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A Pipeline to Identify and Characterize Senescent Cells

Introduction

Senescence is a cell state that contributes to aging, as well as age-related diseases such as Alzheimer's and heart disease. While senescent cells vary in composition, most exhibit these key characteristics: arrest of the cell cycle, changes in metabolism, upregulation of anti-apoptotic pathways, and secretion of various inflammatory molecules, a phenomenon known as the Senescence-Associated Secretory Phenotype (SASP) (Kumari et al., 2021). The senescent cell state is induced by stress. This stress can come from excessive shortening of telomeres (termed replicative senescence), direct DNA damaging events (termed premature senescence), or SASP from other senescent cells.

Through the use of bulk-RNA-seq, several senescent markers have been identified. A notable marker is upregulation of *CDKN2A*, a gene that encodes for the tumor suppressor protein *p16*. Thus, bulk RNA-seq data that exhibits a significant increase in *CDKN2A* expression can indicate senescence in the sample. Other traditional gene expression markers include *CDKN1A* and *LMNB1*, which code for the *p21* and Lamin B1 proteins.

Despite the significance of these bulk RNA-seq senescence markers, these traditional markers are not efficient in determining senescence with precision. For example, non-senescent macrophages as well as pRB-negative cells are known to have high expression levels of *p16*, thus resulting in false positives of senescence (and incorrect targeting in future drug delivery) (Kim & Kim, 2021). In addition, while these bulk RNA-seq senescence markers can detect senescence in the aggregate, these markers are not universally expressed in all senescent cells. This is because senescent cells are heterogeneous; they can express different genes based on cell type and the type of inducing stress. In some settings, senescent cells have no significant differential expression of traditional cellular senescence markers such as *CDKN2A*, *CDKN1A* and *LMNB1*. (Casella et al., 2019; Segura et al., 2017). Conversely, these same studies found hitherto unexpected mRNA transcripts affiliated with DNA damage checkpoints that could serve

as novel indicators of senescence. These findings indicate a need for additional markers of senescence.

Another important issue is the method of analysis. While bulk RNA-seq has been useful in the initial discovery and characterization of traditional senescent cell markers, single cell RNA sequencing (scRNA-seq) may serve as a better alternative for precisely characterizing heterogeneous senescent cell expression profiles. For example, a recent large-scale study was able to find new rare populations of genes associated with aging by performing scRNA-seq analysis on the organs of 19 male and 11 female mice (Kim & Kim, 2021; Tabula Muris Senis Consortium, 2020). In addition, scRNA-seq can explain mechanisms of aging in organs that bulk-RNA-seq cannot. For instance, the question of whether age related changes occur in an organ due to intrinsic changes in gene expression or changes in cell type cannot be answered by bulk-RNA-seq, but can be answered by scRNA-seq (Tabula Muris Senis Consortium, 2020).

Despite the widespread use and success of scRNA-seq in many areas of study since its advent in 2009, scRNA-seq has been used relatively little in the research field of senescence. Few studies have specifically single cell sequenced senescent cells (Kim & Kim, 2021). Even among these few studies, none have the necessary experimental design to allow for fool-proof annotation of senescent cells at the level of single cell resolution, which leads to a catch-22: if there's no consensus method for identifying individual senescent cells at the transcriptome level, there's no reliable way of determining new markers.

The closest study that includes the required information for “ground truth” annotation seems to be the Chen et al. (2022) study. In this study, the authors induced senescence in mouse embryonic fibroblasts (MEFs). They found 6 total clusters and concluded that one of the clusters was likely composed of senescent cells. One challenge with this conclusion is that they only used one pipeline for analysis. Depending upon the type of pipeline used, final results may be different and hence conclusions uncertain. Before the exploration of clusters to search for novel senescent markers can occur, the fundamental classification of cells as being senescent must be robust.

To explore the effect of the analytical pipeline, 64 different pipelines were assembled in this study to determine the consensus among different methods, as well as build the best pipeline for identifying and characterizing senescent cells.

Methods

The publicly available single cell RNA sequencing data from (Chen et al.,2022) was downloaded from the NGDC GSA repository using accession code CRA002582. Following the procedure and parameters of the original study, the sequences were aligned to the reference genome using STAR (Dobin et al., 2012). The aligned sequences were then quantified using Feature counts (Liao et al., 2014). There were 175 single cells left for analysis after these steps.

