Supplementary Information

Huh et al

Feb 13, 2017

Contents

1 Loading relevant data

1.1 Packages used in this analysis

```
library(ape)
library(ade4)
library(beeswarm)
library(boot)
library(compiler)
library(DESeq2)
library(dplyr)
library(edgeR)
library(fpc)
library(gdata)
library(gee)
library(geepack)
library(GGally)
library(ggplot2)
library(gplots)
library(GSEABase)
library(heatmap.plus)
library(HTSanalyzeR)
library(knitr)
library(leaps)
library(lme4)
library(matrixStats)
library(parallel)
library(plyr)
library(RColorBrewer)
library(reshape2)
library(rgl)
library(scatterplot3d)
library(spatstat)
library(TDA)
library(vegan)

knit_hooks$set(webgl = hook_webgl)
# set the colour palette
palette(c( "#FF6FCF", "#8000FF", "#0F82F4", "#008040", "#66FFCC", "#FF0000","#43F708"))
## in the order of :
#   DCIS, HER2, IDC/LUM, PA, NP, TN , unkown

# Colors for heatmap: Rd high and Blue low
HMPallete=brewer.pal(11, "RdBu")
```

1.2 Summary of data-sets used in this study

The analyses conducted in this study come from a number of different sources:

- FACS and I-FISH data (generated in house):
  - FACS was performed on patient samples, separating cells according to CD markers
  - IF for TIGIT and PD1

  - FISH for CCL cluster and ERBB2 was performed on a cohort of DCIS (20 patients) and matched DCIS-IDC (18 samples)
- RNA-seq data:
  - RNA-seq was performed on T-cells isolated from a set of patients in house
  - Public data for IDCs was attained from TCGA
- CN data:
  - CN data for PDL1, CCL, ERBB2 from TCGA
  - CN data for PDL1, CCL, ERBB2 from Oslo

1.3 Publicly available information used in this analysis

Publicly available information from the following sources were used in this study:

1.3.1 Existing immune Databases

Upload comprehensive list of immune related genes (from ImmPort: http://immport.org/immport-open/public/reference/genelists) and from Innate DB: http://www.innatedb.com/annotatedGenes.do?type=innatedb

```
## Antigen_Processing_and_Presentation                    Antimicrobials
##                                 148                               544
##                  BCRSignalingPathway                Chemokine_Receptors
##                                 275                                53
##                           Chemokines                 Cytokine_Receptors
##                                 102                               308
##                            Cytokines                Interferon_Receptor
##                                 456                                 3
##                           Interferons                       Interleukins
##                                  17                                47
##                Interleukins_Receptor     NaturalKiller_Cell_Cytotoxicity
##                                  43                               135
##                  TCRsignalingPathway                 TGFb_Family_Member
##                                 291                                33
##          TGFb_Family_Member_Receptor                 TNF_Family_Members
##                                  12                                12
##         TNF_Family_Members_Receptors
##                                  19
```

1.3.2 Cytotoxic, exhaustion, activation gene Lists:

Obtained from Tirosh, Science 2016; Pardoll and Singer 2016.

```
GeneListAct=read.csv("online_public_data/Supplementary Table 5.csv", header=T, stringsAsFactors = F)
GeneListAct[GeneListAct==""]=NA
GeneListAct=as.list(GeneListAct, na.rm=T)
GeneListAct=lapply(GeneListAct, na.omit)
GeneListAct=lapply(GeneListAct, toupper)
names(GeneListAct)
```

```
## [1] "Activated"   "Inhibitory"  "Cytotoxic"   "Exhausted"   "Naive"
## [6] "Activation"  "Dysfunction"
```

1.3.3 GSEA: Collections from MSigDB

Load collections for GSEA. These were downloaded from MSigDB http://software.broadinstitute.org/gsea/msigdb/: The c2 canonical pathways and c7 immune collection

```
immunec7=getGmt(con="online_public_data/c7.all.v5.1.symbols.gmt", geneIdType=SymbolIdentifier(),
                collectionType=BroadCollection(category="c7"))
CanPath=getGmt(con="online_public_data/c2.cp.v5.1.symbols.gmt", geneIdType=SymbolIdentifier(),
                collectionType=BroadCollection(category="c2"))

# convert to lists:
immunec7entrez=mapIdentifiers(immunec7, EntrezIdentifier('org.Hs.eg.db'))
```

```
## Loading required package: DBI
```

```
##
```

```
immunec7listentrez=geneIds(immunec7entrez)
Canentrez=mapIdentifiers(CanPath, EntrezIdentifier('org.Hs.eg.db'))
Canlistentrez=geneIds(Canentrez)
CanlistSym=geneIds(CanPath)
```

Fetch all immune related genes from canonical pathways:

```
STs=c("IMMUNE", "_IL_[0-9]+", "_IL[0-9]+", "B_CELL", "T_CELL", "LYMPHOCYTE", "BCR", "TCR", "CCR[0-9]",
      "COMPLEMENT", "CXCR[0-9]", "CXCR[0-9]", "TH1", "TH2", "FC_EPSILON", "GRANZYME", "IFN", "IGF",
      "TCR", "MACROPHAGE", "OX40", "PDGF", "T-CELL", "TCELL", "CYTOTOXIC", "T_HELPER", "CTLA4", "ANTIGE
      "INFECTION", "MHC", "INTERFERON", "INTERLEUKIN", "BASOPHIL", "EOSINOPHIL", "MAST_CELL", "DENDRITI

anew=sapply(STs, function(x) grep(x, names(CanlistSym)))
anew2=unlist(anew)

GeneList=stack(CanlistSym[anew2])
write.csv(GeneList, file="output/Canonical_pathways_immune_related.csv")
```

Compile different sources together to get combined immune gene list:

```
CombinedGeneList=data.frame(Symbol=c(as.character(ImmPort$Symbol),
                                     as.character(GeneList$values),
                                     as.character(InnatDB$Gene.Symbol)),
                            Fn=c(as.character(ImmPort$Category), as.character(GeneList$ind),
                                 rep("Innate_Immune_System", length(InnatDB$Gene.Symbol))))
```

1.3.4 Gene Locations

The following file curated from UCSC genome website genome.ucsc.edu identifies the chromosomal position of a number of genes of interest. This will be used in CCL and PDL1 analysis:

```
Genes=read.csv("data/chr17arm.csv", header=T)
Genes
```

```
##       Locus     Start       End Chr
## 1      CCL2  32582296  32584220  17
## 2     CCL13  32683471  32685629  17
## 3      CCL7  32597235  32599261  17
## 4      CCL8  32646066  32648421  17
## 5      CCL4  34431220  34433014  17
```

```
## 6       CCL3  34415603  34417506  17
## 7     CCL3L1  34522268  34524156  17
## 8       CCL5  34198496  34207377  17
## 9      CCL23  34340097  34345005  17
## 10     CCL14  34310692  34313764  17
## 11     CCL15  34324618  34329084  17
## 12     CCL16  34303535  34308523  17
## 13     CCL11  32612687  32615199  17
## 14      CCL1  32687399  32690252  17
## 15     CCL18  34391643  34398841  17
## 16     ERBB2  37856254  37884915  17
## 17    CCL3L3  34623842  34625716  17
## 18     TIGIT 114012154 114029135   3
## 19     PDCD1 242792033 242801058   2
## 20      GZMB  25100161  25103432  14
## 21     CD274   5450503   5470567   9
## 22 PDCD1LG2   5510545   5571282   9
## 23     STAT3  40465343  40540513  17
## 24      JAK2   4985245   5128183   9
```

1.3.5 TCGA data

Copy number, RNA-seq, patient clinical data and GISTIC information was downloaded from firebrowse (firebrowse.org) Here, we load this data:

```
TCGArna=read.delim("online_public_data/RNAseqTCGA.txt")
colnames(TCGArna)=gsub("\\.", "-", colnames(TCGArna))
rownames(TCGArna)=TCGArna$X
TCGArna=TCGArna[ ,-1]

## do a vst transformation
TCGAlogCPM=cpm(TCGArna, log=T, prior.counts=1)
```

Download level 4 GISTIC data from firebrowse.org

```
GisticData=read.delim("online_public_data/TCGA_gistic_lvl_4.txt", sep="\t", header=T)
colnames(GisticData)=gsub("\\.", "-", substr(colnames(GisticData), 1, 12))

NewGistic=GisticData[match(Genes$Locus, GisticData$`Gene-Symbol`),  ]
rownames(NewGistic)=NewGistic$`Gene-Symbol`
NewGistic=NewGistic[ ,-c(1:3)]

# cap the max and min values to ensure colour scheme is ok
NewGistic[which(NewGistic>2, arr.ind = T)]=2
NewGistic[which(NewGistic<(-2), arr.ind=T)]=(-2)
```

Load clinical data:

```
load("online_public_data/TCGAClinInfo_Appended_Mar16.RData")
```

Additional information on Immune infiltration, estimated neoantigen load was obtained from the following studies:

- ESTIMATE (Yoshihara et al, 2013): Supplementary Data 2

```
ESTscore=read.csv("online_public_data/ESTIMATE_scores.csv", header=T)
ESTscore$Description=gsub("\\.", "-", ESTscore$Description)
ESTscore$Description=substr(ESTscore$Description, 3, 14)
summary(ESTscore)
```

```
##  Description         StromalScore      ImmuneScore       ESTIMATEScore
##  Length:1212        Min.   :-3584.1   Min.   :-2449.16   Min.   :-5343.4
##  Class :character   1st Qu.:  448.5   1st Qu.:  -14.98   1st Qu.:  627.3
##  Mode  :character   Median : 1399.8   Median :  794.80   Median : 2266.4
##                     Mean   : 1253.6   Mean   :  975.80   Mean   : 2229.4
##                     3rd Qu.: 2150.0   3rd Qu.: 1792.90   3rd Qu.: 3945.7
##                     Max.   : 4053.4   Max.   : 6062.83   Max.   : 9493.2
```

```
ESTscore$TumPurity=cos(0.6049872018+0.0001467884*ESTscore$ESTIMATEScore)
```

- Estimated neoantigen load from Table S4A from Rooney et al 2015

```
NeoData=read.csv("online_public_data/s4A_mutations_Rooney2015.csv", header=T)
NeoData=NeoData[NeoData$Cancer=="BRCA", ]
summary(NeoData)
```

```
##         PatientID       Cancer          CYT             Total.Mutations
##  TCGA-A1-A0SB:  1   BRCA   :760   Min.   :  0.06302   Min.   :  1.00
##  TCGA-A1-A0SD:  1   BLCA   :  0   1st Qu.:  2.51813   1st Qu.: 24.00
##  TCGA-A1-A0SE:  1   CESC   :  0   Median :  5.46181   Median : 37.00
##  TCGA-A1-A0SF:  1   CRC    :  0   Mean   : 10.24959   Mean   : 52.35
##  TCGA-A1-A0SG:  1   GBM    :  0   3rd Qu.: 11.45561   3rd Qu.: 62.00
##  TCGA-A1-A0SH:  1   HNSC   :  0   Max.   :108.87068   Max.   :393.00
##  (Other)     :754   (Other):  0
##  Predicted.NeoAgs NeoAgs_Observed.Expected AnyVirusPresent    HBV
##  Min.   : 0.000   Min.   :0.0000           Mode :logical   Mode :logical
##  1st Qu.: 4.000   1st Qu.:0.6478           FALSE:759       FALSE:760
##  Median : 7.000   Median :0.9361           TRUE :1         NA's :0
##  Mean   : 9.637   Mean   :1.0185           NA's :0
##  3rd Qu.:11.000   3rd Qu.:1.3023
##  Max.   :64.000   Max.   :5.3781
##  NA's   :25       NA's   :32
##     HPV             EBV
##  Mode :logical   Mode :logical
##  FALSE:760       FALSE:760
##  NA's :0         NA's :0
##
##
##
##
```

2 Patterns in FACS data: Figures 1 and S1

Load FACS data (Supplementary Table 2):

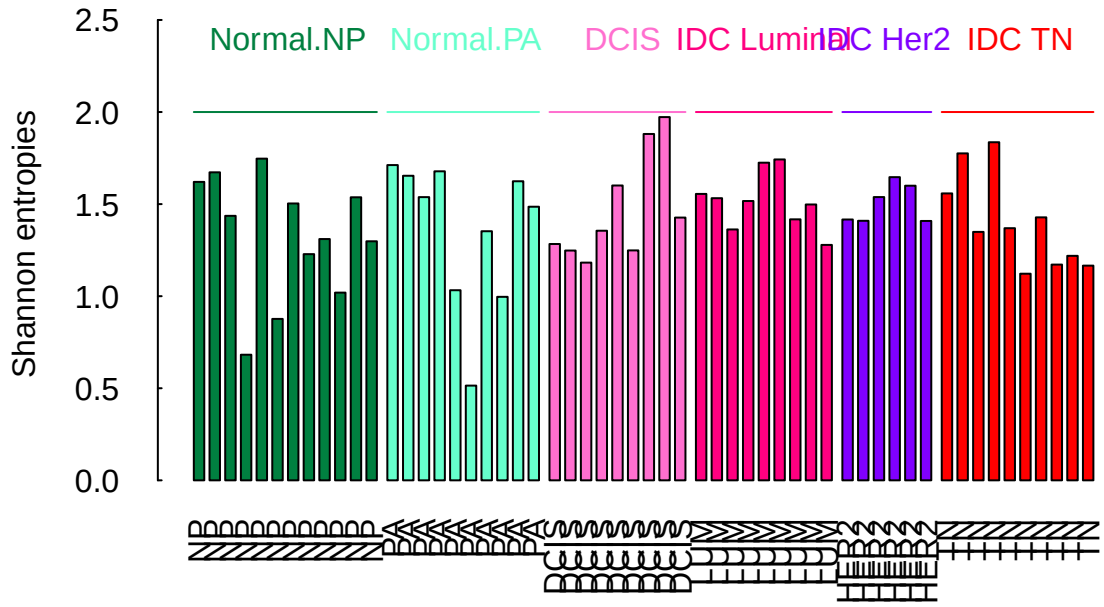
```
facs=read.csv("online_public_data/Supplementary Table 2 FACS data.csv",  row.names=1)
tissues=c(rep("NP", 12), rep("PA", 10), rep("DCIS", length(grep("DCIS", rownames(facs)))),
          rep("LUM",9), rep("HER2", 6), rep("TN", 10))
tissues2=rep("", length(tissues))
```

```
tissues2[grep("stroma", rownames(facs))]="S"
tissues2[grep("org",rownames(facs))]="O"
cells <- colnames(facs)[-1]
#cells <- facs[-1, 1]
```

Was Figure 1 made in R as a barplot?

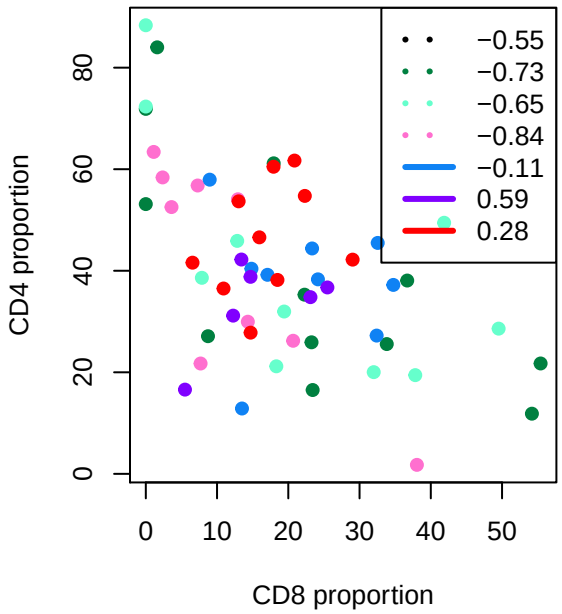
Overview of the Shannon entropies:

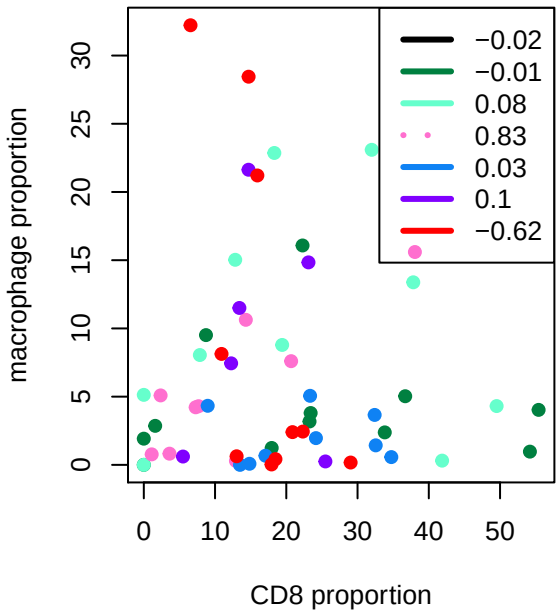
```
colors <- c(rep('#008040',length(which(tissues == "NP"))), rep('#66FFCC', length(which(tissues == "PA"))),
            rep('#FF6FCF', length(which(tissues == "DCIS"))), rep('#FF0080', length(which(tissues == "LU
            rep('#8000FF',  length(which(tissues == "HER2"))), rep('#FF0000', length(which(tissues == "T

diversities <- sapply(1:nrow(facs), function(i) diversity(as.numeric(facs[i, ])))

par(mfrow = c(1, 1), tcl = 0.1, las = 2, oma = c(1, 1, 0, 0))
barplot(diversities, space = c(c(0, rep(0.5, length(which(tissues == "NP")) - 1)),
                               c(1, rep(0.5, length(which(tissues == "PA")) - 1)),
                               c(1, rep(0.5, length(which(tissues == "DCIS")) - 1)),
                               c(1, rep(0.5, length( which(tissues == "LUM")) - 1)),
                               c(1, rep(0.5, length(which(tissues == "HER2")) - 1)),
                               c(1, rep(0.5, length(which(tissues == "TN")) - 1))),
        col = colors, ylab = 'Shannon entropies', names = tissues, ylim = c(0, diversity(rep(1/13, 13))))
text(9, 2.4, 'Normal.NP', col = unique(colors)[1])
lines(c(0, 17.5), c(2, 2), col = unique(colors)[1])
text(26, 2.4, 'Normal.PA', col = unique(colors)[2])
lines(c(18.5, 18.5 + 10 * 1.5 - 0.5), c(2, 2), col = unique(colors)[2])
text(41, 2.4, 'DCIS', col = unique(colors)[3])
lines(c(34, 34 + 9 * 1.5 - 0.5), c(2, 2), col = unique(colors)[3])
text(54.5, 2.4, 'IDC Luminal', col = unique(colors)[4])
lines(c(48, 48 + 9 * 1.5 - 0.5), c(2, 2), col = unique(colors)[4])
text(66, 2.4, 'IDC Her2', col = unique(colors)[5])
lines(c(62, 62 + 6 * 1.5 - 0.5), c(2, 2), col = unique(colors)[5])
text(79, 2.4, 'IDC TN', col = unique(colors)[6])
lines(c(71.5, 71.5 + 10 * 1.5 - 0.5), c(2, 2), col = unique(colors)[6])
```

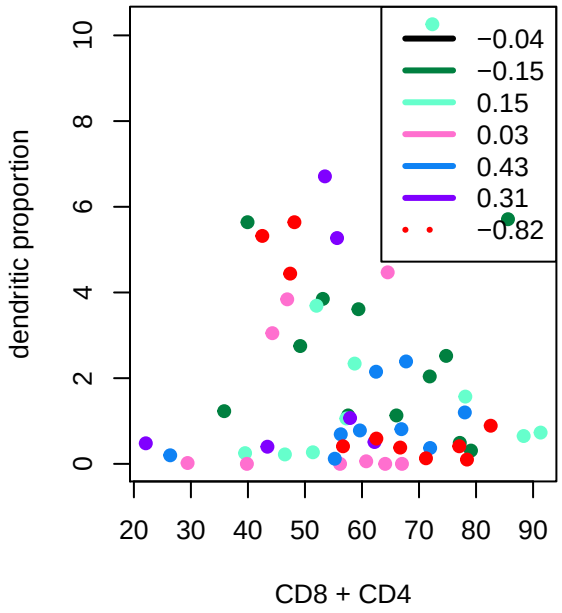


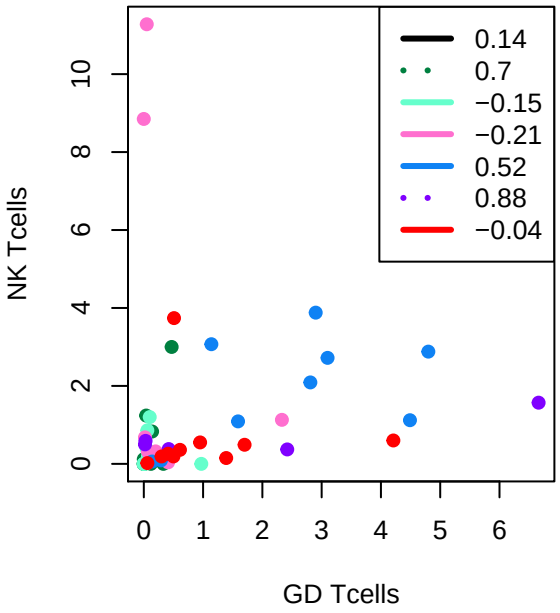
See if there are any associations between the CD4 and CD8 components: Figure S1B

```
par(mfrow=c(2,2))
# plot CD4 vs CD8 colour coded:
plot(facs$CD8..T.cells, facs$CD4..T.cells, col=factor(tissues), xlab="CD8 proportion", ylab="CD4 propor
r1=cor.test(facs$CD8..T.cells, facs$CD4..T.cells)

r2=sapply(unique(tissues), function(x) cor.test(facs$CD8..T.cells[tissues==x],
                                                facs$CD4..T.cells[tissues==x])$p.value)
r3=sapply(unique(tissues), function(x) cor.test(facs$CD8..T.cells[tissues==x],
                                                facs$CD4..T.cells[tissues==x])$estimate)

legend("topright", as.character(c(round(r1$estimate*100)/100, round(r3*100)/100)),
       col=c("black", factor(unique(tissues))), lty=ifelse(c(r1$p.value, r2)<0.05,3, 1), lwd=3)

plot(facs$CD8..T.cells, facs$macrophages, col=factor(tissues), xlab="CD8 proportion",
     ylab="macrophage proportion", pch=19)

s1=cor.test(facs$CD8..T.cells, facs$macrophages)
s2=sapply(unique(tissues), function(x) cor.test(facs$CD8..T.cells[tissues==x],
                                                facs$macrophages[tissues==x])$p.value)
s3=sapply(unique(tissues), function(x) cor.test(facs$CD8..T.cells[tissues==x],
                                                facs$macrophages[tissues==x])$estimate)

legend("topright", as.character(c(round(s1$estimate*100)/100, round(s3*100)/100)),
       col=c("black", factor(unique(tissues))), lty=ifelse(c(s1$p.value, s2)<0.05,3, 1), lwd=3)

plot((facs$CD8..T.cells+facs$CD4..T.cells), facs$dendritic.cells, col=factor(tissues), xlab="CD8 + CD4"

t1=cor.test((facs$CD8..T.cells+facs$CD4..T.cells), facs$dendritic.cells)
t2=sapply(unique(tissues), function(x) cor.test((facs$CD8..T.cells+facs$CD4..T.cells)[tissues==x],
                                                facs$dendritic.cells[tissues==x])$p.value)
t3=sapply(unique(tissues), function(x) cor.test((facs$CD8..T.cells+facs$CD4..T.cells)[tissues==x],
                                                facs$dendritic.cells[tissues==x])$estimate)

legend("topright", as.character(c(round(t1$estimate*100)/100, round(t3*100)/100)),
       col=c("black", factor(unique(tissues))), lty=ifelse(c(t1$p.value, t2)<0.05,3, 1), lwd=3)

plot(facs$gammadelta.Tcells, facs$NK.T.cells, col=factor(tissues), xlab="GD Tcells", ylab="NK Tcells", p

u1=cor.test(facs$gammadelta.Tcells, facs$NK.T.cells)
u2=sapply(unique(tissues), function(x) cor.test(facs$gammadelta.Tcells[tissues==x],
                                                facs$NK.T.cells[tissues==x])$p.value)
u3=sapply(unique(tissues), function(x) cor.test(facs$gammadelta.Tcells[tissues==x],
                                                facs$NK.T.cells[tissues==x])$estimate)

legend("topright", as.character(c(round(u1$estimate*100)/100, round(u3*100)/100)),
       col=c("black", factor(unique(tissues))), lty=ifelse(c(u1$p.value, u2)<0.05,3, 1), lwd=3)
```





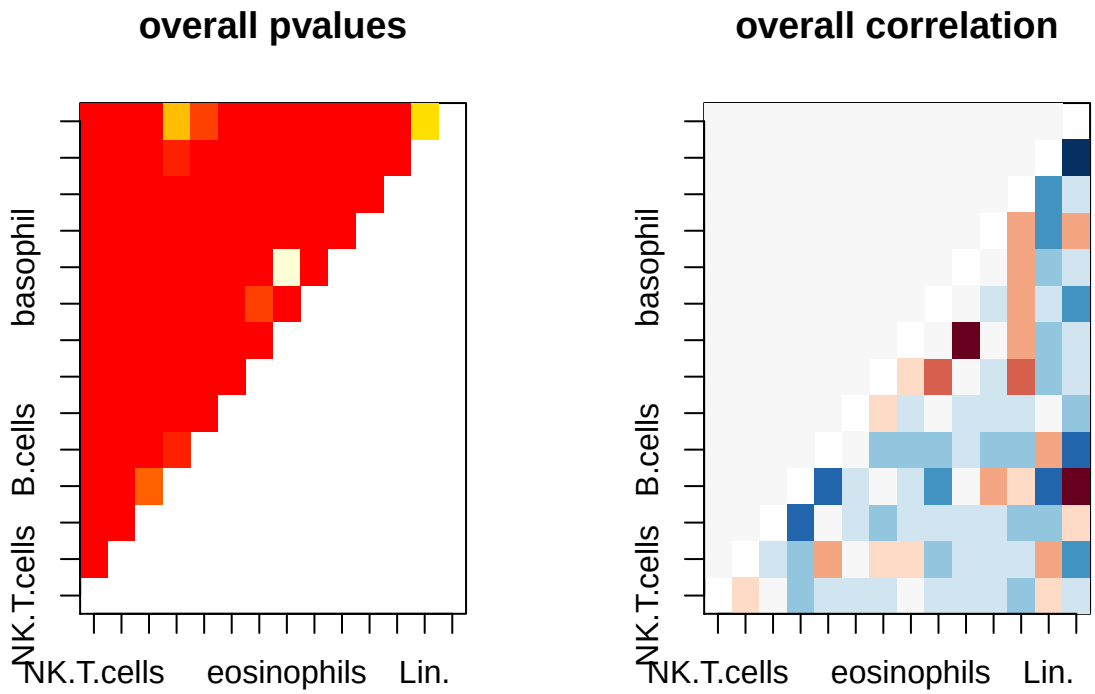




Overall test to look for correlations between the different populations of interest:

```
facs$CD4total=facs$CD8..T.cells+facs$CD4..T.cells
N=ncol(facs)
cor.mat=matrix(NA, N, N)
for (i in 1:N){
  if (i<N){
  for (j in (i+1):N){
    cor.mat[i,j]=cor.test(facs[ ,i], facs[ ,j])$p.value
    cor.mat[j,i]=cor.test(facs[ ,i], facs[ ,j])$estimate
```

```
  }
}}
cor.mat1=upper.tri(cor.mat)*cor.mat
cor.mat1[lower.tri(cor.mat1)]=NA
cor.mat1new=p.adjust(cor.mat1)
cor.mat1new=matrix(cor.mat1new, N, N)
cor.mat2=lower.tri(cor.mat)*cor.mat

par(mfrow=c(1,2))

image(-log10(cor.mat1new), xaxt="n", yaxt="n", main="overall pvalues")
axis(1, seq(0, 1, length=N), colnames(facs))
axis(2, seq(0, 1, length=N), colnames(facs))

image(cor.mat2, main="overall correlation", xaxt="n", yaxt="n", col=HMPallete[11:1])
axis(1, seq(0, 1, length=N), colnames(facs))
axis(2, seq(0, 1, length=N), colnames(facs))
```



Looking at associations in DCIS only

```
pdf('Facs_associations.pdf', useDingbats = FALSE)
par(mfrow=c(2,2))

TestValues=c("DCIS", "LUM", "HER2", "TN", "NP", "PA")

for (k in TestValues){
facs2=facs[tissues==k, ]
cor.mat=matrix(NA, N, N)
for (i in 1:N){
  if (i < N){
  for (j in (i+1):N){
    cor.mat[i,j]=cor.test(facs2[ ,i], facs2[ ,j])$p.value
```

```
    cor.mat[j,i]=cor.test(facs2[ ,i], facs2[ ,j])$estimate
  }
}
}
cor.mat1=upper.tri(cor.mat)*cor.mat
cor.mat1[lower.tri(cor.mat1)]=NA
cor.mat1new=p.adjust(cor.mat1)
cor.mat1new=matrix(cor.mat1new, N, N)
cor.mat2=lower.tri(cor.mat)*cor.mat

image(-log10(cor.mat1new), xaxt="n", yaxt="n", main=sprintf("%s pvalues", k))
axis(1, seq(0, 1, length=N), colnames(facs), las=2)
axis(2, seq(0, 1, length=N), colnames(facs), las=2)

image(cor.mat2, main=sprintf("%s correlation", k), xaxt="n", yaxt="n", col=HMPallete[11:1])
axis(1, seq(0, 1, length=N), colnames(facs), las=2)
axis(2, seq(0, 1, length=N), colnames(facs), las=2)
}
dev.off()
```

```
## pdf
##   2
```

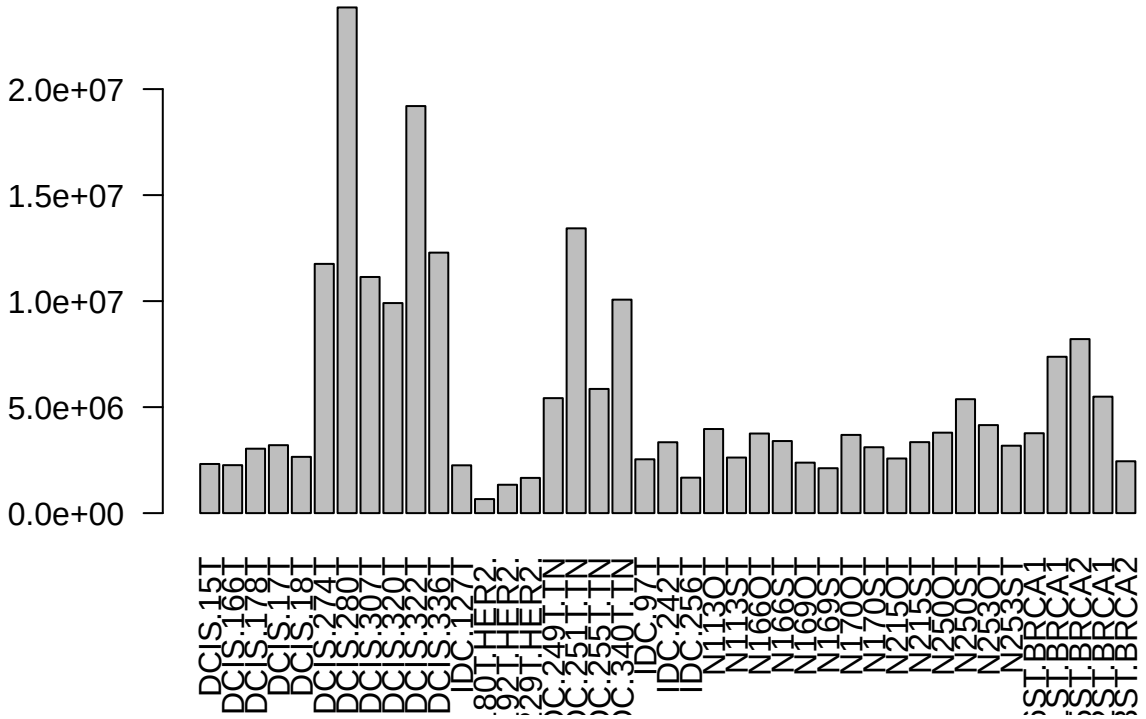
3 Tcell RNA seq data: Figure 2

3.1 Overview of RNA seq data

3.1.1 Loading in data

Load the new GE data. Remove genes with less than 45 counts

```
GEnew=read.csv(file ="data/Leukocytes_RNA-Seq_STAR_htseq-count.csv", header=T, row.names = 1)
QCinfo=GEnew[1:5, ]
GEnew=GEnew[-c(1:5), ]

TotRead=colSums(GEnew)
barplot(TotRead, las=2, main="Total number of reads")
```




```
GeneCounts=which(rowSums(GEnew)<45)
GEnewfilt=GEnew[-GeneCounts, ]
```

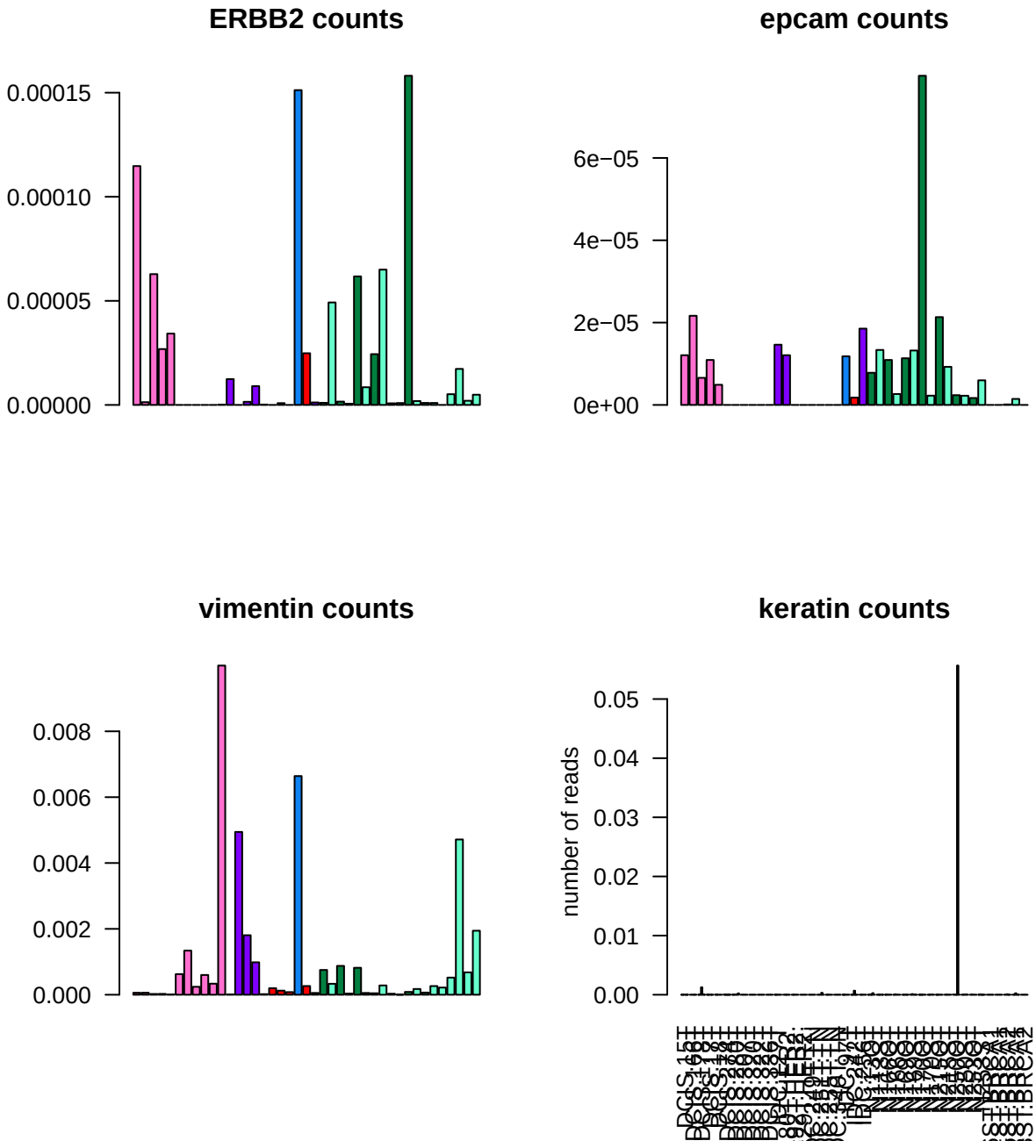
Load in the sample information.

```
SampleInfo=read.csv("data/sample_data.csv", header=T, row.names = 1)
GEnewfiltlog=log2(GEnewfilt+1)
```

3.1.2 Check for epithelial contamination

Check whether there is possible contamination with epithelial cells. For example, in the HER2+ samples, is HER2 expression observed? The markers checked are HER2, Epcam, Vimentin and various keratin. Report the fraction of counts wrt to total reads.

```
par(mfrow=c(2,2))
barplot(as.numeric(GEnew[grep("ERBB2", rownames(GEnew)) ,]/colSums(GEnew)),
        col=as.numeric(SampleInfo$Subtype), las=2, main="ERBB2 counts")

barplot(as.numeric(GEnew[grep("EPCAM", rownames(GEnew)) ,]/colSums(GEnew)),
        col=as.numeric(SampleInfo$Subtype), las=2, main="epcam counts")

barplot(as.numeric(GEnew["VIM",]/colSums(GEnew)), col=as.numeric(SampleInfo$Subtype), las=2,
        main="vimentin counts")

barplot(as.matrix(GEnew[c("KRT5","KRT7","KRT8", "KRT17", "KRT18") ,]/colSums(GEnew)), col=grey.colors(5)
        main="keratin counts", beside=T, ylab="number of reads")
```



Overall, samples do not look contaminated (Epithelial fractions have the most KRT) when assessing total counts. One DCIS sample has high vimentin counts.

Firstly, take into account batch effects: (i) This can be done by subtracting an estimated "batch" value from log-transformed data, or (ii) can be specified in the design model

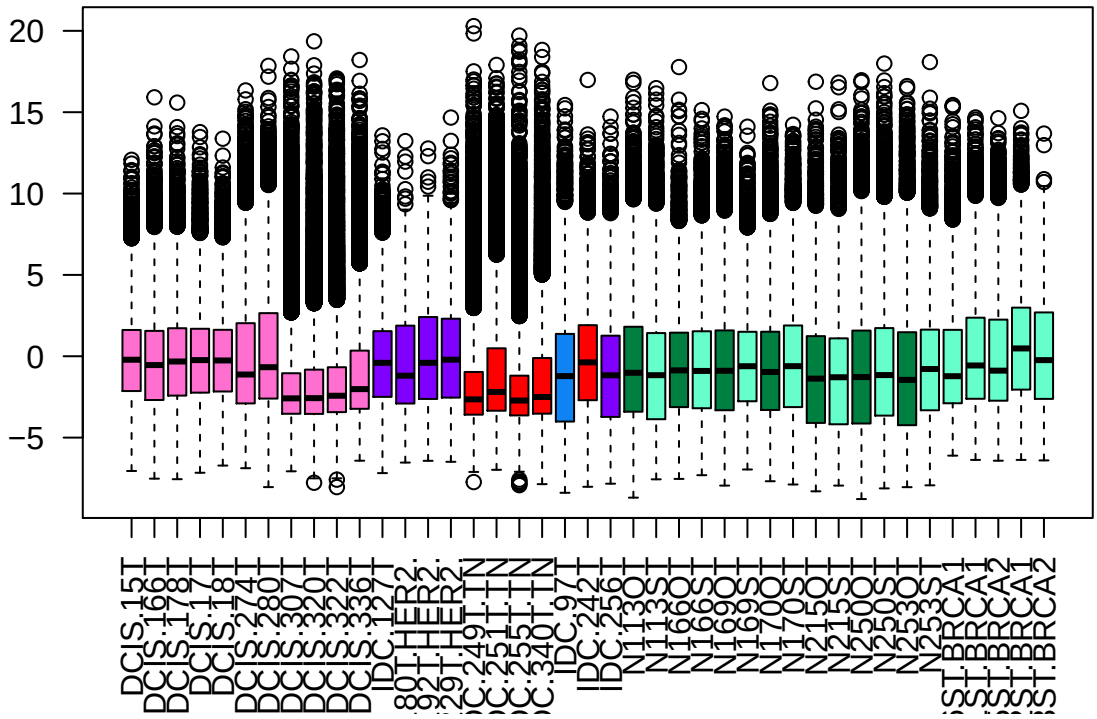
ie. ~ IDCvsDCIS + Batch

3.2 edgeR conversion to logCPM space: Figure 2A

edgeR allows direct correction of Batch effects (on log transformed data) - this is useful for visualisation and clustering.

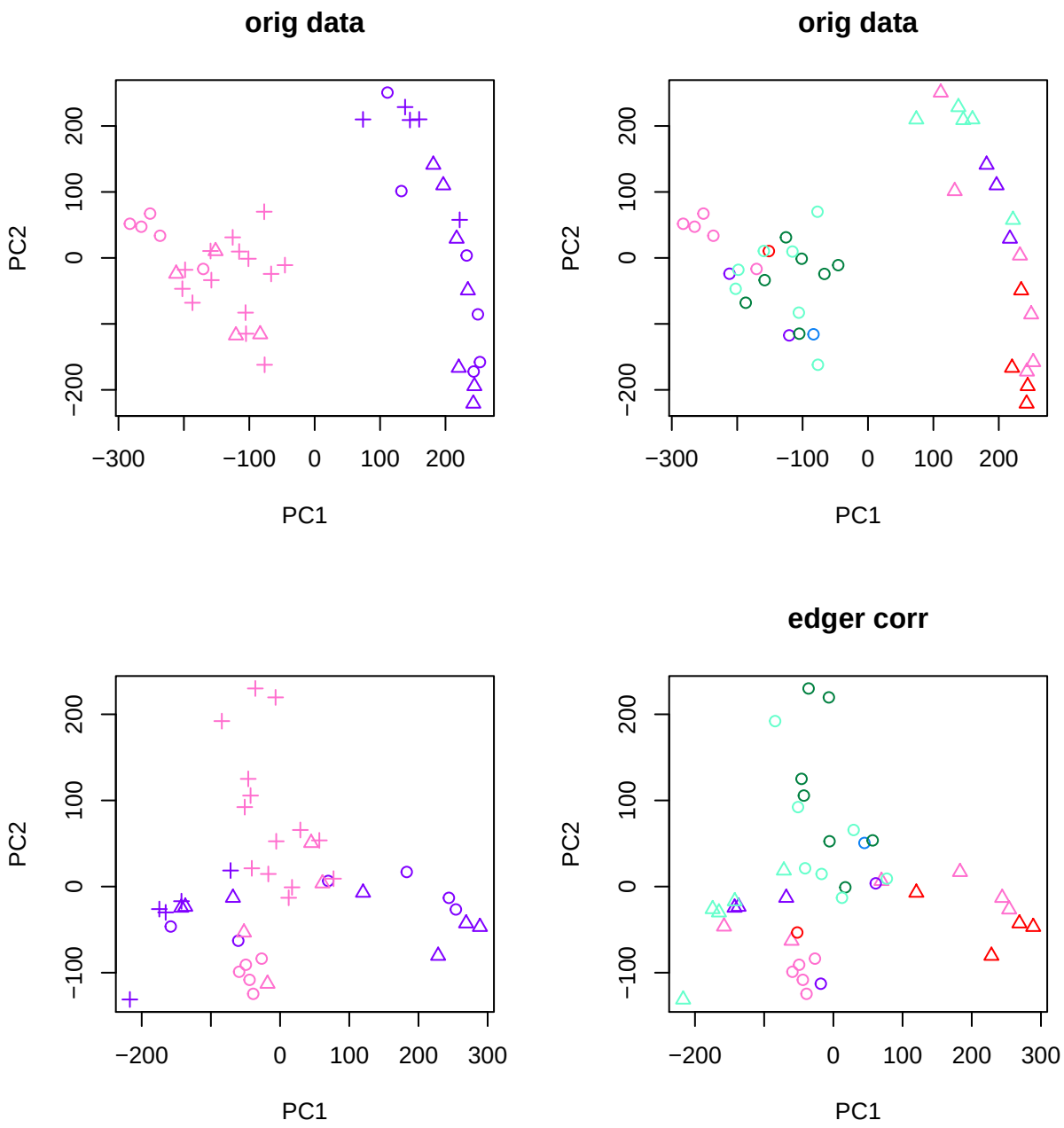
We use edgeR to normalise the counts wrt to total counts. This will be then further corrected to correct for batch effects within the data set:

```
y=DGEList(counts=GEnewfilt)
y2=calcNormFactors(y)
logCPM=cpm(y2, log=T, prior.counts=1)
mod=model.matrix(~Subtype, data=SampleInfo)
logCPM=removeBatchEffect(logCPM, batch=SampleInfo$Batch, design=mod)

# boxplot of the batch corrected expression profiles
boxplot(logCPM, col=SampleInfo$Subtype, las=2)
```



Perform a PCA of the data before and after correction

```
# PCA of the result
par(mfrow=c(2,2))
pca1=prcomp(t(log2(GEnewfilt+1)))
plot(pca1$x[ ,1:2], col=SampleInfo$Batch, pch=as.numeric(SampleInfo$DCISIDC), main="orig data")
plot(pca1$x[ ,1:2], col=SampleInfo$Subtype, pch=as.numeric(SampleInfo$Batch), main="orig data")

pca2=prcomp(t(logCPM), scale=F)
plot(pca2$x[ ,1:2], col=SampleInfo$Batch, pch=as.numeric(SampleInfo$DCISIDC))
plot(pca2$x[ ,1:2], col=SampleInfo$Subtype, pch=as.numeric(SampleInfo$Batch), main="edger corr")
```



Create a 3D plot of the corrected data (FIGURE 2A):

```
SampC=SampleInfo$Subtype
SampC=as.character(SampC)
SampC[SampC=="Str"]="Org"
SampC=factor(SampC, levels=c("DCIS", "HER2", "LUM", "Org", "Str", "TN"))
plot3d(pca2$x[ ,1], pca2$x[ ,2], pca2$x[ ,3],radius=15, type="s", col=as.numeric(SampC),
       xlab = "PC1", ylab = "PC2", zlab="PC3")
rgl.postscript("output/All_samples_pca_3D_edger.pdf", fmt = "pdf")
```

You must enable Javascript to view this page properly.

