Bioconductor Tutorial
Part I

www.bioconductor.org

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References

- Bioconductor www.bioconductor.org
  - software, data, and documentation (vignettes);
  - training materials from short courses;
  - mailing list.
- R www.r-project.org, cran.r-project.org
  - software (CRAN);
  - documentation;
  - newsletter: R News;
  - mailing list.
- Personal
  - www.stat.berkeley.edu/~sandrine
  - www.hsph.harvard.edu/facres/gent.html

Outline

Part I

- Overview of the Bioconductor Project.
- Getting started.
- Pre-processing microarray data: Affymetrix and spotted arrays.
- Differential gene expression.
- Distances, prediction, and cluster analysis.

Part II

- Reproducible research.
- Annotation and metadata.
- Visualization.
- GO: more advanced usage.

Overview of the Bioconductor Project

Bioconductor

- Bioconductor is an open source and open development software project for the analysis of biomedical and genomic data.
- The project was started in the Fall of 2001 and includes 23 core developers in the US, Europe, and Australia.
- R and the R package system are used to design and distribute software.
- Releases
  - v 1.0: May 2nd, 2002, 15 packages.
  - v 1.1: November 18th, 2002, 20 packages.
  - v 1.2: May 28th, 2003, 30 packages.
- ArrayAnalyzer: Commercial port of Bioconductor packages in S-Plus.

Goals

- Provide access to powerful statistical and graphical methods for the analysis of genomic data.
- Facilitate the integration of biological metadata (GenBank, GO, LocusLink, PubMed) in the analysis of experimental data.
- Allow the rapid development of extensible, interoperable, and scalable software.
- Promote high-quality documentation and reproducible research.
- Provide training in computational and statistical methods.

Bioconductor packages

- Bioconductor software consists of R add-on packages.
- An R package is a structured collection of code (R, C, or other), documentation, and/or data for performing specific types of analyses.
- E.g. **`affy`**, **`cluster`**, **`graph`**, **`hexbin`** packages provide implementations of specialized statistical and graphical methods.

Bioconductor packages

Bioconductor provides two main classes of software packages.

- End-user packages:
  - aimed at users unfamiliar with R or computer programming;
  - polished and easy-to-use interfaces to a wide variety of computational and statistical methods for the analysis of genomic data.
- Developer packages: aimed at software developers, in the sense that they provide *software to write software*.

Bioconductor packages

- Data packages:
  - Biological metadata: mappings between different gene identifiers (e.g., AffyID, GO, LocusID, PMID), CDF and probe sequence information for Affy arrays.

    E.g. `hgu95av2`, `GO`, `KEGG`.
  - Experimental data: code, data, and documentation for specific experiments or projects.

    `yeastCC`: Spellman et al. (1998) yeast cell cycle.

    `golubEsets`: Golub et al. (2000) ALL/AML data.
- Course packages: code, data, documentation, and labs for the instruction of a particular course. E.g. `EMBO03` course package.

Bioconductor packages

Release 1.2, May 28th, 2003

- General infrastructure:
  Biobase, DynDoc, reposTools, rhdf5, ruuid, tkWidgets, widgetTools.
- Annotation:
  annotate, AnnBuilder → **data packages.**
- Graphics:
  geneplotter, hexbin.
- Pre-processing Affymetrix oligonucleotide chip data:
  affy, affycomp, affydata, makecdfenv, vsn.
- Pre-processing two-color spotted DNA microarray data:
  limma, marrayClasses, marrayInput, marrayNorm, marrayPlots, marrayTools, vsn.
- Differential gene expression:
  edd, genefilter, limma, multtest, ROC.
- Graphs and networks:
  graph, RBGL, Rgraphviz.
- Analysis of SAGE data: SAGElyzer.

N.B. Many new packages in Bioconductor development version.

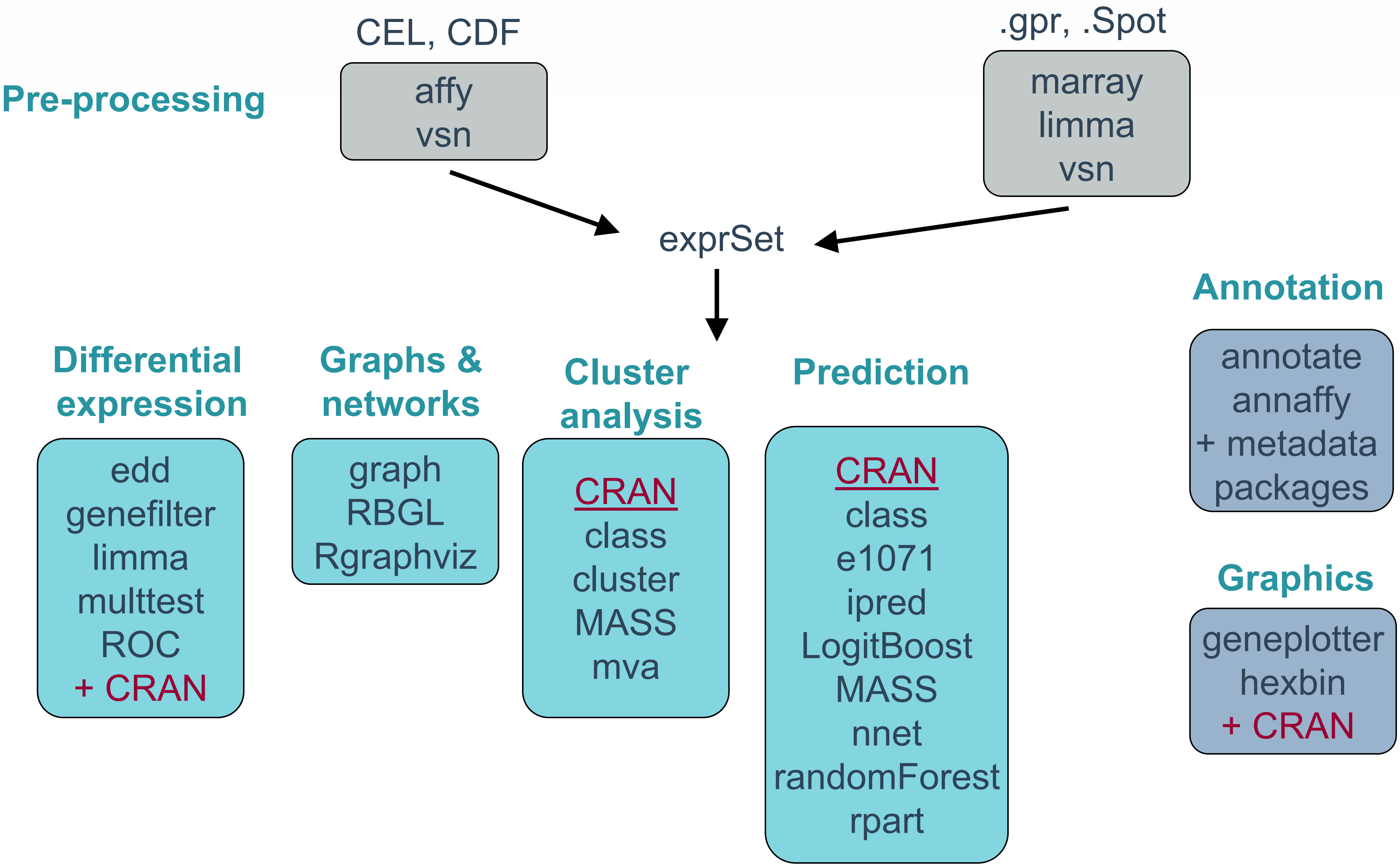
Ongoing efforts

- Variable (feature) selection;
- Prediction;
- Cluster analysis;
- Cross-validation;
- Multiple testing;

Many methods already implemented in CRAN packages.

- Quality measures for microarray data;
- Biological sequence analysis;
- Interactions with MAGE-ML: new **`MAGEML`** package → poster by Durinck, Allemeersch, Moreau, and De Moor;
- etc.

Microarray data analysis



Pre-processing

CEL, CDF

affy
vsn

.gpr, .Spot

marray
limma
vsn

exprSet

Annotation

annotate
annaffy
+ metadata packages

Differential expression

edd
genefilter
limma
multtest
ROC
+ CRAN

Graphs & networks

graph
RBGL
Rgraphviz

Cluster analysis

CRAN
class
cluster
MASS
mva

Prediction

CRAN
class
e1071
ipred
LogitBoost
MASS
nnet
randomForest
rpart

Graphics

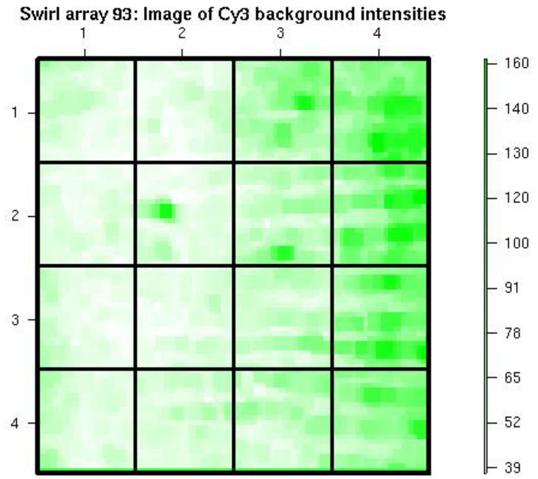
geneplotter
hexbin
+ CRAN

Microarray data analysis

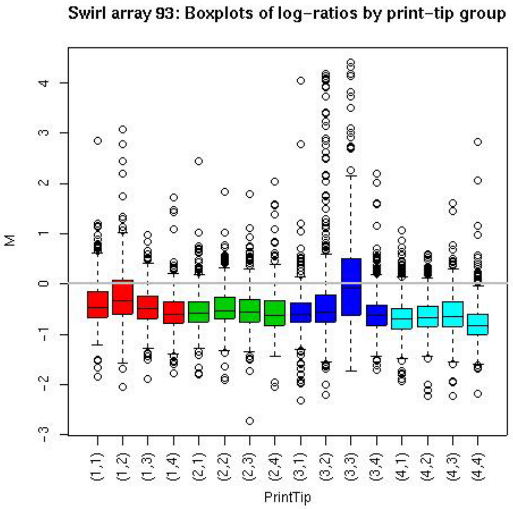
- Pre-processing of
  - spotted array data with `marrayNorm` package;
  - Affymetrix array data with `affy` package.
- List of differentially expressed genes from `genefilter`, `limma`, or `multtest` packages.
- Prediction of tumor class using `randomForest` package.
- Clustering of genes using `cluster` package.
- Use of `annotate` package
  - to retrieve and search PubMed abstracts;
  - to generate an HTML report with links to LocusLink for each gene.

marray packages

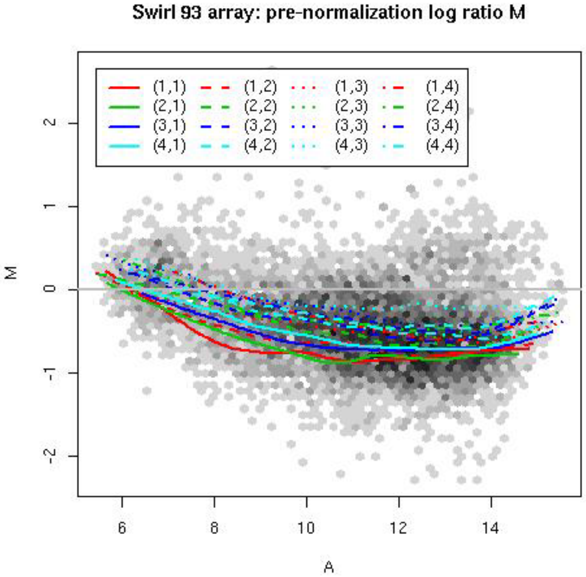
Pre-processing two-color spotted array data:
- diagnostic plots,
- robust adaptive normalization (lowess, loess).


Swirl array 93: Image of Cy3 background intensities

maImage


Swirl array 93: Boxplots of log–ratios by print–tip group

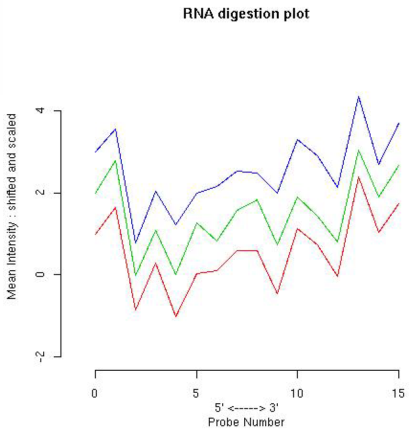
maBoxplot


Swirl 93 array: pre-normalization log ratio M

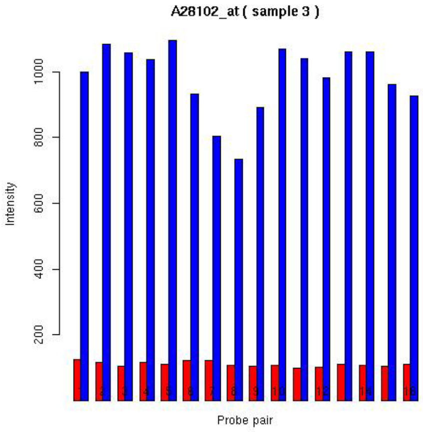
maPlot + hexbin

affy package

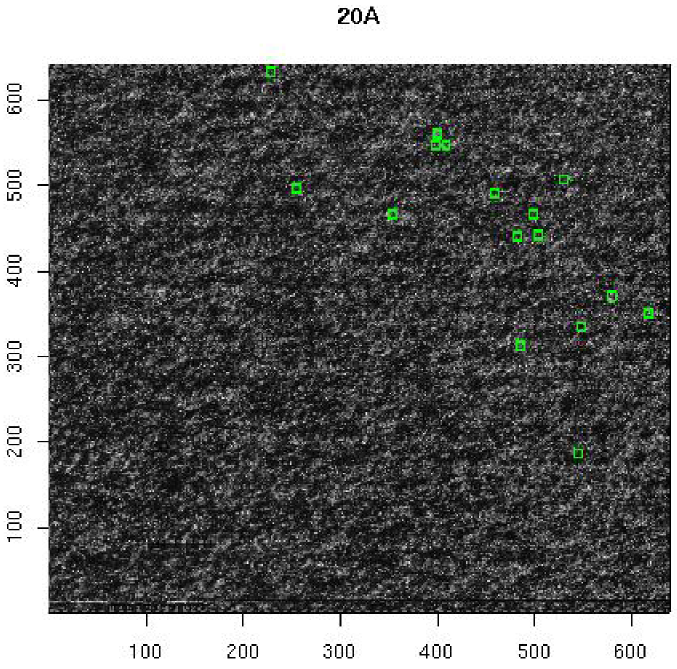
Pre-processing oligonucleotide chip data:
- diagnostic plots,
- background correction,
- probe-level normalization,
- computation of expression measures.



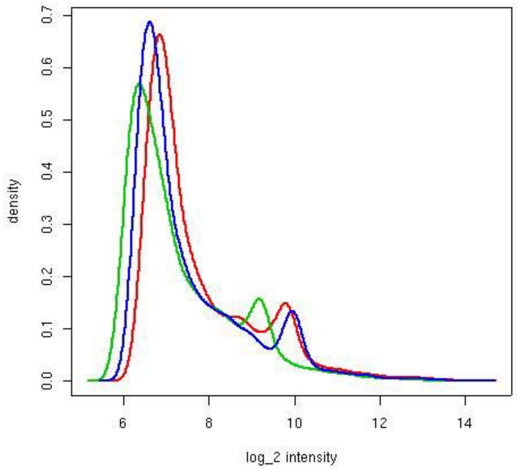
plotAffyRNADeg



barplot.ProbeSet



image

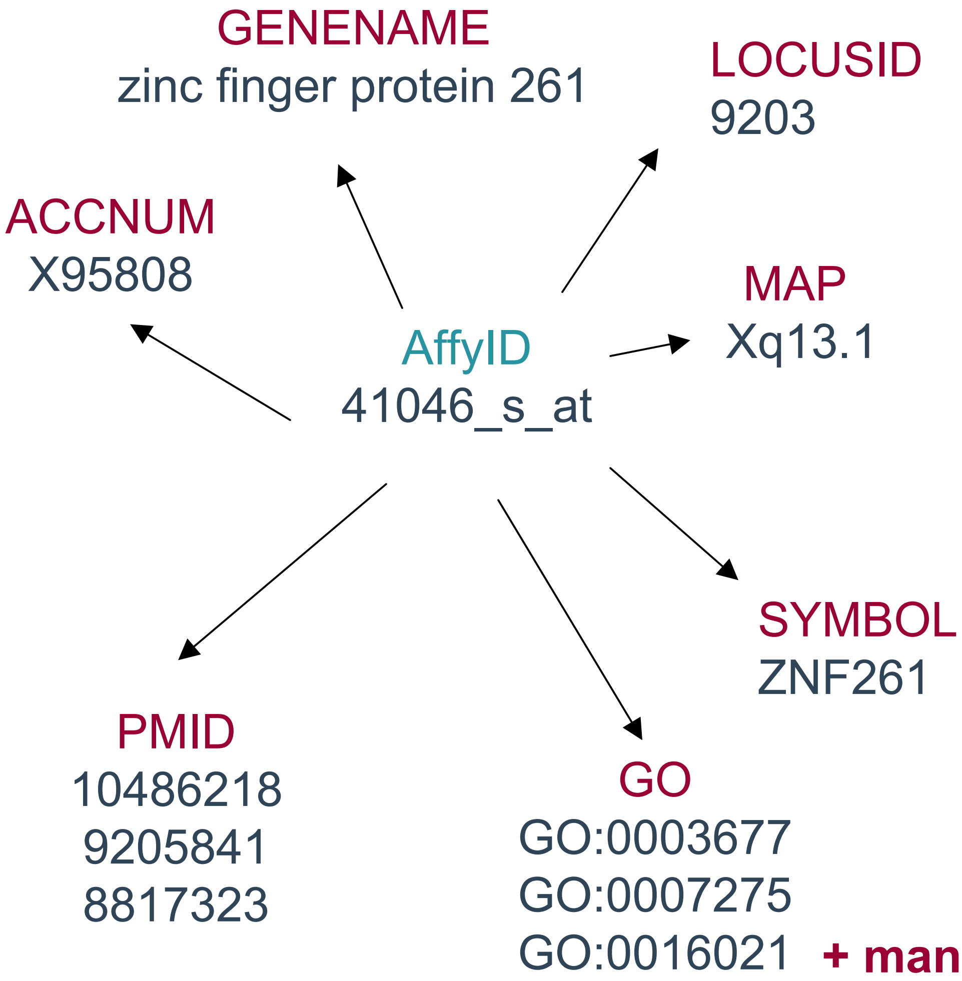


plotDensity

annotate, annafy, and AnnBuilder

Metadata package `hgu95av2`

mappings between different gene identifiers for hgu95av2 chip.



