Basic RNAseq Pipeline

Kathryn Lande

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Load packages and data

```
# install packages if necessary:
if (!require("remotes", quietly = TRUE))
  install.packages("remotes")
if (!require("RNAseqFunctions", quietly = TRUE))
  remotes::install_github("katlande/RNAseq_Functions")
if (!require("ggplot2", quietly = TRUE))
  BiocManager::install("DESeq2")
if (!require("BiocManager", quietly = TRUE))
  install.packages("BiocManager")
if (!require("DESeq2", quietly = TRUE))
  BiocManager::install("DESeq2")

# load necessary packages:
library(RNAseqFunctions)
quietLib("ggplot2") # quietLib loads packages without messages and warnings
quietLib("DESeq2")

# load test data
data(test_counts)
data(test_design)
```

RNAseq experiments require two files. The first is a gene-by-sample matrix of raw count data, that looks something like this:

```
head(test_counts)
```

```
##          ctl1_Young ctl2_Young ctl3_Young ctl1_Old ctl2_Old ctl3_Old trt1_Young
## Mia2           2780       1370       2724     2224     1436     2400       2254
## Abraxas1          0          0         32      174       42       36          0
## Marchf1           2          0         38       14        2        4          0
## Mob3b           392         12        100      234      160      210        178
## Gm10487           0          0          0        0        0        0          0
## Mir145a           0          0          0        0        0        0          0
##          trt2_Young trt3_Young trt1_Old trt2_Old trt3_Old
## Mia2           1934       1888     2728     4364     2522
## Abraxas1         20         94       52       96       24
## Marchf1          14          0       38       32       32
## Mob3b           166         76      154      270      290
## Gm10487           0          0        0        0        0
## Mir145a           0          0        0        0        0
```

The row names are gene or transcript IDs, and the column names are sample names. The included function

homer_to_matrix() will convert the output of Homer's analyzeRepeats.pl into this format. The second file is the meta data, which would look something like this:

```
head(test_design)
```

```
##            treatment   age     group
## ctl1_Young       ctl young young ctl
## ctl2_Young       ctl young young ctl
## ctl3_Young       ctl young young ctl
## ctl1_Old         ctl   old   old ctl
## ctl2_Old         ctl   old   old ctl
## ctl3_Old         ctl   old   old ctl
```

The row names are sample names that match the column names of the counts file, and the column names represent sample information. In thise case, we have information about the treatment and age of our test samples.

Set up data for analysis

Let's make sure samples in our meta data have the same names and occur in the same order as our count data. Samples with mismatched names or those that occur in different orders may cause downstream errors.

```
test_design <- checkMetaData(test_design, test_counts)
```

```
## Data matches!
```

```
# Our files match, so no modification was made.
# Dis-ordered data will be reordered, and mis-matched names will throw a warning.
```

For differential expression analysis with DEseq2, we'll need to combine our count and meta data into a DESeqDataSet object. How we design this object depends on the comparisons we want to make. In the 'design' parameter, we add variables (columns of the meta data) that we want to factor into our analysis. If we want to look at a single variable and controlling for the other (for example, looking at the difference between all young and all old samples, controlling for the effect of the treatment), we would set up an object like this:

```
D1 <- DESeqDataSetFromMatrix(countData = test_counts,
                             colData = test_design,
                             design = as.formula(~age+treatment))
```

However, if you wanted to make more specific comparisons, e.g., young ctl vs. old ctl, you would set up your object like this:

```
D2 <- DESeqDataSetFromMatrix(countData = test_counts,
                             colData = test_design,
                             design = as.formula(~group))
```

