# Confocal epifluorescence detection for microspheres delivered on disposable microfluidic chip

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The laser induced fluorescence (LIF) detection system for 5-$\mu$m microspheres delivered on microfluidic chip is presented employing confocal optical scheme. The parameters of the optical system are specifically optimized for single microsphere detection. With the excitation laser spot size of 4.6 $\mu$m and optical sectioning power of 27 $\mu$m, the lowest concentration detection limit is 0.45 nmol/L, corresponding to only 122 molecules in probe volume. The microsphere detection is carried on successfully with the maximum signal-to-noise ratio (SNR) of 55.7, which provides good detection sensitivity.

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The bottleneck in the development of microchip arrays has prompted the application of microsphere as an alternate research approach in clinical diagnostics in recent years[1]. The fluorescence detection for fluorescein tagged microsphere is widely adopted in immunoassays[2], protein phosphorylation assays[3], and DNA identification[4]. Although the commercial microsphere cytometers, e.g. LiquiChip$^{TM}$, have exceptional performance, they are expensive and bulky. Besides, the risk of cross-contamination remains high because the irreplaceable fluidic system is operated with different samples, which makes the washing procedure repetitive and complex[5]. However, the development of micro total analysis system ($\mu$-TAS) technology and fabrication of microchip[6] with its compact size, little amount of samples and relatively low cost, have given the opportunity to separate fluidic and detection system, showing some superiority over conventional cytometric devices. Several approaches have applied the technology of fibers[7,8], or micro-optical components[9] to compose the optical detection system for microspheres on chip. These applications make the system more integrated, yet sacrificing some detection performance due to the relatively low numerical aperture (NA), small aperture, unrestrictive excitation zone, and large aberrance. In order to increase the detection performance of the optical system, and establish a reasonable interrogation region to validate fluorescent interaction for single microsphere excitation and detection, the confocal epifluorescence system, with its ultra sensitivity and high signal-to-noise ratio (SNR)[10], is still an optimal choice for weak fluorescence pulse detection of single microsphere, which is essential in medical analysis. In this letter, we describe the design of detection system for fluorescein tagged microspheres on disposable mirofluidic chip, evaluate the system detection performance, and demonstrate the effectiveness in cytometric application.

The confocal epifluorecence detection system is schematically shown in Fig. 1. The frame of the optical system was modified from a conventional microscope (Novel Ningbo, China). Microfluidic chip was mounted on $xyz$ translation stage, which has adjustment sensitivity of 1 $\mu$m in vertical ($z$) direction and 10 $\mu$m in horizontal ($xy$) direction. Expanded and collimated excitation beam from 532-nm Nd:YAG laser was reflected by a dichroic mirror which was mounted on the optical axis at an angle of 45°, and focused onto the channel area of chip by a long working distance objective (Planachromat NA = 0.6, 40×, $f_1$ = 4.28 mm, MIC, Chongqing, China). Excitation laser power was measured by an optical power meter (1830-C, Newport, USA). The fluorescence emission was collected by the same objective, passed through the dichroic mirror, and then focused by a single lens ($f_2$ = 240 mm) onto a pinhole at the focal point. An overall magnification of $f_2/f_1$ = 56 was obtained. The pinhole with certain aperture was aligned by an $xyz$ stage with adjustment sensitivity of 10 $\mu$m in $xy$ direction and 0.1 mm in $z$ direction. Two 577.5-nm narrow bandpass filters (10-nm bandpass, 50% maximum transmittance, HB, Shenyang, China) were used behind the pinhole to efficiently block excitation wavelength. A side-window photomultiplier tube (PMT, R3657 with high voltage power supply unit C4710, Hamamatsu, Japan), built in a light-proof box, was placed on top of the microscope. The amplified PMT signal was filtered by elliptic filter (40-Hz cut-off frequency, 20-ms rising time), and processed by A/D card (PCI-9111DG, ADLink, Taiwan, China) at a sampling rate of 67 Hz. All data was smoothed using a five-point box smoothing algorithm in Matlab to suppress noise without affecting pulse width.

