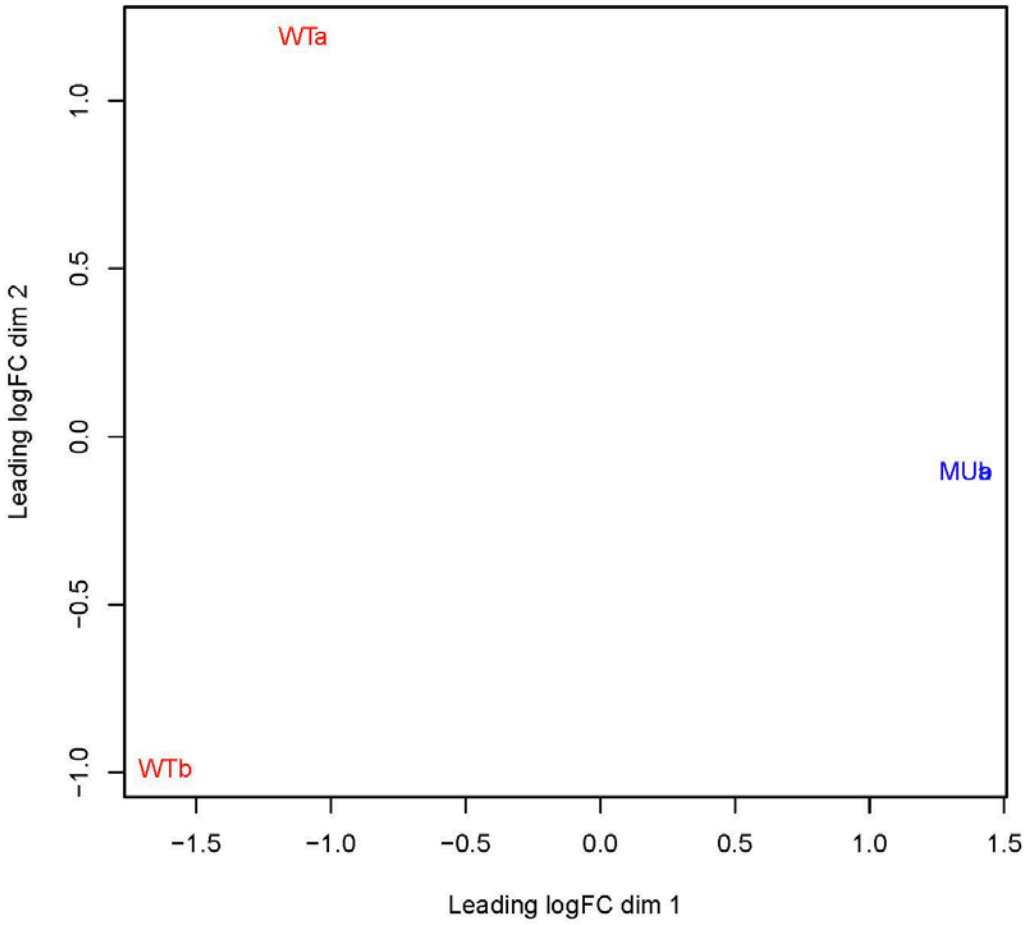
MDS Plot: the two groups are separated well at dim 1



Continue exercise 2
Using EdgeR for DE gene detection

RNA-seq workflow:
http://cbsu.tc.cornell.edu/lab/userguide.aspx

```
library("edgeR")
x <- read.delim("edgeR_count.xls", row.names='Gene')
x <- round(x, 0)
group <- factor(c(1,1,1,2,2,2,3,3,3))
y <- DGEList(counts=x,group=group)
# only keep genes with cpm value greater than 1 in at least 3 samples
keep <-rowSums(cpm(y)>=1) >=3
y<-y[keep,]
y <- calcNormFactors(y)
design<-model.matrix(~0+group)
y <- estimateGLMCommonDisp(y,design)
y <- estimateGLMTrendedDisp(y,design)
y <- estimateGLMTagwiseDisp(y,design)
fit<-glmFit(y,design)
```

To compare 2 vs 1

```
lrt.2v1<-glmLRT(fit,contrast=c(1,-1,0))
top2v1 <-  topTags(lrt.2v1, n=2000)
write.table(top2v1, "diff2-1.txt", sep="\t")
```

To compare 3 vs 1

```
lrt.3v1<-glmLRT(fit,contrast=c(1,0,-1))
top3v1 <-  topTags(lrt.3v1, n=2000)
write.table(top3v1, "diff3-1.txt", sep="\t")
```

To compare 3 vs 2

```
 lrt.3vs2<-glmLRT(fit,contrast=c(0,-1,1))
top3v2 <-  topTags(lrt.3v2, n=2000)
write.table(top3v2, "diff3-2.txt", sep="\t")
```

```
lrt.2v1<-glmLRT(fit,contrast=c(1,-1,0))
```

Use makeContrast:

```
lrt.2v1<-glmLRT(fit, contrast=makeContrasts(Drug.1h-Drug.0h))
```

Use makeContrast in multi-factor analysi:

```
lrt.2v1<-glmLRT(fit, contrast=makeContrasts((Drug.1h-Drug.0h)-(Placebo.1h-Placebo.0h)))
```

Connection between RNA-seq results and Biology

- **RNA-seq results showed that ~300 genes were differentially expressed between condition A and B;**
- **What to do next?**

Public and Commercial Resources of Pathway/Function analysis

- **Public resource:**
  - DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/)

- **Commercial Resource:**
  - BLAST2GO: Bioinformatics Facility has license
  - Ingenuity:

  (License information http://www.biotech.cornell.edu/node/137)

What is Gene Ontology -1

How to describe the function of a gene?

- **Gene description line**

GRMZM2G002950	Putative leucine-rich repeat receptor-like protein kinase family protein
GRMZM2G006470	Uncharacterized protein
GRMZM2G014376	Shikimate dehydrogenase; Uncharacterized protein
GRMZM2G015238	Prolyl endopeptidase
GRMZM2G022283	Uncharacterized protein

- **Pathway (KEGG)**

- **Controlled vocabulary (Gene Ontology)**

What is Gene Ontology -1

How to describe the function of a gene?

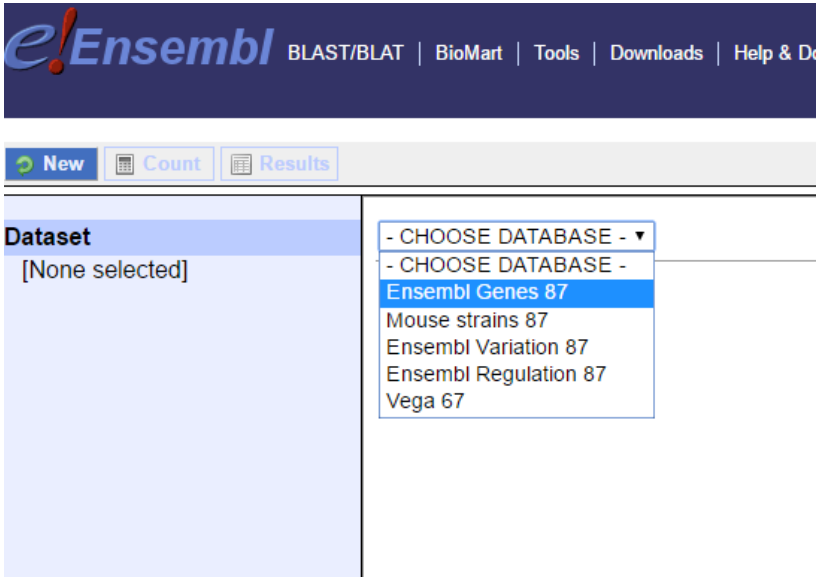
- **Gene description line**
- **Pathway (KEGG)**
- **Controlled vocabulary (Gene Ontology)**

