

Single-cell sequencing

Background

- Most of the biological experiments are performed on “bulk” samples, which contains a large number of cells (millions).
- The high-throughput data we introduced so far are all “bulk” data, which measures the average (gene expression, TF binding, methylation, etc.) of many cells.
- The bulk measurement ignores the inter-cellular heterogeneities:
 - Different cell types.
 - Biological variation among the same type of cell.

Single-cell biology

- The study of individual cells.
- The cells are isolated from multi-cellular organism.
- Experiment is performed for each cell individually.
- Provides more detailed, higher resolution information.
- High-throughput experiments on single cell is possible.

Single cell sequencing

- Perform different types of sequencing at the single-cell level:
 - DNA-seq
 - ATAC-seq
 - BS-seq
 - RNA-seq
- Very active research field in the past few years.
- Major challenges:
 - Cell isolation.
 - Amplification of genomic material.
 - Data analysis.

Basic experimental procedure

- Isolation of single cell. Techniques include
 - Laser-capture microdissection (LCM)
 - Fluorescence-activated cell sorting (FACS)
 - Microfluidics
- Open the cell and obtain DNA/mRNA/etc.
- PCR amplification to get enough materials.
- Perform sequencing.
- Note that single cell sequencing usually has higher error rates than bulk data.

Single cell DNA-seq (scDNA-seq)

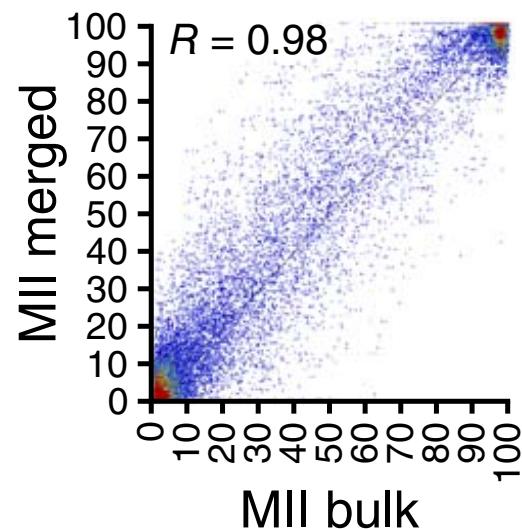
- For a comprehensive review, read *Gawad et al.* (2016) NRG.
- Examples of biological applications:
 - Identify and assemble the genome of unculturable microorganisms.
 - Determine the contribution of intra-tumor genetic heterogeneity in cancer development of treatment response.

scDNA-seq data analysis

- Single cell variant calling:
 - Bulk data can be used as reference to reduce false positives.
 - Combine data from several cells.
 - Software: Monovar (*Zafar et al. 2016 Nat. Method.*)
- Determining genetic relationship among single cells:
 - This is a clustering problem. Cells can be put into groups or a phylogenetic tree based on similarity of variants.
 - Methods are mostly ad hoc.

Single cell BS-seq (scBS-seq)

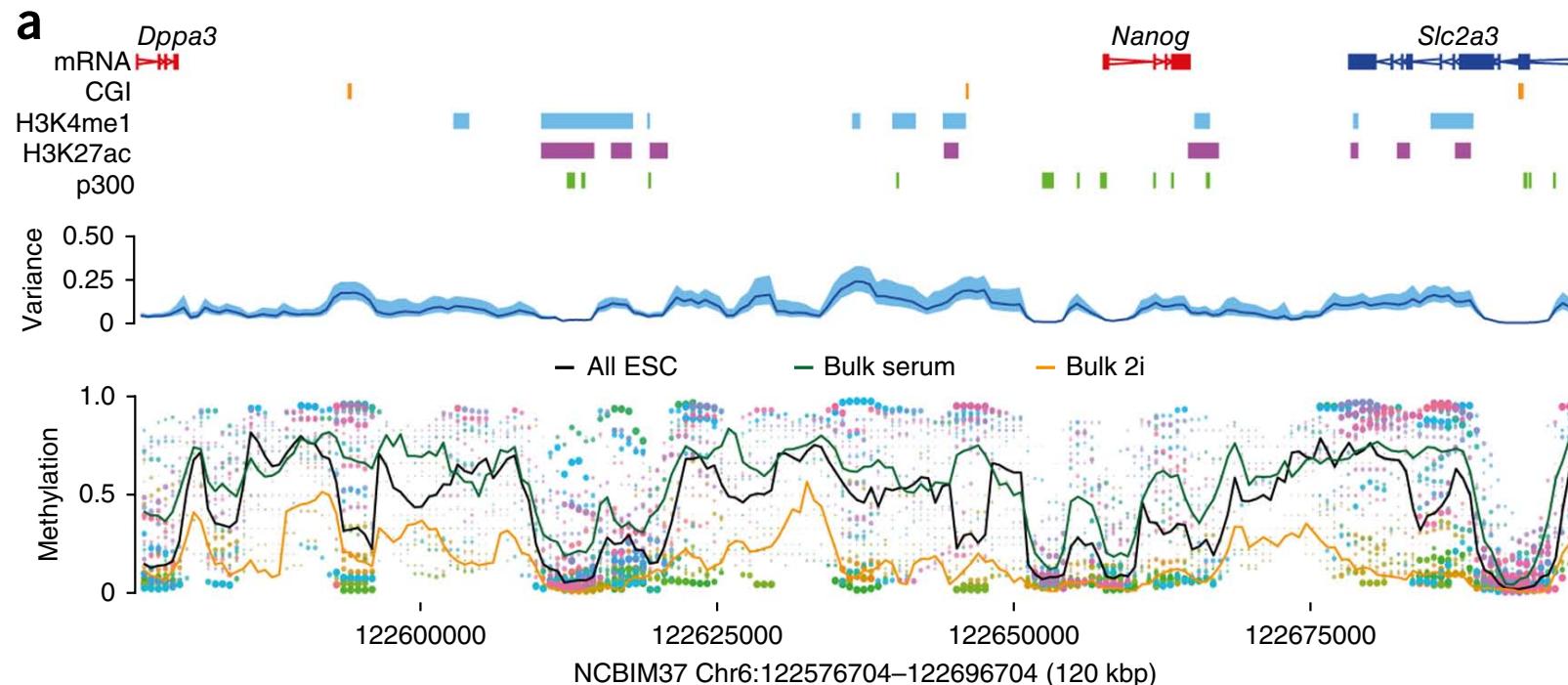
- Similar to scDNA-seq, but with bisulfite treatment before sequencing.
- There's scWGBS and scRRBS.
- The methylation levels from scBS-seq should be 0/1, with some exceptions caused by technical artifacts.
- Merged single cell and bulk data have good correlation.

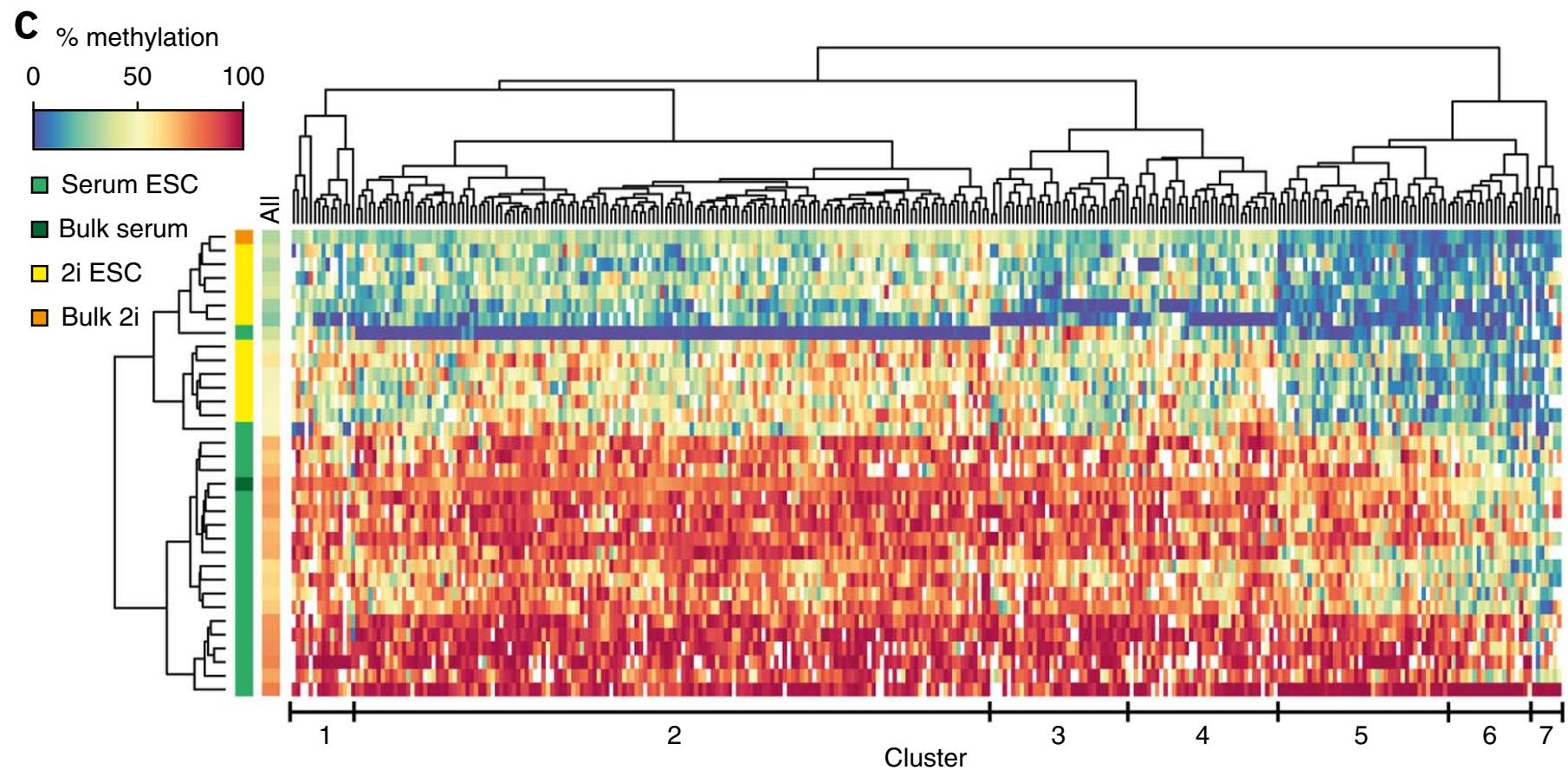


Smallwood et al. 2014, NM

scBS-seq data analysis

- So far the data analysis are mostly descriptive:
 - compute variations among cells
 - Cell clustering
- Lots of rooms for method development



