An evaluation of differential analysis in single-cell RNA-seq analysis

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Overview

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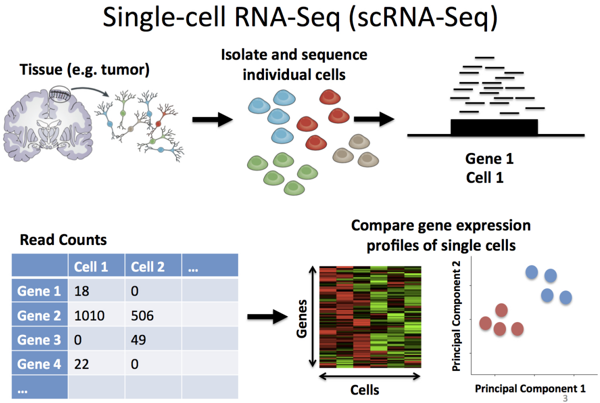
Single-cell RNA Sequencing Overview



Single-cell RNA-Seq (scRNA-Seq)

	Cell 1	Cell 2	...
Gene 1	18	0	
Gene 2	1010	506	
Gene 3	0	49	
Gene 4	22	0	
...			

Figure: *A general overview of scRNA-seq. Source: https : //learn.gencore.bio.nyu.edu/single − cell − rnaseq/*

Use of Single-cell RNA sequencing

- Bulk RNA-seq measure the average level of gene expression of multiple cells
- Single-cell RNA-seq allow us to understand gene expression pattern within the cell
- Single-cell RNA-seq can identify cell heterogeneity, cell population and sub-population
- Single-cell RNA-seq can examine the effects of low copy mRNA distribution and transcriptional regulation

Single-cell RNA Sequencing Applications

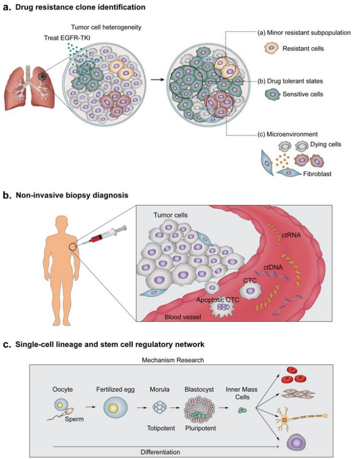


Figure: *Application of single-cell RNA sequencing technology in biological and biomedical research. More could be found from:* $https://www.nature.com/articles/s12276-018-0071-8$

Single-cell RNA sequencing differential analysis methods in Bioconductor/R

- DEsingle (Bioinformatics, 2018)
- Linnorm (Nucleic Acids Research, 2017)
- Monocle2 (Nature Methods, 2017)
- MAST (Genome Biology, 2015)
- DESeq2 (Genome Biology, 2014)

Motivation for evaluating RNA sequencing data analysis methods

- Multiple methods provide inconsistent results for the same dataset
- Exploring false discovery rate control (FDR), sensitivity, specificity, accuracy, and AUC under the ROC curve across different methods for the same dataset
- Proving guidance for investigators to choose appropriate method for their singlle-cell RNA sequencing data analysis

Definition of FDR, Sensitivity, Specificity, Accuracy

	number not rejected	number rejected	
true null hypotheses	U	V	m_0
non-true null hypotheses	T	S	m_1
total	$m - R$	R	m

Table: *Possible outcomes from m hypotheses tests*

$$FDR = E(\frac{V}{R})Pr(R > 0),$$

$$Sensitivity = E(\frac{S}{m_1}), \quad Specificity = E(\frac{U}{m_0})$$

$$Accuracy = E(\frac{U+S}{m})$$

AUC under the ROC curve is calculated using the AUC function in the pROC package in R.

DEsingle

DEsingle use the Zero-Inflated Negative Binomial (ZINB) model to decrible the read counts and excess zeros in single-cell RNA sequencing data. The count data for gth gene in a group of cell are assumed to follow ZINB distribution:

$$Pr(Y_g = y|\theta, r, p) = \theta \times I(y = 0) + (1 - \theta) \times f_{NB}(r, p),$$

where θ is the proportion of constant zeros of gene g in the group of cells, $I(y = 0)$ is an indicator function, $f_{NB}(r, p)$ is the probability mass function of Negative Binomial distribution with parameters r and p.

- DEsingle use likelihood-ratio tests for gene differential analysis.

Linnorm

- Linnorm proposed a novel normalization and transformation method for single-cell RNA-seq analysis.
- The normalization and transformation parameters were calculated based on stably expressed genes across different cells.
- The moderated t test statistics in the limma package for differential analysis through the empirical Bayes approach.

