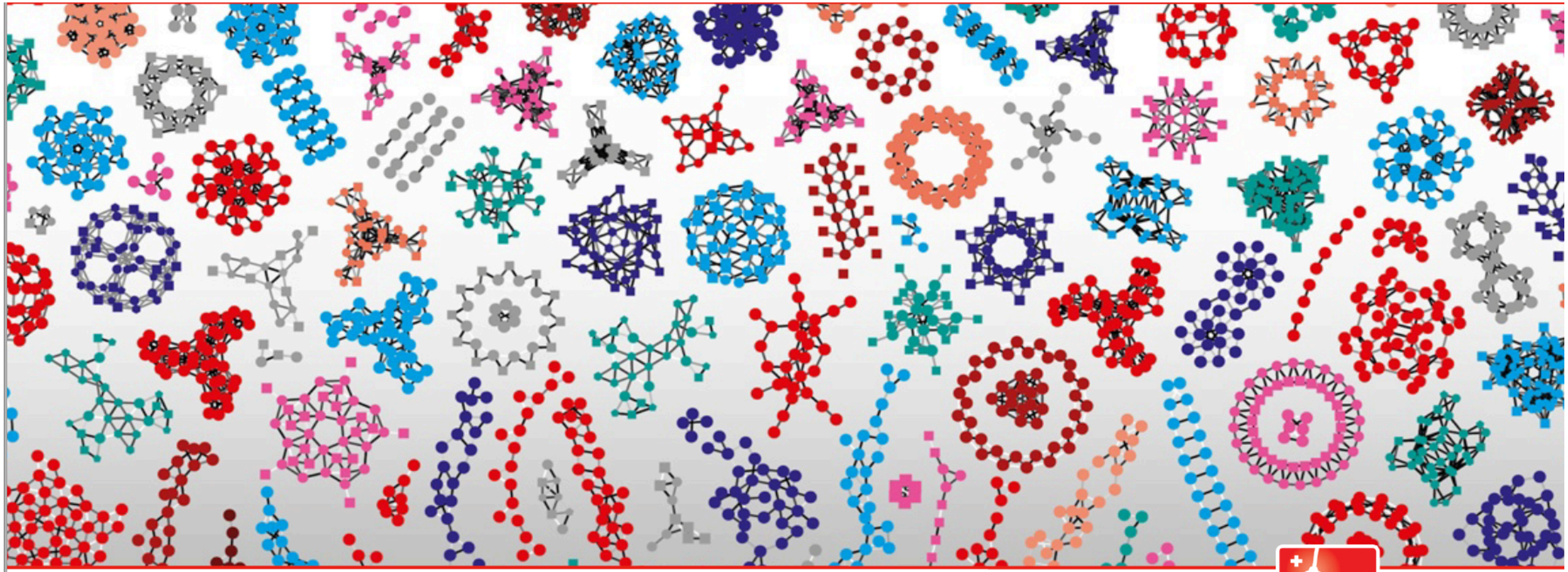


Swiss Institute of Bioinformatics

Single cell transcriptomics data analysis

Day 3

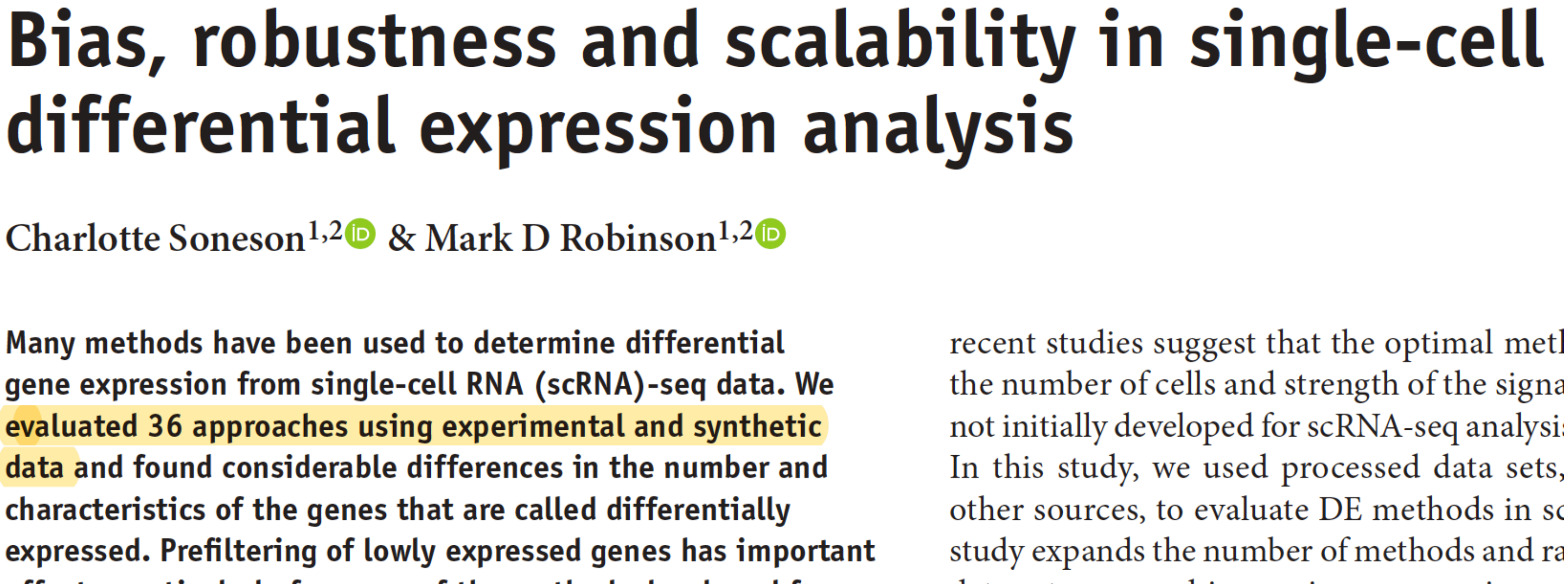
Differential gene expression analysis and enrichment analysis

Differential gene expression (DGE) analysis

- FindAllMarkers(): implemented in Seurat, defaults to Wilcoxon test, to detect genes that are “markers” for cell clusters. Finds genes that are DE between 1 cluster and all other cells.

- FindMarkers(): to perform pairwise DGE analysis, eg between cluster 1 and cluster 2, defaults to Wilcoxon test.

What is the ideal DGE analysis method?



