# Lab: Differential Expression via RNA-Seq Analysis

## IGF RNA-Seq Workshop 2018 (PLPTH885)

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6/7/2018

# Outline

- Differential expression test using DESeq2
- Result visualization
- GO enrichment test

# Lecture assistant

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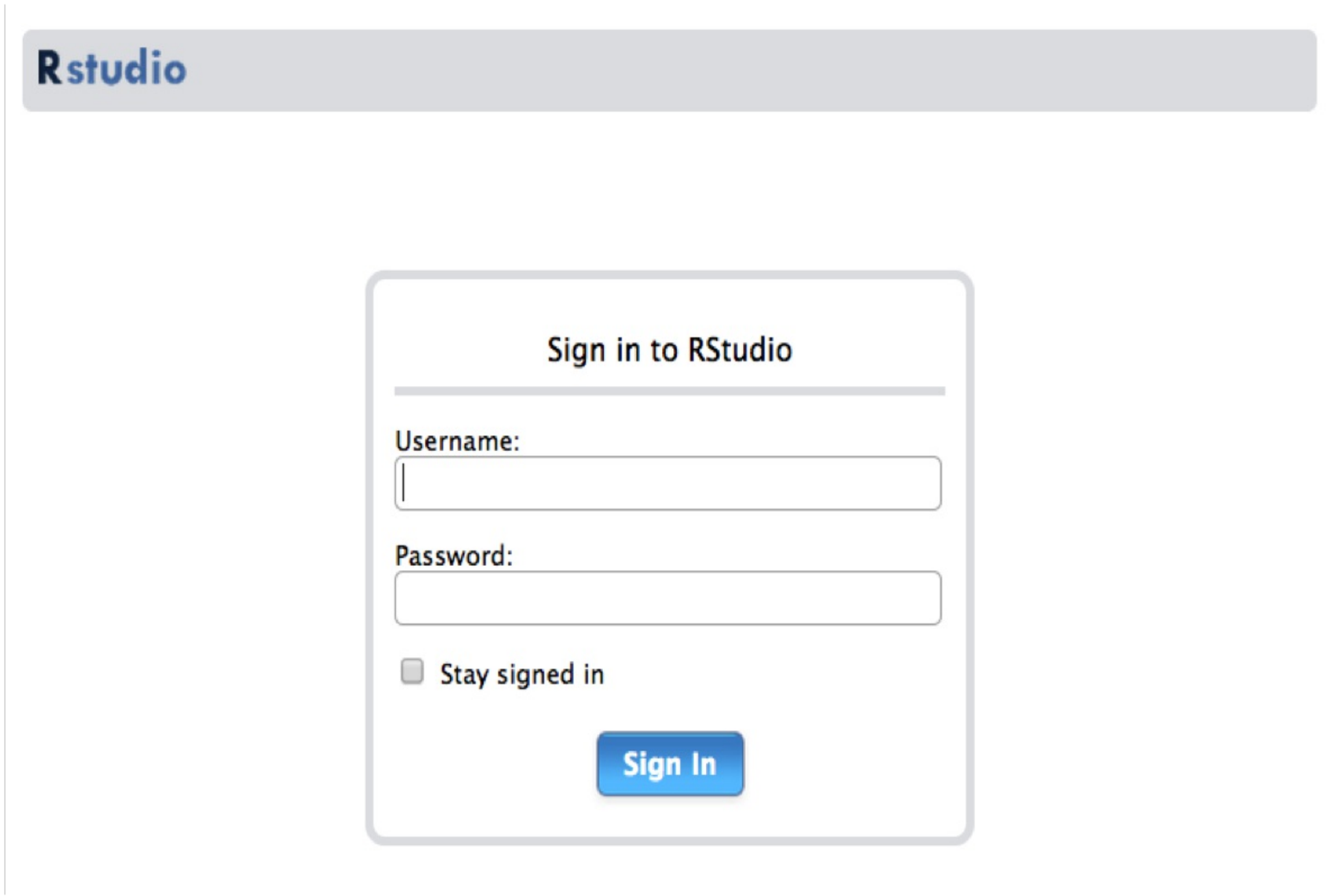
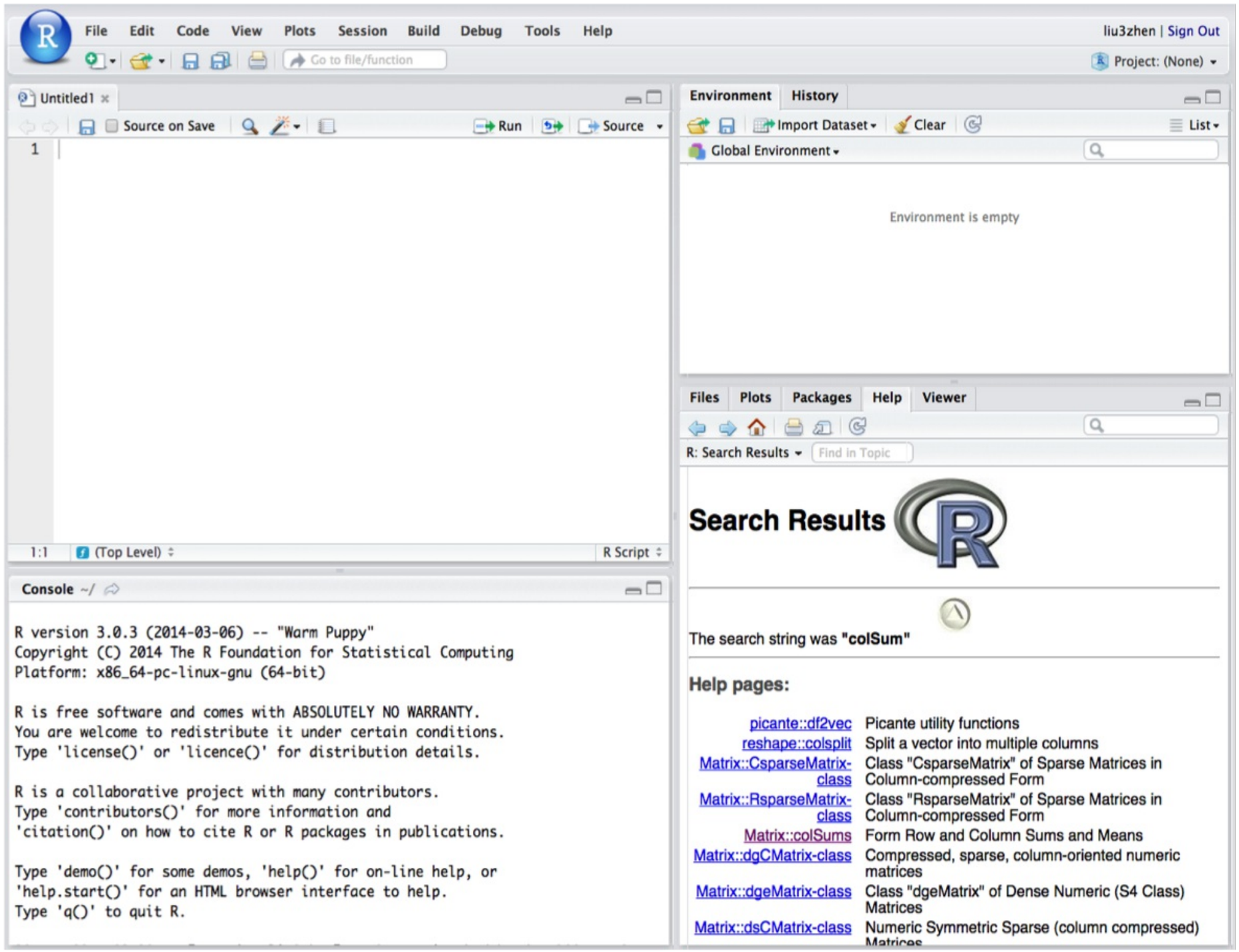
# Rstudio at Beocat