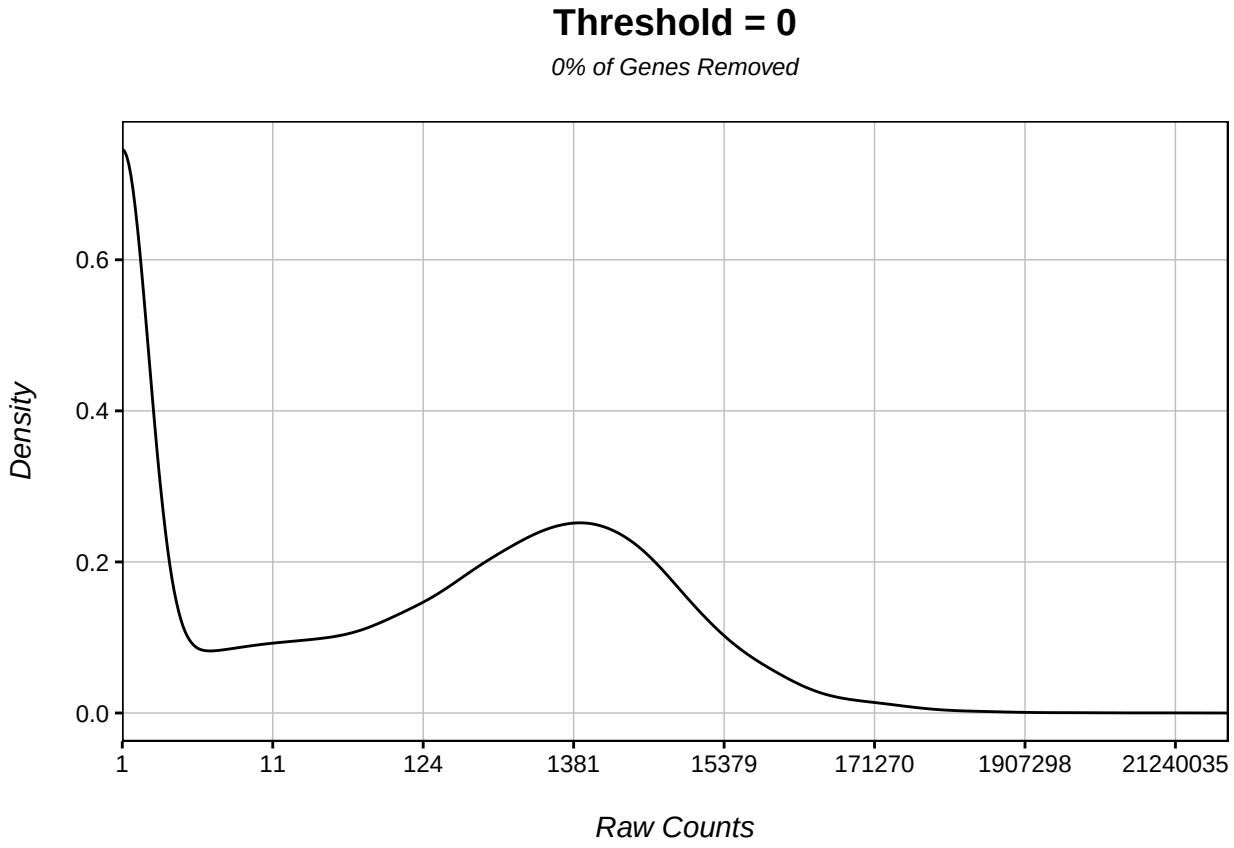
Basically, you will be able to make a comparison of any two levels in any meta data column included in the design, and other columns in the design will be controlled for. The design formula can be made more complex to account for additive and non-additive variables, fixed and random variables, and crossed and nested designs. The design for D1 assumes age and treatment are additive variables in a crossed design. See some examples of other formulas you could set up:

```
design = as.formula(~ factorA:factorB) # returns only the interaction of factorA and factorB.
design = as.formula(~ factorA*factorB) # returns (~ factorA + factorB + factorA:factorB), i.e., non-int
design = as.formula (~ factorB %in% factorA) # when factorB is nested within factorA; this can also be
design = as.formula(~(1|factorA/factorB)) # when factorB is nested and crossed within factorA.
```

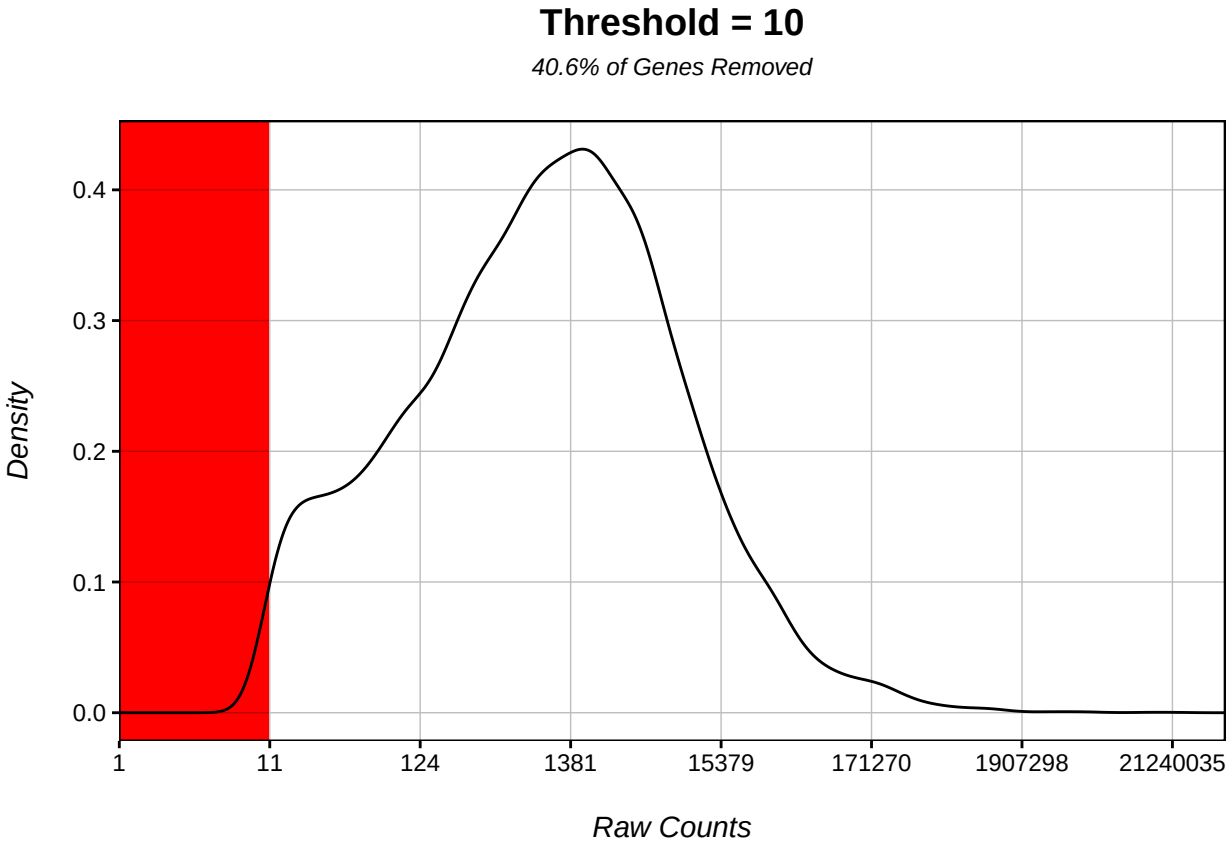
Finally, we will need to identify a minimum read cut-off to remove lowly and non-expressed genes. A large

