limma: Linear Models for Microarray Data User's Guide

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1 Introduction

Limma is a package for the analysis of gene expression microarray data, especially the use of linear models for analysing designed experiments and the assessment of differential expression. Limma provides the ability to analyse comparisons between many RNA targets simultaneously. It has features which make the analyses stable even for experiments with small number of arrays—this is achieved by borrowing information across genes. The normalization and exploratory data analysis functions are for two-colour spotted microarrays. The linear model and differential expression functions apply to all microarrays including Affymetrix and other single-channel microarray experiments.

This guide gives a tutorial-style introduction to the main limma features but does not describe every feature of the package. A full description of the package is given by the individual function help documents available from the R online help system. To access the online help, type `help(package=limma)` at the R prompt or else start the html help system using `help.start()` or the Windows drop-down help menu.

The Bioconductor package marray provides alternative functions for reading and normalizing spotted microarray data. The marray package provides flexible location and scale normalization routines for log-ratios from two-color arrays. The limma package overlaps with marray in functionality but is based on a more general separation between within-array and between-array normalization. If you are using limma in conjunction with the marray package, see Section 10. The Bioconductor package affy provides functions for reading and normalizing Affymetrix microarray data. If you are using the affy package, see Section 7.2 and the relevant case studies.

This guide describes limma as a command-driven package. Packages limmaGUI and affylmGUI are also available which provides graphical user interfaces to the most commonly

used functions in limma (Wettenhall and Smyth, 2004). Both packages are available from Bioconductor or from http://bioinf.wehi.edu.au/limmaGUI. The package limmaGUI is for use with two-color data while affImGUI is for Affymetrix data.

This tutorial was prepared using R Version 1.9.0 for Windows and limma version 1.6.7.

Help with limma is available by sending questions or problems to the Bioconductor mailing list bioconductor@stat.math.ethz.ch.

2 Installation

Limma is a package for the R computing environment and it is assumed that you have already installed R. See the R project at http://www.r-project.org.

Installing from CRAN. Limma is available as a contributed package from the R Project CRAN site. This is the recommended repository from which to obtain limma. If you are using R on a system with a suitable internet connection and with installation privileges on your computer, you should be able to install it via

```
> install.packages("limma")
```

at the R prompt from an internet-connected computer. If you are using Windows, use the drop-down menu [Packages > Install package(s) from CRAN ...].

Installing from WEHI. The limma home page is http://bioinf.wehi.edu.au/limma. The latest version of the package is always available from this page, sometimes a few days ahead of the CRAN site. Unlike CRAN, this page supports only the latest release of R (not the developmental version) and does not provide a Mac package build. You should be able to install limma from this page using

```
> install.packages("limma",contriburl="http://bioinf.wehi.edu.au/limma")
```

at the R prompt.

Installing from Bioconductor. Limma is available as part of the Bioconductor project at http://www.bioconductor.org. Bioconductor works on a 6-monthly official release cycle, lagging each major R release by a few weeks. This means that Bioconductor software is updated only once every six months, unless you are using the developmental version R. Updates of limma between the Bioconductor official releases should be obtained from one of the above two sites.

Change-log. Limma is updated frequently, often a couple of times a week. The change-log can be viewed at http://bioinf.wehi.edu.au/limma/changelog.txt. It can also be viewed from the R prompt. To see the most recent 20 lines type:

```
> changeLog(n=20)
```

Data sets used in this manual. The data sets used in the case study examples can be downloaded from http://bioinf.wehi.edu.au/marray/genstat2002.

3 A Few Preliminaries on R

R is a program for statistical computing. It is a command-driven language meaning that you have to type commands into it rather than pointing and clicking using a mouse. A good way to get started is to type

```
> help.start()
```

at the R prompt or, if you're using Windows, to follow the drop-down menu [Help > Html help]. Following the links [Packages > limma] from the html help page will lead you to the contents page of help topics for commands in limma.

Before you can use any limma commands you have to load the package by typing

```
> library(limma)
```

at the R prompt. You can get help on any function in any loaded package by typing `?` and the function name at the R prompt, for example

```
> ?read.maimages
```

for detailed help on the `read.maimages` function. Anything that you create in R is an "object". Objects might include data sets, variables, functions, anything at all. For example

```
> x <- 2
```

will create a variable `x` and will assign it the value 2. At any stage of your R session you can type

```
> objects()
```

to get a list of all the objects you have created. You see show the contents of any object by typing the name of the object at the prompt, for example either of the following commands will print out the contents of `x`:

```
> show(x)
> x
```

We hope that you can use limma without having to spend a lot of time learning about the R language itself but a little knowledge in this direction will be very helpful, especially when you want to do something not explicitly provided for in limma or in the other Bioconductor packages. For more details about the R language see *An Introduction to R* which is available from the online help.

4 Quick Start

For those who want to see very quickly what a limma analysis might look like for cDNA data, here is a quick analysis of four replicate arrays (including two dye-swaps). The data has been scanned using an Axon scanner, producing a GenePix Array List (GAL) file, and then the intensities have been captured from the images using SPOT software. The GAL file and the image analysis files are in the current working directory of R. For more detail about the data see the Swirl Data example below.

```
          # Read file containing info about the hybridizations,
          # including names of files containing the intensity data
> targets <- readTargets("SwirlSample.txt")
          # Read in the data
> RG <- read.maimages(targets$FileName, source="spot")
          # Read in GAL file containing gene names, only need if using SPOT!
> RG$genes <- readGAL()
          # Set printer layout information
> RG$printer <- getLayout(RG$genes)
          # Print-tip group loess normalization
> MA <- normalizeWithinArrays(RG)
          # Scale normalization between arrays, optional
> MA <- normalizeBetweenArrays(MA)
          # Estimate all the fold changes by fitting a linear model.
          # The design matrix indicates which arrays are dye-swaps
> fit <- lmFit(MA, design=c(-1,1,-1,1))
          # Apply Bayesian smoothing to the standard errors (very important!)
> fit <- eBayes(fit)
> options(digits=3)
          # Show the top 30 genes, control false discovery rate
> topTable(fit, n=30, adjust="fdr")

     Block Row Column      ID   Name     M    A     t  P.Value    B
3721     8   2      1 control   BMP2 -2.21 12.1 -21.1 0.000357 7.96
1609     4   2      1 control   BMP2 -2.30 13.1 -20.3 0.000357 7.78
3723     8   2      3 control   Dlx3 -2.18 13.3 -20.0 0.000357 7.71
1611     4   2      3 control   Dlx3 -2.18 13.5 -19.6 0.000357 7.62
8295    16  16     15 fb94h06 20-L12  1.27 12.0  14.1 0.002067 5.78
7036    14   8      4 fb40h07  7-D14  1.35 13.8  13.5 0.002067 5.54
515      1  22     11 fc22a09 27-E17  1.27 13.2  13.4 0.002067 5.48
5075    10  14     11 fb85f09 18-G18  1.28 14.4  13.4 0.002067 5.48
7307    14  19     11 fc10h09 24-H18  1.20 13.4  13.2 0.002067 5.40
319      1  14      7 fb85a01  18-E1 -1.29 12.5 -13.1 0.002067 5.32
2961     6  14      9 fb85d05 18-F10 -2.69 10.3 -13.0 0.002067 5.29
4032     8  14     24 fb87d12 18-N24  1.27 14.2  12.8 0.002067 5.22
6903    14   2     15 control    Vox -1.26 13.4 -12.8 0.002067 5.20
4546     9  14     10 fb85e07 18-G13  1.23 14.2  12.8 0.002067 5.18
683      2   7     11 fb37b09  6-E18  1.31 13.3  12.4 0.002182 5.02
1697     4   5     17 fb26b10  3-I20  1.09 13.3  12.4 0.002182 4.97
7491    15   5      3 fb24g06  3-D11  1.33 13.6  12.3 0.002182 4.96
4188     8  21     12 fc18d12 26-F24 -1.25 12.1 -12.2 0.002209 4.89
4380     9   7     12 fb37e11  6-G21  1.23 14.0  12.0 0.002216 4.80
3726     8   2      6 control  fli-1 -1.32 10.3 -11.9 0.002216 4.76
2679     6   2     15 control    Vox -1.25 13.4 -11.9 0.002216 4.71
5931    12   6      3 fb32f06  5-C12 -1.10 13.0 -11.7 0.002216 4.63
7602    15   9     18 fb50g12  9-L23  1.16 14.0  11.7 0.002216 4.63
2151     5   2     15 control   vent -1.40 12.7 -11.7 0.002216 4.62
3790     8   4     22 fb23d08  2-N16  1.16 12.5  11.6 0.002221 4.58
7542    15   7      6 fb36g12  6-D23  1.12 13.5  11.0 0.003000 4.27
4263     9   2     15 control   vent -1.41 12.7 -10.8 0.003326 4.13
6375    13   2     15 control   vent -1.37 12.5 -10.5 0.004026 3.91
1146     3   4     18 fb22a12  2-I23  1.05 13.7  10.2 0.004242 3.76
```

```
157      1   7     13 fb38a01   6-I1 -1.82 10.8 -10.2 0.004242 3.75
```

5 Reading Data into Limma

This chapter is for two-color arrays. If you are using Affymetrix arrays, you should use the affy or affyPLM packages to read and normalize the data. If you have single channel arrays others than Affymetrix, you will need to the read the intensity data into your R session yourself using the basic R read functions such as `read.table`. You will need to create a matrix containing the log-intensities with rows for probes and columns are arrays.

5.1 Recommended Files

We assume that an experiment has been conducted with one or more microarrays, all printed with the same library of probes. Each array has been scanned to produce a TIFF image. The TIFF images have then been processed using an image analysis program such a ArrayVision, ImageGene, GenePix, QuantArray or SPOT to acquire the red and green foreground and background intensities for each spot. The spot intensities have then been exported from the image analysis program into a series of text files. There should be one file for each array or, in the case of Imagene, two files for each array.

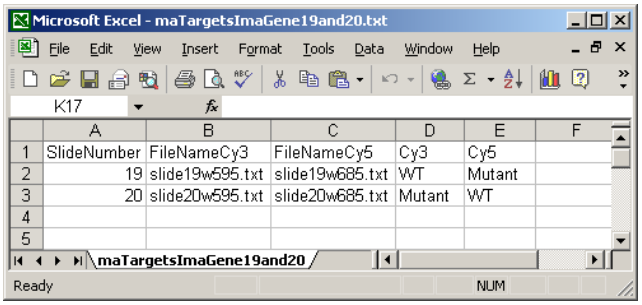
You will need to have the image analysis output files. In most cases these files will include the IDs and names of the probes and possibly other annotation information. A few image analysis programs, for example SPOT, do not write the probe IDs into the output files. In this case you will also need a genelist file which describes the probes. It most cases it is also desirable to have a *Targets File* which describes which RNA sample was hybridized to each channel of each array. A further optional file is the *Spot Types* file which identifies special probes such as control spots.

5.2 The Targets File

The first step in preparing your data is usually to create a Targets File which lists the RNA target hybridized to each channel of each array. It is normally in tab-delimited text format. It should contain a row for each microarray in your experiment. It should contain a FileName column, giving the file from image-analysis containing raw foreground and background intensities for each slide, a Cy3 column giving the RNA type reverse transcribed and labelled with Cy3 dye for that slide (e.g., Wild Type) and a Cy5 column giving the RNA type reverse transcribed and labelled with Cy5 dye for that slide. For ImaGene files, the FileName column is split into a FileNameCy3 column and a FileNameCy5. As well as the essential columns, you can have a Name column giving an alternative slide name to the default name, "Slide n", where n is the SlideNumber and you can have a Date column, listing the date of the hybridization, and as many extra columns as you like, as long as the column names are unique. Some examples are shown below.

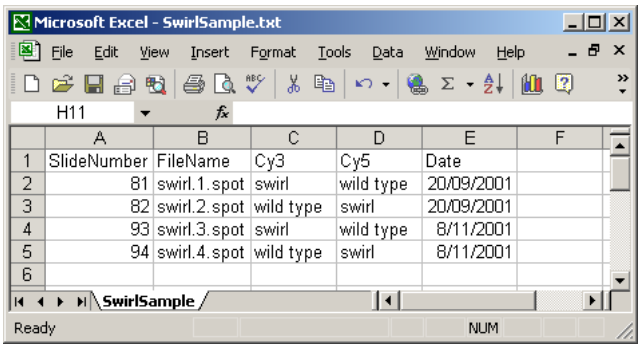
The ImaGene Targets file below shows the special case of the ImaGene image-processing software which gives two (tab-delimited text) output files for each slide, one for the Cy3

(Green) channel and one for the Cy5 (Red) channel. So instead of having a single FileName column, there are two file name columns: a FileNameCy3 column and a FileNameCy5 column.



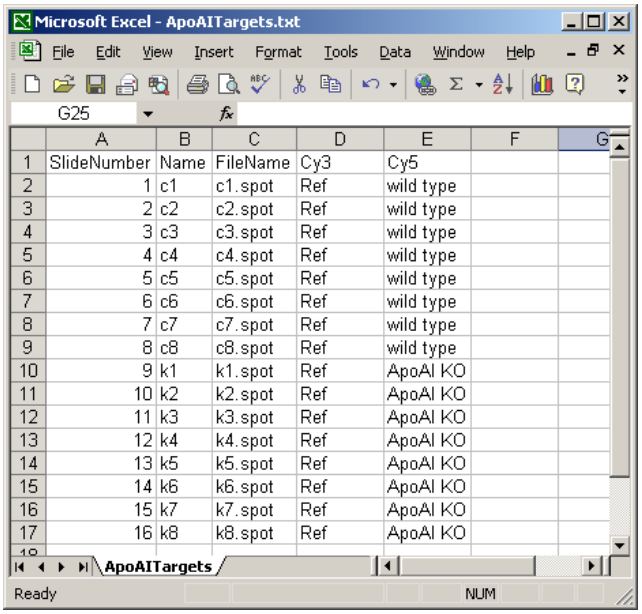
	A	B	C	D	E	F
1	SlideNumber	FileNameCy3	FileNameCy5	Cy3	Cy5	
2	19	slide19w595.txt	slide19w685.txt	WT	Mutant	
3	20	slide20w595.txt	slide20w685.txt	Mutant	WT	
4						
5						

The `Date` column is optional and is not currently used in limma.



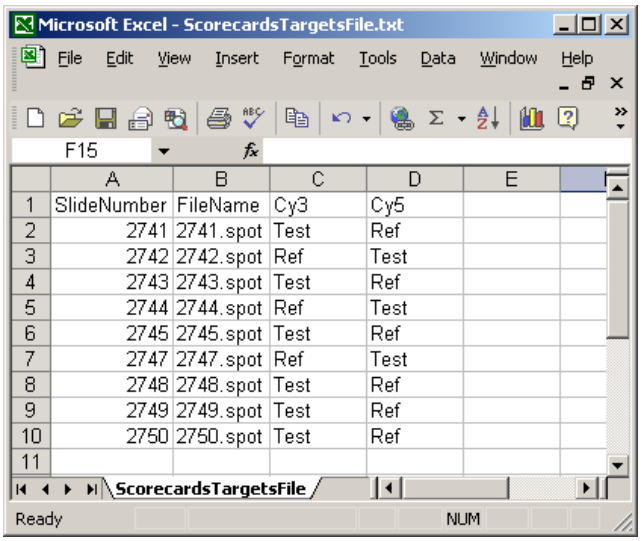
	A	B	C	D	E	F
1	SlideNumber	FileName	Cy3	Cy5	Date	
2	81	swirl.1.spot	swirl	wild type	20/09/2001	
3	82	swirl.2.spot	wild type	swirl	20/09/2001	
4	93	swirl.3.spot	swirl	wild type	8/11/2001	
5	94	swirl.4.spot	wild type	swirl	8/11/2001	
6						

A `Name` column can be included, giving each array a name which can be used for plotting. In this case, a short name is used so that a boxplot of all sixteen arrays can be plotted with labels for all arrays along the horizontal axis. If no `Name` column is given, then a default name will be given to each slide, e.g. "Slide 1".



