Project3 Report

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Introduction

The advent of single-cell RNA sequencing (scRNA-seq)[1] has revolutionized our understanding of cellular biology by enabling gene expression analysis at the single-cell level. In this report, we delve into the analysis of scRNA-seq data from breast cancer tumors, focusing on 558 genes across 716 cells. The primary objective is to employ Principal Component Analysis (PCA) to reduce the dimensionality of our dataset, thereby simplifying the complex gene expression data while retaining the most significant variables for further analysis. By determining the optimal number of principal components, we can capture the essential variability of gene expressions, which is crucial for accurate clustering and analysis.

Following PCA[2], we utilize a Gaussian-Mixture Model (GMM)[3] and an Expectation-Maximization (EM) algorithm[4] to cluster the cells based on their gene expression profiles. This approach helps in identifying distinct cell subtypes within the breast cancer tumors, which may differ in their biological characteristics and behavior. By analyzing these clusters, we aim to uncover specific gene-expression signatures using 'limma' package in 'R'[5] that distinguish between the subpopulations, providing valuable insights into the underlying biology of breast cancer.

This comprehensive approach to analyzing scRNA-seq data underscores the importance of advanced statistical techniques in extracting meaningful information from complex biological datasets, which is fundamental in advancing our understanding of diseases at the molecular level.

Method

Principal Component Analysis (PCA)

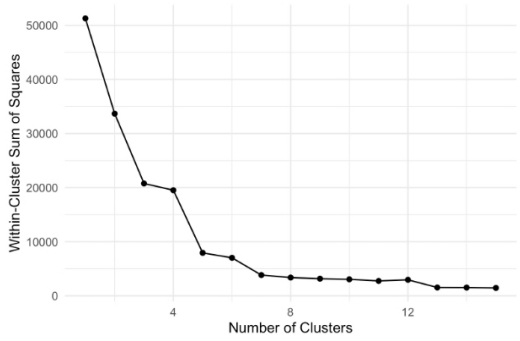
Initially, data is standardized, meaning each feature is transformed to have a mean of zero and a standard deviation of one, using the formula: $x' = \frac{x-\mu_j}{\sigma_j}$ where μ_j and σ_j are the mean and standard deviation of feature j respectively.

Subsequently, the covariance matrix is computed to represent the covariance between variables. For a matrix X with n observations and d variables, the covariance matrix C is: $C = \frac{1}{n-1}X^T X$, where X^T is the transpose of X. Following this, eigenvalues and eigenvectors of C are determined, identifying the principal components, or the directions of maximum variance. For the covariance matrix C, eigenvalues λ and eigenvectors v satisfy: $Cv = \lambda v$.

The principal vectors corresponding to the largest eigenvalues are selected. These are used to create a feature vector V_k for dimension reduction. The original data matrix X is then transformed into a new matrix Y with reduced dimensions: $Y = XV_k$. Matrix Y represents the projection of data onto the top k principal components, effectively reducing data dimensionality while retaining significant variance and minimizing information loss.

K-means

To detect the number of clusters, we first use K-means, the unsupervised learning method, to obtain an approximate range of number of clusters. Based on the two plots below, cluster sizes ranging from $2, 3, \cdots, 10$ is taken into consideration.



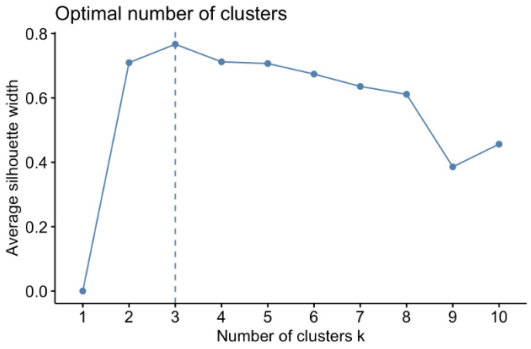


Figure 1 and 2: Comparisons of different cluster sizes

Guassian Mixture Model

After determining the number of components using PCA, the Gaussian Mixture Model (GMM) is introduced as a way of clustering. The main assumption of GMM is that there are a certain number of Gaussian distributions. Furthermore, each of these Gaussian distributions represent a specific cluster. Suppose we have k clusters. Let X_i denote each individual cell, then we have $X \sim N(\mu_j, \Sigma_j)$ with probability p_j, $j = 1, 2, \cdots, k$. The density of X is thus

