Analysing Single-Cell RNA-Seq with R

v2025-06

(Seurat v5)

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Babraham Bioinformatics

Major scRNA Package Systems

https://satijalab.org/seurat/

scater

Single-Cell Analysis Toolkit for Gene Expression Data in R

https://bioconductor.org/packages/release/bioc/html/scater.html

https://cole-trapnell-lab.github.io/monocle3/

https://scanpy.readthedocs.io/en/stable/

What do they provide?

- scRNA Data Structure
  - Data parsing
  - Quantitations
  - Metadata
- Processing methods
  - Normalisation
  - Integration
  - Dimensionality reduction
    - PCA / tSNE / UMAP
- Statistics
  - Enriched genes
  - Differential expression
- Plotting
  - Projections
  - QC
  - Expression graphs

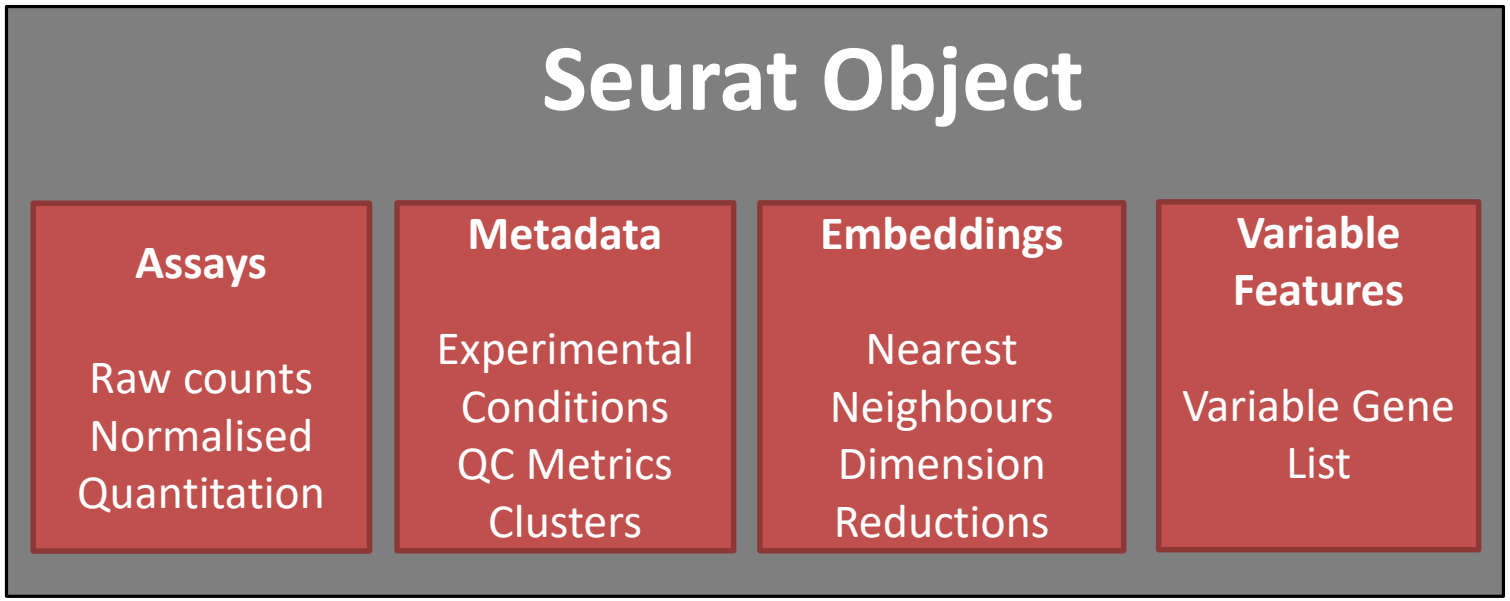
Seurat

- Most popular R framework
  - Well supported and frequently updated
- Good documentation
  - Reference documentation
  - Vignettes and examples
- Easy data model to work with
- Lots of built in functionality
  - Easy to extend to build your own

https://satijalab.org/seurat/

Seurat Data Structure

- Single object holds all data
  - Build from text table or 10X output (feature matrix h5 or raw matrix)



data	S4 [15969 x 5058] (Seurat::Seurat)
assays	list [1]
meta.data	list [5058 x 3] (S3: data.frame)
active.assay	character [1]
active.ident	factor
graphs	list [0]
neighbors	list [0]
reductions	list [0]
project.name	character [1]
misc	list [0]
version	list [1] (S3: package_version, num
commands	list [0]
tools	list [0]

Seurat Metadata

Cell.Barcode <chr>	orig.ident <fctr>	nCount_RNA <dbl>	nFeature_RNA <int>	percent.MT <dbl>	percent.Ribosomal <dbl>
AAACCTGAGAAACCAT-1	course	4410	1176	3.1519274	47.346939
AAACCTGAGATAGCAT-1	course	4470	1482	4.8098434	27.494407
AAACCTGAGCGTGAAC-1	course	2298	870	3.1331593	35.161010
AAACCTGCAACGCACC-1	course	4307	1433	4.2953332	26.259577
AAACCTGCACACCGAC-1	course	2001	669	2.6486757	42.528736
AAACCTGCATCGATTG-1	course	3310	918	3.7764350	48.791541
AAACCTGTCCAGATCA-1	course	3005	1032	2.9284526	38.735441
AAACGGGAGCGCTTAT-1	course	6932	1906	5.5972302	35.776111
AAACGGGAGCTACCTA-1	course	3714	1363	4.2003231	22.240172
AAACGGGAGTGATCGG-1	course	8020	2113	4.3266833	34.501247

- QC
- Conditions
- Clusters

`data[[]]` Access whole table

`data`**`$nCount_RNA`** Access one column

`new_data` **`-> data$new_metric`** Add a column

Seurat Quantitative Data

```
> LayerData(data, layer="counts")
```

Gene <chr>	AACTCAGAGATGTAAC-1 <dbl>	AACTCAGAGTCCAGGA-1 <dbl>	AACTCAGCAAGTAATG-1 <dbl>
AL627309.1	0	0	0
AL627309.5	0	0	0
LINC01409	0	0	0
LINC01128	0	1	0
LINC00115	0	0	0
FAM41C	0	0	0
NOC2L	1	0	0

```
> LayerData(data, layer="data")
```

Gene <chr>	AACTCTTAGCCGGTAA-1 <dbl>	AACTCTTCACTGTTAG-1 <dbl>	AACTCTTCATGCCTAA-1 <dbl>
AL627309.1	0.000000	0.000000	0
AL627309.5	0.000000	0.000000	0
LINC01409	0.000000	0.000000	0
LINC01128	0.000000	0.000000	0
LINC00115	0.000000	0.000000	0
FAM41C	0.000000	0.000000	0
NOC2L	1.228141	1.567888	0

Seurat Dimensionality Reductions

```
> Embeddings(data,reduction = "pca")
                    PC_1       PC_2         PC_3
GAGAAACCAT-1 -4.135099 -8.7585629  0.004920869
GAGATAGCAT-1 10.279728  0.8451562  0.287589575
GAGCGTGAAC-1 -6.002598  4.9504875 -3.022266598
GCAACGCACC-1 10.610838  0.4030928  0.165408128
GCATCGATTG-1 -5.581052 -0.4542359  5.166186308
```

```
> Loadings(data, reduction="pca")
                PC_1        PC_2         PC_3
IGLC3  -0.01326925 -0.07255036 -0.017863727
IGLC1  -0.01039493 -0.05451957 -0.008700478
IGLC2  -0.01569606 -0.08276587 -0.021715297
IGKC   -0.02226017 -0.12668741 -0.038651318
S100A9  0.11325462  0.01836318  0.013403732
```

```
> Embeddings(data,reduction = "tsne")
                        tSNE_1     tSNE_2
AAACCTGAGAGTGAGA-1  14.182877 -12.592458
AAACCTGAGCGAAGGG-1 -24.612876   5.739248
AAACCTGAGCGTCTAT-1  21.212219   4.717356
AAACCTGAGCTACCTA-1   5.228508  21.568443
AAACCTGAGCTCCCAG-1  18.923168   6.299075
```

Variable Gene Information

```
> HVFInfo(data)
```

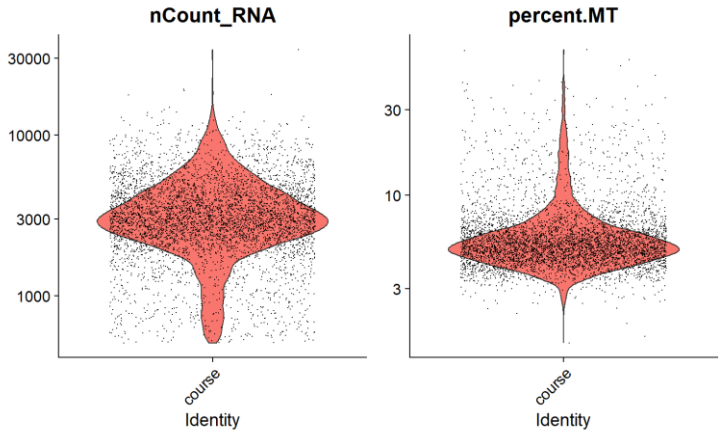
Gene <chr>	mean <dbl>	variance <dbl>	variance.expected <dbl>	variance.standardized <dbl>
AL627309.1	5.077431e-04	5.076141e-04	5.099960e-04	0.9953296
AL627309.5	2.792587e-03	2.785496e-03	2.907594e-03	0.9580070
LINC01409	2.564103e-02	2.498991e-02	2.679890e-02	0.9324974
LINC01128	1.624778e-02	1.801934e-02	1.712920e-02	1.0519658
LINC00115	5.077431e-03	5.560805e-03	5.344264e-03	1.0405185
FAM41C	9.647119e-03	9.556478e-03	1.016386e-02	0.9402406
NOC2L	1.381061e-01	1.342992e-01	1.519011e-01	0.8841229
KLHL17	2.792587e-03	2.785496e-03	2.907594e-03	0.9580070
PLEKHN1	4.061945e-03	4.554345e-03	4.262279e-03	1.0685233
HES4	1.345519e-02	1.987986e-02	1.419635e-02	1.4003494

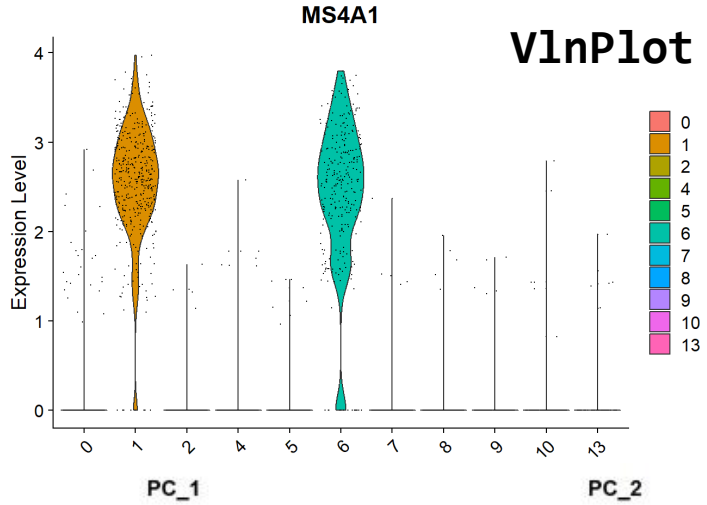
Seurat Methods

- Data Parsing
  - `Read10X`
  - `Read10X_h5*`
  - `CreateSeuratObject`
- Data Normalisation
  - `NormalizeData`
  - `ScaleData`
- Graphics
  - Violin Plot – metadata or expression (`VlnPlot`)
  - Feature plot (`FeatureScatter`)
  - Projection Plot (`DimPlot, DimHeatmap`)
- Dimension reduction
  - `RunPCA`
  - `RunTSNE`
  - `RunUMAP`
- Statistics
  - Select Variable Genes `FindVariableFeatures`
  - Build nearest neighbour graph `FindNeighbors`
  - Build graph based cell clusters `FindClusters`
  - Find genes to classify clusters (multiple tests) `FindMarkers`

