

Brain Cancer Microarray Data Weighted Gene Co-expression Network Analysis R Tutorial

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CONTENTS

This is stolen from: [<https://horvath.genetics.ucla.edu/html/GeneralFramework/GBMTutorialHorvath.pdf>]
This document contains function for carrying out the following tasks

- A) Assessing scale free topology and choosing the parameters of the adjacency function using the scale free topology criterion (Zhang and Horvath 05)
- B) Computing the topological overlap matrix
- C) Defining gene modules using clustering procedures
- D) Summing up modules by their first principal component (first eigengene)
- E) Relating a measure of gene significance to the modules
- F) Carrying out a within module analysis (computing intramodular connectivity) and relating intramodular connectivity to gene significance.
- G) Miscellaneous other functions, e.g. for computing the cluster coefficient.

Downloading the R software

- 1) Go to <http://www.R-project.org>, download R and install it on your computer

After installing R, you need to install several additional R library packages:

To get this tutorial and data files, go to the following webpage [www.genetics.ucla.edu/labs/horvath/CoexpressionNetwork]

Download the zip file containing:

- 1) R function file: “NetworkFunctions.txt”, which contains several R functions needed for Network Analysis.
- 2) The data file “gbm55old_dchip_14kALL_cox_8000mvgenes2.csv”
- 3) Of course, this file: “GBMTutorialHorvath.txt”

(I already did this)

```
source("NetworkFunctions.R")
# read in the R libraries
library(MASS) # standard, no need to install
library(class) # standard, no need to install
library(cluster)
#library(impute) # install it for imputing missing value
library(WGCNA)
options(stringsAsFactors = F)
library(gplots)
```

read in the 8000 most varying genes (GBM microarray data)

```
dat0=read.csv("gbm55old_dchip_14kALL_cox_8000mvgenes2.csv")
# this contains information on the genes
datSummary=dat0[,1:9]
```

The following data frame contains the gene expression data: columns are genes, rows are arrays (samples)

```
datExpr = t(dat0[,10:64])
```

```
no.samples = dim(datExpr)[[1]]
dim(datExpr)
```

```
## [1] 55 8000
```

```
rm(dat0);gc()
```

```
##           used (Mb) gc trigger (Mb) limit (Mb) max used (Mb)
## Ncells    4757390 254.1   8377051 447.4      NA 6253287 334
## Vcells    8734558  66.7   16840304 128.5     65536 12184693   93
```

To choose a cut-off value,

we propose to use the Scale-free Topology Criterion (Zhang and Horvath 2005). Here the focus is on the linear regression model fitting index (denoted below by scale.law.R.2) that quantify the extent of how well a network satisfies a scale-free topology. The function PickSoftThreshold can help one to estimate the cut-off value when using hard thresholding with the step adjacency function. The first column (different from the row numbers) lists the soft threshold Power The second column reports the resulting scale free topology fitting index R^2 (scale.law.R.2) The third column reports the slope of the fitting line. The fourth column reports the fitting index for the truncated exponential scale free model# Usually we ignore it. The remaining columns list the mean, median and maximum connectivity. To a soft threshold (power) with the scale free topology criterion: aim for reasonably high scale free R^2 (column 2), higher than say .80 and negative slope (around -1, col 4). In practice, we pick the threshold by looking for a “kink” in the relationship between R^2 and power, see below.

Soft thresholding

Now we investigate soft thresholding with the power adjacency function

```
powers1=c(seq(1,10,by=1),seq(12,20,by=2))
RpowerTable=pickSoftThreshold(datExpr, powerVector=powers1)[[2]]
```

	Power	SFT.R.sq	slope	truncated.R.sq	mean.k.	median.k.	max.k.
## 1	1	0.0275	0.451	0.979	1640.00	1.60e+03	2700.0
## 2	2	0.2840	-0.851	0.942	527.00	4.79e+02	1310.0
## 3	3	0.7510	-1.420	0.968	214.00	1.74e+02	769.0
## 4	4	0.8980	-1.660	0.975	102.00	7.22e+01	513.0
## 5	5	0.9500	-1.710	0.979	54.90	3.25e+01	373.0
## 6	6	0.9690	-1.660	0.983	32.50	1.58e+01	288.0
## 7	7	0.9720	-1.610	0.980	20.80	8.09e+00	232.0
## 8	8	0.9720	-1.550	0.976	14.10	4.36e+00	193.0
## 9	9	0.9790	-1.500	0.982	10.10	2.43e+00	166.0
## 10	10	0.9810	-1.470	0.984	7.48	1.42e+00	145.0
## 11	12	0.9750	-1.400	0.980	4.52	5.22e-01	114.0
## 12	14	0.9770	-1.360	0.982	2.97	2.08e-01	92.7
## 13	16	0.9700	-1.340	0.971	2.08	8.94e-02	76.9

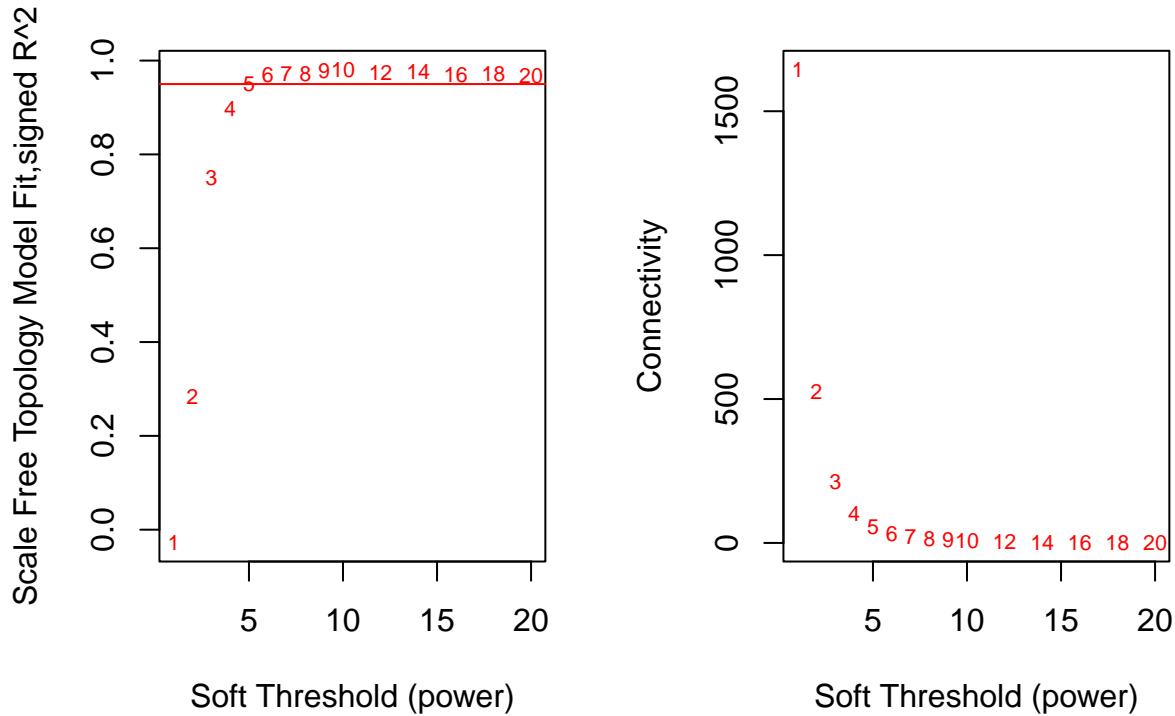
```

## 14     18    0.9720 -1.330      0.980    1.52  4.00e-02   65.3
## 15     20    0.9670 -1.320      0.973    1.14  1.87e-02   56.2
gc()

##           used   (Mb) gc trigger   (Mb) limit   (Mb) max used   (Mb)
## Ncells  4782613 255.5    8377051 447.4        NA 6253287 334.0
## Vcells  8790412 67.1    86874679 662.9      65536 210471029 1605.8

cex1=0.7
par(mfrow=c(1,2))
plot(RpowerTable[,1], -sign(RpowerTable[,3])*RpowerTable[,2],xlab=
"Soft Threshold (power)",ylab="Scale Free Topology Model Fit,signed R^2",type="n")
text(RpowerTable[,1], -sign(RpowerTable[,3])*RpowerTable[,2],
labels=powers1,cex=cex1,col="red")
# this line corresponds to using an R^2 cut-off of h
abline(h=0.95,col="red")
plot(RpowerTable[,1], RpowerTable[,5],xlab="Soft Threshold (power)",ylab="Mean
Connectivity", type="n")
text(RpowerTable[,1], RpowerTable[,5], labels=powers1, cex=cex1,col="red")

```



Note that at power=6, the curve has an elbow or kink, i.e. for this power the scale free topology fit does not improve after increasing the power. This is why we choose beta1=6. Also the scale free topology criterion with a R^2 threshold of 0.95 would lead us to pick a power of 6. Note that there is a natural trade-off between maximizing scale-free topology model fit (R^2) and maintaining a high mean number of connections: parameter values that lead to an R^2 value close to 1 may lead to networks with very few connections. Actually, we consider a signed version of the scale free topology fitting index. Since it is biologically implausible that a network contains more hub genes than non-hub genes, we multiply R^2 with -1 if the slope of the regression line between $\log_{10}(p(k))$ and $\log_{10}(k)$ is positive.

These considerations motivate us to propose the following {scale-free topology criterion} for choosing the parameters of an adjacency function: Only consider those parameter values that lead to a network satisfying scale-free topology at least approximately, e.g. signed $R^2 > 0.80$. In addition, we recommend that the user

take the following additional considerations into account when choosing the adjacency function parameter. First, the mean connectivity should be high so that the network contains enough information (e.g. for module detection). Second, the slope of the regression line should be around -1. When considering the power adjacency functions, we find the relationship between R^2 and the adjacency function parameter (beta) is characterized by a saturation curve type of. In our applications, we use the first parameter value where saturation is reached as long as it is above 0.8. Below we study how the biological findings depend on the choice of the power.

```
beta1=6
```

```
Connectivity=softConnectivity(datExpr, power=beta1)-1
```

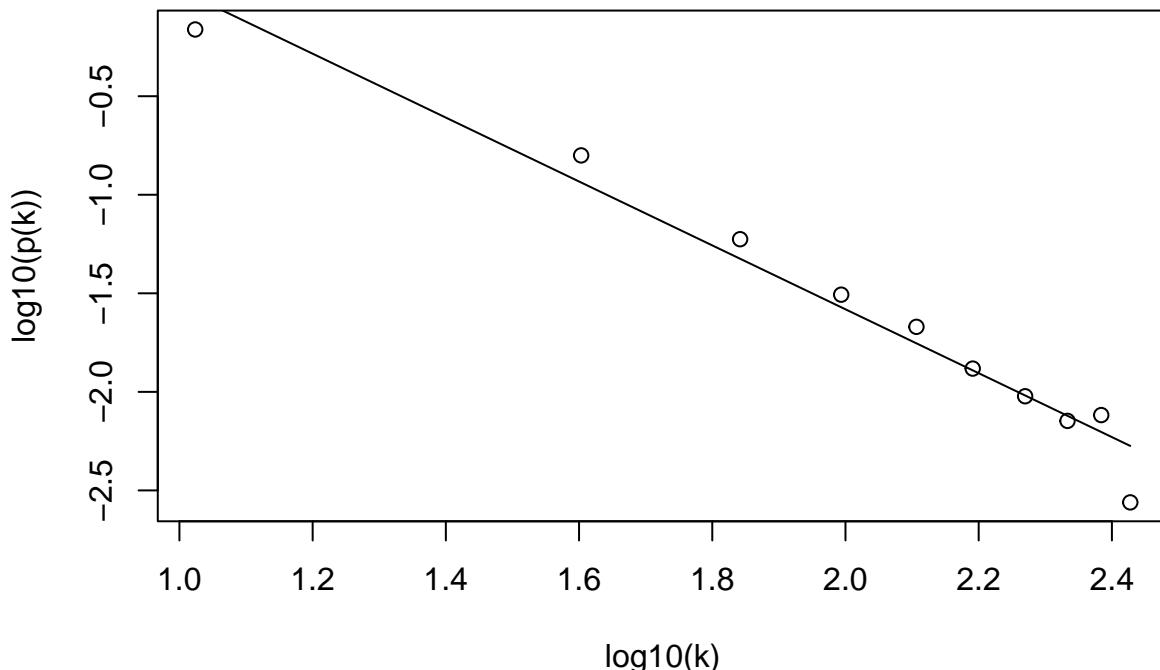
```
## softConnectivity: FYI: connecitvty of genes with less than 19 valid samples will be returned as NA
## ..calculating connectivities..
```

Let's create a scale free topology plot.

The black curve corresponds to scale free topology and the red curve corresponds to truncated scale free topology.

```
par(mfrow=c(1,1))
scaleFreePlot(Connectivity, main=paste("soft threshold, power=", beta1), truncated=F);
```

soft threshold, power= 6 scale $R^2= 0.96$, slope= -1.62



```
##   scaleFreeRsquared slope
## 1                 0.96 -1.62
```

Module Detection

An important step in network analysis is module detection. Here we use methods that use clustering in combination with the topological overlap matrix. This code allows one to restrict the analysis to the most connected genes, which may speed up calculations when it comes to module detection.

```

ConnectivityCut = 3600 # number of most connected genes that will be considered

# Incidentally, in the paper by Mischel et al (2005) we considered all 3600 #genes.
ConnectivityRank = rank(-Connectivity)
restConnectivity = ConnectivityRank <= ConnectivityCut

# thus our module detection uses the following number of genes
sum(restConnectivity)

## [1] 3600

# Now we define the adjacency matrix for the 3600 most connected genes
ADJ= adjacency(datExpr[,restConnectivity],power=beta1)
gc()

##           used   (Mb) gc trigger   (Mb) limit   (Mb) max used   (Mb)
## Ncells    4796714 256.2    8377051 447.4        NA  6253287 334.0
## Vcells    21801510 166.4    69499744 530.3      65536 210471029 1605.8

# The following code computes the topological overlap matrix based on the
# adjacency matrix.
# TIME: This about a few minutes....
dissTOM=TOMdist(ADJ)

## ..connectivity..
## ..matrix multiplication (system BLAS)..
## ..normalization..
## ..done.

gc()

##           used   (Mb) gc trigger   (Mb) limit   (Mb) max used   (Mb)
## Ncells    4797299 256.3    8377051 447.4        NA  6253287 334.0
## Vcells    34762954 265.3    69499744 530.3      65536 210471029 1605.8

```

Now we carry out hierarchical clustering with the TOM matrix.