rstudio.beocat.cis.ksu.edu



# Rstudio interface



# Package installation

```
# DESeq2
source("http://bioconductor.org/biocLite.R")
biocLite("DESeq2")

# GOSeq
biocLite("goseq")

# GO.db
biocLite("GO.db")
```

# preload modules

```
pls="http://129.130.89.83/tmp/public/RNASeq/RNASeq2018/codes/load.R"
source(pls)
```

- panel.cor2
- rnaseq.pca
- normalization

# Read expression data (Read counts per gene)

```
rc <- "http://129.130.89.83/tmp/public/RNASeq/RNASeq2018/data/rc.txt"
grc <- read.delim(rc)
nrow(grc)  # the number of rows/lines
```

```
[1] 22697
```

```
head(grc, 1)  # show the first 1 rows
```

```
              Gene ExonSize ck_rep1 ck_rep2 ck_rep3 trt_rep1 trt_rep2
1 AC147602.5_FG004      483    5480    6075    5934     3370     5784
  trt_rep3
1     6432
```

```
tail(grc, 1)  # show the last 1 rows
```

```
              Gene ExonSize ck_rep1 ck_rep2 ck_rep3 trt_rep1 trt_rep2
22697 GRMZM5G899985      615     267     327     348        83      342
      trt_rep3
22697      403
```

# RPKM normalization

```
datacols <- c("ck_rep1", "ck_rep2", "ck_rep3",
              "trt_rep1", "trt_rep2", "trt_rep3")
lib.sizes <- colSums(grc[, datacols])
exon.sizes <- grc$ExonSize
grcn <- normalization(counts = grc,
                      normcolname = datacols,
                      libsize = lib.sizes,
                      exonsize = exon.sizes,
                      methods = "RPKM")
head(grcn)
```

```
             Gene ExonSize ck_rep1 ck_rep2 ck_rep3 trt_rep1 trt_rep2
1 AC147602.5_FG004      483    5480    6075    5934     3370     5784
2 AC148152.3_FG005     1422     187     295     377      169      158
  trt_rep3 ck_rep1.RPKM ck_rep2.RPKM ck_rep3.RPKM trt_rep1.RPKM
1     6432      854.123      895.760      904.373       567.493
2      563        9.900       14.775       19.516         9.666
  trt_rep2.RPKM trt_rep3.RPKM
1       915.326       916.971
2         8.493        27.262
```

# data organization for DESeq2

- count information

```
geneid <- grc$Gene
in.data <- as.matrix(grc[, 3:8])
head(in.data, 1)
```

```
     ck_rep1 ck_rep2 ck_rep3 trt_rep1 trt_rep2 trt_rep3
[1,]    5480    6075    5934     3370     5784     6432
```

```
# sample names and grouping information (treatment)
sample.ids <- colnames(in.data)
treatment <- c("ck", "ck", "ck", "trt", "trt", "trt")
sample.info <- data.frame(row.names=sample.ids, trt=treatment)
sample.info
```

```
          trt
ck_rep1    ck
ck_rep2    ck
ck_rep3    ck
trt_rep1 trt
trt_rep2 trt
trt_rep3 trt
```

# Differential expression test

```
dds <- DESeqDataSetFromMatrix(countData=in.data,
                              colData=sample.info,
                              formula(~trt))
dds <- DESeq(dds)
```

# DE output

```
res <- results(object = dds)
res <- data.frame(res)
res$Gene <- geneid
res <- res[,c("Gene","baseMean","log2FoldChange","pvalue","padj")]
nrow(res)
```

```
[1] 22697
```

# DE + normalized data

```
### Merge the normalized result with the DE result
out <- merge(grcn, res, by = "Gene")
out <- data.frame(out)
head(out, 2)
```

```
                Gene ExonSize ck_rep1 ck_rep2 ck_rep3 trt_rep1 trt_rep2
1 AC147602.5_FG004      483    5480    6075    5934     3370     5784
2 AC148152.3_FG005     1422     187     295     377      169      158
  trt_rep3 ck_rep1.RPKM ck_rep2.RPKM ck_rep3.RPKM trt_rep1.RPKM
1     6432       854.123       895.760       904.373        567.493
2      563         9.900        14.775        19.516          9.666
  trt_rep2.RPKM trt_rep3.RPKM  baseMean log2FoldChange    pvalue      padj
1       915.326       916.971 5441.6579    -0.13983297 0.4642286 0.8108694
2         8.493        27.262  285.5493     0.03215599 0.9258192 0.9852456
```

