

SIB  
Swiss Institute of  
Bioinformatics

# Enrichment analysis

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# Schedule

- **9:00 - 9:30**
- Introduction
- **9:30 – 10:30**
- Over-representation analysis
- Exercise
- **10:30ish break**
- **10:50 - 12:30**
- Method of gene set enrichment analysis
- Exercise
- **12:30ish - 13:30 lunch break**
- **13:30 - 15:30**
- Visualization of enrichment results
- Exercise
- **15:30ish - 15:50 break**
- **15:50 - 16:50**
- Ontologies and sources of gene sets
- Exercise
- **16:50 - 17:00 Feedback and end of day**

# The Translational Data Science group



- Part of the **SIB Swiss Institute of Bioinformatics**
- Located at the AGORA Cancer Research Center in **Lausanne**
- Provides **the statistics, bioinformatics and computational expertise** to molecular biology and applied research labs.
- Participates in fundamental and translational research by providing expertise in **data analysis** of single-cell and bulk multi-omics, spatial transcriptomics, flow cytometry, etc

For core facility service inquiry: [nadine.fournier@sib.swiss](mailto:nadine.fournier@sib.swiss)

<https://agora-cancer.ch/scientific-platforms/translational-data-science-facility/>

<https://www.sib.swiss/raphael-gottardo-group>

# Tell us about yourself !

- Vevox poll
- Also, write your name and some keywords about yourself and/or your research into the Google doc, to share about yourself.



Photo by National Cancer Institute, Unsplash



Photo by Scott Graham, Unsplash

# Course material

- <https://sib-swiss.github.io/enrichment-analysis-training/>

The screenshot shows a website for an enrichment analysis course. At the top, there is a red header bar with the text "Enrichment analysis". Below the header, on the left, is a sidebar with a navigation menu:

- Home
- Precourse preparations
- Course schedule
- Materials
- Exercises** (this item is highlighted in red)
- Bonus code
- Useful links

The main content area has a title "Exercises" with a pencil icon to its right. Below the title, there is a text block:

In this section, you will find the R code that we will use during the course. We will explain the code and output during correction of the exercises.

Further down, another section titled "Source of data" contains the following text:

We will work with RNA sequencing data generated by [Ercolano et al 2020](#). This study described the transcriptomes of immune cells that are circulating in the blood of humans in healthy conditions. Different types of immune cells circulate in human blood. In this study, 2 cell types were included: Natural Killer (NK) cells and CD4+ T helper (Th) cells. These 2 types of cells have different functions: NK cells provide a rapid response in the innate immune response at the

- **Feedback:** survey at the end of the day about your opinion on this course (link sent by course organizer).

# Credits: 0.25 ECTS

- Please provide answers and R code for an additional exercise (eg 1 Word with answers and figures and 1 script file, or 1 file generated using Rmarkdown)

[https://sib-swiss.github.io/enrichment-analysis-training/exercises/  
#extra-exercise-for-ects-credits](https://sib-swiss.github.io/enrichment-analysis-training/exercises/#extra-exercise-for-ects-credits)

- Sign up for credit by adding your name to the google Doc file (email sent by course organizer)
- Send answers to [tania.wyss@sib.swiss](mailto:tania.wyss@sib.swiss) within 1 week

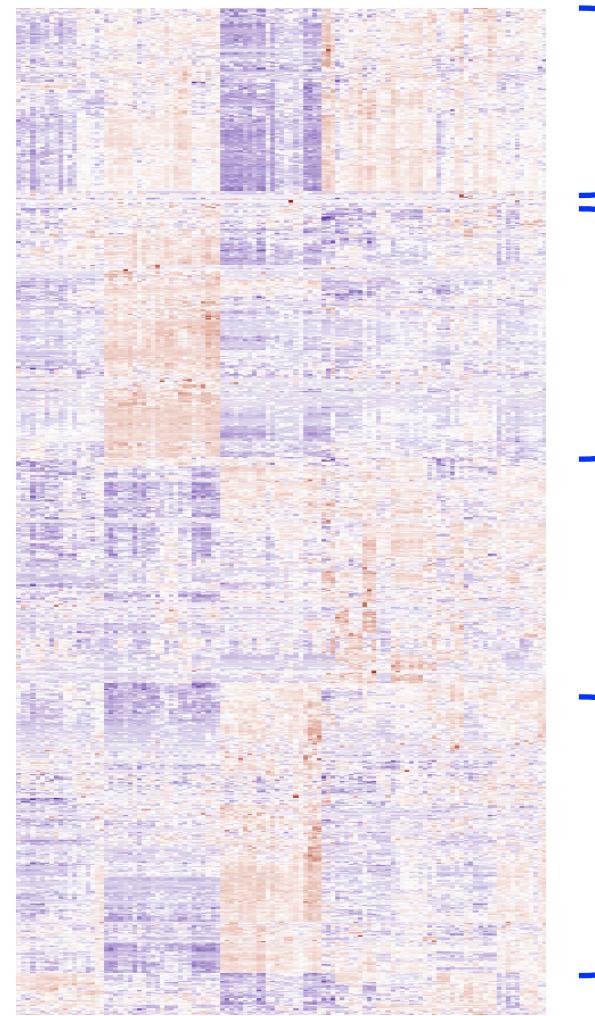
# Questions and Exercises

- Feel free to interrupt with questions by asking them directly or raising your (virtual) hand.
- Use the Q&A in Google Doc (or Zoom chat), we will provide answers
- Add a  when you are done with the current exercise
- Exercises in R:
  - We will try to debug as much as possible
  - We are happy if you share your results or alternative code!



# Why do we perform enrichment analysis?

- Gene expression analysis yields hundreds to thousands of significant genes
  - We need to summarize the information provided by so many genes
  - Understand their biological relationships



IVY GAP: <https://glioblastoma.alleninstitute.org/>

# Differential gene expression analysis : Example of RNAseq

- Use statistics to compare 2 groups:

For each gene  $i$ , is there a significant difference in mean expression between control and patients?

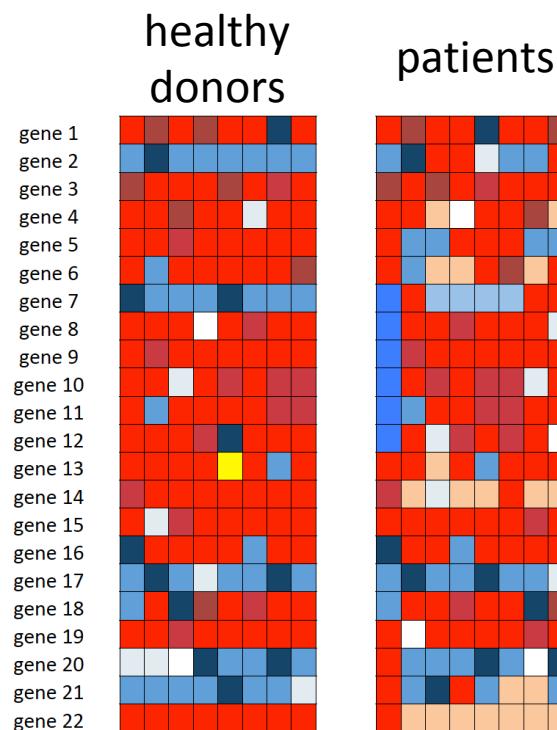
- T-test:

$H_0$ : Healthy donors and patients have similar gene  $i$  expression

$$H_{0i} : \pi_{i1} = \pi_{i2}$$

$H_1$ : Healthy donors and patients don't have a similar gene  $i$  expression

$$H_{1i} : \pi_{i1} \neq \pi_{i2}$$



# T-test in R

```
> t.test(grp1, grp2, paired = F)
```

Welch Two Sample t-test

data: grp1 and grp2

t = -6.3689, df = 8.9195, p-value = 0.0001352

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

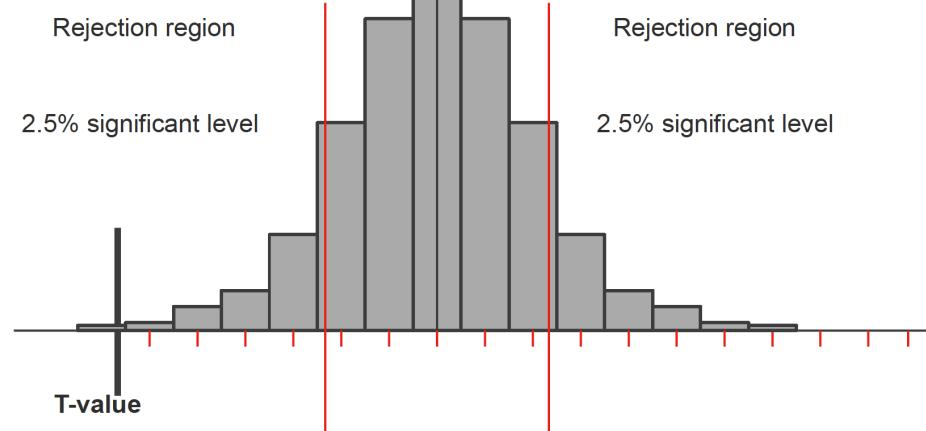
-8.908753 -4.234104

sample estimates:

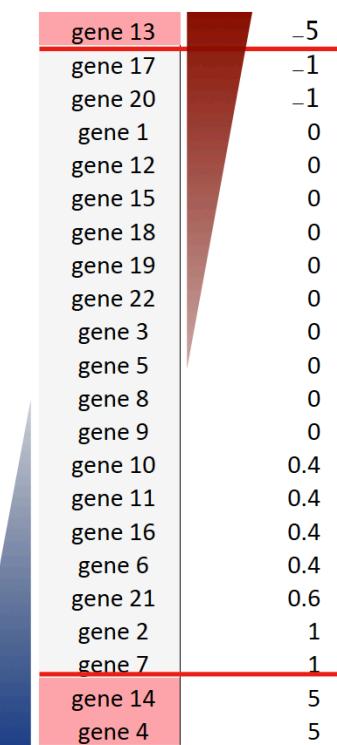
mean of x mean of y

6.00000 12.57143

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{s^2 \left( \frac{1}{n_1} + \frac{1}{n_2} \right)}}$$



sort based  
on T-statistic



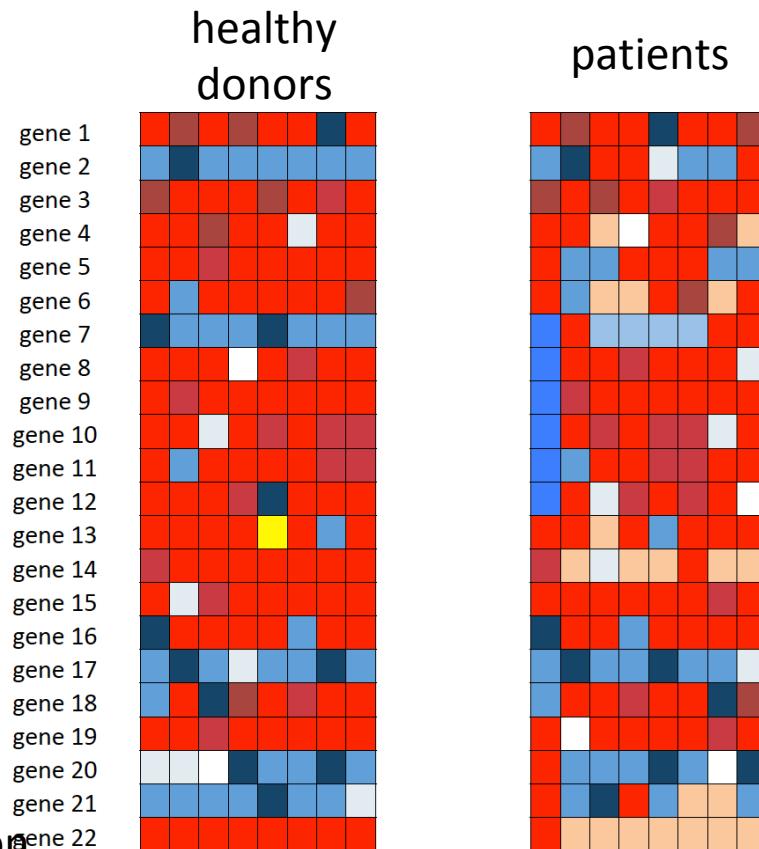
# Differential gene expression analysis using R

- Bioconductor

<https://bioconductor.org/>

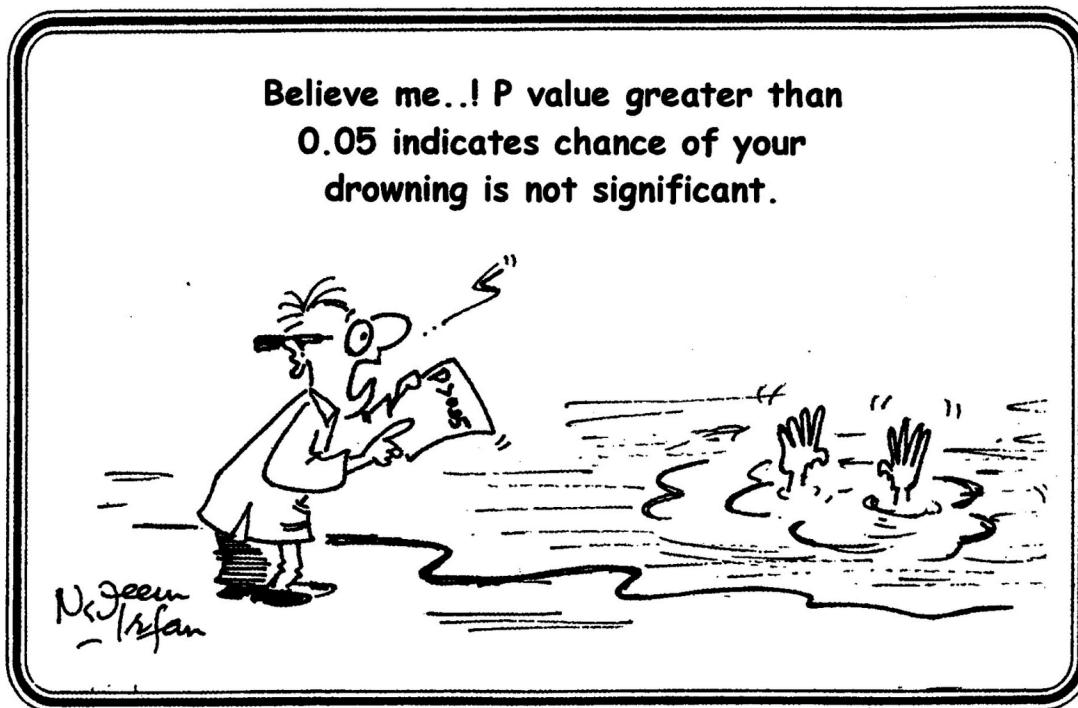
- Several packages :
  - limma: t-test
  - DESeq2: Wald test
  - edgeR: exact test

All allow for simultaneous statistical tests for every gene, together with p-value calculation



# What does $p < 0.05$ mean?

- It implies that it is acceptable to have a 5% probability to incorrectly reject the null hypothesis while it is correct.
- It means that if we repeat an experiment 20 times, we would reject the null hypothesis once because of random error.



