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. The normalization and data analysis functions are for two-colour spotted microarrays. The linear model and differential expression functions apply to all microarrays including Affymetrix and other multi-array oligonucleotide experiments.

The Bioconductor packages marrayClasses, marrayInput and marrayNorm provide alternative functions for reading and normalizing spotted microarray data. If you are using limma in conjunction with these packages, see Section 10. The package affy provides functions for reading and normalizing Affymetrix microarray data. If you are using the affy package, see Sections 7.2 and 11.

This tutorial was prepared using R Version 1.8.0 for Windows and limma version 1.3.0. The latest version of limma is always available from http://www.bioconductor.org under "Developmental Packages" or from http://bioinf.wehi.edu.au/limma/. If you are using Windows, you can install the last official Bioconductor release of limma from the drop-down menu in R, simply select `Packages` then `Install package(s) from Bioconductor...`. Note however that this is updated only once every 6 months. The data sets used in the case study examples can be downloaded from http://bioinf.wehi.edu.au/marray/genstat2002/. Help with limma is available by sending questions or problems to bioconductor@stat.math.ethz.ch.

This guide describes limma as a command-driven package. A menu-driven interface called limmaGUI is also available to most commonly used functions in limma. LimmaGUI is available from http://bioinf.wehi.edu.au/limmaGUI. Although using limmaGUI is easy, installing limmaGUI is at the time of writing a job for an IT professional or for a very experienced computer user because it depends on tck/tk extensions which are not part of standard R.

2. 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. 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.

3. 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 Gene Allocation 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.

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

4. Reading Data into Limma

4.1 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 (i) a file which describes the probes, often called the *Gene List*, and (ii) the image analysis output files. 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.

4.2 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. For example, if the files are SPOT output and have common extension "spot" then they can be read using

```
  files <- dir(pattern="*\\.spot")
  RG <- read.maimages(files, source="spot")
```

The object `files` is then a character vector containing all the spot file names in alphabetical order. If the files are GenePix output files and have extension "gpr" then they can be read using

```
  files <- dir(pattern="*\\.gpr")
  RG <- read.maimages(files, source="genepix")
```

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.

4.3. 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.

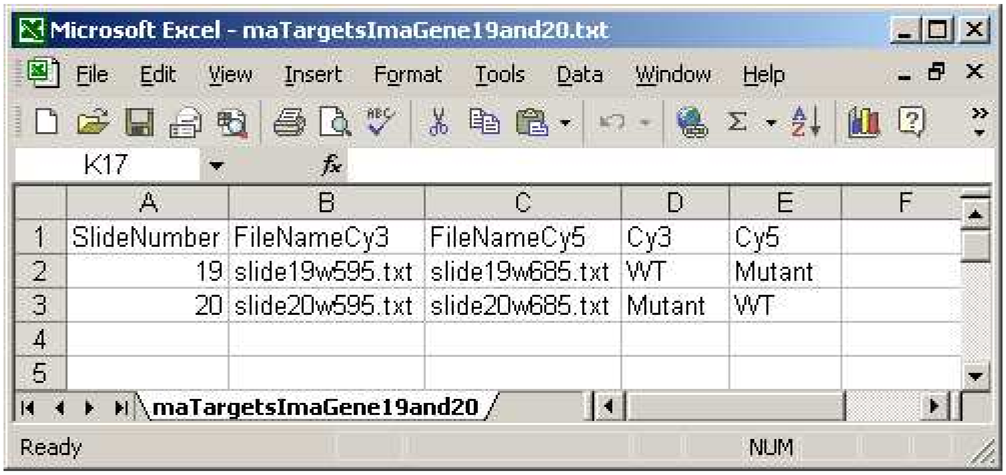
Computing quality weights depends on the image analysis program. Consult the help entry `QualityWeights` to see what quality weight functions are available.

4.4 The Targets File

Although not necessary to use limma it is usually a good idea to construct a targets file which lists the RNA target hybridized to each channel of each array. The Targets File is normally in tab-delimited text format. The file should contain a row for each microarray. It should contain 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.

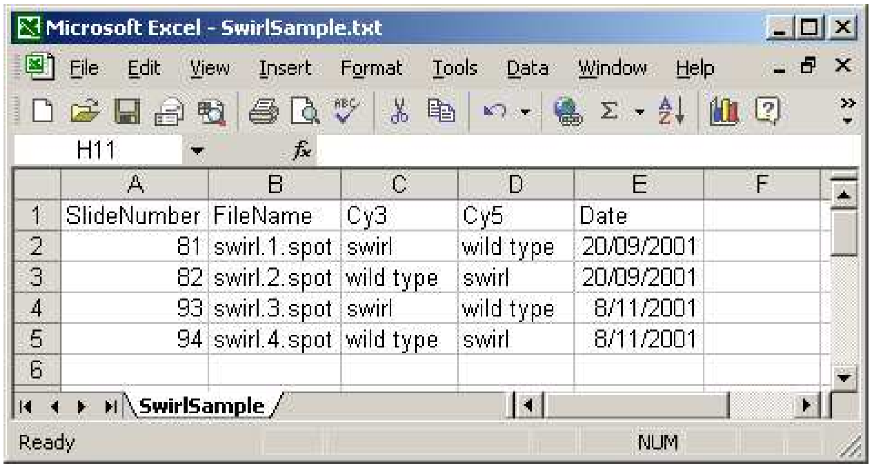
Some examples are shown below.

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.



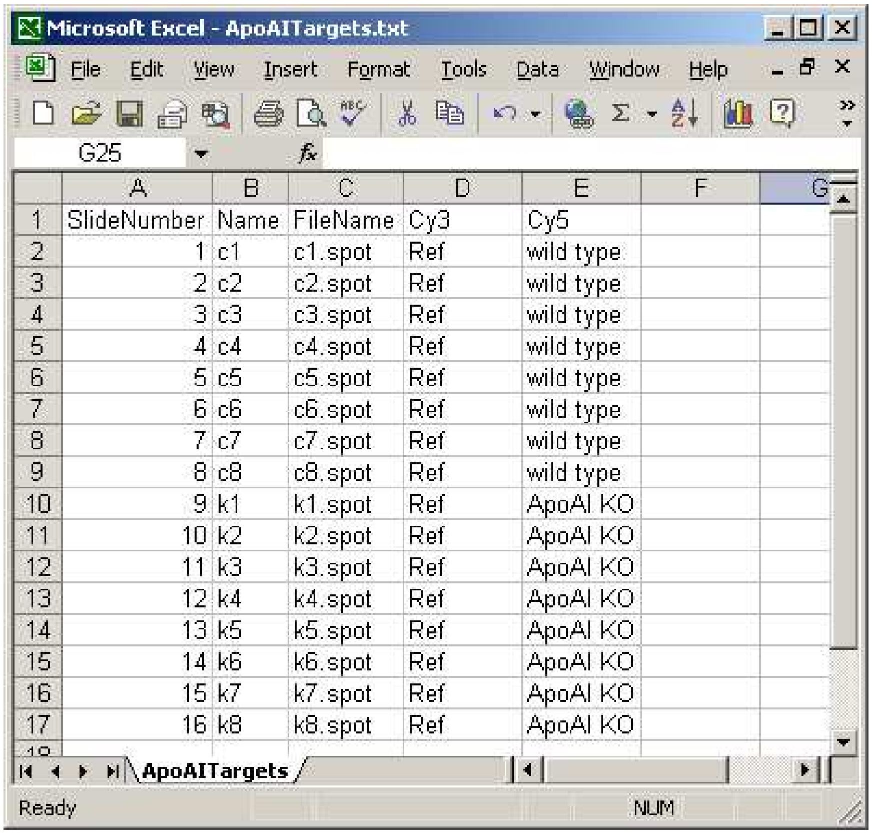
SlideNumber	FileNameCy3	FileNameCy5	Cy3	Cy5
19	slide19w595.txt	slide19w685.txt	WT	Mutant
20	slide20w595.txt	slide20w685.txt	Mutant	WT

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



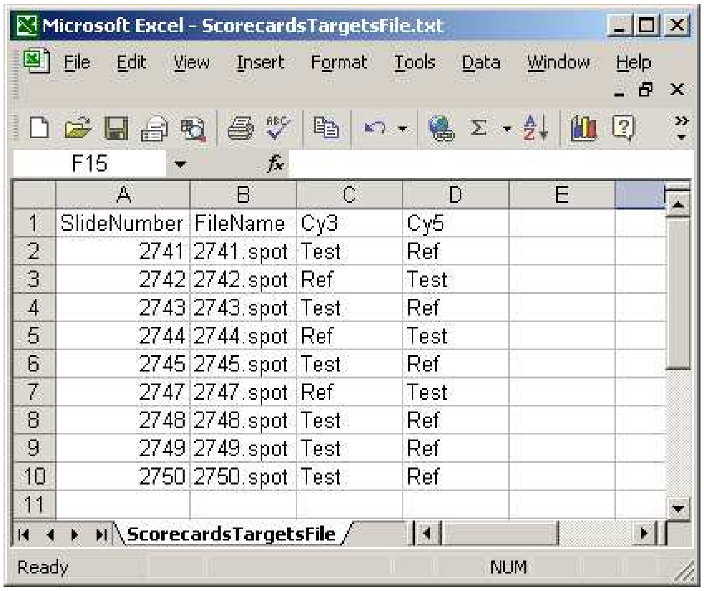
SlideNumber	FileName	Cy3	Cy5	Date
81	swirl.1.spot	swirl	wild type	20/09/2001
82	swirl.2.spot	wild type	swirl	20/09/2001
93	swirl.3.spot	swirl	wild type	8/11/2001
94	swirl.4.spot	wild type	swirl	8/11/2001

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".