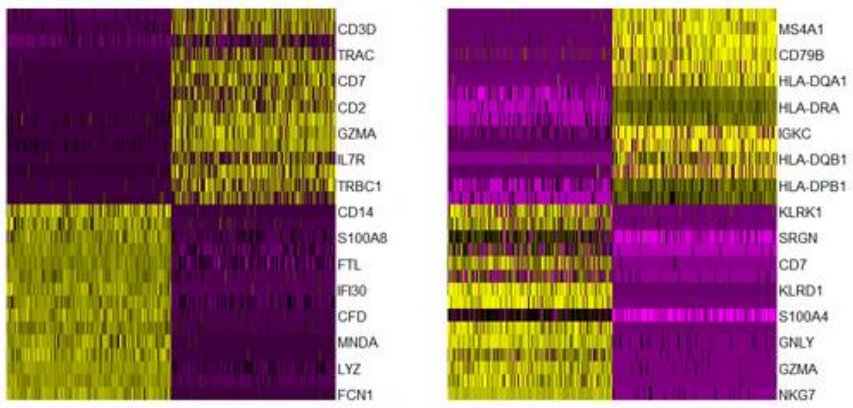
*Requires installing the `hdf5r` package

Seurat Graphs

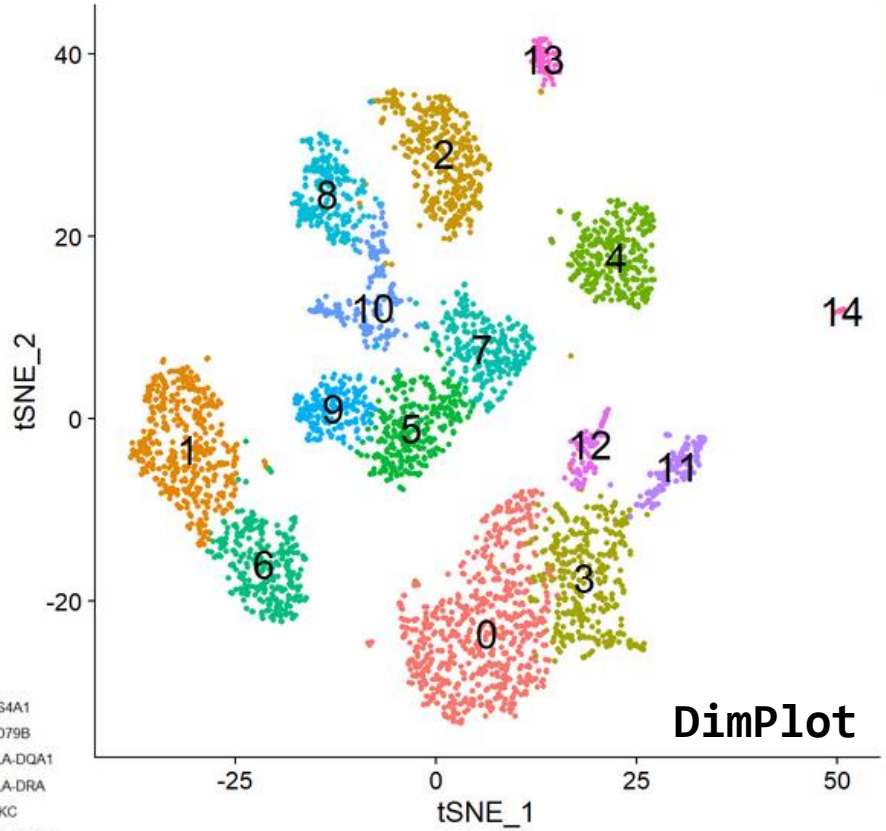




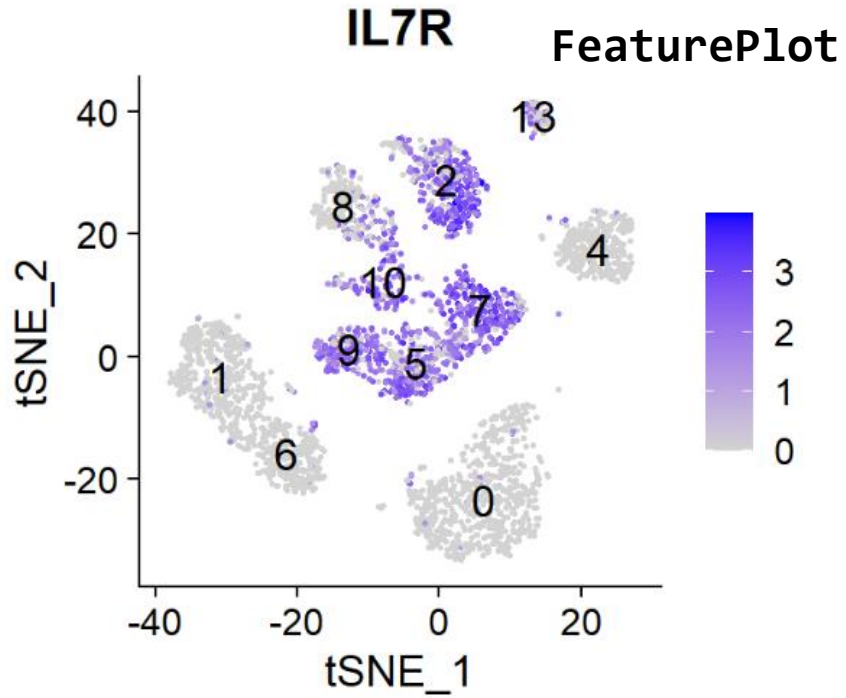
VlnPlot



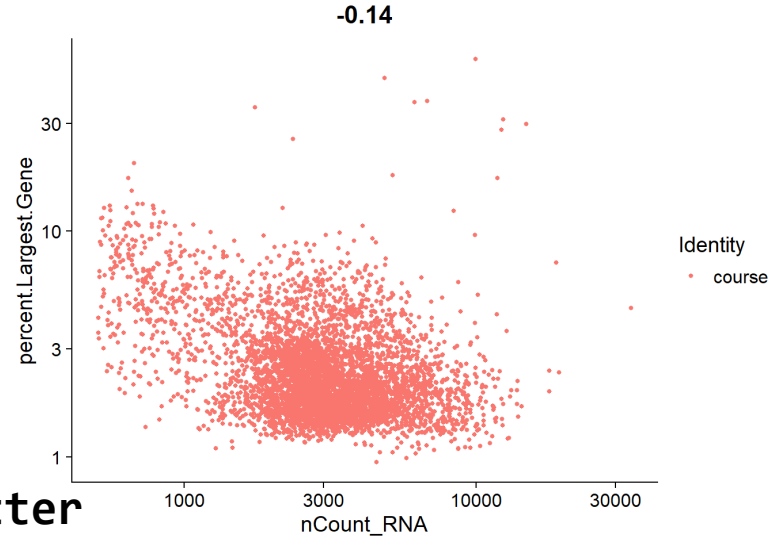
DimHeatmap



DimPlot



FeaturePlot



FeatureScatter

Example 10X Seurat Workflow

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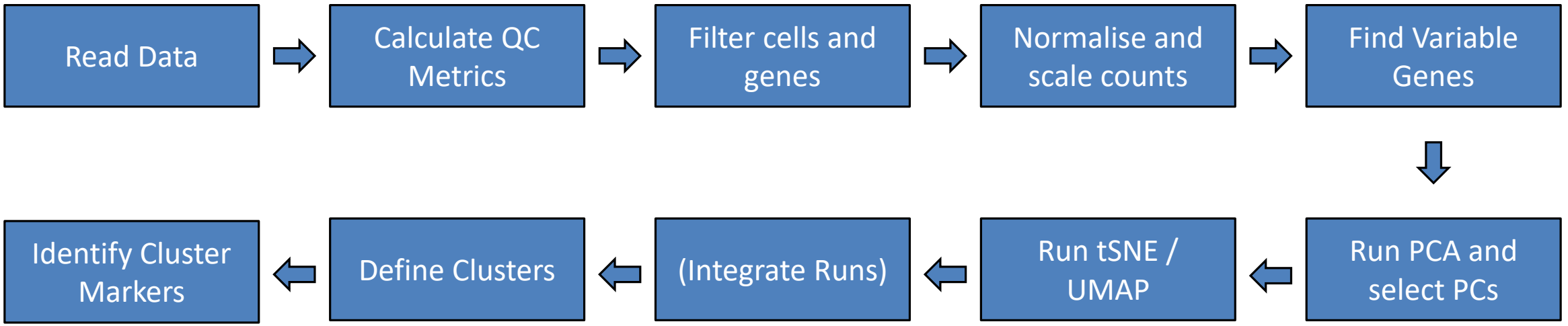
Example Seurat Workflow



Reading Data

```
Read10X_h5("filtered_feature_bc_matrix.h5") -> data

CreateSeuratObject(
      counts=data,
      project="course",
) -> data
```

```
> data
An object of class Seurat
17136 features across 3939 samples within 1 assay
Active assay: RNA (17136 features, 500 variable
features)
 3 layers present: counts, data, scale.data
 2 dimensional reductions calculated: pca, tsne
```

Reading Multiple Files

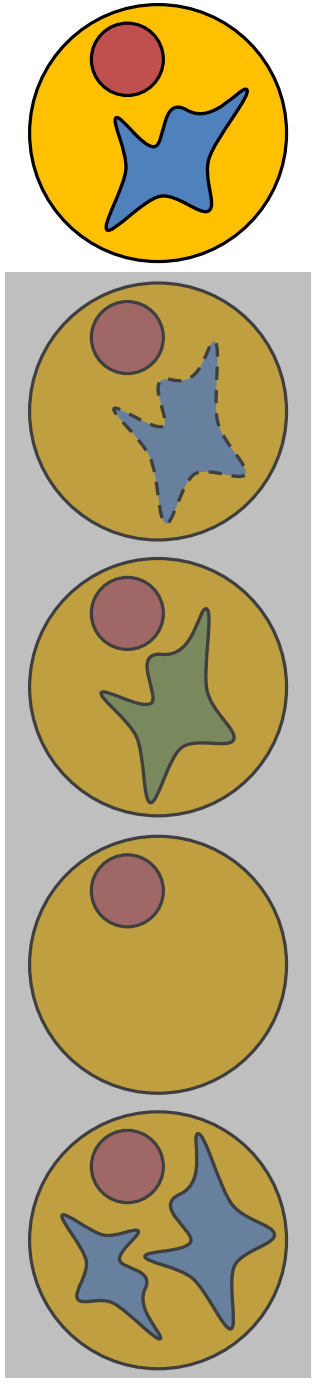
```
CreateSeuratObject(Read10X_h5("SAMPLE1.h5"),project="sample1") -> sample1
CreateSeuratObject(Read10X_h5("SAMPLE2.h5"),project="sample2") -> sample2
CreateSeuratObject(Read10X_h5("SAMPLE3.h5"),project="sample3") -> sample3

merge(
    sample1,
    c(sample2,sample3),
    add.cell.ids=c("sample1","sample2","sample3")
) -> data
```

	orig.ident <chr>	nCount_RNA <dbl>	nFeature_RNA <int>
sample1_AAACCTGAGCTGTTCA-1	sample1	2216	1089
sample1_AAACCTGCAACACGCC-1	sample1	9103	2881
sample1_AAACCTGCATGCAACT-1	sample1	3927	1459
sample1_AAACCTGGTGGTCTCG-1	sample1	6434	2251
sample1_AAACCTGTCATCGATG-1	sample1	2623	1318
sample1_AAACGGGCATCCTTGC-1	sample1	4903	1948
sample1_AAACGGGGTAGAGTGC-1	sample1	2727	934
sample1_AAACGGGGTTGAACTC-1			
sample1_AAAGATGTCACAGGCC-1			
sample1_AAAGCAAAGCGATCCC-1			

