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Comparative Evaluation of Laser-Based Microarray Scanners

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ABSTRACT

Laboratories use different laser-based scanners to scan microarray images. To assess whether results from different scanners are comparable, and thus whether data from different laboratories can be compared, we scanned the same microarray slide with three commercial scanners that use different imaging techniques. After the acquisition of the microarray images produced by the three scanners, the images were quantified using a single imaging software package and protocol. The results were compared, and we found that the data obtained from the three scanners were comparable and that the variations caused by the use of different instruments were negligible, in spite of the fact that the scanners were based on different optical imaging techniques.

INTRODUCTION

Microarray is a powerful technique for profiling gene expression in the functional genomics area. This technique has recently been used to shed light into many biological issues, such as disease gene discovery and the molecular classification of cancers (1,5–7). The effective immobilization of DNAs on coated glass offers numerous advantages over membrane binding, such as low background fluorescence of the glass support and the ability to hybridize with dual labeled target probes (3). Glass slides are mechanically arrayed with thousands of DNA spots by means of a robotic system. The generated microarrays are then subjected to co-hybridization assays using fluorescence-labeled target probes prepared from cellular total RNA. Following hybridization, relative expression levels are determined by establishing a ratio of fluorescence intensity between the two labeled targets. The detection of a microarray image is based on the mea-

surement of its fluorescence intensities, which allows the simultaneous determination of the relative expression levels of all genes represented on the array. The imaging technique requires light of a specific wavelength to excite the fluorophores in the sample, and scanners can accomplish this by using one of two main techniques. Most scanners use laser sources to excite each fluorescent probe, and emitted light is detected by a photomultiplier tube (PMT). In this first method, a laser-based scanning system involves the rapid movement of small points of laser light across the sample, from which the image is then reconstructed. The second imaging technique is based on a charge-coupled device (CCD). Scanners that use this method have a white-light arc lamp as their illumination source. Because white light encompasses all wavelengths in the visible spectrum, it is possible to select the wavelength of choice. The fluorescent light emitted from the sample is collected and imaged using CCD cameras.

Cy5 and Cy3 are two of the most common fluorescent dyes used to label probes. They are excited when the scanner's laser source is set at wavelengths of 633 nm for Cy5 and 543 nm for Cy3. The light emitted is processed through a PMT, and the data collected from each fluorescent channel are stored as an individual 16-bit TIFF file. The images can be scanned at several resolutions, commonly ranging from 5 to 50 μm .

The whole process of microarray technology involves many steps, which is the reason why many of the current protocols are standardized to minimize experimental variations. However, the reliability of the data from experiment to experiment and the comparison of data from laboratory to laboratory or instrument to instrument have not been extensively evaluated. A commonly accepted standard with which to compare the performances of various scanners is presently lacking.

To evaluate the performances of various scanners, we compared images of the same microarray produced by three different laser-based microarray scanners that relied on different optical mechanisms for image processing. The images were quantified, and the data were analyzed using an ArrayVision™

software package (Imaging Research, St. Catharines, ON, Canada), rather than the software packages provided with the instruments, to minimize the variability in data processing. An advantage of this software was that it allowed the simultaneous analysis of multiple arrays, from which comparative expressions profiles were established using the quantitative information extracted by the program.

MATERIALS AND METHODS

Target cDNA spots (4800) were arrayed on a standard glass slide coated with poly-L-lysine. The cDNA was generated by PCR amplification using a Tetrad™ thermalcycler (MJ Research, Waltham, MA, USA) and purified using MAHV N4550 multiscreen-HV, 96-well filtration plates (Millipore, Bedford, MA, USA). The microarray was generated using a Flexys® arrayer with 48 pins (Genomic Solutions, Ann Arbor, MI, USA). The arrayed slides were UV-cross-linked with 650 μJ energy. The array was hybridized with fluorescent Cy3- and Cy5-labeled total RNA probes in ExpressHyb™ solution (Clontech Laboratories, Palo Alto, CA, USA) for 16 h at 42°C in a moist chamber. After the hybridization and washing, the slides were scanned using three different scanners: A, B, and C. Scanner A is a four-laser-based imaging system that uses PMT detectors and has a proprietary dark-field illumination to minimize background signal. Scanner B is a simultaneous dual laser scanner with a large field depth of 60 μm . Scanner C uses patented confocal laser scanning with the capability to calibrate the PMT. ArrayVision was the image-processing software used to quantify images. Statistica™ (StatSoft, Tulsa, OK, USA) was used to carry out statistical data analysis.