# P-value adjustment: what is it?

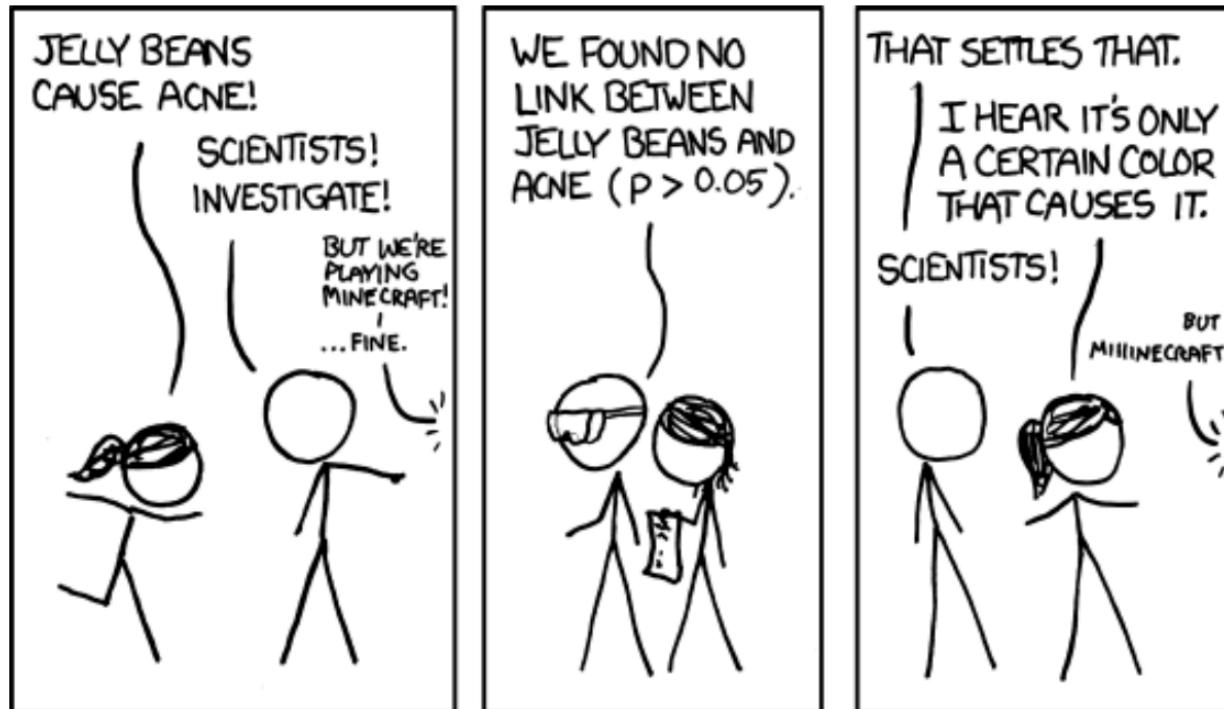


Photo by Patrick Fore on Unsplash

Cartoon: <https://xkcd.com/882/>

Paper on p-value adjustment: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6099145/>

WE FOUND NO  
LINK BETWEEN  
PURPLE JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  


WE FOUND NO  
LINK BETWEEN  
BROWN JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  


WE FOUND NO  
LINK BETWEEN  
PINK JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  


WE FOUND NO  
LINK BETWEEN  
BLUE JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  


WE FOUND NO  
LINK BETWEEN  
TEAL JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  


WE FOUND NO  
LINK BETWEEN  
SALMON JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  


WE FOUND NO  
LINK BETWEEN  
RED JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  


WE FOUND NO  
LINK BETWEEN  
TURQUOISE JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  


WE FOUND NO  
LINK BETWEEN  
MAGENTA JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  


WE FOUND NO  
LINK BETWEEN  
YELLOW JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  


WE FOUND NO  
LINK BETWEEN  
GREY JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  


WE FOUND NO  
LINK BETWEEN  
TAN JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  


WE FOUND NO  
LINK BETWEEN  
CYAN JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  


WE FOUND A  
LINK BETWEEN  
GREEN JELLY  
BEANS AND ACNE  
( $P < 0.05$ ).  


WE FOUND NO  
LINK BETWEEN  
MAUVE JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  

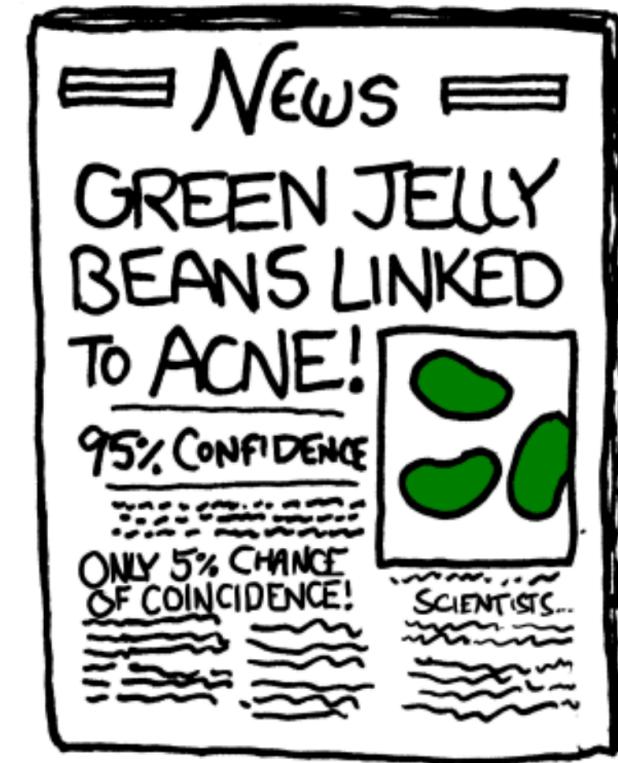

WE FOUND NO  
LINK BETWEEN  
BEIGE JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  


WE FOUND NO  
LINK BETWEEN  
LILAC JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  


WE FOUND NO  
LINK BETWEEN  
BLACK JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  


WE FOUND NO  
LINK BETWEEN  
PEACH JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  


WE FOUND NO  
LINK BETWEEN  
ORANGE JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).  

# Methods of p-value adjustment

- **Bonferroni:** the alpha level is divided by the total number of tests
- if we run  $k=20$  tests:  
 $0.05/k = 0.05/20=0.0025$

Good for small number of tests  
but too conservative for  
thousands of genes

- **Benjamini-Hochberg procedure (BH, decreases the FDR)**
  - Rank the p-values from smallest to largest, adjust less and less as the p-values get larger:  
 $p\text{-value}_1 * (n/1)$   
 $p\text{-value}_2 * (n/2)$   
...  
 $p\text{-value}_k * (n/k) = p\text{-value}_k * 1$
- n= total number of p-values (genes)  
k= rank number of each p-value

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

**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

RNA sequencing pipeline

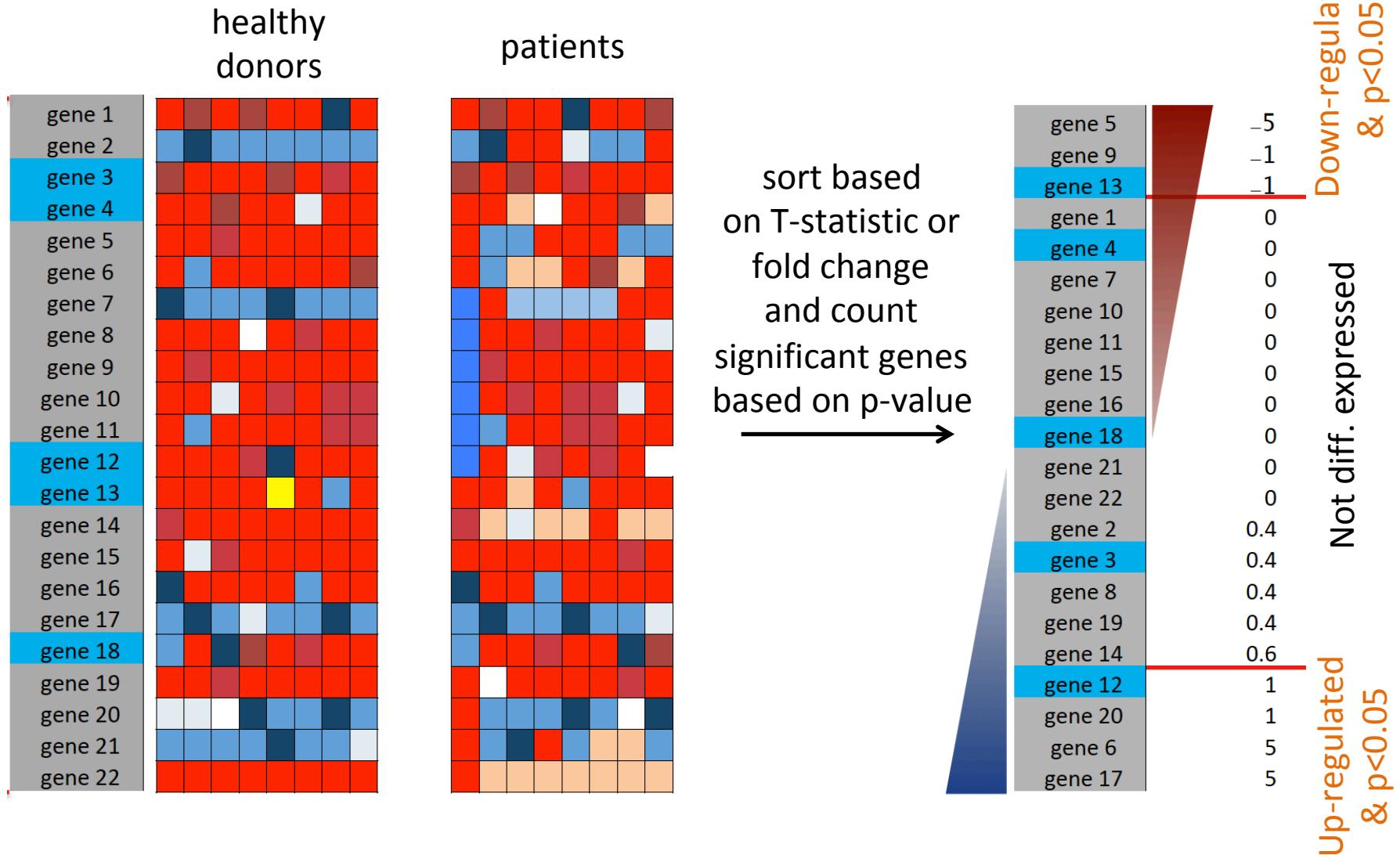
Differential expression analysis

Enrichment analysis

Several methods available, e.g.:

- over-representation analysis (ORA)
- gene set enrichment analysis (GSEA)

# Are the genes belonging to the blue set differentially expressed?



# Fisher's exact test

2x2 count table	Differentially expressed	Not Differentially expressed	total
blue	2	3	5
Not blue	5	12	17
total	7	15	22

contingency table

$H_0$ : The proportion of blue genes differentially expressed is the same as the proportion of blue genes that are not differentially expressed.

$H_1$ : The proportion of blue genes differentially expressed is not the same as the proportion of blue genes that are not differentially expressed.