$$f(\mathbf{x}; \boldsymbol{\mu}, \Sigma) = \frac{\exp(-\frac{1}{2}(\boldsymbol{x} - \boldsymbol{\mu})^T \Sigma^{-1} (\boldsymbol{x} - \boldsymbol{\mu}))}{\sqrt{(2\pi)^p |\Sigma|}}$$

where $\boldsymbol{\mu}$ is p-dimensional mean, and Σ is $p \times p$ variance-covariance matrix;

Through multiplying density of X_i and possibility for each X_i and summing up the product for each cluster, an observed likelihood of $(x_1, ..., x_n)$ can be calculated as below:

$$L(\theta; \mathbf{x}_1, \mathbf{x}_2, ..., \mathbf{x}_n) = \prod_{i=1}^{n} \sum_{j=1}^{k} p_j f(\mathbf{x}_i; \boldsymbol{\mu}_j, \Sigma_j)$$

In order to distinguish which data point belongs to which cluster, a latent variable γ_i is used as the cluster indicator that indicates the probability that each data point belongs to each cluster of the GMM. Hence, let $\gamma_i = (\gamma_{i,1}, ..., \gamma_{i,k}) \in \mathbb{R}^k$ as the cluster indicator of $\mathbf{x}_i$, which takes form $(0, 0, ..., 0, 1, 0, 0)$ with $\gamma_{i,j} = I\{\mathbf{x}_i \text{ belongs to cluster } j\}$. The distribution of γ_i is $f(\gamma_i) = \prod_{j=1}^{k} p_j^{\gamma_{i,j}}$.

Through multiplying the distribution of γ_i with the conditional distribution of $\mathbf{x}_i$ given γ_i, which is $f(\mathbf{x}_i|\gamma_i) = \prod_{j=1}^{k} f(\mathbf{x}_i; \boldsymbol{\mu}_j, \Sigma_j)^{\gamma_{i,j}}$, the complete likelihood function can be calculated as below using the conditional distribution formula: $L(\theta; \mathbf{x}, \gamma) = \prod_{i=1}^{n} \prod_{j=1}^{k} [p_j f(\mathbf{x}_i; \boldsymbol{\mu}_j, \Sigma_j)]^{\gamma_{i,j}}$. Furthermore, the complete log-likelihood becomes:

$$\ell(\theta; \mathbf{x}, \gamma) = \sum_{i=1}^{n} \sum_{j=1}^{k} \gamma_{i,j} [\log p_i + \log f(\mathbf{x}_i; \boldsymbol{\mu}_j, \Sigma_j)] = \sum_{i=1}^{n} \sum_{j=1}^{k} \gamma_{i,j} [\log p_i - 1/2 \log |\Sigma| - 1/2 (\mathbf{x}_i - \boldsymbol{\mu}_j)^\top \Sigma (\mathbf{x}_i - \boldsymbol{\mu}_j)]$$

Expectation-Maximization Algorithm:

E-step

Following, the GMM model is integrated into the Expectation-Maximization algorithm. In the Expectation step, an initial guess of parameters in the GMM model is taken using a pre-determined cluster size (k) value, including the means μ_k, co-variances Σ_k, and mixing coefficients p_k. Using these initial parameters of the GMM model, the responsibilities can be evaluated as following:

$$\gamma_{i,k}^{(t)} = \frac{p_k^{(t)} f(\mathbf{x}_i|\boldsymbol{\mu}_k^{(t)}, \Sigma_k^{(t)})}{\sum_{j=1}^{K} p_j^{(t)} f(\mathbf{x}_i|\boldsymbol{\mu}_j^{(t)}, \Sigma_j^{(t)})}$$

where t denote the t_{th} iteration; In the numerator of this function, the mixing coefficient p_k is multiplied by the possibility density function of X_i under component k. This multiplication results in the prior probability of data point x_i belonging to component k.

