Analysis of publicly available microarray data

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University of Cambridge, Cambridge, UK

Clustering, Classification and Survival analysis

butions by Matt Ritchie, Christina Curtis, Jean Yang and Stephen Eglen)

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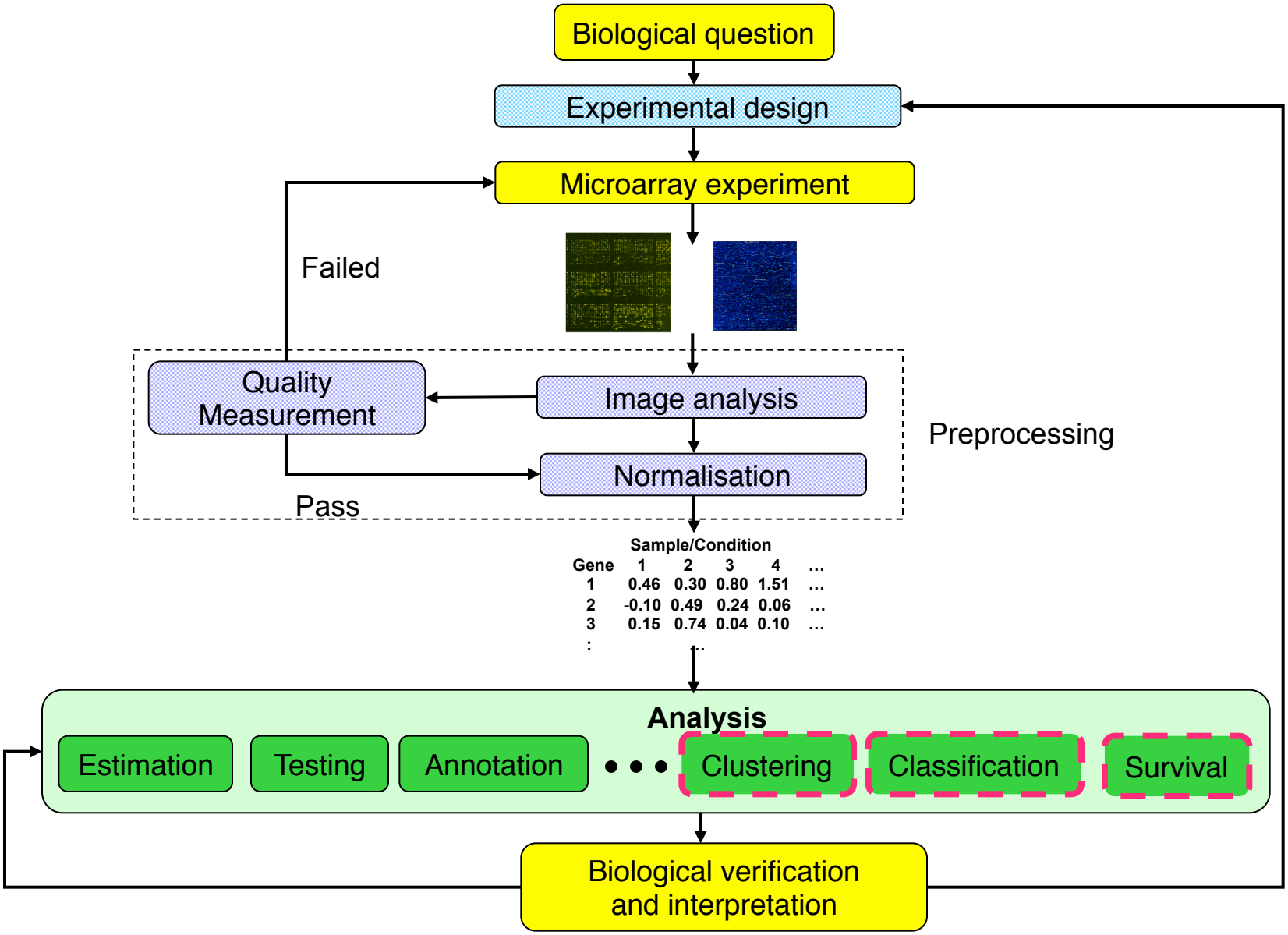

Biological question
Experimental design
Microarray experiment
Failed
Quality Measurement
Image analysis
Normalisation
Pass
Preprocessing
Sample/Condition
Gene 1 2 3 4 …
1 0.46 0.30 0.80 1.51 …
2 -0.10 0.49 0.24 0.06 …
3 0.15 0.74 0.04 0.10 …
Analysis
Estimation
Testing
Annotation
Clustering
Classification
Survival
Biological verification and interpretation

Gene expression as a data matrix

Gene expression data on *p* genes (rows) for *n* samples (columns)

mRNA samples

Genes	sample1	sample2	sample3	sample4	sample5	
1	0.46	0.30	0.80	1.51	0.90	...
2	-0.10	0.49	0.24	0.06	0.46	...
3	0.15	0.74	0.04	0.10	0.20	...
4	-0.45	-1.03	-0.79	-0.56	-0.32	...
5	-0.06	1.06	1.35	1.09	-1.09	...

Gene expression level of gene *i* in mRNA sample *j*

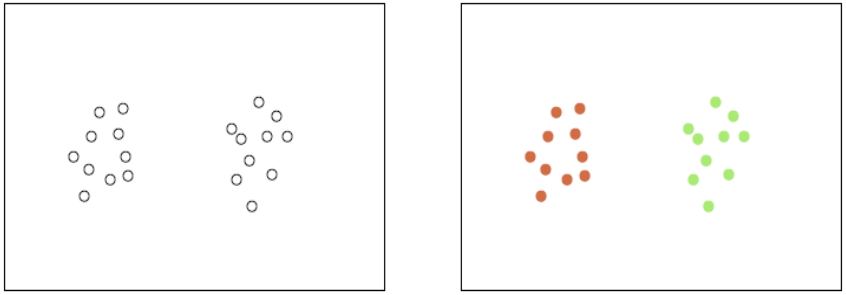
= $\log_2$(Red intensity / Green intensity)

Cluster Analysis

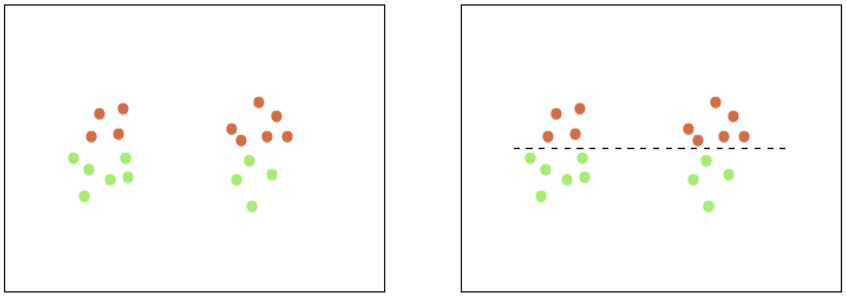
Clustering vs Classification

- **Unsupervised:** classes unknown, want to discover them from the data (cluster analysis)
- **Supervised:** classes are predefined, want to use a (training or learning) set of labelled objects to form a classifier for classification of future observations

Clustering
(Unsupervised)



Classification
(Supervised)



Clustering microarray data: motivation

- Clustering leads to readily interpretable figures and can be helpful for identifying patterns in time or space.

Examples:

- We can **cluster samples** (cols),

  e.g. 1) the identification of new / unknown tumor classes using gene expression profiles.

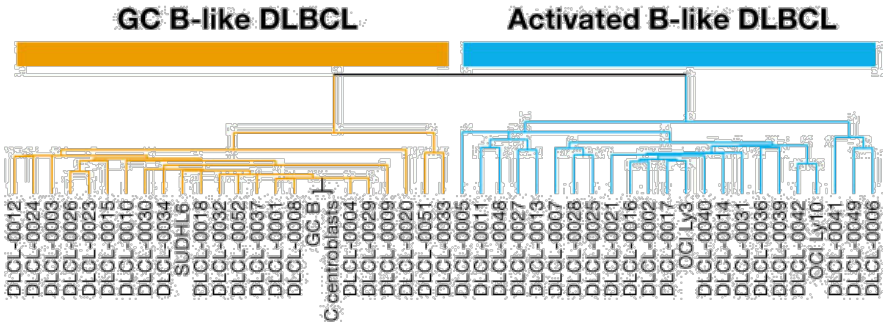
- We can **cluster genes** (rows),

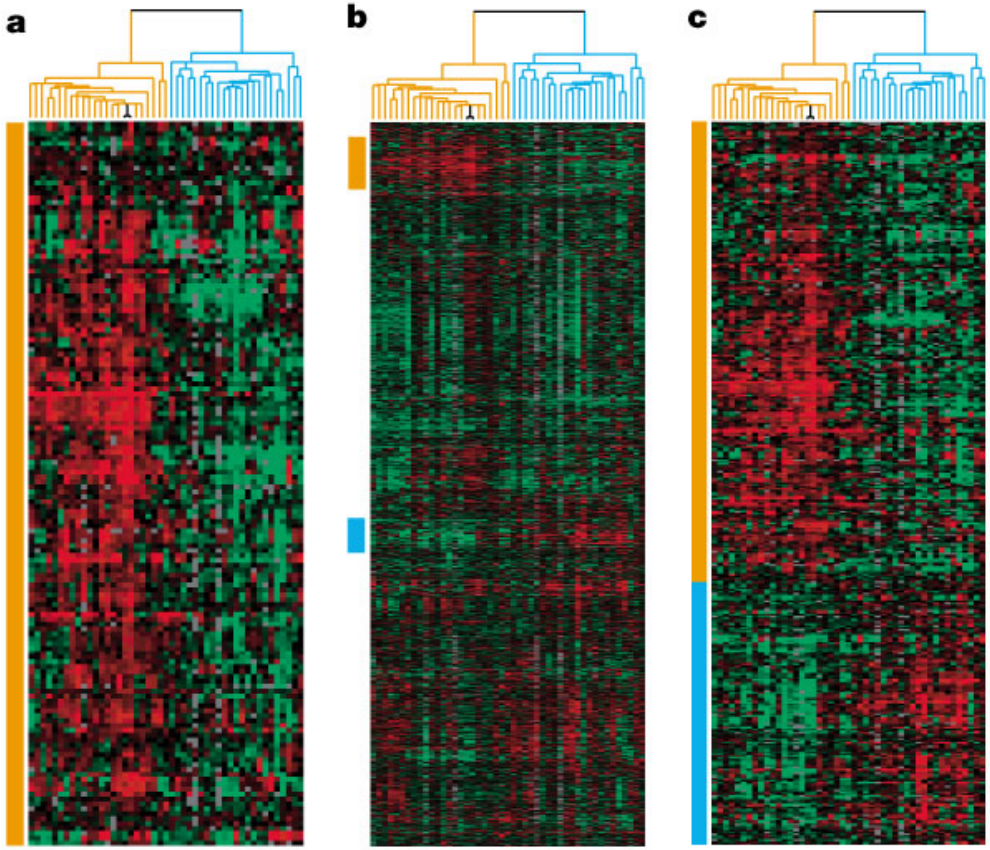
  e.g. 1) using large numbers of yeast experiments, to identify groups of co-regulated genes.

  2) we can cluster genes to reduce redundancy (cf. variable selection) in predictive models.

