Parallel scan hyperspectral fluorescence imaging system
and biomedical application for microarrays

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Abstract: Microarray research offers great potential for analysis of gene
expression profile and leads to greatly improved experimental throughput. A
number of instruments have been reported for microarray detection, such as
chemiluminescence, surface plasmon resonance, and fluorescence markers.
Fluorescence imaging is popular for the readout of microarrays. In this paper
we develop a quasi-confocal, multichannel parallel scan hyperspectral
fluorescence imaging system for microarray research. Hyperspectral imaging
records the entire emission spectrum for every voxel within the imaged area
in contrast to recording only fluorescence intensities of filter-based scanners.
Coupled with data analysis, the recorded spectral information allows for
quantitative identification of the contributions of multiple, spectrally
overlapping fluorescent dyes and elimination of unwanted artifacts. The
mechanism of quasi-confocal imaging provides a high signal-to-noise ratio,
and parallel scan makes this approach a high throughput technique for
microarray analysis. This system is improved with a specifically designed
spectrometer which can offer a spectral resolution of 0.2
nm
, and operates
with spatial resolutions ranging from 2 to 30
µm
. Finally, the application of
the system is demonstrated by reading out microarrays for identification of
bacteria.

1. Introduction
The advent of DNA microarrays has revolutionized the biomedical research and development
in genetic engineering [1-2]. Microarray research, which offers great potential for analysis of
gene expression profile, and leads to greatly improved experimental throughput, has been an
advanced technology for biomedical applications since the end of the 20\textsuperscript{th} century [3-6].

Although microarray technology is of growing importance in various fields of research, it is still young and in need of improvement in many aspects. The operation of the microarray scanners has generated considerable interest in the current study, and a number of methods have been developed for the analysis of microarrays, such as chemiluminescence [7], surface plasmon resonance [8], and fluorescence markers. Fluorescence imaging is popular for the readout of microarrays. Most fluorescence scanners detect the band-integrated emission corresponding to the band-pass filter utilized for each fluorophore [9-11]. In these scanners one filter corresponds to one kind of fluorophore. This approach is a direct imaging of sample plane, however, there are some drawbacks existing in this measurement approach. First, as different fluorescent tags are excited with different wavelengths, and the fluorescence signals are distinguished by band-pass filters, the chosen fluorescent tags should have no spectral overlap of their emission spectra and minimal overlap of their absorption spectra. Second, for these filter-based scanners, it is difficult to determine whether the measured data have been corrupted by some extraneous emission sources. For example, the spot-localized contamination due to spotting chemistry and the emission from substrates affect the determination of genes expression [12], especially for weakly expressed genes. These drawbacks aforementioned limit this approach in reliable and high-accuracy analysis of microarrays.

Hyperspectral imaging overcomes these limitations by recording the entire emission spectrum for every voxel within the imaged area in contrast to recording only fluorescence intensities of filter-based scanners. Hyperspectral approach could detect mixed emission from several fluorescent tags with overlapping emission spectra with only one filter. Multivariate data exploitation is then used for data analysis [13-14]. The wealth of information that is available from spectrally resolving an image makes hyperspectral imaging an extremely attractive technique, and hyperspectral instruments have been reported over the past years [15-18]. In most of these papers, hyperspectral fluorescence imaging systems are employed mainly for imaging biological samples, and some efforts have been devoted to microarray research. While, we attempt to develop a system suitable and optimized for microarrays readout.

We have previously developed a quasi-confocal, parallel scan fluorescence imaging system, which is a filter-based scanner for microarray analysis [11]. Different from laser confocal scanners which are point-focusing of the laser light, quasi-confocal method focuses the laser light to a line, and the whole region of interest is detected with parallel scan of the line light along the direction vertical to the line. Quasi-confocal method provides a high signal-to-noise ratio, and parallel scan offers a high throughput which is important for microarray analysis. Considering the advantages offered by quasi-confocal parallel scan architecture and the great potential of hyperspectral imaging, in this paper we improve the former system and develop a quasi-confocal, multichannel parallel scan hyperspectral system for analysis of microarrays. This system is improved with a specifically designed spectrometer, which can offer a high effective spectral resolution. Two lasers are presently employed in the system, and new laser sources could be easily added to this system for multichannel analysis. The spatial resolution and sensitivity of this system are discussed in detail. Finally, the application of this system is demonstrated by reading out microarrays for
identification of bacteria.