The denominator of this function is the sum of the prior probabilities across all components j (where $j = 1, 2, \cdots, k$). This normalization step ensure that the responsibilities sum up to 1 for each data point.

M-step

Next, in the Maximization step, the parameters of the GMM (means, co-variances, and mixing coefficients) based on the computed responsibilities for each cluster that was obtained from the Expectation step.

First, let $n_k = \sum_{i=1}^{n} \gamma_{i,k}$. The updated mixing coefficient p_k for component k is calculated as the average responsibility assigned to that component: $p_k^{(t+1)} = \frac{n_k}{n}$. The mean μ_k of each component is updated using a weighted average of data points, where the weights are the responsibilities γ_i assigned to each data point for component k: $\boldsymbol{\mu}_k^{(t+1)} = \frac{1}{n_k} \sum_{i=1}^{n} \gamma_{i,k} \mathbf{x}_i$. Using the updated components means, the co-variance matrix Σ_k of each component is updated based on the responsibility-weighted sum of squared distances between the data points and the component means:

$$\Sigma_k^{(t+1)} = \frac{1}{n_k} \sum_{i=1}^{n} \gamma_{i,k} (\mathbf{x}_i - \boldsymbol{\mu}_k^{(t+1)})(\mathbf{x}_i - \boldsymbol{\mu}_k^{(t+1)})^T$$

In each iteration t, the E-M algorithm is repeated to update theses parameters until an optimization of likelihood function is achieved. After an optimization is achieved, the assignment of each data point to a specific cluster is done based on the largest value of latent variable for each cell. If two clusters have the same parameters, the data point would be randomly assigned to one cluster.

Gene-expression signatures

Data Normalization and Precision Weights Estimation

Using the `voom` package, expression data is transformed to log_2 scale, adding 0.5 to address zero counts, and subsequently normalized. This transformation facilitates comparability across different expression levels. Precision weights for each gene are estimated through a linear model, which assesses expression differences across clusters. The `LOWESS` method is applied to predict variance from the square root of residual standard deviations and average log-counts. The inverse of this estimated variance ($\frac{1}{variance}$) serves as a precision weight, correcting for heteroscedasticity and enabling weighted least squares fitting in the subsequent analysis.

Linear Model Fitting and Updating Coefficients

Post-normalization, the `lmFit` function fits linear models row-wise to the expression matrix, using variance-based weights. A design matrix designates cluster memberships per sample, allowing the evaluation of gene expression in relation to cluster variables. To perform specific pairwise cluster comparisons, a contrast matrix is established and integrated into each gene's model through the `contrasts.fit` function, facilitating the calculation of fold changes, F-statistics, and p-values.

Enhancing Statistical Testing

To bolster the robustness of statistical outcomes, Empirical Bayes (EB) methods adjust linear model coefficients by calculating a prior variance distribution based on the variance across all genes and a weighted average. This distribution assists in estimating a posterior variance by amalgamating the prior with individual gene variances, reducing uncertainties in statistical assessments based on small-sample variances.

Identification of DEGs

DEGs are determined by examining fold changes in gene expression (log scale), F-statistics, and p-values. Genes showing significant up-regulation (log FC $>$ 2.5) or down-regulation (log FC $<$ -2.5) with a p-value $<$ 0.01 are classified accordingly. Furthermore, for each cluster, genes are identified as DEGs if they exhibit significant differential expression in at least two contrasts involving the cluster, with $|logFC| > 2$ and p-value $<$ 0.01. The `limma` package orchestrates all steps in this analysis, ensuring a streamlined and efficient identification of gene-expression signatures.

