

PCOT2: Principal Coordinates and Hotelling's T^2 for the analysis of microarray data

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1 Overview

`pcot2` is an R-package for the analysis of groups of genes in microarray experiments. It utilizes inter-gene correlation information to detect significant alterations in the activities of gene sets. Incorporating additional (usually functional) information into the data analysis process allows gene interactions to be investigated in a statistical framework. One of the reasons that gene set analysis is becoming important is that it is suitable for detecting small coordinated changes in expression of groups of genes which are functionally related, which may not be considered significant in a single gene analysis. This vignette gives a tutorial-style introduction to the functions in the `pcot2` package. These functions are used for testing and visualizing changes in expression activity for groups of genes.

2 Example: ALL/AML data

In this example the ALL/AML leukemia data set of Golub *et al.*(1999) is used to illustrate the functionality of the `pcot2` package. This data set contains 38 bone marrow samples obtained from adult leukemia patients, 11 relating to acute myeloid leukemia (AML, class 1) and 27 relating to acute lymphoblastic leukemia (ALL, class 0). Gene expression levels were measured using Affymetrix high density oligonucleotide arrays containing 6817 human genes, of which 3051 genes were considered suitable for analysis by Golub *et al.*(1999) after pre-processing. This data set is available as part of the `multtest` package and gene sets are defined as KEGG pathways using the `hu6800.db` annotation package. Both packages can be downloaded from www.bioconductor.org.

```
> library(pcot2)
> library(multtest)
> library(hu6800.db)
> set.seed(1234567)
```

3 The `pcot2` function

The `pcot2` function implements the PCOT2 testing method, which is a two-stage permutation-based approach for testing changes in activity in pre-specified

gene sets. The function requires at least three inputs: gene expression data, sample class labels, and a gene category indicator matrix. The gene expression data should be in the form of a matrix with no missing values. Data pre-processing (e.g. normalization) must therefore take place before running the PCOT2 analysis.

```
> data(golub)
> rownames(golub) <- golub.gnames[,3]
> colnames(golub) <- golub.cl
```

The class labels represent two distinct experimental conditions (e.g., AML and ALL).

```
> golub.cl

[1] 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1
```

The gene category indicator matrix is designed to indicate presence or absence of genes in the pre-defined gene categories (e.g., gene pathways). The indicator matrix contains rows representing gene identifiers for genes present in the expression data, and columns representing pre-defined group names. The values 1 or 0 indicate the presence or absence of a gene in a particular group.

In this example, the `hu6800.db` annotation package is used to define the KEGG (<http://www.genome.jp/kegg/pathway.html>) pathways for all of 3051 genes in the data. The `getImat` function is used to generate an indicator matrix which includes 65 KEGG pathways containing at least 10 of the total 3051 genes.

```
> KEGG.list <- as.list(hu6800PATH)
> imat <- getImat(golub, KEGG.list, ms=10)
> colnames(imat) <- paste("KEGG", colnames(imat), sep="")
> dim(imat)

[1] 3051 158
```

Permutations are used to produce p -values based on the null distribution of the T^2 statistic. By default `pcot2` will automatically run 1000 permutations. In order to minimize the time taken to build this vignette, only 10 permutations have been performed.

```
> results <- pcot2(golub, golub.cl, imat, iter=10)
```

Comparison: 0-1

The output from the `pcot2` function can contain information on either all pathways or just significantly differentially expressed pathways, based on the value of α used in the function, where α determines the significance threshold for the permutation p -values. For each KEGG pathway, the number of genes in the pathway is listed, along with Hotelling's T^2 statistic. These are followed by parametric p -values for the test statistic, both raw and adjusted. The last two columns provide raw and adjusted permutation-based p -values. The default adjustment method is the false discovery rate controlling method of Benjamini and Yekutieli (2001).

```
> results$res.sig
```

```
[1] Num          T2          P.nor          P.adj          P.permu          P.permu.adj  
<0 rows> (or 0-length row.names)
```

```
> results$res.all
```

