

```

library(dplyr)
library(magrittr)

#####
#### Preprocessing and Normalization
#####
data.dir <- "/path/to/data"
countFile <- file.path(data.dir, "expected_count.txt")
sampleFile <- file.path(data.dir, "samples.txt")
geneFile <- file.path(data.dir, "GFZP01.1.annotation.updated.txt")

counts <- read.table(countFile, sep = "\t", header = TRUE, row.names = 1,
                     stringsAsFactors = FALSE, check.names = FALSE)
counts <- round(counts)

samples <- read.table(sampleFile, sep = "\t", header = TRUE, row.names = 1,
                     stringsAsFactors = FALSE, check.names = FALSE)

genes <- read.table(geneFile, sep = "\t", header = TRUE, row.names = "ID",
                   stringsAsFactors = FALSE, check.names = FALSE, quote = "",
                   comment.char = "")

edgeR.obj <- edgeR::DGEList(counts = counts, samples = samples, genes = genes,
                           remove.zeros = TRUE)
edgeR.obj <- edgeR::calcNormFactors(edgeR.obj)

obj.WE <- obj[, c("O-WE-2", "O-WE-3", "D3-WE-2", "D3-WE-3",
                 "D8-WE-1", "D8-WE-2", "D3-WE-M1", "D3-WE-M2",
                 "D8-WE-M1", "D8-WE-M2")]
obj.ST <- obj[, c("O-ST-2", "O-ST-4", "D3-ST-4", "D3-ST-5",
                 "D8-ST-1", "D8-ST-2", "D3-ST-M1", "D3-ST-M2",
                 "D8-ST-M1", "D8-ST-M2")]

keep.genes.WE <- rowSums(obj.WE$counts == 0) < 6
obj.WE <- obj.WE[keep.genes.WE,, keep.lib.sizes = FALSE]
obj.WE <- edgeR::calcNormFactors(obj.WE, method = "TMM")
keep.genes.ST <- rowSums(obj.ST$counts == 0) < 6
obj.ST <- obj.ST[keep.genes.ST,, keep.lib.sizes = FALSE]

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obj.ST <- edgeR::calcNormFactors(obj.ST, method = "TMM")

saveRDS(obj.WE, file.path(data.dir, "WE.DEGList.rds"))
saveRDS(obj.ST, file.path(data.dir, "ST.DEGList.rds"))

### Heatmap of all profiled genes (Figure 1B)
annotation_color <- list(Day = c("grey90", "#9F9AC8", "#6B52A2"),
  Treatment = c(No = "#263C91", MS275 = "#D04A38"))

pheatmap::pheatmap(log2(edgeR::cpm(obj.WE)+1),
  breaks = seq(-2, 2, length.out = 100),
  cluster_rows = FALSE,
  show_colnames = FALSE,
  show_rownames = FALSE,
  annotation_row = subset(obj.WE$samples, select = c("Day", "Treatment")),
  treeheight_col = 12,
  legend = FALSE,
  annotation_legend = FALSE,
  annotation_names_row = FALSE,
  annotation_colors = annotation_color)

pheatmap::pheatmap(log2(edgeR::cpm(obj.ST)+1),
  breaks = seq(-2, 2, length.out = 100),
  cluster_rows = FALSE,
  show_colnames = FALSE,
  show_rownames = FALSE,
  annotation_row = subset(obj.ST$samples, select = c("Day", "Treatment")),
  treeheight_col = 12,
  legend = FALSE,
  annotation_legend = FALSE,
  annotation_names_row = FALSE,
  annotation_colors = annotation_color)

### PCA analysis (Figure 1C)
col.day <- c("WED0" = "grey70", "STD0" = "grey70",
