```
title: "Affymetrix Microarray Minimal pipeline"
author: "Simon Tomlinson 10/02/2021"
output: html_document
```

Affymetrix Microarray Analysis Basic (Skeleton) Workflow

##Load the required libraries & load the files for the workflow

```
library(limma)
library(affy)
```

```
## Loading required package: BiocGenerics
```

```
##
## Attaching package: 'BiocGenerics'
```

```
## The following object is masked from 'package:limma':
##
##     plotMA
```

```
## The following objects are masked from 'package:stats':
##
##     IQR, mad, sd, var, xtabs
```

```
## The following objects are masked from 'package:base':
##
##     anyDuplicated, aperm, append, as.data.frame, basename, cbind,
##     colnames, dirname, do.call, duplicated, eval, evalq, Filter, Find,
##     get, grep, grepl, intersect, is.unsorted, lapply, Map, mapply,
##     match, mget, order, paste, pmax, pmax.int, pmin, pmin.int,
##     Position, rank, rbind, Reduce, rownames, sapply, setdiff, sort,
##     table, tapply, union, unique, unsplit, which.max, which.min
```

```
## Loading required package: Biobase
```

```
## Welcome to Bioconductor
##
##     Vignettes contain introductory material; view with
##     'browseVignettes()'. To cite Bioconductor, see
##     'citation("Biobase")', and for packages 'citation("pkgname")'.
```

```
library(annotate)
```

```
## Loading required package: AnnotationDbi
```

```
## Loading required package: stats4
```

```
## Loading required package: IRanges
```

```
## Loading required package: S4Vectors
```

```
##
## Attaching package: 'S4Vectors'
```

```
## The following objects are masked from 'package:base':
##
##     expand.grid, I, unname
```

```
## Loading required package: XML
```

```
library(mouse4302.db)# load chip-specific annotation
```

```
## Loading required package: org.Mm.eg.db
```

```
##
```

```
##
```

```
#install.packages("scatterplot3d",repo="http://cran.ma.imperia#l.ac.uk")
#Then load the library
library(scatterplot3d)
```

Load the main data- commented code is just for information

```
system("tar -xvf /shared_files/FGT_T3_GSE10806_RAW.tar")
system("cp  /shared_files/FGT_T3_targets.txt  .")
# Load the target file into an AnnotatedDataFrame object
adf<-read.AnnotatedDataFrame("FGT_T3_targets.txt",header=TRUE,row.names=1,as.is=TRUE)
# Load the expression values of all the CEL files in the targets file
#mydata <- ReadAffy(filenames=pData(adf)$FileName,phenoData=adf)

# Or just to quickly load all CEL files in the R working directory
mydata <- ReadAffy()
# View a summary of the example data
mydata
```

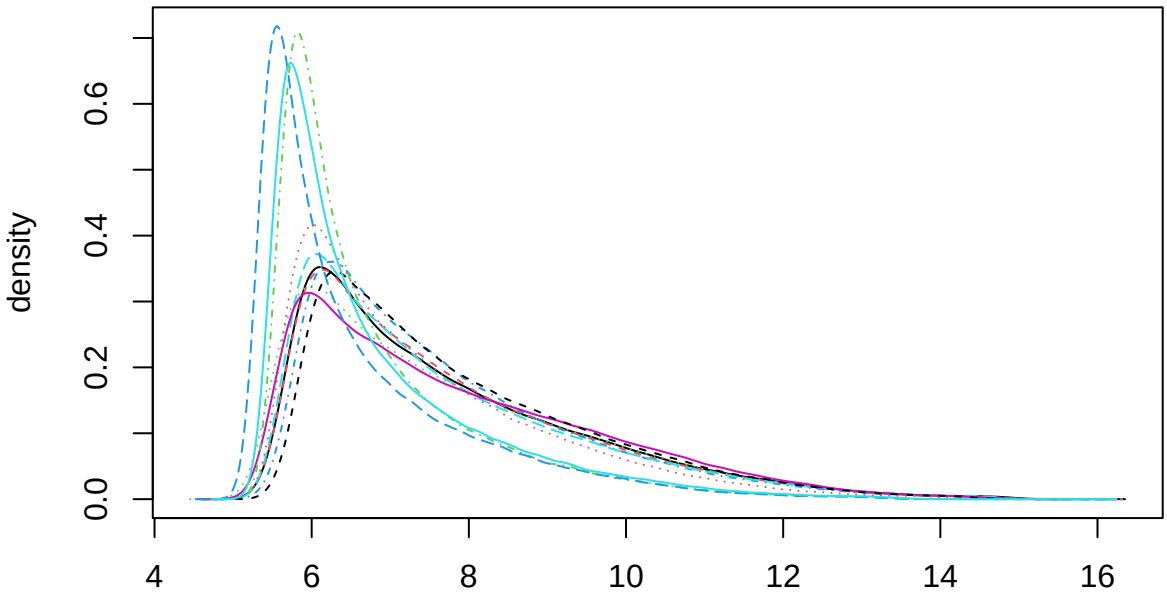
```
## Warning: replacing previous import 'AnnotationDbi::tail' by 'utils::tail' when
## loading 'mouse4302cdf'
```

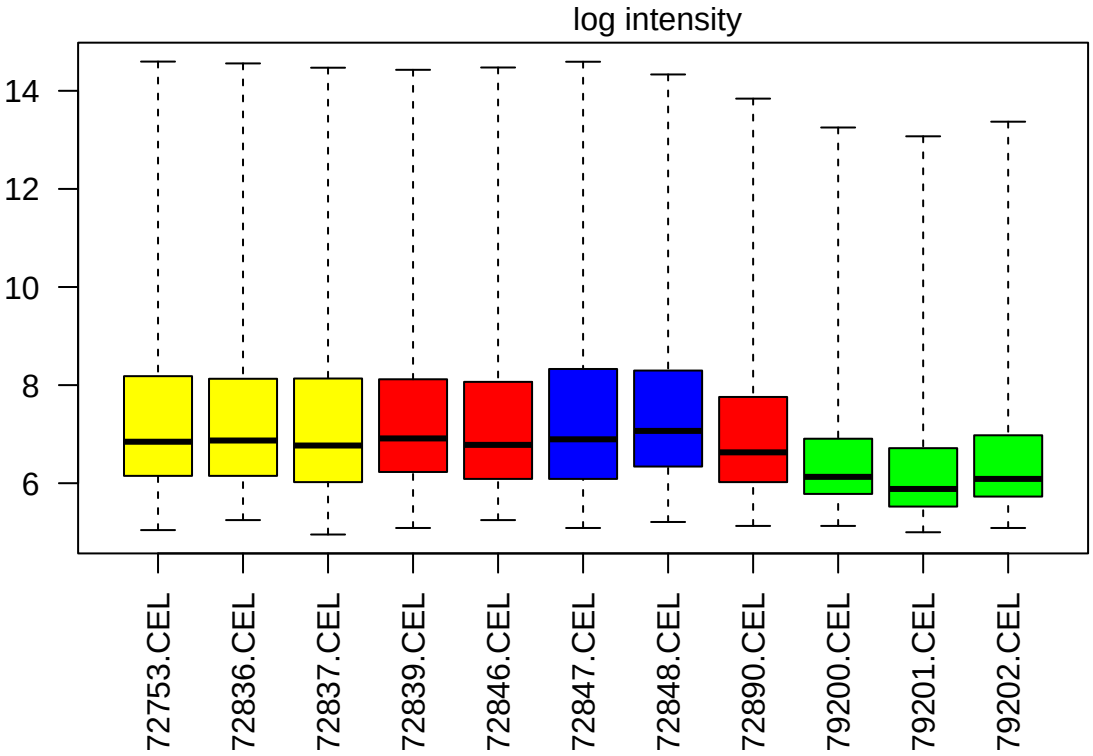
```
## Warning: replacing previous import 'AnnotationDbi::head' by 'utils::head' when
## loading 'mouse4302cdf'
```

```
##
```

```
## AffyBatch object
## size of arrays=1002x1002 features (22 kb)
## cdf=Mouse430_2 (45101 affyids)
## number of samples=11
## number of genes=45101
## annotation=mouse4302
## notes=
```

