limma:
Linear Models for Microarray Data User's Guide

(Now Including RNA-Seq Data Analysis)

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This free open-source software implements academic research by the authors and co-workers. If you use it, please support the project by citing the appropriate journal articles listed in Section 2.1.

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Chapter 1

Introduction

Limma is a package for the analysis of gene expression data arising from microarray or RNA-Seq technologies. A core capability is the use of linear models to assess differential expression in the context of multifactor designed experiments. Limma provides the ability to analyze comparisons between many RNA targets simultaneously. It has features that make the analyses stable even for experiments with small number of arrays—this is achieved by borrowing information across genes. It is specially designed for analysing complex experiments with a variety of experimental conditions and predictors. The linear model and differential expression functions are applicable to data from any microarray platform, including single-channel or two-color microarray platforms. The methods are also applicable to expression data from non-microarray platforms, such as quantitative PCR or RNA-Seq, provided that a suitable matrix of expression values can be provided.

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.

Limma provides a strong suite of functions for reading, exploring and pre-processing data from two-color microarrays. The Bioconductor package marray provides alternative functions for reading and normalizing spotted two-color 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 concept of within-array and between-array normalization as separate steps. If you are using limma in conjunction with marray, see Section 6.4.

Limma can read output data from a variety of image analysis software platforms, including GenePix, ImaGene etc. Either one-channel or two-channel formats can be processed.

The Bioconductor package affy provides functions for reading and normalizing Affymetrix microarray data. Advice on how to use limma with the affy package is given throughout the User's Guide, see for example Section 8.2 and the *E. coli* and estrogen case studies.

Functions for reading and pre-processing expression data from Illumina BeadChips were introduced in limma 3.0.0. See the case study in Section 16.3 for an example of these. Limma can also be used in conjunction with the vst or beadarray packages for pre-processing Illumina data.

From version 3.9.19, limma includes functions to analyse RNA-Seq experiments, demonstrated in Case Study 11.8. The approach is to convert a table of sequence read counts into an expression object which can then be analysed as for microarray data.

This guide describes limma as a command-driven package. Graphical user interfaces to the most commonly used functions in limma are available through the packages limmaGUI [42], for two-color data, or affylmGUI [41], for Affymetrix data. Both packages are available from Bioconductor.

This user's guide should be correct for R Versions 2.8.0 through 3.0.0 and limma versions 2.16.0 through 3.16.0. The limma homepage is `http://bioinf.wehi.edu.au/limma`.

Chapter 2

Preliminaries

2.1 Citing limma

Limma is an implementation of a body of methodological research by the authors and co-workers. Please try to cite the appropriate methodological papers when you use results from the limma software in a publication, as such citations are the main means by which the authors receive professional credit for their work.

If you use limma for differential expression analysis, please cite:

> Smyth, G. K. (2004). Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology*, Vol. 3, No. 1, Article 3.
> `http://www.bepress.com/sagmb/vol3/iss1/art3`

The above article describes the linear modeling approach implemented by `lmFit` and the empirical Bayes statistics implemented by `eBayes`, `topTable` etc.

If you use limma with duplicate spots or technical replication, please cite

> Smyth, G. K., Michaud, J., and Scott, H. (2005). The use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics* **21**(9), 2067–2075.
> `http://bioinformatics.oxfordjournals.org/cgi/content/short/21/9/2067`

The above article describes the theory behind the `duplicateCorrelation` function.

If you use limma for normalization of two-color microarray data, please cite:

> Smyth, G. K., and Speed, T. P. (2003). Normalization of cDNA microarray data. *Methods* **31**, 265–273.

The above article describes the functions `read.maimages`, `normalizeWithinArrays`, `normalizeBetweenArrays` etc, including the use of spot quality weights.

If you use the `backgroundCorrect` function, please cite:

Ritchie, M. E., Silver, J., Oshlack, A., Silver, J., Holmes, M., Diyagama, D., Holloway, A., and Smyth, G. K. (2007). A comparison of background correction methods for two-colour microarrays. *Bioinformatics* 23, 2700–2707.
`http://bioinformatics.oxfordjournals.org/cgi/content/abstract/btm412`

This paper in particular describes the *normexp* background correction method.

If you use limma to estimate array quality weights, please cite:

Ritchie, M. E., Diyagama, D., Neilson, van Laar, R., J., Dobrovic, A., Holloway, A., and Smyth, G. K. (2006). Empirical array quality weights in the analysis of microarray data. *BMC Bioinformatics* **7**, 261.
`http://www.biomedcentral.com/1471-2105/7/261`

The above article describes the functions `arrayWeights`, `arrayWeightsSimple` etc.

If you use limma to pre-process Illumina BeadChip data, please cite:

Shi, W, Oshlack, A, and Smyth, GK (2010). Optimizing the noise versus bias trade-off for Illumina Whole Genome Expression BeadChips. *Nucleic Acids Research* 38, e204.
`http://nar.oxfordjournals.org/content/38/22/e204`

This article describes the `read.ilmn`, `nec` and `neqc` functions.

If you use the separate channel analysis (`lmscFit`), please cite:

Smyth, GK, and Altman, NS (2013). Separate-channel analysis of two-channel microarrays: recovering inter-spot information. *BMC Bioinformatics* 14, 165.
`http://www.biomedcentral.com/1471-2105/14/165`

The limma software itself can be cited as:

Smyth, G. K. (2005). Limma: linear models for microarray data. In: *Bioinformatics and Computational Biology Solutions using R and Bioconductor*, R. Gentleman, V. Carey, S. Dudoit, R. Irizarry, W. Huber (eds.), Springer, New York, pages 397–420.

The above article describes the software package in the context of the Bioconductor project and surveys the range of experimental designs for which the package can be used, including spot-specific dye-effects. The pre-processing capabilities of the package are also described but more briefly, with examples of background correction, spot quality weights and filtering with control spots.

Finally, if you are using one of the menu-driven interfaces to the software, please cite the appropriate one of

Wettenhall, J. M., and Smyth, G. K. (2004). limmaGUI: a graphical user interface for linear modeling of microarray data. *Bioinformatics*, **20**, 3705–3706.

Wettenhall, J. M., Simpson, K. M., Satterley, K., and Smyth, G. K. (2006). affylmGUI: a graphical user interface for linear modeling of single channel microarray data. *Bioinformatics* **22**, 897–899.

2.2 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`. To install the latest version of limma, you will need to be using the latest version of R.

Limma is part of the Bioconductor project at `http://www.bioconductor.org`. (Prior to R 2.6.0, limma was also available from the R project CRAN site.) It is one of a default set of packages installed by `biocLite`. You can install a set of core Bioconductor packages by

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

To get just limma alone (much quicker) you can use

```
> biocLite("limma")
```

This will allow you do to perform many basic analyses, although you'll probably want

```
> biocLite("statmod")
```

as well.

Bioconductor works on a 6-monthly official release cycle, lagging each major R release by a short time. As with other Bioconductor packages, there are always two versions of limma. Most users will use the current official release version, which will be installed by `biocLite` if you are using the current version of R. There is also a developmental version of limma that includes new features due for the next official release. The developmental version will be installed if you are using the developmental version of R. The official release version always has an even second number (for example 3.6.5), whereas the developmental version has an odd second number (for example 3.7.7).

Limma is updated frequently. Once you have installed limma, the change-log can also be viewed from the R prompt. To see the most recent 20 lines type:

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

2.3 How to get help

Most questions about limma will hopefully be answered by the documentation or references. If you've run into a question which isn't addressed by the documentation, or you've found a conflict between the documentation and software itself, then there is an active support community which can offer help.

The authors of the package always appreciate receiving reports of bugs in the package functions or in the documentation. The same goes for well-considered suggestions for improvements.

Any other questions or problems concerning limma should be sent to the Bioconductor mailing list `bioconductor@stat.math.ethz.ch`. To subscribe to the mailing list, see `https://stat.ethz.ch/mailman/listinfo/bioconductor`. Please send requests for general assistance and advice to the mailing list rather than to the individual authors. Users posting to the mailing list for the first time should read the helpful posting guide at `http://www.bioconductor.org/doc/postingGuide.html`. Note that each function in limma has it's own online help page, as described in the next section. Mailing list etiquette requires that you read the relevant help page carefully before posting a problem to the list.

Chapter 3

Quick Start

3.1 A brief introduction to 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. In this guide it will be assumed that you have successfully downloaded and installed R from `http://www.r-project.org`. A good way to get started is to type

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

at the R prompt or, if you're using R for Windows, to follow the drop-down menu items Help ≻ Html help. Following the links Packages ≻ limma from the html help page will lead you to the contents page of help topics for functions 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
```

or equivalently

```
> help("read.maimages")
```

for detailed help on the `read.maimages` function. The individual function help pages are especially important for listing all the arguments which a function will accept and what values the arguments can take.

A key to understanding R is to appreciate that 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 can see 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. For more background on using R for statistical analyses see [5].

3.2 Sample limma Session

This is a quick overview of what an analysis might look like. The first example assumes four replicate two-color arrays, the second and fourth of which are dye-swapped. We assume that the images have been analyzed using GenePix to produce a `.gpr` file for each array and that a targets file `targets.txt` has been prepared with a column containing the names of the `.gpr` files.

```
> library(limma)
> targets <- readTargets("targets.txt")
```

Set up a filter so that any spot with a flag of -99 or less gets zero weight.

```
> f <- function(x) as.numeric(x$Flags > -99)
```

Read in the data.

```
> RG <- read.maimages(targets, source="genepix", wt.fun=f)
```

The following command implements a type of adaptive background correction. This is optional but recommended for GenePix data.

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

Print-tip loess normalization:

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

Estimate the fold changes and standard errors by fitting a linear model for each gene. The design matrix indicates which arrays are dye-swaps.

```
> fit <- lmFit(MA, design=c(-1,1,-1,1))
```

Apply empirical Bayes smoothing to the standard errors.

```
> fit <- eBayes(fit)
```

Show statistics for the top 10 genes.

```
> topTable(fit)
```

The second example assumes Affymetrix arrays hybridized with either wild-type (wt) or mutant (mu) RNA. There should be three or more arrays in total to ensure some replication. The targets file is now assumed to have another column `Genotype` indicating which RNA source was hybridized on each array.

```
> library(gcrma)
> library(limma)
> targets <- readTargets("targets.txt")
```

Read and pre-process the Affymetrix CEL file data.

```
> ab <- ReadAffy(filenames=targets$FileName)
> eset <- gcrma(ab)
```

Form an appropriate design matrix for the two RNA sources and fit linear models. The design matrix has two columns. The first represents log-expression in the wild-type and the second represents the log-ratio between the mutant and wild-type samples. See Section 9.2 for more details on the design matrix.

```
> design <- cbind(WT=1, MUvsWT=targets$Genotype=="mu")
> fit <- lmFit(eset, design)
> fit <- eBayes(fit)
> topTable(fit, coef="MUvsWT")
```

This code fits the linear model, smooths the standard errors and displays the top 10 genes for the mutant versus wild-type comparison.

3.3 Data Objects

There are six main types of data objects created and used in limma:

`EListRaw`. Raw Expression list. A class used to store single-channel raw intensities prior to normalization. Intensities are unlogged. Objects of this class contain one row for each probe and one column for each array. The function `read.ilmn()` for example creates an object of this class.

`EList`. Expression list. Contains background corrected and normalized log-intensities. Usually created from an `EListRaw` objecting using `normalizeBetweenArrays()` or `neqc()`.

`RGList`. Red-Green list. A class used to store raw two-color intensities as they are read in from an image analysis output file, usually by `read.maimages()`.

`MAList`. Two-color intensities converted to M-values and A-values, i.e., to within-spot and whole-spot contrasts on the log-scale. Usually created from an `RGList` using `MA.RG()` or `normalizeWithinArrays()`. Objects of this class contain one row for each spot. There may be more than one spot and therefore more than one row for each probe.

`MArrayLM`. MicroArray Linear Model. Store the result of fitting gene-wise linear models to the normalized intensities or log-ratios. Usually created by `lmFit()`. Objects of this class normally contain one row for each unique probe.

`TestResults`. Store the results of testing a set of contrasts equal to zero for each probe. Usually created by `decideTests()`. Objects of this class normally contain one row for each unique probe.

All these objects can be treated like any list in R. For example, `MA$M` extracts the matrix of M-values if `MA` is an `MAList` object, or `fit$coef` extracts the coefficient estimates if `fit` is an `MArrayLM` object. `names(MA)` shows what components are contained in the object. For those who are familiar with matrices in R, all these objects are also designed to obey many analogies with matrices. In the case of `RGList` and `MAList`, rows correspond to spots and columns to arrays. In the case of `MarrayLM`, rows correspond to unique probes and columns to parameters or contrasts. The functions `summary`, `dim`, `length`, `ncol`, `nrow`, `dimnames`, `rownames`, `colnames` have methods for these classes. For example

```
> dim(RG)
```

```
[1] 11088 4
```

shows that the `RGList` object `RG` contains data for 11088 spots and 4 arrays.

```
> colnames(RG)
```

will give the names of the filenames or arrays in the object, while if `fit` is an `MArrayLM` object then

```
> colnames(fit)
```

would give the names of the coefficients in the linear model fit.

Objects of any of these classes may be subsetted, so that `RG[,j]` means the data for array `j` and `RG[i,]` means the data for probes indicated by the index `i`. Multiple data objects may be combined using `cbind`, `rbind` or `merge`. Hence

```
> RG1 <- read.maimages(files[1:2], source="genepix")
> RG2 <- read.maimages(files[3:5], source="genepix")
> RG <- cbind(RG1, RG2)
```

is equivalent to

```
> RG <- read.maimages(files[1:5], source="genepix")
```

Alternatively, if control status has been set in the `MAList` object then

```
> i <- MA$genes$Status=="Gene"
> MA[i,]
```

might be used to eliminate control spots from the data object prior to fitting a linear model.

Chapter 4

Reading Microarray Data

4.1 Scope of this Chapter

This chapter covers most microarray types other than Affymetrix. To read data from Affymetrix GeneChips, please use the affy, gcrma or aroma.affymetrix packages to read and normalize the data.

4.2 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, ImaGene, 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.

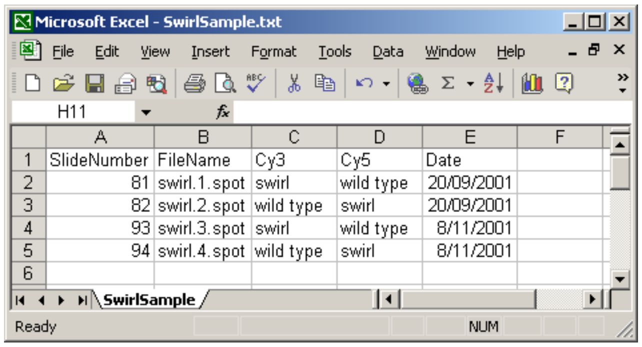
4.3 The Targets Frame

The first step in preparing data for input into limma 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 and should contain a row for each microarray in the experiment. The file can have any name but the default is `Targets.txt`. If it has the default name, it can be read into the R session using

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

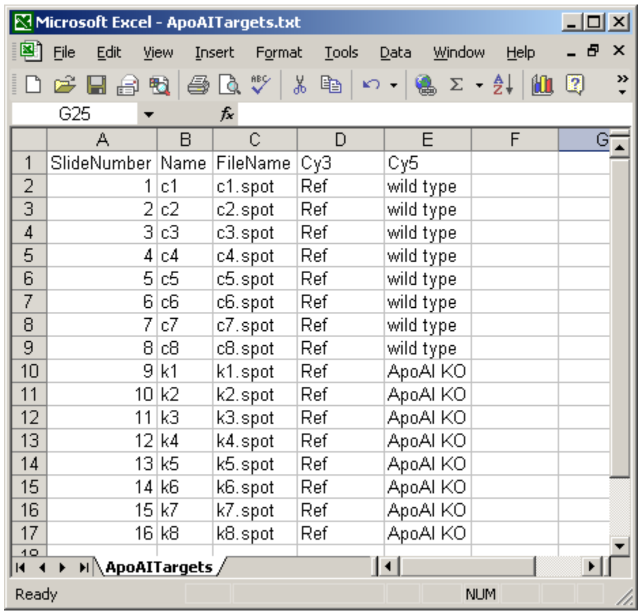
Once read into R, it becomes the *targets frame*.

The targets frame normally contains a `FileName` column, giving the name of the image-analysis output file, a `Cy3` column giving the RNA type labelled with Cy3 dye for that slide and a `Cy5` column giving the RNA type labelled with Cy5 dye for that slide. Other columns are optional. The targets file can be prepared using any text editor but spreadsheet programs such as Microsoft Excel are convenient. The targets file for the Swirl case study includes optional `SlideNumber` and `Date` columns:


Microsoft Excel - SwirlSample.txt
File Edit View Insert Format Tools Data Window Help
H11
SwirlSample
Ready
NUM

	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						

It is often convenient to create short readable labels to associate with each array for use in output and in plots, especially if the file names are long or non-intuitive. A column containing these labels can be included in the targets file, for example the `Name` column used for the Apoa1 case study:


Microsoft Excel - ApoAITargets.txt
File Edit View Insert Format Tools Data Window Help
G25
ApoAITargets
Ready
NUM

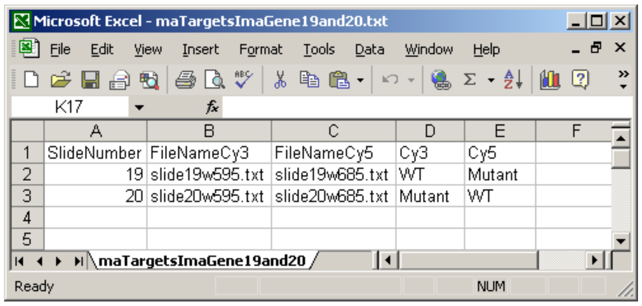
	A	B	C	D	E	F	G
1	SlideNumber	Name	FileName	Cy3	Cy5		
2	1	c1	c1.spot	Ref	wild type		
3	2	c2	c2.spot	Ref	wild type		
4	3	c3	c3.spot	Ref	wild type		
5	4	c4	c4.spot	Ref	wild type		
6	5	c5	c5.spot	Ref	wild type		
7	6	c6	c6.spot	Ref	wild type		
8	7	c7	c7.spot	Ref	wild type		
9	8	c8	c8.spot	Ref	wild type		
10	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		

This column can be used to created row names for the targets frame by

```
> targets <- readTargets("targets.txt", row.names="Name")
```

The row names can be propagated to become array names in the data objects when these are read in.

For ImaGene files, the FileName column is split into a `FileNameCy3` column and a `FileNameCy5` because ImaGene stores red and green intensities in separate files. This is a short example:


Microsoft Excel - maTargetsImaGene19and20.txt
File Edit View Insert Format Tools Data Window Help
K17
maTargetsImaGene19and20
Ready
NUM

	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						

4.4 Reading Two-Color 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")
```

Alternatively, and even more simply, one may give the targets frame itself in place of the `files` argument as

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

In this case the software will look for the column `FileName` in the targets frame.

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

```
> RG <- read.maimages(targets, 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, although other separators can be specified using the `sep=` argument.

Reading data from ImaGene software is a little different to that of other image analysis programs because the red and green intensities are stored in separate files. This means that the targets frame should include two filename columns called, say, `FileNameCy3` and `FileNameCy5`, giving the names of the files containing the green and red intensities respectively. An example is given in Section 4.3. Typical code with ImaGene data might be

```
> targets <- readTargets()
> files <- targets[,c("FileNameCy3","FileNameCy5")]
> RG <- read.maimages(files, source="imagene")
```

For ImaGene data, the `files` argument to `read.maimages()` is expected to be a 2-column matrix of filenames rather than a vector.

The following table gives the default estimates used for the foreground and background intensities:

Source	Foreground	Background
agilent	Median Signal	Median Signal
agilent.mean	Mean Signal	Median Signal
agilent.median	Median Signal	Median Signal
bluefuse	AMPCH	None
genepix	F Mean	B Median
genepix.median	F Median	B Median
genepix.custom	Mean	B
imagene	Signal Mean	Signal Median, or Signal Mean if auto segmentation has been used
quantarray	Intensity	Background
scanarrayexpress	Mean	Median
smd.old	I_MEAN	B_MEDIAN
smd	Intensity (Mean)	Background (Median)
spot	mean	morph
spot.close.open	mean	morph.close.open

The default estimates can be over-ridden by specifying the `columns` argument to `read.maimages()`. Suppose for example that GenePix has been used with a custom background method, and you wish to use median foreground estimates. This combination of foreground and background is not provided as a pre-set choice in limma, but you can specify it by

```
> RG <- read.maimages(files,source="genepix",
+       columns=list(R="F635 Median",G="F532 Median",Rb="B635",Gb="B532"))
```

What should you do if your image analysis program is not in the above list? If the image output files are in standard format, then you can supply the annotation and intensity column names yourself. For example,

```
> RG <- read.maimages(files,
+       columns=list(R="F635 Mean",G="F532 Mean",Rb="B635 Median",Gb="B532 Median"),
+       annotation=c("Block","Row","Column","ID","Name"))
```

is exactly equivalent to `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 acceptable, but extra lines at the end of the file will cause the read to fail.

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 of 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.

4.5 Reading Single-Channel Agilent Intensity Data

Reading single-channel data is similar to two-color data, except that the argument `green.only=TRUE` should be added to tell `read.maimages()` not to expect a red channel. Single-channel Agilent intensities, as produced by Agilent's Feature Extraction software, can be read by

```
> x <- read.maimages(files, source="agilent", green.only=TRUE)
```

or

```
> x <- read.maimages(targets, source="agilent", green.only=TRUE)
```

As for two-color data, the `path` argument is used:

```
> x <- read.maimages(files, source="agilent", path="<directory>", green.only=TRUE)
```

if the data files are not in the current working directory. The `green.only` argument tells `read.maimages()` to output an `EList` object instead an `RGList`. The raw intensities will be stored in the `E` component of the data object, and can be checked for example by

```
> summary(x$E)
```

Agilent's Feature Extraction software has the ability to estimate the foreground and background signals for each spot using either the mean or the median of the foreground and background pixels. The default for `read.maimages` is to read the median signal for both foreground and background. Alternatively

```
> x <- read.maimages(targets, source="agilent.mean", green.only=TRUE)
```

would read the mean foreground signal while still using median for the background. The possible values for `source` are:

Source	Foreground	Background
agilent	Median Signal	Median Signal
agilent.mean	Mean Signal	Median Signal
agilent.median	Median Signal	Median Signal

As for two-color data, the default choices for the foreground and background estimates can be over-ridden by specifying the `columns` argument to `read.maimages()`.

Agilent Feature Extraction output files contain probe annotation columns as well as intensity columns. By default, `read.maimages()` will read the following annotation columns, if they exist: `Row`, `Col`, `Start`, `Sequence`, `SwissProt`, `GenBank`, `Primate`, `GenPept`, `ProbeUID`, `ControlType`, `ProbeName`, `GeneName`, `SystematicName`, `Description`.

See Section 16.4 for a complete worked case study with single-channel Agilent data.

4.6 Reading Illumina BeadChip Data

Illumina whole-genome BeadChips require special treatment. Illumina images are scanned by BeadScan software, and Illumina's BeadStudio or GenomeStudio software can be used to export probe summary profiles. The probe summary profiles are tab-delimited files containing the intensity data. Typically, all the arrays processed at one time are written to a single file, with several columns corresponding to each array. We recommend that intensities should be exported from GenomeStudio without background correction or normalization, as these preprocessing steps can be better done by limma functions. GenomeStudio can also be asked to export profiles for the control probes, and we recommend that this be done as well.

Illumina files differ from other platforms in that each image output file contains data from multiple arrays and in that intensities for control probes are written to a separate file from the regular probes. There are other features of these files that can optionally be used for preprocessing and filtering. Illumina probe summary files can be read by the `read.ilmn` function. A typical usage is

```
> x <- read.ilmn("probe profile.txt", ctrlfiles="control probe profile.txt")
```

where `probe profile.txt` is the name of the main probe summary profile file and `control probe profile.txt` is the name of the file containing profiles for control probes.

If there are multiple probe summary profiles to be read, and the samples are summarized in a targets frame, then the `read.ilmn.targets` function can be used.

Reading the control probe profiles is optional but recommended. If the control probe profiles are available, then the Illumina data can be favorably background corrected and normalized using the `neqc` or `nec` functions. Otherwise, Illumina data is background corrected and normalized as for other single channel platforms.

See Section 16.3 for a fully worked case study with Illumina microarray data.

4.7 Image-derived Spot Quality Weights

Image analysis programs typically output a lot of information, in addition to the foreground and background intensities, which provides information on the quality of each spot. It is sometimes desirable to use this information to produce a quality index for each spot which can be used in the subsequent analysis steps. One approach is to remove all spots from consideration which do not satisfy a certain quality criterion. A more sophisticated approach is to produce a quantitative quality index which can be used to up or downweight each spot in a graduated way depending on its perceived reliability. limma provides an approach to spot weights which supports both of these approaches.

The limma approach is to compute a quantitative quality weight for each spot. Weights are treated similarly in limma as they are treated in most regression functions in R such as `lm()`. A zero weight indicates that the spot should be ignored in all analysis as being unreliable. A weight of 1 indicates normal quality. A spot quality weight greater or less than one will result in that spot being given relatively more or less weight in subsequent analyses. Spot weights less than zero are not meaningful.

The quality information can be read and the spot quality weights computed at the same time as the intensities are read from the image analysis output files. The computation of the quality weights is defined by the `wt.fun` argument to the `read.maimages()` function. This argument is a function which defines how the weights should be computed from the information found in the image analysis files. Deriving good spot quality weights is far from straightforward and depends very much on the image analysis software used. limma provides a few examples which have been found to be useful by some researchers.

Some image analysis programs produce a quality index as part of the output. For example, GenePix produces a column called `Flags` which is zero for a "normal" spot and takes increasingly negative values for different classes of problem spot. If you are reading GenePix image analysis files, the call