The data was then loaded into R (R Core Team (2022)), in which pipeComp (Germain et al., 2020) was used as a framework for the building, organization, and comparison of pipelines. Taking as input different parameters for each computational step, pipeComp runs through all possible combinations of parameters, thus running through all possible pipelines.

The first step, normalization, had 4 different possibilities: seurat normalization (Hao and Hao et al., 2021) , scran normalization (Lun et al.,2016), SCnorm normalization (Bacher et al.,2017), and Linnorm normalization (Yip et al., 2017).

The second step, feature selection, also had 4 different possibilities: vst selection (Hao and Hao et al., 2021), dispersion selection (Hao and Hao et al., 2021), var plot selection (Hao and Hao et al., 2021) , and ROGUE selection (Liu et al., 2020). Each feature selection method only selected the top 2000 highly variable genes.

The third step, clustering, only had one method: seurat clustering using resolution of 1.4 (Hao and Hao et al., 2021).The third step also had an evaluation wrapper that allowed for the generation of the tSNE graphs. In this evaluation step, tSNE plots were generated and saved to a different folder for the 16 different unique combinations.

The fourth step, differential expression testing (DE), had four different possibilities: MAST (Finak et al.,2015), wilcoxon, deseq2 (Love et al., 2014), and bimodal (McDavid et al., Bioinformatics, 2013). DE testing was performed on each individual cluster vs the rest of the data.

The fourth step also had an evaluation wrapper. In this evaluation, the intersection of significant genes for each cluster and a reference list was noted. The cluster with the highest intersection was chosen and also tested for intersection with another reference list.

Results

Pipeline Composition

In total, 64 different combinations of pipelines were applied to the data. A visual of the framework of this study is provided in Figure 1. The four different normalization methods, matched with four feature selection methods and four DE methods gave rise to 64 different pipelines. Different combinations of alignment and quantification methods were not used in order to follow along with the results of the (Chen et al., 2022) study.

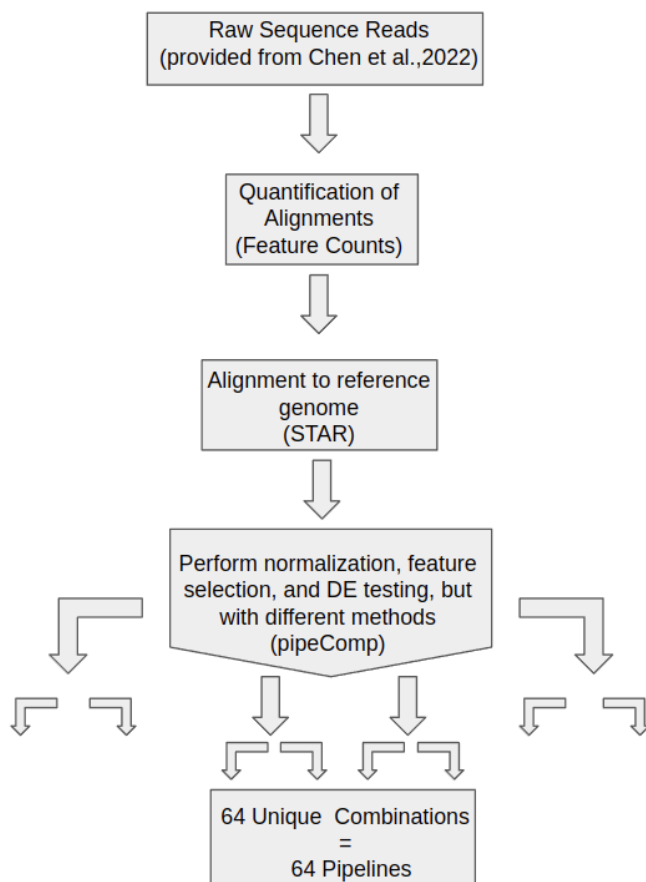


Figure 1: Visual of overall framework of study. The publically available sequence data from Chen et al. was aligned to the reference genome using STAR and quantified using FeatureCounts. The resultant data was then loaded into the pipeComp framework in R.

The constituent runtimes of the pipelines were also tracked. DE testing on the whole was the most computationally intensive task on average.

