

Lab 4 - Gr. 14 - Bioinformatics (732A93)

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Assignment 1

Task:

- Run all the R code and reproduce the graphics (<https://www.bioconductor.org/help/course-materials/2015/Uruguay2015/day3-gene.expression.html>)
- Go carefully through the R code and explain in your words what each step does.

Data import

First, we import the data corresponding to the GEO accession number GSE20986 using the `getGEOSuppFiles()` function. We obtain a .tar file (“GSE20986_RAW.tar”). After extracting its contents, we obtain 12 .CEL.gz files. Consequently, these 12 files are unzipped and the results are put into the `data` directory. The resulting 12 files are .CEL files (cell intensity files).

These files contain microarray data created by Affymetrix DNA microarray image analysis software. Recall that each set of microarray data contains light intensity values, where high intensity corresponds to expressed genes and small intensity to genes that were not expressed.

All the data comes from humans (*Homo sapiens*), from four different tissues: iris, retina, choroid and human umbilical vein. There are three .CEL files per tissue.

References:

- <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20986>
- <https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE20986>
- <https://fileinfo.com/extension/cel>
- <https://www.affymetrix.com/support/developer/powertools/changelog/gcos-agcc/cel.html>
- <https://bioconductor.org/help/course-materials/2009/SeattleApr09/AffyAtoZ/AffymetrixAtoZSlides.pdf>

Creating phenodata

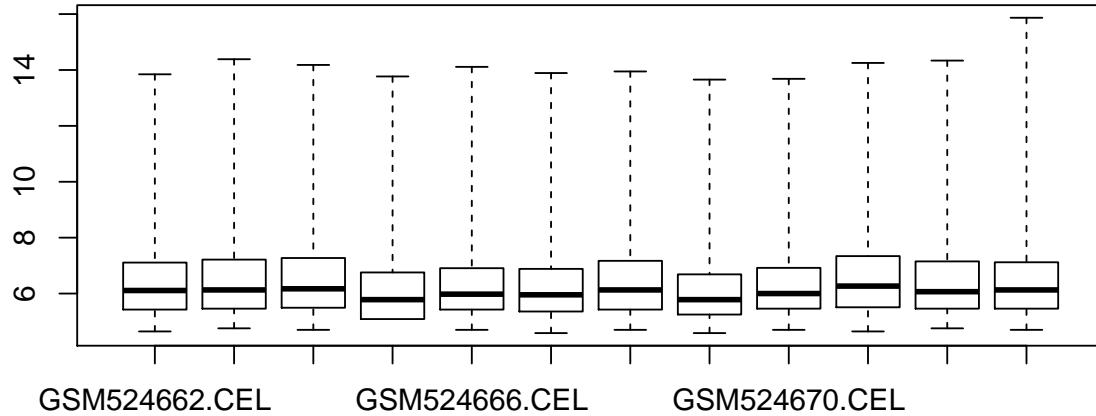
Before we are able to read in the data from all 12 files, we create a list with three objects: The `Name` (for use in the resulting R object), the `FileName` (for finding the corresponding .CEL file) and `Targets` which seems to be some required meta data like the number of files. This list is then written as a .txt file. Finally, the function `read.affy()` from the `simpleaffy` package is called. to actually create the data to be used in R. This function takes phenodata.txt as input to be able to find the .CEL files. The resulting object `celfiles` is of class `AffyBatch` and contains the microarray data.

Simple boxplot

Now, we create a visualization of 12 boxplots, one for every .CEL file, using `celfiles` as input. For this, we use the `boxplot` function from the `BiocGenerics` package. Unfortunately, the x-axis labels are not readable. Therefore, we don't know which boxplots belong to which .CEL file. Therefore, we create an improved boxplot visualization in the next step.

Note: Although light intensity values do have a unit, it does not really matter for the interpretation. All we can do with the values is make relative comparisons within one experiment. Higher values correspond to expressed genes. It is not possible whatsoever, to make comparisons across experiments because the scale can differ. Furthermore, the scale is usually transformed (e.g. with logs), so that absolute interpretations make even less sense.

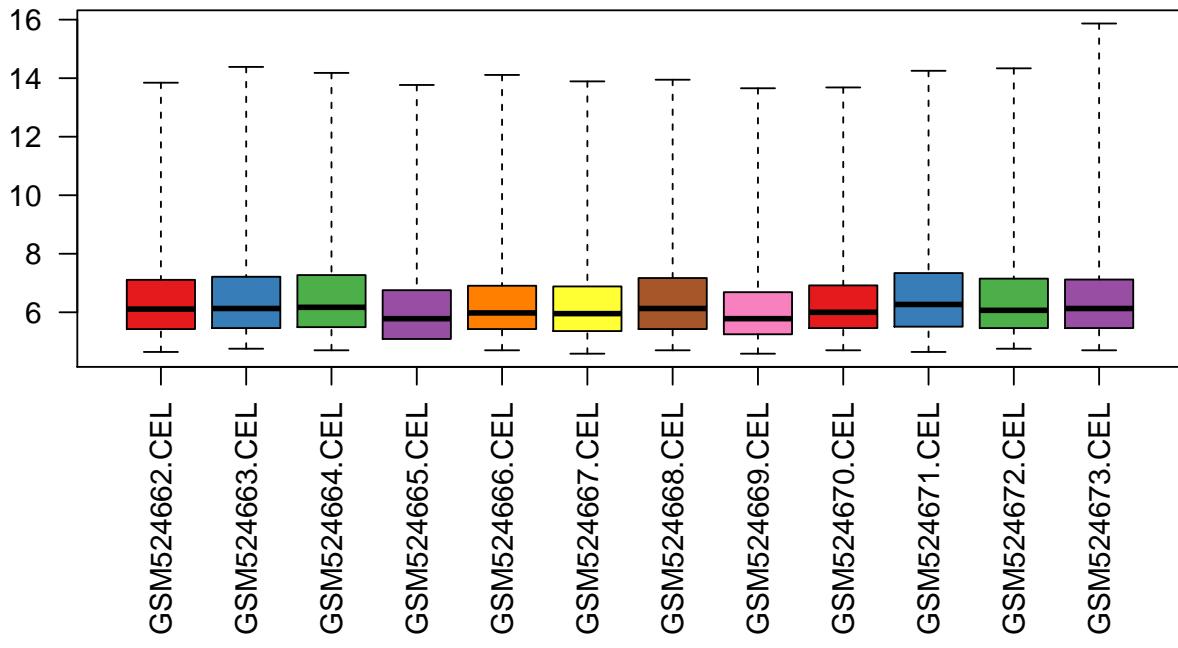
Interpretation: As we can see, the majority of the light intensity values are between 5 and 7. However, there are also some very large light intensity values which correspond to strongly expressed genes. The intensity values in the inter-quartile range (the rectangle boxes in the plots) may correspond to genes that are only expressed to a small extent or even not at all.



Improved boxplot

We now create an improved box plot, in which the x-axis labels are readable.

Note: The colors do not have a special meaning. They do not e.g. correspond to a specific tissue. Instead, there are 8 color values which are used from left to right. After all 8 values have been used, the 1st color is used, then the 2nd color etc.



