

Report 2

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This report will show three different ways to analyse a group of biological data and gene expression. I will demonstrate how to do this in DESeq2, using a random forest, and with tidylo, the weighted log odds ratio

DESeq2

The first thing to do is read in some libraries and data. I'm once again using the pasilla data set

```
library(pasilla)
library(tidyverse)
library(tximeta)
library(tximportData)
library(Rsamtools)
library(DESeq2)
library(magrittr)

pasCts <- system.file("extdata",
                      "pasilla_gene_counts.tsv",
                      package="pasilla", mustWork=TRUE)
pasAnno <- system.file("extdata",
                      "pasilla_sample_annotation.csv",
                      package="pasilla", mustWork=TRUE)
cts <- as.matrix(read.csv(pasCts, sep="\t", row.names="gene_id"))
(coldata <- read.csv(pasAnno, row.names=1))
```

```
##           condition      type number.of.lanes total.number.of.reads
## treated1fb      treated single-read           5           35158667
## treated2fb      treated paired-end           2           12242535 (x2)
## treated3fb      treated paired-end           2           12443664 (x2)
## untreated1fb    untreated single-read           2           17812866
## untreated2fb    untreated single-read           6           34284521
## untreated3fb    untreated paired-end           2           10542625 (x2)
## untreated4fb    untreated paired-end           2           12214974 (x2)
##           exon.counts
## treated1fb      15679615
## treated2fb      15620018
## treated3fb      12733865
## untreated1fb    14924838
## untreated2fb    20764558
## untreated3fb    10283129
## untreated4fb    11653031
```

```
coldata <- coldata[,c("condition", "type")]
coldata$condition <- factor(coldata$condition)
coldata$type <- factor(coldata$type)
```

So, I have `cts` which is a matrix that has a list of samples and `coldata` which contains a summary of the experiment. I'm only looking at `type` and `condition`, so then I will then take the names of the treatment groups and use them as my features in my count matrix. The following manipulation is being done to prepare the data for the next step.

```
rownames(coldata) = sub("fb", "", rownames(coldata))
cts = cts[,rownames(coldata)]
```

Using the `DESeqDataSetFromMatrix` function, I'll pass `DESeq2` my count matrix, `coldata` and a formula. Currently, I'm only going to look at how condition affects the overall gene expression, so that is what I'm making my formula in the `design` argument of the function. Going further, I only want to keep any data where the count data is GEQ 10, just to avoid unnecessary outliers

```
dds = DESeqDataSetFromMatrix(countData = cts,
                             colData = coldata,
                             design = ~ condition)
keep = rowSums(counts(dds)) >= 10
dds = dds[keep,]
```

Before going any further, I want to make sure the system understands that there is a difference between treated and untreated groups, so I'm going to relevel them.

```
dds$condition = relevel(dds$condition, ref = "untreated")
```

Now, I'm going to let `DESeq2` analyse the data. The `results` function will summarise it's findings.

```
dds = DESeq(dds)
(res = results(dds))
```

```
## log2 fold change (MLE): condition treated vs untreated
## Wald test p-value: condition treated vs untreated
## DataFrame with 9921 rows and 6 columns
##           baseMean log2FoldChange    lfcSE      stat    pvalue    padj
##           <numeric>      <numeric> <numeric> <numeric> <numeric> <numeric>
## FBgn0000008    95.14429    0.00227644 0.223729  0.010175 0.9918817 0.997211
## FBgn0000014     1.05652   -0.49512039 2.143186 -0.231021 0.8172987      NA
## FBgn0000017  4352.55357   -0.23991894 0.126337 -1.899041 0.0575591 0.288002
## FBgn0000018   418.61048   -0.10467391 0.148489 -0.704927 0.4808558 0.826834
## FBgn0000024     6.40620    0.21084779 0.689588  0.305759 0.7597879 0.943501
## ...           ...           ...           ...           ...           ...
## FBgn0261570  3208.38861    0.2955329 0.127350  2.3206264 0.020307 0.144240
## FBgn0261572     6.19719   -0.9588230 0.775315 -1.2366888 0.216203 0.607848
## FBgn0261573  2240.97951    0.0127194 0.113300  0.1122634 0.910615 0.982657
## FBgn0261574  4857.68037    0.0153924 0.192567  0.0799327 0.936291 0.988179
## FBgn0261575    10.68252    0.1635705 0.930911  0.1757102 0.860522 0.967928
```

I'm only going to focus on `baseMean`, `log2FoldChange`, and `pvalue` for the time being. The `baseMean` is the average count of genes per sample, `log2FoldChange` takes the change in expression between a treated and untreated group, and takes the \log_2 of that. `pvalue` obviously is the p-value of the hypothesis test between treated and untreated groups.

Now, one of things to notice is the `lfcSE`, the logfold change standard error. Ideally, I want to minimize standard error. There are currently a few ways to shrink the logfold change estimates. A current favorite is the `apegglm` algorithm, as developed in Zhu, Ibrahim, and Love 2018.

```
(resLFC = lfcShrink(dds, coef = "condition_treated_vs_untreated",
                    type = "apeglm"))
```

```
## log2 fold change (MAP): condition treated vs untreated
## Wald test p-value: condition treated vs untreated
## DataFrame with 9921 rows and 5 columns
##           baseMean log2FoldChange      lfcSE      pvalue      padj
##           <numeric>      <numeric> <numeric> <numeric> <numeric>
## FBgn0000008      95.14429      0.00119920  0.151897 0.9918817 0.997211
## FBgn0000014       1.05652     -0.00473412  0.205468 0.8172987      NA
## FBgn0000017    4352.55357     -0.18989990  0.120377 0.0575591 0.288002
## FBgn0000018     418.61048     -0.06995753  0.123901 0.4808558 0.826834
## FBgn0000024       6.40620      0.01752715  0.198633 0.7597879 0.943501
## ...           ...           ...           ...           ...
## FBgn0261570    3208.38861      0.24110290  0.1244469 0.020307 0.144240
## FBgn0261572       6.19719     -0.06576173  0.2141351 0.216203 0.607848
## FBgn0261573    2240.97951      0.01000619  0.0993764 0.910615 0.982657
## FBgn0261574    4857.68037      0.00843552  0.1408267 0.936291 0.988179
## FBgn0261575     10.68252      0.00809101  0.2014704 0.860522 0.967928
```

Same counts and p-values, less standard error

```
resOrdered = resLFC[order(res$pvalue),]
summary(resLFC)
```

```
##
## out of 9921 with nonzero total read count
## adjusted p-value < 0.1
## LFC > 0 (up)      : 518, 5.2%
## LFC < 0 (down)    : 536, 5.4%
## outliers [1]      : 1, 0.01%
## low counts [2]    : 1539, 16%
## (mean count < 6)
## [1] see 'cooksCutoff' argument of ?results
## [2] see 'independentFiltering' argument of ?results
```

How many adjusted p-values are less than 0.1?

```
sum(resLFC$padj < 0.1, na.rm = T)
```

```
## [1] 1054
```

I'll do the same thing with the results function, eliminating anything with a p-value less than 0.95

```
res05 = results(dds, alpha = 0.05)
summary(res05)
```

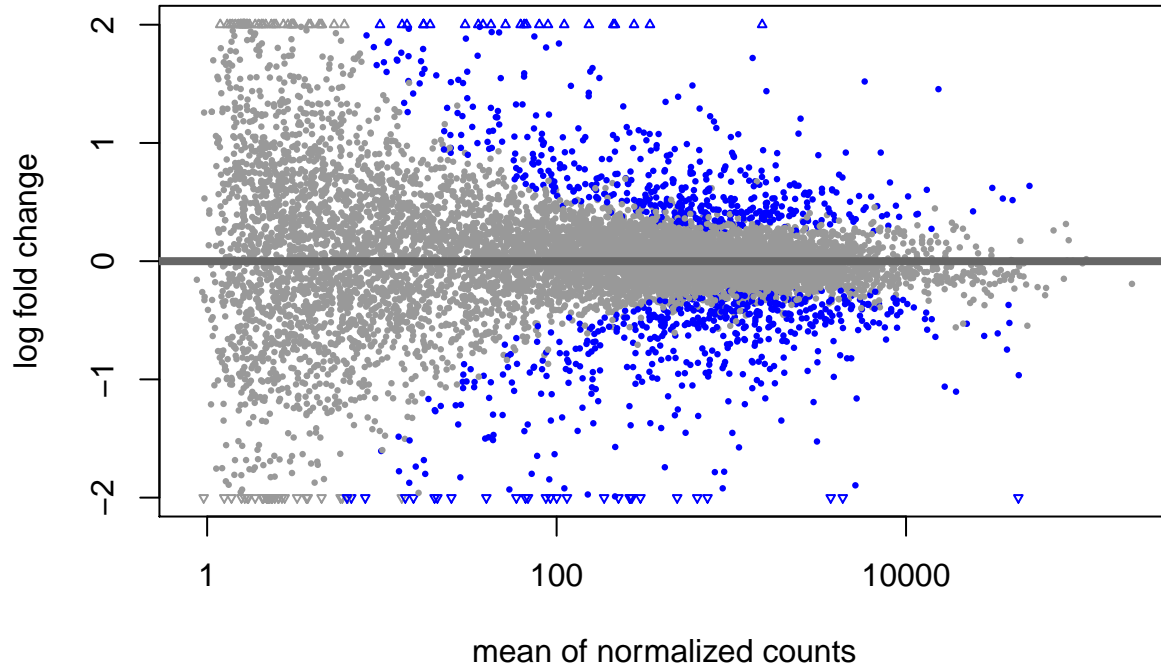
```
##
## out of 9921 with nonzero total read count
## adjusted p-value < 0.05
## LFC > 0 (up)      : 407, 4.1%
## LFC < 0 (down)    : 431, 4.3%
## outliers [1]      : 1, 0.01%
## low counts [2]    : 1347, 14%
## (mean count < 5)
## [1] see 'cooksCutoff' argument of ?results
## [2] see 'independentFiltering' argument of ?results
```

```
sum(res05$padj < 0.05, na.rm = T)
```

```
## [1] 838
```

