UNIVERSITÉ DU LUXEMBOURG

LUXEMBOURG INSTITUTE OF HEALTH
RESEARCH DEDICATED TO LIFE

MISB Course

Transcriptomics (Dr. Stephanie Kreis)

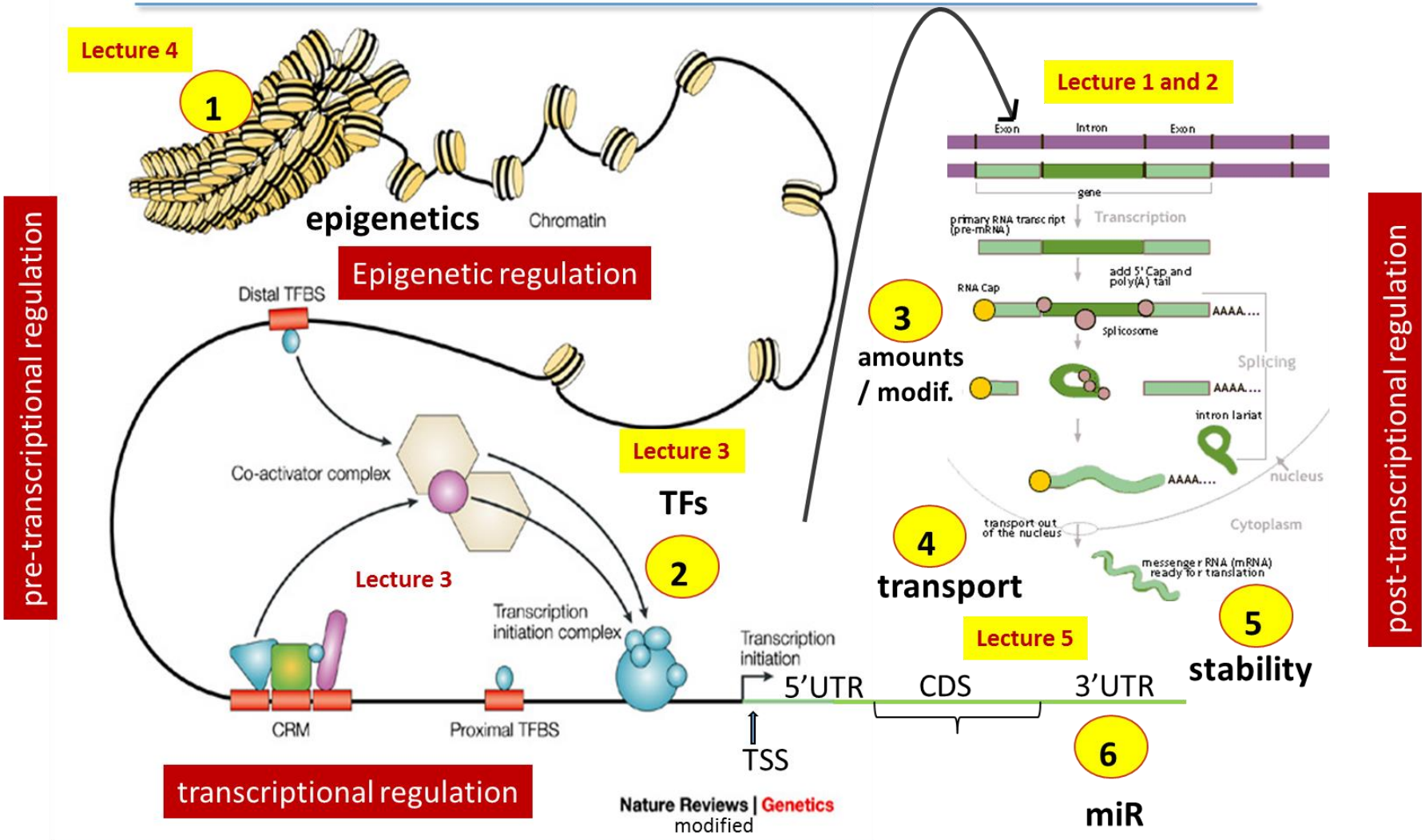
Introduction to Data Analysis in Transcriptomics

Petr Nazarov

petr.nazarov@lih.lu

2019-10-17 & 2019-10-18

Overview of regulation of gene expression

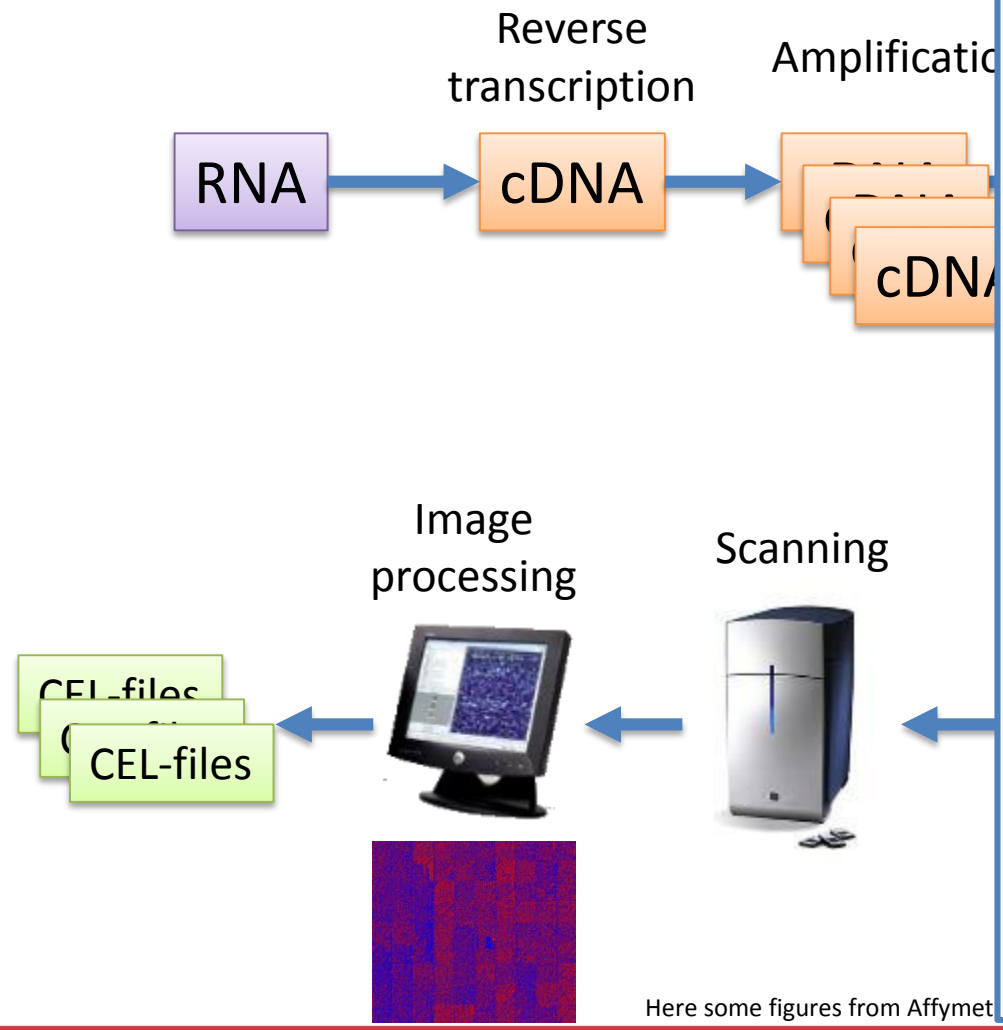


Outline

- **Data overview**
  - Microarrays
  - RNA-seq
- **Exploratory data analysis**
  - PCA
  - clustering
- **Differential expression analysis**
  - multiple hypotheses
  - linear models
- **Classification and marker genes**
- **Enrichment analysis**

Data Overview

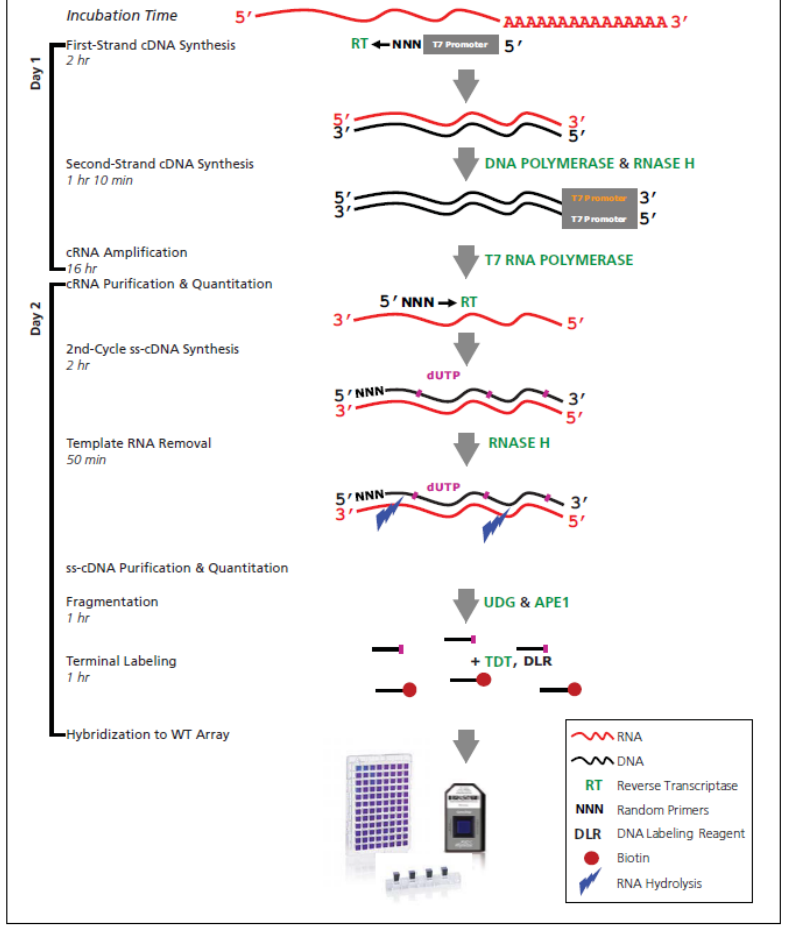
Microarrays



Here some figures from Affymet

Assay Workflow

Figure 1.1 WT PLUS Amplification and Labeling Process



Affymetrix: Probes, Probesets and Transcript clusters

Probes
25-mer sequences targeted on a single region of transcriptome (hopefully)

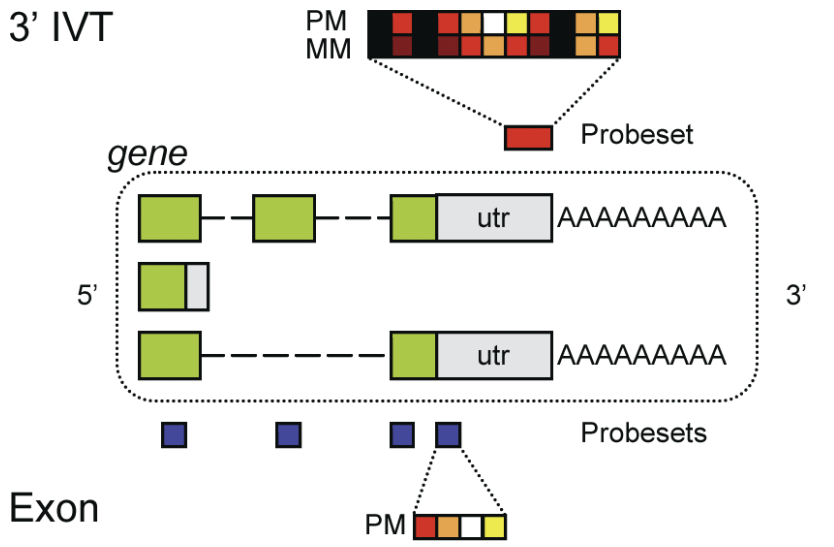
Probesets
groups of closely located or overlapped probes (on average 4 probes)

Exons
Human Exon and HTA arrays allow measuring exon expression

Junctions
HTA arrays allow measuring exon junction expression

Transcript clusters
For majority of features - synonymous to "genes". However, some distinct transcripts of genes are considered as different transcript clusters.

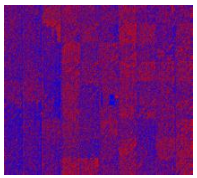
In old versions of Affy arrays (hgu95, hgu133, etc), there were:
PM – perfect match probes
MM – mismatch probes (having replacement in th 13th character)
This was done for background estimation.
But this approach is not used now!!

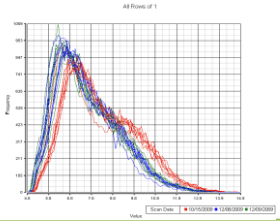


Okoniewski M, Comprehensive Analysis of Affymetrix Exon Arrays Using BioConductor, PLoS CompBiol, 2008

Microarray Data

Analysis Pipeline





Raw CEL files

Text files with $\log_2$ gene (or probeset) expression

Lists of differentially expressed gene

Image Analysis

Pre-processing

Quality control

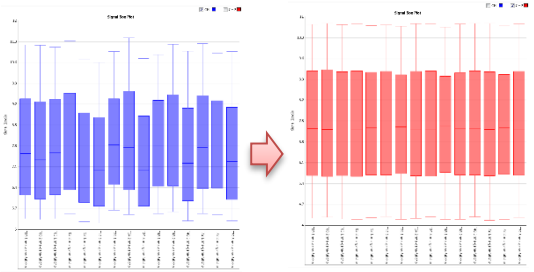
Statistical Analysis

Affymetrix© software

- Background correction
- Normalization
- Summarization

Remove low quality arrays, if necessary

- Significance analysis for differentially expressed genes (DEG)
- Prediction analysis
- Co-regulation analysis
- Etc.



Bioinformatics

Microarray Data

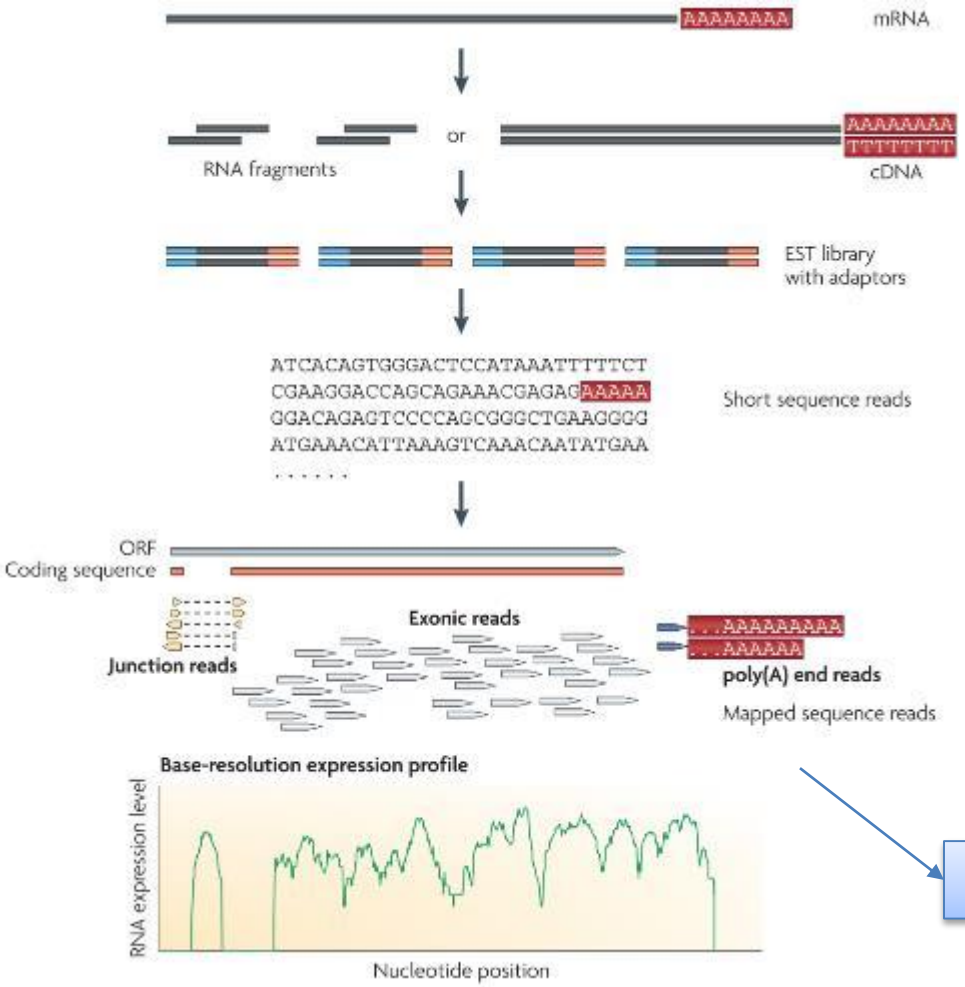
Data Example *(in log scale)*

ID	Gene.Symbol	A1	A2	A3	A4	B1	B2
TC02002853.hg.1	SP110	5.694	5.684	5.719	5.715	7.287	7.288
TC01002850.hg.1	GBP5	3.873	3.839	3.997	3.935	8.699	8.654
TC19000554.hg.1	LGALS17A	3.981	3.967	4.045	4.066	7.887	7.752
TC01006362.hg.1	GBP7	3.862	3.830	3.900	3.881	5.996	6.076
TC16000565.hg.1	SNTB2	7.765	7.734	7.748	7.755	8.973	9.027
TC12000425.hg.1	EIF4B	9.161	9.144	9.150	9.154	8.808	8.811
TC13000383.hg.1	TNFSF13B	3.922	3.890	3.873	3.918	5.151	5.199
TC09000999.hg.1	DDX58	6.629	6.661	6.671	6.598	8.302	8.367
TC06001673.hg.1	ETV7	4.427	4.467	4.434	4.348	6.815	6.713
TC05001767.hg.1	IRF1	5.409	5.470	5.552	5.396	7.988	8.000
TC17000821.hg.1	SSTR2	3.939	3.900	3.922	3.880	5.283	5.360
TC0X001551.hg.1	CLIC2	4.481	4.441	4.388	4.377	6.504	6.416
TC17000705.hg.1	MSI2	6.221	6.201	6.203	6.219	5.832	5.820
TC09000038.hg.1	PDCD1LG2	4.151	4.072	4.219	4.148	6.276	6.330
TC17001523.hg.1	DHX58	4.636	4.581	4.614	4.618	5.526	5.489
TC22000701.hg.1	APOL4	4.866	4.812	4.971	4.828	7.230	7.277
TC02001524.hg.1	ADI1	6.761	6.734	6.760	6.766	6.311	6.313
TC22000700.hg.1	APOL3	5.088	5.080	5.090	5.026	6.715	6.830
TC06000932.hg.1	NUS1	7.870	7.882	7.856	7.871	7.543	7.547
TC14001152.hg.1	GCH1	6.266	6.344	6.268	6.257	7.582	7.551

Here gene expression data are given in $\log_2$ intensity

RNA-Seq Data

Next-Generation Sequencing: RNA-seq



CPM: counts per million nt
TPM: transcripts per million (proportion)
FPKM: fragments per kilobase of exon per million reads mapped
RPKM: reads per ……. (for single-end)