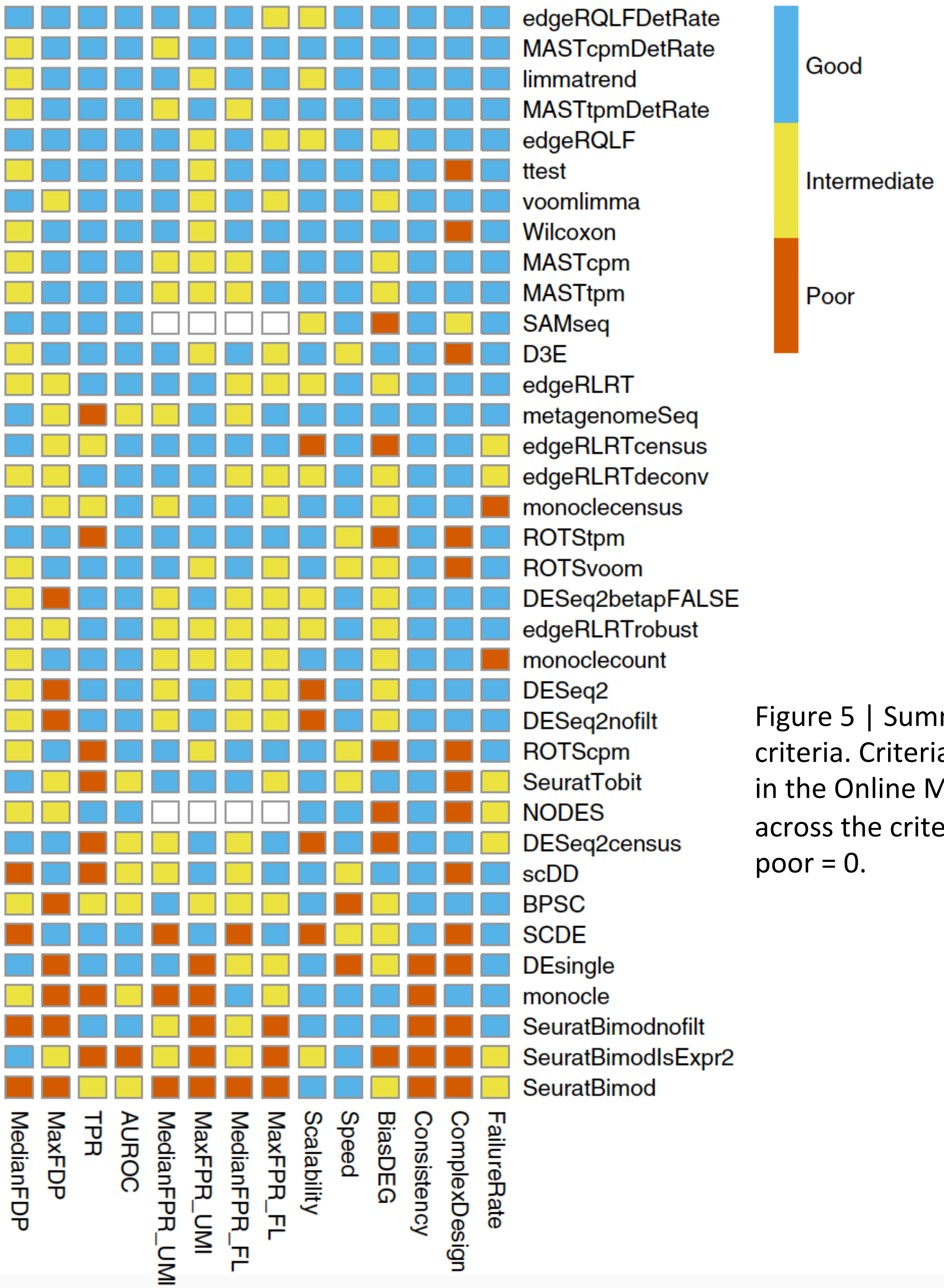
Bias, robustness and scalability in single-cell differential expression analysis

Charlotte Soneson[1,2] & Mark D Robinson[1,2]

Many methods have been used to determine differential gene expression from single-cell RNA (scRNA)-seq data. We evaluated 36 approaches using experimental and synthetic data and found considerable differences in the number and characteristics of the genes that are called differentially expressed. Prefiltering of lowly expressed genes has important

recent studies suggest that the optimal met
the number of cells and strength of the signa
not initially developed for scRNA-seq analysi
In this study, we used processed data sets,
other sources, to evaluate DE methods in s
study expands the number of methods and ra

https://www.nature.com/articles/nmeth.4612



	MedianFDP	MaxFDP	TPR	AUROC	MedianFPR_UMI	MaxFPR_UMI	MedianFPR_FL	MaxFPR_FL	Scalability	Speed	BiasDEG	Consistency	ComplexDesign	FailureRate
edgeRQLFDetRate	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟨	🟨	🟦	🟦	🟦	🟦	🟦
MASTcpmDetRate	🟨	🟦	🟦	🟦	🟨	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦
limmatrend	🟨	🟦	🟦	🟦	🟦	🟨	🟦	🟦	🟨	🟦	🟦	🟦	🟦	🟦
MASTtpmDetRate	🟨	🟦	🟦	🟦	🟨	🟦	🟨	🟦	🟦	🟦	🟦	🟦	🟦	🟦
edgeRQLF	🟦	🟦	🟦	🟦	🟦	🟨	🟦	🟨	🟨	🟦	🟨	🟦	🟦	🟦
ttest	🟨	🟦	🟦	🟦	🟦	🟨	🟦	🟦	🟦	🟦	🟦	🟦	🟧	🟦
voomlimma	🟦	🟨	🟦	🟦	🟦	🟨	🟦	🟨	🟦	🟦	🟨	🟦	🟦	🟦
Wilcoxon	🟨	🟦	🟦	🟦	🟦	🟨	🟦	🟦	🟦	🟦	🟦	🟦	🟧	🟦
MASTcpm	🟨	🟦	🟦	🟦	🟨	🟨	🟨	🟦	🟦	🟦	🟨	🟦	🟦	🟦
MASTtpm	🟨	🟦	🟦	🟦	🟨	🟨	🟨	🟦	🟦	🟦	🟨	🟦	🟦	🟦
SAMseq	🟦	🟦	🟦	🟦	⬜	⬜	⬜	⬜	🟨	🟦	🟧	🟦	🟨	🟦
D3E	🟨	🟦	🟦	🟦	🟦	🟨	🟦	🟨	🟦	🟨	🟦	🟦	🟧	🟦
edgeRLRT	🟨	🟨	🟦	🟦	🟦	🟦	🟨	🟨	🟨	🟦	🟨	🟦	🟦	🟦
metagenomeSeq	🟦	🟨	🟧	🟨	🟨	🟦	🟨	🟦	🟦	🟦	🟦	🟦	🟦	🟦
edgeRLRTcensus	🟦	🟨	🟨	🟦	🟦	🟦	🟦	🟦	🟧	🟦	🟧	🟦	🟦	🟨
edgeRLRTdeconv	🟨	🟨	🟦	🟦	🟦	🟦	🟨	🟨	🟨	🟦	🟨	🟦	🟦	🟨
monoclecensus	🟦	🟨	🟨	🟦	🟨	🟦	🟦	🟨	🟦	🟦	🟨	🟦	🟦	🟧
ROTStpm	🟦	🟦	🟧	🟦	🟦	🟦	🟦	🟦	🟦	🟨	🟧	🟦	🟧	🟦
ROTSvoom	🟨	🟦	🟦	🟦	🟦	🟨	🟦	🟨	🟦	🟨	🟨	🟦	🟧	🟦
DESeq2betapFALSE	🟨	🟧	🟦	🟦	🟨	🟦	🟨	🟨	🟨	🟦	🟨	🟦	🟦	🟦
edgeRLRTrobust	🟨	🟨	🟦	🟦	🟨	🟨	🟨	🟨	🟨	🟦	🟨	🟦	🟦	🟦
monoclecount	🟨	🟦	🟦	🟦	🟨	🟨	🟨	🟨	🟦	🟦	🟨	🟦	🟦	🟧
DESeq2	🟨	🟧	🟦	🟦	🟨	🟦	🟨	🟨	🟧	🟦	🟨	🟦	🟦	🟦
DESeq2nofilt	🟨	🟧	🟦	🟦	🟨	🟦	🟨	🟨	🟧	🟦	🟨	🟦	🟦	🟦
ROTScpm	🟨	🟦	🟧	🟦	🟦	🟨	🟦	🟦	🟦	🟨	🟧	🟦	🟧	🟦
SeuratTobit	🟦	🟨	🟧	🟨	🟦	🟦	🟦	🟨	🟦	🟨	🟦	🟦	🟧	🟨
NODES	🟨	🟨	🟦	🟦	⬜	⬜	⬜	⬜	🟦	🟦	🟧	🟦	🟧	🟨
DESeq2census	🟦	🟦	🟧	🟨	🟨	🟦	🟨	🟦	🟧	🟦	🟧	🟦	🟦	🟨
scDD	🟧	🟦	🟧	🟨	🟨	🟦	🟨	🟦	🟦	🟨	🟦	🟦	🟧	🟦
BPSC	🟨	🟧	🟨	🟨	🟦	🟨	🟨	🟨	🟦	🟧	🟨	🟦	🟦	🟦
SCDE	🟧	🟦	🟦	🟦	🟧	🟦	🟧	🟦	🟧	🟨	🟨	🟦	🟧	🟦
DEsingle	🟦	🟧	🟦	🟦	🟦	🟧	🟨	🟨	🟦	🟧	🟨	🟧	🟧	🟦
monocle	🟨	🟧	🟧	🟨	🟧	🟧	🟦	🟨	🟦	🟦	🟦	🟧	🟦	🟦
SeuratBimodnofilt	🟧	🟧	🟦	🟦	🟨	🟧	🟨	🟧	🟦	🟦	🟦	🟧	🟧	🟦
SeuratBimodIsExpr2	🟦	🟨	🟧	🟧	🟨	🟧	🟨	🟧	🟨	🟦	🟧	🟧	🟧	🟨
SeuratBimod	🟧	🟧	🟨	🟨	🟧	🟧	🟧	🟧	🟦	🟦	🟨	🟧	🟧	🟨

