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Comparison of Commonly Used Methods for Testing Interaction Effect in Time-Course Microarray Experiments

Zaman-Üzeri Mikro-Array Denemelerinde Sıkça Kullanılan Yöntemlerin Karşılaştırılması

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ABSTRACT Objective: The objective of this study is to compare performances of maSigPro, BATS, Repeated Measures ANOVA (RM-ANOVA), and Classical RM-ANOVA, which are commonly used methods, for testing interaction effect in time-course microarray experiments. **Material and Methods:** We generated random time-course gene expression profiles from multivariate normal distribution based on two different covariance structures, three different time-series lengths, and two different profile types. **Results:** The results suggested that the BATS and the maSigPro are generally more superior methods to the ANOVA-based methods in terms of sensitivity and specificity measures. In general, the BATS was more appropriate for medium and especially for long term data sets while maSigPro was appropriate almost for all experimental conditions. On the other hand, the ANOVA-based methods had a good performance especially for the short-term time-course profiles. **Conclusion:** Thus, we concluded that length of time-course measurement and profile types are two major factors affecting the performances of these methods.

Keywords: Gene expression profiles; microarray, time-course data; sensivity specifity; total accurary

ÖZET Amaç: Bu çalışmanın amacı, zaman-üzeri mikro-array denemelerinde sıklıkla kullanılan, maSigPro, BATS, tekrarlanan ölçümler Varyans Analizi, ve Klasik Varyans Analizi yöntemlerinin performanslarının karşılaştırılmasıdır. **Gereç ve Yöntemler:** Bunun için, çok-değişkenli normal dağılımdan, iki değişik covaryans yapısı, üç zaman-serisi uzunluğu ve iki değişik profil tipini temel alarak, rasgele zaman-serisi gen eksprasyonu profilleri ürettik. **Bulgular:** Sonuçlar, BATS ve maSigPro yöntemlerinin, duyarlılık ve özgüllük açısından ANOVA-temelli yöntemlerden daha güçlü olduğunu gösterdi. Genel olarak, BATS yönteminin, orta ve uzun ölçekli zaman-serileri için, maSigPro'nun da, tüm deney şartları için daha uygun oldugu gözlendi. Öbür taraftan, ANOVA temelli yöntemlerin, kısa zaman-serili profiller için iyi performans gösterdiği gözlendi. **Sonuc:** Böylece, zaman-serisi uzunluğunun ve profil tipinin, karşılaştırılan bu yöntemlerin performansında etkili olan iki faktör olduğu sonucuna ulaştık.

Anahtar Kelimeler: Gen ekspresyon profili; mikro-dizin; saman serisi verisi; duyarlılık ve özgüllük; toplam doğruluk

In the last decade, researchers are commonly interested in studying gene expression level changes through time and in evaluating trend differences between the various experimental groups and conditions.[1-4] The main purpose of time-course microarray experiments is to describe the changes in expression levels of genes over time and compare the pattern of that change across genes, tissue types, or experimental conditions.[1,4-12]

These analyses potentially lead to discoveries of genes that may be associated with the likelihood of certain phenotypes and diseases. With the microarray technology, it is possible to measure expression level of thousands of genes simultaneously in time-course microarray experiments, which creates various challenges for statistical analysis of the resulting data. The resulted 'big' data, number of time points as well as the number of replicates, multiplicity of experimental conditions, and dynamic nature of the experiment are some of these challenges.[1,2,6,13-16] Therefore, choosing appropriate statistical methods becomes very critical for statistical analysis stage of such gene expression data sets. Several methods, and their corresponding computational tools, have been proposed in the literature to identify differently expressed gene profiles in time-course microarray experiments. Some of these methods are regression-based (e.g. microarray Significant Profiles (maSigPro), Extraction of Differential Gene Expression (EDGE), maSigProFun and PCA-maSigProFun, some of them are Analysis of Variance (ANOVA)-based (e.g. two-way mixed effects ANOVA, a functional mixed-effect ANOVA, Permutation-based two-way ANOVA, RM-ANOVA) and some of them are Bayesian-based (e.g., Bayesian Analysis of Time Series (BATS), Multivariate Empirical Bayes (MB-statistic)).[1,2,5,18-25] Of these methods, maSigPro, EDGE, BATS, Significance Analysis of Microarrays (SAM), ANOVA-Simultaneous Component Analysis (ASCA), and ANOVA-based methods are the most commonly used ones. The literature suggests that each of these methods has been developed for certain experimental conditions, which limits the utility and generalizability of these methods for other experimental conditions as researchers in different disciplines may consider different factors (e.g. group, time) and experimental conditions according to their study objectives. All these factors may affect the statistical methods that will use for analysis of data sets. Therefore, it is needed to investigate the performances of these tests under different experimental conditions that allow the researchers to choose the most appropriate statistical method for their time-course microarray data analysis.