```
> RG <- read.maimages(files,source="genepix",wt.fun=wtflags(weight=0,cutoff=-50))
```

will read in the intensity data and will compute a matrix of spot weights giving zero weight to any spot with a `Flags`-value less than -50. The weights are stored in the `weights` component of the RGList data object. The weights are used automatically by functions such as `normalizeWithinArrays` which operate on the RG-list.

Sometimes the ideal size, in terms of image pixels, is known for a perfectly circular spot. In this case it may be useful to downweight spots which are much larger or smaller than this ideal size. If SPOT image analysis output is being read, the following call

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

gives full weight to spots with area exactly 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.

The appropriate way to computing spot quality weights depends on the image analysis program used. 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 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.

4.8 Reading Probe Annotation

The `RGList` read by `read.maimages()` will almost always contain a component called `genes` containing the IDs and other annotation information associated with the probes. The only exceptions are SPOT data, `source="spot"`, or when reading generic data, `source="generic"`, without setting the annotation argument, `annotation=NULL`. Try

```
> names(RG$genes)
```

to see if the `genes` component has been set.

If the `genes` component is not set, the probe IDs will need to be read from a separate file. If the arrays have been scanned with an Axon scanner, then the probes IDs will be available in a tab-delimited 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.delim` from R base.

4.9 Printer Layout

The printer layout is the arrangement of spots and blocks of spots on the arrays. Knowing the printer layout is especially relevant for old-style academic spotted arrays printed with a mechanical robot with a multi-tip print-head. The blocks are sometimes called print-tip groups or pin-groups or meta rows and columns. Each block corresponds to a print tip on the print-head used to print the arrays, and the layout of the blocks on the arrays corresponds to the layout of the tips on the print-head. The number of spots in each block is the number of times the print-head was lowered onto the array. Where possible, for example for Agilent, GenePix or ImaGene data, `read.maimages` will set the printer layout information in the component `printer`. Try

```
> names(RG$printer)
```

to see if the printer layout information has been set.

If you've used `readGAL` to set the `genes` component, you may also use `getLayout` to set the `printer` information by

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

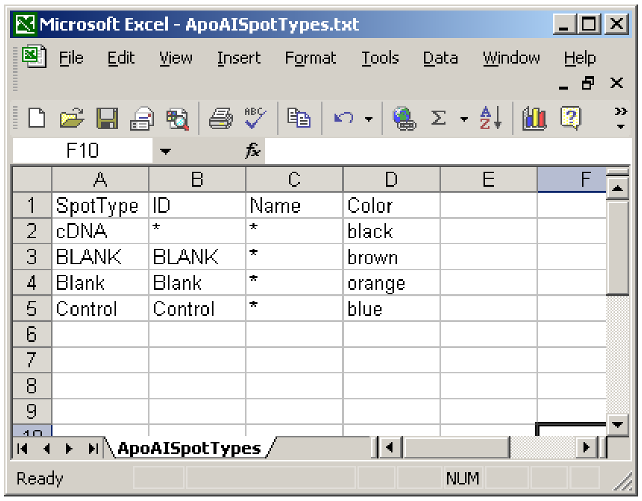
Note this will work only for GenePix GAL files, not for general gene lists.

4.10 The Spot Types File

The Spot Types file (STF) is another optional tab-delimited text file that allows you to identify different types of probes from the entries appearing in the gene list. It is especially useful for identifying different types of control probes. The STF is used to set the control status of each probe on the arrays so that plots may highlight different types of spots in an appropriate way. It is typically used to distinguish control probes from regular probes corresponding to genes, and to distinguish positive from negative controls, ratio from calibration controls and so on. The STF should have a `SpotType` column giving the names of the different spot-types. 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 attributes, such as colors or symbols, to be associated with the spot-types. There is one row for each spot-type to be distinguished.

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. The first row should specify the default spot-type and should have pattern `*` for all the pattern-matching columns.

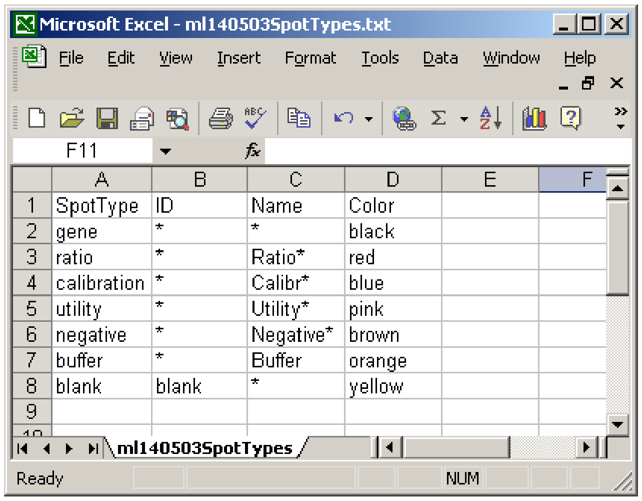
Here is a short STF appropriate for the ApoAI data:


Microsoft Excel - ApoAISpotTypes.txt
File Edit View Insert Format Tools Data Window Help
F10
ApoAISpotTypes
Ready
NUM

	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		

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. This code assumes of that the probe annotation data.frame includes columns `ID` and `Name`. This is usually so if GenePix has been used for the image analysis, but other image analysis software may use other column names.

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


Microsoft Excel - ml140503SpotTypes.txt
File Edit View Insert Format Tools Data Window Help
F11
ml140503SpotTypes
Ready
NUM

	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		

If the STF has default name `SpotTypes.txt` then it can be read using

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

It is typically used as an argument to the `controlStatus()` function to set the status of each spot on the array, for example

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

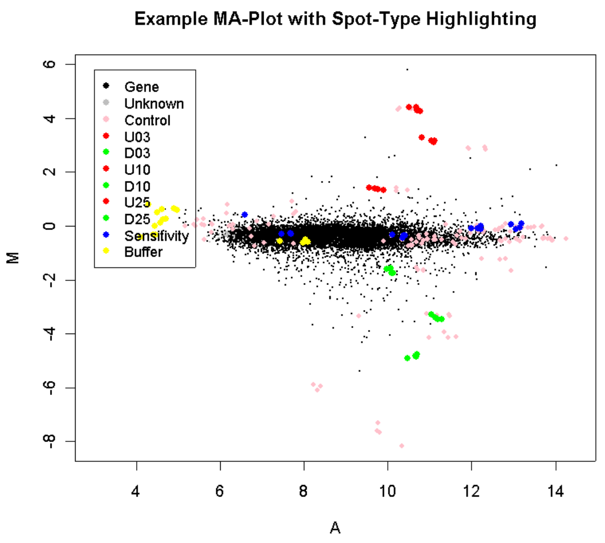
Chapter 5

Quality Assessment

An essential step in the analysis of any microarray data is to check the quality of the data from the arrays. For two-color array data, an essential step is to view the MA-plots of the unnormalized data for each array. The `plotMA()` function produces plots for individual arrays. The `plotMA3by2()` function gives an easy way to produce MA-plots for all the arrays in a large experiment. This functions writes plots to disk as png files, 6 plots to a page.

The usefulness of MA-plots is enhanced by highlighting various types of control probes on the arrays, and this is facilited by the `controlStatus()` funtion. The following is an example MA-Plot for an Incyte array with various spike-in and other controls. (Data courtesy of Dr Steve Gerondakis, Walter and Eliza Hall Institute of Medical Research.) The data shows high-quality data with long comet-like pattern of non-differentially expressed probes and a small proportion of highly differentially expressed probes. The plot was produced using

```
> spottypes <- readSpotTypes()
> RG$genes$Status <- controlStatus(spottypes, RG)
> plotMA(RG)
```



The array includes spike-in ratio controls which are 3-fold, 10-fold and 25-fold up and down regulated, as well as non-differentially expressed sensitivity controls and negative controls.

The background intensities are also a useful guide to the quality characteristics of each array. Boxplots of the background intensities from each array

```
> boxplot(data.frame(log2(RG$Gb)),main="Green background")
> boxplot(data.frame(log2(RG$Rb)),main="Red background")
```

will highlight any arrays unusually with high background intensities.

Spatial heterogeneity on individual arrays can be highlighted by examining imageplots of the background intensities, for example

```
> imageplot(log2(RG$Gb[,1]),RG$printer)
```

plots the green background for the first array. The function `imageplot3by2()` gives an easy way to automate the production of plots for all arrays in an experiment.

If the plots suggest that some arrays are of lesser quality than others, it may be useful to estimate array quality weights to be used in the linear model analysis, see Section 14.

Chapter 6

Pre-Processing Two-Color Data

6.1 Background Correction

The default background correction action is to subtract the background intensity from the foreground intensity for each spot. If the `RGList` object has not already been background corrected, then `normalizeWithinArrays` will do this by default. Hence

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

is equivalent to

```
> RGb <- backgroundCorrect(RG, method="subtract")
> MA <- normalizeWithinArrays(RGb)
```

However there are many other background correction options which may be preferable in certain situations, see Ritchie et al [26].

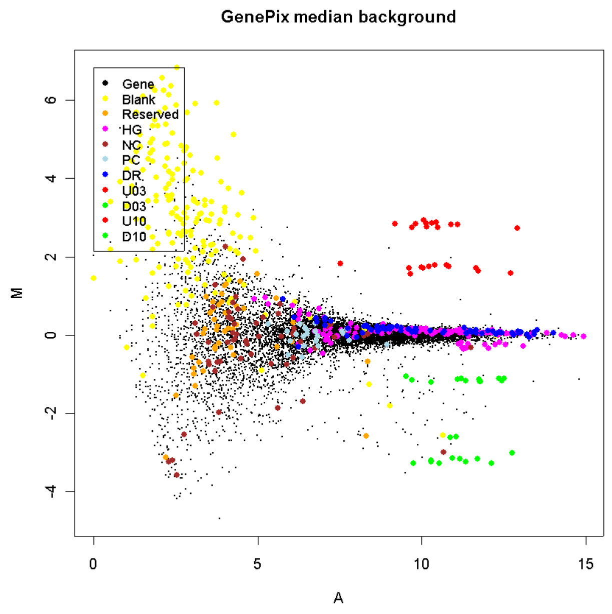
For the purpose of assessing differential expression, we often find

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

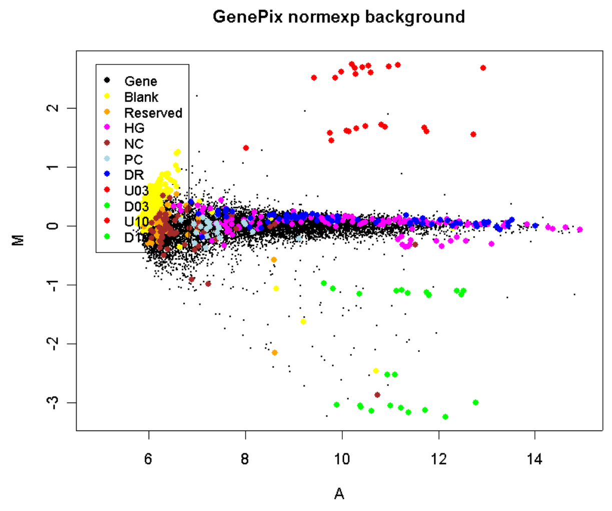
to be preferable to the simple background subtraction when using output from most image analysis programs. This method adjusts the foreground adaptively for the background intensities and results in strictly positive adjusted intensities, i.e., negative or zero corrected intensities are avoided. The use of an offset damps the variation of the log-ratios for very low intensities spots towards zero.

To illustrate some differences between the different background correction methods we consider one cDNA array which was self-self hybridized, i.e., the same RNA source was hybridized to both channels. For this array there is no actual differential expression. The array was printed with a human 10.5k library and hybridized with Jurkatt RNA on both channels. (Data courtesy Andrew Holloway and Dileepa Diyagama, Peter MacCallum Cancer Centre, Melbourne.) The array included a selection of control spots which are highlighted on the plots. Of particular interest are the spike-in ratio controls which should show up and down fold changes of 3 and 10. The first plot displays data acquired with GenePix software and background corrected by subtracting the median local background, which is the default with

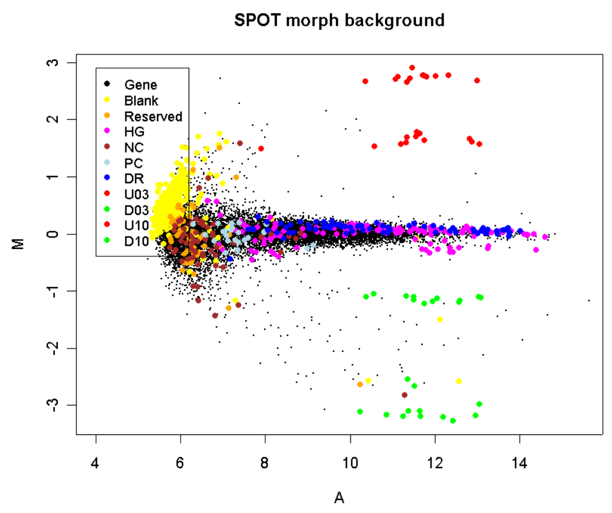
GenePix data. The plot shows the typical wedge shape with fanning of the M-values at low intensities. The range of observed M-values dominates the spike-in ratio controls. The are also 1148 spots not shown on the plot because the background corrected intensities were zero or negative.



The second plot shows the same array background corrected with `method="normexp"` and `offset=50`. The spike-in ratio controls now standout clearly from the range of the M-values. All spots on the array are shown on the plot because there are now no missing M-values.



The third plot shows the same array quantified with SPOT software and with “morph” background subtracted. This background estimator produces a similar effect to that with `normexp`.



The effect of using "morph" background or using `method="normexp"` with an offset is to stabilize the variability of the M-values as a function of intensity. The empirical Bayes methods implemented in the limma package for assessing differential expression will yield most benefit when the variabilities are as homogeneous as possible between genes. This can best be achieved by reducing the dependence of variability on intensity as far as possible [26].

6.2 Within-Array Normalization

Limma implements a range of normalization methods for spotted microarrays. Smyth and Speed [34] describe some of the most commonly used methods. The methods may be broadly classified into methods which normalize the M-values for each array separately (within-array normalization) and methods which normalize intensities or log-ratios to be comparable across arrays (between-array normalization). This section discusses mainly within-array normalization, which all that is usually required for the traditional log-ratio analysis of two-color data. Between-array normalization is discussed further in Section 6.3.

Print-tip loess normalization [46] is the default normalization method and can be performed by

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

There are some notable cases where this is not appropriate. For example, Agilent arrays do not have print-tip groups, so one should use global loess normalization instead:

```
> MA <- normalizeWithinArrays(RG, method="loess")
```

Print-tip loess is also unreliable for small arrays with less than, say, 150 spots per print-tip group. Even larger arrays may have particular print-tip groups which are too small for print-tip loess normalization if the number of spots with non-missing M-values is small for one or more of the print-tip groups. In these cases one should either use global `"loess"` normalization or else use robust spline normalization

```
> MA <- normalizeWithinArrays(RG, method="robustspline")
```

which is an empirical Bayes compromise between print-tip and global loess normalization, with 5-parameter regression splines used in place of the loess curves.

Loess normalization assumes that the bulk of the probes on the array are not differentially expressed. It doesn't assume that that there are equal numbers of up and down regulated genes or that differential expression is symmetric about zero, provided that the loess fit is implemented in a robust fashion, but it is necessary that there be a substantial body of probes which do not change expression levels. Oshlack et al [21] show that loess normalization can tolerate up to about 30% asymmetric differential expression while still giving good results. This assumption can be suspect for boutique arrays where the total number of unique genes on the array is small, say less than 150, particularly if these genes have been selected for being specifically expressed in one of the RNA sources. In such a situation, the best strategy is to include on the arrays a series of non-differentially expressed control spots, such as a titration series of whole-library-pool spots, and to use the up-weighting method discussed below [21]. A whole-library-pool means that one makes a pool of a library of probes, and prints spots from the pool at various concentrations [45]. The library should be sufficiently large than one can be confident that the average of all the probes is not differentially expressed. The larger the library the better. Good results have been obtained with library pools with as few as 500 clones. In the absence of such control spots, normalization of boutique arrays requires specialist advice.

Any spot quality weights found in `RG` will be used in the normalization by default. This means for example that spots with zero weight (flagged out) will not influence the normalization of other spots. The use of spot quality weights will not however result in any spots being removed from the data object. Even spots with zero weight will be normalized and will appear in the output object, such spots will simply not have any influence on the other spots. If you do not wish the spot quality weights to be used in the normalization, their use can be over-ridden using

```
> MA <- normalizeWithinArrays(RG, weights=NULL)
```

The output object `MA` will still contain any spot quality weights found in `RG`, but these weights are not used in the normalization step.

It is often useful to make use of control spots to assist the normalization process. For example, if the arrays contain a series of spots which are known in advance to be non-differentially expressed, these spots can be given more weight in the normalization process. Spots which are known in advance to be differentially expressed can be down-weighted. Suppose for example that the `controlStatus()` has been used to identify spike-in spots which are differentially expressed and a titration series of whole-library-pool spots which should not be differentially expressed. Then one might use

```
> w <- modifyWeights(RG$weights, RG$genes$Status, c("spikein","titration"), c(0,2))
> MA <- normalizeWithinArrays(RG, weights=w)
```

to give zero weight to the spike-in spots and double weight to the titration spots. This process is automated by the `"control"` normalization method, for example

```
> csi <- RG$genes$Status=="titration"
> MA <- normalizeWithinArrays(RG, method="control", controlspots=csi)
```

In general, `csi` is an index vector specifying the non-differentially expressed control spots [21].

The idea of up-weighting the titration spots is in the same spirit as the composite normalization method proposed by [45] but is more flexible and generally applicable. The above code assumes that `RG` already contains spot quality weights. If not, one could use

```
> w <- modifyWeights(array(1,dim(RG)), RG$genes$Status, c("spikein","titration"), c(0,2))
> MA <- normalizeWithinArrays(RG, weights=w)
```

instead.

Limma contains some more sophisticated normalization methods. In particular, some between-array normalization methods are discussed in Section 6.3 of this guide.

6.3 Between-Array Normalization

This section explores some of the methods available for between-array normalization of two-color arrays. A feature which distinguishes most of these methods from within-array normalization is the focus on the individual red and green intensity values rather than merely on the log-ratios. These methods might therefore be called *individual channel* or *separate channel* normalization methods. Individual channel normalization is typically a prerequisite to individual channel analysis methods such as that provided by `lmscFit()`. Further discussion of the issues involved is given by [48]. This section shows how to reproduce some of the results given in [48]. The Apoa1 data set from Section 15.2 will be used to illustrate these methods. We assume that the the Apoa1 data has been loaded and background corrected as follows:

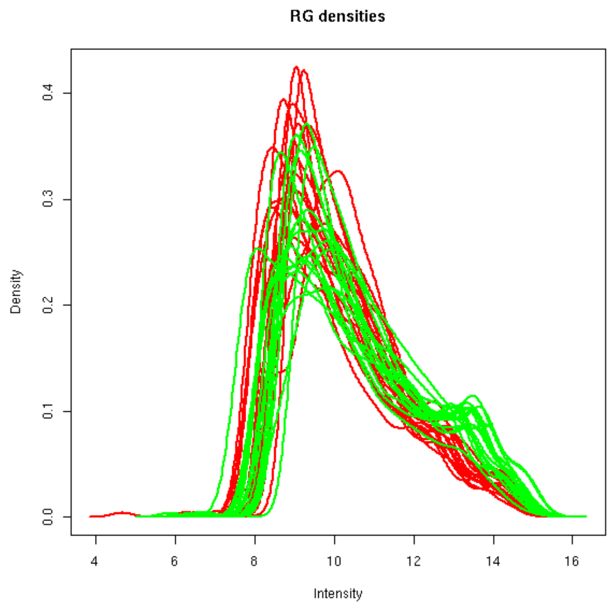
```
> load("Apoa1.RData")
```

An important issue to consider before normalizing between arrays is how background correction has been handled. For between-array normalization to be effective, it is important to avoid missing values in log-ratios which might arise from negative or zero corrected intensities. The function `backgroundCorrect()` gives a number of useful options. For the purposes of this section, the data has been corrected using the `"minimum"` method:

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

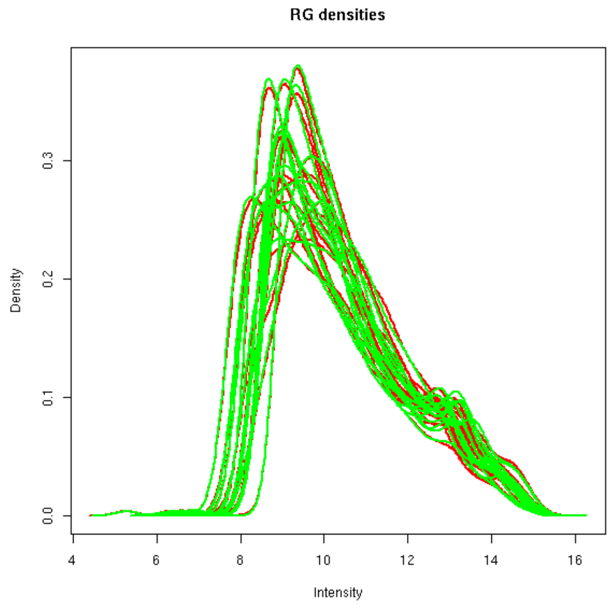
`plotDensities` displays smoothed empirical densities for the individual green and red channels on all the arrays. Without any normalization there is considerable variation between both channels and between arrays:

```
> plotDensities(RG.b)
```



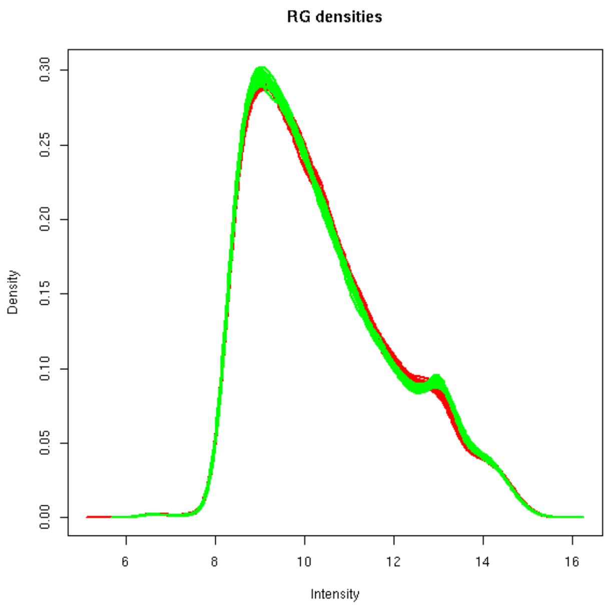
After loess normalization of the M-values for each array the red and green distributions become essentially the same for each array, although there is still considerable variation between arrays:

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



Loess normalization doesn't affect the A-values. Applying quantile normalization to the A-values makes the distributions essentially the same across arrays as well as channels:

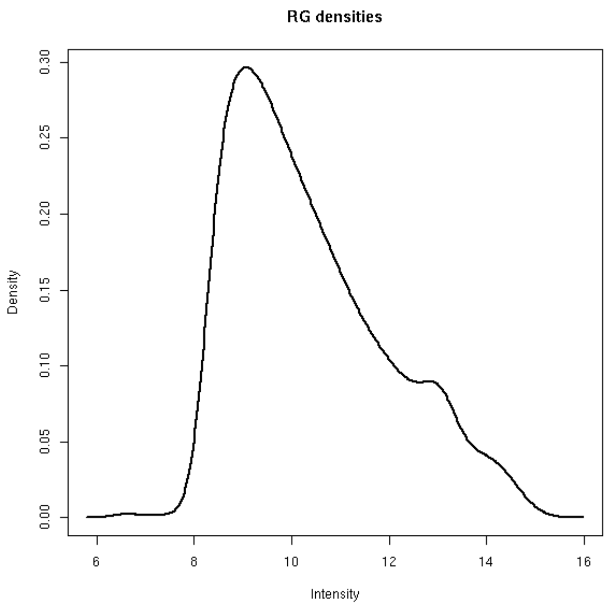
```
> MA.pAq <- normalizeBetweenArrays(MA.p, method="Aquantile")
> plotDensities(MA.pAq)
```



Applying quantile normalization directly to the individual red and green intensities produces a similar result but is somewhat noisier:

```
> MA.q <- normalizeBetweenArrays(RG.b, method="quantile")
> plotDensities(MA.q, col="black")

Warning message:
number of groups=2 not equal to number of col in: plotDensities(MA.q, col = "black")
```



There are other between-array normalization methods not explored here. For example normalizeBetweenArrays with method="vsn" gives an interface to the variance-stabilizing normalization methods of the vsn package.

6.4 Using Objects from the marray Package

The package marray is a well known R package for pre-processing of two-color microarray data. Marray provides functions for reading, normalization and graphical display of data. Marray and limma are both descendants of the earlier and path-breaking sma package available from http://www.stat.berkeley.edu/users/terry/zarray/Software/smacode.html but limma has maintained and built upon the original data structures whereas marray has converted to a fully formal data class representation. For this reason, Limma is backwardly compatible with sma while marray is not.

Normalization functions in marray focus on a flexible approach to location and scale normalization of M-values, rather than the within and between-array approach of limma. Marray provides some normalization methods which are not in limma including 2-D loess normalization and print-tip-scale normalization. Although there is some overlap between the normalization functions in the two packages, both providing print-tip loess normalization, the two approaches are largely complementary. Marray also provides highly developed functions for graphical display of two-color microarray data.

Read functions in marray produce objects of class marrayRaw while normalization produces objects of class marrayNorm. Objects of these classes may be converted to and from limma data objects using the convert package. marrayRaw objects may be converted to RGList objects and marrayNorm objects to MAList objects using the as function. For example, if Data is an marrayNorm object then

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

converts to an MAList object.

marrayNorm objects can also be used directly in limma without conversion, and this is generally recommended. If Data is an marrayNorm object, then

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

fits a linear model to Data as it would to an MAList object. One difference however is that the marray read functions tend to populate the maW slot of the marrayNorm object with qualitative spot quality flags rather than with quantitative non-negative weights, as expected by limma. If this is so then one may need

