# Lab: Differential Expression via RNA-Seq Analysis

## Genomic Technologies Workshop 2022 (PLPTH885)

Sanzhen Liu

6/8/2022

## Outline

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

## Course webpage

RNA-seq DE analysis

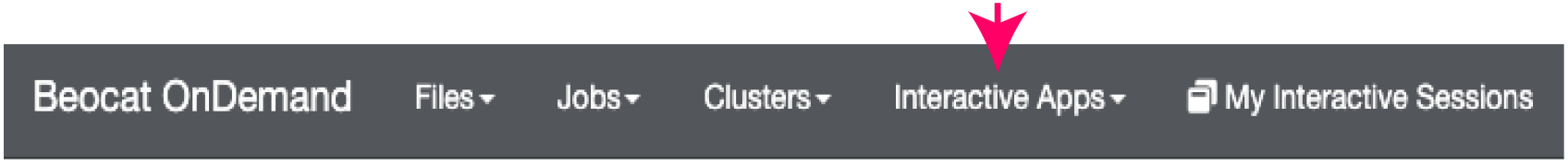
## Lab co-teacher

Dr. Guifang Lin

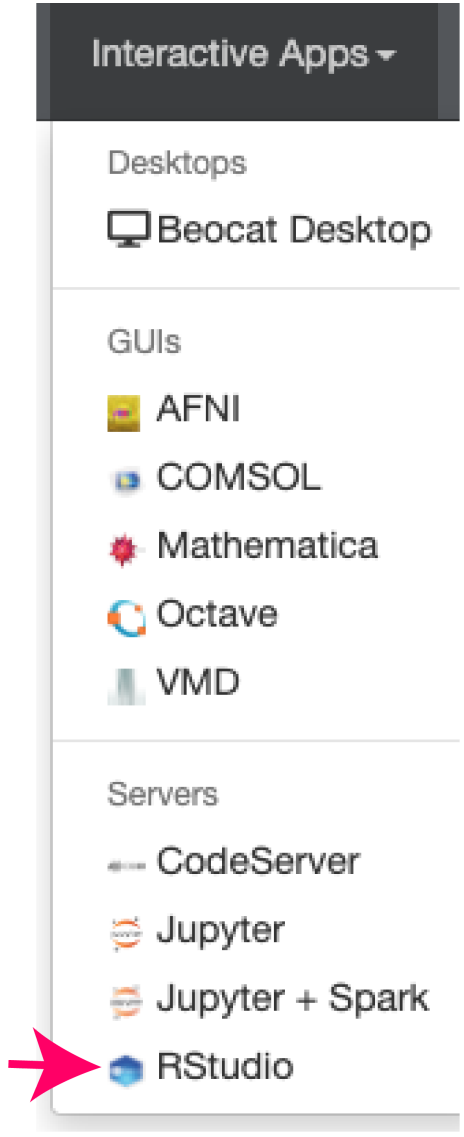
# OnDemand at Beocat

ondemand

login with eID



# Select RStudio



# Request resources

**R version**

4.0.0 (foss-2020a)

This defines the version of R you want to load.