Monocle2

- Monocle2 use census algorithm to convert relative RNA-seq expression levels into relative transcript counts without the need for experimental spike-in controls.
- The census algorithm calculate the total number of single-mRNA genes and divide this number by the fraction of the library contributed by them to estimate the total number of captured mRNAs in the cell and then rescale the transcript per million (TPM) in single cell values into mRNA counts for each gene.
- Monocle2 tests gene differential analysis through a likelihood ratio test for comparing a full generalized linear model with additional effect to a reduced generalized linear model based on negative binomial distributions.

MAST

- MAST propose a hurdle model approach for scRNA-seq data analysis.
- In the scRNA-seq expression data Y_{ig}, the rate of expression and the level of expression for the expressed cells are assumed conditionally independent for each gene g.
- MAST use an indicator variable Z_{ig} to denote whether gene g is expressed in cell i ($z_{ig} = 0$ if $y_{ig} = 0$ and $z_{ig} = 1$ if $y_{ig} > 0$).
- MAST fits a logistic regression for the discrete variable Z and a normal distributed linear model for the continuous variable $(Y|Z = 1)$ independently.

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

DESeq2

- DESeq2 uses a generalized linear model approach to accommodate complex study designs.
- DESeq2 uses a logarithm link between relative gene abundance and design matrix.
- DESeq2 integrates the dispersion estimate and fold change estimate using empirical Bayes approach and test the differential expression using a Wald test.

Simulation Set up

- RNA sequencing count data are generated from negative binomial distributions using RnaXSim function in R
- The means and variance are generated using real RNA sequencing count data
- 1000 genes and 20 independent simulations
- Fraction of differentially expressed genes (π_1) were set at 5%, 10%, 20%, 30%, 40% and 50%
- Sample sizes are 5, 10, and 15 in each group with two-group comparisons

FDR, Sensitivity, Specificity, and Accuracy comparisons for sample size 5 in each group

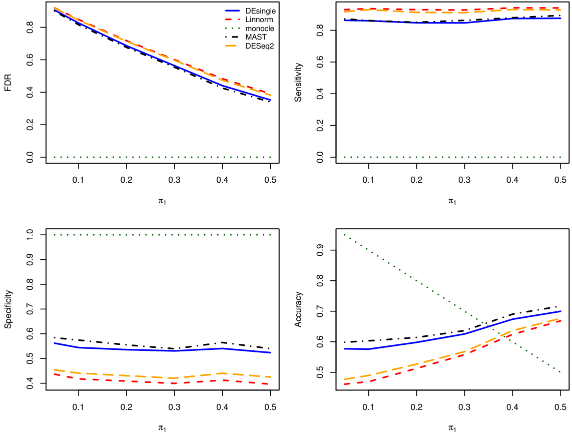


Figure: *FDR, Sensitivity, Specificity, and Accuracy comparisons for sample size* 5 *in each group*

AUC under ROC curves comparisons for sample size 5 in each group

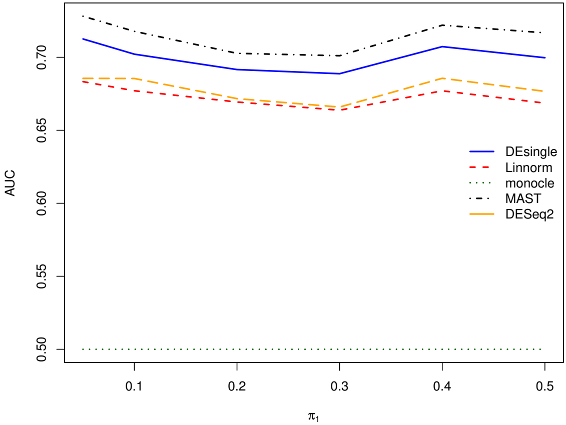


Figure: *AUC under ROC curves comparisons for sample size* 5 *in each group*

Boxplot of comparing performance indicators for sample size of 5 in each group

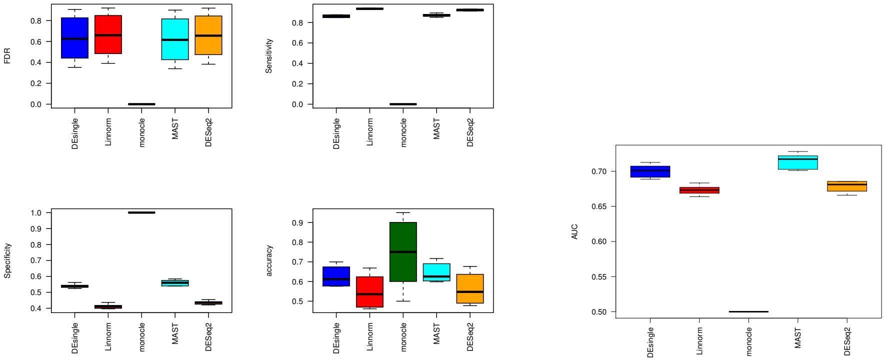


Figure: *Blue: DEsingle; Red: Linnorm; Darkgreen: Monocle; Cyan: MAST; Orange: DESeq2.*

