limma:
Linear Models for Microarray and RNA-Seq Data
User's Guide

Gordon K. Smyth, Matthew Ritchie, Natalie Thorne,
James Wettenhall, Wei Shi and Yifang Hu
Bioinformatics & Computational Biology Division, The Walter and Eliza Hall Institute
of Medical Research, Melbourne, Australia

First edition 2 December 2002

Last revised 19 May 2026

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.

Contents

1 Introduction **5**

2 Preliminaries **7**
2.1 Citing limma . . . 7
2.2 Installation . . . 9
2.3 How to get help . . . 9

3 Quick Start **11**
3.1 A brief introduction to R . . . 11
3.2 Sample limma Session . . . 12
3.3 Data Objects . . . 13

4 Reading Microarray Data **15**
4.1 Scope of this Chapter . . . 15
4.2 Recommended Files . . . 15
4.3 The Targets Frame . . . 15
4.4 Reading Two-Color Intensity Data . . . 17
4.5 Reading Single-Channel Agilent Intensity Data . . . 19
4.6 Reading Illumina BeadChip Data . . . 19
4.7 Image-derived Spot Quality Weights . . . 20
4.8 Reading Probe Annotation . . . 21
4.9 Printer Layout . . . 22
4.10 The Spot Types File . . . 22

5 Quality Assessment **24**

6 Pre-Processing Two-Color Data **26**
6.1 Background Correction . . . 26
6.2 Within-Array Normalization . . . 28
6.3 Between-Array Normalization . . . 30
6.4 Using Objects from the marray Package . . . 32

7 Filtering unexpressed probes **34**

8 Linear Models Overview **36**
8.1 Introduction . . . 36
8.2 Single-Channel Designs . . . 37
8.3 Common Reference Designs . . . 38
8.4 Direct Two-Color Designs . . . 39

9 Single-Channel Experimental Designs **41**
9.1 Introduction . . . 41
9.2 Two Groups . . . 41
9.3 Several Groups . . . 43
9.4 Additive Models and Blocking . . . 43
9.4.1 Paired Samples . . . 43
9.4.2 Blocking . . . 44
9.5 Interaction Models: 2×2 Factorial Designs . . . 44
9.5.1 Questions of Interest . . . 44
9.5.2 Analysing as for a Single Factor . . . 45
9.5.3 A Nested Interaction Formula . . . 46
9.5.4 Classic Interaction Models . . . 46
9.6 Time Course Experiments . . . 48
9.6.1 Replicated time points . . . 48
9.6.2 Many time points . . . 49
9.7 Multi-level Experiments . . . 50

10 Two-Color Experiments with a Common Reference **52**
10.1 Introduction . . . 52
10.2 Two Groups . . . 52
10.3 Several Groups . . . 54

11 Direct Two-Color Experimental Designs **55**
11.1 Introduction . . . 55
11.2 Simple Comparisons . . . 55
11.2.1 Replicate Arrays . . . 55
11.2.2 Dye Swaps . . . 56
11.3 A Correlation Approach to Technical Replication . . . 57

12 Separate Channel Analysis of Two-Color Data **59**

13 Statistics for Differential Expression **61**
13.1 Summary Top-Tables . . . 61
13.2 Fitted Model Objects . . . 62
13.3 Multiple Testing Across Contrasts . . . 63

14 Array Quality Weights **65**
14.1 Introduction . . . 65
14.2 Example 1 . . . 65
14.3 Example 2 . . . 67
14.4 When to Use Array Weights . . . 69

15 RNA-Seq Data **70**
15.1 Introduction . . . 70
15.2 Making a count matrix . . . 70
15.3 Normalization and filtering . . . 70
15.4 Differential expression: limma-trend . . . 71
15.5 Differential expression: voom . . . 71
15.6 Voom with sample quality weights . . . 72
15.7 Differential expression: voomLmFit . . . 74
15.8 Differential splicing . . . 74

16 Two-Color Case Studies **76**
16.1 Swirl Zebrafish: A Single-Group Experiment . . . 76
16.2 Apoa1 Knockout Mice: A Two-Group Common-Reference Experiment . . . 87
16.3 Weaver Mutant Mice: A Composite 2x2 Factorial Experiment . . . 90
16.3.1 Background . . . 90
16.3.2 Sample Preparation and Hybridizations . . . 90
16.3.3 Data input . . . 91
16.3.4 Annotation . . . 92
16.3.5 Quality Assessment and Normalization . . . 92
16.3.6 Setting Up the Linear Model . . . 94
16.3.7 Probe Filtering and Array Quality Weights . . . 95
16.3.8 Differential expression . . . 95
16.4 Bob1 Mutant Mice: Arrays With Duplicate Spots . . . 96

17 Single-Channel Case Studies **100**
17.1 Lrp Mutant *E. Coli* Strain with Affymetrix Arrays . . . 100
17.1.1 Background . . . 100
17.1.2 Downloading the data . . . 100
17.1.3 Background correction and normalization . . . 101
17.1.4 Gene annotation . . . 101
17.1.5 Differential expression . . . 102
17.2 Effect of Estrogen on Breast Cancer Tumor Cells: A 2x2 Factorial Experiment with Affymetrix Arrays . . . 104
17.3 Comparing Mammary Progenitor Cell Populations with Illumina BeadChips . . . 108
17.3.1 Introduction . . . 108
17.3.2 The target RNA samples . . . 109
17.3.3 The expression profiles . . . 110
17.3.4 How many probes are truly expressed? . . . 111
17.3.5 Normalization and filtering . . . 111
17.3.6 Within-patient correlations . . . 112
17.3.7 Differential expression between cell types . . . 112
17.3.8 Signature genes for luminal progenitor cells . . . 113
17.4 Time Course Effects of Corn Oil on Rat Thymus with Agilent 4x44K Arrays . . . 114
17.4.1 Introduction . . . 114
17.4.2 Data availability . . . 114
17.4.3 Reading the data . . . 115

17.4.4 Gene annotation . . . 116
17.4.5 Background correction and normalize . . . 116
17.4.6 Gene filtering . . . 117
17.4.7 Differential expression . . . 117
17.4.8 Gene ontology analysis . . . 118

18 RNA-Seq Case Studies **120**
18.1 Profiles of Yoruba HapMap Individuals . . . 120
18.1.1 Background . . . 120
18.1.2 Data availability . . . 120
18.1.3 Yoruba Individuals and FASTQ Files . . . 120
18.1.4 Mapping reads to the reference genome . . . 122
18.1.5 Annotation . . . 124
18.1.6 DGEList object . . . 125
18.1.7 Filtering . . . 125
18.1.8 Scale normalization . . . 125
18.1.9 Linear modeling . . . 127
18.1.10 Gene set testing . . . 130
18.1.11 Session information . . . 133
18.1.12 Acknowledgements . . . 134
18.2 Differential Splicing after Pasilla Knockdown . . . 134
18.2.1 Background . . . 134
18.2.2 GEO samples and SRA Files . . . 134
18.2.3 Mapping reads to the reference genome . . . 135
18.2.4 Read counts for exons . . . 136
18.2.5 Assemble DGEList and sum counts for technical replicates . . . 136
18.2.6 Gene annotation . . . 137
18.2.7 Filtering . . . 138
18.2.8 Scale normalization . . . 138
18.2.9 Linear modelling . . . 139
18.2.10 Alternate splicing . . . 141
18.2.11 Session information . . . 144
18.2.12 Acknowledgements . . . 145

Chapter 1

Introduction

Limma is a package for the analysis of gene expression data arising from microarray or RNA-seq technologies [29]. A core capability is the use of linear models to assess differential expression in the context of multi-factor 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 quantitative gene expression technology including microarrays, RNA-seq and quantitative PCR. Limma can handle both single-channel and two-color microarrays.

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 17.3 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 4.0.0 and limma versions 2.16.0 through 3.44.0. The limma homepage is `https://bioinf.wehi.edu.au/limma`.

Chapter 2

Preliminaries

2.1 Citing limma

Limma implements a body of methodological research by the authors and co-workers. Please try to cite the appropriate 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.

The limma software package itself can be cited as:

> Ritchie, ME, Phipson, B, Wu, D, Hu, Y, Law, CW, Shi, W, and Smyth, GK (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research* 43(7), e47.

The above article reviews the overall capabilities of the limma package, both new and old.

Other articles describe the statistical methodology behind particular functions of the package. If you use limma for differential expression analysis, please cite:

> Phipson, B, Lee, S, Majewski, IJ, Alexander, WS, and Smyth, GK (2016). Robust hyperparameter estimation protects against hypervariable genes and improves power to detect differential expression. *Annals of Applied Statistics* 10(2), 946–963.

This article describes the linear modeling approach implemented by `lmFit` and the empirical Bayes statistics implemented by `eBayes`, `topTable` etc. It particularly describes `eBayes` with `robust=TRUE` or `trend=TRUE`.

If you use limma for RNA-seq analysis, please cite:

> Law, CW, Chen, Y, Shi, W, and Smyth, GK (2014). Voom: precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biology* 15, R29.

or

> Liu, R, Holik, AZ, Su, S, Jansz, N, Chen, K, Leong, HS, Blewitt, ME, Asselin-Labat, M-L, Smyth, GK, Ritchie, ME (2015). Why weight? Modelling sample and observational level variability improves power in RNA-seq analyses. *Nucleic Acids Research* 43, e97.

or

> Baldoni PL, Chen L, Li M, Chen Y, Smyth GK (2025). Dividing out quantification uncertainty enables assessment of differential transcript usage with limma and edgeR. *Nucleic Acids Research* 53(22), gkaf1305. [

Law et al (2014) describe the voom and limma-trend pipelines for RNA-seq, while Liu et al (2015) describe the `voomWithQualityWeights` function. Baldoni et al (2025) describes the `voomLmFit` and `diffSplice` functions.

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**, 2067–2075.
> `http://www.statsci.org/smyth/pubs/dupcor.pdf`

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

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

> Smyth, G. K., and Speed, T. P. (2003). Normalization of cDNA microarray data. *Methods* **31**, 265–273.
> `http://www.statsci.org/smyth/pubs/normalize.pdf`

> Oshlack, A., Emslie, D., Corcoran, L., and Smyth, G. K. (2007). Normalization of boutique two-color microarrays with a high proportion of differentially expressed probes. *Genome Biology* 8, R2.

The first of these articles describes the functions `read.maimages`, `normalizeWithinArrays` and `normalizeBetweenArrays`. The second describes the use of spot quality weights to normalize on control probes.

The various options provided by the `backgroundCorrect` function are explained by:

> 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.

If you use `arrayWeights` or related functions to estimate sample 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.

If you use the `read.ilmn`, `nec` or `neqc` functions to 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.

The `propexpr` function is explained by

> Shi, W, de Graaf, C, Kinkel, S, Achtman, A, Baldwin, T, Schofield, L, Scott, H, Hilton, D, Smyth, GK (2010). Estimating the proportion of microarray probes expressed in an RNA sample. *Nucleic Acids Research* 38, 2168–2176.

The `lmscFit` function for separate channel analysis of two-color microarray data is explained by:

> Smyth, GK, and Altman, NS (2013). Separate-channel analysis of two-channel microarrays: recovering inter-spot information. *BMC Bioinformatics* 14, 165.

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 we will assume here that you have already installed R (if not, 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`. Like other Bioconductor packages, limma is most easily installed using the BiocManager package:

```
> library(BiocManager)
> install("limma")
```

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

```
> install("statmod")
```

as well. If you're starting from scratch and haven't used any Bioconductor packages before, then you will need to install the BiocManager package itself by `install.packages("BiocManager"` before installing limma.

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 `BiocManager::install` 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. To see what new features were introduced in the latest Bioconductor release, type:

```
> news(package = "limma")
```

There is also a detailed change-log that describes each minor update. To see the most recent 20 lines of the change-log 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 that 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. All other questions or problems concerning limma should be posted to the Bioconductor support site `https://support.bioconductor.org`. Please send requests for general assistance and advice to the support site rather than to the individual authors. Posting questions to the Bioconductor mailing list has a number of advantages. First, the mailing list includes a community of experienced limma users who can answer most common questions. Second, the limma authors try hard to ensure that any user posting to Bioconductor receives assistance. Third, the mailing list allows others with the same sort of questions to gain from the answers. Users posting to the mailing list for the first time will find it helpful to read the posting guide at `http://www.bioconductor.org/help/support/posting-guide`.

Note that each function in limma has its own online help page, as described in the next chapter. If you have a question about any particular function, reading the function's help page will often answer the question very quickly. In any case, it is good etiquette to check the relevant help page first before posting a question to the support site.

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

```
> ?lmFit
```

or equivalently

```
> help("lmFit")
```

for detailed help on the `lmFit` 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 [8].

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.

The options `trend=TRUE` and `robust=TRUE` are also often helpful when running `eBayes`, increasing power for certain types of data. For example:

```
> fit <- eBayes(fit, trend=TRUE, robust=TRUE)
> topTable(fit, coef="MUvsWT")
```

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

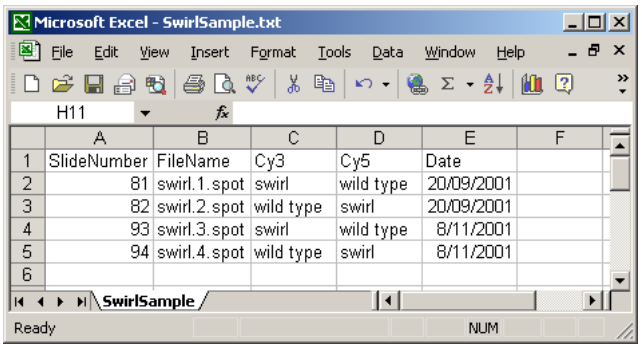
The first step in preparing data for input into limma is usually to create a targets file which lists the RNA target hybridized to each channel of each array. It is normally in tab-delimited text format and should contain a row for each microarray in the experiment. The file can have any name but the 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*.

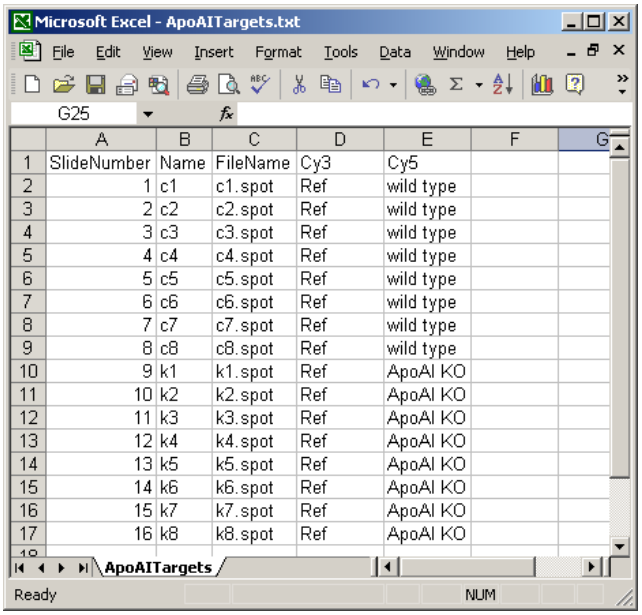
The targets frame normally contains a `FileName` column, giving the name of the image-analysis output file, a `Cy3` column giving the RNA type labeled with Cy3 dye for that slide and a `Cy5`

column giving the RNA type labeled 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:



	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:



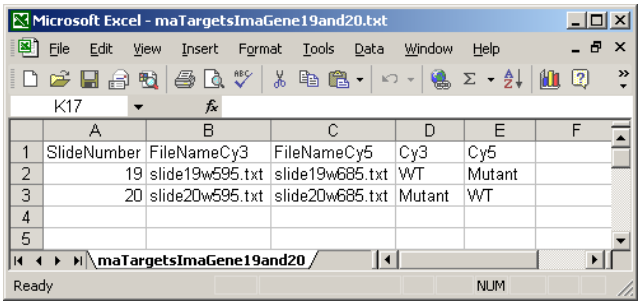
	A	B	C	D	E	F	G
1	SlideNumber	Name	FileName	Cy3	Cy5		
2	1	c1	c1.spot	Ref	wild type		
3	2	c2	c2.spot	Ref	wild type		
4	3	c3	c3.spot	Ref	wild type		
5	4	c4	c4.spot	Ref	wild type		
6	5	c5	c5.spot	Ref	wild type		
7	6	c6	c6.spot	Ref	wild type		
8	7	c7	c7.spot	Ref	wild type		
9	8	c8	c8.spot	Ref	wild type		
10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



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 overridden 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 17.4 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 pre-processing 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 pre-processing 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 17.3 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)
```

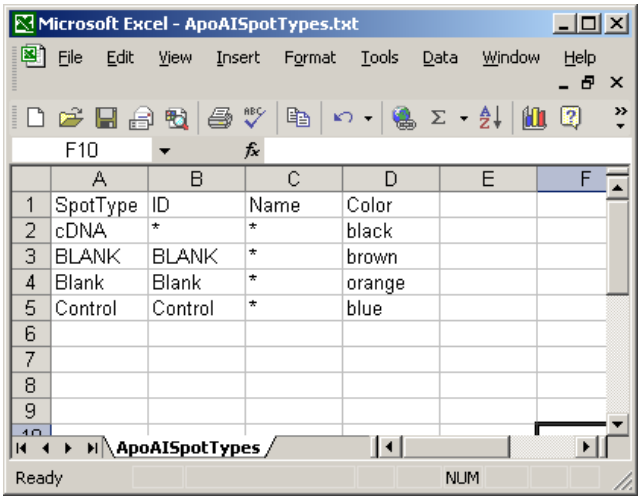
Note this will work only for GenePix GAL files, not for general gene lists.

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.

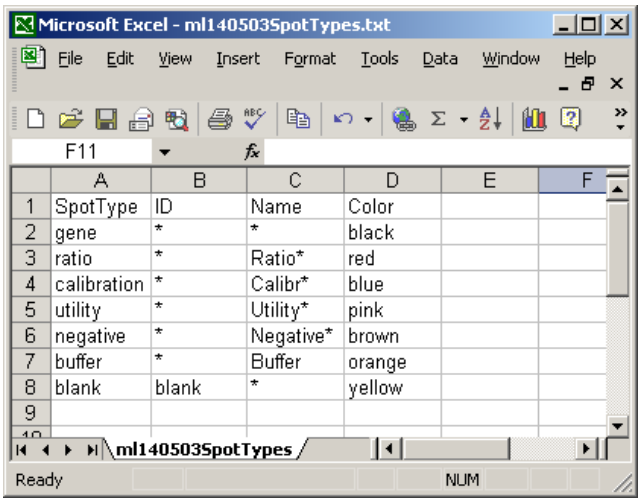
Here is a short STF appropriate for the ApoAI data:



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

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



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

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)
```

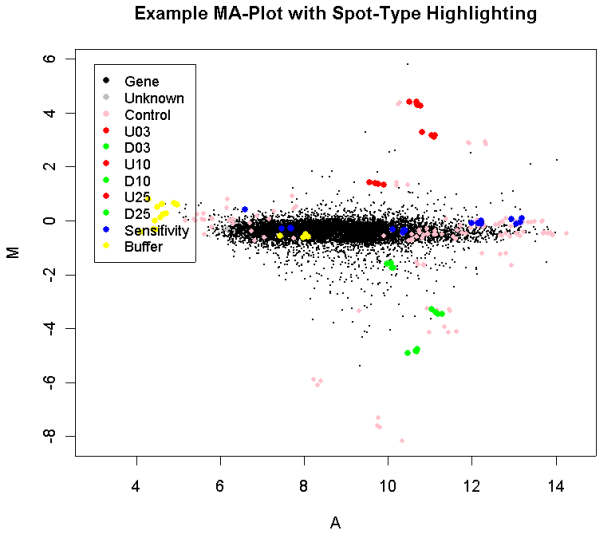
Chapter 5

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 `plotMD()` function produces plots for individual arrays [29]. 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 facilitated by the `controlStatus()` function. 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)
> plotMD(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)
```

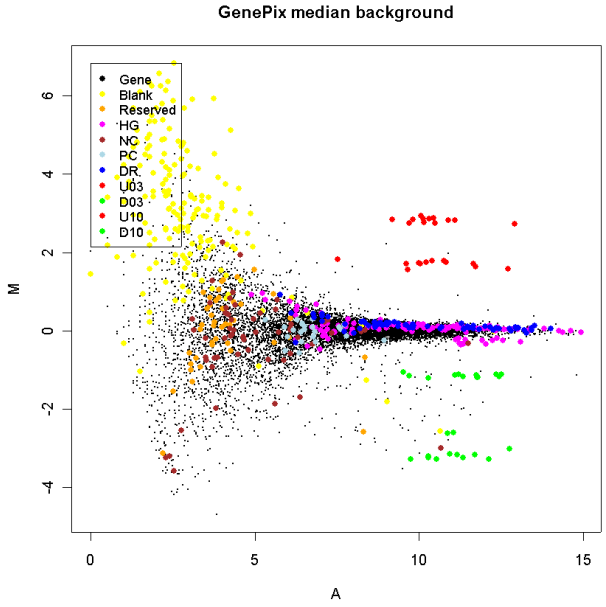
However there are many other background correction options which may be preferable in certain situations, see Ritchie et al [30].

For the purpose of assessing differential expression, we often find

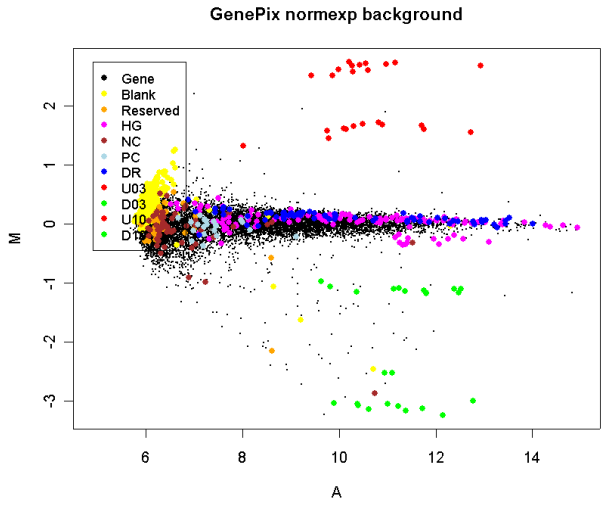
```
> RG <- backgroundCorrect(RG, method="normexp", offset=50)
```

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.

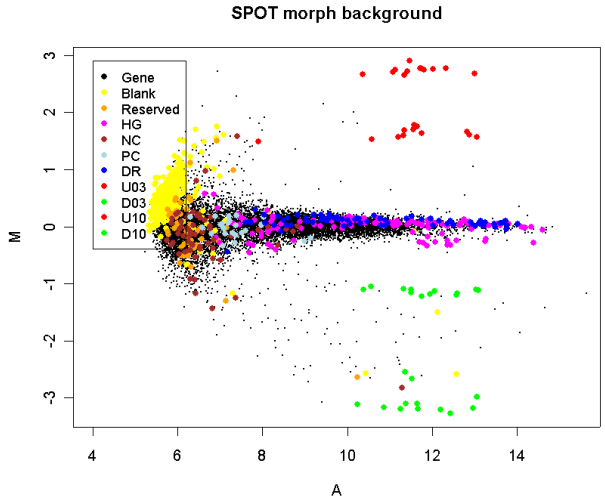
To illustrate some differences between the different background correction methods we consider one cDNA array which was self-self hybridized, i.e., the same RNA source was hybridized to both channels. For this array there is no actual differential expression. The array was printed with a human 10.5k 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 [30].

6.2 Within-Array Normalization

Limma implements a range of normalization methods for spotted microarrays. Smyth and Speed [38] 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 [47] 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 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 [22] 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 [22]. A whole-library-pool means that one makes a pool of a library of probes, and prints spots from the pool at various concentrations [46]. 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 [22].

The idea of up-weighting the titration spots is in the same spirit as the composite normalization method proposed by [46] 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 [49]. This section shows how to reproduce some of the results given in [49]. The Apoa1 data set from Section 16.2 will be used to illustrate these methods. We assume that the Apoa1 data has been loaded and background corrected as follows:

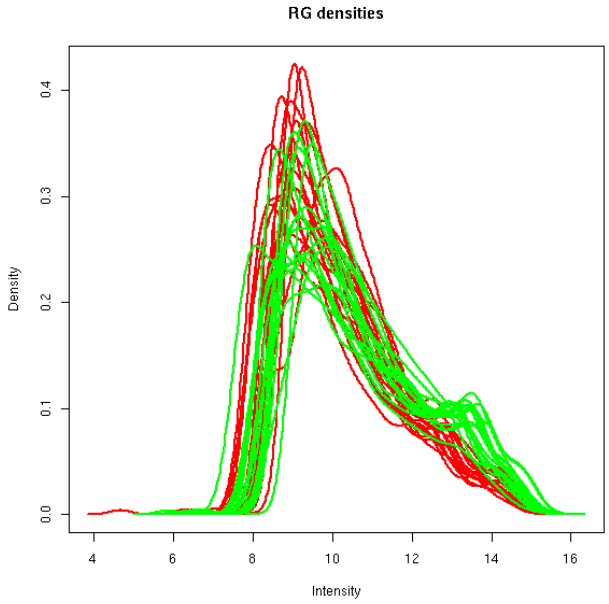
```
> load("Apoa1.RData")
```

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")
```