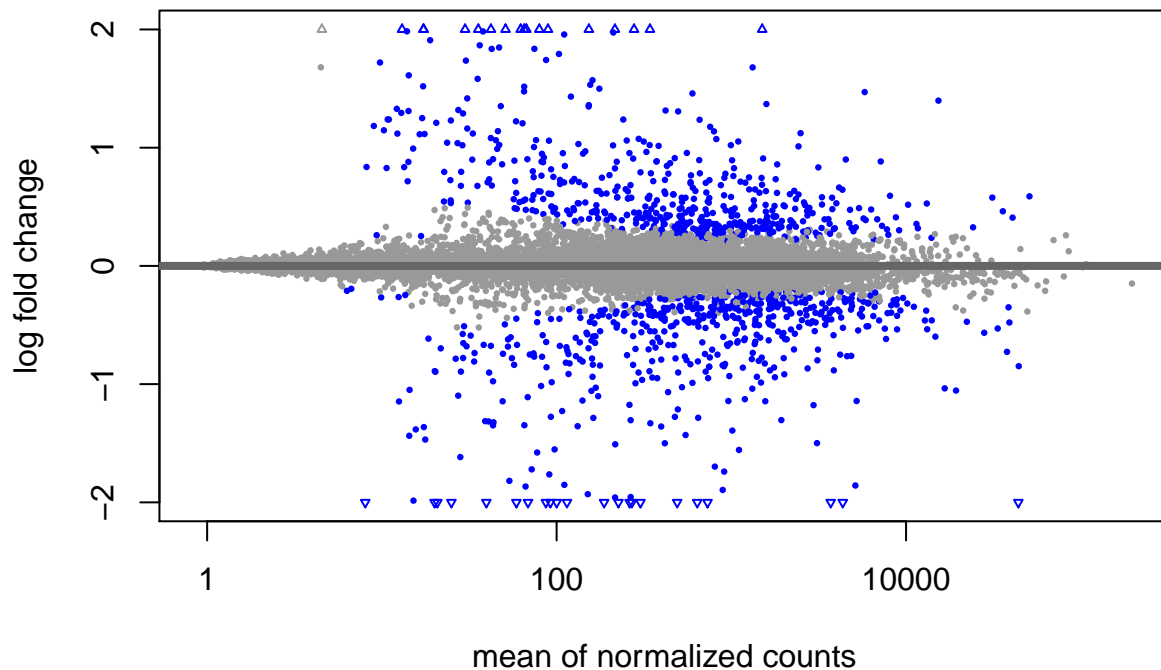
DESeq2 uses the `plotMA` function to plot log2 fold changes to a given variable over the mean of normalized counts for all samples in the data sets. In this case, the condition is going to be the cause of my log2 fold change. If the point is in blue, the adjusted p-value is less than 0.1. Any points that exceed the window parameters are plotted as open triangles, either pointing up or down

```
plotMA(res, ylim = c(-2,2))
```



Using the MA plot with the shrink algorithm, the background noise from low counts goes away and it gives a better visual.

```
plotMA(resLFC, ylim = c(-2,2))
idx = identify(res$baseMean, res$log2FoldChange)
```



```
rownames(res)[idx]
```

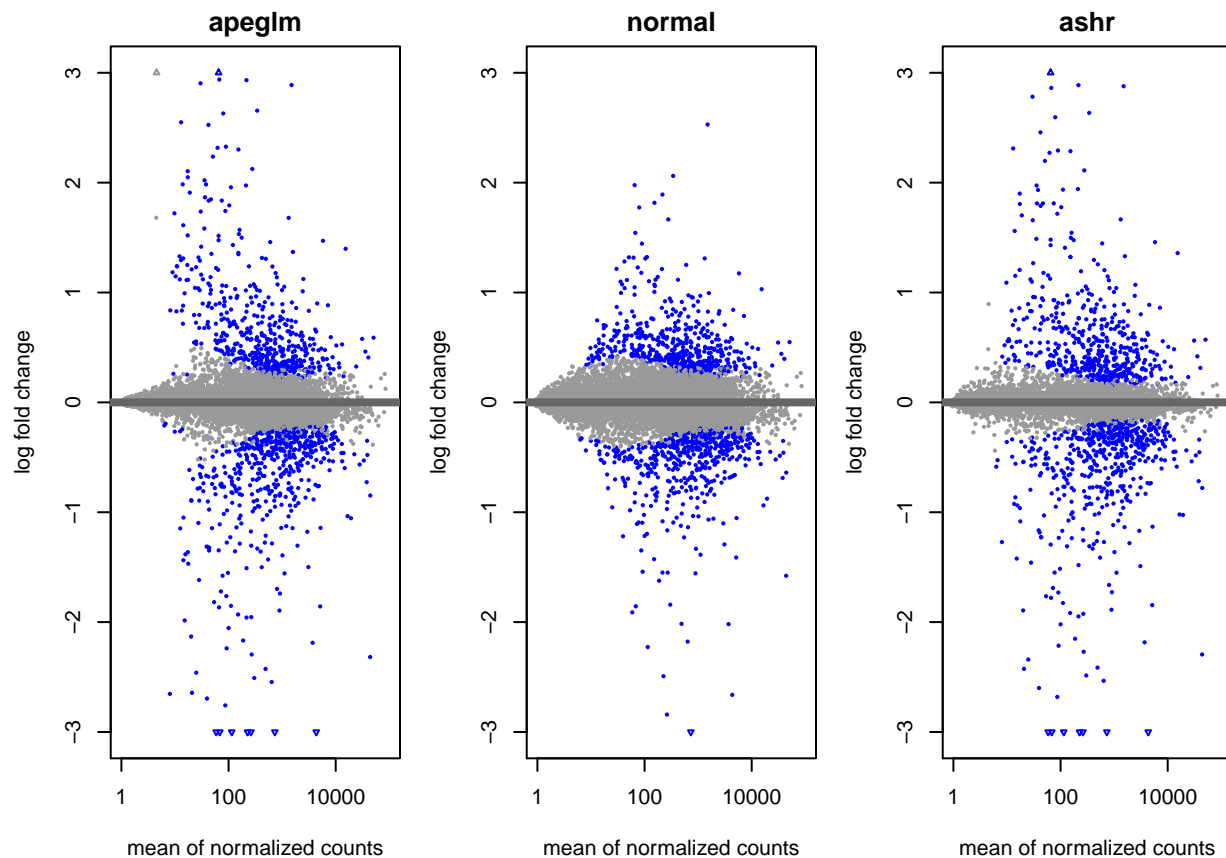
```
## character(0)
```

Of course, there are other algorithms for shrinkage to use. There's the `ashr` algorithm, discussed in Stephens, M (2016), there's also the normal shrinkage algorithm. The normal shrinkage algorithm can also be used

```
resNorm = lfcShrink(dds, coef = 2, type = "normal")  
resAsh = lfcShrink(dds, coef = 2, type = "ashr")
```

Now, to plot all of them

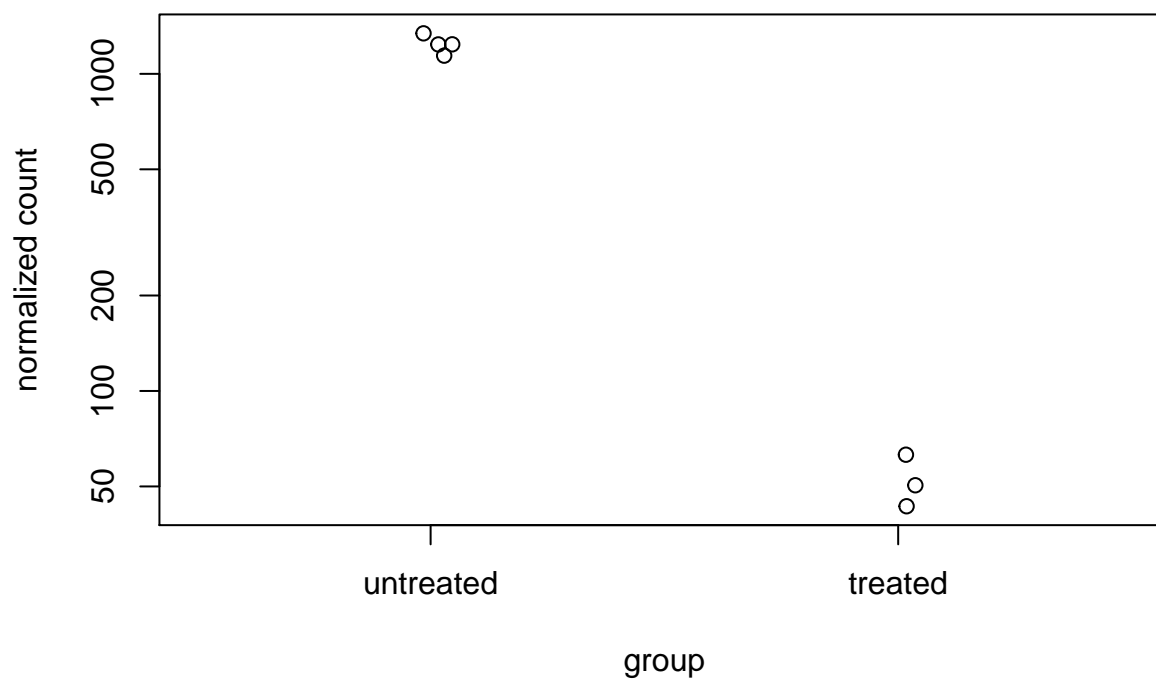
```
par(mfrow = c(1,3), mar = c(4,4,2,1))  
xlim = c(1,1e5); ylim = c(-3,3)  
plotMA(resLFC, xlim = xlim, ylim = ylim, main = "apeglm")  
plotMA(resNorm, xlim = xlim, ylim = ylim, main = "normal")  
plotMA(resAsh, xlim = xlim, ylim = ylim, main = "ashr")
```