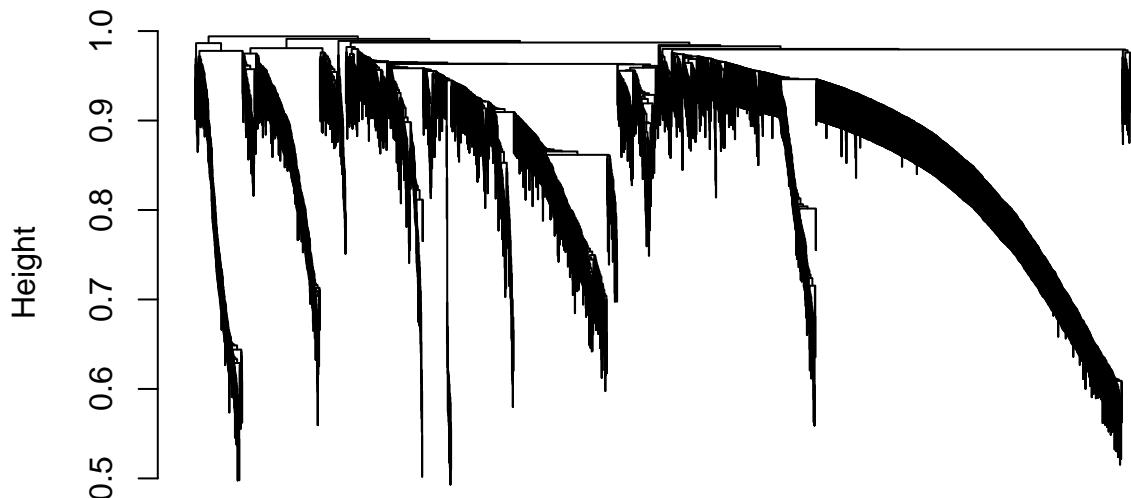
This takes a couple of minutes.

```

hierTOM = hclust(as.dist(dissTOM),method="average");
par(mfrow=c(1,1))
plot(hierTOM,labels=F)

```

Cluster Dendrogram

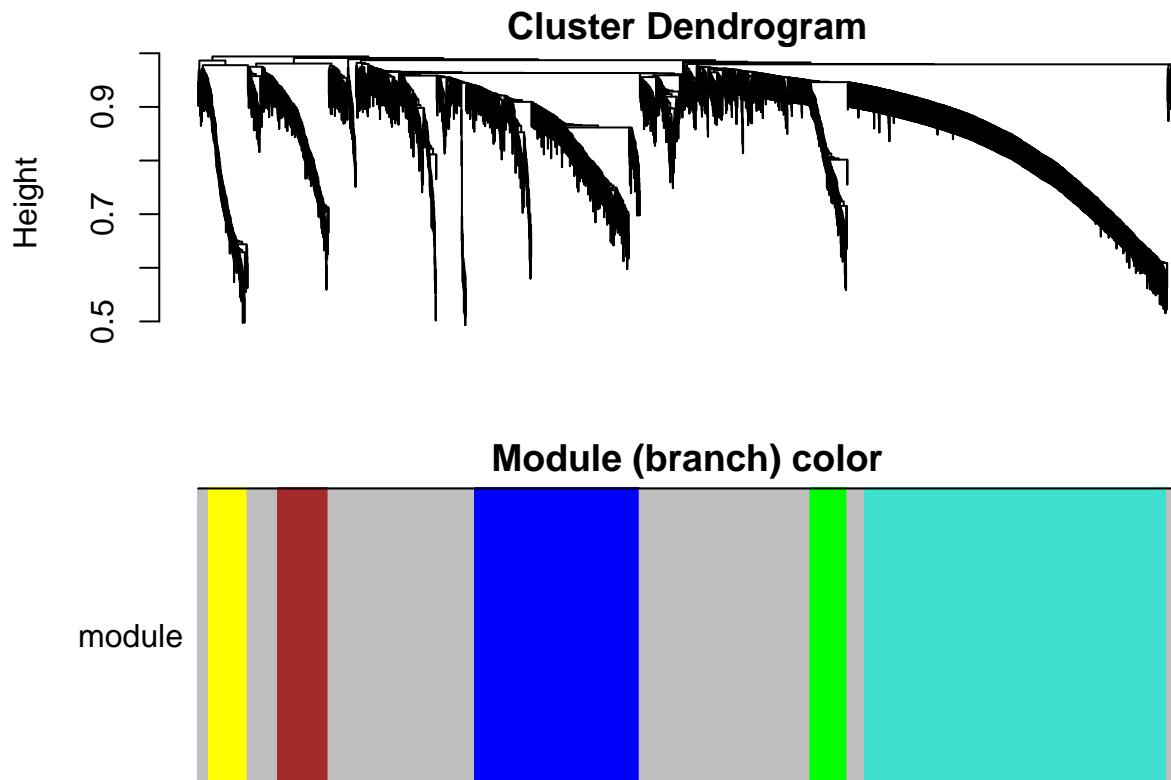


```
as.dist(dissTOM)  
hclust (*, "average")
```

According to our definition, modules correspond to branches of the tree. The question is what height cut-off should be used? This depends on the biology. Large height values lead to big modules, small values lead to small but tight modules. In reality, the user should use different thresholds to see how robust the findings are.

The function `cutreeStaticColor` colors each gene by the branches that result from choosing a particular height cut-off. GREY IS RESERVED to color genes that are not part of any module. We only consider modules that contain at least 125 genes.

```
colorh1= cutreeStaticColor(hierTOM,cutHeight = 0.94, minSize = 125)  
# The above should be identical to colorh1=datSummary$color1[restConnectivity]  
par(mfrow=c(2,1),mar=c(2,4,1,1))  
plot(hierTOM, main="Cluster Dendrogram", labels=F, xlab="", sub="");  
plotColorUnderTree(hierTOM,colors=data.frame(module=colorh1))  
title("Module (branch) color")
```



COMMENTS:

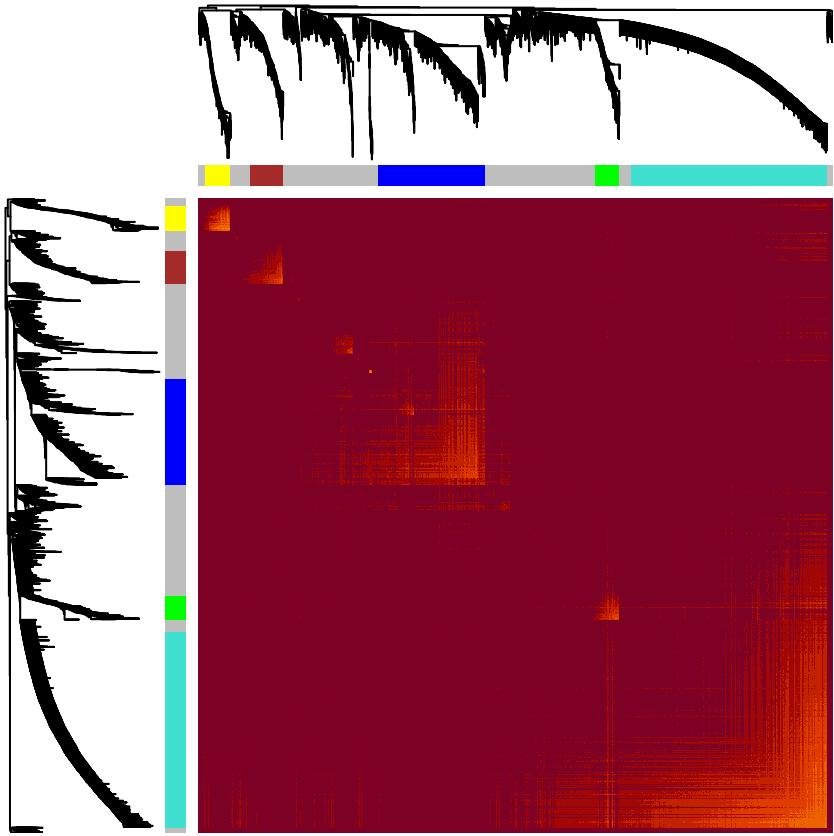
- 1) The colors are assigned based on module size. Turquoise (others refer to it as cyan) colors the largest module, next comes blue, next brown, etc. Just type table(colorh1) to figure out which color corresponds to what module size.
- 2) The minimum module size (minsize1=125) is unusually large. As default, we recommend minsize1=50.
- 3) Here we choose a fixed height cut-off (h1) for cutting off branches. But we have also developed a more flexible method for cutting off branches that adaptively choose a different height for each branch. The resulting dynamic tree cutting algorithm (cutreeDynamic) is described in Langfelder et al (2008).

An alternative view of this is the so called TOM plot that is generated by the function TOMplot

Inputs: TOM distance measure, hierarchical (hclust) object, color

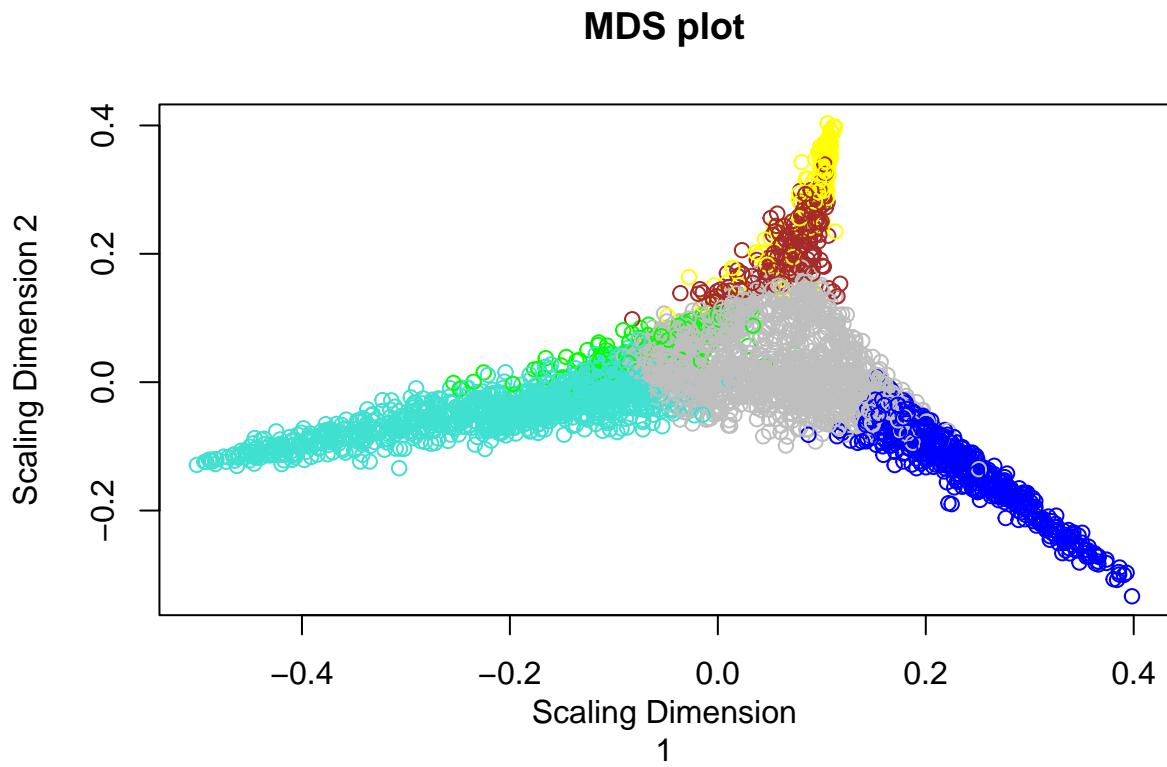
Warning: for large gene sets, say more than 2000 genes this will take a while. I recommend you skip this.

```
TOMplot(dissTOM , hierTOM, colorh1)
```



We also propose to use classical multi-dimensional scaling plots for visualizing the network. Here we chose 2 scaling dimensions. This also takes about 10 minutes...

```
cmd1=cmdscale(as.dist(dissTOM),2)
par(mfrow=c(1,1))
plot(cmd1, col=as.character(colorh1), main="MDS plot", xlab="Scaling Dimension 1", ylab="Scaling Dimension 2")
```



Module significance

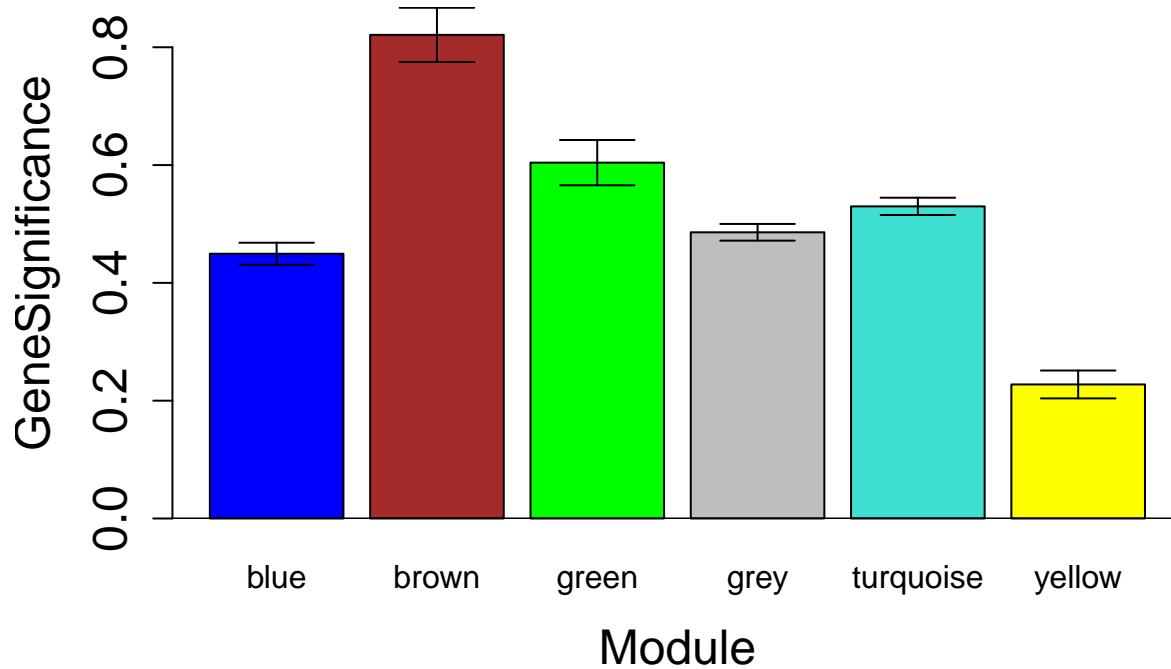
Next we define a gene significance variable as minus log10 of the univariate Cox regression pvalue for predicting survival on the basis of the gene expression info

```
# this defines the gene significance for all genes
GeneSignificanceALL=-log10(datSummary$pCox)
# gene significance restricted to the most connected genes:
GeneSignificance=GeneSignificanceALL[restConnectivity]
```

The function verboseBarplot creates a bar plot that shows whether modules are enriched with essential genes. It also reports a Kruskal Wallis P-value. The gene significance can be a binary variable or a quantitative variable. It also plots the 95% confidence interval of the mean

```
par(mfrow=c(1,1))
verboseBarplot(GeneSignificance,colorh1,main="Module Significance ",
col=levels(factor(colorh1)) ,xlab="Module" )
```

Module Significance $p = 3e-31$



Note that the brown module have a high mean value of gene significance. As aside for the experts, we should mention that the p-value (Kruskal Wallis test) cannot be trusted due to dependence between the genes. The p-value should really be interpreted as a descriptive (not inferential) measure.

