cbcbSEQ: RNAseq analysis for UMD CBCB collaborators

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1 Overview of cbcbSEQ pipeline

The purpose of this pipeline is to streamline the process for analyzing RNA-seq data with potential batch effects. The pipeline includes 1) quantile normalization 2) log-transformation of counts 3) ComBat (location) batch correction 4) voom calculation of weights.

The functions in this package can be grouped into two main categories:

1. The functions used for assessing batch effects.
   - `makeSVD`
   - `pcRes`
   - `plotPC`
2. The functions for removing batch effect and computing weights for limma.
   - `qNorm`
   - `log2CPM`
   - `voomMod`
   - `combatMod`
   - `batchSEQ`

`batchSEQ` is the pipeline function. It combines `qNorm`, `log2CPM`, `voomMod`, and `combatMod` into one step.

Below we will illustrte how to use these functions using the pasilla data set.

note: All the functions in this package have a detailed help file which tells you what kind of objects go in and what kind of objects come out. It is important to look at these help files for each function.

2 Examples of how to use the functions

We will use the `pasilla` dataset found in the `pasilla` package. (This is the same dataset used in the `DESeq` vignette)

```
> require(pasilla)
> # locate the path of the dataset and read in the dataset
> datafile = system.file("extdata/pasilla_gene_counts.tsv", package="pasilla")
> counts = read.table(datafile, header=TRUE, row.names=1)
> head(counts)
```

```
            untreated1 untreated2 untreated3 untreated4 treated1 treated2 treated3
FBgn0000003          0          0          0          0        0        0        1
FBgn0000008         92        161         76         70      140       88       70
FBgn0000014          5          1          0          0        4        0        0
FBgn0000015          0          2          1          2        1        0        0
FBgn0000017       4664       8714       3564       3150     6205     3072     3334
FBgn0000018        583        761        245        310      722      299      308
```

```
> dim(counts)
```

```
[1] 14599     7
```

```
> counts = counts[rowSums(counts) > ncol(counts),]
> dim(counts)
```

```
[1] 10153     7
```

In this dataset there are two biological conditions: treated (3 samples) and untreated (4 samples). Two samples are single-end and the other 4 are paired-end. We will use sigle-end and paired-end as illustration of batch effects. Below is the experiment design matrix (pheno data.frame).

```
> design = data.frame(row.names=colnames(counts),
+                      condition=c("untreated","untreated","untreated",
+                                  "untreated","treated","treated","treated"),
+                      libType=c("single-end","single-end","paired-end",
+                                "paired-end","single-end","paired-end","paired-end"))
> design
```

```
           condition    libType
untreated1 untreated single-end
untreated2 untreated single-end
untreated3 untreated paired-end
untreated4 untreated paired-end
treated1     treated single-end
treated2     treated paired-end
treated3     treated paired-end
```

2.1 Explore data for batch effects

We will begin our analysis by exploring the data for possible/significant batch effects. We implemented here some of the analysis methods outlined in Leek et al. [2].

```
> # load batch package
> require(cbcbSEQ)
> #
> # quantile normalize: adjust counts for library size.
> qcounts = qNorm(counts)
> # convert counts to log2 counts per milliom. (voom scale)
> cpm = log2CPM(qcounts)
> names(cpm)
```

```
[1] "y"        "lib.size"
```

```
> libsize = cpm$lib.size
> cpm = cpm$y
> #
> # PCA analysis
> # returns a list with two components v and d.
> res = makeSVD(cpm)
```

