scRNAseq normalization and gene set selection

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Outline

- Introduction
- Normalization
- Removal of confounders
- Gene set selection

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Why do we need to normalize scRNAseq data?

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Biological and technical variation

- Biological variation:
  - Cell type/state
  - Cell cycle
  - Cell size
  - Sex, Age, …
  - Etc..
- Technical variation
  - Cell quality
  - Library prep efficiency
  - Batch effects
  - Etc…

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Biological and technical variation

- Biological variation:
  - **Cell type/state**
  - Cell cycle
  - Cell size
  - Sex, Age, …
  - Etc..
- Technical variation
  - Cell quality
  - Library prep efficiency
  - Batch effects
  - Etc..

To identify cell types we would like to remove all other sources of variation.

UMIs does not solve the problem

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	Cell-specific effects	Gene-specific effects	Not removed by UMIs
Sequencing depth	✓		✓
Amplification	✓	✓	
Capture and RT efficiency	✓	✓	✓
Gene length		✓	
GC content	✓	✓	✓
mRNA content	✓		✓

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Vallejos et al. Nature Methods 2017

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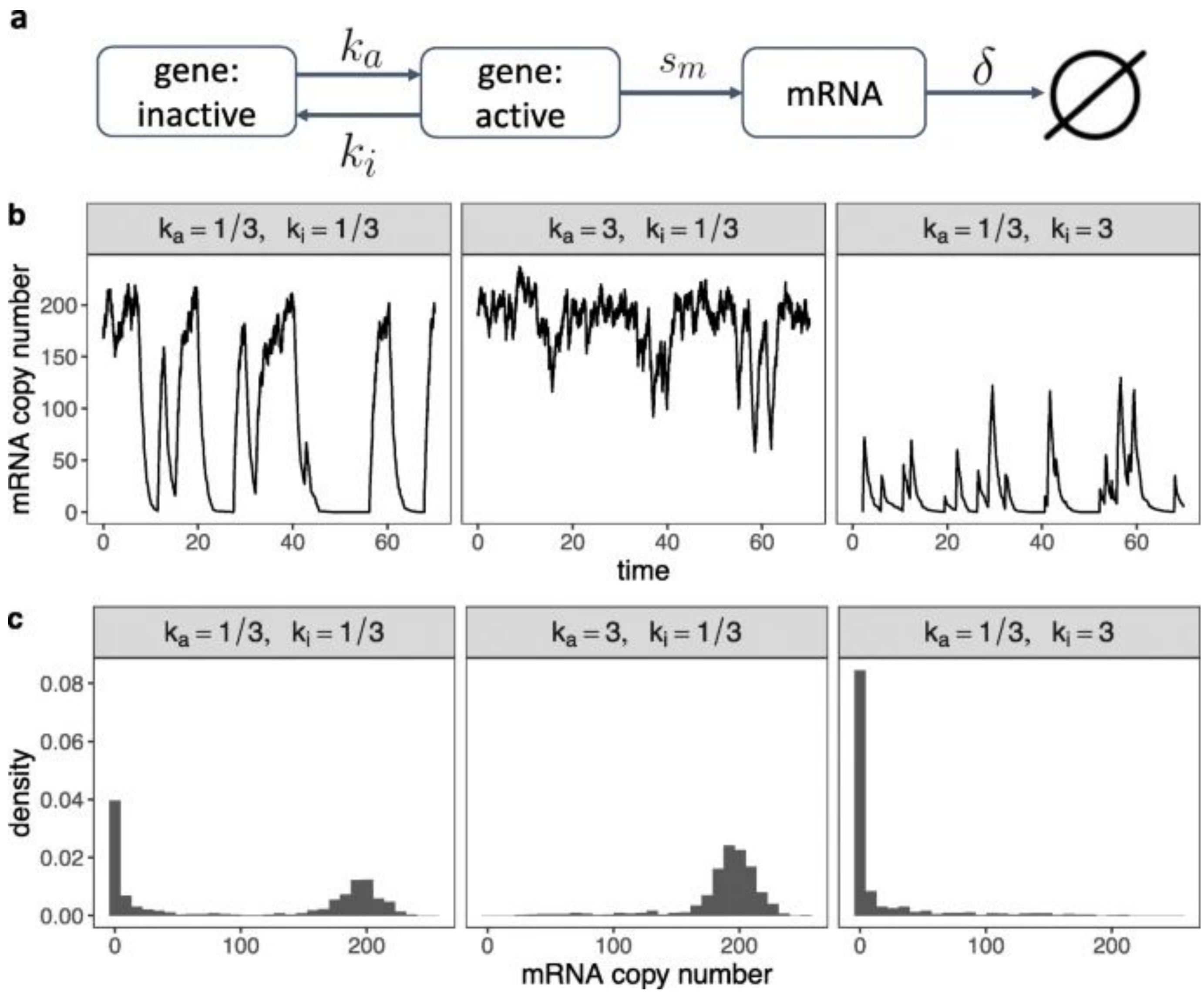
Normalization

- Want to make expression comparable across samples, cells and genes.
- Involves 3 main steps:
  - Scaling
  - Transformation
  - Removal of unwanted variation

Genes with different distributions



(Jiang et al. Genome Biol 2022)

Scaling Normalization

- **Count normalization** –for uneven sequencing depth
- **Gene length normalization** – for differences in gene detection due to gene length (full length methods)
- **Drop-out rate normalization** – for differences in RNA content / drop-out rates

OBS! After scaling we have relative amounts of the different genes, not absolute values.

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Depth normalization

- Assuming same RNA content in all cells – may work well in homogeneous cell population

- In most cases the amount of RNA – and of UMIs/reads differ between cells.

- Also important to check for oulier genes that constitute large proportion of the reads!

Bulk RNAseq methods

- **CPM**: Controls for sequencing depth when dividing by total count
- **RPKM/FPKM**: Controls for sequencing depth and gene length. Good for technical replicates, not good for sample-sample due to compositional bias. Assumes total RNA output is same in all samples.
- **TPM**: Similar to RPKM/FPKM. Corrects for sequencing depth and gene length. Also comparable between samples but no correction for compositional bias.

$$\mathrm{CPM}_i = \frac{X_i}{\frac{N}{10^6}} = \frac{X_i}{N} \cdot 10^6$$

$$\mathrm{FPKM}_i = \frac{X_i}{\left(\frac{\widetilde{l}_i}{10^3}\right)\left(\frac{N}{10^6}\right)} = \frac{X_i}{\widetilde{l}_i N} \cdot 10^9$$

Xi: observed count
li: length of the transcript
N number of fragments sequenced

$$\mathrm{TPM}_i = \frac{X_i}{\widetilde{l}_i} \cdot \left(\frac{1}{\sum_j \frac{X_j}{\widetilde{l}_j}}\right) \cdot 10^6$$

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Transformation Normalization

- Idea is to have a distribution of expression and variance in expression values that best captures biological variation.

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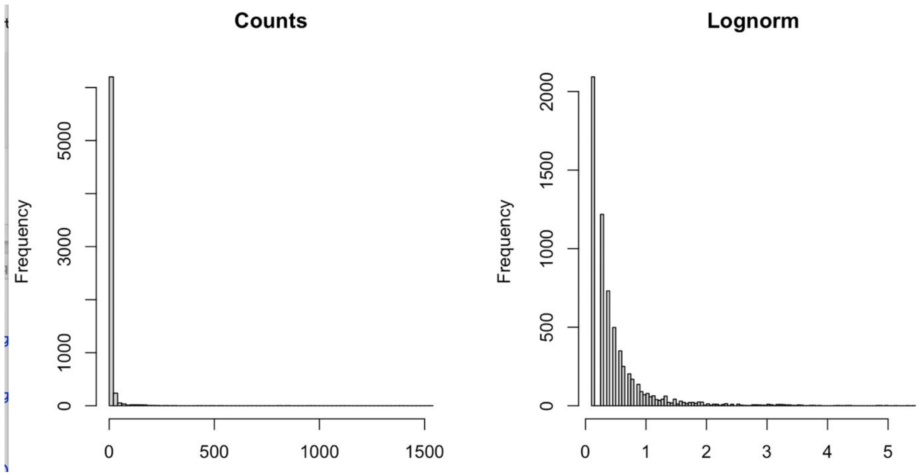
Logtransformation

- Log-transformed values approaches normal distribution for bulk RNAseq data
- For scRNAseq – more similar to zero-inflated binomial
- Still more similar to normal distribution than raw counts.



Bulk RNAseq methods

- **TMM/RLE/MRN**: Improved assumption: The output between samples for a core set only of genes is similar. Corrects for compositional bias. RLE and MRN are very similar and correlates well with sequencing depth. edgeR::calcNormFactors() implements TMM, TMMwzp, RLE & UQ. DESeq2::estimateSizeFactors implements median ratio method (RLE). Does not correct for gene length.

- **VST/RLOG/VOOM**: Variance is stabilised across the range of mean values. For use in exploratory analyses. vst() and rlog() functions from *DESeq2*. voom() function from *Limma* converts data to normal distribution.

Depth normalization and logtransformation in practice:

- The most simple normalization is to divide by sequencing depth * a scale factor and log-transform the data
- Scater **normalize** – uses total counts or provided size factors. Default is return_log = TRUE.
- Seurat **NormalizeData** – returns log-normalized data with scale.factor = 10K by default.
- Scanpy **normalize_per_cell/normalize_total** – normalize by sequencing depth – then need to run **log1p.**

scRNAseq normalization methods

- Deconvolution/Scran (Pooling-Across-Cells)
- SCnorm (Expression-Depth Relation)
- SCTransform
- Census
- Linnorm
- ZINB-WaVE
- BASiCS
- More…