Of course, it could help to plot the counts of reads for a single gene across the groups. `plotCounts` does that. Here, I plotted the gene with the greatest changed between treated and untreated groups based on adjusted p-values.

```
plotCounts(dds, gene = which.min(res$padj), intgroup = "condition")
```

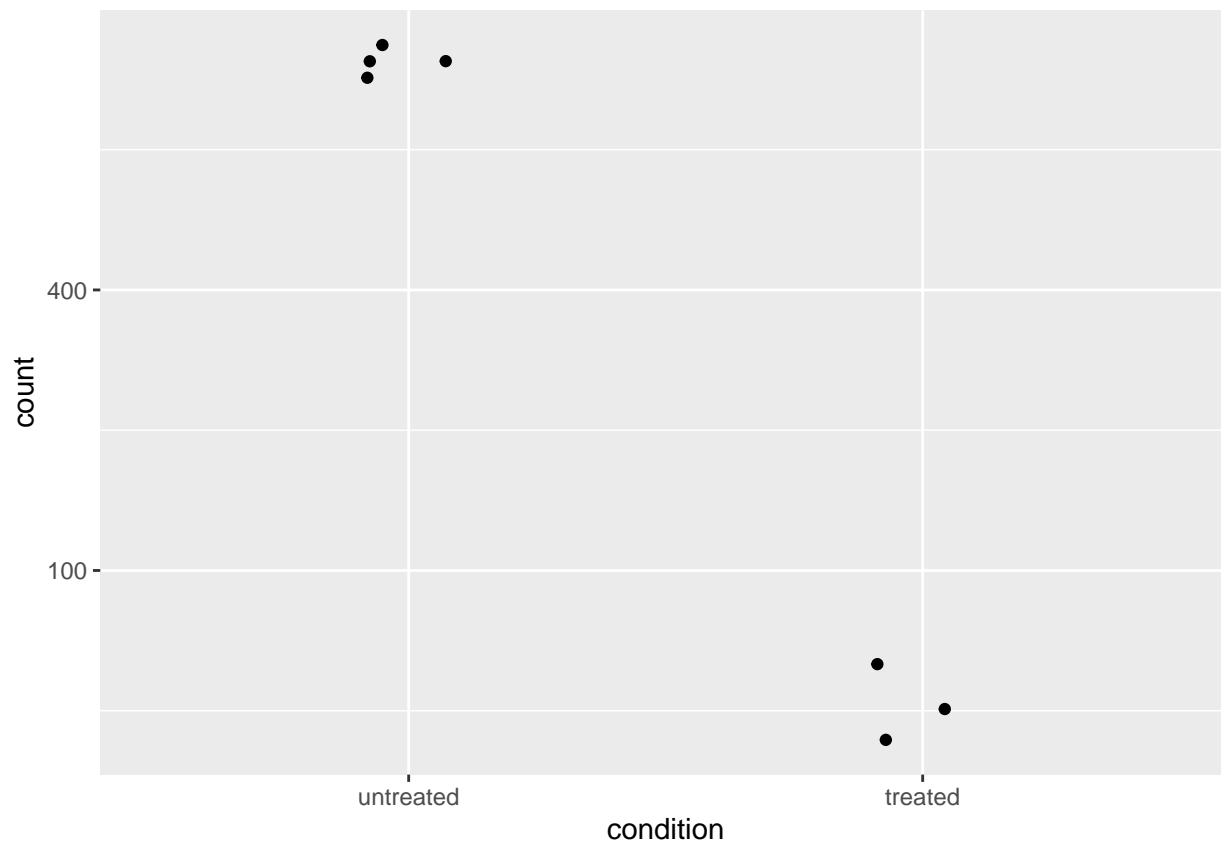
FBgn0039155



This

can also be done in ggplot2

```
d = plotCounts(dds, gene = which.min(res$padj), intgroup = "condition",
               returnData = T)
ggplot(d, aes(x = condition, y = count)) +
  geom_point(position = position_jitter(w=0.1, h = 0)) +
  scale_y_log10(breaks = c(25,100,400))
```



Multi-factor

I can use a lot of the same steps to compare multiple features. In this case, I can also include type of analysis in my comparison of samples

```
ddsMulti = dds
levels(ddsMulti$type) = sub("-", ".", "", levels(ddsMulti$type))
levels(ddsMulti$type)
```

```
## [1] "paired" "single"
```

Just now, I'll change the `design` argument of the DESeq object to include both `condition` and `type`

```
design(ddsMulti) <- formula(~ type + condition)
ddsMulti <- DESeq(ddsMulti)
```

```
resMulti = results(ddsMulti)
head(resMulti)
```

```
## log2 fold change (MLE): condition treated vs untreated
## Wald test p-value: condition treated vs untreated
## DataFrame with 6 rows and 6 columns
##           baseMean log2FoldChange    lfcSE      stat    pvalue    padj
##           <numeric>      <numeric> <numeric> <numeric> <numeric> <numeric>
## FBgn0000008    95.14429   -0.0405571  0.220040 -0.1843169 0.8537648 0.949444
## FBgn0000014     1.05652   -0.0835022  2.075676 -0.0402289 0.9679106      NA
## FBgn0000017 4352.55357   -0.2560570  0.112230 -2.2815471 0.0225161 0.130353
## FBgn0000018  418.61048   -0.0646152  0.131349 -0.4919341 0.6227659 0.859351
## FBgn0000024    6.40620    0.3089562  0.755886  0.4087340 0.6827349 0.887742
```

```
## FBgn0000032 989.72022 -0.0483792 0.120853 -0.4003139 0.6889253 0.890201
```

So, it looks like `type` has no effect. As a follow-up, I can also analyse with just `type` considered

```
resMFTtype <- results(ddsMulti,
                      contrast=c("type", "single", "paired"))
head(resMFTtype)
```

```
## log2 fold change (MLE): type single vs paired
## Wald test p-value: type single vs paired
## DataFrame with 6 rows and 6 columns
##           baseMean log2FoldChange      lfcSE      stat      pvalue      padj
##           <numeric>      <numeric> <numeric> <numeric> <numeric> <numeric>
## FBgn0000008    95.14429    -0.262373  0.218505 -1.200767 0.2298414 0.536182
## FBgn0000014     1.05652     3.289885  2.052786  1.602644 0.1090133      NA
## FBgn0000017 4352.55357    -0.100020  0.112091 -0.892310 0.3722268 0.683195
## FBgn0000018   418.61048     0.229049  0.130261  1.758388 0.0786815 0.291789
## FBgn0000024     6.40620     0.306051  0.751286  0.407369 0.6837368 0.880472
## FBgn0000032   989.72022     0.237413  0.120286  1.973744 0.0484108 0.217658
```

When testing differential expression, typically raw counts and discrete distributions are used. However, for other downstream analyses, like visualisation or clustering, transforming the count data may be beneficial. DESeq2 already uses the log2 fold change as a means of analysis, but there are other ones to consider.

In this discussion, I will consider the variance stabilizing transformation (VST), as noted in Tibshirani (1988); Huber et al. (2003); Anders and Huber (2010) and the regularized logarithm (rlog), as described in Love, Huber, and Anders (2014). These methods take log2 transformed data and normalize the data with respect to specific factors. The key of these transformations is to remove the dependence of the variance of the mean. In particular, there is a higher variance of logarithm of count data when the mean is low. They reduce the variance in comparison to experiment-wide factors.

The code to transform the data with VST and rlog is shown below.

```
vst = vst(dds, blind = F)
rld = rlog(dds, blind = F)
```

One thing to note is the `blind` argument of the functions. This tells the system whether or not to consider parameters of the experiment or not when doing analyses. For instance, in the case of the `pasilla` data, there are known factors in place, like `condition` that can explain the change in gene expression. In this case, it would be best to let `blind = FALSE` because I want the system to consider `condition` when estimating counts.

