

# Single cell RNA sequencing

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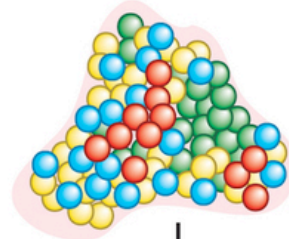
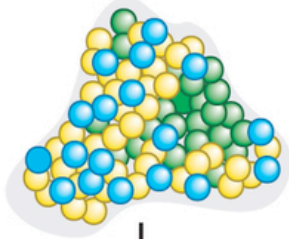
# Outline

- Why single cell gene expression?
- Library preparation methods
- Setting up experiments
- Computational analysis
  - Defining cell types
  - Identifying differentially expressed genes
- Some recent papers

Healthy

Pathological

Tissues

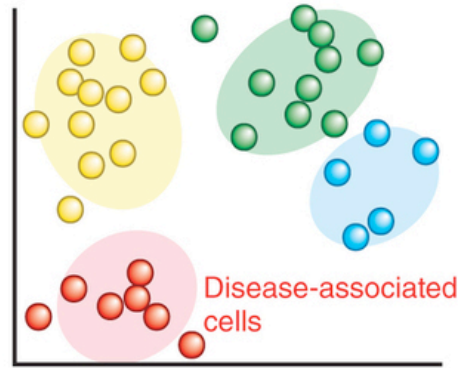
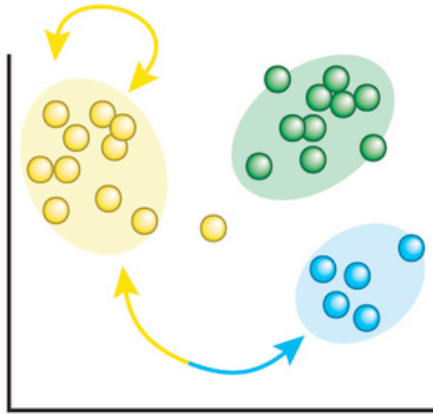


Single-cell RNA-seq



Expression profile clustering

Cell-type maps



Types of analyses



**Within cell type**

- Stochasticity, variability of transcription
- Regulatory network inference
- Allelic expression patterns
- Scaling laws of transcription



**Between cell types**

- Identify biomarkers
- (Post)-transcriptional differences



**Between tissues**

- Cell-type compositions
- Altered transcription in matched cell types

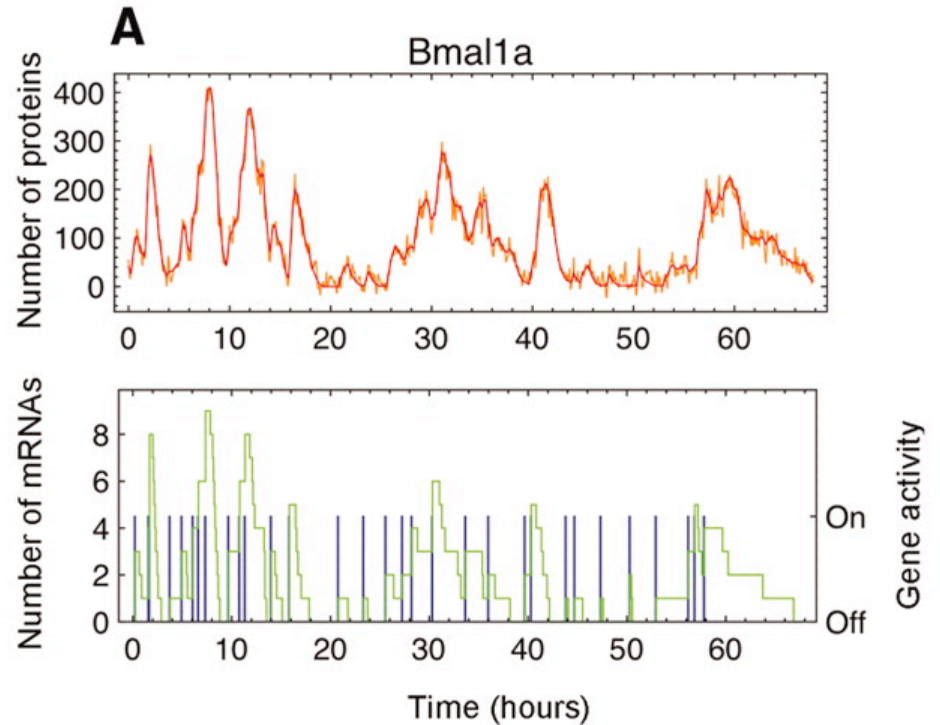
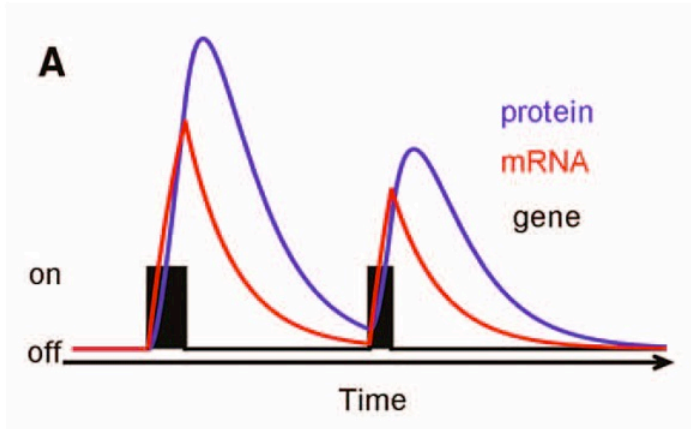
(Sandberg, *Nature Methods* 2014)

# Why single-cell sequencing?

- Understanding heterogeneous tissues
- Identification and analysis of rare cell types
- Changes in cellular composition
- Dissection of temporal changes
- Example of applications:
  - Differentiation trajectories
  - Cancer heterogeneity
  - Neural cell classification
  - Embryonic development
  - Drug treatment response

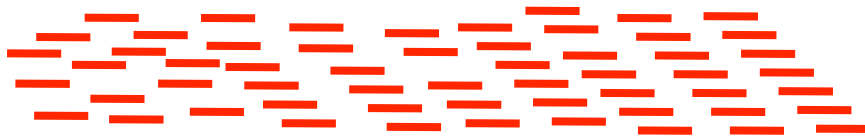


# Transcriptional bursting



- Burst frequency and size is correlated with mRNA abundance
- Many TFs have low mean expression (and low burst frequency) and will only be detected in a fraction of the cells

# scRNA seq methods



SmartSeq2  
(Picelli et al. Nature Methods 2014)



SmartSeq – SMARTer kit  
(Ramsköld et al. Nature Biotech 2012)



Quartz-seq  
(Sasagawa et al. Genome Biology 2013)



Tang et al.  
(Nature methods 2009)



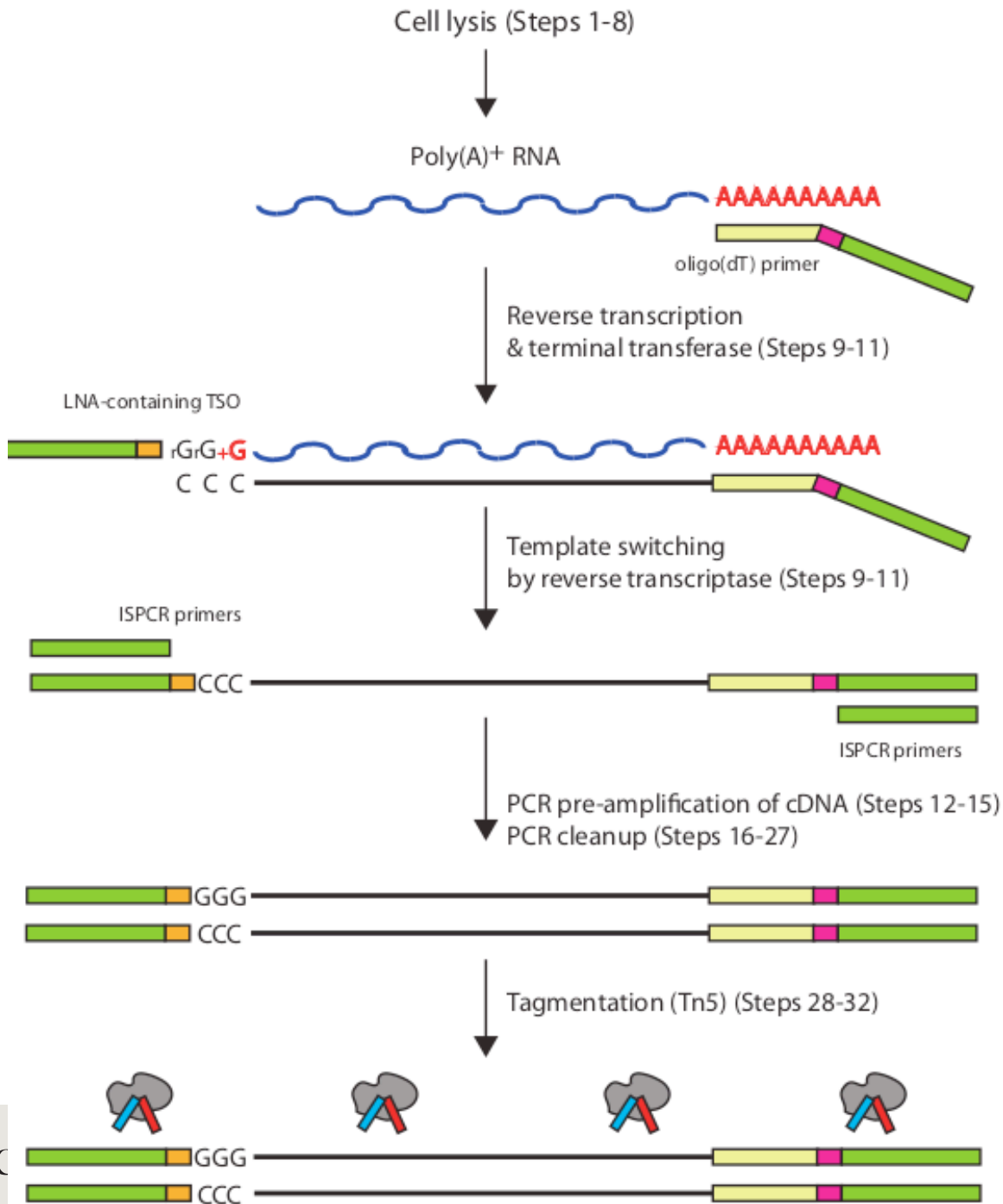
STRT  
(Islam et al. Genome Res 2011)



CEL-Seq  
(Hashimshony et al. Cell Reports 2012)



# SmartSeq2 protocol

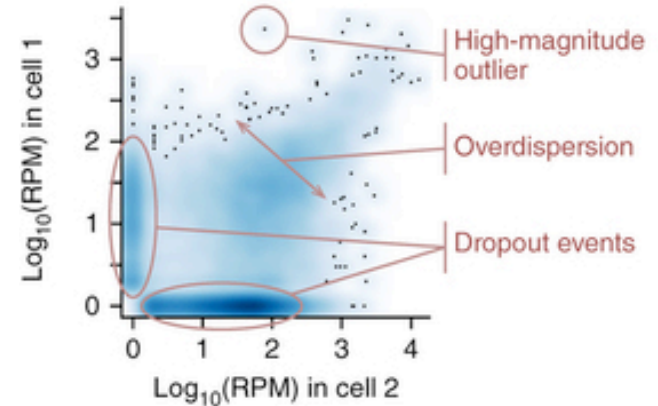


Reverse transcription efficiency limits the detection range – Drop outs

(Picelli et al. *Nature Protocols*, 2014)

# Problems compared to bulk RNA-seq

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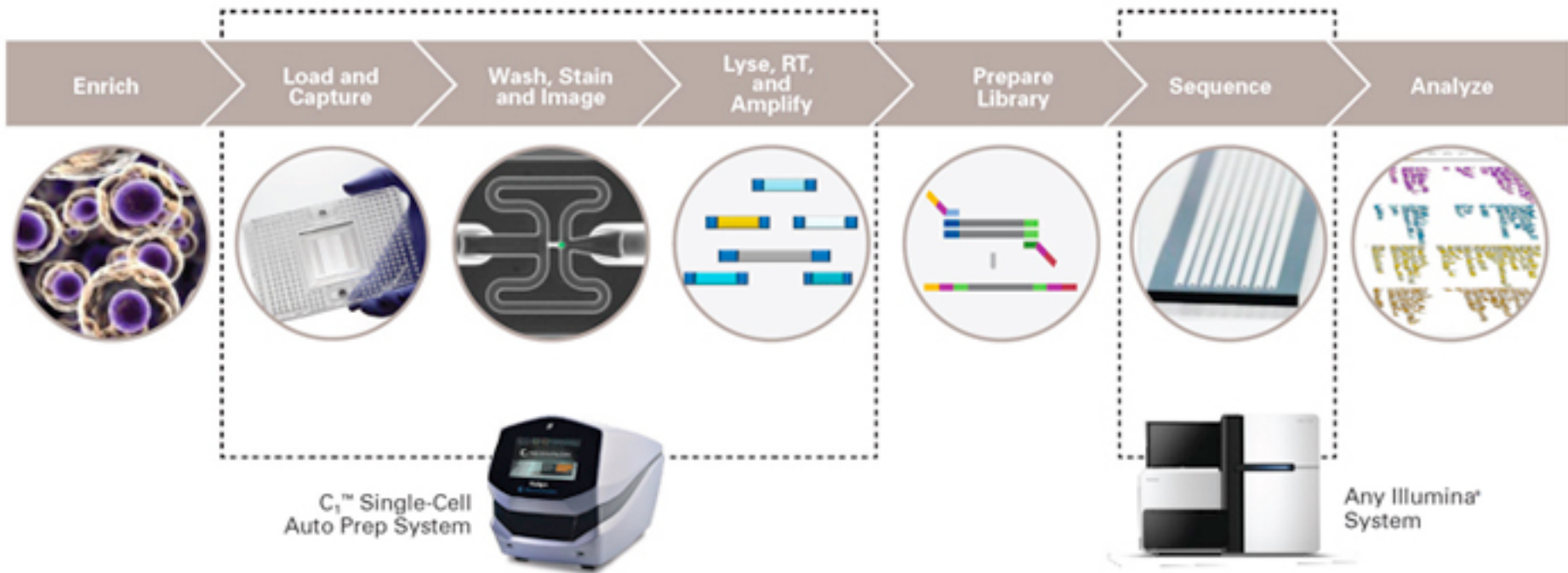