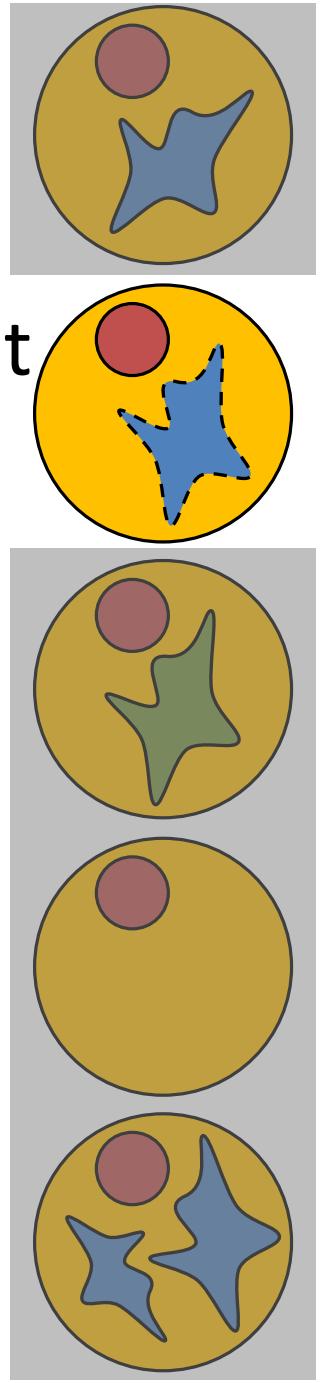
```
An object of class Seurat
33696 features across 6302 samples within 1 assay
Active assay: RNA (33696 features, 0 variable features)
 3 layers present: counts.sample1, counts.sample2, counts.sample3
```

QC – What problems are likely?



- Lysed cells
- Dead or dying cells
- Empty GEMs
- Double (or more) occupied GEMs
- Cells in different cell cycle stages

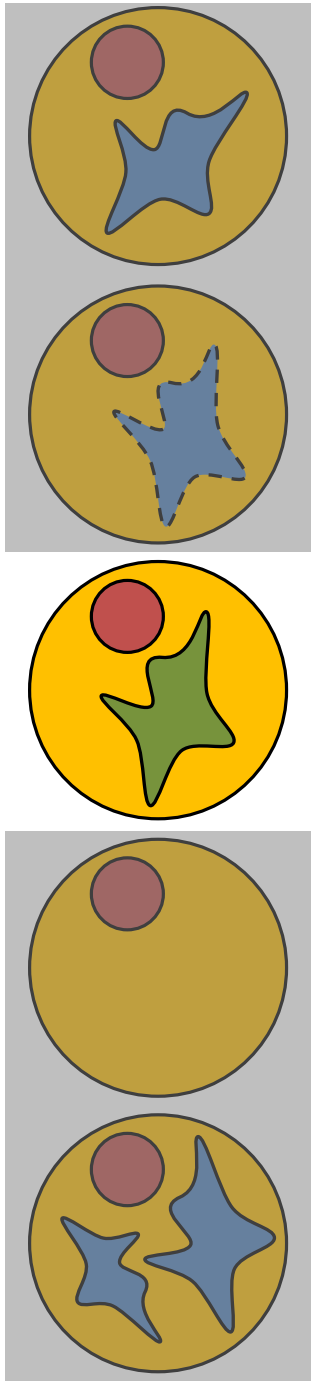
Lysed Cells



- Outer membrane is ruptured – cytoplasmic RNAs leak out
  - Loss of mature RNA, increase in pre-mRNA
  - Lower overall counts/features
  - Increase in nuclear RNAs
    - MALAT1 is an easy marker to use

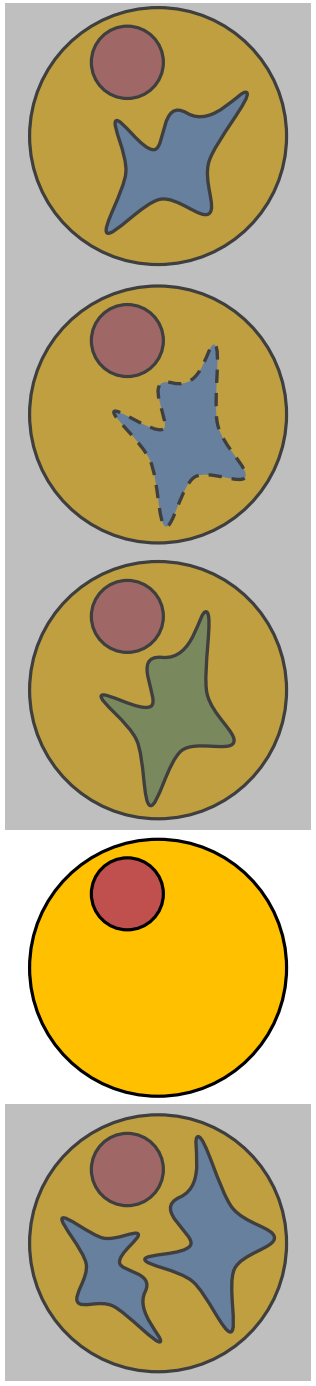
Dead or Dying Cells

- Cells undergoing apoptosis have very different transcriptomes
  - Lower total RNA production
  - Huge upregulation of mitochondrial transcription



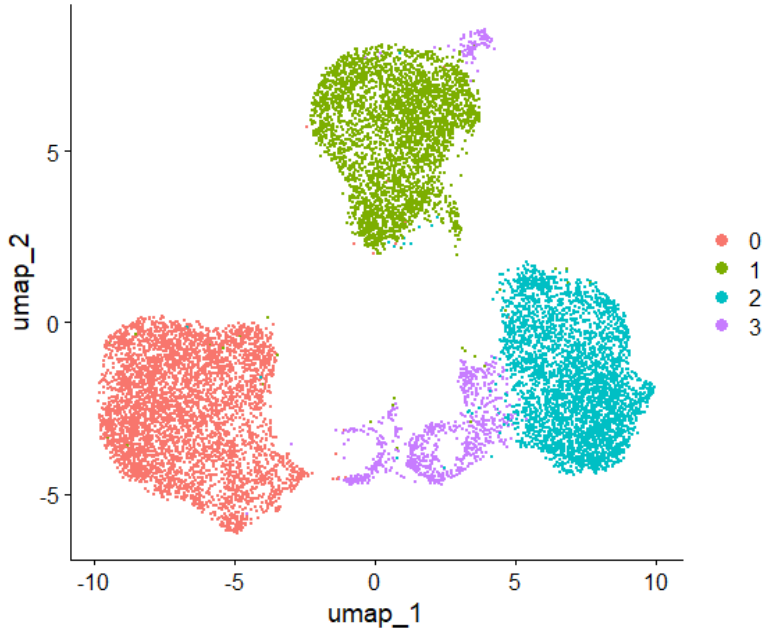
Empty GEMs

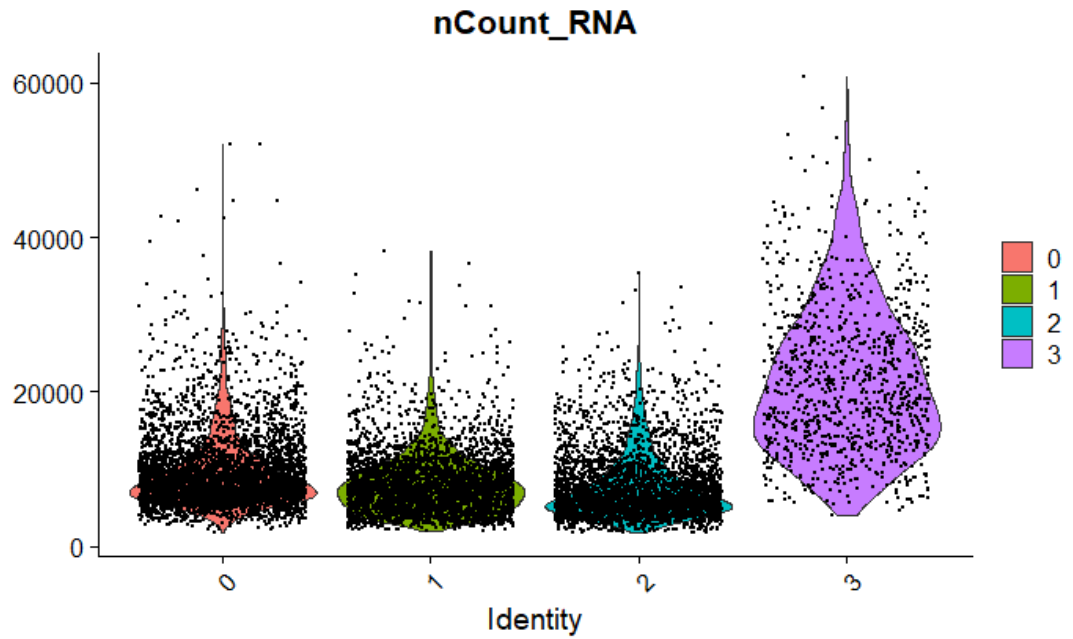
- GEMs containing no cell will still produce some sequence
  - Background RNA in the flow medium
  - Will be worse with higher numbers of lysed cells
- Total amount of signal will be greatly reduced
- Will often cluster together

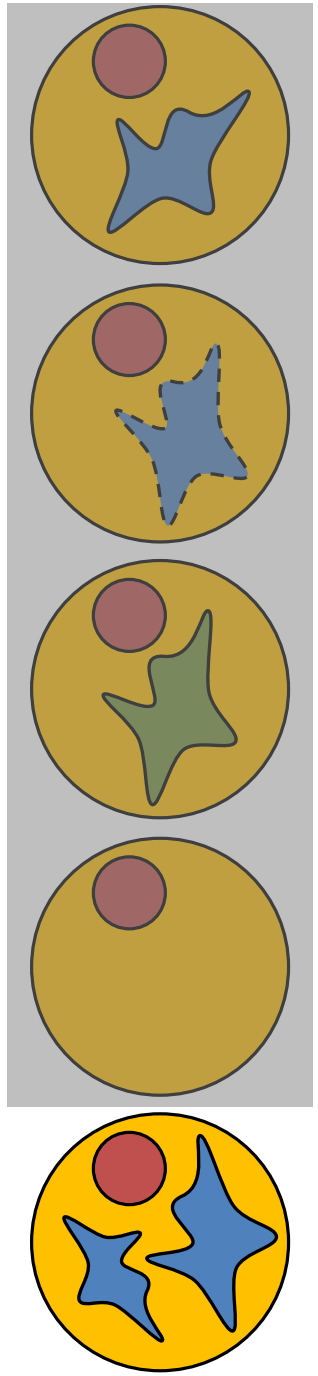


Double occupied GEMs

- Will get a mixed signal from two different cells
- Not as obvious a signal as empty GEMs
  - More UMIs/Features per cell
  - Intermediate clustering