Build Quality Control Plots





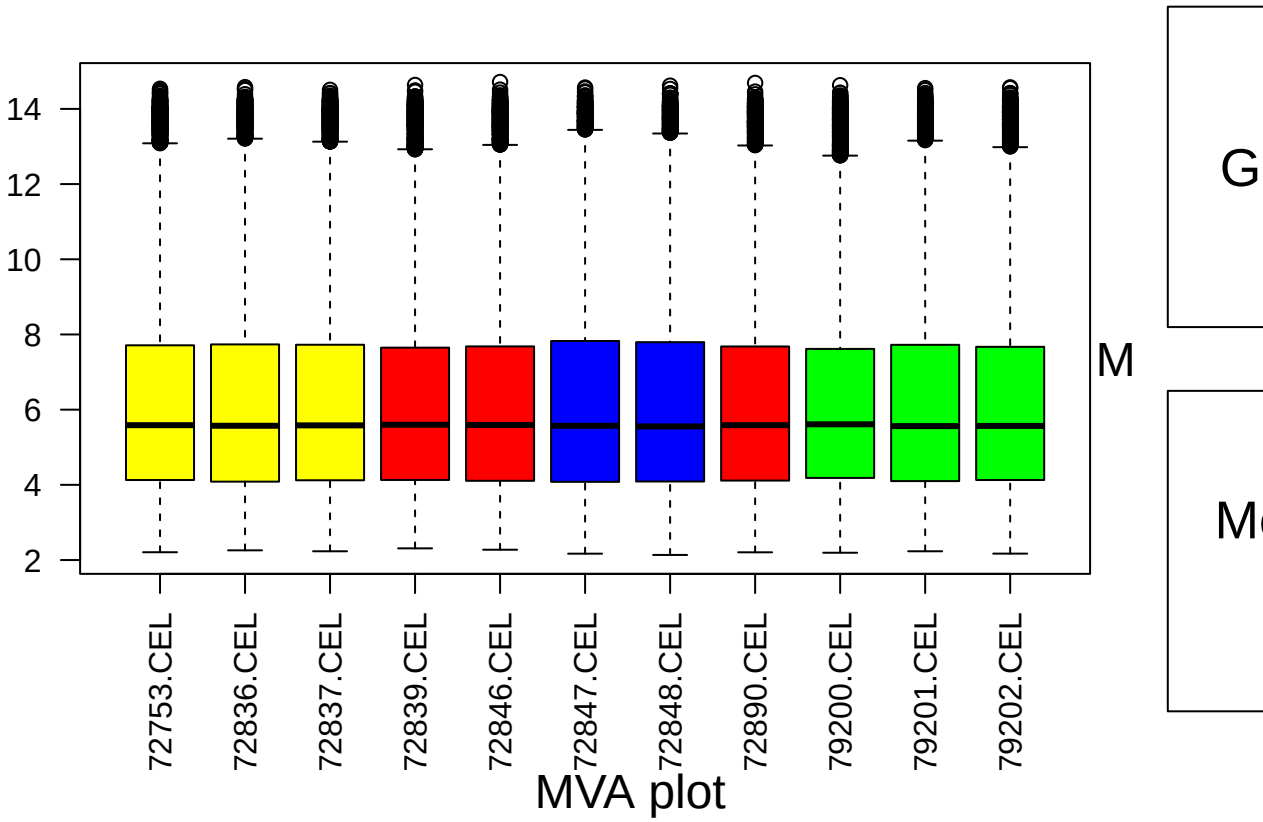
Normalise the data using RMA

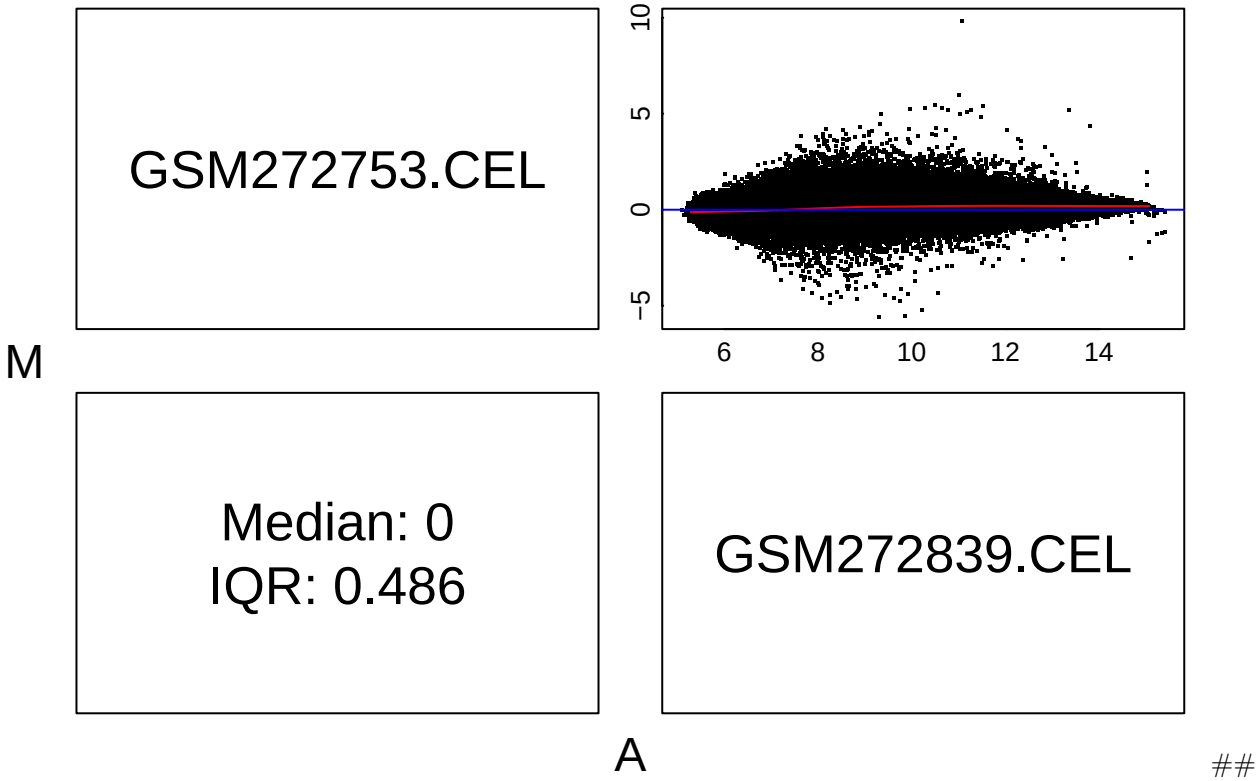
```
## Background correcting
## Normalizing
## Calculating Expression
```

```
## ExpressionSet (storageMode: lockedEnvironment)
## assayData: 45101 features, 11 samples
##   element names: exprs
## protocolData
```

