Gene Expression Reproducibility Analysis for the Treatment of Two Different Batches of *Cordyceps Sinensis* by Loop-design Microarray Experiments

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***Abstract*—The gene expression reproducibility of microarray experiments has long been criticized. Many studies have been undergoing to address this issue. In this study we investigate the reproducibility problem when not only technical variance but also complex biological variances are involved by introducing the treatment of two different batches of Cordyceps sinensis (CS) to immature dendritic cells (DCs) which were isolated from blood samples of different individuals. Cordyceps sinensis, a complex compound, has been commonly used as herbal medicine and a health supplement in China for over two thousand years. In this study, we adopted duplicate sets of loop-design microarray experiments to examine two different batches of CS and analyze the effects of CS on DCs. Immature DCs were treated with CS, lipopolysaccharide (LPS), or LPS/CS for two days, and the gene expression profile were examined using microarrays. The results of two loop-design microarray experiments showed good intersection rates. The expression level of common genes found in both loop-design microarray experiments was consistent, and the R2 was higher than 0.93.**

***Index Terms*—Cordyceps sinensis, microarray, gene expression profile, reproducibility**

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I. Introduction

MICROARRY technology is being applied widely to address increasingly complex scientific questions [1]. Microarray experiments yield lists of tens or hundreds of differentially regulated genes in sets of experiments. However, the presence of dissimilar regulatory patterns among functionally related genes makes it difficult for the biological interpretation of microarray data [2]. This is not surprising, because systematic biases and random variations are inherent in microarray data [1]. A careful experimental design and rigorous statistical analysis can increase the precision of microarray measurements [3], [4]. Moreover, statistical assessment is not only important in data analysis, but also plays a critical role in every stage of the microarray investigative process, including design of the experiment, data preprocessing, evaluation of systematic errors, identification of differentially expressed genes, functional classification, and biological interpretation [3], [4]. Kerr and Churchill first established the loop design for microarray experiments [5]. Previous studies demonstrated that loop design is more efficient than reference design because a range of statistical methods can be employed to increase the statistical power and robustness of microarray data analysis [6], [7]. Additionally, the loop-designed approach has a high hybridizations/nodes ratio that markedly increases the empirical power of microarray measurement [8]. The gene expression reproducibility of microarray experiments has long been criticized. In this study we investigate the reproducibility problem when not only technical variance but also complex biological variances are involved by introducing the treatment of Cordyceps sinensis (CS) to immature dendritic cells (DCs) isolated from blood samples of different individuals. Two kinds of replication are employed for the estimation of variance at different levels: technical and biological replicates. Technical replication is used to estimate system variance such as sample preparation and other effects of artifacts. Biological replication is used to evaluate variance in biological specimens and experimental procedures. Biological variance includes the heterogeneous distribution of cell types of both treating sample, in this study i.e. CS, and the treated sample, in this study i.e. immature DCs.

Cordyceps sinensis (CS) is a species of parasitic fungus on

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the larvae of the Lepidoptera, and has been commonly used as herbal medicine and a health supplement in China for approximately two thousand years [9], [10]. Numerous pharmacological effects of CS have been reported such as anti-tumor [11], [12], immunomodulatory [13]–[15], anti-inflammatory [16]–[18], and anti-oxidant properties [19], [20].

The aim of this study was to examine the gene expression profiles of CS on DCs treated with CS, LPS, and LPS/CS using microarray technology. In each set of microarray experiments, technical replication, performed by identical RNA sampling, in the design of the experiment was employed to estimate systematic variance. For biological replicate, we adopted duplicate sets of loop-design microarray experiments to examine two different batches of CS. We analyzed the common genes found in the two loop-design experiments, which could be used to reduce the variance between different batches of CS. We incorporated technical and biological replication in the design of the experiments to estimate the systematic and biological variance. This was done to ensure more reliable results.

