Dismantling the bulk: examining neuronal heterogeneity using single-cell techniques

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Hour II: Quality control and normalization

The readout from a RNA-Seq experiment is a mixture of a **biological** and **technical** sources.

The goal of quality control is to capture **metrics** and evaluate the **technical quality** of each sample, and help decide which samples to keep and which to exclude from the analysis.

The data from samples with enough quality still carry features that have to do with the technical processing of samples and are unrelated to the biology.

The goal of normalization is to apply a **transformation** to the data to factor out the technical component, making the samples **comparable**.

We will start with quality control, evaluating a set of metrics.

Load libraries

We load R libraries we will use: ggplot2 for general plotting, and scone to evaluate normalization strategies.

```r
library(scone)
library(ggplot2)
```

Load data

gene_counts is a data frame containing unnormalized read counts per gene

ercc_counts is a data frame containing raw read counts per ERCC spike-in

cells is a data frame containing metadata for each cell

```r
gene_counts <- read.csv("../../_m/genes_counts.csv", stringsAsFactors = FALSE, header=TRUE, row.names =
ercc <- read.csv("../../_m/ercc_counts.csv", stringsAsFactors = FALSE, header = TRUE, row.names=1)
cells <- read.csv("../../_m/cell_metadata.csv", stringsAsFactors = FALSE, header = TRUE)

whichTomato <- grep("tdTomato", rownames(ercc))
ercc <- ercc[-whichTomato,]
```

Examine sizes of data frames

gene_counts: 24057 genes x 1679 cells

ercc: 92 spike-ins x 1679 cells

cells: 1679 cells x 16 metadata fields

```r
dim(gene_counts)
```

```
## [1] 24057  1679
```

```r
dim(ercc)
```

```
## [1]   92 1679
```

```r
dim(cells)
```

```
## [1] 1679   16
```

Examine metadata

Some of the metadata fields contain information about the biological sample, some about sequencing metrics.

```r
knitr::kable(head(cells))
```

long_name	cre	collection_date	sequencing_type	total_reads	all_mapped_percent	mRNA_percent	genom
A01101401	Calb2	11/18/2013	hiseq	23770190	93.50	54.43	
A01101402	Calb2	11/18/2013	hiseq	9694719	92.86	45.69	
A01101403	Calb2	11/18/2013	hiseq	5864322	90.55	48.30	
A01101404	Calb2	11/18/2013	hiseq	22102121	93.25	51.41	
A01101405	Calb2	11/18/2013	hiseq	24057147	93.14	51.06	
A01101406	Calb2	11/18/2013	hiseq	24171169	92.18	49.31	

Select fields from metadata that are useful for QC

Select fields related to sequencing and mapping

```r
qc <- cells[,c('total_reads', 'all_mapped_percent', 'mRNA_percent', 'ercc_percent', 'tdt_permillion')]
rownames(qc) <- cells$long_name
```

Add two more fields to the qc dataframe: number of genes detected and number of ERCC spike-ins detected

```r
all.equal(cells$long_name, colnames(gene_counts)) && all.equal(cells$long_name, colnames(ercc))
```

```
## [1] TRUE
```

```r
qc$ercc_detected = colSums(ercc > 0)
```

```r
qc$genes_detected = colSums(gene_counts > 0)
```

Here's how the QC dataframe looks like

```r
knitr::kable(head(qc))
```

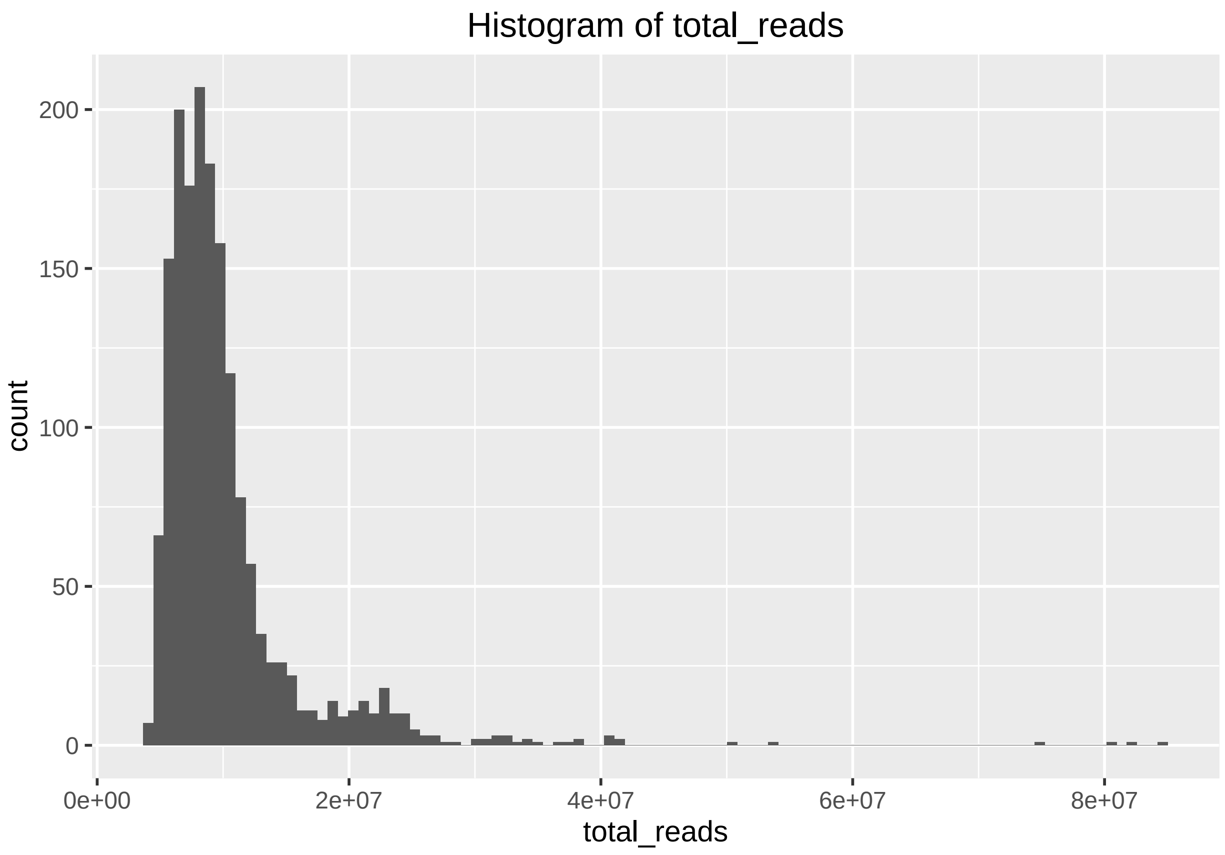
	total_reads	all_mapped_percent	mRNA_percent	ercc_percent	tdt_permillion	ercc_detected	gen
A01101401	23770190	93.50	54.43	4.36	306.1	34	
A01101402	9694719	92.86	45.69	7.84	341.2	29	

	total_reads	all_mapped_percent	mRNA_percent	ercc_percent	tdt_permillion	ercc_detected	gen
A01101403	5864322	90.55	48.30	4.12	106.2	33	
A01101404	22102121	93.25	51.41	4.24	371.1	41	
A01101405	24057147	93.14	51.06	4.98	264.2	34	
A01101406	24171169	92.18	49.31	3.14	205.8	39	

Plot the distribution of these metrics

```
options(repr.plot.width=8, repr.plot.height=8)
```