Have a quick look at the relative levels of CD8 and CD4 across the different samples:

```
ColSide=SampC
levels(ColSide)=palette()[1:6]
```

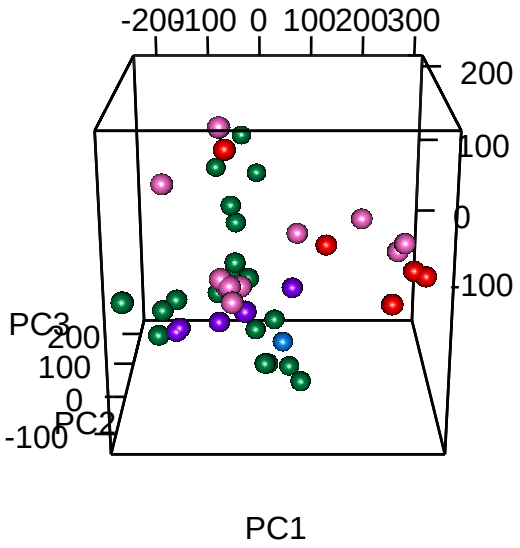
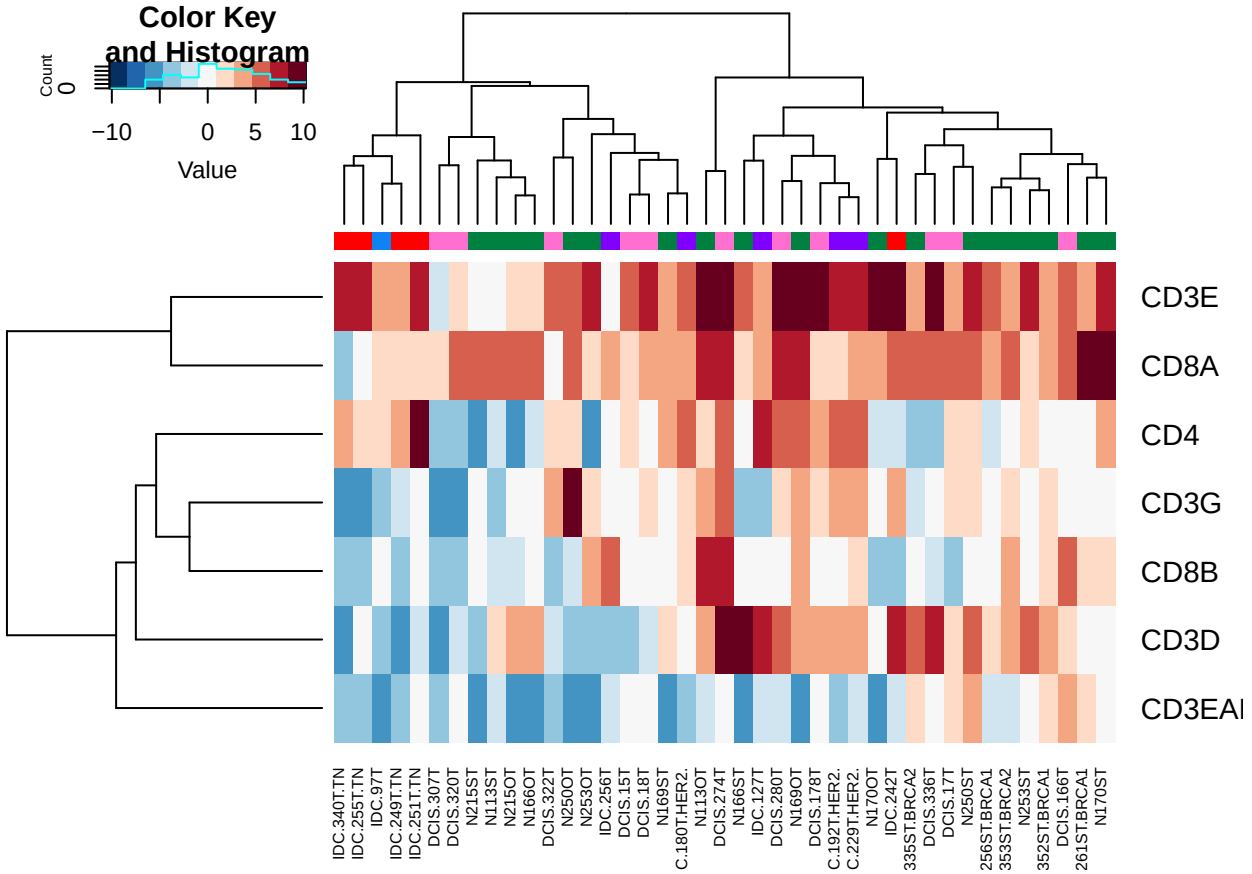
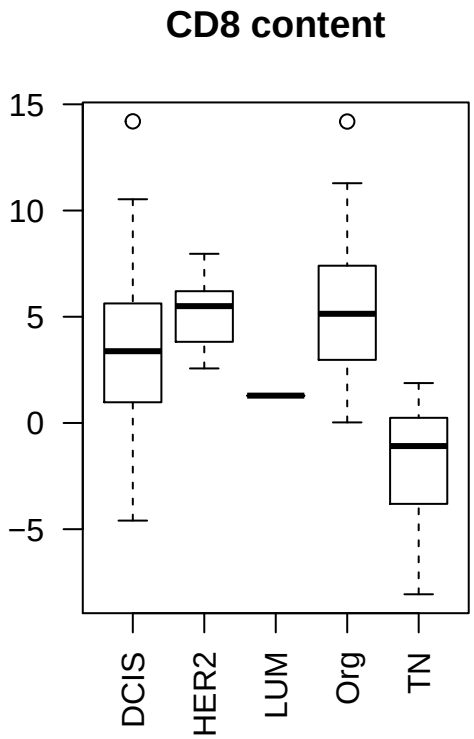


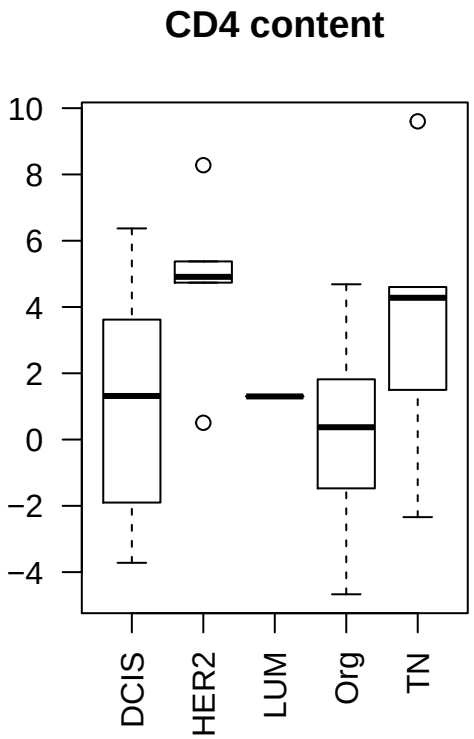
Figure 1: PCA of batch adjusted data

```
ColSide=as.character(ColSide)

# Look for CD4 and CD8 markers:
cdmark=sapply(c("^CD4$", "CD8[A-Z]", "CD3[A-Z]"), function(x) grep(x, rownames(logCPM)))
heatmap.2(logCPM[unlist(cdmark), ], ColSideColors = ColSide, trace="none", col=HMPallete[11:1])
```




```
CDval=colSums(logCPM[c("CD8A", "CD8B"), ])
CDval2=logCPM["CD4", ]
par(mfrow=c(1,2))
boxplot(CDval~factor(SampC), main="CD8 content", las=2)
boxplot(CDval2~factor(SampC), main="CD4 content", las=2)
```





3.3 edgeR: differential analysis

3.3.1 DCIS, IDC and normals

Using edgeR with a linear term for the batch effect, run a DGEA comparing DCIS vs IDC vs Normal. We use a BH-corrected p value of 0.05 to assign significance.

```
mod=model.matrix(~DCISIDC+Batch, data=SampleInfo)
y3=estimateGLMCommonDisp(y2, mod)
y4=estimateGLMTagwiseDisp(y3, mod)
fit=glmFit(y4, mod)

t2=factor(SampleInfo$DCISIDC, levels=c("IDC", "N", "DCIS"))
mod2=model.matrix(~t2+Batch, data=SampleInfo)
fit2=glmFit(y4, mod2)

# Find differences in IDC and DCIS
lrtA=glmLRT(fit, coef=2)
tabAall=topTags(lrtA, adjust.method = "BH", p.value=1, n=23000, sort.by = "none")
tabA=topTags(lrtA, adjust.method = "BH", p.value=0.05, n=10000)
#tabA=tabA$table[abs(tabA$table$logFC)>5, ]

# differences in DCIS and Normal
lrtB=glmLRT(fit, coef=3)
tabBall=topTags(lrtB, adjust.method = "BH", p.value=1, n=23000, sort.by = "none")
tabB=topTags(lrtB, adjust.method = "BH", p.value=0.05, n=10000)
#tabB=tabB$table[abs(tabB$table$logFC)>5, ]

# diff IDC and Normal
lrtC=glmLRT(fit2, coef=2)
```

```
tabCall=topTags(lrtC, adjust.method = "BH", p.value=1, n=23000, sort.by = "none")
tabC=topTags(lrtC, adjust.method = "BH", p.value=0.05, n=10000)
#tabC=tabC$table[abs(tabC$table$logFC)>5, ]
```

There are 2159 differentially expressed genes in the DCISvsIDC, DCIS vs normal and IDC vs Normal comparisons respectively. 138 genes in each set intersect with the Immport database.

3.3.2 Further information on IDC subtype

We can further break down information - firstly, IDC samples can be broken down into predominantly HER2 and Basal groups

```
# Evaluate HER2 vs Basal
SampleInfo$Subtype=relevel((SampleInfo$Subtype),"HER2")
mod=model.matrix(~factor(Subtype)+Batch, data=SampleInfo)
y3=estimateGLMCommonDisp(y2, mod)
y4=estimateGLMTagwiseDisp(y3, mod)
fitD=glmFit(y4, mod)
lrtCS=glmLRT(fitD, coef=6)

tabCS=topTags(lrtCS, adjust.method = "BH", p.value=0.05, n=10000)
tabCSall=topTags(lrtCS, adjust.method = "BH", p.value=1, n=30000, sort.by="none")
```

There are 635 differentially expressed genes in the DCISvsHER2, DCIS vs IDC and HER2vBasal comparisons respectively. 35 genes in each set intersect with the Immport database.

3.3.3 Normal samples

What are the differences between stroma and organoid and parous and nulliparous samples?

```
SampleInfo$Subtype=relevel((SampleInfo$Subtype),"Str")
mod=model.matrix(~factor(Subtype)+Batch, data=SampleInfo)
y3=estimateGLMCommonDisp(y2, mod)
y4=estimateGLMTagwiseDisp(y3, mod)
fitOS=glmFit(y4, mod)
lrtOS=glmLRT(fitOS, coef=5)
tabOS=topTags(lrtOS, adjust.method = "BH", p.value=0.05, n=10000)
tabOSall=topTags(lrtOS, adjust.method = "BH", p.value=1, n=30000, sort.by="none")
```

There are 1742 differentially expressed genes in stroma vs organoid comparison of which 91 genes intersect with the Immport database.

```
SampleInfo$Parity[grep("BRCA", SampleInfo$Sample)]=NA
mod=model.matrix(~factor(Parity), data=SampleInfo)
y3=estimateGLMCommonDisp(y2[ ,!is.na(SampleInfo$Parity)], mod)
y4=estimateGLMTagwiseDisp(y3, mod)
fitPAR=glmFit(y4, mod)
lrtPAR=glmLRT(fitPAR, coef=2)
tabPAR=topTags(lrtPAR, adjust.method = "BH", p.value=0.05, n=10000)
tabPARall=topTags(lrtPAR, adjust.method = "BH", p.value=1, n=30000, sort.by="none")

AllDGEA=cbind(tabAall$table, tabBall$table,tabCall$table,
              tabCSall$table, tabOSall$table, tabPARall$table)
colnames(AllDGEA)=paste(rep(c("DCIS-IDC", "DCIS-Norm", "IDC-Norm", "HER2-Basal", "Str-Org", "Null-Parous
```

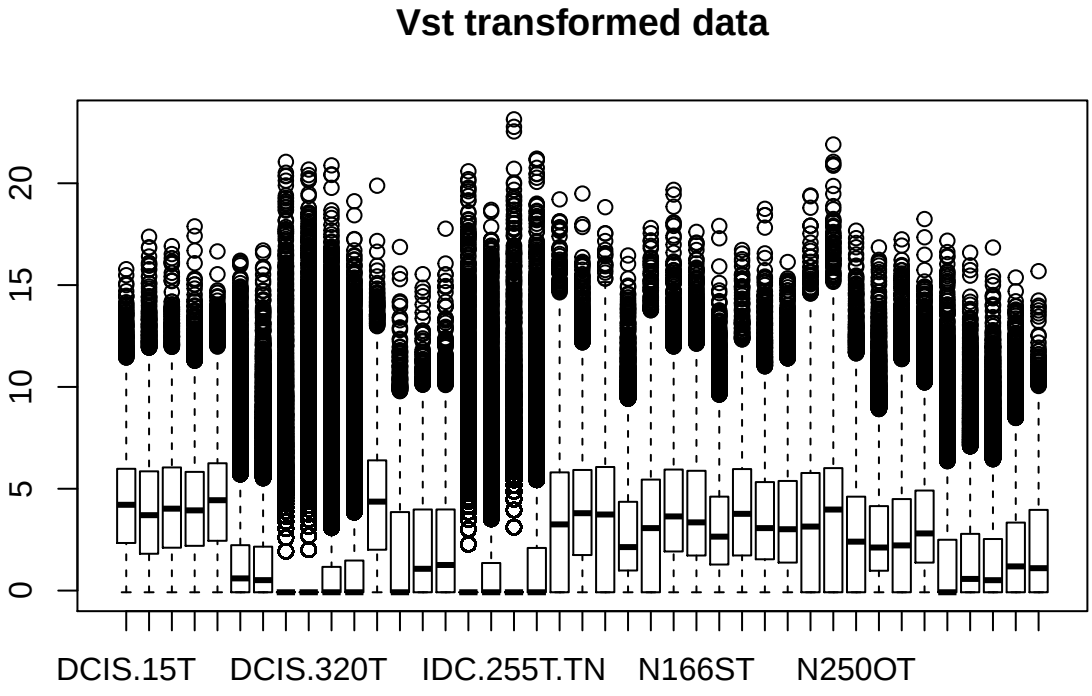
```
x1=sapply(rownames(tabPARall), function(x) grep(x, ImmPort$Symbol))
x2=sapply(x1, length)
x3=sapply(x1, function(x) paste(ImmPort$Category[x], collapse = ", "))
AllDGEA=cbind(AllDGEA, ImmPortCategory=x3)
write.csv(AllDGEA, file="output/Supp_Table_3_Comined_DGEA_analyses.csv")
```

There are 2377 differentially expressed genes in null vs parous comparison of which 131 genes intersect with the Immport database.

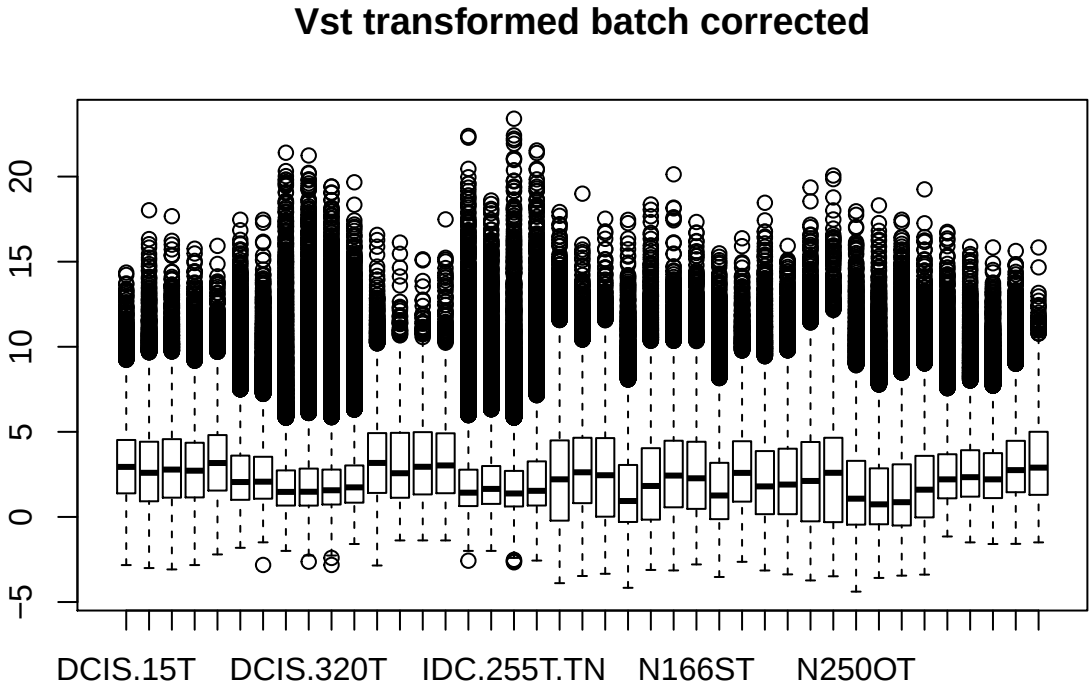
3.4 DESeq2 standardisation

"Standardisation" in DESeq2: Adjust counts for normal counts in DESeq2, and perform a variance Stabilising Transform to adjust the data

```
SampleInfo$Subtype=factor(SampleInfo$Subtype)
SampleInfo$Batch=factor(SampleInfo$Batch)
dds <-DESeqDataSetFromMatrix(countData = GEnewfilt, colData = SampleInfo, design=~Subtype+Batch)
dds=estimateSizeFactors(dds)
dds=estimateDispersions(dds)

ddsCounts=counts(dds, normalized=T)
colnames(ddsCounts)=colnames(GEnewfilt)
ddsCount2=log10(ddsCounts+1)

# can also use a variance Stabilizing Transform for correction
vstNew=varianceStabilizingTransformation(dds)
vstCounts=assay(vstNew)
colnames(vstCounts)=colnames(GEnewfilt)

# perform correction using limma?
mod=model.matrix(~factor(Subtype), data=SampleInfo)
newvstCounts=removeBatchEffect(vstCounts, batch=SampleInfo$Batch, design=mod)

boxplot(vstCounts, main="Vst transformed data")
```




```
boxplot(newvstCounts, main="Vst transformed batch corrected")
```

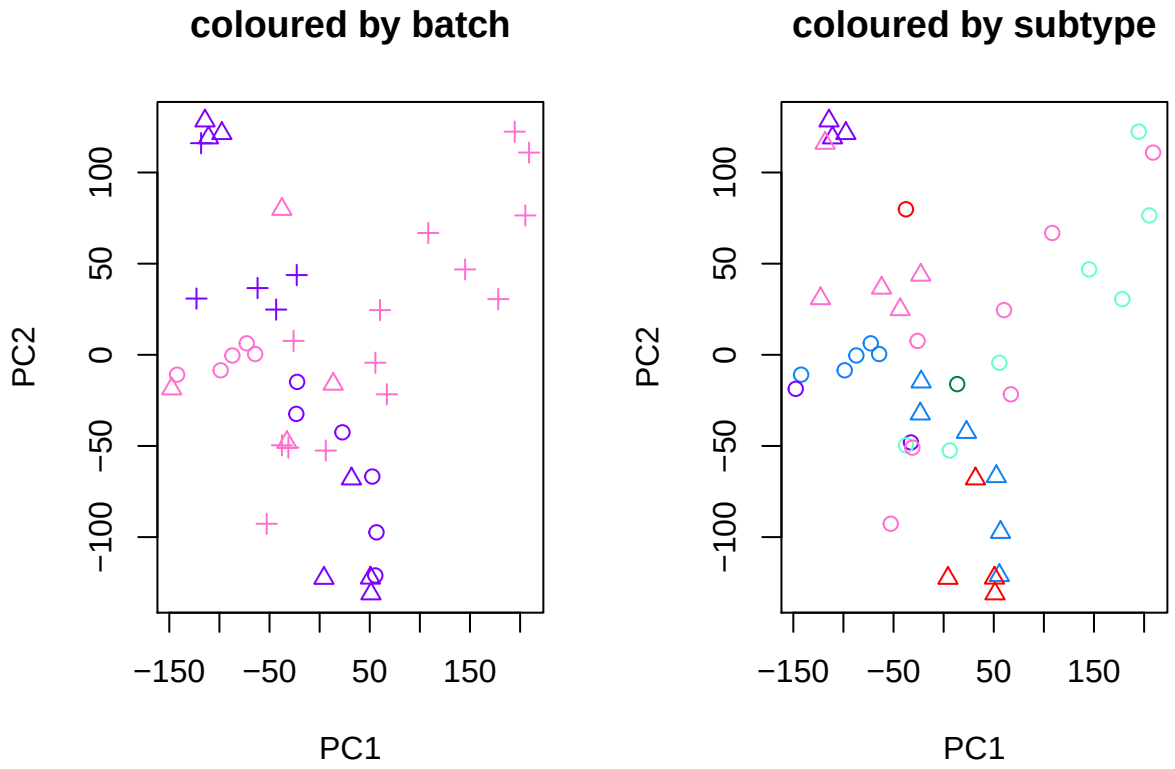


3.5 PCA visualisation

Perform a PCA using the vst transformed batch corrected data

```
pca3=prcomp(t(newvstCounts), scale=F)
# colour by batch
par(mfrow=c(1,2))
plot(pca3$x[ ,1:2], col=SampleInfo$Batch, pch=as.numeric(SampleInfo$DCISIDC), main="coloured by batch")
```

```
plot(pca3$x[ ,1:2], col=SampleInfo$Subtype, pch=as.numeric(SampleInfo$Batch), main="coloured by subtype
```




```
# colour 3d
plot3d(pca3$x[ ,1], pca3$x[ ,2], pca3$x[ ,3],radius=15, type="s",
            col=as.numeric(SampC), xlab = "PC1", ylab = "PC2", zlab="PC3")
rgl.postscript("output/All_samples_pca_vst_transform_DESEQ.pdf", fmt = "pdf")
```

3.6 Differential Gene expression analysis

Run DeSeq2 adding in batch information into the model:

```
dds=DESeq(dds, betaPrior=F)
DEresC1=results(dds, contrast=c("Subtype", "HER2", "TN"))
DEresC2=results(dds, contrast=c("Subtype", "Str", "Org"))

# Run to find DCIS vs IDC
dds1 <-DESeqDataSetFromMatrix(countData = GEnewfilt, colData = SampleInfo, design=~DCISIDC+factor(Batch
dds1=DESeq(dds1, betaPrior=F)
DEresA1=results(dds1, contrast=c("DCISIDC", "DCIS", "IDC"))
DEresA2=results(dds1, contrast=c("DCISIDC", "DCIS", "N"))
DEresA3=results(dds1, contrast=c("DCISIDC", "IDC", "N"))
```

3.7 Figure 2B: Heatmap of differentially expressed genes

How do the gene sets intersect with the EdgeR analysis? Have a look at HER2 vs Basal for example:

```
# HER2 vs Basal
summary(DEresC1)
```

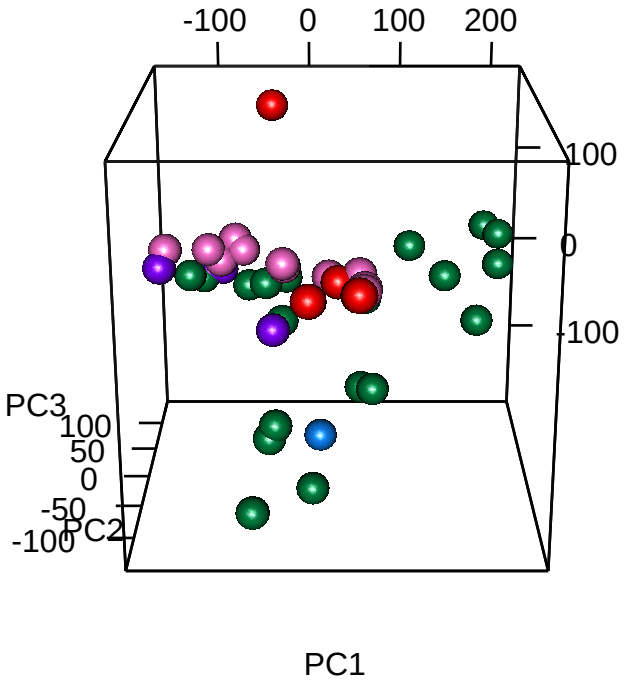


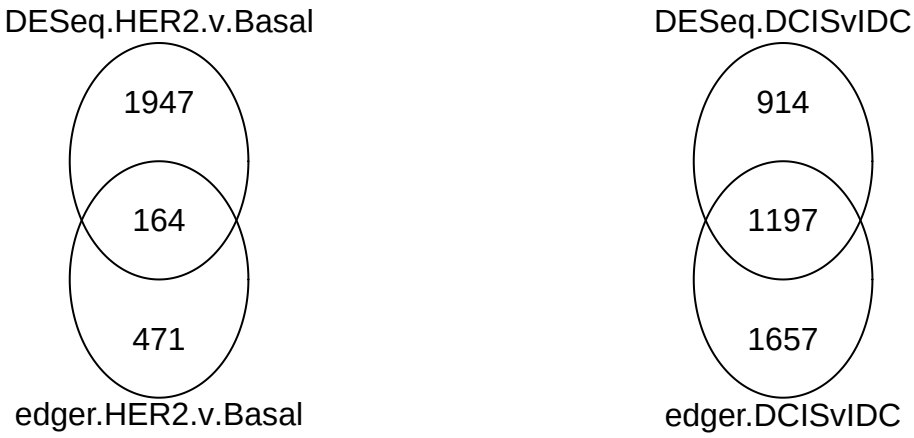
Figure 2: PCA of batch adjusted data

```
##
## out of 21859 with nonzero total read count
## adjusted p-value < 0.1
## LFC > 0 (up)      : 1480, 6.8%
## LFC < 0 (down)    : 924, 4.2%
## outliers [1]      : 3411, 16%
## low counts [2]    : 2242, 10%
## (mean count < 3)
## [1] see 'cooksCutoff' argument of ?results
## [2] see 'independentFiltering' argument of ?results
```

```
GenesDiff=rownames(DEresC1)[which(DEresC1$padj<0.05)]
# DCIS vs IDC
summary(DEresA1)
```

```
##
## out of 21859 with nonzero total read count
## adjusted p-value < 0.1
## LFC > 0 (up)      : 1011, 4.6%
## LFC < 0 (down)    : 1990, 9.1%
## outliers [1]      : 4829, 22%
## low counts [2]    : 2168, 9.9%
## (mean count < 3)
## [1] see 'cooksCutoff' argument of ?results
## [2] see 'independentFiltering' argument of ?results
```

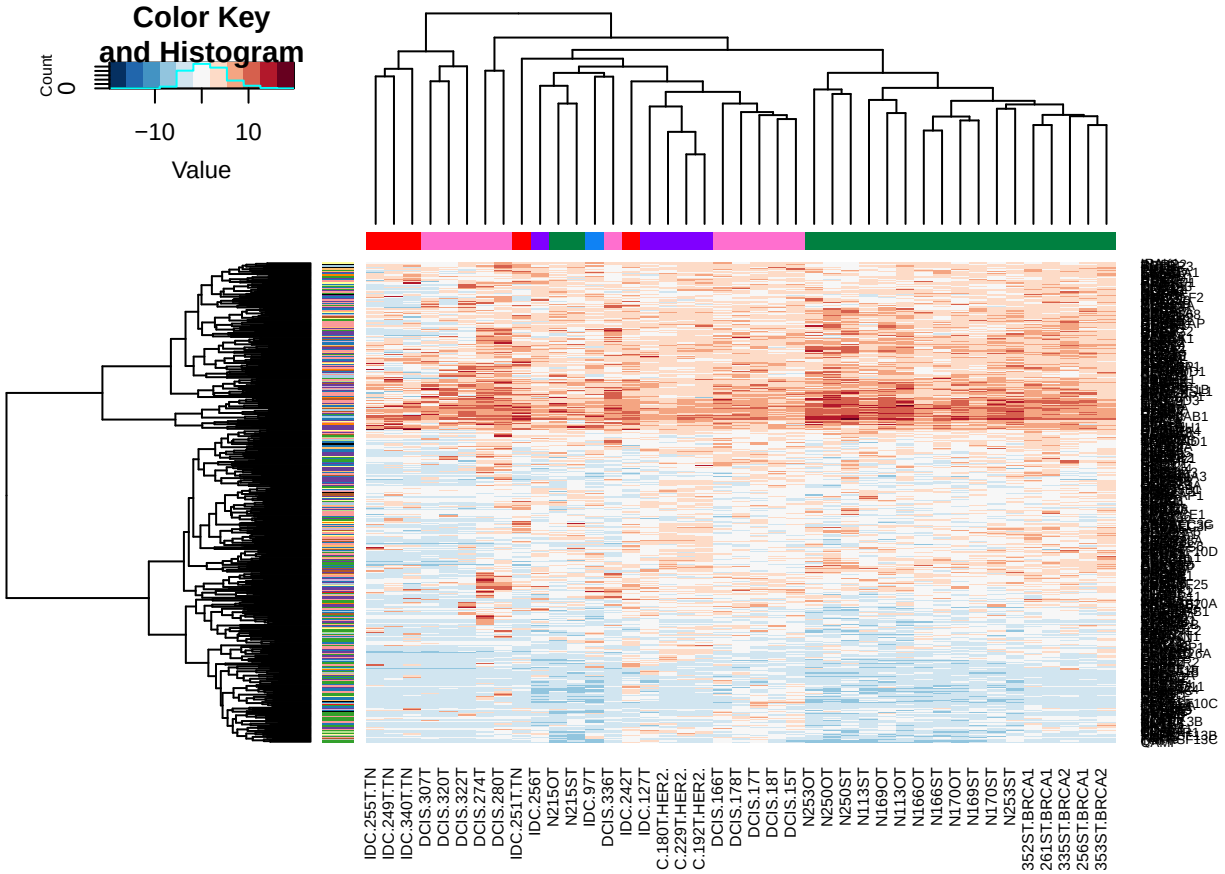
```
GenesDiff=rownames(DEresA1)[which(DEresA1$padj<0.05)]
par(mfrow=c(1,2))
venn(list(edger.HER2.v.Basal=rownames(tabCS), DESeq.HER2.v.Basal=GenesDiff))
venn(list(edger.DCISvIDC=rownames(tabA), DESeq.DCISvIDC=GenesDiff))
```



Approximately half the genes found to be differentially expressed by both DESeq and edger. We can use the intersection of these to construct a heatmap

```
# List of genes differentially expressed in both edger and deseq2
GeneListInt=unique(c(intersect(rownames(tabCS), rownames(DEresC1)[which(DEresC1$padj<0.05)]),
           intersect(rownames(tabA), rownames(DEresA1)[which(DEresA1$padj<0.05 & abs(DEresA1$log2FoldCha
           intersect(rownames(tabB), rownames(DEresA2)[which(DEresA2$padj<0.05 &  abs(DEresA2$log2FoldCh
           intersect(rownames(tabC), rownames(DEresA3)[which(DEresA3$padj<0.05 & abs(DEresA3$log2FoldCha

RowSideColA=factor(CombinedGeneList$Fn[match(GeneListInt, CombinedGeneList$Symbol)])
RowSideColA=as.character(RowSideColA)
```

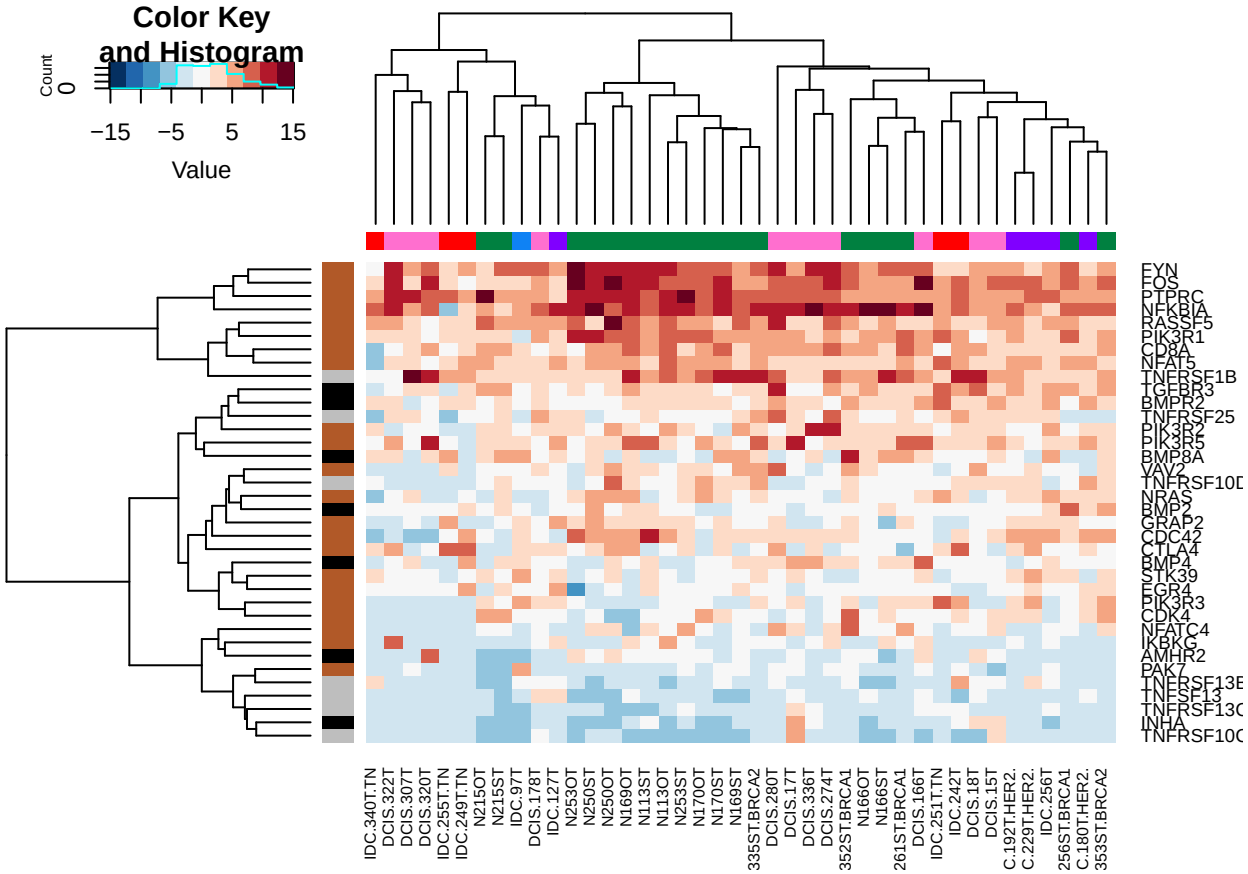
```r
# all genes
# intersection of immune only
x1=which(GeneListInt%in%CombinedGeneList$Symbol)

# fix up category names
RowSideColA=factor(RowSideColA[x1])
RowSideColA2=as.character(RowSideColA)
RowSideColA2[grep("TCR", RowSideColA)]="TCR"
RowSideColA2[grep("IL[0-9]", RowSideColA)]="INTERLEUKIN"
RowSideColA2[grep("Interleukin", RowSideColA)]="INTERLEUKIN"
RowSideColA2[grep("B_CELL", RowSideColA)]="BCR"
RowSideColA2[grep("B_LYMPHOCYTE", RowSideColA)]="BCR"
RowSideColA2[grep("IFN", RowSideColA)]="Interferons"
RowSideColA2[grep("INFECTION", RowSideColA)]="Antimicrobials"
RowSideColA2[grep("TGF", RowSideColA)]="TGF"
RowSideColA2[grep("TNF", RowSideColA)]="TNF"
RowSideColA2[grep("PDGF", RowSideColA)]="PDGF"
RowSideColA2[grep("COMPLEMENT", RowSideColA)]="PDGF"
RowSideColA2[grep("BCR", RowSideColA)]="BCR"
RowSideColA2[grep("CCR", RowSideColA)]="Cytokines"
RowSideColA2[grep("Cytokine", RowSideColA)]="Cytokines"
RowSideColA2[grep("INSULIN", RowSideColA)]="Cytokines"
RowSideColA2[grep("FC", RowSideColA)]="Antimicrobials"
RowSideColA3=RowSideColA2
RowSideColA2=factor(RowSideColA2)
RowSideColA2[RowSideColA2=="NA"]=NA

l1=which(duplicated(CombinedGeneList$Symbol))

lList=unique(CombinedGeneList$Symbol[l1])
GRef=rep(NA, length(lList))

for(i in 1:length(lList)){
  ll1=which(CombinedGeneList$Symbol==lList[i])
  GRef[i]=paste(na.omit(unique(CombinedGeneList$Fn[ll1])), collapse=", ")
}

NewList=data.frame(Symbol=c(as.character(lList),as.character(CombinedGeneList$Symbol[-l1])), Fn=c(as.cha

l2=match(rownames(GEnewfilt), NewList$Symbol)
temp=NewList[l2, ]

write.csv(temp, file="output/immune_annot.csv")

tol14rainbow=c(brewer.pal(12, "Paired"), "black", "grey")
levels(RowSideColA2)=tol14rainbow

heatmap.2(logCPM[GeneListInt[x1], ], scale = "none", col=HMPallete[11:1], ColSideColors = ColSide, trac
```

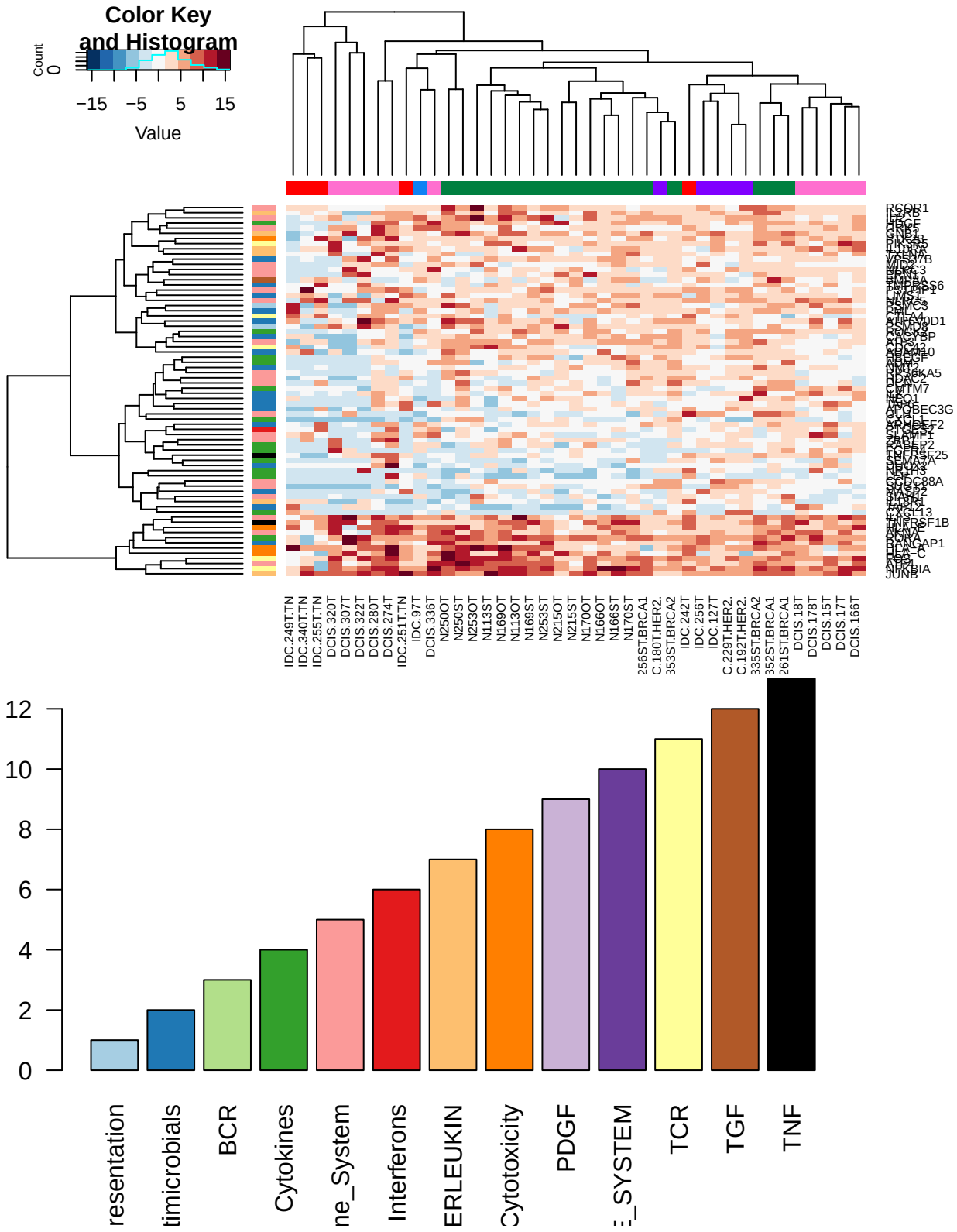



```
l2=which(RowSideColA3=="TCR"|RowSideColA3=="INTER"|
           RowSideColA3=="Cyto"|RowSideColA3=="TGF"|
           RowSideColA3=="TNF"|RowSideColA3=="Inter")

print('look only at genes which are associated with TCell response')
```

```
## [1] "look only at genes which are associated with TCell response"
```

```
t1=heatmap.2(logCPM[GeneListInt[x1[l2]], ], scale = "none", col=HMPallete[11:1], RowSideColors =as.chara
```


Color Key
and Histogram
Count
0
−15
−5
5
15
Value
EYN
FOS
PTPRC
NFKBIA
RASSF5
PIK3R1
CD8A
NFAT5
TNFRSF1B
TGFBR3
BMPR2
TNFRSF25
PIK3R2
PIK3R5
BMP8A
VAV2
TNFRSF10D
NRAS
BMP7
GRAP2
CDC42
CTLA4
BMP4
STK39
EGR4
PIK3R3
CDK4
NFATC4
IKBKG
AMHR2
PAK7
TNFRSF13B
TNFSF13
TNFRSF13C
INHA
TNFRSF10C
IDC.340T.TN
DCIS.322T
DCIS.307T
DCIS.320T
IDC.255T.TN
IDC.249T.TN
N215OT
N215ST
IDC.97T
DCIS.178T
IDC.127T
N253OT
N250ST
N250OT
N169OT
N113ST
N113OT
N253ST
N170OT
N170ST
N169ST
335ST.BRCA2
DCIS.280T
DCIS.17T
DCIS.336T
DCIS.274T
352ST.BRCA1
N166OT
N166ST
261ST.BRCA1
DCIS.166T
IDC.251T.TN
IDC.242T
DCIS.18T
DCIS.15T
C.192T.HER2.
C.229T.HER2.
IDC.256T
256ST.BRCA1
C.180T.HER2.
353ST.BRCA2


Color Key
and Histogram
Count
0
−15
−5
5
15
Value
IDC.249T.TN
IDC.340T.TN
IDC.255T.TN
DCIS.320T
DCIS.307T
DCIS.322T
DCIS.280T
DCIS.274T
IDC.251T.TN
IDC.97T
DCIS.336T
N250OT
N250ST
N253OT
N113ST
N169OT
N113OT
N169ST
N253ST
N215OT
N215ST
N170OT
N166OT
N166ST
N170ST
256ST.BRCA1
C.180T.HER2.
353ST.BRCA2
IDC.242T
IDC.256T
IDC.127T
C.229T.HER2.
C.192T.HER2.
335ST.BRCA2
352ST.BRCA1
261ST.BRCA1
DCIS.18T
DCIS.178T
DCIS.15T
DCIS.17T
DCIS.166T
12
10
8
6
4
2
0
resentation
timicrobials
BCR
Cytokines
ne_System
Interferons
ERLEUKIN
Cytotoxicity
PDGF
E_SYSTEM
TCR
TGF
TNF

4 Gene Set enrichment analysis: Figure 2

4.1 GSEA in HTSanalyzeR

Gene set enrichment analysis is performed using log fold-changes from differential gene expression analysis (from edger analysis).

The set-up is shown below for DCIS vs IDC, this is repeated for all other comparisons: Here it is run with 10 permutations for speed, but run with 100 permutations in this analysis.

```
# define the gene sets
GSEAList=list(Immune=immunec7listentrez, Canonical=Canlistentrez)
# DCIS VS IDC
# Supply phenotypic data (PhData) and hits (differentially expressed genes)
Phdata=tabAall$table$logFC
hits=rownames(tabA)
names(Phdata)=rownames(tabAall)

gscaDvsI=new("GSCA", listOfGeneSetCollections=GSEAList, geneList=Phdata, hits=hits)
gscaDvsI=preprocess(gscaDvsI, species="Hs", initialIDs="Symbol", keepMultipleMappings=T,
                duplicateRemoverMethod='average')
## Change number of Permutations to 100 for real simulation
gscaDvsI<-analyze(gscaDvsI,para=list(nPermutations=10, minGeneSetSize=10,pValueCutoff = 0.05,pAdjustMet
```

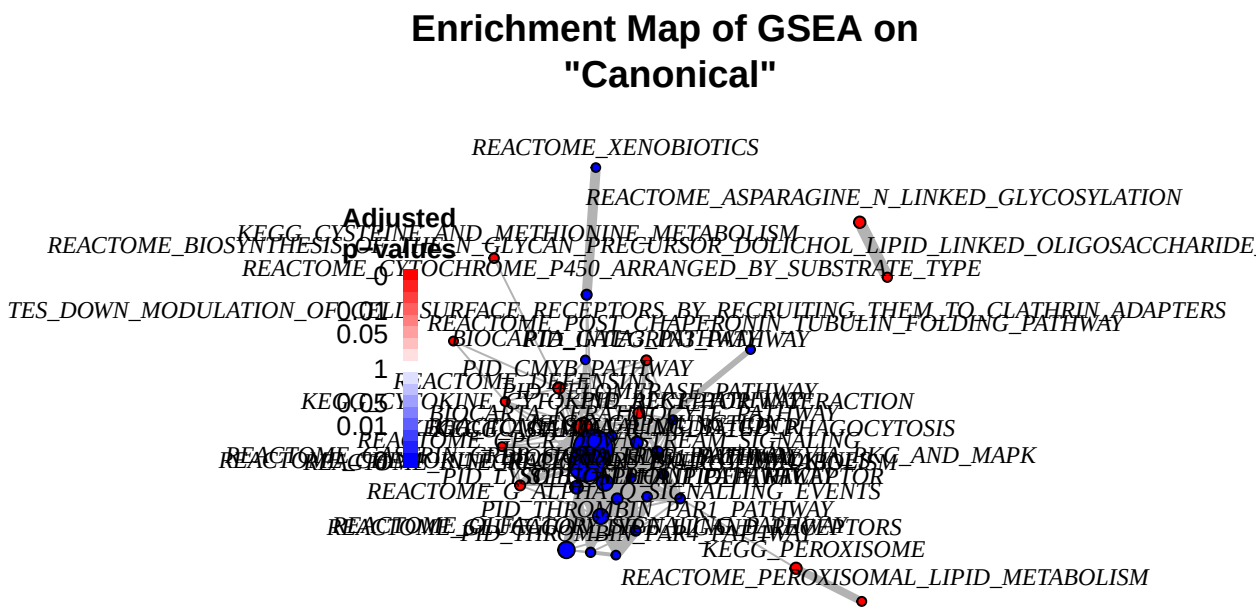
Plot the output for this:

```
load("output/GSEA/example_gsea_DCIS_vs_IDC.RData")
HTSanalyzeR::summarize(gscaD_DvI)
```

```
##
## -No of genes in Gene set collections:
##           input above min size
## Immune     4872           4872
## Canonical  1330           1286
##
##
## -No of genes in Gene List:
##           input valid duplicate removed converted to entrez
## Gene List 22196 22196             22196               22180
##
##
## -No of hits:
##      input preprocessed
## Hits  2840         2835
##
##
## -Parameters for analysis:
##               minGeneSetSize pValueCutoff pAdjustMethod
## HyperGeo Test 10             0.05         BH
##
##      minGeneSetSize pValueCutoff pAdjustMethod nPermutations exponent
## GSEA 10             0.05         BH             100           1
##
##
## -Significant gene sets (adjusted p-value< 0.05 ):
```

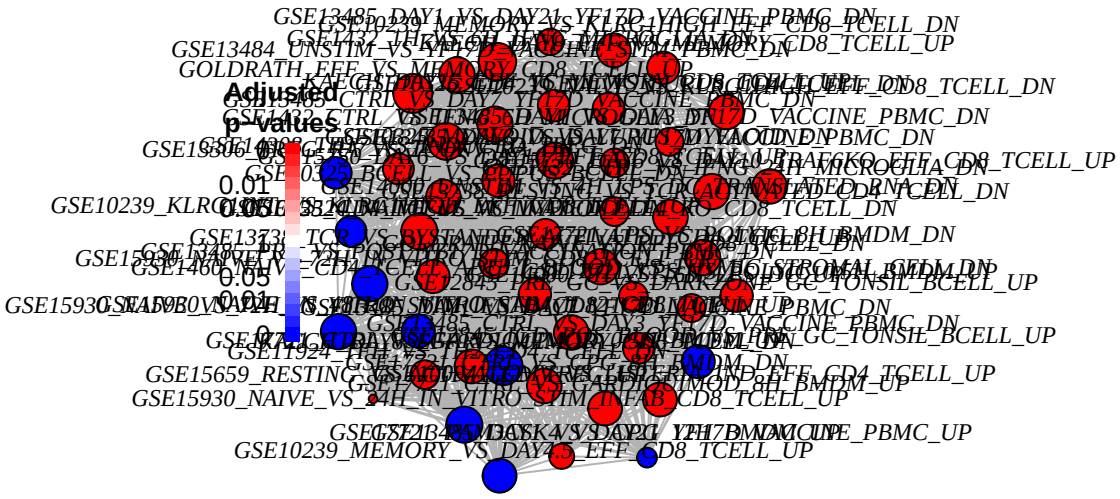
```
##           Immune Canonical
## HyperGeo     358        20
## GSEA         353        38
## Both          39         0
```

```
topTerms_DvsI=getTopGeneSets(gscaD_DvI, "GSEA.results", c("Immune", "Canonical"), allSig=TRUE)

viewEnrichMap(gscaD_DvI, resultName="GSEA.results", "Canonical", ntop=35, allSig=F, gsNameType="id",
              displayEdgeLabel=F)
```




```
## IGRAPH UNW- 35 197 --
## + attr: name (v/c), geneSetSize (v/n), size (v/n), color (v/c),
## | label (v/c), label.dist (v/n), label.cex (v/n), label.font
## | (v/n), label.color (v/c), weight (e/n), color (e/c), width (e/n)
## + edges (vertex names):
## [1] Canonical.KEGG_CYSTEINE_AND_METHIONINE_METABOLISM    --Canonical.PID_CMYB_PATHWAY
## [2] Canonical.KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION--Canonical.KEGG_GAP_JUNCTION
## [3] Canonical.KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION--Canonical.KEGG_ASTHMA
## [4] Canonical.KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION--Canonical.BIOCARTA_KERATINOCYTE_PATHWAY
## + ... omitted several edges
```

```
viewEnrichMap(gscaD_DvI, resultName="GSEA.results", "Immune", ntop=50, allSig=F, gsNameType="id",
              displayEdgeLabel=F)
```




```
## IGRAPH UNW- 50 1125 --
## + attr: name (v/c), geneSetSize (v/n), size (v/n), color (v/c),
## | label (v/c), label.dist (v/n), label.cex (v/n), label.font
## | (v/n), label.color (v/c), weight (e/n), color (e/c), width (e/n)
## + edges (vertex names):
## [1] Immune.KAECH_DAY8_EFF_VS_MEMORY_CD8_TCELL_UP--Immune.KAECH_DAY15_EFF_VS_MEMORY_CD8_TCELL_UP
## [2] Immune.KAECH_DAY8_EFF_VS_MEMORY_CD8_TCELL_UP--Immune.GOLDRATH_NAIVE_VS_EFF_CD8_TCELL_DN
## [3] Immune.KAECH_DAY8_EFF_VS_MEMORY_CD8_TCELL_UP--Immune.GOLDRATH_EFF_VS_MEMORY_CD8_TCELL_UP
## [4] Immune.KAECH_DAY8_EFF_VS_MEMORY_CD8_TCELL_UP--Immune.GSE10239_NAIVE_VS_KLRG1HIGH_EFF_CD8_TCELL_DN
## + ... omitted several edges
```

Note that some immune terms: Toll-like receptor, NK cells, appear to be enriched.