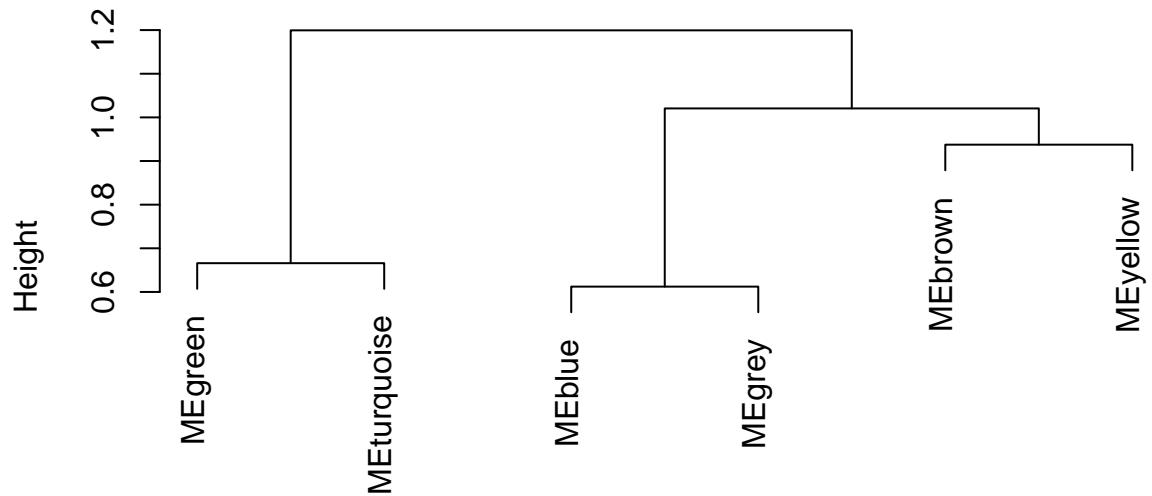
To get a sense of how related the modules are one can summarize each module by its first eigengene (referred to as principal components) and then correlate these module eigengenes with each other.

```
datME=moduleEigengenes(datExpr[,restConnectivity],colorh1)[[1]]
```

We define a dissimilarity measure between the module eigengenes that keeps track of the sign of the correlation between the module eigengenes.

```
dissimME=1-(t(cor(datME, method="p")))/2
hclustdatME=hclust(as.dist(dissimME), method="average" )
par(mfrow=c(1,1))
plot(hclustdatME, main="Clustering tree based on the module eigengenes of modules")
```

Clustering tree based on the module eigengenes of modules

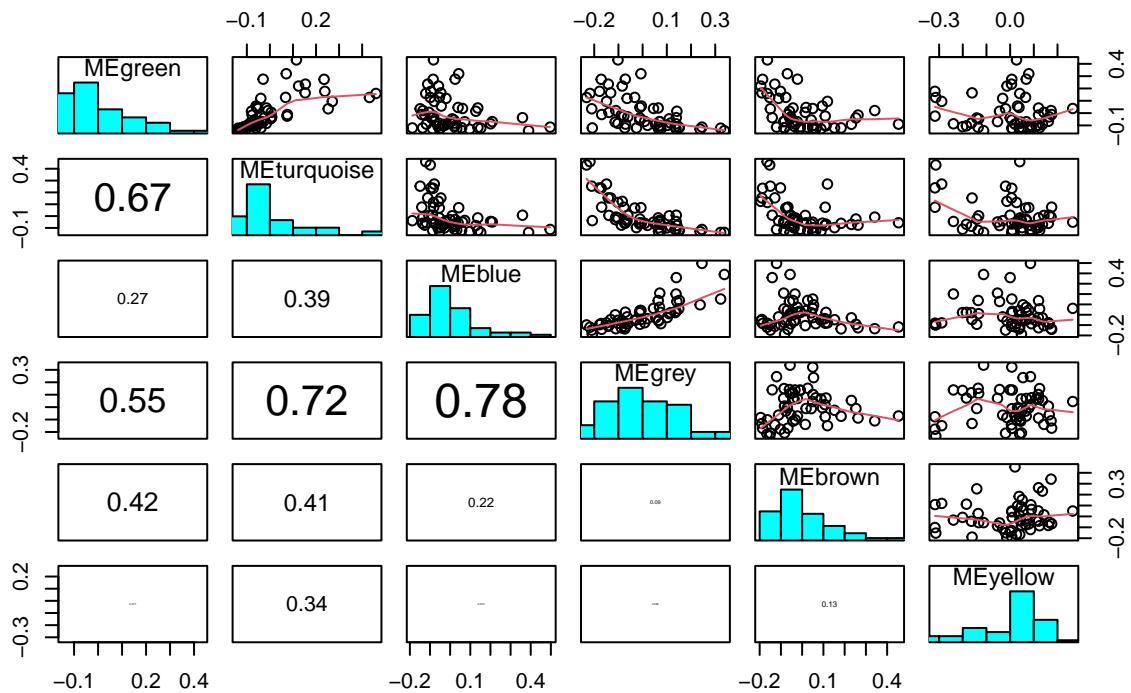


```
as.dist(dissimME)
hclust (*, "average")
```

Now we create scatter plots of the samples (arrays) along the module eigengenes.

```
datMEordered=datME[,hclustdatME$order]
pairs( datMEordered, upper.panel = panel.smooth, lower.panel = panel.cor,
diag.panel=panel.hist ,main="Relation between module eigengenes")
```

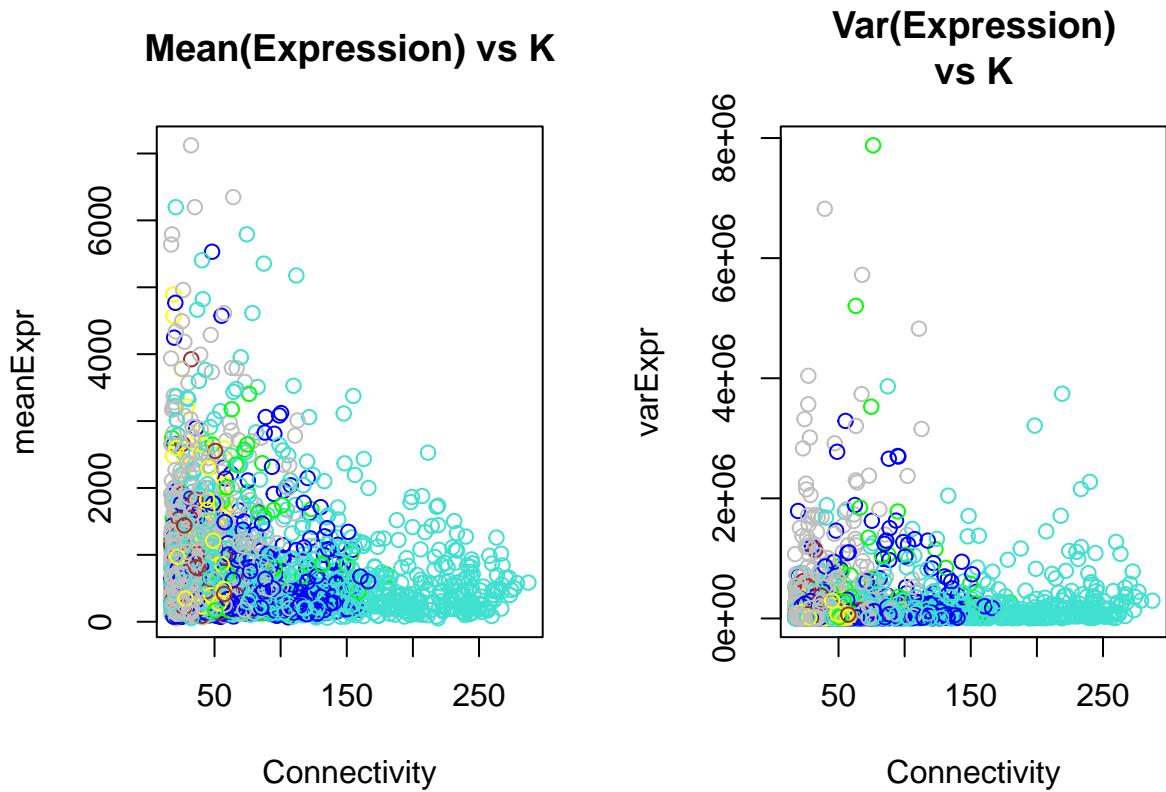
Relation between module eigengenes



Message: the module eigengenes (first PC) of different modules may be highly correlated. WGCNA can be interpreted as a biologically motivated data reduction scheme that allows for dependency between the resulting components. Compare this to principal component analysis that would impose orthogonality between the components. Since modules may represent biological pathways there is no biological reason why modules should be orthogonal to each other. Aside: If you are interested in networks comprised of module eigengenes, the following article may be of interest: Langfelder P, Horvath S (2007) Eigengene networks for studying the relationships between coexpression modules. BMC Systems Biology 2007, 1:54

To study how connectivity is related to mean gene expression or variance of gene expression we create the following plot.

```
mean1=function(x) mean(x,na.rm=T)
var1=function(x) var(x,na.rm=T)
meanExpr=apply( datExpr[,restConnectivity],2,mean1)
varExpr=apply( datExpr[,restConnectivity],2,var1)
par(mfrow=c(1,2))
plot(Connectivity[restConnectivity],meanExpr, col=as.character(colorh1),
main="Mean(Expression) vs K",xlab="Connectivity")
plot (Connectivity[restConnectivity],varExpr, col= as.character(colorh1), main="Var(Expression)
vs K" ,xlab="Connectivity")
```

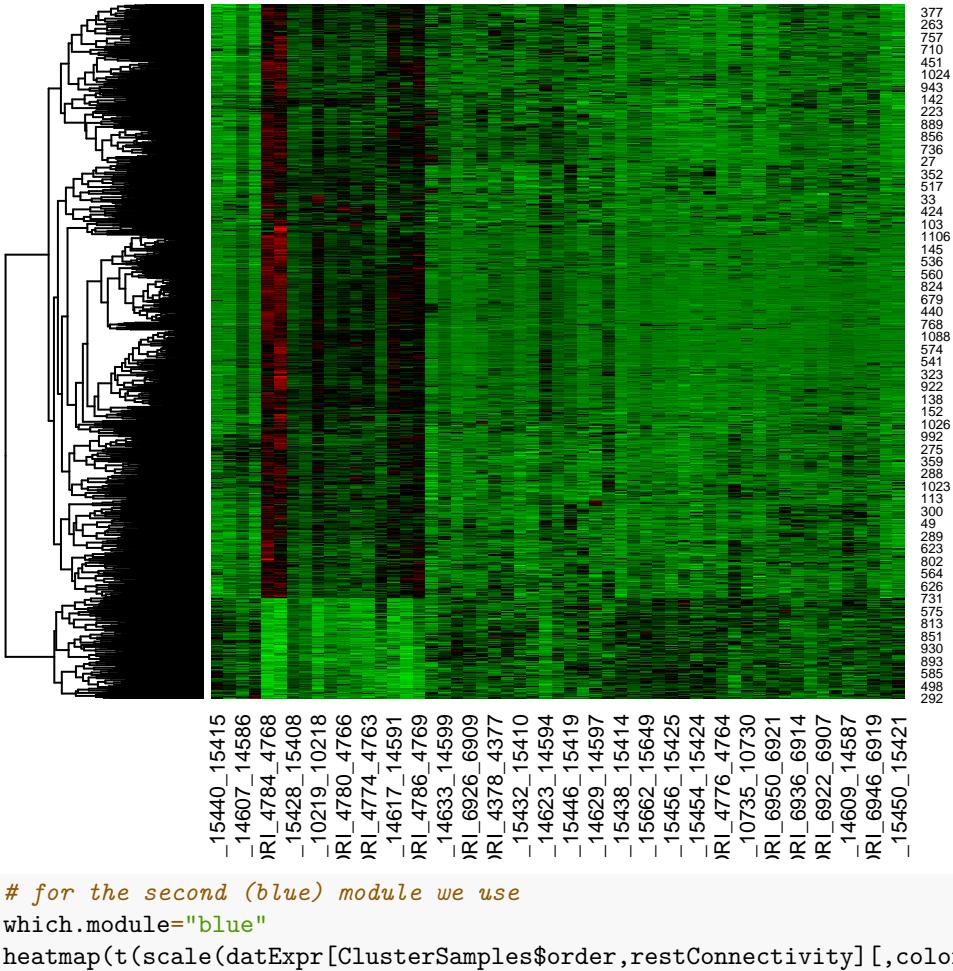


The following produces heatmap plots for each module.

Here the rows are genes and the columns are samples.

Well defined modules results in characteristic band structures since the corresponding genes are highly correlated.

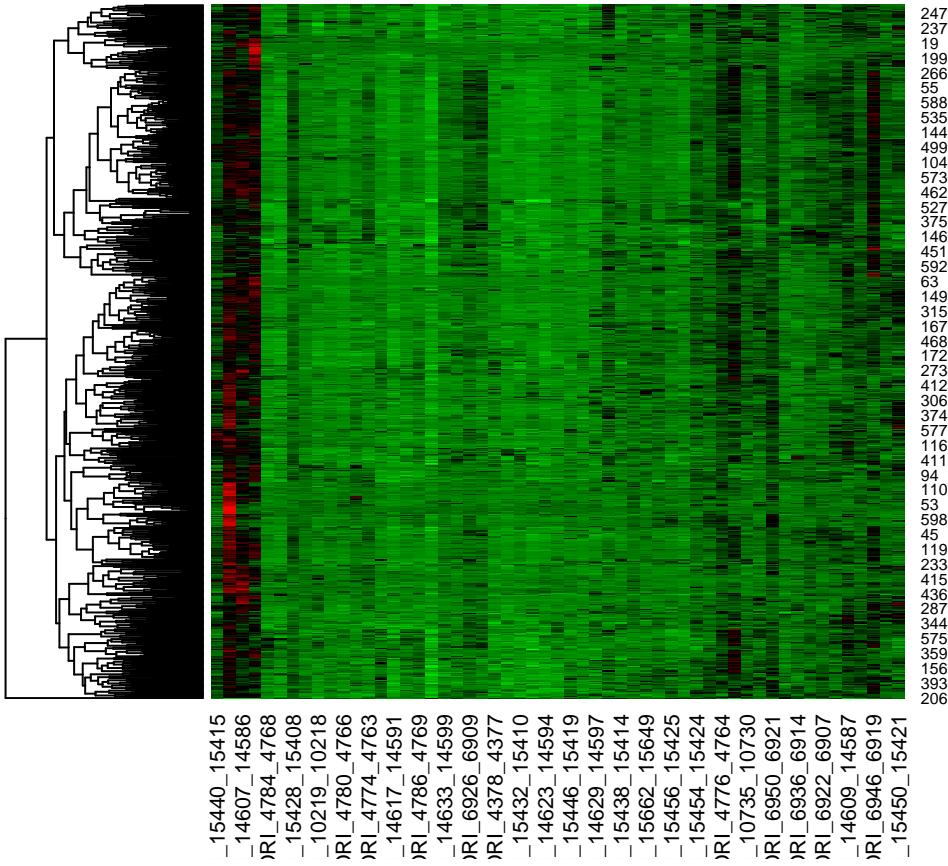
```
par(mfrow=c(2,1), mar=c(1, 2, 4, 1))
ClusterSamples=hclust(dist(datExpr[,restConnectivity]), method="average")
# for the first (turquoise) module we use
which.module="turquoise"
rg=colorpanel(50, low="green", mid="black", high="red")
heatmap(t(scale(datExpr[ClusterSamples$order,restConnectivity][,colorh1==which.module])), col=rg, Colv=NA)
```



```
# for the second (blue) module we use
```

```
which.module="blue"
```

```
heatmap(t(scale(datExpr[ClusterSamples$order,restConnectivity][,colorh1==which.module ])),col=rg,Colv=N)
```



