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
  - Jointly perform several seq
- Very active research field in the past several years.

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)

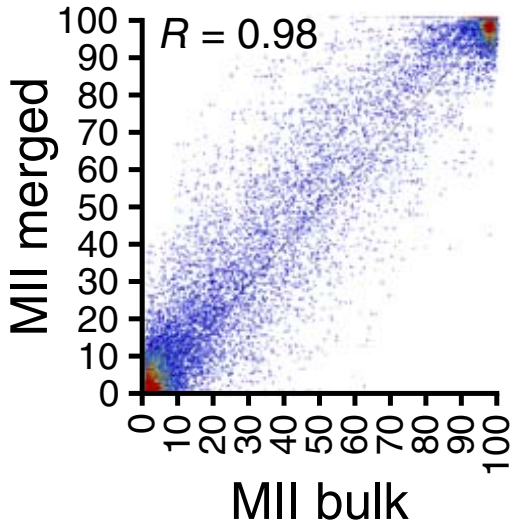
- For a comprehensive review, read Gawad *et al.* (2016) NRG.
- Examples of biological applications:
  - Identify and assemble the genome of unculturable microorganisms.
  - Determine the contribution of intra-tumor genetic heterogeneity in cancer development of treatment response.

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)

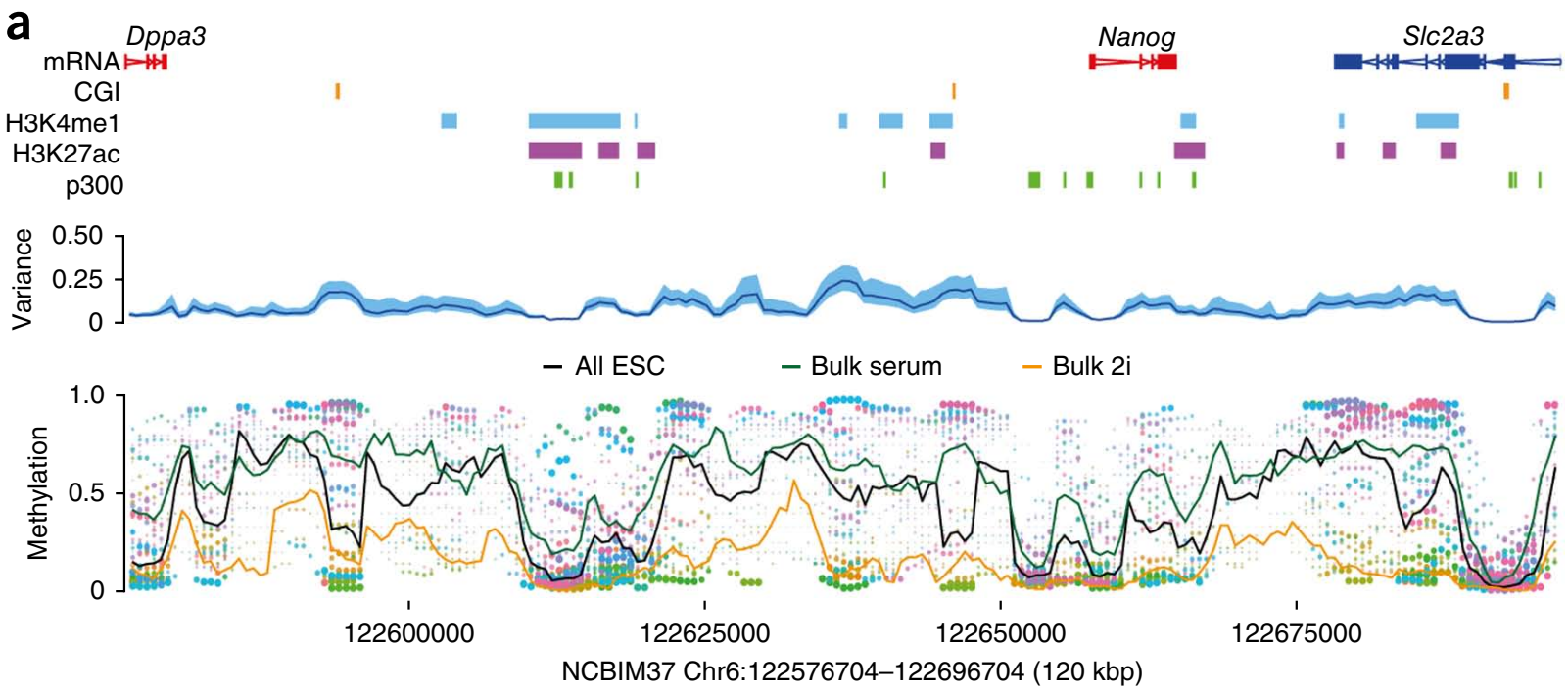
- Similar to scDNA-seq, but with bisulfite treatment before sequencing.
- There's scWGBS and scRRBS.
- The methylation levels from scBS-seq should be 0/1, with some exceptions caused by technical artifacts.
- Merged single cell and bulk data have good correlation.



Smallwood et al. 2014, NM

scBS-seq data analysis

- So far the data analysis are mostly descriptive:
  - compute variations among cells
  - Cell clustering



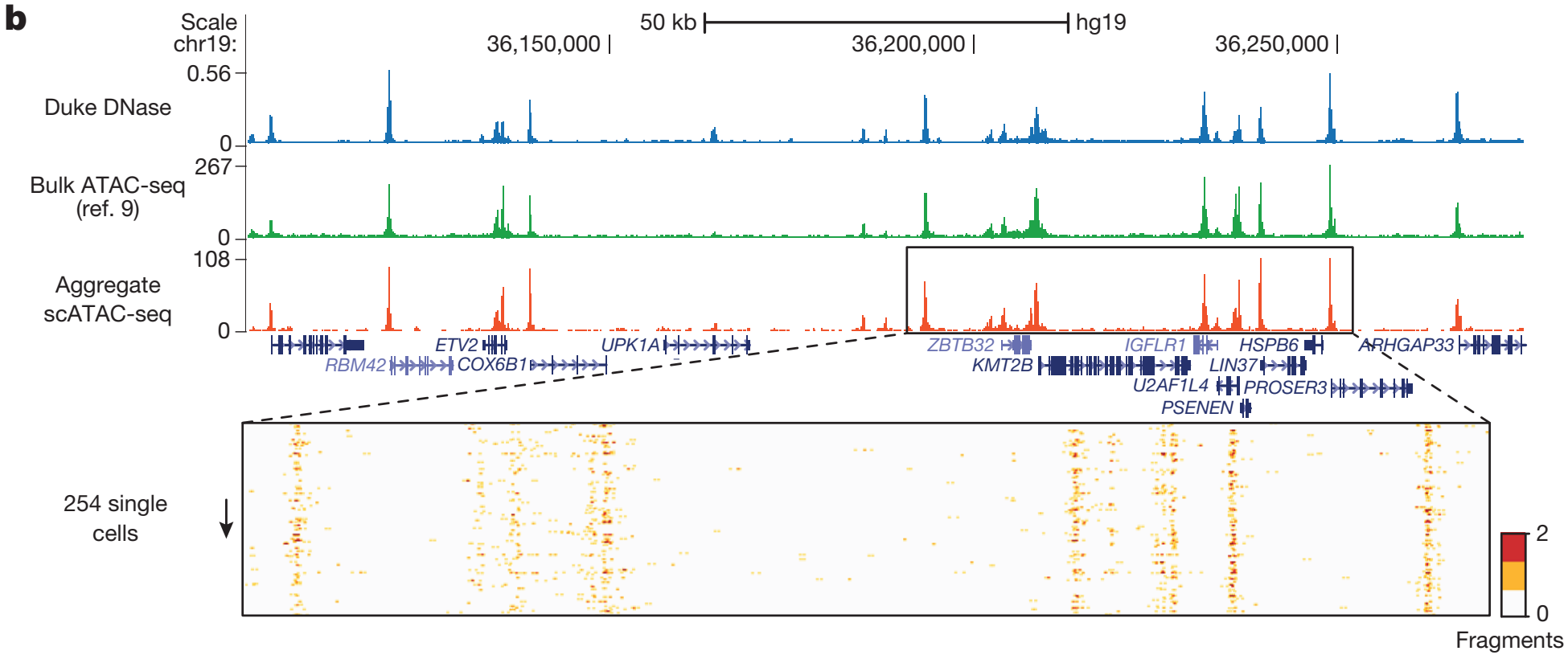
Smallwood et al. 2014, NM

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
  - Pott and Liet (2015) Genome Biology: review