For all samples, the following function will convert the GSEA objects into plots:

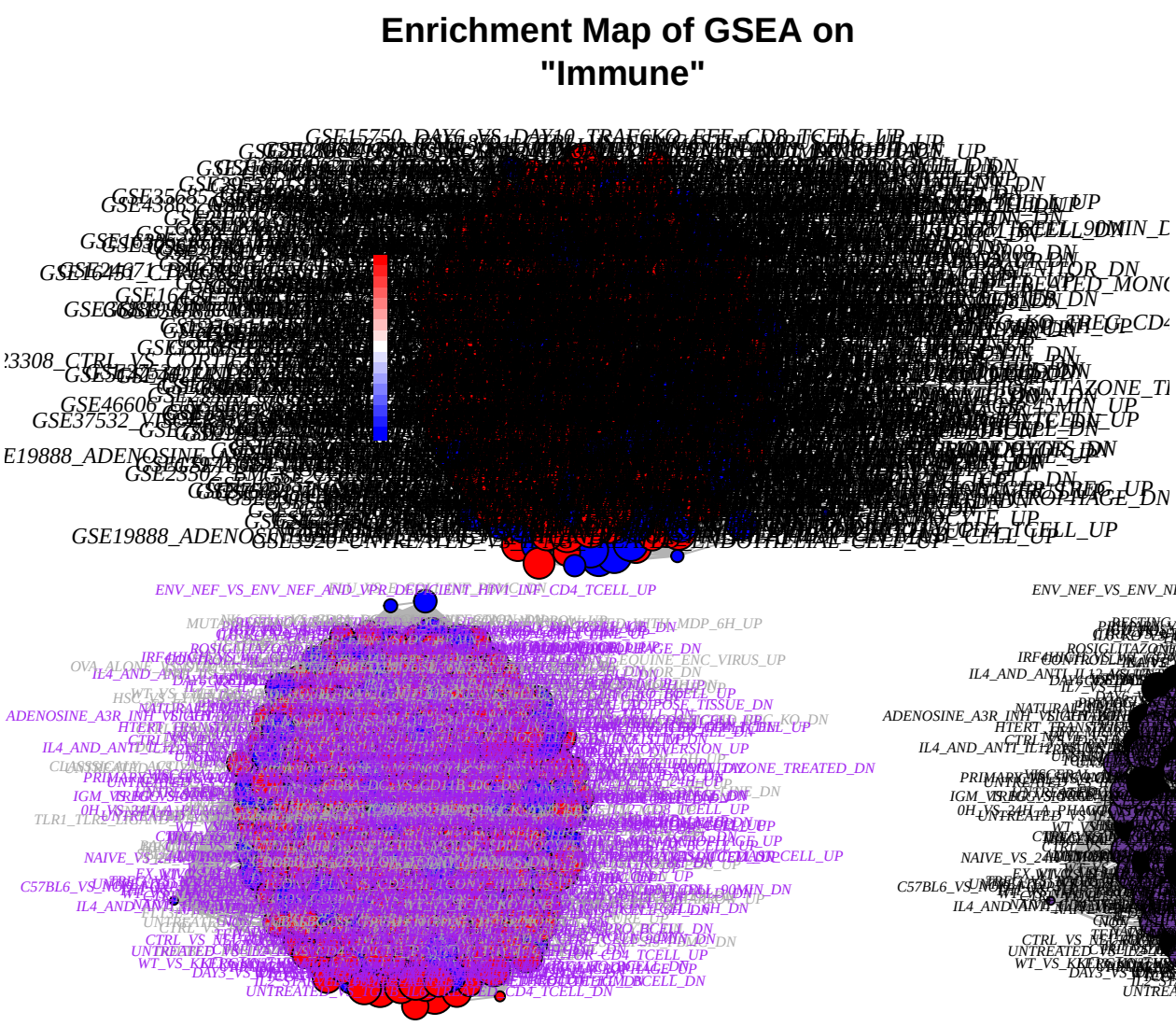
```
# write a function to make the plots, and save the output files from the analysis
# Note that all the functions to print to file have been commented out

SaveGSEAData=function(gsea, FnName="expt1"){
  dir.create(FnName)
  # file lists:
  A1=gsea@result$GSEA.results$Canonical
  D1=gsea@result$GSEA.results$Immune
  write.csv(A1, file=sprintf("%s/GSEA_Canonical_%s.csv",FnName, FnName))
  write.csv(D1, file=sprintf("%s/GSEA_Immune_%s.csv",FnName, FnName))

  # get the number of sig gene sets in each:
  toptabSum=getTopGeneSets(gsea, "GSEA.results", c("Immune","Canonical"), allSig=T)
  nGenes=as.numeric(summary(toptabSum)[ ,1])
  GCatNames=c("IFN", "IL[0-9]","IL_[0-9]", "TNF", "CYTOKINE", "NKCELL", "ANTIGEN", "CHEMOKINE", "TCR",
              "BCR", "CYTOTOXIC", "TGF", "IMMUNE", "TH1", "STAT[0-9]", "INFLAMMATORY", "COMPLEMENT",
              "COAGULATION", "ALLOGRAFT","INTERFERON", "DISEASE","IGG","BCELL", "TCELL", "LYMPHOCYTE",
              "CTL", "TH2", "GRANZYME", "NEUTROPHIL", "T_CELL", "LEUKOCYTE", "B_CELL", "TREG", "INFLUEN
              "FC_", "IGF", "CLASSIC", "COMP_", "THROMBIN", "DEFENSIN", "ASTHMA", "LUPUS", "PDGF")
  Metab=c("PENTOSE", "METAB", "GLYCO", "GLUC","BIOSYNTHESIS")
  DNAnames=c("DNA", "G1", "G2", "MITO", "MEIO", "REPLIC", "REPAIR", "CELL_CYCLE", "TELOMER", "CENTROSOM
```

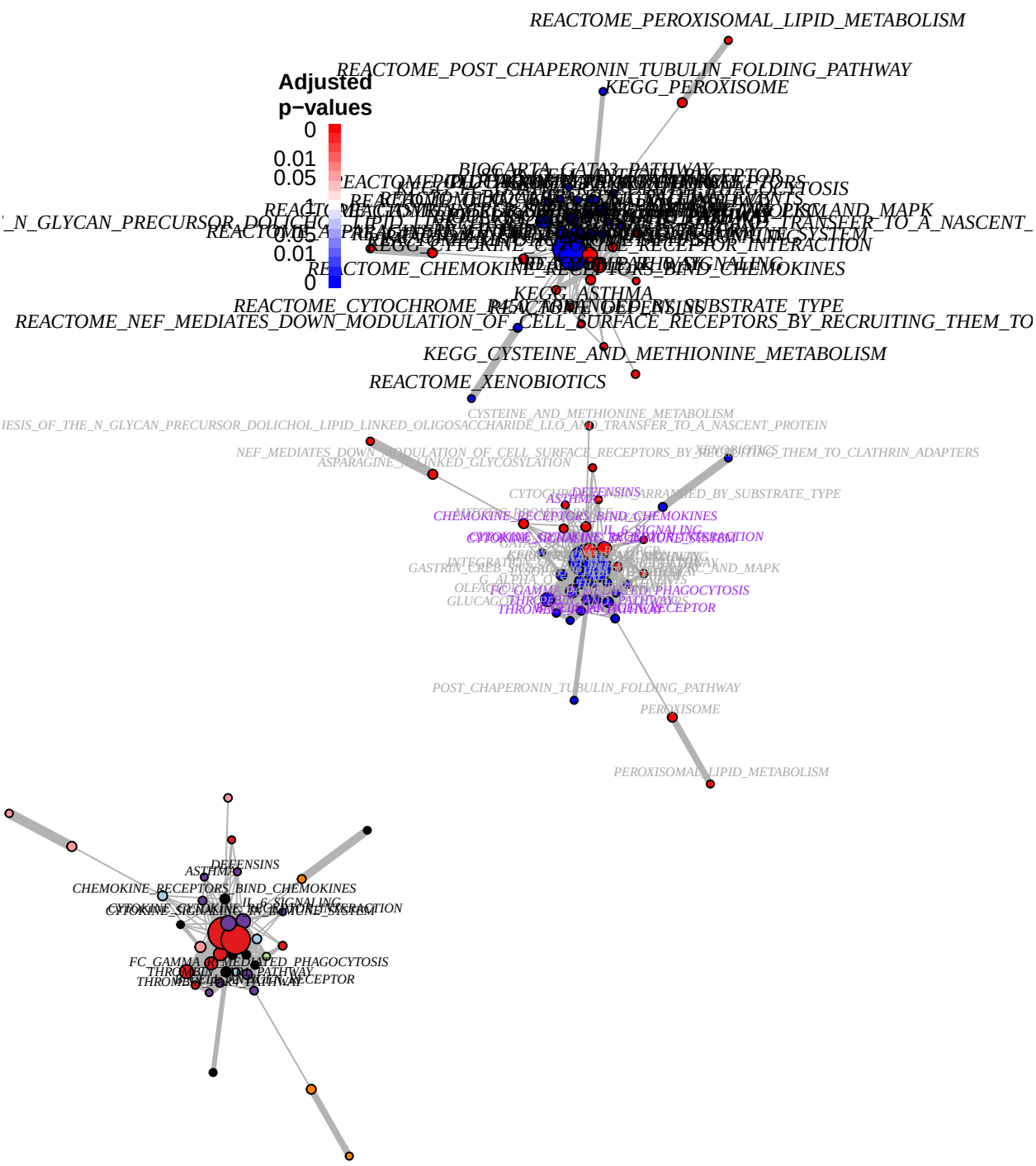
```
  RNAnames=c("RNA", "TRANSCRIPT", "POL", "PROMOTER", "SPLIC", "EXON", "INTRON")

  Cancer=c("CANCER", "CARCIN", "_RB_", "FGFR", "ERBB", "AURORA", "TP53", "ALK")
  Translat=c("TRANSLATION", "RIBOSOME", "43S", "PROTEOL", "PEPTIDE", "EIF", "GOLGI", "PROTEIN")
  Apop=c("APOPTOSIS", "DEATH")
  Signal=c("SECRET", "SIGNAL", "SYNAP", "RECEPTOR", "INTEGRIN", "TRANSDUCTION", "RAB", "NEUROTRANSMIT")
  Elect=c("ELECTRON", "OXID", "ATP", "CYTOCHROM", "PEROXI")
  ECM=c("MATRISOME", "MATRIX", "EXTRACELLULAR", "KERATIN", "MUSCLE")

  TermIdx=list( DNA=DNAnames, RNA=RNAnames, Cancer=Cancer, Protein=Translat,
                Metab=Metab,Secretion=Signal, Apoptosis=Apop, Electron=Elect, ECM=ECM,Imm=GCatNames)
  ColID=brewer.pal(12, "Paired")

  NamesIdx=c("Immune","Canonical")
  for (i in 1:2){
   Plot1=viewEnrichMap(gsea, resultName="GSEA.results", NamesIdx[i], ntop=nGenes[i], allSig=F, gsNameTy
              displayEdgeLabel=F)
  l=layout.fruchterman.reingold(Plot1)
  vertex_attr(Plot1)$label.cex=rep(0.5, nGenes[i])
  vertex_attr(Plot1)$label.dist=rep(0.25, nGenes[i])
  temp1=unlist(regmatches(vertex_attr(Plot1)$label, regexpr("_", vertex_attr(Plot1)$label), invert = TR
  temp1=temp1[seq(2, length(temp1), by=2)]
  vertex_attr(Plot1)$label=temp1
  a1=unique(unlist(sapply(GCatNames, function(x) grep(x, vertex_attr(Plot1)$label))))
  vertex_attr(Plot1)$label.color=rep("darkgrey", nGenes[i])
  if (length(a1)>0){
  vertex_attr(Plot1)$label.color[a1]="purple"
  }
 # pdf(file = sprintf("%s/GSEA_shortenedNames_%s_%s.pdf", FnName, FnName,NamesIdx[i]))
  plot(Plot1, layout=l)
 # dev.off()
  idxNew=lapply(TermIdx, function(x) unlist(sapply(x, function(y) grep(y, vertex_attr(Plot1)$label))))
  idxNew2=sapply(idxNew, unique)
  idxSum=sapply(idxNew2, length)
  idxSum=c(idxSum, nGenes[i])
 write(idxSum, file=sprintf("%s/GSEA_enrichedno_%s_%s.csv",FnName, FnName, NamesIdx[i]))
  vertex_attr(Plot1)$color=rep("black", length(vertex_attr(Plot1)$label))
  for (j in 1:length(idxNew2)){
  vertex_attr(Plot1)$color[unlist(idxNew2[j])]=ColID[j]
  vertex_attr(Plot1)$label.color[unlist(idxNew2[j])]=ColID[j]
  }
 #pdf(file = sprintf("%s/GSEA_coloured_%s_%s.pdf", FnName, FnName,NamesIdx[i]))
  # plot(Plot1, layout=l)
  #dev.off()
   tNames=vertex_attr(Plot1)$label[unlist(idxNew2[10])]
  vertex_attr(Plot1)$label=rep("", nGenes[i])
  vertex_attr(Plot1)$label[unlist(idxNew2[10])]=tNames
  vertex_attr(Plot1)$label.color=rep("black", length(vertex_attr(Plot1)$label))
  #pdf(file = sprintf("%s/GSEA_Nameless_%s_%s.pdf", FnName, FnName,NamesIdx[i]))
  plot(Plot1, layout=l)
  #dev.off()
}
}
```

Using previously run GSEA analysis, we can visualise the output:

```
SaveGSEAData(gscaD_DvI, "DCISvsIDCtest")
```

```
## Warning in dir.create(FnName): 'DCISvsIDCtest' already exists
```





4.2 Immune c7

Note that the immune c7 analysis provides many inter-connected terms. These are representative of studies which compare condition A to B, and the top 100 differentially expressed genes between the two scenarios are

reported as a gene set. This is directional, ie, genes which are more highly expressed in A belong to the "A vs B UP" set, whereas genes more highly expressed in B belong to the "A vs B DOWN" set.

A and B contains information on cell type (eg. B Cells, CD4 cells), activation state and experimental treatment. A separate .Rmd file is provided to create plots based on outputs from GSEA (`summary-immunec7.Rmd`). Examples of plots from DCIS and IDC comparisons are shown below:

4.2.1 Load data of interest

```
GSEAresults=read.csv("output/GSEA/GSEA_immunec7_DCISvsNormal.csv")
PValCutOff=0.05
CompA="DCIS"
CompB="Normal"
```

Note that the script `ImmuneC7Output.R` can be used to derive more information from the gene sets.

```
# Load the Gene Set summary information and index:
GSInfo=read.csv("data/c7_immune_annotated.csv")
NID=GSEAresults$X[which(GSEAresults$Adjusted.Pvalue<PValCutOff)]
NIDmatch=match(NID, GSInfo$GeneSet)
temp=GSInfo[NIDmatch, ]
```

The immune c7 data set was further curated: For example below, the Gene set was separated into GSE number which was used to look up information such as organism, and source of T-cells. Also determined was the celltype (eg. CD4 T cell) and the treatment applied (eg IL3/HIVP17 and LISTERIA). Stimulation refers to whether they are activated, naive or exhausted:

```
GSInfo[8,1:15 ]
```

```
##   X                                          GeneSet      GSE      GrpA
## 8 8 GSE10094_LCMV_VS_LISTERIA_IND_EFF_CD4_TCELL_UP GSE10094 CD4_TCELL
##   TreatA StimA      GrpB   TreatB    StimB  CellType Organ TimeCourse
## 8   LCMV  <NA> CD4_TCELL LISTERIA EFFECTOR CD4_TCELL  <NA>       <NA>
##       Treatment     Organism                         Source
## 8 LCMVvLISTERIA Mus musculus SMARTA TCR transgenic T cells
```

We can have a quick look at the directionality of the different comparison. Here, we compare average DCIS vs Normal expression in samples in gene sets which are of the format A vs B up and down.

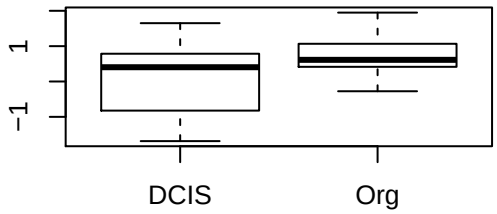
```
l1=which(GSEAresults$Adjusted.Pvalue<0.05)
pos1=which(GSEAresults$Observed.score[l1]>0)
neg1=which(GSEAresults$Observed.score[l1]<0)
up1=grep("UP", temp$GeneSet[l1])
down1=grep("DN", temp$GeneSet[l1])

List=c(intersect(pos1, up1)[1], intersect(pos1, down1)[1],
       intersect(neg1, up1)[1], intersect(neg1, down1)[1])

GSNames=cbind(GeneSet=as.character(temp$GeneSet[l1[List]]), Enrichment=GSEAresults$Observed.score[l1[Lis
```

We will look at the above four gene sets as examples to assess the directionality in DCIS compared to Normal:

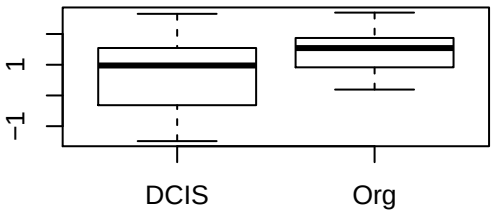
```
subidx=which(SampC%in%c("DCIS", "Org"))
par(mfrow=c(2,2))
for (i in List){
ImmSet=temp[i, "GeneSet"]
x2=which(names(immunec7entrez)==ImmSet)
```

```
GeneListNew=geneIds(immunec7[[x2]])
m2=colMeans(logCPM[na.omit(match(GeneListNew, rownames(logCPM))), subidx])
boxplot(m2~factor(SampC[subidx]), main=sprintf("%s%g", ImmSet, round(GSEAresults$Observed.score[l1[i]]*
}
```

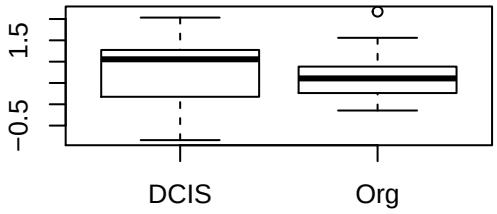
H_DAY8_EFF_VS_DAY15_EFF_CD8_TCELL



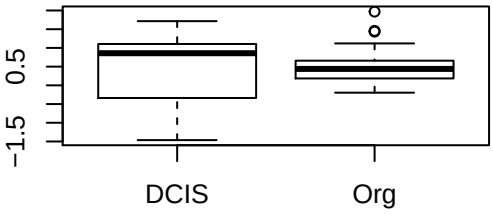
CH_NAIVE_VS_DAY15_EFF_CD8_TCELL



2845_IGD_POS_VS_NEG_BLOOD_BCELL



SE13229_IMM_VS_MATURE_NKCELL_DN

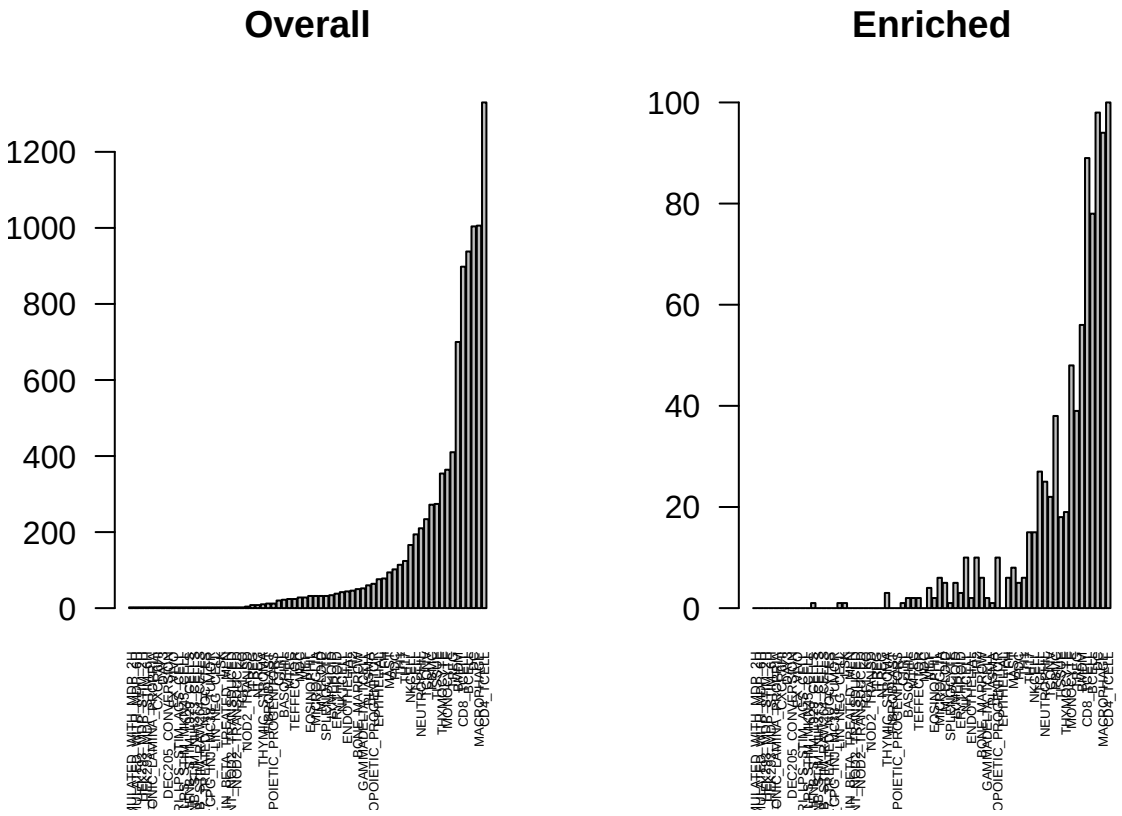


A positive enrichment score means group B (Normal) has higer mean expression compared to the reference (A, DCIS). Also, the gene set directionality is flipped depending on whether a group is "up" or "down". in C vs D up, group B would be more similar to C.

4.3 Cell types enriched

Compare the types of cells (leukocytes) studied in each gene set:

- Overall refers to the overall distribution of gene sets featuring a particular cell type of interest.
- Enriched refers to cell types in gene sets with P-value less than that specified (ie. significant results from GSEA)

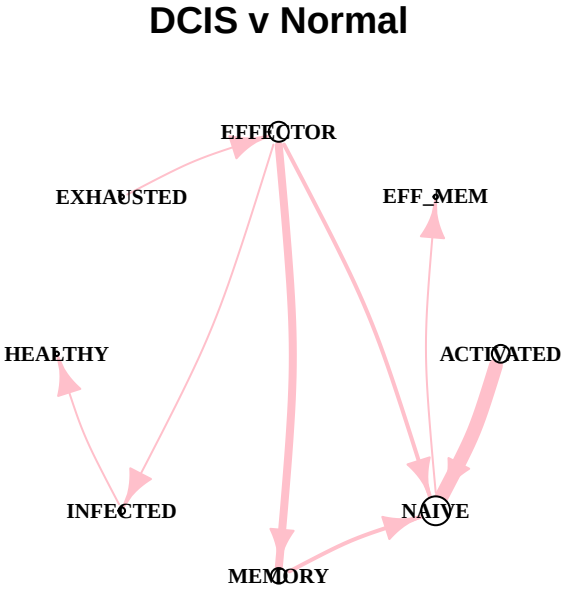


4.3.1 Activation, memory, exhaustion: Fig 2D

Below a function was created to develop a network of the "interaction" between different cell types.

```
ActivationNetwork=function(A, B, GSEAscore, CompA, SearchTerms=NULL){
  library(igraph)
nx=which(GSEAscore<0)
  df=data.frame(StimA=A, StimB=B)
  if (length(nx)>0){
    A2=df$StimA[nx]
    B2=df$StimB[nx]
    df$StimA[nx]=B2
    df$StimB[nx]=A2
  }
   if (!is.null(SearchTerms)){
  a1=sapply(SearchTerms, function(x) grep(x, df[ ,1]))
  b1=sapply(SearchTerms, function(x) grep(x, df[ ,2]))
  xint=intersect(unlist(a1), unlist(b1))
  df=df[xint, ]
}

 # look for na values
  naidx=which(is.na(df$StimA) | is.na(df$StimB))
  if (length(naidx)>0){
    df=df[-naidx, ]
  }
```

```
  allList=factor(c(as.character(df$StimA), as.character(df$StimB)))
  df$StimA=factor(df$StimA, levels=levels(allList))
  df$StimB=factor(df$StimB,levels=levels(allList))

 gr0=graph.edgelist(data.matrix(df), directed=T)
  gr0=igraph::simplify(gr0, remove.loops = T, remove.multiple = F)
 deg=igraph::degree(gr0, mode="all")
 E(gr0)$weight=1
 V(gr0)$size=(deg+1)
 V(gr0)$color="white"
 gr0=igraph::simplify(gr0, remove.multiple = T,  edge.attr.comb="sum")
 V(gr0)$label=levels(df$StimA)
 E(gr0)$width=E(gr0)$weight*2/3

 ## bug here
edge.start <- ends(gr0, es=E(gr0), names=F)
Ecol=rep("pink", nrow(edge.start))
tab0ut=table(df)
tab0ut2=tab0ut-t(tab0ut)
Ecol2=sapply(1:nrow(edge.start), function(x) ifelse(tab0ut2[edge.start[ x,1],edge.start[ x,2]]>0, "pink
Ewidth2=sapply(1:nrow(edge.start), function(x) tab0ut2[edge.start[ x,1],edge.start[ x,2]])
##
E(gr0)$edge.color=Ecol2
E(gr0)$width=Ewidth2
gr02=delete_edges(gr0, E(gr0)[Ecol2=="green"])
#browser()
V(gr02)$size=rowSums(abs(tab0ut2))+1

plot(gr02, layout=layout_in_circle(gr02),vertex.label.color="black",
     edge.color=E(gr02)$edge.color,vertex.label.font=2,
     vertex.label.color="gray", vertex.label.cex=.7, edge.curved=0.1, mark.border=NA,
     edge.arrow.mode=2, edge.arrow.size=1,
     main=sprintf("%s", CompA))

 #plot(gr02, layout=layout_in_circle(gr02),vertex.label.color="black",
 #     edge.color="gray",vertex.label.font=2,
#     vertex.label.color="gray", vertex.label.cex=.7, edge.curved=0.1, mark.border=NA,
#     edge.arrow.mode=3, edge.arrow.size=1,
#     main=sprintf("%s", CompA))

 if (length(naidx)>0){
 ret=list(graph=gr02, data=cbind(df, GSEAscore[-naidx]))
 } else {
 ret=list(graph=gr02, data=df)
 }
  ret
}

a1=ActivationNetwork(temp$StimA,temp$StimB, GSEAresults$Observed.score[which(GSEAresults$Adjusted.Pvalu
```



The node size is reflective of the number of times a stimulation type appears, and the width of lines is reflective of the number of gene sets supporting that 'interaction'. For example, in the above plot a thick blue line points between activated and naive - suggesting that this interaction is downregulated in the DCIS vs IDC comparison (ie. could be higher in IDC compared to DCIS)

```
# Look at the enriched gene sets
summary(a1$data$StimA)
```

```
## ACTIVATED   EFF_MEM  EFFECTOR EXHAUSTED   HEALTHY  INFECTED    MEMORY
##        12         0         9         1         3         8         5
##     NAIVE
##        10
```

```
summary(a1$data$StimB)
```

```
## ACTIVATED   EFF_MEM  EFFECTOR EXHAUSTED   HEALTHY  INFECTED    MEMORY
##         5         1         3         0         4         8         7
##     NAIVE
##        20
```

```
# Also check the type of cells studied
summary(factor(temp[rownames(a1$data), "CellType"]))
```

```
##      BMDM CD4_TCELL CD8_TCELL        DC       MAC    NKCELL     TCELL
##         2         2         7         2         2         1         1
##     TCONV THYMOCYTE      NA's
##         1         1        29
```

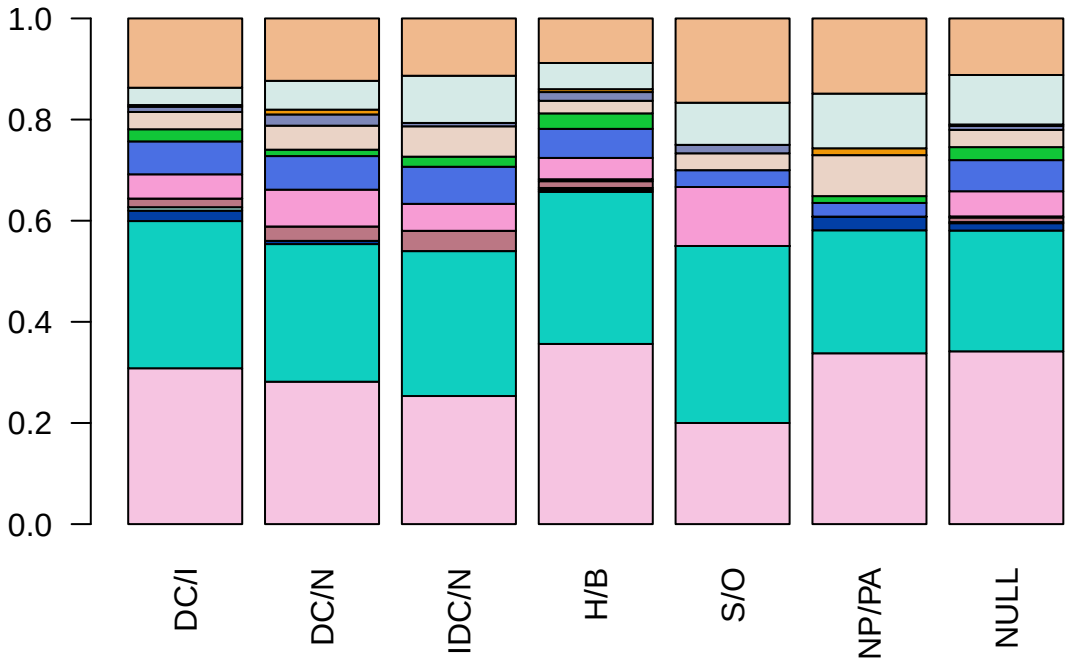
4.3.2 Comparing across different studies: Figure 2C

Load all the results and look at the distribution of cells across different samples:

```
GSEAresults[[1]]=read.csv("output/GSEA/GSEA_immunec7_DCISvsIDC.csv")
GSEAresults[[2]]=read.csv("output/GSEA/GSEA_immunec7_DCISvsNormal.csv")
GSEAresults[[3]]=read.csv("output/GSEA/GSEA_immunec7_IDCvsNormal.csv")
GSEAresults[[4]]=read.csv("output/GSEA/GSEA_immunec7_HER2vsBasal.csv")
GSEAresults[[5]]=read.csv("output/GSEA/GSEA_immunec7_Stroma_organoid.csv")
```

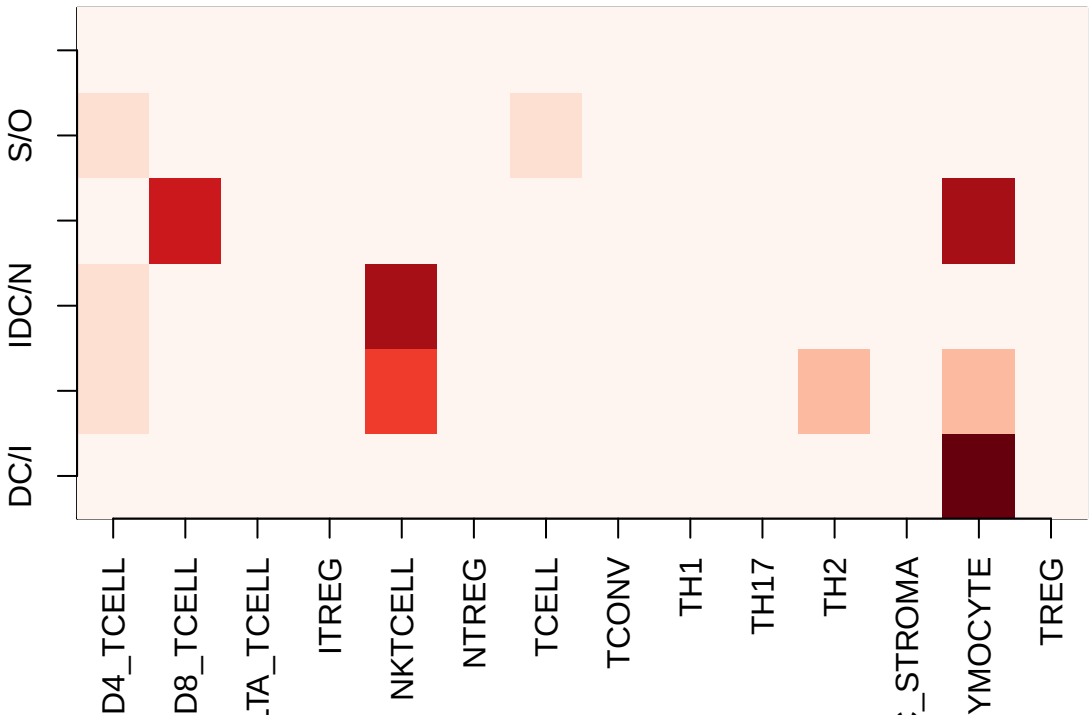
```
GSEAresults[[6]]=read.csv("output/GSEA/GSEA_immunec7_Parity_comparison.csv")
GSEAresults[[7]]=read.csv("output/GSEA/gsca_DCIS_vs_HER2_immunec7.csv")
GSEAresults[[8]]=read.csv("output/GSEA/gsca_DCIS_vs_Basal_immunec7.csv")
```

```
Ctypes=c("TCELL", "^TH", "TREG", "NKTCELL", "TCONV")

Cnames=NA
Ccomp=NA

for(i in 1:6){
  temp1=GSInfo[match(GSEAresults[[i]]$X[which(GSEAresults[[i]]$Adjusted.Pvalue<0.05) ], GSInfo$GeneSet)
Tnull1=unlist(sapply(Ctypes, function(x) grep(x, temp1$GrpA)))
Tnull2=unlist(sapply(Ctypes, function(x) grep(x, temp1$GrpB)))
TnullAll=intersect(Tnull1, Tnull2)
Tnull=c(as.character(temp1$GrpA[TnullAll]), as.character(temp1$GrpB[TnullAll]))
Cnames=c(Cnames, Tnull)
Ccomp=c(Ccomp, rep(i, length(Tnull)))
}

Tnull1=unlist(sapply(Ctypes, function(x) grep(x, GSInfo$GrpA)))
Tnull2=unlist(sapply(Ctypes, function(x) grep(x, GSInfo$GrpB)))
TnullAll=intersect(Tnull1, Tnull2)
Tnull=c(as.character(GSInfo$GrpA[TnullAll]), as.character(GSInfo$GrpB[TnullAll]))

Cnames=c(Cnames, Tnull)
Ccomp=c(Ccomp, rep(7, length(Tnull)))

X1=table(Ccomp, Cnames)
X1=X1/rowSums(X1)
rownames(X1)=c("DC/I", "DC/N", "IDC/N", "H/B", "S/O", "NP/PA", "NULL")

colVals=c("#023FA5","#7D87B9","#BEC1D4","#D6BCC0","#BB7784","#FFFFFF", "#4A6FE3","#8595E1","#B5BBE3","#
colVals=sample(colVals, 24)

barplot(t(X1), col=colVals, las=2)
```



Check for significance across the different cell types

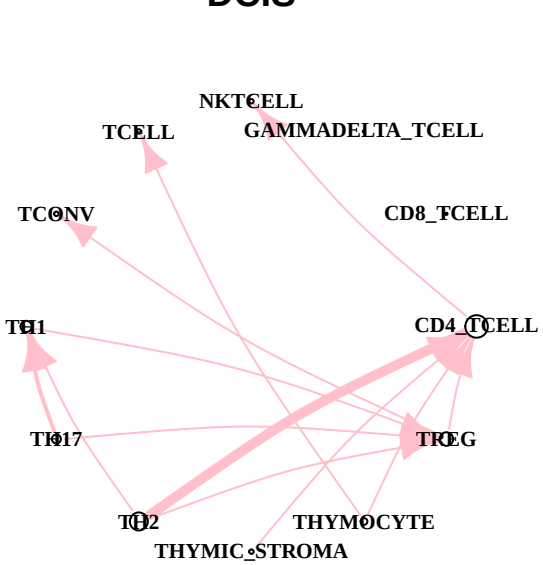
```
L1=table(Ccomp, Cnames)
rownames(L1)=c("DC/I", "DC/N", "IDC/N", "H/B", "S/O", "NP/PA", "NULL")

SigMat=matrix(NA, ncol=ncol(L1), nrow=(nrow(L1)-1))

for (i in 1:6){
  B=c(sum(L1[i, ]), sum(L1[7, ]))
  for (j in 1:ncol(L1)){
    A=c(L1[i,j], L1[7, j])
    SigMat[i,j]=prop.test(A, B)$p.value
  }
}

SigMat2=sapply(1:nrow(SigMat), function(x) p.adjust(SigMat[x, ]))
image(-log10(SigMat2), xaxt="n", yaxt="n", col=brewer.pal(9, "Reds"))
axis(1, at=seq(0, 1, length=ncol(L1)), labels=colnames(L1), cex=0.5, las=2)
axis(2, at=seq(0, 1, length=6), labels=rownames(L1)[1:6], cex=0.5)
```



We can also check the types of T cells enriched in these analyses: eg. for DCIS vs IDC

```
SearchTerms=c("TCELL", "TH1","TH2", "TREG", "TCONV", "THY")
a2=ActivationNetwork(temp$GrpA,temp$GrpB, GSEAresults[[2]]$Observed.score[which(GSEAresults$Adjusted.Pva
```

```
## Warning in Ops.factor(left, right): '<' not meaningful for factors
```




```
V(a2$graph)$size
```

```
##  [1] 10  1  1  2  2  2  5  4  8  2  3  6
```

Run the above analysis for all different test sets:

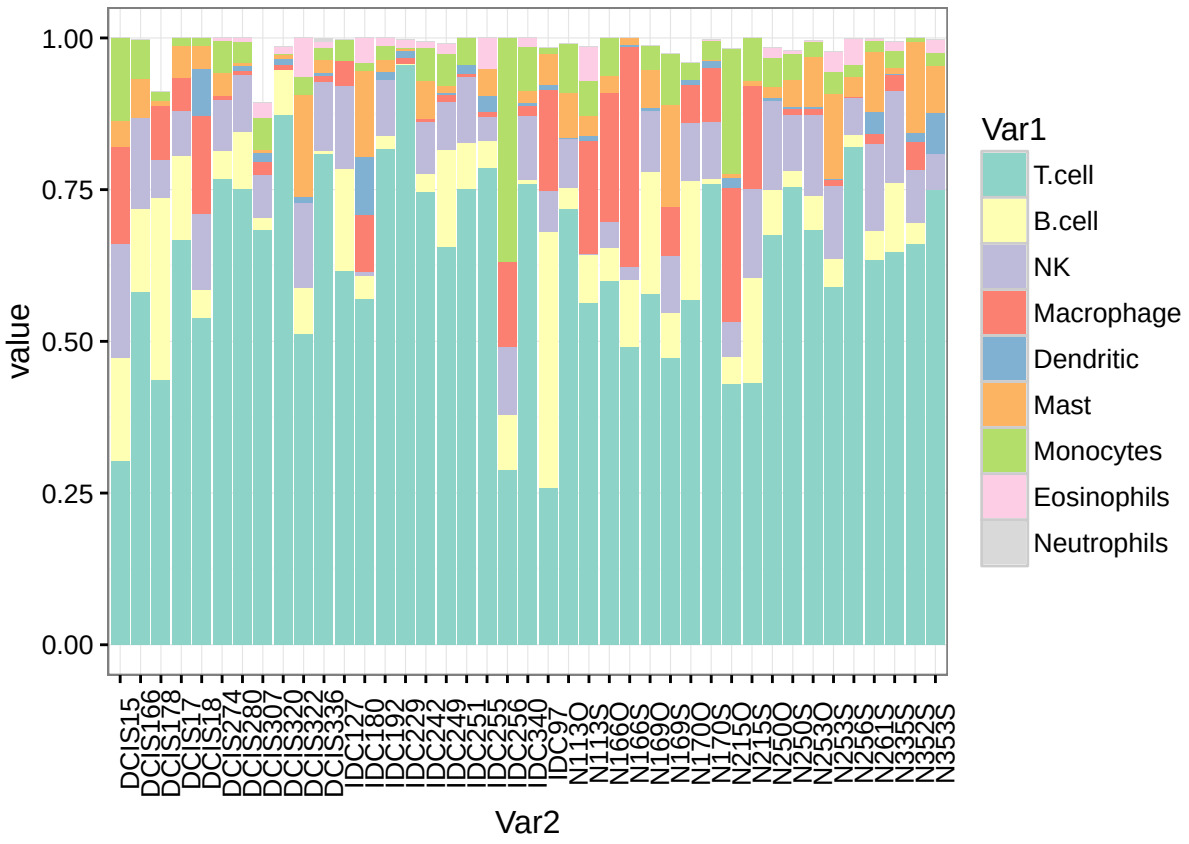
```
pdf("output/GSEA_network_maps.pdf")

CompA=c("DCISvsIDC", "DCISvsNormal", "IDCvsNormal", "HER2vsBasal", "Stroma_organoid.csv", "Parity_compa
#par(mfrow=c(2,2))
par(mfrow=c(1,2))

for (i in 1:length(CompA)){
  PCoff=which(GSEAresults[[i]]$Adjusted.Pvalue<PValCutOff)
  NID=GSEAresults[[i]]$X[PCoff]
  NIDmatch=match(NID, GSInfo$GeneSet)
  temp=GSInfo[NIDmatch, ]

ActivationNetwork(temp$StimA,temp$StimB, GSEAresults[[i]]$Observed.score[which(GSEAresults[[i]]$Adjuste

ActivationNetwork(temp$GrpA,temp$GrpB, GSEAresults[[i]]$Observed.score[which(GSEAresults[[i]]$Adjusted.
}

# Generate a scale matrix to show relative sizes:
m1=factor(c("A",rep("B",3), rep("C",5), rep("D",10), rep("E", 15)))
set.seed(10)
m2=factor(c(sample(c("B","C","D","E"), 1, replace=T), sample(c("C","D","E"), 3, replace=T),
     sample(c("E","D"), 5, replace=T), sample(c("B","C","E"), 10, replace=T),
  sample(c("D"), 15, replace=T)))

levels(m1)=c(1, 4, 11, 24, 20)
levels(m2)=c( 4, 11, 24, 20)
l1=cbind(A=as.factor(m1), B=factor(m2))

out1=ActivationNetwork(m1, m2, rep(1, length(m1)), "scale 1")

dev.off()
```

```
## pdf
##   2
```

4.4 CIBERSORT

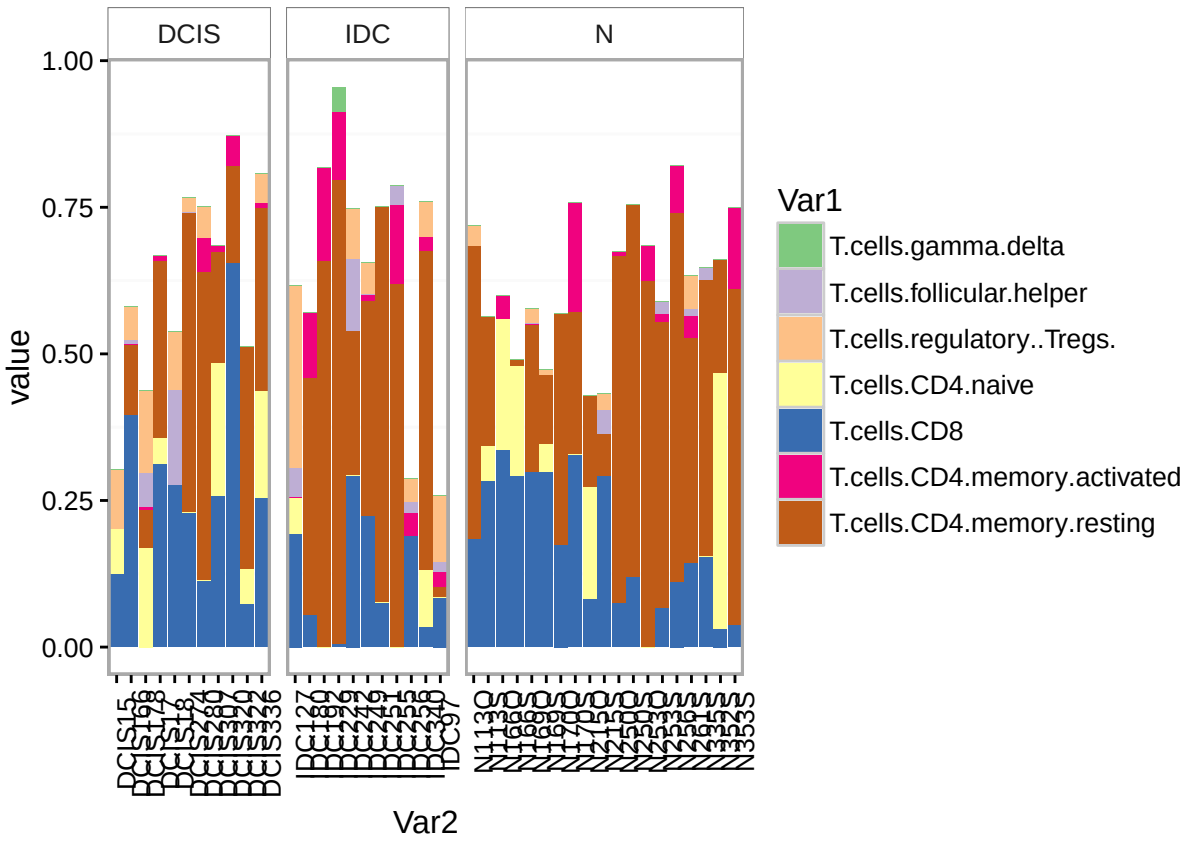
Use CIBERSORT to look at the estimated composition of each sample. This is a deconvolution method based on support-vector regression developed by Neumann et al, Nature Methods 2015 cibersort.stanford.edu.

```
Cres=read.csv("output/GEdata_CIBERSORT_100.csv", header=T, row.names=1)
CresDat=t(Cres[ ,-c(23:25)])

# do a plot of the different lineages, first grouping by cell type
CellList=c("T.cell","B.cell",  "NK","Macrophage", "Dendritic", "Mast")

CresComb=t(sapply(CellList, function(x) colSums(CresDat[grep(x, rownames(CresDat)),  ])))
CresComb=rbind(CresComb, CresDat[c("Monocytes", "Eosinophils", "Neutrophils") ,])

Cmelt=melt(CresComb)
ggplot(Cmelt, aes(x=Var2 , y=value, fill=Var1))+geom_bar(stat="identity")+theme_bw()+theme(axis.text.x =
```



Look at the specific T-cell compositions:

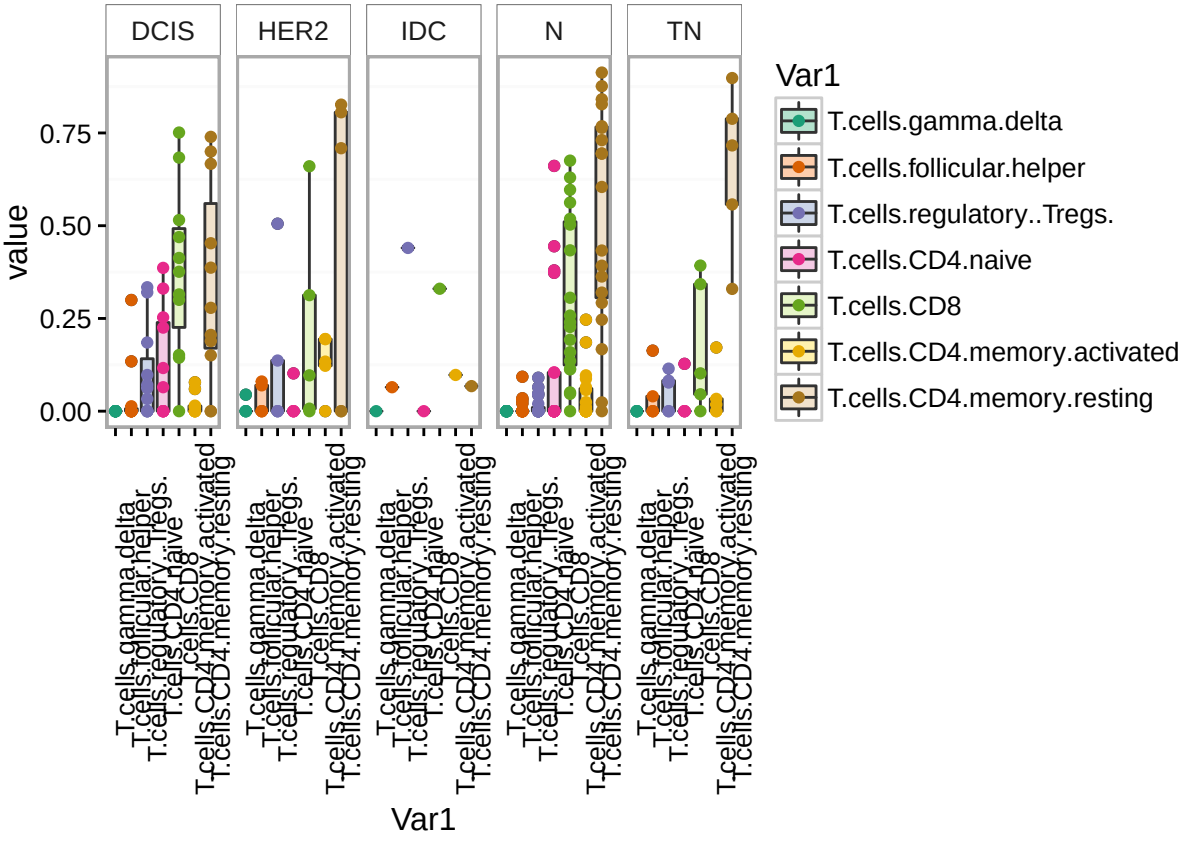
```
CresT=CresDat[grep("T.cell", rownames(CresDat)), ]
CresT2=melt(CresT)
CresT2$type="N"
CresT2$type[grep("DCIS", CresT2$Var2)]="DCIS"
CresT2$type[grep("IDC", CresT2$Var2)]="IDC"
CresT2$Var1 <- factor(CresT2$Var1, levels =
                        c("T.cells.gamma.delta","T.cells.follicular.helper","T.cells.regulatory..Tregs.

ggplot(CresT2, aes(x=Var2 , y=value, fill=Var1))+geom_bar(stat="identity")+theme_bw()+theme(axis.text.x
```



Rescale values to take into account T-cell specific content?

```
Cnames=gsub("\\.", "", rownames(SampleInfo))
Cnames=gsub("T[A-Z0-9]*$", "", Cnames)
l1=Cnames[SampleInfo$Subtype=="HER2"]
l2=Cnames[SampleInfo$Subtype=="TN"]
#l3=Cnames[SampleInfo$Subtype=="Org"]

CresTstd=t(t(CresT)/colSums(CresT))
CresT2std=melt(CresTstd)
CresT2std$type="N"
CresT2std$type[grep("DCIS", CresT2std$Var2)]="DCIS"
CresT2std$type[grep("IDC", CresT2std$Var2)]="IDC"

CresT2std$Var1 <- factor(CresT2std$Var1, levels =
                        c("T.cells.gamma.delta","T.cells.follicular.helper","T.cells.regulatory..Tregs.

#ggplot(CresT2std, aes(x=Var2 , y=value, fill=Var1))+geom_bar(stat="identity")+theme(axis.text.x = elem
```

```
CresT2std$type[which(CresT2std$Var2%in%l2)]="TN"
CresT2std$type[which(CresT2std$Var2%in%l1)]="HER2"
#CresT2std$type[which(CresT2std$Var2%in%l3)]="Org"
ggplot(CresT2std, aes(x=Var1 , y=value, fill=Var1))+geom_boxplot(stat="boxplot")+geom_point(aes(colour =
```



5 Cell activity: exhaustion, cytotoxic signatures: Figures S3, S5

Can the gene expression patterns tell us any information on the "activity" of the T-cells

5.1 Existing Signatures

Existing signatures have been shown in Tirosh 2016, and discussed in Pardell 2015. Here, we compare the expression of different samples

