GEO Database: GSE46268 vitamin D

Jean-Yves Sgro

May 9, 2017

Contents

1 Introduction

This exercise is meant to try knitr (Xie 2016, Xie (2015), Xie (2014)) with a published dataset (Wheelwright et al. 2014) on the GEO database (Barrett et al. 2013).

The title of the dataset is:

Gene expression profile of human monocytes stimulated with all-trans retinoic acid (ATRA) or 1,25a-dihydroxyvitamin D3 (1,25D3)

and is available at the GEO web site at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46268

Data was in the published paper (Wheelwright et al. 2014):

> Wheelwright M, Kim EW, Inkeles MS, De Leon A *et al.* All-trans retinoic acid-triggered antimicrobial activity against Mycobacterium tuberculosis is dependent on NPC2. J Immunol 2014 Mar 1;192(5):2280-90. PMID: 24501203

2 Prerequisites

2.1 CRAN packages

The following R packages are used: `knitr` and other `CRAN` packages will be installed if necessary. Make sure that the "Install Dependencies" button is checked. You can also use line commands such as:

```
install.packages("knitr", repos="http://cran.us.r-project.org")
install.packages("rmarkdown", repos="http://cran.us.r-project.org")
```

2.2 Bioconductor modules

The following BioConductor modules are necessary. To install them run the following code manually if necessary:

```
source("http://bioconductor.org/biocLite.R")
biocLite("GEOquery")
biocLite("limma")
biocLite("Biobase")
biocLite("affy")
```

3 knitr

3.1 Create a new project

An **RStudio** project is a set-up that uses a specific directory to contain the data that we want to use for analysis.

To set-up a new project follow do the following in **RStudio**:

1. Click the `File` menu button, then `New Project`.
2. Click `New Directory`.
3. Click `Empty Project`.
4. Type in the name of the directory to store your project, *e.g.* `VitaminD`.
5. For now don't check "Create a git repository" (should be unselected by default)

6. Click the `Create Project` button.

The project will be saved in the default directory: `/Users/YourName` on the Mac unless you navigate to e.g. the `Desktop` to more easily find the it later.

3.2 R Markdown script file

You can start a new `R Markdown` file with the following menu cascade:

File > New File > R Markdown...

Within the file there will be some examples data.

Remove everything but keep the "header" that is held between 2 lines with dashes `---` as they serve for the final output.

3.2.1 R Markdown

For this exercise you can use very simple `R` Markdown for separating paragraphs with the `#` sign, for example:

```
# Heading 1
## Heading 2
### Heading 3
```

The rest of the text can be simply plain text.

If you need to review R Markdown syntax you can do so from one of the previous exercises Using_RStudio_II.html (short URL: http://bit.ly/21tdUYv ; or check the complete notes from that session at http://bit.ly/1TDrmJB)

For inserting `R` code use the green button with the white **C** and an orange arrow. Or you can write the necessary tick marks by hand. The code "chunk" can have a ***unique*** optional name label:

```
“‘{r optional_name_label }
# This code block starts with 3 backticks and will end the same way.
# Place the R code within these boundaries.
“‘
```

3.3 Caching

Using `knitr` for the exercise allows to use the "**cache**" method which is best used with large data. For these microarray data it saves a minute or two, but for larger datasets it can be a substential time saver.

Here we'll set the cache globally so we don't have to repeat the caching request for each code chunk.

To engage the caching add the following code within your `.Rmd` project file, preferably at the top below the header:

```
“‘{r global_options_settings, include=TRUE, echo=FALSE }
# Global options:
opts_chunk$set(warning=FALSE, message=FALSE, comment="", cache=TRUE)
“‘
```

These global settings make the following changes:

- `warning=FALSE` and `message=FALSE` suppress in the final output any warnings or messages that usually appear in red on the regular `R` console. These can take a lot of space, be unsightly and are not necessary in the final report.

- `comment=""` removes the `##` mark after all output. If you liked that feature, then you can omit this setting.
- `cache=TRUE` is the one that is of interest for large datasets... As you work on your project, if a file, a plot or a dataframe does not need to be changed when you make changes to your file, then the cached data will be used and can save a lot of time.
- Within the `{r}` bracket `global_options_settings` is an optional name for this code "chunk".
- `include=TRUE` means that we want the code to be included/activated.
- `echo=FALSE` means that we don't want anything related to this code to appear within the final report.

There are more caching options or methods detailed at http://yihui.name/knitr/demo/cache/

4 The GEO database

> The Gene Expression Omnibus (GEO) is a public repository that archives and freely distributes microarray, next-generation sequencing, and other forms of high-throughput functional genomic data submitted by the scientific community.

Further information about the database can be found at http://www.ncbi.nlm.nih.gov/geo/info/

A **general overview** of the database can be found at http://www.ncbi.nlm.nih.gov/geo/info/

The data is available in various forms and formats. In summary the various names for data files are summarized in the fillowing table (from http://bit.ly/1LCJOj9 ; Aug 11, 2015 archive: bit.ly/1p2N65D)

Data type	Description
GEO Platform (GPL)	These files describe a particular type of microarray. They are annotation files.
GEO Sample (GSM)	Files that contain all the data from the use of a single chip. For each gene there will be multiple scores including the main one, held in the VALUE column.
GEO Series (GSE)	Lists of GSM files that together form a single experiment.
GEO Dataset (GDS)	These are curated files that hold a summarized combination of a GSE file and its GSM files. They contain normalized expression levels for each gene from each sample (i.e. just the VALUE field from the GSM file).

Today we'll use an experiment on vitamin D stored in a **GSE** file containing multiple samples. Within the database, each of the samples also has an individual **GSM** entry.

5 Explore the GEO page

1. Go to the dataset link GSE46268
2. Scroll down the page and click the [+]**More** at the Samples(12) entry
3. Note the name of the samples and their treatments. We'll find this information again later
4. Note the link named Analyse with GEO2R. **The R code for this exercise is derived from this option.** Check the "R Script" tab to see the code on the GEO web page.
5. You can read information about this service on the About GEO2R page
6. You can also watch the 4 minutes YouTube video titled **GEO2R: Analyze GEO Data** on that same page.

6 Analysis based on GEO2R script

> ***Caveat***: this exercise is meant to explore how the GEO database system of `GEO2R` conducts the computation. The authors of the paper have used other software to draw conclusions on signigicant genes and there is no attempt at this level to reproduce the results of the paper.

6.1 Load the Bioconductor libraries:

```
library(GEOquery)
library(Biobase)
library(limma)
library(affy)
```

6.2 Command getGEO() and R lists

The command `getGEO()` is a function from the packages `GEOquery` (S. Davis and Meltzer 2007) that can download data directly from the GEO database http://www.ncbi.nlm.nih.gov/geo/ .

The help command `?getGEO` provides a description that stating that *It directs the download (if no filename is specified) and parsing of a GEO SOFT format file into an R data structure[..]*

However, the default command as specified in the **Usage** section is:

```
getGEO(GEO = NULL, filename = NULL, destdir = tempdir(), GSElimits=NULL,
GSEMatrix=TRUE,AnnotGPL=FALSE,getGPL=TRUE)
```

The `GSEMatrix=TRUE` option is therefore the default and will open the Matrix format and ***NOT*** the SOFT format as implied by the definition. The result will be a different type of `R` object data structure.

The data exist in various file formats on the GEO database.

Format name	Format
SOFT	Simple Omnibus Format in Text.
MINiML	(MIAME Notation in Markup Language - XML format
Matrix	spreadsheet containing the final, normalized values that are comparable across rows and Samples

The SOFT format contains both data and annotations and therefore the files are larger. The Matrix format is similar but without the annotation.

6.3 Load the dataset

```
gset <- getGEO("GSE46268", GSEMatrix =TRUE)
```

While the format is deemed a "matrix" the `gset` object created is in fact a list that contains one set of experiment and is therefore a list with one element!

We can see that with this code:

```
class(gset)
```

```
[1] "list"
```

```
length(gset)
```

```
[1] 1
```

```
names(gset)
```

```
[1] "GSE46268_series_matrix.txt.gz"
```

Note that the name could have been obtained with the attributes `attr()` function calling on the list item `"names"`.

```
attr(gset,"names")
```

We therefore conclude that **for now** `gset` is a list with one element that was derived from file *GSE46268_series_matrix.txt.gz*.

This result will help explain how the code continues and to better understand that let's make a quick *a parte* into `R` lists.

Let's create a list `L` containing 2 objects, for example a vector of numbers `vn` and a vector of characters `vc`:

```
# Create list L
L  <- list(vn=c(2,3,5), vc=c("sun", "moons"))
# Print list L
L
```

```
$vn
[1] 2 3 5

$vc
[1] "sun"   "moons"
```

```
# Print first item and class of list L
L[1]
```

```
$vn
[1] 2 3 5
```

```
class(L[1])
```

```
[1] "list"
```

```
# Print content of first item of list L and class
L[[1]]
```

```
[1] 2 3 5
```

```
class(L[[1]])
```

```
[1] "numeric"
```

In a similar way, the code continues by checking the length of the `gset` object which is still a ***list***. This conditional statement checks if the length of the list is greater than `1` and saves the result in "index" variable `idx` and checks for the pattern `GPL570` which is the name in **GEO** for the type of microarray that was used in the experiment.

The `gset` object is then overwritten with the experimental data contained within the 1rst object of the list (or more if more than 1 have been detected) :

```
if (length(gset) > 1) idx <- grep("GPL570", attr(gset, "names")) else idx <- 1
gset <- gset[[idx]]
```

We can now check again the class and length of `gset` since this has changed it:

```
class(gset)
```

```
[1] "ExpressionSet"
attr(,"package")
[1] "Biobase"
```

```
length(gset)
```

```
[1] 1
```

```
slotNames(gset)
```

```
[1] "experimentData"    "assayData"         "phenoData"
[4] "featureData"       "annotation"        "protocolData"
[7] ".__classVersion__"
```

The data structure `ExpressionSet` is a kind of data frame that contains the complete information about the experiment. The `names()` command not longer applies as `gset` is no longer a list but the command `slotNames()` applies to dataframe and can be used.

