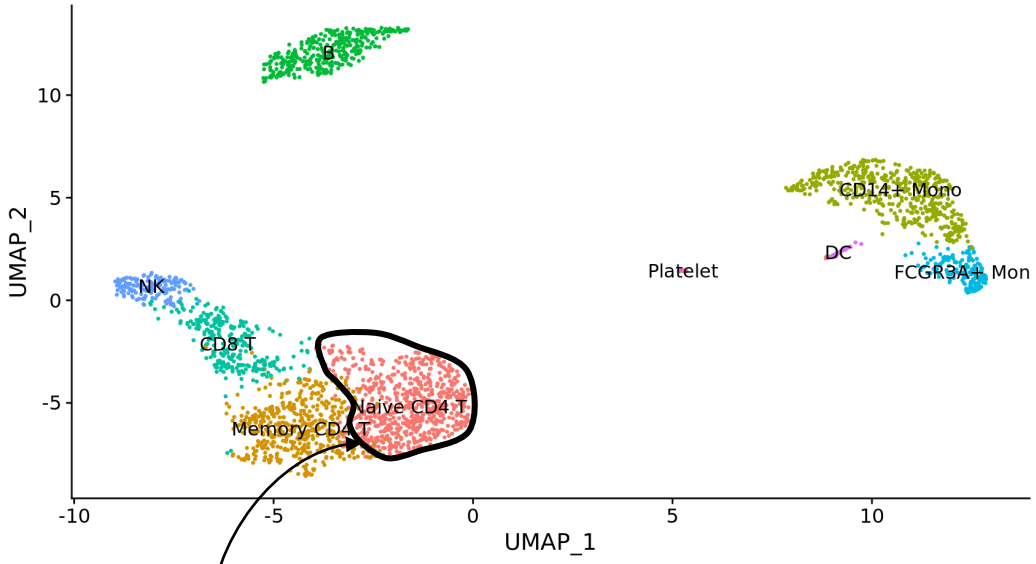
Luecken and Theis (MSB 2019)

DE for cluster annotation

Unannotated clusters



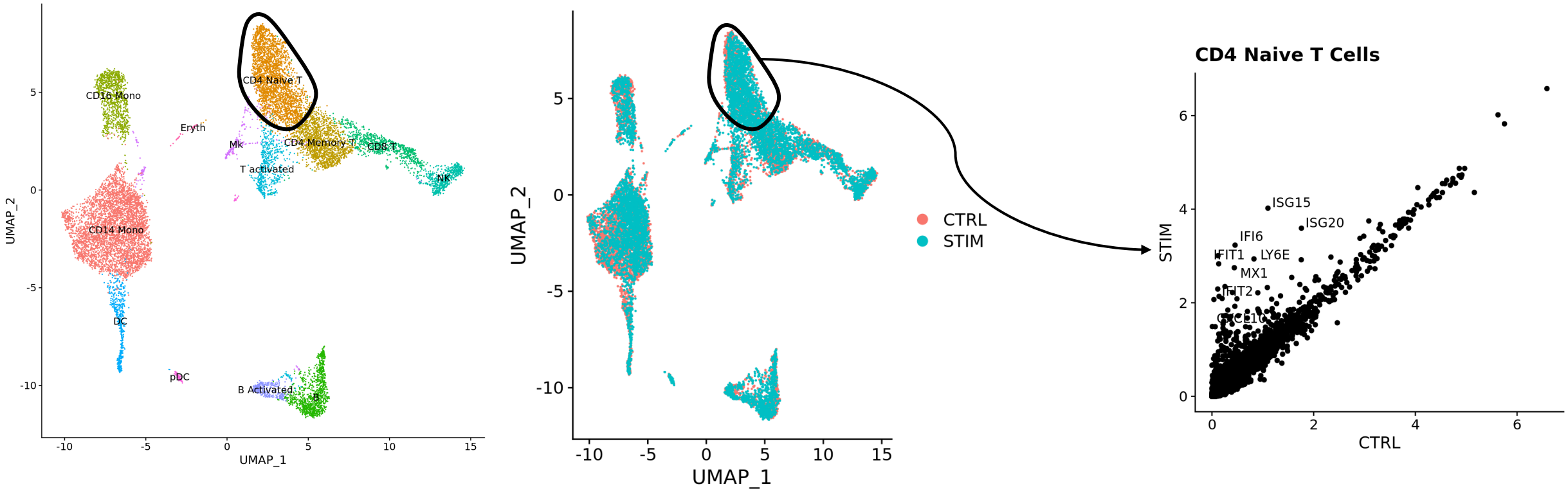
Annotated clusters



Compare *Cluster 0* to all other cells

IL7R
CCR7

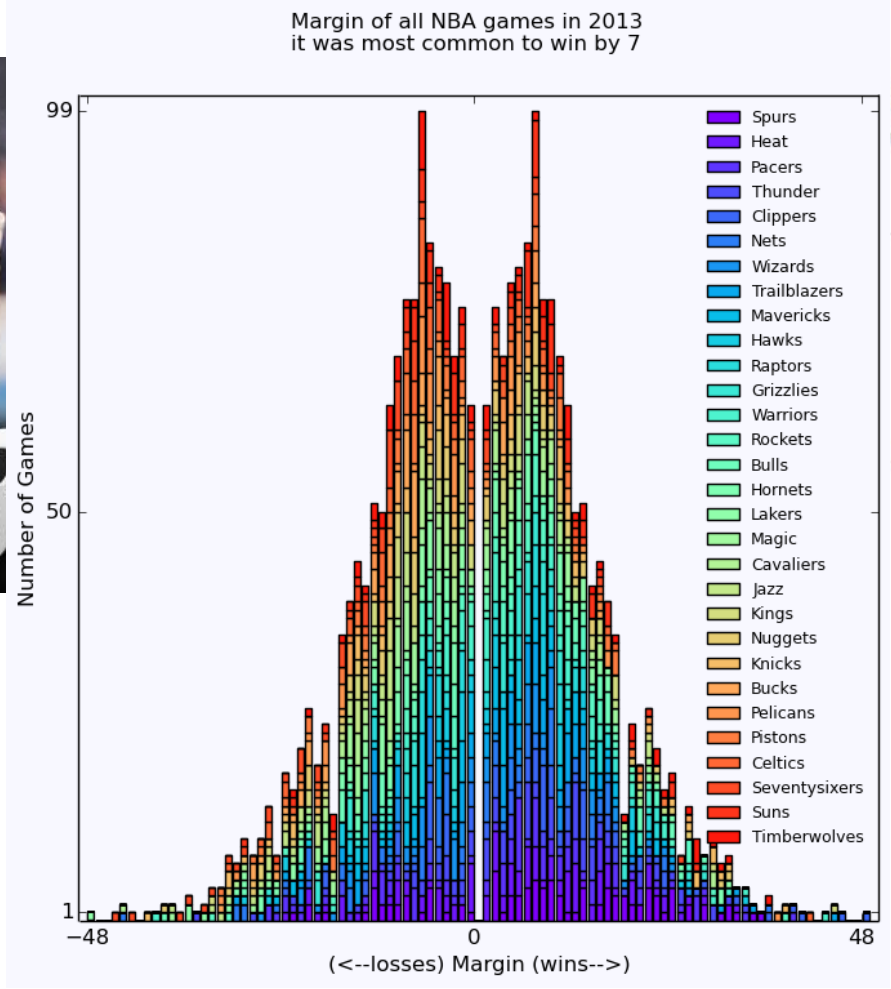
DE for comparing conditions



Outline

- Different methods for differential expression (DE) analysis for scRNA-seq data
- Single-cell DE in practice
- Working with integrated data
- Power analysis for scRNA-seq DE

Is this a large difference?



aptors

und, Game 7 - Raptors won series 4-3

84

89

Differential gene expression

- We need to decide for every gene, if the difference in expression observed between 2 groups is significant

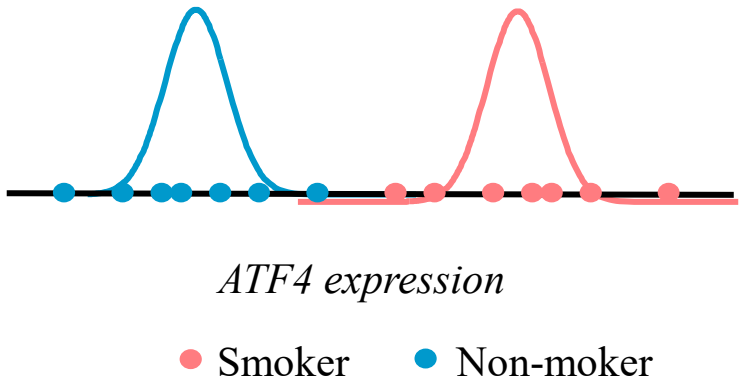
- Significant = greater than it would be expected just due to natural random variation

Example: Is there a difference in *ATF4* expression between smokers and non-smokers?

1. Define hypothesis
   - Null hypothesis (H0): there is no difference in expression
   - Alternative hypothesis (H1): there is a difference in expression

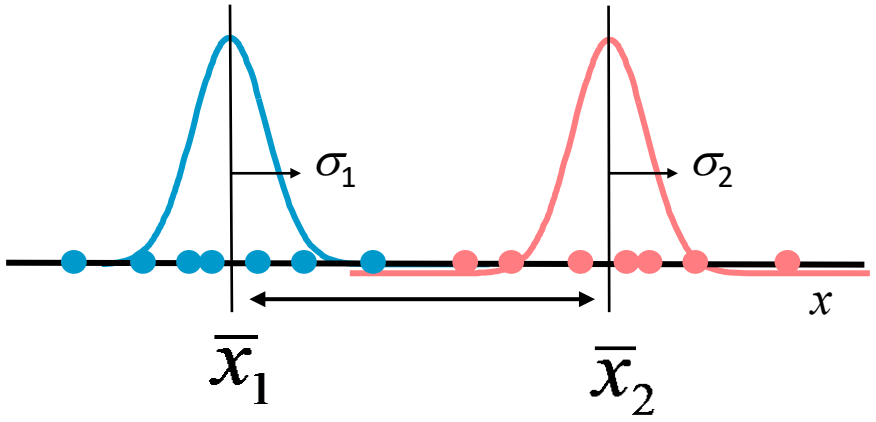
2. Measure some data
   - Expression of *ATF4*

3. Test your hypothesis
   - Compare *ATF4* expression between smokers and non-smokers



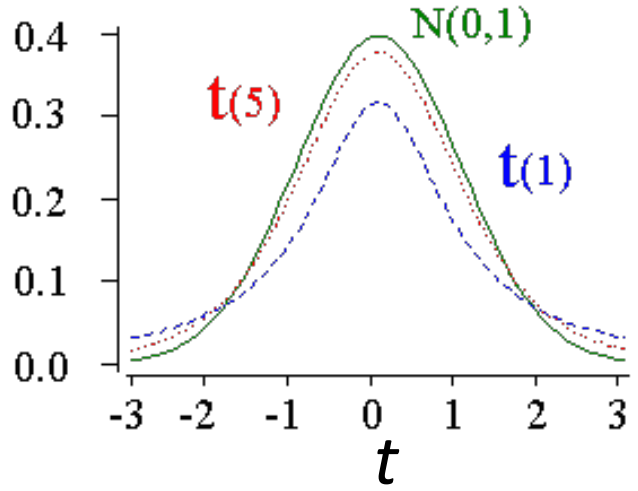
Model the data

- Model the data distribution (e.g. normal)
- Use a statistic to assess the difference (e.g. t-test)



$$\frac{signal}{noise} = \frac{difference\ in\ group\ means}{variability\ in\ groups}$$