# 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	5
Not blue	5	12	17	17
total	7	15	22	22

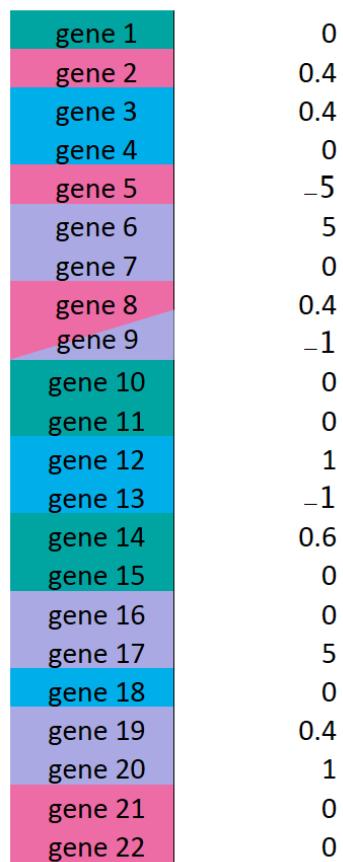
$$2/7 =$$

$$0.29$$

$$3/15 =$$

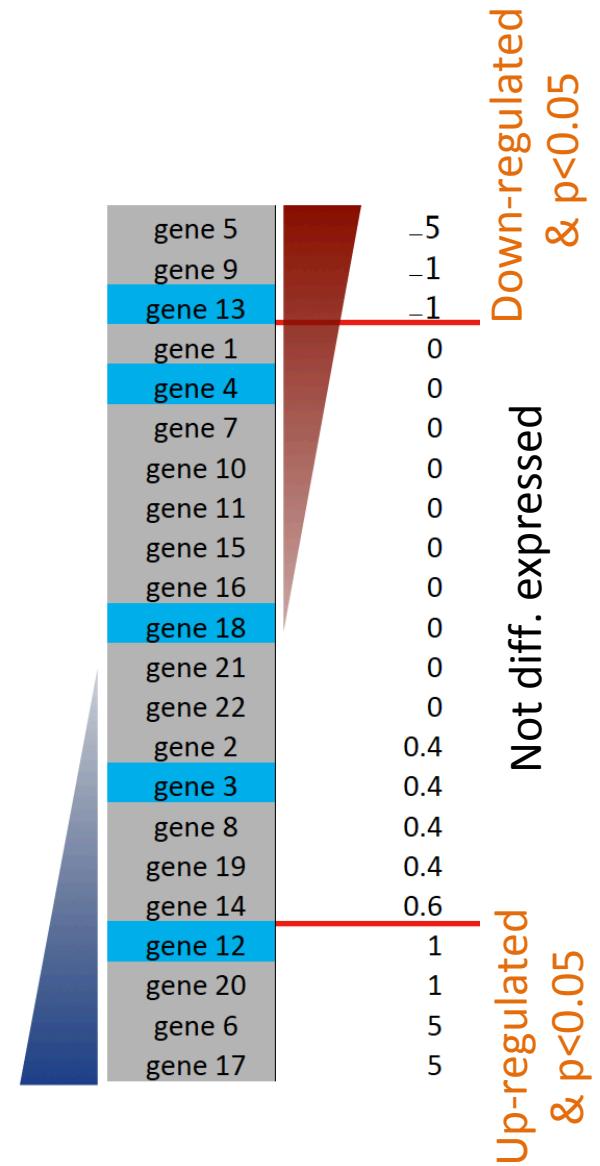
$$0.20$$

# Which gene sets are differentially expressed?



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

⇒ Multiple tests need **p-value adjustment**.



# Enrichment analysis using R: one possibility among others

## clusterProfiler

platforms all rank 41 / 2140 support 1 5 / 2 3 in Bioc 11 years  
build ok updated < 1 week dependencies 125

DOI: [10.18129/B9.bioc.clusterProfiler](https://doi.org/10.18129/B9.bioc.clusterProfiler)  

### A universal enrichment tool for interpreting omics data

Bioconductor version: Release (3.15)

This package supports functional characteristics of both coding and non-coding genomics data for thousands of species with up-to-date gene annotation. It provides a universal interface for gene functional annotation from a variety of sources and thus can be applied in diverse scenarios. It provides a tidy interface to access, manipulate, and visualize enrichment results to help users achieve efficient data interpretation. Datasets obtained from multiple treatments and time points can be analyzed and compared in a single run, easily revealing functional consensus and differences among distinct conditions.

Author: Guangchuang Yu [aut, cre, cph] , Li-Gen Wang [ctb], Erqiang Hu [ctb], Xiao Luo [ctb], Meijun Chen [ctb], Giovanni Dall'Olio [ctb], Wanqian Wei [ctb]

Maintainer: Guangchuang Yu <[guangchuangyu@gmail.com](mailto:guangchuangyu@gmail.com)>

### Built-in functions for enrichment analysis

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

### Built-in GO and KEGG (see later)

- <https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>
- 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)
- Full vignette: <http://yulab-smu.top/clusterProfiler-book/>

# Functions for Fisher test and for ORA with R and clusterProfiler

Fisher exact test (package stats)

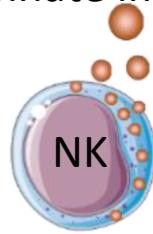
```
fisher.test(x, y = NULL, workspace = 200000, hybrid = FALSE,
            hybridPars = c(expect = 5, percent = 80, Emin = 1),
            control = list(), or = 1, alternative = "two.sided",
            conf.int = TRUE, conf.level = 0.95,
            simulate.p.value = FALSE, B = 2000)
```

enricher(): implementation of hypergeometric test (one-sided Fisher test) for user defined gene list and gene set annotations (package clusterProfiler)

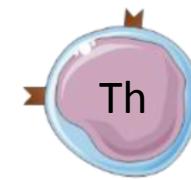
```
enricher(
  gene,
  pvalueCutoff = 0.05,
  pAdjustMethod = "BH",
  universe,
  minGSSize = 10,
  maxGSSize = 500,
  qvalueCutoff = 0.2,
  TERM2GENE,
  TERM2NAME = NA
)
```

Eg genes that are markers of cell clusters of single-cell RNA seq

## Innate immunity



## Adaptive immunity



# Recap and exercise 1

<https://www.mdpi.com/1420-3049/24/24/4530/html>

- Once we have identified differentially expressed (DE) genes, we can use an over-representation analysis to determine whether or not the genes of a gene set of interest are over-represented among the DE genes or not.
- Exercise 1:**
- Results table of differential gene expression analysis between 2 human immune cell types, natural killer (NK) cells and CD4 T helper cells (Th):

ensembl_gene_id	symbol	logFC	t	P.Value	p.adj
ENSG00000000003	TSPAN6	-5.643604444	-4.67212847	4.260000e-05	7.358019e-04
ENSG00000000419	DPM1	-0.181898089	-1.10183079	2.780198e-01	5.176076e-01
ENSG00000000457	SCYL3	0.496987374	1.49103508	1.448691e-01	3.449889e-01
ENSG00000000460	C1orf112	1.121799095	1.44589945	1.570599e-01	3.630935e-01
ENSG00000000938	FGR	10.670687340	7.21234165	1.980000e-08	1.718657e-06
ENSG00000000971	CFH	-3.412927673	-2.78888655	8.480300e-03	4.610083e-02

Positive logFC = higher in NK  
Negative logFC = lower in NK

- Run a **Fisher's exact test** to determine whether genes involved in the **adaptive immune response** are over-represented among the genes up-regulated in Th cells.

RNA sequencing data from:

<https://jlb.onlinelibrary.wiley.com/doi/full/10.1002/JLB.5MA0120-209R?af=R>

<https://ashpublications.org/bloodadvances/article/3/22/3674/428873/CD56-as-a-marker-of-an-ILC1-like-population-with>

# RStudio tour

The screenshot shows the RStudio interface with the following components:

- Code Editor:** Displays an R script titled "ea\_2020\_script.R" containing code related to enrichment analysis.
- Environment Browser:** Shows the global environment with objects like "convert\_ens\_sy..." and "GO\_NK\_Th".
- File Browser:** Shows the contents of a project named "project" with files like ".RData", ".Rhistory", and "EA\_2020\_Exercise\_1.R".
- Console:** Displays R startup messages and a command prompt.

```
1 ##### Enrichment analysis course, SIB, June 26th 2020
2
3
4 # Enrichment analysis, SIB course, June 26th 2020
5
6 # load the packages needed for the R exercise
7 library(clusterProfiler)
8 library(org.Hs.eg.db)
9 library(pathview)
10 # library(biomart)
11
12 # Some reminders about the usage of R:
13
```

/cloud/project/ ↗  
Type 'contributors()' for more information and  
'citation()' on how to cite R or R packages in publications.  
Type 'demo()' for some demos, 'help()' for on-line help, or  
'help.start()' for an HTML browser interface to help.  
Type 'q()' to quit R.

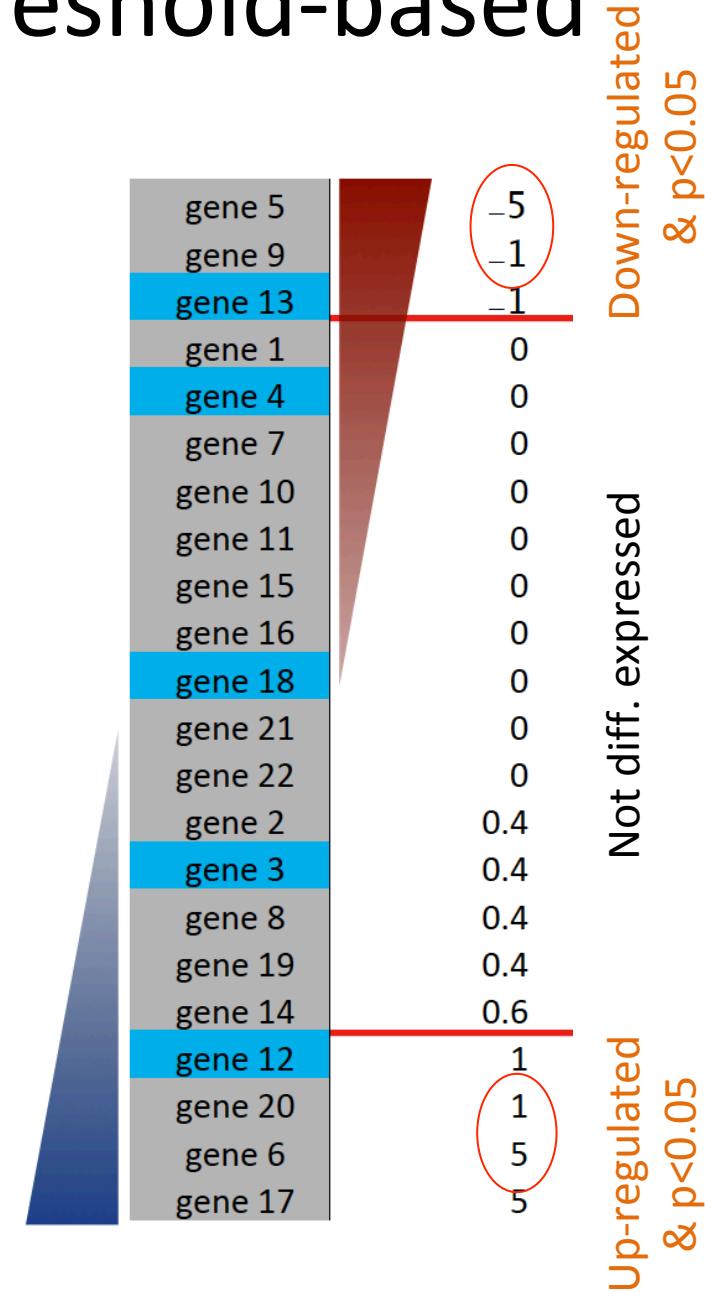
[Workspace loaded from /cloud/project/.RData]

> |

# Fisher's exact test is threshold-based

<i>2x2 count table</i>	Differentially expressed	Not Differentially expressed	total
blue	2	3	5
Not blue	5	12	17
total	7	15	22

Contingency table with count of genes,  
without taking into account the **magnitude**  
of the change of each gene.

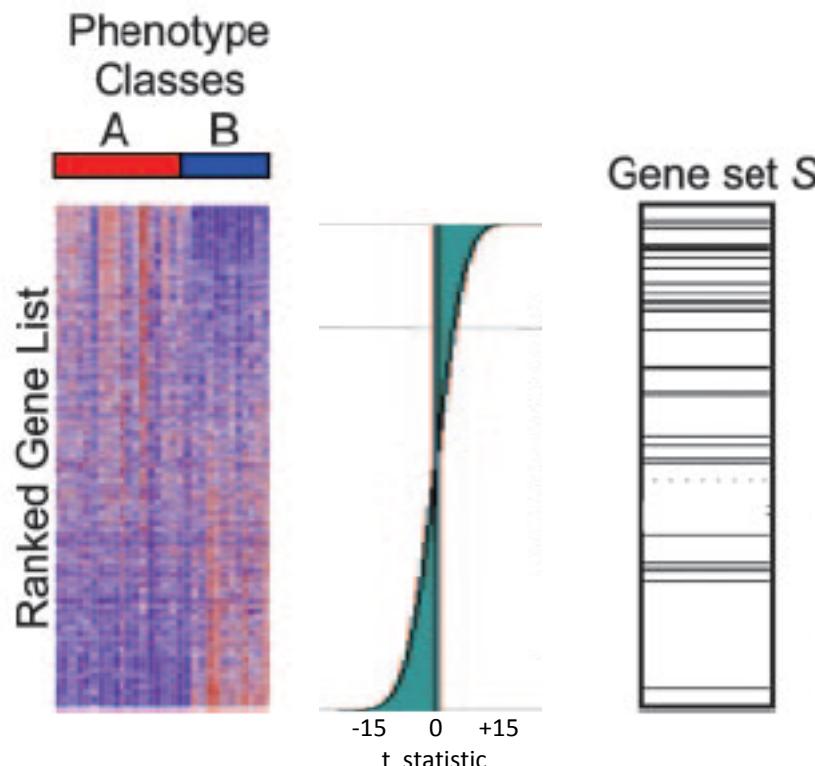


# Gene set enrichment analysis (GSEA)

- **Threshold-free:** the whole list of genes detected in the RNA sequencing experiment is used.
- GSEA is a computational method that determines whether an *a priori* defined set of genes shows statistically significant, concordant differences between two biological states (MSigDB)
- Rank all genes based on score (eg t-statistic) and calculate an enrichment score (ES) that reflects the degree to which the members of a gene set are overrepresented at the top or bottom of the ranked genes.