6.4 Explore the new dataset structure

6.4.1 Dataset dimensions:

```
dim(gset)
```

```
Features  Samples
   54675       12
```

Features are the number of "genes" on the array and we see that there are 54675. We can also see that there are 12 samples.

6.4.2 Dataset structure

Try the following command to get hints from the data structure

```
str(gset)
```

6.5 Samples: "phenotypic" data

"phenotypic" data is the list of samples with their associated attributes and their treatment

The following command will list all attributes:

```
pData(phenoData(gset))
```

We can get the dimensions of the data table with the command:

```
dim(pData(phenoData(gset)))
```

```
[1] 12 38
```

That will tell us that there are **12** rows, corresponding to the number of samples, and there are **38** columns in the table.

We can list the name of the columns in the table with the command:

```
colnames(pData(phenoData(gset)))
```

And we'll print only the first 6: We can list the name of the columns in the table with the command:

```
colnames(pData(phenoData(gset)))[1:6]
```

```
[1] "title"            "geo_accession"    "status"
[4] "submission_date"  "last_update_date" "type"
```

Here is a modified command showing only two columns from the attributes table that seem to be most informative:

```
pData(phenoData(gset))[ , c(12,13)]
```

```
           characteristics_ch1.2                characteristics_ch1.3
GSM1127890   individual: donor 1                   treatment: control
GSM1127891   individual: donor 2                   treatment: control
GSM1127892   individual: donor 3                   treatment: control
GSM1127893   individual: donor 4                   treatment: control
GSM1127894   individual: donor 1   treatment: all-trans retinoic acid
GSM1127895   individual: donor 2   treatment: all-trans retinoic acid
GSM1127896   individual: donor 3   treatment: all-trans retinoic acid
GSM1127897   individual: donor 4   treatment: all-trans retinoic acid
GSM1127898   individual: donor 1 treatment: 1,25a-dihydroxyvitamin D3
GSM1127899   individual: donor 2 treatment: 1,25a-dihydroxyvitamin D3
GSM1127900   individual: donor 3 treatment: 1,25a-dihydroxyvitamin D3
GSM1127901   individual: donor 4 treatment: 1,25a-dihydroxyvitamin D3
```

6.6 Sample labels

```
# make proper column names to match toptable
fvarLabels(gset) <- make.names(fvarLabels(gset))
```

The function `make.names()` is from the `base` pakcage and its descrition is to *make syntactically valid names out of character vectors.*

6.7 Making groups

Below is code to help extract the name of treatment. This is not part of the original `GEO2R` code.

```
# NOTE:
# The following code is added to the GEO2R code to automatically extract
# the name of the treatments from column 13 of the phenoData
tr <- levels(unique(pData(phenoData(gset))[13])[,1])
tr1 <- gsub("treatment: ", "", tr[1])
tr2 <- gsub("treatment: ", "", tr[2])
tr3 <- gsub("treatment: ", "", tr[3])
# The variables tr1, tr2 and tr3 are used wihthin
# the knitr text file to name the treatments in the
# paragraph below
```

The names of treatments are within the 13th column of the `phenoData` information and are saved within object `tr`. The text **"treatment:"** is removed by *gobal substitution* (command `gsub`) to an empty string (`""`) leaving the treatment name available.

Note:In the text below this table, the `.RMD` file used to create this document used this string to write the name of the treatment without any need for manual copy/paste.

Object name	Extracted named treatment
`tr`	treatment: 1,25a-dihydroxyvitamin D3, treatment: all-trans retinoic acid, treatment: control
`tr1`	1,25a-dihydroxyvitamin D3
`tr2`	all-trans retinoic acid
`tr3`	control

Groups were created using the GEO2R web interface which resulted in the following vectors to be created to distinguished three groups labeled `G0`, `G1` and `G2` which, based on the phenotypic data we explored above would correspond to treatments named ***1,25a-dihydroxyvitamin D3***, ***all-trans retinoic acid***, and ***control***.

```
# group names for all samples
sml <- c("G0","G0","G0","G0","G1","G1","G1","G1","G2","G2","G2","G2");
```

This means that the software has idendified 3 groups of 4 that make up our samples! It is not surprising as this set-up had to be done by hand within the web interface.

6.8 Log transform

This is an important step for better statistical evaluation of microarray data.

First the observed expression values are extracted by the function `exprs()` from the `Biobase` package and stored into object `ex` taking care of removing `NA` values (`na.rm=T`). Negative values (for which a log cannot be calculated) are replaced with `NaN` and at the end the original expression values are replaced by their log: `exprs(gset) <- log2(ex)`.

```
# log2 transform
ex <- exprs(gset)
qx <- as.numeric(quantile(ex, c(0., 0.25, 0.5, 0.75, 0.99, 1.0), na.rm=T))
LogC <- (qx[5] > 100) ||
          (qx[6]-qx[1] > 50 && qx[2] > 0) ||
          (qx[2] > 0 && qx[2] < 1 && qx[4] > 1 && qx[4] < 2)
if (LogC) { ex[which(ex <= 0)] <- NaN
  exprs(gset) <- log2(ex) }
```

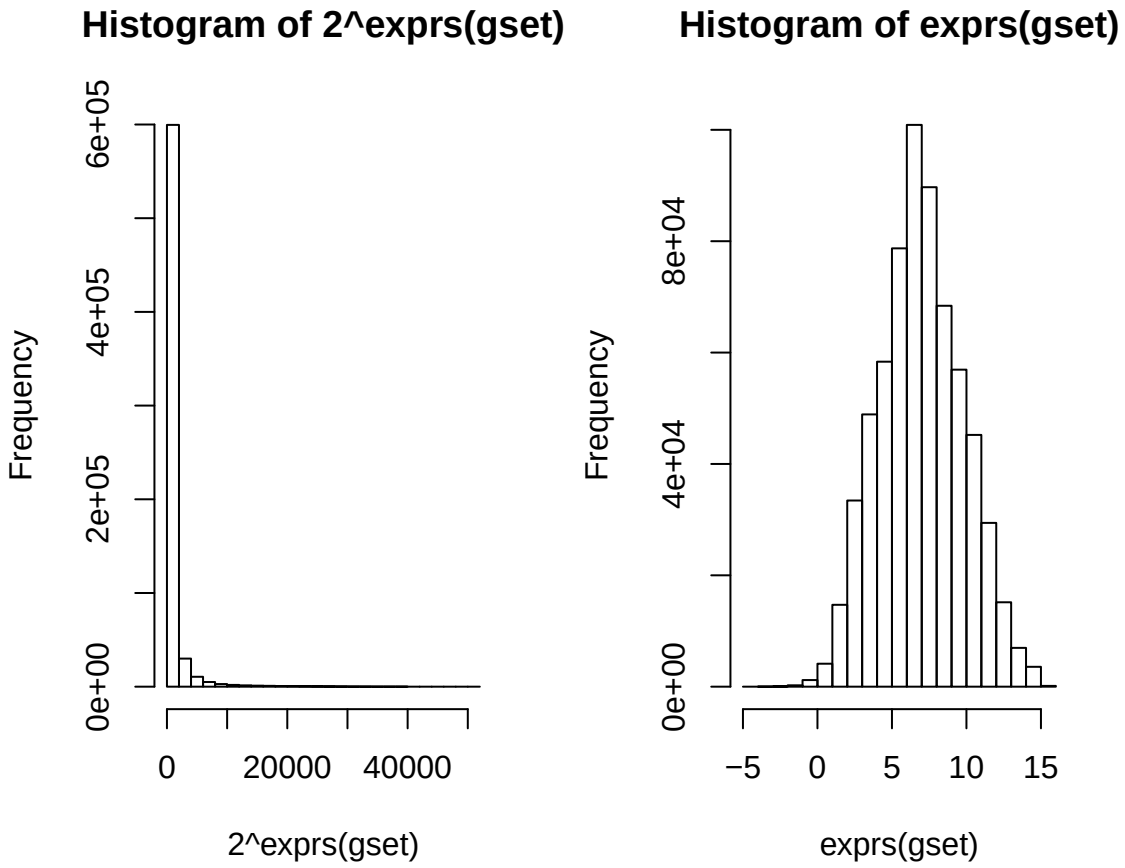
There is some internal calculation on quantiles, and the replacement of negative or zero values (`ex <= 0`) by `NaN` before taking the `log2` of the intensities.

The final result is an ***"expression set"*** which contains the **log2** transform of the intensity values.

The `log` transformation is a necessary step to change the data into a form to which statistics can be better applied: it gives the data a more "Normal" distribution as can be seen by a histogram.

We can compare a histogram of values before and after `log2` transformation:

```
# Split graphics in 2 sections
par(mfrow=c(1,2))
# Before log2 - since it's already been log2 trasnform
# we use 2^ to compute back the original value:
hist(2^exprs(gset), breaks=25)
#After log2
hist(exprs(gset), breaks=25)
```




```
# Return graphics to default
par(mfrow=c(1,1))
```

6.9 Proceed with analysis

Set up the data and proceed with analysis

This uses a method from the `limma` package (Ritchie et al. 2015).

The following code "chunk" prepares an experimental **design matrix** where samples on rows are represented within a matrix by either `1` or `0` and columns are the names of the sample groups `G0`, `G1` and `G2` obtained with the `as.factor()` and `levels()` functions.

The `makeContrasts()` function creates pair-wise comparison between groups.

The `limma` package then uses Baysian statistical methods (`eBayes()`) to fit the data to the model.

The data is adjusted for False Discovery Rate (`adjust="fdr"`) and sorted by column `B` which represents the *log-odds that the gene is differentially expressed.*

Finally, the top `250` "genes" are extracted into a table object `tT` (the default export number is `10`, see `?topTable`.)