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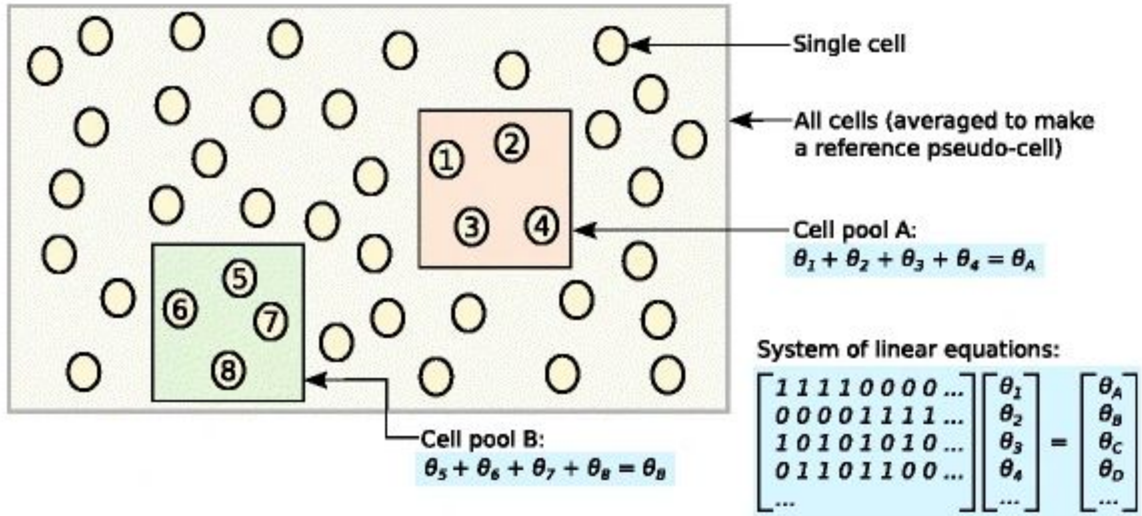
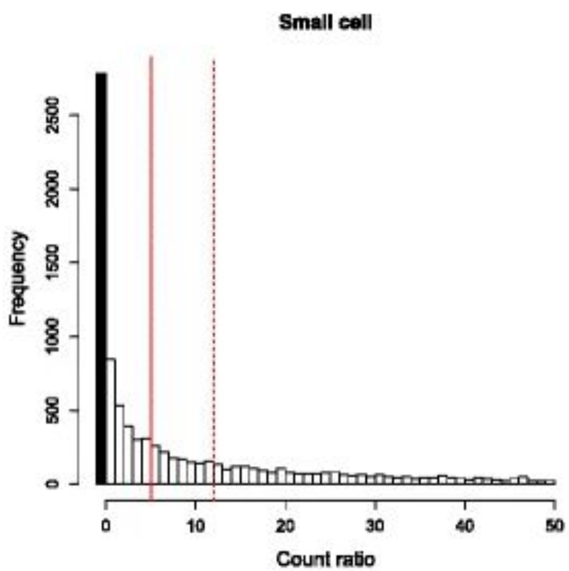
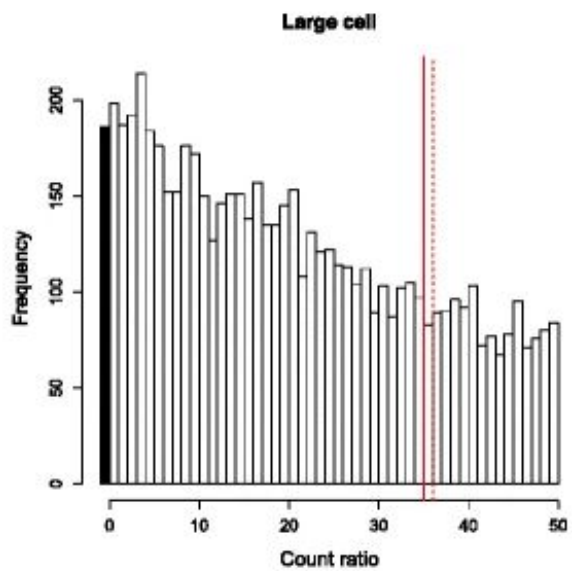
Deconvolution







Lun et al. Genome Biol. 2016

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Scran - computeSumFactors

- Deconvolution with all cells
  - The assumption is that most genes are not differentially expressed (DE) between cells,
- Deconvolution within clusters (FastCluster beforehand)
  - Size factors computed within each cluster and rescaled by normalization between clusters.
  - When many genes are DE between clusters in a heterogeneous population.
- computeSumFactors – will also remove low abundance genes

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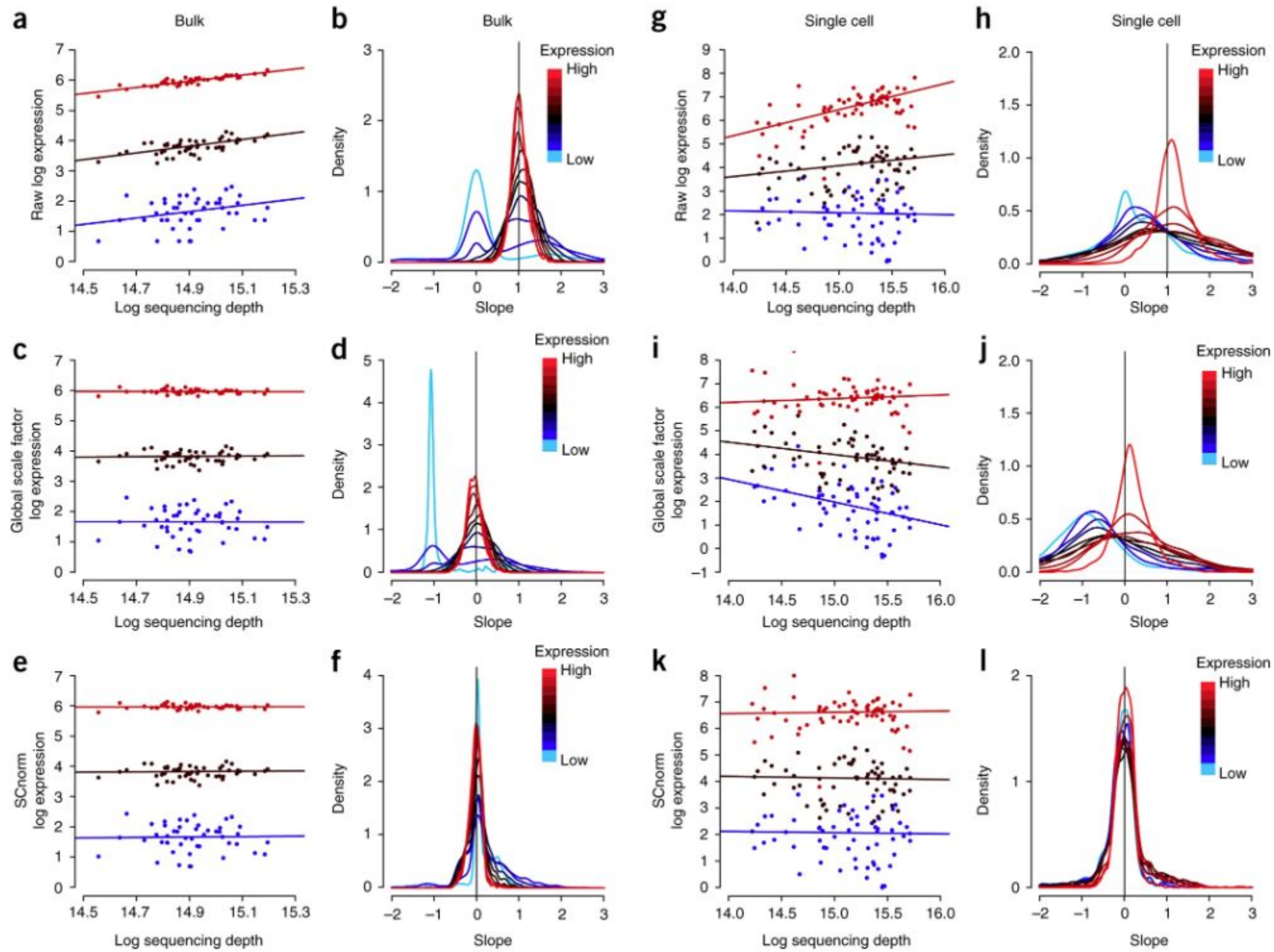
Normalization with gene groups

- Global scale factors may lead to overcorrection for weakly and moderately expressed genes and undercorrection for highly expressed genes.
- It will also differ a lot between cells with high/low total counts.

- Solution: Do normalization for genes at different expression levels – SCNorm & SCTransform

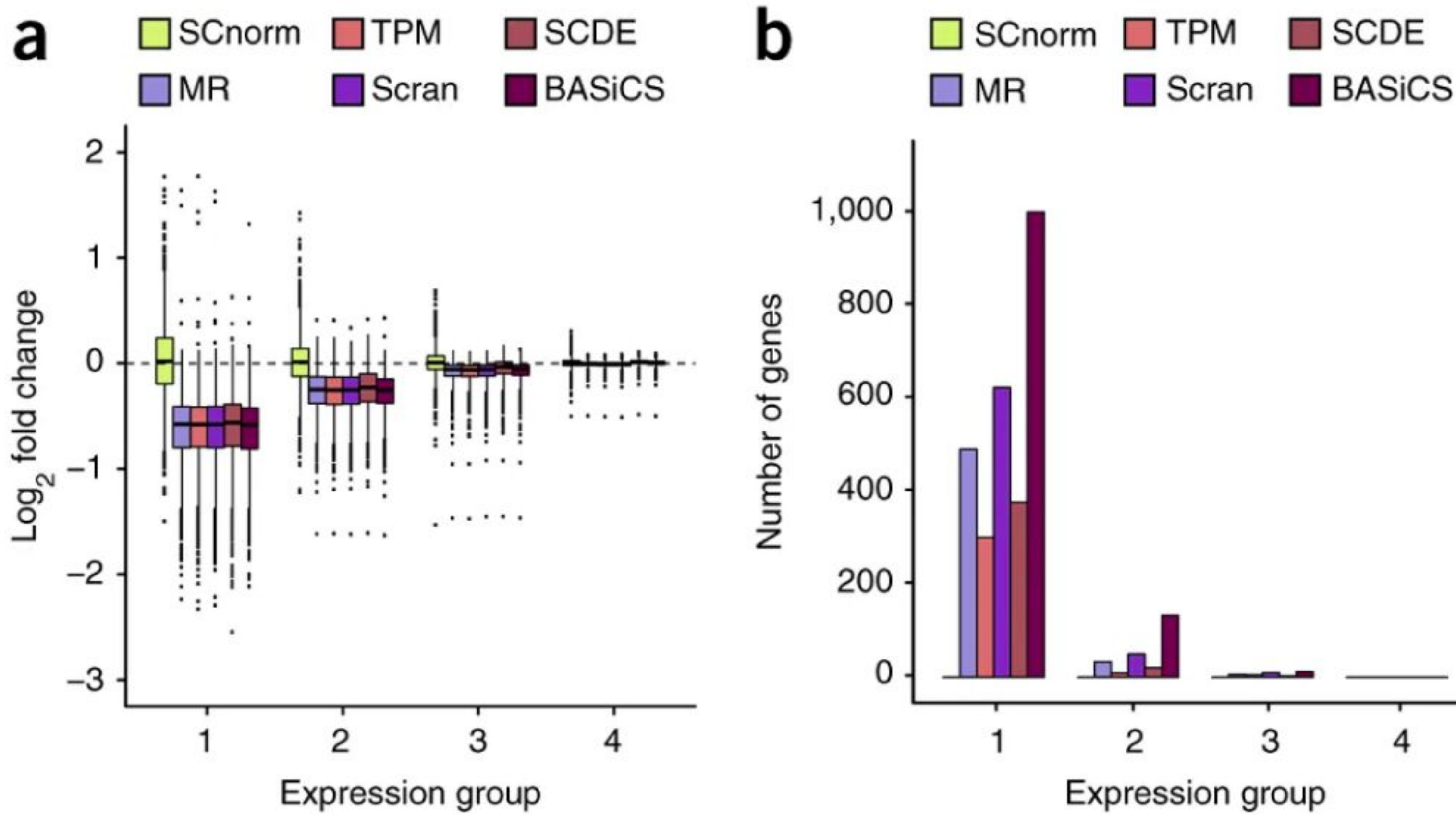
SCNorm: Expression vs. Depth Bias Correction



Quantile regression to estimate the count–depth relationship

Bacher et al. Nature Methods 2017)

SCNorm: Expression vs. Depth Bias Correction



Identical cells in two groups should result in no DE and FC = 1 if normalization was efficient

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(Bacher et al. Nature Methods 2017)