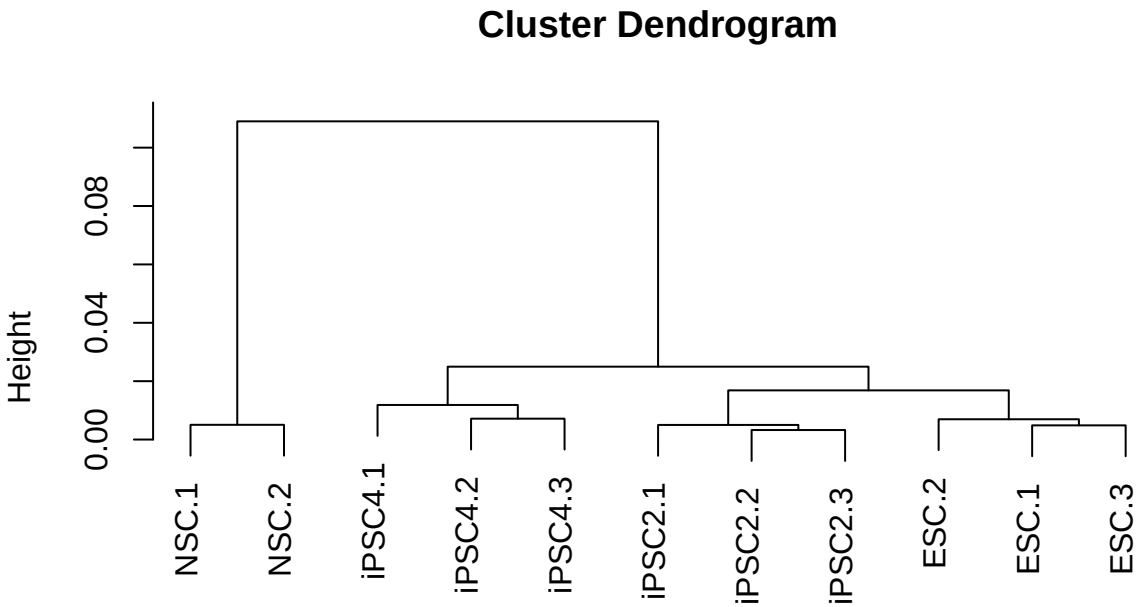
```
##   sampleNames: GSM272753.CEL GSM272836.CEL ... GSM279202.CEL (11 total)
##   varLabels: ScanDate
##   varMetadata: labelDescription
## phenoData
##   sampleNames: GSM272753.CEL GSM272836.CEL ... GSM279202.CEL (11 total)
##   varLabels: sample
##   varMetadata: labelDescription
## featureData: none
## experimentData: use 'experimentData(object)'
## Annotation: mouse4302
```