FDR: **False Discovery Rate**[1] The False discovery rate (FDR) is one way of conceptualizing

[1] https://en.wikipedia.org/wiki/False_discovery_rate

the rate of type I errors in null hypothesis testing when conducting multiple comparisons. FDR-controlling procedures are designed to control the expected proportion of rejected null hypotheses that were incorrect rejections ("false discoveries").(Benjamini and Hochberg 1995)

```
# set up the data and proceed with analysis
fl <- as.factor(sml)
gset$description <- fl
design <- model.matrix(~ description + 0, gset)
colnames(design) <- levels(fl)
fit <- lmFit(gset, design)
cont.matrix <- makeContrasts(G2-G0, G1-G0, G2-G1, levels=design)
fit2 <- contrasts.fit(fit, cont.matrix)
fit2 <- eBayes(fit2, 0.01)
tT <- topTable(fit2, adjust="fdr", sort.by="B", number=250)
```

Side exercise: check content of objects from code above
- `sml`
- `fl`
- `design` before and after `colnames()` change
- `cont.matrix`

The best 250 are kept in the final table. This number is arbitrary, and if left unspecified would provide a table with all results.

We can count the number of columns of the Table:

```
dim(tT)
```

```
[1] 250  23
```

We can see the name of the columns:

```
colnames(tT)
```

```
 [1] "ID"                                "GB_ACC"
 [3] "SPOT_ID"                           "Species.Scientific.Name"
 [5] "Annotation.Date"                   "Sequence.Type"
 [7] "Sequence.Source"                   "Target.Description"
 [9] "Representative.Public.ID"          "Gene.Title"
[11] "Gene.Symbol"                       "ENTREZ_GENE_ID"
[13] "RefSeq.Transcript.ID"              "Gene.Ontology.Biological.Process"
[15] "Gene.Ontology.Cellular.Component" "Gene.Ontology.Molecular.Function"
[17] "G2...G0"                           "G1...G0"
[19] "G2...G1"                           "AveExpr"
[21] "F"                                 "P.Value"
[23] "adj.P.Val"
```

We can view the first 5 witin a table with the command:

```
View(tT[1:5,])
```

We can also view the first **top 3** and specify only a few of the most interesting columns (for printing space sake.)
Note the name of the column headers.

```
head(tT)[1:3,c(2,11:12,17:20, 22:23)]
```

```
               GB_ACC Gene.Symbol ENTREZ_GENE_ID   G2...G0     G1...G0
226099_at    AI924426        ELL2          22936 2.7737814 -0.04501778
206504_at   NM_000782     CYP24A1           1591 9.4846618 -0.88549603
```

```
212685_s_at  AI608789        TBL2          26608 0.4468399  3.36551801
              G2...G1   AveExpr      P.Value    adj.P.Val
226099_at    2.818799 11.341555 6.384254e-10 1.722549e-05
206504_at   10.370158  7.853788 6.519834e-10 1.722549e-05
212685_s_at -2.918678 11.480685 9.451573e-10 1.722549e-05
```

6.10 Update annotations

The annotations are the default options and can be updated with the latest NCBI annotations.

Each microarray has a "platform" ID code specific to that array. Here we find the code for the array with `annotation(gset)` which gives the result ***GPL570*** and this is passed onto the next command to download the corresponsing "platform" file from NCBI/GEO and stored within `platf`.

The operations that follow check the name of "column" information in `tT` and `gset` and "merge" into a final version based on the ***common*** column `ID`.

```
# load NCBI platform annotation
gpl <- annotation(gset)
platf <- getGEO(gpl, AnnotGPL=TRUE)
ncbifd <- data.frame(attr(dataTable(platf), "table"))

# replace original platform annotation
tT <- tT[setdiff(colnames(tT), setdiff(fvarLabels(gset), "ID"))]
tT <- merge(tT, ncbifd, by="ID")
tT <- tT[order(tT$P.Value), ]  # restore correct order
```

Side exercise:
- check the output of commands `colnames(tT)` and `fvarLabels(gset)`
- check the meaning of `setdiff`. (Hint: in package `BiocGenerics`)

With the updated annotations the number of columns has changed:

```
dim(tT)
```

```
[1] 250  28
```

THe new annotation has completly rearranged and changed many of the columns, including the addtion of Gene Ontology.
We can see the name of the columns

```
colnames(tT)
```

```
 [1] "ID"                    "G2...G0"
 [3] "G1...G0"               "G2...G1"
 [5] "AveExpr"               "F"
 [7] "P.Value"               "adj.P.Val"
 [9] "Gene.title"            "Gene.symbol"
[11] "Gene.ID"               "UniGene.title"
[13] "UniGene.symbol"        "UniGene.ID"
[15] "Nucleotide.Title"      "GI"
[17] "GenBank.Accession"     "Platform_CLONEID"
[19] "Platform_ORF"          "Platform_SPOTID"
[21] "Chromosome.location"   "Chromosome.annotation"
[23] "GO.Function"           "GO.Process"
[25] "GO.Component"          "GO.Function.ID"
[27] "GO.Process.ID"         "GO.Component.ID"
```

The statistical columns are now at the begining of the table.

We can print the first 3 with some specific columns. Note that the row names are changed to a number, probably a ranking number.

```
head(tT)[1:3,c(1:10)]
```

```
             ID   G2...G0     G1...G0   G2...G1   AveExpr        F
202   226099_at 2.7737814 -0.04501778  2.818799 11.341555 256.8290
84    206504_at 9.4846618 -0.88549603 10.370158  7.853788 255.8234
122 212685_s_at 0.4468399  3.36551801 -2.918678 11.480685 238.6761
         P.Value    adj.P.Val
202 6.384254e-10 1.722549e-05
84  6.519834e-10 1.722549e-05
122 9.451573e-10 1.722549e-05
                                        Gene.title Gene.symbol
202      elongation factor for RNA polymerase II 2        ELL2
84  cytochrome P450 family 24 subfamily A member 1     CYP24A1
122                        transducin (beta)-like 2        TBL2
```

The file would be written in the current directory.

The table can be made nicer with `kable()`

`kable()` is a`knitr` function to render tables.

```
kable(head(tT)[1:3,c(1:10)])
```

	ID	G2...G0	G1...G0	G2...G1	AveExpr	F	P.Value	adj.P.Val	Gene.title
202	226099_at	2.7737814	-0.0450178	2.818799	11.341555	256.8290	0	1.72e-05	elongation factor
84	206504_at	9.4846618	-0.8854960	10.370158	7.853788	255.8234	0	1.72e-05	cytochrome P450
122	212685_s_at	0.4468399	3.3655180	-2.918678	11.480685	238.6761	0	1.72e-05	transducin (beta)

6.11 Write output results table into a file

The code provided `write.table(tT, file=stdout(), row.names=F, sep="\t")` would print the 250 results onto the console as the given file name is `stdout()`. To write the complete list we would have to update the `number=` to that of the number of "genes" and provide a proper filename such as `"results.txt"` printed with quotes.

7 Box plots

The next portion of the code is meant to create box plots. The code is redundant by downloading again, creating groups again etc. but that makes that chunk of code independent from the rest.
Some slight difference can be noted for the `ex` object created here with a specification as to the ordering of the samples, so that samples are presented together by group.

7.1 The complete GEO2R code:

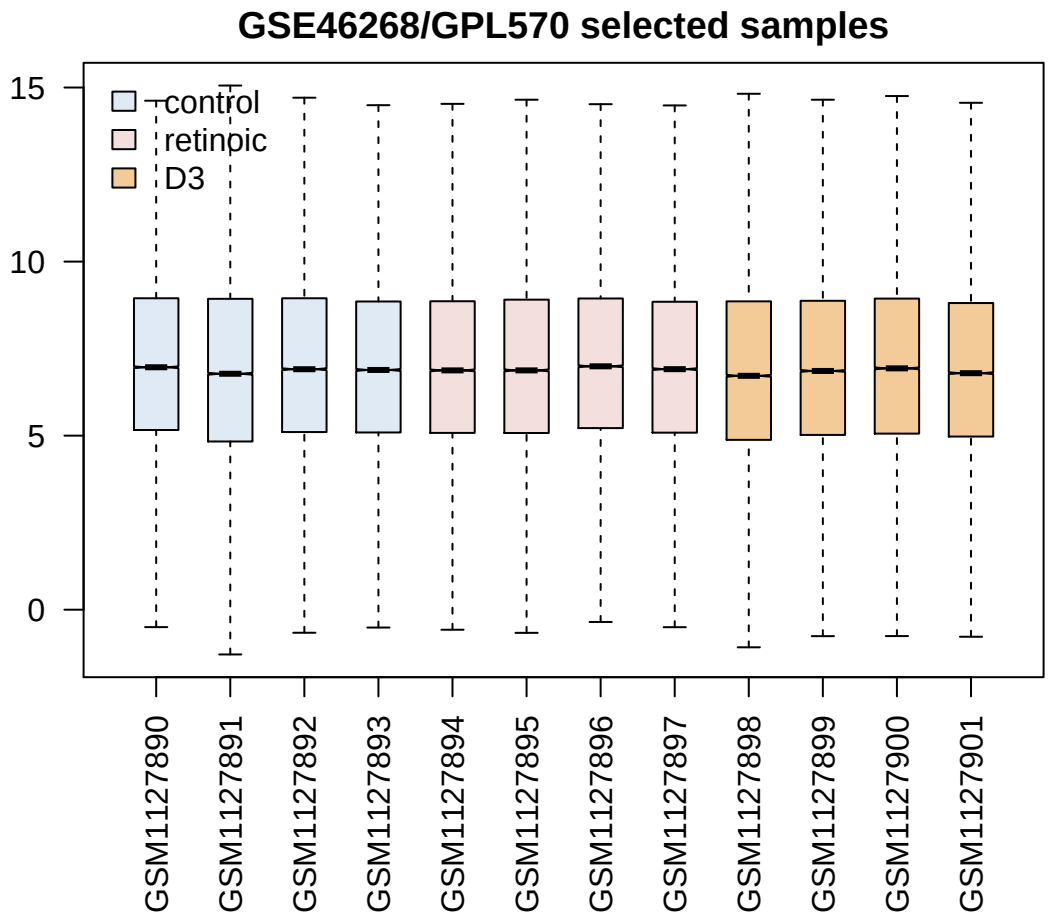
```
# Version info: R 3.2.3, Biobase 2.30.0, GEOquery 2.40.0, limma 3.26.8
################################################################
#   Boxplot for selected GEO samples
library(Biobase)
```

```
library(GEOquery)

# load series and platform data from GEO

gset <- getGEO("GSE46268", GSEMatrix =TRUE)
if (length(gset) > 1) idx <- grep("GPL570", attr(gset, "names")) else idx <- 1
gset <- gset[[idx]]

# group names for all samples in a series
sml <- c("G0","G0","G0","G0","G1","G1","G1","G1","G2","G2","G2","G2")

# order samples by group
ex <- exprs(gset)[ , order(sml)]
sml <- sml[order(sml)]
fl <- as.factor(sml)
labels <- c("control","retinoic","D3")

# set parameters and draw the plot
palette(c("#dfeaf4","#f4dfdf","#f2cb98", "#AABBCC"))
dev.new(width=4+dim(gset)[[2]]/5, height=6)
par(mar=c(2+round(max(nchar(sampleNames(gset)))/2),4,2,1))
title <- paste ("GSE46268", '/', annotation(gset), " selected samples", sep ='')
boxplot(ex, boxwex=0.6, notch=T, main=title, outline=FALSE, las=2, col=fl)
legend("topleft", labels, fill=palette(), bty="n")
```