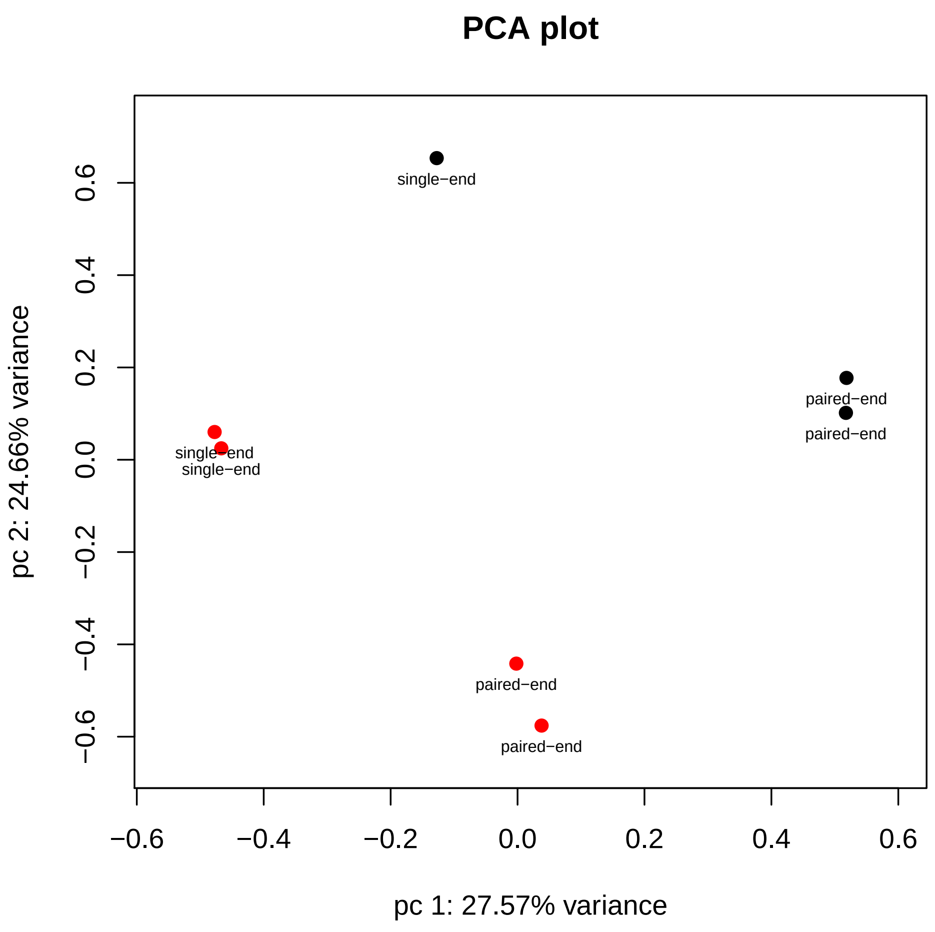
We can now call pcRes and plotPC.

- pcRes: computes variance of each principal component and how they ”correlate” with batch and condition.

  ```
  > pcRes(res$v,res$d, design$condition, design$libType)

    propVar cumPropVar cond.R2 batch.R2
  1   27.57      27.57   48.13    67.00
  2   24.66      52.23   50.74    31.82
  3   15.62      67.85    0.57     0.04
  4   12.15      80.00    0.05     0.35
  5   10.53      90.53    0.14     0.14
  6    9.46      99.99    0.37     0.65
  ```

- plotPC: Plot first 2 principal components. This function works like the regular plot function in R. ie. We can add all the options to make the plot sensible and well labelled. Below is an example:

  ```
  > plotPC(res$v,res$d,
  +        col=design$condition, # color by batch
  +        pch=19, main="PCA plot",
  +        xlim=c(min(res$v[,1])-.08,max(res$v[,1])+.08),
  +              ylim=c(min(res$v[,2])-.08,max(res$v[,2])+.08))
  > text(res$v[,1], res$v[,2], design$libType, pos=1, cex=0.6)
  ```



We see that there is a batch effect in the data. Both in the PCA "correlation" table and the PCA plot.