- Amplification bias
- Drop-out rates
- Stochastic gene expression
- Background noise
- Bias due to cell-cycle, cell size and other factors
- As of now, only polyA transcripts, no method for total RNA sequencing in single cells

(Karchenko et al. *Nature Methods* 2014)

# Isolating single cells

- FACS sorting
- Manual picking
- Fluidigm C1 system
- Dissociation of tissue is a crucial step to minimize leakage and RNA degradation, different depending on tissue type.
- Cell types that are hard to dissociate:
  - Laser capture microscopy (LCM)
  - Nuclei sequencing



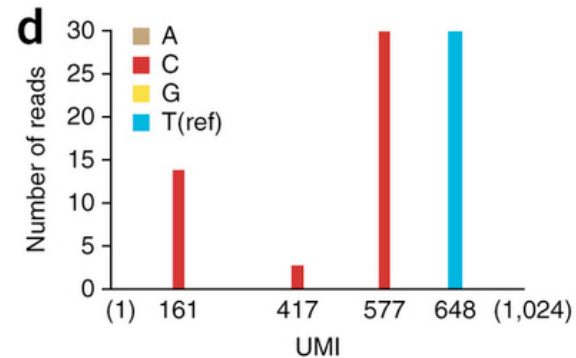
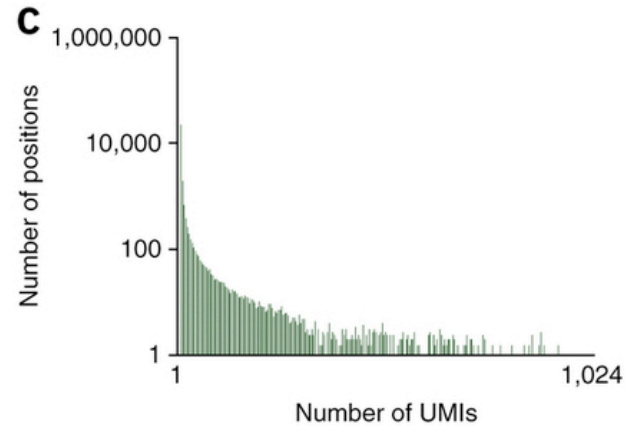
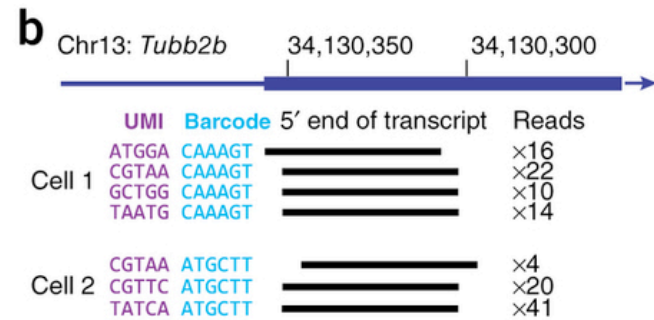
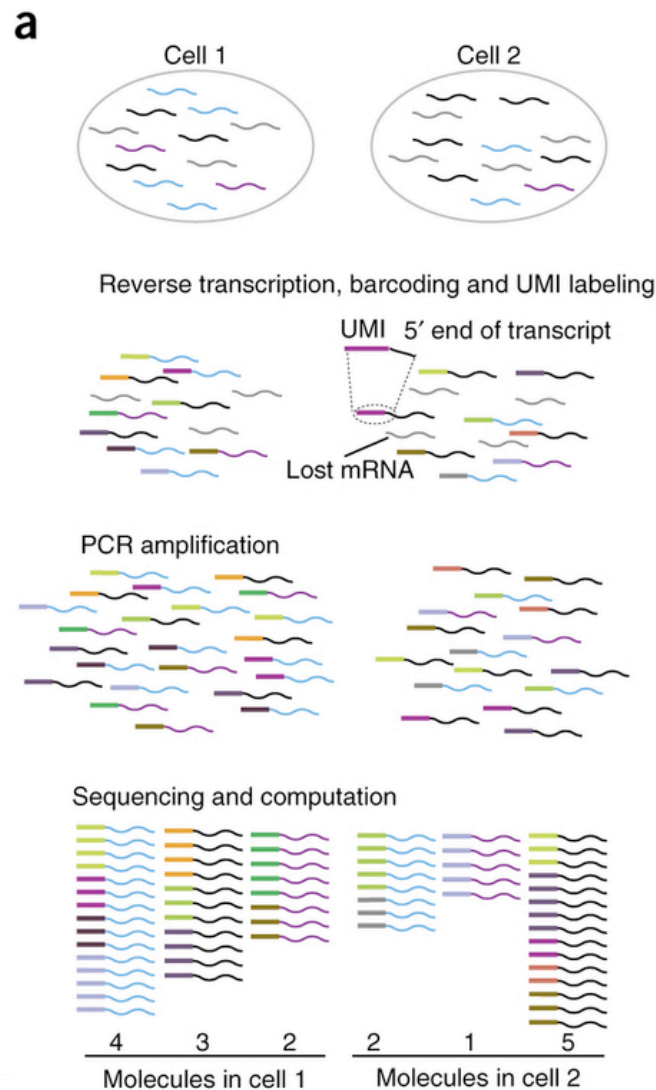
- Fluidigm C1 system

- Limiting factor is size of capture chambers
- Have protocols for running SMARTer, SmartSeq2, CEL-seq & STRT
- Now with 800 cell chips

# Unique molecular identifiers (UMIs) and cellular barcodes

- Cellular barcodes
  - Introduced at RT step with one unique sequence per cell
  - Enables pooling of many libraries into one tube for subsequent steps
- UMIs
  - Introduce random sequences at the beginning of each sequence
  - Reduces effect of amplification bias by removing PCR duplicates
- Implemented with tag-based methods such as STRT and CEL-seq

# Unique molecular identifiers (UMIs) and cellular barcodes



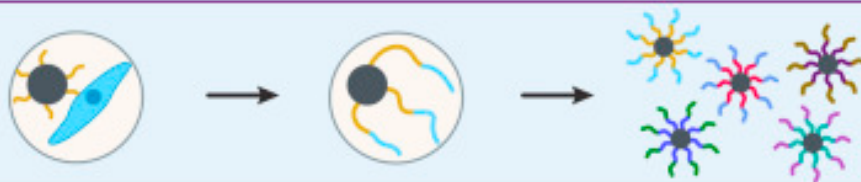
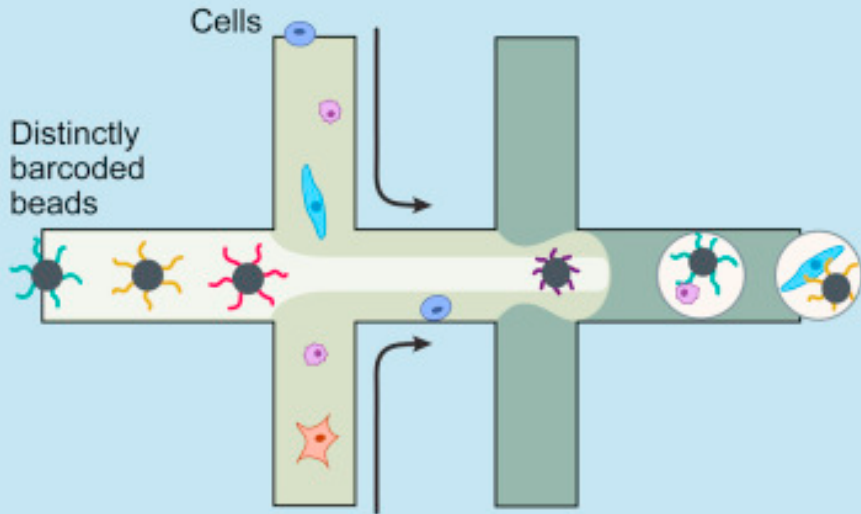


# Small volume approaches

- Volume seem to be a key component in these reactions
  - Smaller volumes give higher detection and better reproducibility
- Smaller volumes = cheaper reagent costs
- Methods for high throughput (1000nds of cells)
- Sequencing cost becomes the bottleneck instead – often shallow sequencing

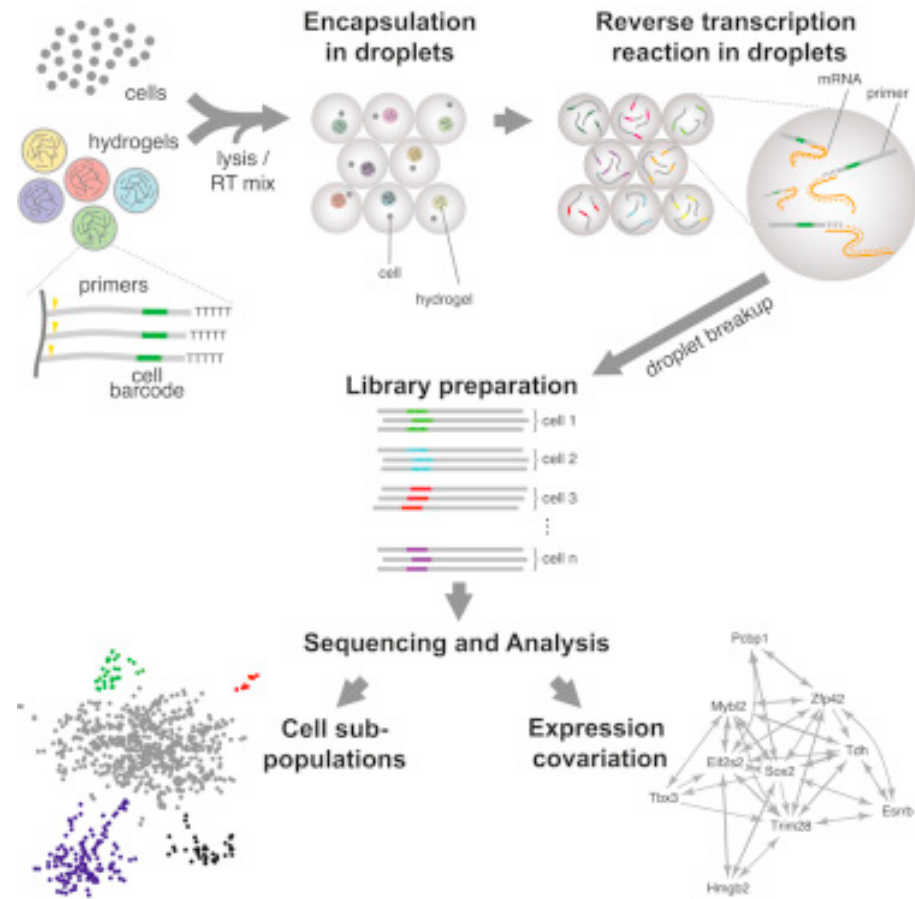
# Droplet / microfluidics approaches

## Drop-seq single cell analysis



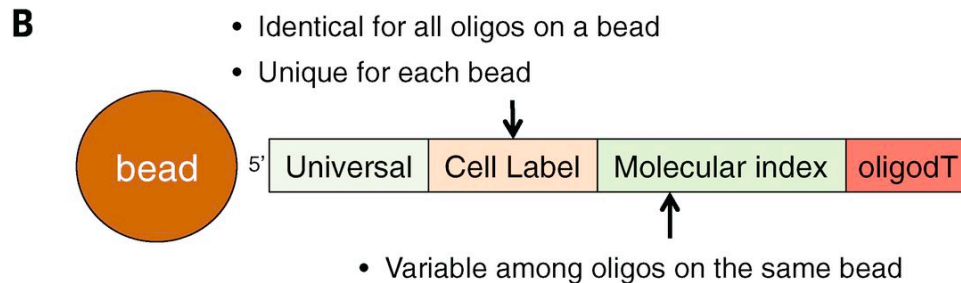
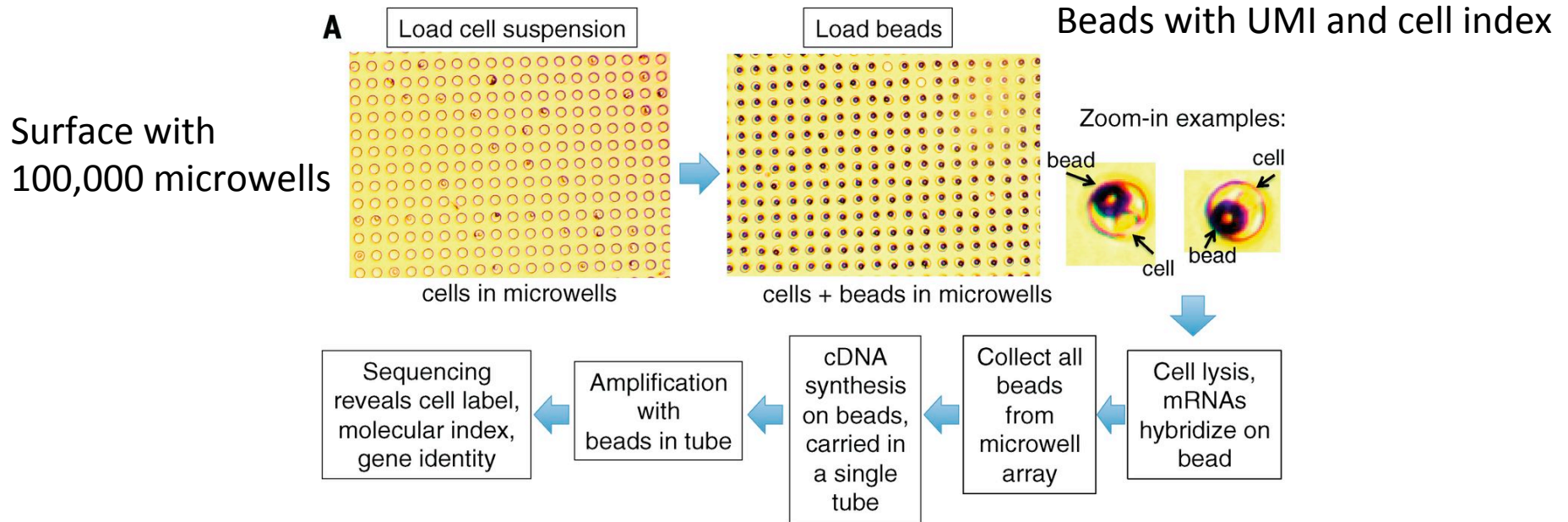
1000s of DNA-barcoded single-cell transcriptomes