Now, the VST stabilises the variance based on the size of the gene. The transformed data is on the log2 scale for large counts

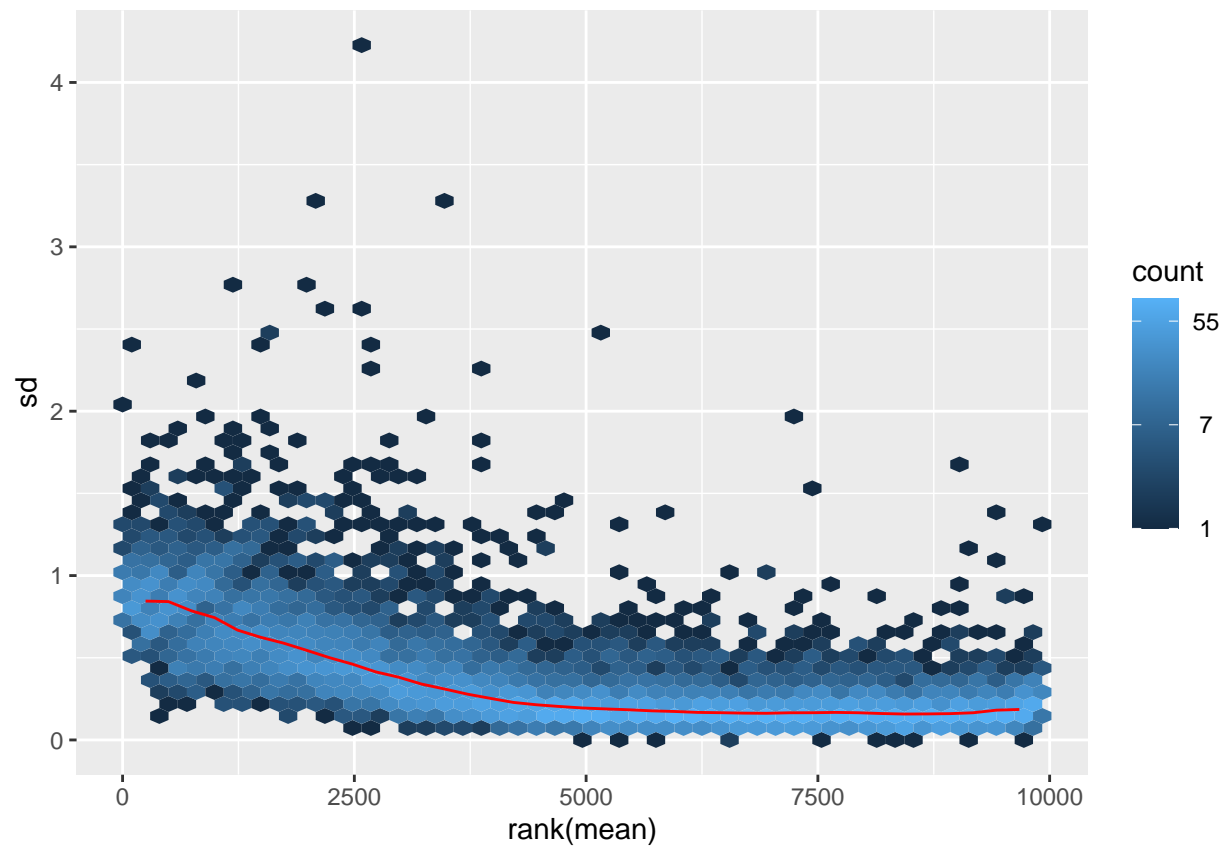
In comparison, the regularized log, takes the original count data to the log2 scale by fitting a model with a term for each sample and a prior distribution based on coefficients estimated from data. The formula is estimated as:

$$\log_2(q_{ij}) = \beta_{i0} + \beta_{ij}$$

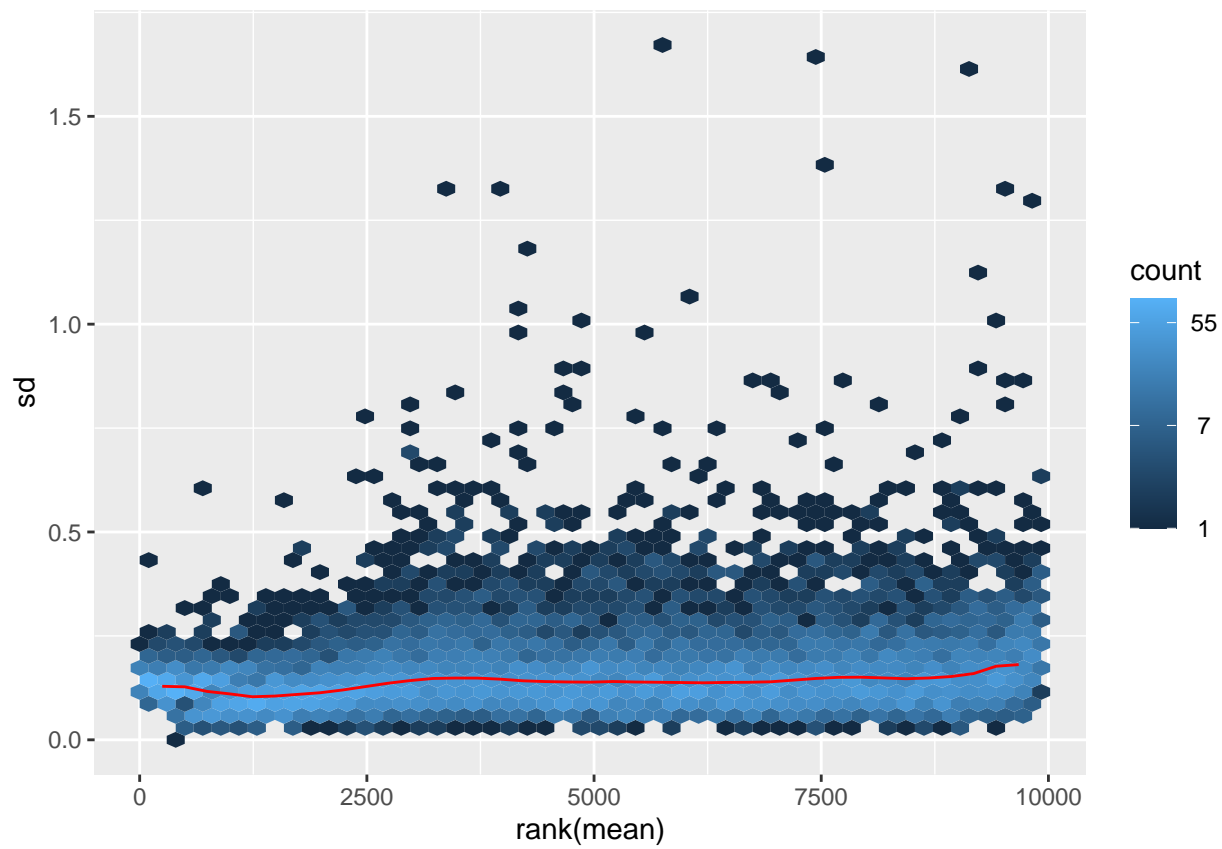
Here, q_{ij} is a parameter proportional to the expected true concentration of fragments for gene i and sample j , β_{i0} is like the background noise (there's always a little bit of error that cannot be explained) and therefore does not undergo shrinkage. β_{ij} is the sample specific coefficient which is shrunk toward zero based on the dispersion mean over the dataset. Because of its nature, `rlog` tends to have a larger effect of shrinkage.

Finally, it's time to plot these transformations. Below is code for transformed data across samples against the mean, using a shifted log transformation, the VST, and the rlog transformation.

