Introduction to Gene Set Enrichment

Lecture Outline

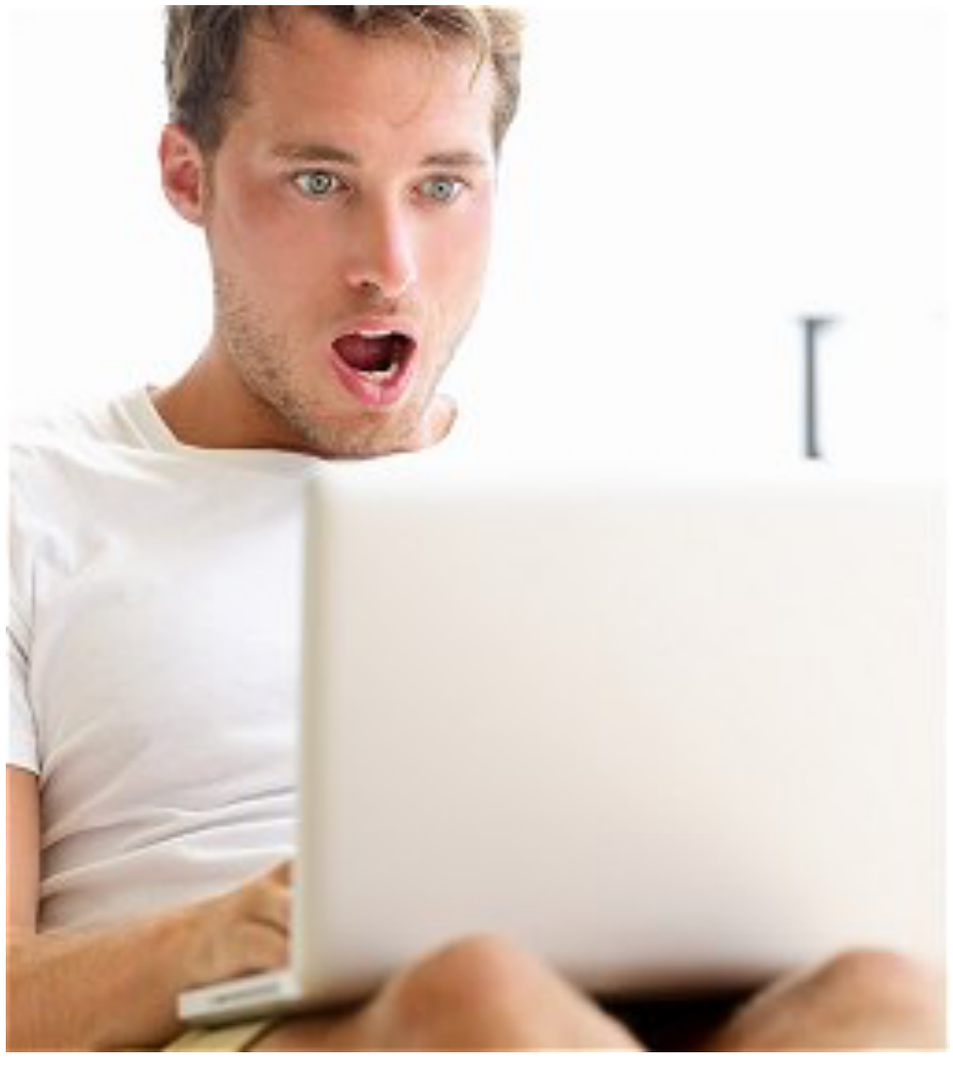
- Some theory:
  - How to analyse large gene lists.
  - The hypergeometric test and Fisher's Exact test
  - Gene set annotations.
  - Some online/R tools
- Exercise:
  - Enrichment of GO-term in FANTOM5 tissue specific genes
- Some more advanced theory.

Interpretation of large gene lists

What to do when you are done?