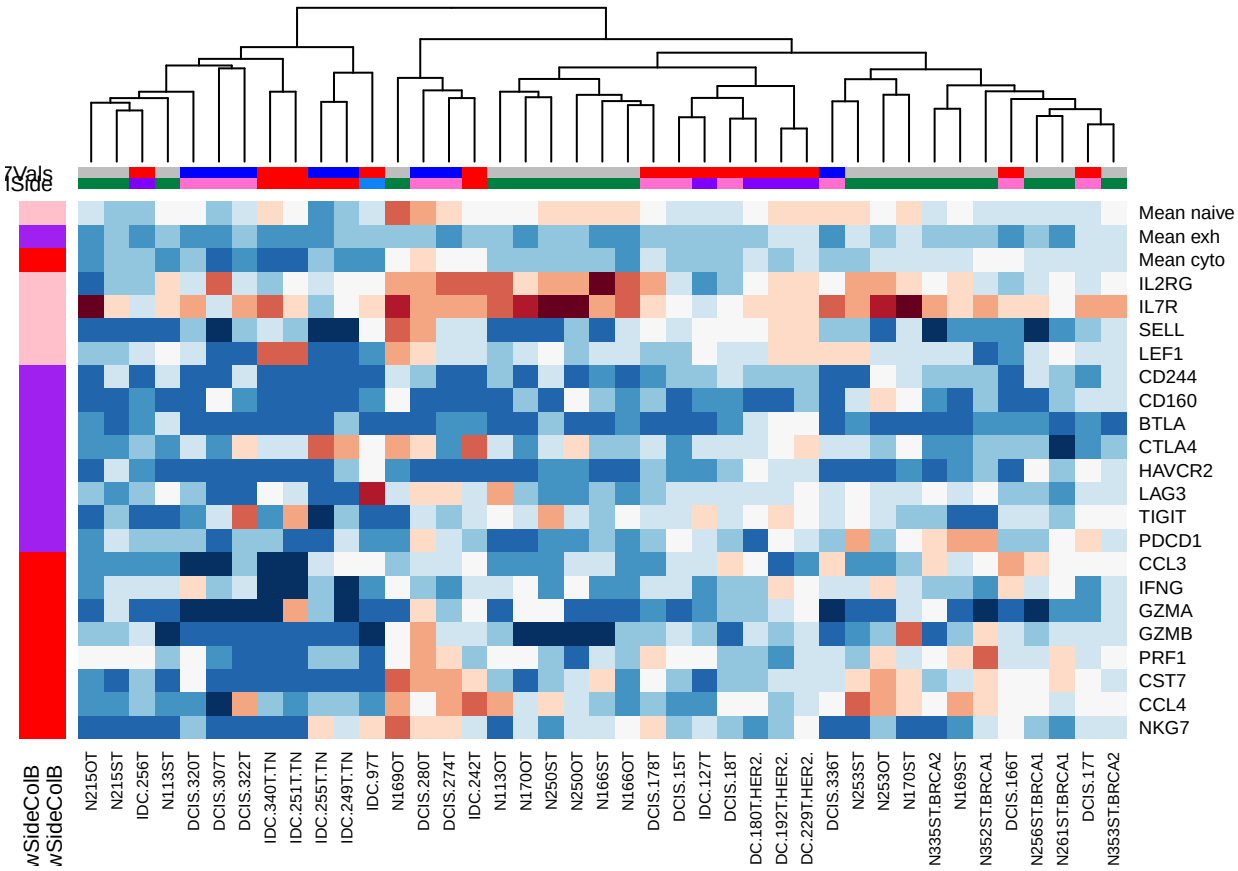
Firstly, we colour according to Ki67 expression:

```
Ki67Vals=rep("blue", length(SampC))
Ki67Vals[which(ddsCount2["MKI67", ]>0.5)]="red"
Ki67Vals[grep("^N", colnames(logCPM))]="grey"
#colnames(logCPM)
#SampC
# centre the gene expression at 0
logCPMNew=sapply(1:ncol(logCPM), function(x) (logCPM[ ,x]-median(logCPM[ ,x])))
```

5.1.1 Tirsoh 2016

```
# Find the cytotoxic genes:
CytoVals=logCPM[GeneListAct$Cytotoxic, ]
ExhVals=logCPM[GeneListAct$Exhausted, ]
```

```
NaiVals=logCPM[GeneListAct$Naive, ]

TMarkers=logCPM[GeneListAct$TCellSpec, ]

MeanVals=rbind(colMeans(CytoVals), colMeans(ExhVals), colMeans(NaiVals) )
rownames(MeanVals)=c("Mean cyto", "Mean exh", "Mean naive")
XTab=rbind(CytoVals, ExhVals, NaiVals, MeanVals)

RowSideColB=c(rep("red", nrow(CytoVals)), rep("purple", nrow(ExhVals)), rep("pink", nrow(NaiVals)), c("

a1=heatmap.plus(rbind(CytoVals,ExhVals, NaiVals, MeanVals), col=HMPallete[11:1], ColSideColors = cbind(
```

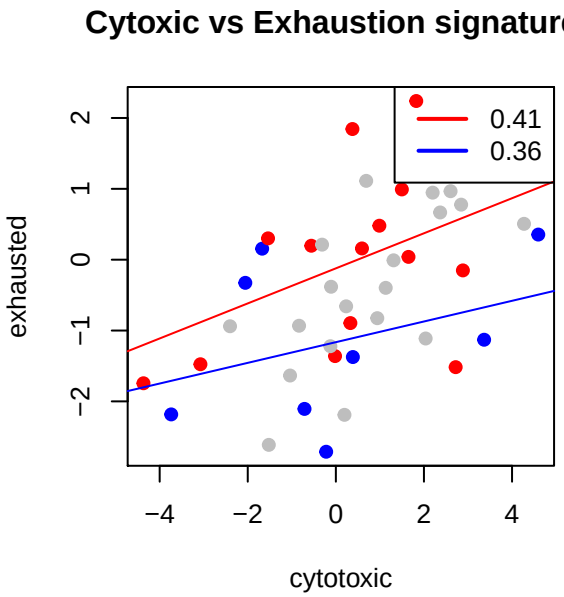


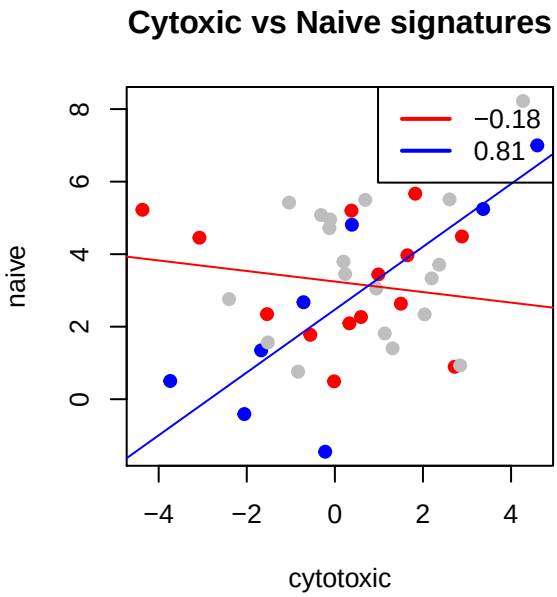
Comparison of exhaustion with cytoxic:

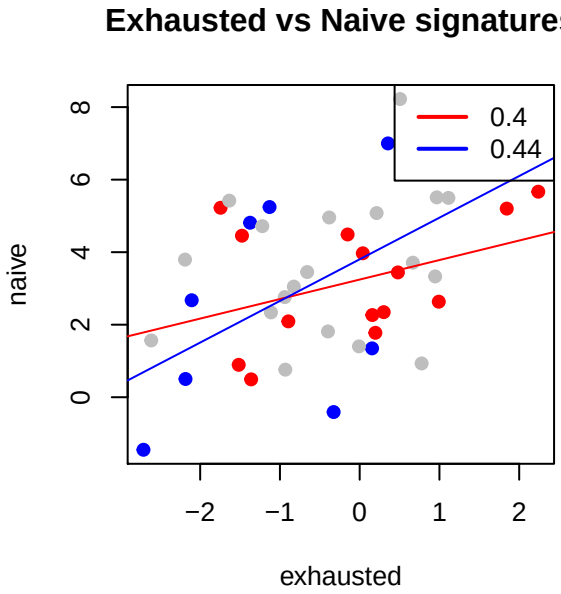
Follow the methods described by Tirosh: compute the average expression of the exhaustion or cytoxtic gene exp and subtract from the naive cells:

```
#MeanV2=t(t(MeanVals)-MeanVals[3, ])
par(mfrow=c(2,2))
plot(MeanVals[1, ], MeanVals[2, ], xlab="cytotoxic", ylab="exhausted", main="Cytoxic vs Exhaustion signa
a1=lm(MeanVals[2,which(Ki67Vals=="red") ]~MeanVals[1, which(Ki67Vals=="red")])
cValR=cor(MeanVals[2,which(Ki67Vals=="red") ], MeanVals[1, which(Ki67Vals=="red")])
abline(a1$coefficients, col="red")
a1=lm(MeanVals[2,which(Ki67Vals=="blue") ]~MeanVals[1, which(Ki67Vals=="blue")])
cValB=cor(MeanVals[2,which(Ki67Vals=="blue") ], MeanVals[1, which(Ki67Vals=="blue")])
abline(a1$coefficients, col="blue")
legend("topright", as.character(c(round(cValR*100)/100, round(cValB*100)/100)), col=c("red", "blue"), lw
```

```
plot(MeanVals[1, ], MeanVals[3, ], xlab="cytotoxic", ylab="naive", main="Cytoxic vs Naive signatures", p
a1=lm(MeanVals[3,which(Ki67Vals=="red") ]~MeanVals[1, which(Ki67Vals=="red")])
cValR=cor(MeanVals[3,which(Ki67Vals=="red") ], MeanVals[1, which(Ki67Vals=="red")])
abline(a1$coefficients, col="red")
a1=lm(MeanVals[3,which(Ki67Vals=="blue") ]~MeanVals[1, which(Ki67Vals=="blue")])
cValB=cor(MeanVals[3,which(Ki67Vals=="blue") ], MeanVals[1, which(Ki67Vals=="blue")])
abline(a1$coefficients, col="blue")
legend("topright", as.character(c(round(cValR*100)/100, round(cValB*100)/100)), col=c("red", "blue"), lw

plot(MeanVals[2, ], MeanVals[3, ], xlab="exhausted", ylab="naive", main="Exhausted vs Naive signatures"
a1=lm(MeanVals[3,which(Ki67Vals=="red") ]~MeanVals[2, which(Ki67Vals=="red")])
cValR=cor(MeanVals[3,which(Ki67Vals=="red") ], MeanVals[2, which(Ki67Vals=="red")])
abline(a1$coefficients, col="red")
a1=lm(MeanVals[3,which(Ki67Vals=="blue") ]~MeanVals[2, which(Ki67Vals=="blue")])
cValB=cor(MeanVals[3,which(Ki67Vals=="blue") ], MeanVals[2, which(Ki67Vals=="blue")])
abline(a1$coefficients, col="blue")
legend("topright", as.character(c(round(cValR*100)/100, round(cValB*100)/100)), col=c("red", "blue"), lw
```







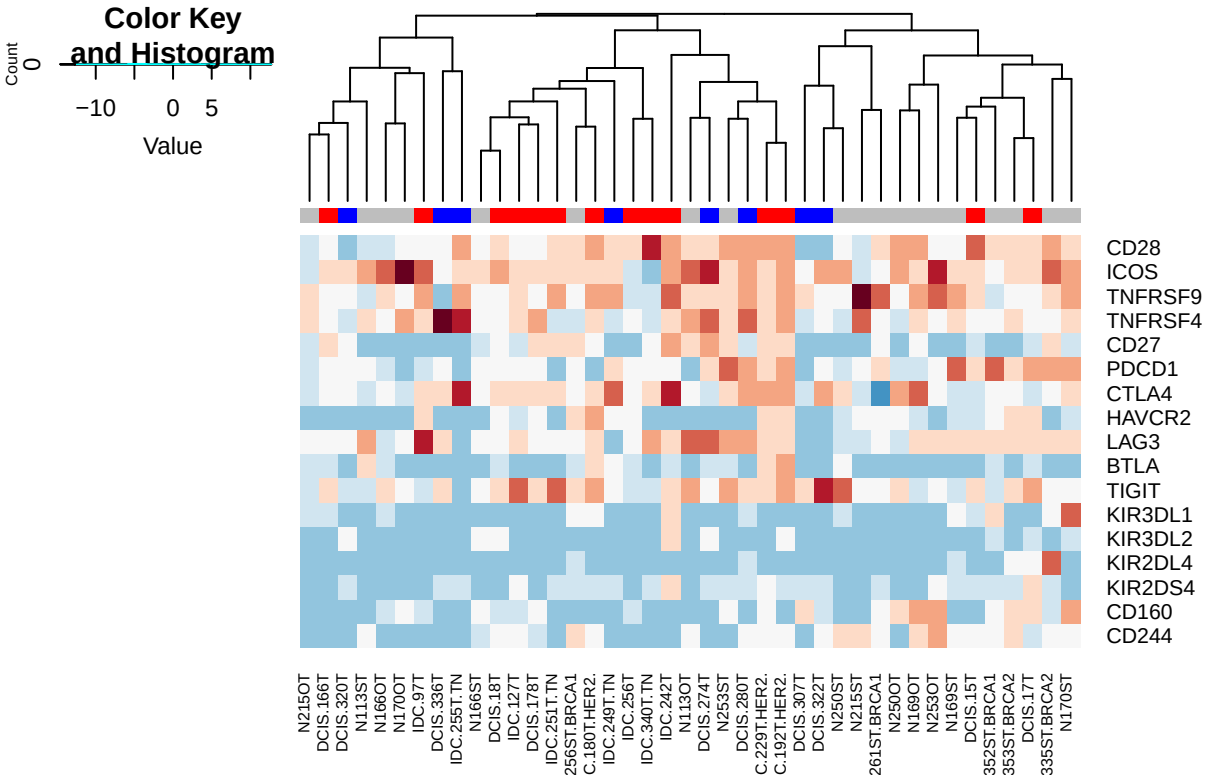
5.1.2 Check-point genes

Pardell has discussed genes involved in T-cell check points: These have directionality - they can either activate a T-cell or inhibit its activity

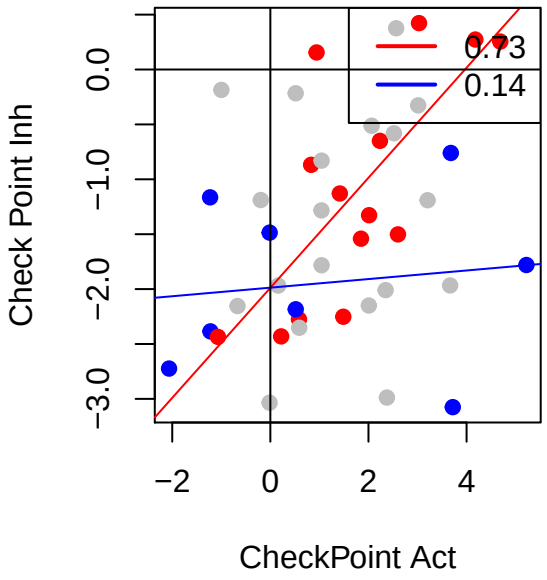
```
glCD=rownames(logCPM)[grep("^CD3[A-Z]$", rownames(logCPM))]

dirnew=c(rep("green", length(GeneListAct$Activated)),
         rep("blue", length(GeneListAct$Inhibit)))

CCVals=logCPM[c(GeneListAct$Activated, GeneListAct$Inhibit),  ]

MeanValsCC=rbind(colMeans(logCPM[GeneListAct$Activated,]),
```

```
                colMeans(logCPM[GeneListAct$Inhibit,]),
                colMeans(logCPM[glCD, ]))
rownames(MeanValsCC)=c("checkUp", "checkDown", "CD")

a1=heatmap.2(CCVals, col=HMPallete[11:1], ColSideColors = Ki67Vals, scale="none",  Rowv=NA, trace="none
```

```
## Warning in heatmap.2(CCVals, col = HMPallete[11:1], ColSideColors =
## Ki67Vals, : Discrepancy: Rowv is FALSE, while dendrogram is `both'.
## Omitting row dendogram.
```

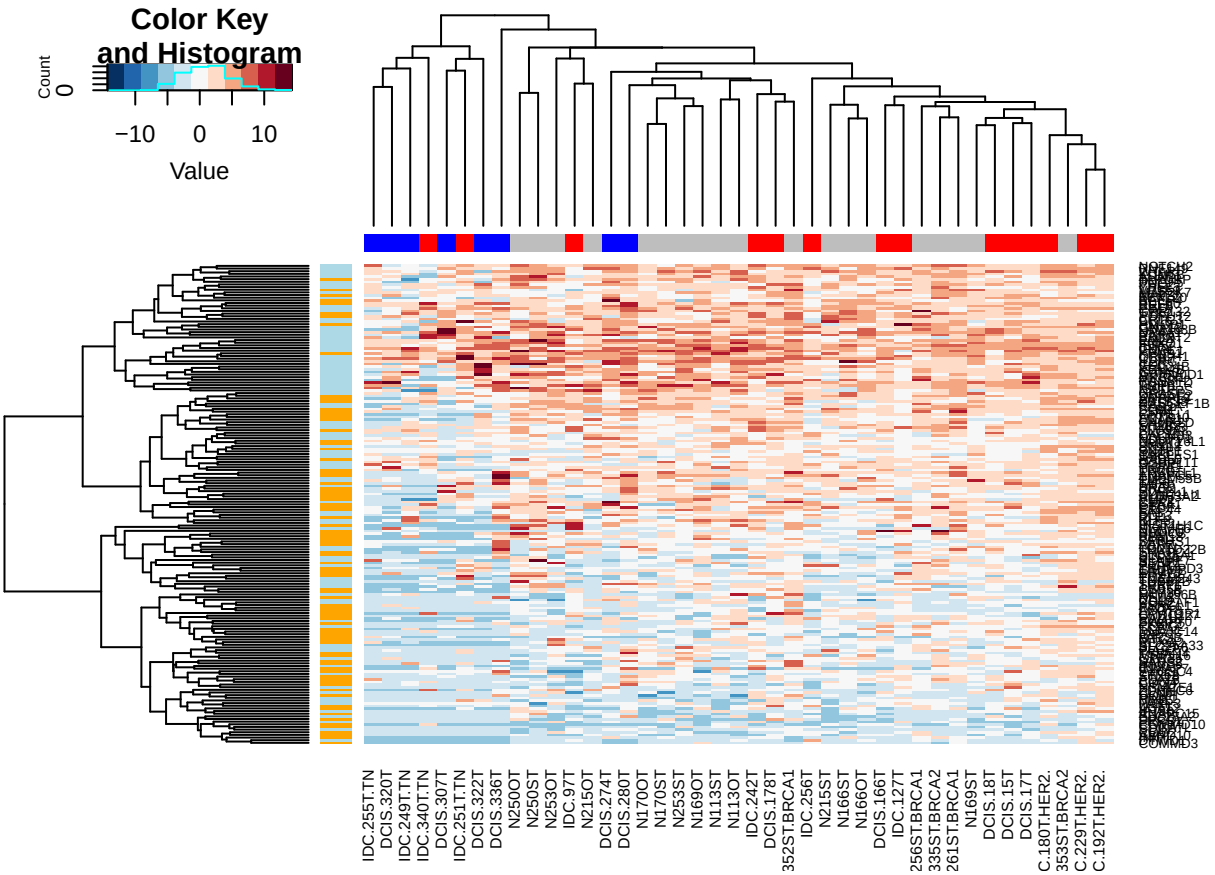



```
par(mfrow=c(1,2))
plot(MeanValsCC[1, ], MeanValsCC[2, ], pch=19, col=Ki67Vals, xlab="CheckPoint Act", ylab="Check Point In
a1=lm(MeanValsCC[2,which(Ki67Vals=="red") ]~MeanValsCC[1, which(Ki67Vals=="red")])
cValR=cor(MeanValsCC[2,which(Ki67Vals=="red") ], MeanValsCC[1, which(Ki67Vals=="red")])
abline(a1$coefficients, col="red")
a1=lm(MeanValsCC[2,which(Ki67Vals=="blue") ]~MeanValsCC[1, which(Ki67Vals=="blue")])
cValB=cor(MeanValsCC[2,which(Ki67Vals=="blue") ], MeanValsCC[1, which(Ki67Vals=="blue")])
abline(a1$coefficients, col="blue")
legend("topright", as.character(c(round(cValR*100)/100, round(cValB*100)/100)), col=c("red", "blue"), lw
abline(h=0, v=0)
```



5.1.3 Singer Signature

```
CCVals=logCPM[na.omit(match(c(GeneListAct$Activation, GeneListAct$Dysfunction), rownames(logCPM))),  ]
RSC=c(rep("orange", sum(GeneListAct$Activation%in%rownames(logCPM))), rep("lightblue", sum(GeneListAct$I
a1=heatmap.2(CCVals, col=HMPallete[11:1], ColSideColors = Ki67Vals, RowSideColors = RSC, scale="none",
```



5.2 Analysis of TIGIT staining the CD3+ cells

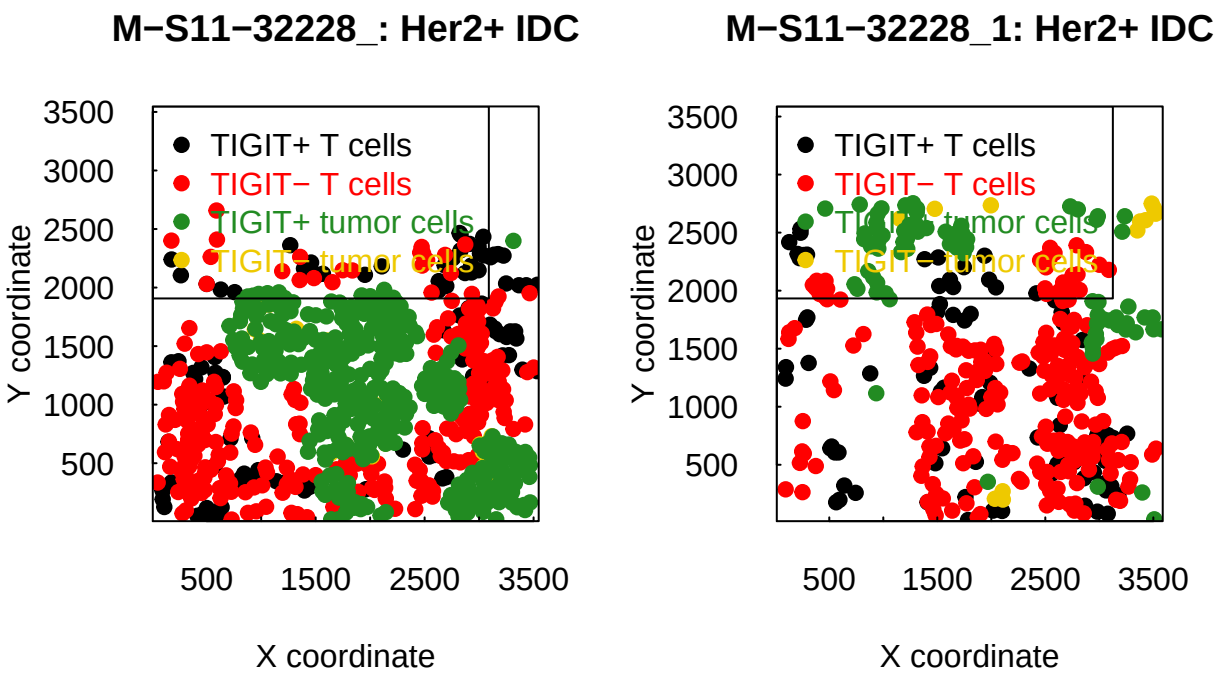
```
tigit <- read.csv('data/TIGIT.csv', stringsAsFactors = FALSE, header = FALSE)[-1, -1]
tigit.indices <- NULL
tigit.cells <- NULL
for (i in 1:nrow(tigit)) {
    for (j in 1:ncol(tigit)) {
        if (is.na(pmatch('M-', tigit[i, j])) == FALSE) {
            tigit.indices <- rbind(tigit.indices, c(i, j))
        }
        if (is.na(pmatch('Her2', tigit[i, j])) == FALSE | is.na(pmatch('HER2', tigit[i, j])) == FALSE |
            tigit.cells <- rbind(tigit.cells, c(tigit[i, j], i, j))
        }
    }
}
tigit.cells <- as.data.frame(data.matrix(tigit.cells), stringsAsFactors = FALSE)

tigit.sample.names <- tigit[tigit.indices]
unique.rows <- c(unique(tigit.indices[, 1]), nrow(tigit) + 1)
tigit.samples <- NULL
for (i in 1:(nrow(tigit.indices))) {
    temp <- tigit[(tigit.indices[i, 1] + 1):(unique.rows[which(unique.rows == tigit.indices[i, 1]) + 1]
    temp.end <- which(temp[, 1] == '')[1]
    if (is.na(temp.end) == FALSE) {
        temp <- temp[1:(temp.end - 1), ]
    }
    temp.col <- temp[1, ]
    temp <- as.data.frame(data.matrix(temp[-1, ]))
    colnames(temp) <- temp.col
    tigit.samples[[i]] <- list()
    tigit.samples[[i]]$table <- temp
    tigit.samples[[i]]$cell <- tigit.cells[tail(which(as.numeric(tigit.cells$V2) <= tigit.indices[i, 1]]
}
Types.tigit <- c('TIGIT+ T cells', 'TIGIT- T cells', 'TIGIT+ tumor cells', 'TIGIT- tumor cells')
Types.tigit.indices <- c(1, 2, 7, 5)
Cols <- c('black', 'red', 'forestgreen', 'gold2')
```

Here, the spatial information from IF is captured, highlighting the different cell populations:

- black: TIGIT + CD3+ TCells
- red: TIGIT- CD3+Tcells
- green: TIGIT+ tumour cells
- yellow: TIGIT - tumour cells

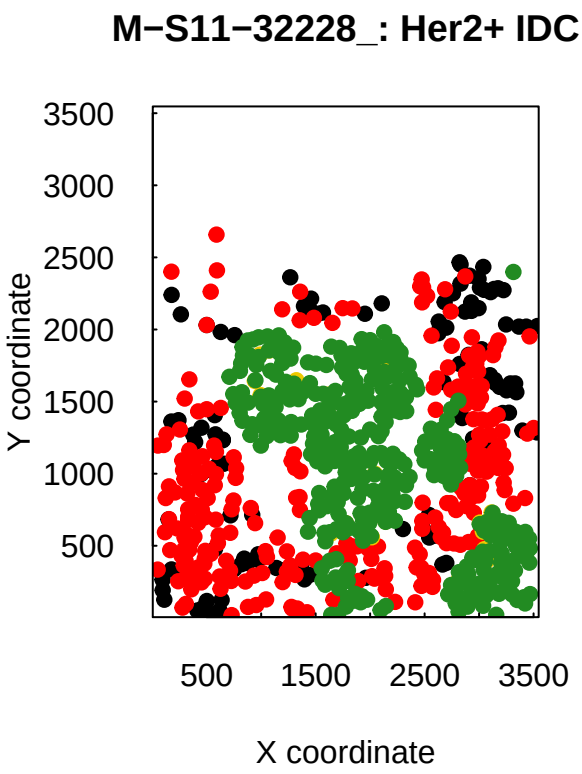
As an example:

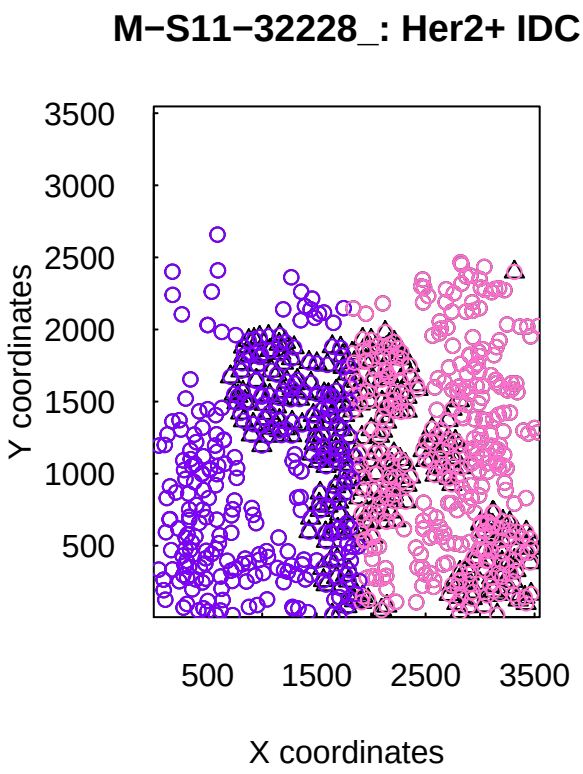
```
## plot TIGIT CD3 positive cells
#pdf('TIGIT CD3.pdf', useDingbats = FALSE)
par(mfrow = c(1, 2), las = 1, tcl = .1, xaxs = 'i', yaxs = 'i')
for (i in 1:2) {
  # change 1 to length(tigit.samples)
    plot(tigit.samples[[i]]$table$X[tigit.samples[[i]]$table$Type %in% Types.tigit.indices], tigit.sampl
    legend('topleft', Types.tigit, col = Cols, text.col = Cols, pch = 19)
}
```

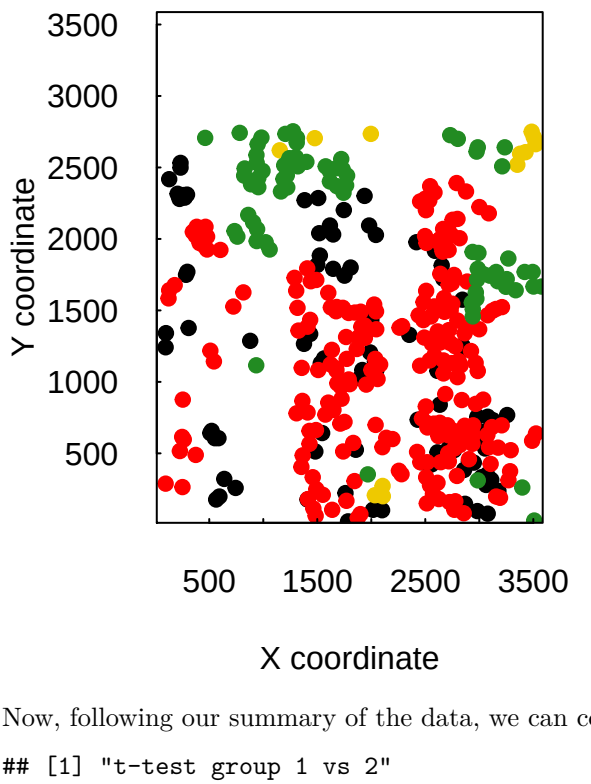


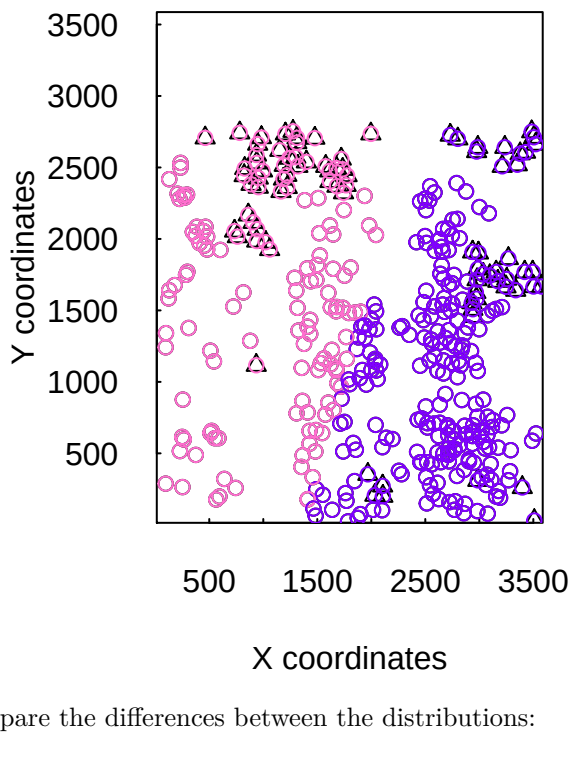
Here, we now analyse the spatial distributions between the different samples: Use k means to using a spatial uniform Poisson process and MC simulation to generate spatial distributions under the null hypothesis (that cells are random) and compared them to our images using the cross K function.

```
set.seed(1234)
distance.tigit <- NULL
par(mfrow = c(1, 2), las = 1, tcl = .1, xaxs = 'i', yaxs = 'i')
for (i in 1:length(tigit.samples)){
    neg.T <- as.data.frame(cbind(tigit.samples[[i]]$table$X[tigit.samples[[i]]$table$Type == 2], tigit.s
    pos.T <- as.data.frame(cbind(tigit.samples[[i]]$table$X[tigit.samples[[i]]$table$Type == 1], tigit.s
    neg.C <- as.data.frame(cbind(tigit.samples[[i]]$table$X[tigit.samples[[i]]$table$Type == 5], tigit.s
    pos.C <- as.data.frame(cbind(tigit.samples[[i]]$table$X[tigit.samples[[i]]$table$Type == 7], tigit.s
    T <- as.data.frame(cbind(tigit.samples[[i]]$table$X[tigit.samples[[i]]$table$Type == 2 | tigit.sampl
    C <- as.data.frame(cbind(tigit.samples[[i]]$table$X[tigit.samples[[i]]$table$Type == 5 | tigit.sampl
    colnames(neg.T) <- colnames(pos.T) <- colnames(neg.C) <- colnames(pos.C) <- colnames(T) <- colnames
    TC <- rbind(T, C)
    km.TC <- kmeans(TC, centers = 2)
    if(i%in%c(1,2)){
    plot(tigit.samples[[i]]$table$X[tigit.samples[[i]]$table$Type %in% Types.tigit.indices], tigit.sampl
    plot(T$x, T$y, pch = 1, main = paste0(tigit.sample.names[i], ': ', tigit.samples[[i]]$cell), xlim =
    points(C$x, C$y, pch = 2)
    points(TC$x, TC$y, col = km.TC$cluster)
    }
    distance.tigit <- rbind(distance.tigit, c(max(length(which(km.TC$cluster[1:nrow(T)] == 1)) / nrow(T
}
```









Now, following our summary of the data, we can compare the differences between the distributions:

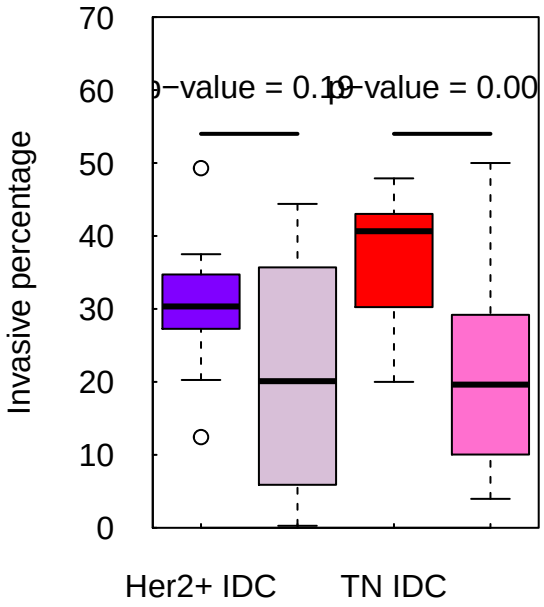
```
## [1] "t-test group 1 vs 2"
```

```
##
##  Welch Two Sample t-test
```

```
##
## data:  distance.tigit[distance.tigit[, 2] < 3, 1] by distance.tigit[distance.tigit[, 2] < 3, 2]
## t = -1.4277, df = 9.0024, p-value = 0.1871
## alternative hypothesis: true difference in means is not equal to 0
## 95 percent confidence interval:
##  -0.24227399  0.05478719
## sample estimates:
## mean in group 1 mean in group 2
##       0.6962007       0.7899441
```

```
## [1] "t-test group 3 vs 4"
```

```
##
##  Welch Two Sample t-test
##
## data:  distance.tigit[distance.tigit[, 2] > 2, 1] by distance.tigit[distance.tigit[, 2] > 2, 2]
## t = -3.177, df = 15.486, p-value = 0.00605
## alternative hypothesis: true difference in means is not equal to 0
## 95 percent confidence interval:
##  -0.25480224 -0.05051996
## sample estimates:
## mean in group 3 mean in group 4
##       0.6365692       0.7892303
```



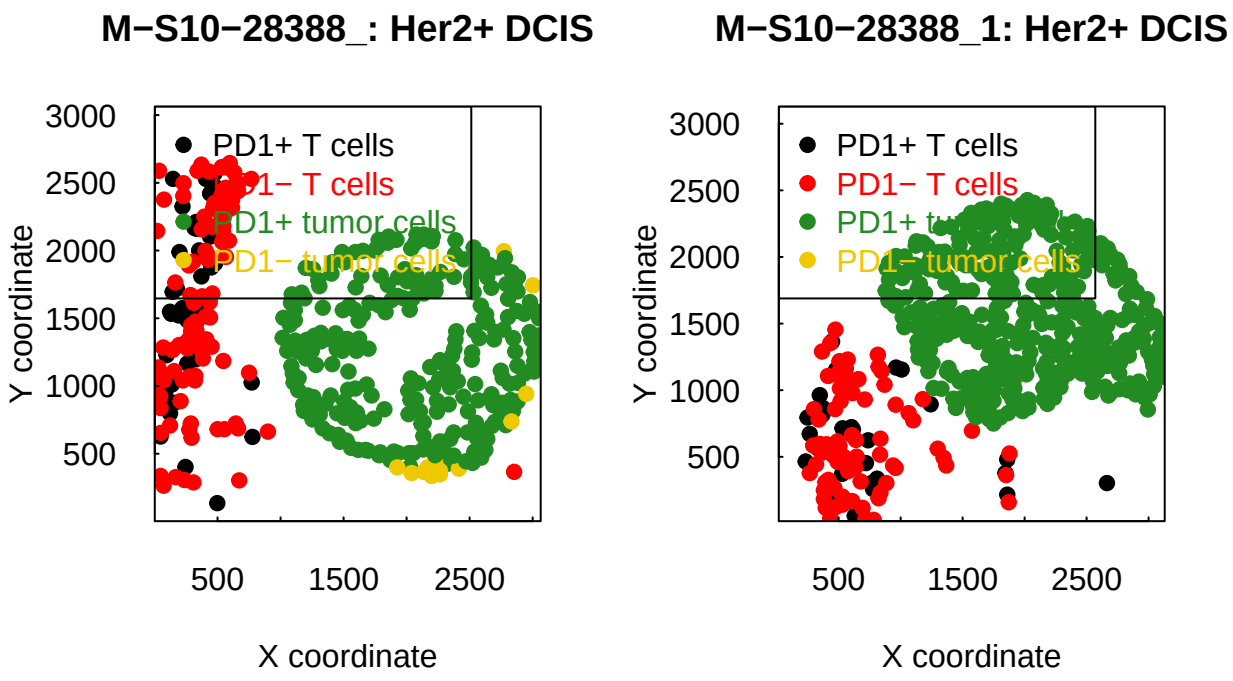
5.3 PD1 staining

Do the same analysis for PD1 staining:

```
pd1 <- read.csv('data/PD1.csv', stringsAsFactors = FALSE, header = FALSE)[-1, -1]
pd1.indices <- NULL
pd1.cells <- NULL
for (i in 1:nrow(pd1)) {
    for (j in 1:ncol(pd1)) {
        if (is.na(pmatch('M-', pd1[i, j])) == FALSE) {
            pd1.indices <- rbind(pd1.indices, c(i, j))
```

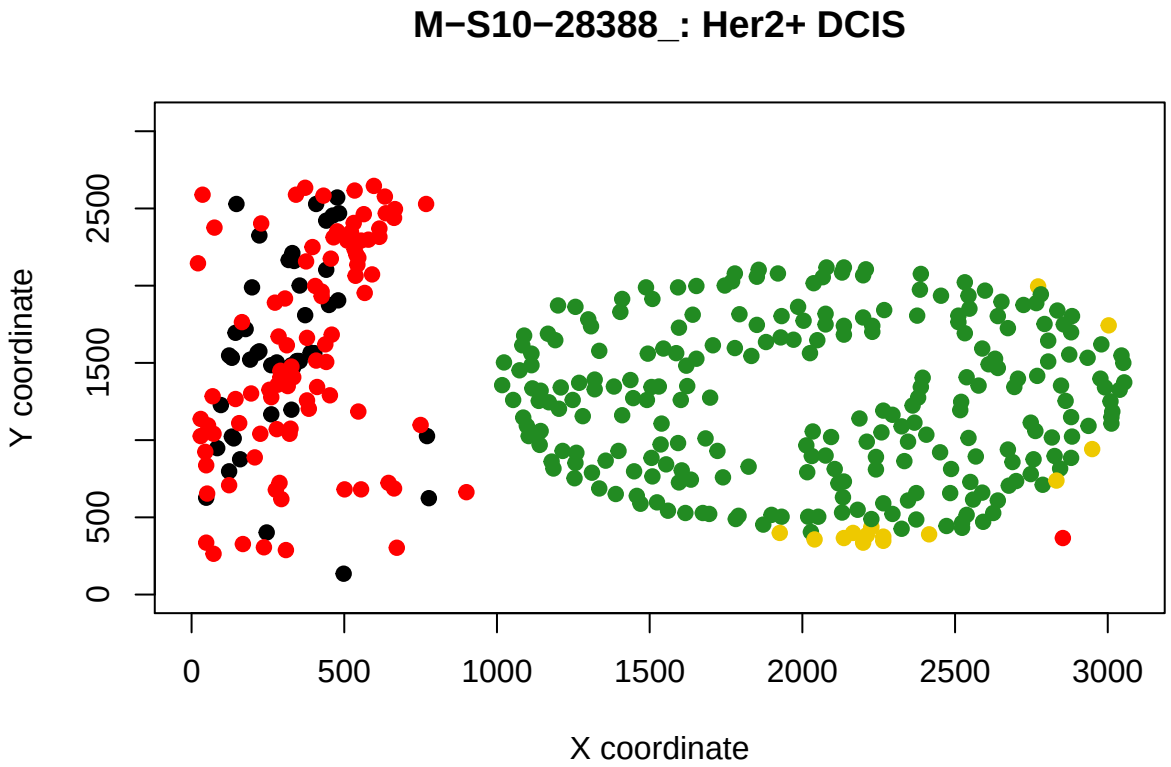
```
        }
        if (is.na(pmatch('Her2', pd1[i, j])) == FALSE | is.na(pmatch('HER2', pd1[i, j])) == FALSE | is.
            pd1.cells <- rbind(pd1.cells, c(pd1[i, j], i, j))
        }
    }
}
pd1.cells <- as.data.frame(data.matrix(pd1.cells), stringsAsFactors = FALSE)
pd1.cells$V1[1] <- "Her2+ DCIS"
pd1.cells$V1[5] <- 'Her2+ DCIS'

pd1.sample.names <- pd1[pd1.indices]
unique.rows <- c(unique(pd1.indices[, 1]), nrow(pd1) + 1)
pd1.samples <- NULL
for (i in 1:(nrow(pd1.indices))) {
    temp <- pd1[(pd1.indices[i, 1] + 1):(unique.rows[which(unique.rows == pd1.indices[i, 1]) + 1] - 1),
    temp.end <- which(temp[, 1] == '')[1]
    if (is.na(temp.end) == FALSE) {
        temp <- temp[1:(temp.end - 1), ]
    }
    temp.col <- temp[1, ]
    temp <- as.data.frame(data.matrix(temp[-1, ]))
    colnames(temp) <- temp.col
    pd1.samples[[i]] <- list()
    pd1.samples[[i]]$table <- temp
    pd1.samples[[i]]$cell <- pd1.cells[tail(which(as.numeric(pd1.cells$V2) <= pd1.indices[i, 1]), n = 1
}
Types.pd1 <- c('PD1+ T cells', 'PD1- T cells', 'PD1+ tumor cells', 'PD1- tumor cells')
Types.pd1.indices <- c(1, 2, 3, 4)
Cols <- c('black', 'red', 'forestgreen', 'gold2')
```

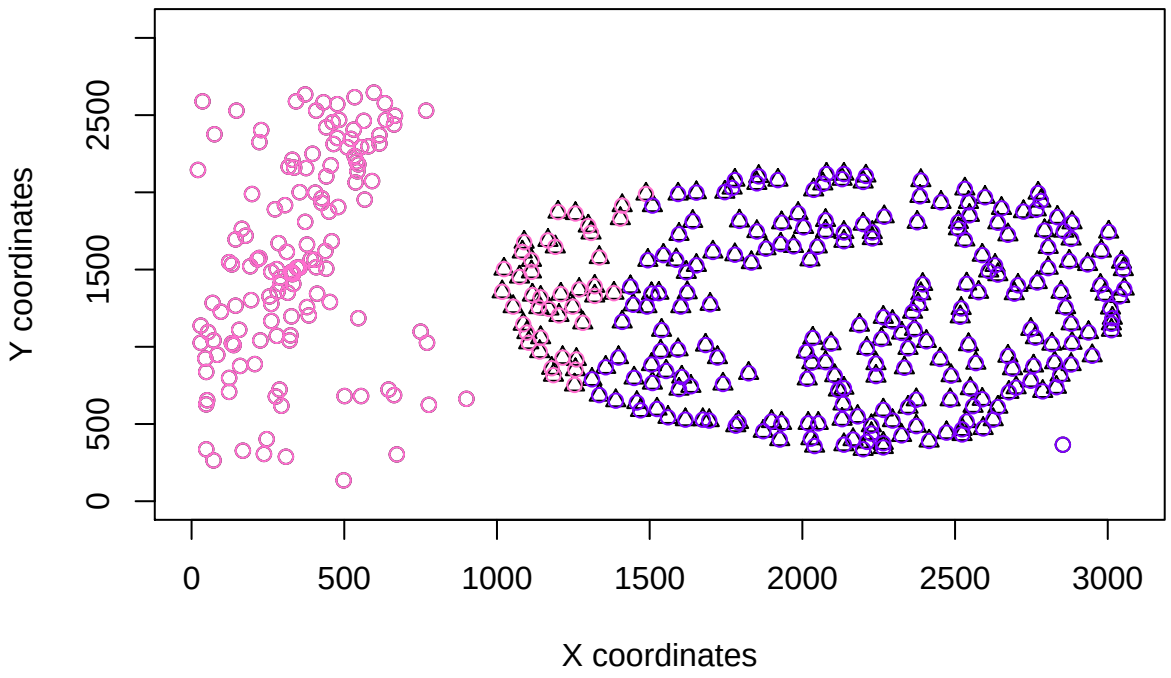
```
## plot pd1 CD3 positive cells
#pdf('pd1 CD3.pdf', useDingbats = FALSE)
par(mfrow = c(1, 2), las = 1, tcl = .1, xaxs = 'i', yaxs = 'i')
for (i in 1:2) {
  # change 1 to length(pd1.samples)
    plot(pd1.samples[[i]]$table$X[pd1.samples[[i]]$table$Type %in% Types.pd1.indices], pd1.samples[[i]]$
    legend('topleft', Types.pd1, col = Cols, text.col = Cols, pch = 19)
}
```



Here, we now analyse the spatial distributions between the different samples: Use k means to using a spatial uniform Poisson process and MC simulation to generate spatial distributions under the null hypothesis (that cells are random) and compared them to our images using the cross K function.

```
set.seed(1234)
distance.pd1 <- NULL
for (i in 1:length(pd1.samples)) {
    neg.T <- as.data.frame(cbind(pd1.samples[[i]]$table$X[pd1.samples[[i]]$table$Type == 2], pd1.samples
    pos.T <- as.data.frame(cbind(pd1.samples[[i]]$table$X[pd1.samples[[i]]$table$Type == 1], pd1.samples
    neg.C <- as.data.frame(cbind(pd1.samples[[i]]$table$X[pd1.samples[[i]]$table$Type == 4], pd1.samples
    pos.C <- as.data.frame(cbind(pd1.samples[[i]]$table$X[pd1.samples[[i]]$table$Type == 3], pd1.samples
    T <- as.data.frame(cbind(pd1.samples[[i]]$table$X[pd1.samples[[i]]$table$Type == 2 | pd1.samples[[i]
    C <- as.data.frame(cbind(pd1.samples[[i]]$table$X[pd1.samples[[i]]$table$Type == 4 | pd1.samples[[i]
    colnames(neg.T) <- colnames(pos.T) <- colnames(neg.C) <- colnames(pos.C) <- colnames(T) <- colnames
    TC <- rbind(T, C)

    if (i==1){
    plot(pd1.samples[[i]]$table$X[pd1.samples[[i]]$table$Type %in% Types.pd1.indices], pd1.samples[[i]]$
    }
    km.TC <- kmeans(TC, centers = 2)
    if (i==1){
    plot(T$x, T$y, pch = 1, main = paste0(pd1.sample.names[i], ': ', pd1.samples[[i]]$cell), xlim = c(mi
    points(C$x, C$y, pch = 2)
    points(TC$x, TC$y, col = km.TC$cluster)
    }
    distance.pd1 <- rbind(distance.pd1, c(max(length(which(km.TC$cluster[1:nrow(T)] == 1)) / nrow(T), le
}
```





Now, following our summary of the data, we can compare the differences between the distributions:

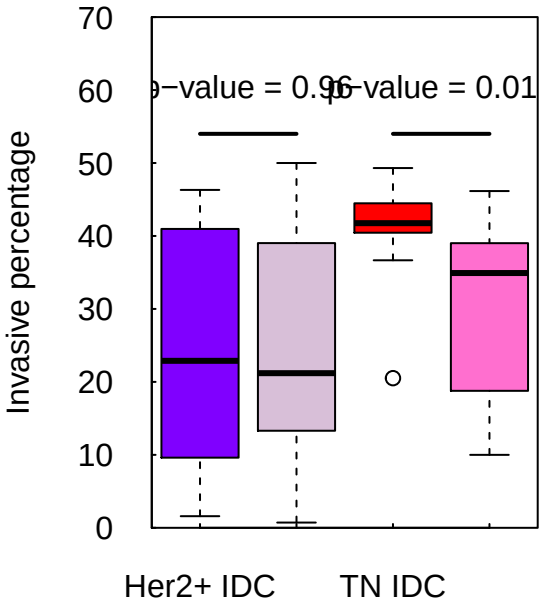
```
## [1] "t-test group 1 vs 2"
```

```
##
##  Welch Two Sample t-test
```

```
##
## data:  distance.pd1[distance.pd1[, 2] < 3, 1] by distance.pd1[distance.pd1[, 2] < 3, 2]
## t = 0.050595, df = 25.583, p-value = 0.96
## alternative hypothesis: true difference in means is not equal to 0
## 95 percent confidence interval:
##  -0.1243100  0.1305789
## sample estimates:
## mean in group 1 mean in group 2
##       0.7674002       0.7642658
```

```
## [1] "t-test group 3 vs 4"
```

```
##
##  Welch Two Sample t-test
##
## data:  distance.pd1[distance.pd1[, 2] > 2, 1] by distance.pd1[distance.pd1[, 2] > 2, 2]
## t = 2.7765, df = 20.24, p-value = 0.01156
## alternative hypothesis: true difference in means is not equal to 0
## 95 percent confidence interval:
##  0.02738358 0.19232719
## sample estimates:
## mean in group 3 mean in group 4
##       0.6996395       0.5897841
```



