Probabilistic clustering of cells using single-cell RNA-seq data

Joy Saha,[1,†] Ridwanul Hasan Tanvir,[1,†] Md. Abul Hassan Samee[2,*] and Atif Rahman[1,*]

[1]Department of Computer Science and Engineering, Bangladesh University of Engineering and Technology, Dhaka, 1000, Bangladesh and
[2]Department of Integrative Physiology, Baylor College of Medicine, Houston, 77030, TX, USA
[†]Contributed equally to this work.
[*]Co-corresponding authors. samee@bcm.edu, atif@cse.buet.ac.bd

Abstract

Single-cell RNA sequencing is a modern technology for analyzing cellular heterogeneity. A key challenge is to cluster a heterogeneous sample of different cell types into multiple different homogeneous groups. Although there exist a number of clustering methods, they do not perform well consistently across various datasets. Moreover, most of them are not based on probabilistic approaches making it difficult to assess uncertainties in their results. Therefore, in spite of having large cell atlases, it is often quite difficult to map cells to types. In addition, many of the methods require prior knowledge such as marker gene information for each type. Also due to technological limitations, dropouts of gene expressions may occur in the data which is not taken into account in other methods. Here we present a probabilistic method named CellHorizon for clustering scRNA-seq data that is based on a generative model, handles dropouts and works without any prior marker gene information. Experiments reveal that our method outperforms current state-of-the-art methods overall on six gold standard datasets.

Key words: Bioinformatics, clustering, dropout, scRNA-seq data

Introduction

Single-cell RNA sequencing (scRNA-seq) technology has become a popular tool to understand the relationship between cells and genes [1]. Even with its growing usage, scRNA-seq data analysis remains a difficult task [24]. There is substantial variability in the data. In addition, the gene expression measurements in the scRNA-seq data are low and sparse, with many "false" zero count observations known as dropout events [17]. The dropout event occurs because of the low RNA capture rate and the low sequencing depth per cell.

Another challenge is that in most scRNA-seq studies, cell types are not known with certainty. Researchers typically use unsupervised clustering techniques to organize cells into sets. Cell types can be identified based on the clustering results [12]. Jaitin *et al.* showed that dissection of tissues into mixtures of cell types is a difficult task [10]. It is a standard procedure to use marker genes after cells are grouped into clusters. Ringeling *et al.* observed that Seurat [5] fails to distinguish CD4 T cells and CD8 T cells based on transcriptomic data alone, Hence, the authors used both marker genes and protein marker expressions (ADT, antibody-derived tags) to estimate cell types. However, they were still unable to label one-fifth of the cells [19].

Unsupervised methods like Leiden [23], Louvain [4] and SC3 [13] are some of the most popular clustering methods. Leiden [23] algorithm, an improved version of Louvain [4] moves its nodes locally to optimize a modularity function. Then it refines the partition and creates an aggregate network. These are computationally very expensive. Another unsupervised clustering method SC3 [13] generates distance matrices like Euclidean, Pearson, and Spearman, and then uses their PCA transformation to get their eigenvectors. Clustering is done on each of these eigenvectors and their consensus is taken as the result. This method requires fixing a large number of parameters upon which the quality of the clustering depends. Most of these parameter values can not be selected intuitively.

An autoencoder-based method scGAC [7] takes the raw-count matrix as input and after normalizing and log transformation it gets the expression matrix. Using auxiliary matrices and PCA, it generates a feature matrix that is optimized by Graph Attentional Autoencoder (GAA). Based on the latent space representation of the GAA, it optimizes a membership matrix based on k-means clustering to assign each cell to one specific cluster. EDClust [26] is a statistical probabilistic clustering method that focuses on subject-specific factors of single-cell data. It uses the Dirichlet-multinomial mixture model to represent expression counts in multi-subject scRNA-seq data. It follows an EM–MM hybrid method. In the E-step, it calculates a posterior probability for cluster assignment and in M-step, it optimizes a loss function. Then to incorporate cell type-specific and subject-specific effects it uses a MM algorithm.