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


Microsoft Excel - ScorecardsTargetsFile.txt
File Edit View Insert Format Tools Data Window Help
F15
fx
ScorecardsTargetsFile
Ready
NUM

	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()`. The file can have any name but the default name is `Targets.txt`. Very often the targets file will be first thing read because it contains the image analysis output file names, e.g.,

```
> targets <- readTargets()
> RG <- read.maimages(targets$FileName)
```

4.5 Reading the Gene List

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()
```

The print layout of the arrays can be extracted from the GAL by

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

Non-Genepix gene lists can be read into R using the function `read.table` from R base. If you have Imagene or SMD image analysis output, then the gene list will be extracted from the image analysis output files by `read.images`.

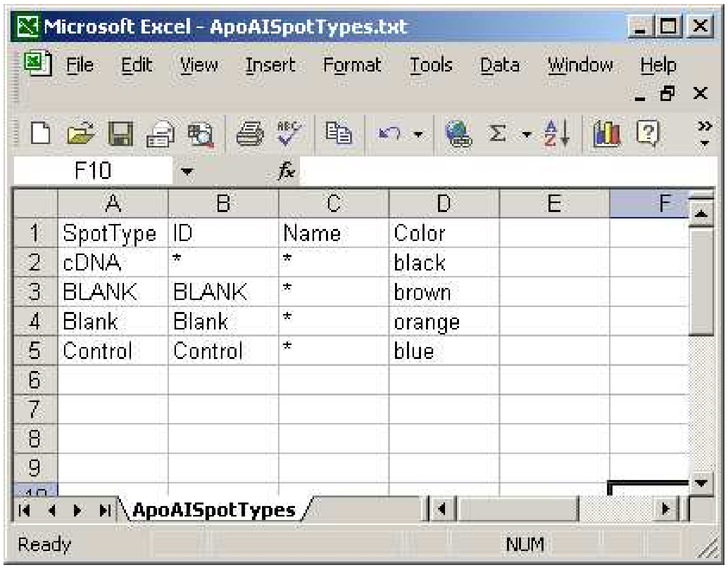
4.6 The Spot Types File

The Spot Types file (another tab-delimited text file) is optional but it is very useful in distinguishing between genes, controls and blanks when using the Color-Coded M A Plot (with legend) feature. Certain spot types (e.g. scorecard controls) can be excluded from a linear model fit if desired. For a given spot type, e.g. "Ratio_control_*", spot sub-types "Ratio_control_1", "Ratio_control_2" can be determined automatically to give a series of box-plots comparing the moderated t-statistics or B statistics (log odds of differential expression) between the controls, which may help in deciding which genes are truly differentially expressed, i.e. what moderated t statistic is significant.

Every SpotType must have a SpotType column, for which each entry should be unique. It must also have an ID column, a Name column and a default Color column. The rows of the SpotTypes file should be read as a list of rules (in order) for defining spot types. First, we say everything is a gene, then we rename certain spots according to what is find in the ID or Name column of the GAL file. The color given here is just a suggested color to associate with the spot type.

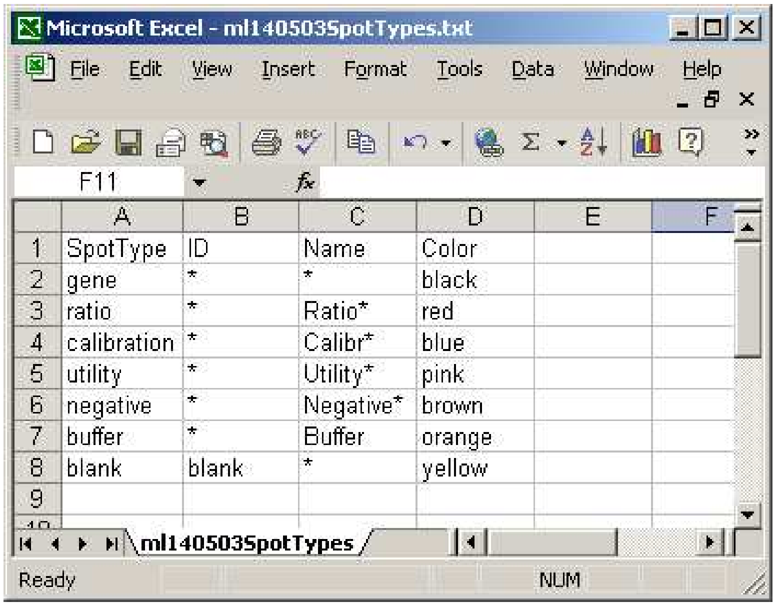
Again, do not change the capitalization or spelling of the column names and do not insert extra spaces.

Here is a short spot types file.



SpotType	ID	Name	Color
cDNA	*	*	black
BLANK	BLANK	*	brown
Blank	Blank	*	orange
Control	Control	*	blue

The spot types file below defines the scorecard control spots. The asterisks are wildcards which can represent anything.



SpotType	ID	Name	Color
gene	*	*	black
ratio	*	Ratio*	red
calibration	*	Calibr*	blue
utility	*	Utility*	pink
negative	*	Negative*	brown
buffer	*	Buffer	orange
blank	blank	*	yellow

You can read the spot types files using readSpotTypes(). For example, if the file has the default name SpotTypes.txt you can use

```
> spottypes <- readSpotTypes()
```

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

```
> RG$genes$Status <- spotStatus(spottypes)
```

5. 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. See the Swirl data case study for examples.

6. Normalization and Background Correction

Limma implements a range of normalization methods for spotted microarrays. Smyth and Speed (2003) describe 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 use

```
> RG <- backgroundCorrect(RG, method="minimum")
```

to reset zero or negative intensities to half the value of the minimum value of the positive intensities. No further background correction will be performed when `normalizeWithinArrays` is used subsequently to normalize the intensities.

Limma contains some more sophisticated normalization methods. Normalization of absolute expression levels as well as just log-ratios is covered in Section 11 at the end of this guide.

7. Differential Expression

7.1 Linear Models

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 (2003) 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.

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 a 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, 2003). 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).

7.2 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-group3, 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 <- classifyTests(fit2)
```

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

```
> vennDiagram(clas)
```

7.3 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 `designMatrix()` function and the targets file. As an example, we consider part of an experiment conducted by Jöelle 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 <- designMatrix(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:

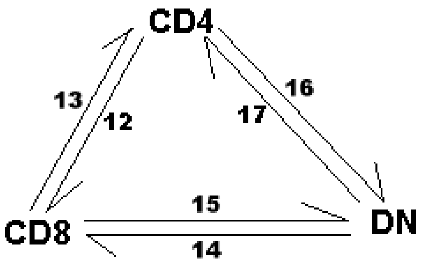
```
> contrast.matrix <- makeContrasts(AML1,CBFb,AML1.CBFb,AML1.CBFb-AML1,AML1.CBFb-
CBFb,levels=design)
> contrast.matrix
          AML1 CBFb AML1.CBFb AML1.CBFb - AML1 AML1.CBFb - CBFb
AML1         1    0         0               -1                0
AML1.CBFb    0    0         1                1   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

7.3 Direct Two-Color Designs

Two-colour designs without a common reference require the most statistical knowledge to choose the appropriate design matrix. 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. Case Studies

8.1. 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
```

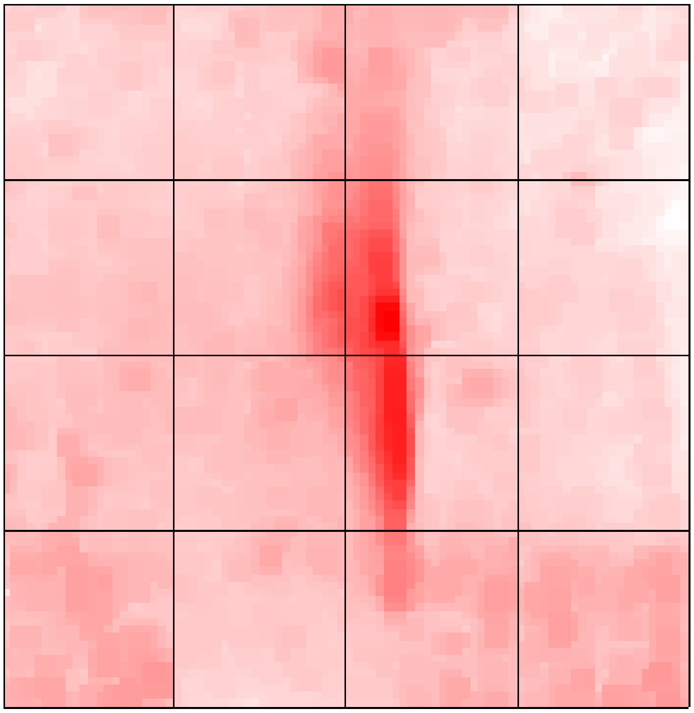
```
18      1   1      18 control    Actin
19      1   1      19 control    3XSSC
20      1   1      20 control    3XSSC
21      1   1      21 control    3XSSC
22      1   1      22 control    3XSSC
23      1   1      23 control    Actin
24      1   1      24 control    Actin
25      1   2       1 control     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")
```



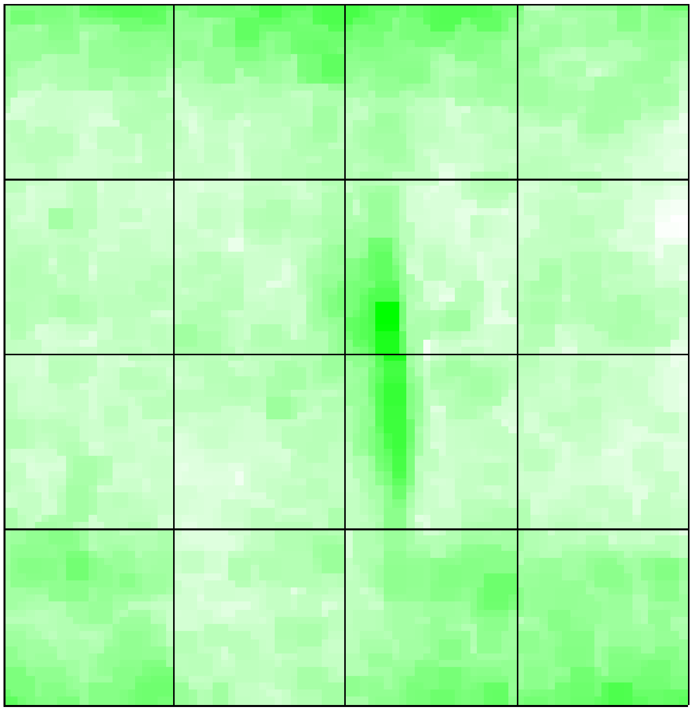
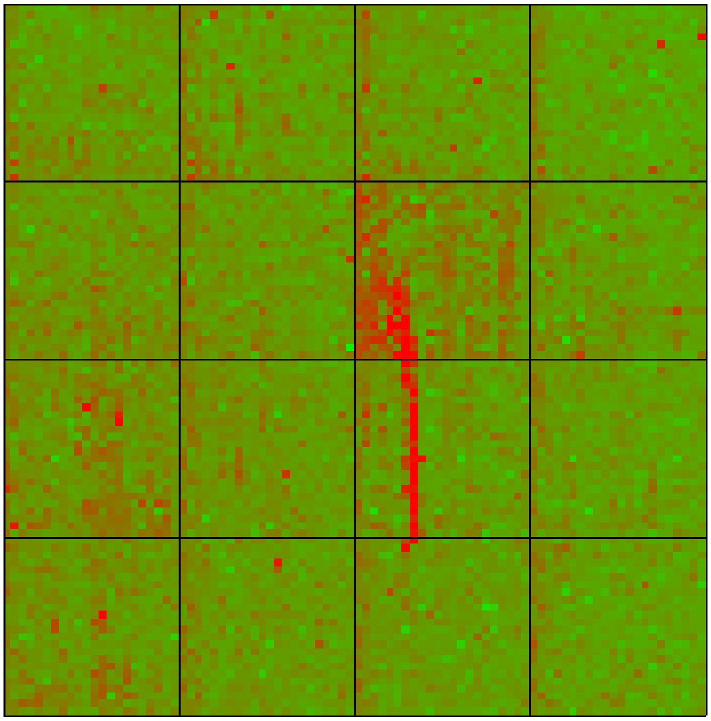