6 TCR clonality

TCR clonality was determined using mixcr (Bolotin, Nat Methods 2015, https://mixcr.readthedocs.io). RNA-seq reads were aligned to a known database of TCR sequences, and are loaded here:

```
fileList <- list.files('output/mixcr/', '.clones.TCR.txt')

combinedDF <- data.frame(stringsAsFactors = FALSE)
droppedSamples <- character()
```

```
for (i in 1:length(fileList))
{
  if (length(readLines(paste('output/mixcr/', fileList[i], sep = ''))) > 0)
  {
    currentDF <- read.table(paste('output/mixcr/', fileList[i], sep = ''), sep = '\t', header = 1, stri
    currentDF <- cbind(Sample = rep(strsplit(fileList[i], '.clones.TCR.txt')[[1]], nrow(currentDF)), cu
    if (nrow(combinedDF) == 0)
    {
      combinedDF <- currentDF
    }
    else
    {
      combinedDF <- rbind(combinedDF, currentDF)
    }
    rm(currentDF)
  }
  else
  {
    droppedSamples <- c(droppedSamples, strsplit(fileList[i], '.clones.TCR.txt')[[1]])
  }
}
combinedDF$Sample <- as.character(combinedDF$Sample)
```

```
countsTable <- ddsCounts[,order(colnames(ddsCounts))]
SampleInfo=SampleInfo[order(rownames(SampleInfo)), ]
SampleInfo$SNames <- gsub("\\.", "", rownames(SampleInfo))
SampleInfo$SNames<-gsub("T[A-ZO-9]*$", "",SampleInfo$SNames)
CNamesCT=gsub("\\.", "", colnames(countsTable))
CNamesCT=gsub("T[A-ZO-9]*$", "",CNamesCT)
```

FIlter out samples which have less than 2 counts, and compute Shannon index for each sample

```
filteredSamples <- unique(combinedDF$Sample)[!unique(combinedDF$Sample) %in% unique(combinedDF[combinedI
droppedSamples <- c(droppedSamples, filteredSamples)
combinedDF <- combinedDF[combinedDF$Clone.count > 1,]

cloneCount <- combinedDF %>% group_by(Sample) %>% count()
```

```
## Warning: failed to assign NativeSymbolInfo for env since env is already
## defined in the 'lazyeval' namespace
```

```
sampleNames <- unique(combinedDF$Sample)
sampleShannonIndex <- lapply(sampleNames, function (x) { diversity(as.numeric(combinedDF[combinedDF$Samp

shannonIndexTable <- data.frame('Sample' = sampleNames, 'ShannonIndex' = as.numeric(sampleShannonIndex)
  'Color' = SampleInfo[ SampleInfo$SNames %in% sampleNames,'finalCol'])
shannonIndexTable$Sample <- as.character(shannonIndexTable$Sample)
shannonIndexTable$SampleType <- as.character(shannonIndexTable$SampleType)
shannonIndexTable$Color <- as.character(shannonIndexTable$Color)

dcisIDCWilcoxP <- wilcox.test(shannonIndexTable[shannonIndexTable$SampleType == 'DCIS','ShannonIndex'],
```

```
## Warning in wilcox.test.default(shannonIndexTable[shannonIndexTable
## $SampleType == : cannot compute exact p-value with ties
```

```
dcisNormalWilcoxP <- wilcox.test(shannonIndexTable[shannonIndexTable$SampleType == 'DCIS','ShannonIndex
```

```
## Warning in wilcox.test.default(shannonIndexTable[shannonIndexTable
## $SampleType == : cannot compute exact p-value with ties
```

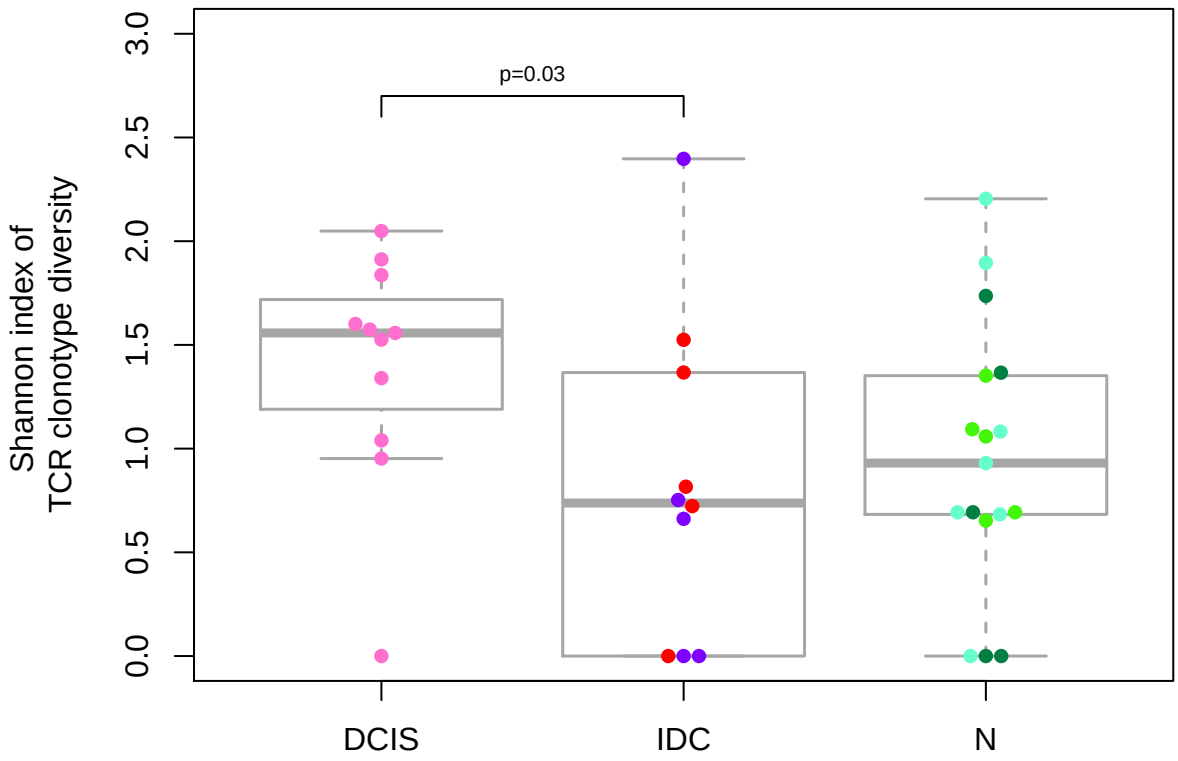
```
idcNormalWilcoxP <- wilcox.test(shannonIndexTable[shannonIndexTable$SampleType == 'IDC','ShannonIndex']
```

```
## Warning in wilcox.test.default(shannonIndexTable[shannonIndexTable
## $SampleType == : cannot compute exact p-value with ties
```

```
pValtToText <- function (pVal)
{
  if (pVal > 0.05) { return('ns') }
  else { paste('p=',round(pVal,2),sep = '') }
}

dcisIDCWilcoxPText <- pValtToText(dcisIDCWilcoxP)
dcisNormalWilcoxPText <- pValtToText(dcisNormalWilcoxP)
idcNormalWilcoxPText <- pValtToText(idcNormalWilcoxP)
```

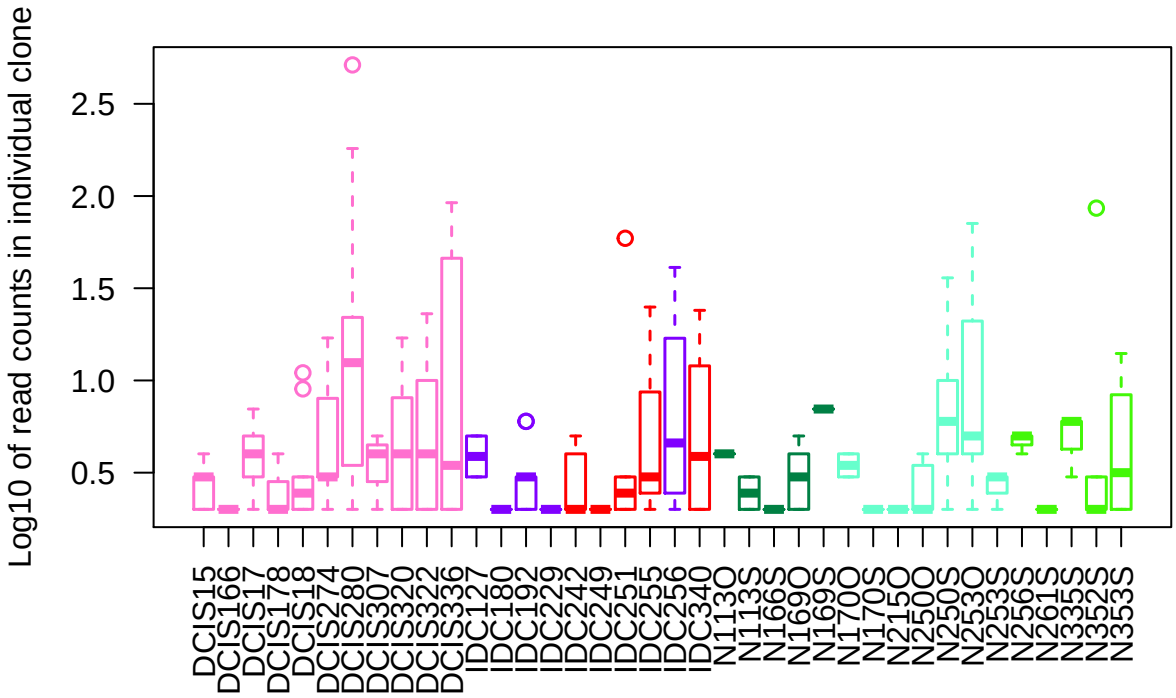
Plot the distribution of diversity across different patient samples:

```
boxplot(ShannonIndex ~ SampleType, data = shannonIndexTable, outline = FALSE, border = c('#a6a6a6'), yli
beeswarm(data = shannonIndexTable, ShannonIndex ~ SampleType, add = TRUE, xlab = '', ylab = '', pch = 1
lines(x=c(1,1,2,2), y=c(2.6,2.7,2.7,2.6))
text(1.5, 2.8, dcisIDCWilcoxPText, cex = 0.7)
```

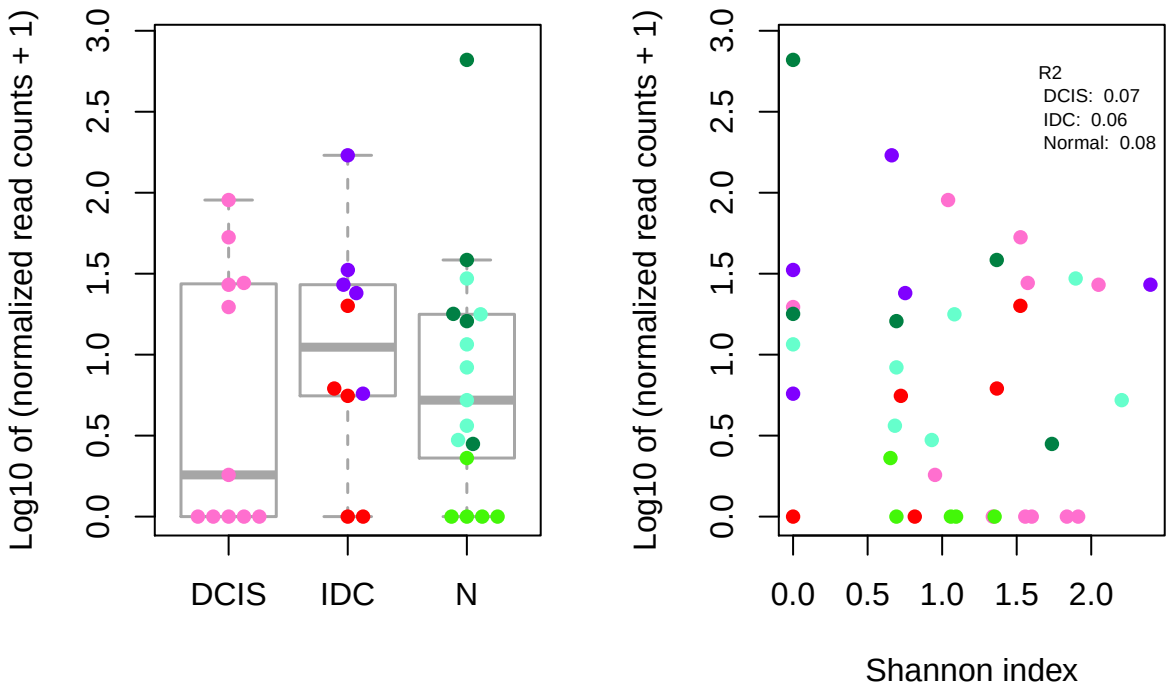


Overview of read counts across different samples:

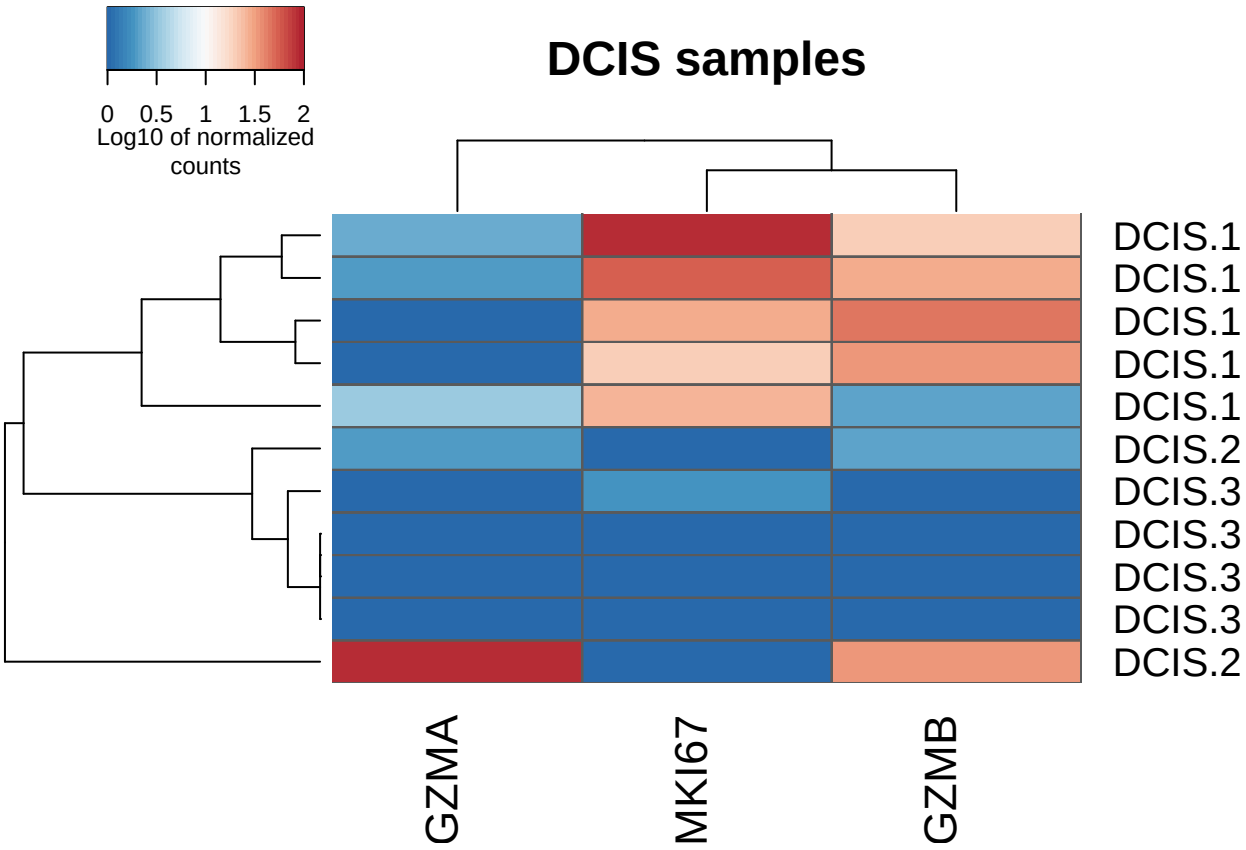
```
boxplot(log10(combinedDF$Clone.count) ~ combinedDF$Sample, las=2, par(mar=c(8,4,2,2)), ylab='Log10 of r
```




```
individualGenePlot <- function(geneName)
{
  currentGeneTable <- data.frame('Gene'=(countsTable[geneName,match(shannonIndexTable$Sample, CNamesCT)]

  par(mfrow = c(1,2))
  boxplot(log10(Gene + 1) ~ SampleType, data = currentGeneTable, outline = FALSE, border = c('#a6a6a6')
  beeswarm(data = currentGeneTable, log10(Gene + 1) ~ SampleType, add = TRUE, xlab = '', ylab = '', pch
  plot(currentGeneTable$ShannonIndex, log10(currentGeneTable$Gene + 1),  xlab = 'Shannon index', ylab =
  #text(currentGeneTable$ShannonIndex, log10(currentGeneTable$Gene + 1), labels = rownames(currentGeneT
  dcisRSq <- round(summary(lm(ShannonIndex ~ log10(Gene + 1), data = currentGeneTable[currentGeneTable$S
  idcRSq <- round(summary(lm(ShannonIndex ~ log10(Gene + 1), data = currentGeneTable[currentGeneTable$Sa
  normalRSq <- round(summary(lm(ShannonIndex ~ log10(Gene + 1), data = currentGeneTable[currentGeneTable
  legend('topright', paste('R2\n', 'DCIS: ', dcisRSq, '\n', 'IDC: ', idcRSq, '\n', 'Normal: ', normalRSq
  title(geneName, outer=TRUE, line=-1)
}

individualGenePlot('MKI67')
```




```
cytotoxicGeneTable <- as.data.frame(t(countsTable[c('MKI67', 'GZMA', 'GZMB'),
                                                  match(shannonIndexTable$Sample, CNamesCT)]))
cytotoxicGeneTable <- log10(cytotoxicGeneTable + 1)
dcisMatCyto <- as.matrix(cytotoxicGeneTable[
  match(shannonIndexTable$Sample[shannonIndexTable$SampleType == 'DCIS'],CNamesCT),])
heatColors <- colorRampPalette(c('#2869ab', '#4594c2', '#94c6de', '#d2e8f2', '#fafafb', '#fbdac8', '#f3
heatmap.2(dcisMatCyto, trace = 'none', density.info='none', breaks = seq(from = 0, to = 2, length.out =
```

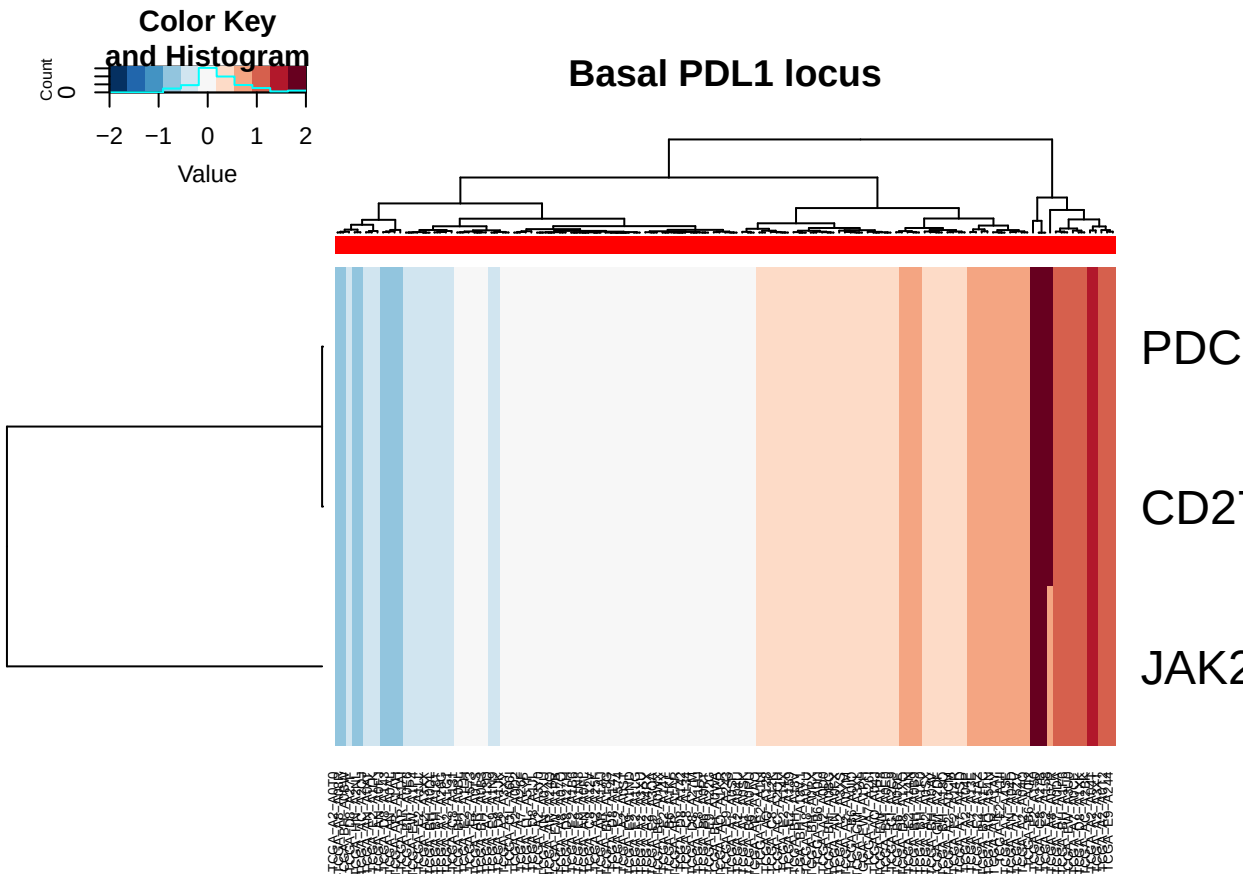


7 PDL1 amplification: Figure 4

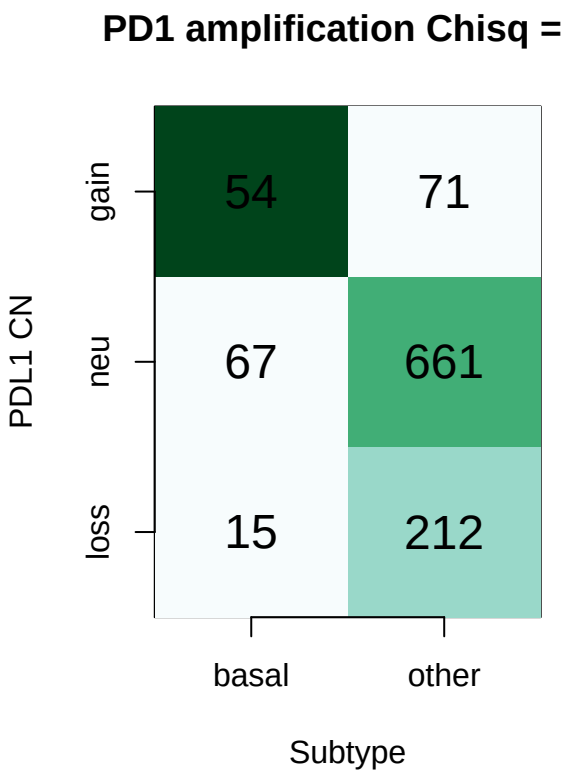
7.1 TCGA data

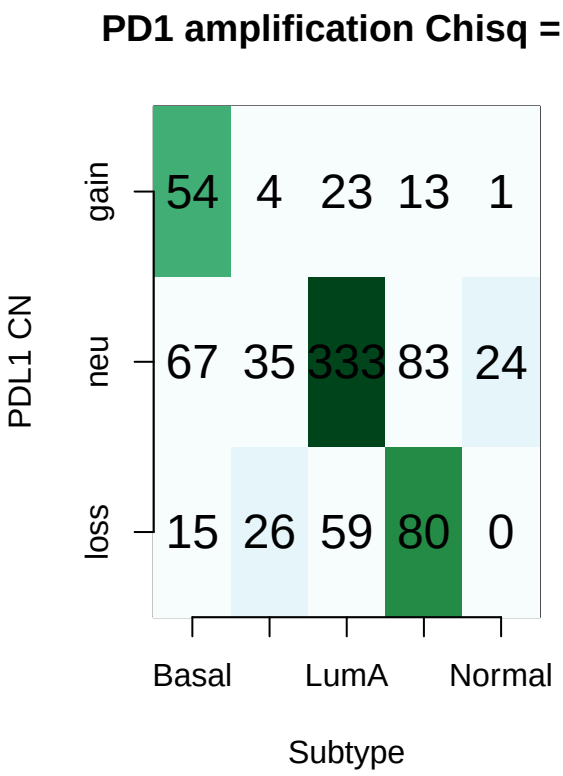
Load the TCGA GISTIC data and assess the frequency of PDL1 amplification in the different subtypes.

```
BasHER2=as.character(TCGAClin$PATIENT_ID[which(TCGAClin$PAM50=="Basal")])
n1=match(BasHER2, colnames(NewGistic))

ColIdNew=as.character(TCGAClin$PAM50[match(colnames(NewGistic), TCGAClin$PATIENT_ID)])
ColIdNew=gsub("Basal", "red", ColIdNew)
ColIdNew=gsub("Her2", "pink", ColIdNew)
ColIdNew=gsub("LumA", "blue", ColIdNew)
ColIdNew=gsub("LumB", "black", ColIdNew)
ColIdNew=gsub("Normal", "green", ColIdNew)

par(mfrow=c(1,2))
heatmap.2(data.matrix(NewGistic[c(21,22,24) ,n1]), col=HMPallete[11:1], scale="none",
          ColSideColors = ColIdNew[n1], trace="none", main="Basal PDL1 locus")
```




```
PDScores=colMeans(NewGistic[c(21:22),])
PDscoresAmp=cut(PDScores, c(-2, -0.3, 0.3, 5), c("loss", "neu", "gain"))
anew=rep("other", length(PDscoresAmp))
anew[n1]="basal"
ContTable(table(anew, PDscoresAmp), title="PD1 amplification", chisqtest = T, xlabL = "Subtype", ylabL =
allSub=TCGAClin$PAM50[match(colnames(NewGistic), TCGAClin$PATIENT_ID)]
ContTable(table(allSub, PDscoresAmp), title="PD1 amplification", chisqtest = T, xlabL = "Subtype", ylabl
```





Make a linear model to explain exhaustion or activation status in these cases

```
ActivationScore=colMedians(TCGAlogCPM[GeneListAct$Activated, ], na.rm=TRUE)
CytoScore=colMeans(TCGAlogCPM[c("IFNG", "GZMB", "GZMA"),], na.rm=TRUE)
ExhScore=colMedians(TCGAlogCPM[GeneListAct$Exhausted, ], na.rm=TRUE)
InhScore=colMedians(TCGAlogCPM[match(GeneListAct$Inhibitory, rownames(TCGAlogCPM)), ], na.rm=TRUE)

MatchData=data.frame(Pat=colnames(NewGistic),CN=PDScores,
                     ActScore=ActivationScore[match(colnames(NewGistic), colnames(TCGAlogCPM))],
                    CytoScore=CytoScore[match(colnames(NewGistic), colnames(TCGAlogCPM))],
                    ExhScore=ExhScore[match(colnames(NewGistic), colnames(TCGAlogCPM))],
                     InhScore=InhScore[match(colnames(NewGistic), colnames(TCGAlogCPM))],
                     Neo=NeoData$Predicted.NeoAgs[match(colnames(NewGistic), NeoData$PatientID)],
                     CNA=TCGAClin$Copy.Number.Alterations[match(colnames(NewGistic), TCGAClin$PATIENT_I
                    PDL1exp=TCGAlogCPM["CD274",match(colnames(NewGistic), colnames(TCGAlogCPM))],
                     Subtype=TCGAClin$PAM50[match(colnames(NewGistic), TCGAClin$PATIENT_ID)],
                     EST=ESTscore$ImmuneScore[match(colnames(NewGistic), ESTscore$Description)],
                    Purity=ESTscore$TumPurity[match(colnames(NewGistic), ESTscore$Description)],
                     Age=as.numeric(as.character(TCGAClin$Age[match(colnames(NewGistic), TCGAClin$PATIE

# model probability of co-amplification
mod1B=glm(CN~log(Neo+1)+CNA+Subtype+EST+log(Age), data=MatchData)
X=summary(mod1B)
X
```

```
##
## Call:
## glm(formula = CN ~ log(Neo + 1) + CNA + Subtype + EST + log(Age),
##     data = MatchData)
##
```

```
## Deviance Residuals:
##      Min        1Q    Median        3Q       Max
## -0.95474  -0.20698   0.03683   0.12238   2.10415
##
## Coefficients:
##                 Estimate Std. Error t value Pr(>|t|)
## (Intercept)    5.997e-01  2.467e-01   2.431   0.0153 *
## log(Neo + 1)  -7.941e-03  2.295e-02  -0.346   0.7295
## CNA           -1.730e-01  8.778e-02  -1.971   0.0492 *
## SubtypeHer2   -3.754e-01  6.174e-02  -6.081 2.09e-09 ***
## SubtypeLumA   -2.963e-01  4.664e-02  -6.353 4.08e-10 ***
## SubtypeLumB   -4.174e-01  4.665e-02  -8.948  < 2e-16 ***
## SubtypeNormal -2.646e-01  1.036e-01  -2.554   0.0109 *
## EST            1.432e-05  1.108e-05   1.293   0.1966
## log(Age)      -8.123e-02  6.098e-02  -1.332   0.1833
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for gaussian family taken to be 0.1274455)
##
##     Null deviance: 92.392  on 631  degrees of freedom
## Residual deviance: 79.399  on 623  degrees of freedom
##   (448 observations deleted due to missingness)
## AIC: 502.51
##
## Number of Fisher Scoring iterations: 2
```

```
# check only in the Basal cases:
mod1B=glm(CN~log(Neo+1)+CNA+EST+log(Age), data=MatchData[MatchData$Subtype=="Basal", ])
X=summary(mod1B)
X
```

```
##
## Call:
## glm(formula = CN ~ log(Neo + 1) + CNA + EST + log(Age), data = MatchData[MatchData$Subtype ==
##     "Basal", ])
##
## Deviance Residuals:
##      Min        1Q    Median        3Q       Max
## -1.18818  -0.27432  -0.09784   0.19276   1.56854
##
## Coefficients:
##                Estimate Std. Error t value Pr(>|t|)
## (Intercept)   5.264e-01  9.663e-01   0.545   0.5871
## log(Neo + 1) -4.008e-02  8.079e-02  -0.496   0.6209
## CNA           7.633e-01  2.970e-01   2.570   0.0116 *
## EST           5.477e-05  3.645e-05   1.503   0.1361
## log(Age)     -1.626e-01  2.319e-01  -0.701   0.4847
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for gaussian family taken to be 0.2755576)
##
##     Null deviance: 29.730  on 104  degrees of freedom
## Residual deviance: 27.556  on 100  degrees of freedom
```

```
##   (294 observations deleted due to missingness)
## AIC: 169.51
##
## Number of Fisher Scoring iterations: 2
```

```
# model activation scores:
## activation score
mod1A=glm(ActScore~CN+CNA+log(Neo+1)+Subtype+EST+log(Age), data=MatchData)
A1=summary(mod1A)
A1
```

```
##
## Call:
## glm(formula = ActScore ~ CN + CNA + log(Neo + 1) + Subtype +
##     EST + log(Age), data = MatchData)
##
## Deviance Residuals:
##      Min        1Q    Median        3Q       Max
## -3.10691  -0.37548   0.09469   0.48926   2.31507
##
## Coefficients:
##                 Estimate Std. Error t value Pr(>|t|)
## (Intercept)    9.031e-01  5.288e-01   1.708   0.0882 .
## CN            -1.526e-01  8.549e-02  -1.785   0.0747 .
## CNA            3.843e-01  1.879e-01   2.046   0.0412 *
## log(Neo + 1)   4.883e-02  4.898e-02   0.997   0.3192
## SubtypeHer2    3.568e-02  1.356e-01   0.263   0.7925
## SubtypeLumA   -2.275e-01  1.027e-01  -2.215   0.0271 *
## SubtypeLumB   -1.546e-01  1.057e-01  -1.462   0.1443
## SubtypeNormal -3.531e-01  2.222e-01  -1.589   0.1125
## EST            8.316e-04  2.367e-05  35.129   <2e-16 ***
## log(Age)      -2.472e-01  1.303e-01  -1.898   0.0582 .
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for gaussian family taken to be 0.580243)
##
##     Null deviance: 1198.26  on 631  degrees of freedom
## Residual deviance:  360.91  on 622  degrees of freedom
##   (448 observations deleted due to missingness)
## AIC: 1461.5
##
## Number of Fisher Scoring iterations: 2
```

```
## cytotoxic score
mod1C=glm(CytoScore~CN+CNA+log(Neo+1)+Subtype+EST+log(Age), data=MatchData)
A2=summary(mod1C)
A2
```

```
##
## Call:
## glm(formula = CytoScore ~ CN + CNA + log(Neo + 1) + Subtype +
##     EST + log(Age), data = MatchData)
##
## Deviance Residuals:
##     Min       1Q   Median       3Q      Max
```

```
## -3.9041  -0.6113   0.1047   0.6749   3.2089
##
## Coefficients:
##                 Estimate Std. Error t value Pr(>|t|)
## (Intercept)   -0.5232948  0.7194227  -0.727  0.46727
## CN            -0.0543780  0.1162940  -0.468  0.64024
## CNA            0.8396006  0.2556012   3.285  0.00108 **
## log(Neo + 1)   0.0625950  0.0666362   0.939  0.34791
## SubtypeHer2   -0.1459251  0.1844564  -0.791  0.42918
## SubtypeLumA   -0.5826575  0.1397108  -4.170 3.47e-05 ***
## SubtypeLumB   -0.4424769  0.1438354  -3.076  0.00219 **
## SubtypeNormal -0.9926956  0.3022355  -3.285  0.00108 **
## EST            0.0012542  0.0000322  38.945  < 2e-16 ***
## log(Age)      -0.2594789  0.1772491  -1.464  0.14372
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for gaussian family taken to be 1.073809)
##
##     Null deviance: 2644.16  on 631  degrees of freedom
## Residual deviance:  667.91  on 622  degrees of freedom
##   (448 observations deleted due to missingness)
## AIC: 1850.5
##
## Number of Fisher Scoring iterations: 2
```

```
## exhaustion score
mod1E=glm(ExhScore~CN+CNA+log(Neo+1)+Subtype+EST+log(Age), data=MatchData)
A3=summary(mod1E)
A3
```

```
##
## Call:
## glm(formula = ExhScore ~ CN + CNA + log(Neo + 1) + Subtype +
##     EST + log(Age), data = MatchData)
##
## Deviance Residuals:
##      Min        1Q    Median        3Q       Max
## -2.49428  -0.46837   0.03612   0.48548   2.09539
##
## Coefficients:
##                 Estimate Std. Error t value Pr(>|t|)
## (Intercept)    1.381e+00  5.119e-01   2.699 0.007144 **
## CN            -1.657e-01  8.274e-02  -2.002 0.045673 *
## CNA            5.169e-01  1.819e-01   2.843 0.004623 **
## log(Neo + 1)   2.227e-02  4.741e-02   0.470 0.638766
## SubtypeHer2   -1.897e-01  1.312e-01  -1.445 0.148909
## SubtypeLumA   -5.587e-01  9.940e-02  -5.620 2.88e-08 ***
## SubtypeLumB   -3.920e-01  1.023e-01  -3.831 0.000141 ***
## SubtypeNormal -4.177e-01  2.150e-01  -1.943 0.052502 .
## EST            9.050e-04  2.291e-05  39.497  < 2e-16 ***
## log(Age)      -4.673e-01  1.261e-01  -3.705 0.000230 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
```

```
## (Dispersion parameter for gaussian family taken to be 0.5435655)
##
##     Null deviance: 1395.5  on 631  degrees of freedom
## Residual deviance:  338.1  on 622  degrees of freedom
##   (448 observations deleted due to missingness)
## AIC: 1420.2
##
## Number of Fisher Scoring iterations: 2
```

```
## inhibition score
mod1I=glm(InhScore~CN+CNA+log(Neo+1)+Subtype+EST+log(Age), data=MatchData)
A4=summary(mod1I)
A4
```

```
##
## Call:
## glm(formula = InhScore ~ CN + CNA + log(Neo + 1) + Subtype +
##     EST + log(Age), data = MatchData)
##
## Deviance Residuals:
##      Min        1Q    Median        3Q       Max
## -2.54739  -0.51755   0.04802   0.53015   2.29021
##
## Coefficients:
##                  Estimate Std. Error t value Pr(>|t|)
## (Intercept)    1.304e+00  5.388e-01   2.421 0.015753 *
## CN            -1.837e-01  8.709e-02  -2.110 0.035285 *
## CNA            3.570e-01  1.914e-01   1.865 0.062625 .
## log(Neo + 1)   2.561e-02  4.990e-02   0.513 0.607946
## SubtypeHer2   -1.313e-01  1.381e-01  -0.951 0.342157
## SubtypeLumA   -4.859e-01  1.046e-01  -4.644 4.17e-06 ***
## SubtypeLumB   -3.739e-01  1.077e-01  -3.471 0.000555 ***
## SubtypeNormal -3.706e-01  2.263e-01  -1.637 0.102052
## EST            9.141e-04  2.412e-05  37.902  < 2e-16 ***
## log(Age)      -5.149e-01  1.327e-01  -3.879 0.000116 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for gaussian family taken to be 0.6022173)
##
##     Null deviance: 1438.40  on 631  degrees of freedom
## Residual deviance:  374.58  on 622  degrees of freedom
##   (448 observations deleted due to missingness)
## AIC: 1484.9
##
## Number of Fisher Scoring iterations: 2
```

Plot PDL1 CN, gene expression and see if there is a relationship with CD8 expression

```
library(fANCOVA)
```

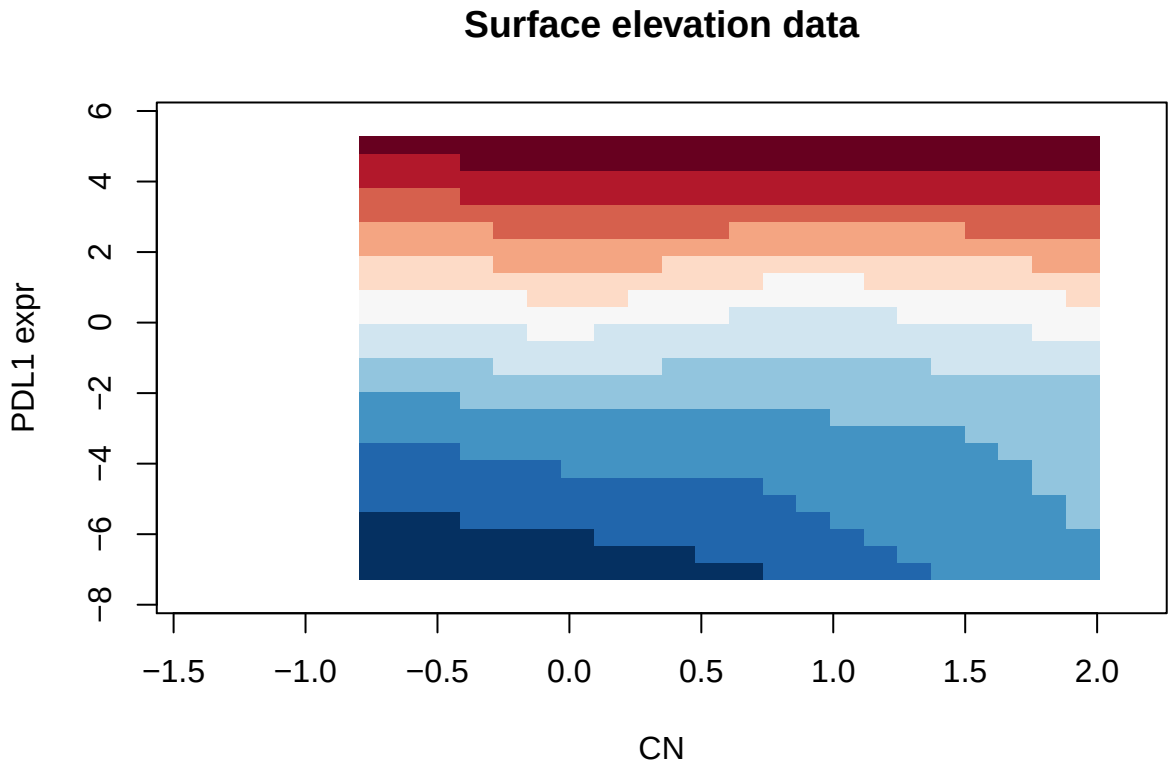
```
## fANCOVA 0.5-1 loaded
```

```
l1=which(MatchData$Subtype=="Basal")

require(geoR)
```

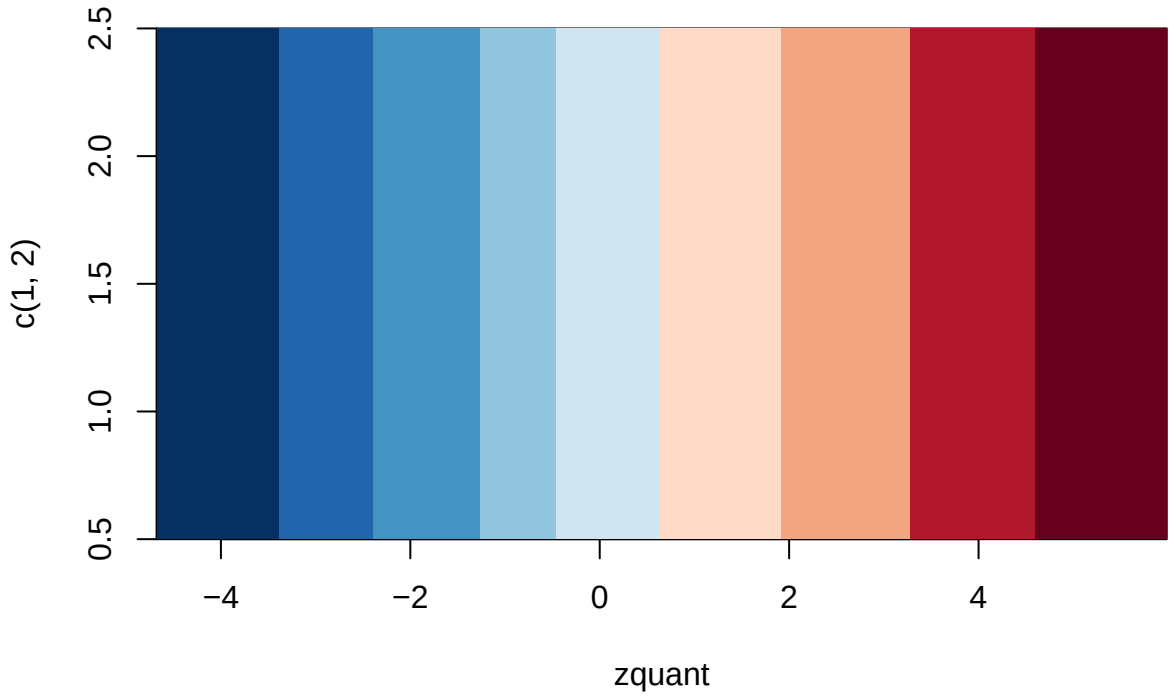
```
## Loading required package: geoR
```

```
## Warning in library(package, lib.loc = lib.loc, character.only = TRUE,
## logical.return = TRUE, : there is no package called 'geoR'
```

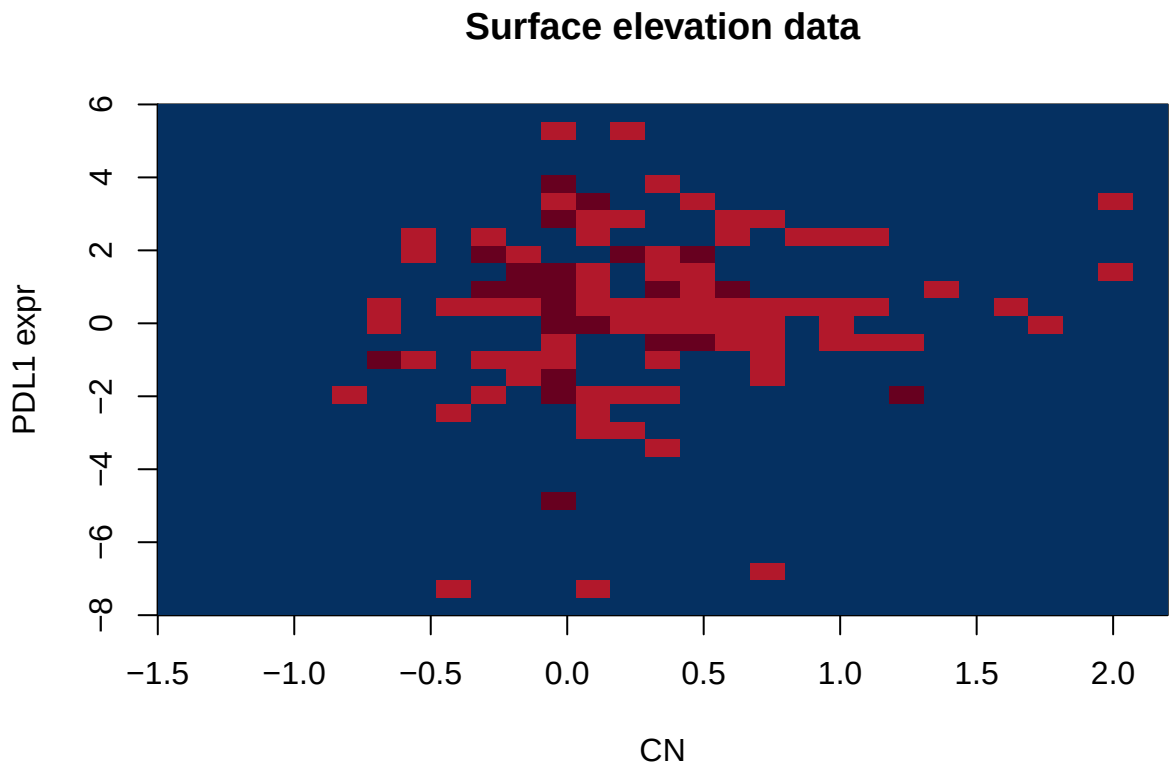
```
elevation.loess = loess(CytoScore~CN*PDL1exp, data = MatchData[l1, ], loess=0.6, degree=1)
elevation.fit = expand.grid(list(CN = seq(-1.5,2.2, length=30), PDL1exp = seq(-8, 6, length=30)))
z = predict(elevation.loess, newdata = elevation.fit)
elevation.fit$z = as.numeric(z)

image(seq(-1.5,2.2, length=30), seq(-8, 6, length=30), z,
  xlab = "CN", ylab = "PDL1 expr",
  main = "Surface elevation data", col=HMPallete[11:1])
```



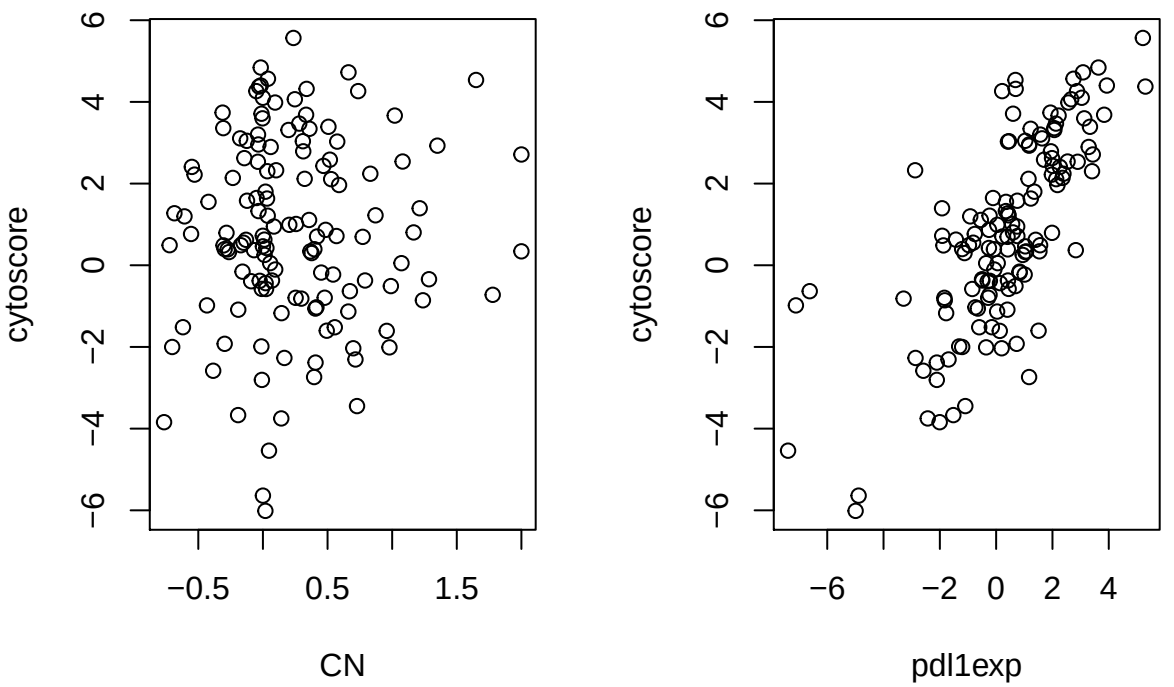

```
# plot the colour bar
zquant=quantile(z, probs=seq(0, 1, length=10), na.rm=TRUE)

image( zquant,c(1,2), cbind(zquant, zquant), col=HMPallete[11:1])
```




```
A=seq(-1.5,2.2, length=30)
B=seq(-8, 6, length=30)
spacing=matrix(NA, length(A)-1, length(B)-1)

for(i in 1:29){
  for(j in 1:29){
    spacing[i,j]=length(which(MatchData$CN[l1]>=A[i] & MatchData$CN[l1]<A[i+1] &
                              MatchData$PDL1exp[l1]>=B[j]&MatchData$PDL1exp[l1]<B[j+1]))
  }
}

spacing[spacing>0]=spacing[spacing>0]+20

image(seq(-1.5,2.2, length=30), seq(-8, 6, length=30), spacing,
  xlab = "CN", ylab = "PDL1 expr",
  main = "Surface elevation data", col=HMPallete[11:1])
```




```
par(mfrow=c(1,2))
plot(MatchData$CN[l1], MatchData$CytoScore[l1], main="CN", ylab="cytoscore", xlab="CN")
plot(MatchData$PDL1exp[l1], MatchData$CytoScore[l1], main="PDL1 GE", ylab="cytoscore", xlab="pdl1exp")
```