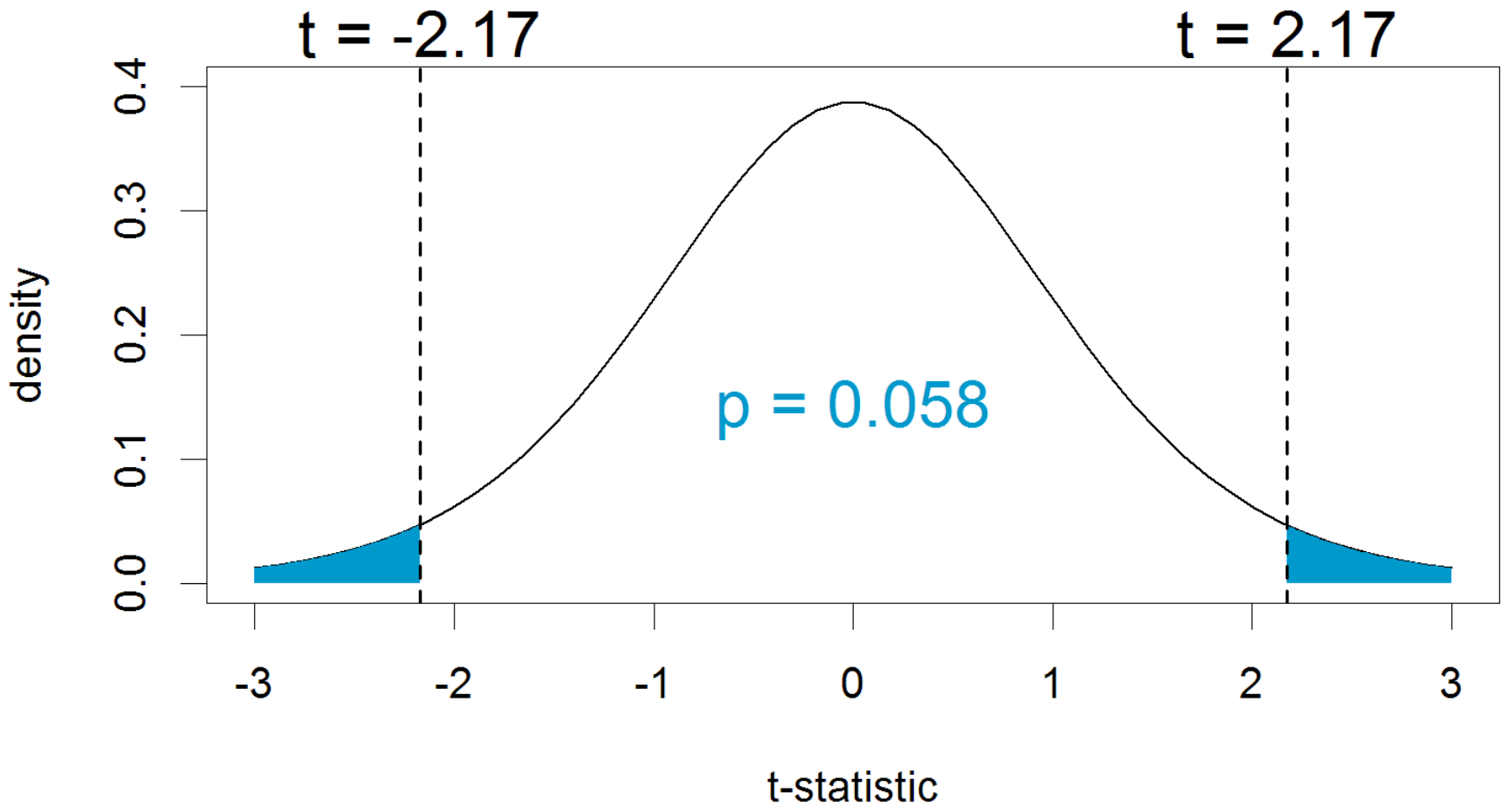
$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{(J_1-1)S_1^2 + (J_2-1)S_2^2}{J_1+J_2-2}\left(\frac{1}{J_1}+\frac{1}{J_2}\right)}}$$



t follows a Student *t*-distribution with J-1 degrees of freedom (DOF)

P-value

Two-sided test
t = 2.17
DOF = 9

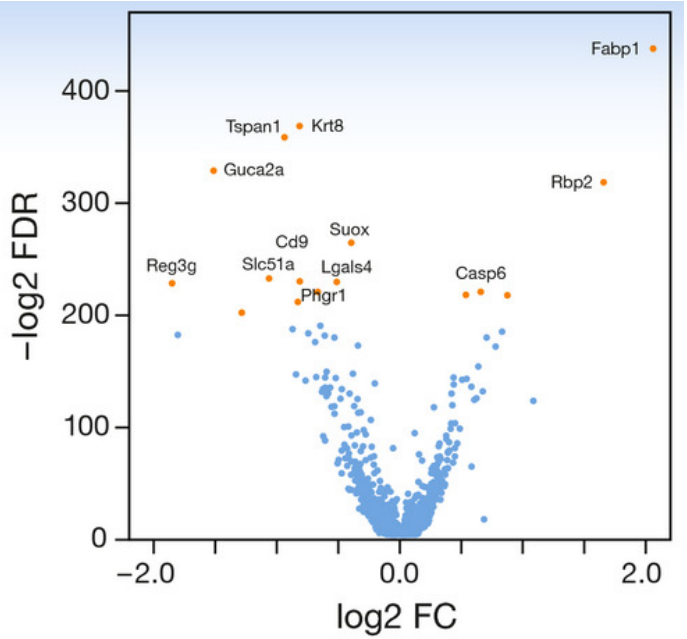


Effect size

- It is also wise to consider the effect size and not only the p-value
  - A very low p-value with a very low effect size is meaningless
- Effect size measure depends on the statistical test used
- E.g. in a t-test, the mean is compared between 2 groups (effect size = difference in the mean)
- Often represented as log fold-change (LFC)

$$lfc = \log_2\left(\frac{\bar{X}_1}{\bar{X}_2}\right)$$

Volcano plot



Luecken and Theis (MSB 2019)

How do we model the data?

- Find an appropriate model (appropriate = fits the data better)

- Use a non-parametric test (no model assumptions)

Non-parametric tests

- Forget about modeling the data, let’s use a non-parametric test.
- No assumption that expression values follow any particular distribution
- Expression values are (generally) converted to ranks and test whether the distribution of ranks for one group are significantly different from the distribution of ranks for the other group.
- Assumption: distributions have the same shape in both groups

Wilcoxon rank-sum test aka Mann-Whitney U test

- H_0: median$_1$ = median$_2$

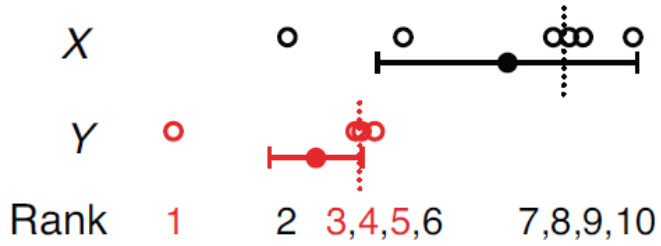
- Start by ranking all values

- Calculate the test statistic:

$$U = W - \frac{n_Y(n_Y+1)}{2}$$

sum of ranks in the smaller-sized sample

The lowest possible rank in the sample with the lower ranks



$$W = 1 + 3 + 4 + 5 = 13$$
$$U' = W - n_Y(n_Y + 1)/2$$
$$= 13 - 10$$
$$= 3$$

For cases in which both samples are larger than 10, the distribution of *U* is approximately normal

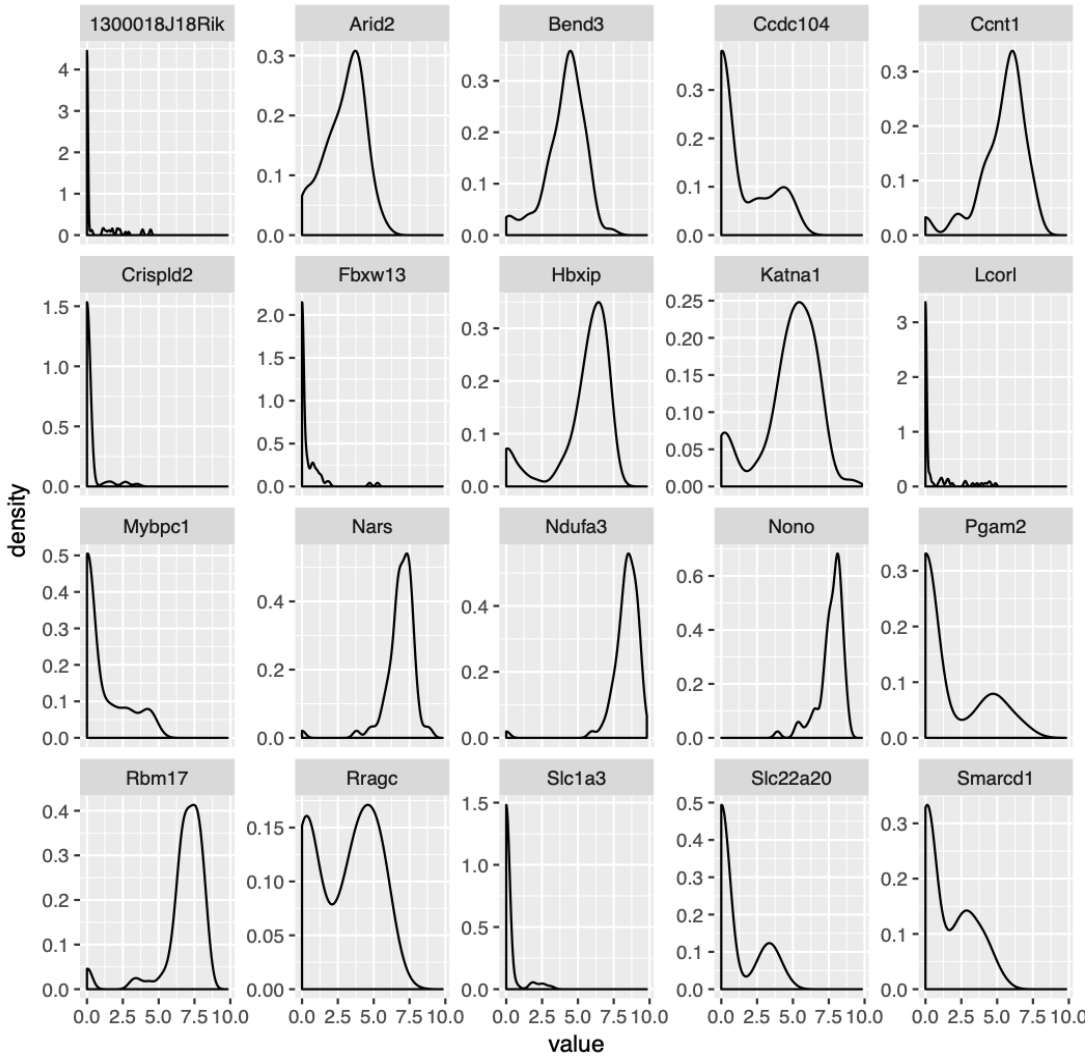
Example from: Krzywinski M, Altman N (2014) Nat Methods 11:467–469.

That must be the solution to everything?

