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Self-self Hybridization as an Alternative Experiment Design to Dye Swap for Two-color Microarrays

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ABSTRACT

Dye-specific bias effects, commonly observed in the *two-color microarray* platform, are normally corrected using the dye swap design. This design, however, is relatively expensive and labor-intensive. We propose a *self-self hybridization design* as an alternative to the dye swap design. In this design, the treated and control samples are labeled with Cy5 and Cy3 (or Cy3 and Cy5), respectively, without dye swap, along with a set of self-self hybridizations on the control sample. We compare this design with the dye swap design through investigation of mouse primary hepatocytes treated with three peroxisome proliferator–activated receptor-alpha (PPARα) agonists at three dose levels. Using Agilent's Whole Mouse Genome microarray, differentially expressed genes (DEG) were determined for both the self-self hybridization and dye swap designs. The DEG concordance between the two designs was over 80% across each dose treatment and chemical. Furthermore, 90% of DEG-associated biological pathways were in common between the designs, indicating that biological interpretations would be consistent. The reduced labor and expense for the self-self hybridization design make it an efficient substitute for the dye swap design. For example, in larger toxicogenomic studies, only about half the chips are required for the self-self hybridization design compared to that needed in the dye swap design.

INTRODUCTION

DNA MICROARRAYS have been widely used for the discovery of the altered profiles of gene expression associated with toxicity and disease (Lander et al., 2001; Shultz et al., 2001; Yeoh et al., 2002), including many cancers (Khan et al., 2001; Perou et al., 2000; Shi et al., 2005c; Sorlie et al., 2001; Tothill et al., 2005; van 't Veer et al., 2002; Yeoh et al., 2002). Microarrays are commonly employed for identifying genes with expression differences in transcript concentrations between the sample classes (e.g., from diseased versus healthy tissue). A list of the most differentially expressed genes (DEG) is used for experiment interpretation. In particular, the list of DEGs, the proteins they encode, and the biological pathways

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involving the genes and proteins provide relevant information on the biological phenomena and associated mechanisms distinguishing the classes. A list of DEGs may also provide a profile or pattern of altered gene expression with predictive value to place an unknown sample within a class.

There are two major types of DNA microarrays—single color and two color—both of which predominately use florescence to measure transcript concentration. With single-color arrays, each sample is hybridized on a separate array containing probe sequences of known mRNA, and thus gives estimations of the absolute value of each gene's expression for each sample. In two-color arrays, each array is typically hybridized with mRNA from both samples to be compared, where each sample is labeled with a different fluorescent dye label. The two-color array thus directly estimates transcript concentration ratios (i.e., each gene's up- or down-regulation).

Two-color arrays for most experiments would require only half the number of arrays of one-color arrays were it not for the need to modify experiment designs to correct for so-called dye bias. Dye bias is a consequence of dyes (usually green [Cy3] and red [Cy5] dyes) having different signal versus transcript concentration dependencies. Importantly, dye bias is gene-specific, sensitive to florescent intensity, and signal versus transcript concentration calibration curves can be nonlinear, especially at lower and higher signal intensities (Cox et al., 2004; Shi et al., 2005b). While identification of DEGs with large differential fold change might not be hindered by dye bias, bias can result in an unacceptable increase in DEG false positives and negatives, especially among genes with lower intensity or smaller fold change.

Dobbins et al. (2003) reviewed design issues for two-color microarray experiments, including design approaches to mitigate dye bias effects. The most prevalent approaches to correct dye bias are the dye swap design and the reference design. AU1

In the dye swap design, two RNA samples, usually control and treatment, are labeled with Cy3 and Cy5, respectively, and then cohybridized on a chip. The process is repeated by switching the dyes of the two samples in a second cohybridization. To minimize the dye effects, the results of the two hybridizations are averaged prior to identifying DEGs (Fig. 1). In the reference design, both treated and control samples are labeled with one dye (usually Cy5), while a reference sample is labeled with the other dye (usually Cy3) and cohybridized. The commonly used reference samples are the mixed RNA from different tissues or cell lines that are commercially available. The DEGs are determined by directly comparing two classes of samples to correct for dye bias on a gene-by-gene basis. F1

Importantly, both the dye swap and reference designs require twice the number of two-color arrays, and thus the same number of arrays, as one color microarray experiments. Additionally, for the dye swap design, doubling the needed RNA could be problematic, particularly in some clinical applications. The reference design is advantageous when RNA is limited, but could confound DEG selection because of greater difficulties in data normalization. The reference design has biological and reference samples that are markedly different, such that the preponderance of genes appear differentially expressed and mostly up- or down-regulated, in violation of fundamental assumptions of some normalization methods.