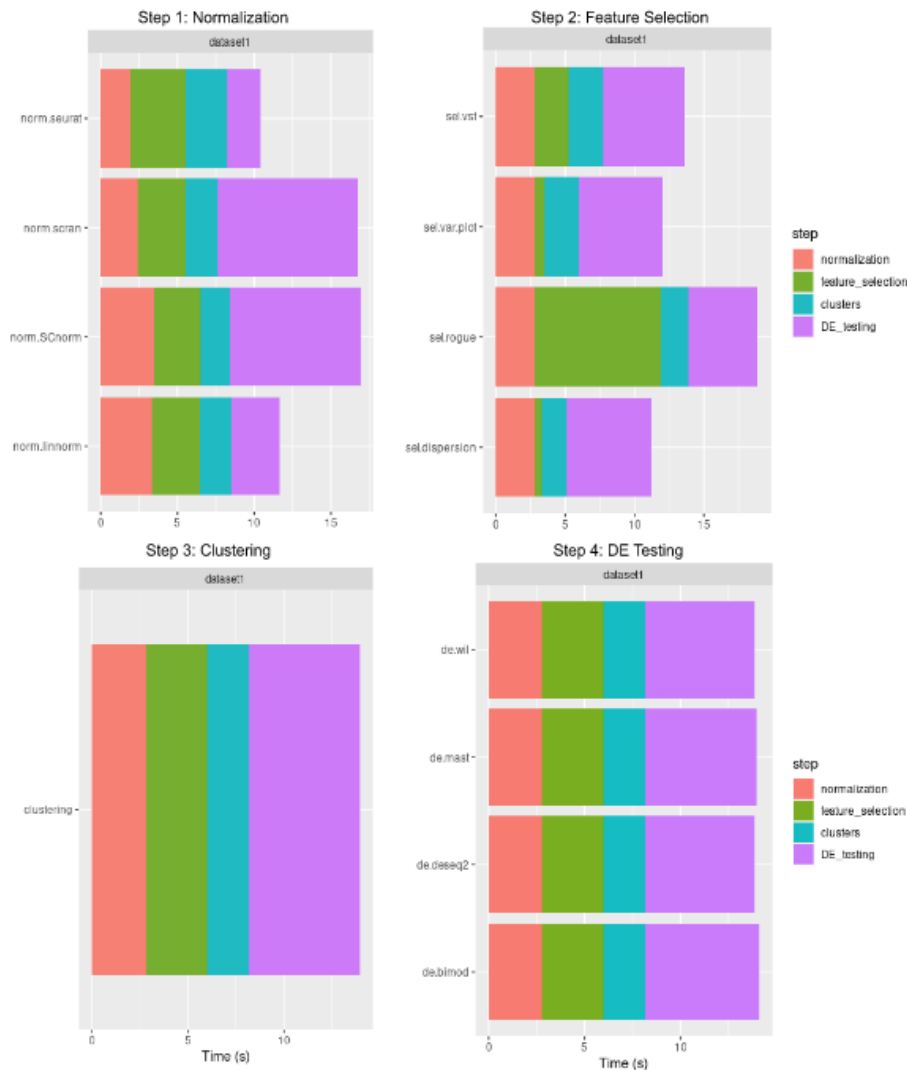


Figure 2: The pipelines were composed of four general steps: normalization, feature selection, clustering, and DE testing. The computational method used for each step had multiple possible combinations. For example, the normalization step could be computed via the scran method, seurat method, linnorm method, or SCnorm method. Aggregated proportion of total runtime for each method is also shown. For example, the normalization step on average contributed to 20% of the total run time when seurat was used, whereas the normalization step on average contributed to 30% of the total run time when linnorm was used.

