Demuxafy

Release 0.0.3

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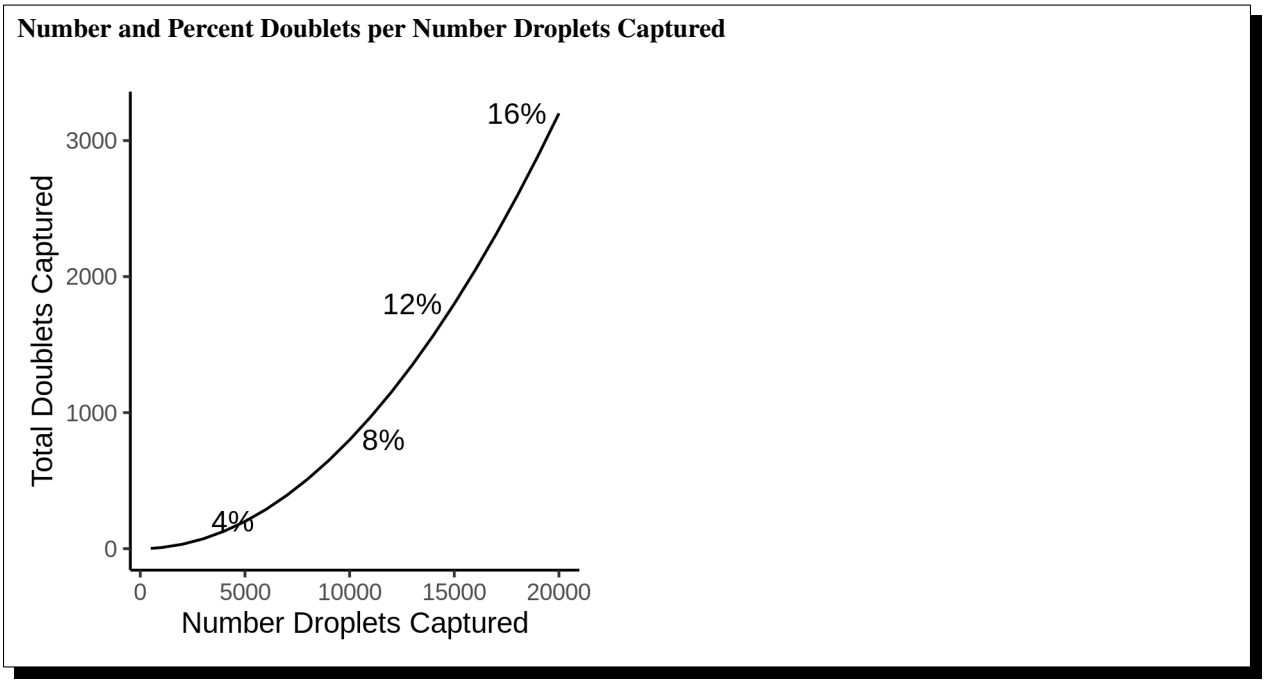
Oct 19, 2021

GENERAL



As droplet-based single cell technologies have advanced, increasingly larger sample numbers have been used to answer research questions at single cell resolution. With a larger number of droplets captured, there is an increase in the proportion of the droplets that are doublets (*Figure 1*).

This has been made possible because, as the droplet-based capture technologies have been optimized, methods to pool and then demultiplex samples - assign droplets to each individual in the pool - have been developed. These multiplexing methods clearly decrease cost and time of scRNA-seq experiments. If left in the dataset, doublets can significantly impact scientific conclusions such identifying spurious cell trajectories or false novel cell types. Therefore, it's crucial to effectively clean datasets prior to downstream analyses.

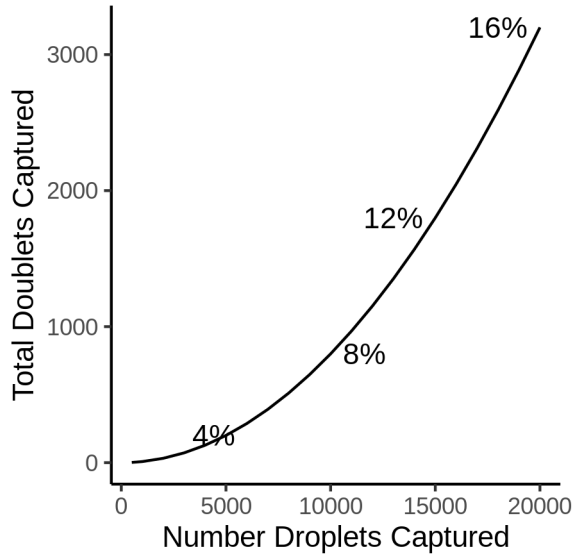
In addition to demultiplexing softwares, there are also doublet detecting softwares that use the transcriptional profiles of droplets to identify doublets by simulating doublets.

CHAPTER

ONE

BACKGROUND

Number and Percent Doublets per Number Droplets Captured



As droplet-based single cell technologies have advanced, increasingly larger sample numbers have been used to answer research questions at single cell resolution. With a larger number of droplets captured, there is an increase in the proportion of the droplets that are doublets (*Figure 1*).

This has been made possible because, as the droplet-based capture technologies have been optimized, methods to pool and then demultiplex samples - assign droplets to each individual in the pool - have been developed. These multiplexing methods clearly decrease cost and time of scRNA-seq experiments. If left in the dataset, doublets can significantly impact scientific conclusions such identifying spurious cell trajectories or false novel cell types. Therefore, it's crucial to effectively clean datasets prior to downstream analyses.

In addition to demultiplexing softwares, there are also doublet detecting softwares that use the transcriptional profiles of droplets to identify doublets by simulating doublets.

CHAPTER

TWO

INSTALLATION

Installation should be pretty painless (we hope). We have provided all the softwares in a singularity image which provides continuity across different computing platforms (see HPCNG Singluarity and Sylabs io for more information on singularity images). The only thing to note before you download this image is that the image is **~6.5Gb** so, depending on the internet speed, it will take **~15-30 min to download**. The good news is that you should only need to do this once unless updates are made to the scripts or image.

Just download the singluarity image with:

```
wget https://www.dropbox.com/s/2v7gv3neseg3g5v/Demuxafy.sif
wget https://www.dropbox.com/s/un1wrt27eab764f/Demuxafy.sif.md5
```

Then you should check to make sure that the image downloaded completely by comparing the image md5sum to the original md5sum. You can do that by running the following commands:

```
md5sum Demuxafy.sif > downloaded_Demuxafy.sif.md5
diff -s Demuxafy.sif.md5 downloaded_Demuxafy.sif.md5
```

If everything was downloaded correctly, that command should report:

```
Files Demuxafy.sif.md5 and downloaded_Demuxafy.sif.md5 are identical
```

Demuxafy software versions - for the curious

Image build date: 11 October, 2021

CHAPTER

THREE

DATA PREPARATION

There isn't a lot of data preparation to be done before running the demultiplexing or doublet detecting softwares.

3.1 Data Required

The demultiplexing and transcriptome-based doublet detecting softwares have different data input requirements:

Software Group	Single Cell Count Data Required	SNP Genotype Data Required
Demultiplexing	✓	✓
Doublet Detecting	✓	

Note: The SNP genotype data can be for multiplexed donors in the pool **OR** it can be publicly available common SNP genotypes which can be downloaded from 1000G (hg19 or hg38) or from HRC (hg19).

There isn't any pre-processing that's needed for the single cell count data. For the demultiplexing softwares, you should filter the SNP genotypes that you will use.

3.2 SNP Genotype Pre-processing

It is best to filter the SNP genotypes for common SNPs (generally > 1% or > 5% minor allele frequency) that overlap exons. Here we provide an example of how to do this filtering. We built the required softwares into the singularity image so you can run these filtering steps with the image.

Note: We have found it best to impute reference SNP genotypes so there are more SNP locations available. If you are using reference SNP genotypes for the donors in your pool, please be sure to impute before filtering.

3.2.1 Filter for Common SNPs

First, filter the SNP genotypes for common SNPs - here we use 5% minor allele frequency.

```
singularity exec Demuxafy.sif bcftools filter --include 'MAF>=0.05' -Oz --output
↪$OUTDIR/common_maf0.05.vcf.gz $VCF
```

Where `$OUTDIR` is the output directory where you want to save the results and `$VCF` is the path to the SNP genotype vcf file.

3.2.2 Filter for SNPs overlapping Exons

Next, filter for the SNPs that overlap exons.

Note: You can get an exon bed using the UCSC table browser (see instructions here) and we have also provided bed files for `hg19` and `hg38`

```
singularity exec Demuxafy.sif vcftools \
  --gzvcf $OUTDIR/common_maf0.05.vcf.gz \
  --max-alleles 2 \
  --remove-indels \
  --bed $BED \
  --recode \
  --recode-INFO-all \
  --out $OUTDIR/common_maf0.05_exon_filtered
```

3.3 Test Dataset

In addition, we have provided test data that you can use.

Information

The test dataset includes 20,982 droplets captured of PBMCs from 13 multiplexed individuals.

3.3.1 10x Directories + Other Necessary Files

We have provided this dataset as the complete dataset which is pretty large (~40Gb tar.gz directory). Therefore, we have also provided the same dataset where the data has been significantly reduced.

Warning: The reduced test dataset may not produce real-world results due to the small size - especially for doublet detecting softwares since the reads have been significantly downsampled to reduce the size.

You can download the dataset with one of the following commands:

Complete Dataset

Reduced Dataset

First, download the dataset and the md5sum:

```
wget https://www.dropbox.com/s/3oujqq98y400rzz/TestData4PipelineFull.tar.gz
wget https://www.dropbox.com/s/5n7u723okkf5m3l/TestData4PipelineFull.tar.gz.md5
```

After downloading the tar.gz directory, it is best to make sure the md5sum of the `TestData4PipelineFull.tar.gz` file matches the md5sum in the `TestData4PipelineFull.tar.gz.md5`:

```
md5sum TestData4PipelineFull.tar.gz > downloaded_TestData4PipelineFull.tar.gz.md5
diff -s TestData4PipelineFull.tar.gz.md5 downloaded_TestData4PipelineFull.tar.gz.md5
```

That should return the following statement indicating that the two md5sums are identical:

```
Files TestData4PipelineFull.tar.gz.md5 and downloaded_TestData4PipelineFull.tar.gz.
↪md5 are identical
```

First, download the reduced dataset and the md5sum:

```
wget https://www.dropbox.com/s/m8u61jn4i1mcktp/TestData4PipelineSmall.tar.gz
wget https://www.dropbox.com/s/ykjg86q3xw39wqr/TestData4PipelineSmall.tar.gz.md5
```

After downloading the tar.gz directory, it is best to make sure the md5sum of the `TestData4PipelineSmall.tar.gz` file matches the md5sum in the `TestData4PipelineSmall.tar.gz.md5`:

```
md5sum TestData4PipelineSmall.tar.gz > downloaded_TestData4PipelineSmall.tar.gz.md5
diff -s TestData4PipelineSmall.tar.gz.md5 downloaded_TestData4PipelineSmall.tar.gz.md5
```

That should return the following statement indicating that the two md5sums are identical:

```
Files TestData4PipelineSmall.tar.gz.md5 and downloaded_TestData4PipelineSmall.tar.gz.
↪md5 are identical
```

3.3.2 Seurat Object

We have also provided a filtered, QC normalized Seurat object (needed for *DoubletFinder* and *DoubletDecon*)

Download the rds object and the md5sum:

```
wget https://www.dropbox.com/s/po4gy2j3eqohhjv/TestData_Seurat.rds
wget https://www.dropbox.com/s/rmix7tt9aw28n7i/TestData_Seurat.rds.md5
```

After downloading the rds.object, it is best to make sure the md5sum of the `TestData_Seurat.rds` file matches the md5sum in the `TestData_Seurat.rds.md5`:

```
md5sum TestData_Seurat.rds > downloaded_TestData_Seurat.rds.md5
diff -s TestData_Seurat.rds.md5 downloaded_TestData_Seurat.rds.md5
```

That should return the following statement indicating that the two md5sums are identical:

```
Files TestData_Seurat.rds.md5 and downloaded_TestData_Seurat.rds.md5 are identical
```

Note: We have used this dataset for each of the tutorials. The example tables in the *Results and Interpretation* sections of each tutorial are the results from this dataset.

CHAPTER

FOUR

SOFTWARE SELECTION RECOMMENDATIONS

Based on our analysis of demultiplexing and doublet detecting softwares, we have generated the following decision tree to help other researchers elect the best set of softwares for their dataset. We have also put together a **selection widget (need to make and add link)** that will take you through each of the decision steps and provide advice regarding which softwares to use based on the characteristics of your dataset.

Insert decision tree figure here.

After you have run the softwares you selected, we have provided a script that will help merge and summarize the results from ethe softwares together. See *Combine Results*.

CHAPTER

FIVE

CONSIDERATIONS FOR OTHER SINGLE CELL DATA TYPES

This workflow was designed for demultiplexing and detecting doublets in scRNA-seq data. However, additional data types are becoming more frequently used - *i.e.* snRNA-seq, snATAC-seq and dual snRNA-seq + scATAC-seq. Based on our experiences with this data we have some recommendations and considerations to take into account when applying demultiplexing and doublet detecting softwares to these data types.

5.1 snRNA-seq

5.1.1 Demultiplexing Softwares

We have not tested any demultiplexing softwares on snRNA-seq data in our hands but we anticipate that it should behave similarly to scRNA-seq. The only difference we would suggest is to filter SNPs overlapping **genes** instead of just overlapping **exons**. If you are running in to any issues or would like a discussion about use of demultiplexing softwares on snRNA-seq data, please feel free to reach out.

5.1.2 Doublet Detecting Softwares

We have not tested doublet detecting softwares on snRNA-seq data but the softwares should work similarly as they do on scRNA-seq data. If you are running in to any issues or would like a discussion about use of doublet detecting softwares on snRNA-seq data, please feel free to reach out.

5.2 snATAC-seq

5.2.1 Demultiplexing Softwares

Demultiplexing snATAC-seq data can be done with the current demultiplexing softwares. However, we note that it is much more memory and time consumptive than scRNA-seq. Additionally, the SNPs should be filtered by SNPs overlapping **peak locations** instead of exon or gene locations. You may even want to filter the SNPs further if you still have many after filtering on minor allele frequency and peak location. We typically aim for ~250,000 SNP. Regardless, since UMI tags aren't used for snATAC-seq data, demultiplexing can take a lot of memory and time.

In addtion, the following flags are required for each of the following softwares to effectively process snATAC-seq data.

Souporcell

- `--no_umi True`

5.2.2 Doublet Detecting Softwares

Technically, doublet detecting softwares cannot be applied to snATAC-seq data as they rely on the unique transcriptomes of each cell type to identify heterotypic doublets. However, if snATAC-seq peaks can be made into a scRNA-seq-like matrices (*i.e.* by linking peaks to genes or some other method), the doublet detecting softwares outlined in this workflow could be applied to snATAC-seq data. This has been shown previously by SnapATAC and ArchR has a method built in that uses a very similar method to *Scrublet*. AMULET is another doublet detecting method that has been developed specifically for snATAC-seq data.

5.3 Combined snRNA-seq + snATAC-seq

5.3.1 Demultiplexing Softwares

We have noticed a higher percentage of ambient RNA from our combined snRNA-seq + scATAC-seq experiments as compared to our scRNA-seq (we haven't tested multiplexed snRNA-seq in our hands) but similar snATAC-seq ambient DNA estimations as detected with *Souporcell* Therefore, we recommend running *Souporcell*, if only to estimate the ambient RNA in your multiplexed pool. If you are doing the demultiplexing with the snRNA-seq results, please see the *snRNA-seq Section*. If you are using the snATAC-seq data for demultiplexing, please see the *snATAC-seq Section*.

5.3.2 Doublet Detecting Softwares

Doublet detecting softwares for the combined snRNA-seq + snATAC-seq should work similarly to the doublet detecting softwares for each assay separately (snRNA-seq and snATAC-seq). However, as noted int the *Demultiplexing Softwares Section* above, we have observed much higher ambient RNA percentages than for either assay run separately. ..Our results (**CITATION**) indicate that increased ambient RNA showed a slight decrease in the MCC and balanced accuracy. However, we did not simulate up to the level of ambient RNA percent that we have observed using this assay.

CHAPTER

SIX

NOTES ABOUT SINGULARITY IMAGES

Singularity images effectively store an operating system with files, softwares etc. that can be easily transported across different operating systems - ensuring reproducibility. Most HPCs have singularity installed making it easy to implement. There are some tips and tricks we have identified through using singularity images that we thought might help new users.

6.1 Tips and Tricks

6.1.1 1. Error: File Not Found

Reason

Singularity only loads the directories directly downstream from where you execute the singularity command. If any of the files that need to be accessed by the command are not downstream of the that location, you will receive an error similar to this one:

```
Failed to open file "/path/to/readfile.tsv" : No such file or directory
```

If you then check for that file:

```
ll /path/to/readfile.tsv
```

We can see that the file does truly exist:

```
-rw-rw-r-- 1 user group 70636291 Dec 21  2020 /path/to/readfile.tsv
```

Solution

The easiest solution to this problem is to "bind" a path upstream of all the files that will need to be accessed by your command:

```
singularity exec --bind /path Demuxafy.sif ...
```

If you don't have access to Singularity on your HPC, you can ask your HPC administrators to install it (see the Singularity page)

CHAPTER

SEVEN

OVERVIEW OF DEMULTIPLEXING SOFTWARES

Demultiplexing softwares use the inherent genetic differences between donors multiplexed in a single pool to assign droplets to each donor and to identify doublets. There are five demultiplexing softwares that have different capabilities and advantages depending on your dataset. As you can see from this table, only *Demuxlet* absolutely requires reference SNP genotypes for the donors multiplexed in your pool. However, *Souporcell* and *Vireo* are also capable of accomodating reference SNP genotypes as well.

Demultiplexing Software	Requires Reference SNP Genotypes	Can Use Reference SNP Genotypes	Estimates Ambient RNA
Demuxlet	✓	✓	
Femuxlet			
scSplit			
Souporcell		✓	✓
Vireo		✓	

We highly recommend using *Souporcell* if only to estimate the percentage of ambient RNA in your pool. As far as we are aware, this is the only software that leverages SNP genotype data to estimate ambient RNA in multiplexed pools and it is helpful to identify high ambient RNA which is sometimes undetectable with basic QC metrics. We view this as suppelementary to other ambient RNA methods that use the transcriptional profile to estimate and remove ambient RNA per droplet.

If you don't know which demultiplexing software(s) to run, take a look at our *Software Selection Recommendations* based on your dataset or use our **add widget link here**

CHAPTER

EIGHT

DEMUXLET TUTORIAL

Demuxlet is a genotype demultiplexing software that requires reference genotypes to be available for each individual in the pool. Therefore, if you don't have reference genotypes, you may want to demultiplex with one of the softwares that do not require reference genotype data (*Freemuxlet*, *scSplit*, *Souporcell* or *Vireo*)

8.1 Data

This is the data that you will need to have preparede to run Demuxlet:

Required

- Reference SNP genotypes for each individual (`$VCF`)
  - Filter for common SNPs (> 5% minor allele frequency) and SNPs overlapping genes
  - Demuxlet is very sensitive to missing data in a vcf so please make sure you only have complete cases in your refernce donor SNP genotype file
- Genotype field in `$VCF` (`$FIELD`)
  - This is `GP` by default but could also be `GT` others
- Barcode file (`$BARCODES`)
- Bam file (`$BAM`)
  - Aligned single cell reads
- Output directory (`$DEMUXLET_OUTDIR`)

Optional

- A text file with the individual ids (`$INDS`)
  - File containing the individual ids (separated by line) as they appear in the vcf file
  - For example, this is the `individual file` for our example dataset

8.2 Run Demuxlet

8.2.1 Poscle Pileup

First we will need to identify the number of reads from each allele at each SNP location:

With `$INDS` file

Without `$INDS` file

The `$INDS` file allows demuxlet to only consider the individual in this pool

```
singularity exec Demuxafy.sif popscle dsc-pileup --sam $BAM --vcf $VCF --group-list
↪$BARCODES --out $DEMUXLET_OUTDIR/pileup --sm-list $INDS
```

This will use all the individuals in your reference SNP genotype `$VCF`. If your `$VCF` only has the individuals multiplexed in your pool, then the `$INDS` file is not required.

```
singularity exec Demuxafy.sif popscle dsc-pileup --sam $BAM --vcf $VCF --group-list
↪$BARCODES --out $DEMUXLET_OUTDIR/pileup
```

If the pileup is successfull, you will have these files in your `$DEMUXLET_OUTDIR`:

```
.
├── pileup.cel.gz
├── pileup.plp.gz
├── pileup.umi.gz
└── pileup.var.gz
```

Additional details about outputs are available below in the *Demuxlet Results and Interpretation*.

8.2.2 Popscle Demuxlet

Once you have run `popscle pileup`, you can demultiplex your samples:

With `$INDS` file

Without `$INDS` file

The `$INDS` file allows demuxlet to only consider the individual in this pool

```
singularity exec Demuxafy.sif popscle demuxlet --plp $DEMUXLET_OUTDIR/pileup --vcf
↪$VCF --field $FIELD --group-list $BARCODES --geno-error-coeff 1.0 --geno-error-
↪offset 0.05 --out $DEMUXLET_OUTDIR/demuxlet --sm-list $INDS
```

This will use all the individuals in your reference SNP genotype `$VCF`. If your `$VCF` only has the individuals multiplexed in your pool, then the `$INDS` file is not required.

```
singularity exec Demuxafy.sif popscle demuxlet --plp $DEMUXLET_OUTDIR/pileup --vcf
↪$VCF --field $FIELD --group-list $BARCODES --geno-error-coeff 1.0 --geno-error-
↪offset 0.05 --out $DEMUXLET_OUTDIR/demuxlet
```

If demuxlet is successfull, you will have these new files in your `$DEMUXLET_OUTDIR`:

```
.
├── demuxlet.best
├── pileup.cel.gz
```

(continues on next page)

(continued from previous page)

```
├── pileup.plp.gz
├── pileup.umi.gz
└── pileup.var.gz
```

Additional details about outputs are available below in the *Demuxlet Results and Interpretation*.