FDR, Sensitivity, Specificity, and Accuracy comparisons for sample size 10 in each group

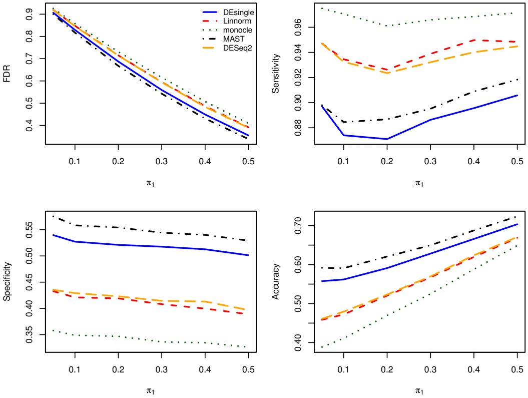


Figure: *Box plots of simulation results for $n = 6$ and equal library size*

AUC under ROC curves comparisons for sample size 10 in each group

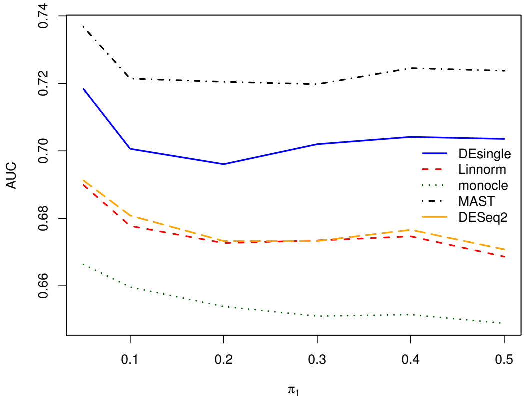


Figure: *AUC under ROC curves comparisons for sample size* 10 *in each group*

Boxplot of comparing performance indicators for sample size of 10 in each group

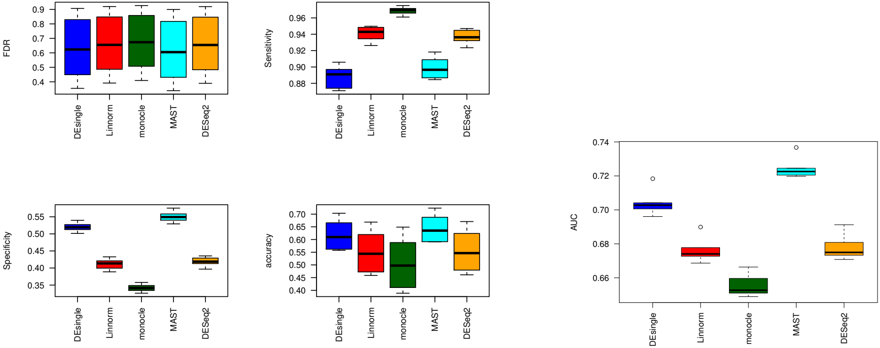


Figure: *Blue: DEsingle; Red: Linnorm; Darkgreen: Monocle; Cyan: MAST; Orange: DESeq2.*

FDR, Sensitivity, Specificity, and Accuracy comparisons for sample size 15 in each group

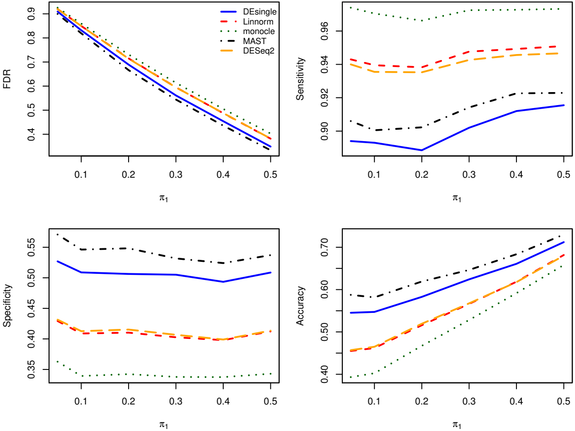


Figure: *FDR, Sensitivity, Specificity, and Accuracy comparisons for sample size* 15 *in each group*