```
> fit <- lmFit(Data, design, weights=NULL)
```

to turn off use of the spot quality weights.

Chapter 7

Filtering

We usually recommend that all probes on the microarray platform be used for the normalization step.

For downstream analysis, it is usually worthwhile to remove probes that appear not be expressed in any of the experimental conditions. This is called filtering. We generally recommend that this is done before the linear modelling and empirical Bayes steps, but after normalization.

There are a number of ways that filtering can be done. One way is to keep probes that are expressed above background on at least n arrays, where n is the smallest number of replicates assigned to any of the treatment combinations. See for example Case studies 15.3 or 15.4 in the limma User's Guide.

Note that filtering methods involving variances should not be used. The limma algorithm analyses the spread of the genewise variances. Any filtering method based on genewise variances will change the distribution of variances, will interfere with the limma algorithm and hence will give poor results.

Chapter 8

Linear Models Overview

8.1 Introduction

The package limma uses an approach called *linear models* to analyze designed microarray experiments. This approach allows very general experiments to be analyzed just as easily as a simple replicated experiment. The approach is outlined in [36, 47]. 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 that 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 be 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 [36]. For arrays with within-array replicate spots, limma uses a pooled correlation method to make full use of the duplicate spots [33].

8.2 Single-Channel Designs

Affymetrix data will usually be normalized using the affy package. We will assume here that the data is available as an `ExpressionSet` 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 analyzed 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 `ExpressionSet` 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(~ 0+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="BH")
```

The outcome of each hypothesis test can be assigned using

```
> results <- decideTests(fit2)
```

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

```
> vennDiagram(results)
```

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 analyzed 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(MA, 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:

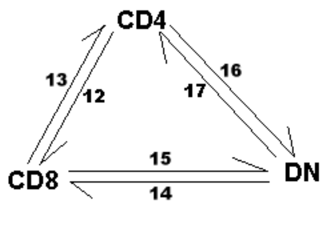
```
> contrast.matrix <- makeContrasts(AML1,CBFb,AML1.CBFb,AML1.CBFb-AML1,AML1.CBFb-CBFb,
+      levels=design)
> contrast.matrix
          AML1 CBFb AML1.CBFb AML1.CBFb - AML1 AML1.CBFb - CBFb
AML1         1    0         0               -1                0
AML1.CBFb    0    0         1                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-color 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
ml12med          12 ml12med.spot CD4 CD8
ml13med          13 ml13med.spot CD8 CD4
ml14med          14 ml14med.spot  DN CD8
ml15med          15 ml15med.spot CD8  DN
ml16med          16 ml16med.spot CD4  DN
ml17med          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 <- modelMatrix(targets, ref="CD4")
Found unique target names:
 CD4 CD8 DN
> design
        CD8 DN
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)
```

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       1      0      1
DN        0      1     -1
```

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

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

Chapter 9

Single-Channel Experimental Designs

9.1 Introduction

Unlike the early days of microarrays, most data is now of the single channel type. Single channel data is generated from popular microarray technologies such as Affymetrix, Illumina or Agilent. The new technology of RNA-Seq also generates single channel data, so everything in this chapter can be applied to RNA-Seq analyses when the data has been pre-processed using the `voom` function [16]. Single-channel data may be analyzed very much like ordinary univariate linear models or analysis of variance. 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.

We will assume for our examples here that the data has been suitably pre-processed normalized and is available as an `ExpressionSet` or `EList` 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)`.

9.2 Two Groups

The simplest possible single channel experiment is to compare two groups. Suppose that we wish to compare two wild type (Wt) mice with three mutant (Mu) mice:

FileName	Target
File1	WT
File2	WT
File3	Mu
File4	Mu
File5	Mu

There are two different ways to form the design matrix. We can either

1. create a design matrix which includes a coefficient for the mutant vs wild type difference, or

2. create a design matrix which includes separate coefficients for wild type and mutant mice and then extract the difference as a contrast.

For the first approach, the treatment-contrasts parametrization, the design matrix should be as follows:

```
> design

       WT MUvsWT
Array1  1      0
Array2  1      0
Array3  1      1
Array4  1      1
Array5  1      1
```

Here the first coefficient estimates the mean log-expression for wild type mice and plays the role of an intercept. The second coefficient estimates the difference between mutant and wild type. Differentially expressed genes can be found by

```
> fit <- lmFit(eset, design)
> fit <- eBayes(fit)
> topTable(fit, coef="MUvsWT", adjust="BH")
```

where `eset` is an `ExpressionSet` or `matrix` object containing the log-expression values. For the second approach, the design matrix should be

```
       WT MU
Array1  1  0
Array2  1  0
Array3  0  1
Array4  0  1
Array5  0  1
```

Differentially expressed genes can be found by

```
> fit <- lmFit(eset, design)
> cont.matrix <- makeContrasts(MUvsWT=MU-WT, levels=design)
> fit2 <- contrasts.fit(fit, cont.matrix)
> fit2 <- eBayes(fit2)
> topTable(fit2, adjust="BH")
```

For the first approach, the treatment-contrasts parametrization, the design matrix can be computed by

```
> design <- cbind(WT=1,MUvsWT=c(0,0,1,1,1))
```

or by

```
> Group <- factor(targets$Target, levels=c("WT","Mu"))
> design <- model.matrix(~Group)
> colnames(design) <- c("WT","MUvsWT")
```

For the second approach, the group-means parametrization, the design matrix can be computed by

```
> design <- cbind(WT=c(1,1,0,0,0,MU=c(0,0,1,1,1))
```

or by

```
> design <- model.matrix(~0+Group)
> colnames(design) <- c("WT","MU")
```

9.3 Several Groups

The above approaches for two groups extend easily to any number of groups. Suppose that three RNA targets to be compared. Suppose that the three targets are called “RNA1”, “RNA2” and “RNA3” and that the column `targets$Target` indicates which one was hybridized to each array. An appropriate design matrix can be created using

```
> f <- factor(targets$Target, levels=c("RNA1","RNA2","RNA3"))
> design <- model.matrix(~0+f)
> colnames(design) <- c("RNA1","RNA2","RNA3")
```

To make all pair-wise comparisons between the three groups one could proceed

```
> fit <- lmFit(eset, design)
> contrast.matrix <- makeContrasts(RNA2-RNA1, RNA3-RNA2, RNA3-RNA1,
+                                  levels=design)
> fit2 <- contrasts.fit(fit, contrast.matrix)
> fit2 <- eBayes(fit2)
```

A list of top genes for RNA2 versus RNA1 can be obtained from

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

The outcome of each hypothesis test can be assigned using

```
> results <- decideTests(fit2)
```

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

```
> vennDiagram(results)
```

The statistic `fit2$F` and the corresponding `fit2$F.p.value` combine the three pair-wise comparisons into one F-test. This is equivalent to a one-way ANOVA for each gene except that the residual mean squares have been moderated between genes. To find genes which vary between the three RNA targets in any way, look for genes with small p-values. To find the top 30 genes:

```
> topTableF(fit2, number=30)
```

9.4 Additive Models and Blocking

9.4.1 Paired Samples

Paired samples occur when we compare two treatments and each sample given one treatment is naturally paired with a particular sample given the other treatment. This is a special case of blocking with blocks of size two. The classical test associated with this situation is the paired *t*-test.

Suppose an experiment is conducted to compare a new treatment (T) with a control (C). Six dogs are used from three sib-ships. For each sib-pair, one dog is given the treatment while the other dog is a control. This produces the targets frame:

FileName	SibShip	Treatment
File1	1	C
File2	1	T
File3	2	C
File4	2	T
File5	3	C
File6	3	T

A moderated paired *t*-test can be computed by allowing for sib-pair effects in the linear model:

```
> SibShip <- factor(targets$SibShip)
> Treat <- factor(targets$Treatment, levels=c("C","T"))
> design <- model.matrix(~SibShip+Treat)
> fit <- lmFit(eset, design)
> fit <- eBayes(fit)
> topTable(fit, coef="TreatT")
```

9.4.2 Blocking

The above approach used for paired samples can be applied in any situation where there are batch effects or where the experiment has been conducted in blocks. The treatments can be adjusted for differences between the blocks by using a model formula of the form:

```
> design <- model.matrix(~Block+Treatment)
```

In this type of analysis, the treatments are compared only within each block.

9.5 Interaction Models: 2×2 Factorial Designs

9.5.1 Questions of Interest

Factorial designs are those where more than one experimental dimension is being varied and each combination of treatment conditions is observed. Suppose that cells are extracted from wild type and mutant mice and these cells are either stimulated (S) or unstimulated (U). RNA from the treated cells is then extracted and hybridized to a microarray. We will assume for simplicity that the arrays are single-color arrays such as Affymetrix. Consider the following targets frame:

FileName	Strain	Treatment
File1	WT	U
File2	WT	S
File3	Mu	U
File4	Mu	S
File5	Mu	S

The two experimental dimensions or *factors* here are Strain and Treatment. Strain specifies the genotype of the mouse from which the cells are extracted and Treatment specifies whether the cells are stimulated or not. All four combinations of Strain and Treatment are observed, so this is a factorial design. It will be convenient for us to collect the Strain/Treatment combinations into one vector as follows:

```
> TS <- paste(targets$Strain, targets$Treatment, sep=".")
> TS

[1] "WT.U" "WT.S" "Mu.U" "Mu.S" "Mu.S"
```

It is especially important with a factorial design to decide what are the comparisons of interest. We will assume here that the experimenter is interested in

1. which genes respond to stimulation in wild-type cells,
2. which genes respond to stimulation in mutant cells, and
3. which genes respond differently in mutant compared to wild-type cells.

as these are the questions which are most usually relevant in a molecular biology context. The first of these questions relates to the `WT.S` vs `WT.U` comparison and the second to `Mu.S` vs `Mu.U`. The third relates to the difference of differences, i.e., `(Mu.S-Mu.U)-(WT.S-WT.U)`, which is called the *interaction* term.

9.5.2 Analysing as for a Single Factor

We describe first a simple way to analyze this experiment using limma commands in a similar way to that in which two-sample designs were analyzed. Then we will go on to describe the more classical statistical approaches using factorial model formulas. All the approaches considered are equivalent and yield identical bottom-line results. The most basic approach is to fit a model with a coefficient for each of the four factor combinations and then to extract the comparisons of interest as contrasts:

```
> TS <- factor(TS, levels=c("WT.U","WT.S","Mu.U","Mu.S"))
> design <- model.matrix(~0+TS)
> colnames(design) <- levels(TS)
> fit <- lmFit(eset, design)
```

This fits a model with four coefficients corresponding to WT.U, WT.S, Mu.U and Mu.S respectively. Our three contrasts of interest can be extracted by

```
> cont.matrix <- makeContrasts(
+     SvsUinWT=WT.S-WT.U,
+     SvsUinMu=Mu.S-Mu.U,
+     Diff=(Mu.S-Mu.U)-(WT.S-WT.U),
+     levels=design)
> fit2 <- contrasts.fit(fit, cont.matrix)
> fit2 <- eBayes(fit2)
```

We can use topTable() to look at lists of differentially expressed genes for each of three contrasts, or else

```
> results <- decideTests(fit2)
> vennDiagram(results)
```

to look at all three contrasts simultaneously.

This approach is recommended for most users, because the contrasts that are being tested are formed explicitly.

9.5.3 A Nested Interaction Formula

Model formulas in R are very flexible, and offer lots of possible shortcuts for setting up the design matrix. However they also require a high level of statistical understanding in order to use reliably, and they are not completely described in the main R documentation. If we only wanted to test the first two questions above, an easy to to setup the design matrix would be to use a nested interaction term:

```
> Strain <- factor(targets$Strain, levels=c("WT","Mu"))
> Treatment <- factor(targets$Treatment, levels=c("U","S"))
> design <- model.matrix(~Strain+Strain:Treatment)
> colnames(design)
[1] "(Intercept)"         "StrainMu"            "StrainWT:TreatmentS" "StrainMu:TreatmentS"
```

The first term in the model formula is an effect for Strain. This introduces an intercept column to the design matrix, which estimates the average log-expression level for wild-type unstimulated cells, and a column for Strain which estimates the mutant vs wildtype difference in the unstimulated state. The second term in the model formula represents the interaction between stimulation and strain. Because there is no main effect for treatment in the model, the interaction is fitted in a nested sense. It introduces a third and a fourth column to the design matrix which represent the effect of stimulation for wild-type and for mutant mice respectively, exactly the same as the contrasts SvsUinWT and SvsUinMu define in the previous section. After

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

then

```
> topTable(fit, coef=3)
```

will find those genes responding to stimulation in wild-type mice, and

```
> topTable(fit, coef=4)
```

will find those genes responding to stimulation in mutant mice. Finally, we could extract the interaction contrast `Diff` considered above by

```
> fit2 <- contrasts.fit(fit, c(0,0,-1,1))
> fit2 <- eBayes(fit2)
> topTable(fit2)
```

This finds genes that respond differently to the stimulus in mutant vs wild-type mice.

9.5.4 Classic Interaction Models

The analysis of factorial designs has a long history in statistics and a system of factorial *model formulas* has been developed to facilitate the analysis of complex designs. It is important to understand though that the above three molecular biology questions do not correspond to any of the classic parametrizations used in statistics for factorial designs. Hence we generally recommend the approaches already considered above for microarray analysis.

Suppose for example that we proceed in the usual statistical way,

```
> design <- model.matrix(~Strain*Treatment)
```

This creates a design matrix which defines four coefficients with the following interpretations:

Coefficient	Comparison	Interpretation
Intercept	`WT.U`	Baseline level of unstimulated WT
StrainMu	`Mu.U-WT.U`	Difference between unstimulated strains
TreatmentS	`WT.S-WT.U`	Stimulation effect for WT
StrainMu:TreatmentS	`(Mu.S-Mu.U)-(WT.S-WT.U)`	Interaction

This is called the *treatment-contrast* parametrization. Notice that one of our comparisons of interest, `Mu.S-Mu.U`, is not represented and instead the comparison `Mu.U-WT.U`, which might not be of direct interest, is included. We need to use contrasts to extract all the comparisons of interest:

```
> fit <- lmFit(eset, design)
> cont.matrix <- cbind(SvsUinWT=c(0,0,1,0),SvsUinMu=c(0,0,1,1),Diff=c(0,0,0,1))
> fit2 <- contrasts.fit(fit, cont.matrix)
> fit2 <- eBayes(fit2)
```

This extracts the WT stimulation effect as the third coefficient and the interaction as the fourth coefficient. The mutant stimulation effect is extracted as the sum of the third and fourth coefficients of the original model. This analysis yields exactly the same results as the previous analysis.

An even more classical statistical approach to the factorial experiment would be to use the *sum to zero* parametrization. In R this is achieved by

```
> contrasts(Strain) <- contr.sum(2)
> contrasts(Treatment) <- contr.sum(2)
> design <- model.matrix(~Strain*Treatment)
```

This defines four coefficients with the following interpretations:

Coefficient	Comparison	Interpretation
Intercept	`(WT.U+WT.S+Mu.U+Mu.S)/4`	Grand mean
Strain1	`(WT.U+WT.S-Mu.U-Mu.S)/4`	Strain main effect
Treatment1	`(WT.U-WT.S+Mu.U-Mu.S)/4`	Treatment main effect
Strain1:Treatment1	`(WT.U-WT.S-Mu.U+Mu.S)/4`	Interaction

This parametrization has many appealing mathematical properties and is the classical parametrization used for factorial designs in much experimental design theory. However it defines only one coefficient which is directly of interest to us, namely the interaction. Our three contrasts of interest could be extracted using

```
> fit <- lmFit(eset, design)
> cont.matrix <- cbind(SvsUinWT=c(0,0,-2,-2),SvsUinMu=c(0,0,-2,2),Diff=c(0,0,0,4))
> fit2 <- contrasts.fit(fit, cont.matrix)
> fit2 <- eBayes(fit2)
```

The results will be identical to those for the previous three approaches.

The various approaches described here for the 2×2 factorial problem are equivalent and differ only in the parametrization chosen for the linear model. The fitted model objects `fit` will differ only in the `coefficients` and associated components. The residual standard deviations `fit$sigma`, residual degrees of freedom `fit$df.residual` and all components of `fit2` will be identical regardless of parametrization used. Since the approaches are equivalent, users are free to choose whichever one is most intuitive or convenient.

9.6 Time Course Experiments

9.6.1 Replicated time points

Time course experiments are those in which RNA is extracted at several time points after the onset of some treatment or stimulation. How best to analyse a time course experiment depends on the nature of the experiment, and especially on the number of distinct time points. We consider first experiments with a relative small number of replicated time points. Simple time course experiments of this type are similar to experiments with several groups covered in Section 9.3.

As an example, we consider here a two-way experiment in which time course profiles are to be compared for two genotypes. Consider the targets frame

FileName	Target
File1	wt.0hr
File2	wt.0hr
File3	wt.6hr
File4	wt.24hr
File5	mu.0hr
File6	mu.0hr
File7	mu.6hr
File8	mu.24hr

The targets are RNA samples collected from wild-type and mutant animals at 0, 6 and 24 hour time points. This can be viewed as a factorial experiment but a simpler approach is to use the group-mean parametrization.

```
> lev <- c("wt.0hr","wt.6hr","wt.24hr","mu.0hr","mu.6hr","mu.24hr")
> f <- factor(targets$Target, levels=lev)
> design <- model.matrix(~0+f)
> colnames(design) <- lev
> fit <- lmFit(eset, design)
```

Which genes respond at either the 6 hour or 24 hour times in the wild-type? We can find these by extracting the contrasts between the wild-type times.

```
> cont.wt <- makeContrasts(
+      "wt.6hr-wt.0hr",
+      "wt.24hr-wt.6hr",
+ levels=design)
> fit2 <- contrasts.fit(fit, cont.wt)
> fit2 <- eBayes(fit2)
> topTableF(fit2, adjust="BH")
```

Any two contrasts between the three times would give the same result. The same gene list would be obtained had `"wt.24hr-wt.0hr"` been used in place of `"wt.24hr-wt.6hr"` for example.

Which genes respond (i.e., change over time) in the mutant?

```
> cont.mu <- makeContrasts(
+      "mu.6hr-mu.0hr",
+      "mu.24hr-mu.6hr",
+ levels=design)
> fit2 <- contrasts.fit(fit, cont.mu)
> fit2 <- eBayes(fit2)
> topTableF(fit2, adjust="BH")
```

Which genes respond *differently* over time in the mutant relative to the wild-type?

```
> cont.dif <- makeContrasts(
+     Dif6hr =(mu.6hr-mu.0hr)-(wt.6hr-wt.0hr),
+     Dif24hr=(mu.24hr-mu.6hr)-(wt.24hr-wt.6hr),
+ levels=design)
> fit2 <- contrasts.fit(fit, cont.dif)
> fit2 <- eBayes(fit2)
> topTableF(fit2, adjust="BH")
```

The method of analysis described in this section was used for a six-point time course experiment on histone deacetylase inhibitors [22].

9.6.2 Many time points

Now we consider an example with many time points for each group. When there are many time points, it is reasonable to assume that expression changes smoothly over time rather than making discrete jumps from one time point to another. This type of time course can be analysed by fitting a temporal trend using a regression spline or a polynomial.

Consider the following targets frame, with 32 rows:

FileName	Group	Time
File1	Control	1
File2	Control	2
⋮	⋮	⋮
File16	Control	16
File17	Treat	1
File18	Treat	2
⋮	⋮	⋮
File32	Treat	16

It might be reasonable to represent a time course for a particular gene in a particular condition using a cubic spline curve with a modest number of knots. Choosing effective degrees of freedom to be in range 3–5 is reasonable. Setup a basis for a natural regression spline:

```
> library(splines)
> X <- ns(targets$Time, df=5)
```

Then fit separate curves for the control and treatment groups:

```
> Group <- factor(targets$Group)
> design <- model.matrix(~Group*X)
> fit <- lmFit(y, design)
> fit <- eBayes(fit)
```

This creates a model with 12 parameters, with the last 5 corresponding to interaction, i.e., to differences in the curves between groups. To detect genes with different time trends for treatment vs control:

```
> topTable(fit, coef=8:12)
```

This conducts a moderated F-test for each gene on 5 df, which can detect very general differences between the treatment and control curves.

Note that for this analysis, it is not necessary to have replicates, nor is it necessary for the two treatment groups to be observed at identical time points.

9.7 Multi-level Experiments

We have considered paired comparisons, and we have considered comparisons between two independent groups. There are however experiments that combine both of these types of comparisons.

Consider a single-channel experiment with the following targets frame:

FileName	Subject	Condition	Tissue
File01	1	Diseased	A
File02	1	Diseased	B
File03	2	Diseased	A
File04	2	Diseased	B
File05	3	Diseased	A
File06	3	Diseased	B
File07	4	Normal	A
File08	4	Normal	B
File09	5	Normal	A
File10	5	Normal	B
File11	6	Normal	A
File12	6	Normal	B

This experiment involves 6 subjects, including 3 patients who have the disease and 3 normal subjects. From each subject, we have expression profiles of two tissue types, A and B.

In analysing this experiment, we want to compare the two tissue types. This comparison can be made within subjects, because each subject yields a value for both tissues. We also want to compare diseased subjects to normal subjects, but this comparison is between subjects.

If we only wanted to compare the two tissue types, we could do a paired samples comparison. If we only wanted to compared diseased to normal, we could do an ordinary two group comparison. Since we need to make comparisons both within and between subjects, it is necessary to treat Patient as a random effect. This can be done in limma using the `duplicateCorrelation` function.

The two experimental factors Condition and Tissue could be handled in many ways. Here we will assume that it is convenient to join the two into a combined factor:

```
> Treat <- factor(paste(targets$Condition,targets$Tissue,sep="."))
> design <- model.matrix(~0+Treat)
> colnames(design) <- levels(Treat)
```

Then we estimate the correlation between measurements made on the same subject:

```
> corfit <- duplicateCorrelation(eset,design,block=targets$Subject)
> corfit$consensus
```

Then this inter-subject correlation is input into the linear model fit:

```
> fit <- lmFit(eset,design,block=targets$Subject,correlation=corfit$consensus)
```

Now we can make any comparisons between the experimental conditions in the usual way, example:

```
> cm <- makeContrasts(
+          DiseasedvsNormalForTissueA = Diseased.A-Normal.A,
+          DiseasedvsNormalForTissueB = Diseased.B-Normal.B,
+          TissueAvsTissueBForNormal = Normal.B-Normal.A,
+          TissueAvsTissueBForDiseased = Diseased.B-Diseased.A,
+ levels=design)
```

Then compute these contrasts and moderated t-tests:

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

Then

```
> topTable(fit2, coef="DiseasedvsNormalForTissueA")
```

will find those genes that are differentially expressed between the normal and diseased subjects in the A tissue type. And so on.

This experiment has two levels of variability. First, there is the variation from person to person, which we call the between-subject strata. Then there is the variability of repeat measurements made on the same subject, the within-subject strata. The between-subject variation is always expected to be larger than within-subject, because the latter is adjusted for baseline differences between the subjects. Here the comparison between tissues can be made within subjects, and hence should be more precise than the comparison between diseased and normal, which must be made between subjects.

Chapter 10

Two-Color Experiments with a Common Reference

10.1 Introduction

Now consider two-color microarray experiments in which a common reference has been used on all the arrays. If the same channel has been used for the common reference throughout the experiment, then the expression log-ratios may be analysed exactly as if they were log-expression values from a single channel experiment. In these cases, the design matrix can be formed as for a single channel experiment.

When the common reference is dye-swapped, the simplest method is to setup the design matrix using the `modelMatrix()` function and the targets file.

10.2 Two Groups

Suppose now that we wish to compare two wild type (Wt) mice with three mutant (Mu) mice using arrays hybridized with a common reference RNA (Ref):

FileName	Cy3	Cy5
File1	Ref	WT
File2	Ref	WT
File3	Ref	Mu
File4	Ref	Mu
File5	Ref	Mu

The interest here is in the comparison between the mutant and wild type mice. There are two major ways in which this comparison can be made. We can either

1. create a design matrix which includes a coefficient for the mutant vs wild type difference, or

2. create a design matrix which includes separate coefficients for wild type and mutant mice and then extract the difference as a contrast.

For the first approach, the design matrix should be as follows

```
> design

       WTvsREF MUvsWT
Array1       1      0
Array2       1      0
Array3       1      1
Array4       1      1
Array5       1      1
```

Here the first coefficient estimates the difference between wild type and the reference for each probe while the second coefficient estimates the difference between mutant and wild type. For those not familiar with model matrices in linear regression, it can be understood in the following way. The matrix indicates which coefficients apply to each array. For the first two arrays the fitted values will be just the `WTvsREF` coefficient, which is correct. For the remaining arrays the fitted values will be `WTvsREF + MUvsWT`, which is equivalent to mutant vs reference, also correct. For reasons that will be apparent later, this is sometimes called the *treatment-contrasts* parametrization. Differentially expressed genes can be found by