In this study, we compared and contrasted the performances of the maSigPro, the BATS, the Repeated Measurement ANOVA (RM-ANOVA), and the Classical RM-ANOVA with Monte Carlo Simulation Methods under different experimental conditions, in terms of sensitivity and specificity measures (Table 1).

MATERIAL AND METHODS

MATERIAL

Simulation Study

For this study, we generated random numbers from multivariate normal distribution based on two most commonly used covariance structures, i.e. unstructured and autoregressive (Table 2) and two different gene profiles (Figure 1). We assumed that there were two conditions or groups, named 'control' and 'treated'. The profile of each gene had three replications under three different experimental time lengths

TABLE 1: Possible outcomes from "m" hypothesis tests of a set of genes (DE= Differentially Expressed).

Truth	Result of Test		Total
	# accept H_0 (Non-DE)	# reject H_0 (DE)	
H_0: Expression profiles are not different	A(TN)	B (FP)	m_0
H_1: Expression profiles are different	C(FN)	D (TP)	m_1
Total	A+C	B+D	m

TABLE 2. Covariance structures considered for the simulation study.

	Unstructured	Autoregressive
Short term (4 time points)	$\begin{bmatrix} 1.00 & 0.80 & 0.70 & 0.75 \\ 0.80 & 1.00 & 0.35 & 0.50 \\ 0.70 & 0.35 & 1.00 & 0.45 \\ 0.75 & 0.50 & 0.45 & 1.00 \end{bmatrix}$	$\begin{bmatrix} 1.00 & 0.80 & 0.64 & 0.51 \\ 0.80 & 1.00 & 0.80 & 0.64 \\ 0.64 & 0.80 & 1.00 & 0.80 \\ 0.51 & 0.64 & 0.80 & 1.00 \end{bmatrix}$
Medium term (6 time points)	$\begin{bmatrix} 1.00 & 0.85 & 0.60 & 0.72 & 0.65 & 0.55 \\ 0.85 & 1.00 & 0.75 & 0.55 & 0.45 & 0.35 \\ 0.60 & 0.75 & 1.00 & 0.60 & 0.50 & 0.30 \\ 0.72 & 0.55 & 0.60 & 1.00 & 0.70 & 0.45 \\ 0.65 & 0.45 & 0.50 & 0.70 & 1.00 & 0.55 \\ 0.55 & 0.35 & 0.30 & 0.45 & 0.55 & 1.00 \end{bmatrix}$	$\begin{bmatrix} 1.00 & 0.85 & 0.72 & 0.61 & 0.52 & 0.44 \\ 0.85 & 1.00 & 0.85 & 0.72 & 0.61 & 0.52 \\ 0.72 & 0.85 & 1.00 & 0.85 & 0.72 & 0.61 \\ 0.61 & 0.72 & 0.85 & 1.00 & 0.85 & 0.72 \\ 0.52 & 0.61 & 0.72 & 0.85 & 1.00 & 0.85 \\ 0.44 & 0.52 & 0.61 & 0.72 & 0.85 & 1.00 \end{bmatrix}$
Long term (8 time points)	$\begin{bmatrix} 1.00 & 0.80 & 0.65 & 0.70 & 0.65 & 0.40 & 0.50 & 0.45 \\ 0.80 & 1.00 & 0.75 & 0.68 & 0.53 & 0.55 & 0.35 & 0.30 \\ 0.65 & 0.75 & 1.00 & 0.70 & 0.60 & 0.60 & 0.44 & 0.40 \\ 0.70 & 0.68 & 0.70 & 1.00 & 0.78 & 0.65 & 0.45 & 0.35 \\ 0.65 & 0.53 & 0.60 & 0.78 & 1.00 & 0.56 & 0.35 & 0.28 \\ 0.40 & 0.55 & 0.60 & 0.65 & 0.56 & 1.00 & 0.75 & 0.43 \\ 0.50 & 0.35 & 0.44 & 0.45 & 0.35 & 0.75 & 1.00 & 0.72 \\ 0.45 & 0.30 & 0.40 & 0.35 & 0.28 & 0.43 & 0.72 & 1.00 \end{bmatrix}$	$\begin{bmatrix} 1.00 & 0.80 & 0.64 & 0.51 & 0.41 & 0.33 & 0.26 & 0.21 \\ 0.80 & 1.00 & 0.80 & 0.64 & 0.51 & 0.41 & 0.33 & 0.26 \\ 0.64 & 0.80 & 1.00 & 0.80 & 0.64 & 0.51 & 0.41 & 0.33 \\ 0.51 & 0.64 & 0.80 & 1.00 & 0.80 & 0.64 & 0.51 & 0.41 \\ 0.41 & 0.51 & 0.64 & 0.80 & 1.00 & 0.80 & 0.64 & 0.51 \\ 0.33 & 0.41 & 0.51 & 0.64 & 0.80 & 1.00 & 0.80 & 0.64 \\ 0.26 & 0.33 & 0.41 & 0.51 & 0.64 & 0.80 & 1.00 & 0.80 \\ 0.21 & 0.26 & 0.33 & 0.41 & 0.51 & 0.64 & 0.80 & 1.00 \end{bmatrix}$

