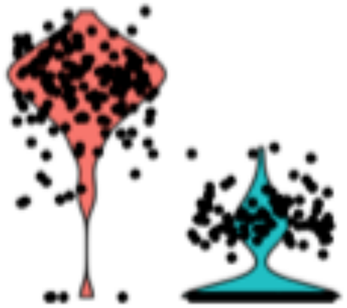
NBIS NATIONAL BIOINFORMATICS INFRASTRUCTURE SWEDEN

SciLifeLab

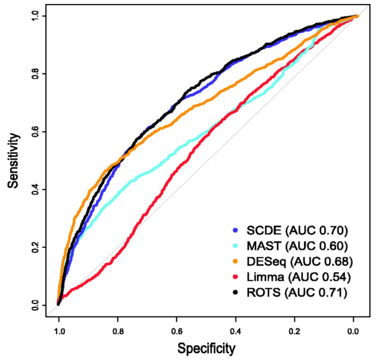
Differential expression analysis

Olga Dethlefsen / Åsa Björklund
NBIS



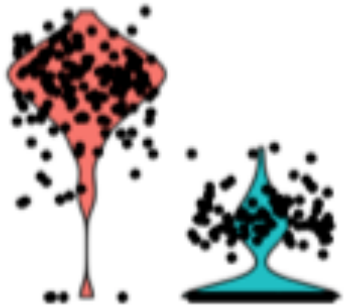
- ❖ **What is “differential expression analysis”**

- ❖ **Common methods**
  - intro to statistical inference



- ❖ **Performance**

- ❖ **Things to think about**



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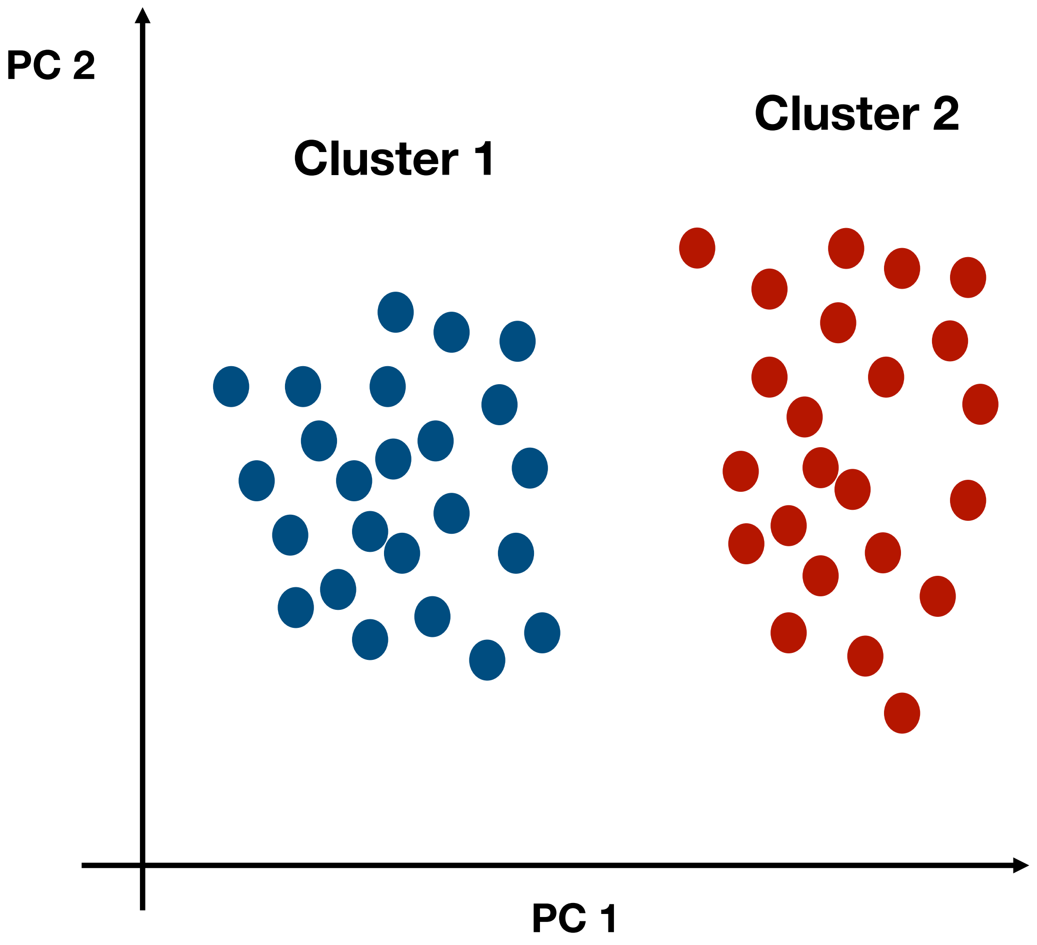

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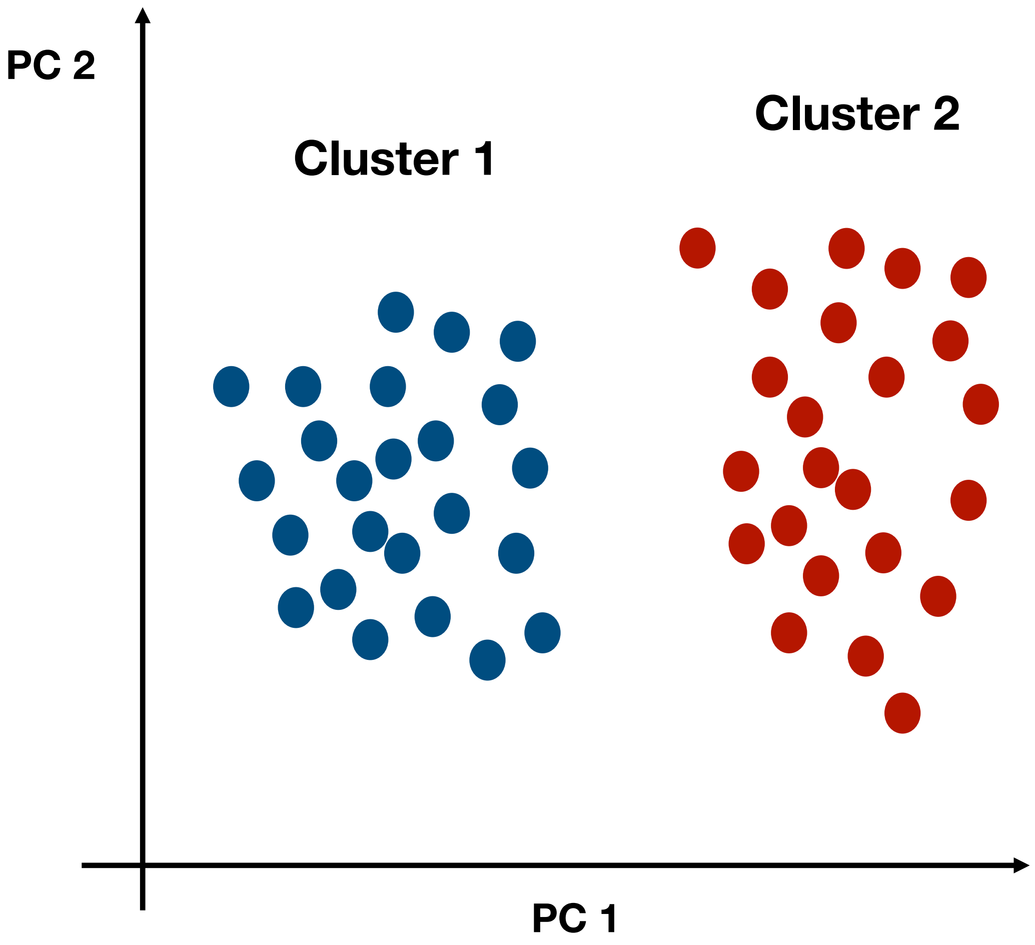
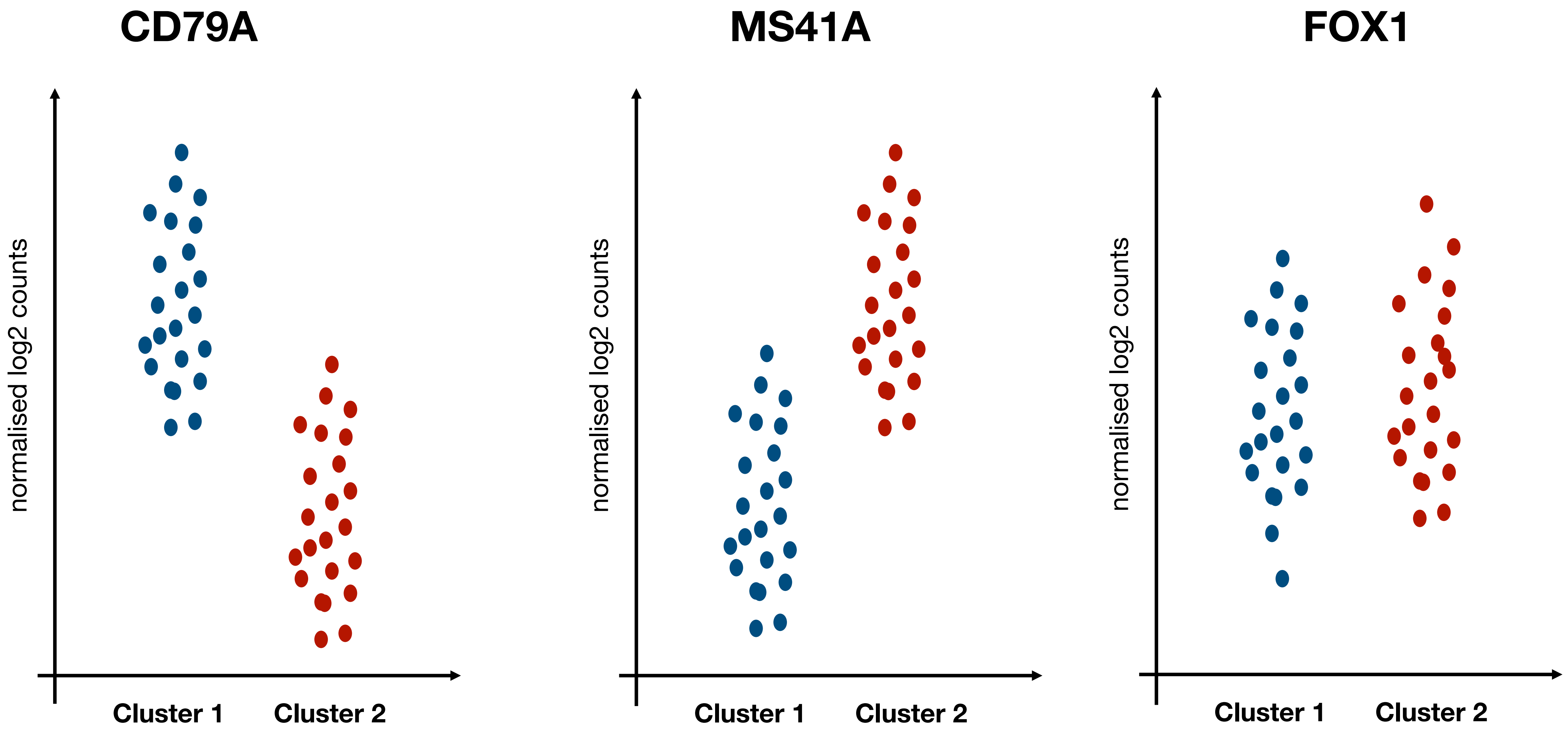
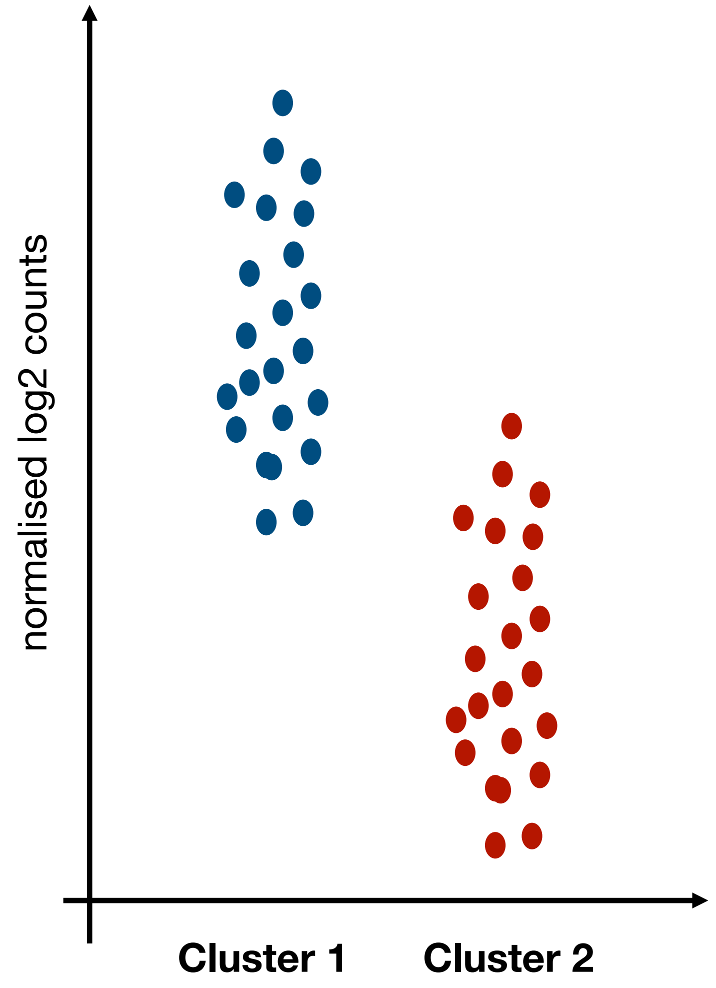


gene	logFC (avg)	p-value
CD79A	2,82	4,73×10^-20
CD79B	2,23	4,07×10^-19
MS4A1	-2,44	4,67×10^-19
CD74	2,07	2,56×10^-17
HLA-DRB1	1,53	5,04×10^-17
IGHM	-3,7	6,00×10^-17
HLA-DPA1	1,45	1,11×10^-16
HLA-DQB1	1,73	2,35×10^-16
⋮		
HLA-DQA1	1,83	3,01×10^-16
HLA-DRA	1,49	4,66×10^-16


CD79A
normalised log2 counts
Cluster 1
Cluster 2
MS41A
normalised log2 counts
Cluster 1
Cluster 2
FOX1
normalised log2 counts
Cluster 1
Cluster 2

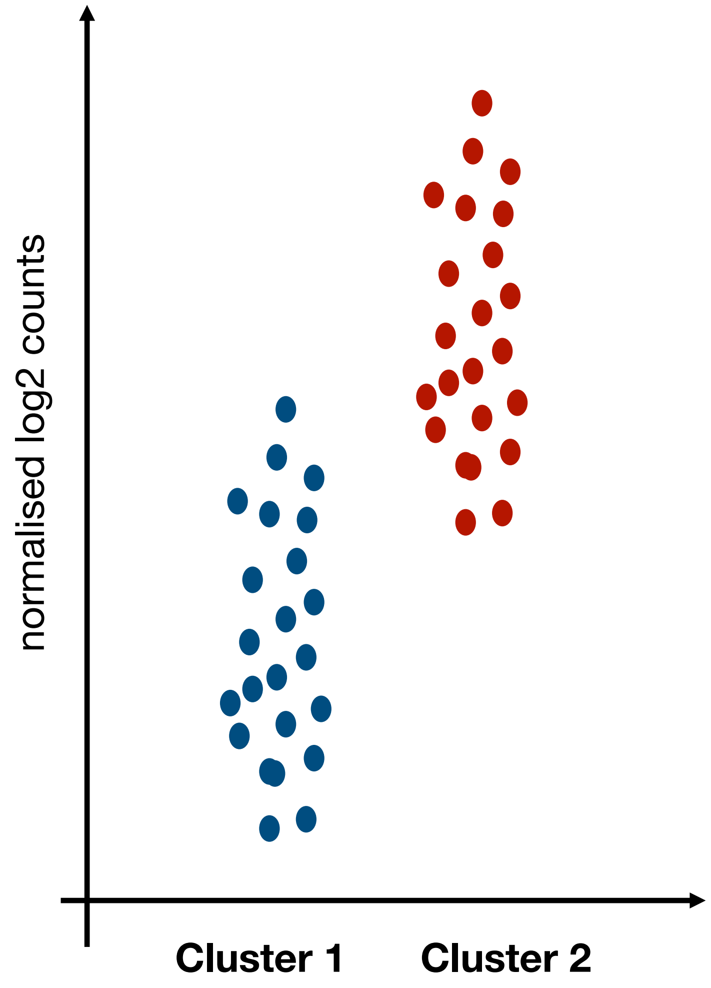
CD79A

?



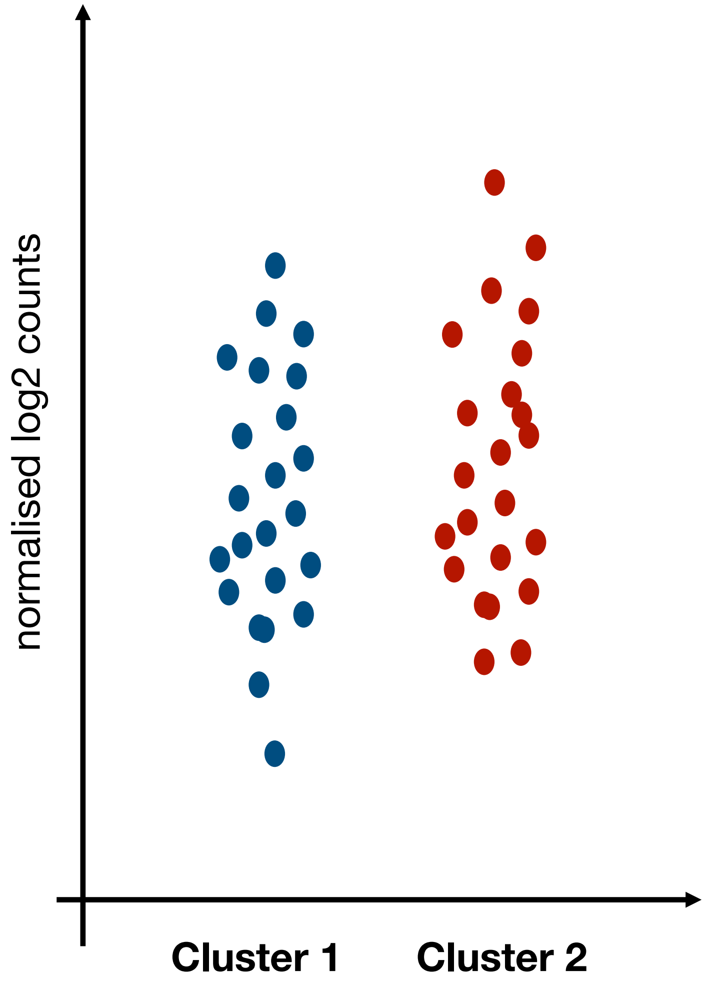
MS41A

?