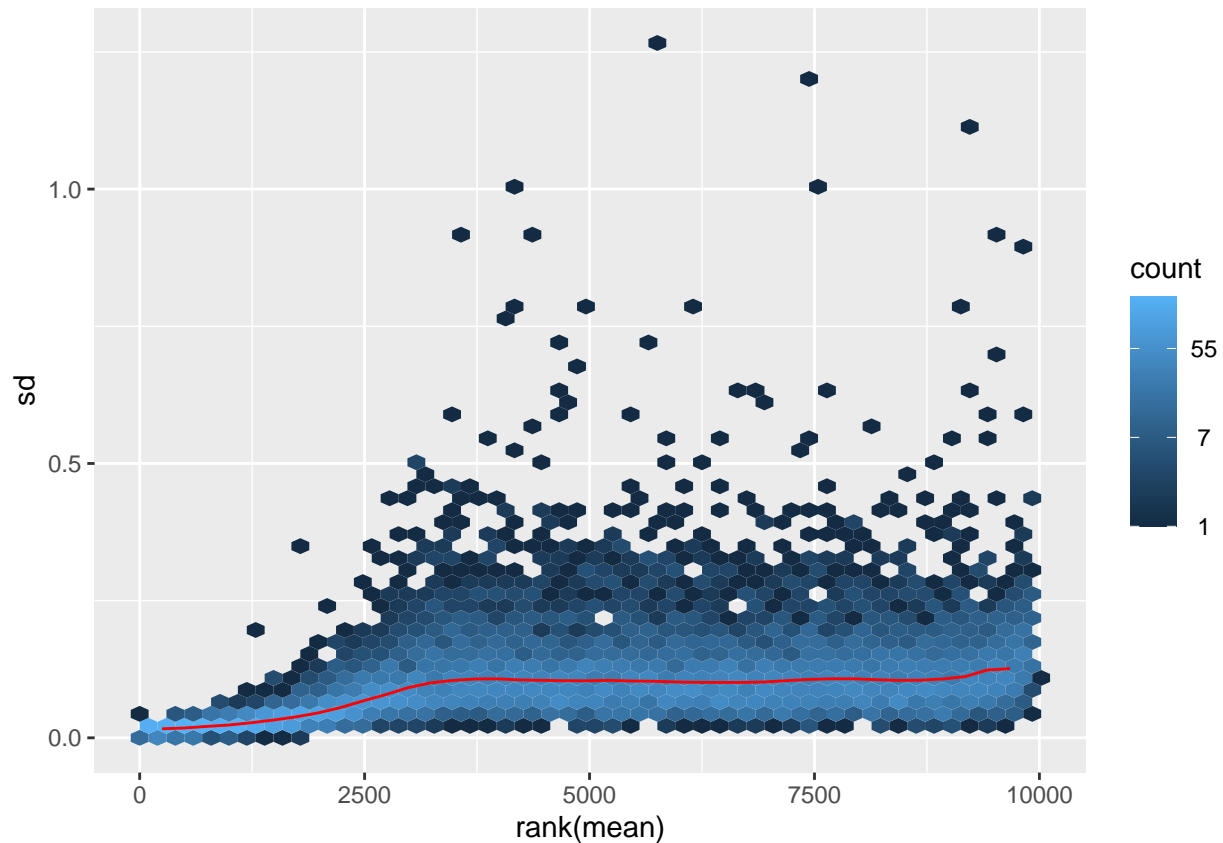

```
ntd = normTransform(dds)
library("vsn")
meanSdPlot(assay(ntd))
```



```
meanSdPlot(assay(vsd))
```



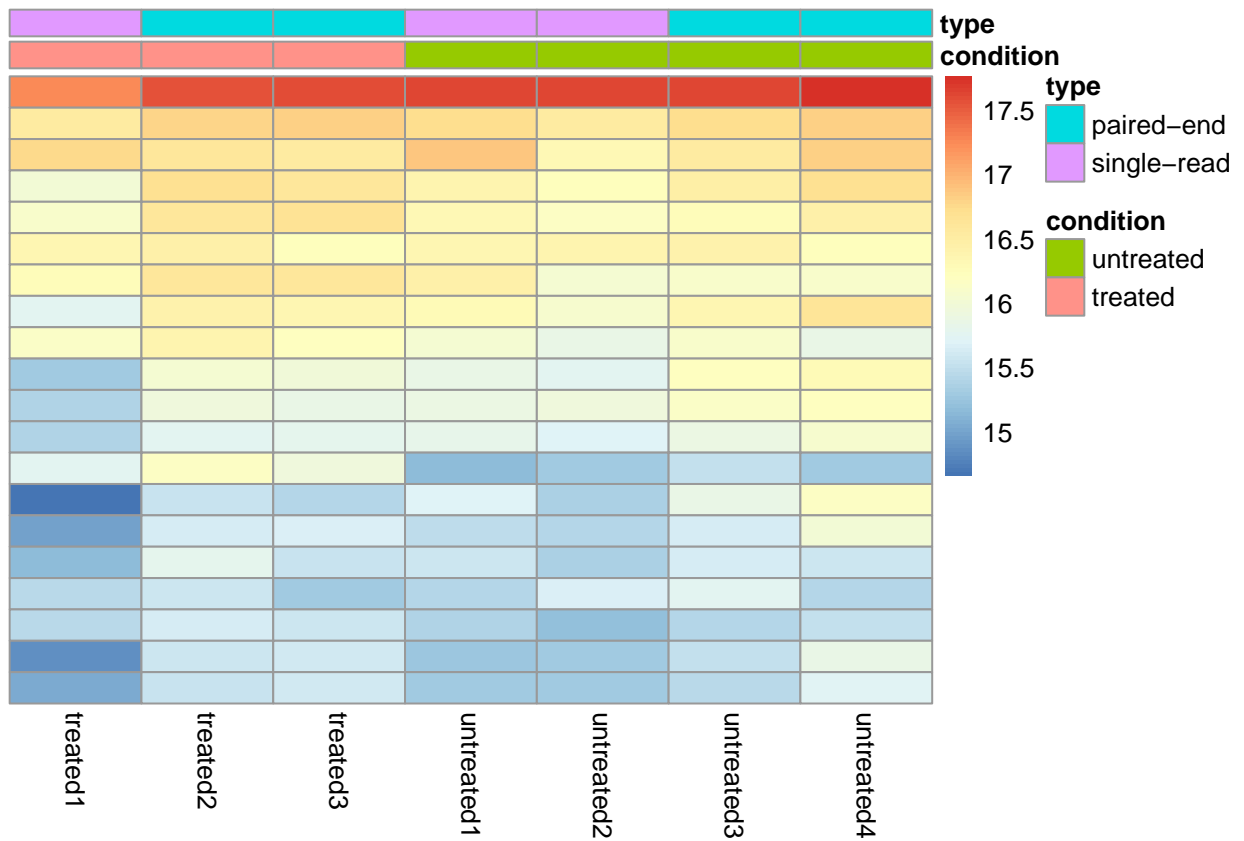
```
meanSdPlot(assay(rld))
```



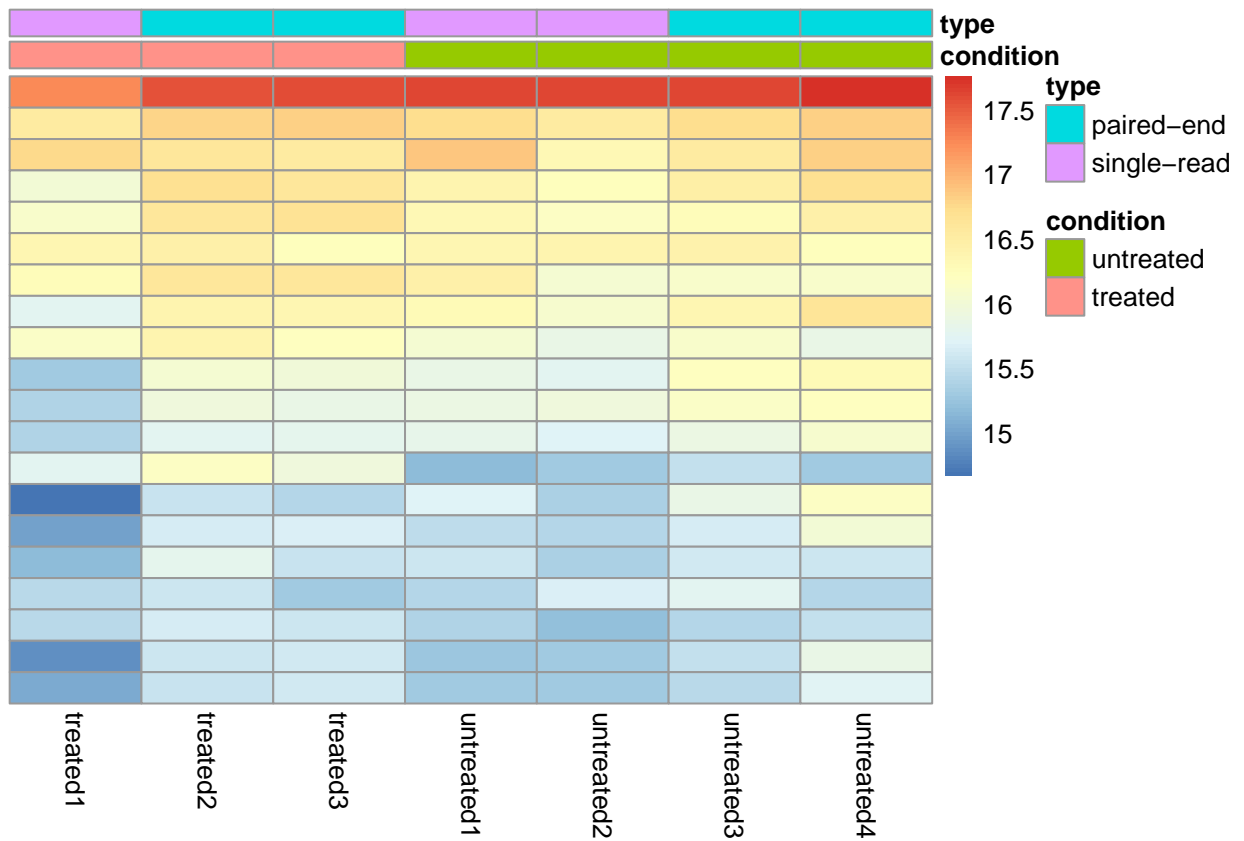
Another thing to do is to assess the quality of the data and remove bad data. I want to look in particular for samples where treatment showed abnormal results and would hurt downstream analyses.