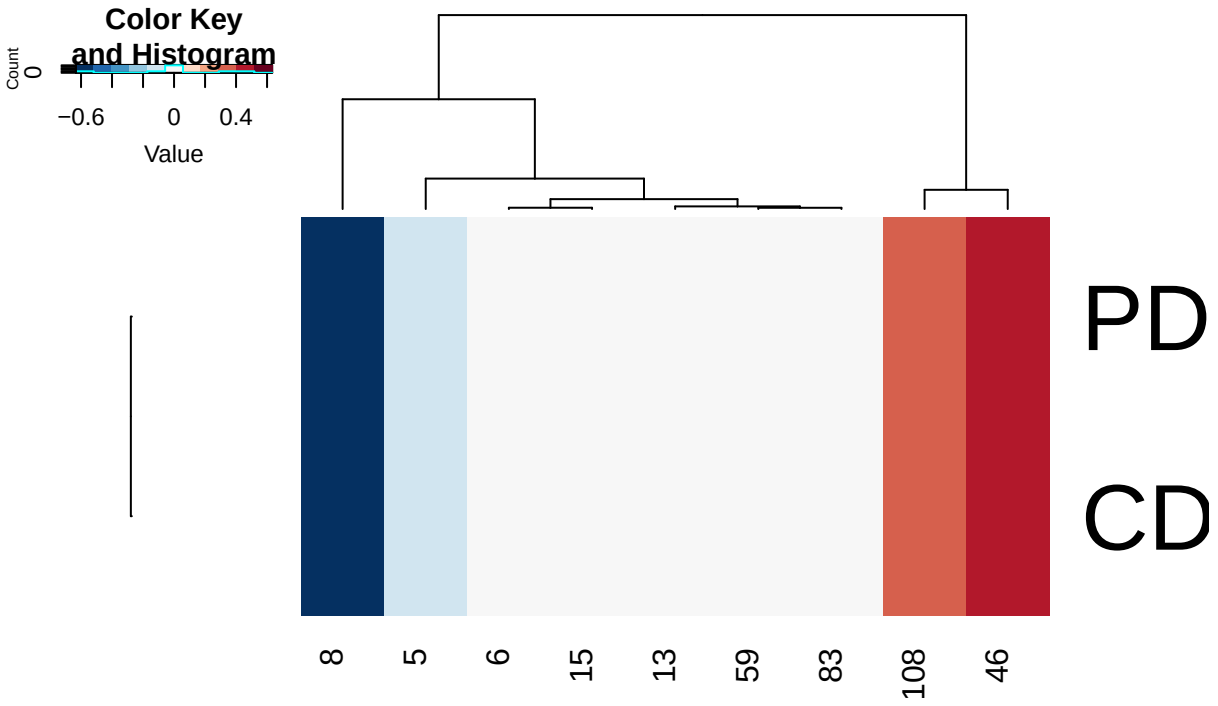


```
a1=lm(CytoScore~CN*PDL1exp+Purity, data=MatchData)
```

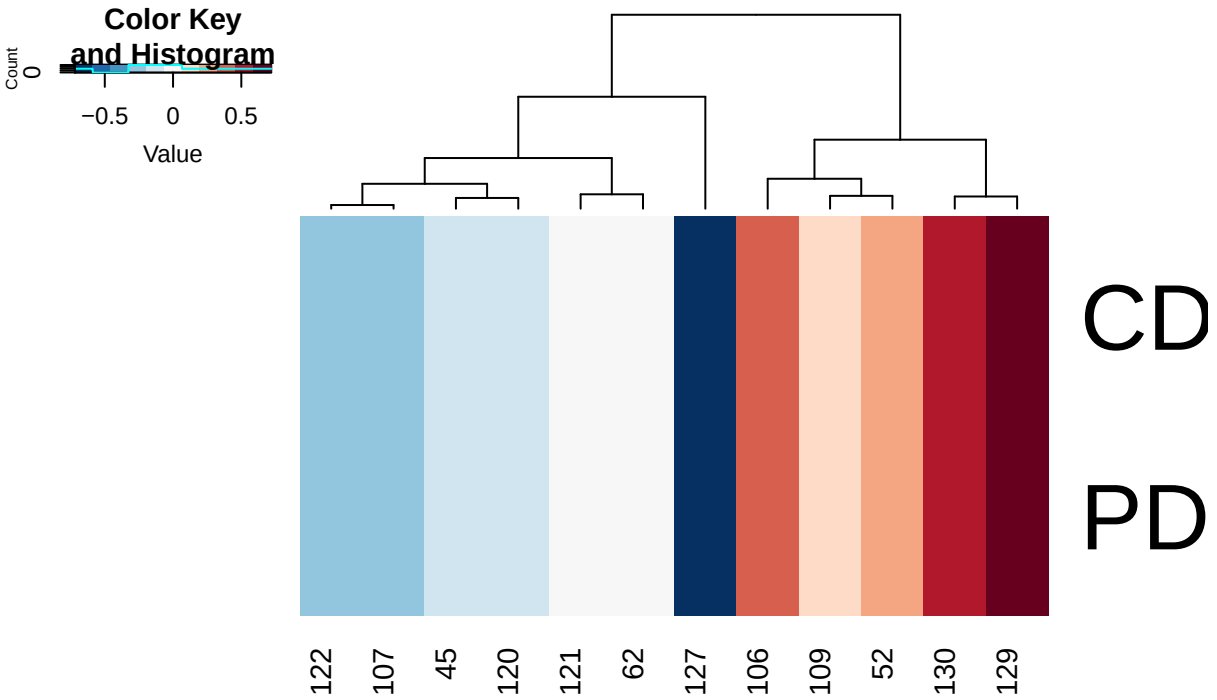
7.2 Oslo cohort

Look at the PD1 locus from data from Oslo:

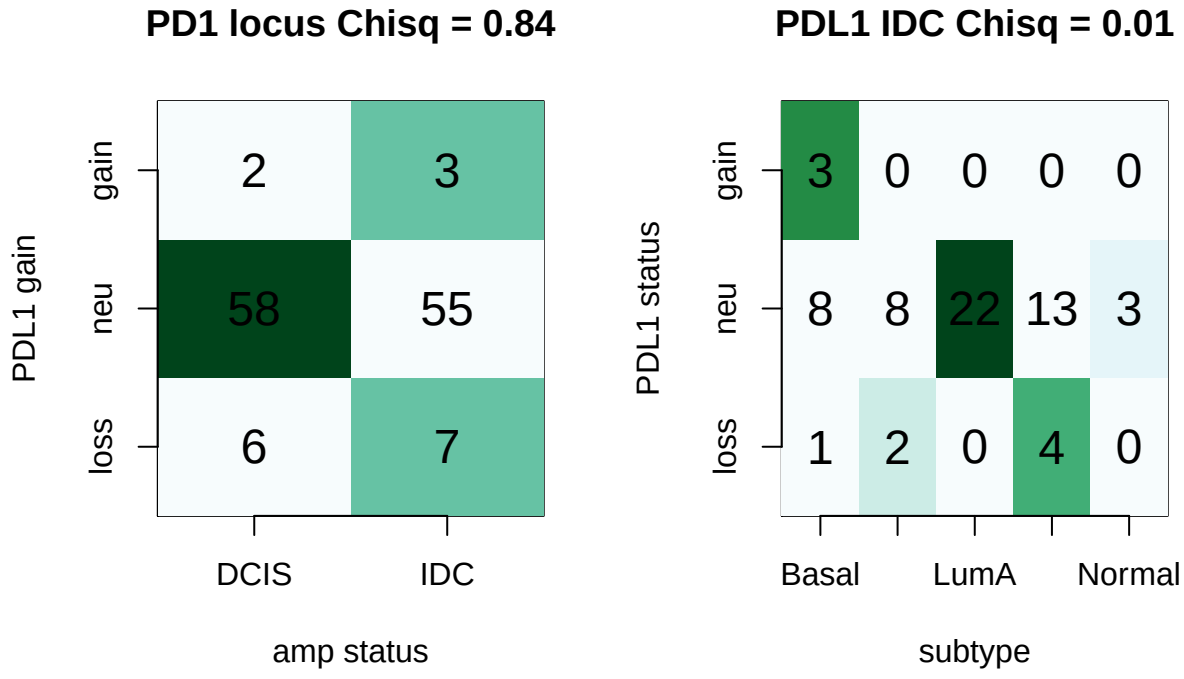
```
OsloGist2=read.csv("data/Oslo_SNP_data/pdls_adj_logr_polyak.csv")

nidx=which(OsloGist2$tissue=="DCIS")
par(mfrow=c(1,2))

OsIdNew=OsloGist2$subtype
OsIdNew=gsub("Basal", "red", OsIdNew)
OsIdNew=gsub("Her2", "pink", OsIdNew)
OsIdNew=gsub("LumA", "blue", OsIdNew)
OsIdNew=gsub("LumB", "blue", OsIdNew)
OsIdNew=gsub("Normal", "green", OsIdNew)

# check the frequency in the different samples
heatmap.2(t(data.matrix(OsloGist2[which(OsloGist2$tissue=="DCIS" & OsIdNew=="red"),c(4:5)])), trace="no
```



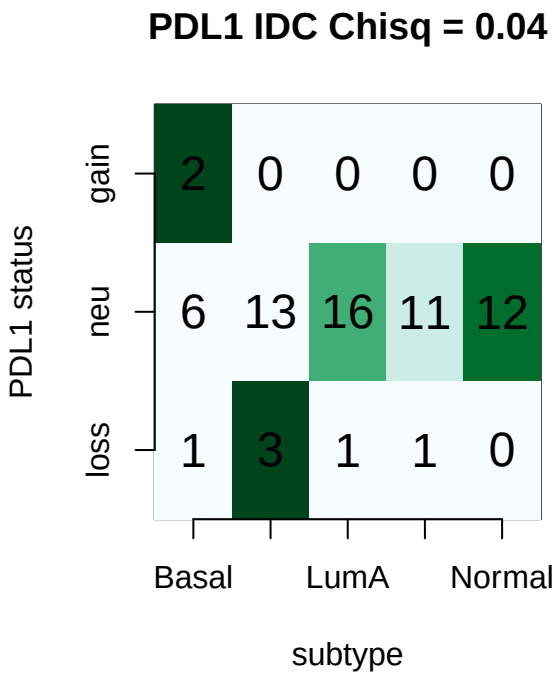

```
heatmap.2(t(data.matrix(OsloGist2[which(OsloGist2$tissue=="IDC" & OsIdNew=="red") ,c(4:5)])), trace="no
```




```
# table of frequencies in DCIS vs IDC
OsloPD1amp=cut(OsloGist2[ ,4], c(-5, -0.3, 0.3, 5), c("loss", "neu", "gain"))
par(mfrow=c(1,2))
ContTable(table(OsloGist2$tissue, OsloPD1amp), title="PD1 locus", chisqtest = T, xlabL = "amp status", y
# table in IDC cases only:
ContTable(table(OsloGist2$subtype[-nidx], OsloPD1amp[-nidx]), title="PDL1 IDC", chisqtest = T, xlabL =
```




```
#table DCIS only
ContTable(table(OsloGist2$subtype[nidx], OsloPD1amp[nidx]), title="PDL1 IDC", chisqtest = T, xlabL = "su
```

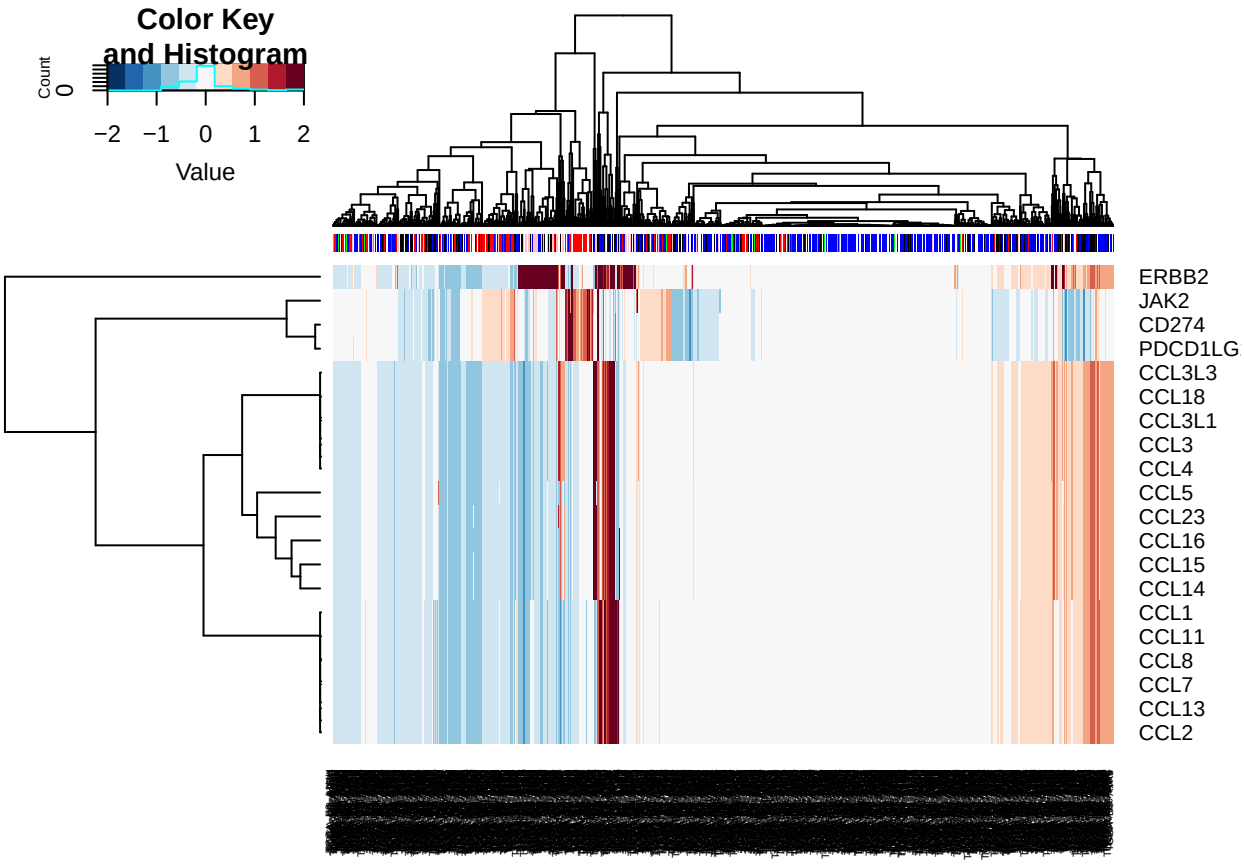


8 Frequency of co-amplification with the CCL data set

8.1 TCGA data set: Figure S7

To do this, load Gistic data from the TCGA and location of genes of interest. Add information on Patient subtype by laoding in TCGA clinical information. (Also available at firebrowse.org)

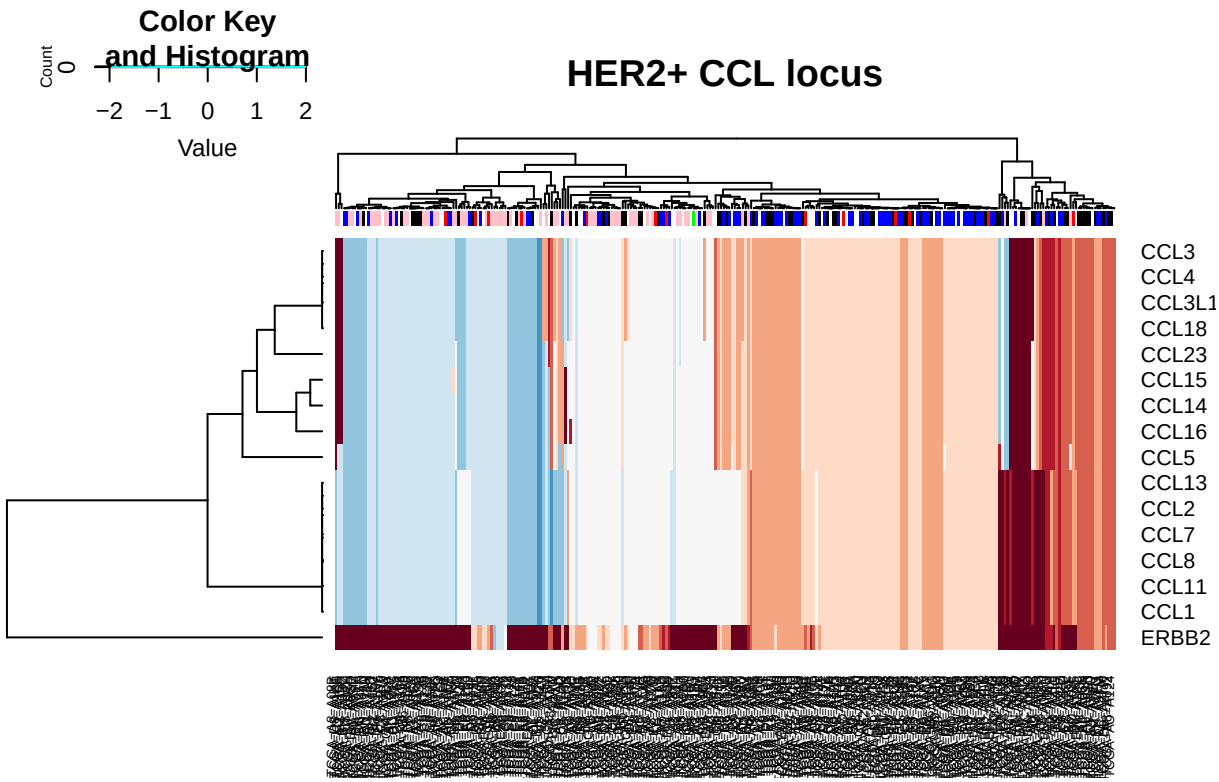
```
heatmap.2(data.matrix(NewGistic[ -c(18:20,23),]), col=HMPallete[11:1], scale="none", ColSideColors = Co
```



8.1.1 CCL coamplification: Figure 5

More closely examine HER2 positive cases, or any with amplification: We will need to specify a cut-off for ERBB2 amplification - here it is set at 0.3, which is often used for Gistic Scores

```
library(gplots)
BasHER2=as.character(TCGAClin$PATIENT_ID[which(TCGAClin$PAM50=="Her2")])
ERBamp=colnames(NewGistic)[which(NewGistic["ERBB2", ]>0.3)]
PatID=unique(c(BasHER2, ERBamp))
n1=match(PatID, colnames(NewGistic))
a2=heatmap.2(data.matrix(NewGistic[c(1:16) ,n1]), col=HMPallete[11:1], scale="none", ColSideColors = Co
```



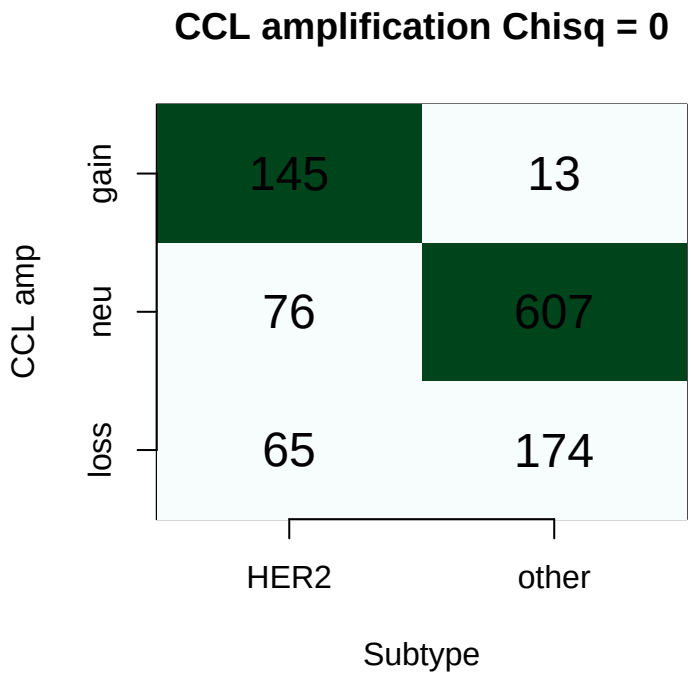

```
colA=rep("black", length(n1))
colA[a2$colInd[1:148]]="pink"
colA[a2$colInd[149:243]]="red"

grpA=colnames(NewGistic[c(1:16) ,n1[a2$colInd[1:148]]])
grpB=colnames(NewGistic[c(1:16) ,n1[a2$colInd[149:243]]])
grpC=colnames(NewGistic[c(1:16) ,n1[a2$colInd[244:286]]])
```

Calculate the chisq values for frequency of co-amplification in HER2 samples

```
PDScores=colMeans(NewGistic[c(1:15),])
PDscoresAmp=cut(PDScores, c(-2, -0.3, 0.3, 5), c("loss", "neu", "gain"))
anew=rep("other", length(PDscoresAmp))
anew[n1]="HER2"
ContTable(table(anew, PDscoresAmp), title="CCL amplification", chisqtest = T, xlabL = "Subtype", ylabL =
```

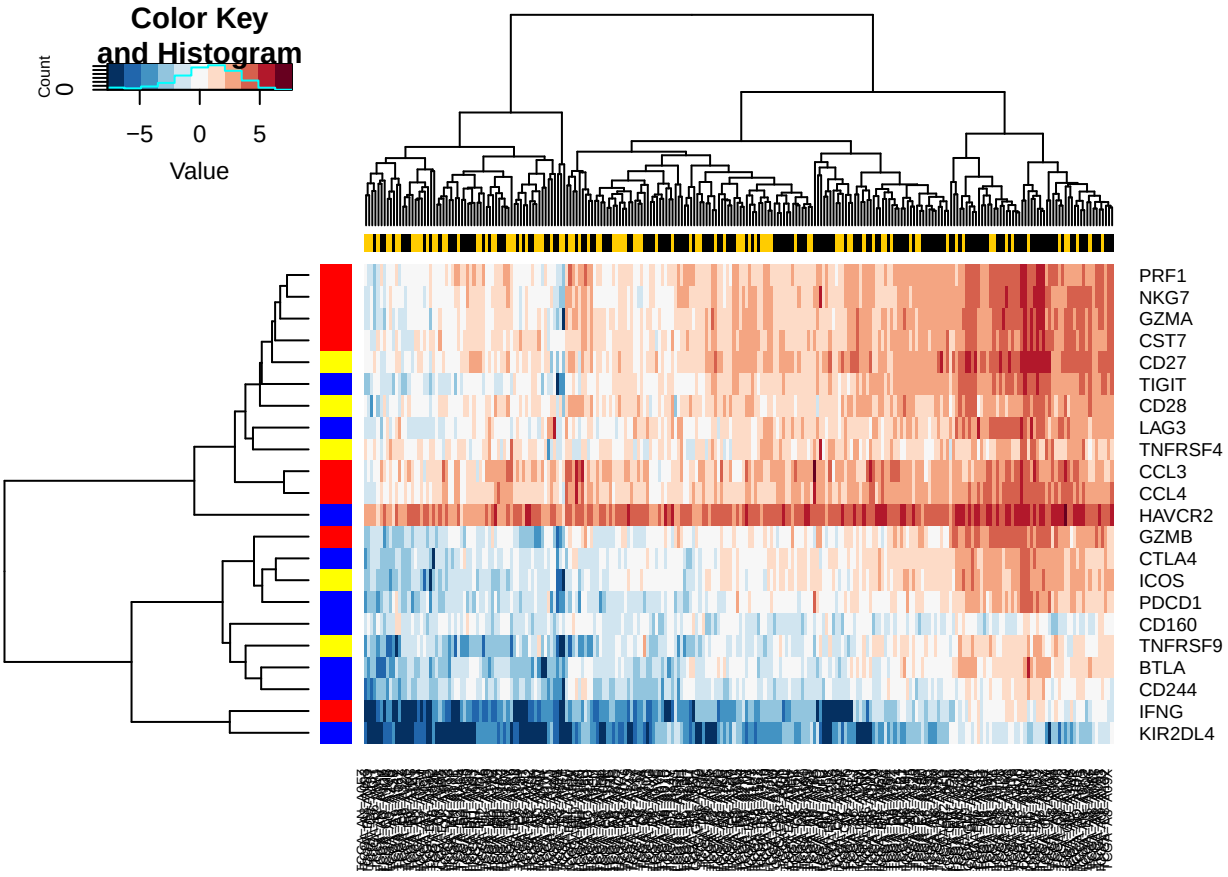
```
## Warning in if (chisqtest == T) {: the condition has length > 1 and only the
## first element will be used
```

```
## Warning in if (chisqtest == T) {: the condition has length > 1 and only the
## first element will be used
```

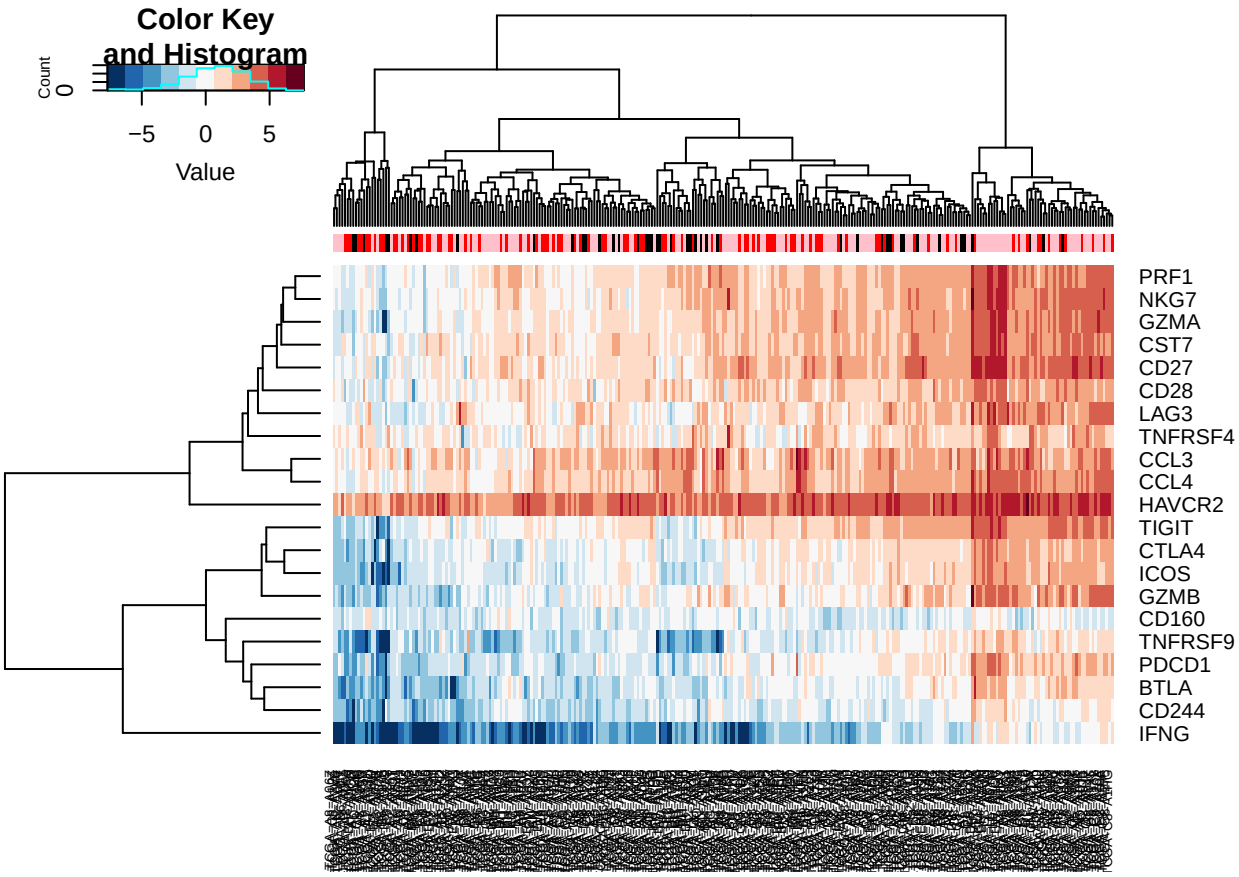


8.2 TCGA RNA-seq data

RNAseq data:



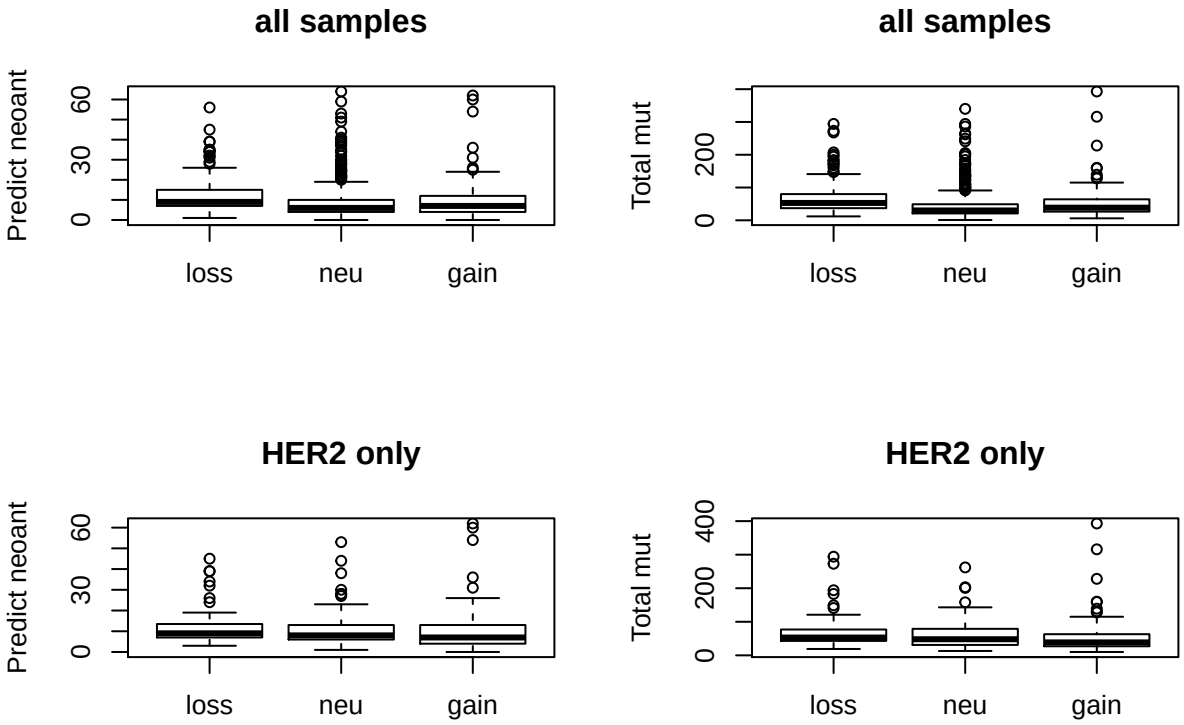
```
# Plot heatmap of activation related genes with HER2/Lum/amp status:
Pid1=match(c(grpA, grpB, grpC), colnames(TCGAlogCPM))
RNA2=TCGAlogCPM[match(c(GeneListAct$Activated, GeneListAct$Exhausted, GeneListAct$Cytotoxic), rownames(T

heatmap.2(RNA2, trace="none", col=HMPallete[11:1], ColSideColors = c(rep("pink", length(grpA)), rep("re
```



8.3 TCGA neoantigen data

This was obtained from supplementary information Table S4A from Rooney et al 2015.

```
mData=match(colnames(NewGistic),NeoData$PatientID)

## construct a boxplot of antigen load for example
par(mfrow=c(2,2))
boxplot(NeoData$Predicted.NeoAgs[mData]~PDscoresAmp, ylab="Predict neoant", main="all samples")
boxplot(NeoData$Total.Mutations[mData]~PDscoresAmp, ylab="Total mut", main="all samples")

## refne search to HER2 cases only
boxplot(NeoData$Predicted.NeoAgs[mData[anew=="HER2"]]~PDscoresAmp[anew=="HER2"], ylab="Predict neoant",
boxplot(NeoData$Total.Mutations[mData[anew=="HER2"]]~PDscoresAmp[anew=="HER2"], ylab="Total mut", main=
```



8.4 Linear model for TCGA dataset

Make a linear model to explain exhaustion or activation status:

```
MatchData=data.frame(Pat=colnames(NewGistic),CN=PDScores,
                      ActScore=ActivationScore[match(colnames(NewGistic), colnames(TCGAlogCPM))],
                     CytoScore=CytoScore[match(colnames(NewGistic), colnames(TCGAlogCPM))],
                     ExhScore=ExhScore[match(colnames(NewGistic), colnames(TCGAlogCPM))],
                      InhScore=InhScore[match(colnames(NewGistic), colnames(TCGAlogCPM))],
                      ERBB2=t(NewGistic["ERBB2", ]),
                      Neo=NeoData$Predicted.NeoAgs[match(colnames(NewGistic), NeoData$PatientID)],
                      CNA=TCGAClin$Copy.Number.Alterations[match(colnames(NewGistic), TCGAClin$PATIENT_I
                      Subtype=TCGAClin$PAM50[match(colnames(NewGistic), TCGAClin$PATIENT_ID)],
                      EST=ESTscore$ImmuneScore[match(colnames(NewGistic), ESTscore$Description)],
                      Age=as.numeric(as.character(TCGAClin$Age[match(colnames(NewGistic), TCGAClin$PATIE

MatchData$Subtype = factor(MatchData$Subtype,levels(MatchData$Subtype)[c(2, 1, 3:5)])

# Model summary:
mod1B=glm(CN~ERBB2+log(Neo+1)+Subtype+log(Age), data=MatchData)
X=summary(mod1B)
X
```

```
##
## Call:
## glm(formula = CN ~ ERBB2 + log(Neo + 1) + Subtype + log(Age),
##     data = MatchData)
##
## Deviance Residuals:
##      Min        1Q    Median        3Q       Max
```

```
## -1.29241  -0.11548  -0.00722   0.13264   2.10180
##
## Coefficients:
##               Estimate Std. Error t value Pr(>|t|)
## (Intercept)   -0.83805    0.26990  -3.105 0.001988 **
## ERBB2          0.22520    0.02407   9.356  < 2e-16 ***
## log(Neo + 1)  -0.05906    0.02321  -2.544 0.011191 *
## SubtypeBasal   0.27087    0.07503   3.610 0.000331 ***
## SubtypeLumA    0.42937    0.06703   6.406 2.95e-10 ***
## SubtypeLumB    0.46717    0.06738   6.933 1.03e-11 ***
## SubtypeNormal  0.33500    0.11849   2.827 0.004844 **
## log(Age)       0.12757    0.06504   1.961 0.050282 .
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for gaussian family taken to be 0.1453391)
##
##     Null deviance: 112.090  on 631  degrees of freedom
## Residual deviance:  90.692  on 624  degrees of freedom
##   (448 observations deleted due to missingness)
## AIC: 584.56
##
## Number of Fisher Scoring iterations: 2
```

```
# Narrow this down to just samples which have the HER2 amplicon
l1=match(c(grpA, grpB, grpC), MatchData$Pat)


# model rna expression expression
## activation signature
mod1A=glm(ActScore~CN+log(Neo+1)+Subtype+EST+log(Age), data=MatchData[l1, ])
summary(mod1A)
```

```
##
## Call:
## glm(formula = ActScore ~ CN + log(Neo + 1) + Subtype + EST +
##     log(Age), data = MatchData[l1, ])
##
## Deviance Residuals:
##     Min       1Q   Median       3Q      Max
## -2.5426  -0.3207   0.0439   0.4428   2.2136
##
## Coefficients:
##                 Estimate Std. Error t value Pr(>|t|)
## (Intercept)    1.484e+00  8.703e-01   1.705   0.0899 .
## CN            -4.647e-02  9.441e-02  -0.492   0.6231
## log(Neo + 1)   2.748e-02  8.158e-02   0.337   0.7367
## SubtypeBasal  -1.701e-02  2.251e-01  -0.076   0.9398
## SubtypeLumA   -1.730e-01  1.409e-01  -1.228   0.2212
## SubtypeLumB   -9.626e-02  1.477e-01  -0.652   0.5154
## SubtypeNormal  5.528e-01  7.084e-01   0.780   0.4363
## EST            7.998e-04  4.273e-05  18.719   <2e-16 ***
## log(Age)      -3.229e-01  2.179e-01  -1.482   0.1402
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
##
## (Dispersion parameter for gaussian family taken to be 0.4854993)
##
##     Null deviance: 275.64  on 188  degrees of freedom
## Residual deviance:  87.39  on 180  degrees of freedom
##   (97 observations deleted due to missingness)
## AIC: 410.57
##
## Number of Fisher Scoring iterations: 2
```

```
## cytotoxic signature
mod1C=glm(CytoScore~CN+log(Neo+1)+Subtype+EST+log(Age), data=MatchData[l1, ])
summary(mod1C)
```

```
##
## Call:
## glm(formula = CytoScore ~ CN + log(Neo + 1) + Subtype + EST +
##     log(Age), data = MatchData[l1, ])
##
## Deviance Residuals:
##      Min        1Q    Median        3Q       Max
## -2.81250  -0.56713   0.04526   0.61313   2.21690
##
## Coefficients:
##                 Estimate Std. Error t value Pr(>|t|)
## (Intercept)   -1.197e+00  1.266e+00  -0.945   0.3458
## CN             1.834e-01  1.373e-01   1.335   0.1834
## log(Neo + 1)   4.330e-02  1.187e-01   0.365   0.7156
## SubtypeBasal   4.127e-02  3.274e-01   0.126   0.8998
## SubtypeLumA   -5.213e-01  2.050e-01  -2.543   0.0118 *
## SubtypeLumB   -8.203e-02  2.148e-01  -0.382   0.7030
## SubtypeNormal -1.000e+00  1.030e+00  -0.971   0.3330
## EST            1.352e-03  6.215e-05  21.759   <2e-16 ***
## log(Age)      -7.301e-02  3.170e-01  -0.230   0.8181
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for gaussian family taken to be 1.027088)
##
##     Null deviance: 728.03  on 188  degrees of freedom
## Residual deviance: 184.88  on 180  degrees of freedom
##   (97 observations deleted due to missingness)
## AIC: 552.19
##
## Number of Fisher Scoring iterations: 2
```

```
## exhaustion signature
mod1E=glm(ExhScore~CN+log(Neo+1)+Subtype+EST+log(Age), data=MatchData[l1, ])
summary(mod1E)
```

```
##
## Call:
## glm(formula = ExhScore ~ CN + log(Neo + 1) + Subtype + EST +
##     log(Age), data = MatchData[l1, ])
##
## Deviance Residuals:
```

```
##      Min        1Q    Median        3Q       Max
## -1.77558  -0.43640  -0.00196   0.55993   1.60795
##
## Coefficients:
##                 Estimate Std. Error t value Pr(>|t|)
## (Intercept)    5.850e-01  9.190e-01   0.637   0.5252
## CN             7.089e-02  9.969e-02   0.711   0.4779
## log(Neo + 1)   1.938e-02  8.615e-02   0.225   0.8223
## SubtypeBasal   1.146e-01  2.377e-01   0.482   0.6304
## SubtypeLumA   -3.321e-01  1.488e-01  -2.232   0.0269 *
## SubtypeLumB   -1.066e-01  1.559e-01  -0.683   0.4953
## SubtypeNormal  2.799e-01  7.481e-01   0.374   0.7087
## EST            9.519e-04  4.512e-05  21.098   <2e-16 ***
## log(Age)      -2.801e-01  2.301e-01  -1.217   0.2251
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for gaussian family taken to be 0.5413373)
##
##     Null deviance: 365.196  on 188  degrees of freedom
## Residual deviance:  97.441  on 180  degrees of freedom
##   (97 observations deleted due to missingness)
## AIC: 431.15
##
## Number of Fisher Scoring iterations: 2
```

```
## inhibition signature
mod1I=glm(InhScore~CN+log(Neo+1)+Subtype+EST+log(Age), data=MatchData[l1, ])
summary(mod1I)
```

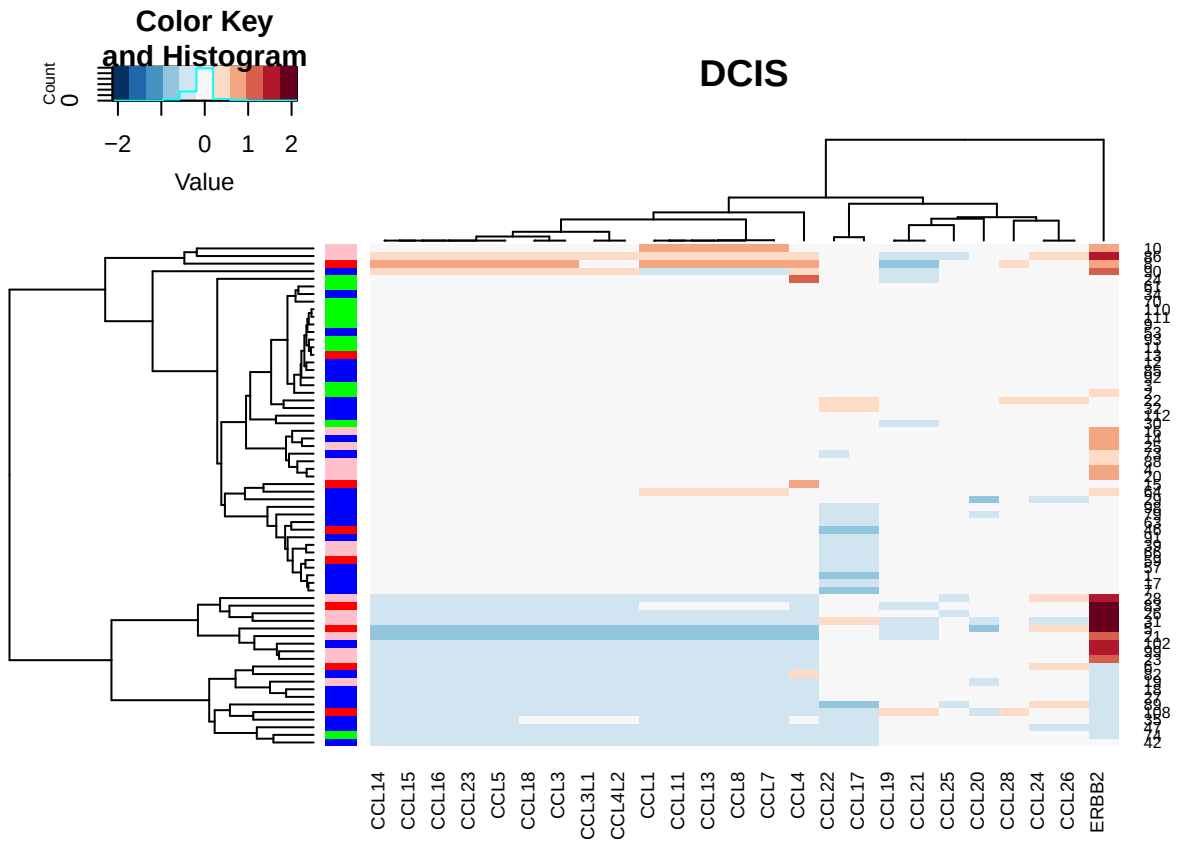
```
##
## Call:
## glm(formula = InhScore ~ CN + log(Neo + 1) + Subtype + EST +
##     log(Age), data = MatchData[l1, ])
##
## Deviance Residuals:
##     Min       1Q   Median       3Q      Max
## -1.7979  -0.4949   0.0000   0.5995   1.7067
##
## Coefficients:
##                 Estimate Std. Error t value Pr(>|t|)
## (Intercept)    0.4847239  0.9449912   0.513   0.6086
## CN             0.0776753  0.1025126   0.758   0.4496
## log(Neo + 1)   0.0182187  0.0885853   0.206   0.8373
## SubtypeBasal  -0.0324532  0.2443827  -0.133   0.8945
## SubtypeLumA   -0.3702346  0.1530340  -2.419   0.0165 *
## SubtypeLumB   -0.1535108  0.1603608  -0.957   0.3397
## SubtypeNormal  0.0543063  0.7692516   0.071   0.9438
## EST            0.0009503  0.0000464  20.483   <2e-16 ***
## log(Age)      -0.3132580  0.2366327  -1.324   0.1872
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for gaussian family taken to be 0.5724283)
##
```

```
##     Null deviance: 371.68  on 188  degrees of freedom
## Residual deviance: 103.04  on 180  degrees of freedom
##   (97 observations deleted due to missingness)
## AIC: 441.7
##
## Number of Fisher Scoring iterations: 2
```

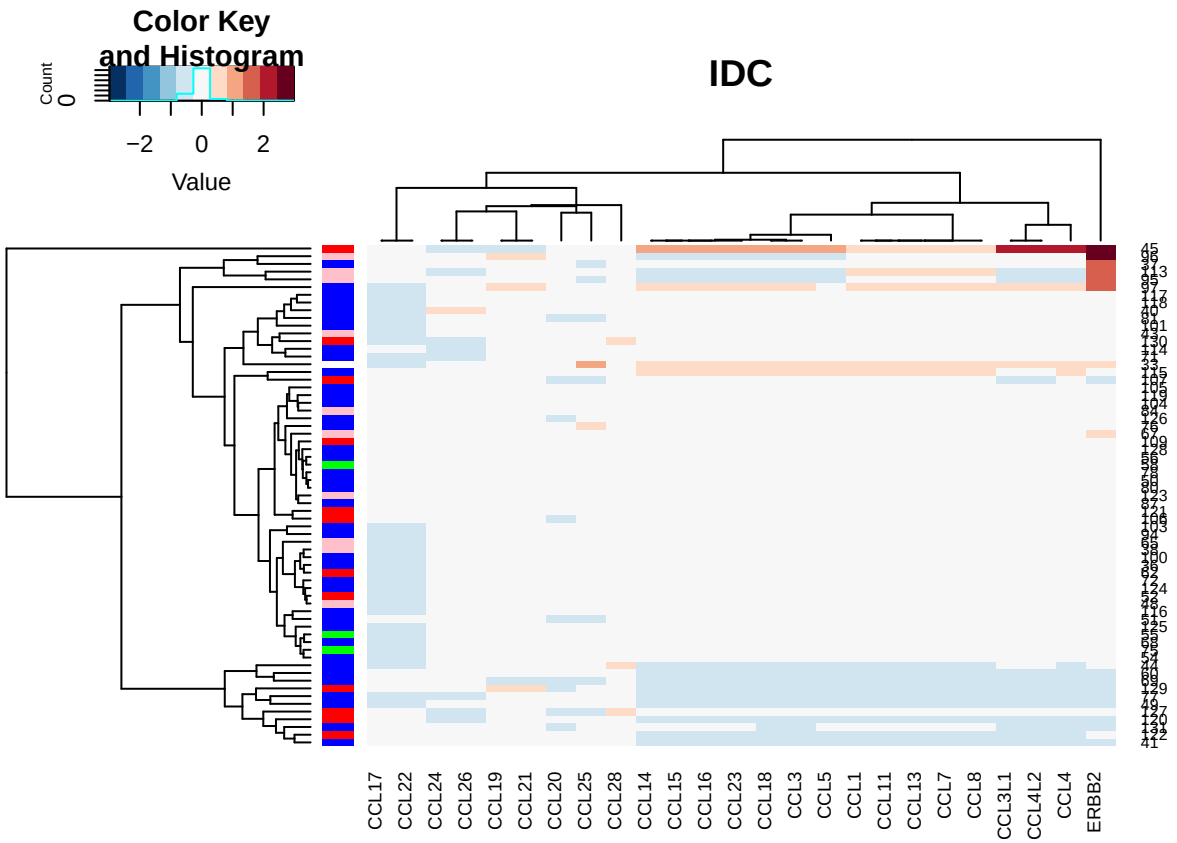
8.5 Oslo Dataset: Figure 4

The Oslo dataset contains CN information from both DCIS and IDC samples. Please contact the corresponding author if you wish to access this information.

```
OsloGist=read.csv("data/Oslo_SNP_data/ccls_her2_adj_cn_polyak.csv")
OsloGistLogR=read.csv("data/Oslo_SNP_data/ccls_her2_adj_logr_polyak.csv")

# heatmap of LogRatios
HER2scale=brewer.pal(5, "Greens")
N2=cut(OsloGistLogR$adj_cn_HER2, c(-5, -0.5, 0.5, 2.5, 10, 100), labels=HER2scale)

nidx=which(OsloGist$tissue=="DCIS")
par(mfrow=c(1,2))

OsIdNew=OsloGistLogR$subtype
OsIdNew=gsub("Basal", "red", OsIdNew)
OsIdNew=gsub("Her2", "pink", OsIdNew)
OsIdNew=gsub("LumA", "blue", OsIdNew)
OsIdNew=gsub("LumB", "blue", OsIdNew)
OsIdNew=gsub("Normal", "green", OsIdNew)

heatmap.2(data.matrix(OsloGistLogR[nidx ,-c(1:7)]), col=HMPallete[11:1],
          scale="none",trace="none", main="DCIS",
          RowSideColors =OsIdNew[nidx])
```




```
heatmap.2(data.matrix(OsloGistLogR[-nidx ,-c(1:7)]), col=HMPallete[11:1],
          scale="none", trace="none", RowSideColors =OsIdNew[-nidx],
          main="IDC")
```



%Calculate the percentages of samples with or without co-amplification? Take a conservative value of 0.1 as amplification or loss?? Also, reanalyse with her2 amplification as value of 3 or above

```
# find in the DCIS case
a1=match(Genes$Locus[1:15], colnames(OsloGistLogR))

OsGismeanl=rowMeans(OsloGistLogR[,na.omit(a1)])
OsGismeanl2=cut(OsGismeanl, c(-5, -0.3, 0.3, 5), c("loss", "neu", "gain"))
HER2CNnew=cut(OsloGistLogR$adj_cn_HER2, c(-10, 2.5, 1000), c("not-amp", "amp"))
# DCIS cases:
table(OsloGistLogR$her2_amplified[nidx], OsGismeanl2[nidx])
```

```
##
##           loss neu gain
##   amp        8   3    0
##   non-amp    9  45    1
```

```
# IDC case:
table(OsloGistLogR$her2_amplified[-nidx], OsGismeanl2[-nidx])
```