🟦 Good

🟨 Intermediate

🟧 Poor

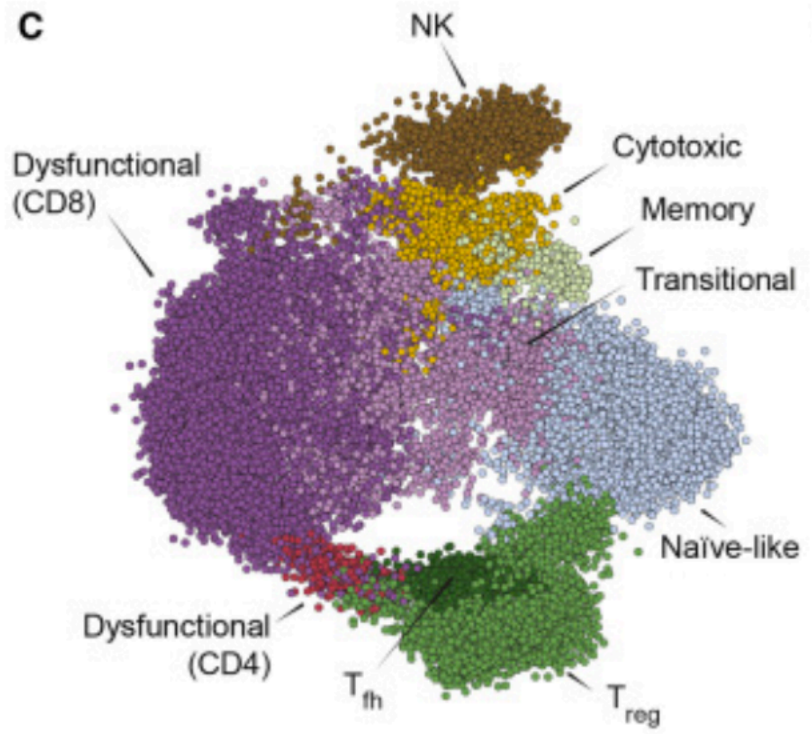
Figure 5 | Summary of DE method performance across all major evaluation criteria. Criteria and cutoff values for performance categories are available in the Online Methods. Methods are ranked by their average performance across the criteria, with the numerical encoding good = 2, intermediate = 1, poor = 0.

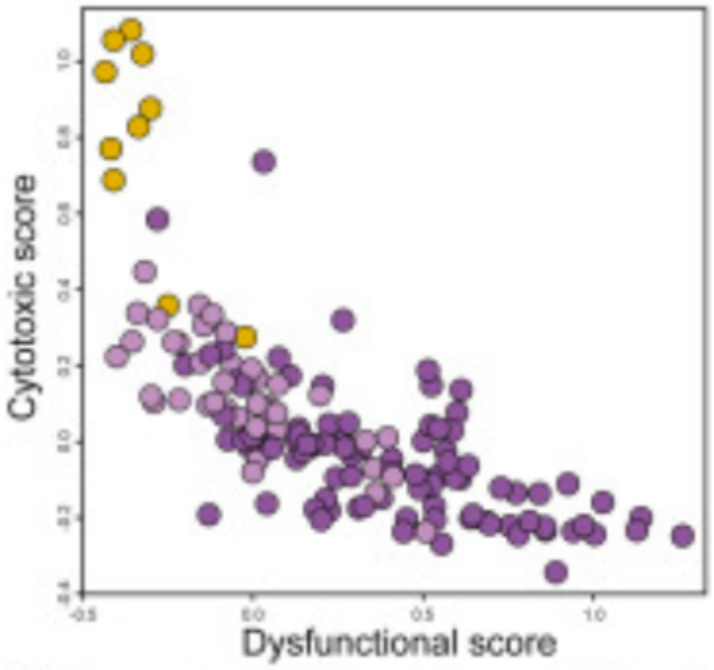
https://github.com/csoneson/conquer_comparison/blob/master/scripts/apply_edgeRQLFDetRate.R

limma or edgeR

- Methods designed for bulk RNA seq analysis
- Can be used to include batch effects in model as covariates
- Can be used to analyze factorial design such as genotype x treatment