Exploring count matrices for quality is best viewed as a heatmap. Below are heatmaps for the regular count matrix, the VST transformed count matrix, and the rlog transformed count matrix

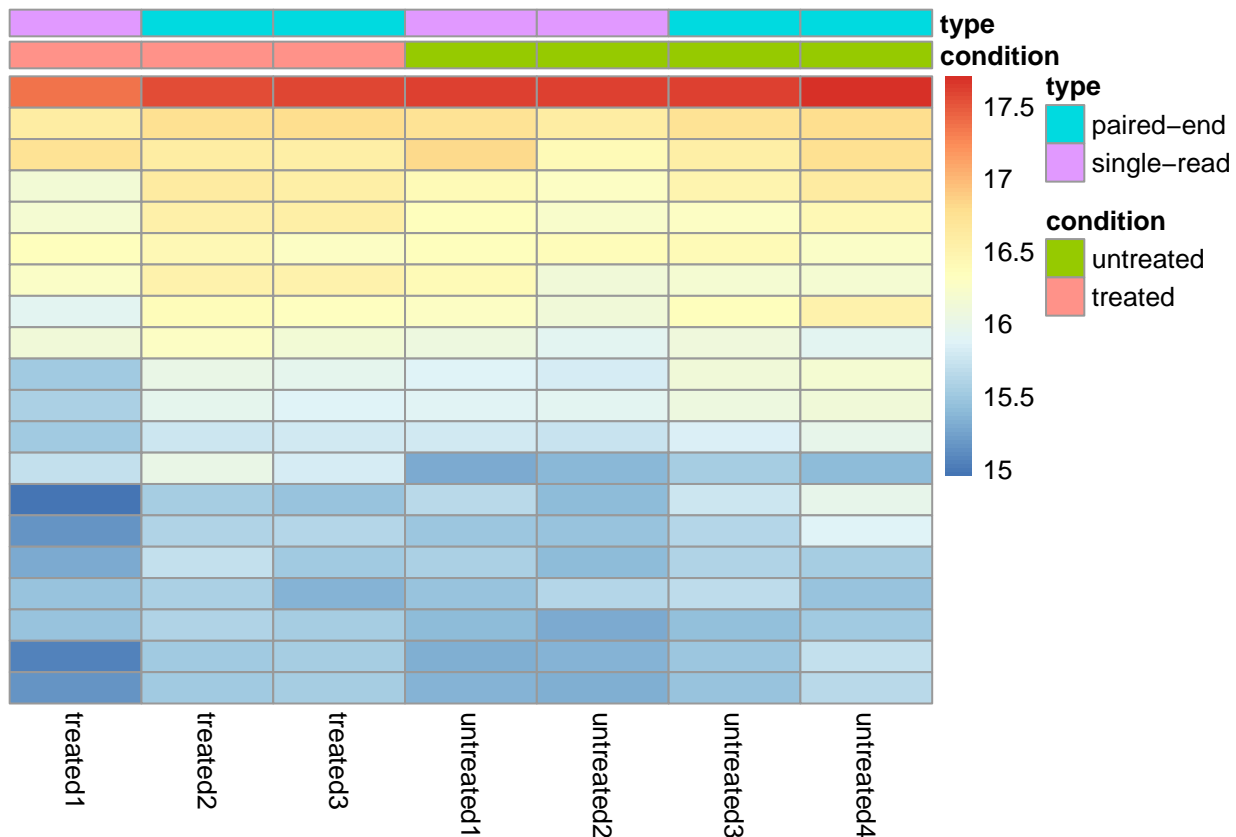
```
library(pheatmap)
select = order(rowMeans(counts(dds, normalized = T)),
               decreasing = T)[1:20]
df = as.data.frame(colData(dds)[,c("condition", "type")])
pheatmap(assay(ntd)[select,], cluster_rows = F, show_rownames = F,
         cluster_cols = F, annotation_col = df)
```



```
pheatmap(assay(vsd)[select,], cluster_rows = F, show_rownames = F,
          cluster_cols = F, annotation_col = df)
```



```
pheatmap(assay(rld)[select,], cluster_rows = F, show_rownames = F,
          cluster_cols = F, annotation_col = df)
```



Sample to sample distances

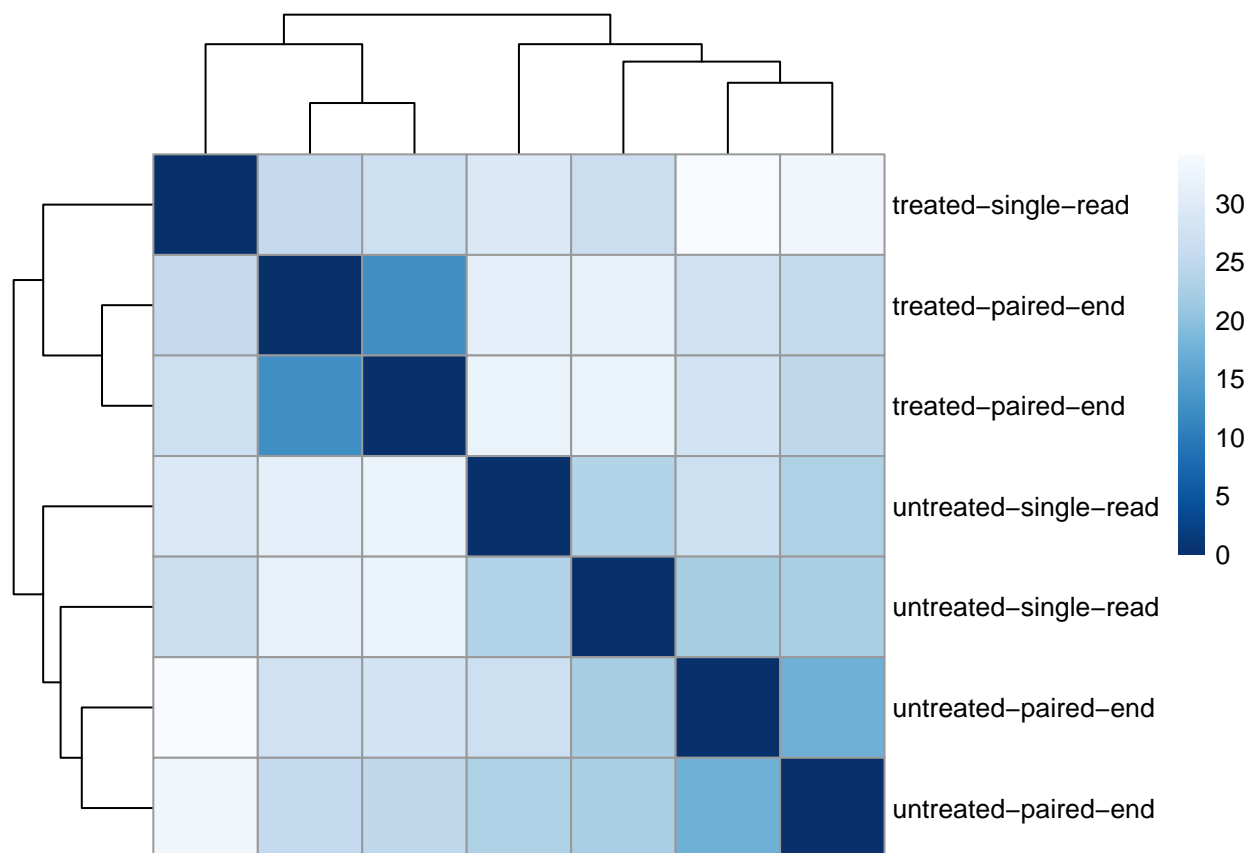
Another use of the transformed data is sample clustering. I can apply the `dist` function to the transpose of my transformed data to get distances between samples

```
(sampleDists = dist(t(assay(vsd))))
```

```
##          treated1 treated2 treated3 untreated1 untreated2 untreated3
## treated2  25.50386
## treated3  26.95680 12.56036
## untreated1 29.40565 31.01013 31.97611
## untreated2 26.55427 31.63020 32.23199  23.69489
## untreated3 34.19351 27.37454 27.85812  26.96435  22.49635
## untreated4 33.10003 25.39556 24.84995  23.17051  22.69880  17.51730
```