2.2 Correct data for batch effects

A standard way of accounting for batch effects in data analysis is to include batch indicators as covariates in a linear model (e.g., in `limma` with weights computed by `voom` to model heteroskedasticity through a mean-variance relationship). However, in some cases we may want to obtain robust estimates of batch effects using a hierarchical model like ComBat [1]. However, we made some modifications to Combat. The most significant is that we do not estimate or adjust for batch scale effect due to heterskedasticity. In order to account for scaling we have to take into account the mean var relationship inherent in this kind of data (we're working on it, but it's not done yet). We adjust data by removing the empirical bayesian estimates of batch location effects.

```
> # combatMod function
> # noScale=TRUE option not to scale adjust
> tmp = combatMod(cpm, batch=design$libType, mod=design$condition, noScale=TRUE)
```

```
Found 2 batches
Found 1  categorical covariate(s)
Standardizing Data across genes
Fitting 'shrunk' batch 1 effects
Fitting 'shrunk' batch 2 effects
Adjusting data for batch effects
```

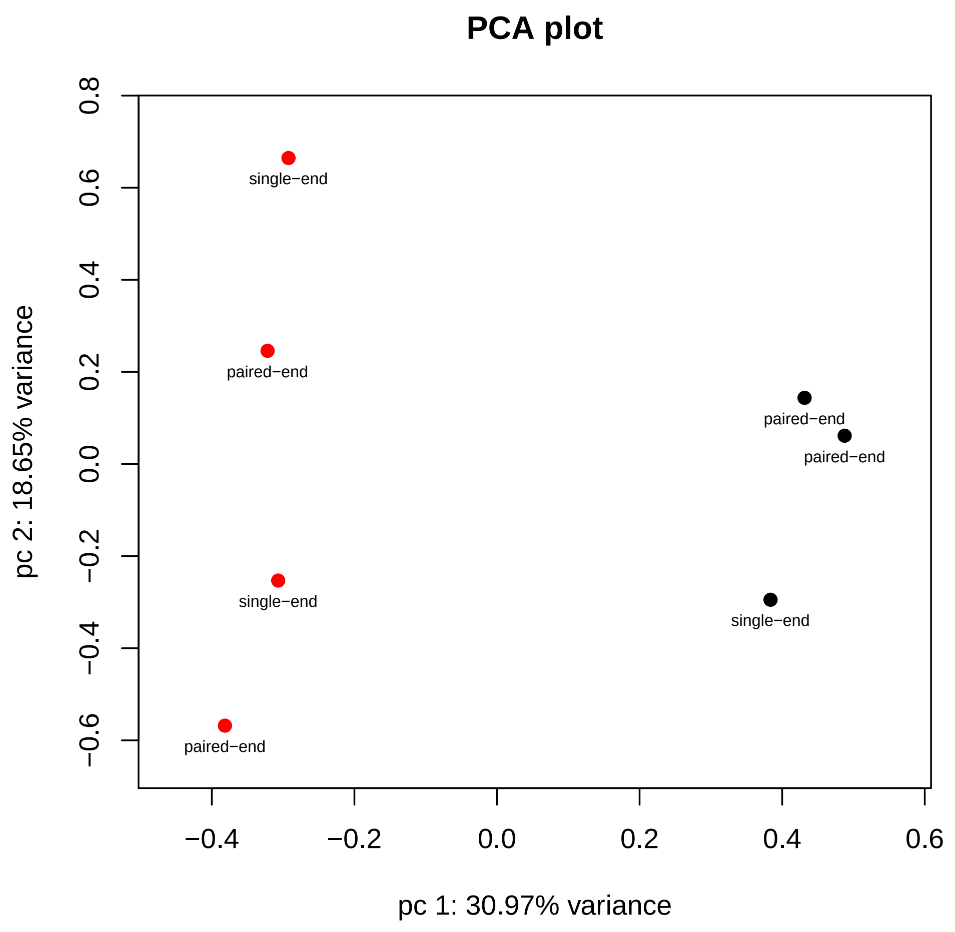
```
> names(tmp)
```

```
[1] "bayesdata" "info"
```

```
> tmp = tmp$bayesdata
> # look at PCA results again
> res = makeSVD(tmp)
> # batch effect is reduced
> pcRes(res$v,res$d, design$condition, design$libType)
```