- This lecture starts when all of the previous lectures have ended:
    - You have performed a high-throughput experiment (RNA-Seq, CAGE, microarray, etc.)
    - You have QC'ed and trimmed your data.
    - You have explored the data (for example using PCA and clustering)
    - You have performed a valid statistical test to obtain lists of "interesting" genes.
- *… What do you do now?*

What you SHOULD NOT do



- It is common to see people to this:
  - *“I looked at my list of 924 genes and saw three genes from my favourite gene family (that I’m doing my thesis on). I therefore conclude that genes from my favourite gene family are the central regulators of the biological response, just as I suspected prior to the experiment.”*
- What is wrong with this approach?

It is extremely easy to over-interpret data such as this!

- There are several problems with this approach:
  - If your list of interesting is long enough, you are bound to choose at least some of your favourite genes by chance.
  - If the favourite gene family is big, the chance of finding these by change is very big.
  - It is very biased to only look at a single gene family - what about categories of genes?
  - It is important not to be biased towards what you expect to see and accept the fact that the experiment might not show what you want/expect it to show.
  - It is easy to tell “stories” - but are they actually true (/ statistically valid)?
- In this lecture we will talk about how to analyse large gene lists, while keeping these important points in mind.

"Ralph's over-interpreting the data again."

Gene set annotations

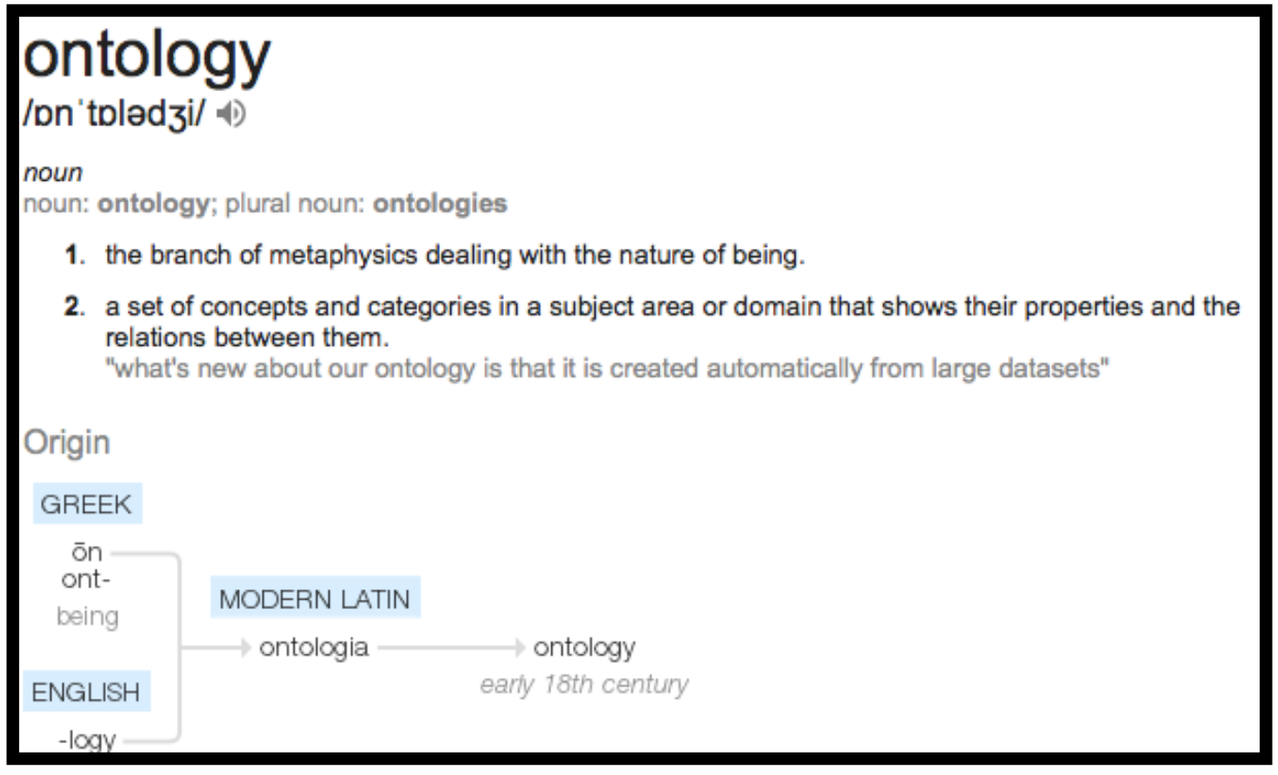
Where to obtain annotations

- The first step in analysing gene tests is to obtain some information about the genes. For example looking at:
    - Gene families
    - Protein domains
    - KEGG pathways
    - Reactome
    - MySigDb
    - Disease-associated genes
    - Gene Ontology (GO) terms

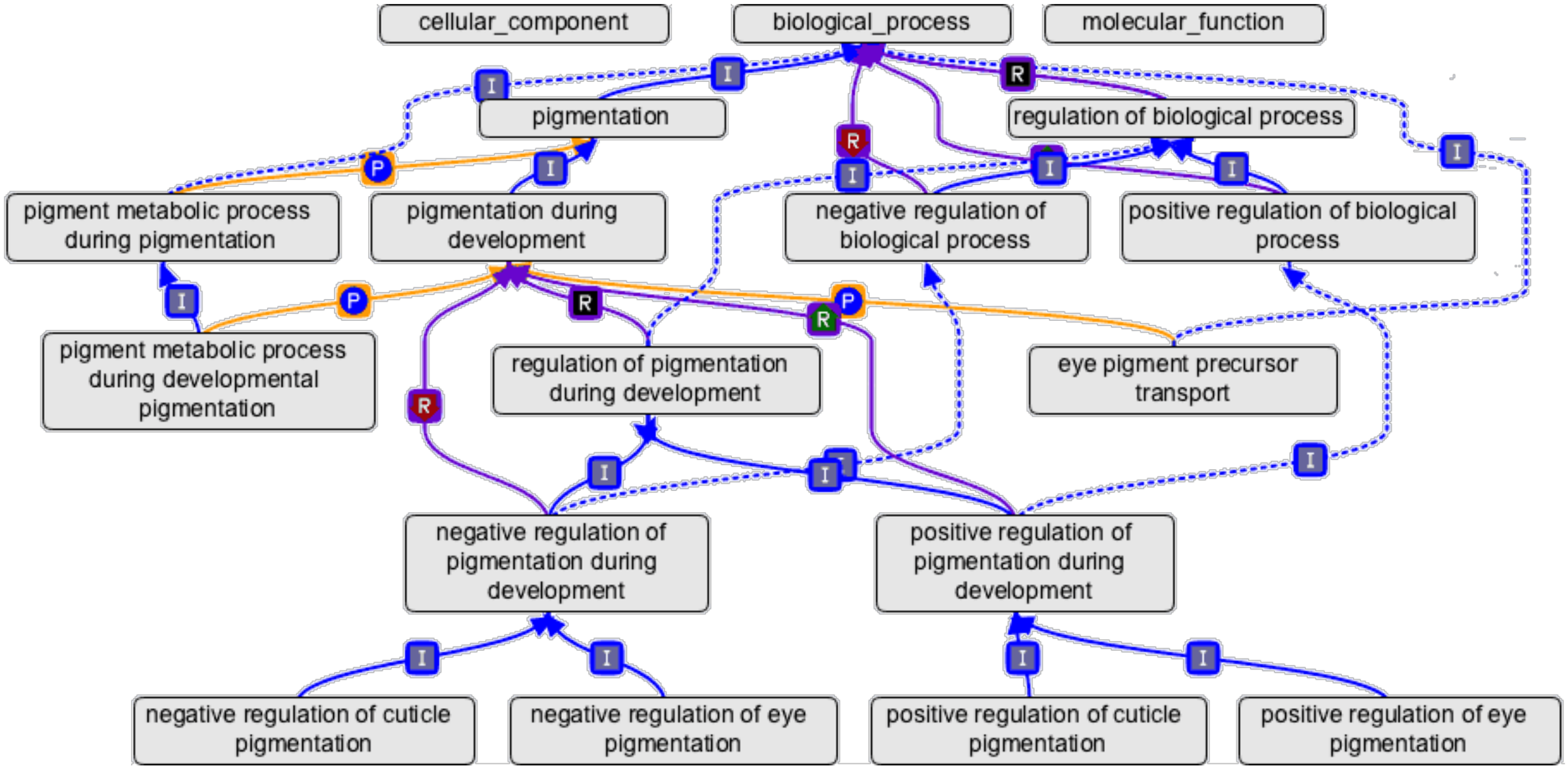
- GO-terms are by far the most widely used, so we will focus at them in this lecture.

