Analysis of Correlated Gene Expression Data on Ordered Categories

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Summary

A bootstrap based methodology is introduced for analyzing repeated measures/longitudinal microarray gene expression data over ordered categories. The proposed non-parametric procedure uses order-restricted inference to compare gene expressions among ordered experimental conditions. The null distribution for determining significance is derived by suitably bootstrapping the residuals. The procedure addresses two potential sources of correlation in the data, namely, (a) correlations among genes within a chip ("intra-chip" correlation), and (b) correlation within subject due to repeated/longitudinal measurements ("temporal" correlation). To make the procedure computationally efficient, the adaptive bootstrap methodology of Guo and Peddada (2008) is implemented such that the resulting procedure controls the false discovery rate (FDR) at the desired nominal level.

Key Words and Phases

Bootstrap residuals; dose-response; gene expression; heteroscedastic gene expression data; longitudinal data; ordered categories; ORIOGEN; time course

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1. INTRODUCTION

Microarray gene expression studies are routinely conducted by researchers to understand changes in expression of thousands of genes under various experimental conditions. In fields such as agriculture, toxicology, cancer research, etc. a common problem of interest is to investigate changes in gene expression over ordered categories. Some examples of ordered categories include dose groups, time points, cancer stages etc. For example, Tamoto et al. (2004) studied the changes in gene expression at various stages of esophageal cancer, while Hamadeh et al. (2004) investigated the effect of Furon on the gene expression of rat liver at various doses of the compound. In a time-course study, Blanding et al. (2007) investigated the changes in gene expression in corn when the plants were exposed to different levels of ultraviolet radiation. Frequently, a goal of such ordered categorical experimental designs is to select statistically significant genes and to cluster them according to their patterns of expression over the ordered categories. As described by several authors (e.g. Blanding et al., 2007) such clusters provide insight into possible co-regulation or functional relationships

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among genes. For convenience, throughout this article we shall use the terms "ordered categories", "dose groups" and "time points" interchangeably.

A typical experimental design consists of a sample of n independent microarrays obtained in the ith ordered category and each array consists of M probes representing several thousands of genes. Thus the data derived from microarray experiments are typically high dimensional (in several thousands) with relatively small sample size. This makes data analysis a challenge as the usual notion of Type I error needs modification and classical methods of analyses need to be modified suitably. The focus of this paper is the case when C, the number of ordered categories, is "small" unlike the usual "long series" time course experiments conducted in the context of cell-cycle experiments. There is no clear definition for what is considered to be "large" or "small" although many authors have used $C > 10$ to denote "large" (Conesa et al., 2006).

For convenience, throughout this paper we shall use the term "probe" and "gene" interchangeably. Also, for convenience we assume that all categories have the same number of subjects or experimental units n (i.e. same sample size). As usually done in other traditional experiments, two types of microarray experiments are commonly conducted: Design I: the samples are independent across ordered categories, and Design II: the samples are correlated across ordered categories as in the case of longitudinal or repeated measurement studies. It is important to recognize that there are two potential sources of correlations. The first source is due to "within chip/intra-chip" correlation among genes. This may be due to some artifact of how each chip was handled or may be due to the natural correlation among genes at a given experimental condition (or category). This intra-chip correlation structure can potentially exist in both Design I and in Design II. The second source of correlation is the temporal correlation in the expression of a gene which arises in Design II.

Due to the underlying correlation structures, statistical methods used for analyzing data from the two designs are different. Numerous statistical and computational methods have been proposed in the literature for analyzing time course gene expression data obtained under Design I. Although some of these methods account for the intra-chip correlation, many do not. Some popular methods of analyses for Design I include, the classical regression based methodology (Liu et al., 2005, Conesa et al., 2006), splines based methods (Luan and Li 2003, 2004, Storey et al., 2005), order-restricted inference based methods (Peddada et al., 2003, 2005, Hu et al., 2005, Simmons and Peddada, 2007, Liu et al. 2009), and Bayesian methods (Ishwaran and Rao, 2003, 2005a, 2005b, Jensen et al., 2009).

As with any longitudinal study, the longitudinal microarray experiments provide an opportunity for researchers to understand the changes in expression over time while accounting for any subject effect. Also, such experiments allow the investigator to estimate inter and intra individual variability in the gene expression. Recently Karlovich et al. (2009) conducted a longitudinal microarray study of normal healthy men and women of different ages to evaluate the changes in the gene expression in venous blood over a period of six months. Their study confirmed that the expression of most of the important genes remained stable during the six month period. In Ferre et al. (2007), the authors conducted a longitudinal study to investigate the changes in gene expression over time in piglets that were given intramuscular injections. Using the resulting gene expression data they identified several pathways involved in post-injection muscle injury. Other interesting longitudinal gene expression data sets may be found at the NCBI's website: http:/www.ncbi.nlm.nih.gov/projects/geo/.

Several methods have been proposed in the literature to analyze longitudinal gene expression data utilizing ideas from linear mixed models or hierarchical Bayesian models to describe the covariance structure. For example, based on Brumback and Rice (1998), Storey et al. (2005) modeled the covariance structure using a linear mixed effects model based on B-Splines. Karlovich et al. (2009) used the standard linear mixed effects model approach, while Marot et al. (2009) extended the structural mixed model approach of Jaffrézic et al. (2007) to longitudinal data.

It is important to note that the intra-chip correlations and temporal correlations may arise in a complex unknown manner and hence are often difficult to model. As a result, often times the correlation models are misspecified. For instance, it may not even be appropriate to assume that all genes have the same temporal correlation structure due to potential heterogeneity among genes. Consequently, it is important to have a methodology which is robust to any hidden/correlation structure among the genes. Motivated by this requirement, in this paper we use a simple bootstrap methodology that accounts for the above correlations non-parametrically without making any modeling assumptions. The proposed methodology, described in Section 2, uses order-restricted inference based test statistics introduced in Peddada et al. (2003, 2005). It is designed to control the false discovery rate (FDR) at the pre-specified nominal level. Results of a small simulation study are provided in Section 3. The proposed method is illustrated in Section 4 using a published data set. Concluding remarks and future research problems in this area are provided in Section 5.