Cluster analysis (not normalized, tissue as labels)

We have extracted the expression matrix (1354896 rows, 12 columns) from the `celfiles` object. Each row corresponds to a light intensity that tells us to what extend a gene is expressed (?). There are 12 columns for cell intensity file.

Now, we compute a distance matrix for these 12 columns, using the `Chebyshev distance` as a metric. The `Chebyshev distance` gives us the maximum difference between any pair of points in two vectors.

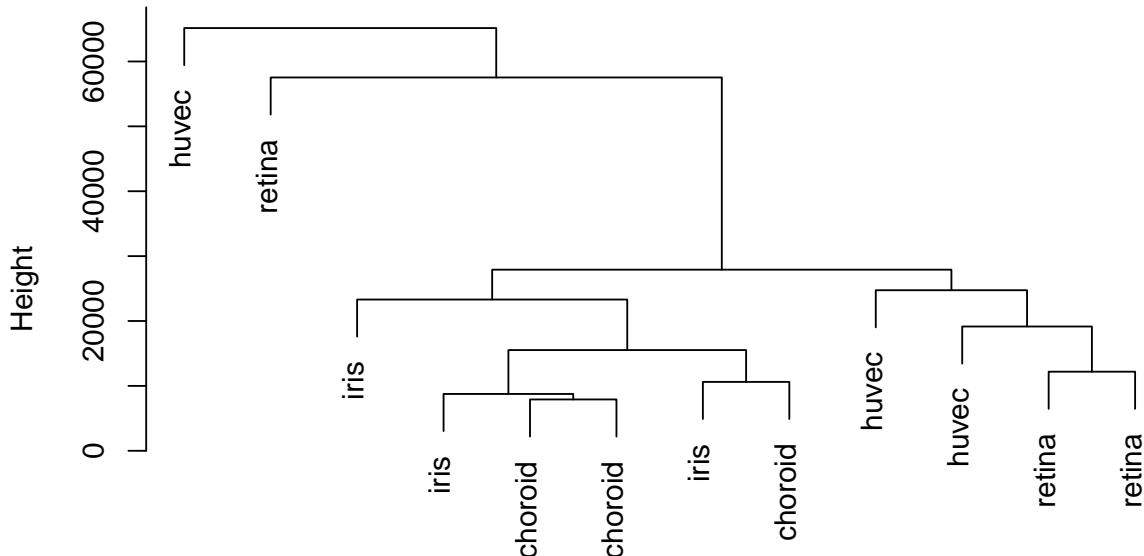
Subsequently, we conduct a hierarchical cluster analysis on this distance matrix and plot the results in a cluster dendrogram. Note that there are 12 leaf nodes in the plotted dendrogram, each node corresponding to one .CEL file. The labels tell us which tissue the .CEL file belonged to.

Note that HCA is done by a stepwise procedure. At each step, the two columns with the smallest dissimilarity are merged. We can therefore e.g. say that the bottom right leaf nodes (retina and retina) had a quite small dissimilarity. Also e.g. the 5th and 6th leaf nodes from the left (choroid and choroid) had a quite small dissimilarity. After all, we can conclude similarities between .CEL files of the same tissue are recognized. However, it is also clear that similarities between different tissues are recognized (such as iris and choroid).

References:

- <https://bioconductor.org/help/course-materials/2009/SeattleApr09/AffyAtoZ/AffymetrixAtoZSlides.pdf>
- <https://stats.stackexchange.com/questions/209606/what-is-maximum-and-its-computation-in-the-function-dist-stats-in>
- https://en.wikipedia.org/wiki/Chebyshev_distance
- <https://stats.stackexchange.com/questions/82326/how-to-interpret-the-dendrogram-of-a-hierarchical-cluster-analysis>
- <http://www.econ.upf.edu/~michael/stanford/maeb7.pdf>

Cluster Dendrogram



```
distance
stats::hclust (*, "complete")
```

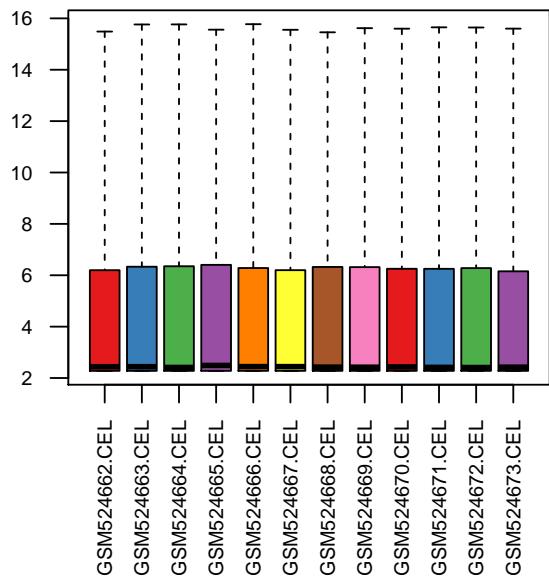
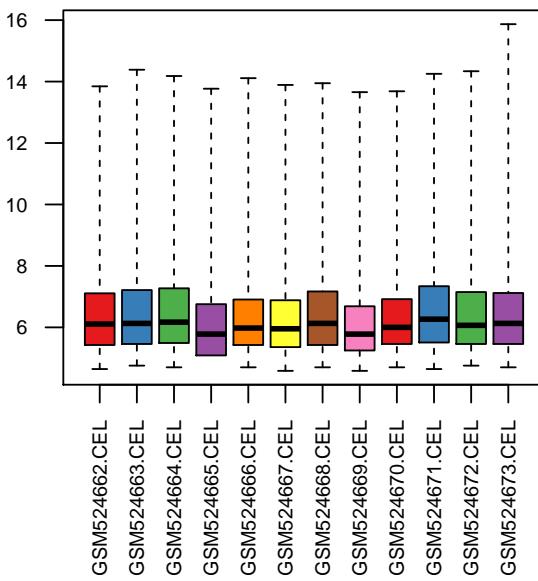
Box Plots with vs. without normalization

The previous box plots (using the `celfiles`, an `AffyBatch` object as input) were not based on normalized features. Here, we use the `gcrma()` function with `celfiles` as input to extract the `ExpressionSet` object `celfiles.gcrma`. When applying the `gcrma()` function, the default parameter `normalize` is set to true. Therefore, we obtain normalized data.

Comparison: After normalizing, we can observe the following.

- Subtle differences in the inter-quartile ranges across samples get reduced. Almost all inter-quartile ranges look very similar in the Post-Normalization plot whereas they noticeably differ in the Pre-Normalization plot.
- The medians decrease from values of around 6 to values of near 2.
- The upper quartile is much more spread out than the lower quartile in Post-Normalization. Likely, the lower quartiles correspond to genes that are not expressed whereas the upper quartiles correspond to genes that are expressed.

```
## Adjusting for optical effect.....Done.
## Computing affinities.Done.
## Adjusting for non-specific binding.....Done.
## Normalizing
## Calculating Expression
```

Post-Normalization**Pre-Normalization**

Cluster analysis (normalized, .CEL names as labels)

Here, we perform another cluster analysis. This time, however, we use the normalized data, which we created for the box plot comparison above. We use the same distance metric for hierarchical clustering as before.

