

# Significance analysis of time course microarray experiments

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**Characterizing the genome-wide dynamic regulation of gene expression is important and will be of much interest in the future. However, there is currently no established method for identifying differentially expressed genes in a time course study. Here we propose a significance method for analyzing time course microarray studies that can be applied to the typical types of comparisons and sampling schemes. This method is applied to two studies on humans. In one study, genes are identified that show differential expression over time in response to *in vivo* endotoxin administration. By using our method, 7,409 genes are called significant at a 1% false-discovery rate level, whereas several existing approaches fail to identify any genes. In another study, 417 genes are identified at a 10% false-discovery rate level that show expression changing with age in the kidney cortex. Here it is also shown that as many as 47% of the genes change with age in a manner more complex than simple exponential growth or decay. The methodology proposed here has been implemented in the freely distributed and open-source EDGE software package.**

aging | differential expression | expression arrays | Q values | time series

The identification of genes that show changes in expression between varying biological conditions is a frequent goal in microarray experiments (1). Differential expression can be studied from a static or temporal viewpoint. In a static experiment, the arrays are obtained irrespective of time, capturing only a single moment of gene expression. In a temporal experiment the arrays are collected over a time course, allowing one to study the dynamic behavior of gene expression. A large amount of work has been done on the problem of identifying differentially expressed genes in static experiments (2). Because the regulation of gene expression is a dynamic process, it is also important to identify and characterize changes in gene expression over time. Here we present a general statistical method that identifies genes differentially expressed over time.

Several clustering methods have been applied to time course microarray data, including hierarchical clustering (3, 4), principal components-based clustering (5), Bayesian model-based clustering (6), and *K*-means clustering of curves (7, 8). None of these clustering methods is directly applicable to identifying genes that show statistically significant changes in expression over time. The *K*-means clustering method has been modified to compare expression over time between two groups (9), but this method can only be applied to a few hundred genes at a time because of the computational cost of fitting a single model to all genes simultaneously (7, 8). This approach also requires that the statistical significance be calculated under the assumption that the clustering model estimated for one of the groups is true, which is nonstandard and potentially problematic.

The method that we propose draws on ideas from the extensive statistical literature on time course data analysis (10, 11), particularly spline-based methods (12–16). It is applicable to detecting changes in expression over time within a single biological group and to detecting differences in the behavior of expression over time between two or more groups. Individuals may be sampled at

multiple time points, or each time point may represent an independently sampled individual. The computational cost is not substantially greater than methods for static experiments, so there is no impeding limit on the number of genes that may be tested. For example, the method is applied here to microarray studies that each measures  $\approx 40,000$  genes.

The proposed method is applied to two recent studies carried out on humans. These studies encompass both types of sampling (longitudinal and independent; discussed below) and both types of differential expression over time (between groups and within a single group). In one study, gene expression was monitored over time in control individuals and endotoxin-treated individuals. Endotoxin [lipopolysaccharide (LPS)] is widely used to study acute inflammatory and immune response; our goal was to understand the mechanism of endotoxin response by identifying genes with expression that is different over time between the treated and untreated groups (17). In a second study, we examined the effect of age on gene expression in the kidney, where samples were obtained from human subjects ranging in age from 27 to 92 years. The goal here was to identify genes whose expression changes significantly with respect to age in human kidney cortex tissue (18). Genes are identified in both studies that corroborate previous findings and provide insights into these problems.

Existing methods for detecting differential expression from static sampling designs (2) are appropriate for comparing unordered categorical conditions, such as three different cancer tumor types (19) or two different treatments (20). It can be shown that these are not generally applicable to time course experiments (see *Supporting Appendix*, which is published as supporting information on the PNAS web site). Typically these existing methods fail to properly use the temporal structure present in the data, either leading to a loss in power or incorrect calculation of significance. The method that we propose is specifically designed for time course experiments; expression over time is modeled flexibly, and statistical significance is calculated while accounting for sources of dependence over time.