**Number of hours**

3

**Number of cores**

1

**Amount of memory**

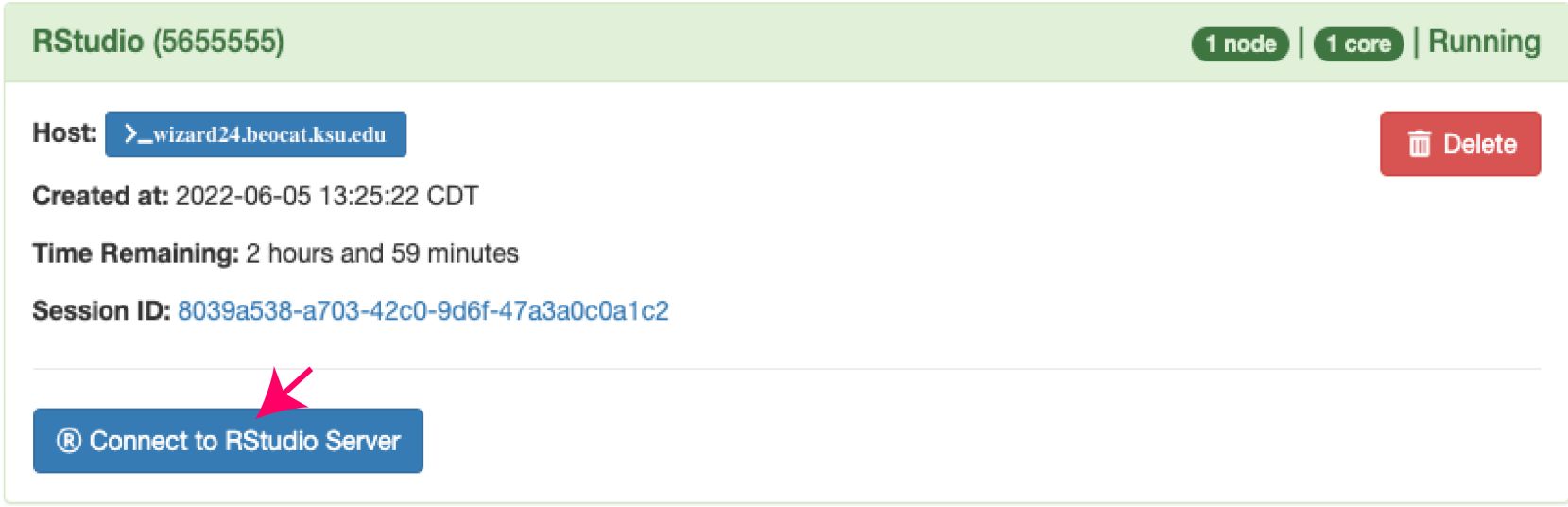
32

The amount of memory (in GB) needed for the whole job

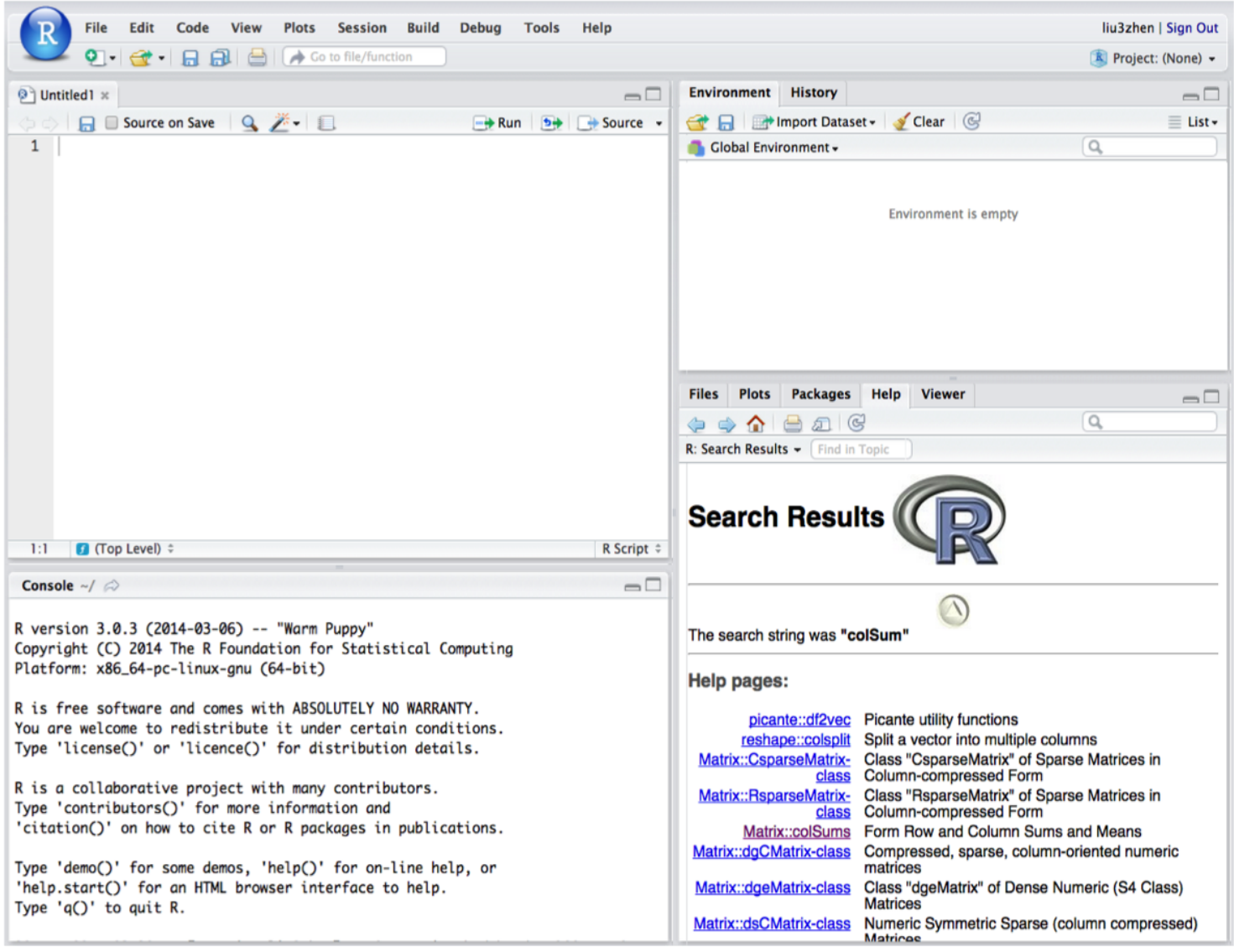
**Job Type**

normal

# Connect to RStudio



# Rstudio interface



# Package installation

```
if (!require("BiocManager", quietly=T))
  install.packages("BiocManager")

if (!require("DESeq2", quietly=T))
  BiocManager::install("DESeq2") # DESeq2

if (!require("goseq", quietly=T))
  BiocManager::install("goseq") # GOSeq

if (!require("GO.db", quietly=T))
  BiocManager::install("GO.db", force=T) # GO.db
```

# preload modules

```
pls=paste0(data_url, "/utils/load.R")
source(pls)
```

- panel.cor2
- rnaseq.pca
- normalization

codes

# Read expression data (Read counts per gene)

```
rc <- paste0(data_url, "/data/rc.txt")
grc <- read.delim(rc)
nrow(grc)  # the number of rows/lines
```

```
[1] 22697
```

| Gene | ExonSize | ck_rep1 | ck_rep2 | ck_rep3 | trt_rep1 | trt_rep2 | trt_rep |
|---|---|---|---|---|---|---|---|
| AC147602.5_FG004 | 483 | 5480 | 6075 | 5934 | 3370 | 5784 | 643 |

| | Gene | ExonSize | ck_rep1 | ck_rep2 | ck_rep3 | trt_rep1 | trt_rep2 | t |
|---|---|---|---|---|---|---|---|---|
| 22697 | GRMZM5G899985 | 615 | 267 | 327 | 348 | 83 | 342 | |