```
> fit <- lmFit(MA, design)
> fit <- eBayes(fit)
> topTable(fit, coef="MUvsWT", adjust="BH")
```

There is no need here to use `contrasts.fit()` because the comparison of interest is already built into the fitted model. This analysis is analogous to the classical *pooled two-sample t-test* except that information has been borrowed between genes.

For the second approach, the design matrix should be

```
       WT MU
Array1  1  0
Array2  1  0
Array3  0  1
Array4  0  1
Array5  0  1
```

The first coefficient now represents wild-type vs the reference and the second represents mutant vs the reference. Our comparison of interest is the difference between these two coefficients. We will call this the *group-means* parametrization. Differentially expressed genes can be found by

```
> fit <- lmFit(MA, design)
> cont.matrix <- makeContrasts(MUvsWT=MU-WT, levels=design)
> fit2 <- contrasts.fit(fit, cont.matrix)
> fit2 <- eBayes(fit2)
> topTable(fit2, adjust="BH")
```

The results will be exactly the same as for the first approach.

The design matrix can be constructed

1. manually,
2. using the limma function `modelMatrix()`, or
3. using the built-in R function `model.matrix()`.

Let `Group` be the factor defined by

```
> Group <- factor(c("WT","WT","Mu","Mu","Mu"), levels=c("WT","Mu"))
```

For the first approach, the treatment-contrasts parametrization, the design matrix can be computed by

```
> design <- cbind(WTvsRef=1,MUvsWT=c(0,0,1,1,1))
```

or by

```
> param <- cbind(WTvsRef=c(-1,1,0),MUvsWT=c(0,-1,1))
> rownames(param) <- c("Ref","WT","Mu")
> design <- modelMatrix(targets, parameters=param)
```

or by

```
> design <- model.matrix(~Group)
> colnames(design) <- c("WTvsRef","MUvsWT")
```

all of which produce the same result. For the second approach, the group-means parametrization, the design matrix can be computed by

```
> design <- cbind(WT=c(1,1,0,0,0),MU=c(0,0,1,1,1))
```

or by

```
> param <- cbind(WT=c(-1,1,0),MU=c(-1,0,1))
> rownames(param) <- c("Ref","WT","Mu")
> design <- modelMatrix(targets, parameters=param)
```

or by

```
> design <- model.matrix(~0+Group)
> colnames(design) <- c("WT","Mu")
```

all of which again produce the same result.

10.3 Several Groups

The above approaches for two groups extend easily to any number of groups. Suppose that the experiment has been conducted to compare three RNA sources, “RNA1”, “RNA2” and “RNA3”. For example the targets frame might be

FileName	Cy3	Cy5
File1	Ref	RNA1
File2	RNA1	Ref
File3	Ref	RNA2
File4	RNA2	Ref
File5	Ref	RNA3

For this experiment the design matrix could be formed by

```
> design <- modelMatrix(targets, ref="Ref")
```

after which the analysis would be exactly as for the equivalent single channel experiment in Section 9.3. For example, to make all pair-wise comparisons between the three groups one could proceed

```
> fit <- lmFit(eset, design)
> contrast.matrix <- makeContrasts(RNA2-RNA1, RNA3-RNA2, RNA3-RNA1, levels=design)
> fit2 <- contrasts.fit(fit, contrast.matrix)
> fit2 <- eBayes(fit2)
```

and so on.

Chapter 11

Direct Two-Color Experimental Designs

11.1 Introduction

Direct two-color designs are those in which there is no common reference, but the RNA samples are instead compared directly by competitive hybridization on the same arrays. Direct two-color designs can be very efficient and powerful, but they require the most statistical knowledge to choose the appropriate design matrix.

11.2 Simple Comparisons

11.2.1 Replicate Arrays

The simplest possible microarray experiment is one with a series of replicate two-color arrays all comparing the same two RNA sources. For a three-array experiment comparing wild type (wt) and mutant (mu) RNA, the targets file might contain the following entries:

FileName	Cy3	Cy5
File1	wt	mu
File2	wt	mu
File3	wt	mu

A list of differentially expressed probes might be found for this experiment by

```
> fit <- lmFit(MA)
> fit <- eBayes(fit)
> topTable(fit)
```

where `MA` holds the normalized data. The default design matrix used here is just a single column of ones. The experiment here measures the fold change of mutant over wild type. Genes which have positive M-values are more highly expressed in the mutant RNA while genes with negative M-values are more highly expressed in the wild type. The analysis is analogous to the classical single-sample *t*-test except that we have used empirical Bayes methods to borrow information between genes.

11.2.2 Dye Swaps

A simple modification of the above experiment would be to swap the dyes for one of the arrays. The targets file might now be

FileName	Cy3	Cy5
File1	wt	mu
File2	mu	wt
File3	wt	mu

Now the analysis would be

```
> design <- c(1,-1,1)
> fit <- lmFit(MA, design)
> fit <- eBayes(fit)
> topTable(fit)
```

Alternatively the design matrix could be set, replacing the first of the above code lines, by

```
> design <- modelMatrix(targets, ref="wt")
```

where `targets` is the data frame holding the targets file information.

If there are at least two arrays with each dye-orientation, then it is possible to estimate and adjust for any probe-specific dye effects. The dye-effect is estimated by an intercept term. If the experiment was

FileName	Cy3	Cy5
File1	wt	mu
File2	mu	wt
File3	wt	mu
File4	mu	wt

then we could set

```
> design <- cbind(DyeEffect=1,MUvsWT=c(1,-1,1,-1))
> fit <- lmFit(MA, design)
> fit <- eBayes(fit)
```

The genes which show dye effects can be seen by

```
> topTable(fit, coef="DyeEffect")
```

The genes which are differentially expressed in the mutant are obtained by

```
> topTable(fit, coef="MUvsWT")
```

The fold changes and significant tests in this list are corrected for dye-effects. Including the dye-effect in the model in this way uses up one degree of freedom which might otherwise be used to estimate the residual variability, but it is valuable if many genes show non-negligible dye-effects.

11.3 A Correlation Approach to Technical Replication

In the previous sections we have assumed that all arrays are biological replicates. Now consider an experiment in which two wild-type and two mice from the same mutant strain are compared using two arrays for each pair of mice. The targets might be

FileName	Cy3	Cy5
File1	wt1	mu1
File2	wt1	mu1
File3	wt2	mu2
File4	wt2	mu2

The first and second and third and fourth arrays are *technical replicates*. It would not be correct to treat this experiment as comprising four replicate arrays because the technical replicate pairs are not independent, in fact they are likely to be positively correlated.

One way to analyze these data is the following:

```
> biolrep <- c(1, 1, 2, 2)
> corfit <- duplicateCorrelation(MA, ndups = 1, block = biolrep)
> fit <- lmFit(MA, block = biolrep, cor = corfit$consensus)
> fit <- eBayes(fit)
> topTable(fit, adjust = "BH")
```

The vector `biolrep` indicates the two blocks corresponding to biological replicates. The value `corfit$consensus` estimates the average correlation within the blocks and should be positive. This analysis is analogous to *mixed model* analysis of variance [19, Chapter 18] except that information has been borrowed between genes. Information is borrowed by constraining the within-block correlations to be equal between genes and by using empirical Bayes methods to moderate the standard deviations between genes [33].

If the technical replicates were in dye-swap pairs as

FileName	Cy3	Cy5
File1	wt1	mu1
File2	mu1	wt1
File3	wt2	mu2
File4	mu2	wt2

then one might use

```
> design <- c(1, -1, 1, -1)
> corfit <- duplicateCorrelation(MA, design, ndups = 1, block = biolrep)
> fit <- lmFit(MA, design, block = biolrep, cor = corfit$consensus)
> fit <- eBayes(fit)
> topTable(fit, adjust = "BH")
```

In this case the correlation `corfit$consensus` should be negative because the technical replicates are dye-swaps and should vary in opposite directions.

This method of handling technical replication using `duplicateCorrelation()` is somewhat limited for two-color experiments. If for example one technical replicate was dye-swapped and the other not,

FileName	Cy3	Cy5
File1	wt1	mu1
File2	mu1	wt1
File3	wt2	mu2
File4	wt2	mu2

then there is no way to use `duplicateCorrelation()` because the technical replicate correlation will be negative for the first pair but positive for the second. In this case, there is no good alternative to treating the technical replicates as if they were biological, so that that the experiment would be analysed as a simple comparison with dye-swaps. Beware however that treating technical replicates as biological gives p-values that are smaller than they should be.

Chapter 12

Separate Channel Analysis of Two-Color Data

Separate channel analysis is a way to analyse two-color data in terms of the individual channel intensities [32]. In effect, separate channel analysis converts a two-color experiment into a single channel experiment with twice as many arrays but with a technical pairing between the two channels that originated from the same array.

Consider an experiment comparing young and old animals for both both wild-type and mutant genotypes.

FileName	Cy3	Cy5
File1	wt.young	wt.old
File2	wt.old	wt.young
File3	mu.young	mu.old
File4	mu.old	mu.young

Each of the arrays in this experiment makes a direct comparison between young and old RNA targets. There are no arrays which compare wild-type and mutant animals. This is an example of an *unconnected* design in that there are no arrays linking the wild-type and mutant targets. It is not possible to make comparisons between wild-type and mutant animals on the basis of log-ratios alone. So to do this it is necessary to analyze the red and green channels intensities separately, i.e., to analyze log-intensities instead of log-ratios. It is possible to do this using a mixed model representation which treats each spot as a randomized block [43, 31]. Limma implements mixed model methods for separate channel analysis which make use of shrinkage methods to ensure stable and reliable inference with small numbers of arrays [31]. Limma also provides between-array normalization to prepare for separate channel analysis, for example

```
> MA <- normalizeBetweenArrays(MA, method="Aquantile")
```

scales the intensities so that A-values have the same distribution across arrays.

The first step in the differential expression analysis is to convert the targets frame to be channel rather than array orientated.

```
> targets2 <- targetsA2C(targets)
> targets2
```

```
        channel.col FileName   Target
File1.1           1    File1 wt.young
File1.2           2    File1   wt.old
File2.1           1    File2   wt.old
File2.2           2    File2 wt.young
File3.1           1    File3 mu.young
File3.2           2    File3   mu.old
File4.1           1    File4   mu.old
File4.2           2    File4 mu.young
```

The following code produces a design matrix with eight rows and four columns:

```
> u <- unique(targets2$Target)
> f <- factor(targets2$Target, levels=u)
> design <- model.matrix(~0+f)
> colnames(design) <- u
```

Inference proceeds as for within-array replicate spots except that the correlation to be estimated is that between the two channels for the same spot rather than between replicate spots.

```
> corfit <- intraspotCorrelation(MA, design)
> fit <- lmscFit(MA, design, correlation=corfit$consensus)
```

Subsequent steps proceed as for log-ratio analyses. For example if we want to compare wild-type young to mutant young animals, we could extract this contrast by

```
> cont.matrix <- makeContrasts("mu.young-wt.young",levels=design)
> fit2 <- contrasts.fit(fit, cont.matrix)
> fit2 <- eBayes(fit2)
> topTable(fit2, adjust="BH")
```

Chapter 13

Statistics for Differential Expression

13.1 Summary Top-Tables

Limma provides functions `topTable()` and `decideTests()` which summarize the results of the linear model, perform hypothesis tests and adjust the p-values for multiple testing. Results include (log2) fold changes, standard errors, t-statistics and p-values. The basic statistic used for significance analysis is the *moderated t-statistic*, which is computed for each probe and for each contrast. This has the same interpretation as an ordinary t-statistic except that the standard errors have been moderated across genes, i.e., shrunk towards a common value, using a simple Bayesian model. This has the effect of borrowing information from the ensemble of genes to aid with inference about each individual gene [36]. Moderated t-statistics lead to p-values in the same way that ordinary t-statistics do except that the degrees of freedom are increased, reflecting the greater reliability associated with the smoothed standard errors. The effectiveness of the moderated t approach has been demonstrated on test data sets for which the differential expression status of each probe is known [11].

A number of summary statistics are presented by `topTable()` for the top genes and the selected contrast. The `logFC` column gives the value of the contrast. Usually this represents a $\log_2$-fold change between two or more experimental conditions although sometimes it represents a $\log_2$-expression level. The `AveExpr` column gives the average $\log_2$-expression level for that gene across all the arrays and channels in the experiment. Column `t` is the moderated t-statistic. Column `P.Value` is the associated p-value and `adj.P.Value` is the p-value adjusted for multiple testing. The most popular form of adjustment is `"BH"` which is Benjamini and Hochberg's method to control the false discovery rate [1]. The adjusted values are often called q-values if the intention is to control or estimate the false discovery rate. The meaning of `"BH"` q-values is as follows. If all genes with q-value below a threshold, say 0.05, are selected as differentially expressed, then the expected proportion of false discoveries in the selected group is controlled to be less than the threshold value, in this case 5%. This procedure is equivalent to the procedure of Benjamini and Hochberg although the original paper did not formulate the method in terms of adjusted p-values.

The B-statistic (`lods` or `B`) is the log-odds that the gene is differentially expressed [36, Section 5]. 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 in the call to `eBayes()`, are expected to be differentially expressed. The p-values and B-statistics will normally rank genes in the same order. In fact, if the data contains no missing values or quality weights, then the order will be precisely the same.

As with all model-based methods, the p-values depend on normality and other mathematical assumptions which are never exactly true for microarray data. It has been argued that the p-values are useful for ranking genes even in the presence of large deviations from the assumptions [35, 33]. Benjamini and Hochberg's control of the false discovery rate assumes independence between genes, although Reiner et al [23] have argued that it works for many forms of dependence as well. The B-statistic probabilities depend on the same assumptions but require in addition a prior guess for the proportion of differentially expressed probes. The p-values may be preferred to the B-statistics because they do not require this prior knowledge.

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 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 denominator degrees of freedom is the same as that of 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.

A frequently asked question relates to the occasional occurrence that all of the adjusted p-values are equal to 1. This is not an error situation but rather an indication that there is no evidence of differential expression in the data after adjusting for multiple testing. This can occur even though many of the raw p-values may seem highly significant when taken as individual values. This situation typically occurs when none of the raw p-values are less than $1/G$, where G is the number of probes included in the fit. In that case the adjusted p-values are typically equal to 1 using any of the adjustment methods except for `adjust="none"`.

13.2 Fitted Model Objects

The output from `lmFit()` is an object of class `MArrayLM`. This section gives some mathematical details describing what is contained in such objects. This section can be skipped by readers not interested in such details.

The linear model for gene j has residual variance σ_j^2 with sample value s_j^2 and degrees of freedom d_j. The output from `lmFit()`, `fit` say, holds the s_j in component `fit$sigma` and the d_j in `fit$df.residual`. The covariance matrix of the estimated $\hat{\boldsymbol{\beta}}_j$ is $\sigma_j^2 C^T (X^T V_j X)^{-1} C$ where V_j is a weight matrix determined by prior weights, any covariance terms introduced by correlation structure and any iterative weights introduced by robust estimation. The square-roots of the diagonal elements of $C^T (X^T V_j X)^{-1} C$ are called unscaled standard deviations and are stored in `fit$stdev.unscaled`. The ordinary t-statistic for the kth contrast for gene j is $t_{jk} = \hat{\beta}_{jk}/(u_{jk}s_j)$ where u_{jk} is the unscaled standard deviation. The ordinary t-statistics can be recovered by

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

after fitting a linear model if desired.

The empirical Bayes method assumes an inverse Chisquare prior for the σ_j^2 with mean s_0^2 and degrees of freedom d_0. The posterior values for the residual variances are given by

$$\tilde{s}_j^2 = \frac{d_0 s_0^2 + d_j s_j^2}{d_0 + d_j}$$

where d_j is the residual degrees of freedom for the jth gene. The output from `eBayes()` contains s_0^2 and d_0 as `fit$s2.prior` and `fit$df.prior` and the $\tilde{s}_j^2$ as `fit$s2.post`. The moderated t-statistic is

$$\tilde{t}_{jk} = \frac{\hat{\beta}_{jk}}{u_{jk}\tilde{s}_j}$$

This can be shown to follow a t-distribution on $d_0 + d_j$ degrees of freedom if $\beta_{jk} = 0$ [36]. The extra degrees of freedom f_0 represent the extra information which is borrowed from the ensemble of genes for inference about each individual gene. The output from `eBayes()` contains the $\tilde{t}_{jk}$ as `fit$t` with corresponding p-values in `fit$p.value`.

13.3 Multiple Testing Across Contrasts

The output from `topTable` includes adjusted p-values, i.e., it performs multiple testing for the contrast being considered. If several contrasts are being tested simultaneously, then the issue arises of multiple testing for the entire set of hypotheses being considered, across contrasts as well as probes. The function `decideTests()` offers a number of strategies for doing this.

The simplest multiple testing method is `method="separate"`. This method does multiple testing for each contrast separately. This method is the default because it is equivalent to using `topTable()`. Using this method, testing a set of contrasts together will give the same results as when each contrast is tested on its own. The great advantage of this method is that it gives the same results regardless of which set of contrasts are tested together. The disadvantage of this method is that it does not do any multiple testing adjustment between contrasts. Another disadvantage is that the raw p-value cutoff corresponding to significance can be very different for different contrasts, depending on the number of DE probes. This method is recommended when different contrasts are being analysed to answer more or less independent questions.

`method="global"` is recommended when a set of closely related contrasts are being tested. This method simply appends all the tests together into one long vector of tests, i.e., it treats all the tests as equivalent regardless of which probe or contrast they relate to. An advantage is that the raw p-value cutoff is consistent across all contrasts. For this reason, `method="global"` is recommended if you want to compare the number of DE genes found for different contrasts, for example interpreting the number of DE genes as representing the strength of the contrast. However users need to be aware that the number of DE genes for any particular contrasts will depend on which other contrasts are tested at the same time. Hence one should include only those contrasts which are closely related to the question at hand. Unnecessary contrasts should be excluded as these would affect the results for the contrasts of interest. Another more theoretical issue is that there is no theorem which proves that `adjust.method="BH"` in combination with `method="global"` will correctly control the false discovery rate for combinations of negatively correlated contrasts, however simulations, experience and some theory suggest that the method is safe in practice.

The `"hierarchical"` method offers power advantages when used with `adjust.method="holm"` to control the family-wise error rate. However its properties are not yet well understood with `adjust="BH"`.

`method="nestedF"` has a more specialised aim to give greater weight to probes which are significance for two or more contrasts. Most multiple testing methods tend to underestimate the number of such probes. There is some practical experience to suggest that `method="nestedF"` gives less conservative results when finding probes which respond to several different contrasts at once. However this method should still be viewed as experimental. It provides formal false discovery rate control at the probe level only, not at the contrast level.

Chapter 14

Array Quality Weights

14.1 Introduction

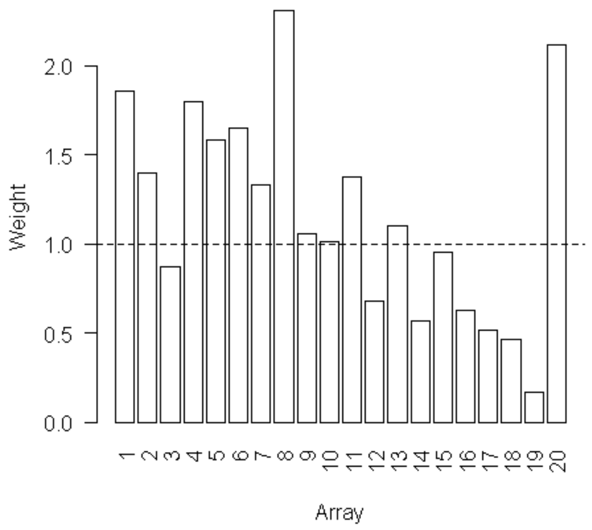
Given an appropriate design matrix, the relative reliability of each array in an experiment can be estimated by measuring how well the expression values for that array follow the linear model. This empirical approach of assessing array quality can be applied to both two-color and single-channel microarray data and is described in [25].

The method is implemented in the `arrayWeights` function, which fits a heteroscedastic model to the expression values for each gene by calling the function `lm.wfit`. (See also `arrayWeightsSimple` which does the same calculation more quickly when there are no probe-level quality weights.) The dispersion model is fitted to the squared residuals from the mean fit, and is set up to have array specific coefficients, which are updated in either full REML scoring iterations, or using an efficient gene-by-gene update algorithm. The final estimates of these array variances are converted to weights which can be used in `lmFit`. This method offers a graduated approach to quality assessment by allowing poorer quality arrays, which would otherwise be discarded, to be included in an analysis but down-weighted.

14.2 Example 1

We consider the array quality weights applied to the spike-in controls from a quality control data set courtesy of Andrew Holloway, Ryan van Laar and Dileepa Diyagama from the Peter MacCallum Cancer Centre in Melbourne. This collection of arrays (described in [25]) consists of 100 replicate hybridizations and we will use data from the first 20 arrays. The object `MAlms` stores the loess normalized data for the 120 spike-in control probes on each array. Since these arrays are replicate hybridizations, the default design matrix of a single column of ones is used.

```
> arrayw <- arrayWeights(MAlms)
> barplot(arrayw, xlab="Array", ylab="Weight", col="white", las=2)
> abline(h=1, lwd=1, lty=2)
```

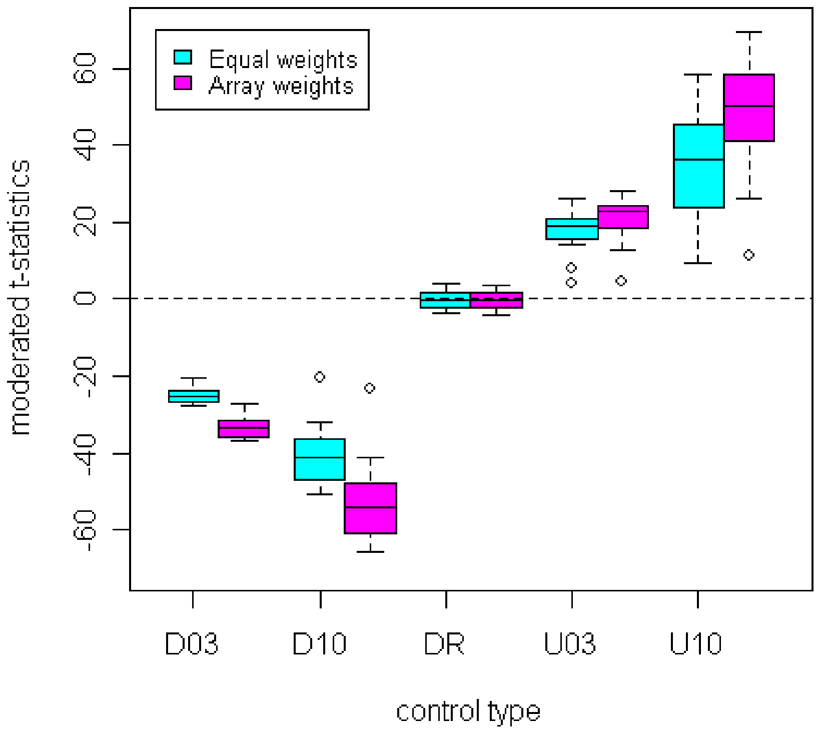


The empirical array weights vary from a minimum of 0.16 for array 19 to a maximum of 2.31 for array 8. These weights can be used in the linear model analysis.

```
> fitw <- lmFit(MAlms, weights=arrayw)
> fitw <- eBayes(fitw)
```

In this example the ratio control spots should show three-fold or ten-fold changes while the dynamic range spots should not be differentially expressed. To compare the moderated t-statistics before and after applying array weights, use the following:

```
> fit <- lmFit(MAlms)
> fit <- eBayes(fit)
> boxplot(fit$t~MAlms$genes$Status, at=1:5-0.2, col=5, boxwex=0.4, xlab="control type",
+        ylab="moderated t-statistics", pch=".", ylim=c(-70, 70), medlwd=1)
> boxplot(fitw$t~MAlms$genes$Status, at=1:5+0.2, col=6, boxwex=0.4,
+       add=TRUE, yaxt="n", xaxt="n", medlwd=1, pch=".")
> abline(h=0, col="black", lty=2, lwd=1)
> legend(0.5, 70, legend=c("Equal weights", "Array weights"), fill=c(5,6), cex=0.8)
```



The boxplots show that the t-statistics for all classes of ratio controls (D03, D10, U03 and U10) move further from zero when array weights are used while the distribution of t-statistics for the dynamic range controls (DR) does not noticeably change. This demonstrates that the array quality weights increase statistical power to detect true differential expression without increasing the false discovery rate.

The same heteroscedastic model can also be fitted at the print-tip group level using the `printtipWeights` function. If there are p print-tip groups across n arrays, the model fitting procedure described in [25] is repeated p times to produce a weight for each print-tip group on each array for use in `lmFit`. This method can be applied to two-color microarray data where the probes are organized into print-tip groups whose size is specified by the printer component of the `MAList`.

14.3 Example 2

Below is an example of applying this method to the Apoa1 data.

