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(~group)

y <- estimateGLMCommonDisp(y,design)

y <- estimateGLMTrendedDisp(y,design)

y <- estimateGLMTagwiseDisp(y,design)

fit<-glmFit(y,design)
```

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")
```

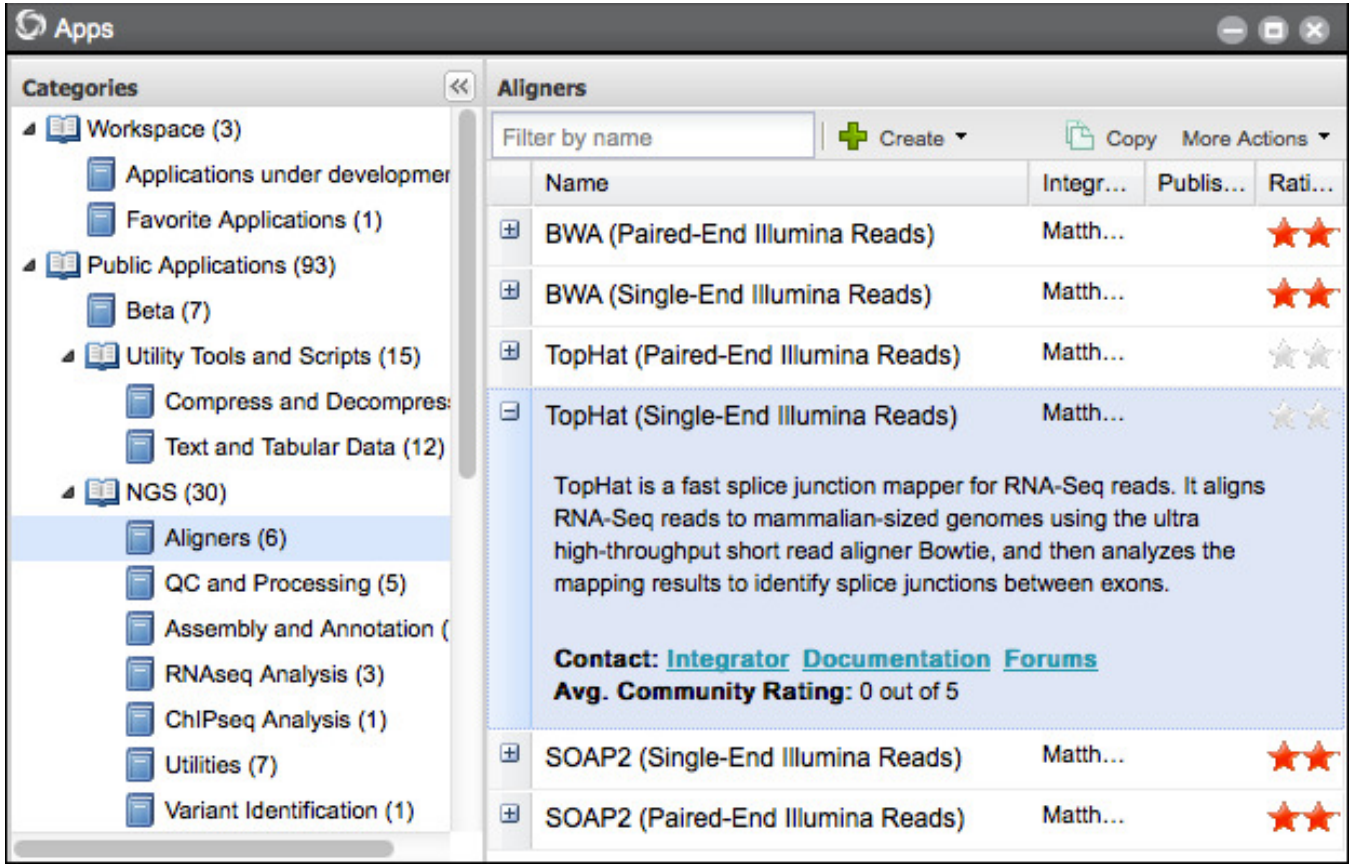
Other computational resources

- **iPlant Discovery Environment**

Tutorials:

General: http://www.iplantcollaborative.org/learning-center/all-tutorials

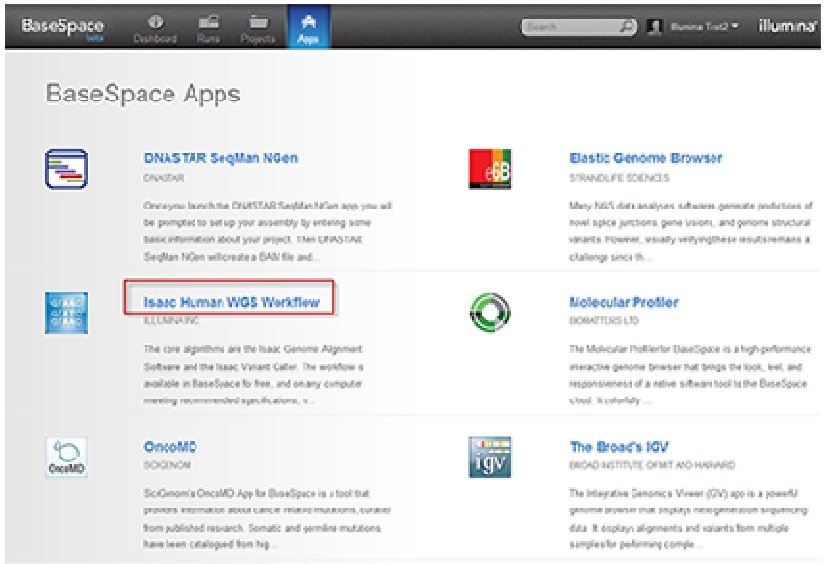
RNA-seqhttp://www.iplantcollaborative.org/learning-center/discovery-environment/de-003-characterizing-differential-expression-rna-seq



Other computational resources

- **Illumina BaseSpace**

https://basespace.illumina.com/home/index



- **Galaxy**

https://usegalaxy.org/

Commercial Software @ Cornell

http://www.biotech.cornell.edu/node/137

- **LaserGene (Ngen)**
- **Geneous**
- **Ingenuity Pathways Analysis**

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?**

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)**

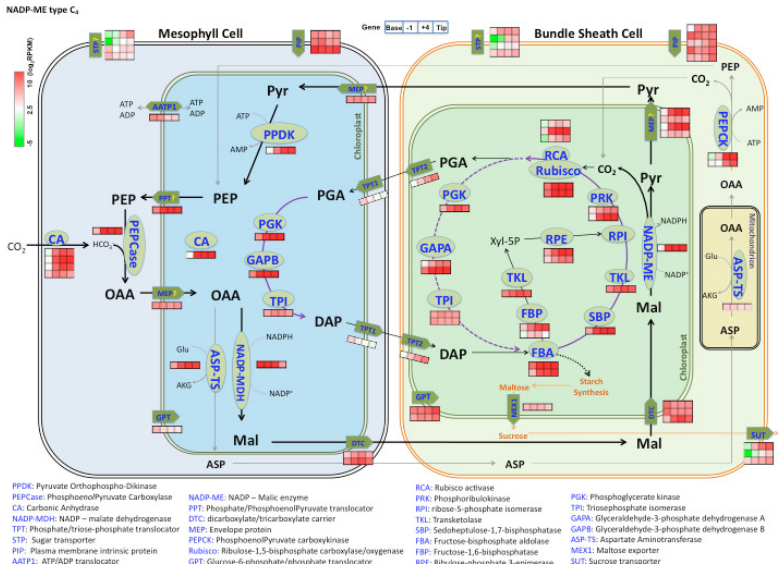
- **Controlled vocabulary (Gene Ontology)**

What is Gene Ontology -1

How to describe the function of a gene?

