

An Introduction to Pathway Enrichment Analysis

Alex Sánchez



*Statistics and Bioinformatics Unit
Vall d'Hebron Institut de Recerca*



*Statistics and Bioinformatics Research Group
Statistics department, Universitat de Barcelona*



Outline

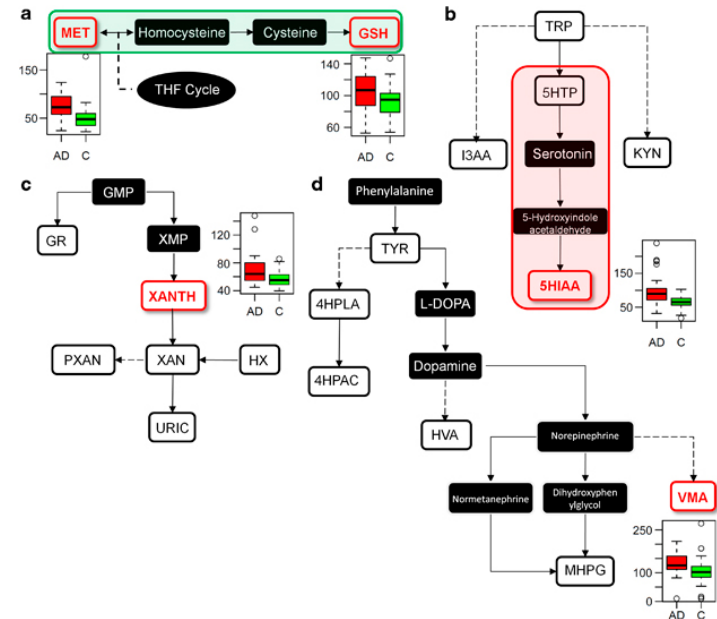
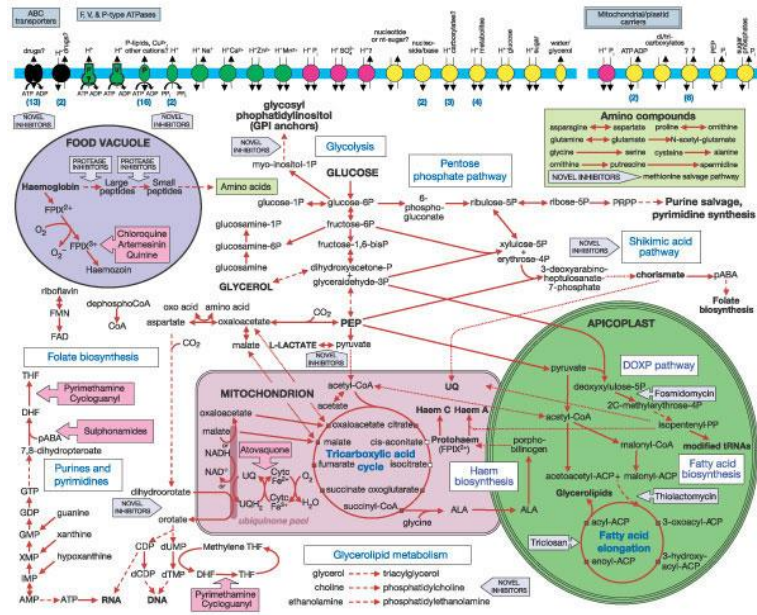
- Presentation
- Introduction and Background
 - Gene lists, Identifiers and Pathway databases
- Pathway Analysis: Methods and Tools
 - Overrepresentation analysis and GSEA
 - Multiple Testing Adjustments
 - Network Visualization and Enrichment Map
- A protocol for Pathway Enrichment Analysis
- A user experience

Introduction & Background

Health, disease and pathways

Metabolism is a complex network of chemical reactions within the confines of a cell that can be analyzed in self-contained parts called **pathways**

One can generally assume that “normal” metabolism is what happens in healthy state or, reciprocally, that disease can *be associated with some type of alteration in metabolism*.



Pathways altered in ALZHEIMER disease

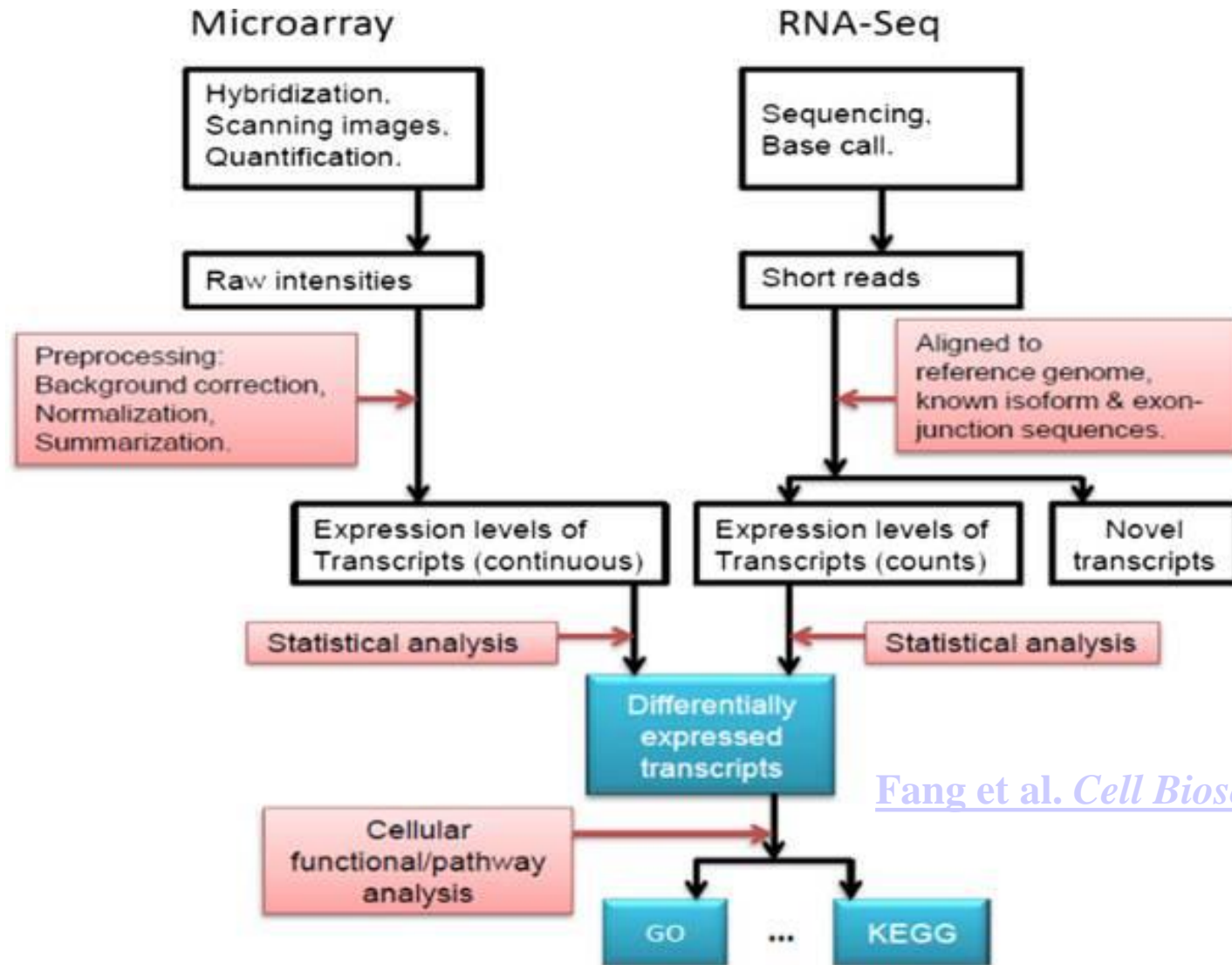
Characterization of disease can be attempted by studying how this affects or disrupts pathways
That's what Pathway Analysis is about (more or less)

Pathway Analysis

- The term Pathway Analysis denotes *any analytic technique that benefits from biological pathway or molecular network information to gain insight into a biological system*. (Creixell et al., Nature Methods 2015 (12 (7)))
- To be more specific, Pathway Analysis methods rely on high throughput information provided by omics technologies to:
 - Contextualize findings to help understand the mechanism of disease
 - Identify genes/proteins associated with the aetiology of a disease
 - Predict drug targets
 - Understand how to therapeutically intervene in disease processes
 - Conduct target literature searches
 - Integrate diverse biological information

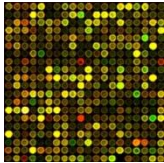
The beginning: *Gene Lists*

The life-cycle of an omics-based study



[Fang et al. Cell Biosci. 2012; 2: 26.](#)

The (in)famous “*where to now?*” question



- You obtained a list of features. What's next?
 - Select some genes for validation?
 - Follow up experiments on some genes/proteins/...?
 - Publish a huge table with all results?
 - Try to learn about **all** features in the list?

| |
|---------|
| GNAQ |
| GNAS |
| DGKZ |
| GUCY1A3 |
| PDE4B |
| PDE4D |
| ATP2A2 |
| ATP2A3 |
| NOS1 |
| CNN1 |
| GSTO1 |
| NOS3 |
| CNN2 |
| MYLK2 |
| CALD1 |
| ACTA1 |
| MYL2 |

| |
|---------|
| GNAQ |
| GNAS |
| DGKZ |
| GUCY1A3 |
| PDE4B |
| PDE4D |
| ATP2A2 |
| ATP2A3 |
| NOS1 |
| CNN1 |
| GSTO1 |
| NOS3 |
| CNN2 |
| MYLK2 |
| CALD1 |
| ACTA1 |
| MYL2 |

my favorite gene

NCBI Resources How To

PubMed GNAQ

US National Library of Medicine
National Institutes of Health

RSS Save search Advanced

Show additional filters

Display Settings: Summary, 20 per page, Sorted by Recently A

Article types
Review
More ...