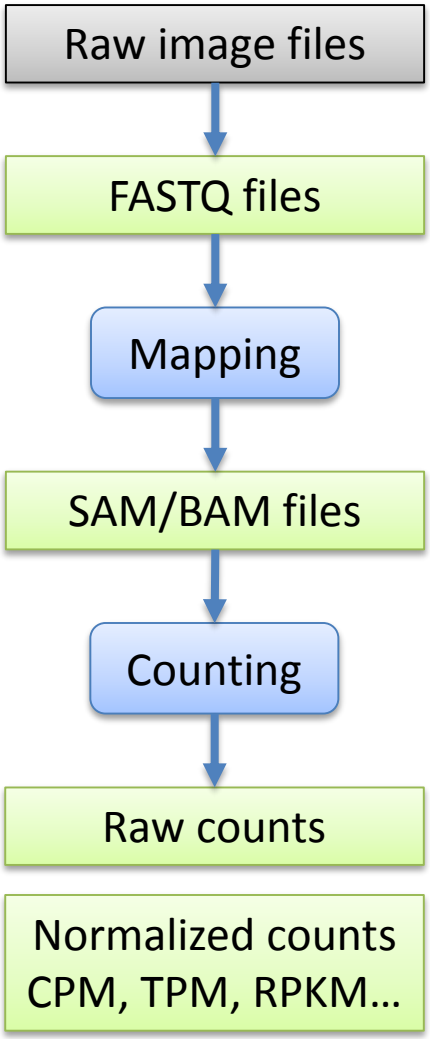
$$\mathrm{CPM}_i = \frac{X_i}{\frac{N}{10^6}} = \frac{X_i}{N} \cdot 10^6 \qquad \mathrm{TPM}_i = \frac{X_i}{\widetilde{l}_i} \cdot \left(\frac{1}{\sum_j \frac{X_j}{\widetilde{l}_j}} \right) \cdot 10^6$$

$$\mathrm{FPKM}_i = \frac{X_i}{\left(\frac{\widetilde{l}_i}{10^3}\right)\left(\frac{N}{10^6}\right)} = \frac{X_i}{\widetilde{l}_i N} \cdot 10^9$$

raw counts → normalized counts, CPM, FPKM, RPKM

Wang Z et al. RNA-Seq: a revolutionary tool for transcriptomics. **Nat Rev Genet. 2009**

RNA-Seq Data



File Types

```
@HWI-ST508:152:D06G9ACXX:2:1101:1160:2042 1:Y:0:ATCACG
NAAGACCGAATTCTCCAAGCTATGGTAAACATTGCACTGGCCTTTCATCTG
+
#11??+2<<<CCB4AC?32@+1@AB1**1?AB<4=4>=BB<9=>?######
```

Read – a short sequence identified in RNA-Seq experiment
Library – set (10^5 – 10^8) of reads from a single sample

```
@HD         VN:1.0 SO:coordinate
@SQ         SN:seq1 LN:5000
@SQ         SN:seq2     LN:5000
@CO         Example of SAM/BAM file format.

B7_591:4:96:693:509 73    seq1       1          99          36M         *
            0             0          CACTAGTGGCTCATTGTAAATGTGTGGTTTAACTCG
                                     <<<<<<<<<<<<<<;<<<<<<<<<5<<<<<;:<;7
            MF:i:18       Aq:i:73    NM:i:0     UQ:i:0      H0:i:1

H1:i:0EAS54_65:7:152:368:113        73         seq1        3           99
            35M           *          0          0
            CTAGTGGCTCATTGTAAATGTGTGGTTTAACTCGT
            <<<<<<<<<<0<<<<655<<7<<<:9<<3/:<6): MF:i:18    Aq:i:66
            NM:i:0        UQ:i:0     H0:i:1     H1:i:0
```

For the list of tools see:
http://en.wikipedia.org/wiki/List_of_RNA-Seq_bioinformatics_tools

Advantage over arrays: you can repeat the pipeline with new knowledge or questions

RNA-Seq Data

Data Example *(in linear scale)*

ID	Gene.Symbol	A1	A2	A3	A4	B1	B2
ENSG00000135899	SP110	32	31	33	33	136	136
ENSG00000154451	GBP5	0	0	0	0	395	383
ENSG00000226025	LGALS17A	0	0	0	0	217	196
ENSG00000213512	GBP7	0	0	0	0	44	47
ENSG00000260873	SNTB2	198	193	195	196	483	502
ENSG00000063046	EIF4B	552	546	548	550	428	429
ENSG00000102524	TNFSF13B	0	0	0	0	16	17
ENSG00000107201	DDX58	79	81	82	77	296	310
ENSG00000010030	ETV7	2	2	2	0	93	85
ENSG00000125347	IRF1	22	24	27	22	234	236
ENSG00000180616	SSTR2	0	0	0	0	19	21
ENSG00000155962	CLIC2	2	2	1	1	71	65
ENSG00000153944	MSI2	55	54	54	54	37	37
ENSG00000197646	PDCD1LG2	0	0	0	0	58	60
ENSG00000108771	DHX58	5	4	4	5	26	25
ENSG00000100336	APOL4	9	8	11	8	130	135
ENSG00000182551	ADI1	88	86	88	89	59	60
ENSG00000128284	APOL3	14	14	14	13	85	94
ENSG00000153989	NUS1	214	216	212	214	167	167
ENSG00000131979	GCH1	57	61	57	56	172	167

Here gene expression data are given in counts

Public Repositories

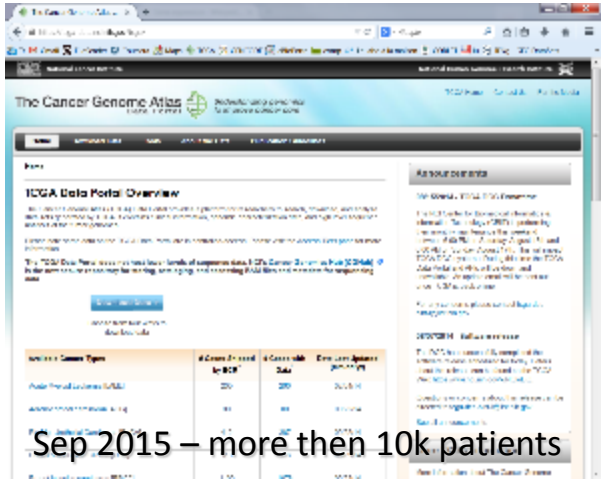
GEO: http://www.ncbi.nlm.nih.gov/gds

Browse Content

Repository Browser

DataSets:	3847
Series:	50810
Platforms:	13387
Samples:	1237318

TCGA: https://tcga-data.nci.nih.gov/tcga/



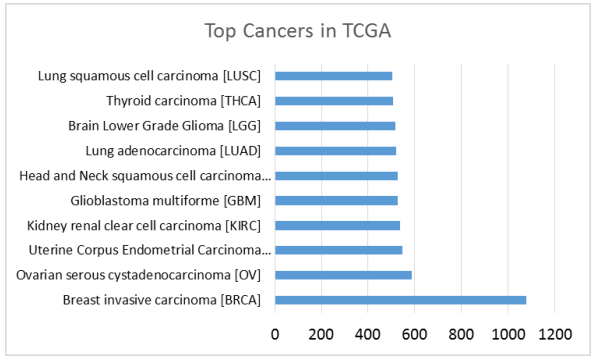
Sep 2015 – more then 10k patients

ArrayExpress: http://www.ebi.ac.uk/arrayexpress/

Data Content

Updated today at 06:00

- 52801 experiments
- 1555904 assays
- 24.99 TB of archived data



Analysis via:
http://www.cbioportal.org/public-portal/

Data Overview

Take Home Messages

- Microarrays **should be normalized** to remove effects of variable RNA content

- Expression-related data in transcriptomics (fluorescence intensity in microarrays and counts in RNAseq) are **strongly right-skewed**. Therefore:
  - For statistics use either precise distribution (negative binomial for RNA-seq) or work with log-transformed data (microarrays).
  - Use log-transformed data for exploratory analysis and visualization

- Main advantage of RNA-seq data: they **can be reprocessed and reused** taking into account new genomic annotation or asking new questions

- Several **large repositories of the data exist**. Before planning your experiments – make a search for existing data

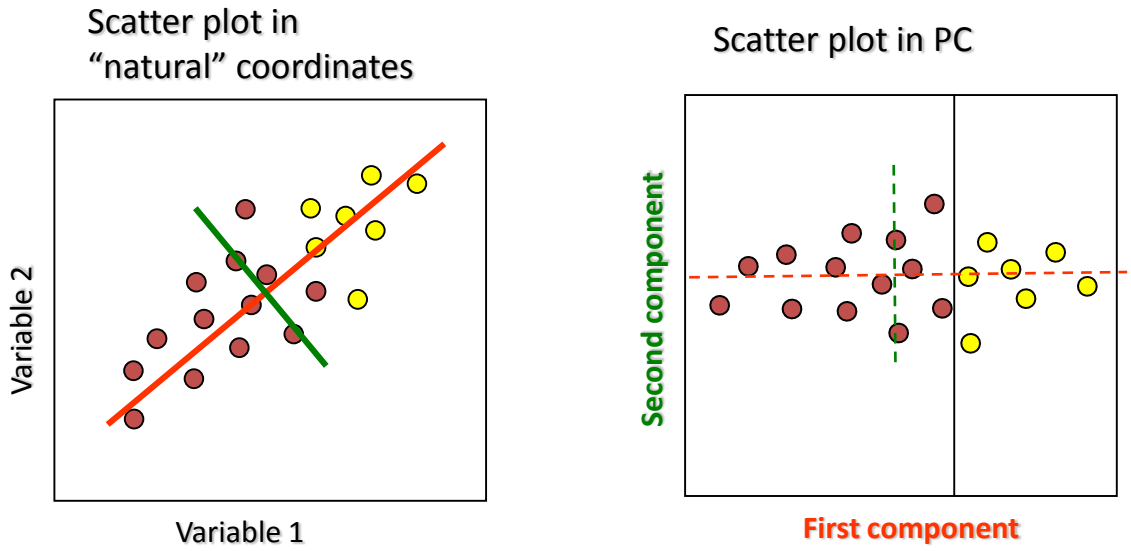
Exploratory Analysis

Principal Component Analysis (PCA)

Principal component analysis (PCA)
is a vector space transform used to reduce multidimensional data sets to lower dimensions for analysis. It selects the **coordinates along which the variation of the data is bigger**.

20000 genes → 2 dimensions

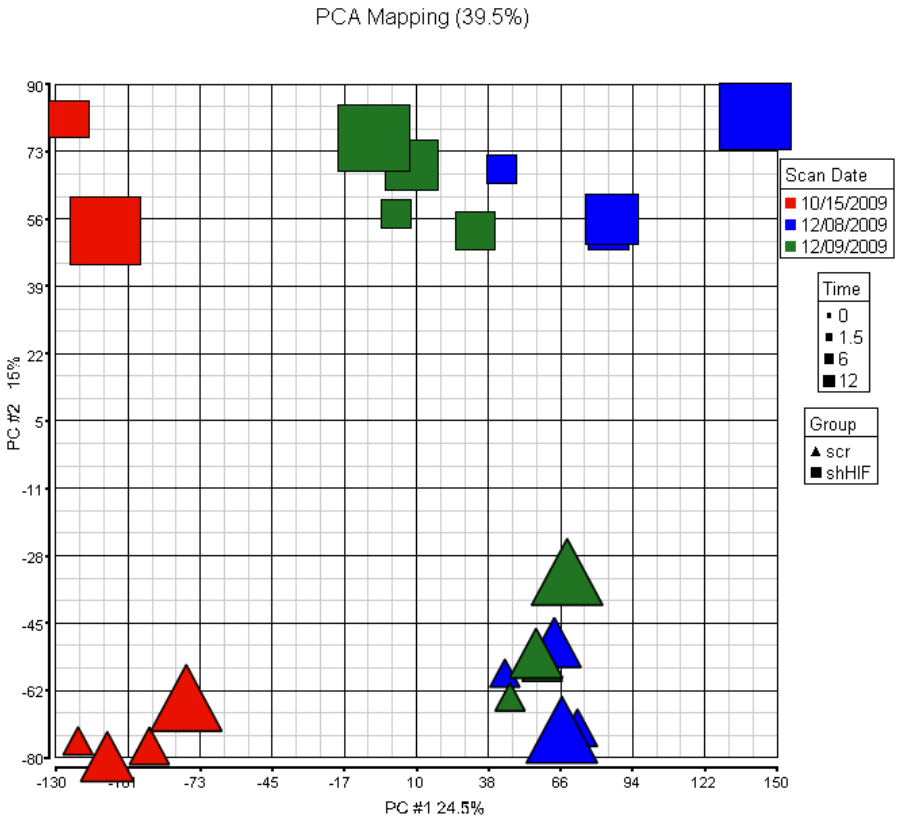
For the simplicity let us consider 2 parametric situation both in terms of data and resulting PCA.

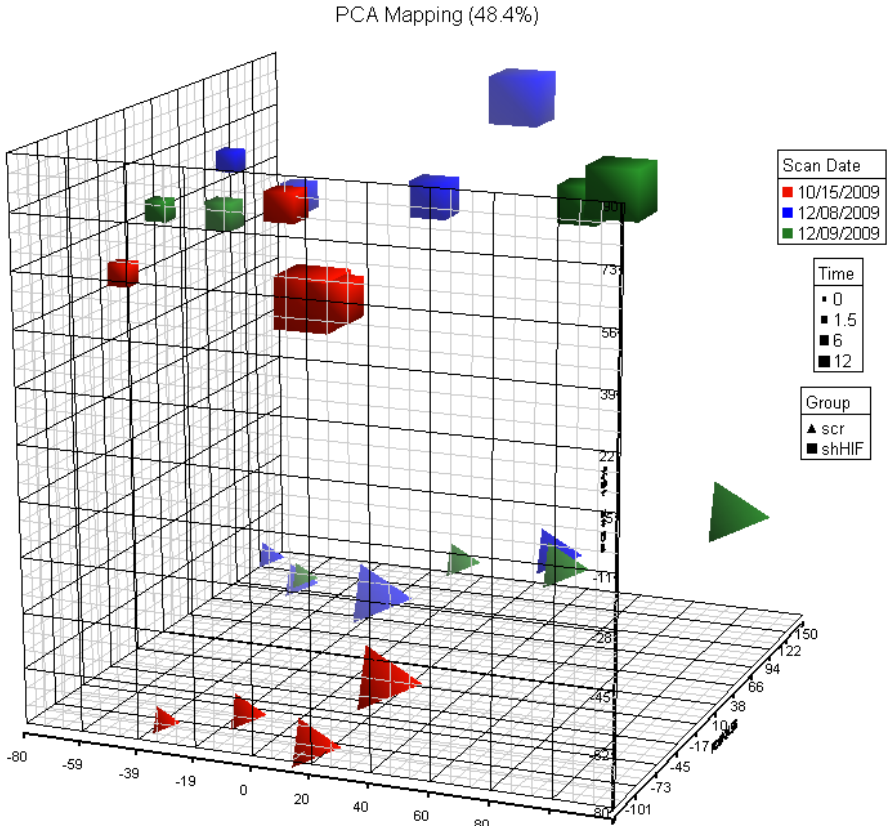


Instead of using 2 "natural" parameters for the classification, we can use the first component!

Exploratory Data Analysis

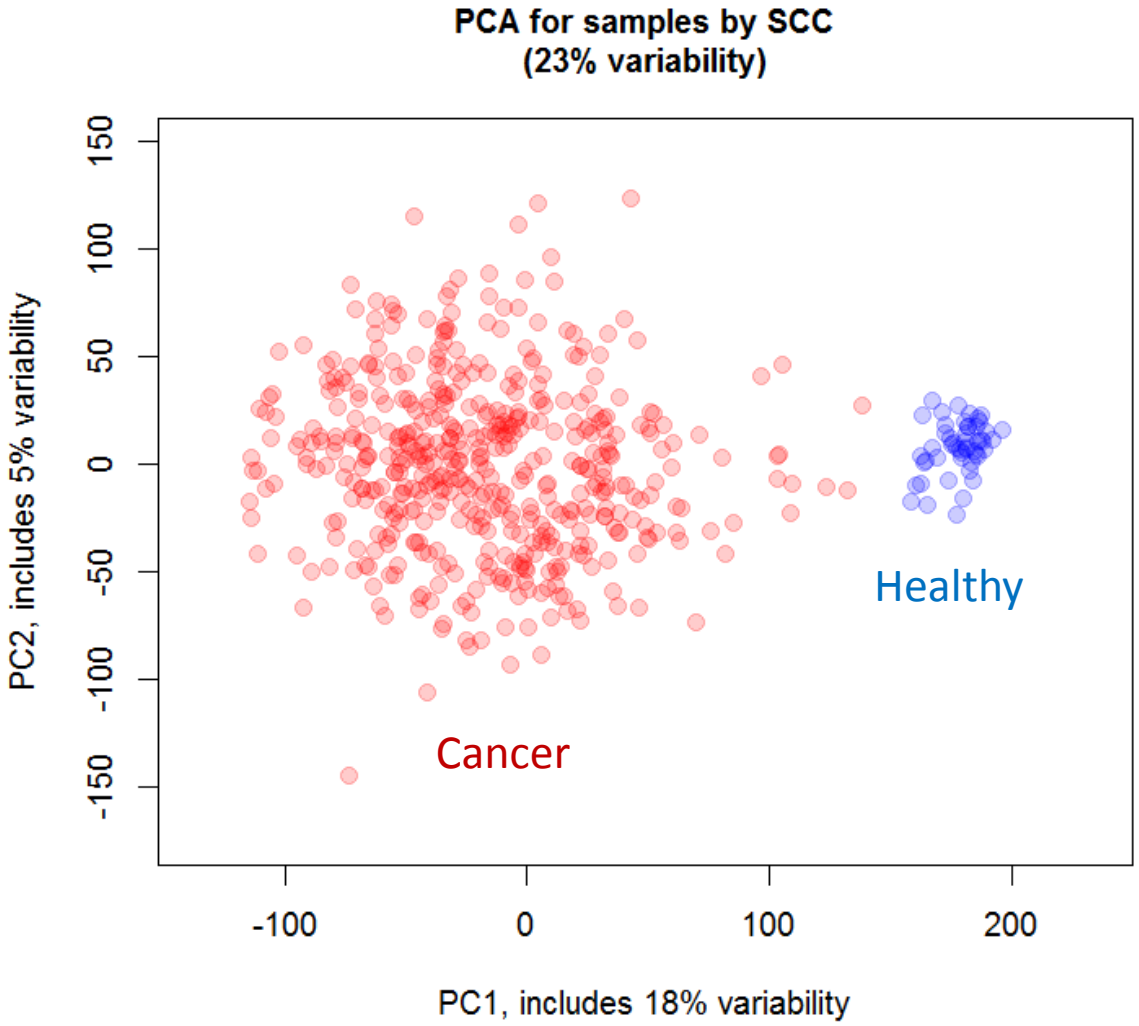
PCA





Exploratory Data Analysis

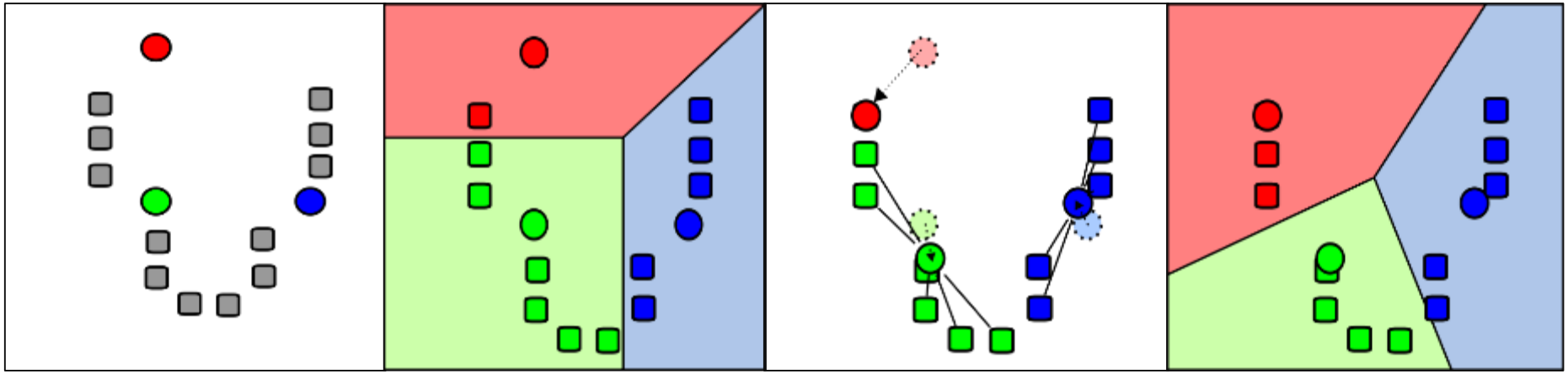
PCA in TCGA (LUSC data)



k-Means Clustering

k-Means Clustering

k-means clustering is a method of cluster analysis which aims to partition n observations into k clusters in which each observation belongs to the cluster with the nearest mean.