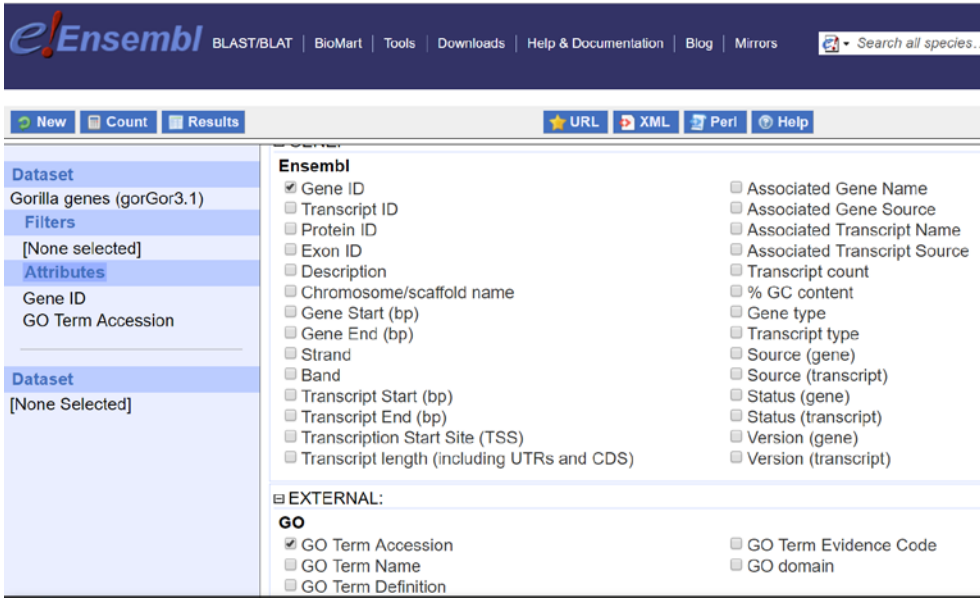
GRMZM5G888620	GO:0003674
GRMZM5G888620	GO:0008150
GRMZM5G888620	GO:0008152
GRMZM5G888620	GO:0016757
GRMZM5G888620	GO:0016758
GRMZM2G133073	GO:0003674
GRMZM2G133073	GO:0016746

How to Get Gene Ontology Data (1)

Publicly available Reference genome

Ensembl BioMart: http://www.ensembl.org





How to Get Gene Ontology Data (2)

Your own reference genome

BLAST2GO on BioHPC Lab

Details for **blast2go** (hide)

Name:	blast2go
Version:	DB: Mar.2016; Software: v1.2.1
OS:	Linux
About:	Gene Ontology annotation and function enrichment analysis.
Added:	4/15/2013 5:20:07 PM
Updated:	4/25/2016 12:13:57 PM
Link:	https://www.blast2go.com/
Manual:	https://www.blast2go.com/images/b2g_pdfs/blast2go_cli_manual.pdf
Download:	https://www.blast2go.com/blast2go-pro/b2g-register-basic
Notes:	

```
################################################

####   Run BLAST on any BioHPC computer   ######
################################################
#you can run blast on any of the biohpc computers, adjust the num_threads based on computer you are
using:general machine: 8; medium memory:24; large memory: 64
# you have an option to use swissprot, refseq or nr for blast database. In most cases swissprot is fast and good
enough. However, if a large percentage of your genes have no blast hits to swissprot, you can try refseq. The nr
database is too big, the blast run would take very long time.
#replace test.fa with your own fasta file. Make sure you are using the right blast software (blastx or blastp). To
save time, it is preferrable to use blastp on protein queries. We recommend to to use TransDecoder software to
identify protein coding sequences from cDNA sequences.
#replace swissprot with nr if you want to blast against nr database
#adjust the blast parameters in blast command
# BLAST might take hours to finish. With nr, it might take days

#commands (use swissprot as an example. To use refseq, replate swissprot with refseq_protein)

cd /workdir/myUserName
cp /shared_data/genome_db/BLAST_NCBI/swissprot* ./

blastp -num_threads 24 -query test.fa -db swissprot -out blastresults.xml  -max_target_seqs 20 -evalue 1e-5 -
outfmt 5  -culling_limit 10 >& blastlogfile &

After this step, the blast result file blastresults.xml will be created. Copy this file to your home directory.



################################################
####   Optional: Run Interproscan on any BioHPC computer   ######
################################################
#you can run interproscan on any of the biohpc computers,

Follow the instruction to run interproscan on BioHPC lab
```

How to do GO analysis?

Using Fisher's Exact Test to identify over represented genes in a pathway or function category

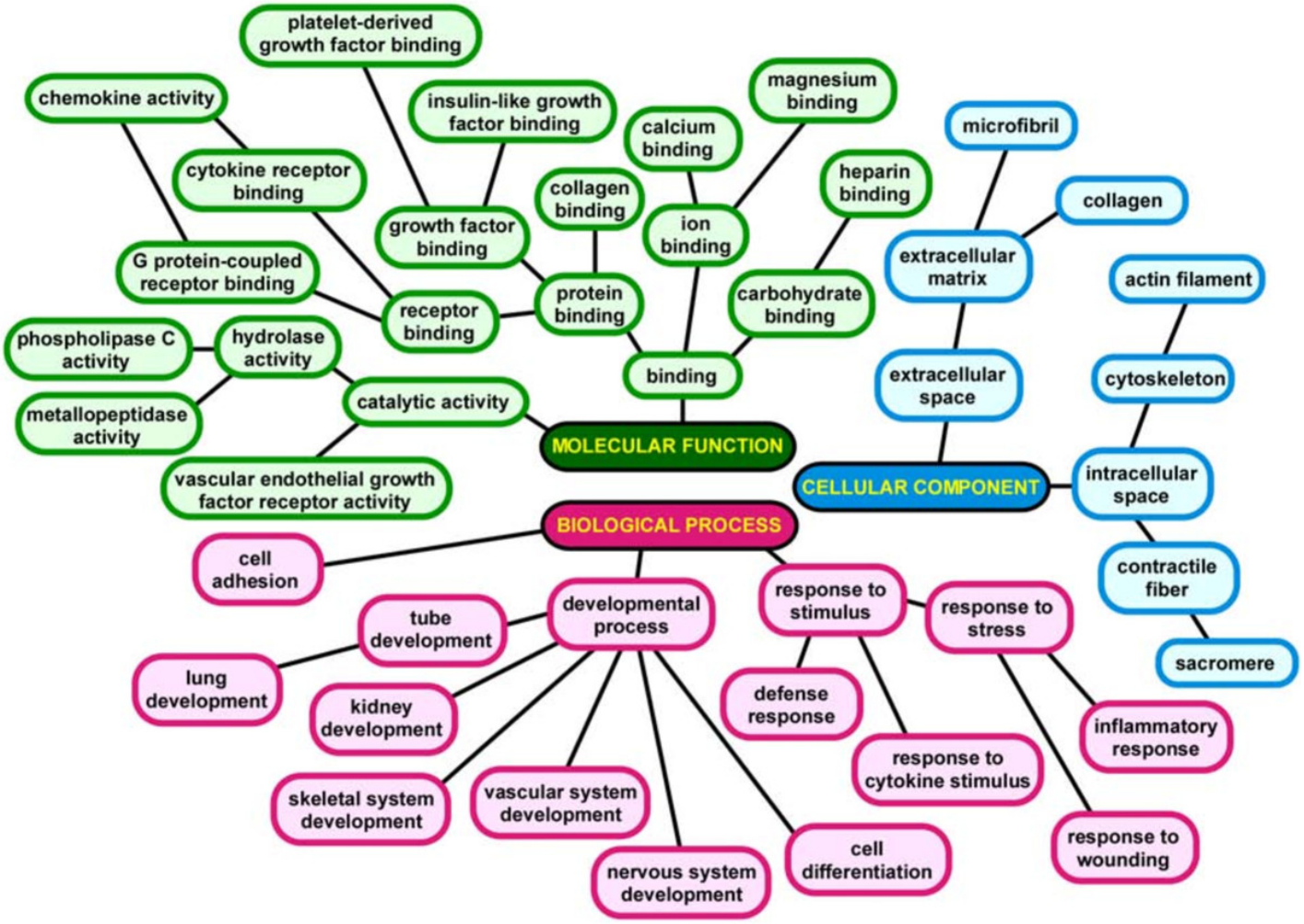
	Genes in the genome	DE genes in a experiment
P53 Pathway	40	3 -1
Not P53 Pathway	29960	297