- **Gene description line**

- **Pathway (KEGG)**



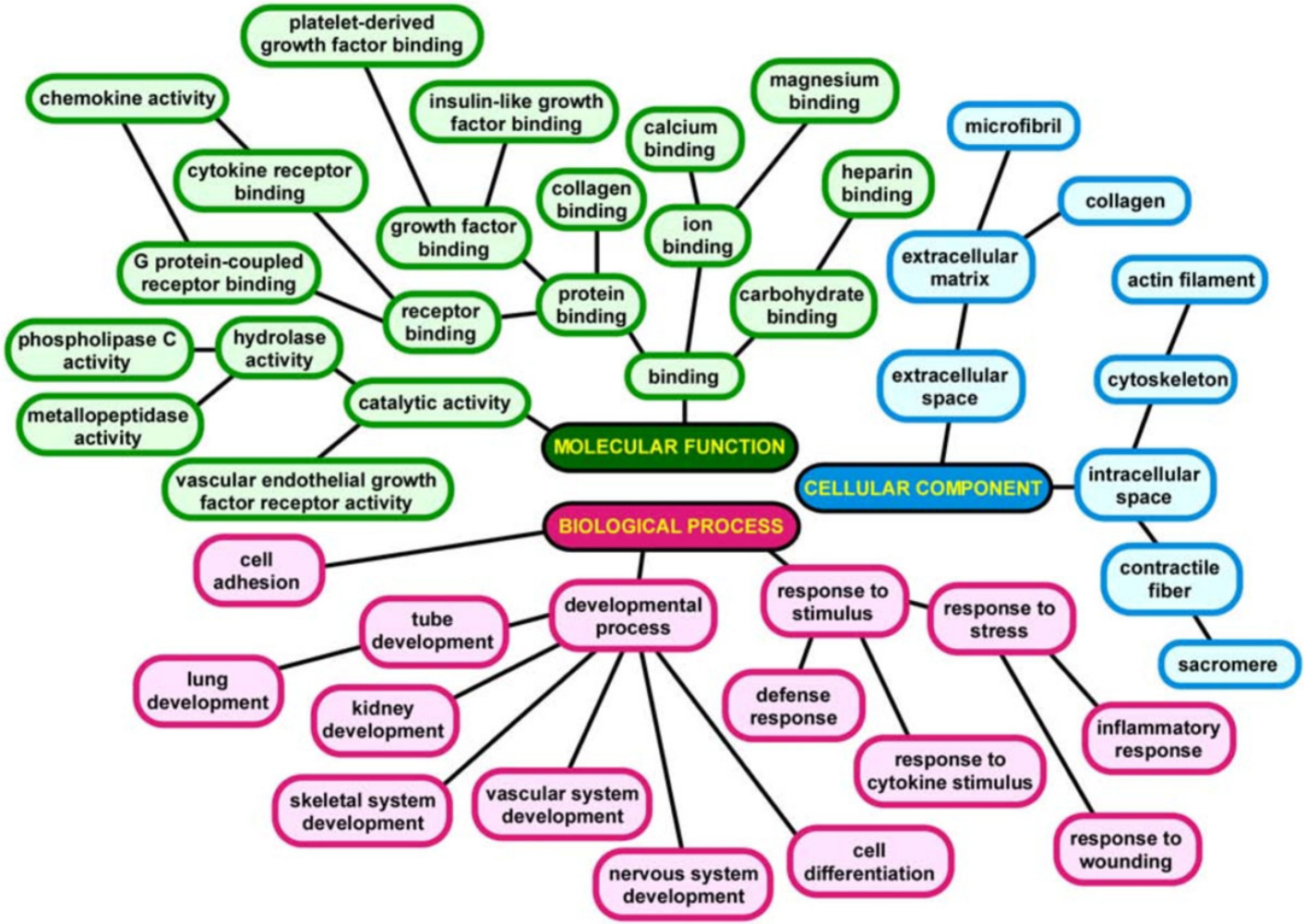
- **Controlled vocabulary (Gene Ontology)**

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

Hierarchical structure of gene ontology?



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

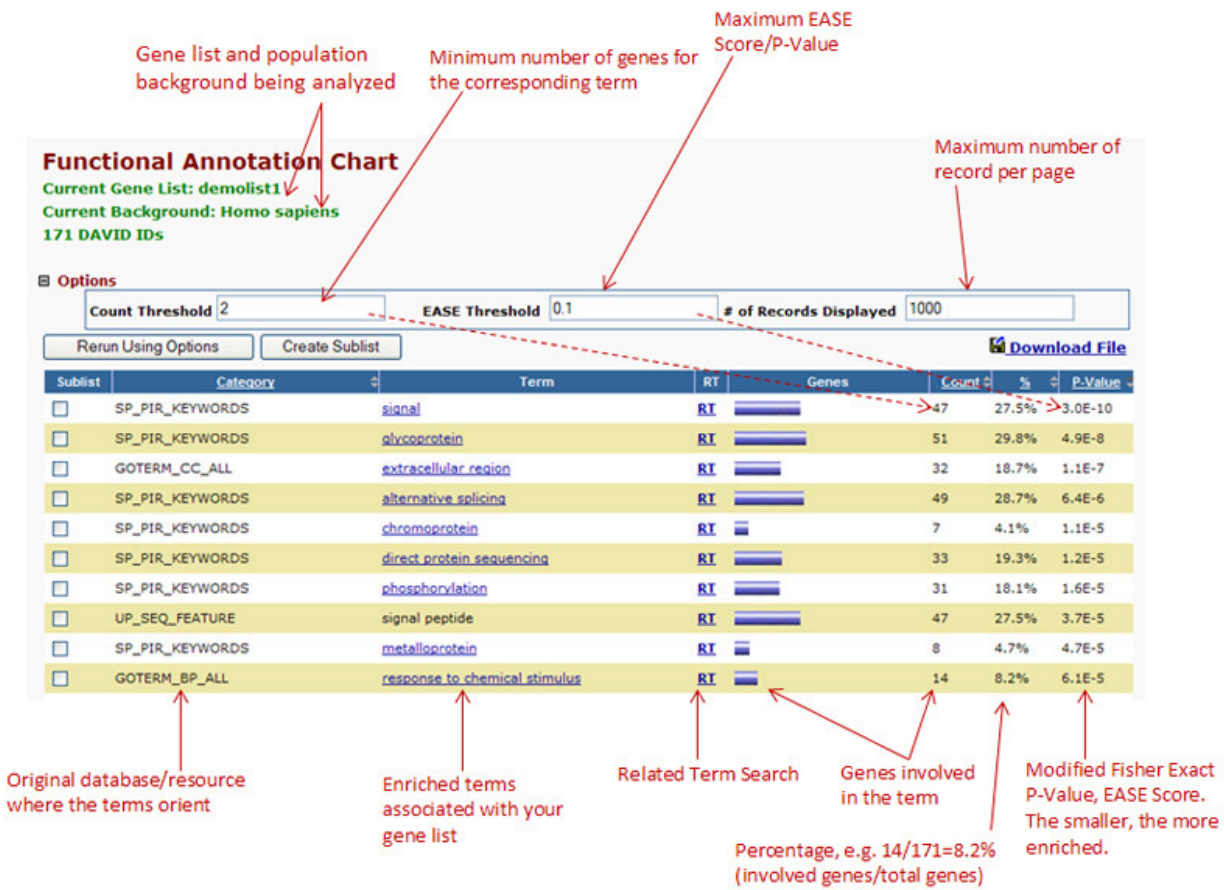
Standard Fisher's exact test: P value= 0.008