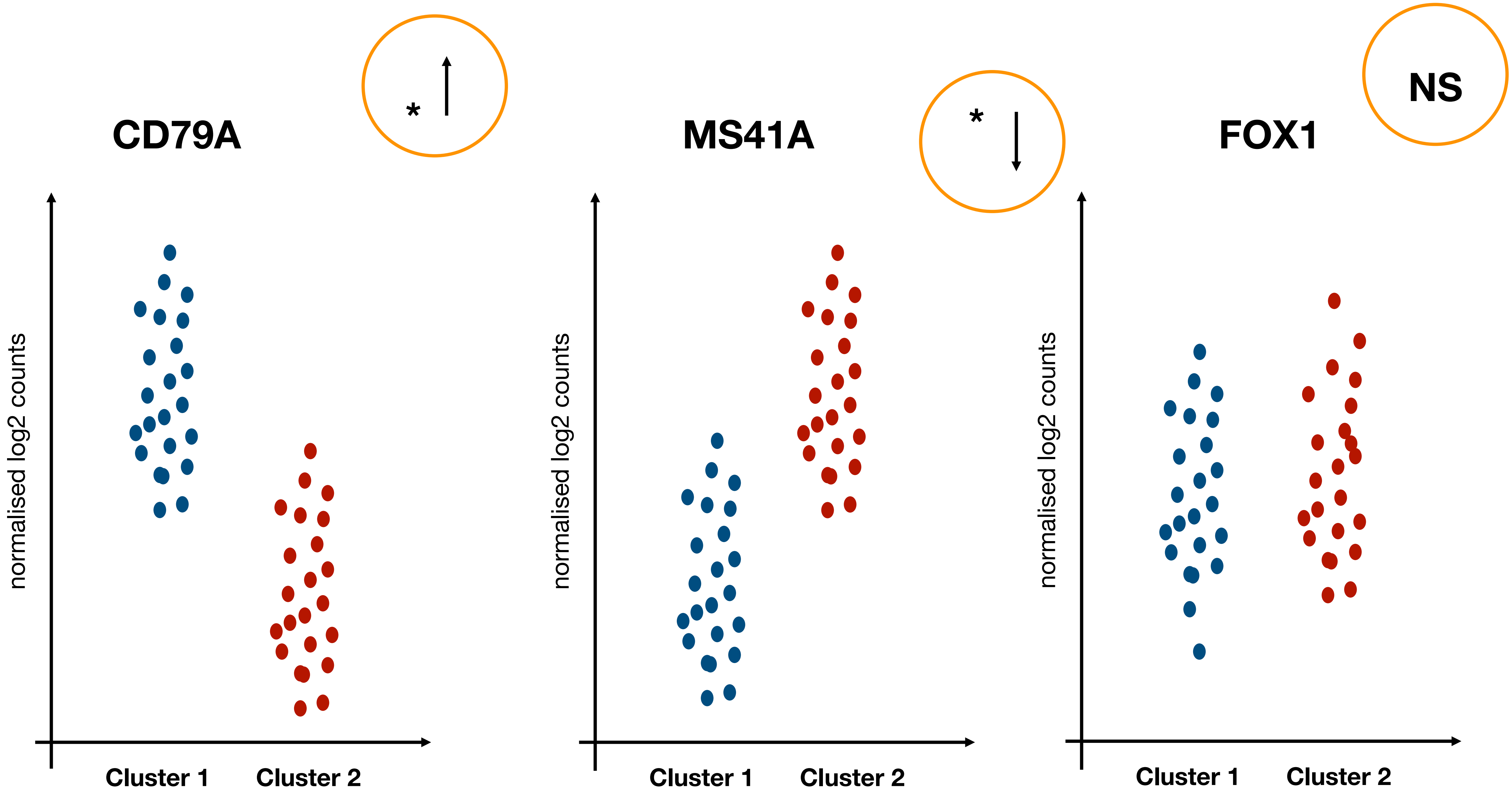


FOX1

?



* ↑ / * ↓ / NS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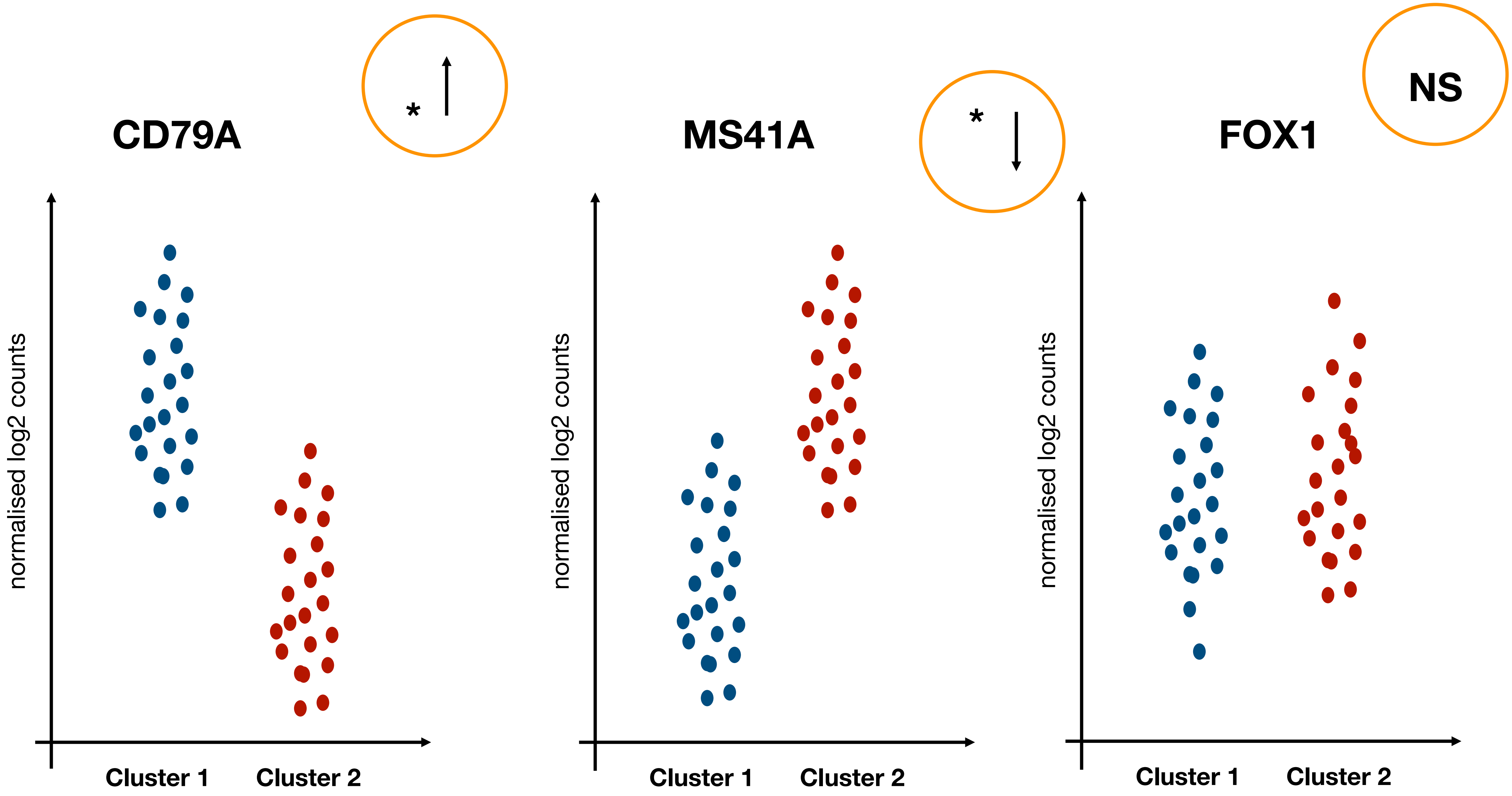
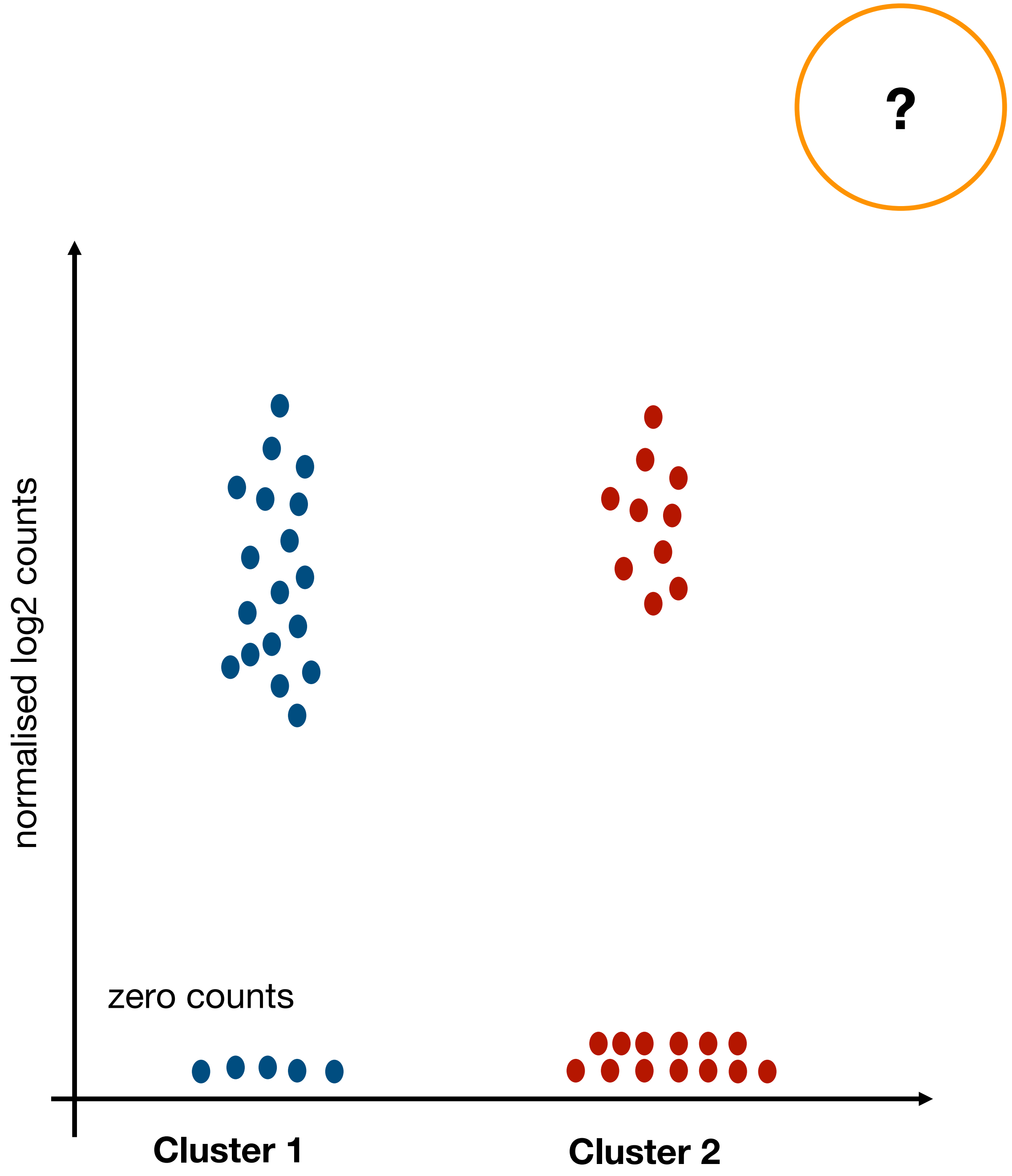
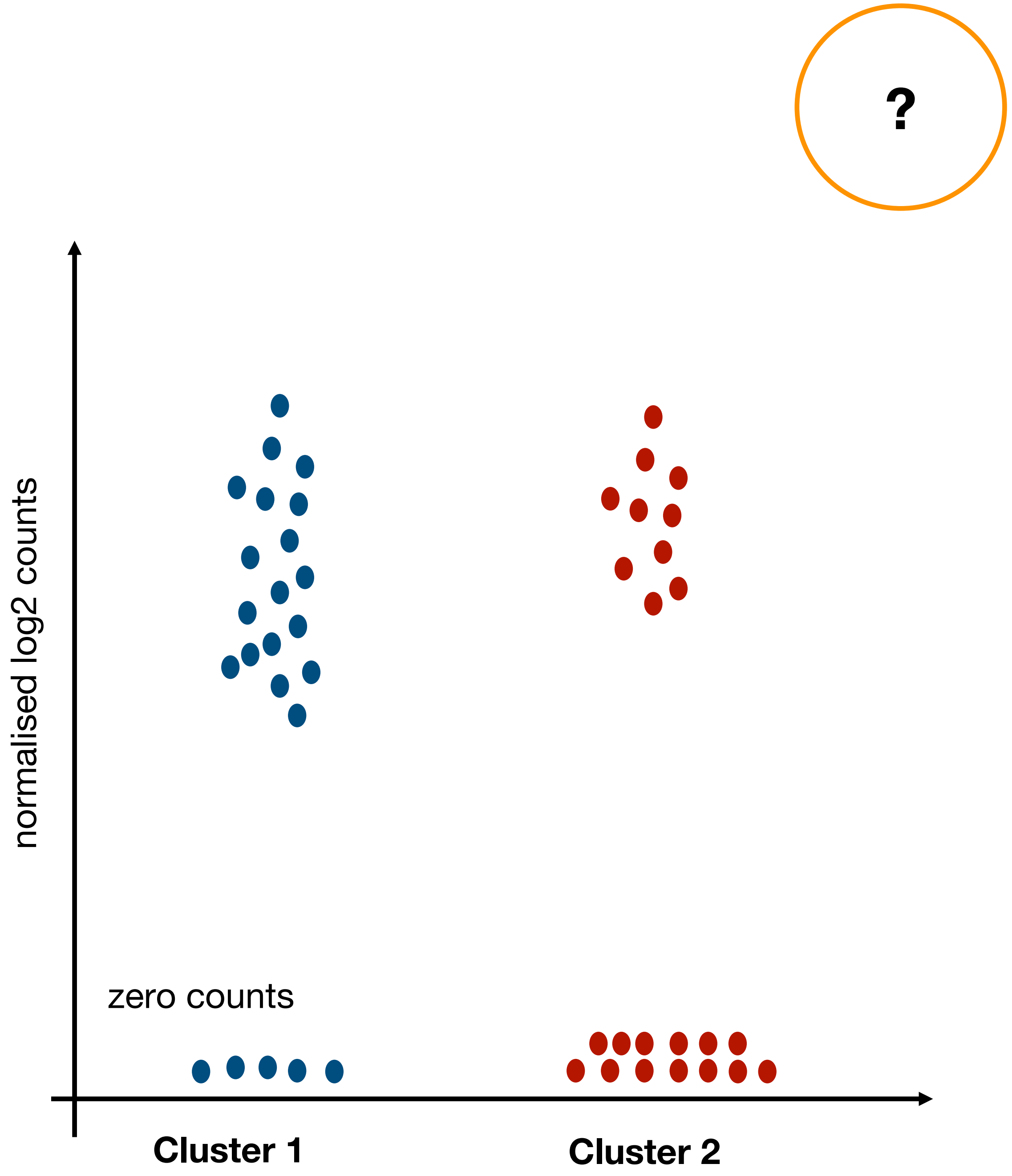
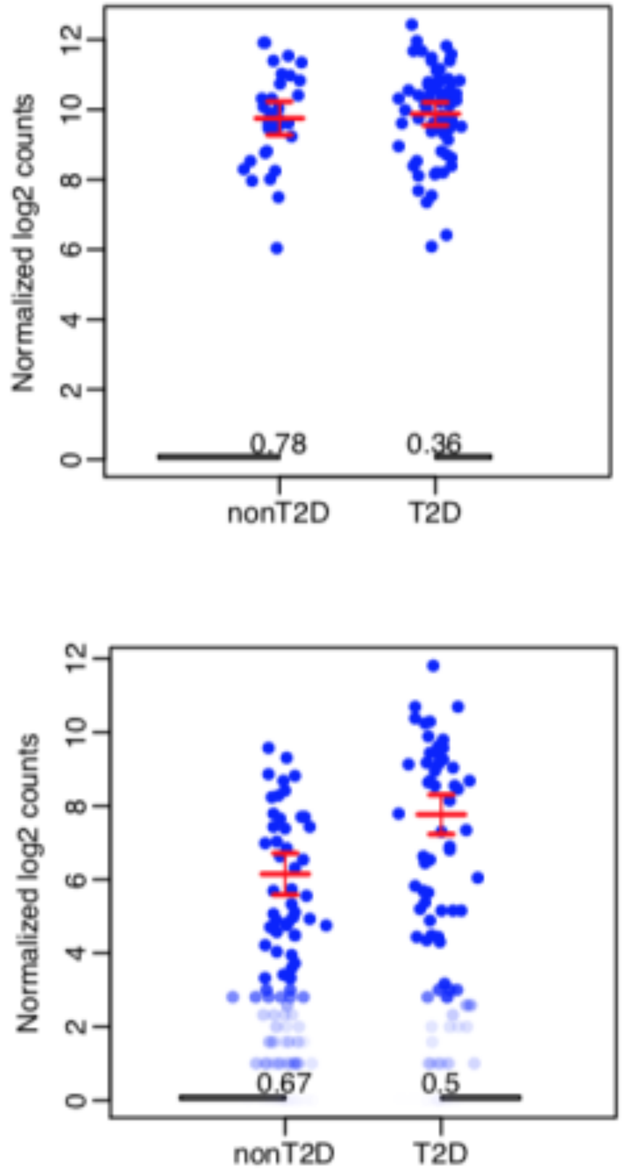