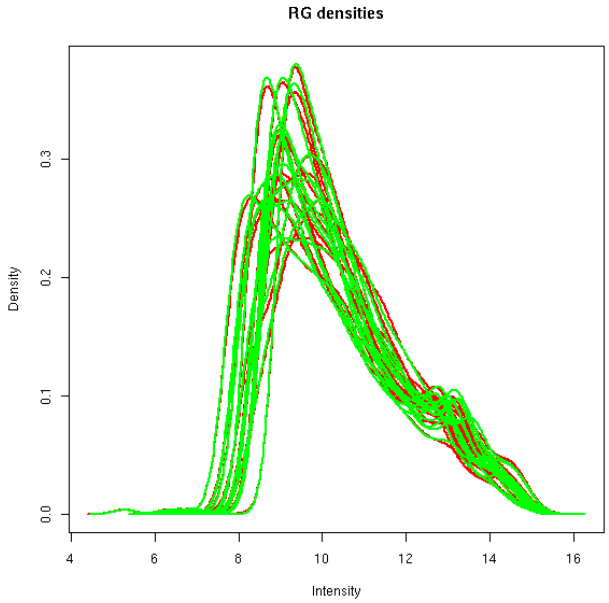
`plotDensities` displays smoothed empirical densities for the individual green and red channels on all the arrays. Without any normalization there is considerable variation between both channels and between arrays:

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



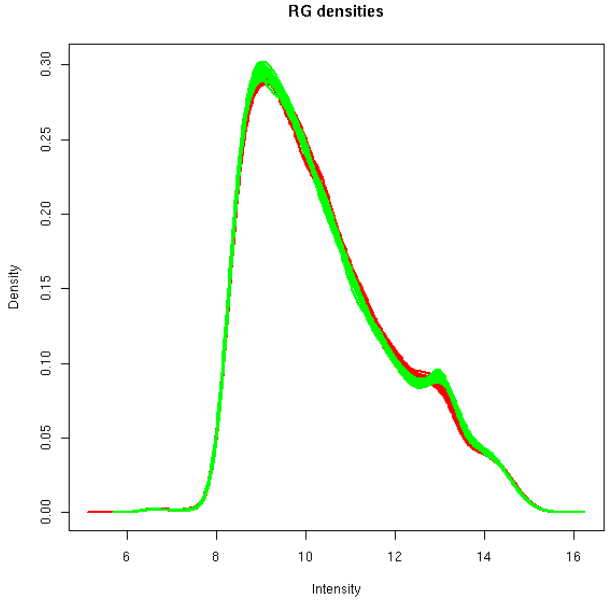
After loess normalization of the M-values for each array the red and green distributions become essentially the same for each array, although there is still considerable variation between arrays:

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



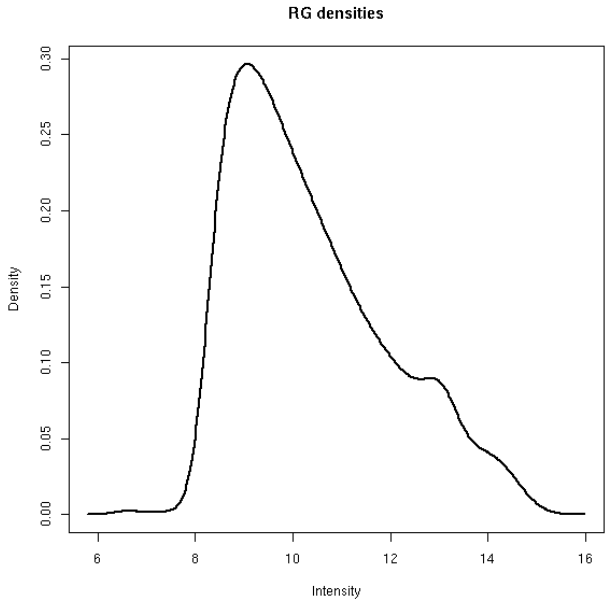
Loess normalization doesn't affect the A-values. Applying quantile normalization to the A-values makes the distributions essentially the same across arrays as well as channels:

```
> 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 unexpressed probes

Sometimes the expression profiling platform (microarray or RNA-seq) includes probes or genes that do not appear to be expressed to a worthwhile degree in any of the RNA samples being compared. This might occur for example for genes that are not expressed in any of the cell types being profiled in the experiment. Genes that are never expressed are, by definition, not differentially expressed, and such genes are unlikely to be of biological interest in a study. In these cases, it may be possible to simplify the differential expression analysis by removing such genes from further consideration early in the analysis. Whether this is worthwhile and how it is done depends on the expression platform.

For two-color microarrays, it is usual to use all the available probes during the background correction and normalization steps. Control probes that don't correspond to genes are usually then removed before the differential expression analysis steps, but no other filtering is done.

For single-channel microarrays, it often is worthwhile to identify and filter unexpressed probes. Some microarray platforms, including Illumina, Agilent and Affymetrix arrays, include control probes from which the background intensity corresponding to unexpressed probes can be estimated. In such cases, it may be useful to keep probes that are expressed above background on at least k arrays, where k is the smallest number of replicates assigned to any of the treatment combinations. Such filtering is done before the linear modeling and empirical Bayes steps but after normalization. Section 17.3.4 gives an example of filtering with Illumina arrays and Section 17.4.6 gives an example with Agilent arrays.

If background control probes are not available, an alternative single-channel approach is to filter probes based on their average log-expression values (`AveExpr`) as computed by `lmFit` and stored as `Amean`. A histogram of the `Amean` values and a sigma vs Amean plot may help identify a cutoff below which `Amean` values can be filtered:

```
fit <- lmFit(y, design)
hist(fit$Amean)
plotSA(fit)
```

Then empirical Bayes and downstream analyses can be conducted on the filtered fit object:

```
keep <- fit$Amean > CutOff
fit2 <- eBayes(fit[keep,], trend=TRUE)
plotSA(fit2)
```

Filtering is most important for RNA-seq. For RNA-seq data is important to filter out genes or exons that are never detected or have very small counts. An effective method is to keep genes or exons that have a worthwhile count, say 5–10 or more, in at least k arrays, where k is the smallest

number of replicates assigned to any of the treatment combinations. The edgeR package provides the `filterByExpr` function to identify genes or exons for filtering, as will be outlined briefly in Section 15.3 [6].

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 [35, 48]. 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 modeled 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 [35, 24]. For arrays with within-array replicate spots, limma uses a pooled correlation method to make full use of the duplicate spots [37].

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:

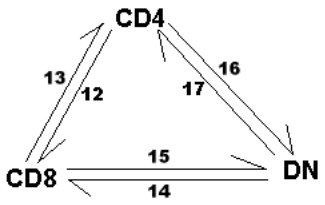
```
> contrast.matrix <- makeContrasts(AML1,CBFb,AML1.CBFb,AML1.CBFb-AML1,AML1.CBFb-CBFb,
+      levels=design)
> contrast.matrix
          AML1 CBFb AML1.CBFb AML1.CBFb - AML1 AML1.CBFb - CBFb
AML1         1    0         0               -1                0
AML1.CBFb    0    0         1                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 [15]. 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:

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

Here, with the `coef` argument not specified, `topTable` will test all coefficients or contrasts found in the linear model object equal to zero. When two or more contrasts are present, then the F-tests for those contrasts are reported. In this case, `fit2` contains three contrasts, so F-tests on 3 degrees of freedom (df) will be reported.

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 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 wild-type 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)
> topTable(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)
> topTable(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)
> topTable(fit2, adjust="BH")
```

The method of analysis described in this section was used for a six-point time course experiment on histone deacetylase inhibitors [23].

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)
```

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 [21, 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 [37].

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)
```

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 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 [36]. 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 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, 36]. 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 [36]. 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)
```

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., squeezed 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 [35, 24]. 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 [13].

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 [2]. 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 [35, 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 [39, 37]. Benjamini and Hochberg's control of the false discovery rate assumes independence between genes, although Reiner et al [27] 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 g has residual variance σ_g^2 with sample value s_g^2 and degrees of freedom d_g. The output from `lmFit()`, `fit` say, holds the s_g in component `fit$sigma` and the d_g in `fit$df.residual`. The covariance matrix of the estimated $\hat{\boldsymbol{\beta}}_g$ is $\sigma_g^2 C^T (X^T V_g X)^{-1} C$ where X is the design matrix and C is the contrast matrix and V_g is a weight matrix. The weight matrix is determined by precision weights specified by the user, any covariance terms introduced by correlation structure and any working weights introduced by robust estimation. The square-roots of the diagonal elements of $C^T (X^T V_g X)^{-1} C$ are called unscaled standard deviations and are stored in `fit$stdev.unscaled`. The ordinary t-statistic for the kth contrast for gene g is $t_{gk} = \hat{\beta}_{gk}/(u_{gk} s_j)$ where u_{gk} is the unscaled standard deviation. The ordinary t-statistics could be recovered by

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

after fitting a linear model, although the moderated versions are always recommended.

The empirical Bayes method assumes a scaled chisquare prior distribution for $1/\sigma_g^2$ with mean $1/s_0^2$ and degrees of freedom d_0. The posterior values for the residual variances are given by

$$\tilde{s}_g^2 = \frac{d_0 s_0^2 + d_j s_g^2}{d_0 + d_g}$$

where d_g is the residual degrees of freedom for the gth gene. Mathematically, $1/\tilde{s}_g^2$ is derived as the posterior mean of $1/\sigma_g^2$ conditional on s_g^2 [35, 24]. 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$s2.post`. If robust empirical Bayes is used [24], then `fit$s2.post` is a vector, otherwise it is a single value.

The moderated t-statistic is

$$\tilde{t}_{gk} = \frac{\hat{\beta}_{gk}}{u_{gk}\tilde{s}_g},$$

which can be shown to follow a t-distribution on $d_0 + d_g$ degrees of freedom if $\beta_{gk} = 0$ [35]. By comparison, an ordinary t-statistic would have only d_g degrees of freedom. The prior degrees of freedom d_0 represent the extra information that is borrowed from the ensemble of genes for inference about each individual gene. The output from `eBayes()` contains the $\tilde{t}_{gk}$ as `fit$t` with corresponding unadjusted p-values in `fit$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 specialized aim to give greater weight to probes that 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

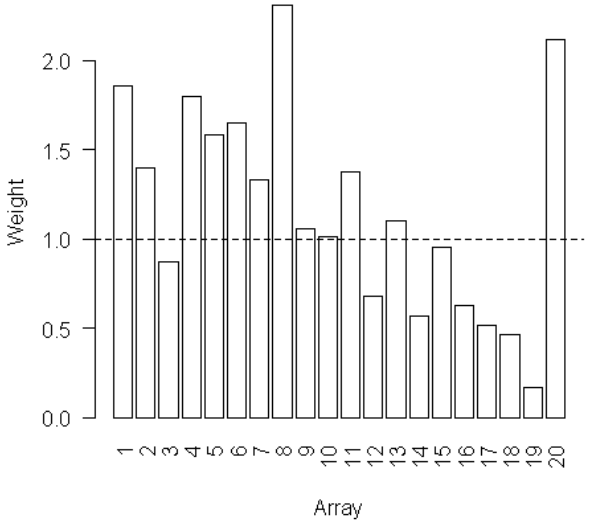
Given an appropriate design matrix, the relative reliability of each array in an experiment can be estimated by measuring how well the expression values for that array follow the linear model. This empirical approach of assessing array quality can be applied to both two-color and single-channel microarray data and is described in [28].

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`. 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 [28]) 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)
```

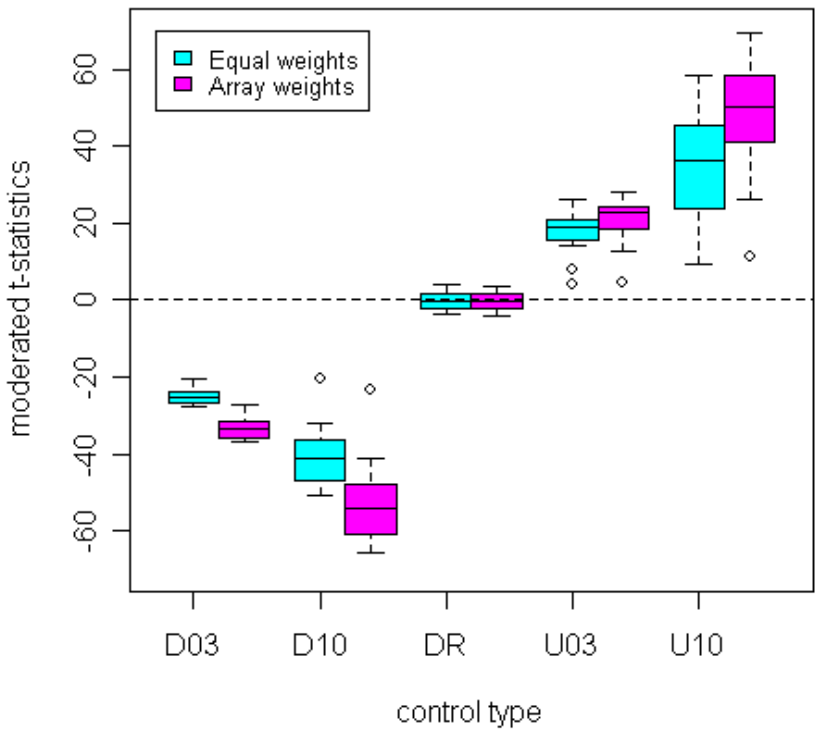


The empirical array weights vary from a minimum of 0.16 for array 19 to a maximum of 2.31 for array 8. These weights can be used in the linear model analysis.

```
> 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 [28] 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])
```

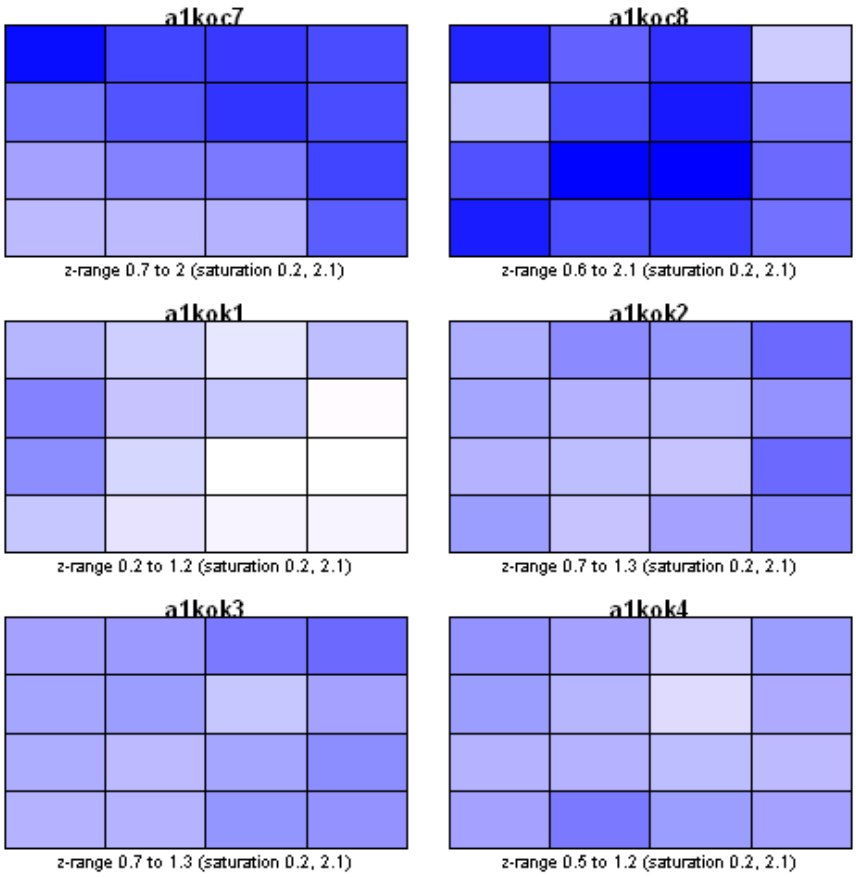


Image plots of the print-tip weights for arrays 7 through to 12 are shown above, with lighter shades indicating print-tip groups which have been assigned lower weights. A corner of array 9 (a1kok1) is measured to be less reproducible than the same region on other arrays, which may be indicative of a 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 16.2).

```
> 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 [10]. 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 down-weighted. 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

RNA-Seq Data

15.1 Introduction

The limma approach to RNA-seq explained in the articles by Law et al [15], Liu et al [19] and Baldoni et al [1]. See also the article by Law et al [14], which gives a complete workflow case study. In the limma approach to RNA-seq, read counts are converted to log2-counts-per-million (logCPM) and the mean-variance relationship is modeled either with precision weights or with an empirical Bayes prior trend. The precision weights approach is called "voom" and the prior trend approach is called "limma-trend" [15]. In either case, the RNA-seq data can be analyzed as if it was microarray data. This means that any of the linear modeling or gene set testing methods in the limma package can be applied to RNA-seq data.

15.2 Making a count matrix

RNA-seq data usually arrives in the form of FastQ or BAM files of unaligned reads. The reads need to be mapped to a reference genome or transcriptome, then summarized at the exon or gene level to produce a matrix of counts. We find the Rsubread package [17] to be convenient, fast and effective for this purpose. Other popular methods include RSEM [16] and HTseq. A runnable example with complete code showing how to use Rsubread with limma is provided at `https://subread.sourceforge.net/RNAseqCaseStudy.html`. Another complete code example is provided by Chen et al [7].

15.3 Normalization and filtering

Once a matrix of read counts `counts` has been created, with rows for genes and columns for samples, it is convenient to create a `DGEList` object using the edgeR package:

```
> dge <- DGEList(counts=counts)
```

The next step is to remove rows that consistently have zero or very low counts. One can for example use

```
> keep <- filterByExpr(dge, design)
> dge <- dge[keep,,keep.lib.sizes=FALSE]
```

where `filterByExpr` is a function in the edgeR package. Here we will assume that filtering has been done.

It is usual to apply scale normalization to RNA-seq read counts, and the TMM normalization method [31] in particular has been found to perform well in comparative studies. This can be applied to the `DGEList` object:

```
> dge <- calcNormFactors(dge)
```

15.4 Differential expression: limma-trend

If the sequencing depth is reasonably consistent across the RNA samples, then the simplest and most robust approach to differential exis to use limma-trend. This approach will usually work well if the ratio of the largest library size to the smallest is not more than about 3-fold.

In the limma-trend approach, the counts are converted to logCPM values using edgeR's `cpm` function:

```
> logCPM <- cpm(dge, log=TRUE, prior.count=3)
```

The prior count is used here to damp down the variances of logarithms of low counts.

The logCPM values can then be used in any standard limma pipeline, using the `trend=TRUE` argument when running `eBayes` or `treat`. For example:

```
> fit <- lmFit(logCPM, design)
> fit <- eBayes(fit, trend=TRUE)
> topTable(fit, coef=ncol(design))
```

Or, to give more weight to fold-changes in the gene ranking, one might use:

```
> fit <- lmFit(logCPM, design)
> fit <- treat(fit, lfc=log2(1.2), trend=TRUE)
> topTreat(fit, coef=ncol(design))
```

15.5 Differential expression: voom

When the library sizes are quite variable between samples, then the voom approach is theoretically more powerful than limma-trend. In this approach, the voom transformation is applied to the normalized and filtered `DGEList` object:

```
v <- voom(dge, design, plot=TRUE)
```

The voom transformation uses the experiment design matrix, and produces an `EList` object.

It is also possible to give a matrix of counts directly to voom without TMM normalization, by

```
> v <- voom(counts, design, plot=TRUE)
```

If the data are very noisy, one can apply the same between-array normalization methods as would be used for microarrays, for example:

```
> v <- voom(counts, design, plot=TRUE, normalize="quantile")
```

After this, the usual limma pipelines for differential expression can be applied, for example:

```
> fit <- lmFit(v, design)
> fit <- eBayes(fit)
> topTable(fit, coef=ncol(design))
```

Or, to give more weight to fold-changes in the ranking, one could use say:

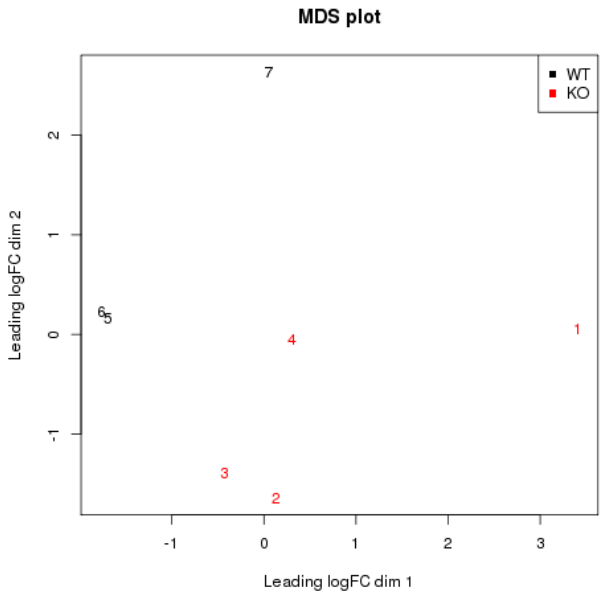
```
> fit <- treat(fit, lfc=log2(1.2))
> topTreat(fit, coef=ncol(design))
```

15.6 Voom with sample quality weights

When a multi-dimensional scaling plot from a designed RNA-seq experiment indicates the presence of outlier samples, it is possible to combine the observation-level weighting strategy used in voom with sample-specific quality weights (as described in the section above on Array Quality Weights) to down-weight outlier samples. This capability is implemented in the `voomWithQualityWeights` function.