chunk of the transcriptome will not be expressed in any given sample, and these genes will create noise if not removed. We can identify a good filtering threshold using the Check_Filter() function:

```
Check_Filter(D1) # this is a density plot of total reads per gene
```




```
Check_Filter(D1, 10) # this is the same density plot, but genes with less than 10 total reads are remov
```



Using a cut-off of 10 sufficiently removes the non-expressed genes.

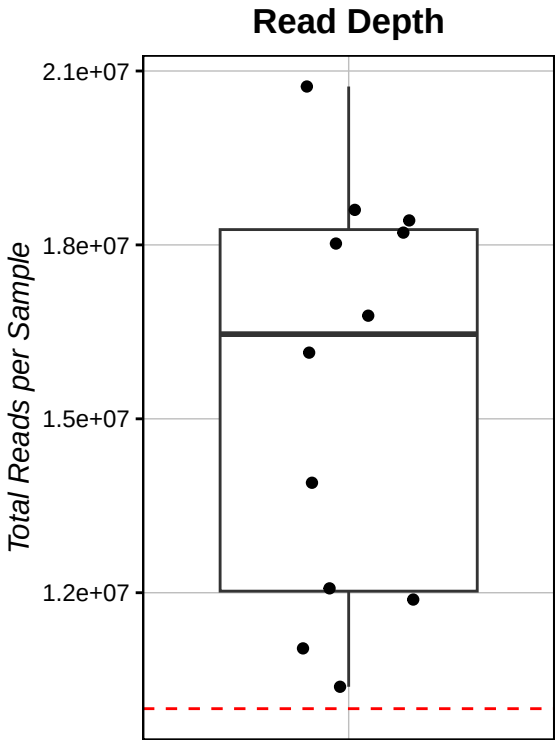
Quality Control

The last step before differential expression analysis is quality control to make sure the data is usable.

Read depth

If too few reads are sequenced in any of our samples, they won't give us reliable information. In general, a minimum read depth of ~10 million is a good threshold for mouse and human. This number will be larger or smaller for larger and smaller transcriptomes, respectively.

```
CheckDepth(test_counts, minDepth = 1e07)
```

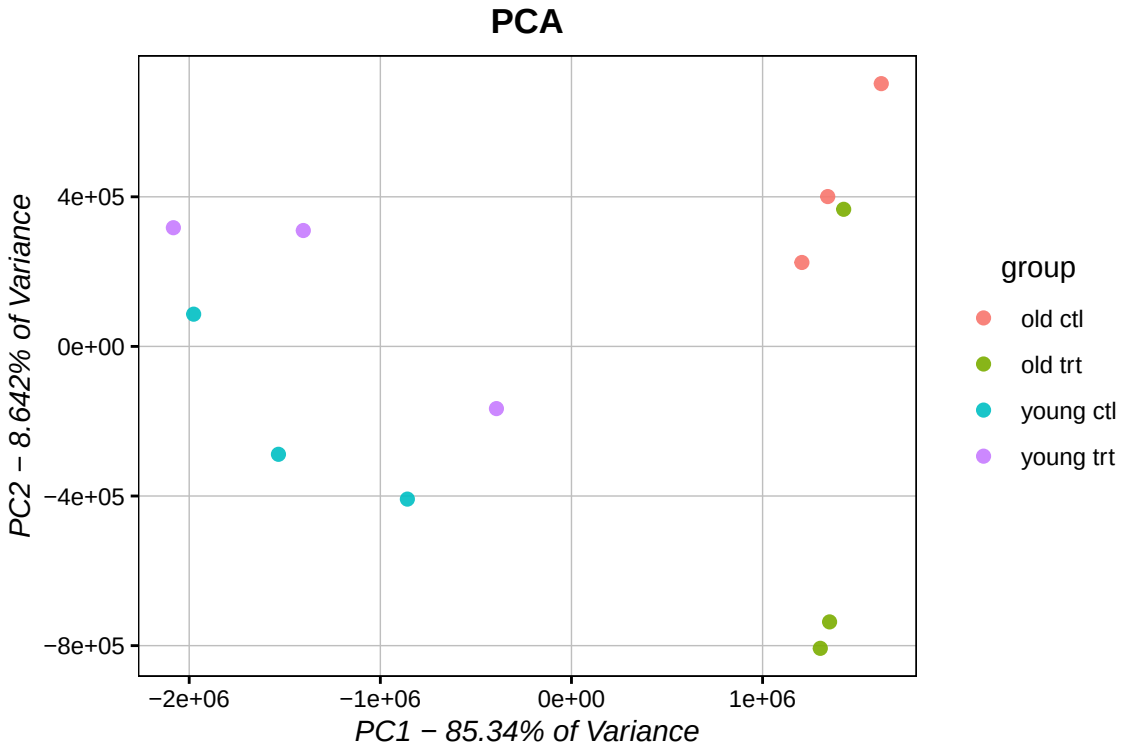



```
# All samples have appropriate read depth above our minimum threshold.
```

