BIOINFORMATICS

Vol. 00 no. 00 2004
Pages 1–11

High throughput screening of co-expressed gene pairs with controlled False Discovery Rate (FDR) and Minimum Acceptable Strength (MAS)

Dongxiao Zhu [a,b]*; *Alfred O Hero* [b] *and Zhaohui S Qin* [c]

[a]*Bioinformatics Program, University of Michigan, Ann Arbor, MI 48109* [b]*Departments of EECS, Biomedical Engineering and Statistics, University of Michigan, Ann Arbor, MI 48105* [c]*Department of Biostatistics, University of Michigan, Ann Arbor, MI 48109*

ABSTRACT

Motivation: Many exploratory microarray data analysis tools such as gene clustering and relevance networks rely on detecting pairwise gene co-expression. Traditional screening of pairwise co-expression either controls biological significance or statistical significance, but not both. The former approach does not provide stochastic error control, and the later approach screens many co-expressions with excessively low correlation.

Methods: We have designed and implemented a statistically sound two-stage co-expression detection algorithm that controls both statistical significance (False Discovery Rate, FDR) and biological significance (Minimum Acceptable Strength, MAS) of the discovered co-expressions. Based on estimation of pairwise gene correlation, the algorithm provides an initial co-expression discovery that controls only FDR, which is then followed by a second stage co-expression discovery which controls both FDR and MAS. It also computes and thresholds the set of FDR p-values for each correlation that satisfied the MAS criterion.

Results: We validated asymptotic null distributions of the Pearson and Kendall correlation coefficients and the two-stage error-control procedure using simulated data. We then used yeast galactose metabolism data (Ideker et al. 2001) to illustrate the advantage of our method for clustering genes and constructing a relevance network. In gene clustering, the algorithm screens a seeded cluster of co-expressed genes with controlled FDR and MAS. In constructing the relevance network, the algorithm discovers a set of edges with controlled FDR and MAS.

Availability: The method has been implemented in an R package "GeneNT" that is freely available from: http://www-personal.umich.edu/~zhud/genent.htm.

Contact: zhud@umich.edu

Supplementary Information: Supplemental material can be found at: http://www-personal.umich.edu/~zhud/genent.htm

*to whom correspondence should be addressed

1 INTRODUCTION

The emergence and development of DNA miroarray technology (Affymetrix oligonucleotide expression arrays and cDNA arrays) enable researchers to interrogate gene expression levels simultaneously on the genome scale (Lockhart *et al*., 1996, Schena *et al*., 1995, DeRisi *et al*., 1997). The development of statistically sound and biologically meaningful techniques to analyze gene expression data is essential for transforming raw experimental data into scientific knowledge. Gene expression data have been subjected to a variety of statistical analyses, such as detecting differentially expressed genes (e.g. Tusher *et al*., 2001, Zareparsi *et al*., 2004), clustering genes/samples (e.g. Eisen *et al*., 1998, McLachlan *et al*., 2002), and cancer classification (e.g. Golub *et al*., 1999, Alizadeh *et al*., 2000).

Detection of co-expressed genes from microarray data has attracted much attention since many co-expressed genes are found to have functional relationships, e.g. lying in the same signal transduction pathway (Eisen *et al*., 1998, DeRisi *et al*., 1997). Hierarchical clustering (Eisen *et al*., 1998) and relevance network construction (Butte and Kohane, 2000, Farkas *et al*., 2003) are two important exploratory techniques. Both of these techniques are based on discovering pairs of co-expressed genes, which is one of the fundamental objectives in functional genomics and system biology. While not necessarily true in many higher Eukaryotes (Boutanaev *et al*., 2002), pairwise gene co-expression as prescribed by the standard two-component model (Nixon *et al*., 1986) characterizes gene co-expression in Bacteria, single-celled Eukaryotes, Archaea and higher Plants (Stock *et al*., 2000).

Clearly, there is a need for statistical methodology for high throughput screening of co-expressed gene pairs with stochastic error and strength of association controls. Two issues have to be considered in developing such a methodology, namely, choice of screening statistic and choice of screening acceptance and rejection criteria.

Several methods have been adopted to measure the strength of association between expression profiles of gene pairs, such

as: Euclidean distance (Tamayo *et al*., 1999), Pearson correlation coefficient (Zhou *et al*., 2002), coherence (Butte *et al*., 2001), mutual information (Butte and Kohane, 2000), edge detection (Filkov *et al*., 2002), and dominant spectral component analysis (Yeung *et al*., 2004). Each of these methods has advantages and disadvantages. To select co-expressed gene pairs, the common practice is to calculate a sample correlation for each pair of genes and then to select the top pairs by correlation thresholding (Butte *et al*., 2000, Zhou *et al*., 2002, and Farkas *et al*., 2003). This approach controls biological significance by screening only strongly correlated pairs, e.g. those exceeding a minimum acceptable strength (MAS) level specified by the threshold. However, it does not account for statistical sampling uncertainty and thus does not control error rate. Another approach (Lee *et al*., 2004) is to control only statistical significance: screen co-expressed gene pairs whose strength of association is different from zero using p-value thresholding, e.g. as determined by a specified level of false discovery rate (FDR). This approach does not control biological significance and can lead to screening-in some weakly correlated gene pairs that are difficult to verify by follow-up experiments such as real time RT-PCR.

Regarding the choice of screening statistic, the Pearson correlation coefficient has been one of the most popular choices because it is easily computed and its performance is often comparable to more complex and computational intense methods (Yeung *et al*., 2004, Kwon *et al*., 2003). However, the Pearson correlation coefficient can only capture linear relationships between gene expression profiles. To circumvent this limitation, we propose to use the nonparametric Kendall rank correlation coefficient that is able to capture both linear and nonlinear associations between gene expression profiles. We decided to explore the Pearson and Kendall correlation coefficient measures because their asymptotic distributions are available, as required by our two-stage screening procedure to be described below.

Regarding the choice of screening acceptance criteria, a two-stage statistical hypothesis testing scheme is applied in order to decide on whether the strength of association is statistically significant at the specified MAS level. The test is non-standard because: 1) MAS is ordinarily greater than 0; 2) many comparisons have to be tested simultaneously. Our method is directly inspired by the two-stage screen methodology of (Hero *et al*., 2004) that controls both False Discovery Rate (FDR) and Minimum Acceptable Difference (MAD) in detecting differentially expressed genes.

We demonstrate the application of our two-stage screening algorithm by constructing relevance networks and clustering co-expressed genes from yeast galactose metabolism data (Ideker *et al*., 2000). This data represents approximately 6200 gene expression levels on two-color cDNA microarrays collected over 20 physiological/genetic conditions (nine mutant and one wild type strains incubated in either GAL-inducing or non-inducing media).

The outline of the paper is as follows. In Section 2, we describe the proposed two-stage multicriteria approach. In Section 3, we first show the approach indeed controls FDR at the specified MAS level using synthetic data, and then illustrate it for yeast galactose metabolism data. In Section 4, we discuss advantages of our method, model assumptions and restrictions.