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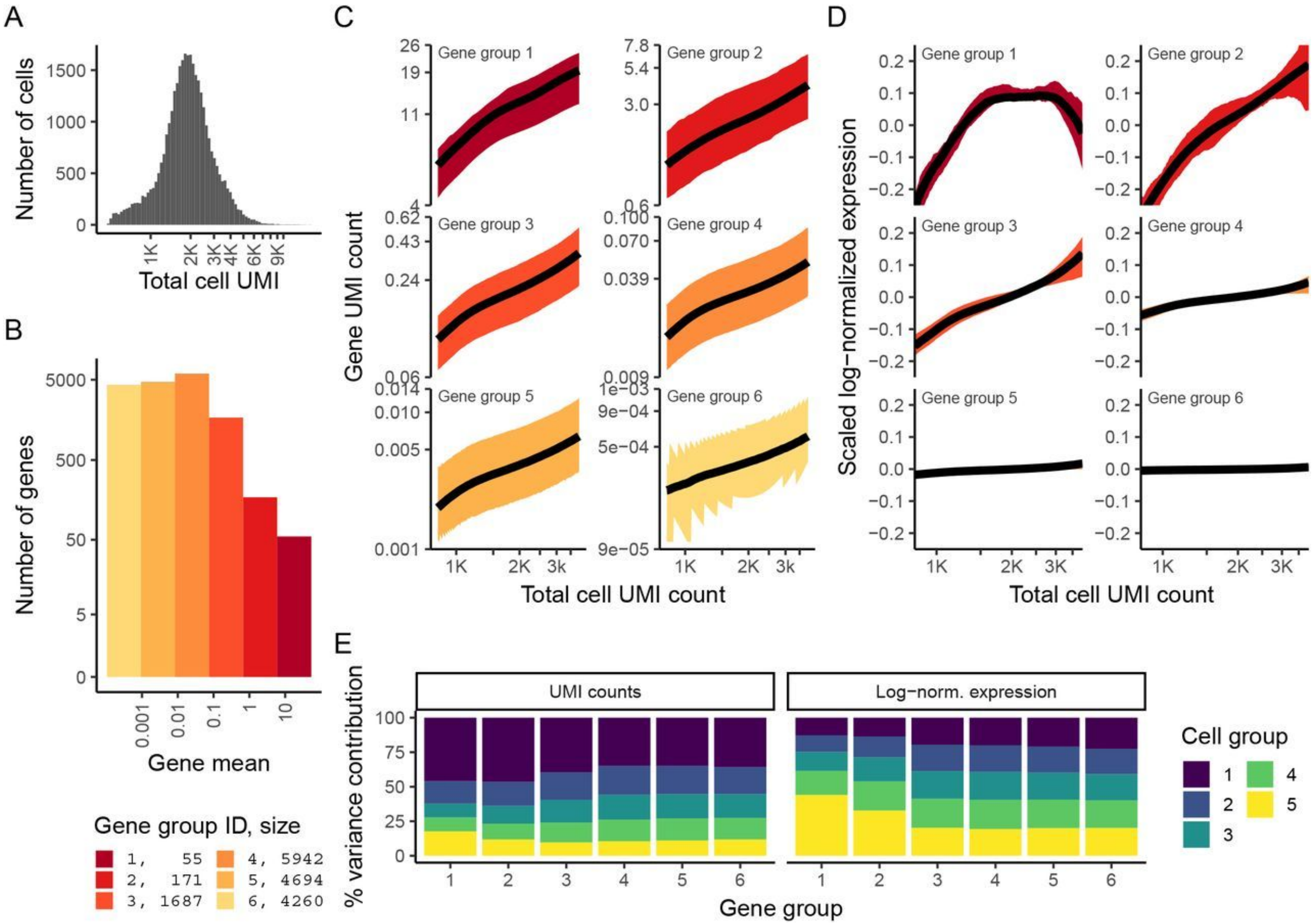
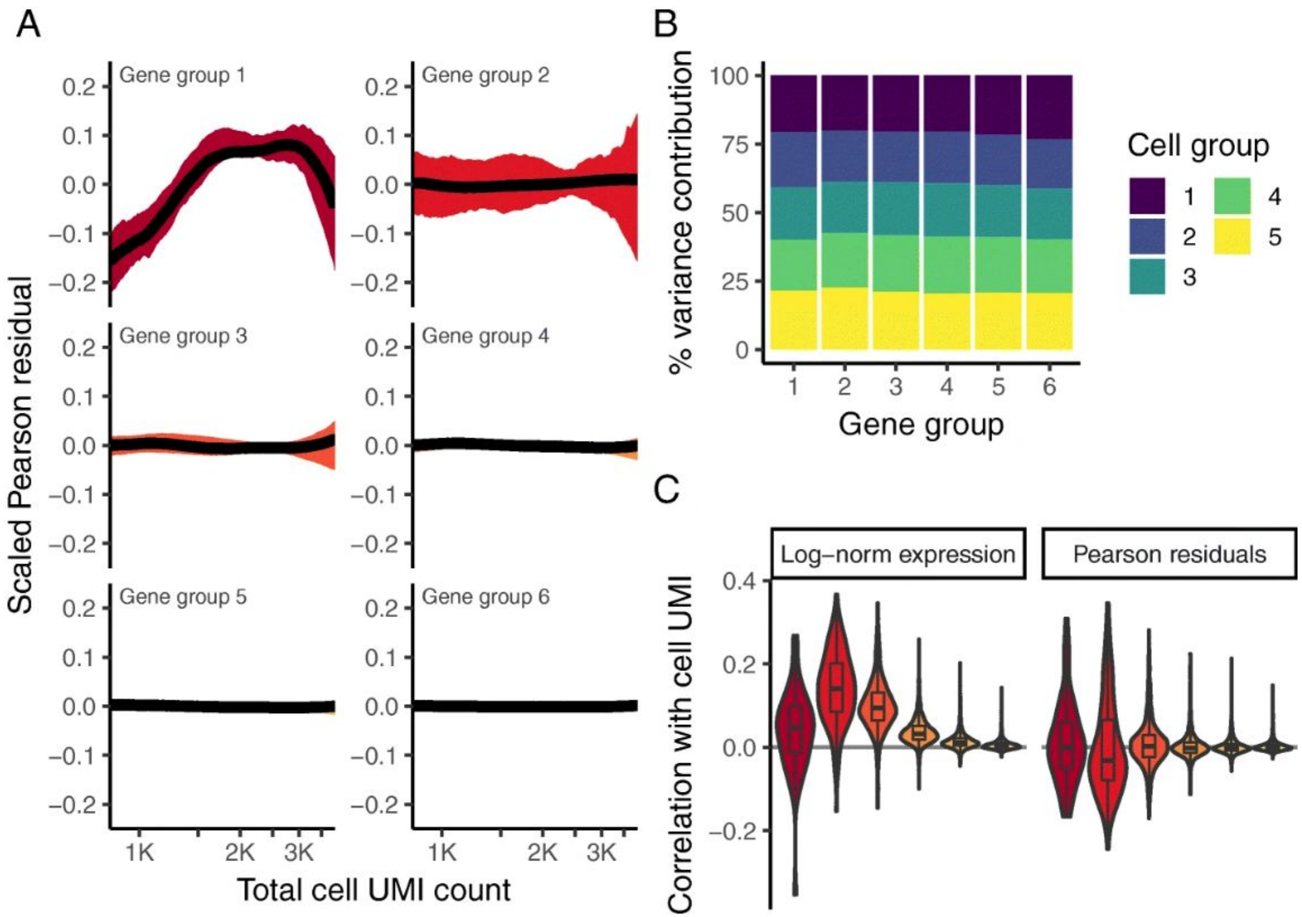
SCTransform (Seurat)



(Hafmeister & Satija Genome Biology 2019)

SCTransform (Seurat)



Pearson residuals from regularized negative binomial (NB) regression

(Hafmeister & Satija Genome Biology 2019)

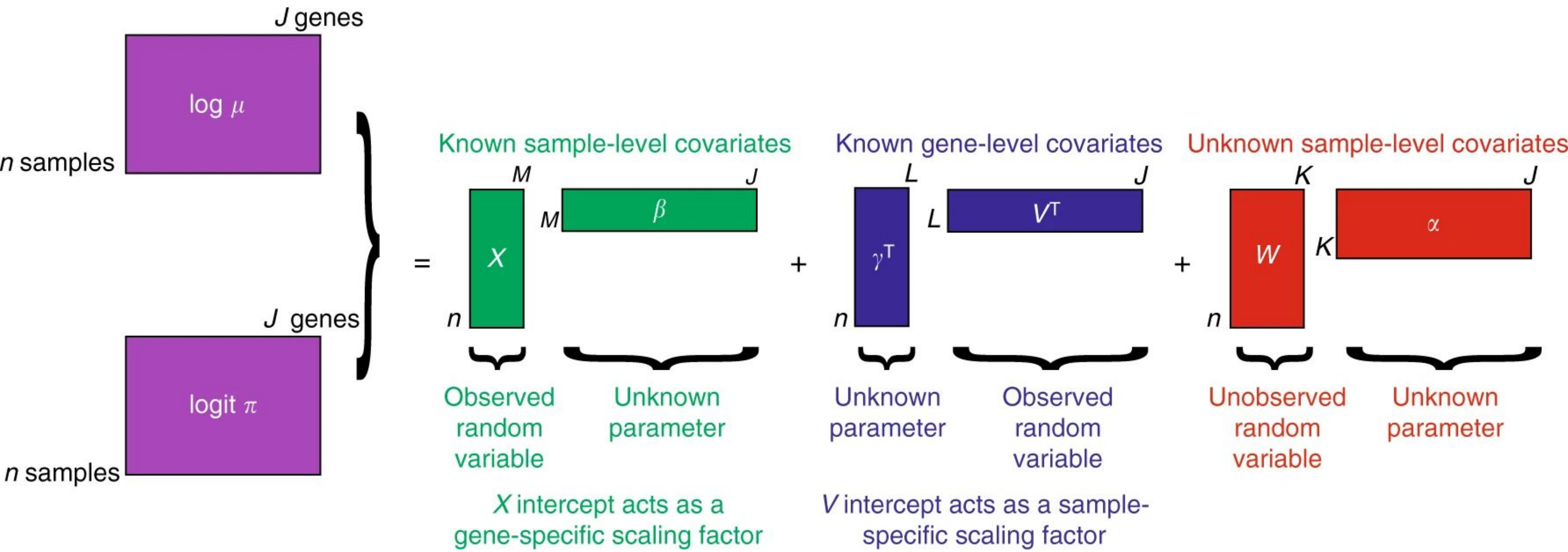
SCTransform (Seurat)

- OBS! SCTransform function in Seurat also does variable gene selection in the same step with a slightly different method than the default in Seurat.
- But you can also specify which genes to run it on.

- You can also run regression of other parameters in the same step.
- Should be run per sample not with all data together.

Zero-Inflated Negative Binomial-based Wanted Variation Extraction (ZINB-WaVE) - NewWave.