	A	B	C	D	E	F	G
1	SlideNumber	Name	FileName	Cy3	Cy5		
2	1	c1	c1.spot	Ref	wild type		
3	2	c2	c2.spot	Ref	wild type		
4	3	c3	c3.spot	Ref	wild type		
5	4	c4	c4.spot	Ref	wild type		
6	5	c5	c5.spot	Ref	wild type		
7	6	c6	c6.spot	Ref	wild type		
8	7	c7	c7.spot	Ref	wild type		
9	8	c8	c8.spot	Ref	wild type		
10	9	k1	k1.spot	Ref	ApoAI KO		
11	10	k2	k2.spot	Ref	ApoAI KO		
12	11	k3	k3.spot	Ref	ApoAI KO		
13	12	k4	k4.spot	Ref	ApoAI KO		
14	13	k5	k5.spot	Ref	ApoAI KO		
15	14	k6	k6.spot	Ref	ApoAI KO		
16	15	k7	k7.spot	Ref	ApoAI KO		
17	16	k8	k8.spot	Ref	ApoAI KO		

The Targets file below is from an experiment with four different RNA sources. The main Targets file is not shown. The one below is used to analyse the spiked-in scorecard controls. Spike-in controls will generally be analysed separately from genes because the follow different rules, e.g. for genes, the log-ratio between A and B plus the log-ratio between B and C should equal the log-ratio between A and C, but for scorecard controls, all three log (red/green) ratios may be the same.



	A	B	C	D	E
1	SlideNumber	FileName	Cy3	Cy5	
2	2741	2741.spot	Test	Ref	
3	2742	2742.spot	Ref	Test	
4	2743	2743.spot	Test	Ref	
5	2744	2744.spot	Ref	Test	
6	2745	2745.spot	Test	Ref	
7	2747	2747.spot	Ref	Test	
8	2748	2748.spot	Test	Ref	
9	2749	2749.spot	Test	Ref	
10	2750	2750.spot	Test	Ref	
11					

The Targets File can be read using `readTargets()`:

```
> targets <- readTargets()
```

The file can have any name but the default name is `Targets.txt`.

5.3 Reading in Intensity Data

Let `files` be a character vector containing the names of the image analysis output files. The foreground and background intensities can be read into an `RGList` object using a command of the form

```
RG <- read.maimages(files, source="<imageanalysisprogram>", path="<directory>")
```

where `<imageanalysisprogram>` is the name of the image analysis program and `<directory>` is the full path of the directory containing the files. If the files are in the current R working directory then the argument `path` can be omitted; see the help entry for `setwd` for how to set the current working directory. The file names are usually read from the Targets File. For example, the Targets File `Targets.txt` is in the current working directory together with the SPOT output files, then one might use

```
> targets <- readTargets()
> RG <- read.maimages(targets$FileName, source="spot")
```

If the files are GenePix output files then they might be read using

```
> RG <- read.maimages(targets$FileName, source="genepix")
```

given an appropriate Targets File. Consult the help entry for `read.maimages` to see which other image analysis programs are supported. Files are assumed by default to be tab-delimited. If the files use a different separator this may be specified using the `sep=` argument. For example if the Genepix files were comma-separated (csv) then the read command would be

```
RG <- read.maimages(files, source="genepix", sep=",")
```

What should you do if your image analysis program is not currently supported by limma? If your output files are of a standard format, you can supply the column names corresponding to the intensities yourself. For example,

```
> RG <- read.maimages(files,
       columns=list(Rf="F635 Mean",Gf="F532 Mean",Rb="B635 Median",Gb="B532 Median"))
```

is exactly equivalent to the earlier command with `source="genepix"`. "Standard format" means here that there is a unique column name identifying each column of interest and that there are no lines in the file following the last line of data. Header information at the start of the file is ok.

It is a good idea to look at your data to check that it has been read in correctly. Type

```
> show(RG)
```

to see a print out the first few lines of data. Also try

```
> summary(RG$R)
```

to see a five-number summary of the red intensities for each array, and so on.

It is possible to read the data in several steps. If `RG1` and `RG2` are two data sets corresponding to different sets of arrays then

```
> RG <- cbind(RG1, RG2)
```

will combine them into one large data set. Data sets can also be subsetted. For example `RG[,1]` is the data for the first array while `RG[1:100,]` is the data on the first 100 genes.

5.4 Spot Quality Weights

It is desirable to use the image analysis to compute a weight for each spot between 0 and 1 which indicates the reliability of the acquired intensities at that spot. For example, if the SPOT image analysis program is used and the size of an ideal perfectly circular spot is known to be 100 pixels, then one might use

```
> RG <- read.maimages(files,source="spot",wt.fun=wtarea(100))
```

The function `wtarea(100)` gives full weight to spots with area 100 pixels and down-weights smaller and larger spots. Spots which have zero area or are more than twice the ideal size are given zero weight. This will create a component called `weights` in the RG list. The weights will be used automatically by functions such as `normalizeWithinArrays` which operate on the RG-list. With GenePix data

```
> RG <- read.maimages(files,source="genepix",wt.fun=wtflags(0.1))
```

will give weight 0.1 to any spot which receives a negative flag from the GenePix program.

The appropriate way to computing spot quality weights depends on the image analysis program that you are using. Consult the help entry `QualityWeights` to see what quality weight functions are available. The `wt.fun` argument is very flexible and allows you to construct your own weights. The `wt.fun` argument can be any function which takes a data set as argument and computes the desired weights. For example, if you wish to give zero weight to all Genepix flags less than -50 you could use

```
> myfun <- function(x) as.numeric(x$Flags > -50.5)
> RG <- read.maimages(files, source="genepix", wt.fun=myfun)
```

The `wt.fun` facility is very general and can be used to compute weights based on any number of columns in the image analysis files. For example, some researchers like to filter out spots if the foreground mean and median from GenePix for a given spot differ by more than a certain threshold, say 50. This could be achieved by

```
> myfun <- function(x, threshold=50) {
+    okred <- abs(x[,"F635 Median"]-x[,"F635 Mean"]) < threshold
+    okgreen <- abs(x[,"F532 Median"]-x[,"F532 Mean"]) < threshold
+    as.numeric(okgreen & okred)
+}
> RG <- read.maimages(files, source="genepix", wt.fun=myfun)
```

Then all the "bad" spots will get weight zero which, in limma, is equivalent to flagging them out. The definition of `myfun` here could be replaced with any other code to compute weights using the columns in the GenePix output files.

5.5 Reading the Gene List

In most cases the RGList `RG` read by `read.maimages()` will contain a component `RG$genes` containing the probe IDs and other probe-specific information. In some cases the `genes` component will be not be set because there is no probe information in the image analysis output files. An example is output from the SPOT program. In such cases, the probe information needs to be read separately.

If the arrays have been scanned with an Axon scanner, then the gene names will be available in a GenePix Array List (GAL) file. If the GAL file has extension "gal" and is in the current working directory, then it may be read into a data.frame by

```
> RG$genes <- readGAL()
```

Non-Genepix gene lists can be read into R using the function `read.table` from R base.

Once the gene array list is available, the print layout of the arrays can be extracted from it by

```
> RG$printer <- getLayout(RG$genes)
```

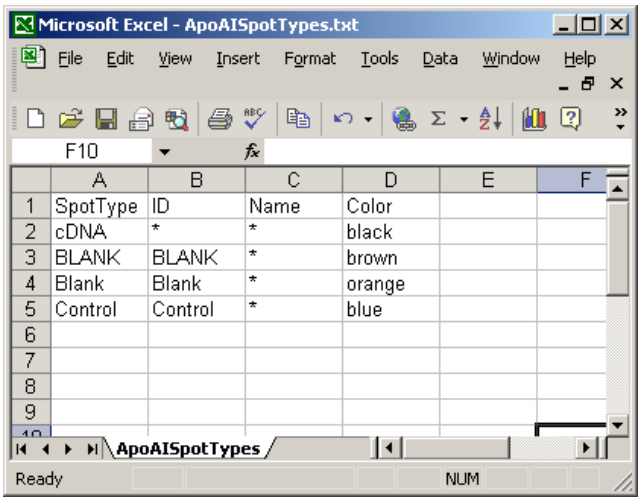
This will set the number of pins, or print-tips, using during the printing of the arrays. This determines the number of what are often called grids or meta rows and columns on the arrays.

5.6 The Spot Types File

The Spot Types file (STF) is another optional tab-delimited text file which allows you to identify different types of spots from the gene list. The STF is typically used to distinguish control spots from those corresponding to genes of interest, to distinguish positive from negative controls, ratio from calibration controls and so on. The SPF should have a column giving the names of the different spot-types. We will assume that this column is called `SpotType`. One or more other columns should have the same names as columns in the gene list and should contain patterns or regular expressions sufficient to identify the spot-type. Any other columns are assumed to contain plotting parameters, such as colors or symbols, to be associated with the spot-types. The STF should have one row for every spot-type which you want to distinguish. The STF is used to set the control status of each spot on the arrays so that plots may highlight different types of spots in an appropriate way.

The STF uses simplified regular expressions to match patterns. For example, `AA*` means any string starting with `AA`, `*AA` means any code ending with `AA`, `AA` means exactly these two letters, `*AA*` means any string containing `AA`, `AA.` means `AA` followed by exactly one other character and `AA\.` means exactly `AA` followed by a period and no other characters. For those familiar with regular expressions, any other regular expressions are allowed but the codes `^` for beginning of string and `$` for end of string should be excluded. Note that the patterns are matched sequentially from first to last, so more general patterns should be included first. For example, it is often a good idea to include a default spot-type as the first line in the STF with pattern `*` for all the pattern-matching columns and with default plotting parameters.

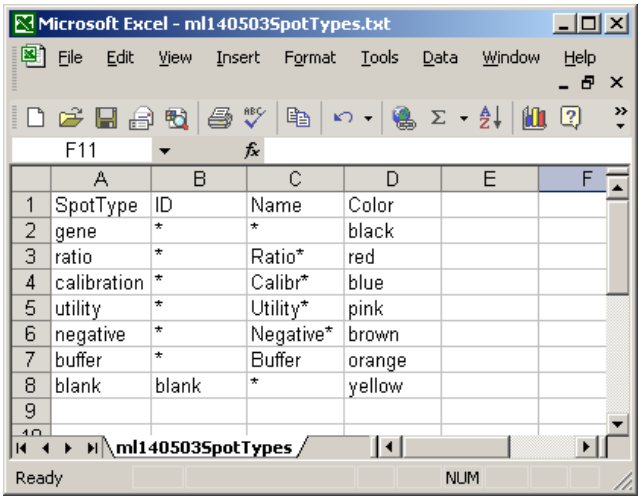
Here is a short STF appropriate for the ApoAI data:



	A	B	C	D	E	F
1	SpotType	ID	Name	Color		
2	cDNA	*	*	black		
3	BLANK	BLANK	*	brown		
4	Blank	Blank	*	orange		
5	Control	Control	*	blue		
6						
7						
8						
9						

In this example, the columns ID and Name are found in the gene-list and contain patterns to match. The asterisks are wildcards which can represent anything. Be careful to use upper or lower case as appropriate and don't insert any extra spaces. The remaining column gives colors to be associated with the different types of points.

Here is a STF below appropriate for arrays with Lucidea Universal ScoreCard control spots.



	A	B	C	D	E	F
1	SpotType	ID	Name	Color		
2	gene	*	*	black		
3	ratio	*	Ratio*	red		
4	calibration	*	Calibr*	blue		
5	utility	*	Utility*	pink		
6	negative	*	Negative*	brown		
7	buffer	*	Buffer	orange		
8	blank	blank	*	yellow		
9						

You can read the STF using `readSpotTypes`. For example, if the file has the default name `SpotTypes.txt` you can use simply

```
> types <- readSpotTypes()
```

The spot types file is used by the `controlStatus()` function to set the status of each spot on the array, for example

```
> RG$genes$Status <- controlStatus(types, RG)
```

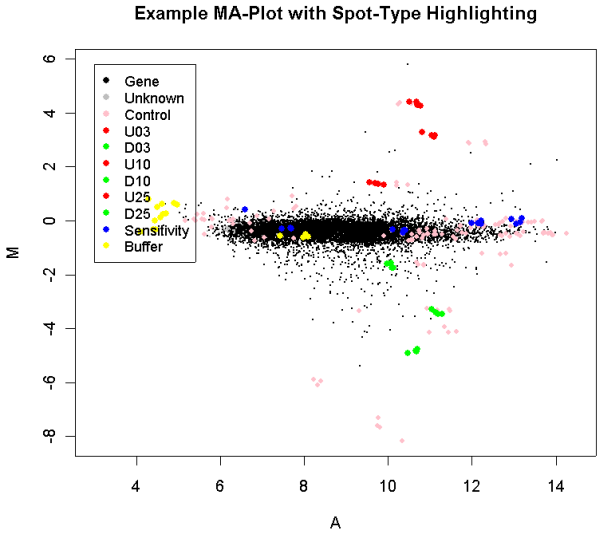
if `RG` is an `RGList` object with the gene-list set. Then

```
> plotMA(RG)
```

will produce an MA-Plot with colors, symbols and sizes and given in the STF.

6 Data Exploration

It is advisable to display your data in various ways as a quality check and to check for unexpected effects. We recommend an imageplot of the raw log-ratios and an MA-plot of the raw data for each array as a minimum routine displays. The following is an example MA-Plot produced by `plotMA` for an `MAList` object with spot-types Gene, Unknown, Control, U03, D03, U10, D10, U25, D25, Sensitivity and Buffer set using a spot-types file and the function `controlStatus` (data not included). This plot shows up and down-regulated spike-in control spots as well as negative controls and calibration or sensitivity controls. See the Swirl data case study for other examples.



7 Normalization and Background Correction

Limma implements a range of normalization methods for spotted microarrays. Smyth and Speed (2003) describe some of the mostly commonly used methods. Most of the examples given in this manual use print-tip loess normalization as the major method. Print-tip loess normalization is performed by

```
> MA <- normalizeWithinArrays(RG)
```

By default, limma will subtract the background from the foreground intensities as part of the normalization process using `normalizeWithinArrays` so there is no need for any special action on the part of users. If you want to over-ride this default background correct, for example to ensure that all the corrected intensities are positive, then use the `backgroundCorrect` function. For example we generally find

```
> RG <- backgroundCorrect(RG, method="normexp", offset=50)
```

to be superior for differential expression purposes to background subtraction with most image analysis programs. This method adjusts the foreground adaptively for the background intensities and results in strictly positive adjusted intensities, i.e., negative intensities are avoided. The use of an offset forces the log-ratios for very low intensities spots towards zero. No further background correction will be performed when `normalizeWithinArrays` is used subsequently to normalize the intensities.

Limma contains some more sophisticated normalization methods. Some between-array normalization methods are discussed in Section 12 of this guide.

8 Linear Models

8.1 Introduction

The package limma uses an approach called *linear models* to analyse designed microarray experiments. This approach allows very general experiments to be analysed just as easily as a simple replicated experiment. The approach is outlined in Smyth (2004) and Yang and Speed (2002). The approach requires one or two matrices to be specified. The first is the *design matrix* which indicates in effect which RNA samples have been applied to each array. The second is the *contrast matrix* which specifies which comparisons you would like to make between the RNA samples. For very simple experiments, you may not need to specify the contrast matrix.

The philosophy of the approach is as follows. You have to start by fitting a linear model to your data which fully models the systematic part of your data. The model is specified by the design matrix. Each row of the design matrix corresponds to an array in your experiment and each column corresponds to a coefficient which is used to describe the RNA sources in your experiment. With Affymetrix or single-channel data, or with two-color with a common reference, you will need as many coefficients as you have distinct RNA sources, no more and no less. With direct-design two-color data you will need one fewer coefficient than you have distinct RNA sources, unless you wish to estimate a dye-effect for each gene, in which case the number of RNA sources and the number of coefficients will be the same. Any set of independent coefficients will do, providing they describe all your treatments. The main purpose of this step is to estimate the variability in the data, hence the systematic part needs to modelled so it can be distinguished from random variation.

In practice the requirement to have exactly as many coefficients as RNA sources is too restrictive in terms of questions you might want to answer. You might be interested in more or fewer comparisons between the RNA source. Hence the contrasts step is provided so that you can take the initial coefficients and compare them in as many ways as you want to answer any questions you might have, regardless of how many or how few these might be.

