RNA-Seq Data Analysis 1

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BIOS 6660

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BASH Scripts from Last Time

> cd /home/teststudent/hw6Test/code

> chmod -R u+wrx *

> ./countRawReads.sh

countRawReads.sh | rawReadCounts.txt

```
1  /path/A1_SO1_S1_R1_001.fastq.gz 150 27014975
2  /path/A1_SO1_S1_R2_001.fastq.gz 150 27014975
3  /path/A2_SO2_S2_R1_001.fastq.gz 150 26326103
4  /path/A2_SO2_S2_R2_001.fastq.gz 150 26326103
5  /path/A3_SO3_S3_R1_001.fastq.gz 150 29626672
```

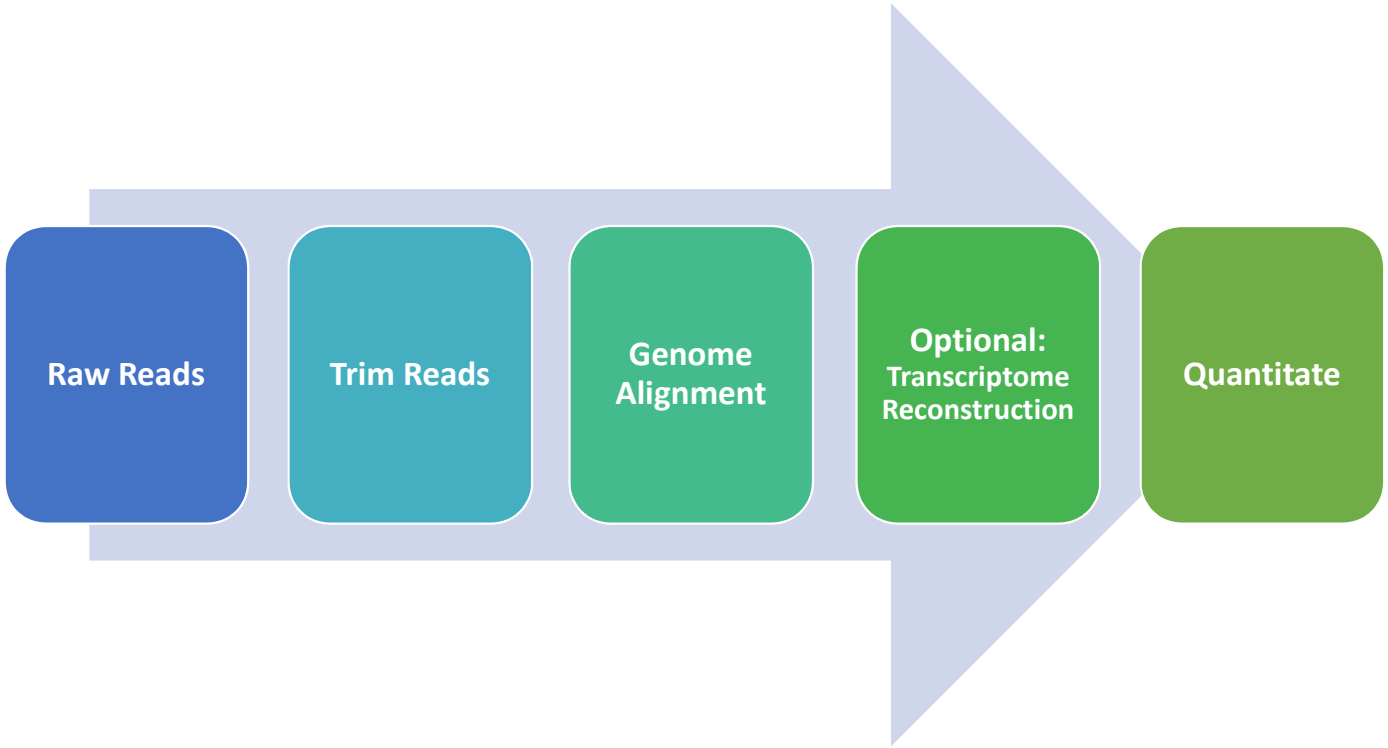
Yampa: /BIOS6660/Homework6/exampleProgramsAndReports

countRawReads.sh

```
1  #!/bin/bash
2  FILES1=/data/home/vanderll/Britt/TagSeq.20181206/rawReads/*.gz
3  for f in $FILES1
4  do
5      gunzip -c $f | awk '/@D00289/ {getline; print length($0)}' | awk -v
       sample="$f" '{sum+=$1} END {print sample,sum/NR,NR}' >> /data/home/
       vanderll/Britt/TagSeq.20181206/data/rawReadCounts.txt
6  done
7
```

Overview From Last Time

- We went from raw RNA-Seq reads to a nice matrix of counts
  - Rows = genes
  - Columns = samples
- Now all the work we will do is in R!

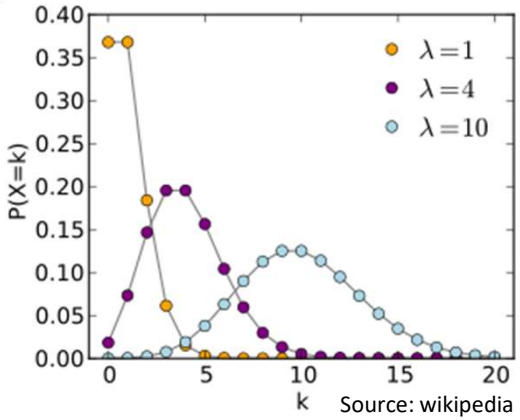


What Type of Data Do We Have?

- We have a matrix of **counts**

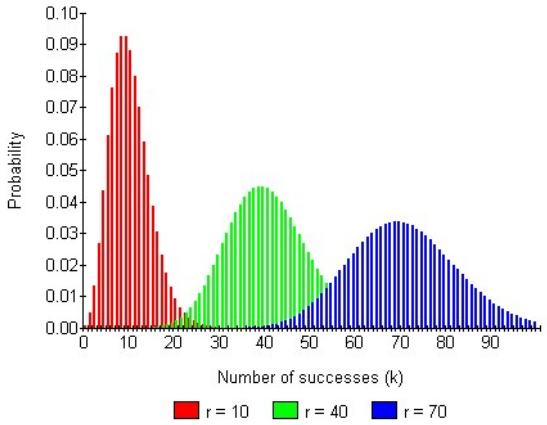
1. Poisson
   - Assuming mean = variance
   - 1 parameter (λ) (Figure k = # occurrences)
2. Negative Binomial
   - Does not make mean = variance assumption
   - 2 parameters:
     - *r* is the total # successes
     - *p* is the probability of success of a single trial

Poisson PDF



Source: wikipedia

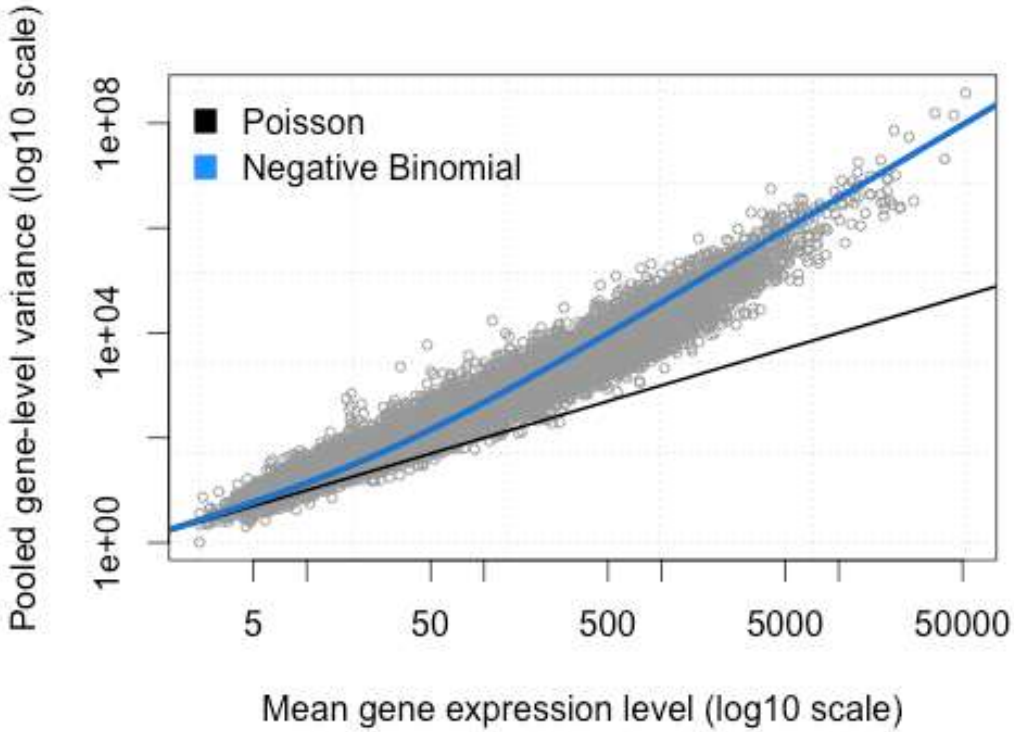
Negative Binomial PDF (p = 0.5)