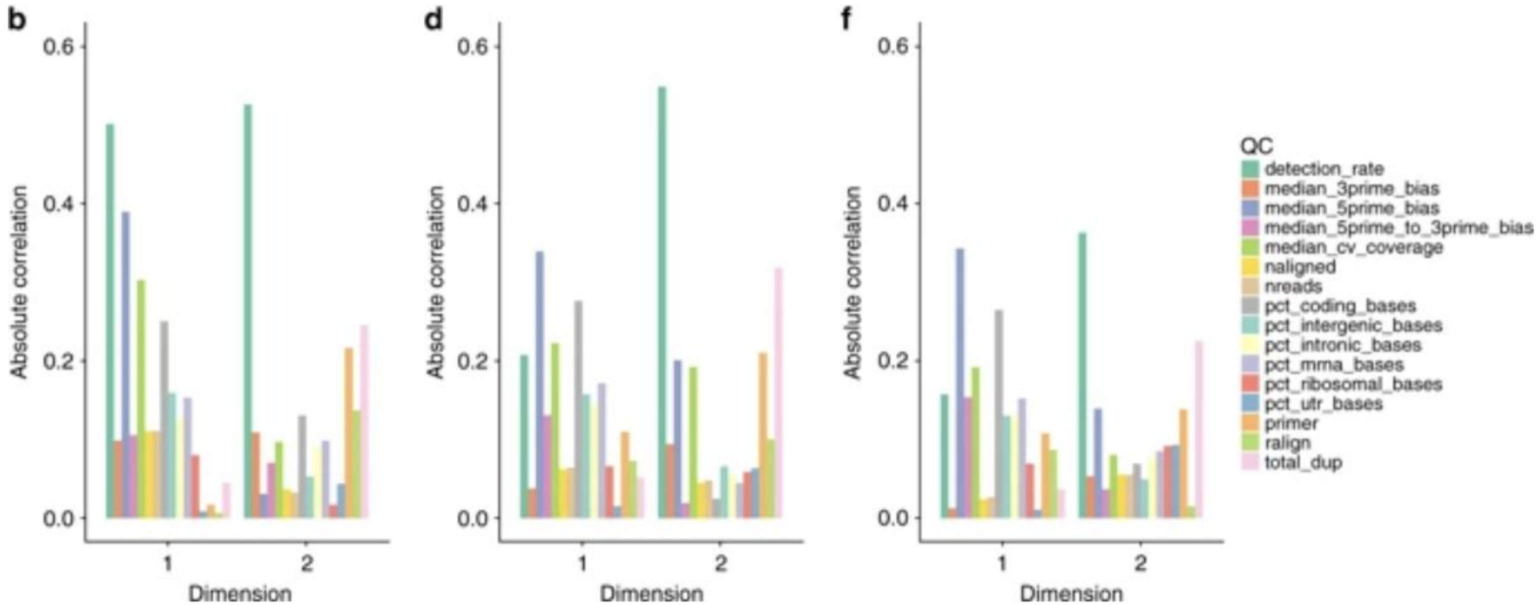
- Both gene-level and sample-level covariates
- Extension of the RUV model

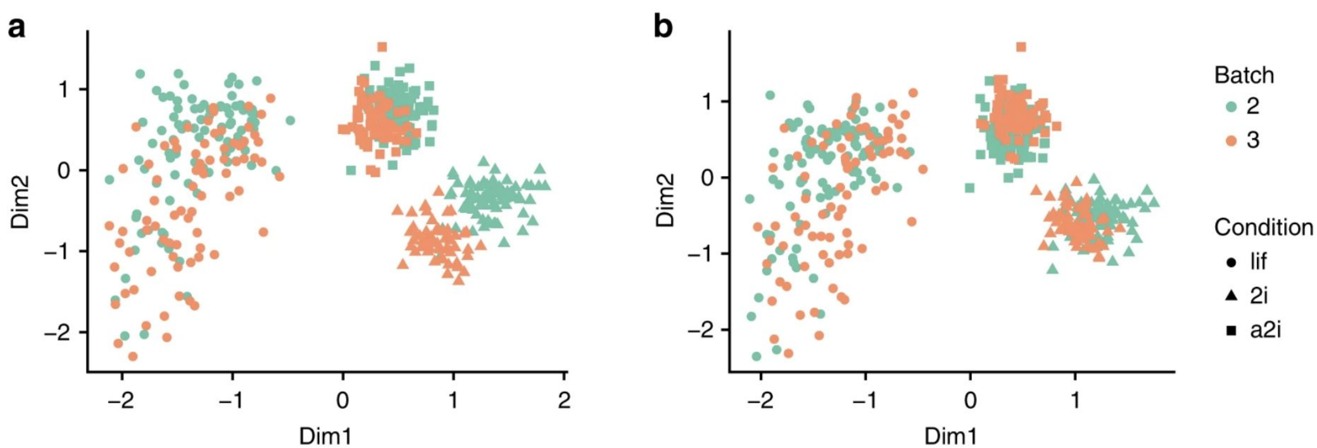


Risso et al. Nat. Comm. 2018

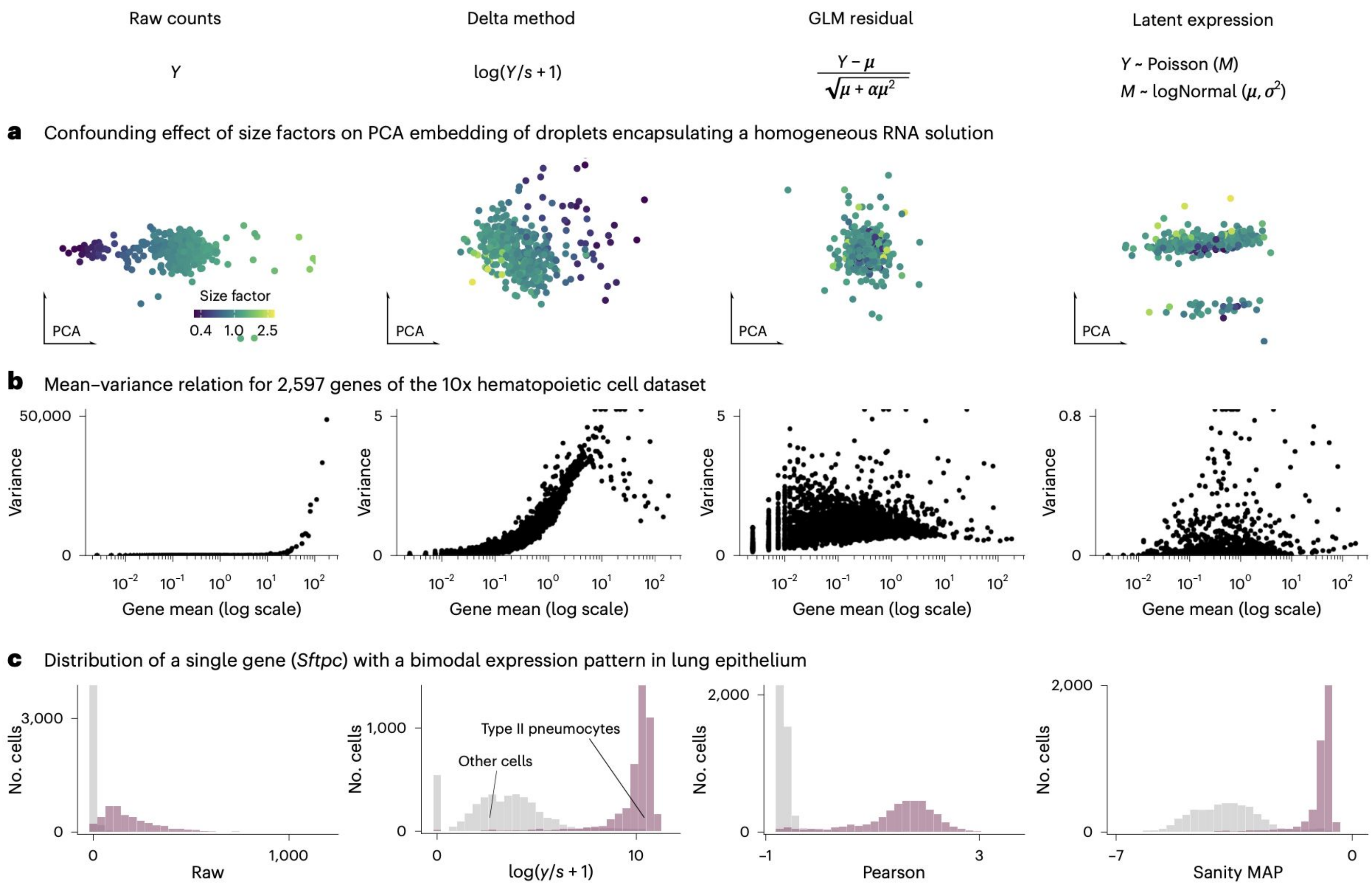
ZINB-WaVE

Reduces technical influence on PCA, also batch effect.