Text availability
Abstract available
Free full text available
Full text available

Publication dates
5 years

See 225 articles about GNAQ gene function
See also: GNAQ guanine nucleotide binding protein (G protein), c
gnaq in Homo sapiens | Mus musculus | Rattus norvegicus | All

Results: 1 to 20 of 114

1. Sturge-Weber Syndrome and Port-Wine Stains Caused by
Shirley MD, Tang H, Gallione CJ, Baugher JD, Frelin LP,
AM, Pevsner J.
N Engl J Med. 2013 May 8. [Epub ahead of print]
Med - as supplied by publisher

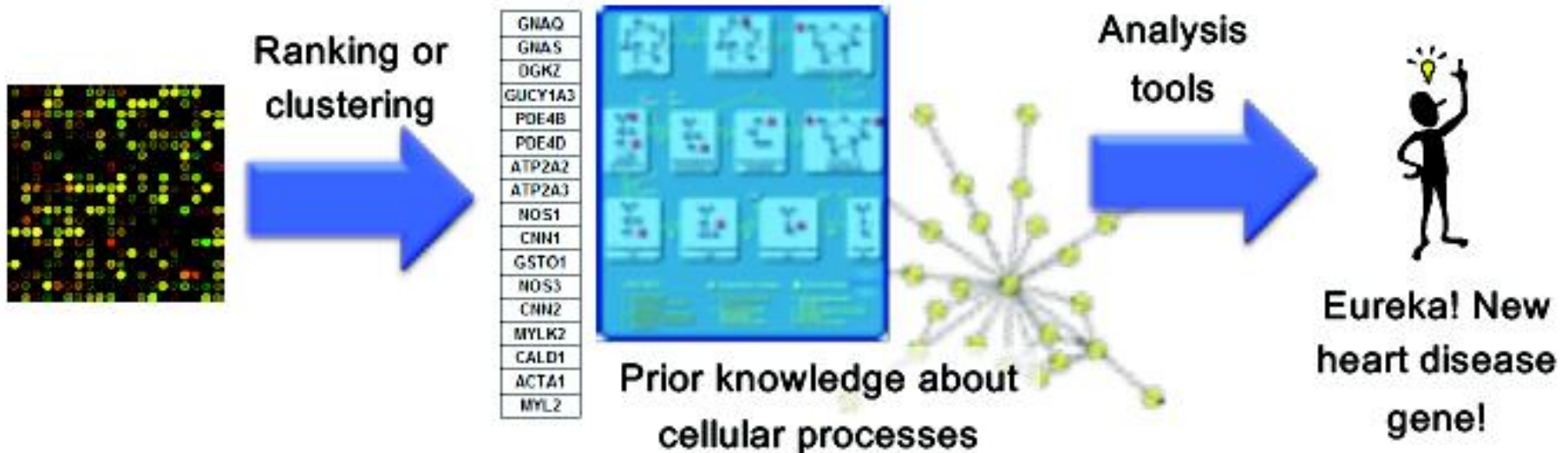


From gene lists to *Pathway Analysis*

- Gene lists are made of individual genes
 - Information about each gene can be extracted from databases.
 - Generically described as ***Gene Annotation***
- Besides, we may obtain information from the analysis of *gene sets*
 - Genes don't act individually, rather in groups
More ***realistic*** approach
 - There are less gene sets than individual genes
Relatively ***simpler*** to manage.
 - Generically described as ***Pathway Analysis***

Pathway Analysis Wishlist

- Tell me what's interesting about these genes
 - Are they enriched in known pathways, complexes, functions



Example 1

- Lists [AvsB](#), [AvsL](#) and [BvsL](#) contain the IDs of genes selected by being differentially expressed between three types of breast cancer tumors.
 - Farmer P, Bonnefoi H, Becette V, Tubiana-Hulin M et al. Identification of molecular apocrine breast tumours by microarray analysis. *Oncogene* 2005 Jul 7;24(29):4660-71. PMID: [15897907](#)
- See the analysis that generates the list in:

[https://github.com/alexsanchezpla/Ejemplo de MDA con Bioconductor](https://github.com/alexsanchezpla/Ejemplo_de_MDA_con_Bioconductor)

Example 2

- Genes with frequent somatic SNVs identified in TCGA exome sequencing data of 3,200 tumors of 12 types
- 127 cancer driver genes displaying higher than expected mutation frequencies were detected using the MuSiC software.
- Genes are ranked in decreasing order of significance and mutation frequency

TP53
PIK3CA
PTEN
APC
VHL
KRAS
MLL3
MLL2
ARID1A
PBRM1
NAV3
EGFR
NF1
PIK3R1
CDKN2A
GATA3
RB1
NOTCH1
FBXW7
CTNNB1
DNMT3A
MAP3K1
FLT3
MALAT1
TSHZ3

Example 3

- Second example is a ranked list of genes obtained from TCGA ovarian cancer dataset.
- Two subgroups - immunoreactive and mesenchymal- were compared.
- The list contains **all genes, not only differentially expressed**, ranked by the value of statistic.

| rank | GeneName | test statistic |
|-------|----------|-------------------|
| 1 | IGDCC3 | 35.5553322839225 |
| 2 | ANTXR1 | 35.3770766531836 |
| 3 | AEBP1 | 33.0690543534961 |
| 4 | FBN1 | 32.1199562790897 |
| 5 | ANGPTL2 | 31.8605806216522 |
| 6 | COL16A1 | 31.7641267462069 |
| 7 | BGN | 31.533826423921 |
| ... | ... | ... |
| 15201 | IRF1 | -14.7629673442493 |
| 15202 | CXCL10 | -14.9827363665643 |
| 15203 | TAP2 | -15.1488606179238 |
| 15204 | UBE2L6 | -15.7162058907796 |
| 15205 | KIAA0319 | -15.7796986548781 |
| 15206 | PSMB8 | -15.7846188665582 |
| 15207 | PSME1 | -16.4510045533584 |
| 15208 | CSAG3 | -16.8014265945244 |
| 15209 | OVGP1 | -17.6903158148446 |
| 15210 | GBP4 | -17.9447602030134 |
| 15211 | TAP1 | -18.0549262210415 |
| 15212 | PSME2 | -18.3639448844986 |
| 15213 | PSMB9 | -18.6614452029879 |

Gene Lists and Annotations

Gene and Protein Identifiers

- Identifiers (IDs) are ideally unique, stable names or numbers that help track database records
 - E.g. Social Insurance Number, Entrez Gene ID 41232
- But, information on features is stored in many databases.
 - Genes have many IDs
- Records for: Gene, DNA, RNA, Protein
 - Important to recognize the correct record type
 - E.g. Entrez Gene records don't store sequence. They link to DNA regions, RNA transcripts and proteins e.g. in RefSeq, which stores sequence.

TP53
PIK3CA
PTEN
APC
VHL
KRAS
MLL3
MLL2
ARID1A
PBRM1
NAV3
EGFR
NF1
PIK3R1
CDKN2A
GATA3
RB1
NOTCH1
FBXW7
CTNNB1
DNMT3A
MAP3K1
FLT3
MALAT1
TSHZ3

Common Identifiers

Gene

Ensembl [ENSG00000139618](#)

Entrez Gene [675](#)

Unigene [Hs.34012](#)

RNA transcript

GenBank [BC026160.1](#)

RefSeq [NM_000059](#)

Ensembl [ENST00000380152](#)

Protein

Ensembl [ENSP00000369497](#)

RefSeq [NP_000050.2](#)

UniProt [BRCA2_HUMAN](#) or
[A1YBP1_HUMAN](#)

IPI [IPI00412408.1](#)

EMBL [AF309413](#)

PDB [1MIU](#)

Species-specific

HUGO HGNC [BRCA2](#)

MGI [MGI:109337](#)

RGD [2219](#)

ZFIN [ZDB-GENE-060510-3](#)

FlyBase [CG9097](#)

WormBase [WBGene00002299](#) or [ZK1067.1](#)

SGD [S000002187](#) or [YDL029W](#)

Annotations

InterPro [IPR015252](#)

OMIM [600185](#)

Pfam [PF09104](#)

Gene Ontology [GO:0000724](#)

SNPs [rs28897757](#)

Experimental Platform

Affymetrix [208368_3p_s_at](#)

Agilent [A_23_P99452](#)

CodeLink [GE60169](#)

Illumina [GI_4502450-S](#)

Red =

Recommended

Identifier Mapping

- There are many IDs!
 - Software tools recognize only a handful
 - May need to map from your gene list IDs to standard IDs
- Four main uses
 - Searching for a favorite gene name
 - Link to related resources
 - Identifier translation
 - E.g. Proteins to genes, Affy ID to Entrez Gene
 - Merging data from different sources
 - Find equivalent records

ID Challenges

- Avoid errors: map IDs correctly
 - Beware of 1-to-many mappings
- Gene name ambiguity – not a good ID
 - e.g. FLJ92943, LFS1, TRP53, p53
 - Better to use the standard gene symbol: TP53
- Excel error-introduction
 - OCT4 is changed to October-4 (paste as text)
- Problems reaching 100% coverage
 - E.g. due to version issues
 - Use multiple sources to increase coverage

Zeeberg BR et al. *Mistaken identifiers: gene name errors can be introduced inadvertently when using Excel in bioinformatics*
BMC Bioinformatics. 2004 Jun 23;5:80

Use ID converters to prepare list

DAVID Bioinformatics Resources 2007
National Institute of Allergy and Infectious Diseases (NIAID), NIH

Gene Accession Conversion Tool

Save the results Submit the converted genes to DAVID for other analytical tools!!