namely short-term (four time points: t1, t2, t3, t4), medium-term (six time points: t1, t2, t3, t4, t5, t6), and long-term (eight time points: t1, t2, t3, t4, t5, t6, t7, t8). Profile 1 represents interaction profile where gene expression does not have a linear relationship with time and the difference between treatment and control decreases as time increases. Profile 2 represents interaction profile where gene expression has linear relationship with time and as time increases, the difference between treatment and control increases. To assess the likelihood of differential expression, we were only interested in Group x Time interaction. Under each simulation scenario and for each replication, 1000 random gene profiles were generated. We randomly selected 25% of those 1000 gene profiles and we shifted the profile to create differently expressed gene profiles, the 'treatment' group, leaving the remaining 75% as control cases. Then, we applied maSigPro, BATS, RM-ANOVA, and Classical Repeated Measurement ANOVA methods to our simulated samples, and the total number of significant genes, number of truly significant genes, and numbers of falsely significant genes were determined. Sensitivity, specificity and total accuracy measures were obtained for each method. We repeated the simulation 100 times in order to estimate the standard error of the measures of comparison.

In Table 1, A is the number of Non-differentially expressed (non-DE) gene profiles that were correctly classified, B is the number of non-DE gene profiles that were incorrectly classified, C is the number of Differentially Expressed (DE) gene profiles that were incorrectly classified, and D is the number of DE gene profiles that were correctly classified.

Performance measures were computed as follows, where the components of each equation are provided in Table 1:

$$\text{False Positive Rate} = \text{FPR} = \frac{B}{m_0}$$

$$\text{False Negative Rate} = \text{FNR} = \frac{C}{m_1}$$

$$\text{Sensitivity} = \frac{D}{m_1} = 1 - \text{FNR}$$

$$\text{Specificity} = \frac{A}{m_0} = 1 - \text{FPR}$$

$$\text{Total Accuracy} = \frac{(A + D)}{m}$$

METHODS CONSIDERED FOR COMPARISON

Various methods, approaches and tools have been developed to analyze time-course microarray data sets. In this study, we considered four of them which freely available and user-friendly namely maSigPro, BATS, RM-ANOVA, and Classical Repeated Measurement ANOVA.[1,2,13,26]

maSigPro (MicoArraySIGnificantPROfiles)

maSigPro is developed[1] for the analysis of single and multiple time-course microarray data sets.[1] The method uses two-step regression strategy. In the first step, differentially expressed genes are selected. The first step of the maSigPro approach applies the least-squares technique to estimate the parameters of the described general regression model for each gene. In the second step, variable selection procedure (stepwise regression) is applied and focused on genes selected in the first step. Once statistically significant gene models have been determined, the regression coefficients of the models can be used to identify the conditions for which genes show statistically significant profile changes. Therefore, the new model is obtained through stepwise regression. Stepwise regression is an iterative regression approach that selects from a pool of potential variables, the 'best' ones (according to a specified criterion) to obtain a better fit to the available data.[1,17]