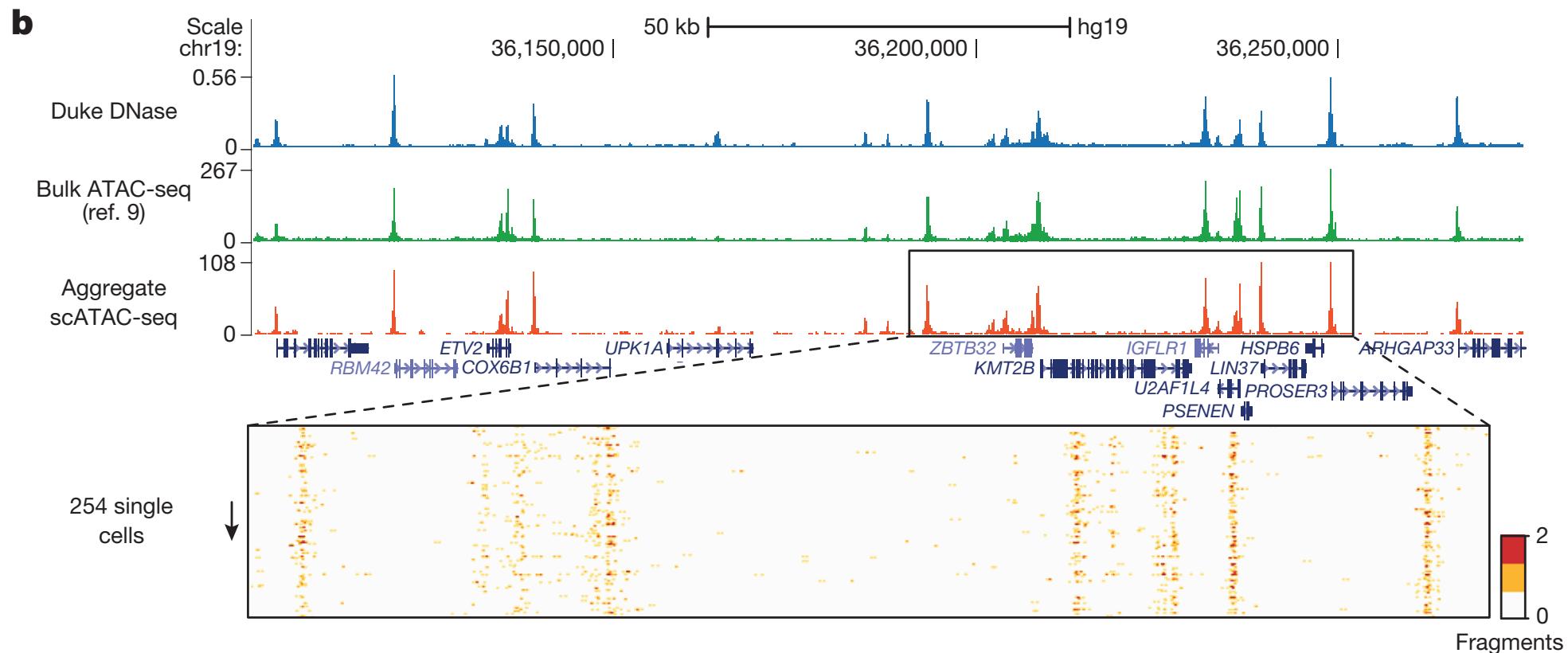


Single cell ChIP/ATAC-seq

- ATAC-seq: similar to DNase-seq, profile the active genomic regions. Data look like ChIP-seq.
- A few papers:
 - Rotem et al. (2015) NBT: scChIP-seq
 - Buenrostro et al. (2015) Nature: scATAC-seq

scChIP/scATAC-seq data

- Aggregated sc data has good agreement with bulk.



- Very sparse: one or a few reads at peak regions.
 - Extremely low signal to noise ratio.
 - Peak calling have to be based on combined data, or rely on other prior information

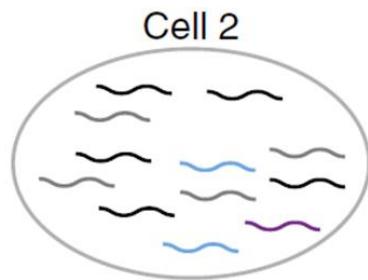
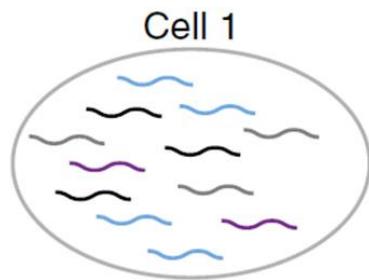
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| Cell2 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 3 | 1 | 0 | 1 | 1 | 0 |
| Cell3 | 2 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 2 | 1 | 0 | 0 |
| ⋮ | 1 | 1 | 2 | 1 | 0 | 2 | 2 | 1 | 0 | 2 | 1 | 0 | 1 | 0 | 0 | 0 | 2 |
| ⋮ | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 3 | 1 | 0 | 1 | 1 | 0 |
| | 2 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 2 | 1 | 0 | 0 |
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Peak1 Peak2 Peak3 ⋮ ⋮ ⋮

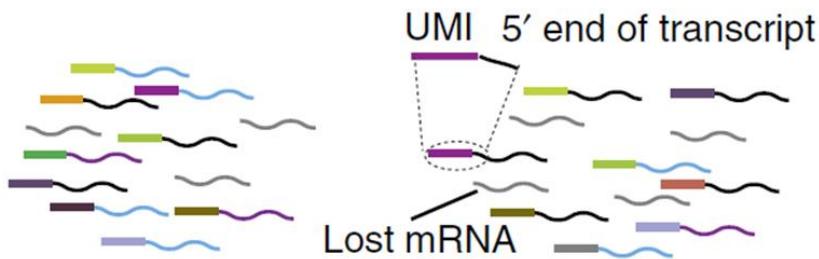
Single cell RNA-seq (scRNA-seq)

- The most active in the sc field.
- Scientific goals:
 - Understand the gene expression heterogeneity within the same sample.
 - Composition of different types of cell in complex tissues, such as brain, cancer, etc.
 - Above can be explored spatially, temporally, or under different biological condition.
- Raw data are the same as bulk RNA-seq, can be aligned using the same software.

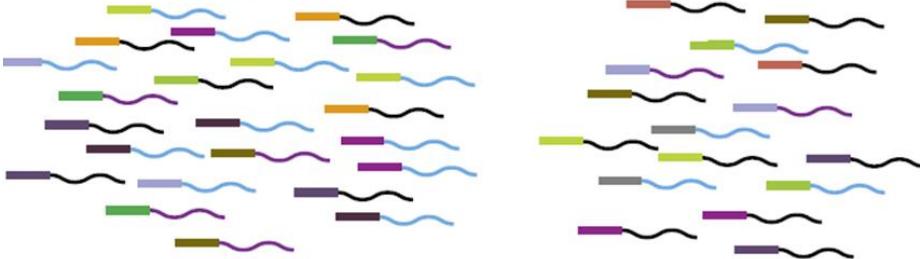
Experimental procedure



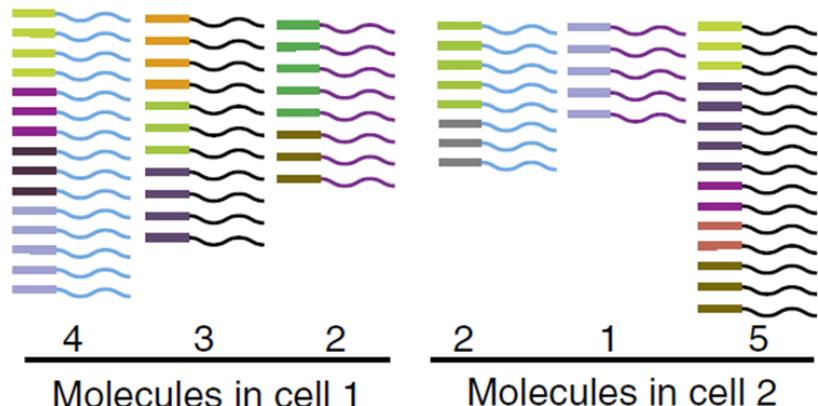
Reverse transcription, barcoding and UMI labeling