If you have data from Affymetrix experiments, from single-channel spotted microarrays or from spotted microarrays using a common reference, then linear modeling is the same as ordinary analysis of variance or multiple regression except that a model is fitted for every gene. With data of this type you can create design matrices as one would do for ordinary modeling with univariate data. If you have data from spotted microarrays using a direct design, i.e., a connected design with no common reference, then the linear modeling approach is very powerful but the creation of the design matrix may require more statistical knowledge.

For statistical analysis and assessing differential expression, limma uses an empirical Bayes method to moderate the standard errors of the estimated log-fold changes. This results in more stable inference and improved power, especially for experiments with small numbers of arrays (Smyth, 2004). For arrays with within-array replicate spots, limma uses a pooled correlation method to make full use of the duplicate spots (Smyth et al, 2003).

8.2 Affymetrix and Other Single-Channel Designs

Affymetrix data will usually be normalized using the affy package. We will assume here that the data is available as an `exprSet` object called `eset`. Such an object will have an slot containing the log-expression values for each gene on each array which can be extracted using `exprs(eset)`. Affymetrix and other single-channel microarray data may be analysed very much like ordinary linear models or anova models. The difference with microarray data is that it is almost always necessary to extract particular contrasts of interest and so the standard parametrizations provided for factors in R are not usually adequate.

There are many ways to approach the analysis of a complex experiment in limma. A straightforward strategy is to set up the simplest possible design matrix and then to extract from the fit the contrasts of interest.

Suppose that there are three RNA sources to be compared. Suppose that the first three arrays are hybridized with RNA1, the next two with RNA2 and the next three with RNA3. Suppose that all pair-wise comparisons between the RNA sources are of interest. We assume that the data has been normalized and stored in an `exprSet` object, for example by

```
> data <- ReadAffy()
> eset <- rma(data)
```

An appropriate design matrix can be created and a linear model fitted using

```
> design <- model.matrix(~ -1+factor(c(1,1,1,2,2,3,3,3)))
> colnames(design) <- c("group1", "group2", "group3")
> fit <- lmFit(eset, design)
```

To make all pair-wise comparisons between the three groups the appropriate contrast matrix can be created by

```
> contrast.matrix <- makeContrasts(group2-group1, group3-group2, group3-group1, levels=design)
> fit2 <- contrasts.fit(fit, contrast.matrix)
> fit2 <- eBayes(fit2)
```

A list of top genes differential expressed in group2 versus group1 can be obtained from

```
> topTable(fit2, coef=1, adjust="fdr")
```

You may classify each gene according to the three pair-wise comparisons using

```
> clas <- classifyTestsF(fit2)
```

A Venn diagram showing numbers of genes significant in each comparison can be obtained from

```
> vennDiagram(clas)
```

8.3 Common Reference Designs

Now consider two-color microarray experiments in which a common reference has been used on all the arrays. Such experiments can be analysed very similarly to Affymetrix experiments except that allowance must be made for dye-swaps. The simplest method is to setup the design matrix using the `modelMatrix()` function and the targets file. As an example, we consider part of an experiment conducted by Joëlle Michaud, Catherine Carmichael and Dr Hamish Scott at the Walter and Eliza Hall Institute to compare the effects of transcription factors in a human cell line. The targets file is as follows:

```
> targets <- readTargets("runxtargets.txt")
> targets
   SlideNumber       Cy3       Cy5
1         2144      EGFP      AML1
2         2145      EGFP      AML1
3         2146      AML1      EGFP
4         2147      EGFP AML1.CBFb
5         2148      EGFP AML1.CBFb
6         2149 AML1.CBFb      EGFP
7         2158      EGFP      CBFb
8         2159      CBFb      EGFP
9         2160      EGFP AML1.CBFb
10        2161 AML1.CBFb      EGFP
11        2162      EGFP AML1.CBFb
12        2163 AML1.CBFb      EGFP
13        2166      EGFP      CBFb
14        2167      CBFb      EGFP
```

In the experiment, green fluorescent protein (EGFP) has been used as a common reference. An adenovirus system was used to transport various transcription factors into the nuclei of HeLa cells. Here we consider the transcription factors AML1, CBFbeta or both. A simple design matrix was formed and a linear model fit:

```
> design <- modelMatrix(targets,ref="EGFP")
> design
   AML1 AML1.CBFb CBFb
1     1         0    0
2     1         0    0
3    -1         0    0
4     0         1    0
5     0         1    0
6     0        -1    0
7     0         0    1
8     0         0   -1
9     0         1    0
10    0        -1    0
11    0         1    0
12    0        -1    0
13    0         0    1
14    0         0   -1
> fit <- lmFit(RG, design)
```

It is of interest to compare each of the transcription factors to EGFP and also to compare the combination transcription factor with AML1 and CBFb individually. An appropriate contrast matrix was formed as follows:

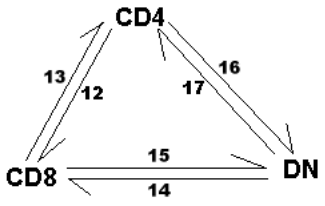
```
> contrast.matrix <- makeContrasts(AML1,CBFb,AML1.CBFb,AML1.CBFb-AML1,AML1.CBFb-CBFb,levels=design)
> contrast.matrix
           AML1 CBFb AML1.CBFb AML1.CBFb - AML1 AML1.CBFb - CBFb
AML1          1    0         0               -1                0
AML1.CBFb     0    0         1                1                1
CBFb          0    1         0                0               -1
```

The linear model fit can now be expanded and empirical Bayes statistics computed:

```
> fit2 <- contrasts.fit(fit, contrasts.matrix)
> fit2 <- eBayes(fit2)
```

8.4 Direct Two-Color Designs

Two-colour designs without a common reference require the most statistical knowledge to choose the appropriate design matrix. A direct design is one in which there is no single RNA source which is hybridized to every array. As an example, we consider an experiment conducted by Dr Mireille Lahoud at the Walter and Eliza Hall Institute to compare gene expression in three different populations of dendritic cells (DC).



Arrow heads represent Cy5, i.e. arrows point in the Cy3 to Cy5 direction.

This experiment involved six cDNA microarrays in three dye-swap pairs, with each pair used to compare two DC types. The design is shown diagrammatically above. The targets file was as follows:

```
> targets
  SlideNumber     FileName Cy3 Cy5
1          12 ml12med.spot CD4 CD8
2          13 ml13med.spot CD8 CD4
3          14 ml14med.spot  DN CD8
4          15 ml15med.spot CD8  DN
5          16 ml16med.spot CD4  DN
6          17 ml17med.spot  DN CD4
```

There are many valid choices for a design matrix for such an experiment and no single correct choice. We chose to setup the design matrix as follows:

```
> design <- cbind("CD8-CD4"=c(1,-1,1,-1,0,0),"DN-CD4"=c(0,0,-1,1,1,-1))
> rownames(design) <- removeExt(targets$FileName)
> design

        CD8-CD4 DN-CD4
ml12med       1      0
ml13med      -1      0
ml14med       1     -1
ml15med      -1      1
ml16med       0      1
ml17med       0     -1
```

In this design matrix, the CD8 and DN populations have been compared back to the CD4 population. The coefficients estimated by the linear model will correspond to the log-ratios of CD8 vs CD4 (first column) and DN vs CD4 (second column). After appropriate normalization of the expression data, a linear model was fit using

```
> fit <- lmFit(MA, design, ndups=2)
```

The use of `ndups` is to specify that the arrays contained duplicates of each gene, see Section 9.

The linear model can now be interrogated to answer any questions of interest. For this experiment it was of interest to make all pairwise comparisons between the three DC populations. This was accomplished using the contrast matrix

```
> contrast.matrix <- cbind("CD8-CD4"=c(1,0),"DN-CD4"=c(0,1),"CD8-DN"=c(1,-1))
> rownames(contrast.matrix) <- colnames(design)
> contrast.matrix
        CD8-CD4 DN-CD4 CD8-DN
CD8-CD4       1      0      1
DN-CD4        0      1     -1
```

The contrast matrix can be used to expand the linear model fit and then to compute empirical Bayes statistics:

```
> fit2 <- constrast.fit(fit, contrast.matrix)
> fit2 <- eBayes(fit2)
```

8.5 Other Designs

There is an enormous variety of possible experimental designs and it isn't possible to give prescriptive rules that will cover all possibilities. Here we consider a less standard design in which three mutant mice are to be compared with three wild type mice. The different mice are biological replicates. Eighteen two-color arrays were used with each mouse appearing on six different arrays. The arrays were arranged in dye-swap pairs although this does not affect the analysis. The contrast of interest is the mutant versus wild type comparison.

```
> targets
      FileName Cy3 Cy5
1391 1391.spot wt1 mu1
1392 1392.spot mu1 wt1
1340 1340.spot wt2 mu1
1341 1341.spot mu1 wt2
1395 1395.spot wt3 mu1
1396 1396.spot mu1 wt3
1393 1393.spot wt1 mu2
1394 1394.spot mu2 wt1
1371 1371.spot wt2 mu2
1372 1372.spot mu2 wt2
1338 1338.spot wt3 mu2
1339 1339.spot mu2 wt3
1387 1387.spot wt1 mu3
1388 1388.spot mu3 wt1
1399 1399.spot wt2 mu3
1390 1390.spot mu3 wt2
1397 1397.spot wt3 mu3
1398 1398.spot mu3 wt3
```

There are several ways to analyse this data using fixed or random effects for the mice. Here we outline a direct approach in which a separate coefficient is fitted for each mouse, and then the contrast of interest is formed between the mice.

```
> design <- modelMatrix(targets, ref="wt1")
> design <- cbind(Dye=1,design)
> colnames(design)
[1] "Dye" "mu1" "mu2" "mu3" "wt2" "wt3"
```

The above code treats the first wild-type mice as a baseline reference, so that columns of the design matrix represent the difference between each of the other mice and wt1. The design matrix also includes an intercept term which represents the dye effect of cy5 over cy3 for each gene. If you don't wish to allow for a dye effect, the second line of code can be omitted.

```
> fit <- lmFit(MA, design)
> cont.matrix <- makeContrasts(muvswt=(mu1+mu2+mu3-wt2-wt3)/3, levels=design)
> fit2 <- contrasts.fit(fit, cont.matrix)
> fit2 <- eBayes(fit2)
```

The contrast defined by the function `makeContrasts` represents the average difference between the mutant and wild-type mice, which is the comparison of interest.

This general approach is applicable to many medium-sized studies involving biological replicates.

9 Statistics for Differential Expression

A number of summary statistics are computed by the `eBayes()` function for each gene and each contrast. The M-value (`M`) is the log2-fold change, or sometimes the log2-expression level,

for that gene. The A-value (`A`) is the the average expression level for that gene across all the arrays and channels. The moderated t-statistic (`t`) is the ratio of the M-value to its standard error. This has the same interpretation as an ordinary t-statistic except that the standard errors have been moderated across genes, effectively borrowing information from the ensemble of genes to aid with inference about each individual gene. The ordinary t-statistics are not usually required or recommended, but they can be recovered by

```
> tstat.ord <- fit$coef / fit$stdev.unscaled / fit$sigma
```

after fitting a linear model. The ordinary t-statistic is on `fit$df.residual` degrees of freedom while the moderated t-statistic is on `fit$df.residual+fit$df.prior` degrees of freedom.

The p-value (`p-value`) is obtained from the moderated t-statistic, usually after some form of adjustment for multiple testing. The most popular form of adjustment is "fdr" which is Benjamini and Hochberg's method to control the false discovery rate. The meaning of the adjusted p-value is as follows. If you select all genes with p-value below a given value, say 0.05, as differentially expression, then the expected proportion of false discoveries in the selected group should be less than that value, in this case less than 5%.

The B-statistic (`lods` or `B`) is the log-odds that that gene is differentially expressed. Suppose for example that B=1.5. The odds of differential expression is exp(1.5)=4.48, i.e, about four and a half to one. The probability that the gene is differentially expressed is 4.48/(1+4.48)=0.82, i.e., the probability is about 82% that this gene is differentially expressed. A B-statistic of zero corresponds to a 50-50 chance that the gene is differentially expressed. The B-statistic is automatically adjusted for multiple testing by assuming that 1% of the genes, or some other percentage specified by the user, are expected to be differentially expressed. If there are no missing values in your data, then the moderated t and B statistics will rank the genes in exactly the same order. Even you do have spot weights or missing data, the p-values and B-statistics will usually provide a very similar ranking of the genes.

Please keep in mind that the moderated t-statistic p-values and the B-statistic probabilities depend on various sorts of mathematical assumptions which are never exactly true for microarray data. The B-statistics also depend on a prior guess for the proportion of differentially expressed genes. Therefore they are intended to be taken as a guide rather than as a strict measure of the probability of differential expression. Of the three statistics, the moderated-t, the associated p-value and the B-statistics, we usually base our gene selections on the p-value. All three measures are closely related, but the moderated-t and its p-value do not require a prior guess for the number of differentially expressed genes.

The above mentioned statistics are computed for every contrast for each gene. The `eBayes()` function computes one more useful statistic. The moderated F-statistic (F) combines the t-statistics for all the contrasts into an overall test of significance for that gene. The moderated F-statistic tests whether any of the contrasts are non-zero for that gene, i.e., whether that gene is differentially expressed on any contrast. The moderated-F has numerator degrees of freedom equal to the number of contrasts and denominator degrees of freedom the same as the moderated-t. Its p-value is stored as `fit$F.p.value`. It is similar to the ordinary F-statistic from analysis of variance except that the denominator mean squares are moderated across genes.

In a complex experiment with many contrasts, it may be desirable to select genes firstly on the basis of their moderated F-statistics, and subsequently to decide which of the individual contrasts are significant for the selected genes. This cuts down on the number of tests which need to be conducted and therefore on the amount of adjustment for multiple testing. The functions `classifyTestsF()` and `decideTests()` are provided for this purpose.

10 Case Studies

10.1 Swirl Zebrafish: A Single-Sample Experiment

In this section we consider a case study in which two RNA sources are compared directly on a set of replicate or dye-swap arrays. The case study includes reading in the data, data display and exploration, as well as normalization and differential expression analysis. The analysis of differential expression is analogous to a classical one-sample test of location for each gene.

In this example we assume that the data is provided as a GAL file called `fish.gal` and raw SPOT output files and that these files are in the current working directory.

Background. The experiment was carried out using zebrafish as a model organism to study the early development in vertebrates. Swirl is a point mutant in the BMP2 gene that affects the dorsal/ventral body axis. The main goal of the Swirl experiment is to identify genes with altered expression in the Swirl mutant compared to wild-type zebrafish.

The hybridizations. Two sets of dye-swap experiments were performed making a total of four replicate hybridizations. Each of the arrays compares RNA from swirl fish with RNA from normal ("wild type") fish. The experimenters have prepared a tab-delimited targets file called "SwirlSamples.txt" which describes the four hybridizations:

```
> targets <- readTargets("SwirlSample.txt")
> targets
  SlideNumber     FileName       Cy3       Cy5      Date
1          81 swirl.1.spot     swirl wild type 2001/9/20
2          82 swirl.2.spot wild type     swirl 2001/9/20
3          93 swirl.3.spot     swirl wild type 2001/11/8
4          94 swirl.4.spot wild type     swirl 2001/11/8
```

We see that slide numbers 81, 82, 93 and 94 were used to make the arrays. On slides 81 and 93, swirl RNA was labelled with green (Cy3) dye and wild type RNA was labelled with red (Cy5) dye. On slides 82 and 94, the labelling was the other way around.