BATS (Bayesian Analysis of Time Series)

BATS software (http://www.na.iac.cnr.it/bats) is free and user-friendly for the analysis of both simulated and real microarray time course experiments. This software allows researchers to automatically identify and rank differentially expressed genes. It also enables one to describe expression profiles of differentially expressed genes. This software has been reported to give satisfactory results especially when number of time point is more than 5.[13]

Repeated Measurement ANOVA for Time-course Microarray Experiments (RM-ANOVA)

RM-ANOVA, unlike the classical F-statistic, determines statistical significance by taking into account the time dependency of the microarray data.[2] The algorithms presented are implemented in R and are freely available upon request.

Classical Repeated Measurement ANOVA or Within-Subject

Repeated Measures ANOVA is a technique that used to test the equality of means when the measurement of the dependent variable is repeated over time or different experimental conditions.[26]

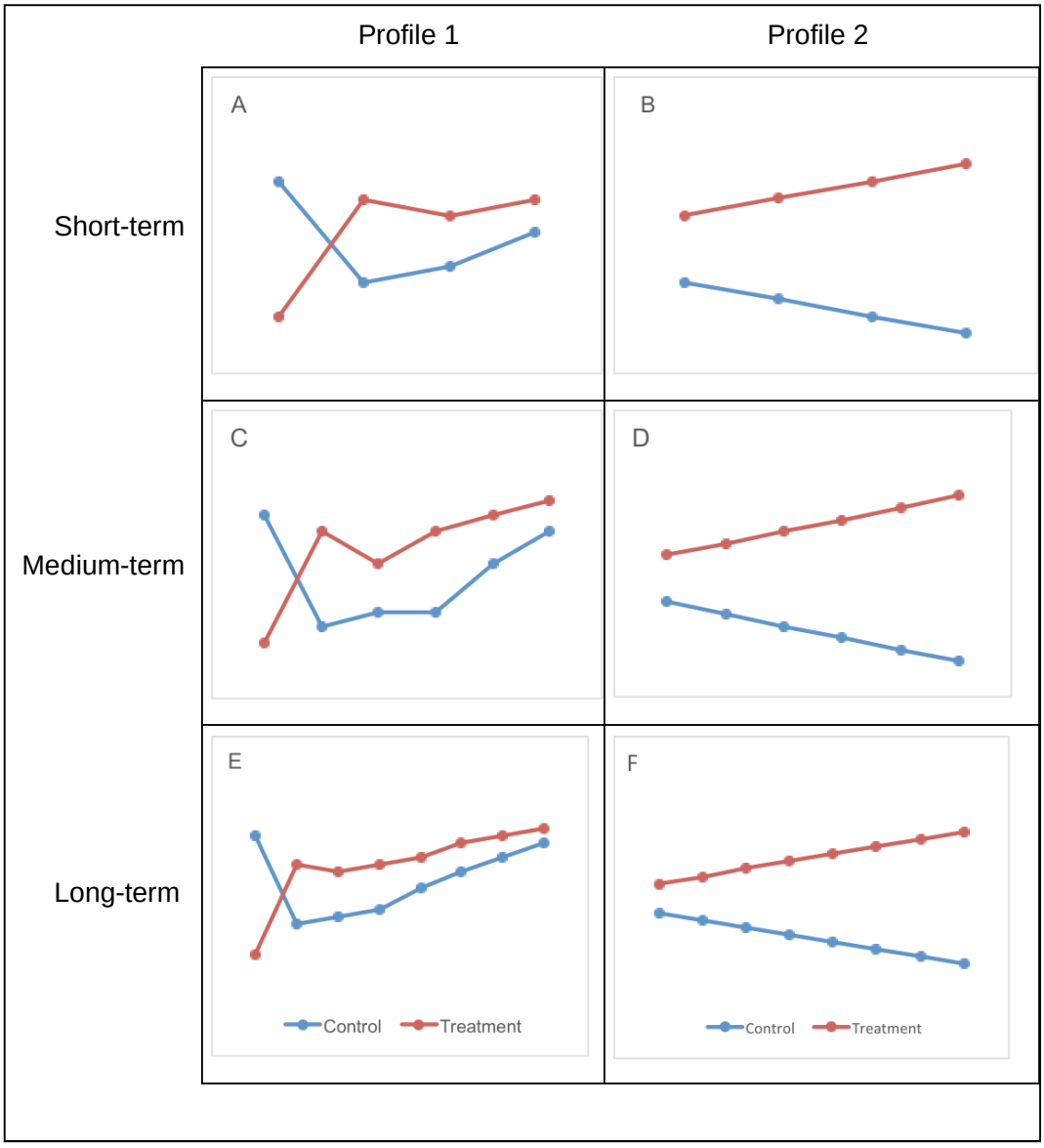

FIGURE 1: Simulation profiles considered for the simulation study. There are two profiles (1 and 2) and three different experiment period lengths (short: 4 time points, medium: 6 time points, and long: 8 time points).

RESULTS

We summarized the simulation results as changes in the sensitivity, specificity and total accuracy values of the methods under different covariance structures, period lengths, and profile types are given in Table 2 and Figure 1.

As seen in Figure 2, there were obvious differences among the four methods in terms of sensitivity (true positives-vertical axis) and 1-specificity (false positives-horizontal axis) combinations depending on the period length and profile type. When the period length was short (4 time points), classical-RM and RM-ANOVA had higher sensitivity or better ability to identify true differentially expressed genes compared to BATS and maSigPro, although this was compromised by lower specificity (i.e. the tests falsely identified more genes that weren't differentially expressed (Figure 2A). Within the short-term