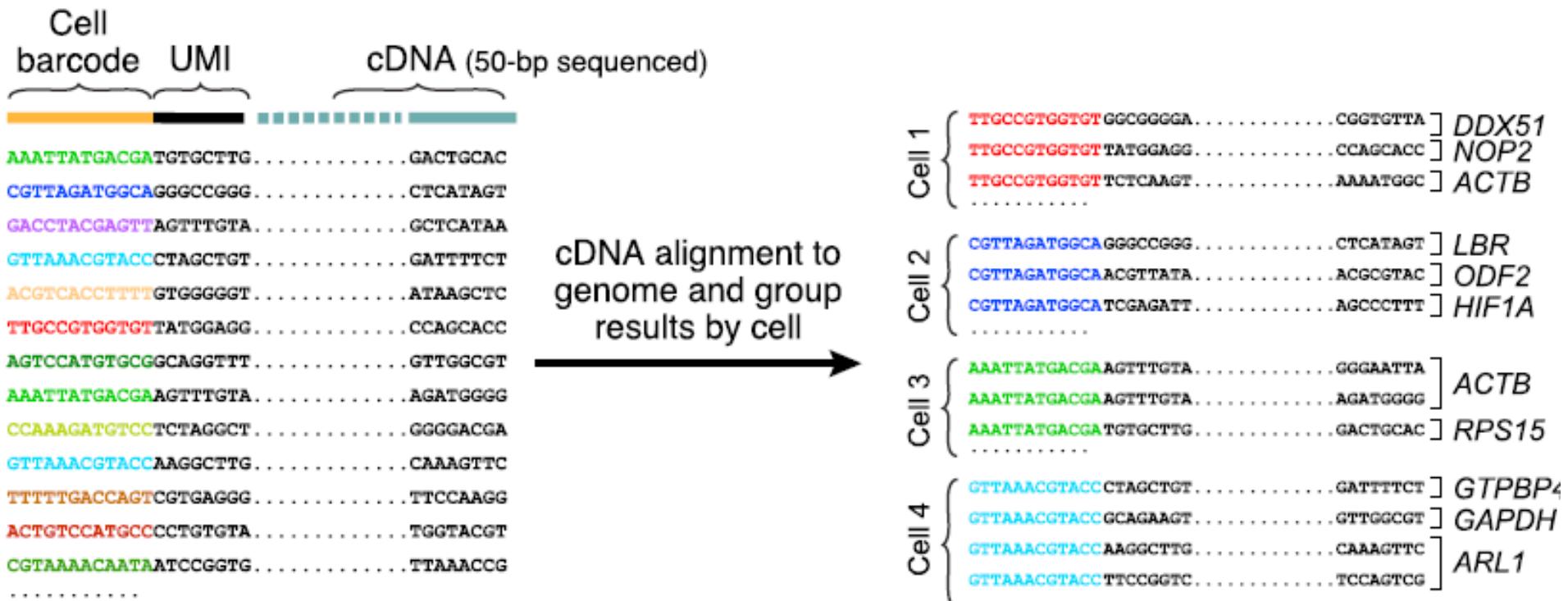
PCR amplification



Sequencing and computation



Saiful Islam ... Sten Linnarsson



Some data characteristics

- Number of transcripts detected is much lower compared to bulk RNA-seq, due to low capture and reverse transcription efficiencies.

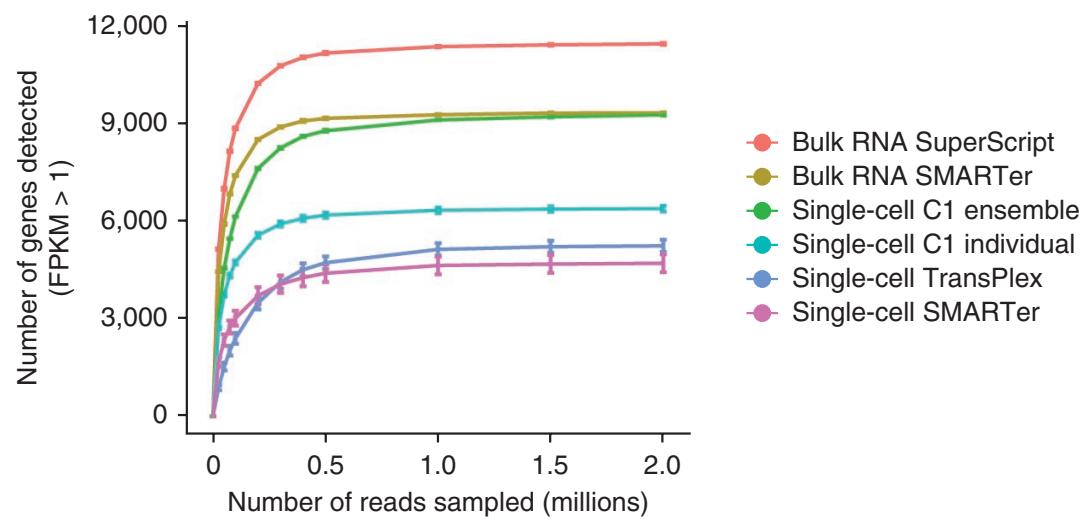
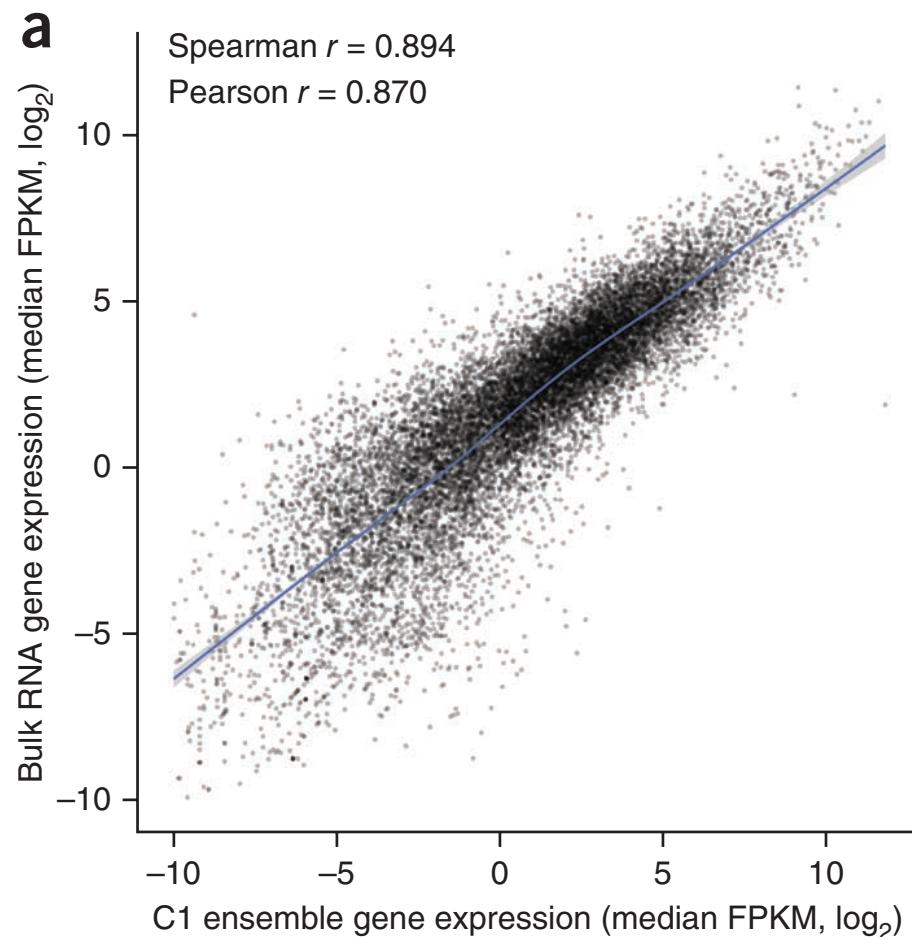
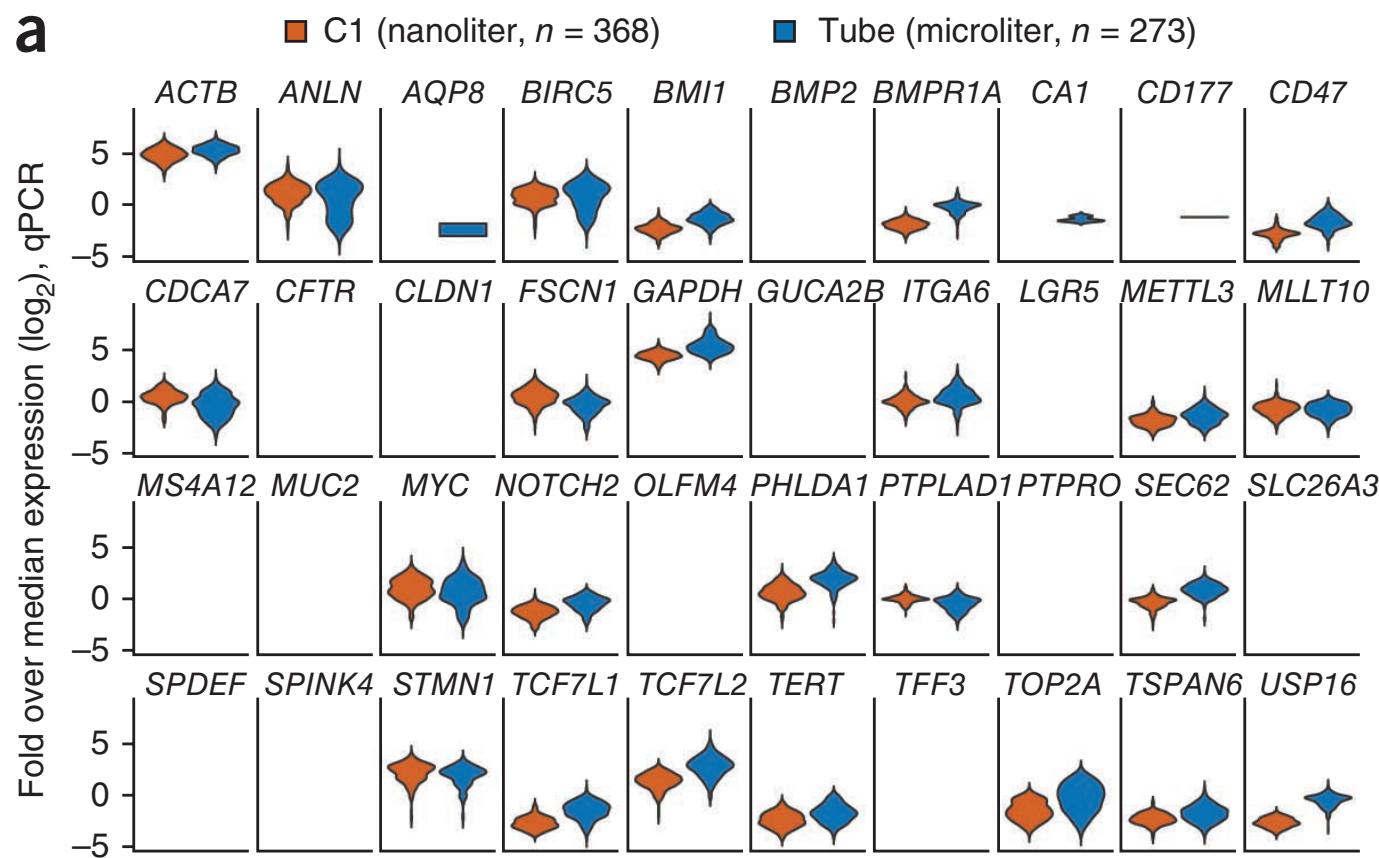