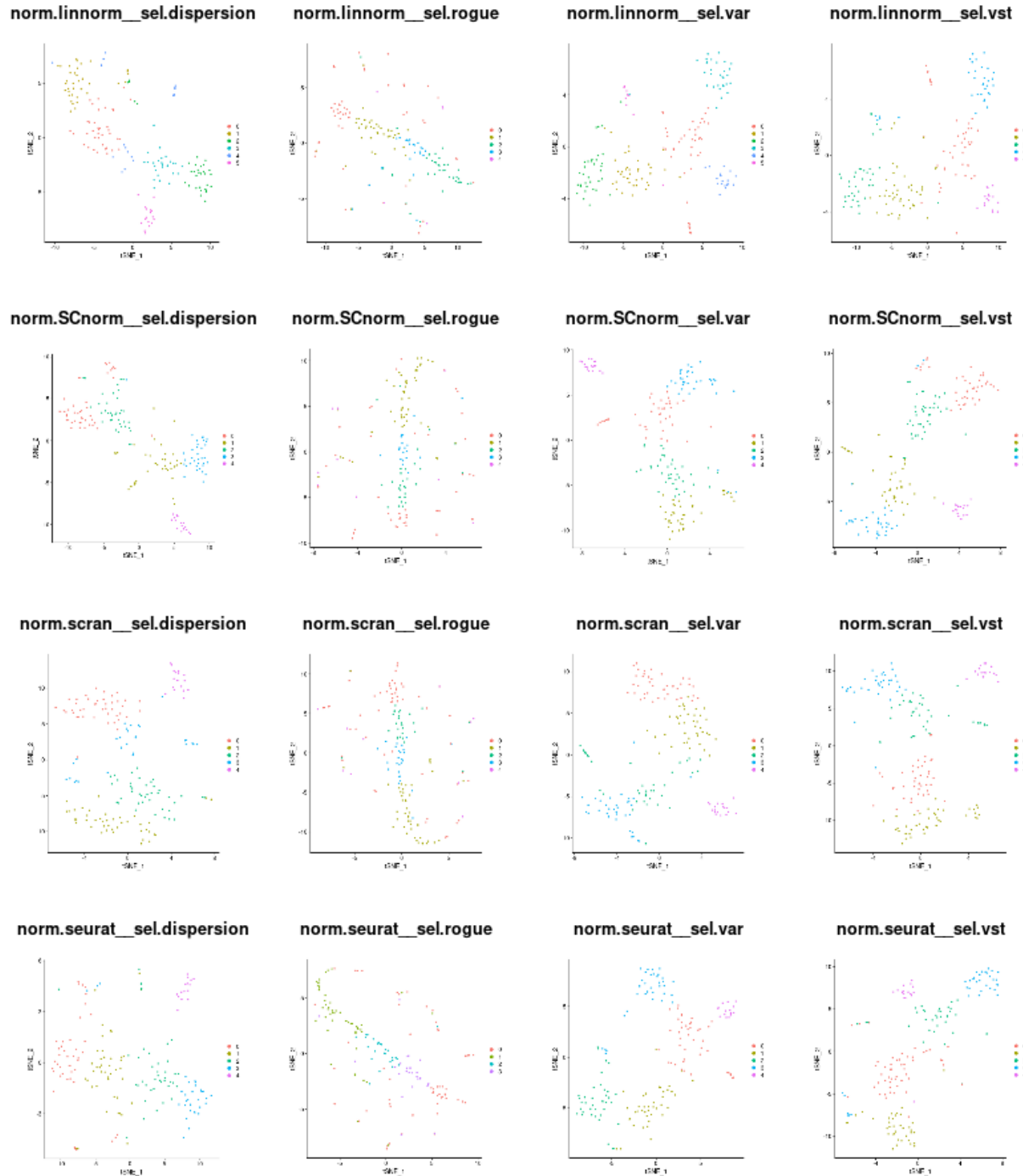


Figure 3: The 16 tSNE dimension reduction plots generated for the 64 pipelines. The tSNE plots were generated at the end of the third step (clustering), before the final step of DE testing. Thus, there are only 16 unique combinations of tSNE plots for the 64 pipelines. As readily seen, depending upon the previous normalization and feature selections step, the tSNE visualizations are very different. This suggests that the resulting clusters compositions may be different as well.

16 unique tSNE plots were generated for the 64 pipelines. Based on the method of normalization and feature selection, the plots appear to be very different. Even among the same normalization method (row in Figure 3), there appeared to be no consistency between the resultant 4 plots when using different feature selection methods. The same is true for feature selection; even when comparing among the same feature selection method (column in Figure 3), the 4 plots were very different based on the previous normalization method. Interestingly, there did appear to be one exception; regardless of normalization method, ROGUE feature selection seemed to yield similar tSNE plots.

Comparison to Chen et al.

Other studies, such as the tabula muris senis (Tabula Muris Senis Consortium, 2020), claim to have captured senescent cells, using bulk RNA-seq senescent markers expressed in certain clusters as validation (and annotation), but this is likely best considered as a preliminary finding. Large studies such as tabula muris senis and tabula sapiens (Tabula Sapiens Consortium, 2022) are composed of many different experiments and thus must employ severe data integration techniques. This data integration likely overcorrects and smooths out important biological signal, thus making it hard to reliably find senescent cells at the single cell level. In addition, some studies have shown that senescent cells can be more similar to their original cell type than to senescent cells of other cell types (Kim & Kim, 2021), meaning studies composed of mixed cells from different cell types (such as these large studies) don't lend to robust annotation of senescent cells.