EASE Score (in red): P value=0.06

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

Tools for function Enrichment analysis

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

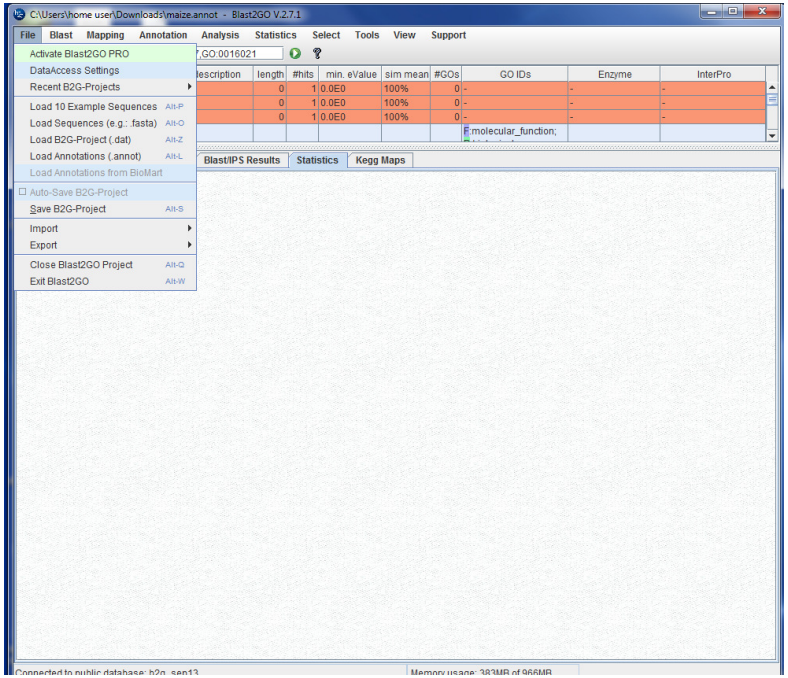


Sublist	Category	Term	RT	Genes	Count	%	P-Value
	SP_PIR_KEYWORDS	signal	RT		47	27.5%	3.0E-10
	SP_PIR_KEYWORDS	glycoprotein	RT		51	29.8%	4.9E-8
	GOTERM_CC_ALL	extracellular region	RT		32	18.7%	1.1E-7
	SP_PIR_KEYWORDS	alternative splicing	RT		49	28.7%	6.4E-6
	SP_PIR_KEYWORDS	chromoprotein	RT		7	4.1%	1.1E-5
	SP_PIR_KEYWORDS	direct protein sequencing	RT		33	19.3%	1.2E-5
	SP_PIR_KEYWORDS	phosphorylation	RT		31	18.1%	1.6E-5
	UP_SEQ_FEATURE	signal peptide	RT		47	27.5%	3.7E-5
	SP_PIR_KEYWORDS	metalloprotein	RT		8	4.7%	4.7E-5
	GOTERM_BP_ALL	response to chemical stimulus	RT		14	8.2%	6.1E-5

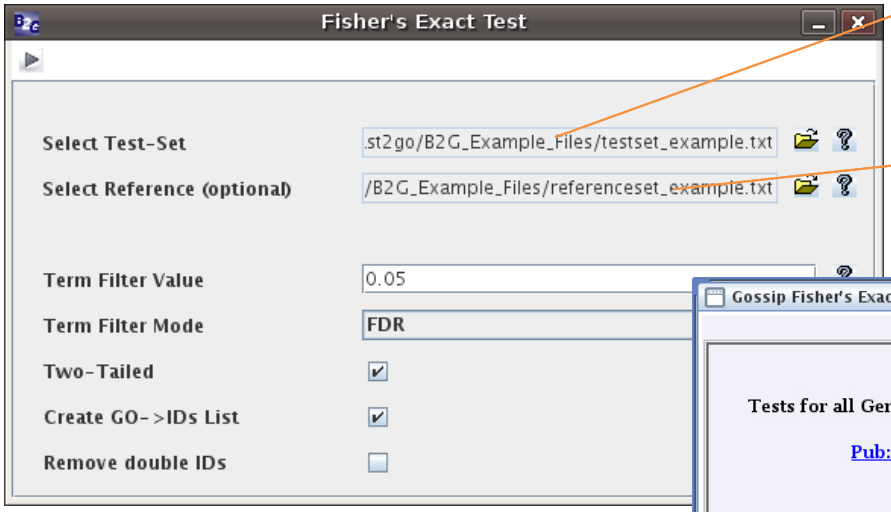
Function Enrichment analysis

- 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

http://www.blast2go.com/data/blast2go/b2g_user_manual_22102013.pdf

Public and Commercial Resources

- **Public resource:**
  - DAVID Bioinformatics Resources

  (http://david.abcc.ncifcrf.gov/)

- **Commercial Resource:**
  - Ingenuity

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

Biological Databases @ Cornell Library

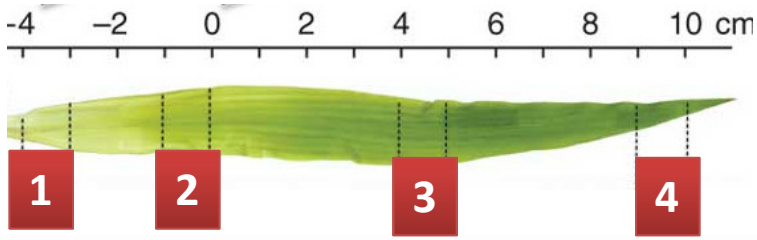
- **KEGG**
  - Biological pathway databases