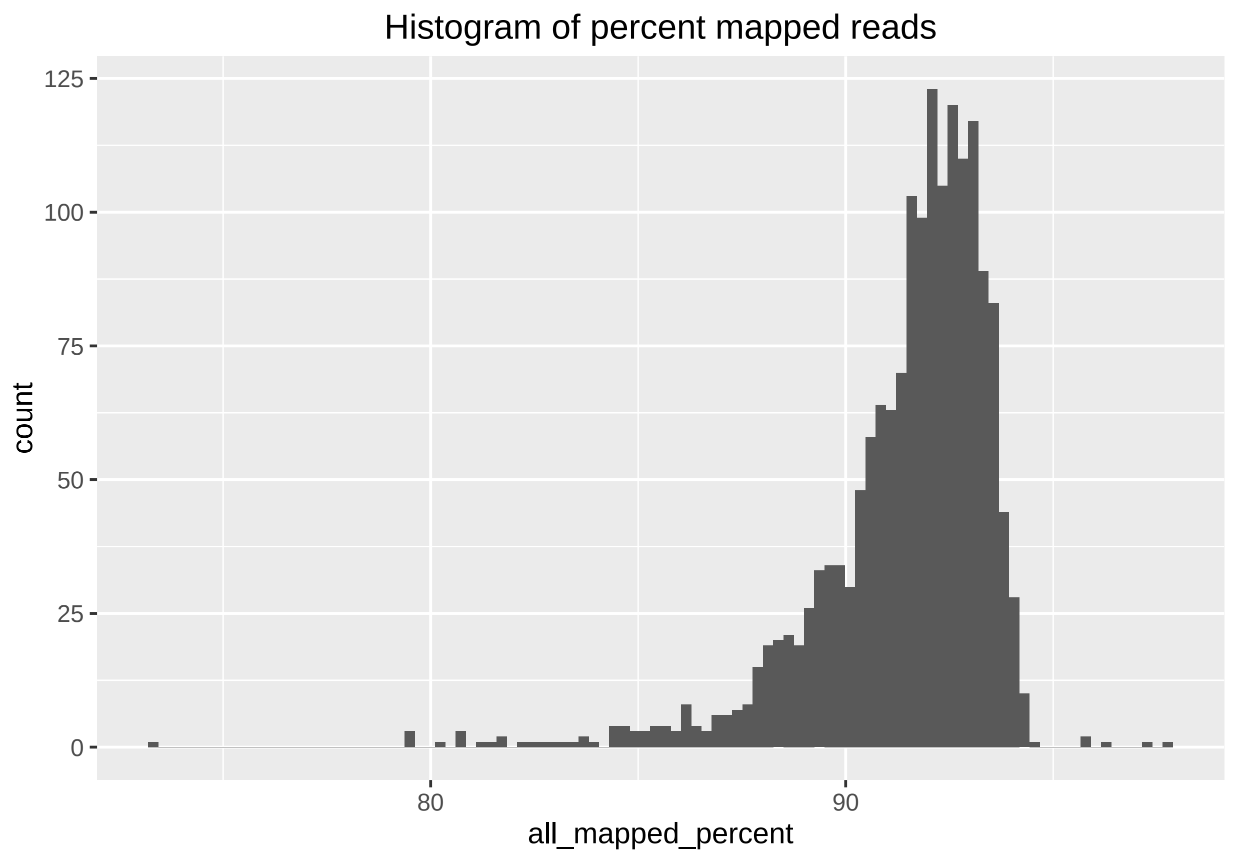
```
ggplot(cells, aes(x=total_reads)) + geom_histogram(bins=100) + ggtitle('Histogram of total_reads')
```



```
summary(qc$total_reads)
```

```
##     Min.  1st Qu.   Median     Mean  3rd Qu.     Max.
##  3782000  6901000  8667000 10240000 10930000 84330000
```

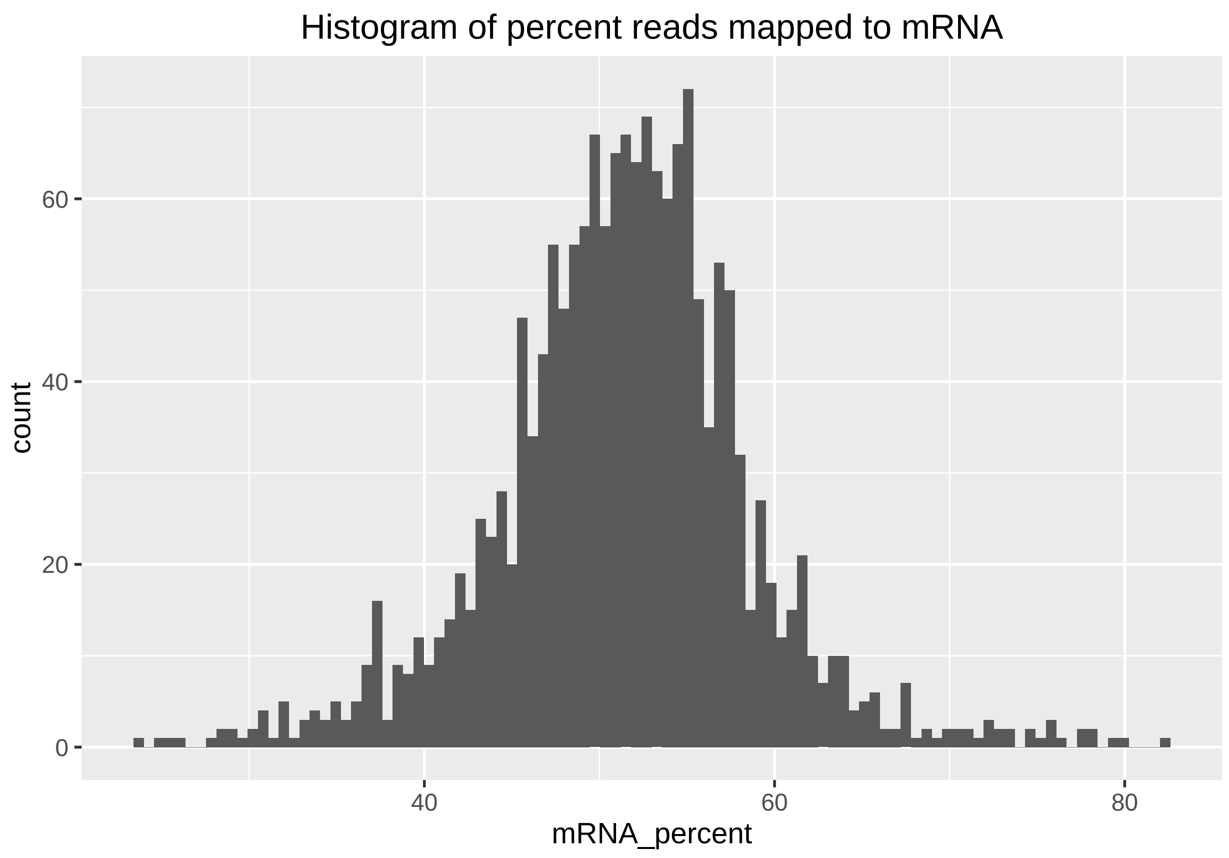
```
ggplot(qc, aes(x=all_mapped_percent)) + geom_histogram(bins=100)  + ggtitle('Histogram of percent mappe
```



```
summary(qc$all_mapped_percent)
```

```
##    Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
##   73.36   90.66   91.95   91.43   92.85   97.80
```

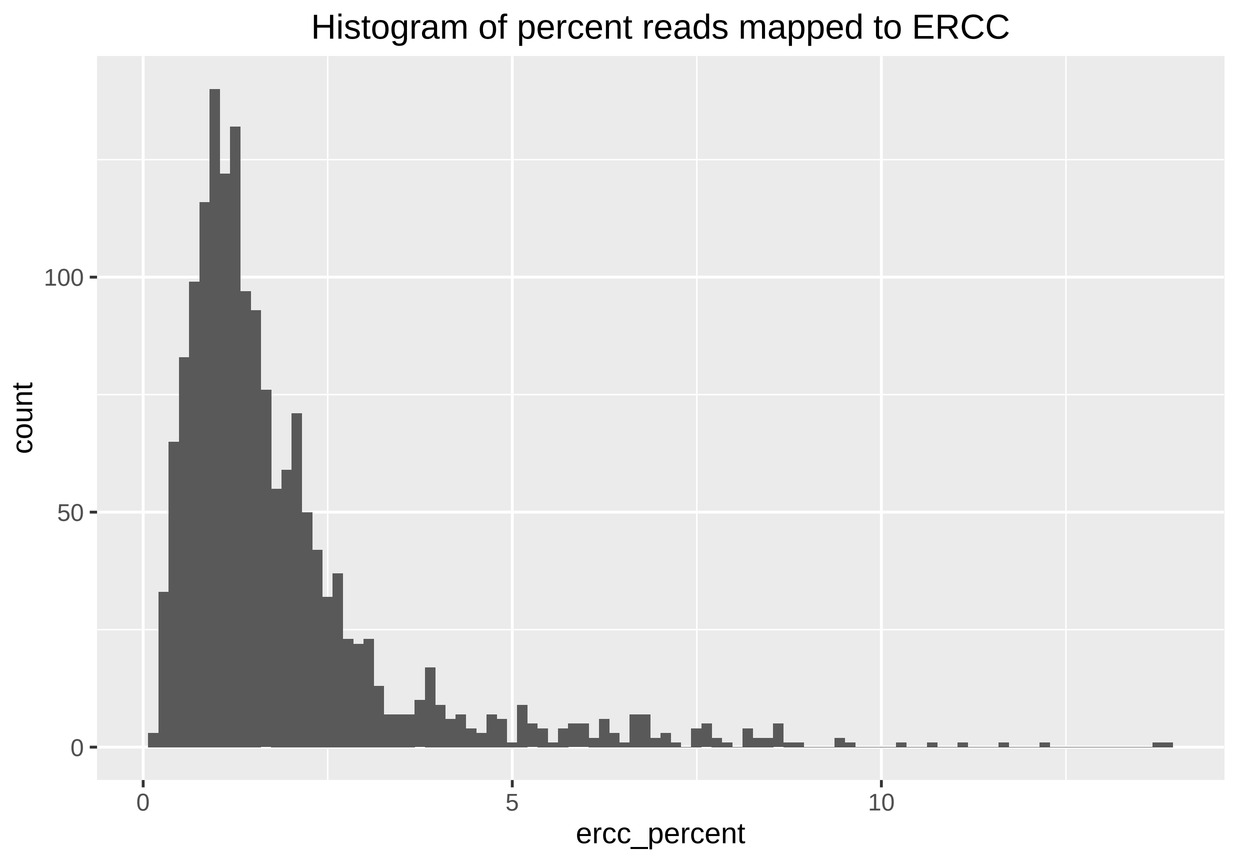
```
ggplot(qc, aes(x=mRNA_percent)) + geom_histogram(bins=100) + ggtitle('Histogram of percent reads mapped
```



```
summary(qc$mRNA_percent)
```

```
##    Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
##   23.94   47.36   51.59   51.39   55.39   82.56
```