GO-terms in a single slide

- Online database:
  - Gene Ontology is a collection of controlled vocabularies describing the biology of a gene product in any organism.
  - Includes both manually curated annotations, as well as electronically inferred.
  - Continuously updated.
- Three domains:
  - Cellular Component (CC)
  - Molecular Function (MF)
  - Biological Process (BP)



GO-term hierarchy



Exercise: 5 minutes

- Go the the GO-term consortium website: http://geneontology.org/ and look up a gene:
  - Left half of class: **DEFA5**
  - Right half of class: **MUC5AC**
- What do the genes do? What GO-terms do they have associated?

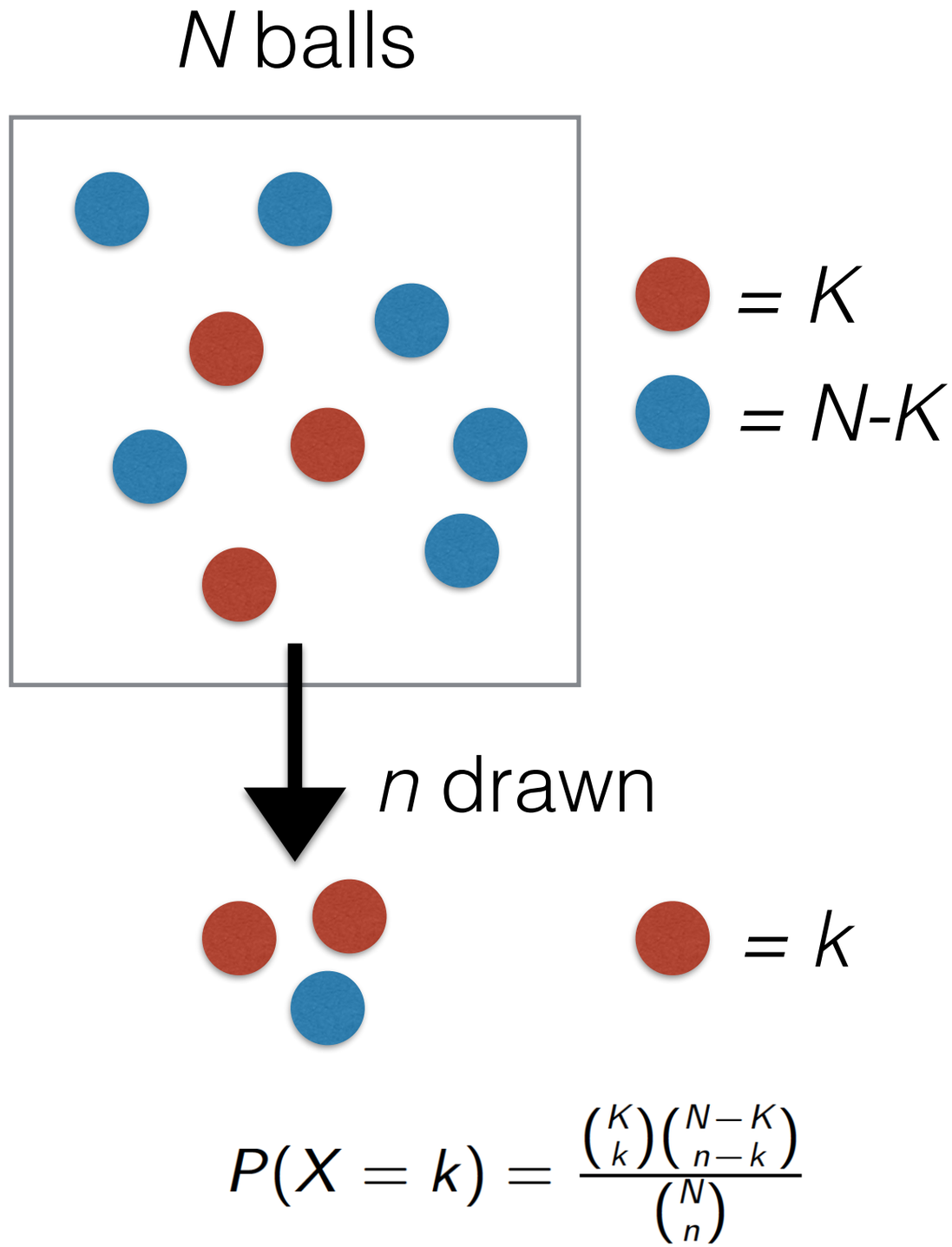
Hypergeometric testing

Intuition

- The question we want to ask is:
    - Are there GO-terms that appear more often in my list of interesting genes than what would be expected by chance (If genes were picked at random)?
- If you remember back to your course(s) in statistics, this is a problem of *sampling without replacement,* which can be described with a *hypergeometric* distribution.

The hypergeometric distribution

- Consider the following example:
  - You have a box containing *N* balls, of which *K* are red and *N* - *K* are blue
  - You draw *n* balls from the box without putting balls back after each draw
  - The hypergeometric distribution describes the probability of drawing *k* red balls.



$$P(X = k) = \frac{\binom{K}{k}\binom{N-K}{n-k}}{\binom{N}{n}}$$

R's hyper

R has excellent built in functions for working with a wide range of probability distributions, including the hypergeometric:

```
# Setting all the variables with some random numbers
N <- 100 # The total number of balls