# significantly DEG

### significant gene sets at different FDRs

```
sum(!is.na(out$padj) & out$padj < 0.05)
```

```
[1] 1200
```

```
sum(!is.na(out$padj) & out$padj < 0.1)
```

```
[1] 1513
```

```
sum(!is.na(out$padj) & out$padj < 0.15)
```

```
[1] 1812
```

```
sum(!is.na(out$padj) & out$padj < 0.2)
```

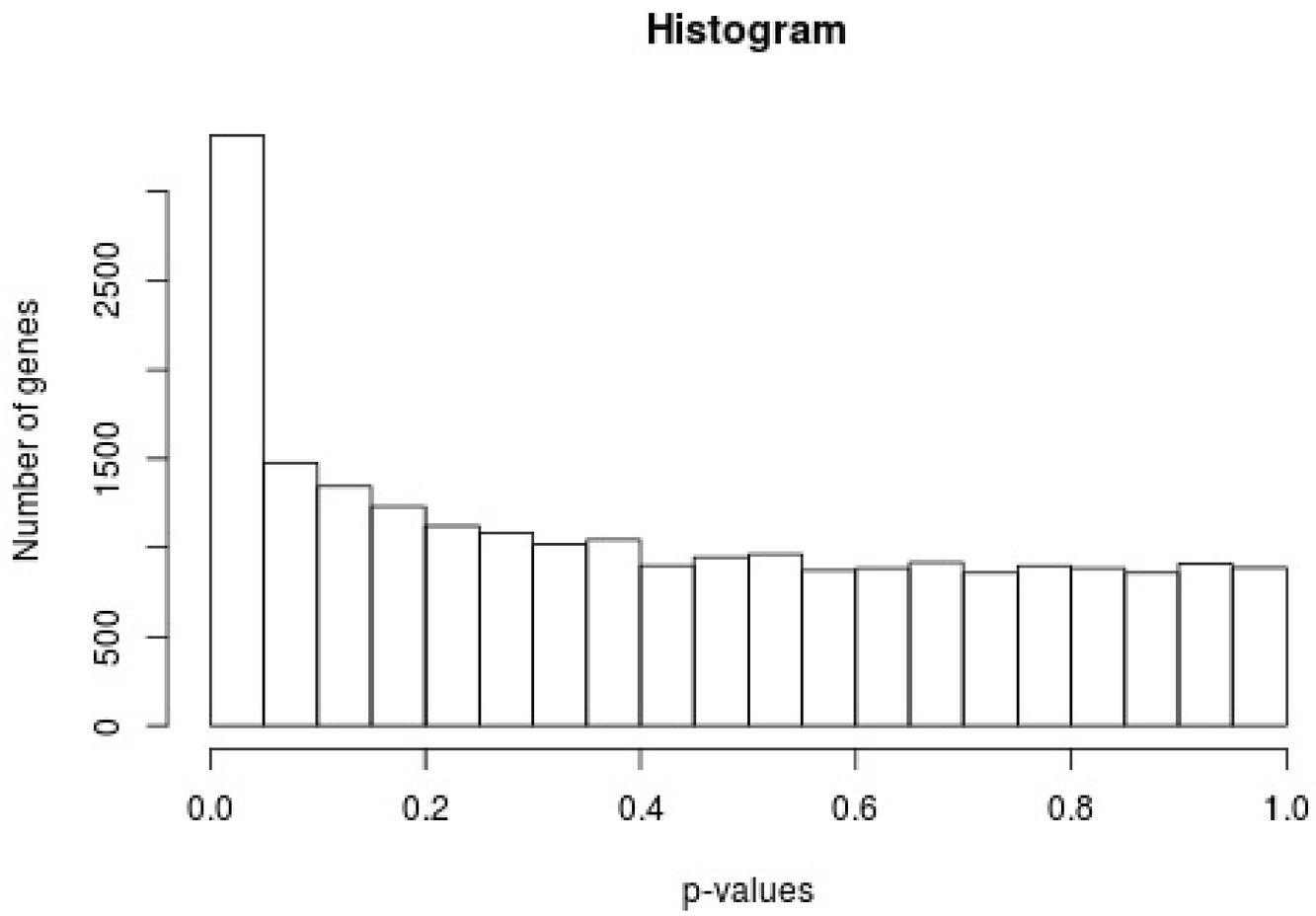
```
[1] 2191
```

# significantly DEG

```
sig <- out[!is.na(out$padj) & out$padj < 0.05, ]
```

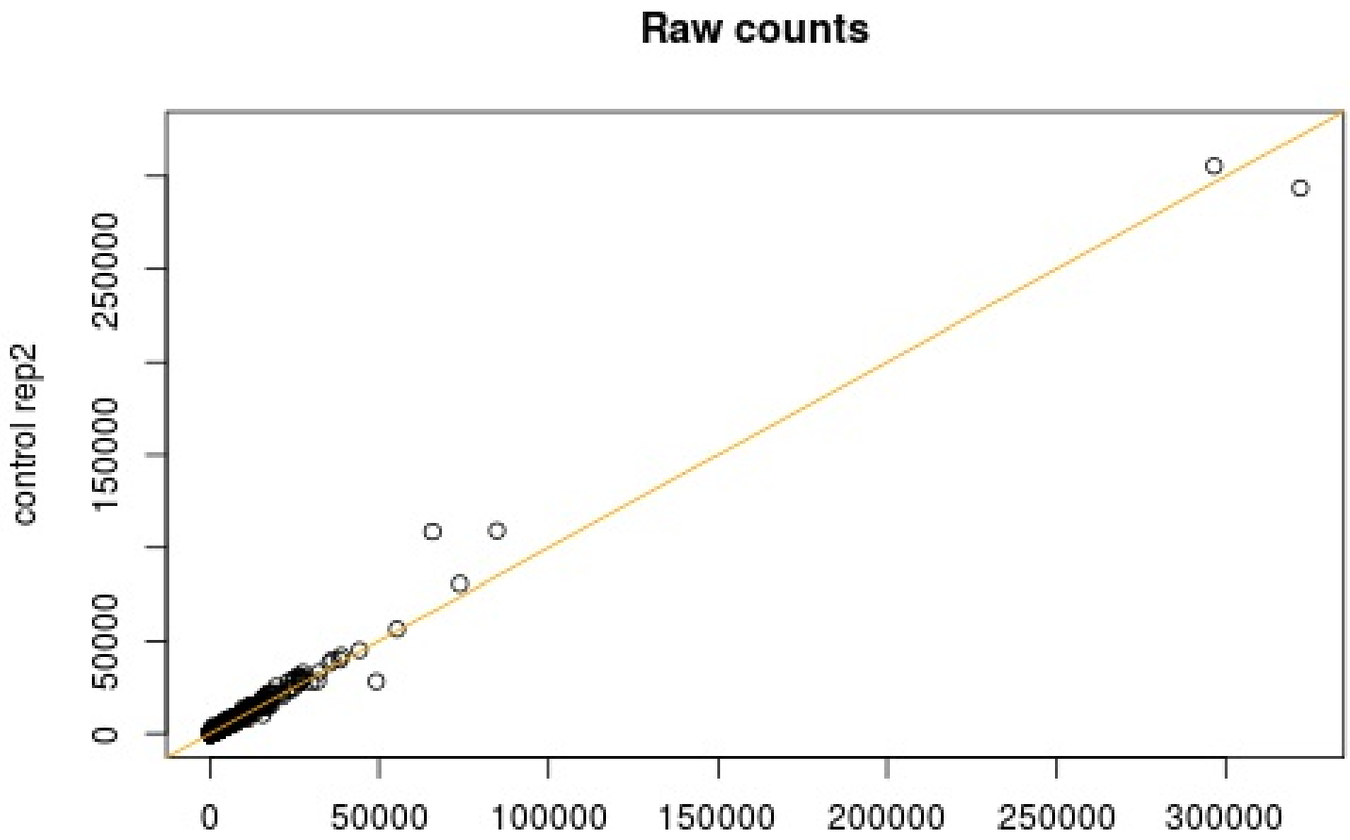
# p-value histogram

```
pvals <- out$pvalue
hist(pvals,main="Histogram",xlab="p-values",ylab="Number of genes")
```