# RPKM normalization

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

| Gene | ExonSize | ck_rep1 | ck_rep2 | ck_rep3 | trt_rep1 | trt_rep2 | trt_rep |
|---|---|---|---|---|---|---|---|
| AC147602.5_FG004 | 483 | 5480 | 6075 | 5934 | 3370 | 5784 | 643 |
| AC148152.3_FG005 | 1422 | 187 | 295 | 377 | 169 | 158 | 56 |

# data organization for DESeq2

- count information

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

| ck_rep1 | ck_rep2 | ck_rep3 | trt_rep1 | trt_rep2 | trt_rep3 |
|---|---|---|---|---|---|
| 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)
```

| | 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)
```

| Gene | ExonSize | ck_rep1 | ck_rep2 | ck_rep3 | trt_rep1 | trt_rep2 |
|---|---|---|---|---|---|---|
| AC147602.5_FG004 | 483 | 5480 | 6075 | 5934 | 3370 | 5784 |
| AC148152.3_FG005 | 1422 | 187 | 295 | 377 | 169 | 158 |

| trt_rep3 | ck_rep1.RPKM | ck_rep2.RPKM | ck_rep3.RPKM | trt_rep1.RPKM |
|---|---|---|---|---|
| 6432 | 854.123 | 895.760 | 904.373 | 567.493 |
| 563 | 9.900 | 14.775 | 19.516 | 9.666 |

| trt_rep2.RPKM | trt_rep3.RPKM | baseMean | log2FoldChange | pvalue | padj |
|---|---|---|---|---|---|
| 915.326 | 916.971 | 5441.6579 | -0.1490702 | 0.4642180 | 0.8049379 |
| 8.493 | 27.262 | 285.5493 | 0.0431574 | 0.9258171 | 0.9845702 |