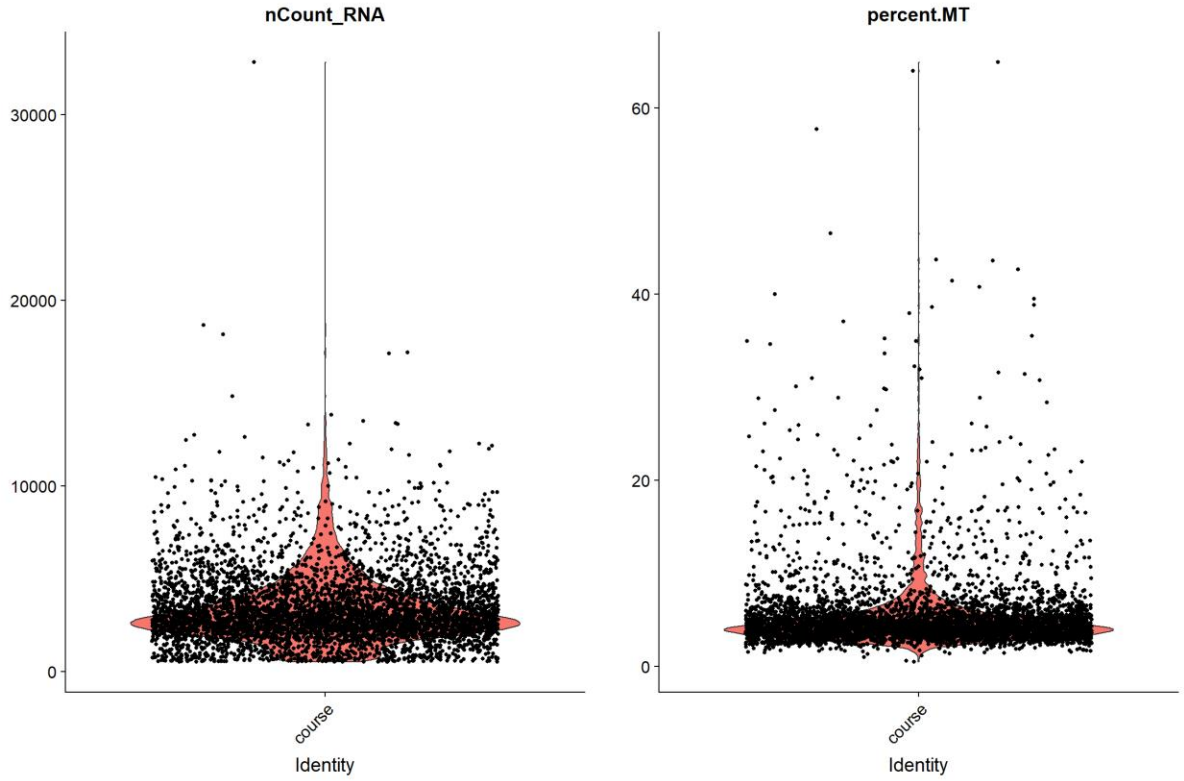






QC and Cell Filtering

- Standard QC Measures
  - Number of observed genes per cell
  - Number of reads per cell
  - Relationship between the two
- Calculated QC Measures
  - Amount of mitochondrial reads
  - Amount of ribosomal reads
  - Marker genes (eg MALAT1)
  - Cell cycle



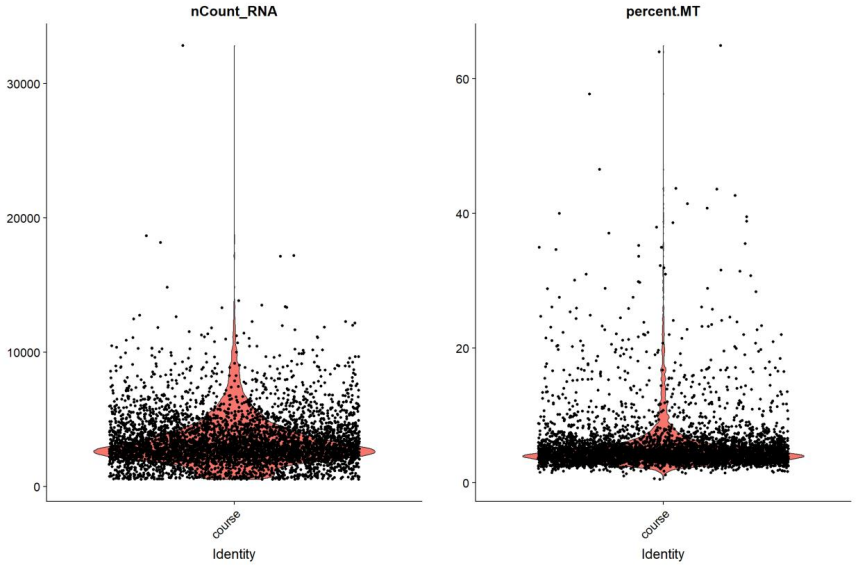
Adding QC Metrics

```
PercentageFeatureSet(
    data,
    pattern="^MT-“
) -> data$percent.MT
```

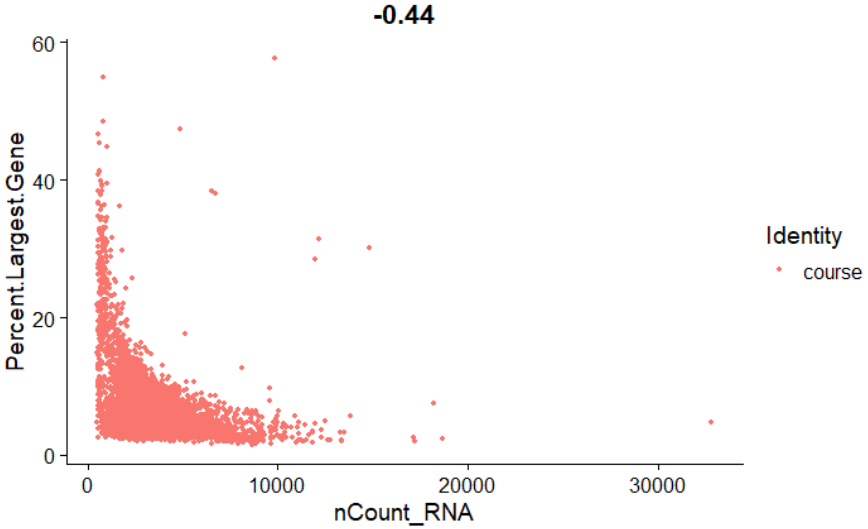
- Mitochondrial
- Immunoglobulin
- Malat1
- Ribosomal

QC and Cell Filtering

```
VlnPlot(
        data,
        features=c("nCount_RNA","percent.MT"),
        log=TRUE
)
```




```
FeatureScatter(
        data,
        feature1 = "nCount_RNA",
        feature2 = "Percent.Largest.Gene“
)
```



Applying Filters

```
subset(
  data,
  nFeature_RNA>750 &
  nFeature_RNA < 2000 &
  percent.MT < 10
) -> data
```

Count Normalisation and Scaling

- Raw counts are biased by total reads per cell
- Counts are more stable on a log scale
- Standard normalisation is just log reads per 10,000 reads

- For PCA counts scale each gene's expression to a z-score
  - Can also use this step to try to regress out unwanted effects

Count Normalisation and Scaling

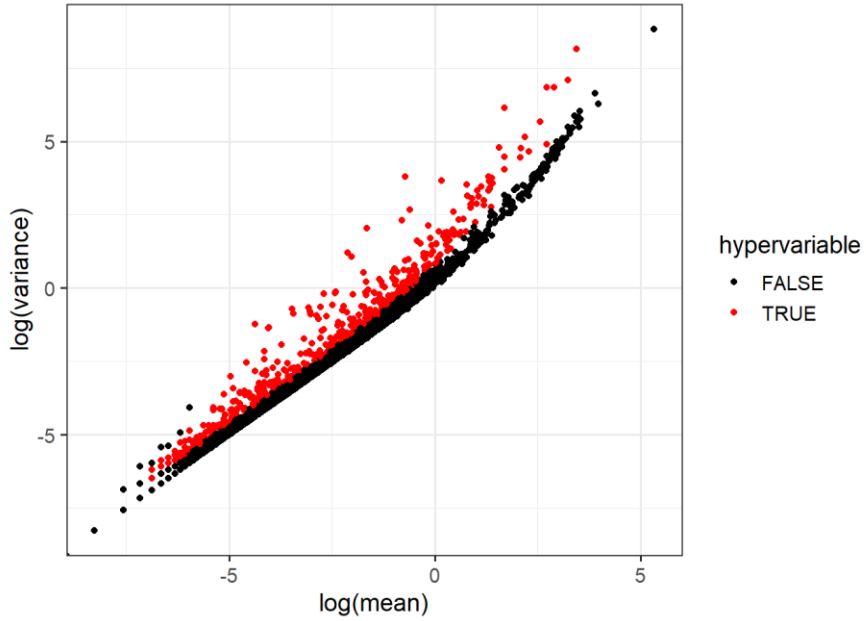
```
NormalizeData(
     data,
     normalization.method = "lognormalize"
) -> data


ScaleData(data) -> data
```

Variable Feature Selection

- Selects a subset of genes to use for downstream analysis
- Identify genes with an unusual amount of variability
- Link the variability with the expression level to find variation which is high in the context of the expression level
- Keep only the most variable genes

```
FindVariableFeatures(
  data,
  selection.method = "vst",
  nfeatures=500
) -> data
```