1) *k* initial "means" (in this case k=3) are randomly selected from the data set (shown in color).

2) *k* clusters are created by associating every observation with the nearest mean.

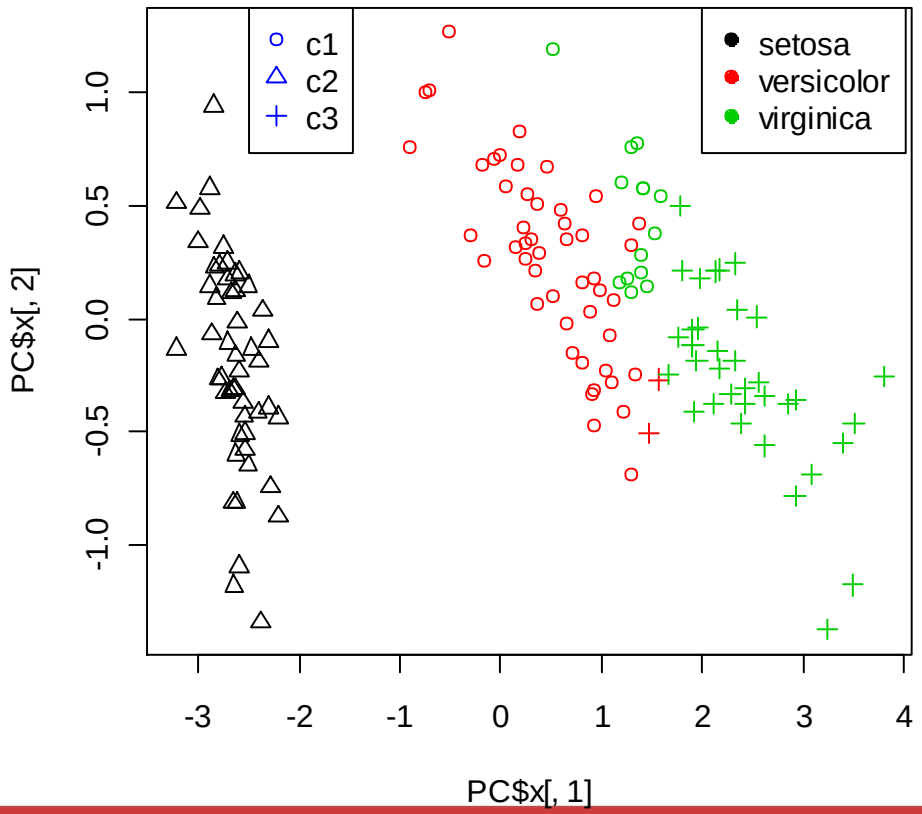
3) *The centroid of each of the k clusters becomes the new means.*

4) Steps 2 and 3 are repeated until convergence has been reached.

http://wikipedia.org

k-Means Clustering: Iris Dataset (Fisher)

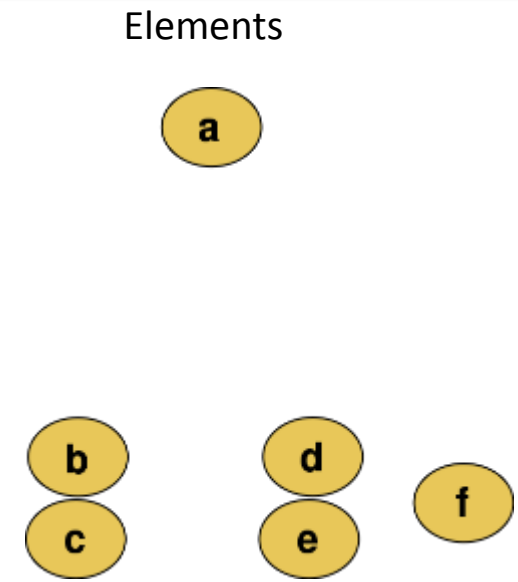
```
clusters = kmeans(x=Data,centers=3,nstart=10)$cluster
plot(PC$x[,1],PC$x[,2],col = classes,pch=clusters)
legend(2,1.4,levels(iris$Species),col=c(1,2,3),pch=19)
legend(-2.5,1.4,c("c1","c2","c3"),col=4,pch=c(1,2,3))
```

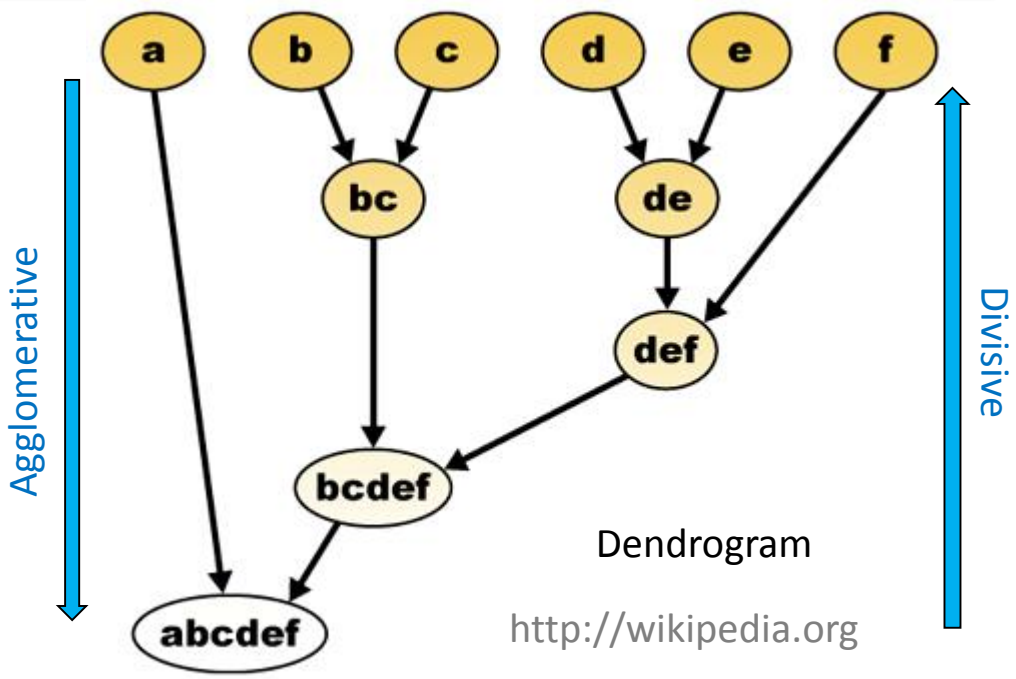


Hierarchical Clustering

Hierarchical Clustering

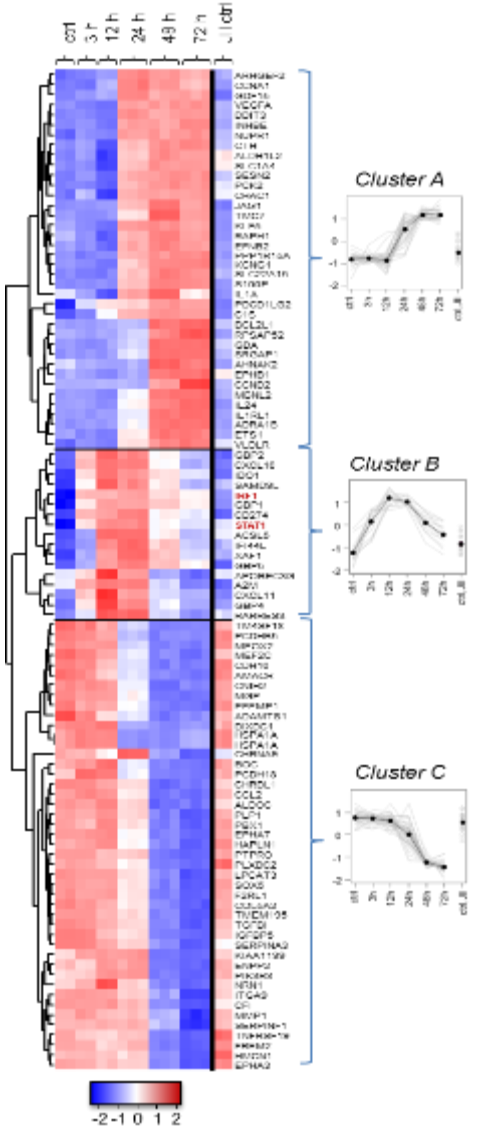
Hierarchical clustering creates a hierarchy of clusters which may be represented in a tree structure called a dendrogram. The root of the tree consists of a single cluster containing all observations, and the leaves correspond to individual observations.
Algorithms for hierarchical clustering are generally either agglomerative, in which one starts at the leaves and successively merges clusters together; or divisive, in which one starts at the root and recursively splits the clusters.

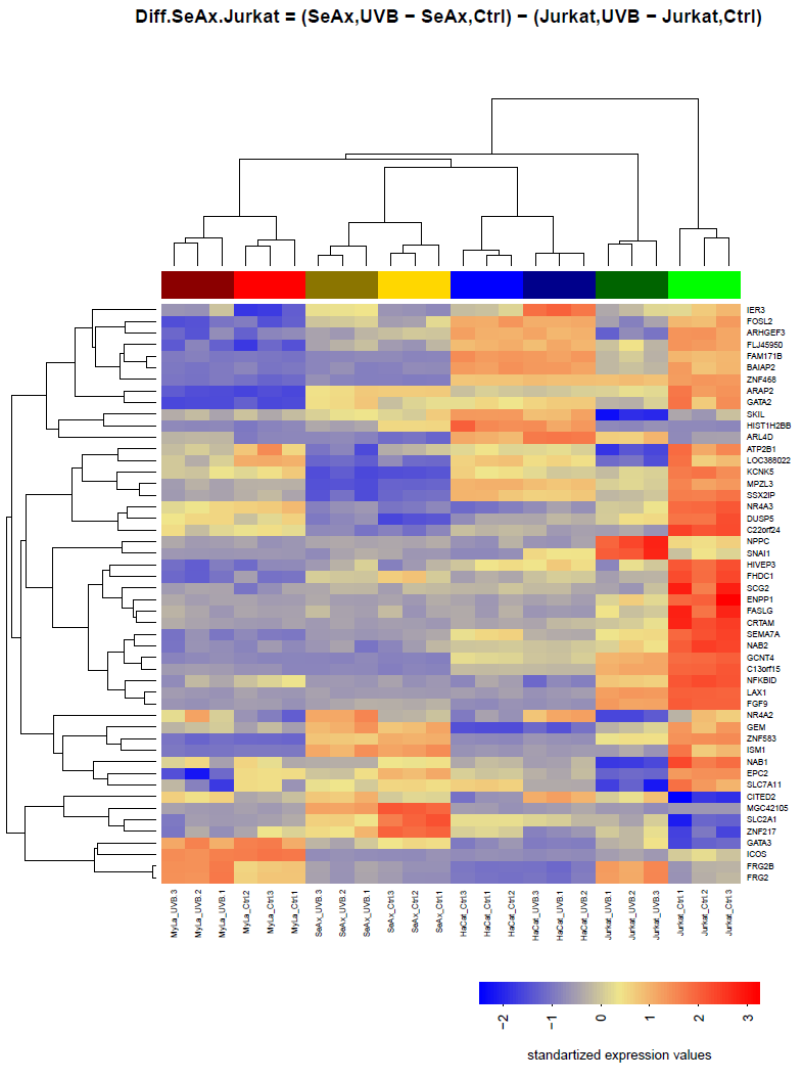




Exploratory Data Analysis

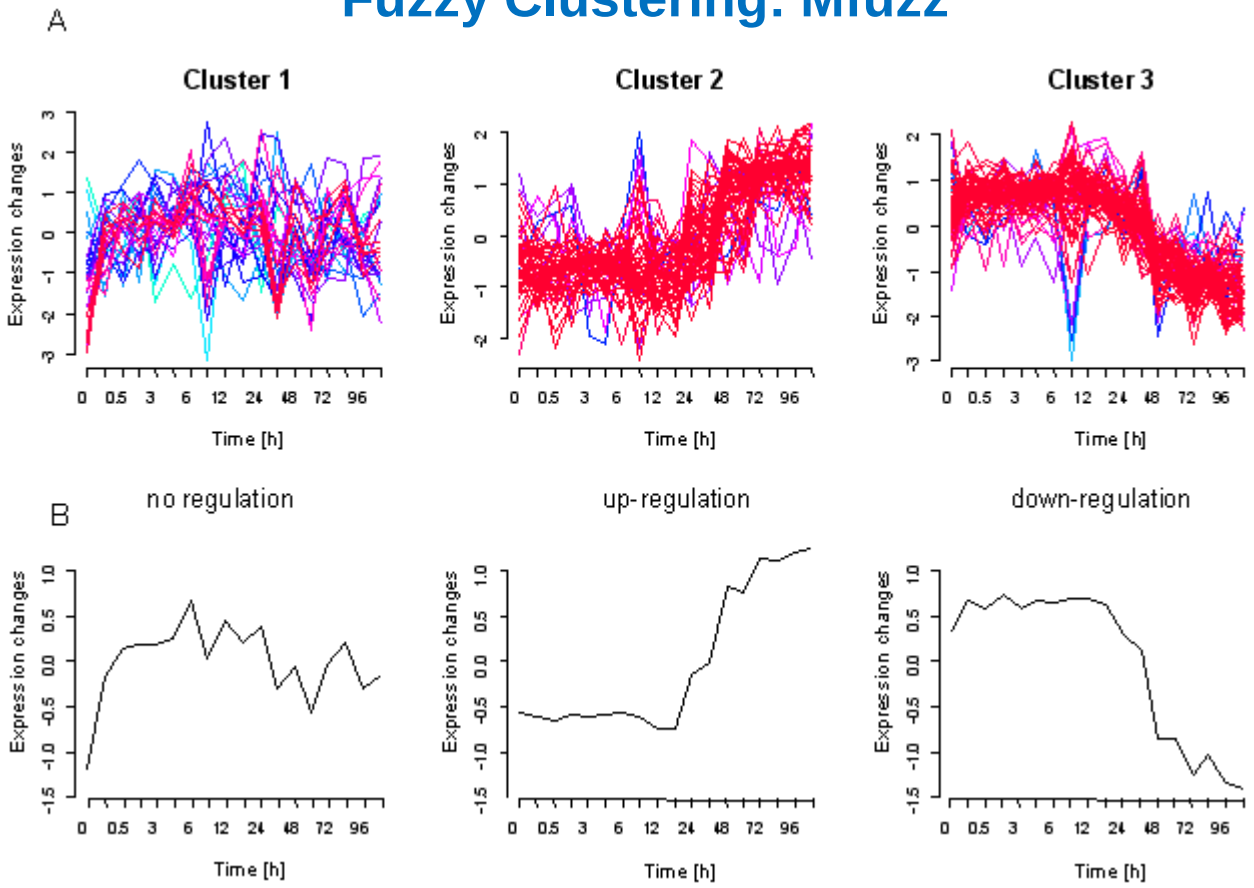
Heatmaps





Exploratory Data Analysis

Fuzzy Clustering: Mfuzz



Take Home Messages

- Start your investigation with **PCA**, which will help
  - **Reduce dimensionality** and **help visualizing** your data
  - See which **factors** may play the **important role** in your data
  - **Find outlier** experiments

- **Clustering** your data decide whether you would like to separate in a fixed number of groups and be **more robust (k-means**) or to a variable number of clusters and be **more flexible (hierarchical)**

- **Heatmap** allows you to visualize profiles of expression **among samples and among genes in one graph**