Plot Normalised Data

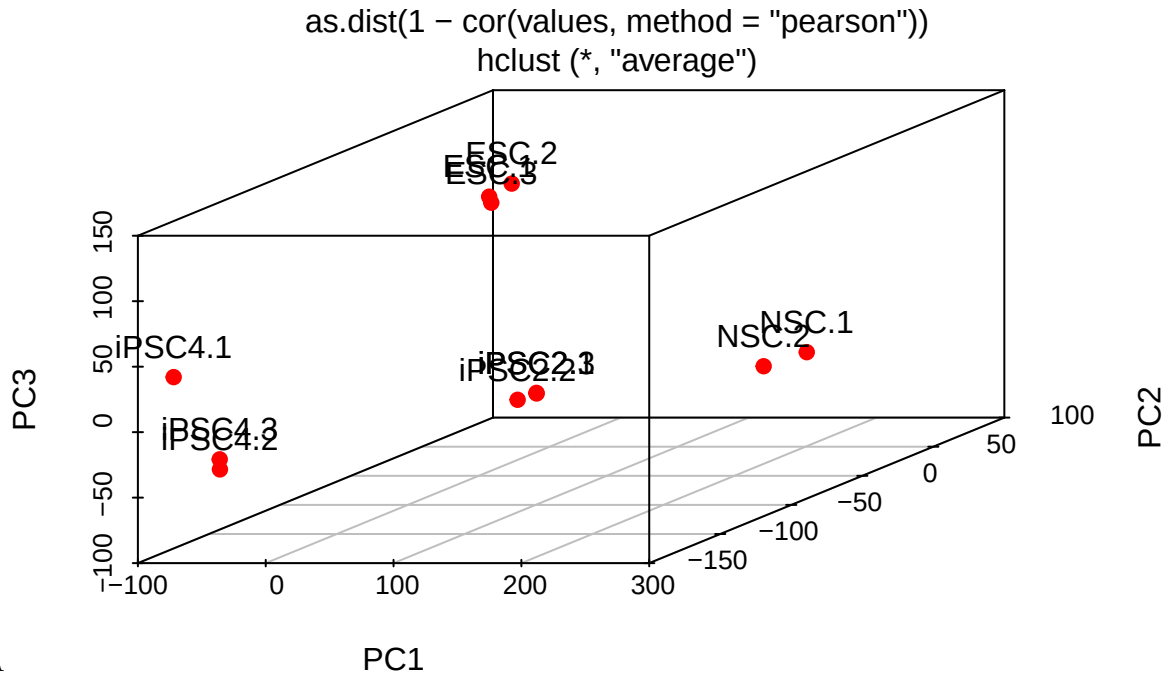




##



Plot Heatmap



Perform PCA

Perform fold filtering

```
#obtaining a matrix of expression values
exprsvals <- exprs(eset)
#RMA outputs log2 data while MAS5 outputs linear data
#To convert from log...
exprsvals10 <-2^exprsvals
#check conversion
exprsvals[1:10,]
```

```
##              GSM272753.CEL GSM272836.CEL GSM272837.CEL GSM272839.CEL
## 1415670_at        9.669418      9.468712      9.840497      9.542554
## 1415671_at       10.804052     10.560056     11.161637     10.006636
## 1415672_at       11.137131     11.004639     11.177958     11.265057
## 1415673_at        9.065874      9.002128      8.882502      9.359967
## 1415674_a_at     10.075517      9.794644      9.838132      9.877397
## 1415675_at        8.808742      8.668103      8.629835      9.120798
## 1415676_a_at     11.802675     11.477381     11.960382     11.134994
## 1415677_at        8.093115      7.773758      8.056709      7.423166
## 1415678_at       10.273131     10.384452     10.557665     10.513949
## 1415679_at       10.886946     10.582367     10.711379     10.492362
##              GSM272846.CEL GSM272847.CEL GSM272848.CEL GSM272890.CEL
## 1415670_at        9.566148      9.556946      9.555320      9.570322
## 1415671_at       10.203007      9.731251      9.542029     10.510781
## 1415672_at       11.253738     11.133394     11.147553     11.388213
## 1415673_at        9.397668      9.355186      9.524904      9.200095
## 1415674_a_at      9.924118      9.680461      9.816652      9.673921
## 1415675_at        9.068031      8.790371      8.846509      8.956731
## 1415676_a_at     11.281870     11.358769     11.387082     11.107652
## 1415677_at        7.416413      8.870749      8.918135      7.399723
## 1415678_at       10.664771     10.095007      9.642961     10.883287
## 1415679_at       10.549639     10.961153     11.149409     10.404885
##              GSM279200.CEL GSM279201.CEL GSM279202.CEL
## 1415670_at        9.997091     10.291382     10.041643
## 1415671_at       10.753063     10.494127     10.333424
## 1415672_at       10.746576     10.887394     10.960298
## 1415673_at        8.500078      8.831864      8.938540
## 1415674_a_at      9.135410      9.433351      9.794644
## 1415675_at        8.847440      9.019164      9.162779
## 1415676_a_at     11.219547     11.548926     11.431180
## 1415677_at        7.072158      7.420528      7.466735
## 1415678_at       10.668909     10.876593     10.378917
## 1415679_at       10.625891     10.558164     10.572555
```

```
#converted
exprsvals10[1:10,]
```

```
##              GSM272753.CEL GSM272836.CEL GSM272837.CEL GSM272839.CEL
## 1415670_at         814.3011      708.5432      916.8216      745.7532
## 1415671_at        1787.9027     1509.7102     2290.8016     1028.7211
## 1415672_at        2252.2186     2054.5958     2316.8651     2461.0480
## 1415673_at         535.9201      512.7556      471.9539      657.0988
## 1415674_a_at      1079.0283      888.1406      915.3197      940.5738
## 1415675_at         448.4310      406.7795      396.1313      556.7162
## 1415676_a_at      3572.3920     2851.2542     3985.0485     2248.8846
## 1415677_at         273.0677      218.8438      266.2632      171.6309
## 1415678_at        1237.4294     1336.6927     1507.2107     1462.2242
## 1415679_at        1893.6399     1533.2386     1676.6650     1440.5079
##              GSM272846.CEL GSM272847.CEL GSM272848.CEL GSM272890.CEL
## 1415670_at         758.0494      753.2296      752.3812      760.2456
## 1415671_at        1178.7210      849.9598      745.4817     1459.0169
## 1415672_at        2441.8139     2246.3920     2268.5474     2680.3630
## 1415673_at         674.4967      654.9251      736.6850      588.1723
## 1415674_a_at       971.5322      820.5579      901.7928      816.8465
## 1415675_at         536.7219      442.7568      460.3251      496.8721
```

```
## 1415676_a_at     2489.8964     2626.2153     2678.2635     2206.6647
## 1415677_at        170.8295      468.1245      483.7556      168.8645
## 1415678_at       1623.3647     1093.7045      799.5036     1888.8425
## 1415679_at       1498.8490     1993.5893     2271.4681     1355.7585
##              GSM279200.CEL GSM279201.CEL GSM279202.CEL
## 1415670_at       1021.9374     1253.1837     1053.9879
## 1415671_at       1725.8164     1442.2710     1290.2406
## 1415672_at       1718.0732     1894.2279     1992.4085
## 1415673_at        362.0583      455.6758      490.6463
## 1415674_a_at      562.3835      691.3876      888.1406
## 1415675_at        460.6221      518.8465      573.1540
## 1415676_a_at     2384.6249     2996.2171     2761.3917
## 1415677_at        134.5649      171.3174      176.8933
## 1415678_at       1628.0272     1880.0990     1331.5738
## 1415679_at       1580.1998     1507.7313     1522.8465
```

```
#More fold filtering
#check order of sample names
mysamples <- sampleNames(eset)
#display the list
mysamples
```

```
##  [1] "GSM272753.CEL" "GSM272836.CEL" "GSM272837.CEL" "GSM272839.CEL"
##  [5] "GSM272846.CEL" "GSM272847.CEL" "GSM272848.CEL" "GSM272890.CEL"
##  [9] "GSM279200.CEL" "GSM279201.CEL" "GSM279202.CEL"
```

```
#it is useful to obtain a vector of ProbeIDs here
probesets <- probeNames(mydata)
#display the first 10 ProbeSets
probesets[1:10]
```

```
##  [1] "1415670_at" "1415670_at" "1415670_at" "1415670_at" "1415670_at"
##  [6] "1415670_at" "1415670_at" "1415670_at" "1415670_at" "1415670_at"
```

```
#Build final fold table
#Calculate the means
#Note mean of the log is not the same as the log of the mean!!
ES.mean <- apply(exprsvals10[,c("GSM272753.CEL", "GSM272836.CEL","GSM272837.CEL")],1,mean)
iPS_OK.mean <- apply(exprsvals10[,c("GSM272839.CEL", "GSM272846.CEL","GSM272890.CEL")],1,mean)
iPS_4F.mean <- apply(exprsvals10[,c("GSM279200.CEL", "GSM279201.CEL","GSM279202.CEL")],1,mean)
NSC.mean <- apply(exprsvals10[,c("GSM272847.CEL","GSM272848.CEL")],1,mean)
#calculate some fold changes
ES_iPS_OK <-ES.mean /iPS_OK.mean
ES_iPS_4F <-ES.mean /iPS_4F.mean
ES_NSC <-ES.mean /NSC.mean
#build a summary table to hold all the data
all.data= cbind(ES.mean,iPS_OK.mean,iPS_4F.mean, NSC.mean, ES_iPS_OK,
ES_iPS_4F, ES_NSC)
#check the column names
colnames(all.data)
```

```
## [1] "ES.mean"     "iPS_OK.mean" "iPS_4F.mean" "NSC.mean"    "ES_iPS_OK"
## [6] "ES_iPS_4F"   "ES_NSC"
```

```
#write the table of means as an output
write.table(all.data,file="group_means.txt", quote=F,
sep="\t",col.names=NA)
```

Beginning statistical analysis

```
#Check original sample order
sampleNames(eset)
```

```
##  [1] "GSM272753.CEL" "GSM272836.CEL" "GSM272837.CEL" "GSM272839.CEL"
##  [5] "GSM272846.CEL" "GSM272847.CEL" "GSM272848.CEL" "GSM272890.CEL"
##  [9] "GSM279200.CEL" "GSM279201.CEL" "GSM279202.CEL"
```

```
#Rename the samples
sampleNames(eset) <-
c("ESC.1","ESC.2","ESC.3","iPS2.2","iPS2.3","NSC.1","NSC.2","iPS2.1","iPS4.1","iPS4.2","iPS.3")
#Check the samples have renamed
sampleNames(eset)
```

```
##  [1] "ESC.1"  "ESC.2"  "ESC.3"  "iPS2.2" "iPS2.3" "NSC.1"  "NSC.2"  "iPS2.1"
##  [9] "iPS4.1" "iPS4.2" "iPS.3"
```

```
##Building annotation for differential gene identification
#establish annotation for MOE430v2
#which annotation do we need
#modified from #http://gettinggeneticsdone.blogspot.co.uk/2012/01/annotating-limma-#results-with-gene.h

eset@annotation
```

```
## [1] "mouse4302"
```

```
#packages in the annotation package
ls("package:mouse4302.db")
```

```
##  [1] "mouse4302"              "mouse4302_dbconn"       "mouse4302_dbfile"
##  [4] "mouse4302_dbInfo"       "mouse4302_dbschema"     "mouse4302.db"
##  [7] "mouse4302ACCNUM"        "mouse4302ALIAS2PROBE"   "mouse4302CHR"
## [10] "mouse4302CHRLENGTHS"    "mouse4302CHRLOC"        "mouse4302CHRLOCEND"
## [13] "mouse4302ENSEMBL"       "mouse4302ENSEMBL2PROBE" "mouse4302ENTREZID"
## [16] "mouse4302ENZYME"        "mouse4302ENZYME2PROBE"  "mouse4302GENENAME"
## [19] "mouse4302GO"            "mouse4302GO2ALLPROBES"  "mouse4302GO2PROBE"
## [22] "mouse4302MAPCOUNTS"     "mouse4302MGI"           "mouse4302MGI2PROBE"
## [25] "mouse4302ORGANISM"      "mouse4302ORGPKG"        "mouse4302PATH"
## [28] "mouse4302PATH2PROBE"    "mouse4302PFAM"          "mouse4302PMID"
## [31] "mouse4302PMID2PROBE"    "mouse4302PROSITE"       "mouse4302REFSEQ"
## [34] "mouse4302SYMBOL"        "mouse4302UNIPROT"
```

```
#build an annotation table
ID <- featureNames(eset)
Symbol <- getSYMBOL(ID, "mouse4302.db")
Name <- as.character(lookUp(ID, "mouse4302.db", "GENENAME"))
tmp <- data.frame(ID=ID, Symbol=Symbol, Name=Name, stringsAsFactors=F)
tmp[tmp=="NA"] <- NA #fix padding with NA characters
#assign as feature data of the current Eset
fData(eset) <- tmp
```