Clustering samples (Example 1)

Subtype discovery:
B-cell lymphoma



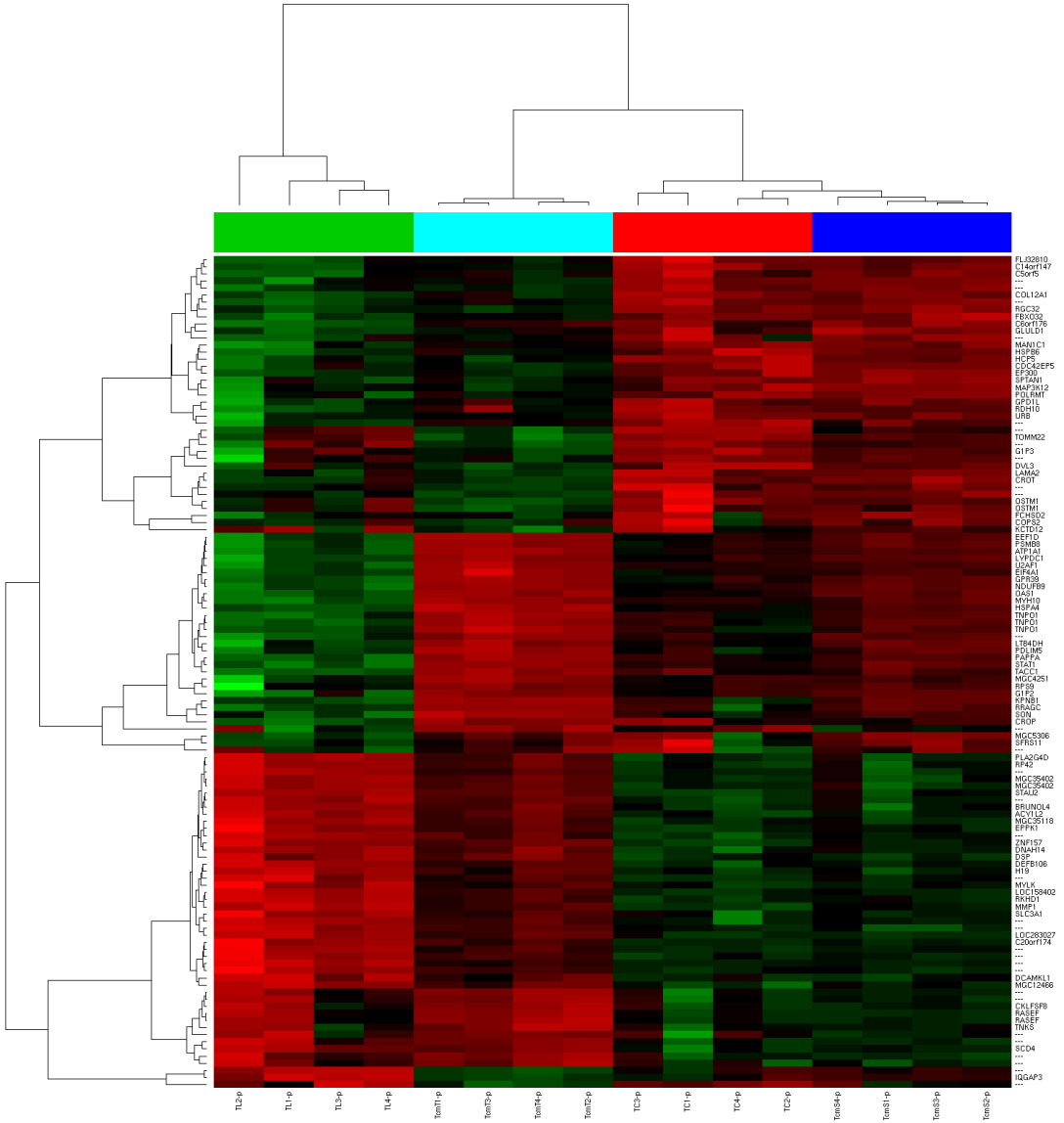


Alizadeh AA, et al. "Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling". *Nature*, 2000 Feb 3;403(6769):503-11

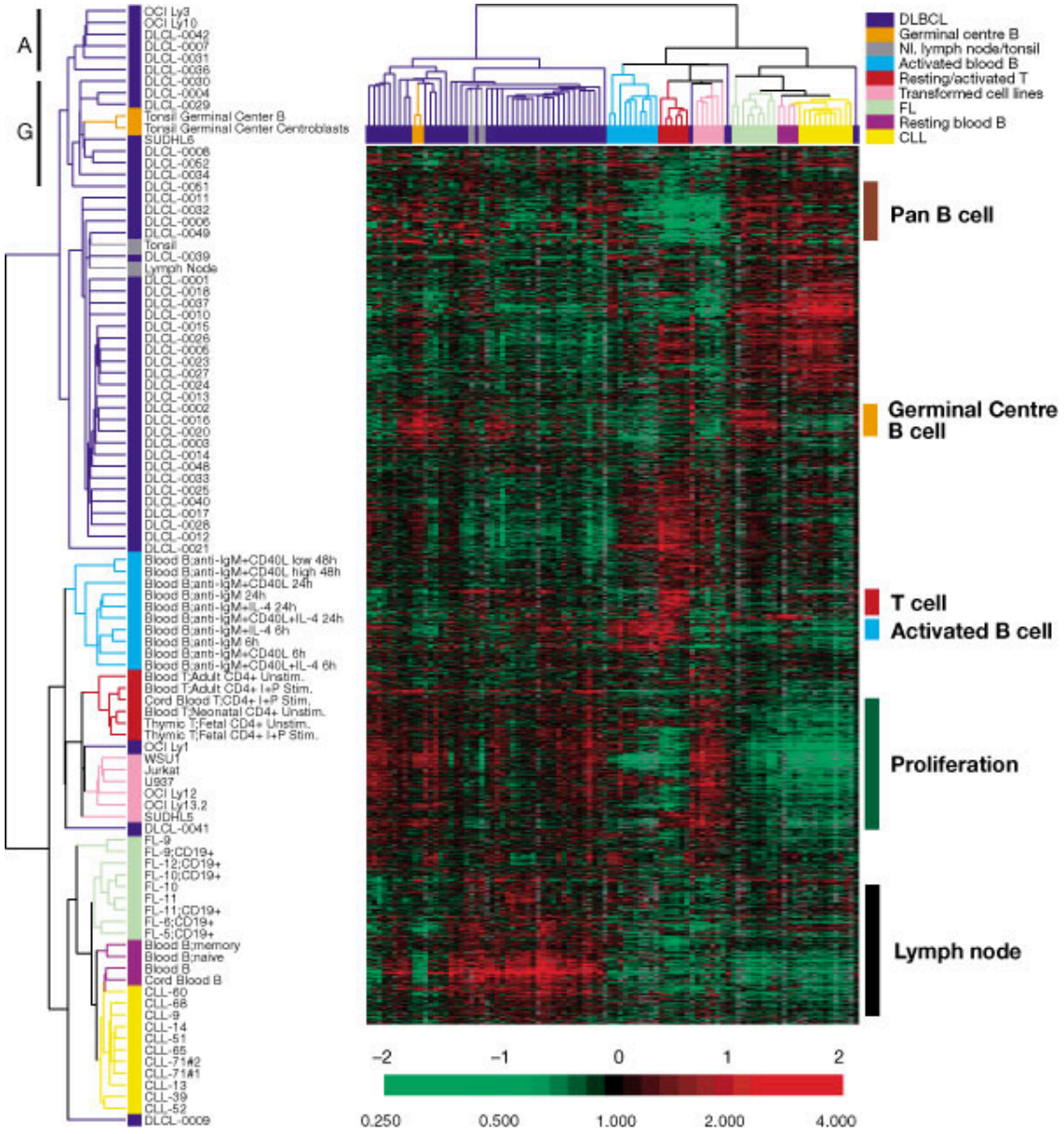
Clustering samples (Example 2)

Quality assessment:

Use clustering to check within/between experiment group variability and potential confounding factors (batch effect, etc)



Clustering samples and genes (Ex.3)



Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling

Alizadeh AA, et al. "Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling". *Nature*, 2000 Feb 3;403(6769):503-11

Steps in a Cluster Analysis

1. Preprocess the data.
2. Choose a dissimilarity measure.
3. Choose a cluster algorithm.
4. Select the number of clusters.
5. Validate the procedure.

Preprocessing

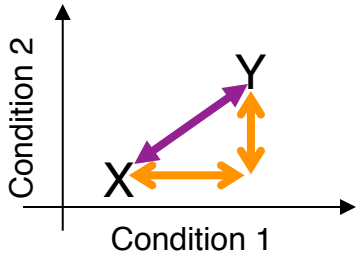
When clustering genes, it is common to pre-process:

- normalise
- filter: remove genes with low variability across samples and many missing values
- impute missing values
- standardise: e.g. zero-mean, unit variance:

$$y_g^* \leftarrow (y_g - \mu_g)/\sigma_g$$

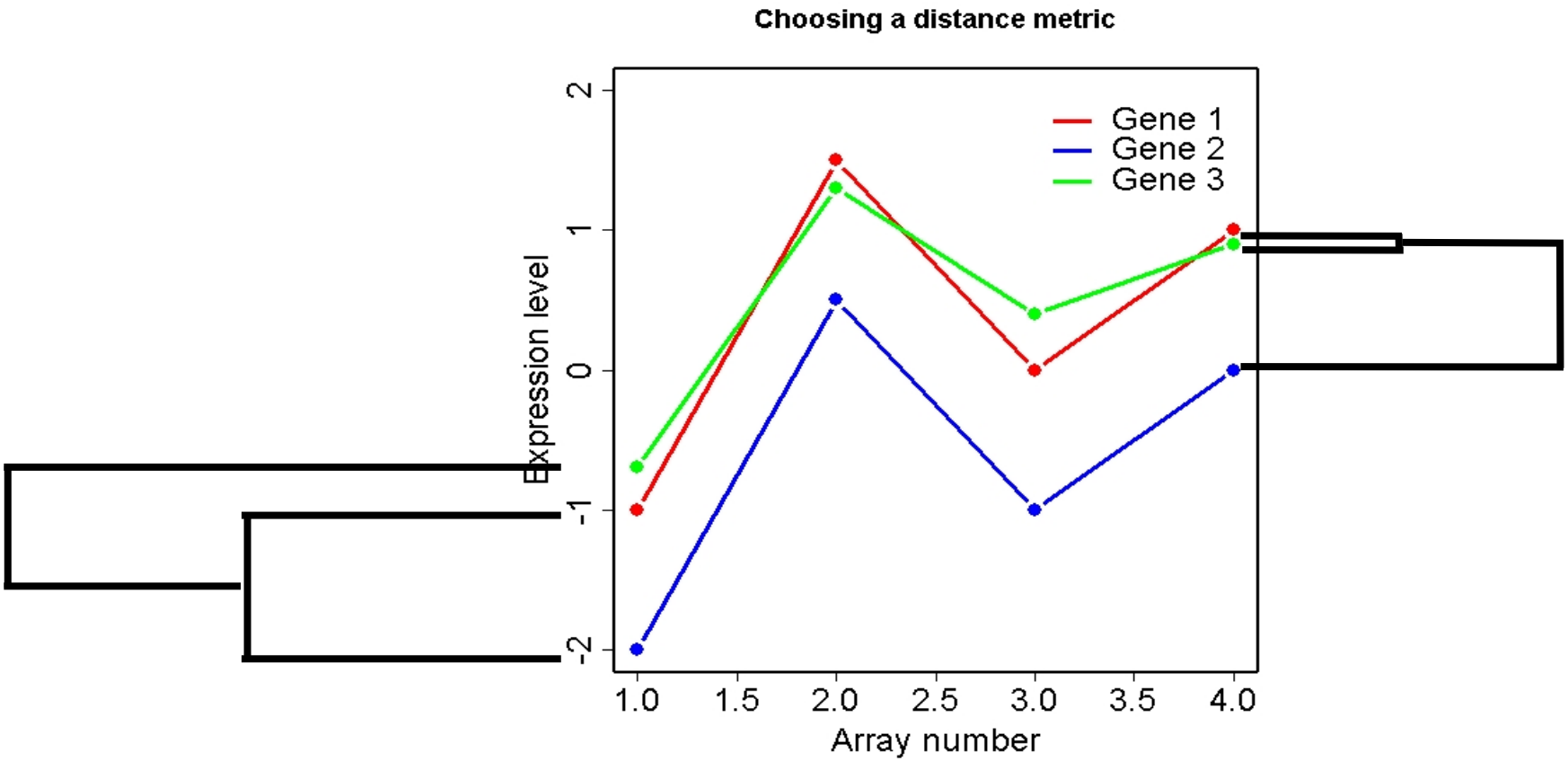
Similarity/Dissimilarity Measures

- Correlation coefficient: *scale invariant*
  - Pearson's correlation: $$s(x_1, x_2) = \frac{\sum_{k=1}^{K} (x_{1k} - \bar{x}_1)(x_{2k} - \bar{x}_2)}{\sqrt{\sum_{k=1}^{K} (x_{1k} - \bar{x}_1)^2 \sum_{k=1}^{K} (x_{2k} - \bar{x}_2)^2}}$$
  - Spearman ρ: Pearson's correlation of ranks
  - Kendall's τ: probability of order concordance
- Distance: *scale dependent*
  - Euclidean distance $$d(X, Y) = \sqrt{\sum_i (x_i - y_i)^2}$$
  - City block (Manhattan) distance $$d(X, Y) = \sum_i |x_i - y_i|$$



