A statistical framework for the design of microarray experiments and effective detection of differential gene expression

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Microarray experiments generate a high data volume. However, often due to financial or experimental considerations, e.g. lack of sample, there is little or no replication of the experiments or hybridizations. These factors combined with the intrinsic variability associated with the measurement of gene expression can result in an unsatisfactory detection rate of differential gene expression (DGE). Our motivation was to provide an easy to use measure of the success rate of DGE detection that could find routine use in the design of microarray experiments or in post-experiment assessment.

In this study, we address the problem of both random errors and systematic biases in microarray experimentation. We propose a mathematical model for the measured data in microarray experiments and on the basis of this model present a t-based statistical procedure to determine DGE. We have derived a formula to determine the success rate of DGE detection that takes into account the number of microarrays, the number of genes, the magnitude of DGE, and the variance from biological and technical sources. The formula and look-up tables based on the formula, can be used to assist in the design of microarray experiments. We also propose an ad hoc method for estimating the fraction of non-differentially expressed genes within a set of genes being tested. This will help to increase the power of DGE detection.

The functions to calculate the success rate of DGE detection have been implemented as a Java application, which is accessible at

http://www.le.ac.uk/mrctox/microarray_lab/Microarray_Softwares/Microarray_Softwares.htm. Supplementary information at ftp://alcyone.mrc.le.ac.uk/ Pub/twg1/BioInf03-0661suppl.pdf

I. INTRODUCTION

Whole genome sequencing and the related development of microarrays have given researchers unprecedented power to simultaneously determine the expressions of many thousands of genes [1]. However, a statistical challenge facing microarray analysis is to identify differential gene expression (DGE) with a high rate of success and low rate of false positives. Such a method is required because of the number of gene expressions being simultaneously determined, and the variation associated with each can give rise to an unacceptably large number of false positives or low successful detection rate. The variations associated with gene expression experiments can be categorized into two sets. First, there are interindividual differences between members of a population, thus sufficient biological individuals should be included in the experiments in order to account for the biological variation. Second, there are always technical errors arising from the experimental procedure, which may be further sub-categorized into random errors and systematic biases. Unlike random errors, which can be reduced by making multiple measurements, systematic biases cannot be reduced by simply doing more measurements, correct experimental designs must be employed to negate them.

One of the most serious sources of systematic bias in microarray experiments (for dual label hybridizations) is the imbalance in the measured fluorescence intensities between the two fluorescent channels [2, 3, 4]. A manifestation of this systematic bias is that when two identical mRNA samples are labelled with different fluorescent dyes and hybridized to the same microarray slide, one channel has a higher average fluorescence level than the other. To complicate matters further the imbalance of the two channels is not uniform, but varies from feature to feature. A feature is the area of fluorescence on a microarray corresponding to one gene and where hybridization of the labelled nucleic acids derived from this gene has taken place [5]. To correct the labelling dye imbalance, different methods of normalizing the microarray data by adjusting the measured fluorescence levels have been proposed [4, 6, 7]. These methods can be roughly classified into two categories. First, global normalization, in which the fluorescence levels of all the features are globally (uniformly) adjusted (by shifting or re-scaling) to fulfill some assumptions about the relative expressions of the genes, e.g. most genes are not differentially expressed between the two samples [6]. However, because global normalization adjusts the fluorescence levels of all features uniformly, it cannot account for the different magnitudes of imbalances from feature to feature, so a second type of normalization method is often employed to take account of this variation. This normalization method adjusts the fluorescence level according to some local properties of the feature spot, e.g. the overall brightness of the spot [4], and usually involves fitting the measured data with a non-linear smoothed curve. The fluorescence level is then adjusted according the smoothed curve, which is assumed to describe the dependence of the imbalance on spot fluorescence intensity. But the fluorescence imbalances between the two channels are more complicated than can be described by a smoothed curve. Due to irregular intrinsic fluorescence of the microarray slide and possibly some gene-specific effect [8, 9], it is unlikely that the fluorescence imbalance can be corrected for all features by the intensitydependent normalization. To correct the fluorescence imbalance of each feature a simple method is to reverse the labelling dyes when hybridizing some microarrays. Kerr et al [10] first proposed an ANOVA model for microarray data, and showed that ANOVA methods can be used to normalize the data and estimate real changes in gene expression. Taking biological variations into account, Dobbin *et al* [2] have addressed the problem of statistical design of reverse dve microarrays to minimize variance with a given number of microarray slides. We have taken the analysis further to address the problem of identifying DGEs with a desired detection power and controlled number of false positives. A model and statistical testing procedure are presented in the following sections to assist research workers in the selection of an appropriate number of microarrays for an experiment in order to achieve the desired detection power, or alternatively in assessing the detection power achievable when the experiment has been done.