period length, interaction profile 2 appeared to give lower sensitivity only in classical-RM. When the period length was medium (6 time points), all methods had comparable sensitivity although maSigPro gave the lowest sensitivity under the profile 1 (less than 0.68) (Figure 2B). BATS gave highest specificity while RM-ANOVA yielded the lowest specificity regardless which profile and covariance structure were used (Figure 2B). When the period length was long (8 time points), BATS performed the best in terms of sensitivity and specificity regardless of the covariance structures and profile types. Sensitivity of Classical-RM and RM-ANOVA improved compare to the medium period length while the sensitivity of maSigPro reduced (Figure 2C). In general, the type of covariance structures did not affect greatly the sensitivity or specificity of any statistical methods. Profile 2, in general, yielded higher sensitivity within each method. Overall, classical-RM and BATS were more sensitive to the profile length and type. The sensitivity of classical-ANOVA, RM-ANOVA and BATS increased as the period length increased and remained unchanged across different covariance structures and profile types. BATS reached the highest sensitivity with long period length (i.e., 8 time points) regardless the profile types. RM-ANOVA appeared to have good sensitivity under medium (6 time points) and long period length. The sensitivity of maSigPro appeared to be optimal under the medium period length condition. In summary, under the long period length, RM-ANOVA and BATS had reached the highest sensitivity while under short (4 time points) and medium period lengths, maSigPro and RM-ANOVA had better sensitivity compare to others.