Gene Accession Conversion Statistics Genes that have been converted. Right-click to Download the list Submit Converted List to DAVID

Conversion Summary

| ID Count | In DAVID DB | Conversion |
|-----------------------------------|-------------|------------|
| 157 IDs | Yes | Successful |
| 0 IDs | Yes | None |
| 0 IDs | No | NA |
| 1 IDs | Ambiguous | Pending |
| Total Unique User IDs: 166 | | |

Summary of Ambiguous Gene IDs

| ID Count | Possible Source | Convert All |
|----------|-----------------|----------------------------------|
| 1 | ENTREZ_GENE_ID | <input checked="" type="radio"/> |
| 1 | GI_ACCESSION | <input type="radio"/> |

Possible Sources For Ambiguous IDs

| Ambiguous ID | Possibility | Convert |
|--------------|----------------|----------------------------------|
| 3538 | ENTREZ_GENE_ID | <input checked="" type="radio"/> |
| 3538 | GI_ACCESSION | <input type="radio"/> |

Genes that have been converted.

| From | To | Species | David Gene Name |
|------------|-------|--------------|---|
| *1112_G_AT | 4684 | HOMO SAPIENS | NEURAL CELL ADHESION MOLECULE 1 |
| *1331_S_AT | 8718 | HOMO SAPIENS | TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY, MEMBER 25 |
| *1355_G_AT | 4915 | HOMO SAPIENS | NEUROTROPHIC TYROSINE KINASE, RECEPTOR, TYPE 2 |
| *1372_AT | 7130 | HOMO SAPIENS | TUMOR NECROSIS FACTOR, ALPHA-INDUCED PROTEIN 6 |
| *1391_S_AT | 1579 | HOMO SAPIENS | CYTOCHROME P450, FAMILY 4, SUBFAMILY A, POLYPEPTIDE 11 |
| *1403_S_AT | 6352 | HOMO SAPIENS | CHEMOKINE (C-C MOTIF) LIGAND 5 |
| *1419_G_AT | 4843 | HOMO SAPIENS | NITRIC OXIDE SYNTHASE 2A (INDUCIBLE, HEPATOCYTES) |
| *1575_AT | 5243 | HOMO SAPIENS | ATP-BINDING CASSETTE, SUB-FAMILY B (MDR/TAP), MEMBER 1 |
| *1645_AT | 2814 | HOMO SAPIENS | KISS-1 METASTASIS-SUPPRESSOR |
| *1786_AT | 10461 | HOMO SAPIENS | C-MER PROTO-ONCOGENE TYROSINE KINASE |
| *1855_AT | 2248 | HOMO SAPIENS | FIBROBLAST GROWTH FACTOR 3 (MURINE MAMMARY TUMOR VIRUS INTEGRATION SITE [V-INT-2...]) |
| *1890_AT | 9518 | HOMO SAPIENS | GROWTH DIFFERENTIATION FACTOR 15 |

g:Profiler

g:GOST Gene Group Functional Profiling
g:Cocoa Compact Compare of Annotations
g:Convert Gene ID Converter
g:Sorter Expression Similarity Search
g:Orth Orthology search
g:SNPense Convert rsID

Welcome! Contact FAQ R / APIs Beta Archive

J. Reimand, T. Arak, P. Adler, L. Kolberg, S. Reisberg, H. Peterson, J. Vilo: g:Profiler -- a web server for functional interpretation of gene lists (2016 update) Nucleic Acids Research

Left Panel **Right Panel**

*Users' decision for ambiguous IDs

Users' input gene IDs Species of converted gene IDs Converted gene IDs Gene

[?] Organism
Homo sapiens

[?] Target database
ENSG

[?] Output type
Table (HTML)

[?] Query (genes, proteins, probes, term)

[?] Interpret query as chromosome
[?] Numeric IDs treated as
AFFY_HUEX_1_0_ST_V2

Convert IDs **Clear**

Example 1: Gene ID conversion with g:Profiler



ID Mapping Services



- g:GOST** Gene Group Functional Profiling
- g:Cocoa** Compact Compare of Annotations
- g:Convert** Gene ID Converter
- g:Sorter** Expression Similarity Search
- g:Orth** Orthology search

Welcome | About | Contact | Beta | Archives | R

J. Reimand, M. Kull, H. Peterson, J. Hansen, J. Vilo: g:Profiler -- a web-based toolset for functional profiling of gene lists from large-scale experiments (2007) NAR 35 W193-W200 [PDF]
 J. Reimand, T. Arak, J. Vilo: g:Profiler -- a web server for functional interpretation of gene lists (2011 update) Nucleic Acids Research 2011; doi: 10.1093/nar/gkr378 [PDF]

[?] **Organism**
 Homo sapiens

[?] **Target database**
 UNIPROTSSWISSPROT

[?] **Output type**
 Table (HTML)

[?] **Query** (genes, proteins, probes, term)
 TP53 MDM2 207105_S_AT P60484

[?] ☐ Interpret query as chromosome ranges

[?] **Numeric IDs treated as**
 AFFY_HUGENE_1_0_ST_V1

Convert IDs Clear

Input gene/protein/transcript IDs (mixed)

Type of output ID

>> Static URL
Come back later

| g# | initial alias >> g:GOST >> g:Sorter >> g:Orth >> g:Cocoa | c# | converted alias >> g:GOST >> g:Sorter >> g:Orth >> g:Cocoa >> Copy values | name >> g:GOST >> g:Sorter >> g:Orth >> g:Cocoa >> Copy values | description | namespace |
|----|--|-----|--|---|---|--|
| 1 | TP53 | 1.1 | P04637 | TP53 | tumor protein p53 [Source:HGNC Symbol;Acc:HGNC:11998] | UNIPROT_GN, ENTREZGENE, VEGA_GENE, DBASS5, DBASS3, HGNC, WIKIGENE |
| 2 | MDM2 | 2.1 | Q00987 | MDM2 | MDM2 proto-oncogene, E3 ubiquitin protein ligase [Source:HGNC Symbol;Acc:HGNC:6973] | UNIPROT_GN, ENTREZGENE, VEGA_GENE, HGNC, WIKIGENE |
| 3 | 207105_S_AT | 3.1 | O00459 | PIK3R2 | phosphoinositide-3-kinase, regulatory subunit 2 (beta) [Source:HGNC Symbol;Acc:HGNC:8980] | AFFY_HG_U133_PLUS_2, AFFY_HG_FOCUS, AFFY_HG_U133A_2, AFFY_HG_U133A |
| 4 | P60484 | 4.1 | P60484 | PTEN | phosphatase and tensin homolog [Source:HGNC Symbol;Acc:HGNC:9588] | UNIPROTSSWISSPROT |

- g:Convert


- <http://biit.cs.ut.ee/gprofiler/gconvert.cgi>

- Ensembl Biomart

- <http://www.ensembl.org>

AFFY_HG_U95C
 AFFY_HG_U95D
 AFFY_HG_U95E
 AFFY_HTA_2_0
 AFFY_HUEX_1_0_ST_V2
 AFFY_HUGENEF1
 AFFY_HUGENE_1_0_ST_V1
 AFFY_HUGENE_2_0_ST_V1
 AFFY_PRIMEVIEW
 AFFY_U133_X3P
 AGILENT_CGH_44B
 AGILENT_SUREPRINT_G3_GE_8X60K
 AGILENT_SUREPRINT_G3_GE_8X60K_V2
 AGILENT_WHOLEGENOME_4X44K_V1
 AGILENT_WHOLEGENOME_4X44K_V2
 ARRAYEXPRESS
 CCDS
 CCDS_ACC
 CHEMBL
 CLONE_BASED_ENSEMBL_GENE
 CLONE_BASED_ENSEMBL_TRANSCRIPT
 CLONE_BASED_VEGA_GENE
 CLONE_BASED_VEGA_TRANSCRIPT
 CODELINK_CODELINK
 DBASS3
 DBASS3_ACC
 DBASS5
 DBASS5_ACC
 EMBL
 ENSG
 ENSP
 ENST
 ENS_HS_TRANSCRIPT
 ENS_HS_TRANSLATION
 ENS_LRQ_GENE
 ENS_LRQ_TRANSCRIPT
 ENTREZGENE
 ENTREZGENE_ACC
 ENTREZGENE_TRANS_NAME
 GO
 GOSLIM_GOA
 HGNC
 HGNC_ACC
 HGNC_TRANS_NAME
 HPA
 HPA_ACC
 ILLUMINA_HUMANHT_12_V3
 ILLUMINA_HUMANHT_12_V4
 ILLUMINA_HUMANREF_8_V3
 ILLUMINA_HUMANWG_6_V1
 ILLUMINA_HUMANWG_6_V2
 ILLUMINA_HUMANWG_6_V3
 MEROPS
 MIM_GENE
 MIM_GENE_ACC
 MIM_MORBID
 MIM_MORBID_ACC
 MIRBASE
 MIRBASE_ACC
 MIRBASE_TRANS_NAME
 OTTG
 OTTP
 OTTT
 PDB
 PHALANX_ONEARRAY
 PROTEIN_ID
 PROTEIN_ID_ACC
 REFSEQ_MRNA
 REFSEQ_MRNA_ACC
 REFSEQ_MRNA_PREDICTED
 REFSEQ_MRNA_PREDICTED_ACC
 REFSEQ_MRNA_PREDICTED_V2

Beware of ambiguous ID mappings



g:Profiler

Welcome! About Contact Beta Archives R

g:GOST Gene Group Functional Profiling

g:Cocoa Compact Compare of Annotations

g:Convert Gene ID Converter

g:Sorter Expression Similarity Search

g:Orth Orthology search

Organism

Homo sapiens

Query (genes, proteins, probes, term)

TP53 MDM2 207105_S_AT P60484

Options

☒ Significant only

☐ Ordered query

☐ No electronic GO annotations

☐ Chromosomal regions

☒ Hierarchical sorting

☐ Hierarchical filtering

Show all terms (no filtering)

Output type

Graphical (PNG)

Show advanced options

☒ Gene Ontology

☒ Biological process

☒ Cellular component

☒ Molecular function

Inferred from experiment [IDA, IPI, IMP, IGI, IEP]

Direct assay [IDA] / Mutant phenotype [IMP]

Genetic interaction [IGI] / Physical interaction [IPI]

Traceable author [TAS] / Non-traceable author [NAS] / Inferred by curator [IC]

Expression pattern [IEP] / Sequence or structural similarity [ISS] / Genomic context [IGC]

Biological aspect of ancestor [IBA] / Rapid divergence [IRD]

Reviewed computational analysis [RCA] / Electronic annotation [IEA]

No biological data [ND] / Not annotated [NA]

Biological pathways ☒ KEGG ☒ Reactome

Regulatory motifs in DNA ☒ TRANSFAC TFBS ☒ miRBase microRNAs

☒ CORUM protein complexes

☒ Human Phenotype Ontology (sequence homologs in other species)

☒ BioGRID protein-protein interaction

[?] or Term ID:

g:Profile! Clear

Example or random query

>> g:Convert
Gene ID Converter

>> g:Orth
Orthology Search

>> g:Sorter
Expression Similarity Search

>> g:Cocoa
Compact Compare of Annotations

>> Static URL
Come back later

Warning: Some gene identifiers are ambiguous. Resolve these manually?