Macosko et al. *Cell* 2015  
 McCarroll, Regev etc. Broad/Harvard



Klein et al. *Cell* 2015  
 Kirschner, Weitz etc. Harvard

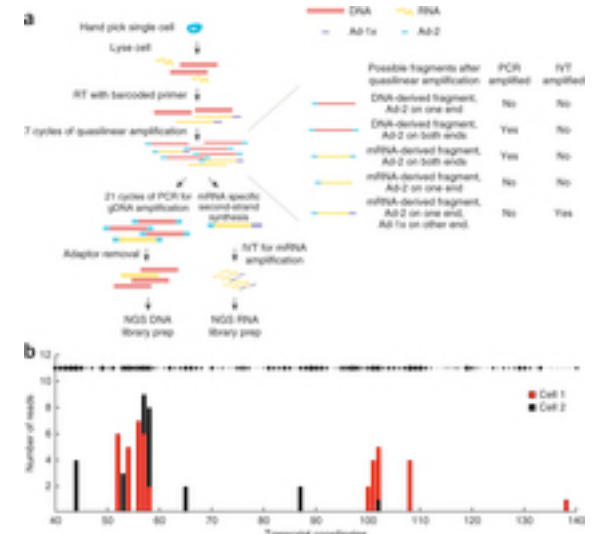
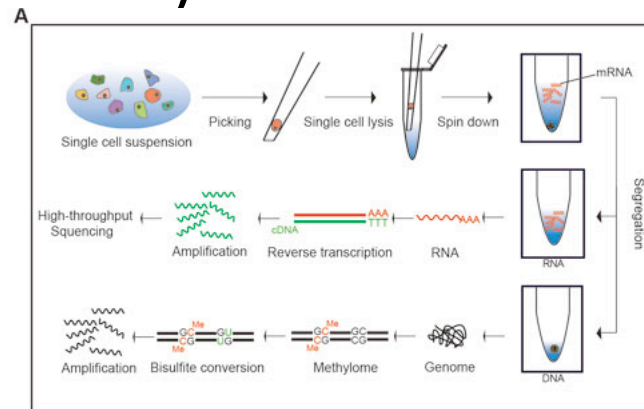
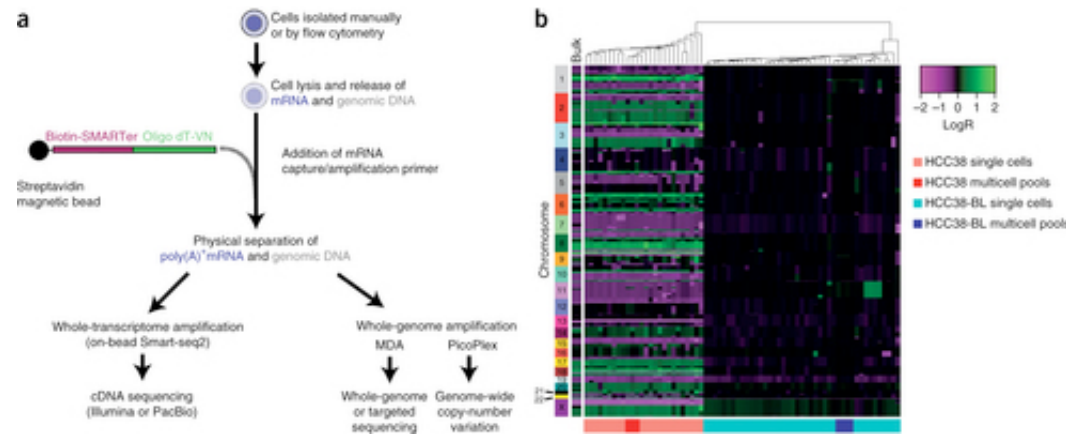
# Cyto-seq method



Presented as targeted sequencing method, but could be used with universal primers.

# Combination with single cell genome sequencing

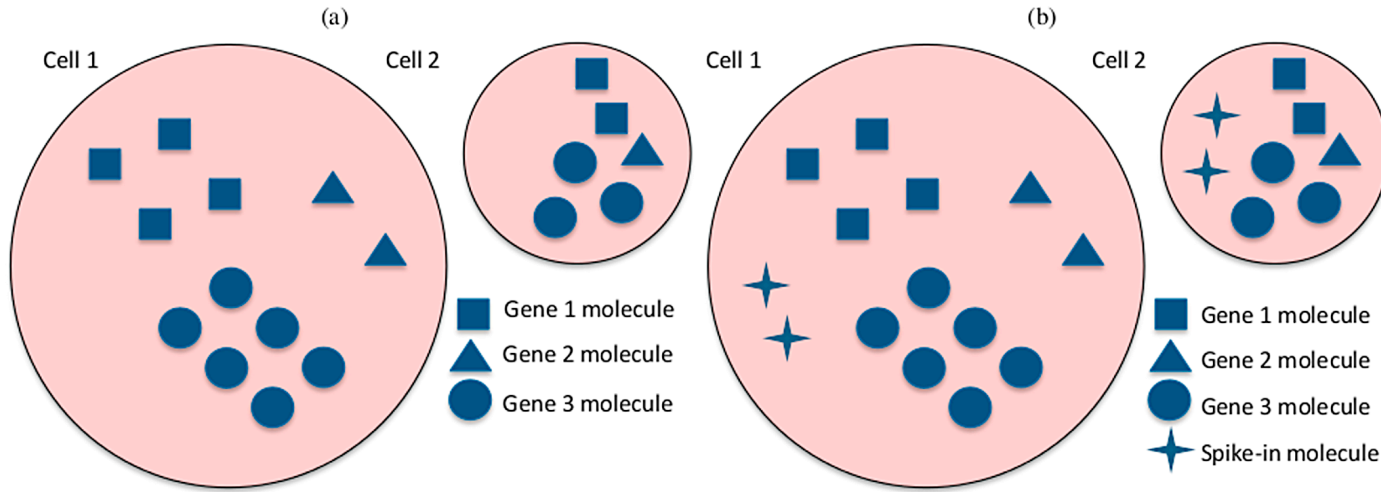
- G&T-seq (Macaulay et al. *Nature Methods* 2015)
- DR-seq (Dey et al. *Nature Biotech* 2015)
- Triple omics (Hou et al. *Cell Research* 2016)



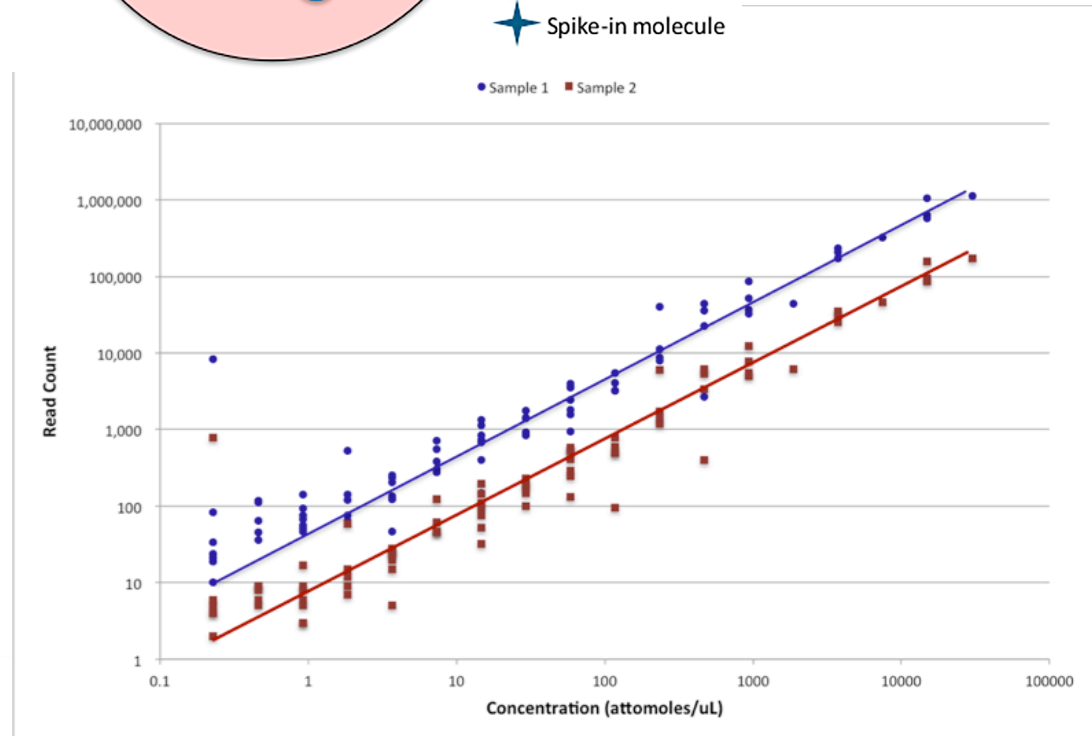
# Spike-in RNAs

- Addition of external controls
- Used to model:
  - technical noise / drop-out rates
  - starting amount of RNA in the cell
- ERCC spike-in most widely used, consists of 48 or 96 mRNAs at 17 different concentrations.
- Add a ratio of about 1:10 to cell RNA.
- Important to add equal amounts to each cell, preferably in the lysis buffer.

# Spike-in RNAs



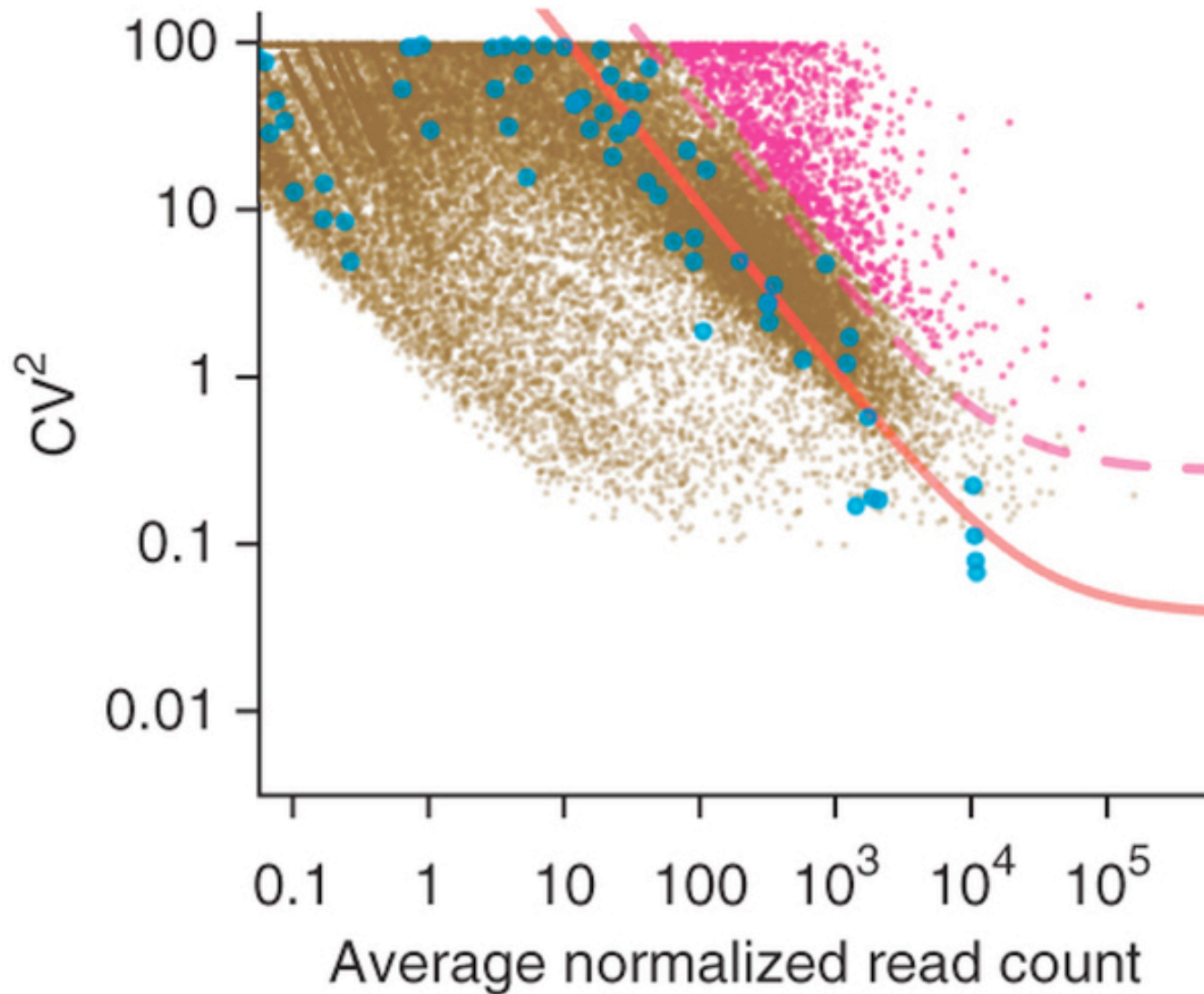
(Vallejos et al. *PLOS Comp Biol* 2015)



(<https://cofactorgenomics.com>)



# Finding biologically variable genes



(Brennecke et al. *Nature Methods* 2013)

# Replicates – how many cells do you have to sequence?

- Recommended to have around 20-30 cells from each cell type
  - A sample with a minor cell type at 5% requires sequencing of 400 cells.
  - Preselecting cells may be necessary, but unbiased cell picking is preferred.
- To study gene expression only, sequencing depth does not have to be deep.
  - Multiplexing of hundreds of samples on one lane is common.
  - For tag-based methods sequencing is often more shallow.
- Possible to have a consultancy session with someone at NBIS for experimental design.