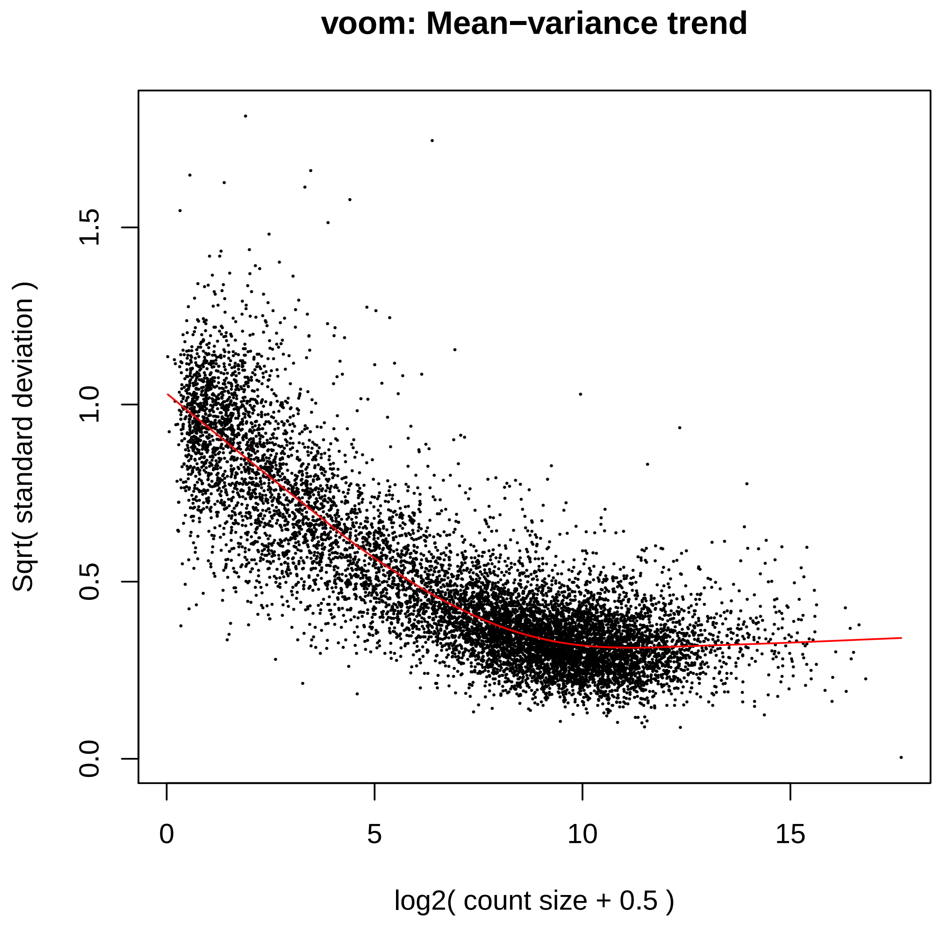
```
  propVar cumPropVar cond.R2 batch.R2
1   30.97      30.97   99.00     2.71
2   18.65      49.62    0.47     0.80
3   14.69      64.31    0.02     5.89
4   12.65      76.96    0.04    10.40
5   12.09      89.05    0.30    46.56
6   10.94      99.99    0.18    33.64
```

```
> plotPC(res$v,res$d,
+        col=design$condition, # color by batch
+        pch=19, main="PCA plot",
+        xlim=c(min(res$v[,1])-.08,max(res$v[,1])+.08),
+        ylim=c(min(res$v[,2])-.08,max(res$v[,2])+.08))
> text(res$v[,1], res$v[,2], design$libType, pos=1, cex=0.6)
```



We are now ready to use `limma` and `voom`. We also modified the `voom` function so it takes data on log-scale as input.

```
> v = voomMod(tmp, model.matrix(~design$condition), lib.size=libsize, plot=TRUE)
```



```
> v
```