```
> ptw <- printtipWeights(MA, design, layout=MA$printer)
> zlim <- c(min(ptw), max(ptw))
> par(mfrow=c(3,2))
> for(i in seq(7,12,by=1))
+        imageplot(ptw[,i], layout=MA$printer, zlim=zlim, main=colnames(MA)[i])
```

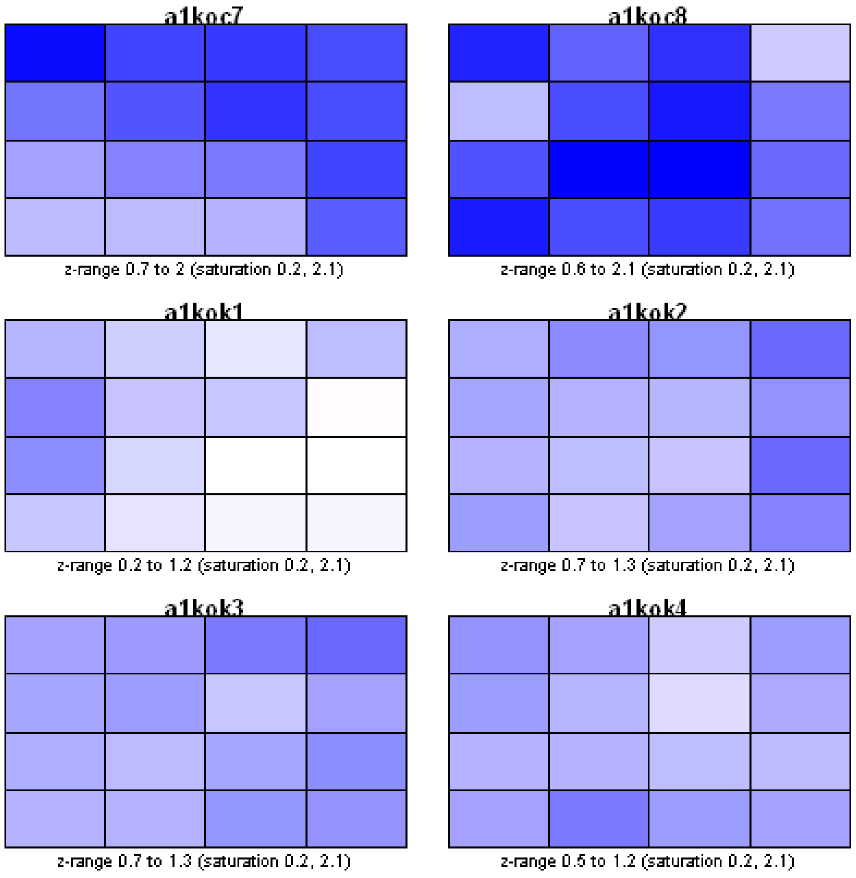


Image plots of the print-tip weights for arrays 7 through to 12 are shown above, with lighter shades indicating print-tip groups which have been assigned lower weights. A corner of array 9 (a1kok1) is measured to be less reproducible than the same region on other arrays, which may be indicative of a spatial artefact. Using these weights in the linear model analysis increases the *t*-statistics of the top ranking genes compared to an analysis without weights (compare the results table below with the table in section 15.2).

```
> fitptw <- lmFit(MA, design, weights=ptw)
> fitptw <- eBayes(fitptw)
> options(digits=3)
> topTable(fitptw,coef=2,number=15,genelist=fitptw$genes$NAME)
                       ID  logFC AveExpr      t  P.Value adj.P.Val       B
2149       ApoAI,lipid-Img -3.151   12.47 -25.64 1.21e-15  7.73e-12 16.4206
540  EST,HighlysimilartoA -2.918   12.28 -14.49 2.22e-11  7.09e-08 12.4699
5356 CATECHOLO-METHYLTRAN -1.873   12.93 -13.16 1.10e-10  2.34e-07 11.5734
4139 EST,WeaklysimilartoC -0.981   12.61 -11.71 7.28e-10  1.16e-06 10.3623
1739     ApoCIII,lipid-Img -0.933   13.74 -10.58 3.66e-09  4.67e-06  9.4155
1496                   est -0.949   12.23  -9.92 9.85e-09  1.05e-05  8.6905
2537 ESTs,Highlysimilarto -1.011   13.63  -9.56 1.75e-08  1.60e-05  8.2587
```

```
4941 similartoyeaststerol -0.873   13.29  -6.88 1.93e-06  1.54e-03  4.6875
947  EST,WeaklysimilartoF -0.566   10.54  -5.08 7.78e-05  5.52e-02  1.6112
2812 5'similartoPIR:S5501 -0.514   11.65  -4.30 4.31e-04  2.75e-01  0.1242
6073          estrogenrec  0.412    9.79   4.21 5.27e-04  3.06e-01 -0.0497
1347 Musmusculustranscrip -0.412   10.18  -4.07 7.09e-04  3.47e-01 -0.3106
634               MDB1376 -0.380    9.32  -4.07 7.11e-04  3.47e-01 -0.3123
2                   Cy5RT  0.673   10.65   4.04 7.61e-04  3.47e-01 -0.3745
5693                Meox2  0.531    9.77   3.84 1.19e-03  4.74e-01 -0.7649
```

For example, the moderated t-statistic of the top ranked gene, Apoa1, which has been knocked-out in this experiment, increases in absolute terms from -23.98 when equal weights are used to -25.64 with print-tip weights. The t-statistic of the related gene ApoCIII also increases in absolute value (moderated t-statistic of -9.83 before weighting and -10.58 after). This analysis provides a further example that a graduated approach to quality control can improve power to detect differentially expressed genes.

14.4 When to Use Array Weights

Array weights are generally useful when there is some reason to expect variable array quality. For example, RNA samples from human clinical patients are typically variable in quality, so array weights might be used routinely with human *in vivo* data, see for example Ellis et al [8]. If array quality plots suggest a problem, then array weights are indicated. If RNA is plentiful, e.g., from cell lines or model organisms, and quality plots of the arrays don't suggest problems, then array weights are usually not needed.

In gross cases where an array is clearly bad or wrong, it should be removed, rather than downweighted. However this action should be reserved for extreme cases.

If most genes are not differentially expressed, then the design matrix for arrayWeights does not need to be as complex as for the final linear model. For example, in a two-group comparison with just 2 replicates in each group, the array weights should be estimated with the default (intercept) design matrix, otherwise each array is compared only to its partner rather than to the other 3 arrays.

Chapter 15

Two-Color Case Studies

15.1 Swirl Zebrafish: A Single-Group 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. The data used for this case study can be downloaded from `http://bioinf.wehi.edu.au/limmaGUI/DataSets.html`.

```
> dir()
[1] "fish.gal"       "swirl.1.spot"    "swirl.2.spot"    "swirl.3.spot"    "swirl.4.spot"
[6] "SwirlSample.txt"
```

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:

```
> library(limma)
> 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, 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 ...
```

```
$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

$source
[1] "spot"
```

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.

Unlike most image analysis software, SPOT does not store probe annotation in the output files, so we have to read it separately. 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
```

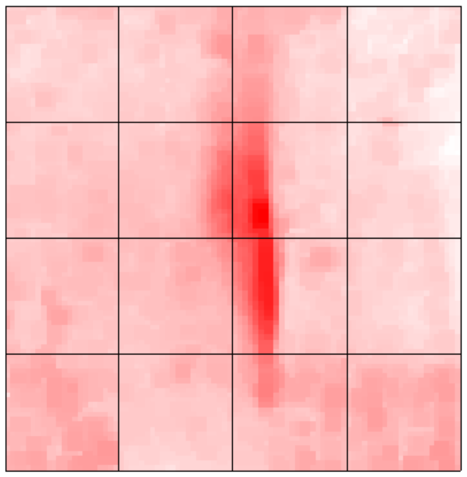
```
29      1   2       5 control ephrinA4
30      1   2       6 control     FGF8
```

Because we are using SPOT output, 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")
```



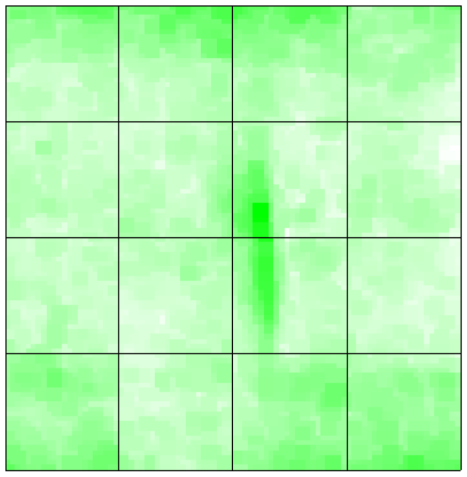
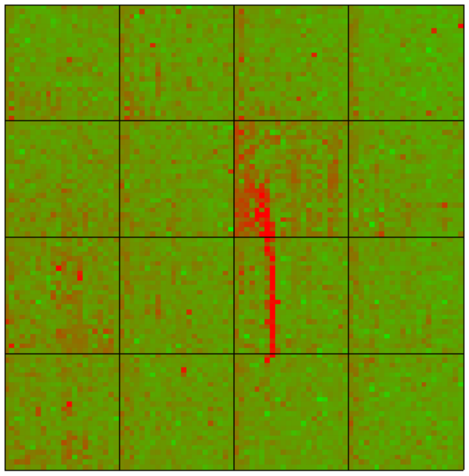


Image plot of the un-normalized log-ratios or M-values for the first array:

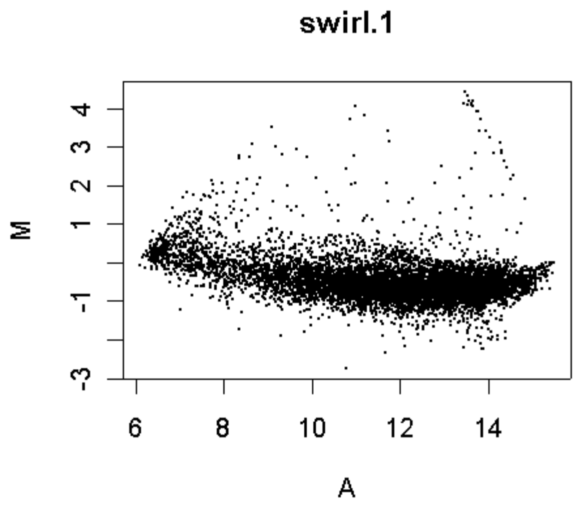
```
> MA <- normalizeWithinArrays(RG, method="none")
> imageplot(MA$M[,1], RG$printer, zlim=c(-3,3))
```



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.

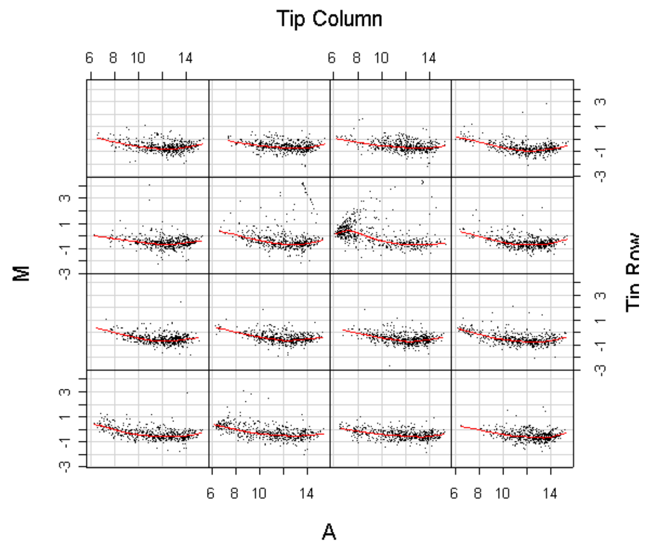
MA-plots. An MA-plot plots the log-ratio of R vs G against the overall intensity of each spot. The log-ratio is represented by the M-value, $M = \log_2(R) - \log_2(G)$, and the overall intensity by the A-value, $A = (\log_2(R) + \log_2(G))/2$. Here is the MA-plot of the un-normalized values for the first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

```
> plotMA(MA)
```



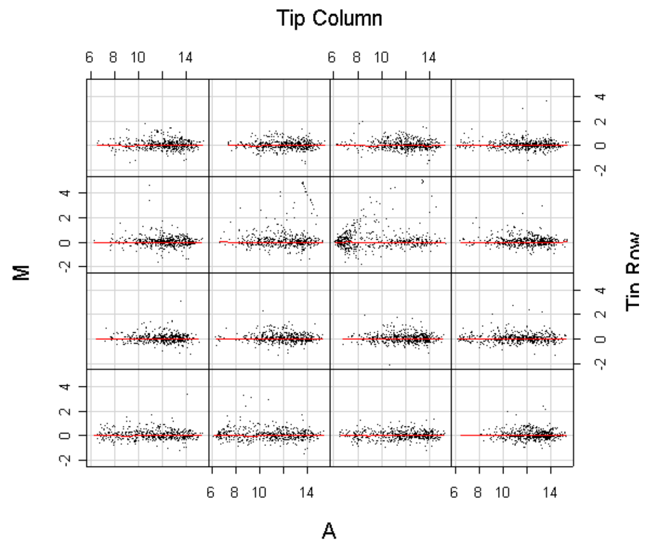
The red streak seen on the image plot can be seen as a line of spots in the upper right of this plot. Now we plot the individual MA-plots for each of the print-tip groups on this array, together with the loess curves which will be used for normalization:

```
> plotPrintTipLoess(MA)
```



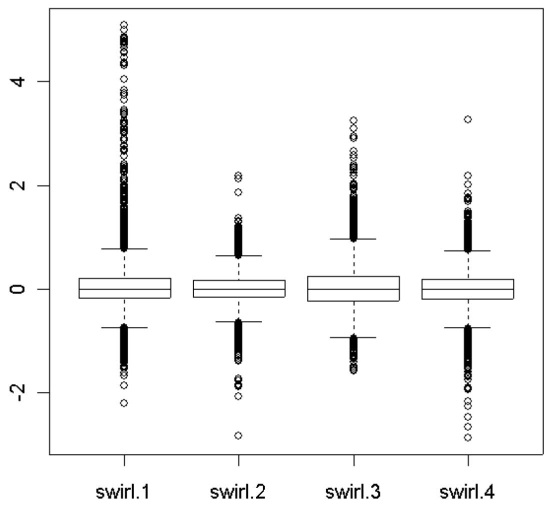
Normalization. Print-tip loess normalization:

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



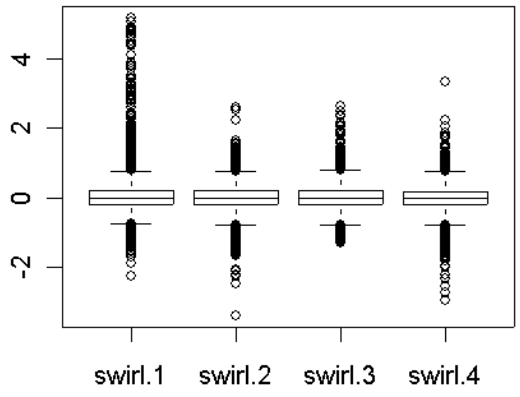
We have normalized the M-values with each array. A further question is whether normalization is required between the arrays. The following plot shows overall boxplots of the M-values for the four arrays.

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



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

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



Note that scale-normalization is not done routinely for all two-color data sets, in fact it is rarely done with newer platforms. However it does give good results on this data set. It should only be done when there is good evidence of a scale difference in the M-values.

Linear model. First setup an appropriate design matrix. The negative numbers in the design matrix indicate the dye-swaps:

```
> design <- modelMatrix(targets, ref="wild type")
Found unique target names:
 swirl wild type
> design
        swirl
swirl.1    -1
swirl.2     1
swirl.3    -1
swirl.4     1
```

Now fit a simple linear model for each gene. This has the effect of estimating the average M-value for each gene, adjusting for the dye-swaps.

```
> fit <- lmFit(MA,design)
> fit
An object of class "MArrayLM"
$coefficients
[1] -0.3556298 -0.3283455 -0.3455845 -0.2254783 -0.3175470
8443 more rows ...

$rank
[1] 1

$assign
NULL

$qr
$qr
     [,1]
[1,]  2.0
[2,] -0.5
[3,]  0.5
[4,] -0.5

$qraux
[1] 1.5

$pivot
[1] 1

$tol
[1] 1e-07

$rank
[1] 1


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

$sigma
```

```
[1] 0.2873571 0.3115307 0.3699258 0.3331054 0.2689609
8443 more elements ...

$cov.coefficients
     [,1]
[1,] 0.25

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

$pivot
[1] 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] 13.44500 13.65700 13.40297 10.71737 10.83383
8443 more elements ...

$method
[1] "ls"

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

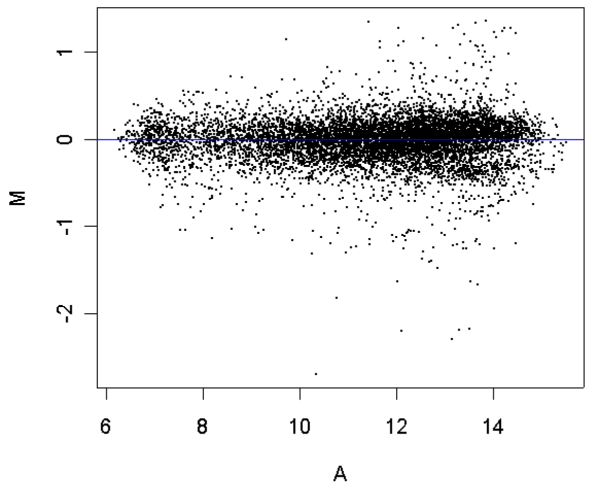
In the above fit object, `coefficients` is the average M-value for each gene and `sigma` is the sample standard deviations for each gene. Ordinary t-statistics for comparing mutant to wt could be computed by

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

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

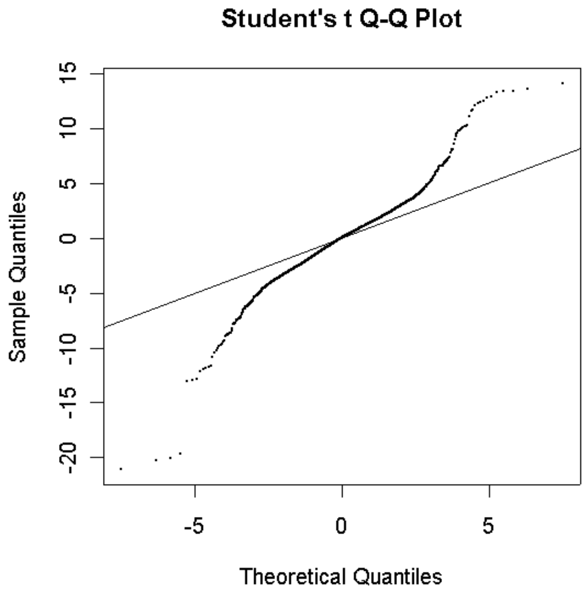
Now create an MA-plot of the average M and A-values for each gene.

```
> plotMA(fit)
> 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)
```



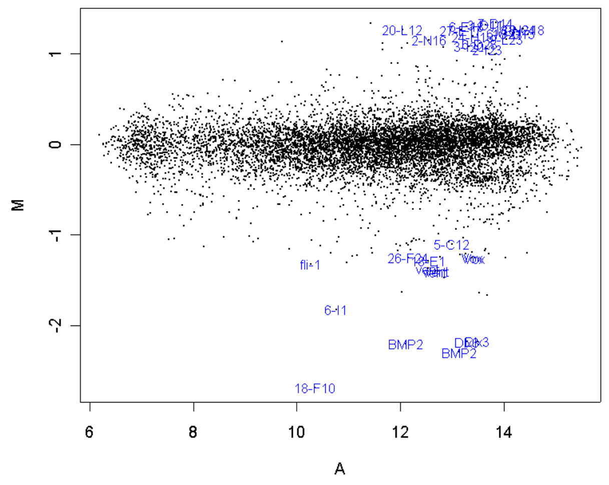
Visually there seems to be plenty of genes which are differentially expressed. We will obtain a summary table of some key statistics for the top genes.

```
> options(digits=3)
> topTable(fit,number=30,adjust="BH")
     Block Row Column      ID   Name logFC AveExpr     t  P.Value adj.P.Val    B
3721     8   2      1 control   BMP2 -2.21    12.1 -21.1 1.03e-07  0.000357 7.96
1609     4   2      1 control   BMP2 -2.30    13.1 -20.3 1.34e-07  0.000357 7.78
3723     8   2      3 control   Dlx3 -2.18    13.3 -20.0 1.48e-07  0.000357 7.71
1611     4   2      3 control   Dlx3 -2.18    13.5 -19.6 1.69e-07  0.000357 7.62
8295    16  16     15 fb94h06 20-L12  1.27    12.0  14.1 1.74e-06  0.002067 5.78
7036    14   8      4 fb40h07  7-D14  1.35    13.8  13.5 2.29e-06  0.002067 5.54
515      1  22     11 fc22a09 27-E17  1.27    13.2  13.4 2.44e-06  0.002067 5.48
5075    10  14     11 fb85f09 18-G18  1.28    14.4  13.4 2.46e-06  0.002067 5.48
7307    14  19     11 fc10h09 24-H18  1.20    13.4  13.2 2.67e-06  0.002067 5.40
319      1  14      7 fb85a01  18-E1 -1.29    12.5 -13.1 2.91e-06  0.002067 5.32
2961     6  14      9 fb85d05 18-F10 -2.69    10.3 -13.0 3.04e-06  0.002067 5.29
4032     8  14     24 fb87d12 18-N24  1.27    14.2  12.8 3.28e-06  0.002067 5.22
6903    14   2     15 control    Vox -1.26    13.4 -12.8 3.35e-06  0.002067 5.20
4546     9  14     10 fb85e07 18-G13  1.23    14.2  12.8 3.42e-06  0.002067 5.18
683      2   7     11 fb37b09  6-E18  1.31    13.3  12.4 4.10e-06  0.002182 5.02
1697     4   5     17 fb26b10  3-I20  1.09    13.3  12.4 4.30e-06  0.002182 4.97
7491    15   5      3 fb24g06  3-D11  1.33    13.6  12.3 4.39e-06  0.002182 4.96
4188     8  21     12 fc18d12 26-F24 -1.25    12.1 -12.2 4.71e-06  0.002209 4.89
4380     9   7     12 fb37e11  6-G21  1.23    14.0  12.0 5.19e-06  0.002216 4.80
3726     8   2      6 control  fli-1 -1.32    10.3 -11.9 5.40e-06  0.002216 4.76
2679     6   2     15 control    Vox -1.25    13.4 -11.9 5.72e-06  0.002216 4.71
5931    12   6      3 fb32f06  5-C12 -1.10    13.0 -11.7 6.24e-06  0.002216 4.63
7602    15   9     18 fb50g12  9-L23  1.16    14.0  11.7 6.25e-06  0.002216 4.63
2151     5   2     15 control   vent -1.40    12.7 -11.7 6.30e-06  0.002216 4.62
3790     8   4     22 fb23d08  2-N16  1.16    12.5  11.6 6.57e-06  0.002221 4.58
7542    15   7      6 fb36g12  6-D23  1.12    13.5  11.0 9.23e-06  0.003000 4.27
4263     9   2     15 control   vent -1.41    12.7 -10.8 1.06e-05  0.003326 4.13
6375    13   2     15 control   vent -1.37    12.5 -10.5 1.33e-05  0.004026 3.91
1146     3   4     18 fb22a12  2-I23  1.05    13.7  10.2 1.57e-05  0.004242 3.76
157      1   7     13 fb38a01   6-I1 -1.82    10.8 -10.2 1.58e-05  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.