Results

Principal Component Analysis (PCA)

The PCA was successfully applied to reduce the dimensions of our data set consisting of 558 genes across 716 cells. The scree plot, used to determine the number of principal components to retain, indicated that the first three components accounted for a significant portion of the variance in the data. These components collectively captured over 60% of the total variance, suggesting that they are significant in representing the data set's complexity.

Guassian Mixture Model and Expectation-Maximization Algorithm:

Through implementing the E-M algorithm is implemented in R (Appendix: E-M Algorithm), updated parameters, including μ_k, Σ_k, and p_k, are obtained for each cluster. The marginal likelihood can be then calculated using the GMM function.

Through comparing the marginal likelihood for cluster sizes ranging from $2, 3, \cdots, 10$ using iteration (Figure 3), a cluster size of 5 is selected for the final GMM model.

cluster	Freq
1	254
2	233
3	208
4	11
5	10

Table 1: Number of data points in each cluster

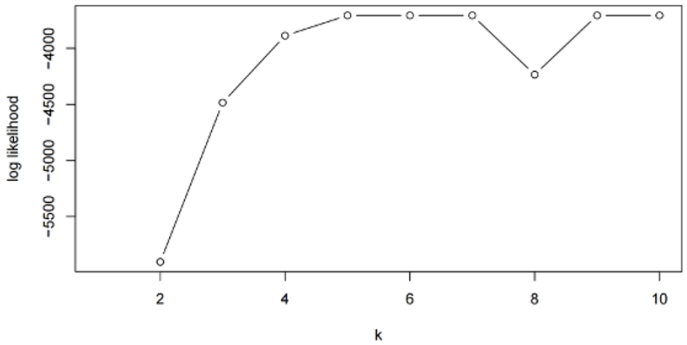


Figure 3: Marginal likelihood for different cluster size

Using the cluster size 5, which data point belongs to each of the 5 clusters could be determined (Table 1).

Gene-expression signatures

After conducting the differential gene expression analysis, 69 DEGs were identified in cluster 1 (19 up regulated, 50 down regulated), 69 DEGs in cluster 2 (17 up regulated, 52 down regulated), 1 DEGs in cluster 3 (1 up regulated, 0 down regulated), 6 DEGs in cluster 4 (6 up regulated, 0 down regulated), and 5 DEGs in cluster 5 (4 up regulated, 1 down regulated).

Fig.4(see Supplementary) displays the significantly differentially expressed genes in several unique pairwise comparisons between each cluster, with red indicating up regulated genes and blue indicating down regulated genes. Additionally, Table 2(see Supplementary) lists top 1 specific genes (with largest |FC|)that are uniquely expressed within a cluster after integrating all comparisons involving that cluster (the complete list of DEGs is provided in Supplementary Tables). These uniquely differentially expressed genes within a cluster can be used to distinguish between different clusters based on changes in their expression levels.

Conclusion

In this project, the PCA effectively reduced the data set's dimensionality, allowing the identification of principal components that capture the major variance within the gene expressions. Three components served as a robust foundation for the subsequent clustering analysis. The use of the GMM to cluster the cells based on their gene expression profiles was pivotal in revealing 5 distinct clusters within the tumor cells. This stratification highlighted the variability in gene expression even among cells that are phenotypically similar, underscoring the complexity of tumor biology.

Moreover, the study identified 381 significantly differentially expressed genes (DEGs) among these clusters, with 185 genes upregulated and 195 downregulated. This detailed gene expression profiling not only enhances our understanding of the molecular underpinnings of breast cancer but also suggests potential targets for therapeutic intervention. Each cluster's unique gene expression signature provides insights into their biological functions and roles in cancer progression.