II. Materials and Methods

A. Reagents

The culture medium was RPMI 1640 (Gibco-BRL, Life Technologies, Paisley, UK) supplemented with 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco-BRL, Life Technologies, Paisley, UK), and 10% heat-inactivated FCS (Hyclone, Logan, UT, USA). Recombinant human GM-CSF and recombinant human IL-4 were purchased from PeproTech (Rocky Hill, NJ, USA). LPS (Escherichia coli serotype O55: B5) was purchased from Sigma (St. Louis, MO, USA). Corning UltraGAPS slides were purchased from Corning Incorporated (Acton, MA, USA). The 3DNA array 900 labeling kit was purchased from Genisphere (Hatfield, PA, USA). The RNeasy mini kit was purchased from Qiagen (Valencia, CA, USA). Human cDNA microarray probe was purchased from Incyte Genomics (Palo Alto, CA, USA) The SuperScript® II was purchased from Gibco-Invitrogen (Carlsbad, CA, USA). The SpotReport™ cDNA Array Validation System was purchased from Stratagene (La Jolla, CA, USA). The Agilent 2100 bioanalyzer and RNA 6000 Nano LabChip kit were purchased from Agilent Technologies (PaloAlto, CA, USA).

B. Preparation of hot-water extracts of CS

Different sample batches of Chinese herbal medicine may have different levels of active ingredients. To reduce the variance of different CS batches, we used two batches of CS in this study. To guarantee the quality of the CS, the genetic variation was analyzed by the DNA sequencing as previously described [21], [22]. The CS extracted with hot water was obtained as previously described [23]. Briefly, CS samples were dried at 45°C in the dark to a constant weight and pulverized. Two grams of the CS sample was dissolved in 40 ml water and hot-water extraction was performed at 90°C for 2 hr. After centrifugation at 3,000 g for 20 min, the supernatant was harvested and sterilized by filtration through a 0.22 μm filter and stored at -20°C until used. To examine potential endotoxin contamination, CS extracts were measured by LAL assay. Results indicated the two batches of CS had undetectable levels (<0.05 endotoxin units/ml) of LPS (data not shown).

C. Generation of human monocyte-derived DCs

Fresh whole blood was obtained from normal volunteers at the Taiwan Blood Center by an Institutional Review Board (IRB) approved procedure issued by National Tsing Hua University, Hsinchu, Taiwan. Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation. Monocytes were purified following the plastic adherence method [24]. A total of 107 cells/well in 6-well flat-bottom plates were incubated in RPMI 1640 culture medium. After 2 h incubation at 37°C in humidified air containing 5 % CO2, nonadherent cells were removed by gentle washing and plastic-adherent cells were used as monocytes. This monocyte population exhibited >90% CD14 positive staining, as revealed by flow cytometric analysis (data not shown). DCs were generated from monocytes that were cultured at 37°C in an incubator with 5% humidified CO2 in RPMI 1640 culture medium that was supplemented with recombinant human GM-CSF 500 U/ml and recombinant human IL-4 1000 U/ml for 6 days. On days 2 and 4, half of the medium was replaced with fresh medium containing recombinant human GM-CSF and recombinant human IL-4. On day 6, immature DCs were reseeded into a 6-well culture plate at a total of 106 cells/well and treated with various concentrations of CS extracts (0 and 1 μg/ml) in the absence or presence of LPS (1 μg/ml) in a culture for 2 days. The viability of the cells under these treatments exceeded 90% (data not shown), based on results of MTT assay that were performed following the manufacturer's instructions (Sigma, St. Louis, MO, USA).

D. Microarray fabrication

A total of 7,334 sequence-verified human cDNA clones, ten Arabidopsis cDNAs (SpotReport™ cDNA Array Validation System) to serve as spike-in controls, and one housekeeping gene (β-actin) to serve as a positive control, were arrayed on Corning UltraGAPS slides. Quadruplicate spotting of 7,334 human cDNA and the 96 spottings of Arabidopsis cDNA and housekeeping genes were performed on every array, to enhance the statistical confidence in the gene expression data. Each array had 32,448 spots. The arrays were post-processed, based on the Corning Instruction Manual for UltraGAPS Coated Slides.