Again, we have 12 leaf nodes. However, the labels are now the names of the .CEL files (instead of their corresponding tissues). Still, we can see that the displayed dendrogram differs from the dendrogram that we received without normalization. This illustrates that normalization is indeed relevant here since it leads to different conclusions.

Cluster Dendrogram

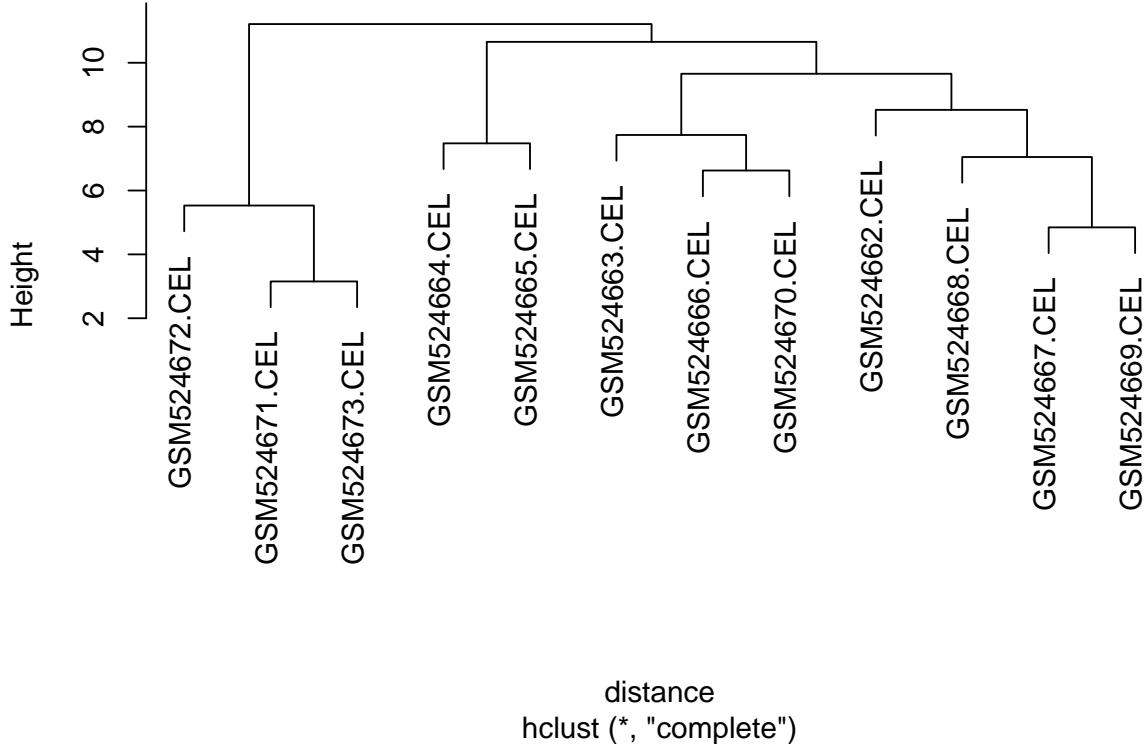


Table 1: Phenodata

Name	FileName	Targets
GSM524662.CEL	GSM524662.CEL	iris
GSM524663.CEL	GSM524663.CEL	retina
GSM524664.CEL	GSM524664.CEL	retina
GSM524665.CEL	GSM524665.CEL	iris
GSM524666.CEL	GSM524666.CEL	retina
GSM524667.CEL	GSM524667.CEL	iris
GSM524668.CEL	GSM524668.CEL	choroid
GSM524669.CEL	GSM524669.CEL	choroid
GSM524670.CEL	GSM524670.CEL	choroid
GSM524671.CEL	GSM524671.CEL	huvec
GSM524672.CEL	GSM524672.CEL	huvec
GSM524673.CEL	GSM524673.CEL	huvec

Linear Regression using Contrast Matrix

First, we create a model matrix. It has four columns, one for each tissue, and twelve rows, one for each sample (i.e. .CEL files). Every row only has a single 1 indicating which tissue the sample belongs to.

Second, we create a contrast matrix (using the model matrix as input to provide the tissue names). This contrast matrix has 4 rows, one for each tissue, and 3 columns, one for each contrast. The three contrasts are **huvec - choroid**, **huvec - retina** and **huvec - iris**. Basically, the **huvec** row contains a value of 1 for all columns (since it is a minuend). The **choroid**, **retina** and **iris** rows have a value of -1 in their

corresponding contrast column (since they are subtrahends in the contrasts).

Third, we use the `lmFit()` function from the `limma` package. It takes the normalized data in form of the `ExpressionSet` object `celfiles.gcrma` and it takes the model matrix `design` which specifies the contrasts that are supposed to be analyzed. The function fits a linear model to the expression data for each sample. According to the documentation, the coefficients of the fitted models describe the differences between the RNA sources hybridized to the arrays.

Fourth, we compute estimated coefficients and standard errors for a given set of contrasts, using the fitted model from the previous step. We also compute various statistics for these coefficients and standard errors such as t-statistics, F-statistics and logodds.

Finally, we print a summary of the results.

```
## [1] "sampleschoroid" "sampleshuvec"    "samplesiris"      "samplesretina"
```

Table 2: Design Matrix

choroid	huvec	iris	retina
0	0	1	0
0	0	0	1
0	0	0	1
0	0	1	0
0	0	0	1
0	0	1	0
1	0	0	0
1	0	0	0
1	0	0	0
0	1	0	0
0	1	0	0
0	1	0	0

```
##      logFC          AveExpr            t        P.Value
## Min. :-9.19111   Min. : 2.279   Min. :-39.77473   Min. :0.0000
## 1st Qu.:-0.05967 1st Qu.: 2.281   1st Qu.:-0.70649  1st Qu.:0.1523
## Median : 0.00000 Median : 2.480   Median : 0.00000 Median :0.5079
## Mean   :-0.02353 Mean   : 4.375   Mean   : 0.07441 Mean   :0.5346
## 3rd Qu.: 0.03986 3rd Qu.: 6.241   3rd Qu.: 0.67455  3rd Qu.:1.0000
## Max.   : 8.67086 Max.   :15.541   Max.   :296.84201 Max.   :1.0000
##
##      adj.P.Val          B      getsymbols
## Min. :0.0000  Min. :-7.710  YME1L1   : 22
## 1st Qu.:0.6036 1st Qu.:-7.710  HFE     : 15
## Median :1.0000  Median :-7.451  CFLAR   : 14
## Mean   :0.7436  Mean   :-6.582  NRP2    : 14
## 3rd Qu.:1.0000 3rd Qu.:-6.498  ARHGEF12: 13
## Max.   :1.0000  Max.   :21.290  (Other) :41857
##                      NA's   :12740
```

Assignment 2

Task:

- Present the variables versus each other original, logscaled and MA-plot for each considered pair both before and after normalization.

- A cluster analysis is performed on the page but not report. Present plots and also draw heatmaps.