## Materials and Methods

**Sample Preparations.** Details on the protocols are described in refs. 17 and 18. For the endotoxin study, four subjects were administered endotoxin and four were administered a placebo. Blood samples were collected before infusion and at 2, 4, 6, 9, and 24 h afterward. The leukocytes were isolated from the blood samples by using a modified lysis protocol (17). Total RNA was extracted by using an RNeasy kit (Qiagen, Valencia, CA). Samples from hours 4 and 6 were unavailable for one of the controls. For the kidney aging study, samples were obtained from normal kidney tissues removed at nephrectomy or renal transplant biopsy from 74 patients ranging in age from 27 to 92 years. The samples were dissected into cortex and medulla sections based on histological evaluation. Each frozen

Abbreviation: LPS, lipopolysaccharide.

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of squares between the curve and the observed expression values, which involves assigning values to only  $\alpha_i$  and  $\beta_i$ . The model fitting procedure for longitudinal sampling is slightly more complicated in that it also takes into account the dependence of measurements within an individual (*Supporting Appendix*). We also developed a method to automatically choose a single value of the basis dimension  $p$  for all genes based on a singular value decomposition of the expression data (*Supporting Appendix*). We found that allowing  $p$  to vary from gene to gene or choosing a single  $p$  in a post hoc fashion lead to anticonservative inflation of significance due to overfitting.

Not only does the basis representation of  $\mu_i(t)$  facilitate model fitting, but it also greatly simplifies the hypothesis tests for differential expression. The tests can now be written in terms of the  $\alpha_i$  and  $\beta_i$  for each gene, which implies that the tests do not depend on specific time points, so general sampling schemes may be analyzed as we have claimed. In the kidney aging study, the null hypothesis of no differential expression is equivalent to restricting  $\mu_i(t)$  to be a constant, and the alternative hypothesis of differential expression allows  $\mu_i(t)$  to be a curve. Thus, the null hypothesis model is fit under the constraint that  $\mu_i(t) = \alpha_i$  and  $\beta_i = 0$ , and the alternative hypothesis model is fit under the general parametrization of  $\mu_i(t)$ . For the endotoxin study, the null hypothesis is that the treated and control groups have equal  $\mu_i(t)$  (i.e., equal  $\alpha_i$  and  $\beta_i$ ), and the alternative hypothesis is that they are not equal. The null hypothesis model is obtained by fitting a curve to the two groups combined, and the alternative hypothesis model is obtained by fitting a separate curve to each group. In this particular study, we were not interested in a difference in the intercepts  $\alpha_i$  because all individuals started out as untreated at time 0. Therefore, the intercept was implicitly assumed to be equal between the two groups under both hypotheses, which comes down to a test for equality of the  $\beta_i$  between the two groups. This equality will not always be the case when testing for between-class temporal differential expression, so we have developed model fitting methods for both scenarios (*Supporting Appendix*).

A statistic for each gene was then formed that quantifies differential expression. The statistic was defined to be an analogue of the  $t$  and  $F$  statistics that are commonly used in static differential expression methods. The statistic compares the goodness of fit of the model under the null hypothesis to that under the alternative hypothesis. First, fitted values from the null and alternative models are calculated for each observed value. The residuals of the model fits are then obtained by subtracting the fitted values from the observed values. Calculating  $SS_i^0$  to be the sum of squares of the residuals obtained from the null model, and  $SS_i^1$  from the alternative model, the statistic for gene  $i$  was constructed as

$$F_i = \frac{SS_i^0 - SS_i^1}{SS_i^1}.$$

This statistic is proportional to the typical  $F$  statistic used in comparing two nested models. The intuition behind the formula is that  $SS_i^0 - SS_i^1$  quantifies the increase in goodness of fit, and dividing this difference by  $SS_i^1$  provides exchangeability of the  $F_i$  across the genes. Justification and exact formulas for all cases can be found in *Supporting Appendix*.