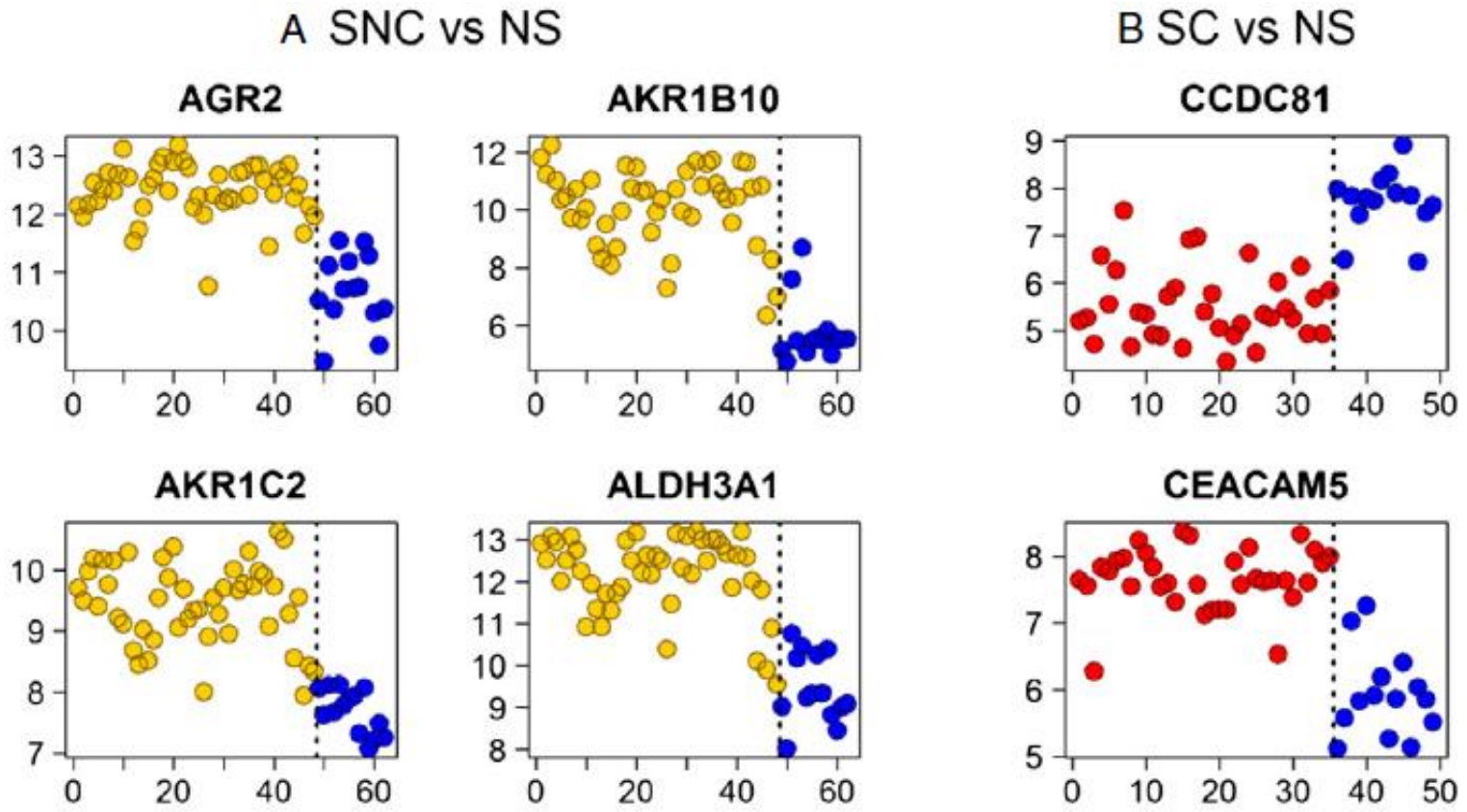
Classification

Classification and Marker Genes

Gene Markers

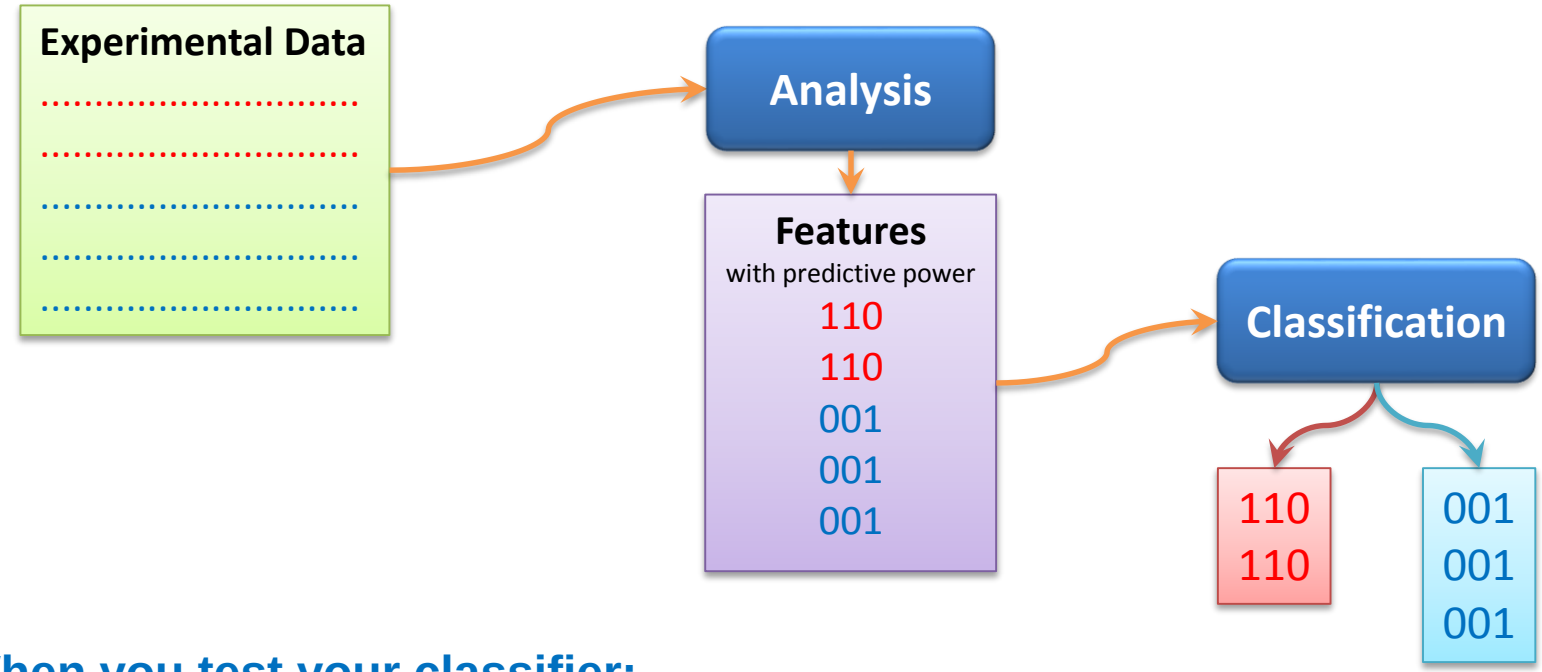
Questions

- Based on which genes or gene sets we can **predict** the group of the samples?
- How reliable is this prediction?

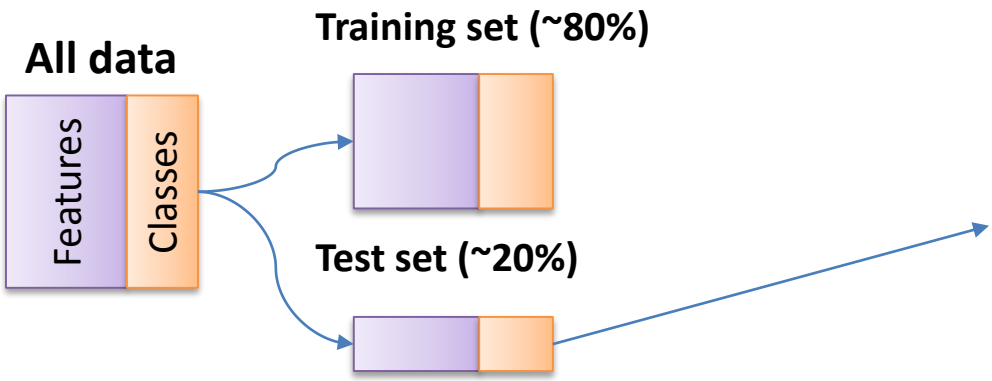


Classification and Marker Genes

General Scheme



When you test your classifier:



Confusion Matrix

	A	B	C
pred.A	50	0	0
pred.B	0	48	2
pred.C	0	2	48

Classification and Marker Genes

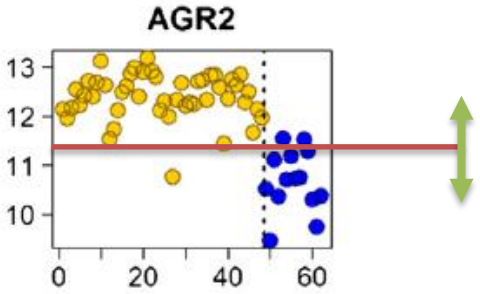
Selection of Features: ROC and AUC

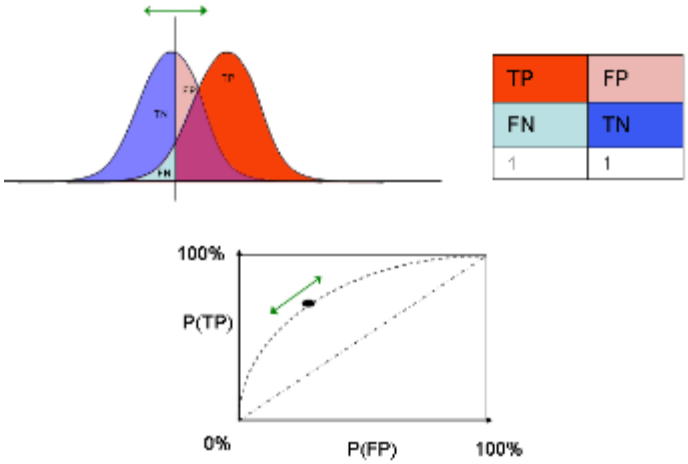
ROC curve
(receiver operating characteristic)
is a graphical plot of the sensitivity, or true positive rate, vs. false positive rate (1-specificity or false positive rate)

AUC
area under ROC curve: 1 – ideal separation, 0.5 – random separation.

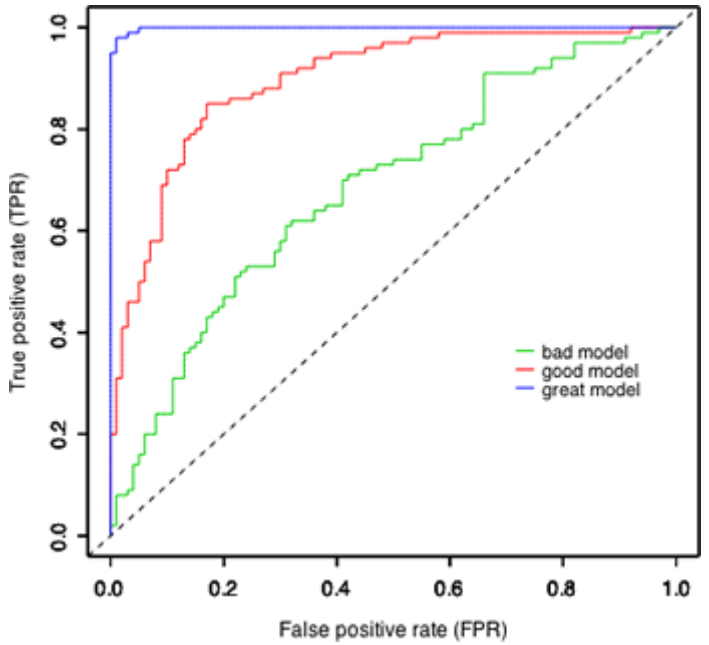
ROC is introduced for 2 classes.

If we have more then 2 classes – create several ROC curves (1 per class)





https://en.wikipedia.org/wiki/Receiver_operating_characteristic

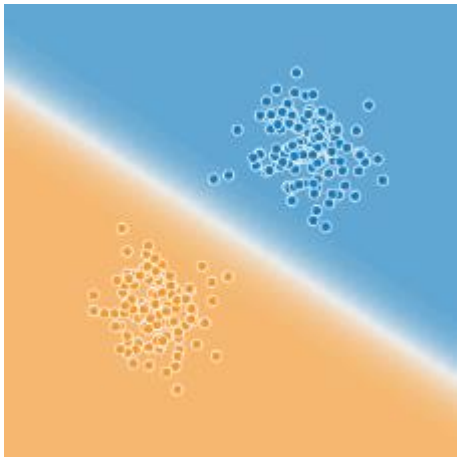


http://www.unc.edu/courses/2010fall/ecol/563/001/docs/lectures/lecture22.htm

Simple Classifier: Logistic Regression

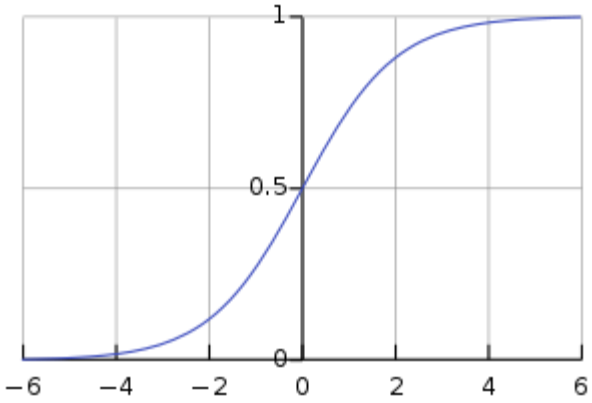
Logistic regression

Linearly combines the features and calculates
1) will divide you data to 2 groups, and
2) has the optimal distance from the closest elements of the groups



Logistic regression: sigmoid function upon linear regression:

$$F(z) = \frac{1}{1 + e^{-(b_1 x_1 + b_2 x_2 + \cdots + b_0)}}$$

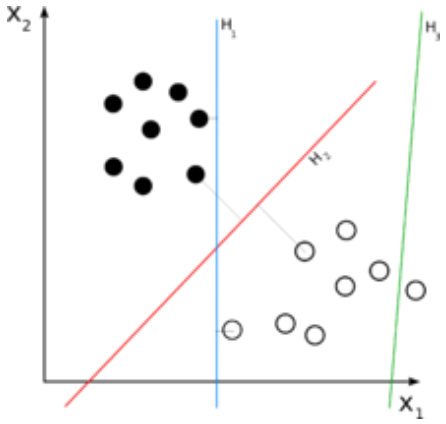


Classification and Marker Genes

More Advanced Classification Methods

Support vector machine (SVM)

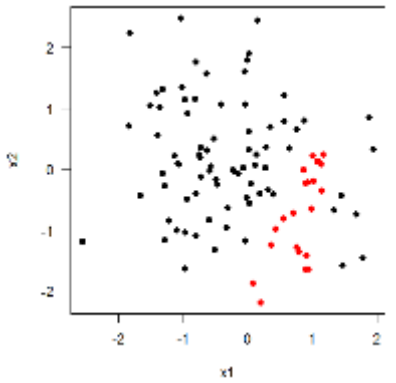
System tries to find a line (hyper plane) which
1) will divide you data to 2 groups, and
2) has the optimal distance from the closest elements of the groups



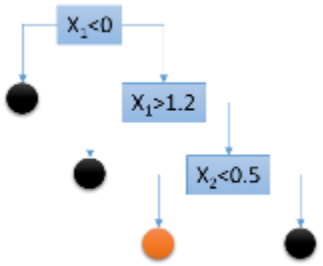
Random Forest (RF)

Makes a set of decision trees (if value x is less then x0 then we choose class A), which is called “forest”. Then vote among the trees.

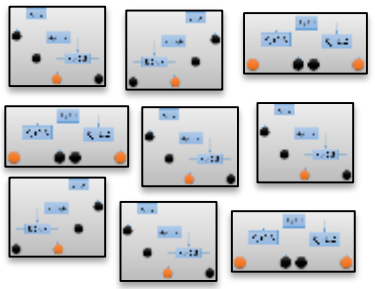
space of features



tree



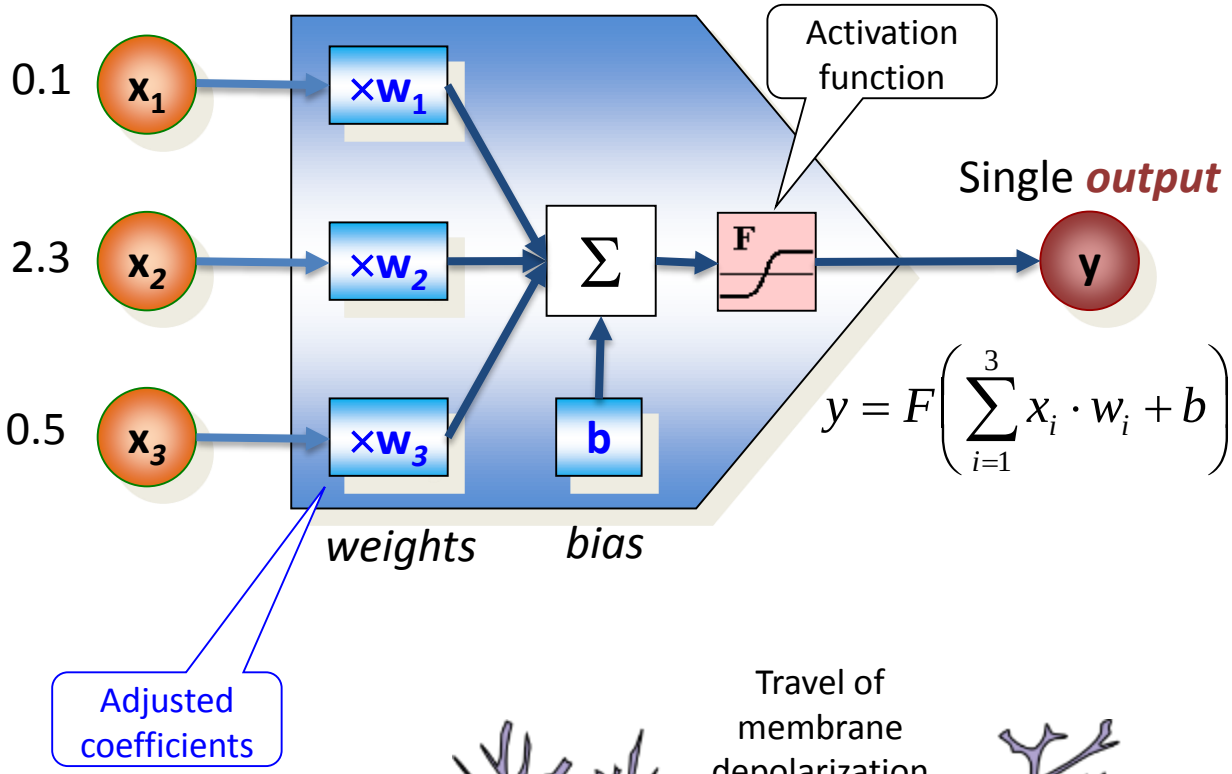
forest

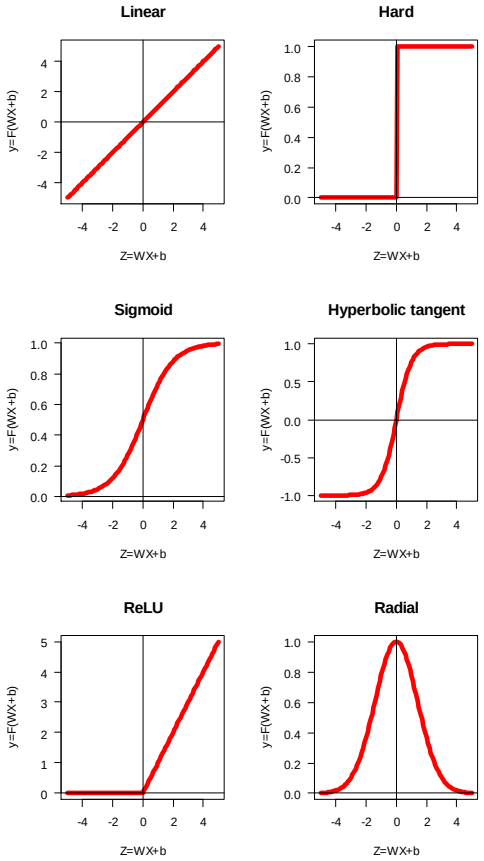


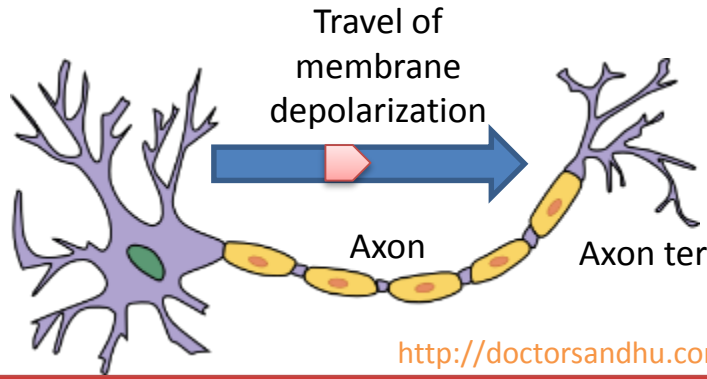
Classification and Marker Genes

Artificial Neuron – a Simple Processing Unit (~ logistic regression)

Multiple ***inputs***







http://doctorsandhu.com/Neuron/Neuron.shtml

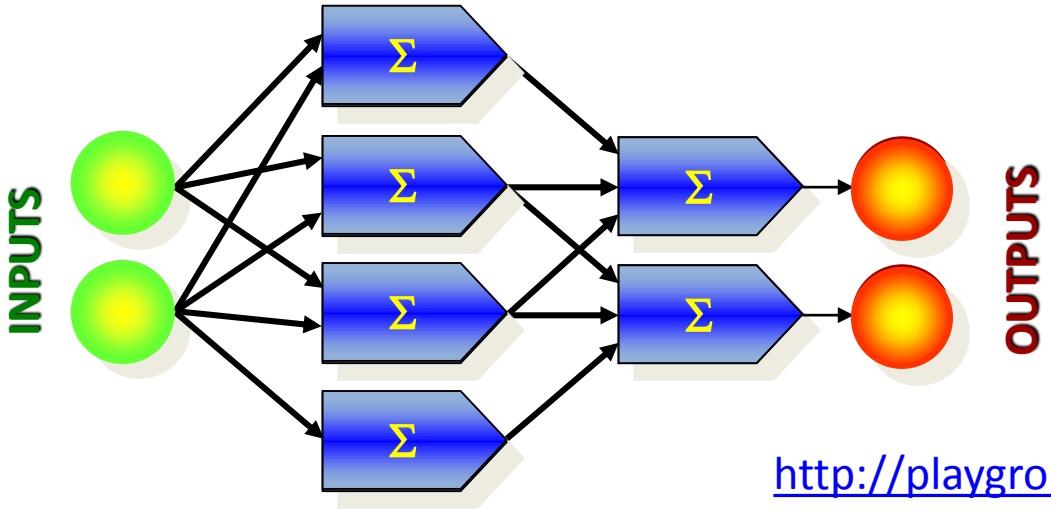
Dendrites: inputs

Classification and Marker Genes

Feed Forward Network (FFN), a.k.a. Multi-layer Perceptron (MLP)

Forward propagation of information

Normalized data: raw, features, variables etc.