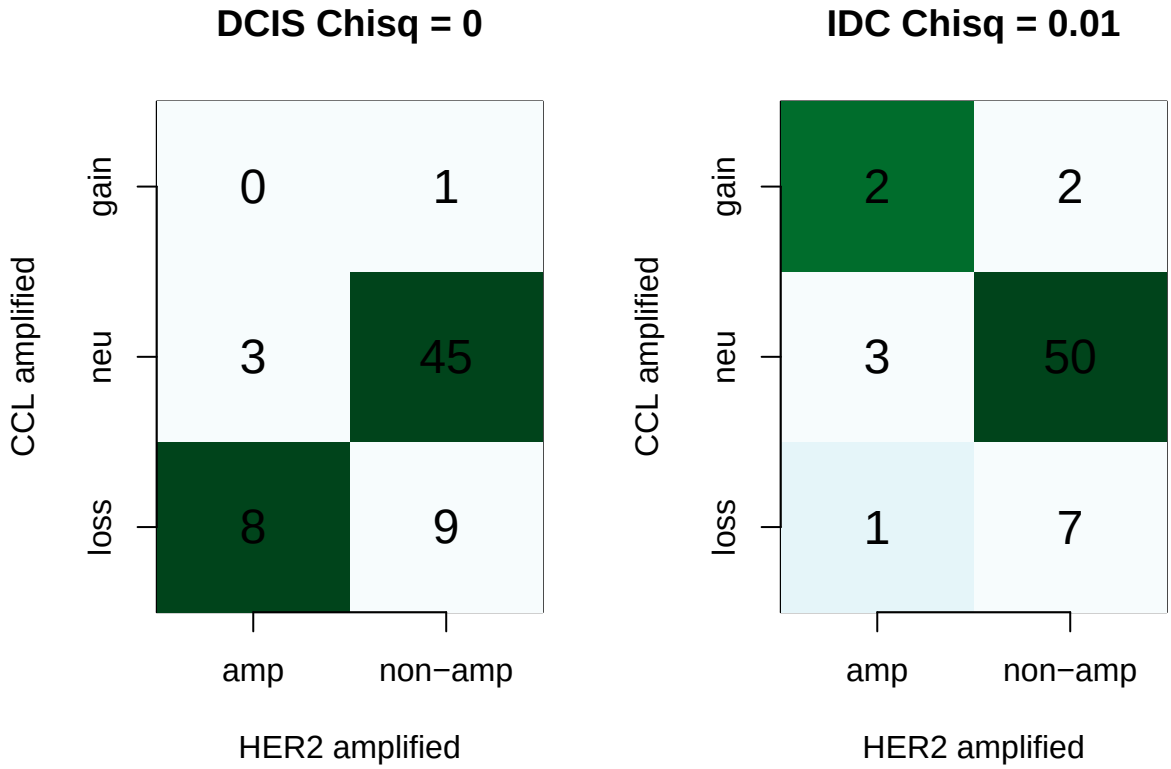
```
##
##           loss neu gain
##   amp        1   3    2
##   non-amp    7  50    2
```

```
par(mfrow=c(1,2))
# cut off of 4 for amplification
ContTable(table(OsloGistLogR$her2_amplified[nidx],OsGismeanl2[nidx]), title = "DCIS", xlabL = "HER2 ampl
```

```
## Warning in if (chisqtest == T) {: the condition has length > 1 and only the
## first element will be used

## Warning in chisq.test(tab): Chi-squared approximation may be incorrect

## Warning in if (chisqtest == T) {: the condition has length > 1 and only the
## first element will be used
```

```
ContTable(table(OsloGistLogR$her2_amplified[-nidx], OsGismeanl2[-nidx]), title = "IDC", xlabL = "HER2 a
```

```
## Warning in if (chisqtest == T) {: the condition has length > 1 and only the
## first element will be used

## Warning in chisq.test(tab): Chi-squared approximation may be incorrect

## Warning in if (chisqtest == T) {: the condition has length > 1 and only the
## first element will be used
```



9 CCL-ERBB2 amplification based on FISH images: Figure 5

9.1 DCIS analysis

First read in pathology information and summary of our image data

```
all.mat<-read.csv("data/DCIS_CCLdata_new.csv", stringsAsFactors = F)
```

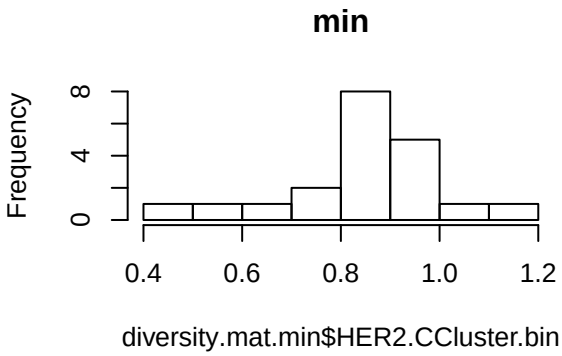
9.1.1 Diversity

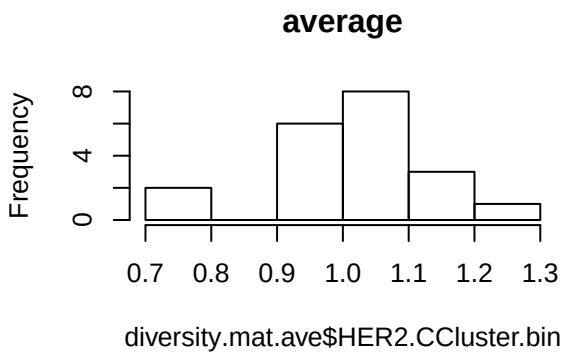
Now compute the diversity for each individual image

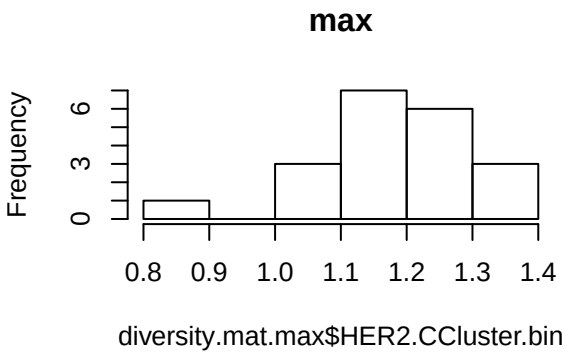
```
attach(all.mat)
diversity.mat <- NULL
for (i in 1:20) {
    indices <- unique(all.mat$index[all.mat$sample_ID == i])
    for (ind in indices) {
      temp.mat <- all.mat[all.mat$index == ind & all.mat$sample_ID == i, ]
      cep17.abs <- table(temp.mat$CEP17)
      her2.abs <- table(temp.mat$HER2)
      ccluster.abs <- table(temp.mat$CCluster)
      her2.ratio <- table(temp.mat$HER2 / temp.mat$CEP17)
      ccluster.ratio <- table(temp.mat$CCluster / temp.mat$CEP17)
      df = as.data.frame(cbind(temp.mat$CCluster, temp.mat$CEP17))
      colnames(df) = c('CCluster', 'CEP17')
      ccluster.uniq <- ddply(df, .(df$CCluster, df$CEP17), nrow)$V1
      df = as.data.frame(cbind(temp.mat$HER2, temp.mat$CEP17))
      colnames(df) = c('HER2', 'CEP17')
      her2.uniq <- ddply(df, .(df$HER2, df$CEP17), nrow)$V1
      her2.ccluster.bin <- c(length(which(temp.mat$her2.bin == TRUE & temp.mat$cluster.bin == TRUE)), le
      df = as.data.frame(cbind(temp.mat$HER2, temp.mat$CCluster))
      colnames(df) = c("HER2", "CCluster")
      her2.ccluster.uniq = ddply(df, .(df$HER2, df$CCluster), nrow)$V1
      her2.ccluster.ratio <- temp.mat$HER2 / temp.mat$CEP17
      diversity.mat <- rbind(diversity.mat, c(i, ind, mean(temp.mat$age), mean(temp.mat$scale.log.age),
    }
}
diversity.mat <- as.data.frame(diversity.mat)
colnames(diversity.mat) <- c('Sample_ID', 'Index', 'age', 'scale.log.age', 'ER', 'PR', 'p53', 'Ki67', '

diversity.mat.ave <- NULL
diversity.mat.max <- NULL
diversity.mat.min <- NULL
for (i in 1:20) {
    temp.div <- diversity.mat[diversity.mat$Sample_ID == i, 3:ncol(diversity.mat)]
    diversity.mat.ave <- rbind(diversity.mat.ave, c(i, apply(temp.div, 2, mean)))
    diversity.mat.max <- rbind(diversity.mat.max, c(i, apply(temp.div, 2, max)))
    diversity.mat.min <- rbind(diversity.mat.min, c(i, apply(temp.div, 2, min)))
}
diversity.mat.ave <- as.data.frame(diversity.mat.ave)
diversity.mat.max <- as.data.frame(diversity.mat.max)
diversity.mat.min <- as.data.frame(diversity.mat.min)

detach(all.mat)
```

Look at the distribution of diversity:

```
par(mfrow=c(2,2))
hist(diversity.mat.min$HER2.CCluster.bin, main="min")
hist(diversity.mat.ave$HER2.CCluster.bin, main="average")
hist(diversity.mat.max$HER2.CCluster.bin, main="max")
```





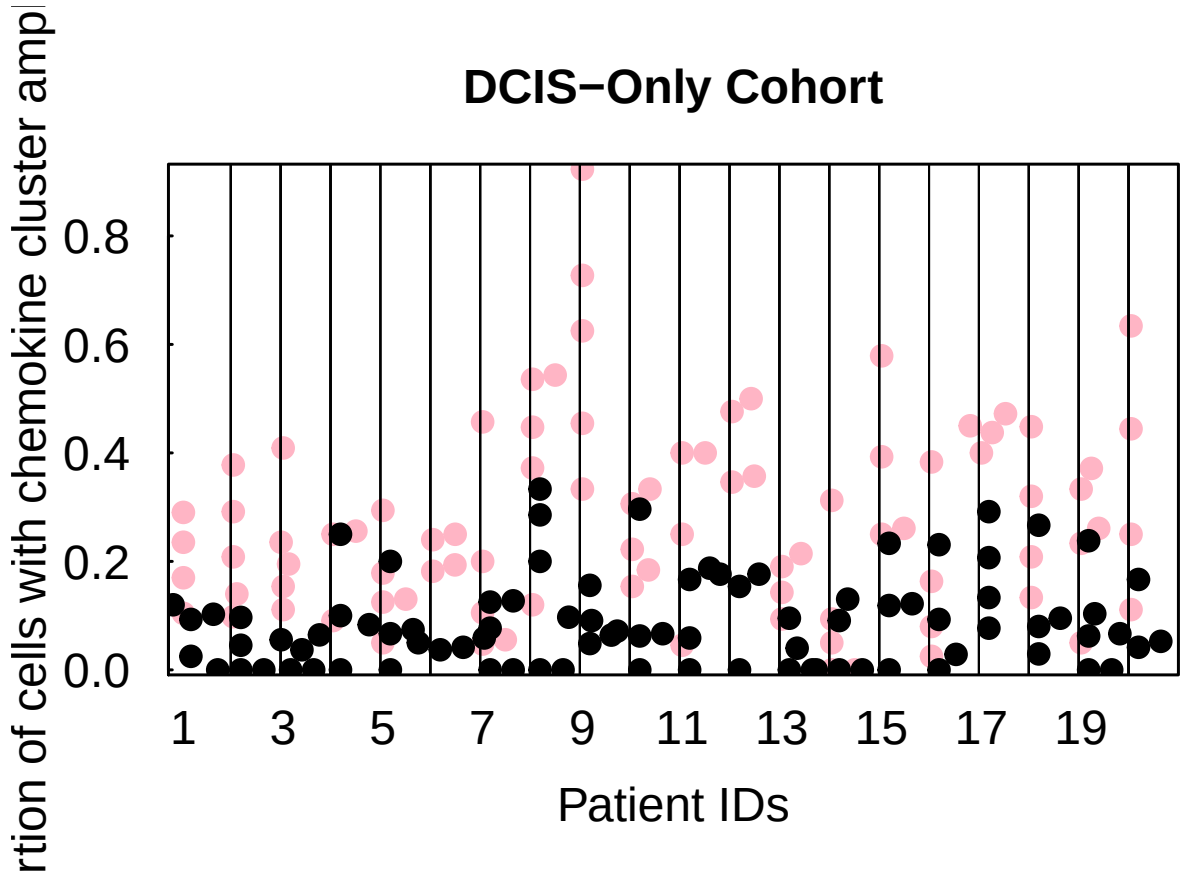



```
attach(all.mat)
prop.mat.sample <- matrix(NA, nrow = 20, ncol = 2)
for (i in 1:20) {
    prop.mat.sample[i, ] <- c(mean(cluster.bin[her2.bin == TRUE & sample_ID == i]), mean(cluster.bin[he
}

prop.mat.slides <- NULL
for (i in 1:20) {
    for (j in unique(index[sample_ID == i])) {
        prop.mat.slides <- rbind(prop.mat.slides, c(i, j, mean(cluster.bin[her2.bin == TRUE & sample_ID
    }
}
colnames(prop.mat.slides) <- c('Sample_ID', 'index', 'Her2.DCIS', 'NoHer2.DCIS')
prop.mat.slides[is.nan(prop.mat.slides) == TRUE] <- NA
prop.mat.slides <- as.data.frame(prop.mat.slides)

detach(all.mat)
```

Plot the distribution of HER2 cells DCIS

```
prop.mat.slides$Her2.Sample_ID <- 20 * prop.mat.slides$Sample_ID - 19
prop.mat.slides$NoHer2.Sample_ID <- 20 * prop.mat.slides$Sample_ID - 16
par(mfrow = c(1, 1), las = 1, cex.axis = 1.5, tcl = 0.1, xaxs = 'i', yaxs = 'i')

beeswarm(prop.mat.slides$Her2.DCIS ~ prop.mat.slides$Her2.Sample_ID,
         col = 'pink1', xlab = 'Patient IDs', ylab = 'Proportion of cells with chemokine cluster amplifi

beeswarm(prop.mat.slides$NoHer2.DCIS ~ prop.mat.slides$NoHer2.Sample_ID, col = 'black', lwd = 1.5, pch =
         cex = 1.5, at = unique(prop.mat.slides$NoHer2.Sample_ID))
for (i in (20 * prop.mat.slides$Sample_ID)) {
```

```
    lines(c(i, i), c(-0.5, 2))
}
```

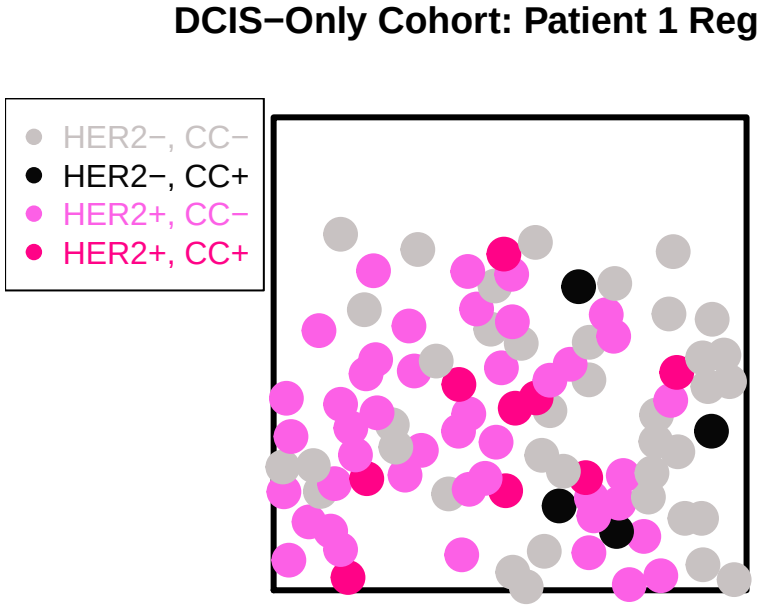



```
#legend('topleft', c('Cells with HER2 amplification', 'Cells without HER2 amplication'), text.col = c('
```

9.1.2 Spatial statistics

Plot an example of the tissue: eg. patient1, region 1

```
i<-1
ind<-1

    temp.mat <- all.mat[all.mat$index == ind & all.mat$sample_ID == i, ]
    temp.mat$m <- 'HER2-, CC-'
    temp.mat$m[temp.mat$her2.bin == TRUE & temp.mat$cluster.bin == TRUE] <- 'HER2+, CC+'
    temp.mat$m[temp.mat$her2.bin == FALSE & temp.mat$cluster.bin == TRUE] <- 'HER2-, CC+'
    temp.mat$m[temp.mat$her2.bin == TRUE & temp.mat$cluster.bin == FALSE] <- 'HER2+, CC-'
    temp.sp <- as.ppp(as.data.frame(cbind(temp.mat$x, temp.mat$y)), c(0, 1, 0, 1))
    temp.sp$m <- as.character(temp.mat$m)
    marks.uniq <- c('HER2-, CC-', 'HER2-, CC+', 'HER2+, CC-', 'HER2+, CC+')
    marks(temp.sp) <- sapply(1:length(temp.mat$m), function(mm) which(marks.uniq == temp.mat$m[mm]))
    colours.uniq <- c('#C8C2C2', '#070707', '#FC60E6', '#FF0387')
    pch.uniq <- c(19, 19, 19, 19)
    plot(temp.sp, border = 'black', cols = function(x) colours.uniq[x], lwd = 3, cex = 2, pch = functio
    legend('topleft', c('HER2-, CC-', 'HER2-, CC+', 'HER2+, CC-', 'HER2+, CC+'), text.col = colours.uni
```

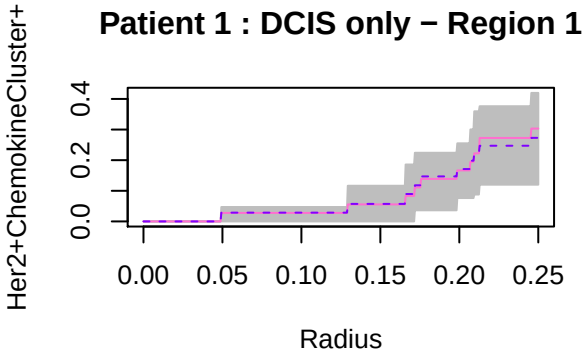


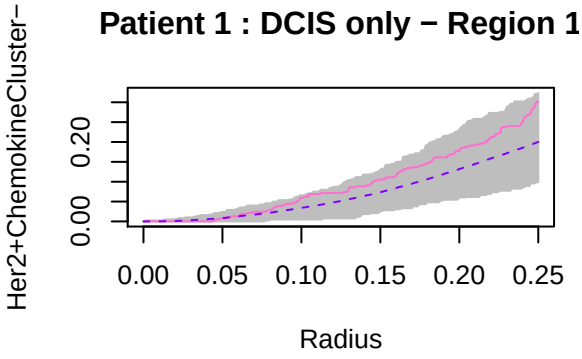
Spatial modelling:

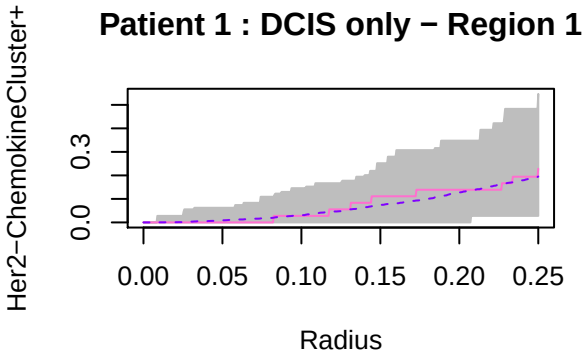
An example for patient 1, region 1:

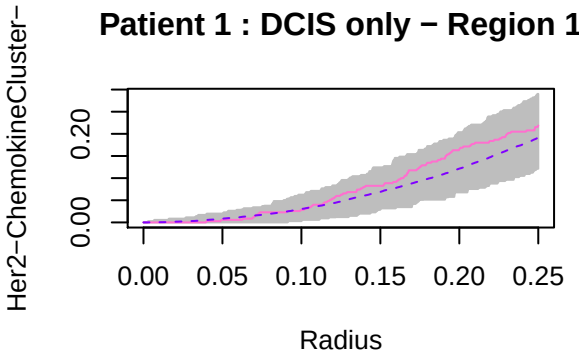
```
set.seed(1206)

genotypes <- c('Her2-ChemokineCluster-', 'Her2+ChemokineCluster+', 'Her2-ChemokineCluster+', 'Her2+Chem
phenotypes <- c('HER2.Neg.Chemokine.Neg', 'HER2.Pos.Chemokine.Pos', 'HER2.Neg.Chemokine.Pos', 'HER2.Pos

pairs1 <- c(2, 2, 2, 2, 4, 4, 4, 4, 3, 3, 3, 3, 1, 1, 1, 1)
pairs2 <- c(2, 4, 3, 1, 4, 3, 1, NA, 3, 1, NA, NA, 1, NA, NA, NA)

eqfun <- function(m1, m2) {
   m1 == m2
}


i<-1
ind<-1
par(mfrow=c(2,2))

temp.mat <- all.mat[all.mat$index == ind & all.mat$sample_ID == i, ]
    temp.mat$m <- 'HER2.Neg.Chemokine.Neg'
    temp.mat$m[temp.mat$her2.bin == TRUE & temp.mat$cluster.bin == TRUE] <- 'HER2.Pos.Chemokine.Pos'
    temp.mat$m[temp.mat$her2.bin == FALSE & temp.mat$cluster.bin == TRUE] <- 'HER2.Neg.Chemokine.Pos'
    temp.mat$m[temp.mat$her2.bin == TRUE & temp.mat$cluster.bin == FALSE] <- 'HER2.Pos.Chemokine.Neg'
    temp.sp <- as.ppp(as.data.frame(cbind(temp.mat$x, temp.mat$y)), c(0, 1, 0, 1))
    marks(temp.sp) <- as.factor(temp.mat$m)
    temp.sp.marks <- as.character(unique(marks(temp.sp)))
    E <- list()
    w <- 1
    for (u in 1:length(pairs1)) {
      if (is.na(pairs2[u]) == TRUE) {
        E[[w]] <- NA
      } else if (!(phenotypes[pairs1[u]] %in% temp.sp.marks) | !(phenotypes[pairs2[u]] %in% temp.sp.mar
```

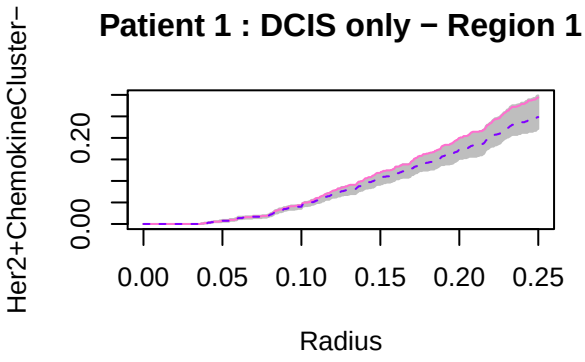
```
      E[[w]] <- NA
    } else {
      E[[w]] <- envelope(temp.sp, Kcross, nsim = nrow(temp.mat), i = phenotypes[pairs1[u]], j = pheno
    }
    w <- w + 1
  }
  w <- w - 1
  # plotting function below:

  for (ww in 1:w) {
    main = paste0('Patient ', i, ' : DCIS only - Region ', which(indices == ind))
    if (mode(E[[ww]]) == "logical") {
      plot(rnorm(100), rnorm(100), type = 'n', main = '', ylim = c(0, 1), axes = FALSE, xlab = '', yla
    } else {
      if (is.nan(E[[ww]]$obs[1]) == TRUE) {
        plot(rnorm(100), rnorm(100), type = 'n', main = '', ylim = c(0, 1), axes = FALSE, xlab = '', y
      } else {
        plot(E[[ww]], main = main, xlab = 'Radius', ylab = paste0(genotypes[pairs1[ww]], ' vs.\n', ge
      }
    }
  }
```

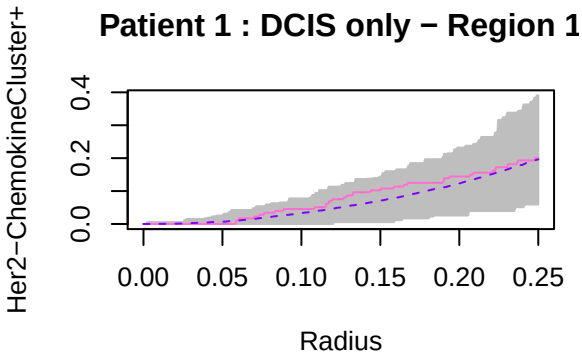


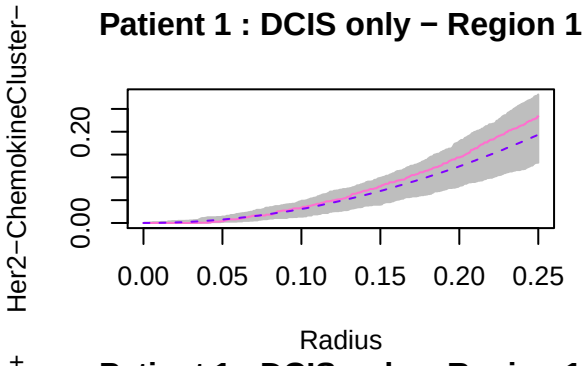


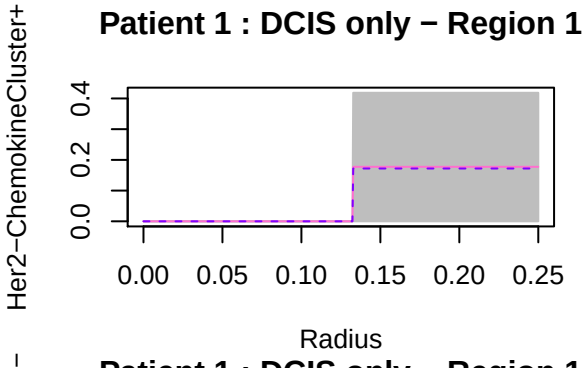


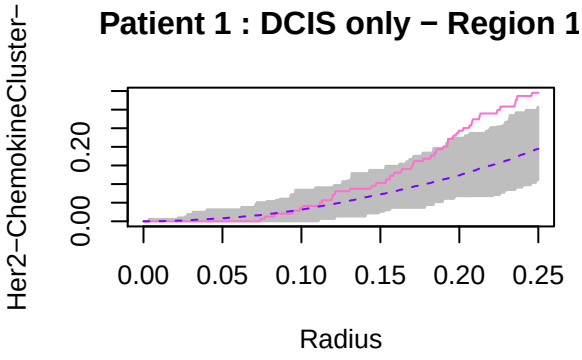


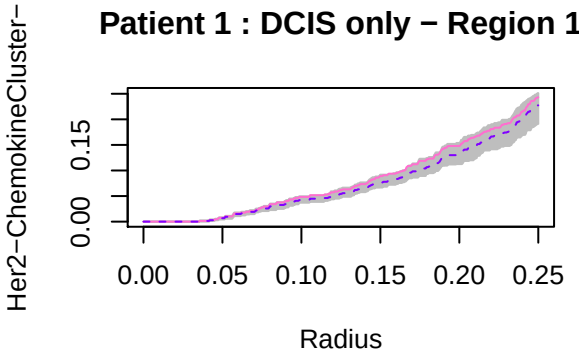












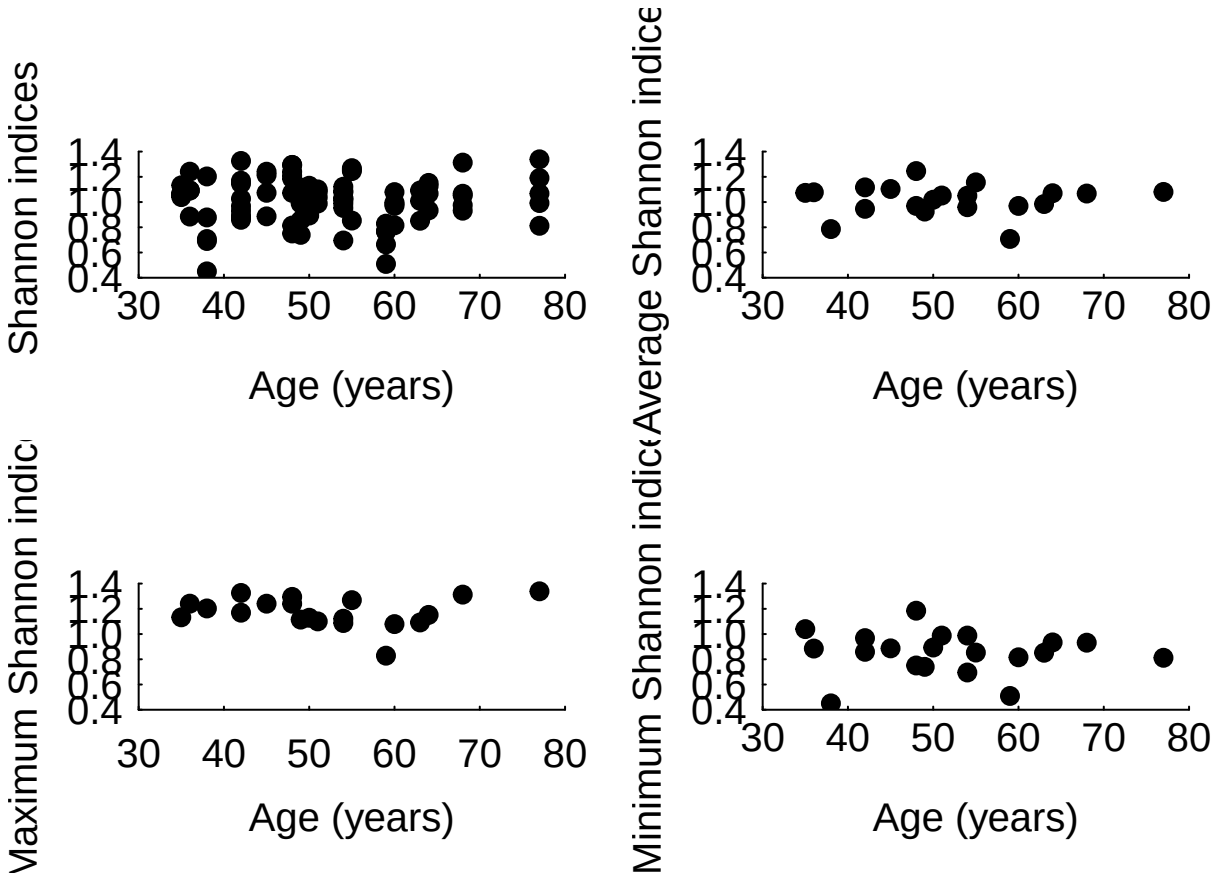
Run for the rest of the cohort, and save to pdf:

```
set.seed(1206)

genotypes <- c('Her2-ChemokineCluster-', 'Her2+ChemokineCluster+', 'Her2-ChemokineCluster+', 'Her2+Chem
phenotypes <- c('HER2.Neg.Chemokine.Neg', 'HER2.Pos.Chemokine.Pos', 'HER2.Neg.Chemokine.Pos', 'HER2.Pos
```

```
pairs1 <- c(2, 2, 2, 2, 4, 4, 4, 4, 3, 3, 3, 3, 1, 1, 1, 1)
pairs2 <- c(2, 4, 3, 1, 4, 3, 1, NA, 3, 1, NA, NA, 1, NA, NA, NA)

eqfun <- function(m1, m2) {
   m1 == m2
}

pdf('output/Spatial_Modeling_DCIS_Only.pdf', height = 16, width = 19.5)
for (i in 1:20) {
  indices <- unique(all.mat$index[all.mat$sample_ID == i])
  for (ind in indices) {
    par(mfrow = c(4, 4), mai = c(1, 1.2, 1, 1))
    temp.mat <- all.mat[all.mat$index == ind & all.mat$sample_ID == i, ]
    temp.mat$m <- 'HER2.Neg.Chemokine.Neg'
    temp.mat$m[temp.mat$her2.bin == TRUE & temp.mat$cluster.bin == TRUE] <- 'HER2.Pos.Chemokine.Pos'
    temp.mat$m[temp.mat$her2.bin == FALSE & temp.mat$cluster.bin == TRUE] <- 'HER2.Neg.Chemokine.Pos'
    temp.mat$m[temp.mat$her2.bin == TRUE & temp.mat$cluster.bin == FALSE] <- 'HER2.Pos.Chemokine.Neg'
    temp.sp <- as.ppp(as.data.frame(cbind(temp.mat$x, temp.mat$y)), c(0, 1, 0, 1))
    marks(temp.sp) <- as.factor(temp.mat$m)
    temp.sp.marks <- as.character(unique(marks(temp.sp)))
    E <- list()
    w <- 1
    for (u in 1:length(pairs1)) {
      if (is.na(pairs2[u]) == TRUE) {
        E[[w]] <- NA
      } else if (!(phenotypes[pairs1[u]] %in% temp.sp.marks) | !(phenotypes[pairs2[u]] %in% temp.sp.mar
        E[[w]] <- NA
      } else {
        E[[w]] <- envelope(temp.sp, Kcross, nsim = nrow(temp.mat), i = phenotypes[pairs1[u]], j = pheno
      }
      w <- w + 1
    }
    w <- w - 1
    # plotting function below:

    for (ww in 1:w) {
      main = paste0('Patient ', i, ' : DCIS only - Region ', which(indices == ind))
      if (mode(E[[ww]]) == "logical") {
        plot(rnorm(100), rnorm(100), type = 'n', main = '', ylim = c(0, 1), axes = FALSE, xlab = '', yla
      } else {
        if (is.nan(E[[ww]]$obs[1]) == TRUE) {
          plot(rnorm(100), rnorm(100), type = 'n', main = '', ylim = c(0, 1), axes = FALSE, xlab = '', y
        } else {
          plot(E[[ww]], main = main, xlab = 'Radius', ylab = paste0(genotypes[pairs1[ww]], ' vs.\n', ge
        }
      }
    }
  }
}
dev.off()
```

9.1.3 Associations between diversity and age: Figure 5

Visualise the data: Association with age



Compute the correlation coefficient and p-value:

```
cor.test(diversity.mat.ave$age, diversity.mat.ave$HER2.CCluster.bin, use="complete")
```

```
##
##  Pearson's product-moment correlation
##
## data:  diversity.mat.ave$age and diversity.mat.ave$HER2.CCluster.bin
## t = 0.071272, df = 18, p-value = 0.944
## alternative hypothesis: true correlation is not equal to 0
## 95 percent confidence interval:
##  -0.4289122  0.4559285
## sample estimates:
##        cor
## 0.01679657
```

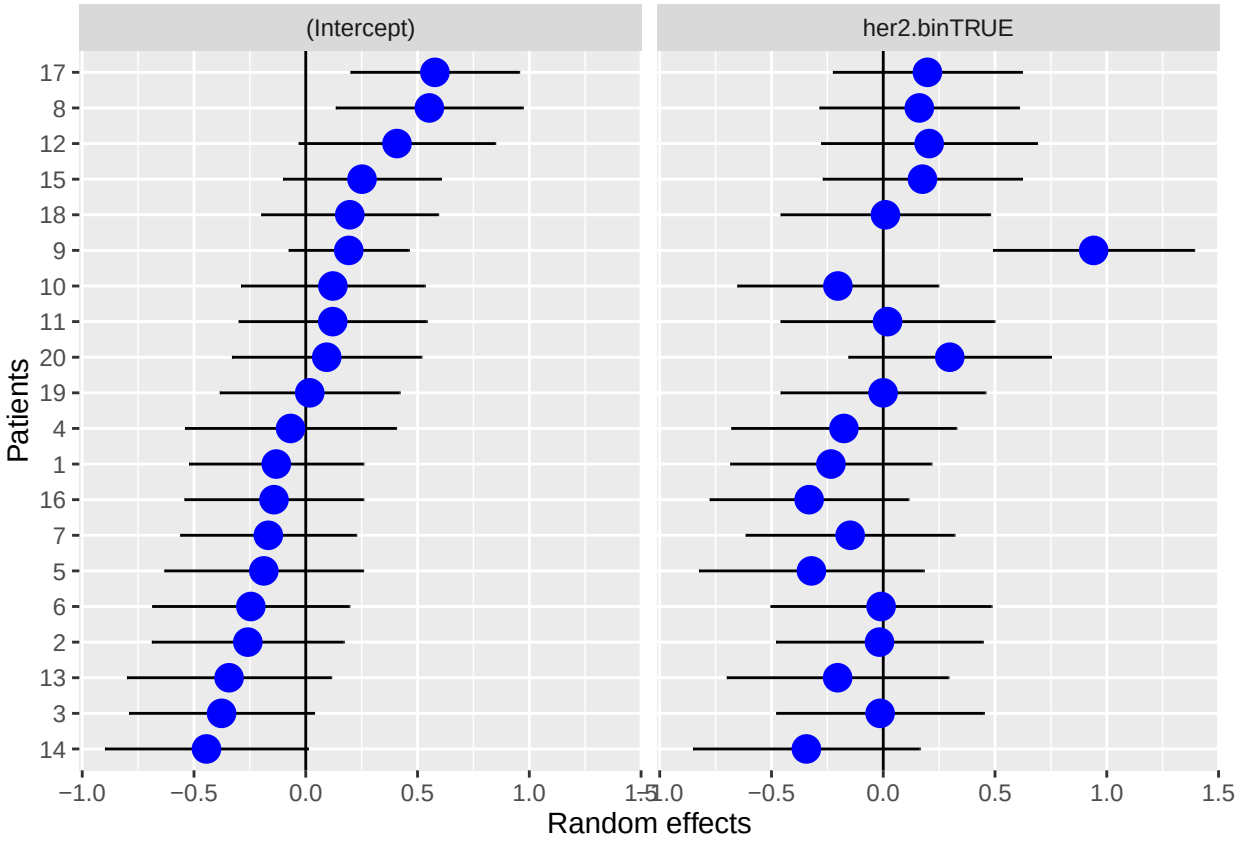
9.1.4 Mixed effects modelling

Mixed effects modelling: (note that the leukocyte data file has been updated to remove samples with poor quality images - S11 25135, 24603 and 24809 are removed)

```
# Mixed effect modeling table
dcis.only.mixed <- glmer(cluster.bin ~ 1 + her2.bin + (1 + her2.bin | sample_ID), data = all.mat, family
```

```
ggCaterpillar(ranef(dcis.only.mixed, condVar = TRUE), QQ = FALSE)
```

```
## $sample_ID
```



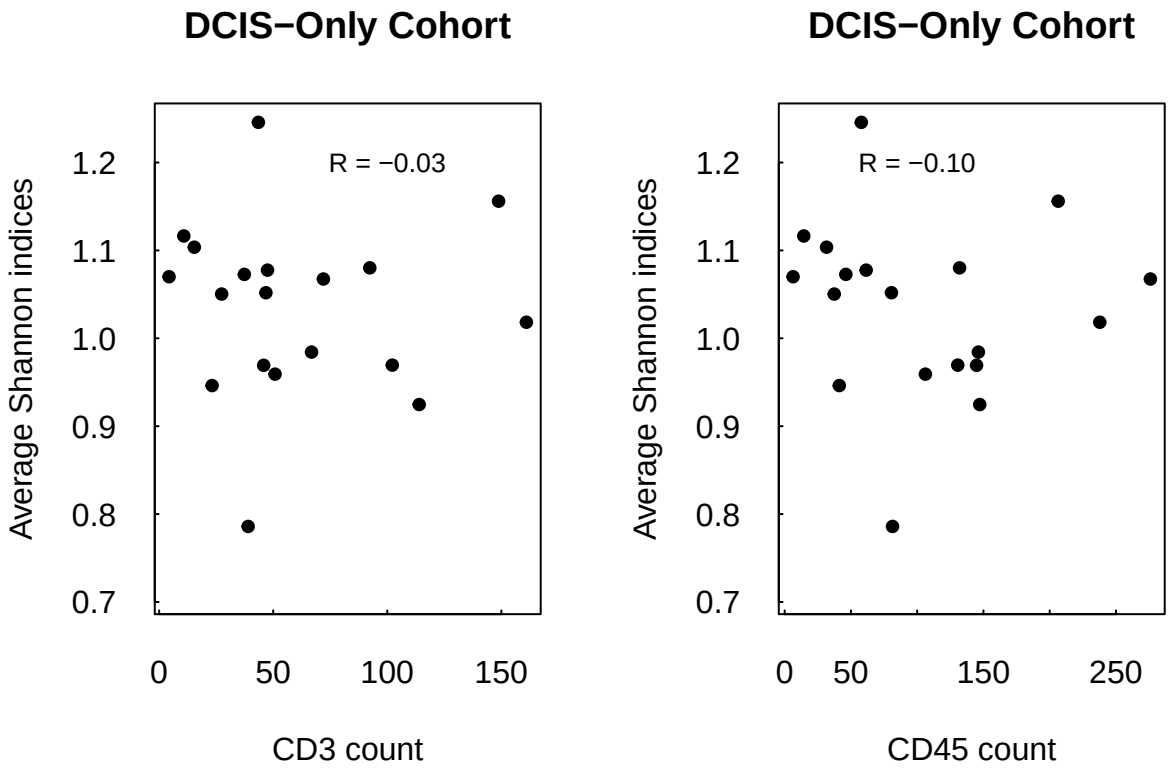

```
summary(dcis.only.mixed)
```

```
## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: binomial  ( logit )
## Formula: cluster.bin ~ 1 + her2.bin + (1 + her2.bin | sample_ID)
##    Data: all.mat
##
##      AIC      BIC   logLik deviance df.resid
##   4412.2   4444.8  -2201.1   4402.2     4979
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -1.0459 -0.4942 -0.3387 -0.2702  4.0595
##
## Random effects:
##  Groups    Name         Variance Std.Dev. Corr
##  sample_ID (Intercept)  0.1287   0.3587
##            her2.binTRUE 0.1398   0.3740   0.11
## Number of obs: 4984, groups:  sample_ID, 20
##
## Fixed effects:
##              Estimate Std. Error z value Pr(>|z|)
```

```
## (Intercept)   -2.3579     0.1141  -20.66   <2e-16 ***
## her2.binTRUE   1.3140     0.1255   10.47   <2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr)
## her2.bnTRUE -0.406
```

9.1.5 Correlations between shannon indicies and CD3 or CD45 counts

```
leukocytes <- read.csv('data/leukocytes_dcis_only.csv', stringsAsFactors = FALSE)
leukocytes <- leukocytes[-which(is.na(leukocytes$image.) == TRUE), ]
leukocytes$cd45 <- as.numeric(leukocytes$cd45)

leukocytes.ave.cd45 <- sapply(1:20, function(i) mean(leukocytes[leukocytes$Pathology.No == i, 3], na.rm
leukocytes.ave.cd3 <- sapply(1:20, function(i) mean(leukocytes[leukocytes$Pathology.No == i, 4], na.rm =
leukocytes.ave.cd3p <- sapply(1:20, function(i) mean(leukocytes[leukocytes$Pathology.No == i, 4] / leuk

par(mfrow = c(1, 2), tcl = 0.1, las = 1)
plot(leukocytes.ave.cd3, diversity.mat.ave$HER2.CCluster.bin, ylab = 'Average Shannon indices', main =
text(100, 1.2, 'R = -0.03', cex = .8)
plot(leukocytes.ave.cd45, diversity.mat.ave$HER2.CCluster.bin, ylab = 'Average Shannon indices', main =
text(100, 1.2, 'R = -0.10', cex = .8)
```



Compute the correlation coefficients and p values

```
cor.test(leukocytes.ave.cd3, diversity.mat.ave$HER2.CCluster.bin)
```

```
##
##  Pearson's product-moment correlation
##
## data:  leukocytes.ave.cd3 and diversity.mat.ave$HER2.CCluster.bin
## t = -0.13629, df = 17, p-value = 0.8932
## alternative hypothesis: true correlation is not equal to 0
## 95 percent confidence interval:
##  -0.4800423  0.4275892
## sample estimates:
##         cor
## -0.03303627
```

```
cor.test(leukocytes.ave.cd45, diversity.mat.ave$HER2.CCluster.bin)
```

```
##
##  Pearson's product-moment correlation
##
## data:  leukocytes.ave.cd45 and diversity.mat.ave$HER2.CCluster.bin
## t = -0.42962, df = 17, p-value = 0.6729
## alternative hypothesis: true correlation is not equal to 0
## 95 percent confidence interval:
##  -0.5327677  0.3678895
## sample estimates:
##        cor
## -0.1036375
```

9.2 IDC cases:

Load the data for the IDC cases:

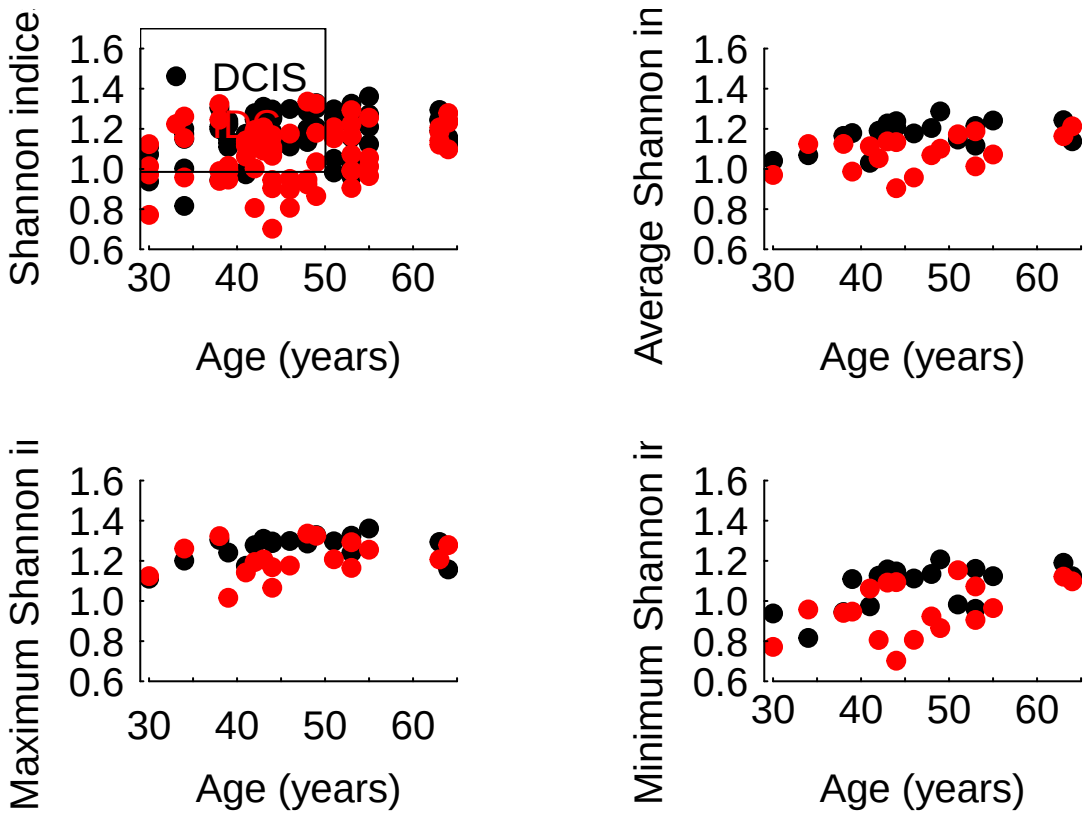
```
all.mat<-read.csv("data/IDC_HER2_CCL_data.csv", stringsAsFactors = F)
all.mat$scale.log.age <- scale(log(all.mat$age))
```

9.2.1 Compute diversity

```
diversity.mat <- NULL
for (i in 1:18) {
    for (t in 0:1) {
        indices <- unique(all.mat$index[all.mat$sample_ID == i & all.mat$Type == t])
        for (ind in indices) {
            temp.mat <- all.mat[all.mat$index == ind & all.mat$sample_ID == i & all.mat$Type == t, ]
            cep17.abs <- table(temp.mat$CEP17)
            her2.abs <- table(temp.mat$HER2)
            ccluster.abs <- table(temp.mat$CCluster)
            her2.ratio <- table(temp.mat$HER2 / temp.mat$CEP17)
            ccluster.ratio <- table(temp.mat$CCluster / temp.mat$CEP17)
            df = as.data.frame(cbind(temp.mat$CCluster, temp.mat$CEP17))
            colnames(df) = c('CCluster', 'CEP17')
            ccluster.uniq <- ddply(df, .(df$CCluster, df$CEP17), nrow)$V1
            df = as.data.frame(cbind(temp.mat$HER2, temp.mat$CEP17))
            colnames(df) = c('HER2', 'CEP17')
            her2.uniq <- ddply(df, .(df$HER2, df$CEP17), nrow)$V1
```

```
            her2.ccluster.bin <- c(length(which(temp.mat$her2.bin == TRUE & temp.mat$cluster.bin == TRU

            df = as.data.frame(cbind(temp.mat$HER2, temp.mat$CCluster))
            colnames(df) = c("HER2", "CCluster")
            her2.ccluster.uniq = ddply(df, .(df$HER2, df$CCluster), nrow)$V1

            her2.ccluster.ratio <- temp.mat$HER2 / temp.mat$CEP17
            if (length(which(her2.ccluster.bin == 0)) > 0) {
                her2.ccluster.bin <- her2.ccluster.bin + 1
            }
            her2.ccluster.bin <- her2.ccluster.bin / sum(her2.ccluster.bin)
            diversity.mat <- rbind(diversity.mat, c(i, t, ind, mean(temp.mat$age), mean(temp.mat$scale.l
        }
    }
}

diversity.mat <- as.data.frame(diversity.mat)
colnames(diversity.mat) <- c('Sample_ID', 'Type', 'Index', 'age', 'scale.log.age', 'ER', 'PR', 'p53', 'K

#pathology$Ki.67 <- sapply(1:nrow(pathology), function(i) as.numeric(sub("%", "", pathology$Ki.67[i])))
diversity.mat.ave <- NULL
diversity.mat.max <- NULL
diversity.mat.min <- NULL
for (i in 1:18) {
    for (t in 0:1) {
        temp.div <- diversity.mat[diversity.mat$Sample_ID == i & diversity.mat$Type == t, 4:ncol(diversi
        diversity.mat.ave <- rbind(diversity.mat.ave, c(i, t, apply(temp.div, 2, mean)))
        diversity.mat.max <- rbind(diversity.mat.max, c(i, t, apply(temp.div, 2, max)))
        diversity.mat.min <- rbind(diversity.mat.min, c(i, t, apply(temp.div, 2, min)))
    }
}

diversity.mat.ave <- as.data.frame(diversity.mat.ave)
diversity.mat.max <- as.data.frame(diversity.mat.max)
diversity.mat.min <- as.data.frame(diversity.mat.min)
colnames(diversity.mat.ave) <- c('Sample_ID', 'Type', 'age', 'scale.log.age', 'ER', 'PR', 'p53', 'Ki67'
colnames(diversity.mat.max) <- c('Sample_ID', 'Type', 'age', 'scale.log.age', 'ER', 'PR', 'p53', 'Ki67'
colnames(diversity.mat.min) <- c('Sample_ID', 'Type', 'age', 'scale.log.age', 'ER', 'PR', 'p53', 'Ki67'
diversity.mat.ave.dcis.idc <- diversity.mat.ave
diversity.mat.min.dcis.idc <- diversity.mat.min
diversity.mat.max.dcis.idc <- diversity.mat.max
```

9.3 Relationship between Diversity and age (Figure 5F):



Compute the correlation coefficient and p value:

```
# DCIS case:
cor.test(diversity.mat.ave$age[diversity.mat.ave$Type == 0], diversity.mat.ave$HER2.CCluster.bin[diversi
         use="complete")
```

```
##
##  Pearson's product-moment correlation
##
## data:  diversity.mat.ave$age[diversity.mat.ave$Type == 0] and diversity.mat.ave$HER2.CCluster.bin[di
## t = 2.0333, df = 16, p-value = 0.05896
## alternative hypothesis: true correlation is not equal to 0
## 95 percent confidence interval:
##  -0.01742104  0.75935855
## sample estimates:
##       cor
## 0.4531345
```

