Phase 1A: RNA-seq data analysis

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Phase 1A raw data was preprocessed at the BIC using our pipeline, implemented according to the MOP. Here we present QC analyses performed on the output of the pipeline.

Input data

```
setwd("/Users/David/Desktop/MoTrPAC/data/pass_1a/rnaseq/")
library(data.table)
library(DESeq2)
library(preprocessCore)
library(ggplot2)

# Data paths
site2fpkm_path = list(
  stanford = "./stanford/rsem_genes_fpkm_pass1a_batch1_Stanford.csv",
  sinai = "./sinai/rsem_genes_fpkm_pass1a_batch1_Sinai.csv"
)
site2genecount_path = list(
  stanford = "./stanford/rsem_genes_count_pass1a_batch1_Stanford.csv",
  sinai = "./sinai/rsem_genes_count_pass1a_batch1_Sinai.csv"
)

# load the metadata
# this is a data frame called rnaseq_meta that contains
# the qc and sample metadata from both sites
load("./rnaseq_meta.RData")
rnaseq_meta$Tissue = tolower(rnaseq_meta$Tissue)
rnaseq_meta$Tissue = gsub(" powder","",rnaseq_meta$Tissue)
rnaseq_meta[rnaseq_meta=="N/A"] = NA
print("Number of samples flagged according to the MOP's thersholds:")
print(sum(rnaseq_meta$IsFlagged))

# read the data in
site2fpkm = list()
site2counts = list()
for(site in names(site2fpkm_path)){
  currfpkm = fread(site2fpkm_path[[site]],header = T,
                   stringsAsFactors = F,data.table = F)
  rownames(currfpkm) = currfpkm[,1]
  currfpkm = currfpkm[,-1]
  site2fpkm[[site]] = currfpkm

  currcounts = fread(site2genecount_path[[site]],
                     header = T,stringsAsFactors = F,data.table = F)
  rownames(currcounts) = currcounts[,1]
  currcounts = currcounts[,-1]
  site2counts[[site]] = currcounts
```

```
}
```

PCA plots

FPKM data

```
#' Takes an FPKM matrix, removes lowly expressed genes and log transform
#' the remaining matrix
#' @return A matrix of log transformed FPKMs
process_fpkm1 <-function(fpkm_matrix, intensity_threshold=0,intensity_pct=0.2){
  lowly_expressed_genes = rowSums(
    fpkm_matrix==intensity_threshold)/ncol(fpkm_matrix) > intensity_pct
  fpkm_matrix = fpkm_matrix[!lowly_expressed_genes,]
  fpkm_matrix = log(fpkm_matrix+1,base = 2)
  return(fpkm_matrix)
}
#' A wrapper for preprocessCore's quantile normalization.
#' Comment: we do not use this by default
run_quantile_normalization<-function(x){
  x = as.matrix(x)
  mode(x) = "numeric"
  newx = preprocessCore::normalize.quantiles.robust(x)
  rownames(newx) = rownames(x)
  colnames(newx) = colnames(x)
  return(newx)
}
# Process the FPKM matrix from each site separately
site_proc_fpkms = lapply(site2fpkm,process_fpkm1)
# Check the dimension of the reduced data
print("FPKM processing done for each site, matrix dim:")
```

```
## [1] "FPKM processing done for each site, matrix dim:"
```

```
print(sapply(site_proc_fpkms,dim))
```

```
##      stanford sinai
## [1,]    13616 11951
## [2,]      320   320
```

```
# Get the shared genes
shared_genes = intersect(rownames(site_proc_fpkms[[1]]),rownames(site_proc_fpkms[[2]]))
print("Number of shared genes that survive the filter above:")
```

```
## [1] "Number of shared genes that survive the filter above:"
```

```
print(length(shared_genes))
```

```
## [1] 11711
```