These unsupervised algorithms often use a low dimensional representation like PCA of count data and cluster the cells based on this. The low-dimensional representations measure linear similarity among cells but fail to capture the latent relationship which leads to sub-optimal clustering [9].

In addition to the clustering methods, there exist a number of supervised and semi-supervised methods like CellAssign [29] and SCINA [31]. Both CellAssign [29] and SCINA [31] require prior information. CellAssign [29] is a probabilistic approach to assign cells to types. Its main focus is to automate the process of annotation rather than clustering cells. For this, it requires additional marker gene information. However, determining the marker genes of a cell type that is not well known is a challenging task. CellAssign [29] also does not handle dropouts which is a key factor in single-cell clustering. Tabula Muris [22], which contained scRNA-seq data for about 120,000 cells from 20 organs and tissue types in mice had a dropout rate of up to 93% [18].

In this work, we propose an unsupervised probabilistic clustering method based on CellAssign [29] that does not require any prior marker gene information and models the expression data using negative binomial distribution. As it is a probabilistic approach it performs well when cells may have characteristics that span multiple clusters and helps us to understand overlapping relationships between clusters. It allows us to capture the uncertainty associated with each cell's assignment to a cluster. It also takes dropout into account by associating a dropout rate with each gene so that, dropout and actual zero value in the expression can be differentiated. Our method also does not require any additional parameter value and it is based on expression count data rather than any low-dimensional representation of it.

Method

In scRNA-seq clustering, given an expression matrix $(n \times g)$ of n cells and g genes, the challenge is to cluster the cells into c different clusters. Our method achieves this through a generative probabilistic approach and using negative binomial distributions to represent the count data. At first, the mean and dispersion parameters of the negative binomial distributions are initialized from the expression matrix. Then an EM algorithm is used to iteratively re-estimate the mean, dispersion and other parameters of the model. The EM algorithm terminates when the model converges. Then the probability for each cell being each type is calculated from the corresponding negative binomial distributions.

Handling Dropouts

One of the key focuses of our method is handling dropouts. ZingeR [2] is a tool to model dropouts. It handles dropouts using a two component mixture model. The dropout is modeled by a point mass at zero and the original count as a negative binomial distribution. Then the probability that a zero expression count belongs to the count component is the negative binomial component divided by the mixture component. Our method uses a dropout parameter d_g for each gene which is the probability of dropout for a gene g.

Generative Model

In the generative model of our algorithm, z or cell type indicator is determined by the prior probabilities π_c. Based on z, we get the parameters of our negative binomial distribution μ and ϕ for each gene. Based on this negative binomial distribution, we sample the initial raw count value y_1. Again d_g or dropout rate is used to determine w which is the probability of count value coming from zero distribution. y is the final count value that is determined by y_1 and this incorporated w.

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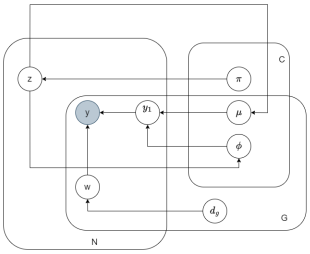
Initialization

For each gene, we calculate the mean and variance column-wise. Then using Equation 1, we calculate the dispersion ϕ.

$$\phi = \frac{\mu^2}{\text{Var} - \mu} \tag{1}$$



Variable	Description
n	Number of cells
g	Number of genes
c	Number of cell types
y_1	Count value before dropout
y_{ng}	Expression count
μ_{ngc}	Mean
ϕ_{ngc}	Dispersion
γ_{nc}	Probability of n^{th} cell being type c
w_{ng}	Probability of zero distribution
d_g	Dropout rate of gene g
π_c	Prior probability of cell type c

Fig. 1. Generative Model

where μ is the mean and Var is the variance. So, μ and ϕ parameters for negative binomial distribution are initialized.