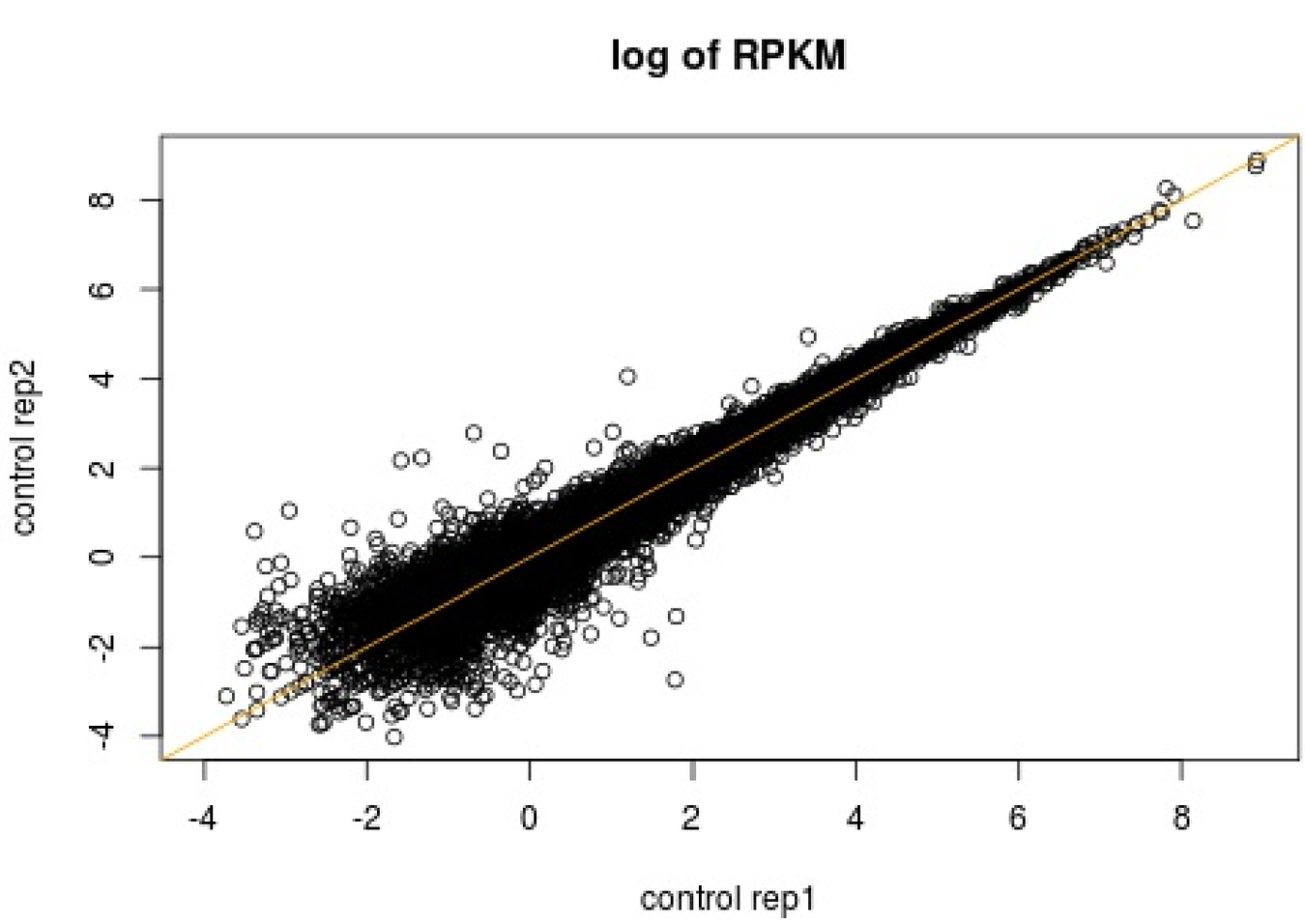
# scatter plot - raw counts

```
x.raw <- out$ck_rep1
y.raw <- out$ck_rep2
drange <- range(x.raw, y.raw, finite=T)
# plot a scatter plot between two samples
plot(x.raw, y.raw, main = "Raw counts",
     xlab="control rep1", ylab="control rep2",
     xlim = drange, ylim = drange)
abline(a = 0, b = 1, col = "orange")
```