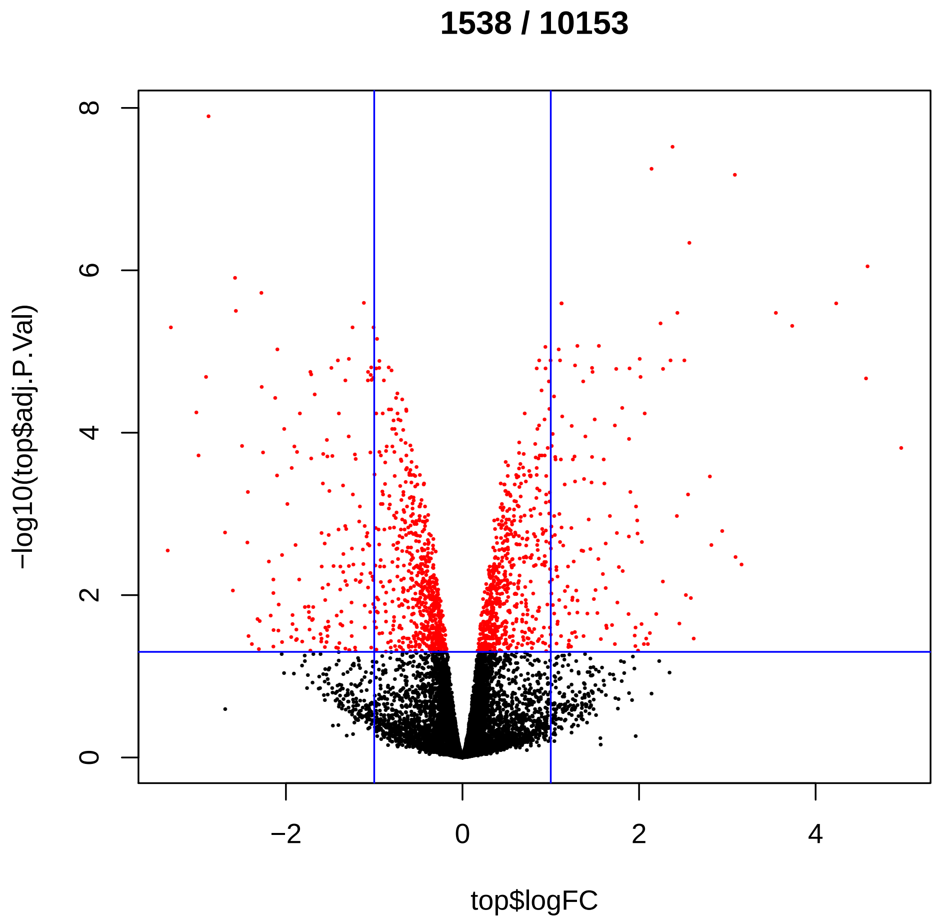
```
An object of class "EList"
$E
            untreated1 untreated2 untreated3 untreated4  treated1   treated2   treated3
FBgn0000008  2.9772407  3.0375781   3.259578   2.852434  2.847232  3.1729673  2.7072849
FBgn0000014 -1.2338011 -4.2500923  -3.970097  -3.970114 -2.500071 -3.9701281 -3.9701469
FBgn0000017  8.3918375  8.6390002   8.703652   8.391730  8.373702  8.3253415  8.3401799
FBgn0000018  5.2548863  5.0144051   5.067631   5.157916  5.045165  5.1067903  5.0125956
FBgn0000024 -0.3373151 -0.9737137  -1.280256  -1.320351 -1.124131 -0.2509431 -0.8702176
10148 more rows ...

$weights
          [,1]      [,2]      [,3]      [,4]       [,5]        [,6]        [,7]
```

```
[1,] 26.520373 26.520219 26.519769 26.520017  24.814440  24.8144274  24.8146913
[2,]  1.017667  1.017662  1.017650  1.017657   0.970376   0.9703756   0.9703826
[3,] 99.583486 99.583548 99.583731 99.583630 100.681377 100.6813824 100.6812770
[4,] 71.619883 71.619617 71.618838 71.619267  69.924363  69.9243415  69.9247981
[5,]  2.838734  2.838720  2.838677  2.838700   3.176932   3.1769302   3.1769600
10148 more rows ...

$design
  (Intercept) design$conditionuntreated
1           1                         1
2           1                         1
3           1                         1
4           1                         1
5           1                         0
6           1                         0
7           1                         0
attr(,"assign")
[1] 0 1
attr(,"contrasts")
attr(,"contrasts")$`design$condition`
[1] "contr.treatment"


$lib.size
untreated1 untreated2 untreated3 untreated4   treated1   treated2   treated3
  13237697   13237599   13237313   13237471   13237605   13237597   13237770
```

```
> fit = lmFit(v)
> eb = eBayes(fit)
> top = topTable(eb, coef=2, n=nrow(v$E))
```