MetaCell - *K*-nn graph partitions





MetaCell method: https://genomebiology.biomedcentral.com/articles/10.1186/s13059-019-1812-2
Application to CD8 T cells: https://www.sciencedirect.com/science/article/pii/S009286741831568X

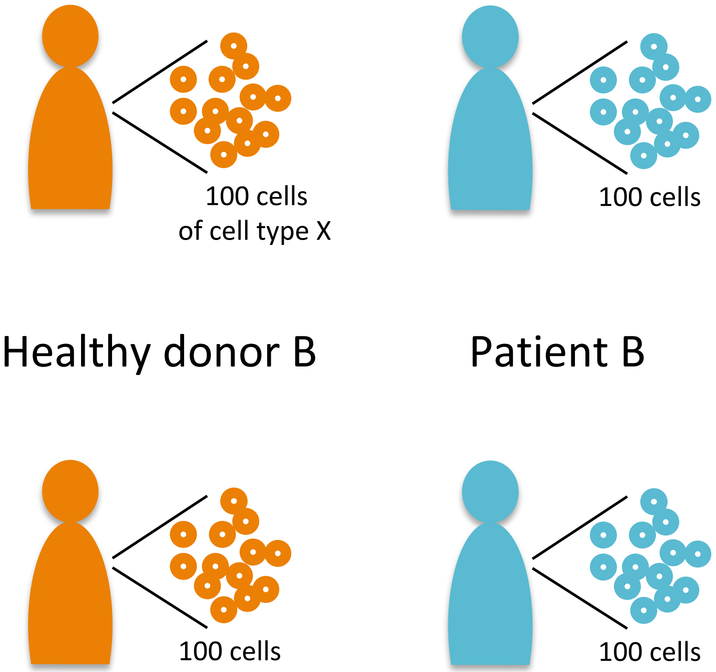
Problem of pseudo-replication?

How many independent replicates do we have,
7 or 2 replicates per ecosystem?

How many independent replicates do we have,
200 or 2 replicates per condition?