K <- 25 # The number of red balls, aka the "successes"
n <- 10 # The number of draws from the box, aka. the "trials"
k <- 4 # The number of observed red balls when drawing

# Using the hypergeometric distribution:
phyper(q=k-1, # We want equal or bigger
            m=K,
            n=N-K,
            k=n,
            lower.tail=FALSE) # P[X > x]
## [1] 0.2146156
```

This means that the chance of drawing 4 (out of 10) or more red balls from a box of 25 (out of 100) is 0.21.

Exercise: 5 minutes

For the following three cases, calculate which was the least likely outcome under random sampling without replacement:

N=100, K=25, n=20, k=15

N=100, K=25, n=30, k=20

N=100, K=25, n=2, k=2

Hypergeometric test

- We can see this probability as a p-value for the following hypotheses:
    - Null hypothesis (H_0): We are sampling red balls at random
    - Alternative hypothesis (H_1): We are sampling *more* red balls than at random.

- Note, that since we have been using the upper tail of the distribution, we are in effect performing a one-sided test. This is often called a test for *enrichment*. Similarly, we could have used the lower tail of the distribution to perform a test for *depletion*.

How would this work with genes?

The above example can be easily translated to genes:

```
# Continuing the example from previously:
N <- 10000 # The total number of genes
K <- 50 # The number of gene belonging to a gene family
n <- 942 # The list of interesting genes
k <- 3 # The number of gene family members in the interesting genes

# Using the hypergeometric distribution:
phyper(q=k-1, m=K, n=N-K, k=n, lower.tail=FALSE) # P[X > x]
## [1] 0.8624552
```

Not very significant!