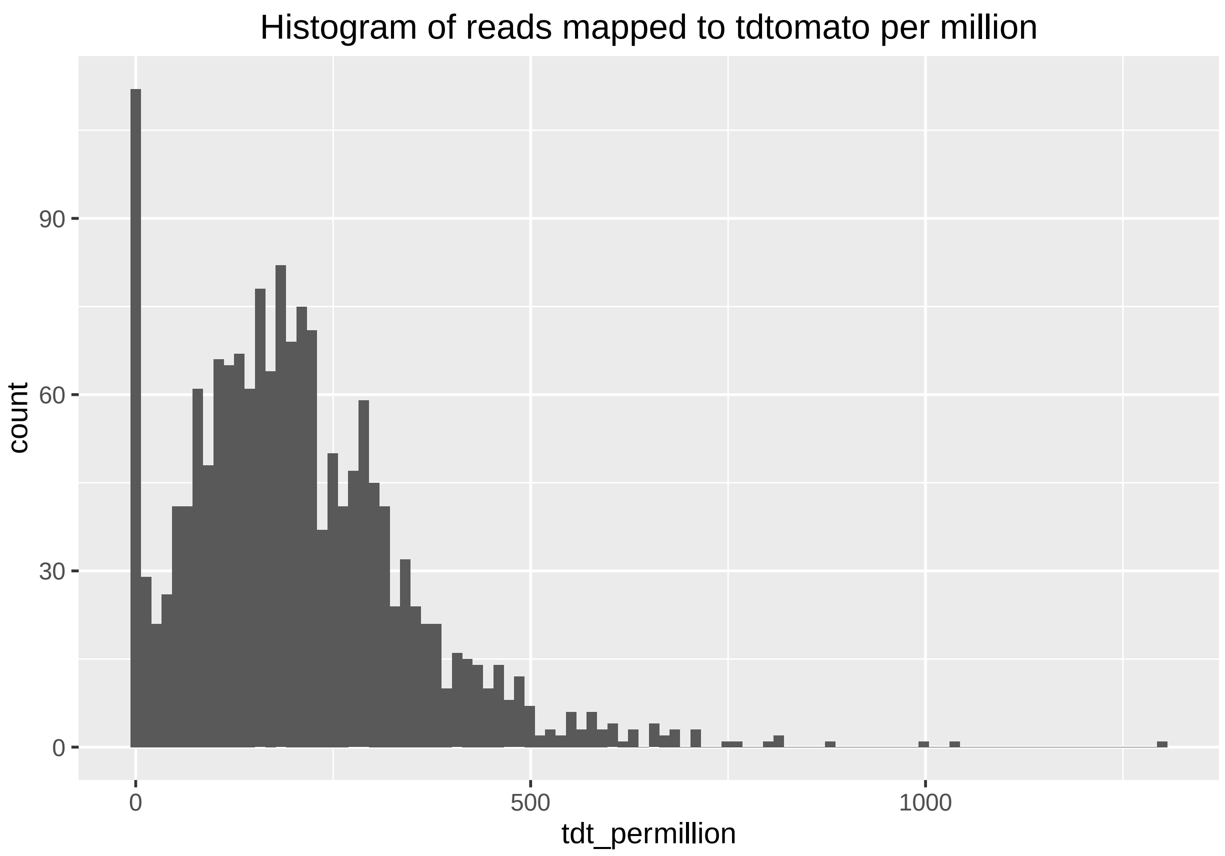
```
ggplot(qc, aes(x=ercc_percent)) + geom_histogram(bins=100) + ggtitle('Histogram of percent reads mapped
```



```
summary(qc$ercc_percent)
```

```
##    Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
##   0.160   0.920   1.370   1.887   2.190  13.900
```

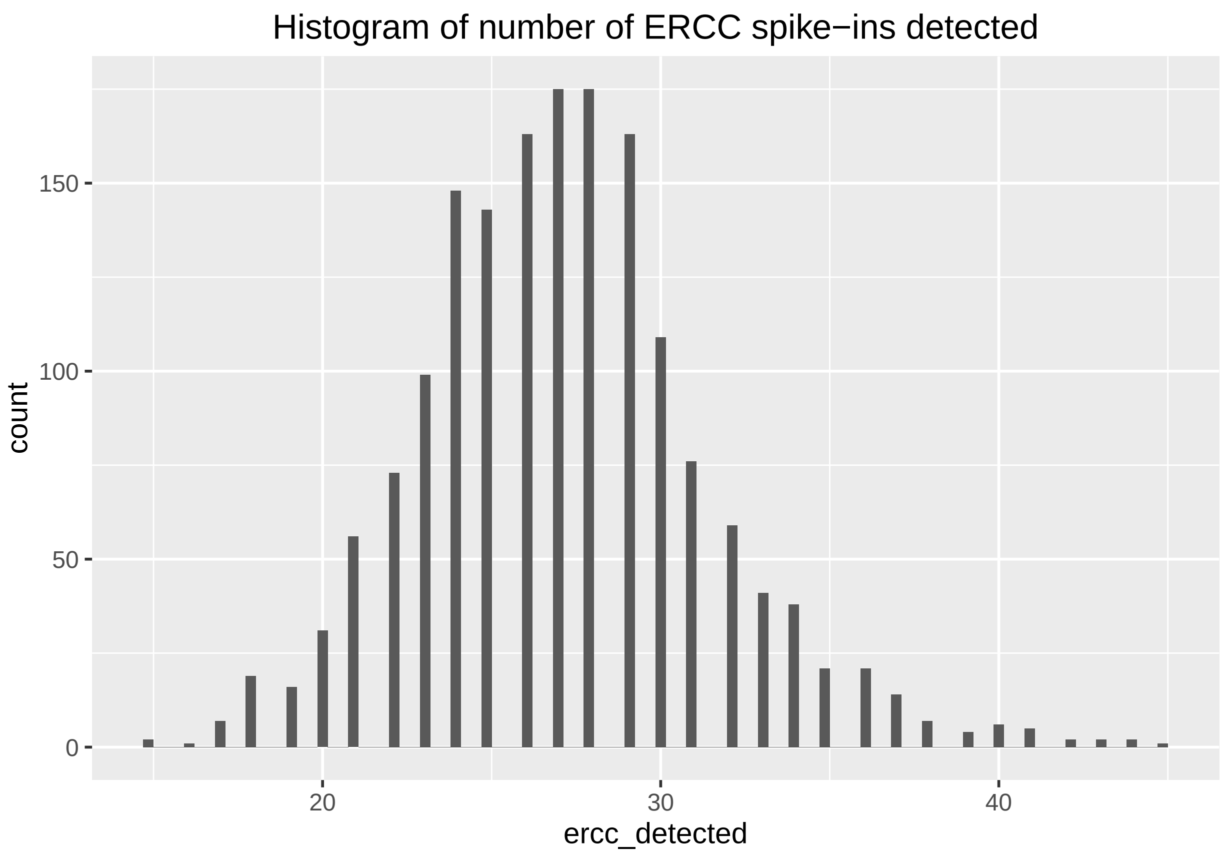
```
ggplot(qc, aes(x=tdt_permillion)) + geom_histogram(bins=100) + ggtitle('Histogram of reads mapped to td
```



```
summary(qc$tdt_permillion)
```

```
##    Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
##     0.0   106.7   185.9   205.7   284.2  1300.0
```