- Many others

Correlation (Pearson) vs Distance (Euclidean)



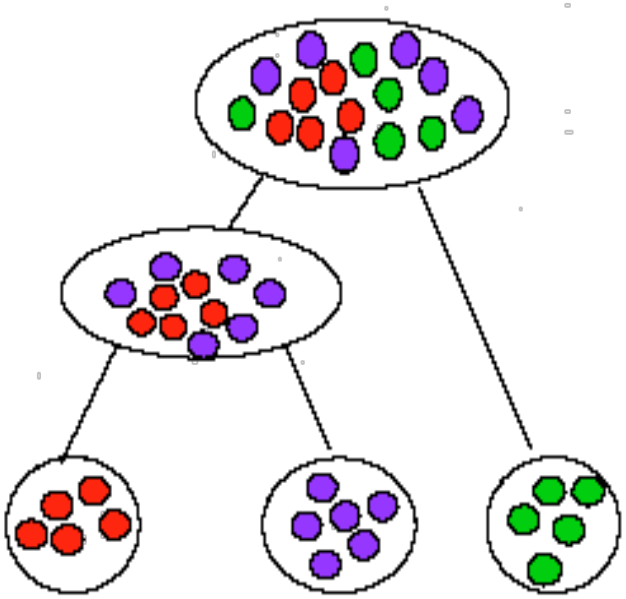
Clustering Algorithms

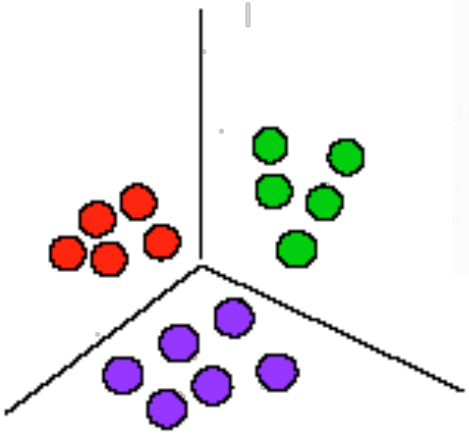
Heuristic

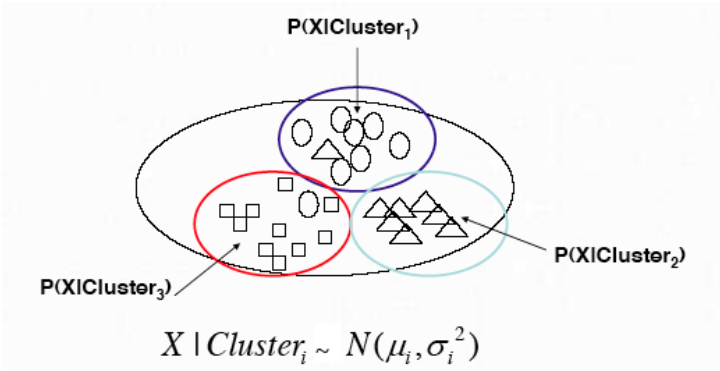
Model-based

Hierarchical

Partitioning







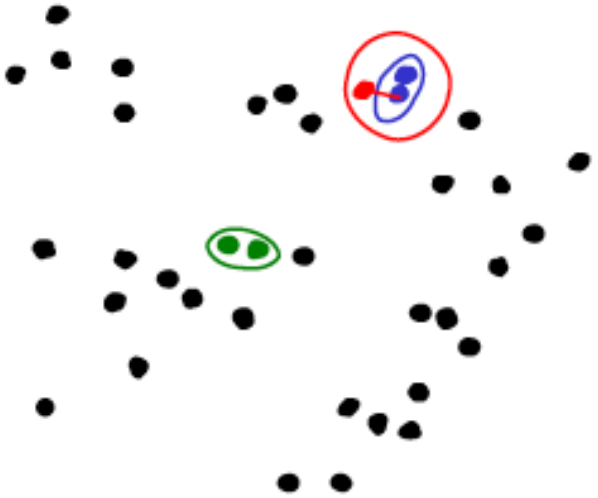
Gaussian mixture

K-means
Self-Organising Maps (SOM)
Partition Around Medoids (PAM)

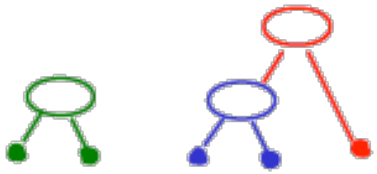
Agglomerative (hierarchical) methods

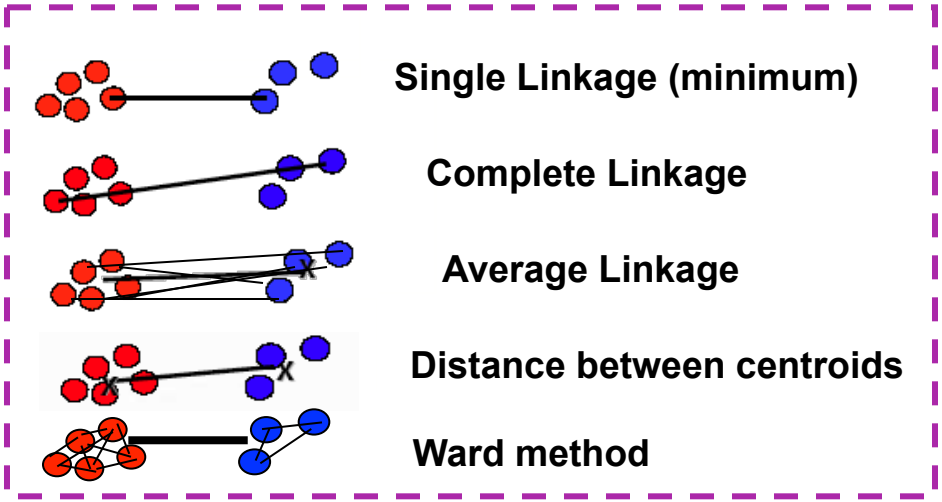
- Start with n mRNA sample (or p gene) clusters
- At each step, merge the two closest clusters using a measure of between-cluster dissimilarity which reflects the shape of the clusters
- The distance between clusters is defined by the method used (e.g., if complete linkage, the distance is defined as the distance between furthest pair of points in the two clusters)

Hierarchical Clustering



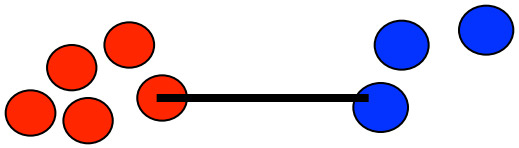
1. Say "Every point is it's own cluster"
2. Find "most similar" pair of clusters
3. Merge it into a parent cluster
4. Repeat



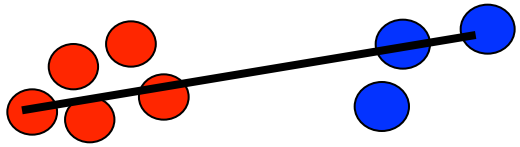


Adapted from A Moore, CMU

Between-cluster dissimilarity measures (Distance between clusters)

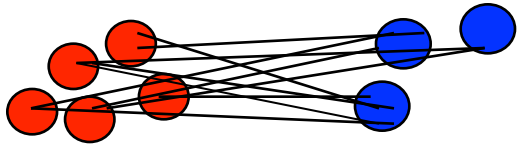


Single
(min. of pairwise distances)
Elongated clusters;
Sensitive to outliers

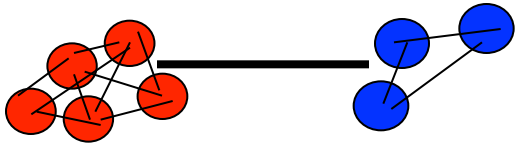


Complete
(max. of pairwise distances)
Compact clusters;
Sensitive to outliers

Distance between centroids

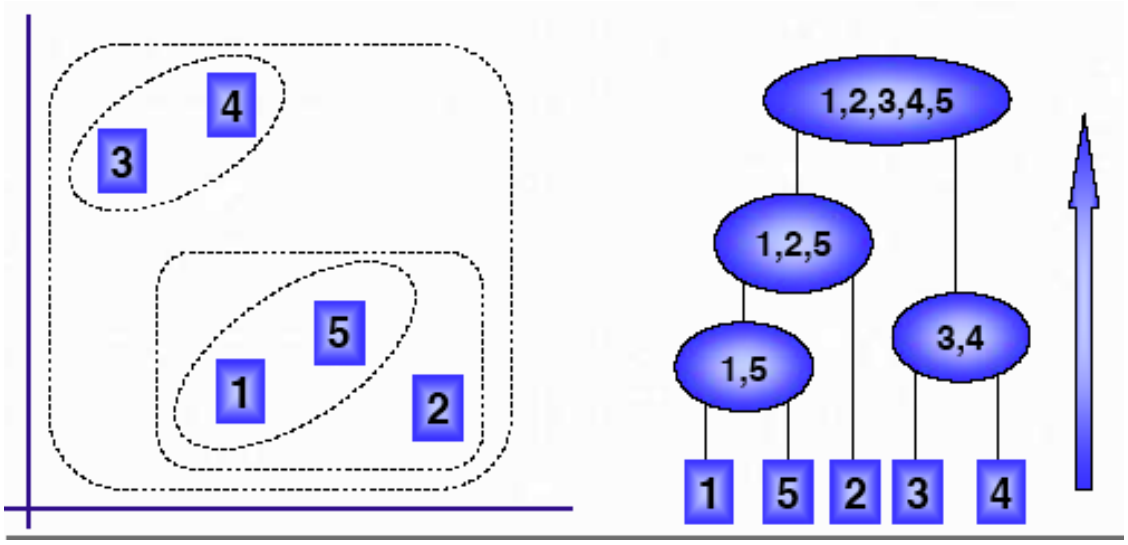


Average linkage
(mean of all pairwise distances)



Ward method
(Between/Within distances)

Hierarchical Clustering

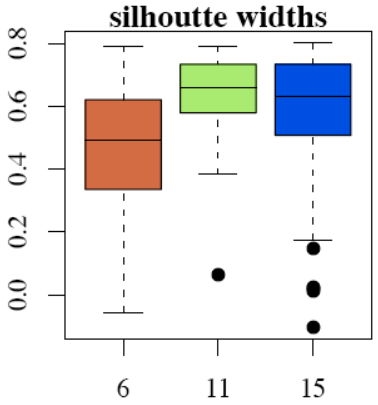


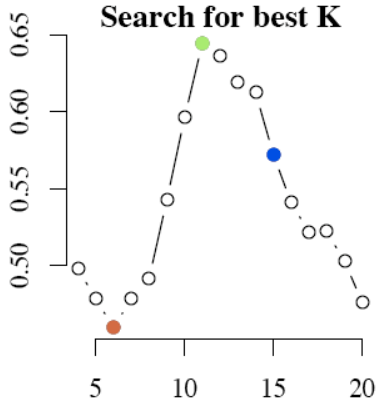
Dendrograms are good visual guides but arbitrary!
Nodes can be reordered. Closer on dendogram ≠ more similar.