Self-self hybridization (SSH) is an experiment where the same RNA sample is labeled separately with Cy3 and Cy5 and cohybridized to the same array. The SSH experiment has been used by a number of researchers, but its advantages have yet to be fully exploited. Previous SSH-based studies have either developed error models and/or estimated false positives using SSH in order to quantify dye bias errors or to derive and apply correction factors to biased expression ratios. (Blangiardo et al., 2006; Liang et al., 2003; Martin-Magniette et al., 2005; Rosenzweig et al., 2004; t Hoen et al., 2004; Yang et al., 2002).

In this paper, we propose a SSH design that utilizes the SSH experiment as a direct alternative to the dye swap design. The SSH design provides the ability to correct for dye bias while avoiding the increased number of needed arrays and amount of RNA required by the dye swap design. The SSH design also avoids the normalization issues that confounds gene selection in the reference design.

The SSH design is somewhat similar to the reference design, but uses true control samples instead of a reference sample. In this design, the treated and control group samples are labeled with Cy5 and Cy3 (or Cy3 and Cy5), respectively, without dye swap. In a separate hybridization, the control group samples are separately labeled with Cy3 and Cy5 and then cohybridized to the same array (Fig. 1). The DEGs are determined by comparing the array experiments of the treated samples with the SSH controls. Since the design uses real biological controls, it avoids the difficulties in the reference design that occurs when the ma-

Hybridization	***(A)** Dye swap design*		***(B)** SSH design*	
Hyb1	Cy3(T1)	Cy3(C)	Cy5(C)	Cy3(C)
Hyb2	Cy5(T1)	Cy5(C)	Cy5(T1)	Cy3(C)
Hyb3	Cy3(T2)	Cy3(C)	Cy5(T2)	Cy3(C)
Hyb4	Cy5(T2)	Cy5(C)	Cy5(T3)	Cy3(C)
Hyb5	Cy3(T3)	Cy3(C)		
Hyb6	Cy5(T3)	Cy5(C)		

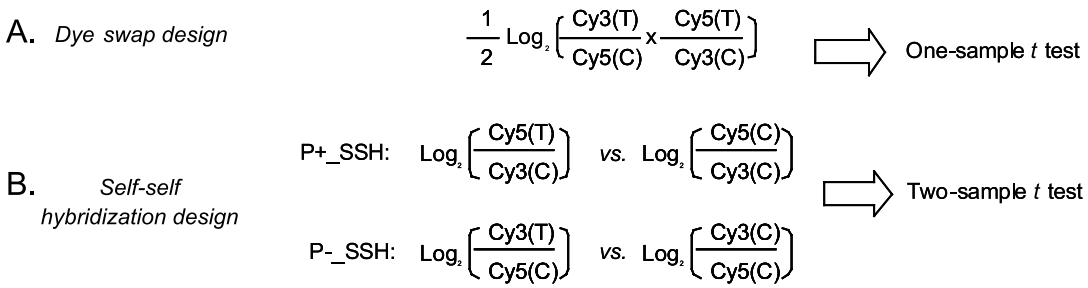

FIG. 1. Schematic comparing (**A**) the dye swap design and (**B**) the self-self hybridization design. For simplicity, each treatment group contains only one sample (denoted as T1, T2, and T3) and the control group contains one sample as well (denoted as C). For the study described in this report, each dose (treatment) group contains three samples (animals) and three dose groups for each chemical treatment, as well as three animal-matched control samples. (**A**) Dye swap design. Each shaded block contains two hybridizations with dye swap; the treatment group (e.g., T1) and the control sample (C) hybridization are repeated with dye labels switched. The ratio of expression between treatment and control is computed as the average of the two hybridization data to compensate for dye bias, as given in the first equation. The differentially expressed genes (DEGs) are calculated from a one-sample *t* test. (**B**) Self-self hybridization design. All treatment samples are labeled with the same dye (e.g., Cy5[T1]), and the control samples with the other dye (e.g., Cy3[C]). In addition, the self-self hybridization is conducted for the control sample only (i.e., control samples are separately labeled with Cy5[C] and Cy3[C]). For each treatment group (e.g., T1) to correct dye bias, the DEGs are determined using a two-sample *t* test, as shown in the second equation, by directly comparing the expression ratio of Cy5(T1)/Cy3(C) with the self-self hybridization expression ratio Cy5(C)/Cy3(C). This design requires lesser arrays compared to the dye swap. This benefit is more pronounced when a multiple dose experiment is conducted, such as the one described in this paper.