- Not really…
- Wilcoxon rank sum test is not as powerful as parametric tests, i.e. it requires more data points to detect the same effects
- Might fail to deal with a large number of tied values, such as the case for zeros in single-cell RNA-seq expression data

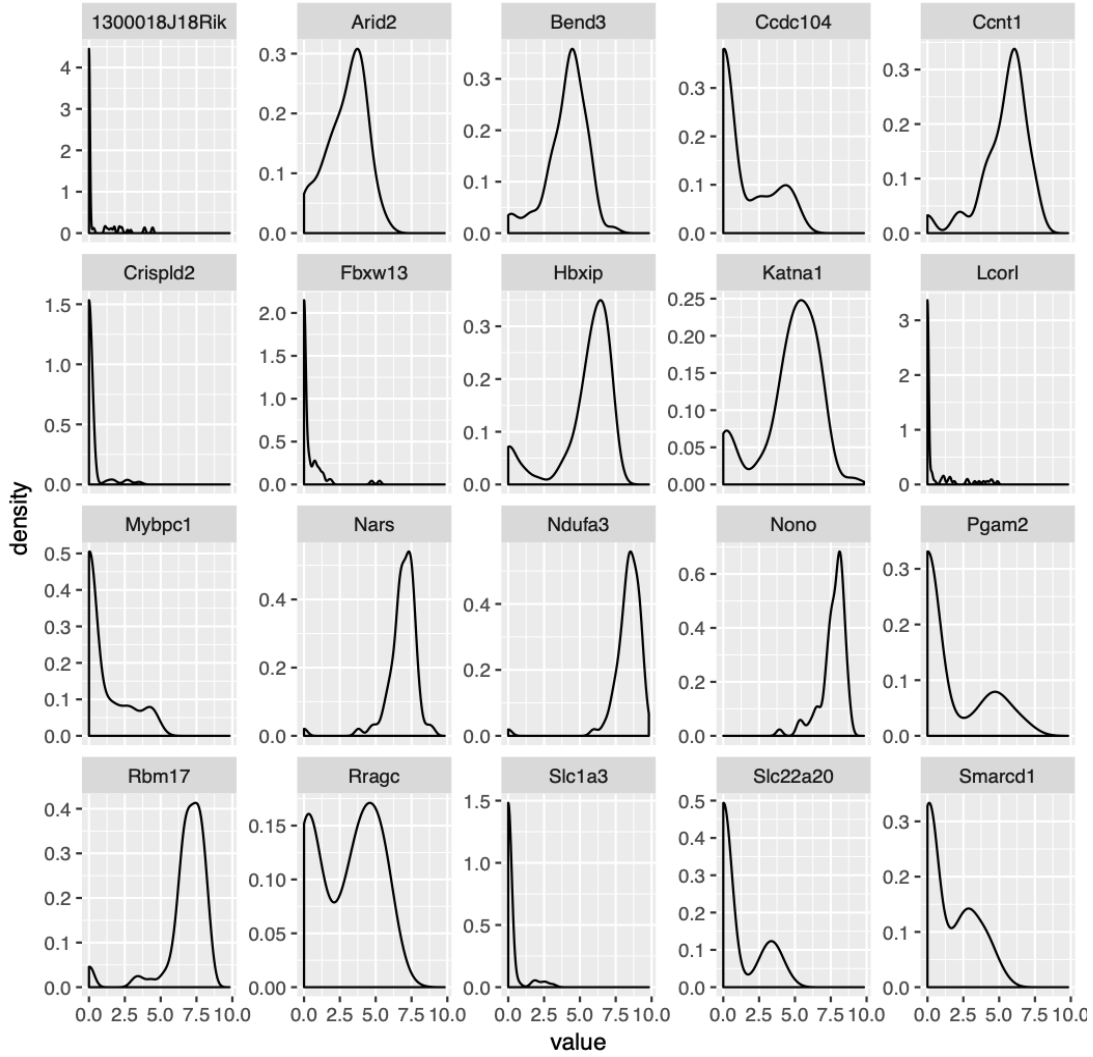
How can we model scRNA-seq data?

- Amplification bias
- Drop-out rates
- Transcriptional bursting
- Background noise
- Bias due to cell-cycle and cell size
- Often clear batch effects
- …



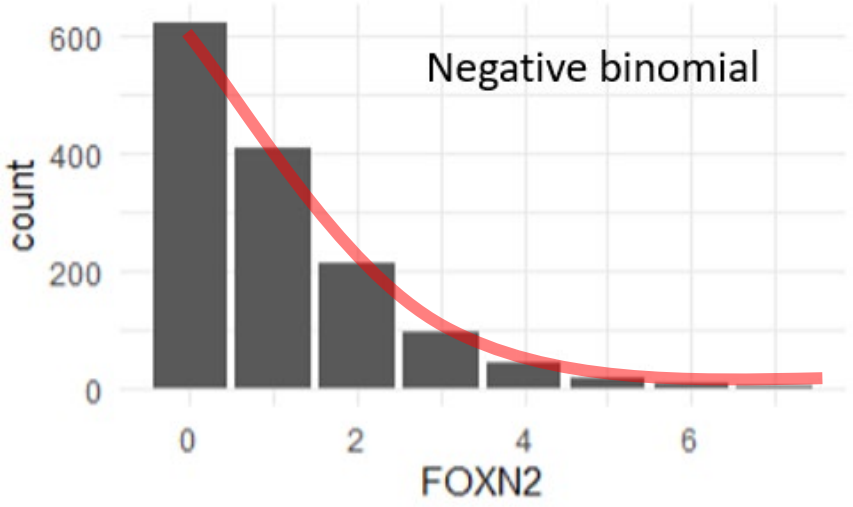
Which distribution would you use to model scRNA-seq data?

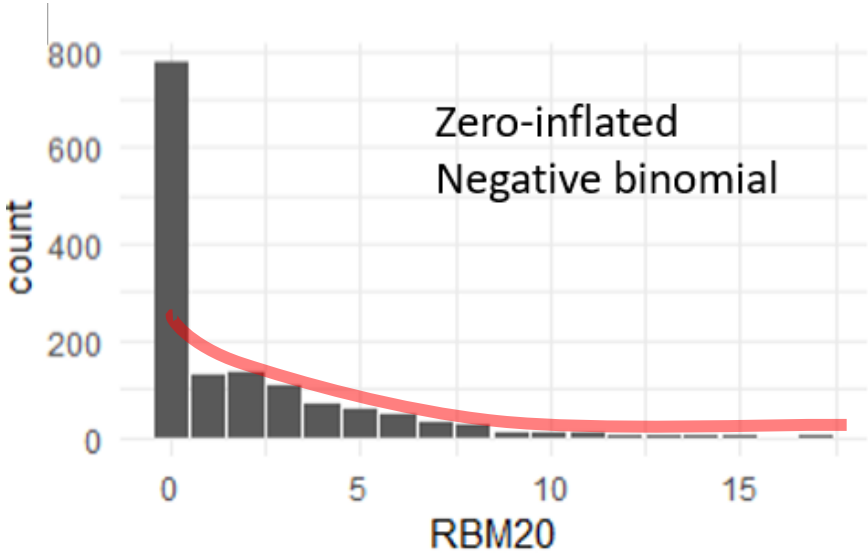
- Binomial
- Negative binomial
- Zero-inflated negative binomial
- Poisson
- …



Is single-cell data zero-inflated?

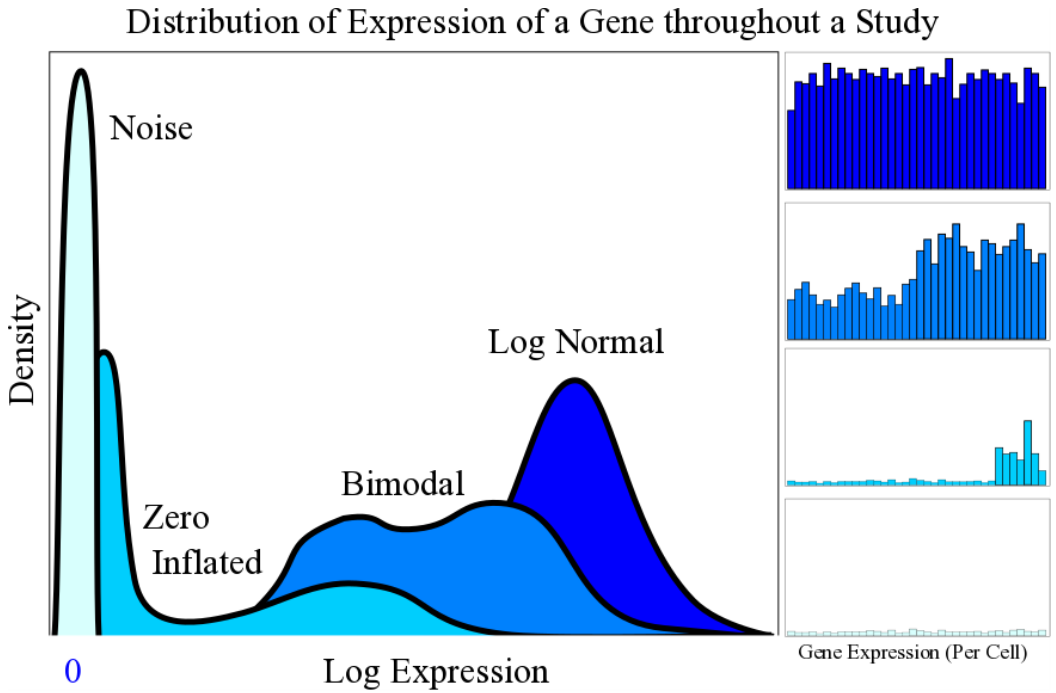
- “More zeros than expected”





MAST (GLM)

- MAST uses a hurdle model (a two-part generalized linear model)
- Part 1: models the discrete expression rate of each gene across cells (is the gene expressed or not?) *-> logistic regression*
- Part 2: models the continuous expression level (conditional on the gene being expressed) *-> linear Gaussian model*
- DE is determined using a likelihood ratio test



Finak et al. (Genome Biol 2015)

Is single-cell data zero-inflated?

Correspondence | Published: 14 January 2020

Droplet scRNA-seq is not zero-inflated

Valentine Svensson

Nature Biotechnology **38**, 147–150 (2020) | Cite this article

11k Accesses | **80** Citations | **89** Altmetric | Metrics

Matters Arising | Published: 01 February 2021

UMI or not UMI, that is the question for scRNA-seq zero-inflation

Yingying Cao, Simo Kitanovski, Ralf Küppers & Daniel Hoffmann

Nature Biotechnology **39**, 158–159 (2021) | Cite this article

Research | Open Access | Published: 27 July 2020

Bayesian model selection reveals biological origins of zero inflation in single-cell transcriptomics

Kwangbom Choi, Yang Chen, Daniel A. Skelly & Gary A. Churchill

Genome Biology **21**, Article number: 183 (2020) | Cite this article

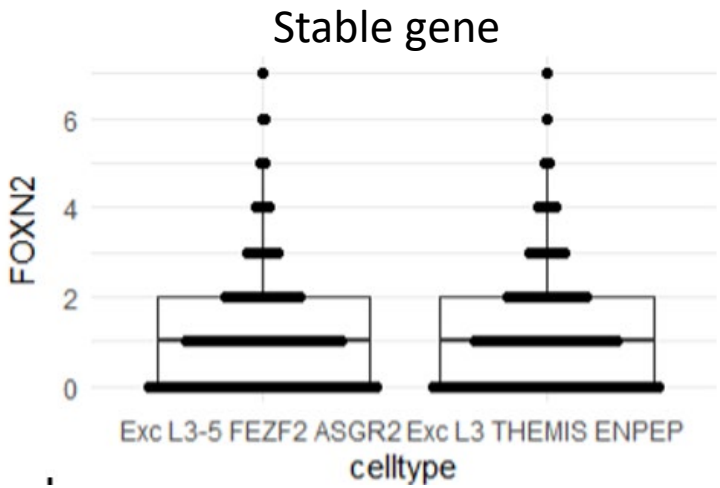
Review | Open Access | Published: 21 January 2022

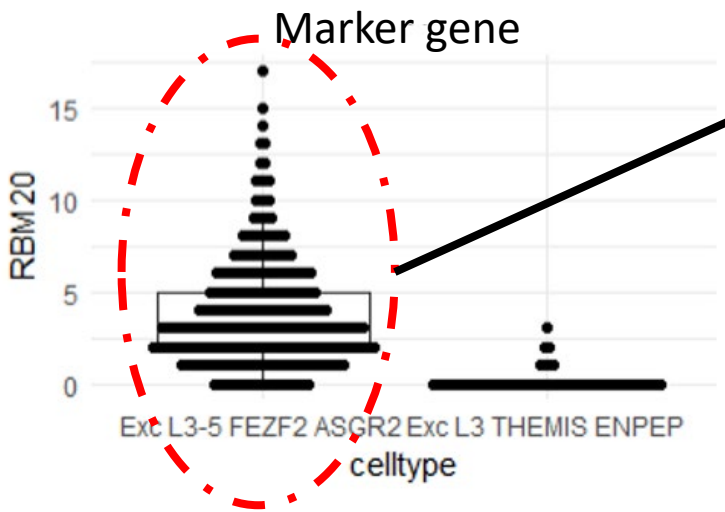
Statistics or biology: the zero-inflation controversy about scRNA-seq data