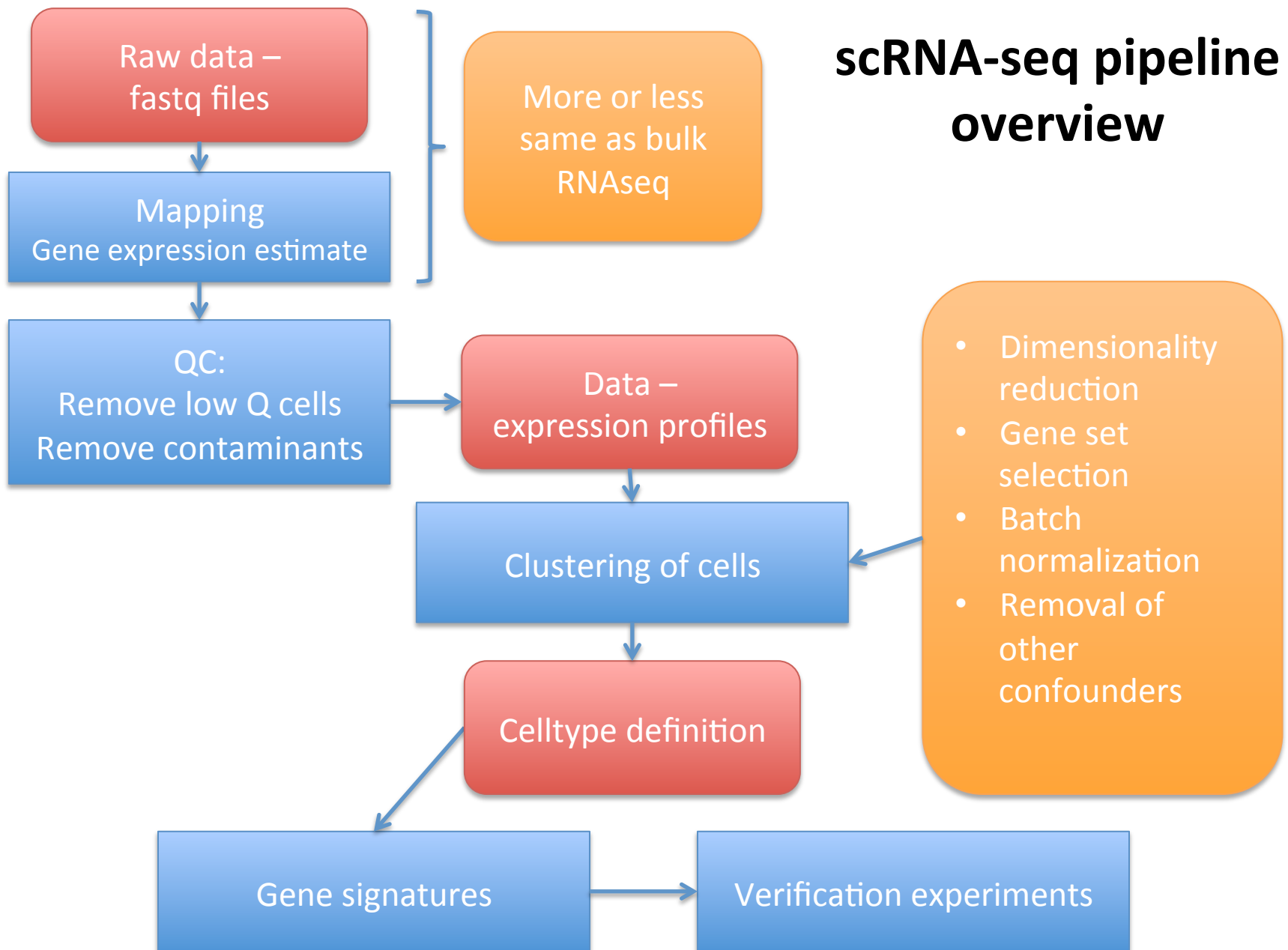
# Which method should I use?

- Full length (SmartSeq2) vs tag-based (CELseq/STRT) methods:
  - Trade-off between throughput and sensitivity
  - Unique molecular identifiers (UMI) implementation with the tag-based methods
- The Single-cell platform currently offers:
  - SmartSeq2 in 384 well format
  - STRT on Fluidigm C1.

# National single cell genomics platform at Scilifelab

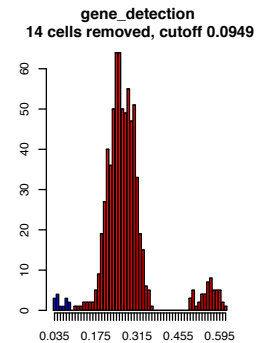
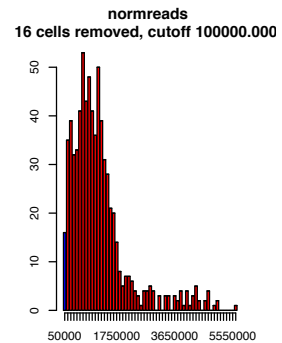
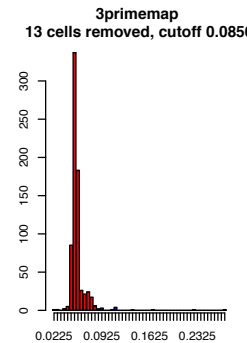
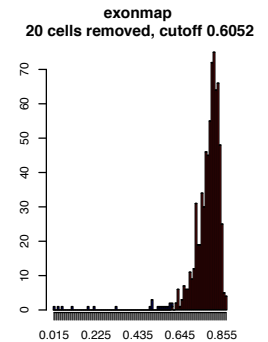
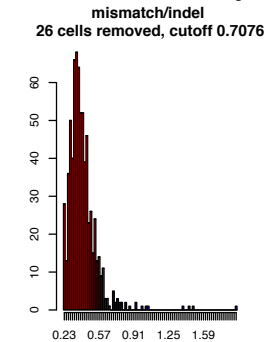
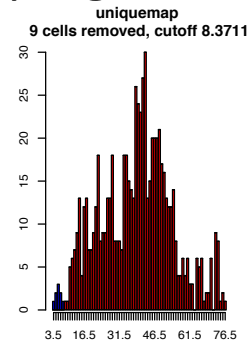
- Uppsala node – microbial single cell genome sequencing
  - <http://www.scilifelab.se/facilities/single-cell/>
  - MDA of whole genomes
  - qPCR of selected target genes
- Stockholm node – eukaryotic single cell RNA / genome sequencing
  - <http://www.scilifelab.se/facilities/eukaryotic-single-cell-genomics/>
  - STRT and cell isolation on Fluidigm C1 system
  - SmartSeq2 on isolated cells on plates
  - MDA whole genome sequencing

# scRNA-seq pipeline overview

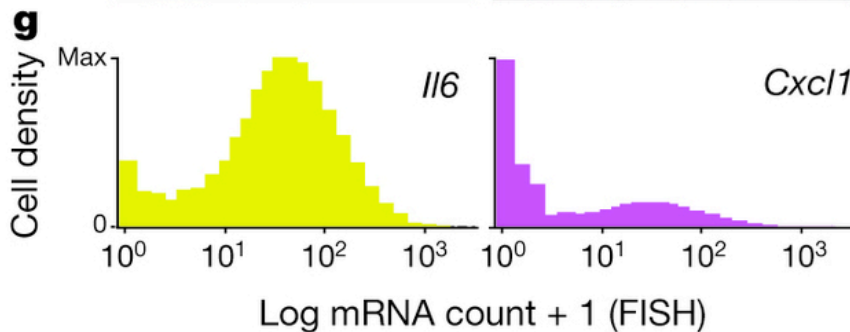
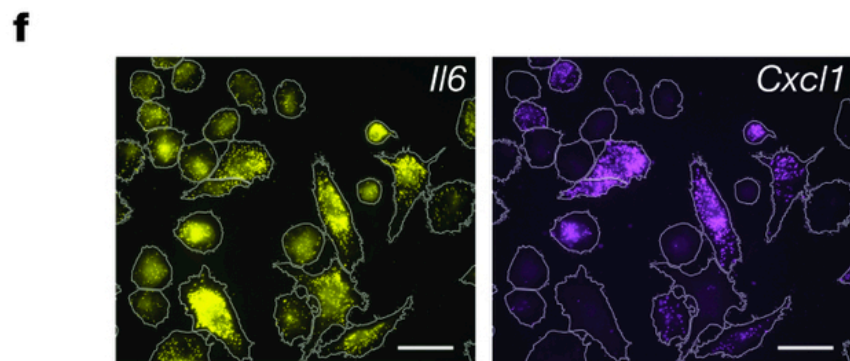
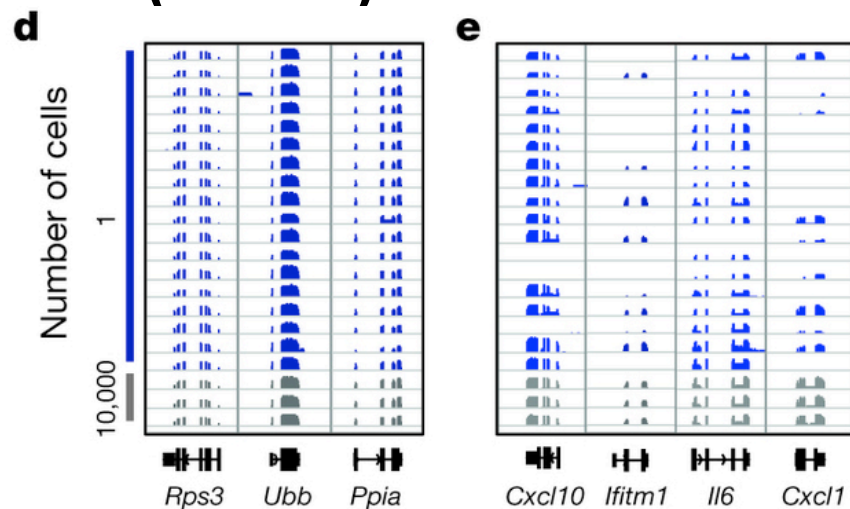
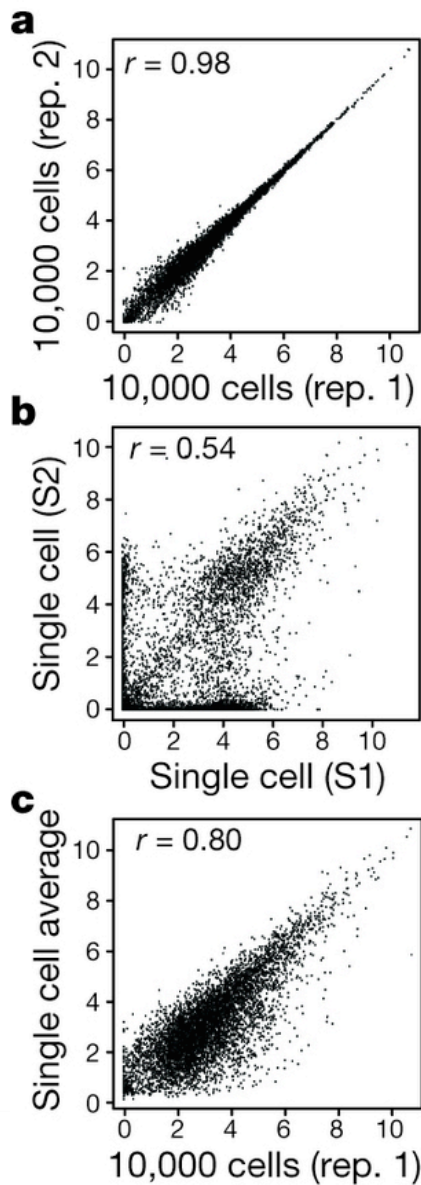


# Quality Control (QC)

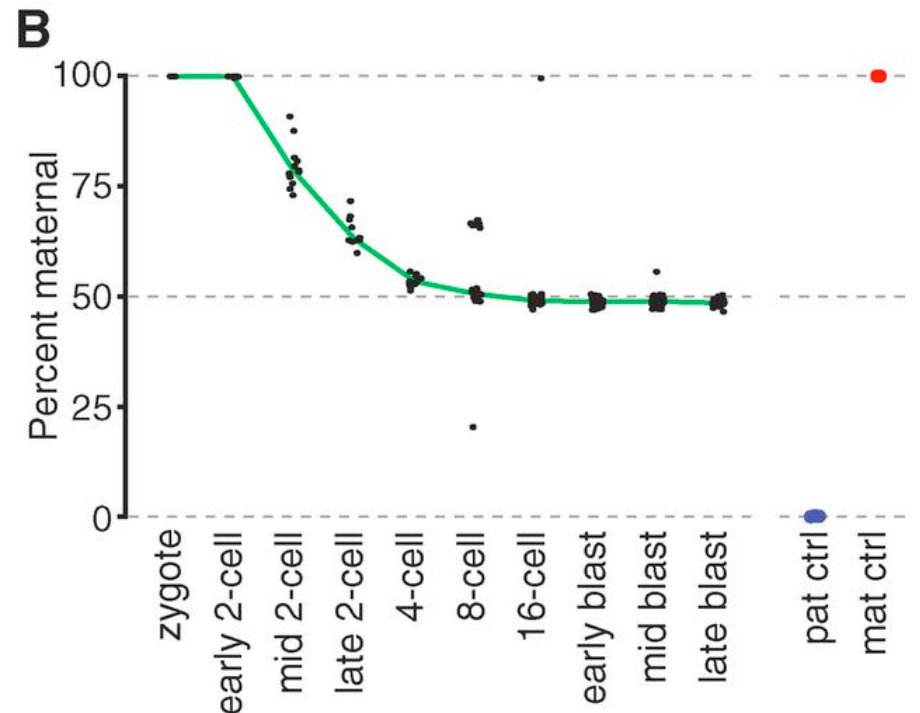
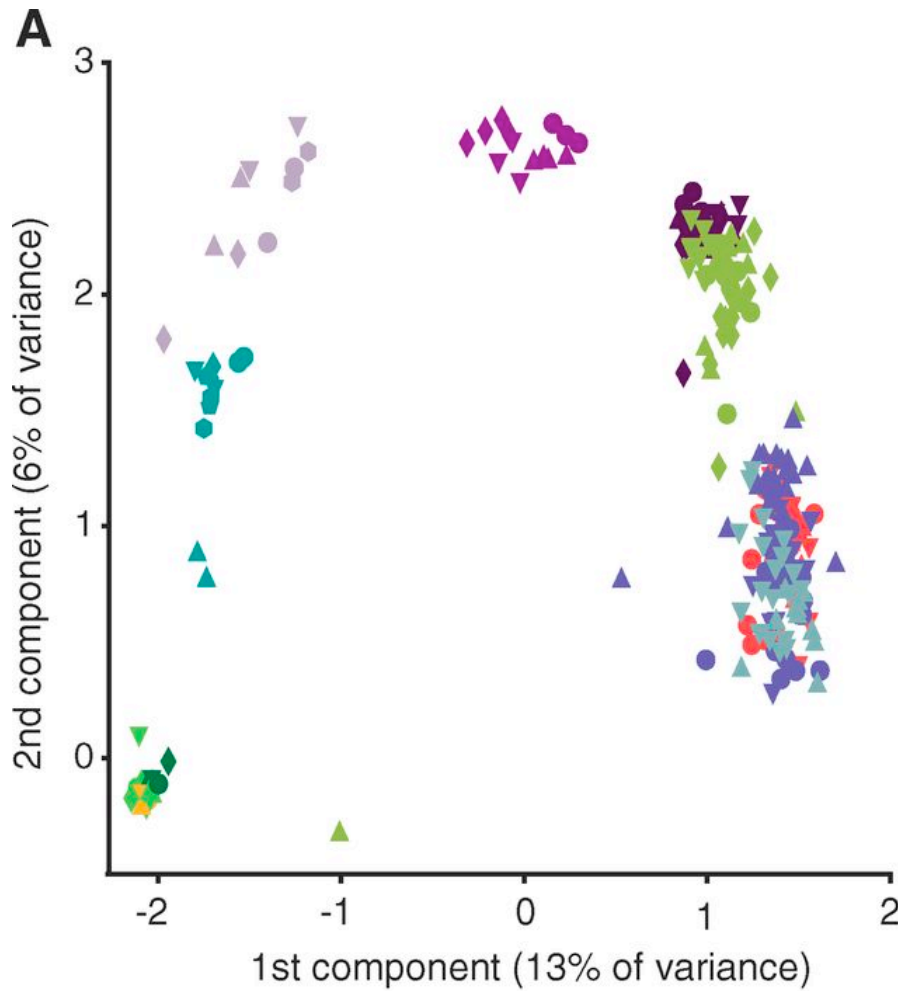
- QC is a crucial step in scRNA-seq - Any experiment will have a number of failed libraries!
- OBS! Smaller celltypes gives lower mapping rates and more primer dimers.
- Look at:
  - Mapping statistics (% uniquely mapping)
  - Mismatch rate
  - Fraction of exon mapping reads
  - 3' bias (degraded RNA)
  - mRNA-mapping reads
  - **Number of detected genes**
  - **Spike-in detection**
  - Mitochondrial read fraction
  - Pairwise correlation to other cells
- Depending on cell type, around 500K exon mapping reads saturates the gene detection (deduced from subsampling).



