ADER17

Functional Enrichment Analysis

Extracting meaning from a list of genes

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Git
CG18519
del
CG13319
RNaseX25
CG15611
CG11309
CG1657
CG6404
CG9987
sfl
CG4300
CG4065
phr6-4
Myo31DF
...
```

- ‘Omics lists of “interesting” genes (e.g., RNAseq, microarrays, proteomics) are essentially meaningless on their own
- We’re typically interested in understanding phenomena at the cellular or organismal level, rather than the gene level

Extracting meaning from a list of genes

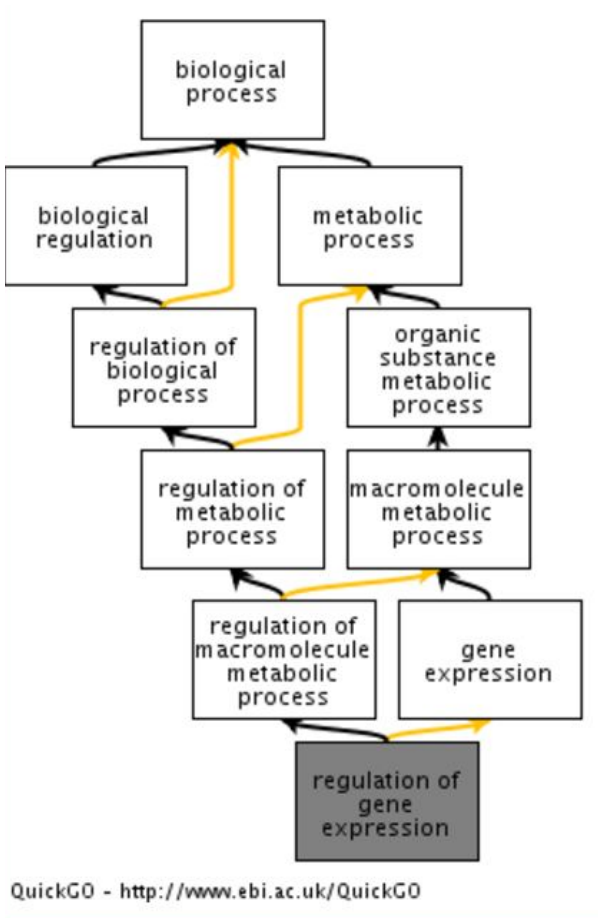
- In order to extract meaning from our list we have to abstract from the gene level to a higher level, in concert with the biological question we are addressing
- We’re focusing on the most common case, which is the functional level, but enrichment analysis can also be applied to other biologically meaningful aspects (e.g., the chromosome level, the transcription regulation level, etc)
- To go from the gene level to the functional level, we need a functional classification of our genes

Functional annotations

- Functional annotations are assignments of functional classifications to genes
- There are multiple functional classifications in use for different functional aspects:
  - Enzymes: EC classification
  - Metabolic pathways: KEGG
- The Gene Ontology (GO) is the most comprehensive functional classification scheme available, and thus is the most commonly used for enrichment analysis