AU5

jority of genes are changed between sample classes. Standard normalization methods can be directly applied in the SSH design.

In this study, we demonstrated the equivalency of the SSH design to the dye swap design based on consistency and stability of DEG lists. Specifically, we compared DEGs from dye swap and SSH designs in a toxicogenomics study where the primary hepatocytes of mice were treated with three peroxisome proliferator–activated receptor-alpha (PPARα) agonists at three different doses. The study showed that the SSH design results in DEGs and biological interpretations were comparable to those in the dye swap design.

Thus, SSH achieves dye bias correction similar to the dye swap design, but does so with a minimal number of additional arrays, RNA, and cost.

MATERIALS AND METHODS

Toxicogenomics study

Three mice were used in this study (Guo et al., 2006) to obtain primary hepatocytes that were treated with three PPARα agonists (i.e., bezafibrate, fenofibrate, and Wy14,643), each at doses of 10, 30, and 100 μM. For each dose of each chemical, the RNA was extracted after 24 h of treatment from three individual mice; that is, there were three biological replicates for each dose of each chemical. Animal-matched control RNA samples were also collected after 24 h of treatment. The gene expression data were obtained from Agilent whole mouse genome oligo microarrays (Mouse 44 K; Agilent Technologies, Santa Clara, CA) with Cy3-labeled, treated samples paired with Cy5-labeled, control samples from the matched animal that were cohybridized. The process was then repeated by switching the dyes between treated and control samples, resulting in 3 (chemicals) $\times$ 3 (doses) $\times$ 3 (animals) $\times$ 2 (dye swaps) = 54 total arrays. In addition, nine SSH experiments were done for each of the three control mice, each with three technical replicates.

Data analysis using ArrayTrack

Data analyses were conducted using FDA microarray software ArrayTrack (Tong et al., 2003; Tong et al., 2004). ArrayTrack is generally available through the FDA website (<http://edkb.fda.gov/webstart/arraytrack>).

ArrayTrack is an integrated software suite designed for management, analysis, and interpretation of microarray experiment data. ArrayTrack is MIAME (minimum information about a microarray experiment) supportive for storing both microarray data and experiment parameters associated with a toxicogenomics study. Many data analysis and visualization tools are available with ArrayTrack, including five normalization methods (including linear & lowess and median scaling used in this study), several statistical approaches for identification of differentially expressed genes, clustering/classification methods (e.g., PCA, HCA), and so forth. Importantly, ArrayTrack provides a rich collection of functional information about genes, proteins, and pathways for facilitating data interpretation that are drawn from various public biological databases. The primary emphasis of ArrayTrack is the direct linking of analysis results with functional information for facilitating the interaction between the choice of analysis methods and the biological relevance of analysis results.

In this study, the raw expression data was first normalized using the ArrayTrack linear and lowess method with a default target value of 1000. Spots with low intensity approaching noise level (i.e., smaller than 500) and the control genes (e.g., spike-in and housekeeping genes) were removed, leaving 25,010 spots for subsequent analysis. The statistical significance (p value) for each gene between two classes for the different experiment design was determined with the Student t test, as illustrated in Figure 1. DEGs were determined using the ArrayTrack volcano plot with default settings of $p < 0.05$ and fold change >1.5. Other selection criteria were also investigated, but the results remained the same.

For the SSH design, two lists of DEGs were generated from the nine SSH data using the two-sample t test. One list was from the nine SSH data with the arrays having control and treatment labeled with Cy3 and Cy5, respectively, and the other compared the arrays having control and treatment labeled with Cy5 and Cy3, respectively. In the dye swap design, the DEGs were determined by averaging each pair of dye-flipped data followed by a one-sample t test based on three biological replicates (Fig. 1).

The common genes shared by the aforementioned three lists of DEGs were determined using the venn diagram function in ArrayTrack. The common pathways shared by the three DEG lists were determined using the common pathway function in ArrayTrack (Fang et al., 2006). The statistical significance of individual pathways was assessed using the Fisher exact test (Zeeberg et al., 2003). The right-sided Fisher exact test with a p value <0.05 indicates that the probability of genes occurring in a pathway by chance alone is $<5\%$.