Figure 5 | Saturation curves for the different sample preparation methods. Each point on the curve was generated by randomly selecting a number of raw reads from each sample library and then using the same alignment pipeline to call genes with mean FPKM >1. Each point represents four replicate subsamplings. Error bars, standard error.

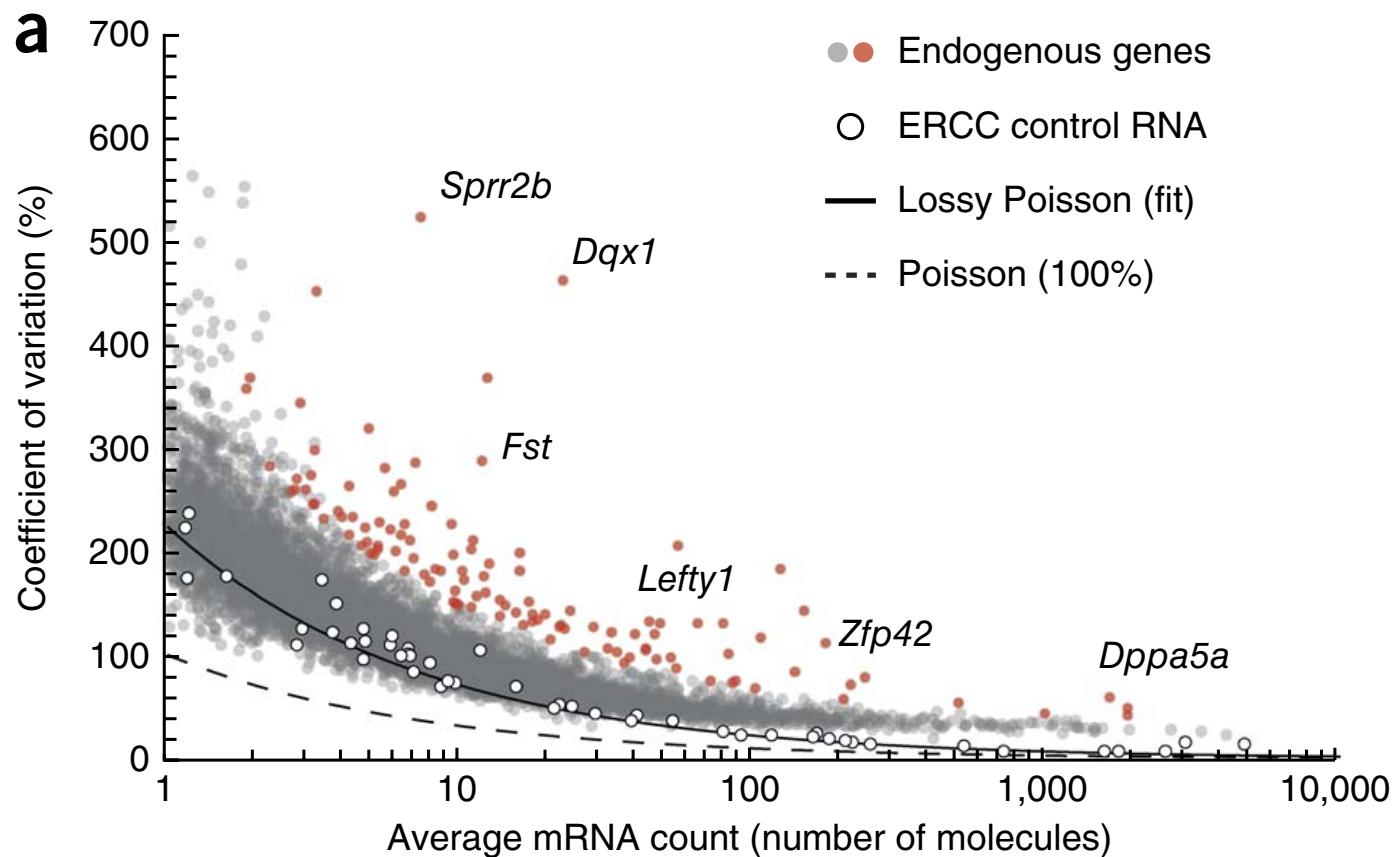
- Bulk and aggregated single cell expressions have good correlation.



- Expression levels for a gene in different cells sometimes show bimodal distribution.

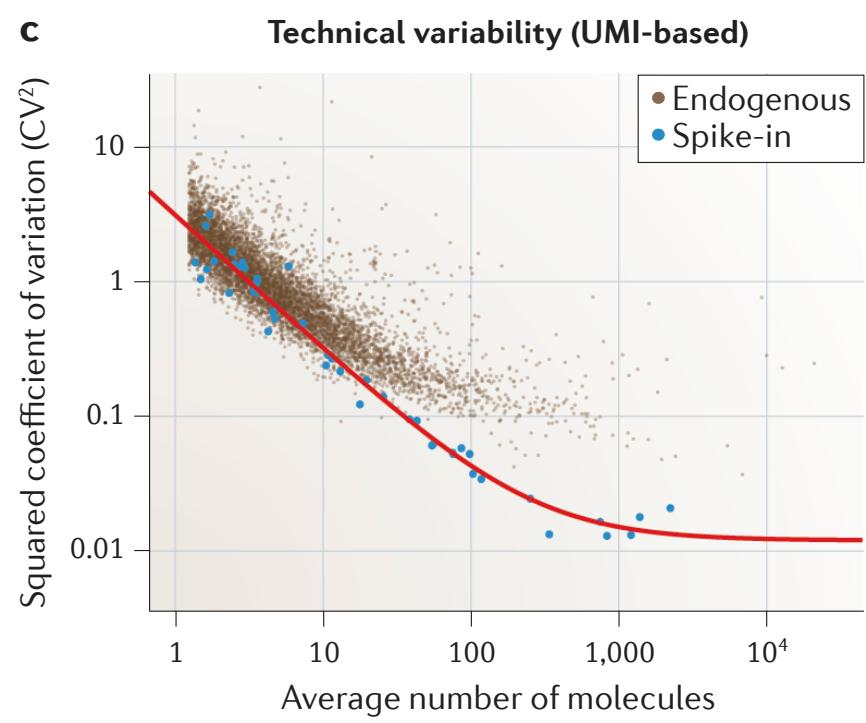
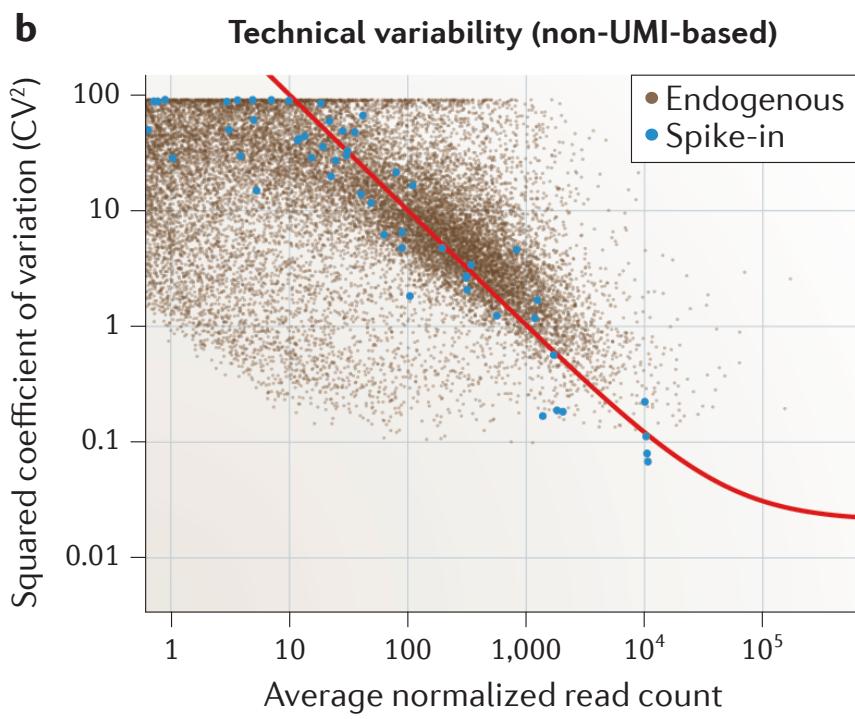


- Negative correlation between mean expression and biological variation (same as in bulk).