AffyID
41046_s_at

GENENAME
zinc finger protein 261

LOCUSID
9203

ACCNUM
X95808

MAP
Xq13.1

SYMBOL
ZNF261

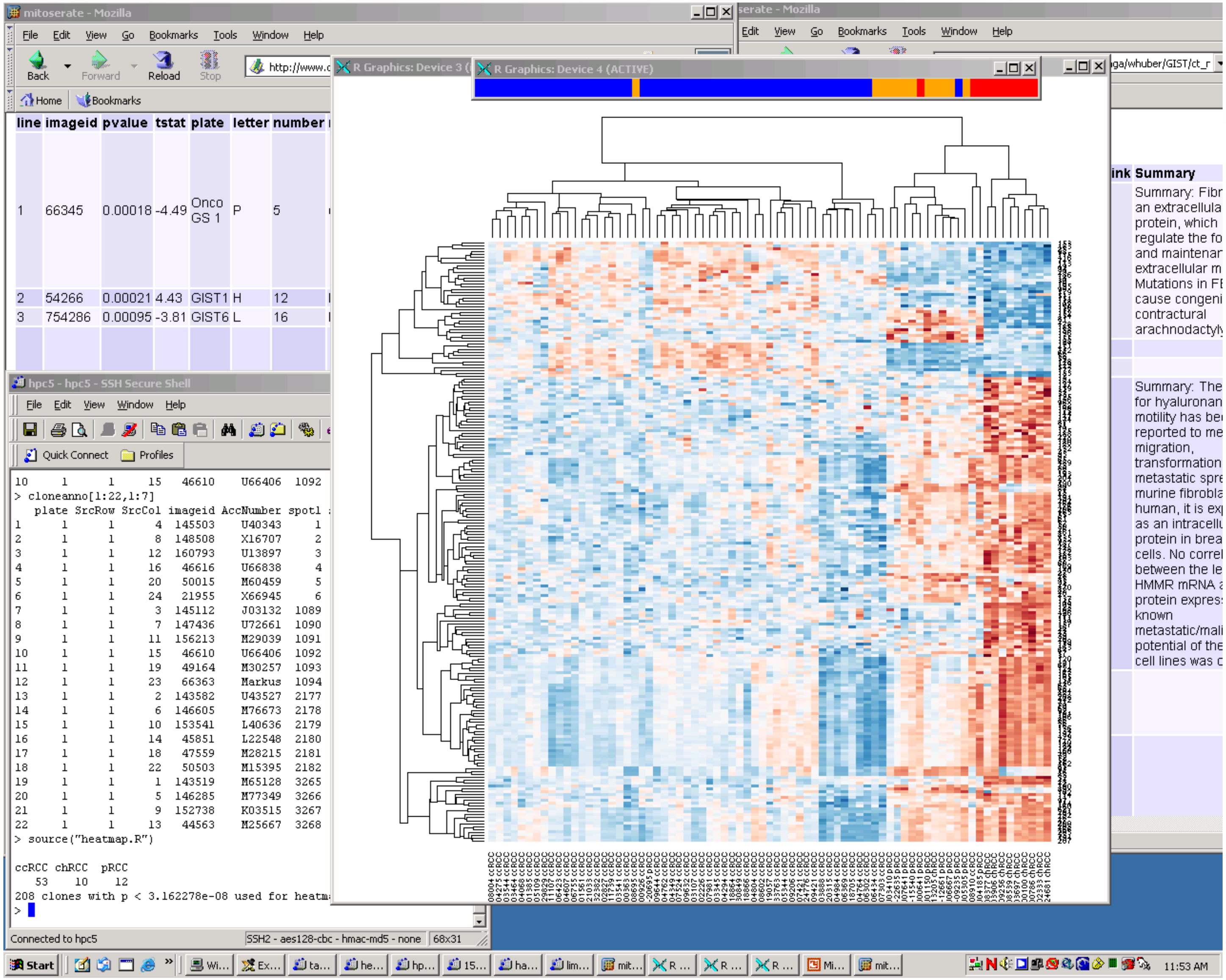
PMID
10486218
9205841
8817323

GO
GO:0003677
GO:0007275
GO:0016021

+ many other mappings

- Assemble and process genomic annotation data from public repositories.
- Build annotation data packages or XML data documents.
- Associate experimental data in real time to biological metadata from web databases such as GenBank, GO, KEGG, LocusLink, and PubMed.
- Process and store query results: e.g., search PubMed abstracts.
- Generate HTML reports of analyses.

mva package



line	imageid	pvalue	tstat	plate	letter	number
1	66345	0.00018	-4.49	Onco GS 1	P	5
2	54266	0.00021	4.43	GIST1	H	12
3	754286	0.00095	-3.81	GIST6	L	16

```
10      1       1      15   46610    U66406  1092
> cloneanno[1:22,1:7]
   plate SrcRow SrcCol imageid AccNumber spotl
1      1      1      4  145503    U40343     1
2      1      1      8  148508    X16707     2
3      1      1     12  160793    U13897     3
4      1      1     16   46616    U66838     4
5      1      1     20   50015    M60459     5
6      1      1     24   21955    X66945     6
7      1      1      3  145112    J03132  1089
8      1      1      7  147436    U72661  1090
9      1      1     11  156213    M29039  1091
10     1      1     15   46610    U66406  1092
11     1      1     19   49164    M30257  1093
12     1      1     23   66363    Markus  1094
13     1      1      2  143582    U43527  2177
14     1      1      6  146605    M76673  2178
15     1      1     10  153541    L40636  2179
16     1      1     14   45851    L22548  2180
17     1      1     18   47559    M28215  2181
18     1      1     22   50503    M15395  2182
19     1      1      1  143519    M65128  3265
20     1      1      5  146285    M77349  3266
21     1      1      9  152738    K03515  3267
22     1      1     13   44563    M25667  3268
> source("heatmap.R")

ccRCC chRCC  pRCC
   53    10    12
208 clones with p < 3.162278e-08 used for heatm
> 
```

ink Summary

Summary: Fibr
an extracellula
protein, which
regulate the fo
and maintenar
extracellular m
Mutations in FE
cause congeni
contractural
arachnodactyl

Summary: The
for hyaluronan
motility has be
reported to me
migration,
transformation
metastatic spre
murine fibrobla
human, it is ex
as an intracellu
protein in brea
cells. No correl
between the le
HMMR mRNA a
protein expres
known
metastatic/mali
potential of the
cell lines was c

heatmap

Data complexity

- Dimensionality.
- Dynamic/evolving data: e.g., gene annotation, sequence, literature.
- Multiple data sources and locations: in-house, WWW.
- Multiple data types: numeric, textual, graphical.

No longer X_{nxp}!

We distinguish between biological metadata and experimental metadata.

Experimental metadata

- Gene expression measures
  - scanned images, i.e., raw data;
  - image quantitation data, i.e., output from image analysis;
  - normalized expression measures, i.e., log ratios or Affy expression measures.
- Reliability/quality information for the expression measures.
- Information on the probe sequences printed on the arrays (array layout).
- Information on the target samples hybridized to the arrays.
- See Minimum Information About a Microarray Experiment (MIAME) standards and new `MAGEML` package.

Biological metadata

- Biological attributes that can be applied to the experimental data.
- E.g. for genes
  - chromosomal location;
  - gene annotation (LocusLink, GO);
  - relevant literature (PubMed).
- Biological metadata sets are large, evolving rapidly, and typically distributed via the WWW.
- Tools: **`annotate`**, **`annaffy`**, and **`AnnBuilder`** packages, and annotation data packages.

OOP

- The Bioconductor project has adopted the object-oriented programming (OOP) paradigm proposed in J. M. Chambers (1998). *Programming with Data*.
- This object-oriented class/method design allows efficient representation and manipulation of large and complex biological datasets of multiple types.
- Tools for programming using the class/method mechanism are provided in the R `methods` package.
- Tutorial:www.omegahat.org/RSMethods/index.html.

OOP: classes

- A class provides a software abstraction of a real world object. It reflects how we think of certain objects and what information these objects should contain.
- Classes are defined in terms of slots which contain the relevant data.
- An object is an instance of a class.
- A class defines the structure, inheritance, and initialization of objects.

OOP: methods

- A method is a function that performs an action on data (objects).
- Methods define how a particular function should behave depending on the class of its arguments.
- Methods allow computations to be adapted to particular data types, i.e., classes.
- A generic function is a dispatcher, it examines its arguments and determines the appropriate method to invoke.
- Examples of generic functions in R include `plot`, `summary`, `print`.

exprSet class

Processed Affymetrix or spotted array data

exprs	Matrix of expression measures, genes x samples
se.exprs	Matrix of SEs for expression measures, genes x samples
phenoData	Sample level covariates, instance of class **phenoData**
annotation	Name of annotation data
description	MIAME information
notes	Any notes

- Use of object-oriented programming to deal with data complexity.
- S4 class/method mechanism (**methods** package).

`marrayRaw` class

Pre-normalization intensity data for a batch of arrays

`maRf`	**`maGf`**	Matrix of red and green foreground intensities
`maRb`	**`maGb`**	Matrix of red and green background intensities
`maW`		Matrix of spot quality weights
`maLayout`		Array layout parameters - **`marrayLayout`**
`maGnames`		Description of spotted probe sequences - **`marrayInfo`**
`maTargets`		Description of target samples - **`marrayInfo`**
`maNotes`		Any notes

AffyBatch class

Probe-level intensity data for a batch of arrays (same CDF)

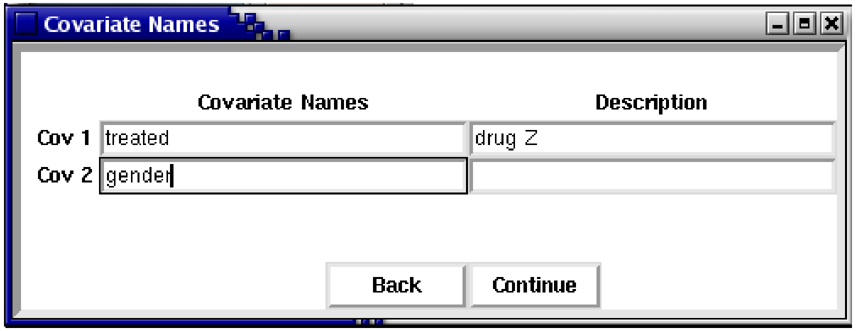
cdfName		Name of CDF file for arrays in the batch
nrow	ncol	Dimensions of the array
exprs	se.exprs	Matrices of probe-level intensities and SEs rows → probe cells, columns → arrays.
phenoData		Sample level covariates, instance of class phenoData
annotation		Name of annotation data
description		MIAME information
notes		Any notes

Widgets

- Widgets. Small-scale graphical user interfaces (GUI), providing point & click access for specific tasks.
- E.g. File browsing and selection for data input, basic analyses.
- Packages:
  - `tkWidgets`: `dataViewer`, `fileBrowser`, `fileWizard`, `importWizard`, `objectBrowser`.
  - `widgetTools`.

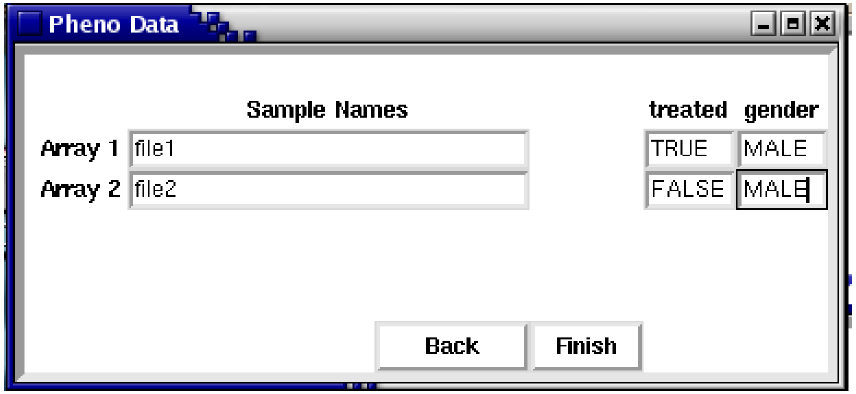
Widgets

Reading in phenoData



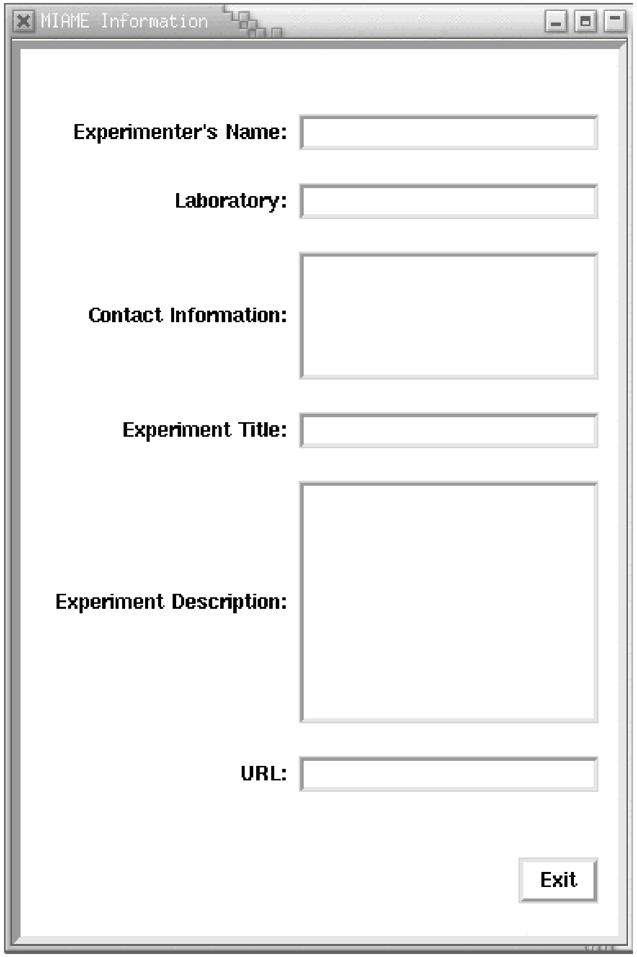
	Covariate Names	Description
Cov 1	treated	drug Z
Cov 2	gender	

tkSampleNames



	Sample Names	treated	gender
Array 1	file1	TRUE	MALE
Array 2	file2	FALSE	MALE

tkphenoData



tkMIAME

Getting Started

Installation

1. Main R software: download from CRAN (cran.r-project.org), use latest release, now 1.7.1.
2. Bioconductor packages: download from Bioconductor (www.bioconductor.org), use latest release, now 1.2.

Available for Linux/Unix, Windows, and Mac OS.

Installation

- After installing R, install Bioconductor packages using `getBioC` install script.
- From R

```
> source("http://www.bioconductor.org/getBioC.R")
> getBioC()
```

- In general, R packages can be installed using the function `install.packages`.
- In Windows, can also use “Packages” pull-down menus.

Installing vs. loading

- Packages only need to be installed once .
- But … packages must be loaded with each new R session.
- Packages are loaded using the function `library`. From R

  ```
   > library(Biobase)
  ```

  or the “Packages” pull-down menus in Windows.
- To update packages, use function `update.packages` or “Packages” pull-down menus in Windows.
- To quit:

  ```
  > q()
  ```

Documentation and help

- R manuals and tutorials:available from the R website or on-line in an R session.
- R on-line help system: detailed on-line documentation, available in text, HTML, PDF, and LaTeX formats.

```
> help.start()
> help(lm)
> ?hclust
> apropos(mean)
> example(hclust)
> demo()
> demo(image)
```

Short courses

- Bioconductor short courses
  - modular training segments on software and statistical methodology;
  - lectures notes, computer labs, and course packages available on WWW for self-instruction.

Vignettes

- Bioconductor has adopted a new documentation paradigm, the vignette.
- A vignette is an executable document consisting of a collection of code chunks and documentation text chunks.
- Vignettes provide dynamic, integrated, and reproducible statistical documents that can be automatically updated if either data or analyses are changed.
- Vignettes can be generated using the `Sweave` function from the R `tools` package.

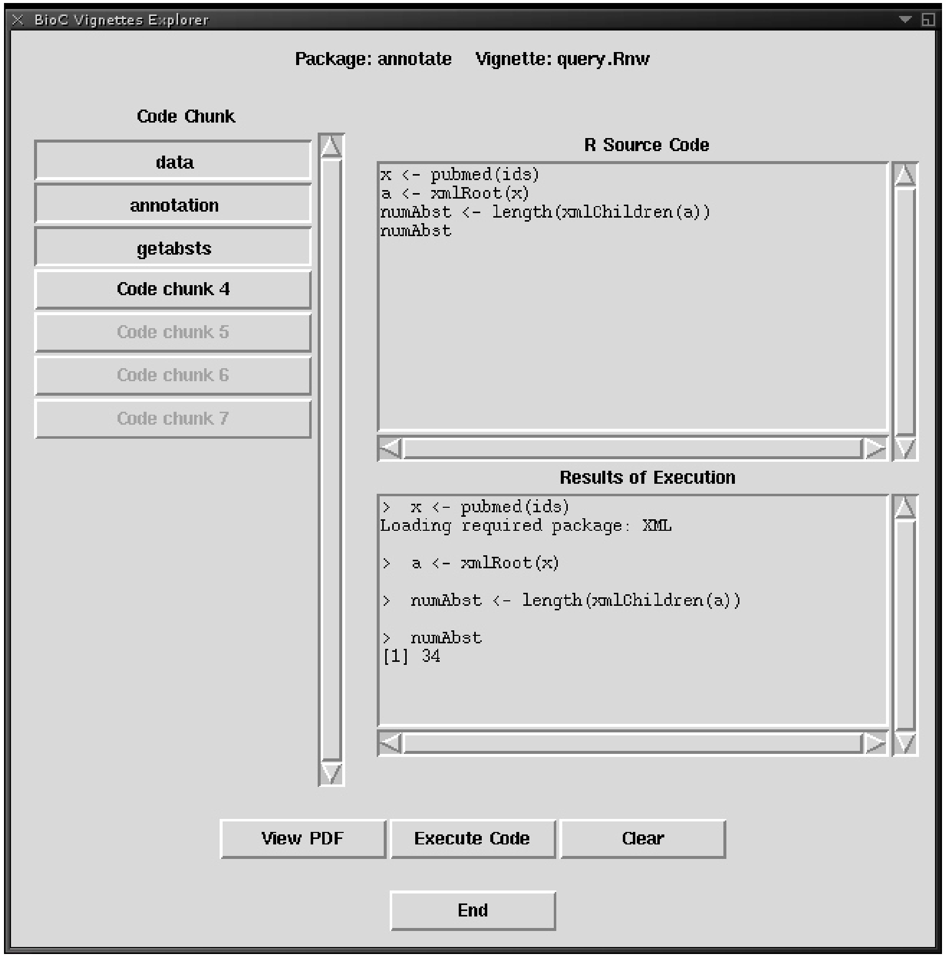
Vignettes

- Each Bioconductor package contains at least one vignette, providing task-oriented descriptions of the package's functionality.
- Vignettes are located in the `doc` subdirectory of an installed package and are accessible from the help browser.
- Vignettes can be used interactively.
- Vignettes are also available separately from the Bioconductor website.

Vignettes

- Tools are being developed for managing and using this repository of step-by-step tutorials
  - `Biobase`: `openVignette` – Menu of available vignettes and interface for viewing vignettes (PDF).
  - `tkWidgets`: `vExplorer` – Interactive use of vignettes.
  - `reposTools`.

Vignettes



Package: annotate Vignette: query.Rnw

```
x <- pubmed(ids)
a <- xmlRoot(x)
numAbst <- length(xmlChildren(a))
numAbst
```

```
>  x <- pubmed(ids)
Loading required package: XML

>  a <- xmlRoot(x)

>  numAbst <- length(xmlChildren(a))

>  numAbst
[1] 34
```

vExplorer

- HowTo's: Task-oriented descriptions of package functionality.
- Executable documents consisting of documentation text and code chunks.
- Dynamic, integrated, and reproducible statistical documents.
- Can be used interactively – **`vExplorer`**.
- Generated using **`Sweave`** (`tools` package).

Sweave

- The Sweave system allows the generation of dynamic, integrated, and reproducible statistical documents intermixing text, code, and code output (textual and graphical).
- Functions are available in the R `tools` package.
- See `? Sweave` and manual www.ci.tuwien.ac.at/~leisch/Sweave/.