8.2.3 Demuxlet Summary

We have provided a script that will summarize the number of droplets classified as doublets, ambiguous and assigned to each donor by Demuxlet and write it to the `$DEMUXLET_OUTDIR`. You can run this to get a fast and easy summary of your results by providing the path to your result file:

```
singularity exec Demuxafy.sif bash Demuxlet_summary.sh $DEMUXLET_OUTDIR/demuxlet.best
```

which will return:

Classification	Assignment N
113_113	1334
349_350	1458
352_353	1607
39_39	1297
40_40	1078
41_41	1127
42_42	1419
43_43	1553
465_466	1094
596_597	1255
597_598	1517
632_633	868
633_634	960
660_661	1362
doublet	3053

or you can write it straight to a file:

```
singularity exec Demuxafy.sif bash Demuxlet_summary.sh $FREEMUXLET_OUTDIR/demuxlet.
↪best > $DEMUXLET_OUTDIR/demuxlet_summary.tsv
```

Note

To check if these numbers are consistent with the expected doublet rate in your dataset, you can use our Doublet Estimation Calculator.

8.3 Demuxlet Results and Interpretation

After running the Demuxlet steps and summarizing the results, you will have a number of files from some of the intermediary steps. Theses are the files that most users will find the most informative:

- `demuxlet.best`
  - Metrics for each droplet including the singelt, doublet or ambiguous assignment (`DROPLET.TYPE`), final assignment (`BEST.GUESS`), log likelihood of the final assignment (`BEST.LLK`) and other QC metrics.

INT_ID	BARCODE	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
0	AAA[illegible] 1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible] 29.42	[illegible]	[illegible] 39.12	[illegible]	[illegible] 33	1	41_41	29.42	597_598	76.24	0.000[illegible]	[illegible]	[illegible] 39.12	[illegible]
1	AAA[illegible] 1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible] 70.61	[illegible]	[illegible] 94.85	[illegible]	[illegible] 74	1	465_466	70.61	42_42	166.61	0.000[illegible]	[illegible]	[illegible] 94.85	[illegible]
2	AAA[illegible] 1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible] 25.05	[illegible]	[illegible] 29.85	[illegible]	[illegible] 28	1	113_113	25.05	349_350	51.63	0.000[illegible]	[illegible]	[illegible] 29.85	[illegible]
3	AAA[illegible] 1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible] 33.14	[illegible]	[illegible] 44.78	[illegible]	[illegible] 36	[illegible]	349_350	33.14	632_633	77.41	0.000[illegible]	[illegible]	[illegible] 44.78	[illegible]
4	AAA[illegible] 1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible] 54.79	[illegible]	[illegible] 72.23	[illegible]	[illegible] 58	[illegible]	632_633	54.79	633_634	163.24	0.000[illegible]	[illegible]	[illegible] 72.23	[illegible]
…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…

8.4 Merging Results with Other Software Restults

We have provided a script that will help merge and summarize the results from multiple softwares together. See *Combine Results*.

8.5 Citation

If you used this workflow for analysis, please reference our paper (REFERENCE) as well as Demuxlet.

CHAPTER

NINE

FREEMUXLET TUTORIAL

Freemuxlet is a genotype-free demultiplexing software that does not require you to have SNP genotypes the donors in your multiplexed capture. In fact, it can't natively integrate SNP genotypes into it's demultiplexing. We have provided some scripts that will help identify clusters from given donors if you do have SNP genotypes but use Freemuxlet. However, it might be better to use a software that is designed integrate SNP genotypes while assigning donor/cluster (*Demuxlet*, *Souporcell* or *Vireo*).

9.1 Data

This is the data that you will need to have preparede to run Freemuxlet:

Required

- Common SNP genotypes vcf (`$VCF`)
  - While not exactly required, using common SNP genotype locations enhances accuracy
    - If you have reference SNP genotypes for individuals in your pool, you can use those
    - If you do not have reference SNP genotypes, they can be from any large population resource (i.e. 1000 Genomes or HRC)
  - Filter for common SNPs (> 5% minor allele frequency) and SNPs overlapping genes
  - Filter for common SNPs (> 5% minor allele frequency) and SNPs overlapping genes
- Barcode file (`$BARCODES`)
- Number of samples in pool (`$N`)
- Bam file (`$BAM`)
  - Aligned single cell reads
- Output directory (`$FREEMUXLET_OUTDIR`)

9.2 Run Freemuxlet

9.2.1 Poscle Pileup

First we will need to identify the number of reads from each allele at each of the common SNP location:

```
singularity exec Demuxafy.sif popscle dsc-pileup --sam $BAM --vcf $VCF --group-list
↪$BARCODES --out $FREEMUXLET_OUTDIR/pileup
```

If the pileup is successfull, you will have these files in your `$FREEMUXLET_OUTDIR`:

```
.
├── pileup.cel.gz
├── pileup.plp.gz
├── pileup.umi.gz
└── pileup.var.gz
```

Additional details about outputs are available below in the *Freemuxlet Results and Interpretation*.

9.2.2 Popscle Freemuxlet

Once you have run `popscle pileup`, you can demultiplex your samples with Freemuxlet:

```
singularity exec Demuxafy.sif popscle freemuxlet --plp $FREEMUXLET_OUTDIR/pileup --
↪out $FREEMUXLET_OUTDIR/freemuxlet --group-list $BARCODES --nsample $N
```

If freemuxlet is successfull, you will have these new files in your `$FREEMUXLET_OUTDIR`:

```
.
├── freemuxlet.clust1.samples.gz
├── freemuxlet.clust1.vcf.gz
├── freemuxlet.lmix
├── pileup.cel.gz
├── pileup.plp.gz
├── pileup.umi.gz
└── pileup.var.gz
```

Additional details about outputs are available below in the *Freemuxlet Results and Interpretation*.

9.2.3 Freemuxlet Summary

We have provided a script that will summarize the number of droplets classified as doublets, ambiguous and assigned to each donor by Freemuxlet and write it to the `$FREEMUXLET_OUTDIR`. You can run this to get a fast and easy summary of your results by providing the result file of interest:

```
singularity exec Demuxafy.sif bash Freemuxlet_summary.sh $FREEMUXLET_OUTDIR/
↪freemuxlet.clust1.samples.gz
```

which will return:

Classification	Assignment N
0	1575
1	1278
10	972
11	1477
12	1630
13	1446
2	1101
3	1150
4	1356
5	1540
6	1110
7	1313
8	1383
9	884
DBL	2767

or you can write it straight to a file:

```
singularity exec Demuxafy.sif bash Freemuxlet_summary.sh $FREEMUXLET_OUTDIR/
↪freemuxlet.clust1.samples.gz > $FREEMUXLET_OUTDIR/freemuxlet_summary.tsv
```

:: Note

To check if these numbers are consistent with the expected doublet rate in your dataset, you can use our Doublet Estimation Calculator.

9.2.4 Correlating Cluster to Donor Reference SNP Genotypes (optional)

If you have reference SNP genotypes for some or all of the donors in your pool, you can identify which cluster is best correlated with each donor in your reference SNP genotypes. We have provided a script that will do this and provide a heatmap correlation figure and the predicted individual that should be assigned for each cluster. You can either run it with the script by providing the reference SNP genotypes (`$VCF`), the cluster SNP genotypes (`$FREEMUXLET_OUTDIR/freemuxletOUT.clust1.vcf.gz`) and the output directory (`$FREEMUXLET_OUTDIR`) You can run this script with:

Note

In order to do this, your `$VCF` must be reference SNP genotypes for the individuals in the pool and cannot be a general vcf with common SNP genotype locations from 1000 Genomes or HRC.

With Script

Run in R

```
singularity exec Demuxafy.sif Assign_Indiv_by_Geno.R -r $VCF -c $FREEMUXLET_OUTDIR/
↪freemuxlet.clust1.vcf.gz -o $FREEMUXLET_OUTDIR
```

To see the parameter help menu, type:

```
singularity exec Demuxafy.sif Assign_Indiv_by_Geno.R -h
```

Which will print:

```
usage: Assign_Indiv_by_Geno.R [-h] -r REFERENCE_VCF -c CLUSTER_VCF -o OUTDIR

optional arguments:
-h, --help            show this help message and exit
-r REFERENCE_VCF, --reference_vcf REFERENCE_VCF
                                                    The output directory where results␣
↪will be saved
-c CLUSTER_VCF, --cluster_vcf CLUSTER_VCF
                                                    A QC, normalized seurat object with
                                                    classificaitons/clusters as Idents().
-o OUTDIR, --outdir OUTDIR
                                                    Number of genes to use in
                                                    'Improved_Seurat_Pre_Process'␣
↪function.
```

You can run the reference vs cluster genotypes manually (possibly because your data doesn't have GT, DS or GP genotype formats) or because you would prefer to alter some of the steps. To run the correlations manually, simply start R from the singularity image:

```
singularity exec Demuxafy.sif R
```

Once, R has started, you can load the required libraries (included in the singularity image) and run the code.

```
.libPaths("/usr/local/lib/R/site-library") ### Required so that libraries are loaded␣
↪from the image instead of locally
library(tidyr)
library(tidyverse)
library(dplyr)
library(vcfR)
library(lsa)
library(ComplexHeatmap)


########## Set up paths and variables ##########

reference_vcf <- "/path/to/reference.vcf"
cluster_vcf <- "/path/to/freemuxlet/out/freemuxletOUT.clust1.vcf.gz"
outdir <- "/path/to/freemuxlet/out/"


########## Set up functions ##########
##### Calculate DS from GP if genotypes in that format #####
calculate_DS <- function(GP_df){
    columns <- c()
    for (i in 1:ncol(GP_df)){
        columns <- c(columns, paste0(colnames(GP_df)[i],"-0"), paste0(colnames(GP_
↪df)[i],"-1"), paste0(colnames(GP_df)[i],"-2"))
    }
    df <- GP_df
    colnames(df) <- paste0("c", colnames(df))
    colnames_orig <- colnames(df)
    for (i in 1:length(colnames_orig)){
        df <- separate(df, sep = ",", col = colnames_orig[i], into = columns[(1+(3*(i-
↪1))):(3+(3*(i-1)))])
    }
    df <- mutate_all(df, function(x) as.numeric(as.character(x)))
    for (i in 1: ncol(GP_df)){
```

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```
        GP_df[,i] <- df[,(2+((i-1)*3))] + 2* df[,(3+((i-1)*3))]
    }
    return(GP_df)
}

pearson_correlation <- function(df, ref_df, clust_df){
    for (col in colnames(df)){
        for (row in rownames(df)){
            df[row,col] <- cor(as.numeric(pull(ref_df, col)), as.numeric(pull(clust_
↪df, row)), method = "pearson", use = "complete.obs")
        }
    }
    return(df)
}


########## Read in vcf files for each of three non-reference genotype softwares ######
↪####
ref_geno <- read.vcfR(reference_vcf)
cluster_geno <- read.vcfR(cluster_vcf)



########## Convert to tidy data frame ##########
####### Identify which genotype FORMAT to use #######
##### Cluster VCF #####
### Check for each of the different genotype formats ##
## DS ##
format_clust=NA
cluster_geno_tidy <- as_tibble(extract.gt(element = "DS",cluster_geno, IDtoRowNames =␣
↪F))
if (!all(colSums(is.na(cluster_geno_tidy)) == nrow(cluster_geno_tidy))){
 ␣
↪message("Found DS genotype format in cluster vcf. Will use that metric for cluster correlation.")
  format_clust = "DS"
}

## GT ##
if (is.na(format_clust)){
  cluster_geno_tidy <- as_tibble(extract.gt(element = "GT",cluster_geno, IDtoRowNames␣
↪= F))
  if (!all(colSums(is.na(cluster_geno_tidy)) == nrow(cluster_geno_tidy))){
   ␣
↪message("Found GT genotype format in cluster vcf. Will use that metric for cluster correlation.")
    format_clust = "GT"

    if (any(grepl("\\|",cluster_geno_tidy[1,]))){
      separator = "|"
      message("Detected | separator for GT genotype format in cluster vcf")
    } else if (any(grepl("/",cluster_geno_tidy[1,]))) {
      separator = "/"
      message("Detected / separator for GT genotype format in cluster vcf")
    } else {
      format_clust = NA
     ␣
↪message("Can't identify a separator for the GT field in cluster vcf, moving on to using GP.")
    }
```

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```
    cluster_geno_tidy <- as_tibble(lapply(cluster_geno_tidy, function(x)
↪{gsub(paste0("0",separator,"0"),0, x)}) %>%
                                lapply(., function(x) {gsub(paste0("0",separator,"1"),1,␣
↪x)}) %>%
                                lapply(., function(x) {gsub(paste0("1",separator,"0"),1,␣
↪x)}) %>%
                                lapply(., function(x) {gsub(paste0("1",separator,"1"),2,␣
↪x)}))

  }
}

## GP ##
if (is.na(format_clust)){
  cluster_geno_tidy <- as_tibble(extract.gt(element = "GP",cluster_geno, IDtoRowNames␣
↪=F))
  if (!all(colSums(is.na(cluster_geno_tidy)) == nrow(cluster_geno_tidy))){
    format_clust = "GP"
    cluster_geno_tidy <- calculate_DS(cluster_geno_tidy)
   ␣
↪message("Found GP genotype format in cluster vcf. Will use that metric for cluster correlation.")

  } else {
   ␣
↪print("Could not identify the expected genotype format fields (DS, GT or GP) in your cluster vcf. F
    q()
  }
}




### Reference VCF ###
### Check for each of the different genotype formats ##
## DS ##
format_ref = NA
ref_geno_tidy <- as_tibble(extract.gt(element = "DS",ref_geno, IDtoRowNames = F))
if (!all(colSums(is.na(ref_geno_tidy)) == nrow(ref_geno_tidy))){
 ␣
↪message("Found DS genotype format in reference vcf. Will use that metric for cluster correlation.")
  format_ref = "DS"
}

## GT ##
if (is.na(format_ref)){
  ref_geno_tidy <- as_tibble(extract.gt(element = "GT",ref_geno, IDtoRowNames = F))
  if (!all(colSums(is.na(ref_geno_tidy)) == nrow(ref_geno_tidy))){
   ␣
↪message("Found GT genotype format in reference vcf. Will use that metric for cluster correlation.")
    format_ref = "GT"

    if (any(grepl("\\|",ref_geno_tidy[1,]))){
      separator = "|"
      message("Detected | separator for GT genotype format in reference vcf")
    } else if (any(grepl("/",ref_geno_tidy[1,]))) {
```

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```
      separator = "/"
      message("Detected / separator for GT genotype format in reference vcf")
    } else {
      format_ref = NA
    ␣
↪message("Can't identify a separator for the GT field in reference vcf, moving on to using GP.")
    }

    ref_geno_tidy <- as_tibble(lapply(ref_geno_tidy, function(x) {gsub(paste0("0",
↪separator,"0"),0, x)}) %>%
                               lapply(., function(x) {gsub(paste0("0",separator,"1"),1,␣
↪x)}) %>%
                               lapply(., function(x) {gsub(paste0("1",separator,"0"),1,␣
↪x)}) %>%
                               lapply(., function(x) {gsub(paste0("1",separator,"1"),2,␣
↪x)}))

  }
}

## GP ##
if (is.na(format_ref)){
  ref_geno_tidy <- as_tibble(extract.gt(element = "GP",ref_geno, IDtoRowNames = F))
  if (!all(colSums(is.na(ref_geno_tidy)) == nrow(ref_geno_tidy))){
    format_clust = "GP"
    ref_geno_tidy <- calculate_DS(ref_geno_tidy)
   ␣
↪message("Found GP genotype format in cluster vcf. Will use that metric for cluster correlation.")

  } else {
   ␣
↪print("Could not identify the expected genotype format fields (DS, GT or GP) in your cluster vcf. F
    q()
  }
}



### Get SNP IDs that will match between reference and cluster ###
## Account for possibility that the ref or alt might be missing
if ((all(is.na(cluster_geno@fix[,'REF'])) & all(is.na(cluster_geno@fix[,'ALT']))) |␣
↪(all(is.na(ref_geno@fix[,'REF'])) & all(is.na(ref_geno@fix[,'ALT'])))){
 ␣
↪message("The REF and ALT categories are not provided for the reference and/or the cluster vcf. Will
  cluster_geno_tidy$ID <- paste0(cluster_geno@fix[,'CHROM'],":", cluster_geno@fix[,
↪'POS'])
  ref_geno_tidy$ID <- paste0(ref_geno@fix[,'CHROM'],":", ref_geno@fix[,'POS'])
} else if (all(is.na(cluster_geno@fix[,'REF'])) | all(is.na(ref_geno@fix[,'REF']))){
 ␣
↪message("The REF categories are not provided for the reference and/or the cluster vcf. Will use the
  cluster_geno_tidy$ID <- paste0(cluster_geno@fix[,'CHROM'],":", cluster_geno@fix[,
↪'POS'],"_", cluster_geno@fix[,'REF'])
  ref_geno_tidy$ID <- paste0(ref_geno@fix[,'CHROM'],":", ref_geno@fix[,'POS'],"_",␣
↪ref_geno@fix[,'REF'])
} else if (all(is.na(cluster_geno@fix[,'ALT'])) | all(is.na(ref_geno@fix[,'ALT']))){
 ␣
↪message("The ALT categories are not provided for the reference and/or the cluster vcf. Will use the
```

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```
  cluster_geno_tidy$ID <- paste0(cluster_geno@fix[,'CHROM'],":", cluster_geno@fix[,
↪'POS'],"_", cluster_geno@fix[,'ALT'])
  ref_geno_tidy$ID <- paste0(ref_geno@fix[,'CHROM'],":", ref_geno@fix[,'POS'],"_",␣
↪ref_geno@fix[,'ALT'])
} else {
 ␣
↪message("Found REF and ALT in both cluster and reference genotype vcfs. Will use chromosome, positi
    cluster_geno_tidy$ID <- paste0(cluster_geno@fix[,'CHROM'],":", cluster_geno@fix[,
↪'POS'],"_", cluster_geno@fix[,'REF'],"_", cluster_geno@fix[,'ALT'])
  ref_geno_tidy$ID <- paste0(ref_geno@fix[,'CHROM'],":", ref_geno@fix[,'POS'],"_",␣
↪ref_geno@fix[,'REF'],"_", ref_geno@fix[,'ALT'])
}


### Update the vcf dfs to remove SNPs with no genotyopes
cluster_geno_tidy <- cluster_geno_tidy[colSums(!is.na(cluster_geno_tidy)) > 0]
ref_geno_tidy <- ref_geno_tidy[colSums(!is.na(ref_geno_tidy)) > 0]



########## Get a unique list of SNPs that is in both the reference and cluster␣
↪genotypes ##########
locations  <- inner_join(ref_geno_tidy[,"ID"],cluster_geno_tidy[,"ID"])
locations <- locations[!(locations$ID %in% locations[duplicated(locations),]$ID),]

########## Keep just the SNPs that overlap ##########
ref_geno_tidy <- left_join(locations, ref_geno_tidy)
cluster_geno_tidy <- left_join(locations, cluster_geno_tidy)

########## Correlate all the cluster genotypes with the individuals genotyped ########
↪##
##### Make a dataframe that has the clusters as the row names and the individuals as␣
↪the column names #####
pearson_correlations <- as.data.frame(matrix(nrow = (ncol(cluster_geno_tidy) -1),␣
↪ncol = (ncol(ref_geno_tidy) -1)))
colnames(pearson_correlations) <- colnames(ref_geno_tidy)[2:(ncol(ref_geno_tidy))]
rownames(pearson_correlations) <- colnames(cluster_geno_tidy)[2:(ncol(cluster_geno_
↪tidy))]
pearson_correlations <- pearson_correlation(pearson_correlations, ref_geno_tidy,␣
↪cluster_geno_tidy)
cluster <- data.frame("Cluster" = rownames(pearson_correlations))
pearson_correlations_out <- cbind(cluster, pearson_correlations)

########## Save the correlation dataframes ##########
write_delim(pearson_correlations_out, file = paste0(outdir,
↪"/ref_clust_pearson_correlations.tsv"), delim = "\t" )


########## Create correlation figures ##########
col_fun = colorRampPalette(c("white", "red"))(101)
pPearsonCorrelations <- Heatmap(as.matrix(pearson_correlations), cluster_rows = T,␣
↪col = col_fun)

########## Save the correlation figures ##########
png(filename = paste0(outdir,"/ref_clust_pearson_correlation.png"), width = 500)
print(pPearsonCorrelations)
dev.off()
```

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```
########## Assign individual to cluster based on highest correlating individual ######
↪####
key <- as.data.frame(matrix(nrow = ncol(pearson_correlations), ncol = 3))
colnames(key) <- c("Genotype_ID","Cluster_ID","Correlation")
key$Genotype_ID <- colnames(pearson_correlations)
for (id in key$Genotype_ID){
    if (max(pearson_correlations[,id]) == max(pearson_correlations[rownames(pearson_
↪correlations)[which.max(pearson_correlations[,id])],])){
        key$Cluster_ID[which(key$Genotype_ID == id)] <- rownames(pearson_
↪correlations)[which.max(pearson_correlations[,id])]
        key$Correlation[which(key$Genotype_ID == id)] <- max(pearson_correlations[,
↪id])
    } else {
        key$Cluster_ID[which(key$Genotype_ID == id)] <- "unassigned"
        key$Correlation[which(key$Genotype_ID == id)] <- NA
    }
}

write_delim(key, file = paste0(outdir,"/Genotype_ID_key.txt"), delim = "\t")
```

After correlating the reference SNP genotypes with the cluster SNP genotypes using either the script or manually, you should have three new files in your `$FREEMUXLET_OUTDIR`:

```
.
├── freemuxlet.clust1.samples.gz
├── freemuxlet.clust1.vcf.gz
├── freemuxlet.lmix
├── freemuxlet_summary.tsv
├── Genotype_ID_key.txt
├── pileup.cel.gz
├── pileup.plp.gz
├── pileup.umi.gz
├── pileup.var.gz
├── ref_clust_pearson_correlation.png
└── ref_clust_pearson_correlations.tsv
```

9.3 Freemuxlet Results and Interpretation

After running the Freemuxlet steps and summarizing the results, you will have a number of files from some of the intermediary steps. Theses are the files that most users will find the most informative:

- `freemuxlet.clust1.samples.gz`
  - Metrics for each droplet including the singelt, doublet or ambiguous assignment (`DROPLET.TYPE`), final assignment (`BEST.GUESS`), log likelihood of the final assignment (`BEST.LLK`) and other QC metrics.

INT_ID	BARCODE	NUM.SNPS	NUM.READS	DROPLET.TYPE	BEST.GUESS	BEST.LLK	NEXT.GUESS	NEXT.LLK	DIFF.LLK.BEST.NEXT	BEST.POSTERIOR	SNG.POSTERIOR	SNG.BEST.GUESS	SNG.BEST.LLK	SNG.NEXT.GUESS	SNG.NEXT.LLK	SNG.ONLY.POSTERIOR	DBL.BEST.GUESS	DBL.BEST.LLK	DIFF.LLK.SNG.DBL
0	GTGAAGGTCCGCTTT-1	600	1500	DBL	12,1	-1001.09	12,4	-1030.21	29.13	-0.00000	6.7e-16	12	-1037.90	1	-1135.80	1.00000	12,1	-1001.09	-36.81
1	CGAGAAGTCTCGCACC-1	654	678	SNG	7,7	-560.30	13,7	-583.64	23.35	-0.00000	1	7	-560.30	13	-650.83	1.00000	13,7	-583.64	23.35
2	CGCTTCAGTCTGCG-1	1029	2847	DBL	9,3	-1651.22	9,6	-1777.52	126.30	-0.00000	1.5e-65	9	-1802.35	3	-1838.25	1.00000	9,3	-1651.22	-151.13
3	CAGCGACTCGTTC-1	167	219	SNG	5,5	-261.97	6,5	-272.51	10.54	-0.00001	1	5	-261.97	6	-303.97	1.00000	6,5	-272.51	10.54
4	CGTAGGGCAGGAT-1	287	465	SNG	1,1	-451.79	4,1	-479.98	28.18	-0.00000	1	1	-451.79	10	-562.57	1.00000	4,1	-479.98	28.18
…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…

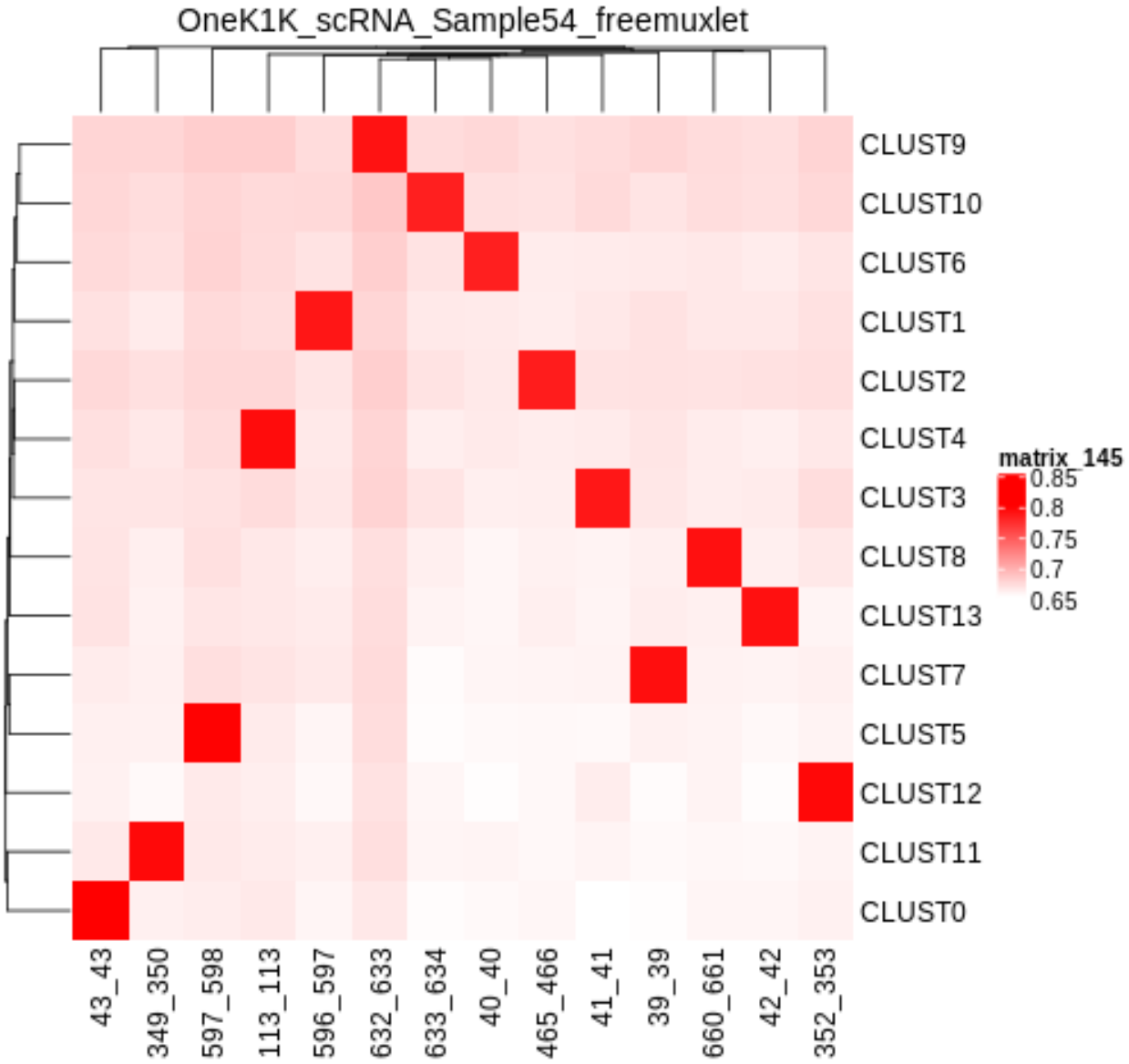
If you ran the `Assign_Indiv_by_Geno.R` script, you will also have the following files:

- `Genotype_ID_key.txt`
  - Key of the cluster and assignments for each individual and the pearson correlation coefficient.

Genotype_ID	Cluster_ID	Correlation
113_113	CLUST4	0.7939599
349_350	CLUST11	0.7954687
352_353	CLUST12	0.7962697
39_39	CLUST7	0.7927807
40_40	CLUST6	0.7833879
41_41	CLUST3	0.7877763
42_42	CLUST13	0.7915233
43_43	CLUST0	0.8008066
465_466	CLUST2	0.7849719
596_597	CLUST1	0.7883125
597_598	CLUST5	0.7996224
632_633	CLUST9	0.7904012
633_634	CLUST10	0.7834359
660_661	CLUST8	0.7914850

- `ref_clust_pearson_correlation.png`
  - Figure of the pearson correlation coefficients for each cluster-individual pair.
- `ref_clust_pearson_correlations.tsv`
  - All of the pearson correlation coefficients between the clusters and the individuals

Cluster	113_113	349_350	352_353	39_39	40_40	…
0	0.671013815501502	0.6867077241784506	0.6966243754688603	0.675970593487308	0.661561196478371	…
1	0.676832450411207	0.6698041245221066	0.6753365794834056	0.674610259343657	0.670220232713515	…
2	0.680371000427	0.6756606413629036	0.6764869329887958	0.674260057528024	0.671247463781301	…
3	0.678245260602395	0.6729013367875029	0.6773636626488672	0.672197934802696	0.667276727783099	…
4	0.793959860486204	0.6714745697877056	0.6713909926031049	0.673064058187681	0.670269016929286	…
…	…	…	…	…	…	…


OneK1K_scRNA_Sample54_freemuxlet
CLUST9
CLUST10
CLUST6
CLUST1
CLUST2
CLUST4
CLUST3
CLUST8
CLUST13
CLUST7
CLUST5
CLUST12
CLUST11
CLUST0
43_43
349_350
597_598
113_113
596_597
632_633
633_634
40_40
465_466
41_41
39_39
660_661
42_42
352_353
matrix_145
0.85
0.8
0.75
0.7
0.65

9.4 Merging Results with Other Software Restults

We have provided a script that will help merge and summarize the results from multiple softwares together. See *Combine Results*.

9.5 Citation

If you used this workflow for analysis, please reference our paper (REFERENCE) as well as Freemuxlet.

CHAPTER

TEN

SCSPLIT TUTORIAL

ScSplit is a reference-free demultiplexing software. If you have reference SNP genotypes, it would be better to use a demultiplexing software that can handle reference SNP genotypes (*Demuxlet*, *Souporcell* or *Vireo*)

10.1 Data

This is the data that you will ned to have prepared to run scSplit:

Required

- Bam file (`$BAM`)
  - Aligned single cell reads
- Genome reference fasta file (`$FASTA`)
- Barcode file (`$BARCODES`)
- Common SNP genotypes vcf (`$VCF`)
  - While not exactly required, using common SNP genotype locations enhances accuracy
    - If you have reference SNP genotypes for individuals in your pool, you can use those
    - If you do not have reference SNP genotypes, they can be from any large population resource (i.e. 1000 Genomes or HRC)
  - Filter for common SNPs (> 5% minor allele frequency) and SNPs overlapping genes
- Number of samples in pool (`$N`)
- Output directory (`$SCSPLIT_OUTDIR`)

10.2 Run ScSplit

10.2.1 Prepare Bam file

First, you will need to prepare the bam file so that it only contains high quality, primarily mapped reads without any PCR duplicated reads.

```
singularity exec Demuxafy.sif samtools view -b -S -q 10 -F 3844 $BAM > $SCSPLIT_
↪OUTDIR/filtered_bam.bam
singularity exec Demuxafy.sif samtools rmdup $SCSPLIT_OUTDIR/filtered_bam.bam
↪$SCSPLIT_OUTDIR/filtered_bam_dedup.bam
singularity exec Demuxafy.sif samtools sort -o $SCSPLIT_OUTDIR/filtered_bam_dedup_
↪sorted.bam $SCSPLIT_OUTDIR/filtered_bam_dedup.bam
singularity exec Demuxafy.sif samtools index $SCSPLIT_OUTDIR/filtered_bam_dedup_
↪sorted.bam
```

After running these bam preparation steps, you will have the following files in your `$SCSPLIT_OUTDIR`:

```
.
├── filtered_bam.bam
├── filtered_bam_dedup.bam
├── filtered_bam_dedup_sorted.bam
└── filtered_bam_dedup_sorted.bam.bai
```

10.2.2 Call Sample SNVs

Next, you will need to identify SNV genotypes in the pooled bam.

```
singularity exec Demuxafy.sif freebayes -f $FASTA -iXu -C 2 -q 1 $SCSPLIT_OUTDIR/
↪filtered_bam_dedup_sorted.bam > $SCSPLIT_OUTDIR/freebayes_var.vcf
singularity exec Demuxafy.sif vcftools --gzvcf $SCSPLIT_OUTDIR/freebayes_var.vcf --
↪minQ 30 --recode --recode-INFO-all --out $SCSPLIT_OUTDIR/freebayes_var_qual30
```

After running these SNV calling steps, you will have the following new files in your `$SCSPLIT_OUTDIR`:

```
.
├── filtered_bam.bam
├── filtered_bam_dedup.bam
├── filtered_bam_dedup_sorted.bam
├── filtered_bam_dedup_sorted.bam.bai
├── freebayes_var_qual30.log
├── freebayes_var_qual30.recode.vcf
└── freebayes_var.vcf
```

10.2.3 Demultiplex with scSplit

The prepared SNV genotypes and bam file can then be used to demultiplex and call genotypes in each cluster.

```
singularity exec Demuxafy.sif scSplit count -c $VCF -v $SCSPLIT_OUTDIR/freebayes_var_
↪qual30.recode.vcf -i $SCSPLIT_OUTDIR/filtered_bam_dedup_sorted.bam -b $BARCODES -r
↪$SCSPLIT_OUTDIR/ref_filtered.csv -a $SCSPLIT_OUTDIR/alt_filtered.csv -o $SCSPLIT_
↪OUTDIR
singularity exec Demuxafy.sif scSplit run -r $SCSPLIT_OUTDIR/ref_filtered.csv -a
↪$SCSPLIT_OUTDIR/alt_filtered.csv -n $N -o $SCSPLIT_OUTDIR
singularity exec Demuxafy.sif scSplit genotype -r $SCSPLIT_OUTDIR/ref_filtered.csv -a
↪$SCSPLIT_OUTDIR/alt_filtered.csv -p $SCSPLIT_OUTDIR/scSplit_P_s_c.csv -o $SCSPLIT_
↪OUTDIR
```

After running these demultiplexing steps, you will have the following new results:

```
.
├── alt_filtered.csv
├── filtered_bam.bam
├── filtered_bam_dedup.bam
├── filtered_bam_dedup_sorted.bam
├── filtered_bam_dedup_sorted.bam.bai
├── freebayes_var_qual30.log
├── freebayes_var_qual30.recode.vcf
├── freebayes_var.vcf
├── ref_filtered.csv
├── scSplit_dist_matrix.csv
├── scSplit_dist_variants.txt
├── scSplit.log
├── scSplit_PA_matrix.csv
├── scSplit_P_s_c.csv
├── scSplit_result.csv
└── scSplit.vcf
```

Additional details about outputs are available below in the *Demuxlet Results and Interpretation*.

10.2.4 ScSplit Summary

We have provided a script that will provide a summary of the number of droplets classified as doublets, ambiguous and assigned to each cluster by scSplit. You can run this to get a fast and easy summary of your results. Just pass the scSplit result file:

```
singularity exec Demuxafy.sif bash scSplit_summary.sh $SCSPLIT_OUTDIR/scSplit_result.
↪csv
```

which will return the following summary:

Classification	Assignment N
DBL	1055
SNG-0	1116
SNG-10	1654
SNG-11	1207
SNG-12	1564
SNG-13	1428
SNG-14	1640
SNG-2	514
SNG-3	1314
SNG-4	1587
SNG-5	1774
SNG-6	1484
SNG-7	1662
SNG-8	1578
SNG-9	1282

You can save the summary to file pointing it to the desired output file:

```
singularity exec Demuxafy.sif bash scSplit_summary.sh $SCSPLIT_OUTDIR/scSplit_result.
↪csv > $SCSPLIT_OUTDIR/scSplit_summary.tsv
```

Note

To check if these numbers are consistent with the expected doublet rate in your dataset, you can use our Doublet Estimation Calculator.

10.2.5 Correlating Cluster to Donor Reference SNP Genotypes (optional)

If you have reference SNP genotypes for some or all of the donors in your pool, you can identify which cluster is best correlated with each donor in your reference SNP genotypes. We have provided a script that will do this and provide a heatmap correlation figure and the predicted individual that should be assigned for each cluster. You can either run it with the script by providing the reference SNP genotypes (`$VCF`), the cluster SNP genotypes (`$SCSPLIT_OUTDIR/scSplit.vcf`) and the output directory (`$SCSPLIT_OUTDIR`) You can run this script with:

Note

In order to do this, your $VCF must be reference SNP genotypes for the individuals in the pool and cannot be a general vcf with common SNP genotype locations from 1000 Genomes or HRC.

With Script

Run in R

```
singularity exec Demuxafy.sif Assign_Indiv_by_Geno.R -r $VCF -c $SCSPLIT_OUTDIR/
↪scSplit.vcf -o $SCSPLIT_OUTDIR
```

To see the parameter help menu, type:

```
singularity exec Demuxafy.sif Assign_Indiv_by_Geno.R -h
```

Which will print:

```
usage: Assign_Indiv_by_Geno.R [-h] -r REFERENCE_VCF -c CLUSTER_VCF -o OUTDIR

optional arguments:
-h, --help            show this help message and exit
-r REFERENCE_VCF, --reference_vcf REFERENCE_VCF
                                                    The output directory where results␣
↪will be saved
-c CLUSTER_VCF, --cluster_vcf CLUSTER_VCF
                                                    A QC, normalized seurat object with
                                                    classificaitons/clusters as Idents().
-o OUTDIR, --outdir OUTDIR
                                                    Number of genes to use in
                                                    'Improved_Seurat_Pre_Process'␣
↪function.
```

You can run the reference vs cluster genotypes manually (possibly because your data doesn't have GT, DS or GP genotype formats) or because you would prefer to alter some of the steps. To run the correlations manually, simply start R from the singularity image:

```
singularity exec Demuxafy.sif R
```

Once, R has started, you can load the required libraries (included in the singularity image) and run the code.

```
.libPaths("/usr/local/lib/R/site-library") ### Required so that libraries are loaded
↪from the image instead of locally
library(tidyr)
library(tidyverse)
library(dplyr)
library(vcfR)
library(lsa)
library(ComplexHeatmap)


########## Set up paths and variables ##########

reference_vcf <- "/path/to/reference.vcf"
cluster_vcf <- "/path/to/scSplit/out/scSplit.vcf"
outdir <- "/path/to/scSplit/out/"


########## Set up functions ##########
##### Calculate DS from GP if genotypes in that format #####
calculate_DS <- function(GP_df){
    columns <- c()
    for (i in 1:ncol(GP_df)){
        columns <- c(columns, paste0(colnames(GP_df)[i],"-0"), paste0(colnames(GP_
↪df)[i],"-1"), paste0(colnames(GP_df)[i],"-2"))
    }
    df <- GP_df
    colnames(df) <- paste0("c", colnames(df))
    colnames_orig <- colnames(df)
    for (i in 1:length(colnames_orig)){
        df <- separate(df, sep = ",", col = colnames_orig[i], into = columns[(1+(3*(i-
↪1))):(3+(3*(i-1)))])
    }
    df <- mutate_all(df, function(x) as.numeric(as.character(x)))
    for (i in 1: ncol(GP_df)){
        GP_df[,i] <- df[,(2+((i-1)*3))] + 2* df[,(3+((i-1)*3))]
    }
    return(GP_df)
}

pearson_correlation <- function(df, ref_df, clust_df){
    for (col in colnames(df)){
        for (row in rownames(df)){
            df[row,col] <- cor(as.numeric(pull(ref_df, col)), as.numeric(pull(clust_
↪df, row)), method = "pearson", use = "complete.obs")
        }
    }
    return(df)
}


########## Read in vcf files for each of three non-reference genotype softwares ######
↪####
ref_geno <- read.vcfR(reference_vcf)
cluster_geno <- read.vcfR(cluster_vcf)
```

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```
########## Convert to tidy data frame ##########
####### Identify which genotype FORMAT to use #######
##### Cluster VCF #####
### Check for each of the different genotype formats ##
## DS ##
format_clust=NA
cluster_geno_tidy <- as_tibble(extract.gt(element = "DS",cluster_geno, IDtoRowNames =␣
↪F))
if (!all(colSums(is.na(cluster_geno_tidy)) == nrow(cluster_geno_tidy))){
 ␣
↪message("Found DS genotype format in cluster vcf. Will use that metric for cluster correlation.")
  format_clust = "DS"
}

## GT ##
if (is.na(format_clust)){
  cluster_geno_tidy <- as_tibble(extract.gt(element = "GT",cluster_geno, IDtoRowNames␣
↪= F))
  if (!all(colSums(is.na(cluster_geno_tidy)) == nrow(cluster_geno_tidy))){
   ␣
↪message("Found GT genotype format in cluster vcf. Will use that metric for cluster correlation.")
    format_clust = "GT"

    if (any(grepl("\\|",cluster_geno_tidy[1,]))){
      separator = "|"
      message("Detected | separator for GT genotype format in cluster vcf")
    } else if (any(grepl("/",cluster_geno_tidy[1,]))) {
      separator = "/"
      message("Detected / separator for GT genotype format in cluster vcf")
    } else {
      format_clust = NA
     ␣
↪message("Can't identify a separator for the GT field in cluster vcf, moving on to using GP.")
    }

    cluster_geno_tidy <- as_tibble(lapply(cluster_geno_tidy, function(x)
↪{gsub(paste0("0",separator,"0"),0, x)}) %>%
                             lapply(., function(x) {gsub(paste0("0",separator,"1"),1,␣
↪x)}) %>%
                             lapply(., function(x) {gsub(paste0("1",separator,"0"),1,␣
↪x)}) %>%
                             lapply(., function(x) {gsub(paste0("1",separator,"1"),2,␣
↪x)}))

  }
}

## GP ##
if (is.na(format_clust)){
  cluster_geno_tidy <- as_tibble(extract.gt(element = "GP",cluster_geno, IDtoRowNames␣
↪=F))
  if (!all(colSums(is.na(cluster_geno_tidy)) == nrow(cluster_geno_tidy))){
    format_clust = "GP"
    cluster_geno_tidy <- calculate_DS(cluster_geno_tidy)
   ␣
↪message("Found GP genotype format in cluster vcf. Will use that metric for cluster correlation.")
```

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```
  } else {
    print("Could not identify the expected genotype format fields (DS, GT or GP) in your cluster vcf. F
    q()
  }
}




### Reference VCF ###
### Check for each of the different genotype formats ##
## DS ##
format_ref = NA
ref_geno_tidy <- as_tibble(extract.gt(element = "DS",ref_geno, IDtoRowNames = F))
if (!all(colSums(is.na(ref_geno_tidy)) == nrow(ref_geno_tidy))){
  message("Found DS genotype format in reference vcf. Will use that metric for cluster correlation.")
  format_ref = "DS"
}

## GT ##
if (is.na(format_ref)){
  ref_geno_tidy <- as_tibble(extract.gt(element = "GT",ref_geno, IDtoRowNames = F))
  if (!all(colSums(is.na(ref_geno_tidy)) == nrow(ref_geno_tidy))){
    message("Found GT genotype format in reference vcf. Will use that metric for cluster correlation.")
    format_ref = "GT"

    if (any(grepl("\\|",ref_geno_tidy[1,]))){
      separator = "|"
      message("Detected | separator for GT genotype format in reference vcf")
    } else if (any(grepl("/",ref_geno_tidy[1,]))) {
      separator = "/"
      message("Detected / separator for GT genotype format in reference vcf")
    } else {
      format_ref = NA
      message("Can't identify a separator for the GT field in reference vcf, moving on to using GP.")
    }

    ref_geno_tidy <- as_tibble(lapply(ref_geno_tidy, function(x) {gsub(paste0("0",separator,"0"),0, x)}) %>%
                               lapply(., function(x) {gsub(paste0("0",separator,"1"),1, x)}) %>%
                               lapply(., function(x) {gsub(paste0("1",separator,"0"),1, x)}) %>%
                               lapply(., function(x) {gsub(paste0("1",separator,"1"),2, x)}))