Differential expression means:

- taking read count data & performing statistical analysis to discover quantitative changes in expression levels between experimental groups (e.g. clusters)
- i.e. to decide whether, for a given gene, an observed difference in read counts is significant (greater than it would be expected just due to natural random variation)

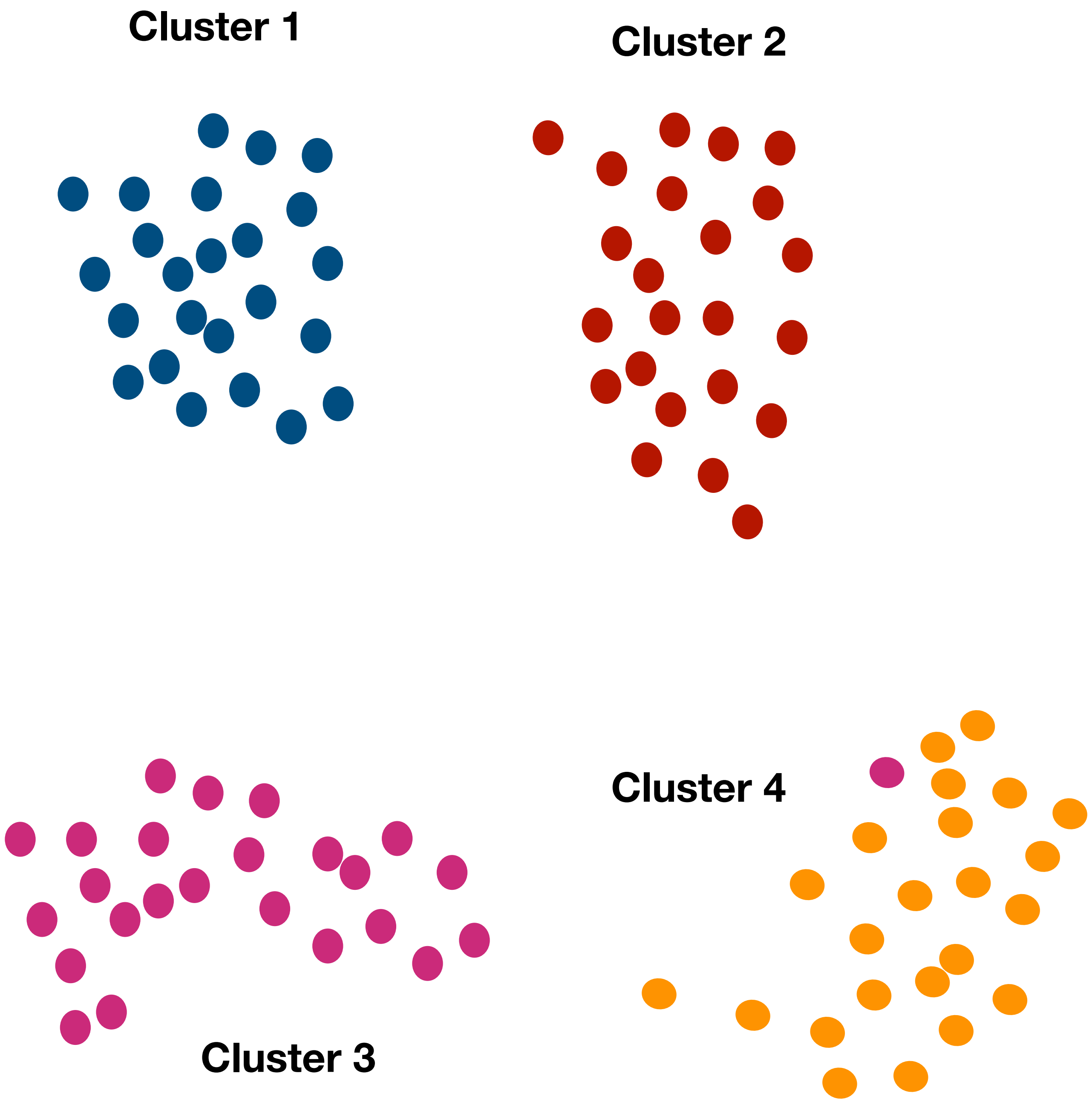

?
normalised log2 counts
zero counts
Cluster 1
Cluster 2

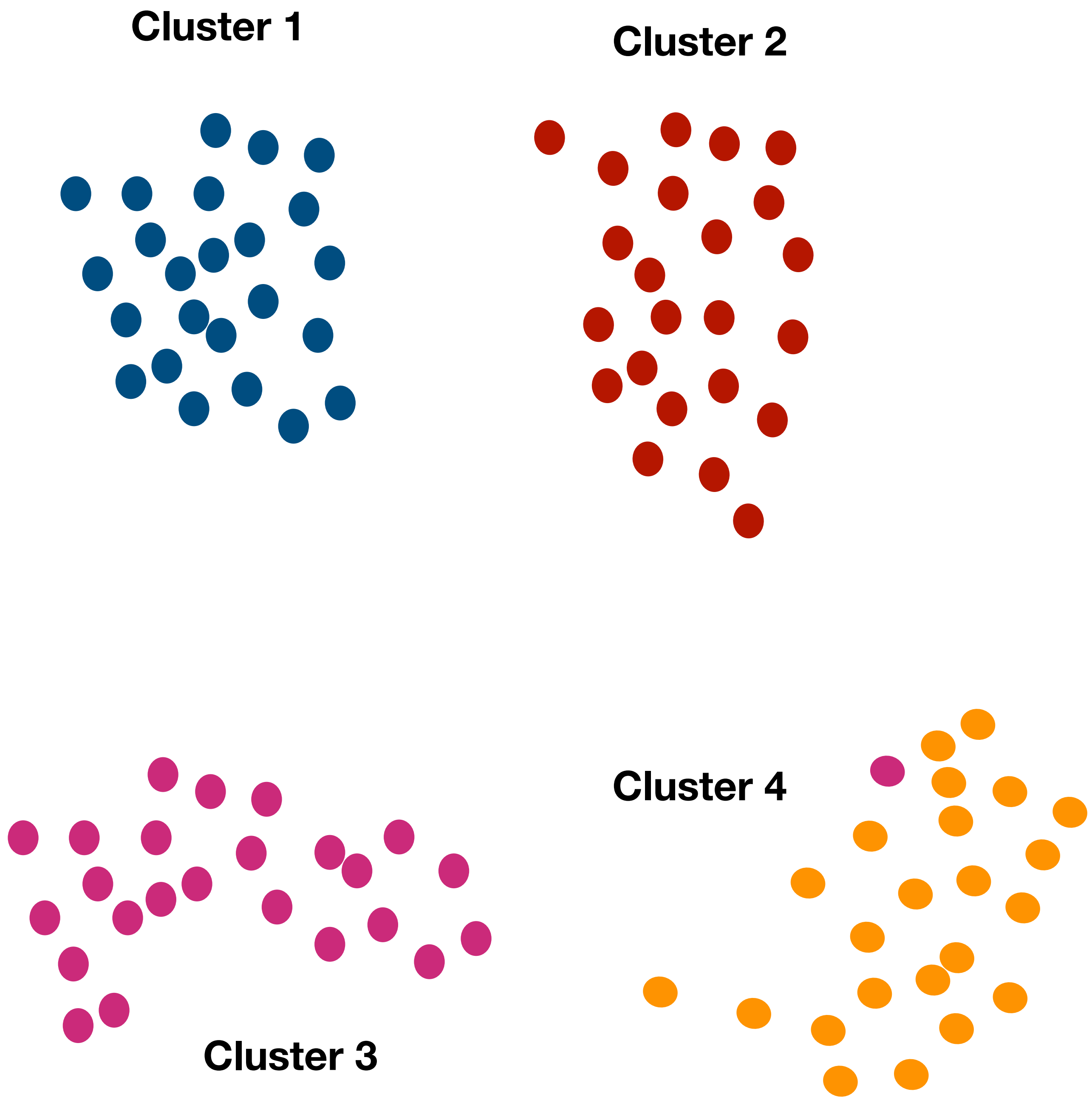




"…most computational methods still stick with the old mentality of viewing differential expression as a simple "up or down" phenomenon. We advocate that we should fully embrace the features of single cell data, which allows us to observe binary (from Off to On) as well as continuous (the amount of expression) regulations."

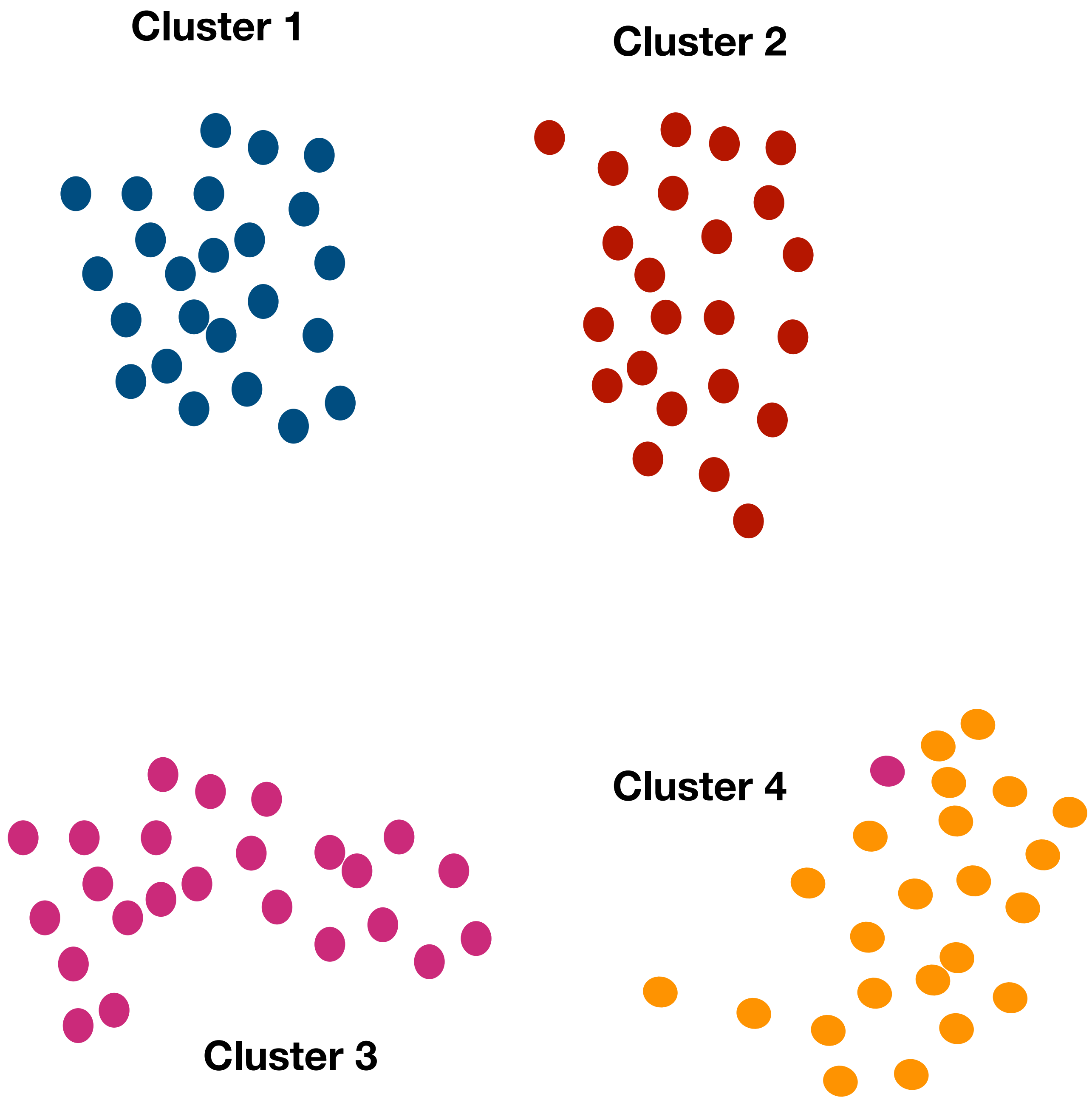
Wu *et al.* (Bioinformatics 2017):
Two phase differential expression


Cluster 1
Cluster 2
Cluster 4
Cluster 3



Differential expression is comparative.
Common comparisons include:

❖ pairwise cluster comparisons,
e.g. c1 vs. c2, c2 vs. c3 etc.



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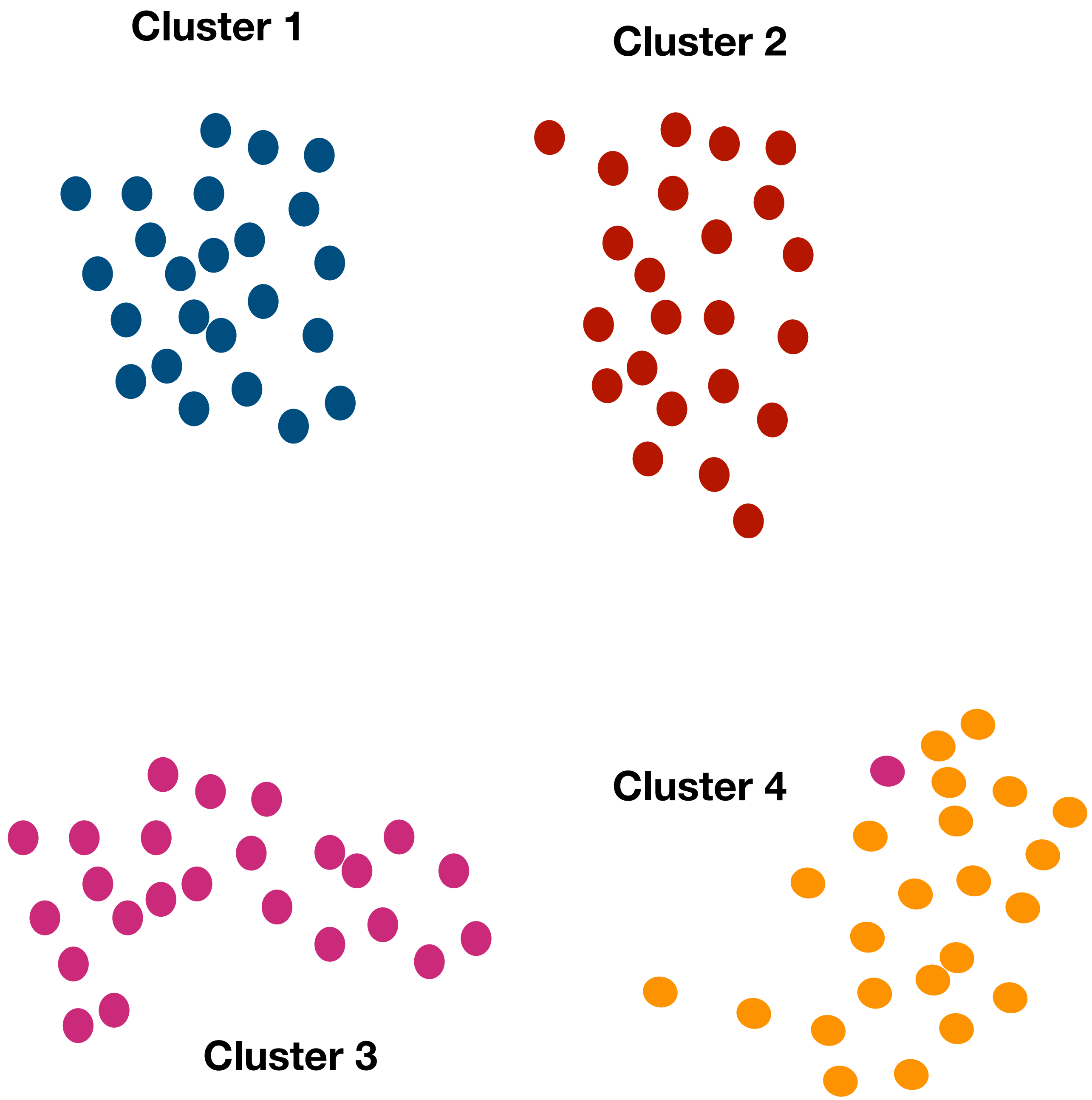
- ❖ pairwise cluster comparisons, e.g. c1 vs. c2, c2 vs. c3 etc.
- ❖ for a given cluster finding ‘marker genes’ that :
    - ❖ DE compared to all cells outside of the cluster
    - ❖ DE compared to at least one other cluster
    - ❖ DE compared to each of the other clusters
    - ❖ DE compare to “most” of the other clusters
    - ❖ DE and up-regulated (up-regulated markers are somehow easier to interpret)