### significant gene sets at different FDRs

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

```
[1] 1261
```

### problem

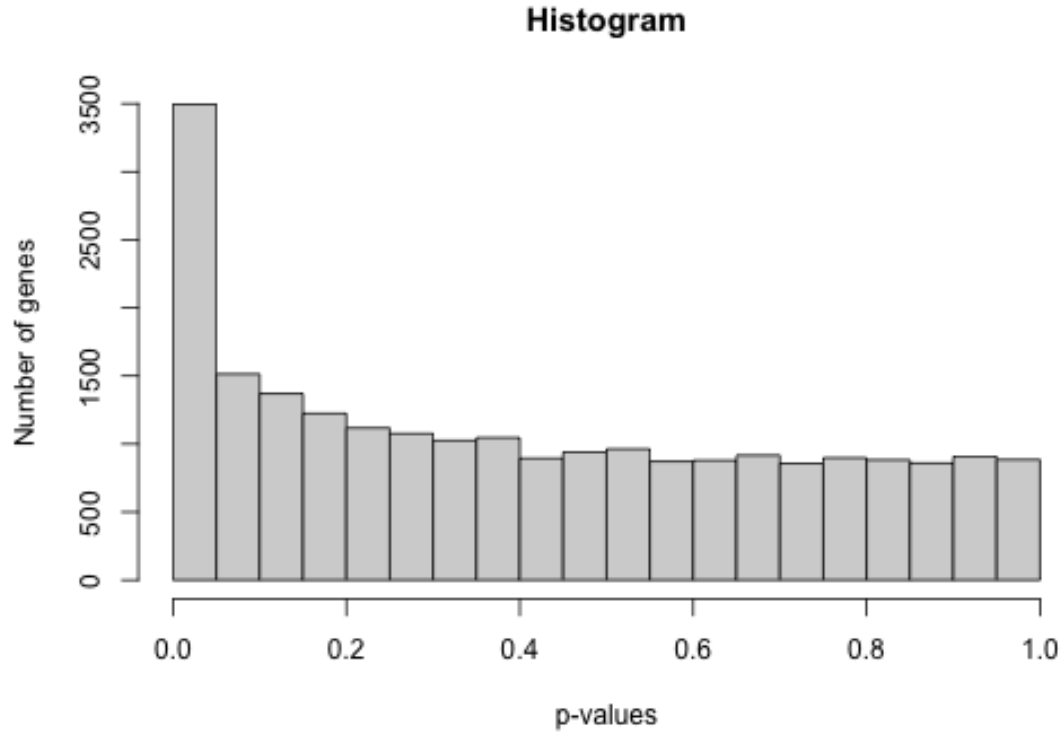
Please revise the code to calculate the number of significant genes with the FDR smaller than 10% and 15%?

# significantly DEG

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

# p-value histogram

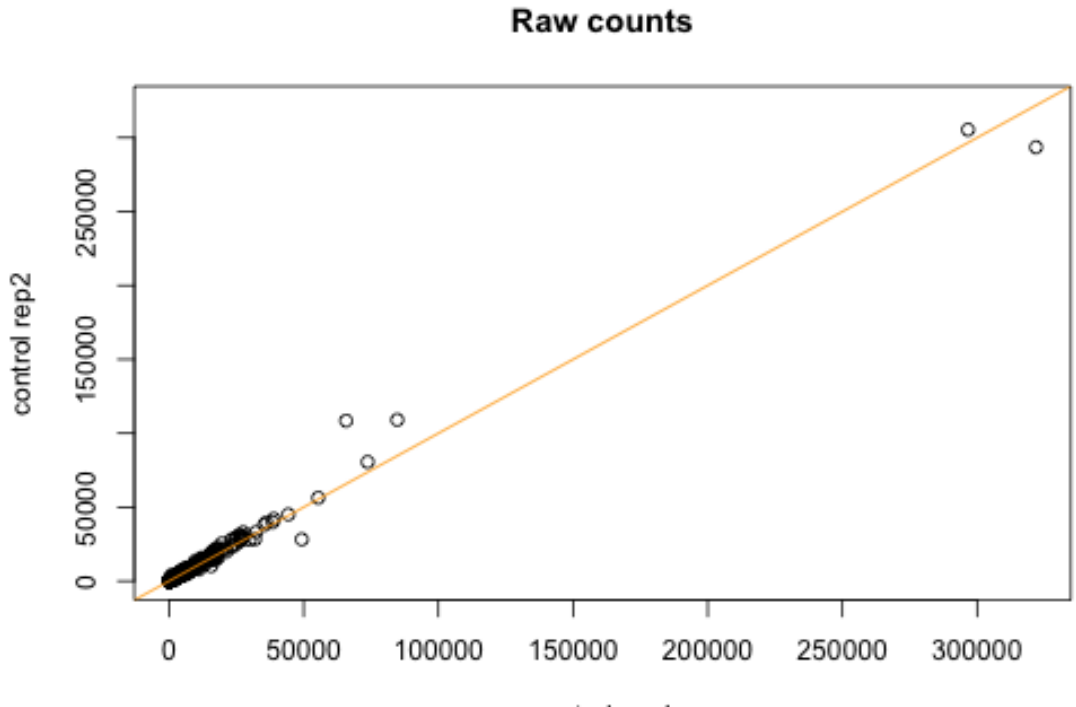
```
pvals <- out$pvalue
hist(pvals, main="Histogram",xlab="p-values",ylab="Number of genes")
```