Assignment 3

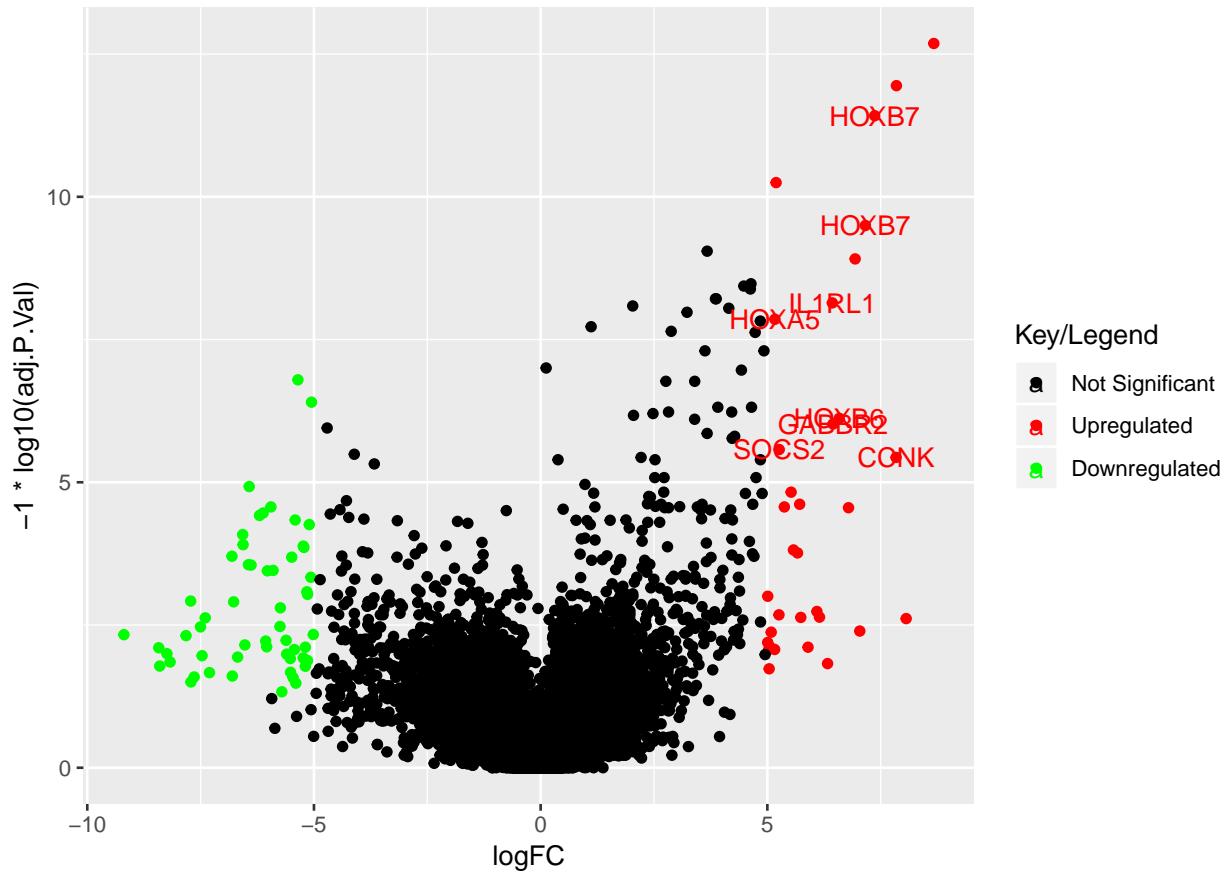
Task:

- Provide volcano plots for the other pairs.
- Indicate significantly differentially expressed genes.
- Explain how they are found.

Volcano Plots

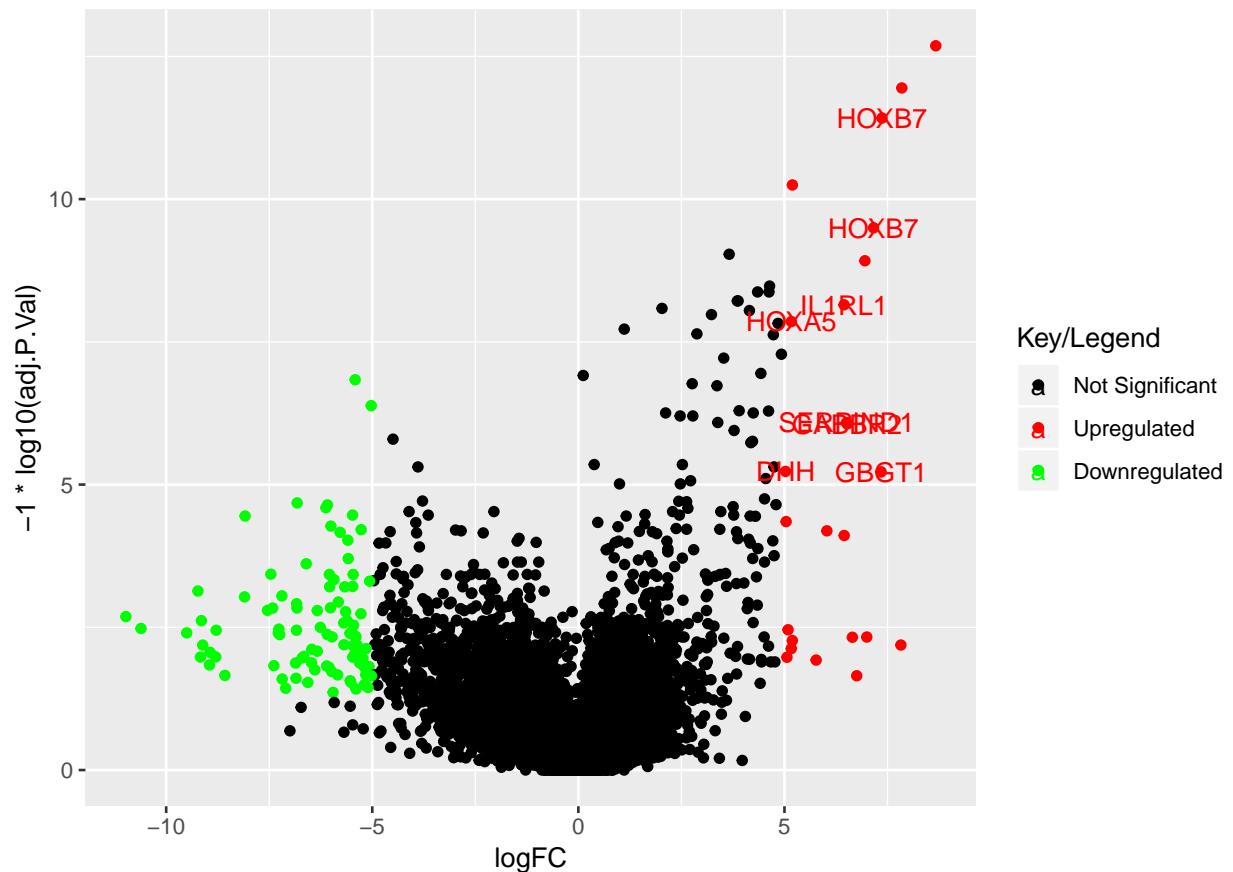
Huvec - Choroid

```
##  
##      1      2      3  
## 54587    33    55
```