EM Algorithm

The EM algorithm [15] or expectation–maximization algorithm is an iterative method of maximizing the likelihood of a statistical model by varying parameter estimation. The E step or expectation step determines an expectation of the log-likelihood of the model using the current estimates of the parameters. The M step or maximization step re-estimates the parameters using a loss function. This two steps are performed iteratively until a convergence criterion is met.

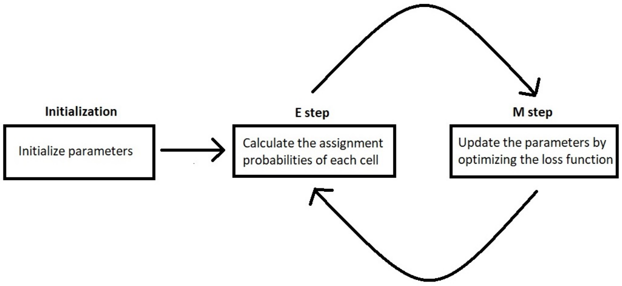

Initialization
Initialize parameters
E step
Calculate the assignment probabilities of each cell
M step
Update the parameters by optimizing the loss function

Fig. 2. EM Algorithm

E Step

In the E step, at first, we define two boolean variables λ_{ng} and τ_{ng} as follows:

$$\lambda_{ng} = \left\{ \begin{array}{ll} 1, & \text{if } y_{ng} = 0 \\ 0, & \text{if } y_{ng} \neq 0 \end{array} \right\}$$

$$\tau_{ng} = \left\{ \begin{array}{ll} 0, & \text{if } y_{ng} \text{ is a true count} \\ 1, & \text{if } y_{ng} \text{ is a dropout} \end{array} \right\}$$

We calculate the probability that a zero value in the gene expression came from dropout w_{ng} as follows:

$$\begin{aligned} w_{ng} &= P(\tau_{ng} = 1) \\ &= \sum_c P(\tau_{ng} = 1 | z_n = c) P(z_n = c) \\ &= \sum_c \frac{d_g * \lambda_{ng}}{d_g + (1 - d_g) NB(\mu_{ngc}, \phi_{ngc})} \gamma_{nc} \end{aligned} \tag{2}$$

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Then we calculate the mean μ_{ngc} of the negative binomial distribution using the following equation:

$$\mu_{ngc} = S_n \times \mu_{gc} \tag{3}$$

where S_n is the size factor of cell n. Using the mean μ_{ngc} and dispersion ϕ_{ngc} we calculate the probability that n^{th} cell is of type c.

$$\begin{aligned}\gamma_{nc} &= P(z_n = c)\\ &= \frac{\prod_g \pi_c(d_g * \lambda_{ng} + (1-d_g)NB(\mu_{ngc}, \phi_{ngc}))}{\sum_c \prod_g \pi_c(d_g * \lambda_{ng} + (1-d_g)NB(\mu_{ngc}, \phi_{ngc}))}\end{aligned} \tag{4}$$

M-step

In the M-step, we minimize the loss function

$$\begin{aligned}Q = &\prod_n \prod_c \prod_g \Big[d_g^{w_{ng}} \{(1-d_g)NB(y_{ng}|\mu_{ngc}, \phi_{ngc})\}^{(1-w_{ng})}\Big]^{\gamma_{nc}}\\ &\times p(\pi)\end{aligned} \tag{5}$$

or equivalently its logarithm

$$\begin{aligned}\log Q = &\sum_n \sum_c \gamma_{nc} \sum_g [w_{ng} \log(d_g) +\\ &(1-w_{ng}) \log\{(1-d_g)NB(y_{ng}|\mu_{ngc}, \phi_{ngc})\}]\\ &+ \log(p(\pi))\end{aligned} \tag{6}$$

using Adam optimizer. The loss function also has two parts: one coming from negative binomial distribution and another from dropout. We update the parameters d_g, μ_{ngc}, ϕ_{ngc}, π_c which are used for the next iteration of EM.