E. RNA extraction and microarray hybridization

DCs were harvested at scheduled sampling times to extract total RNA using an RNeasy mini kit following manufacturer's protocol (Qiagen, Valencia, CA, USA). In addition, we mixed the RNA of three different donors' in each loop-design microarray experiment. The quality of total RNA was evaluated using the Agilent 2100 bioanalyzer

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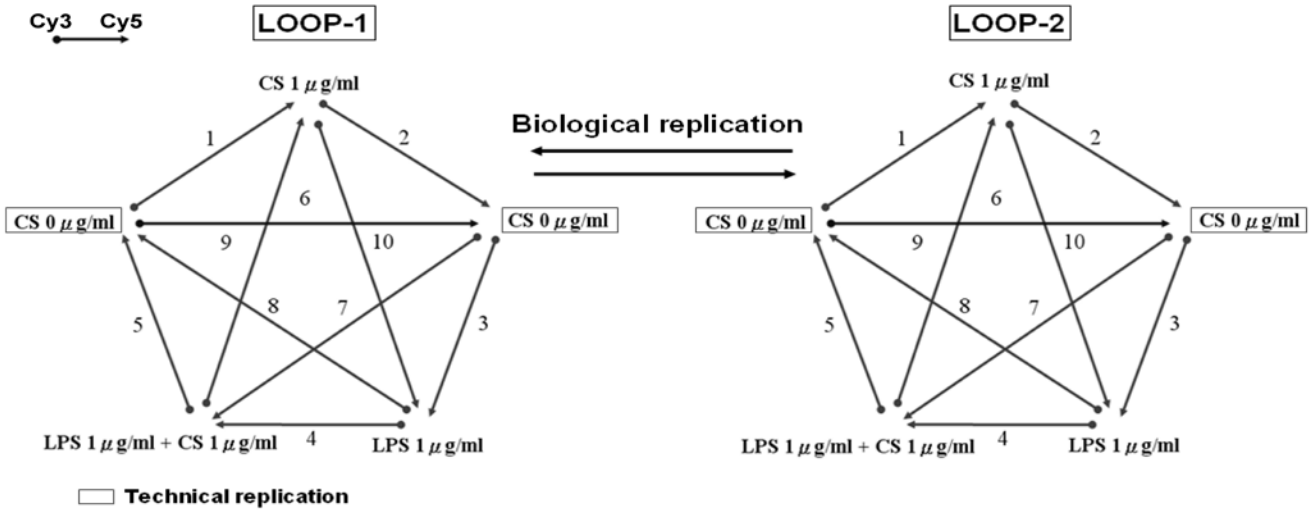

Fig. 1. Design of microarray experiment adopting duplicate sets of loop-design microarray experiments. Each experiment contained ten hybridizations and five experimental conditions, two controls and three treatments. Each mRNA sample was a combination of the mRNA of three donors, performed for biological replication in the loop-design microarray experiment. In addition, we utilized duplicate samples in each experiment for technical replication as well as internal control, estimate technological error and enhance the reliability of data.

with the RNA 6000 Nano LabChip kit. Before reverse transcription, each sample RNA was spiked with a mixture of Arabidopsis mRNAs. Fluorescence-labeled cDNA was conducted using 3DNA Array 900 labeling kit, following the manufacturer's protocols (Genisphere, Hatfield, PA, USA). Reverse transcription was performed using SuperScript II. Hybridization was performed at 65°C in a water bath for 16 to 18 hours, and arrays were washed following the manufacturer's protocol (Corning Life Sciences, New York, NY, USA). The arrays were scanned using the GenePix 4000B scanner (Axon Instruments, Foster City, CA, USA).

F. Microarray data analysis and statistical analysis

Microarray data preprocessing, normalization, and statistical analysis was performed using a bioinformatics software suite called Tsing Hua Engine for Microarray Experiment (THEME) [25]. Spot-screening rules were applied to screen out invalid spots on arrays. The spot-screening rules were as follows. (i) Exclude spots defined as "flag bad" or "absent" in all GPR files; (ii) exclude spots with a diameters of less than 75 μm; (iii) exclude spots with a coefficient of variation (CV) of pixel intensity of over 100% in both channels; (iv) exclude spots whose signal to noise ratios (SNRs) in both channels was less than 2 in loop-1 experiments and less than 3 in loop-2 experiments. The signal to noise ratio is defined as (S-B)/B ("S": mean of pixel intensities of "signal"; "B": median pixel intensity of "background").