Now we extend the color definition to all genes by coloring all non-module genes grey.

```

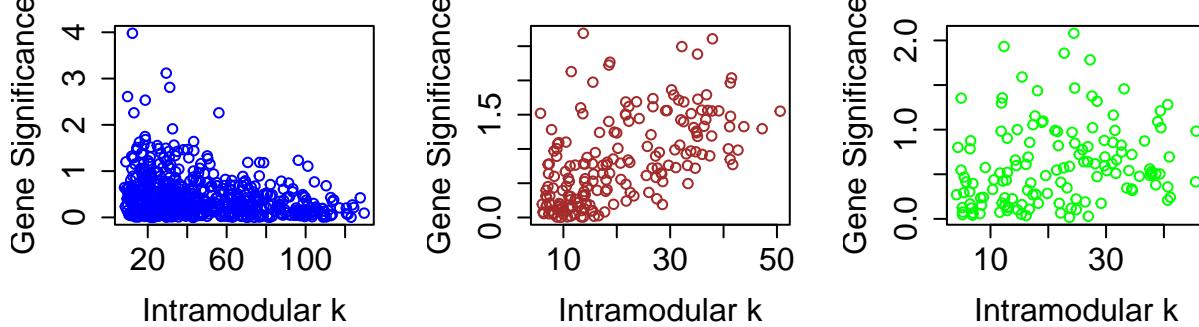
color1=rep("grey",dim(datExpr)[[2]])
color1[restConnectivity]=as.character(colorh1)
# The function intramodularConnectivity computes the whole network connectivity kTotal,
# the within module connectivity (kWithin). kOut=kTotal-kWithin and
# and kDiff=kIn-kOut=2*kIN-kTotal
ConnectivityMeasures=intramodularConnectivity(ADJ,colors=colorh1)
names(ConnectivityMeasures)

## [1] "kTotal"   "kWithin"  "kOut"      "kDiff"

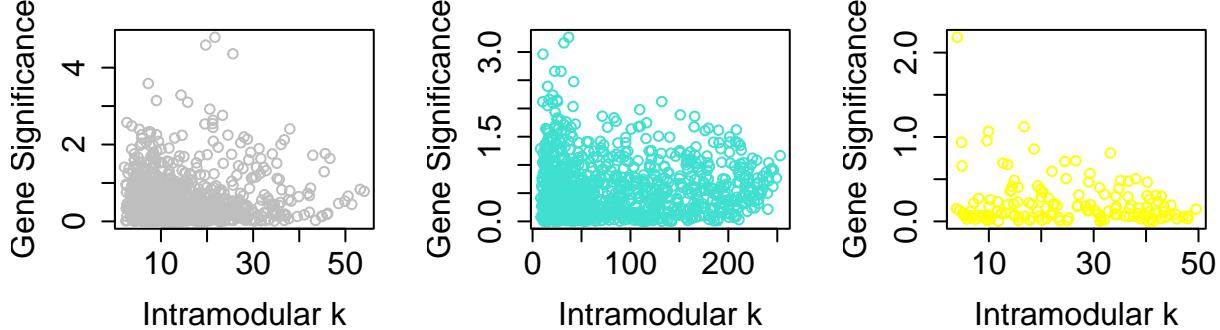
# The following plots show the gene significance vs intramodular connectivity
colorlevels=levels(factor(colorh1))
par(mfrow=c(2,3),mar=c(5, 4, 4, 2) + 0.1)
for (i in c(1:length(colorlevels) ) ) {
whichmodule=colorlevels[[i]];restrict1=colorh1==whichmodule
verboseScatterplot(ConnectivityMeasures$kWithin[restrict1],
GeneSignificance[restrict1],col=colorh1[restrict1],main= paste("set I,",
whichmodule),ylab="Gene Significance",xlab="Intramodular k")
}

```

set I, blue cor=-0.26, p=8.1e-1 set I, brown cor=0.56, p=1.2e-1 set I, green cor=0.21, p=0.0



set I, grey cor=0.06, p=0.02 set I, turquoise cor=0.08, p=0.0 set I, yellow cor=-0.19, p=0.



Generalizing the intramodular connectivity measure to *all* genes. Note that the intramodular connectivity measure is only defined for the genes inside a given module. But in practice it can be very important to measure how connected a given genes is to biologically interesting modules. Toward this end, we define a module eigengene based connectivity measure for each gene as the correlation between a the gene expression and the module eigengene. Specifically,

$kME_{brown}(i)=\text{cor}(x(i), PC_{brown})$

where $x(i)$ is the gene expression profile of the i -th gene and PC_{brown} is the module eigengene of the brown module. Note that the definition does not require that the i -th gene is a member of the brown module.

The following data frame contains the kME values corresponding to each module.

```
datKME=signedKME(datExpr, datME)
#Note we have an intramodular connectivity measure for each color.
names(datKME)
```

```
## [1] "kMEblue"      "kMEbrown"      "kMEgreen"      "kMEgrey"       "kMETurquoise"
## [6] "kMEyellow"
```

Note that the intramodular connectivity has been computed for each of the 8000 genes.

```
dim(datKME)
```

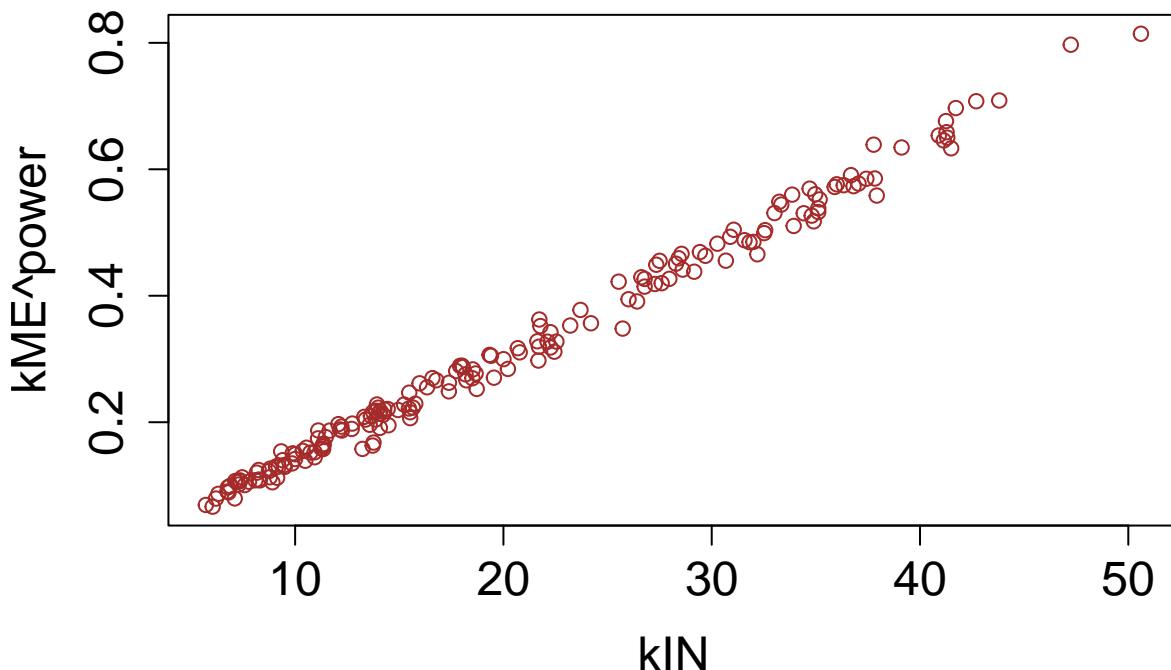
```
## [1] 8000      6
attach(datKME)
```

Question: how do the kME measure relate to the standard intramodular connectivity?

```
whichmodule="brown"
restrictGenes= colorh1== whichmodule
par(mfrow=c(1,1))
```

```
verboseScatterplot(ConnectivityMeasures$kWithin[ restrictGenes],
(datKME$kMEEbrown[restConnectivity][restrictGenes])^beta1 ,xlab="kIN",ylab="kME^power",
col=whichmodule,main="Relation between two measures of intramodular k, ")
```

Relation between two measures of intramodular k, cor=1, p



Note that after raising kME to a power of 6, it is highly correlated with kWithin. A theoretical derivation of this finding can be found in Horvath and Dong (2008).

Question: find genes with high gene significance (Cox-pvalue smaller than 0.05) and high intramodular connectivity in the brown module.

```
attach(datKME)
FilterGenes= GeneSignificanceALL > -log10(0.05) & abs(kMEEbrown)>.85
table(FilterGenes)
```

```
## FilterGenes
## FALSE TRUE
## 7970 30
```

```
datSummary[FilterGenes, ]
```

	gbm133a	Gene.Symbol	LocusLink	pCox	HazardRatio	HRlower95	HRupper95	
##	731	219918_s_at	ASPM	259266	0.04740	1.95	1.100	3.46
##	805	209464_at	AURKB	9212	0.04870	2.21	1.220	4.03
##	1340	214710_s_at	CCNB1	891	0.02790	1.66	0.937	2.94
##	1402	202870_s_at	CDC20	991	0.01090	2.70	1.470	4.96
##	1404	204695_at	CDC25A	993	0.04000	2.09	1.140	3.82
##	1595	204170_s_at	CKS2	1164	0.03720	1.82	1.030	3.22
##	2297	219787_s_at	ECT2	1894	0.02060	2.30	1.260	4.22
##	2630	221591_s_at	FLJ10156	54478	0.01620	1.89	1.070	3.34
##	2671	213007_at	FLJ10719	55215	0.01370	1.92	1.070	3.43
##	2974	202580_x_at	FOXM1	2305	0.02740	1.79	1.020	3.14

## 3360 218662_s_at	HCAP-G	64151	0.04670	1.70	0.957	3.02
## 3475 208808_s_at	HMGB2	3148	0.02750	1.59	0.906	2.80
## 3921 202503_s_at	KIAA0101	9768	0.02760	1.83	1.030	3.25
## 4127 206364_at	KIF14	9928	0.03340	1.81	1.020	3.20
## 4132 218755_at	KIF20A	10112	0.03750	1.81	1.020	3.20
## 4138 218355_at	KIF4A	24137	0.04720	2.52	1.380	4.61
## 4158 218883_s_at	KLIP1	79682	0.02780	1.73	0.975	3.07
## 4164 219306_at	KNSL7	56992	0.01630	2.25	1.250	4.05
## 4166 204162_at	KNTC2	10403	0.02420	1.82	1.030	3.24
## 4168 201088_at	KPNA2	3838	0.02050	2.19	1.210	3.97
## 4481 203362_s_at	MAD2L1	4085	0.02710	1.57	0.895	2.75
## 5245 218039_at	NUSAP1	51203	0.01880	1.84	1.040	3.27
## 5826 218009_s_at	PRC1	9055	0.00247	2.41	1.320	4.41
## 6010 203554_x_at	PTTG1	9232	0.00413	1.97	1.110	3.49
## 6012 208511_at	PTTG2	10744	0.03050	1.60	0.901	2.84
## 6437 201890_at	RRM2	6241	0.01840	1.75	0.982	3.13
## 7450 201292_at	TOP2A	7153	0.02810	1.92	1.080	3.41
## 7453 219148_at	TOPK	55872	0.01930	2.00	1.130	3.54
## 7481 210052_s_at	TPX2	22974	0.00918	1.89	1.070	3.35
## 7624 202954_at	UBE2C	11065	0.00321	2.09	1.150	3.80
##	HRsd color1					
## 731 0.292	brown					
## 805 0.306	brown					
## 1340 0.292	brown					
## 1402 0.311	brown					
## 1404 0.309	brown					
## 1595 0.291	brown					
## 2297 0.308	brown					
## 2630 0.290	brown					
## 2671 0.297	brown					
## 2974 0.288	brown					
## 3360 0.293	brown					
## 3475 0.287	brown					
## 3921 0.292	brown					
## 4127 0.292	brown					
## 4132 0.292	brown					
## 4138 0.307	brown					
## 4158 0.293	brown					
## 4164 0.299	brown					
## 4166 0.292	brown					
## 4168 0.303	brown					
## 4481 0.286	brown					
## 5245 0.293	brown					
## 5826 0.307	brown					
## 6010 0.292	brown					
## 6012 0.293	brown					
## 6437 0.296	brown					
## 7450 0.292	brown					
## 7453 0.291	brown					
## 7481 0.291	brown					
## 7624 0.306	brown					

Comments: The ASPM gene colored in red was the focus of the paper Horvath et al (2006) but there are many other interesting genes.

To illustrate the use of the kME measures, we also address the following questions

Question: Screen for significant genes that have a negative correlation with the brown module eigengene

```
FilterGenes= GeneSignificanceALL > -log10(0.05) & -kMЕbrown> .5 # notice the red minus sign!
table(FilterGenes)
```

```
## FilterGenes
## FALSE TRUE
## 7996    4
datSummary[FilterGenes,]

##          gbm133a Gene.Symbol LocusLink   pCox HazardRatio HRlower95 HRupper95
## 561  206200_s_at      ANXA11      311 0.0132      1.69     0.967     2.97
## 1618 221042_s_at       CLMN      79789 0.0257      1.60     0.906     2.81
## 5567 214152_at        PIGB      9488 0.0396      1.55     0.884     2.71
## 6682 201811_x_at      SH3BP5      9467 0.0162      2.09     1.180     3.69
##          HRsd color1
## 561  0.286   grey
## 1618 0.288   green
## 5567 0.286 turquoise
## 6682 0.290 turquoise
```

Question: Screen for significant genes that are close to the brown module and the green module and far away from the yellow module. Answer

```
FilterGenes= GeneSignificanceALL > -log10(0.05) & abs(kMЕbrown)>.5 & abs(kMЕgreen)>.5
table(FilterGenes)
```

```
## FilterGenes
## FALSE TRUE
## 7996    4
datSummary[FilterGenes,]

##          gbm133a Gene.Symbol LocusLink   pCox HazardRatio HRlower95 HRupper95
## 1422 218399_s_at      CDCA4      55038 0.0438      1.25     0.716     2.18
## 1618 221042_s_at       CLMN      79789 0.0257      1.60     0.906     2.81
## 3244 213170_at        GPX7      2882 0.0199      1.09     0.626     1.91
## 6682 201811_x_at      SH3BP5      9467 0.0162      2.09     1.180     3.69
##          HRsd color1
## 1422 0.284 turquoise
## 1618 0.288   green
## 3244 0.285   grey
## 6682 0.290 turquoise
```

Question: Screen for significant genes that are close to the brown module and far away from the yellow module. Answer

```
FilterGenes= GeneSignificanceALL > -log10(0.05) & abs(kMЕbrown)>.6 & abs(kMЕyellow)<.3
table(FilterGenes)
```

```
## FilterGenes
## FALSE TRUE
## 7949    51
```

How to output the data?

```
datout=data.frame(datSummary, colorNEW=color1, ConnectivityNew=Connectivity,datKME )
write.table(datout, file="OutputCancerNetwork.csv", sep=",", row.names=F)
```

Robustness with regard to the soft threshold

We find that the results of weighted gene co-expression network analysis are highly robust with regard to the soft threshold beta. Here we show some results that demonstrate this point. Now we want to see how the correlation between kWithin and gene significance changes for different SOFT thresholds (powers). This analysis is restricted to the brown module genes.

Also we compare the 2 different connectivity measures: The standard connectivity measure is defined as the row sum of the adjacency matrix. The non-standard connectivity measure (kTOM.IN) is defined as row sum of the topological overlap matrix .