Convergence Criteria

We use two types of convergence criteria for the EM algorithm. The model is assumed to be converged if any of these conditions are met.

- **Max Epoch:** The EM algorithm runs at most 400 epochs, then it stops and returns the results.
- **Early Stopping:** The model is stopped early and returned if for 15 consecutive iterations, the loss does not improve.

Adam Optimizer

Adam optimizer [11] is used in the M step of our algorithm. It is a stochastic gradient descent method and it uses the adaptive estimation of first-order and second-order moments to minimize a loss function. In our method Equation 6 is minimized by Adam optimizer by changing the values of d_g, μ_{ngc}, ϕ_{ngc}, and π_c.

Results

Dataset

The input for our clustering method is a gene expression matrix where each row corresponds to a cell and each column represents a gene. We compared our model with other state-of-the-art models using six gold standard datasets, six silver standard datasets, and one simulated dataset.

Simulated Dataset

We generated a simulated dataset of 5 cell types of 9156 cells and 24 genes. We randomly selected 6 to 12 marker genes for each type of cell. For each gene and each cell type one negative binomial distribution was taken and used to sample the expression count. The mean μ for marker genes was taken two times more than the non-marker genes.

$$\mu_{gc} = \left\{ \begin{array}{ll} rand(10, 20), & \text{if } g \notin marker(c) \\ rand(20, 40), & \text{if } g \in marker(c) \end{array} \right\} \tag{7}$$

To calculate the dispersion parameter ϕ, we randomly generated variance Var.

$$Var_{gc} = rand(100, 500) \tag{8}$$

Then we can calculate ϕ using the following equation:

$$\phi_{gc} = \frac{\mu_{gc}^2}{Var_{gc} - \mu_{gc}} \tag{9}$$

Dropout rate d_g was chosen as follows:

$$d_g = rand(0.1, 0.6) \tag{10}$$

We assumed the size factor was 1 for all cells. Then using μ_{gc} and ϕ_{gc}, negative binomial distributions were created and count values y_{ng} were sampled from them. Dropout rate d_g was used to incorporate the effect of dropout in our simulated dataset.

Gold-standard Datasets

Six gold-standard datasets and six silver-standard datasets were used for our comparison. Goolam, Kolodziejczyk, Yan, Deng, Pollen, and Biase are considered as gold-standard datasets because in these datasets the cell sub-populations were chosen from distinct biological stages or conditions [25].

Table 1. Gold-standard Datasets

Dataset Name	Total Cells	Total Genes	Cell Types
Goolam [9]	124	41428	5
Kolodziejczyk [14]	704	38616	3
Yan [27]	90	20214	6
Deng [8]	268	22431	6
Pollen [16]	301	23730	11
Biase [3]	49	25737	3

Silver-standard Datasets

In addition, we analyze Worm neuron, Human kidney, 10x pbmc, Mouse retina raw, CITE CBMC and TAM FACS datasets. They are considered silver-standard datasets because in these the cells were labeled according to the authors' computational analysis and biological knowledge [25].

Table 2. Silver-standard Datasets

Dataset Name	Total Cells	Total Genes	Cell Types
Worm Neuron [6]	4186	13488	10
Human Kidney [28]	5685	25215	11
10x PBMC [32]	4271	16653	8
Mouse Retina [20]	14653	11422	19
CITE CBMC [21]	8617	2000	15
TAM FACS [30]	110824	22966	126

Preprocessing

We performed four preprocessing steps in our algorithm to filter the data and determine the genes whose expressions are highly differentiable across different cell types.

- Gene filter: We included genes that are expressed in at least three cells.
- Cell filter: A minimum of 200 genes expressed was required for a cell to pass filtering.
- We selected 1% most highly variable genes (HVGs) that have the highest variance across all cells. They have the greatest potential to distinguish between different cell types.
- Finally we applied log normalization to account for high variance in expression count.

Performance Evaluation Metrics

We used Adjusted Rand Index (ARI) an improved version of Rand Index (RI) to compare the performance of our method with other methods using true and predicted cell types. ARI computes the similarity between two clusters on a scale from 0 to 1. ARI scores are higher if the predicted clustering is close to the true clustering.