Each of the four hybridized arrays was scanned on an Axon scanner to produce a TIFF image, which was then processed using the image analysis software SPOT. The data from the arrays are stored in the four output files listed under `FileName`. Now we read the intensity data into an `RGList` object in R. The default for SPOT output is that `Rmean` and `Gmean` are used as foreground intensities and `morphR` and `morphG` are used as background intensities:

```
> RG <- read.maimages(targets$FileName, source="spot")
Read swirl.1.spot
Read swirl.2.spot
```

```
Read swirl.3.spot
Read swirl.4.spot
> RG
An object of class "RGList"
$R
       swirl.1   swirl.2   swirl.3    swirl.4
[1,] 19538.470 16138.720 2895.1600 14054.5400
[2,] 23619.820 17247.670 2976.6230 20112.2600
[3,] 21579.950 17317.150 2735.6190 12945.8500
[4,]  8905.143  6794.381  318.9524   524.0476
[5,]  8676.095  6043.542  780.6667   304.6190
8443 more rows ...

$G
       swirl.1   swirl.2   swirl.3    swirl.4
[1,] 22028.260 19278.770 2727.5600 19930.6500
[2,] 25613.200 21438.960 2787.0330 25426.5800
[3,] 22652.390 20386.470 2419.8810 16225.9500
[4,]  8929.286  6677.619  383.2381   786.9048
[5,]  8746.476  6576.292  901.0000   468.0476
8443 more rows ...

$Rb
     swirl.1 swirl.2 swirl.3 swirl.4
[1,]     174     136      82      48
[2,]     174     133      82      48
[3,]     174     133      76      48
[4,]     163     105      61      48
[5,]     140     105      61      49
8443 more rows ...

$Gb
     swirl.1 swirl.2 swirl.3 swirl.4
[1,]     182     175      86      97
[2,]     171     183      86      85
[3,]     153     183      86      85
[4,]     153     142      71      87
[5,]     153     142      71      87
8443 more rows ...
```

The arrays. The microarrays used in this experiment were printed with 8448 probes (spots), including 768 control spots. The array printer uses a print head with a 4x4 arrangement of print-tips and so the microarrays are partitioned into a 4x4 grid of tip groups. Each grid consists of 22x24 spots that were printed with a single print-tip. The gene name associated with each spot is recorded in a GenePix array list (GAL) file:

```
> RG$genes <- readGAL("fish.gal")
> RG$genes[1:30,]
   Block Row Column      ID     Name
1      1   1      1 control    geno1
2      1   1      2 control    geno2
```

```
3      1   1      3 control    geno3
4      1   1      4 control    3XSSC
5      1   1      5 control    3XSSC
6      1   1      6 control     EST1
7      1   1      7 control    geno1
8      1   1      8 control    geno2
9      1   1      9 control    geno3
10     1   1     10 control    3XSSC
11     1   1     11 control    3XSSC
12     1   1     12 control    3XSSC
13     1   1     13 control     EST2
14     1   1     14 control     EST3
15     1   1     15 control     EST4
16     1   1     16 control    3XSSC
17     1   1     17 control    Actin
18     1   1     18 control    Actin
19     1   1     19 control    3XSSC
20     1   1     20 control    3XSSC
21     1   1     21 control    3XSSC
22     1   1     22 control    3XSSC
23     1   1     23 control    Actin
24     1   1     24 control    Actin
25     1   2      1 control     ath1
26     1   2      2 control    Cad-1
27     1   2      3 control   DeltaB
28     1   2      4 control     Dlx4
29     1   2      5 control ephrinA4
30     1   2      6 control     FGF8
```

The 4x4x22x24 print layout also needs to be set. The easiest way to do this is to infer it from the GAL file:

```
> RG$printer <- getLayout(RG$genes)
```

Image plots. It is interesting to look at the variation of background values over the array. Consider image plots of the red and green background for the first array:

```
> imageplot(log2(RG$Rb[,1]), RG$printer, low="white", high="red")
> imageplot(log2(RG$Gb[,1]), RG$printer, low="white", high="green")
```

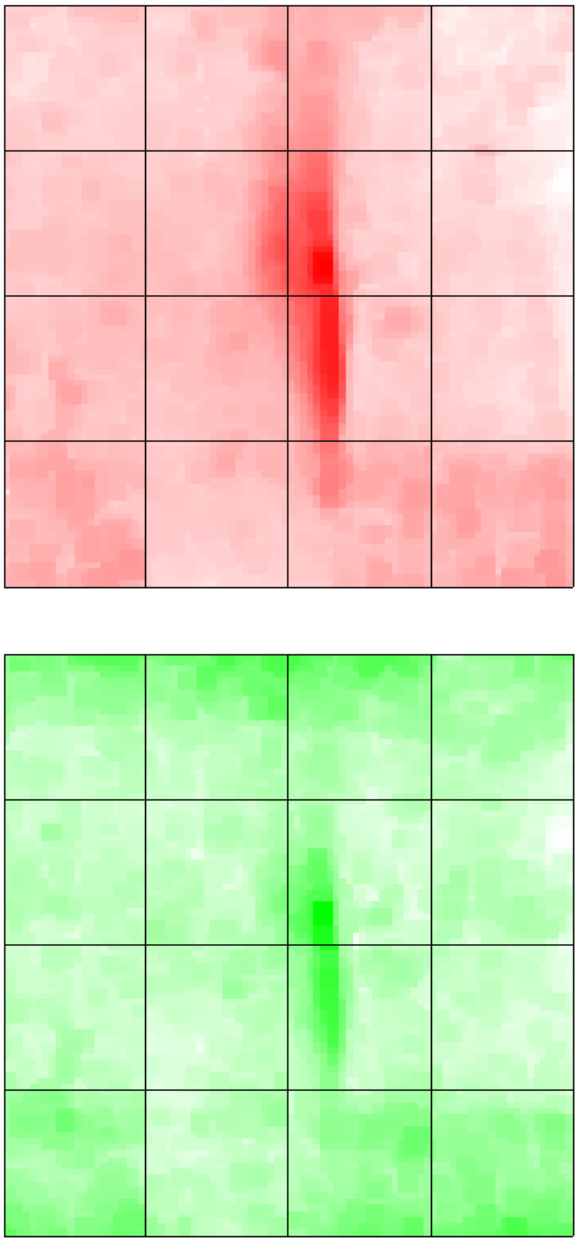
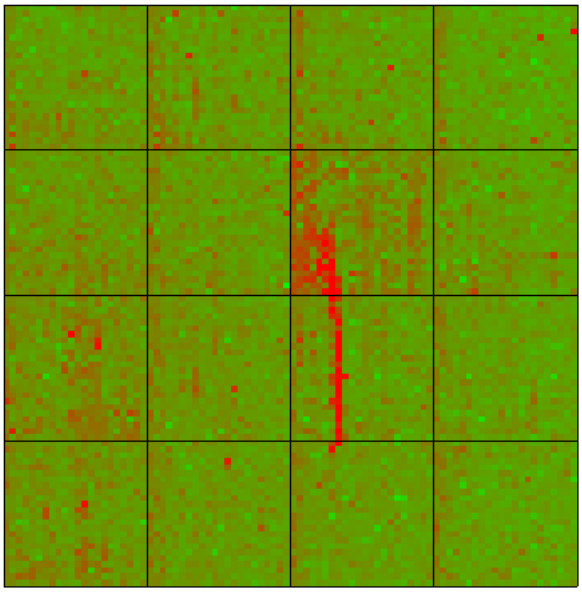


Image plot of the un-normalized log-ratios or M-values for the first array:

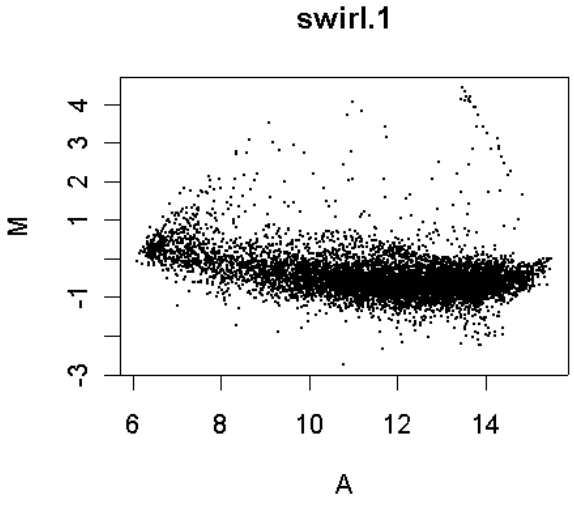
```
> MA <- normalizeWithinArrays(RG, method="none")
> imageplot(MA$M[,1], RG$printer, zlim=c(-3,3))
```



The `imageplot` function lies the slide on its side, so the first print-tip group is bottom left in this plot. We can see a red streak across the middle two grids of the 3rd row caused by a scratch or dust on the array. Spots which are affected by this artefact will have suspect M-values. The streak also shows up as darker regions in the background plots.

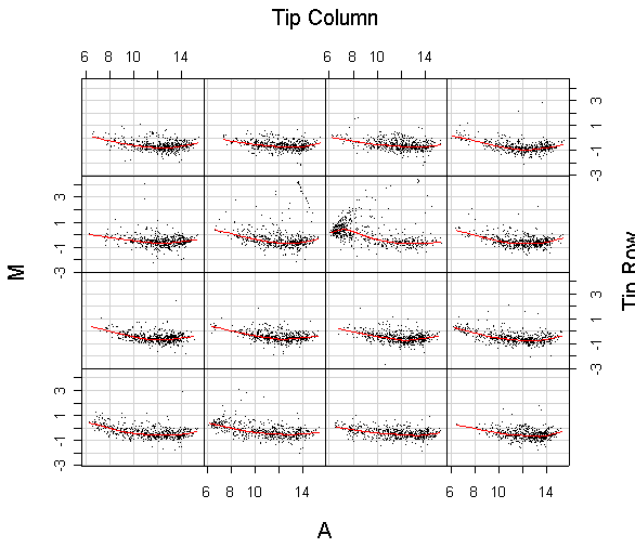
MA-plots. An MA-plot plots the log-ratio of R vs G against the overall intensity of each spot. The log-ratio is represented by the M-value, $M = \log_2(R) - \log_2(G)$, and the overall intensity by the A-value, $A = (\log_2(R) + \log_2(G))/2$. Here is the MA-plot of the un-normalized values for the first array:

```
> plotMA(MA)
```



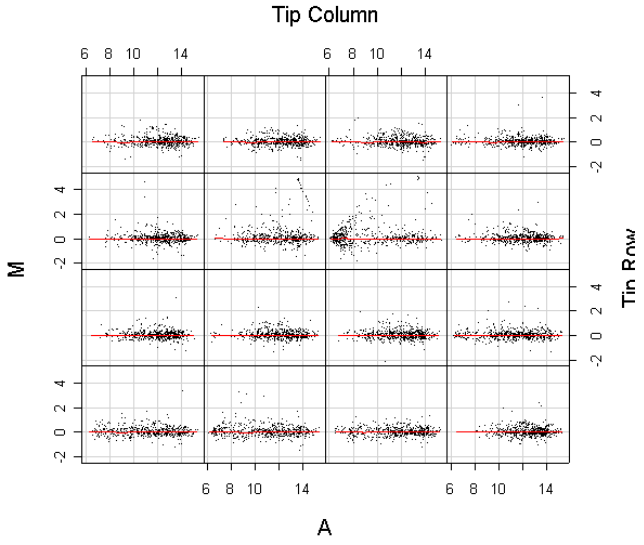
The red streak seen on the image plot can be seen as a line of spots in the upper right of this plot. Now we plot the individual MA-plots for each of the print-tip groups on this array, together with the loess curves which will be used for normalization:

```
> plotPrintTipLoess(MA)
```



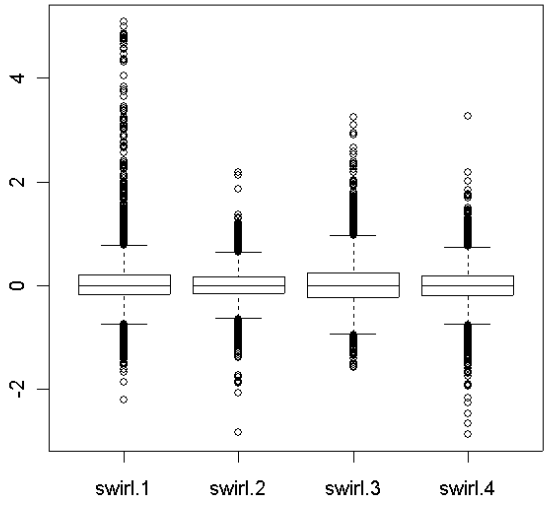
Normalization. Print-tip loess normalization:

```
> MA <- normalizeWithinArrays(RG)
> plotPrintTipLoess(MA)
```



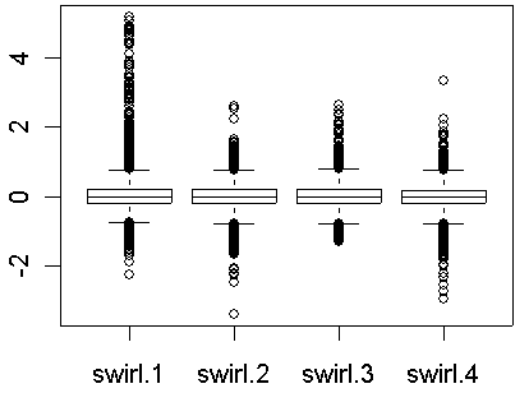
We have normalized the M-values with each array. A further question is whether normalization is required between the arrays. The following plot shows overall boxplots of the M-values for the four arrays.

```
> boxplot(MA$M~col(MA$M),names=colnames(MA$M))
```



There is some evidence that the different arrays have different spreads of M-values, so we will scale normalize between the arrays.

```
> MA <- normalizeBetweenArrays(MA)
> boxplot(MA$M~col(MA$M),names=colnames(MA$M))
```



Linear model. Now estimate the average M-value for each gene. We do this by fitting a simple linear model for each gene. The negative numbers in the design matrix indicate the dye-swaps.

```
> design <- c(-1,1,-1,1)
> fit <- lmFit(MA,design)
> fit
An object of class "MArrayLM"
$coefficients
[1] -0.3943421 -0.3656843 -0.3912506 -0.2505729 -0.3432590
8443 more elements ...

$stdev.unscaled
[1] 0.5 0.5 0.5 0.5 0.5
8443 more elements ...

$sigma
[1] 0.3805154 0.4047829 0.4672451 0.3206071 0.2838043
8443 more elements ...

$df.residual
[1] 3 3 3 3 3
8443 more elements ...

$method
[1] "ls"

$design
     [,1]
[1,]   -1
[2,]    1
[3,]   -1
[4,]    1

$genes
  Block Row Column      ID  Name
1     1   1      1 control geno1
2     1   1      2 control geno2
3     1   1      3 control geno3
4     1   1      4 control 3XSSC
5     1   1      5 control 3XSSC
8443 more rows ...

$Amean
[1] 13.46481 13.67631 13.42665 10.77730 10.88446
8443 more elements ...
```

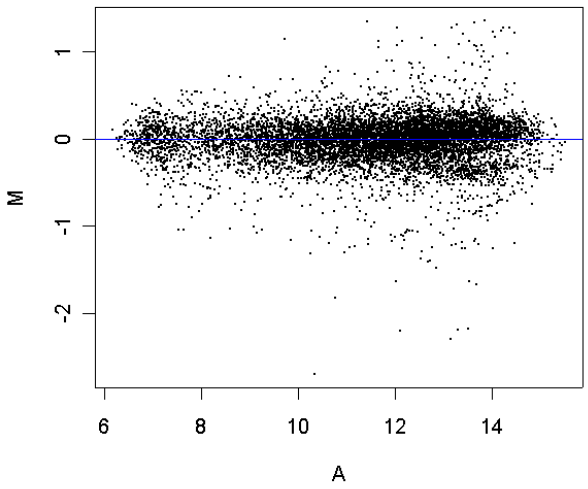
In the above fit object, `coefficients` is the average M-value for each gene and `sigma` is the sample standard deviations for each gene. Ordinary t-statistics for comparing mutant to wt could be computed by

```
> ordinary.t <- fit$coef / fit$stdev.unscaled / fit$sigma
```

We prefer though to use empirical Bayes moderated t-statistics which are computed below.