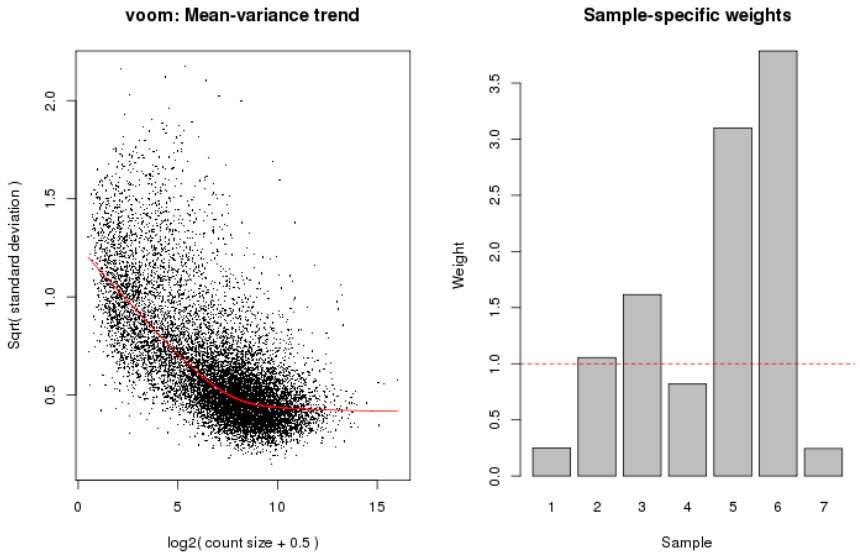
The example below shows its use on an RNA-seq data set where the epigenetic regulator *Smchd1* has been knocked-out in lymphoma cell-lines (GEO series GSE64099) [19]. Overall we obtain more differential expression by applying this combined weighting strategy and the raw p-value and false discovery rate for the *Smchd1* gene, which has been knocked out, is smaller.

```
> plotMDS(x, labels=1:7, col=as.numeric(genotype), main="MDS plot")
> legend("topright", legend=c("WT", "KO"), col=1:2, pch=15)
```




```
> # Analysis with voom only
>  des[1:7,]
  (Intercept) Smchd1nullvsWt
1           1              1
2           1              1
3           1              1
4           1              1
5           1              0
```

```
6           1              0
7           1              0
> v <- voom(x, design=des)
> plotMDS(v, labels=1:7, col=as.numeric(genotype))
> vfit <- lmFit(v)
> vfit <- eBayes(vfit)
> options(digits=3)
> topTable(vfit,coef=2,sort.by="P")
       GeneID    Symbols logFC AveExpr      t  P.Value adj.P.Val    B
74355   74355     Smchd1 -3.12   6.067 -23.35 2.16e-08  0.000266 9.97
18028   18028       Nfib  8.98   1.714  12.60 2.17e-06  0.013355 3.15
75605   75605      Kdm5b -3.55   3.618 -11.75 3.62e-06  0.014857 5.06
667435 667435 Igkv17-121 -5.35  -1.435 -10.22 9.95e-06  0.025513 2.57
381126 381126      Garem  6.17   0.113  10.08 1.10e-05  0.025513 2.35
381413 381413     Gpr176 -4.02   1.328  -9.90 1.25e-05  0.025513 3.39
75033   75033       Mei4  6.44   0.259   9.69 1.45e-05  0.025513 2.23
69136   69136      Tusc1  5.67  -0.184   8.90 2.67e-05  0.040995 1.87
233552 233552      Gdpd5 -2.82   1.948  -8.56 3.49e-05  0.042754 2.81
80890   80890      Trim2 -1.43   4.491  -8.40 4.00e-05  0.042754 2.72
> top <- topTable(vfit,coef=2,number=Inf,sort.by="P")
> sum(top$adj.P.Val<0.05)
[1] 12
> # Analysis with combined voom and sample quality weights
> vwts <- voomWithQualityWeights(x, design=des, normalization="none", plot=TRUE)
> vfit2 <- lmFit(vwts)
> vfit2 <- eBayes(vfit2)
> topTable(vfit2,coef=2,sort.by="P")
       GeneID Symbols logFC AveExpr     t  P.Value adj.P.Val     B
74355   74355  Smchd1 -3.17   6.067 -28.5 1.61e-09  1.98e-05 12.57
18028   18028    Nfib  9.23   1.714  19.0 4.44e-08  2.73e-04  6.91
381126 381126   Garem  6.45   0.113  15.9 1.85e-07  7.58e-04  6.02
75033   75033    Mei4  6.56   0.259  15.0 2.84e-07  8.73e-04  5.83
69136   69136   Tusc1  5.88  -0.184  13.6 6.16e-07  1.11e-03  5.31
54354   54354  Rassf5  5.74   4.554  13.6 6.26e-07  1.11e-03  6.63
75605   75605   Kdm5b -3.80   3.618 -13.5 6.53e-07  1.11e-03  6.67
58998   58998   Pvrl3  7.69   0.961  13.1 8.46e-07  1.11e-03  5.33
320398 320398   Lrig3  7.39   1.584  13.1 8.49e-07  1.11e-03  5.32
17069   17069    Ly6e  2.63   7.605  13.0 9.01e-07  1.11e-03  6.26
> top2 <- topTable(vfit2,coef=2,number=Inf,sort.by="P")
> sum(top2$adj.P.Val<0.05)
[1] 1478
```



15.7 Differential expression: voomLmFit

In recent versions of limma and edgeR, we now recommend `voomLmFit` as the preferred interface for RNA-seq analyses, replacing `voom`, `lmFit` and `voomWithQualityWeights`. `voomLmFit` ensures unbaised estimation of the residual variances, even for genes that have no counts at all in some experimental conditions [20]. `voomLmFit` also automates the estimation of sample weights and intrablock correlations. For example, the Schmd1 analysis with sample weights shown in the previous section could be more efficiently coded by:

```
> vfit2 <- voomLmFit(x, design=des, sample.weights=TRUE)
> vfit2 <- eBayes(vfit2)
> topTable(vfit2, coef=2, sort.by="P")
```

15.8 Differential splicing

limma can also detect genes that how evidence of differential splicing between conditions. One can test for differential splicing associated with any contrast for a linear model [1].

In this case, the matrix of counts should be at the exon level, with a row for each exon. For example,

```
> dge <- DGEList(counts=counts)
> dge$genes$GeneID <- GeneID
```

where `counts` is a matrix of exon-level counts, and GeneID identifies which gene each exon belongs to. Then filter and normalize:

```
> A <- rowSums(dge$counts)
> dge <- dge[A>10,, keep.lib.sizes=FALSE]
> dge <- calcNormFactors(dge)
```

Then apply the voom transformation and fit a linear model:

```
> fit <- voomLmFit(dge, design, plot=TRUE)
```

Now we can test for differential splicing associated with any coefficient in the linear model. First run the diffSplice function:

```
> ex <- diffSplice(fit, geneid="GeneID")
```

Then

```
> topSplice(ex, coef=2, test="simes")
```

will find genes that show evidence of differential splicing associated with the second coefficient in the linear model. The output is similar that from the limma topTable function. More detail can be obtained by

```
> topSplice(ex, coef=2, test="t")
```

which will show individual exons that are enriched or depleted relative to other exons in the same gene. To display the pattern of exons in the top genes:

```
> plotSplice(ex)
```

Chapter 16

Two-Color Case Studies

16.1 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 `https://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 labeled with green (Cy3) dye and wild type RNA was labeled 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")
```

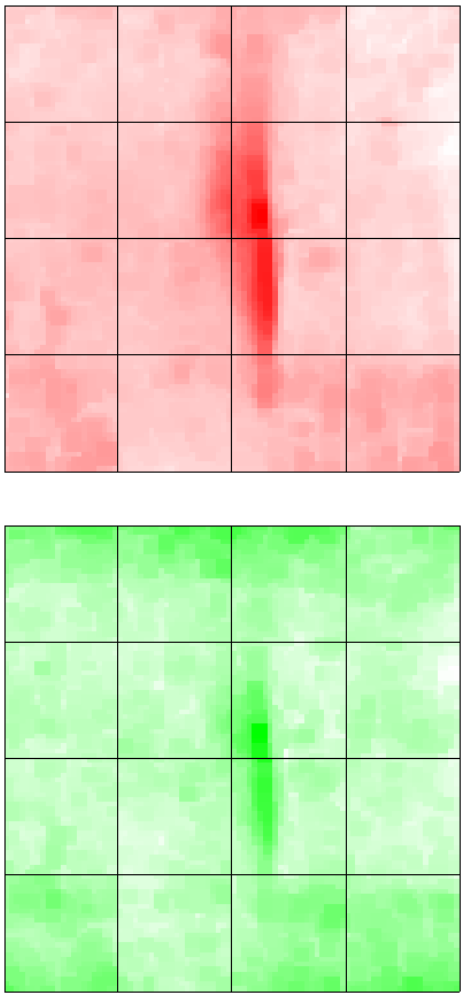
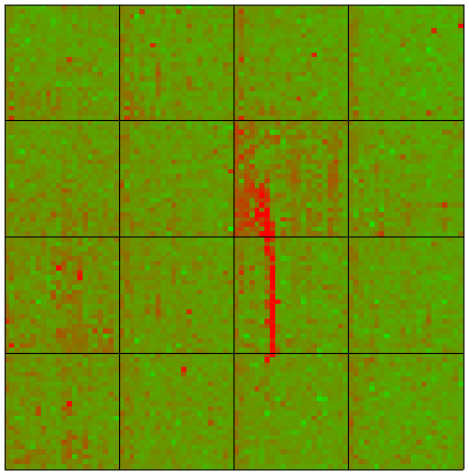


Image plot of the un-normalized log-ratios or M-values for the first array:

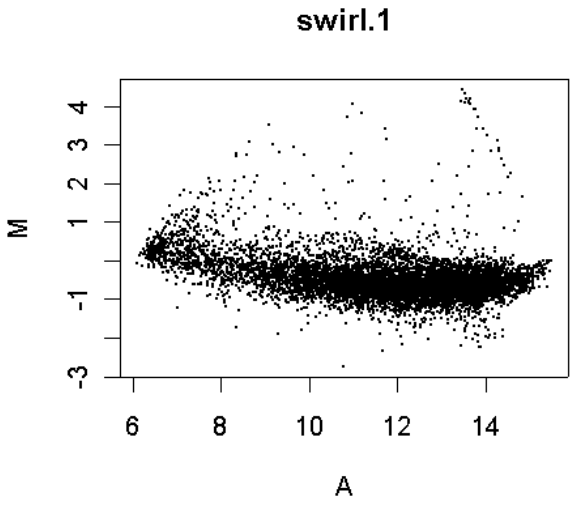
```
> MA <- normalizeWithinArrays(RG, method="none")
> imageplot(MA$M[,1], RG$printer, zlim=c(-3,3))
```



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 artifact will have suspect M-values. The streak also shows up as darker regions in the background plots.

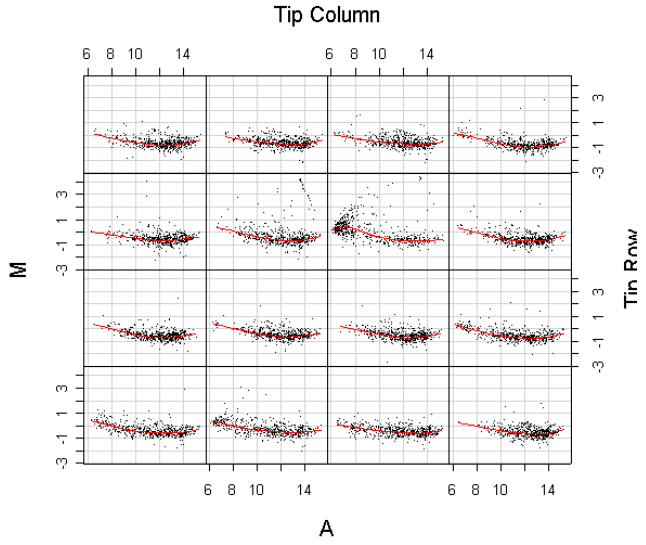
MA-plots. An MA-plot plots the log-ratio of R vs G against the overall intensity of each spot. The log-ratio is represented by the M-value, $M = \log_2(R) - \log_2(G)$, and the overall intensity by the A-value, $A = (\log_2(R) + \log_2(G))/2$. Here is the MA-plot of the un-normalized values for the first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

```
> plotMD(MA)
```



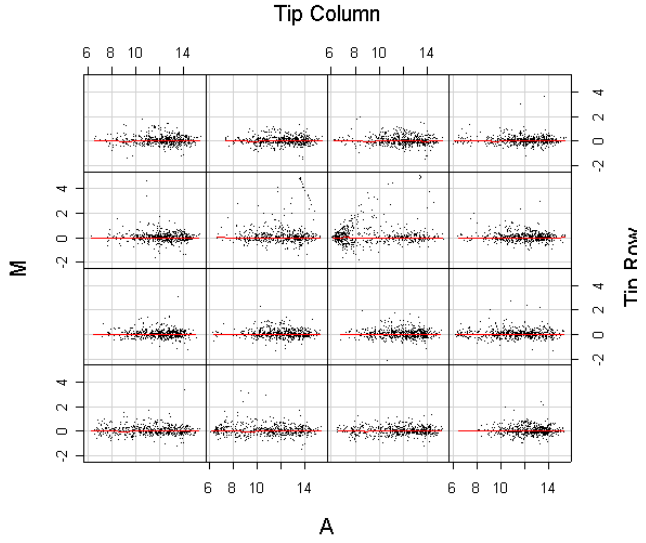
The red streak seen on the image plot can be seen as a line of spots in the upper right of this plot. Now we plot the individual MA-plots for each of the print-tip groups on this array, together with the loess curves which will be used for normalization:

```
> plotPrintTipLoess(MA)
```



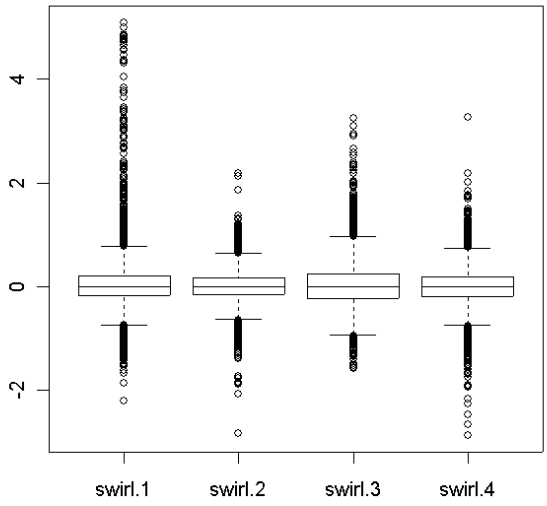
Normalization. Print-tip loess normalization:

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



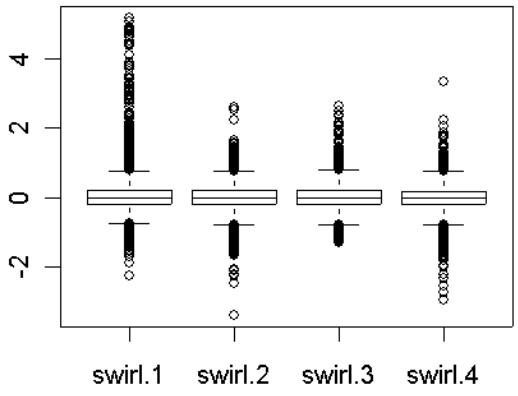
We have normalized the M-values with each array. A further question is whether normalization is required between the arrays. The following plot shows overall boxplots of the M-values for the four arrays.

```
> 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
```

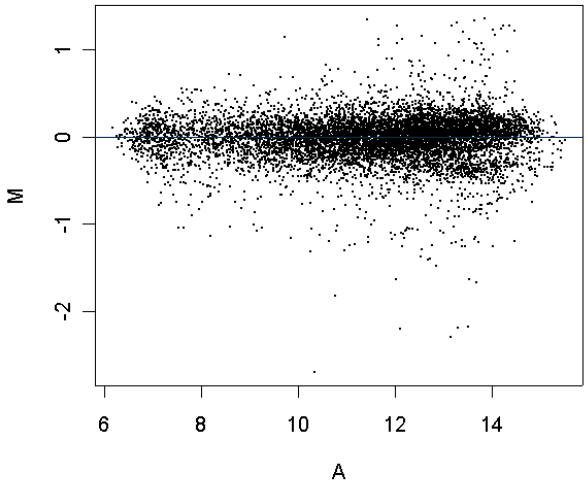
In the above fit object, `coefficients` is the average M-value for each gene and `sigma` is the sample standard deviations for each gene. Ordinary t-statistics for comparing mutant to wt could be computed by

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

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

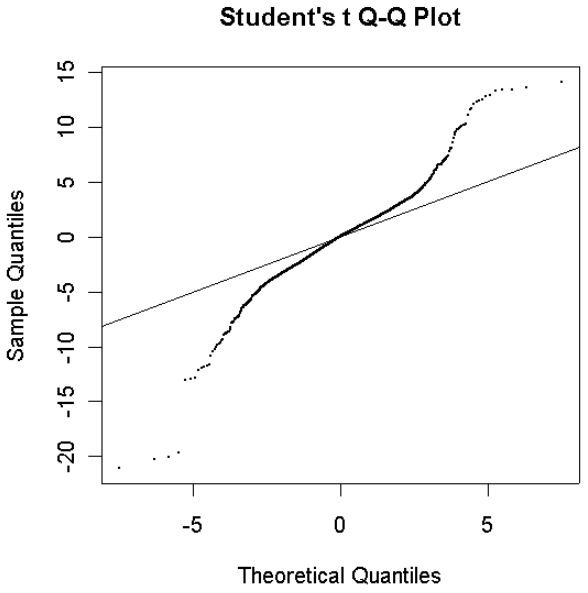
Now create an mean difference plot displaying the log-fold-changes and average A-values for each gene.

```
> plotMD(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 squeezed 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)
```



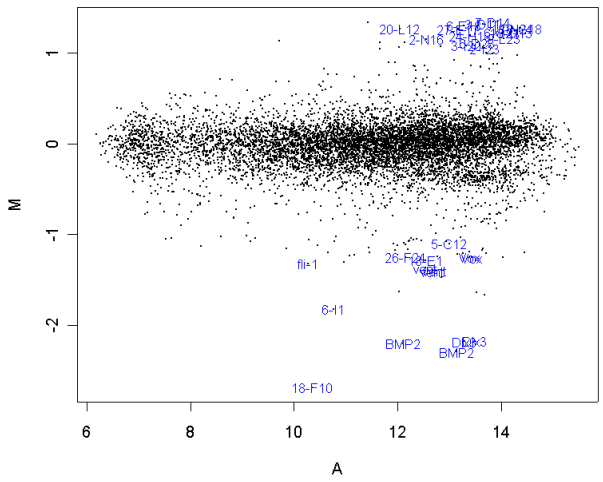
Visually there seems to be plenty of genes which are differentially expressed. We will obtain a summary table of some key statistics for the top genes.

```
> options(digits=3)
> topTable(fit,number=30)
     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.

```
> plotMD(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")
```



16.2 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 `https://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.

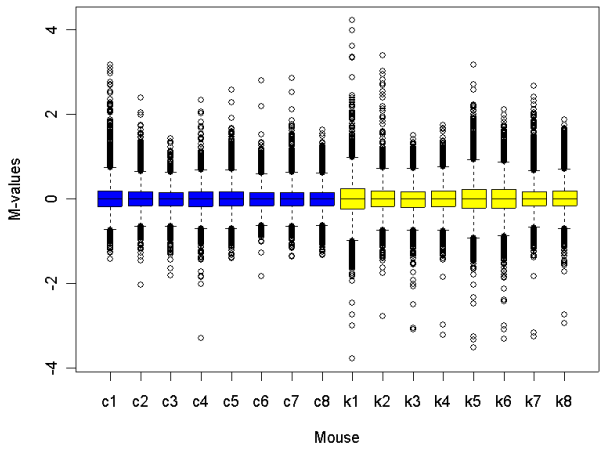
Hybridizations. The experiment compared 8 Apoa1 knockout mice with 8 normal C57BL/6 ("black six") mice, the control mice. For each of these 16 mice, target mRNA was obtained from liver tissue and labelled using a Cy5 dye. The RNA from each mouse was hybridized to a separate microarray. Common reference 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)
```

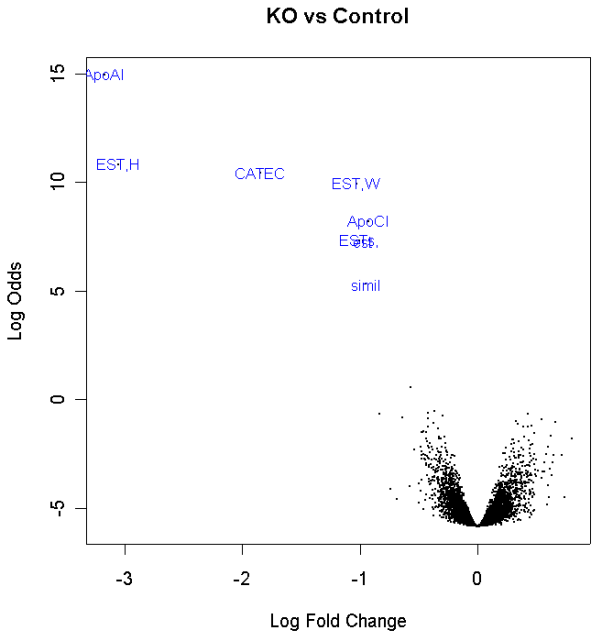
However, the gene annotation is a bit wide for the printed page, so we will tell `topTable()` to show just one column of the annotation information:

```
> 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")
```



16.3 Weaver Mutant Mice: A Composite 2x2 Factorial Experiment

16.3.1 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 [9]. 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.

16.3.2 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.

16.3.3 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)
```

16.3.4 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"]
```

16.3.5 Quality Assessment and Normalization

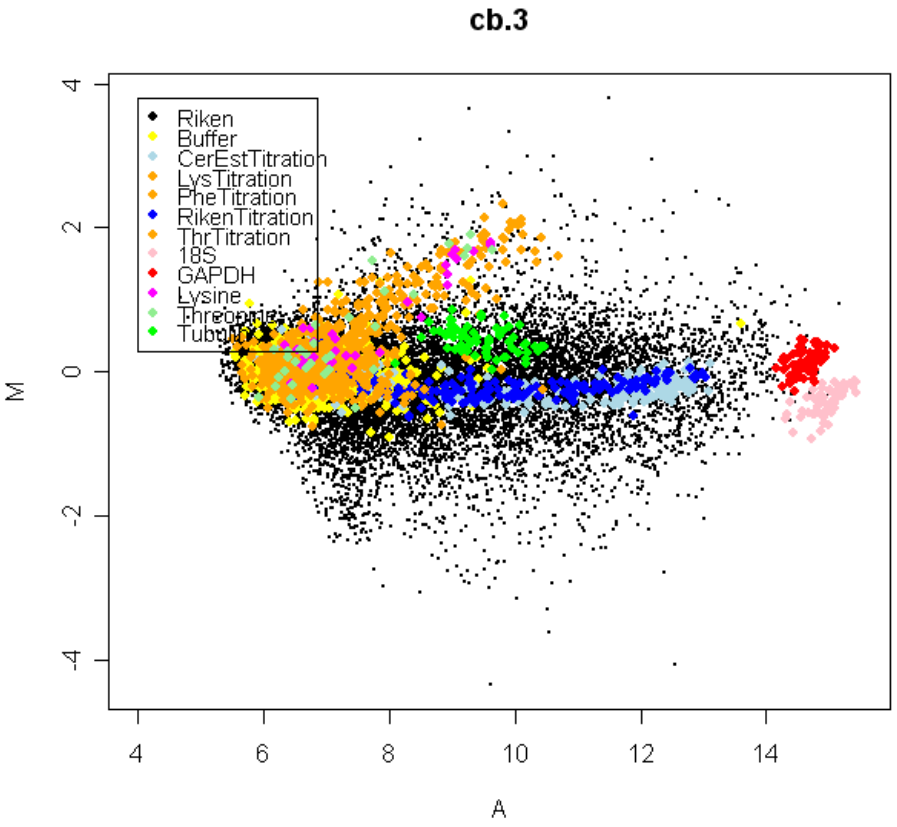
We also read in a spot-types file and set a range of control spots.

```
> 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:

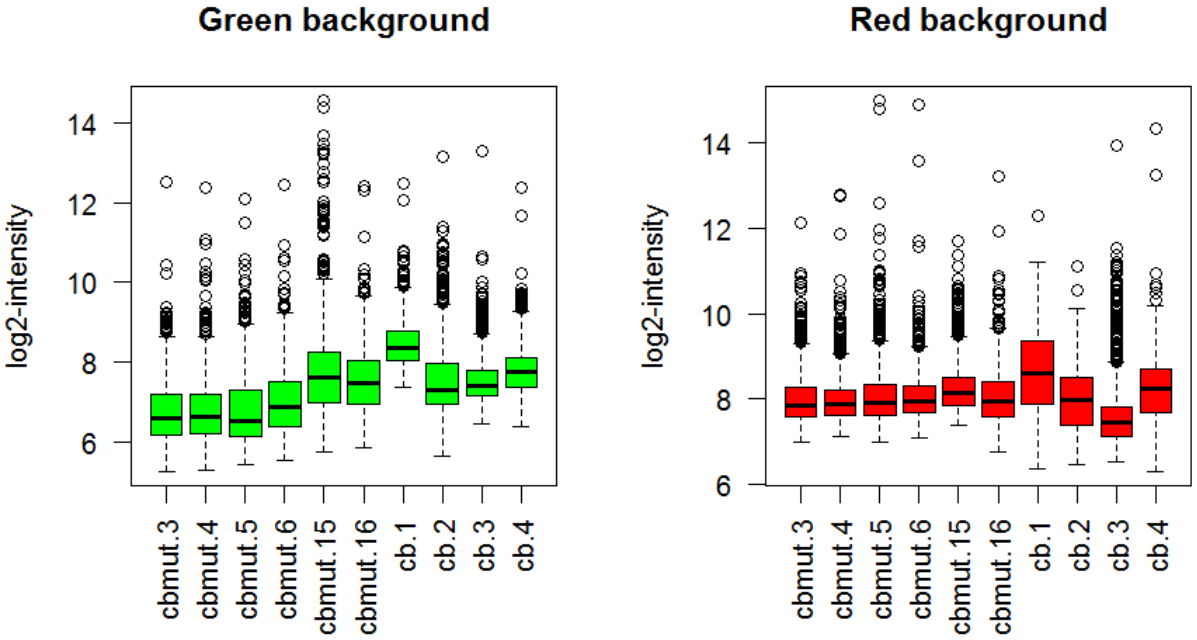
```
> plotMD(RG,column=9,xlim=c(4,15.5))
```



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)
```

16.3.6 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
```

16.3.7 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)
```

16.3.8 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 wild-type 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
```

16.4 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 [37]. 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.

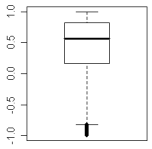
```
> MA <- normalizeWithinArrays(RG)
```

Then remove the control probes:

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

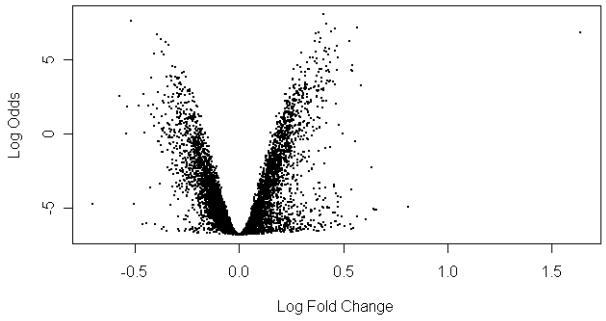
Now estimate the spatial correlation. We estimate a correlation term by REML for each gene, and then take a trimmed mean on the atanh scale to estimate the overall correlation. This command will probably take at least a few minutes depending on the speed of your computer.

```
> 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)
```

```
     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)
```



Chapter 17

Single-Channel Case Studies

17.1 Lrp Mutant *E. Coli* Strain with Affymetrix Arrays

17.1.1 Background

Gene expression in two *Escherichia coli* bacteria strains, labelled lrp+ and lrp-, were compared using eight Affymetrix *E. coli* chips, four chips each for lrp+ and lrp-. The data are from experiments reported in [11], who 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.