Source: systomath

Negative Binomial

- Variance of counts is generally greater than their mean, especially for genes expressed at a higher level.
- This is called **overdispersion**
- 1/r is also sometimes called the “dispersion” or “shape” parameter
- Negative Binomial current choice



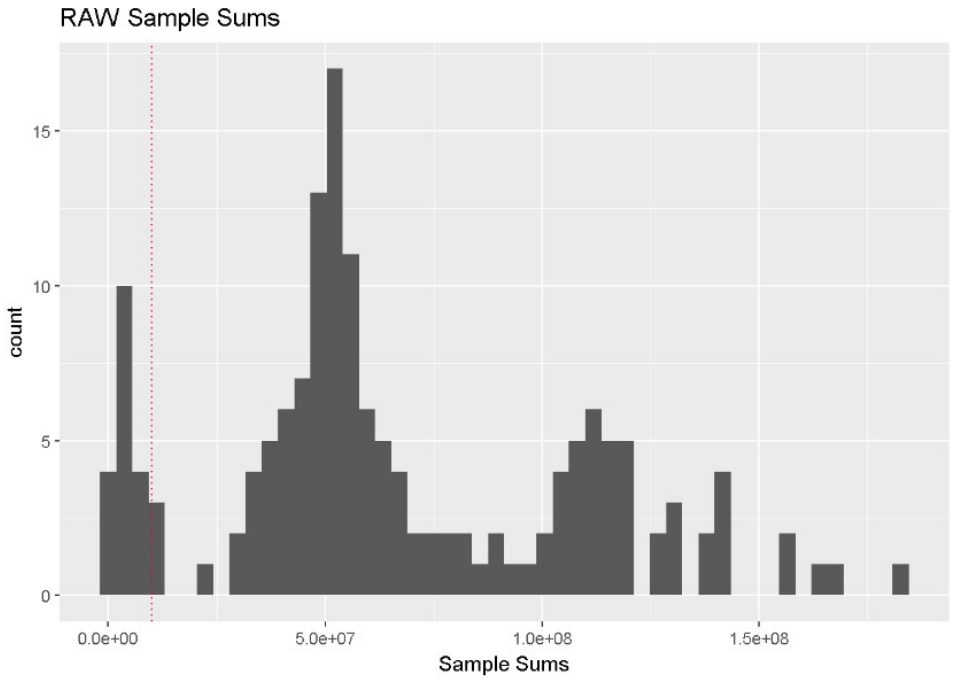
Source: RECount Project

Exploring Data Through Visualization

- Normally, plot your data in a histogram and see what it looks like.
- This is not so easy with any ‘Omics data as you have large number of features (~20,000 genes)
- Next few slides I’m going to walk through some visualizations I look at when analyzing a dataset

Visualizing Data – Library Sizes

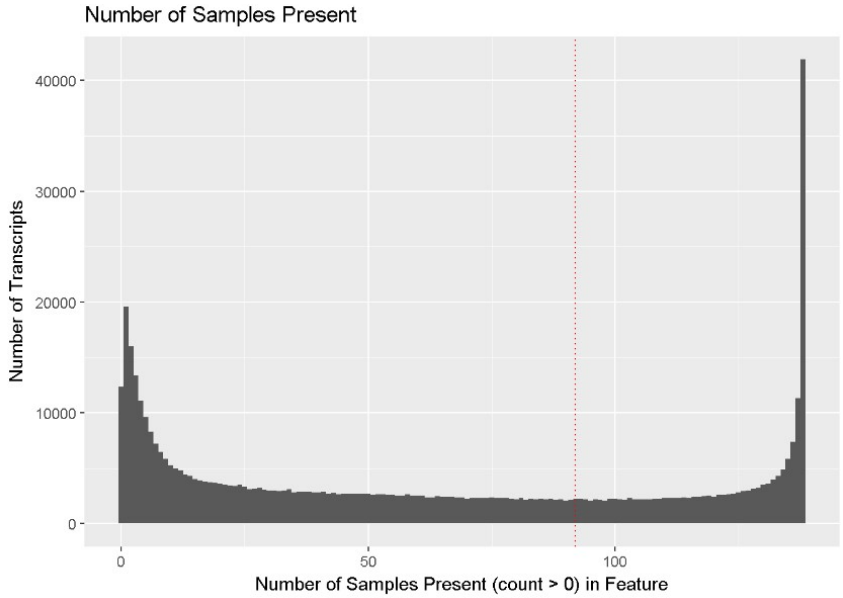
```
qplot(countSums, geom="histogram",main = "RAW Sample Sums", xlab = "Sample Sums", bins=50) + geom_vline(xintercept=10000000, col="red",linetype="dotted")
```



- ggplot2 is a great tool
- Sample-level QC
- In this example I had 158 samples, so it was difficult to look at a table of library sizes

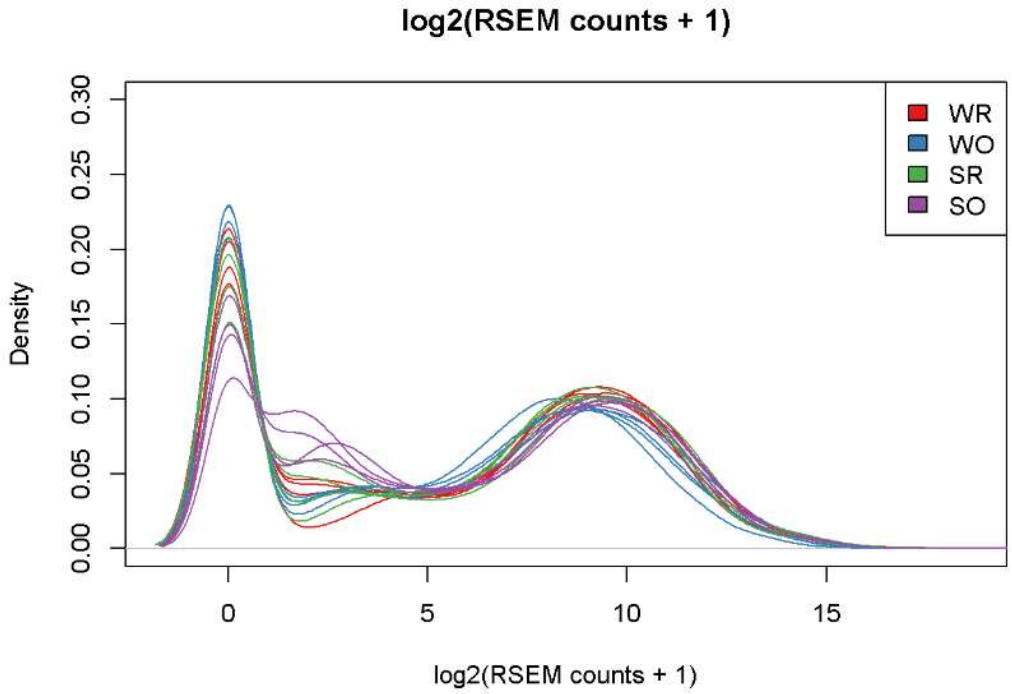
Visualizing Data – Number of 0 Counts

```
presentCount = apply(counts3, 1, function(a) sum(a>0))

qplot(presentCount, geom="histogram",main = "Number of Samples Present", xlab = "Number of Samples Present (coun
t > 0) in Feature",  binwidth = 1) + geom_vline(xintercept=(2/3)*ncol(counts3), col="red",linetype="dotted") + yl
ab("Number of Transcripts")
```



- Feature-level QC
- At the VERY least, you want to remove genes (isoforms) from you dataset that have 0 counts across ALL samples
- Detection Above BackGround (DABG)
- Debated topic

Visualizing Data- Density Plots Example 1



- Density plot for each individual sample
- Colored by group
- Big Spike around 0
  - DABG 0 counts in all samples
- Larger hump later on