II. THE MODEL

The experimental situation analyzed here is one where there are two sample groups. One of the groups might have been subjected to an event such as chemical exposure, the other being a suitable control, or the two groups might be normal and tumor tissues or different organs. For convenience the two groups will be designated as the *treated* and *control* groups.

In cells, the amount of mRNA corresponding to a particular gene is taken to correspond to the expression level of that gene. A microarray is a means to translate the level of mRNA for many genes, which cannot be measured directly, into fluorescence that can be measured directly. The model presented in this paper is designed for experiments where each gene is spotted only once on each microarray, and each individual sample is hybridized only once using one microarray. For the purpose of introduction consider one single feature spot on the microarray. We assume that the log-intensity fluorescence of this feature takes additive contributions from the following sources: the amount of corresponding mRNA in the biological sample, an effect from the quality of the feature spot, an effect from the labelling fluorochrome (including the efficiency of labelling with the fluorochrome, and possible pre-existing intrinsic fluorescence in favor of this fluorochrome), and the random measurement error. Therefore we have the following model

$$G_{v,i,s,c} = I_{v,i} + A_s + D_c + \epsilon_{v,i,s,c},$$
 (1)

where $G_{v,i,s,c}$ is the log-intensity (base 2 logarithms are utilized throughout this paper) of fluorescence of the feature spot;

 $I_{v,i}$ is the expression level of the gene in the *i*th individual sample of group v (v = t for the treated group, or v = c for the control group, and $i = 1 \cdots n$ where n is the number of individuals in each group). $I_{v,i}$ is assumed to

be independently and normally distributed with a mean E_v and a variance σ_v^2 , denoted by $I_{v,i} \sim N(E_v, \sigma_v^2)$;

 A_s is the effect of feature spot quality, which is assumed to be fixed for microarray slide s and independent of fluorescent label used;

 D_c is the effect of the fluorescent label c (c = g for green dye, and c = r for red dye), which is assumed to be fixed with label c and independent of microarray slide;

 $\epsilon_{v,i,s,c}$ is the random error term which is assumed to be independently and normally distributed with a mean 0 and a variance σ_{ϵ}^2 , denoted by $\epsilon_{v,i,s,c} \sim N(0, \sigma_{\epsilon}^2)$.

Note that for each of the features on the microarray the log-intensity is described in the same form Eq.(1). Although the equations are in the same form for each feature the actual values of E_v , σ_v^2 , A_s , D_c , σ_ϵ^2 will be feature dependent.

 E_v is the mean expression level of the gene in the sample group v, so in comparing a gene's expression between the treated and control groups, the quantity of interest is $E_t - E_c$, the magnitude of differential expression. The effects of feature spot quality A_s and fluorescent dye D_c are not of interest and therefore need to be eliminated by a suitable experimental design.

III. EXPERIMENTAL SETUP

Let's introduce the notation (c_j, t_i) to represent a microarray as a result of the following hybridization: individual sample c_j labelled with green dye and individual sample t_i labelled with red dye. Here c and t indicate the sample group while the subscripts index different individuals in each group. As a convention, the first sample in the parenthesis is always labelled with green dye and the second with red dye.

Consider the microarray (c_j, t_i) . Here an individual j is taken from the control group (v = c) and an individual i from the treated group (v = t). RNA is extracted from both and converted to labelled cDNA using fluorescent labels green and red respectively. These are then simultaneously hybridized to the microarray a. This method of labelling (control sample with green and treated sample with red) is referred to as forward labelling. As a result of this experiment we can derive from Eq.(1)

$$\begin{split} G_{c,j,a,g} &= I_{c,j} + A_a + D_g + \epsilon_{c,j,a,g}, \\ G_{t,i,a,r} &= I_{t,i} + A_a + D_r + \epsilon_{t,i,a,r}. \end{split}$$

The difference F_a between the two fluorescence logintensities is therefore

$$F_{a} = G_{t,i,a,r} - G_{c,j,a,g} = I_{t,i} - I_{c,j} + D_{r} - D_{g} + \epsilon_{t,i,a,r} - \epsilon_{c,j,a,g},$$
(2)

and F_a is normally distributed with an expected value (mean) $E_t - E_c + D_r - D_g$ and a variance $\sigma_t^2 + \sigma_c^2 + 2\sigma_{\epsilon}^2$. Note that taking the difference of $G_{c,j,a,g}$ and $G_{t,i,a,r}$ causes the spot effect A_a to be cancelled out and it does not therefore contribute to F_a . However, there is still the labelling fluor effect $D_r - D_g$ to consider. To eliminate this effect microarrays with reverse labelling are required.