```
> plotMA(fit)
> top30 <- order(fit$lods,decreasing=TRUE)[1:30]
> text(fit$Amean[top30],fit$coef[top30],labels=fit$genes[top30,"Name"],cex=0.8,col="blue")
```



15.2 Apoa1 Knockout Mice: A Two-Group Common-Reference 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 `Apoa1.RData`. The data used for this case study can be downloaded from `http://bioinf.wehi.edu.au/limma`.

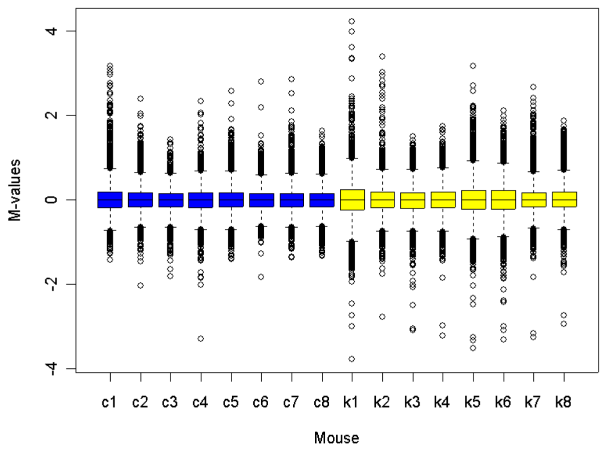
Background. The data is from a study of lipid metabolism by [4]. The apolipoprotein AI (Apoa1) gene is known to play a pivotal role in high density lipoprotein (HDL) metabolism. Mice which have the Apoa1 gene knocked out have very low HDL cholesterol levels. The purpose of this experiment is to determine how Apoa1 deficiency affects the action of other genes in the liver, with the idea that this will help determine the molecular pathways through which Apoa1 operates.

Hybridizations. The experiment compared 8 Apoa1 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	Apoa1 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("Apoa1.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)
```

Normally at this point one would just type

```
> topTable(fit,coef=2)
```

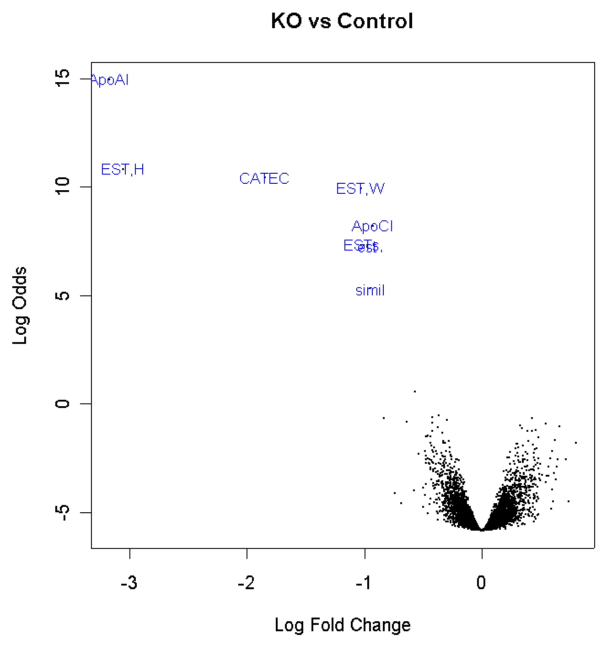
However, the gene annotation is a bit wide for the printed page, so we will tell `topTable()` to show just one column of the annotation information:

```
> topTable(fit,coef=2,number=15,genelist=fit$genes$NAME)
                        ID  logFC AveExpr      t  P.Value adj.P.Val      B
2149      ApoAI,lipid-Img -3.166   12.47 -23.98 4.77e-15  3.05e-11 14.927
540  EST,HighlysimilartoA -3.049   12.28 -12.96 1.57e-10  5.02e-07 10.813
5356 CATECHOLO-METHYLTRAN -1.848   12.93 -12.44 3.06e-10  6.51e-07 10.448
4139 EST,WeaklysimilartoC -1.027   12.61 -11.76 7.58e-10  1.21e-06  9.929
```

```
1739    ApoCIII,lipid-Img -0.933   13.74  -9.84 1.22e-08  1.56e-05  8.192
2537 ESTs,Highlysimilarto -1.010   13.63  -9.02 4.53e-08  4.22e-05  7.305
1496                  est -0.977   12.23  -9.00 4.63e-08  4.22e-05  7.290
4941 similartoyeaststerol -0.955   13.29  -7.44 7.04e-07  5.62e-04  5.311
947  EST,WeaklysimilartoF -0.571   10.54  -4.55 2.49e-04  1.77e-01  0.563
5604                      -0.366   12.71  -3.96 9.22e-04  5.29e-01 -0.553
4140          APXL2,5q-Img -0.420    9.79  -3.93 9.96e-04  5.29e-01 -0.619
6073          estrogenrec  0.421    9.79   3.91 1.03e-03  5.29e-01 -0.652
1337 psoriasis-associated -0.838   11.66  -3.89 1.08e-03  5.29e-01 -0.687
954    Caspase7,heart-Img -0.302   12.14  -3.86 1.17e-03  5.30e-01 -0.757
563  FATTYACID-BINDINGPRO -0.637   11.62  -3.81 1.29e-03  5.30e-01 -0.839
```

Notice that the top gene is Apoa1 itself which is heavily down-regulated. Theoretically the M-value should be minus infinity for Apoa1 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.

```
> volcanoplot(fit,coef=2,highlight=8,names=fit$genes$NAME,main="KO vs Control")
```



15.3 Weaver Mutant Mice: A Composite 2x2 Factorial Experiment

15.3.1 Background

This case study considers a more involved two-color analysis in which the RNA sources have a factorial structure with two factors.

The study examined the development of neurons in wild-type and weaver mutant mice [6]. The weaver mutation 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.

15.3.2 Sample Preparation and Hybridizations

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 aliquots are technical replicates, arising from the same mice and RNA extraction. In our analysis here, we will ignore this complication and will instead treat the aliquots as if they were biological replicates. See Yang and Speed (2002) for a detailed discussion of this issue in the context of this experiment.) A pool of RNA was also made by combining the different RNA samples.

There are four different treatment combinations, P11wt, P11mt, P21wt and P21mt, comprising a 2x2 factorial structure. The RNA samples were hybridized to ten two-color microarrays, spotted with a 20k Riken clone library. There are six arrays comparing the four different RNA sources to the RNA pool, and four arrays making direct comparisons between the four treatment combinations.

The microarray images were scanned using SPOT image analysis software.

15.3.3 Data input

The data used for this case study can be downloaded from `http://bioinf.wehi.edu.au/limma/data/weaverfull.rar`. The data are provided courtesy of Drs Jean Yang and Elva Diaz.

First read in the targets frame:

```
> library(limma)
> targets <- readTargets("targets.txt")
> rownames(targets) <- removeExt(targets$FileName)
> targets
             FileName     Tissue  Mouse   Cy5   Cy3
cbmut.3   cbmut.3.spot Cerebellum Weaver P11wt  Pool
cbmut.4   cbmut.4.spot Cerebellum Weaver P11mt  Pool
cbmut.5   cbmut.5.spot Cerebellum Weaver P21mt  Pool
cbmut.6   cbmut.6.spot Cerebellum Weaver P21wt  Pool
cbmut.15 cbmut.15.spot Cerebellum Weaver P21wt  Pool
cbmut.16 cbmut.16.spot Cerebellum Weaver P21mt  Pool
cb.1         cb.1.spot Cerebellum Weaver P11wt P11mt
cb.2         cb.2.spot Cerebellum Weaver P11mt P21mt
cb.3         cb.3.spot Cerebellum Weaver P21mt P21wt
cb.4         cb.4.spot Cerebellum Weaver P21wt P11wt
```

Exploratory analysis showed that the segmented area for spots for these arrays was quite variable, with a median spot area just over 50 pixels. A small proportion of spots had very small segmented sizes, suggesting that the intensities for these spots might be unreliable. It was therefore decided to set a spot quality weight function, so any spot with an area less than 50 pixels will get reduced weight. The function is set so that any spot with zero area will get zero weight:

```
> wtfun <- function(x) pmin(x$area/50, 1)
```

Then read the SPOT files containing the intensity data using file names recorded in the targets file. The data files are stored in the subdirectory `/spot`:

```
> RG <- read.maimages(targets, source = "spot", path = "spot", wt.fun = wtfun)
Read spot/cbmut.3.spot
Read spot/cbmut.4.spot
Read spot/cbmut.5.spot
Read spot/cbmut.6.spot
Read spot/cbmut.15.spot
Read spot/cbmut.16.spot
Read spot/cb.1.spot
Read spot/cb.2.spot
Read spot/cb.3.spot
Read spot/cb.4.spot
```

Finally, we set the print-tip layout. These arrays were printed using a print-head with 8 rows and 4 columns of print tips:

```
> RG$printer <- list(ngrid.r = 8, ngrid.c = 4, nspot.r = 25, nspot.c = 24)
```

15.3.4 Annotation

Probe annotation is contained a separate file. The rows in the annotation file are as for the intensity data. Columns give Riken chip rearray IDs, GenBank accession numbers and UniGene information.

```
> Annotation <- read.delim("091701RikenUpdatev3.txt", comment.char="", quote="\"",
+                           check.names=FALSE, stringsAsFactors=FALSE)
> names(Annotation)
[1] "ReArrayID"                 "Accession #, GenBank"      "description (Riken)"
[4] "Cluster ID (UniGene)"      "2nd description (UniGene)"
```

For our purposes, we will keep the Riken IDs and GenBank accessions, putting these into the data object:

```
> RG$genes <- Annotation[,c(1,2)]
> colnames(RG$genes) <- c("RikenID","GenBank")
```

Where possible, we find gene symbols corresponding to the GenBank accession numbers, by using the mouse organism package constructed from the NCBI database. Symbols can be found for only a little over 5000 of the probes.

```
> library(org.Mm.eg.db)
```

First we find the Entrez Gene ID for each accession number:

```
> EG.AN <- toTable(org.Mm.egACCNUM)
> i <- match(RG$genes$GenBank, EG.AN[,"accession"])
> EntrezID <- EG.AN[i,"gene_id"]
```

Then convert Entrez Gene IDs to symbols:

```
> EG.Sym <- toTable(org.Mm.egSYMBOL)
> i <- match(EntrezID, EG.Sym[,"gene_id"])
> RG$genes$Symbol <- EG.Sym[i,"symbol"]
```

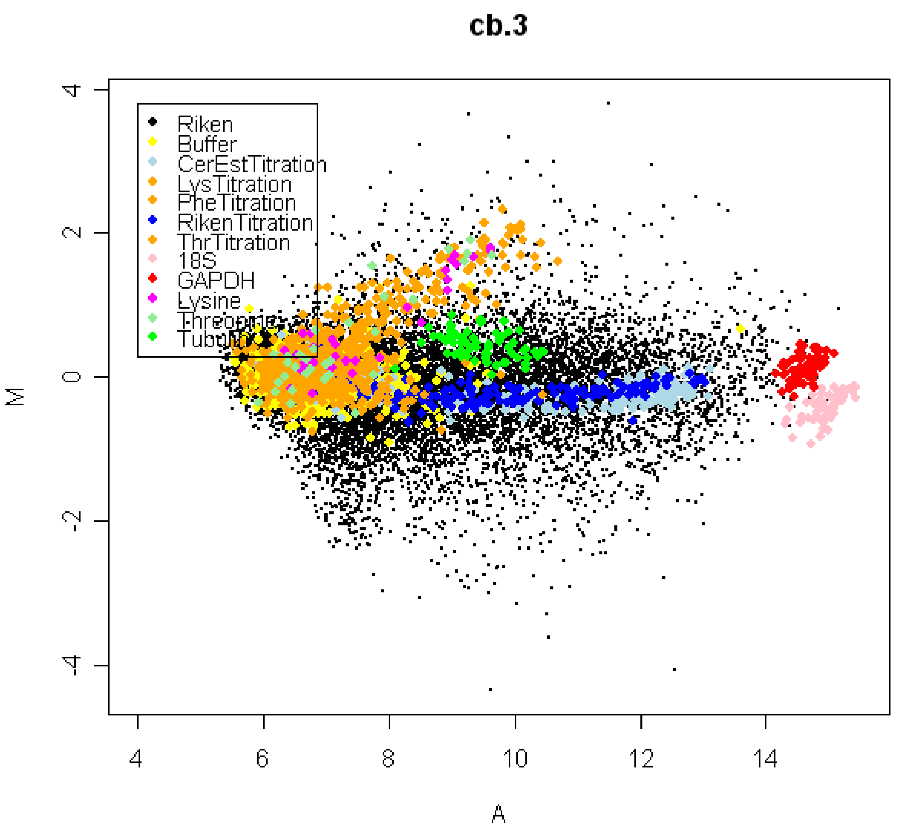
15.3.5 Quality Assessment and Normalization

We also read in a spot-types file and set a range of control spots.

```
> spottypes <- readSpotTypes("spottypes.txt")
> spottypes
          SpotType                  RikenID        col cex
1          Control                        *      green 1.0
2            Riken                       Z*      black 0.2
3           Buffer                  3x SSC     yellow 1.0
4  CerEstTitration             cer est \\(*  lightblue 1.0
5     LysTitration                 Lys \\(*     orange 1.0
6     PheTitration                 Phe \\(*     orange 1.0
7   RikenTitration           Riken est \\(*       blue 1.0
8     ThrTitration                 Thr \\(*     orange 1.0
9              18S      18S \\(0.15ug/ul\\)       pink 1.0
10           GAPDH   GAPDH \\(0.15 ug/ul\\)        red 1.0
11          Lysine   Lysine \\(0.2 ug/ul\\)    magenta 1.0
12       Threonine Threonine \\(0.2ug/ul\\) lightgreen 1.0
13         Tubulin Tubulin \\(0.15 ug/ul\\)      green 1.0
> RG$genes$Status <- controlStatus(spottypes, RG)
Matching patterns for: RikenID
Found 19200 Control
Found 16896 Riken
Found 710 Buffer
Found 192 CerEstTitration
Found 224 LysTitration
Found 260 PheTitration
Found 160 RikenTitration
Found 224 ThrTitration
Found 64 18S
Found 64 GAPDH
Found 32 Lysine
Found 32 Threonine
Found 64 Tubulin
Setting attributes: values col cex
```

MA-plots were examined for all the arrays. Here we give the plot for array 9 only:

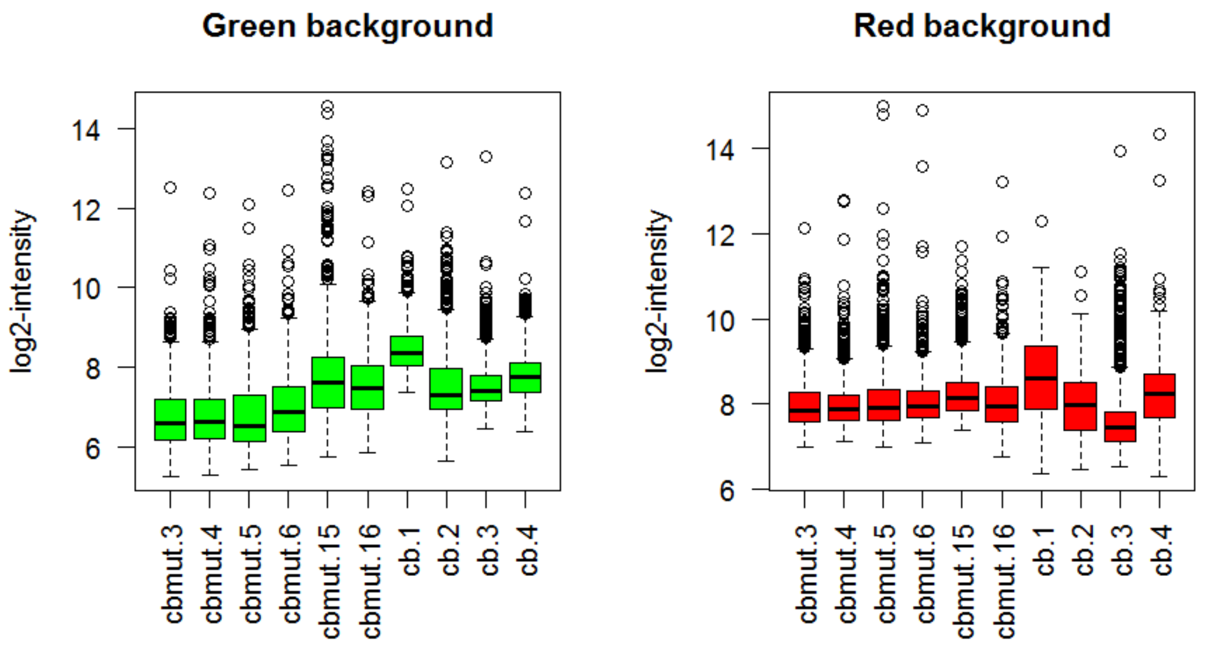
```
> plotMA(RG,array=9,xlim=c(4,15.5))
```



Here Buffer is an obvious negative control while 18S, GAPDH, Lysine, Threonine and Tubulin are single-gene positive controls, sometime called house-keeping genes. RikenTitration is a titration series of a pool of the entire Riken library, and can be reasonably expected to be non-differentially expressed. CerEstTitration is a titration of a pool of a cerebellum EST library. This will show higher expression in later mutant tissues. The Lys, Phe and Thr series are single-gene titration series which were not spiked-in in this case and can therefore be treated as negative controls.

The negative control probe intensities are quite high, especially for the red channel and especially for array 7:

```
> negative <- RG$genes$Status %in% c("Buffer","LysTitration","PheTitration","ThrTitration")
> par(mfrow=c(1,2))
> boxplot(log2(RG$G[negative,]),las=2,main="Green background",ylab="log2-intensity",col="green")
> boxplot(log2(RG$R[negative,]),las=2,main="Red background",ylab="log2-intensity",col="red")
> par(mfrow=c(1,1))
```



Later on, we will investigate setting array quality weights.

Now normalize the data. The Riken titration library, being based on a pool of a large number of non-specific genes, should not be differentially expressed. We can take advantage of this by upweighting these probes in the print-tip normalization step. Here we give double-weight to the titration library probes, although higher weights could also be considered:

```
> w <- modifyWeights(RG$weights, RG$genes$Status, "RikenTitration", 2)
> MA <- normalizeWithinArrays(RG, weights = w)
```

15.3.6 Setting Up the Linear Model

The experiment has a composite design, with some arrays comparing back to the RNA pool as a common reference, and other arrays making direct comparisons between the treatment conditions of interest. The simplest design matrix is that which compares all the RNA samples back to the RNA pool.

```
> design <- modelMatrix(targets, ref = "Pool")
Found unique target names:
 P11mt P11wt P21mt P21wt Pool
```

We also add an intercept term to extract probe-specific dye effects:

```
> design <- cbind(Dye=1,design)
> design
         Dye P11mt P11wt P21mt P21wt
cbmut.3    1     0     1     0     0
cbmut.4    1     1     0     0     0
cbmut.5    1     0     0     1     0
cbmut.6    1     0     0     0     1
cbmut.15   1     0     0     0     1
cbmut.16   1     0     0     1     0
cb.1       1    -1     1     0     0
cb.2       1     1     0    -1     0
cb.3       1     0     0     1    -1
cb.4       1     0    -1     0     1
```

15.3.7 Probe Filtering and Array Quality Weights

First we remove control probes, leaving only the regular probes of the Riken library:

```
> regular <- MA$genes$Status=="Riken"
> MA2 <- MA[regular,]
> MA2$genes$Status <- NULL
```

Then we estimate array quality weights:

```
> aw <- arrayWeights(MA2,design)
> options(digits=3)
> aw
    1     2     3     4     5     6     7     8     9    10
1.175 1.457 0.852 1.216 0.371 1.087 1.325 0.856 1.418 0.869
```

The array weights multiply the spot weights already in the data object:

```
> library(statmod)
> w <- matvec(MA2$weights,aw)
```

15.3.8 Differential expression

Fit the linear model:

```
> fit <- lmFit(MA2, design, weights=w)
```

Now extract all possible comparisons of interest as contrasts. We look for the mutant vs wt comparisons at 11 and 21 days, the time effects for mutant and wt, and the interaction terms:

```
> cont.matrix <- makeContrasts(
+     WT11.MT11=P11mt-P11wt,
+     WT21.MT21=P21mt-P21wt,
+     WT11.WT21=P21wt-P11wt,
+     MT11.MT21=P21mt-P11mt,
+     Int=(P21mt-P11mt)-(P21wt-P11wt),
+     levels=design)
> fit2 <- contrasts.fit(fit, cont.matrix)
> fit2 <- eBayes(fit2)
```

Adjustment for multiple testing, using Benjamini and Hochberg's method to control the false discovery rate at 5% across all genes and all contrasts, leads to the following:

```
> results <- decideTests(fit2,method="global")
> summary(results)
   WT11.MT11 WT21.MT21 WT11.WT21 MT11.MT21   Int
-1        28       136       455       765    74
0      16835     16540     16102     15377 16692
1         33       220       339       754   130
```

The probes that show significant interactions are those which develop differently in the mutant compared to the wildtype between days 11 and 21. To see these:

```
> topTable(fit2,coef="Int")
         RikenID  GenBank    Symbol logFC AveExpr     t  P.Value adj.P.Val    B
14395 ZX00005I07 AV038977      <NA>  2.71   10.52 11.40 8.56e-08   0.00145 7.66
1473  ZX00028J17 AV076735      <NA>  1.92   11.37 10.11 3.18e-07   0.00215 6.65
14288 ZA00003I15 AV010442      <NA>  2.21    9.50  9.94 3.81e-07   0.00215 6.50
1184  ZX00004J09 AK005530 Tnfrsf12a -2.02    8.47 -9.43 6.74e-07   0.00237 6.03
13843 ZX00003J07 AV033559      <NA> -2.97   12.08 -9.33 7.51e-07   0.00237 5.95
14898 ZX00003H24 AK005382 Tnfrsf12a -2.41    9.40 -9.23 8.43e-07   0.00237 5.86
13520 ZX00020K15 AV088030      <NA>  1.83    9.93  9.09 9.88e-07   0.00238 5.73
10916 ZX00023L14 AV104464      <NA>  2.90   10.49  8.93 1.19e-06   0.00252 5.37
12532 ZX00026E06 AV140268      <NA>  2.75   10.55  8.63 1.71e-06   0.00322 5.27
14250 ZX00048F23 AV122249      <NA>  1.89   10.27  8.41 2.23e-06   0.00377 5.04

> sessionInfo()
R version 2.13.0 Patched (2011-04-25 r55638)
Platform: i386-pc-mingw32/i386 (32-bit)

locale:
[1] LC_COLLATE=English_Australia.1252  LC_CTYPE=English_Australia.1252
[3] LC_MONETARY=English_Australia.1252 LC_NUMERIC=C
[5] LC_TIME=English_Australia.1252

attached base packages:
[1] splines   stats     graphics  grDevices utils     datasets  methods   base

other attached packages:
[1] statmod_1.4.11       org.Mm.eg.db_2.5.0   RSQLite_0.9-4        DBI_0.2-5
[5] AnnotationDbi_1.14.0 Biobase_2.12.0       limma_3.9.8
```

15.4 Bob1 Mutant Mice: Arrays With Duplicate 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 [33]. 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.

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

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 Bob1 (aka Pou2af1 or 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 at the Australian Genome Research Facility 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, side by side by rows. The NIA15k probe IDs have been anonymized in the output presented here.

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.

Image analysis. The arrays were image analyzed using SPOT with "morph" background estimation.

The data used for this case study can be downloaded from `http://bioinf.wehi.edu.au/limma/`. The file should be placed in the working directory of your R session. (This case study was last updated on 29 June 2006 using R 2.3.0 and limma 2.7.5.)

```
> library(limma)
> load("Bob.RData")
> objects()
[1] "design" "RG"
> design
[1] -1  1 -1  1
> names(RG)
[1] "R"       "G"       "Rb"      "Gb"      "genes"   "printer"
> RG$genes[1:40,]
   Library             ID
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. The SPOT morph background ensures that the default background subtraction can be used without inducing negative intensities.

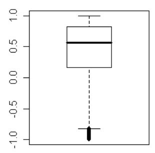
```
> MA <- normalizeWithinArrays(RG)
```

Then remove the control probes:

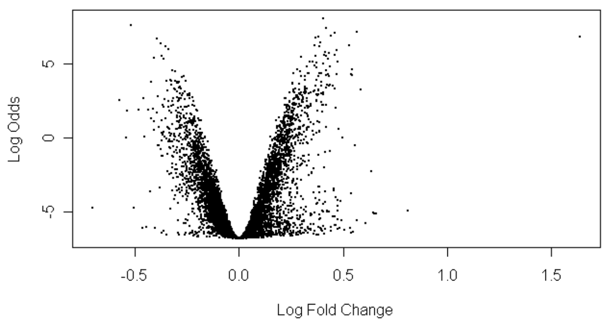
```
> MA2 <- MA[MA$genes$Library=="NIA15k", ]
```

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 will probably take at least a few minutes depending on the speed of your computer.

```
> options(digits=3)
> corfit <- duplicateCorrelation(MA2,design,ndups=2) # A slow computation!
Loading required package: statmod
> corfit$consensus.correlation
[1] 0.575
> boxplot(tanh(corfit$atanh.correlations))
```



```
> fit <- lmFit(MA2,design,ndups=2,correlation=corfit$consensus)
> fit <- eBayes(fit)
> topTable(fit,n=30,adjust="BH")
     Library     ID  logFC AveExpr      t  P.Value adj.P.Val    B
4599  NIA15k H34599  0.404   10.93  12.92 1.34e-07  0.000443 8.02
1324  NIA15k H31324 -0.520    7.73 -12.24 2.23e-07  0.000443 7.57
3309  NIA15k H33309  0.420   10.99  11.99 2.71e-07  0.000443 7.39
440   NIA15k  H3440  0.568    9.96  11.64 3.60e-07  0.000443 7.13
6795  NIA15k H36795  0.460   10.78  11.56 3.85e-07  0.000443 7.07
121   NIA15k  H3121  0.441   10.48  11.31 4.73e-07  0.000443 6.88
2838  NIA15k H32838  1.640   12.74  11.26 4.92e-07  0.000443 6.84
6999  NIA15k H36999  0.381    9.91  11.19 5.21e-07  0.000443 6.79
132   NIA15k  H3132  0.370   10.10  11.17 5.31e-07  0.000443 6.77
6207  NIA15k H36207 -0.393    8.53 -11.06 5.82e-07  0.000443 6.69
7168  NIA15k H37168  0.391    9.88  10.76 7.51e-07  0.000493 6.45
1831  NIA15k H31831 -0.374    9.62 -10.63 8.41e-07  0.000493 6.35
2014  NIA15k H32014  0.363    9.65  10.49 9.54e-07  0.000493 6.23
7558  NIA15k H37558  0.532   11.42  10.49 9.58e-07  0.000493 6.22
4471  NIA15k H34471 -0.353    8.76 -10.41 1.02e-06  0.000493 6.16
126   NIA15k  H3126  0.385   10.59  10.40 1.03e-06  0.000493 6.15
4360  NIA15k H34360 -0.341    9.37 -10.22 1.21e-06  0.000545 6.00
6794  NIA15k H36794  0.472   11.33  10.11 1.35e-06  0.000570 5.90
329   NIA15k  H3329  0.413   11.37   9.97 1.53e-06  0.000612 5.78
5017  NIA15k H35017  0.434   11.41   9.90 1.63e-06  0.000618 5.72
2678  NIA15k H32678  0.461   10.44   9.74 1.90e-06  0.000618 5.57
2367  NIA15k H32367  0.409   10.21   9.72 1.93e-06  0.000618 5.56
1232  NIA15k H31232 -0.372    8.72  -9.70 1.96e-06  0.000618 5.54
111   NIA15k  H3111  0.369   10.42   9.69 1.98e-06  0.000618 5.53
2159  NIA15k H32159  0.418   10.19   9.67 2.03e-06  0.000618 5.51
4258  NIA15k H34258  0.299    9.11   9.62 2.12e-06  0.000622 5.47
3192  NIA15k H33192 -0.410    9.46  -9.55 2.26e-06  0.000638 5.41
6025  NIA15k H36025  0.427   10.37   9.47 2.45e-06  0.000654 5.33
5961  NIA15k H35961 -0.362    8.50  -9.46 2.49e-06  0.000654 5.31
1404  NIA15k H31404  0.474   11.34   9.26 3.00e-06  0.000722 5.13
> volcanoplot(fit)
```


Log Odds
5
0
-5
-0.5
0.0
0.5
1.0
1.5
Log Fold Change

Chapter 16

Single-Channel Case Studies

16.1 Lrp Mutant Ecoli: Two Group Experiment with Affymetrix Arrays

The data are from experiments reported in [10] 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 [10] 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 that 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.