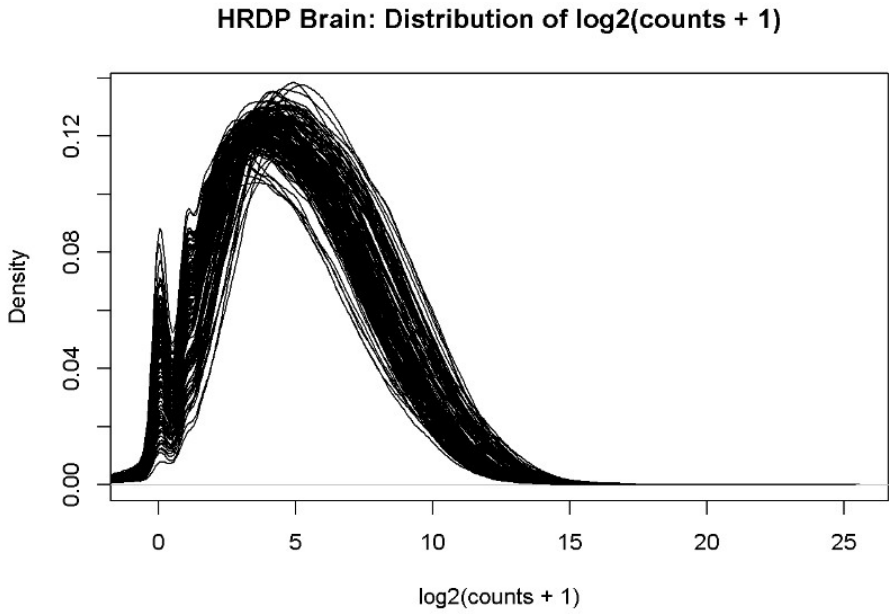
Visualizing Data- Density Plots Example 1 Code

library(RColorBrewer)

```
colorsWant = brewer.pal(9, "Set1")
#Visualizing distributions of samples
par(mfrow=c(1,1))
plot(density(log2(counts2[,1]+1)), main="log2(RSEM counts + 1)", xlab="log2(RSEM counts + 1)", ylim=c
(0,0.3), col=colorsWant[1])
for(i in 2:4) {lines (density(log2(counts2[,i]+1)), col=colorsWant[1])}
for(i in 5:8) {lines (density(log2(counts2[,i]+1)), col=colorsWant[2])}
for(i in 9:12) {lines (density(log2(counts2[,i]+1)), col=colorsWant[3])}
for(i in 13:16) {lines (density(log2(counts2[,i]+1)), col=colorsWant[4])}
legend("topright", c("WR", "WO", "SR", "SO"), fill=colorsWant)
```

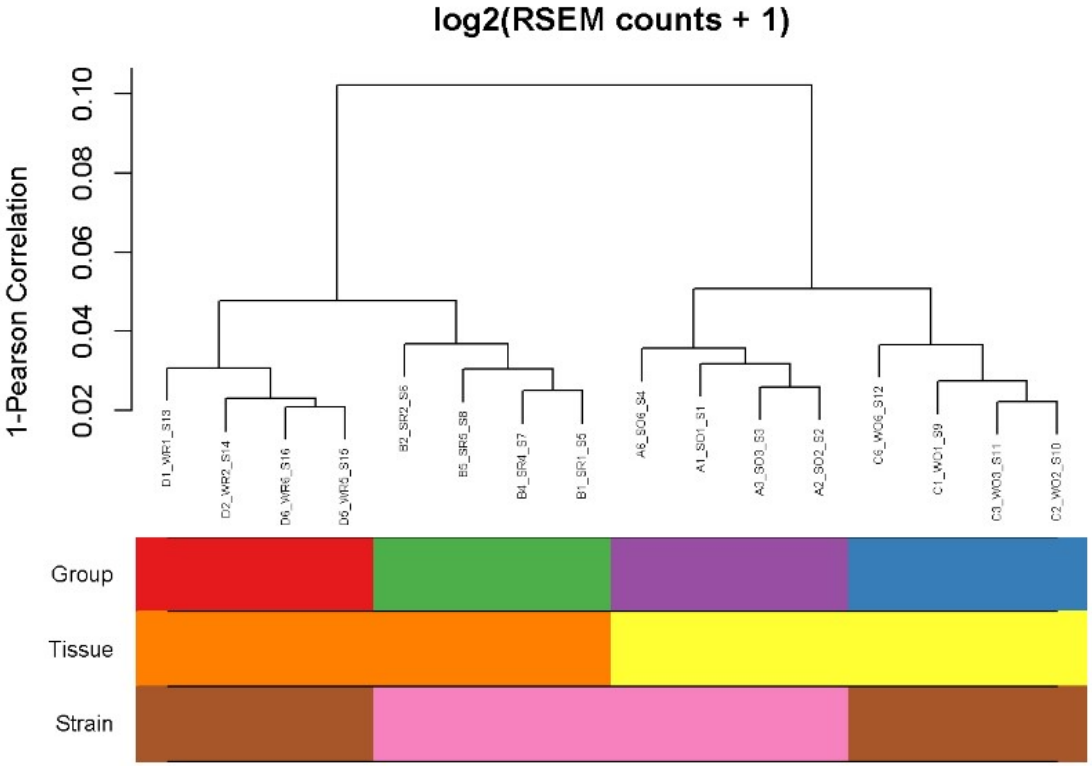
Visualizing Data- Density Plots Example 2

```
plot(density(log2(counts5[,1]+1)), main="HRDP Brain: Distribution of log2(counts + 1)", xlab="log2(co
unts + 1)")
for(i in 2:ncol(counts5)) {lines (density(log2(counts5[,i])))}
```



- Still have a somewhat same shape
- Harsher feature selection on DABG calls
- Smaller spike near 0
- Larger hump later on

Visualizing Data – Dendrogram Example 1



- y-axis is the distance (or dissimilarity)
  - 1-correlation
- Clustering by group really well
  - This is an animal model
- Tissue explains the main distance
  - First break at 0.1
- Strain explains the second most distance
  - Second breaks around 0.05
- Great tool for experiments with small number samples

Visualizing Data- Dendrogram Example 1 Code

library(WGCNA)

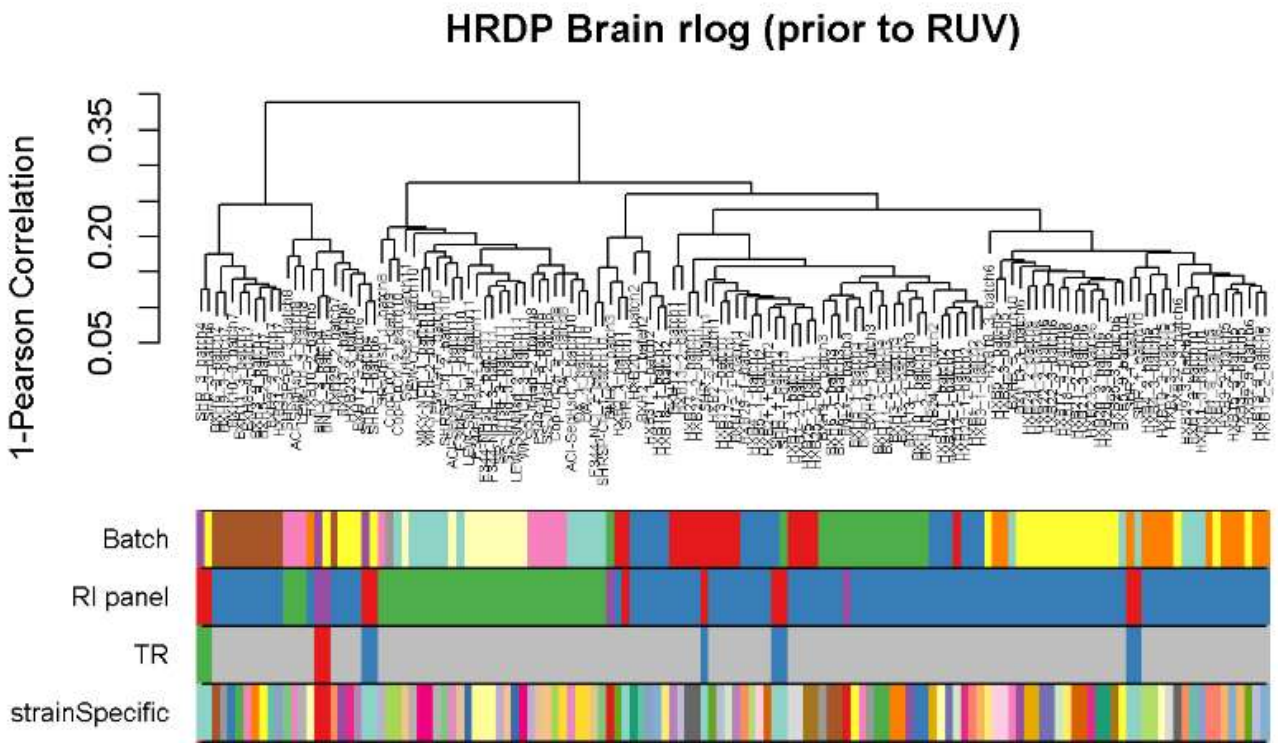
```
counts.hclust = hclust(as.dist(1-cor(log2(counts2+1), method="pearson")))
group.hclust = substr(sapply(strsplit(counts.hclust$labels, split="_", fixed=TRUE), "[[", 2), 1, 2)

colors4plot=c()
for(i in 1:length(group.hclust)){
  if(group.hclust[i]=="WR"){colors4plot[i]=colorsWant[1]}
  if(group.hclust[i]=="WO"){colors4plot[i]=colorsWant[2]}
  if(group.hclust[i]=="SR"){colors4plot[i]=colorsWant[3]}
  if(group.hclust[i]=="SO"){colors4plot[i]=colorsWant[4]}
  }
colors4plot=as.matrix(colors4plot)
colnames(colors4plot) = "Group"
```