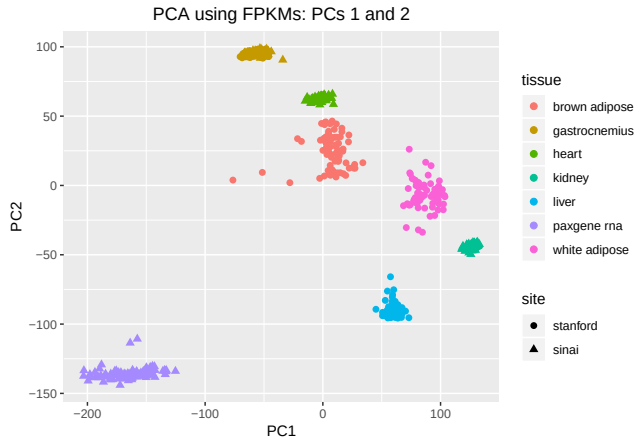
```
proc_fpkms = cbind(site_proc_fpkms[[1]][shared_genes,],site_proc_fpkms[[2]][shared_genes,])
# QC: make sure the metadata and the expression matrix have the same sample id:
print("do we have the same samples in the expression and metadata matrices?")
```

```
## [1] "do we have the same samples in the expression and metadata matrices?"
```

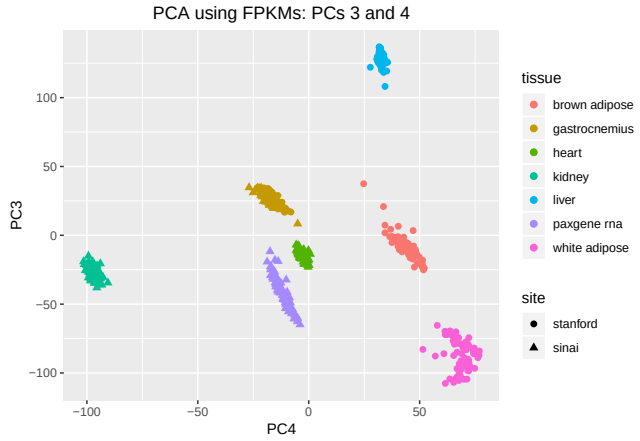
```
print(all(colnames(proc_fpkms) %in% rownames(rnaseq_meta)))
```

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

```
# Run the PCA: try all genes first
fpkm_all = cbind(site2fpkm[[1]],site2fpkm[[2]])
fpkm_all = log(fpkm_all+1,base=2)
fpkm_pca = prcomp(t(fpkm_all))
fpkm_pcax = fpkm_pca$x
df = data.frame(fpkm_pcax[,1:10],
                tissue = rnaseq_meta[rownames(fpkm_pcax),"Tissue"],
                site = rnaseq_meta[rownames(fpkm_pcax),"site"])
ggplot(df,aes(x=PC1, y=PC2,shape=site, color=tissue)) +
  geom_point(size=2) + ggtitle("PCA using FPKMs: PCs 1 and 2") +
  theme(plot.title = element_text(hjust = 0.5))
```

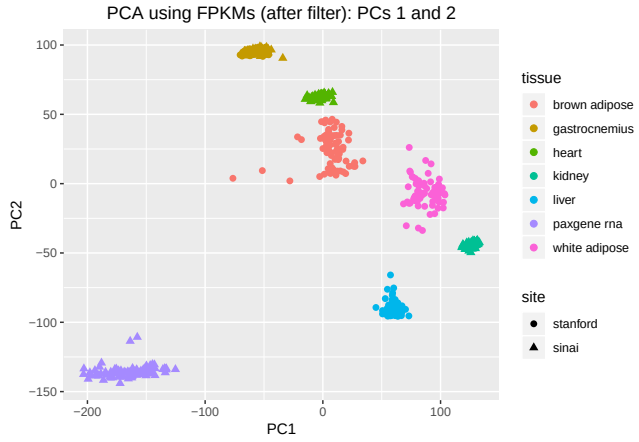



```
ggplot(df,aes(x=PC4, y=PC3,shape=site, color=tissue)) +
  geom_point(size=2) + ggtitle("PCA using FPKMs: PCs 3 and 4") +
  theme(plot.title = element_text(hjust = 0.5))
```