Normalization issues

- scRNA-seq is very noisy.
- Spike-in data is usually available.
 - Spike-ins from the external RNA Control Consortium (ERCC) panel, which contains 92 synthetic spikes based on bacterial genome.
- UMI (unique molecule identifier) is sometimes used to barcode the molecules for estimating amplification noise.
- A combination of spike-in and UMI can potentially be used for data normalization.



Existing work for scRNA-seq normalization

Application Note

Normalization and noise reduction for single cell RNA-seq experiments

Bo Ding^{1,#}, Lina Zheng^{1,#}, Yun Zhu¹, Nan Li¹, Haiyang Jia^{1,2}, Rizi Ai¹, Andre Wildberg¹ and Wei Wang^{1,3*}

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#Equal contribution

Associate Editor: Dr. Ziv Bar-Joseph

- Log-transform FPKM values, denoted by x .
- Assume the expression value, y , follow Gamma distribution. The mean of Gamma is a polynomial function of x : $y = \mu(x)$.

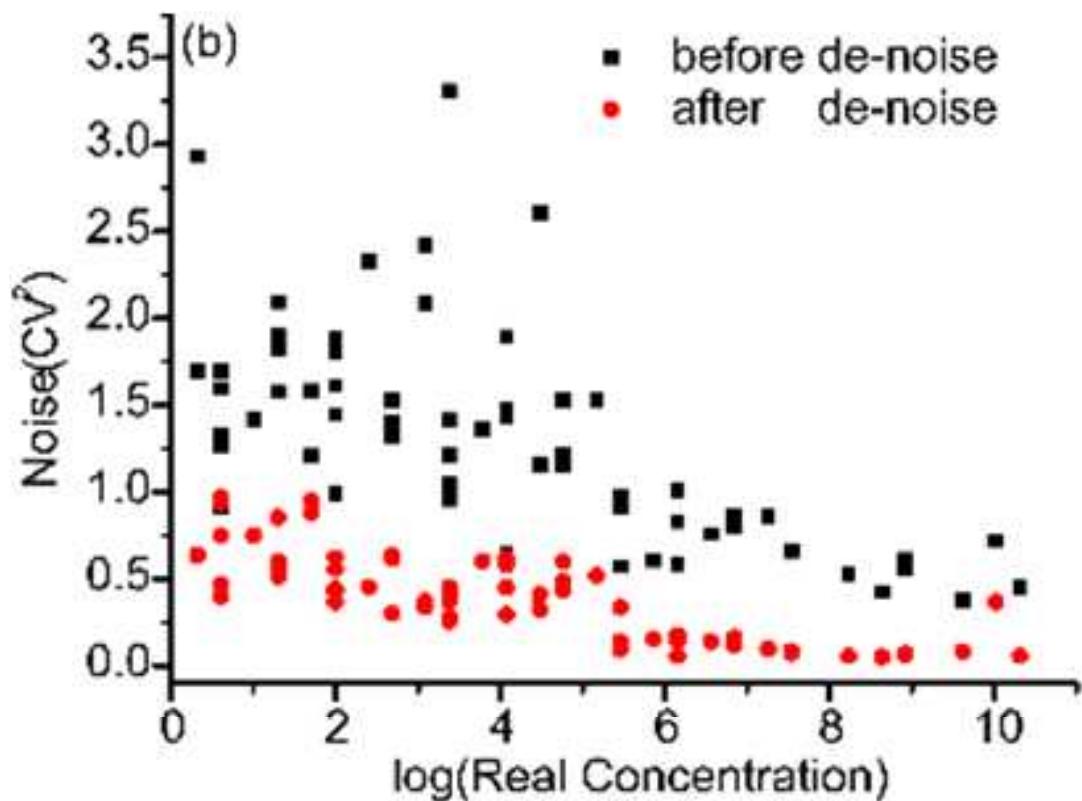
$$\mu(x) = \sum_{i=0}^n \beta_i x^i.$$

The model is the following:

$$y \sim \text{Gamma}(y; \mu(x), \varphi)$$

- Use MLE to estimate parameters based on ERCC data. Then the fitted model is applied to all genes to estimate concentration.

- Results: reduced CV cross cells.



METHOD

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Pooling across cells to normalize single-cell RNA sequencing data with many zero counts

Aaron T. L. Lun^{1,*}, Karsten Bach² and John C. Marioni^{1,2,3*}

- Works for data without spike-in.
- The goal is to estimate a size factor for each cell.
- The idea is to normalize on summed expression values from pools of cells – it's more stable than using individual cell.