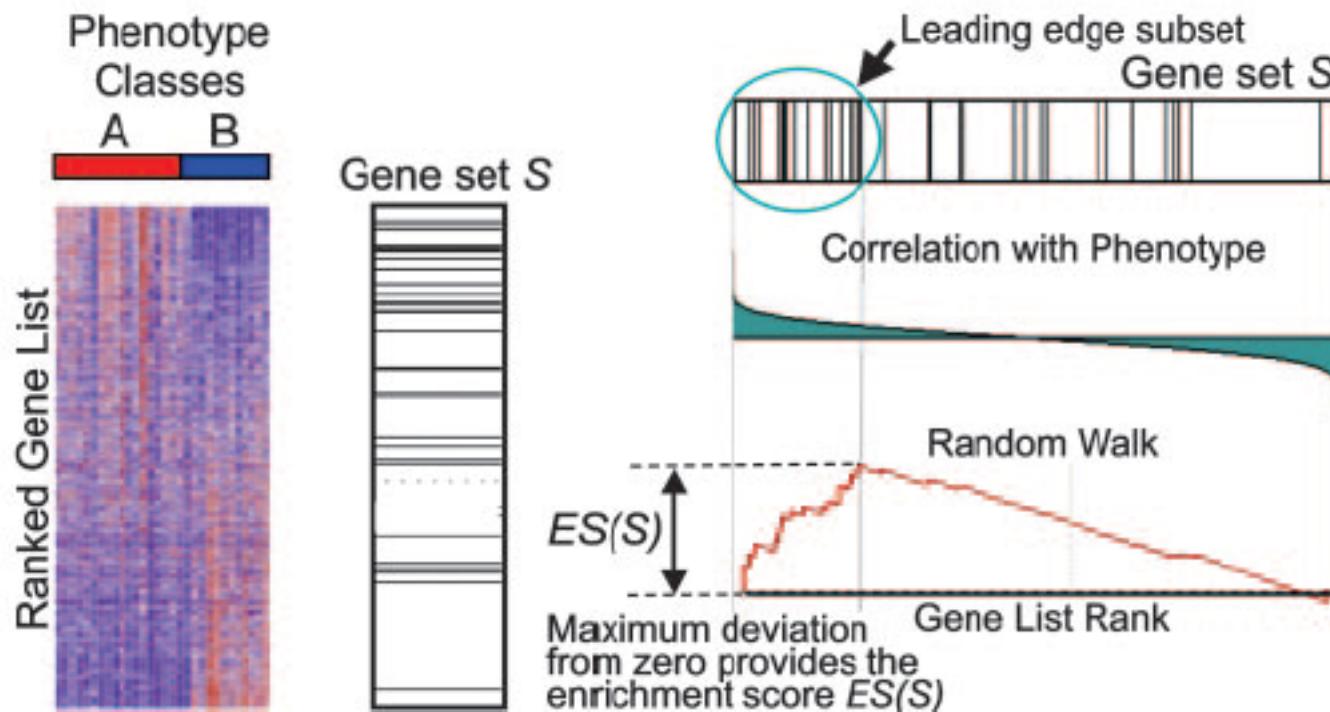
# Method of GSEA

Goal: determine whether the members of a gene set **S** are randomly distributed throughout a ranked gene list or if they are located at the top or bottom of the ranked gene lists



1. Sort the genes based on the t statistic (=weight)

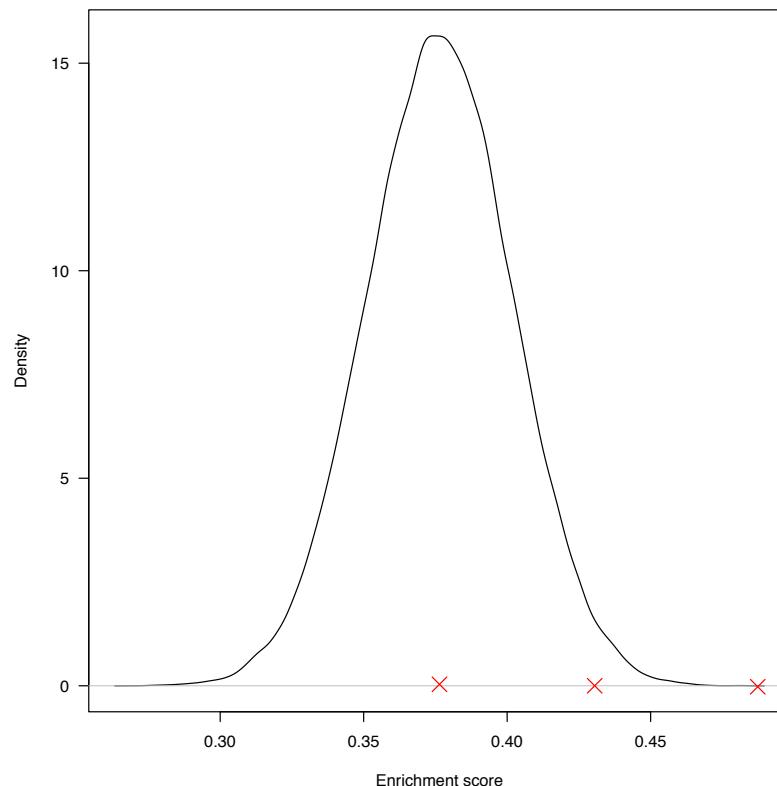
# Method of GSEA



1. Sort the genes based on the t statistic (=weight)
2. Calculate enrichment score ES using weight. The ES for a set is the maximum value reached (pos. or neg.)

# Method of GSEA

1. Sort the genes based on the t statistic (=weight)
2. Calculate enrichment score ES using weight. The ES for a set is the maximum value reached (pos. or neg.)
3. Perform permutations of samples and/or genes to recalculate random ES scores
4. Calculate Normalized ES (NES) and estimate p-value of each gene set based on randomized ES scores
5. Adjust p-value



$$\text{NES} = \frac{\text{actual ES}}{\text{mean(ESs against all permutations of the dataset)}}$$

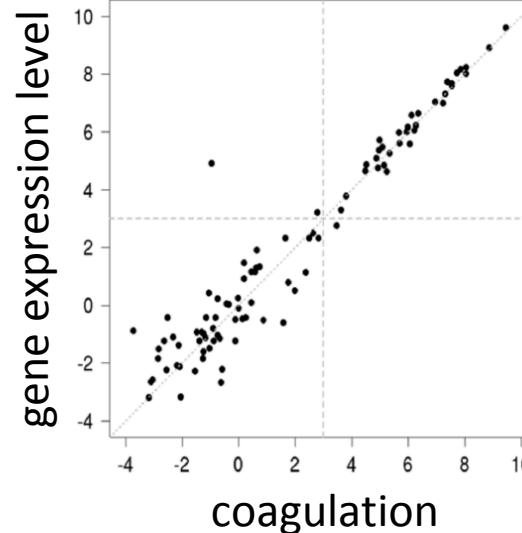
Do not forget p-value  
adjustment if more than 1  
gene set is tested!

NES: 1    NES: 1.16    NES: 1.32  
p: 0.5    p: 0.05    p: 0.001

# Apply GSEA to any type of data or score

- Use t-statistic from paired t-test
- Use F statistic of one way or two way ANOVA
- Use coefficients or p-value of linear model

	Adj. p-value of LM
gene 4	0.0022
gene 13	0.0022
gene 14	0.0022
gene 2	0.19
gene 7	0.19
gene 17	0.19
gene 20	0.19
gene 21	1
gene 6	1
gene 10	1
gene 11	1
gene 16	1
gene 1	1
gene 3	1
gene 5	1
gene 8	1
gene 9	1
gene 12	1
gene 15	1
gene 18	1
gene 19	1
gene 22	1



GSEA for linear model implemented in `romer()` function of the `limma` package

# Functions for GSEA with clusterProfiler

GSEA(): GSEA of user-defined gene sets using all ranked genes

```
GSEA(  
  geneList,  
  exponent = 1,  
  minGSSize = 10,  
  maxGSSize = 500,  
  eps = 1e-10,  
  pvalueCutoff = 0.05,  
  pAdjustMethod = "BH",  
  TERM2GENE,  
  TERM2NAME = NA,  
  verbose = TRUE,  
  seed = FALSE,  
  by = "fgsea",  
  ...  
)
```

TERM2GENE:

term	gene
GOBP_ADAPTIVE_IMMUNE_RESPONSE	ZC3H12A
GOBP_ADAPTIVE_IMMUNE_RESPONSE	ZNF683
GOBP_ADAPTIVE_IMMUNE_RESPONSE	ZP3
GOBP_HAIR_CELL_DIFFERENTIATION	ATOH1
GOBP_HAIR_CELL_DIFFERENTIATION	CDH23
GOBP_HAIR_CELL_DIFFERENTIATION	CLRN1

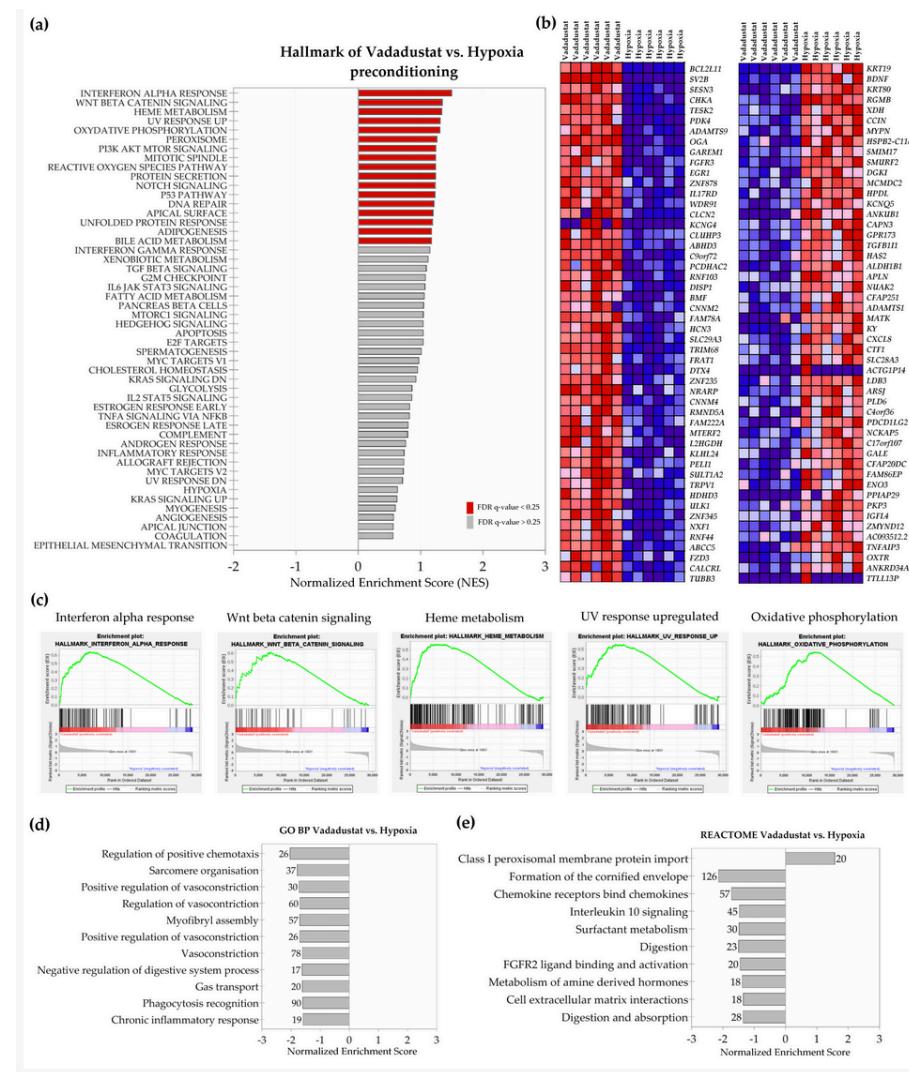
gseGO(): GSEA of GO gene sets using all ranked genes

```
gseGO(  
  geneList,  
  ont = "BP",  
  OrgDb,  
  keyType = "ENTREZID",  
  exponent = 1,  
  minGSSize = 10,  
  maxGSSize = 500,  
  eps = 1e-10,  
  pvalueCutoff = 0.05,  
  pAdjustMethod = "BH",  
  verbose = TRUE,  
  seed = FALSE,  
  by = "fgsea",  
  ...  
)
```

# Recap and exercise 2

- Fisher test is a threshold-based method, while GSEA is a threshold-free enrichment method. Both can be used for single or multiple gene sets.
- Exercise 2: use functions of `clusterProfiler` and data provided in Ex. 1
  - Run a GSEA for the Gene Ontology gene sets (more details on this collection later)
  - Explore the results: how many gene sets are significant? Are the gene sets up-regulated or down-regulated in NK cells?