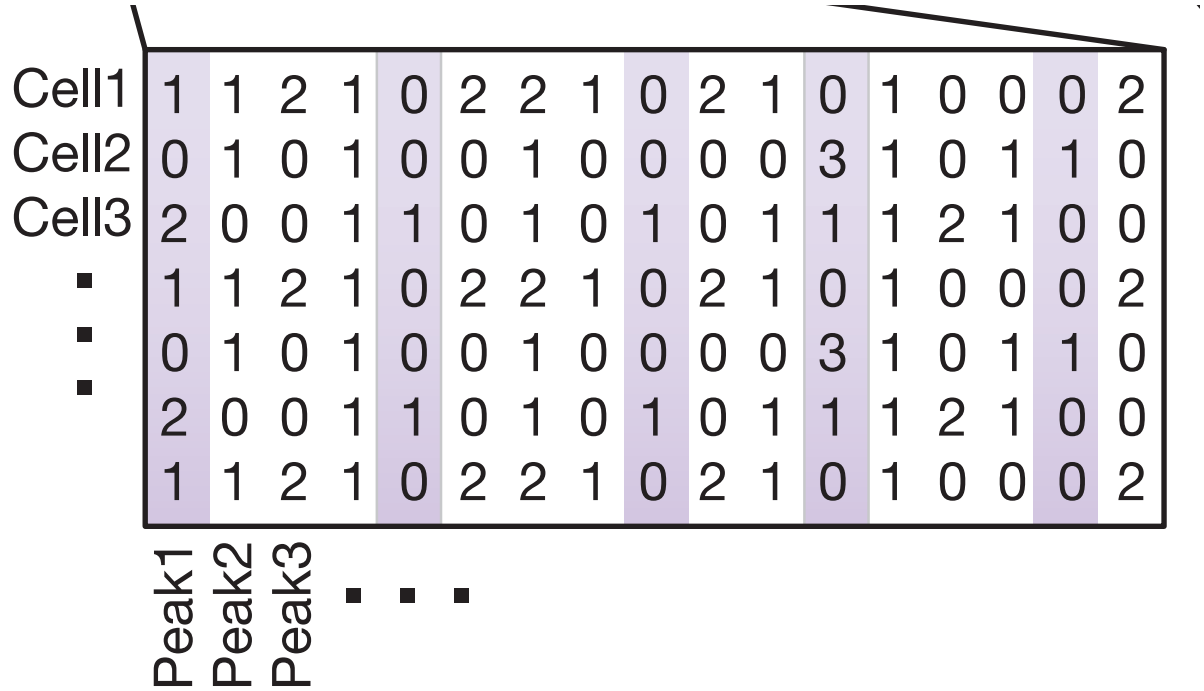
scChIP/scATAC-seq data

- Aggregated sc data has good agreement with bulk.



Buenrostro et al. 2015 Nature

- Very sparse: one 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



Buenrostro et al. 2015 Nature

Single cell RNA-seq (scRNA-seq)

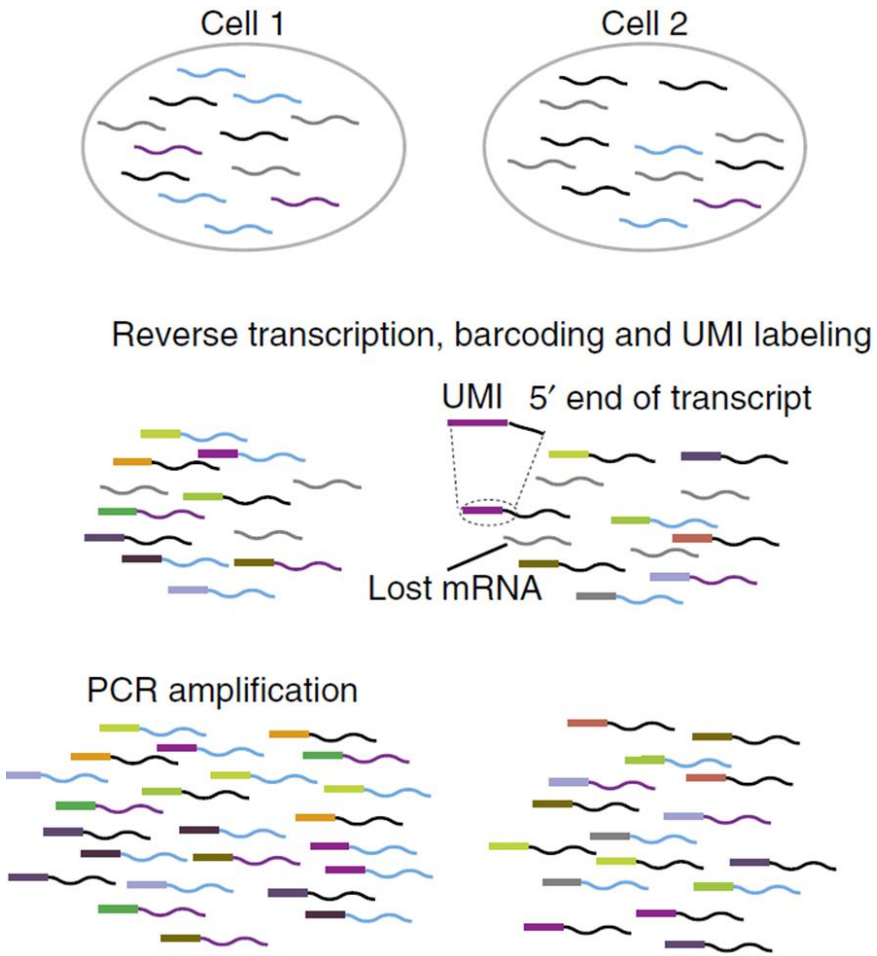
- The most active in the single cell field.
- Scientific goals:
  - Composition of different cell types in complex tissues.
  - New/rare cell type discovery.
  - Gene expression, alternative splicing, allele specific expression at the level of individual cells.
  - Transcriptional dynamics (pseudotime construction).
  - Above can be investigated and compared spatially, temporally, or under different biological condition.

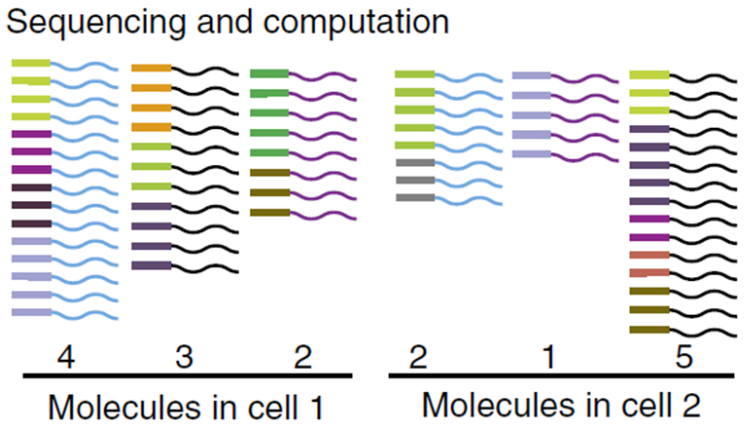
scRNA-seq technologies

- Full-length sequencing, such as Smart-Seq/Smart-Seq2
  - High sequencing depth
  - Better at detecting low expression genes
  - Good for isoform analysis, allele specific expression
- 3’ end sequencing: such as droplet-based (Drop-seq, inDrop, 10x genomics)
  - Many cells, low sequencing depth per cell
  - Good for identifying cell subpopulations

Universal molecular identifier (UMI)

- Short sequence tag added to the mRNA molecular before PCR, for reducing PCR bias.





Saiful Islam ··· Sten Linnarsson

scRNA-seq data analyses questions

- **Data preprocessing**
  - Normalization
  - Batch effect correction
  - Imputation
- **Data analyses**
  - Cell clustering
  - Pseudo-time construction
  - Cell type identification
  - Differential expression
  - Rare cell type discovery; alternative splicing; allele specific expression; RNA velocity
- **Visualization**
  - TSNE and UMAP

scRNA-seq data preprocessing

- Sequence alignment and expression quantification
  - RNA-seq alignment software (Tophat, STAR, HISAT, etc.) can be used
  - Some commercial software, such as Cell Ranger for 10x genomics data.