	Num	T2	P.nor	P.adj	P.permu	P.permu.adj
KEGG04080	56	52.995959	1.322251e-07	3.368299e-06	0.1	0.5865721
KEGG04360	30	35.193509	6.570324e-06	8.614753e-05	0.1	0.5865721
KEGG04010	97	40.757244	1.760778e-06	2.706710e-05	0.1	0.5865721
KEGG04910	55	23.685638	1.437380e-04	1.307707e-03	0.1	0.5865721
KEGG03410	14	40.040059	2.075157e-06	3.135912e-05	0.1	0.5865721
KEGG04650	58	53.920463	1.103493e-07	3.036523e-06	0.1	0.5865721
KEGG05322	45	72.465218	4.146772e-09	3.081016e-07	0.1	0.5865721
KEGG04962	14	25.229090	9.194586e-05	9.108663e-04	0.1	0.5865721
KEGG04510	79	52.030331	1.600398e-07	3.963605e-06	0.1	0.5865721
KEGG04270	44	24.494250	1.135788e-04	1.077294e-03	0.1	0.5865721
KEGG04810	84	42.380783	1.220748e-06	2.015566e-05	0.1	0.5865721
KEGG04060	82	57.371188	5.709109e-08	2.036073e-06	0.1	0.5865721
KEGG04062	87	70.607587	5.610503e-09	3.573047e-07	0.1	0.5865721
KEGG03050	23	26.894107	5.749360e-05	6.030670e-04	0.1	0.5865721
KEGG04110	57	46.327670	5.167040e-07	1.023751e-05	0.1	0.5865721
KEGG03320	18	55.009039	8.939526e-08	2.748410e-06	0.1	0.5865721
KEGG04971	35	20.675694	3.555077e-04	2.962308e-03	0.1	0.5865721
KEGG04972	35	45.037150	6.812727e-07	1.265449e-05	0.1	0.5865721
KEGG04976	20	19.403696	5.288905e-04	4.248228e-03	0.1	0.5865721
KEGG05110	30	24.810971	1.036597e-04	1.004586e-03	0.1	0.5865721
KEGG04146	20	32.548726	1.274396e-05	1.556491e-04	0.1	0.5865721
KEGG00190	43	14.212036	2.959080e-03	2.077390e-02	0.1	0.5865721
KEGG01100	316	68.049116	8.582659e-09	4.546626e-07	0.1	0.5865721
KEGG05010	68	17.102359	1.111225e-03	8.325687e-03	0.1	0.5865721
KEGG05012	43	10.731022	1.040403e-02	6.871201e-02	0.1	0.5865721
KEGG05016	70	31.545201	1.649643e-05	1.935269e-04	0.1	0.5865721
KEGG04142	54	59.905792	3.572803e-08	1.384989e-06	0.1	0.5865721
KEGG03420	15	15.484007	1.909975e-03	1.395831e-02	0.1	0.5865721
KEGG04141	63	41.998038	1.329932e-06	2.121540e-05	0.1	0.5865721
KEGG03018	13	5.431912	8.549321e-02	4.982017e-01	0.1	0.5865721
KEGG04144	53	36.576214	4.692382e-06	6.244297e-05	0.1	0.5865721
KEGG04020	57	34.108620	8.595950e-06	1.110733e-04	0.1	0.5865721
KEGG04666	43	46.505825	4.975229e-07	1.008150e-05	0.1	0.5865721
KEGG05100	31	33.781340	9.329447e-06	1.171555e-04	0.1	0.5865721
KEGG00350	10	5.163053	9.581005e-02	5.546964e-01	0.1	0.5865721
KEGG04514	62	30.108931	2.402932e-05	2.678036e-04	0.1	0.5865721
KEGG04530	36	31.095936	1.854001e-05	2.146764e-04	0.1	0.5865721
KEGG04670	52	33.856570	9.155178e-06	1.166094e-04	0.1	0.5865721
KEGG05160	55	53.505956	1.196423e-07	3.137407e-06	0.1	0.5865721
KEGG03430	13	22.840756	1.844695e-04	1.596806e-03	0.1	0.5865721
KEGG05200	151	67.989561	8.669084e-09	4.546626e-07	0.1	0.5865721
KEGG05210	37	29.737191	2.652154e-05	2.919300e-04	0.1	0.5865721
KEGG05213	28	26.480816	6.452479e-05	6.537458e-04	0.1	0.5865721