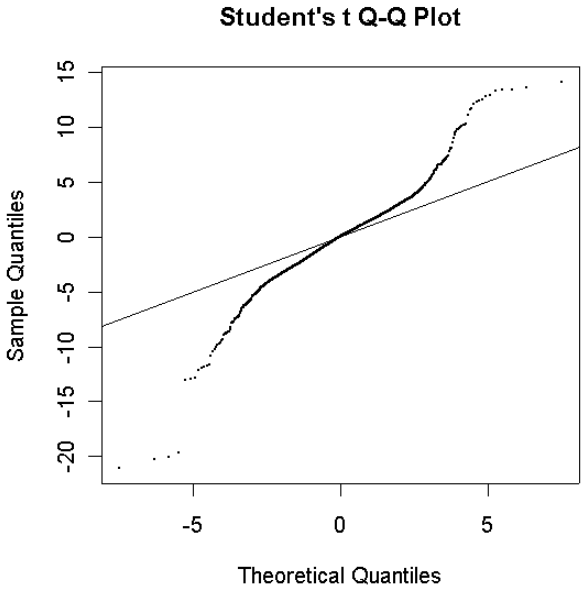
Now create an MA-plot of the average M and A-values for each gene.

```
> M <- fit$coef
> A <- fit$Amean
> plot(A,M,pch=16,cex=0.2)
> abline(0,0,col="blue")
```



Empirical Bayes analysis. We will now go on and compute empirical Bayes statistics for differential expression. The moderated t-statistics use sample standard deviations which have been shrunk towards a pooled standard deviation value.

```
> fit <- eBayes(fit)
> qqt(fit$t,df=fit$df.prior+fit$df.residual,pch=16,cex=0.2)
> abline(0,1)
```



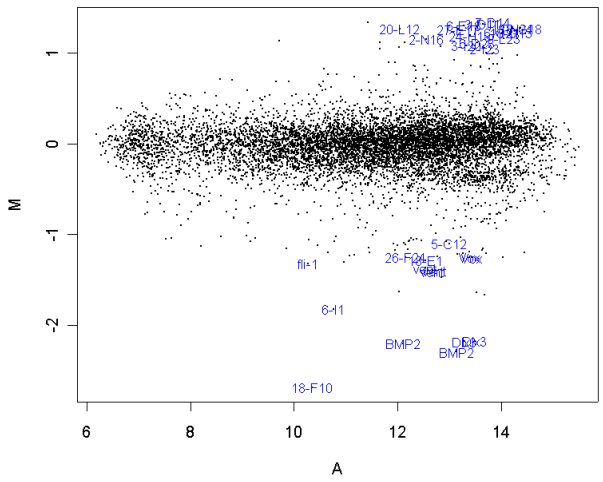
Visually there seems to be plenty of genes which are differentially expressed. We will obtain a summary table of some key statistics for the top genes.

```
> options(digits=3)
> topTable(fit,number=30,adjust="fdr")
     Block Row Column      ID   Name     M    A     t  P.Value    B
3721     8   2      1 control   BMP2 -2.21 12.1 -21.1 0.000357 7.96
1609     4   2      1 control   BMP2 -2.30 13.1 -20.3 0.000357 7.78
3723     8   2      3 control   Dlx3 -2.18 13.3 -20.0 0.000357 7.71
1611     4   2      3 control   Dlx3 -2.18 13.5 -19.6 0.000357 7.62
8295    16  16     15 fb94h06 20-L12  1.27 12.0  14.1 0.002067 5.78
7036    14   8      4 fb40h07  7-D14  1.35 13.8  13.5 0.002067 5.54
515      1  22     11 fc22a09 27-E17  1.27 13.2  13.4 0.002067 5.48
5075    10  14     11 fb85f09 18-G18  1.28 14.4  13.4 0.002067 5.48
7307    14  19     11 fc10h09 24-H18  1.20 13.4  13.2 0.002067 5.40
319      1  14      7 fb85a01  18-E1 -1.29 12.5 -13.1 0.002067 5.32
2961     6  14      9 fb85d05 18-F10 -2.69 10.3 -13.0 0.002067 5.29
4032     8  14     24 fb87d12 18-N24  1.27 14.2  12.8 0.002067 5.22
6903    14   2     15 control    Vox -1.26 13.4 -12.8 0.002067 5.20
4546     9  14     10 fb85e07 18-G13  1.23 14.2  12.8 0.002067 5.18
683      2   7     11 fb37b09  6-E18  1.31 13.3  12.4 0.002182 5.02
1697     4   5     17 fb26b10  3-I20  1.09 13.3  12.4 0.002182 4.97
7491    15   5      3 fb24g06  3-D11  1.33 13.6  12.3 0.002182 4.96
4188     8  21     12 fc18d12 26-F24 -1.25 12.1 -12.2 0.002209 4.89
4380     9   7     12 fb37e11  6-G21  1.23 14.0  12.0 0.002216 4.80
3726     8   2      6 control  fli-1 -1.32 10.3 -11.9 0.002216 4.76
2679     6   2     15 control    Vox -1.25 13.4 -11.9 0.002216 4.71
5931    12   6      3 fb32f06  5-C12 -1.10 13.0 -11.7 0.002216 4.63
7602    15   9     18 fb50g12  9-L23  1.16 14.0  11.7 0.002216 4.63
2151     5   2     15 control   vent -1.40 12.7 -11.7 0.002216 4.62
3790     8   4     22 fb23d08  2-N16  1.16 12.5  11.6 0.002221 4.58
7542    15   7      6 fb36g12  6-D23  1.12 13.5  11.0 0.003000 4.27
4263     9   2     15 control   vent -1.41 12.7 -10.8 0.003326 4.13
6375    13   2     15 control   vent -1.37 12.5 -10.5 0.004026 3.91
1146     3   4     18 fb22a12  2-I23  1.05 13.7  10.2 0.004242 3.76
157      1   7     13 fb38a01   6-I1 -1.82 10.8 -10.2 0.004242 3.75
```

The top gene is BMP2 which is significantly down-regulated in the Swirl zebrafish, as it should be because the Swirl fish are mutant in this gene. Other positive controls also appear in the top 30 genes in terms.

In the table, `t` is the empirical Bayes moderated t-statistic, the corresponding P-values have been adjusted to control the false discovery rate and `B` is the empirical Bayes log odds of differential expression.

```
> ord <- order(fit$lods,decreasing=TRUE)
> top30 <- ord[1:30]
> plot(A,M,pch=16,cex=0.2)
> text(A[top30],M[top30],labels=MA$genes[top30,"Name"],cex=0.8,col="blue")
```



A warning on distributional assumptions. Beware that the p-values given here are intended to be a guide only. In the microarray context it is difficult to verify distributional assumptions, such as normality of the M-values, that the p-values are based on. This is a limitation of all model-based methods for micoarray data. Also beware that the Benjamini and Hochberg method used to control the false discovery rate does assume that the t-statistics for different probes are independent, whereas the t-statistics for different probes are actually somewhat dependent as a result of being based on observations made on the same set of arrays. Rainer et al (2003) have argued that the Benjamini and Hochberg approach is actually quite stable with respect to dependence between the probes, but this remains a somewhat controversial point.

10.2 ApoAI Knockout Data: A Two-Sample Experiment

In this section we consider a case study where two RNA sources are compared through a common reference RNA. The analysis of the log-ratios involves a two-sample comparison of means for each gene.

In this example we assume that the data is available as an RGList in the data file `ApoAI.RData`.

Background. The data is from a study of lipid metabolism by Callow et al (2000). The apolipoprotein AI (ApoAI) gene is known to play a pivotal role in high density lipoprotein (HDL) metabolism. Mice which have the ApoAI gene knocked out have very low HDL cholesterol levels. The purpose of this experiment is to determine how ApoAI deficiency affects the action of other genes in the liver, with the idea that this will help determine the molecular pathways through which ApoAI operates.

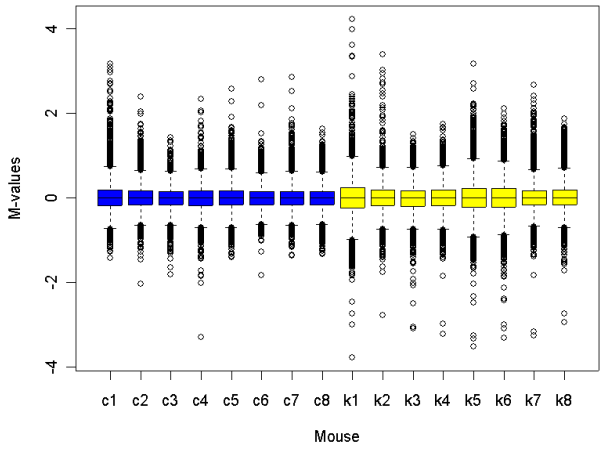
Hybridizations. The experiment compared 8 ApoAI knockout mice with 8 normal C57BL/6 ("black six") mice, the control mice. For each of these 16 mice, target mRNA was obtained from liver tissue and labelled using a Cy5 dye. The RNA from each mouse was hybridized to

a separate microarray. Common reference RNA was labelled with Cy3 dye and used for all the arrays. The reference RNA was obtained by pooling RNA extracted from the 8 control mice.

Number of arrays	Red	Green
8	Normal "black six" mice	Pooled reference
8	ApoAI knockout	Pooled reference

This is an example of a single comparison experiment using a common reference. The fact that the comparison is made by way of a common reference rather than directly as for the swirl experiment makes this, for each gene, a two-sample rather than a single-sample setup.

```
> load("ApoAI.RData")
> objects()
[1] "RG"
> names(RG)
[1] "R" "G" "Rb" "Gb" "printer" "genes" "targets"
> RG$targets
      FileName  Cy3     Cy5
c1 a1koc1.spot Pool C57BL/6
c2 a1koc2.spot Pool C57BL/6
c3 a1koc3.spot Pool C57BL/6
c4 a1koc4.spot Pool C57BL/6
c5 a1koc5.spot Pool C57BL/6
c6 a1koc6.spot Pool C57BL/6
c7 a1koc7.spot Pool C57BL/6
c8 a1koc8.spot Pool C57BL/6
k1 a1kok1.spot Pool ApoAI-/-
k2 a1kok2.spot Pool ApoAI-/-
k3 a1kok3.spot Pool ApoAI-/-
k4 a1kok4.spot Pool ApoAI-/-
k5 a1kok5.spot Pool ApoAI-/-
k6 a1kok6.spot Pool ApoAI-/-
k7 a1kok7.spot Pool ApoAI-/-
k8 a1kok8.spot Pool ApoAI-/-
> MA <- normalizeWithinArrays(RG)
> cols <- MA$targets$Cy5
> cols[cols=="C57BL/6"] <- "blue"
> cols[cols=="ApoAI-/-"] <- "yellow"
> boxplot(MA$M~col(MA$M),names=rownames(MA$targets),col=cols,xlab="Mouse",ylab="M-values")
```



Since the common reference here is a pool of the control mice, we expect to see more differences from the pool for the knock-out mice than for the control mice. In terms of the above plot, this should translate into a wider range of M-values for the knock-out mice arrays than for the control arrays, and we do see this. Since the different arrays are not expected to have the same range of M-values, between-array scale normalization of the M-values is not appropriate here.

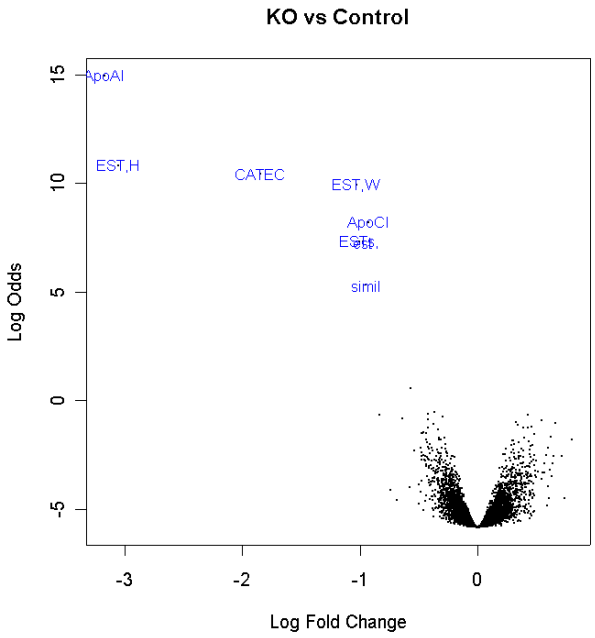
Now we can go on to estimate the fold change between the two groups. In this case the design matrix has two columns. The coefficient for the second column estimates the parameter of interest, the log-ratio between knockout and control mice.

```
> design <- cbind("Control-Ref"=1,"KO-Control"=MA$targets$Cy5=="ApoAI-/-")
> design
      Control-Ref KO-Control
 [1,]           1          0
 [2,]           1          0
 [3,]           1          0
 [4,]           1          0
 [5,]           1          0
 [6,]           1          0
 [7,]           1          0
 [8,]           1          0
 [9,]           1          1
[10,]           1          1
[11,]           1          1
[12,]           1          1
[13,]           1          1
[14,]           1          1
[15,]           1          1
[16,]           1          1
> fit <- lmFit(MA, design)
> fit$coef[1:5,]
     Control-Ref KO-Control
[1,]     -0.6595     0.6393
```

```
[2,]      0.2294     0.6552
[3,]     -0.2518     0.3342
[4,]     -0.0517     0.0405
[5,]     -0.2501     0.2230
> fit <- eBayes(fit)
> options(digits=3)
> topTable(fit,coef=2,number=15,adjust="fdr")
     GridROW GridCOL ROW COL                 NAME TYPE      M      t  P.Value      B
2149       2       2   8   7      ApoAI,lipid-Img cDNA -3.166 -23.98 3.05e-11 14.927
540        1       2   7  15 EST,HighlysimilartoA cDNA -3.049 -12.96 5.02e-07 10.813
5356       4       2   9   1 CATECHOLO-METHYLTRAN cDNA -1.848 -12.44 6.51e-07 10.448
4139       3       3   8   2 EST,WeaklysimilartoC cDNA -1.027 -11.76 1.21e-06  9.929
1739       2       1   7  17    ApoCIII,lipid-Img cDNA -0.933  -9.84 1.56e-05  8.192
2537       2       3   7  17 ESTs,Highlysimilarto cDNA -1.010  -9.02 4.22e-05  7.305
1496       1       4  15   5                 est cDNA -0.977  -9.00 4.22e-05  7.290
4941       4       1   8   6 similartoyeaststerol cDNA -0.955  -7.44 5.62e-04  5.311
947        1       3   8   2 EST,WeaklysimilartoF cDNA -0.571  -4.55 1.77e-01  0.563
5604       4       3   1  18                     cDNA -0.366  -3.96 5.29e-01 -0.553
4140       3       3   8   3         APXL2,5q-Img cDNA -0.420  -3.93 5.29e-01 -0.619
6073       4       4   5   4          estrogenrec cDNA  0.421   3.91 5.29e-01 -0.652
1337       1       4   7  14 psoriasis-associated cDNA -0.838  -3.89 5.29e-01 -0.687
954        1       3   8   9   Caspase7,heart-Img cDNA -0.302  -3.86 5.30e-01 -0.757
563        1       2   8  17 FATTYACID-BINDINGPRO cDNA -0.637  -3.81 5.30e-01 -0.839
```

Notice that the top gene is ApoAI itself which is heavily down-regulated. Theoretically the M-value should be minus infinity for ApoAI because it is the knockout gene. Several of the other genes are closely related. The top eight genes here were confirmed by independent assay subsequent to the microarray experiment to be differentially expressed in the knockout versus the control line.

```
> plot(fit$coef[,2],fit$lods[,2],pch=16,cex=0.2,
       xlab="Log Fold Change",ylab="Log Odds",main="KO vs Control")
> ord <- order(fit$lods[,2],decreasing=TRUE)
> top8 <- ord[1:8]
> text(fit$coef[top8,2],fit$lods[top8,2],labels=substring(genelist[top8,"NAME"],1,5),
       cex=0.8,col="blue")
```



10.3 Ecoli Lrp Data: Affymetrix Data with Two Targets

The data are from experiments reported in Hung et al (2002) and are available from the www site `http://visitor.ics.uci.edu/genex/cybert/tutorial/index.html`.

The data is also available from the ecoliLeucine data package available from the Bioconductor www site under "Experimental Data". Hung et al (2002) state that "The purpose of the work presented here is to identify the network of genes that are differentially regulated by the global E. coli regulatory protein, leucine-responsive regulatory protein (Lrp), during steady state growth in a glucose supplemented minimal salts medium. Lrp is a DNA-binding protein tath has been reported to affect the expression of approximately 55 genes." Gene expression in two E. coli bacteria strains, labelled lrp+ and lrp-, were compared using eight Affymetrix ecoli chips, four chips each for lrp+ and lrp-.