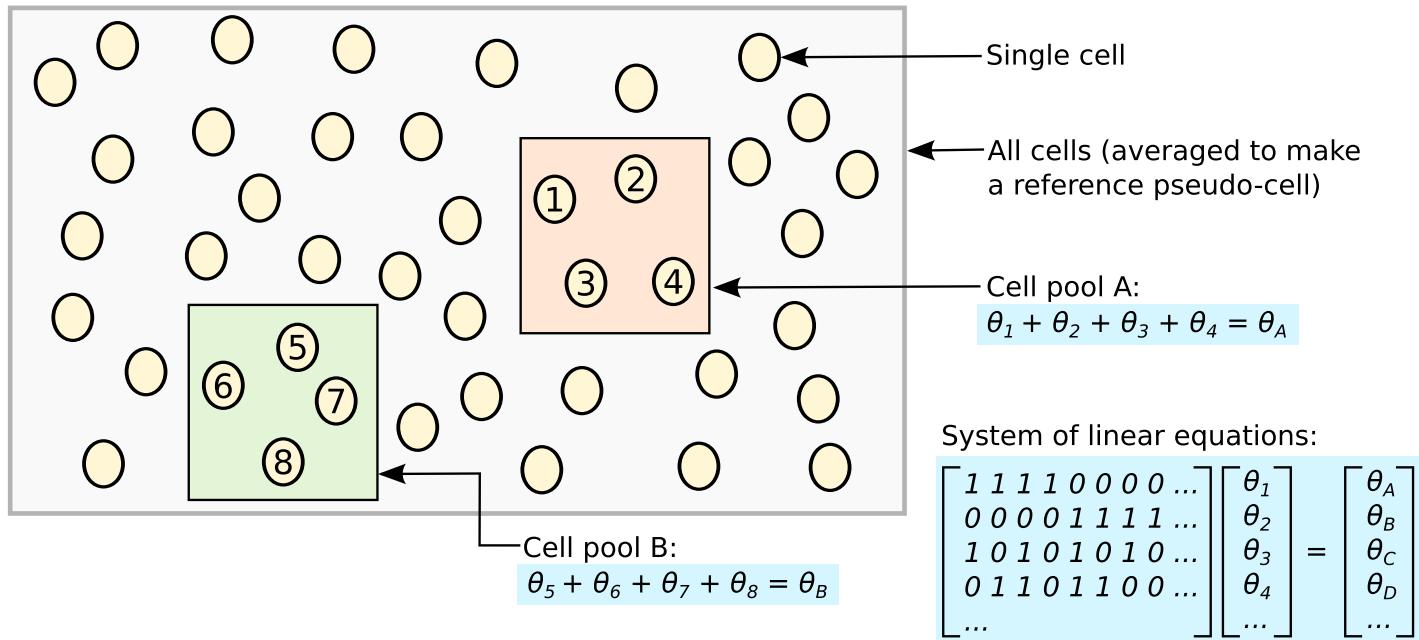


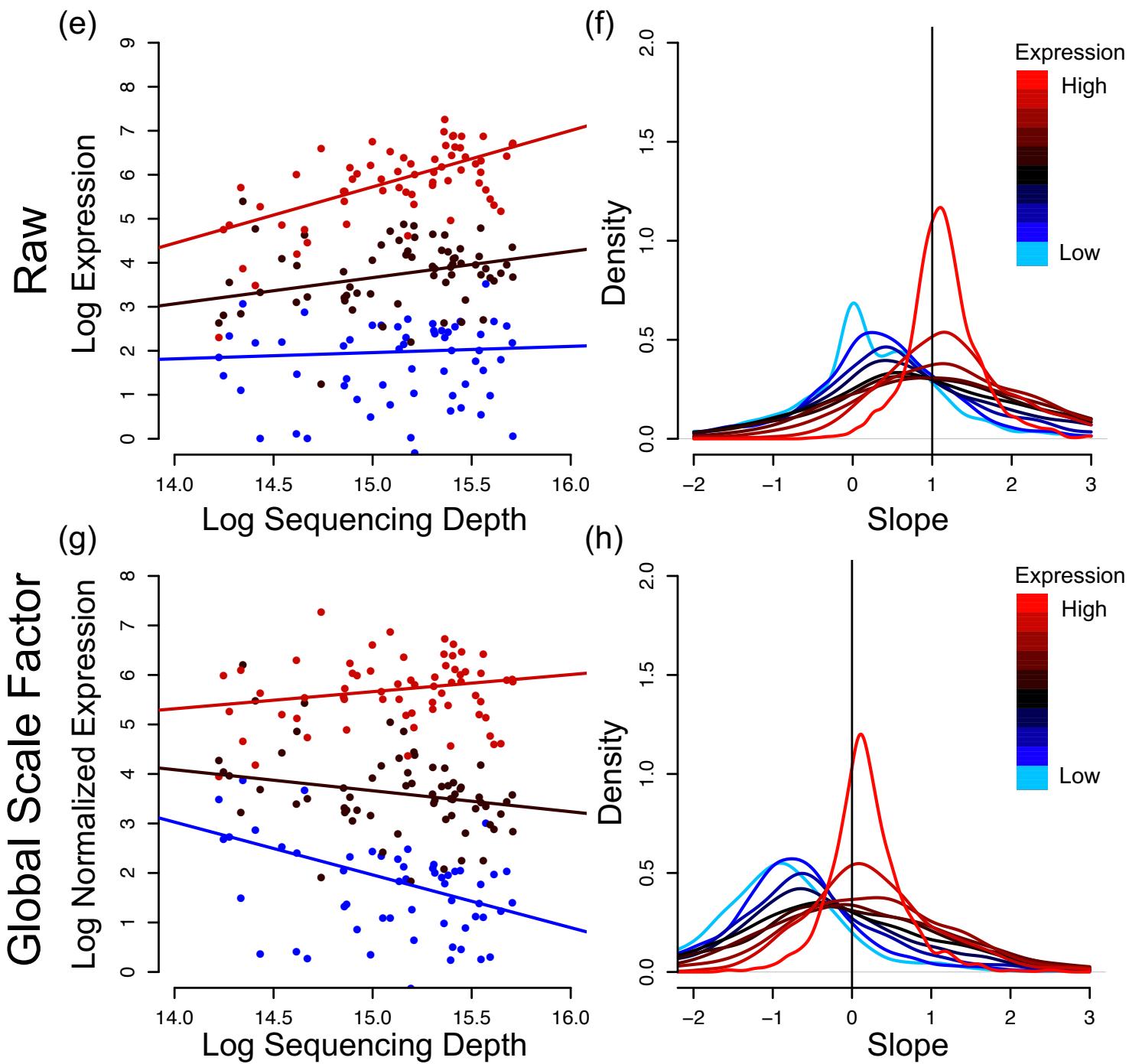
Fig. 3 Schematic of the deconvolution method. All cells in the data set are averaged to make a reference pseudo-cell. Expression values for cells in pool A are summed together and normalized against the reference to yield a pool-based size factor θ_A . This is equal to the sum of the cell-based factors θ_j for cells $j = 1-4$ and can be used to formulate a linear equation. (For simplicity, the t_j term is assumed to be unity here.) Repeating this for multiple pools (e.g., pool B) leads to the construction of a linear system that can be solved to estimate θ_j for each cell j

SCnorm: A quantile-regression based approach for robust normalization of single-cell RNA-seq data

Rhonda Bacher^{1,5}, Li-Fang Chu^{2,5}, Ning Leng², Audrey P. Gasch³, James A. Thomson², Ron M. Stewart², Michael Newton^{1,4}, and Christina Kendziorski^{4*}

- Basic idea: one normalization factor per cell doesn't fit all genes.
- Relationships of read counts and sequencing depths vary and depend on the expression levels.

Single cell



Solution

- Uses quantile regression to estimate the dependence of read counts on sequencing depth for every gene.
- Genes with similar dependence are then grouped, and a second quantile regression is used to estimate scale factors within each group.
- Implemented in software SCnorm.

Differential expression

- Traditional methods test mean changes.
- Due to the bimodal distribution of the GE in scRNA-seq, the consideration and modeling of “drop-out” event (non-expressed) is very important.
- A few existing work, but lots of room for method development.

Bayesian approach to single-cell differential expression analysis

740 | VOL.11 NO.7 | JULY 2014 | NATURE METHODS

Peter V Kharchenko¹⁻³, Lev Silberstein³⁻⁵ &
David T Scadden³⁻⁵

- SCDE (single-cell differential expression).
- Use a mixture of a Poisson with small rate (dropout) and negative binomial (expressed) to model the expression: $p(x | r_c, \Omega_c) = p_d(x)p_{Poisson}(x) + (1 - p_d(x))p_{NB}(x | r_c)$
- The DE is based on Bayesian inference. But the derivation in this paper is messy.

METHOD

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MAST: a flexible statistical framework for assessing transcriptional changes and characterizing heterogeneity in single-cell RNA sequencing data

Greg Finak^{1†}, Andrew McDavid^{1†}, Masanao Yajima^{1†}, Jingyuan Deng¹, Vivian Gersuk², Alex K. Shalek^{3,4,5,6}, Chloe K. Slichter¹, Hannah W. Miller¹, M. Juliana McElrath¹, Martin Prlic¹, Peter S. Linsley²
and Raphael Gottardo^{1,7*}

- MAST: “Model-based Analysis of Single- cell Transcriptomics.”

MAST for DE

- Main ideas:
 - Use $\log_2(\text{TPM}+1)$ as input data
 - Both dropout probability and expression level depends on experimental conditions.

$$\text{logit}(\Pr(Z_{ig} = 1)) = X_i \beta_g^D$$

$$\Pr(Y_{ig} = y | Z_{ig} = 1) = N(X_i \beta_g^C, \sigma_g^2)$$

- Model fitting with some regularization.
- DE is based on chi-square or Wald test.

The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells

Cole Trapnell^{1,2,6}, Davide Cacchiarelli^{1-3,6}, Jonna Grimsby², Prapti Pokharel², Shuqiang Li⁴, Michael Morse^{1,2}, Niall J Lennon², Kenneth J Livak⁴, Tarjei S Mikkelsen¹⁻³ & John L Rinn^{1,2,5}

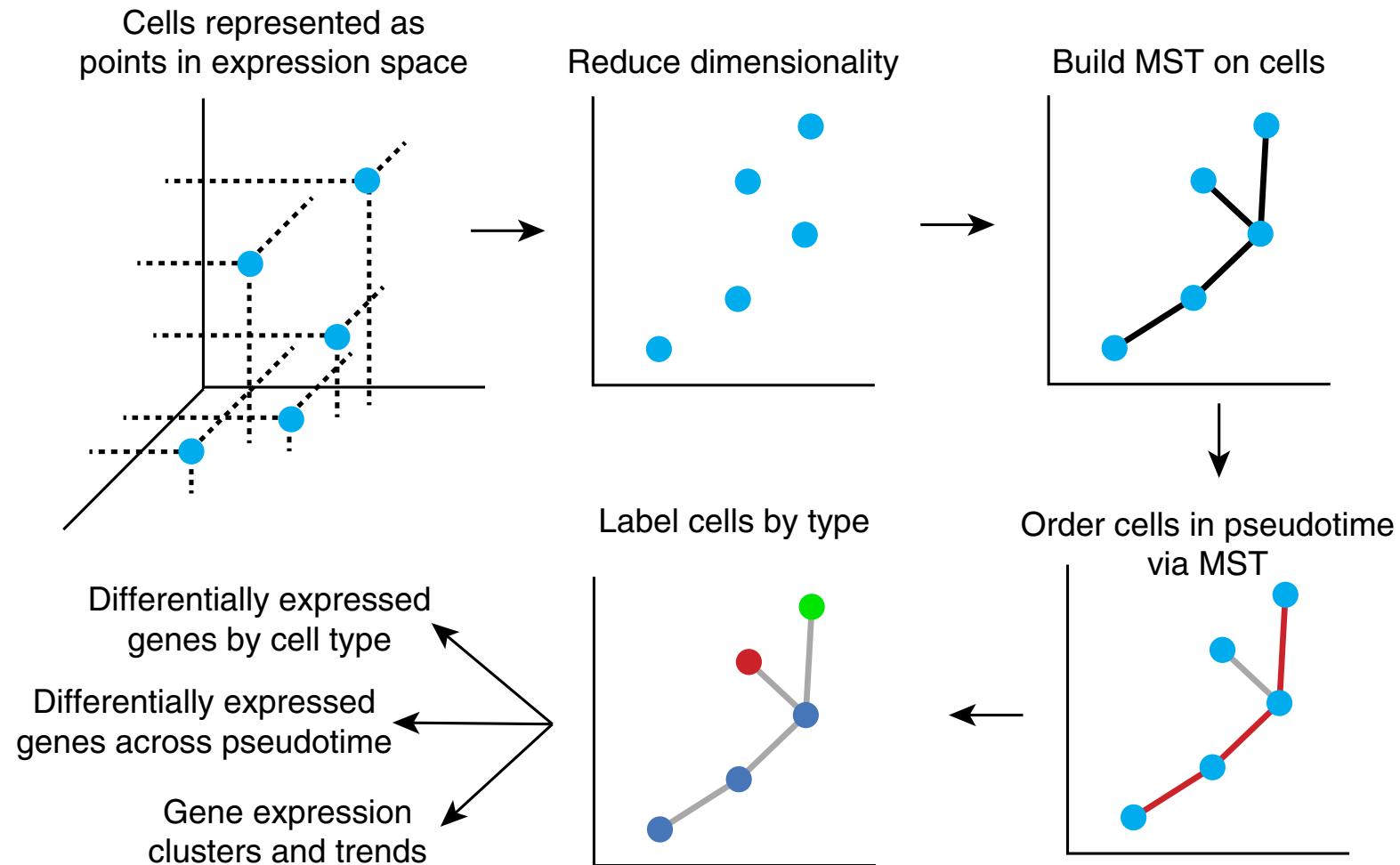
- Monocle: part of “tuxedo suite” for scRNA-seq analysis.
- Works for DE and clustering.
- Main idea for DE:
 - Model data with observed and dropout: $Y = \begin{cases} Y^* & \text{if } Y^* > \lambda \\ \lambda & \text{if } Y^* \leq \lambda \end{cases}$
 - Use a generalized additive model (GAM) for design:
$$g(E(Y)) = \beta_0 + f_1(x_1) + f_2(x_2) + \cdots + f_m(x_m)$$
 - DE is tested from the GAM.