AUC under ROC curves comparisons for sample size 15 in each group

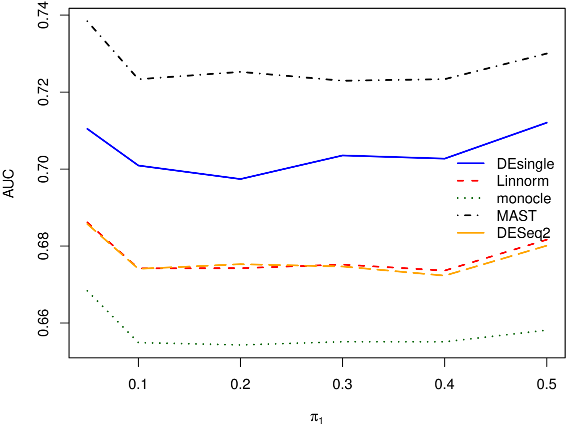


Figure: *AUC under ROC curves comparisons for sample size* 15 *in each group*

Boxplot of comparing performance indicators for sample size of 15 in each group

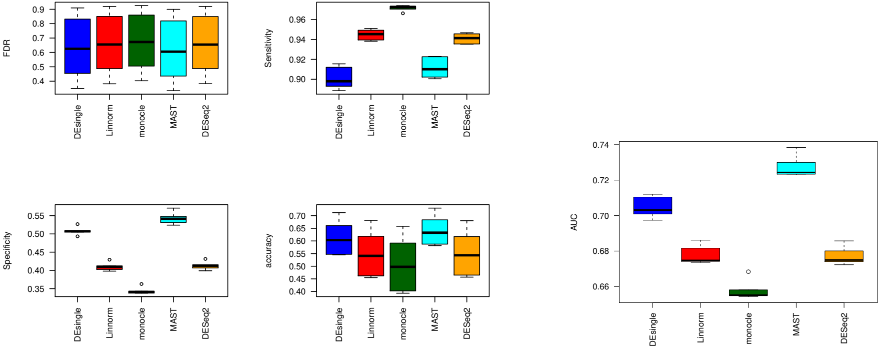


Figure: *Blue: DEsingle; Red: Linnorm; Darkgreen: Monocle; Cyan: MAST; Orange: DESeq2.*

Real data example

- Islam dataset with 92 samples of scRNA-seq raw count data downloaded from GEO website with accession no. GSE29087.
- 48 samples are embryonic stem cells and 44 are embryonic fibroblasts from mouse.
- All methods were used for selecting differentially expressed genes between the two types of cells from 14905 genes.
- The raw *p*-values from all methods were adjusted using the Benjamini-Hochberg procedure to control FDR at 5%.

Empirical power of different scRNA sequencing differential analysis methods

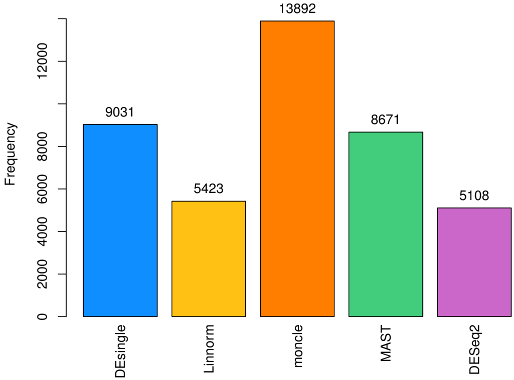
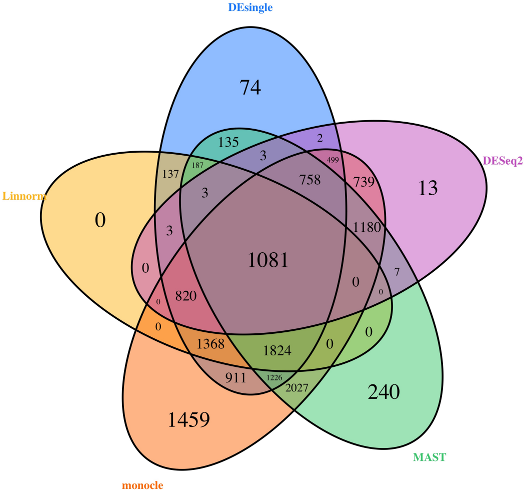


Figure: *DEsingle: dodgerblue; Linnorm: goldenrod1; monocle:darkorange1; MAST:seagreen3; DESeq2:orchid3.*

Venn diagram of selected differentially expressed genes by different scRNA sequencing differential analysis methods



Discussion

- For all methods compared, the FDR seems decrease as the proportions of differential expressed gene increase
- The accuracy of all methods seems increase as the proportions of differential expressed gene increase
- Both sensitivity and specificity seems relatively stable across different proportions of differential expressed genes
- For all five methods compared, AUC under the ROC curve seems relatively stable across different proportions of differential expressed genes

Discussion

- The monocle method has the most significant improvement in the performance indicators with the sample size increases
- All other methods slightly improved with the increase of sample size
- Methods considering modeling the excess zeros in scRNA-seq data seems perform better
- The similar performance of the Linnorm and the DESeq2 methods is likely due to the application of empirical Bayes approach in both methods

Conclusion

- The MAST method has the best AUC under ROC curves among the five methods compared, followed by DEsingle, DEseq2, Linnorm, and monocle.
- Modeling excessive zeros in the scRNA-seq count data slightly improves the performance of differential analysis.

Acknowledge

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