The Gene Ontology

- GO is a functional classification scheme that covers three levels of gene function, called GO types or aspects:
  - Molecular Function: the individual functional level (e.g., GTPase, transcription factor)
  - Biological Process: the cellular and/or organismal functional level (e.g., signalling, muscle development)
  - Cellular Component: the locational level (e.g., cellular membrane, nucleus)
- Each GO type is in essence a (near-)independent classification scheme

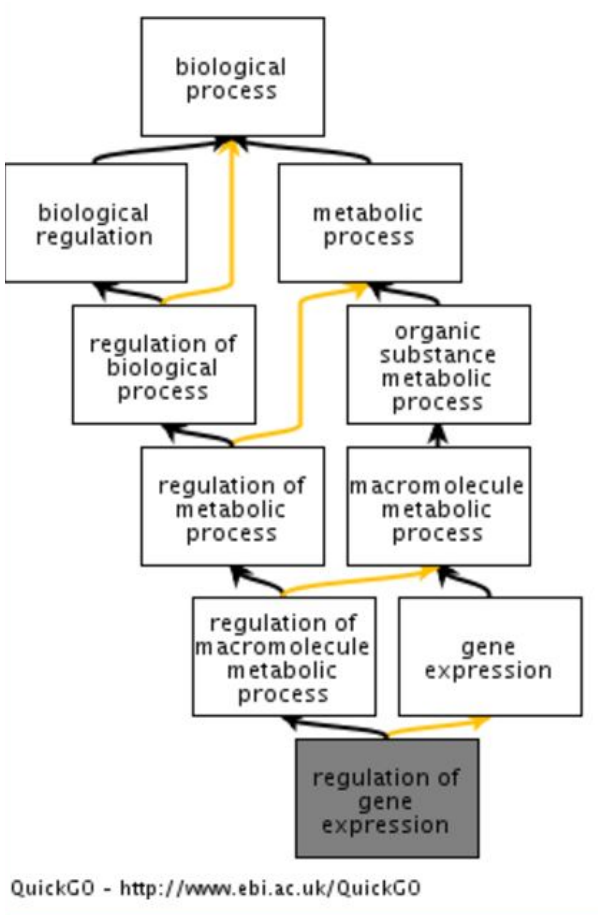


The Gene Ontology

- Each GO type is structured as a directed acyclic graph (i.e., a relaxed hierarchy with multi-parenting)
- In addition to subclass (‘is a’) relations, there are ‘part of’, ‘regulates’, and ‘occurs in’ relations
- Although GO types are ‘is a’ orthogonal, *molecular functions* can be ‘part of’ *biological processes*, and both can ‘occur in’ *cellular components*

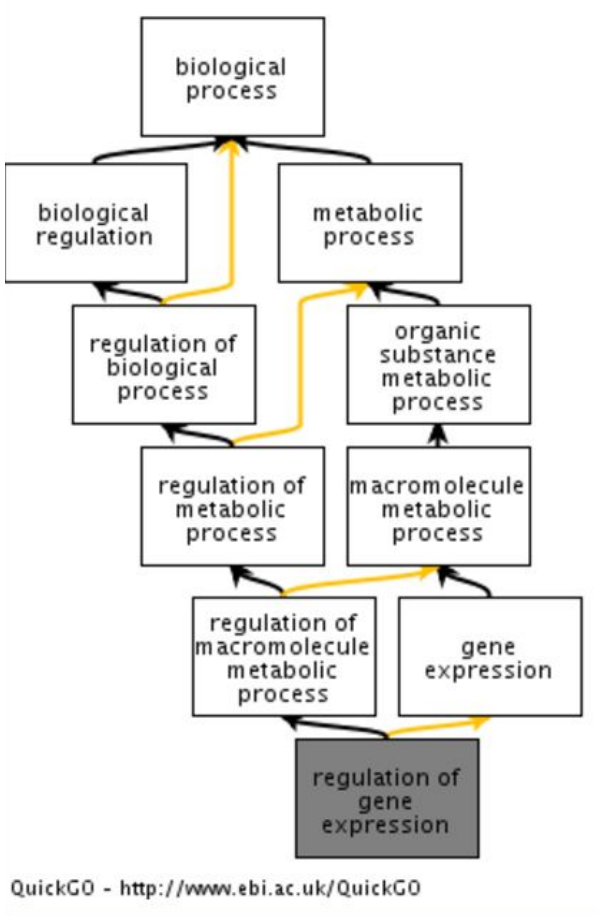
GO slims

- GO slims are ‘trimmed’ versions of GO where the specific fine grained terms have been removed and only broader terms are present
- They usually cover the whole breadth of GO, albeit slims for particular species may exclude sections that are not applicable to that species
- GO slims are useful for giving an overview of the GO annotations of a genome or a large collection of genes, when a broad classification is sufficient



GO annotations