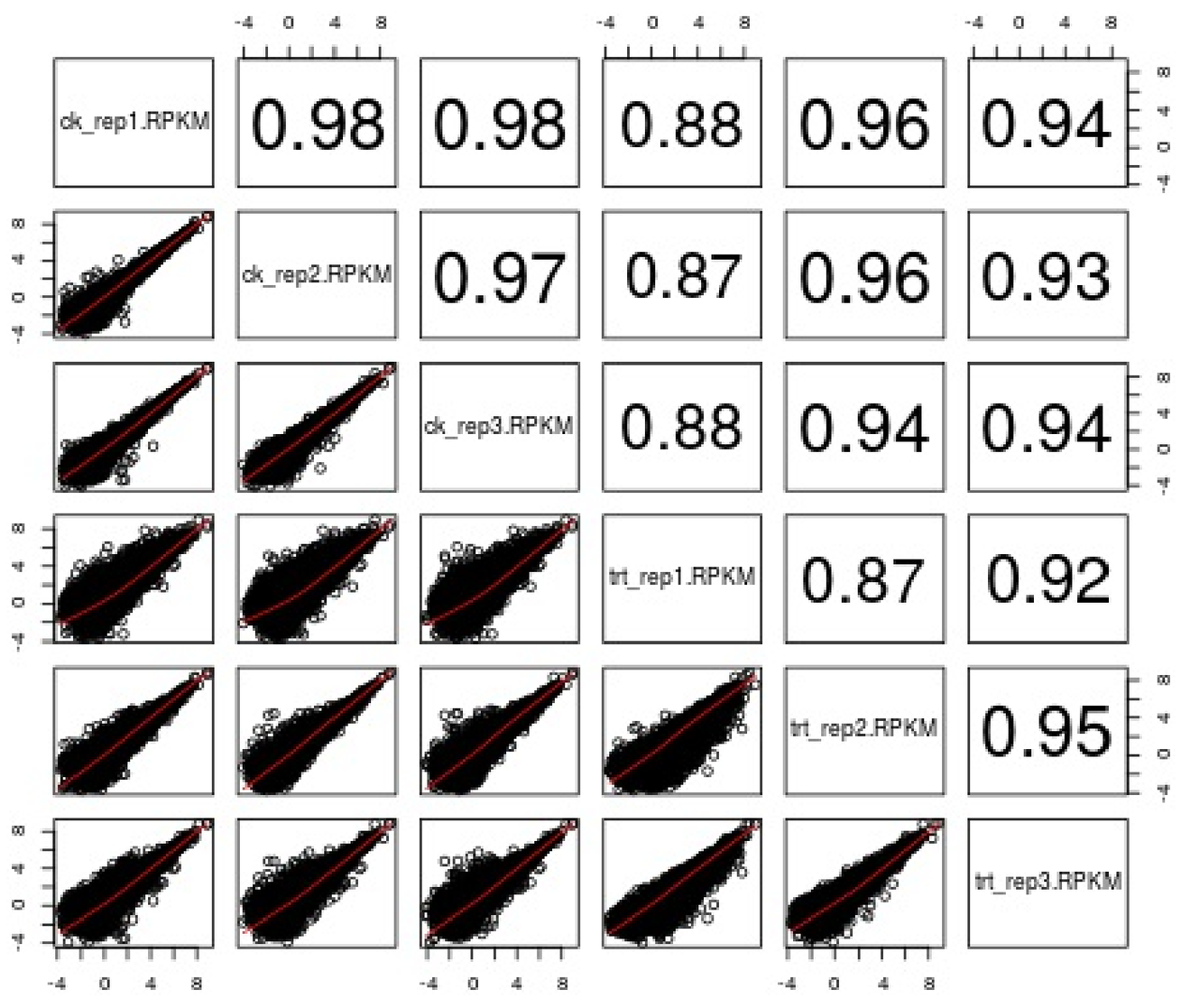
# scatter plot - RPKM

```
x <- log(out$ck_rep1.RPKM)
y <- log(out$ck_rep2.RPKM)
drange <- range(x, y, finite=T)
plot(x, y, main = "log of RPKM",
     xlab = "control rep1", ylab = "control rep2",
     xlim = drange, ylim = drange)
abline(a = 0, b = 1, col = "orange")
```



# pair-wise scatter plots

```
logrpkm <- log(out[, 9:14])
pairs(logrpkm, lower.panel=panel.smooth, upper.panel=panel.cor2)
```

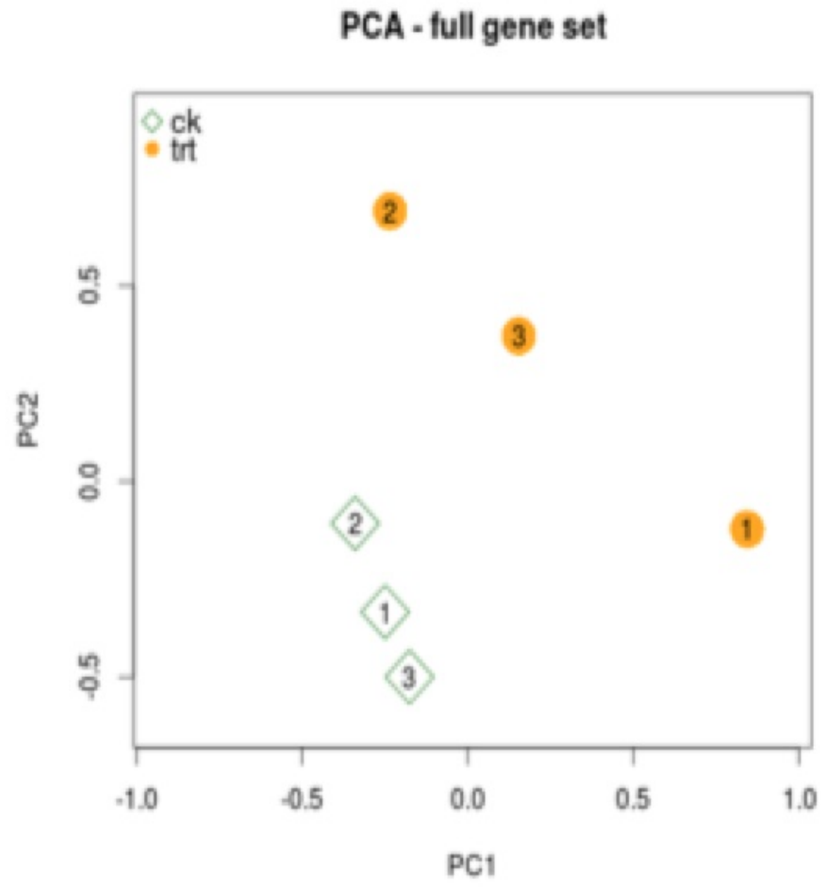


# Principal Component Analysis (PCA)

PCA is a mathematical algorithm that reduces the dimensionality of the data while retaining most of the variation in the data set.