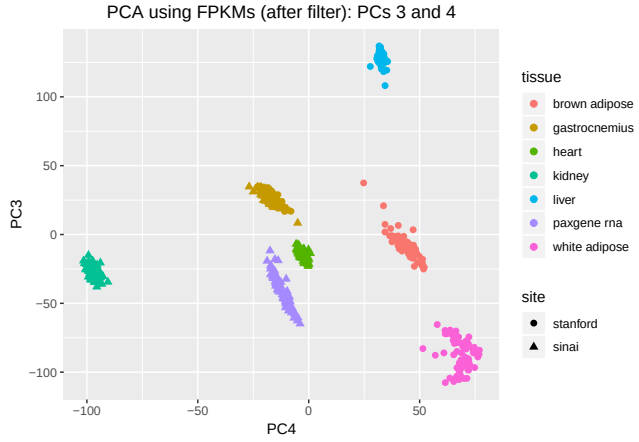



```
# Rerun on the reduced matrix
fpkm_pca = prcomp(t(proc_fpkms),retx = T)
fpkm_pcax = fpkm_pca$x
fpkm_pca_proc = prcomp(t(fpkm_all),retx = T)
fpkm_pca_procx = fpkm_pca_proc$x
df = data.frame(fpkm_pca_procx[,1:10],
```

```
                tissue = rnaseq_meta[rownames(fpkm_pcax),"Tissue"],
                site = rnaseq_meta[rownames(fpkm_pcax),"site"])
ggplot(df,aes(x=PC1, y=PC2,shape=site, color=tissue)) +
  geom_point(size=2) + ggtitle("PCA using FPKMs (after filter): PCs 1 and 2") +
  theme(plot.title = element_text(hjust = 0.5))
```




```
ggplot(df,aes(x=PC4, y=PC3,shape=site, color=tissue)) +
  geom_point(size=2) + ggtitle("PCA using FPKMs (after filter): PCs 3 and 4") +
  theme(plot.title = element_text(hjust = 0.5))
```



Normalized counts

```
# Pipeline 2: work with count data
# Combine the two count matrices
count_matrix = as.matrix(cbind(site2counts[[1]],site2counts[[2]]))
#' Use DESeq2 to estimate sample factors and gene dispersion
#' @return a DESeqDataSet
process_counts<-function(count_matrix){
  mode(count_matrix) = "integer"
  se <- SummarizedExperiment(count_matrix)
  dds <- DESeqDataSet(se, design = ~ 1 )
  #Estimate size factors
  dds <- estimateSizeFactors( dds )
  # Plot the size factors
```

```
  plot(sizeFactors(dds), colSums(counts(dds)))
  abline(lm(colSums(counts(dds)) ~ sizeFactors(dds) + 0))
  dds <- estimateDispersions(dds)
  return(dds)
}

# Process the counts and normalize
dds = process_counts(count_matrix)
# Simple normalization and log transform
# The argument normalized equals true, divides each column by its size factor.
logcounts <- log2( counts(dds, normalized=TRUE) + 1 )
pc <- prcomp( t( logcounts ) )
counts_pcax1 = pc$x
# Try variance stabilizing transformation instead
vsd <- varianceStabilizingTransformation(dds)
pc2 <- prcomp( t( assay(vsd) ) )
counts_pcax2 = pc2$x
# PCA plots
df = data.frame(counts_pcax1[,1:10],
                tissue = rnaseq_meta[rownames(counts_pcax),"Tissue"],
                site = rnaseq_meta[rownames(counts_pcax),"site"])
ggplot(df,aes(x=PC1, y=PC2,shape=site, color=tissue)) +
  geom_point(size=2) + ggtitle("PCA using normalized counts") +
  theme(plot.title = element_text(hjust = 0.5))
df = data.frame(counts_pcax2[,1:10],
                tissue = rnaseq_meta[rownames(counts_pcax),"Tissue"],
                site = rnaseq_meta[rownames(counts_pcax),"site"])
ggplot(df,aes(x=PC1, y=PC2,shape=site, color=tissue)) +
  geom_point(size=2) + ggtitle("PCA using normalized counts (vsd)") +
  theme(plot.title = element_text(hjust = 0.5))
```