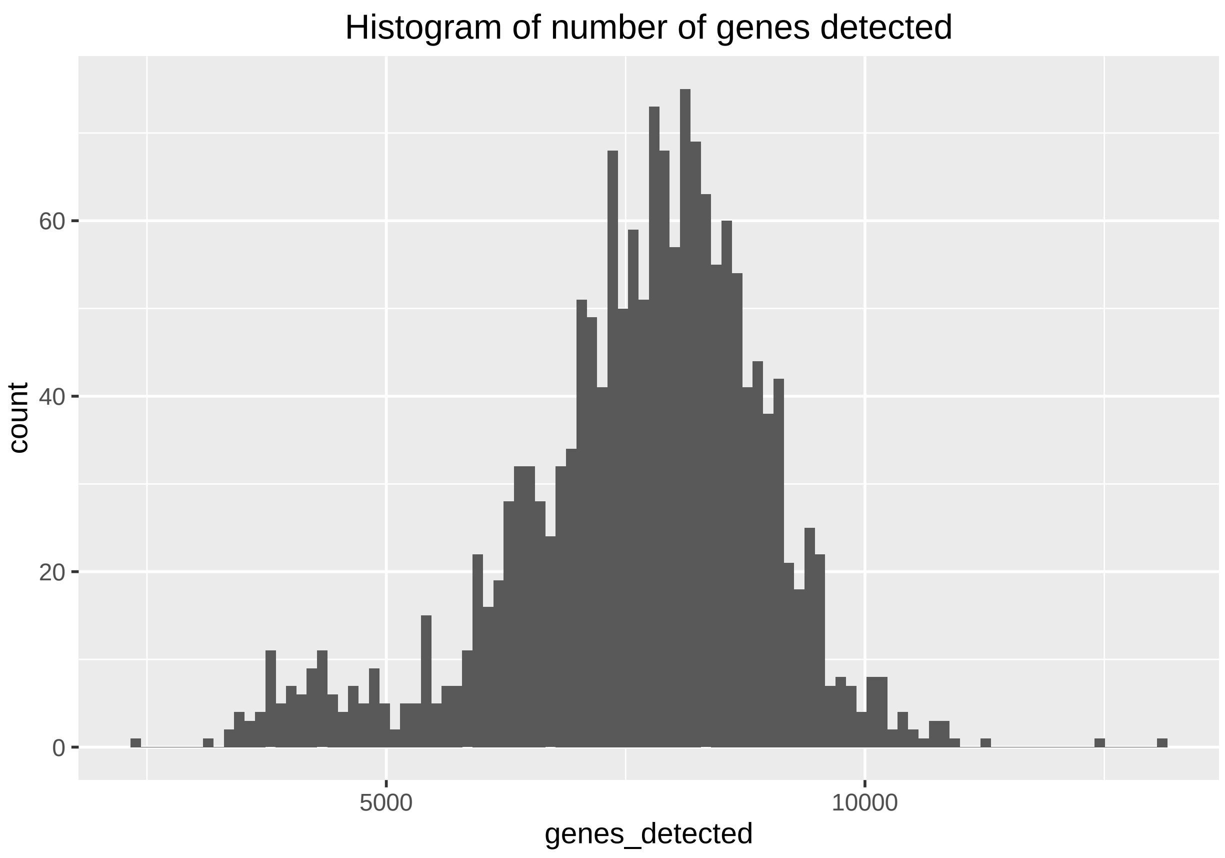
```
ggplot(qc, aes(x=ercc_detected)) + geom_histogram(bins=100) + ggtitle('Histogram of number of ERCC spik
```



```
summary(qc$ercc_detected)
```

```
##    Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
##   15.00   24.00   27.00   27.13   29.00   45.00
```

```
ggplot(qc, aes(x=genes_detected)) + geom_histogram(bins=100) + ggtitle('Histogram of number of genes de
```



```
summary(qc$genes_detected)
```

```
##    Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
##    2402    6966    7811    7626    8508   13120
```

Other metrics commonly used for QC

1. GC content
2. k-mer content
3. 3' bias
4. %reads mapping to mitochondria: high mitochondria/genome ratio suggests apoptotic cell
5. %reads mapping to introns or intergenic regions

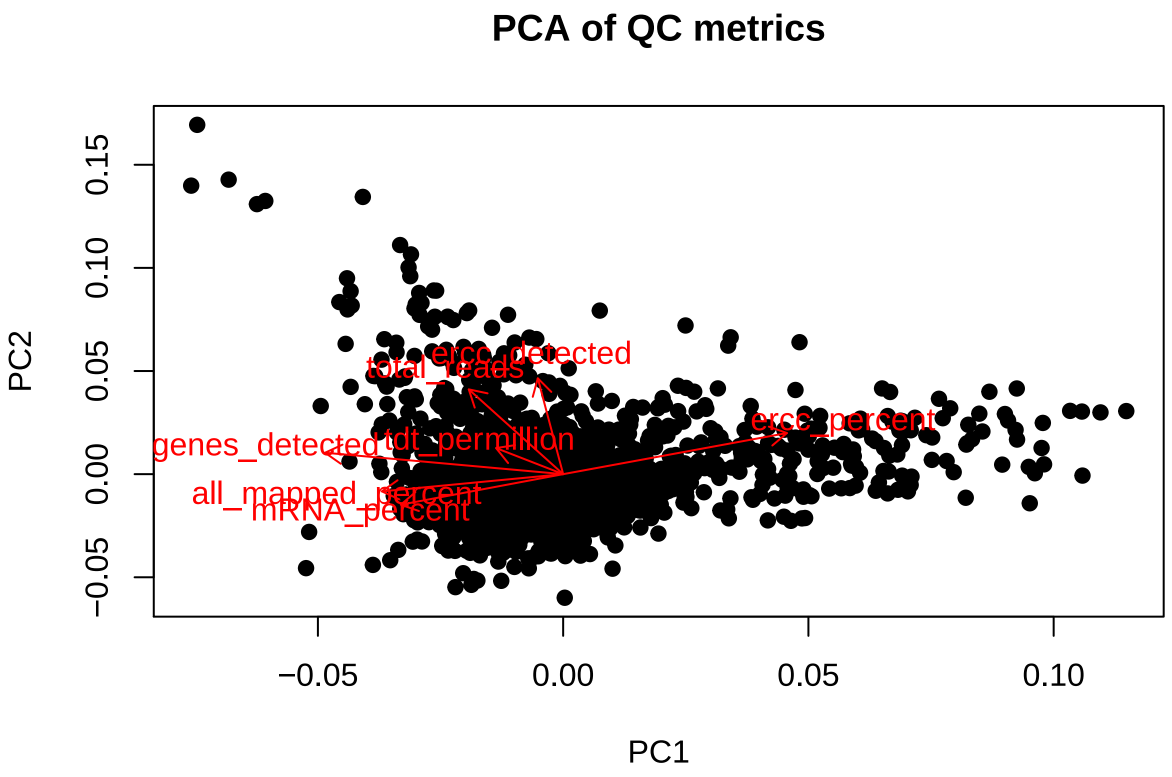
Identifying outlying samples by PCA of QC metrics

We can use PCA to visualize each sample with respect to QC metrics and pick outliers.

```
qc_pc_obj = prcomp(qc, center = TRUE, scale=TRUE)
```

```
#ggplot(as.data.frame(qc_pc_obj$x), aes(x=PC1, y=PC2)) + geom_point()
```

```
qc_bp_obj = biplot_colored(qc_pc_obj, y=1, expand = .8, choices=1:2)
title('PCA of QC metrics')
```



We don't find low quality outliers

The samples on the top left have very high number of reads (and that is good), and this is what causes PC2 to be high.

The authors of the paper had already excluded low-quality samples from this dataset.

Normalization

The purpose of normalization is to make datasets from different cells comparable.

Normalization strategies range from simple scaling (e.g. dividing raw counts by the total number of reads), to a more complex schemes that adjust for batch effects and biological effects.

Too much adjustment can cut away biological signal and/or introduce artifacts.

There is no normalization strategy that is optimal for all datasets.

Many normalization strategies should be considered.

Some common normalization strategies for RNA-Seq

name	long name	notes
RPM	reads per million	(counts / total reads) * 1 million
RPKM or FPKM	fragments per kilobase per million	takes gene aclength into account

name	long name	notes
TPM	transcripts per million	takes gene length into account
FQ	full quantile	match quantiles across samples
UQ	upper quartile	upper quartile of read counts define scaling factor
TMM	trimmed mean of M values (edgeR)	weighted log-fold-change values of a reference sample, removing genes with extreme values
DESeq	DESeq	scales samples to a reference sample based on the geometric mean of read counts across all samples

Testing multiple normalization strategies with SCONE

SCONE (Single-Cell Overview of Normalized Expression) supports a rational, data-driven framework for assessing the efficacy of various normalization workflows, encouraging users to explore trade-offs inherent to their data set prior to finalizing a data normalization strategy. It provides an interface for running multiple normalization workflows in parallel. It also offers tools for ranking workflows and visualizing trade-offs. It imports some common normalization modules used in traditional bulk sequencing, and provide support for integrating user-specified normalization modules.