KEGG05416	41	21.371049	2.871904e-04	2.415622e-03	0.1	0.5865721
KEGG04120	30	13.976193	3.213204e-03	2.220821e-02	0.1	0.5865721
KEGG04974	29	8.159227	2.801678e-02	1.676474e-01	0.1	0.5865721
KEGG04210	41	25.794077	7.829317e-05	7.843300e-04	0.1	0.5865721
KEGG05142	55	49.447678	2.694878e-07	6.322961e-06	0.1	0.5865721
KEGG05014	23	31.606850	1.623516e-05	1.930013e-04	0.1	0.5865721
KEGG05130	24	8.239661	2.713860e-02	1.634898e-01	0.1	0.5865721
KEGG05131	31	55.243538	8.545878e-08	2.721220e-06	0.1	0.5865721
KEGG04115	24	37.099129	4.138379e-06	5.590508e-05	0.1	0.5865721
KEGG04916	32	14.931124	2.307207e-03	1.658937e-02	0.1	0.5865721
KEGG05215	47	53.971118	1.092671e-07	3.036523e-06	0.1	0.5865721
KEGG04310	44	41.315269	1.551142e-06	2.426284e-05	0.1	0.5865721
KEGG04350	23	24.300792	1.201271e-04	1.120171e-03	0.1	0.5865721
KEGG03013	41	12.442917	5.543242e-03	3.716012e-02	0.1	0.5865721
KEGG04145	75	146.861808	4.447553e-13	1.982696e-10	0.1	0.5865721
KEGG04520	32	22.207678	2.229148e-04	1.892843e-03	0.1	0.5865721
KEGG05410	31	18.282987	7.562727e-04	5.863346e-03	0.1	0.5865721
KEGG05414	32	10.341813	1.204386e-02	7.838090e-02	0.1	0.5865721
KEGG00010	37	9.063638	1.964873e-02	1.216569e-01	0.1	0.5865721
KEGG04380	71	32.680575	1.232254e-05	1.525923e-04	0.1	0.5865721
KEGG04620	48	49.019006	2.942818e-07	6.727656e-06	0.1	0.5865721
KEGG04630	53	37.113071	4.124590e-06	5.590508e-05	0.1	0.5865721
KEGG05140	56	41.989367	1.332522e-06	2.121540e-05	0.1	0.5865721
KEGG05145	70	46.045074	5.487504e-07	1.063609e-05	0.1	0.5865721
KEGG05212	42	26.528157	6.367518e-05	6.525532e-04	0.1	0.5865721
KEGG04640	63	123.041575	5.115131e-12	1.520199e-09	0.1	0.5865721
KEGG00980	10	66.696592	1.079104e-08	5.345098e-07	0.1	0.5865721
KEGG00983	12	44.930783	6.971132e-07	1.268447e-05	0.1	0.5865721
KEGG00240	30	74.320240	3.081965e-09	2.747848e-07	0.1	0.5865721
KEGG00480	14	89.964548	3.026550e-10	5.396881e-08	0.1	0.5865721
KEGG00590	16	39.391204	2.410915e-06	3.582578e-05	0.1	0.5865721
KEGG00860	15	49.760861	2.527749e-07	6.091121e-06	0.1	0.5865721
KEGG00030	15	13.506746	3.790243e-03	2.599493e-02	0.1	0.5865721
KEGG00230	51	25.179015	9.327181e-05	9.138480e-04	0.1	0.5865721
KEGG00071	18	39.257416	2.487030e-06	3.635098e-05	0.1	0.5865721
KEGG04920	27	62.446658	2.260875e-08	9.496335e-07	0.1	0.5865721
KEGG05150	32	56.063892	7.306855e-08	2.412858e-06	0.1	0.5865721
KEGG00620	14	24.286911	1.206120e-04	1.120171e-03	0.1	0.5865721
KEGG04930	21	19.258351	5.537710e-04	4.408361e-03	0.1	0.5865721
KEGG04664	36	62.245608	2.343224e-08	9.496335e-07	0.1	0.5865721
KEGG04722	56	56.071300	7.296574e-08	2.412858e-06	0.1	0.5865721
KEGG04912	35	15.060709	2.206856e-03	1.599683e-02	0.1	0.5865721
KEGG00280	19	38.660972	2.858611e-06	4.045568e-05	0.1	0.5865721
KEGG00310	12	28.018168	4.216839e-05	4.529746e-04	0.1	0.5865721
KEGG00380	15	103.491944	5.077894e-11	1.131849e-08	0.1	0.5865721
KEGG00640	14	47.605074	3.946596e-07	8.377961e-06	0.1	0.5865721
KEGG00650	10	4.237641	1.426346e-01	8.204612e-01	0.1	0.5865721
KEGG00020	14	13.152966	4.297080e-03	2.902448e-02	0.1	0.5865721
KEGG04012	38	23.225345	1.645928e-04	1.452962e-03	0.1	0.5865721
KEGG05220	47	39.126096	2.564215e-06	3.687463e-05	0.1	0.5865721