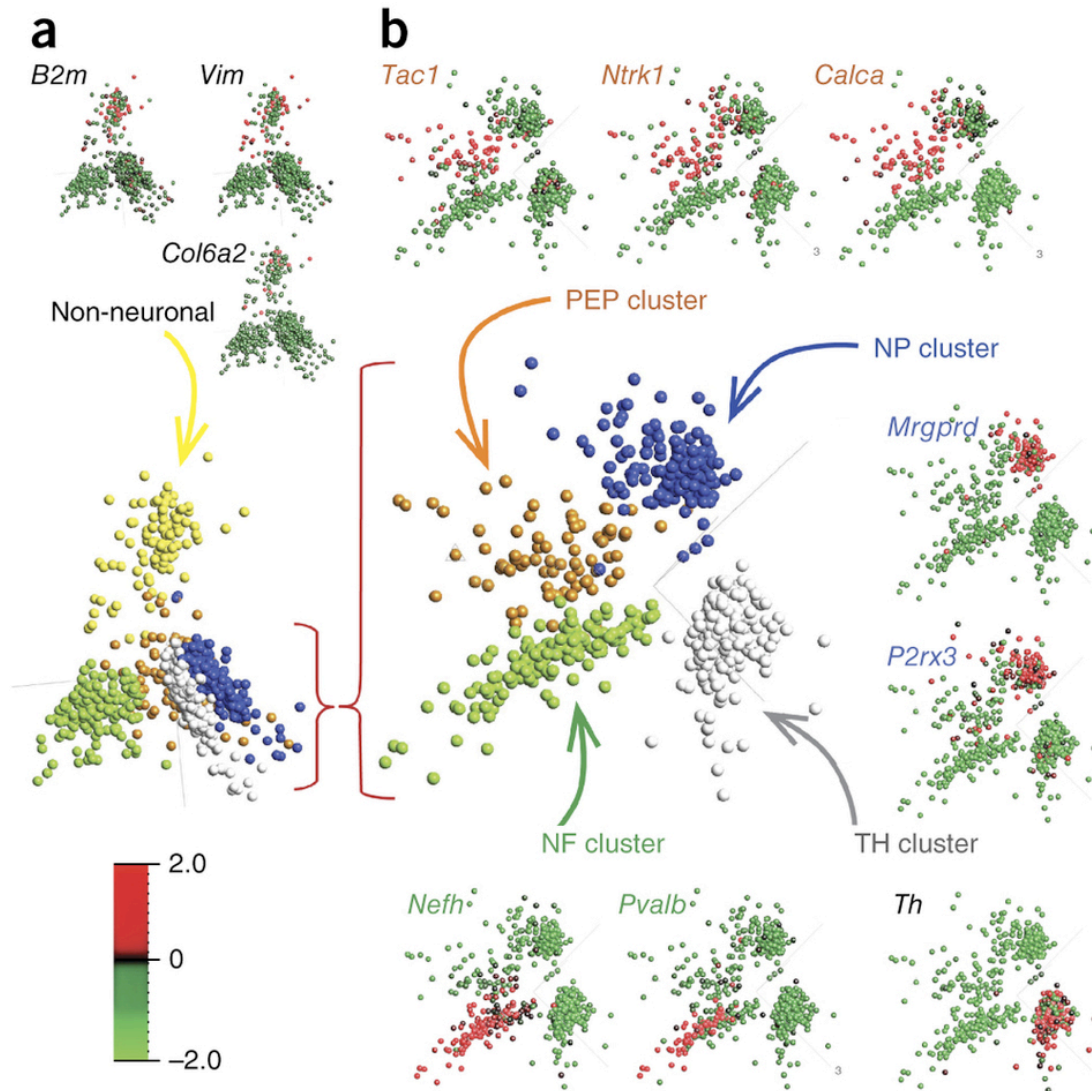
# Example data - mouse bone-marrow-derived dendritic cells (BMDCs)



# Identifying celltypes



# Identifying celltypes





# Identifying celltypes – Dimensionality reduction

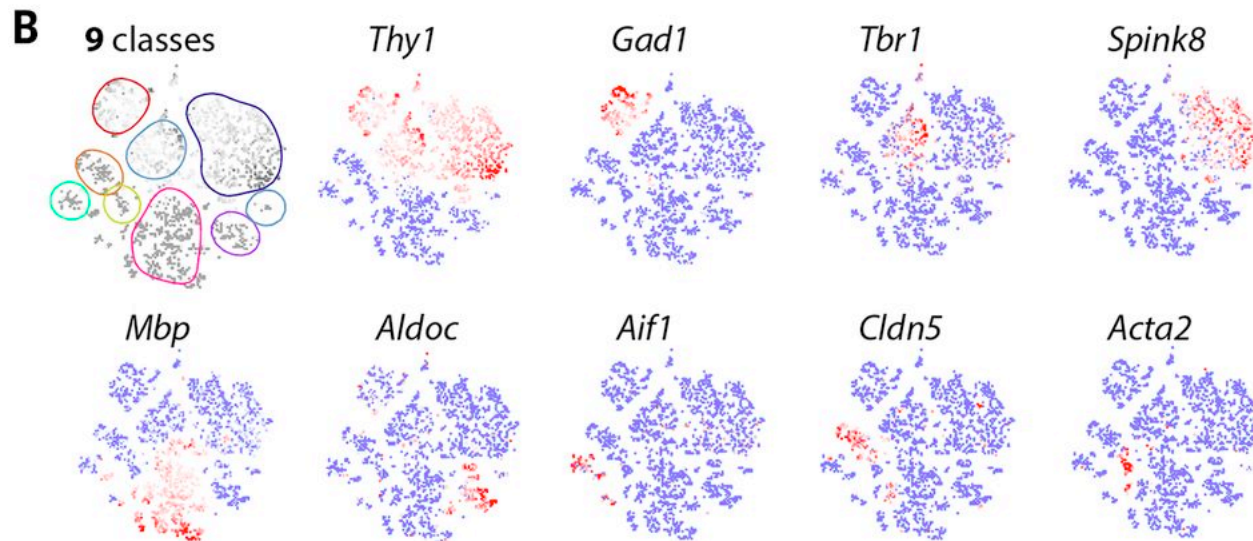
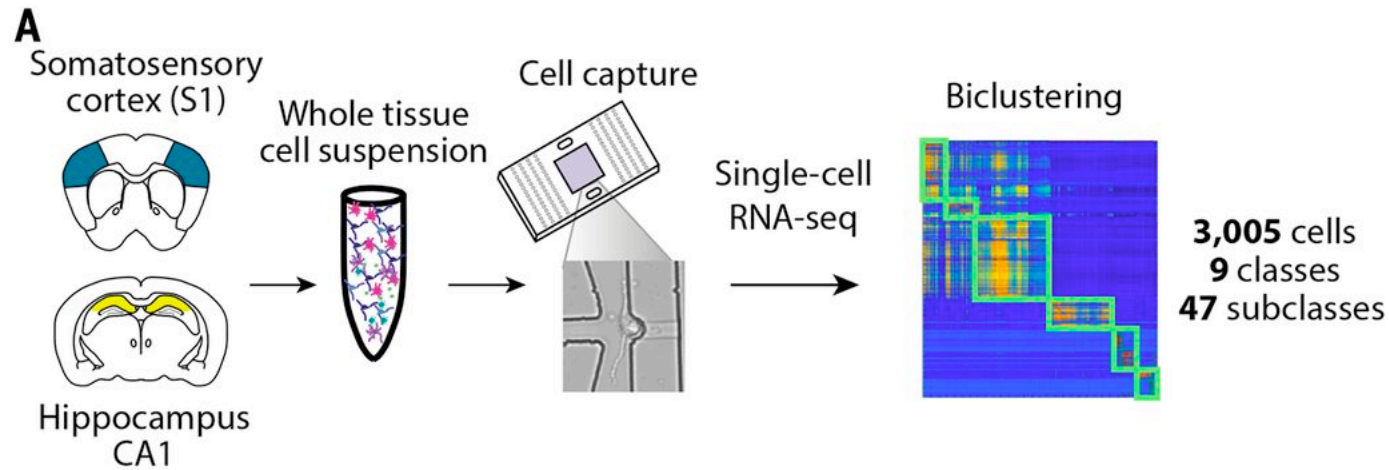
- Many different methods are used:
  - PCA (principal component analysis)
  - ICA (independent component analysis)
  - MDS (multidimensional scaling)
  - Non-linear PCA
  - t-SNE (t-distributed stochastic neighbor embedding)
  - Diffusion maps
  - Network based methods



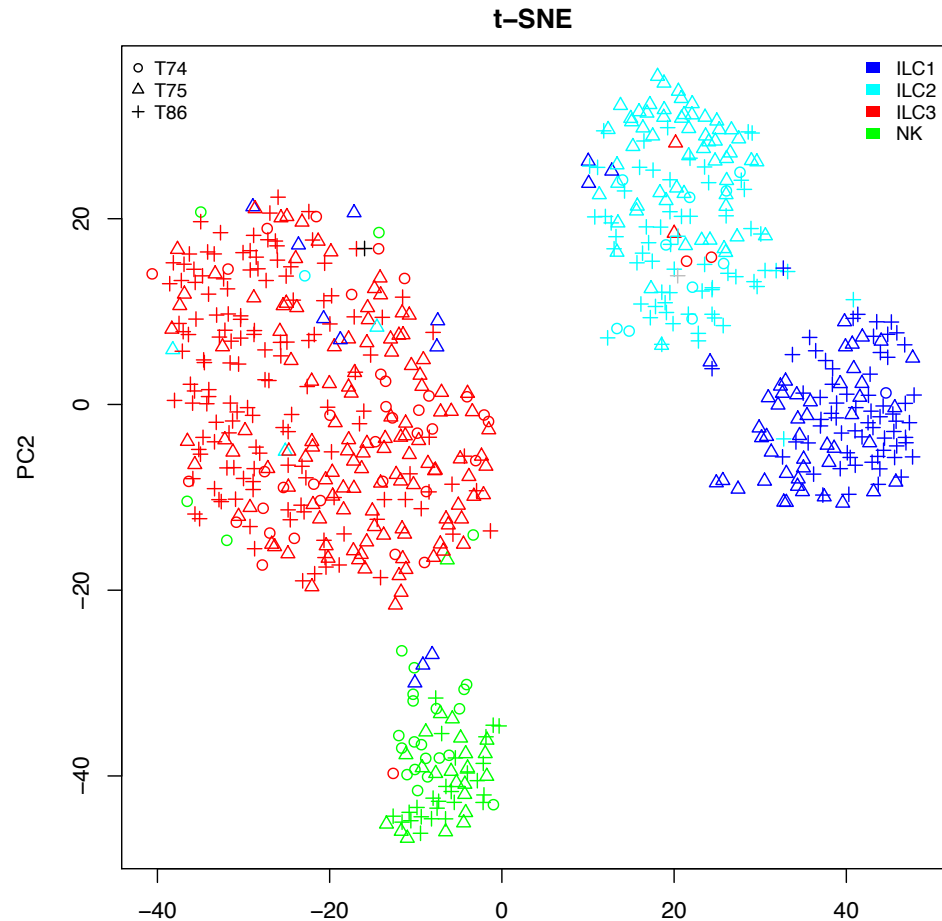
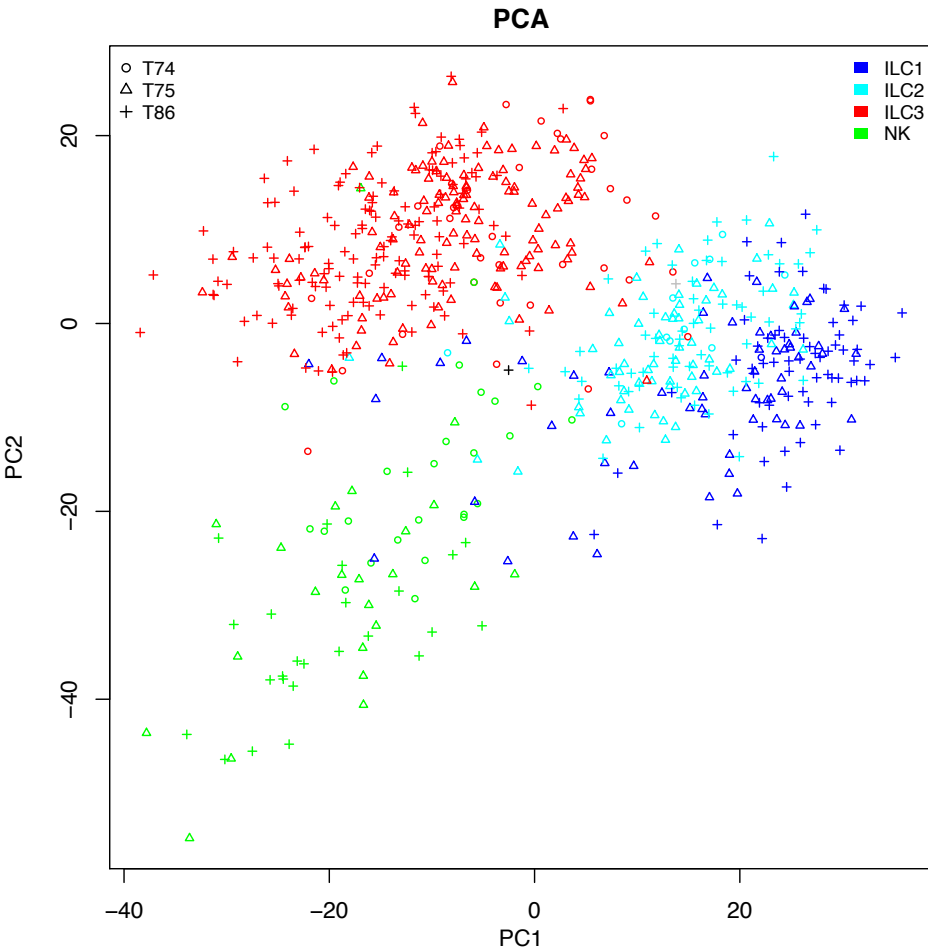
# t-SNE – t-distributed stochastic neighbor embedding