7.2 Continuation

We can start where the first change occurs: with the updating of the `ex` object:

```
##############################################################
#   Boxplot for selected GEO samples
#   (removed chunk)

# order samples by group
ex <- exprs(gset)[ , order(sml)]
sml <- sml[order(sml)]
fl <- as.factor(sml)
labels <- c("control","retinoic","D3")

# set parameters and draw the plot
palette(c("#dfeaf4","#f4dfdf","#f2cb98", "#AABBCC"))
# dev.new(width=4+dim(gset)[[2]]/5, height=6)
par(mar=c(2+round(max(nchar(sampleNames(gset)))/2),4,2,1))
title <- paste ("GSE46268", '/', annotation(gset), " selected samples", sep ='')
boxplot(ex, boxwex=0.6, notch=T, main=title, outline=FALSE, las=2, col=fl)
legend("topleft", labels, fill=palette(), bty="n")
```



The `dev.new()` options shows the plot in a new window on `R` or `RStudio` when running interactively but does **not** include it within an RMarkdown report.

The solution is to simply comment out (`#`) the `dev.new()` line so that the final plot is included in the final report.

Within the `plot()` command the option `las=2` write the names of the samples as vertical labels on the `x` axis.

One important feature to look at in box-plots is the horizontal mark within the boxes that represents the `median` value. The box itself is made of the data from the 25th to the 75th percentile. If the values are similar than it is possible to use the data to compare the samples against each other.

Learn more about box plots at https://en.wikipedia.org/wiki/Box_plot

8 GEO2R ends

The GEO2R `R` scripts only contains the code that we have explored. On the interactive web site, there are other possibilities, for example to show the expression values of specific genes across all samples.

Details of the GEO2R method can be found on the GEO web site at http://www.ncbi.nlm.nih.gov/geo/info/geo2r.html

9 Adding to GEO2R results

There are many other calculations or plots that can be added to the GEO2R analysis. Here are a few examples.

We may use some of the objects defined above as well, for example groups names.

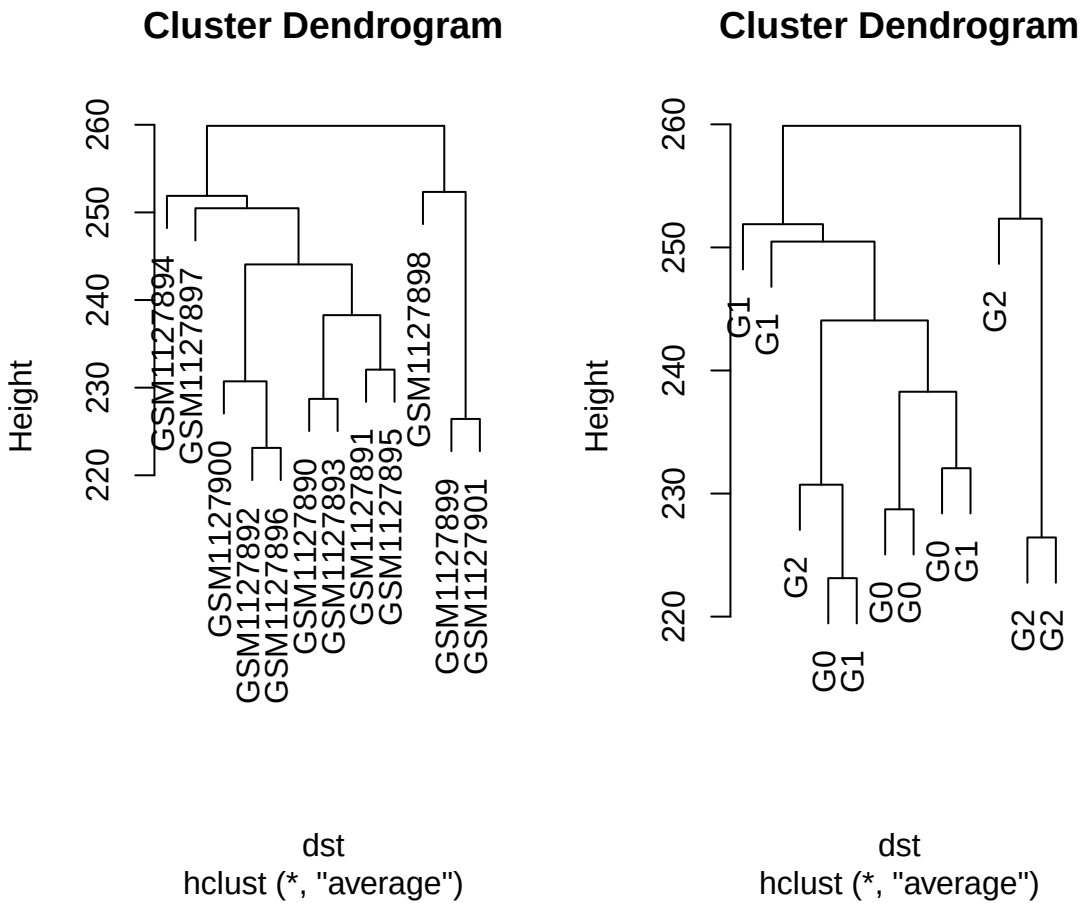
9.1 Simple clustering

A simple cluster of samples can be obtained with:

```
# calculate a distance matrix between each sample (each array)
dst <- dist(t(exprs(gset)))
# Hierarchical cluster analysis on above distance matrix
hh <- hclust(dst, method="average")
```

We can then plot the tree by sample name or by group name using the `fl` object created previously:

```
# We will plot both of them on the same plot
par(mfrow=c(1,2))
# plot default is by sample name
plot(hh)
# label sample by group
plot(hh, label=fl)
```



```
par(mfrow=c(1,1))
```

Ideally all groups would cluster together, but it is common to find, as here, elements of a groups that cluster with anohter.

9.2 Principal Component Analysis

> The central idea of principal component analysis (PCA) is to reduce the dimensionality of a data set consisting of a large number of interrelated variables, while retaining as much as possible of the variation present in the data set. This is achieved by transforming to a new set of variables, the principal components (PCs), which are uncorrelated, and which are ordered so that the first ***few*** retain most of the variation present in all of the original variables[2] (Jolliffe 2002).

PCA can be computed with the `prcomp()` function from the `stats` package installed by default in all `R` installation. There is therefore no package to install.

`prcomp()` will a principal components analysis on the complete set of 54675 genes or probes within the gene expression levels inside the dataset (`exprs(gset)`). Function `t()` transposes the data matrix. Results are stored in object `PC` subsequenlty analysed by the `predict()` function to compute values for each sample. Results are placed into object `scores`.

```
PC=prcomp(t(exprs(gset)))
scores = predict(PC)
```

As stressed in the definition above the first ***few*** components are useful.

> Exercise:
> * Can you determine how many components were calculated?
> * Explore the class and content of `scores`.
> (Hint: `class()` and `dim()`, `print()`)
> Can you print only the values of the first 2 PC?

9.2.1 PCA Plot

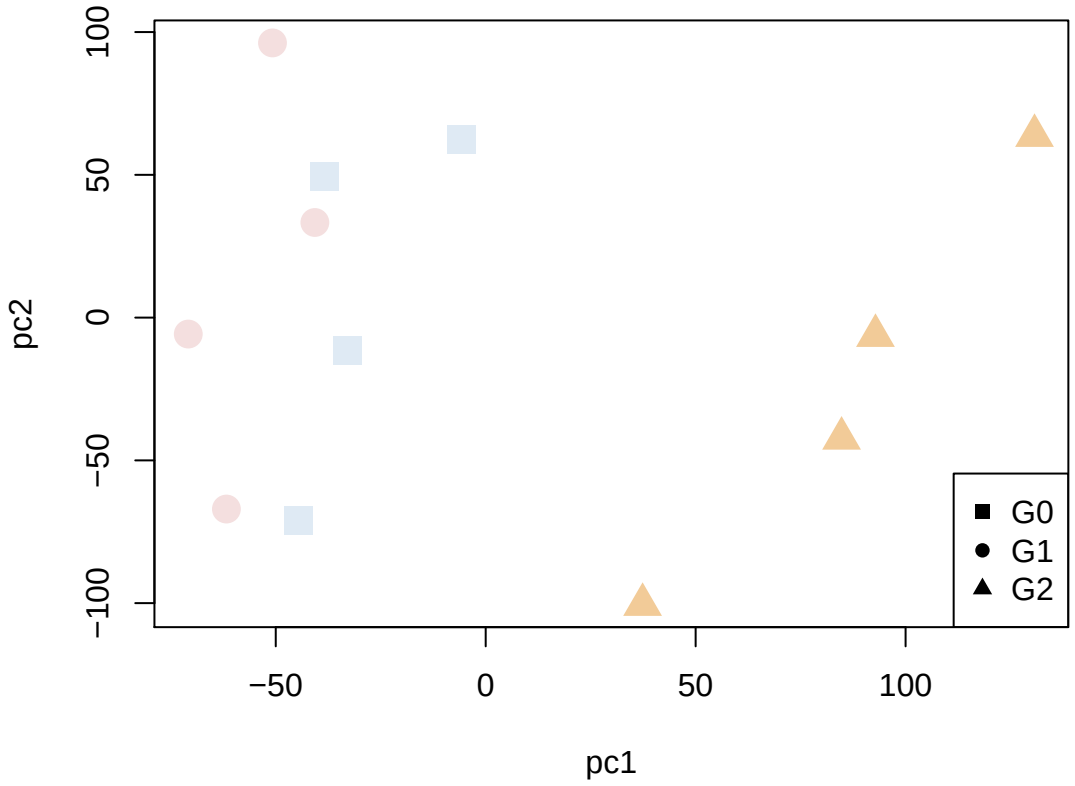
To make the plot code easier to follow it is best to prepare a few variables. Variable `fl` was defined previously as a factor for the groups and is used below to identify the shape of samples on the plot as well as the colors

```
# extract PC1 and PC2
pc1 <- scores[ ,1]
pc2 <- scores[ ,2]
# Create a vector of number for choosing the plot symbol
# We add 14 to reach the symbols that are filled
shape <- as.numeric(fl) + 14
```