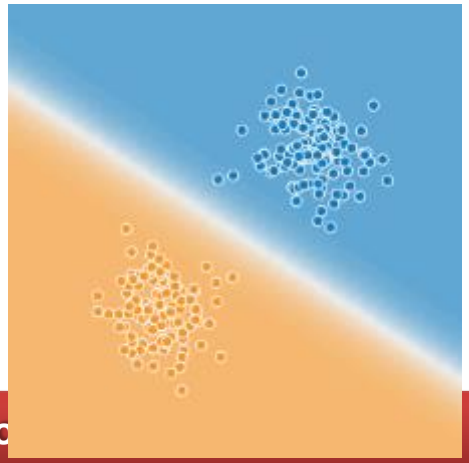


In classification the output is considered as probability of a class (with *softmax*)

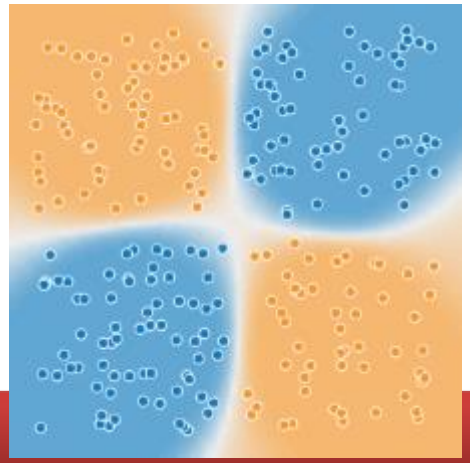
$$p(y_i|X) = \frac{y_i}{\sum y_j}$$

http://playground.tensorflow.org/

1 layer



2 layers



4 layers

Take Home Messages

- Diagnostics & prediction include 3 main steps:
  - 1. Data analysis – transforms data into set of features
  - 2. Select features with predictive properties
  - 3. Use a classification algorithm

- AUC is one of the measures to select genes with strong predictive properties. Ideal AUC = 1, minimal AUC (worst situation) = 0.5

- Classifiers: logistic regression, SVM, RF, neural networks

- When doing classification for a real application - always divide your data in two groups: training and testing subsets to avoid overtraining

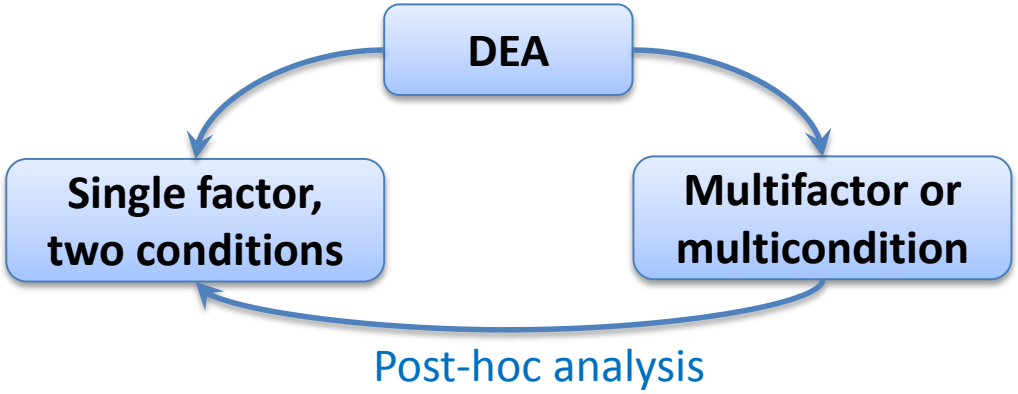
Differential Expression Analysis

Differential Expression Analysis

Basics

Questions

- Which genes have changes in **mean** expression level between conditions?
- How reliable are this observations

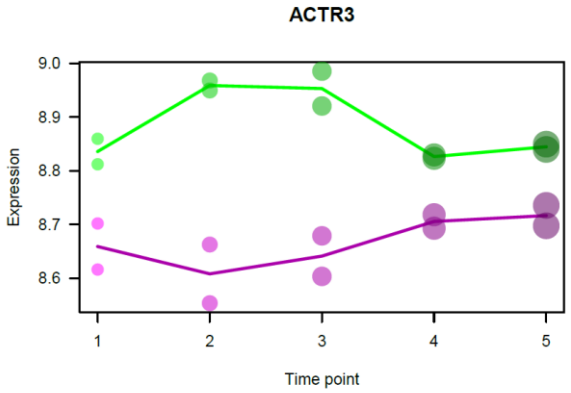


Similar to t-test with Student's statistics: compare means

Similar to ANOVA with Fisher's statistics: compare variances

And do not forget about multiple hypotheses testing

Example: 2 cell lines in time:



Differential Expression Analysis

What is this p-value ?

One-tailed test

A hypothesis test in which rejection of the null hypothesis occurs for values of the test statistic in one tail of its sampling distribution

$H_0: \mu \leq \mu_0$ $\quad$ $H_0: \mu \geq \mu_0$

$H_a: \mu > \mu_0$ $\quad$ $H_a: \mu < \mu_0$

A Trade Commission (TC) periodically conducts statistical studies designed to test the claims that manufacturers make about their products. For example, the label on a large can of Hilltop Coffee states that the can contains 3 pounds of coffee. The TC knows that Hilltop's production process cannot place exactly 3 pounds of coffee in each can, even if the mean filling weight for the population of all cans filled is 3 pounds per can. However, as long as the population mean filling weight is at least 3 pounds per can, the rights of consumers will be protected. Thus, the TC interprets the label information on a large can of coffee as a claim by Hilltop that the population mean filling weight is at least 3 pounds per can. We will show how the TC can check Hilltop's claim by conducting a lower tail hypothesis test.

$\mu_0 = 3$ lbm

Suppose sample of n=36 coffee cans is selected. From the previous studies it's known that $\sigma = 0.18$ lbm

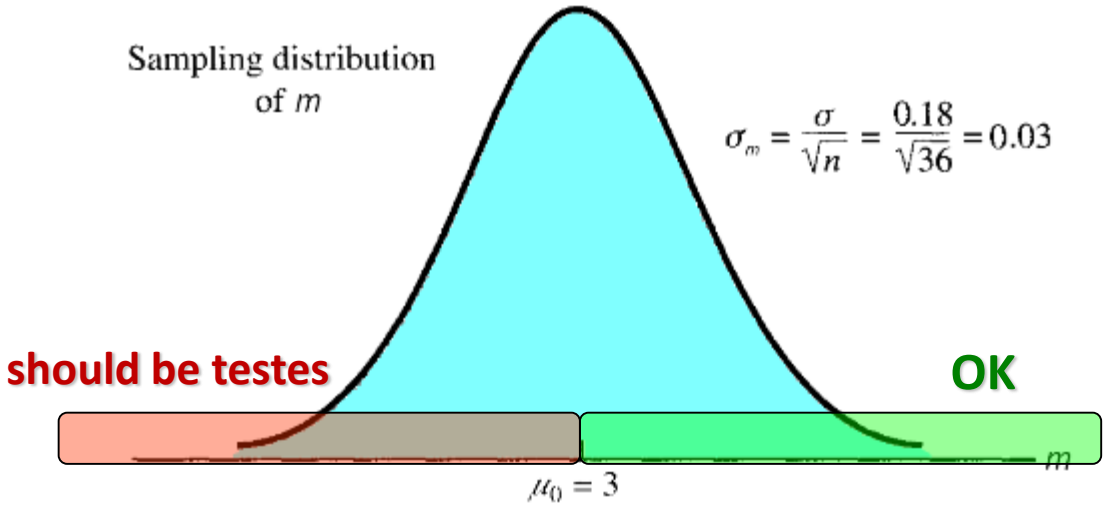
What is this p-value ?

μ_0 = 3 lbm

H_0: $\mu \geq 3$ **no action**

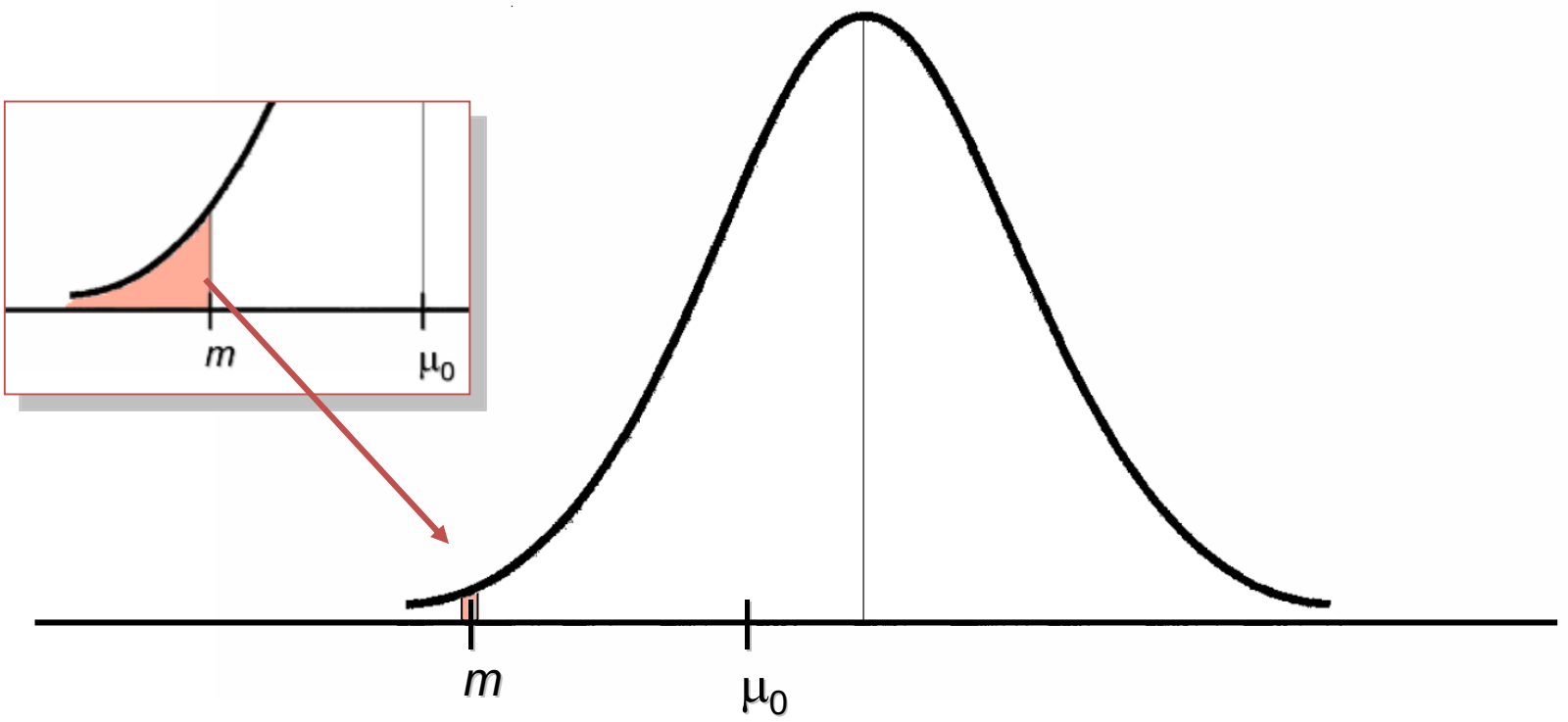
H_a: $\mu < 3$ **legal action**

Let's say: in the extreme case, when μ=3, we would like to be 99% sure that we make no mistake, when starting legal actions against Hilltop Coffee. It means that selected significance level is **α = 0.01**



What is this p-value ?

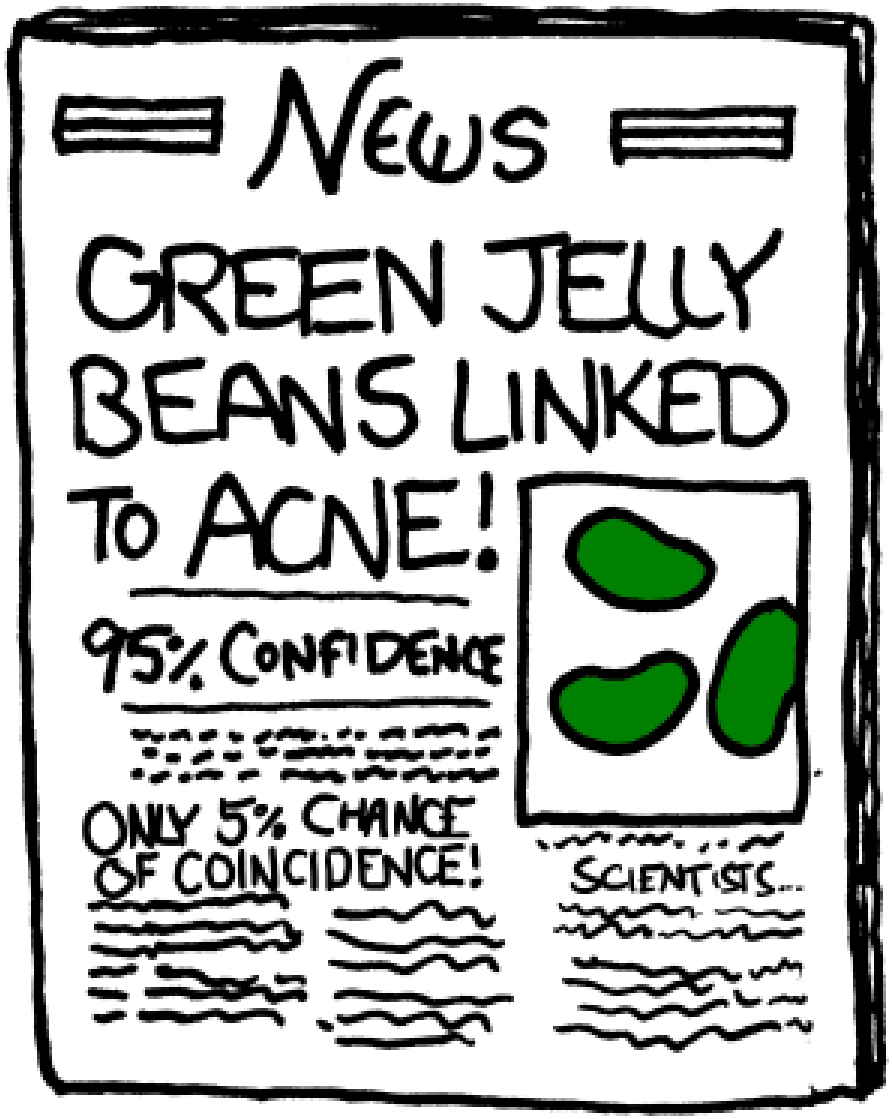
Let's find the probability of observation *m* for all possible $\mu \geq 3$. We start from an extreme case ($\mu=3$) and then probe all possible $\mu>3$. See the behavior of the small probability area around measured *m*. What you will get if you summarize its area for all possible $\mu \geq 3$?