R package on github:

https://github.com/YosefLab/scone

More information and usage examples:

https://niryosef.wordpress.com/tools/scone/

https://www.bioconductor.org/help/course-materials/2016/BioC2016/ConcurrentWorkshops1/Risso/scone.html

How it works

A normalization strategy is composed of a series of steps, each step being optional and having its specific parameters. SCONE tries all combinations of steps and parameters, evaluating many normalization strategies through metrics.

1. imputation - replace zeroes by average values - options: imputation or no imputation
2. scaling - scaling normalization strategy - options: none, UQ, FQ, DeSeq, .
3. RUVg - normalization with housekeeping genes or spike-ing - options: none, k=1, k=2, k=3 (k = number of parameters)
4. batch adjustment - whether to adjust for batch effects - options: yes or no
5. bio adjustment - whether to adjust for biological factors - options: yes or no

RUV: Remove Unwanted Variation from RNA-Seq Data bioconductor package RUVSeq

Risso D, Ngai J, Speed T and Dudoit S (2014). "Normalization of RNA-seq data using factor analysis of control genes or samples." Nature Biotechnology, 32(9), pp. 896–902. In press, http://www.nature.com/nbt/journal/v32/n9/full/nbt.2931.html.

Make sure column names of genes and ercc matrices are compatible

```r
all.equal(colnames(gene_counts), colnames(ercc))
```

```
## [1] TRUE
```

Create a combined matrix of gene and ercc counts

```
gene_and_ercc_counts <- rbind(gene_counts, ercc)
dim(gene_and_ercc_counts)
```

```
## [1] 24149  1679
```

Filter out genes and ERCCs with very low counts over all samples

```
f_gene_and_ercc_counts <- gene_and_ercc_counts[rowSums(gene_and_ercc_counts > 0) >= 50, ]
f_ercc <- rownames(ercc)[rownames(ercc) %in% rownames(f_gene_and_ercc_counts)]
```

Set up biological and batch factors

For **bio**, we use the dissection layer obtained from metadata.

For **batch**, we use the month of sample collection, obtained from metadata.

```
colection_month = gsub("([0-9]+)/[0-9]+/([0-9]+)", "\\1/\\2", cells$collection_date)
batch <- factor(colection_month)
bio <- factor(cells$layer_dissectoin)
```

```
knitr::kable(table(batch, bio))
```

	All	L1	L2/3	L4	L5	L6	L6a	L6b	lower	upper
10/2014	114	0	0	0	0	0	0	28	0	0
11/2013	24	0	0	45	0	0	3	0	16	16
11/2014	154	19	31	0	0	0	35	13	0	0
1/2014	31	0	0	61	23	0	30	0	0	0
12/2013	61	0	0	0	0	0	24	0	0	0
12/2014	8	0	0	0	0	13	0	0	34	72
2/2014	8	0	0	80	0	0	0	0	6	0
2/2015	0	0	0	0	0	0	20	0	0	0
3/2014	31	0	0	23	16	0	0	0	0	0
4/2014	0	0	0	0	101	0	0	0	0	0
5/2014	44	0	0	0	0	0	0	0	0	0
6/2014	16	0	24	0	0	0	0	0	44	45
7/2013	0	0	0	0	3	0	0	0	0	0
7/2014	48	19	0	7	0	0	0	0	44	46
8/2013	0	0	8	8	0	0	0	0	0	0
8/2014	157	0	0	0	0	20	0	0	0	0
9/2014	6	0	0	0	0	0	0	0	0	0

Set up a SCONE run

We use (no_normalization, DESeq, TMM, UQ, FQ) as candidate scaling strategies We use the ERCC spike ins for RUVg

```
params <- scone(expr = as.matrix(f_gene_and_ercc_counts),
                scaling = c(none = identity, deseq = DESEQ_FN, tmm = TMM_FN, uqp = UQ_FN_POS, fq = FQT_
                ruv_negcon = f_ercc, k_ruv = 3,
                k_qc = 0,
                bio = bio, adjust_bio = "yes",
                batch = batch, adjust_batch = "yes",
                run = FALSE)
```

Eliminate combinations of steps that are not meaningful

We don't want to adjust for biological factor unless we also adjust for batch factors.

```
is_screened = (params$adjust_biology == "bio") & (params$adjust_batch != "batch")
params = params[!is_screened,]
```