Standard Fisher's exact test: P value= 0.008

EASE Score (in red): P value=0.06

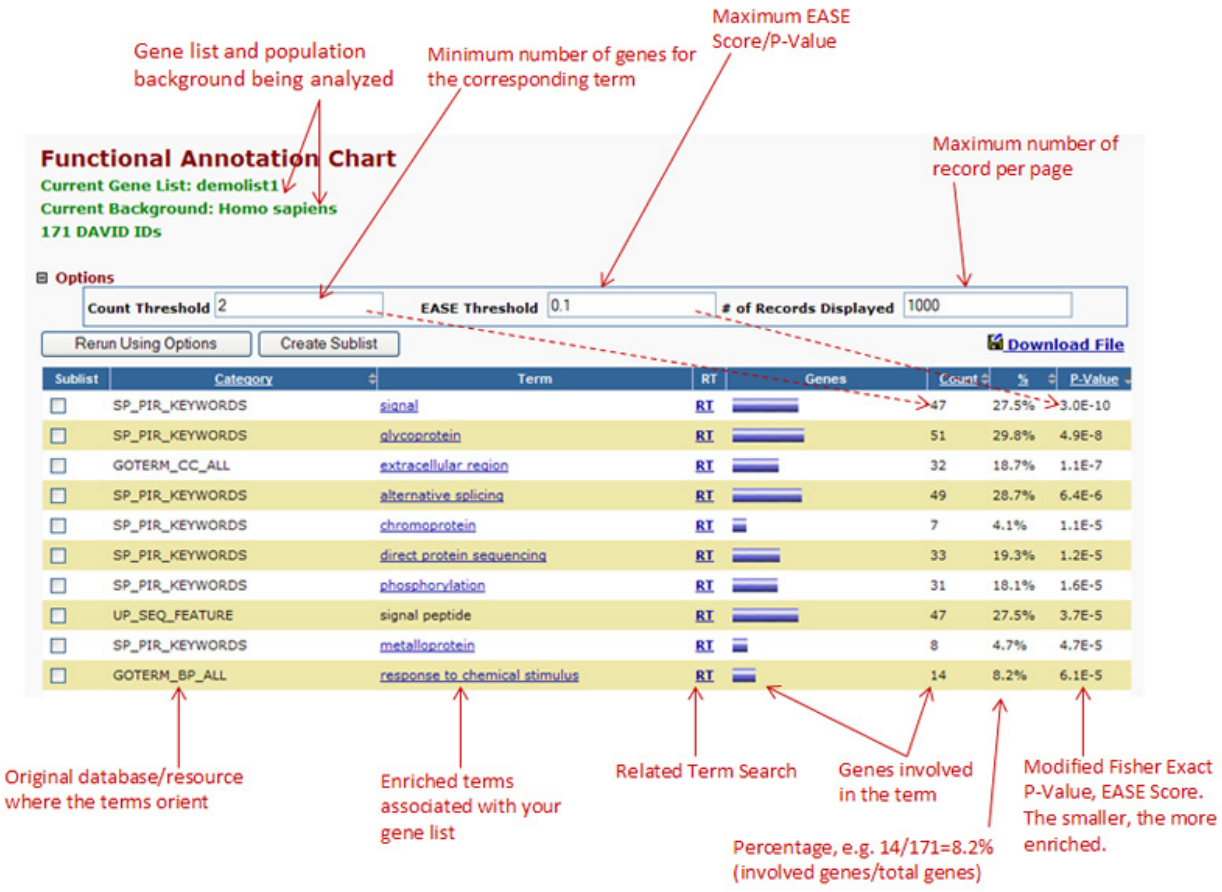
http://david.abcc.ncifcrf.gov/content.jsp?file=functional_annotation.html

Hierarchical structure of gene ontology?



Tools for function Enrichment analysis

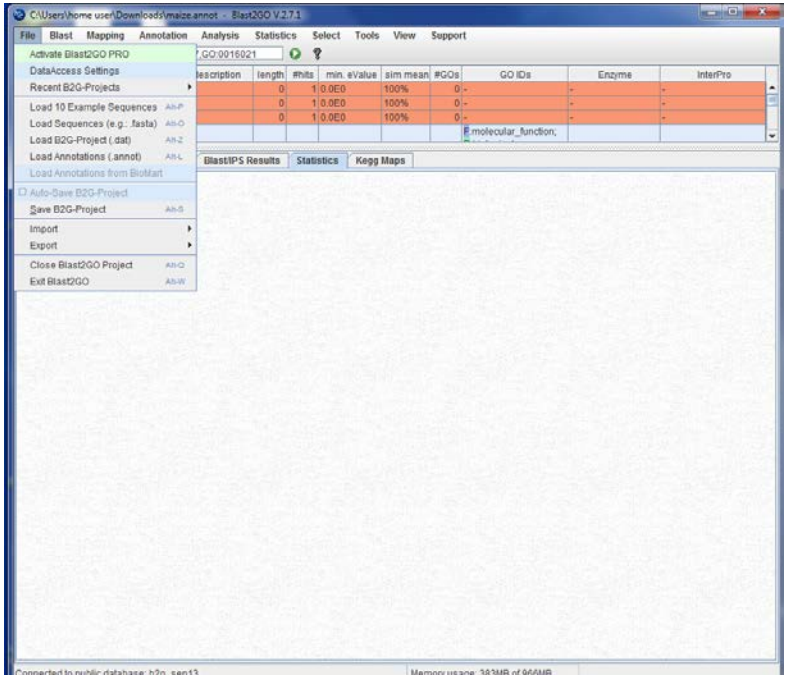
- DAVID
  - Web based (http://david.abcc.ncifcrf.gov/)
  - Recognized Gene IDs are limited



Function Enrichment analysis

- BLAST2GO
  - Flexible input file for reference genome, can do sequence based function annotation