KEGG00564	14	58.921920	4.279369e-08	1.589767e-06	0.1	0.5865721
KEGG05340	25	148.792814	3.701484e-13	1.982696e-10	0.1	0.5865721
KEGG00500	12	28.113816	4.108093e-05	4.466748e-04	0.1	0.5865721
KEGG05120	34	65.157949	1.405379e-08	6.594849e-07	0.1	0.5865721
KEGG05323	44	79.924016	1.294968e-09	1.443225e-07	0.1	0.5865721
KEGG03040	39	17.114706	1.106713e-03	8.325687e-03	0.1	0.5865721
KEGG04660	50	10.494546	1.136995e-02	7.453918e-02	0.1	0.5865721
KEGG00410	12	46.645514	4.830102e-07	1.001504e-05	0.1	0.5865721
KEGG05221	40	37.309442	3.935458e-06	5.482521e-05	0.1	0.5865721
KEGG04340	11	6.073128	6.534459e-02	3.832931e-01	0.1	0.5865721
KEGG05218	31	20.513822	3.737548e-04	3.085518e-03	0.1	0.5865721
KEGG04512	26	24.645916	1.087092e-04	1.042194e-03	0.1	0.5865721
KEGG05146	49	71.444582	4.892881e-09	3.355724e-07	0.1	0.5865721
KEGG05222	46	43.526104	9.470522e-07	1.623811e-05	0.1	0.5865721
KEGG04610	14	72.954692	3.832568e-09	3.081016e-07	0.1	0.5865721
KEGG03030	19	22.769488	1.884236e-04	1.615351e-03	0.1	0.5865721
KEGG04622	20	53.826381	1.123894e-07	3.036523e-06	0.1	0.5865721
KEGG00970	16	23.403392	1.561698e-04	1.392394e-03	0.1	0.5865721
KEGG03015	20	48.276817	3.430849e-07	7.460754e-06	0.1	0.5865721
KEGG04970	38	64.827764	1.488132e-08	6.634013e-07	0.1	0.5865721
KEGG04370	35	31.024253	1.889009e-05	2.159257e-04	0.1	0.5865721
KEGG04662	45	44.427951	7.774477e-07	1.367602e-05	0.1	0.5865721
KEGG00051	16	26.636897	6.176816e-05	6.403703e-04	0.1	0.5865721
KEGG00052	15	19.849740	4.596460e-04	3.759776e-03	0.1	0.5865721
KEGG04114	42	23.562341	1.490349e-04	1.342202e-03	0.1	0.5865721
KEGG04540	35	9.106446	1.932494e-02	1.204889e-01	0.1	0.5865721
KEGG04914	33	18.313146	7.489572e-04	5.857565e-03	0.1	0.5865721
KEGG04070	30	22.848678	1.840354e-04	1.596806e-03	0.1	0.5865721
KEGG04720	36	8.649082	2.309800e-02	1.410544e-01	0.1	0.5865721
KEGG04730	31	78.228678	1.675825e-09	1.660164e-07	0.1	0.5865721
KEGG00561	12	88.191090	3.878922e-10	5.764010e-08	0.1	0.5865721
KEGG00330	21	68.201795	8.365228e-09	4.546626e-07	0.1	0.5865721
KEGG03008	10	14.128713	3.046345e-03	2.121945e-02	0.1	0.5865721
KEGG00520	15	8.466957	2.481070e-02	1.504828e-01	0.1	0.5865721
KEGG04672	24	44.399449	7.822850e-07	1.367602e-05	0.1	0.5865721
KEGG05144	32	81.917469	9.608954e-10	1.223892e-07	0.1	0.5865721
KEGG05310	21	32.129242	1.418916e-05	1.709582e-04	0.1	0.5865721
KEGG05320	25	15.995128	1.606747e-03	1.183933e-02	0.1	0.5865721
KEGG05330	24	19.655395	4.885585e-04	3.959942e-03	0.1	0.5865721
KEGG04612	40	45.349787	6.368783e-07	1.208157e-05	0.1	0.5865721
KEGG04940	24	9.486543	1.668563e-02	1.062624e-01	0.1	0.5865721
KEGG05332	24	10.138221	1.300856e-02	8.404559e-02	0.1	0.5865721
KEGG05143	19	24.060403	1.288248e-04	1.184112e-03	0.1	0.5865721
KEGG05214	39	18.202500	7.761679e-04	5.965717e-03	0.1	0.5865721
KEGG05219	22	48.867088	3.036379e-07	6.768009e-06	0.1	0.5865721
KEGG05223	31	16.965995	1.162369e-03	8.636297e-03	0.1	0.5865721
KEGG00270	13	9.247003	1.830088e-02	1.157225e-01	0.1	0.5865721
KEGG04966	10	42.692106	1.138933e-06	1.915964e-05	0.1	0.5865721
KEGG04621	21	54.823971	9.263717e-08	2.753145e-06	0.1	0.5865721
KEGG04623	17	17.496447	9.763539e-04	7.440231e-03	0.1	0.5865721