2. ANALYSIS OF GENE EXPRESSION DATA USING ORDER RESTRICTED INFERENCE

2.1 Notations and motivation

Let $Y_{g,c,i}$ denote the observed expression of the gth gene $g = 1, 2, \ldots, G$, in the cth category, $c = 1, 2, \ldots, C$, for the ith experimental unit, $i = 1, 2, \ldots, n$, with $E(Y_{g,c,i}) = \mu_{g,c}$. Throughout this paper the terms "profile" and "pattern" will be used interchangeably. A gene g is said to have a "flat" or constant profile across the categories if $\mu_{g,1} = \mu_{g,2} = \ldots = \mu_{g,C}$. This Profile will be denoted by P_0. Typically, researchers are interested in (a) identifying genes that do not have a flat pattern profile across categories, and (b) clustering genes with similar profiles/patterns. For example, $\mu_{g,1} \leq \mu_{g,2} \leq \ldots \leq \mu_{g,c}$ represents a non-decreasing pattern in gene expression over the C ordered categories, whereas $\mu_{g,1} \leq \mu_{g,2} \leq \ldots \leq \mu_{g,C} \geq \mu_{g,c+1} \geq \ldots \geq \mu_{g,C}$ represents an umbrella profile with peak at the cth category. In practice a researcher may be interested in clustering genes that belong to a subset of patterns/profiles which can be enumerated before performing the analysis. These patterns may typically consist of (i) a non-decreasing pattern, (ii) umbrella patterns with peaks at $c = 2, 3, \ldots, C-1$, (iii) a non-increasing pattern, and (iv) inverted umbrella patterns with minimum at $c = 2, 3, \ldots, C-1$. Patterns such as cyclical patterns are of interest some times, but other irregular/arbitrary patterns are not very common. Thus in this paper, and in the companion software that soon will be released (ORIOGEN 3.0), we limit to monotonic patterns, umbrella patterns and single cycle patterns and do not include other arbitrary patterns. Let the collection of all non-null patterns be denoted by $\mathcal{P}$. Then, for each individual gene g one may consider testing the following hypotheses using the classical likelihood ratio test (LRT) provided that the structure of the true temporal covariance matrix is known:

$$H_0: \mu_{g,1} = \mu_{g,2} = \ldots = \mu_{g,C}$$

Against

$$H_a:(\mu_{g,1},\mu_{g,2},\ldots,\mu_{g,C})' \in \mathcal{P}. \tag{1}$$

However, even if one were to know the structure of the temporal covariance matrix, the sample size is typically not large enough to use the asymptotic critical values. Furthermore, it is important to note that such a test does not account for the within intra-chip correlation. Hence one needs to use some version of a resampling procedure to test (1) for all genes.

A possible alternative approach to this problem is to use a parametric model to describe the relationship between the mean expression $\mu_{g,c}$ and c. For example, in a time course experiment or a dose-response study one may consider a regression model to describe the relationship (cf. Liu et al., 2005) and impose a mixed model structure to account for dependence in the data. However, such a model requires the experimenter to know a priori the relationship between the two variables for all genes. Another alternative is to use variations to splines with mixed models as in Storey et al. (2005). Such procedures are generally attractive because of their simplicity but they induce a very specific dependence structure that may not be correct and/or difficult to verify for all genes. Furthermore, such model-based approaches may not be appropriate when c represents an ordered category, such as "tumor stage". For these reasons, Peddada et al. (2003, 2005) used order restricted inference where profiles/patterns among the means are described in terms of mathematical inequalities instead of a parametric model. Hence in this paper we continue with the nonparametric methodology developed in Peddada et al. (2003, 2005) and in ORIOGEN 2.2.1, making no distributional or modeling assumptions and thus providing a large amount of flexibility. As one would expect, a nonparametric procedure such as the procedure proposed here will have smaller power than a parametric procedure when the underlying model is correctly specified. However, since it is hard to justify a particular parametric model in gene expression studies, a nonparametric procedure, such as the one proposed here, may be preferable.

Corresponding to a given inequality pattern, two parameters are said to be *linked* if the inequality between them is specified by the inequality pattern. For example, in an umbrella pattern $\mu_{g,1} \le \mu_{g,2} \le \ldots \le \mu_{g,c} \ge \mu_{g,c+1} \ge \ldots \ge \mu_{g,C}$ the parameters $\mu_{g,1},\mu_{g,2}, \ldots, \mu_{g,c}$ are linked to one another and similarly $\mu_{g,c},\mu_{g,c+1}, \ldots, \mu_{g,C}$ are linked to one another. However, none of the parameters in the collection $\{\mu_{g,1}, \mu_{g,2} \, \mu_{g,c-1}\}$ are linked to any of the parameters in the collection $\{\mu_{g,c+1}, \ldots \mu_{g,C}\}$. A parameter in a given inequality patterns is said to be nodal if it is linked with all parameters $\{ \mu_{g,1}, \mu_{g,2}, \ldots, \mu_{g,C}\}$. Thus in the above umbrella pattern $\mu_{g,c}$ is the only nodal parameter, whereas in a non-decreasing pattern every parameter is a nodal. For a given pattern $P \in \mathcal{P}$, using the estimators derived in Hwang and Peddada (1994), we compute T_P the studentized distance between the farthest linked parameters in P. Thus for example, in the case of umbrella pattern P, $\mu_{g,1} \le \mu_{g,2} \le \ldots \le \mu_{g,c} \ge \mu_{g,c+1} \ge \ldots \ge \mu_{g,C}$ the statistic $T_{g,\,P}$ is given by

$$T_{g,P} = \max \left\{ \frac{\widehat{\mu}_{g,c} - \widehat{\mu}_{g,1}}{S_g \sqrt{\frac{2}{n}}}, \frac{\widehat{\mu}_{g,c} - \widehat{\mu}_{g,C}}{S_g \sqrt{\frac{2}{n}}} \right\} \tag{2}$$