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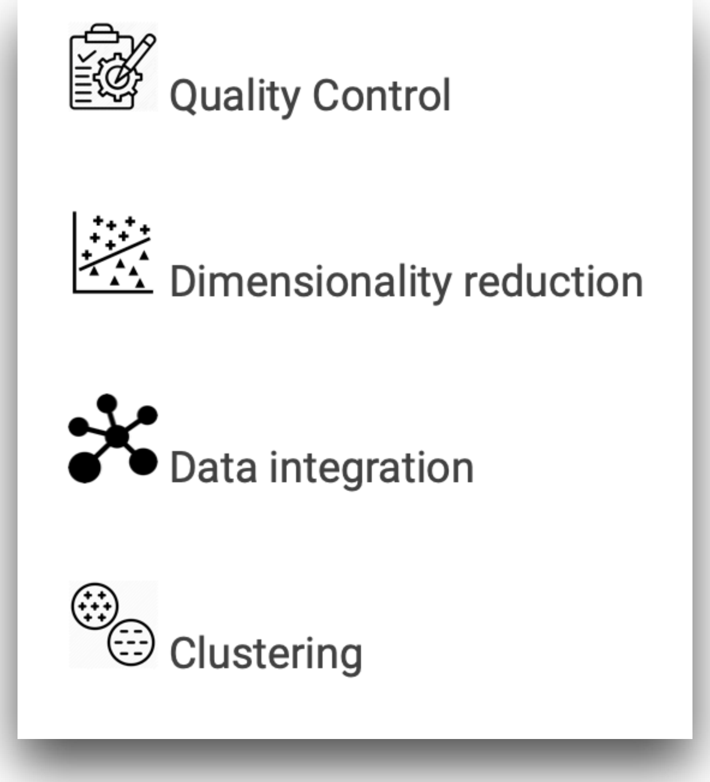
- cell-type comparisons (if cell type is known) (with and without clustering)

❖ Common methods

Context

Context



Quality Control

Dimensionality reduction

Data integration

Clustering

❖ Setting-up data

❖ Quality control and removal of “problematic “ cells

❖ Classification of cell cycle phase

❖ Normalization

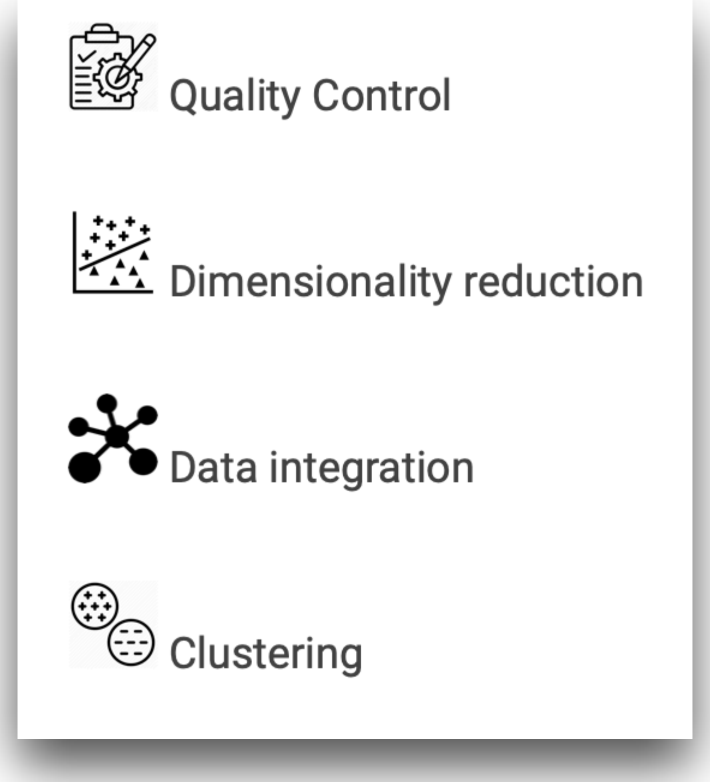
❖ Imputations

❖ Selection of highly variable genes

❖ Data integration

❖ K-means / HCL / graph based clustering

Context



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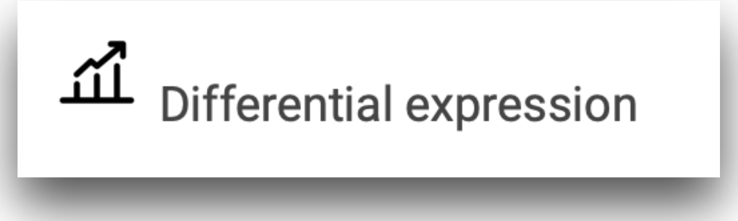

Differential expression

- ❖ Setting-up data
- ❖ Quality control and removal of “problematic “ cells
- ❖ Classification of cell cycle phase
- ❖ Normalization
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Functions

R SEURAT

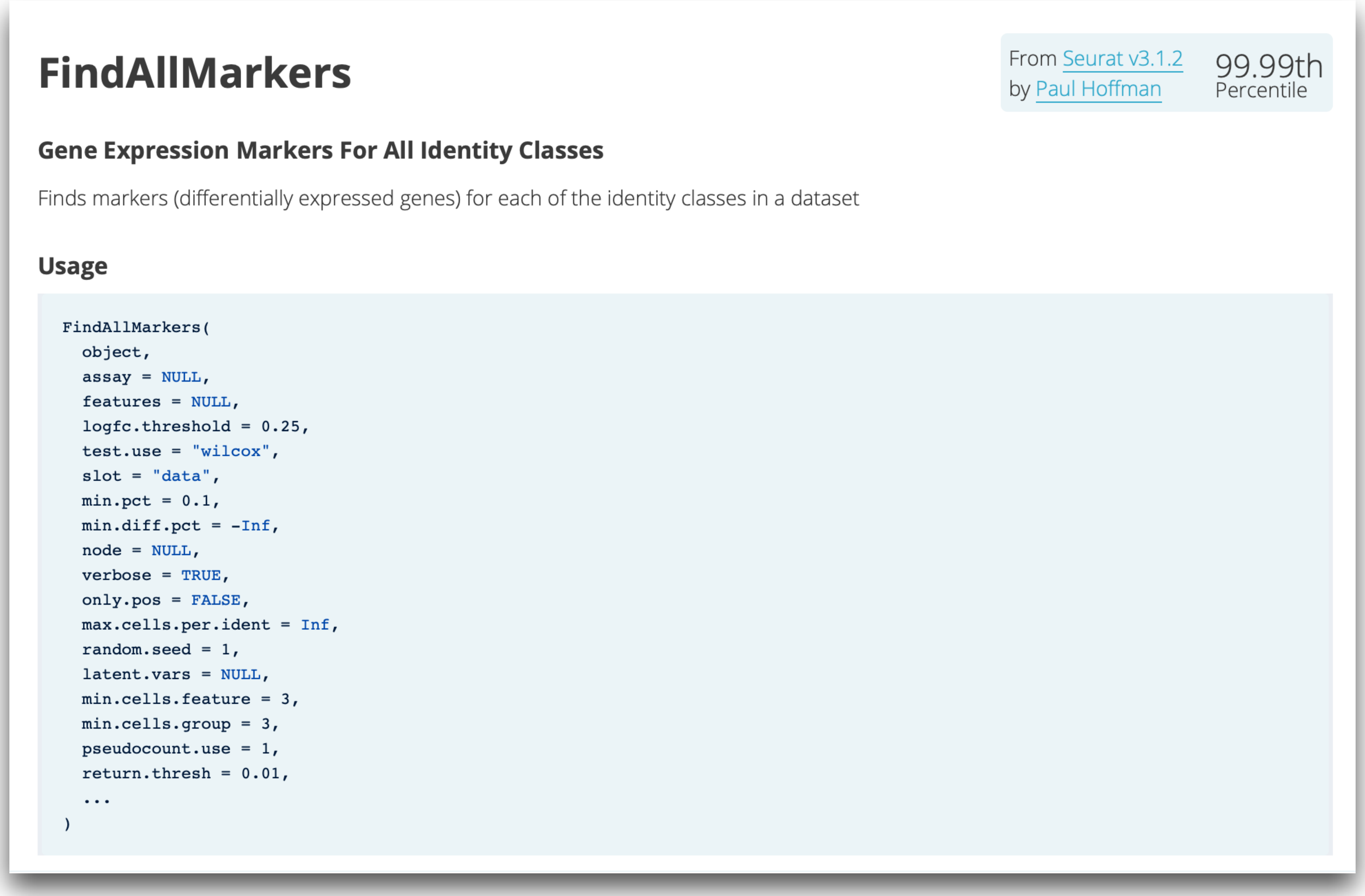
`FindAllMarkers()`

R scran

`findMarkers()`

scanpy

`scanpy.tl.rank_genes_groups()`



FindAllMarkers

From Seurat v3.1.2
by Paul Hoffman

99.99th
Percentile

Gene Expression Markers For All Identity Classes

Finds markers (differentially expressed genes) for each of the identity classes in a dataset

Usage

```
FindAllMarkers(
  object,
  assay = NULL,
  features = NULL,
  logfc.threshold = 0.25,
  test.use = "wilcox",
  slot = "data",
  min.pct = 0.1,
  min.diff.pct = -Inf,
  node = NULL,
  verbose = TRUE,
  only.pos = FALSE,
  max.cells.per.ident = Inf,
  random.seed = 1,
  latent.vars = NULL,
  min.cells.feature = 3,
  min.cells.group = 3,
  pseudocount.use = 1,
  return.thresh = 0.01,
  ...
)
```

test.use Denotes which test to use. Available options are:

- "wilcox" : Identifies differentially expressed genes between two groups of cells using a Wilcoxon Rank Sum test (default)
- "bimod" : Likelihood-ratio test for single cell gene expression, (McDavid et al., Bioinformatics, 2013)
- "roc" : Identifies 'markers' of gene expression using ROC analysis. For each gene, evaluates (using AUC) a classifier built on that gene alone, to classify between two groups of cells. An AUC value of 1 means that expression values for this gene alone can perfectly classify the two groupings (i.e. Each of the cells in cells.1 exhibit a higher level than each of the cells in cells.2). An AUC value of 0 also means there is perfect classification, but in the other direction. A value of 0.5 implies that the gene has no predictive power to classify the two groups. Returns a 'predictive power' (abs(AUC-0.5) * 2) ranked matrix of putative differentially expressed genes.
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- "DESeq2" : Identifies differentially expressed genes between two groups of cells based on a model using DESeq2 which uses a negative binomial distribution (Love et al, Genome Biology, 2014).This test does not support pre-filtering of genes based on average difference (or percent detection rate) between cell groups. However, genes may be pre-filtered based on their minimum detection rate (min.pct) across both cell groups. To use this method, please install DESeq2, using the instructions at https://bioconductor.org/packages/release/bioc/html/DESeq2.html

- ## intro to statistical inference

    - ❖ i.e. to decide whether, for a given gene, an observed difference in read counts is significant (greater than it would be expected just due to natural random variation)

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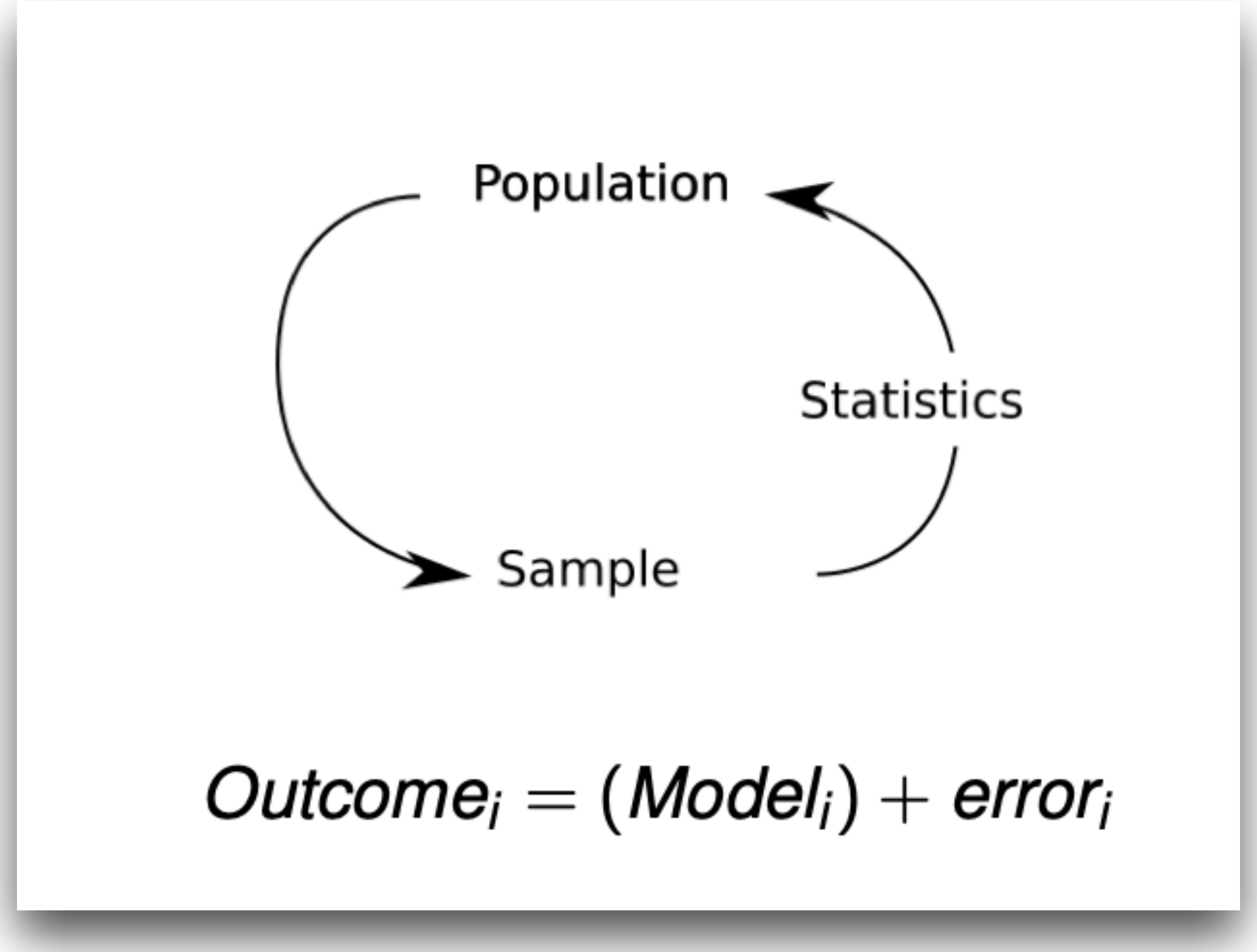

$$Outcome_i = (Model_i) + error_i$$

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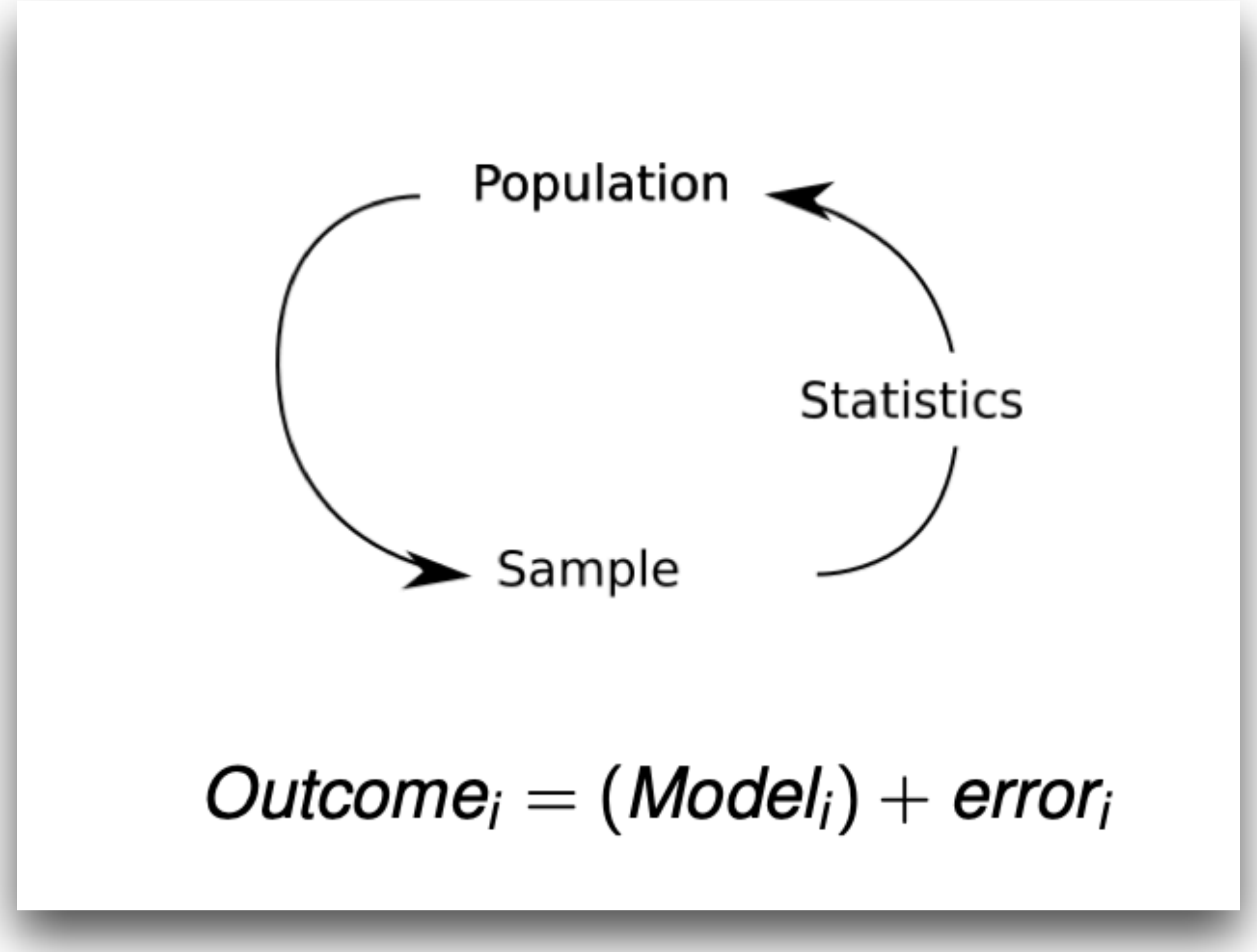

$$Outcome_i = (Model_i) + error_i$$

- we collect data on a sample from a much larger population
- summary statistics lets us to make inferences (conclusions) about the population from which samples was derived
- as well as predict the outcome given a model fitted to the data

e.g.