```
> 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. The package ecolicdf must also be installed, otherwise the `rma()` function will attempt to download and install it for you—without giving you to opportunity to veto the download.

```
> 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.
> 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 $\log_2$-expression of each gene in the lrp- strain. The second coefficient measures the $\log_2$-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="BH")
                                ID logFC AveExpr     t P.Value adj.P.Val     B
4282  IG_821_1300838_1300922_fwd_st -3.32    12.4 -23.1 7.2e-09   5.3e-05 8.017
5365                serA_b2913_st  2.78    12.2  15.8 1.6e-07   6.0e-04 6.603
1389                gltD_b3213_st  3.03    10.9  13.3 6.4e-07   1.6e-03 5.779
4625                 lrp_b0889_st  2.30     9.3  11.4 2.3e-06   4.0e-03 4.911
```

```
1388                  gltB_b3212_st  3.24    10.1  11.1 2.8e-06   4.0e-03  4.766
4609                  livK_b3458_st  2.35     9.9  10.8 3.5e-06   4.0e-03  4.593
4901                  oppB_b1244_st -2.91    10.7 -10.6 4.0e-06   4.0e-03  4.504
4903                  oppD_b1246_st -1.94    10.4 -10.5 4.4e-06   4.0e-03  4.434
5413                  sodA_b3908_st  1.50    10.3   9.7 8.0e-06   6.5e-03  3.958
4900                  oppA_b1243_st -2.98    13.0  -9.1 1.3e-05   9.2e-03  3.601
5217                   rmf_b0953_st -2.71    13.6  -9.0 1.5e-05   9.3e-03  3.474
7300                  ytfK_b4217_st -2.64    11.1  -8.9 1.5e-05   9.3e-03  3.437
5007                  pntA_b1603_st  1.58    10.1   8.3 2.5e-05   1.4e-02  3.019
4281   IG_820_1298469_1299205_fwd_st -2.45    10.7  -8.1 3.1e-05   1.6e-02  2.843
4491                  ilvI_b0077_st  0.95    10.0   7.4 6.3e-05   2.9e-02  2.226
5448                  stpA_b2669_st  1.79    10.0   7.4 6.4e-05   2.9e-02  2.210
611                        b2343_st -2.12    10.8  -7.1 7.9e-05   3.4e-02  2.028
5930                  ybfA_b0699_st -0.91    10.5  -7.0 8.7e-05   3.5e-02  1.932
1435                  grxB_b1064_st -0.91     9.8  -6.9 1.0e-04   3.8e-02  1.810
4634                  lysU_b4129_st -3.30     9.3  -6.9 1.1e-04   3.9e-02  1.758
4829                   ndk_b2518_st  1.07    11.1   6.7 1.2e-04   4.3e-02  1.616
2309 IG_1643_2642304_2642452_rev_st  0.83     9.6   6.7 1.3e-04   4.3e-02  1.570
4902                  oppC_b1245_st -2.15    10.7  -6.3 1.9e-04   5.9e-02  1.238
4490                  ilvH_b0078_st  1.11     9.9   5.9 2.9e-04   8.8e-02  0.820
1178                  fimA_b4314_st  3.40    11.7   5.9 3.2e-04   8.8e-02  0.743
6224                  ydgR_b1634_st -2.35     9.8  -5.8 3.3e-04   8.8e-02  0.722
4904                  oppF_b1247_st -1.46     9.9  -5.8 3.3e-04   8.8e-02  0.720
792                        b3914_st -0.77     9.5  -5.7 3.9e-04   1.0e-01  0.565
5008                  pntB_b1602_st  1.47    12.8   5.6 4.1e-04   1.0e-01  0.496
4610                  livM_b3456_st  1.04     8.5   5.5 4.7e-04   1.1e-01  0.376
5097                  ptsG_b1101_st  1.16    12.2   5.5 4.8e-04   1.1e-01  0.352
4886                  nupC_b2393_st  0.79     9.6   5.5 4.9e-04   1.1e-01  0.333
4898                  ompT_b0565_st  2.67    10.5   5.4 5.6e-04   1.2e-01  0.218
5482                   tdh_b3616_st -1.61    10.5  -5.3 6.3e-04   1.3e-01  0.092
1927        IG_13_14080_14167_fwd_st -0.55     8.4  -5.3 6.4e-04   1.3e-01  0.076
6320                  yeeF_b2014_st  0.88     9.9   5.3 6.5e-04   1.3e-01  0.065
196                   atpG_b3733_st  0.60    12.5   5.2 7.2e-04   1.4e-01 -0.033
954                   cydB_b0734_st -0.76    11.0  -5.0 9.3e-04   1.8e-01 -0.272
1186                  fimI_b4315_st  1.15     8.3   5.0 9.5e-04   1.8e-01 -0.298
4013     IG_58_107475_107629_fwd_st -0.49    10.4  -4.9 1.1e-03   2.0e-01 -0.407
```

The column `logFC` gives the $\log_2$-fold change while the column `AveExpr` gives the average $\log_2$-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 [10]. 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.

16.2 Effect of Estrogen on Breast Cancer Tumor Cells: A 2x2 Factorial Experiment with Affymetrix Arrays

This data is from the estrogen package on Bioconductor. A subset of the data is also analyzed 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 targets 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=targets$filename, celfile.path=datadir)
> eset <- rma(ab)
Background correcting
Normalizing
Calculating Expression
```

By default, the only probe-set annotation contained in `eset` is the Affymetrix ID number. We will add gene symbols to the data object now, so that they will be automatically included in limma results tables later on.

```
> library(annotate)
Loading required package: AnnotationDbi
> library(hgu95av2.db)
Loading required package: org.Hs.eg.db
Loading required package: DBI
> ID <- featureNames(eset)
> Symbol <- getSYMBOL(ID,"hgu95av2.db")
> fData(eset) <- data.frame(Symbol=Symbol)
```

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 $\log_2$-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 way to decide which changes are significant for each gene would be to use Benjamini and Hochberg's method to control the false discovery rate across all the genes and both tests:

```
> results <- decideTests(fit2, method="global")
```

Another method would be to adjust the F-test p-values rather than the t-test p-values:

```
> results <- decideTests(fit2, method="nestedF")
```

Here we use a more conservative method which depends far less on distributional assumptions, which is to make use of control and spike-in probe-sets which theoretically should not be differentially-expressed. The smallest p-value amongst these controls turns out to be about 0.00014:

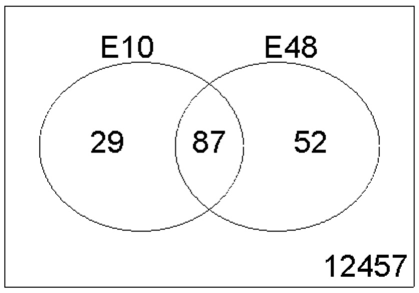
```
> i <- grep("AFFX",featureNames(eset))
> summary(fit2$F.p.value[i])
     Min.   1st Qu.    Median      Mean   3rd Qu.      Max.
0.0001391 0.1727000 0.3562000 0.4206000 0.6825000 0.9925000
```

So a cutoff p-value of 0.0001, say, would conservatively avoid selecting any of the control probe-sets as differentially expressed:

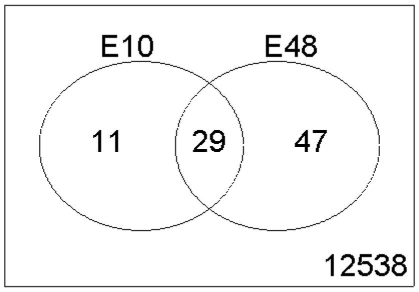
```
> results <- classifyTestsF(fit2, p.value=0.0001)
> summary(results)
   E10   E48
-1    40    76
0  12469 12410
1    116   139

> 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. Estrogen effect at 10 hours:

```
> options(digits=3)
> topTable(fit2,coef="E10",n=20)
             Symbol logFC AveExpr     t  P.Value adj.P.Val    B
39642_at     ELOVL2  2.94    7.88  23.7 4.74e-09  3.13e-05 9.97
910_at          TK1  3.11    9.66  23.6 4.96e-09  3.13e-05 9.94
31798_at       TFF1  2.80   12.12  16.4 1.03e-07  3.51e-04 7.98
41400_at        TK1  2.38   10.04  16.2 1.11e-07  3.51e-04 7.92
40117_at       MCM6  2.56    9.68  15.7 1.47e-07  3.58e-04 7.71
1854_at      MYBL2  2.51    8.53  15.2 1.95e-07  3.58e-04 7.49
39755_at       XBP1  1.68   12.13  15.1 2.05e-07  3.58e-04 7.45
1824_s_at      PCNA  1.91    9.24  14.9 2.27e-07  3.58e-04 7.37
1126_s_at      CD44  1.78    6.88  13.8 4.12e-07  5.78e-04 6.89
1536_at        CDC6  2.66    5.94  13.3 5.80e-07  7.32e-04 6.61
981_at         MCM4  1.82    7.78  13.1 6.46e-07  7.42e-04 6.52
33252_at       MCM3  1.74    8.00  12.6 8.86e-07  9.20e-04 6.25
1505_at        TYMS  2.40    8.76  12.5 9.48e-07  9.20e-04 6.19
34363_at      SEPP1 -1.75    5.55 -12.2 1.14e-06  1.03e-03 6.03
1884_s_at      PCNA  2.80    9.03  12.1 1.26e-06  1.06e-03 5.95
36134_at      OLFM1  2.49    8.28  11.8 1.50e-06  1.19e-03 5.79
37485_at    SLC27A2  1.61    6.67  11.4 1.99e-06  1.48e-03 5.55
239_at         CTSD  1.57   11.25  10.4 4.07e-06  2.66e-03 4.90
38116_at   KIAA0101  2.32    9.51  10.4 4.09e-06  2.66e-03 4.90
40533_at      BIRC5  1.26    8.47  10.4 4.21e-06  2.66e-03 4.87
```

Estrogen effect at 48 hours:

```
> topTable(fit2,coef="E48",n=20)
             Symbol logFC AveExpr     t  P.Value adj.P.Val     B
910_at          TK1  3.86    9.66  29.2 8.27e-10  1.04e-05 11.61
```

```
31798_at      TFF1  3.60   12.12  21.0 1.28e-08  7.63e-05  9.89
1854_at      MYBL2  3.34    8.53  20.2 1.81e-08  7.63e-05  9.64
38116_at  KIAA0101  3.76    9.51  16.9 8.12e-08  2.51e-04  8.48
38065_at      HMGB2  2.99    9.10  16.2 1.12e-07  2.51e-04  8.21
39755_at      XBP1  1.77   12.13  15.8 1.36e-07  2.51e-04  8.05
1592_at      TOP2A  2.30    8.31  15.8 1.39e-07  2.51e-04  8.03
41400_at       TK1  2.24   10.04  15.3 1.81e-07  2.75e-04  7.81
33730_at    GPRC5A -2.04    8.57 -15.1 1.96e-07  2.75e-04  7.74
1651_at      UBE2C  2.97   10.50  14.8 2.39e-07  3.02e-04  7.57
38414_at     CDC20  2.02    9.46  14.6 2.66e-07  3.05e-04  7.48
1943_at      CCNA2  2.19    7.60  14.0 3.72e-07  3.69e-04  7.18
40117_at      MCM6  2.28    9.68  14.0 3.80e-07  3.69e-04  7.17
40533_at     BIRC5  1.64    8.47  13.5 4.94e-07  4.45e-04  6.93
39642_at    ELOVL2  1.61    7.88  13.0 6.71e-07  5.18e-04  6.65
34851_at     AURKA  1.96    9.96  12.8 7.51e-07  5.18e-04  6.55
1824_s_at     PCNA  1.64    9.24  12.8 7.95e-07  5.18e-04  6.50
35995_at     ZWINT  2.76    8.87  12.7 8.32e-07  5.18e-04  6.46
893_at       UBE2S  1.54   10.95  12.7 8.43e-07  5.18e-04  6.45
40079_at    GPRC5A -2.41    8.23 -12.6 8.62e-07  5.18e-04  6.42

> sessionInfo()
R version 3.0.0 (2013-04-03)
Platform: i386-w64-mingw32/i386 (32-bit)

locale:
[1] LC_COLLATE=English_Australia.1252  LC_CTYPE=English_Australia.1252
[3] LC_MONETARY=English_Australia.1252 LC_NUMERIC=C
[5] LC_TIME=English_Australia.1252

attached base packages:
[1] parallel  stats     graphics  grDevices utils     datasets  methods   base

other attached packages:
 [1] hgu95av2.db_2.9.0    org.Hs.eg.db_2.9.0   RSQLite_0.11.3
 [4] DBI_0.2-6            annotate_1.38.0      hgu95av2cdf_2.12.0
 [7] AnnotationDbi_1.22.2 affy_1.38.1          Biobase_2.20.0
[10] BiocGenerics_0.6.0   limma_3.17.7         BiocInstaller_1.10.0

loaded via a namespace (and not attached):
[1] affyio_1.28.0         IRanges_1.18.0        preprocessCore_1.22.0
[4] stats4_3.0.0          tools_3.0.0           XML_3.96-1.1
[7] xtable_1.7-1          zlibbioc_1.6.0
```

16.3 Comparing Mammary Progenitor Cell Populations with Illumina BeadChips

16.3.1 Introduction

This case study examines the expression profiles of adult mammary stem cells and of progenitor and mature mammary lumina cells. The data was first published by Lim et al [17], who used the expression profiles to show that limina progenitor cells are the likely cell of origina for basal-like breast cancer.

The data files used in this case study can be downloaded from `http://bioinf.wehi.edu.au/marray/IlluminaCaseStudy`. The expression data files are provided as gzip files and will need to be uncompressed before they can be used for a limma analysis. To run the analysis described here, download and unzip the data files and set the working directory of R to the folder containing the files.

16.3.2 The target RNA samples

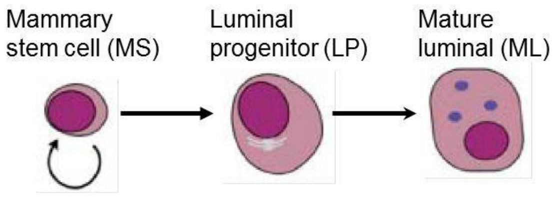
The data consists of three files:

```
> dir()
[1] "control probe profile.txt"
[2] "probe profile.txt"
[3] "Targets.txt"
```

First read the targets file describing the target RNA samples.

```
> library(limma)
> targets <- readTargets()
> targets
   Donor Age CellType
1      1  39       MS
2      1  39   Stroma
3      1  39       ML
4      1  39       LP
5      2  57       MS
6      2  57   Stroma
7      2  57       ML
8      2  57       LP
9      3  21       MS
10     3  21   Stroma
11     3  21       ML
12     3  21       LP
```

Breast tissue was obtained from three healthy human donors who were undergoing reduction mammoplasties. Epithelial cells were sorted into three subpopulations enriched for mammary stem cells (MS), luminal progenitor cells (LP) and mature luminal cells (ML) [17]. The MS, LP and ML cells representative a lineage of luminal cells use to construct the ducts used to transport milk in the breast [39]:



Stromal cells were also profiles as a comparison group. There were therefore four cell populations from each person. RNA was extracted from freshly sorted cells, making twelve RNA samples in total.

16.3.3 The expression profiles

The RNA samples were hybridized to two Illumina HumanWG-6 version 3 BeadChips. Each BeadChip is able to accommodate six samples. The BeadChips images were scanned and summarized using BeadStudio. Un-normalized summary probe profiles were exported from from BeadStudio to tab-delimited text files.

Separate files were written for regular probes and for control probes. The file `probe profile.txt` contains the expression profiles for regular probes, designed to interrogate the expression levels of genes. `control probe profile.txt` contains the profiles of control probes, including negative control probes. Note that BeadStudio by default writes the profiles for all the samples to the same two files.

We read in the expression profiles for both regular and control probes, telling `read.ilm` that we wish to read the detection p-values as well as the expression values:

```
> x <- read.ilmn(files="probe profile.txt",ctrlfiles="control probe profile.txt",
+ other.columns="Detection")
Reading file probeprofile.txt ... ...
Reading file controlprobeprofile.txt ... ...
```

This reads a `EListRaw` object. There are about 750 negative probes and about 49,000 regular probes:

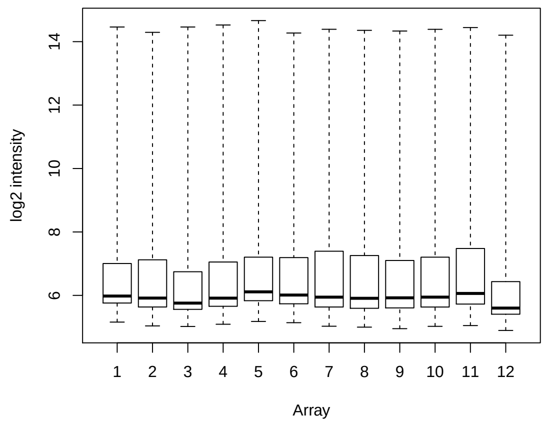
```
> table(x$genes$Status)
             BIOTIN             CY3_HYB        HOUSEKEEPING            LABELING 
                  2                   6                   7                   2 
LOW_STRINGENCY_HYB            NEGATIVE             regular 
                  8                 759               48803 
```

The component `E` contains the expression value for each probe

```
> options(digits=3)
> head(x$E)
                1    2    3    4    5    6    7    8    9   10   11   12
ILMN_1762337 52.3 46.1 54.0 47.7 54.8 47.4 67.4 47.9 40.5 44.7 80.6 42.5
ILMN_2055271 69.9 73.9 58.6 72.4 77.1 82.1 69.1 81.3 79.1 82.5 87.0 60.9
ILMN_1736007 57.5 53.7 53.4 49.4 58.6 59.9 56.4 51.6 58.7 51.7 58.4 43.9
ILMN_2383229 53.6 57.5 48.2 48.2 61.8 64.5 52.7 43.5 65.5 49.8 53.9 39.3
ILMN_1806310 58.1 55.1 50.5 60.0 64.2 58.4 58.0 52.3 56.6 55.6 65.3 46.4
ILMN_1779670 64.5 61.2 52.8 61.9 67.9 59.7 68.2 63.1 65.1 65.7 69.6 52.0
```

The intensities vary from about 5 to 14 on the $\log_2$ scale:

```
> boxplot(log2(x$E),range=0,ylab="log2 intensity")
```



16.3.4 How many probes are truly expressed?

The detection values contain *p*-values for testing whether each probe is more intense than the negative control probes. Small values are evidence that the probe corresponds to a truly expressed gene:

```
> head(x$other$Detection)
                  1     2      3       4      5     6      7      8      9     10     11    12
ILMN_1762337 0.5585 0.675 0.1370 0.60139 0.5776 0.782 0.0503 0.4781 0.9082 0.7145 0.0000 0.460
ILMN_2055271 0.0306 0.000 0.0493 0.00278 0.0364 0.000 0.0391 0.0000 0.0000 0.0000 0.0000 0.000
ILMN_1736007 0.2772 0.292 0.1534 0.48611 0.4112 0.220 0.2318 0.3145 0.1554 0.3774 0.2539 0.360
ILMN_2383229 0.4735 0.187 0.3658 0.56389 0.2951 0.124 0.3408 0.7447 0.0537 0.4680 0.3986 0.747
ILMN_1806310 0.2618 0.248 0.2589 0.12778 0.2196 0.264 0.1955 0.2920 0.1963 0.2382 0.1220 0.203
ILMN_1779670 0.0850 0.113 0.1644 0.10417 0.1469 0.224 0.0461 0.0691 0.0621 0.0655 0.0709 0.058
```

We can go further than this and estimate the overall proportion of the regular probes that correspond to expressed transcript, using the method of Shi et al [29].

```
> pe <- propexpr(x)
> dim(pe) <- c(4,3)
> dimnames(pe) <- list(CellType=c("MS","Stroma","ML","LP"),Donor=c(1,2,3))
> pe
        Donor
CellType     1     2     3
  MS     0.557 0.504 0.529
  Stroma 0.518 0.514 0.514
  ML     0.549 0.495 0.535
  LP     0.555 0.518 0.517
```

The proportion of probes that are expressed varies from 50–56%. The average is 52.5%.

16.3.5 Normalization and filtering

Background correction and normalize:

```
> y <- neqc(x)
```

The `neqc` functions performs normexp background correction using negative controls, then quantile normalizes and finally $\log_2$ transforms [30]. It also automatically removes the control probes, leaving only the regular probes in `y`:

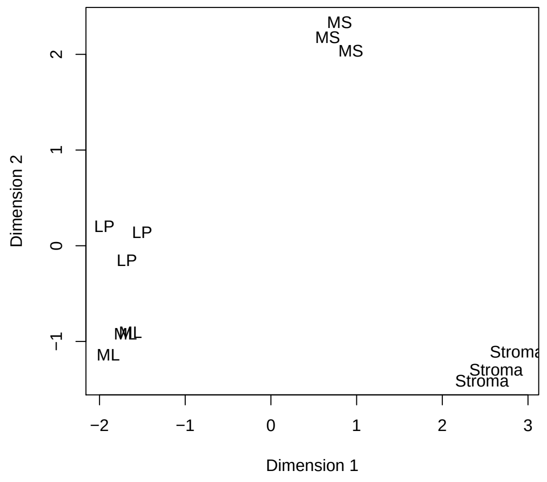
```
> dim(y)
[1] 48803    12
```

Filter out probes that are not expressed. We keep probes that are expressed in at least three arrays according to a detection p-values of 5%:

```
> expressed <- rowSums(y$other$Detection < 0.05) >= 3
> y <- y[expressed,]
> dim(y)
[1] 24691    12
```

A multi-dimensional scaling plot shows that the cell types are cell separated:

```
> plotMDS(y,labels=targets$CellType)
```



16.3.6 Within-patient correlations

The study involves multiple cell types from the same patient. Arrays from the same donor are not independent, so we need to estimate the within-dinor correlation:

```
> ct <- factor(targets$CellType)
> design <- model.matrix(~0+ct)
> colnames(design) <- levels(ct)
> dupcor <- duplicateCorrelation(y,design,block=targets$Donor)
> dupcor$consensus.correlation
[1] 0.134
```

As expected, the within-donor correlation is small but positive.

16.3.7 Differential expression between cell types

Now we look for differentially expressed genes. We make all possible pairwise comparisons between the epithelial cell types, allowing for the correlation within donors:

```
> fit <- lmFit(y,design,block=targets$Donor,correlation=dupcor$consensus.correlation)
> contrasts <- makeContrasts(ML-MS, LP-MS, ML-LP, levels=design)
> fit2 <- contrasts.fit(fit, contrasts)
> fit2 <- eBayes(fit2, trend=TRUE)
> summary(decideTests(fit2, method="global"))
   ML - MS LP - MS ML - LP
-1    3074    2836    1631
0    18088   18707   21307
1     3529    3148    1753
```

Top ten differentially expressed probes between ML and MS:

```
> topTable(fit2, coef=1)
                SYMBOL logFC AveExpr     t  P.Value adj.P.Val    B
ILMN_1766707     IL17B -4.19    5.94 -29.0 2.51e-12  5.19e-08 18.1
ILMN_1706051      PLD5 -4.00    5.67 -27.8 4.20e-12  5.19e-08 17.7
ILMN_1656369    C8orf4  5.24   11.26  25.1 1.36e-11  1.06e-07 16.7
ILMN_2413323       GRP -6.60    6.82 -24.3 2.01e-11  1.06e-07 16.4
ILMN_1787750     CD200 -5.68    6.30 -23.9 2.43e-11  1.06e-07 16.2
ILMN_1669819 LOC402569  2.52    5.50  23.8 2.57e-11  1.06e-07 16.2
ILMN_1777998  ARHGAP25 -4.78    6.26 -23.1 3.50e-11  1.15e-07 15.9
ILMN_1701933      SNCA -5.26    6.27 -22.8 4.19e-11  1.15e-07 15.7
ILMN_1777199       GRP -5.47    6.36 -22.8 4.23e-11  1.15e-07 15.7
ILMN_1708303   CYP4F22  4.09    5.94  22.5 4.76e-11  1.15e-07 15.6
```

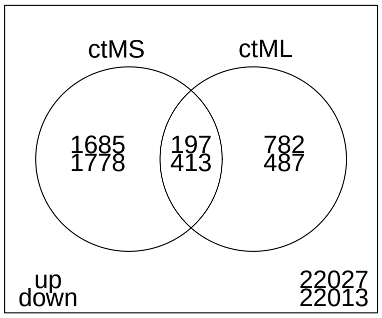
16.3.8 Signature genes for luminal progenitor cells

Now we find genes uniquely expressed in LP cells, as compared to MS and ML. We refit the linear model, making LP the reference cell type:

```
> ct <- relevel(ct, ref="LP")
> design <- model.matrix(~ct)
> fit <- lmFit(y,design,block=targets$Donor,correlation=dupcor$consensus.correlation)
> fit2 <- fit[,c("ctMS","ctML")]
> fit2 <- eBayes(fit2, trend=TRUE)
```

The we find all those genes that are up-regulated in LP vs both MS and ML, using a 2-fold-change and 5% FDR:

```
> results <- decideTests(fit2, lfc=1)
> vennDiagram(results, include=c("up","down"))
```



There are 197 positive signature genes and 413 negative. To see the top positive signature genes with their fold-changes:

```
> LP.sig <- rowSums(results>0)==2
> topTable(fit2[LP.sig,])
                SYMBOL ctMS ctML AveExpr     F  P.Value adj.P.Val
ILMN_1716370      TNS4 4.87 1.47    6.81 156.3 3.31e-09  4.81e-07
ILMN_1810054      CNN1 6.41 1.42    8.47 146.0 4.88e-09  4.81e-07
ILMN_1713744 C14orf132 3.85 1.74    7.34 103.5 3.37e-08  2.21e-06
ILMN_1720513    SETBP1 4.17 1.27    8.27  92.5 6.28e-08  3.09e-06
ILMN_1767662     LASS6 2.60 1.98   10.01  85.2 9.88e-08  3.46e-06
ILMN_1681984   GALNT10 3.54 1.23    8.09  84.2 1.06e-07  3.46e-06
ILMN_1731374       CPE 4.23 3.01    9.12  81.9 1.23e-07  3.46e-06
ILMN_1789639      FMOD 3.57 2.24    8.21  75.7 1.89e-07  4.65e-06
ILMN_1743933     TSHZ3 3.94 1.22    8.93  72.6 2.38e-07  5.21e-06
ILMN_1721876     TIMP2 3.57 1.38   10.10  69.0 3.13e-07  6.17e-06
```

16.4 Time Course Effects of Corn Oil on Rat Thymus with Agilent 4x44K Arrays

This case study analyses a time-course experiment using single-channel Agilent Whole Rat Genome Microarray 4x44K v3 arrays.