2 METHODS

2.1 Measures of the strength of association

There are many possible discriminants for strength of association between two variables, which we generally denote as a real number Γ. Under a Gaussian linear hypothesis, the Pearson correlation coefficient ρ is an appropriate metric. A robust distribution-free alternative is the Kendall rank correlation coefficient (Kendall's τ). The Pearson (Bickel and Doksum, 2000) and Kendall correlation coefficients (Hollander and Wolfe, 1999) are special cases of the generalized correlation coefficient (Daniel, 1944). We define $\{g_p\}_{p=1}^G$ as the indices of G gene probes on the microarray; $\{X_{g_p}\}_{p=1}^G$ as normalized probe responses (random variables); and $\{\{x_{g_{p(n)}}\}_{p=1}^G\}_{n=1}^N$ as realizations of $\{X_{g_p}\}_{p=1}^G$ under N i.i.d. microarray experiments.

2.1.1 Pearson correlation coefficient. The population Pearson correlation coefficient between random variables X_{g_i} and X_{g_j} (defined as long as $\mathrm{var}(X_{g_i})$, $\mathrm{var}(X_{g_j})$ are positive) is:

$$\rho(X_{g_i}, X_{g_j}) = \frac{\mathrm{cov}(X_{g_i}, X_{g_j})}{\sqrt{\mathrm{var}(X_{g_i})\mathrm{var}(X_{g_j})}}. \qquad (1)$$

The sample Pearson correlation coefficient $\hat{\rho}$ is an asymptotically consistent unbiased estimator of ρ:

$$\hat{\rho}_{i,j} = \frac{S_{X_{g_i},X_{g_j}}}{\sqrt{S_{X_{g_i},X_{g_i}} S_{X_{g_j},X_{g_j}}}}, \qquad (2)$$

where $S_{X_{g_i},X_{g_i}}$, $S_{X_{g_j},X_{g_j}}$, and $S_{X_{g_i},X_{g_j}}$ are sample variances and covariances given by

$$S_{X_{g_i},X_{g_i}} = (N-1)^{-1} \sum_{n=1}^{N} (X_{g_i(n)} - \overline{X_{g_i}})^2,$$

$$S_{X_{g_j},X_{g_j}} = (N-1)^{-1} \sum_{n=1}^{N} (X_{g_j(n)} - \overline{X_{g_j}})^2,$$

$$S_{X_{g_i},X_{g_j}} = (N-1)^{-1} \sum_{n=1}^{N} (X_{g_i(n)} - \overline{X_{g_i}})(X_{g_j(n)} - \overline{X_{g_j}}),$$

and $\overline{X_{g_i}} = N^{-1} \sum_{n=1}^{N} X_{g_i(n)}$, $\overline{X_{g_j}} = N^{-1} \sum_{n=1}^{N} X_{g_j(n)}$ are sample means.

2.1.2 Kendall rank correlation coefficient. Kendall's τ statistic is a measure of correlation that captures both linear and non-linear associations. The parameter τ is defined as $\tau = P_+ - P_-$, where, for any two independent pairs of observations $(x_{g_{i(n)}}, x_{g_{j(n)}})$, $(x_{g_{i(m)}}, x_{g_{j(m)}})$ from the population: $P_+ = P[(x_{g_{i(n)}} - x_{g_{i(m)}})(x_{g_{j(n)}} - x_{g_{j(m)}}) \geq 0]$ and $P_- = P[(x_{g_{i(n)}} - x_{g_{i(m)}})(x_{g_{j(n)}} - x_{g_{j(m)}}) < 0]$. An unbiased estimator of τ is given by the Kendall τ statistic:

$$\hat{\tau}_{i,j} = 2 \sum \sum_{1 \leq n \leq m \leq N} \frac{K_{nm}}{N(N-1)}, \qquad (3)$$

here K_{nm} is a indicator variable defined as $K_{nm} = \mathrm{sgn}(x_{g_{i(n)}} - x_{g_{i(m)}})\mathrm{sgn}(x_{g_{j(n)}} - x_{g_{j(m)}})$ for each set of pairs drawn from $\{X_{g_i}\}_{i=1}^{G}$ and $\{X_{g_j}\}_{j=1}^{G}$.

2.2 Hypothesis testing scheme

To screen the strongly co-expressed pairs of G genes on each microarray, we will simultaneously test the $\Lambda = \binom{G}{2}$ pairs of composite hypotheses: $\{H_\lambda, K_\lambda : \lambda = (g_i, g_j)\}$.

$$H_\lambda : \Gamma_{g_i,g_j} \leq cormin \text{ versus } K_\lambda : \Gamma_{g_i,g_j} > cormin, \quad \text{for } g_i \neq g_j, \text{ and } g_i, g_j \in (1, 2, ...G) \qquad (4)$$

where $cormin$ is the specified minimum acceptable strength of correlation. The sample correlation coefficient $\hat{\Gamma}_{i,j}$ ($\hat{\rho}_{i,j}$ or $\hat{\tau}_{i,j}$) could be thresholded to decide on pairwise dependency of two genes in the sample. When we must decide between the null hypothesis H_λ and the alternative hypothesis K_λ based on such a threshold test, there will generally be decision errors in the form of false positives (Type I errors: decide K_λ when H_λ is true) and false negatives (Type II errors: decide H_λ when K_λ is true). The Per Comparison Error Rate (PCER) is defined as the number of Type I errors over the number of independent trials, i.e. the probability of Type I error. The p-value is the probability that a more improbable sample could have been drawn from the population(s) being tested given the assumption that the null hypothesis is true.

For N realizations of any pair of gene probe responses, $\{x_{g_{i(n)}}, x_{g_{j(n)}}\}_{n=1}^{N}$, we first calculate $\hat{\tau}_{i,j}$ or $\hat{\rho}_{i,j}$ respectively. For large N, the PCER p-values for $\rho_{i,j}$ or $\tau_{i,j}$ are:

$$p_{\rho_{i,j}} = 2\left(1 - \Phi\left(\frac{\tanh^{-1}(\hat{\rho}_{i,j})}{(N-3)^{-1/2}}\right)\right) \qquad (5)$$

$$p_{\tau_{i,j}} = 2\left(1 - \Phi\left(\frac{K}{N(N-1)(2N+5)/18^{1/2}}\right)\right) \qquad (6)$$

whrere Φ is the cumulative density function of a standard Gaussian random variable, and $K = \sum \sum_{1 \leq n \leq m \leq N} K_{nm}$. The above expressions are based on asymptotic Gaussian approximations to $\hat{\rho}_{i,j}$ (Bickel and Doksum, 2000) and to $\hat{\tau}_{i,j}$ (Hollander and Wolfe, 1999).

The PCER p-value refers to the probability of Type I error incurred in testing a single pair of hypothesis for a single pair of genes g_i, g_j. It is the probability that purely random effects would have caused g_i, g_j to be erroneously selected based on observing correlation between this pair of genes only. When considering the Λ multiple hypotheses for all possible pairs, two adjusted error rates have frequently been considered in microarray studies. These are family-wise error rate (FWER) and false discovery rate (FDR)(Benjamini and Hochberg, 1995). The FWER is the probability that the test of all Λ pairs of hypotheses yields at least one false positive in the set of declared positive responses. In contrast, the FDR is the average proportion of false positives in the set of declared positive responses. The FDR is dominated by the FWER and is therefore a less stringent measure of significance. As in previous studies (Reiner *et al*., 2003), we adopt the FDR to control statistical significance of the selected gene pair correlations in our screening procedure.