Image plot of the un-normalized log-ratios or M-values for the first array:

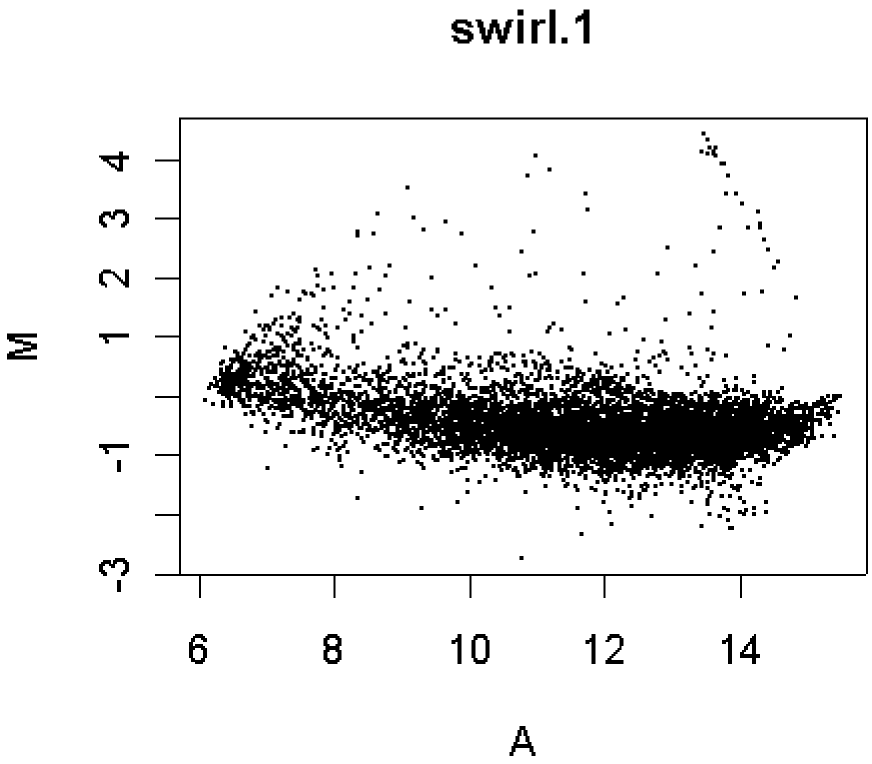
```
> MA <- normalizeWithinArrays(RG, method="none")
> imageplot(MA$M[,1], RG$printer, zlim=c(-3,3))
```



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.

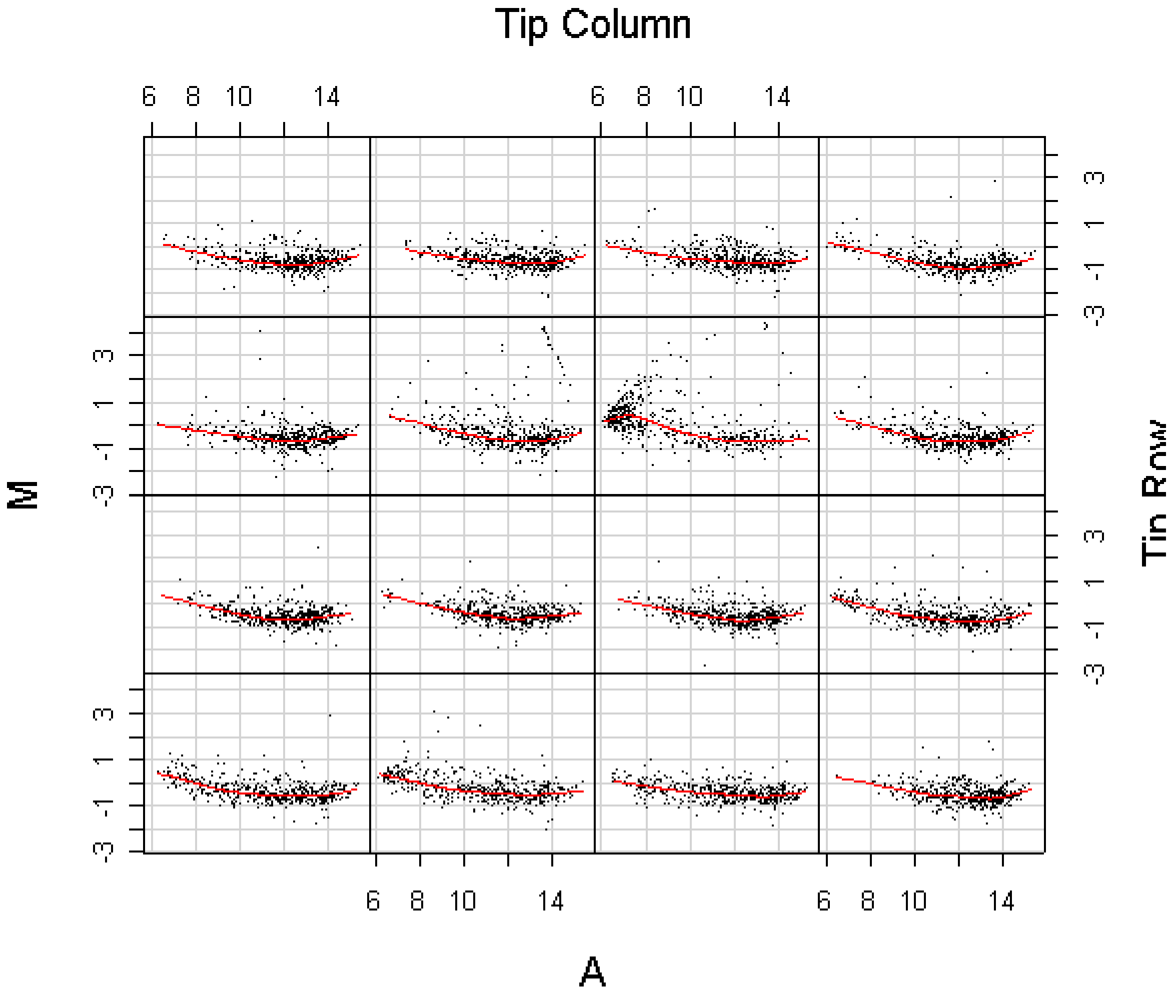
MA-plots. An MA-plot plots the log-ratio of R vs G against the overall intensity of each spot. The log-ratio is represented by the M-value, M = log2(R)-log2(G), and the overall intensity by the A-value, A = log2(R)+log2(G). Here is the MA-plot of the un-normalized values for the first array:

```
> plotMA(MA)
```



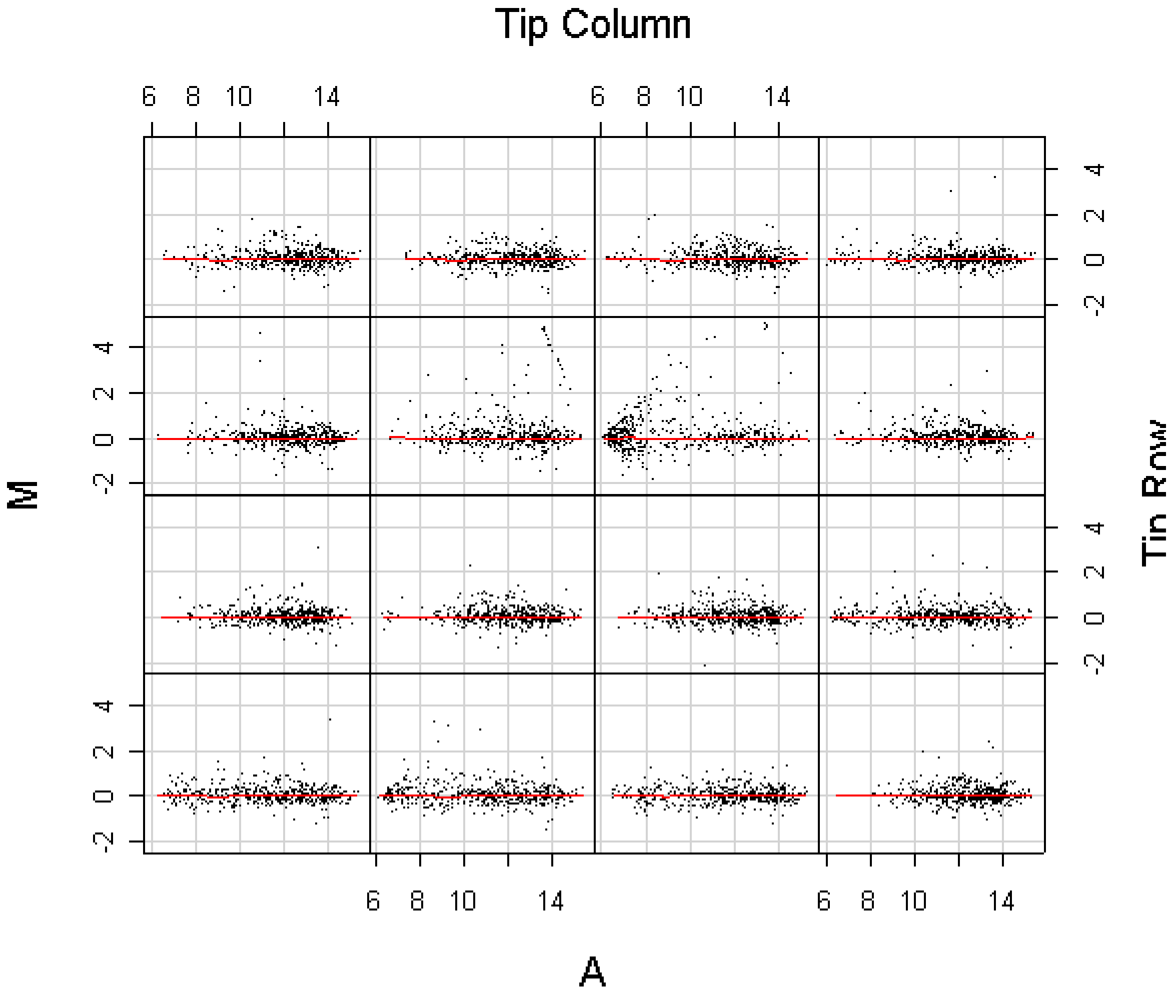
The red streak seen on the image plot can be seen as a line of spots in the upper right of this plot. Now we plot the individual MA-plots for each of the print-tip groups on this array, together with the loess curves which will be used for normalization:

```
> plotPrintTipLoess(MA)
```