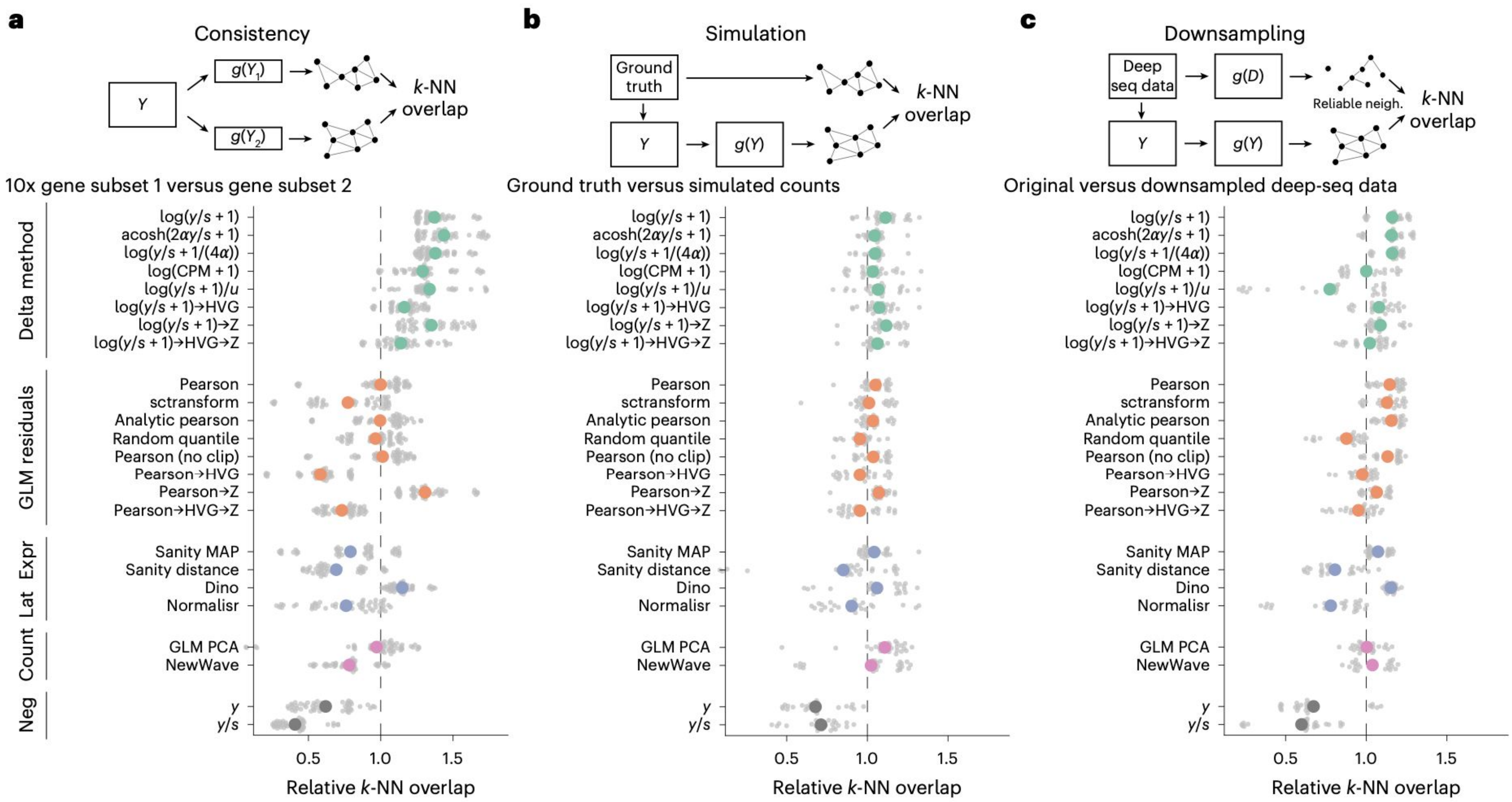


Comparison of transformations for single-cell RNA-seq data



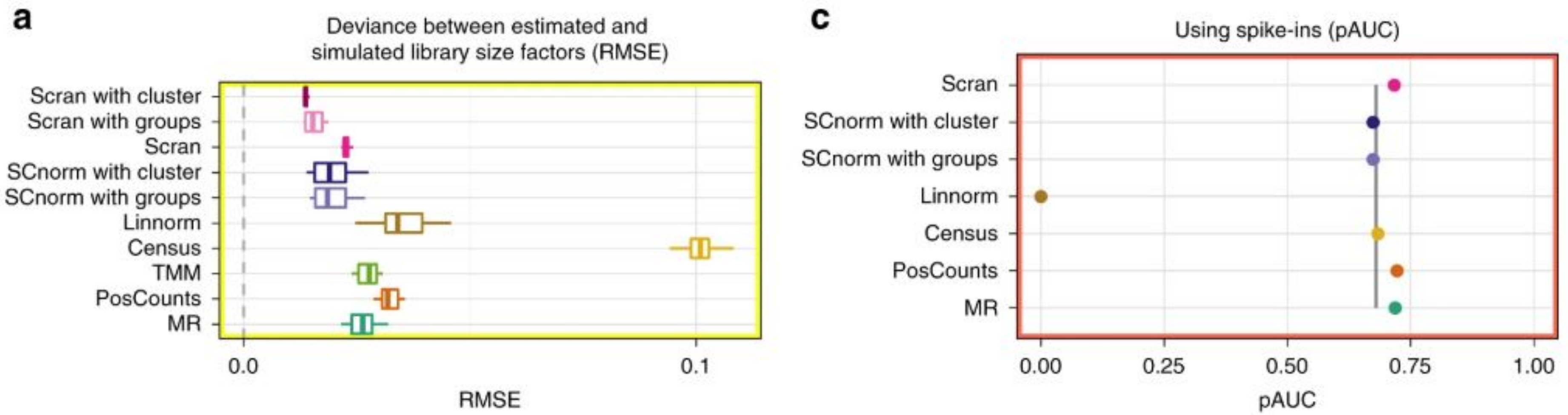
(Ahlmann-Eltze Nat. Methods 2023)

Comparison of transformations for single-cell RNA-seq data



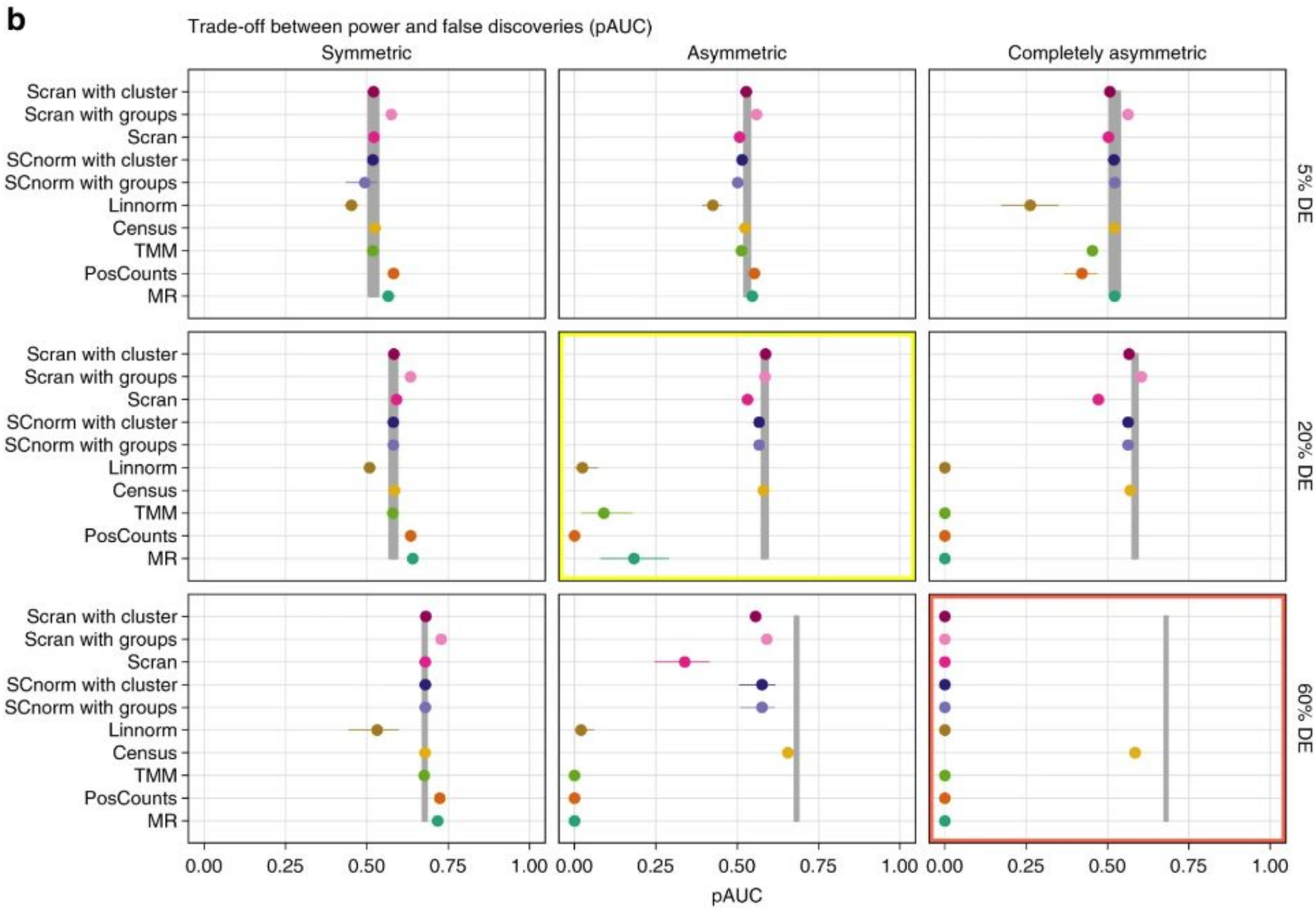
(Ahlmann-Eltze Nat. Methods 2023)

Size factors with different normalizations



Vieth et al. Nature Comm. 2019

DE with different normalizations

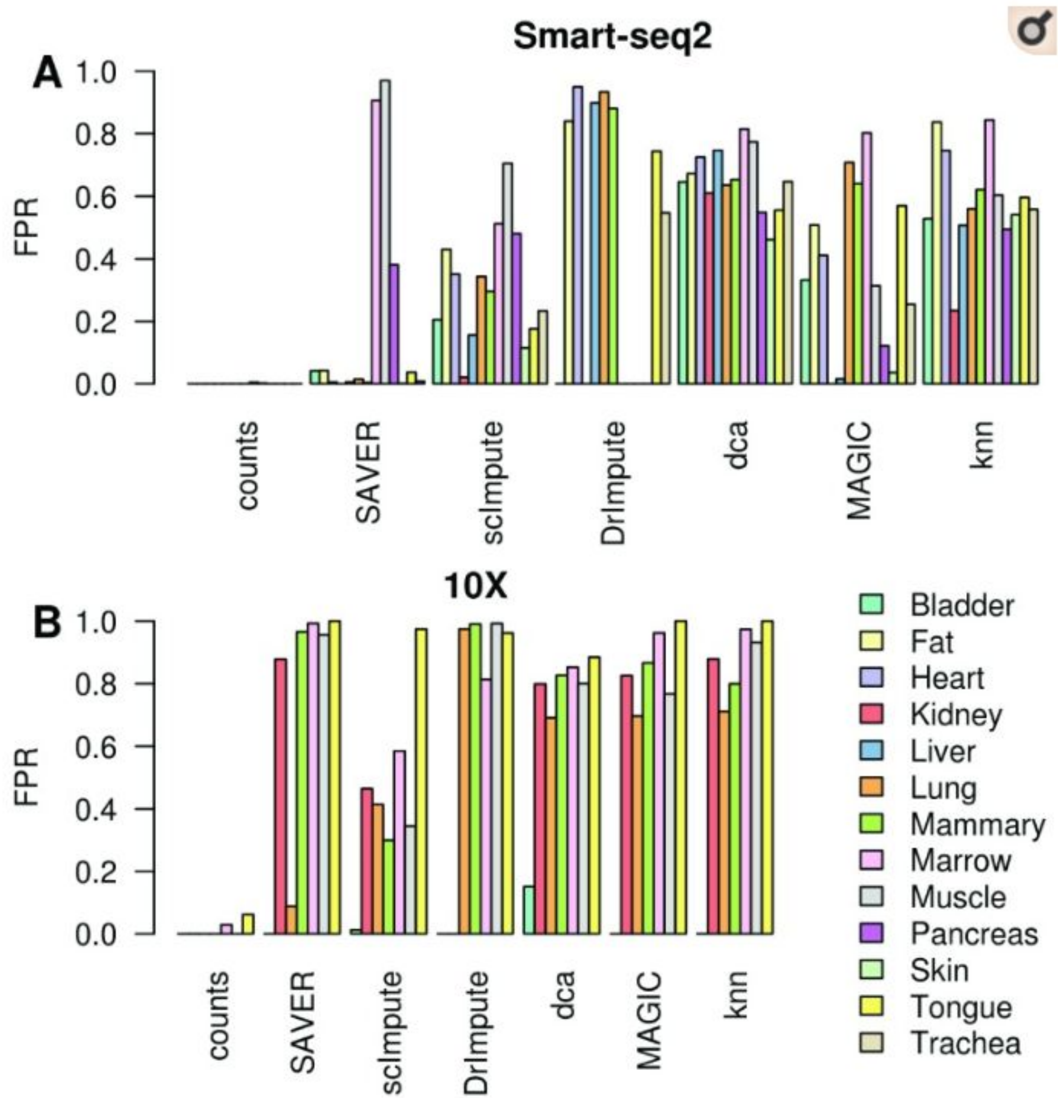


Vieth et al. Nature Comm. 2019

Imputation

- scRNAseq has a lot of zeros in expression matrix
- Common for GWAS data to impute SNPs
- Many methods published:
  - SAVER
  - DrImpute
  - scImpute
  - MAGiC
  - Knn-smooth
  - Deep count autoencoder