Consider the microarray $(t_{i'}, c_{j'})$, where another two individuals, i' from the treated and j' from the control groups are hybridized to another microarray b. On this occasion the individual from the control group j' is labelled with *red* and the individual from the treated group i' with green. This method of labelling (control sample with *red* and treated sample with green) is referred to as reverse labelling. From this microarray b we get:

$$G_{t,i',b,g} = I_{t,i'} + A_b + D_g + \epsilon_{t,i',b,g}, G_{c,j',b,r} = I_{c,j'} + A_b + D_r + \epsilon_{c,j',b,r}.$$

The difference of the two log-intensities is

$$B_{b} = G_{t,i',b,g} - G_{c,j',b,r} = I_{ti'} - I_{cj'} + D_g - D_r + \epsilon_{t,i',b,g} - \epsilon_{c,j',b,r},$$
(3)

and B_b is normally distributed with an expected value $E_t - E_c + D_g - D_r$ and a variance $\sigma_t^2 + \sigma_c^2 + 2\sigma_{\epsilon}^2$.

The quantity F_a (or B_b) is the difference of two logintensities and is therefore equivalent to the logarithm of the ratio of two intensities. Thus F_a (or B_b) is often called the *log-ratio* of a gene. The variance of the log-ratio of a gene, $\sigma_T^2 = \sigma_c^2 + \sigma_t^2 + 2\sigma_\epsilon^2$, is the sum of the biological variance of the control σ_c^2 , the treated σ_t^2 , and the measurement variances associated with them $2\sigma_\epsilon^2$. Hereafter σ_T^2 is referred to as the *total variance* of the log-ratio of the gene.

From Eqs.(2) and (3) it is clear that by combining measurements from both forward and reverse labelled microarrays, it is possible to eliminate the fluorescent label bias. One simple way of doing this is to take the average of Eqs.(2) and (3). The expected value of this average is then $E_t - E_c$, which is the quantity of interest. The above arguments therefore show that to eliminate the spot effect A_s , we need to hybridize the control and treated samples onto the same microarray slide. To cancel out the fluorescent label effect D_c we need to do both forward labelled and reverse labelled microarrays. A general formalism is presented in the following sections to deal with situations where the number of forward labelled microarrays and the number of reverse labelled microarrays are not necessarily the same.

We will consider the following experiment:

$$(c_1, t_1), (c_2, t_2), \cdots, (c_{n_f-1}, t_{n_f-1}), (c_{n_f}, t_{n_f})$$

 $(t_{n_f+1}, c_{n_f+1}), (t_{n_f+2}, c_{n_f+2}), \cdots, (t_{n_f+n_r}, c_{n_f+n_r}).$

In this experiment there are in total $n_f + n_r$ microarrays, n_f of them are forward labelled, and the rest n_r are reverse labelled. In relation to similar studies by other authors[11, 12, 13] using replicated microarrays, this study focuses on a special case of microarray experiment designs, i.e., direct comparison between two groups with biological but no technical replicates in each group. It is a special case of the balanced block design as described by [14]. They have showed that the balanced block design is the most efficient experimental setup when comparing two classes with a given number of microarrays. The limitation of this experimental setup, as Dobbin and Simon pointed out for the balanced block design, is that it is not suitable for clustering analysis.

IV. DETECTING DGEs

A. Hypothesis test

For each gene printed on the microarrays, we want perform a statistical test to determine whether this gene is differentially expressed to a significant degree in the treated group compared to the control group. The null hypothesis is that the gene has the same expression level in the two groups:

Null hypothesis
$$H_0: E_c = E_t$$
 (4)

Alternative hypothesis
$$H_1: E_c \neq E_t$$
 (5)

From each of the n_f forward labelled microarrays an intra-array log-ratio F_i between the treated sample and the control sample is obtained, and similarly from each of the n_r reverse labelled microarrays a log-ratio B_j . Each F_i has an expected value $E_t - E_c + D_r - D_g$, so the average $\overline{F} = \sum_{i=1}^{n_f} F_i/n_f$ has the same expected value. Similarly the average $\overline{B} = \sum_{j=1}^{n_r} B_j/n_r$ has an expected value $E_t - E_c - D_r + D_g$. Averaging \overline{F} and \overline{B} gives

$$R = \frac{\overline{F} + \overline{B}}{2} = \frac{1}{2n_f} \sum_{i=1}^{n_f} F_i + \frac{1}{2n_r} \sum_{j=1}^{n_r} B_j, \qquad (6)$$

which will have an expected value $E_t - E_c$, so R is an unbiased estimator of our quantity of interest. Also R is normally distributed with a variance

$$\sigma_R^2 = \frac{\sigma_T^2}{4} \left(\frac{1}{n_f} + \frac{1}{n_r} \right). \tag{7}$$