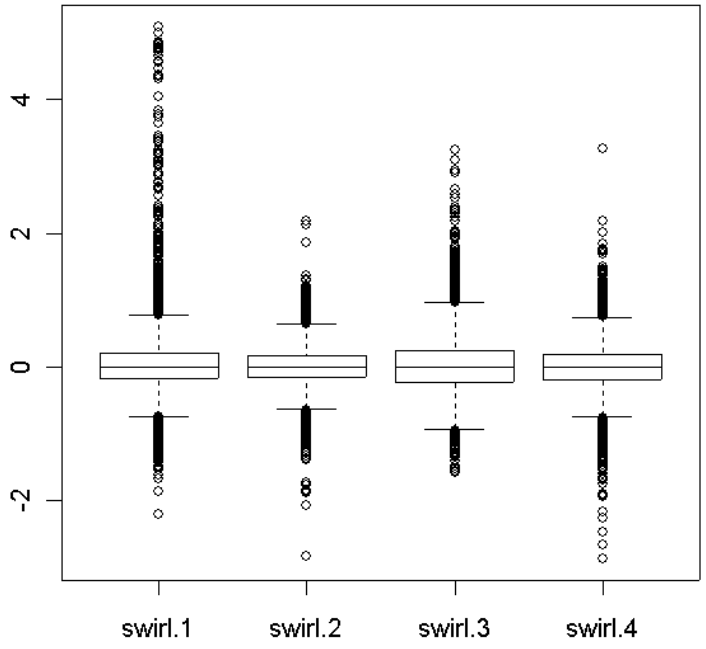
Normalization. Print-tip loess normalization:

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



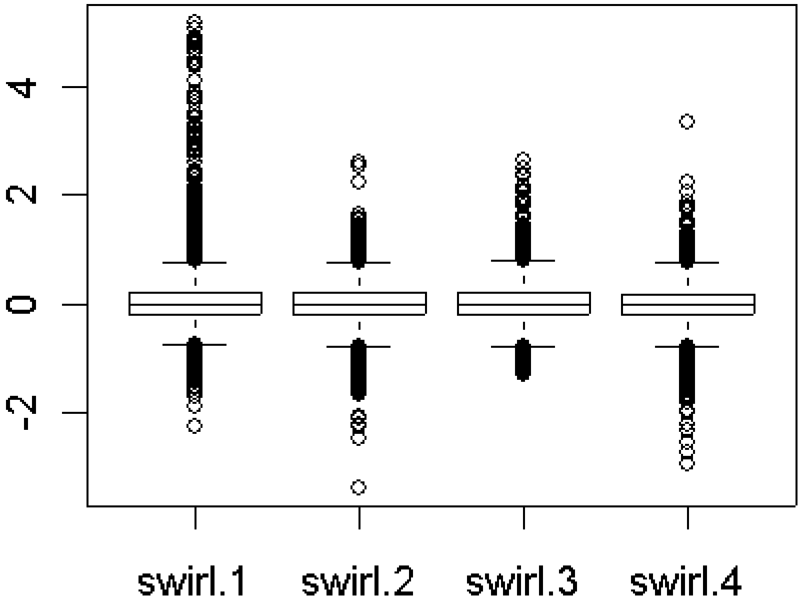
We have normalized the M-values with each array. A further question is whether normalization is required between the arrays. The following plot shows overall boxplots of the M-values for the four arrays.

```
> 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.

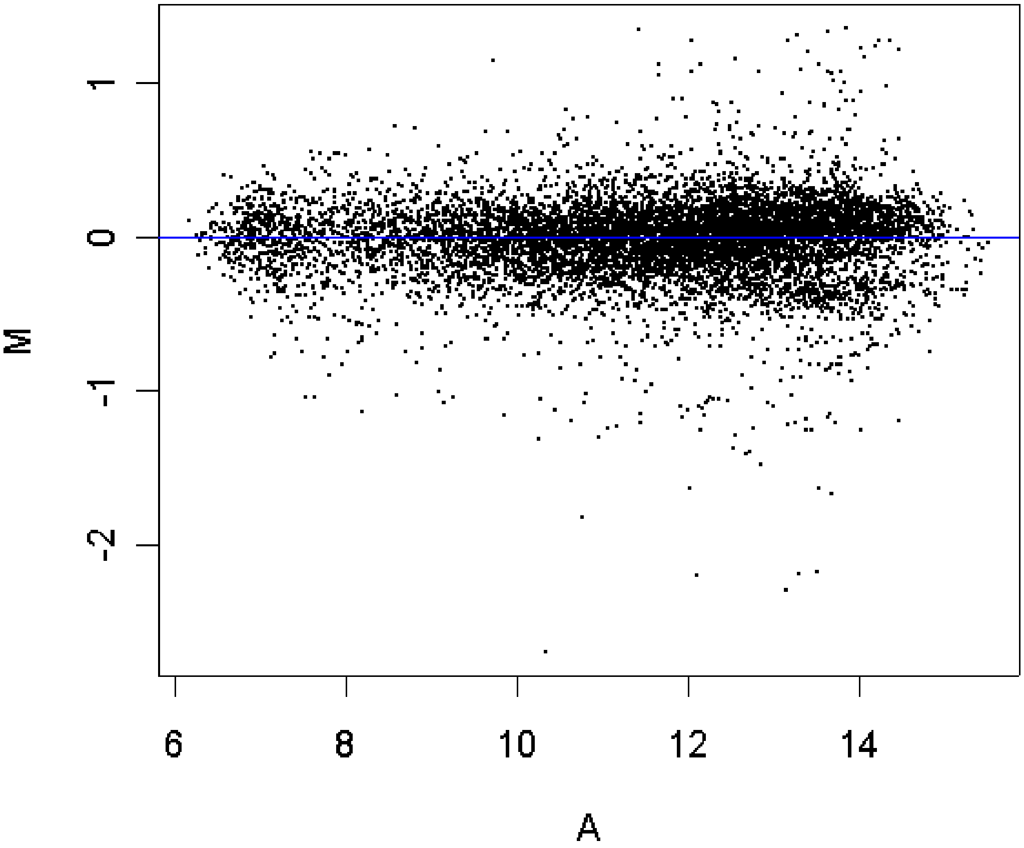
```
> design <- c(-1,1,-1,1)
> fit <- lmFit(MA,design)
> fit
An object of class "MArrayLM"
$coefficients
[1] -0.3943421 -0.3656843 -0.3912506 -0.2505729 -0.3432590
8443 more elements ...

$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
```

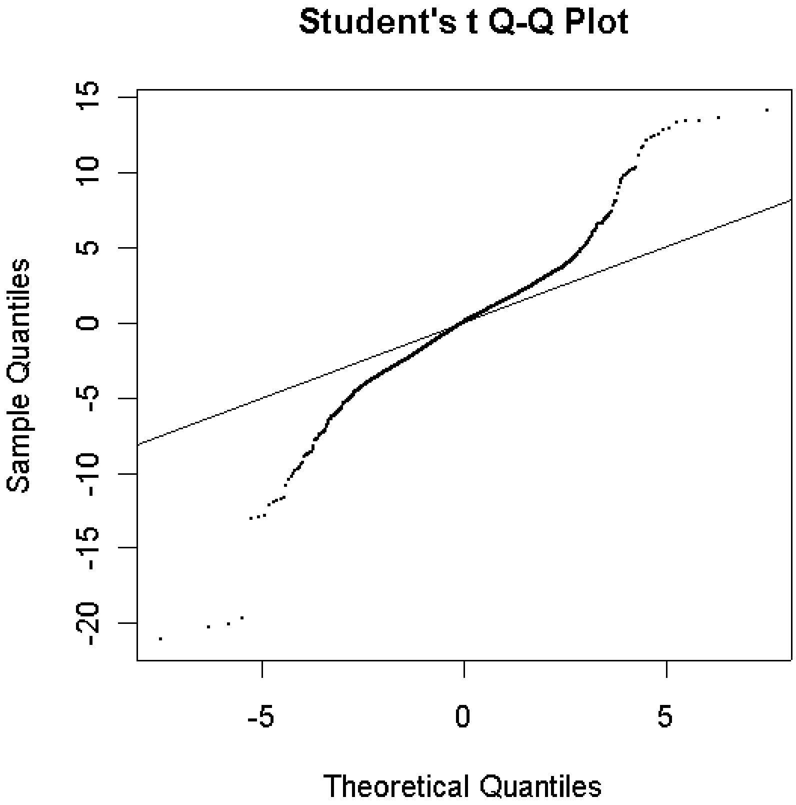
We prefer though to use empirical Bayes moderated t-statistics which are computed below. Now create an MA-plot of the average M and A-values for each gene.

```
> M <- fit$coef
> A <- fit$Amean
> plot(A,M,pch=16,cex=0.1)
> 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=0.1)
> 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.