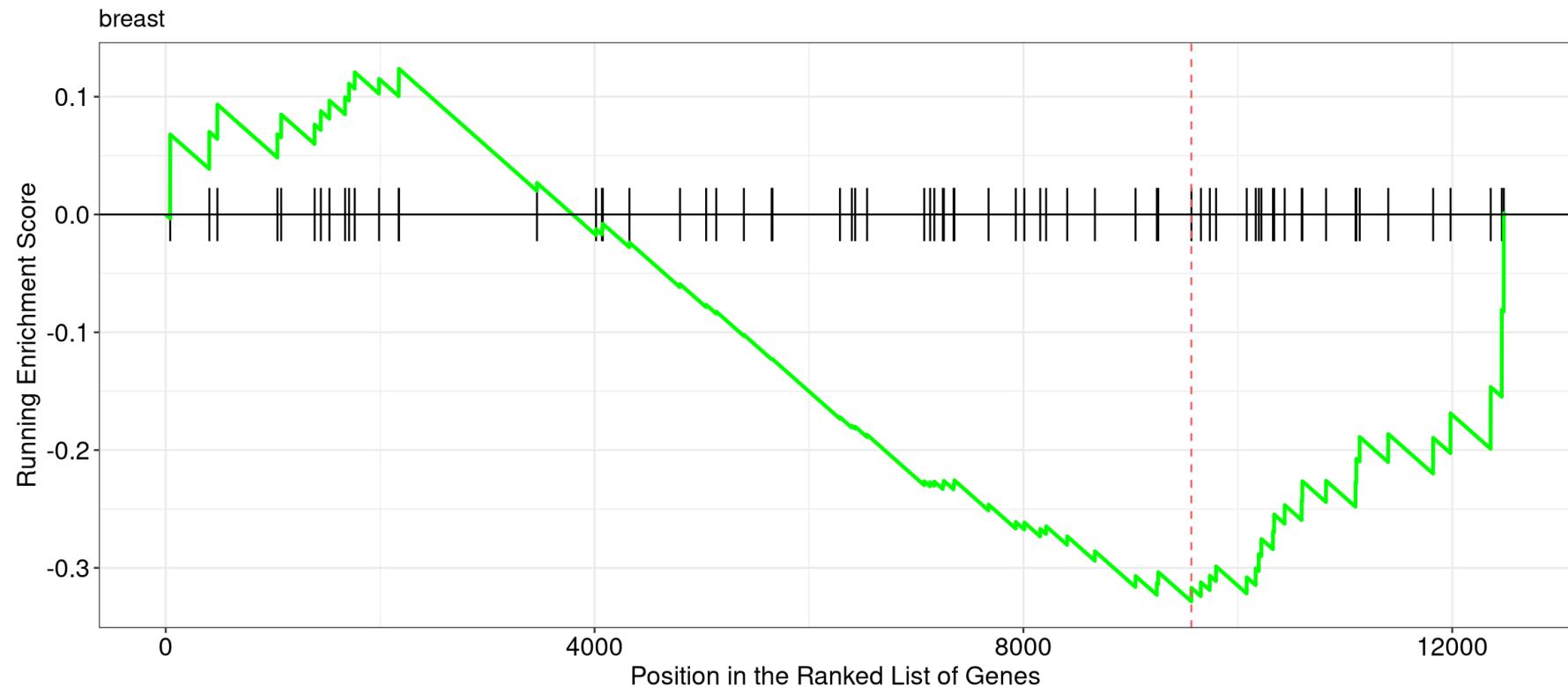
# How to show the results of an enrichment analysis?



# Visualizations available in clusterProfiler

- barcode plot

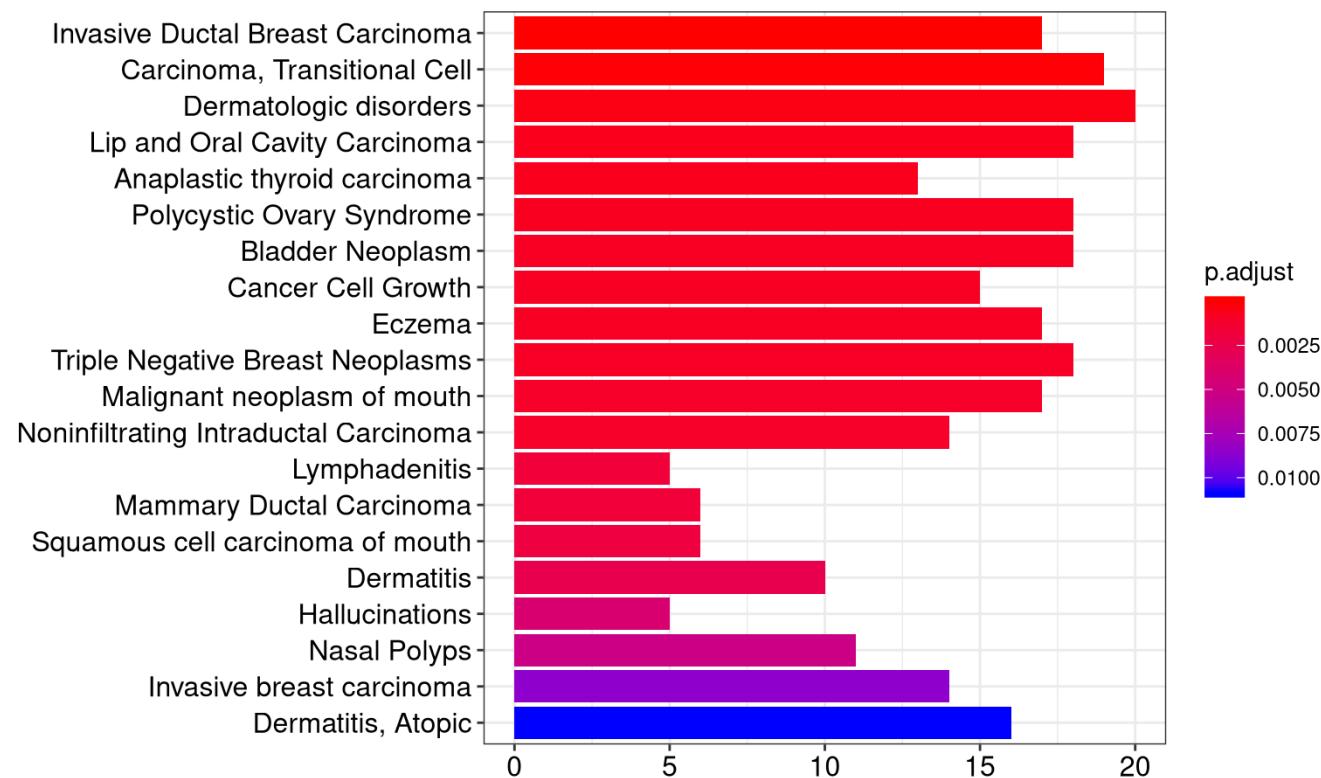
```
gseaplot(h_NK_vs_Th, geneSetID =  
"BREAST", title=" BREAST")
```



# Visualizations available in clusterProfiler

- barplot

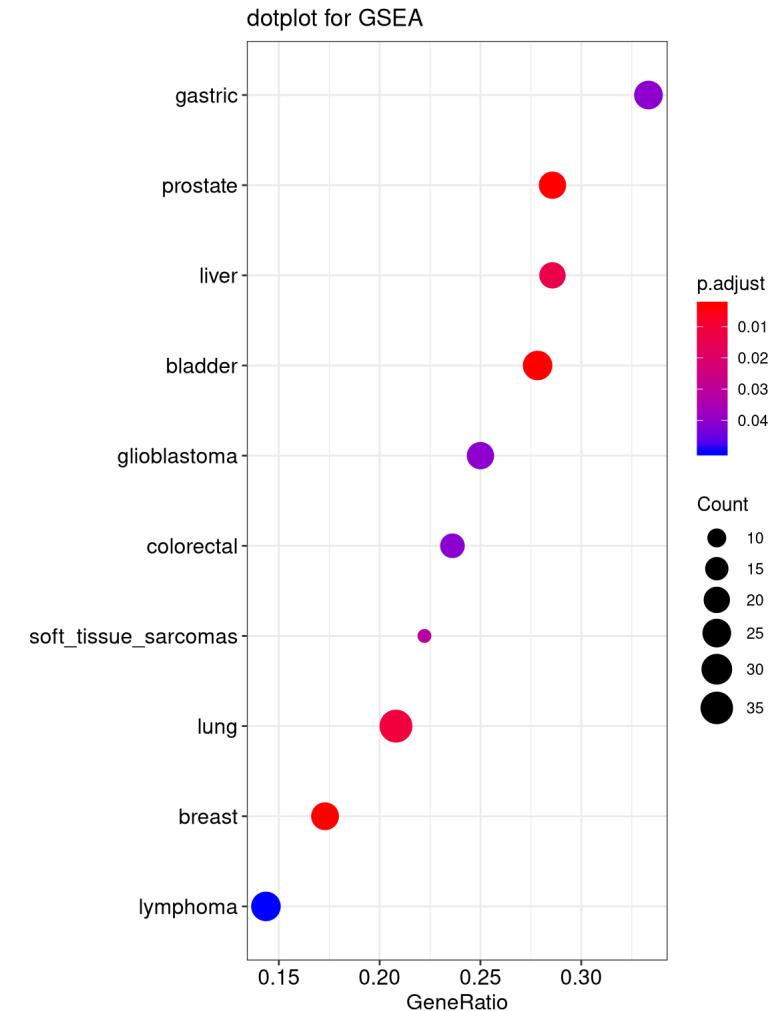
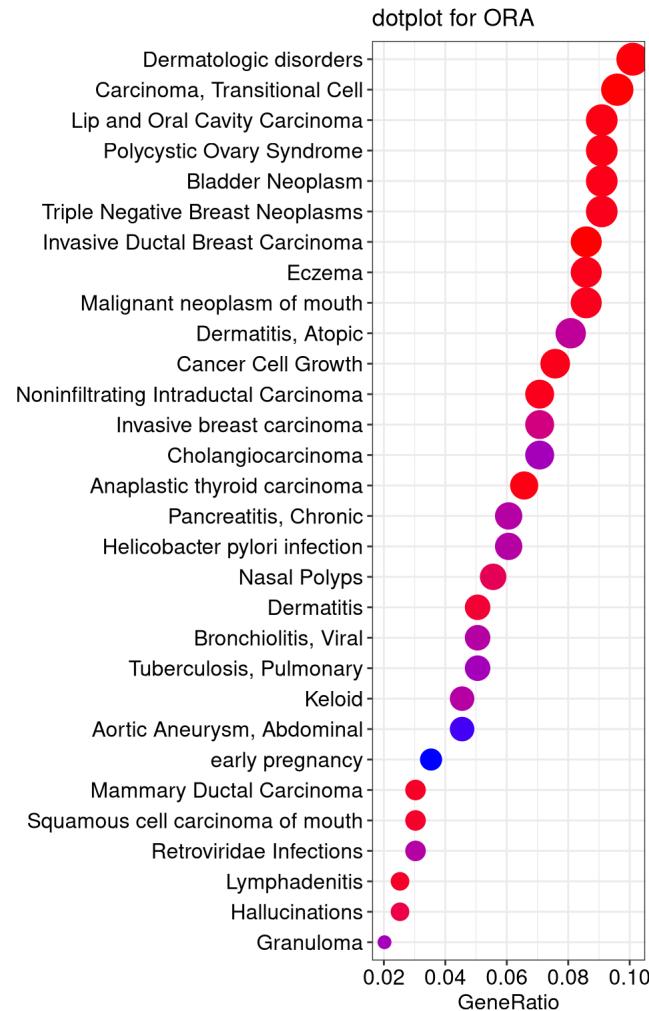
```
ego <- enrichGO(de, OrgDb='org.Hs.eg.db', ont="BP", keyType = "SYMBOL")
barplot(ego, showCategory=20)
```



# Visualizations available in clusterProfiler

- dotplot

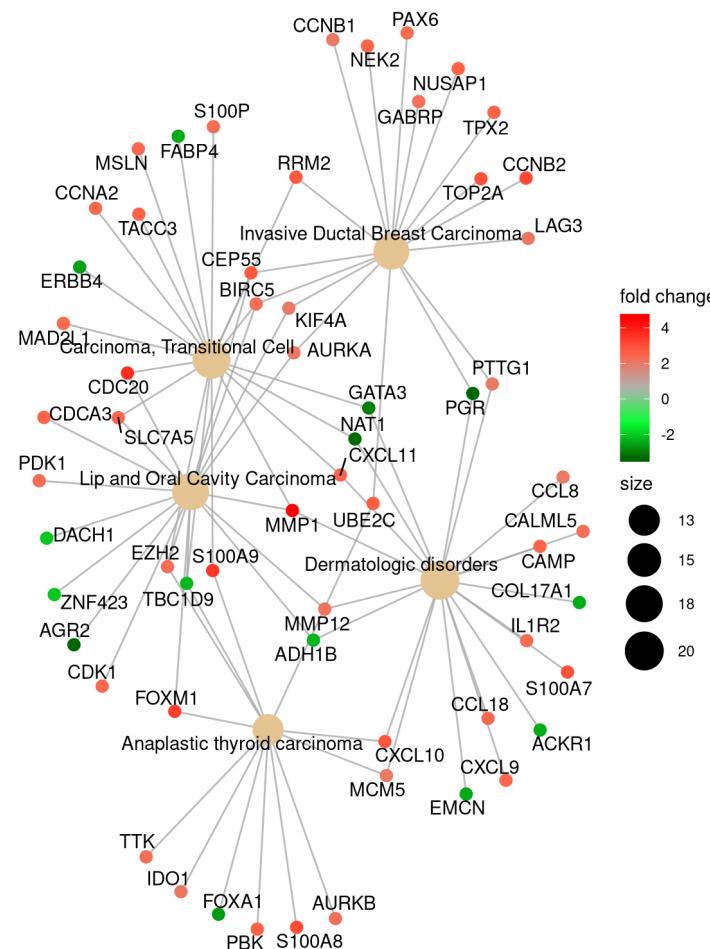
```
ego <- enrichGO(de)
dotplot(ego, showCategory=20)
```