Fisher's Exact Test

A popular way of doing a hypergeometric test is to do a Fisher's Exact test. This tests starts from a 2-by-2 contingency table:

```
# Continuing the example from previous:
N <- 10000 # The total number of genes
K <- 50 # The number of gene belonging to a gene family
n <- 942 # The list of interesting genes
k <- 3 # The number of gene family members in the interesting genes

# Rearrange the data as a 2x2 table
m <- matrix(c(k, K - k, n - k, N - K - n + k),
            2, 2, dimnames = list(c("In List", "Rest"),
                                  c("In Family", "Rest")))
```

```
m
##             In Family  Rest
## In List             3   939
## Rest               47  9011
```

R’s fisher.test

Fisher’s exact test is included in base R:

```
res <- fisher.test(x=m, alternative="greater")

res
##
## Fisher's Exact Test for Count Data
##
## data: m
## p-value = 0.8625
## alternative hypothesis: true odds ratio is greater than 1
## 95 percent confidence interval:
## 0.1612245 Inf
## sample estimates:
## odds ratio
## 0.6125624

res$p.value
## [1] 0.8624552
```

Hypergeometric Test vs Fisher's Exact Test

- What test to use?
  - For single-sided tests, gives identical results.
  - fisher.test allows for easier calculation of two-sided p-values.
  - fisher.test also calculates the odds ratio.
  - It is very simple to make 2-by-2 contingency tables in R using the table()-function, making it easy to apply fisher.test to big datasets.

Online tools

Tools for GO-term enrichment

- While the underlying statistics for GO-term enrichment is rather simple, implementing it in code is a bit tricky.

- There are some issues with matching gene names across different databases.

- Using an online tool can solve both of these issues, at the cost of resorting to “copy-paste” analysis, prone to manual errors.

Popular tools

- Some popular (stand-alone) online tools are:
  - DAVID: https://david.ncifcrf.gov/
  - PANTHER: http://www.pantherdb.org/
  - GOrilla: http://cbl-gorilla.cs.technion.ac.il/
  - gProfiler: http://biit.cs.ut.ee/gprofiler/ ===> My favourite!
  - … and many more!
- Most of the these tools will, in addition to the actual enrichment analysis also help with annotating genes with GO-terms.
- **Small warning:** Be wary of online tools that are no longer kept updated. For example, the popular GOstats tools **(**http://gostats.com/**)** is actually no longer being updated

What should be uploaded to an online tool?

- List of interesting genes or *foreground set*, all with the same id-type (i.e. gene symbol, Entrez ID, etc.)

- A *background set* or *universe.*

- **WARNING**: Many tools will run without a universe input, and instead use the all genes in the genome as background - why is this often a bad idea?

FANTOM5 tissue specific genes - a GO exercise

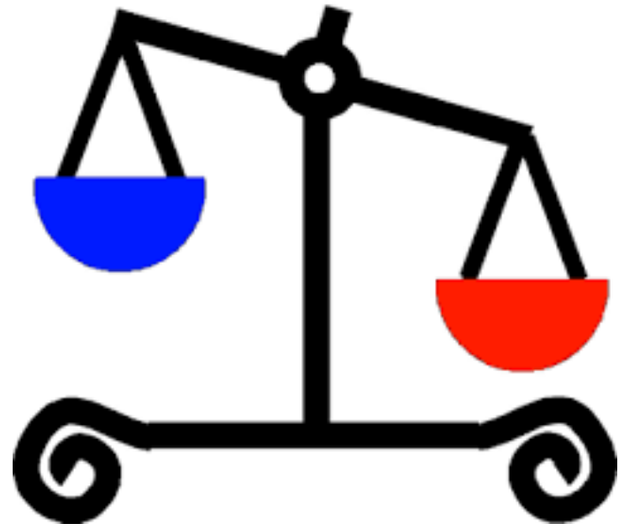
See course web page

Take home messages

- Converting between IDs can be very difficult and can affect results.
- p-values depends a lot on sample sizes.
- GO-enrichments are often ambiguous!
- Be very careful about overinterpretation!