Background. The experiment concerns the effect of corn oil on gene expression in the thymus or rats. The data was submitted by Hong Weiguo to ArrayExpress as series E-GEOD-33005. The description of the experiment reads:

> "To investigate the effects of corn oil (CO), common drug vehicle, on the gene expression profiles in rat thymus with microarray technique. Female Wistar Rats were administered daily with normal saline (NS), CO 2, 5, 10 ml/kg for 14 days, respectively. Then, the thymus samples of rats were collected for microarray test and histopathology examination. The microarray data showed that 0, 40, 458 differentially expressed genes (DEGs) in 2, 5, 10 ml/kg CO group compared to NS

group, respectively. The altered genes were associated with immune response, cellular response to organic cyclic substance, regulation of fatty acid beta-oxidation, et al. However, no obvious histopathologic change was observed in the three CO dosage groups. These data show that 10 ml/kg CO, that dosage has been determined as the vehicle in drug safety assessment, can cause obvious influence on gene expression in rat thymus. Our study suggest that the dosage of CO gavage as the vehicle for water-in-soluble agents in drug development should be no more than 5 ml/kg if agents' molecular effects in thymus want to be assessed. Gene expression in thymus from female Wistar rats daily administered with 2, 5, 10 ml/kg of corn oil or 10 ml/kg of saline by gavage for 14 consecutive days were measured using Agilent Rat Whole Genome 8×60K array."

Data availability. All files files were downloaded from `http://www.ebi.ac.uk/arrayexpress/experiments/E-GEOD-33005`.

Read the sample and data relationship format (SDRF) file. This is equivalent to what is known as the targets file in limma:

```
> SDRF <- read.delim("E-GEOD-33005.sdrf.txt",check.names=FALSE,stringsAsFactors=FALSE)
```

Read data:

```
> x <- read.maimages(SDRF[,"Array Data File"],source="agilent",green.only=TRUE)
Read GSM819076_US10283824_252828210181_S01_GE1_107_Sep09_1_4.txt
Read GSM819075_US10283824_252828210181_S01_GE1_107_Sep09_1_3.txt
Read GSM819074_US10283824_252828210181_S01_GE1_107_Sep09_1_2.txt
Read GSM819073_US10283824_252828210180_S01_GE1_107_Sep09_1_4.txt
Read GSM819072_US10283824_252828210180_S01_GE1_107_Sep09_1_3.txt
Read GSM819071_US10283824_252828210180_S01_GE1_107_Sep09_1_2.txt
Read GSM819070_US10283824_252828210180_S01_GE1_107_Sep09_1_1.txt
Read GSM819069_US10283824_252828210179_S01_GE1_107_Sep09_1_4.txt
Read GSM819068_US10283824_252828210179_S01_GE1_107_Sep09_1_3.txt
Read GSM819067_US10283824_252828210179_S01_GE1_107_Sep09_1_2.txt
Read GSM819066_US10283824_252828210179_S01_GE1_107_Sep09_1_1.txt
Read GSM819065_US10283824_252828210178_S01_GE1_107_Sep09_1_4.txt
Read GSM819064_US10283824_252828210178_S01_GE1_107_Sep09_1_3.txt
Read GSM819063_US10283824_252828210178_S01_GE1_107_Sep09_1_2.txt
Read GSM819062_US10283824_252828210178_S01_GE1_107_Sep09_1_1.txt
Read GSM819061_US10283824_252828210177_S01_GE1_107_Sep09_1_4.txt
Read GSM819060_US10283824_252828210177_S01_GE1_107_Sep09_1_3.txt
Read GSM819059_US10283824_252828210177_S01_GE1_107_Sep09_1_2.txt
Read GSM819058_US10283824_252828210177_S01_GE1_107_Sep09_1_1.txt
```

Background correct and normalize:

```
> y <- backgroundCorrect(x,method="normexp")
Array 1 corrected
Array 2 corrected
Array 3 corrected
Array 4 corrected
Array 5 corrected
Array 6 corrected
Array 7 corrected
Array 8 corrected
Array 9 corrected
Array 10 corrected
Array 11 corrected
Array 12 corrected
Array 13 corrected
Array 14 corrected
Array 15 corrected
Array 16 corrected
Array 17 corrected
Array 18 corrected
Array 19 corrected
> y <- normalizeBetweenArrays(y,method="quantile")
```

Now filter out control probes and low expressed probes. To get an idea of how bright expression probes should be, we compute the 95% percentile of the negative control probes on each array. We keep probes that are at least 10% brighter than the negative controls on at least four arrays (because there are four replicates):

```
> neg95 <- apply(y$E[y$genes$ControlType==-1,],2,function(x) quantile(x,p=0.95))
> cutoff <- matrix(1.1*neg95,nrow(y),ncol(y),byrow=TRUE)
> isexpr <- rowSums(y$E > cutoff) >= 4
> table(isexpr)
isexpr
FALSE  TRUE
11500 32754
```

Regular probes are code as “0” in the ControlType column:

```
> y0 <- y[y$genes$ControlType==0 & isexpr,]
```

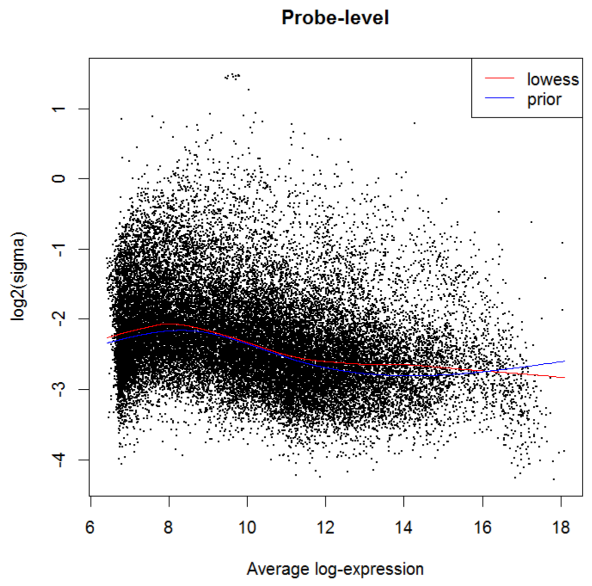
Now we can find genes differentially expressed for the corn oil treatments compared to the saline control:

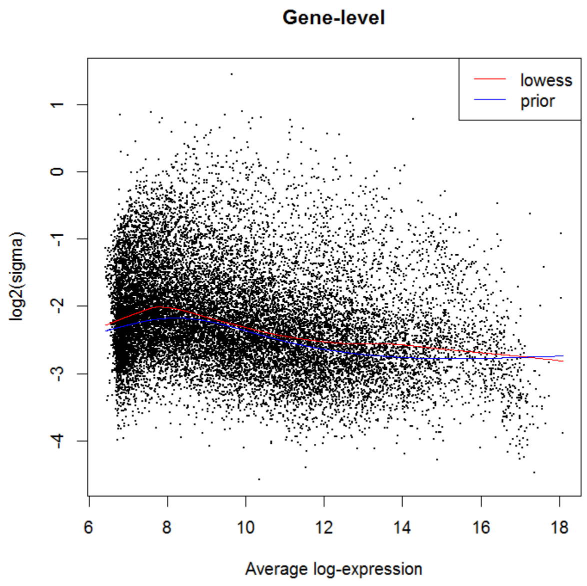
```
> Treatment <- SDRF[,"Characteristics[treatment]"]
> levels <- c("10 ml/kg saline","2 ml/kg corn oil","5 ml/kg corn oil","10 ml/kg corn oil")
> Treatment <- factor(Treatment,levels=levels)
> design <- model.matrix(~Treatment)
> fit <- lmFit(y0,design)
> fit <- eBayes(fit,trend=TRUE)
> plotSA(fit, main="Probe-level")
> summary(decideTests(fit[,-1]))
   Treatment2 ml/kg corn oil Treatment5 ml/kg corn oil Treatment10 ml/kg corn oil
-1                         0                         0                        911
0                      32723                     32723                      30063
1                          0                         0                       1749
```

It appears that only the 10 ml/kg treatment is different from the saline control, however this is quite different.

Same analysis, but now averaging probes for each gene:

```
> yave <- avereps(y0,ID=y0$genes[,"SystematicName"])
> fit <- lmFit(yave,design)
> fit <- eBayes(fit,trend=TRUE)
> plotSA(fit, main="Gene-level")
> summary(decideTests(fit[,-1]))
   Treatment2 ml/kg corn oil Treatment5 ml/kg corn oil Treatment10 ml/kg corn oil
-1                         0                         0                        407
0                      19112                     19112                      17878
1                          0                         0                        827
```





Chapter 17

RNA-Seq Case Studies

17.1 Profiles of Unrelated Nigerian Individuals

17.1.1 Background

RNA-Seq profiles were made from lymphoblastoid cell lines generated as part of the International HapMap project from 69 unrelated Nigerian individuals [13]. RNA from each individual was sequenced on at least two lanes of the Illumina Genome Analyser 2 platform, and mapped reads to the human genome using MAQ v0.6.8.

The study group here is essentially an opportunity sample and the individuals are likely to be genetically diverse. In this analysis we look at genes that are differentially expressed between males and females. This case study requires limma 3.9.19 or later.

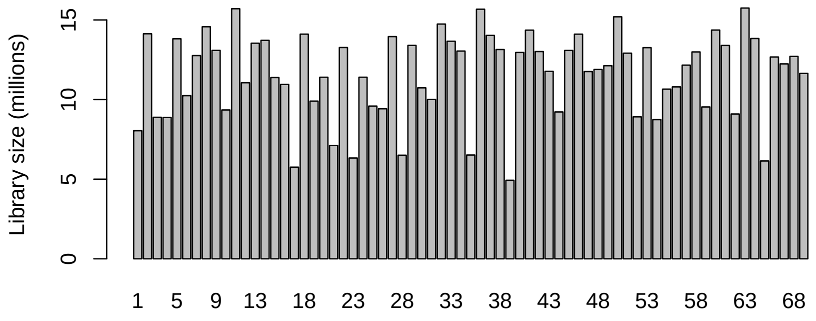
17.1.2 Loading the data

Read counts summarized by Ensembl gene identifiers are available in the tweeDEseqCountData package:

```
> library(tweeDEseqCountData)
> data(pickrell1)
> Counts <- exprs(pickrell1.eset)
> dim(Counts)
[1] 38415    69
> Counts[1:5,1:5]
                NA18486 NA18498 NA18499 NA18501 NA18502
ENSG00000127720       6      32      14      35      14
ENSG00000242018      20      21      24      22      16
ENSG00000224440       0       0       0       0       0
ENSG00000214453       0       0       0       0       0
ENSG00000237787       0       0       1       0       0
```

The library sizes vary from about 5 million to over 15 million:

```
> barplot(colSums(Counts)*1e-6, names=1:69, ylab="Library size (millions)")
```



To demonstrate limma on RNA-Seq data, we will compare female with male individuals.

```
> Gender <- pickrell1.eset$gender
> table(Gender)
Gender
female   male
    40     29
> rm(pickrell1.eset)
> data(genderGenes)
```

Annotation for each Ensemble gene is also available from the tweeDEseqCountData package:

```
> data(annotEnsembl63)
> annot <- annotEnsembl63[,c("Symbol","Chr")]
> annot[1:5,]
                Symbol Chr
ENSG00000252775     U7   5
ENSG00000207459     U6   5
ENSG00000252899     U7   5
ENSG00000201298     U6   5
ENSG00000222266     U6   5
> rm(annotEnsembl63)
```

Note that the BiocGenerics package now defines a `plotMA` function that conflicts with the function of the same name in the limma package. Hence the limma package must be loaded after this point (so that is appears first in the search path), or else the BiocGenerics package needs to be detached:

```
> detach(2)
> detach(2)
> detach(2)
> detach(2)
```

17.1.3 DGEList object

Form a DGEList object combining the counts and associated annotation:

```
> library(edgeR)
> y <- DGEList(counts=Counts, genes=annot[rownames(Counts),])
```

17.1.4 Filtering

Keep genes with least 1 count-per-million reads (cpm) in at least 20 samples:

```
> isexpr <- rowSums(cpm(y)>1) >= 20
```

Keep only genes with defined annotation:

```
> hasannot <- rowSums(is.na(y$genes))==0
> y <- y[isexpr & hasannot,]
> dim(y)
[1] 17310    69
> y$samples$lib.size <- colSums(y$counts)
```

17.1.5 Scale normalization

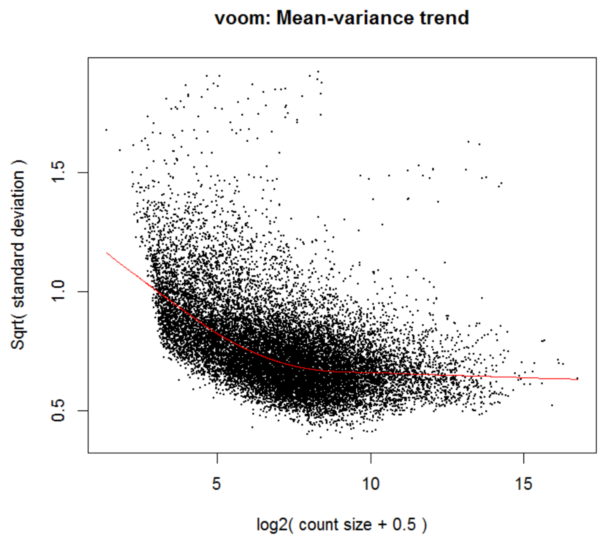
Apply scale normalization:

```
> y <- calcNormFactors(y)
```

17.1.6 Linear modeling

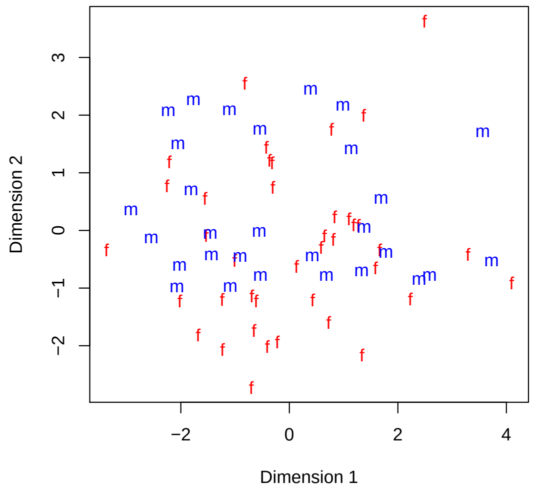
Use `voom()` [16] to convert the read counts to $\log_2$-cpm, with associated weights, ready for linear modelling:

```
> library(limma)
> design <- model.matrix(~Gender)
> v <- voom(y,design,plot=TRUE)
```



Some separation of female and male profiles is evident from an MDS plot, although clear differences seem to rely on only a few score genes.

```
> plotMDS(v,top=50,labels=substring(Gender,1,1),
+        col=ifelse(Gender=="male","blue","red"),gene.selection="common")
```

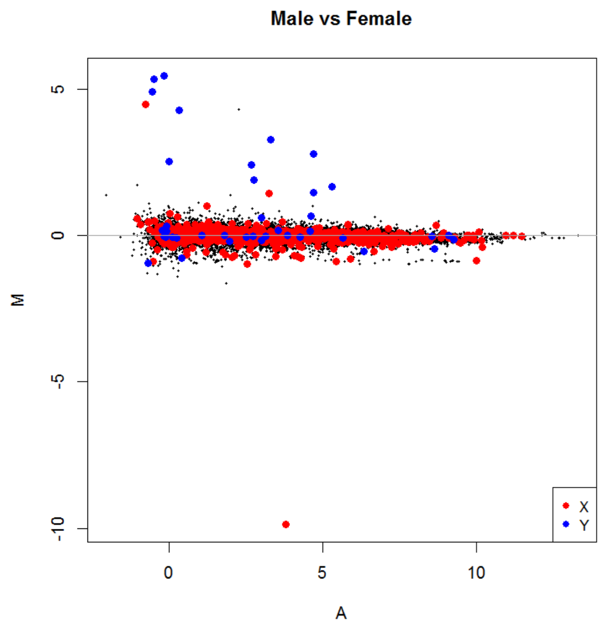


Now find genes differentially expression between male and females. Positive log-fold-changes mean higher in males. The highly ranked genes are mostly on the X or Y chromosomes.

```
> fit <- lmFit(v,design)
> fit <- eBayes(fit)
> options(digits=3)
> topTable(fit,coef=2,n=16,sort="p")
                      Symbol Chr  logFC AveExpr     t  P.Value adj.P.Val    B
ENSG00000229807         XIST   X -9.857  3.8076 -32.5 1.21e-44  2.10e-40 66.5
ENSG00000099749     CYorf15A   Y  4.260  0.3139  27.1 2.40e-39  2.08e-35 64.8
ENSG00000157828       RPS4Y2   Y  3.280  3.3074  26.7 5.88e-39  3.39e-35 73.8
ENSG00000233864       TTTY15   Y  4.896 -0.5546  25.4 1.62e-37  7.00e-34 59.6
ENSG00000198692       EIF1AY   Y  2.397  2.6799  20.5 1.04e-31  3.60e-28 59.1
ENSG00000131002     CYorf15B   Y  5.439 -0.1717  20.2 2.46e-31  7.09e-28 51.1
ENSG00000213318 RP11-331F4.1  16  4.295  2.2647  19.4 3.04e-30  7.51e-27 55.0
ENSG00000165246       NLGN4Y   Y  5.330 -0.4924  17.9 3.59e-28  7.78e-25 45.9
ENSG00000129824       RPS4Y1   Y  2.779  4.7110  17.8 6.17e-28  1.19e-24 52.2
ENSG00000183878          UTY   Y  1.877  2.7422  16.6 3.35e-26  5.81e-23 47.9
ENSG00000012817        KDM5D   Y  1.470  4.7039  14.7 2.33e-23  3.67e-20 42.3
ENSG00000243209   AC010889.1   Y  2.536 -0.0186  14.0 2.97e-22  4.28e-19 36.5
ENSG00000146938       NLGN4X   X  4.472 -0.7808  13.8 6.47e-22  8.62e-19 34.8
ENSG00000067048        DDX3Y   Y  1.670  5.3070  13.3 4.54e-21  5.61e-18 37.3
ENSG00000006757       PNPLA4   X -0.989  2.5333 -10.4 5.09e-16  5.87e-13 25.8
ENSG00000232928 RP13-204A15.4  X  1.434  3.2499  10.2 1.43e-15  1.54e-12 25.0
> fit$df.prior
```

```
[1] 4.6
> summary(decideTests(fit))
   (Intercept) Gendermale
-1         292         43
0          913      17251
1        16105         16

> chrom <- fit$genes$Chr
> plotMA(fit,array=2,status=chrom,values=c("X","Y"),col=c("red","blue"),
+        main="Male vs Female",legend="bottomright")
> abline(h=0,col="darkgrey")
```



17.1.7 Gene set testing

The tweeDEseqCountData package includes a list of genes belonging to the male-specific region of chromosome Y, and a list of genes located in the X chromosome that have been reported to escape X-inactivation. We expect genes in the first list to be up-regulated in males, whereas genes in the second list should be up-regulated in females.

```
> Ymale <- rownames(v) %in% msYgenes
> Xescape <- rownames(v) %in% XiEgenes
```

Roast gene set tests confirm that the male-specific genes are significantly up as a group in our comparison of males with females, whereas the X genes are signficantly down as a group:

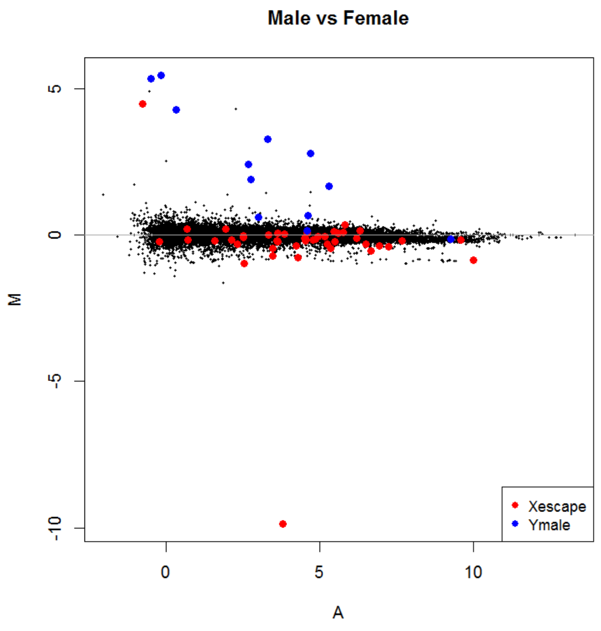
```
> index <- list(Y=Ymale,X=Xescape)
> mroast(v,index,design,nrot=9999)
  NGenes PropDown PropUp Direction PValue   FDR PValue.Mixed FDR.Mixed
Y     12   0.0833 0.9167        Up  2e-04 1e-04        1e-04     5e-05
X     46   0.5217 0.0435      Down  2e-04 1e-04        1e-04     5e-05
```

The results from competitive camera gene sets tests are even more convincing:

```
> camera(v,index,design)
  NGenes Correlation Direction   PValue      FDR
Y     12      0.0836        Up 2.12e-28 4.23e-28
X     46      0.0206      Down 1.14e-10 1.14e-10
```

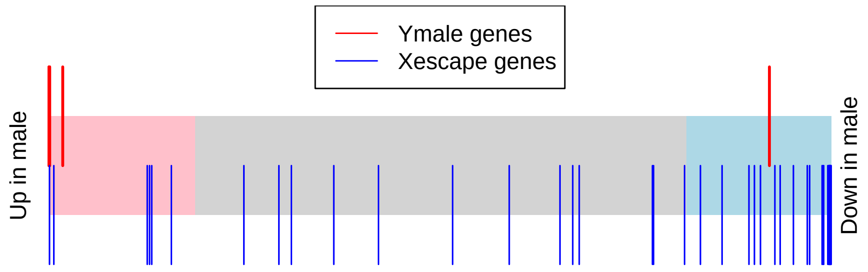
We can highlight the male-specific and X-escape genes on the MA-plot:

```
> status <- rep("Other",nrow(fit))
> status[Ymale] <- "Ymale"
> status[Xescape] <- "Xescape"
> plotMA(fit,array=2,status=status,values=c("Xescape","Ymale"),col=c("red","blue"),
+        main="Male vs Female",legend="bottomright")
> abline(h=0,col="darkgrey")
```



Another way to view the gene sets it to view the ranking of the Y and X specific genes in the differential expression results. This shows that, with a couple of exceptions, the Y specific genes are highly ranked (positive) while the X specific genes are low ranked (negative):

```
> barcodeplot(fit$t[,2],Ymale,Xescape, labels=c("Up in male","Down in male"))
> legend("top",legend=c("Ymale genes","Xescape genes"),lty=1,col=c("red","blue"))
```



```
> sessionInfo()
R version 3.0.0 (2013-04-03)
Platform: i386-w64-mingw32/i386 (32-bit)

locale:
[1] LC_COLLATE=English_Australia.1252  LC_CTYPE=English_Australia.1252
[3] LC_MONETARY=English_Australia.1252 LC_NUMERIC=C
[5] LC_TIME=English_Australia.1252

attached base packages:
[1] parallel  stats     graphics  grDevices utils     datasets  methods   base

other attached packages:
[1] edgeR_3.3.3              limma_3.17.7             tweeDEseqCountData_1.0.9
[4] Biobase_2.20.0           BiocGenerics_0.6.0

loaded via a namespace (and not attached):
[1] tools_3.0.0
```

Notes

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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.

Software Projects Using limma

The limma package is used as a building block or as the underlying computational engine by a number of software projects designed to provide user-interfaces for microarray data analysis including RMAGEML [7], arrayMagic [3], DNMAD [38], GPAP (GenePix Pro Auto-Processor) [40], the KTH Package [28], SKCC WebArray [44], CARMAweb [12] and Pomelo II [20]. The LCBBASE project provides a limma plug-in for the BASE database [18]. The

Stanford Microarray Database `http://genome-www5.stanford.edu` calls out to limma for background correction options.

Citations

Biological studies using the limma package include [9, 27, 24, 2, 22, 37]. Methodological studies using the limma package include [14, 15].

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