There still some areas of this project are expected to be explored in the future study. During the iteration of EM, poor selection may result in NA values and some clusters do not contain any elements. Although genes that are important for differentiating the clusters were identified, as the `limma` package prefers raw count data, the accuracy of the differential expression analysis might be affected due to the original expression data not being raw counts from single-cell sequencing and the normalization process not being explicitly defined. Additionally, while the analysis still relies on linear models to estimate the fold changes of each gene across different clusters, to enhance the accuracy of the results, some nonlinear methods could be applied in the future.

Contribution

Zhuodiao Kuang: Initialized the work; Applied PCA to find components for the further study; Explored gene expression using ANOVA tests; Organized the report. Shaolei Ma: Lead the team; Designed EM algorithm to estimate the GMM model; Connected the work between teammates; Peng Su: Solved the problem of identifying gene-expression signatures with state-of-the-art methods; Found DEGs in clusters; Yilei Yang: Utilized K-means to detect the number of clusters and introduced the Gaussian Mixture Model; Launched the meetings for teammates.

Reference

[1]Levsky, Jeffrey M., et al. "Single-cell gene expression profiling." Science 297.5582 (2002): 836-840.

[2]Abdi, H., & Williams, L. J. (2010). Principal component analysis. Wiley interdisciplinary reviews: computational statistics, 2(4), 433-459.

[3]Reynolds, D. A. (2009). Gaussian mixture models. Encyclopedia of biometrics, 741(659-663).

[4]McLachlan, G. J., & Krishnan, T. (2007). The EM algorithm and extensions. John Wiley & Sons.

[5]Smyth, G. K. (2005). Limma: linear models for microarray data. In Bioinformatics and computational biology solutions using R and Bioconductor (pp. 397-420). New York, NY: Springer New York.

Supplementary

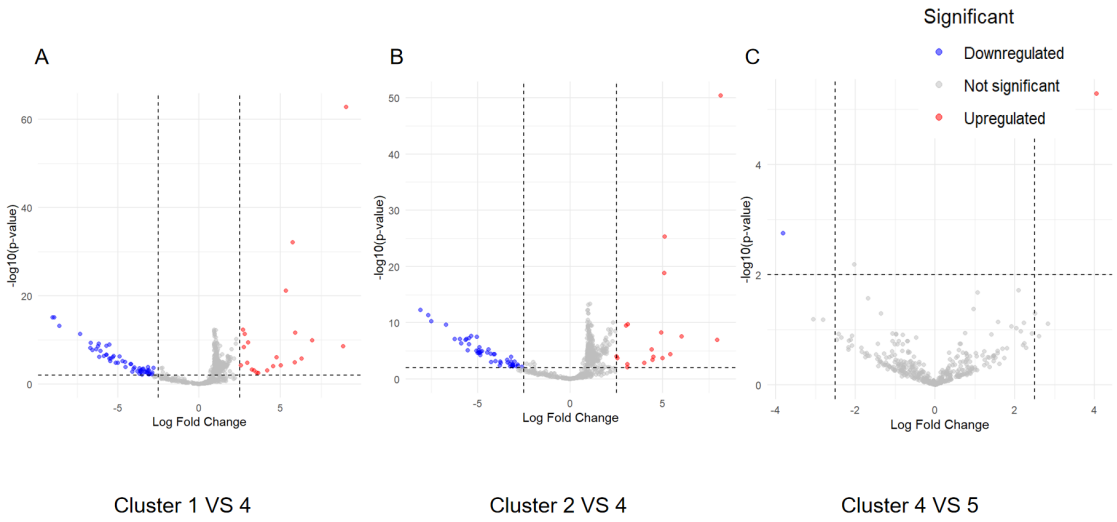


Figure 4: The Volcano plot for unique cluster comparisons. (A) Cluster 1 VS 4, (B) Cluster 2 VS 4, (C) Cluster 4 VS 5.