Some data characteristics

- Data is very sparse, especially for Drop-seq data.
- Number of transcripts detected is much lower compared to bulk RNA-seq, perhaps due to low capture and reverse transcription efficiencies.

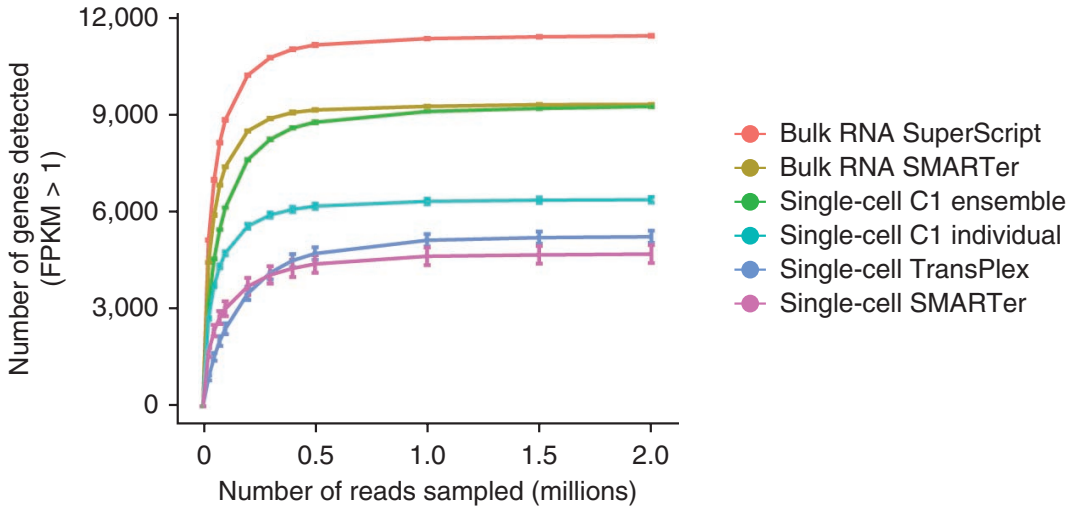
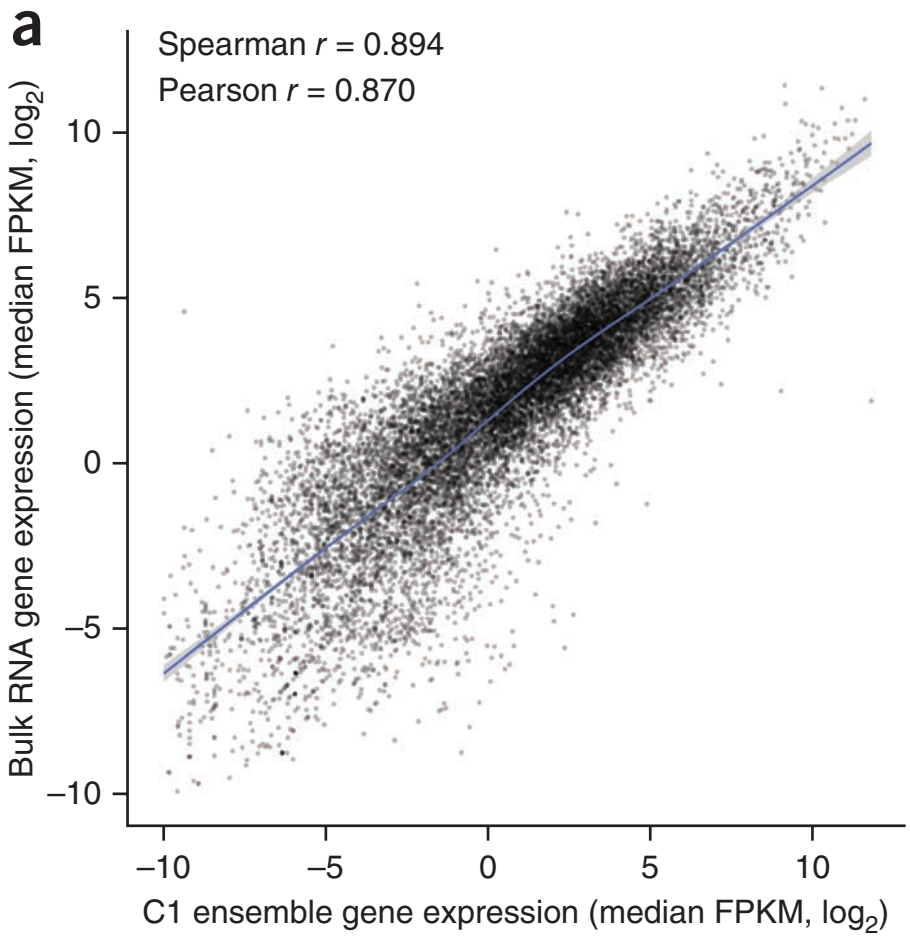


Figure 5 | Saturation curves for the different sample preparation methods. Each point on the curve was generated by randomly selecting a number of raw reads from each sample library and then using the same alignment pipeline to call genes with mean FPKM >1. Each point represents four replicate subsamplings.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

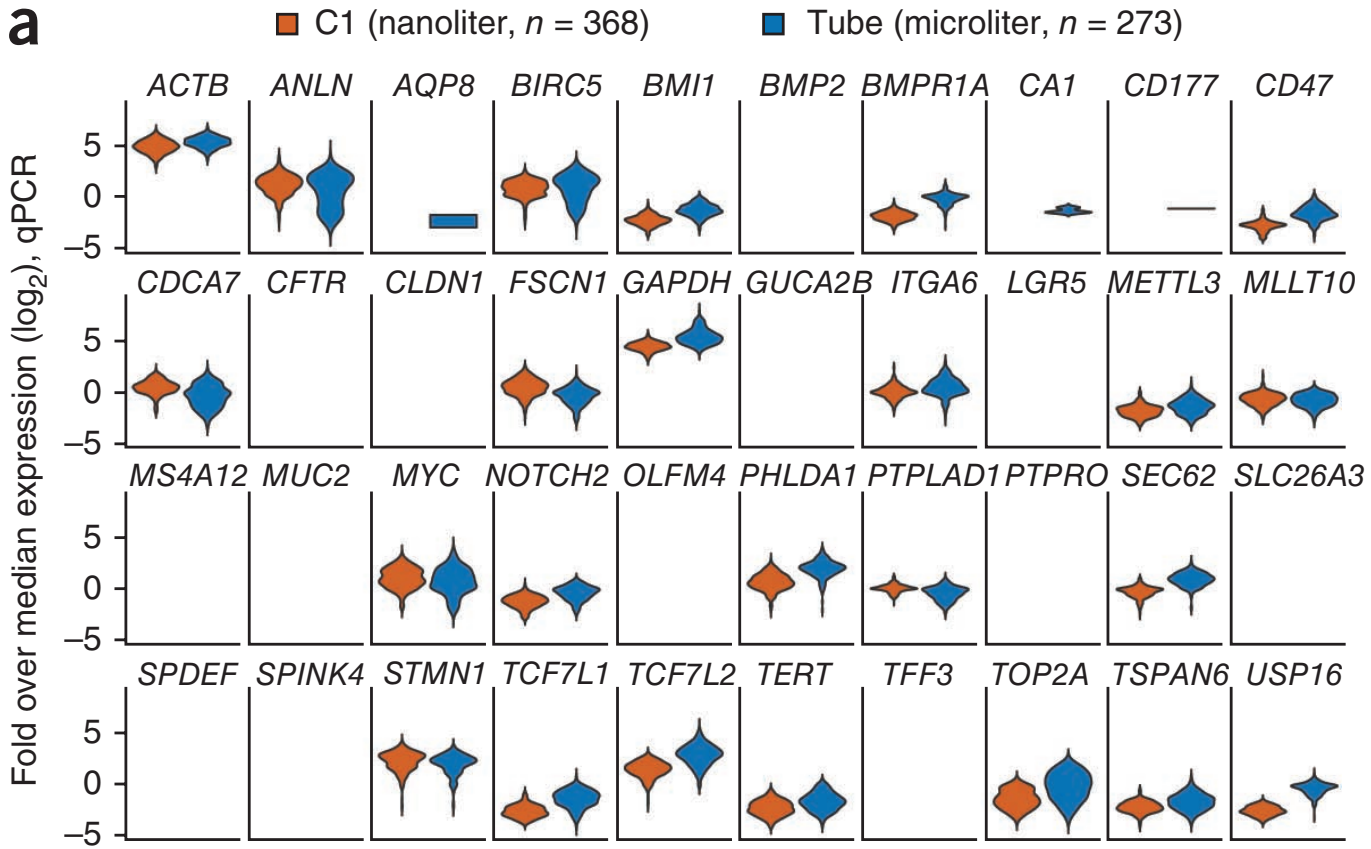
Wu et al. 2013 Nature Method

- Bulk and aggregated single cell expressions have good correlation.