We can now make a plot for the first 2 principal components and then add a legend:

```
plot(pc1, pc2, col=fl, pch=shape, cex=2)
# legend("topright", pch=unique(shape), paste(unique(fl)))
legend("bottomright", pch=unique(shape), paste(unique(fl)))
```

[2] http://www.springer.com/us/book/9780387954424

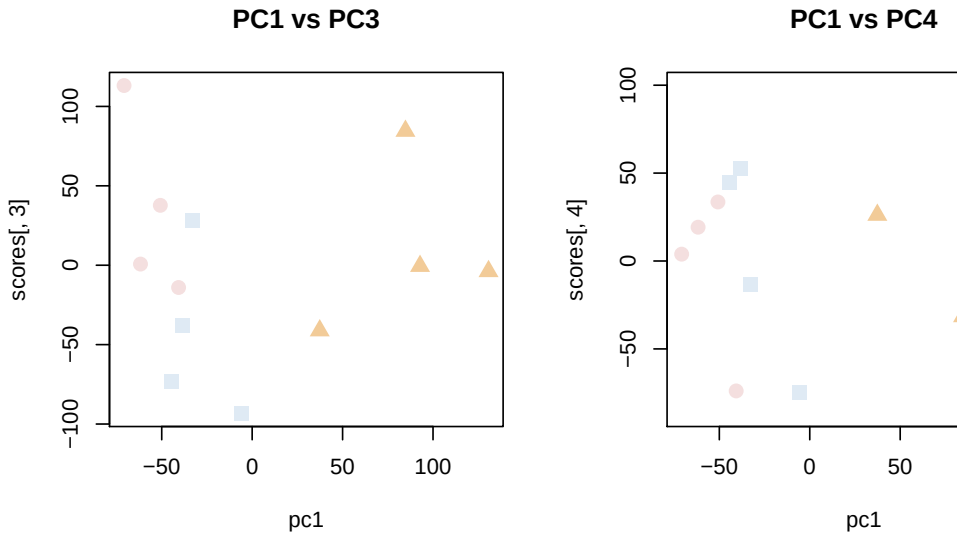


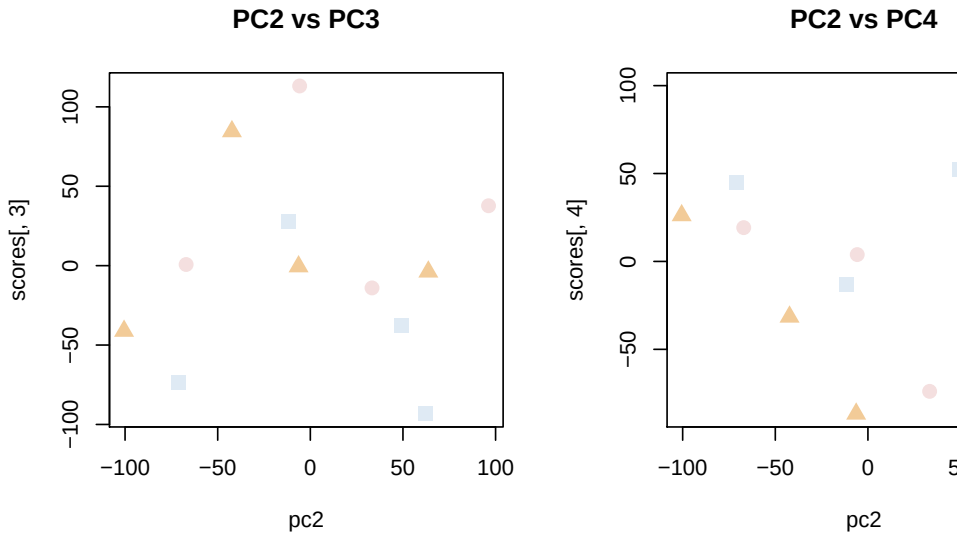
`cex=2` controls the size of the plotted shapes, here making them bigger.

The location of the legend can be controlled by a set of coordinates or predefined locations as used here. See `?legend` for more details.

From this simple plot it seems that group `G2` is more different than the other 2 groups.

Exercise:
* Make a similar plot for PC1 vs PC3
* Make a similar plot for PC1 vs PC4
* What happens if you use PC2 as horizontal axis?
* (Hints: it is not necessary to create `pc` objects, simply use `scores[,3]`, `scores[,4]` etc.)





You would get images similar to this:

Notes: there are methods to plot this data in 3D with interactive (mouse) rotation with the package `rgl` and the `plotlm3d` function[3] but it is also possible to use the `CRAN` package `scatterplot3d` for static plots:

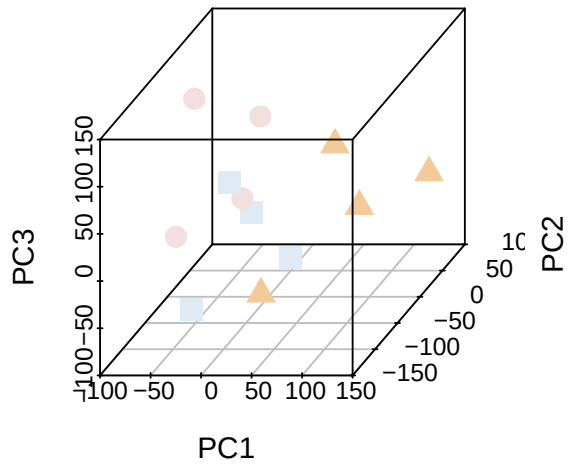
```
# Install scatterplot3d if wanted
install.packages("scatterplot3d", repos="http://cran.us.r-project.org")
```

```
library(scatterplot3d)
#
par(mfrow=c(2,2))

# Angle 50
scatterplot3d(scores[,1], scores[,2], scores[,3], xlab="PC1", ylab="PC2", zlab="PC3", pch=shape, color=

# Angle 40 (default)
scatterplot3d(scores[,1], scores[,2], scores[,3], xlab="PC1", ylab="PC2", zlab="PC3", pch=shape, color=
```

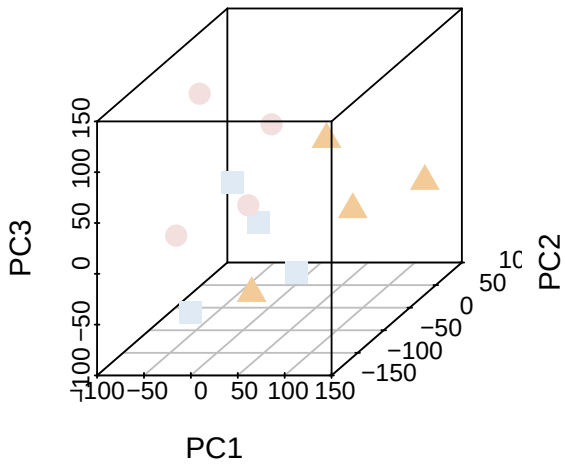
[3] https://github.com/sr1919/R/blob/master/ext/protm3d.R

```
# Angle 30
scatterplot3d(scores[,1], scores[,2], scores[,3], xlab="PC1", ylab="PC2", zlab="PC3", pch=shape, color=

# Angle 20
scatterplot3d(scores[,1], scores[,2], scores[,3], xlab="PC1", ylab="PC2", zlab="PC3", pch=shape, color=
```

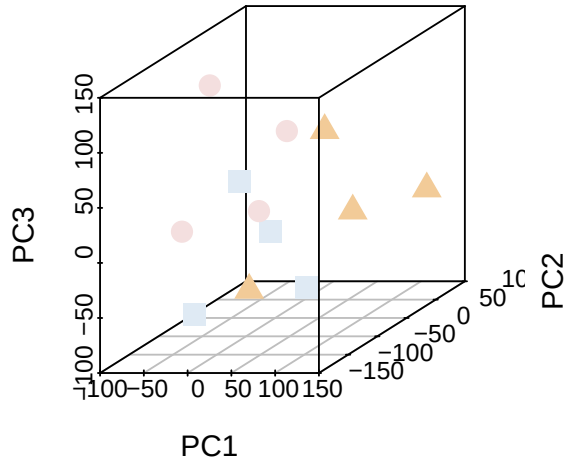
Angle 50



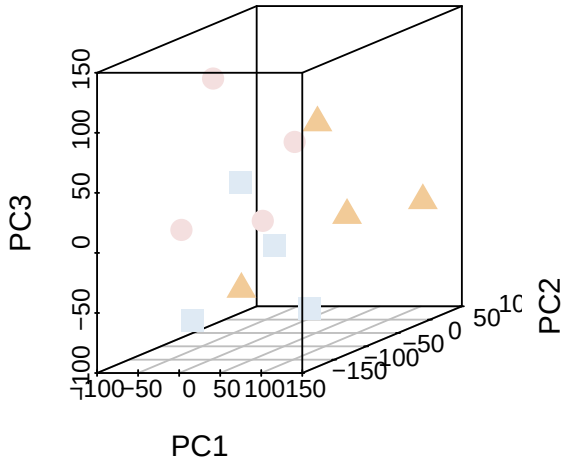
Angle 40 (default)



Angle 30



Angle 20




```
par(mfrow=c(1,1))
```

9.3 Heatmaps

Additional packages needed from both `CRAN` (for `gplots`) and Bioconductor (for `RColorBrewer`). Run the following installation code if necessary:

```
source("http://bioconductor.org/biocLite.R")
biocLite("RColorBrewer")
install.packages("gplots")
```

Load libraries:

```
library(RColorBrewer)
library(gplots)
```

–>

The heatmap can be constructed for each "contrast" for which we calculated a differential expression with `limma` when we constructed the contrast matrix `cont.matrix` during the calculations with `limma`:

```
      Contrasts
Levels G2 - G0 G1 - G0 G2 - G1
    G0      -1      -1       0
    G1       0       1      -1
    G2       1       0       1
```

We can "grab" each contrast name by selecting each element of the column names, for example `colnames(cont.matrix)[2]` yields **G1 - G0** which we can plot below with a false discovery rate (FDR) of 0.01.