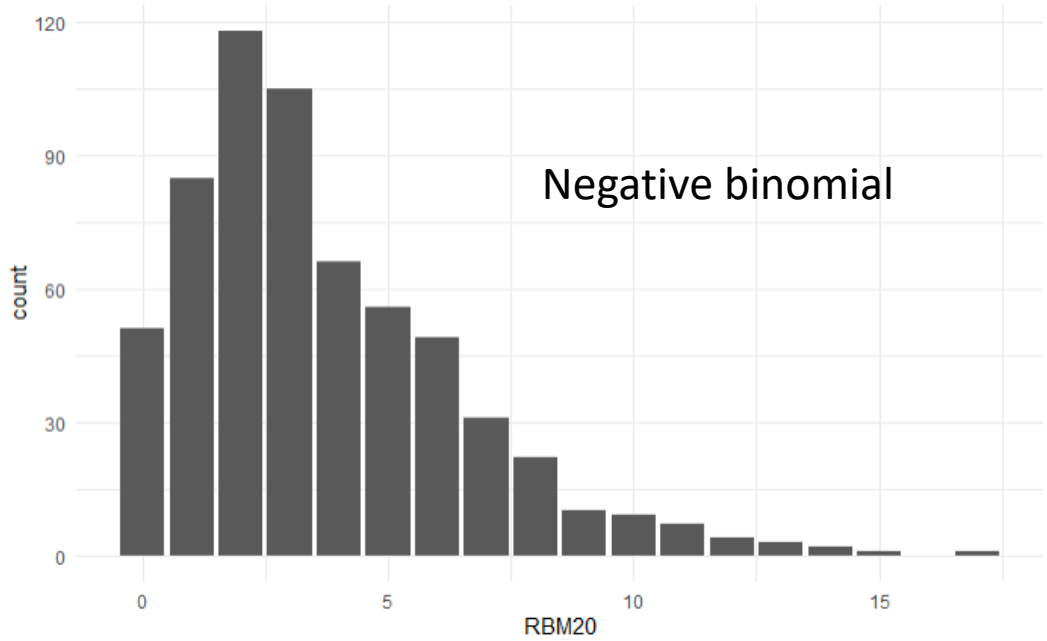
Ruochen Jiang, Tianyi Sun, Dongyuan Song & Jingyi Jessica Li

Genome Biology **23**, Article number: 31 (2022) | Cite this article

Is single-cell data zero-inflated?





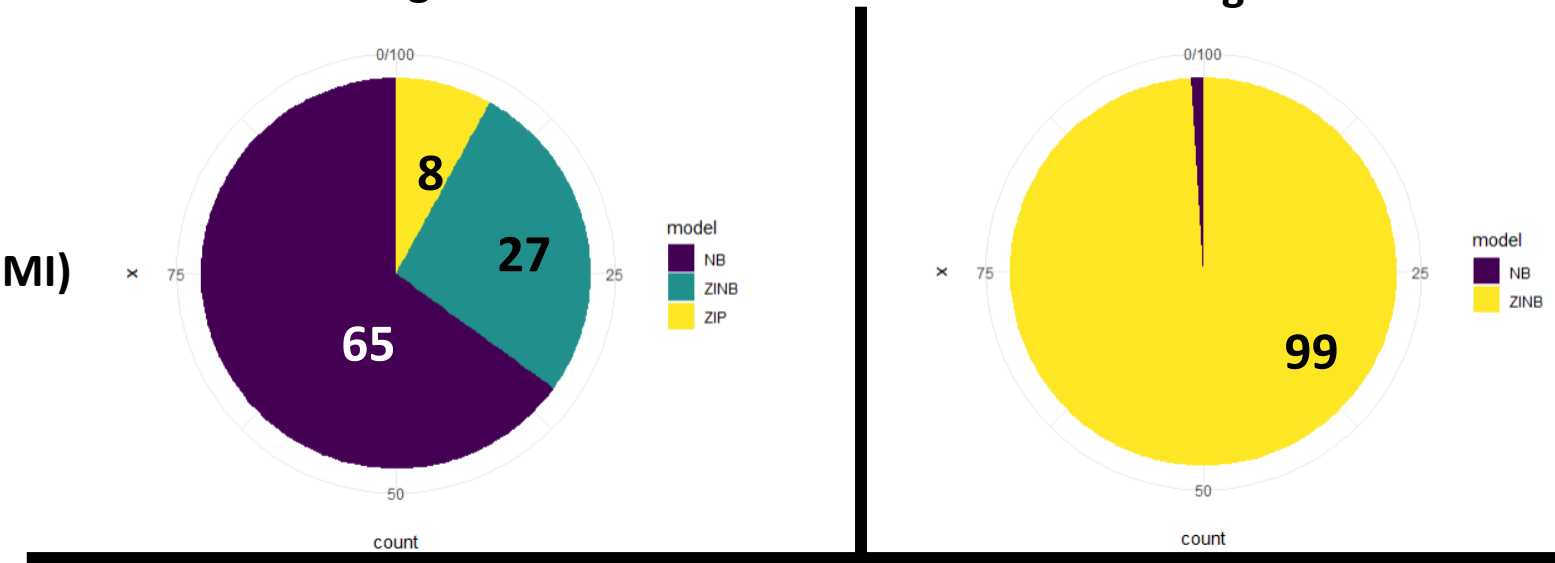


Bouland et al. bioRxiv 2022

Is single-cell data zero-inflated?

Stable genes | **Marker genes**

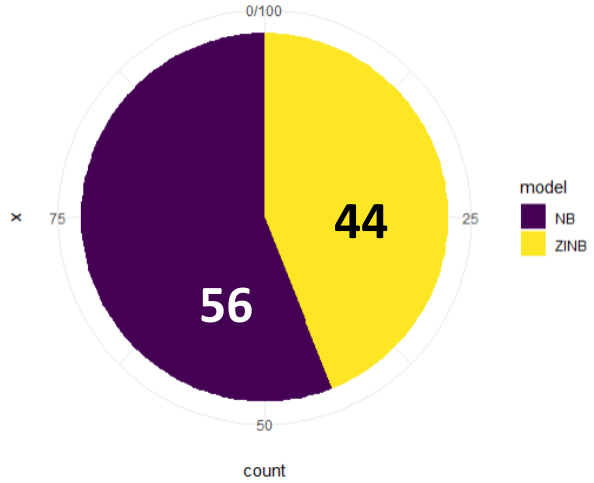
10x (UMI)

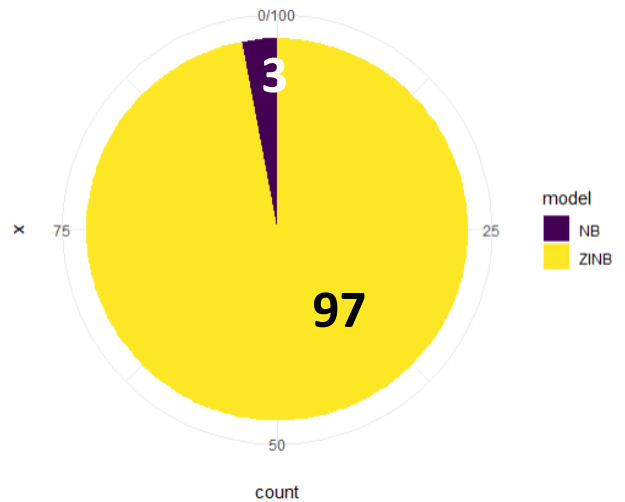


	Non-zero-inflated	Zero-inflated
Stable	65	35
Marker	1	99

$P = 3.02 \times 10^{-25}$
logOR = 5.18

Smart-seq (reads)





	Non-zero-inflated	Zero-inflated
Stable	56	44
Marker	3	97

$P = 5.46 \times 10^{-18}$
logOR = 3.69

Bouland et al. bioRxiv 2022

Is single-cell data zero-inflated?

Some genes are zero-inflated, some are not.

The main driver of zero-inflation is biological heterogeneity.

As such, the presence of a zero is mainly dictated by biology.

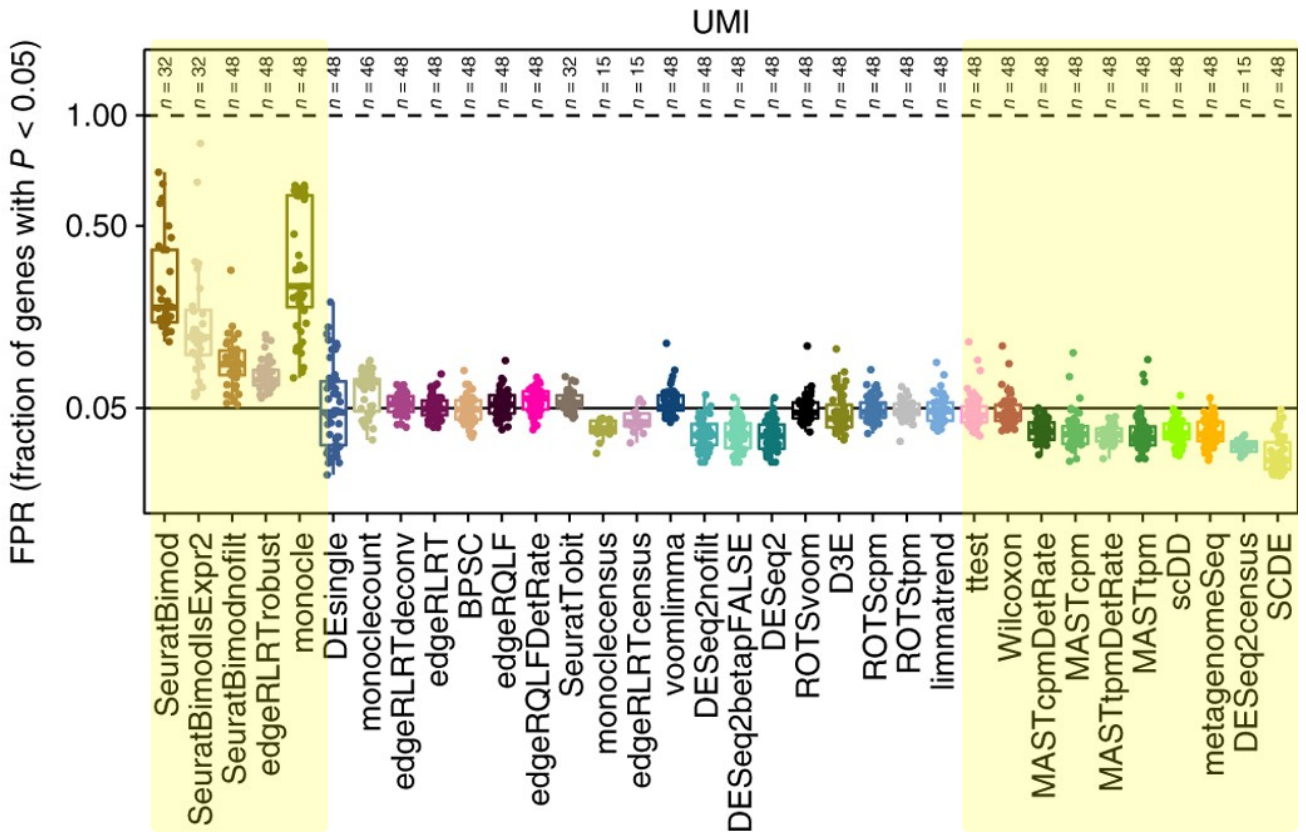
Bouland et al. bioRxiv 2022

Which model is better for DE analysis?