- A GO annotation is the assignment of a GO term to a gene (product)
- A gene may have multiple annotations, even of the same GO type
- According to the **true path rule**, a gene annotated to a term is implicitly annotated to each (‘is a’) ancestor of that term; other relations apply to it accordingly



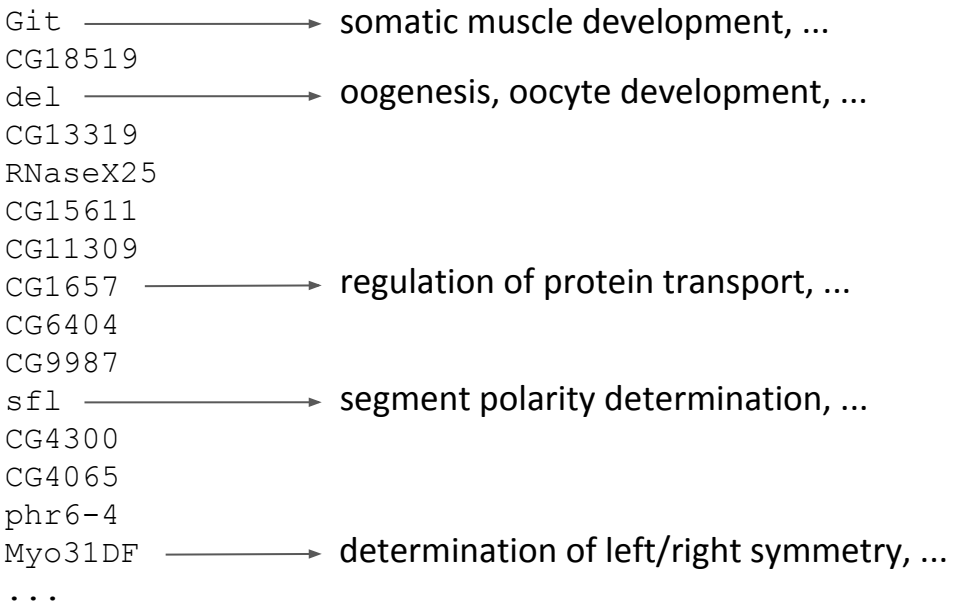
GO annotations

- Are assigned an evidence code that encodes the type of evidence supporting the annotation
- Electronic annotations (IEA) are considered less reliable than manually curated annotations
- Genes can be explicitly annotated to both a term and its ancestor if the annotations have different evidence codes (otherwise it would be redundant)

Getting GO annotations

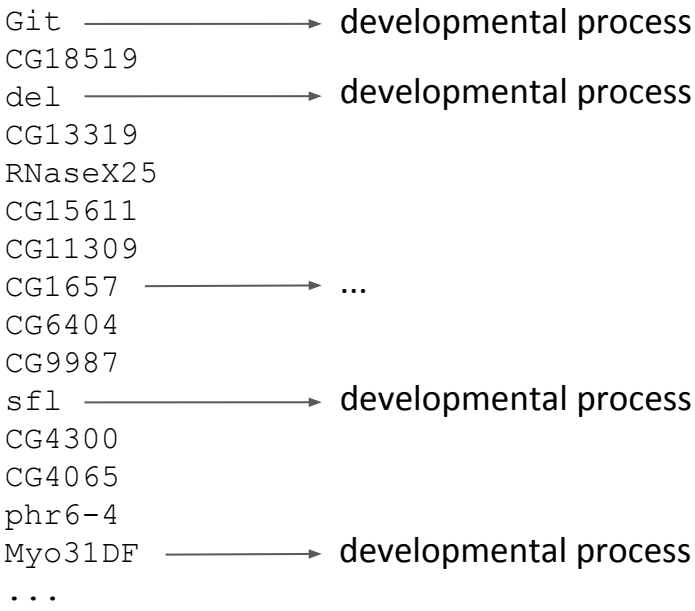
- GO annotations of genes and proteins are available in most major genome databases, in UniProt, and in dedicated search engines such as AmiGO (http://amigo.geneontology.org) and QuickGO (https://www.ebi.ac.uk/QuickGO/)
- You can download complete genome/database annotation sets from:
  - http://www.geneontology.org/page/download-annotations
  - http://www.ensembl.org/biomart

Extracting meaning from a list of genes



- By going from genes to GO annotations, we start to see some meaning
- But there are too many genes to examine manually!!!
- And this doesn't tell us how significant the observed pattern is!!!

Enrichment analysis



- How can we assess if an observed GO term frequency is statistically significant?