Result on Simulated dataset

First, we analyze the simulated dataset to assess how well our method can determine the parameters of our model: mean, dispersion and dropout rate.

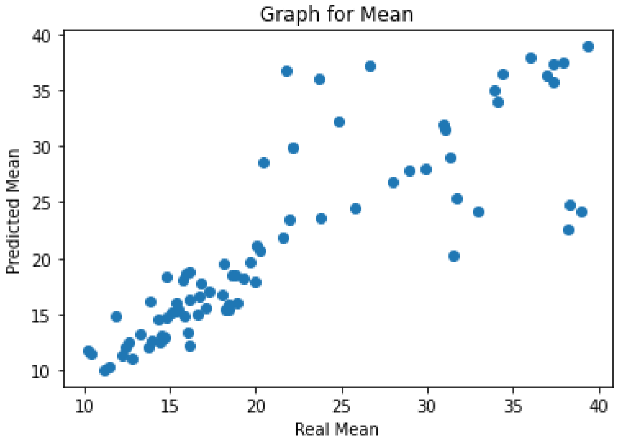

Fig. 3. Real vs Predicted Mean

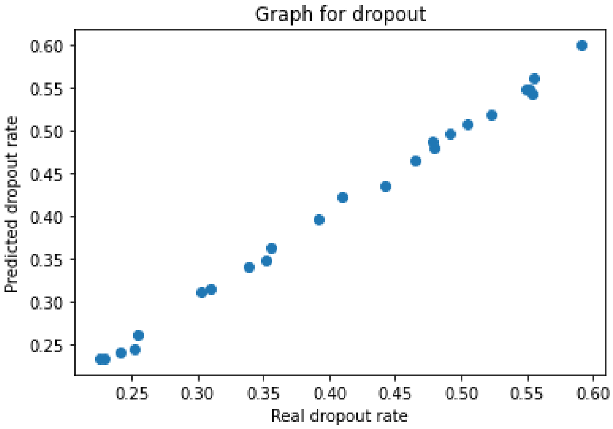

Fig. 4. Real vs Predicted Dropout

From Fig 3 and Fig 4, we find that the predicted mean and dropout are similar to the original mean and dropout. Also, we can observe that the range of the original mean and dropout that is 10-40 and 0.1-0.6 are equal to the range of predicted mean and dropout of 10-40, and 0.1-0.6 respectively. We also find that our method has a very high ARI score of 0.948 in the simulated dataset. We varied the mean and dropout rate of the simulated dataset and found that our method could perform consistently with different ranges of mean, and dropout values. Hence, our method could determine the parameters of the negative binomial distributions precisely and cluster the cells accurately in the simulated dataset.

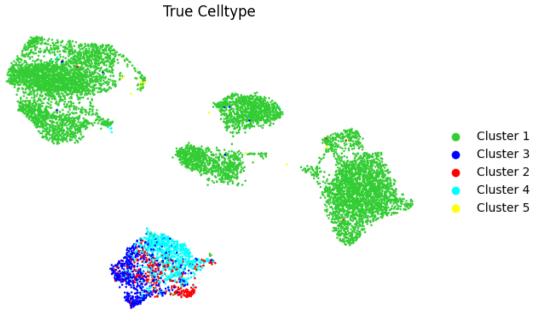

Fig. 5. True celltype

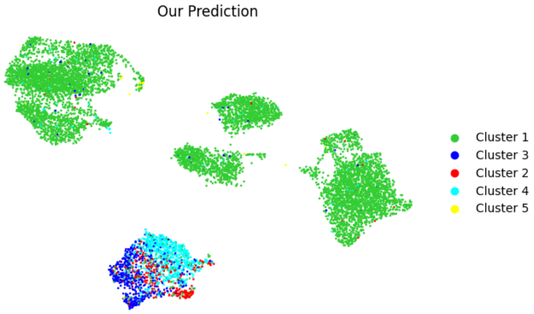

Fig. 6. Predicted celltype

Result on Real dataset

Next we analyze the six gold standard and six silver standard real datasets and compared the ARI of 5 state-of-the-art clustering methods with our method.