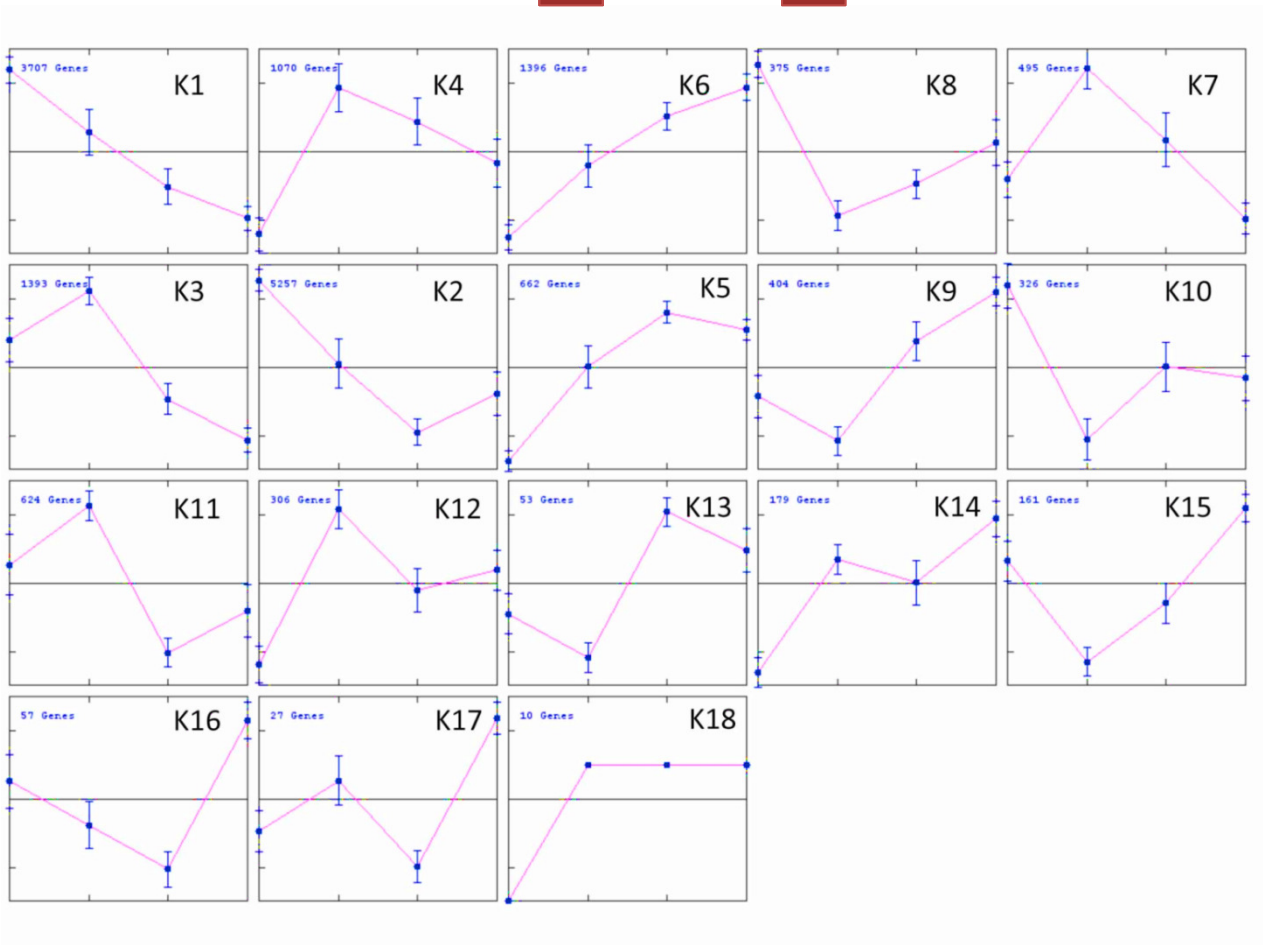
  https://catalog.library.cornell.edu/cgi-bin/Pwebrecon.cgi?BBID=8327047&DB=local

- **TAIR**
  - Arabidopsis

  https://catalog.library.cornell.edu/cgi-bin/Pwebrecon.cgi?BBID=3924196&DB=local

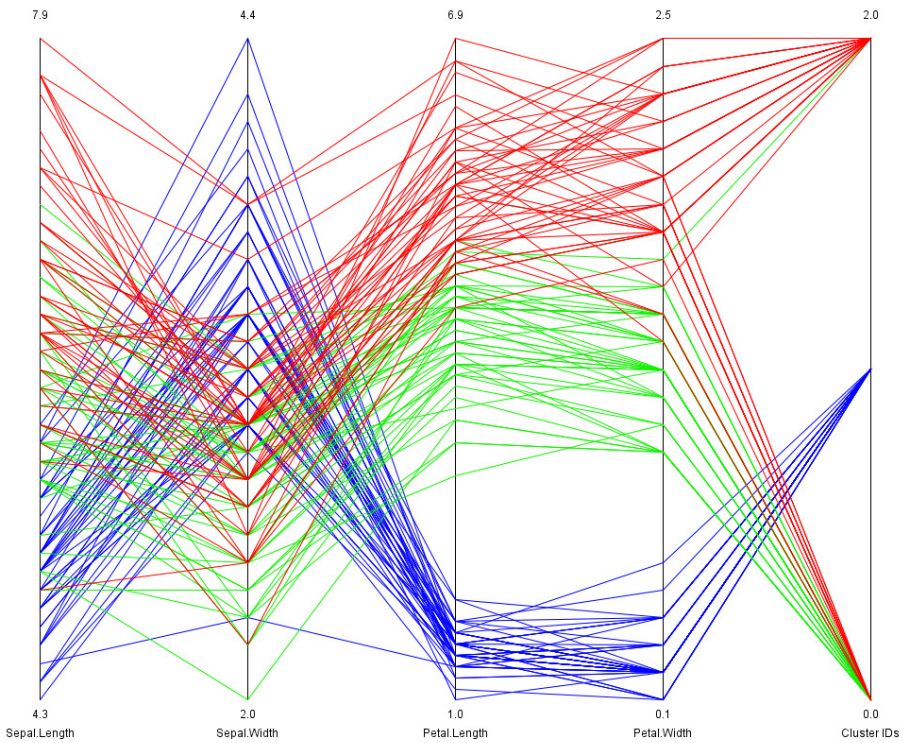
Clustering analysis on multiple conditions of RNA-seq data