  }
}

## GP ##
if (is.na(format_ref)){
  ref_geno_tidy <- as_tibble(extract.gt(element = "GP",ref_geno, IDtoRowNames = F))
```

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```
  if (!all(colSums(is.na(ref_geno_tidy)) == nrow(ref_geno_tidy))){
    format_clust = "GP"
    ref_geno_tidy <- calculate_DS(ref_geno_tidy)
    message("Found GP genotype format in cluster vcf. Will use that metric for cluster correlation.")
  } else {
    print("Could not identify the expected genotype format fields (DS, GT or GP) in your cluster vcf. E
    q()
  }
}



### Get SNP IDs that will match between reference and cluster ###
## Account for possibility that the ref or alt might be missing
if ((all(is.na(cluster_geno@fix[,'REF'])) & all(is.na(cluster_geno@fix[,'ALT']))) |
(all(is.na(ref_geno@fix[,'REF'])) & all(is.na(ref_geno@fix[,'ALT'])))){
  message("The REF and ALT categories are not provided for the reference and/or the cluster vcf. Will
  cluster_geno_tidy$ID <- paste0(cluster_geno@fix[,'CHROM'],":", cluster_geno@fix[,
'POS'])
  ref_geno_tidy$ID <- paste0(ref_geno@fix[,'CHROM'],":", ref_geno@fix[,'POS'])
} else if (all(is.na(cluster_geno@fix[,'REF'])) | all(is.na(ref_geno@fix[,'REF']))){
  message("The REF categories are not provided for the reference and/or the cluster vcf. Will use the
  cluster_geno_tidy$ID <- paste0(cluster_geno@fix[,'CHROM'],":", cluster_geno@fix[,
'POS'],"_", cluster_geno@fix[,'REF'])
  ref_geno_tidy$ID <- paste0(ref_geno@fix[,'CHROM'],":", ref_geno@fix[,'POS'],"_",
ref_geno@fix[,'REF'])
} else if (all(is.na(cluster_geno@fix[,'ALT'])) | all(is.na(ref_geno@fix[,'ALT']))){
  message("The ALT categories are not provided for the reference and/or the cluster vcf. Will use the
  cluster_geno_tidy$ID <- paste0(cluster_geno@fix[,'CHROM'],":", cluster_geno@fix[,
'POS'],"_", cluster_geno@fix[,'ALT'])
  ref_geno_tidy$ID <- paste0(ref_geno@fix[,'CHROM'],":", ref_geno@fix[,'POS'],"_",
ref_geno@fix[,'ALT'])
} else {
  message("Found REF and ALT in both cluster and reference genotype vcfs. Will use chromosome, positi
    cluster_geno_tidy$ID <- paste0(cluster_geno@fix[,'CHROM'],":", cluster_geno@fix[,
'POS'],"_", cluster_geno@fix[,'REF'],"_", cluster_geno@fix[,'ALT'])
  ref_geno_tidy$ID <- paste0(ref_geno@fix[,'CHROM'],":", ref_geno@fix[,'POS'],"_",
ref_geno@fix[,'REF'],"_", ref_geno@fix[,'ALT'])
}


### Update the vcf dfs to remove SNPs with no genotyopes
cluster_geno_tidy <- cluster_geno_tidy[colSums(!is.na(cluster_geno_tidy)) > 0]
ref_geno_tidy <- ref_geno_tidy[colSums(!is.na(ref_geno_tidy)) > 0]



########## Get a unique list of SNPs that is in both the reference and cluster
genotypes ##########
locations  <- inner_join(ref_geno_tidy[,"ID"],cluster_geno_tidy[,"ID"])
```

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```
locations <- locations[!(locations$ID %in% locations[duplicated(locations),]$ID),]

########## Keep just the SNPs that overlap ##########
ref_geno_tidy <- left_join(locations, ref_geno_tidy)
cluster_geno_tidy <- left_join(locations, cluster_geno_tidy)

########## Correlate all the cluster genotypes with the individuals genotyped ########
##
##### Make a dataframe that has the clusters as the row names and the individuals as␣
↪the column names #####
pearson_correlations <- as.data.frame(matrix(nrow = (ncol(cluster_geno_tidy) -1),␣
↪ncol = (ncol(ref_geno_tidy) -1)))
colnames(pearson_correlations) <- colnames(ref_geno_tidy)[2:(ncol(ref_geno_tidy))]
rownames(pearson_correlations) <- colnames(cluster_geno_tidy)[2:(ncol(cluster_geno_
↪tidy))]
pearson_correlations <- pearson_correlation(pearson_correlations, ref_geno_tidy,␣
↪cluster_geno_tidy)
cluster <- data.frame("Cluster" = rownames(pearson_correlations))
pearson_correlations_out <- cbind(cluster, pearson_correlations)

########## Save the correlation dataframes ##########
write_delim(pearson_correlations_out, file = paste0(outdir,
↪"/ref_clust_pearson_correlations.tsv"), delim = "\t" )


########## Create correlation figures ##########
col_fun = colorRampPalette(c("white", "red"))(101)
pPearsonCorrelations <- Heatmap(as.matrix(pearson_correlations), cluster_rows = T,␣
↪col = col_fun)

########## Save the correlation figures ##########
png(filename = paste0(outdir,"/ref_clust_pearson_correlation.png"), width = 500)
print(pPearsonCorrelations)
dev.off()

########## Assign individual to cluster based on highest correlating individual ######
↪####
key <- as.data.frame(matrix(nrow = ncol(pearson_correlations), ncol = 3))
colnames(key) <- c("Genotype_ID","Cluster_ID","Correlation")
key$Genotype_ID <- colnames(pearson_correlations)
for (id in key$Genotype_ID){
    if (max(pearson_correlations[,id]) == max(pearson_correlations[rownames(pearson_
↪correlations)[which.max(pearson_correlations[,id])],])){
        key$Cluster_ID[which(key$Genotype_ID == id)] <- rownames(pearson_
↪correlations)[which.max(pearson_correlations[,id])]
        key$Correlation[which(key$Genotype_ID == id)] <- max(pearson_correlations[,
↪id])
    } else {
        key$Cluster_ID[which(key$Genotype_ID == id)] <- "unassigned"
        key$Correlation[which(key$Genotype_ID == id)] <- NA
    }
}

write_delim(key, file = paste0(outdir,"/Genotype_ID_key.txt"), delim = "\t")
```

10.3 ScSplit Results and Interpretation

After running the scSplit steps and summarizing the results, you will have a number of files from some of the intermediary steps. Theses are the files that most users will find the most informative:

- `scSplit_doublets_singlets.csv`
  - The droplet assignment results. The first column is the droplet barcode and the second column is the droplet type and cluster assignment separated by a dash. For example SNG-9 would indicate that cluster 9 are singlets.

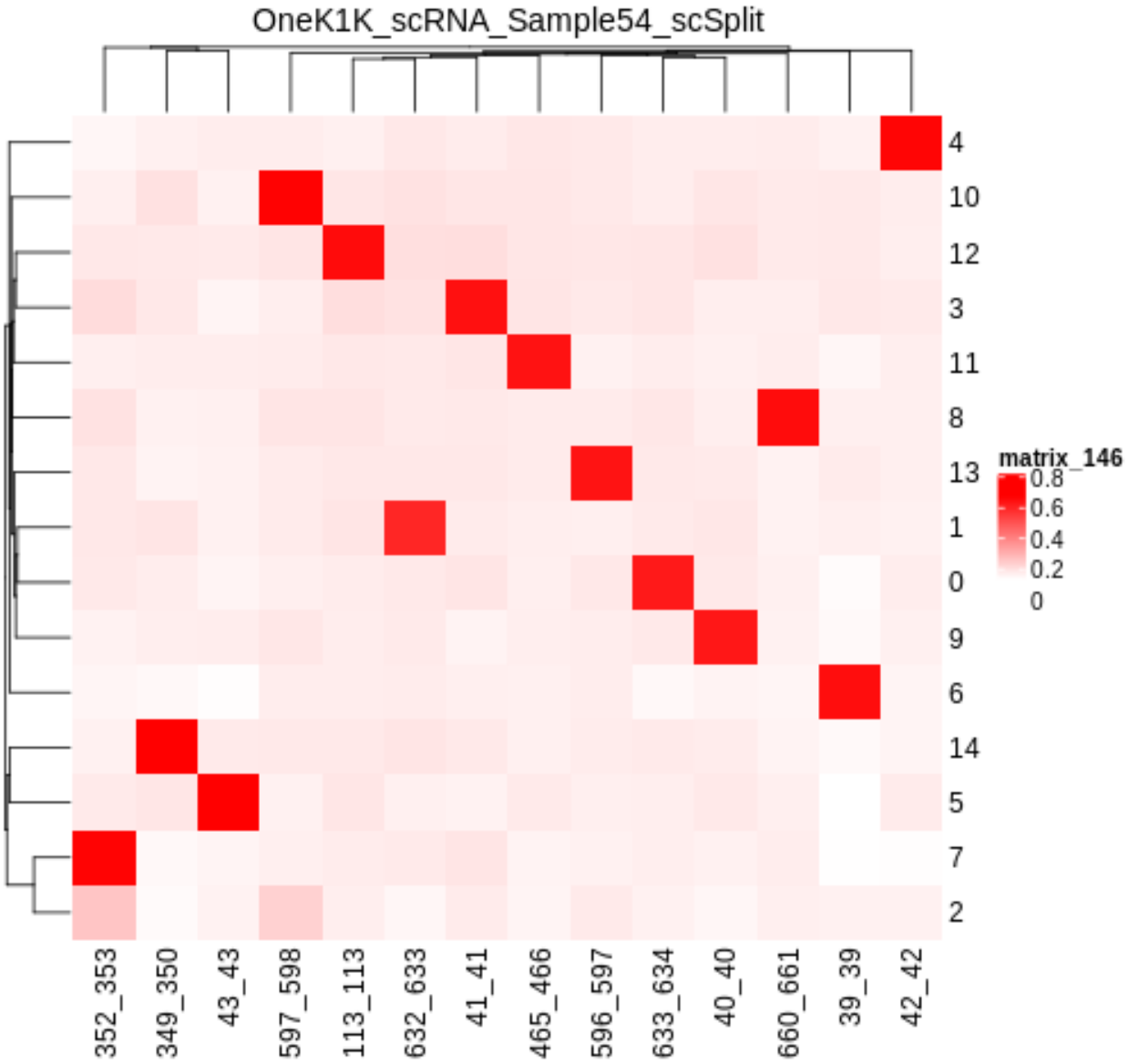
Barcode	Cluster
AAACCTGTCCGAATGT-1	SNG-0
AAACGGGAGTTGAGAT-1	SNG-0
AAACGGGCATGTCTCC-1	SNG-0
AAACGGGTCCACGAAT-1	SNG-0
AAACGGGTCCAGTAGT-1	SNG-0
AAACGGGTCGGCTTGG-1	SNG-0
AAAGATGTCCGAACGC-1	SNG-0
AAAGATGTCCGTCAAA-1	SNG-0
AAAGTAGCATCACGTA-1	SNG-0
...	...

If you ran the `Assign_Indiv_by_Geno.R` script, you will also have the following files:

- `Genotype_ID_key.txt`
  - Key of the cluster and assignments for each individual and the pearson correlation coefficient.

Genotype_ID	Cluster_ID	Correlation
113_113	12	0.6448151
349_350	14	0.6663323
352_353	7	0.6596409
39_39	6	0.6398297
40_40	9	0.6191905
41_41	3	0.6324396
42_42	4	0.6560180
43_43	5	0.6672336
465_466	11	0.6297396
596_597	13	0.6273717
597_598	10	0.6627428
632_633	1	0.5899685
633_634	0	0.6157936
660_661	8	0.6423770

- `ref_clust_pearson_correlation.png`
  - Figure of the pearson correlation coefficients for each cluster-individual pair.
- `ref_clust_pearson_correlations.tsv`
  - All of the pearson correlation coefficients between the clusters and the individuals


OneK1K_scRNA_Sample54_scSplit
4
10
12
3
11
8
13
1
0
9
6
14
5
7
2
352_353
349_350
43_43
597_598
113_113
632_633
41_41
465_466
596_597
633_634
40_40
660_661
39_39
42_42
matrix_146
0.8
0.6
0.4
0.2
0

Cluster	113_113	349_350	352_353	39_39	40_40	...
0	0.18419103983986	0.1653282303206	0.129176272973	0.1553769168058	0.1941075249089	...
1	0.19853015287740	0.13981622074955	0.14924584028347	0.1278557483333	0.1345543339544	...
2	0.17993959098414	0.1054770588338	0.2664128336649	0.1973606484450	0.1463746151608	...
3	0.21286169961530	0.1793251481480	0.2847289916680	0.1934657499878	0.1229216513792	...
4	0.17573820413419	0.1376295040873	0.1642615665946	0.1742799698360	0.1832278541587	...
...	...	...	...	...	...	...

10.4 Merging Results with Other Software Restults

We have provided a script that will help merge and summarize the results from multiple softwares together. See *Combine Results*.

10.5 Citation

If you used this workflow for analysis, please reference our paper (REFERENCE) as well as scSplit.

CHAPTER

ELEVEN

SOUPORCELL TUTORIAL

Souporcell is a genotype-free demultiplexing software that does not require you to have SNP genotypes the donors in your multiplexed capture. However, it can natively integrate SNP genotypes into it's demultiplexing if you have them available for **all** the donors in your pool. If you don't have the reference SNP genotypes for all the donors in your multiplexed pool, we have provided some scripts that will help identify clusters from given donors after running Souporcell without the SNP genotuypes. Depdening on your downstream analyses, if you have reference SNP genotypes for donors in your pool, you could also use *Demuxlet*, or *Vireo*.

One advantage that we have found immensely helpful about Souporcell is that it provides an ambient RNA estimate for the pool. This can be helpful to identify samples that may have high ambient RNA estimates early in the analysis pipeline so that it can be accounted for throughout downstream analyses.

11.1 Data

This is the data that you will need to have preparede to run Souporcell:

Required

- Common SNP genotypes vcf (`$VCF`)
  - While not exactly required, using common SNP genotype locations enhances accuracy
    - If you have reference SNP genotypes for individuals in your pool, you can use those
    - If you do not have reference SNP genotypes, they can be from any large population resource (i.e. 1000 Genomes or HRC)
  - Filter for common SNPs (> 5% minor allele frequency) and SNPs overlapping genes
  - Filter for common SNPs (> 5% minor allele frequency) and SNPs overlapping genes
- Barcode file (`$BARCODES`)
- Number of samples in pool (`$N`)
- Bam file (`$BAM`)
  - Aligned single cell reads
- Reference fasta (`$FASTA`)
  - that your reads were aligned to (or at least the same genome)
- Output directory (`$SOUPORCELL_OUTDIR`)

11.2 Run Souporcell

You can run Souporcell with or without reference SNP genotypes - follow the instructions for each bellow:

Without Reference SNP Genotypes

With Reference SNP Genotypes

If you don't have reference SNP genotypes for all of your donors, you can run souporcell with the following command, providing an appropriate thread number (`$THREADS`) for your system . Don't worry if you only have reference SNP genotypes for a subset of your donors, we have a script that will correlate the cluster and reference SNP genotypes.

```
singularity exec Demuxafy.sif souporcell_pipeline.py -i $BAM -b $BARCODES -f $FASTA -
↪t $THREADS -o $SOUPORCELL_OUTDIR -k $N --common_variants $VCF
```

If you have reference SNP genotypes for **all** of your donors, you can run souporcell with the following command, providing an appropriate thread number (`$THREADS`) for your system and listing the donor ids that correspond in the `$VCF` file

```
singularity exec Demuxafy.sif souporcell_pipeline.py -i $BAM -b $BARCODES -f $FASTA -
↪t $THREADS -o $SOUPORCELL_OUTDIR -k $N --known_genotypes $VCF --known_genotypes_
↪sample_names donor1 donor donor3 donor4
```

If souporcell is successfull, you will have these files in your `$SOUPORCELL_OUTDIR`:

```
.
├── alt.mtx
├── ambient_rna.txt
├── cluster_genotypes.vcf
├── clustering.done
├── clusters.err
├── clusters_tmp.tsv
├── clusters.tsv
├── common_variants_covered_tmp.vcf
├── common_variants_covered.vcf
├── consensus.done
├── depth_merged.bed
├── doublets.err
├── fastqs.done
├── minimap.err
├── ref.mtx
├── remapping.done
├── retag.err
├── retagging.done
├── souporcell_minimap_tagged_sorted.bam
├── souporcell_minimap_tagged_sorted.bam.bai
├── troublet.done
├── variants.done
└── vartrix.done
```

Additional details about outputs are available below in the *Souporcell Results and Interpretation*.

11.2.1 Souporcell Summary

We have provided a script that will provide a summary of the number of droplets classified as doublets, ambiguous and assigned to each cluster by Souporcell. You can run this to get a fast and easy summary of your results by providing the souporcell result file:

```
singularity exec Demuxafy.sif bash souporcell_summary.sh $SOUPORCELL_OUTDIR/clusters.
↪tsv
```

which should print:

Classification	Assignment N
0	1441
1	980
10	1285
11	1107
12	1315
13	1529
2	1629
3	1473
4	1381
5	1360
6	1157
7	892
8	1111
9	1565
doublet	2757

or you can write the results to file:

```
singularity exec Demuxafy.sif bash souporcell_summary.sh $SOUPORCELL_OUTDIR/clusters.
↪tsv > $SOUPORCELL_OUTDIR/souporcell_summary.tsv
```

Note

To check if these numbers are consistent with the expected doublet rate in your dataset, you can use our Doublet Estimation Calculator.

If the souporcell summary is successfull, you will have this new file in your `$SOUPORCELL_OUTDIR`:

```
.
├── alt.mtx
├── ambient_rna.txt
├── cluster_genotypes.vcf
├── clustering.done
├── clusters.err
├── clusters_tmp.tsv
├── clusters.tsv
├── common_variants_covered_tmp.vcf
├── common_variants_covered.vcf
├── consensus.done
├── depth_merged.bed
├── doublets.err
```

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```
├── fastqs.done
├── minimap.err
├── ref.mtx
├── remapping.done
├── retag.err
├── retagging.done
├── souporcell_minimap_tagged_sorted.bam
├── souporcell_summary.tsv
├── troublet.done
├── variants.done
└── vartrix.done
```

Additional details about outputs are available below in the *Souporcell Results and Interpretation*.

11.2.2 Correlating Cluster to Donor Reference SNP Genotypes (optional)

If you have reference SNP genotypes for some or all of the donors in your pool, you can identify which cluster is best correlated with each donor in your reference SNP genotypes. We have provided a script that will do this and provide a heatmap correlation figure and the predicted individual that should be assigned for each cluster. You can either run it with the script by providing the reference SNP genotypes (`$VCF`), the cluster SNP genotypes (`$SOUPORCELL_OUTDIR/cluster_genotypes.vcf`) and the output directory (`$SOUPORCELL_OUTDIR`) You can run this script with:

Note

In order to do this, your $VCF must be reference SNP genotypes for the individuals in the pool and cannot be a general vcf with common SNP genotype locations from 1000 Genomes or HRC.