The null distribution of these statistics is calculated through a data resampling method called the bootstrap (24), where residuals from the alternative model are resampled and added back to the null model to simulate the case where there is no differential expression (*Supporting Appendix*). We note that our resampling scheme takes into account dependence between time points when the study involves longitudinal sampling. The observed statistics and null statistics were used to estimate a  $Q$  value for each gene (*Supporting Appendix*), which estimates the false-discovery rate incurred when calling the gene significant (23, 25).

**Table 1. A comparison of the number of probe sets called significant as found by the proposed method, a standard  $t$  test, and a SAM test**

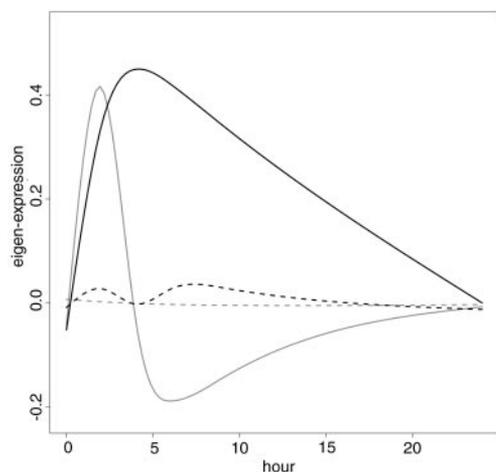
Q value cutoff, %	Proposed method		t test		SAM	
	Eight subjects, 46 arrays	Four subjects, 16 arrays	0 h vs. 2 h	0 h vs. 4 h	0 h vs. 2 h	0 h vs. 4 h
	1	7,409	548	0	0	0
2	9,188	2,683	226	91	65	0
3	10,467	4,392	1,678	837	1,756	695
4	11,642	5,859	2,524	1,826	2,535	1,718
5	12,720	7,229	3,202	2,686	3,101	2,694

Shown is a comparison of the proposed method to a standard  $t$  test and a SAM test, which is a  $t$  test with adaptive asymmetric thresholding. The ratio of the number of probe sets called significant is an estimate of the increase in power that our procedure provides. For the static methods, eight subjects and 16 arrays were used to compare two time points (0 h vs. 2 h and 0 h vs. 4 h). The proposed method was compared to the static methods in two ways. First, all 8 subjects and 46 arrays were used, potentially giving our method an advantage due to a decrease in technical replication variance. Second, only four subjects were used at times 0, 4, 6, and 24 h for a total of 16 arrays, giving the static methods a substantial advantage in that they have twice as many biological replicates. The fairest comparison lies in between these two, although both applications outperform the static methods substantially.

**Analysis of Systemic Inflammatory Response Induced by LPS.** In the endotoxin study there are 4,163, 7,409, and 12,720 significant probe sets at respective  $Q$ -value cut-offs of 0.1%, 1%, and 5%. These results indicate that an endotoxin injection causes a profound gene expression response in blood leukocytes, which is expected for a number of reasons. First, *in vivo* administration of LPS invokes an acute systemic inflammation that dramatically perturbs the body's physiology. Second, some differential expression may be due to changing distributions of cell populations in the blood in addition to transcriptional changes (17). Also, measuring differential expression over time is a more sensitive study design than the typical static design; any change over time qualifies as differential expression. A  $t$  test (23) and the SAM software (20, 23) were used to test for differential expression between 0 and 2 h and between 0 and 4 h. The significance results are displayed in Table 1, where it can be seen that an actual time course analysis offers a sizeable increase in statistical power over a static design analysis.

Under the assumption that the control individuals have flat expression over time, it would have sufficed to simply test the endotoxin individuals for within-class temporal differential expression. However, we found that, among the controls, at least 6% of the probe sets show within-class temporal differential expression. We also tested the endotoxin-treated individuals for within-class temporal differential expression and found the significance to be inflated to unrealistic levels. Therefore, it is necessary to include both treated and control individuals in a test for between-class temporal differential expression to identify genes differentially expressed because of LPS injection.