The microfluidic chip was fabricated by bonding two

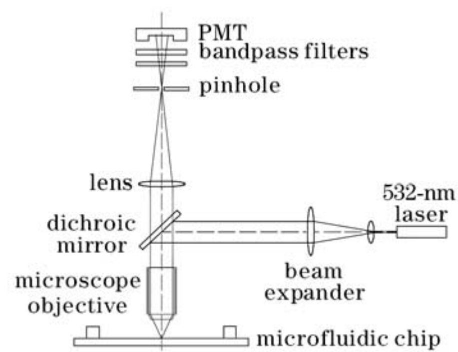


Fig. 1. Schematic diagram of the confocal epifluorecence detection system.

pieces of glass together, with a cross-format channel etched in the bottom plate. The channel was 30-$\mu$m-deep, 110-$\mu$m-wide at the top, and 50-$\mu$m-wide at the bottom. Microspheres of 5 $\mu$m in diameter were tagged in streptavidin-R-PE (Qiagen, USA) solution, which has fluorescence emission maximum at 578 nm. The flow in microchannel was driven by gravity generated by liquid level difference. Under the hydrodynamic focusing by buffer flow, sample flow containing microspheres in suspension was confined in the central area of the channel. The velocity of the flow was estimated to be 0.071 mm/s by measuring the time for a microsphere passing through the viewfield of the microscopy which was 0.2 mm in diameter.

The selection of microscope objective determines not only the collection efficiency of the fluorescence, but also the excitation laser spot size. A large NA objective makes it efficient to collect fluorescence but reduces the size of excitation laser spot, which decreases the laser excitation volume and increases the detection limit of the system. In our detection system, however, to detect a single 5-$\mu$m fluorescent microsphere was the ultimate goal. A relatively large laser spot may increase the risk of exciting more than one microspheres simultaneously, which should be avoided. Therefore, we chose an objective of 0.6 NA and long working distance of 3 mm, which enables microscope to detect fluorescence through 1.5-mm-thick cover glass.

The size of excitation laser spot is theoretically defined by the diffraction limit of objective aperture, which is expressed by

$$d = \frac{1.22\lambda}{\mathrm{NA}}, \quad (1)$$

where $d$ is the diameter of the focused laser spot on focal plane, and $\lambda$ is the excitation wavelength. Under this equation, $d$ was calculated to be 1.08 $\mu$m. However, in practice, both the aberrance and under-filling of back aperture make the laser spot larger. From the photograph of the focused excitation laser spot (Fig. 2), we can observe the diameter of 4.6 $\mu$m, similar to the size of microsphere. Given the excitation laser power of 2 mW, the excitation laser intensity was calculated to be $1.2 \times 10^4$ W/cm$^2$.

To determine the excitation volume of laser in microchannel, the excitation area is approximated as a cylinder with 4.6-$\mu$m diameter (the laser spot size) and 30-$\mu$m height (the channel depth). The expansion percentage of laser spot on bottom of the channel was no more than 40% due to the Gaussian beam propagation equation

$$[w(\Delta z)]^2 = w_0^2 \left[1 + \left(\frac{\lambda \Delta z}{\pi w_0^2}\right)^2\right], \quad (2)$$

where $w_0$ is the laser spot radius in focal plane, and $\Delta z$ is the distance from focal plane. Hence, the calculation of excitation volume under this assumption was about 0.5 pL.

For confocal optical system, the ability of optical sectioning is an important system feature, which is defined as vertical displacement given by the full-width at half-maximum (FWHM) of the axial response of the system[11,12]. Employing Wilson's model when $\lambda_{\mathrm{em}}/\lambda_{\mathrm{ex}} = 1$, the 100-, 200-, 400-, 600-$\mu$m pinholes have the FWHM displacements of 8, 14, 28, 41 $\mu$m, utilizing a 40×, 0.6 NA objective[10]. In our system with 30-$\mu$m channel depth, 400-$\mu$m pinhole was clearly the optimum choice. To evaluate the optical sectioning power of our confocal system, a 417-$\mu$m pinhole was mounted and 0.25-$\mu$g/mL streptavidin-R-PE solution was flushed through channel. The microfluidic chip was scanned vertically from focus. From Fig. 3, the FWHM displacement was estimated to be $(27 \pm 0.1)$ $\mu$m.

To detect fluorescent microspheres in solution, the detection limit was another critical factor. In order to determine the lowest concentration limit for instrumental response to the fluorescent reagent, streptavidin-R-PE solutions with various concentrations were flushed through the channel with flow velocity of 0.071 mm/s. The laser power was fixed to 2 mW, 417-$\mu$m pinhole was chosen, and PMT was biased at 932 V. All data were smoothed by a five-point box, and the collection time was about 30 s.