2.3 Two-stage screening procedure

Select a level α of FDR and a level $cormin$ of MAS significance levels. We use a modified version of the two-stage screening procedure proposed for gene screening by (Hero *et al*., 2004). This procedure consists of two stages, summarized in Fig 1.

Stage I. For each gene pair $\lambda = (g_i, g_j)$ in the set $\mathcal{G}$ of all $\Lambda = \binom{G}{2}$ gene pairs, test the simple null hypothesis:

$$H_\lambda : \Gamma_{g_i,g_j} = 0 \text{ versus } K_\lambda : \Gamma_{g_i,g_j} \neq 0, \quad \text{for } g_i \neq g_j, \text{and } g_i, g_j \in (1, 2, ...G) \qquad (7)$$

at FDR level α. The step-down procedure of Benjamini and Hochberg (Benjamini and Hochberg, 1995) is used to accomplish this.

Stage II. Suppose a number Λ_1 pairs of genes, denoted by the set $\mathcal{G}_1 \subset \mathcal{G}$, pass the Stage I procedure. In Stage II, we first construct asymptotic PCER Confidence Intervals (PCER-CI's): $I^\lambda(\alpha)$ for each Γ (ρ or τ) in subset $\mathcal{G}_1$. We convert these PCER-CI's into FDR Confidence Intervals (FDR-CI's): $I^\lambda(\alpha) \rightarrow I^\lambda(\Lambda_1\alpha/\Lambda)$ using the procedure in (Benjamini and Yekutieli, 2004). A gene pair in subset $\mathcal{G}_1$ is declared to be both statistically significant and biologically significant if its FDR-CI does not intersect the MAS interval $[-cormin, cormin]$ (see Fig 5). The set of all such gene pairs is called $\mathcal{G}_2$.

In many practical situations, the experimenter may not be comfortable in specifying a MAS or FDR criterion in advance. In this situation, it is useful to solve the inverse problem: what is the most stringent pair of criteria (α , $cormin$) that would cause a particular subset of gene pairs to be included in the screen $\mathcal{G}_2$. The inverse screening procedure is displayed in Fig 2.

Stage I (step-down): control of FDR at MAS = 0.

1. Specify FDR level α and MAS level $cormin$.
2. Compute a list of PCER p-values: $p_1, p_2, ..., p_\Lambda$ corresponding to $\Lambda = \binom{G}{2}$ pairs of composite hypotheses: $\{H_\lambda, K_\lambda : \lambda = (g_i, g_j)\}$ from $\{\hat{\rho}_{i,j}\}$ or $\{\hat{\tau}_{i,j}\}$.
3. Sort the list of PCER p-values in increasing order, i.e. $p_{(1)}, p_{(2)}, ..., p_{(\Lambda)}$.
4. Find the index k_0 where $k_0 = max\{k : p_{(k)} \leq \frac{k\alpha}{\Lambda\nu}\}$.
5. Set initial screening $\mathcal{G}_1$ as those $k_0 = \Lambda_1$ gene pairs having p-values: $p_{(1)}, p_{(2)}, ..., p_{(k_0)}$.

In step 4, $\nu = 1$ if the test statistics can be assumed statistically independent or positively dependent, where $\nu = \frac{1}{\sum_{\lambda=1}^{\Lambda} \lambda^{-1}}$ under the general dependency assumption.

Stage II: control of FDR and MAS = $cormin$.

1. Construct Λ_1 different $(1-\alpha)\times 100\%$ PCER-CI's $I^\lambda(\alpha)$ for ρ or τ of each gene pair in $\mathcal{G}_1$ (Appendix 5.1).
2. Convert these PCER-CI's into Λ_1 different $(1-\alpha)\times 100\%$ FDR-CI's using formula (Benjamini and Yekutieli, 2004): $I^\lambda(\alpha) \rightarrow I^\lambda(\Lambda_1\alpha/\Lambda)$.
3. Select the subset $\mathcal{G}_2$ containing Λ_2 of Λ_1 gene pairs whose FDR-CI's do not intersect $[-cormin, cormin]$.

Fig. 1. Two-stage direct screening procedure yields a subset $\mathcal{G}_2$ of all possible gene pairs $\mathcal{G}$ whose strength of association exceeds MAS level $cormin$ at FDR level α.

1. Compute a list of PCER p-values: $p_1, p_2, ..., p_\Lambda$ corresponding to $\Lambda = \binom{G}{2}$ pairs of composite hypotheses: $\{H_\lambda, K_\lambda : \lambda = (g_i, g_j)\}$ from $\{\hat{\rho}_{i,j}\}$ or $\{\hat{\tau}_{i,j}\}$.
2. Sort the list of PCER p-values in increasing order, i.e. $p_{(1)}, p_{(2)}, ..., p_{(\Lambda)}$.

for any gene pair $\lambda_0 \in \{g_i, g_j\}_{i,j=1}^G$:

- Find the minimal $\alpha = \alpha(\lambda_0)$ such that the PCER-CI $I^{\lambda_0}(\alpha)$ does not intersect $[-cormin, cormin]$.
- Compute the integer index $N(\alpha(\lambda_0)) = \sum_{k=1}^{\Lambda} I(p_{(k)})k \leq \alpha(\lambda_0))$, where $I(A)$ is an indicator function of the truth of statement A. The FDR p-value of the gene pair λ_0 is then simply p_i, where $i = N(\alpha(\lambda_0))$.

endfor

Fig. 2. Inverse screening procedure allows the FDR p-value of a gene pair's (λ_0) strength of association to be computed.

3 RESULTS

3.1 Validating the two-stage algorithm

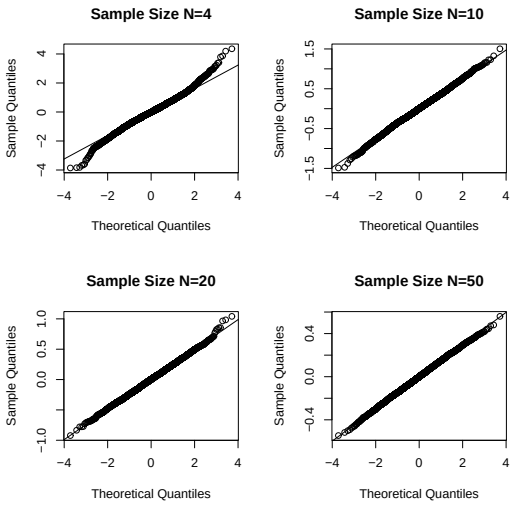
3.1.1 Validating asymptotic null distribution. Here we verify that the proposed two-stage algorithm controls FDR at a specified MAS level using simulated data. Since the p-values are based on asymptotic distribution approximations (eq. 5 and eq. 6), we display in Fig 3a the goodness of fit of the $\hat{\rho}$ sampling distribution to the Gaussian distribution using QQ plots. Note that there is good agreement to the Gaussian distribution for $N \geq 10$. Moreover, since the construction of confidence intervals requires estimation of sampling distribution variance, the accuracy of the variance approximation is vital. This can be evaluated by the mean squared approximation error (MSE) at the sample size N:

$$MSE_\rho^{(N)} = \Lambda^{-1} \sum_{1\leq i<j\leq G} (S^{(N)}_{\tanh^{-1}(\hat{\rho}_{i,j})} - (N-3)^{-1/2})^2, \tag{8}$$