The following code assumes that the data files for the eight chips are in your current working directory. The E. coli CDF is also assumed to be available, either the Ecoli CDF data package from Bioconductor is installed or the file Ecoli.CDF is in your current working directory.

```
> dir()
[1] "Ecoli.CDF"          "nolrp_1.CEL"        "nolrp_2.CEL"
[4] "nolrp_3.CEL"        "nolrp_4.CEL"        "wt_1.CEL"
[7] "wt_2.CEL"           "wt_3.CEL"           "wt_4.CEL"
```

The data is read and normalized using the affy package.

```
> library(affy)
Welcome to Bioconductor
        Vignettes contain introductory material.  To view,
```

```
        simply type: openVignette()
        For details on reading vignettes, see
        the openVignette help page.
> Data <- ReadAffy()
> eset <- rma(Data)
Background correcting
Normalizing
Calculating Expression
> pData(eset)
            sample
nolrp_1.CEL      1
nolrp_2.CEL      2
nolrp_3.CEL      3
nolrp_4.CEL      4
wt_1.CEL         5
wt_2.CEL         6
wt_3.CEL         7
wt_4.CEL         8
```

Now we consider differential expression between the lrp+ and lrp- strains.

```
> strain <- c("lrp-","lrp-","lrp-","lrp-","lrp+","lrp+","lrp+","lrp+")
> design <- model.matrix(~factor(strain))
> colnames(design) <- c("lrp-","lrp+vs-")
> design
  lrp- lrp+vs-
1    1       0
2    1       0
3    1       0
4    1       0
5    1       1
6    1       1
7    1       1
8    1       1
attr(,"assign")
[1] 0 1
attr(,"contrasts")
attr(,"contrasts")$"factor(strain)"
[1] "contr.treatment"
```

The first coefficient measures log2-expression of each gene in the lrp- strain. The second coefficient measures the log2-fold change of lrp+ over lrp-, i.e., the log-fold change induced by lrp.

```
> fit <- lmFit(eset, design)
> fit <- eBayes(fit)
> options(digits=2)
> topTable(fit, coef=2, n=40, adjust="fdr")
                        ProbeSetID     M    A     t P.Value      B
4282  IG_821_1300838_1300922_fwd_st -3.32 12.4 -23.1 5.3e-05  8.017
5365                  serA_b2913_st  2.78 12.2  15.8 6.0e-04  6.603
1389                  gltD_b3213_st  3.03 10.9  13.3 1.6e-03  5.779
```

```
4625                   lrp_b0889_st  2.30  9.3  11.4 4.0e-03  4.911
1388                  gltB_b3212_st  3.24 10.1  11.1 4.0e-03  4.766
4609                  livK_b3458_st  2.35  9.9  10.8 4.0e-03  4.593
4901                  oppB_b1244_st -2.91 10.7 -10.6 4.0e-03  4.504
4903                  oppD_b1246_st -1.94 10.4 -10.5 4.0e-03  4.434
5413                  sodA_b3908_st  1.50 10.3   9.7 6.5e-03  3.958
4900                  oppA_b1243_st -2.98 13.0  -9.1 9.2e-03  3.601
5217                   rmf_b0953_st -2.71 13.6  -9.0 9.3e-03  3.474
7300                  ytfK_b4217_st -2.64 11.1  -8.9 9.3e-03  3.437
5007                  pntA_b1603_st  1.58 10.1   8.3 1.4e-02  3.019
4281  IG_820_1298469_1299205_fwd_st -2.45 10.7  -8.1 1.6e-02  2.843
4491                  ilvI_b0077_st  0.95 10.0   7.4 2.9e-02  2.226
5448                  stpA_b2669_st  1.79 10.0   7.4 2.9e-02  2.210
611                        b2343_st -2.12 10.8  -7.1 3.4e-02  2.028
5930                  ybfA_b0699_st -0.91 10.5  -7.0 3.5e-02  1.932
1435                  grxB_b1064_st -0.91  9.8  -6.9 3.8e-02  1.810
4634                  lysU_b4129_st -3.30  9.3  -6.9 3.9e-02  1.758
4829                   ndk_b2518_st  1.07 11.1   6.7 4.3e-02  1.616
2309 IG_1643_2642304_2642452_rev_st  0.83  9.6   6.7 4.3e-02  1.570
4902                  oppC_b1245_st -2.15 10.7  -6.3 5.9e-02  1.238
4490                  ilvH_b0078_st  1.11  9.9   5.9 8.8e-02  0.820
1178                  fimA_b4314_st  3.40 11.7   5.9 8.8e-02  0.743
6224                  ydgR_b1634_st -2.35  9.8  -5.8 8.8e-02  0.722
4904                  oppF_b1247_st -1.46  9.9  -5.8 8.8e-02  0.720
792                        b3914_st -0.77  9.5  -5.7 1.0e-01  0.565
5008                  pntB_b1602_st  1.47 12.8   5.6 1.0e-01  0.496
4610                  livM_b3456_st  1.04  8.5   5.5 1.1e-01  0.376
5097                  ptsG_b1101_st  1.16 12.2   5.5 1.1e-01  0.352
4886                  nupC_b2393_st  0.79  9.6   5.5 1.1e-01  0.333
4898                  ompT_b0565_st  2.67 10.5   5.4 1.2e-01  0.218
5482                   tdh_b3616_st -1.61 10.5  -5.3 1.3e-01  0.092
1927       IG_13_14080_14167_fwd_st -0.55  8.4  -5.3 1.3e-01  0.076
6320                  yeeF_b2014_st  0.88  9.9   5.3 1.3e-01  0.065
196                   atpG_b3733_st  0.60 12.5   5.2 1.4e-01 -0.033
954                   cydB_b0734_st -0.76 11.0  -5.0 1.8e-01 -0.272
1186                  fimI_b4315_st  1.15  8.3   5.0 1.8e-01 -0.298
4013     IG_58_107475_107629_fwd_st -0.49 10.4  -4.9 2.0e-01 -0.407
```

The column M gives the log2-fold change while the column A gives the average log2-intensity for the probe-set. Positive M-values mean that the gene is up-regulated in lrp+, negative values mean that it is repressed.

It is interesting to compare this table with Tables III and IV in Hung et al (2002). Note that the top-ranked gene is an intergenic region (IG) tRNA gene. The knock-out gene itself is in position four. Many of the genes in the above table, including the ser, glt, liv, opp, lys, ilv and fim families, are known targets of lrp.

10.4 Estrogen Data: A 2x2 Factorial Experiment with Affymetrix Arrays

This data is from the estrogen package on Bioconductor. A subset of the data is also analysed in the factDesign package vignette. To repeat this case study you will need to have the R packages affy, estrogen and hgu95av2cdf installed.

The data gives results from a 2x2 factorial experiment on MCF7 breast cancer cells using Affymetrix HGU95av2 arrays. The factors in this experiment were estrogen (present or absent) and length of exposure (10 or 48 hours). The aim of the study is the identify genes which respond to estrogen and to classify these into early and late responders. Genes which respond early are putative direct-target genes while those which respond late are probably downstream targets in the molecular pathway.

First load the required packages:

```
> library(limma)
> library(affy)
Welcome to Bioconductor
         Vignettes contain introductory material.  To view,
         simply type: openVignette()
         For details on reading vignettes, see
         the openVignette help page.
> library(hgu95av2cdf)
```

The data files are contained in the 'extdata' directory of the estrogen package:

```
> datadir <- file.path(.find.package("estrogen"),"extdata")
> dir(datadir)
 [1] "00Index"       "bad.cel"       "high10-1.cel"  "high10-2.cel"  "high48-1.cel"
 [6] "high48-2.cel"  "low10-1.cel"   "low10-2.cel"   "low48-1.cel"   "low48-2.cel"
[11] "phenoData.txt"
```

The target file is called phenoData.txt. We see there are two arrays for each experimental condition, giving a total of 8 arrays.

```
> targets <- readTargets("phenoData.txt",path=datadir,sep="",row.names="filename")
> targets
             filename estrogen time.h
low10-1   low10-1.cel   absent     10
low10-2   low10-2.cel   absent     10
high10-1 high10-1.cel  present     10
high10-2 high10-2.cel  present     10
low48-1   low48-1.cel   absent     48
low48-2   low48-2.cel   absent     48
high48-1 high48-1.cel  present     48
high48-2 high48-2.cel  present     48
```

Now read the cel files into an AffyBatch object and normalize using the `rma()` function from the affy package:

```
> ab <- ReadAffy(filenames=file.path(datadir,targets$filename))
> eset <- rma(ab)
Background correcting
Normalizing
Calculating Expression
```

There are many ways to construct a design matrix for this experiment. Given that we are interested in the early and late estrogen responders, we can choose a parametrization which includes these two contrasts.

```
> treatments <- factor(c(1,1,2,2,3,3,4,4),labels=c("e10","E10","e48","E48"))
> contrasts(treatments) <- cbind(Time=c(0,0,1,1),E10=c(0,1,0,0),E48=c(0,0,0,1))
> design <- model.matrix(~treatments)
> colnames(design) <- c("Intercept","Time","E10","E48")
```

The second coefficient picks up the effect of time in the absence of estrogen. The third and fourth coefficients estimate the log2-fold change for estrogen at 10 hours and 48 hours respectively.

```
> fit <- lmFit(eset,design)
```

We are only interested in the estrogen effects, so we choose a contrast matrix which picks these two coefficients out:

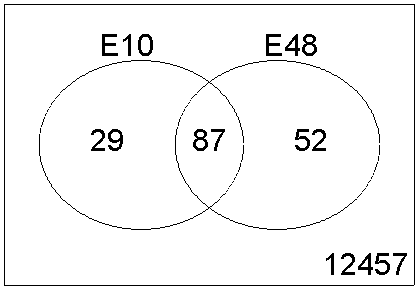
```
> cont.matrix <- cbind(E10=c(0,0,1,0),E48=c(0,0,0,1))
> fit2 <- contrasts.fit(fit, cont.matrix)
> fit2 <- eBayes(fit2)
```

We can examine which genes respond to estrogen at either time using the moderated F-statistics on 2 degrees of freedom. The moderated F p-value is stored in the component `fit2$F.p.value`. What p-value cutoff should be used? One guide is to examine the control and spike-in probe-clusters which theoretically should not be differentially expressed. We find that the smallest p-value amongst these is about 0.00014. So a cutoff p-value of 0.0001, say, would be below any of these.

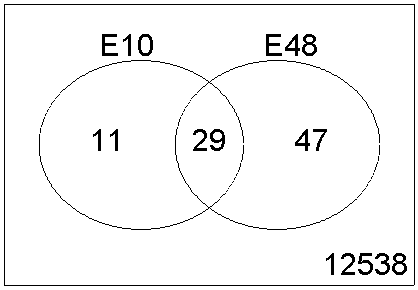
```
> i <- grep("AFFX",geneNames(eset))
> summary(fit2$F.p.value[i])
     Min.   1st Qu.    Median      Mean   3rd Qu.      Max.
0.0001391 0.1727000 0.3562000 0.4206000 0.6825000 0.9925000
```

Now we consider those genes with moderated F-statistics with p-values below 0.0001, and classify these according to whether they are significantly up or down regulated at the early or late times:

```
> results <- classifyTestsF(fit2, p.value=0.0001)
> table(E10=results[,1],E48=results[,2])
    E48
E10  -1    0     1
  -1    29    11     0
  0     47 12370    52
  1      0    29    87
> vennDiagram(results,include="up")
```




```
> vennDiagram(results,include="down")
```



We see that 87 genes were up regulated at both 10 and 48 hours, 29 only at 10 hours and 52 only at 48 hours.. Also, 29 genes were down-regulated throughout, 11 only at 10 hours and 47 only at 48 hours. No genes were up at one time and down at the other.

`topTable` gives a detailed look at individual genes. The leading genes are clearly significant, even using the default p-value adjustment method, which is the highly conservative Holm's method.

```
> options(digits=3)
> topTable(fit2,coef="E10",n=20)
             ID     M     A     t  P.Value    B
9735   39642_at  2.94  7.88  23.7 5.99e-05 9.97
12472    910_at  3.11  9.66  23.6 6.26e-05 9.94
1814   31798_at  2.80 12.12  16.4 1.29e-03 7.98
11509  41400_at  2.38 10.04  16.2 1.41e-03 7.92
```

```
10214  40117_at  2.56  9.68  15.7 1.86e-03 7.70
953     1854_at  2.51  8.53  15.2 2.46e-03 7.49
9848   39755_at  1.68 12.13  15.1 2.59e-03 7.45
922   1824_s_at  1.91  9.24  14.9 2.86e-03 7.37
140   1126_s_at  1.78  6.88  13.8 5.20e-03 6.89
580     1536_at  2.66  5.94  13.3 7.30e-03 6.61
12542    981_at  1.82  7.78  13.1 8.14e-03 6.52
3283   33252_at  1.74  8.00  12.6 1.12e-02 6.25
546     1505_at  2.40  8.76  12.5 1.20e-02 6.19
4405   34363_at -1.75  5.55 -12.2 1.44e-02 6.03
985   1884_s_at  2.80  9.03  12.1 1.59e-02 5.95
6194   36134_at  2.49  8.28  11.8 1.90e-02 5.79
7557   37485_at  1.61  6.67  11.4 2.50e-02 5.55
1244     239_at  1.57 11.25  10.4 5.14e-02 4.90
8195   38116_at  2.32  9.51  10.4 5.16e-02 4.90
10634  40533_at  1.26  8.47  10.4 5.31e-02 4.87
> topTable(fit2,coef="E48",n=20)
             ID     M     A     t  P.Value     B
12472    910_at  3.86  9.66  29.2 1.04e-05 11.61
1814   31798_at  3.60 12.12  21.1 1.62e-04  9.89
953     1854_at  3.34  8.53  20.2 2.29e-04  9.64
8195   38116_at  3.76  9.51  16.9 1.02e-03  8.48
8143   38065_at  2.99  9.10  16.2 1.42e-03  8.21
9848   39755_at  1.77 12.13  15.8 1.72e-03  8.05
642     1592_at  2.30  8.31  15.8 1.76e-03  8.03
11509  41400_at  2.24 10.04  15.3 2.29e-03  7.81
3766   33730_at -2.04  8.57 -15.1 2.48e-03  7.74
732     1651_at  2.97 10.50  14.8 3.02e-03  7.57
8495   38414_at  2.02  9.46  14.6 3.36e-03  7.48
1049    1943_at  2.19  7.60  14.0 4.69e-03  7.18
10214  40117_at  2.28  9.68  14.0 4.79e-03  7.16
10634  40533_at  1.64  8.47  13.5 6.24e-03  6.93
9735   39642_at  1.61  7.88  13.0 8.46e-03  6.65
4898   34851_at  1.96  9.96  12.8 9.47e-03  6.55
922   1824_s_at  1.64  9.24  12.8 1.00e-02  6.50
6053   35995_at  2.76  8.87  12.7 1.05e-02  6.46
12455    893_at  1.54 10.95  12.7 1.06e-02  6.45
10175  40079_at -2.41  8.23 -12.6 1.09e-02  6.42
```

10.5 Weaver Mutant Data: A 2x2 Factorial Experiment with Two-Color Data

This case study considers a more involved analysis in which the sources of RNA have a factorial structure. In this example we assume that data is available as an `RGList`.

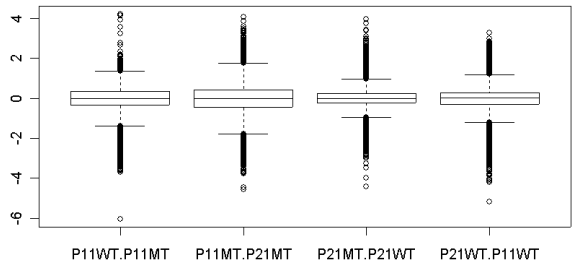
Background. This is a case study examining the development of certain neurons in wild-type and weaver mutant mice from Diaz et al (2002). The weaver mutant affects cerebellar granule neurons, the most numerous cell-type in the central nervous system. Weaver mutant mice are characterized by a weaving gait. Granule cells are generated in the first postnatal week in the external granule layer of the cerebellum. In normal mice, the terminally differentiated granule

cells migrate to the internal granule layer but in mutant mice the cells die before doing so, meaning that the mutant mice have strongly reduced numbers of cells in the internal granule layer. The expression level of any gene which is specific to mature granule cells, or is expressed in response to granule cell derived signals, is greatly reduced in the mutant mice.