Is there a difference in height between students taking scRNA-seq course in 2019 and 2020?

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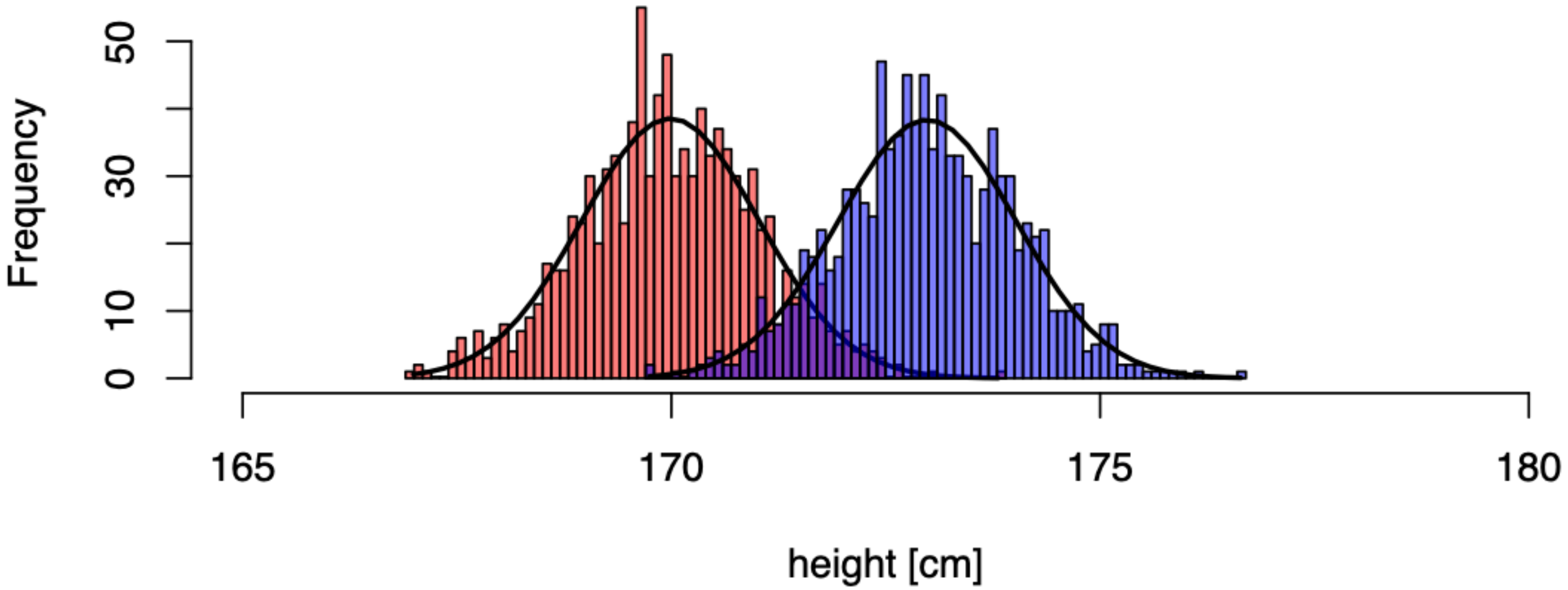

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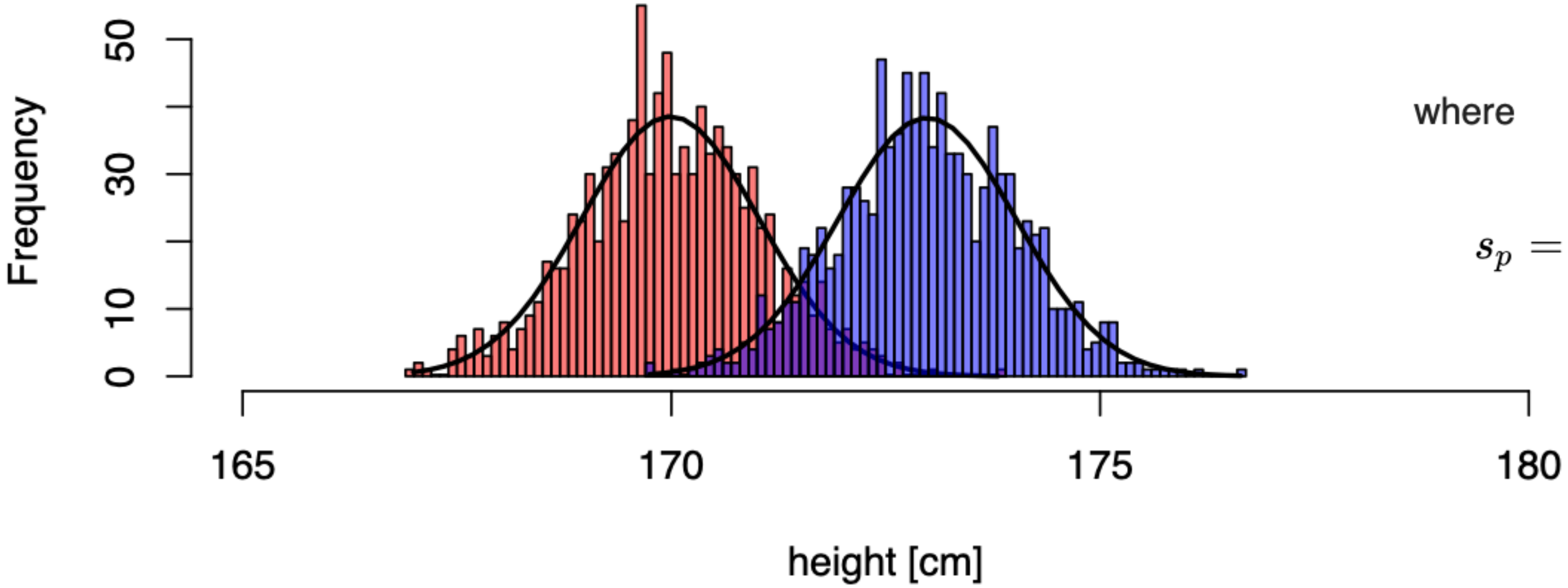

$$t = \frac{\bar{X}_1 - \bar{X}_2}{s_p \sqrt{\frac{2}{n}}}$$

where

$$s_p = \sqrt{\frac{s_{X_1}^2 + s_{X_2}^2}{2}}.$$

The observed value, here of mean difference, form basis of observed test statistics. A test statistics enables us to carry out a hypothesis test, which is a formal procedure to decide between the null and alternative hypotheses.

Important implication:

The better model fits to the data the better (more accurate) statistics

Generic non-parametric methods

when we cannot fit a model to our data

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✓ they test whether the distribution of ranks for one group are significantly different from the distribution of ranks for the other group

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Gene-wise null hypothesis:
it is equally like that a randomly selected cell from group 1 will have higher or lower expression of the gene than a randomly selected cell from group 2

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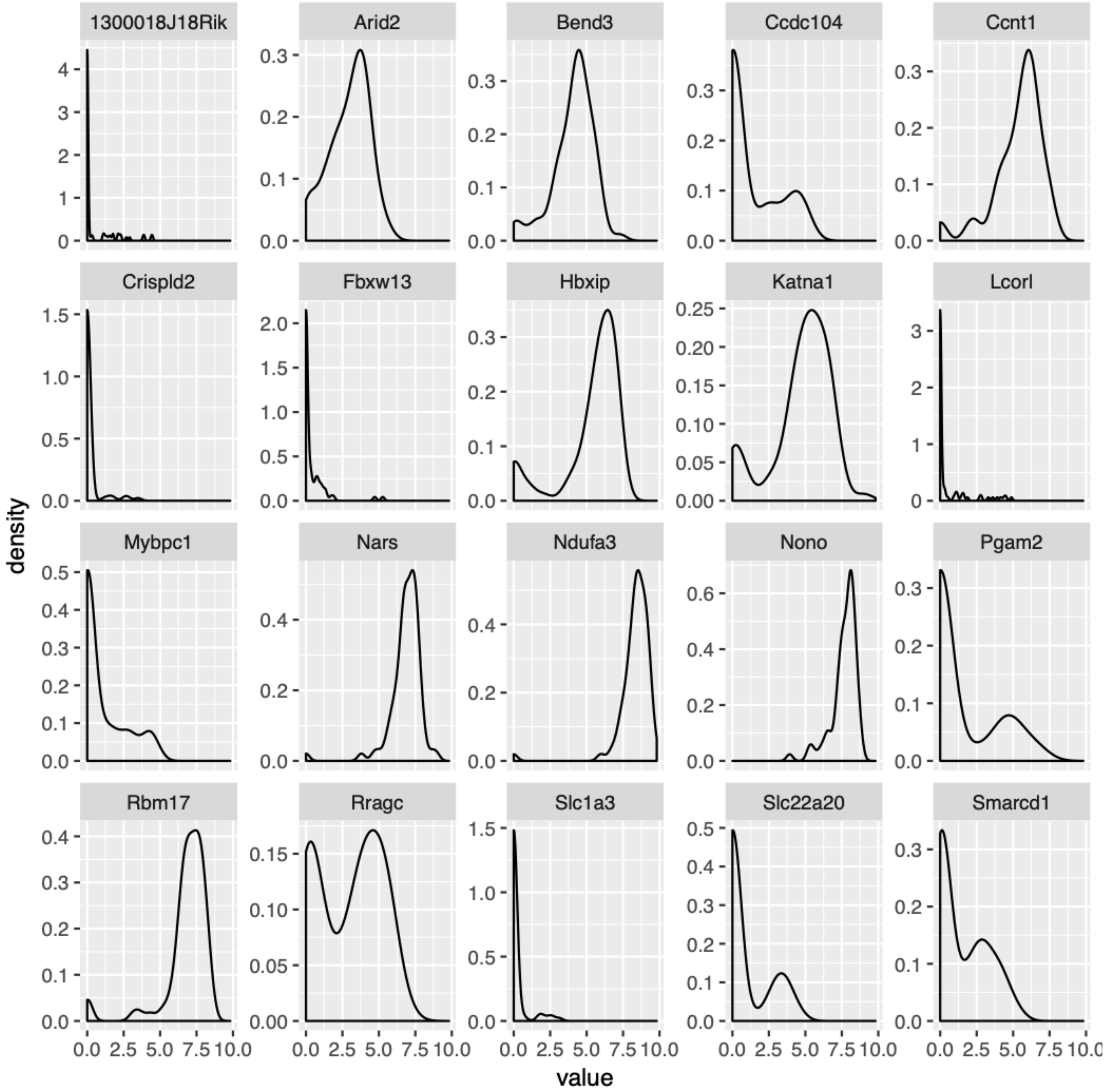
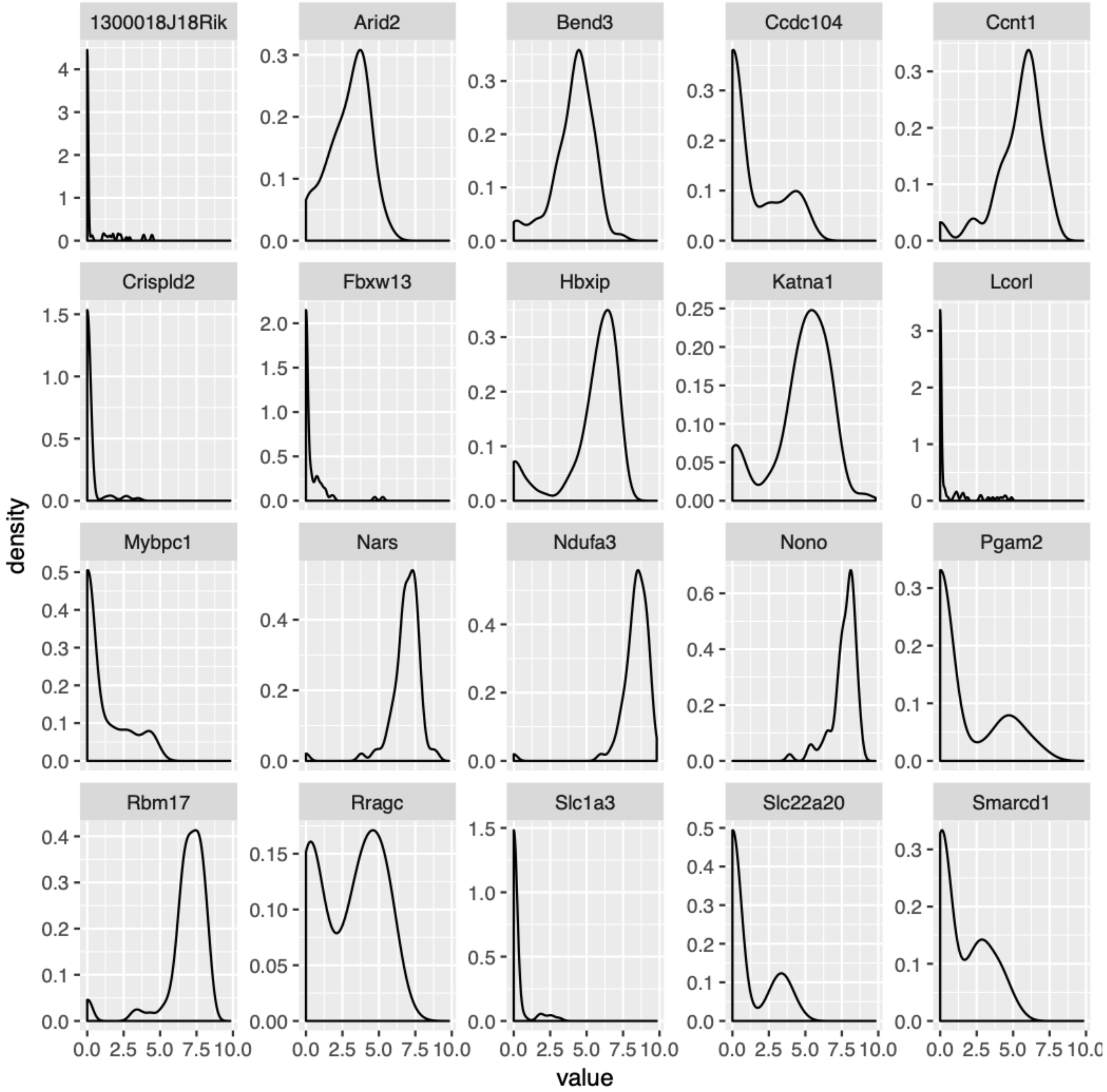
Why special distributions?



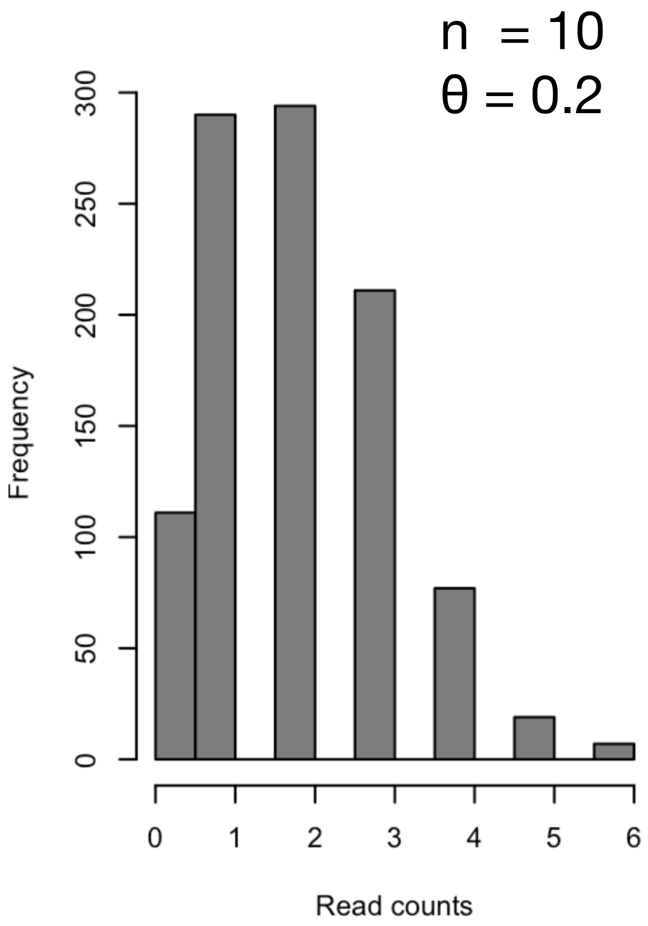
Why special distributions?

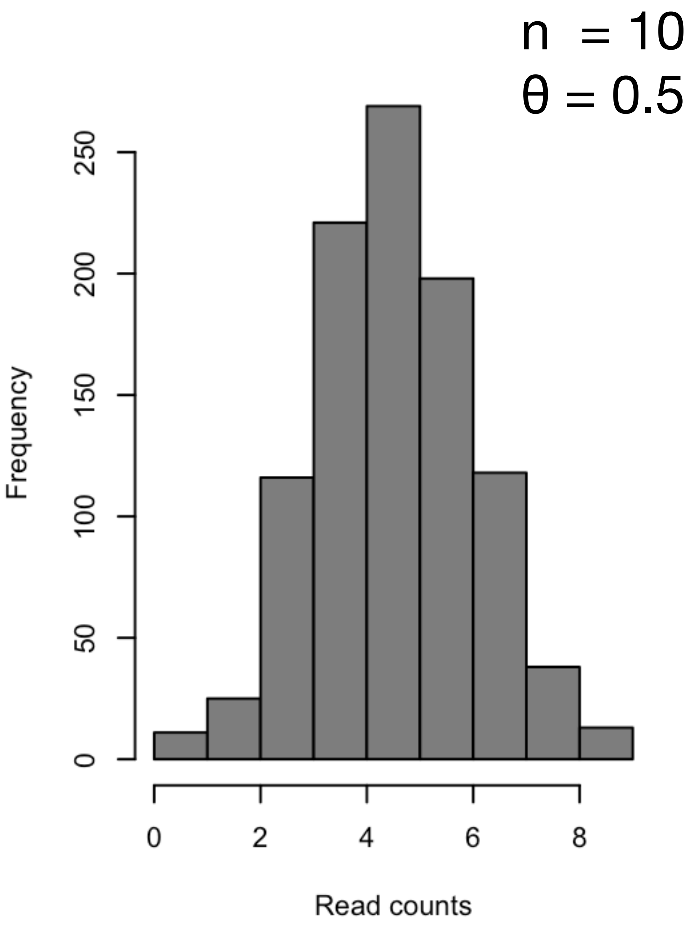


- high noise levels (technical and biological factors)
- low library sizes
- low amount of available mRNAs results in amplification biases and “dropout events”
- 3’ bias, partial coverage, uneven depth
- stochastic nature of transcription
- multimodality in gene expression; presence of multiple possible cell states within a cell population

What kind of distributions?