Sample name format: A1_SO1_S1

…

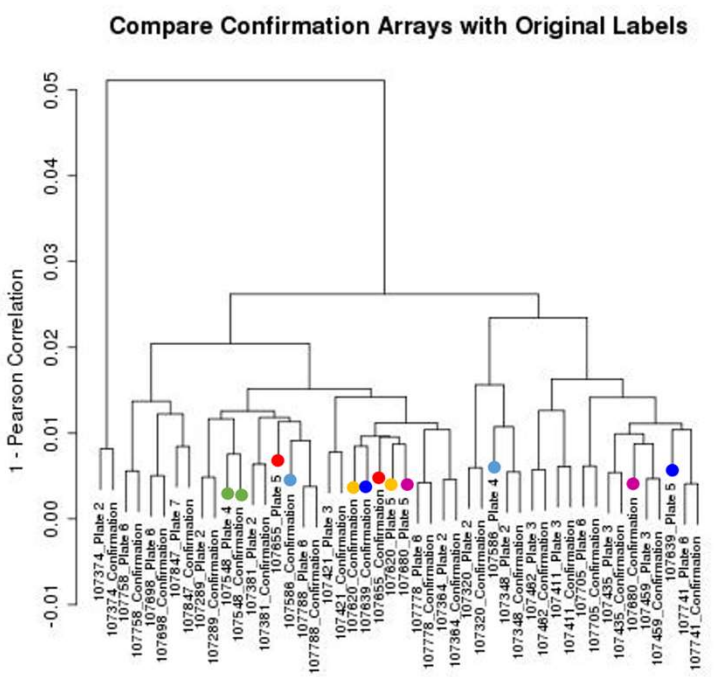
```
par(mfrow=c(1,1))
plotDendroAndColors(dendro=counts.hclust, colors=cbind(colors4plot, tissues4plot, strain4plot), main=
"log2(RSEM counts + 1)", ylab="1-Pearson Correlation",cex.dendroLabels = 0.45)
```

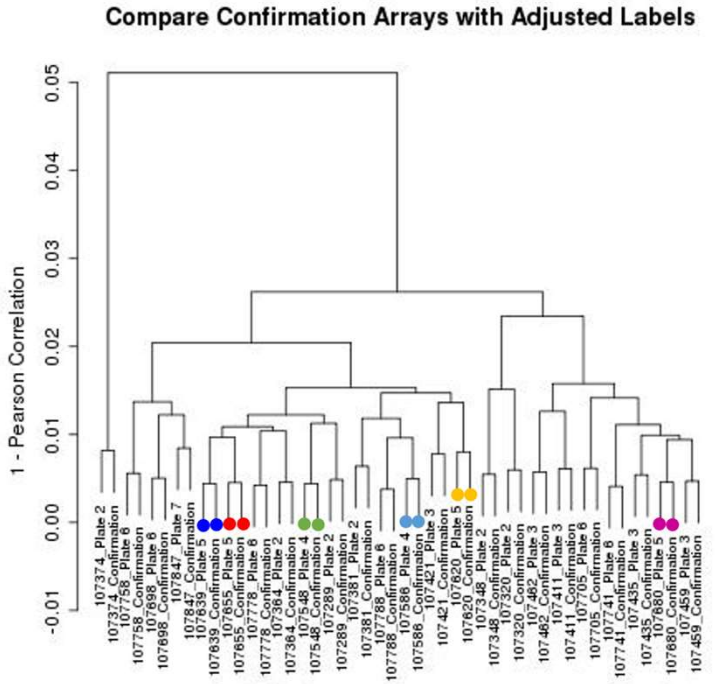
Visualizing Data – Dendrogram Example 2



- Take away from this is there are some major batch effects we need to deal with
- Good to identify sample outliers
- Hard to actually see grouping with some many samples

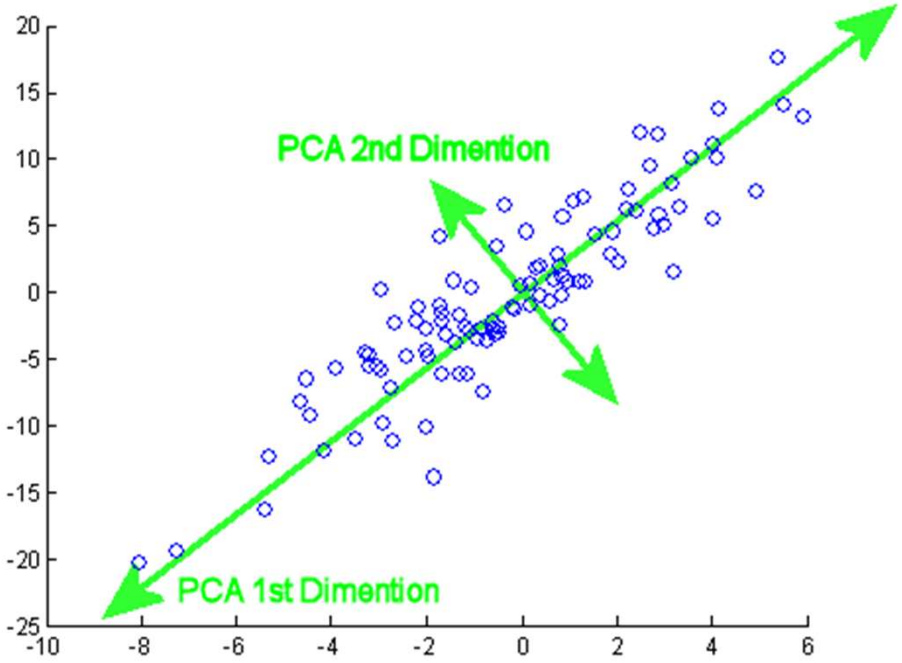
Visualizing Data – Dendrogram Example 3





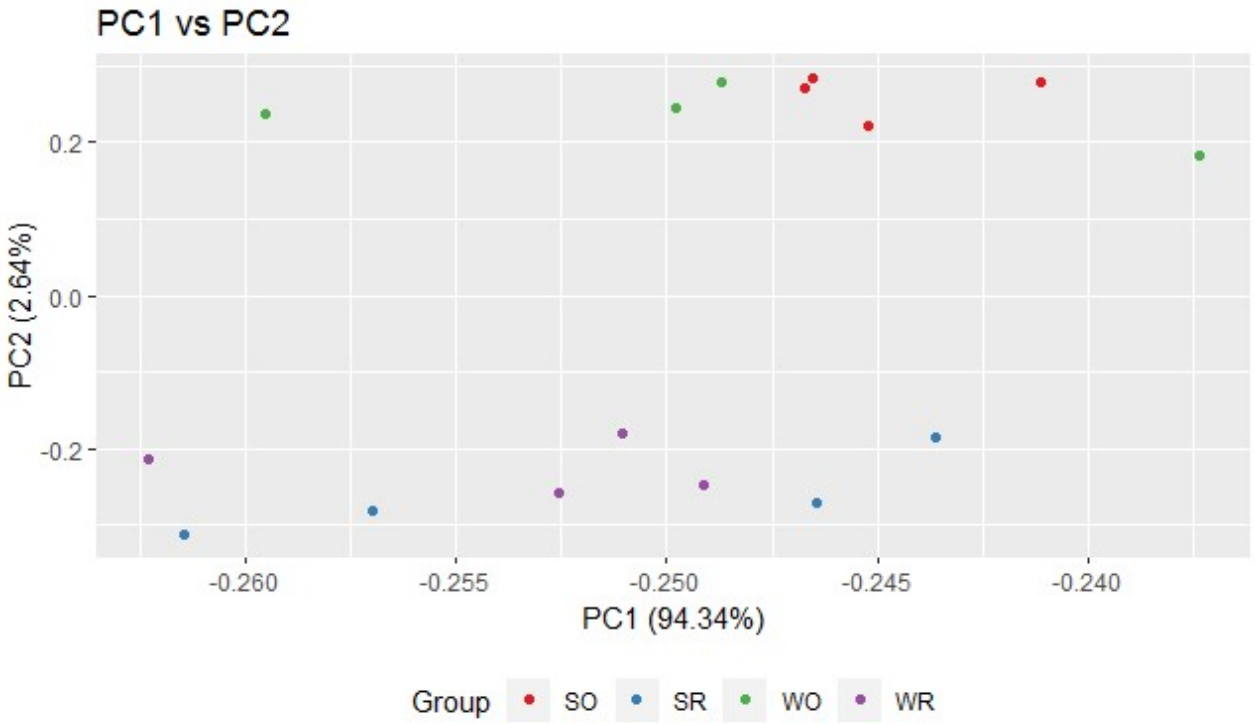
Principal Component Analysis (PCA)

- Good way to summarize your data
- Linear combinations of data which explain the most variance
- PC1 explains the most variance in your data, PC2 the second most
- Easy example with 2D space, what about 20K dimensions!