Variable Features for Multiple Samples

- Can just find variable features across all cells

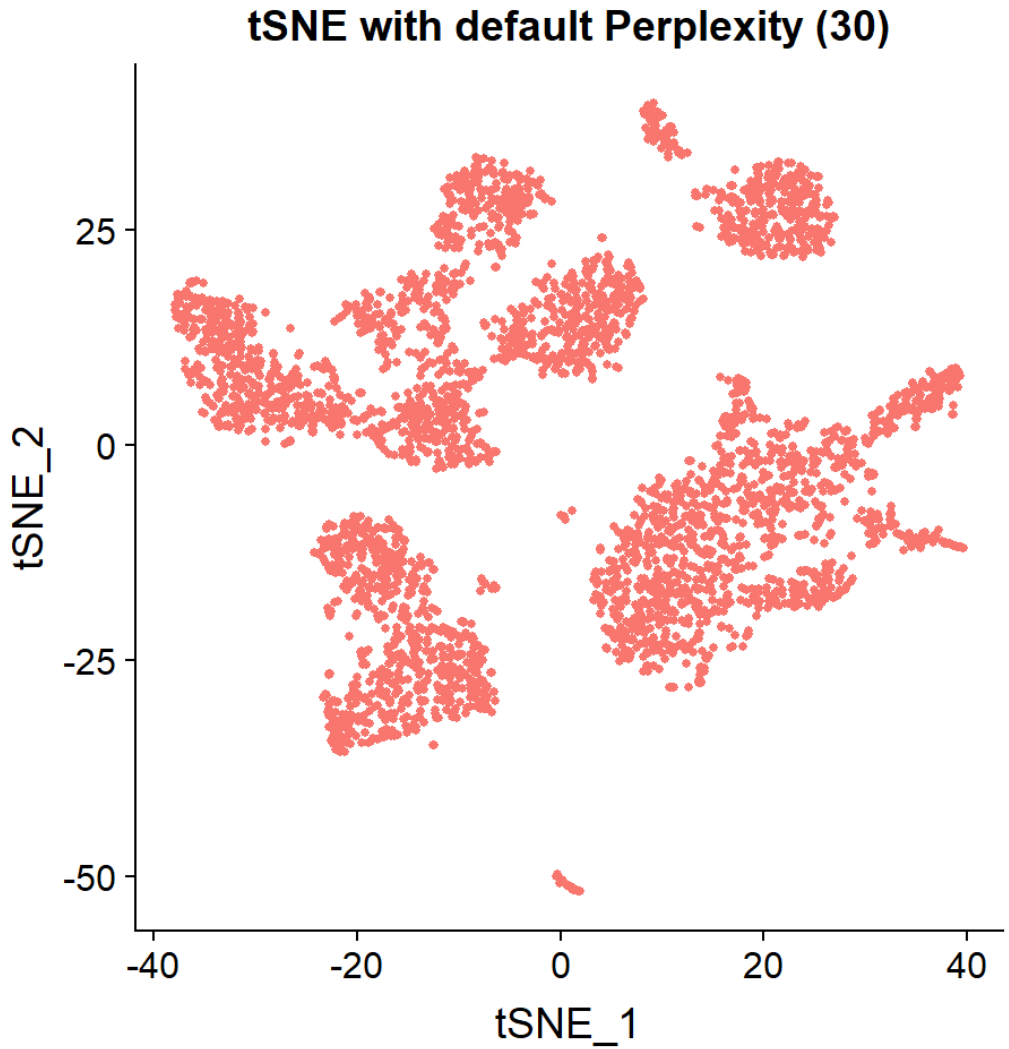
```
lapply(unique(data$orig.ident), function(x) {
    data |>
    subset(orig.ident==x) |>
    FindVariableFeatures() |>
    HVFInfo() |>
    as_tibble(rownames="Gene") |>
    add_column(orig.ident=x)}) -> variability_data

do.call(bind_rows,variability_data) -> variability_data
```

- Compare Variance
- Select Common Variable Features

Dimensionality Reduction

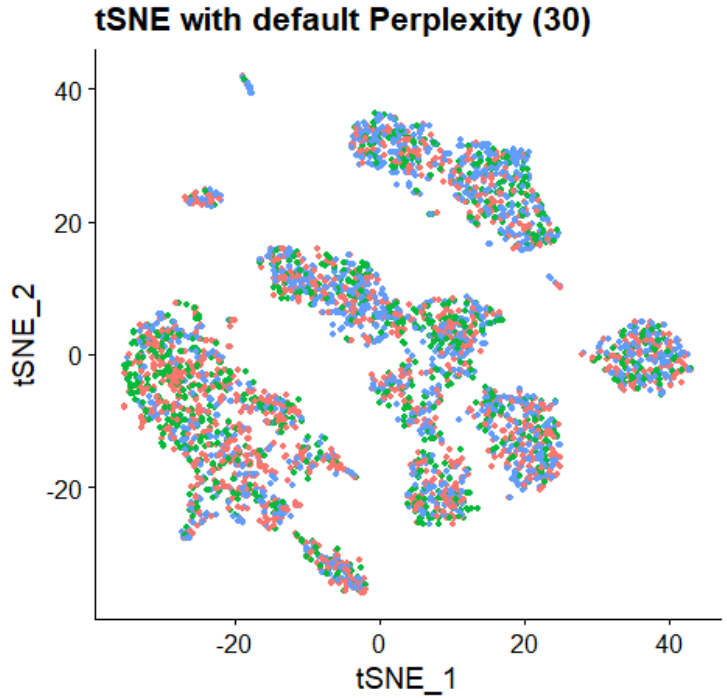
- Start with PCA on the normalised, filtered (both cells and genes), scaled data
- Scree / Elbow plot to decide how many PCs are informative
- Pass only the interesting PCs to subsequent tSNE or UMAP reduction to get down to 2 dimensions

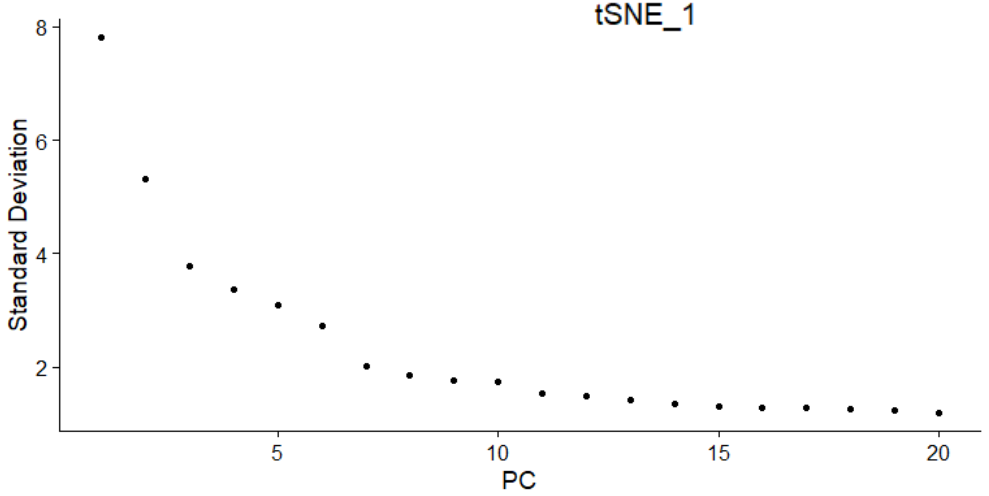


Dimensionality Reduction

```
RunPCA(
      data,
      features=VariableFeatures(data)
) -> data
```

```
RunTSNE(
   data,
   dims=1:15,
   seed.use = saved.seed,
   perplexity=30
) -> data
```





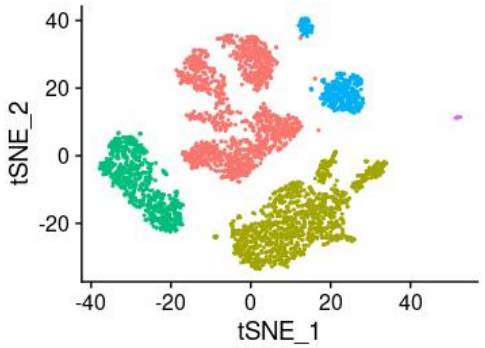
Defining clusters

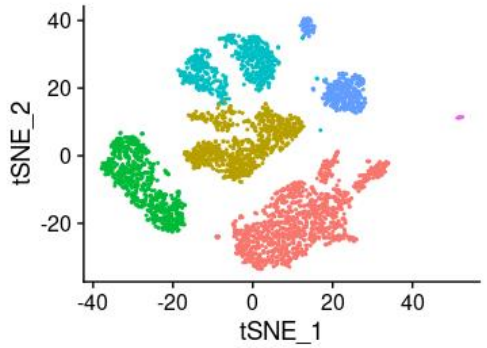
- Construct nearest neighbour graph
  - Constructed from PCA
  - Same dimensions as tSNE/UMAP

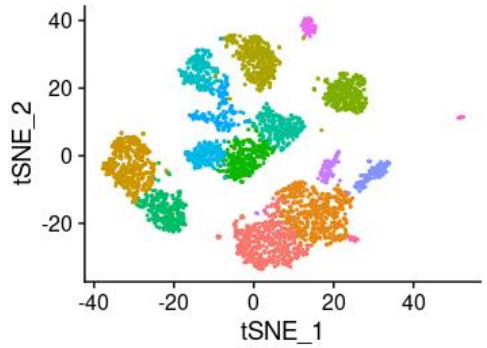
```
FindNeighbors(
      data,
      dims=1:15
) -> data
```

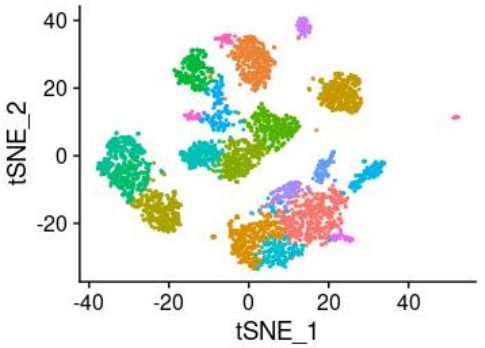
- Find clusters
  - All cells are classified
  - Graph Based (Louvain) Clustering
  - Resolution (0.01 - 5) defines granularity

```
FindClusters(
      data,
      resolution = 0.5
) -> data
```