RESULTS

The dye swap design required two complete sets of hybridizations. One contained control and treated samples labeled with Cy3 and Cy5, respectively (called polarity +, or P+ hereafter), and the other contained hybridization data from control and treatment samples that were reversely labeled with Cy5 and Cy3, respectively (called polarity −, or P− hereafter). As depicted in Figure 1, the DEGs were determined based on each gene's average differential expression of the dye swap pair (P+ and P−). For the dye swap design, 3 (chemicals) × 3 (doses) × 3 (animals) × 2 (dye swaps) = 54 arrays were needed to derive the DEGs.

In the SSH design, two DEG lists were determined. One DEG list was derived by comparing nine SSHs and data from P+ (called P+_SSH hereafter), and the other list was obtained by comparing the SSH data with the hybridization data from P (called P−_SSH hereafter). For the SSH design, 27 arrays from control-treated hybridizations (i.e., half the number of arrays used in the dye swap design) plus nine arrays from control-control hybridizations were used for either P+_SSH or P−_SSH.

T1

Table 1 provides the number of DEGs obtained for each of three doses of three chemicals from both the SSH and dye swap designs, along with the number of DEGs that are in common in the two designs. The total number of DEGs from the SSH design (P+_SSH and P−_SSH) is similar to that from the dye swap design, indicating that both designs have similar sensitivity for DEG identification. Importantly, DEG concordance between the designs is high. The P+_SSH and dye swap DEGs overlap by >80% overlap. The DEG overlap between the P−_SSH and dye swap results is similarly high. A stringent test of DEG list consistency can be made by comparing common genes between P+_SSH and P−_SSH (Table 1, penultimate column) with common genes between P+_SSH and P−_SSH and dye swap (Table 1, final column), which are practically identical. Also note that DEG concordances between any two of three analyses (P+_SSH, P−_SSH, and dye swap) are higher for higher doses, possibly because the signal-to-noise ratio increases with increasing dose.

The dependency of DEG list stability to normalization method was also investigated. The results from the linear and lowess normalization, median scaling methods, and raw data were compared to determine if normalization affected the concordance between SSH and dye swap designs. In general, the linear and lowess normalization resulted in higher concordance than median normalization, indicating that median scaling is not an efficient method to minimize the variability associated with dye bias in this experiment and that linear and lowess normalization is preferable for two-color array data.

The consistency of results in terms of biological context was also evaluated. More specifically, biological pathways associated with genes identified as differentially expressed were compared using the common pathway tool available in ArrayTrack. Figure 2 shows the common pathways shared by the three analyses (P+_SSH, P−_SSH, and dye swap) associated with the 100 μM bezafibrate treatment. In this analysis, each DEG list (having 285, 235, and 256 DEGs from P+_SSH, P−_SSH, and dye swap, respectively, as shown in Table 1) was mapped to KEGG pathways. Sixty-four, 63, and 64 pathways associated with P+_SSH, P−_SSH, and dye swap, respectively, were identified, of which 59 pathways were shared by the three analyses. Of the 59 pathways, 24 were determined to be statistically significant based on the Fisher exact test ($p < 0.05$) (Table 3). Similar results were obtained for other chemicals and treatments (results not shown).

F2
T2
AU2

T3

DISCUSSION

The SSH design was investigated as an alternative to the dye swap design using data from a toxicogenomics study. Primary hepatocytes from mice were treated with three PPARα agonists (bezafibrate, fenofibrate, and Wy14,643) at three dose levels (10, 30, and 100 μM). Gene expression data for each dose of each chemical treatment was generated using Agilent's two-color microarray platform. Both SSH and dye swap design experiments were independently carried out so that results in terms of significant gene selection could be directly compared.