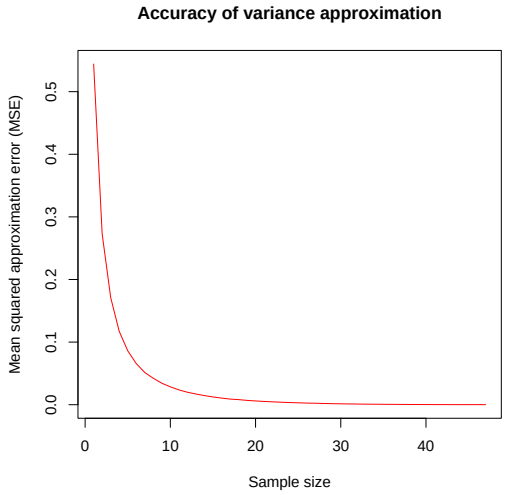
$$MSE_\tau^{(N)} = \Lambda^{-1} \sum_{1\leq i<j\leq G} (S^{(N)}_{\hat{\tau}_{i,j}} - (\frac{2}{N(N-1)} \frac{2(N-2)}{N(N-1)^2} \sum_{i=1}^{N} (C_r - \overline{C}) + 1 - \hat{\tau}))^2, \tag{9}$$

where $S^{(N)}_{\tanh^{-1}(\hat{\rho}_{i,j})}$ and $S^{(N)}_{\hat{\tau}_{i,j}}$ denote standard errors of $\tanh^{-1}(\hat{\rho}_{i,j})$ and $\hat{\tau}_{i,j}$ at the sample size N. The definitions of C_r and $\overline{C}$ can be found in Appendix 5.1. The $\hat{\rho}$ variance approximations are seen to be in good agreement even for small sample sizes ($N > 10$) from Fig 3b.

3.1.2 Validating the error control procedure. In order to validate our FDR and MAS error control procedure, we simulated pairwise gene expression data based on known population covariances (Appendix 5.2). The actual FDR at a MAS level is calculated as a ratio of the number of screened gene pairs whose corresponding population correlation parameters $\Gamma_{i,j}$ are less than the MAS level specified, divided by the total number of screened gene pairs. The actual MAS is the minimum true discovery of population correlation $\Gamma_{i,j}$ among the screened pairs. We specified 16 pairs of (FDR,MAS) criteria (Four FDR levels: 0.2, 0.4, 0.6, 0.8; Four



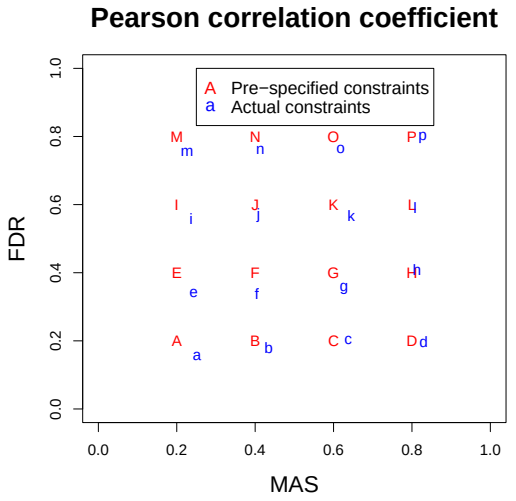
(a)



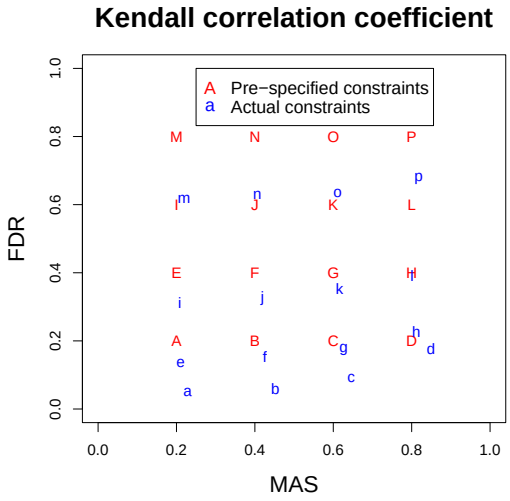
(b)

Fig. 3. Verification of Gaussian null sampling distribution and variance approximation for Pearson correlation coefficient (eq. 8). (a) QQ plot of transformed sampling distribution of Pearson correlation coefficient $\hat{\rho}$ versus Gaussian distribution. (b) Mean squared approximation errors (MSE) of the variances of transformed sample Pearson correlation coefficients $\hat{\rho}$.

MAS levels: 0.2, 0.4, 0.6, 0.8), and each is plotted as a different upper case English alphabet (Red) in Fig 4. The 16 corresponding pairs of actual (FDR,MAS) criteria are also shown in Fig 4 using the same set of lower case English alphabets (Blue). It can be observed that generally the actual FDR's (lower case) fall below the specified constraint (upper case) and the actual MAS's (lower case) fall above the specified constraints (upper case). Any deviations of actual FDR's and MAS's from their specified levels are due to the conservative asymptotic approximation (eq.5 and eq.6). Observe that use of Kendall correlation (Fig 4b) leads to more significant overestimation of error rates than the Pearson correlation (Fig 4a). Overestimation of error rates will translate into a reduction of power in discovering co-expressed pairs at the specified levels.

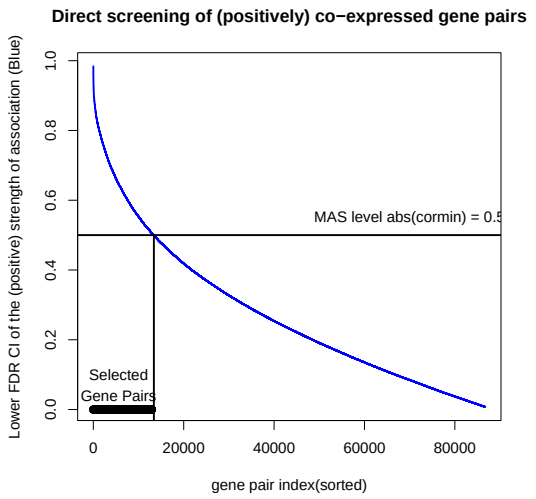


(a)

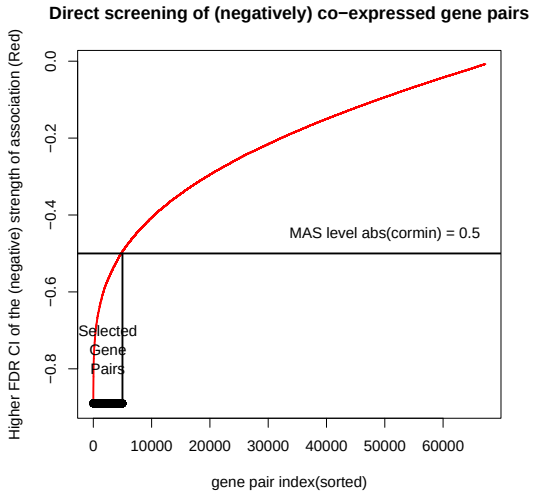


(b)

Fig. 4. Verification of two-stage error control procedure based on Pearson correlation coefficient (a) and Kendall correlation coefficient (b). Sample size $N = 20$.