Attempt to automatically resolve symbols using a namespace (percentage of ambiguous symbols resolved in brackets):

207105_S_AT

- ☐ ENSG00000268173 (PIK3R2, 26 GO annot.) - phosphoinositide-3-kinase, regulatory subunit 2 (beta) [Source:HGNC Symbol;Acc:HGNC:8980]
- ☐ ENSG00000105647 (PIK3R2, 26 GO annot.) - phosphoinositide-3-kinase, regulatory subunit 2 (beta) [Source:HGNC Symbol;Acc:HGNC:8980]

Resubmit query

Recommendations

- For proteins and genes
 - (doesn't consider splice forms)
 - Map everything to Entrez Gene IDs or Official Gene Symbols using an appropriate tool, such as gProfiler, DAVID or Biomart.
- If 100% coverage desired, manually curate missing mappings using multiple resources
- Be careful of Excel auto conversions – especially when pasting large gene lists!
 - Remember to format cells as 'text' before pasting

Pathway and Gene Sets databases

Where is pathway information? (1)

- Most common sources*
 - Gene Ontology: Biological process,
 - Pathway databases:
 - Reactome : <http://reactome.org>
 - <http://www.pathguide.org>
 - MSigDB:
<http://www.broadinstitute.org/gsea/msigdb/>
 - <http://www.pathwaycommons.org/>

[*Comparison of human cell signaling pathway databases—evolution, drawbacks and challenges](#)

Where is pathway information? (2)

- Other annotations
 - Gene Ontology molecular function, cell location
 - Chromosome position
 - Disease association
 - DNA properties (TF binding sites, gene structure (intron/exon), SNPs, ...)
 - Transcript properties (Splicing, 3' UTR, microRNA binding sites, ...)
 - Protein properties (Domains, 2ry and 3ry structure, PTM sites)
 - Interactions with other genes

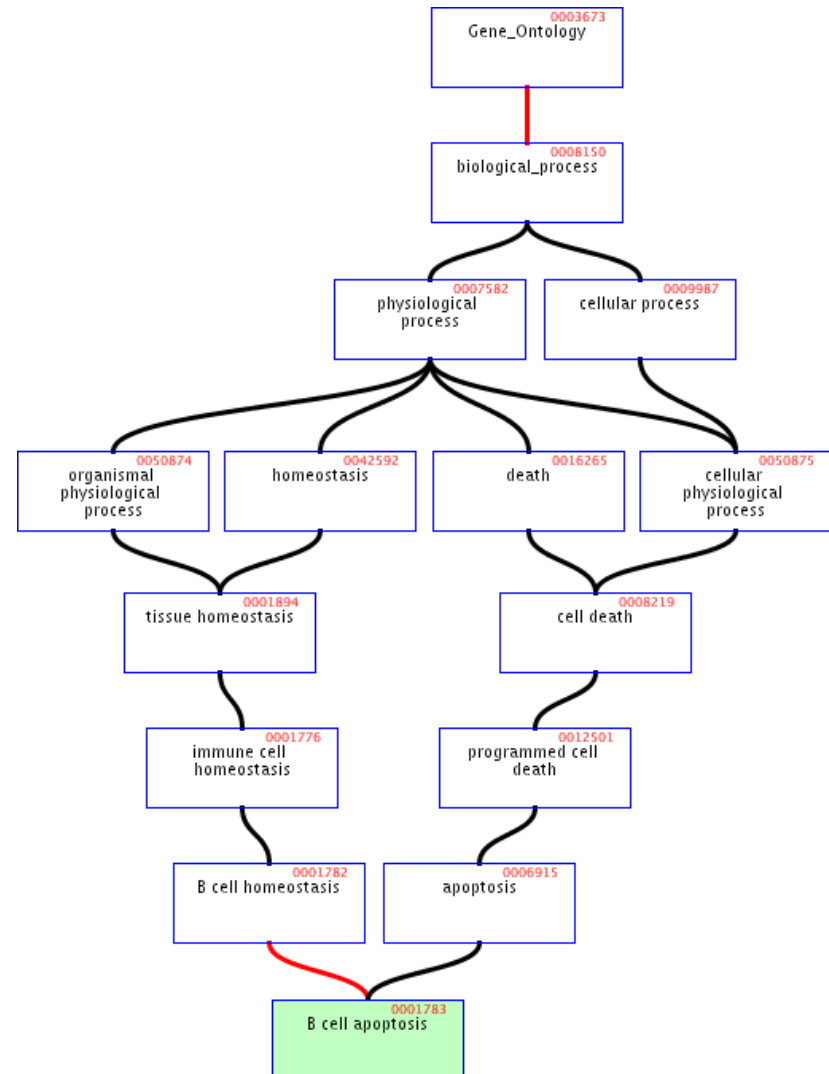
[*Comparison of human cell signaling pathway databases—evolution, drawbacks and challenges](#)

What is the Gene Ontology (GO)?

- Set of biological phrases (terms) which are applied to genes:
 - protein kinase, apoptosis, membrane
- An ontology is not a dictionary
 - Dictionary: A collection of term definitions,
 - Alphabetic organization
 - Ontology: A formal system for describing knowledge
 - Hierarchical organization
- <http://geneontology.org/>

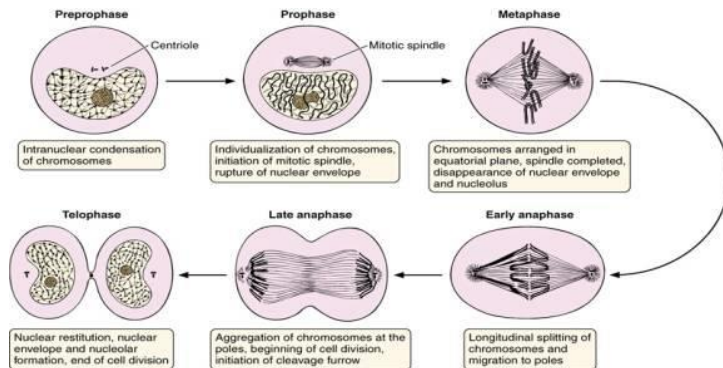
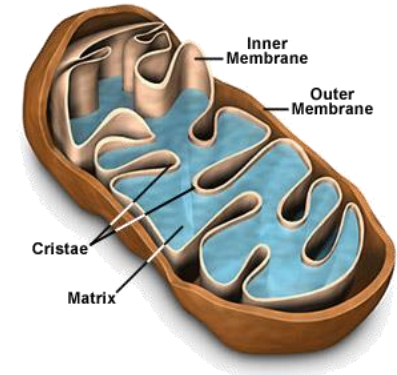
GO Structure

- Terms are related within a hierarchy
 - is-a
 - part-of
- Describes multiple levels of detail of gene function
- Terms can have more than one parent or child

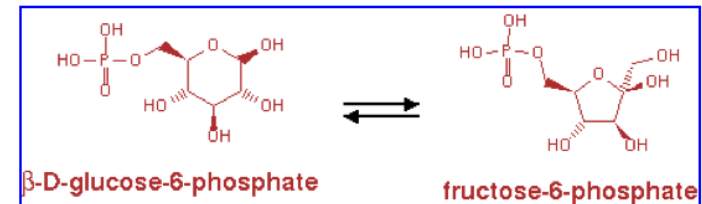


What is covered by the GO?

- GO terms divided into three aspects:
 - cellular component
 - molecular function
 - biological process



**Cell
division**



**glucose-6-phosphate
isomerase activity**

Annotation Sources

- Manual annotation
 - Curated by scientists
 - High quality
 - Small number (time-consuming to create)
 - Reviewed computational analysis
- Electronic annotation
 - Annotation derived without human validation
 - Computational predictions (accuracy varies)
 - Lower 'quality' than manual codes
- Key point: be aware of annotation origin

Evidence Types

- **Experimental Evidence Codes**
 - EXP: Inferred from Experiment
 - IDA: Inferred from Direct Assay
 - IPI: Inferred from Physical Interaction
 - IMP: Inferred from Mutant Phenotype
 - IGI: Inferred from Genetic Interaction
 - IEP: Inferred from Expression Pattern



- **Computational Analysis Evidence Codes**
 - ISS: Inferred from Sequence or Structural Similarity
 - ISO: Inferred from Sequence Orthology
 - ISA: Inferred from Sequence Alignment
 - ISM: Inferred from Sequence Model
 - IGC: Inferred from Genomic Context
 - RCA: inferred from Reviewed Computational Analysis



- **Author Statement Evidence Codes**
 - TAS: Traceable Author Statement
 - NAS: Non-traceable Author Statement
- **Curator Statement Evidence Codes**
 - IC: Inferred by Curator
 - ND: No biological Data available