  "WED3" = "#98CFE3", "STD3" = "#98CFE3",
  "WED8" = "#FF989F", "STD8" = "#FF989F",
  "WED3MS" = "#017AB3", "STD3MS" = "#017AB3",

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"WED8MS" = "#F61F35", "STD8MS" = "#F61F35")
```

```
pca <- prcomp(t(log2(edgeR::cpm(obj.WE)+1)), center = TRUE, scale. = TRUE)
eigs <- pca$sdev^2
pc.1 <- eigs[1] * 100 / sum(eigs)
pc.2 <- eigs[2] * 100 / sum(eigs)
```

```
xlab <- paste("PC", pc[1], " (", format(pc.1, digit = 2, nsmall = 2), "%)", sep
= "")
ylab <- paste("PC", pc[2], " (", format(pc.2, digit = 2, nsmall = 2), "%)", sep
= "")
```

```
par(mgp = c(1, 0.3, 0))
plot(pca$x[,c(1,2)], xlab = "", ylab = "", col = "white",
     xaxt = "n", yaxt = "n", tck = -0.01, main = "Epidermis")
points(pca$x[,c(1,2)], pch = 16, col = col.day[obj.WE$samples$Condition], cex =
16)
```

```
text(pca$x[,1], pca$x[,2] + 1.5, labels = p.text, col = col, cex = 1.0)
```

```
axis(1, tck = -0.01, cex.axis = 0.6)
axis(2, las = 2, tck = -0.01, cex.axis = 0.6)
```

```
mtext(xlab, side = 1, line = 1.5, cex = 0.75)
mtext(ylab, side = 2, line = 1.5, cex = 0.75)
box()
```

```
pca <- prcomp(t(log2(edgeR::cpm(obj.ST)+1)), center = TRUE, scale. = TRUE)
eigs <- pca$sdev^2
pc.1 <- eigs[1] * 100 / sum(eigs)
pc.2 <- eigs[2] * 100 / sum(eigs)
```

```
xlab <- paste("PC", pc[1], " (", format(pc.1, digit = 2, nsmall = 2), "%)", sep
= "")
ylab <- paste("PC", pc[2], " (", format(pc.2, digit = 2, nsmall = 2), "%)", sep
= "")
```

```
par(mgp = c(1, 0.3, 0))
```

```
plot(pca$x[,c(1,2)], xlab = "", ylab = "", col = "white",
     xaxt = "n", yaxt = "n", tck = -0.01, main = "Soft tissue")
points(pca$x[,c(1,2)], pch = 16, col = col.day[obj.ST$samples$Condition], cex =
16)
```

```
text(pca$x[,1], pca$x[,2] + 1.5, labels = p.text, col = col, cex = 1.0)
```

```
axis(1, tck = -0.01, cex.axis = 0.6)
axis(2, las = 2, tck = -0.01, cex.axis = 0.6)
```

```
mtext(xlab, side = 1, line = 1.5, cex = 0.75)
mtext(ylab, side = 2, line = 1.5, cex = 0.75)
box()
```

```
#####
#### Differential expression analysis
#####
group <- factor(obj.WE$samples$Condition)
```

```
## c: without MS-275; t: with MS-275
## 0, 3 and 8: 0 dpa, 3 dpa, and 8 dpa
```

```
design <- model.matrix(~0 + group)
colnames(design) <- gsub("group", "", colnames(design))
contrast.matrix <- limma::makeContrasts(
  c3.c0 = WED3-WED0,
  c8.c0 = WED8-WED0,
  c8.c3 = WED8-WED3,
  t3.c0 = WED3MS-WED0,
  t8.c0 = WED8MS-WED0,
  t8.t3 = WED8MS-WED3MS,
  t3.c3 = WED3MS-WED3,
  t8.c8 = WED8MS-WED8,
  levels = design)
```

```
v <- limma::voom(obj.WE, design)
vfit <- limma::lmFit(v, design)
vfit <- limma::contrasts.fit(vfit, contrasts = contrast.matrix)
```

```

efit <- limma::eBayes(vfit)
limma.res <- list()

for (tag in colnames(contrast.matrix)){