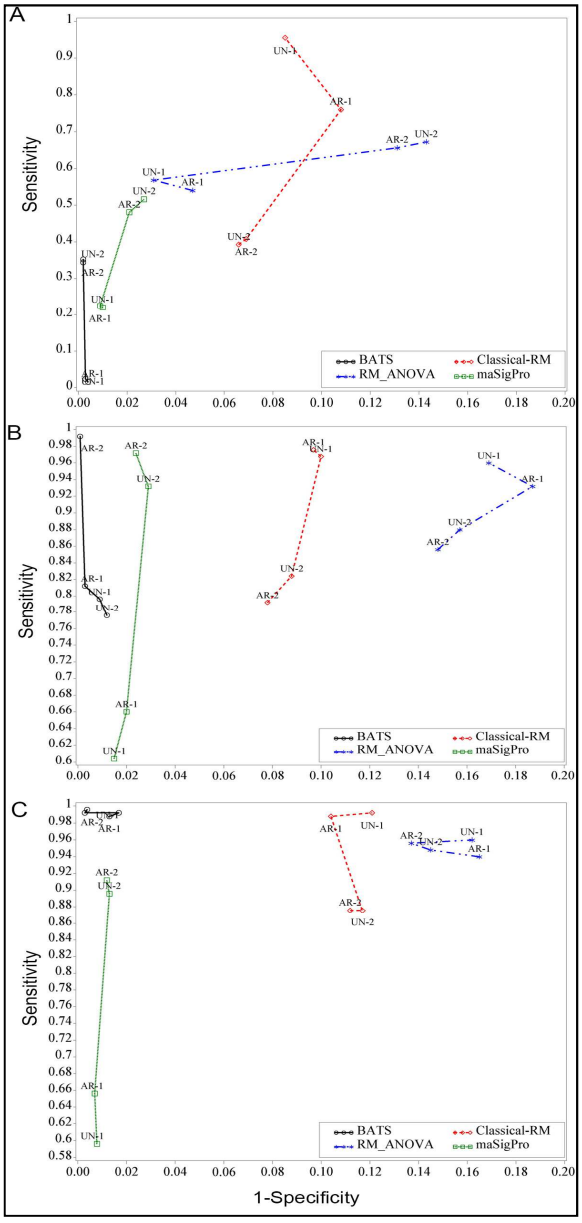

FIGURE 2: Sensitivity and specificity of BATS, Classical-RM, RM-ANOVA, and maSigPro under different covariance matrix profiles (AR: autoregressive, UN: unstructured), interaction profile (1 and 2) and time lengths (A) short-term period, (B) medium-term period and (C) long-term period (see Figure 1 and Table 2 for details).

In terms of specificity, these methods appeared not to be affected by the period length and the profile type. We classified these methods into two groups: ANOVA-based methods (RM-ANOVA and the Classical-RM) and the other methods (the BATS and the maSigPro). The specificity of the BATS and the maSigPro were consistently higher than those of the RM-ANOVA and the Classical-RM. Therefore, we concluded that the BATS and the maSigPro can be considered more specific than the ANOVA-based methods under these experimental conditions.