Another problem is that it's well-documented that senescent cells accumulate with age, but the actual proportion of senescent cells in aging tissue is very small. One can (for proof of concept) imagine that the proportion of senescent cells increases from 0.01% to 0.1% from young to old, but this 0.1% still remains very difficult to detect at the single cell level. Furthermore, many of the markers used to characterize senescent cells are simply stress related markers. Older tissue indeed may have more stress than younger tissue, but the presence of these stress related markers alone are not enough to prove senescence, at least at the single cell level.

In the Chen et al. study (2022), only one cell type was analyzed: mouse embryonic fibroblast cells (MEFs). These cells were induced to senesce via oxidative stress. The cells were then passaged until half of the cells were positive in SA- β -Gal staining, at which point they were

then sequenced. This means that unlike the aforementioned studies, the data this study generated likely has a much larger proportion of senescent cells. After clustering their results, they annotated one of the clusters as having senescent characteristics by finding the intersection of significantly expressed genes in that cluster and a reference gene list composed of senescent markers. This reference gene list was composed of senescent markers gathered from the CellAge website (Avelar et al., 2018) and, critically, genes from a previous bulk study (Chen et al., 2018) the authors performed on senescent MEFs vs non senescent MEFs. They also used another gene list composed of cell cycle markers from the (Mackosko et al., 2015) study. In summary, the specificity of these parameters allowed the authors of this study a better (albeit imperfect) way of annotating senescent cells than other methods at the single cell level.

Following the same procedure but applying the 64 different pipelines to the data, the fraction of genes in both reference lists that were also in the senescent cluster were plotted in Figure 4. This was done to check for agreement among pipelines as well as identify an ideal pipeline (see methods for more details).