The number of genes defined here is the number of "probesets" representing the genes onto the array.

The object `completeTopTable` will contain the differential expression table values for the selected contrast.

From this table we can select only those genes/probesets that match the chosen criterion that FDR should be less than the defined cut-off value, for example `FDR < 0.01`.

This creates the object `selected` which is a logical list of all the genes with `TRUE` or `FALSE` and can be used as a "selector" to select the same genes within the `gset` expression values that are then stored in object `esetSel`.

```
# Define FDR cut-off, typically 0.05 or 0.01
FDR_cutoff <- 0.01

# Calculate the number of genes
numGenes <- nrow(exprs(gset))

# Extract a "contrast" from the contrast matrix
contrast <- colnames(cont.matrix)[2]

# Select the differential expression for this specific contrast
# for all genes and sorted by columns "B" which represents
# the log-odds that a gene is differentially expressed.
completeTopTable <- topTable(fit2,coef=contrast, adjust="fdr", sort.by="B", number=numGenes)

# Create a logical selector containing TRUE or FALSE
# that defines if the gene meets the criterion about FDR
selected <- completeTopTable$adj.P.Val < FDR_cutoff

# Create a subset of the expression set only for selected genes
```

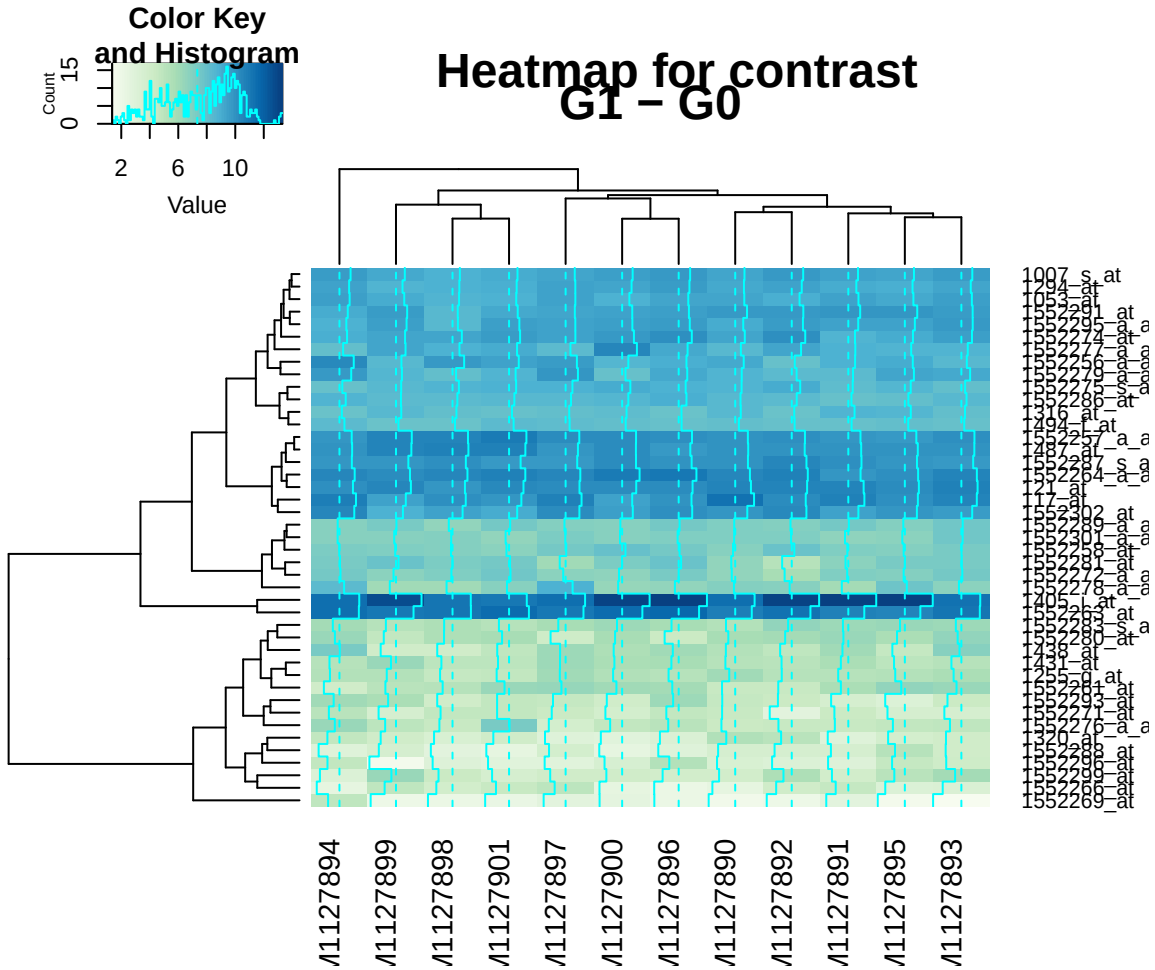
```
esetSel <- gset[selected]

# Check some esetSel properties. Dimensions
dim(esetSel)
```

```
Features  Samples
      43       12
```

```
# Calculate the number of genes that are selected. This number
# should be the same as the number of "Features" above.
sum(selected)
```

```
[1] 43
```

```
# Create a heatmap with heatmap.2 that allows more colors
# color gradient to represent the expression values of gene
# heatmap.2(exprs(esetSel))
hmcol <- colorRampPalette(brewer.pal(9,"GnBu"))(100)
heatmap.2(exprs(esetSel), col=hmcol, main=c("     Heatmap for contrast " , contrast ))
```



The color of the plotted genes depends on the palette chosen. By default the colors would be red-to-yellow but here we access one of the "green-blue" palettes from the `RColorBrewer` package. See `?brewer.pal` for all the color palettes descriptions.

From the total number of genes (54675) there are **43** that are selected for the plot. The number of genes is

the number of "probesets" onto the array.

The name of the Affymetrix probeset is listed on the right hand side of the heatmap. The function help (`?heatmap.2`) reveals that the annotation of the "genes" is by default the row names of the object plotted (`rownames(x)` for object `x`) and indeed:

```
rownames(esetSel)
```

```
 [1] "1007_s_at"    "1053_at"      "117_at"       "121_at"
 [5] "1255_g_at"    "1294_at"      "1316_at"      "1320_at"
 [9] "1405_i_at"    "1431_at"      "1438_at"      "1487_at"
[13] "1494_f_at"    "1552256_a_at" "1552257_a_at" "1552258_at"
[17] "1552261_at"   "1552263_at"   "1552264_a_at" "1552266_at"
[21] "1552269_at"   "1552271_at"   "1552272_a_at" "1552274_at"
[25] "1552275_s_at" "1552276_a_at" "1552277_a_at" "1552278_a_at"
[29] "1552279_a_at" "1552280_at"   "1552281_at"   "1552283_s_at"
[33] "1552286_at"   "1552287_s_at" "1552288_at"   "1552289_a_at"
[37] "1552291_at"   "1552293_at"   "1552295_a_at" "1552296_at"
[41] "1552299_at"   "1552301_a_at" "1552302_at"
```

Therefore, more manipulation is necessary to obtain the name gene symbol rather than the probeset to be listed.

9.3.1 Finding the gene symbols

First we have to wonder and verify the `class` of object `esetSel` which should be an "annotated data frame for expression sets".

```
class(esetSel)
```

```
[1] "ExpressionSet"
attr(,"package")
[1] "Biobase"
```

This is the main final type of data structure for many microarray experiment based on the Affymetrix platform. It contains the data and other annotations listed in various "slots."

```
slotNames(esetSel)
```

```
[1] "experimentData"    "assayData"         "phenoData"
[4] "featureData"       "annotation"        "protocolData"
[7] ".__classVersion__"
```

We have explored before how to look at, extract and modify the `phenoData`. For this subset, the complete phenotypic data information would have been transfered. You can verify that with the commands that we know, for example `pData(esetSel)`.