- Method often used in single cell proteomics.
- **Step 1** – probability distribution for all pairs in PCA space with N principal components
- **Step 2** – dimensionality reduction with similar probability distribution and minimization of divergence between distributions
- Implementations in R:
  - tsne
  - Rtsne (Barnes-Hut t-SNE)
- For other languages (python, java, matlab, C++ etc.):
  - <http://lvdmaaten.github.io/tsne/>

# t-SNE – t-distributed stochastic neighbor embedding

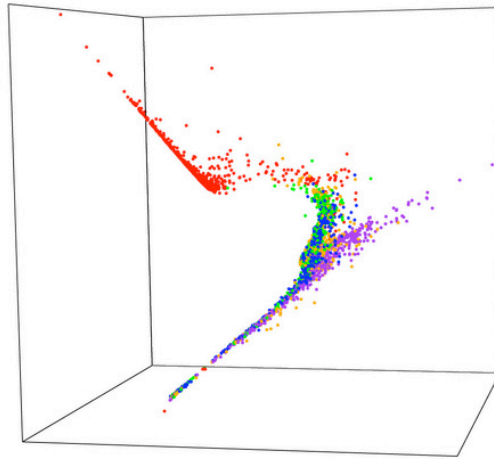


# t-SNE vs PCA dimensionality reduction

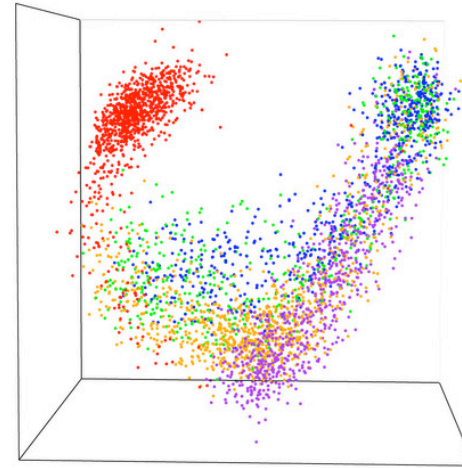


# More dimensionality reductions

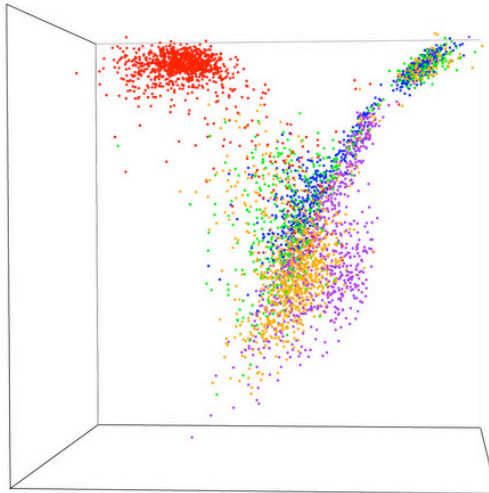
Diffusion map



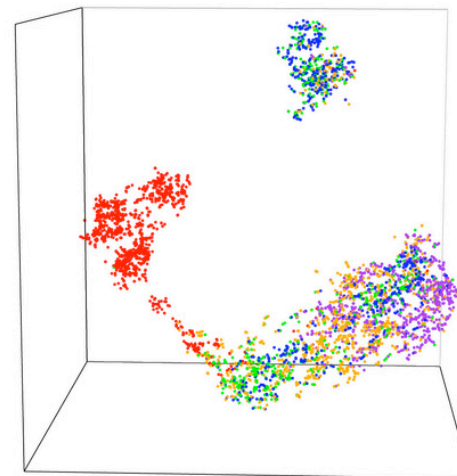
PCA



ICA



tSNE



# Identifying celltypes - Clustering

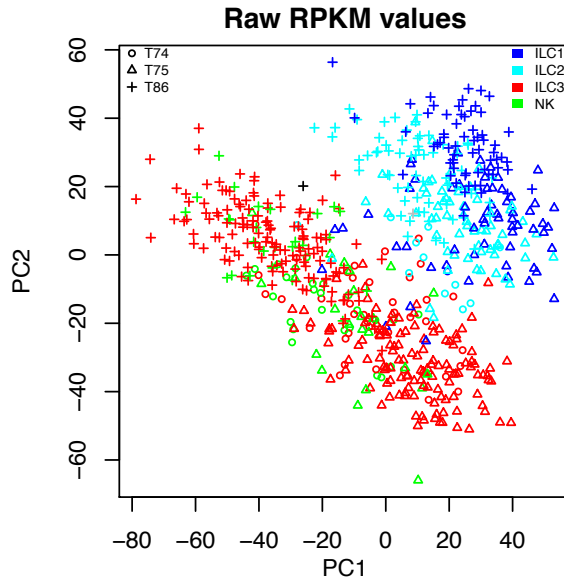
- Clustering based on
  - rpkms/counts – Euklidian distances
  - Pairwise correlations
  - PCA or other dimensionality reduction method
- Method of choice: hierarchical, k-means, biclustering
- Some programs:
  - WGCNA
  - BackSPIN
  - Pagoda
  - DBscan
- OBS! Outlier removal as an initial step may be necessary, especially with PCA-based clustering or similar.

# Data bias

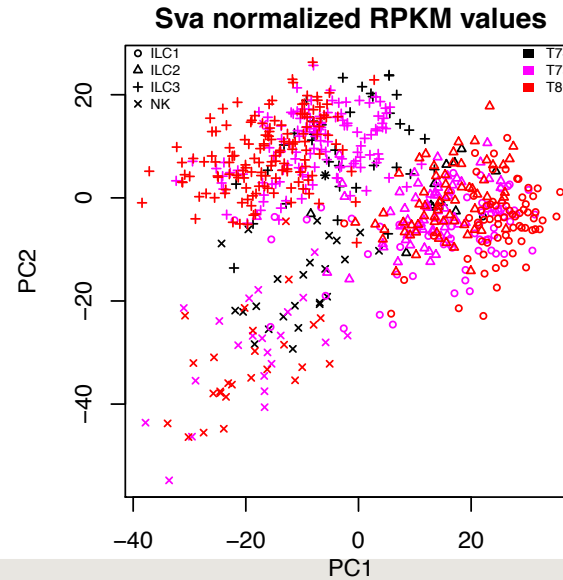
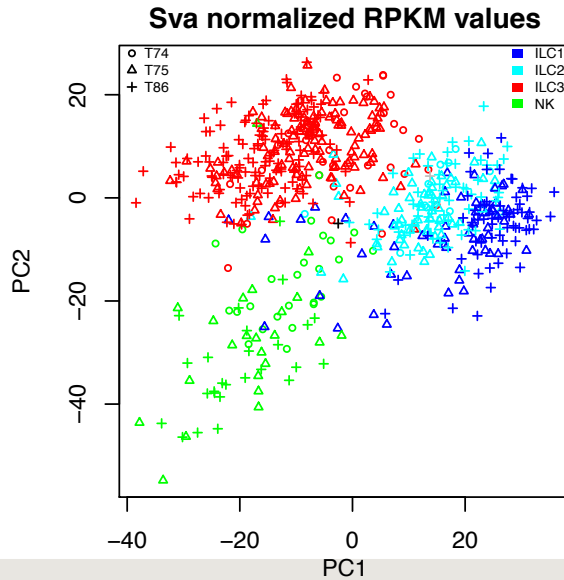
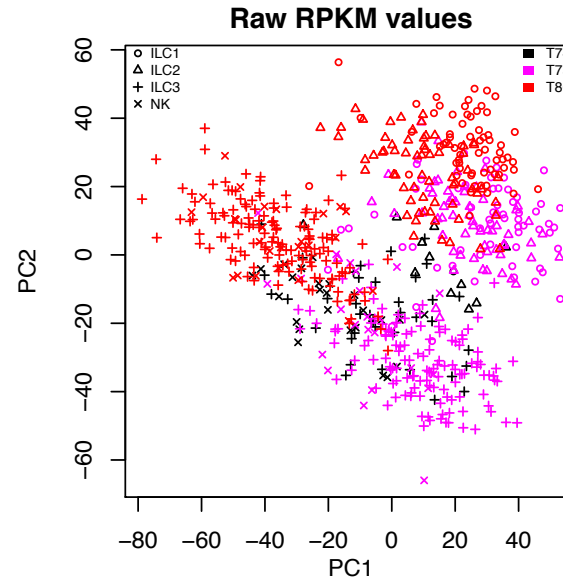
- May need to do data transformation before clustering/PCA
  - Batch effect removal (SVA ComBat function)
  - Remove cell-cycle effects or size bias (scLVM package)
  - RT efficiency / drop-out rate (SCDE package)
  - Technical noise (BASiCS package, GRM, Brenneke method)

# Batch normalization with SVA function ComBat

Color by celltype



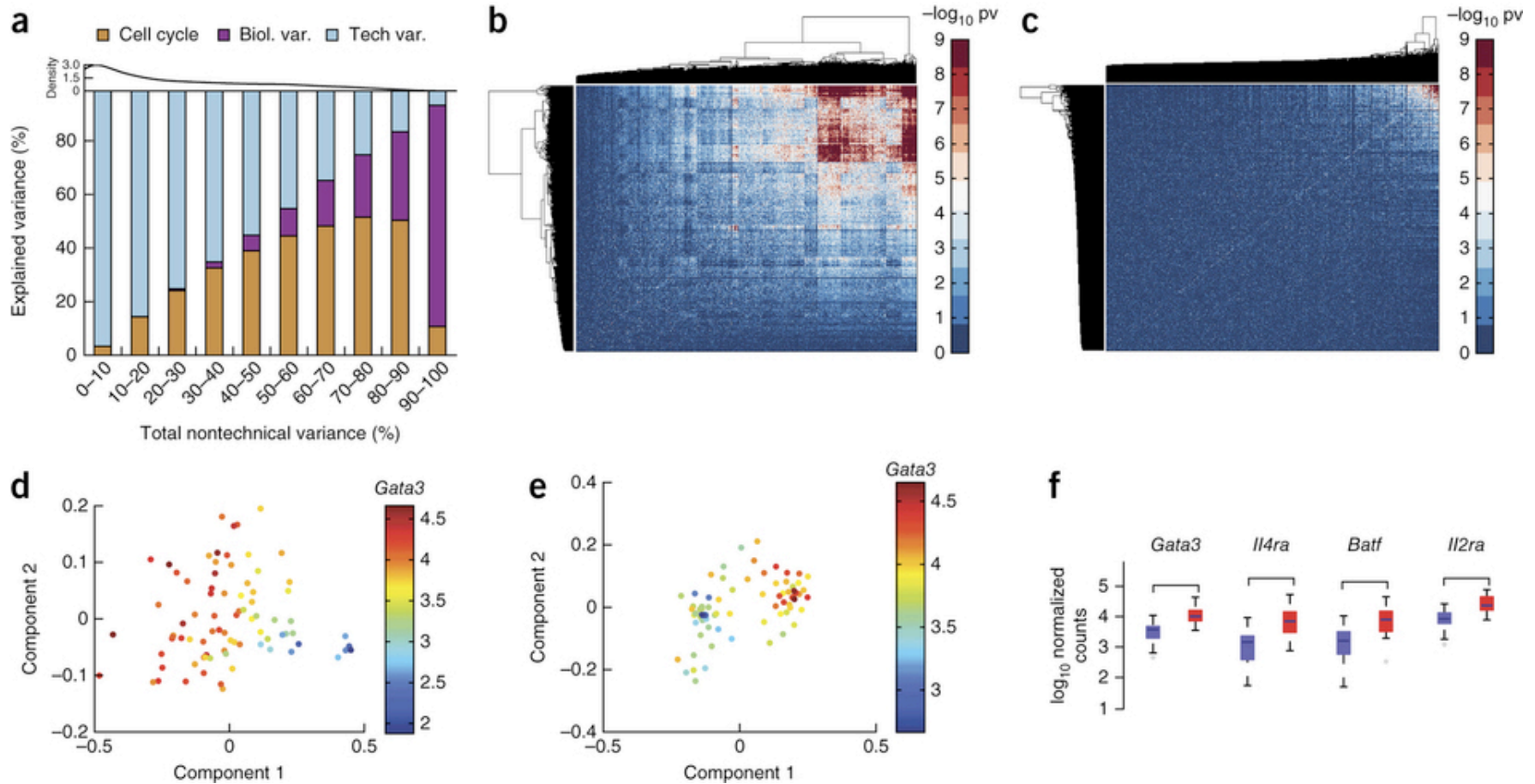
Color by donor





# scLVM - Marioni lab

<https://github.com/PMBio/scLVM>





# Preselection of a gene set

- In most cases, all genes are not used in PCA/ clustering.
- Filtering based on:
  - Biologically variable genes (Brenneke method based on spike-in data) or top variable genes if no spike-in data.
  - Genes expressed in X cells.
  - Filter out genes with correlation to few other genes
  - Prior knowledge / annotation
  - DE genes from bulk experiments
  - Top PCA loadings

# Detecting differentially expressed genes

- Parametric methods like EdgeR & DESeq not suitable for scRNAseq since the parameter assumptions in those methods does not apply here.
- Can use non-parametric methods like SAMseq