To get a broad picture of the behavior of differentially expressed genes, a singular value decomposition was performed on the 4,163 most significant probe sets. Fig. 3 shows the top two eigen-genes that explain 66% and 16% of the variance, respectively. The relevant information is the shape of each eigen-gene, not the magnitude or direction of differential expression. Among the 4,163 most significant probe sets, 27% are up-regulated as the eigen-gene is drawn; 73% are down-regulated, which is simply the reflection of the eigen-gene's shape across the time axis. The second eigen-gene shows more complex behavior, where changes in expression occur in both directions over the time course. We identified 2,914 unique genes among the 4,136 probe sets; 756 showed up-regulation during the time course, and 2,158 showed down-regulation. Global func-

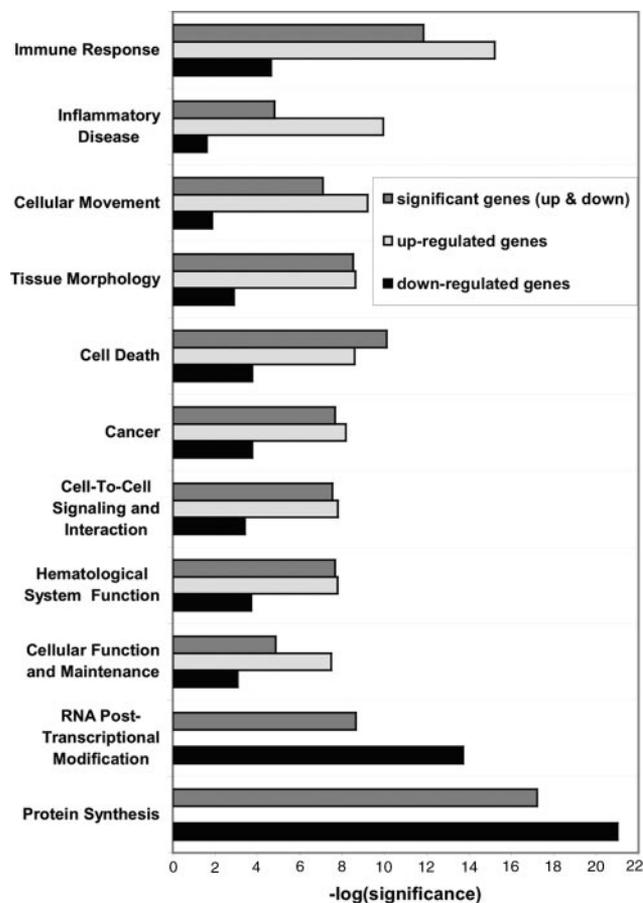


**Fig. 3.** Smoothed version of the top two eigen-genes obtained from probe sets significant at  $Q \leq 0.1\%$  in the endotoxin study. The solid lines are smoothers fit to endotoxin-treated individuals, and the dashed lines are fit to the control individuals. The first eigen-gene explains 66% of the variance and is represented by the black lines. The second eigen-gene explains 16% of the variance and is represented by the gray lines.

tional analysis was performed by using a gene ontology built from experimental evidence compiled in the Ingenuity Pathways Knowledge Base (Fig. 4 and Data Set 1, which is published as supporting information on the PNAS web site).