17.1.2 Downloading the data

The raw data files can be downloaded from `http://bioinf.wehi.edu.au/limma/`. The following code assumes that the files in your current working directory:

```
> dir()
 [1] "Escherichia_coli_str._K-12_substr._MG1655.gene_info.gz" "nolrp_1.CEL"
 [3] "nolrp_2.CEL"                                            "nolrp_3.CEL"
 [5] "nolrp_4.CEL"                                            "targets.txt"
 [7] "wt_1.CEL"                                               "wt_2.CEL"
 [9] "wt_3.CEL"                                               "wt_4.CEL"
```

The file `targets.txt` describes the design of the experiment:

```
> library(limma)
> targets <- readTargets("targets.txt")
> targets
           FileName Strain Experiment
nolrp_1 nolrp_1.CEL   lrp-          1
nolrp_2 nolrp_2.CEL   lrp-          2
nolrp_3 nolrp_3.CEL   lrp-          3
nolrp_4 nolrp_4.CEL   lrp-          4
wt_1       wt_1.CEL   lrp+          1
wt_2       wt_2.CEL   lrp+          2
```

```
wt_3       wt_3.CEL   lrp+          3
wt_4       wt_4.CEL   lrp+          4
```

The `lrp-` samples are from the Lrp-deficient strain while the `lrp+` are from the wild-type strain. The data was collected in four batches or "experiments".

17.1.3 Background correction and normalization

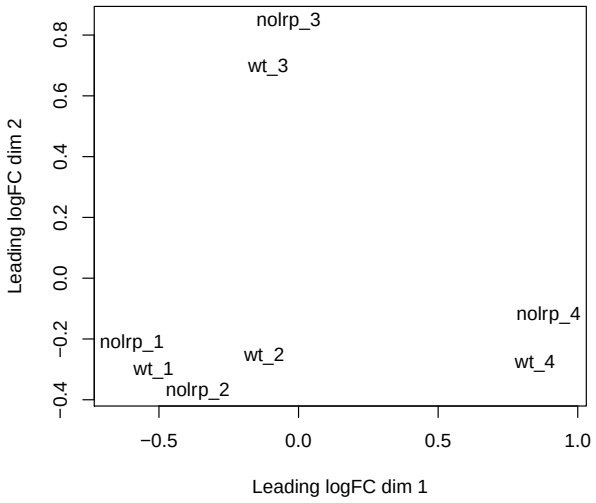
The data is read and normalized using the affy package. The package ecolicdf must also be installed, otherwise the `rma()` function will attempt to download and install it for you.

```
> library(affy)
> eset <- justRMA(filenames = targets$FileName)
> colnames(eset) <- row.names(targets)
> head(exprs(eset))
              nolrp_1 nolrp_2 nolrp_3 nolrp_4  wt_1  wt_2  wt_3  wt_4
aas_b2836_st     8.53    9.20    8.18    8.01  8.89  8.52  8.28  8.11
aat_b0885_st     8.12    8.76    7.96    8.37  7.88  8.61  7.74  8.52
abc_b0199_st    10.65   10.31   10.17   11.60 10.44 10.58 10.20 11.48
abrB_b0715_st    9.32    9.16    9.68    9.20  9.21  9.28  9.65  9.94
accA_b0185_st   11.28   11.38   10.93   10.64 11.22 11.41 11.14 10.82
accB_b3255_st   12.52   11.81   12.32   12.69 12.65 12.29 12.59 12.66
```

The rownames of the expression matrix combine the gene symbols (`aas`) with an open reading frame identifier (`b2836`). This format is idiosyncratic to this historical GeneChip.

An MDS plot shows clearly that there is a batch effect between the four experiments:

```
> plotMDS(eset)
```



17.1.4 Gene annotation

The gene information file `Escherichia_coli_str._K-12_substr._MG1655.gene_info.gz` was downloaded from the NCBI ftp site at `ftp://ftp.ncbi.nlm.nih.gov/gene/DATA/GENE_INFO`. We can use this file to update the gene annotation.

First we extract the old gene symbols from the rownames:

```
> Alias <- sub("_.*", "", row.names(eset))
> Alias[1:5]
[1] "aas"  "aat"  "abc"  "abrB" "accA"
```

Then we use limma function `alias2SymbolUsingNCBI` to update the old gene symbols to current symbols and associated information. The information is stored in the `fData` slot of the ExpressionSet:

```
> GeneInfo <- "Escherichia_coli_str._K-12_substr._MG1655.gene_info.gz"
> fData(eset) <- alias2SymbolUsingNCBI(Alias, GeneInfo,
+                required=c("GeneID","Symbol","type_of_gene"))
> head(fData(eset))
     GeneID Symbol   type_of_gene
2495 947315    aas protein-coding
719  945490    aat protein-coding
153  944896   metN protein-coding
554  945321   abrB protein-coding
152  944895   accA protein-coding
2922 947758   accB protein-coding
```

We remove all probes that do not have an Entrez Gene ID and Symbol:

```
> HasSymbol <- !is.na(fData(eset)$Symbol)
> eset <- eset[HasSymbol,]
> dim(eset)
Features  Samples
    3709        8
```

17.1.5 Differential expression

Make factors for the Experiment and Strain:

```
> Exp <- factor(targets$Experiment)
> Strain <- factor(targets$Strain, levels=c("lrp+","lrp-"))
```

Then construct the design matrix, with Experiment and Strain. This will allow us to test for Strain differences adjusting for differences between the Experiments:

```
> design <- model.matrix(~Exp+Strain)
```

Now we can run the differential expression pipeline:

```
> fit <- lmFit(eset, design)
> fit <- eBayes(fit, trend=TRUE, robust=TRUE)
> results <- decideTests(fit)
> summary(results)
       (Intercept) Exp2 Exp3 Exp4 Strainlrp-
Down             0   92  382  714        101
NotSig           0 3601 3249 2859       3526
Up            3709   16   78  136         82
```

There are 82 up-regulated and 101 down-regulated genes in the Lrp- strain at FDR$<$ 0.05. Including Experiment as a batch was also clearly important.

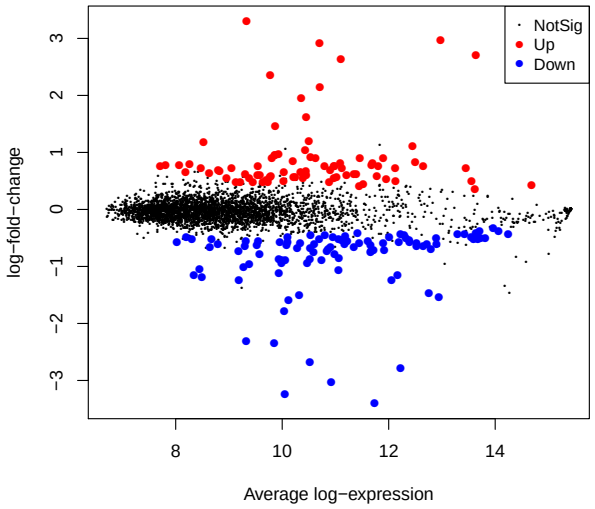
The top 30 differentially expressed genes can be seen:

```
> topTable(fit, coef="Strainlrp-", n=30)
              GeneID Symbol   type_of_gene  logFC AveExpr      t  P.Value adj.P.Val     B
oppA_b1243_st 945830   oppA protein-coding  2.976   12.97  24.64 7.39e-12  1.33e-08 17.11
rmf_b0953_st  945567    rmf protein-coding  2.705   13.64  24.08 9.74e-12  1.33e-08 16.89
ytfK_b4217_st 948736   ytfK protein-coding  2.641   11.10  23.88 1.08e-11  1.33e-08 16.80
oppB_b1244_st 945823   oppB protein-coding  2.913   10.70  21.57 3.67e-11  3.40e-08 15.77
serA_b2913_st 945258   serA protein-coding -2.776   12.22 -21.13 4.71e-11  3.50e-08 15.56
ompT_b0565_st 945185   ompT protein-coding -2.673   10.52 -20.25 7.86e-11  4.86e-08 15.11
gltD_b3213_st 947723   gltD protein-coding -3.033   10.92 -19.35 1.40e-10  7.44e-08 14.60
ydgR_b1634_st 947436   dtpA protein-coding  2.348    9.78  16.90 6.79e-10  3.15e-07 13.16
pntB_b1602_st 946144   pntB protein-coding -1.470   12.76 -14.66 3.61e-09  1.49e-06 11.59
gltB_b3212_st 947724   gltB protein-coding -3.236   10.05 -16.95 6.07e-09  2.25e-06 11.12
lrp_b0889_st  949051    lrp protein-coding -2.304    9.33 -13.74 7.73e-09  2.61e-06 10.85
oppD_b1246_st 945802   oppD protein-coding  1.944   10.36  12.86 1.66e-08  5.13e-06 10.11
stpA_b2669_st 947130   stpA protein-coding -1.789   10.04 -12.25 2.89e-08  8.18e-06  9.56
livK_b3458_st 947964   livK protein-coding -2.352    9.85 -13.11 3.09e-08  8.18e-06  9.55
ilvC_b3774_st 948286   ilvC protein-coding -1.541   12.94 -11.40 7.31e-08  1.81e-05  8.65
sodA_b3908_st 948403   sodA protein-coding -1.505   10.32 -10.26 2.14e-07  4.97e-05  7.56
ptsG_b1101_st 945651   ptsG protein-coding -1.156   12.17 -10.10 2.55e-07  5.55e-05  7.39
ndk_b2518_st  945611    ndk protein-coding -1.066   11.06  -9.82 3.51e-07  7.22e-05  7.06
pntA_b1603_st 946628   pntA protein-coding -1.585   10.12  -9.57 4.71e-07  9.20e-05  6.76
oppC_b1245_st 945810   oppC protein-coding  2.146   10.71  10.97 1.33e-06  2.28e-04  5.99
ilvH_b0078_st 947267   ilvH protein-coding -1.110    9.94  -8.87 1.05e-06  1.96e-04  5.94
glnA_b3870_st 948370   glnA protein-coding -1.246   12.06  -8.63 1.41e-06  2.28e-04  5.65
livH_b3457_st 947965   livH protein-coding -1.240    9.19  -8.63 1.41e-06  2.28e-04  5.65
artI_b0863_st 948988   artI protein-coding  1.114   12.45   8.48 1.72e-06  2.65e-04  5.45
clpA_b0882_st 945764   clpA protein-coding  0.906   11.90   8.35 2.02e-06  2.91e-04  5.28
dnaK_b0014_st 944750   dnaK protein-coding  0.722   13.45   8.34 2.04e-06  2.91e-04  5.27
ybfA_b0699_st 947452   ybfA protein-coding  0.911   10.54   8.00 3.18e-06  4.37e-04  4.81
livM_b3456_st 947966   livM protein-coding -1.041    8.45  -7.83 3.97e-06  5.26e-04  4.59
yhiP_b3496_st 948006   dtpB protein-coding  1.189    8.52   7.62 5.28e-06  6.76e-04  4.29
livF_b3454_st 947961   livF protein-coding -1.004    9.28  -7.57 5.62e-06  6.83e-04  4.23
```

Lrp itself is the 11th gene in the list.

We can plot the fold-changes for the significant genes:

```
> plotMD(fit, coef="Strainlrp-", status=results)
```



17.2 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:

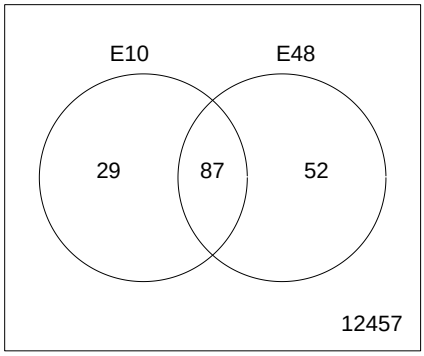
```
> results <- decideTests(fit2, method="nestedF")
```

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:

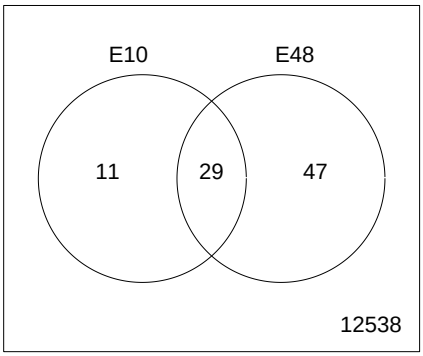
```
> i <- grep("AFFX",featureNames(eset))
> summary(fit2$F.p.value[i])
     Min.   1st Qu.    Median      Mean   3rd Qu.      Max.
0.0001391 0.1727000 0.3562000 0.4206000 0.6825000 0.9925000
```

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
```

17.3 Comparing Mammary Progenitor Cell Populations with Illumina BeadChips

17.3.1 Introduction

This case study examines the expression profiles of adult mammary stem cells and of progenitor and mature mammary luminal cells. The data was first published by Lim et al [18], who used the expression profiles to show that luminal progenitor cells are the likely cell of origin 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.

17.3.2 The target RNA samples

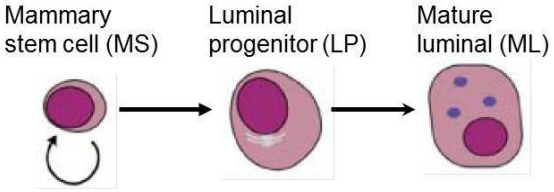
The data consists of three files:

```
> 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) [18]. The MS, LP and ML cells representative a lineage of luminal cells use to construct the ducts used to transport milk in the breast [40]:



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.

17.3.3 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 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:

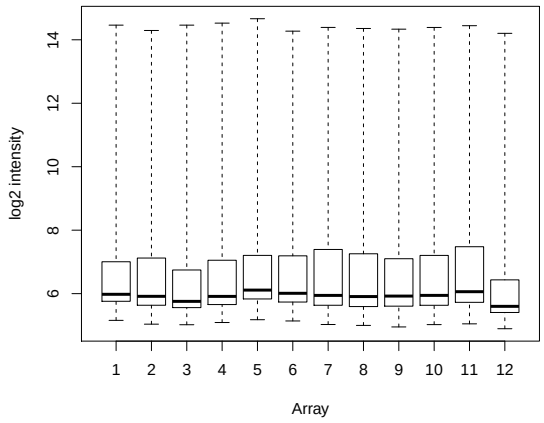
```
> table(x$genes$Status)
            BIOTIN            CY3_HYB       HOUSEKEEPING           LABELING
                 2                  6                  7                  2
LOW_STRINGENCY_HYB           NEGATIVE            regular
                 8                759              48803
```

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")
```



17.3.4 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 [32].

```
> 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%.

17.3.5 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 [33]. It also automatically removes the control probes, leaving only the regular probes in `y`:

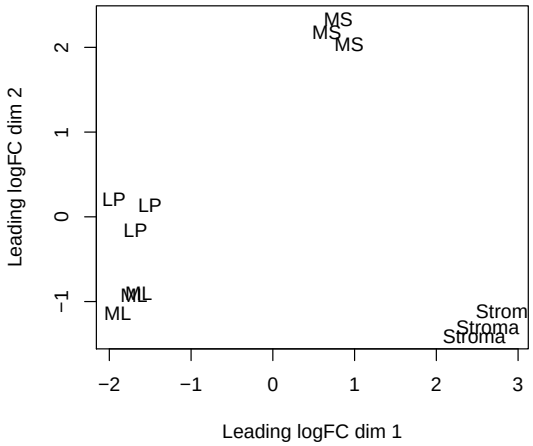
```
> dim(y)
[1] 48803    12
```

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)
```



17.3.6 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-donor 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.

17.3.7 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
Down      3074    2836    1631
NotSig   18088   18707   21307
Up        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
```

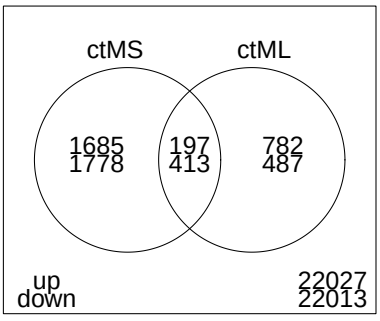
17.3.8 Signature genes for luminal progenitor cells

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
```

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

17.4.1 Introduction

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

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."

17.4.2 Data availability

All files were downloaded from `http://www.ebi.ac.uk/arrayexpress/experiments/E-GEOD-33005`. The data is also available as GEO series GSE33005.

Using R, the data can be downloaded to your working directory by:

```
> URL <- "https://www.ebi.ac.uk/arrayexpress/files/E-GEOD-33005"
> SDRF.file <- "E-GEOD-33005.sdrf.txt"
> Data.file <- "E-GEOD-33005.raw.1.zip"
> download.file(paste(URL,SDRF.file,sep="/"), SDRF.file)
> download.file(paste(URL,Data.file,sep="/"), Data.file)
> unzip(Data.file)
```

17.4.3 Reading the data

First we 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)
> SDRF[,c("Array Data File","Characteristics[treatment]")]
                                          Array Data File Characteristics[treatment]
1  GSM819076_US10283824_252828210181_S01_GE1_107_Sep09_1_4.txt          10 ml/kg corn oil
2  GSM819075_US10283824_252828210181_S01_GE1_107_Sep09_1_3.txt          10 ml/kg corn oil
3  GSM819074_US10283824_252828210181_S01_GE1_107_Sep09_1_2.txt           5 ml/kg corn oil
4  GSM819073_US10283824_252828210180_S01_GE1_107_Sep09_1_4.txt           2 ml/kg corn oil
5  GSM819072_US10283824_252828210180_S01_GE1_107_Sep09_1_3.txt           2 ml/kg corn oil
6  GSM819071_US10283824_252828210180_S01_GE1_107_Sep09_1_2.txt            10 ml/kg saline
7  GSM819070_US10283824_252828210180_S01_GE1_107_Sep09_1_1.txt            10 ml/kg saline
8  GSM819069_US10283824_252828210179_S01_GE1_107_Sep09_1_4.txt            10 ml/kg saline
9  GSM819068_US10283824_252828210179_S01_GE1_107_Sep09_1_3.txt          10 ml/kg corn oil
10 GSM819067_US10283824_252828210179_S01_GE1_107_Sep09_1_2.txt          10 ml/kg corn oil
11 GSM819066_US10283824_252828210179_S01_GE1_107_Sep09_1_1.txt          10 ml/kg corn oil
12 GSM819065_US10283824_252828210178_S01_GE1_107_Sep09_1_4.txt           5 ml/kg corn oil
13 GSM819064_US10283824_252828210178_S01_GE1_107_Sep09_1_3.txt           5 ml/kg corn oil
14 GSM819063_US10283824_252828210178_S01_GE1_107_Sep09_1_2.txt           5 ml/kg corn oil
15 GSM819062_US10283824_252828210178_S01_GE1_107_Sep09_1_1.txt           2 ml/kg corn oil
16 GSM819061_US10283824_252828210177_S01_GE1_107_Sep09_1_4.txt           2 ml/kg corn oil
17 GSM819060_US10283824_252828210177_S01_GE1_107_Sep09_1_3.txt           2 ml/kg corn oil
18 GSM819059_US10283824_252828210177_S01_GE1_107_Sep09_1_2.txt            10 ml/kg saline
19 GSM819058_US10283824_252828210177_S01_GE1_107_Sep09_1_1.txt            10 ml/kg saline
```

We are interested in the treatment column:

```
> Treatment <- SDRF[,"Characteristics[treatment]"]
```

We set the saline control to be the first level of the Treatment factor.

```
> 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)
```

Next read the intensity data:

```
> x <- read.maimages(SDRF[,"Array Data File"],
+           source="agilent", green.only=TRUE, other.columns="gIsWellAboveBG")
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
```

Note that we have read in the extra column `gIsWellAboveBG`, which records whether the intensity of each spot is considered above the background level for that array. This column will help us later with probe filtering.

The data has 44,254 probes and 19 arrays:

```
> dim(x)
[1] 44254    19
```

17.4.4 Gene annotation

We can use the annotation package for this type of Agilent array, `RnAgilentDesign028282.db`, to get gene symbols and Entrez Gene Ids from the probe Ids:

```
> library(RnAgilentDesign028282.db)
> x$genes$EntrezID <- mapIds(RnAgilentDesign028282.db, x$genes$ProbeName,
+                            keytype="PROBEID", column="ENTREZID")
> x$genes$Symbol <- mapIds(RnAgilentDesign028282.db, x$genes$ProbeName,
+                            keytype="PROBEID", column="SYMBOL")
> x$genes[201:205,]
    Row Col ControlType    ProbeName SystematicName EntrezID Symbol
201   3  34           0 A_42_P642757      NM_031235    81918  Pard3
202   3  35           0 A_64_P066694   A_64_P066694     <NA>   <NA>
203   3  36           0 A_42_P699201      XM_227798   295538 Depdc1
204   3  37           0 A_42_P591944      NM_172093    94164   Hbg1
205   3  38           0 A_44_P115293   NM_001108294   360611  Copz2
```

17.4.5 Background correction and normalize

We use normexp background correction followed by quantile normalization:

```
> 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")
```

17.4.6 Gene filtering

We will filter out control probes as indicated by the `ControlType` column:

```
> NotControl <- y$genes$ControlType==0L
```

We will also filter out probes with no Entrez Gene Id or Symbol

```
> NoSymbol <- is.na(y$genes$Symbol)
```

Finally, we will filter probes that don't appear to be expressed. We keep probes that are above background on at least four arrays (because there are four replicates of each treatment):

```
> IsExpr <- rowSums(y$other$gIsWellAboveBG > 0) >= 4
```

Now we select the probes to keep in a new data object `yfilt`:

```
> yfilt <- y[NotControl & !NoSymbol & IsExpr, ]
> dim(yfilt)
[1] 27024    19
```

To be tidy, we remove annotation columns we no longer need:

```
> yfilt$genes <- yfilt$genes[,c("ProbeName","Symbol","EntrezID")]
> head(yfilt$genes)
      ProbeName  Symbol EntrezID
13 A_64_P002176   Wdfy3   305164
14 A_42_P664913 Ankrd37   361149
15  A_43_P13320    Ifng    25712
18  A_43_P11804     Ptn    24924
19 A_44_P808710     Rd3   684158
20 A_64_P142111  Gxylt1   300173
```

17.4.7 Differential expression

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

```
> design <- model.matrix(~Treatment)
> fit <- lmFit(yfilt,design)
> fit <- eBayes(fit,trend=TRUE,robust=TRUE)
> summary(decideTests(fit[,-1]))
       Treatment2 ml/kg corn oil Treatment5 ml/kg corn oil Treatment10 ml/kg corn oil
Down                           0                        0                       756
NotSig                     27024                    27024                     24734
Up                             0                        0                      1534
```

It appears that only the 10 ml/kg treatment is different from the saline control. The top 20 genes for the 10 ml/kg treatment are as follows:

```
> topTable(fit,coef=4,n=20)
        ProbeName  Symbol  EntrezID logFC AveExpr     t  P.Value adj.P.Val    B
28763 A_44_P552452  RT1-Bb    309622  9.33    8.78  45.3 1.68e-21  4.55e-17 32.4
9680  A_44_P991565     Bad     64639  3.73    7.91  39.1 2.03e-20  2.74e-16 31.5
42069 A_64_P160096    Mei1    315162  3.84    7.67  32.6 7.49e-19  6.74e-15 29.5
12513 A_42_P638620    Lcn2    170496  3.83    8.09  32.1 1.01e-18  6.84e-15 29.3
6942  A_42_P667782 Fastkd2    301463 -1.70   12.44 -24.4 2.08e-16  1.12e-12 25.7
4631  A_64_P006625   RT1-A 100188935  2.65   13.17  24.1 2.61e-16  1.17e-12 25.6
40353 A_64_P149280   Vegfb     89811 -3.26    8.69 -21.2 3.31e-15  1.28e-11 23.6
655   A_42_P667782 Fastkd2    301463 -1.44   12.13 -20.5 6.40e-15  2.16e-11 23.1
23459 A_42_P667782 Fastkd2    301463 -1.64   12.53 -20.3 7.21e-15  2.16e-11 23.0
22161 A_64_P154811 Ccdc146    499980  1.79    6.97  20.1 9.05e-15  2.44e-11 22.8
37136 A_42_P667782 Fastkd2    301463 -1.78   12.32 -20.0 1.00e-14  2.46e-11 22.7
10116 A_42_P667782 Fastkd2    301463 -1.71   12.49 -19.4 1.71e-14  3.85e-11 22.3
36556 A_42_P667782 Fastkd2    301463 -1.81   12.36 -18.9 2.98e-14  6.20e-11 21.8
29421 A_42_P667782 Fastkd2    301463 -1.72   12.53 -18.3 5.52e-14  1.06e-10 21.3
23088 A_64_P163386   Uqcc6    691921  1.49   11.22  18.0 7.19e-14  1.30e-10 21.1
35094 A_64_P054586   Usp9x    363445 -1.59    9.35 -17.8 9.48e-14  1.60e-10 20.9
13512 A_42_P667782 Fastkd2    301463 -1.56   12.64 -17.3 1.62e-13  2.58e-10 20.4
11619 A_64_P107239   A4gnt    685758  1.17    6.46  16.8 2.66e-13  3.99e-10 20.0
22476 A_42_P667782 Fastkd2    301463 -1.55   12.65 -16.5 3.81e-13  5.42e-10 19.7
371   A_64_P059545   Mlycd     85239 -2.43   13.28 -16.4 5.91e-13  7.98e-10 19.3
```