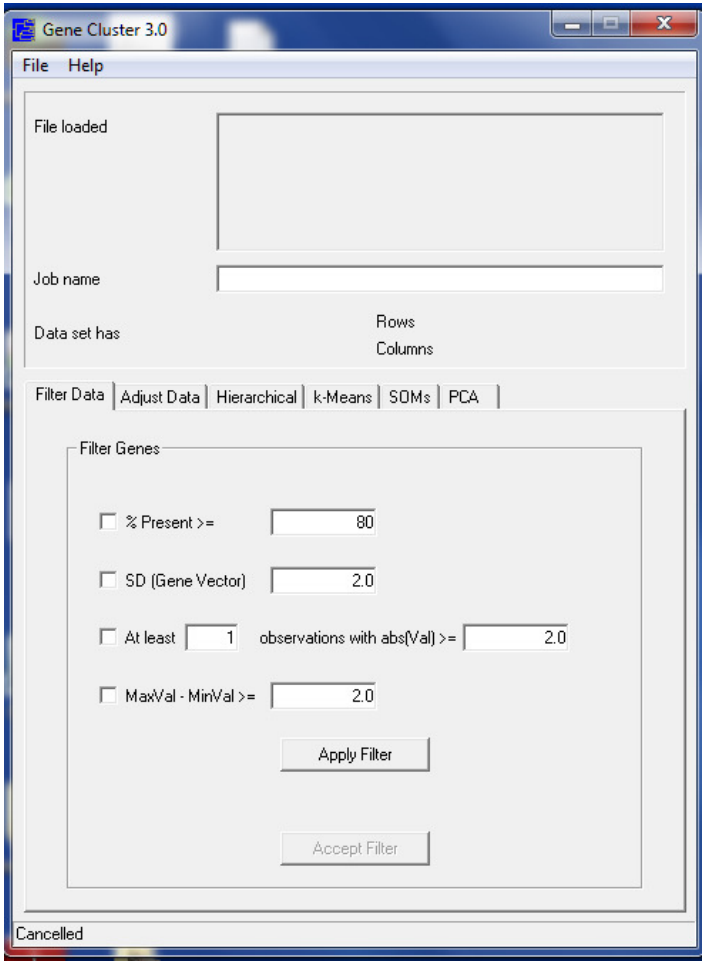
Clustering analysis

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



* Add 1 to each FPKM value before loading into Cluster

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

Alternative software

- **Gene-E**

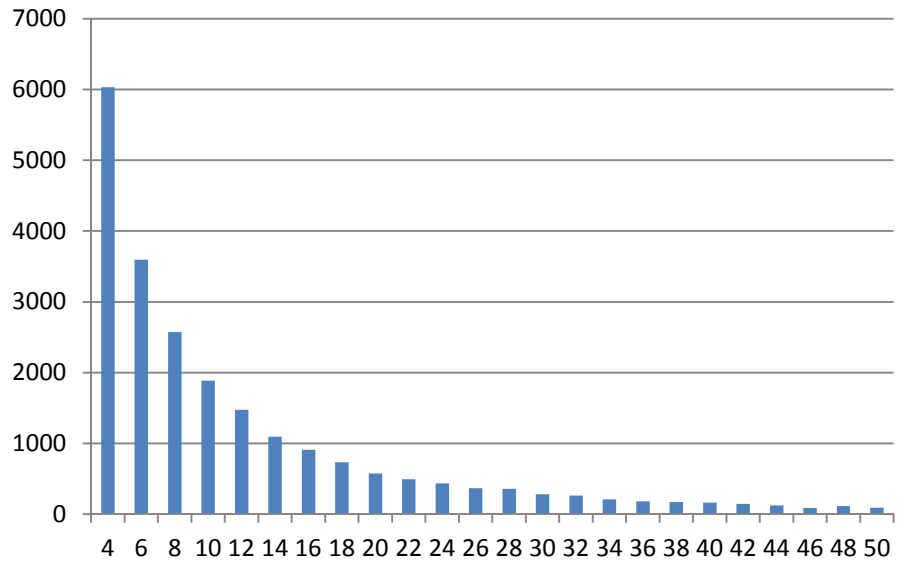
http://www.broadinstitute.org/cancer/software/GENE-E/

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

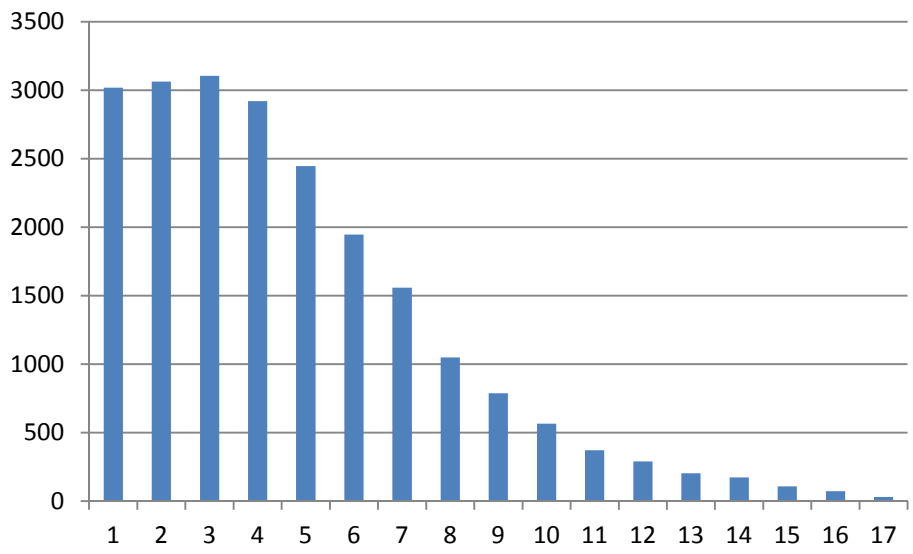
Prepare data for clustering

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

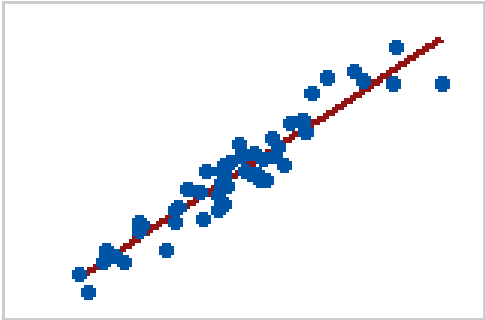
- Low expressed genes;
- Invariant genes.

Construction of pairwise distance matrix of all genes

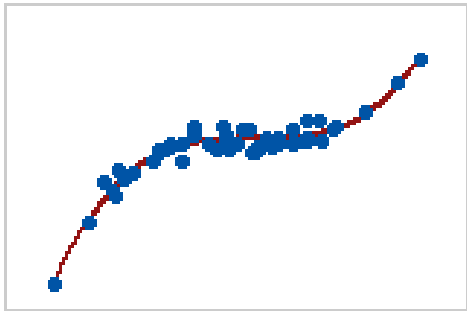
Pearson : **Linear correlation (Default)**