$Table 2: Top 1 DEGs with largest logFC (absolute value) in clusters$

cluster1 up	cluster4 up	cluster5 up	cluster1 down
Cyp4b1	Reg1	Aqp1	Crabp1

up1
Cyp4b1
Fabp4
Rgs4
Rgs5
Higd1b
Myl9
Kcnj8
Tinagl1
Sncg
Acta2
Tmem37
Ephx3
Rgs16
Errfi1
X1810011H11Rik
Ifitm1
Ankrd37
Actg2
Arhgdib
Dmp1
H2.M9
Emcn

down1
Lum
Mfap5
Fbln2
Crabp1
Dcn
Smoc2
Lrrc15
Cpxm2
Itm2a
Fbln1
Rbp1
Rnase4
Gja1
Serpinf1
Cilp
Serpina3n
Ly6a
Olfml3
Cpxm1
Tnfaip6
Hsd11b1
Il33
Pdgfrl
Ndufa4
Ly6e
Igfbp6

down1
Apod
Fndc1
Col1a1
Mfap4
Col3a1
Cxcl14
Lox
Itgbl1
Pi16
Eln
Cpz
Mmp2
Col1a2
Sfrp2
Ly6c1
Gas6
Ctla2a
Mgst1
Omd
Cd34
Htra3
Ccl8
Cthrc1
Fxyd6
Ccl11
Dpt

up2
Fabp4
Rgs4
Rgs5
Higd1b
Myl9
Kcnj8
Tinagl1
Sncg
Acta2
Tmem37
Ephx3
Rgs16
Errfi1
X1810011H11Rik
Ifitm1
Ankrd37
Actg2
Arhgdib
Dmp1
H2.M9
Emcn
Tspan7

up2
Ly6k

down2
Lum
Mfap5
Fbln2
Dcn
Smoc2
Lrrc15
Itm2a
Fbln1
Rbp1
Rnase4
Gja1
Serpinf1
Cilp
Serpina3n
Ly6a
Olfml3
Cpxm1
Tnfaip6
Hsd11b1
Il33
Ndufa4
Ly6e
Igfbp6
Apod
Fndc1
Col1a1
Mfap4
Cxcl14
Lox
Itgbl1
Pi16
Eln
Cpz
Mmp2
Sfrp2
Ly6c1
Gas6
Ctla2a
Mgst1
Cd34
Htra3
Ccl8
Cthrc1
Fxyd6
Ccl11
Dpt

up3
Fabp4
Rgs4
Rgs5
Higd1b
Myl9
Kcnj8
Tinagl1
Sncg
Acta2
Tmem37
Ephx3
Rgs16
Errfi1
X1810011H11Rik
Ifitm1
Ankrd37
Actg2
Arhgdib
Dmp1
Cd36
Ccrl2
H2.M9
Cpa1
Emcn

up4
Dpt
Ccl11
Fxyd6
Reg1
Cthrc1
Ccl8
Sfrp1
Reg3g
Htra3
Efemp1
Cd34
Cxcl12
Omd
Mgst1
Ctla2a
Gas6
Ly6c1
Sfrp2
Col1a2
Mmp2
Lrrn4cl
Cd55
Cpz
Eln

up4
Pi16
Itgbl1
Lox
Cxcl14
Mfap4
Col1a1
Fndc1
Apod
Igfbp6
Ly6e
Ndufa4
Pdgfrl
Il33
Hsd11b1
Tnfaip6
Cpxm1
Olfml3
Ly6a
Serpina3n
Cilp
Serpinf1
Gja1
Rnase4
Rbp1
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Smoc2
Dcn
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Lum

up5
Dpt
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Mgst1
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Ctla2a
Gas6

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Ly6c1
Sfrp2
Col1a2
Mmp2
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Cpxm1
Olfml3
Ly6a
Serpina3n
Cilp
Serpinf1
Gja1
Rnase4
Rbp1
Fbln1
Itm2a
Cpxm2
Lrrc15
Smoc2
Dcn
Crabp1
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Mfap5
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Tmem37
Acta2
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Tinagl1
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Myl9
Higd1b
Rgs5
Rgs4
Fabp4