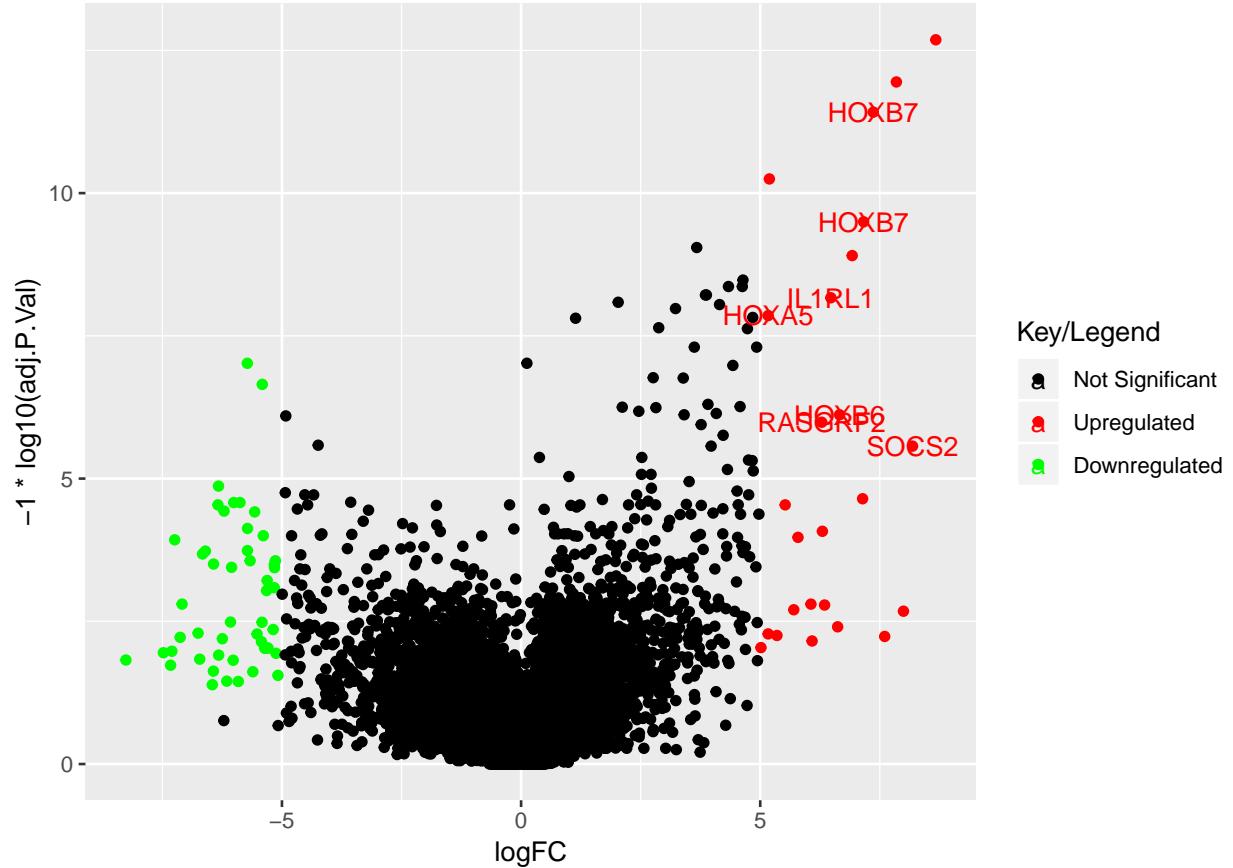
Huvec - Retina

```
##  
##      1      2      3  
## 54557    24    94
```



Huvec - Iris

```
##  
##      1     2     3  
## 54601   25   49
```



Significantly Differentially Expressed Genes

We observe the following differentially expresses genes:

```
## [1] "HOXB7"      "IL1RL1"      "HOXA5"      "HOXB6"      "GABBR2"      "SOCS2"
## [7] "CCNK"        "SERPINID1"   "DHH"        "GBGT1"      "RASGRF2"
```

Explanation How They Are Found

A volcano plot prints significance against fold-change. A fold-change is a measurement between two variables and is used as a measurement between how much they changed during measurements. So if we put on those values on the y- and x-axis we will get a view of the statistical significance and the magnitude of the a change. This enables the viewers to quickly recognize not only significant, but also “strong influencing” genes.

Therefore we have the \log_2 of the fold-change on the x-axis and the $-\log_{10}$ of the p-value on the y-axis. Thus interesting data points are those which are far to the top (p-value) and far to the left or right (significant change in the fold-change). In this one we can observe another feature of the data expressed in color. Here it is the regulation of the data.

The datapoints (genes) we obtained are those which are far to the right and in the upper part of the volcano plot. We used the provided code filtering those and adding the names to them as the separator. All obtained genes are genes which are upregulated.



Figure 1: HOXB7

Assignment 4

Task:

- Try to find more information on the genes that are reported to be significantly differentially expressed. Report in your own words on what you find.
- Report all the Gene Ontology (GO) terms associated with each gene.
- Are any of the GO terms common between genes?
- If so do the common GO terms seem to be related to anything particular?
- Try to present GO analysis in an informative manner, if possible visualize.

More Information About The Genes

Comment: Most information is taken from GeneCards and Wikipedia, the figures are all taken from Wikipedia. We tried to describe the genes in our own words, but some descriptions are so bio heavy in their expression that we just quoted them as we are not sure how to deliver the same and correct amount of information in our own words.

HOXB7

The full name of this gene is *Homeobox protein Hox-B7* and is a member of the Antp homeobox family. The homeobox family is a cluster of genes which are used during the early embryological development. This family is interesting to inspect due to the fact that their sequences are conserved and widely used by living taxa. The whole homobox family is part of the human genome.

This gene is member of the homobox B cluster and found on chromosome 17.

The encoded nuclear protein functions as a sequence-specific transcription factor that is involved in cell proliferation and differentiation. Increased expression of this gene is associated with some cases of melanoma and ovarian carcinoma.

From: <https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd>ShowDetailView&TermToSearch=3217>

<https://www.genecards.org/cgi-bin/carddisp.pl?gene=HOXB7>

IL1RL1

The full name if this gene is *Interleukin 1 receptor-like 1* and is a member of the Toll-like receptor which are a part of the innate immune system. It is found on chromosome 2 and is 40,536 bases long and encodes a

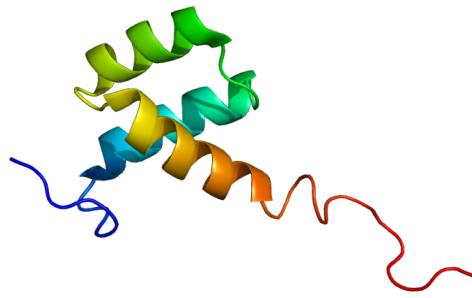


Figure 2: HOXA5



Figure 3: HOXB6

protein of 556 amino acids. It seems to be important for T helper cells type 2.

<https://www.genecards.org/cgi-bin/carddisp.pl?gene=IL1RL1>

HOXA5

Homeobox protein Hox-A5 is part of the A cluster on chromosome 7 and “encodes a DNA-binding transcription factor which may regulate gene expression, morphogenesis, and differentiation.” (from: <https://www.ncbi.nlm.nih.gov/gene/3202>). It’s possible that this gene plays a role in tumorigenesis. It upregulates the tumor suppressor p53 which is also used as an example in the Book “Concepts in Bioinformatics and Genomics.”