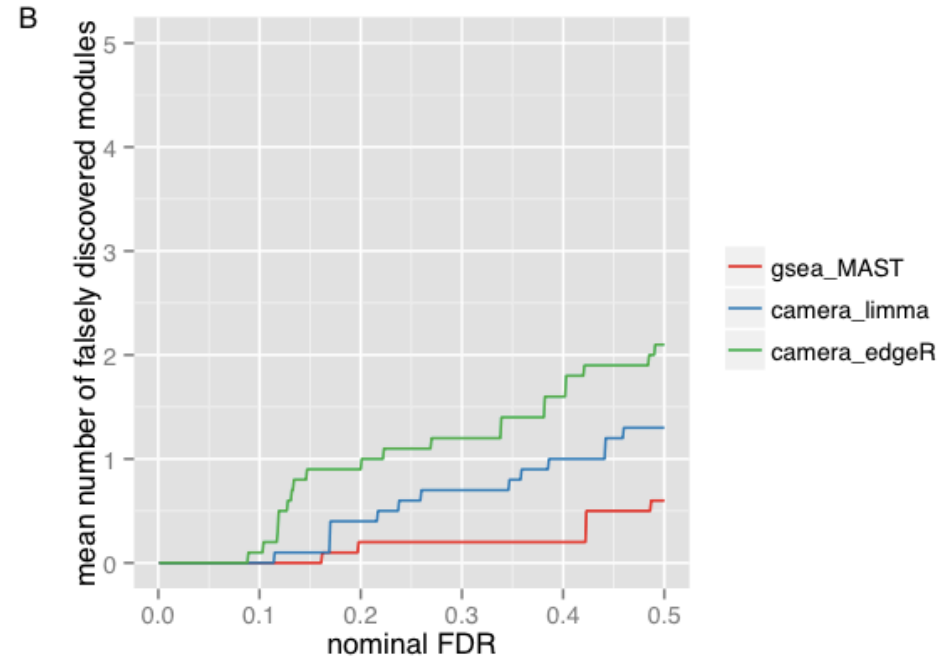
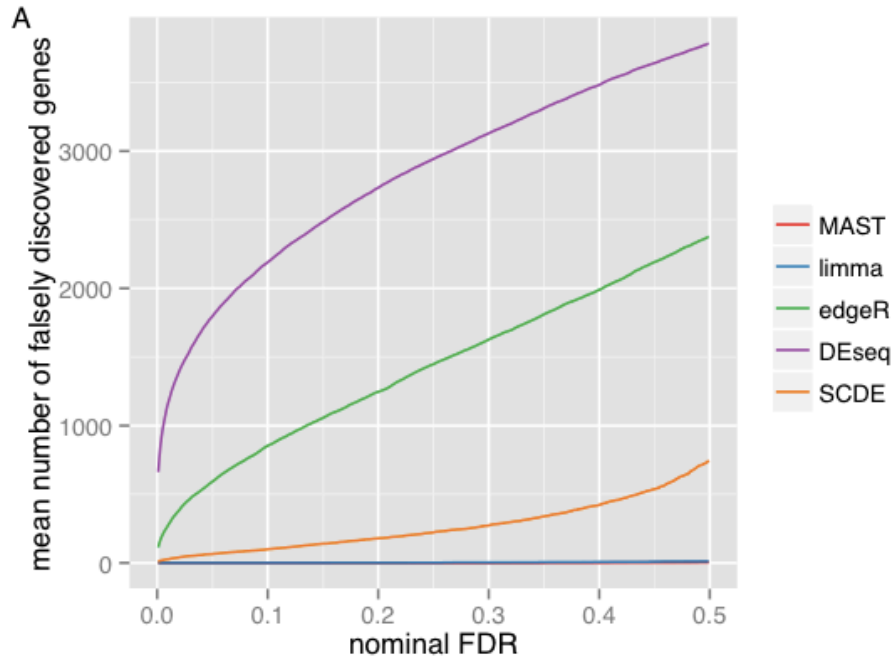
# Detecting differentially expressed genes

- Available single cell DE methods:
  - SingleCellAssay – developed for qPCR experiments
  - Monocle package
  - Single Cell Differential Expression - SCDE
  - Model-based Analysis of Single-cell Transcriptomics – MAST
  - SAMstrt – extension to SAMseq with spike-in normalization
  - Many other recent publications.....
- Some studies use PCA contribution (loadings) or gene clustering to define celltype specific genes with no statistical DE test at all.

# Comparison of DE detection methods

1429	631	399	1049	741	SAM
631	661	375	647	557	SCA
399	375	689	619	362	SCDE
1049	647	619	2780	755	DESEQ
741	557	362	755	794	MONOCLE
SAM	SCA	SCDE	DESEQ	MONOCLE	

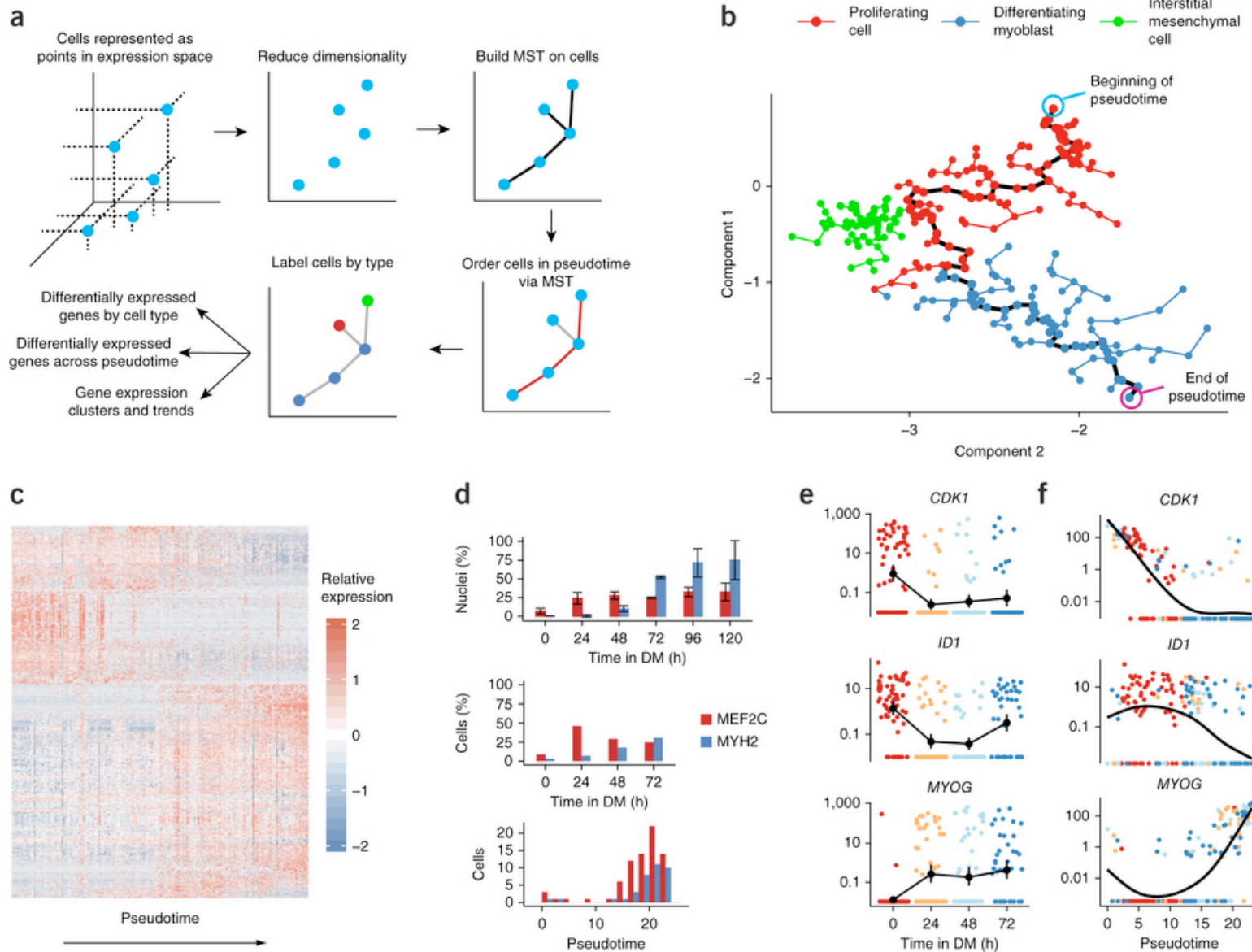
# High false discovery rate for DESeq and EdgeR



Detected DE genes and gene sets using randomly permuted cells from unstimulated MAIT cells

# Pseudotime ordering - Monocle

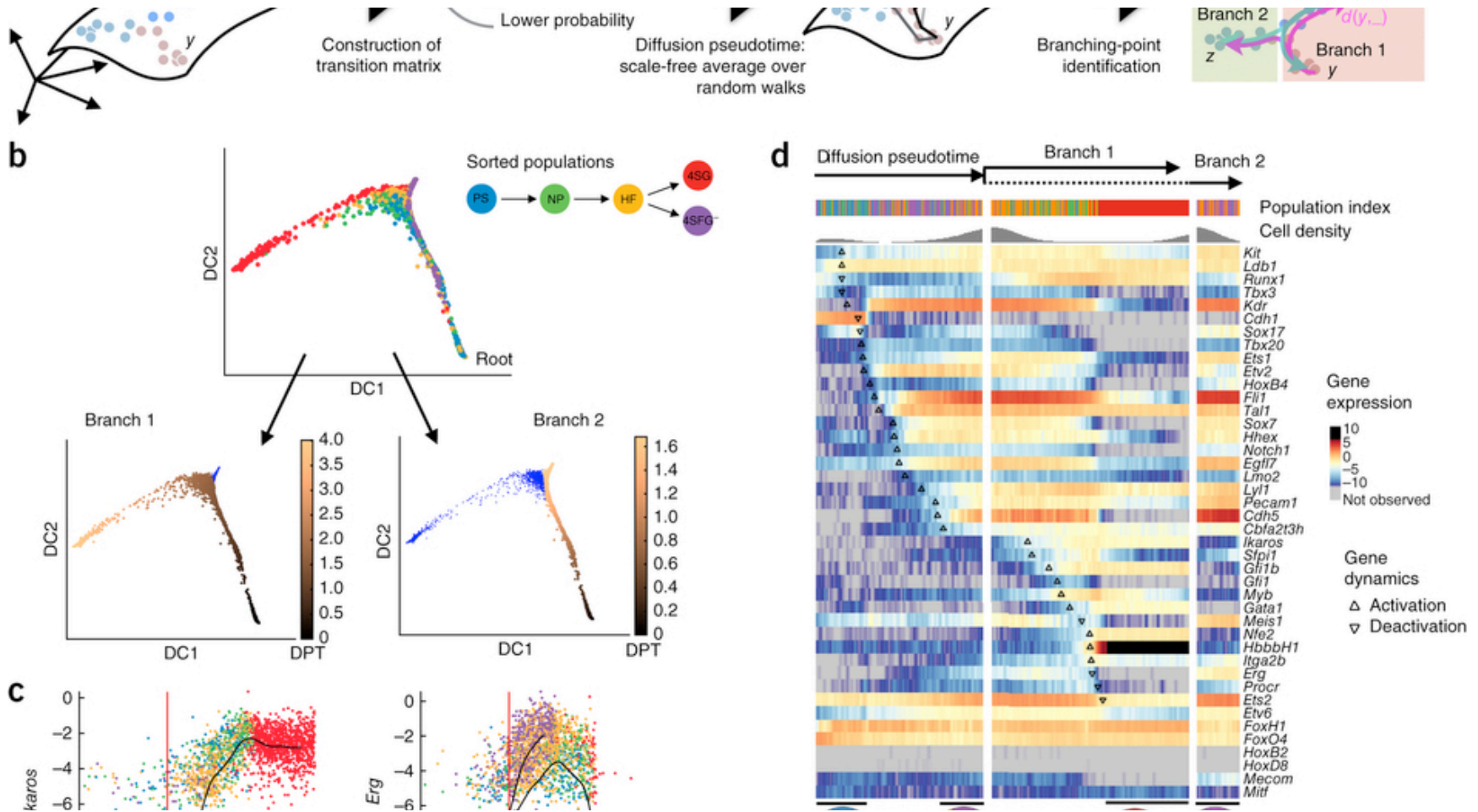
The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells.  
 Trapnell et al. *Nature Biotechnology* 32, 381–386 (2014)