where $\hat{\mu}_{g,i}, i = 1,2, \ldots C$, is the constrained estimator of $\mu_{g,c}$ under the constraint P derived according to the Hwang and Peddada (1994) methodology, ignoring correlations in the data. It is important to note that one could use restricted maximum likelihood estimators (RMLE) of $\mu_{g,c}$ instead of the point estimators proposed in Hwang and Peddada (1994) (ignoring the

correlation structure). Perhaps iterative algorithms along the lines of Shi (1994), Shi and Jiang (1998) and Hoferkamp and Peddada (2002) may be developed to the present context which not only estimate the mean parameters but also estimate the underlying covariance matrix iteratively. Although in theory such an iterative algorithm may be appealing, it can be computationally expensive. Furthermore, as noted in Hwang and Peddada (1994), the RMLE need not perform well as an estimator for individual components of the mean vector. For these reasons, we use the estimator proposed in Hwang and Peddada (1994), ignoring correlations in the data. As usual, S_g^2 denotes the pooled sample variance for the gth gene $g = 1,2, \ldots G$. Again, one may replace S_g^2 by the true standard error of the numerator in (2). However, the derivation of such a quantity is not straightforward and since our test relies on bootstrap the choice of the correct denominator is very important. As in Peddada et al. (2003, 2005), for a gene g, the test statistic for testing (1) is defined as:

$$T_g = \max_{P \in \mathcal{P}} T_{g,P}$$

(3)

Thus neither in the computation of the order-restricted estimators, nor in the construction of the above test statistic do we incorporate the underlying correlation structure. However, in the spirit of the popular generalized estimating equations (GEE) methodology, the correlation in the data will be accounted in the derivation of the bootstrap null distribution (3), which is described in Section 2.2.

Once the null hypothesis is rejected for a gene g according to the methodology described in Section 2.2, we assign it to the pattern P in $\mathcal{P}$ which corresponds to $\max_{P \in \mathcal{P}} T_{g,P}$.

2.2 The bootstrap methodology

In Peddada et al. (2003, 2005) and in ORIOGEN 2.2.1, for a gene g, the null distribution of $T_g = \max_{P \in \mathcal{P}} T_{g,P}$ was derived by assigning simple random samples (with replacement) of size n, from the combined sample of size $n \times C$, to each ordered category and by computing the statistic $T_g^* = \max_{P \in \mathcal{P}} T_{g,P}^*$. The process was repeated a large number of times B which yielded the null distribution for gene g. The above bootstrap methodology assumes that for a gene g, under the null hypothesis, its expressions across the C categories are independently and identically distributed. In the context of longitudinal data such an assumption is not valid. Under the null hypothesis we can only assume that the means across categories are equal, but cannot assume independence or that the variances are equal. A simple method to relax these assumptions is by bootstrapping the residuals as shown below. Bootstrapping residuals is widely used in literature because of the flexibility it provides to deal with heterogeneity in the data (Efron and Tibshirani, 1993).

Let $\overline{\overline{Y}}_{g,\cdot,\cdot}$ denote the grand sample mean of the gth gene $g = 1,2, \ldots, G$, averaged over all experimental units and categories and let $\overline{Y}_{g,c}$ denote its sample mean in the cth category, $c = 1,2, \ldots, C$. Let $\hat{\varepsilon}_{g,c,i} = Y_{g,c,i} - \overline{Y}_{g,c,\cdot}$ denote the residual corresponding to the ith experimental unit in the cth category. Let the jth simple random sample of n experimental units (with replacement) from $\{1,2, \ldots, n\}$ be denoted by $\{j_1, j_2, \ldots, j_n\}$. Thus we are resampling the experimental units. Then for gene g, the bootstrap sample that honors the null hypothesis while maintaining any dependence structure in the data is given by $Y_{g,c,j_i}^* = \overline{\overline{Y}}_{g,\cdot,\cdot} + \widehat{\varepsilon}_{g,c,j_i}$, $i = 1,2, \ldots, n$, $c = 1,2, \ldots, C$. Using this bootstrap sample we construct the statistic $T_g^* = \max_{P \in \mathcal{P}} T_{g,P}^*$ which yields the bootstrap null distribution. Rather than fixing the number of bootstrap samples B a priori, we use the adaptive bootstrap methodology of Guo and Peddada (2008)

which reduces the total number of bootstraps while controlling the false discovery rate at the desired nominal level α.

Although this manuscript is written for analyzing longitudinal gene expression data, the residual bootstrap methodology described above is readily applicable for analyzing cross-sectional ordered gene expression data when the variance of expression for a gene is not constant across ordered categories (known as heteroscedasticity). Since the sample experimental unit is not observed in all categories in a cross-sectional data, the above

bootstrap sampling scheme can be modified as follows. As above, let $\overline{\overline{Y}}_{g,.,.}$ denote the grand sample mean of the gth gene $g = 1, 2, \ldots, G$, averaged over all experimental units and categories and let $\overline{Y}_{g,c}$ denote its sample mean in the cth category, $c = 1, 2, \ldots, C$. Let $\hat{\varepsilon}_{g,c,i} = Y_{g,c,i} - \overline{Y}_{g,c,.}$ denote the residual corresponding to the ith experimental unit in the cth category. For the cth category, let j^{th} simple random sample of n experimental units (with replacement) from $\{1, 2, \ldots, n\}$ be denoted by $\{j_{c,1}\, j_{c,2}, \ldots, J_{c,n}\}$. Thus we are resampling the experimental units within each category separately rather than using the same set of sampling units across all categories as was done above for the longitudinal data. Then for gene g, the bootstrap sample that honors the null hypothesis while maintaining any

dependence structure in the data is given by $Y^{*}_{g,c,j_{c,i}} = \overline{\overline{Y}}_{g,.,.} + \widehat{\varepsilon}_{g,c,j_{c,i}}$, $i = 1, 2, \ldots, n$, $c = 1, 2, \ldots,$

C. Using this bootstrap sample we construct the bootstrap statistic $T^{*}_{g} = \max_{P \in \mathcal{P}} T^{*}_{gP.}$ which yields the bootstrap null distribution. Thus the proposed methodology is applicable for heteroscedastic gene expression data.