Binomial





$$\text{Bi}(n, \theta)$$

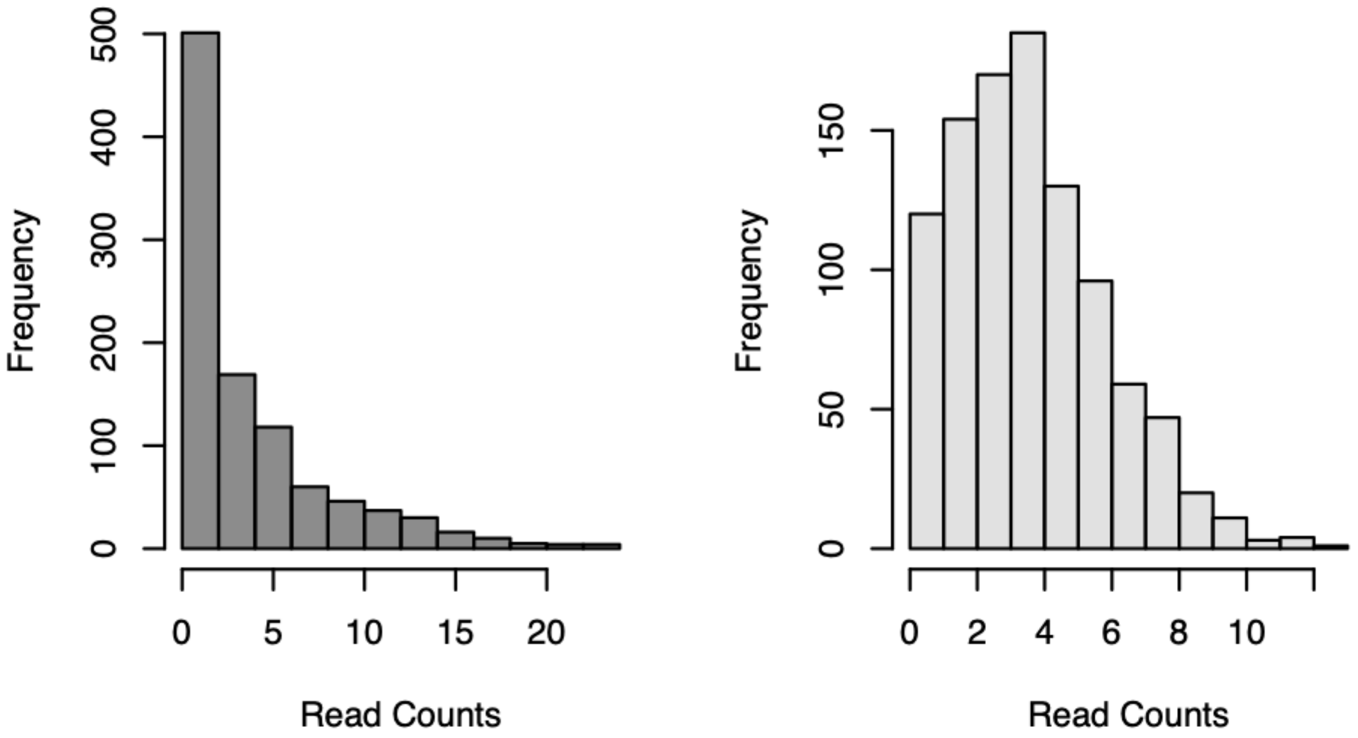
discrete probability distribution of the number of success in a sequence of *n* independent experiments;
θ - probability of success

Used to compare proportions of zeros.

Gene-wise null hypothesis:
probability of being expressed is the same in group 1 and group 2

avail in scran

Negative binomial



$$NeBi = (\mu, \delta^2)$$

$$\mu = mu$$

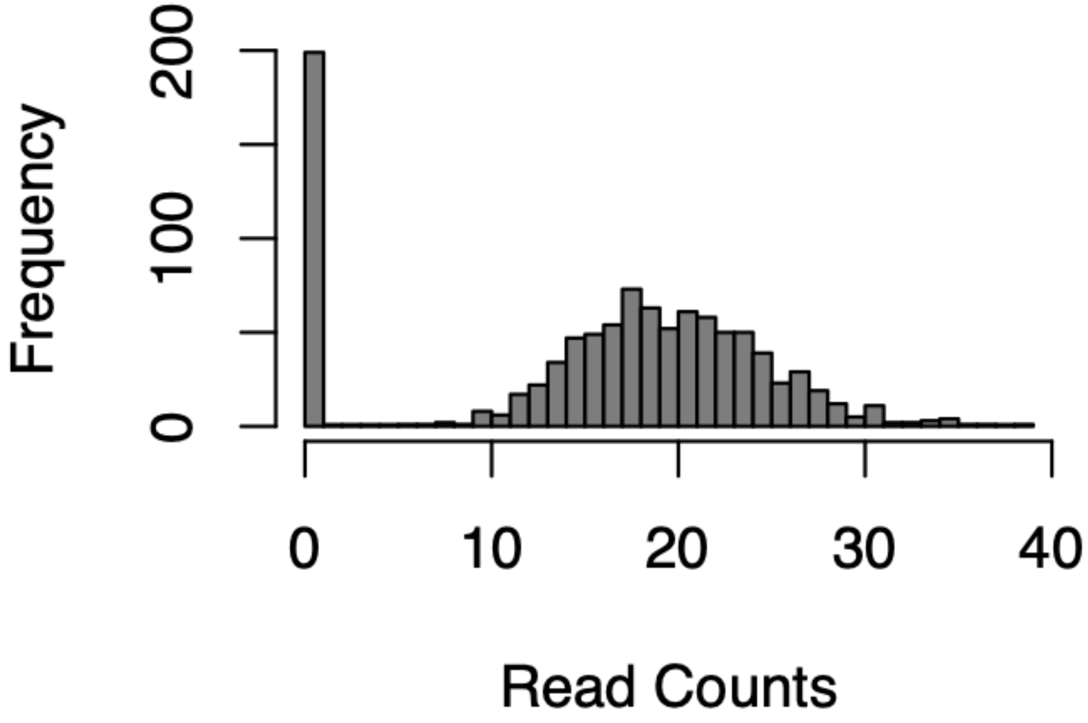
$$\delta^2 = mu + mu^2 / size$$

μ **mean expression**

δ^2 **dispersion, which is inversely related to the variance**

NeBi fits bulk RNA-seq data very well and it is used for most statistical methods designed for such data. In addition, it has been show to fit the distribution of molecule counts obtained from data tagged by unique molecular identifiers (UMIs) quite well (Grun et al. 2014, Islam et al. 2011).

zero-inflated negative binomial



$$NeBi = (\mu, \delta^2)$$

$$\mu = mu * (1 - d)$$

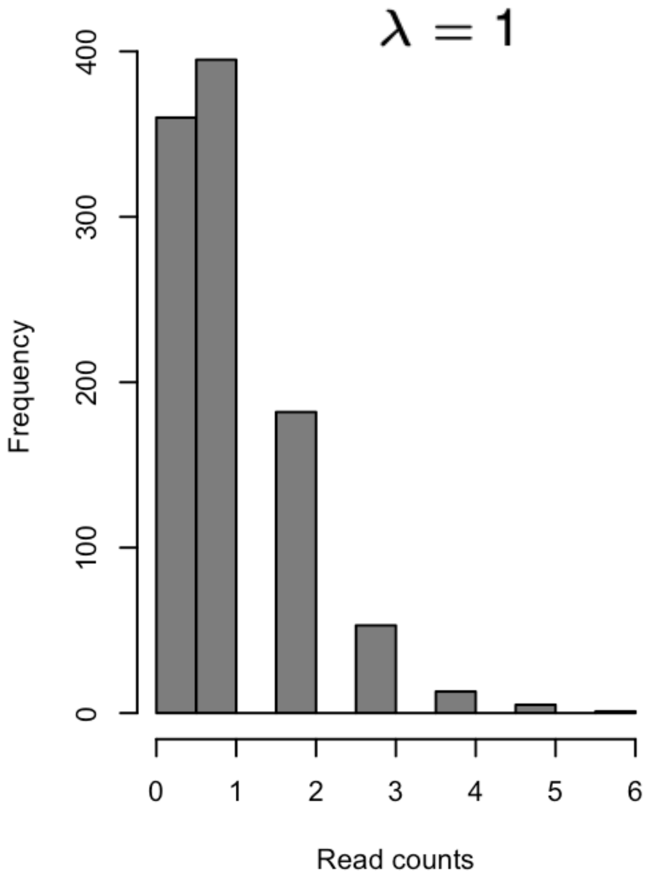
$$\delta^2 = \mu * (1 - d) * (1 + d * \mu + \mu / size)$$

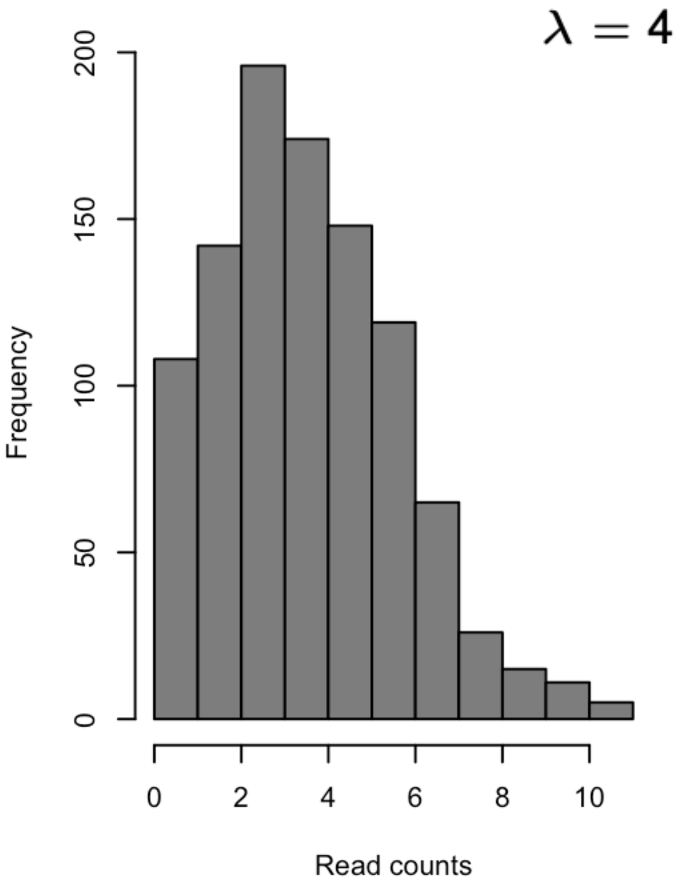
d, dropout rate.

The dropout of a gene is strongly correlated with the mean expression of the gene. Different zero-inflated negative binomial models use different relationships between mean expression and dropout rate.

Implemented in MAST, SCDE

Poisson





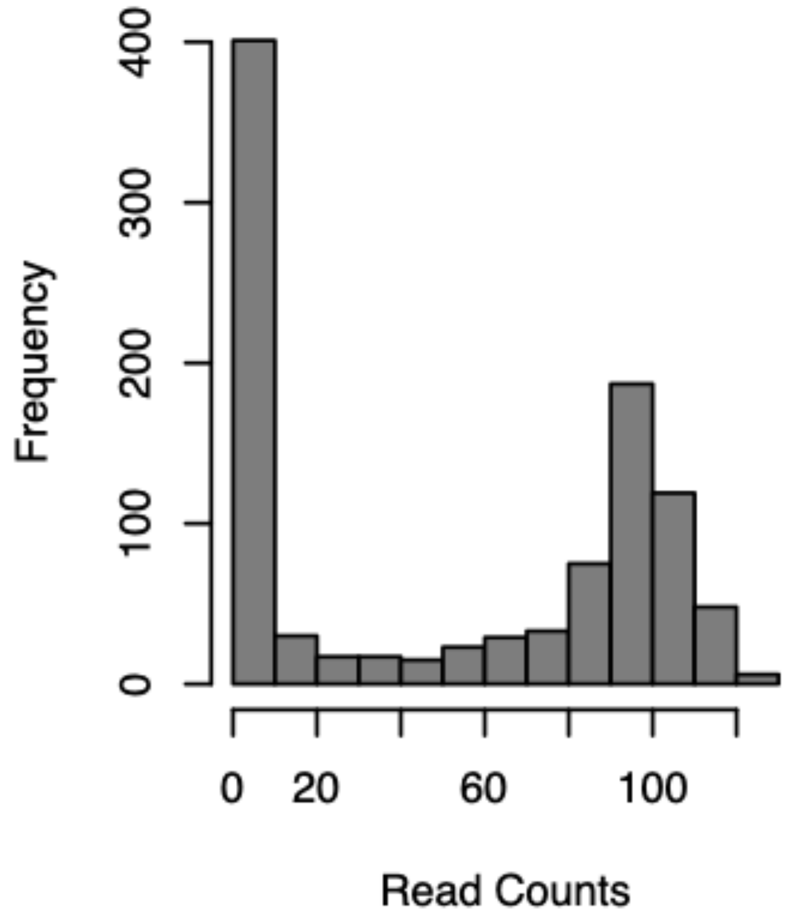
$\mathrm{Poi}(\lambda)$

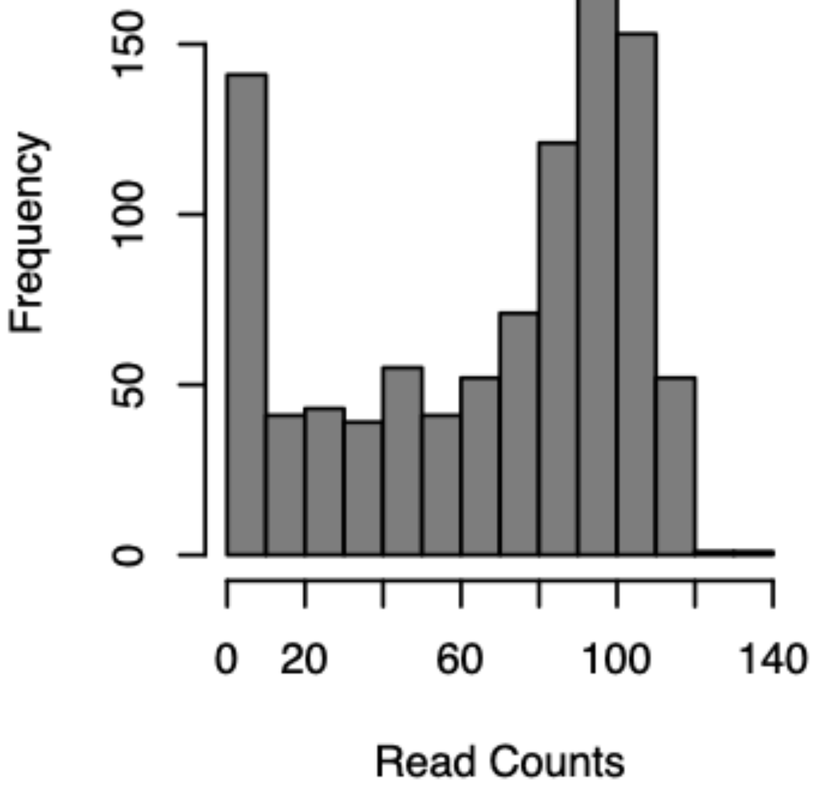
discrete probability distribution that expresses the probability of a given number of events occurring in a fixed interval of time or space if these events occur with a known constant mean rate, *lambda*, and independently of the time since the last event

Poisson-Beta

$$\mu = g * a/(a+b)$$

$$\delta^2 = g^2 * a * b/((a+b+1) * (a+b)^2)$$





a: the rate of activation of transcription
b: the rate of inhibition of transcription
g: the rate of transcript production while transcription is active at the locus

implemented in BPSC

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MAST

- uses generalized linear hurdle model
- designed to account for stochastic dropouts and bimodal expression distribution in which expression is either strongly non-zero or non-detectable
- The rate of expression ***Z***, and the level of expression ***Y***, are modeled for each gene ***g***, indicating whether gene ***g*** is expressed in cell ***i*** (i.e., $Z_{ig} = 0$ if $y_{ig} = 0$ and $z_{ig} = 1$ if $y_{ig} > 0$)
- A logistic regression model for the discrete variable ***Z*** and a Gaussian linear model for the continuous variable (Y|Z=1):