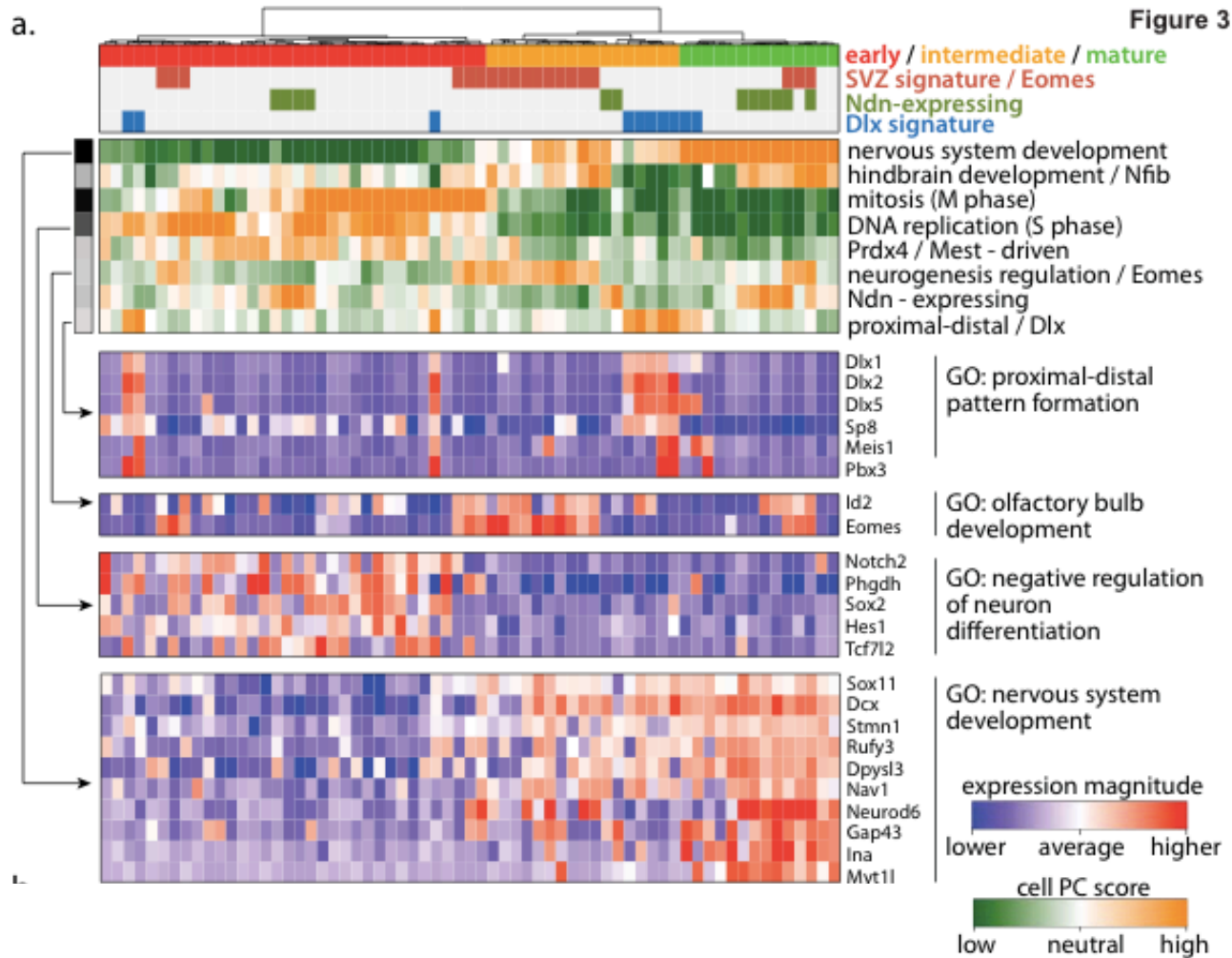
# Diffusion pseudotime

## Diffusion pseudotime robustly reconstructs lineage branching

Haghvadi et al Nature Methods 2016



# Pagoda – Pathway And Geneset OverDispersion Analysis

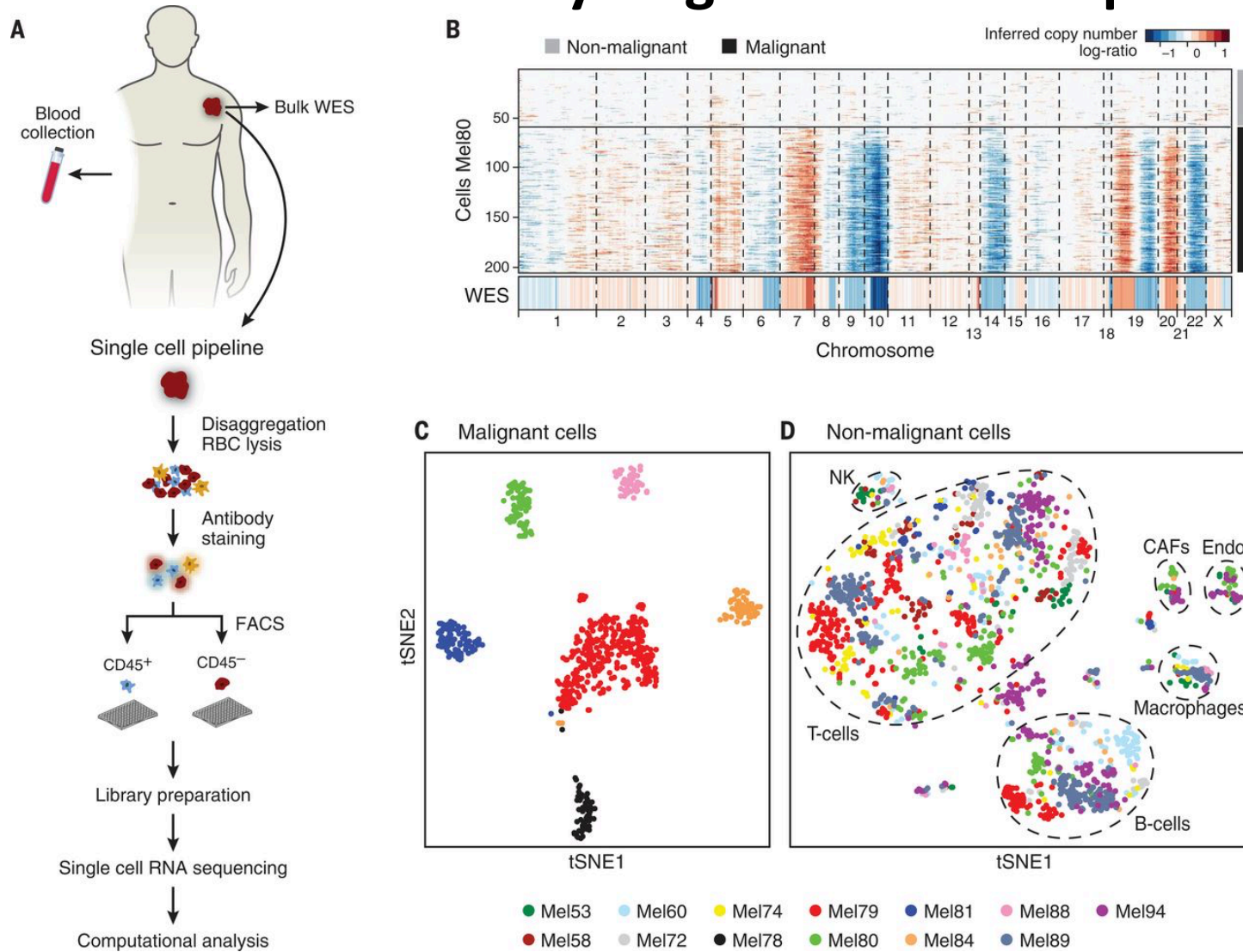




# Additional analyses

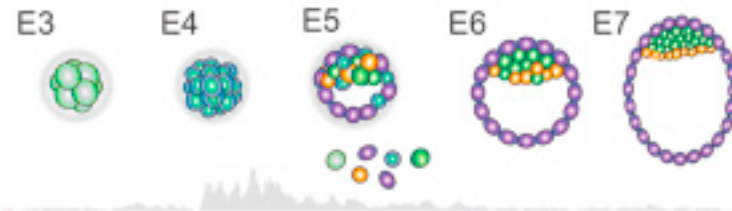
- Alternative splicing
- Allelic expression
- Copy-number variation
- Alternative splicing and allelic expression requires full length methods.
  - But only works for highly expressed genes with good read coverage
  - Must be careful to take into consideration the drop-out rate, a unique splice form/allele in a single cell may actually be a detection issue.

# Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq

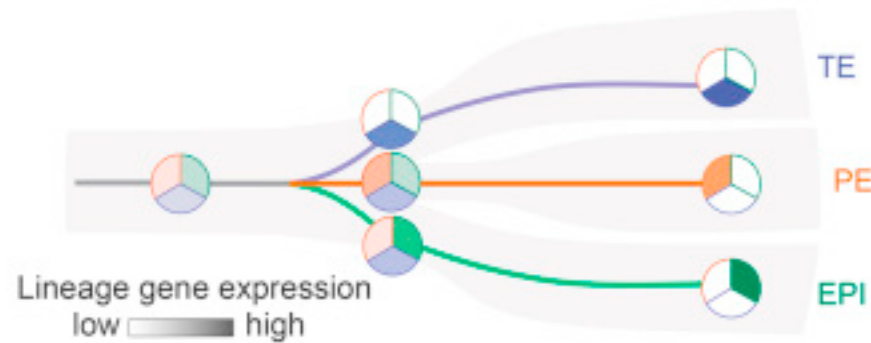


# Single-Cell RNA-Seq Reveals Lineage and X Chromosome Dynamics in Human Preimplantation Embryos

1529 single-cell RNA-seq libraries from 88 human embryos



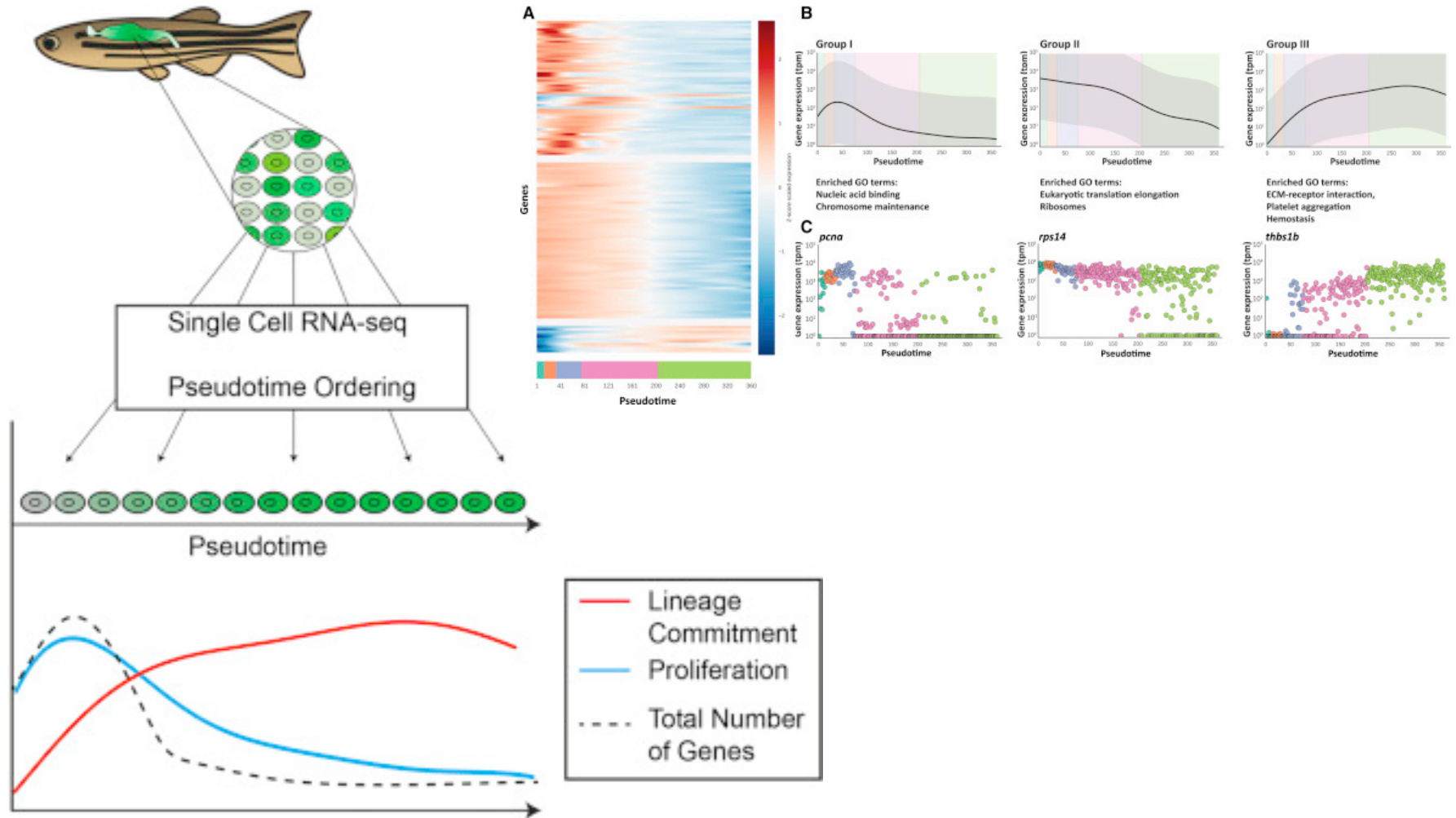
Initial co-expression and concurrent lineage formation



X-chromosome dosage compensation



# Single-Cell RNA-Sequencing Reveals a Continuous Spectrum of Differentiation in Hematopoietic Cells



# Conclusions

- For diverse cell-types often straight forward to group cells into clusters and detect differentially expressed genes.
- For highly similar subtypes or with subtle changes in cellular states – feature selection and different clustering methods may be required.
- PCA or other dimensionality reduction technique to get to know your data and what biases you .

# Tools for single cell analysis

- Tutorial from Harvard WS:
  - <http://pklab.med.harvard.edu/scw2015/>
- For differential expression:
  - SCDE: <http://pklab.med.harvard.edu/scde/index.html>
  - SCA: <https://github.com/RGLab/SingleCellAssay>
  - MAST: <https://github.com/RGLab/MAST>
  - SAMseq: <http://cran.r-project.org/web/packages/samr>
- For clustering etc.:
  - Monocle: <https://github.com/cole-trapnell-lab/monocle-release>
  - Rtsne: <http://cran.r-project.org/web/packages/Rtsne>
  - Sincell: <http://master.bioconductor.org/packages/devel/bioc/html/sincell.html>
  - scLVM: <https://github.com/PMBio/scLVM>
  - BASiCS: <https://github.com/catavallejos/BASiCS>
  - Pagoda: <http://pklab.med.harvard.edu/scde>
  - Seurat toolkit: <http://www.satijalab.org/seurat.html>
  - Sincera pipeline: <https://research.cchmc.org/pbge/sincera.html>
  - SimpleSingleCell pipeline: <https://www.bioconductor.org/help/workflows/simpleSingleCell/>

# Recommended reading

- **Single-cell sequencing-based technologies will revolutionize whole-organism science.** Shapiro et al. *Nature Reviews Genetics*, 14, 618–630 (2013)
- **Computational and analytical challenges in single-cell transcriptomics.** Stegle et al. . *Nature Reviews Genetics*, 16(3) (2015)
- **Entering the era of single-cell transcriptomics in biology and medicine.** Sandberg, *Nature Methods*, 11(1): 22-4 (2014)
- **Accounting for technical noise in single-cell RNA-seq experiments.** Brennecke et al. *Nature Methods* 10, 1093–1095 (2013)
- **Bayesian approach to single-cell differential expression analysis.** Karchenko et al. *Nature Methods* (2014)
- **The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells.** Trapnell et al. *Nature Biotechnology* 32, 381–386 (2014)
- **Data exploration, quality control and testing in single-cell qPCR-based gene expression experiments.** McDavid et al. *Bioinformatics* 29.4 (2013): 461-467.
- **Quantitative single-cell RNA-seq with unique molecular identifiers.** Islam et al. *Nature Methods* 11, 163–166 (2014)
- **Full-length mRNA-Seq from single-cell levels of RNA and individual circulating tumor cells.** Ramsköld et al. *Nature Biotechnology* 30, 777–782 (2012)
- **Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells.** Shalek et al. *Nature* 498, 236–240 (13 June 2013)
- **Validation of noise models for single-cell transcriptomics.** Grun et al. *Nature Methods* 11, 637–640 (2014)
- **Smart-seq2 for sensitive full-length transcriptome profiling in single cells.** Picelli et al. *Nature Methods* (2013).
- **SAMstr: statistical test for differential expression in single-cell transcriptome with spike-in normalization.** Katayama et al. *Bioinformatics*. 2013 Nov 15;29(22):2943-5