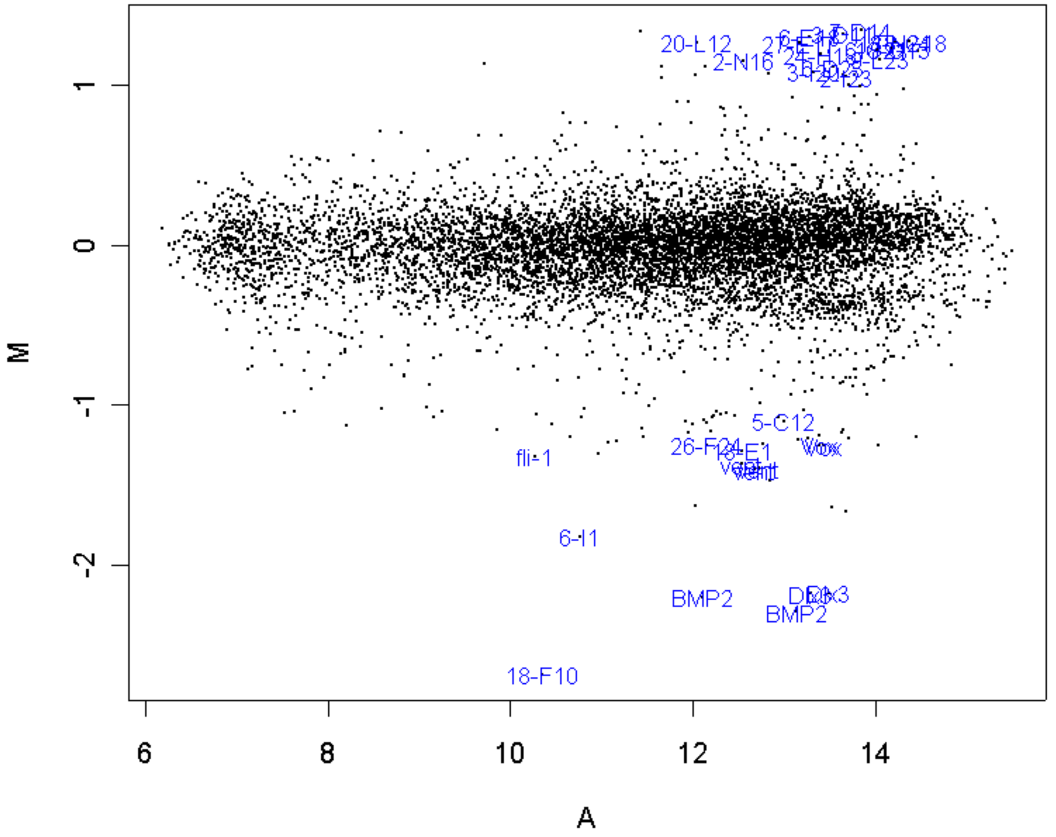
```
> options(digits=3)
> topTable(fit,number=30,adjust="fdr")
     Block Row Column      ID   Name     M    A     t  P.Value    B
3721     8   2      1 control   BMP2 -2.21 12.1 -21.1 0.000357 7.96
1609     4   2      1 control   BMP2 -2.30 13.1 -20.3 0.000357 7.78
3723     8   2      3 control   Dlx3 -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 50 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. Beware that the Benjamini and Hochberg method used to control the false discovery rate assumes independent statistics which we do not have here (see `help(p.adjust)`).

```
> ord <- order(fit$lods,decreasing=TRUE)
> top30 <- ord[1:30]
> plot(A,M,pch=16,cex=0.1)
> text(A[top30],M[top30],labels=MA$genes[top30,"Name"],cex=0.8,col="blue")
```



8.2 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 RG list in the data file `ApoAI.RData`.

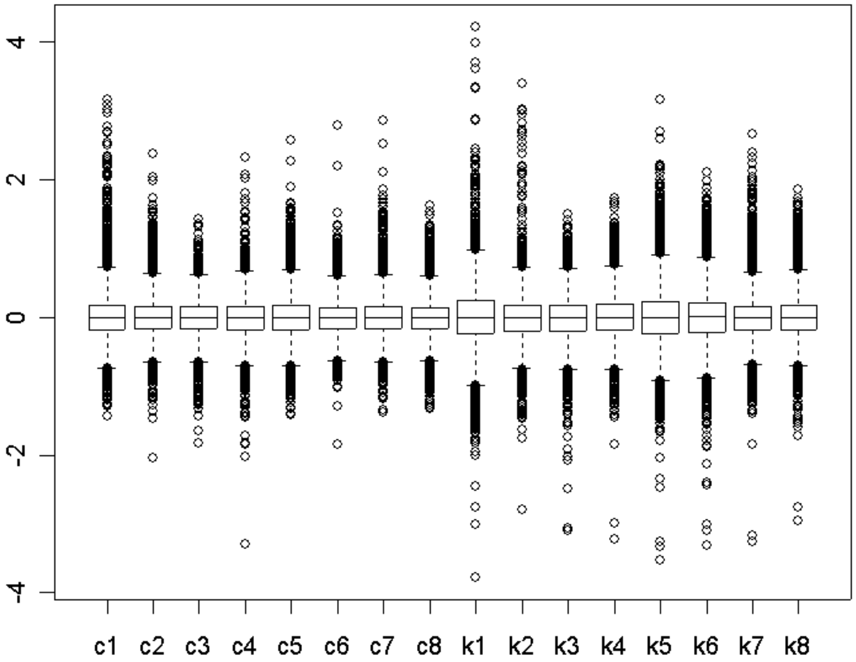
Background. The data is from a study of lipid metabolism by Callow et al (2000). The apolipoprotein AI (ApoAI) gene is known to play a pivotal role in high density lipoprotein (HDL) metabolism. Mouse which have the ApoAI gene knocked out have very low HDL cholesterol levels. The purpose of this experiment 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] "design"   "genelist" "layout"   "RG"
> RG$R[1:4,]
       c1      c2      c3      c4      c5      c6      c7     c8      k1      k2      k3
1 2765.58 1768.22 1440.54  763.06 2027.94  864.05  958.68 644.58  747.11 1388.79 1588.76
2 2868.43 2277.18 1599.92 1238.33 1513.43 1079.33 1228.66 757.33 1930.25 2093.00 1369.81
3 1236.32 1546.84 2639.45  999.48 3689.67 1505.20  785.10 994.86  753.52 1300.00 1301.61
4  383.62  532.50  323.55  585.14  250.74  566.58  409.18 417.79  829.82  402.84  513.91
       k4      k5      k6      k7      k8
1 1280.17 1881.72 1733.53 1170.84 1512.45
2 1071.17 3218.58 2451.04 1605.00 1700.82
3 3292.26 1149.23 3424.30 1901.06 2200.82
4  459.69  391.09  601.00  438.03  507.25
> MA <- normalizeWithinArrays(RG,layout)
> boxplot(MA$M~col(MA$M),names=colnames(RG$R))
```



The differences in scale are moderate, so we won't scale normalize between arrays.

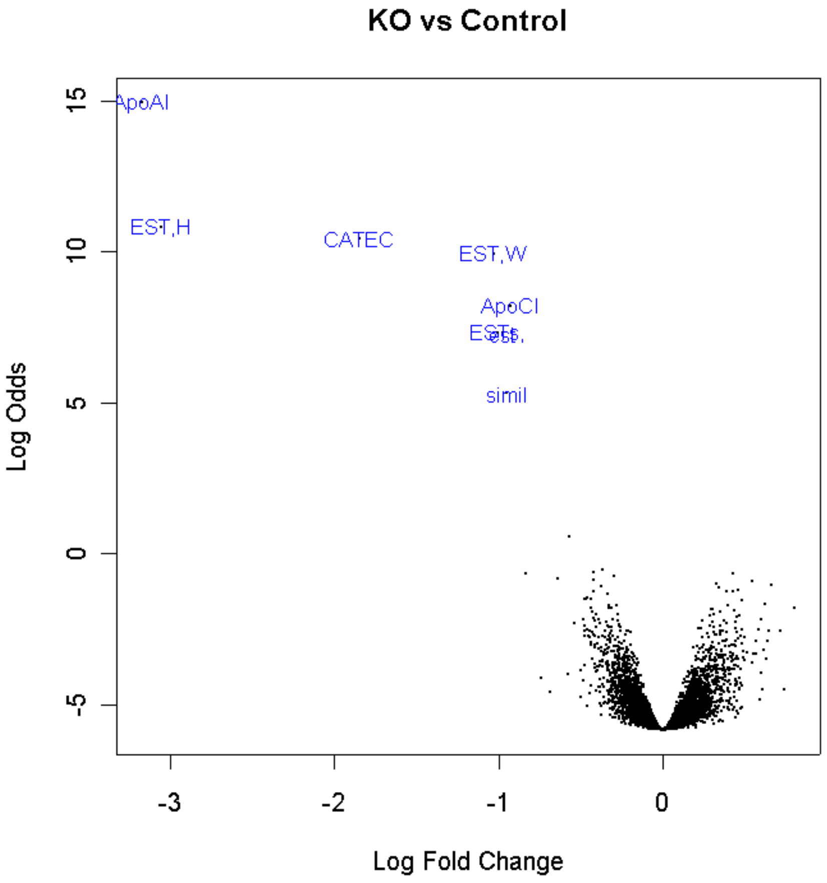
Now we can go on to estimate the fold change between the two groups. In this case the design matrix has two columns. The coefficient for the second column estimates the parameter of interest, the log-ratio between knockout and control mice.

```
> design
   Control-Ref KO-Control
c1           1          0
c2           1          0
c3           1          0
c4           1          0
c5           1          0
c6           1          0
c7           1          0
c8           1          0
k1           1          1
k2           1          1
k3           1          1
k4           1          1
k5           1          1
k6           1          1
k7           1          1
k8           1          1
> fit <- lm.series(MA$M,design)
> fit$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
> eb <- ebayes(fit)
> options(digits=3)
> toptable(coef=2,number=15,genelist=genelist[,1:6],fit=fit,eb=eb,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$coef[,2],eb$lods[,2],pch=16,cex=0.1,xlab="Log Fold Change",
ylab="Log Odds",main="KO vs Control")
> ord <- order(eb$lods[,2],decreasing=TRUE)
> top8 <- ord[1:8]
> text(fit$coef[top8,2],eb$lods[top8,2],labels=substring(genelist[top8,"NAME"],1,5),
cex=0.8,col="blue")
```



8.3 Weaver Mutant Data: A Factorial Experiments

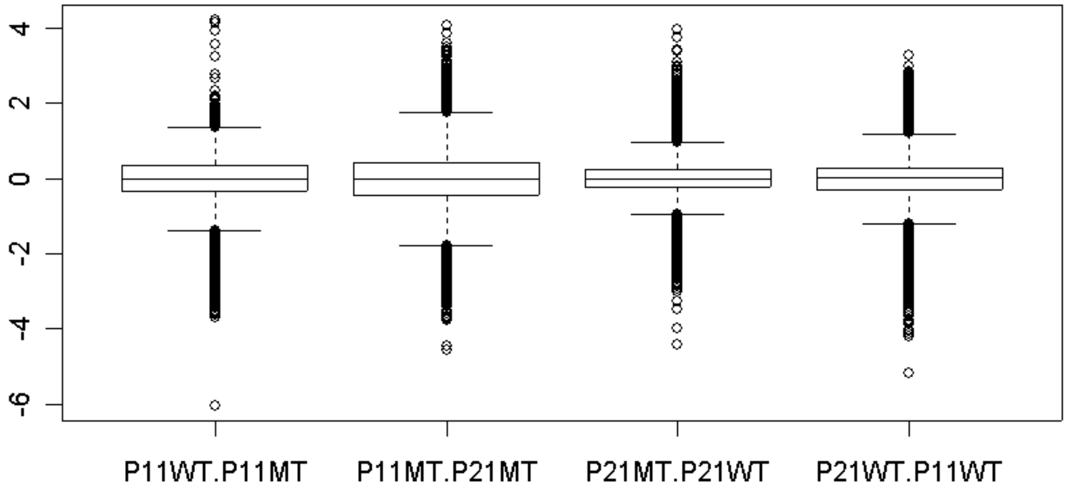
This case study considers a more involved analysis in which the sources of RNA have a factorial structure. In this example we assume that data is available as an `RGList`.