**problem**
Please modify the plot code to change the figure title to "DE"

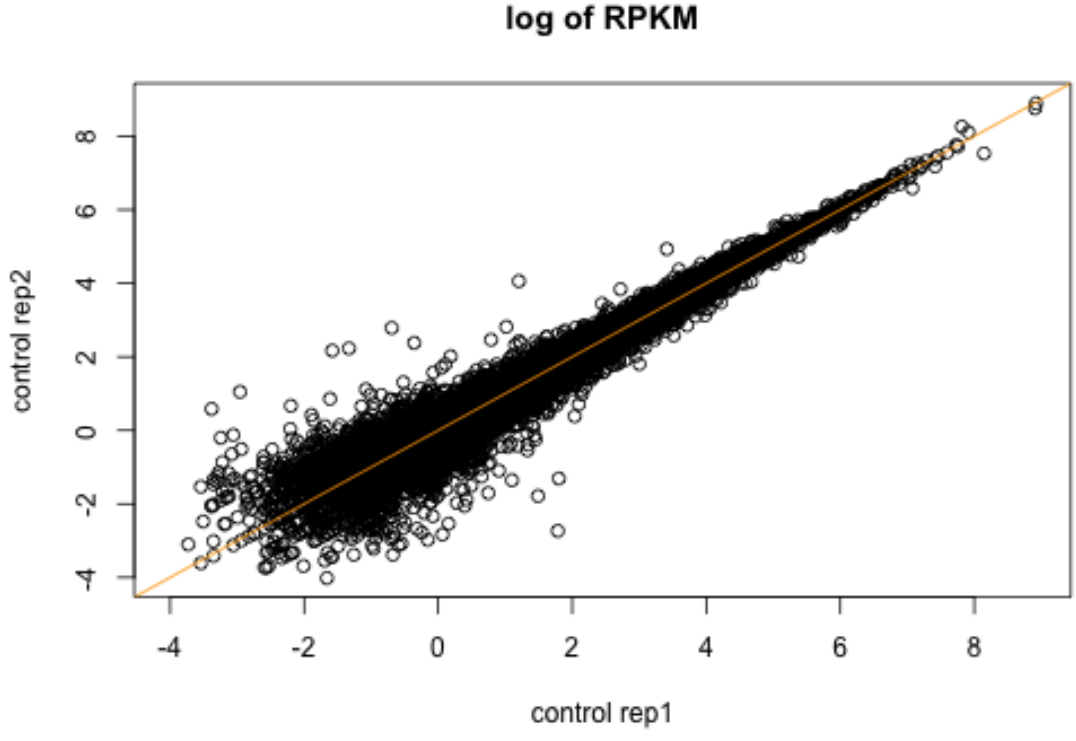
# scatter plot - raw counts

```
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")
```