- **IEA: Inferred from electronic annotation**



<http://www.geneontology.org/GO.evidence.shtml>

Pathway Analysis

Overrepresentation Analysis

Gene Set Enrichment Analysis

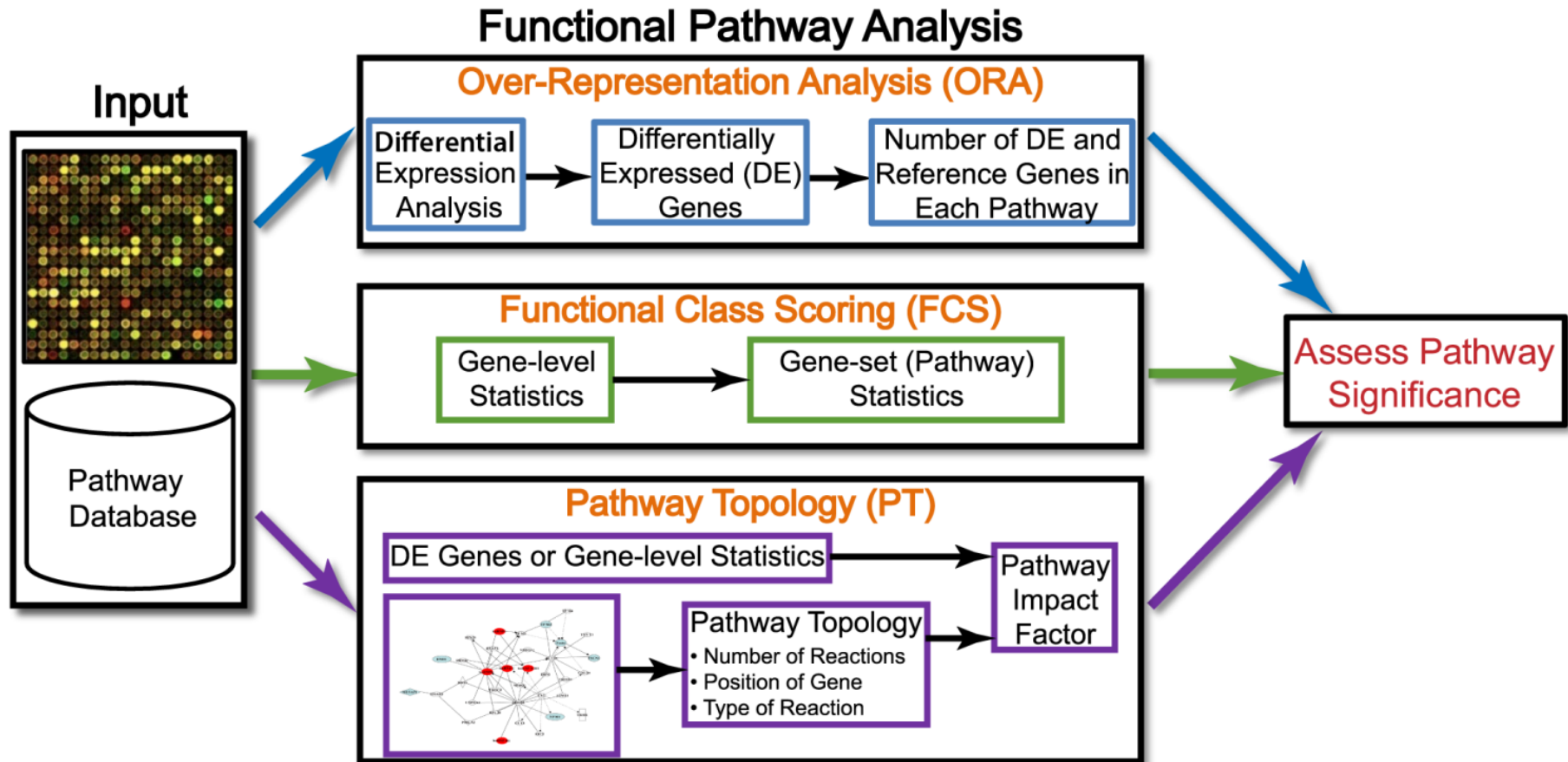
Pathway Analysis

- *“Any type of analysis that involves pathway or information”*
 - Most popular type is ***over-representation analysis***, but many others exist.
- Intended to gain insight into ‘omics’ data. E.g:
 - Identifying a master regulator gene,
 - Finding drug targets,
 - Characterizing pathways active in a sample.

Benefits of Pathway Analysis

- Relatively easy to interpret
 - Familiar concepts e.g. cell cycle
- Identifies possible causal mechanisms
- Predicts new roles for genes
- Improves statistical power
 - Fewer tests, aggregates data from multiple genes into one pathway
- More reproducible
 - E.g. gene expression signatures
- Facilitates integration of multiple data types

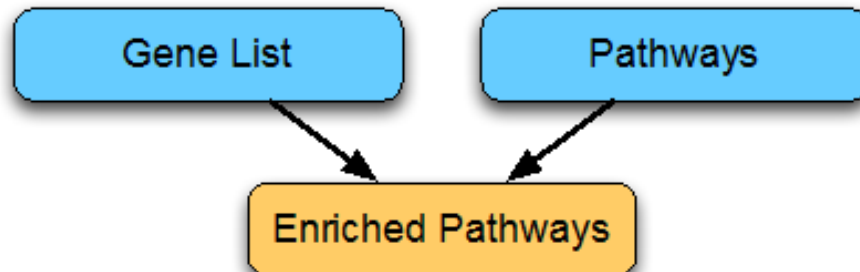
Types of Pathway Analysis



Analysis of *thresholded* lists
with *Enrichment Analysis*
(also called Overrepresentation A.)

Over-representation analysis

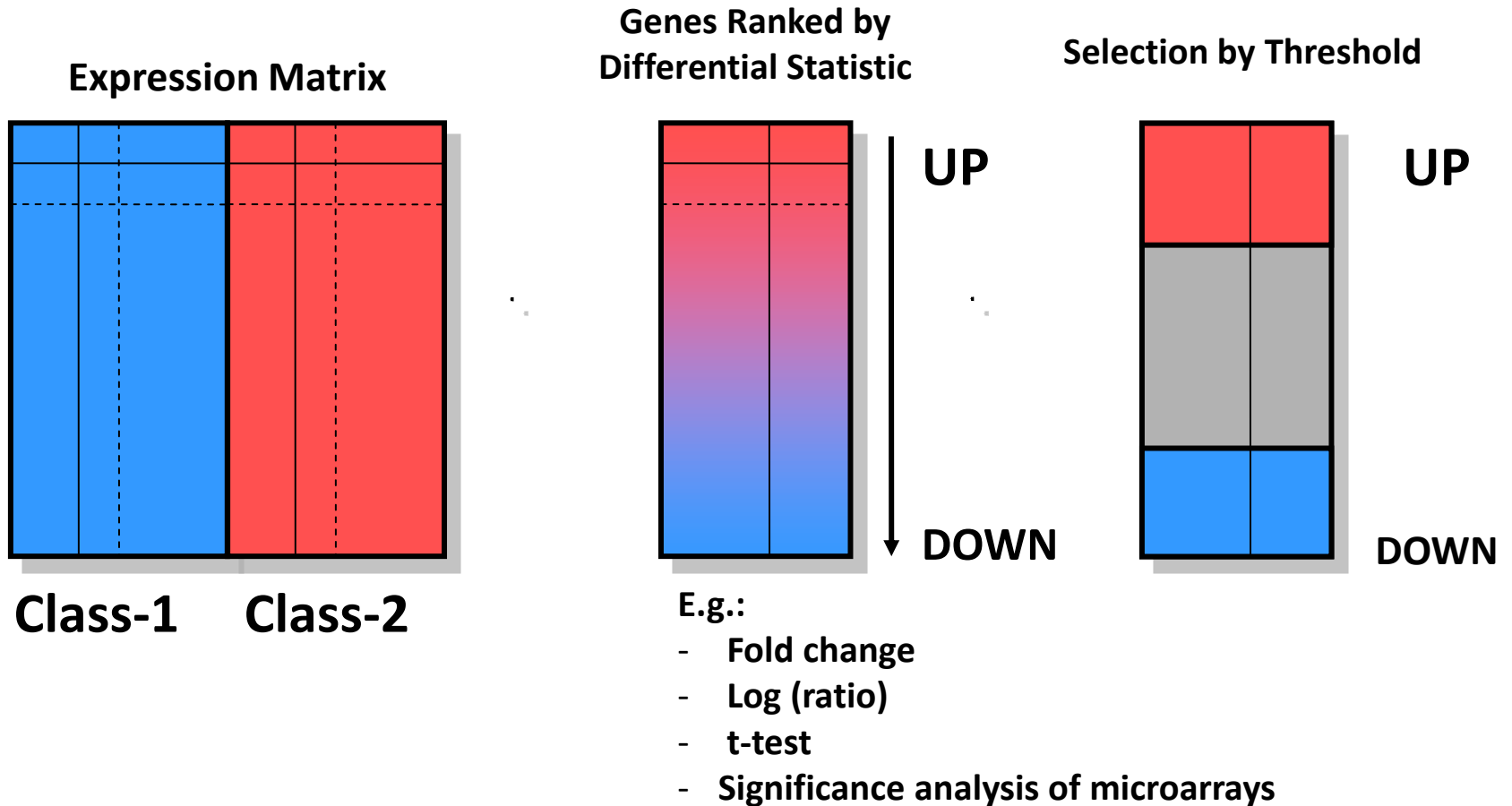
- Combines
 - Gene (feature) lists \leftarrow (Gen)omic experiment
 - Pathways and other gene annotations
 - Gene Ontology
 - Reactome
 - Pathway commons



Over-representation analysis

- Given:
 1. Gene list: e.g. RRP6, MRD1, RRP7, RRP43, RRP42 (yeast)
 2. Gene sets or annotations: e.g. Gene ontology, transcription factor binding sites in promoter
- Question: *Are any of the gene annotations surprisingly enriched in the gene list?*
- Details:
 1. Where do the gene lists come from?
 2. How to assess “surprisingly” (statistics)
 3. How to adjust for test multiplicity?