Plot results as a volcano plot

```
> sel = top$adj.P.Val < 0.05
> plot(top$logFC, -log10(top$adj.P.Val), pch=16, cex=0.3,
+      main=paste(sum(sel), "/", length(sel)),col=ifelse(sel,"red","black"))
> abline(v=c(-1,1), h=-log10(0.05), col="blue")
```



Let us now compare the results to what we get when we adjust for batch in the model

```
> cond=design$condition
> batch=design$libType
> mod = model.matrix(~cond+batch ,
+                     contrasts.arg=list(cond="contr.treatment", batch="contr.sum"))
> v1 = voom(counts, mod)
> fit1 = lmFit(v1)
> eb1 = eBayes(fit1)
> top1 = topTable(eb1, coef=2, n=nrow(v1$E))
```

Compare results:

```
> tab = merge(top[,c("ID", "adj.P.Val")], top1[,c("ID", "adj.P.Val")], by="ID")
> as.data.frame(table(combat = tab[,2] < 0.05, model = tab[,3] < 0.05))
```

```
  combat model Freq
1  FALSE FALSE 8516
2   TRUE FALSE  401
3  FALSE  TRUE   99
4   TRUE  TRUE 1137
```

After correction with modified ComBat, there are a few more differentially abundant genes.

References

[1] W Evan Johnson, Cheng Li, and Ariel Rabinovic. Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics (Oxford, England)*, 8(1):118–127, January 2007.

[2] Jeffrey T Leek, Robert B Scharpf, Héctor Corrada Bravo, David Simcha, Benjamin Langmead, W Evan Johnson, Donald Geman, Keith Baggerly, and Rafael A Irizarry. Tackling the widespread and critical impact of batch effects in high-throughput data. *Nature reviews Genetics*, 11(10):733–739, October 2010.

SessionInfo

- R version 3.0.1 (2013-05-16), `x86_64-apple-darwin10.8.0`
- Locale: `en_US.utf-8/en_US.utf-8/en_US.utf-8/C/en_US.utf-8/en_US.utf-8`
- Base packages: base, datasets, graphics, grDevices, methods, parallel, stats, utils
- Other packages: Biobase 2.20.0, BiocGenerics 0.6.0, BiocInstaller 1.10.1, cbcbSEQ 0.9, corpcor 1.6.6, DESeq 1.12.0, devtools 1.2, DEXSeq 1.6.0, lattice 0.20-15, limma 3.16.5, locfit 1.5-9.1, mgcv 1.7-22, pasilla 0.2.16, preprocessCore 1.22.0, RColorBrewer 1.0-5, sva 3.6.0
- Loaded via a namespace (and not attached): annotate 1.38.0, AnnotationDbi 1.22.6, biomaRt 2.16.0, Biostrings 2.28.0, bitops 1.0-5, DBI 0.2-7, digest 0.6.3, evaluate 0.4.3, genefilter 1.42.0, geneplotter 1.38.0, GenomicRanges 1.13.4, grid 3.0.1, httr 0.2, hwriter 1.3, IRanges 1.19.1, Matrix 1.0-12, memoise 0.1, nlme 3.1-109, RCurl 1.95-4.1, Rsamtools 1.12.3, RSQLite 0.11.4, splines 3.0.1, statmod 1.4.17, stats4 3.0.1, stringr 0.6.2, survival 2.37-4, tools 3.0.1, whisker 0.3-2, XML 3.95-0.2, xtable 1.7-1, zlibbioc 1.6.0