3. SIMULATION STUDY

A simulation study was conducted to evaluate the performance of the proposed methodology in terms of the control of false discovery rate (FDR) and the power in identifying the true non-nulls. We compared our procedure with the EDGE procedure developed by Storey et al. (2005). We generated data according to the following linear mixed effects model which is somewhat consistent with the underlying assumptions of Storey's methodology (Storey et al., 2005).

$Y_{g,c,i} = \theta_{g,c} + \alpha_i + \varepsilon_{g,c,i}$, $g = 1, 2, \ldots, G$, $c = 1, 2, \ldots, C$, $i = 1, 2, \ldots, n$, where

$$\alpha_i | \lambda \overset{iid}{\sim} N(0, \lambda), \lambda \sim Gamma(a_1, a_2) \tag{4}$$

$$\varepsilon_{g,c,i} \overset{iid}{\sim} N(0, \sigma^2 \gamma_{g,c,i}), \gamma_{g,c,i} \overset{ind}{\sim} Gamma(d_{1,g,c}, d_{2,g,c}). \tag{5}$$

Note that (4) induces intra-chip dependence as well as temporal dependence and (5) induces heteroscedasticity. All random variables described above are assumed to be independently distributed. We considered an experiment where microarray data were obtained on a random sample of $n = 6$ subjects over $C = 6$ ordered categories. Each microarray chip consisted of $G = 16000$ probes of which 12000 had null pattern (i.e., $\theta_{g,c} = 0$ $g = 1, 2, \ldots, 12000$, $c = 1, 2, \ldots,$ 6) and 4000 had non-null patterns described in Table 1. Each non-null pattern had 400 probes. Patterns of parameters in equations (4) and (5) that describe the variability and correlation structure are provided in Table 2. Although a broad collection of patterns of various parameters were investigated, we provide a small subset of our simulation study.

A software package called ORIOGEN 3.0 was developed by SDP and programmed by SH for the methodology described in this paper and is currently in use. We used this software for simulations reported in this paper. The results are based on 10,000 bootstrap samples per gene. The nominal FDR level was set at 0.05. Similarly, EDGE 1.1.291 was used to evaluate the procedure of Storey et al. (2005). Throughout this paper EDGE 1.1.291 will be referred to EDGE and ORIOGEN 3.0 will be referred to as ORIOGEN. Since EDGE uses q-values, we evaluated EDGE at a nominal q-value of 0.05, which in our experience approximates an FDR nominal level of 0.05. We implemented EDGE using 500 bootstrap samples.

Based on the results of the simulation study for the 8 cases summarized in Table 3 and others (not reported in the paper) we find that the proposed methodology controls the FDR at the nominal level of 0.05, although it can be conservative. In our experience its FDR never exceeded the nominal level. Although the EDGE procedure of Storey et al. (2005) enjoys larger power than the proposed methodology, it tends to have an inflated FDR especially when there is a significant amount of heteroscedasticity among the null genes.

Note that in an ideal simulation study we should repeat this simulation for several thousand random realizations for each case considered in Table 2. However, neither ORIOGEN nor EDGE is designed to run a simulation study and it is practically impossible to run ORIOGEN and EDGE one random realization at a time for a large number of times. However, we arbitrarily selected case 3 in Table 2 and generated 5 sets of microarrays with 16000 genes each and ran ORIOGEN and EDGE 5 times each. The estimated FDRs and powers for each of the 5 sets of random realizations are provided in Table 3 in the row corresponding to case 3. From these numbers it is clear that the FDR estimates for ORIOGEN and EDGE are fairly stable with standard deviations of 0.0085 and 0.0061, respectively.

4. ILLUSTRATION

We also illustrate the proposed methodology by re-analyzing the data of Ferre et al. (2007). They observed that patients receiving treatments/drugs by intramuscular (IM) injections may experience some adverse reactions or outcomes. Among infants IM injections could lead to serious complications. Since not much was known about the molecular pathways involved in local skeletal muscle injury due to IM injections, Ferre et al. conducted a longitudinal gene expression study using 10 male piglets weighing between 23 and 32 kg. Each piglet was injected with 4ml of Propylene Glycol at 4 time points (6 hours, 2 days, 7 days, and 21 days before sacrifice) in the lumbar region and cDNA microarrays were obtained using injected muscles and the non-injected sites. Thus on each piglet 5 microarray chips were obtained, one at the baseline and one each at 6 hours, 2 days, 7 days and 21 days before sacrifice, respectively. In the analysis reported in their paper, Ferre et al. analyzed 1651 clones that correspond to genes with known accession numbers. They discovered 324 genes to have "variable expression" using a p-value of 0.0125. Of these 324, they found 153 to have a differential expression with a fold change more than 1.5 (in either direction) in at least one time interval. The standard t-test was used for making each pair-wise comparison between neighboring time intervals. Using these results, a "global" pattern of expression for each gene over the 21 day time interval was provided. Thus, among genes that were declared to be significant, Ferre et al. were interested in identifying their pattern of expression over time. Furthermore from the summaries provided in their paper, it seems that the authors were interested in clustering genes with similar pattern of expression. Professors Lefebvre and Liaubet, authors of the above publication, provided us the log-transformed normalized gene expression data, which is also available at http:/www.ncbi.nlm.nih.gov/geo/. with GEO accession number GSE3217, so that we may re-analyze the data using the methodology described in this paper.