vs

Spearman: **Ranked correlation**

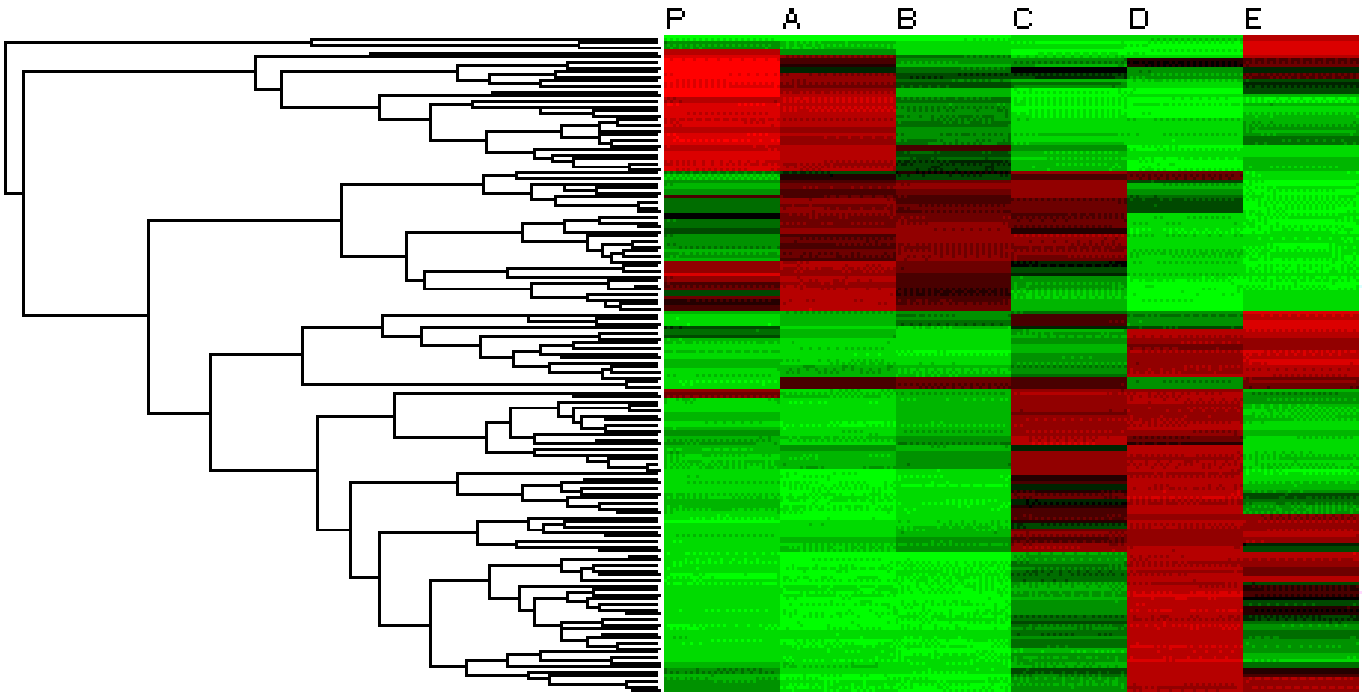


Use Pearson



Use Spearman

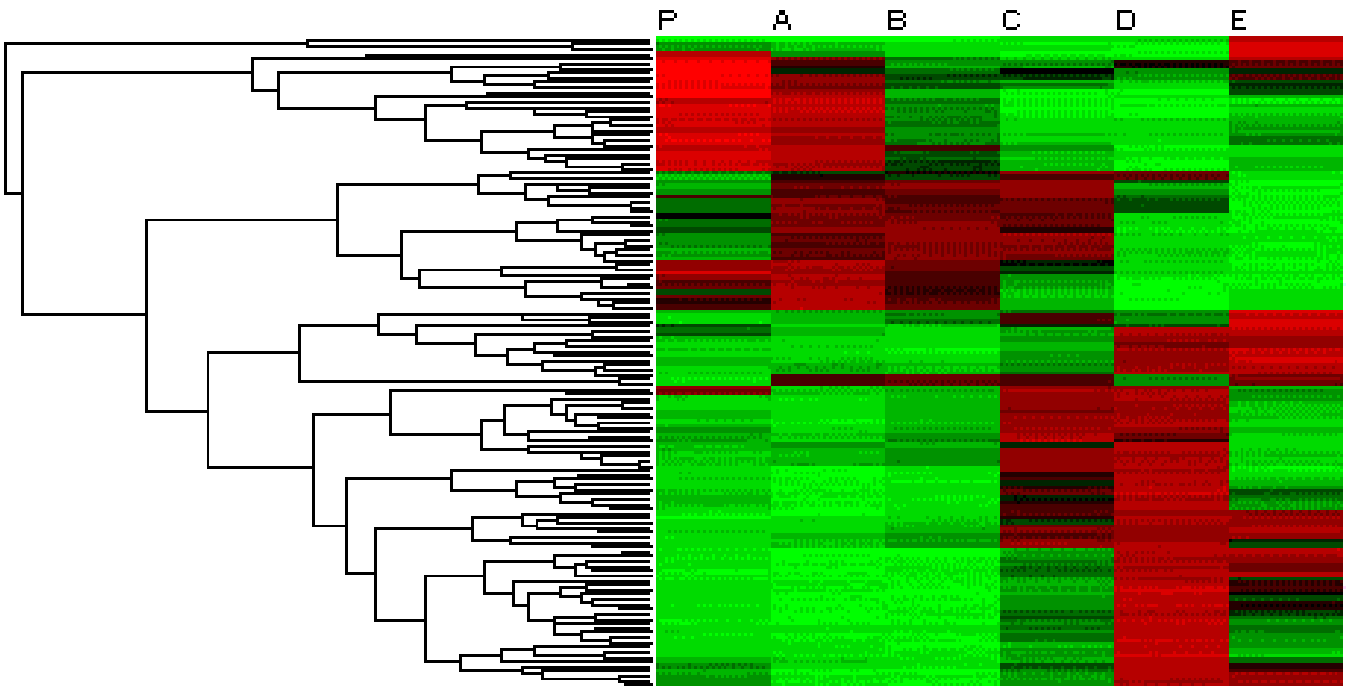
Hierarchical clustering

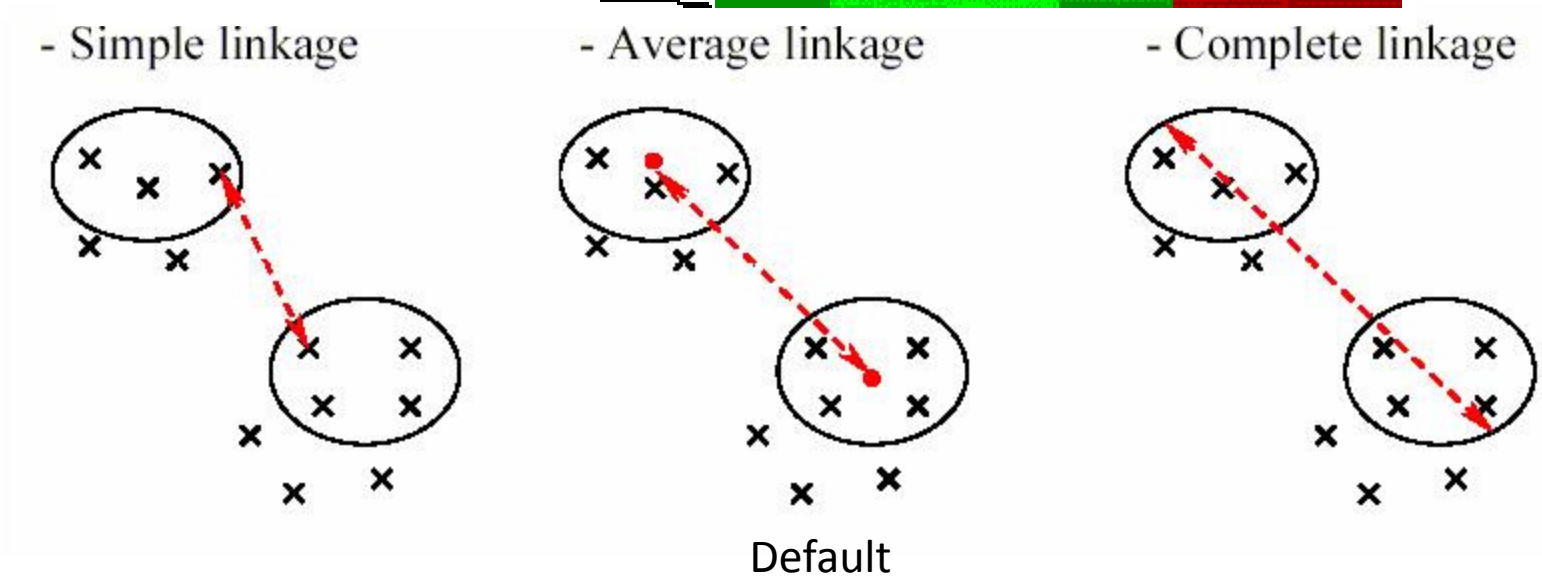


http://compbio.pbworks.com/w/page/16252903/Microarray%20Clustering%20Methods%20and%20Gene%20Ontology