Extensions of the hypergeometric test

- Use a “biased urn” model:
  - Some genes have a higher chance of being samples
  - Tools: goSeq, limma’s goana
- Use a more sophisticated FDR correction:
  - GO-term enrichments p-values are not really independent, since the same gene can be in multiple of the same groups.
  - Take the GO-term hierarchy into accounts when accounting for multiple testing



Threshold-free approaches

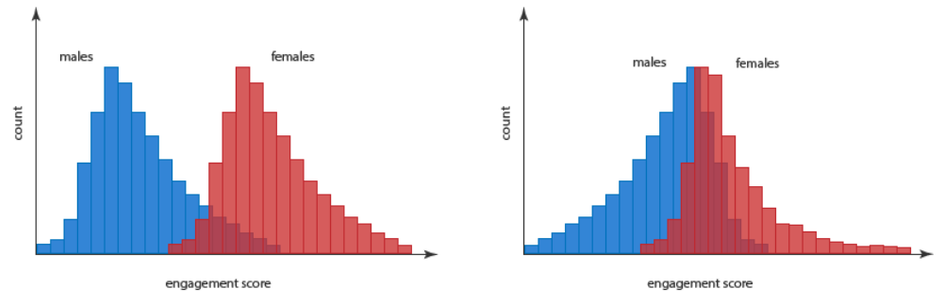
- In many cases the “interesting vs rest” is artificial, (i.e. unregulated vs non-regulated).

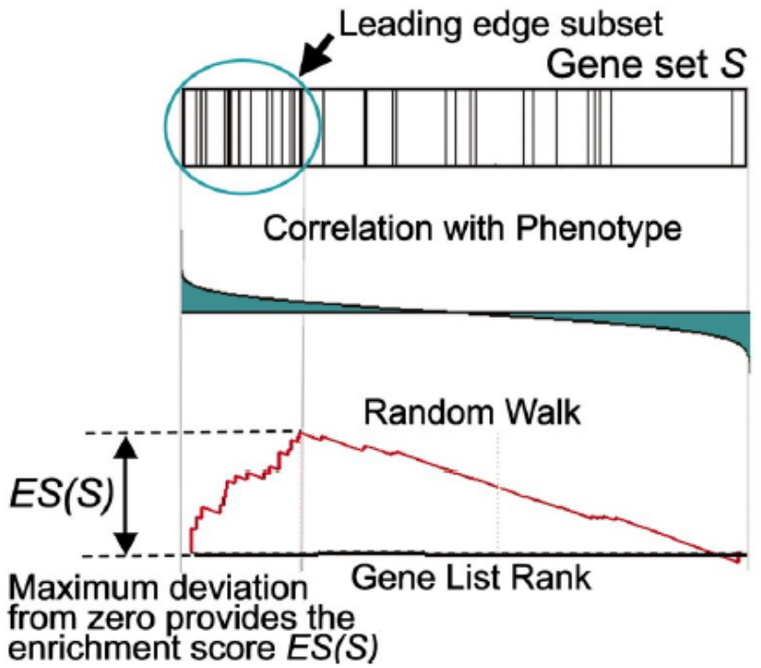
- Real signal is smooth across genes - i.e. some genes are VERY upregulated, some only a little.

- Calculate some statistics for each gene (logFC, t-statistics, etc), and perform a test (i.e. Wilcox or t-test) for difference between genes in and outside of a geneset.

- Some statistical issues arise when there is intergene correlation within gene sets. This can be corrected using Variance Inflation Factors (i.e. limma’s CAMERA)

- The popular GSEA method uses a running-sum maximum instead of simple test.





Subramanian et al., 2005, fig 1.

More detailed statistics

- Goeman *et al* 2008 (http://www.ncbi.nlm.nih.gov/pubmed/17303618) introduced the statistical foundation for gene set testing:
  - **Competitive tests:** Is a gene set enriched relative to other genes?
    - *Permutation over genes*
    - Hypergeometric and friends + Foreground/background methods
    - Issues with intergene correlations
  - **Self-contained tests:** Is a gene set associated with the outcome in an absolute sense (without looking at other genes)?
    - *Permutation over samples.*
    - More advanced statistics: globalTest and limma's ROAST.
    - Preserves intergene correlation, but suffers from issues with low sample size.