2. Methods and materials

2.1. Constitution of the hyperspectral scanner

Figure 1 shows the optical layout of the system. A He-Ne laser with a wavelength of 632.8 nm and a power of 12.5 mW, a solid state laser with a wavelength of 473 nm and a power of 12.5 mW, are used as light sources. Two lasers are present here, however, new lasers could be easily added to the system to enlarge spectral ranges for multichannel detection. The laser fluence is adjusted to fit experimental conditions by use of a filter wheel populated with a set of neutral-density filters. The laser light is broadened by a beam expander and directed through a cylindrical lens (f = 30 mm) for the desired line illumination. Then a dichroic mirror which is characterized by high reflectivity at the laser wavelength and high transparency at longer wavelengths, is used to direct the laser light through the scan lens (f = 50 mm). For higher resolution and fluorescence collection efficiencies, apochromatic objectives with high numerical apertures (NA) are used.

The scan lens focuses the incident laser light to a line approximately 5 mm long on the surface of the microarray mounted on the scan stage, which is capable of 80 mm of travel in x direction and 60 mm of travel in y direction. Fluorescence excited by the focused line light is collected by the scan lens and directed through the dichroic mirror. A band-pass filter is used to further eliminate unwanted scattered and reflected laser light. An imaging lens (f = 80 mm) is then used to focus the fluorescence signals for a line image, which is relayed to the entrance slit of the spectrometer for the acquisition of the spectrally dispersed image. The spectrometer, which is able to offer an effective spectral resolution of 0.2 nm, is specifically designed for this system. A CCD array (Canon 500D) records these spectrally dispersed images for further data analysis. Then the whole plane is detected with parallel scan along the direction vertical to the focused line. The system achieves so-called "quasi-confocal" imaging because some light from adjacent voxels along the illuminated line could mix at the CCD.
array. While vertical to the illuminated line, light from adjacent voxels is efficiently rejected.

2.2. Fabrication of microarrays

Throughout this study, we prepare microarrays ourselves to test the characterization and the imaging capability of the system. For the fabrication of the DNA-arrays, the magnetron sputtering gold films are used as substrates. The fluorophores labeled DNA strands are thiolated for covalent bonding to substrates.

The gold film needs to be cleaned thoroughly before the printing of sample spots. Firstly, the gold film is cleaned with a solution consisting of H$_2$O$_2$ (30%), NH$_3$ (30%) and deionized water in a 1:1.5 ratio for 10 min [19]. Then, the film is dipped into acetone and ethanol for 10 min oscillating successively. After that, the film is thoroughly rinsed by deionized water and then dried and stored until further use.

The fluorescence labeled DNA is dissolved with ultra pure water to prepare solutions with different concentrations. When all above are ready, the gold film is put into a wet box, which provides a wet environment for the whole process of array printing. In our study, printing of the array is carried out manually. After that, the gold film, kept in the wet box, is put into the electric heating constant temperature incubator (Shanghai permanent science and technology the Limited company, DHP-9052) for 2h incubation at 37 °C. Then the DNA array is ready for optical scan.

Equation (1) shows the calculation of sample density ($D_s$) for the printed spots:

$$D_s = V_p \times M_p \times N_A / A_o,$$

(1)

where $N_A$ is the Avogadro’s constant, $V_p$ is the extracted volume for one spot, $M_p$ is the concentration of fluorescent dye with the unit of µmol/L, and $A_o$ is the area of one spot on the sensing plane. Thus the unit of sample density is the number of fluorescence molecules per square micro (fluors/µm$^2$). The spots with variant sample densities can be acquired by adjusting concentrations of fluorescent dye.