Sample clustering

We expect samples to group together based on meta data variables. We can confirm this with a PCA.

```
customPCA(D1, # DESeqDataSet
          test_design, # meta data
          ptcol = "group", # colour the points based on this column of test_design
          filter_threshold = 10) # the threshold we determined previously
```



Here we can see that a massive portion of the transcriptional variance is explained by age (~85%). There is also separation between treatment and control samples within age groups, but it's a little noisier. We can expect that there will be a lot more differentially expressed genes between young and old than there will be between treatment and control.

PCAs can also be used to identity outliers (single samples that cluster strangely), and potential sample swaps (e.g., one young sample that clusters with old samples and one old sample that clusters with young samples on the same PCA *might* indicate that the sample IDs of these two samples were swapped).

Differential Expression

There are many tools for calcualting differential expression, but in this tutorial we'll be using DESeq2. The following function will filter, normalize, and run DESeq2 on a DESeqDataSet object:

```
Dnorm1 <- DESeq_and_Filt(D1, filter_thresh = 10,  minSamples = 2) # minimum of 2 of samples with > 0 re
Dnorm2 <- DESeq_and_Filt(D2, filter_thresh = 10, minSamples = 2)
```

These Dnorm objects contain differential expression information for all possible comparisons based on the design of the input object. Dnorm1 contains a comparison of ages controlling for treatment, and a comparison of treatments controlling for ages. Dnorm2 contains all pairwise comparisons of the 'group' column in the metadata: 'young trt' vs. 'old trt', 'young trt' vs. 'young ctl', etc. We can extract these differential expression statistics into a data.frame using the makeComp() function:

```
young_old_all <- makeComp(Dnorm1, var = "age", up="old", down="young") # var = a variable in 'design';
ctl_trt_all <- makeComp(Dnorm1, "treatment", "trt", "ctl")

# just looking age the effect of treatment within age groups:
young_old_ctl <- makeComp(Dnorm2, "group", "old ctl", "young ctl")
young_old_trt <- makeComp(Dnorm2, "group", "old trt", "young trt")

# These dataframes look like this:
head(young_old_all)
```

```
##                  baseMean log2FoldChange     lfcSE      stat       pvalue
## Mia2           2218.33888      0.2801533 0.2607599 1.0743726 0.2826556995
## Abraxas1         47.41611      1.6237780 1.2882173 1.2604846 0.2074946102
## Marchf1          13.38804      0.9157091 1.4988117 0.6109567 0.5412282314
## Mob3b           177.04860      0.6666551 0.5956179 1.1192663 0.2630265330
## 1810034E14Rik    23.16251      4.1775213 1.2011733 3.4778672 0.0005054203
## Crat            589.43951      0.7836559 0.4124474 1.9000142 0.0574312521
##                       padj           Gene Dir
## Mia2           0.416958384           Mia2 old
## Abraxas1       0.331624129       Abraxas1 old
## Marchf1        0.662170454        Marchf1 old
## Mob3b          0.395290232          Mob3b old
## 1810034E14Rik 0.002492322 1810034E14Rik old
## Crat           0.125180326           Crat old
```

The two most important values for differential expression are log2FoldChange, the magnitude of change, and pAdj, the adjusted p-value of differential expression. Typically, differentially expressed genes (DEGs) are defined as pAdj<=0.05 and absolute(log2FC) > 0.5 or > 1. Let's see how many DEGs we get from each of these comparisons:

```
getDEGs(young_old_all, lfc = 1) # absolute log2FC cut-off of 1. pAdj cut-off is set automatically to 0.
```

```
## [1] "4361 DEGs: 2995 upregulated; 1366 downregulated."
```

```
getDEGs(ctl_trt_all, lfc = 1)
```

```
## [1] "340 DEGs: 203 upregulated; 137 downregulated."
```

```
getDEGs(young_old_ctl, lfc = 1)
```