Tissue dissection and RNA preparation. At each time point (P11 = 11 days postnatal and P21 = 21 days postnatal) cerebella were isolated from two wild-type and two mutant littermates and pooled for RNA isolation. RNA was then divided into aliquots and labelled before hybridizing to the arrays. (This means that different hybridizations are biologically related through using RNA from the same mice, although we will ignore this here. See Yang and Speed (2002) for a detailed discussion of this issue in the context of this experiment.)

Hybridizations. We have just four arrays each comparing two out of the four treatment combinations of time (11 days or 21 days) by genotype (wild-type or mutant). This has the structure of a 2x2 factorial experiment.

```
> objects()
[1] "designIA" "designMt" "gal" "layout" "RG" "Targets"
> Targets
   FileName        Name   Cy5   Cy3
1 cb.1.spot P11WT.P11MT P11WT P11MT
2 cb.2.spot P11MT.P21MT P11MT P21MT
3 cb.3.spot P21MT.P21WT P21MT P21WT
4 cb.4.spot P21WT.P11WT P21WT P11WT
> MA <- normalizeWithinArrays(RG,layout)
> boxplot(MA$M~col(MA$M),names=Targets$Name)
```



First we consider a classical interaction parametrization.

```
> designIA
            TimeWt Mutant11 I/A
P11WT.P11MT      0       -1   0
P11MT.P21MT     -1        0  -1
P21MT.P21WT      0        1   1
P21WT.P11WT      1        0   0
```

TimeWt is late vs early time for the wild-type mice. Mutant11 is mutant vs wild-type at the early time. The third column estimates the interaction between time and genotype.

```
> fitIA <- lmFit(MA,designIA)
> ebIA <- ebayes(fitIA)
> options(digits=3)
> toptable(coef="I/A",n=10,genelist=gal,fit=fitIA,eb=ebIA,adjust="fdr")
           ID            Name    M     t P.Value     B
7737    RIKEN           Z6801 6.49 12.95   0.886 -4.03
780     RIKEN            Z636 6.57 12.67   0.886 -4.03
4063    RIKEN           Z3559 6.41 12.37   0.886 -4.03
3627  Control              L1 6.08 11.89   0.886 -4.03
3084    RIKEN           Z2652 4.88  9.38   1.000 -4.04
16230 Control  T7/SP6 7- Vrg2 6.00  9.12   1.000 -4.05
12537   RIKEN          Z11025 5.03  9.03   1.000 -4.05
2866    RIKEN           Z2506 4.19  8.46   1.000 -4.05
11430 Control T7/SP6 5- msx 1 3.31  6.40   1.000 -4.08
15590   RIKEN          Z13718 3.17  5.88   1.000 -4.10
```

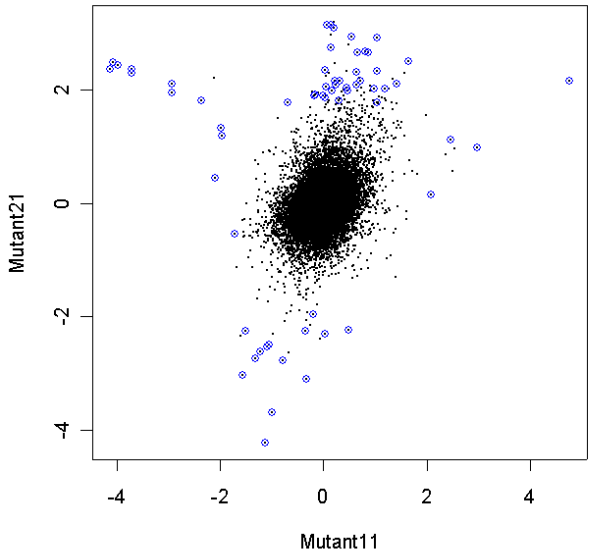
With only four arrays there is only one residual df for the linear model, so even large M-values and t-statistics are not significant after adjusting for multiple testing. There are differentially expressed genes here, although it is difficult to confirm it from the four arrays that we are using for this exercise.

Consider another parametrization.

```
> designMt
            Mutant11 Mutant21 TimeMt
P11WT.P11MT       -1        0      0
P11MT.P21MT        0        0     -1
P21MT.P21WT        0        1      0
P21WT.P11WT        1       -1      1
```

Here Mutant21 is mutant vs wild-type at the later time and TimeMt is late vs early time for the mutant mice.

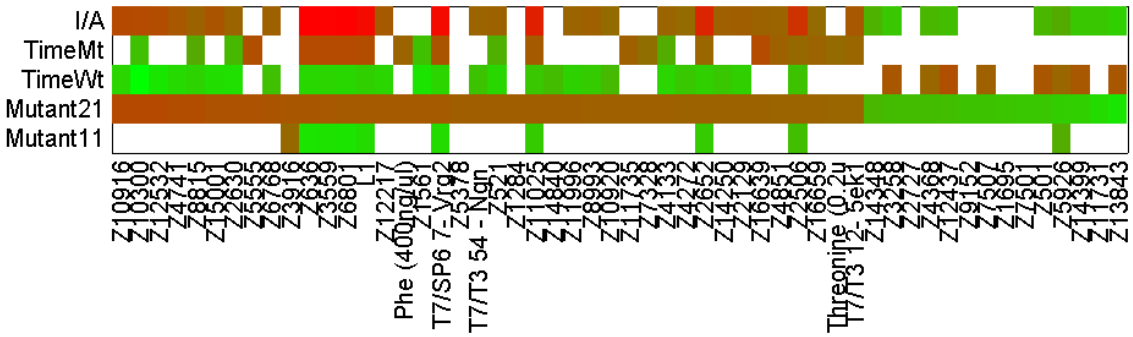
```
> fitMt <- lm.series(MA$M,designMt)
> ebMt <- ebayes(fitMt)
> plot(fitMt$coef[,"Mutant11"],fitMt$coef[,"Mutant21"],pch=16,cex=0.2,
       xlab="Mutant11",ylab="Mutant21")
> sel <- abs(ebMt$t[,"Mutant11"])>4 | abs(ebMt$t[,"Mutant21"])>4
> points(fitMt$coef[sel,"Mutant11"],fitMt$coef[sel,"Mutant21"],col="blue")
```



This scatterplot allows the genes to be visually clustered according to whether they are differentially expressed in the mutant at the two times.

We will now collate the results of the two fits.

```
> fit <- fitIA
> fit$coefficients <- cbind(fitMt$coef,fitIA$coef)
> fit$coefficients <- fit$coef[,c(1,2,4,3,6)]
> fit$coef[1:5,]
     Mutant11 Mutant21  TimeWt TimeMt    I/A
[1,]  -0.5396   0.1670  1.3362  2.043 0.7066
[2,]   0.2481   0.8601 -0.9112 -0.299 0.6120
[3,]  -1.1368  -0.5642 -0.0119  0.561 0.5726
[4,]  -1.0166  -0.5837  0.0837  0.517 0.4329
[5,]   0.0135   0.0614  0.3701  0.418 0.0479
> fit$stdev.unscaled <- cbind(fitMt$std,fitIA$std)
> fit$stdev.unscaled <- fit$std[,c(1,2,4,3,6)]
> fit$std[1:5,]
     Mutant11 Mutant21 TimeWt TimeMt I/A
[1,]    0.866    0.866  0.866  0.866   1
[2,]    0.866    0.866  0.866  0.866   1
[3,]    0.866    0.866  0.866  0.866   1
[4,]    0.866    0.866  0.866  0.866   1
[5,]    0.866    0.866  0.866  0.866   1
> eb <- ebayes(fit)
> heatdiagram(abs(eb$t),fit$coef,"Mutant21",names=gal$Name)
```



This heat diagram shows the expression profiles for all genes judged to be differentially expressed ($|t| > 4$) with respect to Mutant21. The genes are sorted from left to right in terms of their coefficients for Mutant21, with red meaning up-regulation and green meaning down-regulation. It is especially interesting to see that genes which are up-regulated (red) in the mutant at 21 days are those which have decreasing expression in the wild-type over time, and those which are down-regulated (green) in the mutant are those which increase over time in the wild-type. The mutant is not participating in normal development between 11 and 21 days in respect of these genes.

11 Within-Array Replicate Spots

In this section we consider a case study in which all genes (ESTs and controls) are printed more than once on the array. This means that there is both within-array and between-array replication for each gene. The structure of the experiment is therefore essentially a randomized block experiment for each gene. The approach taken here is to estimate a common correlation for all the genes for between within-array duplicates. The theory behind the approach is explained in Smyth, Michaud and Scott (2003). This approach assumes that all genes are replicated the same number of times on the array and that the spacing between the replicates is entirely regular.

11.1 Example. Bob Mutant Data

In this example we assume that the data is available as an RG list.

Background. This data is from a study of transcription factors critical to B cell maturation by Lynn Corcoran and Wendy Dietrich at the WEHI. Mice which have a targeted mutation in the Bob (OBF-1) transcription factor display a number of abnormalities in the B lymphocyte compartment of the immune system. Immature B cells that have emigrated from the bone marrow fail to differentiate into full fledged B cells, resulting in a notable deficit of mature B cells.

Arrays. Arrays were printed with expressed sequence tags (ESTs) from the National Institute of Aging 15k mouse clone library, plus a range of positive, negative and calibration controls.

The arrays were printed using a 48 tip print head and 26x26 spots in each tip group. Data from 24 of the tip groups are given here. Every gene (ESTs and controls) was printed twice on each array.

Hybridizations. A retrovirus was used to add Bob back to a Bob deficient cell line. Two RNA sources were compared using 2 dye-swap pairs of microarrays. One RNA source was obtained from the Bob deficient cell line after the retrovirus was used to add GFP ("green fluorescent protein", a neutral protein). The other RNA source was obtained after adding both GFP and Bob protein. RNA from Bob+GFP was labelled with Cy5 in arrays 2 and 4, and with Cy3 in arrays 1 and 4.

```
> objects()
[1] "design" "gal"    "layout" "RG"
> design
[1] -1  1 -1  1
> gal[1:40,]
   Library            Name
1  Control       cDNA1.500
2  Control       cDNA1.500
3  Control Printing.buffer
4  Control Printing.buffer
5  Control Printing.buffer
6  Control Printing.buffer
7  Control Printing.buffer
8  Control Printing.buffer
9  Control       cDNA1.500
10 Control       cDNA1.500
11 Control Printing.buffer
12 Control Printing.buffer
13 Control Printing.buffer
14 Control Printing.buffer
15 Control Printing.buffer
16 Control Printing.buffer
17 Control       cDNA1.500
18 Control       cDNA1.500
19 Control Printing.buffer
20 Control Printing.buffer
21 Control Printing.buffer
22 Control Printing.buffer
23 Control Printing.buffer
24 Control Printing.buffer
25 Control       cDNA1.500
26 Control       cDNA1.500
27  NIA15k             H31
28  NIA15k             H31
29  NIA15k             H32
30  NIA15k             H32
31  NIA15k             H33
32  NIA15k             H33
33  NIA15k             H34
34  NIA15k             H34
```

```
35  NIA15k            H35
36  NIA15k            H35
37  NIA15k            H36
38  NIA15k            H36
39  NIA15k            H37
40  NIA15k            H37
```

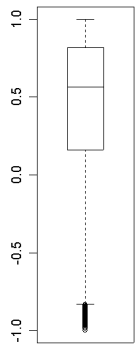
Although there are only four arrays, we have a total of eight spots for each gene, and more for the controls. Naturally the two M-values obtained from duplicate spots on the same array are highly correlated. The problem is how to make use of the duplicate spots in the best way. The approach taken here is to estimate the spatial correlation between the adjacent spots using REML and then to conduct the usual analysis of the arrays using generalized least squares.

First normalize the data using print-tip loess regression.

```
> MA <- normalizeWithinArrays(RG,layout)
```

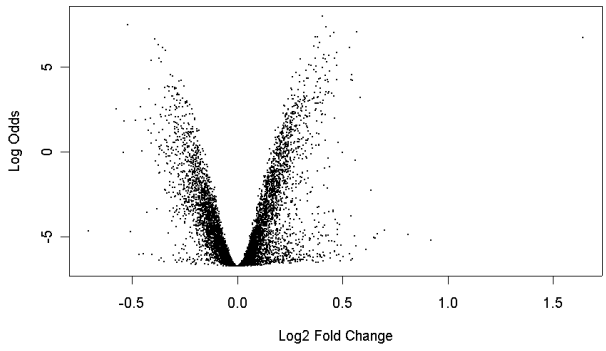
Now estimate the spatial correlation. We estimate a correlation term by REML for each gene, and then take a trimmed mean on the atanh scale to estimate the overall correlation. This command takes a lot of time, perhaps as much as an hour for a series of arrays.

```
> cor <- duplicateCorrelation(MA,design,ndups=2) # This is a slow computation!
> cor$consensus.correlation
[1] 0.571377
> boxplot(cor$all.correlations)
```




```
> fit <- lmFit(MA,design,ndups=2,correlation=0.571377)
> fit <- eBayes(fit)
> topTable(fit,n=30,adjust="fdr")
     Name          M          t      P.Value        B
1  H34599  0.4035865  13.053838 0.0004860773 7.995550
2  H31324 -0.5196599 -12.302094 0.0004860773 7.499712
3  H33309  0.4203320  12.089742 0.0004860773 7.352862
4   H3440  0.5678168  11.664229 0.0004860773 7.049065
```

```
5  H36795  0.4600335  11.608550 0.0004860773 7.008343
6   H3121  0.4408640  11.362917 0.0004860773 6.825927
7  H36999  0.3806754  11.276571 0.0004860773 6.760715
8   H3132  0.3699805  11.270201 0.0004860773 6.755881
9  H32838  1.6404839  11.213454 0.0004860773 6.712681
10 H36207 -0.3930972 -11.139510 0.0004860773 6.656013
11 H37168  0.3909476  10.839880 0.0005405097 6.421932
12 H31831 -0.3738452 -10.706775 0.0005405097 6.315602
13 H32014  0.3630416  10.574797 0.0005405097 6.208714
14 H34471 -0.3532587 -10.496483 0.0005405097 6.144590
15 H37558  0.5319192  10.493157 0.0005405097 6.141856
16  H3126  0.3849980  10.467091 0.0005405097 6.120389
17 H34360 -0.3409371 -10.308779 0.0005852911 5.988745
18 H36794  0.4716704  10.145670 0.0006399135 5.850807
19  H3329  0.4125222  10.009042 0.0006660758 5.733424
20 H35017  0.4337911   9.935639 0.0006660758 5.669656
21 H32367  0.4092668   9.765338 0.0006660758 5.519781
22 H32678  0.4608290   9.763809 0.0006660758 5.518423
23 H31232 -0.3717084  -9.758581 0.0006660758 5.513778
24  H3111  0.3693533   9.745794 0.0006660758 5.502407
25 H34258  0.2991668   9.722656 0.0006660758 5.481790
26 H32159  0.4183633   9.702614 0.0006660758 5.463892
27 H33192 -0.4095032  -9.590227 0.0007130533 5.362809
28 H35961 -0.3624470  -9.508868 0.0007205823 5.288871
29 H36025  0.4265827   9.503974 0.0007205823 5.284403
30  H3416  0.3401763   9.316136 0.0008096722 5.111117
> plot(fit$coef,eb$lods,xlab="Log2 Fold Change",ylab="Log Odds",pch=16,cex=0.2)
```



12 Using limma with the marray Package

The package marray is designed to read and normalize cDNA data. The marray package provides location and scale normalization of M-values, rather than the within and between-array approach of limma, and provides some normalization methods which are not in limma. Data input using the marray packages produces a data object of class `marrayRaw`. Normalization using marrayNorm will produce a data object of class `marrayNorm`. Suppose that you have an `marrayNorm` object called `Data`.

The marrayNorm data object may be used directly in the lmFit function in limma, for example

```
> fit <- lmFit(Data, design)
```

after which one proceeds exactly as in previous sections.