P(*m*) for all possible $\mu \geq \mu_0$ is equal to *P*(*x*<*m*) for an extreme case of $\mu=\mu_0$

Differential Expression Analysis

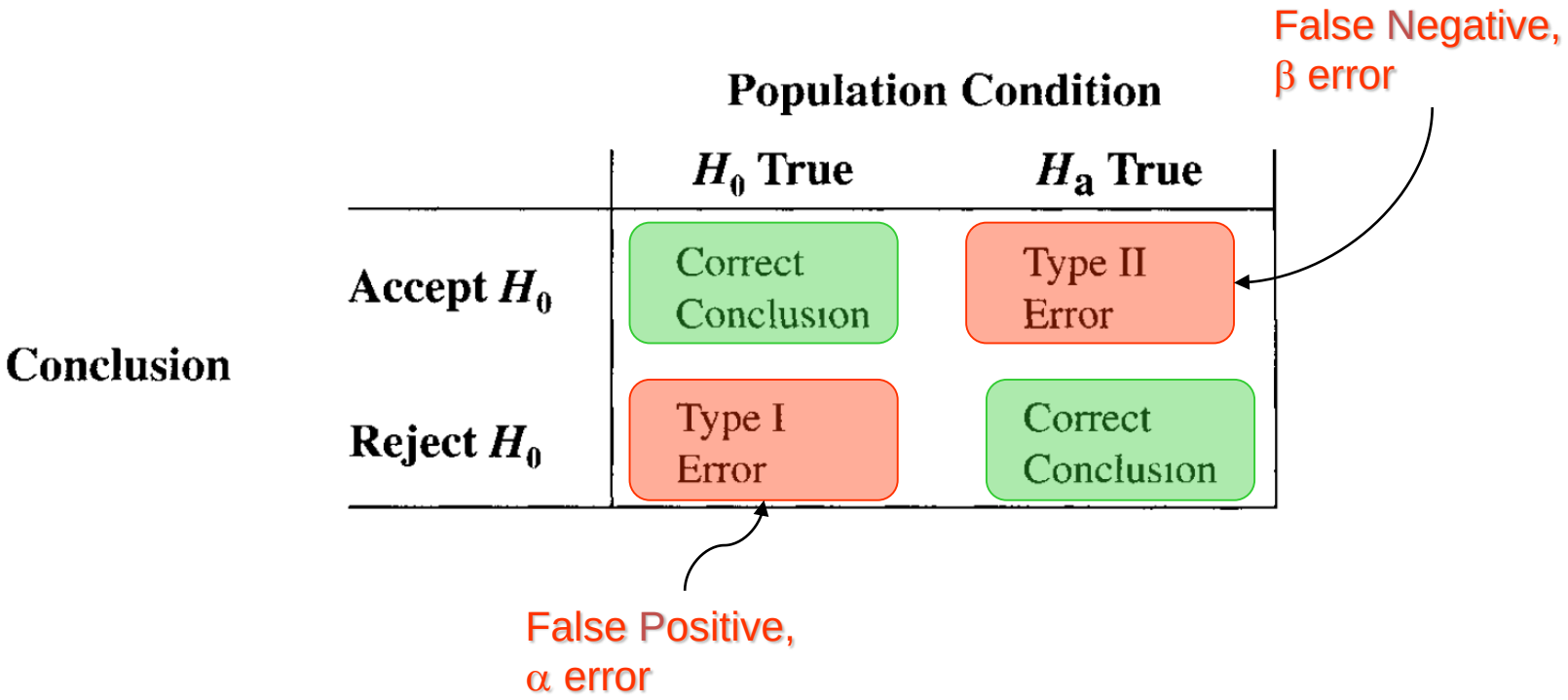
Example



http://www.xkcd.com/882/

Differential Expression Analysis

Multiple Hypotheses



Probability of an error in a multiple test:

$1-(0.95)^{\text{number of comparisons}}$

Multiple Hypotheses: False Discovery Rate

False discovery rate (FDR)

FDR control is a statistical method used in multiple hypothesis testing to correct for multiple comparisons. In a list of rejected hypotheses, FDR controls the expected proportion of incorrectly rejected null hypotheses (type I errors).

Conclusion	Population Condition: H_0 is TRUE	H_0 is FALSE	Total
Accept H_0 (non-significant)	U	T	$m - R$
Reject H_0 (significant)	V	S	R
Total	m_0	$m - m_0$	m

$$FDR = E\left(\frac{V}{V+S}\right)$$

Differential Expression Analysis

False Discovery Rate: Benjamini & Hochberg

Assume we need to perform *m* = 100 comparisons, and select maximum **FDR = α = 0.05**

$$FDR = E\left(\frac{V}{V+S}\right)$$

Expected value for FDR < α if $P_{(k)} < \frac{k}{m}\alpha$ ⇒ $\frac{mP_{(k)}}{k} < \alpha$

```
p.adjust(pv, method="fdr")
```

Theoretically, the sign should be "≤".
But for practical reasons it is replaced by "<"

Familywise Error Rate (FWER)

Bonferroni – simple, but too stringent, not recommended

$$mP_{(k)} < \alpha$$

Holm-Bonferroni – a more powerful, less stringent but still universal FWER

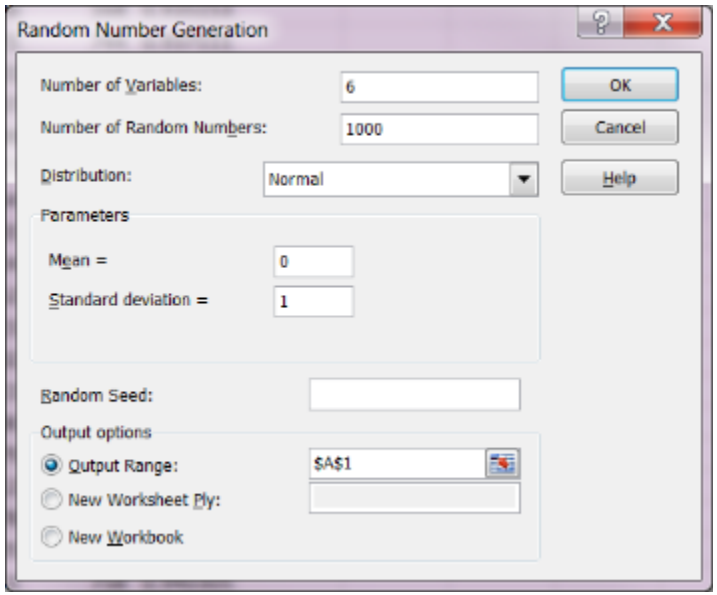
$$(m+1-k)P_{(k)} < \alpha$$

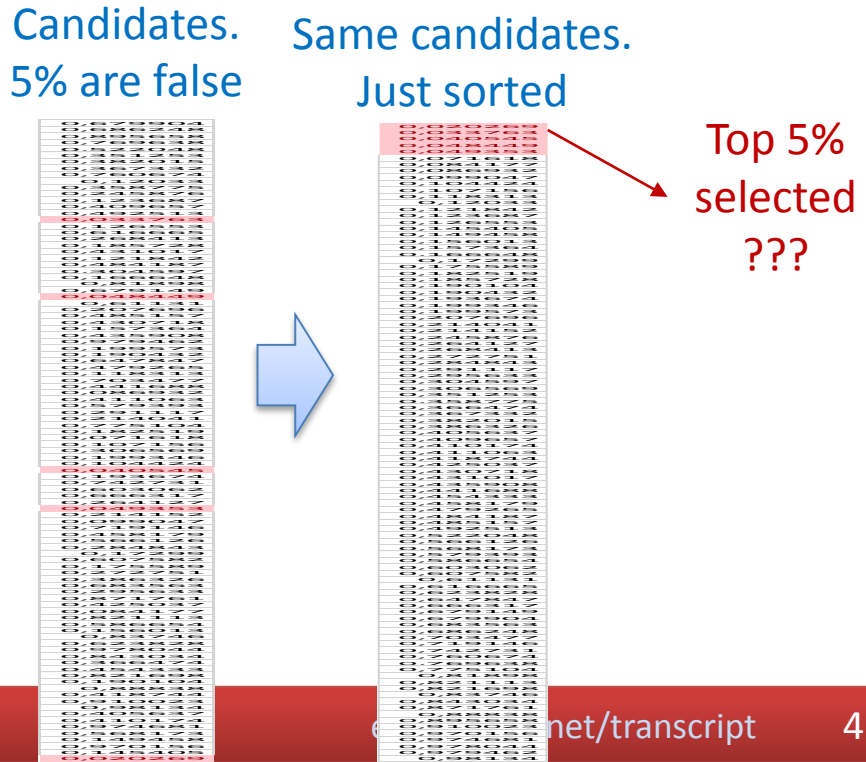
Differential Expression Analysis

Why is it so important to correct p-values?..

Let's generate a completely random experiment (Excel)

- Generate 6 columns of normal random variables (1000 points/candidates in each).
- Consider the first 3 columns as "treatment", and the next 3 columns as "control".
- Using t-test calculate p-values b/w "treatment" and "control" group. How many candidates have p-value<0.05 ?
- Calculate FDR. How many candidates you have now?





Differential Expression Analysis

Linear Models

Many conditions

We have measurements for 5 conditions. Are the means for these conditions equal?

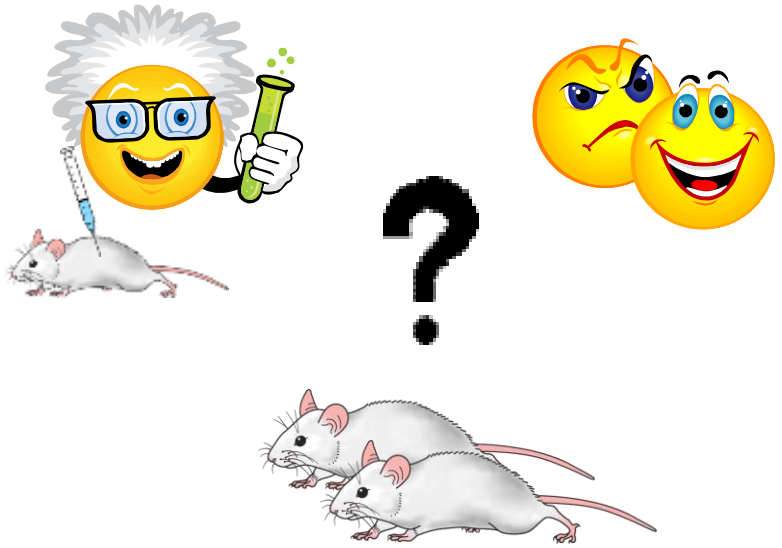
Many factors

We assume that we have several factors affecting our data. Which factors are most significant? Which can be neglected?

If we would use pairwise comparisons, what will be the probability of getting error?

Number of comparisons: $C_2^5 = \frac{5!}{2!3!} = 10$

Probability of an error: $1-(0.95)^{10} = 0.4$



ANOVA
example from Partek™

http://easylink.playstream.com/affymetrix/ambsymposium/partek_08.wvx

Linear Models

As part of a long-term study of individuals 65 years of age or older, sociologists and physicians at the Wentworth Medical Center in upstate New York investigated the relationship between geographic location and depression. A sample of 60 individuals, all in reasonably good health, was selected; 20 individuals were residents of Florida, 20 were residents of New York, and 20 were residents of North Carolina. Each of the individuals sampled was given a standardized test to measure depression. The data collected follow; higher test scores indicate higher levels of depression.

Q: Is the depression level same in all 3 locations?

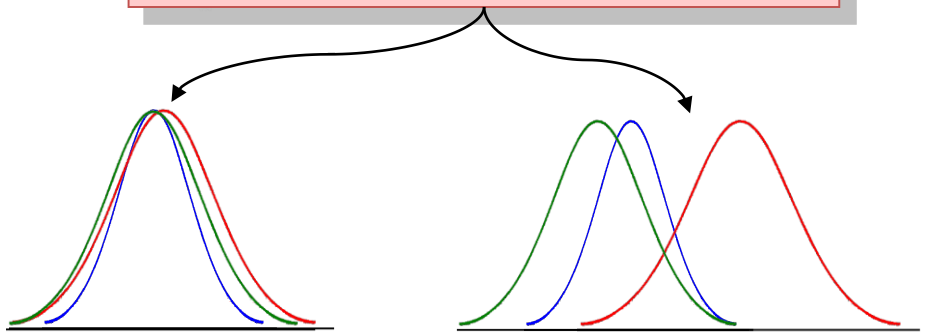
depression.txt

1. Good health respondents

Florida	New York	N. Carolina
3	8	10
7	11	7
7	9	3
3	7	5
8	8	11
8	7	8
...	...	...

H_0: $\mu_1 = \mu_2 = \mu_3$

H_a: not all 3 means are equal

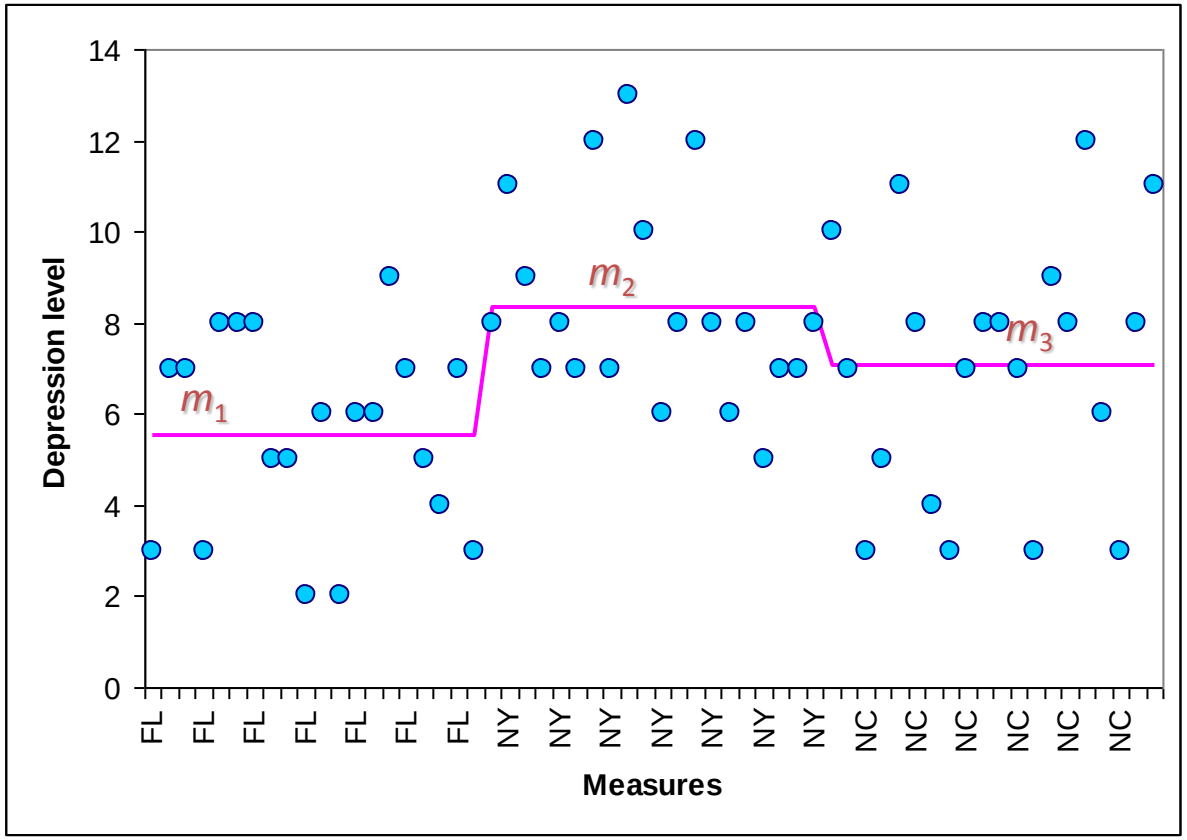


Differential Expression Analysis

Linear Models

H_0: $\mu_1 = \mu_2 = \mu_3$

H_a: not all 3 means are equal



Differential Expression Analysis

LIMMA & EdgeR : Linear Models for Microarrays

$$Y_{ij} = \mu_I + A_j + B_j + A_j*B_j + \epsilon_{ij}$$

i – gene index
j – sample index

A_j*B_j – effect which cannot be explained by superposition A and B

Limma – R package for DEA in microarrays based on linear models.

It is similar to t-test / ANOVA but using all available data for variance estimation, thus it has higher power when number of replicates is limited

edgeR – R package for DEA in RNA-Seq, based on linear models and negative binomial distribution of counts.

Better noise model results in higher power detecting differentially expressed genes

negative binomial process – number of tries before success: rolling a die until you get 6

Differential Expression Analysis

Take Home Messages

- When doing multiple hypothesis testing and selecting only those elements which are significantly – always use FDR (or other, like FWER) correction!
  - the simplest correction – multiply p-value by the number of genes. Is it still significant? The best correction – use FDR or FWER

- DEA provides the genes which have variability in **mean** gene expression between condition
  - => more data you have, smaller differences you will be able to see

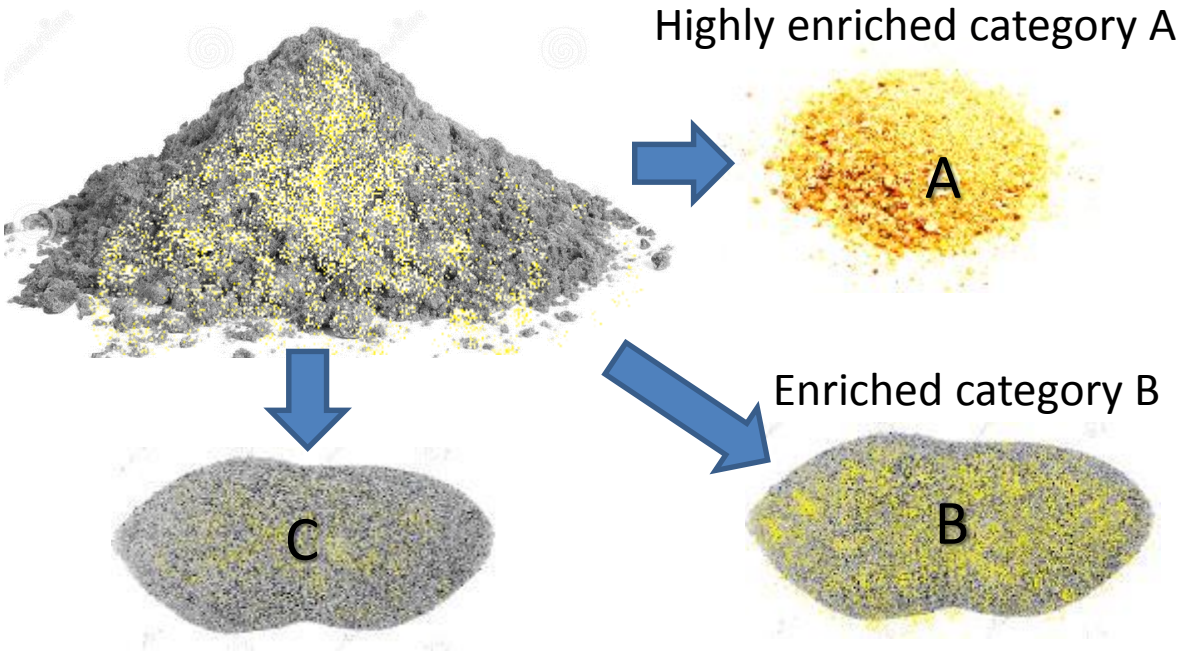
- Several factors can be taken into account in ANOVA approach. This will give you insight into significance of each experimental factor but at the same time will correct batch effects and allow answering complex questions (remember shoes affecting ladies…).

Enrichment Analysis

Enrichment Analysis

1. Category Enrichment Analysis

Are interesting genes overrepresented in a subset corresponding to some biological process?