Center the data to median value to get green-red color visualization

Linkage criteria in hierarchical clustering



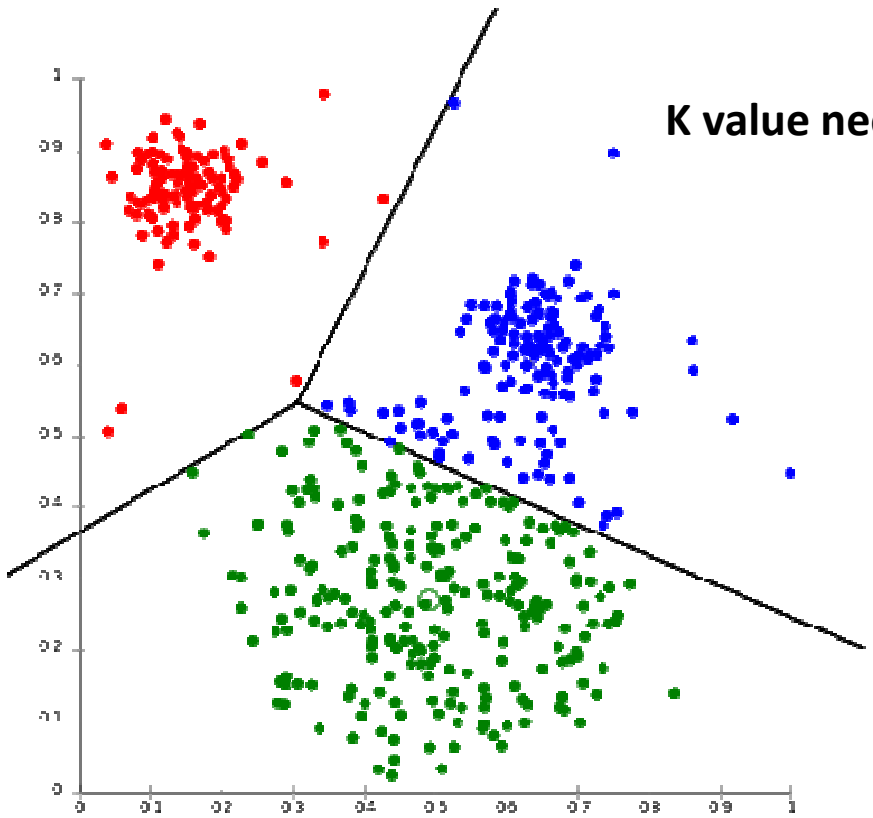


Default

Visualize the clustering results with Treeview

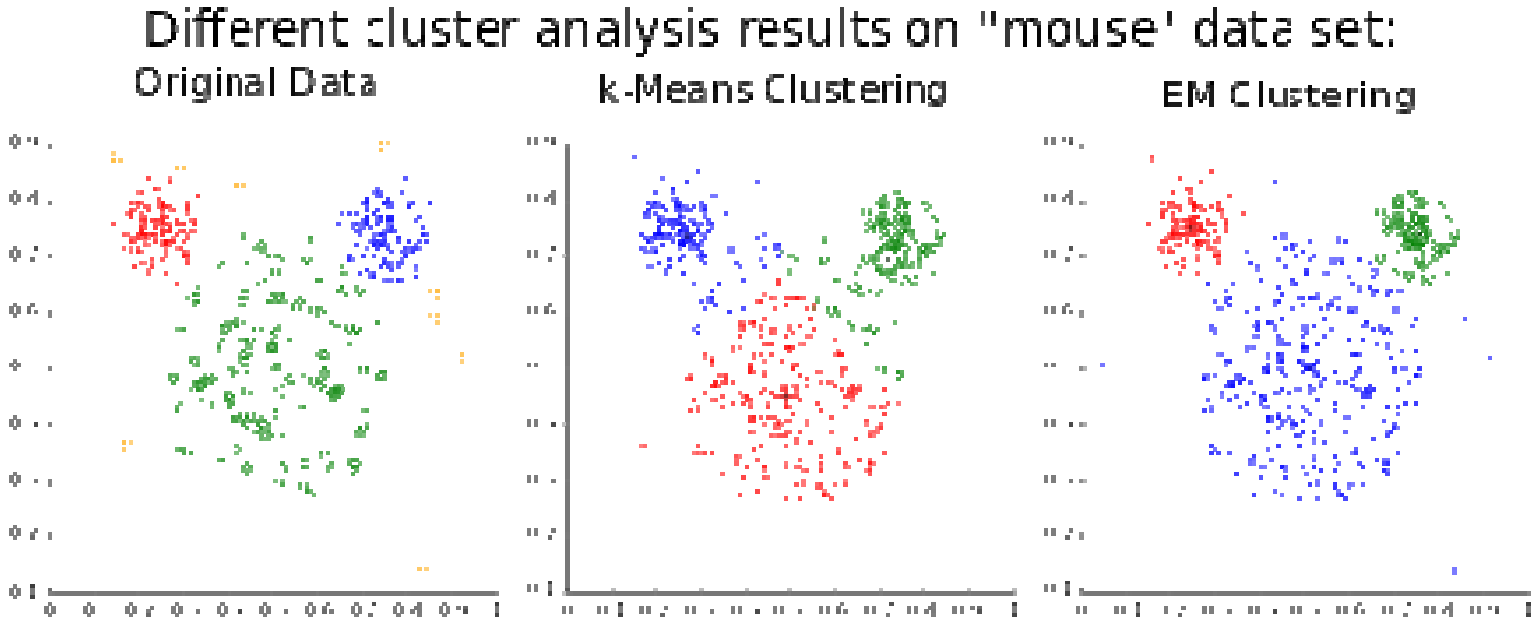
The software has functions to select nodes and export genes in selected node.

K-means clustering



K value need to be pre-specified

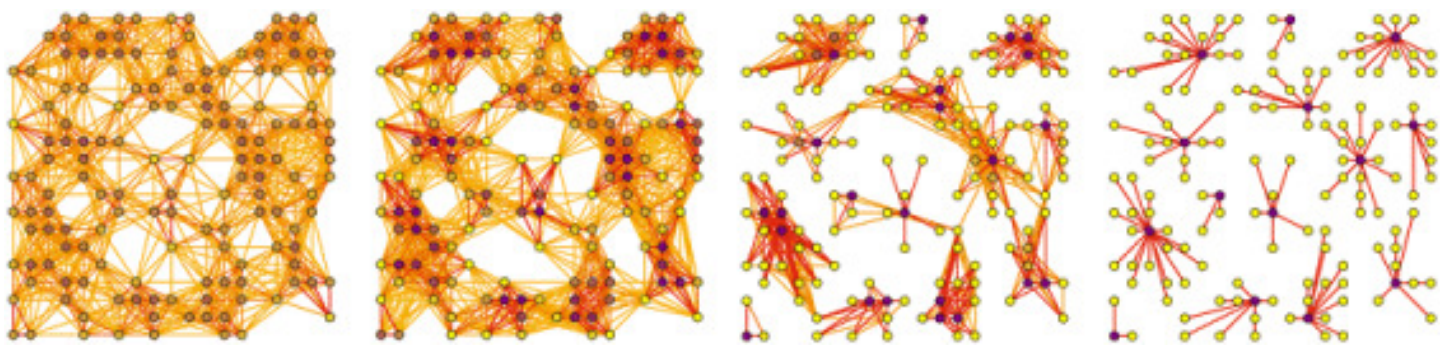
The tendency of *k*-means to produce equal-sized clusters leads to bad results