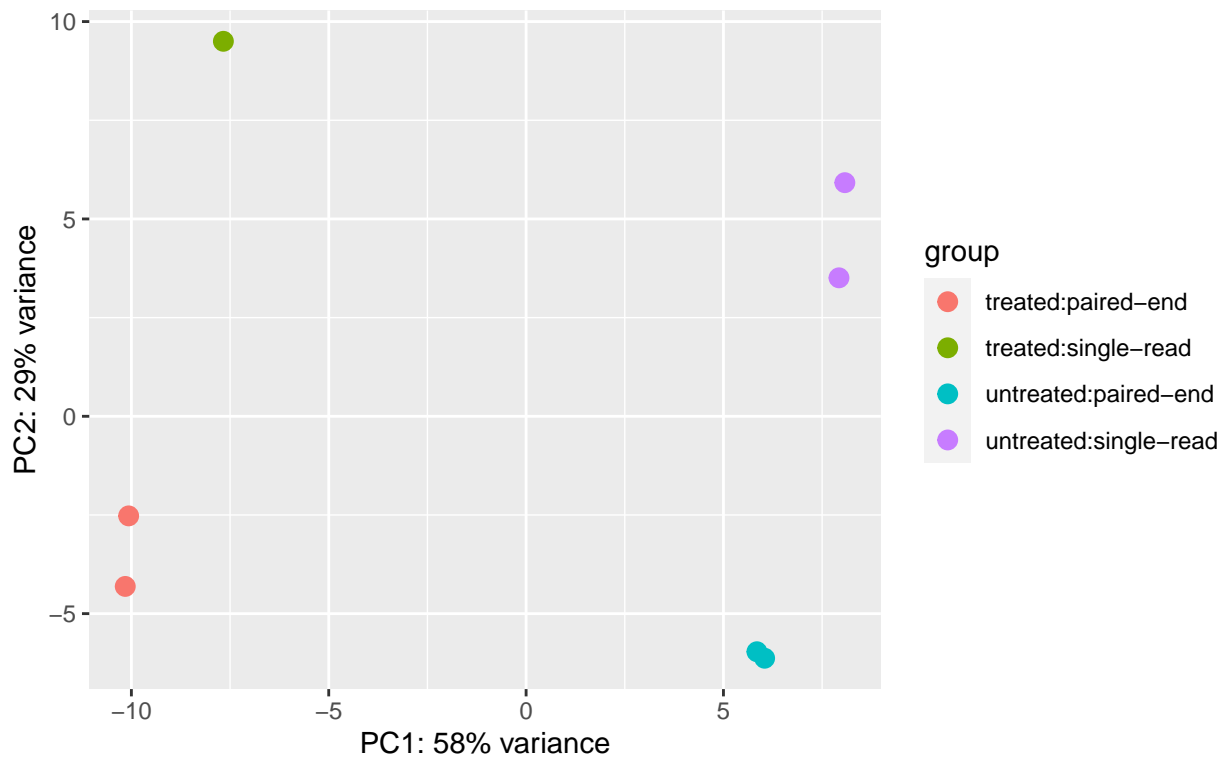
Then I can make a heatmap of the distances between samples.

```
library(RColorBrewer)
sampleDistMatrix = as.matrix(sampleDists)
rownames(sampleDistMatrix) = paste(vsd$condition, vsd$type, sep = "-")
colnames(sampleDistMatrix) = NULL
colors = colorRampPalette(rev(brewer.pal(9, "Blues")))(255)
pheatmap(sampleDistMatrix,
          clustering_distance_rows = sampleDists,
          clustering_distance_cols = sampleDists,
          col = colors)
```

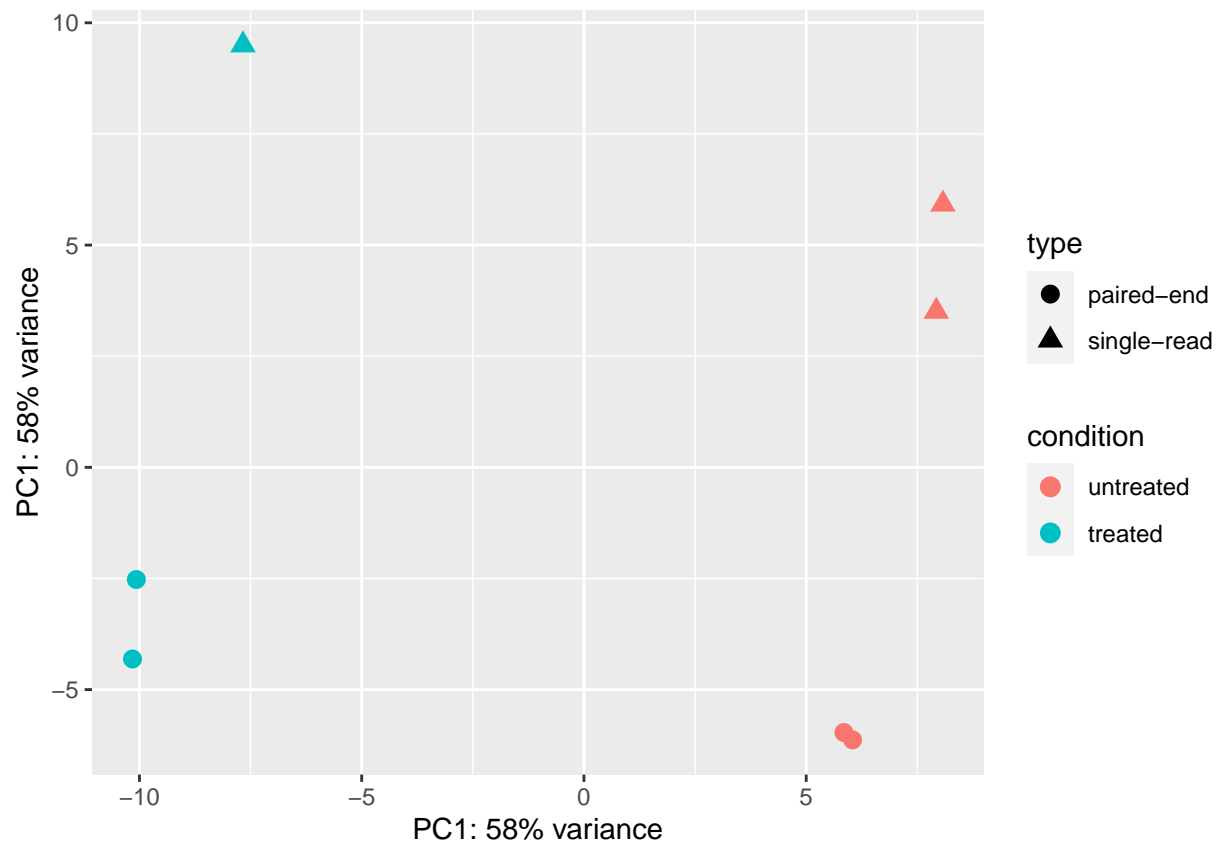


Related to the distance matrix is the PCA plot. This is useful for visualising the overall effect of experiment parameters.

```
plotPCA(vsd, intgroup = c("condition", "type"))
```



```
# Using ggplot
pcaData = plotPCA(vsd, intgroup = c("condition", "type"), returnData=T)
percentVar = round(100*attr(pcaData, "percentVar"))
ggplot(pcaData, aes(PC1, PC2, color = condition, shape = type)) +
  geom_point(size=3) +
  xlab(paste0("PC1: ", percentVar[1], "% variance")) +
  ylab(paste0("PC2: ", percentVar[2], "% variance")) +
  coord_fixed()
```

There's a wealth of even more information about DESeq2, but I think this is a good stopping place

Random Forest

The second test I'm using is the random forest. What I've found in my research is that this works a lot better as a selection algorithm of factors after running the results. I can take the average count of each gene, and build the random forest based on which treatment group gives the greatest change in expression. First thing I need to do is sort of clean up the data. I want to match up my original genes with there respective **baseMean** from the running the **DESeq** function. I converted everything to a tibble as it's a little bit easier to work with. Anywhere where the system didn't come up with a **baseMean** for a gene, I'm going to toss that out.

```
library(rsample)
library(randomForest)
library(randomForestExplainer)

RF = merge(cts, resMulti, by = "row.names", all.x = TRUE)
RF = as_tibble(RF)
(RF %<>% filter(!is.na(baseMean)) %>%
  dplyr::select(`Row.names`,
    untreated1, untreated2, untreated3, untreated4,
    treated1, treated2, treated3,
    baseMean))
```

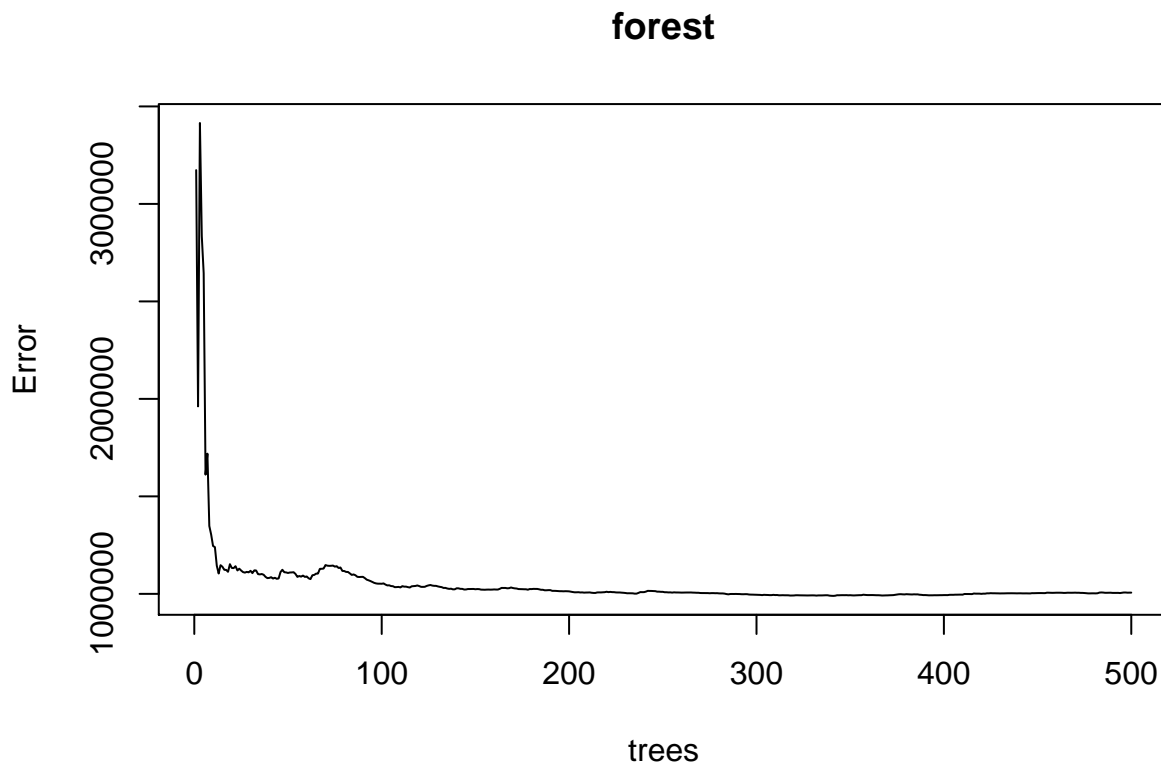
```
## # A tibble: 9,921 x 9
##   Row.names untreated1 untreated2 untreated3 untreated4 treated1 treated2
##   <I<chr>>      <int>      <int>      <int>      <int>      <int>      <int>
## 1 FBgn0000~      92        161         76         70        140         88
## 2 FBgn0000~       5          1          0          0          4          0
```

```
## 3 FBgn0000~      4664      8714      3564      3150      6205      3072
## 4 FBgn0000~       583       761       245       310       722       299
## 5 FBgn0000~        10        11         3         3        10         7
## 6 FBgn0000~     1446     1713      615      672     1698      696
## 7 FBgn0000~        15        25         9         5        20        14
## 8 FBgn0000~    101664    120163    45880    53201    127363    76099
## 9 FBgn0000~    33402    41118    16007    18360    56048    31421
## 10 FBgn0000~       21        63        15        13        64        28
## # ... with 9,911 more rows, and 2 more variables: treated3 <int>,
## #   baseMean <dbl>
```