17.4.8 Gene ontology analysis

```
> g <- goana(fit, coef=4, species="Rn", geneid="EntrezID")
> topGO(g,n=20,truncate="50")
                                                    Term Ont    N  Up Down     P.Up  P.Down
GO:0006955                                immune response  BP 1430 147   59 1.83e-08 0.01410
GO:0042611                             MHC protein complex  CC   23  11    0 6.37e-08 1.00000
GO:0009605                    response to external stimulus  BP 2110 197   82 9.40e-08 0.01676
GO:0005576                             extracellular region  CC 1544 153   45 9.98e-08 0.70056
GO:0006691                    leukotriene metabolic process  BP   30  12    2 1.83e-07 0.23877
GO:0050776                    regulation of immune response  BP  790  90   36 1.91e-07 0.01359
GO:0032101   regulation of response to external stimulus  BP  973 104   45 4.78e-07 0.00469
GO:0002376                           immune system process  BP 2092 192   79 5.00e-07 0.03467
GO:0031347                  regulation of defense response  BP  739  84   32 5.52e-07 0.03602
GO:0009607                     response to biotic stimulus  BP 1440 141   56 7.04e-07 0.04523
GO:0042612                     MHC class I protein complex  CC   14   8    0 7.38e-07 1.00000
GO:0005615                           extracellular space  CC 1167 118   37 1.36e-06 0.47360
GO:0019370                leukotriene biosynthetic process  BP   15   8    0 1.49e-06 1.00000
GO:0098553     lumenal side of endoplasmic reticulum membrane  CC   15   8    0 1.49e-06 1.00000
GO:0002684       positive regulation of immune system process  BP  941  99   36 1.89e-06 0.11328
GO:0006954                           inflammatory response  BP  730  81   33 2.32e-06 0.01966
GO:0044419 biological process involved in interspecies int...  BP 1493 142   60 3.28e-06 0.02188
GO:0050727           regulation of inflammatory response  BP  355  47   14 3.70e-06 0.21597
GO:0051707                    response to other organism  BP 1400 134   56 4.70e-06 0.02861
GO:0009986                                  cell surface  CC  828  88   41 4.92e-06 0.00209

> sessionInfo()
R version 4.6.0 (2026-04-24 ucrt)
Platform: x86_64-w64-mingw32/x64
Running under: Windows 11 x64 (build 26200)

Matrix products: default
  LAPACK version 3.12.1
```

```
locale:
[1] LC_COLLATE=English_Australia.utf8  LC_CTYPE=English_Australia.utf8
[3] LC_MONETARY=English_Australia.utf8 LC_NUMERIC=C
[5] LC_TIME=English_Australia.utf8

time zone: Australia/Sydney
tzcode source: internal

attached base packages:
[1] stats4    stats     graphics  grDevices utils     datasets  methods   base

other attached packages:
[1] RnAgilentDesign028282.db_3.2.3 org.Rn.eg.db_3.23.0           AnnotationDbi_1.74.0
[4] IRanges_2.46.0                 S4Vectors_0.50.1              Biobase_2.72.0
[7] BiocGenerics_0.58.1            generics_0.1.4                limma_3.69.0

loaded via a namespace (and not attached):
 [1] crayon_1.5.3      vctrs_0.7.3       httr_1.4.8        cli_3.6.6         rlang_1.2.0
 [6] otel_0.2.0        DBI_1.3.0         png_0.1-9         GO.db_3.23.1      bit_4.6.0
[11] statmod_1.5.2     Biostrings_2.80.0 KEGGREST_1.52.0   Seqinfo_1.2.0     fastmap_1.2.0
[16] memoise_2.0.1     compiler_4.6.0    RSQLite_3.52.0    blob_1.3.0        pkgconfig_2.0.3
[21] XVector_0.52.0    R6_2.6.1          splines_4.6.0     tools_4.6.0       bit64_4.8.0
[26] cachem_1.1.0
```

Chapter 18

RNA-Seq Case Studies

18.1 Profiles of Yoruba HapMap Individuals

18.1.1 Background

RNA-seq profiles were made of cell lines derived from B-lymphocytes from 69 different Yoruba individuals from Ibadan, Nigeria [25] [26]. The profiles were generated as part of the International HapMap project [12]. RNA from each individual was sequenced on at least two lanes of an Illumina Genome Analyser 2.

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 female.

This case study requires limma 3.9.19 or later.

18.1.2 Data availability

The raw FASTQ files used for this example are available from the URLs given below. For those who wish to skip the alignment and count summarization steps, the `DGEList` object `y` used for the statistical analysis can be downloaded from `http://bioinf.wehi.edu.au/limma/`.

18.1.3 Yoruba Individuals and FASTQ Files

Sequence reads of the Yoruba HapMap cell lines are available at Pritchard Lab's eQTL resources at `http://eqtl.uchicago.edu/RNA_Seq_data/unmapped_reads/` in FASTQ format. Sample information are recorded at Pritchard Lab's eQTL resources at `http://eqtl.uchicago.edu/RNA_Seq_data/list_lanes_pickrell_2010_plosgenetics`. Genders of the individuals are available at the International HapMap Project NHGRI Repository at `http://ccr.coriell.org/`.

We will only use the read sequenced at Argonne National Laboratory. All lanes from the Argonne center were sequenced with 46 bp reads.

```
> Targets <- read.delim("targets.txt", sep = "\t")
> Targets
```

```
    center                        flow_cell lane individual concentration cellline gender
1  argonne              090617_HGAC_S100091    5    NA18486           3.5  NA18486   Male
2  argonne              090330_HGAC_S100065    3  NA18498_2           3.5  NA18498   Male
3  argonne 090218_HGAC_S100047_PIPELINE_V2    7    NA18498           2.5  NA18498   Male
```

4	argonne	090312_HGAC_S100055	5	NA18499	3.5	NA18499	Female
5	argonne	090113_HGAC_S100037_PIPELINE_V2	5	NA18501	1.5	NA18501	Male
6	argonne	090722_HGAC_S1000102	5	NA18502_2	3.5	NA18502	Female
7	argonne	090417_HGAC_S100073	5	NA18502	3.5	NA18502	Female
8	argonne	090421_HGAC_S100075	3	NA18504_2	3.5	NA18504	Male
9	argonne	090218_HGAC_S100047_PIPELINE_V2	1	NA18504	2.5	NA18504	Male
10	argonne	090722_HGAC_S1000102	8	NA18505_2	3.5	NA18505	Female
11	argonne	090417_HGAC_S100073	3	NA18505	3.5	NA18505	Female
12	argonne	090421_HGAC_S100075	6	NA18507	3.5	NA18507	Male
13	argonne	090417_HGAC_S100073	6	NA18508	3.5	NA18508	Female
14	argonne	090520_HGAC_S100083	8	NA18510	3.5	NA18510	Male
15	argonne	090330_HGAC_S100065	1	NA18511	3.5	NA18511	Female
16	argonne	090421_HGAC_S100075	8	NA18516_2	3.5	NA18516	Male
17	argonne	090218_HGAC_S100047_PIPELINE_V2	2	NA18516	2.5	NA18516	Male
18	argonne	090205_HGAC_S100044_PIPELINE_V2	7	NA18517_2	2.5	NA18517	Female
19	argonne	090205_HGAC_S100044_PIPELINE_V2	6	NA18517	2.5	NA18517	Female
20	argonne	090417_HGAC_S100073	8	NA18519	3.5	NA18519	Male
21	argonne	090330_HGAC_S100065	2	NA18520	3.5	NA18520	Female
22	argonne	090218_HGAC_S100047_PIPELINE_V2	3	NA18522	2.5	NA18522	Male
23	argonne	090617_HGAC_S100091	8	NA18523	3.5	NA18523	Female
24	argonne	090520_HGAC_S100083	5	NA18852	3.5	NA18852	Female
25	argonne	090414_HGAC_S100072	7	NA18853	3.5	NA18853	Male
26	argonne	090520_HGAC_S100083	6	NA18855	3.5	NA18855	Female
27	argonne	090722_HGAC_S1000102	7	NA18856_2	3.5	NA18856	Male
28	argonne	090113_HGAC_S100037_PIPELINE_V2	1	NA18856	1.5	NA18856	Male
29	argonne	090421_HGAC_S100075	1	NA18858	3.5	NA18858	Female
30	argonne	091013_HGAC_S1000131	5	NA18859	3.5	NA18859	Male
31	argonne	090330_HGAC_S100065	8	NA18861	3.5	NA18861	Female
32	argonne	090414_HGAC_S100072	6	NA18862	3.5	NA18862	Male
33	argonne	090520_HGAC_S100083	7	NA18870	3.5	NA18870	Female
34	argonne	090617_HGAC_S100091	1	NA18871	3.5	NA18871	Male
35	argonne	090312_HGAC_S100055	1	NA18909	3.5	NA18909	Female
36	argonne	090617_HGAC_S100091	7	NA18912_2	3.5	NA18912	Female
37	argonne	090113_HGAC_S100037_PIPELINE_V2	6	NA18912	1.5	NA18912	Female
38	argonne	090414_HGAC_S100072	8	NA18913	3.5	NA18913	Male
39	argonne	090414_HGAC_S100072	5	NA18916	3.5	NA18916	Female
40	argonne	091013_HGAC_S1000131	2	NA19092_2	3.5	NA19092	Male
41	argonne	090218_HGAC_S100047_PIPELINE_V2	5	NA19093	2.5	NA19093	Female
42	argonne	090312_HGAC_S100054	3	NA19098	3.5	NA19098	Male
43	argonne	090205_HGAC_S100044_PIPELINE_V2	8	NA19099	2.5	NA19099	Female
44	argonne	090205_HGAC_S100044_PIPELINE_V2	1	NA19101	2.5	NA19101	Male
45	argonne	091013_HGAC_S1000131	8	NA19102_3	3.5	NA19102	Female
46	argonne	090113_HGAC_S100037_PIPELINE_V2	7	NA19102	1.5	NA19102	Female
47	argonne	090330_HGAC_S100065	6	NA19108	3.5	NA19108	Female
48	argonne	090421_HGAC_S100075	7	NA19114	3.5	NA19114	Female
49	argonne	090218_HGAC_S100047_PIPELINE_V2	8	NA19116	2.5	NA19116	Female
50	argonne	090113_HGAC_S100037_PIPELINE_V2	2	NA19119	1.5	NA19119	Male
51	argonne	090417_HGAC_S100073	2	NA19127	3.5	NA19127	Female
52	argonne	090205_HGAC_S100044_PIPELINE_V2	2	NA19128	2.5	NA19128	Male
53	argonne	090205_HGAC_S100044_PIPELINE_V2	3	NA19130	2.5	NA19130	Male
54	argonne	090330_HGAC_S100065	5	NA19131	3.5	NA19131	Female
55	argonne	090722_HGAC_S1000102	2	NA19137	3.5	NA19137	Female
56	argonne	090417_HGAC_S100073	7	NA19138	3.5	NA19138	Male
57	argonne	090417_HGAC_S100073	1	NA19140	3.5	NA19140	Female
58	argonne	091013_HGAC_S1000131	6	NA19141	3.5	NA19141	Male
59	argonne	090312_HGAC_S100055	8	NA19143	3.5	NA19143	Female
60	argonne	090312_HGAC_S100054	5	NA19144	3.5	NA19144	Male

```
61 argonne             090312_HGAC_S100055    2    NA19147          3.5  NA19147 Female
62 argonne             090312_HGAC_S100055    3    NA19152          3.5  NA19152 Female
63 argonne             090312_HGAC_S100054    8    NA19153          3.5  NA19153   Male
64 argonne             090617_HGAC_S100091    3    NA19159          3.5  NA19159 Female
65 argonne             090520_HGAC_S100083    3  NA19160_2          3.5  NA19160   Male
66 argonne             090421_HGAC_S100075    5    NA19160          3.5  NA19160   Male
67 argonne             090617_HGAC_S100091    6  NA19171_2          3.5  NA19171   Male
68 argonne 090113_HGAC_S100037_PIPELINE_V2    4    NA19171          1.5  NA19171   Male
69 argonne 090218_HGAC_S100047_PIPELINE_V2    6    NA19172          2.5  NA19172 Female
70 argonne             090330_HGAC_S100065    7    NA19190          3.5  NA19190 Female
71 argonne             090414_HGAC_S100072    2    NA19192          3.5  NA19192   Male
72 argonne             090520_HGAC_S100083    2    NA19193          3.5  NA19193 Female
73 argonne            090722_HGAC_S1000102    3  NA19200_2          3.5  NA19200   Male
74 argonne 090113_HGAC_S100037_PIPELINE_V2    3    NA19200          1.5  NA19200   Male
75 argonne             090312_HGAC_S100054    6    NA19201          3.5  NA19201 Female
76 argonne 090205_HGAC_S100044_PIPELINE_V2    4    NA19203          2.5  NA19203   Male
77 argonne             090617_HGAC_S100091    2    NA19204          3.5  NA19204 Female
78 argonne            091013_HGAC_S1000131    7  NA19206_2          3.5  NA19206 Female
79 argonne            091013_HGAC_S1000131    3    NA19207          3.5  NA19207   Male
80 argonne             090312_HGAC_S100055    7    NA19209          3.5  NA19209 Female
81 argonne             090312_HGAC_S100054    7    NA19210          3.5  NA19210   Male
82 argonne             090414_HGAC_S100072    3    NA19222          3.5  NA19222 Female
83 argonne             090421_HGAC_S100075    2    NA19225          3.5  NA19225 Female
84 argonne             090312_HGAC_S100054    1    NA19238          3.5  NA19238 Female
85 argonne             090312_HGAC_S100054    2    NA19239          3.5  NA19239   Male
86 argonne             090414_HGAC_S100072    1    NA19257          3.5  NA19257 Female
```

18.1.4 Mapping reads to the reference genome

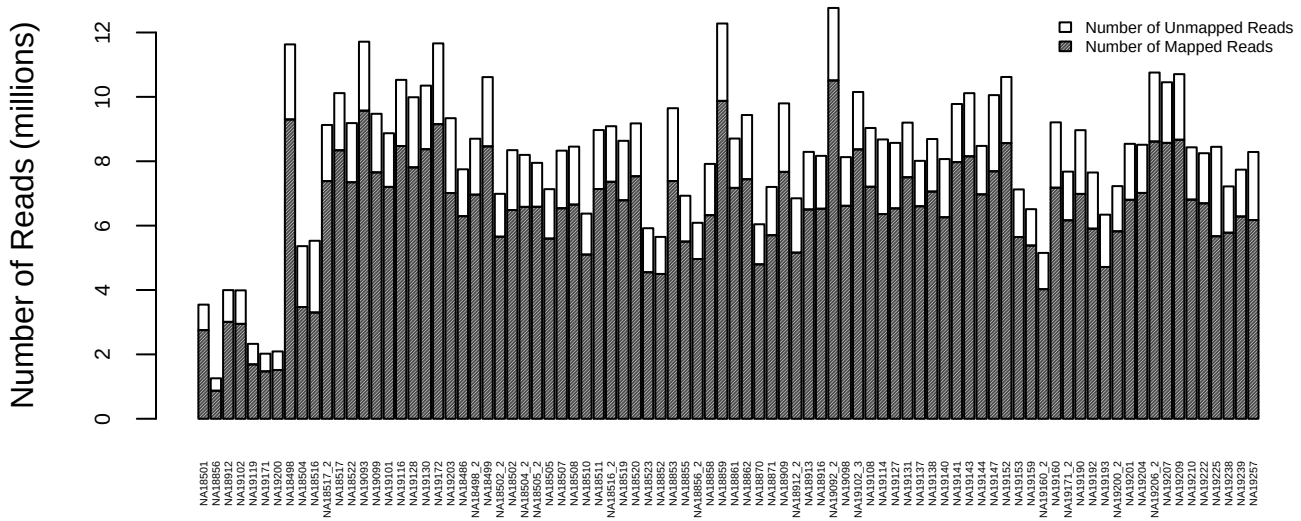
Rsubread 1.14.2 [17] was used to perform read alignment and to summarize read counts at the gene level. The reference genome used in read alignment was GRCh37 (hg19 Genome Reference Consortium Human Build 37), and the annotations used in read summarization were NCBI RefSeq Build 37.2. FASTA files representing the Homo sapiens genome were downloaded from UCSC Human Genome at `http://hgdownload.soe.ucsc.edu/goldenPath/hg19/`.

```
> library(Rsubread)
> FASTQFiles <- paste0(Targets$individual, "_", Targets$center, ".fastq.gz")
> BAMFiles <- paste0(Targets$individual, "_", Targets$center, ".subread.bam")
> buildindex(basename="hg19_subread",reference="GRCh37.fa")
> align("hg19_subread", readfile1 = FASTQFiles, input_format = "gzFASTQ", output_file = BAMFiles)
> gene <- featureCounts(BAMFiles, useMetaFeatures=TRUE, annot.inbuilt="hg19", allowMultiOverlap=TRUE)
> propmap <- propmapped(BAMFiles)
```

Show the number of mapped reads. The average proportion of mapped reads is 0.786. Samples are ordered by the concentration of the cDNA using for sequencing.

```
> summary(propmap[,"PropMapped"])

   Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
  0.597   0.778   0.798   0.786   0.810   0.828


> Targets$concentration <- factor(Targets$concentration)
> o <- order(Targets$concentration)
> barplot(as.matrix(t(cbind(propmap[o,"NumMapped"]*1e-6,
```

```
+         (propmap[o,"NumTotal"]-propmap[o,"NumMapped"])*1e-6))),
+         ylab="Number of Reads (millions)", names = Targets$individual[o],
+         las=3, cex.names=0.3, cex.axis=0.7, density = c(80,0),
+         legend = c("Number of Mapped Reads", "Number of Unmapped Reads"),
+         args.legend = list(x = "topright",cex = 0.5, bty = "n"))
```

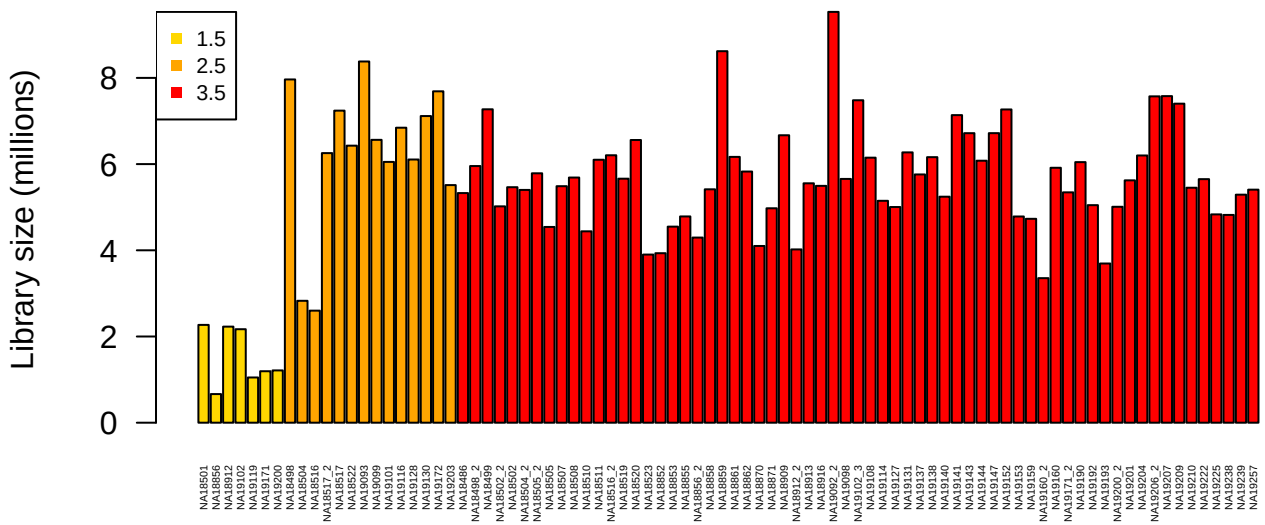


Load the gene level counts.

```
> Counts <- gene$counts
> dim(Counts)

[1] 25702    86

> Counts[1:5,1:5]

          NA18486 NA18498_2 NA18498 NA18499 NA18501
653635         12         8      19      17       5
100422834       0         0       0       0       0
645520          0         0       0       0       0
79501           0         0       0       0       0
729737          6         6       2       6       4
```

The library sizes vary from over 0.6 million to over 9 million. Samples are ordered by the concentration of the cDNA (pM) using for sequencing.

```
> col.concent <- c("gold" , "orange", "red")
> barplot(colSums(Counts[,o])*1e-6, names = Targets$individual[o], ylab="Library size (millions)",
+         col = col.concent[Targets$concentration][o], las = 2, cex.names = 0.3)
> legend("topleft", legend = c("1.5","2.5","3.5"), col = col.concent, pch = 15, cex = 0.7)
```



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

```
> table(Targets$gender[!duplicated(Targets$cellline)])

Female   Male
    41     33
```

18.1.5 Annotation

Annotation for each Entrez Gene ID from the NCBI gene info file.

```
> library(limma)
> columns <- c("GeneID","Symbol", "chromosome", "type_of_gene")
> GeneInfo <- read.columns("130811-Homo_sapiens.gene_info",required=columns, stringsAsFactors = FALSE)
> m <- match(gene$annotation$GeneID, GeneInfo$GeneID)
> Ann <- cbind(gene$annotation[, c("GeneID", "Chr", "Length")],
+              GeneInfo[m, c("Symbol", "type_of_gene")])
> Ann$Chr <- unlist(lapply(strsplit(Ann$Chr, ";"), function(x) paste(unique(x), collapse = "|")))
> Ann$Chr <- gsub("chr", "", Ann$Chr)
> head(Ann)

         GeneID Chr Length     Symbol   type_of_gene
28373    653635   1   1769     WASH7P         pseudo
37837 100422834   1    138 MIR1302-10        miscRNA
27617    645520   1   1130    FAM138A        miscRNA
14957     79501   1    918      OR4F5 protein-coding
29525    729737   1   3402  LOC729737        miscRNA
NA    100507658   1    793       <NA>           <NA>
```

18.1.6 DGEList object

Form a DGEList object combining the counts and associated annotation:

```
> library(edgeR)
> y <- DGEList(counts = Counts, genes = Ann)
```

18.1.7 Filtering

Keep genes with total counts more than 50.

```
> A <- rowSums(y$counts)
> isexpr <- A > 50
```

Keep only genes with defined annotation:

```
> hasannot <- rowSums(is.na(y$genes)) == 0
> y <- y[isexpr & hasannot, , keep.lib.size = FALSE]
> dim(y)

[1] 16918    86
```