With Script

Run in R

```
singularity exec Demuxafy.sif Assign_Indiv_by_Geno.R -r $VCF -c $SOUPORCELL_OUTDIR/
↪cluster_genotypes.vcf -o $SOUPORCELL_OUTDIR
```

To see the parameter help menu, type:

```
singularity exec Demuxafy.sif Assign_Indiv_by_Geno.R -h
```

Which will print:

```
usage: Assign_Indiv_by_Geno.R [-h] -r REFERENCE_VCF -c CLUSTER_VCF -o OUTDIR

optional arguments:
-h, --help            show this help message and exit
-r REFERENCE_VCF, --reference_vcf REFERENCE_VCF
                                                The output directory where results␣
↪will be saved
-c CLUSTER_VCF, --cluster_vcf CLUSTER_VCF
                                                A QC, normalized seurat object with
                                                classificaitons/clusters as Idents().
-o OUTDIR, --outdir OUTDIR
                                                Number of genes to use in
                                                'Improved_Seurat_Pre_Process'␣
↪function.
```

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You can run the reference vs cluster genotypes manually (possibly because your data doesn't have GT, DS or GP genotype formats) or because you would prefer to alter some of the steps. To run the correlations manually, simply start R from the singularity image:

```
singularity exec Demuxafy.sif R
```

Once, R has started, you can load the required libraries (included in the singularity image) and run the code.

```
.libPaths("/usr/local/lib/R/site-library") ### Required so that libraries are loaded␣
↪from the image instead of locally
library(tidyr)
library(tidyverse)
library(dplyr)
library(vcfR)
library(lsa)
library(ComplexHeatmap)


########## Set up paths and variables ##########

reference_vcf <- "/path/to/reference.vcf"
cluster_vcf <- "/path/to/souporcell/out/cluster_genotypes.vcf"
outdir <- "/path/to/souporcell/out/"


########## Set up functions ##########
##### Calculate DS from GP if genotypes in that format #####
calculate_DS <- function(GP_df){
    columns <- c()
    for (i in 1:ncol(GP_df)){
        columns <- c(columns, paste0(colnames(GP_df)[i],"-0"), paste0(colnames(GP_
↪df)[i],"-1"), paste0(colnames(GP_df)[i],"-2"))
    }
    df <- GP_df
    colnames(df) <- paste0("c", colnames(df))
    colnames_orig <- colnames(df)
    for (i in 1:length(colnames_orig)){
        df <- separate(df, sep = ",", col = colnames_orig[i], into = columns[(1+(3*(i-
↪1))):(3+(3*(i-1)))])
    }
    df <- mutate_all(df, function(x) as.numeric(as.character(x)))
    for (i in 1: ncol(GP_df)){
        GP_df[,i] <- df[,(2+((i-1)*3))] + 2* df[,(3+((i-1)*3))]
    }
    return(GP_df)
}

pearson_correlation <- function(df, ref_df, clust_df){
    for (col in colnames(df)){
        for (row in rownames(df)){
            df[row,col] <- cor(as.numeric(pull(ref_df, col)), as.numeric(pull(clust_
↪df, row)), method = "pearson", use = "complete.obs")
        }
    }
    return(df)
```

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```
}


########## Read in vcf files for each of three non-reference genotype softwares ######
↪####
ref_geno <- read.vcfR(reference_vcf)
cluster_geno <- read.vcfR(cluster_vcf)



########## Convert to tidy data frame ##########
####### Identify which genotype FORMAT to use #######
##### Cluster VCF #####
### Check for each of the different genotype formats ##
## DS ##
format_clust=NA
cluster_geno_tidy <- as_tibble(extract.gt(element = "DS",cluster_geno, IDtoRowNames =␣
↪F))
if (!all(colSums(is.na(cluster_geno_tidy)) == nrow(cluster_geno_tidy))){
 ␣
↪message("Found DS genotype format in cluster vcf. Will use that metric for cluster correlation.")
  format_clust = "DS"
}

## GT ##
if (is.na(format_clust)){
  cluster_geno_tidy <- as_tibble(extract.gt(element = "GT",cluster_geno, IDtoRowNames␣
↪= F))
  if (!all(colSums(is.na(cluster_geno_tidy)) == nrow(cluster_geno_tidy))){
   ␣
↪message("Found GT genotype format in cluster vcf. Will use that metric for cluster correlation.")
    format_clust = "GT"

    if (any(grepl("\\|",cluster_geno_tidy[1,]))){
      separator = "|"
      message("Detected | separator for GT genotype format in cluster vcf")
    } else if (any(grepl("/",cluster_geno_tidy[1,]))) {
      separator = "/"
      message("Detected / separator for GT genotype format in cluster vcf")
    } else {
      format_clust = NA
     ␣
↪message("Can't identify a separator for the GT field in cluster vcf, moving on to using GP.")
    }

    cluster_geno_tidy <- as_tibble(lapply(cluster_geno_tidy, function(x)
↪{gsub(paste0("0",separator,"0"),0, x)}) %>%
                                   lapply(., function(x) {gsub(paste0("0",separator,"1"),1,␣
↪x)}) %>%
                                   lapply(., function(x) {gsub(paste0("1",separator,"0"),1,␣
↪x)}) %>%
                                   lapply(., function(x) {gsub(paste0("1",separator,"1"),2,␣
↪x)}))

  }
}
```

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```
## GP ##
if (is.na(format_clust)){
  cluster_geno_tidy <- as_tibble(extract.gt(element = "GP",cluster_geno, IDtoRowNames␣
↪=F))
  if (!all(colSums(is.na(cluster_geno_tidy)) == nrow(cluster_geno_tidy))){
    format_clust = "GP"
    cluster_geno_tidy <- calculate_DS(cluster_geno_tidy)
   ␣
↪message("Found GP genotype format in cluster vcf. Will use that metric for cluster correlation.")

  } else {
   ␣
↪print("Could not identify the expected genotype format fields (DS, GT or GP) in your cluster vcf. F
    q()
  }
}




### Reference VCF ###
### Check for each of the different genotype formats ##
## DS ##
format_ref = NA
ref_geno_tidy <- as_tibble(extract.gt(element = "DS",ref_geno, IDtoRowNames = F))
if (!all(colSums(is.na(ref_geno_tidy)) == nrow(ref_geno_tidy))){
 ␣
↪message("Found DS genotype format in reference vcf. Will use that metric for cluster correlation.")
  format_ref = "DS"
}

## GT ##
if (is.na(format_ref)){
  ref_geno_tidy <- as_tibble(extract.gt(element = "GT",ref_geno, IDtoRowNames = F))
  if (!all(colSums(is.na(ref_geno_tidy)) == nrow(ref_geno_tidy))){
   ␣
↪message("Found GT genotype format in reference vcf. Will use that metric for cluster correlation.")
    format_ref = "GT"

    if (any(grepl("\\|",ref_geno_tidy[1,]))){
      separator = "|"
      message("Detected | separator for GT genotype format in reference vcf")
    } else if (any(grepl("/",ref_geno_tidy[1,]))) {
      separator = "/"
      message("Detected / separator for GT genotype format in reference vcf")
    } else {
      format_ref = NA
     ␣
↪message("Can't identify a separator for the GT field in reference vcf, moving on to using GP.")
    }

    ref_geno_tidy <- as_tibble(lapply(ref_geno_tidy, function(x) {gsub(paste0("0",
↪separator,"0"),0, x)}) %>%
                               lapply(., function(x) {gsub(paste0("0",separator,"1"),1,␣
↪x)}) %>%
                               lapply(., function(x) {gsub(paste0("1",separator,"0"),1,␣
↪x)}) %>%
```

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```
                              lapply(., function(x) {gsub(paste0("1",separator,"1"),2,␣
↪x)}))

  }
}

## GP ##
if (is.na(format_ref)){
  ref_geno_tidy <- as_tibble(extract.gt(element = "GP",ref_geno, IDtoRowNames = F))
  if (!all(colSums(is.na(ref_geno_tidy)) == nrow(ref_geno_tidy))){
    format_clust = "GP"
    ref_geno_tidy <- calculate_DS(ref_geno_tidy)
   ␣
↪message("Found GP genotype format in cluster vcf. Will use that metric for cluster correlation.")

  } else {
   ␣
↪print("Could not identify the expected genotype format fields (DS, GT or GP) in your cluster vcf. P
    q()
  }
}



### Get SNP IDs that will match between reference and cluster ###
## Account for possibility that the ref or alt might be missing
if ((all(is.na(cluster_geno@fix[,'REF'])) & all(is.na(cluster_geno@fix[,'ALT']))) |␣
↪(all(is.na(ref_geno@fix[,'REF'])) & all(is.na(ref_geno@fix[,'ALT'])))){
 ␣
↪message("The REF and ALT categories are not provided for the reference and/or the cluster vcf. Will
  cluster_geno_tidy$ID <- paste0(cluster_geno@fix[,'CHROM'],":", cluster_geno@fix[,
↪'POS'])
  ref_geno_tidy$ID <- paste0(ref_geno@fix[,'CHROM'],":", ref_geno@fix[,'POS'])
} else if (all(is.na(cluster_geno@fix[,'REF'])) | all(is.na(ref_geno@fix[,'REF']))){
 ␣
↪message("The REF categories are not provided for the reference and/or the cluster vcf. Will use the
  cluster_geno_tidy$ID <- paste0(cluster_geno@fix[,'CHROM'],":", cluster_geno@fix[,
↪'POS'],"_", cluster_geno@fix[,'REF'])
  ref_geno_tidy$ID <- paste0(ref_geno@fix[,'CHROM'],":", ref_geno@fix[,'POS'],"_",␣
↪ref_geno@fix[,'REF'])
} else if (all(is.na(cluster_geno@fix[,'ALT'])) | all(is.na(ref_geno@fix[,'ALT']))){
 ␣
↪message("The ALT categories are not provided for the reference and/or the cluster vcf. Will use the
  cluster_geno_tidy$ID <- paste0(cluster_geno@fix[,'CHROM'],":", cluster_geno@fix[,
↪'POS'],"_", cluster_geno@fix[,'ALT'])
  ref_geno_tidy$ID <- paste0(ref_geno@fix[,'CHROM'],":", ref_geno@fix[,'POS'],"_",␣
↪ref_geno@fix[,'ALT'])
} else {
 ␣
↪message("Found REF and ALT in both cluster and reference genotype vcfs. Will use chromosome, positi
    cluster_geno_tidy$ID <- paste0(cluster_geno@fix[,'CHROM'],":", cluster_geno@fix[,
↪'POS'],"_", cluster_geno@fix[,'REF'],"_", cluster_geno@fix[,'ALT'])
  ref_geno_tidy$ID <- paste0(ref_geno@fix[,'CHROM'],":", ref_geno@fix[,'POS'],"_",␣
↪ref_geno@fix[,'REF'],"_", ref_geno@fix[,'ALT'])
}
```

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```
### Update the vcf dfs to remove SNPs with no genotyopes
cluster_geno_tidy <- cluster_geno_tidy[colSums(!is.na(cluster_geno_tidy)) > 0]
ref_geno_tidy <- ref_geno_tidy[colSums(!is.na(ref_geno_tidy)) > 0]



########## Get a unique list of SNPs that is in both the reference and cluster␣
↪genotypes ##########
locations  <- inner_join(ref_geno_tidy[,"ID"],cluster_geno_tidy[,"ID"])
locations <- locations[!(locations$ID %in% locations[duplicated(locations),]$ID),]

########## Keep just the SNPs that overlap ##########
ref_geno_tidy <- left_join(locations, ref_geno_tidy)
cluster_geno_tidy <- left_join(locations, cluster_geno_tidy)

########## Correlate all the cluster genotypes with the individuals genotyped ########
↪##
##### Make a dataframe that has the clusters as the row names and the individuals as␣
↪the column names #####
pearson_correlations <- as.data.frame(matrix(nrow = (ncol(cluster_geno_tidy) -1),␣
↪ncol = (ncol(ref_geno_tidy) -1)))
colnames(pearson_correlations) <- colnames(ref_geno_tidy)[2:(ncol(ref_geno_tidy))]
rownames(pearson_correlations) <- colnames(cluster_geno_tidy)[2:(ncol(cluster_geno_
↪tidy))]
pearson_correlations <- pearson_correlation(pearson_correlations, ref_geno_tidy,␣
↪cluster_geno_tidy)
cluster <- data.frame("Cluster" = rownames(pearson_correlations))
pearson_correlations_out <- cbind(cluster, pearson_correlations)

########## Save the correlation dataframes ##########
write_delim(pearson_correlations_out, file = paste0(outdir,
↪"/ref_clust_pearson_correlations.tsv"), delim = "\t" )


########## Create correlation figures ##########
col_fun = colorRampPalette(c("white", "red"))(101)
pPearsonCorrelations <- Heatmap(as.matrix(pearson_correlations), cluster_rows = T,␣
↪col = col_fun)

########## Save the correlation figures ##########
png(filename = paste0(outdir,"/ref_clust_pearson_correlation.png"), width = 500)
print(pPearsonCorrelations)
dev.off()

########## Assign individual to cluster based on highest correlating individual ######
↪####
key <- as.data.frame(matrix(nrow = ncol(pearson_correlations), ncol = 3))
colnames(key) <- c("Genotype_ID","Cluster_ID","Correlation")
key$Genotype_ID <- colnames(pearson_correlations)
for (id in key$Genotype_ID){
    if (max(pearson_correlations[,id]) == max(pearson_correlations[rownames(pearson_
↪correlations)[which.max(pearson_correlations[,id])],])){
        key$Cluster_ID[which(key$Genotype_ID == id)] <- rownames(pearson_
↪correlations)[which.max(pearson_correlations[,id])]
        key$Correlation[which(key$Genotype_ID == id)] <- max(pearson_correlations[,
↪id])
    } else {
```

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```
        key$Cluster_ID[which(key$Genotype_ID == id)] <- "unassigned"
        key$Correlation[which(key$Genotype_ID == id)] <- NA
    }
}

write_delim(key, file = paste0(outdir,"/Genotype_ID_key.txt"), delim = "\t")
```

After correlating the cluster and reference donor SNP genotypes, you should have the new results in your directory:

If the souporcell summary is successfull, you will have this new file in your `$SOUPORCELL_OUTDIR`:

```
.
├── alt.mtx
├── ambient_rna.txt
├── cluster_genotypes.vcf
├── clustering.done
├── clusters.err
├── clusters_tmp.tsv
├── clusters.tsv
├── common_variants_covered_tmp.vcf
├── common_variants_covered.vcf
├── consensus.done
├── depth_merged.bed
├── doublets.err
├── fastqs.done
├── Genotype_ID_key.txt
├── Individual_genotypes_subset.vcf.gz
├── minimap.err
├── ref.mtx
├── ref_clust_pearson_correlation.png
├── ref_clust_pearson_correlations.tsv
├── remapping.done
├── retag.err
├── retagging.done
├── souporcell_minimap_tagged_sorted.bam
├── souporcell_summary.tsv
├── troublet.done
├── variants.done
└── vartrix.done
```

Additional details about outputs are available below in the *Souporcell Results and Interpretation*.

11.3 Souporcell Results and Interpretation

After running the Souporcell steps and summarizing the results, you will have a number of files from some of the intermediary steps. Theses are the files that most users will find the most informative:

- To check if these numbers are consistent with the expected doublet rate in your dataset, you can use our Expected Doublet Estimation Calculator.
- `clusters.tsv`
  - The Souporcell droplet classifications with the log probabilitites of each donor and doublet vs singlet.

barcode	status	assignment	log_prob_singleton	log_prob_doublet	cluster0	cluster1	cluster2	cluster3	cluster4	cluster5	cluster6	cluster7	cluster8	cluster9	cluster10	cluster11	cluster12	cluster13
AAACCTGAGATAGCAT-1	singlet	6	-47.49[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]9218
AAACCTGAGCAGCGTA-1	singlet	[illegible]	-102.8[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]9155
AAACCTGAGCGATGAC-1	singlet	5	-39.97[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]20557
AAACCTGAGCGTAGTG-1	singlet	3	-66.73[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]20117
…	…		…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…

- `ambient_rna.txt`
  - The estimated ambient RNA percent in the pool. We typically see < 5% for scRNA-seq PBMCs and < 10% for other scRNA-seq cell types.

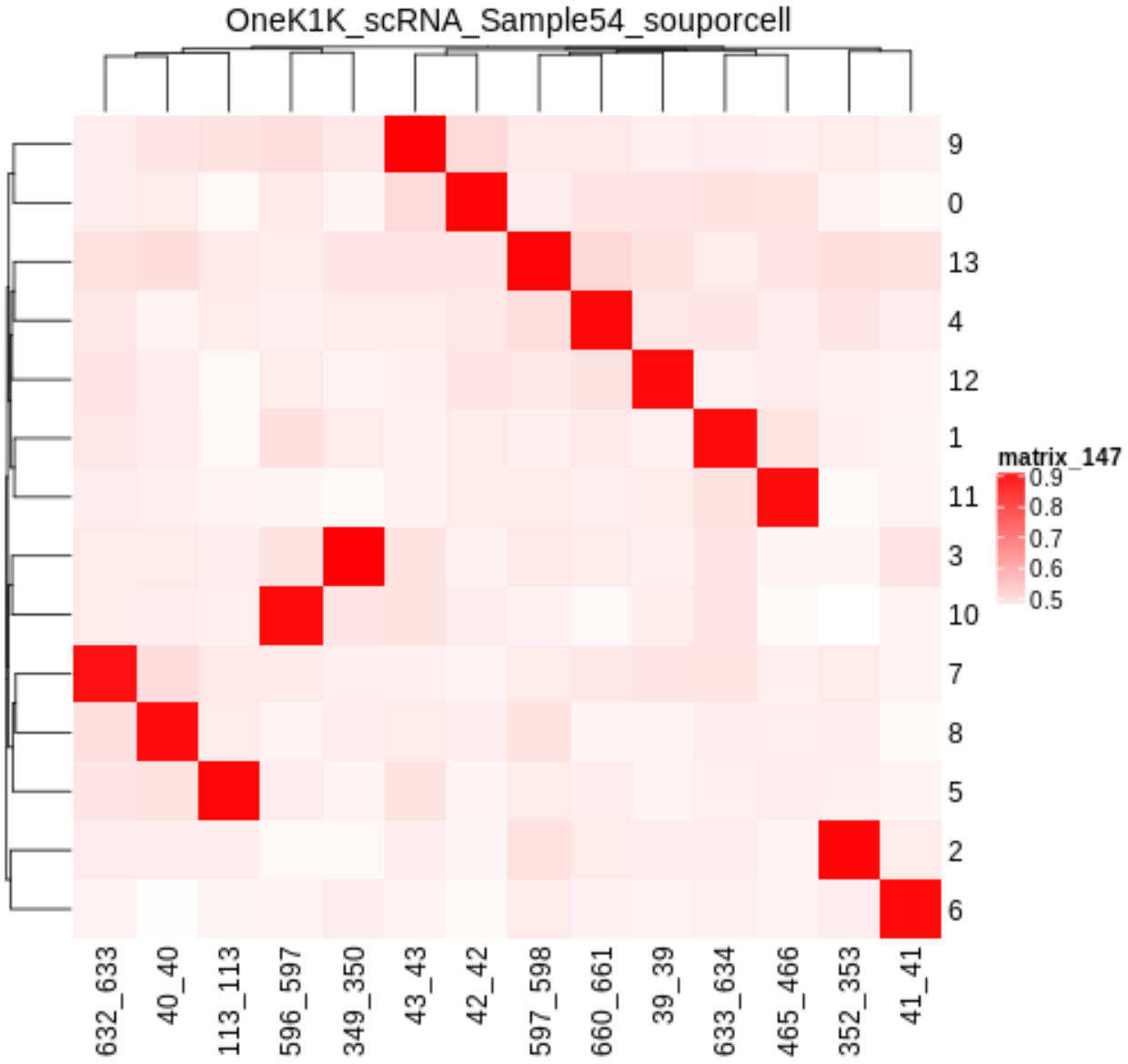
```
ambient RNA estimated as 4.071468697320357%
```

If you ran the `Assign_Indiv_by_Geno.R` script, you will also have the following files:

- `Genotype_ID_key.txt`
  - Key of the cluster and assignments for each individual and the pearson correlation coefficient.

Genotype_ID	Cluster_ID	Correlation
113_113	5	0.9365902
349_350	3	0.9484794
352_353	2	0.9385500
39_39	12	0.9325007
40_40	8	0.9252865
41_41	6	0.9282633
42_42	0	0.9387788
43_43	9	0.9497327
465_466	11	0.9234109
596_597	10	0.9277824
597_598	13	0.9435752
632_633	7	0.9179054
633_634	1	0.9222734
660_661	4	0.9368751

- `ref_clust_pearson_correlation.png`
  - Figure of the pearson correlation coefficients for each cluster-individual pair.
- `ref_clust_pearson_correlations.tsv`
  - All of the pearson correlation coefficients between the clusters and the individuals


OneK1K_scRNA_Sample54_souporcell
9
0
13
4
12
1
11
3
10
7
8
5
2
6
matrix_147
0.9
0.8
0.7
0.6
0.5
632_633
40_40
113_113
596_597
349_350
43_43
42_42
597_598
660_661
39_39
633_634
465_466
352_353
41_41

Cluster	113_113	349_350	352_353	39_39	40_40	…
0	0.45780872413920145	0.4589573335017816	0.46351292453350486	0.4892672061488018	0.484187144110379	…1
1	0.4570643404384028	0.48252804452734604	0.42750261879732254	0.46781878069650948	0.48011647970997	…36
2	0.4760176832308064	0.45281488606508193	0.38550003666007247	0.47703829279476647	0.4763977156991785	…5
3	0.4771709808299329	0.48479435206736645	0.45983613637668247	0.46988325938270249	0.482277958757972	…8
4	0.48518729333467052	0.48480637867430175	0.47508275654324042	0.48900594491809126	0.471006755998	…44
…	…	…	…	…	…	…

11.4 Merging Results with Other Software Restults

We have provided a script that will help merge and summarize the results from multiple softwares together. See *Combine Results*.

11.5 Citation

If you used this workflow for analysis, please reference our paper (REFERENCE) as well as Souporcell.

CHAPTER

TWELVE

VIREO TUTORIAL

Vireo is a flexible demultiplexing software that can demutliplex without any reference SNP genotypes, with reference SNP genotypes for a subset of the donors in the pool or no reference SNP genotypes. If you have reference SNP genotypes for **all** of the donors in your pool, you could also use *Demuxlet* or *Souporcell*. If you don't have reference SNP genotypes, you could alternatively use *Freemuxlet* or *ScSplit*.

12.1 Data

This is the data that you will need to have preparede to run Vireo:

Required

- Common SNP genotypes vcf (`$VCF`)
  - If you have reference SNP genotypes for individuals in your pool, you can use those
    - For Vireo you should only have the donors that are in this pool in the vcf file
  - If you do not have reference SNP genotypes, they can be from any large population resource (i.e. 1000 Genomes or HRC)
  - Filter for common SNPs (> 5% minor allele frequency) and SNPs overlapping genes
  - Filter for common SNPs (> 5% minor allele frequency) and SNPs overlapping genes
- Barcode file (`$BARCODES`)
- Number of samples in pool (`$N`)
- Bam file (`$BAM`)
  - Aligned single cell reads
- Output directory (`$VIREO_OUTDIR`)

12.2 Run Vireo

12.2.1 CellSNP Pileup

First, you need to count the number of alleles at each SNP in each droplet using cellSNP-lite:

```
singularity exec Demuxafy.sif cellsnp-lite -s $BAM -b $BARCODES -O $VIREO_OUTDIR -R
↪$VCF -p 20 --minMAF 0.1 --minCOUNT 20
```

You can alter the `-p`, `--minMAF` and `--minCOUNT` parameters to fit your data and your needs. We have found these settings to work well with our data

If the pileup is successfull, you will have this new file in your `$VIREO_OUTDIR`:

```
.
├── cellSNP.base.vcf
├── cellSNP.samples.tsv
├── cellSNP.tag.AD.mtx
├── cellSNP.tag.DP.mtx
└── cellSNP.tag.OTH.mtx
```

Additional details about outputs are available below in the *Vireo Results and Interpretation*.

Vireo expects the *cellSNP.base.vcf* input to be gzipped. We have contacted the developers to update this issue, but until `cellSNP-lite` outputs, we will need to add one extra step to prepare for the demultiplexing step:

```
singularity exec Demuxafy.sif bgzip $VIREO_OUTDIR/cellSNP.base.vcf
```

12.2.2 Demultiplex with Vireo

Next, we can use the cellSNP results to demultiplex the data with Vireo. As already mentioned, you can use Vireo with multiple different levels of reference SNP genotypes. We've provided an example command for each of these differing amounts of donor SNP genotype data.

With SNP Genotype Data for All Donors

With SNP Genotype Data for Some Donors

Without Donor SNP Genotype Data

You will need to provide which genotype measure (`$FIELD`) is provided in your donor SNP genotype file (GT, GP, or PL); default is PL.

Note

For Vireo you should only have the donors that are in this pool in the vcf file.

Recommended

Vireo runs more efficiently when the SNPs from the donor `$VCF` have been filtered for the SNPs identified by `cellSNP-lite`. Therefore, it is highly recommended subset the vcf first:.

If your `$VCF` file is bgzipped (*i.e.* ends in `.vcf.gz`), you can use `bcftools` to filter your `$VCF` for the positions in `$VIREO_OUTDIR/cellSNP.base.vcf`:

```
singularity exec Demuxafy.sif bcftools view $VCF -R $VIREO_OUTDIR/cellSNP.
↪base.vcf.gz -Ov -o $VIREO_OUTDIR/donor_subset.vcf
```

If your `$VCF` file is **not** bgzipped (*i.e.* ends in `.vcf`), you can use `bedtools` to filter your `$VCF` for the positions in `$VIREO_OUTDIR/cellSNP.base.vcf`:

```
singularity exec Demuxafy.sif bedtools intersect -a $VCF -b $VIREO_OUTDIR/
↪cellSNP.base.vcf.gz -wa -header > $VIREO_OUTDIR/donor_subset.vcf
```

To run Vireo with reference SNP genotype data for your donors (ideally filtered as shown above):

```
singularity exec Demuxafy.sif vireo -c $VIREO_OUTDIR -d $VIREO_OUTDIR/donor_subset.
↪vcf -o $VIREO_OUTDIR -t $FIELD
```

Note

For Vireo you should only have the donors that are in this pool in the vcf file. It assumes that `$N` is larger than the number of donors in the `$VCF`

Recommended

Vireo runs more efficiently when the SNPs from the donor `$VCF` have been filtered for the SNPs identified by `cellSNP-lite`. Therefore, it is highly recommended subset the vcf as follows first:

```
singularity exec Demuxafy.sif bcftools view $VCF -R $VIREO_OUTDIR/cellSNP.
↪base.vcf -Oz -o $VIREO_OUTDIR/donor_subset.vcf
```

```
singularity exec Demuxafy.sif vireo -c $VIREO_OUTDIR -d $VIREO_OUTDIR/donor_subset.
↪vcf -o $VIREO_OUTDIR -t $FIELD -N $N
```

```
singularity exec Demuxafy.sif vireo -c $VIREO_OUTDIR -o $VIREO_OUTDIR -N $N
```

If Vireo is successfull, you will have these new files in your `$VIREO_OUTDIR`:

```
.
├── cellSNP.base.vcf
├── cellSNP.samples.tsv
├── cellSNP.tag.AD.mtx
├── cellSNP.tag.DP.mtx
├── cellSNP.tag.OTH.mtx
├── donor_ids.tsv
├── donor_subset.vcf
├── fig_GT_distance_estimated.pdf
├── _log.txt
├── prob_doublet.tsv.gz
├── prob_singlet.tsv.gz
└── summary.tsv
```

Additional details about outputs are available below in the *Vireo Results and Interpretation*.

12.3 Vireo Results and Interpretation

After running the Vireo steps, you will have a number of files in your `$VIREO_OUTDIR`. Theses are the files that most users will find the most informative:

- `summary.tsv`
  - A summary of the droplets assigned to each donor, doublets and unassigned.

Var1	Freq
113_113	1342
349_350	1475
352_353	1619
39_39	1309
40_40	1097
41_41	1144
42_42	1430
43_43	1561
465_466	1104
596_597	1271
597_598	1532
632_633	871
633_634	967
660_661	1377
doublet	2770
unassigned	113

    - To check whether the numbe of doublets identified by Vireo is consistent with the expected doublet rate based on the number of droplets that you captured, you can use our Expected Doublet Estimation Calculator.

- `donor_ids.tsv`
  - The classification of each droplet, and some droplet metrics.

cell	donor_id	prob_max	prob_doublet	n_vars	best_singlet	best_doublet
AAACCTGAGATAGCAT-1	41_41	1.00e+00	9.13e-09	115	41_41	40_40,41_41
AAACCTGAGCAGCGTA-1	465_466	1.00e+00	5.03e-17	239	465_466	349_350,43_43
AAACCTGAGCGATGAC-1	113_113	1.00e+00	7.57e-07	98	113_113	113_113,633_634
AAACCTGAGCGTAGTG-1	349_350	1.00e+00	8.07e-07	140	349_350	349_350,597_598
AAACCTGAGGAGTTTA-1	632_633	1.00e+00	5.99e-11	177	632_633	40_40,113_113
AAACCTGAGGCTCATT-1	39_39	1.00e+00	4.44e-06	110	39_39	39_39,40_40

12.4 Merging Results with Other Software Restults

We have provided a script that will help merge and summarize the results from multiple softwares together. See *Combine Results*.

12.5 Citation

If you used this workflow for analysis, please reference our paper (REFERENCE) as well as Vireo.

CHAPTER

THIRTEEN

OVERVIEW OF DOUBLET DETECTING SOFTWARES

Transcrition-based doublet detection softwares use the transcriptomic profiles in each cell to predict whether that cell is a singlet or doublet. Most methods simulate doublets by adding the transcritional profiles of two droplets in your pool together. Therefore, these approaches assume that only a small percentage of the droplets in your dataset are doublets. The table bellow provides a comparison of the different methods.

Doublet Detecting Software	QC Filtering Required	Requires Pre-clustering	Doublet Detecting Method
DoubletDecon		✓	Deconvolution based on clusters provided.
DoubletDetection			Iterative boost classifier to classify doublets.
DoubletFinder	✓		Identify ideal cluster size and call expected number of droplets with highest number of simulated doublet neighbors as doublets.
scDblFinder			Gradient boosted trees trained with number neighboring doublets and QC metrics to classify doublets
Scds			**cxds**: Uses genes pairs that are typically not expressed in the same droplet to rank droplets based on coexpression of all pairs. **bcds**: Uses highly variable genes and simulated doublets to train a binary classificaiton algorithm and return probability of droplet being a doublet.
Scrublet			Identifies the number of neighboring simulated doublets for each droplet and uses bimodal distribution of scores to classify singlets and doublets.
Solo			Simulates doublets and fits a two-layer neural network.

If you don't know which demultiplexing software(s) to run, take a look at our *Software Selection Recommendations* based on your dataset or use our **add widget link here**

CHAPTER

FOURTEEN

DOUBLETDECON TUTORIAL

DoubletDecon is a transcription-based doublet detction software that uses deconvolution to identify doublets using the *R* statistical software. We have provided a wrapper script that takes common arguments for DoubletDecon and also provide example code for you to run manually if you prefer.

14.1 Data

This is the data that you will need to have preparede to run DoubletDecon:

Required

- A QC-filtered and normalized seurat object (`$SEURAT_OBJ`)
  - If you run DoubletDecon manually, you can use any data format of interest and read in with a method that works for your data.
- Output directory (`$DOUBLETDECON_OUTDIR`)

14.2 Run DoubletDecon

You can either run DoubletDecon with the wrapper script we have provided or you can run it manually if you would prefer to alter more parameters.

With Wrapper Script

Run in R

Note

Since it is hard to predict the correct *rhop* to use for each dataset, we typically run a range. For example: 0.6, 0.7, 0.8, 0.9, 1, and 1.1. Then we select the results that predict the number of doublets closest to the expected doublet number. You can estimate that number with our **doublet calculator** The *rhop* paramenter can be set with `-r` or `--rhop` in the command below.

```
singularity exec Demuxafy.sif DoubletDecon.R -o $DOUBLETDECON_OUTDIR -s $SEURAT_OBJ
```

You can provide many other parameters as well which can be seen from running a help request:

```
singularity exec image DoubletDecon.R -h

usage: DoubletDecon.R [-h] -o OUT -s SEURAT_OBJECT [-g NUM_GENES] [-r RHOP]
                [-p SPECIES] [-n NCORES] [-c REMOVECC] [-m PMF]
                [-f HEATMAP] [-t CENTROIDS] [-d NUM_DOUBS] [-5 ONLY50]
                [-u MIN_UNIQ]

optional arguments:
  -h, --help            show this help message and exit
  -o OUT, --out OUT     The output directory where results will be saved
  -s SEURAT_OBJECT, --seurat_object SEURAT_OBJECT
                        A QC, normalized seurat object with
                        classificaitons/clusters as Idents().
  -g NUM_GENES, --num_genes NUM_GENES
                        Number of genes to use in
                        'Improved_Seurat_Pre_Process' function.
  -r RHOP, --rhop RHOP  rhop to use in DoubletDecon - the number of SD from
                        the mean to identify upper limit to blacklist
  -p SPECIES, --species SPECIES
                        The species of your sample. Can be scientific species
                        name, KEGG ID, three letter species abbreviation, or
                        NCBI ID.
  -n NCORES, --nCores NCORES
                        The number of unique cores you would like to use to
                        run DoubletDecon. By default, uses one less than
                        available detected.
  -c REMOVECC, --removeCC REMOVECC
                        Whether to remove clusters enriched in cell cycle
                        genes.
  -m PMF, --pmf PMF     Whether to use unique gene expression in doublet
                        determination.
  -f HEATMAP, --heatmap HEATMAP
                        Whether to generate heatmaps.
  -t CENTROIDS, --centroids CENTROIDS
                        Whether to use centroids instead of medoids for
                        doublet detecting.
  -d NUM_DOUBS, --num_doubs NUM_DOUBS
                        The nunmber of doublets to simulate for each cluster
                        pair.
  -5 ONLY50, --only50 ONLY50
                        Whether to only compute doublets as 50:50 ratio.
                        Default is to use other ratios as well.
  -u MIN_UNIQ, --min_uniq MIN_UNIQ
                        Minimum number of unique genes to rescue a cluster
                        identified as doublets.
```

First, you will have to start R. We have built R and all the required software to run DoubletDecon into the singularity image so you can run it directly from the image.

```
singularity exec Demuxafy.sif R
```

That will open R in your terminal. Next, you can load all the libraries and run DoubletDecon.

```
.libPaths("/usr/local/lib/R/site-library") ### This is required so that R uses the␣
↪libraries loaded in the image and not any local libraries
library(DoubletDecon)
library(tidyverse)
```

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```
library(Seurat)
library(ggplot2)
library(data.table)

## Set up variables ##
out <- "/path/to/doubletdecon/outdir"
seurat_object <- "/path/to/preprocessed/seurat_object.rds"




## make sure the directory exists ###
dir.create(out, recursive = TRUE)

## Read in Data ##
seurat <- readRDS(seurat_object)

## Preprocess ##
processed <- Improved_Seurat_Pre_Process(seurat, num_genes=50, write_files=FALSE)

## Run Doublet Decon ##
results <- Main_Doublet_Decon(rawDataFile = processed$newExpressionFile,
  groupsFile = processed$newGroupsFile,
  filename = "DoubletDecon_results",
  location = paste0(out, "/"),
  fullDataFile = NULL,
  removeCC = FALSE,
  species = "hsa",
  rhop = 0.9,                           ## We recommend testing multiple rhop␣
↪parameters to find which fits your data the best
  write = TRUE,
  PMF = TRUE,
  useFull = FALSE,
  heatmap = FALSE,
  centroids=FALSE,
  num_doubs=100,
  only50=FALSE,
  min_uniq=4,
  nCores = 1)




doublets <- read.table(paste0(out, "/Final_doublets_groups_DoubletDecon_results.txt"))
doublets$Barcode <- gsub("\\.", "-",rownames(doublets))
doublets$DoubletDecon_DropletType <- "doublet"
doublets$V1 <- NULL
doublets$V2 <- NULL


singlets <- read.table(paste0(out,␣
↪"/Final_nondoublets_groups_DoubletDecon_results.txt"))
singlets$Barcode <- gsub("\\.", "-",rownames(singlets))
singlets$DoubletDecon_DropletType <- "singlet"
singlets$V1 <- NULL
singlets$V2 <- NULL
```

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```
doublets_singlets <- rbind(singlets,doublets)

fwrite(doublets_singlets, paste0(out, "/DoubletDecon_doublets_singlets.tsv"), sep =␣
↪"\t", append = FALSE)


### Make a summary of the number of singlets and doublets
summary <- as.data.frame(table(doublets_singlets$DoubletDecon_DropletType))
colnames(summary) <- c("Classification", "Droplet N")
fwrite(summary, paste0(out,"/DoubletDecon_doublet_summary.tsv"), sep = "\t", append =␣
↪FALSE)
```

14.3 DoubletDecon Results and Interpretation

After running the DoubletDecon, you will have multiple files in the `$DOUBLETDECON_OUTDIR`:

```
.
├── data_processed_DoubletDecon_results.txt
├── data_processed_reclust_DoubletDecon_results.txt
├── DoubletDecon_doublets_singlets.tsv
├── DoubletDecon_doublet_summary.tsv
├── DoubletDecon_results.log
├── DRS_doublet_table_DoubletDecon_results.txt
├── DRS_results_DoubletDecon_results.txt
├── Final_doublets_exp_DoubletDecon_results.txt
├── Final_doublets_groups_DoubletDecon_results.txt
├── Final_nondoublets_exp_DoubletDecon_results.txt
├── Final_nondoublets_groups_DoubletDecon_results.txt
├── groups_processed_DoubletDecon_results.txt
├── groups_processed_reclust_DoubletDecon_results.txt
├── new_PMF_results_DoubletDecon_results.txt
├── resultsreadable_synths.txt
└── Synth_doublet_info_DoubletDecon_results.txt
```

DoubletDecon puts most of the results in multiple separate files. However, the wrapper script and the example code has some steps to combine these results together into a single file, which will likely be the most informative output. These are the files that we think will be the most helpful for users:

- `DoubletDecon_doublet_summary.tsv`
  - A sumamry of the number of singlets and doublets predicted by DoubletDecon.

Classification	Droplet N
doublet	1510
singlet	19470

    * To check whether the numbe of doublets identified by DoubletDecon is consistent with the expected doublet rate expected based on the number of droplets that you captured, you can use our Expected Doublet Estimation Calculator.

- `DoubletDecon_doublets_singlets.tsv`
  - The per-barcode singlet and doublet classification from DoubletDecon.

Barcode	DoubletDecon_DropletType
AAACCTGAGCAGCGTA-1	singlet
AAACCTGAGCGATGAC-1	singlet
AAACCTGAGCGTAGTG-1	singlet
AAACCTGAGGCTCATT-1	singlet
AAACCTGAGTAGCCGA-1	singlet
...	...

14.4 Merging Results with Other Software Restults

We have provided a script that will help merge and summarize the results from multiple softwares together. See *Combine Results*.

14.5 Citation

If you used this workflow for analysis, please reference our paper (REFERENCE) as well as DoubletDecon.

CHAPTER

FIFTEEN

DOULBETDETECTION TUTORIAL

DoubletDetection is a transcription-based doublet detection software. This was one of the better-performing doublet detecting softwares that we identified in our paper (**CITE**) and it is also relatively fast to run. We have provided a wrapper script that enables DoubletDetection to be easily run from the command line but we also provide example code so that users can run manually as well depending on their data.

15.1 Data

This is the data that you will need to have preparede to run DoubletDetection:

Required

- A counts matrix (`$MATRIX`)
  - DoubletDetection expects counts to be in the cellranger output format (directory containint `barcodes.tsv`, `genes.tsv` and `matrix.mtx` **or** `barcodes.tsv.gz`, `features.tsv.gz` and `matrix.mtx.gz`)
    - If you don't have your data in this format, you can run DoubletDetection manually in python and load the data in using a method of your choosing.

Optional

- Output directory (`$DOUBLETDETECTION_OUTDIR`)
  - If you don't provide an `$DOUBLETDETECTION_OUTDIR`, the results will be written to the present working directory.

15.2 Run DoubletDetection

You can either run DoubletDetection with the wrapper script we have provided or you can run it manually if you would prefer to alter more parameters.

With Wrapper Script

Run in python

```
singularity exec Demuxafy.sif DoubletDetection.py -m $MATRIX -o $DOUBLETDETECTION_
↪OUTDIR
```

To see all the parameters that this wrapper script will accept, run:

```
singularity exec Demuxafy.sif DoubletDetection.py -h


usage: DoubletDetection.py [-h] -m COUNTS_MATRIX [-b BARCODES] [-o OUTDIR]
                           [-i N_ITERATIONS] [-p PHENOGRAPH]
                           [-s STANDARD_SCALING] [-t P_THRESH]
                           [-v VOTER_THRESH]

wrapper for DoubletDetection for doublet detection from transcriptomic data.

optional arguments:
  -h, --help            show this help message and exit
  -m COUNTS_MATRIX, --counts_matrix COUNTS_MATRIX
                        cell ranger counts matrix.mtx
  -b BARCODES, --barcodes BARCODES
                        File containing droplet barcodes. Use barcodes from
                        provided 10x dir by default.
  -o OUTDIR, --outdir OUTDIR
                        The output directory; default is current working
                        directory
  -i N_ITERATIONS, --n_iterations N_ITERATIONS
                        Number of iterations to use; default is 50
  -p PHENOGRAPH, --phenograph PHENOGRAPH
                        Whether to use phenograph (True) or not (False);
                        default is False
  -s STANDARD_SCALING, --standard_scaling STANDARD_SCALING
                        Whether to use standard scaling of normalized count
                        matrix prior to PCA (True) or not (False); default is
                        True
  -t P_THRESH, --p_thresh P_THRESH
                        P-value threshold for doublet calling; default is
                        1e-16
  -v VOTER_THRESH, --voter_thresh VOTER_THRESH
                        Voter threshold for doublet calling; default is 0.5
```

To run DoubletDetection manually, first start python from the singularity image (all the required software have been provided in the image)

```
singularity exec Demuxafy.sif python
```

Now, python will open in your terminal and you can run the DoubletDetection code. Here is an example:

```
import os
import numpy as np
import doubletdetection
import tarfile
import matplotlib
matplotlib.use('PDF')
import matplotlib.pyplot as plt
import sys
import pandas as pd

# Load read10x function from mods directory

mods_path = "/opt/Demultiplexing_Doublet_Detecting_Docs/mods" ## custom script for␣
↪loading 10x data in python
```

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```
sys.path.append(mods_path)
import read10x

### Set up parameters and variables ###
counts_matrix = "/path/to/counts/matrix.mtx"
outdir = "/path/to/doublet/detection/outdir"


if not os.path.isdir(outdir):
  os.mkdir(outdir)


### Read in data ###
raw_counts = read10x.import_cellranger_mtx(counts_matrix)

try:
  barcodes_df = read10x.read_barcodes(counts_matrix + "/barcodes.tsv.gz")
except:
  try:
    barcodes_df = read10x.read_barcodes(counts_matrix + "/barcodes.tsv")
  except:
    ␣
↪print("No barcode file in provided counts matrix directory. Please double check the directory or pr

print('Counts matrix shape: {} rows, {} columns'.format(raw_counts.shape[0], raw_
↪counts.shape[1]))

# Remove columns with all 0s
zero_genes = (np.sum(raw_counts, axis=0) == 0).A.ravel()
raw_counts = raw_counts[:, ~zero_genes]
print('Counts matrix shape after removing unexpressed genes: {} rows, {} columns'.
↪format(raw_counts.shape[0], raw_counts.shape[1]))

clf = doubletdetection.BoostClassifier(n_iters=50, use_phenograph=True, standard_
↪scaling=False, verbose = True)
doublets = clf.fit(raw_counts).predict(p_thresh=1e-16, voter_thresh=50)

results = pd.Series(doublets, name="DoubletDetection_DropletType")
dataframe = pd.concat([barcodes_df, results], axis=1)
dataframe.DoubletDetection_DropletType = dataframe.DoubletDetection_DropletType.
↪replace(1.0, "doublet")
dataframe.DoubletDetection_DropletType = dataframe.DoubletDetection_DropletType.
↪replace(0.0, "singlet")

dataframe.to_csv(os.path.join(outdir,'DoubletDetection_doublets_singlets.tsv'), sep =␣
↪"\t", index = False)


### Figures ###
doubletdetection.plot.convergence(clf, save=os.path.join(outdir,
↪'convergence_test.pdf'), show=False, p_thresh=1e-16, voter_thresh=0.5)

f3 = doubletdetection.plot.threshold(clf, save=os.path.join(outdir,
↪'threshold_test.pdf'), show=False, p_step=6)


### Make summary of singlets and doublets and write to file ###
```

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```
summary = pd.DataFrame(dataframe.DoubletDetection_DropletType.value_counts())
summary.index.name = 'Classification'
summary.reset_index(inplace=True)
summary = summary.rename({'DoubletDetection_DropletType': 'Droplet N'}, axis=1)

summary.to_csv(os.path.join(outdir,'DoubletDetection_summary.tsv'), sep = "\t", index␣
↪= False)
```

15.3 DoubletDetection Results and Interpretation

After running the DoubletDetection, you will have multiple files in the `$DOUBLETDETECTION_OUTDIR`:

```
.
├── convergence_test.pdf
├── DoubletDetection_doublets_singlets.tsv
├── DoubletDetection_summary.tsv
└── threshold_test.pdf
```

We have found these to be the most helpful:

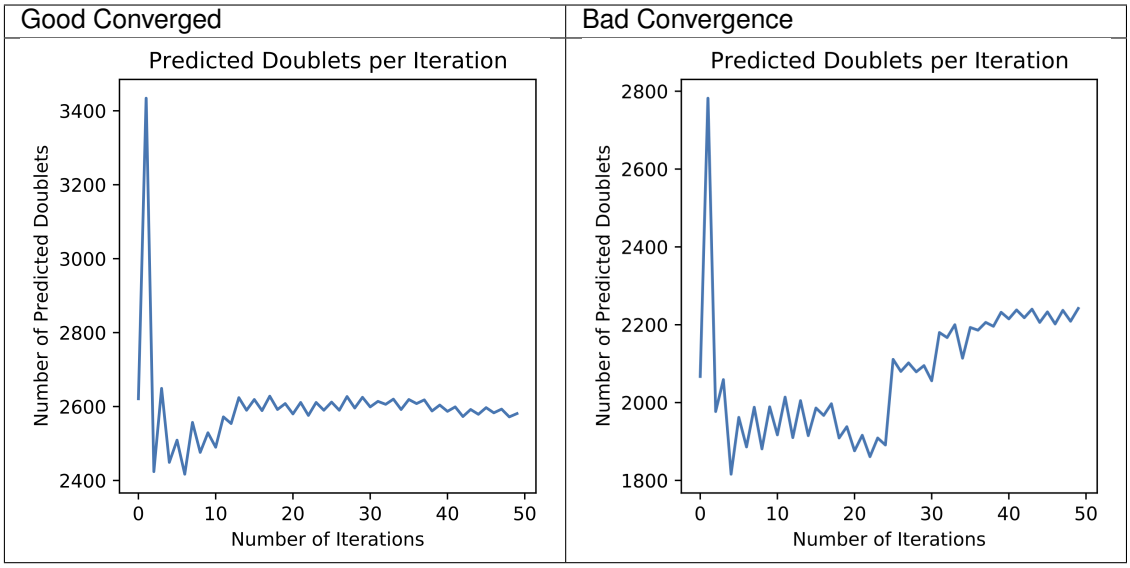
- `DoubletDetection_summary.tsv`
  - A sumamry of the number of singlets and doublets predicted by DoubletDetection.

DoubletDetection_DropletType	Droplet N
doublet	2594
singlet	18388

  - To check whether the numbe of doublets identified by DoubletDetection is consistent with the expected doublet rate expected based on the number of droplets that you captured, you can use our Expected Doublet Estimation Calculator.
- `DoubletDetection_doublets_singlets.tsv`
  - The per-barcode singlet and doublet classification from DoubletDetection.

Barcode	DoubletDetection_DropletType
AAACCTGAGATAGCAT-1	singlet
AAACCTGAGCAGCGTA-1	singlet
AAACCTGAGCGATGAC-1	singlet
AAACCTGAGCGTAGTG-1	singlet
AAACCTGAGGAGTTTA-1	singlet
AAACCTGAGGCTCATT-1	singlet
AAACCTGAGGGCACTA-1	singlet
...	...

- `convergence_test.pdf`
  - The expectation is that after multiple rounds, the expected number of doublets will converge. If that is not the case, we suggest that you run DoubletDetection for more iterations (try 150, or even 250 if that isn't convincing).
  - Here are two figures - one of a sample that came to convergence after 50 iterations (left) and one that did not (right)



15.4 Merging Results with Other Software Restults

We have provided a script that will help merge and summarize the results from multiple softwares together. See *Combine Results*.

15.5 Citation

If you used this workflow for analysis, please reference our paper (REFERENCE) as well as DoubletDetection.

CHAPTER

SIXTEEN

DOUBLETFINDER TUTORIAL

DoubletFinder is a transcription-based doublet detection software that uses simulated doublets to find droplets that has a high proportion of neighbors that are doublets. We have provided a wrapper script that takes common arguments for DoubletFinder and we alsp provide an example script that you can run manually in R if you prefer.

16.1 Data

This is the data that you will need to have preparede to run DoubletFinder:

Required

- A QC-filtered and normalized seurat object (`$SEURAT_OBJ`)
  - If you run DoubletFinder manually, you can use any data format of interest and read in with a method that works for your data.
- Output directory (`$DOUBLETFINDER_OUTDIR`)
- Expected number of doublets (`$DOUBLETS`)
  - This can be calculated based on the number of droplets captured using our **doublet calculator**

16.2 Run DoubletFinder

You can either run DoubletFinder with the wrapper script we have provided or you can run it manually if you would prefer to alter more parameters.

With Wrapper Script

Run in R

```
singularity exec Demuxafy.sif DoubletFinder.R -o $DOUBLETFINDER_OUTDIR -s $SEURAT_OBJ␣
↪-c TRUE -d $DOUBLETS
```

You can provide many other parameters as well which can be seen from running a help request:

```
singularity exec Demuxafy.sif DoubletFinder.R -h


usage: DoubletFinder.R [-h] -o OUT -s SEURAT_OBJECT -c SCT -d DOUBLET_NUMBER [-p PCS]␣
↪[-n PN]a@brenner-fpoptional arguments:
```

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```
          -h, --help            show this help message and exit
  -o OUT, --out OUT     The output directory where results will be saved
  -s SEURAT_OBJECT, --seurat_object SEURAT_OBJECT
                        A QC, normalized seurat object with
                        classificaitons/clusters as Idents().
  -c SCT, --sct SCT     Whether sctransform was used for normalization.
  -d DOUBLET_NUMBER, --doublet_number DOUBLET_NUMBER
                        Number of expected doublets based on droplets
                        captured.
  -p PCS, --PCs PCS     Number of PCs to use for 'doubletFinder_v3' function.
  -n PN, --pN PN        Number of doublets to simulate as a proportion of the
                        pool size.
```

First, you will have to start R. We have built R and all the required software to run DoubletFinder into the singularity image so you can run it directly from the image.

```
singularity exec Demuxafy.sif R
```

That will open R in your terminal. Next, you can load all the libraries and run DoubletFinder.

```
.libPaths("/usr/local/lib/R/site-library") ### This is required so that R uses the␣
↪libraries loaded in the image and not any local libraries
library(Seurat)
library(ggplot2)
library(DoubletFinder)
library(dplyr)
library(tidyr)
library(tidyverse)

## Set up parameters ##
out <- "/path/to/doubletfinder/outdir"
seurat_object <- "/path/to/preprocessed/seurat_object.rds"
doublet_number <- 3200

## make sure the directory exists ###
dir.create(out, recursive = TRUE)

## Add max future globals size for large pools
options(future.globals.maxSize=(850*1024^2))

### Read in the data
seurat <- readRDS(seurat_object)


## pK Identification (no ground-truth) ---------------------------------------------
↪---------------------------------------
sweep.res.list <- paramSweep_v3(seurat, PCs = 1:10, sct = TRUE)
sweep.stats <- summarizeSweep(sweep.res.list, GT = FALSE)
bcmvn <- find.pK(sweep.stats)
plot <- ggplot(bcmvn, aes(pK, BCmetric)) +
    geom_point()
ggsave(plot, filename = paste0(out,"/pKvBCmetric.png"))

## Homotypic Doublet Proportion Estimate -------------------------------------------
↪---------------------------------------
annotations <- Idents(seurat)
```

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```
homotypic.prop <- modelHomotypic(annotations)
nExp_poi <- doublet_number
print(paste0("Expected number of doublets: ", doublet_number))
nExp_poi.adj <- round(doublet_number*(1-homotypic.prop))

## Run DoubletFinder with varying classification stringencies ----------------------
↪----------------------------------------
seurat <- doubletFinder_v3(seurat, PCs = 1:10, pN = 0.25, pK = as.numeric(as.
↪character(bcmvn$pK[which(bcmvn$BCmetric == max(bcmvn$BCmetric))])), nExp = nExp_poi.
↪adj, reuse.pANN = FALSE, sct = TRUE)
doublets <- as.data.frame(cbind(colnames(seurat), seurat@meta.data[,
↪grepl(paste0("pANN_0.25_",as.numeric(as.character(bcmvn$pK[which(bcmvn$BCmetric ==␣
↪max(bcmvn$BCmetric))]))), colnames(seurat@meta.data))], seurat@meta.data[,
↪grepl(paste0("DF.classifications_0.25_",as.numeric(as.character(bcmvn$pK[which(bcmvn
↪$BCmetric == max(bcmvn$BCmetric))]))), colnames(seurat@meta.data))]))
colnames(doublets) <-  c("Barcode","DoubletFinder_score","DoubletFinder_DropletType")
doublets$DoubletFinder_DropletType <- gsub("Singlet","singlet",doublets$DoubletFinder_
↪DropletType) %>% gsub("Doublet","doublet",.)

write_delim(doublets, file = paste0(out,"/DoubletFinder_doublets_singlets.tsv"),␣
↪delim = "\t")

### Calculate number of doublets and singlets ###
summary <- as.data.frame(table(doublets$DoubletFinder_DropletType))
colnames(summary) <- c("Classification", "Droplet N")
write_delim(summary, paste0(out,"/DoubletFinder_doublet_summary.tsv"), "\t")
```

16.3 DoubletFinder Results and Interpretation

After running the DoubletFinder, you will have multiple files in the `$DOUBLETFINDER_OUTDIR`:

```
.
├── DoubletFinder_doublets_singlets.tsv
├── DoubletFinder_doublet_summary.tsv
└── pKvBCmetric.png
```

Here's a more detailed description of the contents of each of those files:

- `DoubletFinder_doublet_summary.tsv`
  - A sumamry of the number of singlets and doublets predicted by DoubletFinder.

Classification	Droplet N
doublet	3014
singlet	16395

    * To check whether the numbe of doublets identified by DoubletFinder is consistent with the expected doublet rate expected based on the number of droplets that you captured, you can use our Expected Doublet Estimation Calculator.

- `DoubletFinder_doublets_singlets.tsv`
  - The per-barcode singlet and doublet classification from DoubletFinder.

Barcode	DoubletFinder_score	DoubletFinder_DropletType
AAACCTGAGATAGCAT-1	0.206401766004415	singlet
AAACCTGAGCAGCGTA-1	0.144039735099338	singlet
AAACCTGAGCGATGAC-1	0.191501103752759	singlet
AAACCTGAGCGTAGTG-1	0.212472406181015	singlet
AAACCTGAGGAGTTTA-1	0.242273730684327	singlet
AAACCTGAGGCTCATT-1	0.211368653421634	singlet
AAACCTGAGGGCACTA-1	0.626379690949227	doublet
...	...	...

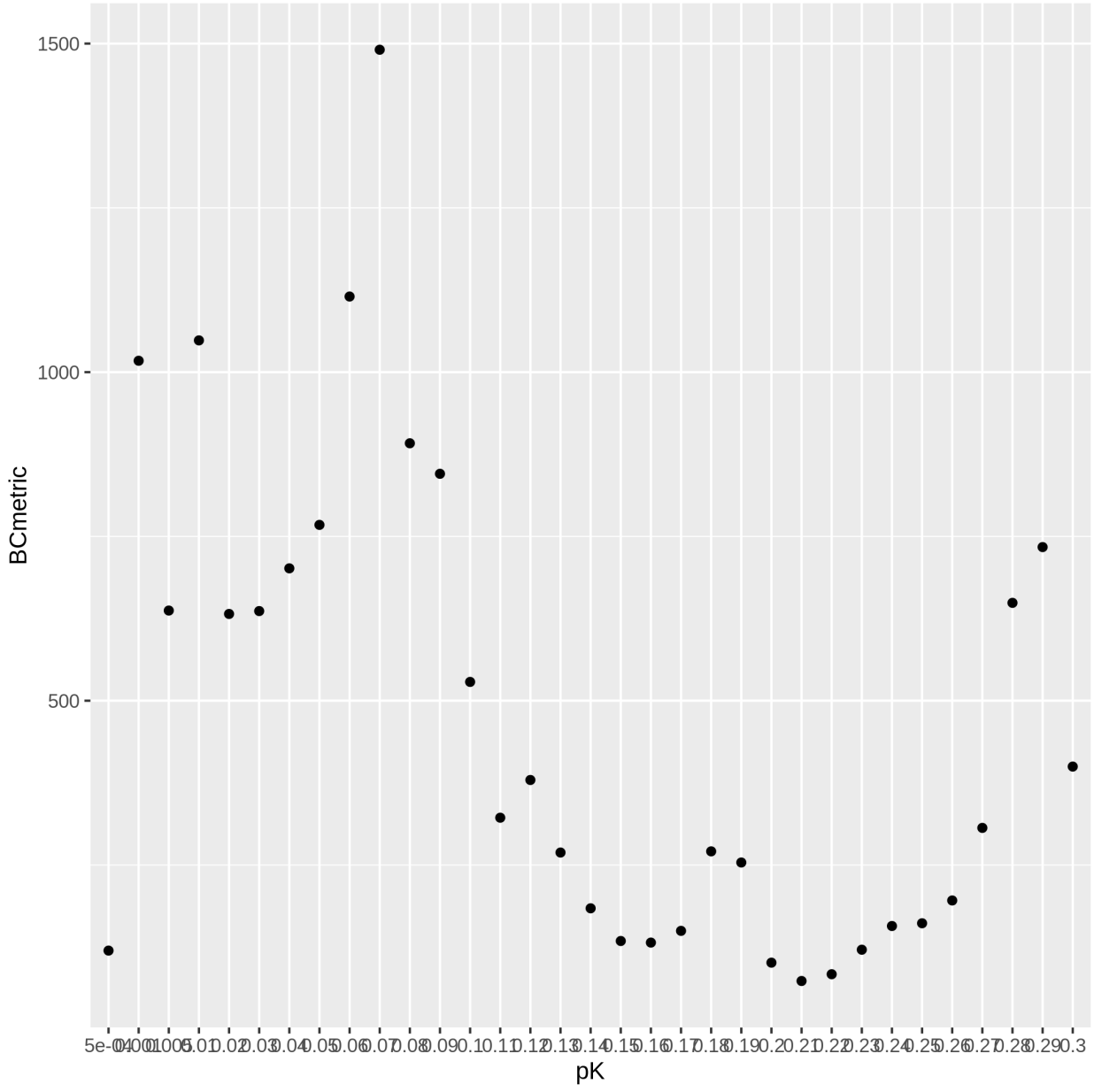
- `pKvBCmetric.png`
  - This is the metric that DoubletFinder uses to call doublets and singlets. Typically the `pK` value at the maximum `BC` value is the best doublet calling threshold.
  - If you do not have a clear `BC` maximum, see responses from the DoubletFinder developer here and here for possible solutions.

16.4 Merging Results with Other Software Restults

We have provided a script that will help merge and summarize the results from multiple softwares together. See *Combine Results*.

16.5 Citation

If you used this workflow for analysis, please reference our paper (REFERENCE) as well as DoubletFinder.


1500
1000
500
BCmetric
pK

CHAPTER

SEVENTEEN

SCDBLFINDER TUTORIAL

scDblFinder is a transcriptome-based doublet detecting method that uses doublet simulation from droplets in the dataset to identify doublets. We have provided a wrapper script that takes common arguments for scDblFinder and also provide example code for you to run manually if you prefer.

17.1 Data

This is the data that you will need to have preparede to run scDblFinder:

Required

- A counts matrix (`$MATRIX`)
  - DoubletDetection expects counts to be in the cellranger output format (directory containint `barcodes.tsv`, `genes.tsv` and `matrix.mtx` **or** `barcodes.tsv.gz`, `features.tsv.gz` and `matrix.mtx.gz`)
    - If you don't have your data in this format, you can run scDblFinder manually in R and load the data in using a method of your choosing.
- Output directory (`$SCDBLFINDER_OUTDIR`)
  - If you don't provide an `$SCDBLFINDER_OUTDIR`, the results will be written to the present working directory.

17.2 Run scDblFinder

You can either run scDblFinder with the wrapper script we have provided or you can run it manually if you would prefer to alter more parameters.

With Wrapper Script

Run in R

```
singularity exec Demuxafy.sif scDblFinder.R -o $SCDBLFINDER_OUTDIR -t $MATRIX
```

First, you will have to start R. We have built R and all the required software to run scDblFinder into the singularity image so you can run it directly from the image.

```
singularity exec Demuxafy.sif R
```

That will open R in your terminal. Next, you can load all the libraries and run scDblFinder.

```
.libPaths("/usr/local/lib/R/site-library") ### This is required so that R uses the libraries loaded in the image and not any local libraries
library(scDblFinder)
library(Seurat)
library(SingleCellExperiment)
library(tidyverse)

## Set up variables and parameters ##
out <- "/path/to/scds/outdir/"
tenX_matrix <- "/path/to/counts/matrix/dir/"

dir.create(out, recursive = TRUE)
print(paste0("Using the following counts directory: ", tenX_matrix))



### Read in data as an sce object ###
counts <- Read10X(tenX_matrix, gene.column = 1)
sce <- SingleCellExperiment(list(counts=counts))


## Calculate doublet ratio ###
doublet_ratio <- ncol(sce)/1000*0.008


### Calculate Singlets and Doublets ###
sce <- scDblFinder(sce, dbr=doublet_ratio)



### Make a dataframe of the results ###
results <- data.frame("Barcode" = rownames(colData(sce)), "scDblFinder_DropletType" = sce$scDblFinder.class, "scDblFinder_Score" = sce$scDblFinder.score)


write_delim(results, path = paste0(out,"/scDblFinder_doublets_singlets.tsv"), delim = "\t")

### Calculate number of doublets and singlets ###
summary <- as.data.frame(table(results$scDblFinder_DropletType))
colnames(summary) <- c("Classification", "Droplet N")
write_delim(summary, paste0(out,"/scDblFinder_doublet_summary.tsv"), "\t")
```

17.3 scDblFinder Results and Interpretation

After running the scDblFinder with the wrapper script or manually you should have two files in the `$SCDBLFINDER_OUTDIR`:

```
.
├── scDblFinder_doublets_singlets.tsv
└── scDblFinder_doublet_summary.tsv
```

Here's a more detaild description of each of those files:

- `scDblFinder_doublet_summary.tsv`

  - A sumamry of the number of singlets and doublets predicted by scDblFinder.

Classification	Droplet N
doublet	3323
singlet	17659

    * To check whether the numbe of doublets identified by scDblFinder is consistent with the expected doublet rate expected based on the number of droplets that you captured, you can use our Expected Doublet Estimation Calculator.

- `scDblFinder_doublets_singlets.tsv`
  - The per-barcode singlet and doublet classification from scDblFinder.

Barcode	scDblFinder_DropletType	scDblFinder_Score
AAACCTGAGATAGCAT-1	singlet	0.0033526041079312563
AAACCTGAGCAGCGTA-1	doublet	0.9937564134597778
AAACCTGAGCGATGAC-1	singlet	5.045032594352961e-
AAACCTGAGCGTAGTG-1	singlet	0.007504515815526247
AAACCTGAGGAGTTTA-1	singlet	0.00835108570754528
AAACCTGAGGCTCATT-1	singlet	0.028838597238063812
AAACCTGAGGGCACTA-1	doublet	0.9985504746437073
AAACCTGAGTAATCCC-1	singlet	0.005869860760867596
...	...	...

17.4 Merging Results with Other Software Restults

We have provided a script that will help merge and summarize the results from multiple softwares together. See *Combine Results*.

17.5 Citation

If you used this workflow for analysis, please reference our paper (REFERENCE) as well as scDblFinder.

CHAPTER

EIGHTEEN

SCDS TUTORIAL

scds is a transcription-based doublet detection software that uses two different methods to detect doublets - cxds and bcds. The cxds method uses marker genes that are not co-expressed to identify droplets that are likely doublets. bcds simulates doublet by adding droplet transcriptomes together and then uses variable genes to identify the probability a droplet is a doublet with a binary classification algorithm. We typically use the combined score of these two methods but they can be use separately as well. We have provided a wrapper script that takes common arguments for scds and we alsp provide an example script that you can run manually in R if you prefer.

18.1 Data

This is the data that you will need to have preparede to run scds:

Required

- A counts matrix (`$MATRIX`)
  - Scds expects counts to be in the cellranger output format (directory containing `barcodes.tsv`, `genes.tsv` and `matrix.mtx` **or** `barcodes.tsv.gz`, `features.tsv.gz` and `matrix.mtx.gz`)
    - If you don't have your data in this format, you can run scds manually in R and load the data in using a method of your choosing.
- Output directory (`$SCDS_OUTDIR`)
  - If you don't provide an `$SCDS_OUTDIR`, the results will be written to the present working directory.

18.2 Run scds

You can either run scds with the wrapper script we have provided or you can run it manually if you would prefer to alter more parameters.

With Wrapper Script

Run in R

```
singularity exec Demuxafy.sif scds.R -o $SCDS_OUTDIR -t $MATRIX
```

First, you will have to start R. We have built R and all the required software to run scds into the singularity image so you can run it directly from the image.

```
singularity exec Demuxafy.sif R
```

That will open R in your terminal. Next, you can load all the libraries and run scds.

```
.libPaths("/usr/local/lib/R/site-library") ### This is required so that R uses the␣
↪libraries loaded in the image and not any local libraries
library(dplyr)
library(tidyr)
library(tidyverse)
library(scds)
library(Seurat)
library(SingleCellExperiment)

## Set up variables and parameters ##
out <- "/path/to/scds/outdir/"
tenX_matrix <- "/path/to/counts/matrix/dir/"

## Read in data
counts <- Read10X(as.character(tenX_matrix), gene.column = 1)

## Account for possibility that not just single cell data
if (is.list(counts)){
  sce <- SingleCellExperiment(list(counts=counts[[grep("Gene", names(counts))]]))
} else {
  sce <- SingleCellExperiment(list(counts=counts))
}

## Annotate doublet using binary classification based doublet scoring:
sce = bcds(sce, retRes = TRUE, estNdbl=TRUE)

## Annotate doublet using co-expression based doublet scoring:
try({
    sce = cxds(sce, retRes = TRUE, estNdbl=TRUE)
})

### If cxds worked, run hybrid, otherwise use bcds annotations
if ("cxds_score" %in% colnames(colData(sce))) {
    ## Combine both annotations into a hybrid annotation
    sce = cxds_bcds_hybrid(sce, estNdbl=TRUE)
    Doublets <- as.data.frame(cbind(rownames(colData(sce)), colData(sce)$hybrid_score,
↪ colData(sce)$hybrid_call))
} else {
    print("this pool failed cxds so results are just the bcds calls")
    Doublets <- as.data.frame(cbind(rownames(colData(sce)), colData(sce)$bcds_score,␣
↪colData(sce)$bcds_call))
}

## Doublet scores are now available via colData:
colnames(Doublets) <- c("Barcode","scds_score","scds_DropletType")
Doublets$scds_DropletType <- gsub("FALSE","singlet",Doublets$scds_DropletType)
Doublets$scds_DropletType <- gsub("TRUE","doublet",Doublets$scds_DropletType)

message("writing output")
write_delim(Doublets, paste0(out,"/scds_doublets_singlets.tsv"), "\t")


summary <- as.data.frame(table(Doublets$scds_DropletType))
```

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```
colnames(summary) <- c("Classification", "Droplet N")
write_delim(summary, paste0(out,"/scds_doublet_summary.tsv"), "\t")
```

18.3 scds Results and Interpretation

After running the scds with the wrapper script or manually you should have two files in the `$SCDS_OUTDIR`:

```
.
├── scds_doublets_singlets.tsv
└── scds_doublet_summary.tsv
```

- `scds_doublet_summary.tsv`
  - A sumamry of the number of singlets and doublets predicted by scds.

Classification	Droplet N
doublet	2771
singlet	18211

    * To check whether the numbe of doublets identified by scds is consistent with the expected doublet rate expected based on the number of droplets that you captured, you can use our Expected Doublet Estimation Calculator.

- `scds_doublets_singlets.tsv`
  - The per-barcode singlet and doublet classification from scds.