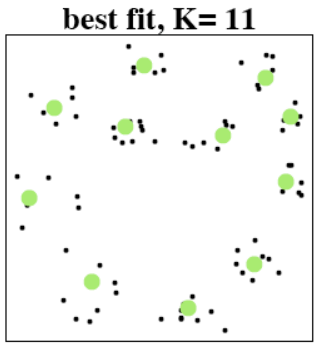
Optimal number of clusters

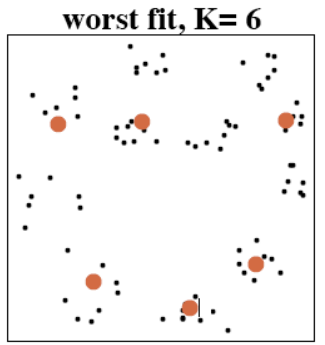
- Silhouette width (from PAM). Given K, for each $\mathbf{x}_i$ calculate:
  1. Within-cluster dissimilarity:
     $a_i = \langle dist(\mathbf{x}_i, \mathbf{x}_j) \rangle, \qquad j \in$ all vectors in same cluster as i.
  2. For all other clusters C_k not containing $\mathbf{x}_i$, compute:
     $d(i, C_k) = \langle dist(\mathbf{x}_i, \mathbf{x}_j) \rangle, j \in$ all vectors in cluster C_k.
  3. Between-cluster dissimilarity:
     $b_i = \min\ d(i, C_k)$
  4. Silhouette width: $sil_i = \frac{b_i - a_i}{max(a_i, b_i)}$
- $\overline{sil} = \langle sil_i \rangle_i$
- Choose K to maximise $\overline{sil}$

(Slide from Stephen Eglen)









Cluster validation

Biological

- enrichment of functional categories within clusters

Statistical

- try different algorithms
- sensitivity analysis
- IGP: In Group Proportion measure (Kapp and Tibshirani).

Clustering software in R/Bioconductor

Package	Function	What
stats	hclust	hierarchical clustering
	heatmap	color image with dendrogram
	kmeans	k-means
	dist	distance
mclust		model-based clustering
class	SOM	self-organizing maps
cluster	pam	partition around medoids
	clara, fanny, diana, agnes, mona,	hierarchical clust. (divisive, agglomerative)
	silhouette	silhouette (choose number of clusters)
	daisy	distance
hopach		hierarchical ordered partitioning and collasping hybrid
pvclust		hierarchical with cluster reliability assessment
e1071	bclust, cmeans	
bioDist		additional distance functions
clusterRepro	clusterRepro	IGP measure for cluster validation

Clustering - Summary

- Useful as **exploratory/visualisation** tools
- Usually outside the normal framework of statistical inference
- Choice of metric, methods and parameters usually guided by prior knowledge about the question …

  The result is guided by what you are looking for!

- Be aware …
  - Clustering cannot NOT work. Always produce some clusters!

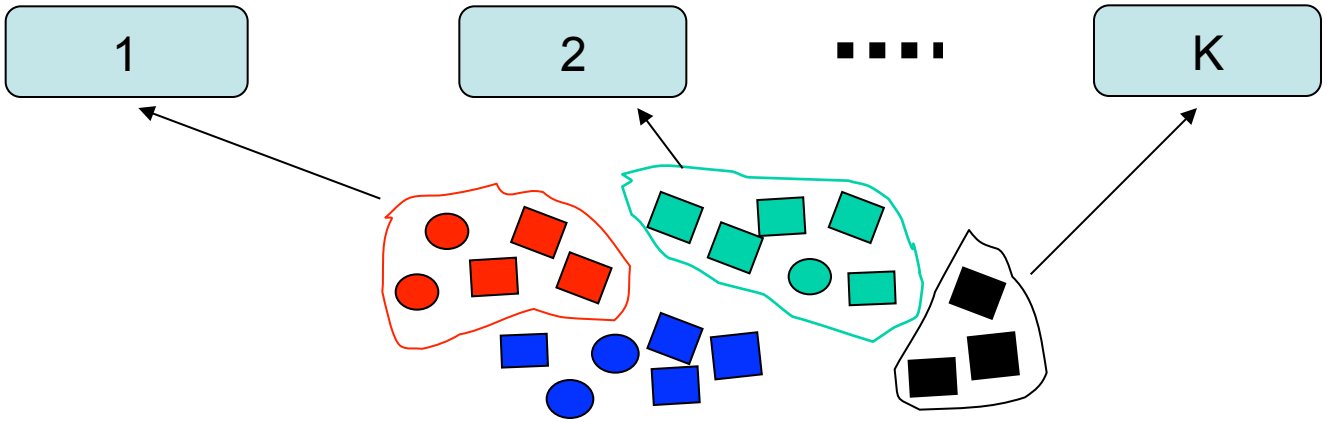
Classification

Discrimination - basic principles

• Each object associated with a class label (or **response**) **Y** ∈ {1, 2, …, K} and a feature vector (vector of predictor variables) of G measurements:

$\mathbf{X} = (X_1, \ldots, X_G)$

Aim: predict Y from **X**.



Predefined Class {1,2,…K}

Objects

Y = Class Label

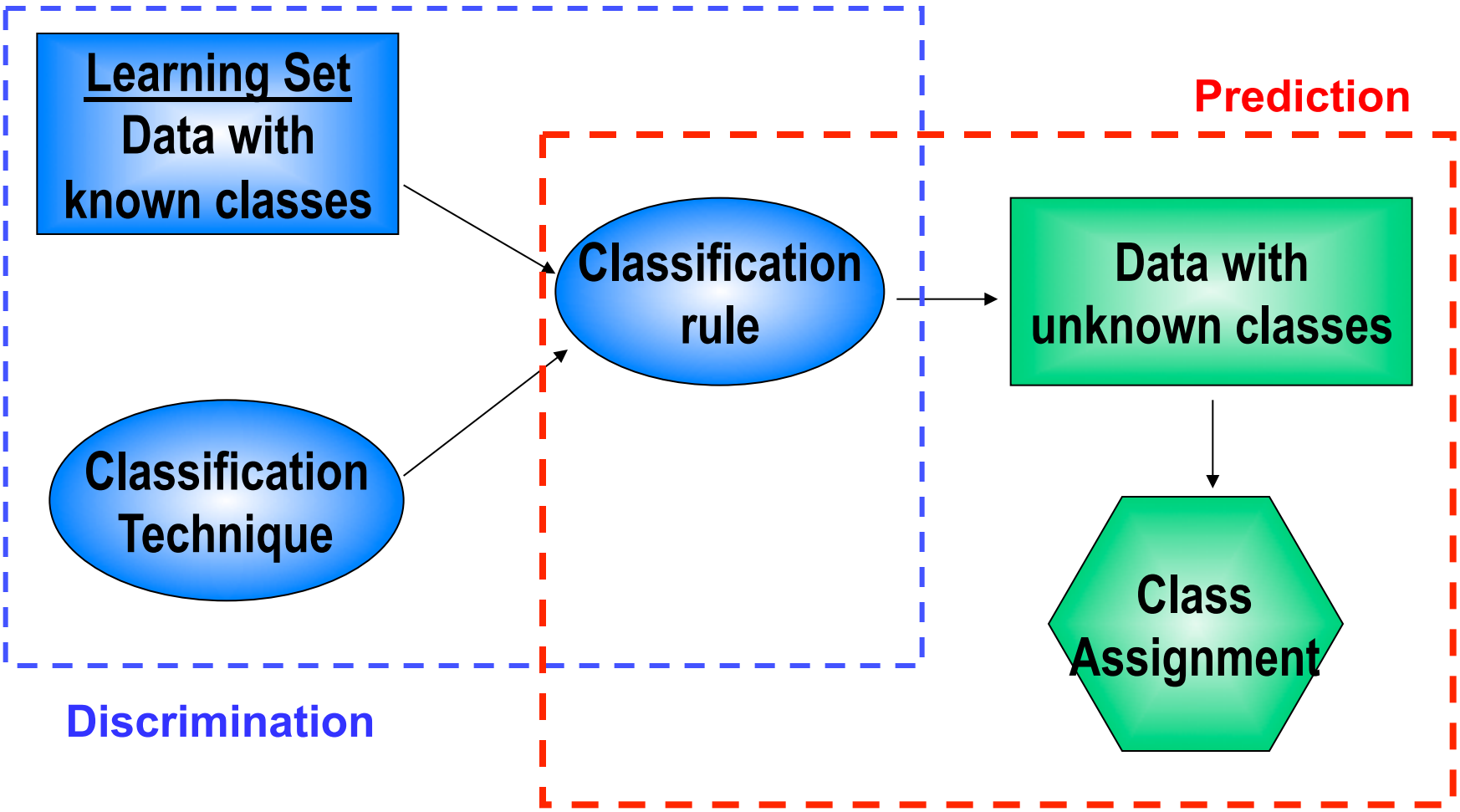
X = Feature vector {colour, shape}

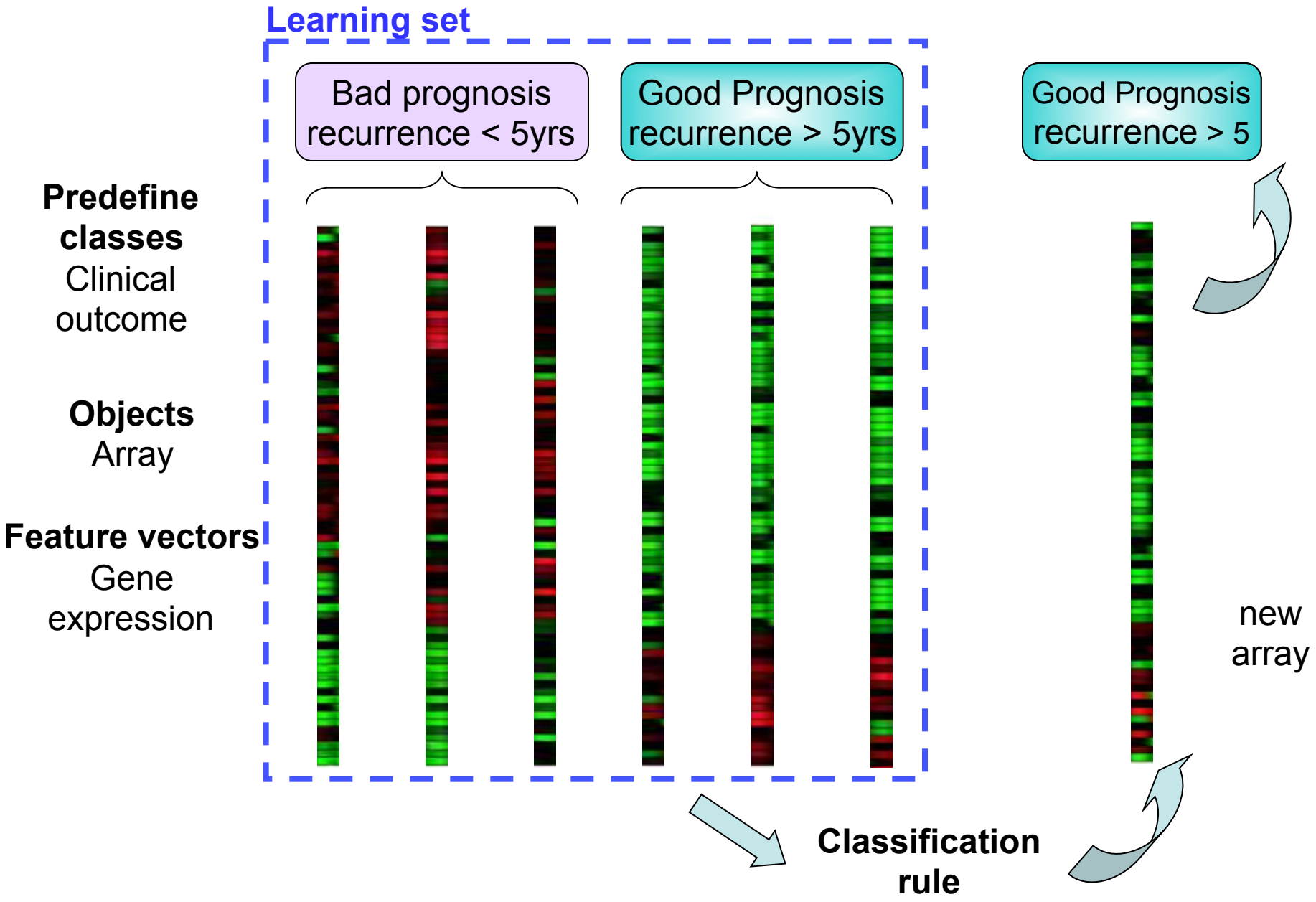
Classification rule ?