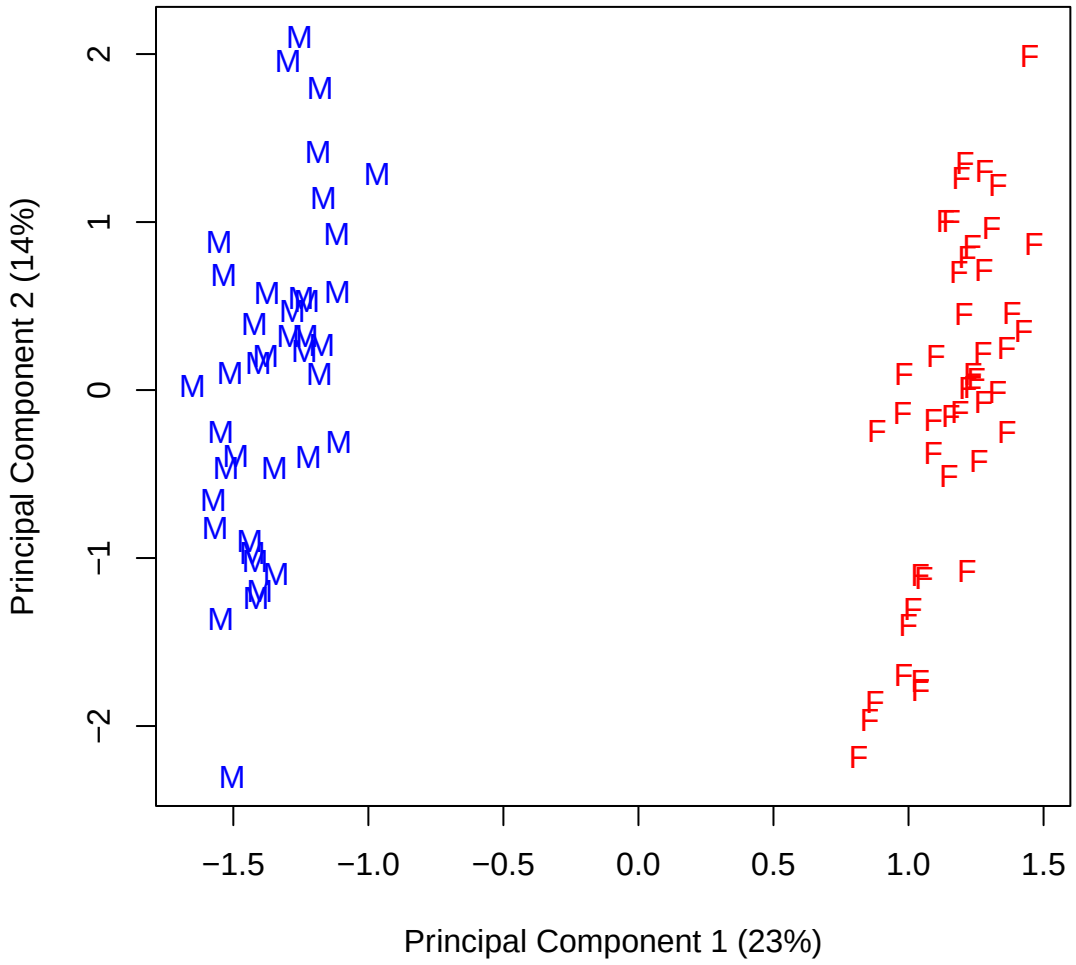
18.1.8 Scale normalization

Apply scale normalization:

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

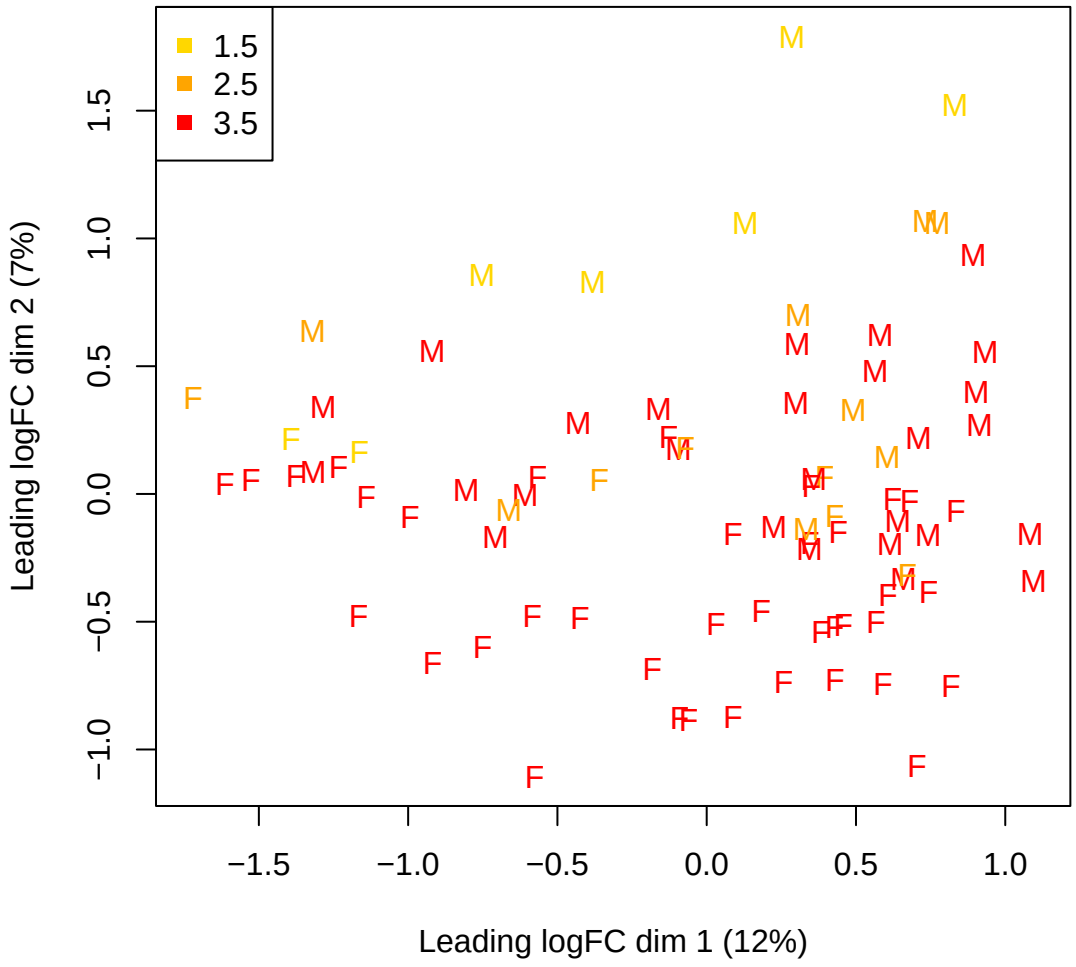
Separation of female and male profiles is evident from an MDS plot.

```
> Gender <- substring(Targets$gender,1,1)
> plotMDS(y, labels=Gender, top=50, col=ifelse(Gender=="M","blue","red"), gene.selection="common",
+         prior.count = 5)
```



Separation of low cDNA concentration from high cDNA concentration is evident from an MDS plot.

```
> plotMDS(y, labels=Gender, col=col.concent[Targets$concentration], prior.count=5)
> legend("topleft", legend = c("1.5","2.5","3.5"), col = col.concent, pch = 15)
```



18.1.9 Linear modeling

Create design matrix.

```
> design <- model.matrix(~ gender + concentration, data = Targets)
```

Use `voom()` [15] to convert the read counts to $\log_2$-cpm, with associated weights, ready for linear modelling:

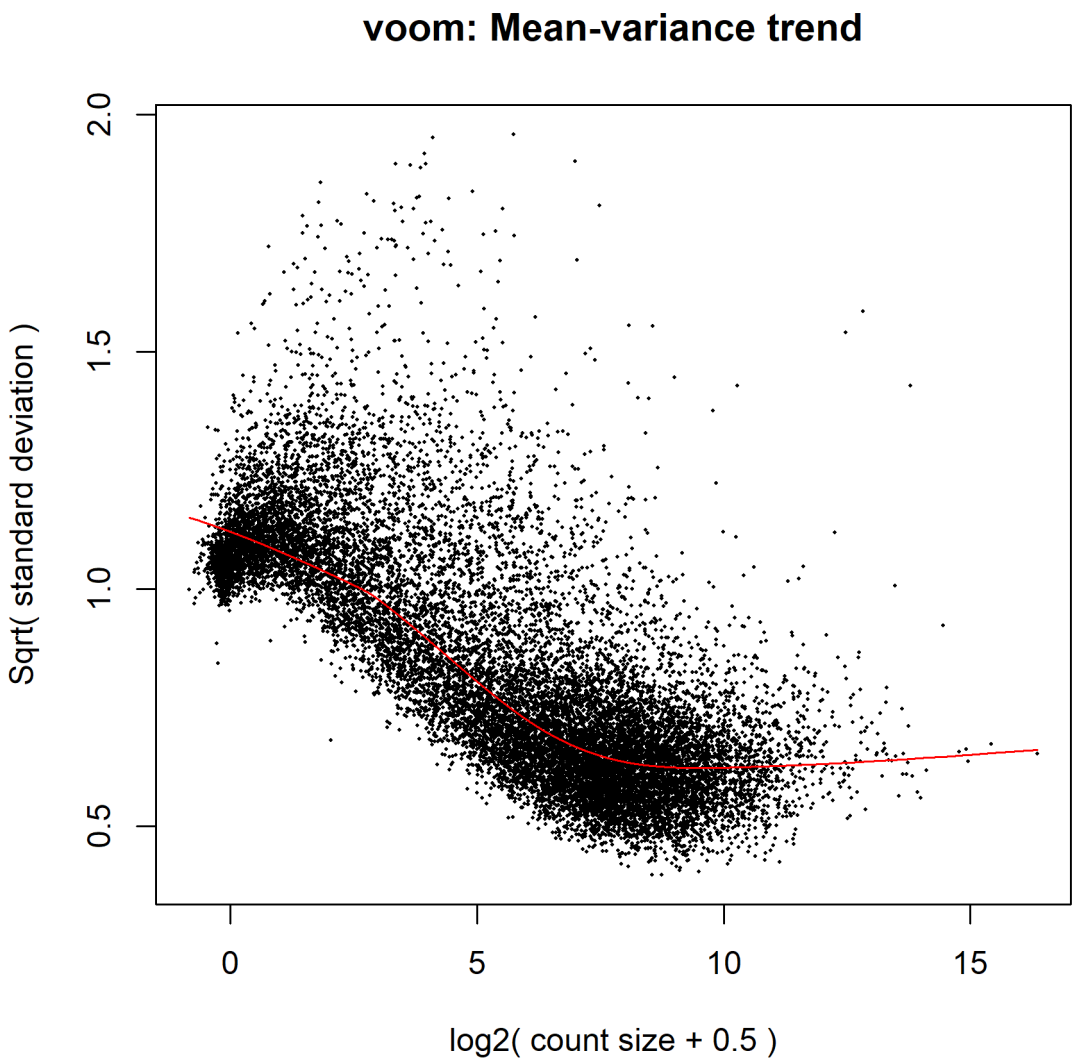
```
> v <- voom(y, design)
```

Estimate the correlation between the duplicated cell lines that were sequenced more than once.

```
> cor <- duplicateCorrelation(v, design, block = Targets$cellline)
> cor$consensus

[1] 0.446
```

The intra cell line correlation will change the voom weights slightly, so we run `voom` a second time:

```
> v <- voom(y, design, plot = TRUE, block = Targets$cellline, correlation = cor$consensus)
```



Similary, we run update the correlation for the new voom weights:

```
> cor <- duplicateCorrelation(v, design, block = Targets$cellline)
> cor$consensus

[1] 0.446
```

The correlation has hardly changed on the second iteration.

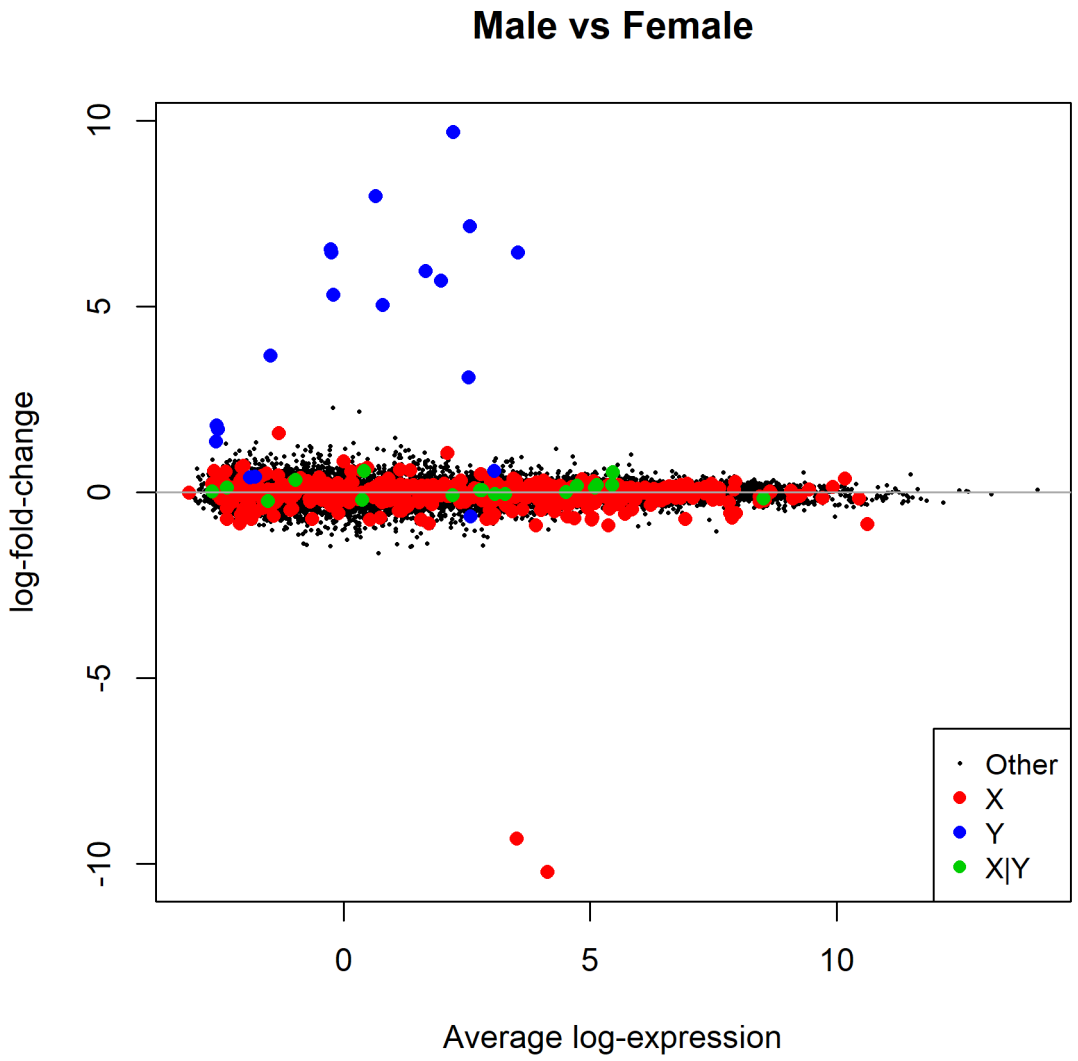
Now find genes differentially expression between male and females. Positive log-fold-changes mean higher in males.

```
> fit <- lmFit(v, design, block = Targets$cellline, correlation = cor$consensus)
> fit <- eBayes(fit)
> summary(decideTests(fit))

       (Intercept) genderMale concentration2.5 concentration3.5
Down          1464         27             1469             4105
NotSig        3352      16869            13622             9704
Up           12102         22             1827             3109

> topTable(fit, coef = "genderMale", n = Inf, sort = "p", p = 0.05)[,-1]

          Chr Length       Symbol   type_of_gene   logFC AveExpr      t  P.Value adj.P.Val       B
64595       Y   5242       TTTY15        miscRNA   6.529  -0.258  52.97 3.63e-68  6.14e-64 123.983
6192        Y    897       RPS4Y1 protein-coding   9.691   2.224  44.08 1.91e-61  1.62e-57 117.215
9086        Y   1390       EIF1AY protein-coding   7.962   0.659  42.44 4.52e-60  2.55e-56 113.661
8284        Y   5581        KDM5D protein-coding   7.148   2.566  41.47 3.08e-59  1.30e-55 115.802
8653        Y   4795        DDX3Y protein-coding   6.439   3.547  39.30 2.68e-57  9.08e-54 114.155
7503        X  19271         XIST        miscRNA -10.223   4.135 -37.53 1.20e-55  3.37e-52 107.001
8287        Y  10040        USP9Y protein-coding   5.693   1.982  36.51 1.14e-54  2.75e-51 107.303
7404        Y   7055          UTY protein-coding   5.945   1.670  34.29 1.93e-52  4.08e-49 102.661
246126      Y    865      TXLNG2P         pseudo   5.316  -0.209  32.57 1.25e-50  2.36e-47  96.469
22829       Y   6276       NLGN4Y protein-coding   6.454  -0.241  32.14 3.65e-50  6.18e-47  95.755
9383        X  37027         TSIX        miscRNA  -9.314   3.511 -32.02 4.99e-50  7.67e-47  96.703
7544        Y   5616          ZFY protein-coding   5.029   0.799  30.89 8.90e-49  1.25e-45  94.753
5616        Y   7217         PRKY         pseudo   3.090   2.542  23.92 4.22e-40  5.50e-37  79.334
140032      Y    888       RPS4Y2 protein-coding   3.670  -1.476  19.07 6.85e-33  8.28e-30  60.881
8226        X   2447        HDHD1 protein-coding  -0.900   5.373 -10.09 2.59e-16  2.92e-13  26.572
8242        X   6862        KDM5C protein-coding  -0.688   7.881  -9.70 1.59e-15  1.68e-12  24.728
100506003   Y    525      ZFY-AS1        miscRNA   1.689  -2.543   9.67 1.83e-15  1.82e-12  24.206
7403        X   5772        KDM6A protein-coding  -0.689   5.044  -9.62 2.30e-15  2.16e-12  24.416
8228        X   2901       PNPLA4 protein-coding  -0.889   3.901  -9.52 3.72e-15  3.31e-12  24.028
246119      Y   1628       TTTY10        miscRNA   1.793  -2.575   9.28 1.14e-14  9.66e-12  22.508
7543        X   7883          ZFX protein-coding  -0.739   5.039  -8.56 3.52e-13  2.83e-10  19.434
6191        X    956       RPS4X protein-coding  -0.857  10.630  -8.55 3.70e-13  2.85e-10  19.339
5613        X   6084         PRKX protein-coding  -0.584   5.717  -8.04 4.07e-12  2.99e-09  16.967
1964        X   4431       EIF1AX protein-coding  -0.699   4.679  -7.05 4.03e-10  2.84e-07  12.512
389906      X   1791    LOC389906         pseudo  -0.701   3.021  -6.23 1.57e-08  1.06e-05   9.120
55787       X   4396        TXLNG protein-coding  -0.442   5.405  -6.04 3.63e-08  2.36e-05   8.016
1654        X   5399        DDX3X protein-coding  -0.573   7.960  -5.99 4.64e-08  2.91e-05   7.722
1968        X   3465       EIF2S3 protein-coding  -0.570   7.845  -5.66 1.89e-07  1.14e-04   6.344
10943       X   4954         MSL3 protein-coding  -0.721   6.933  -5.49 3.96e-07  2.31e-04   5.625
554203      X   1672          JPX        miscRNA  -0.739   1.572  -5.19 1.38e-06  7.78e-04   4.975
56999       3   7313      ADAMTS9 protein-coding   1.304  -2.386   5.04 2.45e-06  1.34e-03   4.576
2941        6   1341        GSTA4 protein-coding  -1.453  -0.270  -4.88 4.76e-06  2.52e-03   3.918
4267      X|Y   2474         CD99 protein-coding   0.535   5.466   4.85 5.30e-06  2.72e-03   3.145
159013      X   4250      CXorf38 protein-coding  -0.329   5.569  -4.69 1.00e-05  4.88e-03   2.523
286554      Y   3668       BCORP1         pseudo   1.370  -2.576   4.69 1.01e-05  4.88e-03   3.262
23136      18   4446      EPB41L3 protein-coding   2.276  -0.215   4.67 1.09e-05  5.13e-03   3.122
27334       X   1790       P2RY10 protein-coding  -0.469   5.853  -4.64 1.24e-05  5.58e-03   2.308
8233        X   1512        ZRSR2 protein-coding  -0.525   4.282  -4.63 1.25e-05  5.58e-03   2.443
139341      X   1170       FUNDC1 protein-coding  -0.525   3.419  -4.57 1.57e-05  6.82e-03   2.359
100506661   X   1203 LOC100506661        miscRNA  -0.695   0.751  -4.54 1.80e-05  7.61e-03   2.610
8935        7   3971         SKAP2 protein-coding   0.455   5.566   4.44 2.66e-05  1.10e-02   1.581
1237        3   1487          CCR8 protein-coding  -1.393   1.085  -4.43 2.73e-05  1.10e-02   2.187
57502       X   5815       NLGN4X protein-coding   1.585  -1.307   4.35 3.73e-05  1.47e-02   2.022
3552        2   2928          IL1A protein-coding  -1.439   1.379  -4.09 9.48e-05  3.65e-02   0.980
6399        X   2860      TRAPPC2 protein-coding  -0.467   3.629  -4.08 9.76e-05  3.67e-02   0.575
```

```
10333       4   5890         TLR6 protein-coding  -0.473   4.510  -4.04 1.16e-04  4.27e-02   0.279
284486      1   1146        THEM5 protein-coding  -1.074   0.264  -4.03 1.19e-04  4.30e-02   0.863
152273      3   5897         FGD5 protein-coding   1.033  -2.187   4.01 1.27e-04  4.49e-02   0.914
80086       2   1380       TUBA4B          pseudo   0.833   1.659   4.00 1.32e-04  4.57e-02   0.602
```

As expected, the highly ranked genes are mostly on the X or Y chromosomes.

```
> chrom <- fit$genes$Chr
> plotMD(fit, column=2, status=chrom, values=c("X","Y", "X|Y"),
+      hl.col=c("red","blue", "green3"), main="Male vs Female",legend="bottomright")
> abline(h=0,col="darkgrey")
```



18.1.10 Gene set testing

Get the list of genes belonging to the male-specific region of chromosome Y [34], and a list of genes located in the X chromosome that have been reported to escape X-inactivation [5]. The gender

genes are available at `http://bioinf.wehi.edu.au/software/GenderGenes/`. 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.

```
> load(url("http://bioinf.wehi.edu.au/software/GenderGenes/GenderGenes.RData"))
> Ymale <- v$genes$GeneID %in% msYgenes
> Xescape <- v$genes$GeneID %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 significantly down as a group [44]:

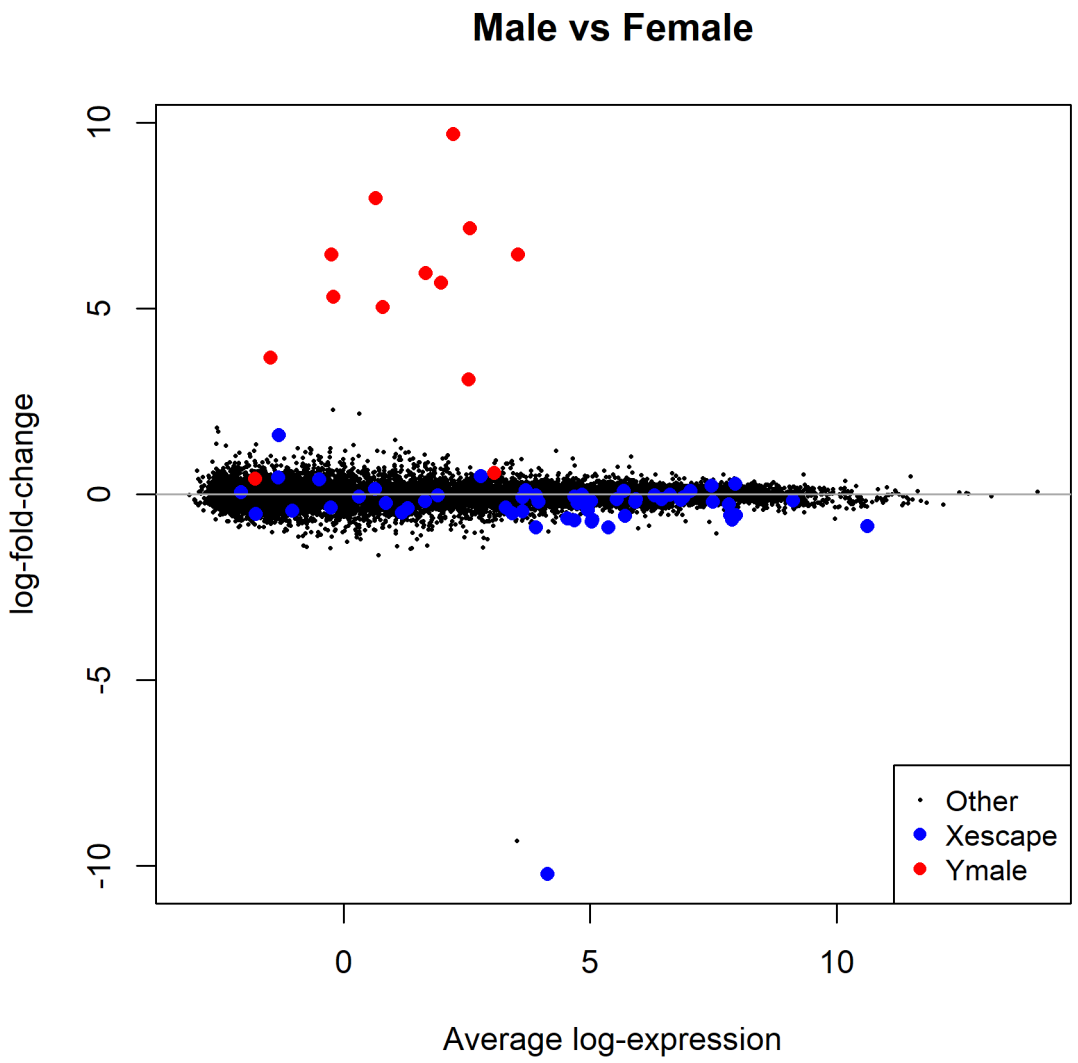
```
> index <- list(Y = Ymale, X = Xescape)
> mroast(v,index,design,2, block = Targets$cellline, correlation = cor$consensus, nrot=9999)