Wu et al. 2013 Nature Method

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



Wu et al. 2013 Nature Method

Data normalization

- scRNA-seq is very noisy.
- Spike-in data is usually available.
  - Spike-ins from the external RNA Control Consortium (ERCC) panel contains 92 synthetic spikes based on bacterial genome with known expression level.
- UMI is helpful for removing amplification noise.
- A combination of spike-in and UMI can potentially be used for data normalization.

Application Note

Normalization and noise reduction for single cell RNA-seq experiments

Bo Ding[1,#], Lina Zheng[1,#], Yun Zhu[1], Nan Li[1], Haiyang Jia[1,2], Rizi Ai[1], Andre Wildberg[1] and Wei Wang[1,3*]

[1]Department of Chemistry and Biochemistry, University of California, La Jolla, CA 92093, USA,

[2] College of Computer Science and Technology, Jilin University, Changchun 130012, China.

[3]Department of Cellular and Molecular Medicine, University of California, La Jolla, CA 92093, USA,

[#]Equal contribution

Associate Editor: Dr. Ziv Bar-Joseph

ABSTRACT

- Assume the log-transform FPKM value is a function of the true expressions.
- Use MLE to estimate parameters based on ERCC controls. Then the fitted model is applied to all genes to estimate concentration.

Lun *et al. Genome Biology* (2016) 17:75
DOI 10.1186/s13059-016-0947-7

Genome Biology

METHOD Open Access

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

Aaron T. L. Lun[1*], Karsten Bach[2] 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.
- Bioconductor package **scran.**

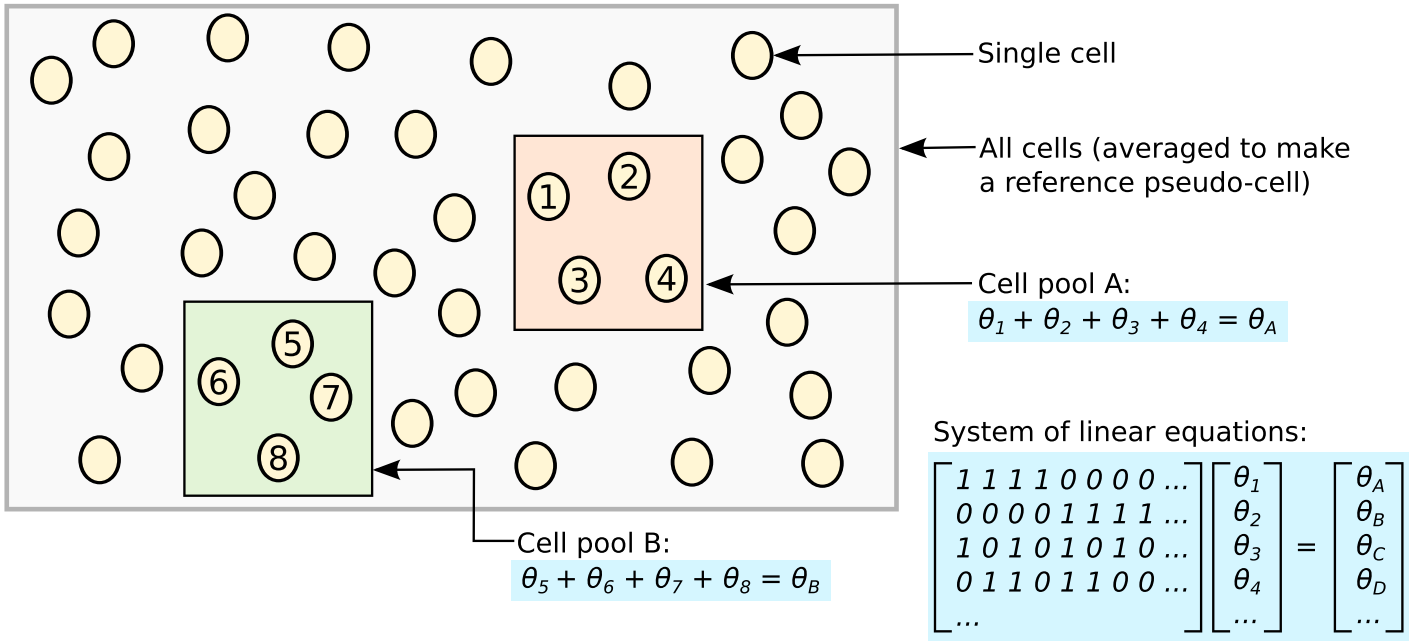


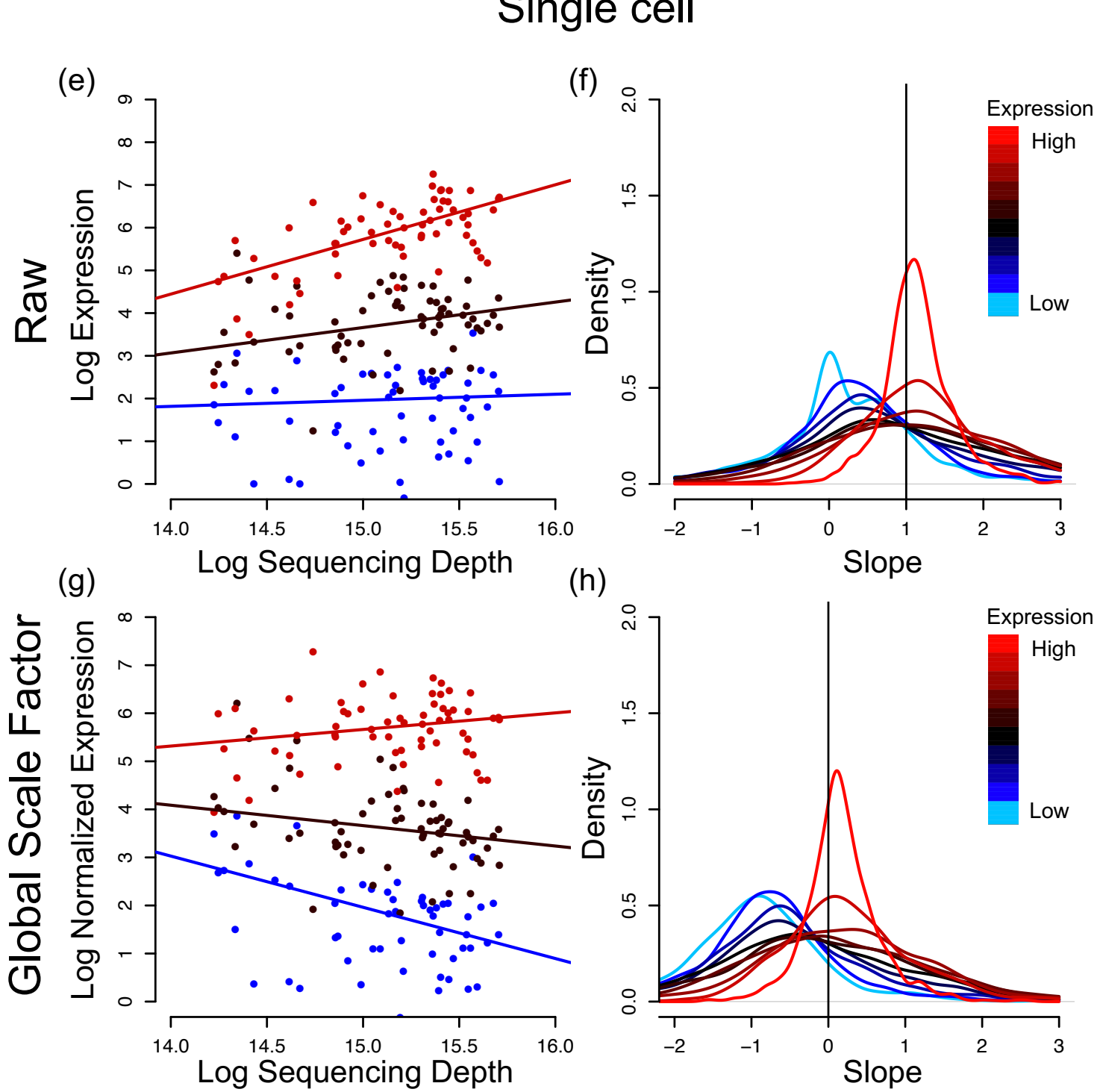
Fig. 3 Schematic of the deconvolution method. All cells in the data set are averaged to make a reference pseudo-cell. Expression values for cells in pool A are summed together and normalized against the reference to yield a pool-based size factor θ_A. This is equal to the sum of the cell-based factors 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: robust normalization of single-cell RNA-seq data

584 | VOL.14 NO.6 | JUNE 2017 | NATURE METHODS

Rhonda Bacher[1,5], Li-Fang Chu[2,5], Ning Leng[2], Audrey P Gasch[3], James A Thomson[2], Ron M Stewart[2], Michael Newton[1,4] & 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
(e)
(f)
Raw
Log Expression
Log Sequencing Depth
Density
Slope
Expression
High
Low
(g)
(h)
Global Scale Factor
Log Normalized Expression
Log Sequencing Depth
Density
Slope
Expression
High
Low

SCnorm 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.
- Bioconductor package **SCnorm**.