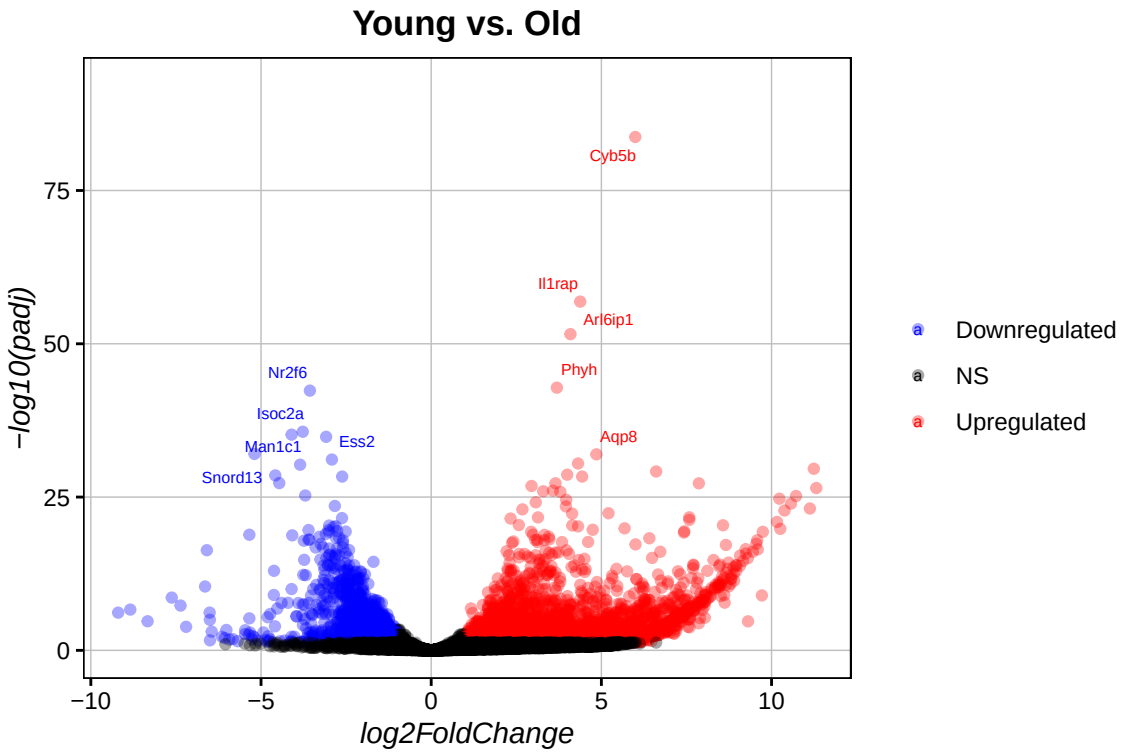
```
## [1] "2623 DEGs: 1683 upregulated; 940 downregulated."
```

```
getDEGs(young_old_trt, lfc = 1)
```

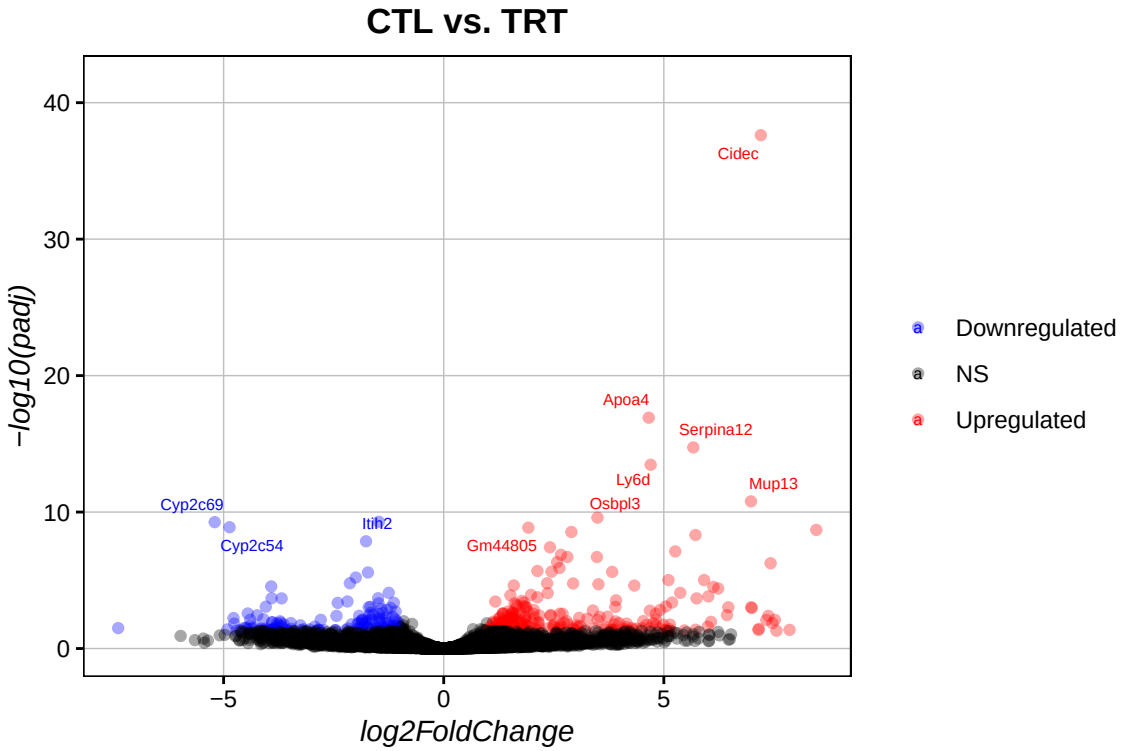
```
## [1] "3761 DEGs: 2652 upregulated; 1109 downregulated."
```