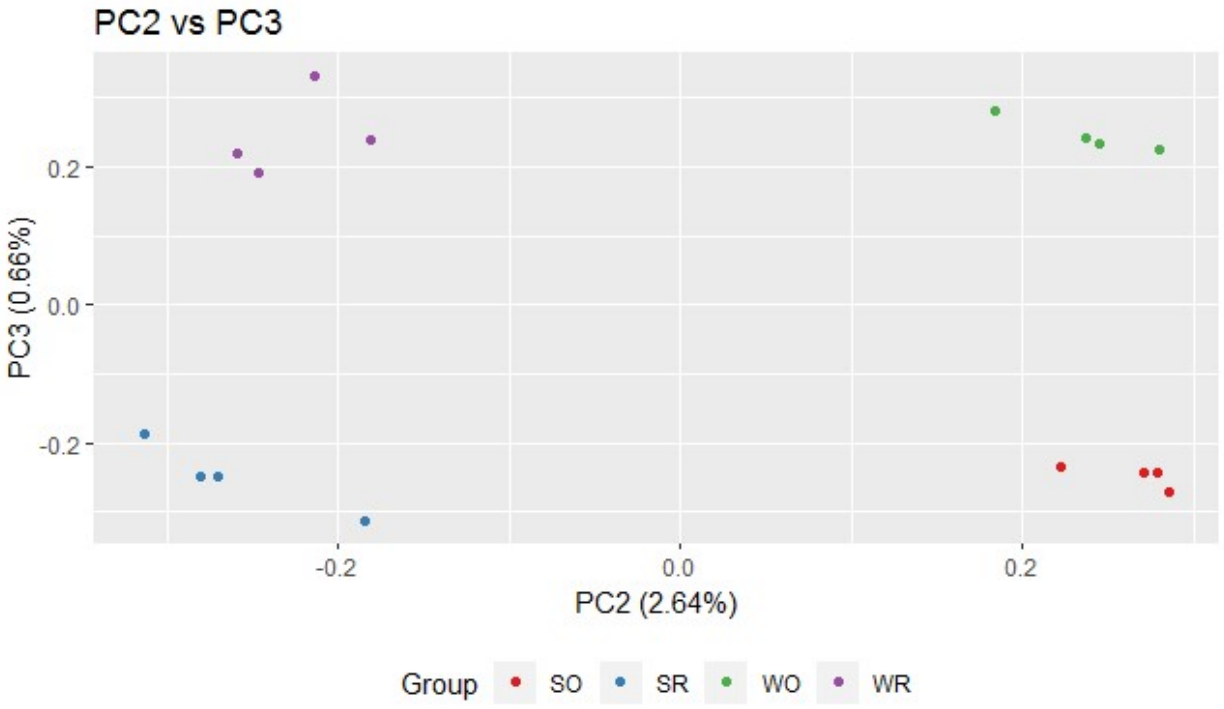
Source: stackExchange: making-sense-of-principal-component-analysis-eigenvectors-eigenvalues

Visualizing Data – PC Plots Example 1



- # points = # samples
- This is the same data from the 16 sample dendrogram
- Large separation from tissue type on PC2
- For a homogeneous design (controlled genetic background), you do see a large % variance explained by PC1

Visualizing Data – PC Plots Example 1 Cont.



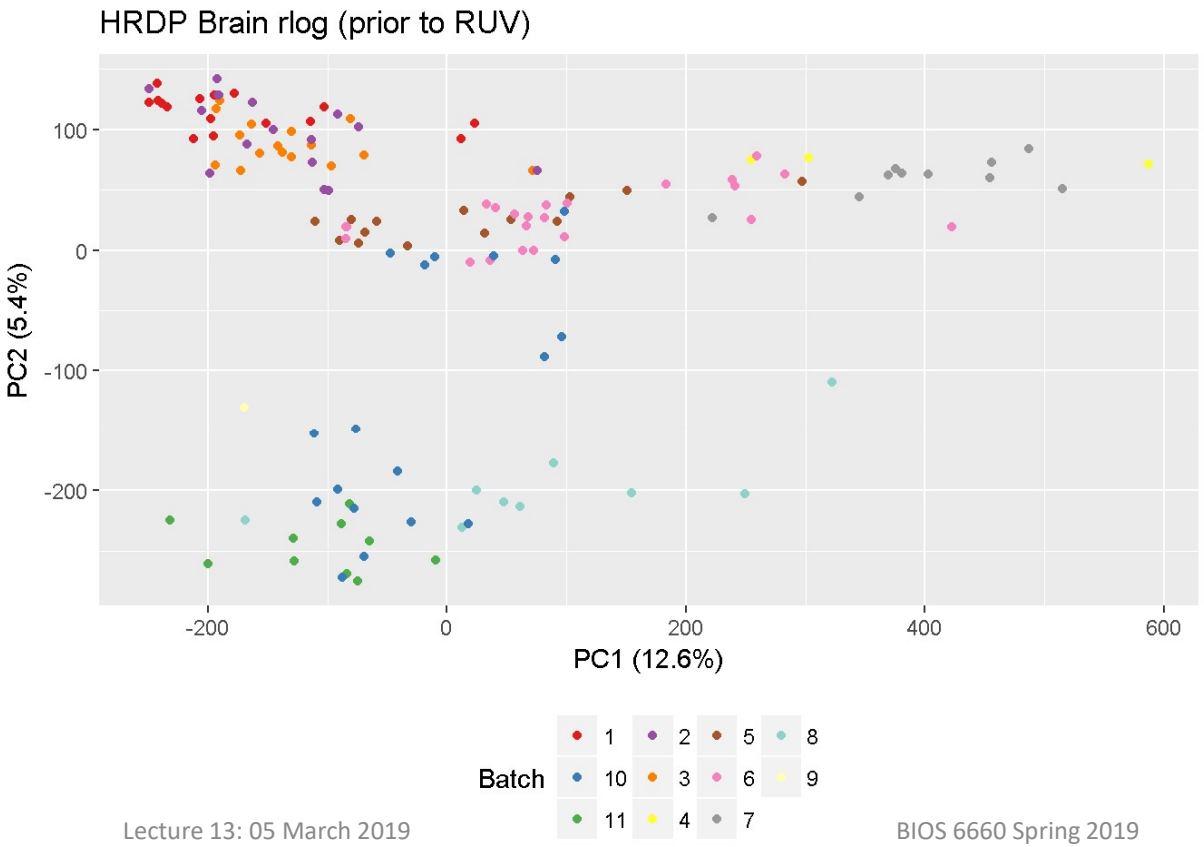
- Here you are seeing great separation of groups.
- You normally don't see this on PC1
- PC2 describes tissue differences (the 2nd letter in group)
- PC3 describes the strain differences (the 1st letter in group)

Visualizing Data – PC Plots Example1 Code

library(ggplot2)
library(RColorBrewer)