X = {red, square}

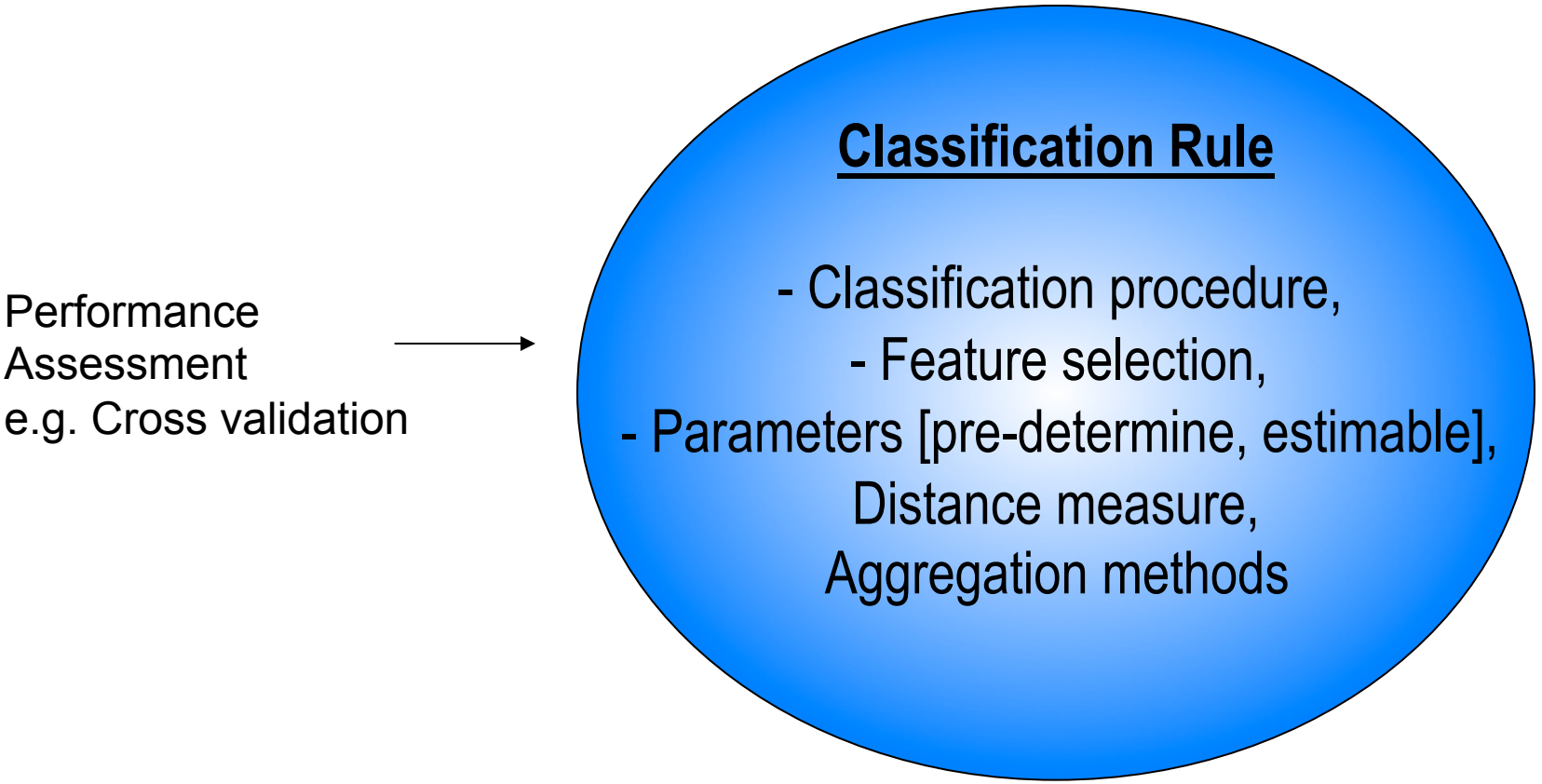
Y = ?

Classification





van 't Veer LJ, et al. "Gene expression profiling predicts clinical outcome of breast cancer". *Nature*, 2002 Jan 31;415(6871):530-6.



- One can think of the classification rule as a black box, some methods provide more insight into the box.
- Performance assessment needs to be looked at for all classification rules.

Why feature selection?

- Removing variables that are noise with respect to the outcome leads to better classification performance
- May provide useful insights into etiology of a disease
- Can eventually lead to a diagnostic test (e.g., "breast cancer chip")

Common methods

Many classifiers available including:

- Linear Discriminant Analysis (LDA).
- Logistic regression.
- Single/multi-layer neural networks.
- Nearest-neighbour methods (k-NN).
- Classification and regression trees (CART).
- Prediction Analysis for microarrays (PAM)
- Many others: Support Vector Machines (SVM), Bayesian networks, logic regression…

Discriminant Analysis

- Assumption: data follows a **multivariate normal distribution**:

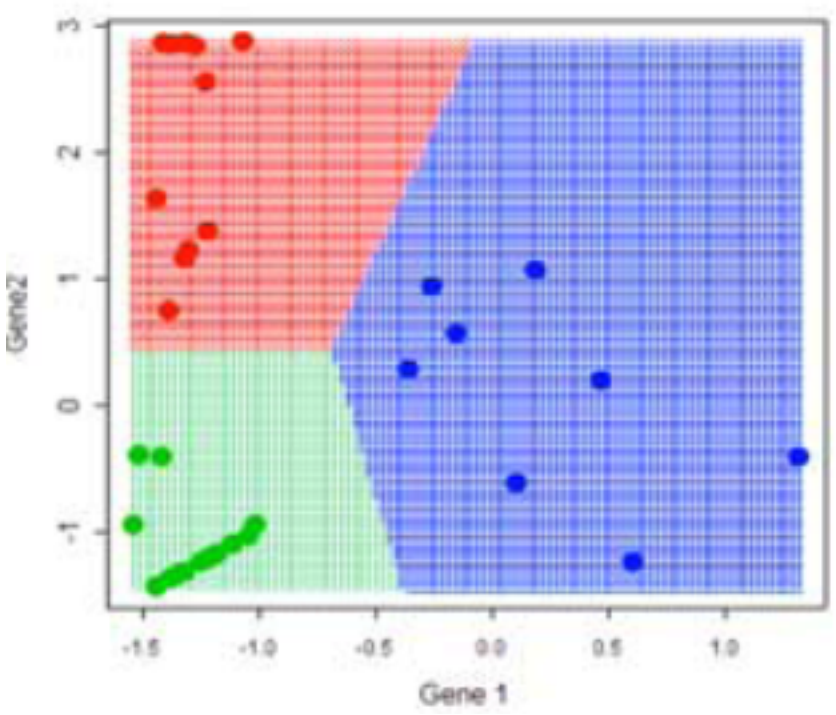
$$(X \mid Y = k) \sim N(\mu_k, \Sigma_k)$$

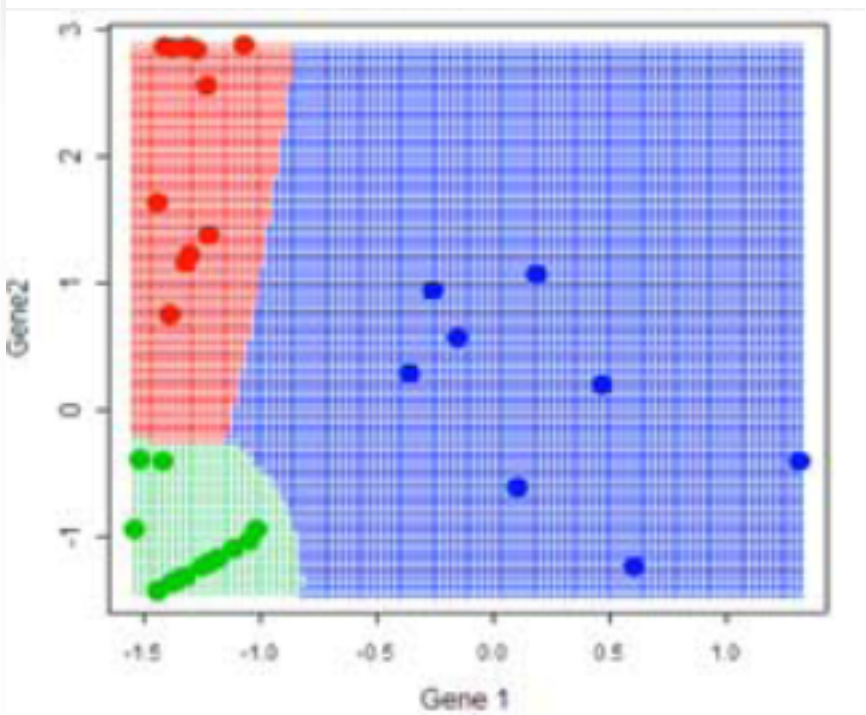
- Classification rule:

$$C(X) = \arg\min_k \left\{ (X - \mu_k)\Sigma_k^{-1}(X - \mu_k)^T + \log|\Sigma_k| \right\}$$

In general, this is a quadratic rule

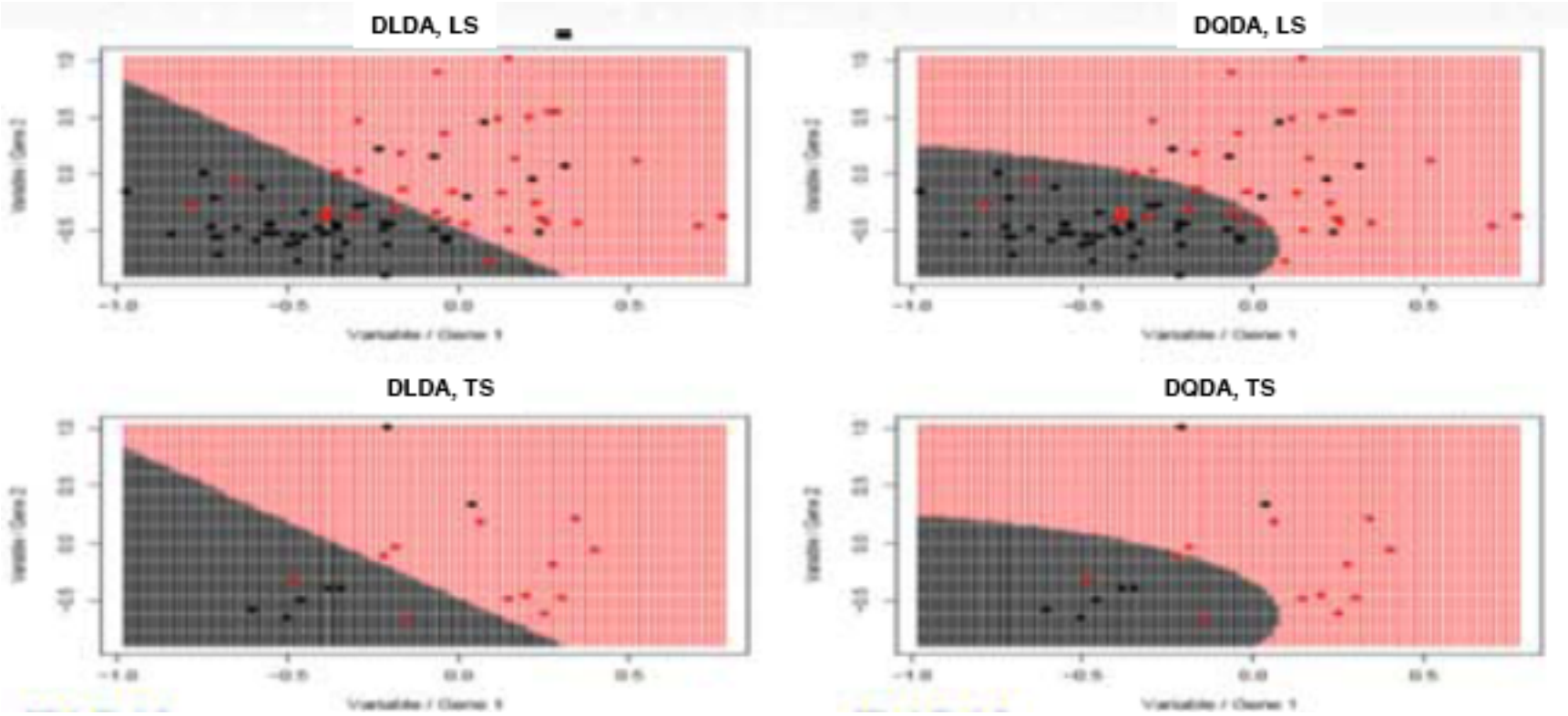
Discriminant analysis: example(I)