(a)



(b)

Fig. 5. Curves specify lower endpoints (a) and upper endpoints (b) of the 5% FDR-CI's on the positive Pearson correlation coeffi cients (a) and negative Pearson correlation coeffi cients (b) for the galactose metabolism study. Only those gene pairs whose FDR-CI's do not intersect $[-cormin, cormin]$ are selected by the second stage of screening. When the MAS strength of association criterion is $cormin = 0.5$, these gene pairs are obtained by thresholding the curves as indicated.

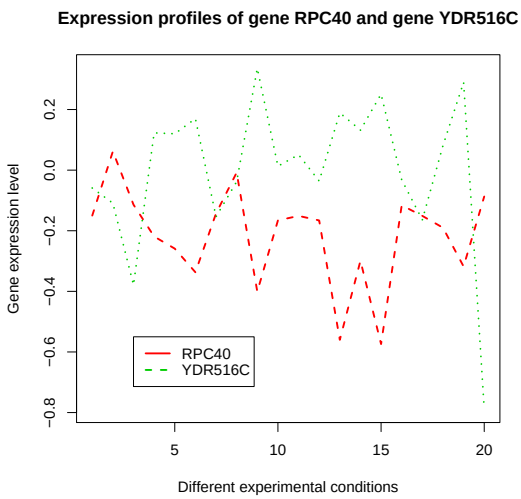
3.2 Constructing relevance networks with controlled FDR and MAS

For the yeast galactose metabolism dataset, a subset of 997 differentially expressed genes were identified by Ideker et al using a generalized likelihood ratio test procedure (Ideker *et al*., 2000). Genes having a likelihood ratio statistic $\lambda \leq 45$ were selected as differentially expressed, i.e. whose mRNA levels differed significantly from the reference under one or more treatments.

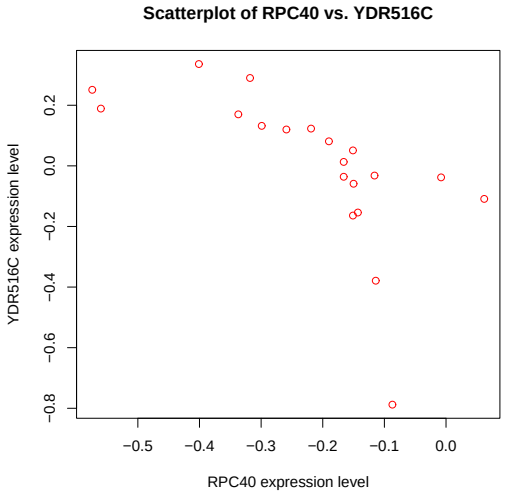
Fig 5a and 5b illustrate the direct implementation of the two-stage procedure to screen positively or negatively correlated gene pairs based on the Pearson correlation coefficient. The direct screening procedure is constrained by FDR level $\alpha = 0.05$ and MAS level $cormin = 0.5$. Stage I of the screen discovered $\Lambda_1 = 153,983$ out of $\Lambda = \binom{997}{2} = 496,506$ gene pairs having FDR ≤ 0.05, leaving 153,983 correlation coefficients for which FDR-CI's must be constructed. Recall that gene pair passes the Stage II screening if the FDR-CI does not intersect the interval $[-0.5, 0.5]$. $\Lambda_2 = 18,135$ of the 153,983 gene pairs passed the Stage II screening and were declared to be both "biologically" and "statistically" significant. Similarly, using Kendall correlation coefficient, there were $\Lambda_1 = 95,205$ gene pairs that passed the Stage I screen, and only $\Lambda_2 = 3,552$ gene pairs passed the Stage II screen constrained by the same MAS and FDR criteria as above (STable 1).

Although for Gaussian distributed pairs the Kendall rank correlation coefficient has lower discovery power compared to the Pearson correlation coefficient, our screening procedure was nevertheless able to pull out many non-linearly correlated gene pairs that were missed by the Pearson correlation procedure. For example, the link between gene "RPC40" and gene "YDR516C" passed both Stage I and II screening ($\alpha = 0.015$, $cormin = 0.5$) when using Kendall correlation coefficient ($\hat{\tau}$ =-7.5e-01, FDR p-value = 6.2e-04, FDR-CI = [-9.7e-01, -5.4e-01]), but they failed to pass even the first screening when the Pearson correlation coefficient was used ($\hat{\rho}$ =-6.3e-01, FDR p-value = 1.2e-02). From the scatter plot, we can observe an obvious non-linear correlation for this gene pair (Fig 6). The poor linear fit can be verified by fitting a simple linear regression model and observing $R^2 = 0.36$.

Relevance networks are implemented as a graph where n nodes (genes) are connected by p sets of edges (co-expressions). Each of the p sets of edges are discovered using a different similarity measure (Butte *et al*., 2000, Butte and Kohane, 2000). Therefore, our constructed networks are mixed networks with $p = 2$ in which edges are discovered using either Pearson correlation coefficients or Kendall correlation coefficients constrained by the same set of (FDR,MAS) criteria. In relevance networks, genes that are of considerable interest to the biologist are "hub genes" such as RPL33A and RPS4A in Fig 7. Hub genes are best connected genes that dominate a large part of the network topology (Jeong *et al*., 2001, Barabàsi, 2004). We constructed five such networks that are constrained by five pairs of constraints (FDR ≤ 0.05, $cormin = 0.5, 0.6, 0.7, 0.8, 0.9$). Most of the "hub genes" in each discovered network fall into two categories: "RPL" and "RPS". The former encodes "Ribosome Protein Large (60S) subunit," and the latter encodes "Ribosome Protein Small (40S) subunit". Both of these categories are structural



(a)



(b)

Fig. 6. A pair of non-linearly correlated genes.

components of the ribosome that is responsible for protein biosynthesis. Protein biosynthesis plays the central role in galactose metabolism because galactose is not a primary carbon source for yeast, and different types of proteins including transporters, enzymes, and regulators have to be synthesized upon induction (Wieczorke *et al*., 1999). We ranked the "hub genes" by calculating and sorting average rank of each "hub gene" over five networks (Table 1, STable 2). Interestingly, the list of "hub genes" contains many hypothetical Open Reading Frames (ORFs)(STable 2), which are presumably indispensable for galactose metabolism (Jeong *et al*., 2001).

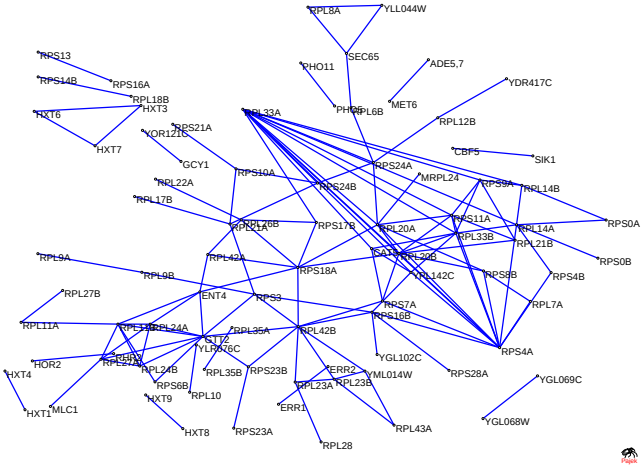


Fig. 7. Network topology visualization. The network is discovered by constraining FDR $\leq 5\%$ at a MAS level of 0.9. No significant negative correlation is discovered at this level. The graph is drawn using Pajek (Batagelj and Mrvar, 1998).