- Biological ground truth is difficult to define!
- Simulations: possible, but then results depend on the model used
- Rely on data with some known effects
  - E.g. Matched bulk RNA-seq dataset in the same purified cell type, exposed to the same perturbation under identical experimental conditions

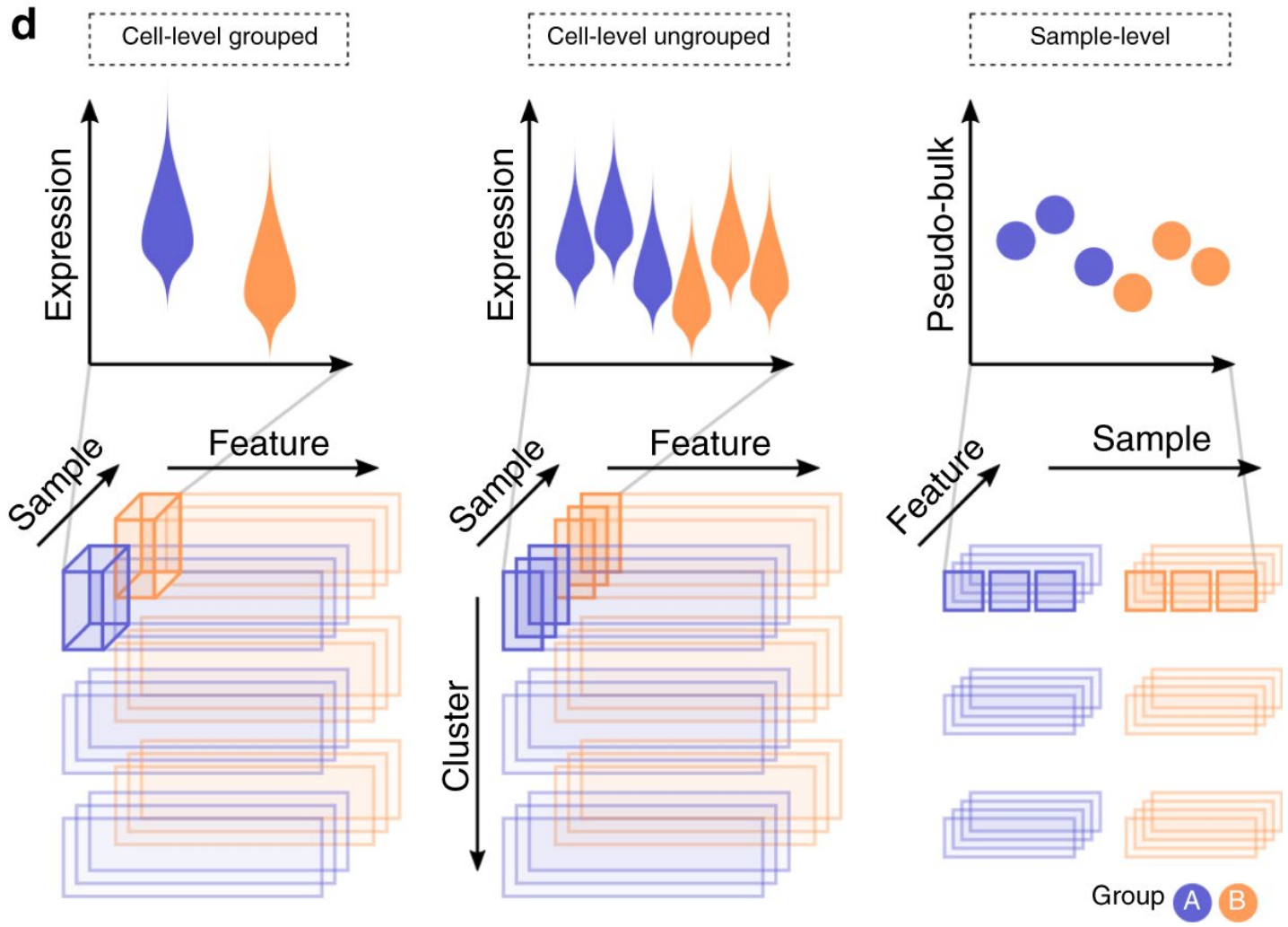
Comparing cell types (to identify markers)

- Overall, MAST, Wilcoxon, t-test outperformed other methods
- bulk RNA-seq analysis methods do not perform worse than scRNA-seq-specific methods
- Did not consider multi-sample setups



Soneson & Robinson, Nature Methods 2018

Comparing conditions



Cromwell et al., Nature Communications 2020

Benchmarking based on multi-sample setups

Using simulated data

Article | Open Access | Published: 30 November 2020

muscat detects subpopulation-specific state transitions from multi-sample multi-condition single-cell transcriptomics data

Helena L. Crowell, Charlotte Soneson, Pierre-Luc Germain, Daniela Calini, Ludovic Collin, Catarina Raposo, Dheeraj Malhotra & Mark D. Robinson

Nature Communications **11**, Article number: 6077 (2020) | Cite this article

7161 Accesses | **23** Citations | **48** Altmetric | Metrics

Recommends **pseudobulk approaches**

Article | Open Access | Published: 02 February 2021

A practical solution to pseudoreplication bias in single-cell studies

Kip D. Zimmerman, Mark A. Espeland & Carl D. Langefeld

Nature Communications **12**, Article number: 738 (2021) | Cite this article

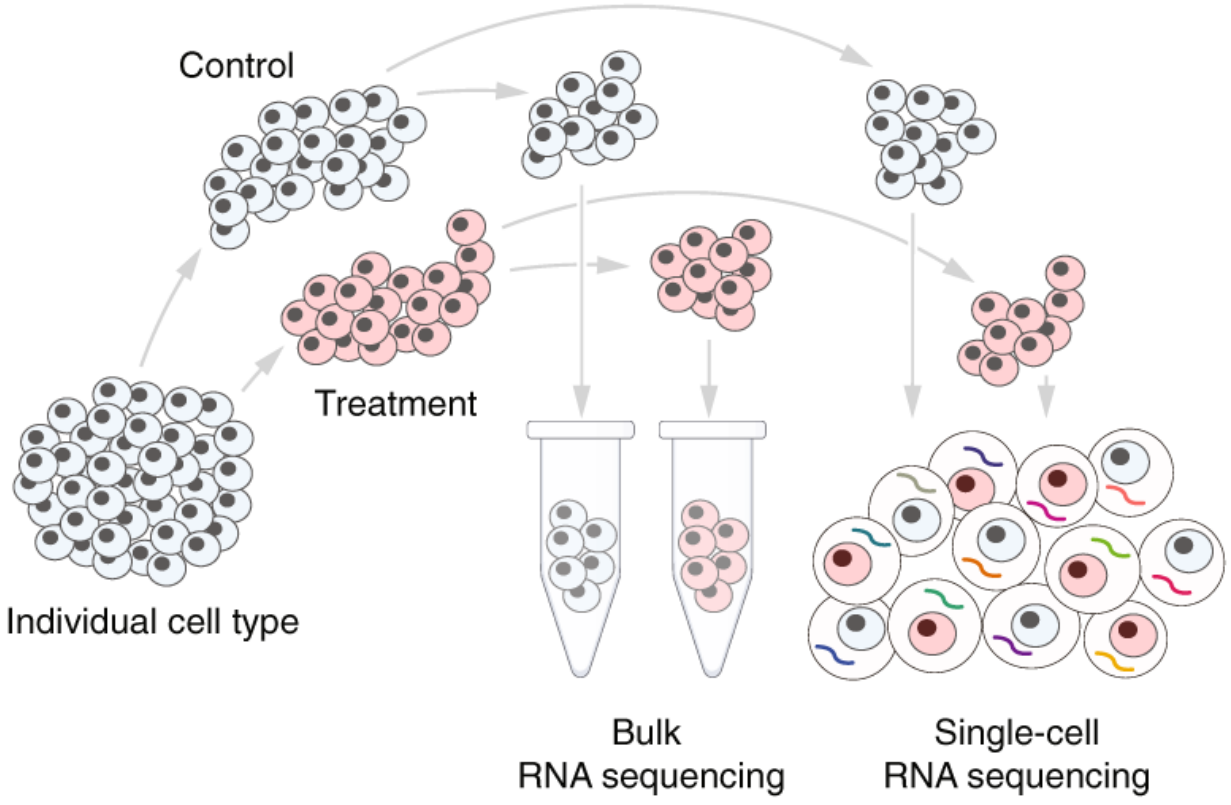
5078 Accesses | **4** Citations | **6** Altmetric | Metrics

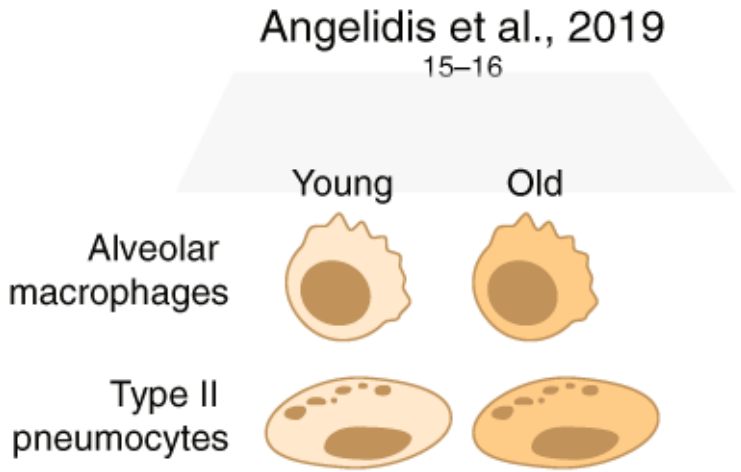
Recommends **generalized linear mixed models**

They use different simulation models!