Linear discriminant analysis (LDA)	Quadratic discriminant analysis (QDA)
Same covariance matrix for all groups	Different covariance matrix for all groups

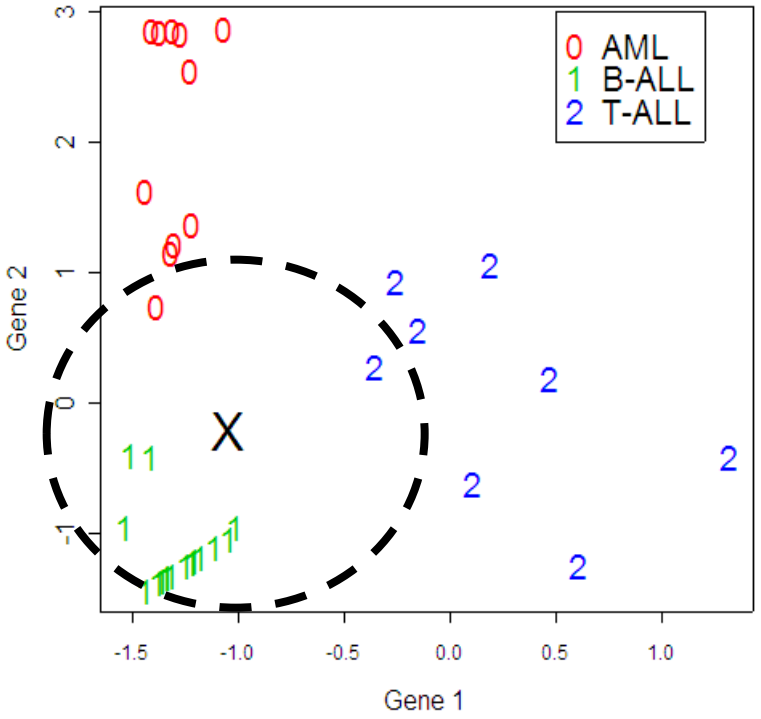
Discriminant analysis: example(II)



Diagonal Linear discriminant analysis (DLDA)	Diagonal Quadratic discriminant analysis (QLDA)
Same (diagonal) covariance matrix for all groups	Different (diagonal) covariance matrix for all groups

k nearests neighbours

- Based on a measure of distance between observations (e.g. Euclidean distance or one minus correlation).
- k-nearest neighbor rule classifies an observation **X** as follows:
  - find the k observations in the learning set **closest** to **X**
  - predict the class of **X** by **majority vote**, i.e. choose the class that is most common among those k observations.
- The number of neighbors k can be chosen by **cross-validation**.

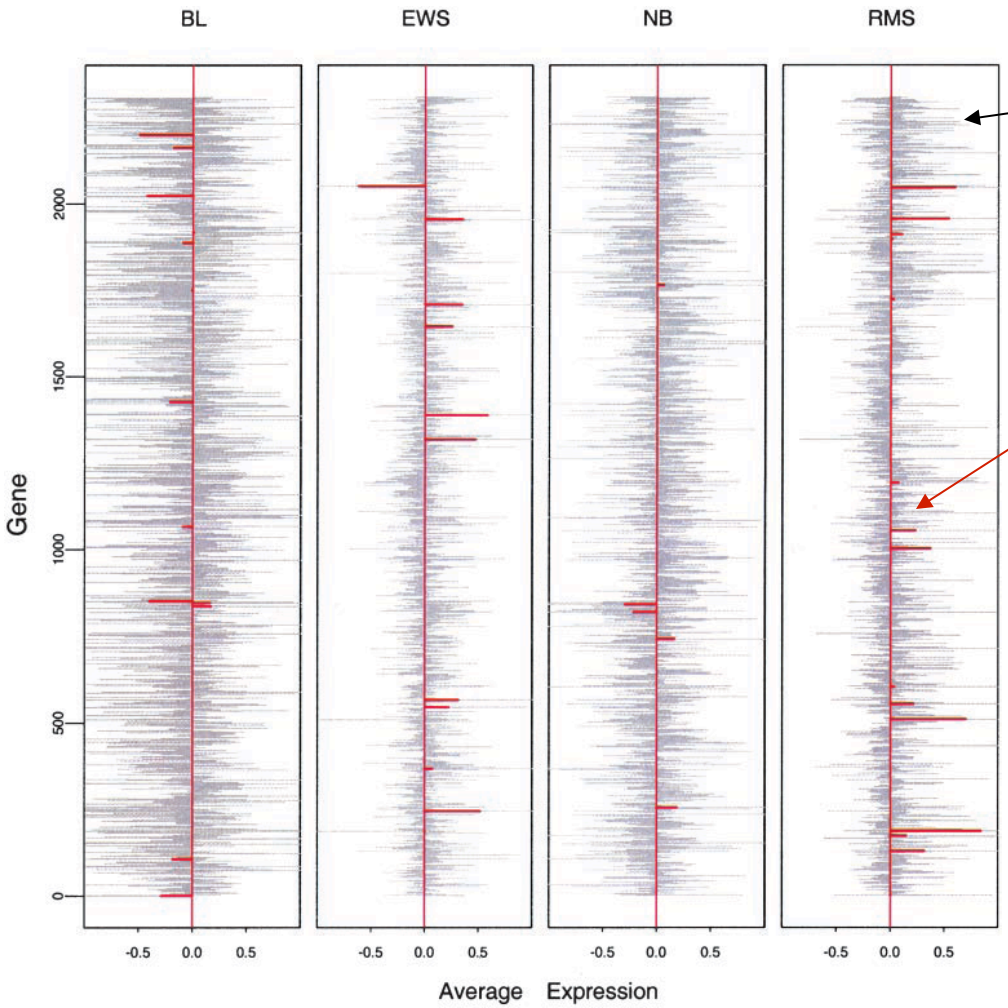


Classification trees

- A tree is a partition of the feature space.
- We can compare trees with their misclassification rate.
- Interactions between variables and monotonic transformations are handed automatically.
- Trees are "pruned" to avoid overfitting.
- A set of trees can be assembled into **random forests**.

PAM

- Tibshirani et al., 2002.
- Uses **nearest shrunken centroids**.



Standardized centroid (average expression of the gene divided by the intra-class s.d.)

Shrunken centroid: each centroid is shrunken towards de overall centroid for all classes.

Each sample is assigned to the class whose centroid is closest to it.

It is easy to interpret and incorporates automatic feature selection.

Common problems

- over-fitting

  (with enough genes, you can perfectly classify random data)
- bias

  (observational/confounding: sample handling, background differences between classes - sex, age)
- results can be sensitive to tuning parameters, standardization methods, feature selection
- interpretability of classifier (no black box)

  (how to make sense, biologically)

Important to assess performance of classifier using independent data set

Performance Assessment

- Any **classification rule** needs to be evaluated for its performance on the future samples. It is almost never the case in microarray studies that a large independent population-based collection of samples is available at the time of initial classifier-building phase.

- One needs to estimate future performance based on what is available: often the same set that is used to build the classifier.

- Assessing performance of the classifier based on
  - Cross-validation
  - Test set
  - Independent testing on future dataset

Estimation of error rates (I)

- **Apparent error rate:** misclassification error on the samples of the dataset used to build the classification rule. It is downward biased.
- **Estimation based on a test sample:** the sample is divided (randomly) in two subsets: training sample, to build the classifier, and test sample, to estimate the error rate in classifying those samples. We lose sample size.
- **K-fold cross-validation:** the sample is divided in K groups of roughly the same size. Sequentially, the rule is obtained leaving one set out and the error is estimated on this subset. The error rate is averaged on the K estimates.

Estimation of error rates (II)

- **Leave-one-out cross-validation:** special case with K=n. Cross-validation methods are computationally intensive.
- **Bootstrap:** A bootstrap sample (sample with replacement from the original dataset) is generated as the training sample. The test sample is formed by the observations not selected for the bootstrap sample. This procedure is repeated P times and the error is averaged.
- **0.632 Estimator:** Efron, 1986.

$$\hat{e} = 0.632\varepsilon_B + 0.368\varepsilon_{App}$$

Selection bias

- Filter approach: select the genes that are relevant for the prediction (F-ratio, Wilcoxon test, …) and use these genes to build the classifier. Then, estimate the error with cross-validation.
- This approach leads to a **downward bias**: the genes were selected using all samples, including the ones used to test the rule.
- Solution: use **cross-validation on the whole process** (gene selection and prediction).
- We can even add another layer in the cross-validation: selecting the number of genes that leads to lower error rate (finding the best subset among subsets).
- Web application **tnasas**: http://tnasas.bioinfo.cnio.es

Classification - Summary

- Many methods available.
- No Free Lunch Theorem: No classifier is superior to the others in all scenarios.
- Some methods are black boxes.
- It is crucial to obtain unbiased estimations of the error rate.

Classification software in R/ Bioconductor

Package	Function	What
MASS	lda	linear discriminant analysis
	qda	quadratic discriminant analysis
class	knn	K-nearest neighbour
e1071	svm	support vector machines
rpart		classification & regression trees
tree		
nnet	nnet	neural networks
ipred	slda, cv, bagging	
pamr	pamr.train, pamr.cv	
randomForest		
MLInterfaces	function names as above, add 'B' to end	
	xval	cross validation

Survival Analysis

Survival Analysis

- Analysis of **failure times** (events).
- The response variable is **time until the event**.
- Examples of events: death, metastasis, relapse…
- In **microarray studies**, we are usually interested in finding **signatures** (sets) of genes that are related to **prognosis.**

Censoring

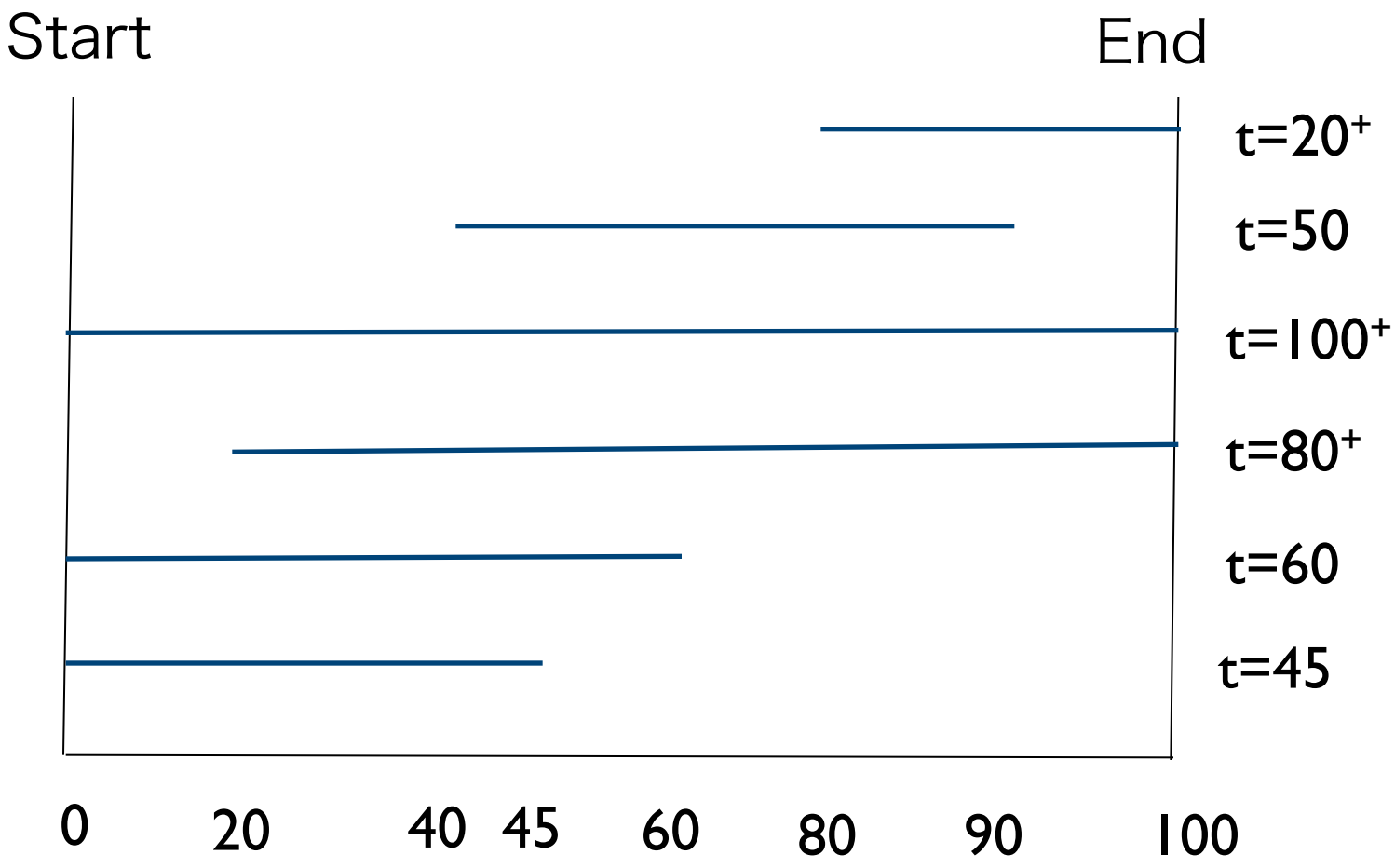
- Times are usually **censored**: we are not able to observe the failure times for all individuals.
- **Interval-censoring**: the event has occurred within an interval of time.
- **Left censoring**: the event has occurred before a certain time.
- **Left truncation**: an unknown number of subjects failed before a certain time, but they never got into the study.