Table 3 shows the ARI of the methods on the gold standard datasets. As the cell sub-populations were chosen from distinct biological stages or conditions in gold datasets, they are more reliable than silver datasets where the labels were given using authors' computational analysis. Our method outperforms all the other methods on average in 6 gold datasets. In the Goolam and Deng datasets, our method has a significantly higher ARI score than the second highest performing methods Leiden and scGAC respectively. Our method showed poor performance in only the Biase dataset. A possible reason is the number of cells is small in this dataset which may lead to inaccurate estimation of parameters.

Table 3. Gold-standard Datasets ARI Comparison

Dataset	Secuer	Louvain	Leiden	SC3	scGAC	Our Method
Goolam [9]	0.4673	0.4584	0.5709	0.4820	0.5554	0.8602
Kolod [14]	0.5064	0.4448	0.4452	0.5279	0.6017	0.5112
Yan [27]	0.7917	0.8278	0.9233	0.6383	0.8955	0.8451
Deng [8]	0.3747	0.4571	0.4683	0.3892	0.5596	0.6743
Pollen [16]	0.8508	0.8508	0.8508	0.8976	0.5857	0.7902
Biase [3]	1.0000	0.8986	0.9483	1.0000	1.0000	0.6120
Average	0.6651	0.6562	0.7011	0.6559	0.6996	0.7155

In the case of silver datasets Securer, Louvain and scGAC (Tab 4) failed to run TAM FACS due to very high memory demands. Moreover, scGAC could not run Mouse Retina.

Table 4. Silver-standard Datasets ARI Comparison

Dataset	Secuer	Louvain	Leiden	SC3	scGAC	Our Method
Worm Neuron [6]	0.451	0.508	0.434	0.209	0.431	0.250
Human Kidney [28]	0.629	0.588	0.570	0.236	0.528	0.360
10x PBMC [32]	0.643	0.681	0.679	0.448	0.654	0.581
Mouse Retina [20]	0.634	0.689	0.691	0.224	—	0.529
CITE CBMC [21]	0.495	0.569	0.487	0.388	0.612	0.542
TAM FACS [30]	—	—	0.144	0.020	—	0.037
Average	—	—	0.501	0.254	—	0.383

Running time

Table 5 shows running times of the methods. We find that our method is the third fastest method overall. The two methods faster than our method are SC3 and Secuer. However, their ARI scores are less than our method. Hence, no other method performed better in less time than our method.

Table 5. Gold-standard Datasets Time Comparison in milliseconds

Dataset	Secuer	Louvain	Leiden	SC3	scGAC	Our Method
Goolam [9]	2849	23869	18972	1117	39492	11265
Kolod [14]	7284	24419	13336	4941	136261	12962
Yan [27]	3968	23779	12347	771	33388	10776
Deng [8]	5712	25895	12238	2294	41409	11407
Pollen [16]	4108	24800	11932	2400	44244	12307
Biase [3]	3836	22516	12440	666	28768	11513
Average	4626	24213	13544	2031	53927	11705

Discussion

We generated the PCA visualization of the Gold datasets and analyzed the discrepancy between our clustering and ground truth celltypes visually. Moreover, we determined the probable marker gene list using both true celltypes and our predicted celltypes using scanpy. We calculated the number of common marker genes between these two lists for all possible combination of true celltypes and predicted celltypes.