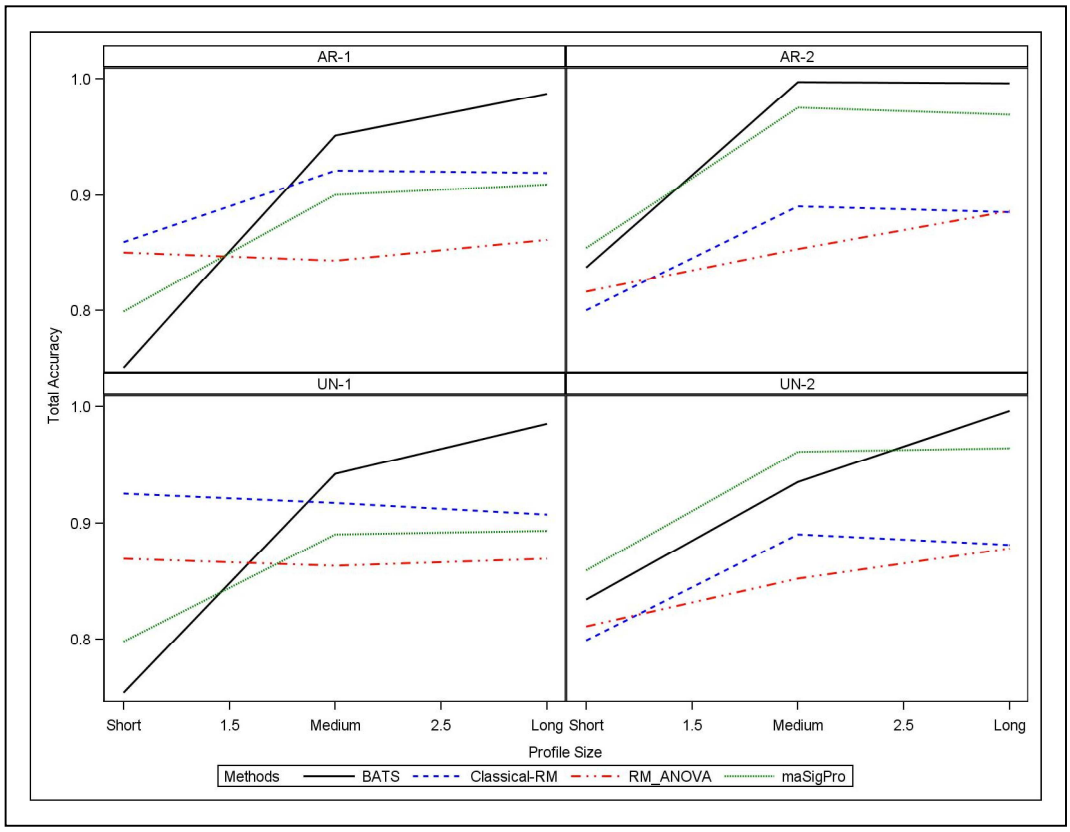

FIGURE 3: Total accuracy of different statistical methods under different time lengths and covariance matrix profiles. AR: autoregressive, UN: unstructured (see Figure 1 and Table 2 for details).

The performance of these methods would not be fully evaluated if we just considered the sensitivity and specificity. Thus, total accuracy, a joint summary of sensitivity and specificity, was also examined. As seen in Figure 3, the total accuracy values of all the methods except for maSigPro were affected by the time-course length. For the short time course experiment, BATS had the lowest performance in terms of total accuracy, as it would need more data for the data to be able to have higher weights on the posterior distribution compared to the weight of the prior distribution. However, as expected, the performance of the BATS got improved as the period length extended. Although BATS was a bit superior to the maSigPro for longer term profiles, maSigPro was more robust for the profile length and thus had good performance in general. The ANOVA-based methods had good performance under short-term, especially for RM-ANOVA, but did not retain their good performance under the medium and long-term cases. As a result, we concluded that maSigPro had good performance in general. BATS was more suitable method for analyzing medium and especially long-term data sets while ANOVA-based methods were more suitable for analyzing short-term time-course microarray data sets. Comparisons of the methods regarding the total accuracy measure based on period length and profile type (Figure 3) suggested that the period length (short, medium, and long) and the profile types (AR-1, AR-2, UN-1, UN-2) are both factors that affect the performances of the four methods. We also conclude that none of the methods is uniformly the best, BATS was the best method in terms of total accuracy regardless of type of profile when the period length was long. This was true also in

the case of the medium period length design except when the profile type was UN-2, maSigPro was better than BATS. For short profiles, none of the methods was best under any of these four profiles.

DISCUSSION

Microarray technology enables us to measure expression levels for thousands of genes simultaneously, but it is a significant statistical challenge to identify differentially expressed genes that could be the potential underlying mechanism in differing phenotypes and diseases. Therefore, using more appropriate statistical methods for analyzing time-course microarray data sets is critical to obtain reliable results. In this project, we compared the performances of 4 commonly used statistical methods under different experimental conditions through extensive simulation. We used sensitivity, specificity, and total accuracy measures to describe and compare diagnostic tests.

Our results suggested that BATS and maSigPro are generally more superior methods to the ANOVA-based methods in analyzing time-course microarray data sets. Although the BATS did not perform well for analyzing the short-term time-course microarray data sets as it required more data for the data to overweight the prior distribution specifications in obtaining the posterior distribution, it worked well for medium- and especially for long-term data sets. BATS is a free user-friendly software for the analysis of both simulated and real microarray time course experiments. It was also reported to be particularly suitable for time course experiments where at least 5–6 time points were available, which corresponds to our 'medium-term' profiles simulated. BATS is also robust against various technical difficulties that arise in time-course microarray experiments, missing data, non-uniform sampling intervals and replication status.[13] maSigPro is a powerful method for the analysis of time course microarray data. The method is very efficient tool in filtering out non-significant genes, fitting a model to the experimental conditions, and allowing the researchers to visualization of significant profiles.[1]Conesa et al. also reported that since the maSigPro is two-stage regression based methods, in experiments expanding a larger number of time points, more complex expression patterns could be expected and that may result in failures in identifying some of differently expressed genes. Although our findings are generally supportive of the findings reported by previous studies, there are also some differences between our findings and theirs.[1,5,13] We think that differences in the experimental conditions as well as in the definition of period length possibly resulted in these differences. For example in the literature, it is seen that generally two period lengths have been defined as a short-term (between 3 and 6 time points) and long-term (more than 6 time points).[1] On the other hand, we defined three different period lengths namely short (3-4 time points), medium (5-7 time points), and long-term (≥8 time points) to obtain more detail information.