Method of the analysis:
Fisher's exact test

Someone grabs "randomly" 20 balls from a box with 100x ● and 100x ●

How surprised will you be if he grabbed

●●●●●●●●●●●●●●●●●●●●

(17 red , 3 green)

sand belongs to: http://www.dreamstime.com/photos-images/pile-sand.html ;)))

Enrichment Analysis

1. Category Enrichment Analysis

Fisher's exact test: based on hypergeometrical distributions

Hypergeometrical: distribution of objects taken from a "box", without putting them back

$$P = 1 - \sum_{i=0}^{k-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

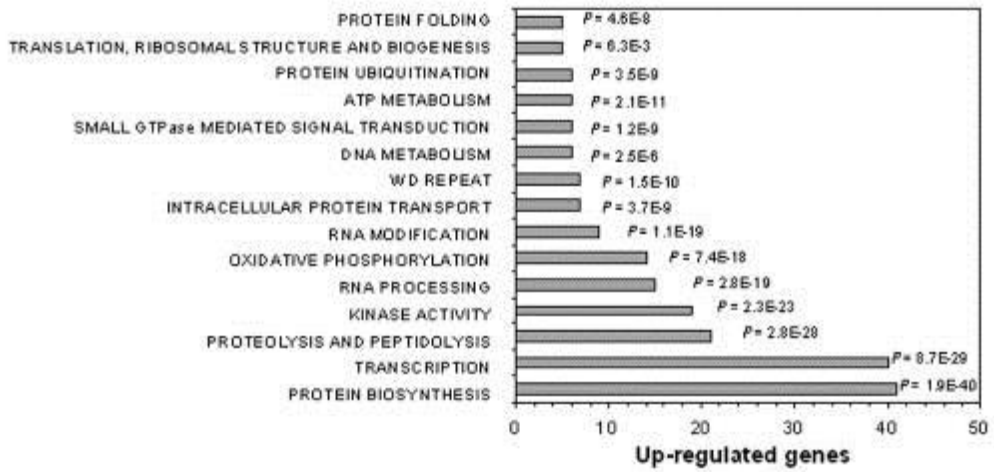
N: total number of genes

M: total number of genes annotated with this term

n: number of genes in the list

k: number of genes in the list annotated with this term

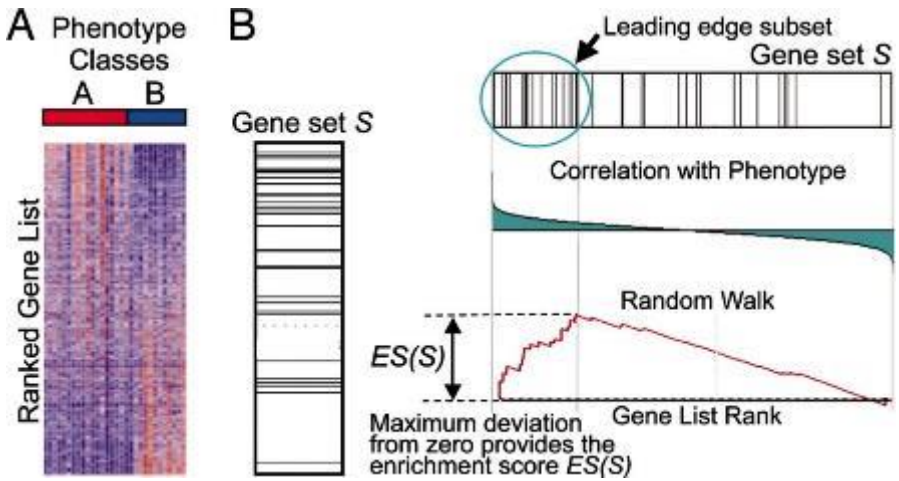
$$C_k^n = C_n^k = \binom{n}{k} = \frac{n!}{k!\,(n-k)!}$$

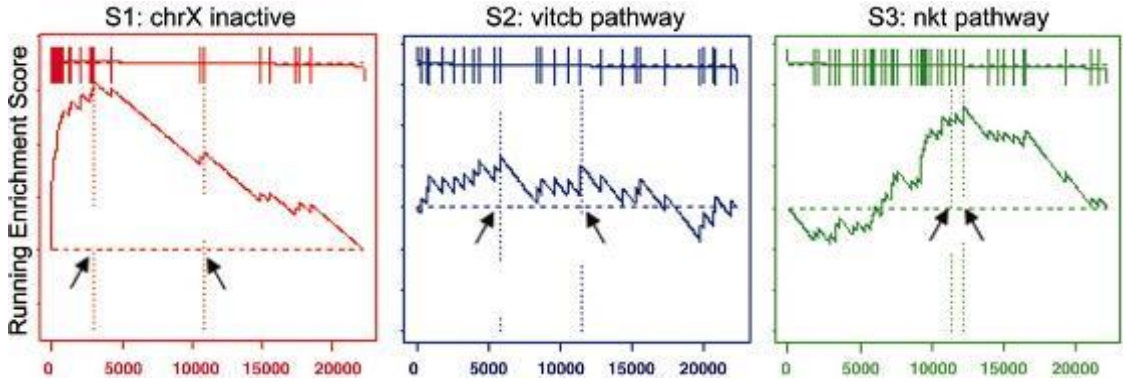


Okamoto et al. Cancer Cell International 2007 7:11 doi:10.1186/1475-2867-7-11

2. Gene Set Enrichment Analysis (GSEA)

Is direction of genes in a category random?





A. Subramanian et al. PNAS **2005**,102,43

Enrichment Analysis

Take Home Messages

- To find biological meaning of the significantly regulated genes use enrichment analysis methods linking known groups of genes to DEA results

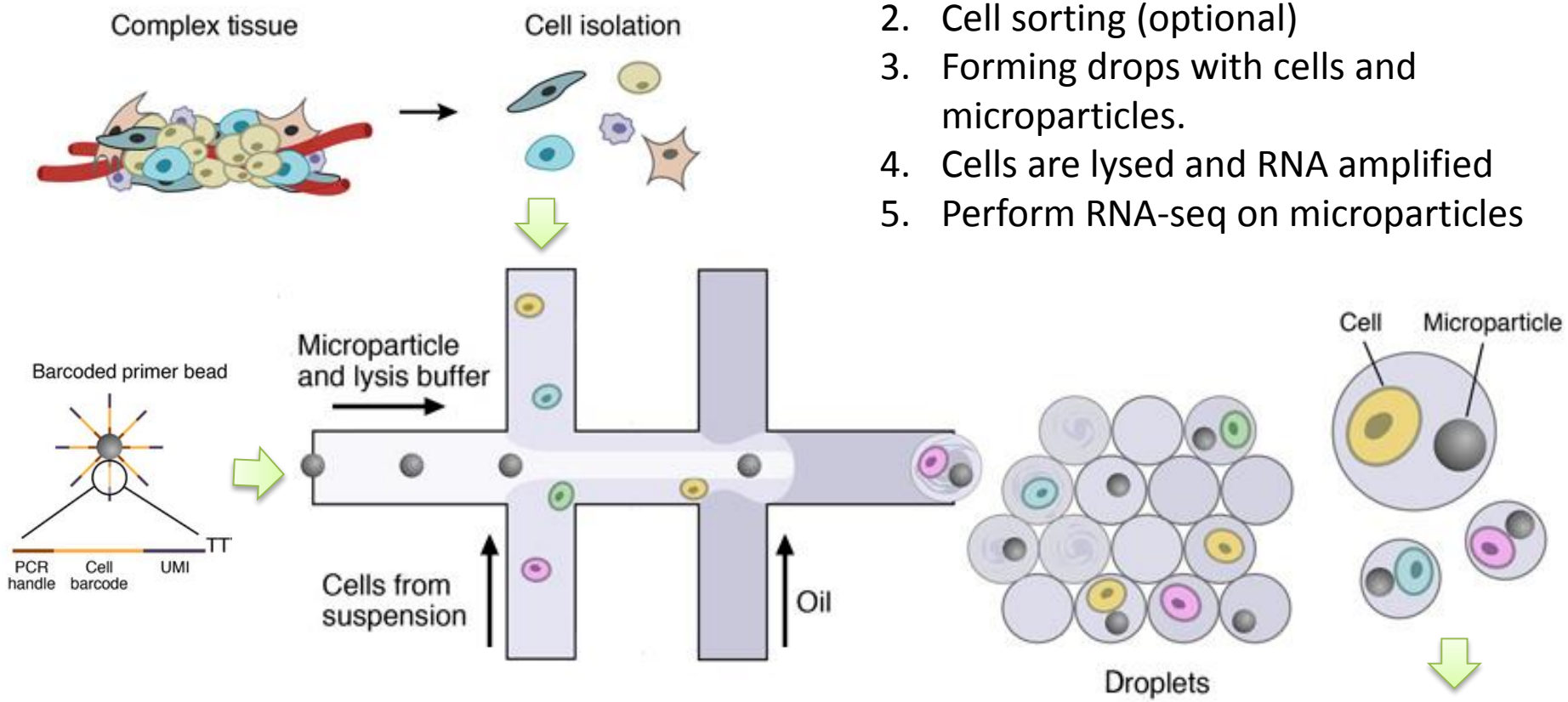
- Enriched categories are usually more robust then individual genes

Single Cell Transcriptomics

Single Cell Transcriptomics

Single Cell Transcriptomics – one of the method to handle the tissue heterogeneity problem.

1. Cell dissociation
2. Cell sorting (optional)
3. Forming drops with cells and microparticles.
4. Cells are lysed and RNA amplified
5. Perform RNA-seq on microparticles



Each microparticle contains more than 10^8 individual primers that share the same “PCR handle” and “cell barcode”, but have different unique molecular identifiers (UMIs).

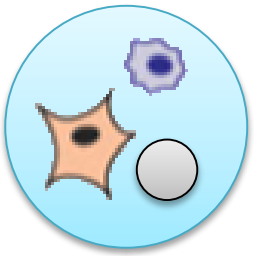
https://www.elveflow.com/microfluidic-tutorials/microfluidic-reviews-and-tutorials/drop-seq/

Single Cell Data Properties

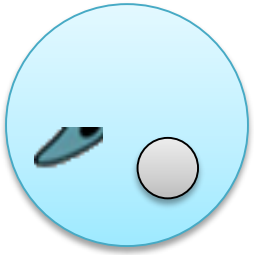
Ideal: one bead - one cell

What you have in practice:

no cell, floating RNA

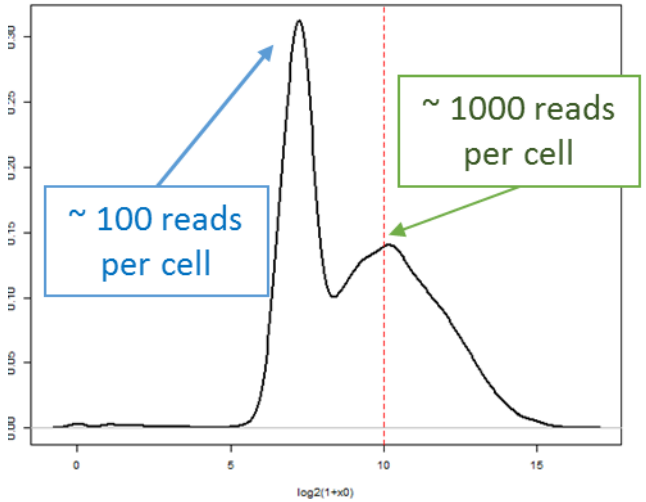


two cells



some cellular debris: often mitochondria

Number of “reads” (detected RNA fragments) per cell



Therefore:

1. Single-cell RNA-seq data are sparse (many zeros) and large (expect to have 10^2-10^4 cells x 10^3-10^4 genes).
2. Filtering is unavoidable and often remove majority of “cells”.
3. Standard normalization methods are questionable.

Single Cell Data Properties

PCA of SC RNA-seq data

- PCA captures variability => distant data points have larger effect
- PC1 always captures number of reads per cell – this is the largest effect (even after normalization)
- Biologists do not like it as the density of points is not constant ☺

We need a method that is going to:

- puts the similar objects together
- produces the picture with constant density
- is easy to understand ☺

t-SNE

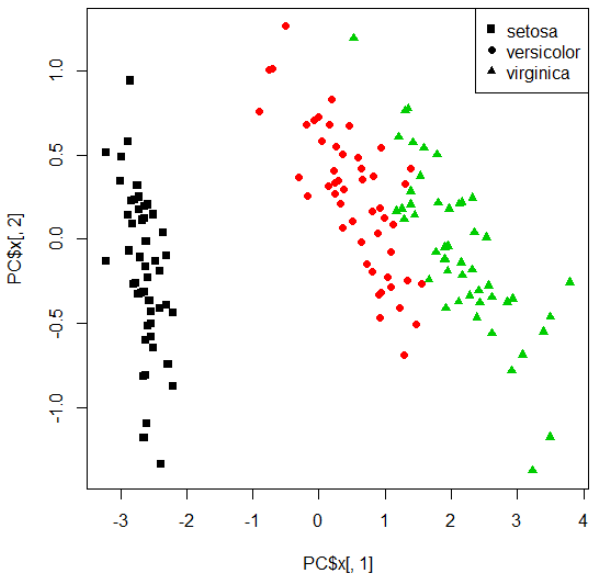
Visualization of large datasets

t-SNE is an iterative non-linear transformation that search for objects representation in 2D space by:

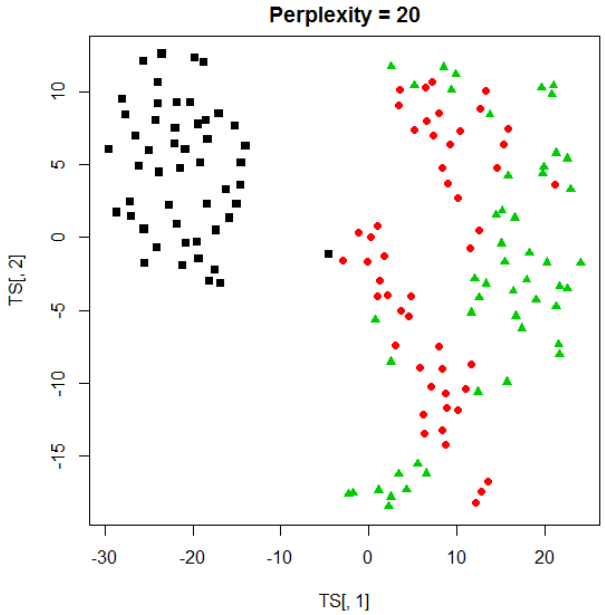
1) placing the similar objects together
2) controlling the density of the obtained clusters

Unlike PCA, distant objects are not influencing t-SNE!

PCA



t-SNE



Pro:

- easy to understand
- no effect of outliers

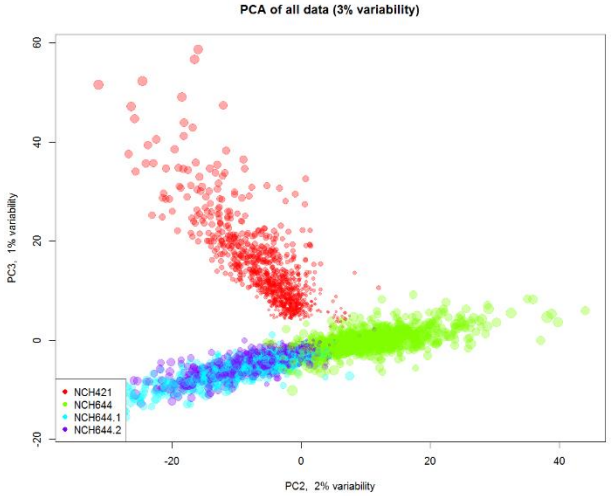
Con:

- depends on init.estim.
- can be over-interpreted !
- depends on *perplexity* parameter

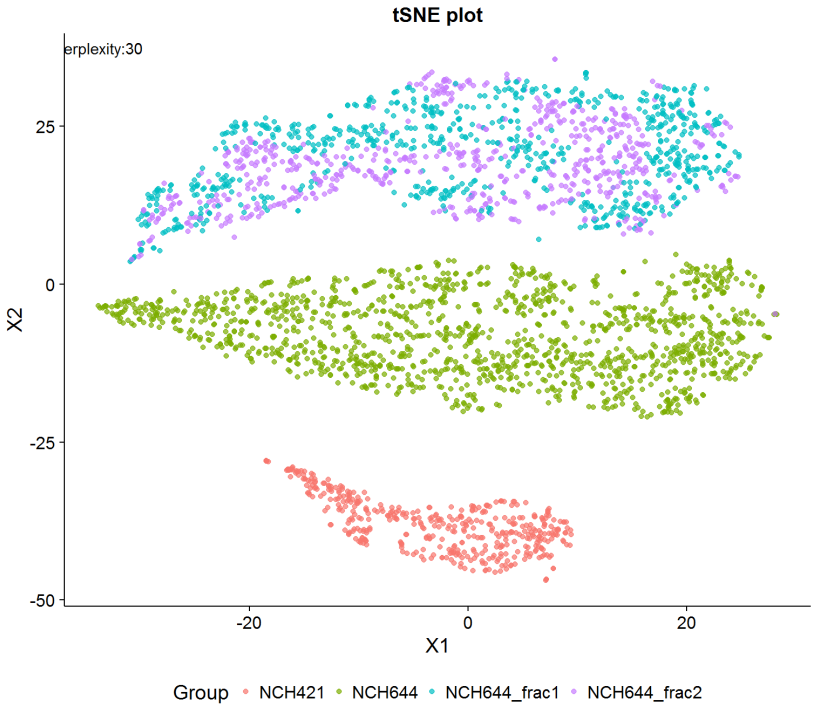
Play with t-SNE here: https://distill.pub/2016/misread-tsne/

t-SNE for single cell transcriptomics

PCA plots



t-SNE plot



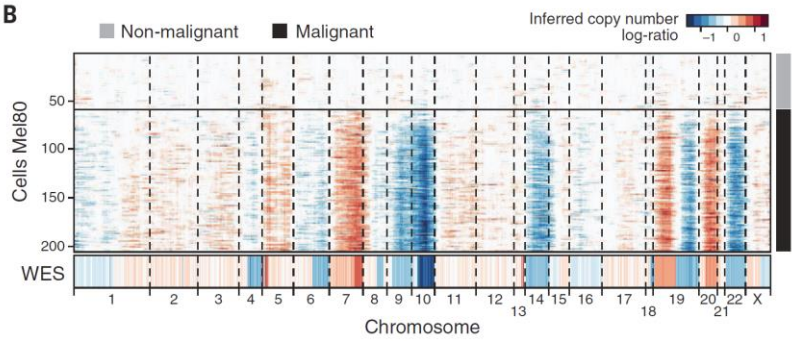
t-SNE for single cell transcriptomics

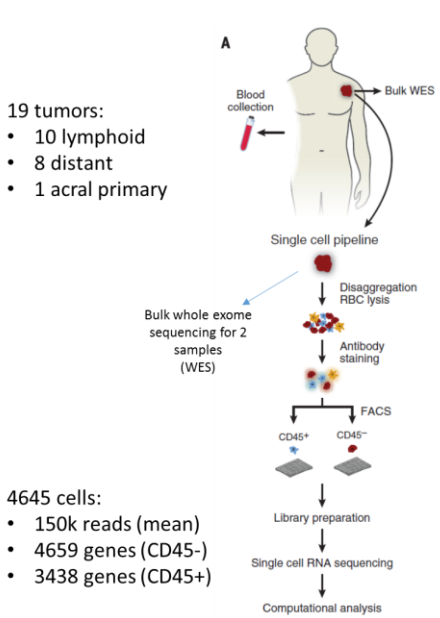
RESEARCH ARTICLES

CANCER GENOMICS

Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq

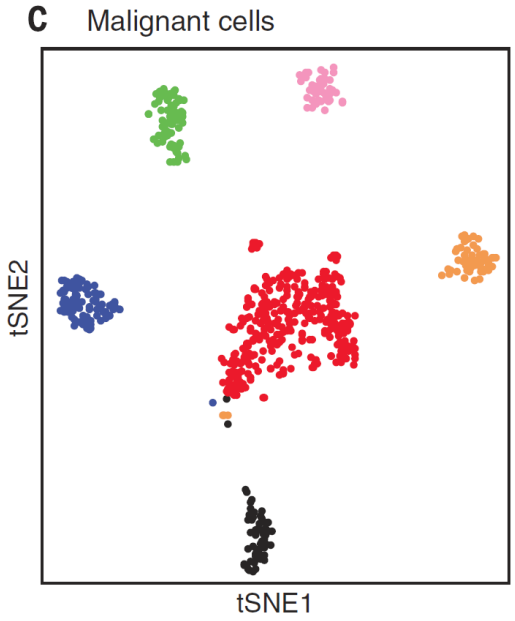
Itay Tirosh,[1*] Benjamin Izar,[1,2,3*†‡] Sanjay M. Prakadan,[1,4,5,6]
Marc H. Wadsworth II,[1,4,5,6] Daniel Treacy,[1] John J. Trombetta,[1] Asaf Rotem,[1,2,3]