Forest A Grassland A

Forest B Grassland B



Healthy donor A

100 cells
of cell type X

Patient A

100 cells

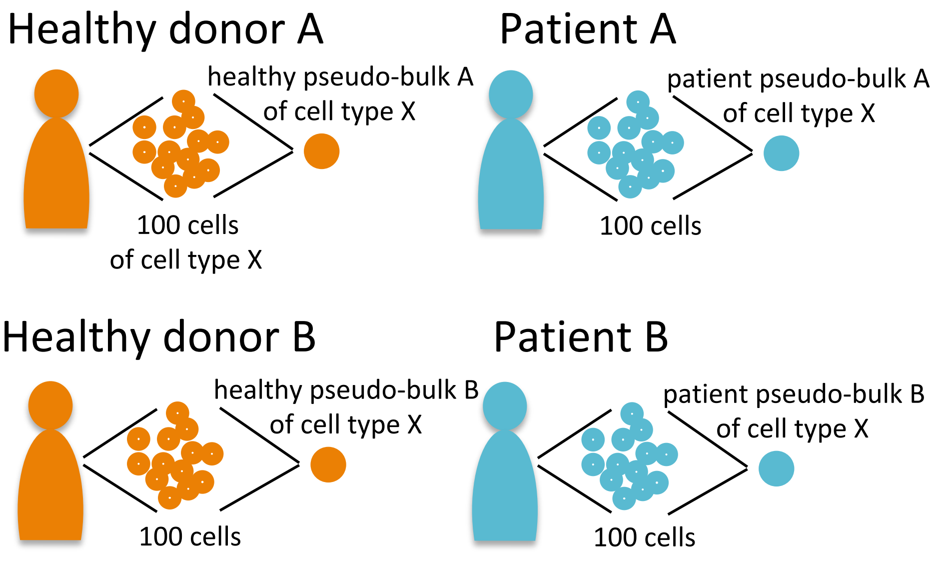
Healthy donor B

100 cells

Patient B

100 cells

Pseudo-bulk DE analysis: muscat



Healthy donor A

healthy pseudo-bulk A
of cell type X

100 cells
of cell type X

Patient A

patient pseudo-bulk A
of cell type X

100 cells

Healthy donor B

healthy pseudo-bulk B
of cell type X

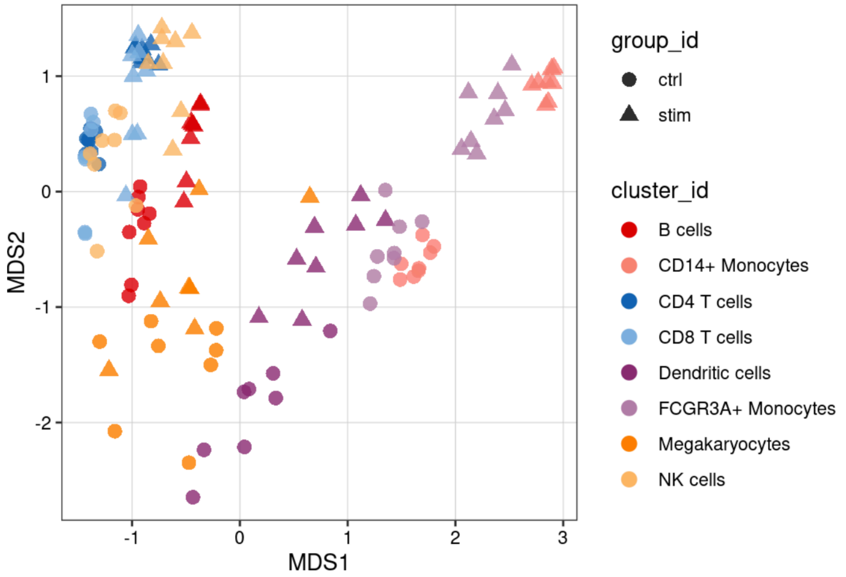
100 cells

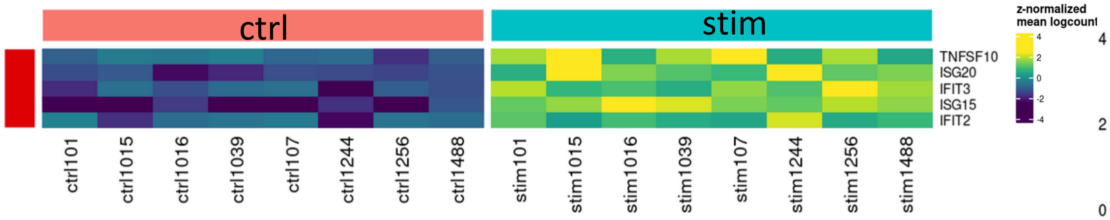
Patient B

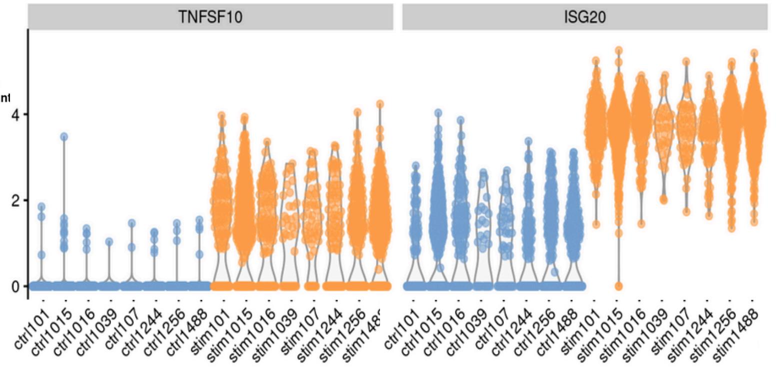
patient pseudo-bulk B
of cell type X

100 cells

One pseudo-bulk per cell type per sample



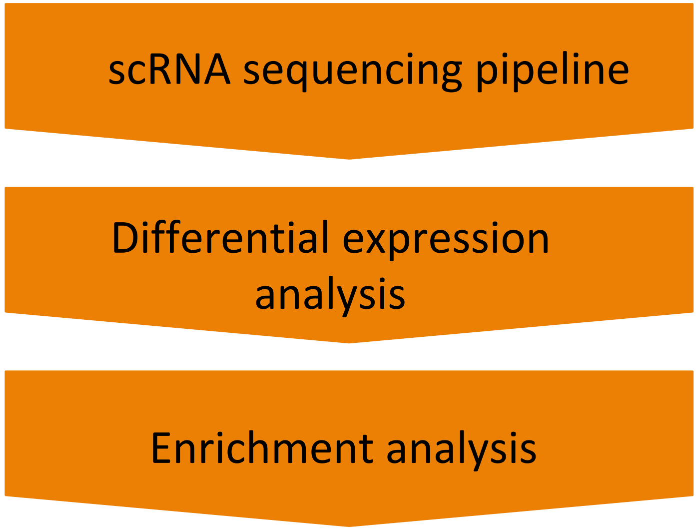




https://www.bioconductor.org/packages/release/bioc/vignettes/muscat/inst/doc/analysis.html

Question on DE analysis

Once we have identified DE genes, what do we do?



scRNA sequencing pipeline

Differential expression analysis

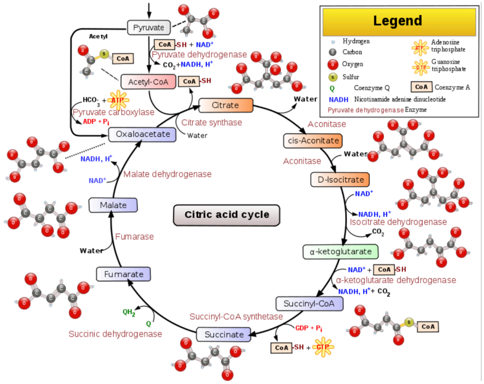
Enrichment analysis

Several methods available, *e.g.*:
- over-representation analysis (ORA)
- gene set enrichment analysis (GSEA)

Goal: to gain biologically-meaningful insights from long gene lists

- test if differentially expressed genes are enriched in genes associated with a particular function
- approaches: test a small number of gene sets, or a large collection of gene sets

What is a gene set?

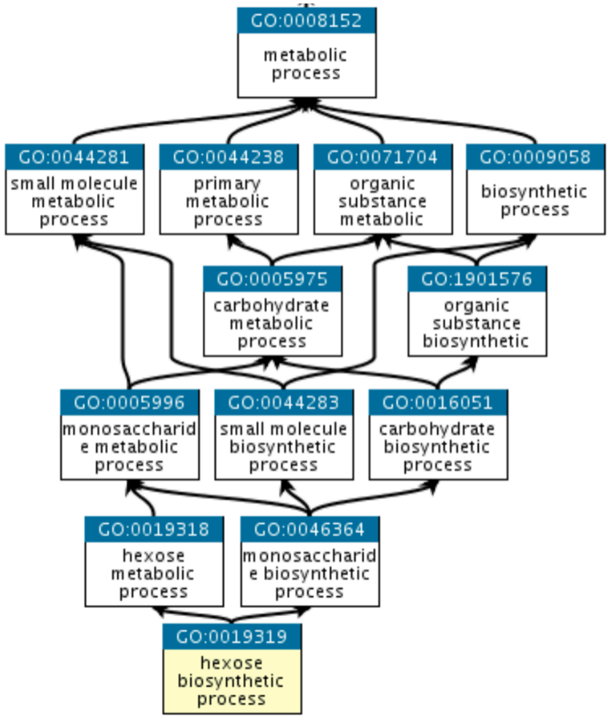


https://en.wikipedia.org/wiki/Citric_acid_cycle
Narayanese, WikiUserPedia, YassineMrabet, TotoBaggins –
http://biocyc.org/META/NEW-IMAGE?type=PATHWAY&object=TCA. I
mage adapted from Image:Citric acid cycle noi.svg CC BY-SA 3.0

- Genes working together in a pathway (e.g. energy release through Krebs cycle)
- Genes located in the same compartment in a cell (e.g. all proteins located in the cell nucleus)
- Proteins that are all regulated by a same transcription factor
- Custom gene list that comes from a publication and that are down-regulated in a mutant
- List of genes that contain SNPs associated with a disease
- ⋯ etc!
- Several gene sets are grouped into Knowledge bases

Gene ontology

- http://geneontology.org/



The mission of the GO Consortium is to develop a comprehensive, computational model of biological systems, ranging from the molecular to the organism level, across the multiplicity of species in the tree of life.