Benchmarking based on multi-sample setups

Using scRNA-seq + bulk data



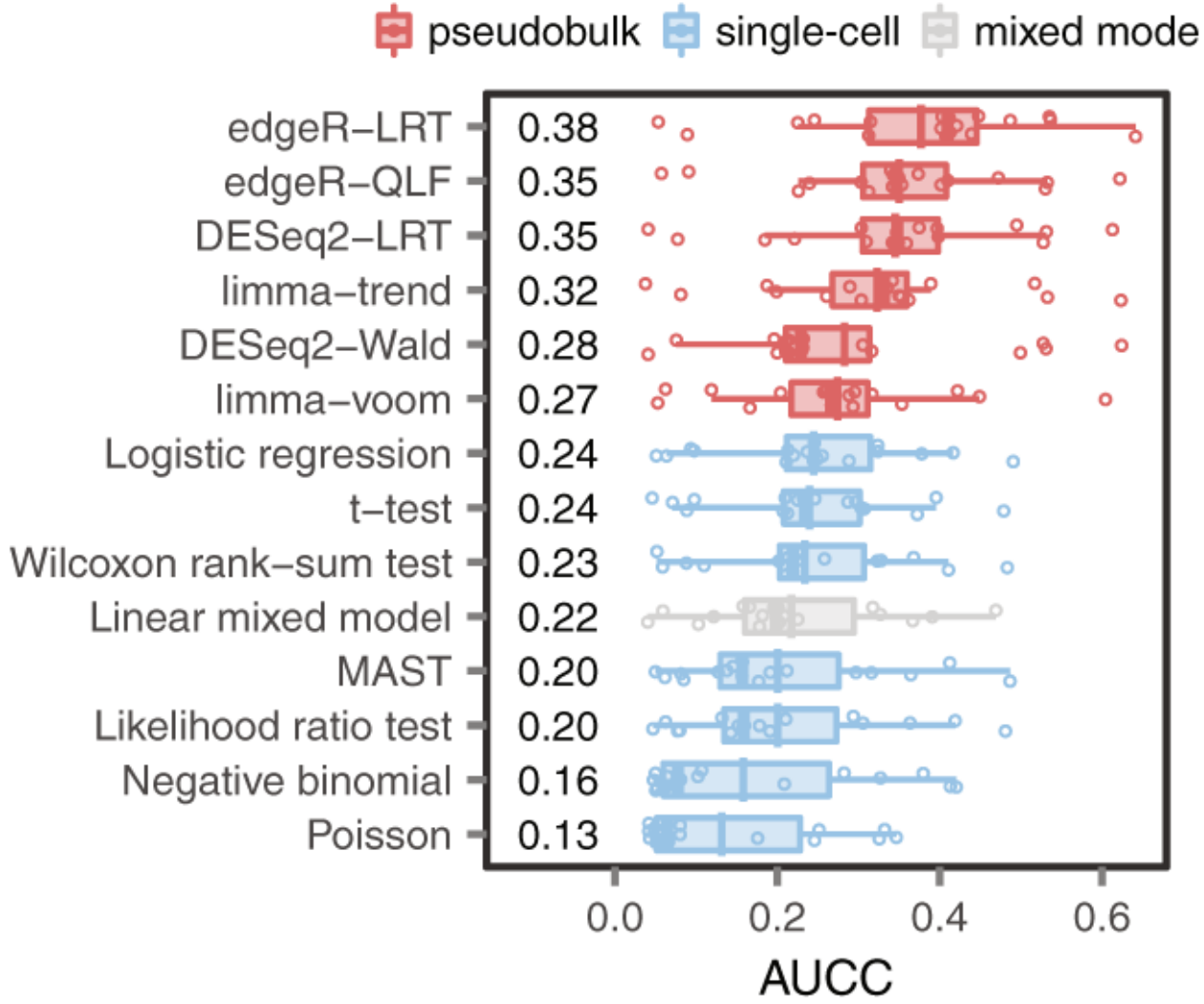


Squair et al., Nature Communications 2021

Benchmarking based on multi-sample setups

Using scRNA-seq + bulk data

- Pseudobulk methods are better
- Accounting for variation between biological replicates determines the performance of single-cell DE methods



Squair et al., Nature Communications 2021

Single-cell DE in practice

Single-cell DE in practice

Seurat

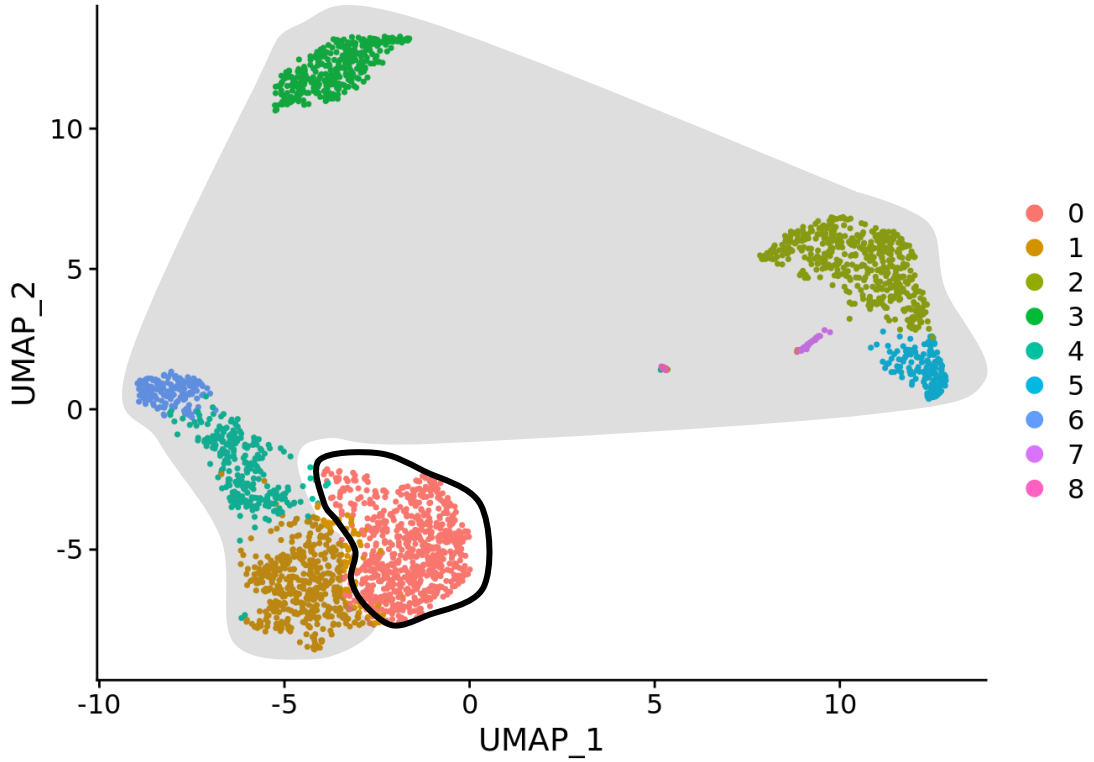
- "wilcox" : Wilcoxon rank sum test (default)
- "bimod" : Likelihood-ratio test for single cell feature expression, (McDavid et al., Bioinformatics, 2013)
- "roc" : Standard AUC classifier
- "t" : Student's t-test
- "poisson" : Likelihood ratio test assuming an underlying negative binomial distribution. Use only for UMI-based datasets
- "negbinom" : Likelihood ratio test assuming an underlying negative binomial distribution. Use only for UMI-based datasets
- "LR" : Uses a logistic regression framework to determine differentially expressed genes. Constructs a logistic regression model predicting group membership based on each feature individually and compares this to a null model with a likelihood ratio test.
- "MAST" : GLM-framework that treates cellular detection rate as a covariate (Finak et al, Genome Biology, 2015) (Installation instructions)
- "DESeq2" : DE based on a model using the negative binomial distribution (Love et al, Genome Biology, 2014) (Installation instructions)

https://satijalab.org/seurat/v3.1/de_vignette.html

Identifying cluster markers

- Approach 1: one-vs-all (default is Seurat)

- Limitations:
  - Sensitive to the population composition (one dominant population can drive marker selection for every other cluster)

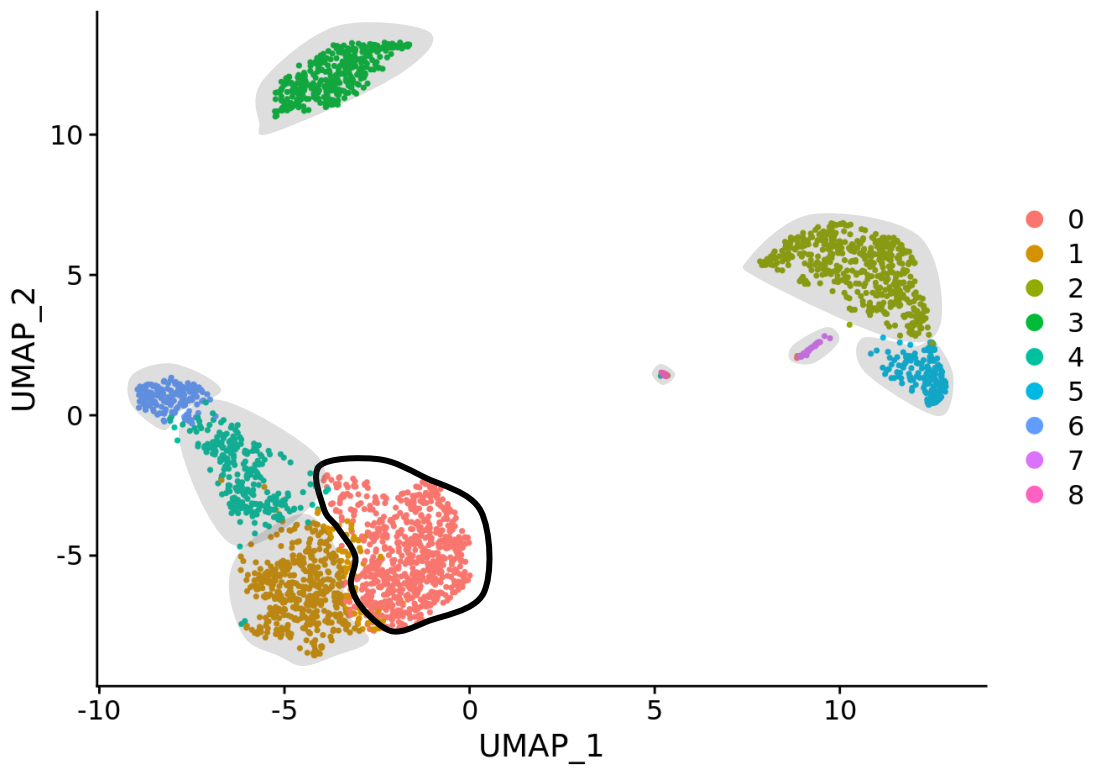


Identifying cluster markers

- Approach 2: multiple pairwise comparisons (default in scran)

- Strategies to combine results:
  - Prioritize genes significant in ***any*** pairwise comparison -> focuses on combinations of genes that (together) drive separation of a cluster from the others
  - Prioritize genes significant in ***all*** pairwise comparisons -> explicitly favors genes that are uniquely expressed in a cluster (too stringent)

- Limitations:
  - How to combine and report results?
  - Slow



Additional (practical) considerations

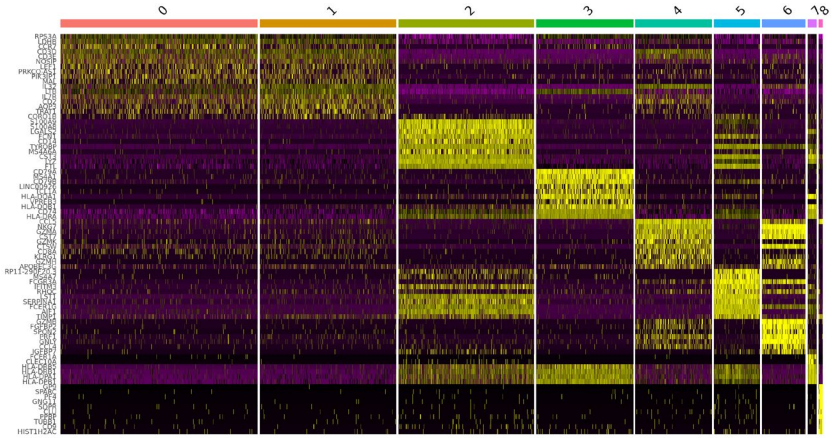
- Focus on ***positive*** markers only
  - It is difficult to interpret and experimentally validate the absence of expression

- Focus on genes with ***large effect size*** (log fold-change, LFC)
  - More biologically interesting markers (e.g. possible to validate with qPCR)
  - Faster testing (in Seurat)

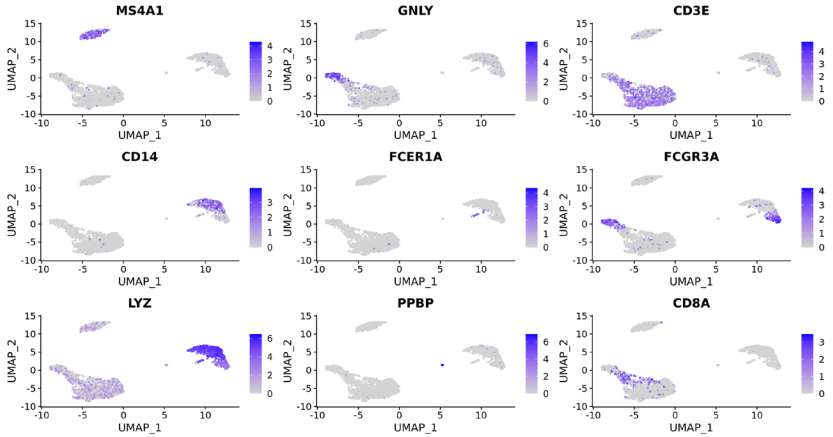
- Filter genes that are very infrequently detected in either group of cells
  - Seurat: `min.pct, logfc.threshold, min.diff.pct, max.cells.per.ident`