Wikipedia: K-means_clustering

Co-expression network modules

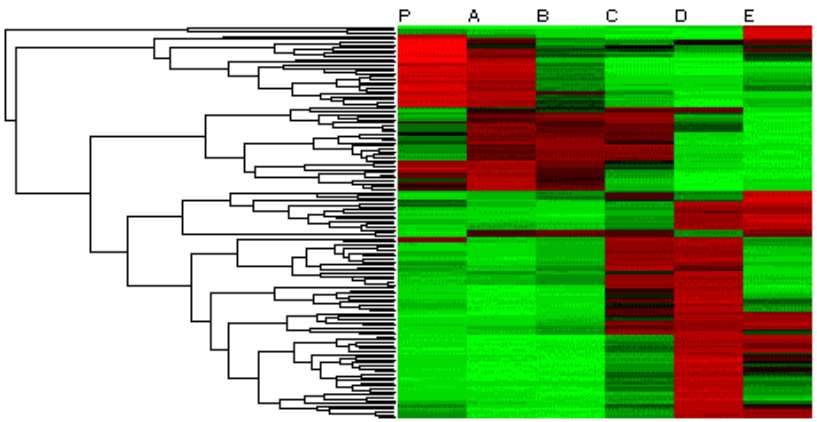
1. **MCL (Markov Cluster Algorithm)**
   - Easy to use interface: only need a distance matrix and inflation value

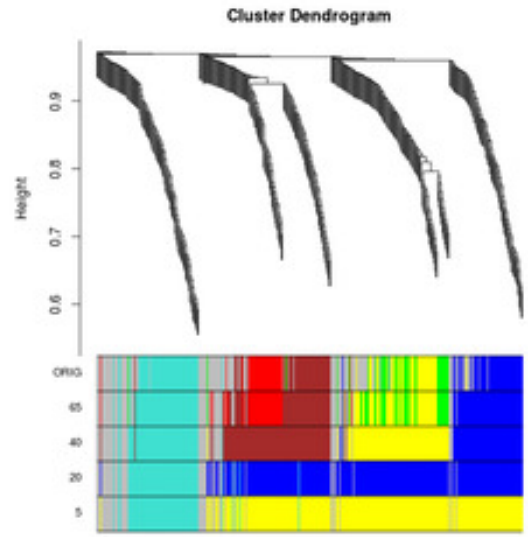


Co-expression network modules

2. WGCNA (weighted correlation network analysis)

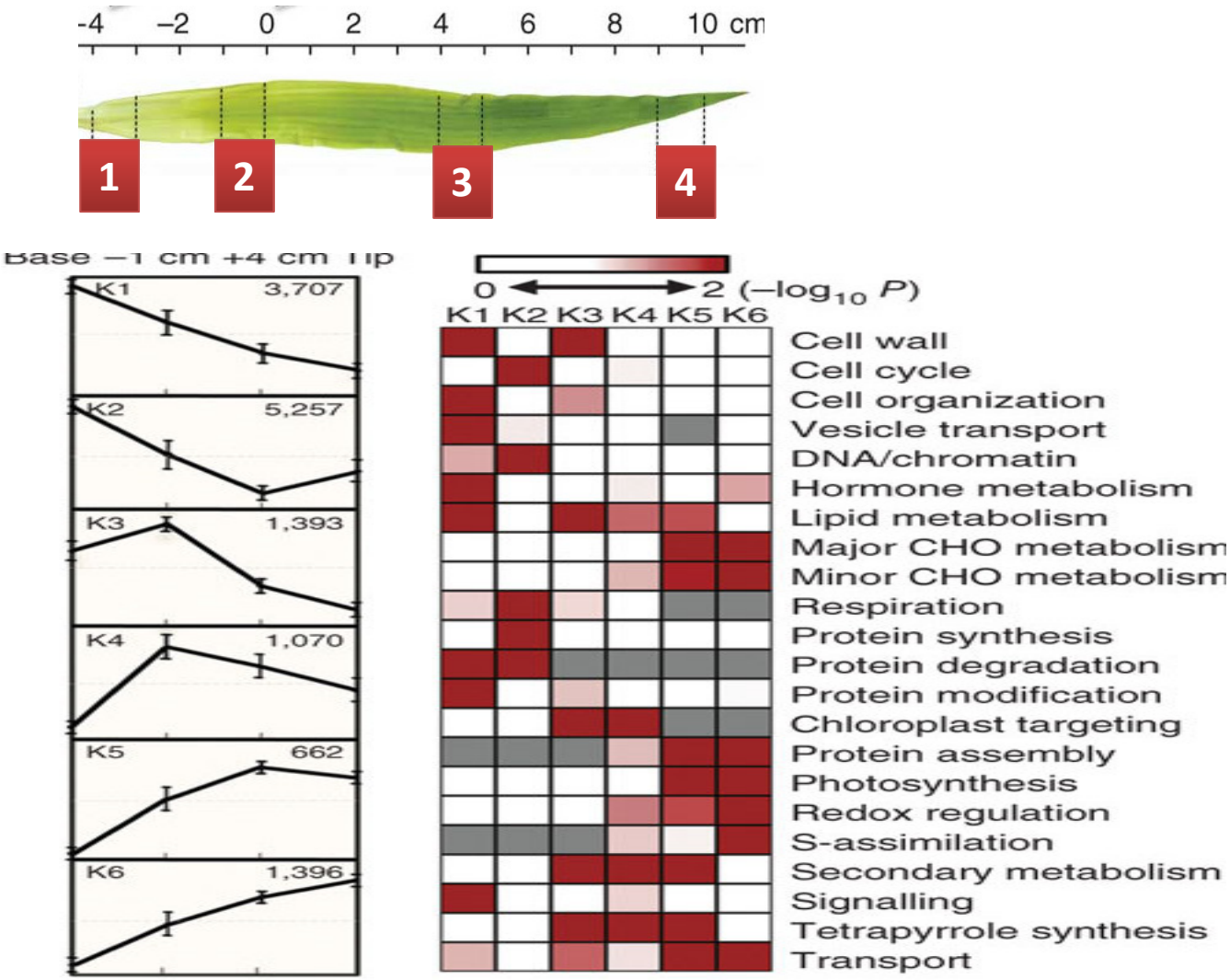
- transform the initial distance matrix into Topological Overlap Matrix





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:
  - Cuffdiff result: genes.fpkm_tracking
  - Rice Gene Ontology annotation file: rice.annot created with Ensembl BioMart.
- Tasks:
  - Hierarchical clustering
  - K-means clustering
  - Function enrichment analysis with BLAST2GO