KEGG04330	16	14.667409	2.526630e-03	1.787870e-02	0.1	0.5865721
KEGG04964	11	27.049375	5.506555e-05	5.844746e-04	0.1	0.5865721
KEGG04150	18	11.009560	9.376387e-03	6.238723e-02	0.1	0.5865721
KEGG04973	20	13.182805	4.251686e-03	2.893709e-02	0.1	0.5865721
KEGG05216	19	30.751272	2.028858e-05	2.289758e-04	0.1	0.5865721
KEGG05020	21	14.773131	2.436126e-03	1.737620e-02	0.1	0.5865721
KEGG04740	10	9.033627	1.987914e-02	1.222347e-01	0.1	0.5865721
KEGG04742	10	9.165107	1.889037e-02	1.186088e-01	0.1	0.5865721
KEGG00562	15	18.867003	6.271148e-04	4.948044e-03	0.1	0.5865721
KEGG00510	15	7.675775	3.396901e-02	2.019094e-01	0.2	1.0000000
KEGG00250	12	10.116799	1.311470e-02	8.412183e-02	0.2	1.0000000
KEGG04960	19	6.414720	5.672222e-02	3.349201e-01	0.3	1.0000000
KEGG04260	29	2.355025	3.299165e-01	1.000000e+00	0.4	1.0000000
KEGG05412	26	3.301194	2.153740e-01	1.000000e+00	0.5	1.0000000
KEGG05211	31	2.628229	2.913801e-01	1.000000e+00	0.5	1.0000000

In the `pcot2` function, the T^2 statistic can be calculated in two ways, using either a pooled estimate of correlation for the two classes (default) or an un-pooled estimate. And users can set `var.equal=F` if the correlation structure is assumed to differ across the two classes.