The performances of the ANOVA-based methods are generally inferior to those of the BATS and the maSigPro. In general, the ANOVA-based methods have a good performance for short-term data sets. However, when the period length extends, the performances of these tests get worse although their performance should get better as the increasing time-course length is expected to impact the power positively under the normal circumstances. Based on these results it is possible to conclude that for the short-term time-course microarray data sets, the ANOVA-based methods may be preferred to the BATS. However, the ANOVA model is one of the most criticized methodologies, used in many papers as a comparative method to introduce a novel technique. It is argued that he ANOVA model does not take the temporal ordering into account. Especially during the last decade, since the majority of authors have generally concentrated on the regression-based and Bayesian-based methods, the studies about ANO-

VA-based methods are limited. ANOVA-based methods can still be very useful when very short time course experiments have to be analysed (up to about 4–5 time points), however the shortcoming of these approaches is that they ignore the biological temporal structure of the data producing results that are invariant under permutation of the time points.[13,21] In addition, it is suggested that ANOVA should be applied on background-normalized data.[27] Another functional ANOVA mixed-effect model was developed and reported to have higher power than those of EDGE and classical two-way ANOVA.[23] A general statistical method based on repeated measures (RM) ANOVA was proposed for detecting changes in microarray expression over time within a single biological group. This method outperformed EDGE, SAM, and Oriogen methods under different experimental conditions.[2]

Results of this project also suggested that the performances of the methods are affected by different factors where the two major factors are the period length and profile types. On the other hand, even though the period length and the profile type are two important factors that affect the performances of the methods, they may not be enough to evaluate the performances of these methods in full detail. Since gene expression levels in a given cell can be influenced by different factors, it may be necessary to consider some other new factors especially population prevalence values, changes in number of replication, effect of missing value, effect of outliers as well. That is, multi-factors time course experiments are needed. The multiple series time-course microarray experiments are useful approaches for exploring biological processes. However, the large amount of data, multiplicity of experimental conditions and the dynamic nature of the experiments pose great challenges to data analysis.[1,6,28] Therefore, in the light of the results of this study, we think that new and more comprehensive studies are needed to:

a) Investigate the effect of the factors that affect the performances of the methods more detail,

b) Determine optimum factor levels (e.g. determining optimum period length, profile type, etc.),

c) Compare the performances of these methods under different population prevalence values

d) Investigate the effects of missing values,

e) Investigate the effects of outliers, and

e) Investigate the possibility of develop a new novel method.

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Conflict of Interest

Authors declared no conflict of interest or financial support.

Authorship Contributions

Idea/Concept: Constructing the hypothesis or idea of research and/or article: *Quynh Tran, Mehmet Mendes;* ***Design: Planning methodology to reach the conclusions:*** *Quynh Tran, Mehmet Mendes, Mehmet Koçak;* ***Control/Supervision: Organizing, supervising the course of progress and taking the responsibility of the research/study:*** *Mehmet Kocak, Mehmet Mendes;* ***Data Collection and/or Processing Taking responsibility in patient follow-up, collection of relevant biological materials, data management and reporting, execution of the experiments:*** *Quynh Tran, Mehmet Mendes;* ***Analysis and/or Interpretation: Taking responsibility in logical interpretation and conclusion of the results:*** *Quynh Tran, Mehmet Mendes, Mehmet Koçak;* ***Literature Review: Taking responsibility in necessary literature review for the study:*** *Mehmet Mendes, Quynh Tran;* ***Writing the Article: Taking responsibil-***

ity in the writing of the whole or important parts of the study: *Mehmet Mendes, Mehmet Koçak;* ***Critical Review: Reviewing the article before submission; Scientifically besides spelling and grammar:*** *Mehmet Koçak, Mehmet Mendes;* ***References and Fundings: Providing personnel, environment, financial support tools that are vital:*** *Mehmet Mendes, Mehmet Koçak.*

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