When the total number of microarrays $n_f + n_r$ is fixed, the variance of R is minimized at $n_f = n_r$, so whenever possible, equal numbers of forward and reverse labelled microarrays should be combined. The variances σ_c^2 , σ_t^2 , and σ_ϵ^2 are unknowns, but fortunately there is no need to estimate them individually. For the purposes of identifying differential gene expression, estimating σ_T^2 as a whole is sufficient and σ_T^2 can be estimated using its un-biased estimator

$$s^{2} = \frac{1}{n_{f} + n_{r} - 2} \left[\sum_{i=1}^{n_{f}} (F_{i} - \overline{F})^{2} + \sum_{j=1}^{n_{r}} (B_{j} - \overline{B})^{2} \right]$$
(8)

and $(n_f + n_r - 2)s^2/\sigma_T^2$ will follow the χ^2 distribution with $n_f + n_r - 2$ degrees of freedom, independent of \overline{F} and $\overline{B}[15]$, thus s^2 is independent of R. Note that in order to estimate $E_t - E_c$ and σ_T^2 properly it is necessary that $n_f \geq 1$, $n_r \geq 1$, and $n_f + n_r > 2$. In other words there must be at least one forward and one reverse labelled microarray, and at least three microarrays in total. It is then apparent that

$$t = \frac{R - (E_t - E_c)}{s\sqrt{\frac{1}{4}\left(\frac{1}{n_f} + \frac{1}{n_r}\right)}}$$
(9)

is distributed as the Student's t distribution with $n_f + n_r - 2$ degrees of freedom. In testing the null hypothesis Eq.(4), we insert $E_t = E_c$ into Eq.(9) and thus our test statistic t_0 is defined as,

$$t_0 = \frac{R}{s\sqrt{\frac{1}{4}\left(\frac{1}{n_f} + \frac{1}{n_r}\right)}}$$
(10)

Note that there is now no unknown quantity in Eq.(10). Under the null hypothesis that $E_t = E_c$, t_0 follows the Student's distribution with $n_f + n_r - 2$ degrees of freedom. Based on the value of t_0 the p-value of the test can be calculated. If the p-value calculated is larger than some pre-set threshold P_{th} , the null hypothesis is accepted that the gene has the same level of expression in both the control and treated groups. If the calculated p-value is smaller than the threshold P_{th} , it is declared that the test for this gene is *positive*, in the sense that its expression level in the treated group is different from that in the control group. Then depending on the sign of t_0 the gene is either designated as up ($t_0 > 0$) or down regulated ($t_0 < 0$).

B. Setting the threshold p-value

A t test is performed for each gene, which is then declared as differentially expressed, or not, according to the above criteria. By adjusting the value of threshold P_{th} a control can be exerted on the number of false DGE calls made. By definition, p-value is the probability of observing a value of the statistic as extreme or more extreme than the observed value, under the condition that the null hypothesis is true. For each gene whose null hypothesis is true (we call each such gene a *null gene*), its p-value is uniformly distributed in (0,1). Therefore the probability that a null gene's p-value is smaller than P_{th} is just P_{th} . Suppose that in a total number N genes, N_0 are null genes. When every gene on the microarray is tested, the number of false DGE calls O_{fp} will has an expected value $N_0 P_{th}$. So if one decides to tolerate an expected number ${\cal N}_{fp}$ false DGEs the threshold p-value should be set at $P_{th} = N_{fp}/N_0$. However, in reality only N is known and not N_0 and therefore, it is necessary to make an estimation of N_0 or N_0/N . Some methods for estimating N_0/N are discussed in Sec. VB.

Once the threshold value P_{th} is set, the ability to detect genuine DGE, i.e. a gene with $E_t \neq E_c$, depends on the following factors: the magnitude of differential expression $E_t - E_c$, the total variance in one microarray experiment σ_T^2 , and the number of forward and reverse labelled microarrays. Among these factors, the ones over which experimental control is exercised are n_f and n_r . In general the larger n_f and n_r , the more powerful will be the statistical testing. The key question is therefore, how many forward and reverse labelled microarrays are required in order to achieve a desired power of DGE detection with control on the number of false DGE calls? Based on the standard normal Z test, several authors have presented results on calculating the number of microarrays needed to achieve given statistical power while controlling false positive rate [2, 16]. These results would be applicable if we knew σ_T^2 for each gene. In reality though the variances cannot be assumed known, and more often than not, the number of microarrays used to estimate the variances is rather small. It is therefore necessary to use t-based test rather than the standard normal test. Other authors have also presented approximate formulas [17, 18] for calculating the power of the traditional two-sample t test with equal variance. In this paper we present an exact formula for calculating the power of the t-based statistical test developed here.