Right censoring

- **Right censoring**: the event has not occurred up to a certain time.
  - Type I censoring: the study finishes at a pre-specified time (but the censoring can vary between subjects).
  - Type II censoring: the study finishes after a fixed number of events.

- **Assumption**: censoring is **non informative** about the event (for example, patients are not removed from the study because of a worsening condition).

Type I Censoring



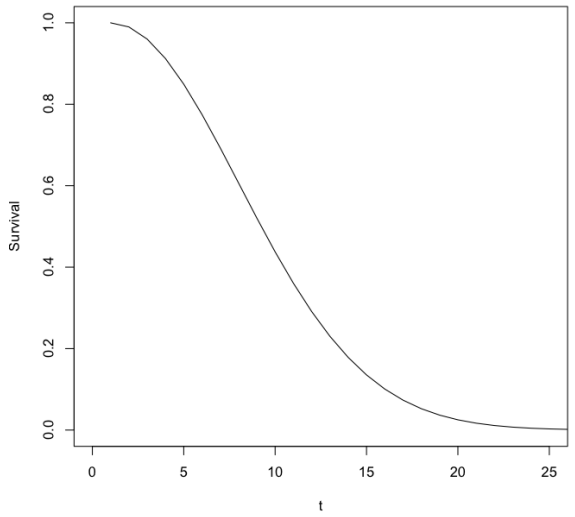
Functions of interest

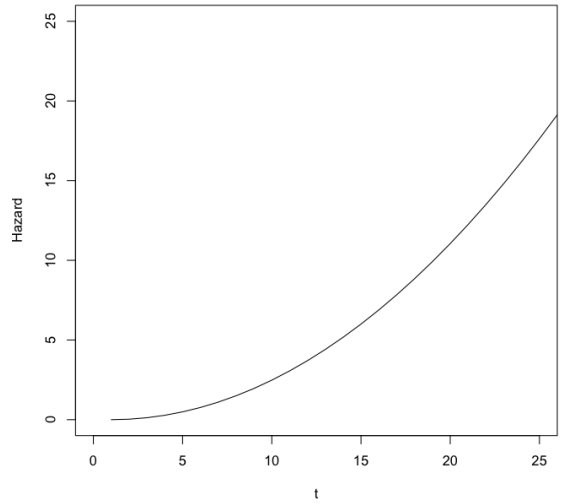
- **Survival function:**

$$S(t) = P(T > t) = 1 - F(t)$$

- **Hazard function:**

$$\lambda(t) = \lim_{u \to 0} \frac{P(t < T \leq t + u \mid T > t)}{u} = \frac{f(t)}{S(t)}$$





Distributions of interest: exponential, Weibull, lognormal…

Kaplan-Meier Estimator

- **Empirical survival function** when censoring is present.

$$S_{KM}(t) = \prod_{i:t_i<t} (1 - d_i / n_i)$$

d_i is the number of failures at t_i

n_i is the number of subjects at risk at t_i

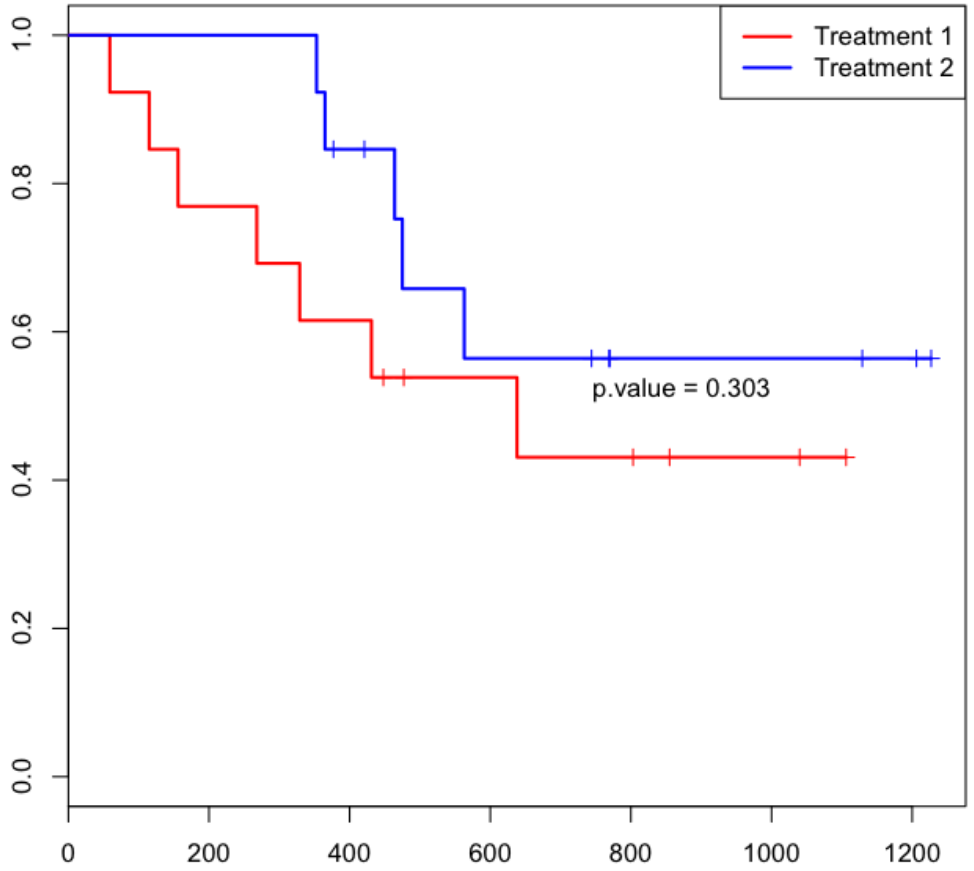
Day	Subjects at risk	Deaths	Censored	Cumulative Survival
12	100	1	0	99/100=0.99
30	99	2	1	97/99 x 0.99=0.97
60	96	0	3	96/96 x 0.97 = 0.97
72	93	3	0	

Table 16.1, Harrell.

Log rank test

- Tests **differences between the survival** functions for two or more groups.

Compares observed and expected events in each group



Cox model

- **Semiparametric proportional hazards model.**

$$\lambda(t \mid X) = \lambda(t)\exp(X\beta)$$

• Uses a partial likelihood to estimate β

• No assumptions about the shape of the underlying hazard, but the relative hazard function must be constant through time. The predictors have the same effect on the hazard function at all values of t.

• The model can be extended to include strata and time-dependent covariates.

R functions and packages

- Package survival:

Surv(time,status)	Define survival (time, censoring)
survfit()	Kaplan-Meier estimator
survdiff()	Log-rank test
coxph()	Cox model

- Package Design *(Harrell)*
- Signs web application (http://signs.bioinfo.cnio.es/))

Principal Components Analysis

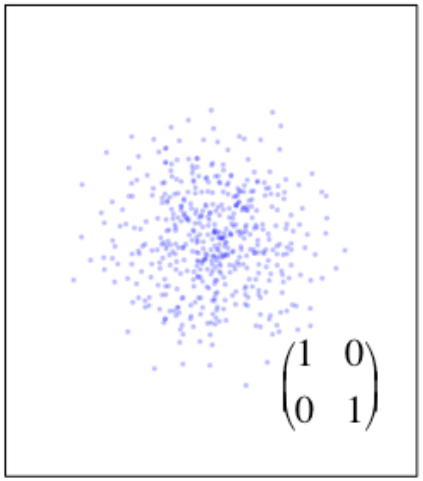
Dimension reduction

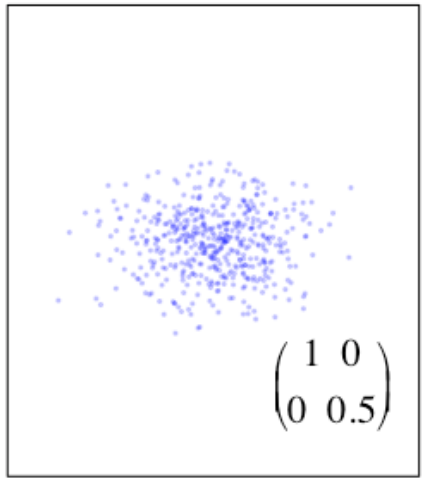
- **Problem**: Many variables, often correlated - how do we remove redundancy to give a smaller, more manageable set of variables

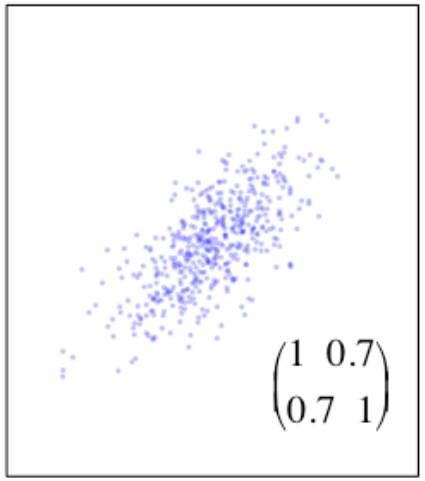
Solution: dimension reduction techniques

- Principal Components Analysis (PCA) - `princomp()` in R
- Singular Value Decomposition (SVD) - `svd()` in R
- Gene shaving