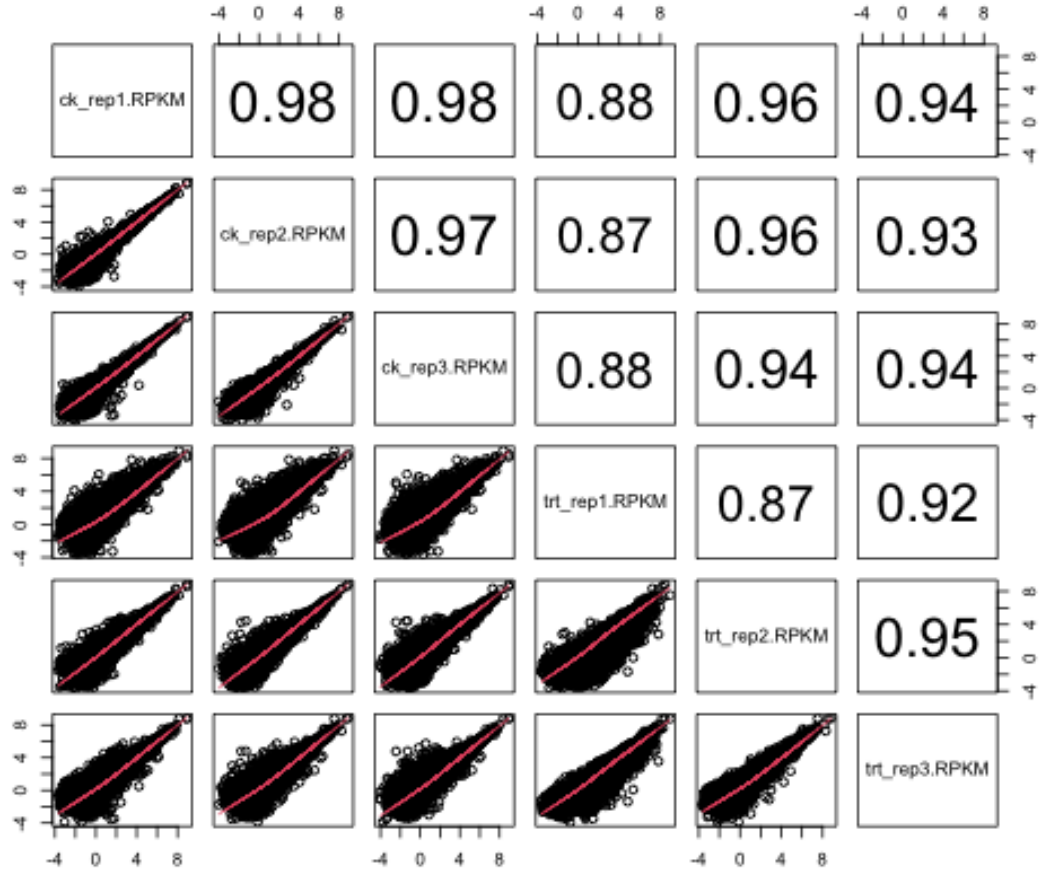
# scatter plot - RPKM

```
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)
```