C. Determination of the threshold t-value

When the numbers of forward and reverse labelled microarrays are given, setting P_{th} is equivalent to setting a threshold, say $|\xi|$, for the statistics t_0 defined in Eq.(10). With this threshold t-value, our criteria for claiming a DGE is as follows: If $t_0 > |\xi|$, the gene is claimed as up-regulated $(E_t - E_c > 0)$; if $t_0 < -|\xi|$, it is claimed as down-regulated $(E_t - E_c < 0)$. So the rate at which false positive claims are made is

$$P_{th} = \int_{-\infty}^{-|\xi|} \rho_{n_f + n_r - 2}(t_0) dt_0 + \int_{|\xi|}^{\infty} \rho_{n_f + n_r - 2}(t_0) dt_0$$
$$= 2 \int_{-\infty}^{-|\xi|} \rho_{n_f + n_r - 2}(t_0) dt_0 = 2T_{n_f + n_r - 2}(-|\xi|) (11)$$

where $\rho_r(x)$ is the probability density function (PDF) of the Student's distribution with r degrees of freedom, and $T_r(.)$ is the cumulative probability distribution function (CDF) for the Student's t distribution. It is therefore apparent that the threshold t-value $|\xi|$ can be obtained by solving the equation $2T_{n_f+n_r-2}(-|\xi|) = P_{th}$ with a given false positive rate P_{th} .

D. Successful detection rate

The successful detection rate is the rate at which DGE is correctly identified (either up-regulated or downregulated). If a gene has $E_t - E_c = \mu > 0$, the successful detection rate for this gene is the probability that $t_0 > |\xi|$ is observed. On the other hand, if a gene has $E_t - E_c = \mu < 0$, the successful detection rate equals the probability that $t_0 < -|\xi|$ is observed. It can be shown (see supplementary information I) that in both cases, the rate at which the genes behavior is correctly identified, i.e. $\mu > 0$ or $\mu < 0$, can be described by the following equation

$$S\left(n_{f}, n_{r}, \frac{|\mu|}{\sigma_{T}}, |\xi|\right) = \int_{0}^{\infty} p_{n_{f}+n_{r}-2}(Y) \times \Phi\left[-|\xi|\sqrt{\frac{Y}{n_{f}+n_{r}-2}} + 2\left(\frac{|\mu|}{\sigma_{T}}\right)\sqrt{\frac{n_{f}n_{r}}{n_{f}+n_{r}}}\right] dY, (12)$$

where $p_r(Y)$ is the PDF for the χ^2 distribution with r degrees of freedom, and $\Phi(.)$ is the CDF for the standard normal distribution.

Therefore the successful detection rate S is a function of n_f , n_r , $|\mu|/\sigma_T$, and $|\xi|$, where $|\xi|$ can be obtained by solving Eq.(11) at a given P_{th} . Eventually, S is a function of P_{th} , n_f , n_r , and $|\mu|/\sigma_T$.

E. Usage of the S function

We have implemented the calculation of the S function as a Java application, which is accessible through the URL given in the abstract. Two look-up tables also are provided in the supplementary for some typical results of S for quick reference. Experiment designers can use these to find the value of S at given parameters n_f , n_r , $|\mu|/\sigma_T$, and P_{th} , thus get some general idea of what percentage of truly DGEs can be detected by their experimental design.

The applicability of the S function can be seen from two perspectives. First, for the user who has not carried out any microarray experiments on their system before, the total variances (σ_T^2) will be completely unknown. In this situation the S function can serve as a post-experiment assessment to inform the user of the detection rate in their experiment based on the observed values of R and s^2 from the measurements. For example, 3 forward and 3 reverse labelled microarrays, with 5000 genes printed on each microarray, were used in a experiment. The tolerance for false positives is set at $N_{fp} = 2$, and for simplicity the threshold p-value is set as $P_{th} = 2/5000$. If most genes have an s^2 around 1, then the typical value of σ_T^2 for the set of genes is 1. We can now ask: for genes with two-fold differential expression and typical variance, what percentage of them can be correctly detected by this experiment? Remembering that a two-fold differential expression corresponds to $\mu = E_t - E_c = 1$ or $\mu = E_t - E_c = -1$, we have $|\mu| = 1$ and $\sigma_T = 1$. Using the S calculator or the look-up tables (Supplementary Table I) we find that the successful detection rate for $n_f = 3$, $n_r = 3$, $P_{th} = 2/5000$, and $|\mu|/\sigma_T = 1$ is 9.08×10^{-3} , which means that in this experiment only 0.908% of genes with two-fold DGE and