2.3. Hyperspectral data analysis

Analysis of hyperspectral data is crucial to the fluorescence image reconstruction and gene expression. Prior to analysis of the hyperspectral data, calibration data frames of the system are obtained by use of mercury argon calibration source (Ocean Optics). The acquired spectral data can be used to calibrate the wavelength scale and remove the slight influence of image curvature due to the spectrometer with the linear interpolation method.
To illustrate the hyperspectral data analysis, a sensing model of manually printed sample spots is prepared, as shown in figure 2(a). Cy5 (absorption/emission: 649/667 nm) and Dylight 680 (absorption/emission: 680/715 nm) are employed as fluorescent tags, which are corresponding to red laser excitation. Figure 2(b) shows one frame of data corresponding to the vertical line in figure 2(a). Each row represents the spectrum of one certain point in the line region, thus the entire picture of figure 2(b) is the spectra of illuminated line region. Figure 2(c) shows the intensity curves of the rows marked in figure 2(b), which are normalized for comparison. As can be seen from figure 2(c), the normalized spectra of these two dyes are overlapped. For every voxel on the array plane, the intensity of each emission source is calculated by integration of corresponding spectrum. The whole image of the array is acquired by analysis of all the data frames obtained during optical scan.

3. System Characterization

3.1. Performance of the hyperspectral scanner

The spatial resolution of this hyperspectral system is characterized using a U. S. Air Force standard resolution target (Edmund Optics) with broadband illumination. As mentioned above, this system is designed to enable simple changes of scan lens to obtain different resolutions for variant applications. Figure 3(a) (left) is an image of the resolution target obtained by an achromatic lens with the focus length of 50 mm. As can be seen from this image, element 1 of group 4 of the resolution target, corresponding to 16.0 line pairs/mm (~31 µm), is resolved in both the horizontal and the vertical direction. For higher resolution, a 10x, NA 0.25 objective is also available, and the corresponding image is shown in figure 3(a) (right). In this case, element 4 of group 5, corresponding to 45.3 line pairs/mm (~11 µm), can be resolved. The spot diameters and center-to-center spacings of commercial microarrays are of the order of ~100 µm, thus the 10~30 µm resolution is good and adequate for microarray analysis. A 40x, NA 0.65 objective is used for an even higher resolution of ~2 µm and higher fluorescence collecting efficiencies than 10x objective. Thus the resolution of this hyperspectral system ranges from 2 to 30 µm. However, as the length of the scan line, corresponding to the throughput within a single scan, decreases with the use of high
magnification objectives, we should choose the spatial resolution according to the size of the array.

![Figure 3](image)

**Figure 3.** The spatial resolution and sensitivity of the system. (a) Scanning images of the standard resolution target. Left: 30-µm resolution; right: 10-µm resolution. (b) Left: the scanning image of a spot; middle and right: intensity graphs of the marked rows A and B, respectively. The values of $s_n$, $b_n$ and $\sigma_b$ are given in the graphs.

The sensitivity of fluorescence imaging is calculated as follows [20-21]:

$$S_d = D_s / \text{SNR}, \quad (2)$$

$$\text{SNR} = (s_n - b_n) / \sigma_b, \quad (3)$$

where $S_d$ is the detection sensitivity, $D_s$ is the sample density and SNR is the signal-to-noise ratio. Eq. (3) shows the calculation of SNR [22], where $s_n$ is the average intensity obtained from the spot of interest, $b_n$ is the average intensity of background, and $\sigma_b$ is the standard deviation of background. We use a spot with the sample density of 30 fluors/µm$^2$ for illustration, as shown in figure 3(b) (left). To demonstrate the signal intensity and background noise level, intensity graphs of the marked rows A and B are plotted to the right of the spot image, and the values of $s_n$, $b_n$ and $\sigma_b$ are given in the graphs. Calculated by Eq. (3), an SNR of approximately 48 is derived, thus the detection sensitivity
is 0.63 fluors/µm² according to Eq. (2). The sensitivity can be further improved by pixel binning method [11]. The sensitivity achieved from this hyperspectral fluorescence imaging system is comparable to that of commercial microarray scanners, thus it is adequate for analysis of most microarrays.

3.2. multi-channel fluorescence imaging

![Figure 4](image)

**Figure 4.** Hyperspectral imaging of spectrally overlapping fluorescent dyes. (a) Design of an array. (b) Image of Cy5. (c) Image of Dylight 680. (d) Image of the whole sample plane. (e) and (f) Multivariate data analysis for the determination of the ratios of each dye. Red curve: the weighted spectrum for Cy5; blue curve: the weighted spectrum for Dylight 680; brown curve: the raw spectrum for a certain point of the mixture spots; Black curve: the calculated spectrum as a sum of these two weighted spectra.