In the first step of the PCOT2 analysis, the dimensionality of the gene expression data is reduced via principal coordinates. The default dimensionality in the `pcot2` function is set as `ncomp=2`. In the second step of the PCOT2 analysis, the distances between the transformed groups are calculated via euclidean distances by default. Other distances (e.g., correlation or Spearman distances) can also be used by defining `dist.method` in the function. A permutation p -value for each category is calculated by re-arranging the sample labels. The permutations can also be performed by permuting rows (genes), using `permu='ByRow'`.

Table 1 lists computation times (in minutes) required to run 1000 permutations of the `pcot2` function on the AML/ALL data under various parameter configurations. The two machines used were a 3.2GHz Pentium 4 with 1Gb RAM running Microsoft Windows XP and R 2.1.0 (PC), and a 1.70GHz Pentium M with 256Mb of RAM running Fedora Core 3 and R 2.2.0 (Unix).

Table 1: *Computation times (minutes, 1000 permutations)*

Changes	PC machine	UNIX machine
default setting	5.6	6.8
var.equal=F	5.5	6.8
comp=8	6	7.6
dist.method="euclidean"	4.8	6
permu="ByRow"	5.6	6.8

4 The `corplot` and `corplot2` functions

The `corplot` and `corplot2` functions enable visualization of both correlation and gene expression information for a particular gene category, in particular the groups identified as being differentially expressed. The plot produced by the `corplot` function displays the pooled correlation calculated from the two classes,

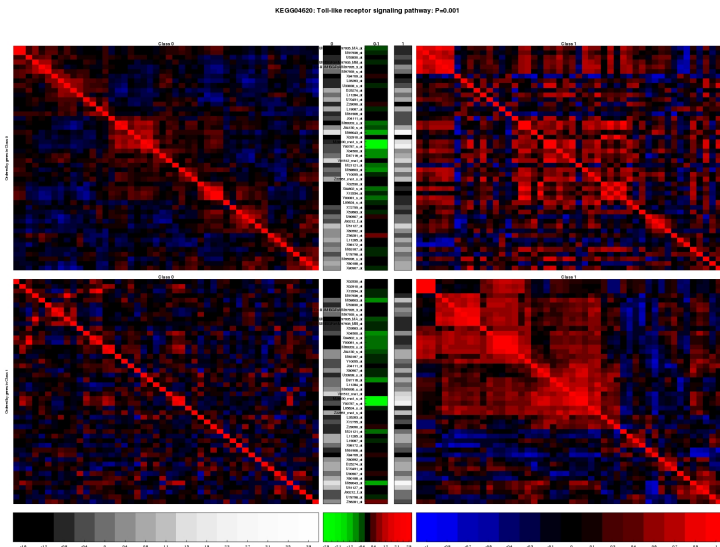


Figure 1: KEGG04620

while the `corplot2` function produces a plot based on unpooled correlation. Gene names can be added to the plot using `add.name=T` (default). The font size can be changed by setting the `font.size` argument. The `main` option specifies the title of the plot.

```
> sel <- c("04620", "04120")
> pvalue <- c(0.001, 0.72)
> library(KEGG.db)
> pname <- unlist(mget(sel, env=KEGGPATHID2NAME))
> main <- paste("KEGG", sel, ": ", pname, ": ", "P=", pvalue, sep="")
> for(i in 1:length(sel)){
+   fname <- paste("corplot2-KEGG", sel[i], ".jpg", sep="")
+   jpeg(fname, width=1600, height=1200, quality=100)
+   selgene <- rownames(imat)[imat[,match(paste("KEGG", sel, sep="")[i], colnames(imat))]==1]
+   corplot2(golub, selgene, golub.cl, main=main[i])
+   dev.off()
+ }
```