with typical variance 1 can be detected, the remaining 99% are missed. If the same question was asked about genes with four-fold DGE and one decides to tolerate $N_{fp} = 8$ false positive claims and the threshold is set at $P_{th} = 8/5000$, then the successful detection rate for $P_{th} = 8/5000$, $n_f = 3$, $n_r = 3$, and $|\mu|/\sigma_T = 2$ is 0.217, which means 21.7% of them are successfully detected. If the detection rate is unsatisfactory, then more forward and reverse microarray datasets need to be added.

Second, if there is some general knowledge of total variance from previous experiments or other sources, then a target for the detection rate can be set. In this case, the S function will assist in the determination of how many forward and reverse microarrays are required in the experiment. For example, if from previous experience we know that the typical value of the total variance for the set of genes under consideration is $\sigma_T^2 = 0.25$, which gives $\sigma_T = 0.5$; A microarray experiment is now designed to identify DGEs between the treated and the control with a tolerance of 8 false positive claims out of 5000 genes being tested with $P_{th} = 8/5000$ for simplicity; The preset target is that after this experiment no less than 60%of genes with two-fold DGE and with typical variance should be detected; How many forward and reverse labelled microarrays are needed? As before, two-fold DGE corresponds to $|\mu| = 1$, so one has $|\mu|/\sigma_T = 2$. Using the look-up tables (Supplementary Table II, in the $|\mu|/\sigma_T = 2$ panel and $P_{th} = 8/5000$ column), one finds that the row $n_f = n_r = 4$ gives a detection rate S = 0.605which is closest to meet the target. Therefore 4 forward and 4 reverse labelled microarrays are required in this experiment.

V. CONTROLLING FALSE POSITIVES

A. Procedures

In this section, we explore further on how to effectively control false positives in a multiple test situation. Generally speaking, all different multiple-testing methods eventually amount to effectively setting a threshold p-value, and then rejecting all the null hypothesis with p-value below this threshold. For example, the classical Bonferroni multiple-testing procedure controls family-wise error rate at α by setting the threshold $P_{th} = \alpha/N$, where Nis the total number of hypothesis tested. In this study, we aim to control the number false positives such that the expectation of O_{fp} equals N_{fp} , our pre-set target. As discussed in Section IV B, to achieve this, we should set $P_{th} = N_{fp}/N_0$, which requires an estimation of N_0 or N_0/N , the fraction of null genes in the set.

We present three procedures here for setting P_{th} to control false positives:

Procedure A: Suppose we have made an estimation of N_0/N as c, then set $P_{th} = N_{fp}/(cN)$. The method for calculating c will be discussed below.

Procedure B: Set $P_{th} = N_{fp}/N$. This can be seen as using c = 1 as the crudest estimation of N_0/N .

Procedure C: Suppose genes are sorted by their ascending p-values, so that $p_1 \leq p_2 \leq p_3 \leq \cdots \leq p_N$, where p_i is the p-value for gene *i*. Set $P_{th} = p_{i^*}$, where i^* is the largest index satisfying $p_i[N - i + \min(i, N_{fp})] \leq N_{fp}$. This can be seen as estimating N_0/N by $c = [N - i^* + \min(i^*, N_{fp})]/N$. The idea behind this is that if gene i^* and all genes indexed below it are to be declared DGEs, these genes should not contribute to the fraction of null genes. Thus this represents some improvement over the crudest estimation c = 1.

We have performed simulations to compare the performances of the three procedures. Procedure A allows us to achieve the highest rate of DGE detection among the three, and the observed false positives O_{fp} matches our preset target N_{fp} statistically. Procedure B does not estimate N_0/N effectively, and it is the most conservative procedure. So Procedure A is recommended over C and B (See Supplementary for details on simulation procedures and data).