The logarithm of the ratios for all valid spots on each array was normalized by pin-wise normalization [26]. After data preprocessing, the normalized log ratios of the cDNAs were processed using a log linear model, which was described in our previous study [2]. 5550 and 5843 genes satisfied the selection criteria in the two biological replication loop experiments. F test was used to identify differentially expressed genes. Differentially regulated cDNA clones were identified by applying Bonferroni-adjusted $P< 0.05$ for each null hypothesis in combination with at least a 1.5-fold change [2]. According to the technical replication experiments, a 1.5-fold change, reached 1% false discover rate, and was adopted as another selection criterion. The microarray data is available at GEO (GSE24191).

III. Results

A. Duplicate sets of loop-design microarray experiments

Two types of replication were used in the study: (1) technical replication: identical RNA samples were performed on multiple microarrays as shown in Fig. 1; (2) biological replication: each loop-design microarray experiment was used to assay different batches of CS (Fig. 1). In addition, we mixed the RNA of three different donors in each loop-design microarray experiment, to reduce individual variations. In the present study, we adopted duplicate sets of loop-design microarray experiments to examine the two batches of CS. Each loop-design microarray experiment contained ten hybridizations and five experimental conditions, two controls and three treatments (Fig. 1).

B. Good intersection rate in two loop-design microarray experiments

Table I shows the number of genes differentially expressed in two biological replication experiments treated with CS, LPS, and LPS/CS. In experiment loop-1, we found 605, 776, and 725 differentially regulated genes treated with CS, LPS, and LPS/CS, respectively (Table I). In addition, we found 688, 754, and 748 differentially regulated genes treated with CS, LPS, and LPS/CS in experiment loop-2, respectively (Table I). The number of common genes between two loop microarray experiments treated with CS, LPS, and LPS/CS was 406, 490, and 453, respectively (Table I). The results indicated that the intersectional rate in two loop-design microarray experiments was higher than 60% treated with CS,

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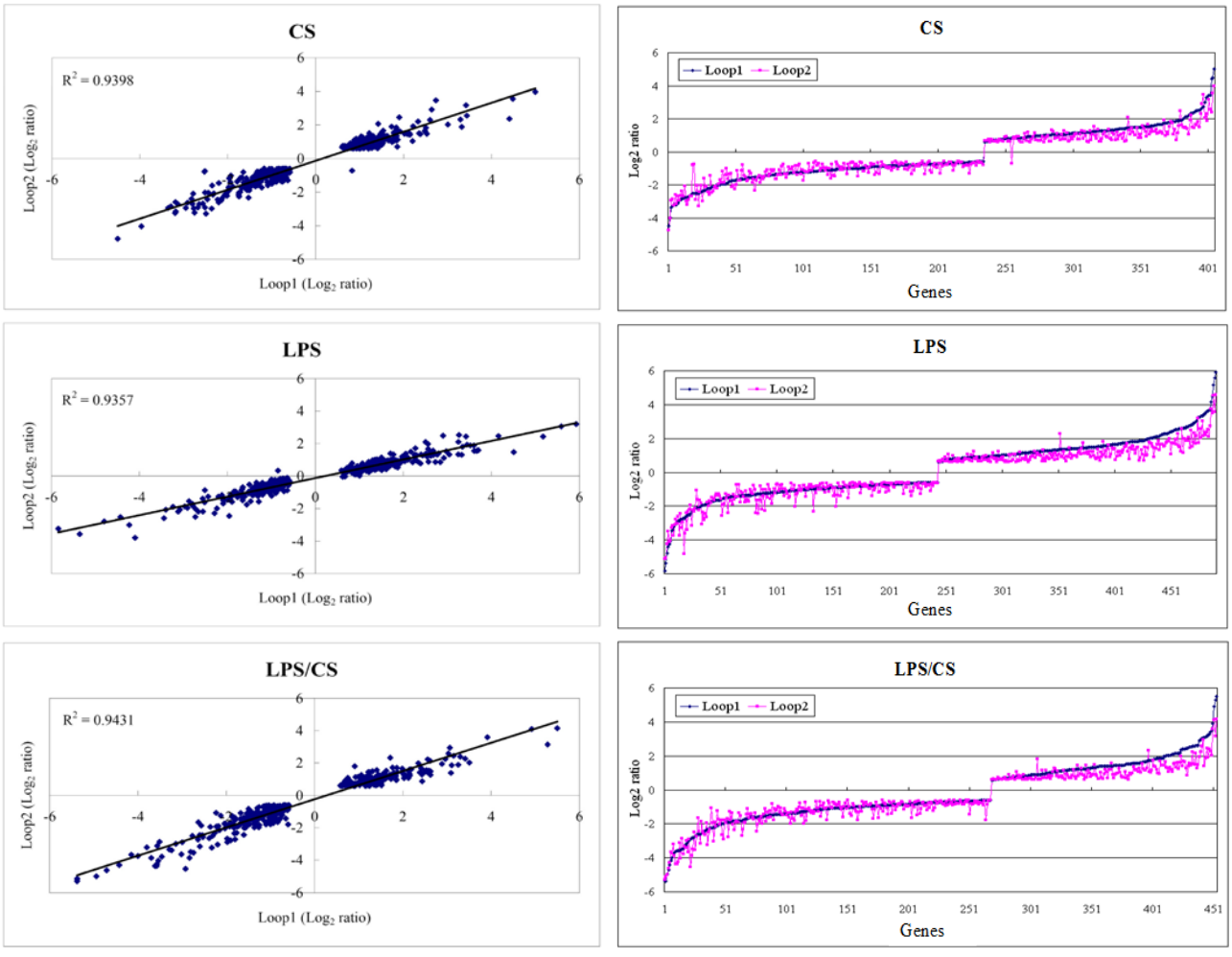
LPS, and LPS/CS (Table I). The expression level of common genes between two loop-design microarray experiments was consistent and the R2 was higher than 0.93 (Fig. 2(a)). To avoid variances from different batches of CS, different donors, and experimental manipulations, we used common genes between two loop experiments following treatment with CS, LPS, and LPS/CS for analysis. Figure 2(b) is another way to display the same sets of data as shown in Fig. 2(a). The log2 ratios of common genes of Loop1 is plotted in order (from smallest to largest) from left to right in purple and the log2 ratio of the corresponding gene of Loop2 is plotted accordingly in pink. It shows good agreement of the common genes between two loops.