Batch effect correction

- Batch effect in scRNA-seq can be severe.
- It's difficult to randomize the design, i.e., batch is often confounded with individual, so it causes trouble for analyzing data from multiple individuals (more on this later).
- There are a number of methods specifically designed for scRNA-seq:
  - MNN (Haghverdi et al. 2018 Nat. Biotech.)
  - BUSseq (Song et al. 2020 Nat. Comm.)
  - scBatch (Fei and Yu, 2020 Bioinformatics)

Data imputation

- scRNA-seq has lots of missing data (dropout).
- Imputing the missing data help the downstream analyses.
- There are a number of methods:
  - SAVER (Huang et al. 2018 Nat. Methods)
  - ScImpute (Li et al. 2018 Nat. Comm.)
  - MAGIC (van Dijk et al. 2018 Cell)
  - SCRABBLE (Peng et al. 2019 GB)

General strategy for imputation

- The problem is similar to a “recommendation system”.
  - First compute the similarities among genes and cells.
  - To impute one element, borrow information from similar gene/cell.
- Use with caution:
  - It can enhance “wrong” signals.

Data analyses tasks

- Cell clustering
- Pseudotime construction
- Cell type identification
- Differential expression
- Rare cell type discovery
- Alternative splicing
- Allele specific expression
- RNA velocity

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.

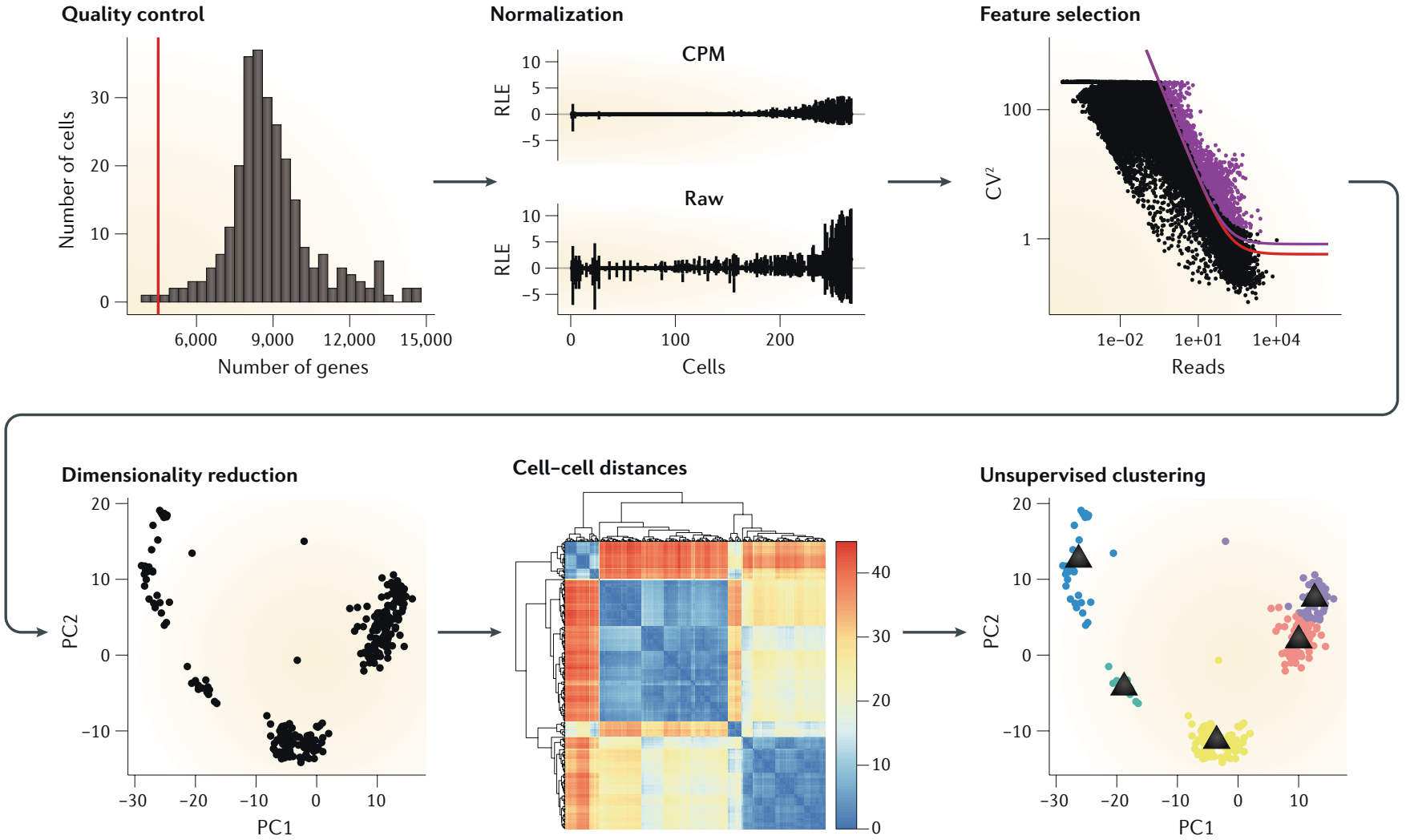
Cell clustering methods

- Many methods available
  - SC3, Seurat, TSCAN, Monocle, CIDR, …
  - Comprehensively compared in Duo et. al (2018) F1000 Research.
  - According to my experience: SC3 has the best performance, but is the slowest.

and robust [73]. Due to the heavy time consuming nature of consensus clustering, a rule of thumb for unsupervised single cell clustering is to use single-cell consensus clustering (SC3, integrated in Scater [52]) when the number of cells is < 5000 but use Seurat instead when there are more than 5000 cells.