Benjamini and Hochberg [19] proposed the FDR approach to control the false discovery rate (FDR) at q by setting $P_{th} = i^*q/N$, where i^* is the largest index satisfying $p_i \leq iq/N$. The false discovery rate was defined as the expectation of the ratio of false to total positives, i.e., $q \equiv E(O_{fp}/i^*)$. When the FDR procedure controls false discovery rate at q, the observed false discovery rate O_{fp}/i^* should have value around q, i.e., $q \approx O_{fp}/i^*$, which gives $P_{th} = i^*q/N \approx O_{fp}/N$. The expectation of the threshold p-value under the FDR procedure is therefore $E(P_{th}) \approx E(O_{fp}/N) = N_{fp}/N$. It is thus clear that the FDR procedure of [19] is on average equivalent to Procedure B in this section.

B. Estimating N_0/N

Pounds and Morris [20] recently proposed the use of a beta-uniform mixture (BUM) function to approximate the distribution of p-values from a set of genes tested, and estimate the fraction of null genes in the set. Here we propose another method to estimate N_0/N , which does not requires the BUM form of distribution of p-values. The aim was to achieve a more accurate estimation of the fraction of null genes. As in [20], we wanted to extract a uniform density from the observed distribution of p-values. To achieve this, the genes were first sorted by their ascending p-values, so that $p_1 \leq p_2 \leq p_3 \leq \cdots \leq p_N$, where p_i is the p-value for gene *i*. Then an empirical cumulative distribution of p-values can be easily obtained by plotting i/N versus p_i . The idea was to find a straight line tangent to the cumulative distribution curve with minimum slope. Taking into account that the cumulative distribution curve is a non-decreasing function ending at the point (1.0, 1.0), the minimum slope was found as follows. Each point $(p_i, i/N)$ on the cumulative distribution plot was connected with the ending point (1.0, 1.0) with a straight line, and the slope of the line calculated as $c_i = (1.0 - i/N)/(1.0 - p_i)$. Then the minimum of c_i at a given range of p-value, say $P_l \leq p_i \leq P_u$, was found

$$c_{min} = \min_{i} (c_i \mid P_l \le p_i \le P_u). \tag{13}$$

 c_{\min} can be used as our estimation of the fraction of null genes in the set.

We have carried out simulations to test the performance of Eq.(13), and found that it tends to underestimate the true value of N_0/N . Instead, using median slope as the estimation of N_0/N gives more accurate results than the minimum slope. We thus use the following equation to estimate the fraction of null genes

$$c_{mid} = \text{median}(c_i \mid P_l \le p_i \le P_u). \tag{14}$$

In a recent paper [21], Storey and Tibshirani used a natural cubic spline to fit the data of c_i as a function of p_i for a given range of p-values, then took the value of the spline at p = 1 as the estimation of N_0/N . We compared the Storey-Tibshirani method with Eq.(14), an advantage of the latter is that it is computationally much simpler than the Storey-Tibshirani method. As can be seen from Table I, both our method and the Storey-Tibshirani method become more accurate as N and/or N_0/N increases, and in all the cases our method gives slightly better results, as indicated by the coefficient of variation.

As for the values of P_l and P_u , a practical guidance for choosing them is to set P_l a value between 0.4 and 0.5, and P_u between 0.9 and 0.95. In fact, Eq.(14) gives quite robust results with respect to changing the values of P_l and P_u within the recommended range. For a set of simulation tests with true null fraction 0.8, using $(P_l, P_u) = (0.4, 0.95)$ gives $c_{mid} = 0.800 \pm 0.023$, while using $(P_l, P_u) = (0.5, 0.9)$ gives $c_{mid} = 0.800 \pm 0.024$.

The method here to estimate N_0/N does not depend on the specific form of statistical tests being used, as long as the p-values pertaining to the tests are obtained. But similar to the BUM method and the the Storey-Tibshirani method, the method we are proposing here also implicitly assumes that the multiple test statistics are independent, or at least the true null statistics are independent. In the context of microarray experiments, this would require that the null genes' expressions are independent of each other. This may be not realistic, thus the estimation of the fraction of null genes based on these methods will be less accurate. An extreme example is when all the null genes in each biological sample behave in a concerted manner, and all the non-null genes express in a synchronized way, then the p-values we observe will be concentrated on two separate points, one for all the null genes and one for the non-null genes. Such a situation will defy all the methods for estimating N_0/N discussed here. Estimating the fraction of null genes with possibly strong inter-gene dependence is an important issue, and probably a very difficult one, especially without specifying their structure of interdependence beforehand.

This is beyond the scope of current study, and is an issue worth of future investigation and continuous efforts. Until further statistical advances are made in this respect, the method we proposed in this paper can serve as an approximation for estimating the fraction of null statistics.