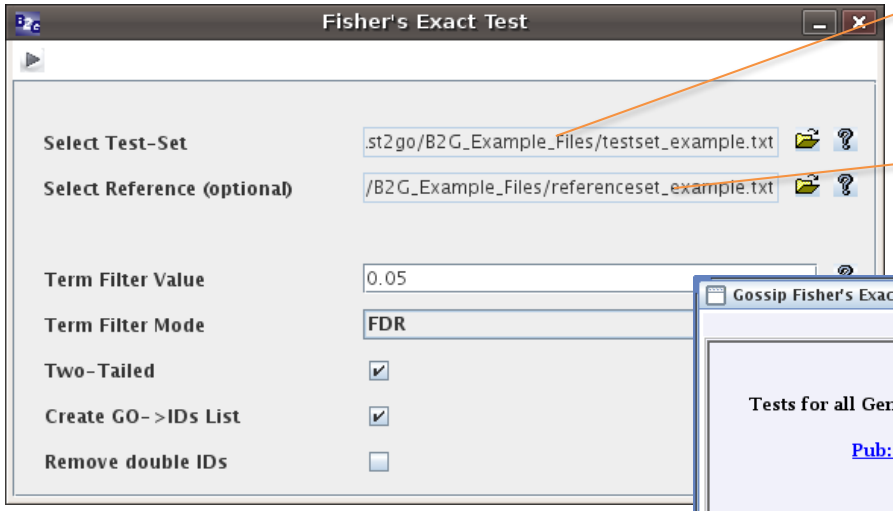
    Input file: Sequence FASTA, BLAST results, GO annotation file
  - Do Fisher's Exact test with a graphic user interface



Fisher's Exact Test with BLAST2GO

Genes in test set

Genes in reference set (filtered gene list)



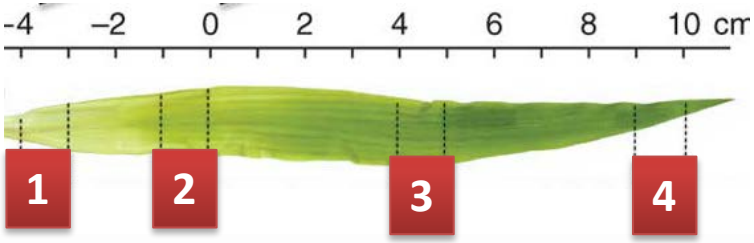
Gossip Fisher's Exact Test Results: testset_example.txt

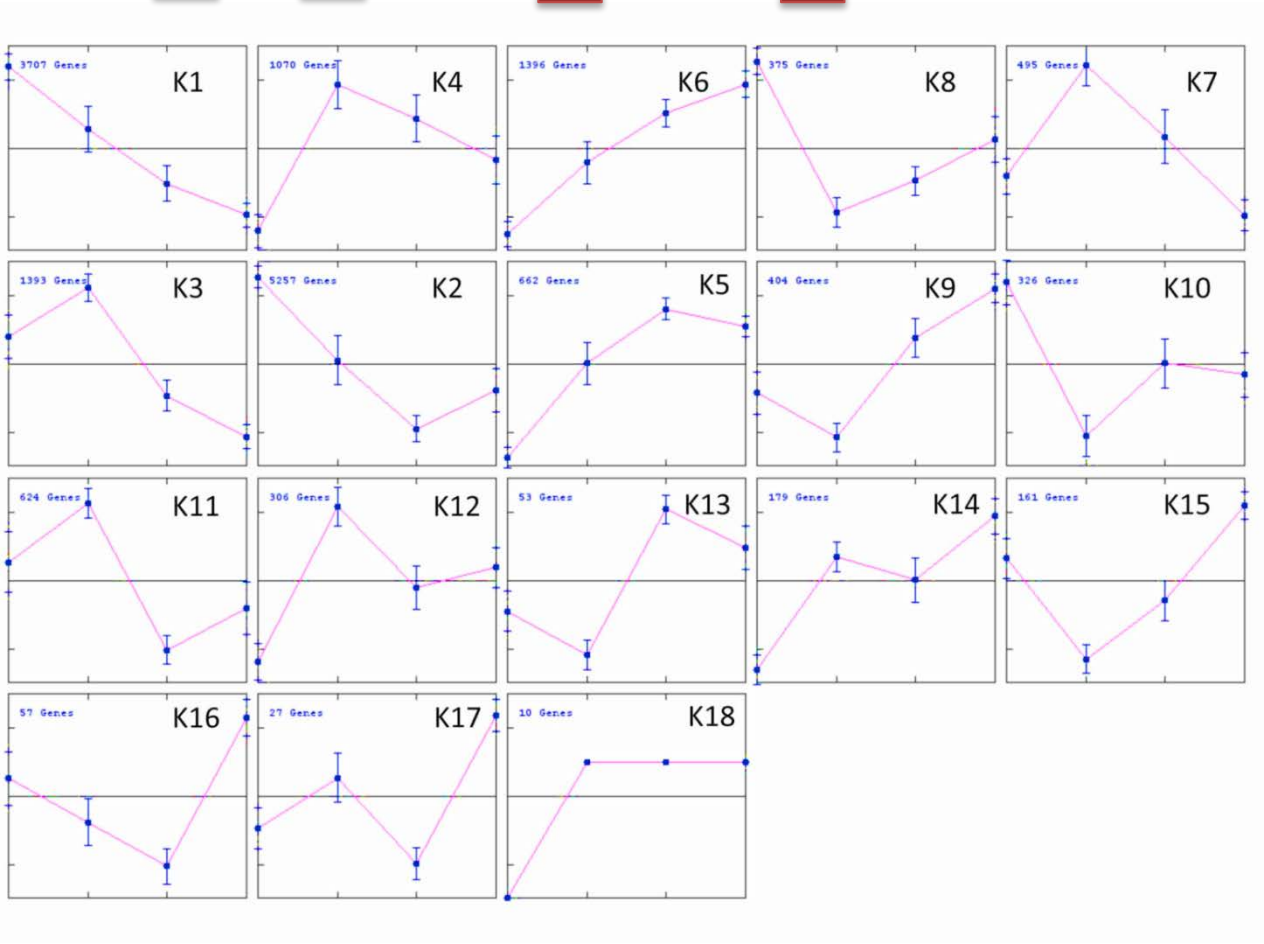
GOSSIP
Test-Set: testset_example.txt
Tests for all Gene Ontology terms if they are enriched in a test group when compared to a reference group using Fisher's exact test with multiple testing correction.
Pub: Biological Profiling of Gene Groups utilizing Gene Ontology A Statistical Framework
Poster: GOSSIP: Biological Profiling of Gene Groups utilizing Gene Ontology
by Nils Blthgen, Karsten Brand, Hanspeter Herzel, Dieter Beule