Imputation can introduce false correlations



Andrews et al. F1000research 2018

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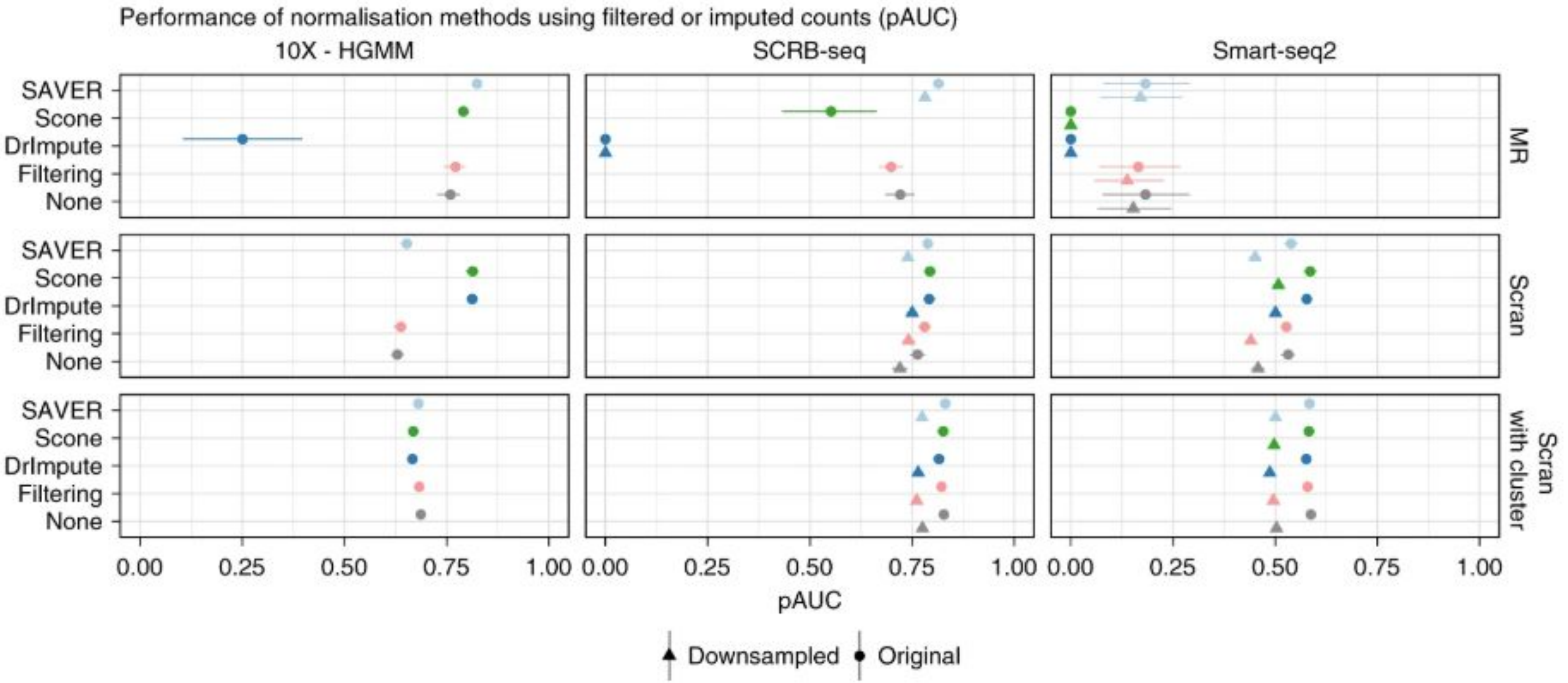
Imputation has little effect on DE detection



Vieth et al. Nature Comm. 2019

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Scaling data – Z-score transformation

- Z-score transformation - linearly transform data to a mean of zero and a standard deviation of 1 - also called **centering** and **scaling**

- PCA or any other type of analysis will be dominated by highly expressed genes with high variance.
- It can be wise to center and scale each gene before performing PCA

What normalization should you use?

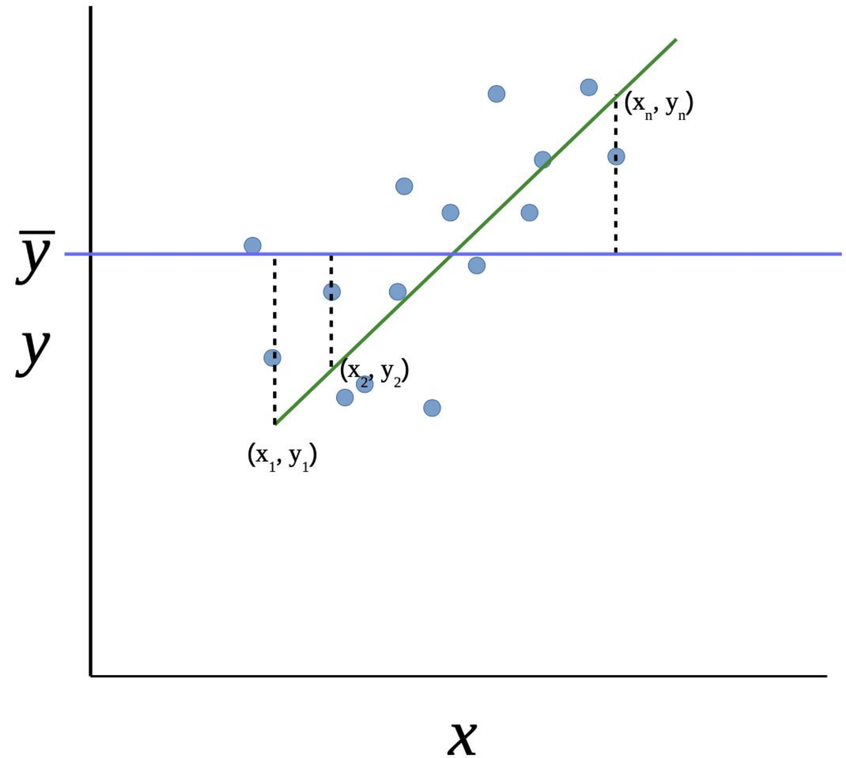
- Normalization has big impact on differential gene expression, but not as much on clustering
- In most cases it is enough to do sequence depth normalization and log-transformation.
- When working with highly similar subtypes of the same celltype, or with celltypes of very different sizes, individual size factors could help.
- Binning by gene level (SCTransform) helps to remove the effect of different gene detection across cells.

Confounding factors

- Any source of variation that you do not expect to give separation of the cell types.
  - Cell cycle
  - Cell size
  - Sequencing depth
  - Cell quality
  - Batch
  - More…

Linear regression

- Fit a line to the gene expression vs variable of interest
- Calculate residuals
- Remove variance explained by the variable of interest by taking the residuals.
- Multiple linear regression if multiple factors.



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Other tools to remove unwanted variance

- RUVseq() or svaseq()
- Linear models with e.g. removeBatchEffect() in limma or scater
- ComBat() in sva
- Tools like SCTransform, ZIMB-WaVE does regression in the same step.

What confounders should you remove?

- Percent mitochondrial reads – often correlates with quality of cell
- Sequencing depth
- Gene detection rate – relates to amount of RNA per cell.
- Cell cycle
- Batch effects (Sample, sort date, sex, etc.)
  - in most cases it is better to use an integration tool.

What confounders should you remove?

ALWAYS check QC parameters in PCA/tSNE/UMAP and see how they influence your data.

BUT, be careful that your confounders are not related to your biological question!

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Scaling and regression in practice

- Seurat **ScaleData:** does Z-score transformation and regression of variables in **vars.to.regress.** Can use **linear** (default), **poisson** or **negbiom** models.

- Scran: runs scaling but not centering automatically in PCA step. **trendVar** function estimates unwanted variation either with a **design** matrix or with **block** factors. **decomposeVar** or **denoisePCA** to remove unwanted variation.

- Scanpy: **pp.regress_out** and **pp.scale** functions.

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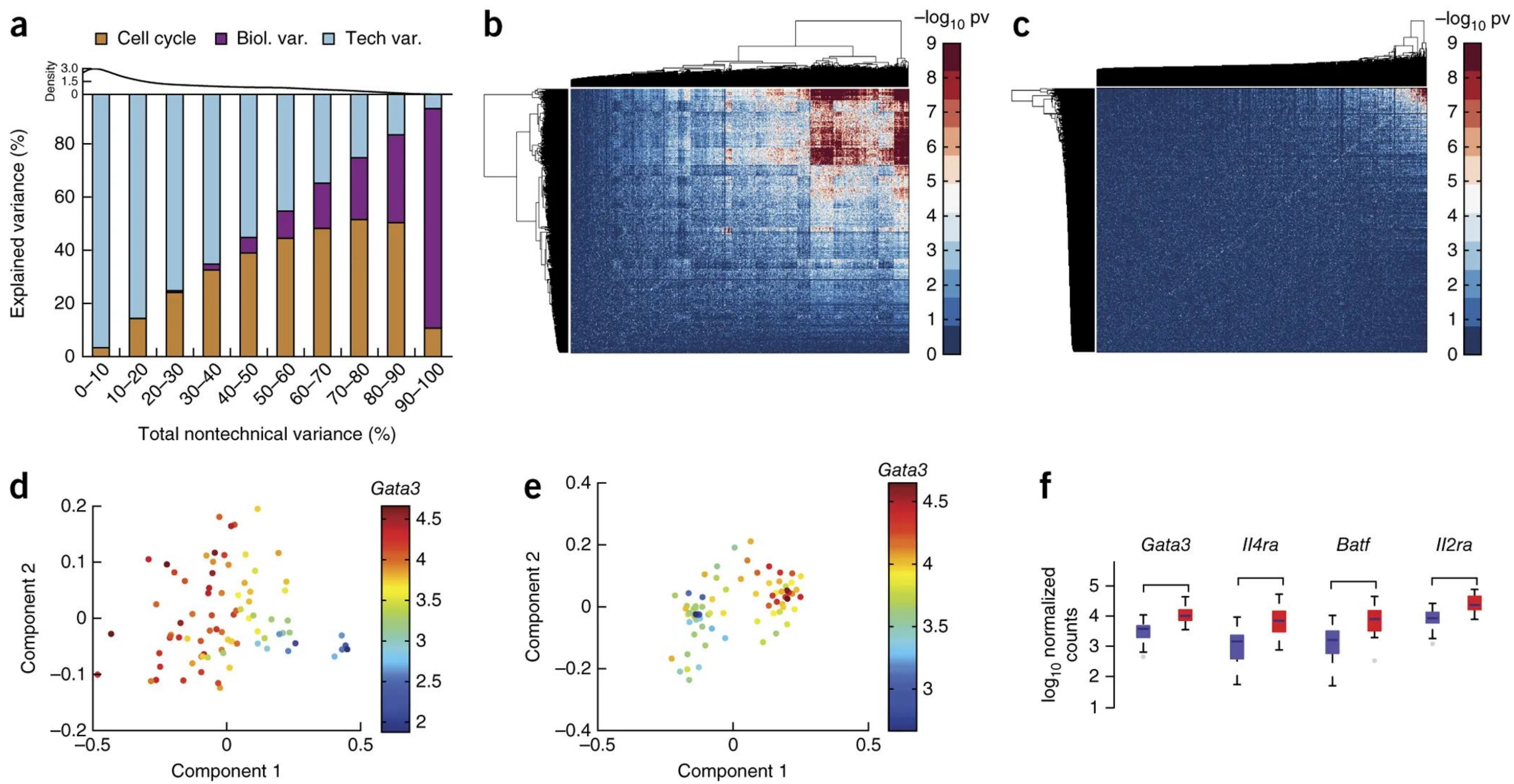
Cell cycle effect