| | Control | | | Treatment | | |
|---|---|---|---|---|---|---|
| Gene | Rep1 | Rep2 | Rep3 | Rep1 | Rep2 | Rep3 |
| 1 | 2679 | 2360 | 2573 | 2563 | 3398 | 3012 |
| 2 | 177 | 161 | 171 | 154 | 137 | 152 |
| 3 | 381 | 371 | 397 | 541 | 723 | 635 |
| ... | | | | | | |
| 20000 | 990 | 1073 | 1236 | 850 | 672 | 859 |

Normalized and standardized data



# function / module

You can write your own function:
fun_name <- function (…) { … }

```
gpa_improve <- function(gpa, rate) {
### gpa: a numeric vector for GPAs
### rate: percentage for the improvement
    new.gpa <- gpa * (1 + rate)
    new.gpa[new.gpa > 4] <- 4
    return(new.gpa)
}

### running the function
our.gpa <- c(3.8, 3.3, 2.8, 3.1)
gpa_improve(our.gpa, 0.1)
```

```
[1] 4.00 3.63 3.08 3.41
```

```
gpa_improve(our.gpa, 0.2)
```

```
[1] 4.00 3.96 3.36 3.72
```

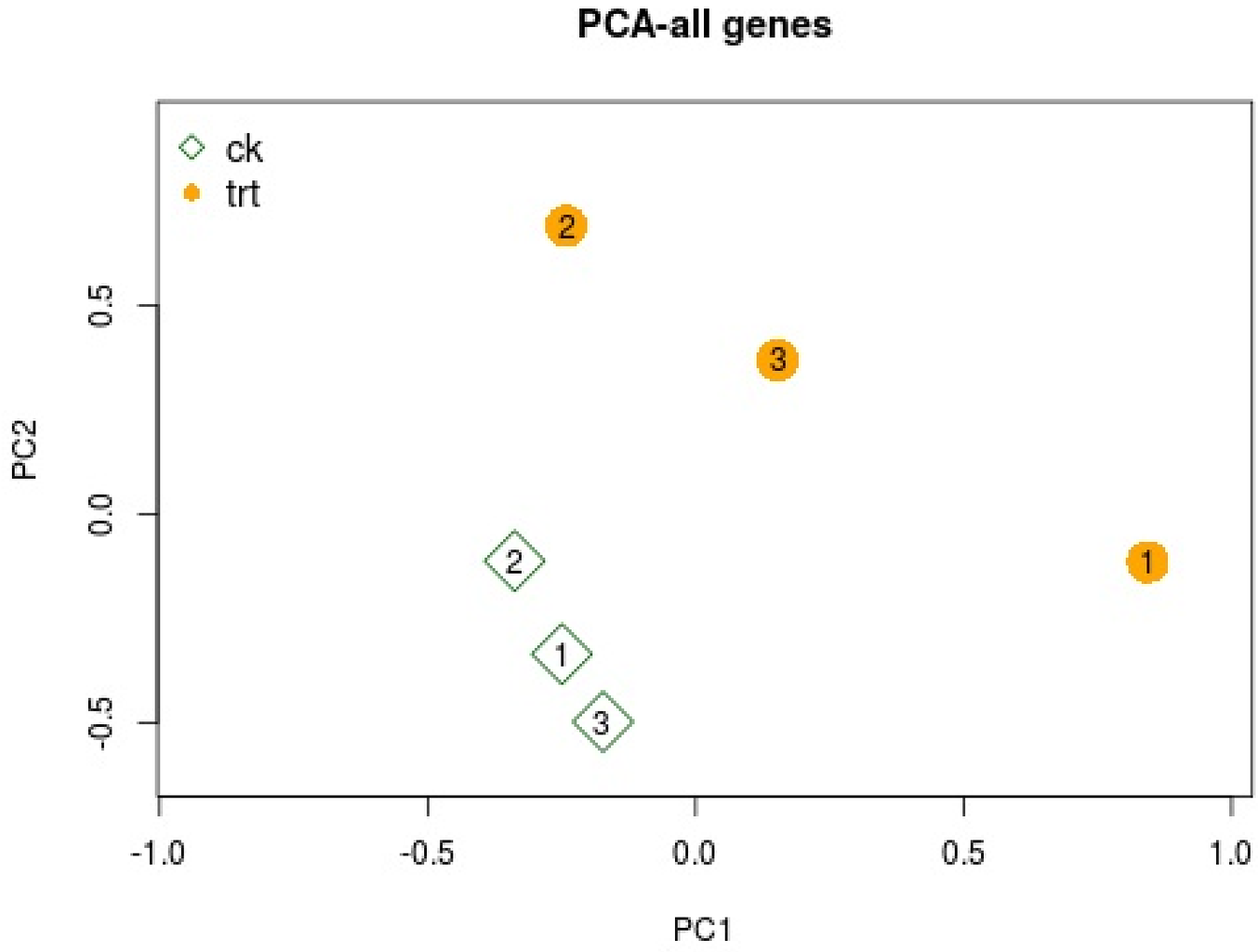
# PCA function

### principal component analysis and ploting

```
rnaseq.pca <- function(norm.data,
  norm.feature="RPKM",
    group.feature,
    title="",
  shape.code=NULL,
    mean.cutoff=0.1,
    colors=NULL,
    scaling=T, ...) {
    …
}
```