GO Term	Name	FDR	FWER	single test p-Value	# in test group	# in reference group	# non annot test	# non annot reference group	Over/Under
GO:0044464	cell part	5.85654E-4	2.92787E-4	1.53838E-4	29	166	32	60	under
GO:0005623	cell	5.85654E-4	2.92787E-4	1.53838E-4	29	166	32	60	under
GO:0003824	catalytic activity	0.0067865	0.0050773	9.6063E-4	18	119	43	107	under
GO:0006790	sulfur metabolic process	0.0097901	0.00258308	8.84665E-5	8	2	53	224	over
GO:0004364	glutathione transferase activity	0.0097901	0.0152647	3.79698E-4	5	0	56	226	over
GO:0042221	response to chemical stimulus	0.0097901	0.0156898	3.91899E-4	17	21	44	205	over
GO:0006749	glutathione metabolic process	0.0097901	0.0187977	4.39258E-4	6	1	55	225	over

https://www.blast2go.com/images/b2g_pdfs/b2g_user_manual.pdf

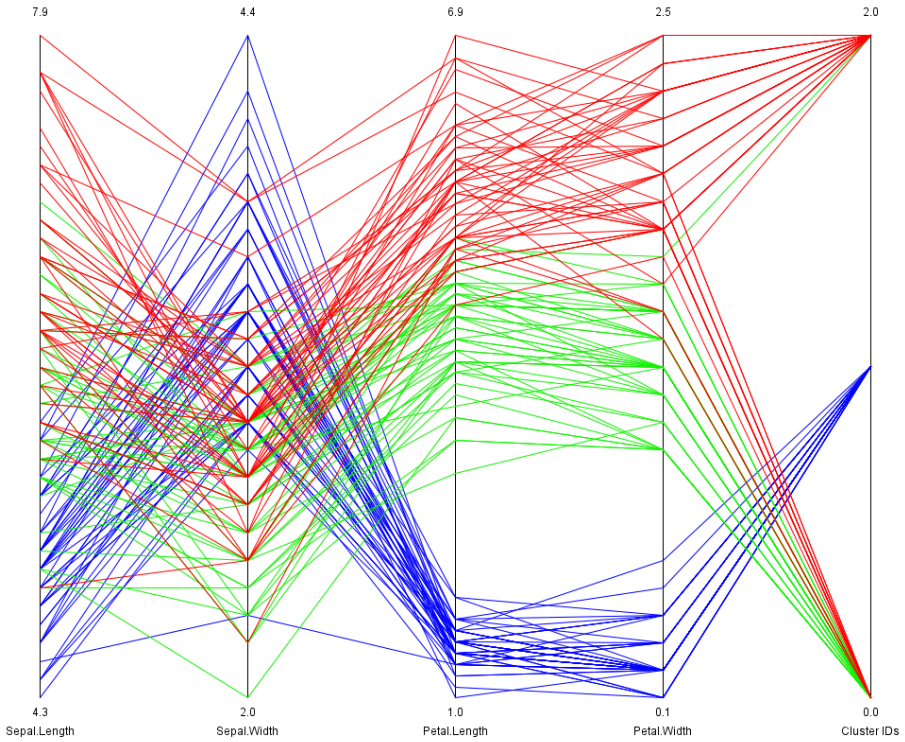
Clustering analysis on multiple conditions of RNA-seq data





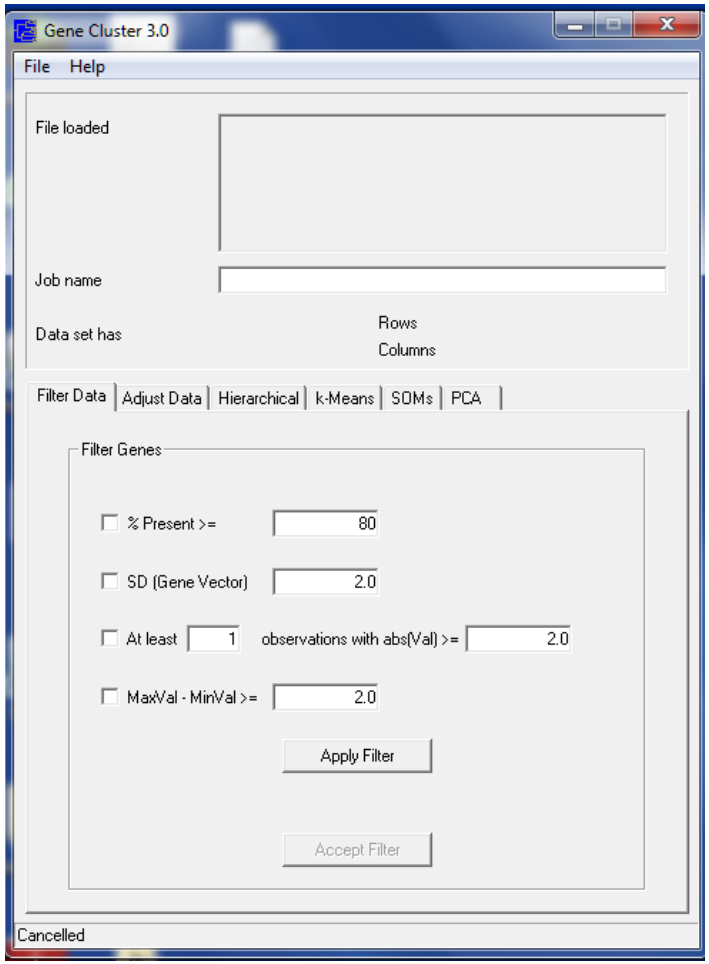
Clustering analysis

1. Hierarchical
2. K-means
3. Co-expression network



Using free software Cluster 3.0 for hierarchical and k-means clustering

http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm



tracking_id	s1_FPKM	s2_FPKM	s3_FPKM	s4_FPKM
AC148152.3_FG001	1	1	1.085823	1.237447
AC148152.3_FG002	1	1	1	1
AC148152.3_FG005	1.054317	6.65432	1.089866	1
AC148152.3_FG006	1.044314	1.223353	1	1
AC148152.3_FG007	1	1	1	1
AC148152.3_FG008	3.13339	20.1778	68.1838	88.5417
AC148167.6_FG001	17.603	43.4081	54.7869	37.5133
AC149475.2_FG002	149.468	10.75707	14.3301	11.8052
AC149475.2_FG003	101.308	34.2556	30.6524	20.2889
AC149475.2_FG004	1.053882	1	1	1

* Add 1 to each FPKM value before loading into Cluster

Alternative software

- **Gene-E**

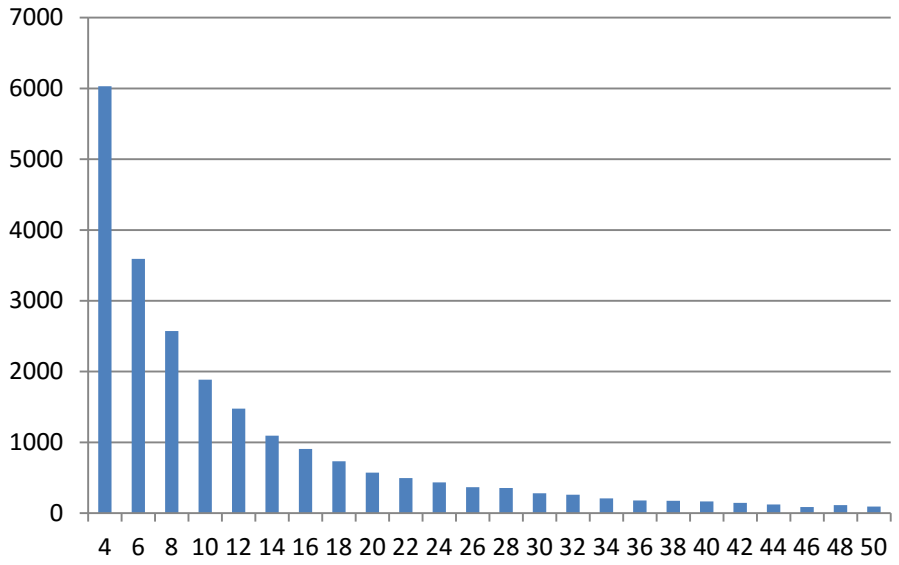
http://www.broadinstitute.org/cancer/software/GENE-E/