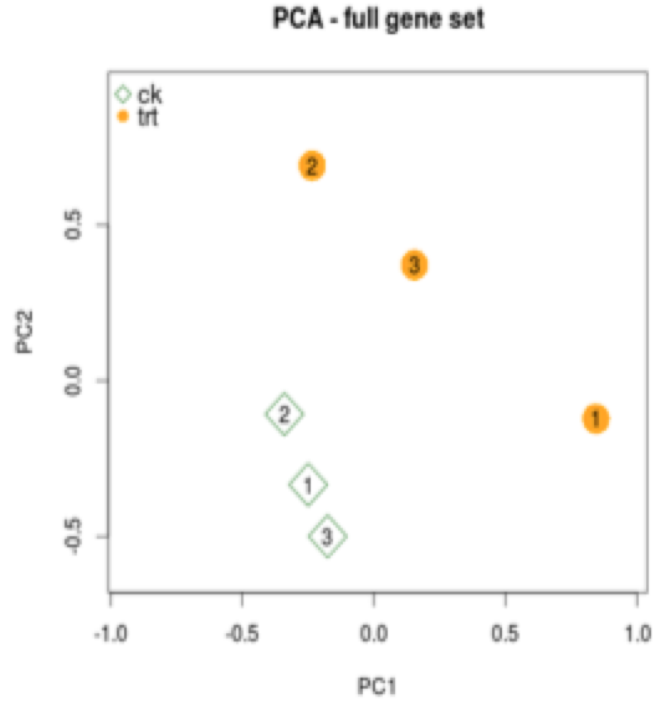


# Principal Component Analysis (PCA)

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
```

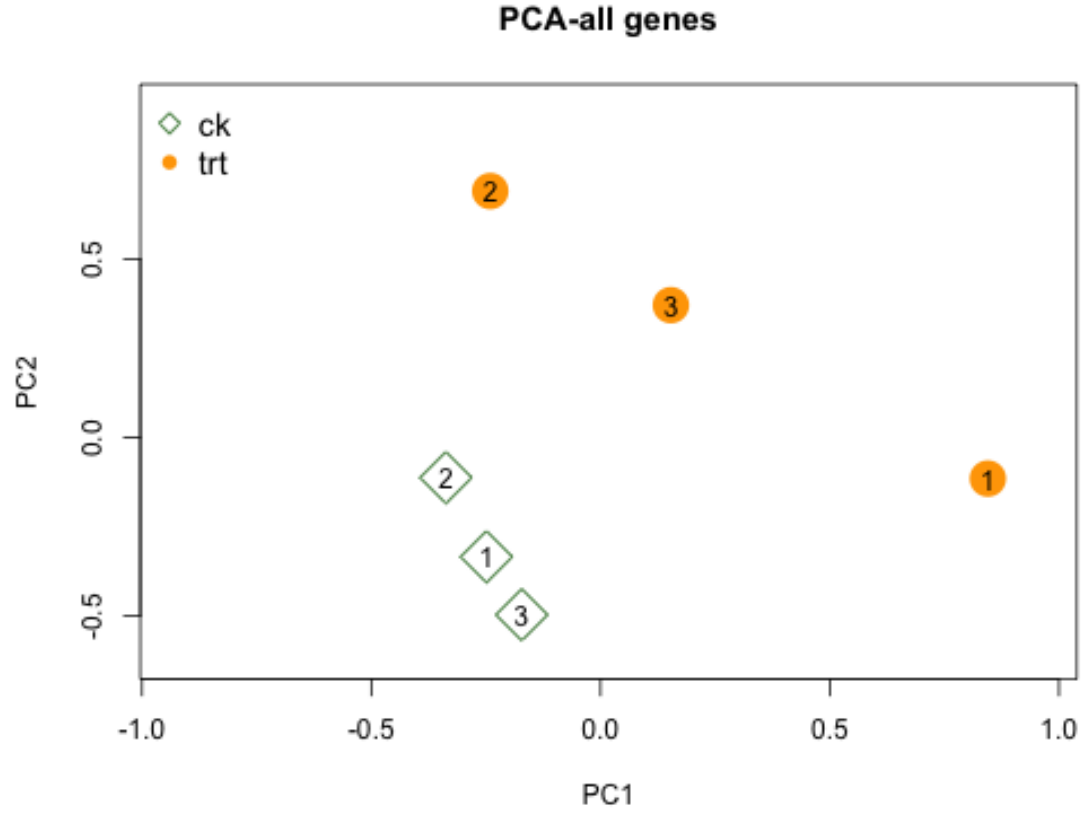
# PCA function

**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, ...) {
    …
}
```

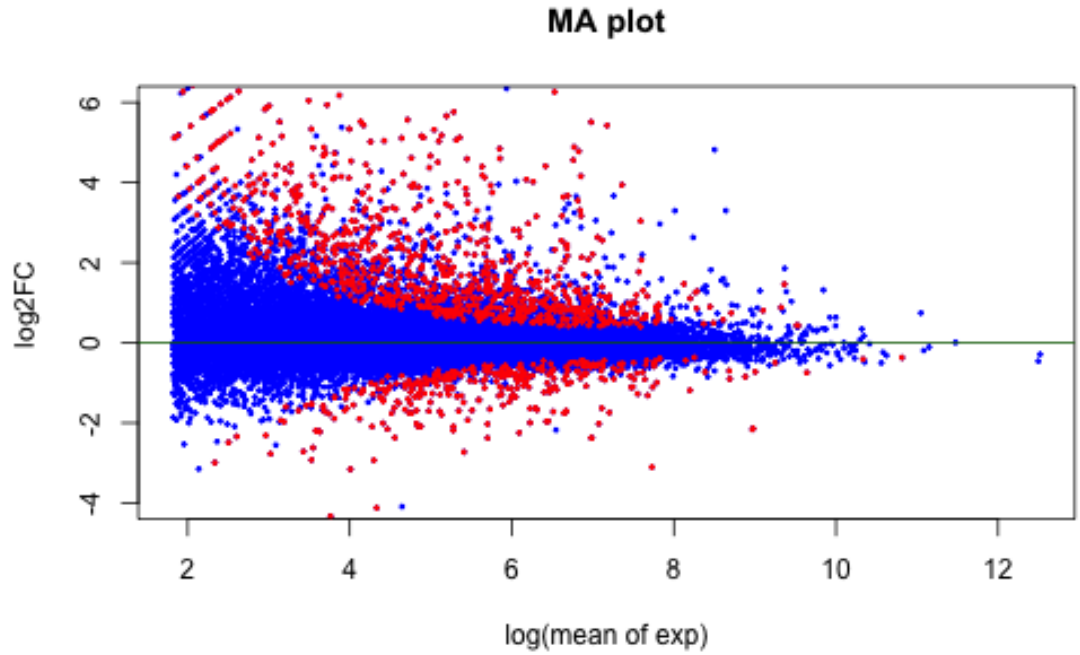
# PCA plotting

```
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"))
```