Goolam dataset

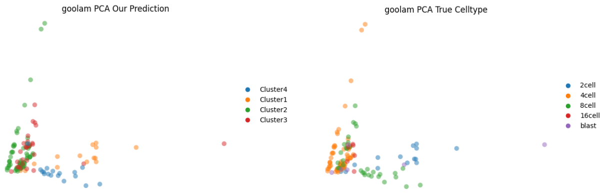

Fig. 7. Goolam pca

We can see in the true celltype, the 8 cell cluster is separated into two parts. But in our prediction, those two parts are clustered into different groups and also in the PCA visualization they appear to belong to different clusters. Moreover, there is a celltype blast in the true celltype which is a cluster of very few cells scattered around. As few scattered cells rarely make any cluster, our method did not consider it as a separate cluster. In further analysis, we found that blast has the lowest or second lowest number of common marker genes with all predicted clusters. This is possibly why blast was not considered as a different cluster by our method.

Kolodziejczyk dataset

Our method merges some a2i cells into 2i cells. This is due to the fact that between 2i and a2i cells, the Spearman correlation coefficient of mean gene expression levels is very high (0.95). It is also reflected in the common marker list. For lif celltype, cluster 3 has the highest number of common marker genes much more than other clusters but for 2i and a2i both cluster 1 and cluster 2 have roughly the same number of common marker genes.

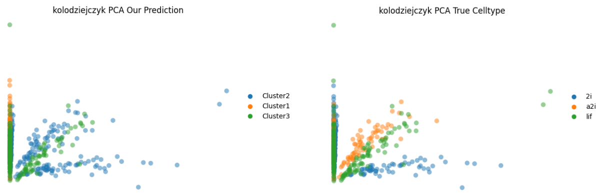

Fig. 8. Kolodziejczyk pca

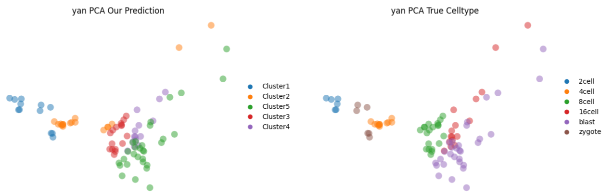

Fig. 9. Yan pca

Yan dataset

We can see that in true celltype the zygote cluster is divided into two parts and another cluster 4 cell is placed between them. Our prediction combines these two parts with cluster 1.

Deng dataset

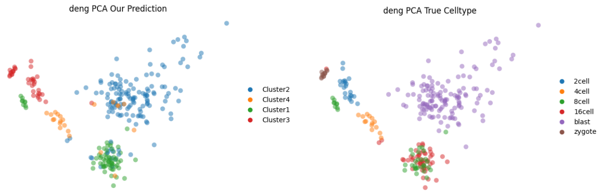

Fig. 10. Deng pca

In the Deng dataset, we see that in true celltype the 16 cells and 8 cells are overlapping with each other. Our prediction does not have any overlapping and considers all of them as cluster 1.

Biase dataset

Biase is a small dataset containing only 49 cells. Our ARI was the lowest in the Biase dataset. It is due to the fact that fitting a distribution with such small number of samples is relatively hard.

Conclusion

Here we have presented a probabilistic unsupervised clustering algorithm for single-cell RNA-seq data based on a generative model. It models the expression data using negative binomial distributions. The method does not use any prior marker gene information. We also consider and tackle the effect of dropout to get a better quality clustering. We tested our method on the simulated dataset to evaluate the accuracy of parameter estimation and find that it can accurately estimate the means and dropout rates of genes. We then compared our method with other methods on Gold and Silver datasets. It was shown that our method is consistent and overall outperforms other methods in the gold datasets.

In future, a number of directions may be explored. We have used our method only for detecting clusters. But we can easily modify it to track the progress of cell development. Also, in the simulated dataset we have successfully estimated the mean and dropout parameters. However. there is scope for improvement in the estimation of the dispersion parameter. The method may enable finding the characteristics of unknown cell types from a heterogeneous cell population. So we may be able to detect new types of cells as well as subclusters.

Again, in tumor and cancer cells, not all the cells are malignant. There may be healthy cells beside so we would use the clustering algorithm to detect outliers. Doublet detection can be another promising aspect of our method as it can use the advantage of probabilistic assignment.

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