RESULTS AND DISCUSSION

Table 1 briefly summarizes the comparison of technical specifications of the three instruments. Scanner A, which was a sequential scanner (scans one channel after another), combined dark-field imaging and laser-beam

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Table 1. Comparison of the Specifications of the Three Scanners

Specifications	Scanner A	Scanner B	Scanner C
Imaging	Dark-Field	Simultaneous	Confocal
Field Focus (μm)	"unknown"	60 (thick specimen)	10 (small depth)
Scan Resolution (μm)	10, 20, and 50	10 and 40	5, 10, 20, and 50
Scanning Parameters	PMT adjustment	PMT adjustment	PMT and laser power adjustment
File Format	TIFF	TIFF	TIFF
Dimensions (In.)	36 x 24 x 22	12 x 18 x 7	30 x 16 x 14

scanning with PMT detection. A dark-field approach angles the laser beam so that the back-scattered laser beam is prevented from reaching the PMT. Consequently, the only photons to reach the detector are those emitted by the cDNA molecules, rendering this particular technique extremely useful in

terms of the high contrast and improved signal it provides. Likewise, dark-field imaging improves both the sensitivity and the dynamic range of the instrument. As a drawback, this technology yields images that are dimmer than those from scanners using other techniques and thus requires longer expo-

sure to obtain a good signal. In the process, there is a greater level of photobleaching of the fluorescent signal.

Scanner B carried out the simultaneous imaging of the two fluorescent probes; hence, its scanning process was fast. However, this increased speed was achieved at the expense of an enhancement of both signal and background. Likewise, although simultaneous imaging avoids pixel shifting, a large data file of 64 MB is generated. With this scanner, a full-power laser beam that scans across the entire slide is used. The fluorescence of Cy3 and Cy5 are measured at 532 and 635 nm, respectively. Although the emission maximum of Cy3 is 543 nm, a lower wavelength is chosen to achieve a better spectral separation of the two fluorophores and to reduce any potential cross-talk during the simultaneous scanning process. If other fluorescent probes are used, their emission wave-

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A

B

Figure 1. Correlation analysis among three scanners. (A) 20- μm and (B) 10- μm resolution. The correlation coefficients (r) are labeled in each of the comparisons. R_A , R_B , and R_C in the figure axis represent data obtained from scanners A, B, and C, respectively. $\text{Log}_{10}(R)$ is the logarithm-transformed intensity ratios of the two channels. A linear regression line is shown in each plot.

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lengths also have to be separated enough to avoid cross-talk.

Scanner C used confocal imaging technology. With this type of imaging, the laser source is focused to a point on the sample to be imaged at a depth of 10 μm . An upstream detector allows the detection of photons emitted from the area on the sample upon which the pinhole is focused. In this manner, only the "in focus" light (from the microarray spots) makes it into the detector, rendering a presumably high signal-to-noise ratio. The scanning procedure is single-pass.

Based on an understanding of the different imaging techniques used by the three scanners, we first expected to find differences in their quantification of data. Instead of using the acquisition and analysis software provided by each manufacturer of the instruments, the 16-bit image files were retrieved by ArrayVision as TIFF files and analyzed after setting up the array's alignment parameters. The protocol selected for analysis provided: (i) measurements of volumes, defined as the density of each spot multiplied by its area; (ii) background (background volume), defined as the corners between spots, with the size of each background spot being four pixels; (iii) sVol, defined as the background corrected volume, (i.e., the volume of the spot minus the background volume); and (iv) ratio, defined as the data from each spot in one channel image divided by the data from the corresponding spot in another channel.

The ratio of sVols from two channels (Cy3 and Cy5) was used as the relative expression level (R). Because the two sets of quantitative data from each of the channels varied over a large range, we carried out the comparison and correlation analyses after transforming the data into a logarithmic value (2,4).

A detailed comparison of data pairs was carried out using logarithm-transformed intensity ratios, defined as

$$\log_{10} \left(\frac{R_1}{R_2} \right) \quad [\text{Eq. 1}]$$

where

$$R_1 = \left(\frac{\text{Cy5}_{\text{sVol}}}{\text{Cy3}_{\text{sVol}}} \right)_{\text{Instrument1}} \text{ and } R_2 = \left(\frac{\text{Cy5}_{\text{sVol}}}{\text{Cy3}_{\text{sVol}}} \right)_{\text{Instrument2}} \quad [\text{Eq. 2}]$$

Figure 1, A and B, presents the comparisons between the logarithm₁₀-based ratios for all three scanners. The array data obtained from a low-resolution (20 μm) scan are illustrated in Figure 1A, while Figure 1B reflects the data obtained from a 10- μm resolution scan. High correlation coefficients (r) between each pair of scanners implied that the data produced by the different scanners were in agreement. The data obtained at the higher resolution (10 μm) were better correlated than data obtained at the lower resolution (20 μm). The correlation coefficient for a channel-to-channel comparison between the instruments was greater than 0.95.

It is worth noting that when images and data were obtained using the same scanner at different times, the correlation coefficient was about 0.93. This number fell within the range of variation observed for the data obtained from the different scanners (r = 0.90–0.96). Thus, any variability observed was not a result of the instrument used.

We also compared the data analysis of the differentially expressed genes of the two co-hybridized samples acquired from the three scanners. We used a threshold ratio of greater than 3.0. Among the top-ranked 160 genes, there was 95% agreement with the genes from the three analyses. Thus, the three instruments are identifying the same genes as differentially expressed.

Although the three commonly used laser scanners differed in a number of technical features, the gene expression data obtained from them were very similar. A practical consequence of this observation is that data produced by different laser scanners from various laboratories can be reliably compared and analyzed.

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