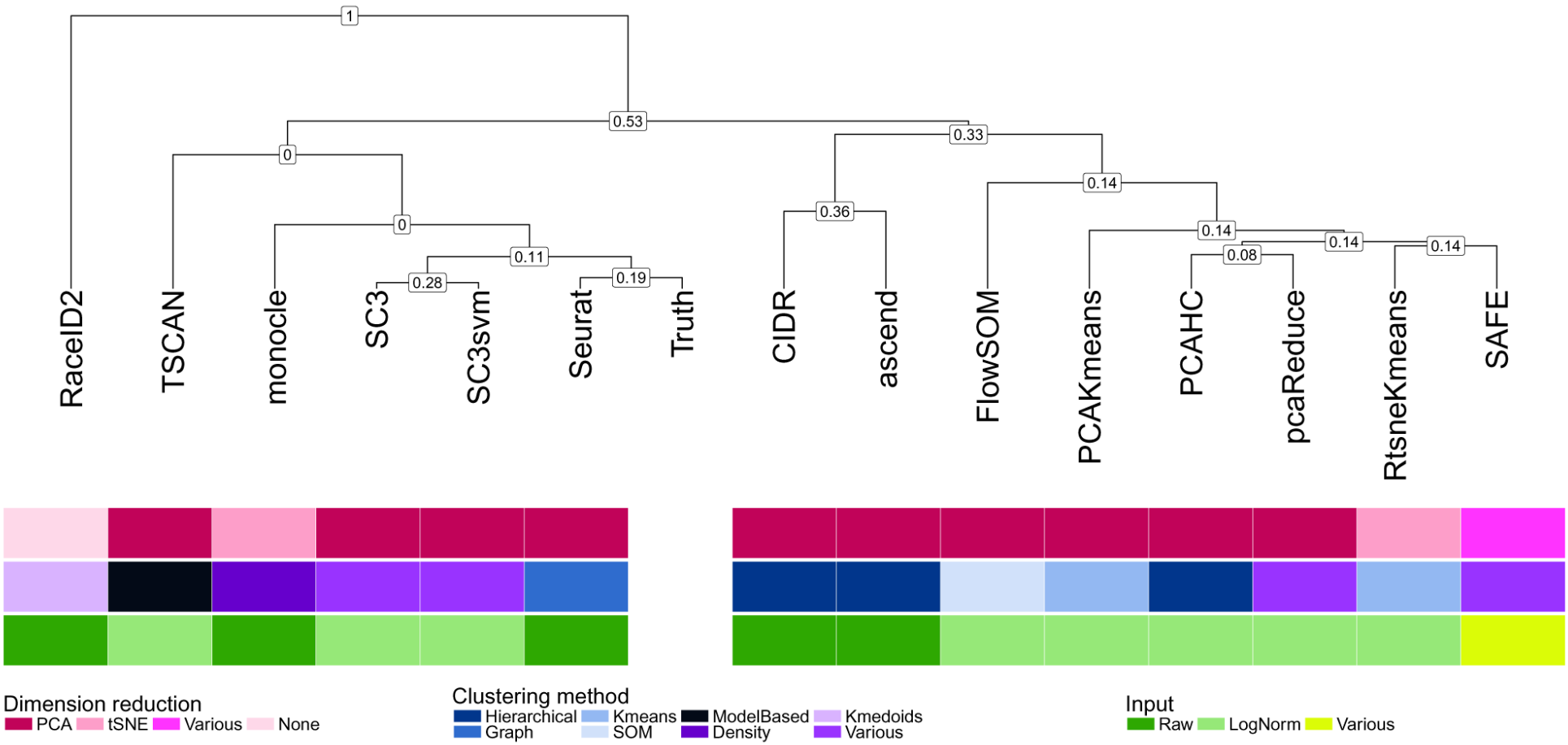
Mu et al. Genomics Proteomics Bioinformatics (2019)

Essence of the clustering methods



Kiselev et al. (2019) Nat. Rev. Genet.

Cell clustering methods



Duo et. al (2018) F1000 Research

Example codes for SC3

```
sce = SingleCellExperiment(
    assays = list(
        counts = as.matrix(counts),
        logcounts = log2(as.matrix(counts) + 1)
    )
)
sce = sc3_prepare(sce)
if( missing(K) ) { ## estimate number of clusters
    sce = sc3_estimate_k(sce)
    K = metadata(sce)$sc3$k_estimation
}

sce = sc3_calc_dists(sce)
sce = sc3_calc_transfs(sce)
sce = sc3_kmeans(sce, ks = K)
sce = sc3_calc_consens(sce)
result = colData(sce)[,1]
```

Example code for Seurat

```
seuset = CreateSeuratObject( counts )
seuset = NormalizeData(object = seuset)
seuset = FindVariableFeatures(object = seuset)
seuset = ScaleData(object = seuset)
seuset = RunPCA(object = seuset)
seuset = FindNeighbors(object = seuset)
seuset = FindClusters(object = seuset)
Result = seuset@active.ident
```

Pseudotime construction

- This belongs to the “clustering” category.
- Instead of putting cells into independent, exchangeable groups, it orders the cells by underlying temporal stage (estimated).
- Methods/tools:
  - Monocle/monocle2: Trapnell et al. (2014) Nat. Biotechnol; Qiu et al. (2017) Nat. Methods.
  - Waterfall: Shin et al. (2015) Cell Stem Cell
  - Wanderlust: Bendall et al. (2014) Cell
  - TSCAN: Ji et al. (2016) NAR

Pseudotime construction method

General steps:

1. Select informative genes.
2. Dimension reduction of GE.
3. Cluster the cells based on reduced data. Often want to over-cluster them to have many groups.
4. Construct a MST (miminum spanning tree) from the clustering results.
5. Map cells to the MST.

Cell clustering for multiple samples

- When scRNA-seq data are from multiple samples, batch effects could have significant impact on the results.
- Cells from the same sample, instead of the same cell type form different sample, can cluster together.
- Possible solution:
  - Remove batch effect then cluster: MNN + SC3
  - Jointly model cell type and sample effect: BAMM-SC (Sun et al. 2019, Nat. Comm)
- Still an open problem.

Cell type annotation

- Another paradigm to identify cell type.
- Cell clustering:
  - Cluster cells to multiple clusters (unsupervised). then assign cell type for each cluster.
- Cell type assignment:
  - Directly assign each cell to a cell type.
  - Requires some reference, or training data (supervised).
  - Potentially work better for data from multiple samples.
  - Can incorporate the hierarchy in cell types.
  - Cannot identify new cell types (restricted to the known cell types in the reference).

Cell annotation methods

- Pre-train a classifier using training set with generic machine learning methods: SVM, LDA, RF, kNN, RF
  - scmap (Kiselev et al. 2018 Nat. Methods)
  - CaSTLe (Lieberman et al. 2018 Plos One)
  - Garnett (Pliner et al. 2019 Nat. Methods)
  - CHETAH (Kanter et al. 2019 Nucleic Acids Research)
- Marker-based classifier
  - CellAssign (Zhang et al. 2019 Nat. Methods)
- Comprehensively compared in Abdelaal et al. Genome Biology 2019
- Annotation performance is a trade-off between accuracy and un-assigned rate

scmap: projection of scRNA-seq data across datasets

- Correlation based assignment
- User can specify a threshold. Cells below the threshold are "unassigned"

```
sce <- SingleCellExperiment(assays =
    list(normcounts = as.matrix(trainmat)),
    colData = DataFrame(cell_type1 = trainlabel))
logcounts(sce) <- log2(normcounts(sce) + 1)
rowData(sce)$feature_symbol <- rownames(sce)
sce <- selectFeatures(sce, suppress_plot = TRUE)

sce_test <- SingleCellExperiment(assays =
    list(normcounts = as.matrix(testmat)),
    colData = DataFrame(cell_type1 = testlabel))
logcounts(sce_test) <- log2(normcounts(sce_test) + 1)
rowData(sce_test)$feature_symbol <- rownames(sce_test)

sce <- indexCluster(sce)
scmapCluster_results <- scmapCluster(projection = sce_test,
        index_list = list(metadata(sce)$scmap_cluster_index))
```

CHETAH: a selective, hierarchical cell type identification method for single-cell RNA sequencing

- Adopt a hierarchical structure when assign the cells
- Allow intermediate or unassigned categories
- Especially good when cells of unknown type are encountered, e.g. tumor

```
sce_train <- SingleCellExperiment(assays =
    list(counts = as.matrix(trainmat)),
        colData = DataFrame(celltypes=trainlabel))

sce_test <- SingleCellExperiment(assays =
    list(counts = as.matrix(testmat)),
         colData = DataFrame(celltypes = testlabel))

#run classifier
test <- CHETAHclassifier(input = sce_test,
                         ref_cells = sce_train)
test$celltype_CHETAH
```

Differential expression (DE)

- DE analysis is the most important task for bulk expression data (microarray or RNA-seq).
- DE in scRNA-seq is a little different:
  - Traditional methods test mean changes, while the consideration and modeling of “drop-out” event (non-expressed) is important in sc data.
  - Considering cell types: can compare cross cell types or compare the same cell type cross biological conditions.

DE methods

- SCDE (Kharchenko et al. 2014 Nat. Methods)
- MAST (Finik et al. 2015 GB)
- SC2P (Wu et al. 2018 Bioinformatics)
- Seurat and monocle also provides DE functions.
- Bulk methods (DESeq, edgeR) are sometimes used.
- A comparison paper: Soneson and Robinson (2018) Nat. Methods

Finak *et al. Genome Biology* (2015) 16:278
DOI 10.1186/s13059-015-0844-5

Genome Biology

METHOD Open Access

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 Deng[1], Vivian Gersuk[2], Alex K. Shalek[3,4,5,6], Chloe K. Slichter[1], Hannah W. Miller[1], M. Juliana McElrath[1], Martin Prlic[1], Peter S. Linsley[2] and Raphael Gottardo[1,7*]

- MAST: "Model-based Analysis of Single- cell Transcriptomics."
- Bioconductor package **MAST**.