Fig. 7 presents the discovered network topology with a FDR level of 5% (5% discovered edges are expected to be false positive) at the MAS level of $cormin = 0.9$. The network is composed of 89 connected vertices and 132 edges. Similar to some other biological networks, the network marginal degree distributions appear to be of the form of a power-law. This was tested by verifying goodness of fit to the log-transformed power-law model ($R^2 = 0.95$) i.e., $\log P(D_i) = -\gamma \log D_i + \log \eta + \varepsilon_i$ (Barabàsi, 2004). Here γ and η are shape and intercept parameters, i is the index of a gene in the network, ε_i is a residual fitting error, D_i is the number of edges (degree) of ith gene and $P(D_i)$ is the corresponding probability.

3.3 Clustering co-expressed genes

Inspired by the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990), and based on the "guilt-by-association" assumption (Eisen *et al*., 1998), we applied the two-stage screening procedure to cluster co-expressed genes with controlled FDR and MAS. We sought to demo its application in metabolic pathway discovery by "rediscovering" the extensively studied galactose metabolic pathway, which consists of at least three types of genes including transporter genes (GAL2, HXTs etc), enzyme genes (GAL1, GAL7, GAL10 etc) and transcription factor genes (GAL4, GAL80, GAL3 etc). Some other genes are also involved in galactose metabolism but their roles are not entirely clear (Rohde *et al*., 2000, Ideker *et al*., 2001). Therefore, our aims are not only to validate our procedure by rediscovering known co-expressed

Table 1. Top ten "hub genes". The rank of each gene is the average rank over five different networks. Each of five networks is constrained by a different pair of (FDR,MAS) criteria. The highest ranked gene is the most connected and stable gene under varying constraints of (FDR,MAS).

Gene Name	Average Rank
RPL42B	4.2
RPS16B	6.2
RPL14A	7.4
RPS3	7.4
GTT2	8.0
RPS4A	9.8
RPL33A	11.6
RPL23B	15.4
RPS7A	15.8
RPS4B	17.2

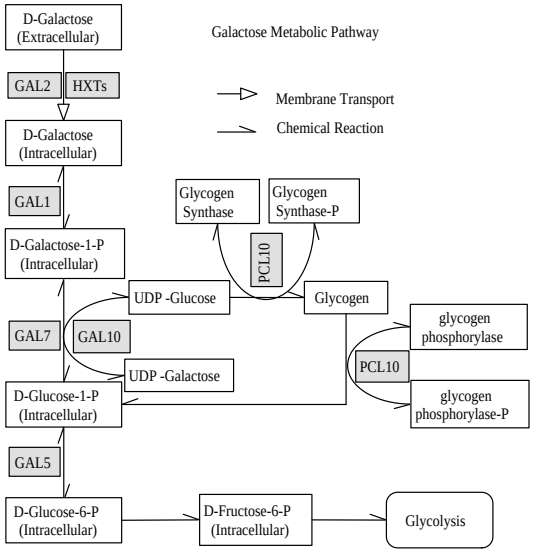


Fig. 8. Diagram of the structural module of the galactose metabolic pathway. The shaded squares denote the genes whose gene products lie in the galactose metabolic pathway "rediscovered" by our algorithm.

genes pairs, but also to discover some unknown genes in the pathway.

We selected gene "GAL7" as the "seed gene" which encodes the UDP-glucose-hexose-1-phosphate uridylyltransferase (EC 2.7.7.12). The enzyme catalyzes the transformation of Galactose-1-P into Glucose-1-P, and the latter enters the glycolysis pathway through relocating the phosphate group. Many genes lying in the galactose metabolic pathway are rediscovered by our technique under the relative stringent criteria ($\alpha = 0.05,\ cormin = 0.2$) (Fig 8). Known transcription factor genes (GAL4 and GAL80) were not discoverable from this microarray experiment as the GAL4 and GAL80 expressions are time shifted and only one time sample was included. The two-stage procedure also discovers some unknown genes that we hypothesize to be relevant to galactose metabolism (STable 3). The pathways discovered using other "seed genes" in the pathway such as GAL1 and GAL10 gave similar results (STable 4).

3.4 Performance comparison

In Table 2 and Table 3, we compare the performance of the proposed two-stage FDR-CI screening algorithm (labeled "FDR-CI" in the tables), with two other commonly used algorithms, called thresholded FDR (labeled "FDR" in the tables) and thresholded MAS (labeled "MAS" in the tables). All three algorithms aim to control MAS at a level of $cormin = 0.5$. The two-stage FDR-CI and thresholded FDR algorithms aim to control FDR at a level of $\alpha = 0.05$ in addition to MAS. Both of these latter algorithms were implemented as two-stage algorithms with common Stage I, which is to select pairs of genes $\mathcal{G}_1$ that pass the test of association with $cormin = 0$ at a FDR level of 5%. Stage II of the two-stage FDR-CI algorithm selects $\mathcal{G}_2$ as a subset of $\mathcal{G}_1$ at the specified FDR-CI level of 5%. Stage II of the thresholded FDR algorithm simply selects a subset of $\mathcal{G}_1$ having a strength of association greater than 0.5. The single-stage thresholded MAS algorithm selects a subset of the original 496,506 gene pairs by thresholding Pearson correlation $\hat{\rho}_{i,j} \geq 0.5$ (Table 2) and Kendall coefficient $\hat{\tau}_{i,j} \geq 0.5$ (Table 3) without attemping to control FDR.

The number of screened and discovered gene pairs for the three algorithms is indicated in the first two columns of Table 2 and Table 3. The maximum and median of the FDR p-values of the discovered gene pairs are indicated in the third and fourth columns for each algorithm. The last column indicates the average length of the FDR-CI's on correlation coefficients of the discovered gene pairs. We conclude from Table 2 and Table 3 that the proposed two-stage FDR-CI algorithm outperforms the other algorithms in terms of: (1) maintaining the FDR requirement that false positives not exceed 5% (column 4); (2) ensuring a substantially lower median FDR p-value than the others (column 5); (3) discovering genes that have tighter (on the average) confidence intervals on biologically significant (i.e. $\Gamma \geq 0.5$) correlation coefficients (column 6).

4 DISCUSSION

In this paper, we presented a two-stage procedure for screening co-expressed gene pairs that controls both biological and

Table 2. Performance comparison for three algorithms based on Pearson correlation coefficient for selecting gene pairs with a MAS level of 0.5. Thresholded MAS and thresholded FDR are significantly worse in terms of statistical significance (p-value) than the proposed two-stage FDR-CI algorithm (columns 4 and 5). Furthermore, the average length of the CI's on ρ's of the discovered gene pairs are shorter for the two-stage FDR-CI algorithm than for the other algorithms (column 6).

	# Screened	# Discovered	Max(Pv)	Meidan(Pv)	AvgFDRCI
MAS	496,506	174,830	2.5e-02	2.1e-03	6.5e-01
FDR	153,983	153,983	1.6e-02	1.4e-03	6.3e-01
FDR-CI	153,983	18,135	1.3e-05	1.3e-06	3.3e-01

Table 3. Performance comparison for three algorithms based on Kendall's τ statistic for selecting gene pairs with a MAS level of 0.5. Thresholded MAS and thresholded FDR are significantly worse in terms of statistical significance (p-value) than the proposed two-stage FDR-CI algorithm (columns 4 and 5). Furthermore, the average length of the CI's on τ's of the discovered gene pairs are shorter for the two-stage FDR-CI algorithm than for the other algorithms (column 6).