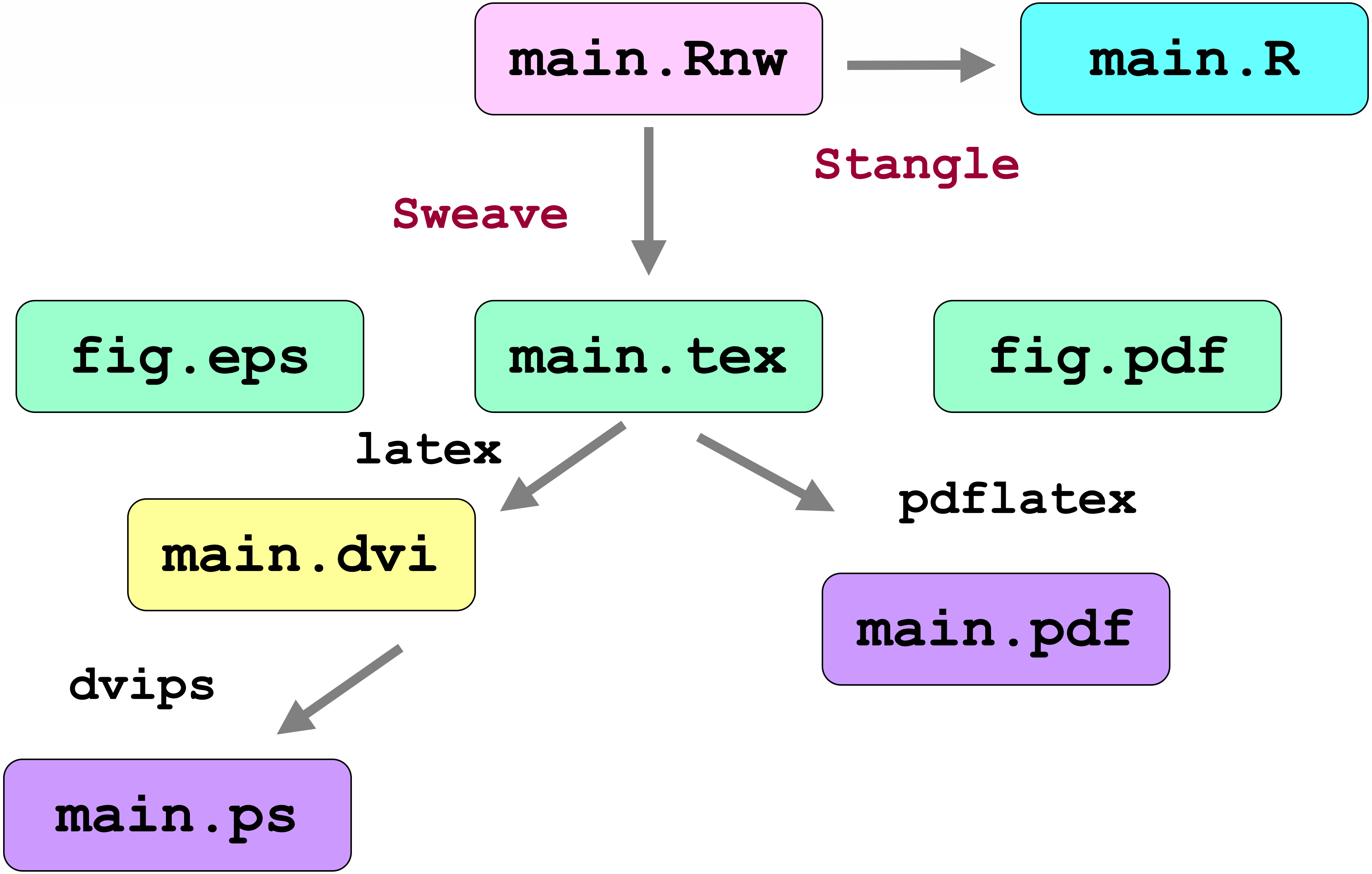
Sweave: input

- Input: a text file which consists of a sequence of code chunks and documentation text chunks (noweb file).
  - Documentation chunks
    - start with `@`
    - text in a markup language like LaTeX.
  - Code chunks
    - start with `<<name>>=`
    - R or S-Plus code.
  - File extension: `.rnw`, `.Rnw`, `.snw`, `.Snw`.

Sweave: output

- Output: a single document, e.g., `.tex` file or `.pdf` file containing
  - the documentation text,
  - the R code,
  - the code output: text and graphs.
- The document can be automatically regenerated whenever the data, code, or documentation text change.
- `Stangle` or `tangleToR`: extract only the code chunks.

Sweave



main.Rnw

main.R

Stangle

Sweave

fig.eps

main.tex

fig.pdf

latex

main.dvi

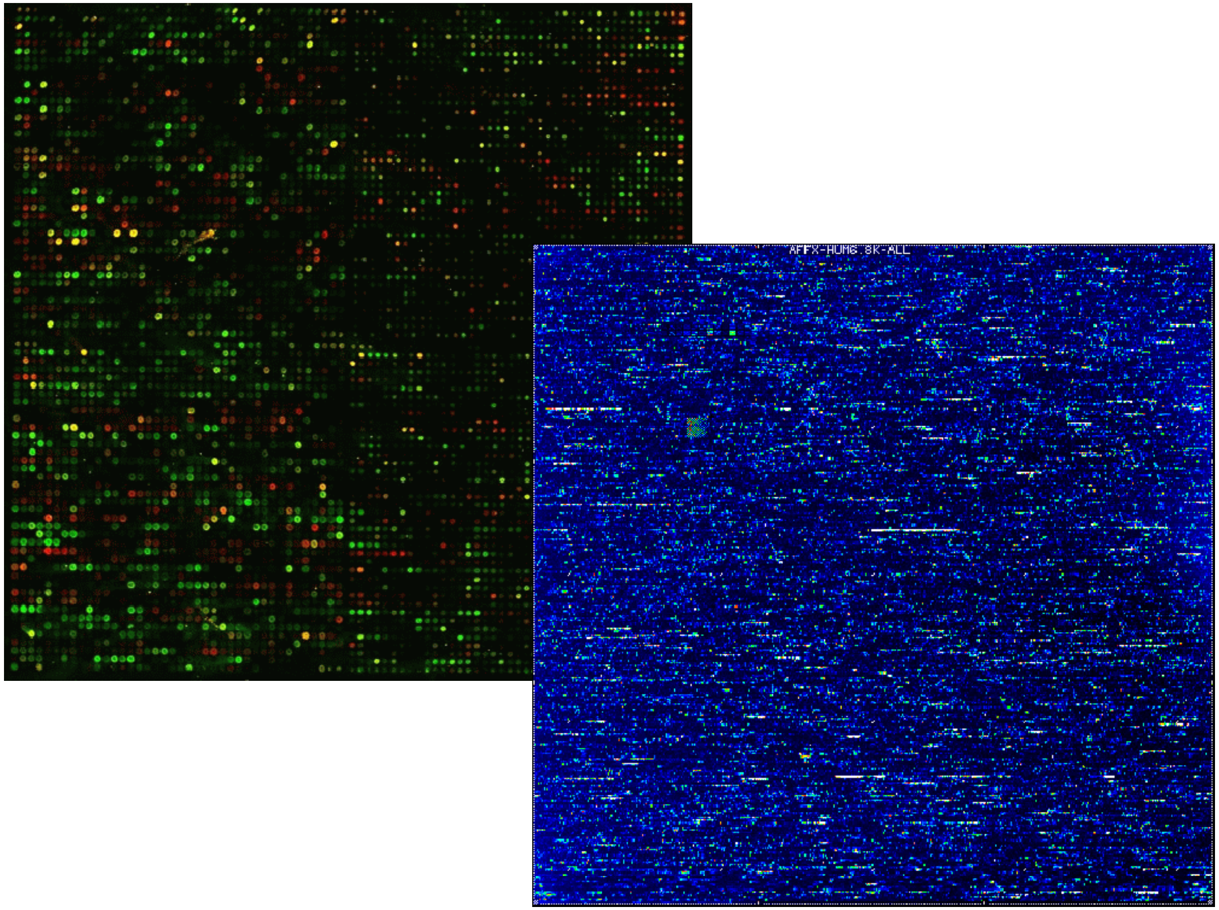
pdflatex

main.pdf

dvips

main.ps

Pre-processing



Pre-processing packages

- `affy`: Affymetrix oligonucleotide chips.
- `marray`, `limma`: Spotted DNA microarrays.
- `vsn`: Variance stabilization for both types of arrays.

Reading in intensity data, diagnostic plots, normalization, computation of expression measures.

The packages start with very different data structures, but produce similar objects of class **`exprSet`**.

One can then use other Bioconductor and CRAN packages, e.g., **`mva`**, **`genefilter`**, **`geneplotter`**.

marray packages

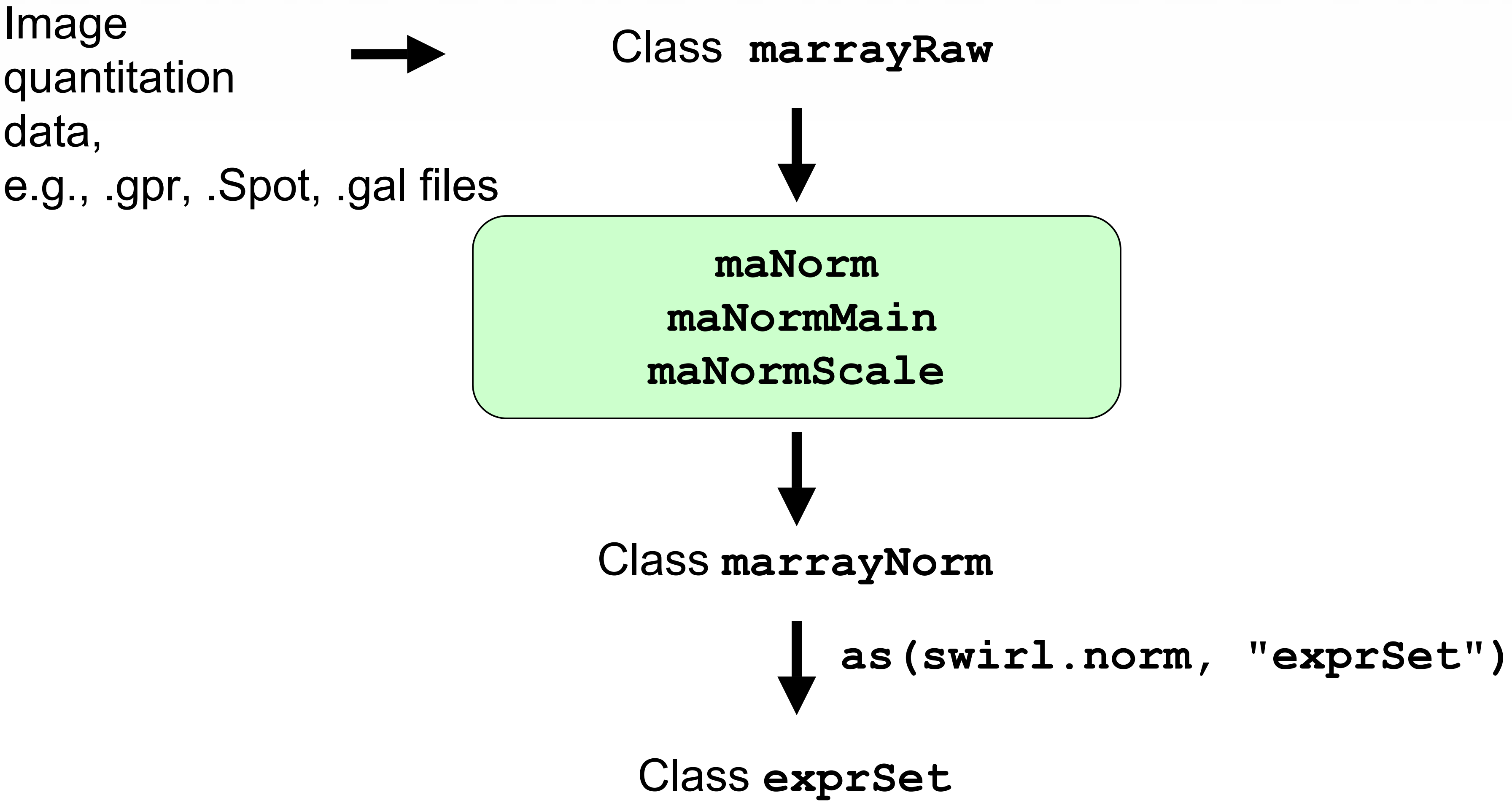


Image quantitation data,
e.g., .gpr, .Spot, .gal files

Class `marrayRaw`

`maNorm`
`maNormMain`
`maNormScale`

Class `marrayNorm`

`as(swirl.norm, "exprSet")`

Class `exprSet`

Save data to file using `write.exprs` or continue analysis using other Bioconductor and CRAN packages

`marray` packages

- `marrayClasses`:
  - class definitions for spotted DNA microarray data;
  - basic methods for manipulating microarray objects: printing, plotting, subsetting, class conversions, etc.
- `marrayInput`:
  - reading in intensity data and textual data describing probes and targets;
  - automatic generation of microarray data objects;
  - widgets for point & click interface.
- `marrayPlots`: diagnostic plots.
- `marrayNorm`: robust adaptive location and scale normalization procedures (lowess, loess).
- `marrayTools`: miscellaneous tools for spotted array data.

marrayLayout class

Array layout parameters

`maNspots`		Total number of spots
`maNgr`	`maNgc`	Dimensions of grid matrix
`maNsr`	`maNsc`	Dimensions of spot matrices
`maSub`		Current subset of spots
`maPlate`		Plate IDs for each spot
`maControls`		Control status labels for each spot
`maNotes`		Any notes

marrayRaw class

Pre-normalization intensity data for a batch of arrays

maRf	**maGf**	Matrix of red and green foreground intensities
maRb	**maGb**	Matrix of red and green background intensities
maW		Matrix of spot quality weights
maLayout		Array layout parameters - **marrayLayout**
maGnames		Description of spotted probe sequences - **marrayInfo**
maTargets		Description of target samples - **marrayInfo**
maNotes		Any notes

`marrayNorm` class

Post-normalization intensity data for a batch of arrays

`maA`	Matrix of average log intensities, A
`maM`	Matrix of normalized intensity log ratios, M
`maMloc` `maMscale`	Matrix of location and scale normalization values
`maW`	Matrix of spot quality weights
`maLayout`	Array layout parameters - `marrayLayout`
`maGnames`	Description of spotted probe sequences - `marrayInfo`
`maTargets`	Description of target samples - `marrayInfo`
`maNormCall`	Function call
`maNotes`	Any notes

`marrayInput` package

- `marrayInput` provides functions for reading microarray data into R and creating microarray objects of class **`marrayLayout`**, **`marrayInfo`**, and **`marrayRaw`**.
- Input
  - Image quantitation data, i.e., output files from image analysis software.

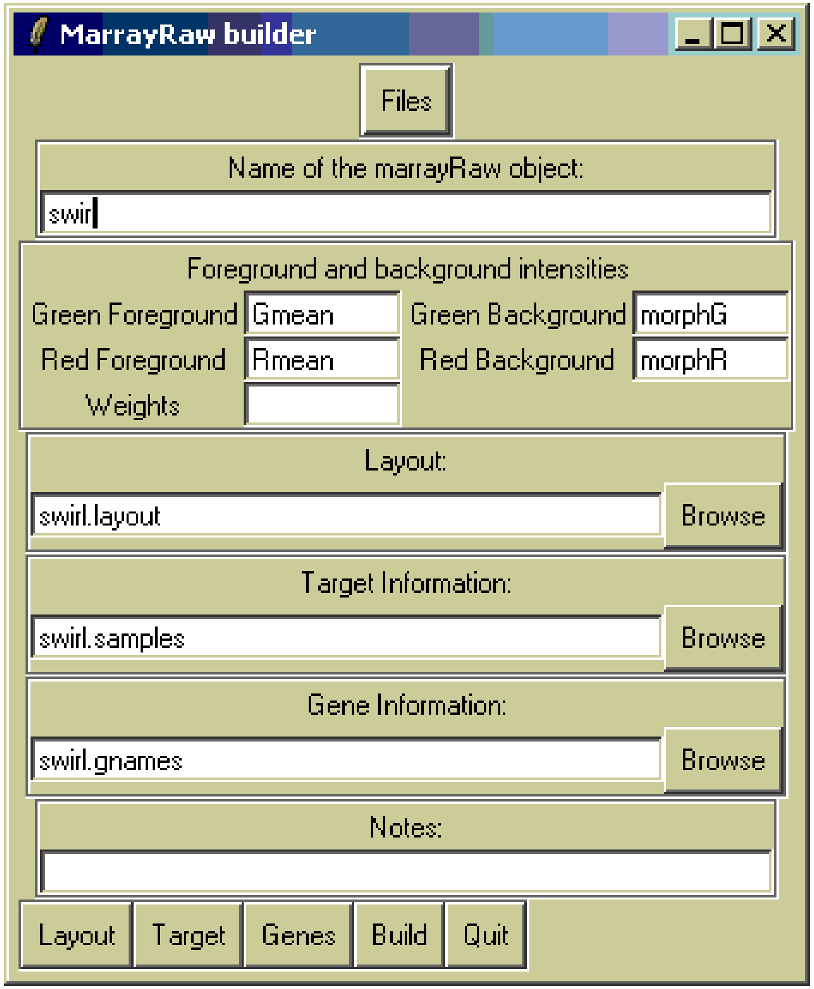
    E.g. **`.gpr`** for **`GenePix`**, **`.spot`** for **`Spot`**.
  - Textual description of probe sequences and target samples.

    E.g. gal files, god lists.

marrayInput package

- Widgets for graphical user interface

  widget.marrayLayout, **widget.marrayInfo**, **widget.marrayRaw**.



Name of the marrayRaw object: swir

Foreground and background intensities

Green Foreground: Gmean
Green Background: morphG
Red Foreground: Rmean
Red Background: morphR
Weights:

Layout: swirl.layout

Target Information: swirl.samples

Gene Information: swirl.gnames

Notes:

`marrayPlots` package

- See `demo(marrayPlots)`.
- Diagnostic plots of spot statistics.

  E.g. Red and green log intensities, intensity log ratios M, average log intensities A, spot area.

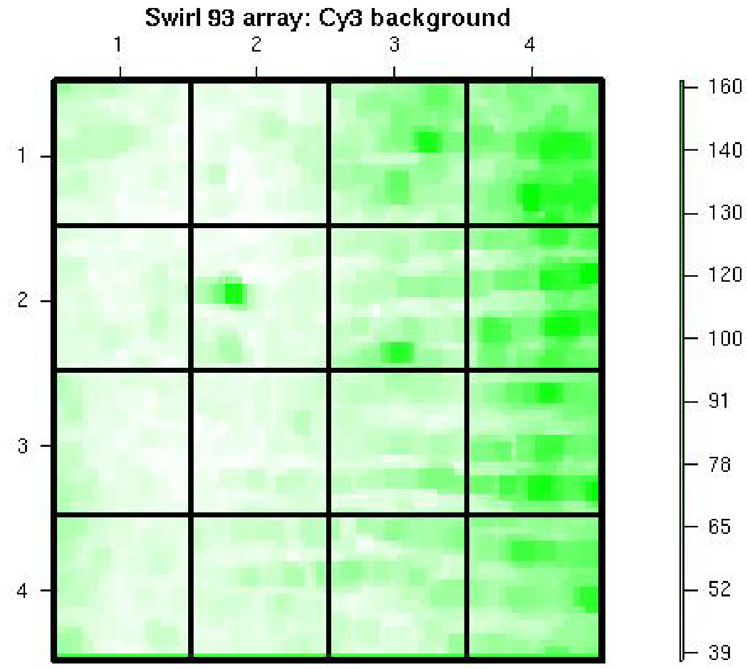
  – `maImage`: 2D spatial color images.