Now we want to see how the correlation between kWithin and gene significance changes for different powers beta within the BROWN module.

```
corhelp=cor(datExpr[,restConnectivity],use="pairwise.complete.obs")
whichmodule="brown"
datconnectivitiesSoft=data.frame(matrix(666,nrow=sum(colorh1==whichmodule),ncol=length(powers1)))
names(datconnectivitiesSoft)=paste("kWithinPower",powers1,sep="")
for (i in c(1:length(powers1)) ) {
  datconnectivitiesSoft[,i]=apply(abs(corhelp[colorh1==whichmodule,
  colorh1==whichmodule])^powers1[i],1,sum)
  SpearmanCorrelationsSoft=signif(cor(GeneSignificance[ colorh1==whichmodule],
  datconnectivitiesSoft, method="s",use="p"))
# Here we use the new connectivity measure based on the topological overlap matrix
datKTOM.IN=data.frame(matrix(666,nrow=sum(colorh1==whichmodule),ncol=length(powers1)))
names(datKTOM.IN)=paste("omegaWithinPower",powers1,sep="")
for (i in c(1:length(powers1)) ) {
  datconnectivitiesSoft[,i]=apply(
  1-TOMdist(abs(corhelp[colorh1==whichmodule, colorh1==whichmodule])^powers1[i])
  ,1,sum)}

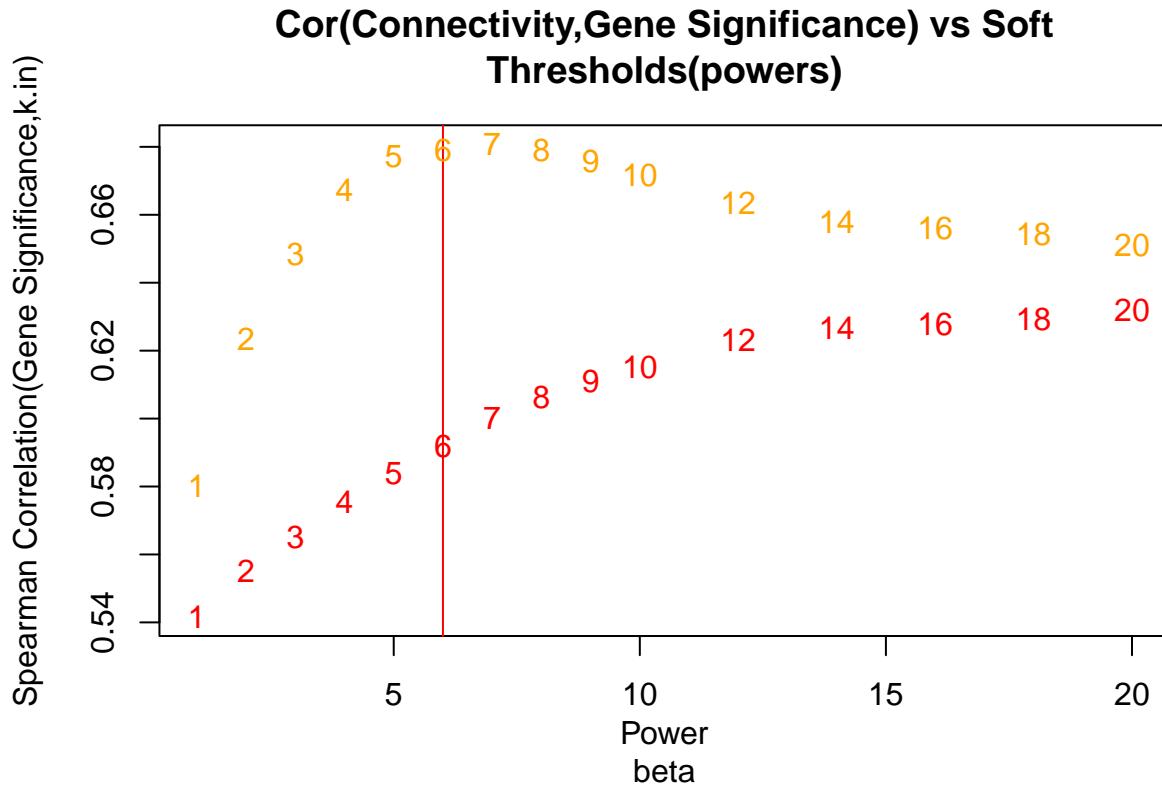
## ..connectivity..
## ..matrix multiplication (system BLAS)..
## ..normalization..
## ..done.
## ..connectivity..
## ..matrix multiplication (system BLAS)..
## ..normalization..
## ..done.
## ..connectivity..
## ..matrix multiplication (system BLAS)..
## ..normalization..
## ..done.
## ..connectivity..
## ..matrix multiplication (system BLAS)..
## ..normalization..
## ..done.
## ..connectivity..
## ..matrix multiplication (system BLAS)..
## ..normalization..
```



```

points(powers1, SpearmanCorrelationskTOMSoft, type="n")
text(powers1, SpearmanCorrelationskTOMSoft, labels=powers1, col="orange")

```

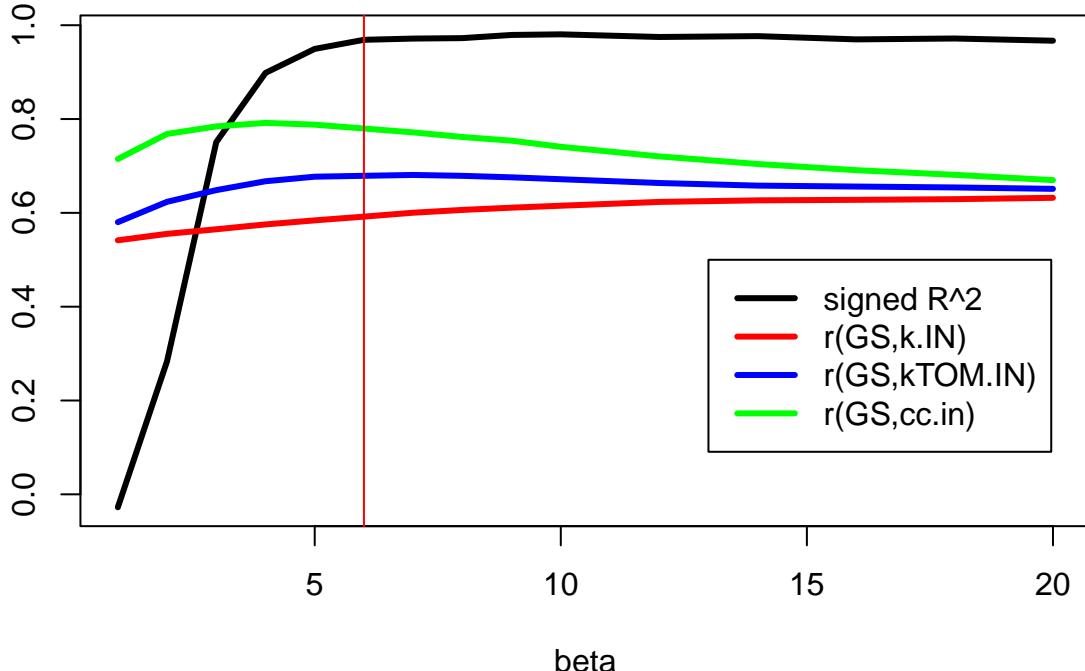


Now we define the intramodular clustering coefficient (also see the section below)

```

datCCinSoft=data.frame(matrix(666,nrow=sum(colorh1==whichmodule),ncol=length(powers1)))
names(datCCinSoft)=paste("CCinSoft",powers1,sep="")
for (i in c(1:length(powers1)) ) {
datCCinSoft[,i]= clusterCoef(abs(corhelp[colorh1==whichmodule,
colorh1==whichmodule])^powers1[i])
}
SpearmanCorrelationsCCinSoft=as.vector(signif(cor(GeneSignificance[ colorh1==whichmodule],
datCCinSoft, method="s",use="p")))
dathelpSoft=data.frame(signedRsquared=-sign(RpowerTable[,3])*RpowerTable[,2], corGSKINSoft=
as.vector(SpearmanCorrelationsSoft), corGSWINSoft=
as.vector(SpearmanCorrelationskTOMSoft), corGCCSSoft=as.vector(SpearmanCorrelationsCCinSoft))
matplot(powers1,dathelpSoft,type="l",lty=1,lwd=3,col=c("black","red","blue","green"),ylab="",xlab="beta
abline(v=6,col="red")
legend(13,0.5, c("signed R^2","r(GS,k.IN)","r(GS,kTOM.IN)","r(GS,cc.in)"),
col=c("black","red","blue","green"), lty=1,lwd=3,ncol = 1, cex=1)

```



Comment:

the intramodular cluster coefficient (green line) achieves the highest correlation with the gene significance. The TOM based intramodular connectivity kTOM.in (blue line) is superior to the standard connectivity measure k.in (red line) in this application. The vertical line corresponds to the power picked by the scale free topology criterion. The scale free topology criterion leads to near optimal biological signal when using kTOM.IN.

CAVEAT: It is worth mentioning that in other real data sets k.in outperforms cc.in and kTOM.IN.

Computation of the cluster coefficient in the weighted network.

The clustering coefficient measures the cliquishness of a gene. Many references use this concept. For our definition of the clustering coefficient in weighted networks consult Zhang and Horvath (2005) and Dong and Horvath (2007).

Here we study how the clustering coefficient depends on the connectivity.

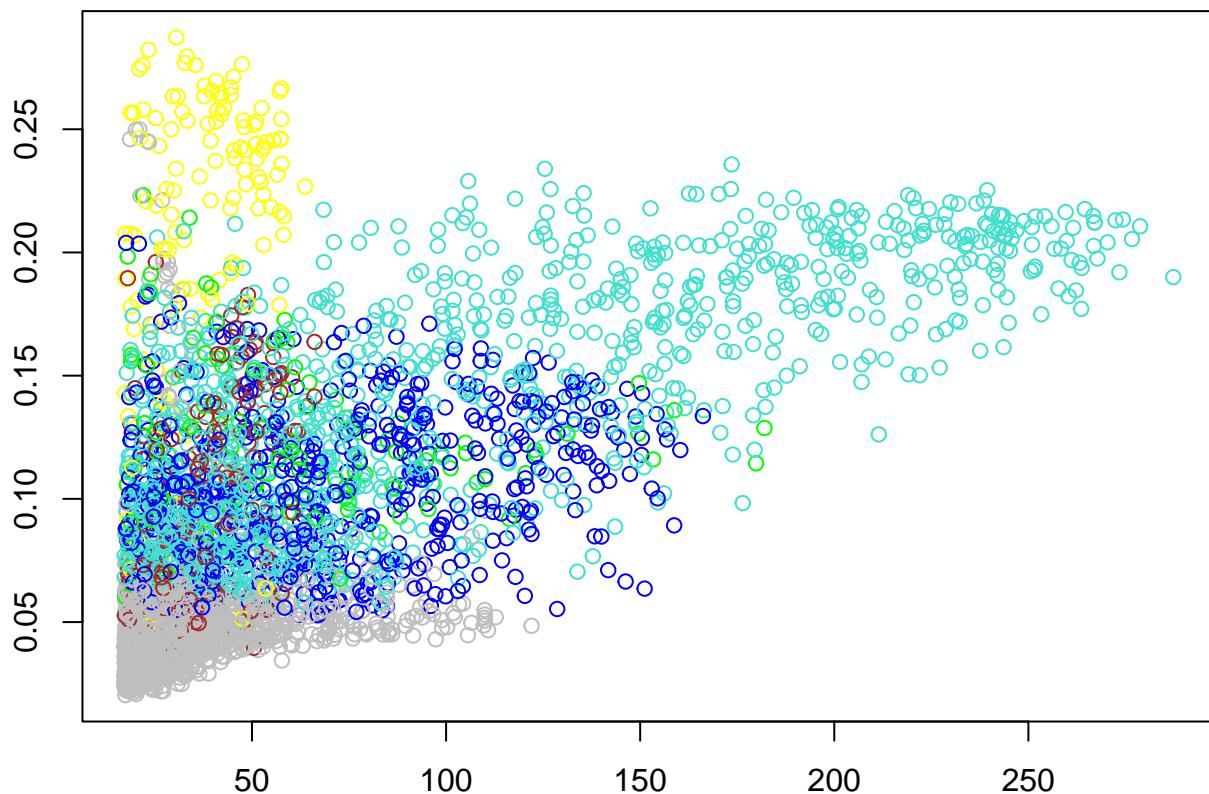
Since this is computationally intensive (around 15 minutes), we recommend to skip it.

```
CC= clusterCoef(ADJ)
gc()
```

```
##           used   (Mb) gc trigger   (Mb) limit   (Mb)  max used   (Mb)
## Ncells  4851720 259.2    8377051 447.4        NA  8377051 447.4
## Vcells 47977622 366.1   120386756 918.5      65536 210471029 1605.8
```

Now we plot cluster coefficient versus connectivity for all genes

```
par(mfrow=c(1,1),mar=c(2,2,2,1))
plot(Connectivity[restConnectivity],CC,col=as.character(colorh1),xlab="Connectivity",ylab="Cluster Coefficient")
```



This compute the correlation between cluster coefficient and connectivity within each module.

```
restHub= Connectivity[restConnectivity]>0
by(data.frame(CC=CC[restHub], k=Connectivity[restConnectivity][restHub]),
  INDICES=colorh1[restHub], FUN=cor)
```

```
## colorh1[restHub]: blue
##          CC      k
## CC 1.000000 0.242394
## k   0.242394 1.000000
##
## -----
## colorh1[restHub]: brown
##          CC      k
## CC 1.0000000 0.4765162
## k   0.4765162 1.0000000
##
## -----
## colorh1[restHub]: green
##          CC      k
## CC 1.00000000 0.06135442
## k   0.06135442 1.00000000
##
## -----
## colorh1[restHub]: grey
##          CC      k
## CC 1.0000000 0.1347935
## k   0.1347935 1.0000000
##
## -----
## colorh1[restHub]: turquoise
##          CC      k
## CC 1.000000 0.732844
```

```

## k  0.732844 1.000000
## -----
## colorh1[restHub]: yellow
##      CC          k
## CC 1.000000 0.484205
## k   0.484205 1.000000

```

Unweighted analysis

To construct an unweighted network (hard thresholding), we consider the following vector of potential thresholds.

```
thresholds1= c(seq(.1,.5, by=.1), seq(.55,.95, by=.05) )
```

To choose a cut-off value, we propose to use the Scale-free Topology Criterion (Zhang and Horvath 2005). Here the focus is on the linear regression model fitting index (denoted below by scale.law.R.2) that quantify the extent of how well a network satisfies a scale-free topology. The function PickHardThreshold can help one to estimate the cut-off value when using hard thresholding with the step adjacency function. The first column lists the threshold (“cut”), the second column lists the corresponding p-value based on the Fisher transform. The third column reports the resulting scale free topology fitting index R^2. The fourth column reports the slope of the fitting line. The fifth column reports the fitting index for the truncated exponential scale free model. Usually we ignore it. The remaining columns list the mean, median and maximum connectivity. To pick a hard threshold (cut) with the scale free topology criterion: aim for high scale free R^2 (column 3), high connectivity (col 6) and negative slope (around -1, col 4).

```
RdichotTable=WGCNA::pickHardThreshold(datExpr, cutVector=thresholds1)[[2]]
```

```

##      Cut  p.value SFT.R.sq  slope. truncated.R.2  mean.k. median.k. max.k.
## 1  0.10 4.63e-01  0.86900  6.5600          0.945 5610.000    5680  6780
## 2  0.20 1.39e-01  0.71900  1.7900          0.953 3530.000    3580  5520
## 3  0.30 2.47e-02  0.00696  0.0695          0.955 1960.000    1890  4200
## 4  0.40 2.25e-03  0.65400 -0.8370          0.941 947.000     786  2940
## 5  0.50 8.72e-05  0.83300 -1.2100          0.914 395.000     232  1860
## 6  0.55 1.13e-05  0.87100 -1.2300          0.904 243.000     110  1410
## 7  0.60 1.02e-06  0.86100 -1.2400          0.870 145.000      43  1080
## 8  0.65 5.92e-08  0.96100 -1.1100          0.952 85.900      14  795
## 9  0.70 1.93e-09  0.97300 -1.0500          0.966 50.200       4  616
## 10 0.75 2.88e-11  0.98400 -1.0100          0.983 28.900       1  480
## 11 0.80 1.40e-13  0.95300 -1.0300          0.940 15.700       0  383
## 12 0.85 0.00e+00  0.97600 -1.0400          0.972 7.200       0  262
## 13 0.90 0.00e+00  0.98000 -1.0900          0.976 2.170       0  154
## 14 0.95 0.00e+00  0.94000 -1.2600          0.965 0.194       0   47
gc()

##           used   (Mb) gc trigger   (Mb) limit   (Mb)  max used   (Mb)
## Ncells  4853357 259.2    8377051 447.4        NA 8377051 447.4
## Vcells 47983066 366.1   120386756 918.5      65536 210471029 1605.8