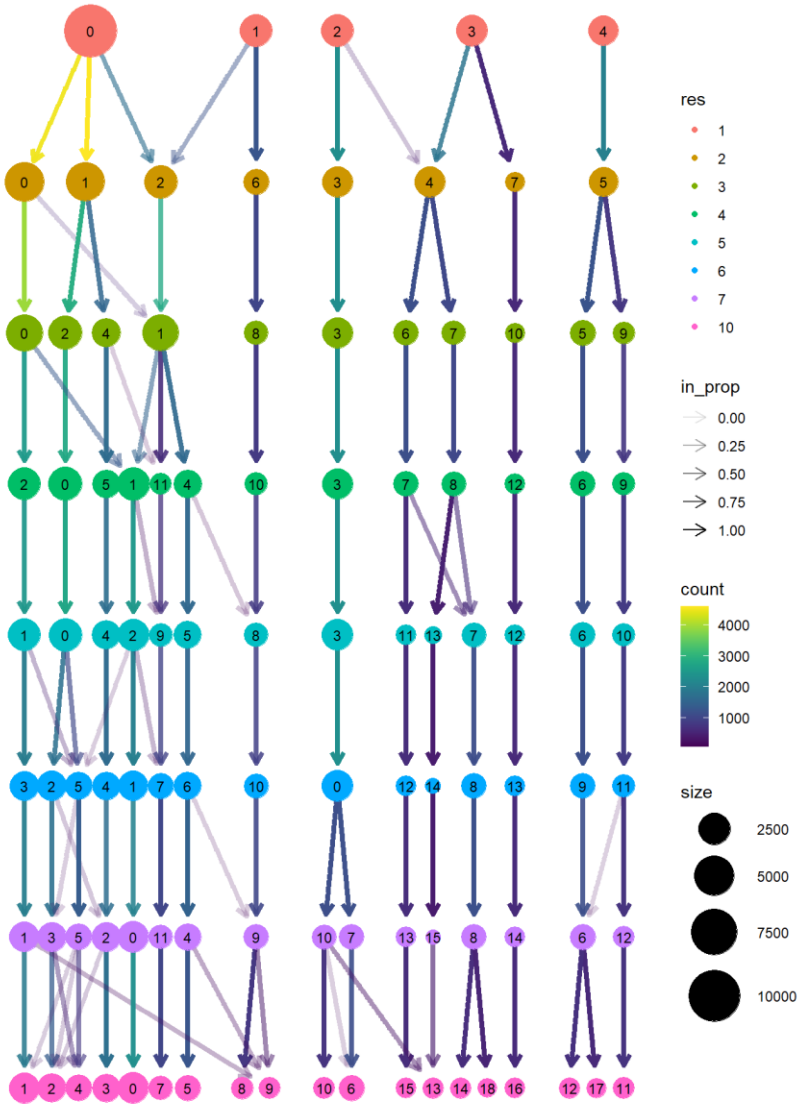


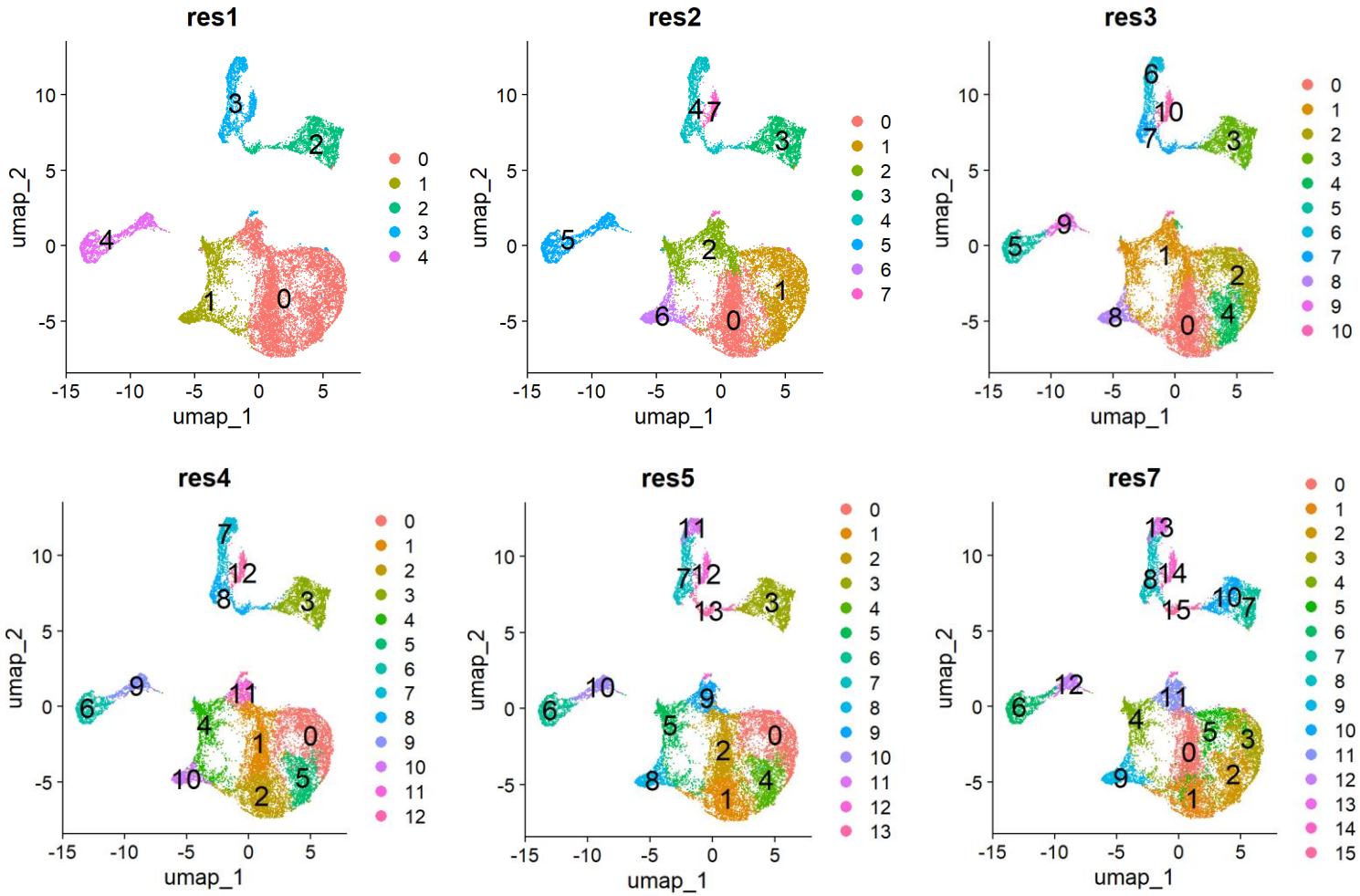






Clustree to see effect of resolution



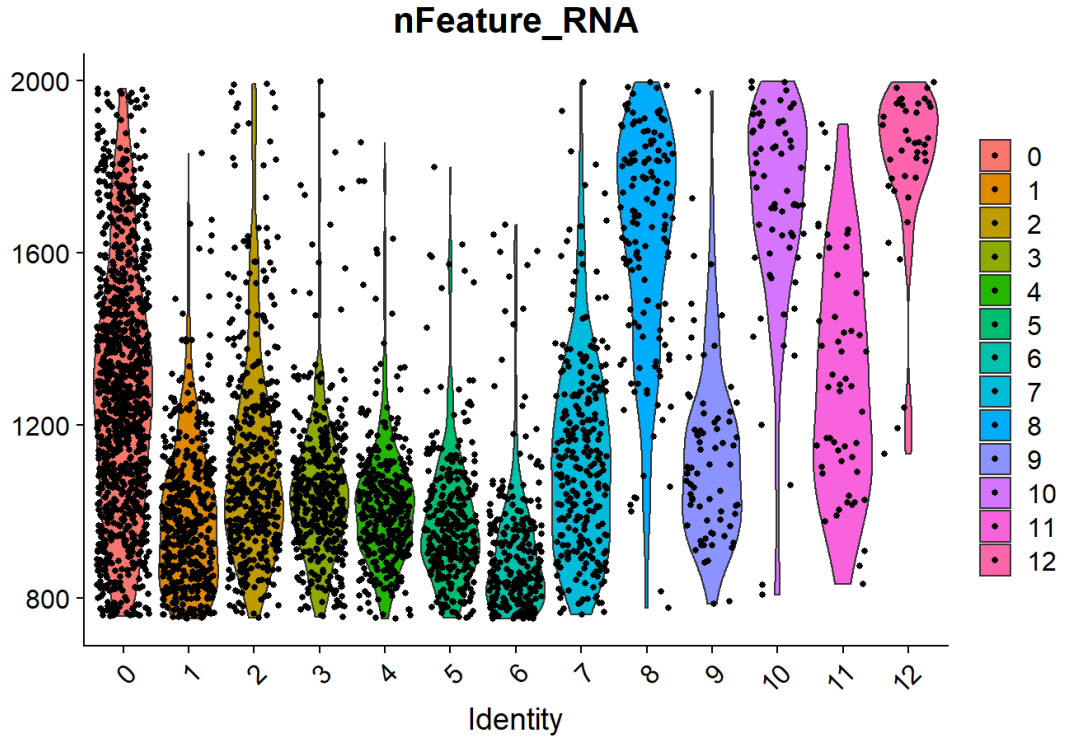


https://github.com/lazappi/clustree

Comparing Properties of Clusters

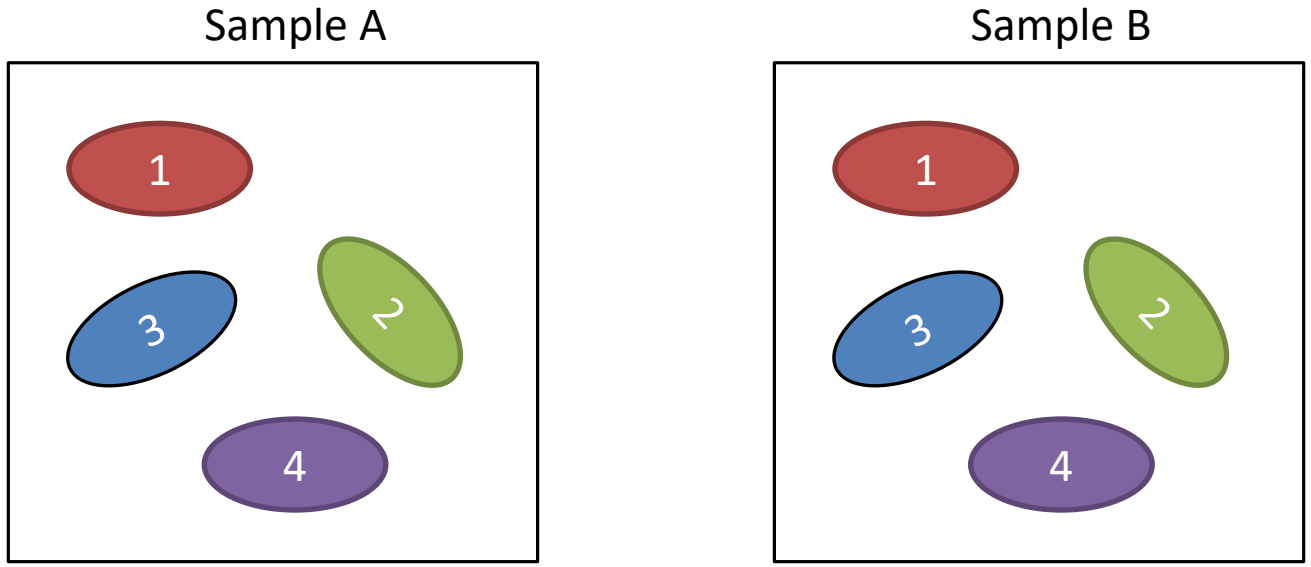
```
VlnPlot(data,features="nFeature_RNA")
```

- We want to know that clusters are occurring because of biological changes, not technical differences
- We plot QC metrics for clusters
  - Read/Gene counts
  - Mitochondrion
  - MALAT1
- Can remove suspect clusters




```
subset(data, !Seurat_clusters %in% c(8,10,12)) -> data
```

Statistical Analysis



- Cluster 1 vs Clusters [2,3,4]
- Cluster 1 vs Cluster 3

- Cluster A1 vs Cluster B1

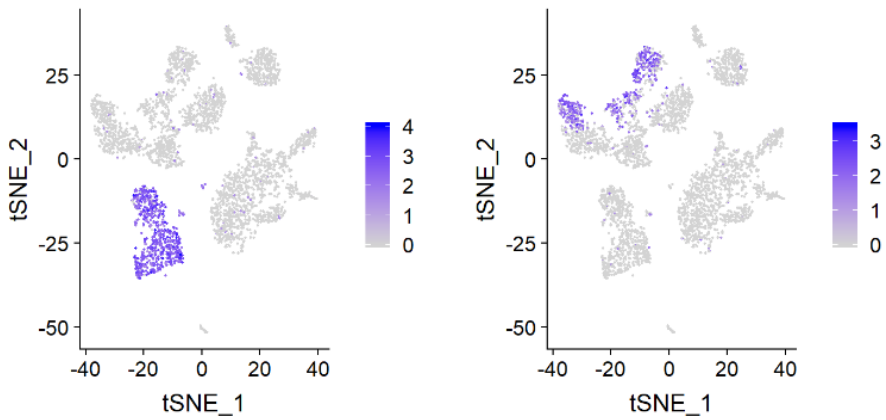
Types of Statistics

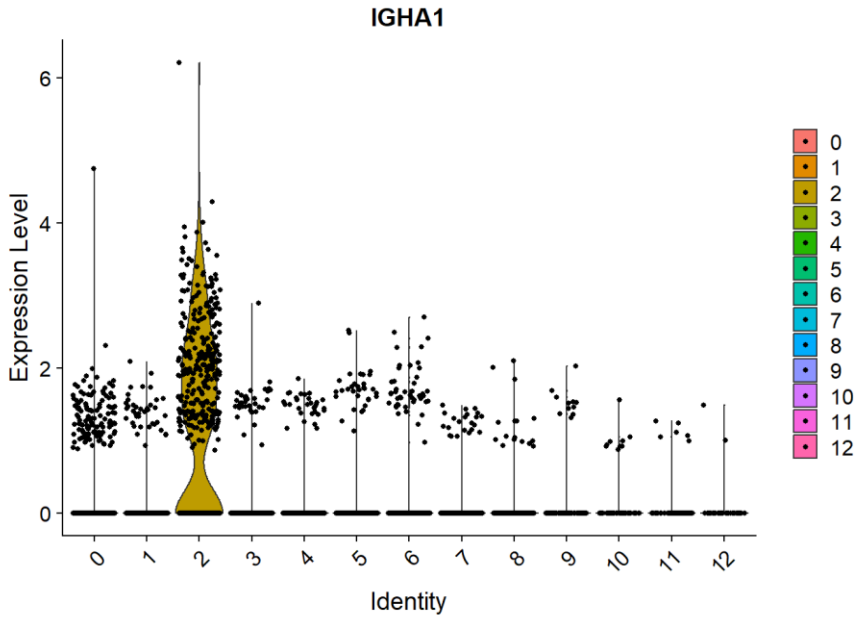
- Non Parametric Stats
  - Default test
  - Wilcoxon Rank Sum
  - Semi Quantitative
  - Each Cell is a replicate
  - Highly Powered
  - Over powered?
- ROC Analysis
  - Not a stats test
  - How well can each gene separate the groups
  - Values 0 – 1
  - 0.5 is worst
  - 0 or 1 are perfect separation
- DESeq Stats
  - Only with replicates
  - Aggregate counts per cluster and sample
  - Standard RNA-Seq analysis
  - Works well if your data supports it