Statistical analysis using Limma

```
#Build the design matrix
design <- model.matrix(~-1+factor(c(1,1,1,2,2,3,3,2,4,4,4)))
colnames(design) <- c("ESC","iPS2","NSC","iPS4")
```

```
#Check it makes sense
sampleNames(eset)
```

```
##  [1] "ESC.1"  "ESC.2"  "ESC.3"  "iPS2.2" "iPS2.3" "NSC.1"  "NSC.2"  "iPS2.1"
##  [9] "iPS4.1" "iPS4.2" "iPS.3"
```

```
#output the design matrix
design
```

```
##    ESC iPS2 NSC iPS4
## 1    1    0   0    0
## 2    1    0   0    0
## 3    1    0   0    0
## 4    0    1   0    0
## 5    0    1   0    0
## 6    0    0   1    0
## 7    0    0   1    0
## 8    0    1   0    0
## 9    0    0   0    1
## 10   0    0   0    1
## 11   0    0   0    1
## attr(,"assign")
## [1] 1 1 1 1
## attr(,"contrasts")
## attr(,"contrasts")$`factor(c(1, 1, 1, 2, 2, 3, 3, 2, 4, 4, 4))`
## [1] "contr.treatment"
```

```
#This instructs Limma which comparisons to make
contrastmatrix <- makeContrasts(ESC-iPS2,ESC-NSC,ESC-iPS4,
levels=design)
contrastmatrix
```

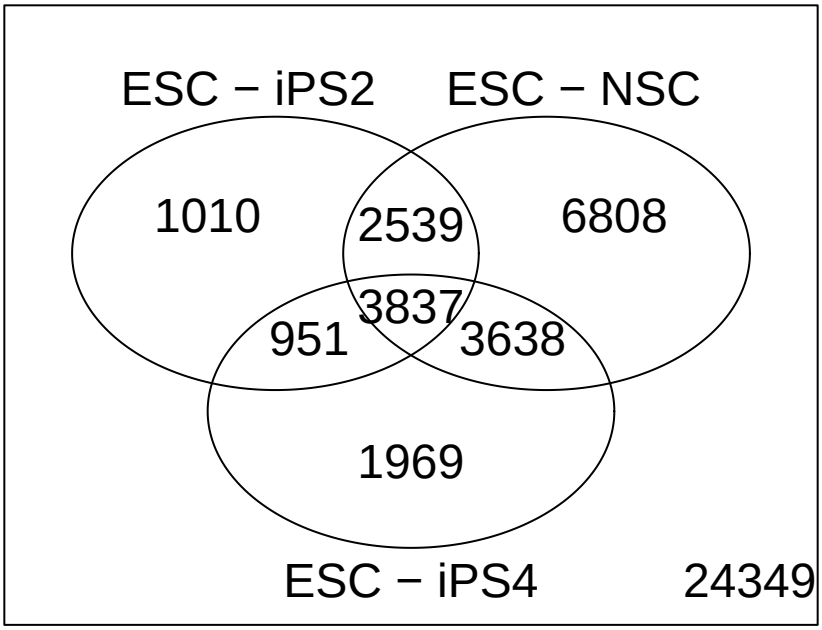
```
##       Contrasts
## Levels ESC - iPS2 ESC - NSC ESC - iPS4
##   ESC           1         1          1
##   iPS2         -1         0          0
##   NSC           0        -1          0
##   iPS4          0         0         -1
```

```
#issue these commands to fit the model
#and make the contrasts
fit <- lmFit(eset, design)

fit2 <- contrasts.fit(fit, contrastmatrix)

#this last part essentially moderates the t-statistic using
#the borrowed variance approach described in class
fit2 <- eBayes(fit2)

#get the results
topTable(fit2,coef=1,adjust="fdr")
```

```
##                         ID   Symbol
## 1421144_at     1421144_at  Rpgrip1
## 1437967_at     1437967_at  Ccdc141
## 1425137_a_at 1425137_a_at   H2-Q10
```

```
## 1425427_at     1425427_at  Gm20767
## 1425220_x_at 1425220_x_at AF067061
## 1445226_at     1445226_at AI506816
## 1418704_at     1418704_at  S100a13
## 1430979_a_at 1430979_a_at    Prdx2
## 1420773_at     1420773_at  Usp17la
## 1454971_x_at 1454971_x_at  Tsc22d1
##                                                                  Name
## 1421144_at   retinitis pigmentosa GTPase regulator interacting protein 1
## 1437967_at                             coiled-coil domain containing 141
## 1425137_a_at                    histocompatibility 2, Q region locus 10
## 1425427_at                                       predicted gene, 20767
## 1425220_x_at                                   cDNA sequence AF067061
## 1445226_at                                expressed sequence AI506816
## 1418704_at                             S100 calcium binding protein A13
## 1430979_a_at                                          peroxiredoxin 2
## 1420773_at                         ubiquitin specific peptidase 17-like A
## 1454971_x_at                                 TSC22 domain family, member 1
##                  logFC   AveExpr         t      P.Value    adj.P.Val        B
## 1421144_at    5.177794 10.718373  39.80404 2.024468e-14 9.130555e-10 21.15821
## 1437967_at    3.890379  5.694028  28.66822 1.120095e-12 1.526453e-08 18.52879
## 1425137_a_at -3.651515  5.599389 -28.25430 1.337197e-12 1.526453e-08 18.39780
## 1425427_at   -3.426349  4.074569 -27.77323 1.648182e-12 1.526453e-08 18.24169
## 1425220_x_at -3.548330  6.182014 -27.71308 1.692261e-12 1.526453e-08 18.22187
## 1445226_at   -4.509854  7.539736 -27.12318 2.198722e-12 1.539552e-08 18.02388
## 1418704_at    3.648166  7.897353  26.78701 2.558847e-12 1.539552e-08 17.90804
## 1430979_a_at -3.163601  7.443417 -26.64406 2.730852e-12 1.539552e-08 17.85810
## 1420773_at   -4.061445  5.430320 -26.19480 3.357846e-12 1.682691e-08 17.69845
## 1454971_x_at  2.297421 11.820596  25.59797 4.442621e-12 1.946981e-08 17.47982
```

```
myresults <-topTable(fit2,coef=1, adjust="fdr", number=nrow(eset))
write.table(myresults,"myresults.txt")

#make a venn diagram
clas <- classifyTestsF(fit2)
vennDiagram(clas)
```