```

Let's plot the scale free topology model fitting index (R^2) versus the cut-off tau. However, the R^2 values of those cut-offs that lead to a negative slope have been pre-multiplied by -1.

```
cex1=0.7
gc()
```

```

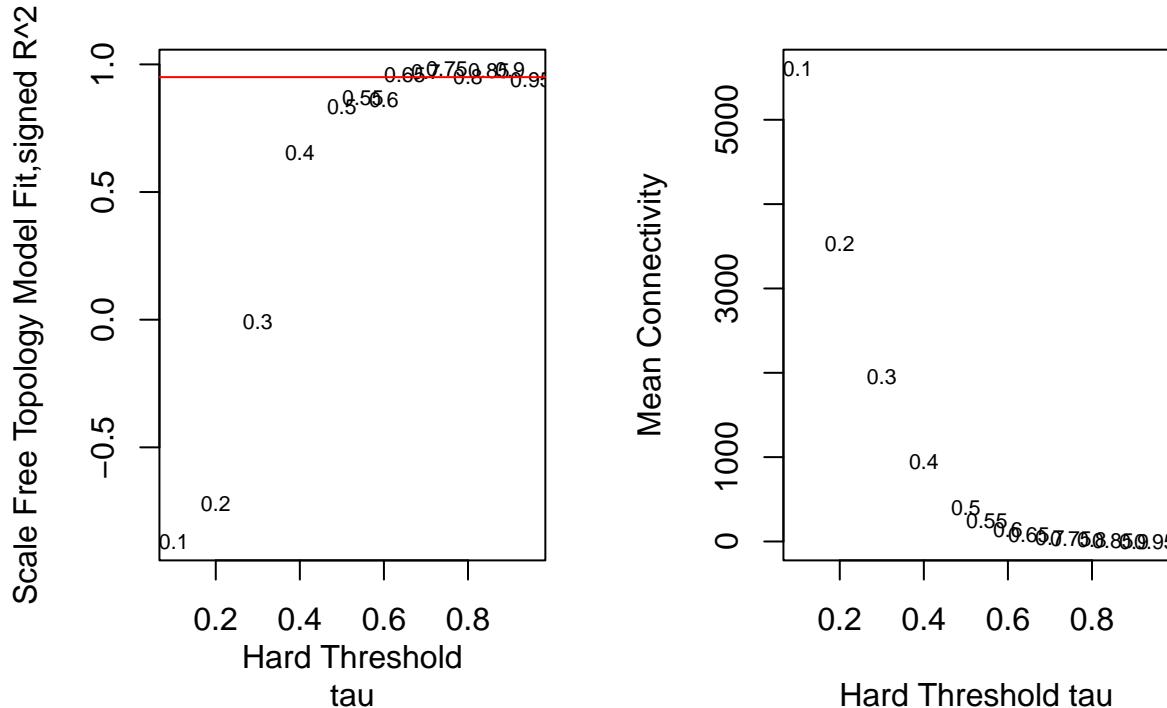
##           used   (Mb) gc trigger   (Mb) limit   (Mb)  max used   (Mb)
## Ncells  4853506 259.3    8377051 447.4        NA 8377051 447.4

```

```

## Vcells 47984441 366.1 120386756 918.5      65536 210471029 1605.8
par(mfrow=c(1,2))
plot(RdichotTable[,1], -sign(RdichotTable[,4])*RdichotTable[,3], xlab="Hard Threshold tau", ylab="Scale Free Topology Model Fit,signed R^2", type="n")
text(RdichotTable[,1], -sign(RdichotTable[,4])*RdichotTable[,3] , labels=thresholds1, cex=cex1)
# this line corresponds to using an R^2 cut-off of h
abline(h=0.95, col="red")
plot(RdichotTable[,1], RdichotTable[,6], xlab="Hard Threshold tau", ylab="Mean Connectivity", type="n")
text(RdichotTable[,1], RdichotTable[,6] , labels=thresholds1, cex=cex1)

```



To choose a cut-off value tau, we propose to use the Scale-free Topology Criterion (Zhang and Horvath 2005). Here the focus is on the linear regression model fitting index (denoted as scale.law.R.2) that quantify the extent of how well a network satisfies a scale-free topology. We choose the cut value (tau) of 0.7 for the correlation matrix since this is where the R^2 curve seems to saturates. The red line corresponds to R^2=0.95. From the above table, we find that the resulting slope looks OK (negative and around -1), and the mean number of connections looks good Below we investigate different choices of tau.

Now we want to see how the correlation between kWithin and gene significance changes for different hard threshold values tau within the BROWN module.

```

corhelp=cor(datExpr[,restConnectivity], use="pairwise.complete.obs")
whichmodule="brown"
# the following data frame contains the intramodular connectivities
# corresponding to different hard thresholds
datconnectivitiesHard=data.frame(matrix(666,nrow=sum(colorh1==whichmodule),ncol=length(thresholds1)))
names(datconnectivitiesHard)=paste("kWithinTau",thresholds1,sep="")
for (i in 1:length(thresholds1)) {
  datconnectivitiesHard[,i]=apply(abs(corhelp[colorh1==whichmodule,
  colorh1==whichmodule])>=thresholds1[i],1,sum)}
SpearmanCorrelationsHard=signif(cor(GeneSignificance[ colorh1==whichmodule],
datconnectivitiesHard, method="s",use="p"))

```

Now we define the new connectivity measure omega based on the TOM matrix. It simply considers TOM as adjacency matrix...

```
datkTOMINHard<-data.frame(matrix(666,nrow=sum(colorh1==whichmodule),ncol=length(thresholds1)))
names(datkTOMINHard)=paste("omegaWithinHard",thresholds1,sep="")
for (i in c(1:length(thresholds1)) ) {
  datconnectivitiesHard[,i]=apply(
    1-TOMdist(apply(abs(corrhelp[colorh1==whichmodule,
    colorh1==whichmodule])>thresholds1[i],2,as.numeric)),1,sum)

  ## ..connectivity..
  ## ..matrix multiplication (system BLAS)..
  ## ..normalization..
  ## ..done.
  ## ..connectivity..
  ## ..matrix multiplication (system BLAS)..
  ## ..normalization..
  ## ..done.
  ## ..connectivity..
  ## ..matrix multiplication (system BLAS)..
  ## ..normalization..
  ## ..done.
  ## ..connectivity..
  ## ..matrix multiplication (system BLAS)..
  ## ..normalization..
  ## ..done.
  ## ..connectivity..
  ## ..matrix multiplication (system BLAS)..
  ## ..normalization..
  ## ..done.
  ## ..connectivity..
  ## ..matrix multiplication (system BLAS)..
  ## ..normalization..
  ## ..done.
  ## ..connectivity..
  ## ..matrix multiplication (system BLAS)..
  ## ..normalization..
  ## ..done.
  ## ..connectivity..
  ## ..matrix multiplication (system BLAS)..
  ## ..normalization..
  ## ..done.
  ## ..connectivity..
  ## ..matrix multiplication (system BLAS)..
  ## ..normalization..
  ## ..done.
  ## ..connectivity..
  ## ..matrix multiplication (system BLAS)..
  ## ..normalization..
  ## ..done.
  ## ..connectivity..
  ## ..matrix multiplication (system BLAS)..
  ## ..normalization..
  ## ..done.
  ## ..connectivity..
  ## ..matrix multiplication (system BLAS)..
  ## ..normalization..
  ## ..done.
  ## ..connectivity..
  ## ..matrix multiplication (system BLAS)..
  ## ..normalization..
  ## ..done.
```

```

## ..done.
## ..connectivity..
## ..matrix multiplication (system BLAS)..
## ..normalization..
## ..done.
## ..connectivity..
## ..matrix multiplication (system BLAS)..
## ..normalization..
## ..done.
## ..connectivity..
## ..matrix multiplication (system BLAS)..
## ..normalization..
## ..done.

SpearmanCorrelationskTOMHard<-as.vector(signif(cor(GeneSignificance[
colorh1==whichmodule], datconnectivitiesHard, method="s",use="p")))

```

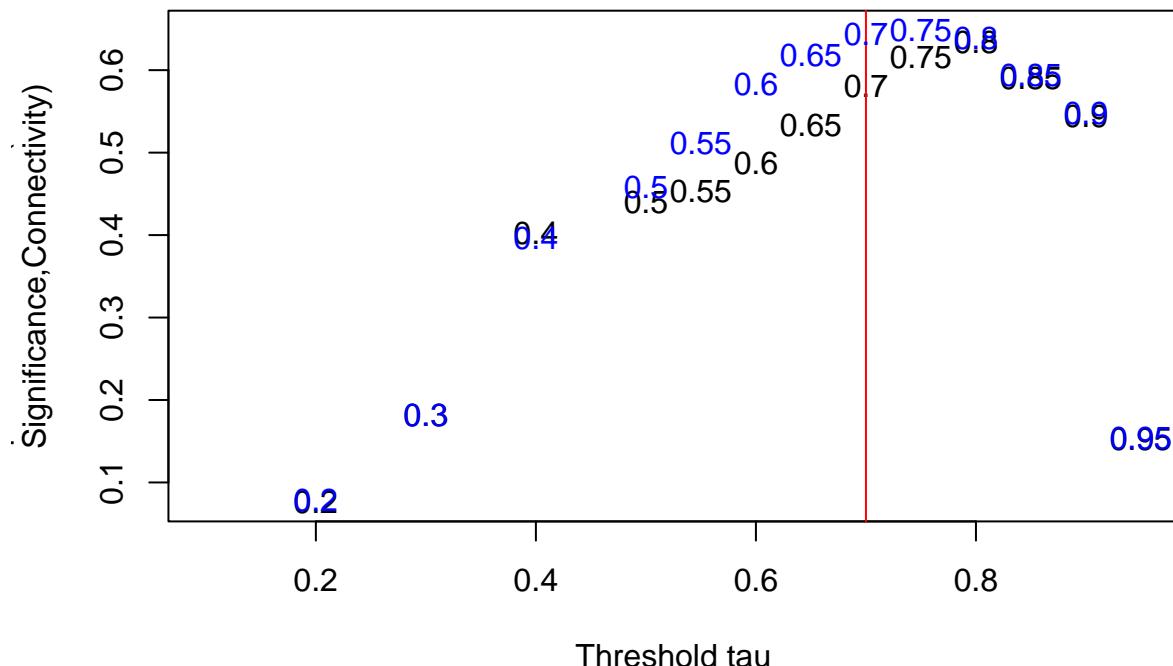
Now we compare the performance of the 2 connectivity measures (k.in and kTOM.IN) across different hard thresholds when it comes to predicting prognostic genes in the brown module

```

par(mfrow=c(1,1), mar=c(5, 4, 4, 2) +0.1)
plot(thresholds1, SpearmanCorrelationsHard, main=
"Cor(Connectivity, Gene Significance) vs Hard Thresholds", ylab="Spearman Correlation(Gene
Significance,Connectivity)", xlab="Threshold tau", type="n",
ylim=range(c(SpearmanCorrelationsHard, SpearmanCorrelationskTOMHard),na.rm=T))
text(thresholds1, SpearmanCorrelationsHard, labels=thresholds1,col="black")
# this draws a vertical line at the tau that was chosen by the
# scale free topology criterion.
abline(v=0.7,col="red")
points(thresholds1, SpearmanCorrelationskTOMHard, type="n")
text(thresholds1, SpearmanCorrelationskTOMHard, labels=thresholds1,col="blue")

```

Cor(Connectivity, Gene Significance) vs Hard Thresholds

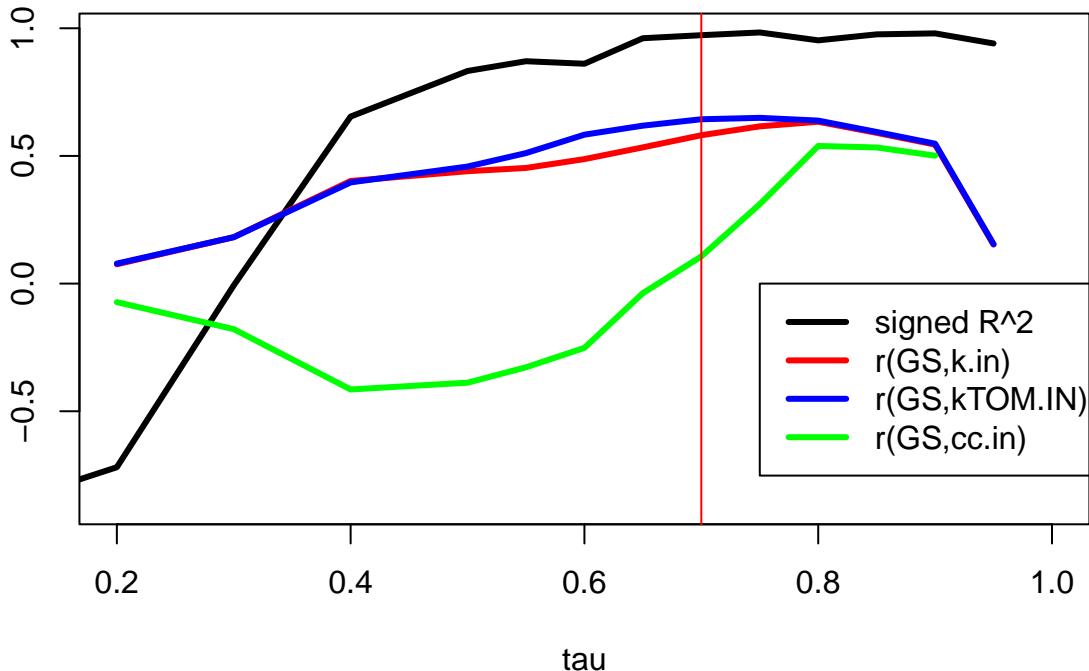


Now we define the intramodular cluster coefficient

```
datCCinHard=data.frame(matrix(666,nrow=sum(colorh1==whichmodule),ncol=length(thresholds1)))
names(datCCinHard)=paste("CCinHard",thresholds1,sep="")
for (i in 1:length(thresholds1)) {
datCCinHard[,i]= clusterCoef(apply(abs(corhelp[colorh1==whichmodule,
colorh1==whichmodule])>thresholds1[i],2,as.numeric))}
SpearmanCorrelationsCCinHard=as.vector(signif(cor(GeneSignificance[ colorh1==whichmodule],
datCCinHard, method="s",use="p")))
```