- **Bioconductor: hclust & kmeans**
  - Free R package

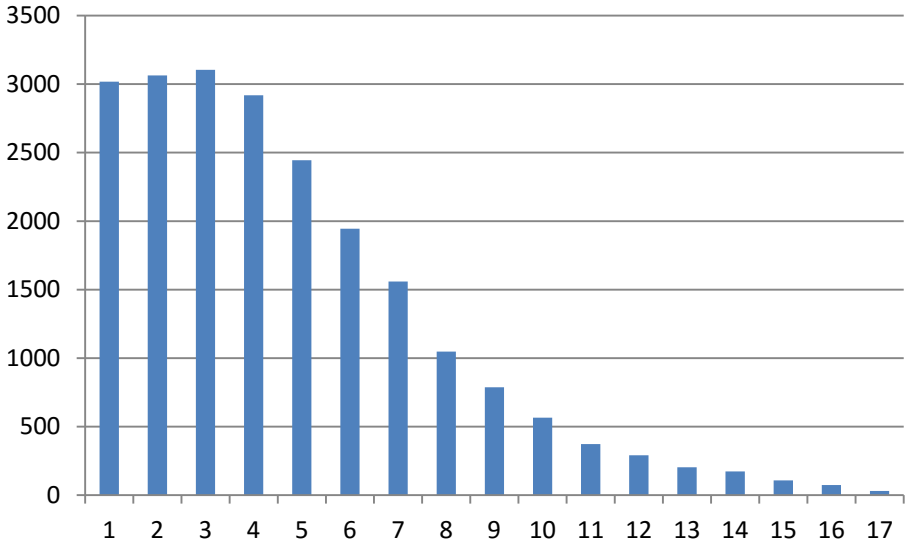
Prepare data for clustering

LOG transformation of FPKM (or CPM) value to improve the distribution

FPKM



Log2(FPKM)



To Avoid log(0), using Excel to add 1 to all FPKM values before loading to Cluster.

Filter data

To make the analysis computational feasible on a desktop computer, pre-filter the data to remove

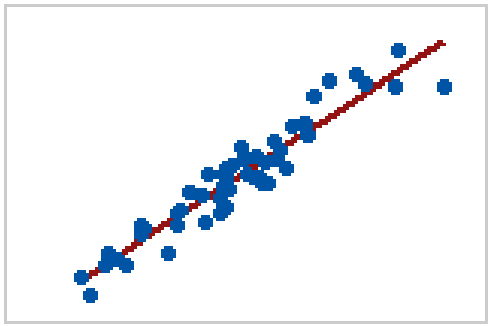
- Low expressed genes;
- Invariant genes.

Construction of pairwise distance matrix of all genes

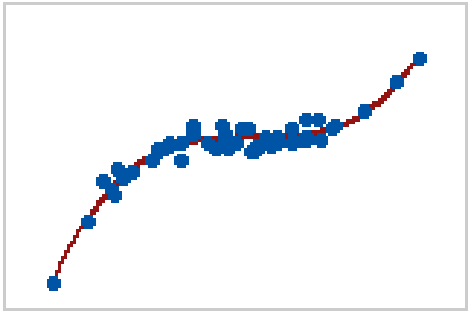
Pearson : **Linear correlation (Default)**

vs

Spearman: **Ranked correlation**



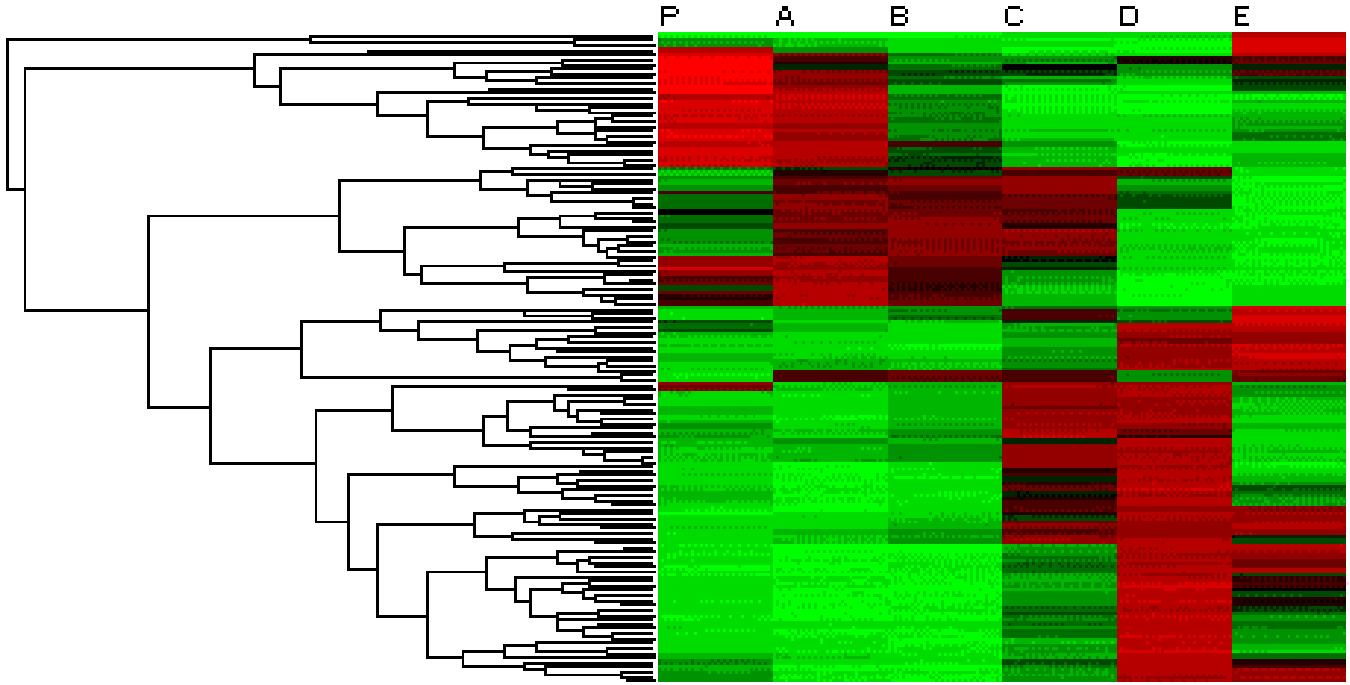
Use Pearson



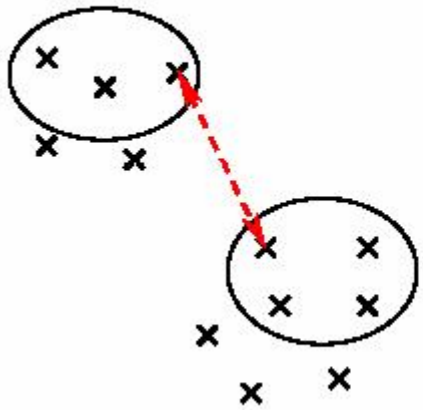
Use Spearman

Hierarchical clustering

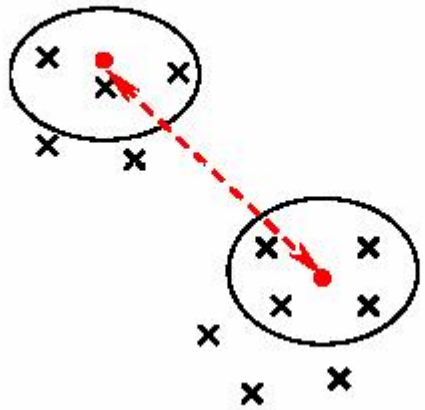
Linkage criteria in hierarchical clustering