TABLE I
NUMBERS OF GENES DIFFERENTIALLY EXPRESSED IN TWO BIOLOGICAL REPLICATION EXPERIMENTS TREATED WITH CS. LPS. AND LPS/CS

Treatment	CS	LPS	LPS/CS
Genes identified from Loop1 experiment	605	776	725
Genes identified from Loop2 experiment	688	754	748
Common genes	406	490	453
Intersection rate	62.7%	64.0%	61.5%

Intersection rate was calculated by the following equation: common genes/((Loop1 genes+Loop2 genes)/2).



(a) (b)

Fig. 2. (a) Linear regression of the expression level of common genes found in both sets of loop microarray experiments. The Number of common genes found in both loop microarray experiments under CS, LPS, and LPS/CS treatment are 406, 490, and 453 respectively. Loop1 and loop2 log2 ratio denotes the expression ratio between control and treatments (CS, LPS, and LPS/CS) regarding duplicate sets of loop microarray experiments. R2, square of the Pearson correlation coefficient, in the three lists of common genes are 0.9398, 0.9357, and 0.9431, respectively. (b) Another way to display the same sets of data as (a), the log2 ratios of common genes of Loop1 is plotted in order (from smallest to largest) from left to right in purple and the log2 ratio of the corresponding gene of Loop2 is plotted accordingly in pink. It shows good agreement of the common genes between two loops.

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IV. Discussion

The loop-design microarray experiments in this study involved technical and biological replication. The technical replication helped us to define the threshold of a 1.5 fold change, reached 1% false discover rate. To reduce the individual variations, each mRNA sample was derived from a combination the mRNA of three donors. The biological replication in this study involved two sets of microarray experiments performed by different individuals using different batches of CS. Through such conscientious controls, our microarray results showed a good intersection rate in two loop-design microarray experiments, with good consistency among the expression of common genes. These results indicate that the data derived from this microarray arrangement is highly accurate and reliable.

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