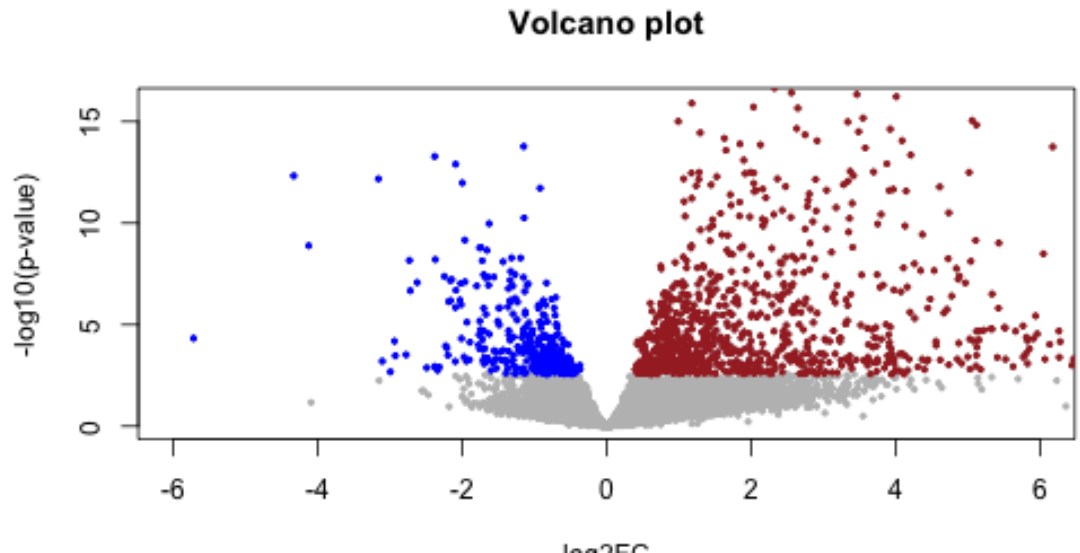
# MA plot

```
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")
```

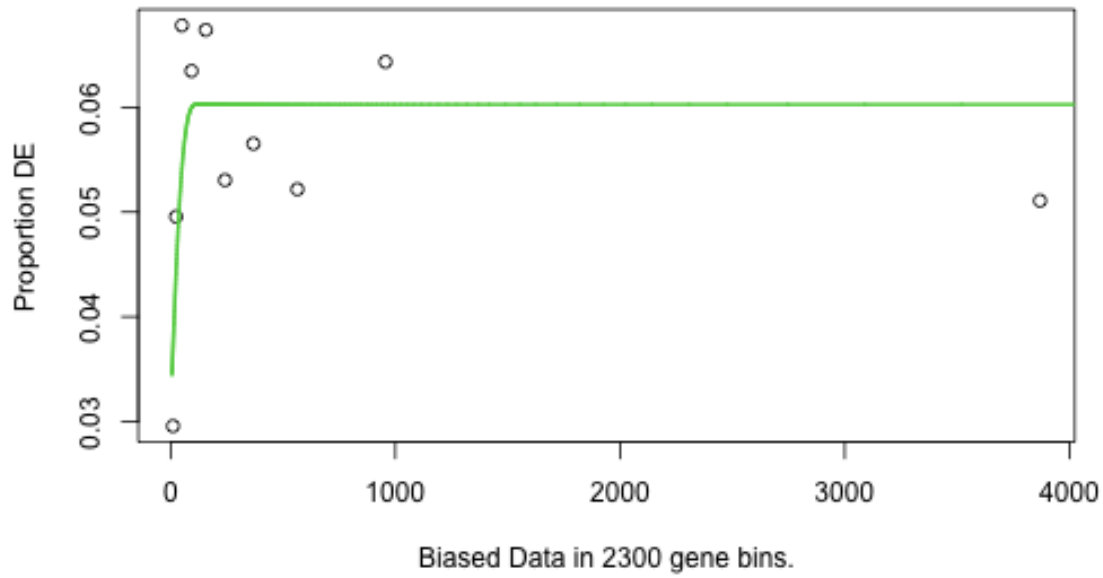


# Gene ontology (GO) enrichment analysis

- 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=paste0(data_url, "/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 enrichment
```

| | **208** |
|---|---|
| category | GO:0004175 |
| over_represented_pvalue | 0.000999001 |
| under_represented_pvalue | 1 |
| numDEInCat | 16 |
| numInCat | 31 |
| term | endopeptidase activity |
| ontology | 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

DESeq2 tutorial

# Contact information

Sanzhen Liu
Plant Pathology
4729 Throckmorton Plant Sciences Center
Manhattan, KS 66506-5502
phone: 785-532-1379

liu3zhen@ksu.edu
twitter: liu3zhen

# Bioinformatics Applications

PLPTH813, Spring 2022