Carry out Functional Enrichment analysis

```
Mm.H <- readRDS("/shared_files/MSigDB/Mm.h.all.v7.1.entrez.rds")

#Check that you have the required objects
ls()
```

```
##  [1] "adf"            "all.data"       "clas"           "colours"
##  [5] "contrastmatrix" "design"         "ES_iPS_4F"      "ES_iPS_OK"
##  [9] "ES_NSC"         "ES.mean"        "eset"           "exprsvals"
## [13] "exprsvals10"    "fit"            "fit2"           "hc"
## [17] "ID"             "iPS_4F.mean"    "iPS_OK.mean"    "Mm.H"
## [21] "mydata"         "myresults"      "mysamples"      "Name"
## [25] "NSC.mean"       "pca"            "probesets"      "s3d"
## [29] "s3d.coords"     "Symbol"         "tmp"            "values"
```

```
#Show the full contents of the annotation package
ls("package:mouse4302.db")
```

```
##  [1] "mouse4302"              "mouse4302_dbconn"       "mouse4302_dbfile"
##  [4] "mouse4302_dbInfo"       "mouse4302_dbschema"     "mouse4302.db"
##  [7] "mouse4302ACCNUM"        "mouse4302ALIAS2PROBE"   "mouse4302CHR"
## [10] "mouse4302CHRLENGTHS"    "mouse4302CHRLOC"        "mouse4302CHRLOCEND"
## [13] "mouse4302ENSEMBL"       "mouse4302ENSEMBL2PROBE" "mouse4302ENTREZID"
## [16] "mouse4302ENZYME"        "mouse4302ENZYME2PROBE"  "mouse4302GENENAME"
## [19] "mouse4302GO"            "mouse4302GO2ALLPROBES"  "mouse4302GO2PROBE"
## [22] "mouse4302MAPCOUNTS"     "mouse4302MGI"           "mouse4302MGI2PROBE"
## [25] "mouse4302ORGANISM"      "mouse4302ORGPKG"        "mouse4302PATH"
## [28] "mouse4302PATH2PROBE"    "mouse4302PFAM"          "mouse4302PMID"
## [31] "mouse4302PMID2PROBE"    "mouse4302PROSITE"       "mouse4302REFSEQ"
## [34] "mouse4302SYMBOL"        "mouse4302UNIPROT"
```

```
#Show the annotation keys in this database
keytypes(mouse4302.db)
```

```
##  [1] "ACCNUM"       "ALIAS"        "ENSEMBL"      "ENSEMBLPROT"  "ENSEMBLTRANS"
##  [6] "ENTREZID"     "ENZYME"       "EVIDENCE"     "EVIDENCEALL"  "GENENAME"
## [11] "GENETYPE"     "GO"           "GOALL"        "IPI"          "MGI"
## [16] "ONTOLOGY"     "ONTOLOGYALL"  "PATH"         "PFAM"         "PMID"
## [21] "PROBEID"      "PROSITE"      "REFSEQ"       "SYMBOL"       "UNIPROT"
```

```
sampleNames(eset)
```

```
##  [1] "ESC.1"  "ESC.2"  "ESC.3"  "iPS2.2" "iPS2.3" "NSC.1"  "NSC.2"  "iPS2.1"
##  [9] "iPS4.1" "iPS4.2" "iPS.3"
```

Process annotation for functional enrichment

```
#Here we select from the annotation a number of keys with the primary key being PROBEID
res <- select(mouse4302.db, keys = rownames(eset), columns = c("ENTREZID", "ENSEMBL","SYMBOL"), keytype=
```

```
## 'select()' returned 1:many mapping between keys and columns
```

```
#View the top of the table
head(res)
```

```
##        PROBEID ENTREZID            ENSEMBL   SYMBOL
## 1   1415670_at    54161 ENSMUSG00000030058    Copg1
## 2   1415671_at    11972 ENSMUSG00000013160 Atp6v0d1
## 3   1415672_at    57437 ENSMUSG00000015341   Golga7
## 4   1415673_at   100678 ENSMUSG00000029446     Psph
## 5 1415674_a_at    60409 ENSMUSG00000032112  Trappc4
## 6   1415675_at    13481 ENSMUSG00000026810     Dpm2
```

```
#find the index of each row of the expression set in the #annotation object res
idx <- match(rownames(eset), res$PROBEID)
#Use the index to set the phenotypic data in the ExpressionSet
fData(eset) <- res[idx, ]
head(fData(eset), 10)
```

```
##         PROBEID ENTREZID            ENSEMBL   SYMBOL
## 1    1415670_at    54161 ENSMUSG00000030058    Copg1
## 2    1415671_at    11972 ENSMUSG00000013160 Atp6v0d1
## 3    1415672_at    57437 ENSMUSG00000015341   Golga7
## 4    1415673_at   100678 ENSMUSG00000029446     Psph
## 5  1415674_a_at    60409 ENSMUSG00000032112  Trappc4
## 6    1415675_at    13481 ENSMUSG00000026810     Dpm2
## 7  1415676_a_at    19173 ENSMUSG00000022193    Psmb5
## 8    1415677_at    52585 ENSMUSG00000002332    Dhrs1
## 9    1415678_at    19042 ENSMUSG00000021096    Ppm1a
## 10   1415679_at    66340 ENSMUSG00000036835   Psenen
```

```
#Find all rows that don't have an EntrezID and remove then
eset_t<-eset[is.na(fData(eset)$ENTREZID)==0,]
```