Intrinsically Ferre et al. (2007) were interested in identifying the time course pattern for each gene. For this reason, we considered the following patterns for each gene:

- Increasing pattern:

  $P_1: \mu_{g,base} \leq \mu_{g,6hrs} \leq \mu_{g,2days} \leq \mu_{g,7\ days} \leq \mu_{g,21days}$

- Umbrella pattern with peak at 6 hours:

  $P_2: \mu_{g,base} \leq \mu_{g,6hrs} \geq \mu_{g,2days} \geq \mu_{g,7days} \geq \mu_{g,21\ day}$

- Umbrella pattern with peak at day 2:

  $P_3: \mu_{g,base} \leq \mu_{g,6hrs} \leq \mu_{g,2days} \geq \mu_{g,7days} \geq \mu_{g,21days}$

- Umbrella pattern with peak at day 7:

  $P_4: \mu_{g,base} \leq \mu_{g,6hrs} \leq \mu_{g,2days} \leq \mu_{g,7days} \geq \mu_{g,21days}$

- Decreasing pattern:

  $P_5: \mu_{g,base} \geq \mu_{g,6hrs} \geq \mu_{g,2days} \geq \mu_{g,7days} \geq \mu_{g,21days}$

- Inverted umbrella pattern with minimum at 6 hours:

  $P_6: \mu_{g,base} \geq \mu_{g,6hrs} \leq \mu_{g,2days} \leq \mu_{g,7days} \leq \mu_{g,21days}$

- Inverted umbrella pattern with minimum at day 2:

  $P_7: \mu_{g,base} \geq \mu_{g,6hrs} \geq \mu_{g,2days} \leq \mu_{g,7days} \leq \mu_{g,21days}$

- Inverted umbrella pattern with minimum at day 7:

  $P_8: \mu_{g,base} \geq \mu_{g,6hrs} \geq \mu_{g,2days} \geq \mu_{g,7days} \leq \mu_{g,21days}$

Thus for each gene g we tested $H_0: \mu_{g,1} = \mu_{g,6hrs} = \mu_{g,2days} = \mu_{g,7days} = \mu_{g,21days})$ against the

alternative hypothesis $H_{g,a}: (\mu_{g,base}, \mu_{g,6hrs}, \mu_{g,2days}, \mu_{g,7days}, \mu_{g,21days})' \in \mathcal{P}$ where $\mathcal{P} = \bigcup_{i=1}^{8} P_i$. If the null hypothesis was rejected then the gene was assigned to the pattern P_j with the largest goodness of fit statistic defined in (1).

Since multiple hypotheses were being tested, we selected significant genes at an FDR of 0.05 using 10,000 bootstrap samples. Our procedure discovered a total of 429 probes. In Table 4 we provide the list of all genes selected by ORIOGEN according to the 8 different time course patterns. In many cases multiple probes of the same gene were selected. In many cases the probes of the same gene displayed different time course patterns of expression. In Table 4 corresponding to each gene we provide the number of probes that were identified in a given pattern. There were several probes for which the gene names were not known. The plot of mean expression of each probe was plotted according to the pattern of expression. These plots are provided in Figure 1.

Genes that were also identified by Ferre et al. (2007) are denoted by the asterisk (*) in Table 4. Interestingly, more than 85% of the genes identified by Ferre et al. were also identified by our procedure. The gene expression patterns were also consistent between the two methods, although the patterns shown by Ferre et al are less specific than the ORIOGEN patterns. For example, the Ferre et al pattern indicated by a single down arrow followed by three horizontal arrows could fall into any of the ORIOGEN patterns with a decreasing first segment: overall decreasing pattern or any of the inverted umbrella patterns. Several genes identified by our methodology were not identified by Ferre et al. (2007). These are also provided in Table 4 without the asterisk. There were a few probes whose gene names were ambiguous and hence were not listed in tables provided in this paper.

A large proportion of genes selected by ORIOGEN displayed either an umbrella pattern in expression with maximum at day 7 or an inverted umbrella with minimum at day 7. This is interesting in view of the pathology findings reported in Ferre et al. (2007). On day 21, the authors observed dense fibrous and collagenous tissues in the injected areas with re-generating myocites, consistent with a repairing process following muscle injury. The top 10 biological functions (At FDR = 0.05) these genes involved in are provided in Figure 3 (using Ingenuity Pathway Analysis). The lengths of horizontal bars represent the log-p value associated with the category. As expected, these genes are largely involved in functions such as skeletal muscle development, tissue morphology, tissue development etc. These results are obtained using Ingenuity Pathway Analysis software (Ingenuity Inc.)

Ferre et al. (2007), who did not control for multiple testing, found only two genes to be differentially expressed between day 7 and 21. Consequently, it is not surprising that in the Principal Component Analysis (PCA) plot in Figure 2, based on genes with fold change exceeding 1.5, day 7 and day 21 samples are clustered together. Samples from the remaining time points seem to be well clustered according within their groups. Although we see two potential outliers at the baseline which cluster with 6 hours time point. Here fold change for a given pattern is defined to be the exponential of the mean distance between the farthest linked points. For example, in the case of umbrella pattern with peak at 6 hours, for a gene g, we define fold change as $\exp(\max(\hat{\mu}_{g,6hrs} - \hat{\mu}_{g,base}, \hat{\mu}_{g,6hrs} - \hat{\mu}_{g,21days}))$.

Our procedure did not identify 21 genes that were identified by the original authors (see Table 5). However, it is interesting to see that according our test these genes had p-values ranging from 0.012 to 0.392.

Note that there are several differences between the two methods. Firstly, the two methods are testing different types of hypotheses and hence it is not surprising to find some differences. Our method tests for trend in expression over time whereas the original authors performed pairwise comparisons. Secondly, our method controls the overall FDR whereas the original paper did not control for multiple testing. Lastly, our method accounts for the heteroscedasticity, any correlations within chip and also accounts for within animal correlation over time.

5. DISCUSSION

In this article we described a nonparametric bootstrap methodology based on the residuals for analyzing gene expression data which accounts for potential correlations among genes within a chip as well as temporal correlations due to repeated measurements on the same subject. The proposed methodology uses order-restricted inference based techniques developed in Peddada et al. (2003 in Peddada et al. (2006). A methodology is also proposed which is suitable for cross-sectional data when the variance within gene is not constant across ordered categories (heteroscedasticity). One could argue that it would be better to use order restricted estimates that account for the underlying covariance matrices. However there are three problems with that approach, firstly, as demonstrated in Hwang and Peddada (1994), the classical restricted maximum likelihood estimators (RMLE) may fail even if the covariance matrix is known. Secondly, typically the covariance matrix is unknown, and in gene expression studies it needs to be estimated using substantially smaller sample size relative to the number of genes. Thirdly, the correlation structure among the genes is typically unknown to the researcher which makes it difficult to model. Our hope (which is confirmed in our modest simulation study) is that bootstrapping the residuals results in a methodology which is robust to underlying correlation structures. However, we do believe that there is room for improvement in the proposed methodology and is worth exploring new methods. For instance, one could explore modifications to the proposed nonparametric