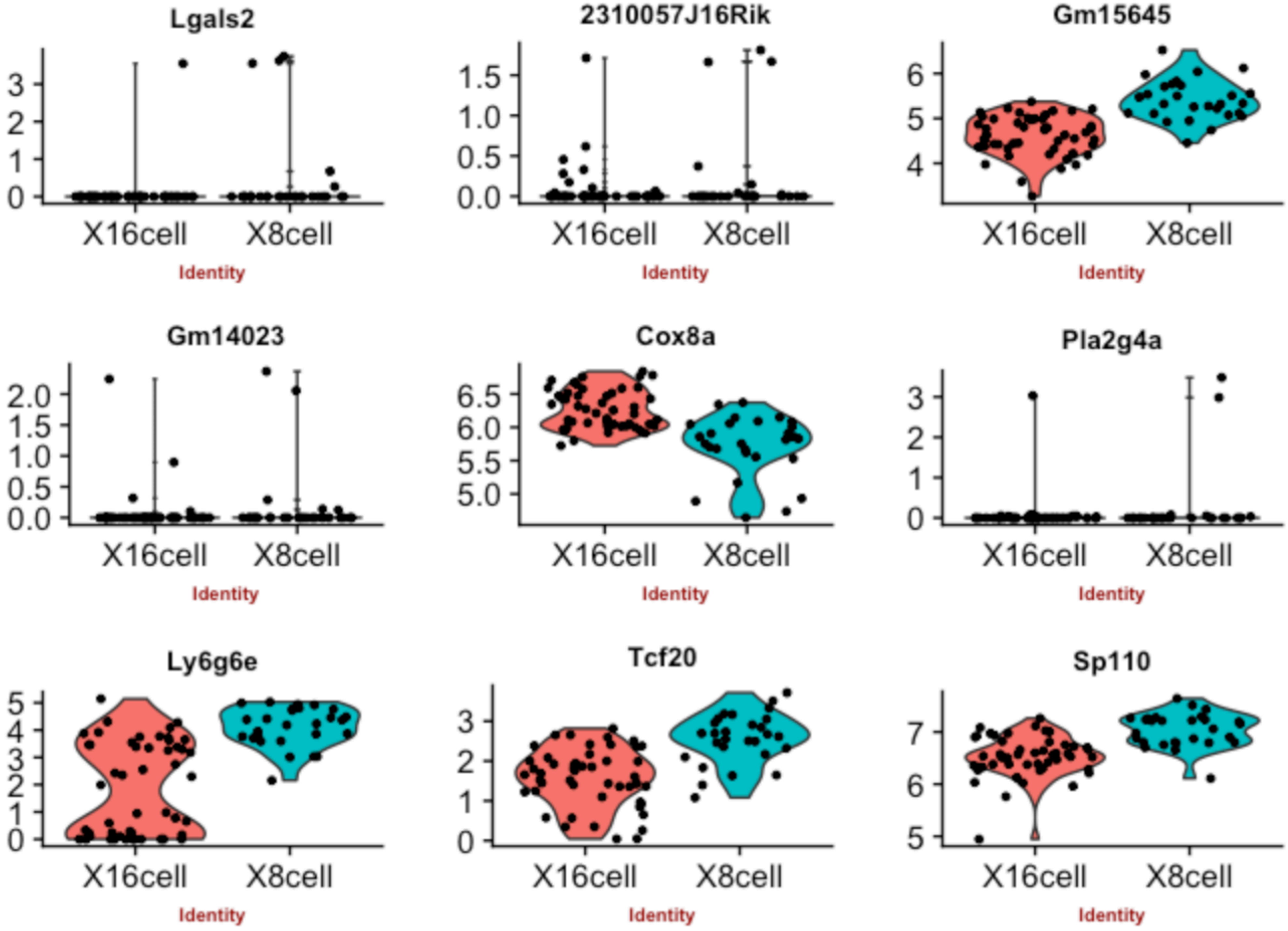
$$logit(P_r(Z_{ig} = 1)) = X_i \beta_g^D$$
$$P_r(Y_{ig} = Y|Z_{ig} = 1) = N(X_i \beta_g^C, \sigma_g^2), \text{ where } X_i \text{ is a design matrix}$$

- Model parameters are fitted using an empirical Bayesian framework
- Allows for a joint estimate of nuisance and treatment effects
- DE is determined using the likelihood ratio test

So what's really important?

- Models can get quite complicated but in Seurat / Scran / Scanpy default methods are set to t-test and wilcox
- It's important to understand what are we trying to compare, e.g. mean expressions, or probability of being expressed
- It's important to understand the data
- It's important to assess and validate the results

What’s important: assessing results



❖ Performance

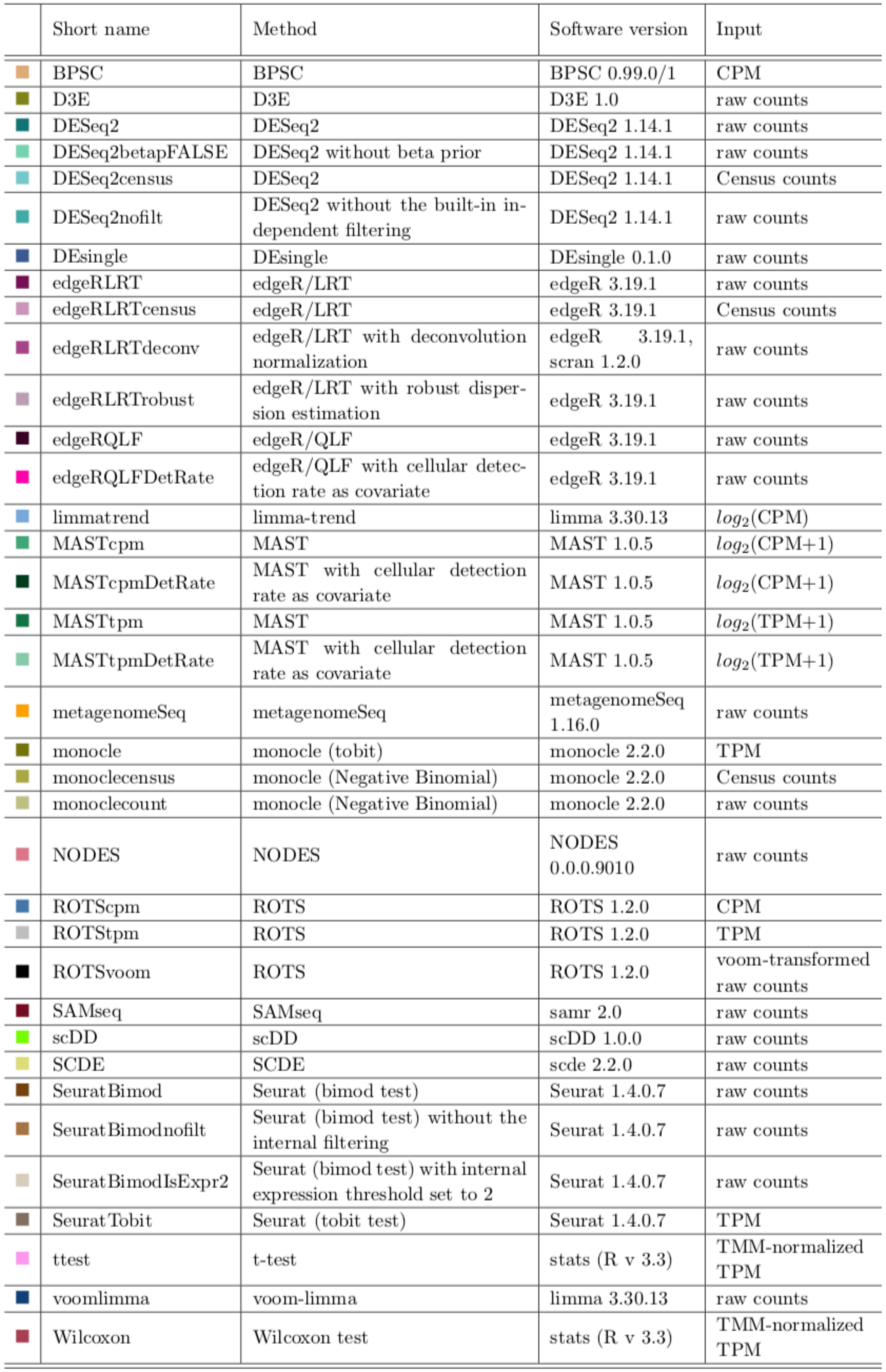
Why is it hard to say which method is best?

Performance

No ground truth data available

- **Known data:**
  - using data we know something about to get “positive controls”
- **Simulated data:**
  - null-data sets by re-sampling, modelling datasets based on various distributions
- **Compare:**
  - comparing between numbers and ranks of DEs
- **Investigating results:**
  - how does the expression and distributions of detected DEs look like?

Performance



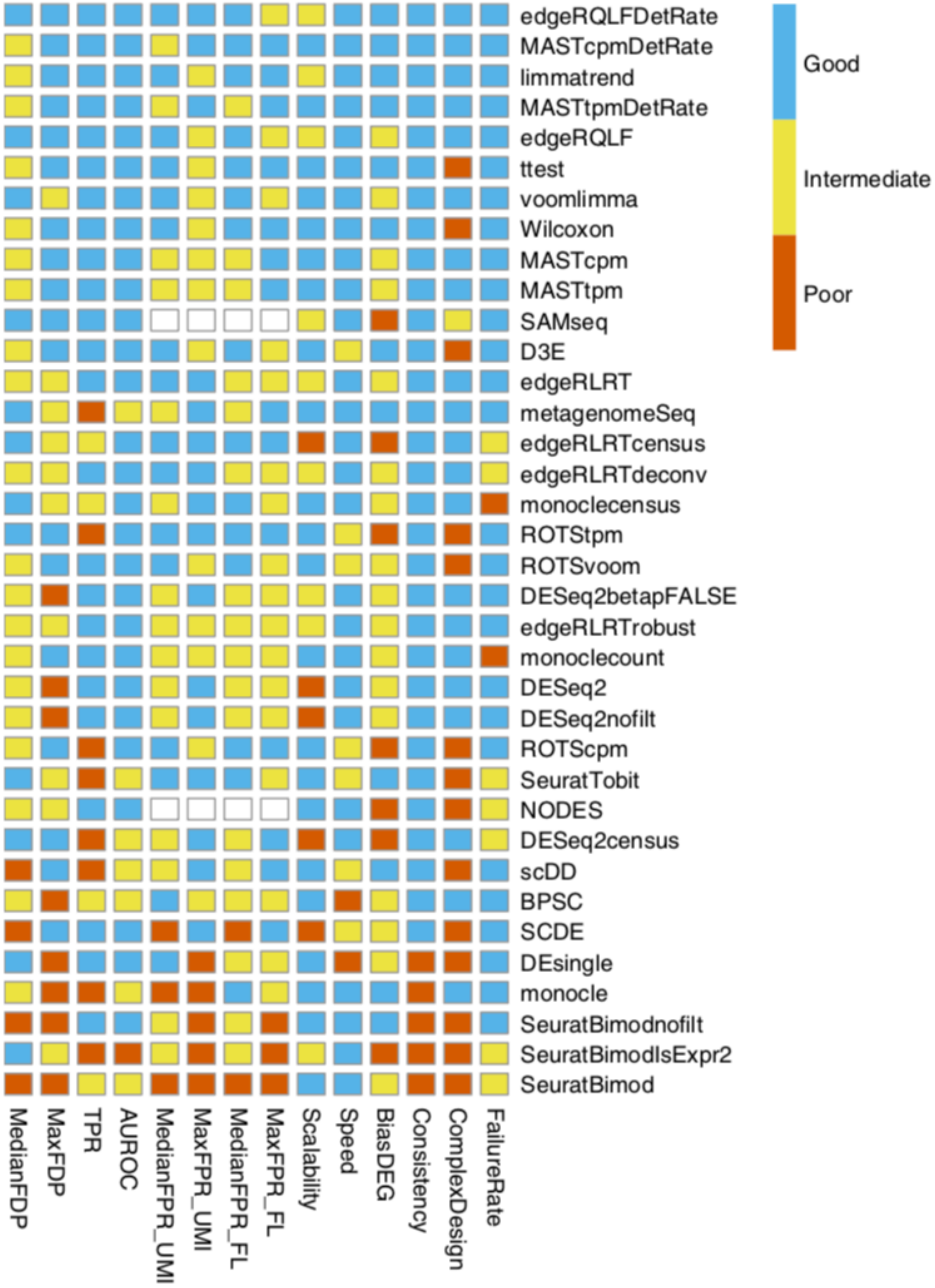
	Short name	Method	Software version	Input
🟧	BPSC	BPSC	BPSC 0.99.0/1	CPM
🟩	D3E	D3E	D3E 1.0	raw counts
🟦	DESeq2	DESeq2	DESeq2 1.14.1	raw counts
🟩	DESeq2betapFALSE	DESeq2 without beta prior	DESeq2 1.14.1	raw counts
🟦	DESeq2census	DESeq2	DESeq2 1.14.1	Census counts
🟦	DESeq2nofilt	DESeq2 without the built-in independent filtering	DESeq2 1.14.1	raw counts
🟦	DEsingle	DEsingle	DEsingle 0.1.0	raw counts
🟪	edgeRLRT	edgeR/LRT	edgeR 3.19.1	raw counts
🟪	edgeRLRTcensus	edgeR/LRT	edgeR 3.19.1	Census counts
🟪	edgeRLRTdeconv	edgeR/LRT with deconvolution normalization	edgeR 3.19.1, scran 1.2.0	raw counts
🟪	edgeRLRTrobust	edgeR/LRT with robust dispersion estimation	edgeR 3.19.1	raw counts
⬛	edgeRQLF	edgeR/QLF	edgeR 3.19.1	raw counts
🟥	edgeRQLFDetRate	edgeR/QLF with cellular detection rate as covariate	edgeR 3.19.1	raw counts
🟦	limmatrend	limma-trend	limma 3.30.13	log_2(CPM)
🟩	MASTcpm	MAST	MAST 1.0.5	log_2(CPM+1)
🟩	MASTcpmDetRate	MAST with cellular detection rate as covariate	MAST 1.0.5	log_2(CPM+1)
🟩	MASTtpm	MAST	MAST 1.0.5	log_2(TPM+1)
🟩	MASTtpmDetRate	MAST with cellular detection rate as covariate	MAST 1.0.5	log_2(TPM+1)
🟧	metagenomeSeq	metagenomeSeq	metagenomeSeq 1.16.0	raw counts
🟩	monocle	monocle (tobit)	monocle 2.2.0	TPM
🟨	monoclecensus	monocle (Negative Binomial)	monocle 2.2.0	Census counts
🟨	monoclecount	monocle (Negative Binomial)	monocle 2.2.0	raw counts
🟥	NODES	NODES	NODES 0.0.0.9010	raw counts
🟦	ROTScpm	ROTS	ROTS 1.2.0	CPM
⬜	ROTStpm	ROTS	ROTS 1.2.0	TPM
⬛	ROTSvoom	ROTS	ROTS 1.2.0	voom-transformed raw counts
🟥	SAMseq	SAMseq	samr 2.0	raw counts
🟩	scDD	scDD	scDD 1.0.0	raw counts
🟨	SCDE	SCDE	scde 2.2.0	raw counts
🟫	SeuratBimod	Seurat (bimod test)	Seurat 1.4.0.7	raw counts
🟫	SeuratBimodnofilt	Seurat (bimod test) without the internal filtering	Seurat 1.4.0.7	raw counts
⬜	SeuratBimodIsExpr2	Seurat (bimod test) with internal expression threshold set to 2	Seurat 1.4.0.7	raw counts
🟫	SeuratTobit	Seurat (tobit test)	Seurat 1.4.0.7	TPM
🟪	ttest	t-test	stats (R v 3.3)	TMM-normalized TPM
🟦	voomlimma	voom-limma	limma 3.30.13	raw counts
🟥	Wilcoxon	Wilcoxon test	stats (R v 3.3)	TMM-normalized TPM

Bias, robustness and scalability in single-cell differential expression and analysis:

- 36 statistical approaches for DE analysis to compare the expression levels in the two groups of cells
- based on 9 data sets, with 11 - 21 separate instances (sample size effect)
- extensive evaluation of metrics incl. number of genes found, characteristics of the false positive detections, robustness of methods, similarities between methods