To investigate the multichannel, hyperspectral imaging performance of the system, we design a sensing model for demonstration, as shown in figure 4(a). For all the spots in the first column, only Cy5 labeled DNA is used, and only Dylight 680 labeled DNA is applied in the second column. These two cases correspond to the multicolor labeling strategy which is generally utilized in gene expression research [23]. In order to mimic heterozygotes [24] and eliminate unwanted artifacts, such as emission from substrates and spot-localized contamination, a mixture of these two dyes is applied in the third column. Figure 4(b) and (c) show the fluorescence images of Cy5 and Dylight 680, respectively. The whole image of the sample plane is acquired by combining the concentration maps of these two dyes, as shown in figure 4(d). The raw emission spectra (brown color) for two points A and B in figure 4(d) are shown in figure 4(e) and (f). Although the same Cy5-Dylight 680 ratio is applied for spots in the third column, the spectra of the points within one spot vary from each other due to the...
inhomogeneities of distributions of fluorescence molecules. With multivariate data analysis, the weight of each dye is determined. In figure 4(e) and (f), the red and blue curves correspond to the weighted spectra of Cy5 and Dylight 680, respectively, and the black curve is a sum of these two weighted spectra. As can be seen from figure 4(e) and (f), the sum curves (black) correspond well to the raw spectra (brown) of the two randomly chosen points, indicating that the entire hyperspectral image stack could be modeled using linear admixtures of these two dyes. For presentation, only two dyes are applied here corresponding to a single laser, the scanning image demonstrates the ability of this hyperspectral scanner to identify multiple fluorescent labels with spectral analysis.

4. Biomedical Application of Microarrays

The identification of bacteria using gene chips is performed by this quasi-confocal, parallel scan hyperspectral fluorescence imaging system as an example of biomedical application of microarrays.

A gold film covered quartz glass is used as the substrate, which is carefully cleaned before the printing of probes. Then two \(5 \times 4\) probe arrays are printed manually on the substrates. All the probes are thiolated for covalent bonding to gold films. As shown in figure 5(a), the array comprises one row of Escherichia coli probes, one row of \(\beta\)-hemolytic streptococcus probes, one row of negative probes as quality report marks of the chip hybridization, and two rows of positive probes. For the validation of results on the chip, the positive probes should show a high fluorescent intensity signal, and the negative probes should show a low (or zero) signal.

Throughout this study, Cy5 is used for positive and \(\beta\)-hemolytic streptococcus DNA fragments, and Dylight 680 is for Escherichia coli. Figure 5(b) shows the detection results with no microbe present in the sample. As expected, the positive probes report a high fluorescent intensity signal, while the negative probes show a blank signal. Figure 5(c) shows the detection results with only Escherichia coli present in a sample. Besides positive probes, only the row of Escherichia coli probes shows a high signal, corresponding well with expectation. The experimental results indicate that the target genes are selectively hybridized to the probes. This hyperspectral system generates high quality images and can be successfully applied in biomedical research.

5. Conclusion
In this paper, we have proposed a quasi-confocal, multichannel parallel scan hyperspectral fluorescence imaging system. This system employs a pushbroom scan architecture with the line focusing of the excitation laser and records the spectrum for every voxel of the excited line region, and the whole sample plane is detected with parallel scan vertical to the scanning line. Coupled with multivariate data analysis, this system is capable of quantitative analysis of the concentrations of all the emitting species for any pixel on the sample plane. The spatial resolution of this system ranges from 2 to 30 µm. Although the quasi-confocal mechanism limits the system in extreme high-resolution microscopic imaging, the spatial resolution of this system is particularly suitable for imaging of microarrays. The sensitivity of this system is 0.63 fluors/µm², which is adequate and good for microarray analysis. The application of this system is demonstrated by reading multicolor microarrays for identification of bacteria. We believe that the proposed hyperspectral system can be used to build a commercially valuable device for microarrays analysis application.

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