Obtaining the gene lists



Assessing “surprisingly”

- Given a gene list, “gl”, and a gene set, “GS”, check:
- Is the % of genes in “gl” annotated in “GS” the same as the % of genes globally annotated in “GS”?
 - If both percentages are similar → *No Enrichment*
 - If the % of genes annotated in “GS” is greater in “gl” than in the rest of genes → *“gl” is enriched in “GS”*

[illegible]

Examples

| | Differentially expressed (gl_1) | Not differentially expressed | TOTAL |
|--------------------|-------------------------------------|-------------------------------------|-------|
| In Gene Set (GS1) | 10 | 30 | 40 |
| Not In Gene Set | 390 | 3570 | 3960 |
| TOTAL | 400 | 3600 | 4000 |
| % of gl_1 in GS1 | $10/400=0.025$ | $30/3600=0.00833$ | |

$0.025 \gg 0.00833 \rightarrow "gl_1" \text{ is enriched in "GS}_1"$

| | Differentially expressed (gl_2) | Not differentially expressed | TOTAL |
|--------------------------------|-------------------------------------|---------------------------------|-------|
| In Gene Set (GS2) | 10 | 30 | 40 |
| Not In Gene Set | 390 | 1220 | 1610 |
| TOTAL | 400 | 1500 | 1650 |
| % of gl_2 in GS ₂ | $10/400=0.025$ | $30/1500=0.2$ | |

$0.025 \approx 0.02 \rightarrow \text{Can't say that "gl}_2" \text{ is enriched in "GS}_2"$

Assessing significance: Fisher test

- The examples shows two cases
 - One where percentages are quite different
 - Another where percentages are similar
- How can we set a threshold to decide that the difference is “big enough” to call it “Enriched”
 - Use Fisher Test or, equivalently,
 - a test to compare proportions or
 - a hypergeometric test.

Assessing significance: Fisher test (1)

```
> GOnnnnnCounts<- matrix(c(10, 30, 390, 3570),
+       nrow = 2, byrow=TRUE,
+       dimnames = list(GeneSet = c("In Gene Set", "Not in Gene Set"),
+       Test =c("Differentially expressed", "Not Dif. Expr.")))
> GOnnnnnCounts
```

| GeneSet | Test | |
|-----------------|--------------------------|----------------|
| | Differentially expressed | Not Dif. Expr. |
| In Gene Set | 10 | 30 |
| Not in Gene Set | 390 | 3570 |

```
> fisher.test(GOnnnnnCounts, alternative = "greater")
```

Fisher's Exact Test for Count Data

```
data: GOnnnnnCounts
p-value = 0.004836
alternative hypothesis: true odds ratio is greater than 1
95 percent confidence interval:
 1.508343      Inf
sample estimates:
odds ratio
 3.049831
```

P-value small, odds-ratio high → List is *surprisingly* enriched in Gene Set

Assessing significance: Fisher test (2)

```
> GOnnnnnCounts<-matrix(c(10,30,390,1220), nrow=2, byrow=TRUE,  
+                         dimnames=list(  
+                         GeneSet=c("In Gene Set", "Not in Gene Set"),  
+                         Test=c("Diff.expressed", "Not diff.expr.")))  
> GOnnnnnCounts
```

| | Test | |
|-----------------|----------------|----------------|
| GeneSet | Diff.expressed | Not diff.expr. |
| In Gene Set | 10 | 30 |
| Not in Gene Set | 390 | 1220 |

```
> fisher.test(GOnnnnnCounts, alternative="greater")
```

Fisher's Exact Test for Count Data

```
data: GOnnnnnCounts  
p-value = 0.517  
alternative hypothesis: true odds ratio is greater than 1  
95 percent confidence interval:  
 0.5149828      Inf  
sample estimates:  
odds ratio  
 1.042711
```

P-value not small, odds-ratio approx. 1 → List is not *surprisingly* enriched in Gene Set

Recipe for gene list enrichment test

- **Step 1:** Define **gene list** (e.g. thresholding analyzed list) and **background list**,
- **Step 2:** Select gene sets to test for enrichment,
- **Step 3:** Run enrichment tests and correct for multiple testing, if necessary,
- **Step 4:** Interpret your enrichments
- **Step 5:** Publish! ;)

Possible problems with gene list test

- No “natural” value for the threshold
- Possible loss of statistical power due to thresholding
 - No resolution between significant signals with different strengths
 - Weak signals neglected
- Different results at different threshold settings
- Based on the wrong assumption of independent gene (or gene group) sampling, which increases false positive predictions

Analysis of ranked gene lists with
Gene Set Enrichment Analysis
(also called Functional Class Scoring)

Gene Sets

- A gene set
 - a group of genes with related functions.
 - sets of genes or pathways, for their association with a phenotype.
 - Examples: metabolic pathway, protein complex, or GO (gene ontology) category.
- Identified from a prior biological knowledge.
- May better reflect the true underlying biology.
- May be more appropriate units for analysis.

Gene Sets

Each row represents one gene set

| | A | B | C | D | E | F | G |
|----|----------|------------------|-----------|----------|-----------|----------|---------|
| 1 | chr10q24 | Cytogenetic band | PITX3 | SPFH1 | NEURL | C10orf12 | NDUFB8 |
| 2 | chr5q23 | Cytogenetic band | ALDH7A1 | IL13 | 8-Sep | IRF1 | ACSL6 |
| 3 | chr8q24 | Cytogenetic band | HAS2 | LRRC14 | TSTA3 | DGAT1 | RECQL4 |
| 4 | chr16q24 | Cytogenetic band | RPL13 | GALNS | FANCA | CPNE7 | COTL1 |
| 5 | chr13q14 | Cytogenetic band | AKAP11 | ARL11 | ATP7B | C13orf1 | C13orf9 |
| 6 | chr7p21 | Cytogenetic band | ARL4A | SCIN | GLCC1 | SP8 | SOSTDC1 |
| 7 | chr10q23 | Cytogenetic band | SNCG | FER1L3 | C10orf116 | HHEX | TNKS2 |
| 8 | chr14q12 | Cytogenetic band | C14orf125 | FOXG1C | HECTD1 | SCFD1 | AP4S1 |
| 9 | chr13q13 | Cytogenetic band | ALG5 | RFXAP | DCAMKL1 | MAB21L1 | STOML3 |
| 10 | chr1p34 | Cytogenetic band | JMJD2A | MRPS15 | HIVEP3 | GJB3 | CDCA8 |
| 11 | chr10q21 | Cytogenetic band | MBL2 | C10orf70 | DNAJC12 | BICC1 | CXXC6 |

If editing in excel, watch out for its tendency to auto-format gene sets (SEP8 becomes 8-Sep)

First column are gene set names. Duplicates are not allowed

Second column contains a brief description. Its optional – you can fill in a dummy field (e.g. “na”)

Unequal lengths (i.e # of genes) is allowed

| MSigDB Collection | Subcollection | No. Gene Sets |
|-----------------------------|---|---------------|
| C1: positional gene sets | | 326 |
| C2: curated gene sets | CGP: chemical and genetic perturbations | 3402 |
| | CP: Canonical pathways | 1320 |
| | KEGG/Biocarta/REACTOME | |
| C3: motif gene sets | MIR: microRNA targets | 221 |
| | TFT: transcription factor targets | 615 |
| | CGN: cancer gene neighborhoods | 427 |
| C4: computational gene sets | CM: cancer modules | 431 |
| | BP: GO biological process | 825 |
| C5: GO gene sets | CC: GO cellular component | 233 |
| | MF: GO molecular function | 396 |
| C6: oncogenic signatures | | 189 |
| C7: immunologic signatures | | 1910 |
| Total | | 10295 |

Gene Set (Enrichment) Analysis

- Mootha (2003) as an alternative to ORA.
- It aims to identify gene sets with *subtle but coordinated expression changes* that cannot be detected by ORA methods.
 - Weak changes in individual genes gathered to large gene sets can show a significant pattern.
- Results not affected by arbitrarily chosen cutoffs.
- It does not provide information as detailed as ORA

The GSEA method

- Original GSEA method is based on comparing, for each gene group, the distribution of the test statistic within the group with the overall distribution of those statistics, i.e. the calculated for all genes.
- To do this, test statistics are ranked (from biggest to smallest) and **for each gene set** a running sum is computed such that
 - If a gene is in the gene set add a certain quantity (moderate)
 - If a gene is not in the gene set, subtract a (small) quantity
- The distribution of the running sum is compared with that of the random walk using a Kolmogorov-Smirnov test (K-S test) statistic
- P-values are computed based on a randomization.

Calculating enrichment score (ES)