Check the identified markers

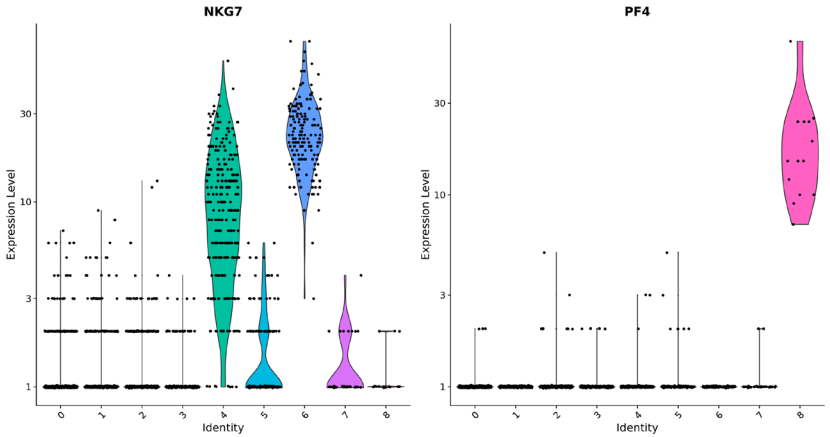
Heatmap



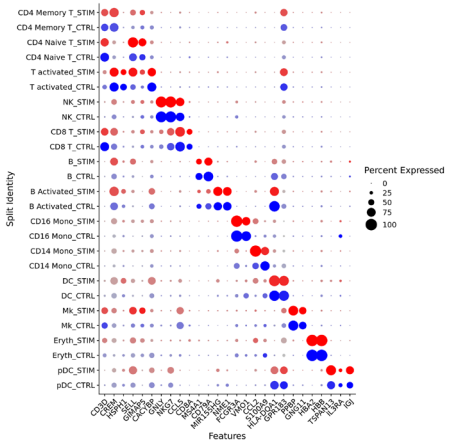
Overlap on tSNE/UMAP



Violinplot



Dotplot



Invalidity of p-values

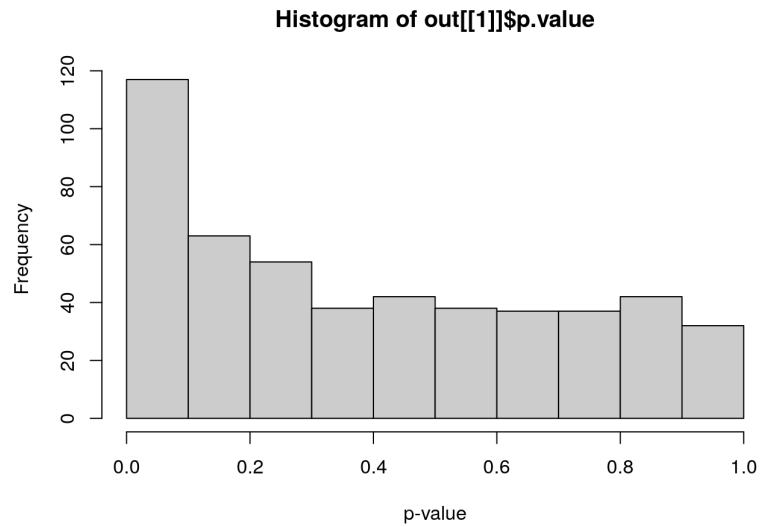
- DE analysis to detect marker genes between clusters is statistically flawed!

Amezquita et al. (Nature Methods 2019)

Invalidity of p-values

- Simulate i.i.d. normal values
- perform k-means clustering
- test for DE between clusters
- Plot the distribution of the resulting p-values

```r
library(scran)
set.seed(0)
y <- matrix(rnorm(100000), ncol=200)
clusters <- kmeans(t(y), centers=2)$cluster
out <- findMarkers(y, clusters)
hist(out[[1]]$p.value, col="grey80", xlab="p-value")
```



- heavily skewed towards low values -> we can detect “significant” differences between clusters even in the absence of any real substructure in the data.

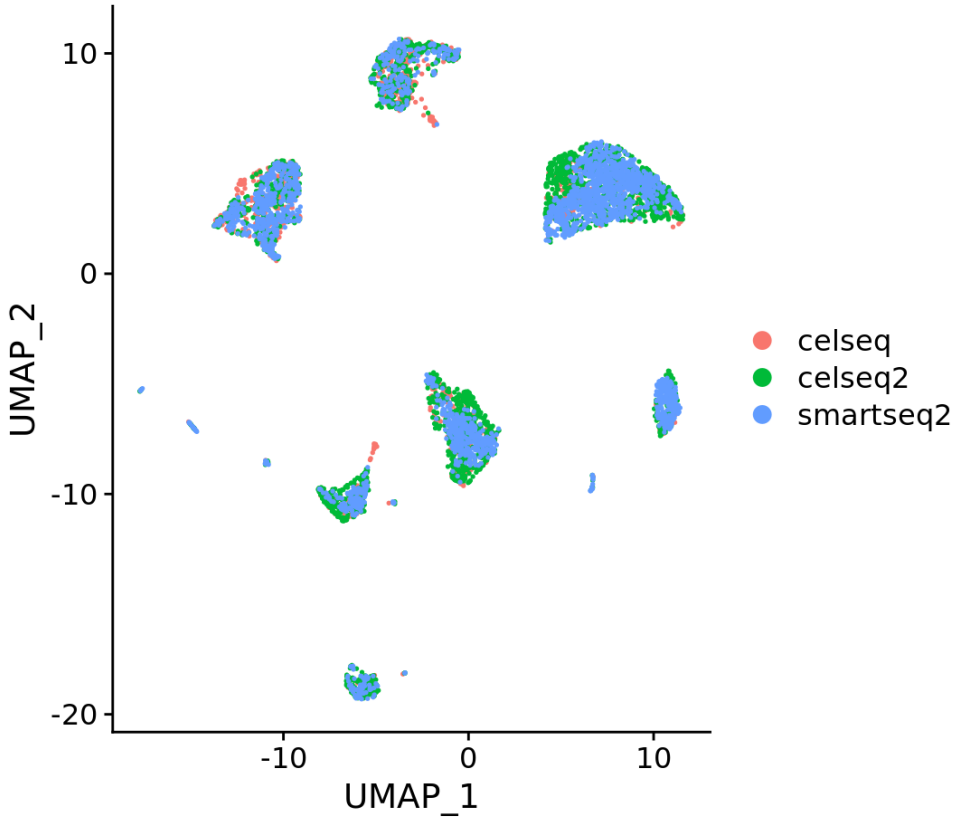
Amezquita et al. (Nature Methods 2019)

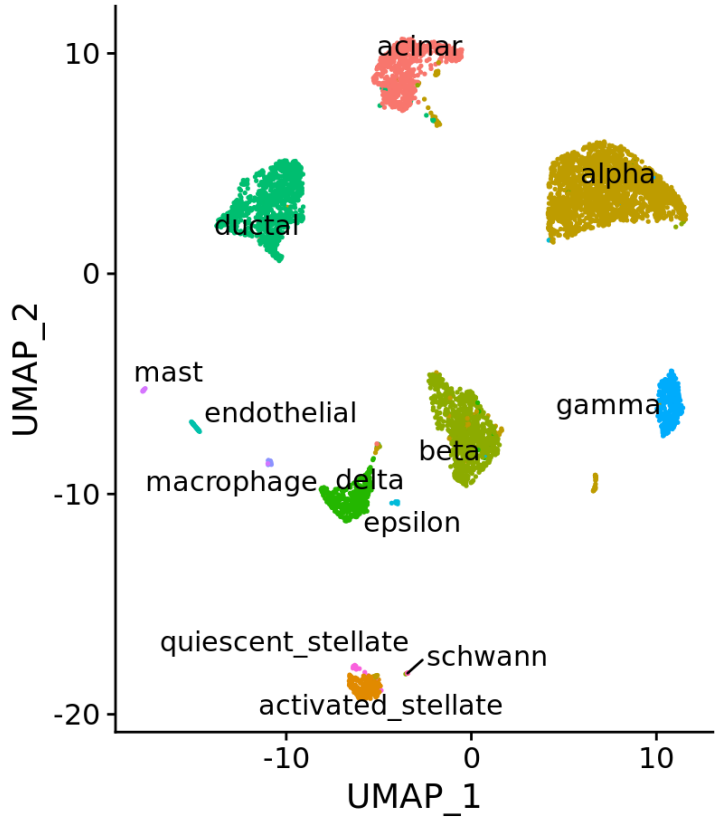
Invalidity of p-values

- DE analysis to detect marker genes between clusters is statistically flawed!
- DE analysis is performed on the same data used to obtain the clusters (data snooping) -> testing for DE genes between clusters will inevitably yield some significant results (that is how the clusters were defined).
- For marker gene detection, this effect is largely harmless as the p-values are used only for ranking.
- However, it becomes an issue when the p-values are used to define “significant differences” between clusters

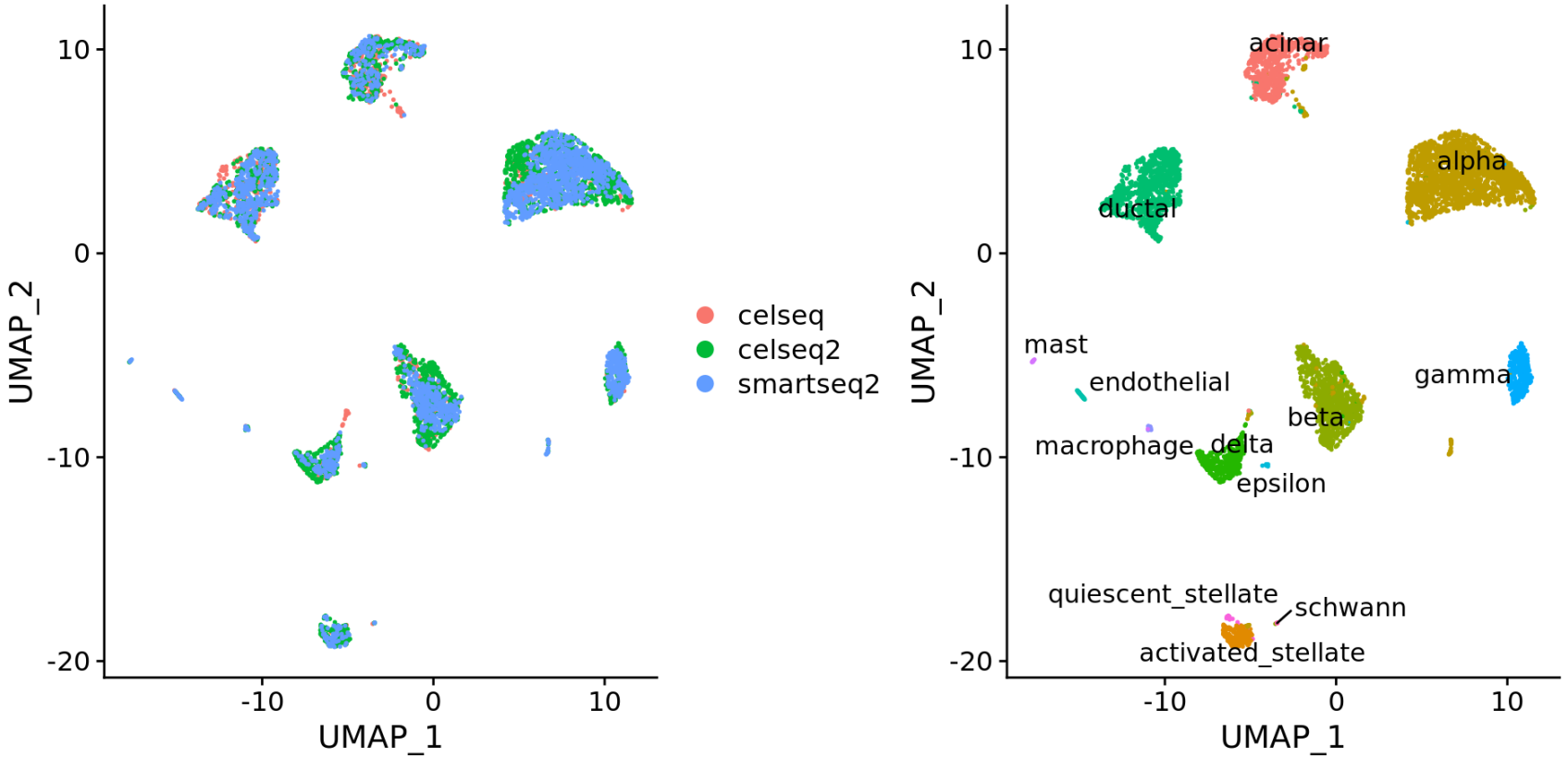
Amezquita et al. (Nature Methods 2019)