# Visualizations available in clusterProfiler

```
cnetplot(edox, categorySize="pvalue", foldChange=geneList)
```

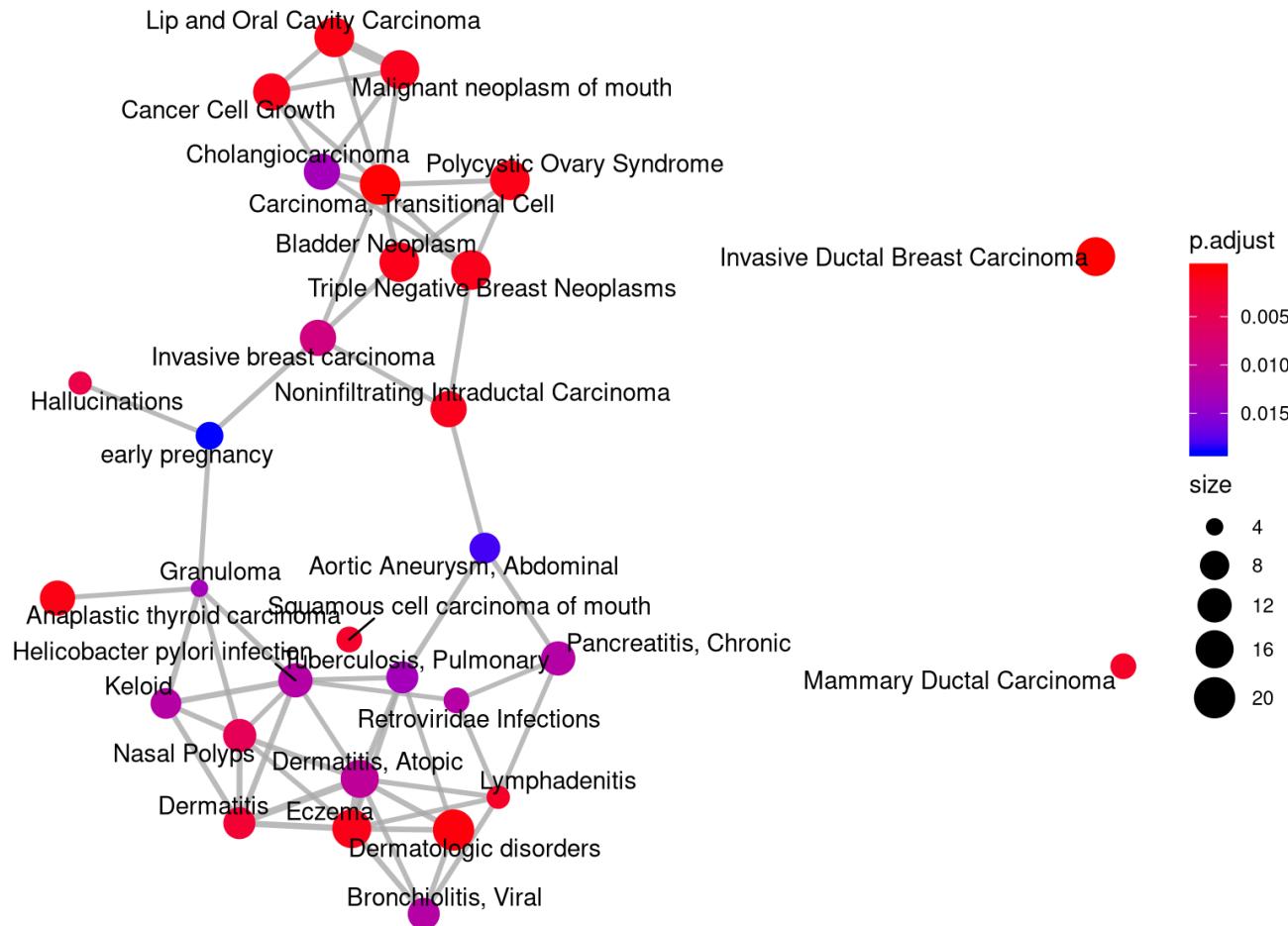
- Gene-concept network



# Visualizations available in clusterProfiler

- Enrichment map

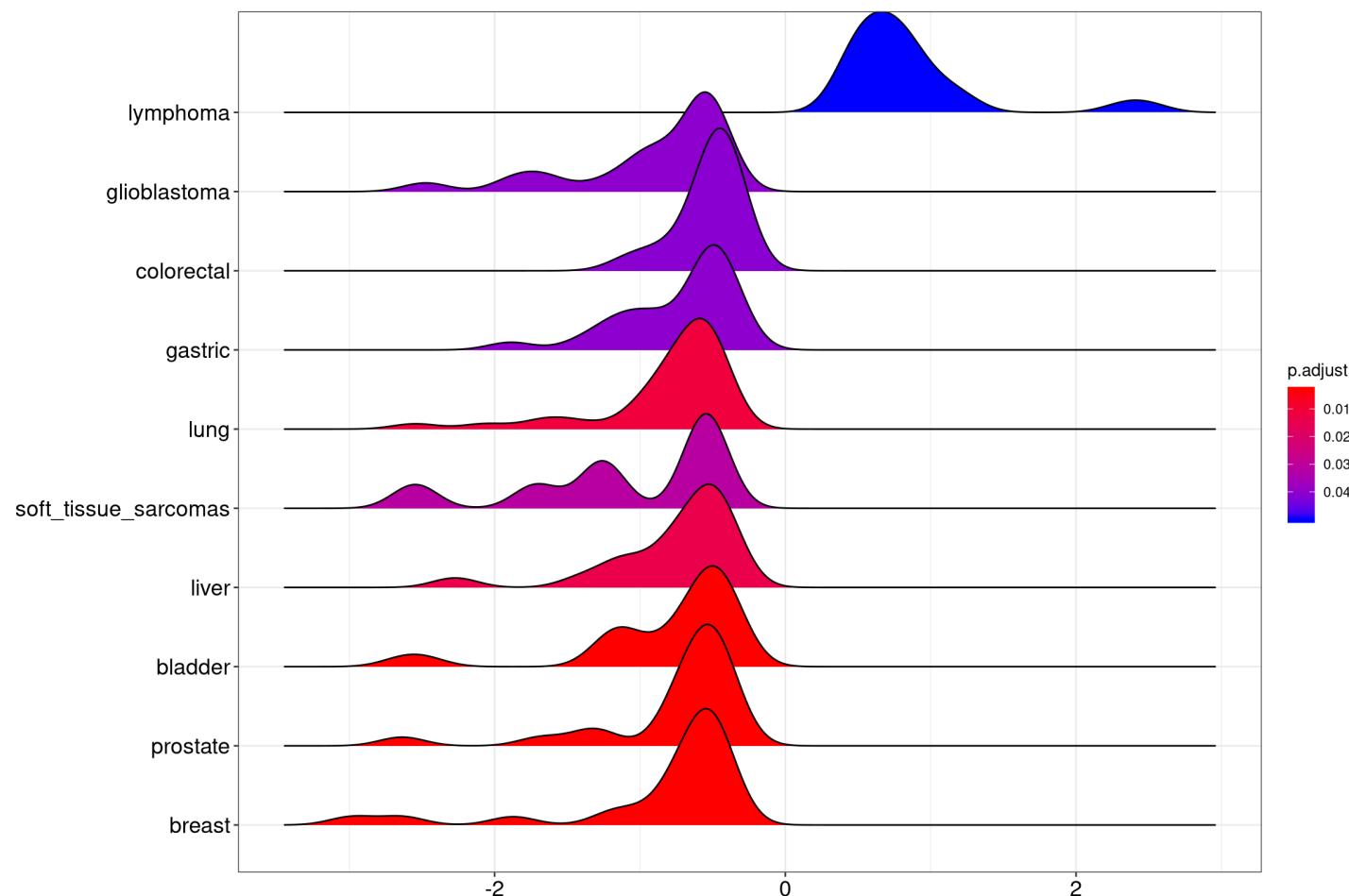
```
ego <- enrichGO(de)
emapplot(ego)
```



# Visualizations available in clusterProfiler

- Ridgeplot

```
ego <- gseGO(de)  
ridgeplot(ego)
```



# Recap and Exercise 3

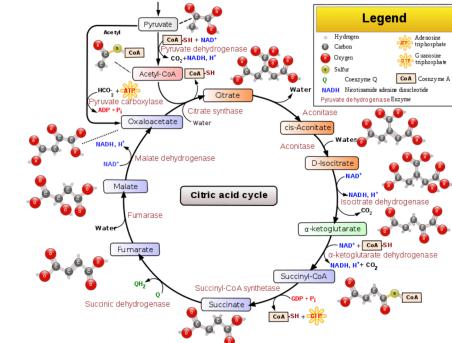
Several visualization methods can be used to represent the results, either for single gene sets (barcode plot) or for several gene sets (barplots, etc).

## Exercise 3: Create figures for the enrichment results:

- barplot of  $-\log_{10}(p\text{-value})$  of the p-values of the top 10 GO gene sets, or of positive and negative NES values
- Enrichment maps, gene-concept networks, ridge plots, etc

# What is a gene set?

[https://en.wikipedia.org/wiki/Citric\\_acid\\_cycle](https://en.wikipedia.org/wiki/Citric_acid_cycle)



- 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 SNPs associated with a disease
- ... etc!
- Several gene sets are grouped into Knowledge bases

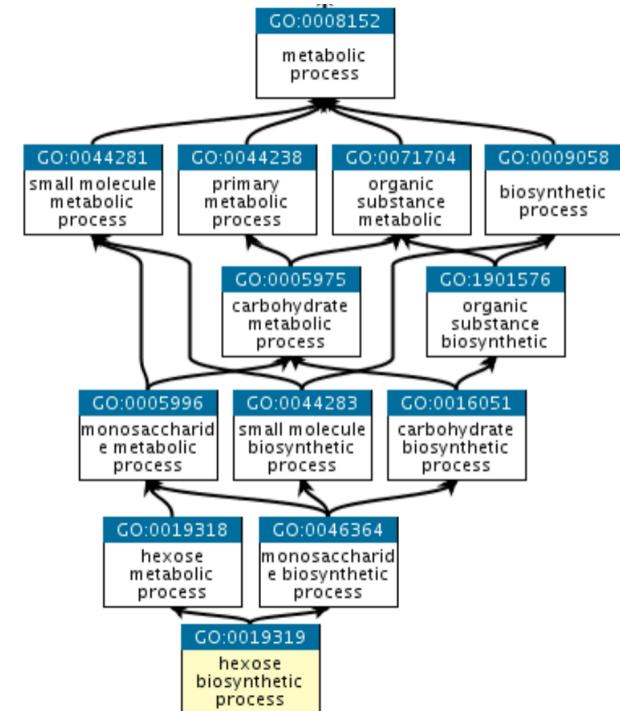
# Gene ontology

- <http://geneontology.org/>

Collaborative effort to address the need for consistent descriptions of gene products across databases

- GO Consortium: 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
- GO terms = GO categorizations
- GO term: each with a name (DNA repair) and a unique accession number (GO:0005125)

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



Not covered today: SetRank (cran), GOSemSim (bioconductor), Revigo (<http://revigo.irb.hr/>)

# Gene ontology

**GO ontologies: GO terms organized in 3 independent controlled vocabularies**

- **Molecular function:** represents the biochemical activity of the gene product, such activities could include "ligand", "GTPase", and "transporter".
- **Cellular component:** refers to the location in the cell of the gene product. Cellular components could include "nucleus", "lysosome", and "plasma membrane".
- **Biological process:** refers to the biological role involving the gene or gene product, and could include "transcription", "signal transduction", and "apoptosis". A biological process generally involves a chemical or physical change of the starting material or input.

# KEGG

<https://www.genome.jp/kegg/pathway.html>

Bi-directional eg mTOR signaling

KEGG Databases Mapper Auto annotation Kanehisa Lab



## KEGG PATHWAY Database

Wiring diagrams of molecular interactions, reactions and relations

KEGG2 PATHWAY BRITE MODULE KO GENES COMPOUND DISEASE DRUG

Select prefix   Enter keywords   Help

[ New pathway maps | Update history ]

### Pathway Maps

**KEGG PATHWAY** is a collection of manually drawn [pathway maps](#) representing our knowledge of the molecular interaction, reaction and relation networks for:

#### 1. Metabolism

Global/overview Carbohydrate Energy Lipid Nucleotide Amino acid Other amino Glycan  
Cofactor/vitamin Terpenoid/PK Other secondary metabolite Xenobiotics Chemical structure