- We need to compute the probability of said frequency arising from chance
- This is an application of Fisher's exact test, which in the genomic context is typically called enrichment analysis

The statistics behind enrichment analysis

- To compute the probability of observing a given frequency by chance, we can employ the hypergeometric distribution, for which we need to know:
  - The sample frequency: number of genes in the set annotated with the term
  - The sample size: total number of genes (with any annotation) in the set
  - The population frequency: number of genes in the population* annotated with the term
  - The population size: total number of genes (with any annotation) in the population*

* the total set of genes involved in the study: all expressed genes in the case of RNAseq, genes contained in the microarray in the case of microarray studies, etc

The statistics behind enrichment analysis

- To assess significance, we need to compute the probability of observing **at least** the given frequency by chance
- This is given by the sum of hypergeometric probabilities P($x = i$) with i ranging from the sample frequency to the minimum of the sample size and the population frequency
- The resulting probability is the p-value in Fisher's exact test
- We typically consider significant, events with p-value ≤ 0.001

Correcting for multiple tests

- In enrichment analysis, we typically want to perform multiple tests – as many as the number of functional aspects of the genes in our set
- The p-value corresponds to the probability of making a type I error, i.e., erroneously rejecting the null hypothesis (in our case, that the observation is due to chance)
- If we made 1000 tests, we would expect to obtain a p-value of 0.001 in one of them by chance alone
- Thus, when performing multiple tests, it is necessary to correct the statistics

Correcting for multiple tests

- **Family-wise error rate (FWER)**: control the probability of making at least one false discovery − more conservative but safer
    - Bonferroni correction: multiply the p-values by the number of tests to obtain corrected p-values
- **False discovery rate (FDR)**: control the ratio of false discoveries − more powerful
    - Benjamini-Hochberg correction: step-wise correction that produces q-values, which indicate the ratio of false discoveries you are accepting if you reject the null hypothesis

GO enrichment analysis

- GO is hierarchical, and we must consider both direct and inherent annotations when doing enrichment analysis
- This enables integration: with specific terms like “somatic muscle development” and “segment polarity determination”, we would be unable to find a pattern, but we can find it with the more generic “developmental process”
- However, it also makes the analysis of the results more difficult: we often get enriched terms at several levels of specificity, many of which are interrelated, and this is not always readily apparent

GO enrichment analysis

- Multiple test corrections should be apply to multiple tests in a family:
  - The three GO types correspond to different families, and thus should be treated separately for the purpose of multiple test corrections
- Some hierarchically related tests are redundant:
  - If the study frequency of “DNA binding” is the same as that of “binding”, then testing the latter is unnecessary – it can only be significant if the former is also significant, and the more specific the term the more meaning we can derive

What to test for enrichment

- Biological process is typically the most interesting GO type to test for enrichment, but molecular function and cellular component may also be relevant for validation in particular studies (e.g., in a proteomics study where you sampled only membrane proteins, you should check for enrichment of “cellular membrane”)

- In gene expression studies, we can analyze all differentially expressed genes together, or separate them into overexpressed and underexpressed genes, analyze them separately, and compare the results − both approaches may make sense, depending on the study and the goal of the analysis

Tools for enrichment analysis

- Web tools:
  - GOrilla: http://cbl-gorilla.cs.technion.ac.il/
- Galaxy tools:
  - GOEnrichment [IGC]
  - Ontologizer
- R tools:
  - gsea
  - GOstats
  - topGO

Interpreting enrichment analysis results

- **Statistically significant ≠ biologically meaningful**
- More specific terms are generally more meaningful, and are easier to interpret
- Generic terms are often challenging to interpret, but this does not mean they are meaningless
- Related terms should be considered together

Interpreting enrichment analysis results

- Broader experiments will generally lead to results that are more difficult to interpret
- Some subsets (e.g., only underexpressed or only overexpressed genes) may be more meaningful or easier to interpret than others

Top enriched GO terms, mouse iPSCs vs ESCs up
single-organism process
oxidation-reduction process
single-organism metabolic process

Top enriched GO terms, mouse iPSCs vs ESCs down
modulation of synaptic transmission
regulation of nervous system development
behavior

Interpreting enrichment analysis results

- Graph views can help interpret the results
- But GO is large, and broad experiments tend to produce thousands of differentially expressed genes, resulting in hundreds of enriched terms and **very large graphs**