Here are the strategies to be tested

```
params
```

```
##                                     imputation_method scaling_method
## none,none,no_uv,no_bio,no_batch                  none           none
## none,deseq,no_uv,no_bio,no_batch                 none          deseq
## none,tmm,no_uv,no_bio,no_batch                   none            tmm
## none,uqp,no_uv,no_bio,no_batch                   none            uqp
## none,fq,no_uv,no_bio,no_batch                    none             fq
## none,none,ruv_k=1,no_bio,no_batch                none           none
## none,deseq,ruv_k=1,no_bio,no_batch               none          deseq
## none,tmm,ruv_k=1,no_bio,no_batch                 none            tmm
## none,uqp,ruv_k=1,no_bio,no_batch                 none            uqp
## none,fq,ruv_k=1,no_bio,no_batch                  none             fq
## none,none,ruv_k=2,no_bio,no_batch                none           none
## none,deseq,ruv_k=2,no_bio,no_batch               none          deseq
## none,tmm,ruv_k=2,no_bio,no_batch                 none            tmm
## none,uqp,ruv_k=2,no_bio,no_batch                 none            uqp
## none,fq,ruv_k=2,no_bio,no_batch                  none             fq
## none,none,ruv_k=3,no_bio,no_batch                none           none
## none,deseq,ruv_k=3,no_bio,no_batch               none          deseq
## none,tmm,ruv_k=3,no_bio,no_batch                 none            tmm
## none,uqp,ruv_k=3,no_bio,no_batch                 none            uqp
## none,fq,ruv_k=3,no_bio,no_batch                  none             fq
## none,none,no_uv,no_bio,batch                     none           none
## none,deseq,no_uv,no_bio,batch                    none          deseq
## none,tmm,no_uv,no_bio,batch                      none            tmm
## none,uqp,no_uv,no_bio,batch                      none            uqp
## none,fq,no_uv,no_bio,batch                       none             fq
## none,none,ruv_k=1,no_bio,batch                   none           none
## none,deseq,ruv_k=1,no_bio,batch                  none          deseq
## none,tmm,ruv_k=1,no_bio,batch                    none            tmm
## none,uqp,ruv_k=1,no_bio,batch                    none            uqp
## none,fq,ruv_k=1,no_bio,batch                     none             fq
## none,none,ruv_k=2,no_bio,batch                   none           none
## none,deseq,ruv_k=2,no_bio,batch                  none          deseq
## none,tmm,ruv_k=2,no_bio,batch                   none          tmm
## none,uqp,ruv_k=2,no_bio,batch                   none          uqp
## none,fq,ruv_k=2,no_bio,batch                    none           fq
## none,none,ruv_k=3,no_bio,batch                  none         none
## none,deseq,ruv_k=3,no_bio,batch                 none        deseq
## none,tmm,ruv_k=3,no_bio,batch                   none          tmm
## none,uqp,ruv_k=3,no_bio,batch                   none          uqp
## none,fq,ruv_k=3,no_bio,batch                    none           fq
## none,none,no_uv,bio,batch                       none         none
## none,deseq,no_uv,bio,batch                      none        deseq
## none,tmm,no_uv,bio,batch                        none          tmm
## none,uqp,no_uv,bio,batch                        none          uqp
## none,fq,no_uv,bio,batch                         none           fq
## none,none,ruv_k=1,bio,batch                     none         none
## none,deseq,ruv_k=1,bio,batch                    none        deseq
## none,tmm,ruv_k=1,bio,batch                      none          tmm
## none,uqp,ruv_k=1,bio,batch                      none          uqp
## none,fq,ruv_k=1,bio,batch                       none           fq
## none,none,ruv_k=2,bio,batch                     none         none
## none,deseq,ruv_k=2,bio,batch                    none        deseq
## none,tmm,ruv_k=2,bio,batch                      none          tmm
## none,uqp,ruv_k=2,bio,batch                      none          uqp
## none,fq,ruv_k=2,bio,batch                       none           fq
## none,none,ruv_k=3,bio,batch                     none         none
## none,deseq,ruv_k=3,bio,batch                    none        deseq
## none,tmm,ruv_k=3,bio,batch                      none          tmm
## none,uqp,ruv_k=3,bio,batch                      none          uqp
## none,fq,ruv_k=3,bio,batch                       none           fq
##                                     uv_factors adjust_biology adjust_batch
## none,none,no_uv,no_bio,no_batch          no_uv         no_bio     no_batch
## none,deseq,no_uv,no_bio,no_batch         no_uv         no_bio     no_batch
## none,tmm,no_uv,no_bio,no_batch           no_uv         no_bio     no_batch
## none,uqp,no_uv,no_bio,no_batch           no_uv         no_bio     no_batch
## none,fq,no_uv,no_bio,no_batch            no_uv         no_bio     no_batch
## none,none,ruv_k=1,no_bio,no_batch      ruv_k=1         no_bio     no_batch
## none,deseq,ruv_k=1,no_bio,no_batch     ruv_k=1         no_bio     no_batch
## none,tmm,ruv_k=1,no_bio,no_batch       ruv_k=1         no_bio     no_batch
## none,uqp,ruv_k=1,no_bio,no_batch       ruv_k=1         no_bio     no_batch
## none,fq,ruv_k=1,no_bio,no_batch        ruv_k=1         no_bio     no_batch
## none,none,ruv_k=2,no_bio,no_batch      ruv_k=2         no_bio     no_batch
## none,deseq,ruv_k=2,no_bio,no_batch     ruv_k=2         no_bio     no_batch
## none,tmm,ruv_k=2,no_bio,no_batch       ruv_k=2         no_bio     no_batch
## none,uqp,ruv_k=2,no_bio,no_batch       ruv_k=2         no_bio     no_batch
## none,fq,ruv_k=2,no_bio,no_batch        ruv_k=2         no_bio     no_batch
## none,none,ruv_k=3,no_bio,no_batch      ruv_k=3         no_bio     no_batch
## none,deseq,ruv_k=3,no_bio,no_batch     ruv_k=3         no_bio     no_batch
## none,tmm,ruv_k=3,no_bio,no_batch       ruv_k=3         no_bio     no_batch
## none,uqp,ruv_k=3,no_bio,no_batch       ruv_k=3         no_bio     no_batch
## none,fq,ruv_k=3,no_bio,no_batch        ruv_k=3         no_bio     no_batch
## none,none,no_uv,no_bio,batch             no_uv         no_bio        batch
## none,deseq,no_uv,no_bio,batch            no_uv         no_bio        batch
## none,tmm,no_uv,no_bio,batch              no_uv         no_bio        batch
## none,uqp,no_uv,no_bio,batch              no_uv         no_bio        batch
## none,fq,no_uv,no_bio,batch               no_uv         no_bio        batch
## none,none,ruv_k=1,no_bio,batch        ruv_k=1         no_bio        batch
## none,deseq,ruv_k=1,no_bio,batch       ruv_k=1         no_bio        batch
## none,tmm,ruv_k=1,no_bio,batch         ruv_k=1         no_bio        batch
## none,uqp,ruv_k=1,no_bio,batch         ruv_k=1         no_bio        batch
## none,fq,ruv_k=1,no_bio,batch          ruv_k=1         no_bio        batch
## none,none,ruv_k=2,no_bio,batch        ruv_k=2         no_bio        batch
## none,deseq,ruv_k=2,no_bio,batch       ruv_k=2         no_bio        batch
## none,tmm,ruv_k=2,no_bio,batch         ruv_k=2         no_bio        batch
## none,uqp,ruv_k=2,no_bio,batch         ruv_k=2         no_bio        batch
## none,fq,ruv_k=2,no_bio,batch          ruv_k=2         no_bio        batch
## none,none,ruv_k=3,no_bio,batch        ruv_k=3         no_bio        batch
## none,deseq,ruv_k=3,no_bio,batch       ruv_k=3         no_bio        batch
## none,tmm,ruv_k=3,no_bio,batch         ruv_k=3         no_bio        batch
## none,uqp,ruv_k=3,no_bio,batch         ruv_k=3         no_bio        batch
## none,fq,ruv_k=3,no_bio,batch          ruv_k=3         no_bio        batch
## none,none,no_uv,bio,batch               no_uv            bio        batch
## none,deseq,no_uv,bio,batch              no_uv            bio        batch
## none,tmm,no_uv,bio,batch                no_uv            bio        batch
## none,uqp,no_uv,bio,batch                no_uv            bio        batch
## none,fq,no_uv,bio,batch                 no_uv            bio        batch
## none,none,ruv_k=1,bio,batch           ruv_k=1            bio        batch
## none,deseq,ruv_k=1,bio,batch          ruv_k=1            bio        batch
## none,tmm,ruv_k=1,bio,batch            ruv_k=1            bio        batch
## none,uqp,ruv_k=1,bio,batch            ruv_k=1            bio        batch
## none,fq,ruv_k=1,bio,batch             ruv_k=1            bio        batch
## none,none,ruv_k=2,bio,batch           ruv_k=2            bio        batch
## none,deseq,ruv_k=2,bio,batch          ruv_k=2            bio        batch
## none,tmm,ruv_k=2,bio,batch            ruv_k=2            bio        batch
## none,uqp,ruv_k=2,bio,batch            ruv_k=2            bio        batch
## none,fq,ruv_k=2,bio,batch             ruv_k=2            bio        batch
## none,none,ruv_k=3,bio,batch           ruv_k=3            bio        batch
## none,deseq,ruv_k=3,bio,batch          ruv_k=3            bio        batch
## none,tmm,ruv_k=3,bio,batch            ruv_k=3            bio        batch
## none,uqp,ruv_k=3,bio,batch            ruv_k=3            bio        batch
## none,fq,ruv_k=3,bio,batch             ruv_k=3            bio        batch
```

This is the call to SCONE.

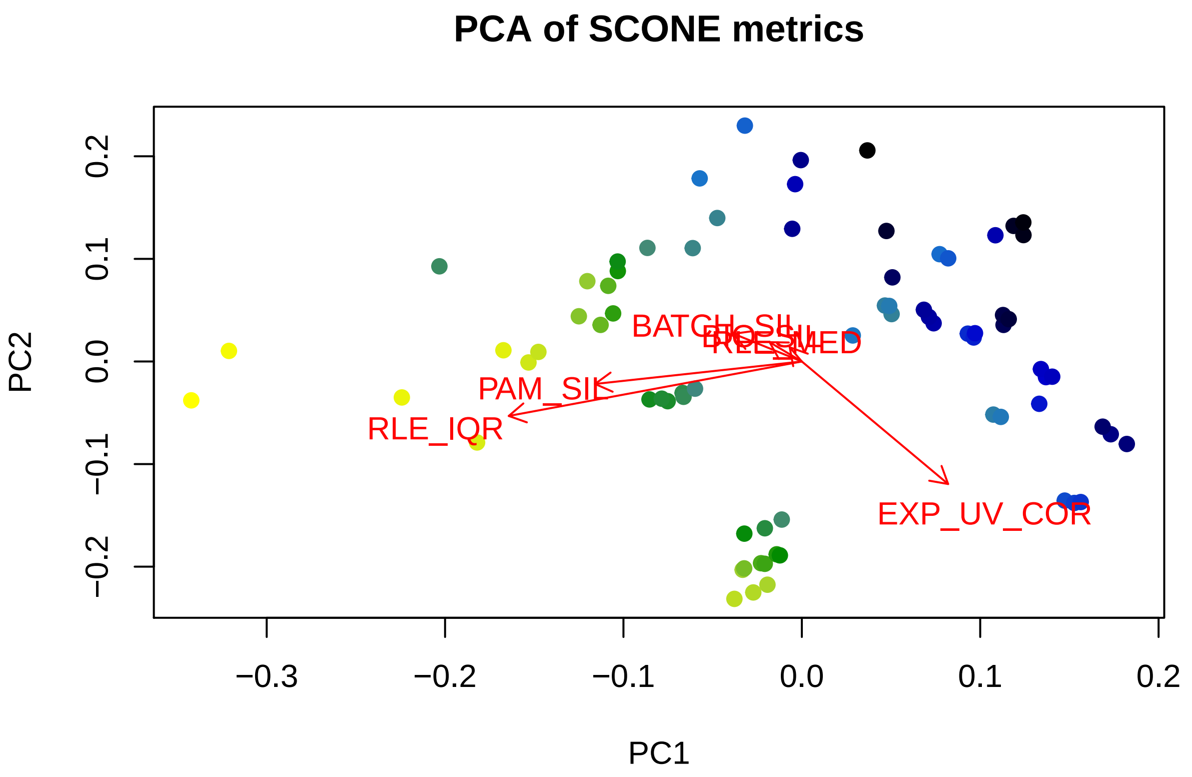
It took about 1 hour in a 16-core machine and 48G memory. We are loaded precomputed results here.

```
#res <- scone(expr = as.matrix(f_gene_and_ercc_counts),
#                 scaling = c(none = identity, deseq = DESEQ_FN, tmm = TMM_FN, uqp = UQ_FN_POS, fq = FQT
#                 ruv_negcon = f_ercc, k_ruv = 3,
#                 k_qc = 0,
#                 bio = bio, adjust_bio = "yes",
#                 batch = batch, adjust_batch = "yes",
#                 run = TRUE, eval_kclust = 2:3)

load('res.Rdata')
```

```
scores = res$scores[, !(colnames(res$scores) %in% c('EXP_QC_COR', 'EXP_WV_COR', 'mean_score'))]
```

```
pc_obj = prcomp(scores, center = TRUE, scale = FALSE)
```

```r
bp_obj = biplot_colored(pc_obj, y = -res$scores[,'mean_score'],expand = .6)
title('PCA of SCONE metrics')
```