Site comparison using the gastro samples

Load the data

```
# tissue vector - used below for getting the subset of
# gastro samples
tissue = rnaseq_meta$Tissue
names(tissue) = rownames(rnaseq_meta)
# get the gastro samples from each site
gastro_fpkm = lapply(site2fpkm,
    function(x,y)x[,grepl("gastro",y[colnames(x)],ignore.case = T)],y=tissue)
print("Gastro samples, data dim:")
```

```
## [1] "Gastro samples, data dim:"
```

```
print(sapply(gastro_fpkm,dim))
```

```
##      stanford sinai
## [1,]    32883 32883
## [2,]       80    80
```

```r
# Process the FPKM data matrix from each site separately
gastro_fpkm_processed = lapply(gastro_fpkm,process_fpkm1)
print("Filtered FPKM data (separately for each site):")
```

```
## [1] "Filtered FPKM data (separately for each site):"
```

```r
print(sapply(gastro_fpkm_processed,dim))
```

```
##      stanford sinai
## [1,]    12977 14218
## [2,]       80    80
```

```r
shared_genes = intersect(rownames(gastro_fpkm_processed[[1]]),
                         rownames(gastro_fpkm_processed[[2]]))
print("Number of shared genes that survive the filter above:")
```

```
## [1] "Number of shared genes that survive the filter above:"
```

```r
print(length(shared_genes))
```

```
## [1] 12966
```

```r
# Merge the datasets, store in a single data frame
gastro_fpkm_mat = cbind(gastro_fpkm_processed[[1]][shared_genes,],
                        gastro_fpkm_processed[[2]][shared_genes,])

# Analysis of the metadata
gastro_metadata = rnaseq_meta[colnames(gastro_fpkm_mat),]
# We by default keep the vial sample id, which is different even if
# the biospecimen id is the same.
# This vector keeps the BID+PIDs
sample_id = paste(gastro_metadata$BID,gastro_metadata$PID,sep=";")
names(sample_id) = rownames(gastro_metadata)
print("Do we have a copy from each site?")
```

```
## [1] "Do we have a copy from each site?"
```

```r
all(table(sample_id)==2) # QC: make sure we have two copies for each id
```

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

```r
# Reorder the data by the site and sample id
gastro_metadata = gastro_metadata[order(gastro_metadata$site,sample_id),]
sample_id = sample_id[rownames(gastro_metadata)]
```

Simple QC scores comparison

```r
metadata2site_pval = c()
site_ind = gastro_metadata$site==gastro_metadata$site[1]
# We go over all numeric columns in the metadata matrix and use
# a paired Wilcoxon test to estimate site differences
for(col in names(gastro_metadata)){
  x = gastro_metadata[[col]]
  if(! mode(x)=="numeric"){next}
  # data are ordered by site and sample id, which keeps the correct
  # order for the paired test
```

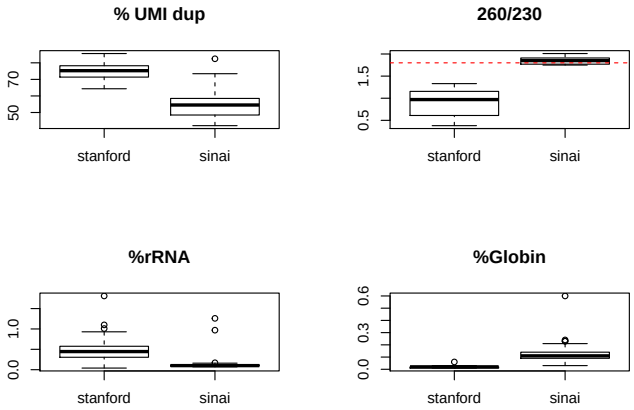
```
  x1 = x[site_ind];x2 = x[!site_ind] # define the two vectors
  if(!is.numeric(x1) || !is.numeric(x2)){next}
  sd1 = sd(x1,na.rm = T);sd2=sd(x2,na.rm = T)
  if(is.na(sd1)||is.na(sd2)){next}
  if(sd1==0 || sd2==0){next}
  # Need to try, some numeric columns are constants or have NAs
  metadata2site_pval[col] = wilcox.test(x1,x2,paired=T)$p.value
}
# Take the top 30 significant columns
selected_qc_comparisons = sort(metadata2site_pval)[1:30]
# Some of the columns are not informative (e.g., date)
# Take the "pct_" columns and print the p-values
print("Top pct_ qc scores that differ between sites:")
```

```
## [1] "Top pct_ qc scores that differ between sites:"
```

```
print(selected_qc_comparisons[grepl("pct_",names(selected_qc_comparisons))])
```