Statistical analysis of differences between clusters

```
FindMarkers(
      data,
      ident.1 = 2,
      ident.2 = 6,
      test.use = "roc",
      only.pos = TRUE
)
```

```
FindAllMarkers(
      data,
      group.by ="seurat_clusters",
      test.use = "roc",
      only.pos = TRUE
)
```





Wang *et al. BMC Bioinformatics* (2019) 20:40
https://doi.org/10.1186/s12859-019-2599-6

BMC Bioinformatics

RESEARCH ARTICLE Open Access

Comparative analysis of differential gene expression analysis tools for single-cell RNA sequencing data

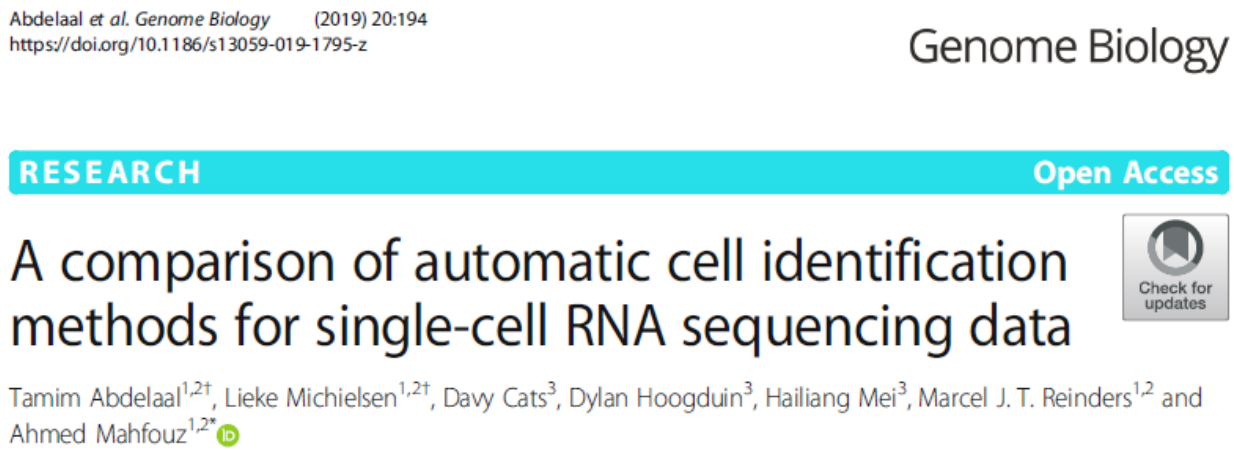
Tianyu Wang[1], Boyang Li[2], Craig E. Nelson[3] and Sheida Nabavi[4*]

Conclusions: In general, agreement among the tools in calling DE genes is not high. There is a trade-off between true-positive rates and the precision of calling DE genes. Methods with higher true positive rates tend to show low precision due to their introducing false positives, whereas methods with high precision show low true positive rates due to identifying few DE genes. We observed that current methods designed for scRNAseq data do not tend to show better performance compared to methods designed for bulk RNAseq

Automated Cell Assignment

- Can automatically assign cell identities to clusters
- Need a source of marker genes
  - Result of a previous run/experiment
  - Publicly available data (https://azimuth.hubmapconsortium.org/)

- Many packages to do this
  - SCINA has worked well for us
  - Azimuth built into Seurat


Abdelaal *et al. Genome Biology* (2019) 20:194
https://doi.org/10.1186/s13059-019-1795-z

Genome Biology

RESEARCH Open Access

A comparison of automatic cell identification methods for single-cell RNA sequencing data

Tamim Abdelaal[1,2†], Lieke Michielsen[1,2†], Davy Cats[3], Dylan Hoogduin[3], Hailiang Mei[3], Marcel J. T. Reinders[1,2] and Ahmed Mahfouz[1,2*]

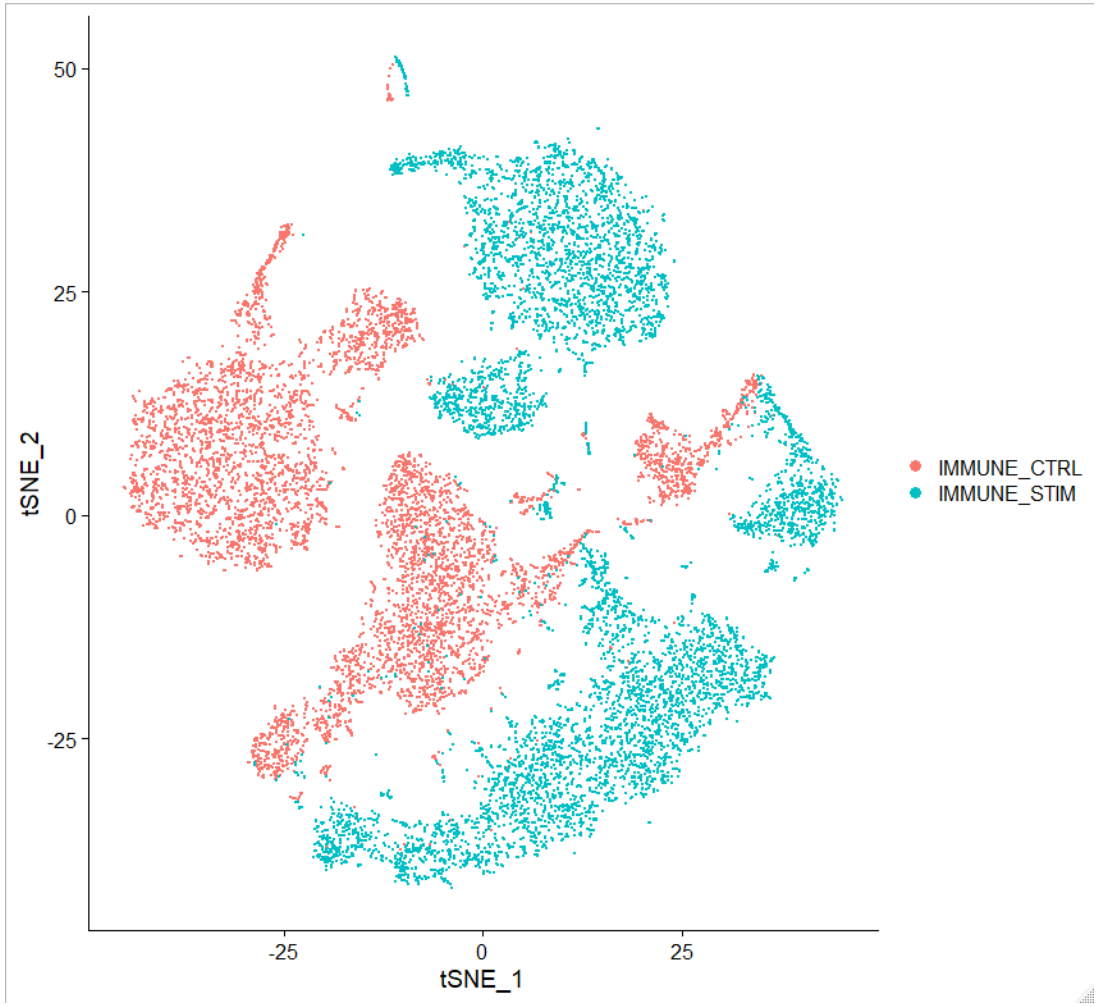
Integrating Multiple Runs

- When multiple runs are combined (eg Unstim and Stim), the batch differences between the runs can overwhelm the biological differences

- Raw comparisons can therefore miss changes between what are actually matched subgroups

Raw merged runs

- Two PBMC populations run at different times
- tSNE spread coloured by library
- Little to no overlap between cell populations



Integrating Runs

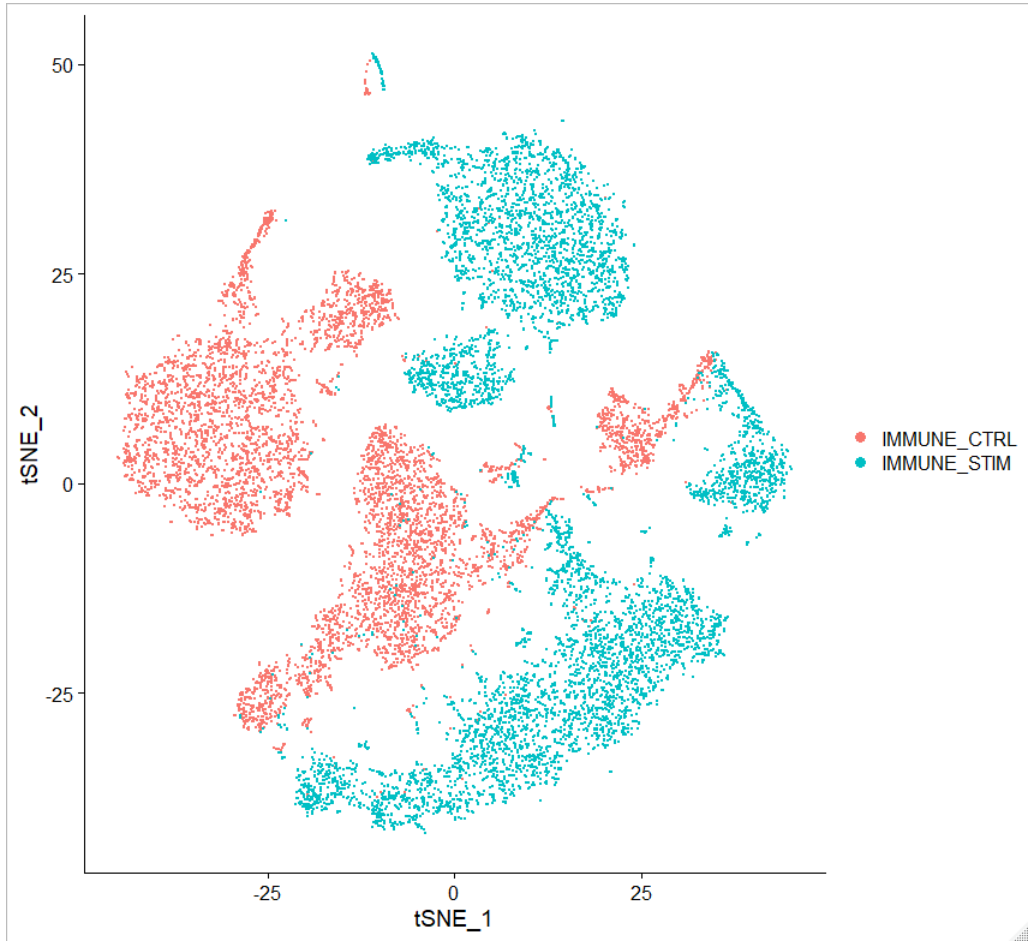
- Split the layers based on the metadata

```
split(data[["RNA"]], f = data$Batch) -> data[["RNA"]]
```