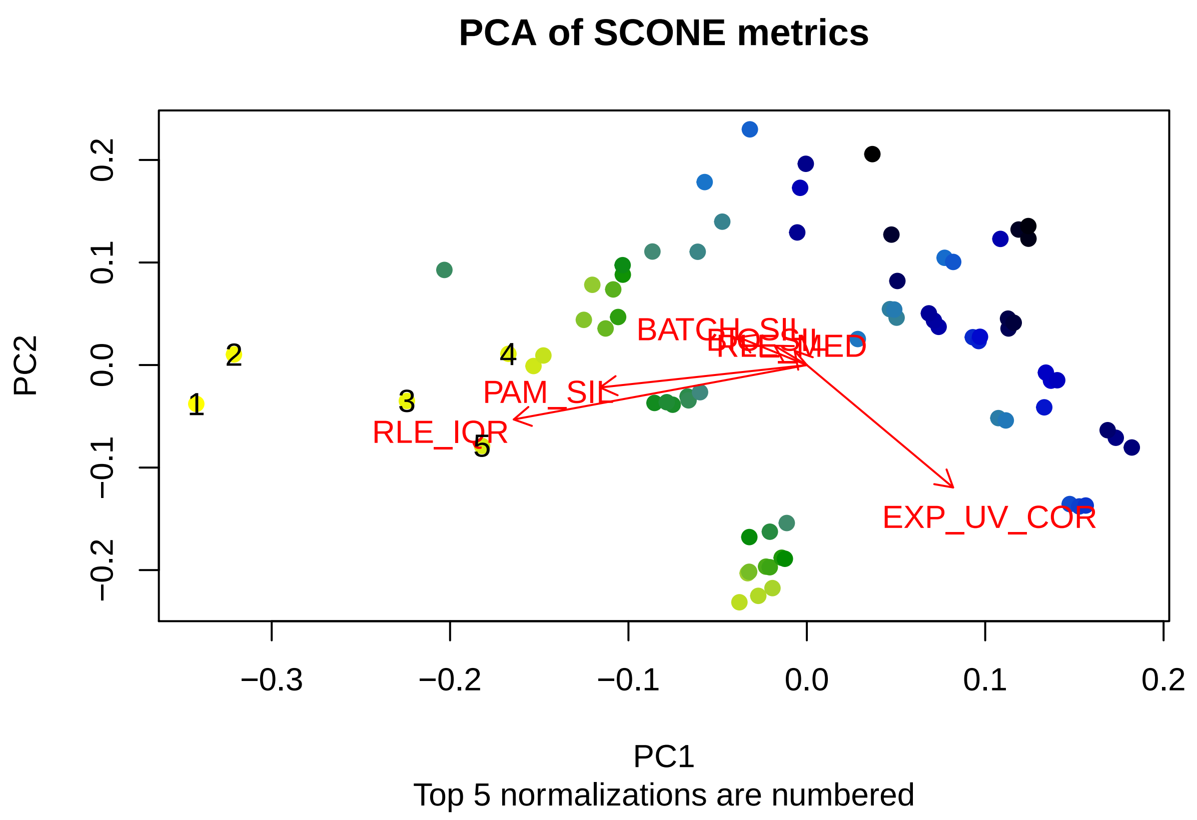


High score normalizations

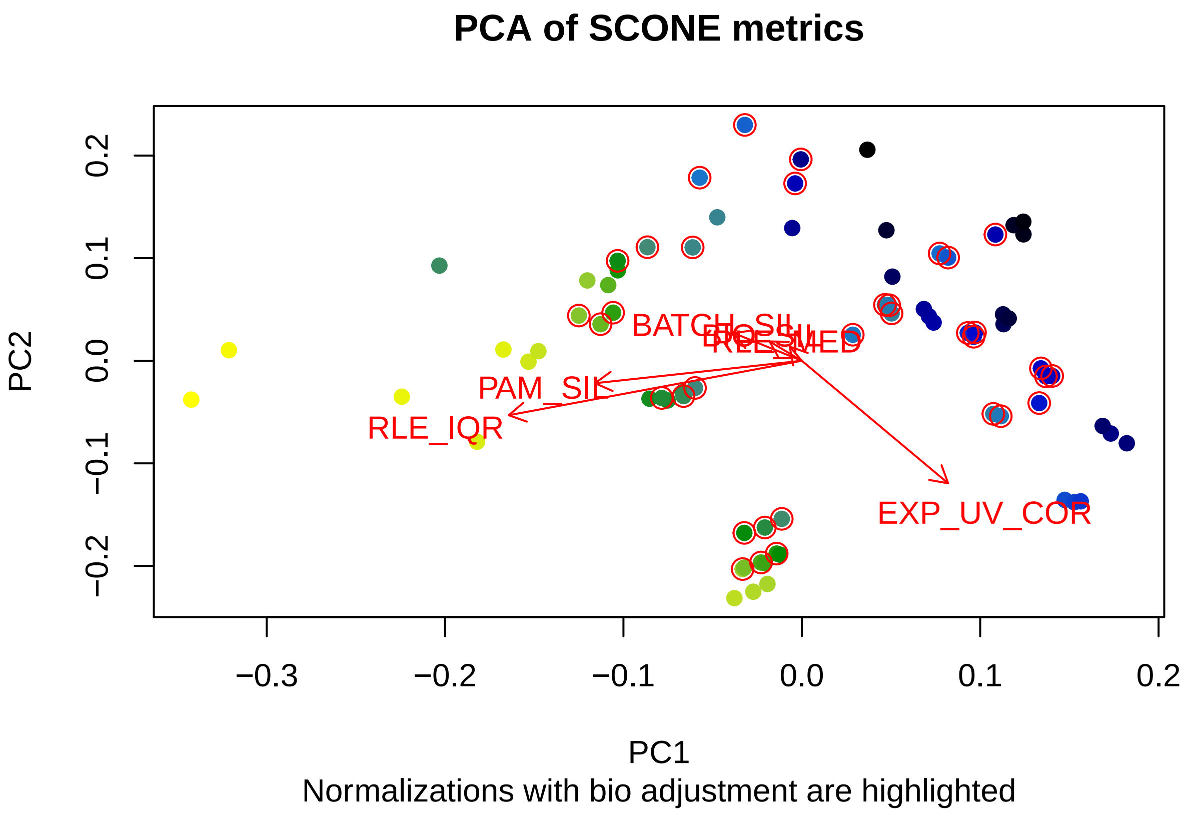
```r
knitr::kable(head(res$scores))
```

	BIO_SIL	BATCH_SIL	PAM_SIL	EXP_QC_COR	EXP_UV_COR
none,deseq,no_uv,no_bio,no_batch	-0.1376663	0.3991757	0.5022178	NA	-0.2892409
none,fq,no_uv,no_bio,no_batch	-0.1417659	0.4081318	0.5101578	NA	-0.3048712
none,uqp,no_uv,no_bio,no_batch	-0.1498649	0.3589001	0.4226251	NA	-0.2534073
none,deseq,ruv_k=1,no_bio,no_batch	-0.1397361	0.3583659	0.4723546	NA	-0.2450541
none,none,no_uv,no_bio,no_batch	-0.1586934	0.3363167	0.4312794	NA	-0.2145279
none,deseq,ruv_k=2,no_bio,no_batch	-0.1396720	0.3556571	0.4712522	NA	-0.2316802

```r
bp_obj = biplot_colored(pc_obj, y = -res$scores[,'mean_score'],expand = .6)

#points(t(bp_obj[grepl("none,deseq,no_uv,no_bio,no_batch",rownames(bp_obj)),]), pch = 1, col = "red", c
#points(t(bp_obj[grepl("none,none,no_uv,no_bio,no_batch",rownames(bp_obj)),]), pch = 1, col = "blue", c
#points(t(bp_obj[grepl("none,deseq,ruv_k=1,no_bio,no_batch",rownames(bp_obj)),]), pch = 1, col = "blue"

text(bp_obj[1:5,], labels=1:5)

title('PCA of SCONE metrics', sub='Top 5 normalizations are numbered')
```