As we predicted from the PCA, we see many more DEGs with age than we do with treatment. We can also look at DEGs using volcano plots:

```
eRupt(young_old_all, FCsig = 1)+ggtitle("Young vs. Old")
```



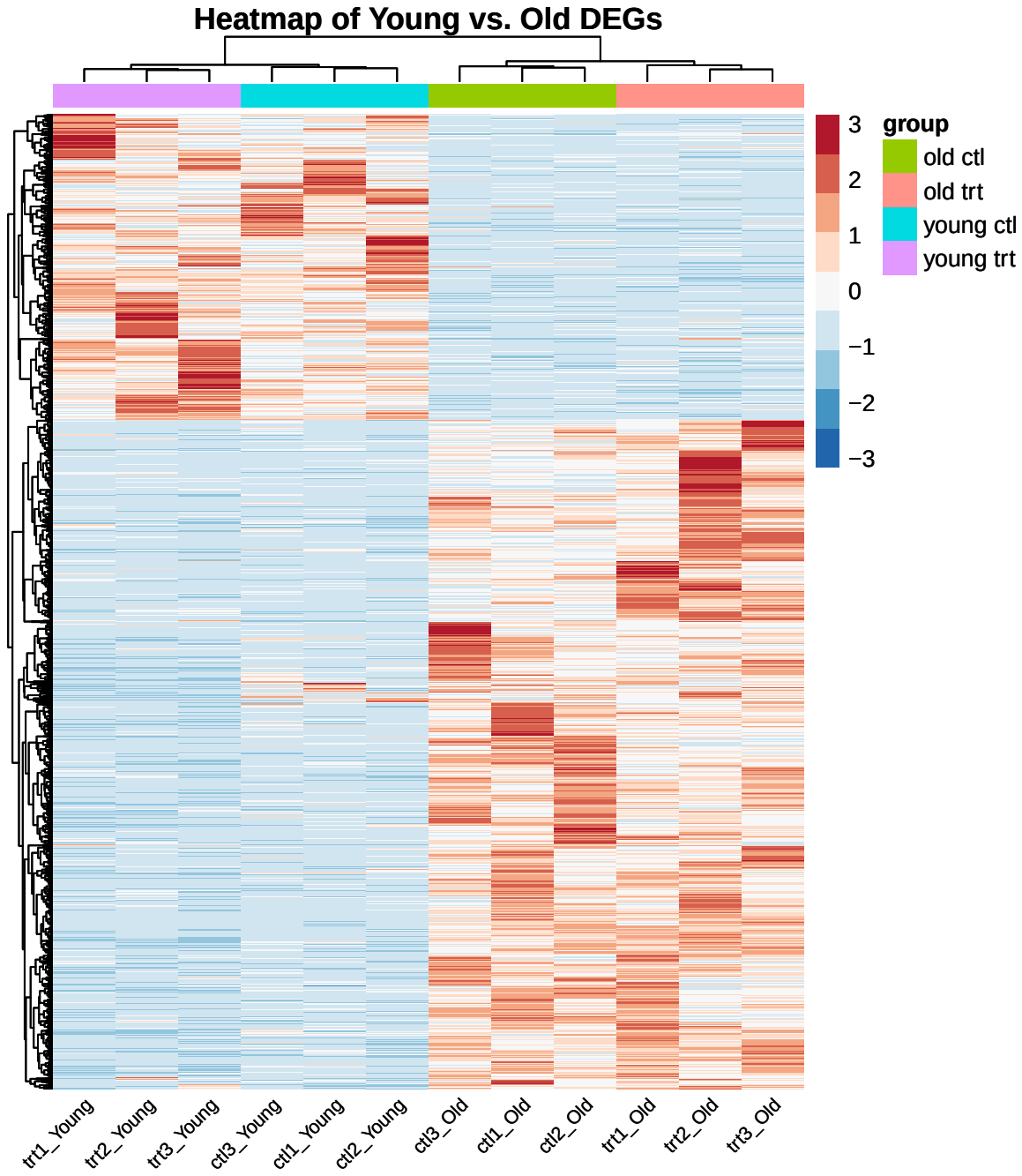

```
eRupt(ctl_trt_all, FCsig = 1)+ggtitle("CTL vs. TRT")
```



We may want to visualize our differentially expressed genes with a heatmap:

```
normalized_counts <- filt.norm(D1, thresh=10) # generate a data.frame of normalized counts
YOAdegs <- getDEGs(young_old_all, lfc = 1, direction = "all", mode = "vector") # a vector all DEGs in t
```