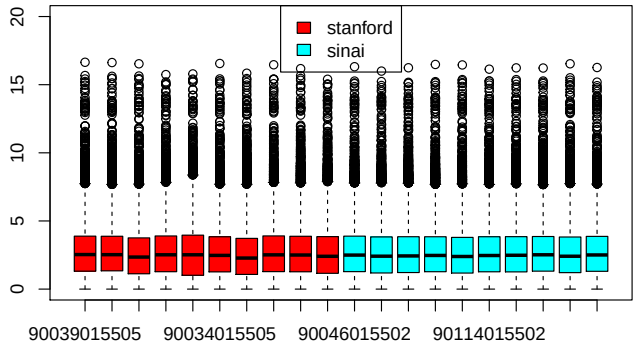
```
##      pct_unmapped_other              pct_globin    pct_adapter_detected
##            4.280729e-15            7.749430e-15            7.985442e-15
##                 pct_utr       pct_trimmed_bases          pct_picard_dup
##            7.989667e-15            7.993189e-15            8.628287e-15
##        pct_dup_sequence             pct_umi_dup             pct_coding
##            8.957509e-15            1.003521e-14            1.081158e-14
##                pct_chrX pct_multimapped_toomany                pct_rRNA
##            1.790211e-13            1.014781e-12            2.552097e-12
##     pct_uniquely_mapped         pct_multimapped                pct_mrna
##            3.844703e-12            7.674417e-12            3.366564e-10
##            pct_intronic          pct_intergenic                pct_chrM
##            5.172570e-10            6.216488e-09            4.088722e-08
##             pct_chrAuto   pct_unmapped_tooshort              pct_contig
##            3.172734e-07            4.687337e-07            4.814240e-03
##                  pct_GC
##            6.067464e-02
```

```
# Comparison 1: selected qc scores
par(mfrow=c(2,2))
boxplot(pct_umi_dup~site,data=gastro_metadata,main="% UMI dup")
boxplot(r_260_230~site,data=gastro_metadata,main = "260/230");abline(h = 1.8,lty=2,col="red")
boxplot(pct_rRNA~site,data=gastro_metadata,main="%rRNA")
boxplot(pct_globin~site,data=gastro_metadata,main="%Globin")
```