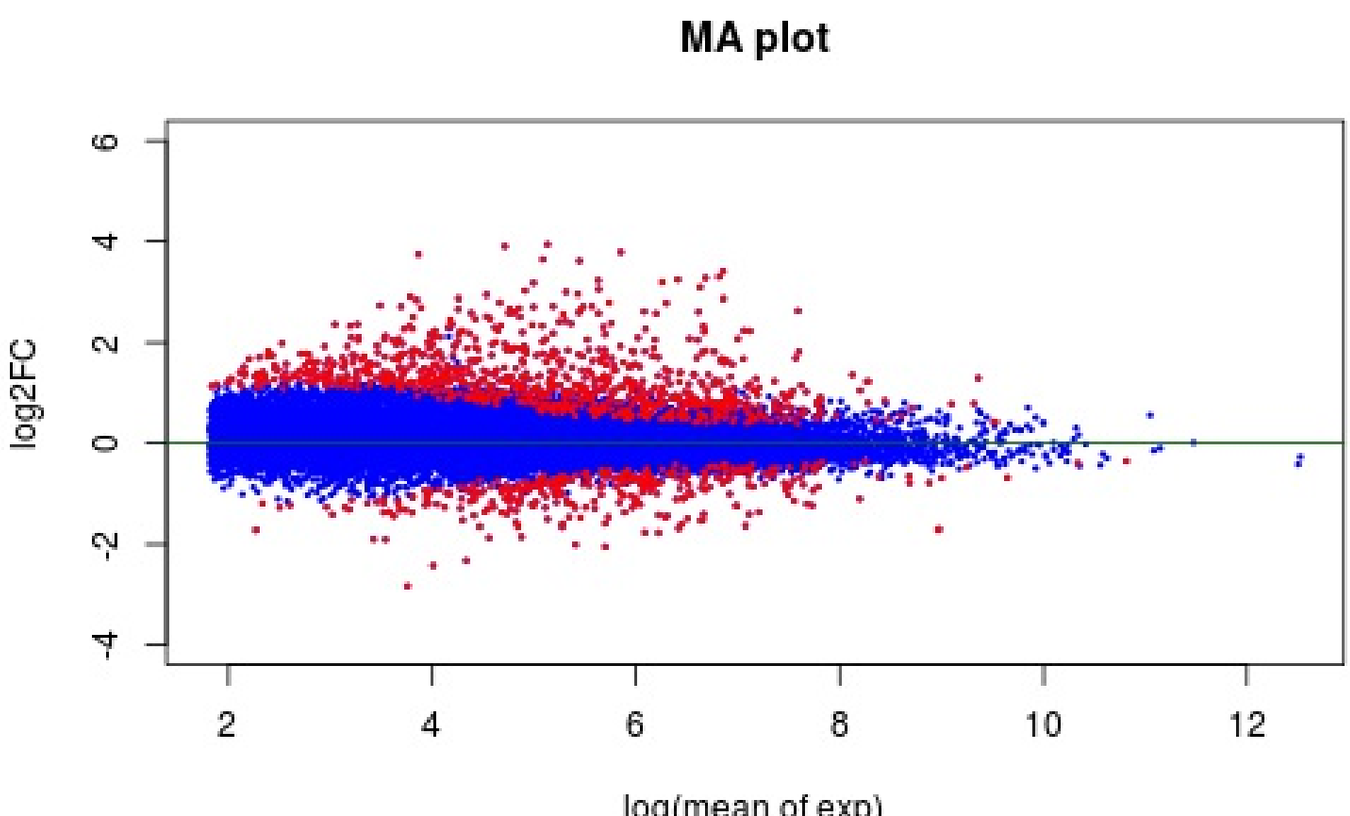
# PCA plotting

```
rnaseq.pca(out, norm.feature="RPKM", group.feature=c("ck", "trt"),
           cex=3, shape.code=c(5, 16), mean.cutoff=0.1, scaling = T,
           title="PCA-all genes", colors=c("dark green","orange"))
```



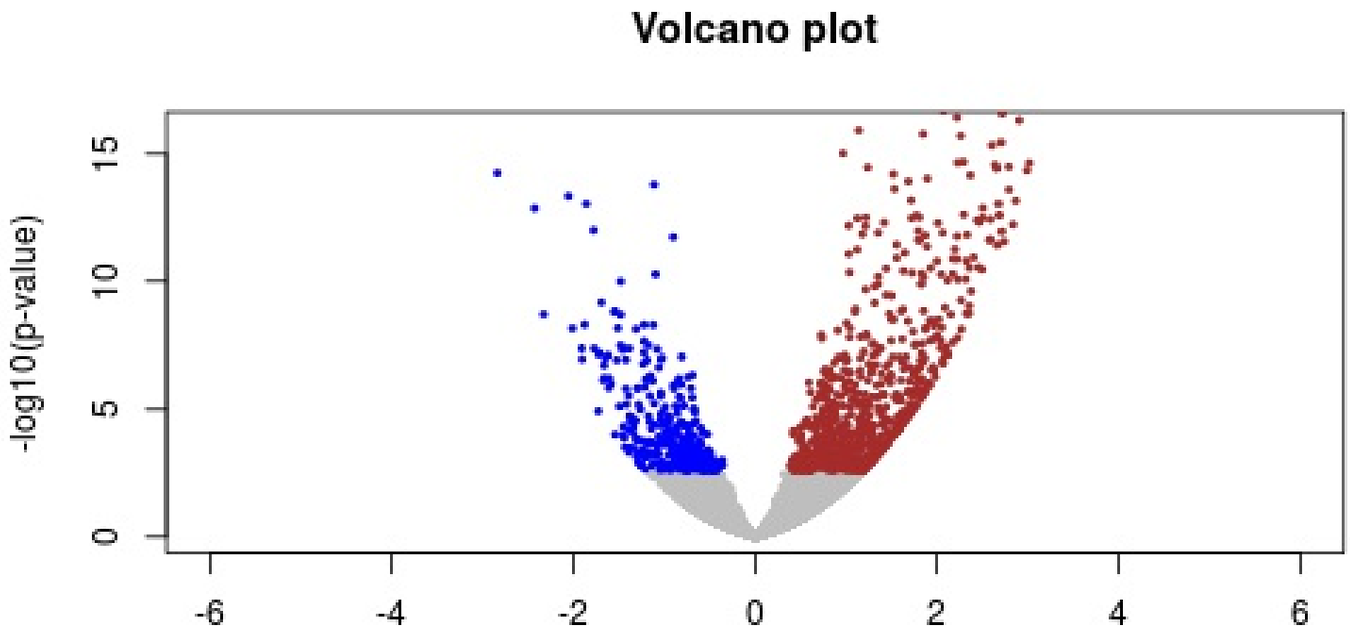
# MA plot

```
aval <- log(out$baseMean)
mval <- out$log2FoldChange
sig.set <- which(out$padj < 0.05)
plot(aval, mval, main="MA plot",
     xlab="log(mean of exp)", ylab="log2FC",
     ylim=c(-4, 6), pch=19, cex=0.3, col="blue")
# add points and a horizontal line
points(aval[sig.set], mval[sig.set], pch=19,cex=0.3,col="red")
abline(h = 0, cex = 1, col = "dark green")
```