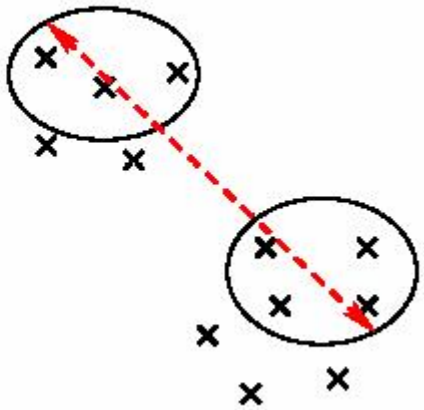
- Simple linkage



- Average linkage



- Complete linkage

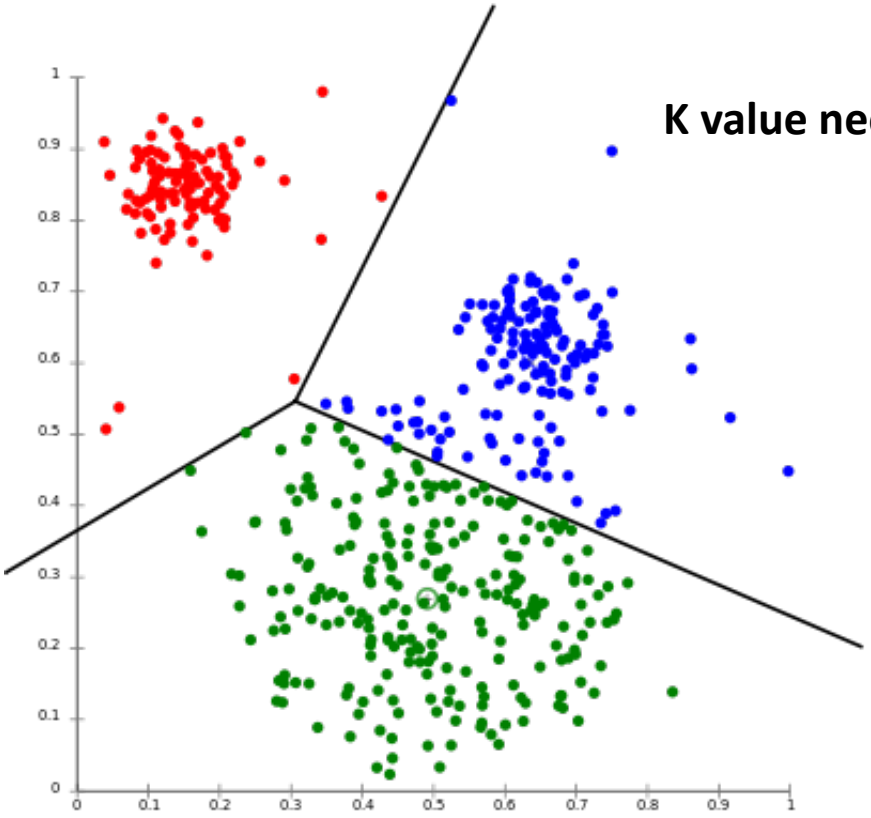


Default

Visualize the clustering results with Treeview

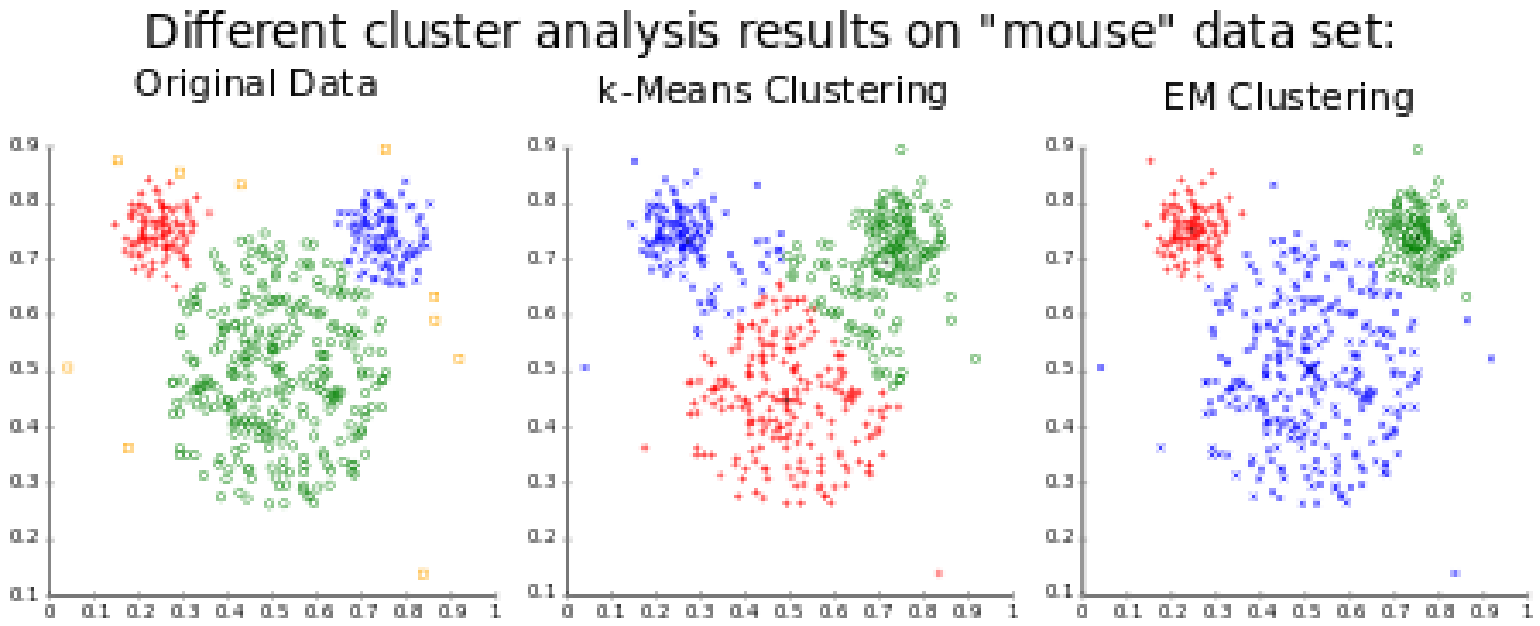
The software has functions to select nodes and export genes in selected node.