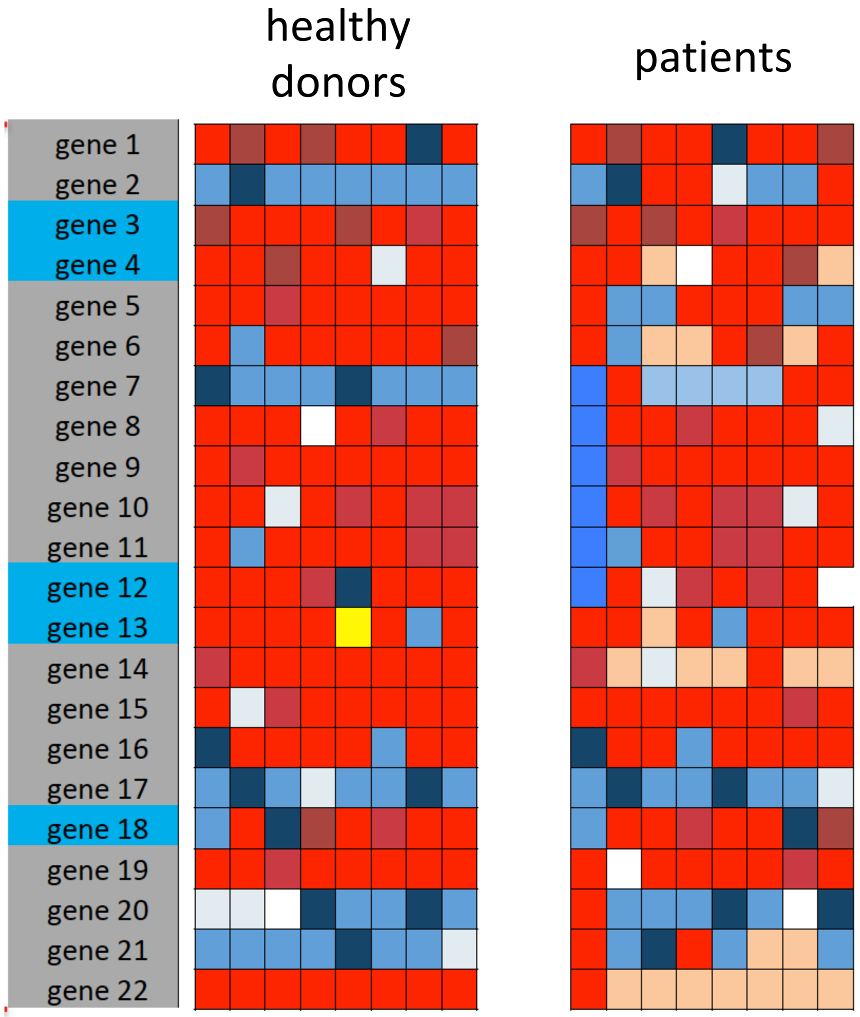
The Gene Ontology (GO) knowledgebase is the world's largest source of information on the functions of genes.

Different ontologies:
- Biological processes
- Cellular components
- Molecular functions

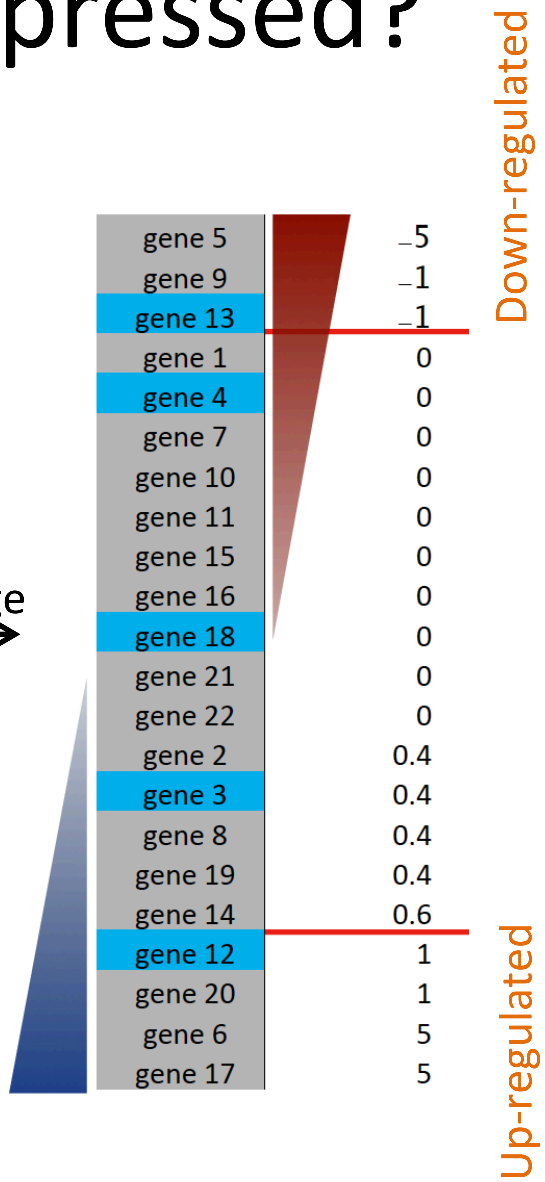
Sources of gene sets

- Online:
- MSigDB: database containing several types of gene set lists
- https://www.gsea-msigdb.org/gsea/msigdb/index.jsp
  - GO
  - hallmark
  - published gene sets
- KEGG (bi-directional eg mTOR signaling): https://www.kegg.jp/kegg/pathway.html
- Reactome https://reactome.org/
- WikiPathways https://www.wikipathways.org/index.php/WikiPathways

Are the genes belonging to the blue set differentially expressed?



sort based on fold change →



gene 5	-5
gene 9	-1
gene 13	-1
gene 1	0
gene 4	0
gene 7	0
gene 10	0
gene 11	0
gene 15	0
gene 16	0
gene 18	0
gene 21	0
gene 22	0
gene 2	0.4
gene 3	0.4
gene 8	0.4
gene 19	0.4
gene 14	0.6
gene 12	1
gene 20	1
gene 6	5
gene 17	5

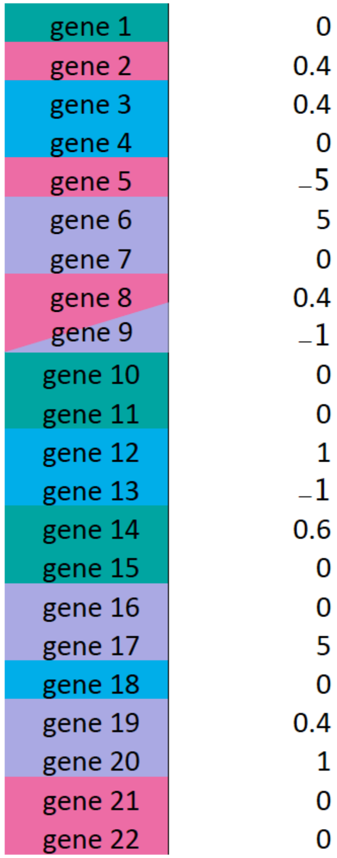
Fisher’s exact test in R

```
> cont.table<-matrix(c(2,3,5,12), ncol=2, byrow = T)
> fisher.test(cont.table)

	Fisher's Exact Test for Count Data

data:  cont.table
p-value = 1
alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:
  0.1012333 18.7696686
sample estimates:
odds ratio
   1.56456
```

2X2 count table	**Differentially expressed**	**Not Differentially expressed**	**total**
blue	2	3	**5**
Not blue	5	12	**17**
total	**7**	**15**	**22**

	2/7 = 0.29	3/15 = 0.20	

Which gene sets are differentially expressed?