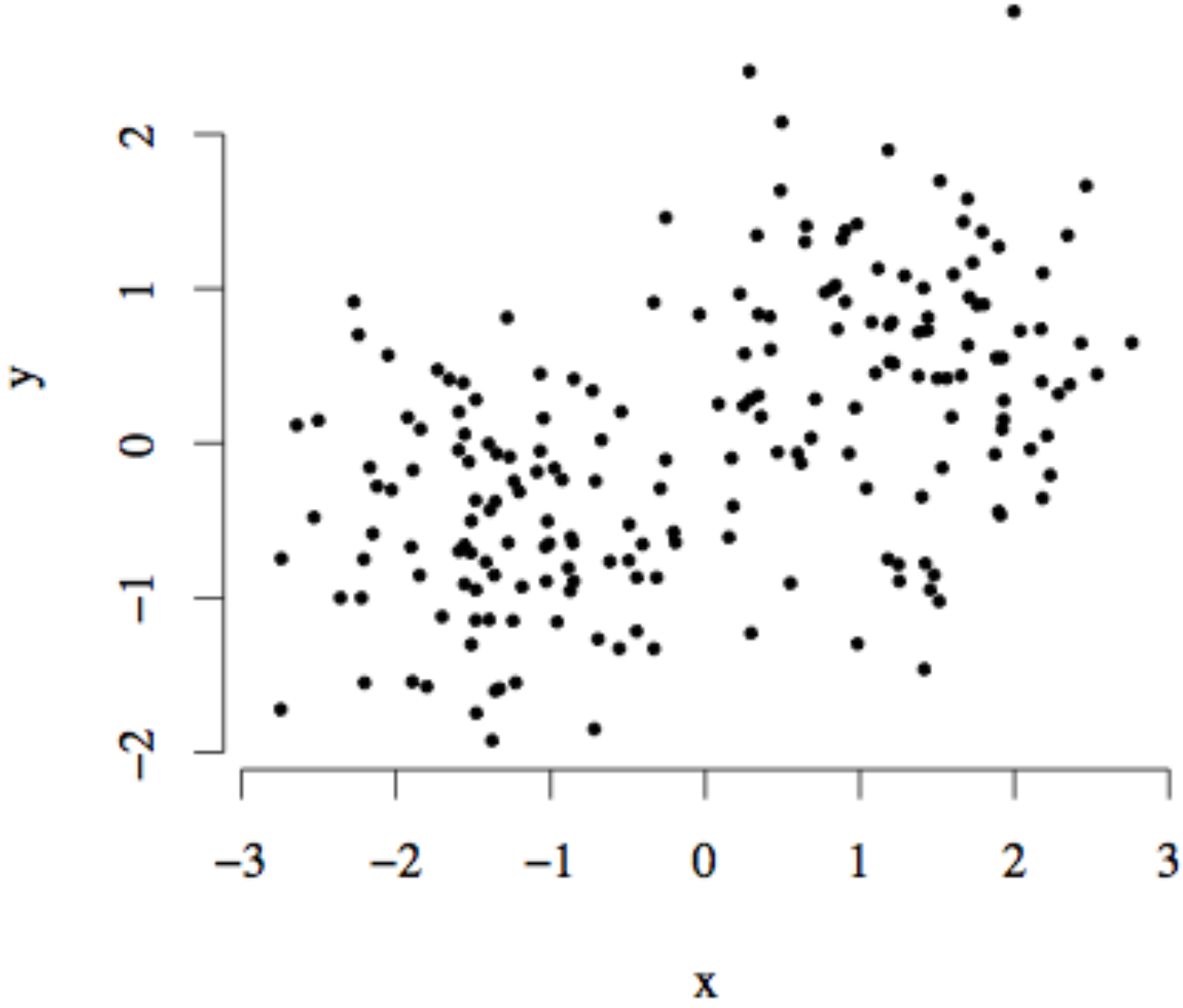
Example: covariance matrices



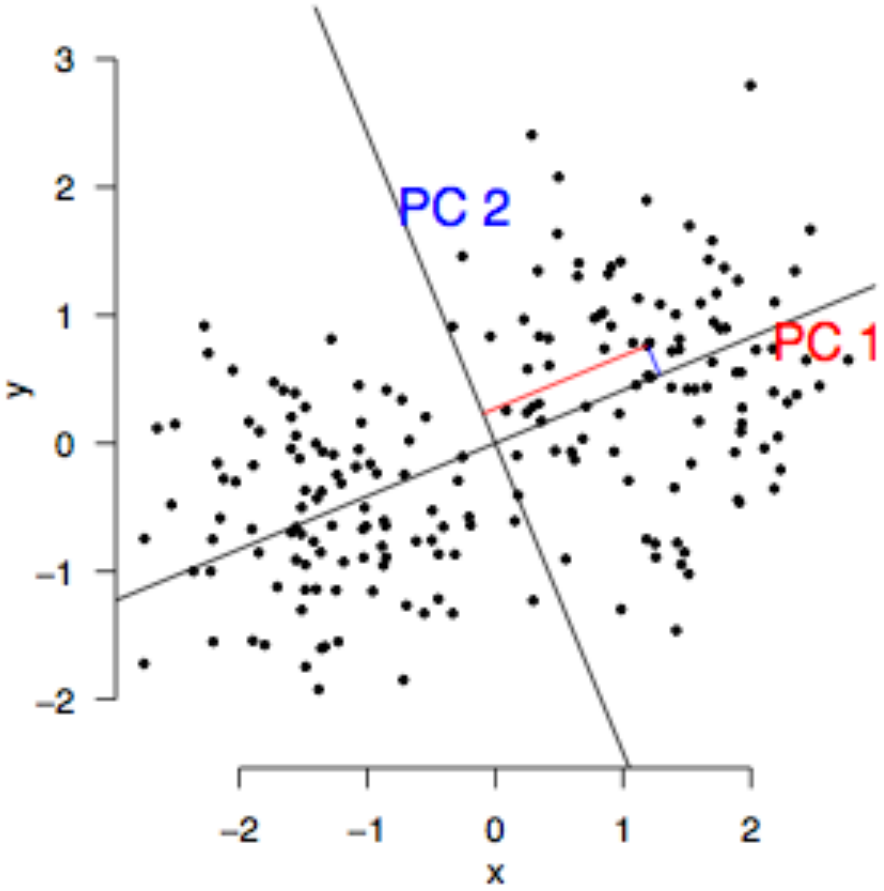


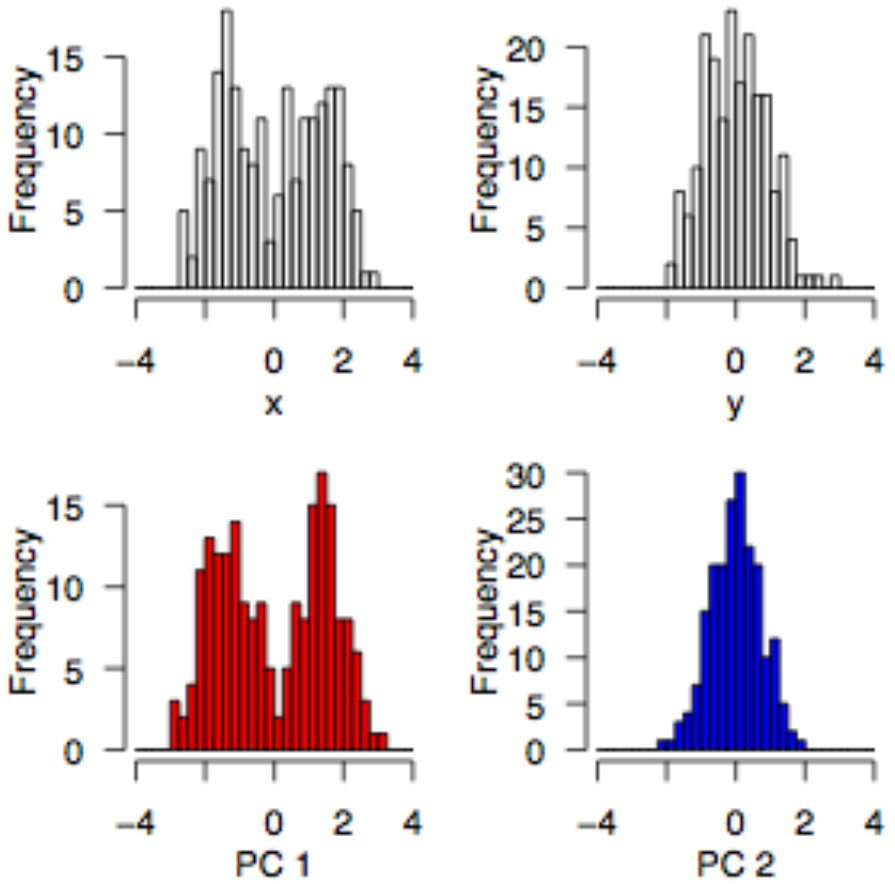


PCA in 2-dimensions



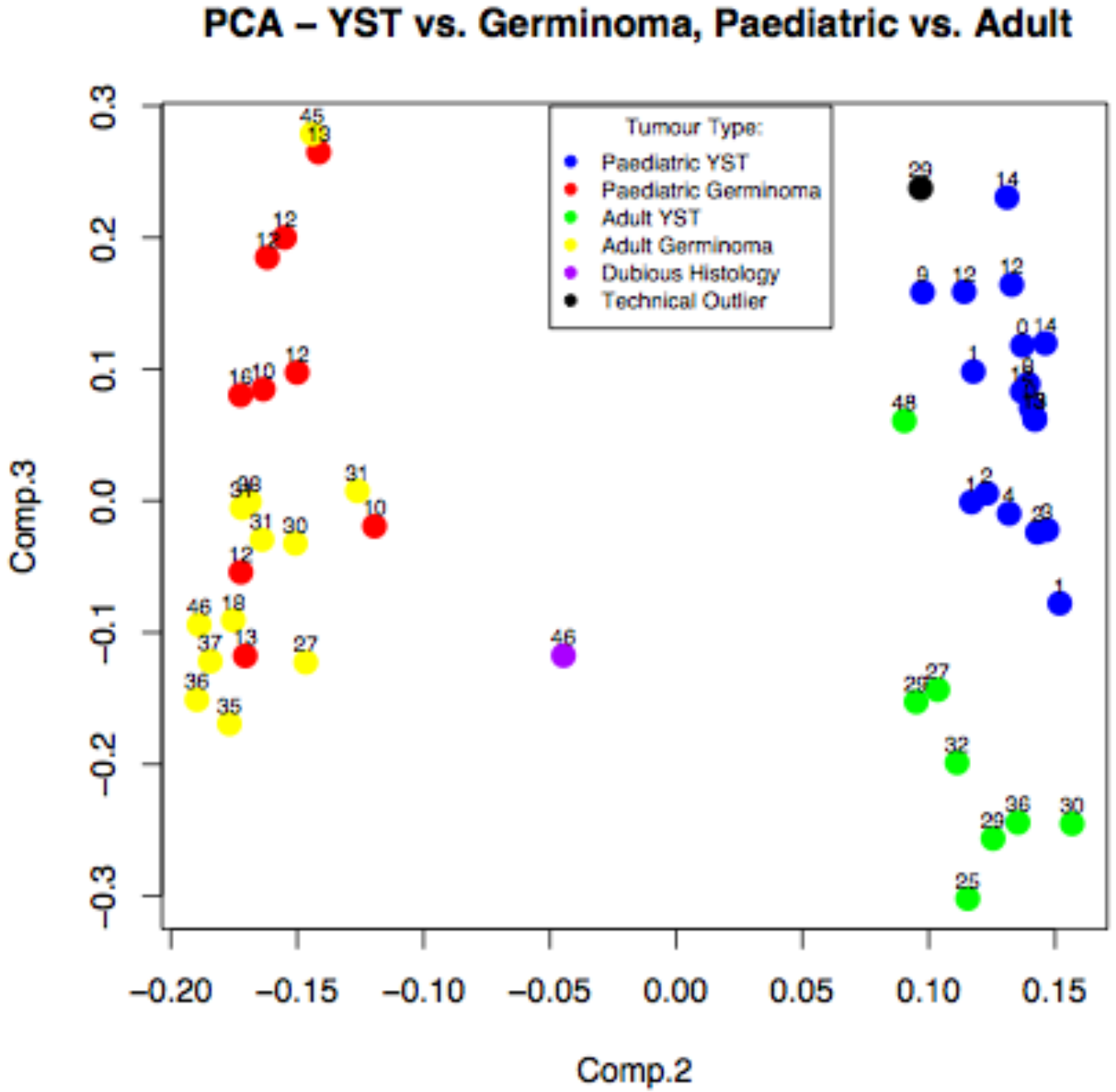
PCA in 2-dimensions





They are linear combinations of the original variables

PCA example


PCA – YST vs. Germinoma, Paediatric vs. Adult
Tumour Type:
Paediatric YST
Paediatric Germinoma
Adult YST
Adult Germinoma
Dubious Histology
Technical Outlier
Comp.3
Comp.2
0.3
0.2
0.1
0.0
−0.1
−0.2
−0.3
−0.20
−0.15
−0.10
−0.05
0.00
0.05
0.10
0.15

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