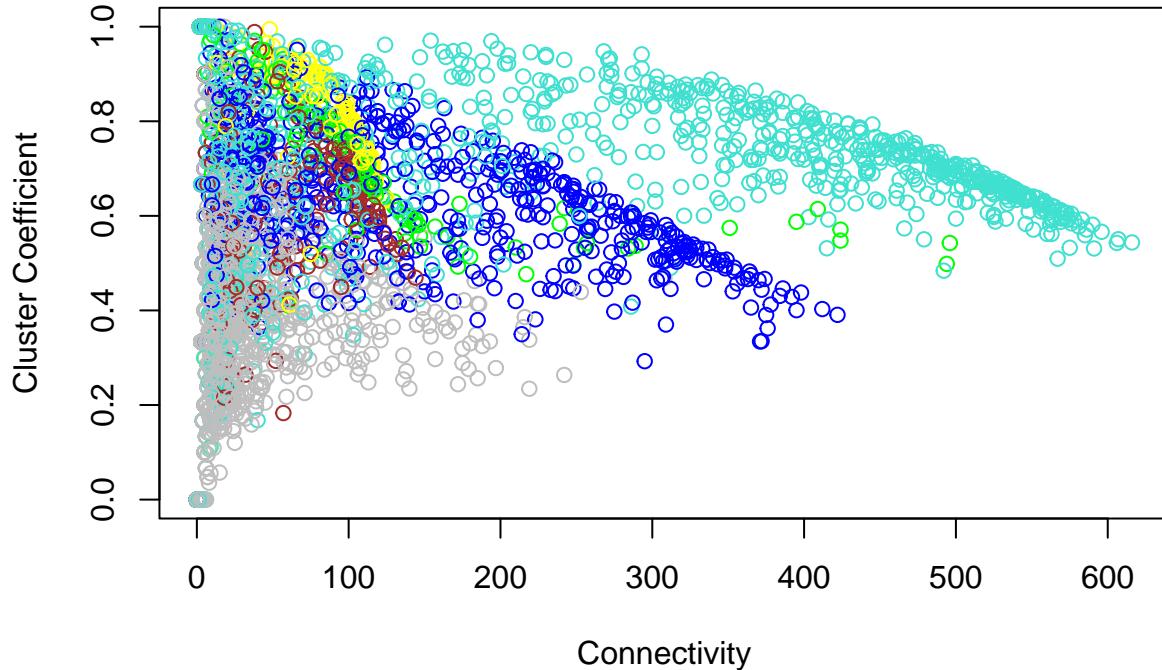
Now we compare the performance of the connectivity measures (k.in, kTOM.IN, cluster coefficience) across different hard thresholds when it comes to predicting prognostic genes in the brown module

```
dathelpHard=data.frame(signedRsquared=-sign(RdichotTable[,4])*RdichotTable[,3],
corGSkINHard =as.vector(SpearmanCorrelationsHard), corGSwINHard=
as.vector(SpearmanCorrelationskTOMHard), corGSCCHard=as.vector(SpearmanCorrelationsCCinHard)
matplot(thresholds1,dathelpHard,type="l",lty=1,lwd=3,col=c("black","red","blue","green"),ylab="",
,xlab="tau",xlim=c(.2,1))
legend(0.75,0, c("signed R^2","r(GS,k.in)","r(GS,kTOM.IN)","r(GS,cc.in)"),
col=c("black","red","blue","green"), lty=1,lwd=3,ncol = 1, cex=1)
abline(v=.7,col="red")
```



Note that very high or very small threshold values lead to a small correlation, i.e. a diminished biological signal. The red line corresponds to the threshold that was picked using the scale free topology criterion. The scale free topology criterion picked a threshold that leads to very high significant correlation between node connectivity and gene significance.

```
AdjMathHARD=abs(cor(datExpr[,restConnectivity]))>0.70+0.0
diag(AdjMathHARD)=0
cluster.coefrestHARD= clusterCoef(AdjMathHARD)
ConnectivityHARD= apply(AdjMathHARD,2,sum)
par(mfrow=c(1,1))
plot(ConnectivityHARD,cluster.coefrestHARD,col=as.character(colorh1),xlab="Connectivity",ylab="Cluster 0")
```



Now we correlate the cluster coefficient with connectivity by module in the unweighted network

```
restHub=ConnectivityHARD>100
by(data.frame(CC= cluster.coefrestHARD[restHub], k=ConnectivityHARD[restHub]),
  INDICES=colorh1[restHub], FUN=cor)
```

```
## colorh1[restHub]: blue
##          CC            k
## CC  1.0000000 -0.5704594
## k   -0.5704594  1.0000000
## -----
## colorh1[restHub]: brown
##          CC            k
## CC  1.0000000 -0.759792
## k   -0.759792  1.0000000
## -----
## colorh1[restHub]: green
##          CC            k
## CC  1.0000000 -0.5427443
## k   -0.5427443  1.0000000
## -----
## colorh1[restHub]: grey
##          CC            k
## CC  1.0000000 -0.205176
## k   -0.205176  1.0000000
## -----
## colorh1[restHub]: turquoise
##          CC            k
## CC  1.0000000 -0.4295943
## k   -0.4295943  1.0000000
## -----
## colorh1[restHub]: yellow
##          CC            k
```

```

## CC  1.0000000 -0.9097497
## k   -0.9097497  1.0000000

```

Let's compare a summary of soft thresholding to one of hard thresholding

```

apply(datHelpHard, 2, summary)

## $signedRsquared
##      Min. 1st Qu. Median    Mean 3rd Qu.    Max.
## -0.8686  0.6988  0.9056  0.5993  0.9698  0.9835
##
## $corGSKINHard
##      Min. 1st Qu. Median    Mean 3rd Qu.    Max.    NA's
## 0.07569 0.40205 0.48756 0.43782 0.58108 0.63378      1
##
## $corGSwINHard
##      Min. 1st Qu. Median    Mean 3rd Qu.    Max.    NA's
## 0.07837 0.39628 0.54797 0.46577 0.61856 0.64919      1
##
## $corGSCCHard
##      Min. 1st Qu. Median    Mean 3rd Qu.    Max.    NA's
## -0.41462 -0.27069 -0.05500 0.02667 0.35852 0.53909      2

```

- Using the Max. values, we find that when it comes to correlating gene significance with a centrality measure (connectivity or cluster coefficient), the soft intramodular cluster coefficient is most highly correlated with gene significance. Next comes the soft TOM based connectivity (wIN), then kIN. In other applications, the cluster coefficient is not the best centrality measure. But please let us know if you find empirical evidence that the cluster coefficient is a good centrality measure. In the latest version of the manuscript Zhang and Horvath (2005) we present a theoretical argument that shows that is a weak positive correlation between intramodular cluster coefficient and intramodular connectivity in weighted networks. In contrast, one finds a negative correlation between cluster coefficient and connectivity in unweighted networks (see the plot in the appendix).
- We find that soft thresholding is superior to hard thresholding especially for low values of the scale free topology R^2 .
- In our opinion, soft centrality (connectivity) measures are better than hard measures because they are relatively robust with respect to the parameter of the adjacency function. For soft thresholding even choosing a power of beta=1 leads to a good biological signal (correlation). In contrast, choosing a hard threshold of tau=0.2 leads to a much reduced biological signal. Robustness is a very attractive property in this type of analysis since picking parameters of the adjacency function is rather ad-hoc.
- The Scale free topology criterion leads to estimates of the adjacency function that often have good biological signal.

APPENDIX: Constructing an unweighted networks and comparing it to the weighted nework.

Here we study whether the ‘soft’ modules of the unweighted network described above can also be found in the unweighted network

Recall that the soft module assignment in the 3600 most connected genes is given by

```
colorh1=as.character(datSummary$color1[restConnectivity])
```

Let's define the adjacency matrix of an unweighted network

```

ADJ= abs(cor(datExpr[,restConnectivity],use="p"))>0.7
ADJ <- apply(ADJ,2,as.numeric)
gc()

##           used   (Mb) gc trigger   (Mb) limit   (Mb) max used   (Mb)
## Ncells    4855659 259.4    8377051 447.4        NA  8377051 447.4
## Vcells   60964501 465.2   120386756 918.5      65536 210471029 1605.8
# This is the unweighted connectivity
k=as.vector(apply(ADJ,2,sum))

```

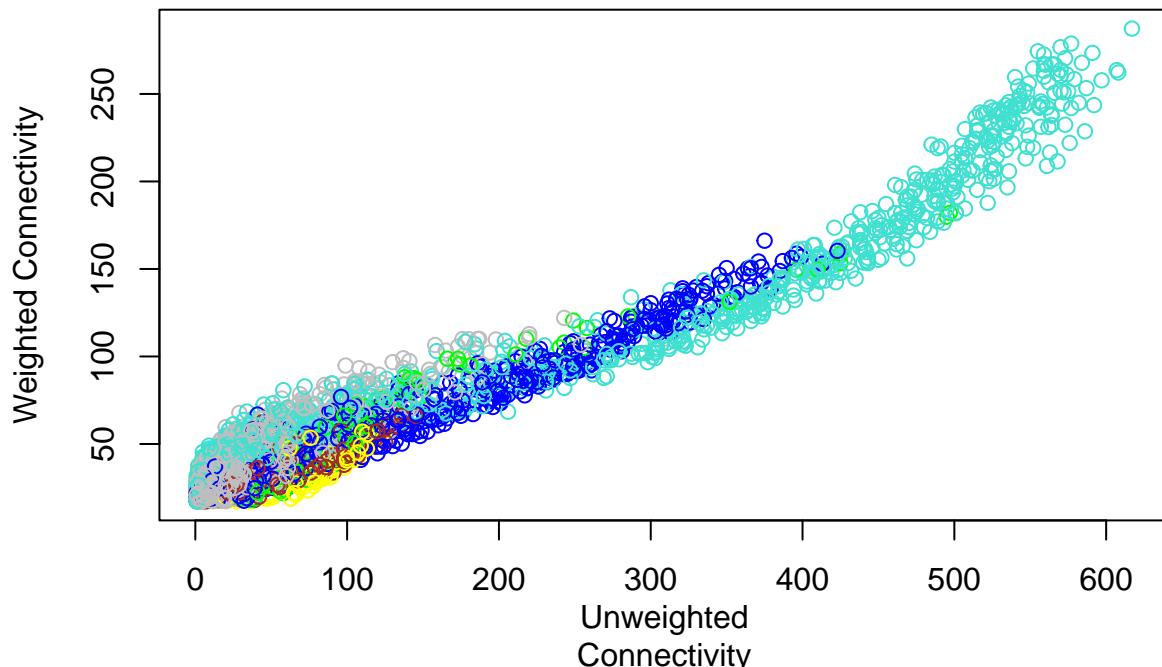
Let's compare weighted to unweighted connectivity in a scatter plot

```

plot(k, Connectivity[restConnectivity],xlab="Unweighted
Connectivity",ylab="Weighted Connectivity",main=paste( "correlation =
",signif(cor(k,Connectivity[restConnectivity]),2)),col=colorh1)

```

**correlation =
0.97**



Comments:

- the connectivity measures is highly preserved between weighted and unweighted networks as long as the scale free topology criterion is used for network construction. It is re-assuring that the biological findings will be robust with respect to the network construction method
- The genes with the highest whole network connectivity are contained in the turquoise module, which happens to be the largest module. The second most connected genes are in the blue module, which is the second largest module, etc.

The following code computes the topological overlap matrix based on the adjacency matrix

TIME: Takes about 10 minutes....

```
dissTOM=TOMdist(ADJ)
```

```

## ..connectivity..
## ..matrix multiplication (system BLAS)..
## ..normalization..

```

```

## ..done.
gc()

##           used   (Mb) gc trigger   (Mb) limit   (Mb) max used   (Mb)
## Ncells  4855605 259.4    8377051 447.4      NA  8377051 447.4
## Vcells 60967770 465.2   120386756 918.5     65536 210471029 1605.8

```

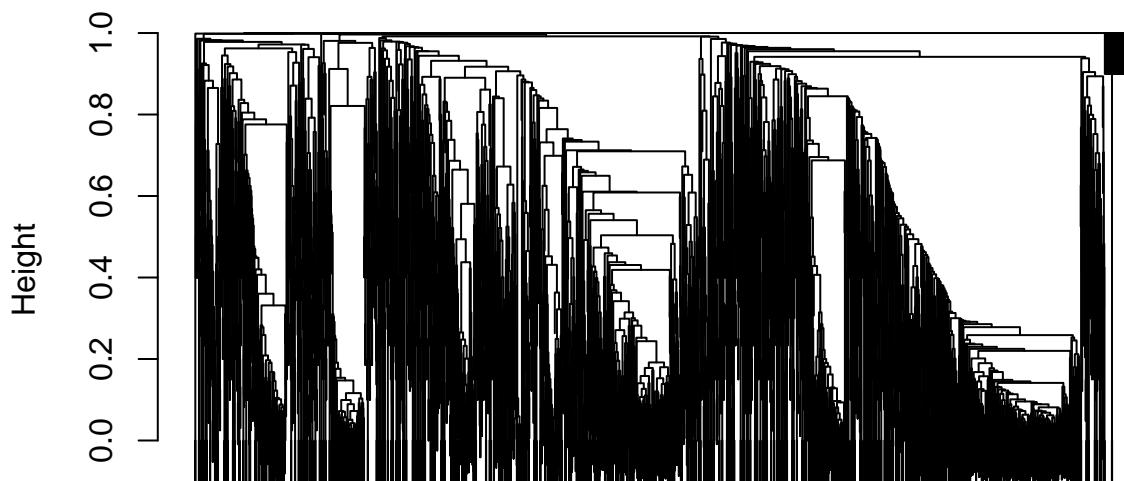
Now we carry out hierarchical clustering with the TOM matrix. Branches of the resulting clustering tree will be used to define gene modules.

```

hierTOM = hclust(as.dist(dissTOM),method="average");
par(mfrow=c(1,1))
plot(hierTOM,labels=F)

```

Cluster Dendrogram



```

as.dist(dissTOM)
hclust (*, "average")

```

By our definition, modules correspond to branches of the tree. The function modulecolor2 colors each gene by the branches that result from choosing a particular height cut-off. GREY IS RESERVED to color genes that are not part of any module. We only consider modules that contain at least 125 genes.

```
colorh2=as.character(modulecolor2(hierTOM,h1=.75, minsize1=125))
```