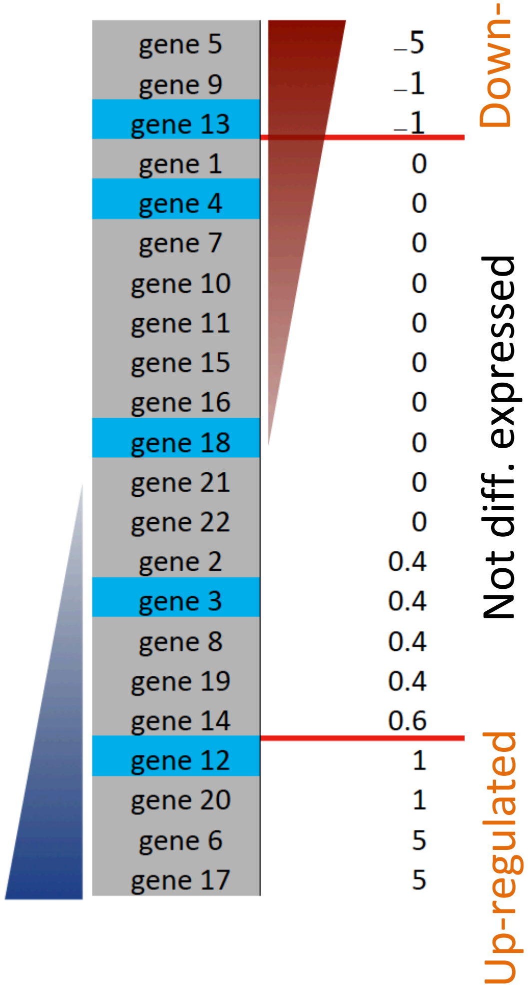


gene 1	0
gene 2	0.4
gene 3	0.4
gene 4	0
gene 5	–5
gene 6	5
gene 7	0
gene 8	0.4
gene 9	–1
gene 10	0
gene 11	0
gene 12	1
gene 13	–1
gene 14	0.6
gene 15	0
gene 16	0
gene 17	5
gene 18	0
gene 19	0.4
gene 20	1
gene 21	0
gene 22	0

Run individual Fisher's exact tests for each gene set, blue, pink, purple, green

⇒Multiple tests need p-value adjustment.

⇒Fisher test is threshold-based



gene 5	–5
gene 9	–1
gene 13	–1
gene 1	0
gene 4	0
gene 7	0
gene 10	0
gene 11	0
gene 15	0
gene 16	0
gene 18	0
gene 21	0
gene 22	0
gene 2	0.4
gene 3	0.4
gene 8	0.4
gene 19	0.4
gene 14	0.6
gene 12	1
gene 20	1
gene 6	5
gene 17	5

Over-representation analysis using R: One possibility among many

clusterProfiler

platforms all
rank 41 / 1905
posts 17 / 0.7 / 2 / 1
in Bioc 9 years
build warnings
updated before release
dependencies 126

DOI: 10.18129/B9.bioc.clusterProfiler

statistical analysis and visualization of functional profiles for genes and gene clusters

Built-in gene sets for human, mouse, yeast, etc

Built-in GO and KEGG (see later)

- ***G Yu***, LG Wang, Y Han, QY He. clusterProfiler: an R package for comparing biological themes among gene clusters. ***OMICS: A Journal of Integrative Biology*** 2012, 16(5):284-287. doi:[10.1089/omi.2011.0118](http://dx.doi.org/10.1089/omi.2011.0118)

Functions for over-representation analysis

Fisher exact test (package stats)

```
fisher.test(x, y = NULL, workspace = 200000, hybrid = FALSE,
            hybridPars = c(expect = 5, percent = 80, Emin = 1),
            control = list(), or = 1, alternative = "two.sided",
            conf.int = TRUE, conf.level = 0.95,
            simulate.p.value = FALSE, B = 2000)
```

Over-representation analysis (similar to Fisher test) for built-in GO gene sets:

```
enrichGO(gene, OrgDb, keyType = "ENTREZID", ont = "MF",
  pvalueCutoff = 0.05, pAdjustMethod = "BH", universe,
  qvalueCutoff = 0.2, minGSSize = 10, maxGSSize = 500,
  readable = FALSE, pool = FALSE)
```

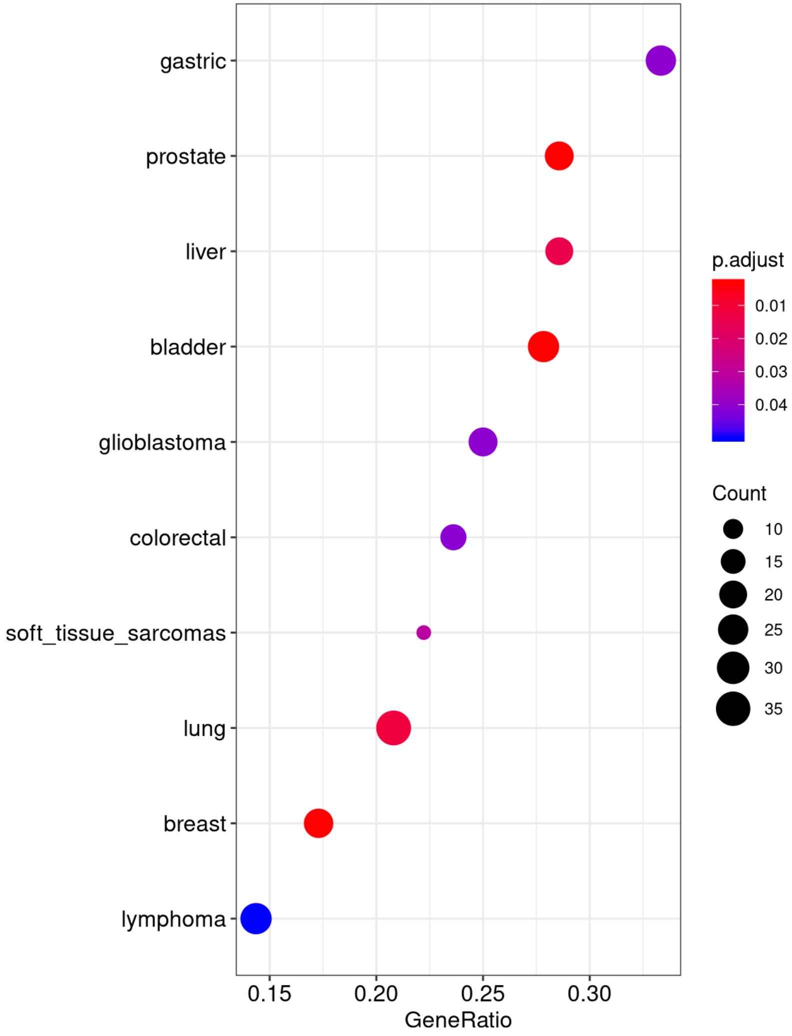
enricher(): similar enrichGO() but for user defined gene sets

```
enricher(gene, pvalueCutoff = 0.05, pAdjustMethod = "BH", universe,
  minGSSize = 10, maxGSSize = 500, qvalueCutoff = 0.2, TERM2GENE,
  TERM2NAME = NA)
```