The "slot" of interest now is `featureData` which contains the actual data. While we used a helper function `pData()` before, we can also access slot information with help of the `@` nomenclature as shown below:

```
esetSel@featureData
```

```
An object of class 'AnnotatedDataFrame'
  featureNames: 1007_s_at 1053_at ... 1552302_at (43 total)
  varLabels: ID GB_ACC ... Gene.Ontology.Molecular.Function (16
    total)
  varMetadata: Column Description labelDescription
```

However, this compact output is not yet very useful and we need a little more work to get to what we need. For example it would be nice to know the name of the various columns that make up this table of data:

```
colnames(esetSel@featureData)
```

```
 [1] "ID"                                 "GB_ACC"
 [3] "SPOT_ID"                            "Species.Scientific.Name"
 [5] "Annotation.Date"                    "Sequence.Type"
 [7] "Sequence.Source"                    "Target.Description"
 [9] "Representative.Public.ID"           "Gene.Title"
[11] "Gene.Symbol"                        "ENTREZ_GENE_ID"
[13] "RefSeq.Transcript.ID"               "Gene.Ontology.Biological.Process"
[15] "Gene.Ontology.Cellular.Component" "Gene.Ontology.Molecular.Function"
```

We should now be able to access and list the gene symbols, and we use the `$` annotation to extract these values:

```
esetSel@featureData$Gene.Symbol
```

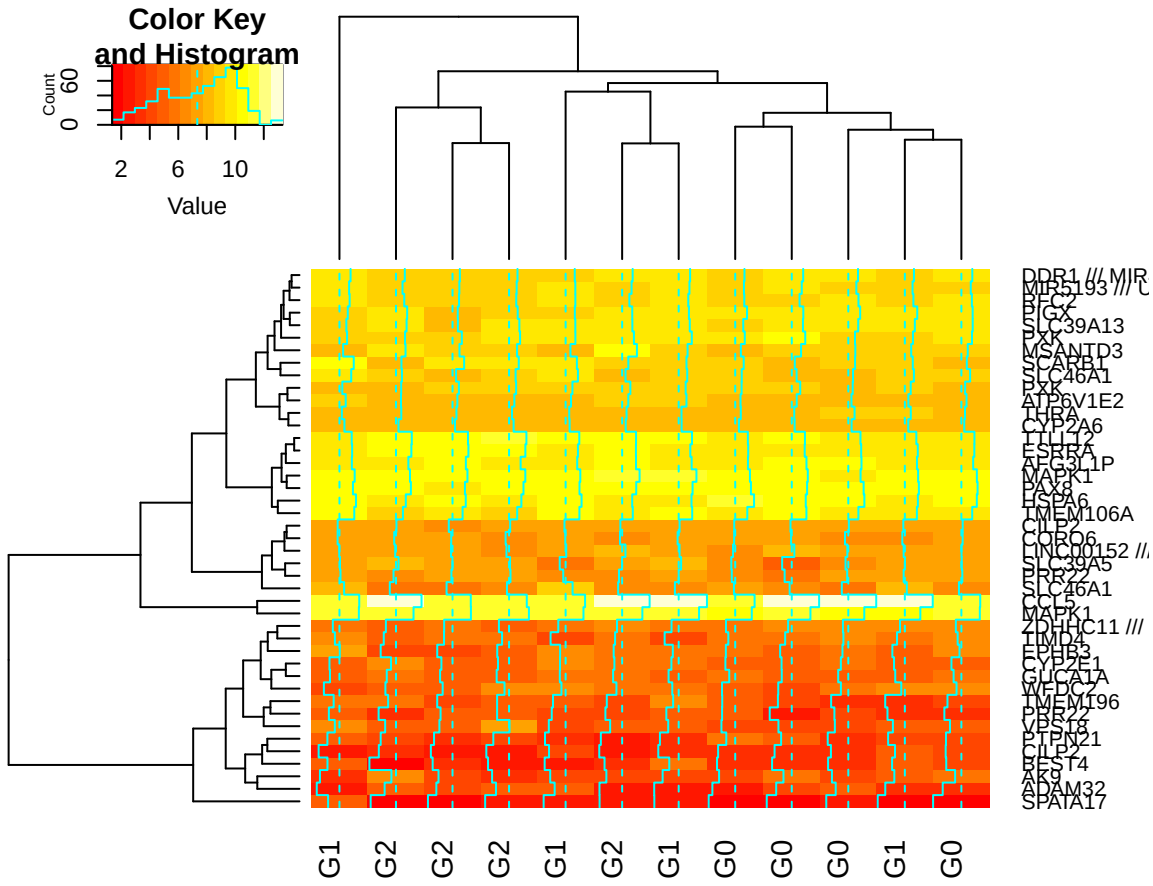
```
 [1] DDR1 /// MIR4640          RFC2
 [3] HSPA6                     PAX8
 [5] GUCA1A                    MIR5193 /// UBA7
 [7] THRA                      PTPN21
 [9] CCL5                      CYP2E1
[11] EPHB3                     ESRRA
[13] CYP2A6                    SCARB1
[15] TTLL12                    LINC00152 /// LOC101930489
[17] WFDC2                     MAPK1
[19] MAPK1                     ADAM32
[21] SPATA17                   PRR22
[23] PRR22                     PXK
[25] PXK                       VPS18
[27] MSANTD3                   SLC46A1
[29] SLC46A1                   TIMD4
[31] SLC39A5                   ZDHHC11 /// ZDHHC11B
[33] ATP6V1E2                  AFG3L1P
[35] CILP2                     CILP2
[37] PIGX                      TMEM196
[39] SLC39A13                  BEST4
[41] AK9                       CORO6
[43] TMEM106A
23521 Levels: ADAM32 AFG3L1P AK9 ALG10 ARMCX4 ATP6V1E2 BEST4 ... ZZZ3
```

Now that we know where these are and how to access them we should be able to change the annotation of the heatmap with these gene symbols:

```
heatmap.2(exprs(esetSel), labRow=esetSel@featureData$Gene.Symbol)
```

But we can take this opportunity to also change the label for the samples and use the group label instead by using the `sml` object containing the list of groups and passing this information with the `labCol` paramter:

```
heatmap.2(exprs(esetSel), labRow=esetSel@featureData$Gene.Symbol, labCol=sml)
```



Note that the heatmap colors are that of the default since we have not specified here the color command as previously using `col=hmcol` in the command.

We can finalize this version of the heatmap by adding one more optional parameter named `ColSideColors` to color a horizontal side-bar to distinguish the groups. This is an optional parameter.

We know that the groups are contained within the object `sml`, an object of class "character" that we need to transform into numeric values that can be factors. The `as.numeric()` function alone applied to `sml` would yield a new vector of only `NA` values. That is why we first apply the function `as.factor()` to force this output:

```
as.numeric(sml)
```

```
 [1] NA NA NA NA NA NA NA NA NA NA NA NA
```

```
as.numeric(as.factor(sml))
```

```
 [1] 1 1 1 1 2 2 2 2 3 3 3 3
```

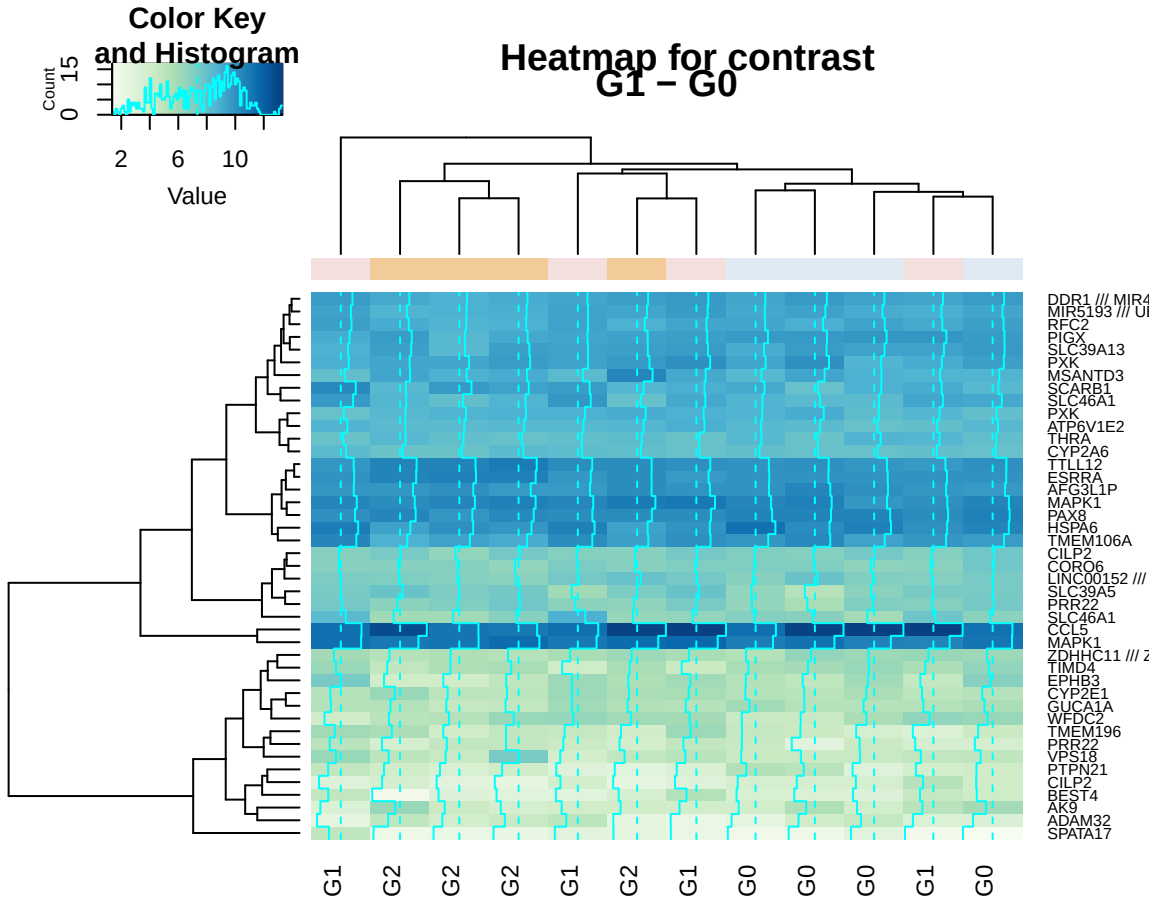
Now that we have a vector reprenting the groups as numbers, we need to choose a palette and pass these values. We can store this into a new object that we'll use the in final heatmap command:

```
# prepare vector of colors for heatmap
colside <- palette(brewer.pal(8,"Dark2"))[as.numeric(as.factor(sml))]
# print values
colside
```

```
 [1] "#DFEAF4" "#DFEAF4" "#DFEAF4" "#DFEAF4" "#F4DFDF" "#F4DFDF" "#F4DFDF"
 [8] "#F4DFDF" "#F2CB98" "#F2CB98" "#F2CB98" "#F2CB98"
```

We can now make a final heatmap with all the additions we made, starting from a previous command:

```
heatmap.2(exprs(esetSel), col=hmcol, main=c("     Heatmap for contrast " , contrast ), labRow=esetSel@f
```



9.3.2 Exercise

Can you add color sides for the rows?