	# Screened	# Discovered	Max(Pv)	Meidan(Pv)	AvgFDRCI
MAS	496,506	31,151	2.0e-02	6.4e-03	6.3e-01
FDR	95,205	31,151	2.0e-02	6.4e-03	6.3e-01
FDR-CI	95,205	3,552	1.4e-03	4.3e-04	4.1e-01

statistical significance. For the discovered co-expressions, our method also provides an "accuracy" assessment of the strength of association by constructing confidence intervals for the strength of each edge. Indeed, for the typically small sample size microarray data, a simultaneous confidence interval is useful to characterize reliability of the reported strength of association. We illustrated two potential applications of our algorithm to discovering relevance networks and to clustering genes, in which the algorithm provides the error rate control at a biologically detectable level.

The algorithm is sufficiently general to be applied to many different correlation measures, e.g. Spearman's or Hotelling's dependency statistics. The algorithm can also be extended to different frameworks such as Gaussian Graphic Models (GGM) in which partial correlation coefficients are used as the dependency measures (Whittaker, 1990). Different groups have developed appraches to infer GGM from small sample size microarray data (Wang *et al*., 2003, Schafer and Strimmer, 2004, Dobra *et al*., 2004). Schafer and Strimmer recently presented a procedure that is based on the bootstrap estimator of the partial correlation coefficient (Schafer and Strimmer, 2004). Our two-stage algorithm has been extended to the GGM framework to control biological significance in addition to statistical significance, and the implementations are included in our R package "GeneNT" (available from http://www-personal.umich.edu/ zhud/genent.htm).

The scope of application of our statistical analysis is explicitly that of random sampled, complete observational data. In this paper, we are not concerned with developing models of causal gene networks. This would require a different experimentation and interventation approach to understand directional influences, rather than the simple observational, random sampling paradigm adopted here (Dobra *et al*., 2004).

The two-stage procedures can be applied under the independency/positive dependency or the general dependency assumptions (Benjamini and Hochberg, 1995, Benjamini and Yekutieli, 2001). The implementation of the general dependency procedure ($\nu = \frac{1}{\sum_{\lambda=1}^{\lambda} \lambda^{-1}}$) causes loss of discovery power. The assumption of independence may not be critical in the discovery of relevance networks since biological networks are typically very sparse (Yeung *et al*., 2002).

REFERENCES

Alizadeh,A.A., Eisen,M.B., Davis,R.E., Ma,C., Lossos,I.S., Rosenwald,A., Boldrick,J.C., Sabet,H., Tran,T., Yu,X., Powell,J.I., Yang,L., Marti,G.E., Moore,T., Hudson,J. Jr., Lu,L., Lewis,D.B., Tibshirani,R., Sherlock,G., Chan,W.C., Greiner,T.C., Weisenburger,D.D., Armitage,J.O., Warnke,R., Levy,R., Wilson,W., Grever,M.R., Byrd,J.C., Botstein,D., Brown,P.O. and Staudt,L.M. (2000) Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature*, **403**, 503-11.

Altschul,S., Gish,W., Miller,W., Myers,E.W. and Lipman,D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.*, **215**, 403-410.

Barab`asi,A. (2004) Network biology: understanding the cell's functional organization. *Nat.Rev.Genet.*, **5**, 101-113.

Batagelj,A. and Mrvar,A. (1998) Pajek - Program for large network analysis. *Connections*, **21**, 47-57.

Benjamini,Y. and Hochberg,Y. (1995) Controlling the false discovery rate - a practical and powerful approach to multiple testing. *J Roy Stat Soc B Met*, **57**, 289-300.

Benjamini,Y. and Yekutieli,D. (2001) The control of the false discovery rate in multiple testing under dependency. *Ann Stat*, **29**, 1165-1188.

Benjamini,Y. and Yekutieli,D. (2004) False discovery rate adjusted multiple confidence intervals for selected parameters. Submitted to *Journal of American Statistical Association*.

Boutanaev,A., Kalmykova,A., Shevelyov,Y.Y. and Nurminsky,D.I. (2002) Large clusters of co-expressed genes in the $Drosophila$ genome. *Nature*, **420**, 666-9.

Bickel,P.J. and Doksum,K.A. (2000) Mathematical statistics: basic ideas and selected topics. 2nd Edition. *Prentice Hall*, Upper Saddle River, NJ, USA.

Butte,A. and Kohane,I.S. (2000) Mutual information relevance networks: functional genomic clustering using pairwise entropy measurements. *Pacific Symposium on Biocomputing*, **5**, 415-426.

Butte,A., Tamayo,P. Slonim,D., Golub,T.R. and Kohane,I.S. (2000) Discovering functional relationships between RNA expression and chemotherapeutic susceptibility using relevance networks. *Proc Natl Acad Sci USA*, **97**, 12182-6.

Butte,A., Bao,L., Reis,B.Y., Watkins,T.W. and Kohane,I.S. (2001) Comparing the similarity of time-series gene expression using signal processing metrics. *J Biomed Inform*, **34**, 396-405.

Daniel, H. (1944) The relation between measures of correlation in the universe of sample permutations. *Biometrika*, **33**, 129-135.

DeRisi,J., Iyer,V., and Brown,P.O. (1997) Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science*, **278**, 680-686.

Dobra,A., Hans,C., Nevins,R., Yao,G. and West,M. (2004) Sparse graphical models for exploring gene expression data. *Journal of Multivariate Analysis*, **90**, 196-212.

Eisen,M., Spellman,P., Brown,P.O., Botstein,D. (1998) Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA*, **95**, 14863-8.

Filkov,V., Skiena,S. and Zhi,J. (2002) Methods for analysis of microarray time-series data. *Journal of Computational Biology*, **9**, 317-330.

Farkas,I., Jeong,H., Vicsek.,T., Barabasi,A.L. and Oltvai,Z.N. (2003) The topology of transcription regulatory network in the yeast, $Saccharomyces\ cerevisiae$. *Physica A*, **318**, 601-612.

Golub,T., Slonim,D., Tamayo,P., Huard,C., Gaasenbeek,M., Mesirov,J.P., Coller,H., Loh,M.L., Downing,J.R., Caligiuri,M.A., Bloomfi eld,C.D. and Lander,E.S. (1999) Molecular classifi cation of cancer: class discovery and class prediction by gene expression monitoring. *Science*, **286**, 531-7.

Hero,A.O., Fleury,G., Mears,A. and Swaroop,A. (2004) Multicriteria gene screening for analysis of differential expression with DNA microarrays. *EURASIP Journal on Applied Signal Processing*, **1**, 43-52.

Hollander,A. and Wolfe,D. (1999) Nonparametric statistical methods. *Wiley-Interscience*, Hoboken, NJ, USA.

Ideker,T., Thorsson,V., Siegel, A.F. and Hood, L.E. (2000) Testing for differentially expressed genes by maximum-likelihood analysis of microarray data. *Journal of Computational Biology*, **7**, 805-817.

Ideker,T., Thorsson,V., Ranish,J.A., Christmas,R., Buhler,J., Eng,J.K., Bumgarner,R., Goodlett,D.R., Aebersold,R. and Hood,L. (2001) Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. *Science*, **292**, 929-34.