Cell clustering

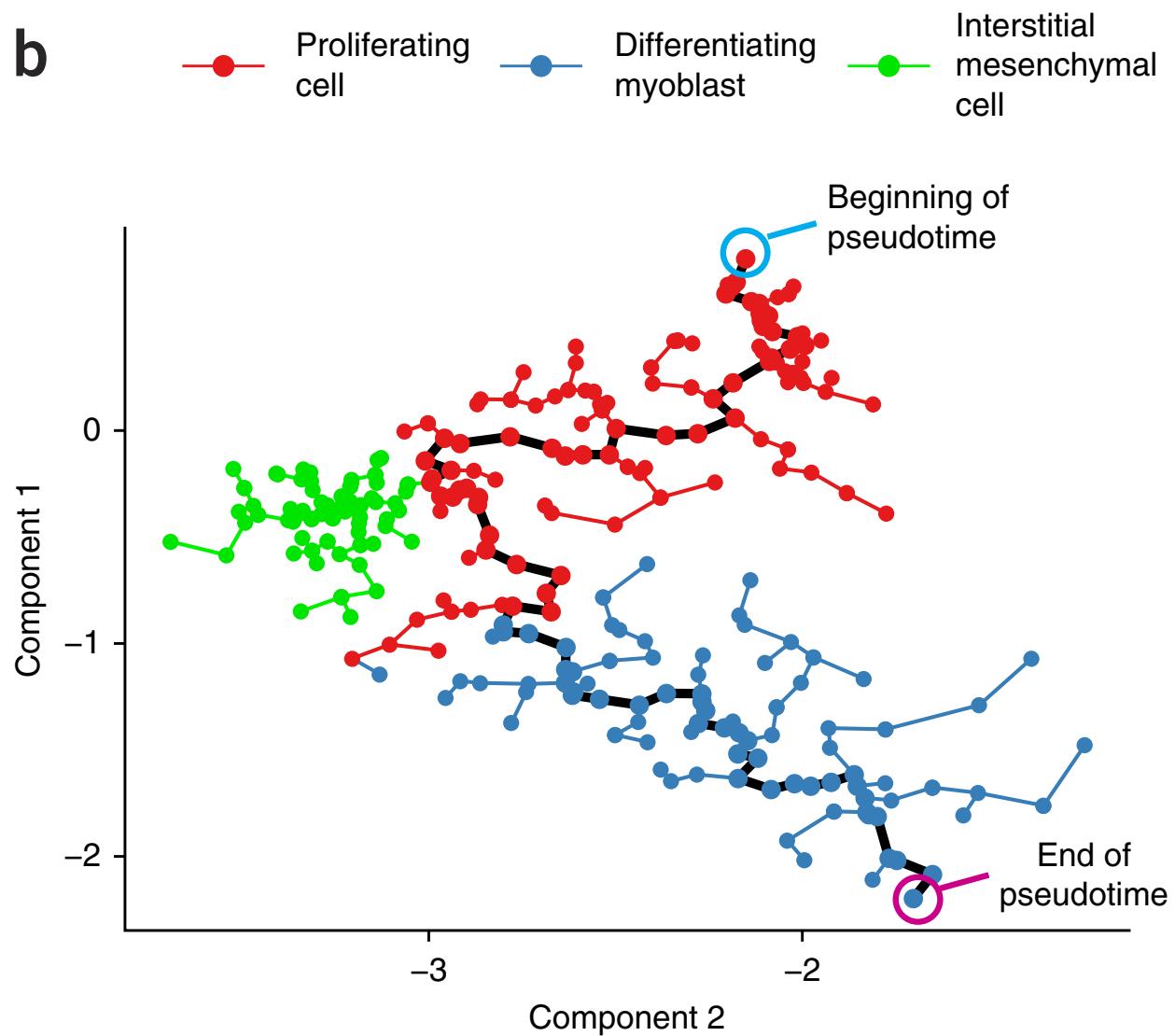
- Perhaps the most active topic in scRNA-seq.
- The goals include:
 - Cluster cells into subgroups.
 - Model temporal transcriptomic dynamics: reconstruct “pseudo-time” for cells. This is useful for understanding development or disease progression.
- Traditional method like k-means or hierarchical clustering need to be used with caution due to dropout events.

Monocle

a



Monocle result



Use Monocle Bioconductor package

First create a CellDataSet object using newCellDataSet function, then:

- Differential expression using differentialGeneTest.
- Cell ordering (pseudo-time estimation). This contains three steps:
 - Select a list of genes (often the DE genes) used for cell ordering. Use setOrderingFilter function to set that.
 - Dimension reduction using reduceDimension function.
 - Cell ordering using orderCells function.

```
### Create data object
pd <- new("AnnotatedDataFrame", data = sample_sheet)
fd <- new("AnnotatedDataFrame", data = gene_annotation)
dataobj <- newCellDataSet(as.matrix(expr_matrix),
                         phenoData = pd, featureData = fd)

### DE test
diff_test_res <- differentialGeneTest(dataobj,
                                         fullModelFormulaStr=GE~cond",
                                         reducedModelFormulaStr="GE~1")

### cell ordering
ordering_genes <- row.names(subset(diff_test_res, qval < 0.1))
dataobj <- setOrderingFilter(dataobj, ordering_genes)
dataobj <- reduceDimension(dataobj, use_irlba=FALSE)
dataobj <- orderCells(dataobj, num_paths=2, reverse=TRUE)
plot_spanning_tree(dataobj)
```

Other similar software

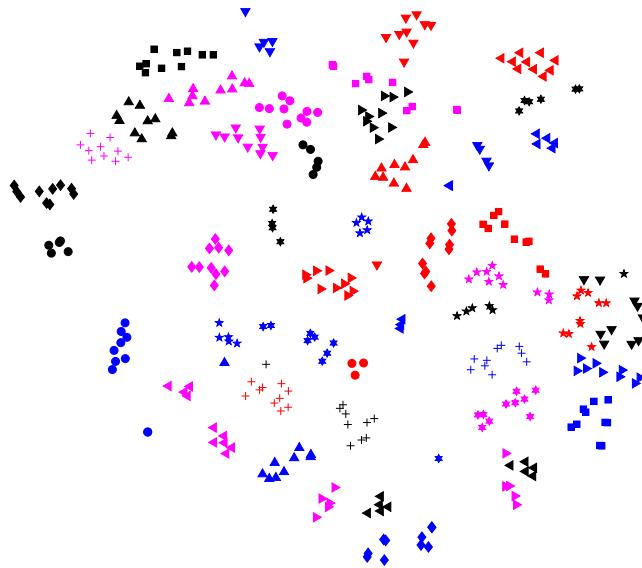
- Waterfall: Shin et al. (2015) Cell Stem Cell
- Wanderlust: Bendall et al. (2014) Cell
- TSCAN: Ji et al. (2016) NAR
- Ideas are similar:
 - Select informative genes.
 - Dimension reduction of GE.
 - Cluster the cells based on reduced data. Often want to over-cluster them to have many groups.
 - Construct a MST (mimumum spanning tree) from the clustering results.
 - Map cells to the MST.

Detect rare cell type

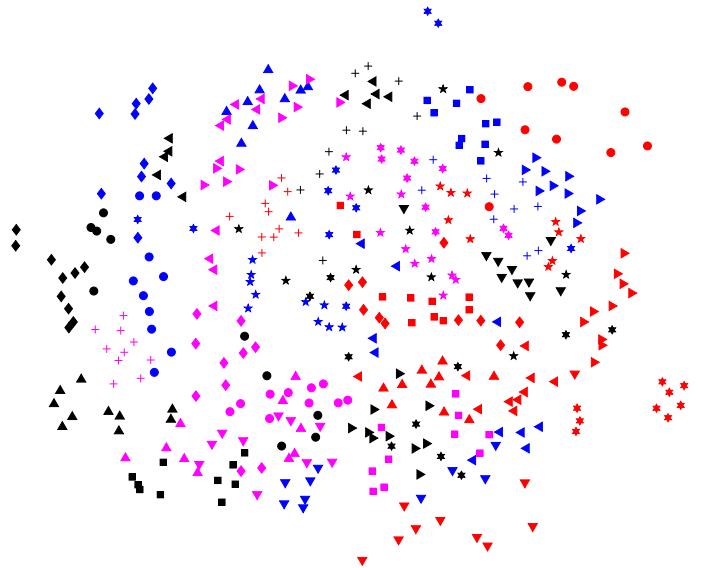
- Rare cell are “outliers” in the data.
- RaceID (Grun et al. 2015 Nature):
 - Normalize and log-transformed data.
 - Filter cells and genes
 - K-means clustering
 - Detect outliers from k-means result.
- GiniClust (Jiang et al. 2016 GB):
 - Difference is the gene filtering. It uses gini-index instead of variance to select genes.

t-SNE: a useful visualization tool

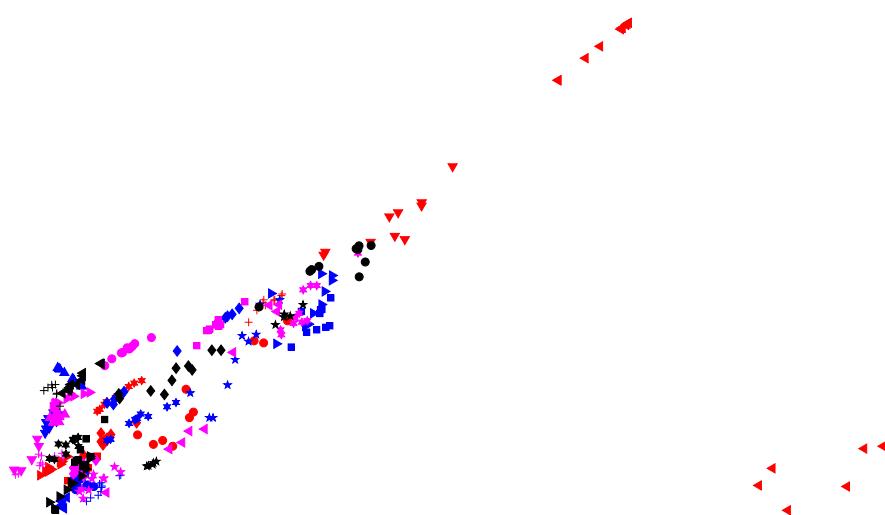
- t-SNE (t-distributed stochastic neighbor embedding): visualize high-dimensional data on 2-/3-D map.
- When project high-dimensional data into lower dimensional space, preserve the distances among data points.
 - This alleviate the problem that many clusters overlap on low dimensional space.
- Try to make the pairwise distances of points similar in high and low dimension.
- This is used in almost all scRNA-seq data visualization.
- Has “tsne” package on CRAN.