  limma.res[[tag]] <- subset(limma::topTable(efit, coef=tag, number = Inf),
    select = c("Transcript", "Symbol", "logFC", "P.Value", "adj.P.Val"))
}

saveRDS(limma.res, file = file.path(data.dir, "limma.res.WE.rds"))

## ST
group <- factor(obj.WE$samples$Condition)

design <- model.matrix(~0 + group)
colnames(design) <- gsub("group", "", colnames(design))
contrast.matrix <- limma::makeContrasts(
  c3.c0 = STD3-STD0,
  c8.c0 = STD8-STD0,
  c8.c3 = STD8-STD3,
  t3.c0 = STD3MS-STD0,
  t8.c0 = STD8MS-STD0,
  t8.t3 = STD8MS-STD3MS,
  t3.c3 = STD3MS-STD3,
  t8.c8 = STD8MS-STD8,
  levels = design)

v <- limma::voom(obj.ST, design)
vfit <- limma::lmFit(v, design)
vfit <- limma::contrasts.fit(vfit, contrasts = contrast.matrix)
efit <- limma::eBayes(vfit)
limma.res <- list()

for (tag in colnames(contrast.matrix)){
  limma.res[[tag]] <- subset(limma::topTable(efit, coef=tag, number = Inf),
    select = c("Transcript", "Symbol", "logFC", "P.Value", "adj.P.Val"))
}

saveRDS(limma.res, file = file.path(data.dir, "limma.res.ST.rds"))

```

```
#####
#### Identification of temporal-expression clusters
#####
## Averaging the expression value of two replicates
avgByGroup <- function(exp, group){
  out <- c()
  fs <- unique(group)
  for (f in fs){
    m <- apply(exp[,group == f], 1, mean)
    out <- cbind(out, m)
  }
  colnames(out) <- fs
  out
}

exprs.WE.avg <- avgByGroup(log2(edgeR::cpm(obj.WE)) + 1, obj.WE$Condition)
exprs.ST.avg <- avgByGroup(log2(edgeR::cpm(obj.ST)) + 1, obj.ST$Condition)

collectDiffGenes <- function(limma.obj, tags){
  genes <- c()
  for (tag in tags){
    dat <- limma.obj[[tag]]
    gl <- with(dat, rownames(dat[P.Value < 0.05,]))
    genes <- c(genes, gl)
  }
  genes <- unique(genes)
  genes
}

## Only considering the differentially expressed genes
tags <- c("c3.c0", "c8.c3", "t3.c0", "t8.t3", "c8.c0", "t8.c0")

genes.WE <- file.path(data.dir, "limma.res.WT.rds") %>% readRDS() %>%
collectDiffGenes(tags)
genes.ST <- file.path(data.dir, "limma.res.ST.rds") %>% readRDS() %>%
collectDiffGenes(tags)
```

```

gene.df.WE.filter <- gene.df.WE[genes.WE,]
gene.df.ST.filter <- gene.df.WE[genes.ST,]

exprs.WE.filter <- exprs.WE.avg[genes.WE,]
exprs.ST.filter <- exprs.ST.avg[genes.ST,]

exprs.WE.Ctrl <- exprs.WE.filter[, c("WED0", "WED3", "WED8")]
exprs.WE.MS <- exprs.WE.filter[, c("WED0", "WED3MS", "WED8MS")]

exprs.ST.Ctrl <- exprs.ST.filter[, c("STD0", "STD3", "STD8")]
exprs.ST.MS <- exprs.ST.filter[, c("STD0", "STD3MS", "STD8MS")]

## Cluster analysis using Mfuzz
library(Mfuzz)

processESET <- function(exprs){
  eset <- new("ExpressionSet", exprs=exprs)
  eset.s <- standardise(eset)
  return(eset.s)
}

runMfuzz <- function(exprs, c=8){
  eset.s <- processESET(exprs)
  m1 <- mestimate(eset.s)
  cl <- mfuzz(eset.s, m=m1, c=c)