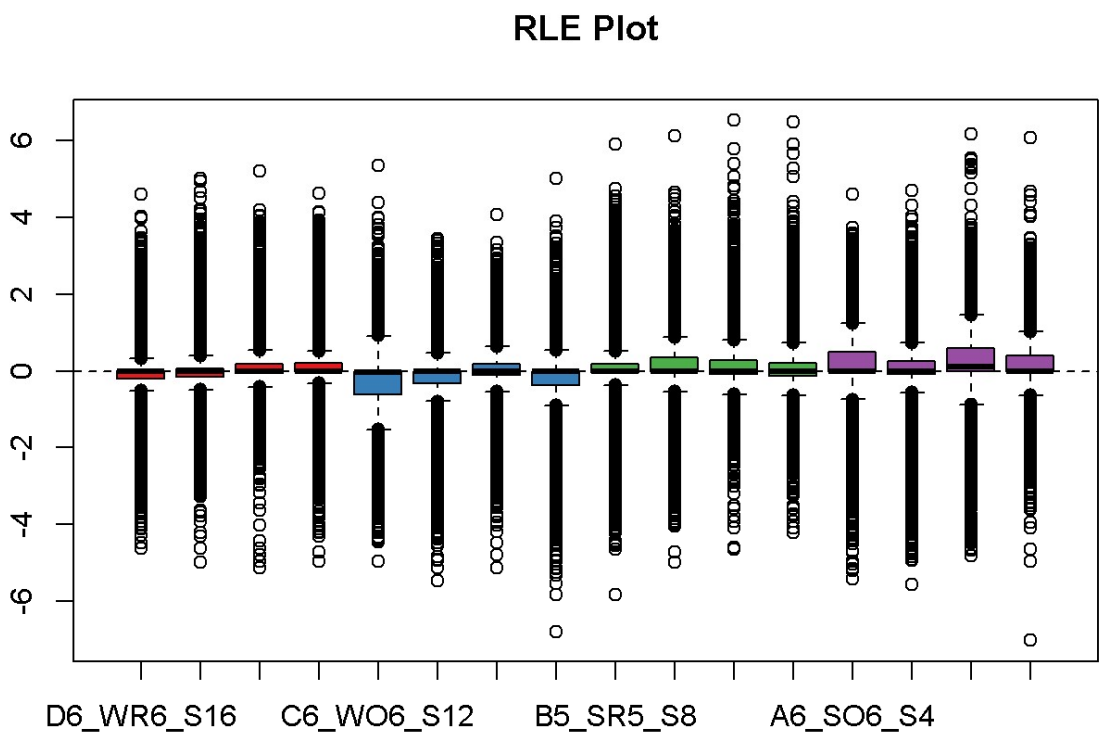
```
#perform pca
pca.results = prcomp(log2(counts2+1))
#get results you want
propEx = summary(pca.results)$importance #proportion var explained
toPlot = data.frame(pca.results$rotation[,1:2]) #PC1 and PC2
toPlot$group = substr(sapply(strsplit(rownames(toPlot), split="_", fixed=TRUE), "[[", 2), 1, 2)

#make plot
colors= brewer.pal(9,"Set1") #define colors
orig = ggplot(toPlot, aes(x=PC1, y=PC2, color=as.character(group))) + geom_point() +
  scale_color_manual(values=colors[c(1:4)])+
  xlab(paste("PC1 (", round(100*propEx[2,"PC1"], 2), "%)", sep="")) +
  ylab(paste("PC2 (", round(100*propEx[2,"PC2"], 2), "%)", sep="")) +
  ggtitle("PC1 vs PC2") +
  #theme(legend.position="none")
  labs(color="Group")+
  theme(legend.position="bottom")
orig
```

Visualizing Data – PC Plots Example 2



- Lot more samples
- Batch effects are clearly an issue
- PC1 explains lower % variance
  - Find this is the case the more complex the study design

Visualizing Data – RLE Plots



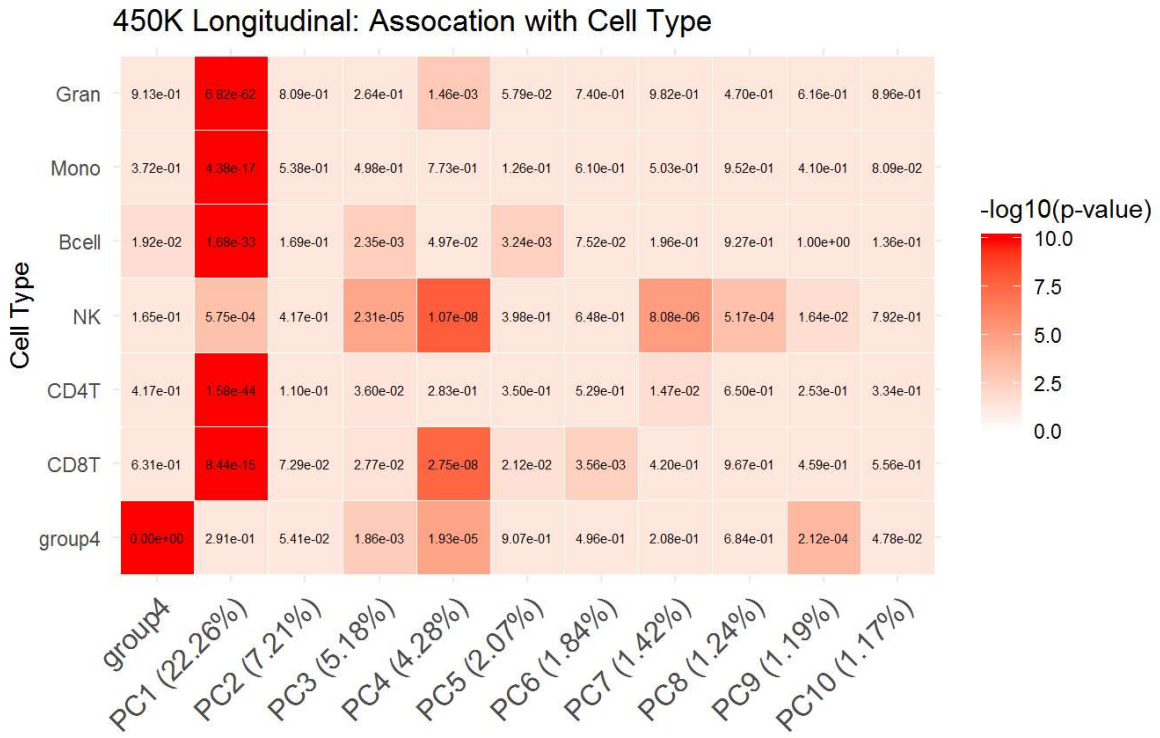
- Relative Log Expression
- For each gene, calculation the median logged expression
- These boxplots are the differences from the median value
- Originally looked at for microarrays, but the theory still stands
- Want the median RLE near 0
- ASSUMING # up-regulated genes = # down-regulated genes
  - Majority of genes DO NOT CHANGE in the system you are questioning

Visualizing Data – RLE Plot Code

library(EDASeq)
library(RColorBrewer)

```
x = substr(sapply(strsplit(colnames(counts2), split="_", fixed=TRUE), "[[", 2), 1, 2)

x.col=c()
for(i in 1:length(x)){
  if(x[i]=="WR"){x.col[i]=colorsWant[1]}
  if(x[i]=="WO"){x.col[i]=colorsWant[2]}
  if(x[i]=="SR"){x.col[i]=colorsWant[3]}
  if(x[i]=="SO"){x.col[i]=colorsWant[4]}
  }
set = newSeqExpressionSet(as.matrix(round(counts2)), phenoData=data.frame(x, row.names=colnames(count
s)))
par(mfrow=c(1,1))
plotRLE(set, colLabel=x, main="RLE Plot", col=x.col)
```

Visualizing Data – Diagnostic Heatmap



Visualizing Data – Diagnostic Heatmap Code

```
#toPlot is a dataframe with 4 columns: PC, Cell Type, estimate and pvalue
toPlot$minus.log10pval[which(toPlot$pvalue>0.05)]=1.3
toPlot$pvalsToPrint = formatC(toPlot$pvalue, format = "e", digits = 2)

ggheatmap <- ggplot(toPlot, aes(PC, CellType, fill = minus.log10pval))+
 geom_tile(color = "white")+
   ggtitle("450K Longitudinal: Assocation with Cell Type") +
 scale_fill_gradient2(low = "white", high = "red", mid="white", na.value = "red",
   midpoint = 0.05, limit = c(0,10), space = "Lab",
    name="-log10(p-value)") +
  theme_minimal()+ # minimal theme
 theme(axis.text.x = element_text(angle = 45, vjust = 1,
    size = 12, hjust = 1))+
  xlab("") +
  scale_x_discrete(labels=c("group4", paste("PC", c(1:10), " (", round(eig_pc_proportion[1:10], 2), "%)", sep=""))) +
  ylab("Cell Type") +
 coord_fixed()
# Print the heatmap
ggheatmap + geom_text(label = toPlot$pvalsToPrint, size=2)
```

Relative Abundance

- RNA-Seq is a **relative abundance** measurement technology

Gene	Sample 1 Absolute Abundance	Sample 1 Relative Abundance	Sample 2 Absolute Abundance	Sample 2 Relative Abundance
A	20	10%	20	5%
B	20	10%	20	5%
C	20	10%	20	5%
D	20	10%	20	5%
E	20	10%	20	5%
F	100	50%	300	75%
	TOTAL 200 counts		TOTAL 400 counts	

Library Sizes Matter

Normalization Methods

- Need to do this to make samples comparable across features and samples
- Common Methods:
  - RPKM/FPKM
  - TPM
  - Quantile
  - TMM (EDASeq)
  - DESeq Median of Ratios (geometric mean & scaling factor)
  - RUV

RPKM/FPKM

- Reads Per Kilobase per Million or Fragments PKM
- One of the 1st normalization methods
- Adjusts for
  - Sequencing depth (or library size)
  - Length of gene (longer gene more reads)

1. Count up total number reads in sample and divide by a million (per million scaling factor)
2. Divide read counts by "per million" scaling factor. Normalizing for sequencing depth (RPM, reads per million)
3. Divide RPM by length of gene in Kb, this give you RPKM

- FPKM is for paired-end reads, really so the 2 reads from a single fragment of RNA is not counted twice.