Jeong,H., Mason,S., Barabasi,A.L. and Oltvai,Z.N. (2001) Lethality and centrality in protein networks. *Nature*, **411**, 41-42.

Kwon,A., Holger,H. and Ng,R. (2003) Inference of transcriptional regulation relationships from gene expression data. *Bioinformatics*, **19**, 905-912.

Lee,H., Hsu,A., Sajdak,J., Qin,J. and Pavlidis,P. (2004) Coexpression analysis of human genes across many microarray data sets. *Genome Res*, **14**, 1085-1094.

Lockhart,D., Dong,H., Byrne,M.C., Follettie,M.T., Gallo,M.V., Chee,M.S., Mittmann,M., Wang,C., Kobayashi,M., Horton,H. and Brown,E.L. (1996) Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nature Biotechnology*, **14**, 1675-1680.

McLachlan,G., Bean,R. and Peel,D. (2002) A mixture model-based approach to the clustering of microarray expression data. *Bioinformatics*, **18**, 413-422.

Nixon,T., Ronson,C. and Ausubel,F.M. (1986) Two-component regulatory systems responsive to environmental stimuli share strongly conserved domains with the nitrogen assimilation regulatory genes ntrB and ntrC. *Proc Natl Acad Sci USA*, **83**, 7850-7854.

Reiner,A., Yekutieli,D. and Benjamini,Y. (2003) Identifying differentially expressed genes using false discovery rate controlling procedures. *Bioinformatics*, **19**, 386-375.

Rohde,J., Trinh,J. and Sadowski,I. (2000) Multiple signals regulate GAL transcription in yeast. *Mol Cell Biol*, **20**, 3880-6.

Schena,M., Shalon,D., Davis,R.W. and Brown,P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science*, **270**, 467-470.

Schafer,J., and Strimmer,K. (2004) An empirical Bayes approach to inferring large-scale gene association networks. *Bioinformatics*, **1**, 1-13.

Stock,M., Victoria,L. and Goudreau,P.N. (2000) Two-component signal transduction. *Annual Review of Biochemistry*, **69**, 183-215.

Tamayo,P., Slonim,D., Mesirov,J., Zhu,Q., Kitareewan,S., Dmitrovsky,E., Lander,E.S., Golub,T.R. (1999) Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic differentiation. *Proc Natl Acad Sci USA*, **96**, 2907-2912.

Tusher,V., Tibshirani,R. and Chu,G. (2001) Signifi cance analysis of microarrays applied to the the ionizing radiation response. *Proc Natl Acad Sci USA*, **98**, 5116-5121.

Wang,J., Myklebost,O. and Hovig,E. (2003) MGraph: graphical models for microarray data analysis. *Bioinformatics*, **19**, 2210-1.

Wieczorke,R., Krampe,S., Weierstall,T., Freidel,K., Hollenberg,C.P. and Boles,E. (1999) Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in $Saccharomyces\ cerevisiae$. *FEBS Lett*, **464**, 123-128.

Whittaker,J. (1990) Graphic models in applied multivariate statistics. *Wiley*, New York, USA.

Yeung,L., Szeto,L., Liew,A.W. and Yan,H. (2004) Dominant spectral component analysis for transcriptional regulations using microarray time-series data. *Bioinformatics*, **20**, 742-9.

Yeung,M., Tegner,J. and Collins,J.J. (2002) Reverse engineering gene networks using singular value decomposition and robust regression. *Proc Natl Acad Sci USA*, **99**, 6163-6168.

Zareparsi,S., Hero,A.O., Zack,D.J., Williams,R. and Swaroop,A. (2004) Seeing the unseen: Microarray-based gene expression profi ling in vision. *Invest Ophthalmol Vis Sci.*, **45**, 2457-2462.

Zhou,X., Kao,M. and Wong,W.H. (2002) Transitive functional annotation by shortest path analysis of gene expression data. *Proc Natl Acad Sci USA*, **99**, 12783-12788.

5 APPENDIX

5.1 Construct PCER-CI for ρ

Based on the fact that z ($z = \tanh^{-1}(\hat{\rho})$) is the monotonic function of $\hat{\rho}$, the asymptotic PCER $(1-\alpha)\times 100\%$ Confidence Interval: $I^{\lambda}(\alpha)$ on each true Pearson correlation coefficient ρ of the set $\mathcal{G}_1$ is: $\tanh(z - \frac{z_{\alpha/2}}{(N-3)^{1}/2}) \leq \rho \leq (z + \frac{z_{\alpha/2}}{(N-3)^{1}/2})$, where $P(N(0,1) > z_{\alpha/2}) = \alpha/2$.

5.2 Construct PCER-CI for τ

The asymptotic PCER $(1-\alpha)\times 100\%$ Confidence Interval: $I^{\lambda}(\alpha)$ on each true Kendall correlation coefficient τ of the set $\mathcal{G}_1$ is constructed as follows:

- Compute $C_r = \sum_{\substack{t=1 \\ t\neq r}}^{N} Q((X_r,Y_r),(X_t,Y_t))$, for $r = 1,2,...,N.$, where $Q((a,b),(c,d))$ is given by:

$$Q((a,b),(c,d)) = \begin{cases} 1 & \text{if } (d-b)(c-a) > 0, \\ 0 & \text{if } (d-b)(c-a) = 0, \\ -1 & \text{if } (d-b)(c-a) < 0. \end{cases} \tag{10}$$

- Let $\bar{C} = \frac{1}{N}\sum_{r=1}^{N} C_r$ and define $\hat{\sigma}_\tau = \frac{2}{N(N-1)}\frac{2(N-2)}{N(N-1)}\sum_{i=1}^{N} (C_r - \bar{C})^2 + 1 - \hat{\tau}^2]$

- $I^{\lambda}(\alpha) : \hat{\tau} - z_{\alpha/2}\hat{\sigma}_\tau \leq \tau \leq \hat{\tau} + z_{\alpha/2}\hat{\sigma}_\tau$.

5.3 Simulation of pairwise vectors based on pre-specified population covariances

5.3.1 Pearson correlation coefficient ρ .

- Specify a covariance matrix $\mathbf{V}$ and a mean vector μ.

- Form the Cholesky decomposition of $\mathbf{V}$, i.e. find the lower triangular matrix L such that $\mathbf{V} = LL^T$.

- Simulate a vector $\mathbf{z}$ with independent $N(0,1)$ elements.

- A vector simulated from the required multivariate normal distribution is then given by $\mu + L\mathbf{z}$.

5.3.2 Kendall's τ .

- Specify a value for τ.

- Simulate an $N \times N$ indicator matrix M given τ as follows:

$$M[n,m]_{1\leq n<m\leq N} = \begin{cases} 1 & \text{if Bernulli}(\frac{1+\tau}{2}) \text{ is TRUE}, \\ -1 & \text{if Otherwise.} \end{cases} \tag{11}$$

- Simulate i.i.d pairs (X_r, Y_r) $(r = 1,2,...,N)$ according to M matrix and definition

$$Q((a,b),(c,d)) = \begin{cases} 1 & \text{if } (d-b)(c-a) > 0, \\ -1 & \text{if } (d-b)(c-a) < 0. \end{cases} \tag{12}$$

No tied observations are generated. Alternatively, $\hat{\tau}$ can be directly calculated from the indicator matrix M without generating the i.i.d pairs (eq. 3).