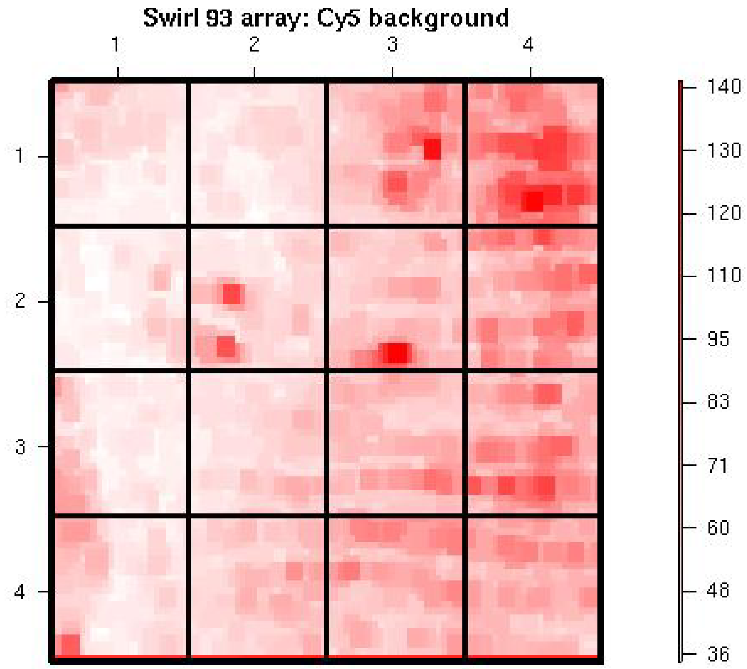
  – `maBoxplot`: boxplots.

  – `maPlot`: scatter-plots with fitted curves and text highlighted.
- Stratify plots according to layout parameters such as print-tip-group, plate.

  E.g. MA-plots with loess fits by print-tip-group.

2D spatial images
`maImage`





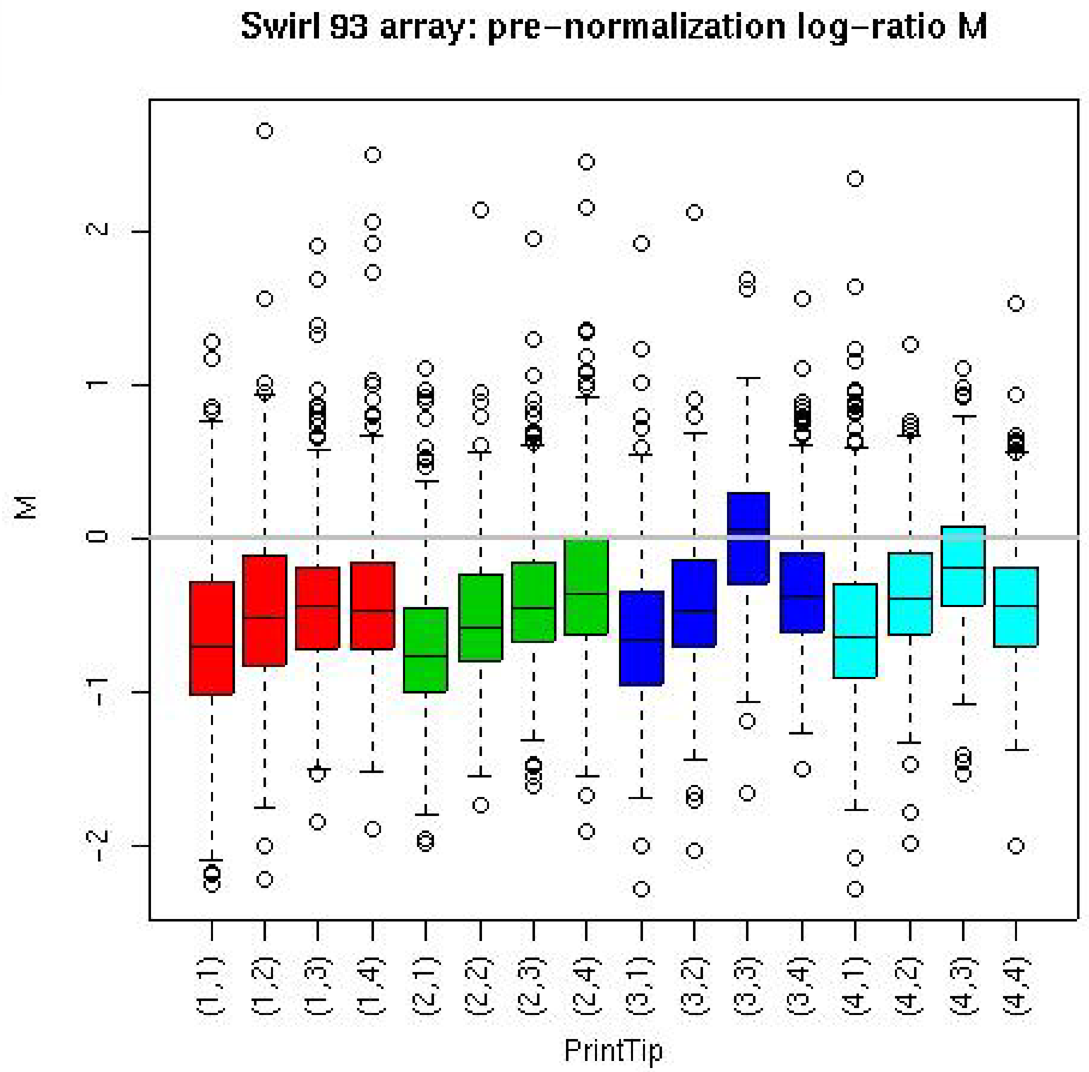
Cy3 background intensity

Cy5 background intensity

Boxplots by print-tip-group

maBoxplot

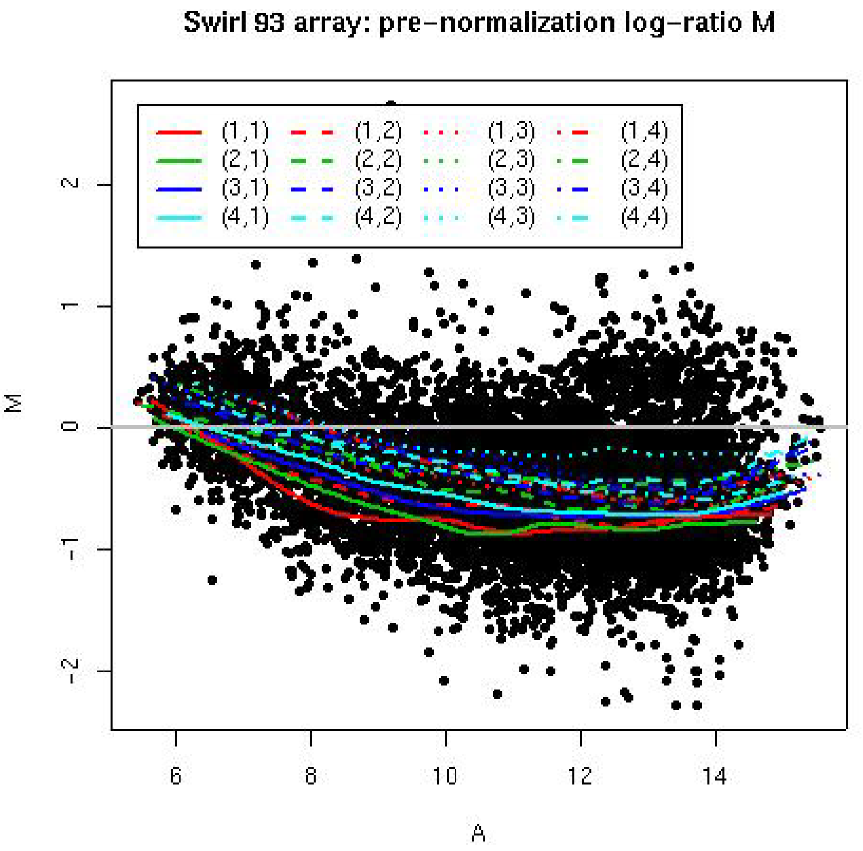
Intensity
log ratio, M

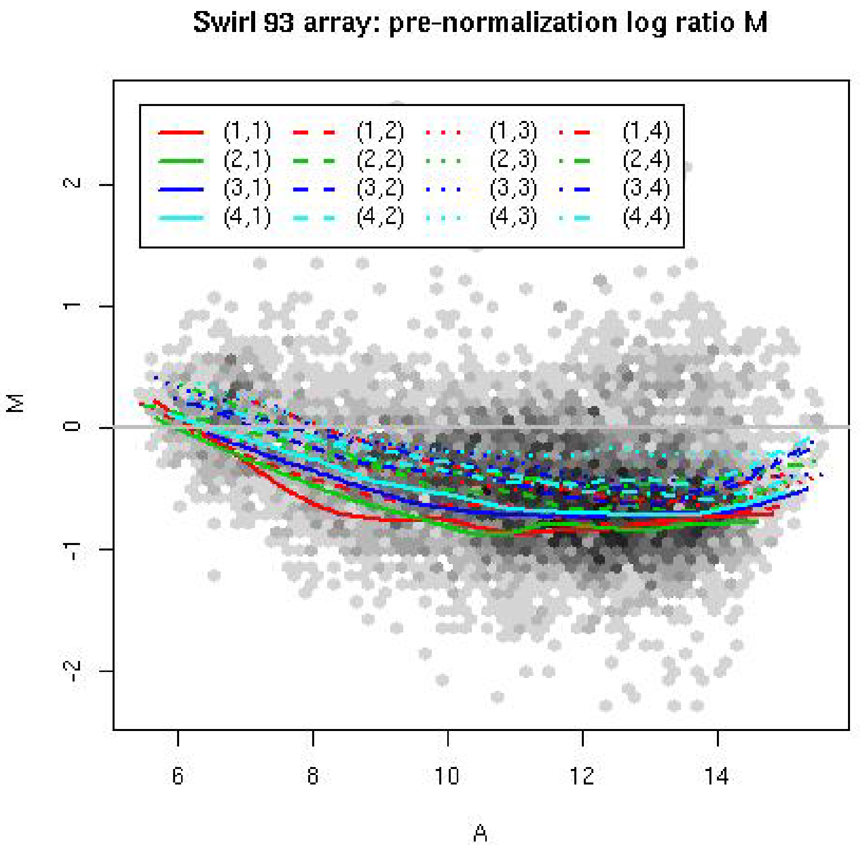


MA-plot by print-tip-group

`maPlot`

$$M = \log_2 R - \log_2 G \text{ vs. } A = (\log_2 R + \log_2 G)/2$$


Swirl 93 array: pre–normalization log–ratio M


Swirl 93 array: pre-normalization log ratio M

`hexbin`

marrayNorm package

- maNormMain: main normalization function, robust adaptive location and scale normalization (lowess, loess) for batch of arrays
  - intensity or A-dependent location normalization (maNormLoess);
  - 2D spatial location normalization (maNorm2D);
  - median location normalization (maNormMed);
  - scale normalization using MAD (maNormMAD);
  - composite normalization;
  - your own normalization function.
- maNorm: simple wrapper function.
- maNormScale: simple wrapper function for scale normalization.

`marrayTools` package

- The `marrayTools` package provides additional functions for handling two-color spotted microarray data.
- The `spotTools` and `gpTools` functions start from Spot and GenePix image analysis output files, respectively, and automatically
  - read in these data into R,
  - perform standard normalization (within print-tip-group loess),
  - create a directory with a standard set of diagnostic plots (jpeg format) and tab delimited text files of quality measures, normalized log ratios M, and average log intensities A.

`swirl` dataset

- Microarray layout:
  - 8,448 probes (768 controls);
  - 4 x 4 grid matrix;
  - 22 x 24 spot matrices.
- 4 hybridizations: swirl mutant vs. wild type mRNA.
- Data stored in object of class **`marrayRaw`**

```
> data(swirl)
> maInfo(maTargets(swirl))[,3:4]
experiment Cy3 experiment Cy5
1          swirl      wild type
2      wild type          swirl
3          swirl      wild type
4      wild type          swirl
```

Affymetrix chips

- Each gene or portion of a gene is represented by 16 to 20 oligonucleotides of 25 base-pairs, i.e., 25-mers.

- Probe: a 25-mer.
- Perfect match (PM): A 25-mer complementary to a reference sequence of interest (e.g., part of a gene).
- Mismatch (MM): same as PM but with a single homomeric base change for the middle (13th) base (transversion purine <-> pyrimidine, G <->C, A <->T) .
- Probe-pair: a (PM,MM) pair.
- Probe-pair set: a collection of probe-pairs (16 to 20) related to a common gene or fraction of a gene.
- Affy ID: an identifier for a probe-pair set.
- The purpose of the MM probe design is to measure non-specific binding and background noise.

Affymetrix chips

GeneChip® Expression Array Design

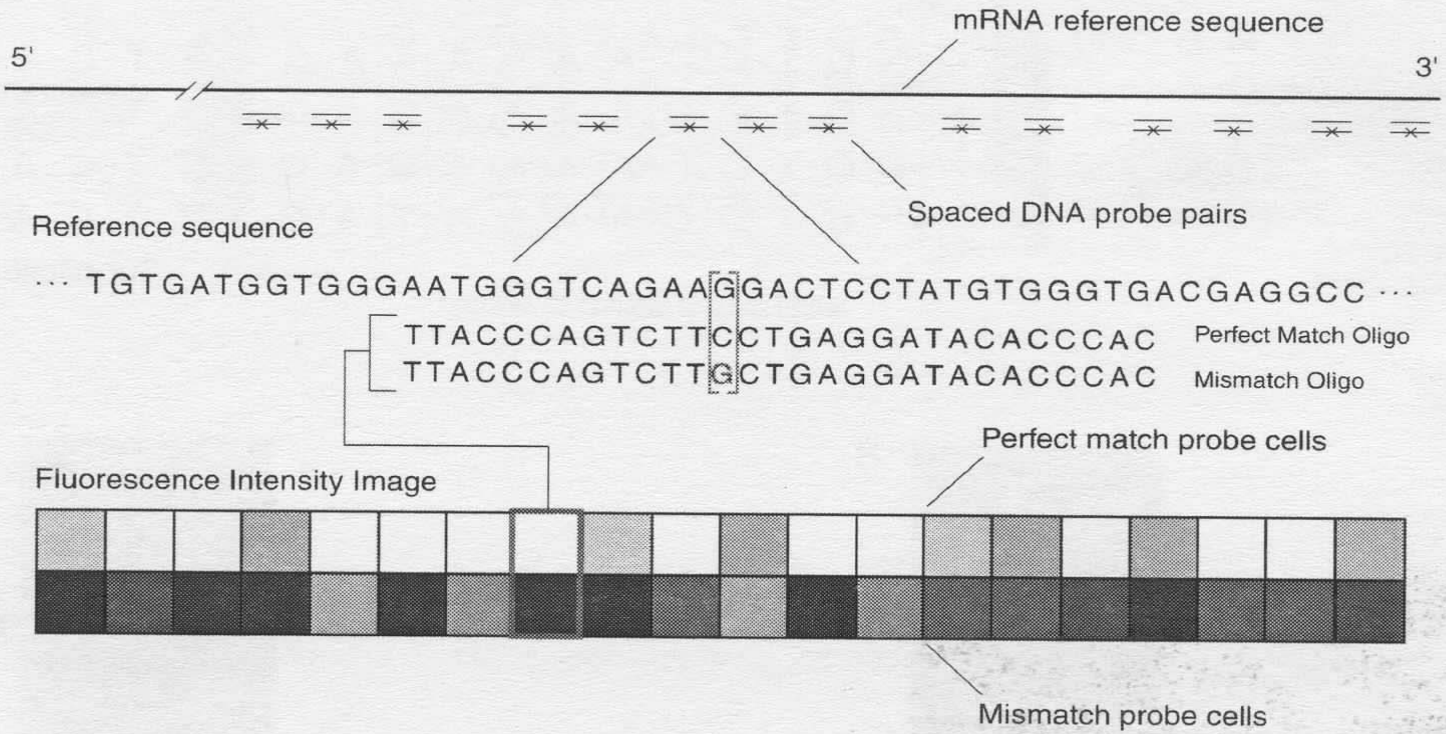
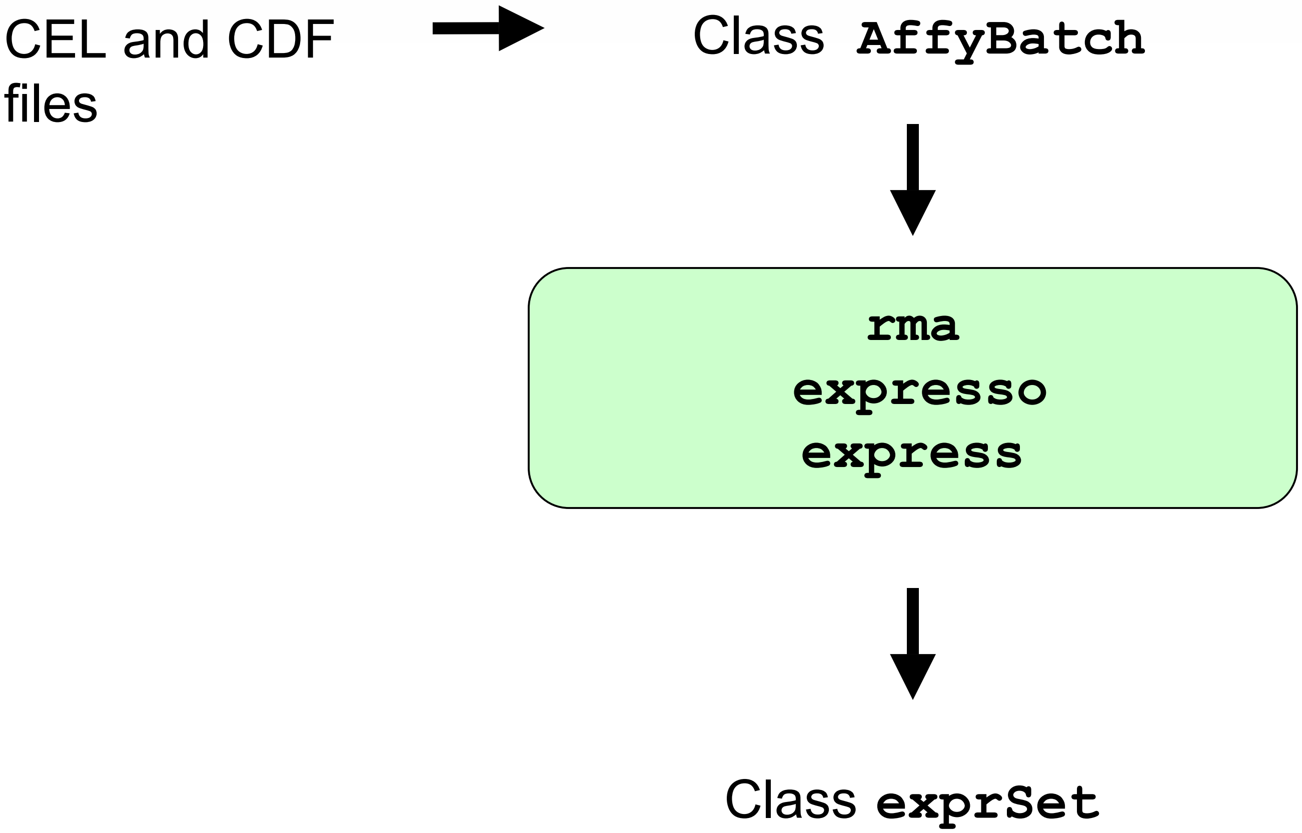

mRNA reference sequence
5'
3'
Spaced DNA probe pairs
Reference sequence
··· TGTGATGGTGGGAATGGGTCAGAAGGACTCCTATGTGGGTGACGAGGCC ···
TTACCCAGTCTTCCTGAGGATACACCCAC
Perfect Match Oligo
TTACCCAGTCTTGCTGAGGATACACCCAC
Mismatch Oligo
Perfect match probe cells
Fluorescence Intensity Image
Mismatch probe cells

Figure 1-3 Expression tiling strategy

Affymetrix chips

- **`DAT`** file: Image file, ~10^7 pixels, ~50 MB.
- **`CEL`** file: Cell intensity file, probe level PM and MM values.
- **`CDF`** (Chip Description File): Describes which probes belong to which probe-pair set and the location of the probes.

affy package



CEL and CDF files

Class **AffyBatch**

rma
expresso
express

Class **exprSet**

Save data to file using **write.exprs** or continue analysis using other Bioconductor and CRAN packages

`affy` package

- Class definitions for probe-level data: **`AffyBatch`**, **`ProbSet`**, **`Cdf`**, **`Cel`**.
- Basic methods for manipulating microarray objects: printing, plotting, subsetting.
- Functions and widgets for data input from **`CEL`** and **`CDF`** files, and automatic generation of microarray data objects.
- Diagnostic plots: 2D spatial images, density plots, boxplots, MA-plots.