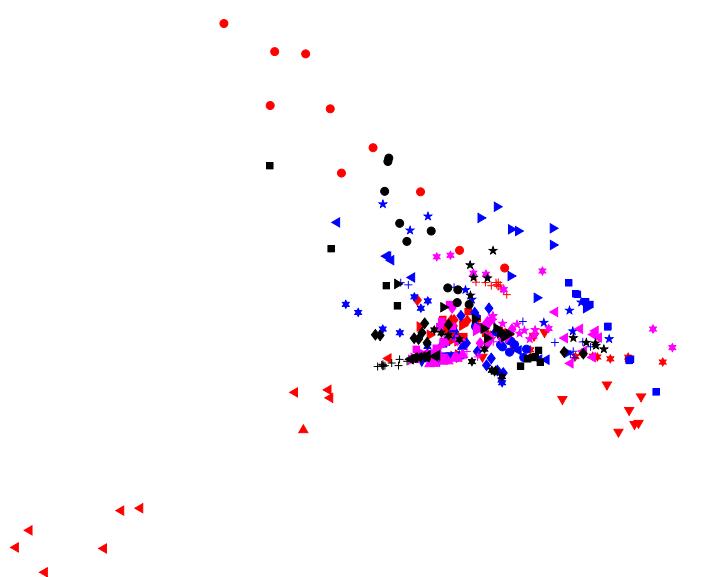
(a) Visualization by t-SNE.



(b) Visualization by Sammon mapping.



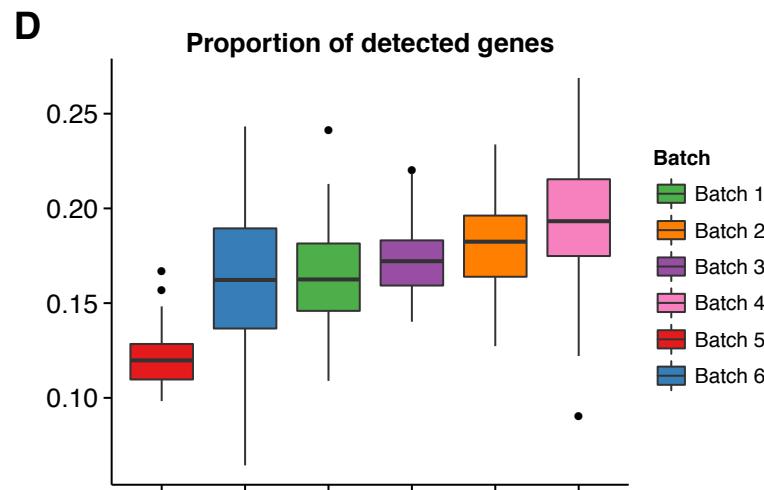
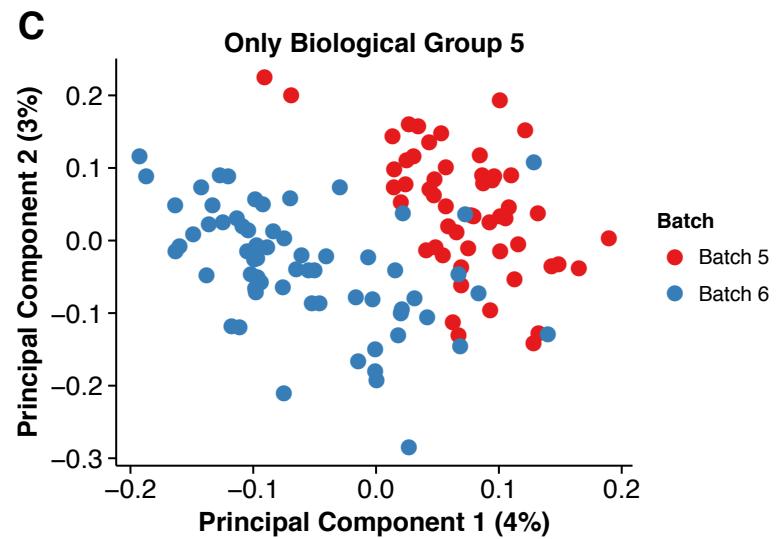
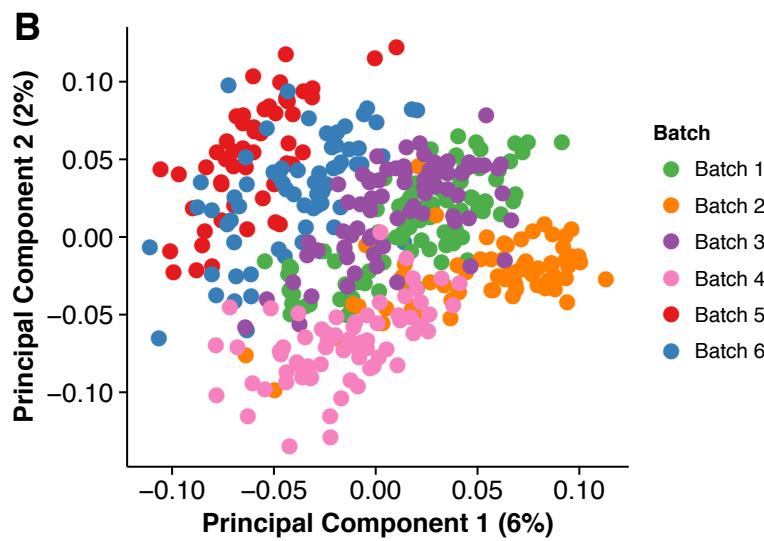
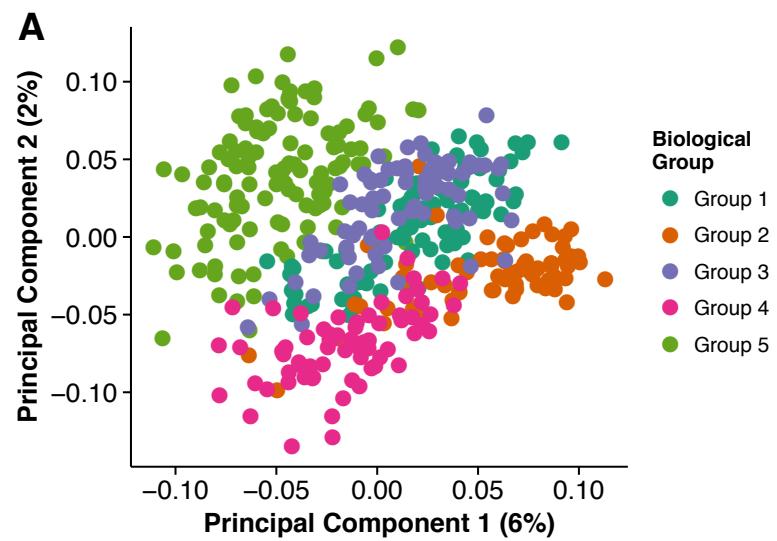
(c) Visualization by Isomap.



(d) Visualization by LLE.

Batch effect in scRNA-seq

(Hicks et al. 2016, bioRxiv)



Summary for scRNA-seq

- The main interests are inter-cellular heterogeneity, expression dynamics, cell type discovery.
- Statistical questions include normalization, differential expression and clustering.
- Batch effect could be a huge problem, and difficult to overcome.
- Rooms for model development.

Single cell GE microarray



Single cell-derived clonal analysis of human glioblastoma links functional and genomic heterogeneity

Mona Meyer^{a,1}, Jüri Reimand^{b,c,1}, Xiaoyang Lan^{a,b}, Renee Head^a, Xueming Zhu^a, Michelle Kushida^a, Jessica C. Pressey^e, Anath C. Lionel^{b,f}, Ian D. Clarke^{a,g}, Michael Cusimano^h, Jeremy A. Squireⁱ, Stephen Mark Bernstein^j, Melanie A. Woodin^e, Gary D. Bader^{b,c,2}, and Peter B. Dirks^{a,b,k,2}

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Single cell lncRNA

Genome Biology



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Localization and abundance analysis of human lncRNAs at single-cell and single-molecule resolution

Genome Biology

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Published online: 29 January 2015

Grand summary for scSeq

- Single-cell biology reveals a lot of information that can't be detected from bulk data.
- Data are much noisier, and more difficult to analyze.
- Methods are still under-developed, but quickly catching up.