DE with integrated data





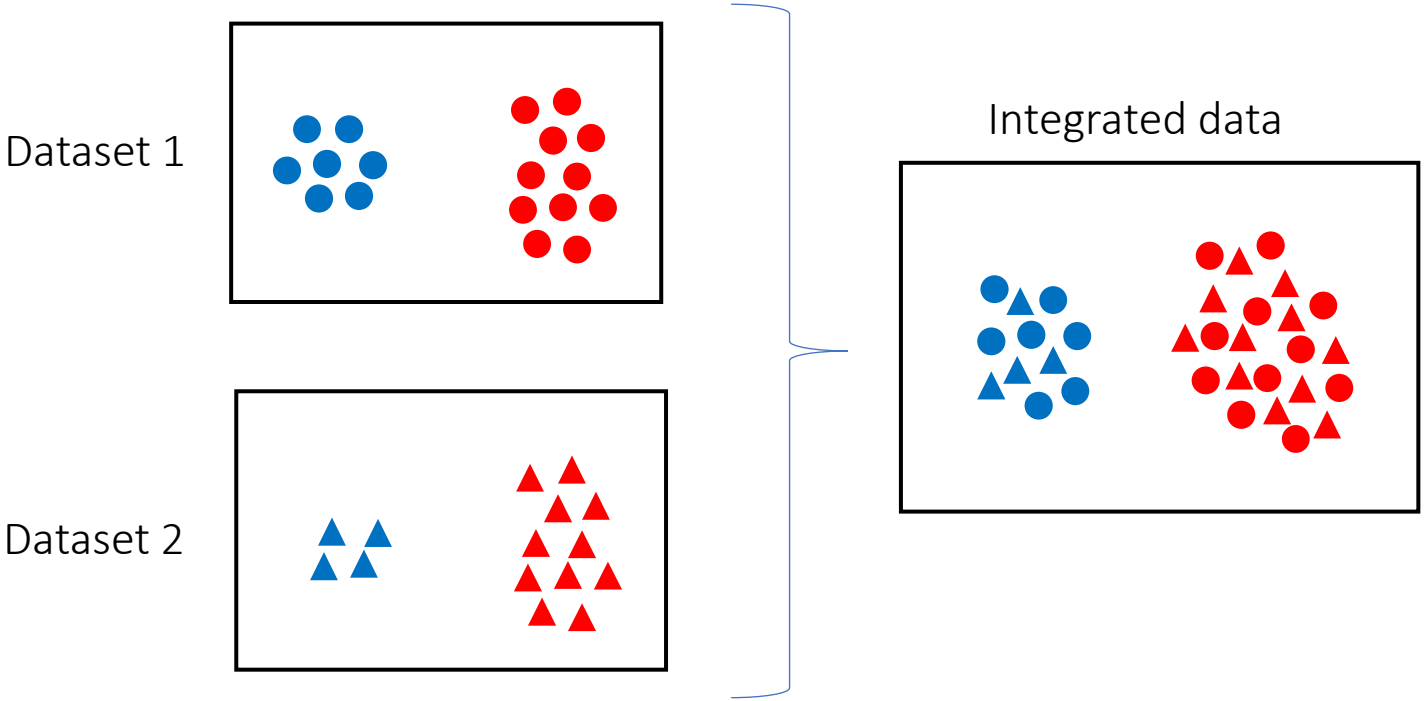
DE with integrated data

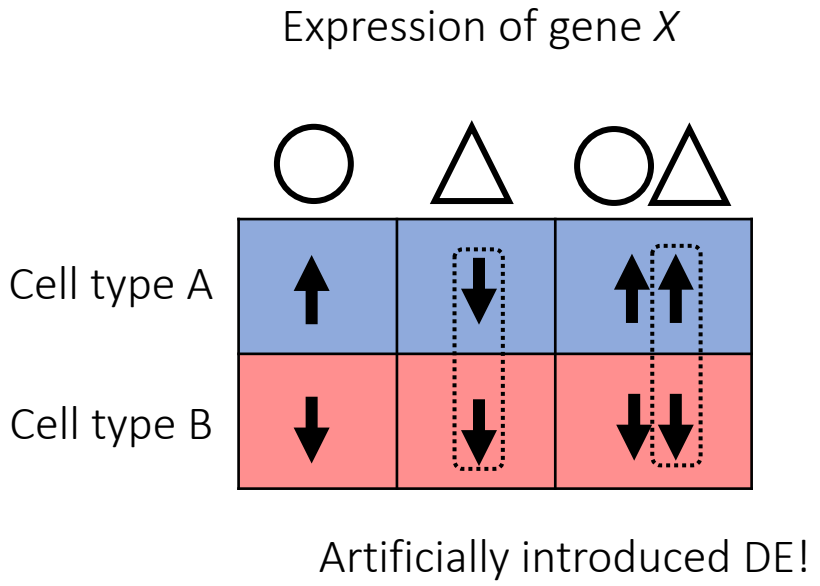


Uncorrected, measured data should be used for DE testing

Why uncorrected values?

- Correction algorithms are not obliged to preserve the magnitude or direction of differences in per-gene expression when attempting to align multiple batches.





Amezquita et al. (Nature Methods 2019)

How to perform DE with integrated data?

- Perform DE using the uncorrected values, separately per batch and combine p-values using meta-analysis.

- Similar to incorporating covariates in bulk DE analysis

- Penalizes genes with inconsistent DE across batches

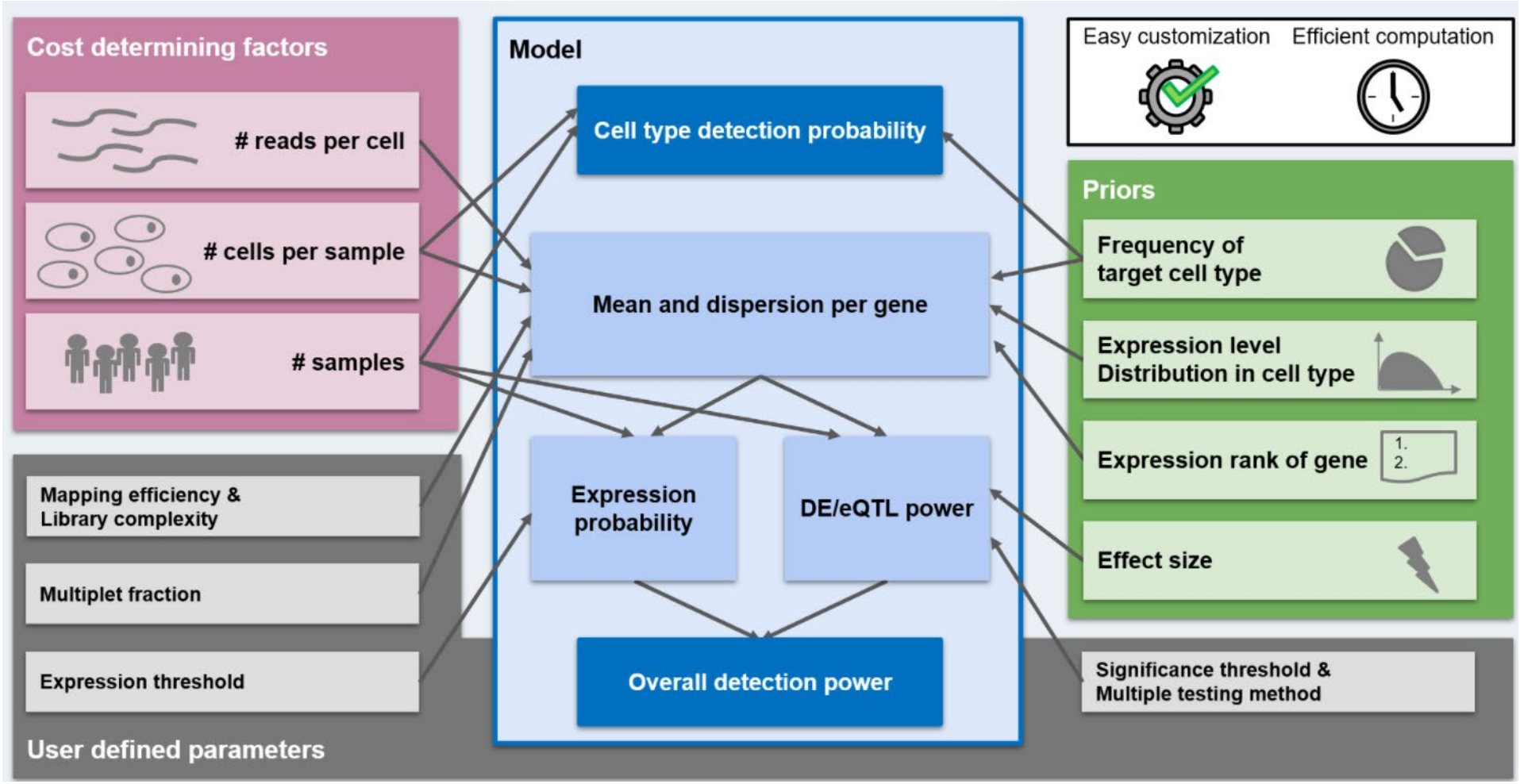
- In practice:
  - Seurat, use the `FindConservedMarkers` function
  - scran, incorporating batches as blocks in the `findMarkers` function

Power analysis for scRNA-seq DE

Power analysis for scRNA-seq DE

- What is the best possible experiment we can do for a limited budget
- Given a certain budget, how many ***samples***, how many ***cells per sample***, and how many ***reads per cell*** are required to detect DEGs given certain assumptions about the expected effect sizes
- Available tools:
  - PowsimR
  - scDesign
  - scPower
  - …

scPower



Website: http://scpower.helmholtz-muenchen.de/
R package: https://github.com/heiniglab/scPower

scPower

- Uses prior knowledge based on published data (website) or your own pilot data (R package)
- General recommendation: shallow sequencing of a large number of cells per individual (many DE scenarios, different platforms)
- Results based on using Negative binomial regression to detect DEGs, results might be different for other tests/models!

Schmid et al. (bioRxiv 2020)

To summarize

- Differential expression can be used to identify cell type markers or to compare biological conditions
- For marker gene identification (comparing cell types), MAST and Wilcoxon rank-sum test perform well
- P-values obtained from cell type comparisons are statistically invalid
- For comparisons between conditions, it is better to use pseudobulk approaches to account for variation between biological replicates
- DE testing should not be performed on batch-corrected data, but instead on measured data with technical covariates included in the model

Before you go…

- Mini-symposium tomorrow
- RStudio Cloud will be accessible **until 17 October 2022**.
- All course materials (lectures, markdown files, data,…) are available: https://github.com/LeidenCBC/MGC-BioSB-SingleCellAnalysis2022
- Don’t forget to return the evaluation forms after the mini-symposium.

Thank You!

 a.mahfouz@lumc.nl

 mahfouzlab.org

 @ahmedElkoussy