In our experiment, the average background signal from phosphate buffered saline (PBS) buffer was $(30.6 \pm 2.6)$ mV, while 0.025-, 0.25-, 0.625-, and 1-$\mu$g/mL (0.45, 4.5, 11.4, and 18.2 nmol/L) streptavidin-R-PE solutions gave the fluorescent signal of $(43.4 \pm 5.7)$, $(188.3 \pm 14.7)$, $(461.4 \pm 20.9)$, and $(767.4 \pm 27.8)$ mV respectively (see Figs. 4 and 5), corresponding to the corrected SNR values of 2.25, 10.73, 20.61, and 26.50.

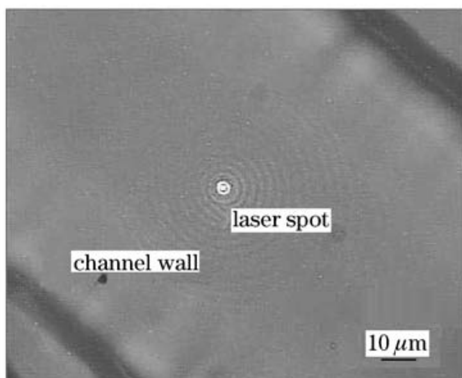

Fig. 2. Photograph of the focused excitation laser spot on the microchannel of the chip.

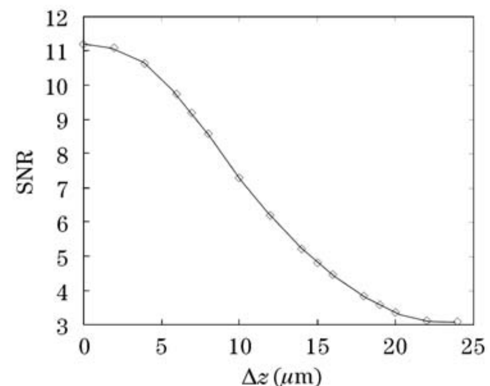

Fig. 3. Corrected SNR versus vertical displacement $\Delta z$ of the chip from focus for a 417-$\mu$m pinhole.

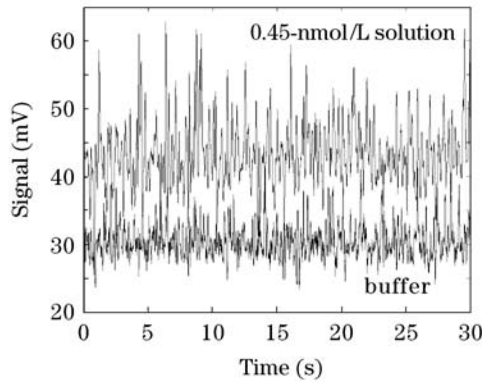


Fig. 4. Signal for continuous flow of PBS buffer and 0.45-nmol/L streptavidin-R-PE solution.

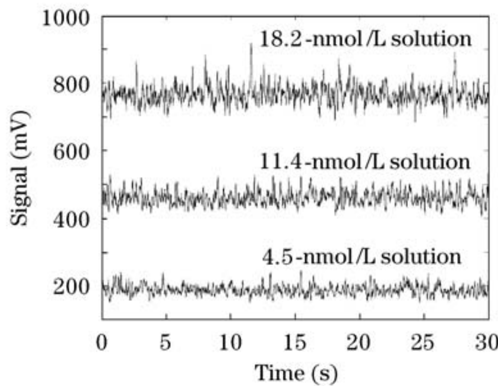


Fig. 5. Signal for continuous flow of 4.5-, 11.4-, 18.2-nmol/L streptavidin-R-PE solutions.

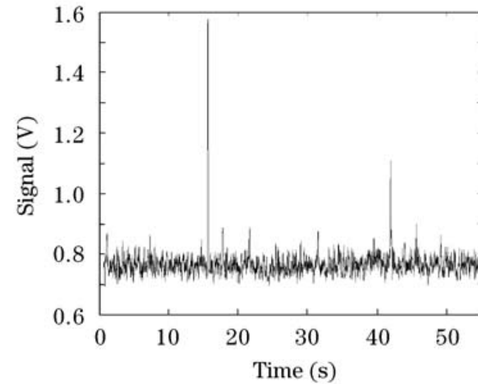


Fig. 6. Pulse signal for continuous flow containing 5-$\mu$m microspheres.