The argument `inputP` allows users to input the p -values of individual genes calculated using other approaches, such as the `limma` package (Smyth *et al.*, 2004), allowing the results from both per-gene and per-pathway analysis to be printed on a single plot. To allow users to identify genes from in correlation image plots, the argument `gene.locator=T` allows the selection of interesting (e.g., highly correlated and differential expressed between two classes) genes by clicking beginning and end points on the main diagonal of the image plots. This prints the identifiers for the selected genes. Further details of this functionality are provided in the `HowToUseGeneLocator.pdf` document. The usage of `corplot2` is similar to that for the `corplot` function.

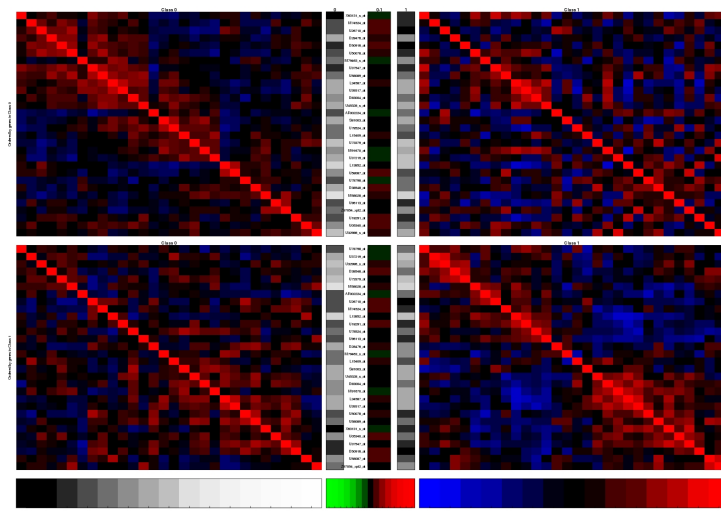


Figure 2: KEGG04120

5 The aveProbes function

In Affymetrix gene expression data, a unique gene can often link to multiple probe sets, with such genes then having a greater influence on the pathway analysis (particularly if the gene is differentially expressed). In order to solve this problem, the `aveProbe` function is provided to change the multiple probe data to the unique gene data by taking the median of the probe values. This function can be used to transform both expression data and the indicator matrix by providing a vector of unique gene identifiers.

```
> pathlist <- as.list(hu6800PATH)
> pathlist <- pathlist[match(rownames(golub), names(pathlist))]
> ids <- unlist(mget(names(pathlist), env=hu6800SYMBOL))
> #### transform data matrix only ####
> newdata <- aveProbe(x=golub, ids=ids)$newx
> #### transform both data and imat ####
> output <- aveProbe(x=golub, imat=imat, ids=ids)
> newdata <- output$newx
> newimat <- output$newimat
> newimat <- newimat[,apply(newimat, 2, sum)>=10]
> dim(newdata)

[1] 2560 38

> dim(newimat)

[1] 2560 151
```

After the multiple probe data set has been changed to the unique gene symbol data, further analysis such as testing and visualizing pathways can be done on the new data set.

References

- [1] Benjamini,B.Y. and Yekutieli,D. (2001) The control of the false discovery rate in multiple testing under dependency. *The Annals of Statistics*, **29**, 1165-1188.
- [2] Gentleman,R.C., Carey,V.J., Bates,D.M., Bolstad,B., Dettling,M., Dudoit,S., Ellis,B., Gautier,L., Ge,Y., Gentry,J. *et al.* (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biology*, **5**, R80.
- [3] Golub,T.R., Slonim,D.K., Tamayo,P., Huard,C., Gaasenbeek,M., Mesirov,J.P., Coller,H., Loh,M.L., Downing,J.R., Caligiuri,M.A. *et al.* (1999) Molecular Classification of Cancer: Class Discovery and Class Prediction by Gene Expression Monitoring, *Science*, **286**, 531-537.
- [4] Smyth,G.K. (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology*, **3**, No.1, Article 3.