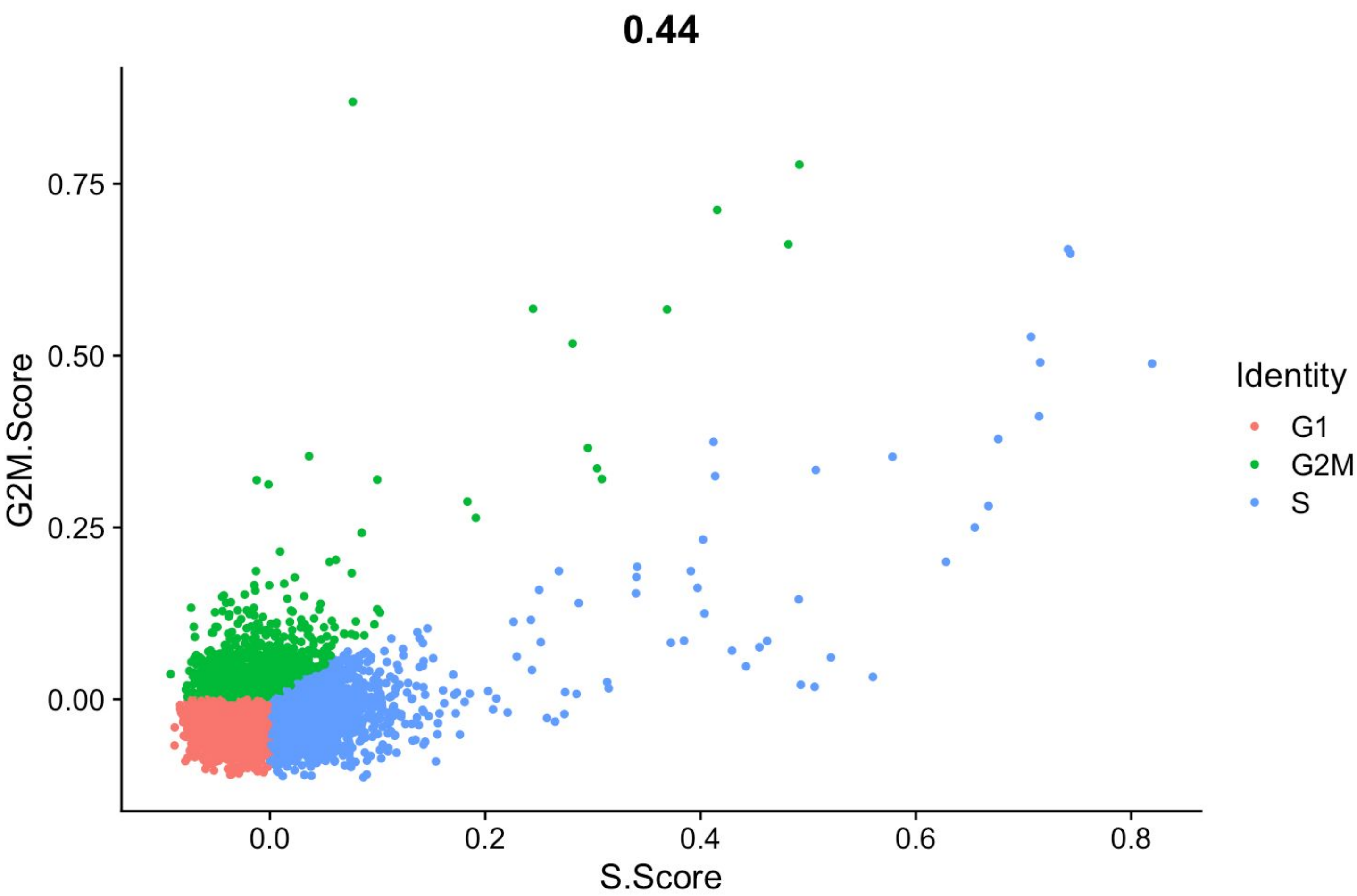


Buettner et al. Nature Biotech. 2019

Predict cell cycle stage / scores

- Seurat – **CellCycleScoring** – builds on G2M- & S-phase human gene lists from Tirosh et al. paper
- Scran – **cyclone** function – trained on mouse cell cycle sorted cells. Uses relative expression of pairs of genes.
- Scanpy - **tl.score_genes_cell_cycle** – uses same gene list as Seurat

OBS! Seurat ”Phase” predictions use a fixed cutoff.




```
FeatureScatter(data, "S.Score","G2M.Score", group.by =
"Phase")
```

Cell cycle removal

- Regression on cell cycle scores.
  - Either with S.Score and G2M.Score
  - Or with Diff = S.Score – G2M.Score
- scLVM - Designed for cell-cycle variation correction. Also has correction of other confounding variables.
- ccRemover (stable version from CRAN). “ccRemover outperforms scLVM slightly.”
- Oscope
- reCAT

Selecting genes

- Excluding invariable genes that do not contribute informative/interesting information
  - Improved signal to noise ratio
  - Reduced computational requirements

- Highly variable genes (HVGs)
- Correlated gene pairs/groups
- Top PCA loadings

Variable gene selection

- **Genes which behave differently from a null model describing technical noise**
  - Mean-variance trend: genes with higher than expected variance
  - Coefficient of variation (Brennecke et al. 2013)
- **High dropout genes**
  - Number of zeros unexpectedly high compared to null model

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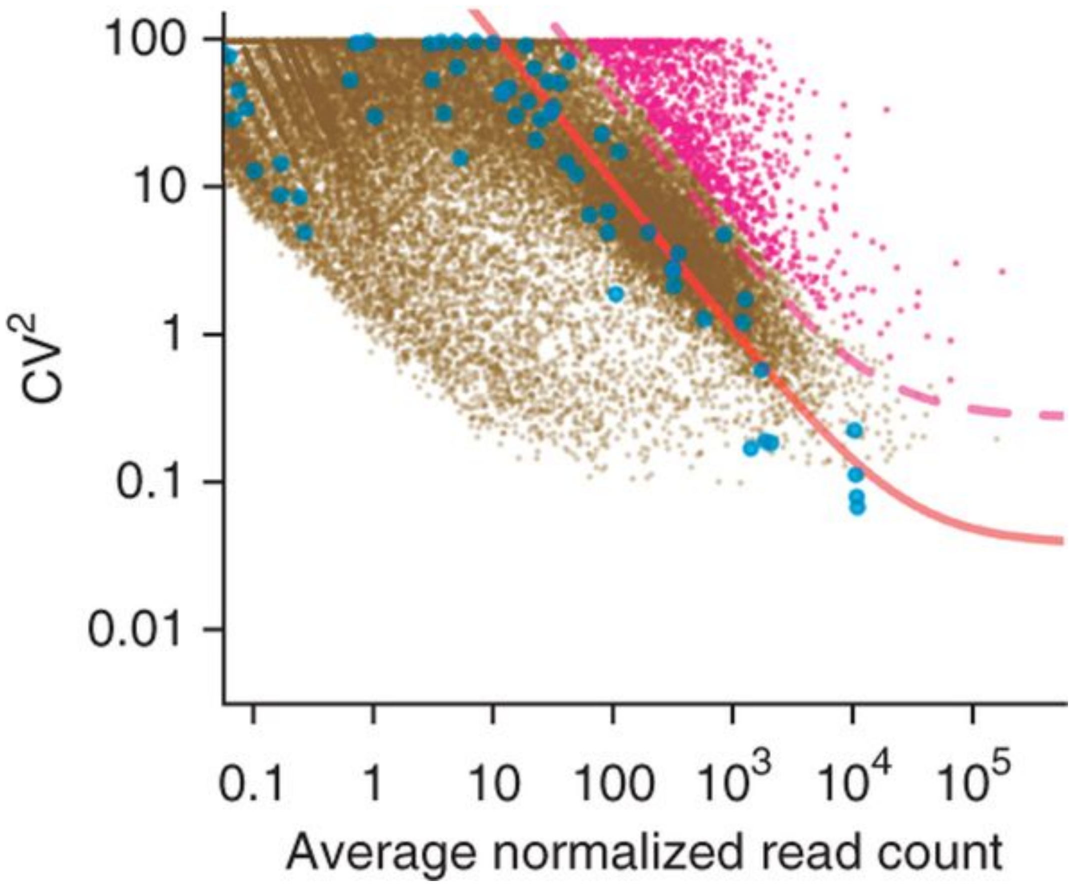
Highly variable genes (HVGs)



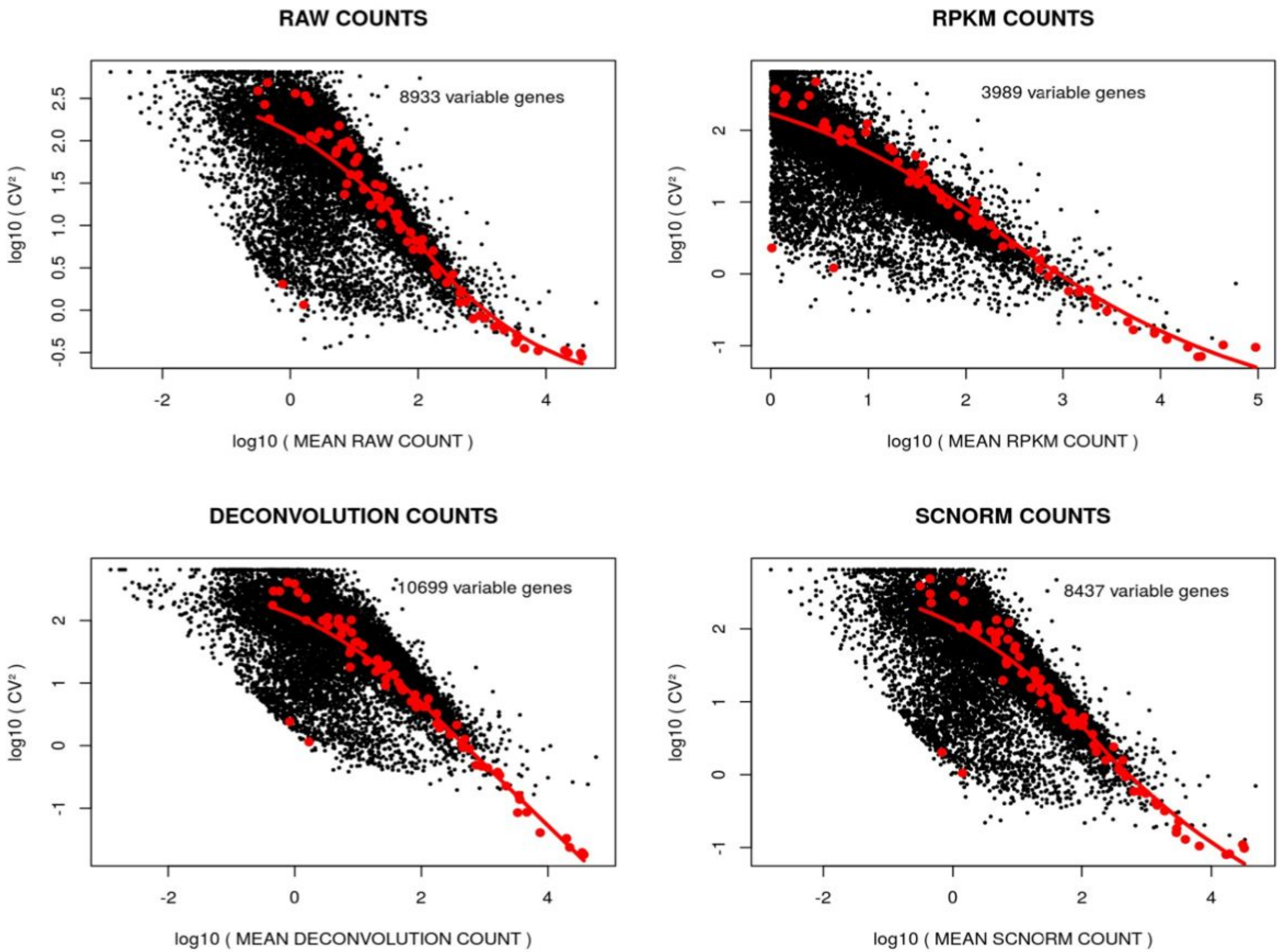
$$CV = \frac{var}{mean} = \frac{\sigma}{\mu}$$