<https://www.genecards.org/cgi-bin/carddisp.pl?gene=HOXA5>

HOXB6

Homeobox protein Hox-B6 is found in a cluster of the homobox B genes also on chromosome 17. It’s involved in the development of lung, skin and has been localized in nucleus as well as in cytoplasm.

<https://www.genecards.org/cgi-bin/carddisp.pl?gene=HOXB6>

GABBR2

Gamma-aminobutyric acid (GABA) B receptor, 2 (GABAB2) is a G-Protein, also called GPCR which is a family of proteins. They’re also called “seven-transmembrane receptors” as they pass through the cell membrane seven times.

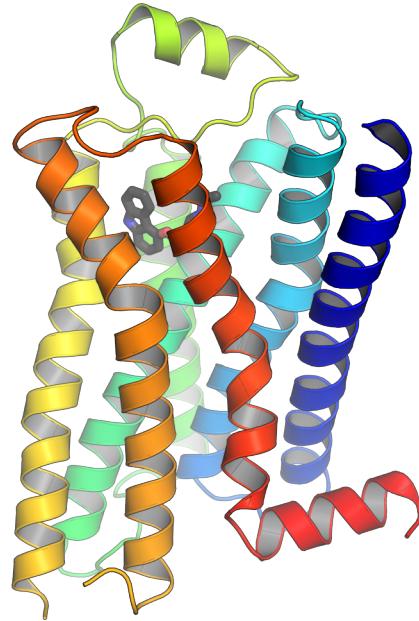


Figure 4: G-Protein

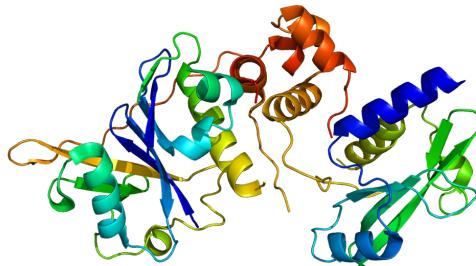


Figure 5: SOCS2

GABBR2 build some kind of messenger system for B-type receptors which inhibit neural activity. These messenger systems regulate the release of neurotransmitters and the activity of ion channels.

<https://www.genecards.org/cgi-bin/carddisp.pl?gene=GABBR2>

SOCS2

Suppressor of cytokine signaling 2 is found on chromosome 12 and is part of the SOCS family of proteins which are a part of the feedback system that regulates signal transduction. It seems to be a negative regulator.

Diseases associated with SOCS2 are Essential Thrombocythemia and Vertebral Artery Occlusion.

<https://www.genecards.org/cgi-bin/carddisp.pl?gene=SOCS2>

CCNK

Cyclin-K is found on chromosome 14 and is a member of the transcription cyclin family.

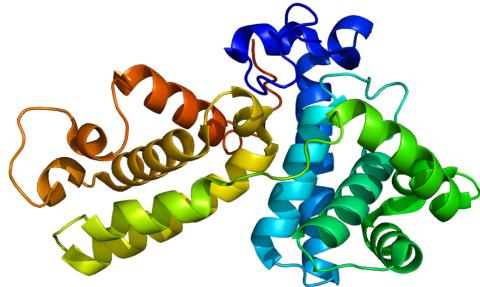


Figure 6: CCNK

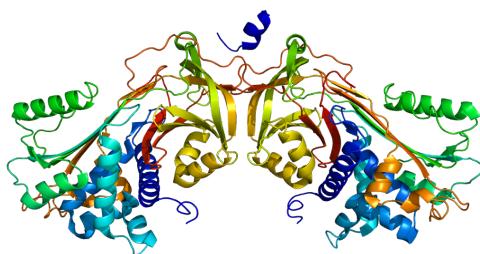


Figure 7: SERPIND1

These cyclins may regulate transcription through their association with and activation of cyclin-dependent kinases (CDK) that phosphorylate the C-terminal domain (CTD) of the large subunit of RNA polymerase II. This gene product may play a dual role in regulating CDK and RNA polymerase II activities.

<https://www.genecards.org/cgi-bin/carddisp.pl?gene=CCNK>

SERPIND1

Heparin cofactor II (HCII) is found on chromosome 22 and belongs to the serpin gene superfamily. They play a role in processes like inflammation, blood clotting, and cancer metastasis. SERPIND1 is a Protein Coding gene.

<https://www.genecards.org/cgi-bin/carddisp.pl?gene=SERPIND1>

DHH

Desert hedgehog homolog is a member of the hedgehog family which encodes signaling molecules that regulate morphogenesis. It is located on chromosome 12 and may be involved in gonadal differentiation and perineurial development.

<https://www.genecards.org/cgi-bin/carddisp.pl?gene=DHH>

GBGT1

Globoside alpha-1,3-N-acetylgalactosaminyltransferase 1 is located on chromosome 9 and encodes for a member of the histo-blood group ABO gene family.

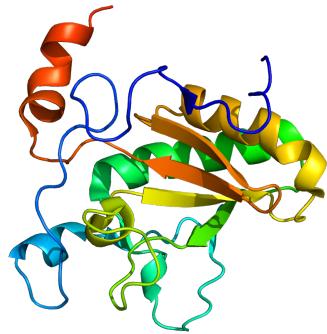


Figure 8: DHH

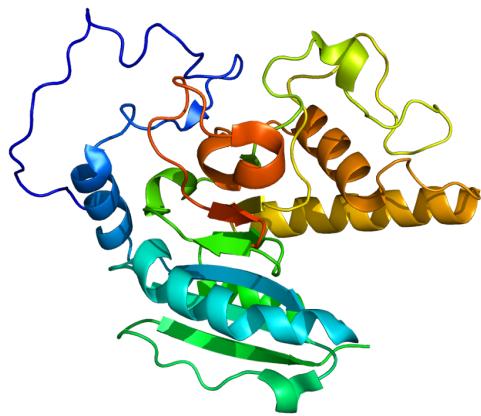


Figure 9: ABO

expression of this protein may determine host tropism to microorganisms. Alternative splicing results in multiple transcript variants

<https://www.genecards.org/cgi-bin/carddisp.pl?gene=GBGT1>

RASGRF2

Ras-specific guanine nucleotide-releasing factor 2 is located on chromosome 5 and variations of it seem to be related to propensity to binge drink by teenagers.

Functions as a calcium-regulated nucleotide exchange factor activating both Ras and RAC1 through the exchange of bound GDP for GTP.