Soneson & Robinsons, Nature Methods, 2018

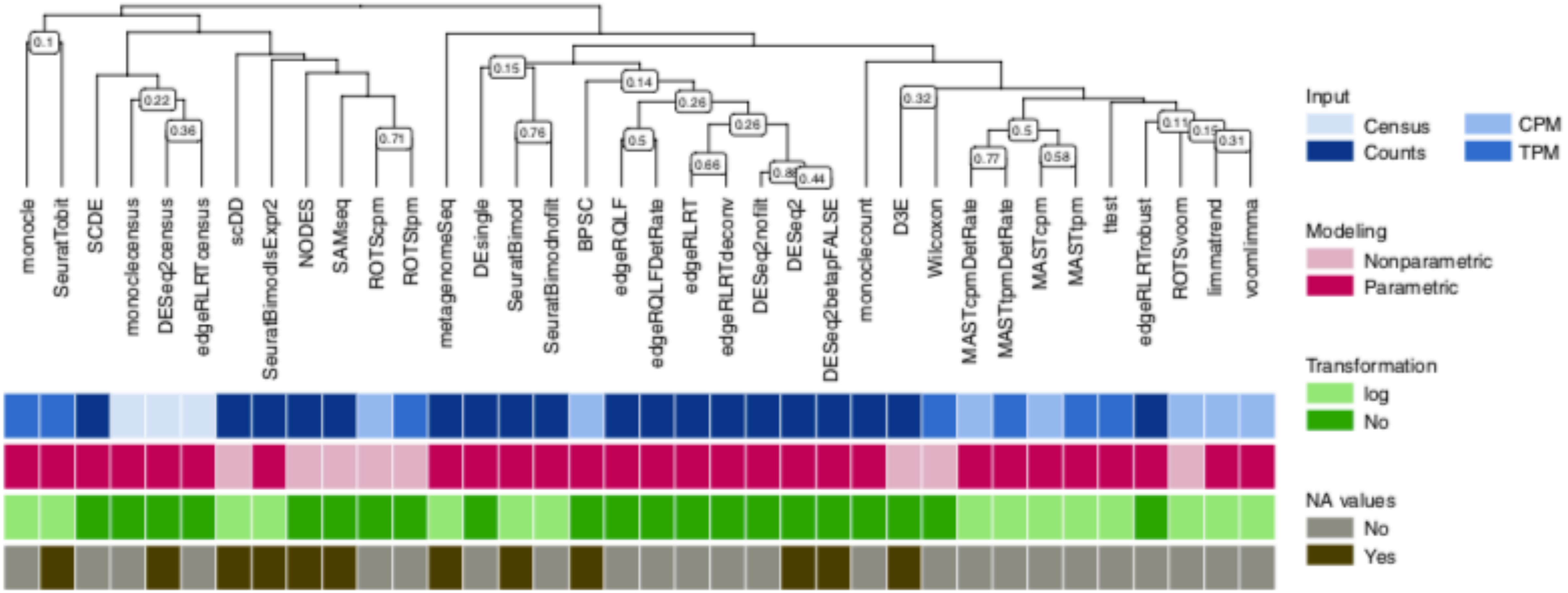
Performance



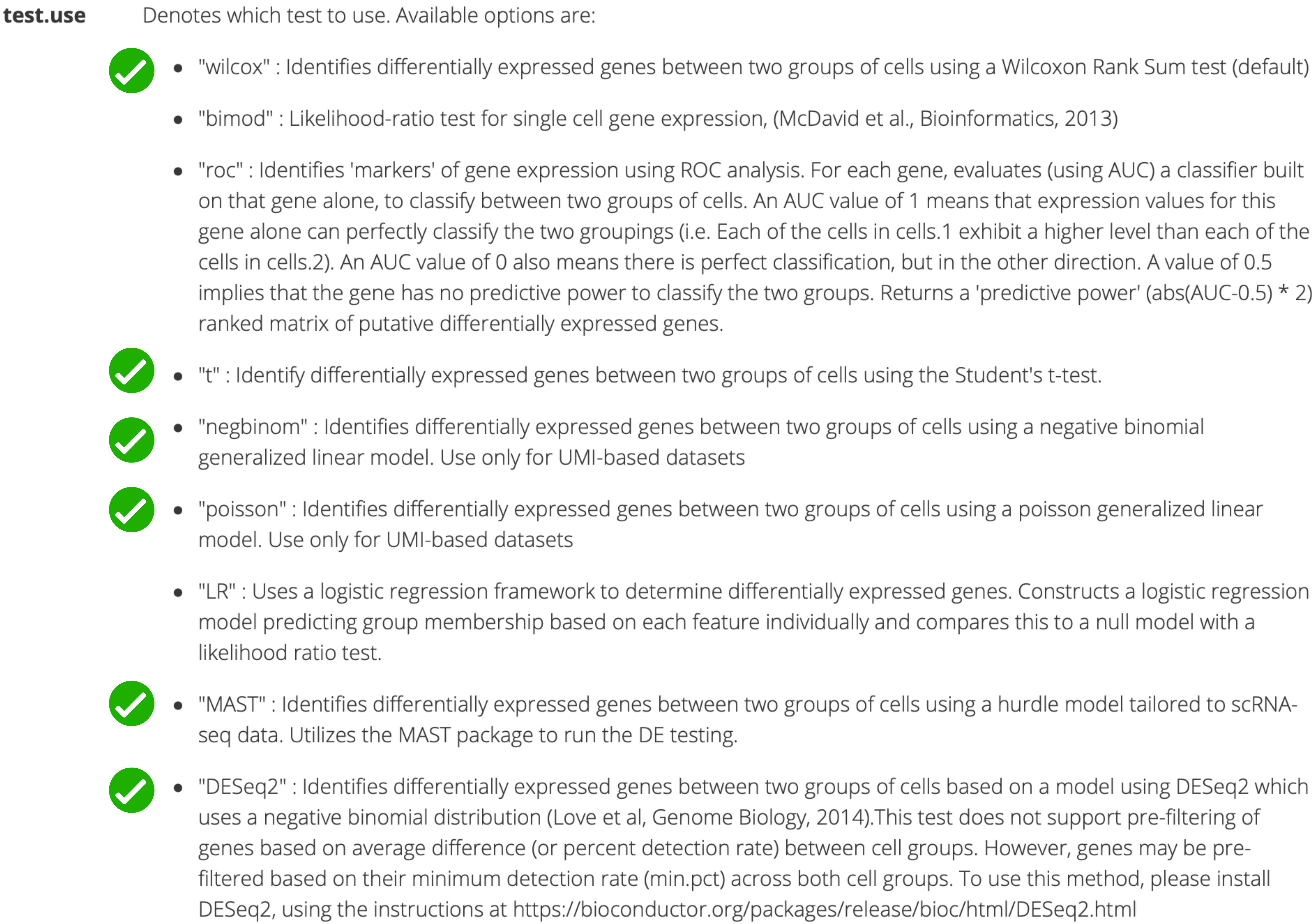
Some highlights:

- t-test and Wilcoxon work well, given at least few dozens cells to compare
- bulk RNA-seq analysis methods do not generally perform worse than those specifically developed for scRNA-seq
- Filtering out lowly expressed genes in quite important for good performance of bulk methods (edgeR, DEseq2)

Performance



Finally



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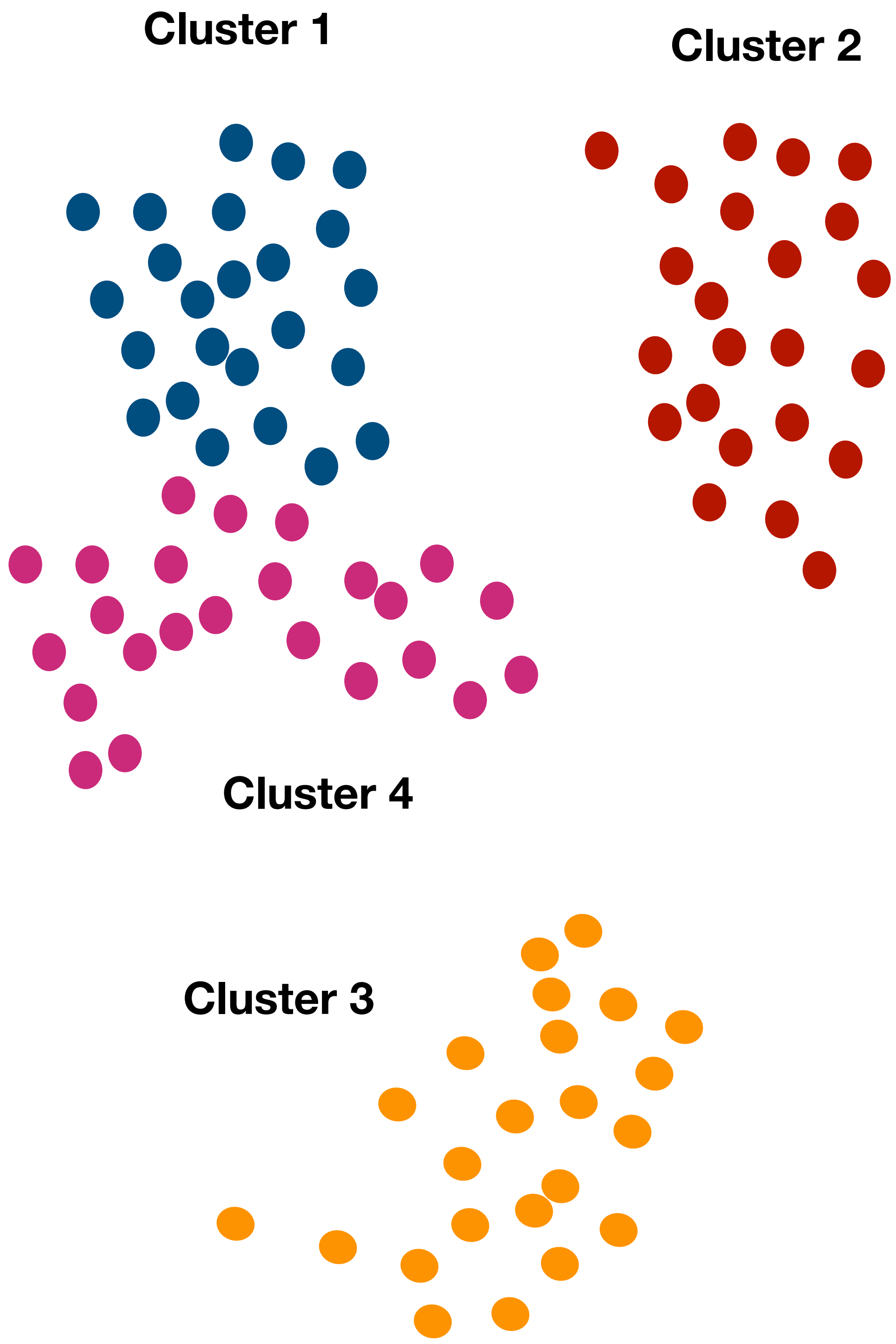
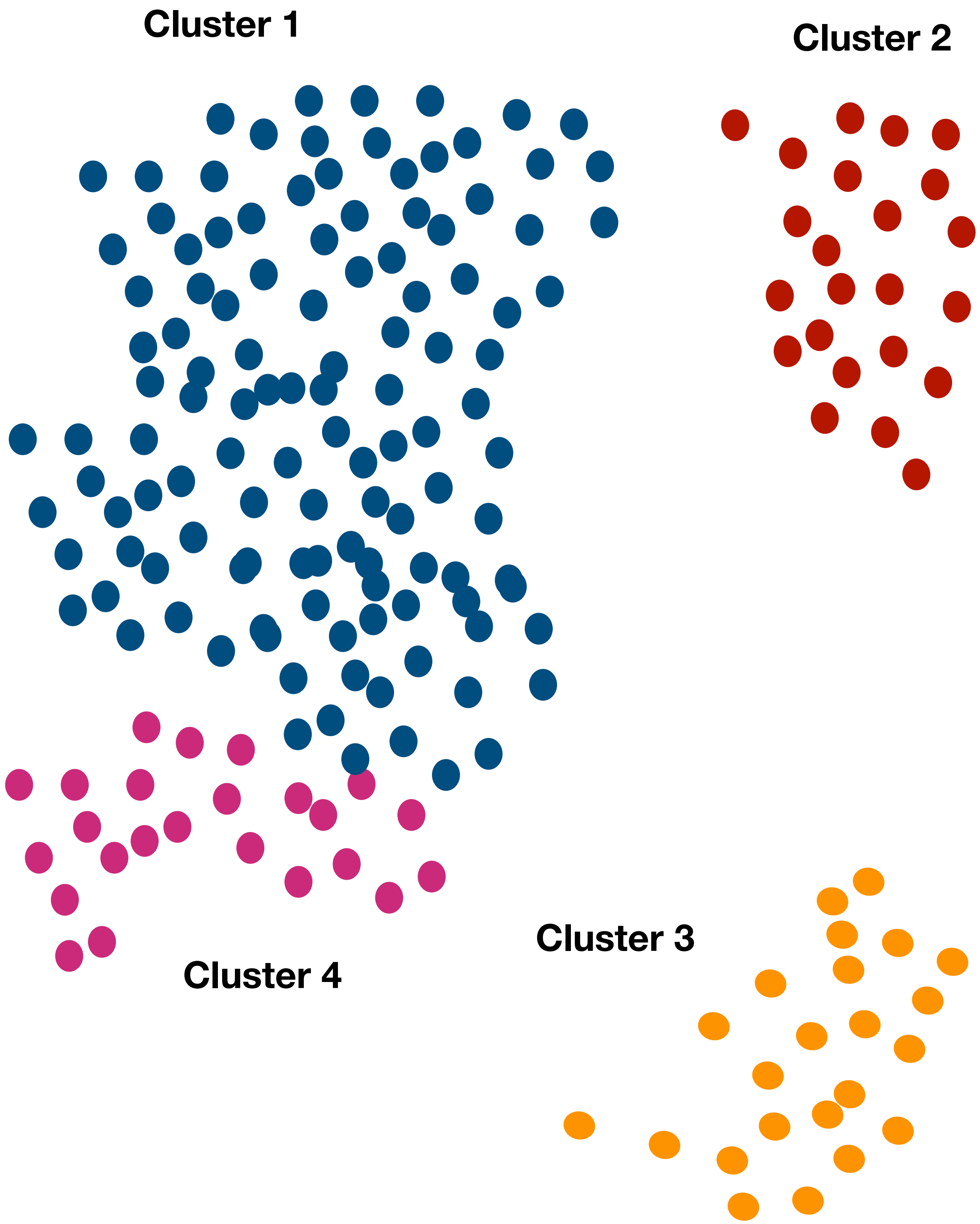
Things to think about



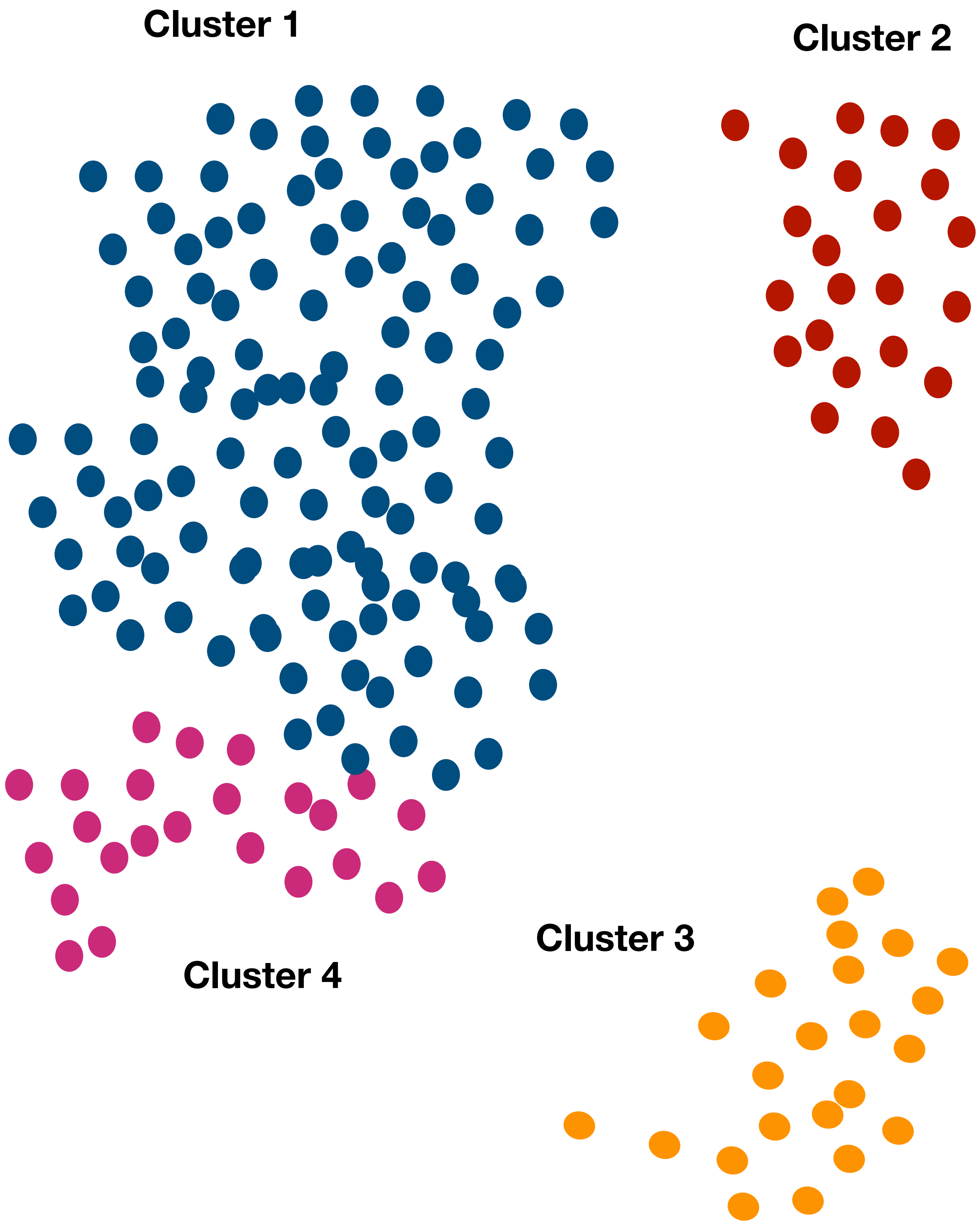
- Balanced cluster sizes
- Highly similar clusters

Things to think about



- Balanced cluster sizes
- Highly similar clusters

Things to think about



Balanced cluster sizes:

- Cluster1 will domate all 1-vs-rest comparisons.
- Probably good idea to subsample
- Be aware the subsampling strategies in Seurat only does it per test.

Highly similar clusters:

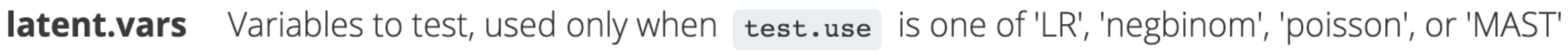
- Will have most of their DEGs overlapping.

Things to think about

- Always go back to RNA assay (or similar) for doing differential expression.
- Depending on the method you chose use: counts, normalised counts or lognormalized counts.
- Normalization strategy has a big influence on the results in differential expression, size factors may help.
  - E.g comparing celltype with few expressed genes vs a cell type with many genes.

Things to think about

- Batch effects:
  - Always check if the DEGs you get are just unregulated in one of the batches.
  - OBS! Use a test that can control for batch effects.



latent.vars Variables to test, used only when `test.use` is one of 'LR', 'negbinom', 'poisson', or 'MAST'

Things to think about

- How many cells do you need to get reliable detection of differential expression?
  - Highly expressed genes - probably 10-20 cells is enough
  - Lowly expressed genes, at least 20 cells, but probably 50 are needed
- Depends on the sensitivity of the lib. prep. method and how distinct the cell types you are comparing are.
  - E.g:
    - Macrophage vs T-cell (different)
    - CD8 T-cell vs CD4 T-cell (similar)

Things to think about

- A lot of what you get will be noise. Take two random set of cells and run DE and you probably with have a few significant genes with most of the commonly used tests.

Thank you for your attention!