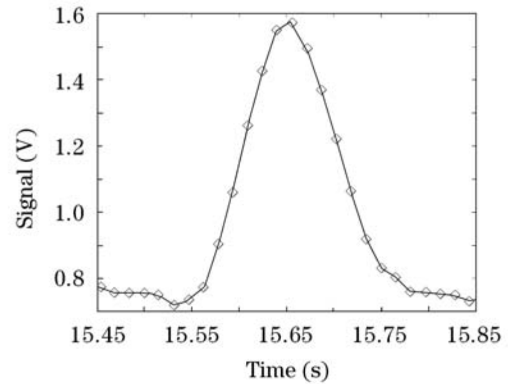


Fig. 7. Detailed pulse profile at 15.5 s.

In order to determine the number of detected fluorescein molecules over a given acquisition time, it is necessary to evaluate the probe volume which is defined by the overlapping of detection volume and excitation volume. The excitation volume, as discussed above, is defined as a cylinder with diameter of excitation laser spot size and height of the channel depth. The detection volume can also be estimated with the pinhole, overall magnification and the optical sectioning power. A 417-$\mu$m pinhole, with system magnification of 56, opened a detection window of 7.45 $\mu$m in diameter on the focal plane. Given the height of 27-$\mu$m FWHM displacement, the detection volume was estimated to be 1.2 pL. These two volumes led to the probe cylinder with diameter of laser spot size (4.6 $\mu$m) and height of the optical sectioning (27 $\mu$m). Thus, the probe volume was estimated to be 0.45 pL, with the cross-sectional area of 124 $\mu m^2$ across the channel.

In 0.025-$\mu$g/mL (0.45 nmol/L) streptavidin-R-PE solution detection, the number of fluorescein molecules in probe volume was about 122. With the flow velocity of 0.071 mm/s, the volumetric flow through the cross-sectional area of probe volume was 8.8 pL/s, corresponding to 48 molecules over the rising time of 20 ms, which was considered to be sensitive enough for single microsphere detection with at least $1 \times 10^3$ fluorescein molecules tagged on the surface.

During microspheres detection, 1-$\mu$g/mL (18.2 nmol/L) streptavidin-R-PE solution containing microspheres in suspension was injected in sample well, while the buffer well was filled with 1-$\mu$g/mL streptavidin-R-PE solution to confine mirospheres in central area of the channel. Excitation laser was focused on center of the channel. The fluorescent signal of two microspheres passing through the excitation area at 15.5 and 42 s was shown in Fig. 6. The PMT pulse response with peak of 1.58 V at 15.5 s was obtained, while the average background fluorescent signal was 0.76 V. Given the background buffer signal of 30.6 mV and the noise signal of 27.8 mV, the SNR of this signal pulse was 55.7, which was high enough for instrumental detection.

Detailed fluorescent pulse profile at 15.5 s was shown in Fig. 7. The fluorescent emission began when the microsphere entered into the excitation laser spot, and ended at the point of departure. The bottom width of the signal pulse was 0.22 s, corresponding to the microsphere's flow distance of $2d_{\mathrm{sphere}} + d_{\mathrm{laserspot}} = 14.6$ $\mu$m. The velocity of microsphere could be evaluated to be 0.067 mm/s, fitting well with the estimated velocity of 0.071 mm/s.

The fluorescent pulse profile at 41.5 s had a relatively low peak of 1.1 V and a short bottom width of 0.14 s, indicating that the microsphere's flow route slightly deviate from the center of excitation laser spot. Other signal pulses in Fig. 6 could be considered to be the random noise fluctuation because of their low peak voltages and short pulse widths of no more than 0.05 s.

Based on above discussion, the designed confocal microscope system was proved to be a sensitive system with low detection limit and high SNR response. The microspheres detection was carried on successfully, indi-

cating that the system be promising in detecting small fluorescent particles in microchannel of the microfluidic chip. Based on this optical system, with the improvement of mircobeads delivery mechanism and the signal processing performance of circuit, it is not difficult to realize a detecting system with high speed and high accuracy.

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