DEG identification is one of the most important objectives in the application of DNA microarrays. The best method to obtain a stable (or reproducible) list of DEGs is a current area of scientific debate and even

Table 1. Comparison of Number and Overlap of DEGs Between Self-self Hybridization and Dye Swap Designs

Chemical	Doses (μM)	No. of DEGs[a]			Common DEGs (%)[b]			
		P+_SSH	*P−_SSH*	*Dye swap*	*P+_SSH and dye swap*	*P−_SSH and dye swap*	*P+_SSH and P−_SSH*	*All three*
Bezafibrate	10	7	7	7	7 (100)	5 (71)	5 (71)	5
	30	54	59	52	45 (87)	44 (85)	37 (71)	37
	100	285	235	256	235 (92)	223 (87)	204 (80)	204
Fenofibrate	10	14	13	14	12 (86)	11 (76)	10 (71)	10
	30	86	79	72	61 (85)	60 (83)	50 (69)	50
	100	257	265	246	213 (87)	222 (90)	190 (77)	190
Wy 14,643	10	56	75	61	48 (79)	54 (89)	43 (69)	42
	30	108	74	92	85 (92)	63 (68)	57 (62)	57
	100	233	251	231	210 (91)	209 (90)	189 (82)	189

[a]Identification of differentially expressed genes (DEGs) is outlined in Figure 1 using a combination of $p < 0.05$ and fold change > 1.5.

[b]The percentage overlapping (in parentheses) between any pairs of DEGs was calculated using the number of DEGs from the dye swap design as the denominator.

Table 2. Effect of Normalization Method and Numbers of DEGs, and Common, Overlapping DEGs Derived from P+_SSH, P−_SSH, and Dye Swap Designs for 100 μm Bezafibrate Treatment

Normalization	*Doses (μM)*	*No. of DEGs*[a]			*Common DEGs (%)*[b]			
		P+_SSH	*P−_SSH*	*Dye swap*	*P+_SSH and dye swap*	*P−_SSH and dye swap*	*P+_SSH and P−_SSH*	*All three*
Linear and lowness	10	7	7	7	7 (100)	5 (71)	5 (71)	5
	30	54	59	52	45 (87)	44 (85)	37 (71)	37
	100	285	235	256	235 (92)	223 (87)	204 (80)	204
Raw	10	6	12	5	2 (40)	5 (100)	2 (40)	2
	30	66	18	33	27 (82)	16 (48)	13 (39)	13
	100	335	180	308	228 (74)	163 (53)	124 (40)	124
Median	10	8	9	4	3 (75)	3 (75)	3 (75)	3
	30	40	42	28	23 (82)	17 (61)	12 (43)	12
	100	247	220	240	157 (65)	166 (69)	111 (46)	111

Calculations are described in Table 1.

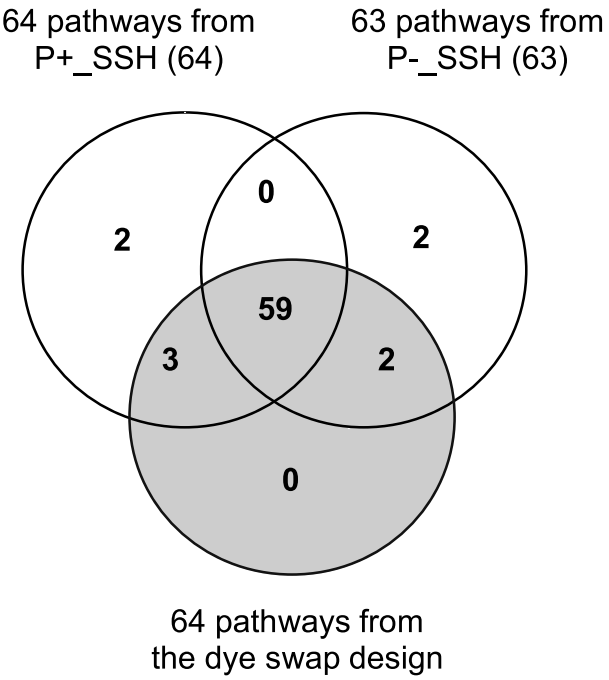

FIG. 2. Common pathways shared by the dye swap and self-self hybridization designs (P+_SSH and P−_SSH) for the 100 μM bezafibrate treatment of a mouse. KEGG pathways were used in this comparison.

contention (Marshall, 2004; Shi et al., 2005a; Tan et al., 2003). The number of DEGs derived from the SSH design is comparable to that with the dye swap design, though the two designs compensate very differently for dye bias. It is interesting to compare the SSH DEG list with lists derived from using only one half the dye swap arrays (i.e., P+ and P− in this study). Applying the same statistical criteria for gene selection, many more genes were identified for either P+ or P− than the SSH design (about 1.4- to 8-fold more genes; results not shown). The need for dye bias correction by experiment design is readily apparent. Without correction, the DEG list is substantially longer, and the increased number of DEGs is almost entirely false positives. Since SSH and dye swap yielded a high 80% concordance in DEGs, the SSH design can be viewed as an economical means of minimizing false positives compared with the dye swap design.