K-means clustering



K value need to be pre-specified

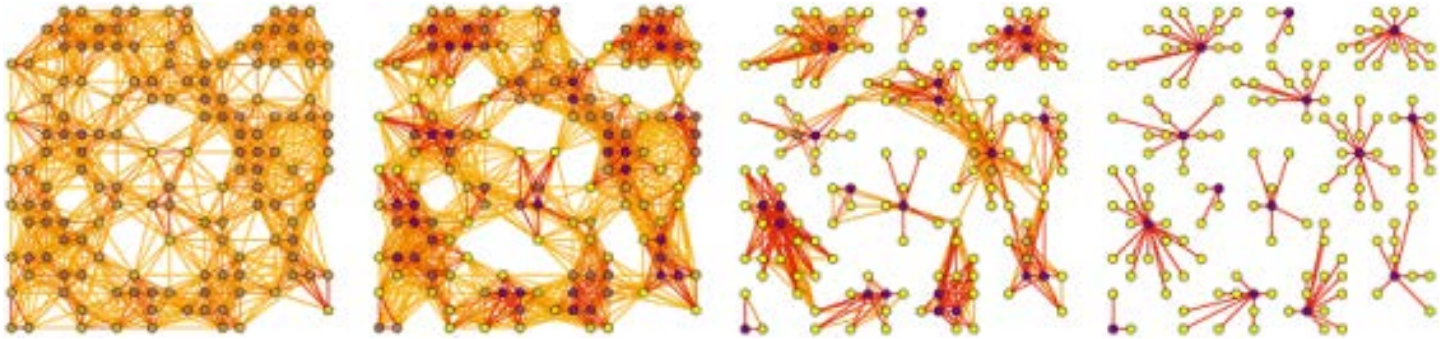
The tendency of *k*-means to produce equal-sized clusters leads to bad results



Wikipedia: K-means_clustering

Co-expression network modules

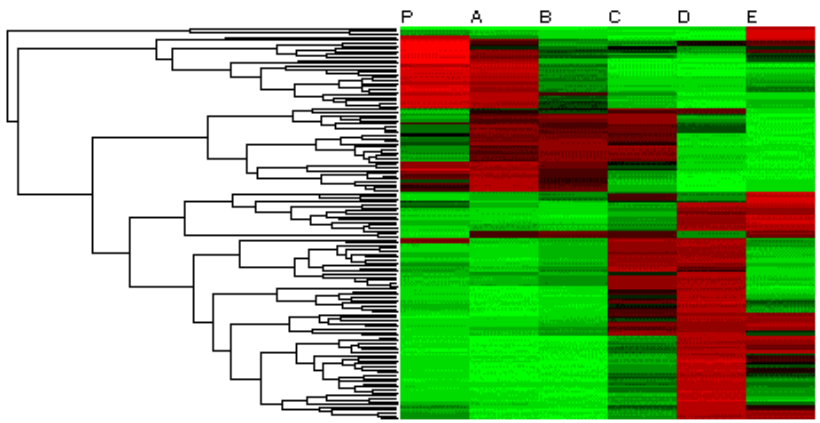
1. **MCL (Markov Cluster Algorithm)**
   - Easy to use interface: only need a distance matrix and inflation value

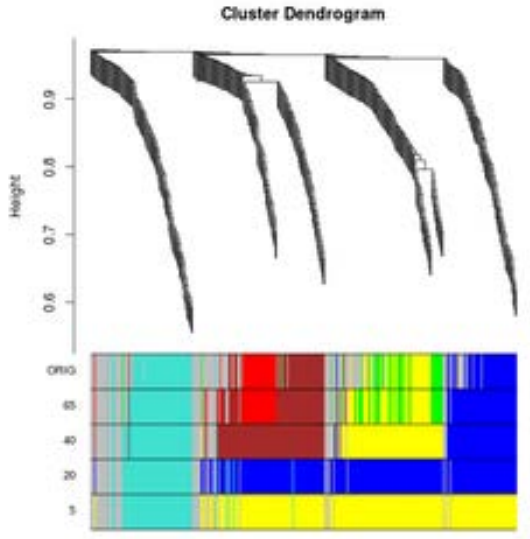


Co-expression network modules

2. WGCNA (weighted correlation network analysis)

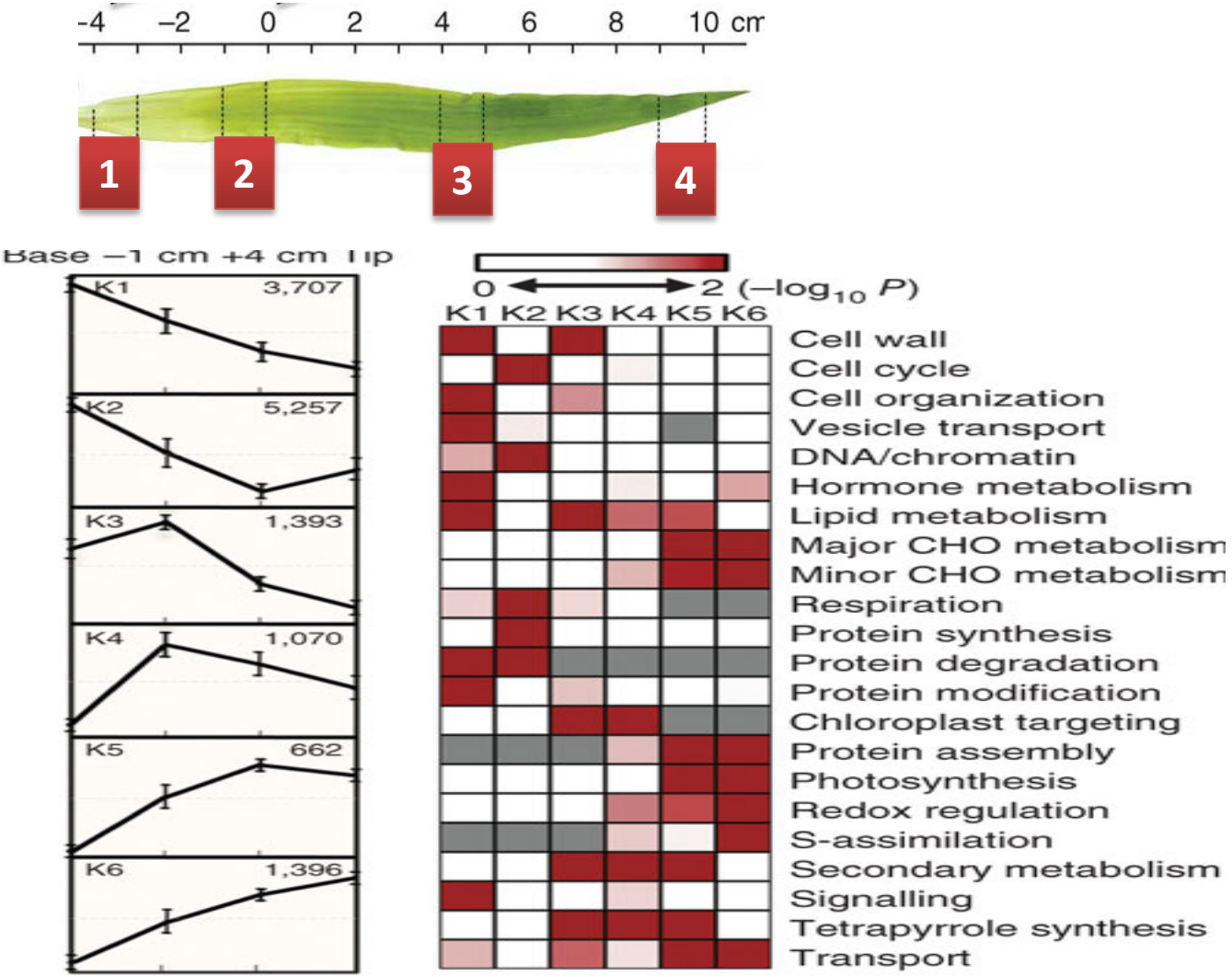
- transform the initial distance matrix into Topological Overlap Matrix





http://rgm3.lab.nig.ac.jp/RGM/R_image_list?package=WGCNA&init=true

Presentation of the results, an example



Nature Genetics 42, 1060–1067 (2010)

Homework

- Clustering and Function enrichment analysis.
- Starting file:
  - Normalized Read Count file: genes.txt
  - Rice Gene Ontology annotation file: rice.annot created with Ensembl BioMart.
- Tasks:
  - Hierarchical clustering
  - K-means clustering
  - Function enrichment analysis with BLAST2GO