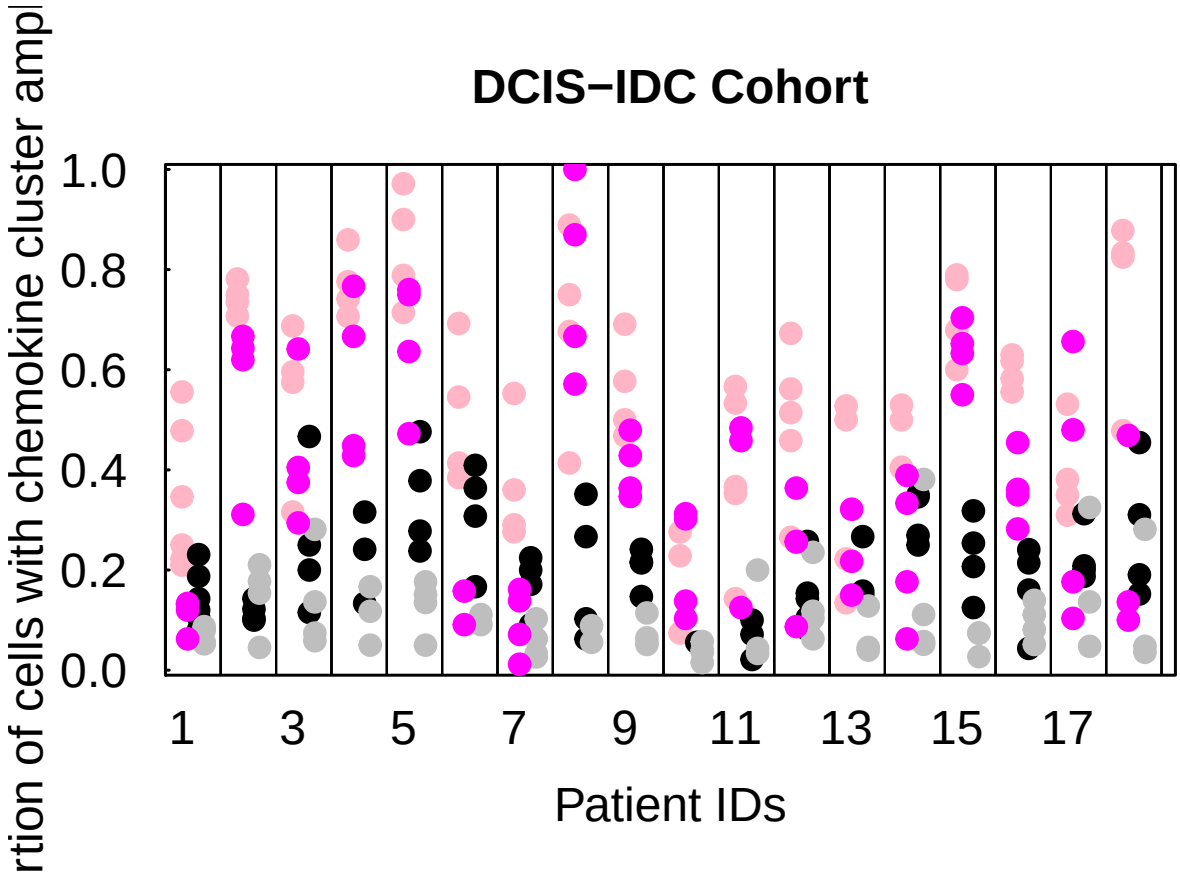
```
# IDC case:
cor.test(diversity.mat.ave$age[diversity.mat.ave$Type == 1], diversity.mat.ave$HER2.CCluster.bin[diversi
         use="complete")
```

```
##
##  Pearson's product-moment correlation
##
## data:  diversity.mat.ave$age[diversity.mat.ave$Type == 1] and diversity.mat.ave$HER2.CCluster.bin[di
## t = 2.0168, df = 16, p-value = 0.06082
```

```
## alternative hypothesis: true correlation is not equal to 0
## 95 percent confidence interval:
##  -0.02110128  0.75779549
## sample estimates:
##      cor
## 0.450204
```

9.3.1 Summary of amplification diversity the data sets

```
attach(all.mat)
prop.mat.slides <- NULL
for (i in 1:18) {
    for (j in unique(index[sample_ID == i])) {
        prop.mat.slides <- rbind(prop.mat.slides, c(i, j, mean(cluster.bin[her2.bin == TRUE & Type == 0
    }
}
colnames(prop.mat.slides) <- c('Sample_ID', 'index', 'Her2.DCIS', 'NoHer2.DCIS', 'Her2.IDC', 'NoHer2.IDC
prop.mat.slides[is.nan(prop.mat.slides) == TRUE] <- NA
prop.mat.slides <- as.data.frame(prop.mat.slides)


prop.mat.slides$Her2.DCIS.Sample_ID <- 20 * prop.mat.slides$Sample_ID - 19
prop.mat.slides$Her2.IDC.Sample_ID <- 20 * prop.mat.slides$Sample_ID - 17
prop.mat.slides$NoHer2.DCIS.Sample_ID <- 20 * prop.mat.slides$Sample_ID - 13
prop.mat.slides$NoHer2.IDC.Sample_ID <- 20 * prop.mat.slides$Sample_ID - 11
par(mfrow = c(1, 1), las = 1, cex.axis = 1.5, tcl = 0.1, xaxs = 'i', yaxs = 'i')
beeswarm(prop.mat.slides$Her2.DCIS ~ prop.mat.slides$Her2.DCIS.Sample_ID, col = 'pink1', xlab = 'Patien
beeswarm(prop.mat.slides$NoHer2.DCIS ~ prop.mat.slides$NoHer2.DCIS.Sample_ID, col = 'black', lwd = 1.5,
beeswarm(prop.mat.slides$NoHer2.IDC ~ prop.mat.slides$NoHer2.IDC.Sample_ID, col = 'grey', lwd = 1.5, pch
beeswarm(prop.mat.slides$Her2.IDC ~ prop.mat.slides$Her2.IDC.Sample_ID, col = 'magenta', lwd = 1.5, pch
for (i in (20 * prop.mat.slides$Sample_ID)) {
    lines(c(i-5, i-5), c(-0.5, 2))
}
```




```
detach(all.mat)
```

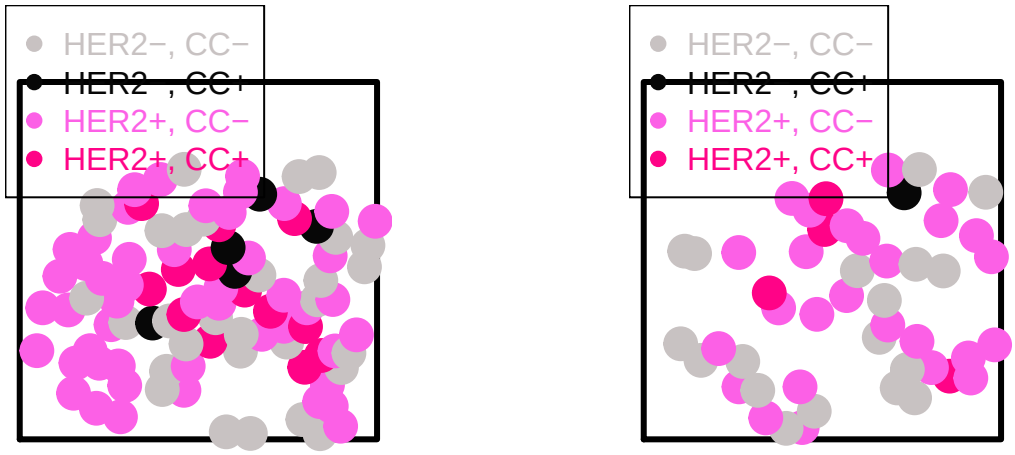
9.3.2 Spatial statistics:

As an example for DCIS-IDC patient 1, region 1, we can firstly plot an example of the tissue, highlighting differences in HER2/CCL status

```
i=1
ind<-1

par(mfrow=c(1,2))

  for (t in 0:1) {
      temp.mat <- all.mat[all.mat$index == ind & all.mat$sample_ID == i & all.mat$Type==t, ]
      temp.mat$m <- 'HER2-, CC-'
      temp.mat$m[temp.mat$her2.bin == TRUE & temp.mat$cluster.bin == TRUE] <- 'HER2+, CC+'
      temp.mat$m[temp.mat$her2.bin == FALSE & temp.mat$cluster.bin == TRUE] <- 'HER2-, CC+'
      temp.mat$m[temp.mat$her2.bin == TRUE & temp.mat$cluster.bin == FALSE] <- 'HER2+, CC-'
      temp.sp <- as.ppp(as.data.frame(cbind(temp.mat$x, temp.mat$y)), c(0, 1, 0, 1))
      temp.sp$m <- as.character(temp.mat$m)

      marks.uniq <- c('HER2-, CC-', 'HER2-, CC+', 'HER2+, CC-', 'HER2+, CC+')
      marks(temp.sp) <- sapply(1:length(temp.mat$m), function(mm) which(marks.uniq == temp.mat$m[mm]))
      if (t == 0) {
        colours.uniq <- c('#C8C2C2', '#070707', '#FC60E6', '#FF0387')
        pch.uniq <- c(19, 19, 19, 19)
```

```
      plot(temp.sp, border = 'black', cols = function(x) colours.uniq[x], lwd = 3, cex = 2, pch = fun
      legend('topleft', c('HER2-, CC-', 'HER2-, CC+', 'HER2+, CC-', 'HER2+, CC+'), text.col = colours
    } else {
      colours.uniq <- c('#C8C2C2', '#070707', '#FC60E6', '#FF0387')
      pch.uniq <- c(19, 19, 19, 19)
      plot(temp.sp, border = 'black', cols = function(x) colours.uniq[x], lwd = 3, cex = 2, pch = fun
      legend('topleft', c('HER2-, CC-', 'HER2-, CC+', 'HER2+, CC-', 'HER2+, CC+'), text.col = colours
    }
  }
```

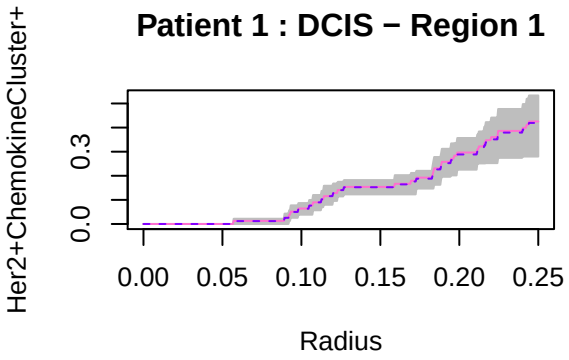
CIS−IDC Cohort: Patient 1 DCIS Re CIS−IDC Cohort: Patient 1 IDC Reg

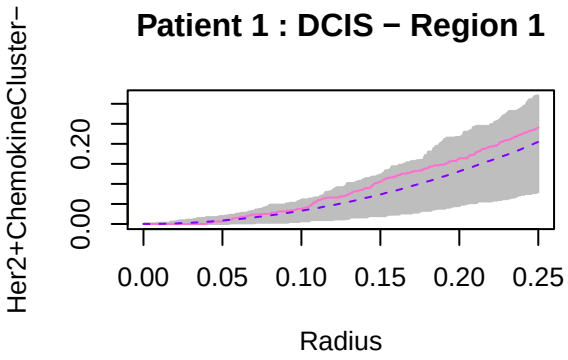


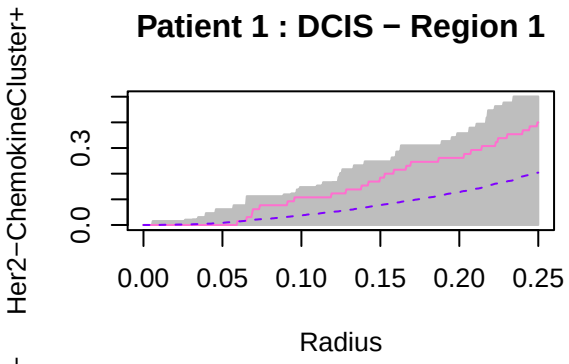
There are four different genotypes, and 4 different phenotypes: 16 combinations to try in terms of spatial clustering

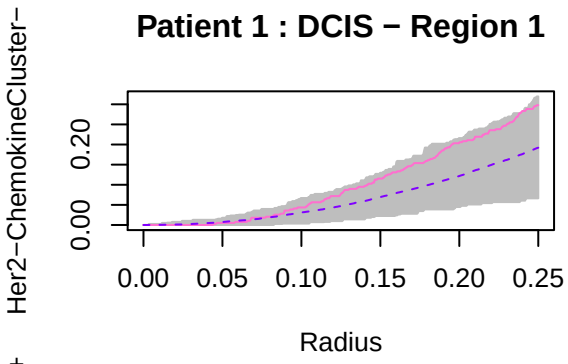
```
genotypes <- c('Her2-ChemokineCluster-', 'Her2+ChemokineCluster+', 'Her2-ChemokineCluster+', 'Her2+Chem
phenotypes <- c('HER2.Neg.Chemokine.Neg', 'HER2.Pos.Chemokine.Pos', 'HER2.Neg.Chemokine.Pos', 'HER2.Pos

pairs1 <- c(2, 2, 2, 2, 4, 4, 4, 4, 3, 3, 3, 3, 1, 1, 1, 1)
pairs2 <- c(2, 4, 3, 1, 4, 3, 1, NA, 3, 1, NA, NA, 1, NA, NA, NA)

eqfun <- function(m1, m2) {
   m1 == m2
}

set.seed(1206)
i <- 1
ind <-1
 par(mfrow = c(2, 2))

for (t in 0:1) {
      temp.mat <- all.mat[all.mat$index == ind & all.mat$sample_ID == i & all.mat$Type == t, ]
      temp.mat$m <- 'HER2.Neg.Chemokine.Neg'
      temp.mat$m[temp.mat$her2.bin == TRUE & temp.mat$cluster.bin == TRUE] <- 'HER2.Pos.Chemokine.Pos'
      temp.mat$m[temp.mat$her2.bin == FALSE & temp.mat$cluster.bin == TRUE] <- 'HER2.Neg.Chemokine.Pos'
      temp.mat$m[temp.mat$her2.bin == TRUE & temp.mat$cluster.bin == FALSE] <- 'HER2.Pos.Chemokine.Neg'
      temp.sp <- as.ppp(as.data.frame(cbind(temp.mat$x, temp.mat$y)), c(0, 1, 0, 1))
      marks(temp.sp) <- as.factor(temp.mat$m)
      temp.sp.marks <- as.character(unique(marks(temp.sp)))
```

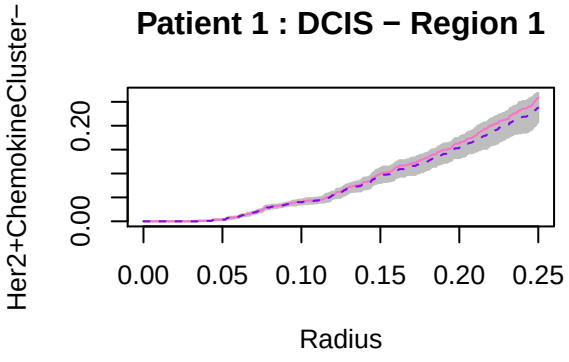
```
      E <- list()
      w <- 1
      for (u in 1:length(pairs1)) {
        if (is.na(pairs2[u]) == TRUE) {
          E[[w]] <- NA
        } else if (!(phenotypes[pairs1[u]] %in% temp.sp.marks) | !(phenotypes[pairs2[u]] %in% temp.sp.ma
          E[[w]] <- NA
        } else {
          E[[w]] <- envelope(temp.sp, Kcross, nsim = nrow(temp.mat), i = phenotypes[pairs1[u]], j = phen
        }
        w <- w + 1
      }
      w <- w - 1
      if (i==1){
      #  ww <- 1
      if (t == 0) {
        for (ww in 1:w) {
          main = paste0('Patient ', i, ' : DCIS - Region ', which(indices == ind))
          if (mode(E[[ww]]) == "logical") {
            plot(rnorm(100), rnorm(100), type = 'n', main = '', ylim = c(0, 1), axes = FALSE, xlab = ''
          } else {
            if (is.nan(E[[ww]]$obs[1]) == TRUE) {
              plot(rnorm(100), rnorm(100), type = 'n', main = '', ylim = c(0, 1), axes = FALSE, xlab =
            } else {
              plot(E[[ww]], main = main, xlab = 'Radius', ylab = paste0(genotypes[pairs1[ww]], ' vs.\n'
            }
          }
      }} else {
        for (ww in 1:w) {
          main = paste0('Patient ', i, ' : IDC - Region ', which(indices == ind))
          if (mode(E[[ww]]) == "logical") {
            plot(rnorm(100), rnorm(100), type = 'n', main = '', ylim = c(0, 1), axes = FALSE, xlab = ''
          } else {
            if (is.nan(E[[ww]]$obs[1]) == TRUE) {
              plot(rnorm(100), rnorm(100), type = 'n', main = '', ylim = c(0, 1), axes = FALSE, xlab =
            } else {
              plot(E[[ww]], main = main, xlab = 'Radius', ylab = paste0(genotypes[pairs1[ww]], ' vs.\n'
            }
          }
        }
    }
  }
}
```

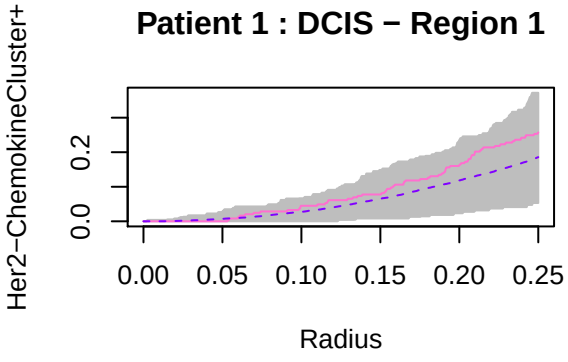

Patient 1 : DCIS − Region 1
Her2+ChemokineCluster+
0.0
0.3
0.00
0.05
0.10
0.15
0.20
0.25
Radius

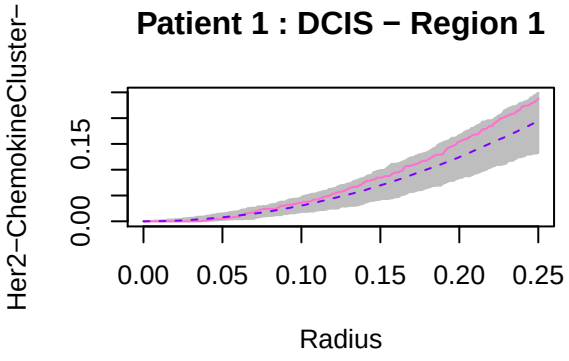

Patient 1 : DCIS − Region 1
Her2+ChemokineCluster−
0.00
0.20
0.00
0.05
0.10
0.15
0.20
0.25
Radius

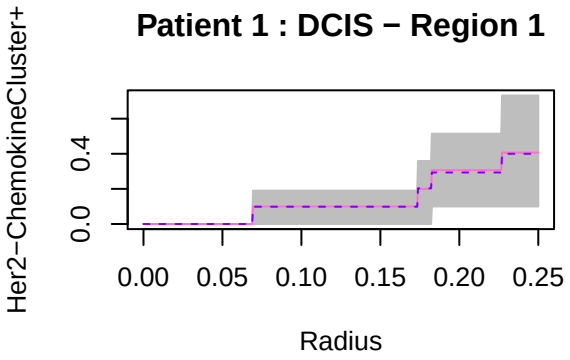

Patient 1 : DCIS − Region 1
Her2−ChemokineCluster+
0.0
0.3
0.00
0.05
0.10
0.15
0.20
0.25
Radius

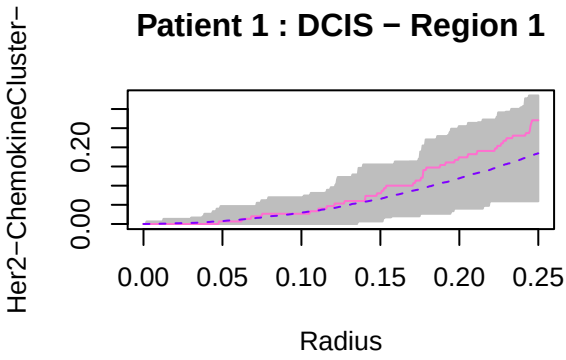

Patient 1 : DCIS − Region 1
Her2−ChemokineCluster−
0.00
0.20
0.00
0.05
0.10
0.15
0.20
0.25
Radius

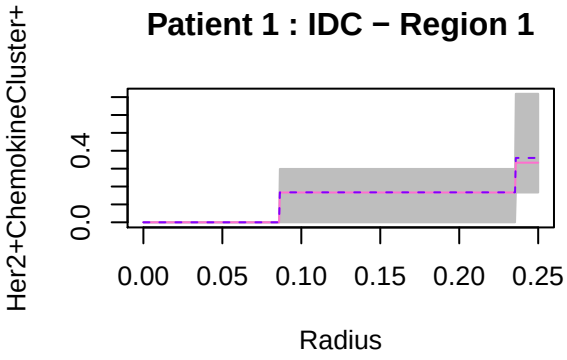

Patient 1 : DCIS − Region 1
Her2+ChemokineCluster−
0.00
0.20
0.00
0.05
0.10
0.15
0.20
0.25
Radius

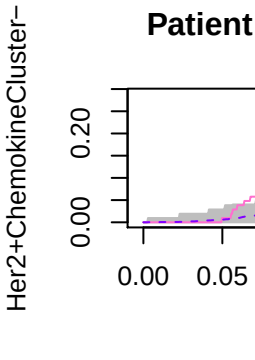

Patient 1 : DCIS − Region 1
Her2−ChemokineCluster+
0.0
0.2
0.00
0.05
0.10
0.15
0.20
0.25
Radius

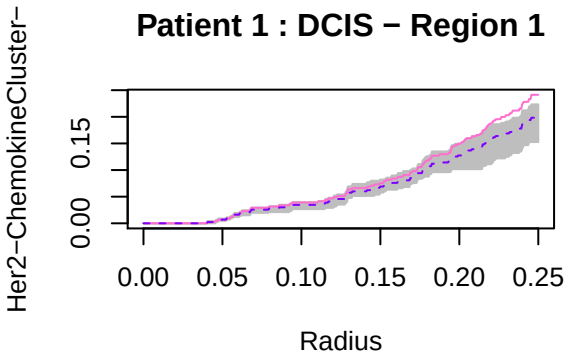

Patient 1 : DCIS − Region 1
Her2−ChemokineCluster−
0.00
0.15
0.00
0.05
0.10
0.15
0.20
0.25
Radius

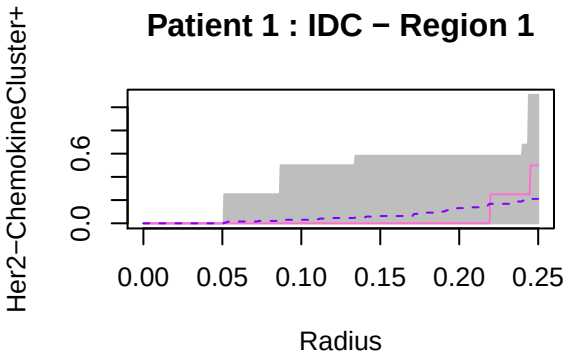

Patient 1 : DCIS − Region 1
Her2−ChemokineCluster+
0.0
0.4
0.00
0.05
0.10
0.15
0.20
0.25
Radius

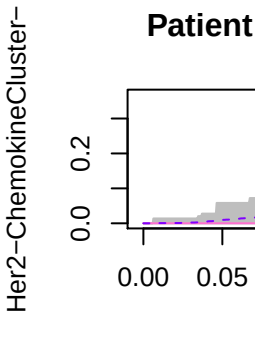

Patient 1 : DCIS − Region 1
Her2−ChemokineCluster−
0.00
0.20
0.00
0.05
0.10
0.15
0.20
0.25
Radius

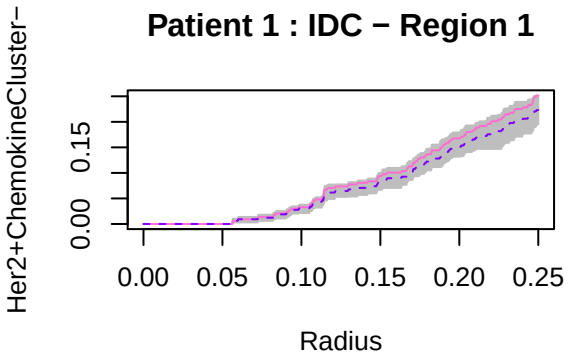

Patient 1 : IDC − Region 1
Her2+ChemokineCluster+
0.0
0.4
0.00
0.05
0.10
0.15
0.20
0.25
Radius

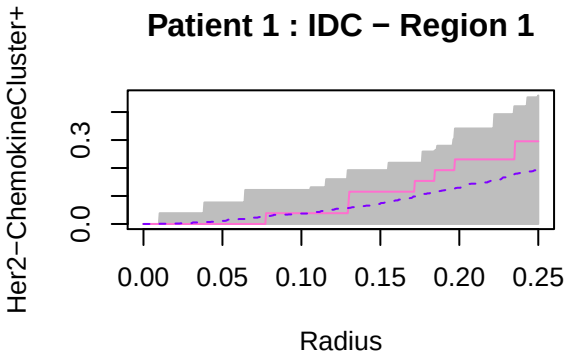

Patient
Her2+ChemokineCluster−
0.00
0.20
0.00
0.05

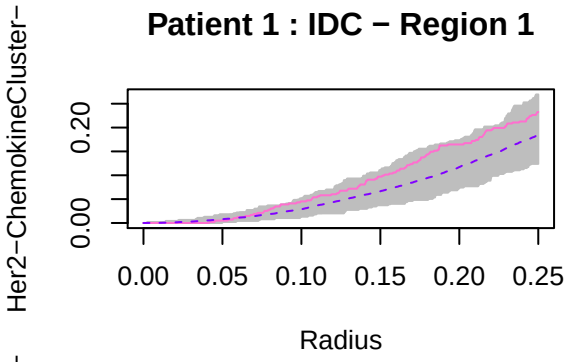

Patient 1 : DCIS − Region 1
Her2−ChemokineCluster−
0.00
0.15
0.00
0.05
0.10
0.15
0.20
0.25
Radius

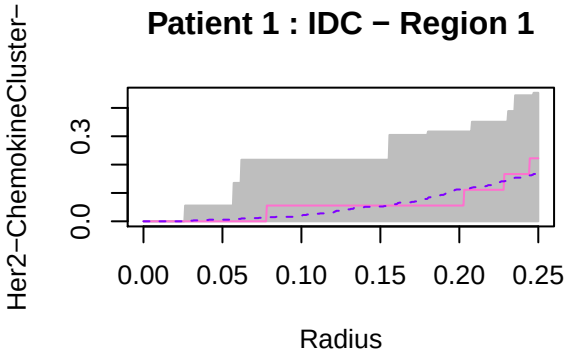

Patient 1 : IDC − Region 1
Her2−ChemokineCluster+
0.0
0.6
0.00
0.05
0.10
0.15
0.20
0.25
Radius

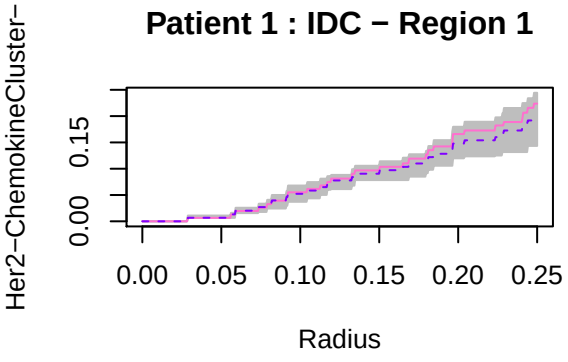

Patient
Her2−ChemokineCluster−
0.0
0.2
0.00
0.05











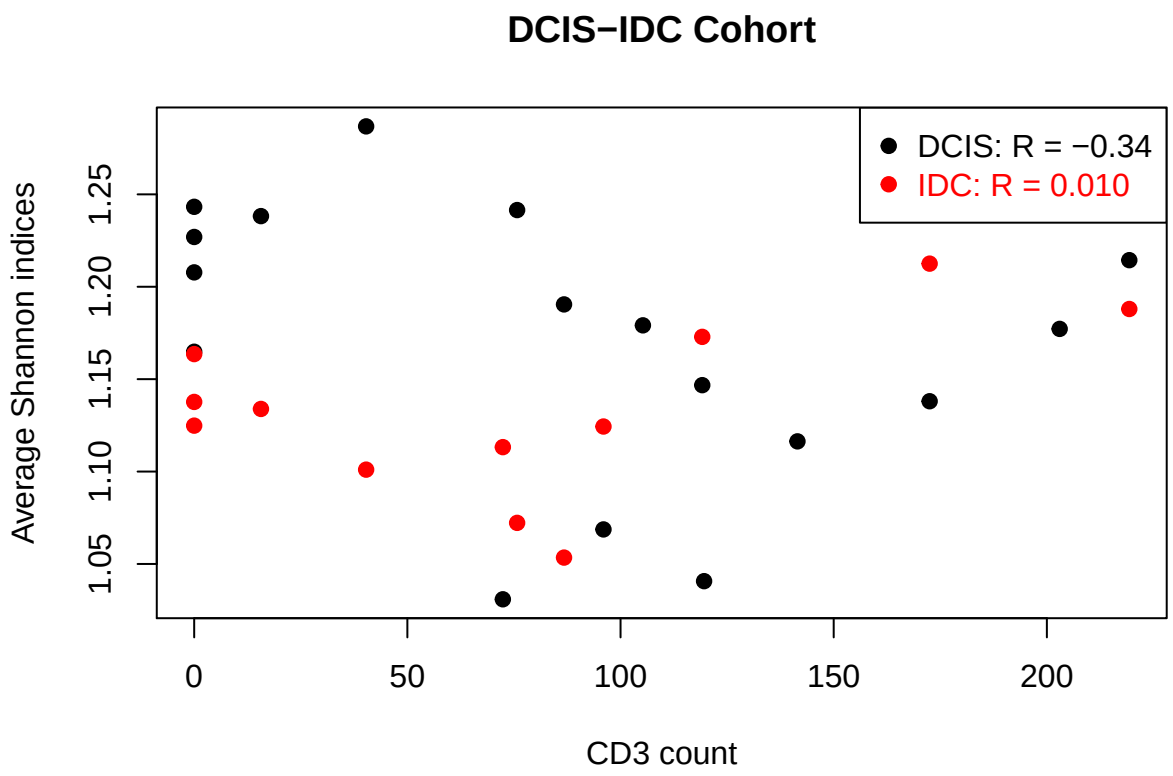

```
# Run this when compile for the final time
# as examples, plot Patient 1

pdf('output/Spatial_Modeling_DCIS_IDC.pdf', height = 16, width = 19.5)
for (i in 1:18) {
  for (t in 0:1) {
    indices <- unique(all.mat$index[all.mat$sample_ID == i & all.mat$Type == t])
    for (ind in indices) {
      par(mfrow = c(4, 4), mai = c(1, 1.2, 1, 1))
      temp.mat <- all.mat[all.mat$index == ind & all.mat$sample_ID == i & all.mat$Type == t, ]
      temp.mat$m <- 'HER2.Neg.Chemokine.Neg'
      temp.mat$m[temp.mat$her2.bin == TRUE & temp.mat$cluster.bin == TRUE] <- 'HER2.Pos.Chemokine.Pos'
      temp.mat$m[temp.mat$her2.bin == FALSE & temp.mat$cluster.bin == TRUE] <- 'HER2.Neg.Chemokine.Pos'
      temp.mat$m[temp.mat$her2.bin == TRUE & temp.mat$cluster.bin == FALSE] <- 'HER2.Pos.Chemokine.Neg'
      temp.sp <- as.ppp(as.data.frame(cbind(temp.mat$x, temp.mat$y)), c(0, 1, 0, 1))
      marks(temp.sp) <- as.factor(temp.mat$m)
      temp.sp.marks <- as.character(unique(marks(temp.sp)))
      E <- list()
```

```
      w <- 1
      for (u in 1:length(pairs1)) {
        if (is.na(pairs2[u]) == TRUE) {
          E[[w]] <- NA
        } else if (!(phenotypes[pairs1[u]] %in% temp.sp.marks) | !(phenotypes[pairs2[u]] %in% temp.sp.ma
          E[[w]] <- NA
        } else {
          E[[w]] <- envelope(temp.sp, Kcross, nsim = nrow(temp.mat), i = phenotypes[pairs1[u]], j = phen
        }
        w <- w + 1
      }
      w <- w - 1
      if (i==1){
      #  ww <- 1
      if (t == 0) {
        for (ww in 1:w) {
          main = paste0('Patient ', i, ' : DCIS - Region ', which(indices == ind))
          if (mode(E[[ww]]) == "logical") {
            plot(rnorm(100), rnorm(100), type = 'n', main = '', ylim = c(0, 1), axes = FALSE, xlab = ''
          } else {
            if (is.nan(E[[ww]]$obs[1]) == TRUE) {
              plot(rnorm(100), rnorm(100), type = 'n', main = '', ylim = c(0, 1), axes = FALSE, xlab =
            } else {
              plot(E[[ww]], main = main, xlab = 'Radius', ylab = paste0(genotypes[pairs1[ww]], ' vs.\n'
            }
          }
      }} else {
        for (ww in 1:w) {
          main = paste0('Patient ', i, ' : IDC - Region ', which(indices == ind))
          if (mode(E[[ww]]) == "logical") {
            plot(rnorm(100), rnorm(100), type = 'n', main = '', ylim = c(0, 1), axes = FALSE, xlab = ''
          } else {
            if (is.nan(E[[ww]]$obs[1]) == TRUE) {
              plot(rnorm(100), rnorm(100), type = 'n', main = '', ylim = c(0, 1), axes = FALSE, xlab =
            } else {
              plot(E[[ww]], main = main, xlab = 'Radius', ylab = paste0(genotypes[pairs1[ww]], ' vs.\n'
            }
          }
        }
      }
    }
  }
}}
dev.off()
```

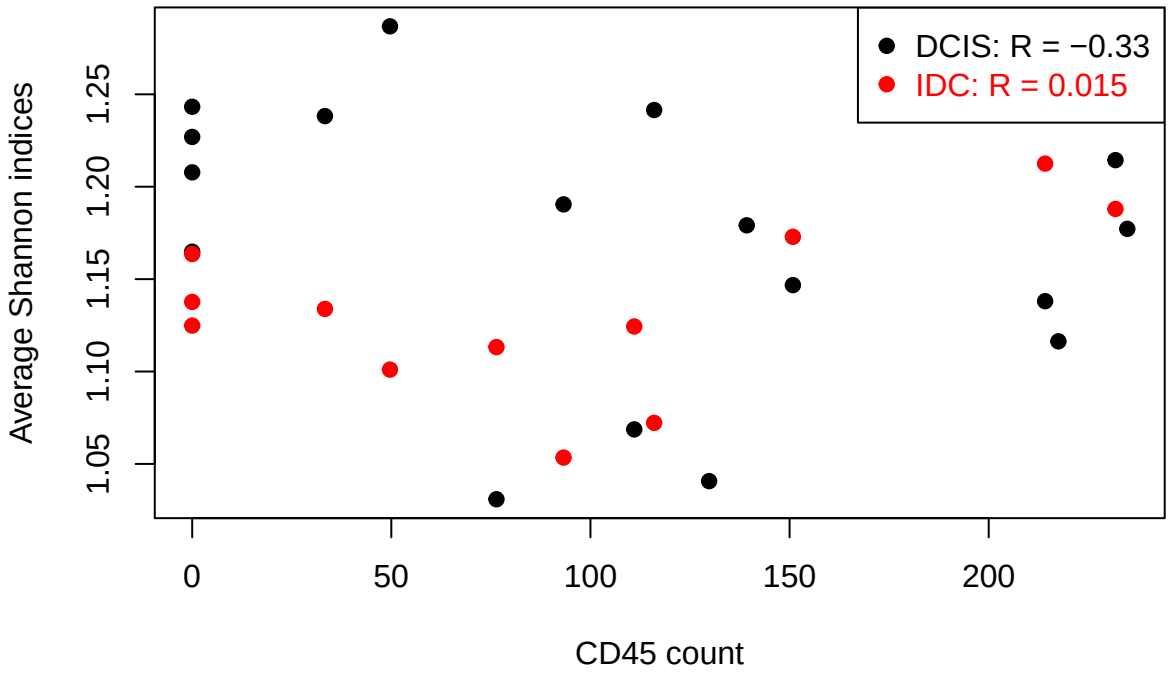
Average Shannon indicies and CD3 or CD45 counts:

```
leukocytes <- read.csv('data/leukocytes_dcis_idc.csv', stringsAsFactors = FALSE)
leukocytes <- leukocytes[-which(is.na(leukocytes$image.) == TRUE), ]
leukocytes <- leukocytes[-which(leukocytes$cd45 == 'IDC'), ]

leukocytes$cd45 <- as.numeric(leukocytes$cd45)
leukocytes.ave.cd45 <- sapply(1:18, function(i) mean(leukocytes[leukocytes$Pa == i, 3], na.rm = TRUE))
leukocytes.ave.cd3 <- sapply(1:18, function(i) mean(leukocytes[leukocytes$Pa == i, 4], na.rm = TRUE))
```

```
leukocytes.ave.cd3p <- sapply(1:18, function(i) mean(leukocytes[leukocytes$Pa == i, 4] / leukocytes[leu

plot(leukocytes.ave.cd3, diversity.mat.ave$HER2.CCluster.bin[diversity.mat.ave$Type == 0], ylab = 'Avera
points(leukocytes.ave.cd3, diversity.mat.ave$HER2.CCluster.bin[diversity.mat.ave$Type == 1], col = 'red
legend('topright', c(expression(paste('DCIS: ', R, ' = -0.34', sep = '')), expression(paste('IDC: ', R,
```




```
plot(leukocytes.ave.cd45, diversity.mat.ave$HER2.CCluster.bin[diversity.mat.ave$Type == 0], ylab = 'Ave
points(leukocytes.ave.cd45, diversity.mat.ave$HER2.CCluster.bin[diversity.mat.ave$Type == 1], col = 're
legend('topright', c(expression(paste('DCIS: ', R, ' = -0.33', sep = '')), expression(paste('IDC: ', R,
```




```
#CD3
## DCIS sections:
cor.test(leukocytes.ave.cd3, diversity.mat.ave$HER2.CCluster.bin[diversity.mat.ave$Type == 0])

##
##  Pearson's product-moment correlation
##
## data:  leukocytes.ave.cd3 and diversity.mat.ave$HER2.CCluster.bin[diversity.mat.ave$Type == leukocyt
## t = -1.3611, df = 15, p-value = 0.1936
## alternative hypothesis: true correlation is not equal to 0
## 95 percent confidence interval:
##  -0.7005631  0.1773442
## sample estimates:
##        cor
## -0.3315628
## IDC:
cor.test(leukocytes.ave.cd3, diversity.mat.ave$HER2.CCluster.bin[diversity.mat.ave$Type == 1])

##
##  Pearson's product-moment correlation
##
## data:  leukocytes.ave.cd3 and diversity.mat.ave$HER2.CCluster.bin[diversity.mat.ave$Type == leukocyt
## t = -0.0060727, df = 15, p-value = 0.9952
## alternative hypothesis: true correlation is not equal to 0
## 95 percent confidence interval:
##  -0.4818496  0.4794381
## sample estimates:
##          cor
## -0.001567962
```

```
# CD45
## DCIS sections:
cor.test(leukocytes.ave.cd45, diversity.mat.ave$HER2.CCluster.bin[diversity.mat.ave$Type == 0])
```

```
##
##  Pearson's product-moment correlation
##
## data:  leukocytes.ave.cd45 and diversity.mat.ave$HER2.CCluster.bin[diversity.mat.ave$Type == leukocyt
## t = -1.2869, df = 15, p-value = 0.2176
## alternative hypothesis: true correlation is not equal to 0
## 95 percent confidence interval:
##  -0.6912162  0.1948405
## sample estimates:
##       cor
## -0.3153351
```

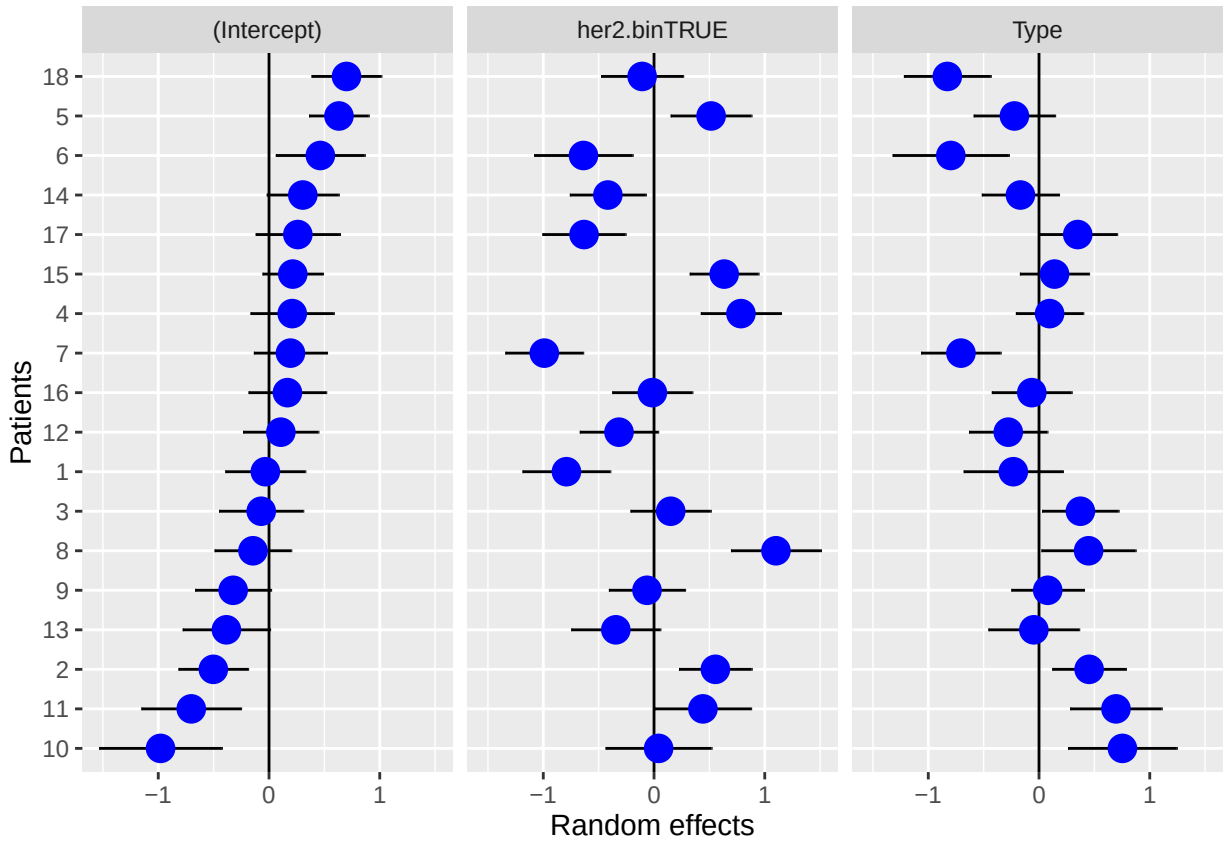
```
## IDC:
cor.test(leukocytes.ave.cd45, diversity.mat.ave$HER2.CCluster.bin[diversity.mat.ave$Type == 1])
```

```
##
##  Pearson's product-moment correlation
##
## data:  leukocytes.ave.cd45 and diversity.mat.ave$HER2.CCluster.bin[diversity.mat.ave$Type == leukocyt
## t = -0.11182, df = 15, p-value = 0.9124
## alternative hypothesis: true correlation is not equal to 0
## 95 percent confidence interval:
##  -0.5025342  0.4581395
## sample estimates:
##        cor
## -0.02886036
```

9.3.3 Generalised linear model

```
model.final <- glmer(cluster.bin ~ 1 + her2.bin + Type + scale(log(age)) + ER + ER * her2.bin + (1 + her
ggCaterpillar(ranef(model.final, condVar = TRUE), QQ = FALSE)
```

```
## $sample_ID
```




```
age.patients = sapply(1:18, function(i) mean(all.mat$scale.log.age[all.mat$sample_ID == i]))
summary(model.final)
```

```
## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: binomial  ( logit )
## Formula: cluster.bin ~ 1 + her2.bin + Type + scale(log(age)) + ER + ER *
##     her2.bin + (1 + her2.bin + Type | sample_ID)
##    Data: all.mat
##
##      AIC      BIC   logLik deviance df.resid
##  10910.8  10997.7  -5443.4  10886.8    10353
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -2.2110 -0.6571 -0.3567  0.7795  4.9703
##
## Random effects:
##  Groups    Name         Variance Std.Dev. Corr
##  sample_ID (Intercept)  0.2269   0.4764
##            her2.binTRUE 0.3654   0.6045   -0.26
##            Type         0.2518   0.5018   -0.72  0.51
## Number of obs: 10365, groups:  sample_ID, 18
##
## Fixed effects:
##                 Estimate Std. Error z value Pr(>|z|)
## (Intercept)      -1.4230     0.2024  -7.030 2.06e-12 ***
```

```
## her2.binTRUE      1.3197     0.2699   4.890 1.01e-06 ***
## Type             -0.8664     0.1299  -6.669 2.58e-11 ***
## scale(log(age))   0.2480     0.1098   2.258   0.0239 *
## ER               -0.1295     0.2200  -0.588   0.5562
## her2.binTRUE:ER   0.5284     0.3086   1.712   0.0869 .
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr) hr2.TRUE Type   sc(()) ER
## her2.bnTRUE -0.163
## Type        -0.389  0.239
## scal(lg(g)) -0.305  0.002   -0.021
## ER          -0.791  0.059   -0.016  0.351
## hr2.TRUE:ER  0.048 -0.823    0.001  0.031 -0.064
```

10 R Session Info

```
sessionInfo()
```

```
## R version 3.3.1 RC (2016-06-17 r70798)
## Platform: x86_64-apple-darwin13.4.0 (64-bit)
## Running under: OS X 10.11.6 (El Capitan)
##
## locale:
## [1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8
##
## attached base packages:
##  [1] parallel  stats4    compiler  stats     graphics  grDevices utils
##  [8] datasets  methods   base
##
## other attached packages:
##  [1] org.Hs.eg.db_3.2.3         RSQLite_1.0.0
##  [3] DBI_0.4-1                  fANCOVA_0.5-1
##  [5] vegan_2.4-2                lattice_0.20-33
##  [7] permute_0.9-4              TDA_1.5
##  [9] spatstat_1.48-0            rpart_4.1-10
## [11] nlme_3.1-128               scatterplot3d_0.3-37
## [13] rgl_0.95.1441              reshape2_1.4.1
## [15] RColorBrewer_1.1-2         plyr_1.8.4
## [17] matrixStats_0.51.0         lme4_1.1-12
## [19] Matrix_1.2-6               leaps_3.0
## [21] knitr_1.14                 HTSanalyzeR_2.24.0
## [23] igraph_1.0.1               heatmap.plus_1.3
## [25] GSEABase_1.32.0            graph_1.48.0
## [27] annotate_1.48.0            XML_3.98-1.4
## [29] AnnotationDbi_1.32.3       gplots_3.0.1
## [31] ggplot2_2.1.0              GGally_1.1.0
## [33] geepack_1.2-1              gee_4.13-19
## [35] gdata_2.17.0               fpc_2.1-10
## [37] edgeR_3.12.1               limma_3.28.21
## [39] dplyr_0.4.3                DESeq2_1.12.4
```

```
## [41] SummarizedExperiment_1.2.3 Biobase_2.30.0
## [43] GenomicRanges_1.24.2       GenomeInfoDb_1.8.7
## [45] IRanges_2.6.1              S4Vectors_0.10.3
## [47] BiocGenerics_0.18.0        boot_1.3-18
## [49] beeswarm_0.2.3             ade4_1.7-4
## [51] ape_3.5
##
## loaded via a namespace (and not attached):
##  [1] backports_1.0.2       Hmisc_3.17-4          lazyeval_0.2.0
##  [4] splines_3.3.1         BiocParallel_1.4.3    digest_0.6.9
##  [7] BiocInstaller_1.22.3  htmltools_0.3.5       magrittr_1.5
## [10] tensor_1.5            cluster_2.0.4         prada_1.46.0
## [13] colorspace_1.2-6      rrcov_1.3-11          rglwidget_0.1.1434
## [16] RCurl_1.95-4.8        jsonlite_1.0          genefilter_1.52.1
## [19] survival_2.39-4       polyclip_1.5-6        gtable_0.2.0
## [22] zlibbioc_1.16.0       XVector_0.12.1        RankProd_2.42.0
## [25] kernlab_0.9-25        cellHTS2_2.34.1       prabclus_2.2-6
## [28] DEoptimR_1.0-4        abind_1.4-3           scales_0.4.0
## [31] futile.options_1.0.0  vsn_3.38.0            mvtnorm_1.0-5
## [34] Rcpp_0.12.5           xtable_1.8-2          foreign_0.8-66
## [37] mclust_5.2.2          preprocessCore_1.32.0 Formula_1.2-1
## [40] htmlwidgets_0.6       acepack_1.3-3.3       modeltools_0.2-21
## [43] reshape_0.8.5         flexmix_2.3-13        nnet_7.3-12
## [46] deldir_0.1-12         locfit_1.5-9.1        labeling_0.3
## [49] munsell_0.4.3         tools_3.3.1           evaluate_0.9
## [52] stringr_1.0.0         yaml_2.1.13           goftest_1.0-4
## [55] robustbase_0.92-6     caTools_1.17.1        RBGL_1.46.0
## [58] mime_0.4              formatR_1.4           BioNet_1.32.0
## [61] biomaRt_2.26.1        affyio_1.40.0         geneplotter_1.48.0
## [64] pcaPP_1.9-60          stringi_1.1.1         futile.logger_1.4.1
## [67] trimcluster_0.1-2     nloptr_1.0.4          markdown_0.7.7
## [70] data.table_1.9.6      bitops_1.0-6          httpuv_1.3.3
## [73] R6_2.1.2              latticeExtra_0.6-28   affy_1.48.0
## [76] KernSmooth_2.23-15    gridExtra_2.2.1       lambda.r_1.1.7
## [79] MASS_7.3-45           gtools_3.5.0          assertthat_0.1
## [82] chron_2.3-47          Category_2.36.0       rprojroot_1.1
## [85] diptest_0.75-7        mgcv_1.8-12           grid_3.3.1
## [88] class_7.3-14          minqa_1.2.4           rmarkdown_1.3
## [91] shiny_0.13.2
```