The ultimate goal of using the DNA microarray is to gain insight into the biological phenomena that distinguishes sample classes. An effective and commonly employed means to examine biological context is identifying and examining the biological pathways known to be associated with the DEGs (Kanehisa, 2002; Karchin et al., 2002). Pathways identified in the P+_SSH/P−_SSH and dye swap designs had more than 90% commonality (Table 3). Additionally 24 of 59 common pathways were statistically significant across these three experiments. Most of the common pathways involved lipid metabolism, consistent with the known mode of action of the three test chemicals. The results demonstrate that the biological interpretation based on common pathways is consistent between the SSH design and dye swap designs.

Fewer arrays are needed for the SSH design than the dye swap design. The number arrays required for the dye swap design is dependent on the number of treated RNA samples (N). More accurately, two times the number ($2N$) of arrays are needed regardless of the number of control samples in the dye swap design. For the SSH design, however, the required number of arrays is dependent on both number of treated (N) and control (M) samples, where M is the number of control samples for which the SSHs are conducted. Two extreme cases illustrate the potential savings of using SSH. When only one control is needed for an experiment in which treated samples are obtained at multiple dose points, about half of the arrays are needed for the SSH design as that for the dye swap design. On the other extreme, if a matched control for each treated sample is used (i.e., $N = M$), the required arrays are equal in the SSH and dye swap designs. The

TABLE 3. STATISTICALLY SIGNIFICANT KEGG PATHWAYS SHARED BY THREE LISTS OF DEGS DERIVED FROM P+_SSH, P−_SSH, AND DYE SWAP FOR 100 μm BEZAFIBRATE TREATMENT

Title	P *values*[a]
Fatty acid metabolism	10^{-8}
Fatty acid elongation in mitochondria	1.29×10^{-7}
Valine, leucine and isoleucine degradation	4.96×10^{-5}
Glycolysis/gluconeogenesis	4.97×10^{-5}
Benzoate degradation via hydroxylation	8.18×10^{-5}
Caprolactam degradation	8.67×10^{-5}
Propanoate metabolism	1.135×10^{-5}
Bile acid biosynthesis	2.12×10^{-5}
Limonene and pinene degradation	6.68×10^{-4}
Lysine degradation	9.85×10^{-4}
Tryptophan metabolism	2.34×10^{-3}
Alkaloid biosynthesis II	2.48×10^{-3}
Pyruvate metabolism	3.47×10^{-3}
beta-alanine metabolism	5.82×10^{-3}
gamma-hexachlorocyclohexane degradation	6.14×10^{-3}
Pentose phosphate pathway	7.11×10^{-3}
Butanoate metabolism	7.20×10^{-3}
Carbon fixation	7.45×10^{-3}
1- and 2-methylnaphthalene degradation	0.0330
Peptidoglycan biosynthesis	0.0336
Nitrogen metabolism	0.0406
Fructose and mannose metabolism	0.0415
Tyrosine metabolism	0.0434
Glycerolipid metabolism	0.0443

[a]Fisher exact test, $p < 0.05$.

benefit of using the SSH design over the dye swap design can be best realized when $M < N$. Thus, the SSH design is especially efficient for toxicogenomics studies where only a few controls are commonly used. Using a pooled control rather than matched controls is maximally advantageous.

CONCLUSIONS

One of the most common uses of DNA microarrays is to determine DEGs for subsequent use in biological interpretation or signature identification. To derive a reliable DEG list using the two-color microarray type, the experiment design must enable correction of the inevitable dye bias. To date, the dye swap design is commonly recommended for the correction of the dye bias for two-color array. In this study, we proposed a SSH design that utilizes the SSH data as an alternative solution to the dye swap design. We demonstrated that the SSH design is comparable to the dye swap design in terms of identified DEGs and the biological interpretations linked to those genes, while requiring far fewer arrays for many types of studies.

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FANG

AU1
“Dobbins” or Dobbin—see ref. list.

AU2
Check where Table 2 should be cited. Table 3 appears to be correctly cited here, but Table 2 has not been cited yet.

AU3
Dobbin or Dobbins—see text citation.

AU4
Provide update info on publication.

AU5
; OK as added?