TPM

- Transcripts Per Million
- Very similar to RPKM/FPKM but order of operations are switched
- Adjusts for length of gene first, then sequencing depth

1. Divide counts by length of gene in Kb (RPK)
2. Count up all the RPK values in a sample and divide this number by 1 million (this is the per million scalilng factor).
3. Divide the RPK values by “per million” scaling factor, this gives you TPM.

- Preferred over RPKM/FPKM since using TPM the total sum of gene TPM will be the same across all samples (i.e. really have the same denominator)
- RSEM outputs TPM

RPKM vs TPM example

Counts Matrix

Gene	Rep 1	Rep 2	Rep 3
A (2kb)	10	12	30
B (4kb)	20	25	60
C (1kb)	5	8	15
D (10kb)	0	0	1
TOTAL	**35**	**45**	**106**

Note: In this sample I used per 10 instead of per 1 million just to make the numbers easier to look at

Source: RNASeq blog

Your total is the sample across samples, so it's easier to compare across samples

RPKM Matrix

Gene	Rep 1	Rep 2	Rep 3
A (2kb)	1.43	1.33	1.42
B (4kb)	1.43	1.39	1.42
C (1kb)	1.43	1.78	1.42
D (10kb)	0	0	0.009
TOTAL	**4.29**	**4.5**	**4.269**

TPM Matrix

Gene	Rep 1	Rep 2	Rep 3
A (2kb)	3.33	2.96	3.326
B (4kb)	3.33	3.09	3.326
C (1kb)	3.33	3.95	3.326
D (10kb)	0	0	0.02
TOTAL	**10**	**10**	**10**

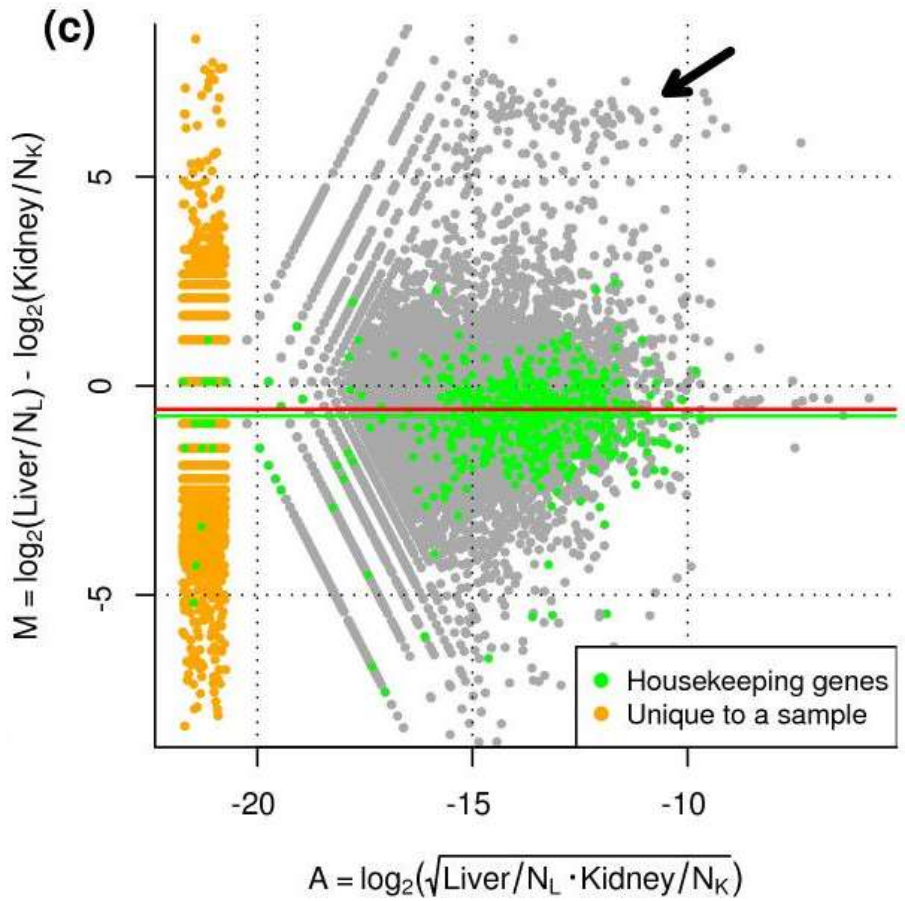
Quantile Normalization

- Making your distributions identical in statistical properties (i.e. the quantiles will be the same)
- Ranking the genes for each sample
- Get a rank expression level then each rank gets a new expression level

Rank	Sample 1	Sample 2	….	Sample N	Normalized Value
1	0	1	…	0	Mean 1st row
2	3	2	…	2	Mean 2nd row
…	…	…	….	….	…
G	10002	9999	…	9232	Mean Gth row

TMM (EDASeq)

- Weighted Trimmed Mean of Log Expression Ratios (M values)
- Very similar to a Bland-Altman plot
- Call A (x-axis) the reference sample (geometric mean of 2 samples)
- Red line should be at 0
- Where the line is the normalization factor



Source: Robinson et al, 2010

DESeq Median of Ratios

1. Normalized Count = count / geometric mean of that gene

Gene	Sample 1	Sample 2	Geometric Mean
A	100	50	**70.7**
B	300	220	**256.9**

→

Gene	Sample 1	Sample 2
A	1.41	0.71
B	1.17	0.86

2. Within sample normalization factor by taking the median across all genes.

Sample 1 normalization factor = median(c(1.41, 1.17)) = 1.29

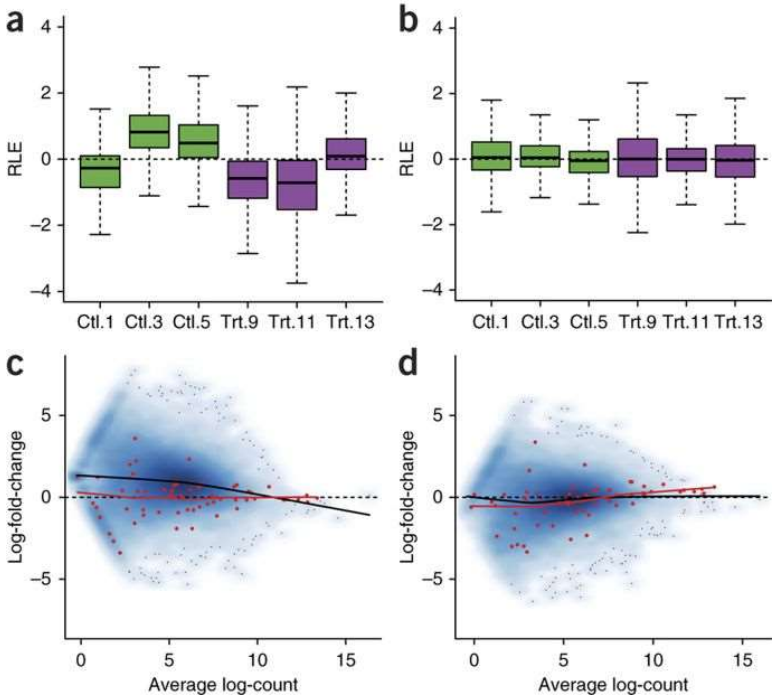
Sample 2 normalization factor = median(c(0.71, 0.86)) = 0.785

3. Final Normalized Matrix

Gene	Sample 1	Sample 2
A	100/1.29 = **77.5**	50/0.785 = **63.7**
B	300/1.29 = **232.6**	220/0.785 =**280.3**

RUV

- Remove Unwanted Variation
- Idea is to use control genes that do not change in your design
  - Spike-ins
  - Biological knowledge
  - Empirical Method
- Uses factor analysis (very similar to PCA)
- Depending on how many control genes you have, you can adjust out for as many factors (k)