  NGenes PropDown PropUp Direction PValue   FDR PValue.Mixed FDR.Mixed
Y     13    0.000 1.0000        Up  1e-04 1e-04        1e-04     1e-04
X     54    0.574 0.0926      Down  1e-04 1e-04        1e-04     1e-04
```

The results from competitive camera gene sets tests are even more convincing [45]:

```
> camera(v,index,design,2)

  NGenes Direction    PValue       FDR
Y     13        Up 2.40e-262 4.80e-262
X     54      Down  5.58e-26  5.58e-26
```

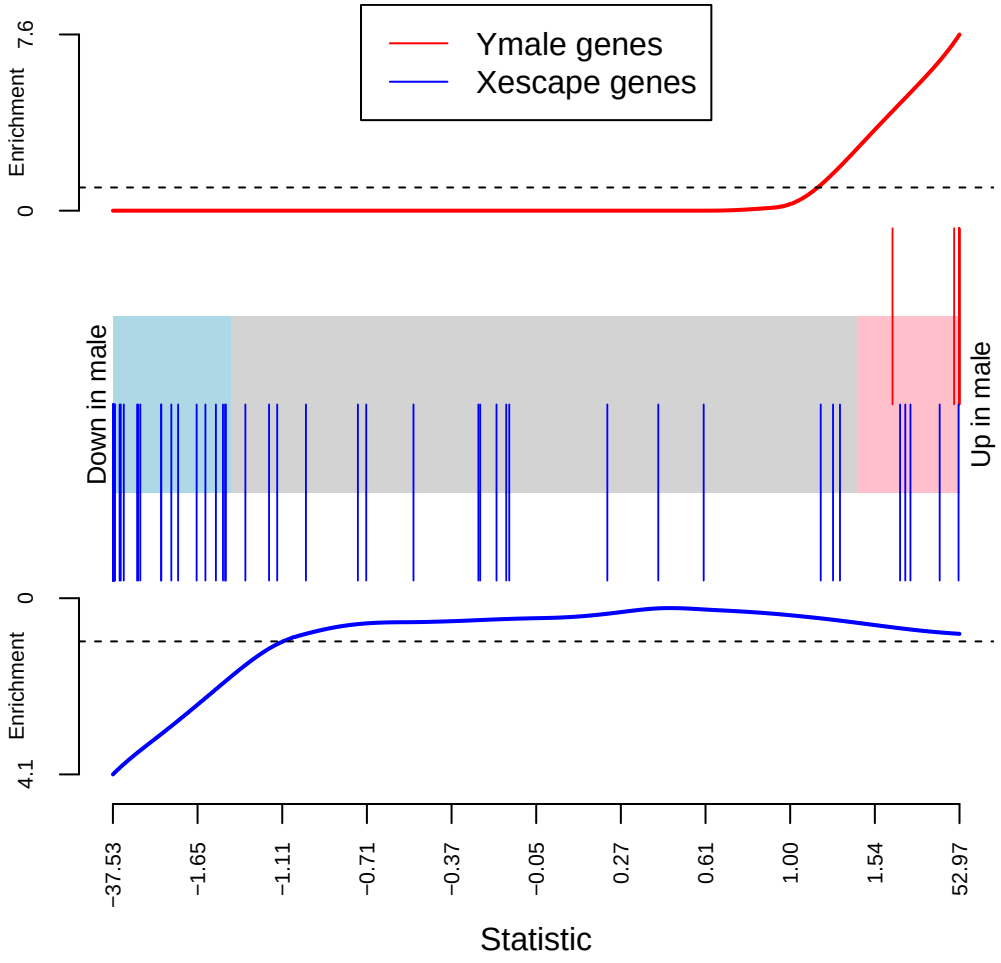
We can highlight the male-specific and X-escape genes on the mean-difference plot:

```
> status <- rep("Other",nrow(fit))
> status[Ymale] <- "Ymale"
> status[Xescape] <- "Xescape"
> plotMD(fit,column=2,status=status,values=c("Xescape","Ymale"),hl.col=c("blue","red"),
+      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("Down in male","Up in male"))
> legend("top",legend=c("Ymale genes","Xescape genes"),lty=1,col=c("red","blue"))
```



18.1.11 Session information

```
> sessionInfo()

R version 4.1.0 (2021-05-18)
Platform: x86_64-w64-mingw32/x64 (64-bit)
Running under: Windows 10 x64 (build 19042)

Matrix products: default

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] stats     graphics  grDevices utils     datasets  methods   base

other attached packages:
[1] edgeR_3.34.0 knitr_1.33   limma_3.49.1

loaded via a namespace (and not attached):
 [1] compiler_4.1.0  magrittr_2.0.1  tools_4.1.0     Rcpp_1.0.7      stringi_1.6.2   highr_0.9
 [7] grid_4.1.0      locfit_1.5-9.4  stringr_1.4.0   xfun_0.24       statmod_1.4.36  lattice_0.20-44
[13] evaluate_0.14
```

18.1.12 Acknowledgements

Thanks for Yang Liao for mapping the reads and running featureCounts.

18.2 Differential Splicing after Pasilla Knockdown

18.2.1 Background

This case study re-analyzes data produced by Brooks et al [3].

The proteins NOVA1 and NOVA2 are known to regulate splicing in mammals. Brooks et al used *Drosophila melanogaster* as a model system, and used an RNA interference system (RNAi) to knock down the expression of the *D. melanogaster* ortholog of NOVA1 and NOVA2, which is Pasilla (ps). The experiment compared treated and untreated cells from the S2-DRSC cell line.

In this case study we find exons and genes that are differentially expressed after ps knockdown, and we find genes are that are differentially spliced in the knockdown samples as compared to wild-type.

For completeness, we will describe all the steps in the analysis from raw data files including mapping and alignment. If you want to skip the mapping and read counting steps, go straight to Section 18.2.6 where the statistical analysis begins.

18.2.2 GEO samples and SRA Files

Brooks et al [3] deposited data for six RNA samples to GEO `http://www.ncbi.nlm.nih.gov/geo`. We have prepared the GEO accession numbers and titles in a csv file:

```
> library(limma)
> GEO <- readTargets("GEO-samples.csv", sep=",")
> GEO

        GEO                 Title Pasilla
1 GSM461176   S2_DRSC_Untreated-1  Normal
2 GSM461177   S2_DRSC_Untreated-3  Normal
3 GSM461178   S2_DRSC_Untreated-4  Normal
4 GSM461179 S2_DRSC_CG8144_RNAi-1    Down
5 GSM461180 S2_DRSC_CG8144_RNAi-3    Down
6 GSM461181 S2_DRSC_CG8144_RNAi-4    Down
```

There are three untreated biological samples, in which ps should be expressed at normal levels, and three treated biological samples, in which ps should be expressed at reduced levels.

While GEO records the sample information, the sequencing data file are actually held on the NCBI Short Read Archive (SRA). The RNA samples were sequenced on an Illumina Genome Analyzer II. Multiple sequencing runs were used for several of the samples, resulting in a total of 20 SRA files:

```
> SRA <- readTargets("SRA-Files.csv", sep=",")
> SRA
```

```
         SRA       GEO                  Title  RunDate FlowCellID Type ReadLength
1  SRR031708 GSM461176   S2_DRSC_Untreated-1  7/15/08  308T2AAXX   SE         45
2  SRR031709 GSM461176   S2_DRSC_Untreated-1  7/15/08  308T2AAXX   SE         45
3  SRR031710 GSM461176   S2_DRSC_Untreated-1  8/15/08  30AYWAAXX   SE         45
4  SRR031711 GSM461176   S2_DRSC_Untreated-1  8/15/08  30AYWAAXX   SE         45
5  SRR031712 GSM461176   S2_DRSC_Untreated-1  8/15/08  30AYWAAXX   SE         45
6  SRR031713 GSM461176   S2_DRSC_Untreated-1  8/15/08  30AYWAAXX   SE         45
7  SRR031714 GSM461177   S2_DRSC_Untreated-3 11/14/08  30MNEAAXX   PE         37
8  SRR031715 GSM461177   S2_DRSC_Untreated-3 12/23/08  30M2BAAXX   PE         37
9  SRR031716 GSM461178   S2_DRSC_Untreated-4 12/23/08  30M2BAAXX   PE         37
10 SRR031717 GSM461178   S2_DRSC_Untreated-4 12/23/08  30M2BAAXX   PE         37
11 SRR031718 GSM461179 S2_DRSC_CG8144_RNAi-1  7/15/08  308T2AAXX   SE         45
12 SRR031719 GSM461179 S2_DRSC_CG8144_RNAi-1  7/18/08  308UEAAXX   SE         45
13 SRR031720 GSM461179 S2_DRSC_CG8144_RNAi-1  8/15/08  30AYWAAXX   SE         45
14 SRR031721 GSM461179 S2_DRSC_CG8144_RNAi-1  8/15/08  30AYWAAXX   SE         45
15 SRR031722 GSM461179 S2_DRSC_CG8144_RNAi-1  8/15/08  30AYWAAXX   SE         45
16 SRR031723 GSM461179 S2_DRSC_CG8144_RNAi-1  8/21/08  308A0AAXX   SE         45
17 SRR031724 GSM461180 S2_DRSC_CG8144_RNAi-3 12/23/08  30M2BAAXX   PE         37
18 SRR031725 GSM461180 S2_DRSC_CG8144_RNAi-3 12/23/08  30M2BAAXX   PE         37
19 SRR031726 GSM461181 S2_DRSC_CG8144_RNAi-4 12/23/08  30M2BAAXX   PE         37
20 SRR031727 GSM461181 S2_DRSC_CG8144_RNAi-4 12/23/08  30M2BAAXX   PE         37
```

Run dates are in American format, so that `7/15/08` denotes 15 July 2008. All runs in July and August 2008 used single end (SE) sequencing with 45 base-pair reads, while the later runs used paired end (PE) sequencing with 37 bp reads.

18.2.3 Mapping reads to the reference genome

The reads were mapped to the reference *D. melanogaster* genome using Rsubread [17].

First the SRA format files need to be converted to FASTQ format, which is the *de facto* standard for representing high-throughput sequencing data. The was done using the SRA Toolkit version 2.3.4. Specifically the Unix SRA toolkit command `fastq-dump --split-spot --split-files` was used to produce one FASTQ file for each single-end SRA file and two FASTQ files for each paired-end SRA file.

Second, FASTA files representing the *D. melanogaster* genome were downloaded from `ftp://ftp.ncbi.nlm.nih.gov/genomes/Drosophila_melanogaster/RELEASE_5_48`. There are five FASTA files corresponding to chromosome arms:

```
> DM <- readTargets("FASTA-GFF-files.csv",sep=",")
> DM
```

```
                FASTA                 GFF Chromosome
1 CHR_2/NT_033778.fna CHR_2/NT_033778.gff      Chr2R
2 CHR_2/NT_033779.fna CHR_2/NT_033779.gff      Chr2L
3 CHR_3/NT_033777.fna CHR_3/NT_033777.gff      Chr3R
4 CHR_3/NT_037436.fna CHR_3/NT_037436.gff      Chr3L
5 CHR_4/NC_004353.fna CHR_4/NC_004353.gff       Chr4
6 CHR_X/NC_004354.fna CHR_X/NC_004354.gff       ChrX
```

The five *.fna files were concatenated into a single FASTA-format file Drosophila_ref.fasta, and an index file was built using Rsubread

```
> library(Rsubread)
> buildindex(basename="index",reference="Drosophila_ref.fasta")
```

Third, reads were aligned to the genome. The SE reads were mapped using:

```
> align("index",SE_fastq_files,input_format="FASTQ")
```

where SE_fastq_files is a character vector containing the names of the SE FASTQ files. The PE reads were mapped using:

```
> align("index",PE_fastq_files1,PE_fastq_files2,input_format="FASTQ")
```

where PE_fastq_files1 and PE_fastq_files2 are vectors containing the names of the PE FASTQ files. These commands produce BAM files containing aligned reads (SE) or fragments (PE).

18.2.4 Read counts for exons

Next we counted the number of reads or fragments overlapping each annotated exon of each gene. GFF files containing gene and exon annotation were downloaded from ftp://ftp.ncbi.nlm.nih.gov/genomes/Drosophila_melanogaster/RELEASE_5_48. There is one GFF file for each chromosome arm as shown above.

The five *.gff files were concatenated into one file, and repeated exons instances of the same exon (same start and stop position) were removed, to create a data.frame of start/stop positions called unique.gff. SE reads were counted by:

```
> fc_SE <- featureCounts(SE_bam_files, annot.ext="unique.gff",
+                 isGTFAnnotationFile=TRUE,
+                 GTF.featureType="exon", GTF.attrType="ID",
+                 useMetaFeatures=FALSE, allowMultiOverlap=TRUE)
```

where SE_bam_files is a vector of BAM file names for the SE reads. PE reads were counted by

```
> fc_PE <- featureCounts(PE_bam_files, annot.ext="unique.gff",
+                 isGTFAnnotationFile=TRUE,
+                 GTF.featureType="exon", GTF.attrType="ID",
+                 useMetaFeatures=FALSE, allowMultiOverlap=TRUE,
+                 isPairedEnd=TRUE)
```

where PE_bam_files is a vector of BAM file names for the PE reads.

18.2.5 Assemble DGEList and sum counts for technical replicates

We assemble all the counts into an edgeR DGEList object:

```
> library(edgeR)
> y.all <- DGEList(counts=cbind(fc_SE$counts,fc_PE$counts), genes=fc_SE$annotation)
> dim(y.all)

[1] 74184    20

> head(y.all$genes)
```

```
       GeneID         Chr  Start    End Strand Length
138088  30970 NC_004354.3 138094 139379      -   1286
138087  30970 NC_004354.3 139445 139611      -    167
138089  30970 NC_004354.3 139445 139889      -    445
138086  30970 NC_004354.3 139713 139889      -    177
138091  30971 NC_004354.3 140011 141629      +   1619
138092  30971 NC_004354.3 142415 144271      +   1857
```

The annotation includes Entrez ID and the length, chromosome and start and stop position of each exon.

Resort back to original SRA order:

```
> y.all <- y.all[,SRA$SRA]
```

Then we collapse the data so that there is a single column for each GEO sample by summing the counts over the technical replicates:

```
> y <- sumTechReps(y.all, ID=SRA$GEO)
> y$samples

          group lib.size norm.factors
GSM461176     1 31007529            1
GSM461177     1 13040952            1
GSM461178     1 15030819            1
GSM461179     1 28143539            1
GSM461180     1 14901292            1
GSM461181     1 16264066            1

> colnames(y) <- c("N1","N3","N4","D1","D3","D4")
```

18.2.6 Gene annotation

Annotation for *D. melanogaster* genes was downloaded from `ftp://ftp.ncbi.nlm.nih.gov/gene/DATA/GENE_INFO/Invertebrates`. We now add selected annotation columns to the DGEList object:

```
> ncbi.L1 <- readLines("Drosophila_melanogaster.gene_info", n = 1)
> ncbi.colname <- unlist(strsplit(substring(ncbi.L1, 10, 234), ' '))
> ncbi <- read.delim("Drosophila_melanogaster.gene_info",
+         skip=1, header=FALSE, stringsAsFactors=FALSE)
> colnames(ncbi) <- ncbi.colname
> m <- match(y$genes$GeneID, ncbi$GeneID)
> y$genes$Chr <- ncbi$chromosome[m]
> y$genes$Symbol <- ncbi$Symbol[m]
> y$genes$Strand <- NULL
> head(y$genes)

       GeneID Chr  Start    End Length Symbol
138088  30970   X 138094 139379   1286 CG3038
138087  30970   X 139445 139611    167 CG3038
138089  30970   X 139445 139889    445 CG3038
138086  30970   X 139713 139889    177 CG3038
138091  30971   X 140011 141629   1619    G9a
138092  30971   X 142415 144271   1857    G9a
```

18.2.7 Filtering

Keep exons that have more than 1 cpm in at least 3 samples.

```
> isexpr <- rowSums(cpm(y) > 1) >=3
> y <- y[isexpr,,keep.lib.sizes=FALSE]
```

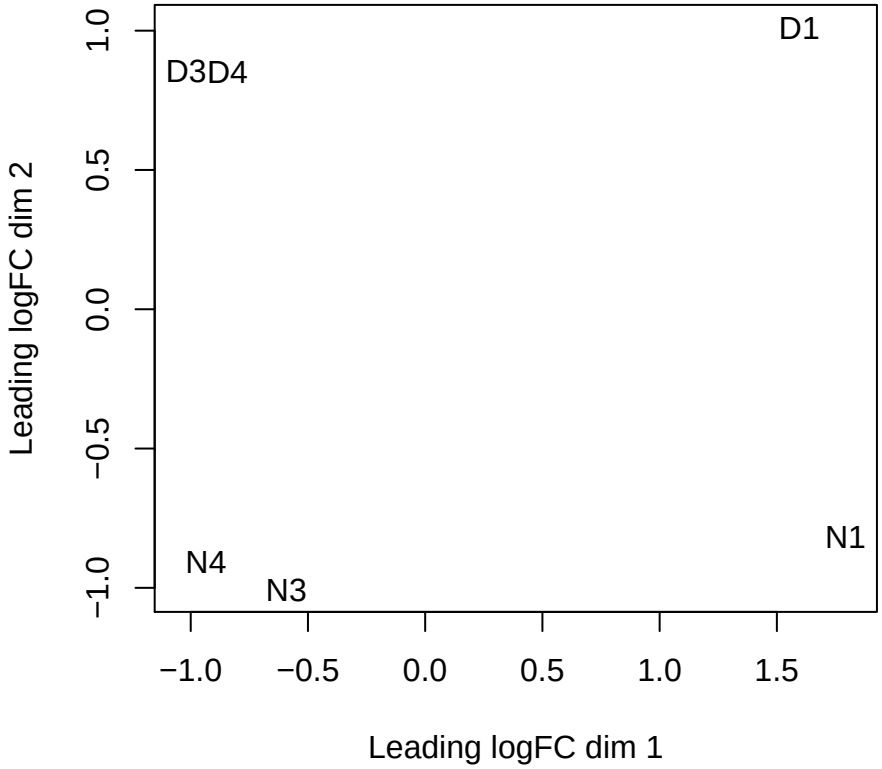
18.2.8 Scale normalization

Apply TMM normalization.

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

   group lib.size norm.factors
N1     1 30872843        0.955
N3     1 12962245        1.031
N4     1 14908555        0.976
D1     1 27989806        1.005
D3     1 14760887        1.022
D4     1 16172265        1.014
```

An MDS plot shows clear separation of the Pasilla down vs normal samples, but also a batch effect associated with sequencing type and date:

```
> plotMDS(y)
```



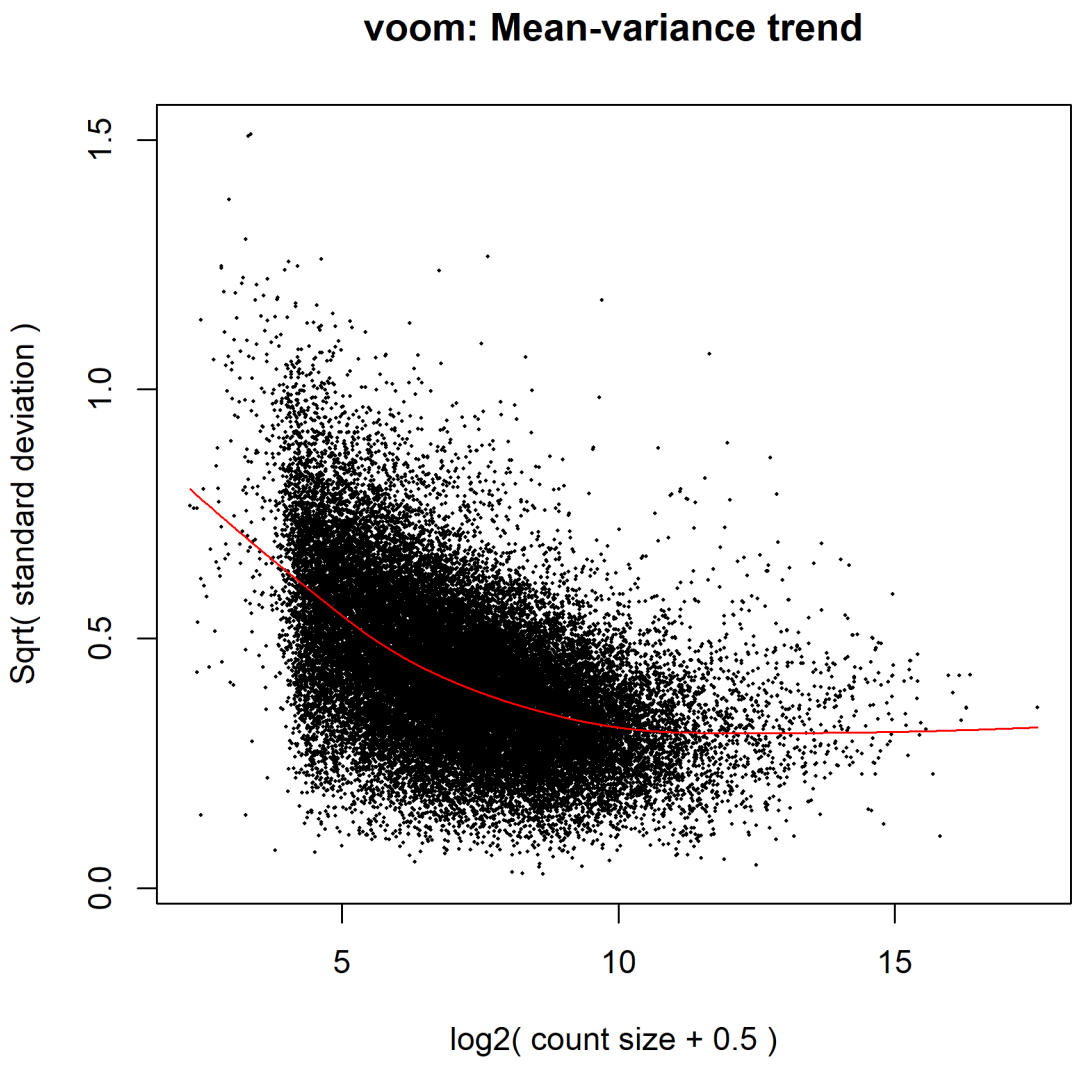
18.2.9 Linear modelling

Create design matrix:

```
> Batch <- factor(c(1,3,4,1,3,4))
> Pasilla <- factor(GEO$Pasilla,levels=c("Normal","Down"))
> design <- model.matrix(~ Batch + Pasilla)
```

Apply voom to convert the read counts to log2-cpm with associated weights:

```
> v <- voom(y,design,plot=TRUE)
```



Linear modelling:

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

Test for differentially expressed exons:

```
> fit.de <- eBayes(fit, robust=TRUE)
> topTable(fit.de, coef="PasillaDown")
```

	GeneID	Chr	Start	End	Length	Symbol	logFC	AveExpr	t	P.Value	adj.P.Val	B
150709	32007	X	10674926	10676128	1203	sesB	-3.26	6.41	-32.1	4.45e-14	8.35e-10	22.3
150713	32007	X	10675026	10676128	1103	sesB	-3.26	6.41	-32.1	4.48e-14	8.35e-10	22.3
96435	43230	3R	22694960	22695852	893	BM-40-SPARC	-2.47	10.50	-28.2	2.51e-13	2.61e-09	20.9
150697	32008	X	10672987	10673728	742	Ant2	2.85	5.53	28.0	2.80e-13	2.61e-09	20.6
96434	43230	3R	22695915	22696094	180	BM-40-SPARC	-2.27	8.11	-26.9	4.72e-13	3.52e-09	20.3
96433	43230	3R	22697648	22697717	70	BM-40-SPARC	-2.17	6.27	-23.5	2.70e-12	1.46e-08	18.6
107856	44030	3L	2561932	2562843	912	msn	-2.46	5.12	-23.5	2.80e-12	1.46e-08	18.5

```
70750   44258  3R  5271691  5272628    938          ps -2.27    5.53 -23.1 3.38e-12  1.46e-08 18.4
11333   44548  2R  6407125  6408782   1658        lola  2.25    5.73  23.1 3.54e-12  1.46e-08 18.3
150702  32008   X 10674230 10674694    465        Ant2  2.97    3.89  21.9 6.82e-12  2.34e-08 17.4
```

```
> summary(decideTests(fit.de))

       (Intercept) Batch3 Batch4 PasillaDown
Down           443   5380   6070        2062
NotSig        3388  26386  25184       33346
Up           33427   5492   6004        1850
```

18.2.10 Alternate splicing

Test for differences in exon retention between Pasilla down vs normal:

```
> ex <- diffSplice(fit[,"PasillaDown"], geneid = "GeneID", exonid = "Start")

Total number of exons:  37258
Total number of genes:  8192
Number of genes with 1 exon:  1619
Mean number of exons in a gene:  5
Max number of exons in a gene:  56
```

Show top genes that show differential splicing.