Visualizations available in clusterProfiler

- dotplot

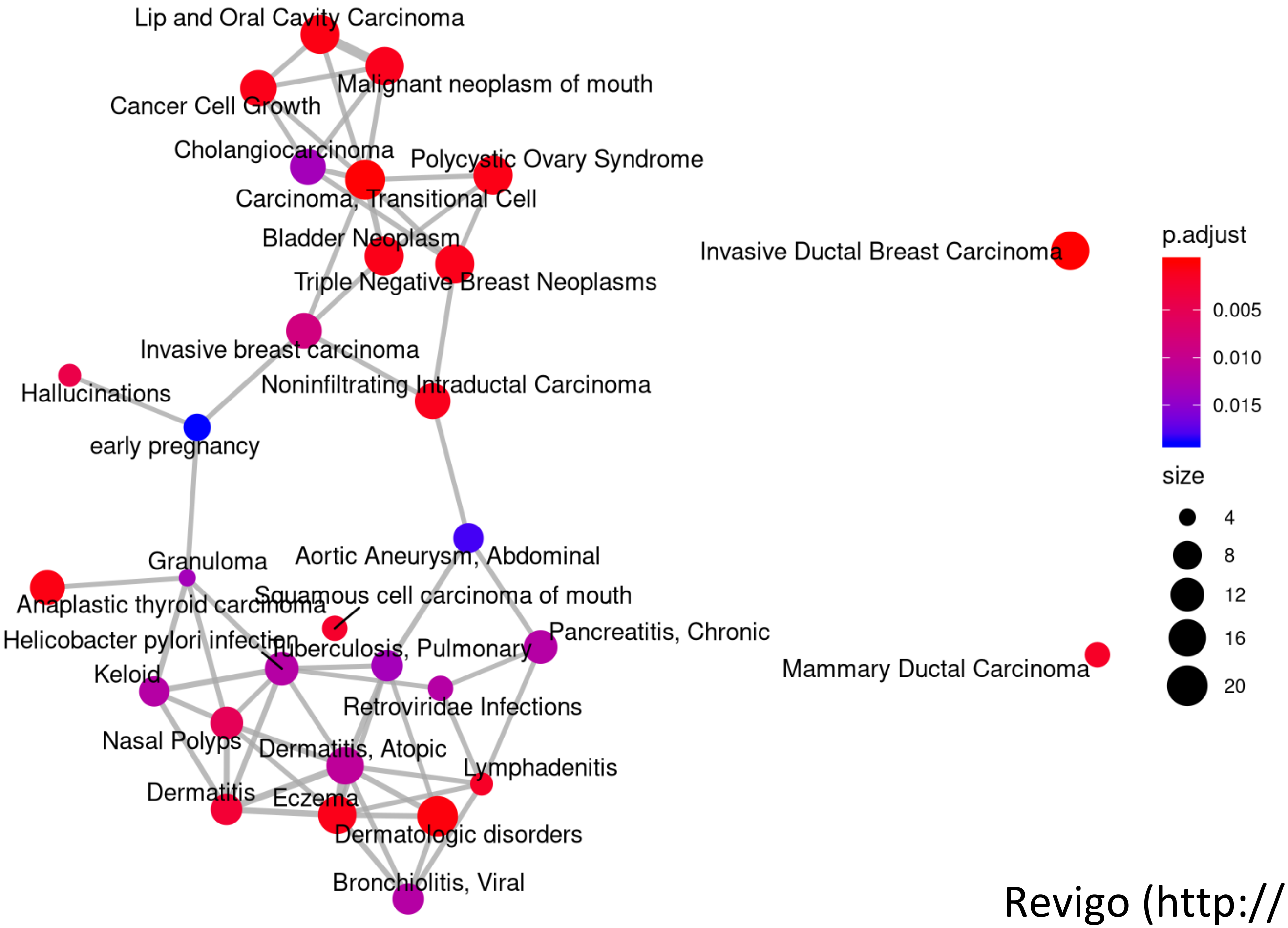
```
ego <- enrichGO(de)
dotplot(ego, showCategory=10)
```



Visualizations available in clusterProfiler

```
ego <- enrichGO(de)
emapplot(pairwise_termsim(ego))
```

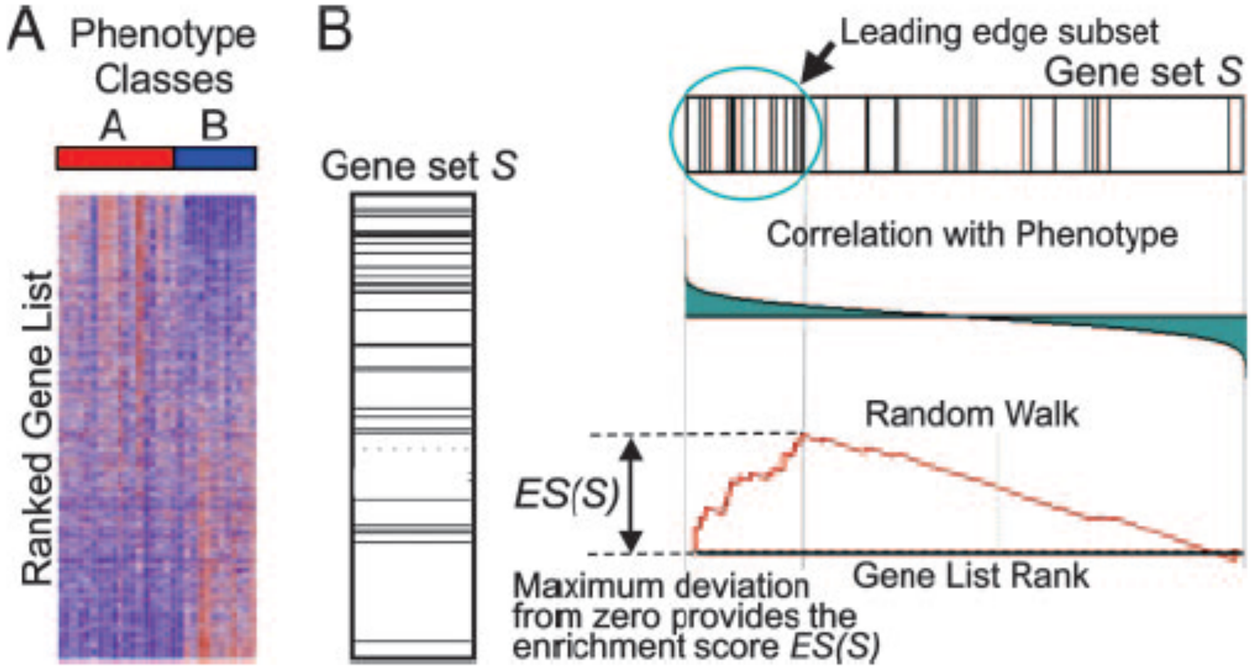
- Enrichment map



Revigo (http://revigo.irb.hr/)

Gene set enrichment analysis (GSEA)

Can be performed if you have statistics for all genes detected in the scRNAseq dataset, when using limma or edgeR



clusterProfiler:

- gseGO(): GSEA of GO terms using all ranked genes
- gseKEGG(): GSEA of KEGG pathways using all ranked genes
- GSEA(): GSEA of custom gene set collection using all ranked genes

Subramanian et al 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. PNAS 102(43):15545-50 doi: 10.1073/pnas.0506580102

Question on enrichment analysis