Can you plot the other contrasts?

9.4 MA Plot

We have seen MA plots in the previous workshop.

> An improved method, which is basically a scaled, 45 degree rotation of the R vs. G plot is an MA-plot.[2] The MA-plot is a plot of the distribution of the red/green intensity ratio ('M') plotted by the average intensity ('A'). M and A are defined by the following equations. Review: https://en.wikipedia.org/wiki/MA_plot

The MA plots were defined at a time where the experimental and control experiments were both on the same array and labelled with red and green dyes but the same principles and calculations can apply to simple

arrays.

`A` is the average log-expression and is used as the horizontal axis.

`M` is the log fold-change and is used on the vertical axis.

Horizontal lines are often shown at `-1` and `1` for $-log_2(2)$ and $+log_2(2)$ representing the **2-fold** change limit.

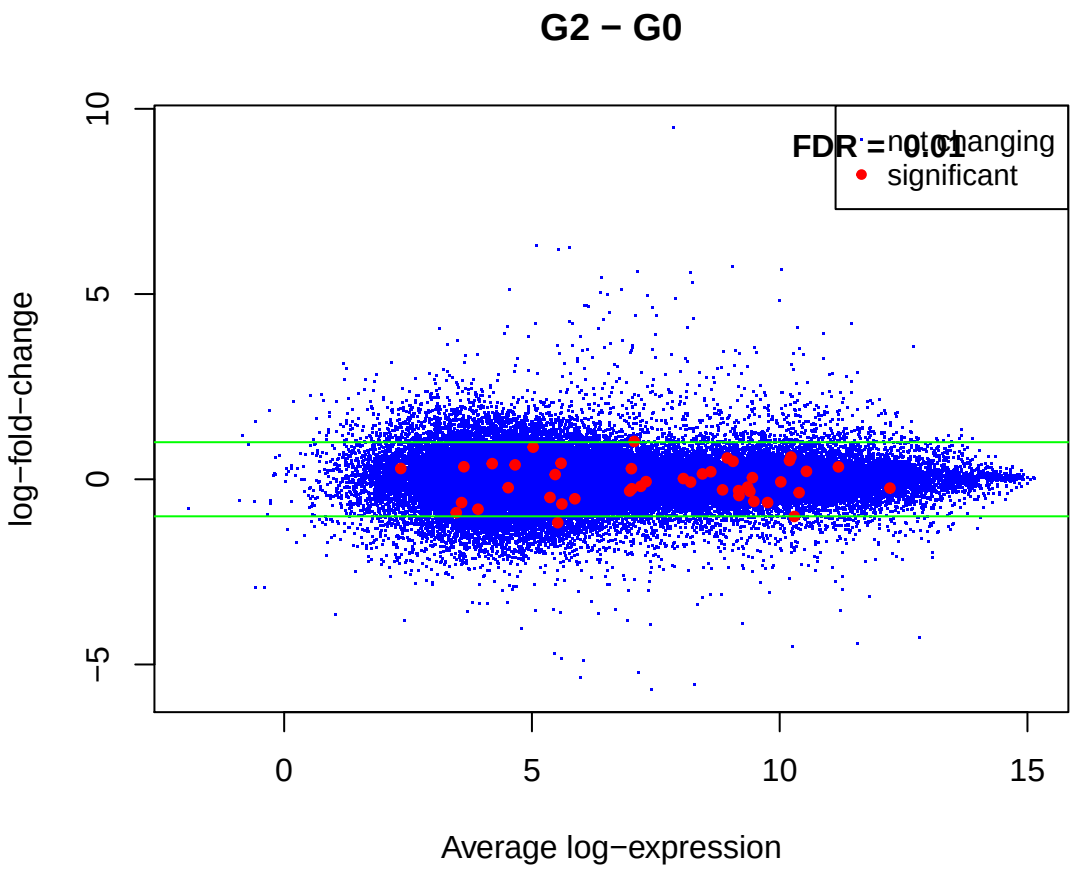
9.4.1 Plot our selected genes

With the same `selected` genes we can also create an MA plot for the same contrast.

The simplest command would be `plotMA(fit2[,1])` to plot the first contrast (`[,1]`) but we can make it a bit better and mark significant genes.

We prepare an object named `status` to define if the gene is "significant" and this information will be automatically be plotted (top left) together with the legend.

```
# Prepare the "status" parameter to distinguish
# differentially expressed genes on the plot
# Check the number of genes (probesets)
number_genes <- dim(exprs(gset))[1]
# create a new object as long as the number of gens
# Each position will contain "" (nothing) at first
status <- character (length=number_genes)
# fill object with "not changing" at every position
status <- rep ("not changing", number_genes)
# add a name attribute, in the form of gene number
names (status) <- seq (1, number_genes, 1)
# change the value to "significant" for the selected genes
status [selected] <- "significant"

# Make the MA plot using the "status" option to show selected genes
# The "values" option can separate options to be plotted
# (Compare with the simpler plot command below.)
# The plot symbols are chosen with the "pch" option.
plotMA(fit2[,1], status=status, values=c("not changing", "significant"), col=c("blue","red"), pch=c(46,1
# Simpler plots
# plotMA(fit2[,1]) # no colors
# plotMA(fit2[,1], status=status, col=c("red","blue"))
# Add text info
text(x=12, y=9, labels=paste("FDR = ", FDR_cutoff), col="black", font=2)
# Add horizontal lines at +-log2(2) to mark 2X fold change
abline(h=c(1,-1), col="green")
```



9.4.2 Exercise

Can you plot the other contrasts?

Can you alter colors?

Can you change plot symbols?

10 Online practical exercises for microarray data analysis

A document titled "*A Tutorial Review of Microarray Data Analysis*" provides statistical background on analysis and is accompanied by a series of exercises.

The document is available in PDF at http://www.ub.edu/stat/docencia/bioinformatica/microarrays/ADM/slides/A_Tutorial_Review_of_Microarray_data_Analysis_17-06-08.pdf (and is archived at: http://bit.ly/1QAuuT9)

The practical exercises page is at http://www.ub.edu/stat/docencia/bioinformatica/microarrays/ADM/practicalExs.htm (archived: http://bit.ly/1Lbo91D)

11 Session info

```
sessionInfo()
```

```
R version 3.3.3 (2017-03-06)
Platform: x86_64-apple-darwin13.4.0 (64-bit)
Running under: OS X El Capitan 10.11.6

locale:
[1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8

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

other attached packages:
[1] gplots_3.0.1         RColorBrewer_1.1-2   scatterplot3d_0.3-40
[4] affy_1.52.0          limma_3.30.13        GEOquery_2.40.0
[7] Biobase_2.34.0       BiocGenerics_0.20.0  knitr_1.15.1

loaded via a namespace (and not attached):
 [1] Rcpp_0.12.10          magrittr_1.5          zlibbioc_1.20.0
 [4] R6_2.2.0              highr_0.6             stringr_1.2.0
 [7] httr_1.2.1            caTools_1.17.1        tools_3.3.3
[10] KernSmooth_2.23-15    gtools_3.5.0          htmltools_0.3.6
[13] yaml_2.1.14           rprojroot_1.2         digest_0.6.12
[16] preprocessCore_1.36.0 affyio_1.44.0         codetools_0.2-15
[19] bitops_1.0-6          RCurl_1.95-4.8        evaluate_0.10
[22] rmarkdown_1.5         gdata_2.17.0          stringi_1.1.5
[25] BiocInstaller_1.24.0  backports_1.0.5       XML_3.98-1.7
```

References

Barrett, T., S. E. Wilhite, P. Ledoux, C. Evangelista, I. F. Kim, M. Tomashevsky, K. A. Marshall, et al. 2013. "NCBI GEO: archive for functional genomics data sets–update." *Nucleic Acids Res.* 41 (Database issue): D991–995.

Benjamini, Yoav, and Yosef Hochberg. 1995. "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing." *J. Roy. Statist. Soc. Ser. B* 57 (1): 289–300. http://links.jstor.org/sici?sici=0035-9246(1995)57:1<289:CTFDRA>2.0.CO;2-E.

Davis, Sean, and Paul Meltzer. 2007. "GEOquery: A Bridge Between the Gene Expression Omnibus (Geo) and Bioconductor." *Bioinformatics* 14: 1846–7.

Jolliffe, I.T. 2002. *Principal Component Analysis*. 2nd ed. Boca Raton, Florida: Springer-Verlag New York. http://www.springer.com/us/book/9780387954424.

Ritchie, Matthew E, Belinda Phipson, Di Wu, Yifang Hu, Charity W Law, Wei Shi, and Gordon K Smyth. 2015. "limma Powers Differential Expression Analyses for RNA-Sequencing and Microarray Studies." *Nucleic Acids Research* 43 (7): e47.

Wheelwright, M., E. W. Kim, M. S. Inkeles, A. De Leon, M. Pellegrini, S. R. Krutzik, and P. T. Liu. 2014. "All-trans retinoic acid-triggered antimicrobial activity against Mycobacterium tuberculosis is dependent on

NPC2." *J. Immunol.* 192 (5): 2280–90.

Xie, Yihui. 2014. "Knitr: A Comprehensive Tool for Reproducible Research in R." In *Implementing Reproducible Computational Research*, edited by Victoria Stodden, Friedrich Leisch, and Roger D. Peng. Chapman; Hall/CRC. http://www.crcpress.com/product/isbn/9781466561595.

———. 2015. *Dynamic Documents with R and Knitr*. 2nd ed. Boca Raton, Florida: Chapman; Hall/CRC. http://yihui.name/knitr/.

———. 2016. *Knitr: A General-Purpose Package for Dynamic Report Generation in R*. http://yihui.name/knitr/.