I'll then take the counts for the respective treatment groups and build my random forest based on regression parameters. I'll then plot the amount of variance over the progression of the number of trees made.

```
set.seed(5391)
(forest = randomForest(baseMean ~., data = RF))

##
## Call:
## randomForest(formula = baseMean ~ ., data = RF)
##               Type of random forest: regression
##               Number of trees: 500
## No. of variables tried at each split: 2
##
##               Mean of squared residuals: 1006190
##               % Var explained: 95.48
plot(forest)
```



I will admit, this one is still a work in progress, I'm still thinking about which data from the set I should use to build the data. In addition, the parameter of **baseMean** being estimated by the treatment groups may not be the best way of going about it.

Weighted Logged Odds

The first thing I did was to read the data into the system. I have a count matrix and a file with the annotations for the data.

```
library("pasilla")
library(tidyverse)
library(tidylo)
library(tidyuesdayR)
library(tidytext)

pasCTS = system.file("extdata",
                     "pasilla_gene_counts.tsv",
                     package = "pasilla", mustWork = TRUE)

pasAnno = system.file("extdata",
                     "pasilla_sample_annotation.csv",
                     package = "pasilla", mustWork = TRUE)
```

I'm going to read my count matrix as a data frame and then take a look at the data

```
cts = as.data.frame(read.csv(pasCTS, sep = "\t"))
head(cts, 4)
```

```
##      gene_id untreated1 untreated2 untreated3 untreated4 treated1 treated2
## 1 FBgn0000003         0         0         0         0         0         0
## 2 FBgn0000008        92        161         76         70        140        88
## 3 FBgn0000014         5         1         0         0         4         0
## 4 FBgn0000015         0         2         1         2         1         0
##      treated3
## 1           1
## 2          70
## 3           0
## 4           0
```

I'll now use the principles of tidy data to arrange the data properly

```
cts2 = cts %>% pivot_longer(untreated1:treated3, names_to = "condition", values_to = "counts")
head(cts2, 10)
```

```
## # A tibble: 10 x 3
##   gene_id      condition  counts
##   <chr>      <chr>      <int>
## 1 FBgn0000003 untreated1         0
## 2 FBgn0000003 untreated2         0
## 3 FBgn0000003 untreated3         0
## 4 FBgn0000003 untreated4         0
## 5 FBgn0000003 treated1         0
## 6 FBgn0000003 treated2         0
## 7 FBgn0000003 treated3          1
## 8 FBgn0000008 untreated1        92
## 9 FBgn0000008 untreated2       161
## 10 FBgn0000008 untreated3        76
```

Now, apply the weighted log odds from the `tidylo` package. Now, the vignette tells me to use the `count` function to count the data. However, I'm already given the exon counts, so I'm going to set my *n* to the counts column. Finally, I'll arrange the data by the greatest to least log odds ratio

```
n = cts2$counts
cts2 = cts2 %>% bind_log_odds(gene_id, condition, n)
cts2 %>% arrange(-log_odds_weighted)
```

```
## # A tibble: 102,193 x 4
```

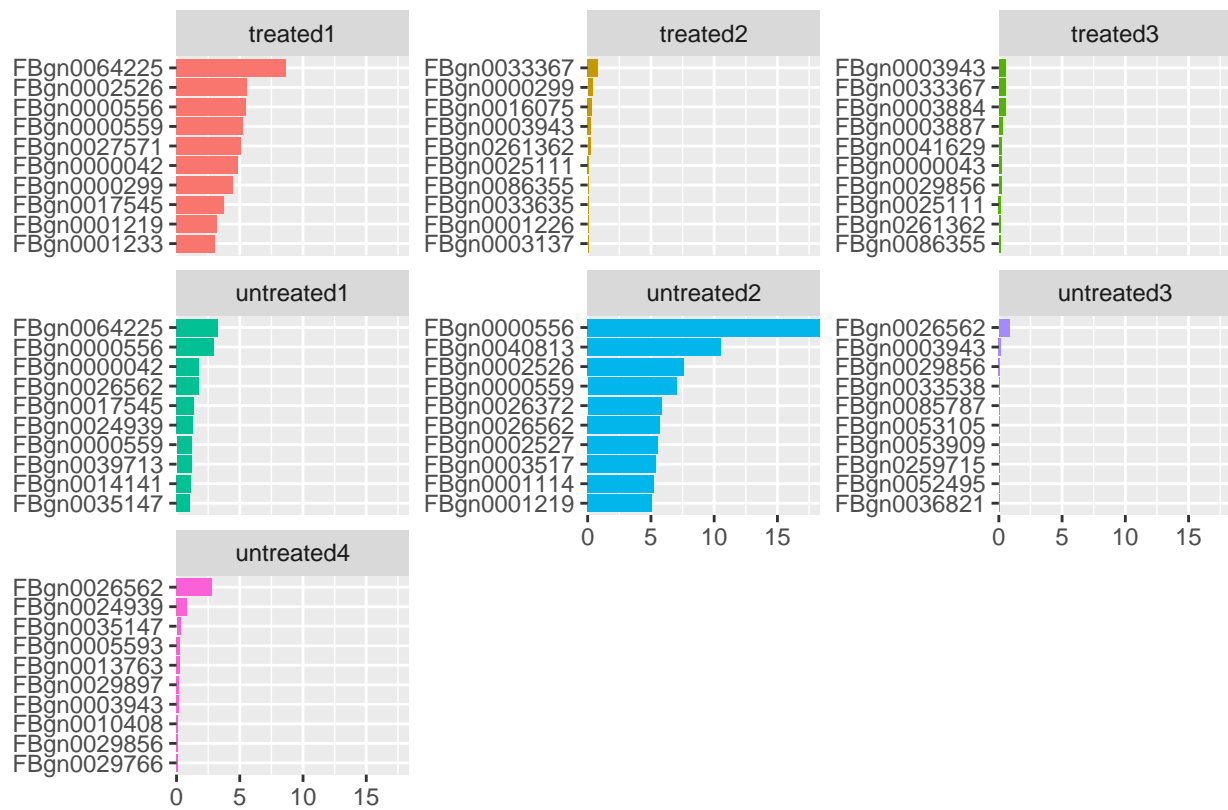
	gene_id	condition	counts	log_odds_weighted
	<chr>	<chr>	<int>	<dbl>
## 1	FBgn0000556	untreated2	360330	18.4
## 2	FBgn0040813	untreated2	139905	10.6
## 3	FBgn0064225	treated1	180986	8.62
## 4	FBgn0002526	untreated2	153771	7.60
## 5	FBgn0000559	untreated2	171656	7.03
## 6	FBgn0026372	untreated2	113692	5.84
## 7	FBgn0026562	untreated2	91503	5.73
## 8	FBgn0002527	untreated2	87970	5.58
## 9	FBgn0002526	treated1	136813	5.58
## 10	FBgn0000556	treated1	253500	5.48

```
## # ... with 102,183 more rows
```

And now to visualise the data, grouping by the sample with the largest log odds ratio based on treatment group

```
conditions <- c("untreated1", "untreated2", "untreated3", "untreated4",
               "treated1", "treated2", "treated3")

cts2 %>%
  group_by(condition) %>%
  top_n(10) %>%
  ungroup() %>%
  mutate(weight = reorder_within(gene_id, log_odds_weighted, condition)) %>%
  ggplot(aes(log_odds_weighted, weight, fill = condition)) +
  geom_col(show.legend = FALSE) +
  facet_wrap(~condition, scales = "free_y") +
  scale_y_reordered() +
  scale_x_continuous(expand = c(0, 0)) +
  labs(y = NULL, x = "Weighted log odds (empirical Bayes)")
```



Weighted log odds (empirical Bayes)