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 <- lm.series(MA$M,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
```

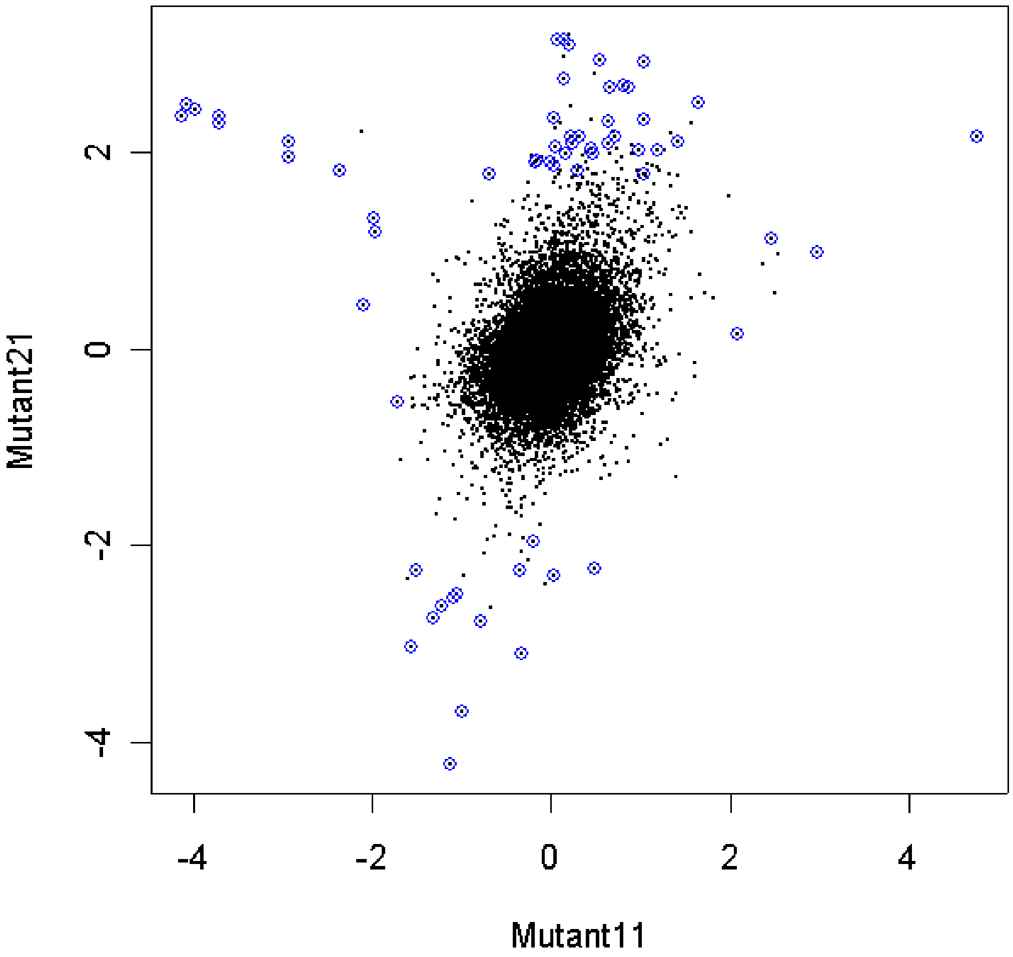
With only four arrays there is only one residual df for the linear model, so even large M-values and t-statistics are not significant after adjusting for multiple testing. There are differentially expressed genes here, although it is difficult to confirm it from the four arrays that we are using for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

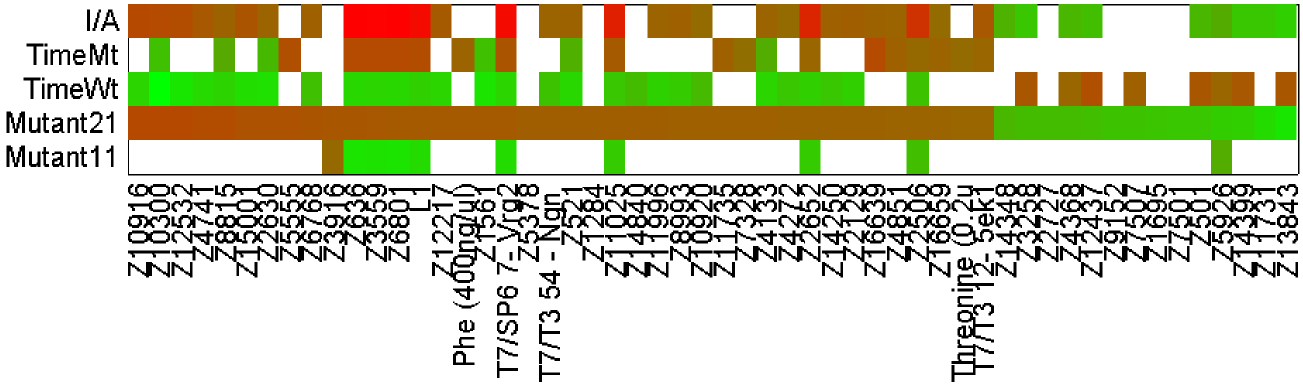
```
> fitMt <- lm.series(MA$M,designMt)
> ebMt <- ebayes(fitMt)
>
plot(fitMt$coef[,"Mutant11"],fitMt$coef[,"Mutant21"],pch=16,cex=0.1,xlab="Mutant1
1",ylab="Mutant21")
> sel <- abs(ebMt$t[,"Mutant11"])>4 | abs(ebMt$t[,"Mutant21"])>4
> points(fitMt$coef[sel,"Mutant11"],fitMt$coef[sel,"Mutant21"],col="blue")
```



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.

9. 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.

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
```

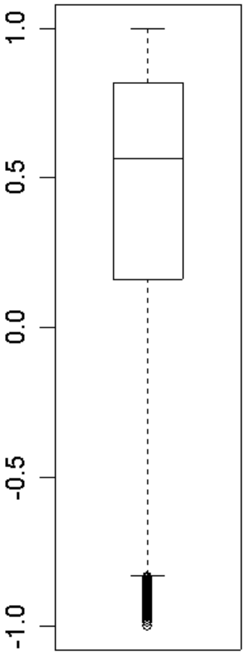
Although there are only four arrays, we have a total of eight spots for each gene, and more for the controls. Naturally the two M-values obtained from duplicate spots on the same array are highly correlated. The problem is how to make use of the duplicate spots in the best way. The approach taken here 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)
```

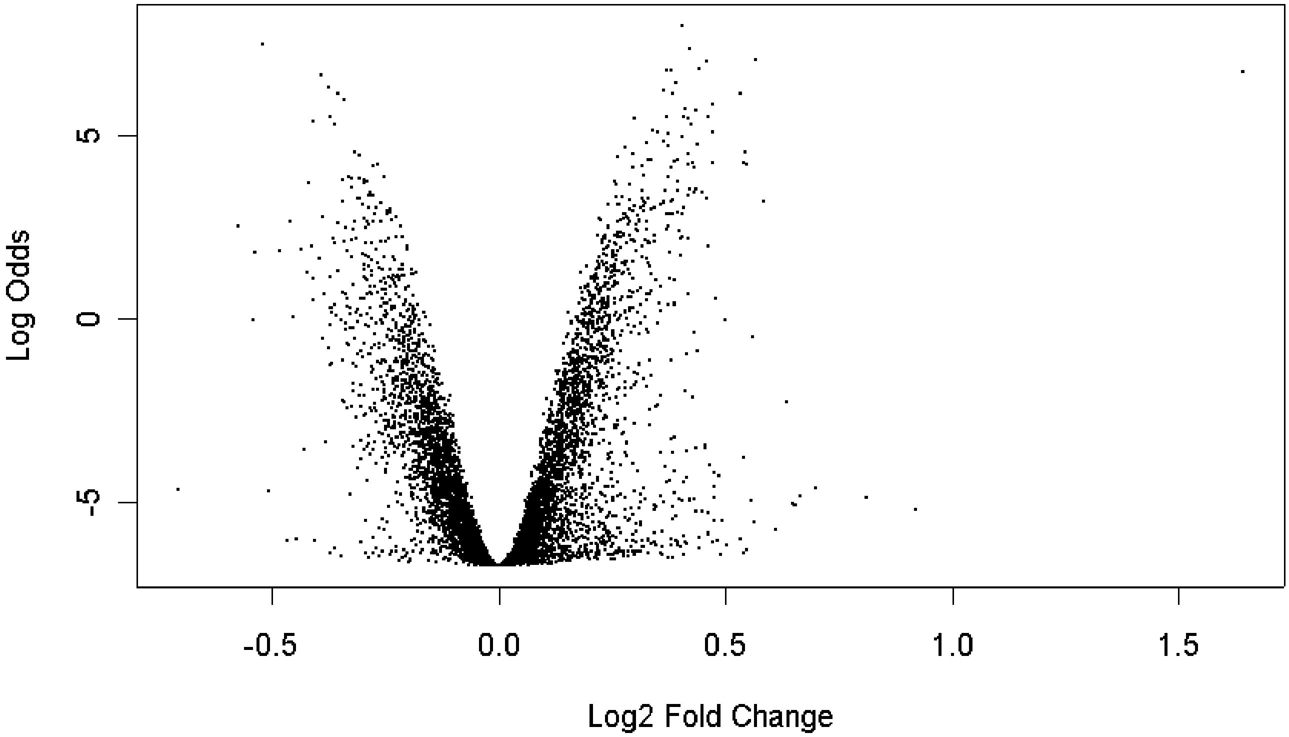
Now estimate the spatial correlation. We estimate a correlation term by REML for each gene, and then take a trimmed mean on the atanh scale to estimate the overall correlation. This command takes a lot of time, perhaps as much as an hour for a series of arrays.