MAST for DE

- Main ideas:
  - Use log2(TPM+1) as input data
  - Both dropout probability and expression level depends on experimental conditions.

$$logit\left(Pr(Z_{ig}=1)\right)=\mathrm{X_i}\,\beta_{\mathrm{g}}^{\mathrm{D}}$$

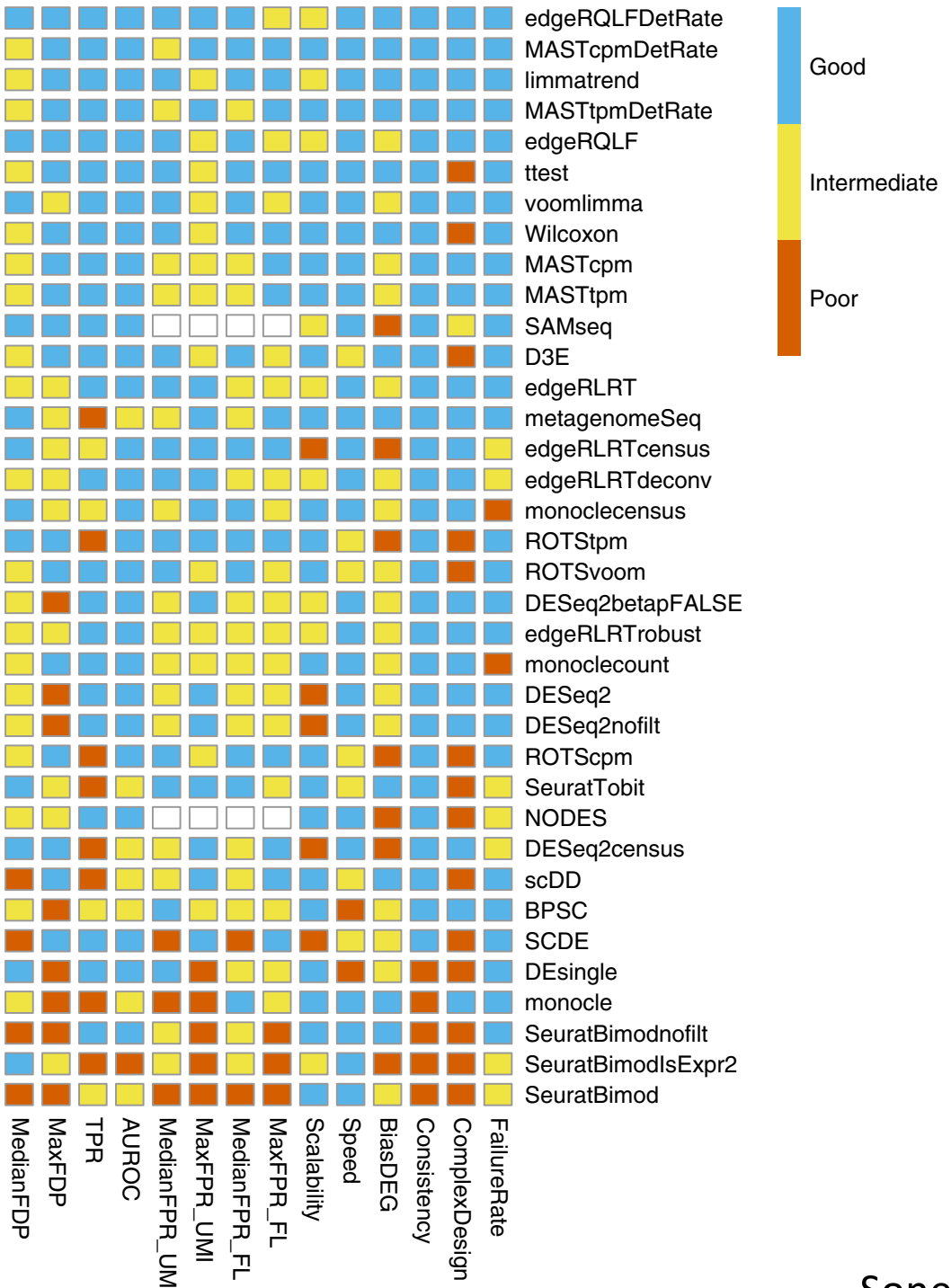
$$\Pr\left(Y_{ig}=y|Z_{ig}=\ 1\right)=\ \mathrm{N}\left(\mathrm{X_i}\beta_{\mathrm{g}}^{\mathrm{C}},\ \sigma_g^2\right)$$

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

Example codes for MAST

- Start from log TPM and biological condition

```
sca <- FromMatrix(ltpm,
       cData=data.frame(celltype))
cdr2 <- colSums(assay(sca)>0)
colData(sca)$cngeneson <- scale(cdr2)
thres <- thresholdSCRNACountMatrix(assay(sca),
       nbins=200, min_per_bin=30)
assays(sca) <- list(thresh=thres$counts_threshold,
              tpm=assay(sca))
## fit model and perform test
fit <- zlm(~celltype, sca)
lrt <- lrTest(fit, "celltype")
```



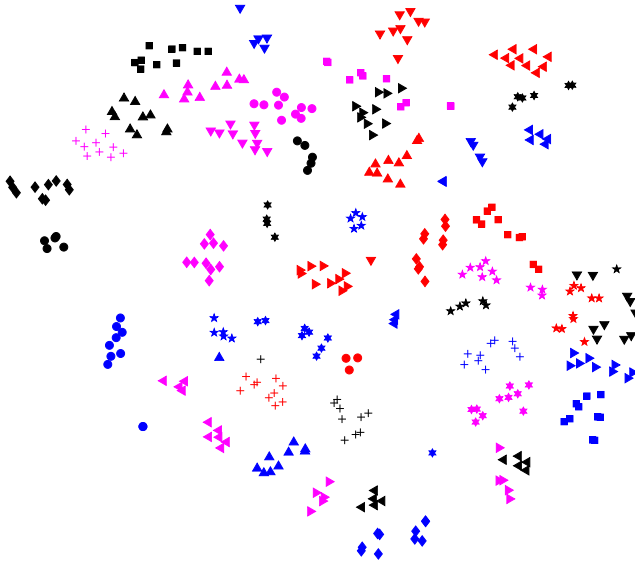
Soneson and Robinson (2018) Nat. Methods

Visualization

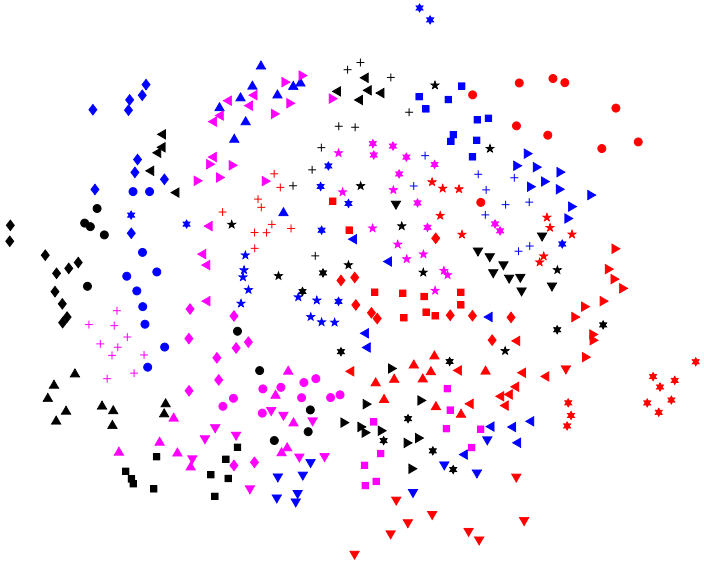
- TSNE
- UMAP

t-SNE: a useful visualization tool

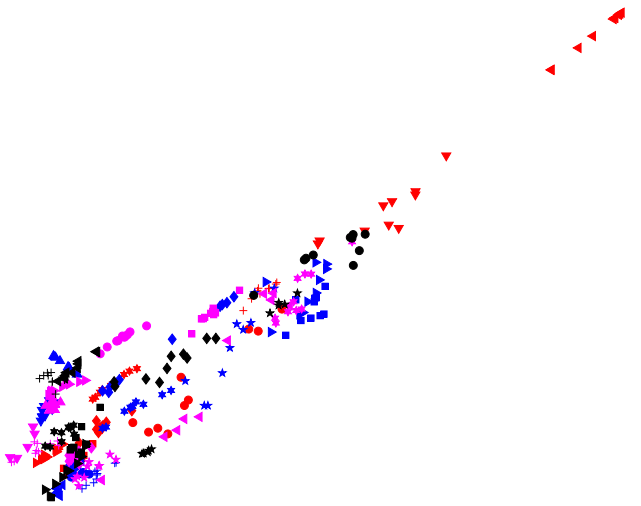
- t-SNE (t-distributed stochastic neighbor embedding): visualize high-dimensional data on 2-/3-D map.
- When project high-dimensional data into lower dimensional space, preserve the distances among data points.
  - This alleviate the problem that many clusters overlap on low dimensional space.
- Try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