Create a running sum statistic based on the following

If gene p is not in set S , then add

$$X_i = -\sqrt{\frac{N_S}{N - N_S}}$$

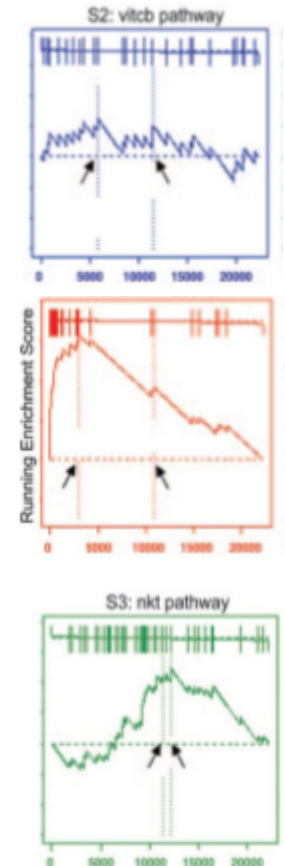
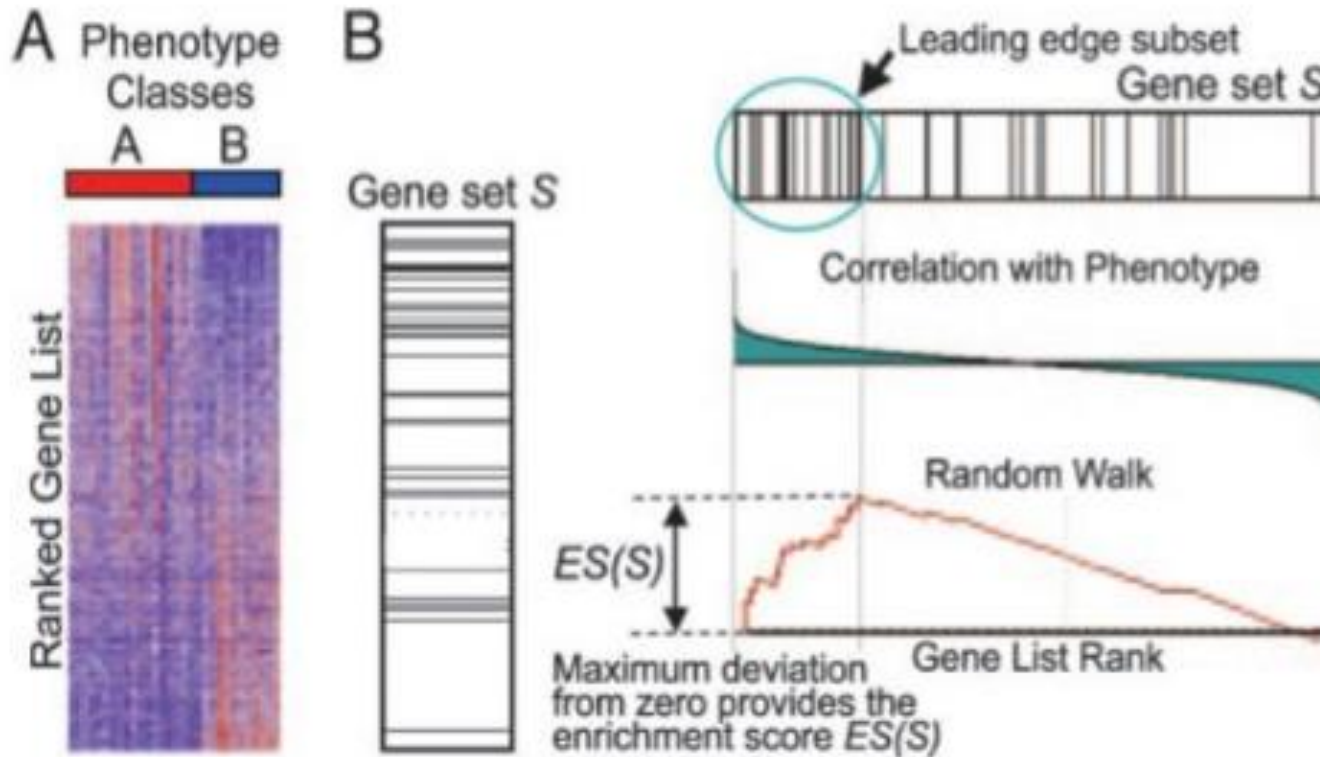
If gene p is in set S , then add

$$X_i = \sqrt{\frac{N - N_S}{N_S}}$$

This creates a running sum

The maximum sum over the whole list L is the Enrichment Score
MES

The GSEA method



Recipe for ranked list enrichment test

- **Step 1:** Rank ALL your genes,
- **Step 2:** Select gene sets to test for enrichment,
- **Step 3:** Run enrichment tests and correct for multiple testing, if necessary,
- **Step 4:** Interpret your enrichments
- **Step 5:** Publish! ;)

GSEA variants

- GSEA is not free from criticisms
 - Use of KS test
 - Null hypothesis is not clear
- Many alternative available
 - Efron's GSA
 - Limma's ROAST
 - Irizarry's simple GSA based on Wilcoxon...

Multiple test adjustments

Why we need to “adjust”

- We use a statistical test to decide if a gene list is “surprisingly” enriched in a Gene Set.
 - We use “surprisingly” instead of “significantly”
- Remember that when doing statistical tests one can be right or wrong differently.
 - Right
 - Rejecting the null hypothesis (H_0) when it is false
 - Not rejecting H_0 when it is true
 - Wrong
 - Rejecting the null hypothesis (H_0) when it is true
 - Not rejecting H_0 when it is false

Errors and Successes in tests: Type I and type II errors

| Actual Situation “Truth” | | |
|--------------------------|--|--|
| Decision \ | H_0 True | H_0 False |
| Do Not Reject H_0 | Correct Decision $1 - \alpha$ | Incorrect Decision Type II Error β |
| Rejct H_0 | Incorrect Decision Type I Error α | Correct Decision $1 - \beta$ |

$$\alpha = P(\text{Type I Error}) \quad \beta = P(\text{Type II Error})$$

Testing repeatedly

- Omics studies are “high throughput”
 - Selecting genes: One test per each gene
 - Finding enriched gene sets: One test per each gene set
- Doing many tests means facing repeatedly the probability of making one false positive.
 - As the number of tests increases →
 - The chance of observing at least one false positive is going to increase too.

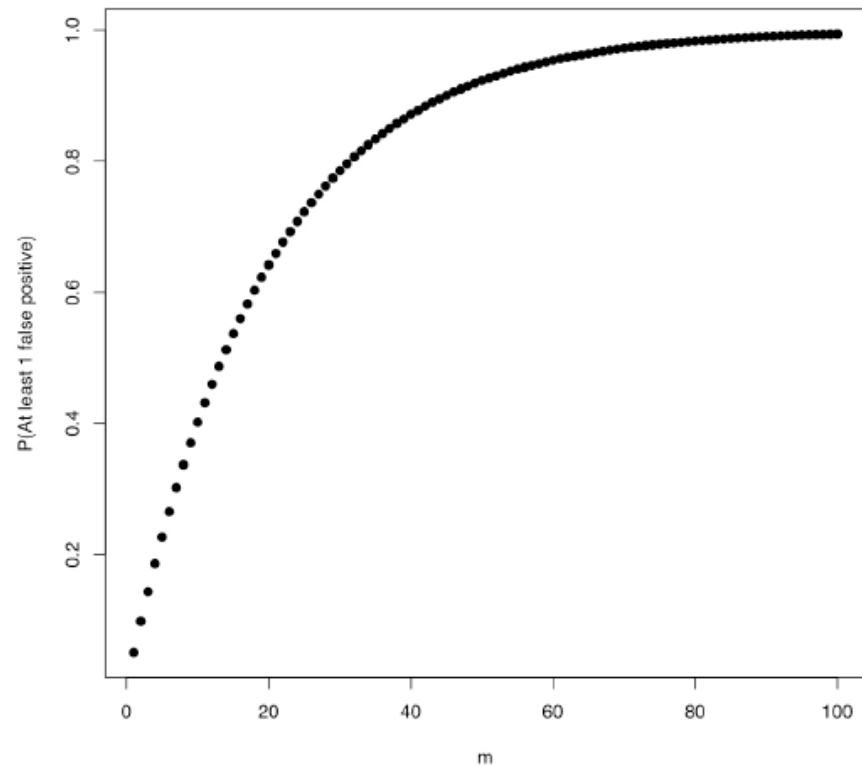
Why multiple testing matters

- The probability of observing one false positive if testing once is:
 - $P(\text{Making a type I error}) = \alpha$
 - $P(\text{not making a type I error}) = 1 - \alpha$
- Now imagine we perform m tests independently
 - $P(\text{not making a type I error in } m \text{ tests}) = (1 - \alpha)^m$
 - $P(\text{making at least a type I error in } m \text{ tests}) = 1 - (1 - \alpha)^m$
- As m increases the probability of having at least one type error tends to increase

Type I error not useful in multiple testing

Probability of At Least 1 False Positive

| Number of tests: m | P(making at least a type I error) = $1-(1-\alpha)^m$ |
|--------------------|--|
| 1 | 0.050000 |
| 2 | 0.097500 |
| 3 | 0.142625 |
| 4 | 0.185494 |
| 5 | 0.226219 |
| 6 | 0.264908 |
| 7 | 0.301663 |
| 8 | 0.336580 |



How can we deal with this issue?

- Controlling for type I error is not feasible if many tests.
- Idea: Modify α (or alternatively the p-value) so the error probability is ***controlled overall***
- This may mean different things:
 1. The probability of at least one error in m tests is $< \alpha$
 2. The expected number of false positives is below a fixed threshold.
- ...

Controlling the FWER: *Bonferroni*

If M = # of annotations tested:

Corrected P-value = M x original P-value

Corrected P-value is greater than or equal to the probability that ***one or more of the observed enrichments*** could be due to random draws.

The jargon for this correction is “controlling for the *Family-Wise Error Rate (FWER)*”

Bonferroni correction caveats

- Bonferroni correction is very stringent and can “wash away” real enrichments leading to false negatives,
- Often one is willing to accept a less stringent condition, the “false discovery rate” (FDR), which leads to a gentler correction when there are real enrichments.