bootstrap by using a suitable parametric bootstrap. Secondly, the test statistic may be modified by using a better denominator than used in this paper. Note that the standard deviation used in the denominator is not a consistent estimator of the true variance estimator of the numerator. Unfortunately, it is practically a challenging problem to derive the moments of order restricted estimates. Consequently, we are using a pooled sample variance estimator. In a bootstrap setting it may be reasonable to use such a scaling factor. However, we believe there is an opportunity to improve the methodology by using a better scaling factor than one used here. The resulting methodologies would have applications in the analysis of other high dimensional data. It would also be interesting to extend the proposed methodology to deal with designs with more than one explanatory variable, possibly some having continuous covariates. The present framework would allow such generalizations, although they may be non-trivial.

Acknowledgments

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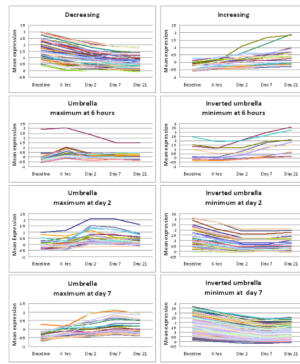
Figure 1.
Time course plots of mean expression of all significant probes clustered according to their pattern.
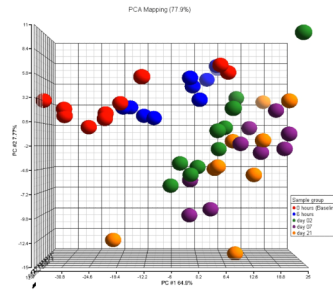
Figure 2.
PCA plots based on probes selected by ORIOGEN with fold change larger than 1.5
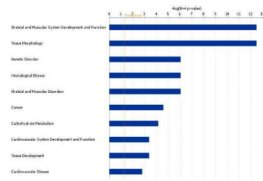
Figure 3.
Top ten biological functions of genes with umbrella or inverted umbrella pattern at day 7.

Table 1

Patterns of mean expressions of non-null probes

Pattern name	Mean vector $(\theta_1, \theta_2, \theta_3, \theta_4, \theta_5, \theta_6)$
Increasing	$(0,0.5,1,1.5,2,2.5)$
Deceasing	$(0,-0.5,-1,-1.5,-2,-2.5)$
Umbrella Peak at 2	$(0,0.5,0,-0.5,-1,-1.5)$
Umbrella Peak at 3	$(0,0.5,1,0.5,0,-0.5)$
Umbrella Peak at 4	$(0,0.5,1,1.5,1,0.5)$
Umbrella Peak at 5	$(0,0.5,1,1.5,2,1.5)$
Inverted Umbrella Min at 2	$(0,-0.5,0,0.5,1,1.5)$
Inverted Umbrella Min at 3	$(0,-0.5,-1,0-.5,0,0.5)$
Inverted Umbrella Min at 4	$(0,-0.5,-1,-1.5,-1,-0.5)$
Inverted Umbrella Min at 5	$(0,-0.5,-1,-1.5,-2,-1.5)$

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Table 2

Patterns of parameters describing different variance structures. Within each case, for each value of σ, 6000 null probes and 2000 non-null probes were selected. Thus each case consists of a total of 16000 probes per microarray.

Case	$(d_{1g,c}, d_{2g,c})$ $(a_1, a_2) = (5, 0.2)$		σ
	Null	**Non-null**	
1	$(c, 0.2)$	$(\sqrt{c}, 1/c)$	$\frac{0.2}{1}$
2	$(c, 0.2)$	$(c, 1/c)$	$\frac{0.2}{1}$
3	$(c^2, 0.2)$	$(\sqrt{c}, 1/c)$	$\frac{0.2}{1}$
4	$(c^2, 0.2)$	$(\sqrt{c}, 1/c)$	$\frac{0.2}{1}$

Case	$(d_{1g,c}, d_{2g,c})$ $(a_1, a_2) = (5, 1)$		σ
	Null	**Non-null**	
5	$(2, .1c)$	$(2, .1|.1 + \theta_{g,c}|)$	$\frac{0.2}{1}$
6	$(2, .1c)$	$(2, .1|.1 + \theta_{g,c}|^2)$	$\frac{0.2}{1}$
7	$(2, .1c^2)$	$(2, 1|.1 + \theta_{g,c}|)$	$\frac{0.2}{1}$
8	$(2, .1c^2)$	$(2, .1|.1 + \theta_{g,c}|^2)$	$\frac{0.2}{1}$

Table 3

FDR and Power comparisons between ORIOGEN and EDGE

Case	ORIOGEN		EDGE	
	FDR	**Power**	**FDR**	**Power**
1	0.022	0.795	0.054	0.926
2	0.020	0.616	0.048	0.765
3	0.038*(.051, .035, .028, .041, .036)	0.79*(.82, .81, .74, .80, .78)	0.091*(.096, .082, .097, .091, .090)	0.918*(.919, .935, .912, .911, .912)
4	0.043	0.640	0.098	0.712
5	0.015	0.937	0.052	0.989
6	0.020	0.774	0.051	0.918
7	0.027	0.940	0.080	0.988
8	0.033	0.774	0.085	0.916

*
average of the numbers that are in the parenthesis

Table 4

List of selected genes clustered according to the pattern of expression.