```
> cor <- dupcor.series(MA$M,design,ndups=2) # This is a very slow computation!
> cor$cor
[1] 0.571377
> boxplot(cor$cor.genes)
```



```
> fit <- gls.series(MA$M,design,ndups=2,correlation=0.571377)
> eb <- ebayes(fit)
> genenames <- uniquegenelist(gal[,"Name"],ndups=2)
> toptable(number=30,genelist=genenames,fit=fit,eb=eb,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=0.1)
```



10. Using limma with the marray Packages

The packages marrayClasses, marrayInput, marrayNorm and marrayTools are designed to read and normalize cDNA data. The marrayNorm package provides some normalization methods which are not provided by limma. Normalization using marrayNorm will produce a data object of class `marrayNorm`. Suppose that you have an `marrayNorm` object called `N`. The data may be converted into an `MAList` suitable for further manipulation in limma using

```
> MA <- as.MAList(N)
```

Even without conversion, the `marrayNorm` object may be used directly in the `lmFit` function in limma, for example

```
fit <- lmFit(N, design)
```

after which one proceeds exactly as in previous sections.

Note that there are no facilities for importing `marrayRaw` objects into limma. This means that, if you have read your data into R using the marrayInput package, you should use marrayNorm rather than limma for normalization. After normalization, you are free to use limma for analysis of differential expression.

11. Affymetrix and Single-Color Arrays

Normalization of Affymetrix data using functions in the package `affy` will produce a data object of class `exprSet` or of `AffyBatch` which inherits from `exprSet`. Objects of class exprSet may be used directly in the `lmFit` function in limma. Let `eset` be the `exprSet` object. A linear model may be fitted using

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

See Section 7.2 for more details.

12. Single-Channel Normalization for Two-Color Arrays

We provide a short background on the topic of single-channel normalization for two color arrays. Throughout this section the ApoAI data set will be used to demonstrate single-channel normalization.

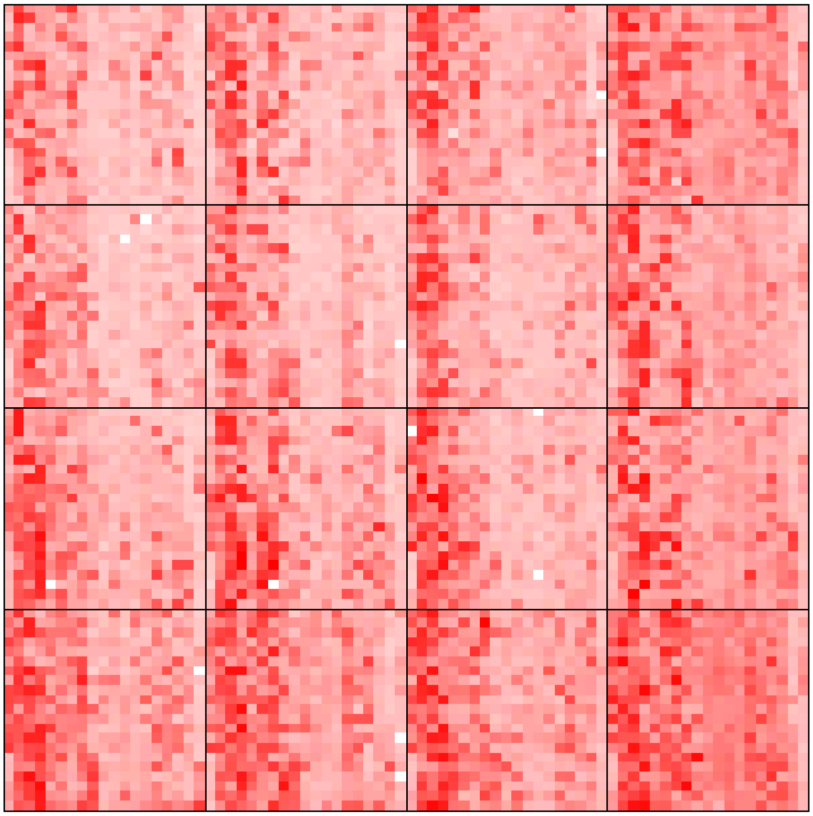
Load the ApoAI data and perform background correction on the `RGList` data object:

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

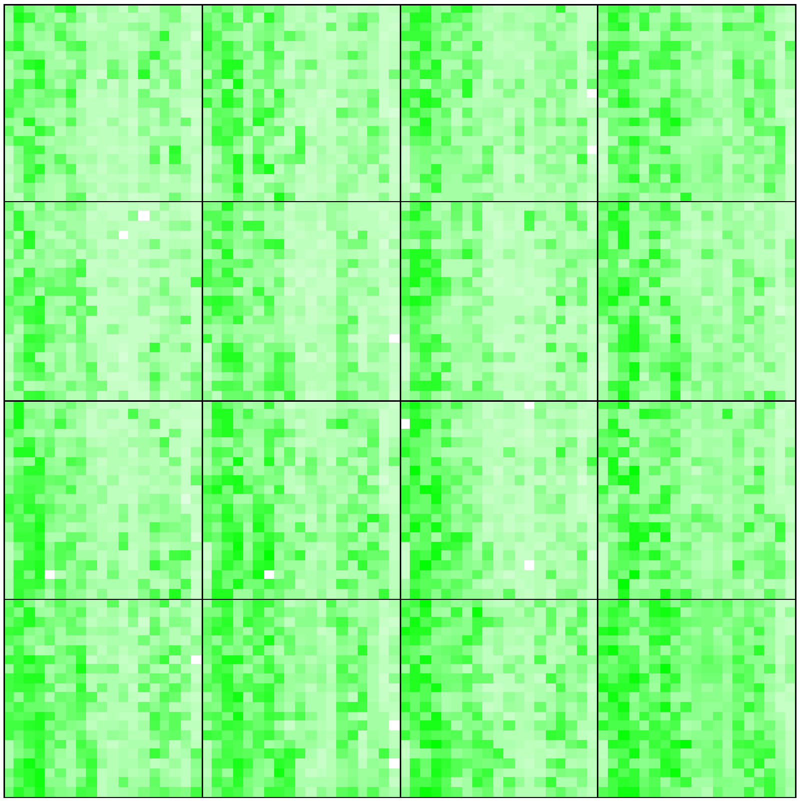
cDNA (or oligo) microarrays compare the gene expression between two different sources of RNA for thousands of genes simultaneously. In general, the log-ratio of spot intensities for the red and green channels form the primary data used for downstream analysis. Thus traditional normalization methods, 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.

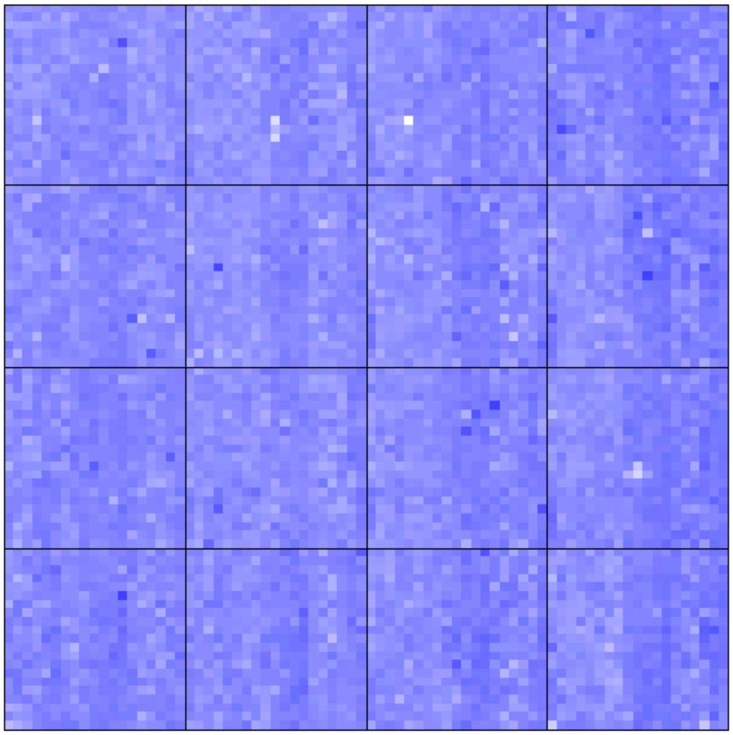
```
> imageplot(log(RG.b$R[,4],2), layout, low="white", high="red")
```



```
> 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=F` 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)
```

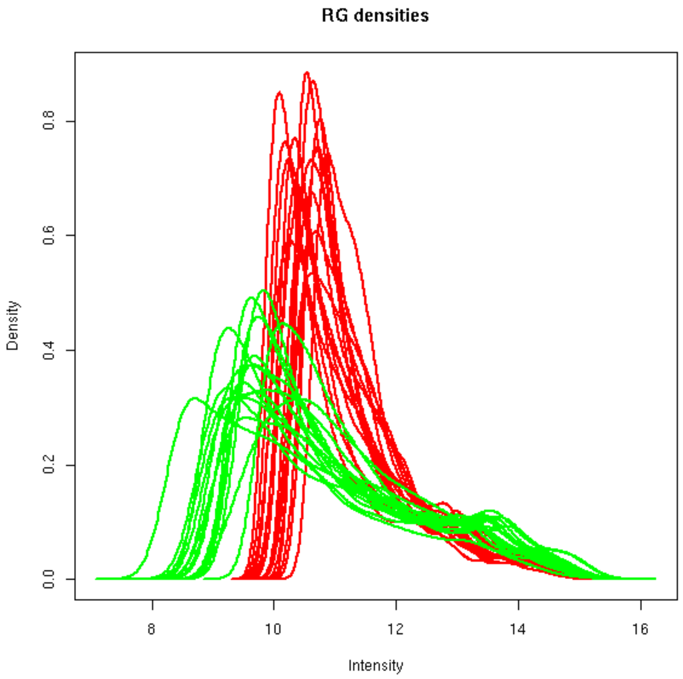
At any stage we can recover the RGList of normalized single-channels using RG.MA. RG.MA(MA.p) would give us within-array only normalized single-channels. Next we perform between array normalization of the single-channels. We use the function normalizeBetweenArrays which takes and returns an MAList. normalizeBetweenArrays 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))
```