False discovery rate (FDR)

- FDR is *the expected **proportion** of “False Positives” that is of the observed enrichments due to random chance.*
- Compare to Bonferroni correction which is a bound on *the probability that **any one** of the observed enrichments could be due to random chance.*
- Typically FDR corrections are calculated using the Benjamini-Hochberg procedure.
- FDR threshold is often called the “q-value”

Benjamini-Hochberg example I

| Rank | Category | (Nominal) P-value |
|------|---------------------------------|----------------------|
| 1 | <i>Transcriptional</i> | 0.001 |
| 2 | <i>regulation</i> | 0.002 |
| 3 | <i>Transcription factor</i> | 0.003 |
| 4 | <i>Initiation of</i> | 0.0031 |
| 5 | <i>transcription</i> | 0.005 |
| ... | <i>Nuclear localization</i> | ... |
| | <i>Chromatin modification</i> | |
| 52 | ... | 0.97 |
| 53 | <i>Cytoplasmic localization</i> | 0.99 |
| | <i>Translation</i> | |

Sort P-values of all tests in decreasing order

Benjamini-Hochberg example II

| Rank | Category | (Nominal) P-value | Adjusted P-value |
|------|------------------------------------|----------------------|-----------------------|
| 1 | <i>Transcriptional regulation</i> | 0.001 | 0.001 x 53/1 = 0.053 |
| 2 | <i>Transcription factor</i> | 0.002 | 0.002 x 53/2 = 0.053 |
| 3 | <i>Initiation of transcription</i> | 0.003 | 0.003 x 53/3 = 0.053 |
| 4 | <i>Nuclear localization</i> | 0.0031 | 0.0031 x 53/4 = 0.040 |
| 5 | <i>Chromatin modification</i> | 0.005 | 0.005 x 53/5 = 0.053 |
| ... | ... | ... | ... |
| 52 | <i>Cytoplasmic localization</i> | 0.97 | 0.985 x 53/52 = 1.004 |
| 53 | <i>Translation</i> | 0.99 | 0.99 x 53/53 = 0.99 |

Adjusted P-value is “nominal” P-value times # of tests divided by the rank of the P-value in sorted list

$$\text{Adjusted P-value} = \text{P-value} \times [\# \text{ of tests}] / \text{Rank}$$


Benjamini-Hochberg example III

| Rank | Category | (Nominal) P-value | Adjusted P-value | FDR / Q-value |
|------|------------------------------------|----------------------|------------------------------|------------------|
| 1 | <i>Transcriptional regulation</i> | 0.001 | $0.001 \times 53/1 = 0.053$ | 0.040 |
| 2 | <i>Transcription factor</i> | 0.002 | $0.002 \times 53/2 = 0.053$ | 0.040 |
| 3 | <i>Initiation of transcription</i> | 0.003 | $0.003 \times 53/3 = 0.053$ | 0.040 |
| 4 | <i>Nuclear localization</i> | 0.0031 | $0.0031 \times 53/4 = 0.040$ | 0.040 |
| 5 | <i>Chromatin modification</i> | 0.005 | $0.005 \times 53/5 = 0.053$ | 0.053 |
| ... | ... | ... | ... | ... |
| 52 | <i>Cytoplasmic localization</i> | 0.97 | $0.985 \times 53/52 = 1.004$ | 0.99 |
| 53 | <i>Translation</i> | 0.99 | $0.99 \times 53/53 = 0.99$ | 0.99 |

Q-value (or FDR) corresponding to a nominal P-value is the smallest adjusted P-value assigned to P-values with the same or larger ranks.

Benjamini-Hochberg example III

P-value threshold for FDR < 0.05 (Nominal)



| Rank | Category | P-value | Adjusted P-value | FDR / Q-value |
|------|------------------------------------|---------|-----------------------|---------------|
| 1 | <i>Transcriptional regulation</i> | 0.001 | 0.001 x 53/1 = 0.053 | 0.040 |
| 2 | <i>Transcription factor</i> | 0.002 | 0.002 x 53/2 = 0.053 | 0.040 |
| 3 | <i>Initiation of transcription</i> | 0.003 | 0.003 x 53/3 = 0.053 | 0.040 |
| 4 | <i>Nuclear localization</i> | 0.0031 | 0.0031 x 53/4 = 0.040 | 0.040 |
| 5 | <i>Chromatin modification</i> | 0.005 | 0.005 x 53/5 = 0.053 | 0.053 |
| ... | ... | ... | ... | ... |
| 52 | <i>Cytoplasmic localization</i> | 0.97 | 0.985 x 53/52 = 1.004 | 0.99 |
| 53 | <i>Translation</i> | 0.99 | 0.99 x 53/53 = 0.99 | 0.99 |

Red: non-significant

Green: significant at FDR < 0.05


P-value threshold is highest ranking P-value for which corresponding Q-value is below desired significance threshold

Reducing adjustment stringency

- The adjustment to the P-value threshold depends on the # of tests that you do,
- So, no matter what, *the more tests you do, the more sensitive the test needs to be*
- Can control the stringency by ***reducing the number of tests:***
 - Don't use all collections of Gene Sets available
 - Restrict testing to the appropriate GO annotations;
 - Filter gene sets by size

Tools for Pathway Analysis

R/Bioconductor



Bioconductor
OPEN SOURCE SOFTWARE FOR BIOINFORMATICS

[Home](#) [Install](#) [Help](#) [Developers](#) [About](#)

[Home](#) » [BiocViews](#)

All Packages

Bioconductor version 3.10 (Release)
Autocomplete biocViews search:

▼ Software (1823)

- ▶ AssayDomain (732)
- ▼ BiologicalQuestion (756)
 - AlternativeSplicing (38)
 - Coverage (117)
 - DifferentialExpression (315)
 - DifferentialMethylation (42)
 - DifferentialPeakCalling (12)
 - DifferentialSplicing (30)
 - DNA3DStructure (5)
 - DriverMutation (1)
 - FunctionalPrediction (18)
 - GeneFusionDetection (1)
 - GenePrediction (17)
 - GeneRegulation (88)
 - GeneSetEnrichment (120)
 - GeneSignaling (6)

Packages found under GeneSetEnrichment:
Rank based on number of downloads: lower numbers are more frequently downloaded.
Show **All** ▼ entries Search table:

| Package | Maintainer | Title | Rank |
|---------------------------------|--|---|------|
| limma | Gordon Smyth | Linear Models for Microarray Data | 13 |
| edgeR | Yunshun Chen, Aaron Lun, Mark Robinson, Gordon Smyth | Empirical Analysis of Digital Gene Expression Data in R | 24 |
| fgsea | Alexey Sergushichev | Fast Gene Set Enrichment Analysis | 41 |
| DOSE | Guangchuang Yu | Disease Ontology Semantic and Enrichment analysis | 42 |
| clusterProfiler | Guangchuang Yu | statistical analysis and visualization of functional profiles for genes and gene clusters | 44 |
| enrichplot | Guangchuang Yu | Visualization of Functional Enrichment Result | 49 |
| GSEABase | Bioconductor Package Maintainer | Gene set enrichment data structures and methods | 53 |
| pathview | Weijun Luo | a tool set for pathway based data integration and visualization | 64 |
| Category | Bioconductor Package Maintainer | Category Analysis | 68 |
| GOstats | Bioconductor Package Maintainer | Tools for manipulating GO and microarrays | 79 |

March 2017:

February 2020:

74 packages under the view “Gene Set Enrichment”

120 packages under the view “Gene Set Enrichment”

Other (non-R) pathway analysis tools

- DAVID
- Pathway Painter
- Babelomics
- GenMAPP ([www. genmapp.com](http://www.genmapp.com))
- WikiPathways ([www. wikipathways.org](http://www.wikipathways.org))
- cPath (cbio.mskcc.org/cpath)
- BioCyc (www.biocyc.org)
- Pubgene (www.pubgene.org)
- PANTHER ([www. pantherdb.org](http://www.pantherdb.org))
- WebGestalt (bioinfo.vanderbilt.edu/webgestalt/)
- ToppGeneSuite ([/toppgene.cchmc.org/](http://toppgene.cchmc.org/))
- GeneGo/MetaCore (www.genego.com)
- Ingenuity Pathway Analysis (www.ingenuity.com)
- Pathway Studio ([www. ariadnegenomics.com](http://www.ariadnegenomics.com))

Summary

- Pathway Analysis is a useful approach to help gain biological understanding from omics-based studies.
- There are many ways, many methods, many tools
- Choice of the method should be guided by
 - a combination of availability, ease of use and usefulness ,
 - Usually obtained from a good understanding of how it
- Different methods may yield different results
 - Worth checking!

References

- Efron, Bradley, and Robert Tibshirani. 2007. "On Testing the Significance of Sets of Genes." *The Annals of Applied Statistics* 1 (1): 107–29. doi:10.1214/07-AOAS101.
- Irizarry, Rafael A., Chi Wang, Yun Zhou, and Terence P. Speed. 2009. "Gene Set Enrichment Analysis Made Simple." *Statistical Methods in Medical Research* 18 (6): 565–75. doi:10.1177/0962280209351908.
- Khatri, Purvesh, and Sorin Drăghici. 2005. "Ontological Analysis of Gene Expression Data: Current Tools, Limitations, and Open Problems." *Bioinformatics (Oxford, England)* 21 (18): 3587–95. doi:10.1093/bioinformatics/bti565.
- Khatri, Purvesh, Marina Sirota, and Atul J. Butte. 2012. "Ten Years of Pathway Analysis: Current Approaches and Outstanding Challenges." *PLOS Computational Biology* 8 (2): e1002375. doi:10.1371/journal.pcbi.1002375.
- Maciejewski, Henryk. 2014. "Gene Set Analysis Methods: Statistical Models and Methodological Differences." *Briefings in Bioinformatics* 15 (4): 504–18. doi:10.1093/bib/bbt002.
- Mootha, Vamsi K., Cecilia M. Lindgren, Karl-Fredrik Eriksson, Aravind Subramanian, Smita Sihag, Joseph Lehar, Pere Puigserver, et al. 2003. "PGC-1 α -Responsive Genes Involved in Oxidative Phosphorylation Are Coordinately Downregulated in Human Diabetes." *Nature Genetics* 34 (3): 267–73. doi:10.1038/ng1180.
- Pan, Kuang-Hung, Chih-Jian Lih, and Stanley N. Cohen. 2005. "Effects of Threshold Choice on Biological Conclusions Reached during Analysis of Gene Expression by DNA Microarrays." *Proceedings of the National Academy of Sciences of the United States of America* 102 (25): 8961–65. doi:10.1073/pnas.0502674102.
- Subramanian, Aravind, Pablo Tamayo, Vamsi K. Mootha, Sayan Mukherjee, Benjamin L. Ebert, Michael A. Gillette, Amanda Paulovich, et al. 2005. "Gene Set Enrichment Analysis: A Knowledge-Based Approach for Interpreting Genome-Wide Expression Profiles." *Proceedings of the National Academy of Sciences of the United States of America* 102 (43): 15545–50. doi:10.1073/pnas.0506580102.
- the Mutation Consequences and Pathway Analysis working group of the International Cancer Genome Consortium. 2015. "Pathway and Network Analysis of Cancer Genomes." *Nature Methods* 12 (7): 615–21. doi:10.1038/nmeth.3440.