The most significant functional groups from the 756 up-regulated genes take part in immune response (121 genes), inflammatory disease (41 genes), cellular movement (84 genes), tissue morphology (72 genes), and cell death (144 genes). These groups are consistent with an intense response of leukocytes to LPS. The apparent expression of many elements of inflammation are up-regulated, including secretive cytokines, chemokines, and associated proteins (CCL20, CCL3, CCL4, CXCL16, CXCL2, IL-1A, IL-1B, IL-8, TNF, TNF-SF13B, IL-1RAP, IL-1RN, TNF-AIP3, and TNF-AIP6); their membrane receptors (CCR1, IL-10RB, IL-18R1, IL-18RAP, IL-1R1, IL-1R2, IL-4R, IL-8RA, IL-9R, and TNF-RSF6), Toll-like receptors (TLR4, TLR5, TLR6, and TLR8), Fc receptors (FC-AR, FC-ER1G, FC-GR1A, FC-GR2A, and FC-GR2B), IFN receptors (IFN-GR1, IFN-GR2, and IFN-AR1), protein tyrosine phosphatases (PTP-4A1, PTP-N2, PTP-N22, PTP-NS1, PTP-RC, PTP-RJ, PTP-RN2, and PTP-RO), Janus tyrosine kinases/signal transducers and activators of transcription (JAK2, JAK3, STAT2, STAT3, STAT5A, and STAT5B), and NF $\kappa$ B/I $\kappa$ B proteins (NF $\kappa$ B-2, NF $\kappa$ B-1A, and NF $\kappa$ B-1E). These and other elevated genes participate in the activation of innate immune response and many functions of leukocytes, including cell movement of leukocytes, infiltration, migration, phagocytosis, activation, chemotaxis, proliferation, and recruitment. The most significantly up-regulated gene is ORM1, a key acute phase plasma protein.

The most significant functional groups from the genes showing down-regulation are protein synthesis (83 genes), including genes of elongation initiation factors, ribosomal proteins, mitochondrial ribosomal protein; and RNA posttranscriptional modification (60 genes), including genes such as heterogeneous nuclear ribonucleoproteins, small nuclear ribonucleoproteins, and splicing factors. Members of RNA polymerase II are also decreased. Correspondingly, genes involved in oxidative phosphorylation, such as mitochondrial complexes I (NDUF), II (SDH), III (UQCR), and IV (COX) are down-regulated. This concerted suppression of the cell's protein syntheses, transcription programs, and energy productions may reflect a generalized stress response in blood leukocytes. Interestingly, expression levels of members associated with the



**Fig. 4.** Functional analysis of genes found to be significant at  $Q \leq 0.1\%$  in the endotoxin study. Shown are representative functions differentially enriched [difference in  $\log(\text{significance}) > 4$ ] between the groups of up-regulated (light gray) and down-regulated (black) genes. The significance values of these functions in the combined group are shown in dark gray.

MHC class II are also suppressed, consistent with the reduced antigen-presenting capability after LPS shock.

Taking these results together, we observed a comprehensive transcriptional response to LPS stimulation, where leukocyte cells reallocate resources and up-regulate transcription of genes involved in innate immune and defense mechanisms.

**Analysis of Age-Related Genes in the Kidney Cortex.** We detected substantial differential expression in this study, although not nearly as much as in the previous study. At  $Q$ -value cutoffs of 5% and 10%, there are 187 and 436 significant probe sets, respectively. Among the 436 probe sets significant at  $Q \leq 10\%$ , 320 unique genes were identified, with 240 show increasing expression with age and 80 show decreasing expression.

A functional analysis was also performed on these age-regulated genes (Data Set 2, which is published as supporting information on the PNAS web site). The most significant functional group is immune response (69 genes), which includes genes associated with the complement component (C1QA, C1QB, C1QG, C1QR1, C1R, C1S, and C4A), cytokines, chemokines, and receptors (CCL2, CCL20, CCR1, CX3CR1, CXCL9, CXCR4, IL10RB, CSF1R, and CSF2RB), and the MHC II complex (FCER1G, HLA-DMA, HLA-DPA1, HLA-DQB1, HLA-DRB1, and HLA-DRB3). All but three of these genes show up-regulated expression with age, which suggests an increased abundance of immune response genes in the kidney cortex, perhaps as a result of a low level of inflammation over life or as a compensation for the decreased immune function

in older age. Renal infiltration with immunocompetent cells, which is known to correlate with renal diseases and increase over age, can also account for some of these observed changes.