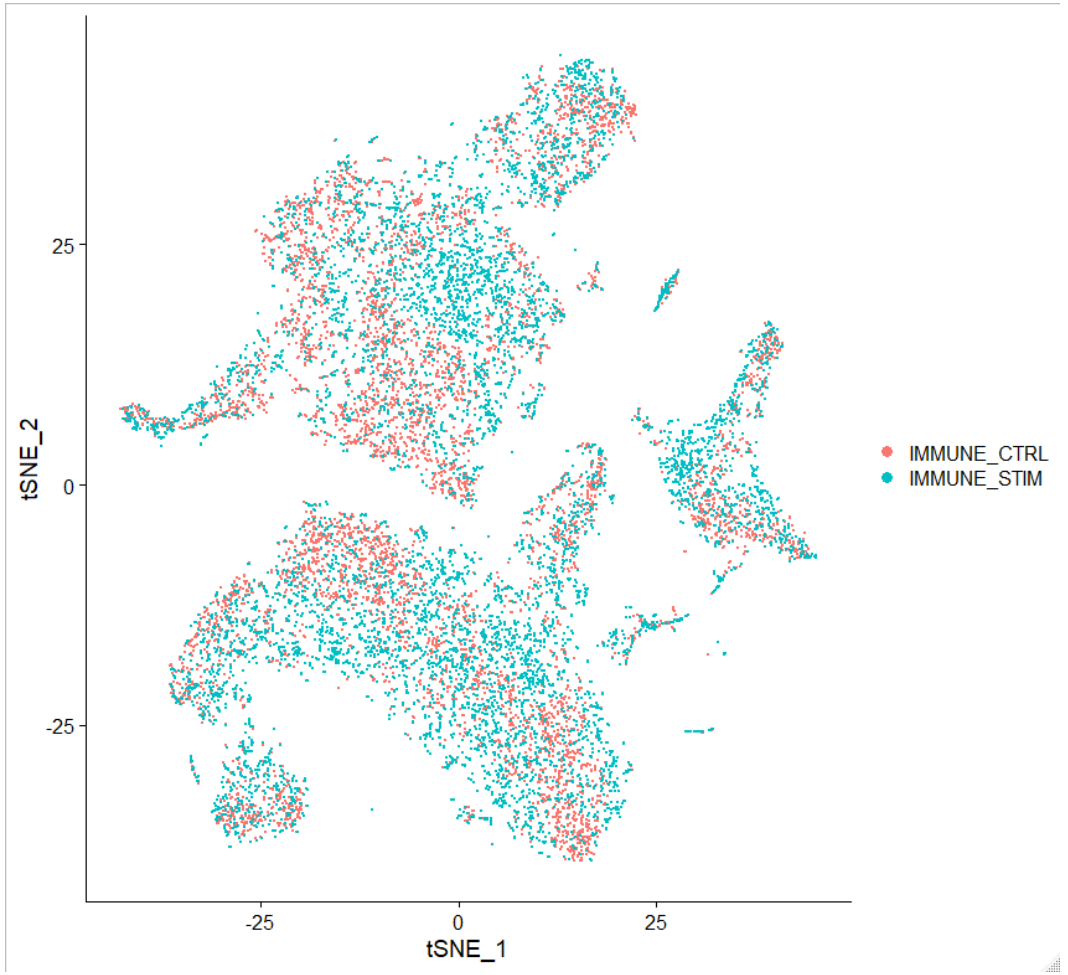
- Rerun Normalisation, Variable Features, Scaling, PCA
- Create a new integrated layer

```
IntegrateLayers(
  object = data, method = RPCAIntegration,
  orig.reduction = "pca", new.reduction ="integrated.rpca",
  verbose = FALSE
) -> data
```

Integrating Runs

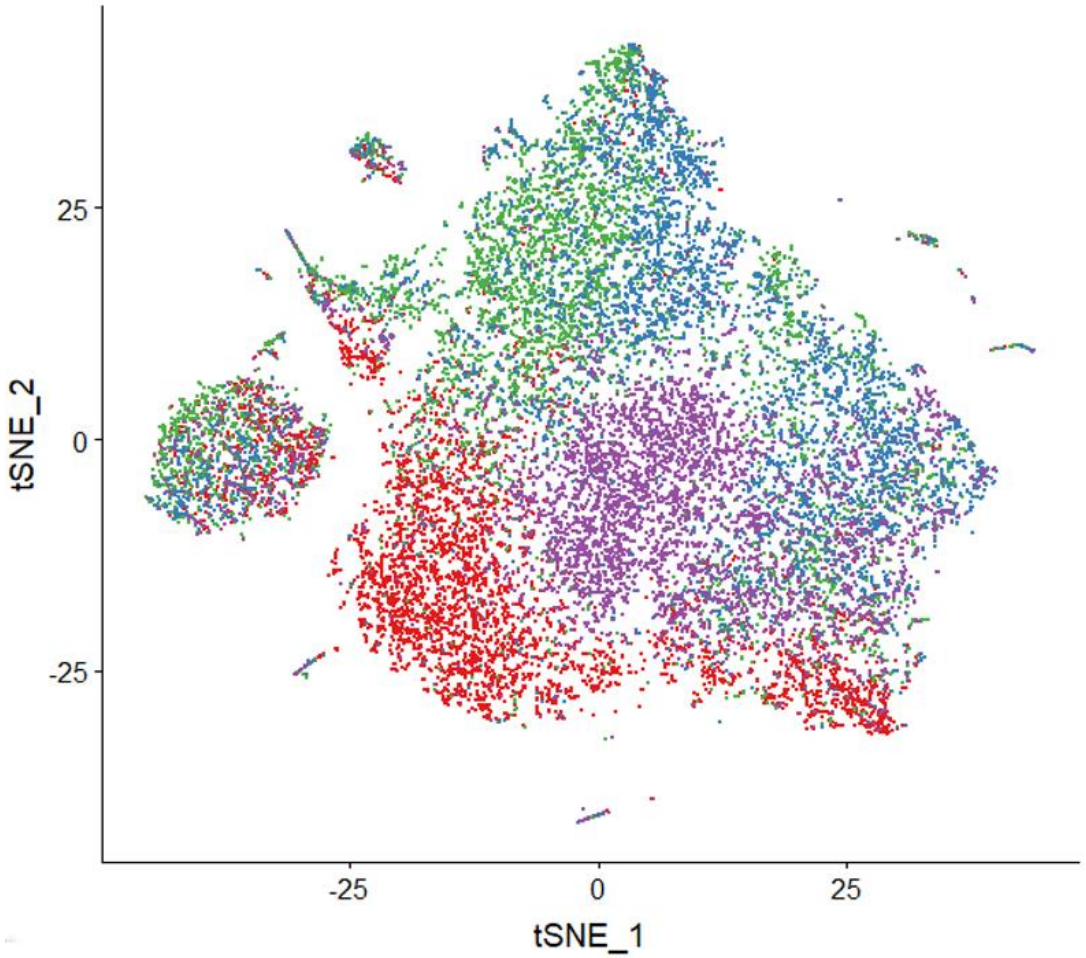


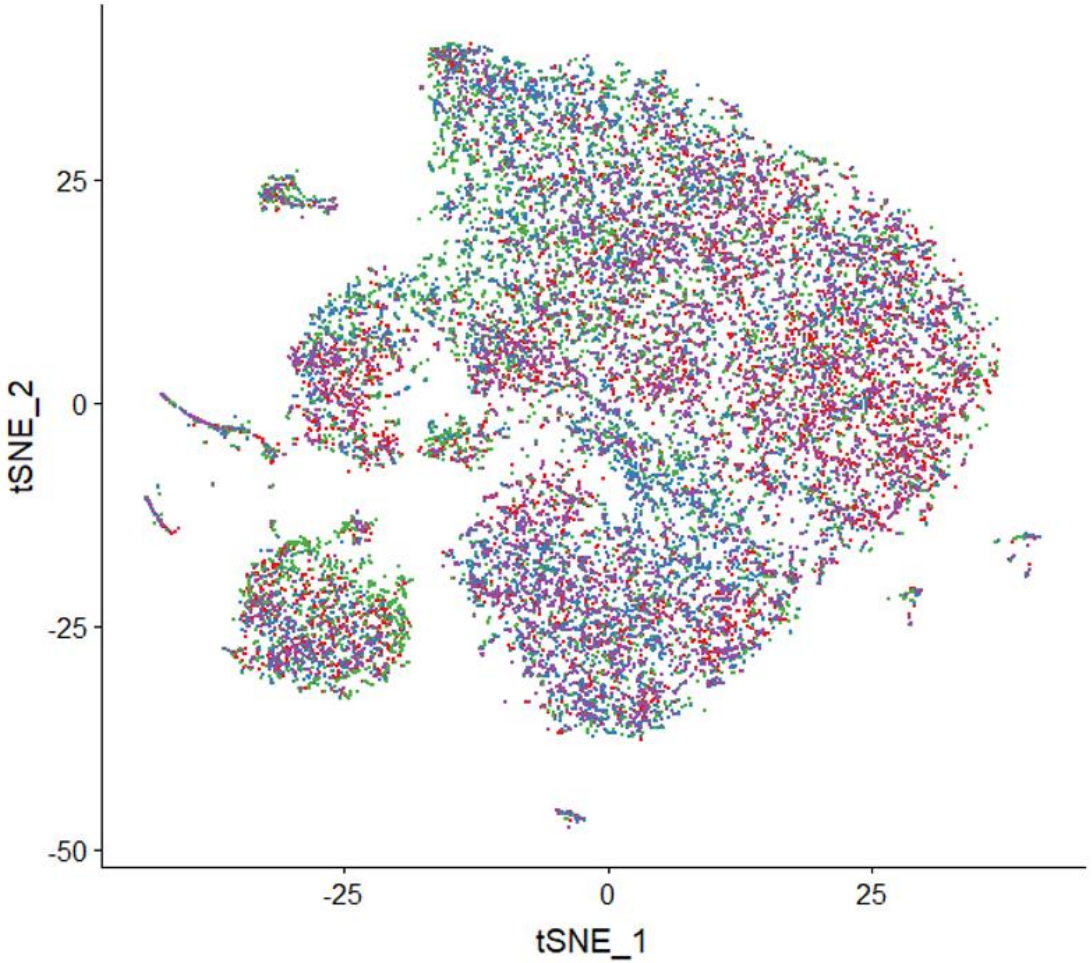
Raw



Anchored

Over-Integration





Exercise – Using Seurat to analyse 10X data