`affy` package

- Background estimation.
- Probe-level normalization: quantile and curve-fitting normalization (Bolstad et al., 2003).
- Expression measures: MAS 4.0 AvDiff, MAS 5.0 Signal, MBEI (Li & Wong, 2001), RMA (Irizarry et al., 2003).
- Main functions: `ReadAffy`, `rma`, `expresso`, `express`.

AffyBatch class

Probe-level intensity data for a batch of arrays (same CDF)

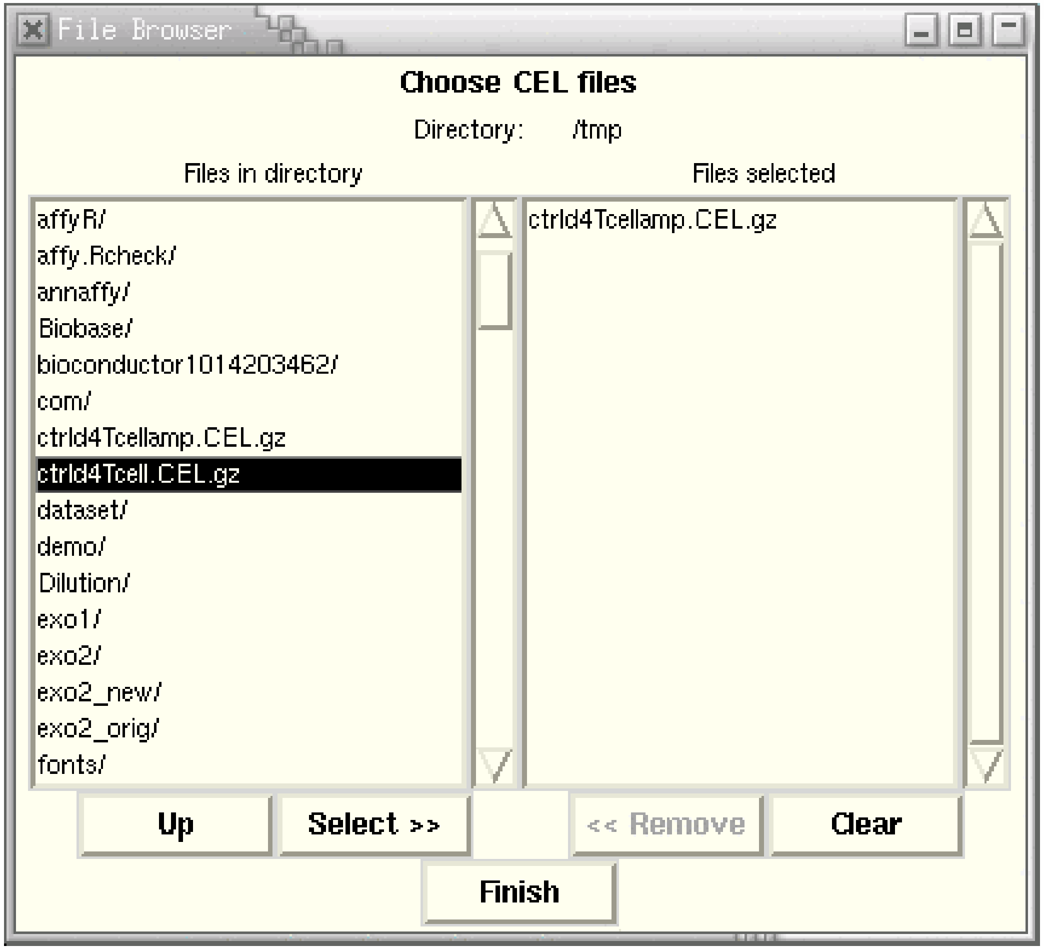
cdfName		Name of CDF file for arrays in the batch
nrow	ncol	Dimensions of the array
exprs	se.exprs	Matrices of probe-level intensities and SEs rows → probe cells, columns → arrays.
phenoData		Sample level covariates, instance of class phenoData
annotation		Name of annotation data
description		MIAME information
notes		Any notes

Other `affy` classes

- `ProbeSet`: PM, MM intensities for individual probe sets.
  - `pm`: matrix of PM intensities for one probe set, rows → 16-20 probes, columns → arrays.
  - `mm`: matrix of MM intensities for one probe set, rows → 16-20 probes, columns → arrays.

    Apply `probeset` to `AffyBatch` object to get a list of `ProbeSet` objects.
- `Cel`: Single array cel intensity data.
- `Cdf`: Information contained in a `CDF` file.

Reading in data: `ReadAffy`



Choose CEL files

Directory: /tmp

Files in directory

```
affyR/
affy.Rcheck/
annaffy/
Biobase/
bioconductor1014203462/
com/
ctrld4Tcellamp.CEL.gz
ctrld4Tcell.CEL.gz
dataset/
demo/
Dilution/
exo1/
exo2/
exo2_new/
exo2_orig/
fonts/
```

Files selected

```
ctrld4Tcellamp.CEL.gz
```

Creates object
of class **`AffyBatch`**

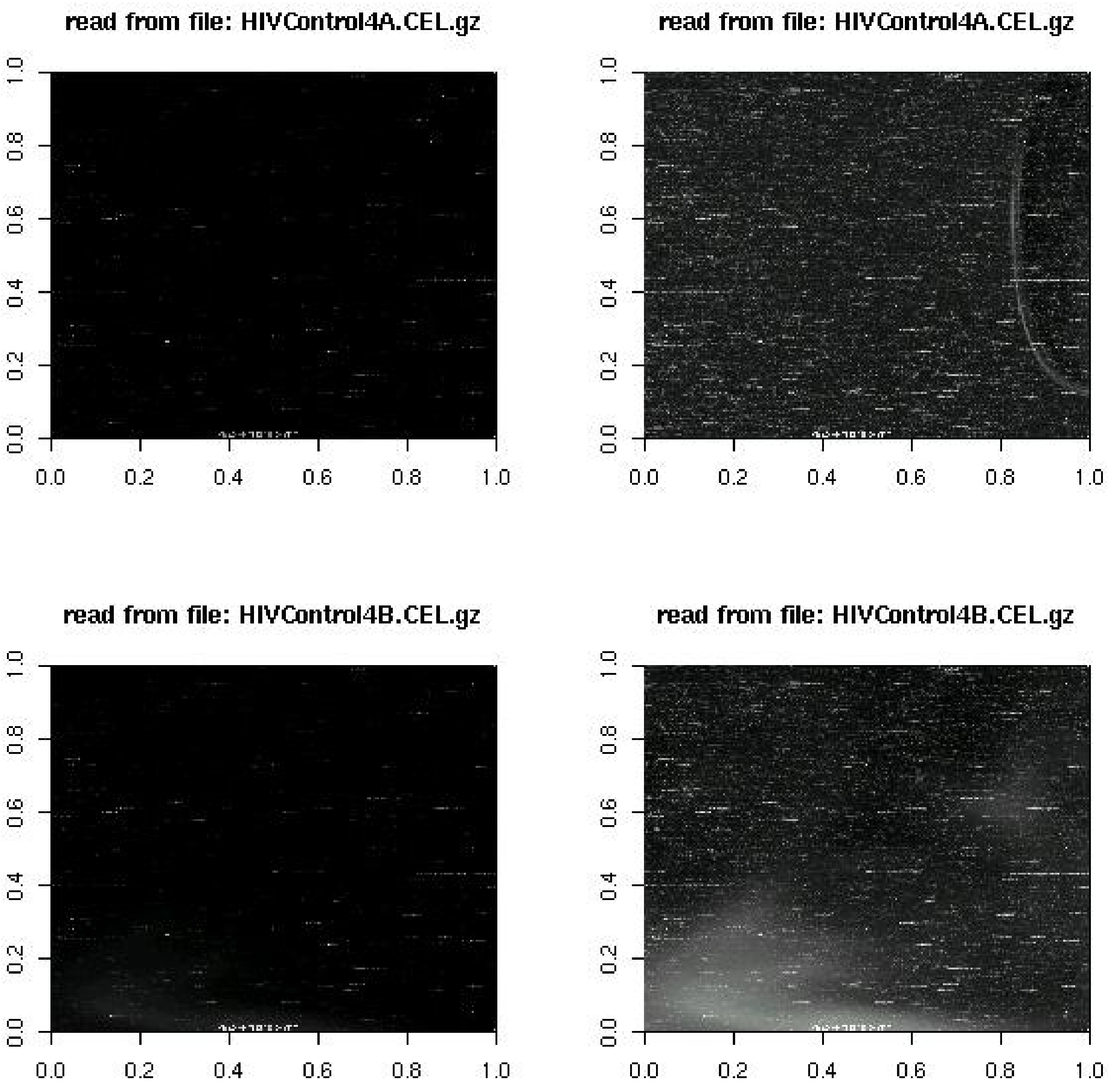
Accessing PM/MM data

- **`probeNames`**: method for accessing AffyIDs corresponding to individual probes.
- **`pm`**, **`mm`**: methods for accessing probe-level PM and MM intensities → probes x arrays matrix.
- Can use on **`AffyBatch`** objects.

Diagnostic plots

- See `demo(affy)`.
- Diagnostic plots of probe-level intensities, PM and MM.
  - `image`: 2D spatial color images of log intensities (**`AffyBatch`**, **`Cel`**).
  - `boxplot`: boxplots of log intensities (**`AffyBatch`**).
  - `mva.pairs`: scatter-plots with fitted curves (apply **`exprs`**, **`pm`**, or **`mm`** to **`AffyBatch`** object).
  - `hist`: density plots of log intensities (**`AffyBatch`**).

image



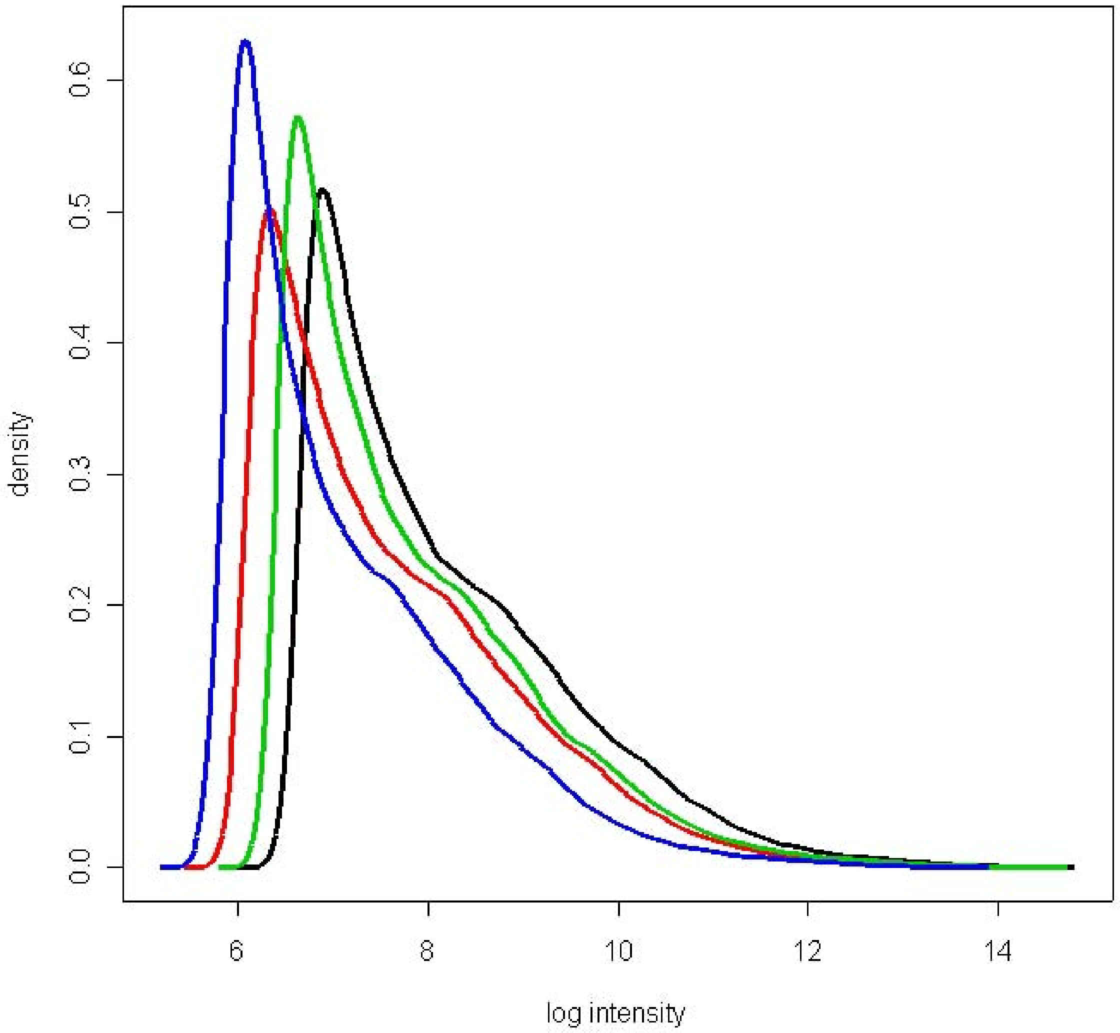
read from file: HIVControl4A.CEL.gz

read from file: HIVControl4A.CEL.gz

read from file: HIVControl4B.CEL.gz

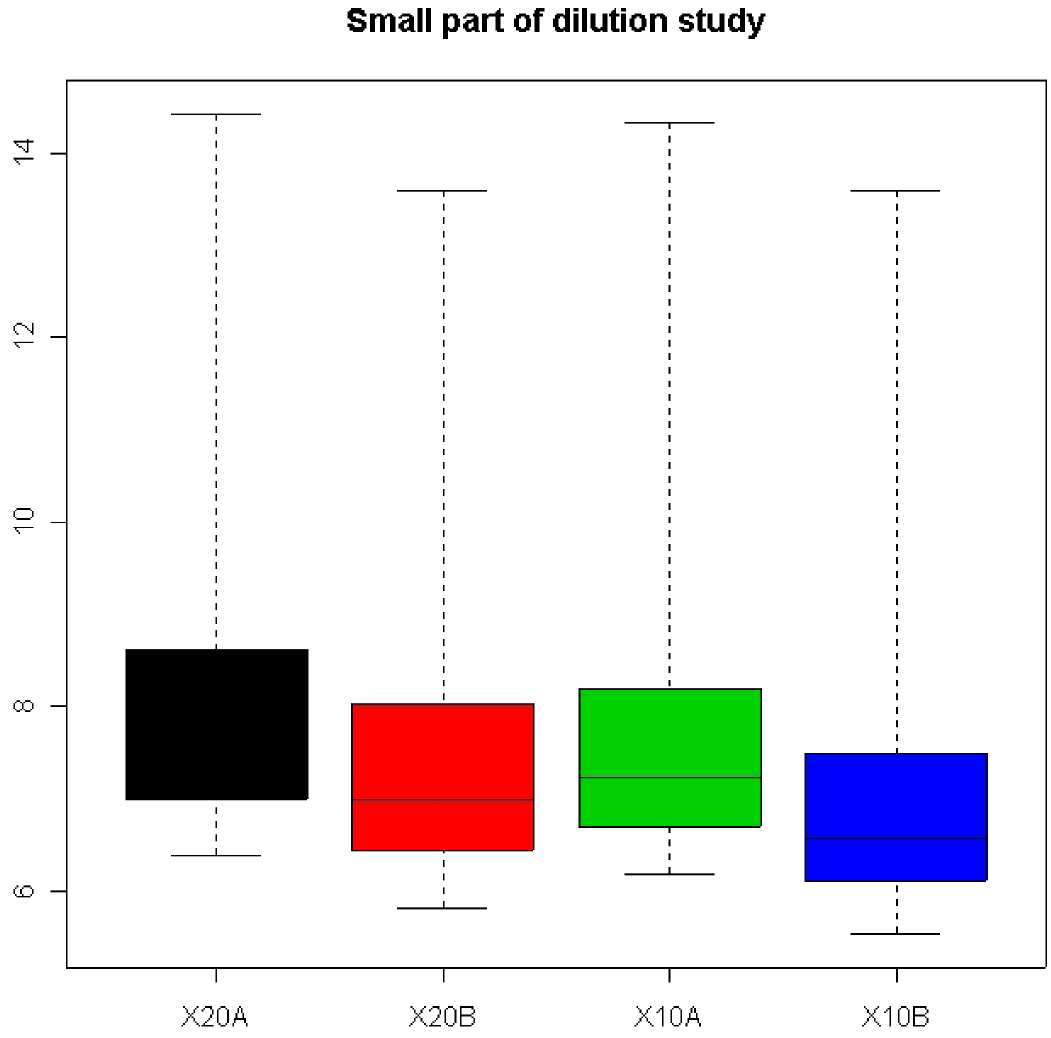
read from file: HIVControl4B.CEL.gz

hist



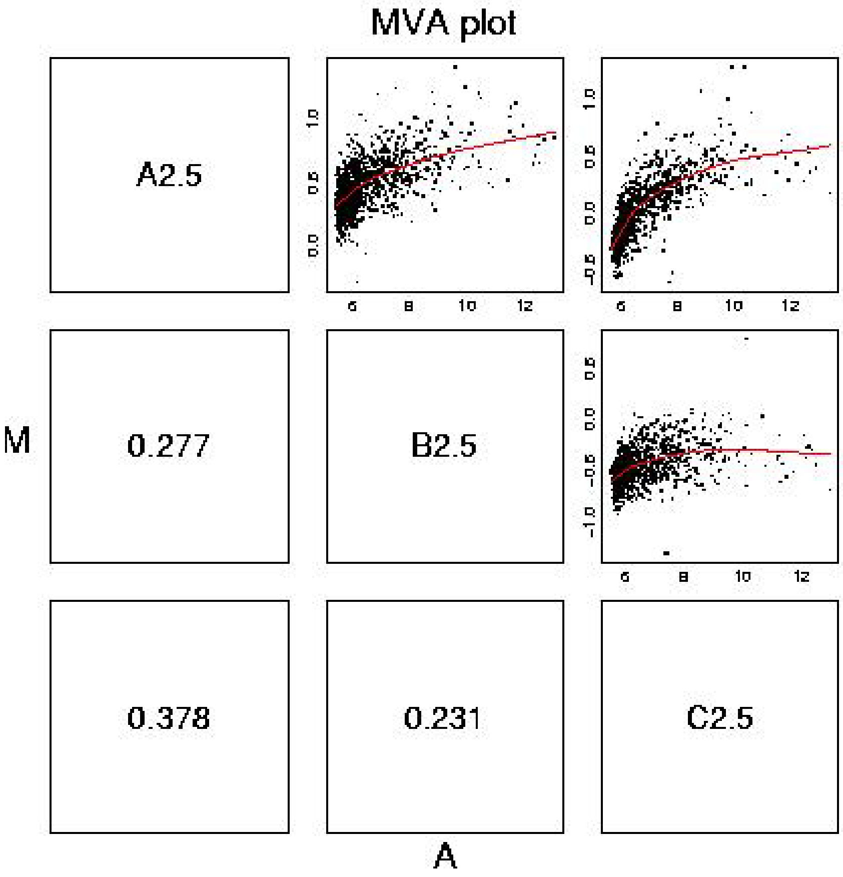
```
hist(Dilution,col=1:4,type="l",lty=1,lwd=3)
```