Functional Enrichment Analysis

```
#convert to indexes
H.indices <- ids2indices(Mm.H,fData(eset_t)$ENTREZID)
#Pick the most suitable enrichment analysis tool to find #enrichment signatures in the data and run thi
```

```r
#I just run mroast here as an example- justify the selection of this method!

#if you want to run mroast
results <-mroast(eset_t,index=H.indices,design=design,contrast=contrastmatrix[,1],adjust.method = "BH")
#if you want to run camera
#results <-camera(eset_t,index=H.indices,design=design,contrast=contrastmatrix[,1],adjust.method = "BH"
#if you want to run romer
#results <-romer(eset_t,index=H.indices,design=design,contrast=contrastmatrix[,1],adjust.method = "BH")
#View the results
results
```

```
##                                                NGenes  PropDown     PropUp
## HALLMARK_MYC_TARGETS_V1                           609 0.4548440 0.05582923
## HALLMARK_UNFOLDED_PROTEIN_RESPONSE                348 0.3477011 0.13793103
## HALLMARK_SPERMATOGENESIS                          450 0.2422222 0.17333333
## HALLMARK_PROTEIN_SECRETION                        327 0.1712538 0.32415902
## HALLMARK_APICAL_SURFACE                           149 0.1073826 0.26174497
## HALLMARK_MYC_TARGETS_V2                           128 0.5390625 0.07812500
## HALLMARK_ANGIOGENESIS                             157 0.3375796 0.15286624
## HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION        638 0.3652038 0.13949843
## HALLMARK_HEME_METABOLISM                          628 0.1608280 0.27866242
## HALLMARK_PANCREAS_BETA_CELLS                      131 0.2137405 0.12213740
## HALLMARK_ALLOGRAFT_REJECTION                      553 0.2332731 0.13562387
## HALLMARK_G2M_CHECKPOINT                           708 0.3206215 0.15677966
## HALLMARK_TNFA_SIGNALING_VIA_NFKB                  529 0.2079395 0.29300567
## HALLMARK_E2F_TARGETS                              596 0.3456376 0.13590604
## HALLMARK_INTERFERON_ALPHA_RESPONSE                256 0.2343750 0.14062500
## HALLMARK_P53_PATHWAY                              612 0.1911765 0.26797386
## HALLMARK_COMPLEMENT                               648 0.2330247 0.16512346
## HALLMARK_CHOLESTEROL_HOMEOSTASIS                  200 0.2200000 0.29000000
## HALLMARK_APOPTOSIS                                476 0.2689076 0.22478992
## HALLMARK_DNA_REPAIR                               376 0.3164894 0.13829787
## HALLMARK_HEDGEHOG_SIGNALING                       135 0.1851852 0.29629630
## HALLMARK_MITOTIC_SPINDLE                          805 0.1913043 0.23105590
## HALLMARK_ESTROGEN_RESPONSE_LATE                   561 0.1853832 0.27807487
## HALLMARK_MTORC1_SIGNALING                         633 0.2654028 0.21484992
## HALLMARK_COAGULATION                              364 0.2252747 0.16208791
## HALLMARK_UV_RESPONSE_UP                           610 0.2442623 0.19672131
## HALLMARK_INTERFERON_GAMMA_RESPONSE                519 0.2177264 0.14643545
## HALLMARK_PI3K_AKT_MTOR_SIGNALING                  391 0.2404092 0.18158568
## HALLMARK_APICAL_JUNCTION                          729 0.2304527 0.17009602
## HALLMARK_ESTROGEN_RESPONSE_EARLY                  654 0.2125382 0.27675841
## HALLMARK_INFLAMMATORY_RESPONSE                    551 0.2050817 0.16333938
## HALLMARK_IL6_JAK_STAT3_SIGNALING                  209 0.2248804 0.13397129
## HALLMARK_OXIDATIVE_PHOSPHORYLATION                455 0.2065934 0.25934066
## HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY          162 0.1666667 0.23456790
## HALLMARK_HYPOXIA                                  648 0.2114198 0.28086420
## HALLMARK_XENOBIOTIC_METABOLISM                    563 0.2202487 0.23090586
## HALLMARK_GLYCOLYSIS                               628 0.2531847 0.20382166
## HALLMARK_IL2_STAT5_SIGNALING                      625 0.1984000 0.25600000
## HALLMARK_NOTCH_SIGNALING                          115 0.2000000 0.21739130
## HALLMARK_TGF_BETA_SIGNALING                       234 0.1965812 0.25641026
## HALLMARK_MYOGENESIS                               692 0.2052023 0.23121387
## HALLMARK_ADIPOGENESIS                             638 0.2288401 0.23197492
```

```
## HALLMARK_ANDROGEN_RESPONSE                    396 0.2424242 0.22727273
## HALLMARK_PEROXISOME                           336 0.2410714 0.21130952
## HALLMARK_BILE_ACID_METABOLISM                 267 0.1985019 0.19101124
## HALLMARK_KRAS_SIGNALING_DN                    712 0.1516854 0.14185393
## HALLMARK_KRAS_SIGNALING_UP                    629 0.2289348 0.21144674
## HALLMARK_UV_RESPONSE_DN                       751 0.2330226 0.21704394
## HALLMARK_FATTY_ACID_METABOLISM                438 0.2260274 0.20547945
## HALLMARK_WNT_BETA_CATENIN_SIGNALING           151 0.1788079 0.18543046
##                                          Direction PValue        FDR
## HALLMARK_MYC_TARGETS_V1                       Down 0.0010 0.01250000
## HALLMARK_UNFOLDED_PROTEIN_RESPONSE            Down 0.0010 0.01250000
## HALLMARK_SPERMATOGENESIS                      Down 0.0010 0.01250000
## HALLMARK_PROTEIN_SECRETION                      Up 0.0015 0.01562500
## HALLMARK_APICAL_SURFACE                         Up 0.0020 0.01607143
## HALLMARK_MYC_TARGETS_V2                       Down 0.0025 0.01607143
## HALLMARK_ANGIOGENESIS                         Down 0.0025 0.01607143
## HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION    Down 0.0035 0.01805556
## HALLMARK_HEME_METABOLISM                        Up 0.0035 0.01805556
## HALLMARK_PANCREAS_BETA_CELLS                  Down 0.0040 0.01875000
## HALLMARK_ALLOGRAFT_REJECTION                  Down 0.0045 0.01931818
## HALLMARK_G2M_CHECKPOINT                       Down 0.0070 0.02788462
## HALLMARK_TNFA_SIGNALING_VIA_NFKB                Up 0.0075 0.02788462
## HALLMARK_E2F_TARGETS                          Down 0.0140 0.04910714
## HALLMARK_INTERFERON_ALPHA_RESPONSE            Down 0.0215 0.07083333
## HALLMARK_P53_PATHWAY                            Up 0.0380 0.11796875
## HALLMARK_COMPLEMENT                           Down 0.0500 0.14632353
## HALLMARK_CHOLESTEROL_HOMEOSTASIS                Up 0.0635 0.17569444
## HALLMARK_APOPTOSIS                            Down 0.0700 0.18355263
## HALLMARK_DNA_REPAIR                           Down 0.0830 0.20416667
## HALLMARK_HEDGEHOG_SIGNALING                     Up 0.0860 0.20416667
## HALLMARK_MITOTIC_SPINDLE                        Up 0.1235 0.28011364
## HALLMARK_ESTROGEN_RESPONSE_LATE                 Up 0.1405 0.29218750
## HALLMARK_MTORC1_SIGNALING                     Down 0.1405 0.29218750
## HALLMARK_COAGULATION                          Down 0.1560 0.31150000
## HALLMARK_UV_RESPONSE_UP                       Down 0.1675 0.32163462
## HALLMARK_INTERFERON_GAMMA_RESPONSE            Down 0.1835 0.33750000
## HALLMARK_PI3K_AKT_MTOR_SIGNALING              Down 0.1930 0.33750000
## HALLMARK_APICAL_JUNCTION                      Down 0.2055 0.33750000
## HALLMARK_ESTROGEN_RESPONSE_EARLY                Up 0.2080 0.33750000
## HALLMARK_INFLAMMATORY_RESPONSE                Down 0.2095 0.33750000
## HALLMARK_IL6_JAK_STAT3_SIGNALING              Down 0.2260 0.35273437
## HALLMARK_OXIDATIVE_PHOSPHORYLATION              Up 0.2360 0.35719697
## HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY        Up 0.2485 0.36507353
## HALLMARK_HYPOXIA                                Up 0.2940 0.41964286
## HALLMARK_XENOBIOTIC_METABOLISM                  Up 0.3130 0.43437500
## HALLMARK_GLYCOLYSIS                           Down 0.3335 0.45033784
## HALLMARK_IL2_STAT5_SIGNALING                    Up 0.3495 0.45953947
## HALLMARK_NOTCH_SIGNALING                      Down 0.5020 0.62708333
## HALLMARK_TGF_BETA_SIGNALING                     Up 0.5145 0.62708333
## HALLMARK_MYOGENESIS                             Up 0.5160 0.62708333
## HALLMARK_ADIPOGENESIS                           Up 0.5270 0.62708333
## HALLMARK_ANDROGEN_RESPONSE                      Up 0.5625 0.65377907
## HALLMARK_PEROXISOME                           Down 0.5920 0.67244318
## HALLMARK_BILE_ACID_METABOLISM                   Up 0.6820 0.75750000
```

```
## HALLMARK_KRAS_SIGNALING_DN                        Down 0.6990 0.75951087
## HALLMARK_KRAS_SIGNALING_UP                        Down 0.7675 0.81096939
## HALLMARK_UV_RESPONSE_DN                           Down 0.7855 0.81096939
## HALLMARK_FATTY_ACID_METABOLISM                    Down 0.7950 0.81096939
## HALLMARK_WNT_BETA_CATENIN_SIGNALING               Down 0.8265 0.82650000
##                                                PValue.Mixed FDR.Mixed
## HALLMARK_MYC_TARGETS_V1                               5e-04     5e-04
## HALLMARK_UNFOLDED_PROTEIN_RESPONSE                    5e-04     5e-04
## HALLMARK_SPERMATOGENESIS                              5e-04     5e-04
## HALLMARK_PROTEIN_SECRETION                            5e-04     5e-04
## HALLMARK_APICAL_SURFACE                               5e-04     5e-04
## HALLMARK_MYC_TARGETS_V2                               1e-03     1e-03
## HALLMARK_ANGIOGENESIS                                 5e-04     5e-04
## HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION            5e-04     5e-04
## HALLMARK_HEME_METABOLISM                              5e-04     5e-04
## HALLMARK_PANCREAS_BETA_CELLS                          5e-04     5e-04
## HALLMARK_ALLOGRAFT_REJECTION                          5e-04     5e-04
## HALLMARK_G2M_CHECKPOINT                               1e-03     1e-03
## HALLMARK_TNFA_SIGNALING_VIA_NFKB                      5e-04     5e-04
## HALLMARK_E2F_TARGETS                                  5e-04     5e-04
## HALLMARK_INTERFERON_ALPHA_RESPONSE                    5e-04     5e-04
## HALLMARK_P53_PATHWAY                                  5e-04     5e-04
## HALLMARK_COMPLEMENT                                   5e-04     5e-04
## HALLMARK_CHOLESTEROL_HOMEOSTASIS                      5e-04     5e-04
## HALLMARK_APOPTOSIS                                    5e-04     5e-04
## HALLMARK_DNA_REPAIR                                   1e-03     1e-03
## HALLMARK_HEDGEHOG_SIGNALING                           5e-04     5e-04
## HALLMARK_MITOTIC_SPINDLE                              5e-04     5e-04
## HALLMARK_ESTROGEN_RESPONSE_LATE                       5e-04     5e-04
## HALLMARK_MTORC1_SIGNALING                             5e-04     5e-04
## HALLMARK_COAGULATION                                  5e-04     5e-04
## HALLMARK_UV_RESPONSE_UP                               5e-04     5e-04
## HALLMARK_INTERFERON_GAMMA_RESPONSE                    5e-04     5e-04
## HALLMARK_PI3K_AKT_MTOR_SIGNALING                      5e-04     5e-04
## HALLMARK_APICAL_JUNCTION                              5e-04     5e-04
## HALLMARK_ESTROGEN_RESPONSE_EARLY                      5e-04     5e-04
## HALLMARK_INFLAMMATORY_RESPONSE                        5e-04     5e-04
## HALLMARK_IL6_JAK_STAT3_SIGNALING                      5e-04     5e-04
## HALLMARK_OXIDATIVE_PHOSPHORYLATION                    5e-04     5e-04
## HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY              5e-04     5e-04
## HALLMARK_HYPOXIA                                      5e-04     5e-04
## HALLMARK_XENOBIOTIC_METABOLISM                        5e-04     5e-04
## HALLMARK_GLYCOLYSIS                                   5e-04     5e-04
## HALLMARK_IL2_STAT5_SIGNALING                          5e-04     5e-04
## HALLMARK_NOTCH_SIGNALING                              5e-04     5e-04
## HALLMARK_TGF_BETA_SIGNALING                           5e-04     5e-04
## HALLMARK_MYOGENESIS                                   5e-04     5e-04
## HALLMARK_ADIPOGENESIS                                 5e-04     5e-04
## HALLMARK_ANDROGEN_RESPONSE                            5e-04     5e-04
## HALLMARK_PEROXISOME                                   5e-04     5e-04
## HALLMARK_BILE_ACID_METABOLISM                         5e-04     5e-04
## HALLMARK_KRAS_SIGNALING_DN                            5e-04     5e-04
## HALLMARK_KRAS_SIGNALING_UP                            5e-04     5e-04
## HALLMARK_UV_RESPONSE_DN                               5e-04     5e-04
```

```
## HALLMARK_FATTY_ACID_METABOLISM                    5e-04     5e-04
## HALLMARK_WNT_BETA_CATENIN_SIGNALING               1e-03     1e-03
```

```
#Use help for other parameters. Note we might decide to use #exactly the same model as our differential
#sv <- squeezeVar(fit$sigma^2,df=fit$df.residual)

write.table(results,"enrichment.txt",sep="\t")
#You can then examine the results in "enrichment.txt".  It is a text file.  It can be downloaded to vie
```