Barcode	scds_score	scds_DropletType
AAACCTGAGATAGCAT-1	0.116344358493288	singlet
AAACCTGAGCAGCGTA-1	0.539856378453988	singlet
AAACCTGAGCGATGAC-1	0.0237184380134577	singlet
AAACCTGAGCGTAGTG-1	0.163695865366576	singlet
AAACCTGAGGAGTTTA-1	0.11591462421927	singlet
AAACCTGAGGCTCATT-1	0.0479944175570073	singlet
AAACCTGAGGGCACTA-1	0.374426050641161	singlet
AAACCTGAGTAATCCC-1	0.247842972104563	singlet
...	...	...

18.4 Merging Results with Other Software Restults

We have provided a script that will help merge and summarize the results from multiple softwares together. See *Combine Results*.

18.5 Citation

If you used this workflow for analysis, please reference our paper (REFERENCE) as well as scds.

CHAPTER

NINETEEN

SCRUBLET TUTORIAL

Scrublet is a transcription-based doublet detecting software. We have provided a wrapper script that enables Scrublet to be easily run from the command line but we also provide example code so that users can run manually as well depending on their data.

19.1 Data

This is the data that you will need to have preparede to run Scrublet:

Required

- A counts matrix (`$MATRIX`)
  - DoubletDetection expects counts to be in the cellranger output format (directory containint `barcodes.tsv`, `genes.tsv` and `matrix.mtx` **or** `barcodes.tsv.gz`, `features.tsv.gz` and `matrix.mtx.gz`)
    - If you don't have your data in this format, you can run Scrublet manually in python and load the data in using a method of your choosing.

Optional

- Output directory (`$SCRUBLET_OUTDIR`)
  - If you don't provide an `$SCRUBLET_OUTDIR`, the results will be written to the present working directory.

19.2 Run Scrublet

You can either run Scrublet with the wrapper script we have provided or you can run it manually if you would prefer to alter more parameters.

Note

It is a good idea to try multiple different percentile variable numbers. We typically try, 80, 85, 90 and 95. Then we choose the one that has the best defined bimodal distribution based on the `doublet_score_histogram.png` (see *Scrublet Results and Interpretation* for details).

With Wrapper Script

Run in python

```
singularity exec Demuxafy.sif Scrublet.py -m $MATRIX -o $SCRUBLET_OUTDIR
```

To see all the parameters that this wrapper script will accept, run:

```
singularity exec Demuxafy.sif Scrublet.py -h


usage: scrublet.py [-h] -m COUNTS_MATRIX [-b BARCODES] [-r SIM_DOUBLET_RATIO]
        [-c MIN_COUNTS] [-e MIN_CELLS]
        [-v MIN_GENE_VARIABILITY_PCTL] [-p N_PRIN_COMPS]
        [-t SCRUBLET_DOUBLET_THRESHOLD] [-o OUTDIR]

wrapper for scrublet for doublet detection of transcriptomic data.

optional arguments:
-h, --help            show this help message and exit
-m COUNTS_MATRIX, --counts_matrix COUNTS_MATRIX
            cell ranger counts matrix directory
-b BARCODES, --barcodes BARCODES
            barcodes.tsv or barcodes.tsv.gz from cellranger
-r SIM_DOUBLET_RATIO, --sim_doublet_ratio SIM_DOUBLET_RATIO
            Number of doublets to simulate relative to the number
            of observed transcriptomes.
-c MIN_COUNTS, --min_counts MIN_COUNTS
            Used for gene filtering prior to PCA. Genes expressed
            at fewer than min_counts in fewer than min_cells are
            excluded.
-e MIN_CELLS, --min_cells MIN_CELLS
            Used for gene filtering prior to PCA. Genes expressed
            at fewer than min_counts in fewer than are excluded.
-v MIN_GENE_VARIABILITY_PCTL, --min_gene_variability_pctl MIN_GENE_VARIABILITY_PCTL
            Used for gene filtering prior to PCA. Keep the most
            highly variable genes in the top
            min_gene_variability_pctl percentile), as measured by
            the v-statistic [Klein et al., Cell 2015].
-p N_PRIN_COMPS, --n_prin_comps N_PRIN_COMPS
            Number of principal components used to embed the
            transcriptomes priorto k-nearest-neighbor graph
            construction.
-t SCRUBLET_DOUBLET_THRESHOLD, --scrublet_doublet_threshold SCRUBLET_DOUBLET_THRESHOLD
            Manually Set the scrublet doublet threshold location.
            For running a second time if scrublet incorreclty
            places the threshold the first time
-o OUTDIR, --outdir OUTDIR
            The output directory
```

To run Scrublet manually, first start python from the singularity image (all the required software have been provided in the image)

```
singularity exec Demuxafy.sif python
```

Now, python will open in your terminal and you can run the Scrublet code. Here is an example:

```
import sys
import os
import scrublet as scr
```

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```
import scipy.io
import matplotlib
matplotlib.use('AGG')
import matplotlib.pyplot as plt
import numpy as np
import pandas as pd
import umap
import numba
import numba.typed

# Get path of mods directory from current script directory
mods_path = "/opt/Demultiplexing_Doublet_Detecting_Docs/mods"
sys.path.append(mods_path)
import read10x

## Set up parameters and variables ##
counts_matrix_dir = "/path/to/counts/matrix/dir/"
outdir = "/path/to/doublet/detection/outdir"

if not os.path.isdir(outdir):
  os.mkdir(outdir)


plt.rc('font', size=14)
plt.rcParams['pdf.fonttype'] = 42

## Basic run with scrublet
counts_matrix = read10x.import_cellranger_mtx(counts_matrix_dir)

try:
  barcodes_df = read10x.read_barcodes(counts_matrix_dir + "/barcodes.tsv.gz")
except:
  try:
    barcodes_df = read10x.read_barcodes(counts_matrix_dir + "/barcodes.tsv")
  except:
    
↪print("No barcode file in provided counts matrix directory. Please double check the directory or pr



dbl_rate = counts_matrix.shape[0]/1000 * 0.008
print('Counts matrix shape: {} rows, {} columns'.format(counts_matrix.shape[0],␣
↪counts_matrix.shape[1]))
scrub = scr.Scrublet(counts_matrix, expected_doublet_rate=dbl_rate, sim_doublet_ratio␣
↪= 2)
doublet_scores, predicted_doublets = scrub.scrub_doublets(min_counts=3,
                                                          min_cells=3,
                                                          min_gene_variability_
↪pctl=85,
                                                          n_prin_comps=30)


### Plotting and saving
scrub.plot_histogram();
plt.savefig(os.path.join(outdir,'doublet_score_histogram.png'))
print('Running UMAP...')
scrub.set_embedding('UMAP', scr.get_umap(scrub.manifold_obs_, 10, min_dist=0.3))
```

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```
print('Done.')
scrub.plot_embedding('UMAP', order_points=True);
plt.savefig(os.path.join(outdir,'UMAP.png'))

results = pd.Series(scrub.predicted_doublets_, name="scrublet_DropletType")
scores = pd.Series(scrub.doublet_scores_obs_, name="scrublet_Scores")
dataframe = pd.concat([barcodes_df, results, scores], axis=1)
dataframe.scrublet_DropletType = dataframe.scrublet_DropletType.replace(True,␣
↪"doublet")
dataframe.scrublet_DropletType = dataframe.scrublet_DropletType.replace(False,␣
↪"singlet")

dataframe.to_csv(os.path.join(outdir,'scrublet_results.tsv'), sep = "\t", index =␣
↪False)


### Make summary of singlets and doublets and write to file ###
summary = pd.DataFrame(dataframe.scrublet_DropletType.value_counts())
summary.index.name = 'Classification'
summary.reset_index(inplace=True)
summary = summary.rename({'scrublet_DropletType': 'Droplet N'}, axis=1)

summary.to_csv(os.path.join(outdir,'scrublet_summary.tsv'), sep = "\t", index = False)
```

19.3 Scrublet Results and Interpretation

After running the Scrublet, you will have four files in the `$SCRUBLET_OUTDIR`:

```
.
├── doublet_score_histogram.png
├── scrublet_results.tsv
├── scrublet_summary.tsv
└── UMAP.png
```

We have found these to be the most helpful:

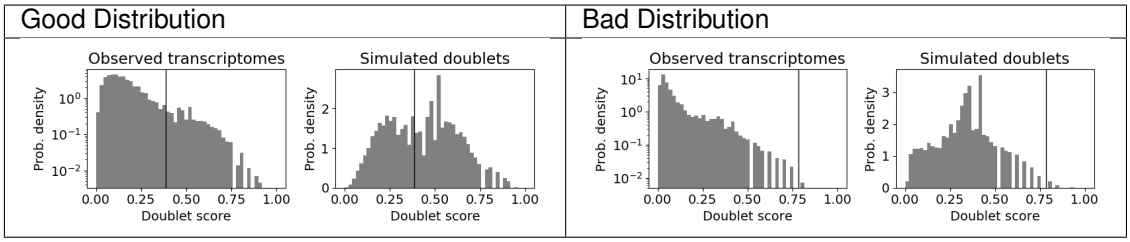
- `scrublet_summary.tsv`
  - A sumamry of the number of singlets and doublets predicted by Scrublet.

scrublet_DropletType	Droplet N
doublet	1851
singlet	19131

  - To check whether the number of doublets identified by Scrublet is consistent with the expected doublet rate expected based on the number of droplets that you captured, you can use our Expected Doublet Estimation Calculator.
- `scrublet_results.tsv`

Barcode	scrublet_DropletType	scrublet_Scores
AAACCTGAGATAGCAT-1	singlet	0.0545
AAACCTGAGCAGCGTA-1	singlet	0.1179
AAACCTGAGCGATGAC-1	singlet	0.1356
AAACCTGAGCGTAGTG-1	singlet	0.0844
AAACCTGAGGAGTTTA-1	singlet	0.0958
AAACCTGAGGCTCATT-1	singlet	0.1329
AAACCTGAGGGCACTA-1	doublet	0.4474
...	...	...

- `doublet_score_histogram.png`
  - This is the method that Scrublet uses to identify doublets - it assumes a bimodal distribution of doublet scores. Those droplets with lower scores should be singlets and those with higher scores should be doublets. It identifies the correct threshold by identifying the minimum of the bimodal distribution of simulated doublets (right).
  - However, sometimes there is not a good bimodal distribution and sometimes you will have to set the threshold manually.
  - Here is an example of a good distribution (left) and a bad distribution (left)



    - In the case of the left sample, we would rerun with different parameters to try to get a better distribution and possibly manually set the threshold to ~0.2 depending on the results. In the event that we can't achieve a clear bimodal distribution, we don't use scrublet for doublet detecting.

19.4 Merging Results with Other Software Restults

We have provided a script that will help merge and summarize the results from multiple softwares together. See *Combine Results*.

19.5 Citation

If you used this workflow for analysis, please reference our paper (REFERENCE) as well as Scrublet.

CHAPTER

TWENTY

SOLO TUTORIAL

solo is a transcription-based doublet detecting software that was one of the better transcription-based doublet detecting softwares that we tested (CITATION)

20.1 Data

This is the data that you will need to have preparede to run solo:

Required

- Parameter json file (`$JSON`)
  - Solo has provided an `example file` that we have found to work well for most of our data.
- Counts (`$COUNTS`)
  - This can be a h5ad file, loom file, or 10x counts matrix directory (containing `barcodes.tsv`, `genes.tsv` and `matrix.mtx` **or** `barcodes.tsv.gz`, `features.tsv.gz` and `matrix.mtx.gz`)
- Barcode files (`barcodes.tsv`)
- Output directory (`$SOLO_OUTDIR`)

Optional

- Expected number of doublets (`$N_DOUB`)

20.2 Run solo

```
singularity exec Demuxafy.sif solo -o $SOLO_OUTDIR -e $N_DOUB -j $JSON -d $COUNTS
```

Solo also has additional paramters that can be seen with:

```
singularity exec Demuxafy.sif solo -h

usage: solo [-h] -j MODEL_JSON_FILE -d DATA_PATH
          [--set-reproducible-seed REPRODUCIBLE_SEED]
          [--doublet-depth DOUBLET_DEPTH] [-g] [-a] [-o OUT_DIR]
          [-r DOUBLET_RATIO] [-s SEED] [-e EXPECTED_NUMBER_OF_DOUBLETS] [-p]
```

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```
          [-recalibrate_scores] [--version]

optional arguments:
  -h, --help            show this help message and exit
  -j MODEL_JSON_FILE    json file to pass VAE parameters (default: None)
  -d DATA_PATH          path to h5ad, loom, or 10x mtx dir cell by genes
                        counts (default: None)
  --set-reproducible-seed REPRODUCIBLE_SEED
                        Reproducible seed, give an int to set seed (default:
                        None)
  --doublet-depth DOUBLET_DEPTH
                        Depth multiplier for a doublet relative to the average
                        of its constituents (default: 2.0)
  -g                    Run on GPU (default: True)
  -a                    output modified anndata object with solo scores Only
                        works for anndata (default: False)
  -o OUT_DIR
  -r DOUBLET_RATIO      Ratio of doublets to true cells (default: 2)
  -s SEED               Path to previous solo output directory. Seed VAE
                        models with previously trained solo model. Directory
                        structure is assumed to be the same as solo output
                        directory structure. should at least have a vae.pt a
                        pickled object of vae weights and a latent.npy an
                        np.ndarray of the latents of your cells. (default:
                        None)
  -e EXPECTED_NUMBER_OF_DOUBLETS
                        Experimentally expected number of doublets (default:
                        None)
  -p                    Plot outputs for solo (default: False)
  -recalibrate_scores   Recalibrate doublet scores (not recommended anymore)
                        (default: False)
  --version             Get version of solo-sc (default: False)
```

If solo runs correctly, you should have the following files and directory structure in your `$SOLO_OUTDIR`:

```
.
├── classifier
│   ├── attr.pkl
│   ├── model_params.pt
│   └── var_names.csv
├── is_doublet.csv
├── is_doublet.npy
├── is_doublet_sim.npy
├── latent.npy
├── logit_scores.csv
├── logit_scores.npy
├── logit_scores_sim.npy
├── no_updates_softmax_scores.csv
├── no_updates_softmax_scores.npy
├── no_updates_softmax_scores_sim.npy
├── preds.csv
├── preds.npy
├── smoothed_preds.npy
├── softmax_scores.csv
├── softmax_scores.npy
└── vae
    ├── attr.pkl
```

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```
    ├── model_params.pt
    └── var_names.csv
```

20.2.1 Solo Summary

We have provided a script that will summarize the number of droplets classified as doublets and singlets by solo and write it to the `$SOLO_OUTDIR`. This script also combines some of the solo outputs into a single file that can be more easily used for downstream analyses. You can run this to get a fast and easy summary of your results with:

```
singularity exec Demuxafy.sif solo_summary.py -b $BARCODES -s $SOLO_OUTDIR
```

If successful, you should have two new files in your `$SOLO_OUTDIR`:

```
.
├── classifier
│   ├── attr.pkl
│   ├── model_params.pt
│   └── var_names.csv
├── is_doublet.csv
├── is_doublet.npy
├── is_doublet_sim.npy
├── latent.npy
├── logit_scores.csv
├── logit_scores.npy
├── logit_scores_sim.npy
├── no_updates_softmax_scores.csv
├── no_updates_softmax_scores.npy
├── no_updates_softmax_scores_sim.npy
├── preds.csv
├── preds.npy
├── smoothed_preds.npy
├── softmax_scores.csv
├── softmax_scores.npy
├── solo_results.tsv
├── solo_summary.tsv
└── vae
    ├── attr.pkl
    ├── model_params.pt
    └── var_names.csv
```

20.3 Solo Results and Interpretation

solo puts most of the results in multiple separate files. However, the wrapper script and the example code has some steps to combine these results together into a single file, which will likely be the most informative output.

- `solo_summary.tsv`
  - A sumamry of the number of singlets and doublets predicted by solo.

Classification	Droplet N
singlet	17461
doublet	3521

  - To check whether the numbe of doublets identified by solo is consistent with the expected doublet rate expected based on the number of droplets that you captured, you can use our Expected Doublet Estimation Calculator.

- `solo_results.tsv`
  - The per-barcode singlet and doublet classification from solo.

Barcode	solo_DropletType	solo_DropletScore
AAACCTGAGATAGCAT-1	singlet	-8.442187
AAACCTGAGCAGCGTA-1	singlet	-2.8096201
AAACCTGAGCGATGAC-1	singlet	-2.8949203
AAACCTGAGCGTAGTG-1	singlet	-5.928284
AAACCTGAGGAGTTTA-1	doublet	0.2749935
AAACCTGAGGCTCATT-1	singlet	-5.2726507
AAACCTGAGGGCACTA-1	singlet	-0.65760195
AAACCTGAGTAATCCC-1	singlet	-3.5948637
...	...	...

20.4 Merging Results with Other Software Restults

We have provided a script that will help merge and summarize the results from multiple softwares together. See *Combine Results*.

20.5 Citation

If you used this workflow for analysis, please reference our paper (REFERENCE) as well as solo.

CHAPTER

TWENTYONE

COMBINING RESULTS

After you have run each of the Demultiplexing and Doublet Detecting softwares you would like, it is helpful to convert them to similar nomenclarture and combine the results into a single dataframe. In addition, we have found it helpful to generate summaries of each of the combinations of softwares identified. To help streamline this process, we have provided a script that will easily integrate all the softwares you have run into a single dataframe and to generate a summary file for all the software combinations and if you ran demultiplexing softwares, it will also generate a demultiplexing summary file for the individual and cluster assignments from the demultiplexing softwares.

21.1 Data

In order to use our script to combine the results from the various demultiplexing and doublet detecting softwares, you need the following:

Required

- Output directory (`$OUTDIR`)
- Path to results of each of the softwares you would like to merge into a single dataframe.
  - You need to provide the path to at least one software result, otherwise, it will not run.

21.2 Merging Results with Combine_Results.r

The script has multiple options to provide the paths to each of the software results you would like to run. To see each of the options, simply run:

```
singularity exec Demuxafy.sif Combine_Results.R -h
```

Providing the possible parameter options:

```
usage: Combine_Results.R
     [-h] -o OUT [-d DEMUXLET] [-f FREEMUXLET] [-g FREEMUXLET_ASSIGNMENTS]
     [-s SCSPLIT] [-w SCSPLIT_ASSIGNMENTS] [-u SOUPORCELL]
     [-x SOUPORCELL_ASSIGNMENTS] [-v VIREO] [-e DOUBLETDECON]
     [-t DOUBLETDETECTION] [-i DOUBLETFINDER] [-n SCDBLFINDER] [-c SCDS]
     [-r SCRUBLET] [-l SOLO]

optional arguments:
  -h, --help            show this help message and exit
```

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```
  -o OUT, --out OUT     The file where results will be saved
  -d DEMUXLET, --demuxlet DEMUXLET
                        Path to demuxlet results. Only use this option if you
                        want to include the demuxlet results.
  -f FREEMUXLET, --freemuxlet FREEMUXLET
                        Path to freemuxlet results. Only use this option if
                        you want to include the freemuxlet results.
  -g FREEMUXLET_ASSIGNMENTS, --freemuxlet_assignments FREEMUXLET_ASSIGNMENTS
                        Path to freemuxlet cluster-to-individual assignments.
                        Only use this option if have used reference SNP
                        genotypes to assign individuals to clusters for the
                        freemuxlet results.
  -s SCSPLIT, --scSplit SCSPLIT
                        Path to scSplit results. Only use this option if you
                        want to include the scSplit results.
  -w SCSPLIT_ASSIGNMENTS, --scSplit_assignments SCSPLIT_ASSIGNMENTS
                        Path to scSplit cluster-to-individual assignments.
                        Only use this option if you have used reference SNP
                        genotypes to assign individuals to clusters for the
                        scSplit results.
  -u SOUPORCELL, --souporcell SOUPORCELL
                        Path to souporcell results. Only use this option if
                        you want to include the souporcell results.
  -x SOUPORCELL_ASSIGNMENTS, --souporcell_assignments SOUPORCELL_ASSIGNMENTS
                        Path to souporcell cluster-to-individual assignments.
                        Only use this option if you have used reference SNP
                        genotypes to assign individuals to clusters for the
                        souporcell results.
  -v VIREO, --vireo VIREO
                        Path to vireo results. Only use this option if you
                        want to include the vireo results.
  -e DOUBLETDECON, --DoubletDecon DOUBLETDECON
                        Path to DoubletDecon results. Only use this option if
                        you want to include the DoubletDecon results.
  -t DOUBLETDETECTION, --DoubletDetection DOUBLETDETECTION
                        Path to DoubletDetection results. Only use this option
                        if you want to include the DoubletDetection results.
  -i DOUBLETFINDER, --DoubletFinder DOUBLETFINDER
                        Path to DoubletFinder results. Only use this option if
                        you want to include the DoubletFinder results.
  -n SCDBLFINDER, --scDblFinder SCDBLFINDER
                        Path to scDblFinder results. Only use this option if
                        you want to include the scDblFinder results.
  -c SCDS, --scds SCDS  Path to scds results. Only use this option if you want
                        to include the scds results.
  -r SCRUBLET, --scrublet SCRUBLET
                        Path to scrublet results. Only use this option if you
                        want to include the scrublet results.
  -l SOLO, --solo SOLO  Path to solo results. Only use this option if you want
                        to include the solo results.
```

An example command that combines *Demuxlet* results, *Souporcell* results, *Solo* results and *Scds* results would look like this:

```
singularity exec Demuxafy.sif Combine_Results.R \
  -o $OUTDIR/combined_results.tsv \
```

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```
  --demuxlet $DEMUXLET_OUTDIR \
  --souporcell $DEMUXLET_OUTDIR \
  --solo $SOLO_OUTDIR \
  --scds $SCDS_OUTDIR \
```

Note

The path to the directories will work if the file names are the expected file names. However, if you used a different file naming convention or changed the names, you can also provide the full path to the exact file for each software.

21.3 Results and Interpretation

After running the `Combine_Results.R` script, you should have three (or two if you didn't have any demultiplexing softwares)

```
.
├── combined_results_demultiplexing_summary.tsv
├── combined_results_summary.tsv
└── combined_results.tsv
```

Note

You will only have the `combined_results_demultiplexing_summary.tsv` file if you included demultiplexing softwares.

21.4 Citation

If you used this workflow for analysis, please reference our paper (REFERENCE).

CHAPTER

TWENTYTWO

SUPPORT

If you're having trouble with any part of the Demultiplexing and Doublet Detecting Pipeline, feel free to submit an issue.