Fit a gamma generalized linear model

No ERCCs?
-> estimate technical noise based on all genes

(Brennecke et al. *Nature Methods* 2013)

HVGs with spike-in controls – normalization matters



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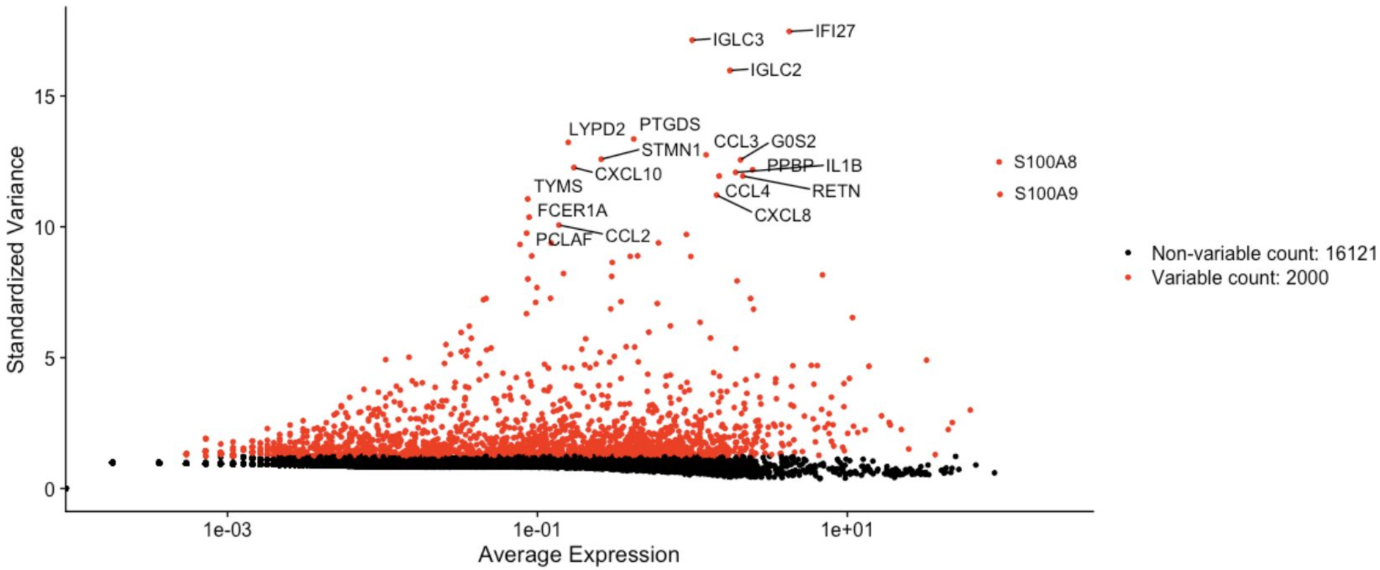
Varaiable gene selection in practise:

- Seurat: FindVariableFeatures
- Fits a line to the relationship of log(variance) and log(mean) using local polynomial regression (loess). Then standardizes the feature values using the observed mean and expected variance. Feature variance is then calculated on the standardized values after clipping to a maximum.



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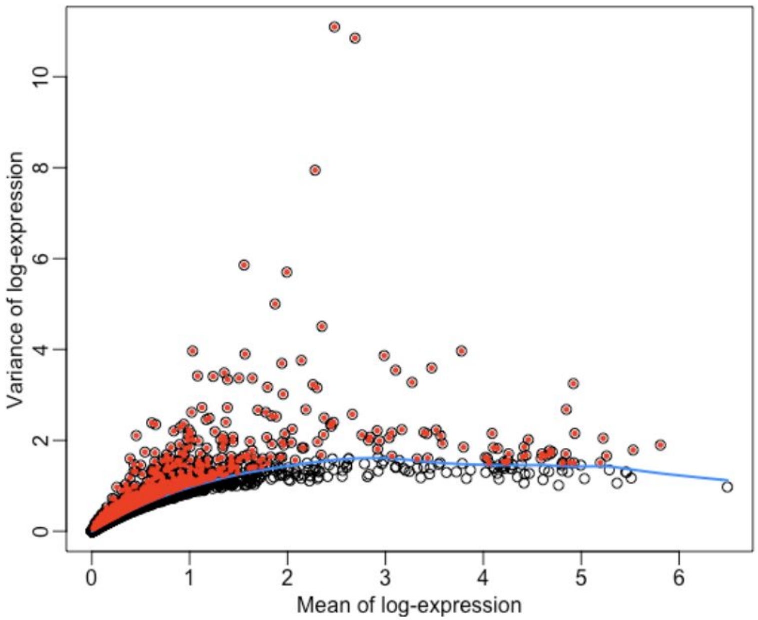
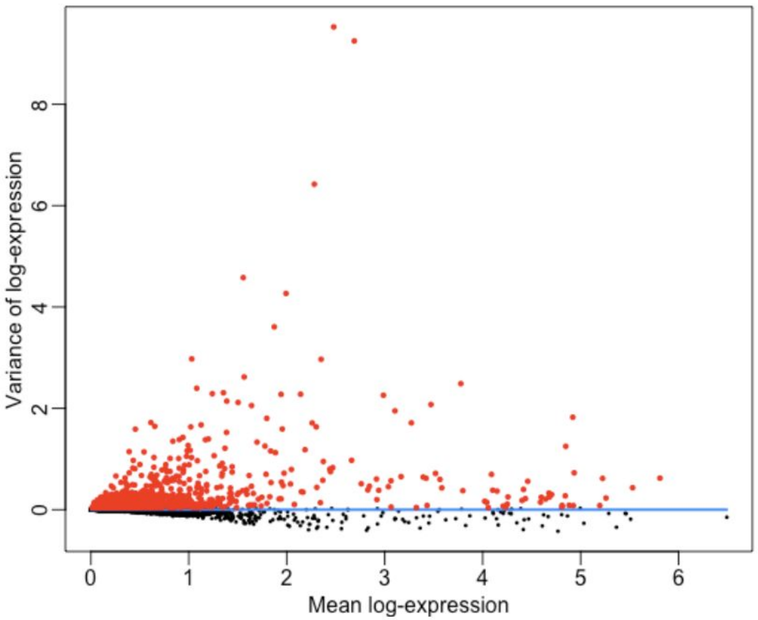
Varaiable gene selection in practise:

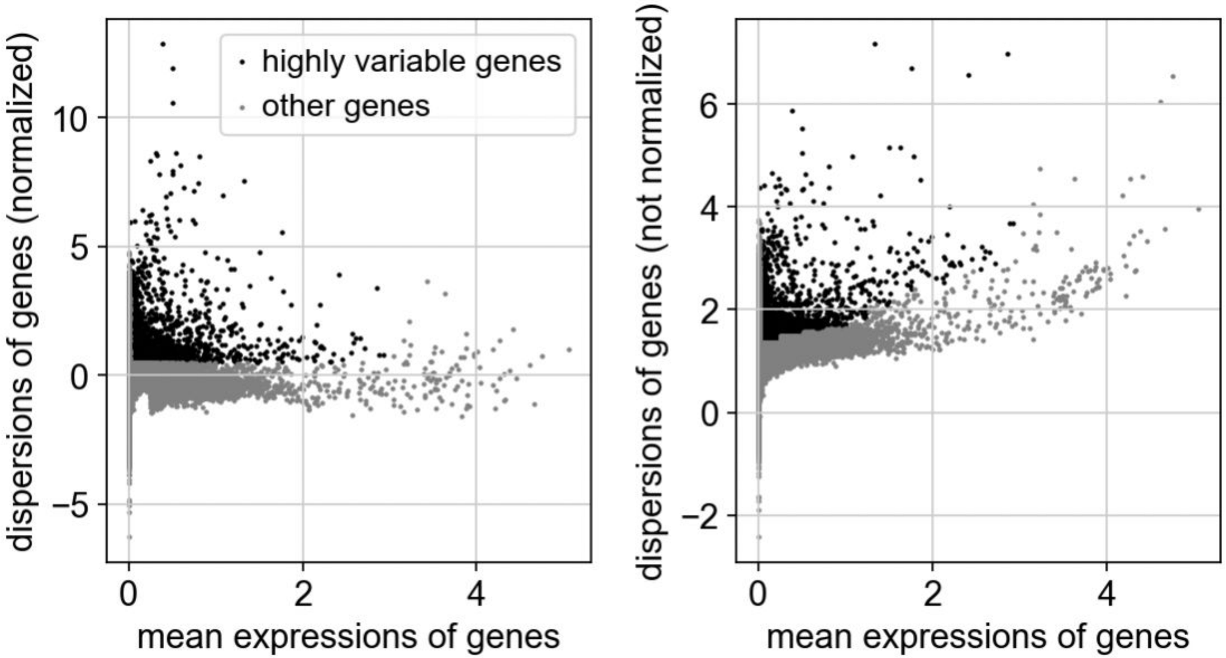
- Scran: ModelGeneVar & getTopHVGs
- Model the variance of the log-expression profiles for each gene, decomposing it into technical and biological components based on a fitted mean-variance trend.
- Can include blocking parameters in “design”.





Varaiable gene selection in practise:

- Scanpy: sc.pp.highly_variable_genes
- Implements same method as Seurat
- Can specify "batch_key" and calculate per batch then combine the values.



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Conclusions

- Normalization has impact on differential gene expression.
- Many different methods to remove unwanted variance – often an important step!
- Selection of variable genes is important to remove noise in the data. Always subset genes before running PCA/clustering.
- Always aim for same sequencing depth in all samples – to avoid at least one confounding factor.

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Do not worry!

If you have distinct celltypes – the clustering will be the same regardless of how you treat the data.

But, for subclustering of similar celltypes normalization and removal of confounders may be crucial.