The following table shows that there is fairly high agreement between the soft and the hard module assignments

```
table(colorh1,colorh2)
```

	colorh2	blue	brown	green	grey	turquoise	yellow
## colorh1	blue	457	0	0	149	0	0
##	brown	0	159	0	26	0	0
##	green	0	0	0	5	0	131
##	grey	68	7	0	1321	15	7
##	turquoise	0	1	0	278	817	16

```

##      yellow      0      0    132     11      0      0
#Rand index to measure agreement between the clusterings
randIndex(table(colorh1,colorh2))

## [1] 0.5727022

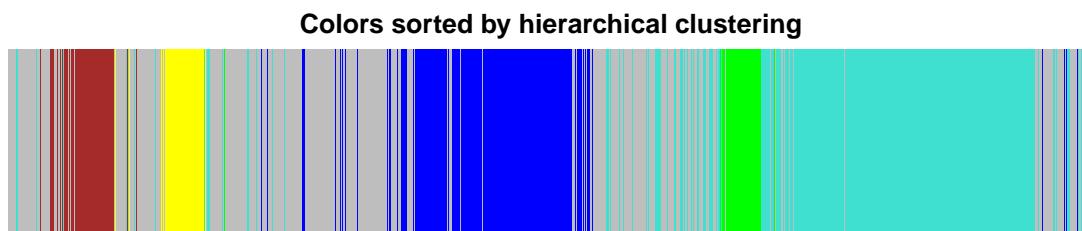
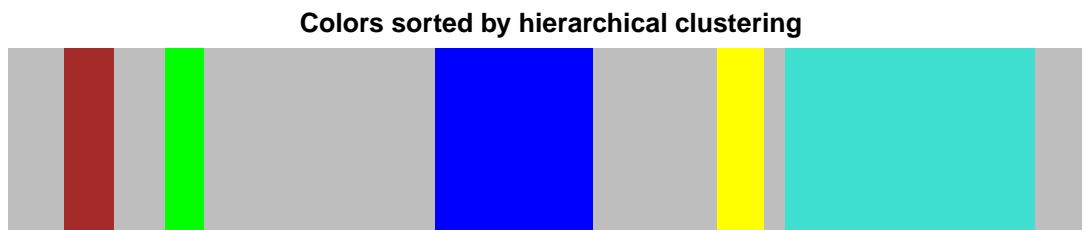
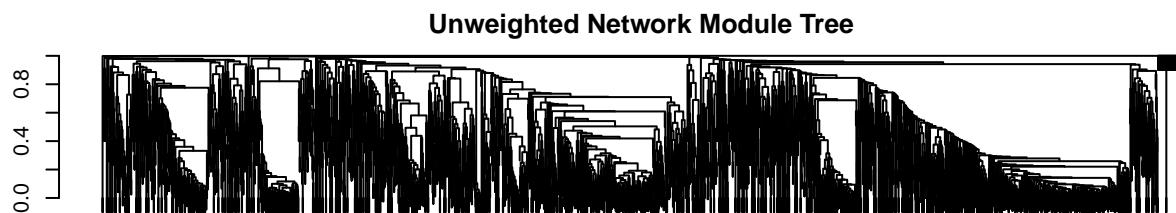
```

Note that the brown module in the weighted network (colorh1) is a little bit larger than the corresponding module in the unweighted network. But the point is that it is highly preserved. Since this module is of biological interest, the good news is that the biological findings are robust with respect to the network construction method as long as the scale free topology criterion is used to construct the network.

```

par(mfrow=c(3,1), mar=c(2,2,2,1))
plot(hierTOM, main="Unweighted Network Module Tree ", labels=F, xlab="",
sub="");
hclustplot1(hierTOM, colorh2, main="Colored by Unweighted modules")
hclustplot1(hierTOM, colorh1, main="Colored by Weighted modules")

```

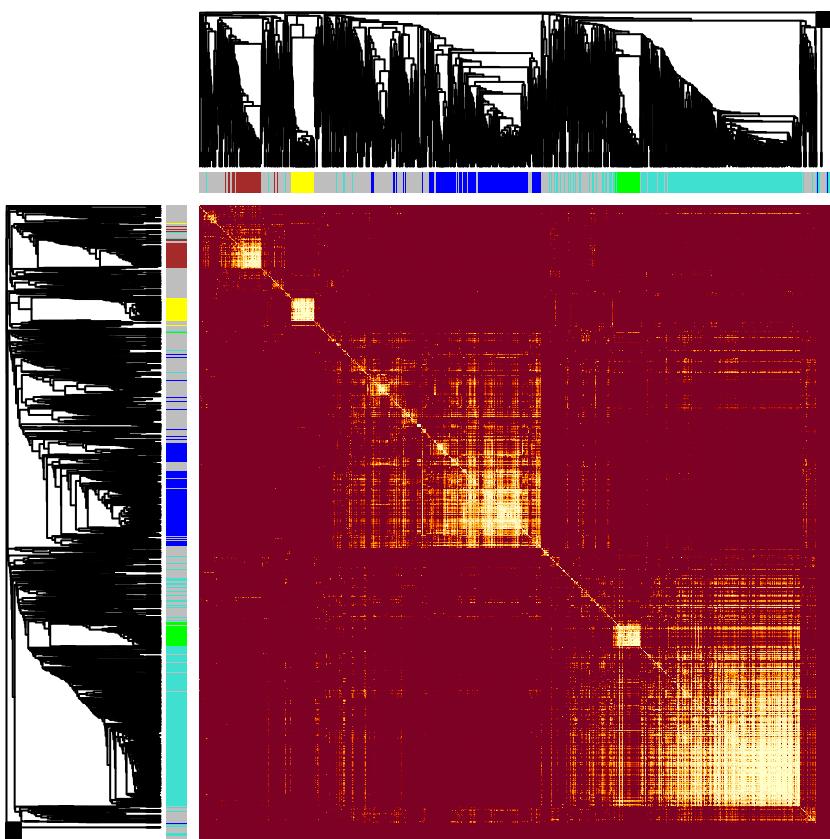


An alternative view of this is the so called TOM plot that is generated by the function TOMplot

Inputs: TOM distance measure, hierarchical (hclust) object, color

Here we use the unweighted module tree but color it by the weighted modules.

```
TOMplot(dissTOM , hierTOM, as.character(datSummary$color1[restConnectivity]))
```



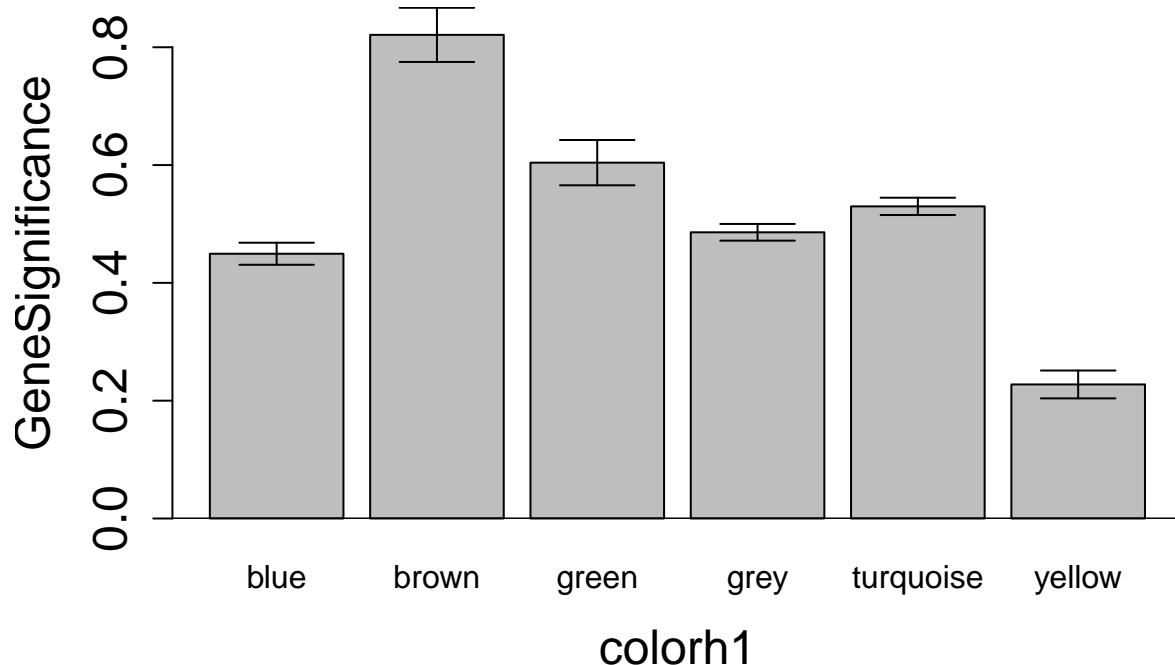
```
gc()
```

```
##           used   (Mb) gc trigger   (Mb) limit   (Mb) max used   (Mb)
## Ncells  5743311 306.8    8377051  447.4        NA  8377051  447.4
## Vcells 87949229 671.0   144544107 1102.8      65536 210471029 1605.8
```

Comment: module assignment is highly preserved.

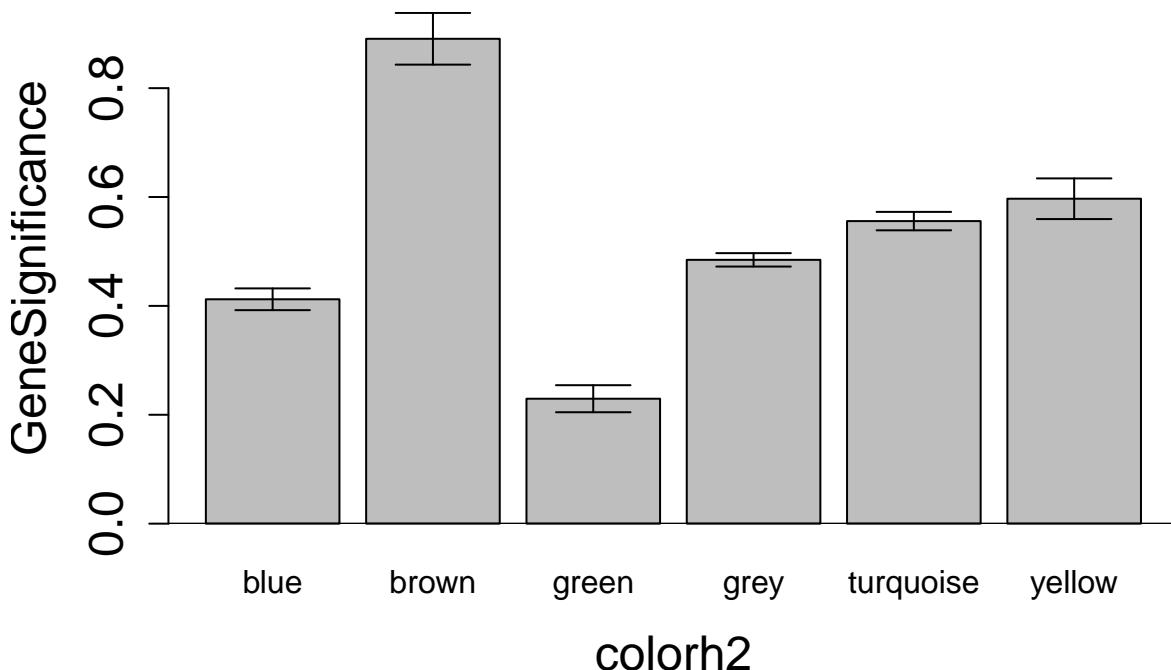
```
verboseBarplot(GeneSignificance,colorh1,main="Gene Significance in Soft Modules")
```

Gene Significance in Soft Modules $p = 3e-31$



```
verboseBarplot(GeneSignificance,colorh2,main="Gene Significance in Hard Modules")
```

Gene Significance in Hard Modules $p = 5.9e-41$



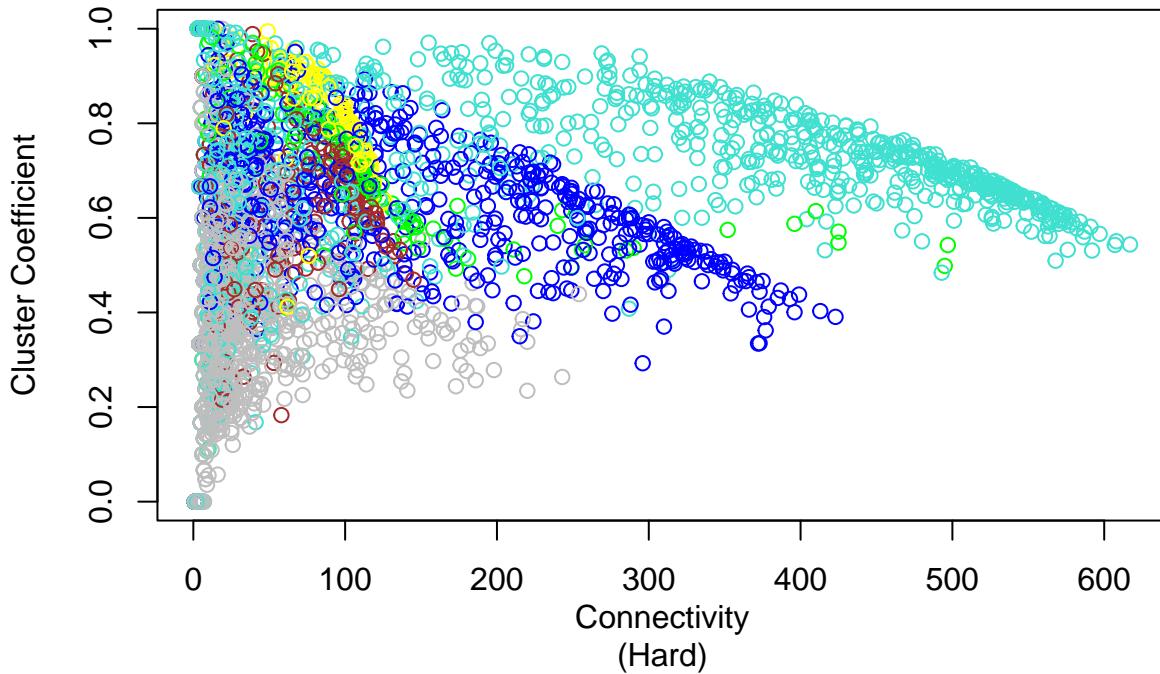
Now we compute the cluster coefficient. Since this is computationally intensive, we recommend to skip it.

```
CC= clusterCoef(ADJ)  
gc()
```

```

##          used   (Mb) gc trigger   (Mb) limit (Mb) max used   (Mb)
## Ncells  4856317 259.4    8377051  447.4        NA  8377051  447.4
## Vcells 60974079 465.2   148737040 1134.8       65536 210471029 1605.8
plot(k,CC,col= as.character(datSummary$color1[restConnectivity]),xlab="Connectivity
(Hard)",ylab="Cluster Coefficient")

```



Comment: for unweighted networks there is an inverse relationship between cluster coefficient and connectivity. This is different from the case of weighted networks. In our opinion, this inverse relationship is an artifact of hard thresholding, see Zhang and Horvath (2005).

Now we study how intramodule connectivity relates to gene significance

```
ConnectivityMeasures=intramodularConnectivity(ADJ,colorh2)
```

The following plots would show the gene significance vs intromodular connectivity

```

par(mfrow=c(2,3))
colorlevels=unique(colorh2)
whichmodule=colorlevels[[1]];restrict1=colorh2==whichmodule
verboseScatterplot(ConnectivityMeasures$kWithin[restrict1],
GeneSignificance[restrict1],col=colorh2[restrict1],main=whichmodule)
whichmodule= colorlevels[[2]];restrict1=colorh2==whichmodule
verboseScatterplot(ConnectivityMeasures$kWithin[restrict1],
GeneSignificance[restrict1],col=colorh2[restrict1],main=whichmodule)
whichmodule= colorlevels[[3]];restrict1=colorh2==whichmodule
verboseScatterplot(ConnectivityMeasures$kWithin[restrict1],
GeneSignificance[restrict1],col=colorh2[restrict1],main=whichmodule)
whichmodule= colorlevels[[4]];restrict1=colorh2==whichmodule
verboseScatterplot(ConnectivityMeasures$kWithin[restrict1],
GeneSignificance[restrict1],col=colorh2[restrict1],main=whichmodule)
whichmodule= colorlevels[[5]];restrict1=colorh2==whichmodule
verboseScatterplot(ConnectivityMeasures$kWithin[restrict1],
GeneSignificance[restrict1],col=colorh2[restrict1],main=whichmodule)
whichmodule= colorlevels[[6]];restrict1=colorh2==whichmodule

```

```
verboseScatterplot(ConnectivityMeasures$kWithin[restrict1],  
GeneSignificance[restrict1], col=colorh2[restrict1], main=whichmodule)
```