Source: Risso et al, 2014

Normalization Methods Summary

- DO NOT USE RPKM/FPKM
- Preferably keep data as count data and use either DESeq or EDASeq
  - These packages allow you to analyze the data using a GLM with assumption data is distributed with NB distribution
  - However, they don't allow you to have any random effects
- RUV is a newer exciting method
  - Selecting number factors to adjust out (k) is not straight forward
  - Many times used in conjunction with others

Differential Expression

- Basic analysis to find differences in expression between a group(s).
- Common Analysis Techniques:
  - T-test
  - ANOVA
  - Linear Regression
  - Linear Mixed-Effect Models
  - Generalized Linear Models (GLM)
    - Link function so you can run data that is non-normal in a linear model structure
    - DESeq & EDASeq

Normally Distributed Data
(TPM and/or transformation)

DESeqDataSet Format

- S4 Type of object
- Stores the input values (raw counts), intermediate calculations (any normalization) and results of an analysis of differential expression (from DESeq or DESeq2)

```
dds2 = DESeqDataSetFromMatrix(countData = as.matrix(round(counts2)),
      colData = data.frame(group, tissue, strain),
      design = ~ strain*tissue)
```

- If you used HTSeq (python module) to generate counts, you can use DESeqDataSetFromHTSeqCount() to generate object

DESeqDataSet Format

```
> dds2
class: DESeqDataSet
dim: 15127 16
metadata(1): version
assays(1): counts
rownames(15127): FBgn0000003 FBgn0000008 ... FBgn0267794 FBgn0267795
rowData names(0):
colnames(16): D6_WR6_S16 D5_WR5_S15 ... A2_SO2_S2 A1_SO1_S1
colData names(3): group tissue strain
```

rawCountMatrix <- counts(dds2)
metaData <- colData(dds2)

DESeq2 Analysis - LRT example

#performs median of ratios normalization

dds2 = estimateSizeFactors(dds2)

#if you want to extract normalized count matrix

normalizedCounts = counts(dds2, normalized=TRUE)

#perform a likelihood ratio test (LRT) to see any strain effect

```
> dds2 = DESeq(dds2,test="LRT",reduced= ~ tissue, fitType="local")
using pre-existing size factors
estimating dispersions
gene-wise dispersion estimates
mean-dispersion relationship
final dispersion estimates
fitting model and testing
> |
```

DESeq2 Analysis – LRT example

```
> strainEffects = results(dds2, independentFiltering=FALSE)
> head(strainEffects)
log2 fold change (MLE): strainWhiteEyed.tissueRetina
LRT p-value: '~ strain * tissue' vs '~ tissue'
DataFrame with 6 rows and 6 columns
                   baseMean     log2FoldChange             lfcSE              stat
                  <numeric>          <numeric>         <numeric>         <numeric>
FBgn0000003 699.145888361084  -0.58771314044907 0.693613552000856 2.61212587909614
FBgn0000008 2954.45567305398 -0.895597749628288 0.250017864368627 14.7137213480259
FBgn0000014 8.21095639029486   1.24812287239828  1.96457143573063 2.64892501978943
FBgn0000015 3.79786939660886   4.08364954314244  2.65369361537612 2.26799339087655
FBgn0000017  5671.9724202071 -0.410359966390261 0.274445280382548 4.97547427058328
FBgn0000018 113.196237762821 -0.418801337528481 0.377321859183718 3.18711565071041
                      pvalue               padj
                   <numeric>          <numeric>
FBgn0000003     0.27088444817034   0.607569563854795
FBgn0000008 0.000638198832163481 0.00746430224491593
FBgn0000014    0.265945863950927   0.601885243945011
FBgn0000015    0.321744767444056    0.67099517415778
FBgn0000017   0.0830977930453044   0.294911094150426
FBgn0000018    0.203201369150486   0.515821492054677
> |
```

DESeq2 Analysis – single coefficient example

```
> dds.inter = DESeqDataSetFromMatrix(countData = as.matrix(round(counts2)),
+                 colData = data.frame(group, tissue, strain),
+                 design = ~ strain*tissue)
converting counts to integer mode
Warning message:
In DESeqDataSet(se, design = design, ignoreRank) :
  some variables in design formula are characters, converting to factors
> dds.inter = DESeq(dds.inter)
estimating size factors
estimating dispersions
gene-wise dispersion estimates
mean-dispersion relationship
final dispersion estimates
fitting model and testing
> interactionEffect = results(dds.inter, name="strainWhiteEyed.tissueRetina",
                 independentFiltering=FALSE)
```

DESeq2 Analysis – single coefficient example

```
> head(interactionEffect)
log2 fold change (MLE): strainWhiteEyed.tissueRetina
Wald test p-value: strainWhiteEyed.tissueRetina
DataFrame with 6 rows and 6 columns
                   baseMean     log2FoldChange             lfcSE                stat
                  <numeric>          <numeric>         <numeric>           <numeric>
FBgn0000003 699.145888361084 -0.587558882866976 0.632132296455124 -0.929487207285395
FBgn0000008 2954.45567305398 -0.895529730693888 0.270234151087478  -3.31390287678331
FBgn0000014 8.21095639029486   1.24910089991614  2.02038098532741   0.61825017607445
FBgn0000015 3.79786939660886    4.0786729090029  2.74465670088278   1.48604118966538
FBgn0000017  5671.9724202071 -0.410361872640175 0.293033034643545  -1.40039457714845
FBgn0000018 113.196237762821 -0.418655048790763 0.392255275774135  -1.06730253140516
                      pvalue              padj
                   <numeric>         <numeric>
FBgn0000003    0.352636651914663  0.920261409774183
FBgn0000008 0.000920034588316088 0.0399724537563497
FBgn0000014    0.536410439428845  0.992603703499596
FBgn0000015    0.137268226396593   0.66180229649985
FBgn0000017     0.16139519302746   0.70510860946658
FBgn0000018     0.28583524538277  0.864727389563077
>
```

DESeq2 Analysis – Adjusting for Covariate

```
> dds.cov = DESeqDataSetFromMatrix(countData = as.matrix(round(counts2)),
+                 colData = data.frame(group, tissue, strain),
+                 design = ~  tissue + strain)
converting counts to integer mode
Warning message:
In DESeqDataSet(se, design = design, ignoreRank) :
  some variables in design formula are characters, converting to factors
> dds.cov = DESeq(dds.cov)
estimating size factors
estimating dispersions
gene-wise dispersion estimates
mean-dispersion relationship
final dispersion estimates
fitting model and testing
> strain.adjForTissue = results(dds.cov)
>
```

DESeq2 Analysis – Adjusting for Covariate

```
> strain.adjForTissue = results(dds.cov)
> head(strain.adjForTissue)
log2 fold change (MLE): strain WhiteEyed vs Sevenless
Wald test p-value: strain WhiteEyed vs Sevenless
DataFrame with 6 rows and 6 columns
                   baseMean      log2FoldChange             lfcSE                stat
                  <numeric>           <numeric>         <numeric>           <numeric>
FBgn0000003 699.145888361084   0.481065789920314 0.315813565099327    1.52325879279129
FBgn0000008 2954.45567305398   0.180576506548756 0.173719729223854    1.03947034315294
FBgn0000014 8.21095639029486   -1.51479593858499 0.982522214724989   -1.54174217730943
FBgn0000015 3.79786939660886 -0.0561051226257818  1.37892407109896 -0.0406876084054923
FBgn0000017  5671.9724202071   0.227839409937837 0.152736766573332    1.49171293231775
FBgn0000018 113.196237762821   0.264910424695025  0.19763036163017     1.3404338407818
                     pvalue              padj
                  <numeric>         <numeric>
FBgn0000003 0.127693972189392 0.353716203604252
FBgn0000008 0.298586043835595 0.567851795440525
FBgn0000014 0.123136258247327 0.346438222750232
FBgn0000015 0.967544940475883 0.987751006971347
FBgn0000017 0.135774417382166 0.365765984149215
FBgn0000018 0.180104339899211 0.430512887063497
```

results() has a name option for selecting coefficient, but default it automatically returns last coefficient in model

References

Robinson MD, Oshlack A. *A scaling normalization method for differential expression analysis of RNA-seq data.* Genome Biol. **2010**;11(3):R25.

Anders S, Huber W. *Differential expression analysis for sequence count data.* Genome Biol. **2010**;11(10):R106.

Risso D, Ngai J, Speed T, Dudoit S (2014). "Normalization of RNA-seq data using factor analysis of control genes or samples." *Nature Biotechnology*, **32**(9), 896–902.