Clones with decreasing pattern		Clones with increasing pattern over time	
Gene	**Gene Id**	**Gene**	**Gene Id**
26S proteasome non-ATPase regulatory subunit 2	Psmd2	6-phosphofructokinase type C	PFKP
Actin alpha skeletal muscle	Acta1 (2) *	6S ribosomal protein L27a	Rpl27a
Band 4.1-like protein 2	EPB41L2 *	Calcyclin	S1A6
Creatine kinase M chain	CKM *	Cell division protein kinase 4	CDK4
Cytochrome c oxidase polypeptide VIII heart mitochondrial precursor	COX8H (2) *	Collagen alpha 1	COL6A1 *
		Collagen alpha 2	COL1A2 *
Fructose-bisphosphate aldolase A	ALDOA (5) *	DNA replication licensing factor MCM2	MCM2
Glyceraldehyde-3-phosphate dehydrogenase	GAPD (2) *	DNA-binding protein inhibitor ID-3	ID3
Glycerol-3-phosphate dehydrogenase [NAD+] cytoplasmic	Gpd1 *	Fibronectin	FN1 *
		Galectin-1	LGALS1 (2)
Glycogen phosphorylase muscle form	PYGM *	Histone H3.3	
Importin 13	Ipo13	Homo sapiens annexin A2	ANXA2
Myosin heavy chain skeletal muscle juvenile	MYH4 *	Hydroxyacylglutathione hydrolase	Hagh
Myosin light chain 1 skeletal muscle isoform	MYL1	Ligand of Numb-protein X 2	Lnx2
Myosin light polypeptide 3	MYL3	Lipid phosphate phosphohydrolase 1 lipoprotein lipase	PPAP2A LPL
Myosin regulatory light chain 2, skeletal muscle isoform	Mylpf (2) *	Protein phosphatase 1 regulatory subunit 12A	PPP1R12A
NADH-ubiquinone oxidoreductase MLRQ subunit	NDUFA4	Septin 4	4 S E P
Phosphoglycerate mutase 2	PGAM2 (2) *	Splicing factor 3B subunit 1	SF3B1
Protein C2orf142 homolog precursor	C2orf142	Tumor suppressor p53-binding protein 1	
Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	ATP2A1 *	Tyrosine-protein kinase JAK1 Vimentine	JAK1 (2)
Selenoprotein W	SEPW1	Zinc finger protein 14	ZNF14
Triosephosphate isomerase	TPI1		
Tropomyosin 1 alpha chain	TPM1 (3) *	**Clones with minimum at 6 hours**	
Tropomyosin alpha 3 chain	TPM3		
Troponin C skeletal muscle	TNNC2 *	72 kDa type IV collagenase precursor	MMP2 *
Troponin C slow skeletal and cardiac muscles		Collagen alpha 1	COL1A1 *
Troponin T fast skeletal muscle isoforms		Collagen alpha 2	COL1A2 *
Troponin T slow skeletal muscle isoforms	? (3)	Jerky protein	Jrk
		Pyruvate kinase isozymes M1/M2 secreted protein, acidic, cysteine-rich	PKM2 *
Clones with peak at 6 hours		Sus scrofa H19 gene	SPARC *
4S ribosomal protein S16	Rps16 *		
Ankyrin 1	Ank1	**Clones with minimum at day 2**	

Clones with decreasing pattern		Clones with increasing pattern over time	
Gene	**Gene Id**	**Gene**	**Gene Id**
Ankyrin repeat domain protein 2	Ankrd2 (2) [*]		
Ankyrin repeat domain protein 6	Ankrd6 [*]	Actin alpha skeletal muscle	Acta1 (4) [*]
BAG-family molecular chaperone regulator-3	BAG3 [*]	Alpha-actinin 3	Acta3 (2) [*]
Cytochrome c oxidase polypeptide III	MT-CO3 [*]	Conserved oligomeric Golgi complex component 4	COG4
GTP-binding protein RAD	RRAD [*]	Creatine kinase M chain	CKM [*]
Heat shock 7 kDa protein 1B	HSPA1B [*]	Enigma homolog	Enh
Multisynthetase complex auxiliary component p38	JTV1 [*]	Fructose-bisphosphate aldolase A	ALDOA (2) [*]
Oligodendrocyte transcription factor 2	Olig2	Glycerol-3-phosphate dehydrogenase [NAD+] cytoplasmic	Gpd1 [*]
Pantophysin	SYPL [*]		
Tyrosine-protein kinase JAK1	JAK1	Glycogen phosphorylase muscle form	PYGM [*]
Ubiquitin-conjugating enzyme	CDC34	Muscle type phosphofructokinase	M-PFK [*]
E2-32 kDa complementing		Myoglobin	MB
		Myosin heavy chain cardiac muscle beta isoform	MYH7 (2) [*]
Clones with peak at day 2		Myosin heavy chain skeletal muscle adult 2	MYH2 [*]
		Myosin heavy chain skeletal muscle juvenile	MYH4 [*]
Adenylyl cyclase-associated protein 1	CAP1	Myosin regulatory light chain 2 ventricular/cardiac muscle isoform	MYL2
Alpha crystallin B chain			
Beta-2-microglobulin precursor	B2M (2) [*]	Myosin regulatory light chain 2, skeletal muscle isoform	Mylpf [*]
Calgizzarin	S1A11	Nebulin	NEB [*]
Calnexin precursor	CANX	Phosphoglycerate mutase 2	PGAM2 (5) [*]
Calpain small subunit 1	CAPNS1 [*]	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	ATP2A1 [*]
Cofilin non-muscle isoform	CFL1 (3) [*]	Selenoprotein W	SEPW1
Desmin	DES [*]	Triosephosphate isomerase	TPI1 [*]
DNA repair protein RAD51 homolog 1	RAD51	Troponin C skeletal muscle	TNNC2 (2) [*]
Dynein light chain 2A cytoplasmic	Dncl2a	Troponin T fast skeletal muscle isoforms beta/alpha	? (3)
Ferritin heavy chain	FTH [*]	Ubiquitin-like protein FUBI	FAU
Ferritin light chain	FTL (2) [*]		
ferritin, heavy polypeptide 1	FTH1	**Clones with minimum at day 7**	
Gamma-aminobutyric acid	Gabarap		
receptor associated protein		4S ribosomal protein S2	Rps2
Glia maturation factor gamma	GMFG (2) [*]	5′	Nt5m
Heat shock protein HSP 9-alpha	HSPCA [*]	6-phosphofructokinase muscle type	PFKM
High mobility group protein 2	HMGB2	Actin alpha skeletal muscle	Acta1 (32) [*]
Legumain precursor	LGMN [*]	Actin aortic smooth muscle	Acta2