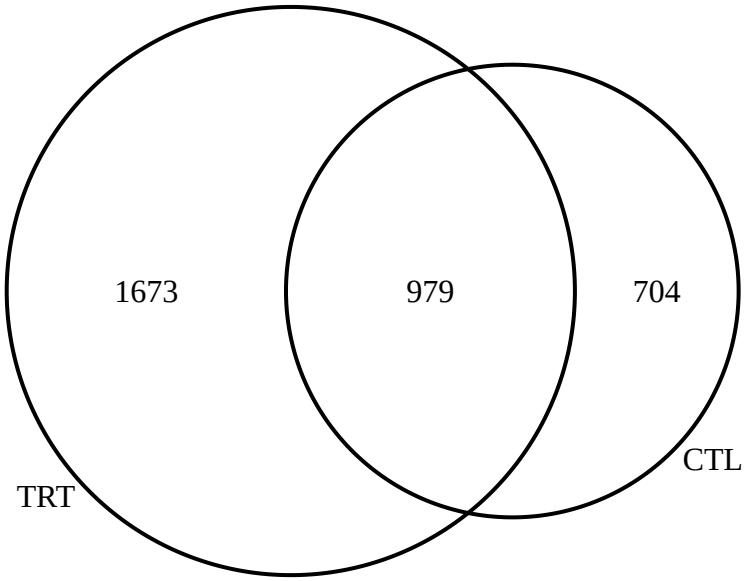
```
# This is a pipe to pheatmap:
Reliable_heatmap(normalized_counts, # values to plot
                 genes = YOAdegs, # only plot these genes
                 sample_meta = test_design, # meta data
                 annotation = T, # whether to add colour coding based on the meta data
                 annots = "group", # which columns of the meta data to colour code
                 rowlabs = F, # whether to add row text
                 title = "Heatmap of Young vs. Old DEGs")
```



If we want to check how much overlap we see between DEGs from multiple comparisons, we can make a venn diagram:

```
old_ctl <- getDEGs(young_old_ctl, lfc = 1, direction = "up", mode = "vector") # a vector all age-upregu
old_trt <- getDEGs(young_old_trt, lfc = 1, direction = "up", mode = "vector") # a vector all age-upregu

display_venn(list(old_ctl, old_trt), # a list of gene names to overlap
             category.names = c("CTL", "TRT")) # names of the gene sets
```



Many of the genes upregulated with age are common to both control and treatment samples, but these samples also seem to have unique aging signatures as well.

Functions of Differentially Expressed Genes

Now that we know which genes change under different conditions, we can check to see what functions/pathways they belong to. *For the rest of the tutorial, we'll focus only on the young_old_all data.*

```
ORA <- WebGestalt_Pipe("ORA", # type of enrichment; either 'ORA' or 'GSEA'
                young_old_all, # makeComp() output file
                FC = 1, # absolute log2FC for significance
                DB = "geneontology_Biological_Process_noRedundant", # which ontology to look at. Here w
                species = "mmusculus", # your species; this data is from mouse. Mouse is also the defau
                projectName = "YO_ORA") # This function will save the raw data to a folder with this na
```

The ORA results tell us which biological processes contain more DEGs than we expect by random chance, here's a bit of what these results look like:

```
head(ORA[c(3,8,10)])
```

```
##                                    description enrichmentRatio         FDR
## 1   nucleoside monophosphate metabolic process       -1.985967 0.001221773
## 2    nucleoside triphosphate metabolic process       -1.967510 0.001311558
## 101                  muscle tissue development        1.481263 0.005423823
## 102                             molting cycle        1.952426 0.006918359
## 104                     epidermis development        1.565358 0.018244517
## 103             glycerolipid metabolic process        1.429082 0.018244517
```

Here we see a significant increase in muscle tissue development and molting cycle genes with age (positive enrichment), and a significant decrease in nucleoside mono- and tri-phosphate metabolic process genes with age (negative enrichment).

Looking at gene sets of interest

You may be interested in the behaviour of a specific set of genes. In this example, we'll look at the following set of genes that's related to oxidative phosphorylation:

```
OxPhos <- c("Ndufb10", "Ndufa10", "Ndufb9", "Atp5e", "Uqcrq", "Atp5o", "Ndufv3",
            "Ppa1", "Ppa2", "Ndufb3", "Atp5a1", "Cox4i1", "Uqcr10", "Ndufa12",
            "Cox5a", "Cox5b", "Cox7c", "Cox7b", "Cox17", "Atp6v1b2", "Atp5k",
            "Ndufs4", "Atp5h", "Cox7a1", "Lhpp", "Ndufs3", "Ndufa1", "Cox6a1")
```

First, we can check if these genes are enriched in either the up- or down-regulated DEGs. The function SetEnrichment() prints two values. The first is the p-value of enrichment (Fisher's Exact Test), the second is the enrichment ratio. If the enrichment ratio is above 1, the genes are enriched in the DEGs. If they are below 1, they are depleted from the DEGs.

```
oldDEGs <- getDEGs(young_old_all, lfc = 1, direction = "up", mode = "vector")
youngDEGs <- getDEGs(young_old_all, lfc = 1, direction = "down", mode = "vector")

SetEnrichment(OxPhos, # Genes of interest
              oldDEGs, # All DEGs
              young_old_all$Gene) # All filtered genes
```

```
## [1] 0.007146275 0.119003439
```

```
# p=0.007146275, enrichment ratio=0.119003439
# this means OxPhos genes are significantly depleted from the upregulated genes

SetEnrichment(OxPhos, # Genes of interest
              youngDEGs, # All DEGs
              young_old_all$Gene) # All filtered genes
```

```
## [1] 1.349370e-21 7.005344e+01
```