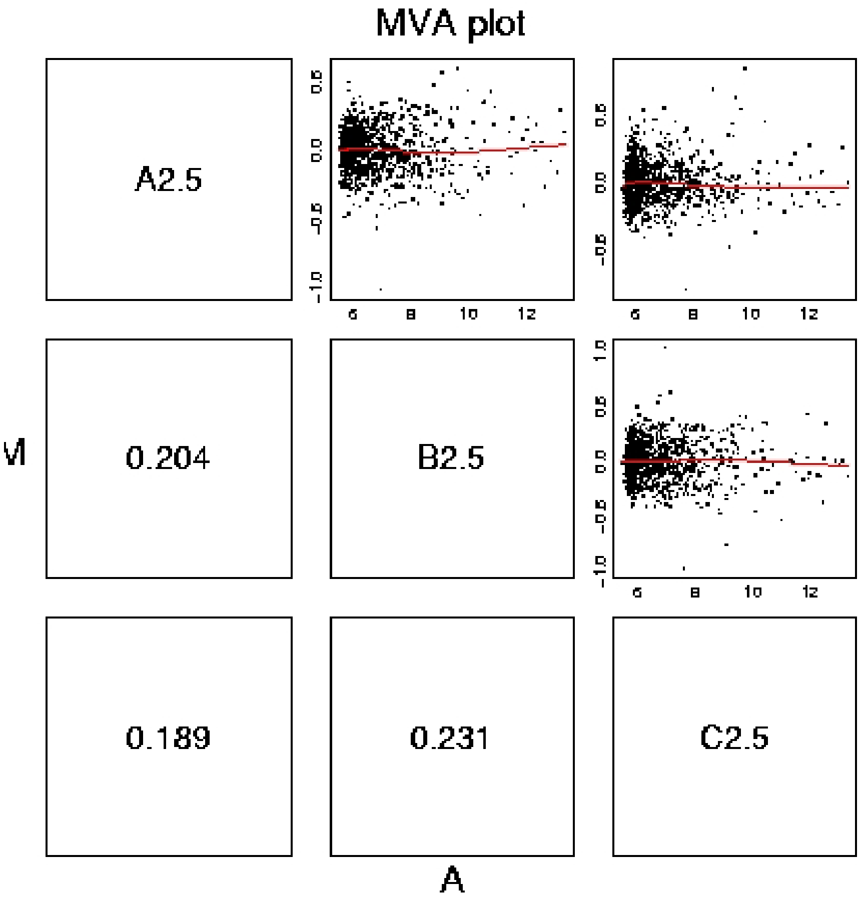
boxplot



```
boxplot(Dilution,col=1:4)
```

mva.pairs

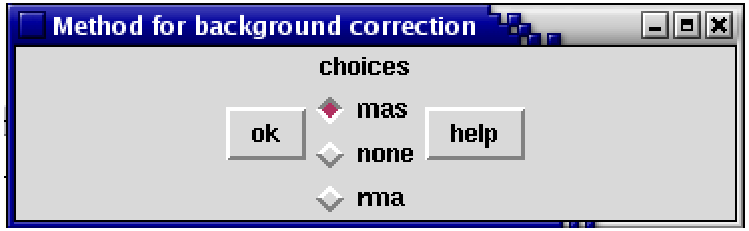

MVA plot
A2.5
B2.5
C2.5
M
0.277
0.378
0.231
A
6
8
10
12
-0.5
0.0
0.5
1.0
-1.0


MVA plot
A2.5
B2.5
C2.5
M
0.204
0.189
0.231
A
6
8
10
12
-1.0
-0.5
0.0
0.5
1.0

Expression measures

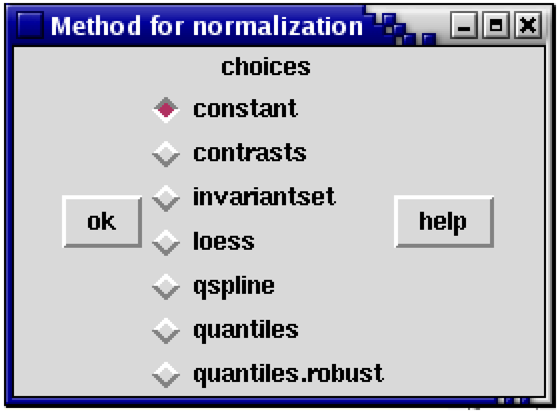
- `expresso`: Choice of common methods for
  - background correction: `bgcorrect.methods`
  - normalization: `normalize.AffyBatch.methods`
  - probe specific corrections: `pmcorrect.methods`
  - expression measures: `express.summary.stat.methods`.
- `rma`: Fast implementation of RMA (Irizarry et al., 2003): model-based background correction, quantile normalization, median polish expression measures.
- `express`: Implementing your own methods for computing expression measures.
- `normalize`: Normalization procedures in `normalize.AffyBatch.methods` or `normalize.methods(object)`.

Expression meassures:
`expresso`


Method for background correction

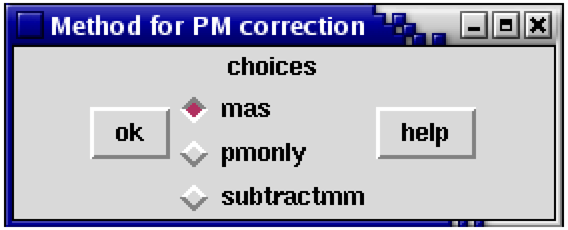
choices

- ● mas
- ○ none
- ○ rma


Method for normalization

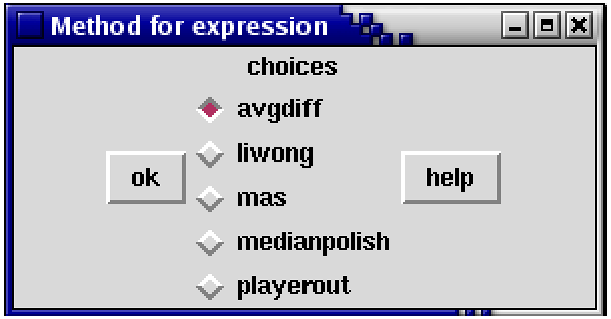
choices

- ● constant
- ○ contrasts
- ○ invariantset
- ○ loess
- ○ qspline
- ○ quantiles
- ○ quantiles.robust


Method for PM correction

choices

- ● mas
- ○ pmonly
- ○ subtractmm


Method for expression

choices

- ● avgdiff
- ○ liwong
- ○ mas
- ○ medianpolish
- ○ playerout

```
expresso(widget=TRUE)
```

Probe sequence analysis

- Examine probe intensities based on location relative to 5’ end of the RNA sequence of interest.
- Expect probe intensities to be lower at 5’ end compared to 3’ end of mRNA.
- E.g.

```
deg <- AffyRNAdeg(Dilution)
plotAffyRNAdeg(deg)
```

`CDF` data packages

- Data packages containing **`CDF`** information are available at www.bioconductor.org.
- Packages contain environment objects, which provide mappings between AffyIDs and matrices of probe locations,

  rows → probe-pairs,

  columns → PM, MM

  (e.g., 20X2 matrix for hu6800).
- **`cdfName`** slot of **`AffyBatch`**.
- **`makecdfenv`** package.

Other packages

- `affycomp`: assessment of Affymetrix expression measures.
- `affydata`: sample Affymetrix datasets.
- `annaffy`: annotation functions.
- `gcrma`: background adjustment using sequence information.
- `makecdfenv`: creating CDF environments and packages.

Differential Gene Expression

Combining data across arrays

Data on *G* genes for *n* arrays

→ *G x n* genes-by-arrays data matrix

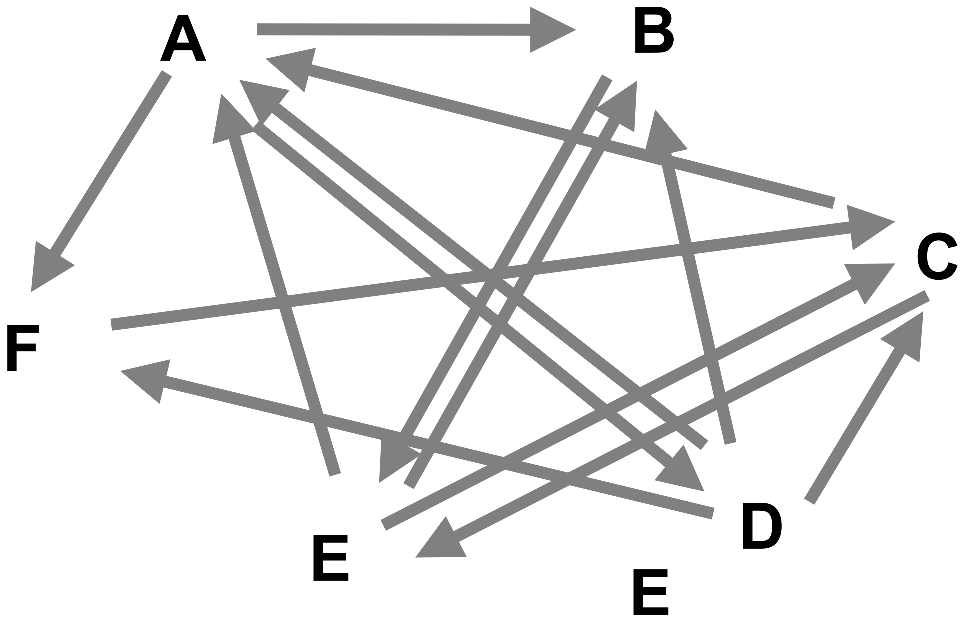
Arrays

		Array1	Array2	Array3	Array4	Array5	...
Genes	Gene1	0.46	0.30	0.80	1.51	0.90	...
	Gene2	-0.10	0.49	0.24	0.06	0.46	...
	Gene3	0.15	0.74	0.04	0.10	0.20	...
	Gene4	-0.45	-1.03	-0.79	-0.56	-0.32	...
	Gene5	-0.06	1.06	1.35	1.09	-1.09	...
	...	...	...	...	...	...	

M = $\log_2$(Red intensity / Green intensity)
expression measure, e.g., from RMA.

Combining data across arrays

… but the columns have structure, determined by the experimental design.



Combining data across arrays

- *Spotted array factorial experiment*. Each column corresponds to a pair of mRNA samples with different drug x dose x time combinations.
- *Clinical trial.* Each column corresponds to a patient, with associated clinical outcomes, such as survival and response to treatment.
- Linear models and extensions thereof can be used to effectively combine data across arrays for complex experimental designs.

Gene filtering

- A very common task in microarray data analysis is gene-by-gene selection.
- Filter genes based on
  - data quality criteria, e.g., absolute intensity or variance;
  - subject matter knowledge;
  - their ability to differentiate cases from controls;
  - their spatial or temporal expression patterns.
- Depending on the experimental design, some highly specialized filters may be required and applied sequentially.

Gene filtering

- *Clinical trial.* Filter genes based on association with survival, e.g., using a Cox model.
- *Factorial experiment.* Filter genes based on interaction between two treatments, e.g., using 2-way ANOVA.
- *Time-course experiment*. Filter genes based on periodicity of expression pattern, e.g., using Fourier transform.

genefilter package

- The genefilter package provides tools to sequentially apply filters to the rows (genes) of a matrix or of an exprSet object.
- There are two main functions, filterfun and genefilter, for assembling and applying the filters, respectively.
- Any number of functions for specific filtering tasks can be defined and supplied to filterfun.

  E.g. Cox model p-values, coefficient of variation.

`genefilter`: separation of tasks

1. Select/define functions for specific filtering tasks.
2. Assemble the filters using the `filterfun` function.
3. Apply the filters using the `genefilter` function → a logical vector, where **`TRUE`** indicates genes that are retained.
4. Apply this vector to the **`exprSet`** object to obtain a microarray object corresponding to the subset of interesting genes.

genefilter:
supplied filters

- kOverA – select genes for which k samples have expression measures larger than A.
- gapFilter – select genes with a large IQR or gap (jump) in expression measures across samples.
- ttest – select genes according to t-test nominal p-values.
- Anova – select genes according to ANOVA nominal p-values.
- coxfilter – select genes according to Cox model nominal p-values.

genefilter: custom filters

- It is very simple to write your own filters -- use the supplied filtering functions as templates.
- The basic idea is to rely on lexical scoping to provide values (bindings) for the variables that are needed to do the filtering.

genefilter:
How to?

1. First, build the filters

   ```
   f1 <- anyNA
   f2 <- kOverA(5, 100)
   ```

2. Next, assemble them in a filtering function

   ```
   ff <- filterfun(f1,f2)
   ```

3. Finally, apply the filtering function

   ```
   wh <- genefilter(marrayDat, ff)
   ```

4. Use **wh** to obtain a microarray object for the relevant gene subset

   ```
   mySub <- marrayDat[wh,]
   ```

Differential expression

- Identify genes whose expression levels are associated with a response or covariate of interest
  - clinical outcome such as survival, response to treatment, tumor class;
  - covariate such as treatment, dose, time.
- Estimation: estimate effects of interest and variability of these estimates.

  E.g. Slope, interaction, or difference in means.
- Testing: assess the statistical significance of the observed associations.

Multiple hypothesis testing

- Large multiplicity problem: thousands of hypotheses are tested simultaneously!
  - Increased chance of false positives.
  - E.g. Chance of at least one p-value < α for G independent tests is $1-(1-\alpha)^G$

    and converges to one as G increases.

    For G=1,000 and $\alpha = 0.01$, this chance is 0.9999568!
  - Individual p-values of 0.01 no longer correspond to significant findings.

- Need to adjust for multiple testing when assessing the statistical significance of the observed associations.

Multiple hypothesis testing

- Define an appropriate Type I error or false positive rate.
- Apply multiple testing procedures that
  - control this error rate under the true unknown data generating distribution,
  - are powerful (few false negatives),
  - take into account the joint distribution of the test statistics.
- Report adjusted p-values for each gene which reflect the overall Type I error rate for the experiment.
- Use resampling methods to deal with the unknown joint distribution of the test statistics.

multtest package

- Multiple testing procedures for controlling
  - Family-Wise Error Rate (FWER): Bonferroni, Holm (1979), Hochberg (1986), Westfall & Young (1993) maxT and minP;
  - False Discovery Rate (FDR): Benjamini & Hochberg (1995), Benjamini & Yekutieli (2001).
- Tests based on t- or F-statistics for one- and two-factor designs.
- Permutation procedures for estimating adjusted p-values.
- Fast permutation algorithm for minP adjusted p-values.
- Documentation: tutorial on multiple testing.

`limma` package

- Fitting of gene-wise linear models to estimate log ratios between two or more target samples simultaneously: `lm.series`, `rlm.series`, `glm.series` (handle replicate spots).
- `ebayes`: moderated t-statistics and log-odds of differential expression by empirical Bayes shrinkage of the standard errors towards a common value.

Distances, Prediction, and Cluster Analysis

Supervised vs. unsupervised learning

- Unsupervised learning a.k.a. cluster analysis
  - the classes are unknown a priori;
  - the goal is to discover these classes from the data.
- Supervised learning a.k.a. class prediction
  - the classes are predefined;
  - the goal is to understand the basis for the classification from a set of labeled objects and to build a predictor for future unlabeled observations.

- *Details in lectures from Dec. 2002 course at Fred Hutchinson Cancer Research Center.*

Distances

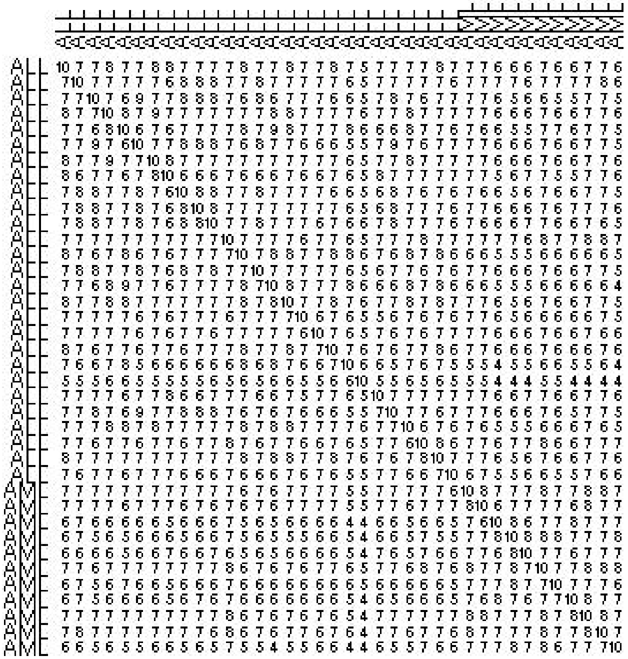
- Microarray data analysis often involves
  - clustering genes and/or samples;
  - classifying genes and/or samples.
- Both types of analyses are based on a measure of distance (or similarity) between genes or samples.
- R has a number of functions for computing and plotting distance and similarity matrices.

Distances

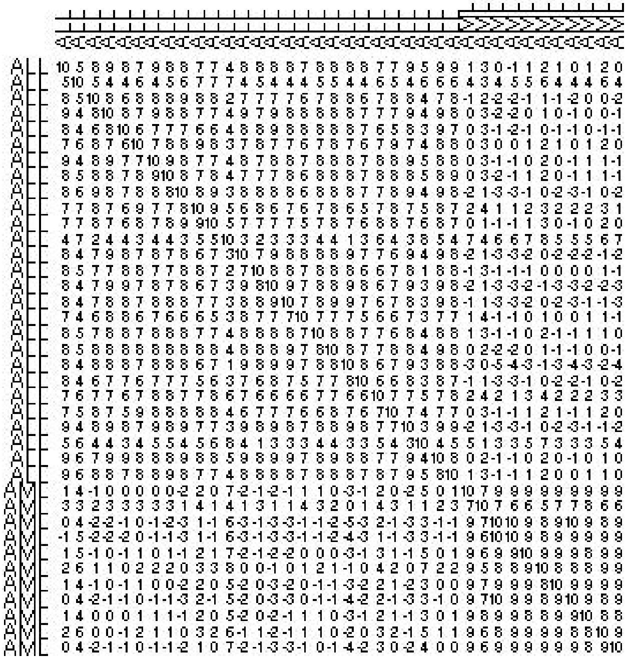
- Distance functions
  - `dist` (`mva`): Euclidean, Manhattan, Canberra, binary;
  - `daisy` (`cluster`).
- Correlation functions
  - `cor`, `cov.wt`.
- Plotting functions
  - `image`;
  - `plotcorr` (`ellipse`);
  - `plot.cor`, `plot.mat` (`sma`).