VI. DISCUSSION

The data volume generated by microarray studies combined with the intrinsic variability of the system demands that rigorous statistical analysis be applied to the data to avoid the problem of false positives and/or low successful rate in DGE detection. In this study we have taken into account all the major variables associated with microarray data. The procedure proposed in this paper deals with fluorescent label bias often present in microarray experiments. A t statistic has been derived for hypothesis testing based on a model that describes each gene individually with its own set of parameters. An advantage of this design is that if there exists any fluorescent biases $(D_q \neq D_r)$ for some genes they will be corrected by the reverse labelling procedure. For genes with no fluorescent bias (for example, some genes may have $D_g = D_r$) the method will perform equally satisfactorily.

In this work, we have adopted the normality assumption, which leads to the test statistic t_0 following the Students' t distribution under the null hypothesis. Thus the successful detection rate S can be calculated in closed form. While the normality assumption seems reasonable with common technologies, especially for the measurement error ϵ_{visc} , large scale replicate experiments have not yet been performed to make a precise assessment [1]. If normality is not met, R defined in Eq.(6) will continue to be an unbiased estimator of the quantity of interest but t_0 will not follow Students' t distribution. In this case some non-parametric methods [22, 23, 24, 25] could be employed. While those methods can be readily applied to microarrays with a common reference design, where the systematic dye bias subtracts out in the calculation of the test statistic, the application of those methods to the direct comparison design needs to be further developed and investigated. If non-parametric methods have to be used the rate of successful detection cannot be as readily calculated as in Eq.(12).

In the published literature it is a common practice to apply some form of normalization (global or local) to remove systematic biases before the statistical analysis of microarray data. Here we are proposing to remove much of the systematic bias by experimental means, i.e. by a dye-swapping procedure. Since the model deals with the fluorescent bias for each gene individually, no other local normalization procedure (e.g. LOWESS [7]) should be applied before the statistical testing procedure given here. However, some form of global normalization is appropriate, such as that utilized by Pollack *et al* [26], or that described in [6], where the log-ratios in a microarray dataset are globally shifted so that the most probable value of log-ratio becomes 0. The purpose of global normalization is to adjust the effect of global factors that could generally affect the fluorescence, such as a difference between the overall concentrations of two mRNAs, and possibly the difference of photo-amplifier voltages used between the two fluorescent channels when the microarray image was scanned. All the local feature-specific bias is looked after by the reverse labelling and statistical testing procedure proposed here.

Finally a word for the overworked bench researcher facing the prospect of multiple hybridizations in order to achieve a reasonably high level of S without having to contend with an unsatisfactory false positive rate. What can be regarded as reasonable? This depends on the desired outcome of the experiment. If for example the interest is in defining genes which might give rise to differential susceptibility, then there will be a desire to have a high value of S in order not to miss any potential candidate genes. There would be two ways of achieving this, either by increasing the number of hybridizations or by accepting a higher false positive rate. In an experiment such as the one described then the candidate genes will probably be verified by other methods downstream. Therefore the balance is driven by the need to achieve a high S and the decision is between whether it is more economical to use more microarrays, or put more resource into downstream verification. Where no downstream verification of DGEs identified in a microarray experiment are proposed then it is essential to maintain a low value of false positive rate, at the expense of S if the total number of microarrays is limiting. This study does not seek to put a figure on the number of microarrays that should be hybridized in an experiment. Rather a framework is provided for the experiment designer to decide on the number of microarrays to hybridize taking into account the system, availability of sample, downstream analysis primarily and the objective of the experiment.

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TABLE I: The fraction of null genes as estimated by Eq.(14) (c_{mid}) and by the Storey–Tibshirani method (π_0) . Parameters used are: $\mu = 1$, $\sigma_T = 0.5$, $n_f = 2$, $n_\tau = 2$, $P_l = 0.4$, $P_u = 0.95$. Results are based on 16 simulations for each cell in the table. cv, the coefficient of variation, is defined as the standard deviation divided by the true value of null fraction, N_0/N .

		N = 100			N = 500			N = 1000			N = 5000		
N_0/N		mean	stdev	cv	mean	stdev	cv	mean	stdev	cv	mean	stdev	cv
0.2	c_{mid}	0.186	0.040	0.200	0.205	0.017	0.085	0.197	0.013	0.067	0.201	0.009	0.044
0.2	π_0	0.158	0.109	0.544	0.209	0.061	0.307	0.178	0.042	0.212	0.203	0.017	0.087
0.8	c_{mid}	0.767	0.112	0.140	0.807	0.047	0.059	0.805	0.031	0.038	0.800	0.023	0.029
0.8	π_0	0.724	0.284	0.355	0.785	0.097	0.121	0.792	0.064	0.080	0.807	0.060	0.075