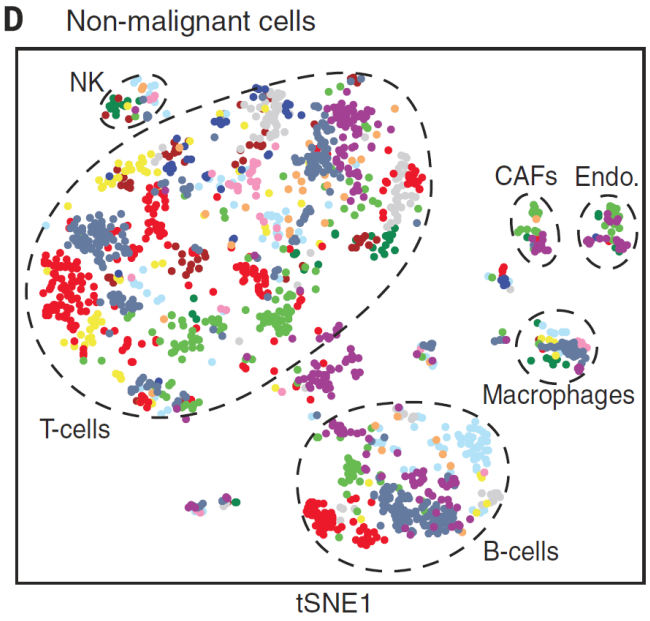




19 tumors:
- 10 lymphoid
- 8 distant
- 1 acral primary

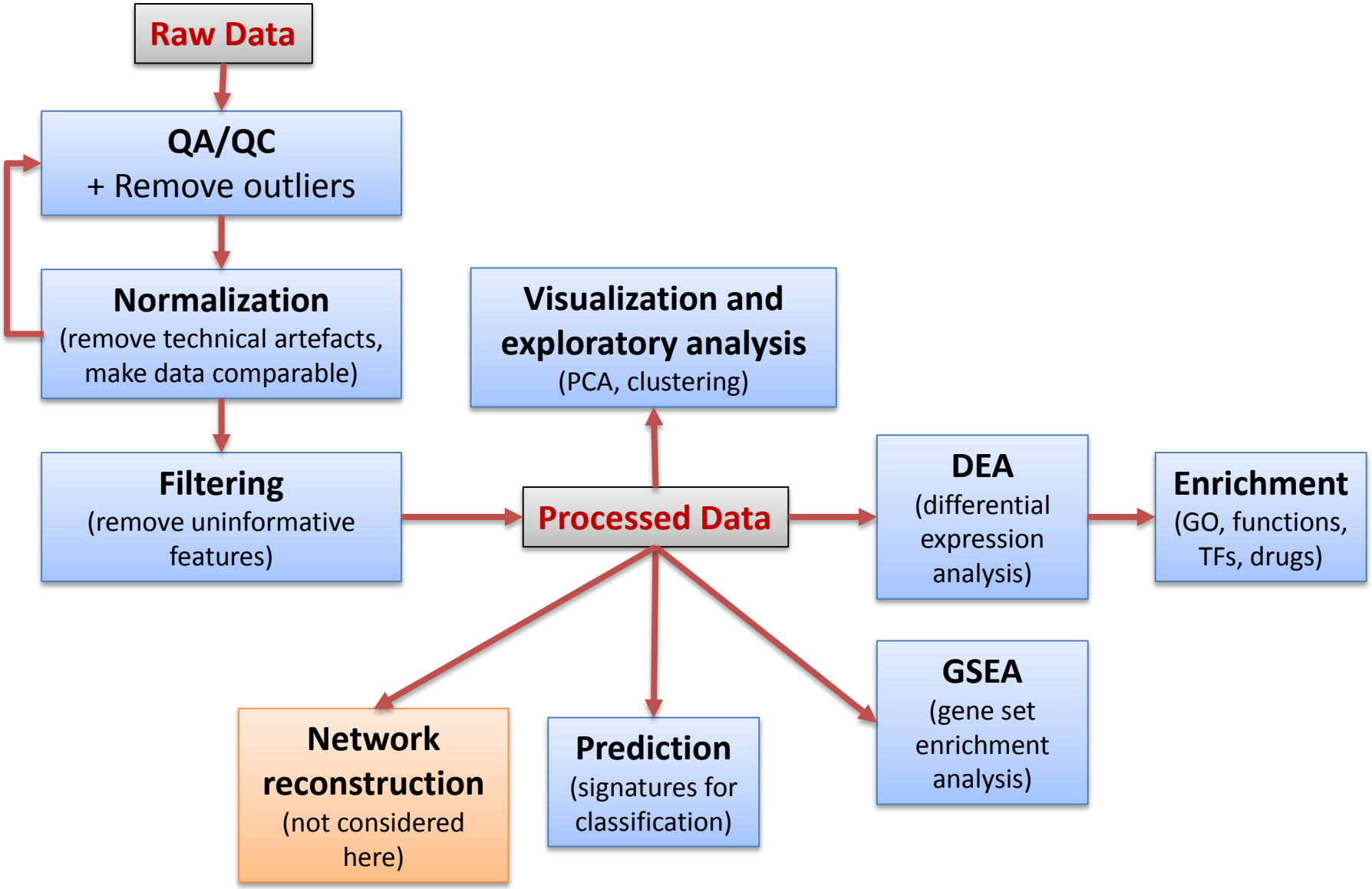
4645 cells:
- 150k reads (mean)
- 4659 genes (CD45-)
- 3438 genes (CD45+)





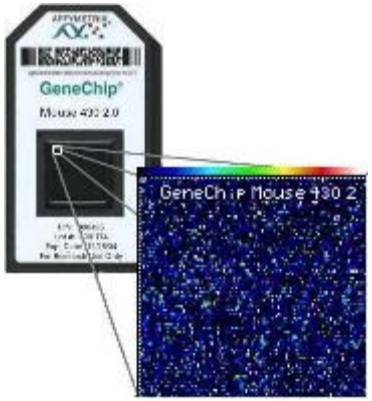
Mel53 Mel60 Mel74 Mel79 Mel81 Mel88 Mel94
Mel58 Mel72 Mel78 Mel80 Mel84 Mel89

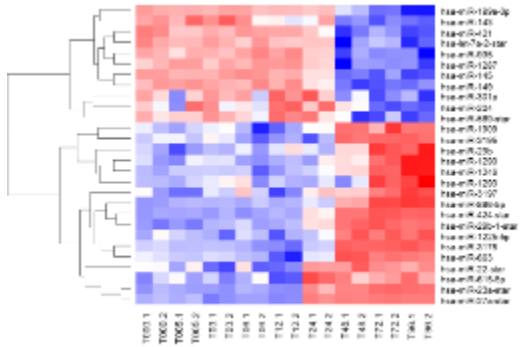
Summary


Raw Data
QA/QC
+ Remove outliers
Normalization
(remove technical artefacts, make data comparable)
Filtering
(remove uninformative features)
Visualization and exploratory analysis
(PCA, clustering)
Processed Data
DEA
(differential expression analysis)
Enrichment
(GO, functions, TFs, drugs)
GSEA
(gene set enrichment analysis)
Prediction
(signatures for classification)
Network reconstruction
(not considered here)

Questions ?

Thank you for your attention !





Practice

Practice in Transcriptomics Data Analysis

Please visit: http://edu.sablab.net/transcript/
and follow the instructions

Task1. Simple analysis in Excel

lusc20.txt

lusc20.xlsx

TCGA (LUSC) database extract:

- 20 normal lung tissues
- 20 squamous cell carcinoma tissues

Task2. Analysis in TAC software

SCC CEL files

TAC software

Affymetrix HTA 2.0 arrays on:

- 10 normal lung tissues
- 10 squamous cell carcinoma tissues

Tissues are paired!

Task3. Analysis in TAC software (optional)

Affymetrix HuGene arrays on A375 cell line under IFNg treatment

Task1. Differential Expression Analysis

Example: let's make it easy

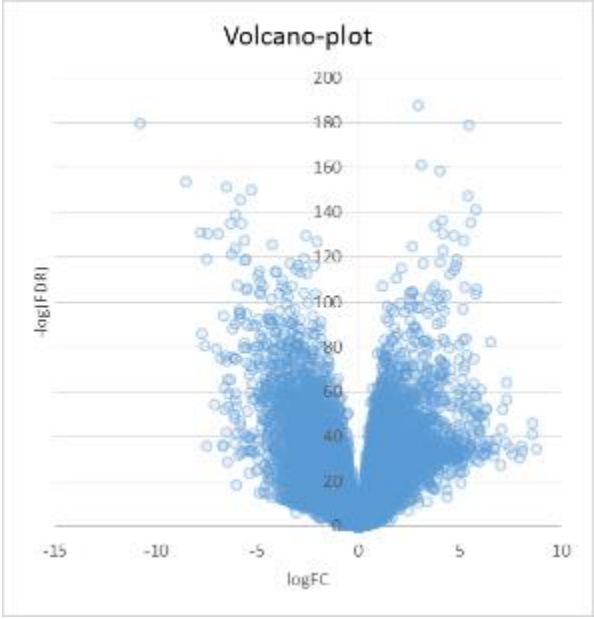
http://edu.sablab.net/transcript/lusc20.xlsx

1. Find genes significantly differentially expressed in SCC vs normal tissue
   - apply t-test. Same or different variance?
   - perform FDR correction
   - Keep genes with FDR > 0.001

2. Calculate mean logFC and keep only genes with |logFC| > 2

3. Make a "volcano plot":
   -log10(FDR) vs LogFC

4. Save lists of up and down regulate genes –
we shall need them



Task 1. Enrichment Analysis

LUSC Example

http://edu.sablab.net/transcript/lusc20.txt

http://amp.pharm.mssm.edu/Enrichr/

http://biocompendium.embl.de/

http://string-db.org

0. Prepare lists of DE genes…

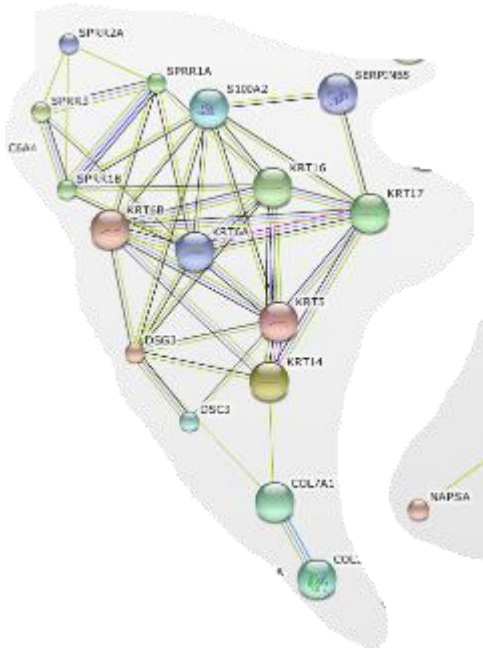
1. Put up-regulated into **enrich**

3. Check: Down CMAP, Disease Signatures from GEO up,

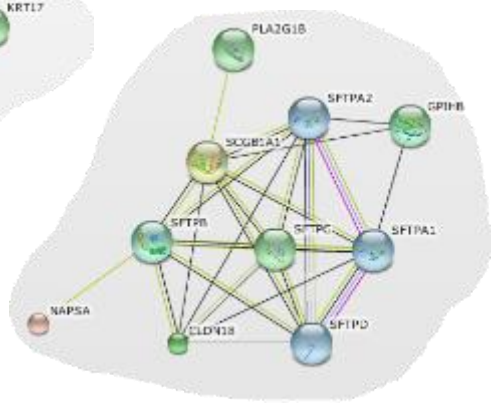
4. Try **biocompendium**

5. Put top 100 genes into String to see PP-interactions

Up regulated



Down regulated



Task 3. Enrichment Analysis

Example: GO enrichment

http://edu.sablab.net/transcript

Strategy 1:
Take all DEG and use them in enrichment.

- Safe
- No additional assumptions
- Cannot distinguish ↑ and ↓ functions

Strategy 2:
Separate DEG to down- and up- regulated genes. Then perform independent enrichment by these 2 groups

- Can be biased (gene can be ↑↓)
- Assume ↑gene => ↑function
- Can distinguish ↑ and ↓ functions

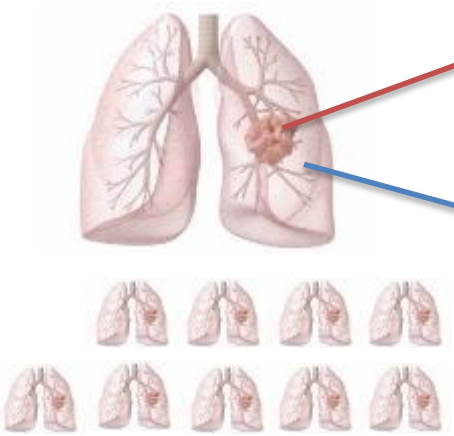
Enrichr
http://amp.pharm.mssm.edu/Enrichr

BioCompendium
http://biocompendium.embl.de/

Task2. Practical Preview: SCC Dataset

Lung SCC cancer, 9 patients, 18 samples

9 patients with lung squamous cell carcinoma



Unité INSERM, University of Reims
Prof. Ph. Birembaut

18 samples:

tumour

adj. normal

Affymetrix HTA 2.0 Arrays

100 ng

Microarray analysis with Affymetrix HTA v.2

CEL

TAC software

normalization, probe summarization analysis

Gene expression

Exon expression

Junction expression

BMC Genomics. 2017 Jun 6;18(1):443. doi: 10.1186/s12864-017-3819-y.

RNA sequencing and transcriptome arrays analyses show opposing results for alternative splicing in patient derived samples.

Nazarov PV[1], Muller A[2], Kaoma T[2], Nicot N[2], Maximo C[2], Birembaut P[3], Tran NL[4], Dittmar G[2], Vallar L[2].

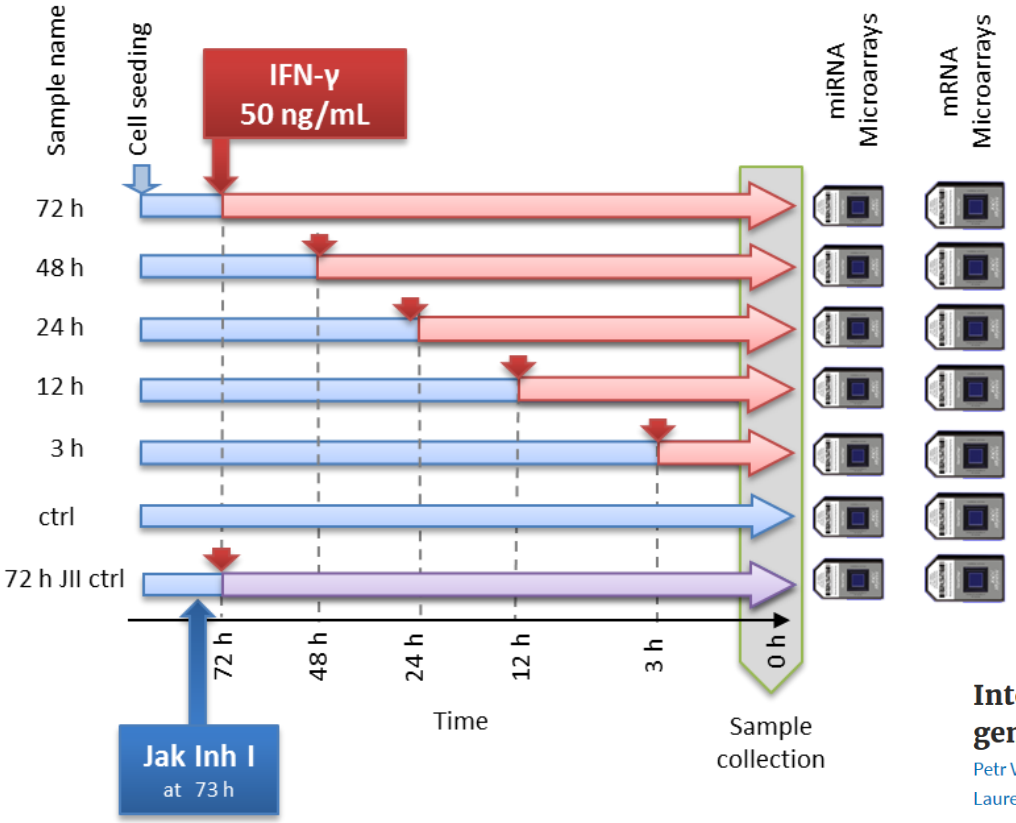
Data: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE84784

Soft: Transcriptome Analysis Console [TAC]

http://www.qmedicine.co.in/top%20health%20topics/L/Lung%20Cancer.html

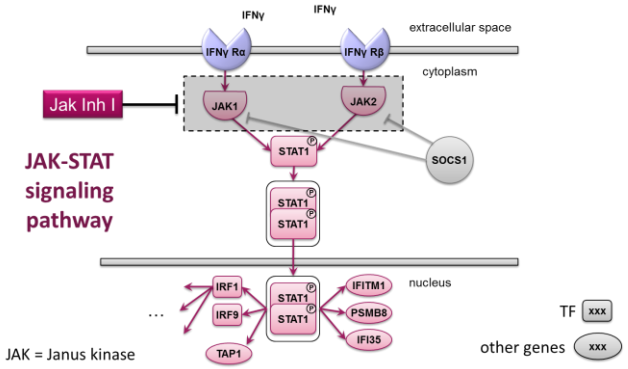
Task 3. Practical Preview: Dataset IFNg

Human melanoma **A375** cells were seeded together and cultured until sample collection. Cells were **IFNγ**-stimulated at different time points.



mRNA

Affymetrix HuGene 1.0 ST arrays



Interplay of microRNAs, transcription factors and target genes: linking dynamic expression changes to function

Petr V. Nazarov, Susanne E. Reinsbach, Arnaud Muller, Nathalie Nicot, Demetra Philippidou, Laurent Vallar, Stephanie Kreis

Nucleic Acids Research, Volume 41, Issue 5, 1 March 2013, Pages 2817–2831, https://doi.org/10.1093

Data: https://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-3720/

Soft: Transcriptome Analysis Console [TAC]