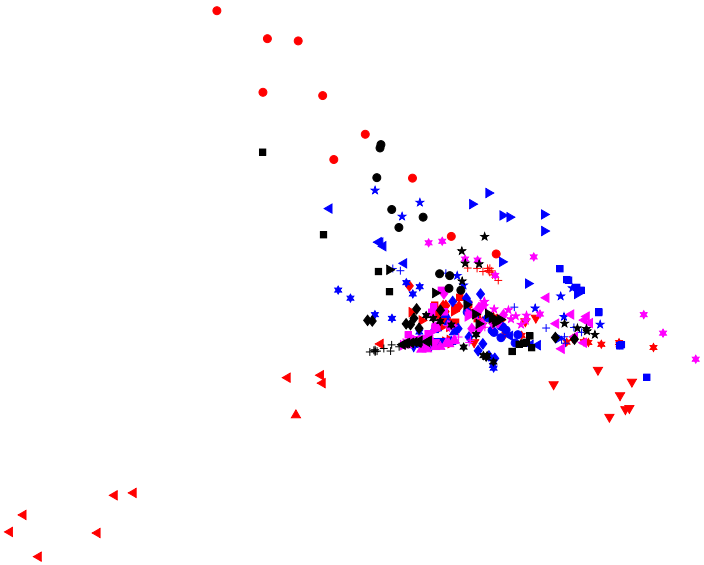
(a) Visualization by t-SNE.



(b) Visualization by Sammon mapping.



(c) Visualization by Isomap.



(d) Visualization by LLE.

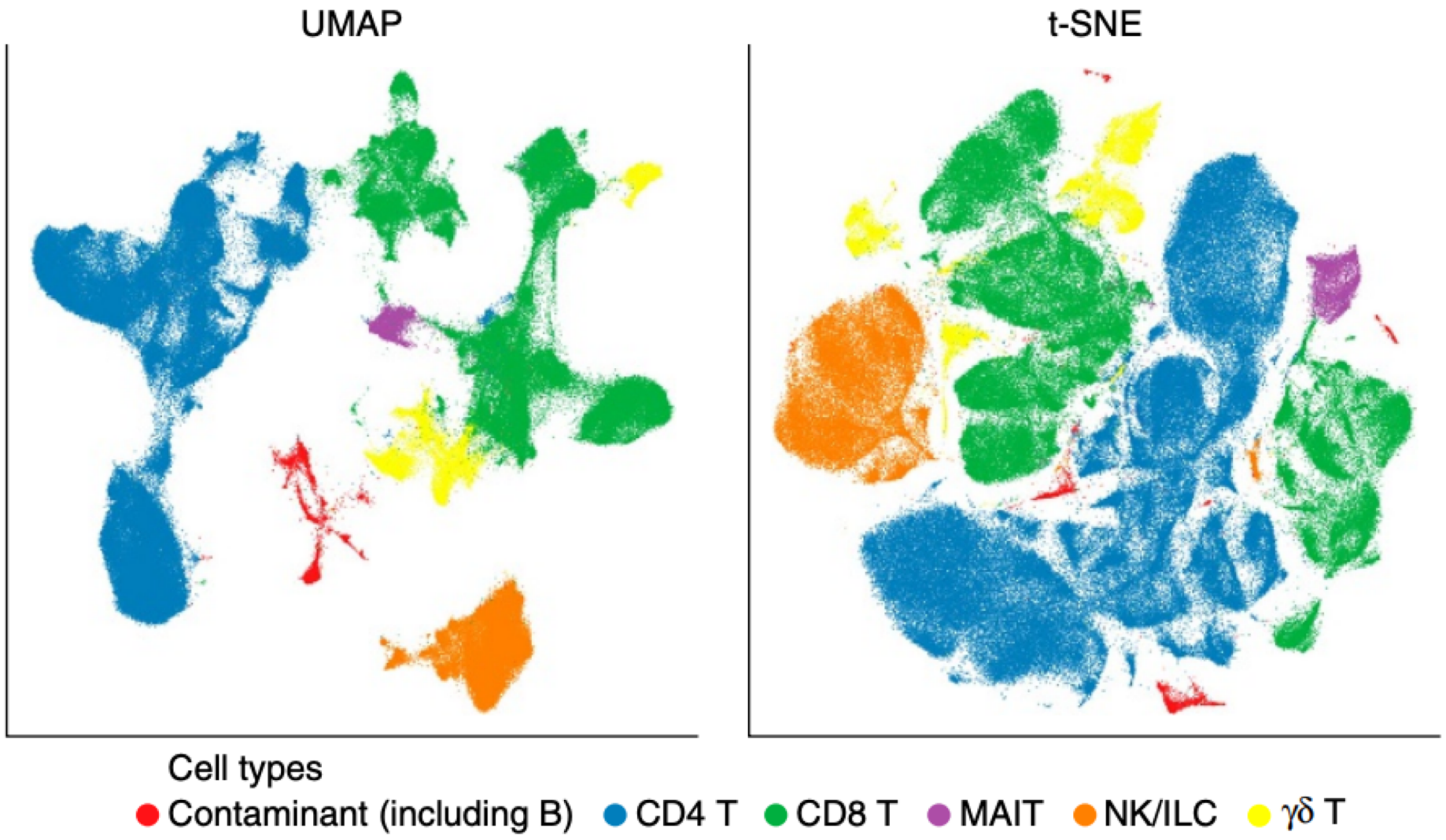
Example code for t-SNE

```
library(Rtsne)
tsne_model_1 = Rtsne(datamatrix,  check_duplicates=FALSE, pca=TRUE,
                perplexity=30, theta=0.5, dims=3)
tsne_out = as.data.frame(tsne_model_1$Y)

pdf(”your_figure_name.pdf", width = 5, height = 5)
par(mar = c(2.4, 2.4, 0.5, 0.5), mgp = c(1.2, 0.4, 0))
plot(tsne_out$V1, tsne_out$V2,  pch = 19, cex = 0.4, col = mycolor)
legend("bottomleft", col = mycolor,  legend = uniqCT, pch = 19,
       cex = 0.5, bty = "n")
dev.off()
```

UMAP: a newer (and better?) visualization tool

- UMAP (uniform manifold approximation and projection): a recently developed dimension reduction tool
- "*Comparing the performance of UMAP with five other tools, we find that UMAP provides the fastest run times, highest reproducibility and the most meaningful organization of cell clusters.* " ---- Betcht et al. 2018 Nat Biotech
- "*UMAP, which is based on theories in Riemannian geometry and algebraic topology, has been developed, and soon demonstrated arguably better performance than t-SNE due to its higher efficiency and better preservation of continuum.*" ---- Mu et al. 2018 GBP
- Has "umap" package on CRAN.



Betcht et al. 2018 Nat Biotech

Example code for UMAP

```
library(umap)
sim_umap <- umap(datamatrix)
sim_umap2 <- sim_umap$layout
colnames(sim_umap2) <- c("UMAP1", "UMAP2")

pdf(”your_figure_name.pdf", width = 5, height = 5)
par(mar = c(2.4, 2.4, 0.5, 0.5), mgp = c(1.2, 0.4, 0))
plot(sim_umap2[,1], sim_umap2[,2], pch = 19, cex = 0.4, col = mycolor)
legend("bottomleft", col = mycolor, legend = uniqCT, pch = 19,
            cex = 0.5, bty = "n")
dev.off()
```

Summary for scRNA-seq

- The main interests are inter-cellular heterogeneity, expression dynamics, cell type discovery, etc.
- Many statistical methods and computational tools for different biological questions.
  - Data pre-processing: normalization, batch effect, imputation
  - Cell clustering and cell type annotation
  - Differential expression

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.
- Some rooms for method development, but very competitive.