<https://www.genecards.org/cgi-bin/carddisp.pl?gene=RASGRF2>

Gene Ontology Terms

Order of information: GO ID Qualified GO term Evidence PubMed IDs

HOXB7

- GO:0000978 RNA polymerase II proximal promoter sequence-specific DNA binding IDA 8756643
- GO:0000981 RNA polymerase II transcription factor activity, sequence-specific DNA binding ISA
- GO:0001077 transcriptional activator activity, RNA polymerase II proximal promoter sequence-specific DNA binding IDA 8756643
- GO:0003677 DNA binding IEA
- GO:0003700 DNA binding transcription factor activity NAS 1678287

IL1RL1

- GO:0002113 interleukin-33 binding IEA
- GO:0002114 interleukin-33 receptor activity IEA
- GO:0004896 cytokine receptor activity TAS 10191101
- GO:0004908 interleukin-1 receptor activity IEA
- GO:0005057 obsolete signal transducer activity, downstream of receptor TAS

HOXA5

- GO:0000978 RNA polymerase II proximal promoter sequence-specific DNA binding IDA 10879542
- GO:0000981 RNA polymerase II transcription factor activity, sequence-specific DNA binding NAS 19274049
- GO:0001077 transcriptional activator activity, RNA polymerase II proximal promoter sequence-specific DNA binding IDA 10879542
- GO:0003677 DNA binding IDA 8657138
- GO:0003700 DNA binding transcription factor activity IDA 10879542

HOXB6

- GO:0000981 RNA polymerase II transcription factor activity, sequence-specific DNA binding NAS 19274049
- GO:0003677 DNA binding IEA
- GO:0003700 DNA binding transcription factor activity IEA
- GO:0003723 RNA binding HDA 22681889
- GO:0005515 protein binding IPI 25416956

GABBR2

- GO:0005737 cytoplasm IEA
- GO:0005886 plasma membrane TAS
- GO:0005887 integral component of plasma membrane IDA,TAS 9872316

- GO:0016020 membrane IEA
- GO:0016021 integral component of membrane IEA

SOCs2

- GO:0004860 protein kinase inhibitor activity IBA
- GO:0005070 SH3/SW2 adaptor activity TAS 9344848
- GO:0005131 growth hormone receptor binding NAS 12135564
- GO:0005159 insulin-like growth factor receptor binding IPI 9727029
- GO:0005515 protein binding IPI 11781573

CCNK

- GO:0004674 protein serine/threonine kinase activity TAS
- GO:0004693 cyclin-dependent protein serine/threonine kinase activity IDA 9632813
- GO:0005515 protein binding IPI 16189514
- GO:0008353 RNA polymerase II carboxy-terminal domain kinase activity IDA 9632813
- GO:0016538 cyclin-dependent protein serine/threonine kinase regulator activity IBA

SERPIND1

- GO:0004866 endopeptidase inhibitor activity TAS 2647747
- GO:0004867 serine-type endopeptidase inhibitor activity IBA
- GO:0008201 heparin binding IEA
- GO:0030414 peptidase inhibitor activity IEA

DHH

- GO:0005113 patched binding IEA
- GO:0005509 calcium ion binding IDA 19561611
- GO:0005515 protein binding IPI 19561611
- GO:0008233 peptidase activity IEA
- GO:0008270 zinc ion binding IDA 19561611

GBGT1

- GO:0016740 transferase activity IEA
- GO:0016757 transferase activity, transferring glycosyl groups IBA,IEA
- GO:0016758 transferase activity, transferring hexosyl groups IEA
- GO:0046872 metal ion binding IEA

- GO:0047277 globoside alpha-N-acetylgalactosaminyltransferase activity IBA

RASGRF2

- GO:0005085 guanyl-nucleotide exchange factor activity TAS,IEA
- GO:0005088 Ras guanyl-nucleotide exchange factor activity TAS,IMP
- GO:0005089 Rho guanyl-nucleotide exchange factor activity IEA,TAS
- GO:0005515 protein binding IEA
- GO:0005516 calmodulin binding IEA

Gene Ontology Analysis

Appendix

```

knitr::opts_chunk$set(fig.width = 7, fig.height = 5, echo = FALSE,
                      warning = FALSE, message = FALSE)

# All provided Links:
# R Bio: Untangling Genomes
# https://www.bioconductor.org/help/course-materials/ 2015/Uruguay2015/
# Step by Step HUVEC and OVE
# https://www.bioconductor.org/help/course-materials/2015/Uruguay2015/ day3-gene.expression.html
# Cell Data:
# ftp://ftp.ncbi.nlm.nih.gov/geo/series/GSE20nnn/GSE20986/suppl/
# Description of Cell Data:
# https://www.ncbi.nlm.nih.gov/geo/query/acc. cgi?acc=GSE20986
# Additional
# https://www.bioconductor.org/help/course-materials/ 2015/Uruguay2015/day5-data_analysis.html
# Explanations Graphic
# https://www.bioconductor.org/help/course-materials/2015/Uruguay2015/V6-RNASeq.html

library(ggplot2)

# Use this if BiocManager is not installed
#if (!requireNamespace("BiocManager", quietly = TRUE))
#  install.packages("BiocManager")
library("BiocManager")

# BiocManager packages
# BiocManager::install("GEOquery", version = "3.8")
# BiocManager::install("simpleaffy", version = "3.8")
# BiocManager::install("RColorBrewer", version = "3.8")
# BiocManager::install("affyPLM", version = "3.8")
# BiocManager::install("limma", version = "3.8")
# BiocManager::install("annotate", version = "3.8")
# BiocManager::install("hgu133plus2.db", version = "3.8")
library(GEOquery)

```

```

library(simpleaffy)
library(RColorBrewer)
library(affyPLM)
library(limma)
library(hgu133plus2.db)
library(annotation)

# -----
# Question 1
# -----

# Data import ----

# Get the Data
x = getGEOSuppFiles("GSE20986")
x

# Untar and Unzip
# DONT ADD THE FILE TO GIT IT'S 60MB!
untar("GSE20986/GSE20986_RAW.tar", exdir = "data")
cels = list.files("data/", pattern = "[gz]")
sapply(paste("data", cels, sep = "/"), gunzip)

# Creating phenodata -----
# It's a matrix with two columns. Each row has the same entries in their cells,
# which is the filename. The first column is called 'Name' and the second one
# is called 'FileName'.
phenodata = matrix(rep(list.files("data", pattern = "[CEL]"), 2), ncol =2)

# class(phenodata) # "matrix"

phenodata <- as.data.frame(phenodata)
colnames(phenodata) <- c("Name", "FileName")
# Adding a new column with the target
phenodata$Targets <- c("iris",
                      "retina",
                      "retina",
                      "iris",
                      "retina",
                      "iris",
                      "choroid",
                      "choroid",
                      "choroid",
                      "huvec",
                      "huvec",
                      "huvec")

# Writes the dataframe to a .txt file
write.table(phenodata, "data/phenodata.txt", quote = F, sep = "\t",
            row.names = F)

```

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# Now the data is read again and a boxplot is created
celfiles <- simpleaffy::read.affy(covdesc = "phenodata.txt", path = "data")

# Simple box plot -----
# Creates a boxplot for the log base 2 intensities including pm (perfect) and
# mm (mismatch)
BiocGenerics::boxplot(celfiles)

# Improved box plot -----
# Create "nice looking" color palettes. So this array actually has hex-color
# values inside.
cols = brewer.pal(8, "Set1")

# Help page: exprs, AffyBatch-method
# The columns are the different .CEL files and hold the data. It's a class re-
# presentation for the probe level data. The main component are the intensities
# from multiple arrays of the save CDF type.
eset <- affy::exprs(celfiles)
samples <- celfiles$Targets
# colnames(eset) # Print colnames