Correlation matrices

Correlation matrix for ALL AML data
G=3,051 genes

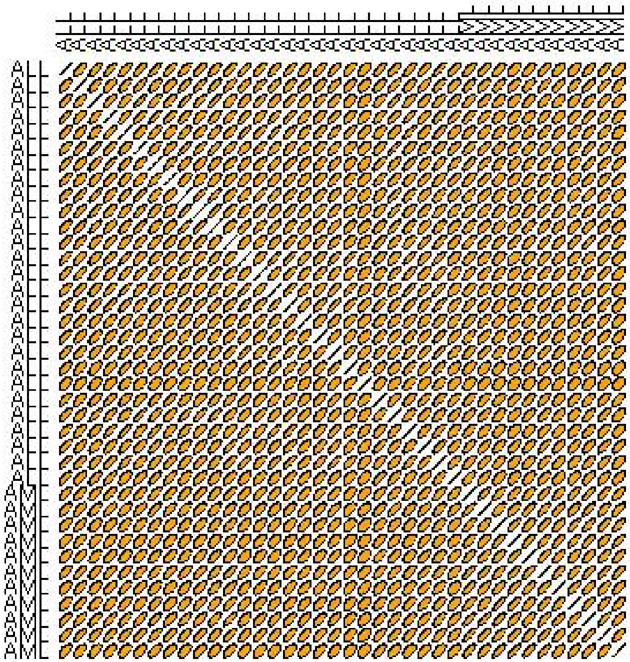


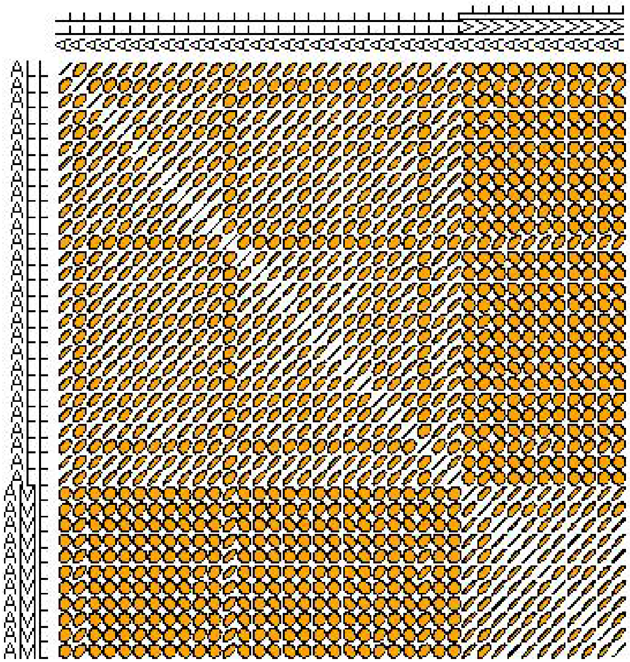
Correlation matrix for ALL AML data
G=39 genes with maxT adjusted p-value < 0.01



`plotcorr` function from `ellipse` package

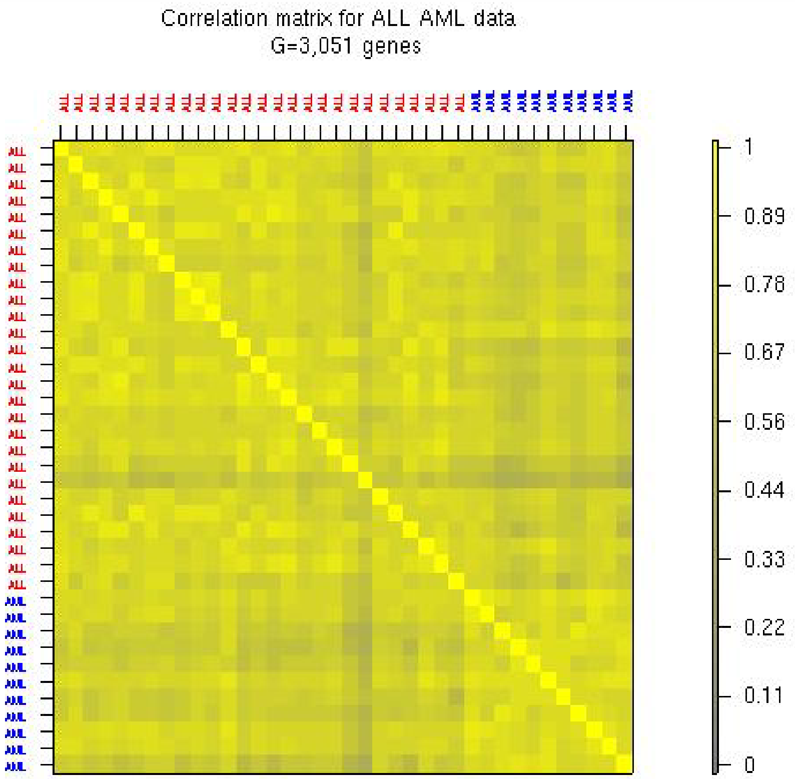
Correlation matrices


Correlation matrix for ALL AML data
G=3,051 genes

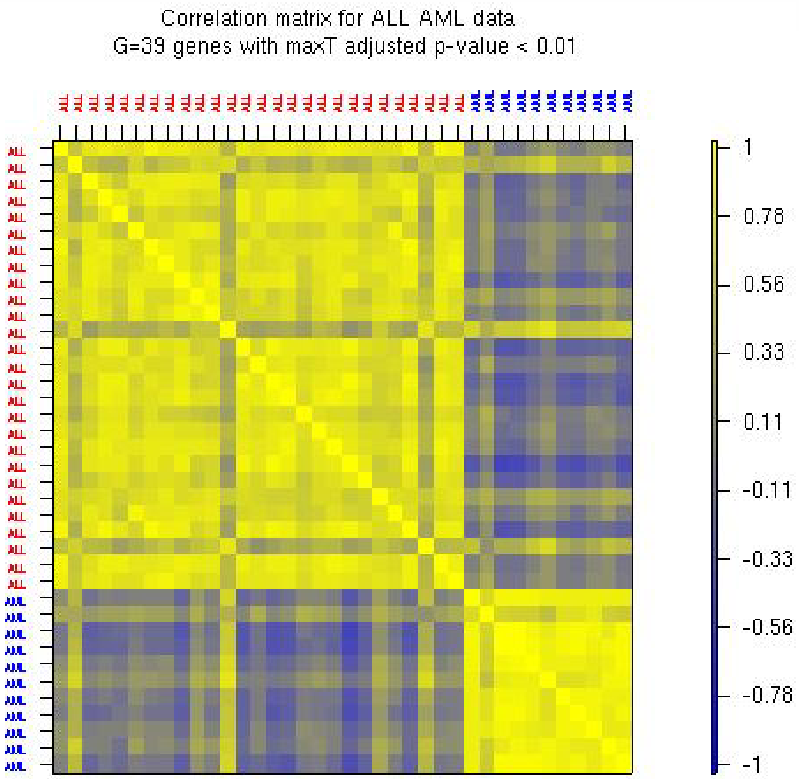

Correlation matrix for ALL AML data
G=39 genes with maxT adjusted p-value < 0.01

`plotcorr` function from `ellipse` package

Correlation matrices



Correlation matrix for ALL AML data
G=3,051 genes



Correlation matrix for ALL AML data
G=39 genes with maxT adjusted p-value < 0.01

`plot.cor` function from `sma` package

Multidimensional scaling

- Given any *n x n* distance matrix *D*, multidimensional scaling (MDS) is concerned with identifying *n* points in Euclidean space with a similar distance structure *D'*.
- The purpose is to provide a lower dimensional representation of the distances which conveys information on the relationships between the *n* objects, such as the existence of clusters or one-dimensional structure in the data (e.g., seriation).

MDS

- There are different approaches for reducing dimensionality, depending on how one defines similarity between the old and new distance matrices for the *n* objects, i.e., depending on the objective or stress function *S* that one seeks to minimize.
  - Least-squares scaling

    $$S(D,D') = \left(\sum (d_{ij} - d'_{ij})^2\right)^{1/2}$$

  - Sammon mapping places more emphasis on smaller dissimilarities (and hence should be preferred for clustering methods)
  - $$S(D,D') = \sum (d_{ij} - d'_{ij})^2 / d_{ij}$$
  - Shepard-Kruskal non-metric scaling is based on ranks, i.e., the order of the distances is more important than their actual values.

MDS and PCA

- When the distance matrix *D* is the Euclidean distance matrix between the rows of an *n x m* matrix *X*, there is a duality between principal component analysis (PCA) and MDS.
- The *k*-dimensional classical solution to the MDS problem is given by the centered scores of the *n* objects on the first *k* principal components.
- The classical solution of MDS in *k*-dimensional space minimizes the sum of squared differences between the entries of the new and old distance matrices, i.e., is optimal for least-squares scaling.

MDS

- As with PCA, the quality of the representation will depend on the magnitude of the first *k* eigenvalues.
- One should choose a value for *k* that is small enough for ease of representation, but also corresponds to a substantial "proportion of the distance matrix explained".

MDS

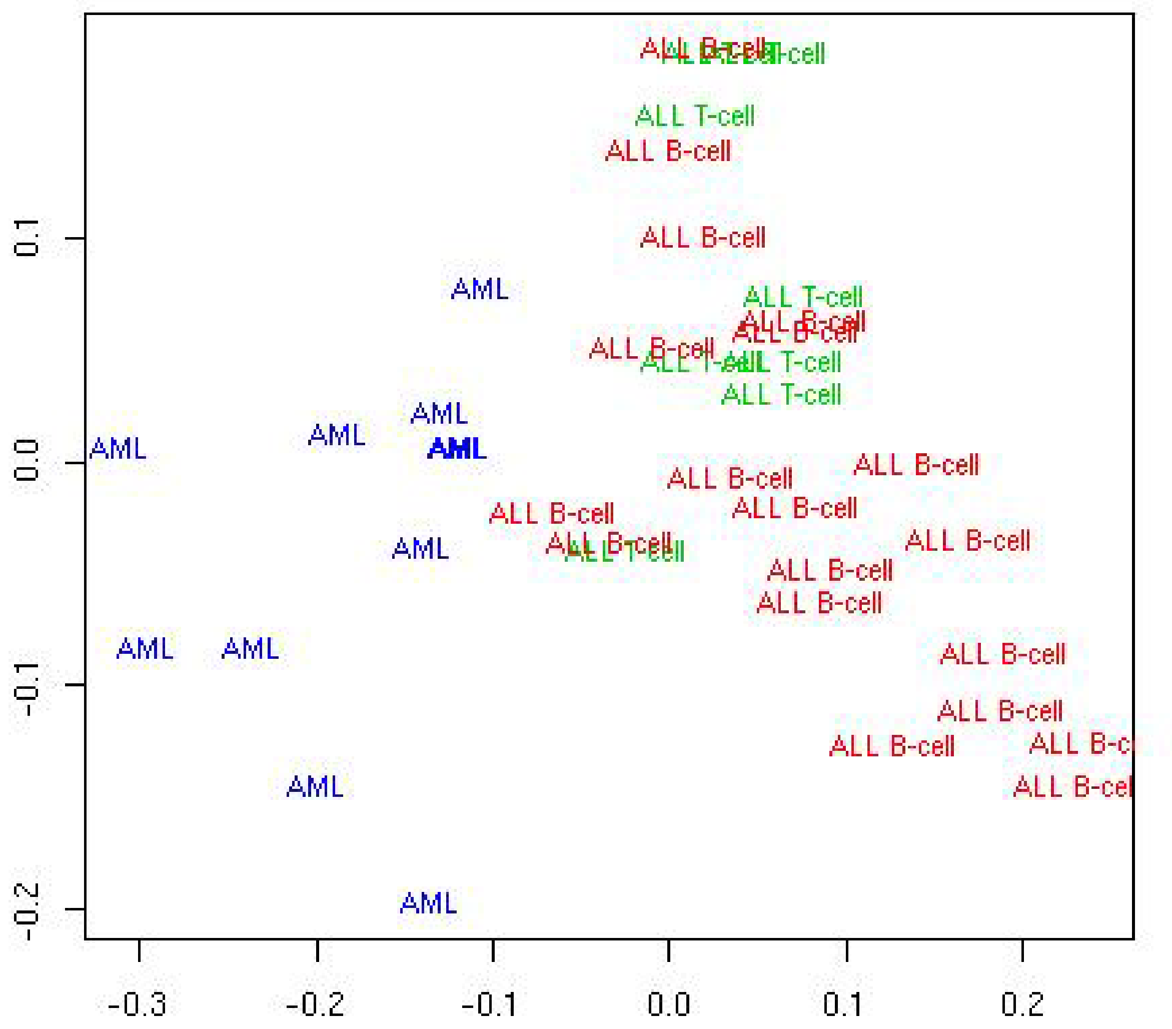
- **N.B.** The MDS solution reflects not only the choice of a distance function, but also the features selected.
- If features (genes) are selected to separate the data into two groups (e.g., on the basis of two-sample t-statistics), it should come as no surprise that an MDS plot has two groups. In this instance, MDS is not a confirmatory approach.

R MDS software

- `cmdscale`: Classical solution to MDS, in package `mva`.
- `sammon`: Sammon mapping, in package `MASS`.
- `isoMDS`: Shepard-Kruskal's non-metric MDS, in package `MASS`.

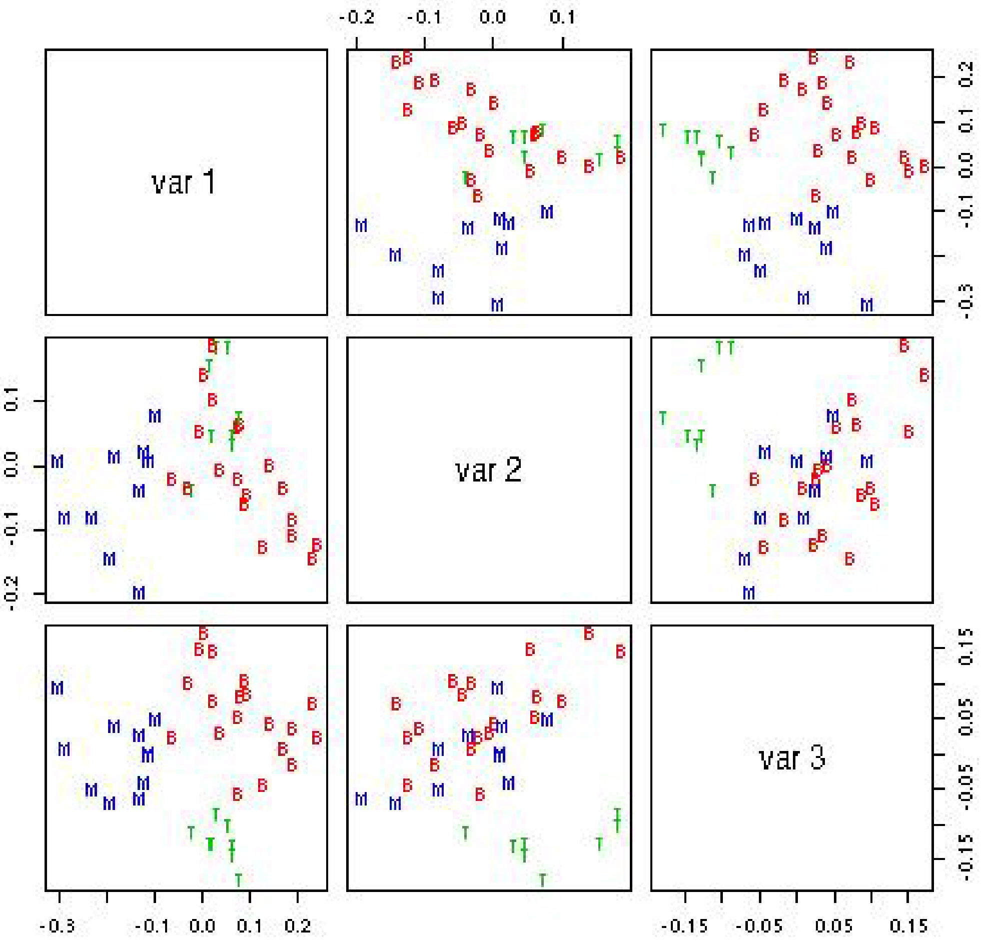
Classical MDS

MDS for ALL AML data, correlation matrix, G=3,051 genes, k=2



Classical MDS

MDS for ALL AML data, correlation matrix, G=3,051 genes, k=3



$$\frac{|\lambda_1|+|\lambda_2|}{\sum|\lambda_i|}=43\%$$

$$\frac{|\lambda_1|+|\lambda_2|+|\lambda_3|}{\sum|\lambda_i|}=55\%$$

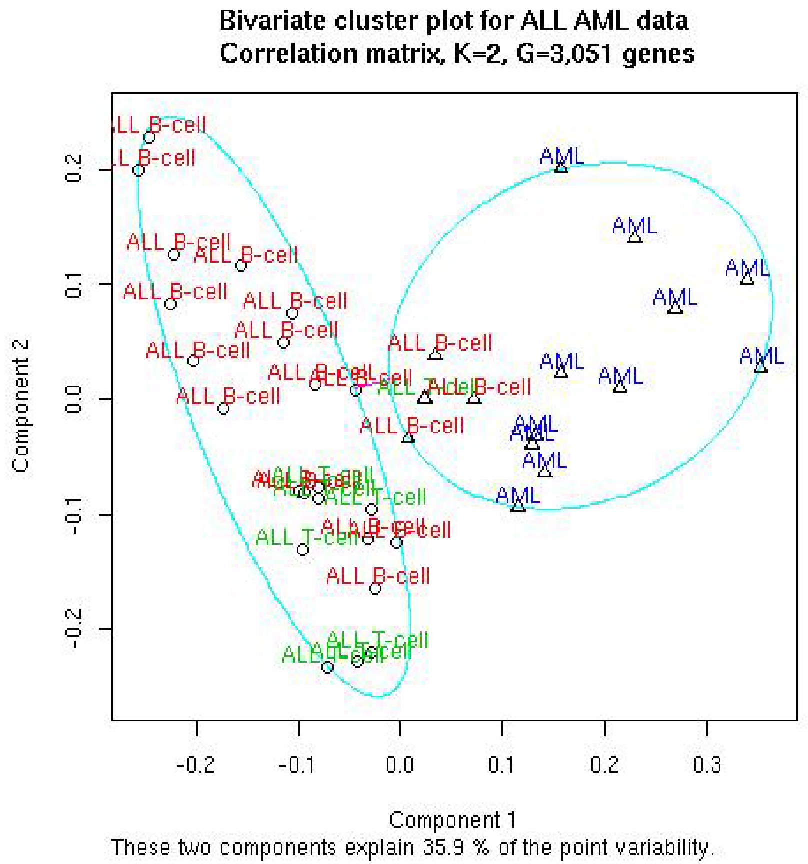
R cluster analysis packages

- `cclust`: convex clustering methods.
- `class`: self-organizing maps (`SOM`).
- `cluster`:
  - AGglomerative NESting (`agnes`),
  - Clustering LARe Applications (`clara`),
  - DIvisive ANAlysis (`diana`),
  - Fuzzy Analysis (`fanny`),
  - MONothetic Analysis (`mona`),
  - Partitioning Around Medoids (`pam`).
- `e1071`:
  - fuzzy C-means clustering (`cmeans`),
  - bagged clustering (`bclust`).
- `flexmix`: flexible mixture modeling.
- `fpc`: fixed point clusters, clusterwise regression and discriminant plots.
- `GeneSOM`: self-organizing maps.
- `mclust, mclust98`: model-based cluster analysis.
- `mva`:
  - hierarchical clustering (`hclust`),
  - k-means (`kmeans`).
- Specialized summary, plot, and print methods for clustering results.

Download from CRAN

PAM

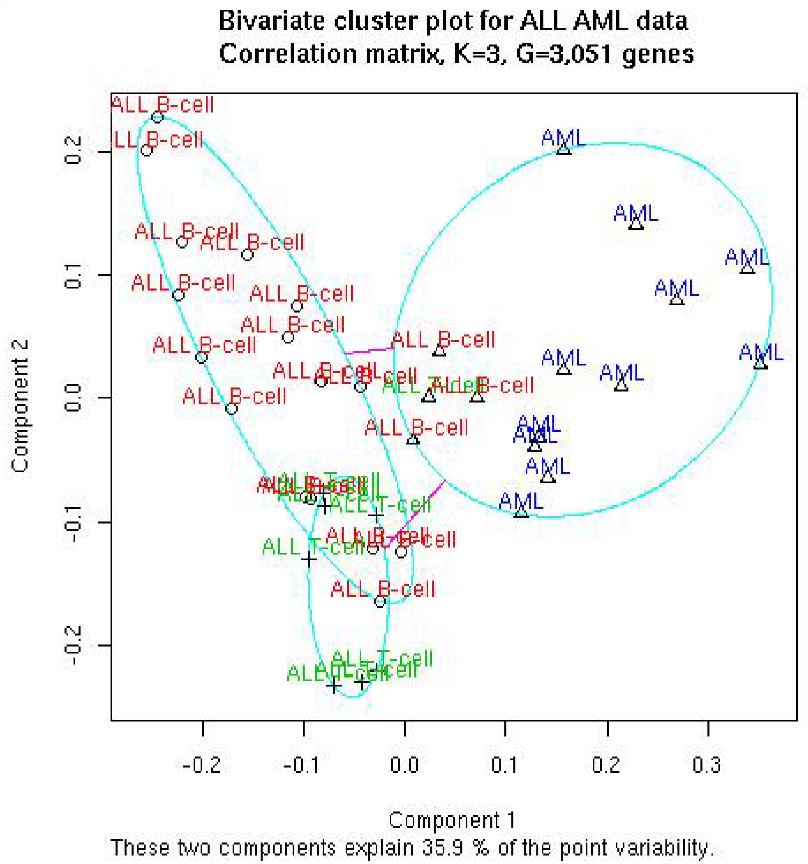
K=2



Bivariate cluster plot for ALL AML data
Correlation matrix, K=2, G=3,051 genes

These two components explain 35.9 % of the point variability.