#### 2. Genetic Information Processing

#### 3. Environmental Information Processing

#### 4. Cellular Processes

#### 5. Organismal Systems

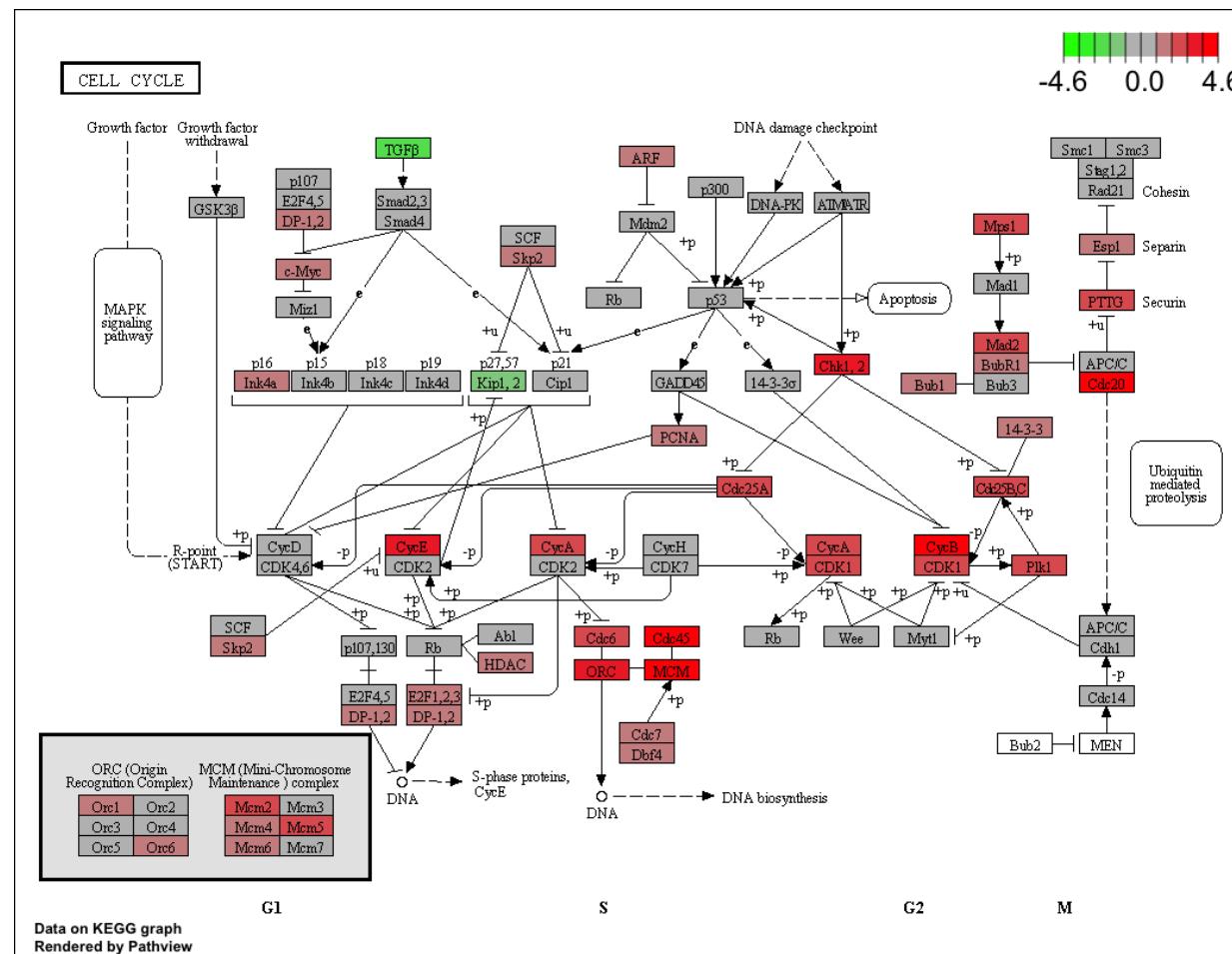
#### 6. Human Diseases

#### 7. Drug Development

KEGG PATHWAY is the reference database for pathway mapping in **KEGG Mapper**.

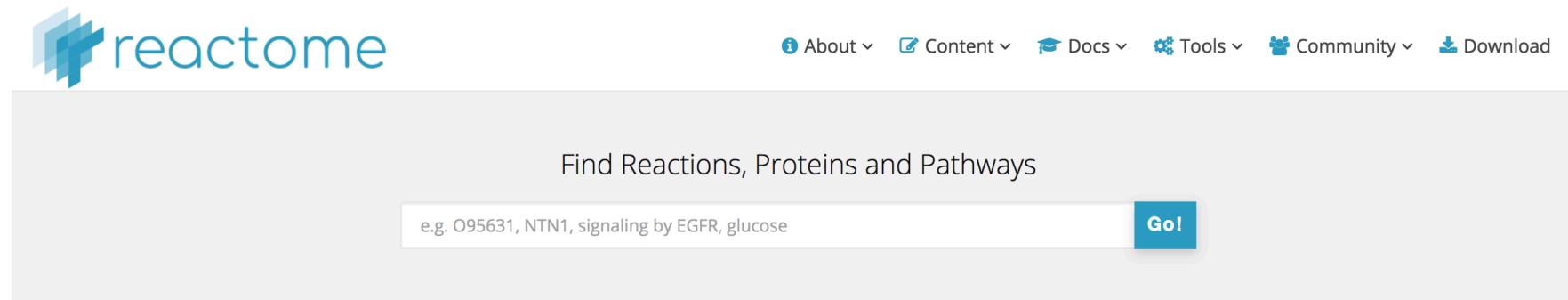
# Visualizations available - pathview package

```
hsa04110 <- pathview(gene.data = geneList, pathway.id = "hsa04110", species = "hsa",
limit = list(gene=max(abs(geneList)), cpd=1))
```



# Reactome

<https://reactome.org/>



The screenshot shows the Reactome website homepage. At the top left is the Reactome logo. To its right are navigation links: About, Content, Docs, Tools, Community, and Download. Below this is a search bar with the placeholder "Find Reactions, Proteins and Pathways" and a "Go!" button. Underneath the search bar are four large blue icons representing different tools: "Pathway Browser" (a tree diagram), "Analysis Tools" (a bar chart), "ReactomeFIViz" (a network graph), and "Documentation" (a document icon).

Tool	Description
Pathway Browser	Visualize and interact with Reactome biological pathways
Analysis Tools	Merges pathway identifier mapping, over-representation, and expression analysis
ReactomeFIViz	Designed to find pathways and network patterns related to cancer and other types of diseases
Documentation	Information to browse the database and use its principal tools for data analysis

# MSigDB

<https://www.gsea-msigdb.org/gsea/msigdb/index.jsp>

**H**

**hallmark gene sets** are coherently expressed signatures derived by aggregating many MSigDB gene sets to represent well-defined biological states or processes.

**C1**

**positional gene sets** for each human chromosome and cytogenetic band.

**C2**

**curated gene sets** from online pathway databases, publications in PubMed, and knowledge of domain experts.

**C3**

**regulatory target gene sets** based on gene target predictions for microRNA seed sequences and predicted transcription factor binding sites.

**C4**

**computational gene sets** defined by mining large collections of cancer-oriented microarray data.

**C5**

**ontology gene sets** consist of genes annotated by the same ontology term.

**C6**

**oncogenic signature gene sets** defined directly from microarray gene expression data from cancer gene perturbations.

**C7**

**immunologic signature gene sets** represent cell states and perturbations within the immune system.

**C8**

**cell type signature gene sets** curated from cluster markers identified in single-cell sequencing studies of human tissue.

Download gmt files with version number:

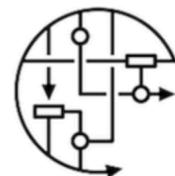
<https://www.gsea-msigdb.org/gsea/downloads.jsp>

The Hallmark collection:

<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4707969/>

# WikiPathways

<https://www.wikipathways.org/index.php/WikiPathways>



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- WikiPathways RDF
- Embed code

activity

- Browse pathways
- Recent changes
- New releases
- New pathways
- Edit pathways

page discussion view source history Log in / create account

**Share your pathway knowledge in the fight against COVID-19**

ACCESS the rapidly growing collection of COVID-19 pathways, CONTRIBUTE your time and domain knowledge about pathway biology as a pathway author, and USE these pathways in your research.

**Welcome to WikiPathways**

WikiPathways is a database of biological pathways maintained by and for the scientific community.

Read about our 12-year journey so far and [official exit from beta](#) or our [2021 NAR paper](#).

**Find Pathways**

**Search**

Search

You can search by:

- Pathway name (*Apoptosis*)
- Gene or protein name (*p53*)
- Any page content (*cancer*)

**Browse**

**Browse pathways**

**Browse by species and category**

**Get Pathways**

**Download**

**Growth**

COVID-19, thrombosis and anticoagulation (Homo sapiens)

**Today's Featured Pathway**

COVID-19, thrombosis and anticoagulation (Homo sapiens)

COVID-19, thrombosis and anticoagulation

**Curator of the Week**

Kristina Hanspers (Gladstone Institutes)

# GSEA of other gene sets in R

ClusterProfiler: GSEA for KEGG pathways

```
gseKEGG(geneList, organism = "hsa", keyType = "kegg", exponent = 1,
  nPerm = 1000, minGSSize = 10, maxGSSize = 500,
  pvalueCutoff = 0.05, pAdjustMethod = "BH", verbose = TRUE,
  use_internal_data = FALSE, seed = FALSE, by = "fgsea")
```

Import a .gmt file of gene sets and convert to format needed for clusterProfiler

```
read.gmt(gmtfile)

> head(term2gene_h)
      ont      gene
1 HALLMARK_TNFA_SIGNALING_VIA_NFKB JUNB
2 HALLMARK_TNFA_SIGNALING_VIA_NFKB CXCL2
3 HALLMARK_TNFA_SIGNALING_VIA_NFKB ATF3
4 HALLMARK_TNFA_SIGNALING_VIA_NFKB NFKBIA
5 HALLMARK_TNFA_SIGNALING_VIA_NFKB TNFAIP3
6 HALLMARK_TNFA_SIGNALING_VIA_NFKB PTGS2
```

conversion of gene ID types with clusterProfiler (or biomaRt package)

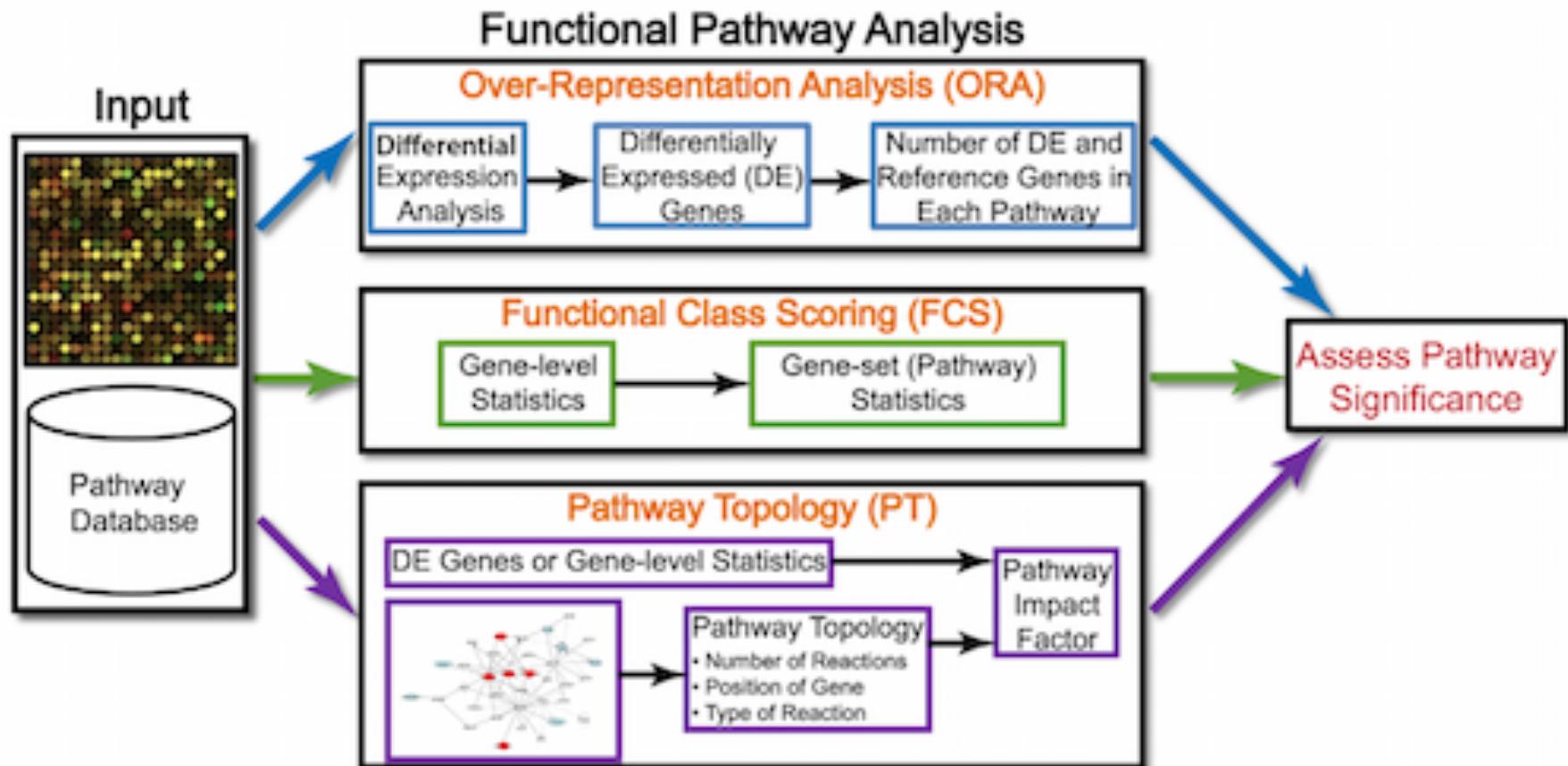
```
bitr(geneID, fromType, toType, OrgDb, drop = TRUE)
```

biomaRt: <https://bioconductor.org/packages/release/bioc/html/biomaRt.html>

# Recap and exercise 4

- We have seen how to perform GSEA using the built-in GO gene sets. Please perform GSEA with the built-in KEGG pathways, as well as with the hallmark gene sets obtained from MSigDB.
- Exercise 4: use functions of clusterProfiler and data provided in Ex. 1, and hallmark gene sets downloaded from MSigDB
  - First convert the gene symbols to EntrezID to perform a GSEA of KEGG pathways (with argument minGSSize=30).
  - Explore the results. Is there a KEGG immune-related gene set coming up? Is there a KEGG Natural killer gene set coming up?
  - Import the hallmark gene sets and run a GSEA. How many significant gene sets are there?