Session Information

```
sessionInfo()
```

```
## R version 4.2.2 (2022-10-31)
## Platform: x86_64-pc-linux-gnu (64-bit)
## Running under: Ubuntu 22.04.4 LTS
##
## Matrix products: default
## BLAS:   /usr/lib/x86_64-linux-gnu/blas/libblas.so.3.10.0
## LAPACK: /usr/lib/x86_64-linux-gnu/lapack/liblapack.so.3.10.0
##
## 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] stats4    stats     graphics  grDevices utils     datasets  methods
## [8] base
##
## other attached packages:
##  [1] mouse4302cdf_2.18.0  scatterplot3d_0.3-43 mouse4302.db_3.13.0
##  [4] org.Mm.eg.db_3.16.0  annotate_1.76.0      XML_3.99-0.14
##  [7] AnnotationDbi_1.60.2 IRanges_2.32.0       S4Vectors_0.36.2
## [10] affy_1.76.0          Biobase_2.58.0       BiocGenerics_0.44.0
## [13] limma_3.54.2
##
## loaded via a namespace (and not attached):
##  [1] Rcpp_1.0.10            highr_0.10             compiler_4.2.2
##  [4] BiocManager_1.30.20    GenomeInfoDb_1.34.9    XVector_0.38.0
##  [7] bitops_1.0-7           tools_4.2.2            zlibbioc_1.44.0
## [10] digest_0.6.31          bit_4.0.5              RSQLite_2.3.0
## [13] evaluate_0.20          memoise_2.0.1          preprocessCore_1.60.2
## [16] pkgconfig_2.0.3        png_0.1-8              rlang_1.1.2
## [19] DBI_1.1.3              cli_3.6.1              rstudioapi_0.15.0
## [22] yaml_2.3.7             xfun_0.41              fastmap_1.1.1
## [25] GenomeInfoDbData_1.2.9 httr_1.4.5             knitr_1.42
## [28] Biostrings_2.66.0      vctrs_0.6.5            bit64_4.0.5
## [31] R6_2.5.1               rmarkdown_2.20         blob_1.2.4
## [34] htmltools_0.5.5        KEGGREST_1.38.0        xtable_1.8-4
## [37] RCurl_1.98-1.10        cachem_1.0.7           crayon_1.5.2
```

```
## [40] affyio_1.68.0
```