# Volcano plot

```
log2val <- out$log2FoldChange  # log2 fold change
mlogP <- -log10(out$pvalue) # transformed p-values
### plot
plot(log2val, mlogP, main="Volcano plot",
     xlab="log2FC", ylab="-log10(p-value)",
     pch=19, cex=0.4,col="grey", xlim=c(-6,6), ylim=c(0,16))
### highlight
up <- which(out$padj<0.05 & log2val > 0) # up
down <- which(out$padj<0.05 & log2val < 0)  # down
points(log2val[up], mlogP[up], pch=19,cex=0.4,col="brown")
points(log2val[down], mlogP[down],pch=19,cex=0.4,col="blue")
```

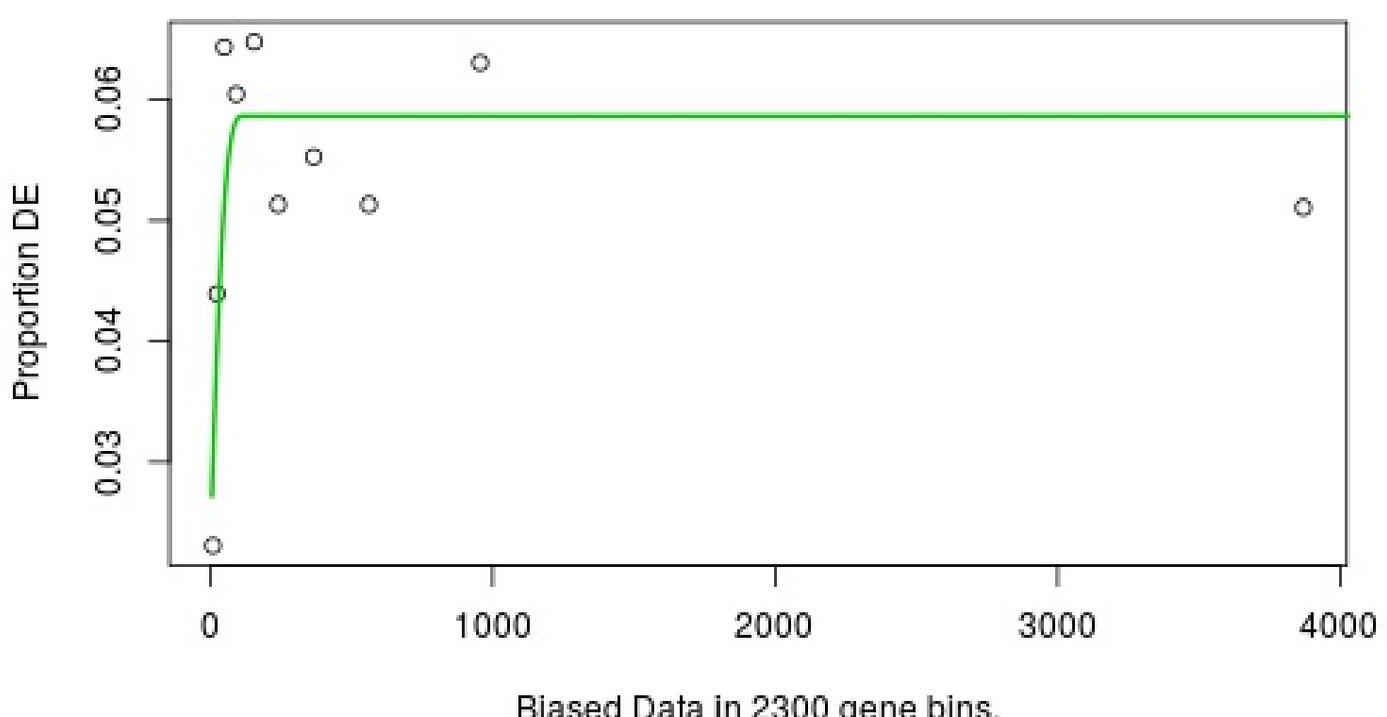


# Gene ontology (GO) enrichment analysis

- a gene and GO association table
- a list of all genes
- a list of significant genes
- mean or total gene read counts per gene (**optional**)

# GOSeq (I)

```
gdbf="http://129.130.89.83/tmp/public/RNASeq/RNASeq2018/data/go.txt"
godb <- read.delim(gdbf)
geneid <- as.character(out$Gene)  # gene vector
# a vector to indicate if the gene is DE (0 or 1)
de.vector <- as.integer(!is.na(out$padj) & out$padj < 0.05)
names(de.vector) <- geneid
countbias <- out$baseMean # total raw reads per gene
# bias fitting
pwf.counts <- nullp(DEgenes=de.vector, bias.data=countbias)
```



# GOSeq (II)

```
go <- goseq(pwf=pwf.counts, gene2cat=godb, method="Sampling",
            repcnt = 1000, use_genes_without_cat = F)  # GO enrichmer
```

```
head(go, 1)
```

```
     category over_represented_pvalue under_represented_pvalue numDEInCat
208 GO:0004175                0.000999001                     1         16
    numInCat                       term ontology
208       31 endopeptidase activity       MF
```

```
example.go <- GOTERM[["GO:0004175"]]  # GO information
Definition(example.go)  # return GO definition
```

```
[1] "Catalysis of the hydrolysis of internal, alpha-peptide bonds in a polype
```

# Summary of the analyzing procedure

1. Read counts per gene
2. DE analysis based on the experimental design
3. Examine results (p-value distribution, number of significant genes)
4. Gene Ontology enrichment test

# Contact information

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# Bioinformatics Applications

PLPTH813, Spring 2019