  return(cl)
}

if (TRUE){
  ## WE
  tmp1 <- exprs.WE.Ctrl
  row.names(tmp1) <- paste(row.names(tmp1), "ctrl", sep = "_")
  tmp2 <- exprs.WE.MS
  row.names(tmp2) <- paste(row.names(tmp2), "ms", sep = "_")

  tmp.WE <- rbind(tmp1, tmp2)

  mfobj.WE <- runMfuzz(tmp.WE, c = 8)

```

```

saveRDS(mfobj.WE, file.path(data.dir, "WE.MFuzz.rds"))
png(file.path(analysis.dir, "Mfuzz_WE.origin.png"), width = 32, height = 12,
units = "cm", res = 1200)
par(mar = c(4, 4, 2, 2))
mfuzz.plot2(processESET(tmp.WE), cl=mfobj.WE, time.labels=c("D0", "D3",
"D8"),
x11 = FALSE, cex.lab = 1, cex.main = 1, cex.axis = 1,
min.mem = 0.2, mfrow = c(2, 4))
dev.off()

## ST
tmp1 <- exprs.ST.Ctrl
row.names(tmp1) <- paste(row.names(tmp1), "ctrl", sep = "_")
tmp2 <- exprs.ST.MS
row.names(tmp2) <- paste(row.names(tmp2), "ms", sep = "_")

tmp.ST <- rbind(tmp1, tmp2)

mfobj.ST <- runMfuzz(tmp.ST, c = 8)
#mfuzz.plot(processESET(tmp.ST), cl=mfobj.ST, time.labels=c("D0", "D3",
"D8"),
# min.mem = 0.2, mfrow = c(4, 2))
saveRDS(mfobj.ST, file.path(data.dir, "ST.MFuzz.rds"))
png(file.path(analysis.dir, "Mfuzz_ST.origin.png"), width = 32, height = 12,
units = "cm", res = 1200)
par(mar = c(4, 4, 2, 2))
mfuzz.plot2(processESET(tmp.ST), cl=mfobj.ST, time.labels=c("D0", "D3",
"D8"),
x11 = FALSE, cex.lab = 1, cex.main = 1, cex.axis = 1,
min.mem = 0.2, mfrow = c(2, 4))
dev.off()
}

## Re-name cluster numbers to make expression pattern consistency
if (TRUE){
mfobj.WE <- readRDS(file.path(data.dir, "WE.MFuzz.rds"))
order.WE <- c(1, 4, 2, 5, 8, 6, 3, 7)
new.label.WE <- c(1, 3, 7, 2, 4, 6, 8, 5)

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```

mfobj.WE.edit <- mfobj.WE
mfobj.WE.edit$size <- mfobj.WE.edit$size[order.WE]
mfobj.WE.edit$membership <- mfobj.WE.edit$membership[, order.WE]
colnames(mfobj.WE.edit$membership) <- c(1:8)
cluster.WE <- factor(mfobj.WE$cluster)
levels(cluster.WE) <- new.label.WE
cluster.WE <- as.character(cluster.WE)
cluster.WE <- as.numeric(cluster.WE)
names(cluster.WE) <- names(mfobj.WE$cluster)
mfobj.WE.edit$cluster <- cluster.WE
saveRDS(mfobj.WE.edit, file.path(data.dir, "WE.MFuzz.modified.rds"))

```

```

mfobj.ST <- readRDS(file.path(data.dir, "ST.MFuzz.rds"))
order.ST <- c(2, 1, 8, 6, 5, 7, 3, 4)
new.label.ST <- c(2, 1, 7, 8, 5, 4, 6, 3)
mfobj.ST.edit <- mfobj.ST
mfobj.ST.edit$size <- mfobj.WE.edit$size[order.ST]
mfobj.ST.edit$membership <- mfobj.ST.edit$membership[, order.ST]
colnames(mfobj.ST.edit$membership) <- c(1:8)
cluster.ST <- factor(mfobj.ST$cluster)
levels(cluster.ST) <- new.label.ST
cluster.ST <- as.character(cluster.ST)
cluster.ST <- as.numeric(cluster.ST)
names(cluster.ST) <- names(mfobj.ST$cluster)
mfobj.ST.edit$cluster <- cluster.ST
saveRDS(mfobj.ST.edit, file.path(data.dir, "ST.MFuzz.modified.rds"))

```

```

}
```