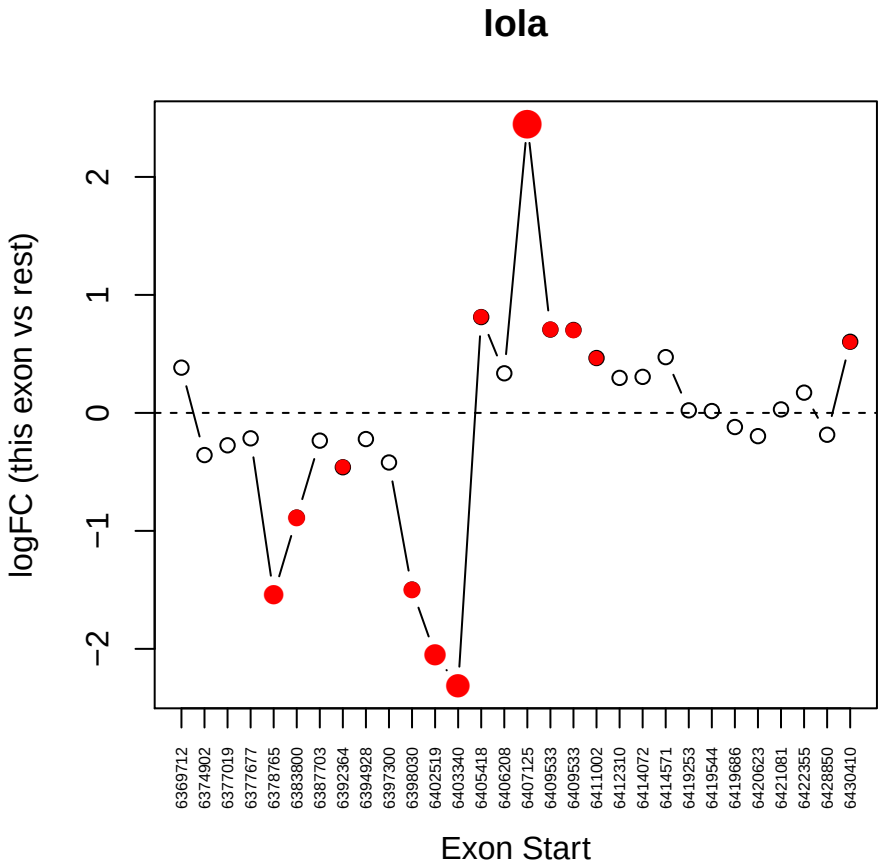
```
> topSplice(ex, test="simes", n=20)

        GeneID Chr    Symbol NExons  P.Value      FDR
11214    44548  2R      lola     30 5.52e-32 3.63e-28
95956    44448  3R     scrib     35 1.99e-20 4.41e-17
141235   45320   X      trol     44 2.01e-20 4.41e-17
16060    36542  2R      shot     38 5.12e-18 7.80e-15
107810   44030  3L       msn     24 5.93e-18 7.80e-15
19880    36773  2R        Dg     15 5.15e-17 5.64e-14
32242    37893  2R      slik     19 1.93e-16 1.81e-13
41795  3771968  2L   Msp-300     33 2.27e-16 1.86e-13
82117    42130  3R       osa     17 1.14e-15 8.34e-13
150694   32008   X      Ant2      5 6.26e-14 4.12e-11
52823    34652  2L     vir-1      7 4.62e-13 2.76e-10
115767   38879  3L       pbl     12 7.60e-13 4.16e-10
163416   32817   X CrebB-17A     12 1.22e-11 6.16e-09
110493   38491  3L       ens     16 2.31e-11 1.08e-08
2032   2768716  2R       mim     25 1.51e-10 6.60e-08
166094   33098   X   CG32521      8 1.63e-10 6.69e-08
131170   40205  3L   CG42674     16 2.16e-10 8.36e-08
109416   38418  3L       kst     18 3.92e-10 1.43e-07
150710   32007   X      sesB      7 1.06e-09 3.49e-07
526      35494  2R  laccase2      9 1.08e-09 3.49e-07
```

```
> topSplice(ex, test="F", n=20)

        GeneID Chr      Symbol NExons      F  P.Value      FDR
141235   45320   X        trol     44  30.87 1.93e-40 1.27e-36
11214    44548  2R        lola     30  41.80 4.81e-33 1.58e-29
95956    44448  3R       scrib     35  16.20 1.53e-23 3.35e-20
```

```
41795  3771968  2L      Msp-300     33  13.81 2.66e-20 4.37e-17
32242    37893  2R         slik     19  26.39 1.91e-18 2.52e-15
16060    36542  2R         shot     38   9.43 1.51e-17 1.65e-14
166094   33098   X      CG32521      8  65.84 3.26e-14 3.06e-11
19880    36773  2R           Dg     15  18.78 6.19e-13 5.08e-10
150694   32008   X         Ant2      5 119.76 8.01e-13 5.85e-10
2032   2768716  2R          mim     25   9.55 1.42e-12 9.32e-10
107810   44030  3L          msn     24   9.91 1.68e-12 1.00e-09
82117    42130  3R          osa     17  12.87 2.03e-11 1.11e-08
52823    34652  2L        vir-1      7  44.35 2.92e-11 1.43e-08
150710   32007   X         sesB      7  44.17 3.05e-11 1.43e-08
115767   38879  3L          pbl     12  15.22 8.55e-10 3.75e-07
163416   32817   X    CrebB-17A     12  14.92 1.10e-09 4.52e-07
134207   40464  3L        Ten-m     12  13.79 2.99e-09 1.16e-06
108973   38376  3L       BtbVII     10  16.87 3.81e-09 1.39e-06
11103    36104  2R G-oalpha47A     13  11.96 5.66e-09 1.96e-06
139657   44505   X           br     13  11.50 9.41e-09 3.09e-06
```

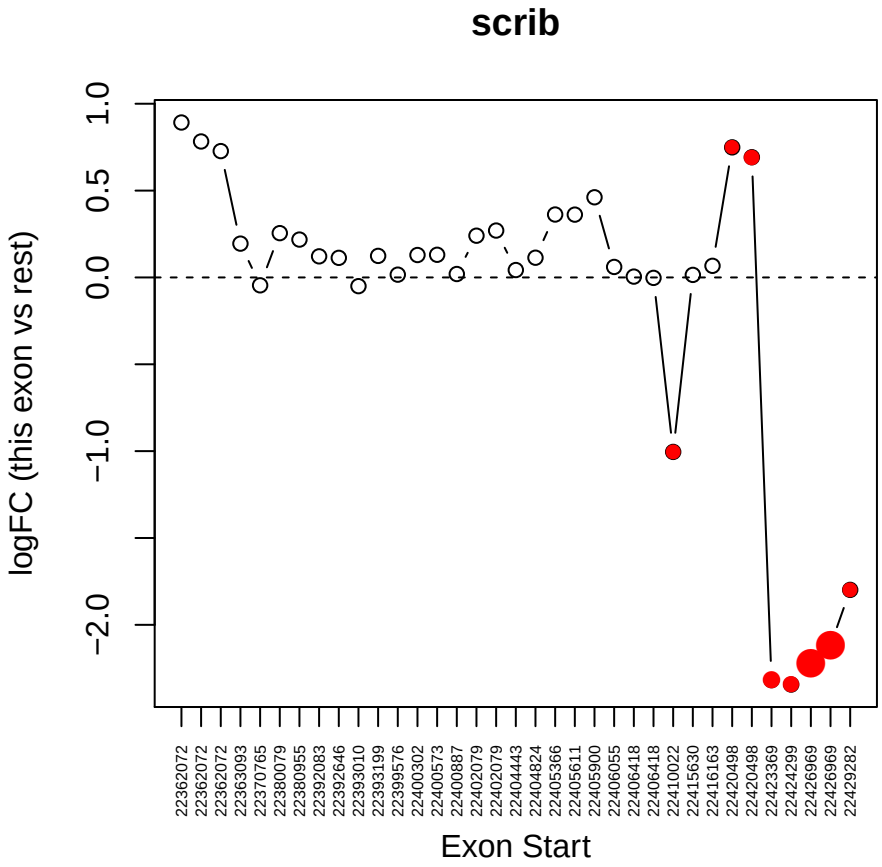
We can plot all the exons for the most differentially spliced genes:

```
> plotSplice(ex, geneid="lola", genecol="Symbol")
```

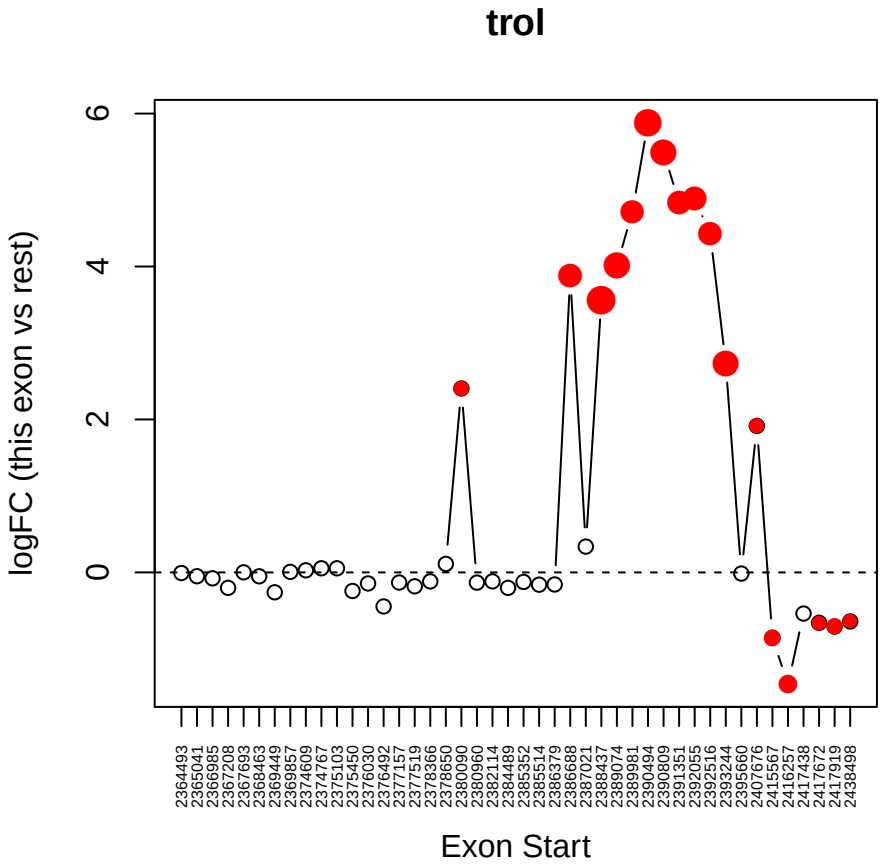


This plot highlights exons that are individually significant in that they have fold changes larger or smaller than most other exons.

```
> plotSplice(ex, geneid="scrib", genecol="Symbol")
```




```
> plotSplice(ex, geneid="trol", genecol="Symbol")
```



We can see that a block of five or six exons at the right end of the trol gene are relatively lost when Pasilla is down, at the expense of an earlier block of twelve exons which become relatively more highly expressed when Pasilla is down. Most exons in the first half of the gene behave similarly to each other. This gene is on the negative strand, so transcription is from right to left. Thus figure can be compared to Supplementary Figure 8A in Brooks et al [3]. The gene trol was identified by Brooks et al to have a novel set of coordinately regulated exons. Brooks et al said:

> "A particularly striking example involving these unannotated splice junctions is found in the trol gene in which a group of nine contiguous exons, which are annotated as being constitutive, are coordinately skipped in untreated cells ... but coordinately included in the ps(RNAi) cells ..."

18.2.11 Session information

```
> sessionInfo()

R version 3.6.1 (2019-07-05)
Platform: x86_64-w64-mingw32/x64 (64-bit)
Running under: Windows 10 x64 (build 15063)

Matrix products: default
```

```
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] stats     graphics  grDevices utils     datasets  methods   base

other attached packages:
[1] edgeR_3.27.13 limma_3.41.15

loaded via a namespace (and not attached):
[1] compiler_3.6.1  tools_3.6.1     Rcpp_1.0.2      grid_3.6.1      locfit_1.5-9.1  statmod_1.4.32
[7] lattice_0.20-38
```

18.2.12 Acknowledgements

Thanks for Yang Liao for mapping the reads and running featureCounts.

Notes

Acknowledgements

Thanks to Jean Yee Hwa Yang and Sandrine Dudoit for the first three data sets. The Swirl zebrafish data were provided by Katrin Wuennenburg-Stapleton from the Ngai Lab at UC Berkeley. Laurent Gautier made the ecoliLeucine data set available on Bioconductor. Lynn Corcoran provided the Bob1 Mutant data. Andrew Holloway, Ryan van Laar and Dileepa Diyagama provided the quality control data set. Marnie Blewitt and Natasha Jansz provided the Smchd1 RNA-seq data.

Many current and former members of the Smyth Lab (students, programmers, postdocs) have contributed to the development of particular functions in limma as acknowledged in the limma package description and on the function help pages.

The limma package has benefited from many people who have made suggestions or reported bugs including Kemal Akat, Naomi Altman, Gioia Altobelli, James Arnold, Alain Bateman, Ido Ben-Zvi, Henrik Bengtsson, Lourdes Peña Castillo, Dongseok Choi, Marcus Davy, Simon de Bernard, Ramon Diaz-Uriarte, Lars Eijssen, Pär Engström, Egil Ferkingstad, Anthoula Gaigneaux, Robert Gentleman, Guido Hooiveld, Wolfgang Huber, Derek Janszen, William Kenworthy, Axel Klenk, Kevin Koh, Erik Kristiansson, Mette Langaas, Michael Lawrence, Gildas Le Corguille, Gregory Lefebvre, Philip Lijnzaad, Andrew Lynch, James MacDonald, Martin Maechler, Aaron Mackey, Steffen Moeller, Duarte Molha, Florian Nigsch, Ashley Ng, Mario Novkovic, Ron Ophir, Francois Pepin, Sergi Sayols Puig, Hubert Rehrauer, Davide Risso, Mark Robinson, Ken Simpson, Dario Strbenac, Laurentiu Adi Tarca, Dan Tenenbaum, Gregory Theiler, Ryan C. Thompson, Joern Toedling, Michael Turewicz, Björn Usadel, Chris Wilkinson, Beth Wilmot, Jean Yee Hwa Yang, John Zhang.

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 many software projects designed to provide user-interfaces for microarray or RNA-seq data analysis.

Bibliography

1. Baldoni, P.L., Chen, L., Li, M., Chen, Y., and Smyth, G.K. (2025). Dividing out quantification uncertainty enables assessment of differential transcript usage with limma and edgeR. *Nucleic Acids Research* 53, gkaf1305.

2. Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B* 57, 289–300.

3. Brooks, A.N., Yang, L., Duff, M.O., Hansen, K.D., Park, J.W., Dudoit, S., Brenner, S.E., and Graveley, B.R. (2011). Conservation of an RNA regulatory map between Drosophila and mammals. *Genome Res* 21, 193–202.

4. Callow, M.J., Dudoit, S., Gong, E.L., Speed, T.P., and Rubin, E.M. (2000). Microarray expression profiling identifies genes with altered expression in HDL deficient mice. *Genome Research* 10, 2022–2029.

5. Carrel, L. and Willard, H.F. (2005). X-inactivation profile reveals extensive variability in X-linked gene expression in females. *Nature* 434, 400–404.

6. Chen, Y., Chen, L., Lun, A.T.L., Baldoni, P.L., and Smyth, G.K. (2025). edgeR v4: powerful differential analysis of sequencing data with expanded functionality and improved support for small counts and larger datasets. *Nucleic Acids Research* 53, gkaf018.

7. Chen, Y., Lun, A.T.L., and Smyth, G.K. (2016). From reads to genes to pathways: differential expression analysis of RNA-seq experiments using Rsubread and the edgeR quasi-likelihood pipeline. *F1000Research* 5, 1438.

8. Dalgaard, P. (2002). *Introductory Statistics with R.* Springer, New York.

9. Diaz, E., Ge, Y., Yang, Y.H., Loh, K.C., Serafini, T.A., Okazaki, Y., Hayashizaki, Y., Speed, T., P., Ngai, J., and Scheiffele, P. (2002). Molecular analysis of gene expression in the developing pontocerebellar projection system. *Neuron* 36, 417–434.

10. Ellis, L., Pan, Y., Smyth, G.K., George, D., McCormack, C., Williams-Truax, R., Mita, M., Beck, J., Burris, H., Ryan, G., et al. (2008). Histone deacetylase inhibitor panobinostat induces clinical responses with associated alterations in gene expression profiles in cutaneous T-cell lymphoma. *Clinical Cancer Research* 14, 4500–4510.

11. Hung, S., Baldi, P., and Hatfield, G.W. (2002). Global gene expression profiling in Escherichia coli K12: The effects of leucine-responsive regulatory protein. *Journal of Biological Chemistry* 277, 40309–40323.

12. International HapMap Consortium, T. (2005). A haplotype map of the human genome. *Nature* 437, 1299–1320.

13. Irizarry, R. (2005). From CEL files to annotated lists of interesting genes. In R. Gentleman, V. Carey, S. Dudoit, R. Irizarry, and W. Huber, editors, *Bioinformatics and Computational Biology Solutions using R and Bioconductor*, pages 431–442. Springer, New York.

14. Law, C.W., Alhamdoosh, M., Su, S., Smyth, G.K., and Ritchie, M.E. (2016). RNA-seq analysis is easy as 1-2-3 with limma, Glimma and edgeR. *F1000Research* 5, 1408.

15. Law, C.W., Chen, Y., Shi, W., and Smyth, G.K. (2014). Voom: precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biology* 15, R29.

16. Li, B. and Dewey, C.N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12, 323.

17. Liao, Y., Smyth, G.K., and Shi, W. (2019). The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. *Nucleic Acids Research* 47, e47.

18. Lim, E., Vaillant, F., Wu, D., Forrest, N., Pal, B., Hart, A., Asselin-Labat, M., Gyorki, D., Ward, T., Partanen, A., et al. (2009). Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. *Nature Medicine* 15, 907–913.

19. Liu, R., Holik, A.Z., Su, S., Jansz, N., Chen, K., Leong, H.S., Blewitt, M.E., Asselin-Labat, M.L., Smyth, G.K., and Ritchie, M.E. (2015). Why weight? modelling sample and observational level variability improves power in RNA-seq analyses. *Nucleic Acids Research* 43, e97.

20. Lun, A.T. and Smyth, G.K. (2017). No counts, no variance: allowing for loss of degrees of freedom when assessing biological variability from RNA-seq data. *Statistical Applications in Genetics and Molecular Biology* 16, 83–93.

21. Milliken, G.A. and Johnson, D.E. (1992). *Analysis of Messy Data, Volume 1: Designed Experiments*. Chapman & Hall, New York.

22. Oshlack, A., Emslie, D., Corcoran, L.M., and Smyth, G.K. (2007). Normalization of boutique two-color microarrays with a high proportion of differentially expressed probes. *Genome Biology* 8, R2.

23. Peart, M., Smyth, G.K., Van Laar, R., Bowtell, D., Richon, V., Marks, P., Holloway, A., and Johnstone, R. (2005). Identification and functional significance of genes regulated by structurally different histone deacetylase inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* 102, 3697–3702.

24. Phipson, B., Lee, S., Majewski, I.J., Alexander, W.S., and Smyth, G.K. (2016). Robust hyperparameter estimation protects against hypervariable genes and improves power to detect differential expression. *Annals of Applied Statistics* 10, 946–963.

25. Pickrell, J.K., Marioni, J.C., Pai, A.A., Degner, J.F., Engelhardt, B.E., Nkadori, E., Veyrieras, J.B., Stephens, M., Gilad, Y., and Pritchard, J.K. (2010). Understanding mechanisms underlying human gene expression variation with RNA sequencing. *Nature* 464, 768–772.

26. Pickrell, J.K., Pai, A.A., Gilad, Y., and Pritchard, J.K. (2010). Noisy splicing drives mRNA isoform diversity in human cells. *PLoS Genetics* 6, e1001236.

27. Reiner, A., Yekutieli, D., and Benjamini, Y. (2003). Identifying differentially expressed genes using false discovery rate controlling procedures. *Bioinformatics* 19, 368–375.

28. Ritchie, M., Diyagama, D., Neilson, J., Van Laar, R., Dobrovic, A., Holloway, A., and Smyth, G.K. (2006). Empirical array quality weights in the analysis of microarray data. *BMC Bioinformatics* 7, 261.

29. Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research* 43, e47.

30. Ritchie, M.E., Silver, J., Oshlack, A., 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.

31. Robinson, M.D. and Oshlack, A. (2010). A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biology* 11, R25.

32. Shi, W., De Graaf, C.A., Kinkel, S.A., Achtman, A.H., Baldwin, T., Schofield, L., Scott, H.S., Hilton, D.J., and Smyth, G.K. (2010). Estimating the proportion of microarray probes expressed in an RNA sample. *Nucleic Acids Research* 38, 2168–2176.

33. Shi, W., Oshlack, A., and Smyth, G.K. (2010). Optimizing the noise versus bias trade-off for Illumina Whole Genome Expression Beadchips. *Nucleic Acids Research* 38, e204.

34. Skaletsky, H., Kuroda-Kawaguchi, T., Minx, P.J., Cordum, H.S., Hillier, L., Brown, L.G., Repping, S., Pyntikova, T., Ali, J., Bieri, T., Chinwalla, A., Delehaunty, A., Delehaunty, K., Du, H., Fewell, G., Fulton, L., Fulton, R., Graves, T., Hou, S.F., Latrielle, P., Leonard, S., Mardis, E., Maupin, R., McPherson, J., Miner, T., Nash, W., Nguyen, C., Ozersky, P., Pepin, K., Rock, S., Rohlfing, T., Scott, K., Schultz, B., Strong, C., Tin-Wollam, A., Yang, S.P., Waterston, R.H., Wilson, R.K., Rozen, S., and Page, D.C. (2003). The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature* 423, 825–837.

35. Smyth, G.K. (2004). Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology* 3, Article 3.

36. Smyth, G.K. and Altman, N.S. (2013). Separate-channel analysis of two-channel microarrays: recovering inter-spot information. *BMC Bioinformatics* 14, 165.

37. Smyth, G.K., Michaud, J., and Scott, H.S. (2005). Use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics* 21, 2067–2075.

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

39. Smyth, G.K., Yang, Y., and Speed, T. (2003). Statistical issues in cDNA microarray data analysis. *Methods in Molecular Biology* 224, 111–136.

40. Visvader, J.E. (2009). Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. *Genes & development* 23, 2563–2577.

41. 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.

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

43. Wolfinger, R.D., Gibson, G., Wolfinger, E.D., Bennett, L., Hamadeh, H., Bushel, P., Afshari, C., and Paules, R.S. (2001). Assessing gene significance from cDNA microarray expression data via mixed models. *Journal of Computational Biology* 8, 625–637.

44. Wu, D., Lim, E., Vaillant, F., Asselin-Labat, M., Visvader, J.E., and Smyth, G.K. (2010). ROAST: rotation gene set tests for complex microarray experiments. *Bioinformatics* 26, 2176–2182.

45. Wu, D. and Smyth, G.K. (2012). Camera: a competitive gene set test accounting for inter-gene correlation. *Nucleic Acids Research* 40, e133.

46. Yang, Y.H., Dudoit, S., Luu, P., Lin, D.M., Peng, V., Ngai, J., and Speed, T.P. (2002). Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Research* 30, e15.

47. Yang, Y.H., Dudoit, S., Luu, P., and Speed, T.P. (2001). Normalization for cDNA microarray data. In M.L. Bittner, Y. Chen, A.N. Dorsel, and E.R. Dougherty, editors, *Microarrays: Optical Technologies and Informatics*, pages 141–152. Proceedings of SPIE, Volume 4266, San Jose, CA.

48. Yang, Y.H. and Speed, T.P. (2003). Design and analysis of comparative microarray experiments. In T.P. Speed, editor, *Statistical Analysis of Gene Expression Microarray Data*, pages 35–91. Chapman & Hall/CRC Press.

49. Yang, Y.H. and Thorne, N.P. (2003). Normalization for two-color cDNA microarray data. In D.R. Goldstein, editor, *Science and Statistics: A Festschrift for Terry Speed*, pages 403–418. Institute of Mathematical Statistics Lecture Notes – Monograph Series, Volume 40.