Clones with decreasing pattern		Clones with increasing pattern over time	
Gene	**Gene Id**	**Gene**	**Gene Id**
Peptidyl-prolyl cis-trans isomerase A	PPIA [*]	Adenylate kinase isoenzyme 1	AK1
Protein K4		Alpha-actinin 3	Acta3 (6) [*]
Tubulin alpha-ubiquitous chain		ATP synthase delta chain mitochondrial precursor	ATP5D
U4/U6 small nuclear ribonucleoprotein Prp3	Prpf3 (2)	Band 4.1-like protein 2	EPB41L2 [*]
		Beta enolase	ENO3 (11) [*]
Clones with peak at day 7		Calsequestrin skeletal muscle isoform precursor	CASQ1
26S protease regulatory subunit 6B		Carboxy-terminal domain RNA polymerase II polypeptide A small phosphatase 2	CTDSP2 [*]
6S ribosomal protein L18	RPL18 [*]	Creatine kinase M chain	CKM (16) [*]
ADP-ribosylation factor-like protein 7	Arl7	Cytochrome c oxidase polypeptide VIa-heart mitochondrial precursor	COX6A2
Calmodulin	CALM2		
calponin 2	CNN2	Cytochrome c1 heme protein mitochondrial precursor	CYC1
Co-chaperone protein HscB mitochondrial precursor	HSCB	FKSG26 protein	FKSG26 [*]
		Fructose-bisphosphate aldolase A	ALDOA (6) [*]
Collagen alpha 1	COL6A1 [*]	Glyceraldehyde-3-phosphate dehydrogenase	GAPD (12) [*]
Elongation factor 1-alpha 1	EEF1A1 [*]	Glycogen phosphorylase muscle form	PYGM (4) [*]
Enabled protein homolog	ENAH	GPD;	
Eukaryotic translation initiation factor 2 subunit 1	Eif2s1	Hypothetical protein B495.5 in chromosome II	
Formin-binding protein 3	FNBP3	11Importin 13	IPO13
Guanine nucleotide-binding protein G	GNB2	L-lactate dehydrogenase B chain	LDHB
Heterogeneous nuclear ribonucleoprotein K		Myomesin 2	MYOM2
IGF1;		Myosin heavy chain cardiac muscle beta isoform	MYH7 (2) [*]
Leukocyte elastase inhibitor	SERPINB1	Myosin heavy chain skeletal muscle adult 1	MYH1 (7) [*]
Metastasis-associated protein MTA1	Mta1	Myosin heavy chain skeletal muscle juvenile	MYH4 (9) [*]
Palmitoyl-protein thioesterase 2 precursor	PPT2	Myosin light polypeptide 3	MYL3 (2)
Pig complement cytolysis inhibitor		Myosin-binding protein C fast-type	MYBPC2 (2) [*]
Pleckstrin 2	PLEK2	NADH-ubiquinone oxidoreductase 19 kDa subunit	NDUFA8 [*]
Potential carboxypeptidase-like	CPXM2	Oxytocin receptor	OXTR
protein X2 precursor		Peroxiredoxin 2	
Retinol dehydrogenase type III		Phosphoglycerate mutase 2	PGAM2 (6) [*]
Retrovirus-related Pol polyprotein LINE-1	Pol	Proteasome activator complex subunit 3	PSME3
S1 calcium-binding protein A16	S1A16	Protein C1orf8 precursor	C1orf8
Syntaxin-1	STX1	Protein kinase C and casein kinase	PACSIN3
TBC1 domain family, member 15	TBC1D15	substrate in neurons protein 3	
Thiosulfate sulfurtransferase	TST	Protein phosphatase inhibitor 1	Ppp1r1a
Thymosine beta 4	TMSB4 [*]	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	ATP2A1 [*]
Tryptophanyl-tRNA synthetase	WARS	Sterol regulatory element binding protein-1	SREBF1

Clones with decreasing pattern		Clones with increasing pattern over time	
Gene	**Gene Id**	**Gene**	**Gene Id**
Tubulin alpha-3 chain	TUBA3	Telethonin	TCAP
		Tropomyosin 1 alpha chain	TPM1 (4) [*]
		Troponin C skeletal muscle	TNNC2 (22) [*]
		Troponin I fast skeletal muscle	Tnni2
		Troponin T fast skeletal muscle isoforms	? (2)
		Troponin T fast skeletal muscle isoforms beta/alpha	
		Ubiquitin-conjugating enzyme E2-32 kDa complementing	CDC34

[*] genes also identified by Ferre et al. (2007)

If the number of clones are more than 1 then the number is within parenthesis ( ) next to the Gene Id

Table 5

Genes that were identified by Ferre et al. (2007) and not by ORIOGEN

Gene Name	Gene ID	P-value according to ORIOGEN
4S ribosomal protein S11	Rps11	0.146
4S ribosomal protein S15	Rps15	0.117
4S ribosomal protein S19	Rps19	0.392
4S ribosomal protein S26	Rps26	0.043
4S ribosomal protein S3	RPS3	0.045
4S ribosomal protein S5	RPS5	0.058
6S ribosomal protein L11	Rpl11	0.062
6S ribosomal protein L19	Rpl19	0.012
6S ribosomal protein L23a	Rpl23a	0.046
6S ribosomal protein L8	Rpl8	0.069
Actinin alpha 3	ACTN3	0.068
ATP synthase alpha chain heart isoform mitochondrial precursor	ATP5A1	0.062
Carboxypeptidase D precursor	CPD	0.036
Cytochrome c oxidase polypeptide VIIa-liver/heart mitochondrial precursor	COX7A2	0.015
Inhibitor of carbonic anhydrase precursor	ICA	0.034
Major seminal plasma glycoprotein PSP-I precursor		0.027
NF-kappaB inhibitor-like protein 1	NFKBIL1	0.036
Nucleoprotein TPR	TPR	0.060
Peptidyl-prolyl cis-trans isomerase like 2	PPIL2	0.017
Trifunctional purine biosynthetic protein adenosine-3 [Includes: Phosphoribosylamine--glycine ligase	GART	0.021
Tropomyosin beta chain	Tpm2	0.018