# The colnames are set to our targets
colnames(eset) <- samples

# The .CEL files and their content is being plotted. It's basically the same
# plot as before but this time with fancy colors
par(mar=c(8,2,2,2)) # bottom, left, top and right margins
BiocGenerics::boxplot(celfiles, col = cols, las = 2)

# Creating a distance matrix
distance <- stats::dist(t(eset), method = "maximum")

# "Hierarchical cluster analysis on a set of dissimilarities and methods for analyzing it."
clusters <- stats::hclust(distance)

# Plot the clusters in a tree diagram
# (https://en.wikipedia.org/wiki/Dendrogram)
plot(clusters)

# Box Plots with vs. without normalization -----
# "Robust Multi-Array expression measure using sequence information"
# Converts an AffyBatch into an ExpressionSet by using RMA (robust multi-array)
# expression measure with the help of probe sequence
celfiles.gcrma = gcrma::gcrma(celfiles) # Biobase / ExpressionSet

# Two Boxplots showing different .CELS before and after normalization
par(mfrow=c(1,2),

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    mar=c(10,2,2,2)) # bottom, left, top and right margins
boxplot(celfiles.gcrma, col = cols, las = 2.5, cex.axis = 0.7, main = "Post-Normalization");
boxplot(celfiles, col = cols, las = 2.5, cex.axis = 0.7, main = "Pre-Normalization")

# Turns of the "devices"
# dev.off()

# Create a distance matrix
distance <- dist(t(exprs(celfiles.gcrma)), method = "maximum")

# Performs a hierachical cluster analysis.
clusters <- hclust(distance)

# Plots the clusters, which is a Dendrogram again
plot(clusters)
knitr:::kable(phenodata, caption = "Phenodata")

# Create a model matrix
samples <- as.factor(samples) # Factor of length 12 with tissue names
design <- model.matrix(~0+samples) # Creates model matrix by expanding factors
colnames(design) # "sampleschoroid" "sampleshuvec" "samplesiris" "samplesretina"
colnames(design) <- c("choroid", "huvec", "iris", "retina") # change colnames
knitr:::kable(design, caption = "Design Matrix") # Print design matrix

# Create a contrast matrix from the model matrix
contrast.matrix = makeContrasts(huvec_choroid = huvec - choroid,
                                 huvec_retina = huvec - retina,
                                 huvec_iris = huvec - iris,
                                 levels = design)

# Fits a linear model for each gene given a series of arrays
# Results from the RMA and the contrast.matrix
fit = limma:::lmFit(celfiles.gcrma, design)

# Use the model to fit microdata data (coefficients, errors)
huvec_fit <- contrasts.fit(fit, contrast.matrix)

# Calculates t-statistics, F-statistics and logodds for the fitted data
huvec_ebay <- eBayes(huvec_fit)

# Creates a list of probe name for 100000 entries. topTable takes the top-ranked
# genes from a linear fit
probenames.list <- rownames(topTable(huvec_ebay, number = 100000))

# Get symbols
getsymbols <- getSYMBOL(probenames.list, "hgu133plus2")

# And get the 100000 by the coefficient huvec_choroid
results <- topTable(huvec_ebay, number = 100000, coef = "huvec_choroid")

# Add the symbols to this
results <- cbind(results, getsymbols)

```

```

# Prints the summary of our results
summary(results)

# "Extract Data" depending on the p value and logFC
results$threshold <- "1"
a <- subset(results, adj.P.Val < 0.05 & logFC > 5)
results[rownames(a), "threshold"] <- "2"
b <- subset(results, adj.P.Val < 0.05 & logFC < -5)
results[rownames(b), "threshold"] <- "3"

# -----
# Question 2
# -----

# -----
# Question 3
# -----

significant_genes = c()

# -----
# Huvec - Choroid
# -----

current_genes = subset(results, logFC > 5 & -1*log10(adj.P.Val) > 5)
significant_genes = c(significant_genes, as.vector(current_genes$getsymbols))

table(results$threshold)

# Make a volcano plot
volcano <- ggplot(data = results,
                     aes(x = logFC, y = -1*log10(adj.P.Val),
                         colour = threshold,
                         label = getsymbols))
volcano <- volcano +
  geom_point() +
  scale_color_manual(values = c("black", "red", "green"),
                     labels = c("Not Significant", "Upregulated", "Downregulated"),
                     name = "Key/Legend")
volcano +
  geom_text(data = subset(results, logFC > 5 & -1*log10(adj.P.Val) > 5),
            aes(x = logFC, y = -1*log10(adj.P.Val), colour = threshold,
                label = getsymbols))

# These are the other combinatin where we need volcano plots for
# huvec_retina
# huvec_iris

# -----
# Huvec - Retina
# -----

results <- topTable(huvec_ebay, number = 100000, coef = "huvec_retina")

```

```

results <- cbind(results, getsymbols)

current_genes = subset(results, logFC > 5 & -1*log10(adj.P.Val) > 5)
significant_genes = c(significant_genes, as.vector(current_genes$getsymbols))

results$threshold <- "1"
a <- subset(results, adj.P.Val < 0.05 & logFC > 5)
results[rownames(a), "threshold"] <- "2"
b <- subset(results, adj.P.Val < 0.05 & logFC < -5)
results[rownames(b), "threshold"] <- "3"
table(results$threshold)

# Make a volcano plot
volcano2 <- ggplot(data = results,
                     aes(x = logFC, y = -1*log10(adj.P.Val),
                         colour = threshold,
                         label = getsymbols))

volcano2 <- volcano2 +
  geom_point() +
  scale_color_manual(values = c("black", "red", "green"),
                     labels = c("Not Significant", "Upregulated", "Downregulated"),
                     name = "Key/Legend")

volcano2 +
  geom_text(data = subset(results, logFC > 5 & -1*log10(adj.P.Val) > 5),
            aes(x = logFC, y = -1*log10(adj.P.Val), colour = threshold,
                label = getsymbols))

# -----
# Huvec - Iris
# -----

results <- topTable(huvec_ebay, number = 100000, coef = "huvec_iris")
results <- cbind(results, getsymbols)

current_genes = subset(results, logFC > 5 & -1*log10(adj.P.Val) > 5)
significant_genes = c(significant_genes, as.vector(current_genes$getsymbols))

results$threshold <- "1"
a <- subset(results, adj.P.Val < 0.05 & logFC > 5)
results[rownames(a), "threshold"] <- "2"
b <- subset(results, adj.P.Val < 0.05 & logFC < -5)
results[rownames(b), "threshold"] <- "3"
table(results$threshold)

# Make a volcano plot
volcano2 <- ggplot(data = results,
                     aes(x = logFC, y = -1*log10(adj.P.Val),
                         colour = threshold,
                         label = getsymbols))

```

```

volcano2 <- volcano2 +
  geom_point() +
  scale_color_manual(values = c("black", "red", "green"),
                     labels = c("Not Significant", "Upregulated", "Downregulated"),
                     name = "Key/Legend")

volcano2 +
  geom_text(data = subset(results, logFC > 5 & -1*log10(adj.P.Val) > 5),
            aes(x = logFC, y = -1*log10(adj.P.Val), colour = threshold,
                label = getsymbols))

significant_genes = unique(significant_genes)
significant_genes = significant_genes[!is.na(significant_genes)]
print(significant_genes)

# -----
# Question 4
# -----

```