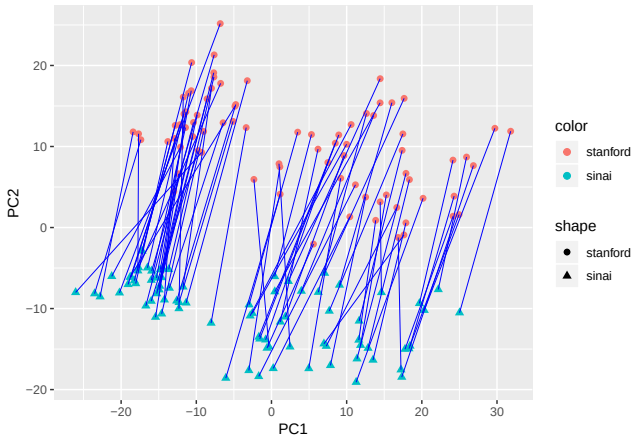


FPKM data comparison

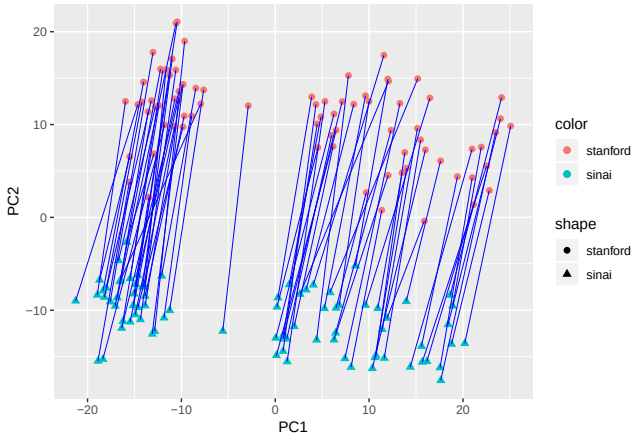
```
# Comparison 2: boxplots
#' Helper function to get a color set by a discrete vector
get_cols_vector_from_names<-function(v,pl_func = topo.colors){
  v = as.character(v)
  vals = unique(v)
  cols = pl_func(length(vals))
  names(cols) = vals
  newv = cols[v]
  return(list(newv,cols))
}
currcols = get_cols_vector_from_names(rnaseq_meta[colnames(gastro_fpkm_mat),"site"],rainbow)
# Select a set of samples for the plot (too many samples otherwise)
inds_for_boxplot = c(1:10,81:90)
boxplot(gastro_fpkm_mat[,inds_for_boxplot],
        col=currcols[[1]][inds_for_boxplot],
        ylim = c(0,20)) # extend lim to have room for legend
legend(x="top",names(currcols[[2]]),fill=currcols[[2]])
```




```
# Comparison 3: PCA
# Attempt 1: Take the FPKM data and run the PCA
gastro_fpkm_pca = prcomp(t(gastro_fpkm_mat))
gastro_fpkm_pcax = gastro_fpkm_pca$x
df = data.frame(gastro_fpkm_pcax[,1:10],
                shape=gastro_metadata$site, color=gastro_metadata$site,
                sample = sample_id[rownames(gastro_fpkm_pcax)])
df = df[order(df$sample),]
# Add lines between matching samples (i.e., same sample, different site)
ggplot(df,aes(x=PC1, y=PC2, shape=shape, color=color,group=sample)) +
  geom_point(size=2) + geom_path(size=0.1,color="blue")
```




```
# Retry - quantile normalization before PCA (slightly cleaner)
gastro_fpkm_mat_q = run_quantile_normalization(gastro_fpkm_mat)
gastro_fpkm_pca = prcomp(t(gastro_fpkm_mat_q))
gastro_fpkm_pcax = gastro_fpkm_pca$x
df = data.frame(gastro_fpkm_pcax[,1:10],
                shape=gastro_metadata$site, color=gastro_metadata$site,
                sample = sample_id[rownames(gastro_fpkm_pcax)])
df = df[order(df$sample),]
# Add lines between matching samples (i.e., same sample, different site)
ggplot(df,aes(x=PC1, y=PC2, shape=shape, color=color,group=sample)) +
  geom_point(size=2) + geom_path(size=0.1,color="blue")
```



Look at the sample correlation

```
par(mfrow=c(1,2))
# Raw FPKMs
x1 = site2fpkm[[1]][,colnames(gastro_fpkm_processed[[1]])]
x2 = site2fpkm[[2]][,colnames(gastro_fpkm_processed[[2]])]
x1 = x1[,order(sample_id[colnames(x1)])]
x2 = x2[,order(sample_id[colnames(x2)])]
print("Do we have the same mapped sample id in the matrices?")
```

```
## [1] "Do we have the same mapped sample id in the matrices?"
```

```r
print(all(sample_id[colnames(x1)] == sample_id[colnames(x2)]))
```

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

```r
corrs = cor(x1,x2,method="spearman")
l = list(
  within = diag(corrs),
  between = corrs[lower.tri(corrs,diag = F)]
)
boxplot(l,col=c("gray","green"),
        main="Sample corr (Spearman), raw FPKM data",
        cex.main=0.9)

# Processed FPKMs - take the expressed genes
corrs = cor(x1[shared_genes,],x2[shared_genes,],method="spearman")
l = list(
  within = diag(corrs),
  between = corrs[lower.tri(corrs,diag = F)]
)
boxplot(l,col=c("gray","green"),
        main="Sample corr (Spearman), filtered FPKM data",
        cex.main=0.9)
```