The aging process has previously been shown to involve a widespread and complicated alteration in gene expression (18). In our analysis, significant genes were identified that have a variety of cell-to-cell signaling and interaction (68 genes). For example, 34 genes are involved in the quantity or mobilization of calcium and 14 genes in tyrosine phosphorylation. Fifty-nine genes were identified to be involved in apoptosis and cell death. The expression levels of apoptosis-enhancer proteins MYC (C-MYC), SP1 (Sp1 transcription factor), caspase 1, and ubiquitin D increase with age in the kidney, whereas the antiapoptosis protein HSPA9B (mortalin-2) decreases with age. However, the expression of BCL2A1, an inhibitor of the intrinsic apoptosis pathway, is observed to be increasing with age (26).

Gene expression may change with age in response to declining kidney function and increased susceptibility to kidney disease. Interestingly, a group of 12 genes known to be localized in mitochondria (HSPA9B, COX8A, COX7C, AKAP1, BCKDHA, CLPP, FDX1, AMT, DBT, ATP5G3, AK2, and NME4) are identified as significant, and their expression level uniformly declines in older age. A number of age-regulated genes are also known to be involved in renal diseases, including C1QA, CCR1, LYN, PTPRC, and TNFSF13B, which all show increasing expression with age (27–31). The expression of TRPM6 (a transient receptor potential cation channel) negatively correlates with age, and mutations of this gene cause hypomagnesemia with secondary hypocalcemia (32).

We used a 4-dimensional basis in the significance analysis, although other choices of  $p$  are possible. A 1-dimensional basis is equivalent to identifying differential expression by fitting a linear regression and testing whether the slope is zero. Applied to log-transformed expression data, a linear regression is only able to identify genes that show simple exponential behavior in their differential expression. By testing the null hypothesis of linear or flat log-transformed expression, we found that at least 25% of the 35,068 probe sets are differentially expressed in a manner more complex than an exponential function of age. Among the 417 most significant probe sets when using the 1-dimensional basis, it is estimated that at least 47% of these are more complex than simple exponential behavior. At  $Q \leq 5\%$ , just over half of the genes found to be significant using the 4-dimensional basis are also significant when using the 1-dimensional basis. Therefore, there is potentially a functional class of genes showing simple exponential temporal differential expression and another functional class showing substantially more complex differential expression.

## Discussion

We have proposed a significance method to identify differentially expressed genes in time course microarray experiments and applied it to studies involving two types of sampling and both types of temporal differential expression. The method may also be applied to more complicated situations, where three or more groups are compared, for example. We have justified the method in terms of well established statistical concepts (*Supporting Appendix*).

Temporally differentially expressed genes were identified in two time course microarray studies on humans. The human *in vivo* model of bacterial endotoxin represents a unique opportunity to examine the onset of systemic inflammation in blood leukocytes. Our analysis suggests that a genome-wide transcriptional response takes place where blood leukocytes reallocate resources and up-regulate transcription of genes involved in innate immune and defense mechanisms. The inclusion of controls in this study played an important role, in which the controls themselves showed differential expression over time. In the clinical setting, comparing a treatment group to time 0 does not provide a proper control over the entire time course. The significance analysis of the kidney aging study indicates that a large proportion of genes have expression that increases with age, including those involved in signal transduction, cell growth, and genome stability. Genes related to metabolism were found to be decreasing with age. It was also shown that a large proportion of gene expression changing with age does so in a manner more complex than exponential growth. Therefore, in general it is important to use flexible models when identifying genes that are temporally differentially expressed.

We have developed a freely available point-and-click software package called EDGE that includes this methodology and new methodology for static experiments. EDGE is available from J.D.S. upon request.

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