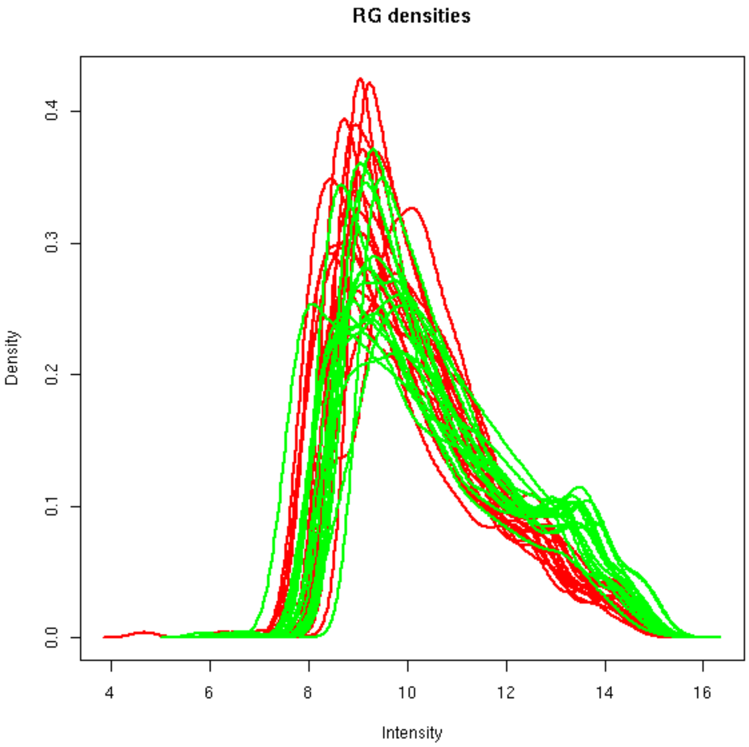
We find that **pq** and **MpAq** work quite well. Next we show some plots of the single-channel log-intensity densities which illustrate the results of the different single-channel normalization methods. We use the function plotDensities which will take either an RGList or an MAList . The form of the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

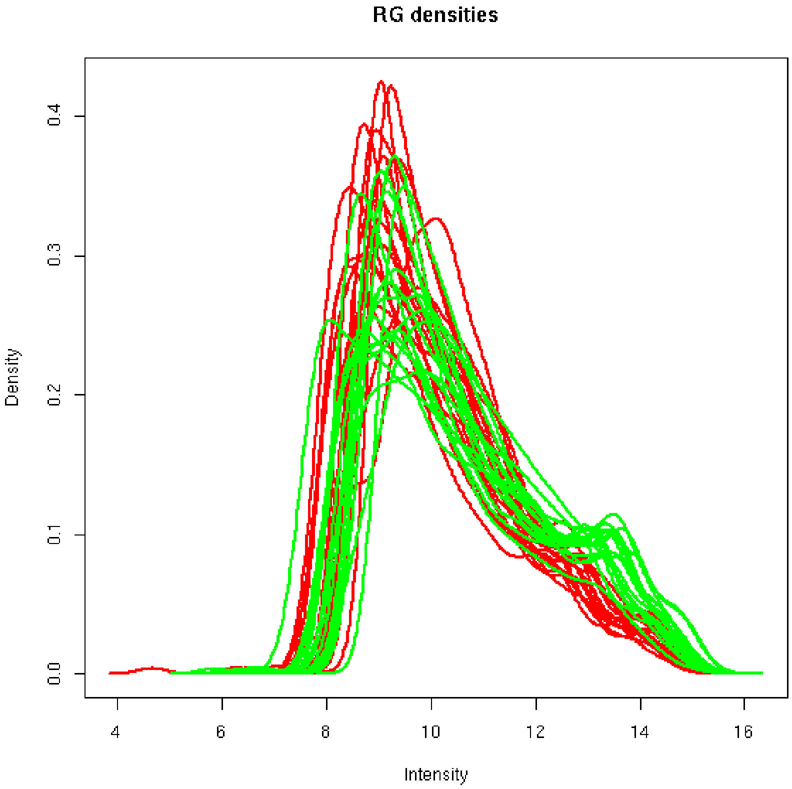
```
 > plotDensities(RG, log.transform=TRUE)
```



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

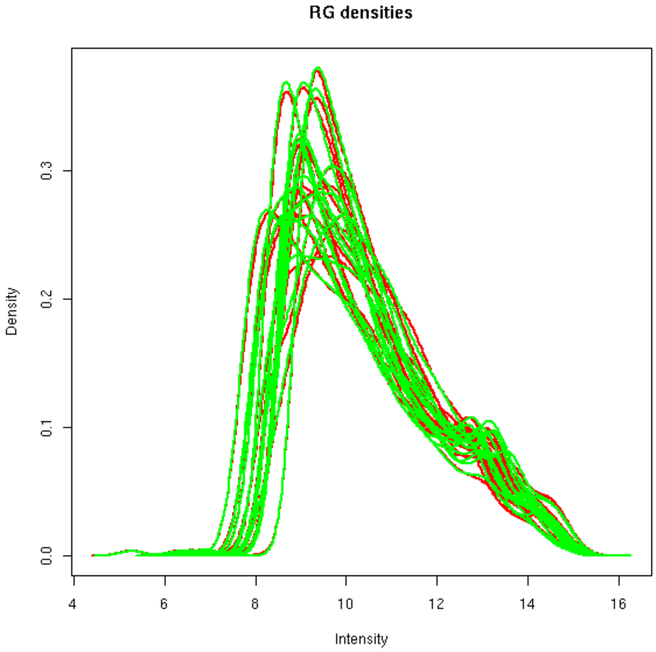


```
> plotDensities(MA.n)
```



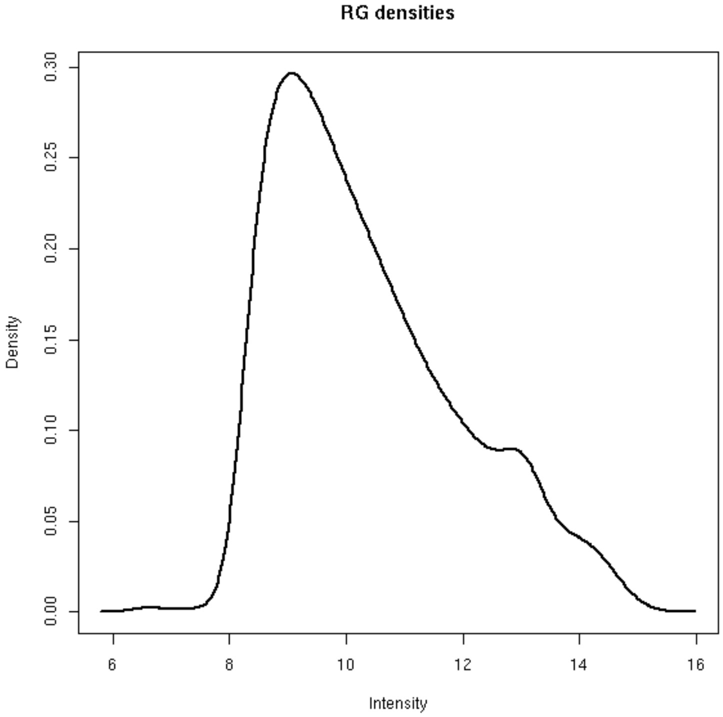
`Printtiploess` makes the single-channels within arrays similar:

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

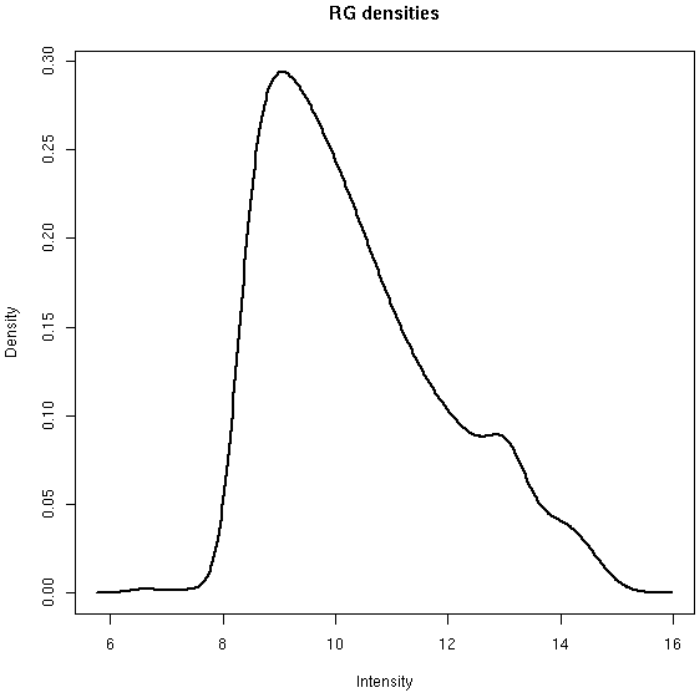


All the single-channels have the same distribution.

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

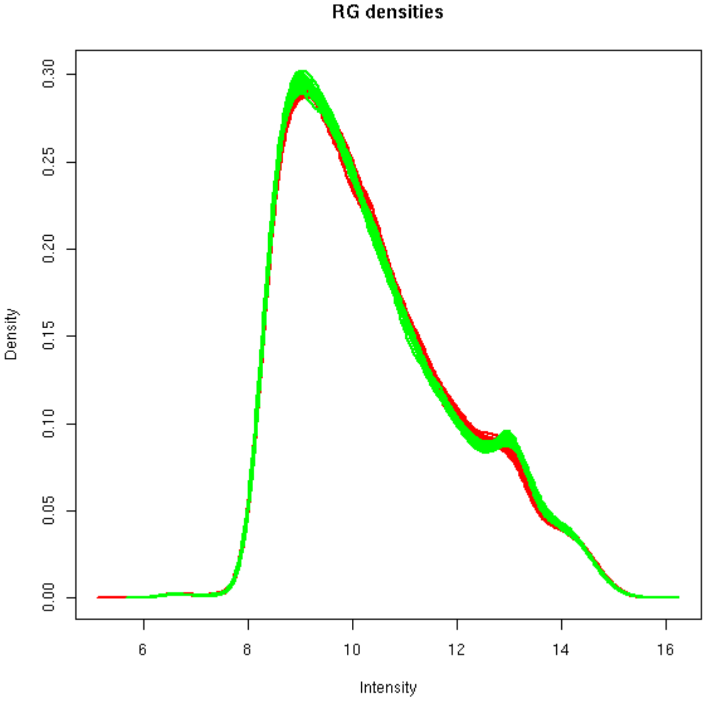


```
> plotDensities(MA.pq, col="black")
```



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

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



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