# Functional analysis



# Functional analysis: Pathway topology tools

Signaling pathway impact analysis (SPIA)

Identification of dys-regulated pathways: taking into account gene interaction information + fold changes and adjusted p-values from differential expression analysis

KEGG pathway	P <sub>NDE</sub>	P <sub>PERT</sub>	P <sub>G</sub>	P <sub>FDR</sub>	P <sub>FWER</sub>	Status
Focal adhe..4510	0.0001	0.0000	0.0000	0.000000	0.00000	Act.
ECM-recept..4512	0.0001	0.0004	0.0000	0.00001	0.00002	Act.
PPAR signa..3320	0.0000	0.1240	0.0000	0.00011	0.00034	Inh.
Alzheimers..5010	0.0000	0.7260	0.0001	0.00059	0.00235	Act.
Adherens j..4520	0.0001	0.0852	0.0001	0.00090	0.00452	Act.
Axon guida..4360	0.0002	0.2324	0.0006	0.00487	0.02922	Act.
MAPK signa..4010	0.0001	0.7112	0.0007	0.00504	0.03527	Inh.
Tight junc..4530	0.0007	0.5156	0.0032	0.02073	0.16585	Act.

$$P_{NDE} = P(X \geq N_{DE} | H_0)$$

P<sub>PERT</sub>: probability to observe a larger perturbation than observed

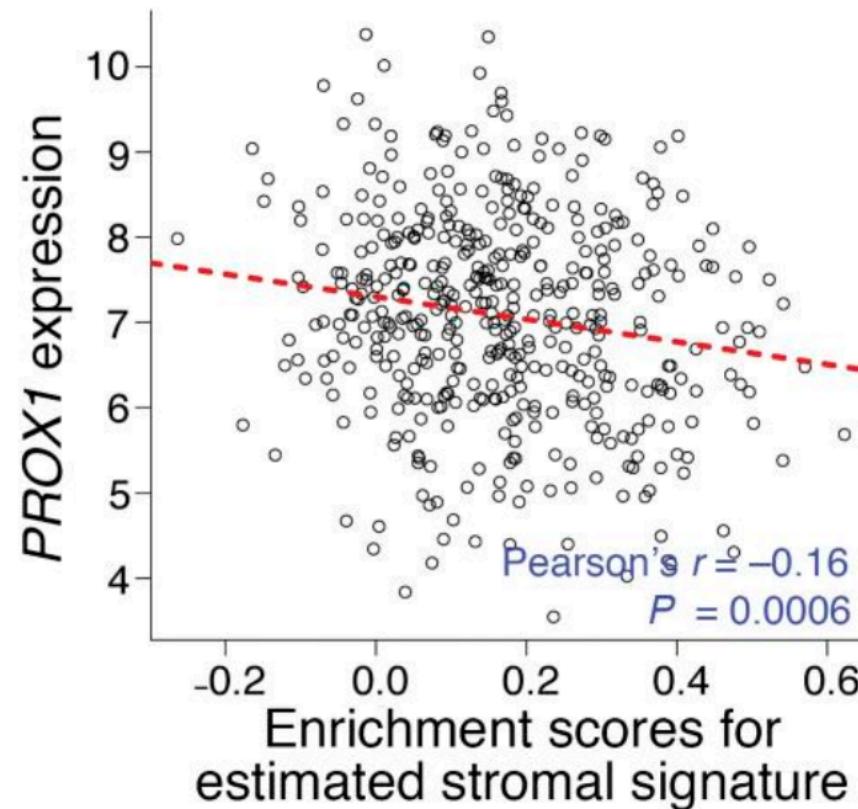
P<sub>G</sub>: combination of P<sub>NDE</sub> and P<sub>PERT</sub>

P<sub>FDR</sub>: adjusted FDR p-value

P<sub>FWER</sub>: adjusted FDR p-value (more conservative)

<https://bioconductor.org/packages/release/bioc/html/SPIA.html>

# Single-sample gene set variation analysis



GSVA:

<https://bioconductor.org/packages/release/bioc/html/GSVA.html>

<https://www.jci.org/articles/view/129558>

# Credits: 0.25 ECTS

- Please provide answers and R code for an additional exercise (eg 1 Word with answers and figures and 1 script file, or 1 file generated from Rmarkdown)

[https://sib-swiss.github.io/enrichment-analysis-training/exercises/  
#extra-exercise-for-ects-credits](https://sib-swiss.github.io/enrichment-analysis-training/exercises/#extra-exercise-for-ects-credits)

- Sign up for credit by adding your name to the google Doc file (email sent by course organizer)
- Send answers to [tania.wyss@sib.swiss](mailto:tania.wyss@sib.swiss) within 1 week

Thank you for your attention!

Please fill in the **feedback** sent by the course organizer.

We thank Isabelle Dupanloup and Linda Dib for providing course material



# Bioconductor

- **GO.db** : A set of annotation maps describing the entire Gene Ontology assembled using data from GO
- **gskb: mouse**
  - mm\_GO: gene sets from Gene Ontology for mouse (*Mus musculus*)
  - mm\_location: Gene sets based on chromosomal location
  - mm\_metabolic: metabolic pathways
  - mm\_miRNA: Target genes of microRNAs, predicted or experimentally verified
  - mm\_pathway: Curated pathways
  - mm\_TF: Transcription factor target genes.
  - mm\_other
- **KEGG.db**: A set of annotation maps for KEGG assembled using data from KEGG

# Note on gene ID conversion

When converting, not all genes have a matching ID type, or some genes have duplicated IDs (eg symbols).

- A good idea is to obtain Ensembl ID-to-symbol conversion for your reference genome version:
- Download gtf from Ensembl:  
<http://www.ensembl.org/index.html>
- [ftp://ftp.ensembl.org/pub/release-100/gtf/homo\\_sapiens/](ftp://ftp.ensembl.org/pub/release-100/gtf/homo_sapiens/)
- Download as a data frame with refGenome package (archived)
- <https://cran.r-project.org/web/packages/refGenome/index.html>

# Differential gene expression analysis

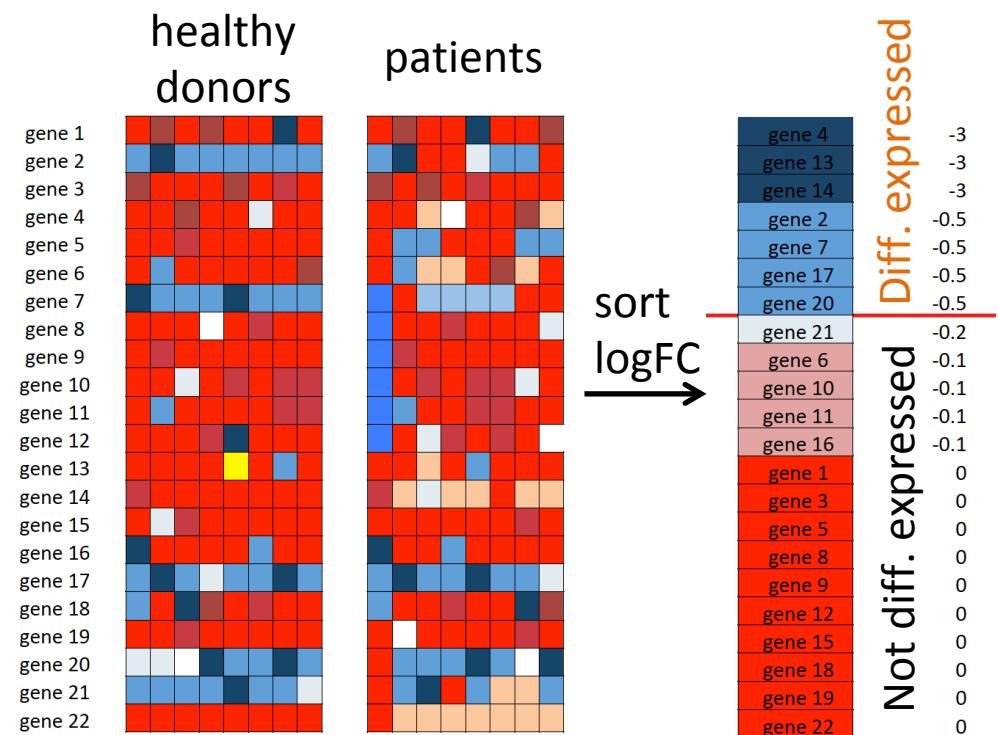
- Comparing 2 groups:

For each gene  $i$ , is there a **difference** in expression between control and patients?

- Fold change in genomics:

$$\log_2 \text{of ratios} = \log \text{fold change}$$

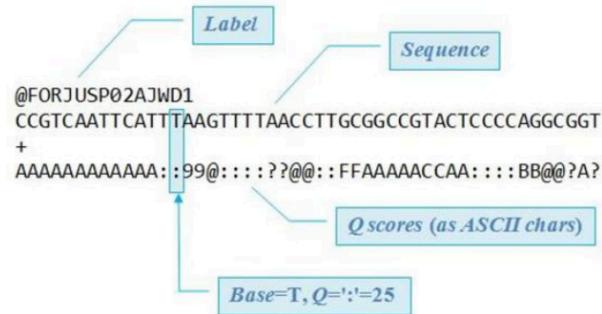
$$\log(\pi_{i1}/\pi_{i2}) = \log(\pi_{i1}) - \log(\pi_{i2})$$



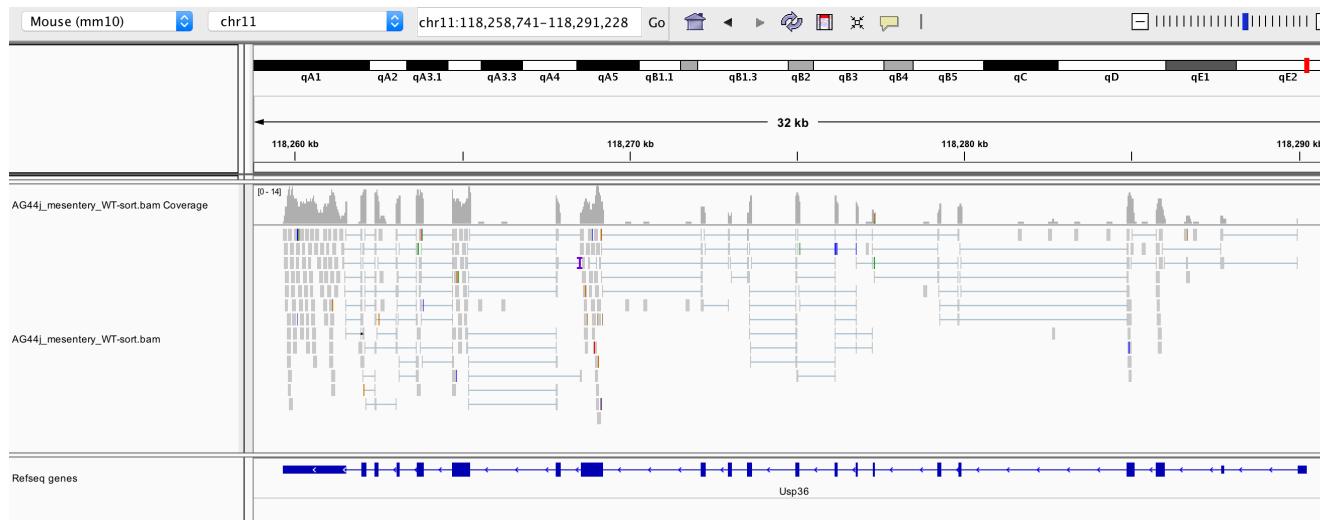
# Typical RNA sequencing analysis workflow

fastq file:

```
@HWI-M01141:63:A4NDL:1:1101:14849:1418 1:N:0:TATAGCGAGACACCGT  
NACGAAGGGTCAAGCGTTACTCGGAATTACTGGCGTAAGCGTGCCTAGGTGGTTT/  
+  
#>>>A??FAA1BGGEGGAAFGCA@BFF1D2BCF/EEG/DBEE/E?GAEEFGAEAFGJ  
@HWI-M01141:63:A4NDL:1:1101:13802:1421 1:N:0:TATAGCGAGACACCGT  
NACGGAGGGTCAAGCGTTAACCGAATTACTGGCGTAAGCGCACGCAGCGGTGTTT/  
+  
#>>AAABBBABBGGGGGGGG?FHGGGGGGHHHHHHHHGGGGH  
@HWI-M01141:63:A4NDL:1:1101:15928:1426 1:  
NACGTAGGGTCCAGCGTTAACCGAATTACTGGCGTAAA/  
+  
#>>AABFB@FBGGGGGGGGGGHGGGGFHHHHHHHGGGGH  
@HWI-M01141:63:A4NDL:1:1101:14861:1431 1:  
NACGAAGGGTCAAGCGTTACTCGGAATTACTGGCGTAAA/  
+  
#>>AAAABBFABGGGGGGCEGHGGEFFHHHHHHGGGGH  
@HWI-M01141:63:A4NDL:1:1101:15264:1465 1:  
NACGTAGGGTGCAGCGTTCTCGGAATTACTGGCGTAAA/  
+
```



Filter quality  
Align to ref. genome



count reads  
→ per gene

Downstream  
statistical analysis:  
R: import  
counts table

# Some additional resources

- g:Profiler - <http://biit.cs.ut.ee/gprofiler/index.cgi>
- DAVID - <http://david.abcc.ncifcrf.gov/tools.jsp>
- clusterProfiler - <http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>
- GeneMANIA - <http://www.genemania.org/>
- GenePattern - <http://www.broadinstitute.org/cancer/software/genepattern/> (need to register)
- WebGestalt - <http://bioinfo.vanderbilt.edu/webgestalt/> (need to register)
- AmiGO - <http://amigo.geneontology.org/amigo>
- ReviGO (visualizing GO analysis, input is GO terms) - <http://revigo.irb.hr/>
- WGCNA - <http://www.genetics.ucla.edu/labs/horvath/CoexpressionNetwork>
- GSEA - <http://software.broadinstitute.org/gsea/index.jsp>
- SPIA - <https://www.bioconductor.org/packages/release/bioc/html/SPIA.html>
- GAGE/Pathview - <http://www.bioconductor.org/packages/release/bioc/html/gage.html>