K=3



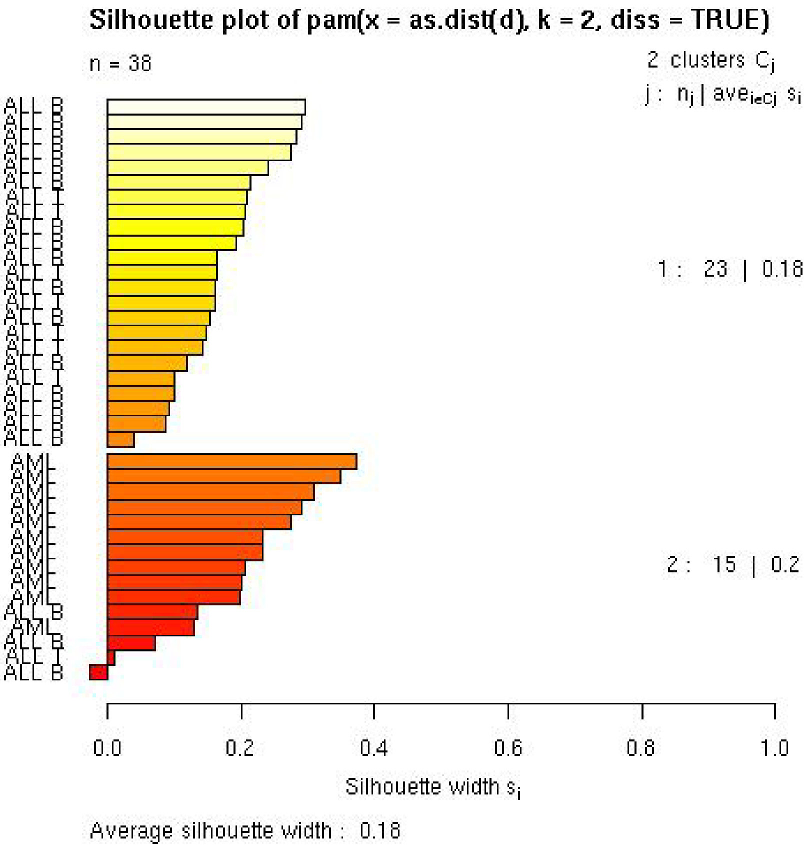
Bivariate cluster plot for ALL AML data
Correlation matrix, K=3, G=3,051 genes

These two components explain 35.9 % of the point variability.

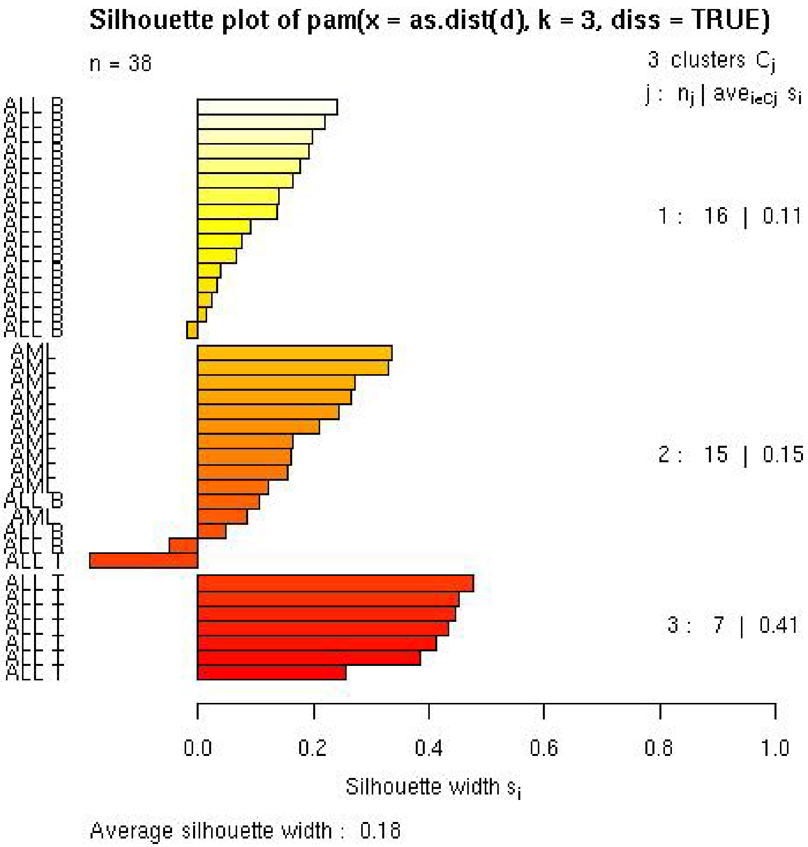
`pam` and `clusplot` functions from `cluster` package

PAM

K=2

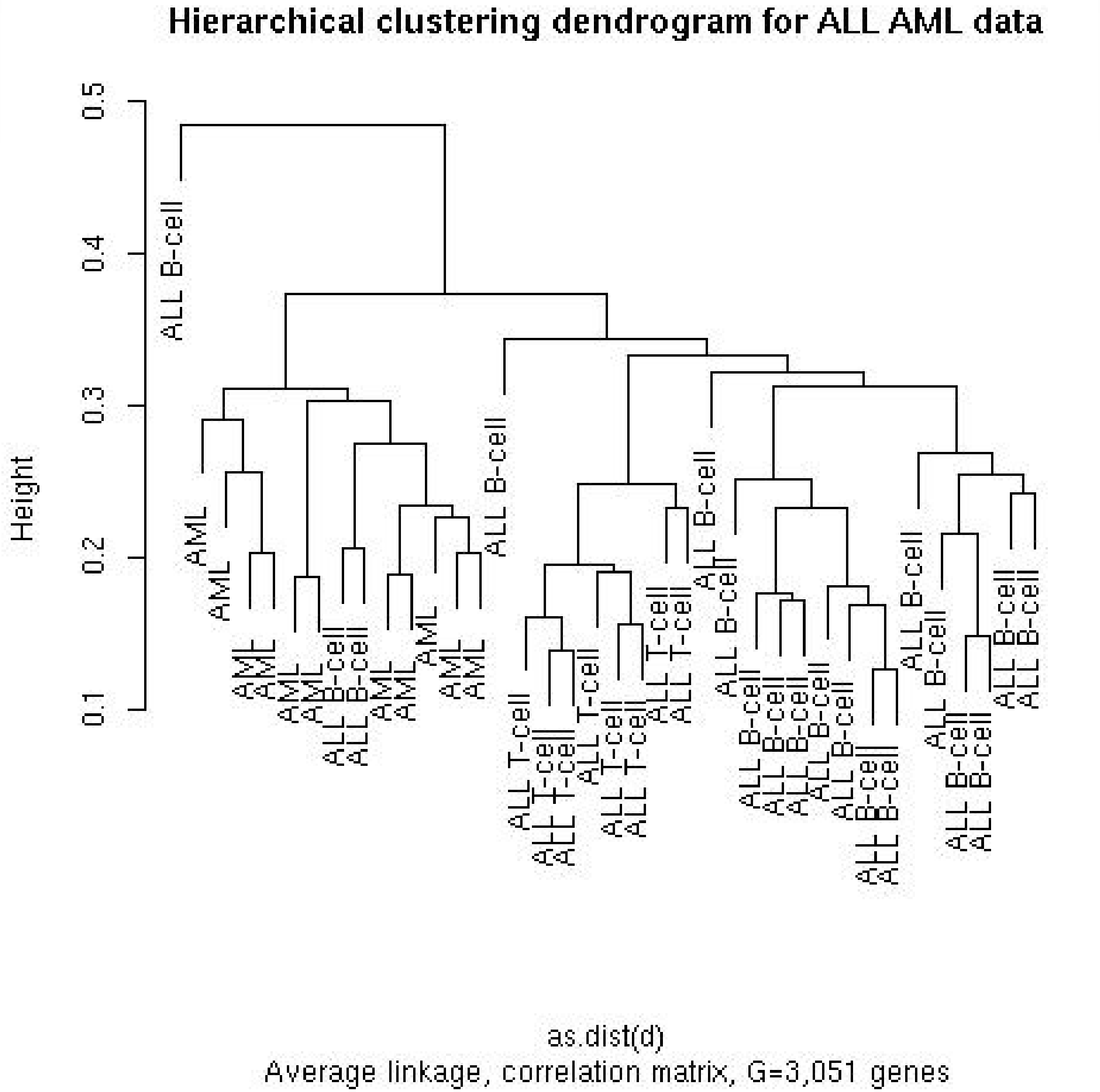


K=3



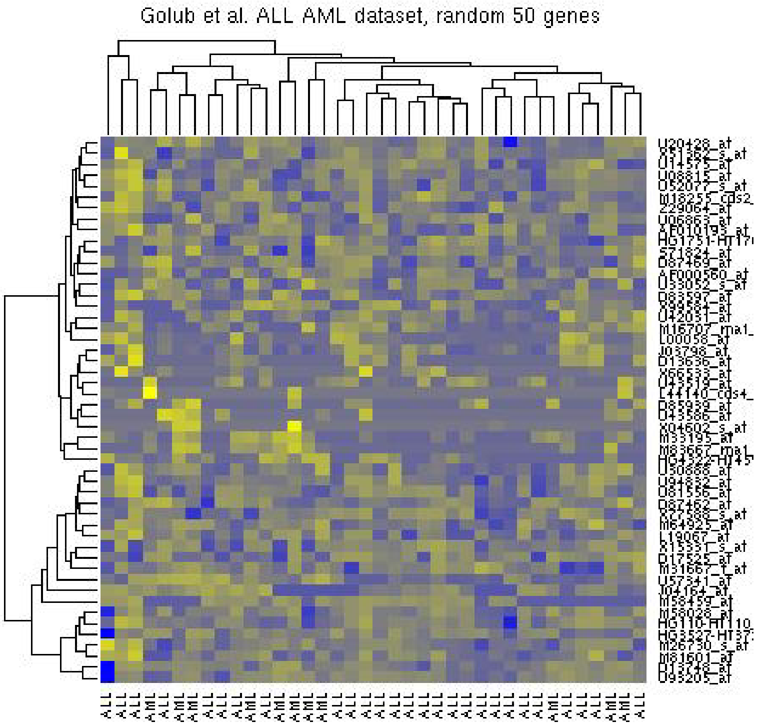
`pam` and `plot` functions from `cluster` package

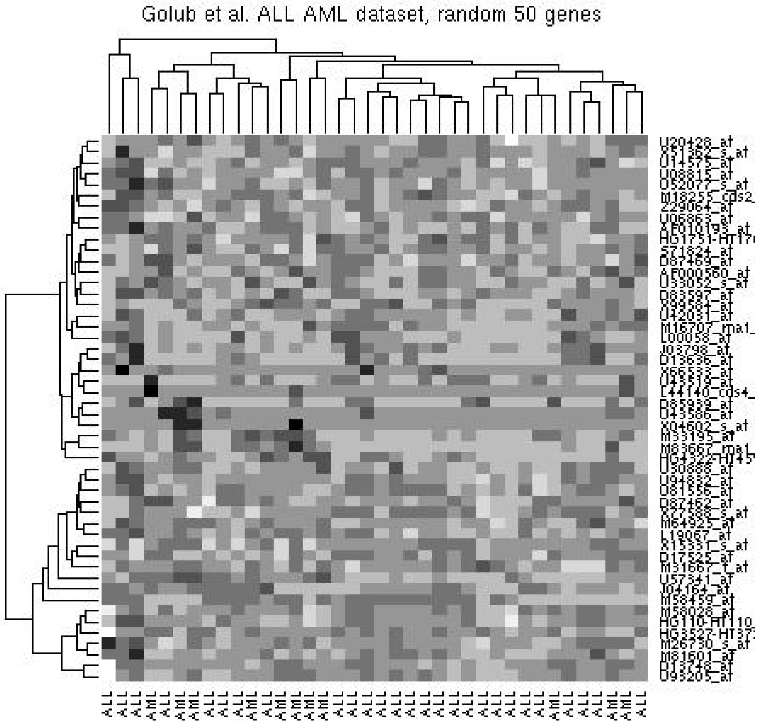
Hierarchical clustering



`hclust` function from `mva` package

Heatmaps





`heatmap` function from `mva` package

Dendrograms

- **N.B.** While dendrograms are appealing because of their apparent ease of interpretation, they can be misleading.
- First, the dendrogram corresponding to a given hierarchical clustering is not unique, since for each merge one needs to specify which subtree should go on the left and which on the right --- there are 2^*(n-1)* choices.
- The default in the R function **`hclust`** is to order the subtrees so that the tighter cluster is on the left.

Dendrograms

- Second, dendrograms *impose* structure on the data, instead of *revealing* structure in these data.
- Such a representation will be valid only to the extent that the pairwise distances possess the hierarchical structure imposed by the clustering algorithm.

Dendrograms

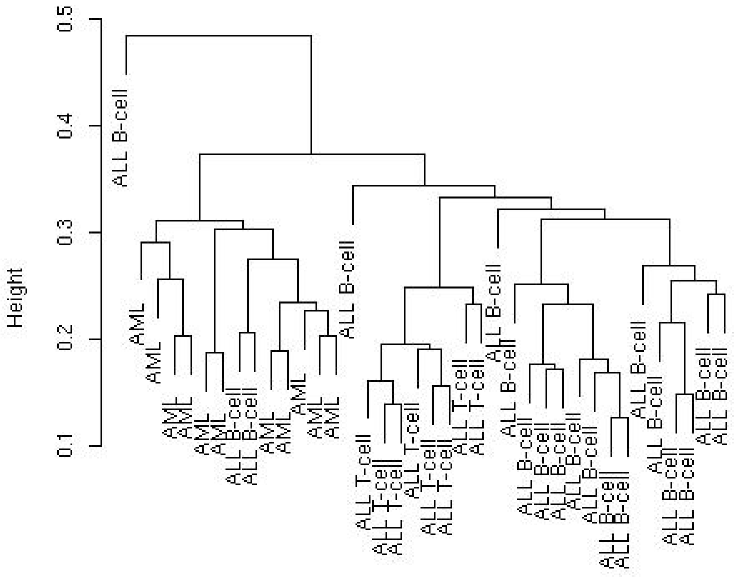
- The cophenetic correlation coefficient can be used to measure how well the hierarchical structure from the dendrogram represents the actual distances.
- This measure is defined as the correlation between the *n(n-1)/2* pairwise distances between observations and their cophenetic dissimilarities, i.e., the between cluster distances at which two observations are first joined together in the same cluster.
- Function **`cophenetic`** in **`mva`** package.

Dendrograms

Original data,
coph corr = 0.74.

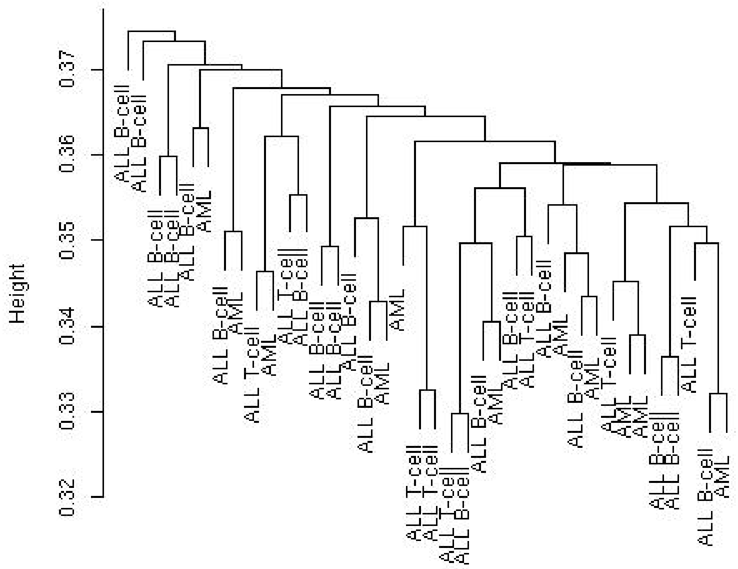
Randomized data
(perm. wi features),
coph corr = 0.57.

Hierarchical clustering dendrogram for ALL AML data



as.dist(d)
Average linkage, correlation matrix, G=3,051 genes

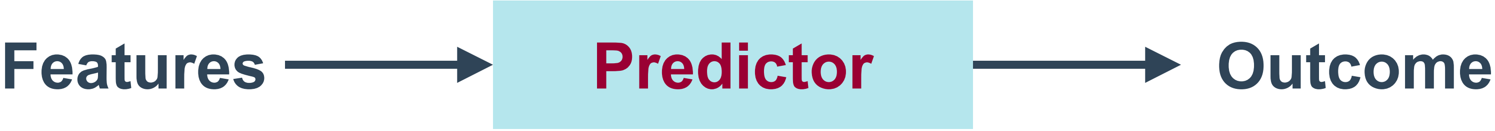
Hierarchical clustering dendrogram for randomized ALL AML data



as.dist(d0)
Average linkage, correlation matrix, G=3,051 genes

Prediction

- Predict an outcome on the basis of observable explanatory variables or features.



Features → Predictor → Outcome

- Outcome:
  - Polychotomous: tumor class, type of bacterial infection, response to treatment --- classifier.
  - Continuous: survival.
  - Possibly censored!
- Features: gene expression measures, covariates such as age, sex.

Class prediction

- Old and extensive literature on class prediction, in statistics and machine learning.
- Examples of classifiers
  - nearest neighbor classifiers (k-NN);
  - discriminant analysis: linear, quadratic, logistic;
  - neural networks;
  - classification trees;
  - support vector machines.
- Aggregated classifiers: bagging and boosting.
- Comparison on microarray data:

  simple classifiers like k-NN and naïve Bayes perform remarkably well.

R class prediction packages

Download from CRAN

- `class`:
  - k-nearest neighbor (`knn`),
  - learning vector quantization (`lvq`).
- `classPP`: projection pursuit.
- `e1071`: support vector machines (`svm`).
- `ipred`: bagging, resampling based estimation of prediction error.
- `knnTree`: k-nn classification with variable selection inside leaves of a tree.
- `LogitBoost`: boosting for tree stumps.
- `MASS`: linear and quadratic discriminant analysis (`lda`, `qda`).
- `mlbench`: machine learning benchmark problems.
- `nnet`: feed-forward neural networks and multinomial log-linear models.
- `pamR`: prediction analysis for microarrays.
- `randomForest`: random forests.
- `rpart`: classification and regression trees.
- `sma`: diagonal linear and quadratic discriminant analysis, naïve Bayes (`stat.diag.da`).

Performance assessment

- Classification error rates, or related measures, are usually reported
  - to compare the performance of different classifiers;
  - to support statements such as "*clinical outcome X for cancer Y can be predicted accurately based on gene expression measures*".
- Classification error rates can be estimated by resampling, e.g., bootstrap or cross-validation.

Performance assessment

- It is essential to take into account feature selection and other training decisions in the error rate estimation process.

  E.g. Number of neighbors in k-NN, kernel in SVMs.

- Otherwise, error estimates can be severely biased downward, i.e., overly optimistic.

Other important issues

- Loss function;
- Censoring;
- Standardization;
- Distance function;
- Feature selection;
- Class priors;
- Binary vs. polychotomous classification.