Alternatively, you may convert marray data objects to limma data objects using the convert package. marrayRaw objects may be converted to RGList objects and marrayNorm objects to MAList objects:

```
> library(convert)
> MA <- as(Data, "MAList")
```

13 Between Array Normalization of Two-Color Arrays

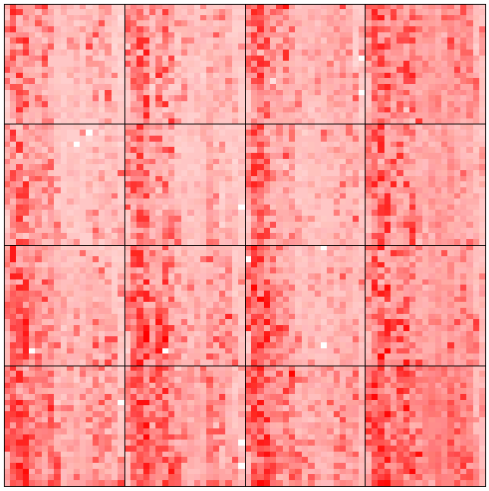
This section explores some of the methods available for between-array of two-color arrays. A feature which distinguishes most of these methods from within-array normlization is the focus on the individual red and green intensity values rather than merely on the M-values or log-ratios. These methods might therefore be called *individual channel* or *separate channel* normalization methods. The ApoAI data set from Section 10.2 will be used to illustrate these methods. We assume that the the ApoAI data has been loaded and background corrected as follows:

```
> load("ApoAI.RData")
> RG.b <-backgroundCorrect(RG,method="minimum")
```

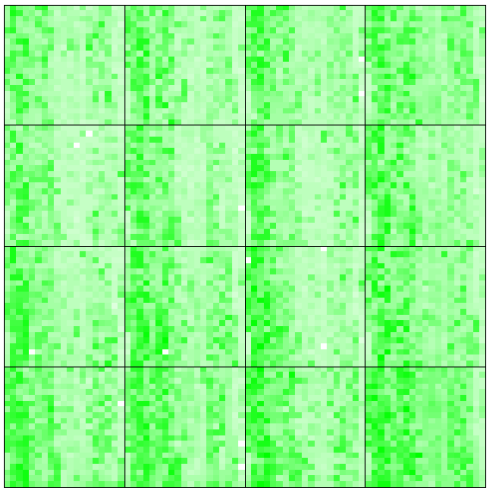
Two-color microarrays compare the gene expression between two different sources of RNA for thousands of genes simultaneously. In general, the log-ratio of spot intensities for the red and green channels form the primary data used for downstream analysis. Thus traditional normalization methods, which remove systematic variation in microarray data, focus on adjusting the log-ratios within each slide. However sometimes it is desirable to work with single-channel (log-intensity) data rather than the log-ratios and so new techniques for normalizing such single-channel data have been investigated. In the current literature there has been limited attention given to single-channel normalization despite many groups basing their entire analyses on single channel data. Single-channel data display a higher level of systematic variation than that observed in log-ratio data.

For example below are imageplots of the log-intensity single-channels and the log-ratio for a single array from the ApoAI data set. (The imageplots below are based on non-normalised background corrected data). Clearly some of the systematic spatial variation is cancelled out by forming the log-ratio. This is just a simple demonstration of how M-values are less noisy than single-channels.

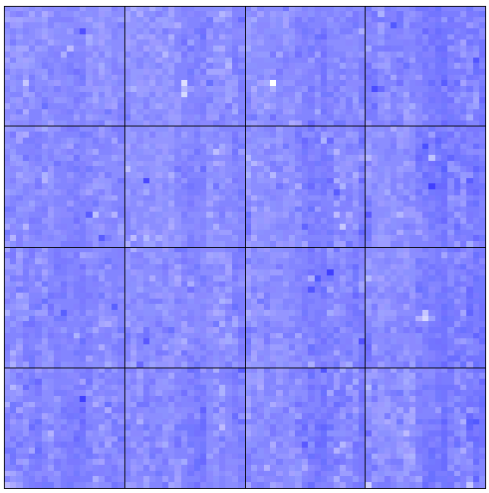
```
> imageplot(log(RG.b$R[,4],2), layout, low="white", high="red")
```



```
> imageplot(log(RG.b$G[,4],2), layout, low="white", high="green")
```



```
> imageplot(log(MA.n$M[,4],2), layout, low="white", high="blue")
```



It should be noted that analysing log-ratios corresponds to doing all analysis on the basis of within-array contrasts while the single-channel approach gives the possibility of recovering information from the between-array variation. This should only be considered after careful single-channel normalization to remove uncontrolled systematic effects at the array level. Yang and Thorne (2003) provides an outline of the motivations for performing single-channel (log-intensity) analysis. We currently perform single-channel normalization using a quantile method based on Bolstad *et al.*'s quantile normalization of high density oligonucleotide data). In the following we demonstrate within-slide and between-slide single-channel normalization routines. We use the ApoAI data set to illustrate the methods.

We perform the normalization of single-channel data using methods in the `normalizeWithinArrays` and `normalizeBetweenArrays` functions.

Note that `RG.b` contains unlogged single-channel intensities and `normalizeWithinArrays` expects its input `RGlist` to be unlogged. There is an argument `log.transform=FALSE` which needs to be implemented if the `RGlist` supplied is already logged. The following command creates an `MAList` containing non-normalized background corrected values.

```
> MA.n <-normalizeWithinArrays(RG.b,layout,method="n")
```

Next we normalize the M-values via the default within array normalization of `printtiploess` (we could have use the method `loess` instead, but we find that `printtiploess` is often a good choice since it acts as a proxy for spatial normalization of the Mvalues.

```
> MA.p <-normalizeWithinArrays(RG.b,layout)
```

At any stage we can recover the `RGList` of normalized single-channels using `RG.MA`. `RG.MA(MA.p)` would give us within-array only normalized single-channels. Next we perform between array normalization of the single-channels. We use the function `normalizeBetweenArrays` which takes and returns an `MAList`. `normalizeBetweenArrays` forms an RG matrix when implementing the `quantile` normalization method on the single-channels; and although it returns an `MAList` the single-channel normalised values can be obtained by using the function `RG.MA`. We show how to implement the following between array normalization methods respectively, quantile normalization between all single-channels only (**q**); quantile normalization after printtiploess normalization within arrays (**pq**); quantile normalization between the arrays on the `Aq` values which is then combined with the within array printtiploess normalization `Mp` to give `MpAq`. Notice that for `MpAq` we have mixed and matched different within and between array normlizations to create a simultaneous within and between array single-channel normalization method.

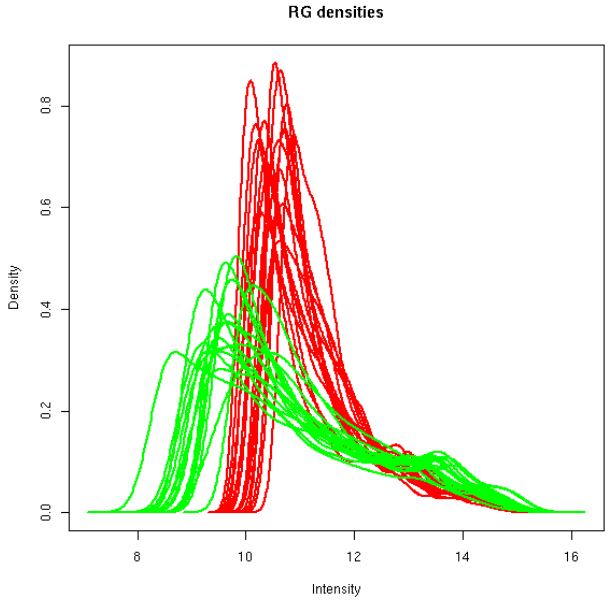
```
> MA.q <- normalizeBetweenArrays(MA.n, method="quantile")
> MA.pq <- normalizeBetweenArrays(MA.p, method="quantile")
> MA.Aq <- normalizeBetweenArrays(MA.n, method="Aquantile")
> MA.MpAq <- new("MAList", list(M=MA.p$M, A=MA.Aq$A))
```

We find that **pq** and **MpAq** work quite well. Next we show some plots of the single-channel log-intensity densities which illustrate the results of the different single-channel normalization methods. We use the function `plotDensities` which will take either an `RGList` or

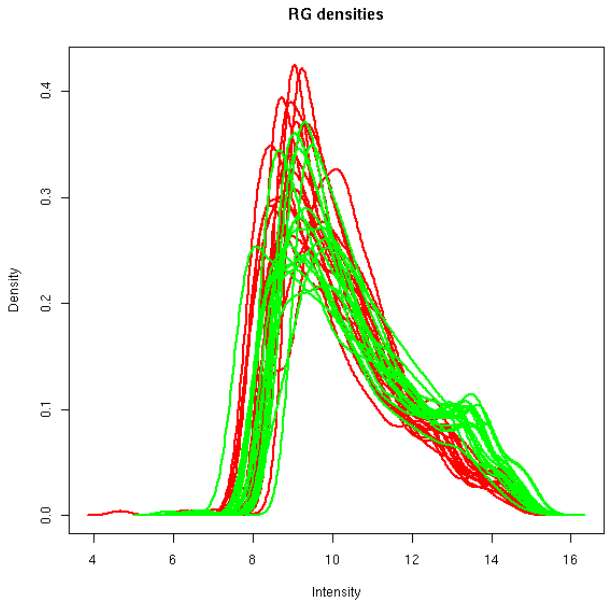
an `MAList` . The form of the call is: `plotDensities(object, log.transform = FALSE, arrays = NULL, singlechannels = NULL, groups = NULL, col = NULL)`. The default usage of `plotDensities` results in red/green coloring of the densities.

Without any background correction there is a significant difference between the red and green single-channel intensity distributions:

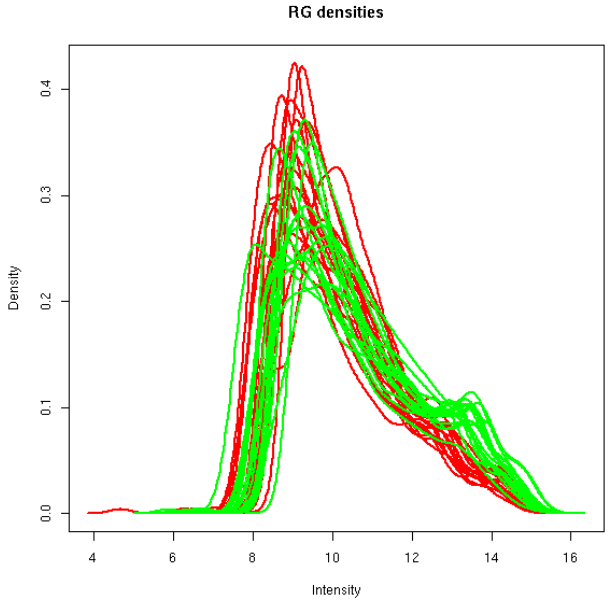
```
> plotDensities(RG, log.transform=TRUE)
```




```
> plotDensities(RG.b, log.transform=TRUE)
```

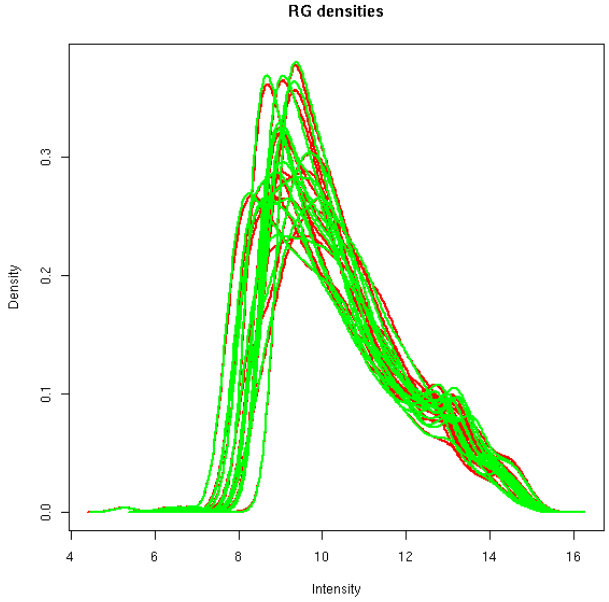


```
> plotDensities(MA.n)
```



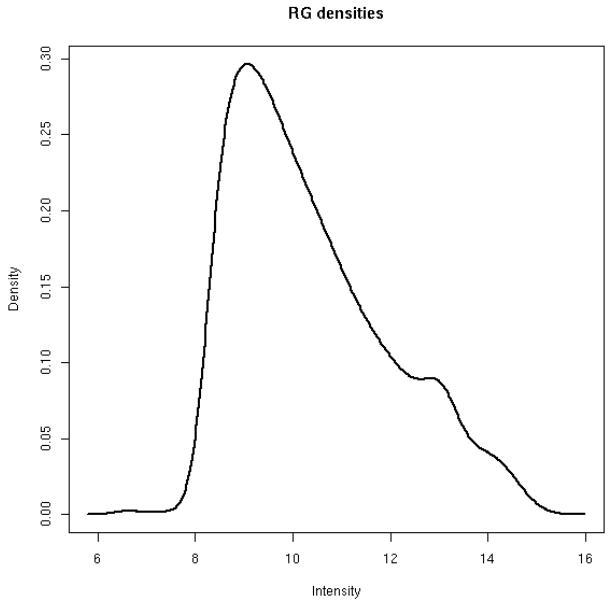
`Printtiploess` makes the single-channels within arrays similar:

```
> plotDensities(MA.p)
```

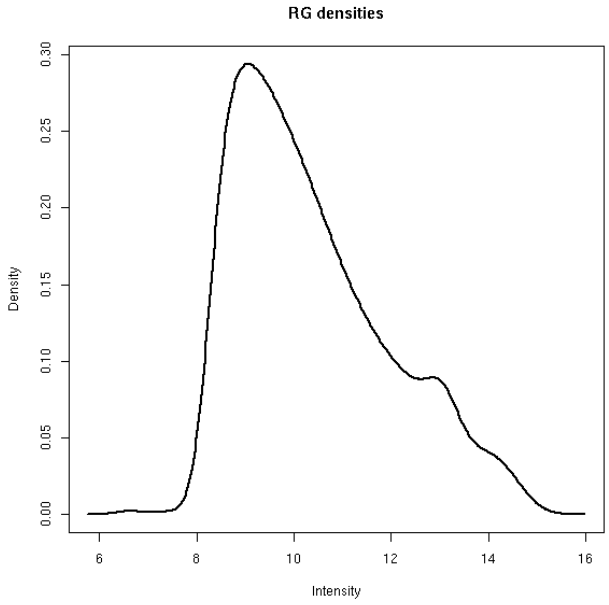


All the single-channels have the same distribution.

```
> plotDensities(MA.q, col="black")
```

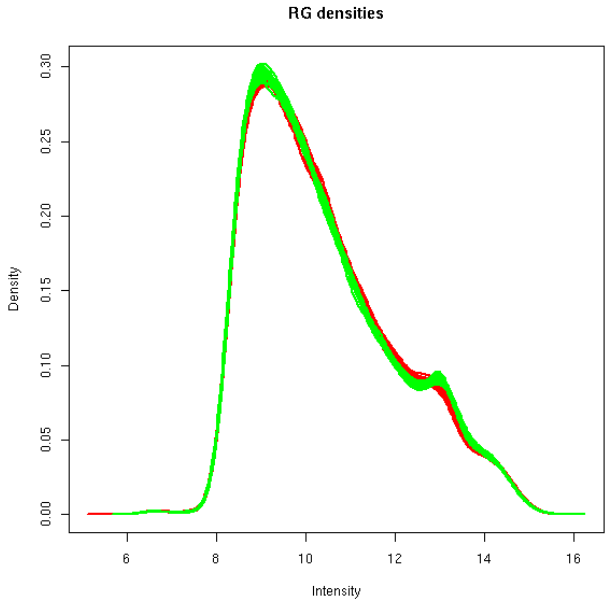



```
> plotDensities(MA.pq, col="black")
```



MpAq gives very similar results as **pq**.

```
> plotDensities(MA.MpAq)
```



Conventions

Where possible, limma tries to use the convention that class names are in upper CamelCase, i.e., the first letter of each word is capitalized, while function names are in lower camelCase, i.e., first word is lowercase. When periods appear in function names, the first word should be an action while the second word is the name of a type of object on which the function acts.

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Published Articles Using limma

The following articles are not cited in the User's Guide but are examples of biologically orientated publications which use the limma package.

1. Golden, T., R., and Melov, S. (2004). Microarray analysis of gene expression with age in individual nematodes. *Aging Cell* 3, 111–124. doi:10.1111/j.1474-9728.2004.00095.x Online: `http://www.blackwell-synergy.com/links/doi/10.1111/j.1474-9728.2004.00095.x` (Published June 2004)

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