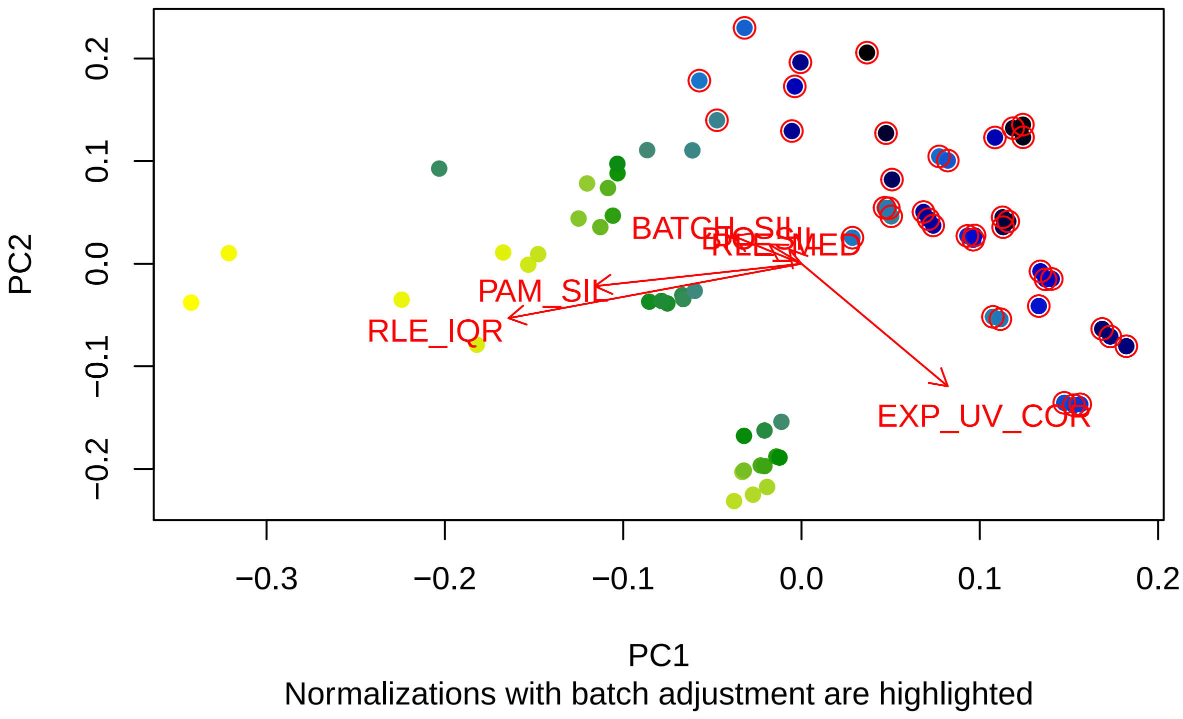


```
bp_obj = biplot_colored(pc_obj, y = -res$scores[,'mean_score'],expand = .6)

points(bp_obj[grepl(",bio",rownames(bp_obj)),], pch = 1, col = "red", cex = 1.5)

title('PCA of SCONE metrics', sub='Normalizations with bio adjustment are highlighted')
```



```
bp_obj = biplot_colored(pc_obj, y = -res$scores[,'mean_score'],expand = .6)

points(bp_obj[grepl(",batch",rownames(bp_obj)),], pch = 1, col = "red", cex = 1.5)

title('PCA of SCONE metrics', sub='Normalizations with batch adjustment are highlighted')
```

PCA of SCONE metrics



SCONE's metrics

BIO_SIL. The average silhouette width of clusters defined by bio, defined with respect to a Euclidean distance metric over the first 3 expression PCs. Positive signature.

BATCH_SIL. The average silhouette width of clusters defined by batch, defined with respect to a Euclidean distance metric over the first 3 expression PCs. Negative signature.

PAM_SIL. The maximum average silhouette width of clusters defined by PAM clustering, defined with respect to a Euclidean distance metric over the first 3 expression PCs. Positive signature.

EXP_QC_COR. Maximum squared Spearman correlation between first 3 expression PCs and first k_qc QPCs. Negative signature.

EXP_UV_COR. Maximum squared Spearman correlation between first 3 expression PCs and first 3 PCs of the negative control (specified by eval_negcon or ruv_negcon by default) sub-matrix of the original (raw) data. Negative signature.

EXP_WV_COR. Maximum squared Spearman correlation between first 3 expression PCs and first 3 PCs of the positive control (specified by eval_poscon) sub-matrix of the original (raw) data. Positive signature.

RLE_MED. The mean squared median Relative Log Expression (RLE). Negative signature.

RLE_IQR. The mean inter-quartile range (IQR) of the RLE. Negative signature.

Discussion

It seems like simple normalization strategies like DESeq, and quantile normalization perform better in this dataset.

One possibility is that DESeq's assumption that most genes are not DE across samples is valid. This "internal normalization" migh be better for this dataset than ERCC spike-ins. A median of 27 (out of 93) ERCC spike-ins are detected per cell. Non-DE genes provide a bigger set of genes for normalization.

Adjustment for biological and batch factors did not perform well. These asjustments were also based on ERCC. Using another set of housekeeping genes might improve the performance.

It is important to compare many normalization strategies.

```r
sessionInfo()
```

```
## R version 3.3.1 (2016-06-21)
## Platform: x86_64-pc-linux-gnu (64-bit)
## Running under: Arch Linux
##
## locale:
##  [1] LC_CTYPE=en_US.UTF-8       LC_NUMERIC=C
##  [3] LC_TIME=en_US.UTF-8        LC_COLLATE=en_US.UTF-8
##  [5] LC_MONETARY=en_US.UTF-8    LC_MESSAGES=en_US.UTF-8
##  [7] LC_PAPER=en_US.UTF-8       LC_NAME=C
##  [9] LC_ADDRESS=C               LC_TELEPHONE=C
## [11] LC_MEASUREMENT=en_US.UTF-8 LC_IDENTIFICATION=C
##
## attached base packages:
## [1] stats     graphics  grDevices utils     datasets  methods   base
##
## other attached packages:
## [1] ggplot2_2.1.0 scone_0.0.7   rmarkdown_1.0
##
## loaded via a namespace (and not attached):
##  [1] segmented_0.5-1.4          bitops_1.0-6
##  [3] matrixStats_0.50.2         RColorBrewer_1.1-2
##  [5] prabclus_2.2-6             GenomeInfoDb_1.8.7
##  [7] tools_3.3.1                R6_2.1.3
##  [9] KernSmooth_2.23-15         DBI_0.5-1
## [11] BiocGenerics_0.18.0        colorspace_1.2-6
## [13] trimcluster_0.1-2          nnet_7.3-12
## [15] Biobase_2.32.0             formatR_1.4
## [17] rtracklayer_1.32.2         labeling_0.3
## [19] diptest_0.75-7             caTools_1.17.1
## [21] scales_0.4.0               DEoptimR_1.0-6
## [23] mvtnorm_1.0-5              robustbase_0.92-6
## [25] genefilter_1.54.2          DESeq_1.24.0
## [27] stringr_1.1.0              digest_0.6.10
## [29] Rsamtools_1.24.0           mixtools_1.0.4
## [31] R.utils_2.3.0              XVector_0.12.1
## [33] htmltools_0.3.5            limma_3.28.21
## [35] highr_0.6                  RSQLite_1.0.0
## [37] shiny_0.14                 hwriter_1.3.2
## [39] mclust_5.2                 BiocParallel_1.6.6
## [41] gtools_3.5.0               R.oo_1.20.0
## [43] RCurl_1.95-4.8             magrittr_1.5
## [45] modeltools_0.2-21          Matrix_1.2-7.1
## [47] Rcpp_0.12.7                munsell_0.4.3
## [49] S4Vectors_0.10.3           R.methodsS3_1.7.1
## [51] stringi_1.1.1              yaml_2.1.13
## [53] edgeR_3.14.0                MASS_7.3-45
## [55] SummarizedExperiment_1.2.3 zlibbioc_1.18.0
## [57] flexmix_2.3-13              rhdf5_2.16.0
## [59] gplots_3.0.1                plyr_1.8.4
## [61] grid_3.3.1                  parallel_3.3.1
## [63] gdata_2.17.0                miniUI_0.1.1
## [65] lattice_0.20-34             Biostrings_2.40.2
## [67] splines_3.3.1               GenomicFeatures_1.24.5
## [69] annotate_1.50.0             EDASeq_2.6.2
## [71] knitr_1.14                  GenomicRanges_1.24.3
## [73] boot_1.3-18                 fpc_2.1-10
## [75] geneplotter_1.50.0          biomaRt_2.28.0
## [77] stats4_3.3.1                XML_3.98-1.4
## [79] evaluate_0.9                ShortRead_1.30.0
## [81] latticeExtra_0.6-28         httpuv_1.3.3
## [83] gtable_0.2.0                kernlab_0.9-24
## [85] aroma.light_3.2.0           mime_0.5
## [87] xtable_1.8-2                class_7.3-14
## [89] survival_2.39-5             RUVSeq_1.6.2
## [91] GenomicAlignments_1.8.4     AnnotationDbi_1.34.4
## [93] IRanges_2.6.1               cluster_2.0.4
```