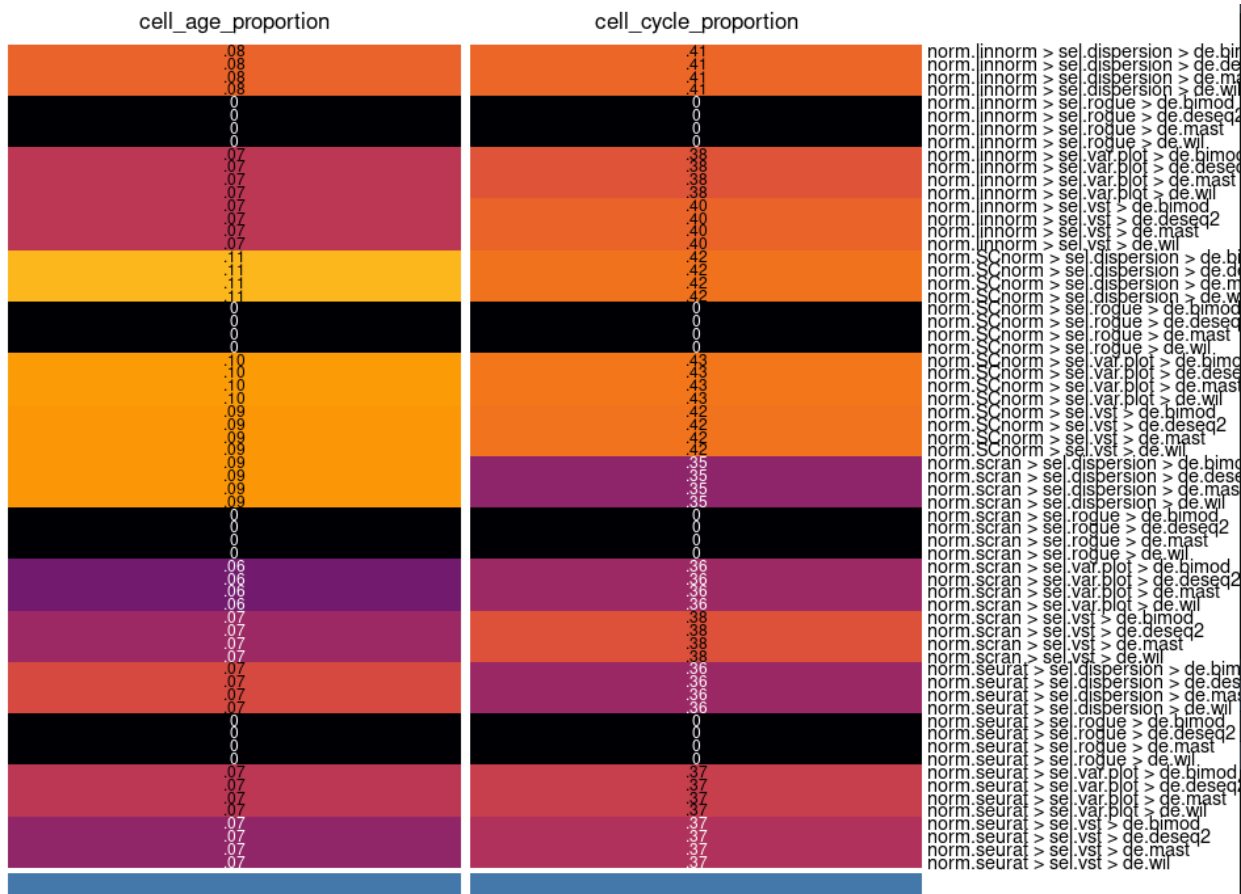


Figure 4: The proportion of genes in the reference lists that were identified by the pipelines. One reference list was composed of genes associated with senescence (see methods), the other was a list composed of genes associated with the cell cycle.

As seen in Figure 4, there was a general agreement among the CellAge gene reference list (composed of genes from CellAge website as well as the genes from the bulk study) and the cell cycle gene reference list, which is to be expected; senescent cells express phenotypes that halt their cell cycle progression. There were a few exceptions though; for example, pipelines such as the `norm.SCnorm > sel.var.plot > de.bimod` pipeline had a larger intersection with the CellAge gene reference list than the cell cycle reference list.

Interestingly, despite the ROGUE selection method yielding robust tSNE plots (as discussed in the last section), every pipeline that used ROGUE was unable to identify even a single gene on both of the reference lists. However, ROGUE uses a non-conventional method to identify highly variable genes, so ROGUE's apparent failure might be due to inherent bias in the reference gene lists themselves.

The success of the pipelines at identifying reference genes seems to be most affected by the normalization steps and feature selection step; the method of DE testing seemed to play very little role, as seen by color composition in Figure 4.

It seems like the best pipeline for identifying reference genes on the CellAge list was the `norm.SCnorm > sel.dispersion` pipeline, with any of the DE methods as the third parameter performing equivalently. The best pipeline for identifying reference genes on the cell cycle list was the `norm.SCnorm > sel.var.plot` pipeline, with any of the DE methods as the third parameter performing equivalently.

Discussion

The purpose of this study was to compare the robustness of pipelines at identifying senescent cells, as well as search for an ideal pipeline for this dataset. The data was taken from the Chen et al. (2022) study for a number of factors that allowed for increased confidence in senescent cell cluster annotation.

Normalization was found to have an important effect on the final results, whereas DE testing appeared to not have much effect. This was consistent with the results of a benchmarking

study (Vieth et al., 2019), in which they found that normalization was the overall most influential step in over 1000 different pipelines, whereas DE testing was the least influential step.

The norm.SCnorm > sel.dispersion > (any of the DE methods) pipelines and the norm.SCnorm > sel.var.plot > (any of the DE methods) pipelines were the best pipelines for identifying reference genes on the CellAge and cell cycle list, respectively. It should be mentioned that these pipelines had no way of controlling FDR in the DE methods. Theoretically, a very insensitive DE method may not be better overall but would appear to perform the best due to identifying a higher number of significant genes, thus increasing the likelihood that these genes are in the references list. However, the DE method appeared to play very little role, so not being able to control FDR did not appear to be problematic.

Another important point is that the pipeline for this dataset is unlikely to be the ideal pipeline on all other datasets. This dataset was relatively small, with only 175 cells. A larger dataset or even a dataset composed of different cells may actually perform better with a different pipeline. That being said, the eventual goal is to develop a robust pipeline that can work on a large number of datasets. It was found that the ROGUE feature selection method was the most robust software tool, in terms of results in both tSNE plot and final results.

In conclusion, data from the Chen et al. (2022) study was used for more confident annotation of senescent cells, at least more confident than using data from perhaps the Tabula Muris Senis study (Tabula Muris Senis Consortium, 2020). It was found that the first step of data normalization had the greatest final effect on the final results. This result advances the field by showing that careful attention to the pipeline is critical for confident, reproducible results. Additional critical resources to advance the field include more studies using single cell RNA sequencing on specifically senescent cells. Secondly, there needs to be a methodology that provides the required “ground truth” annotation of senescent cells to which the pipeline performance can be compared with. With such tools and a robust analytical pipeline, senescent cells can be better characterized at the single cell level.

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