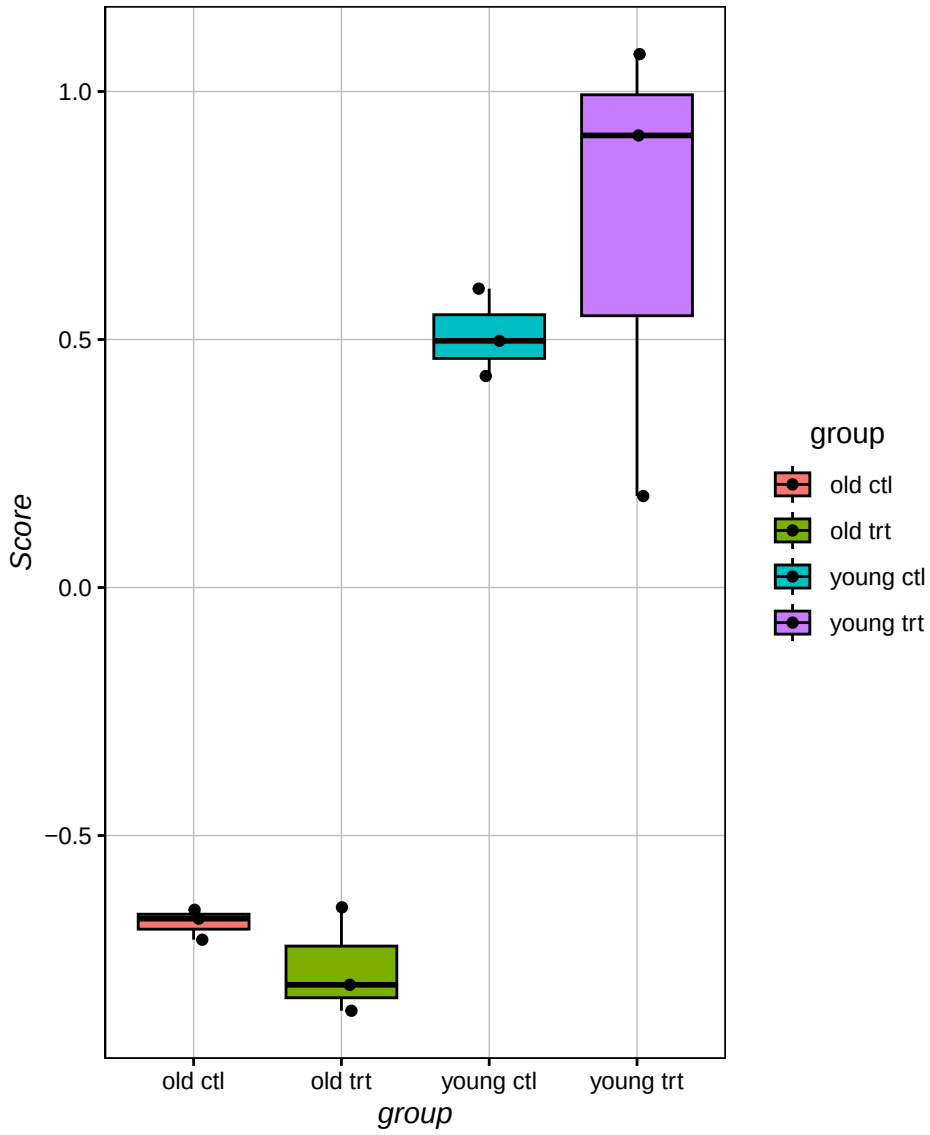
```
# p=1.349370e-21, enrichment ratio=70.05344
# this means OxPhos genes are extremely significantly enriched in the downregulated genes!
```

We can also calculate set expression scores for each of our samples. These values reflect the average relative expression of all genes in a set, and as such take *normalized counts* as an input (do not use raw counts or FPKMs).

```
OxPhos_Scores <- SetScores(normalized_counts, OxPhos, test_design, out="data.frame")
head(OxPhos_Scores)
```

```
##       Sample treatment   age     group      Score
## 1   ctl1_Old       ctl   old   old ctl -0.6496537
## 2 ctl1_Young       ctl young young ctl  0.4973454
## 3   ctl2_Old       ctl   old   old ctl -0.6675475
## 4 ctl2_Young       ctl young young ctl  0.6024897
## 5   ctl3_Old       ctl   old   old ctl -0.7100029
## 6 ctl3_Young       ctl young young ctl  0.4264215
```

```
# plot the scores:
ggplot(OxPhos_Scores, aes(x=group, y=Score, fill=group))+
  geom_boxplot(colour="black")+ # boxplots of score for each group
  geom_jitter(height = 0, width=0.075)+ # one point for each sample
  Ol_Reliable() # custom plot theme
```



Here we see that age has a strong influence on oxphos gene expression, while treatment does not. An important caveat about these plots is that the score value is relative, and will change if different samples are used for the input. *You cannot compare scores that are generated in two separate commands.*