Spatially modulated fluorescence emission from moving particles

Cite as: Appl. Phys. Lett. **94**, 041107 (2009); https://doi.org/10.1063/1.3070536
Submitted: 24 September 2008 . Accepted: 21 December 2008 . Published Online: 27 January 2009

Peter Kiesel, Michael Bassler, Markus Beck, and Noble Johnson

Spatially modulated fluorescence emission from moving particles

Peter Kiesel,[a] Michael Bassler, Markus Beck, and Noble Johnson
Palo Alto Research Center Inc., 3333 Coyote Hill Road, Palo Alto, California 94304, USA

(Received 24 September 2008; accepted 21 December 2008; published online 27 January 2009)

An optical detection technique for a flow cytometer is described, which delivers high signal-to-noise discrimination without precision optics to enable a flow cytometer that can combine high performance, robustness, compactness, low cost, and ease of use. The enabling technique is termed "spatially modulated emission" and generates a time-dependent signal as a continuously fluorescing bioparticle traverses a predefined pattern for optical transmission. Correlating the detected signal with the known pattern achieves high discrimination of the particle signal from background noise. The technique is demonstrated with measurements of fluorescent beads flowing through a microfluidic chip. © *2009 American Institute of Physics*. [DOI: 10.1063/1.3070536]

Fluorescence-based flow cytometers are complex optofluidic systems extensively used in medical research and clinical diagnostics laboratories to measure chemical and or physical characteristics of biological cells as they are transported in a fluid stream.[1,2] All such systems use the same basic optical configuration, namely, intense illumination of the bioparticle as it speeds (e.g., 6 m/s) through a highly localized spot, which generally involves an intense laser source, an elaborate arrangement of precision optics, and a sensitive detector in order to measure fluorescence and scattered light. Typically, one or more lasers, microscope objectives with large numerical apertures (NAs), dichroic filters, and photomultiplier tubes are used. The focused excitation beam is required to achieve high spatial resolution (particle discrimination) and usable sensitivity since the signal is proportional to the photon flux density. In addition to minimizing particle coincidence, the narrow excitation (or emission) aperture also serves to reduce background sources. Since the detection volume is determined by the focused-spot diameter (e.g., 50 μm) which the particles traverse rapidly (e.g., transit times of $\sim$10 μs), achieving useful signal-to-noise ratio is demanding particularly for weakly fluorescing cells. The size, position, and flow speed of the particle stream have to be accurately controlled, which are typically realized by hydrodynamic focusing with significant amounts of sheath liquid. Through critical system design and incorporation of multiple stages of sophisticated and costly components, such instruments can achieve high sensitivity for multiple-parameter analysis of different bioparticles. However, the conventional approach is not readily extendable to point-of-care (POC) applications, where high performance, robustness, compactness, low cost, and ease of use are required in a single instrument. Such instruments are needed, for example, in resource-limited settings for monitoring the concentration of CD4 T-lymphocytes in blood for effective treatment of HIV-infected persons.[3]

In this paper we introduce an optical detection technique that delivers high effective sensitivity [i.e., high signal-to-noise (S/N) discrimination] without complex optics or bulky expensive light sources to enable a flow cytometer that can meet the instrument requirements for POC diagnostics applications. The enabling innovation, which we term the "spatially modulated emission technique," is based on establishing relative movement between a fluorescing bioparticle and a patterned environment to produce a time-dependent signal that is analyzed with correlation techniques. The advantage is high discrimination of the particle signal from the background noise. The benefits arise from the ability to replace expensive bulky components with inexpensive ones that can be readily integrated on a fluidic chip and by eliminating the need for critical optical alignment. In addition, the technique offers high spatial resolution for particle differentiation.

The spatially modulated emission technique can be viewed as the application of the principles of spread spectrum technology to the field of flow cytometry with unanticipated benefits. Spread spectrum has a long history from early applications in radio-controlled systems and radar to current applications that include wireless digital and analog communications (e.g., wireless Ethernet and cell phones) and global navigation.[4,5] Features of spread spectrum include resistance to interference and interception and increased transmission capacity, which relate to its inherent S/N discrimination capability.

The basic concept and benefit of imposing spatial modulation on the fluorescence emission from a moving particle are schematically illustrated in Fig. 1. The arrangement for a conventional flow cytometer is shown in Fig. 1(a) with the

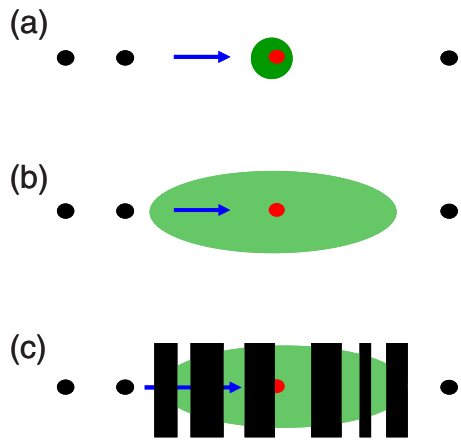

FIG. 1. (Color online) Schematic diagrams illustrating the basic concept of spatially modulated collection of optically stimulated fluorescence emission (red) from particles flowing in a fluid stream: (a) optical excitation (green) with a highly focused excitation spot (conventional approach), (b) a hypothetical large excitation zone to increase fluorescence-collection integration time, and (c) patterned fluorescence-collection zone superimposed over a large excitation zone.

[a]Electronic mail: peter.kiesel@parc.com.

fluorescence emission optically excited within a highly focused spot. The advantages of this approach include strong signal, high S/N, and good particle separation. However, realizing these benefits requires high photon flux densities (i.e., intense light sources, precision optics, and critical optical alignment), with the risk of saturation effects, and accurate control of both the flow path and speed of the particle. A conceivable partial fix for these disadvantages is shown in Fig. 1(b) with a large excitation zone to increase integration time for emission collection. While allowing lower excitation flux densities, with less saturation, and eliminating critical optical alignment, the fluorescence signal and the S/N would be concomitantly lower and particle separation would be poorer than in the conventional approach. The spatial modulation technique is illustrated in Fig. 1(c) with a patterned collection zone superimposed on a large excitation zone. The time-dependent signal is analyzed with standard correlation techniques. This yields improved S/N discrimination and high spatial resolution with neither precision optics nor critical alignment, while using low excitation flux densities. In addition, the technique yields particle speed to enable volumetric calibration and simple fluidic handling.

The key features of spread spectrum can be illustrated with the spatially modulated emission technique. The bioparticle is continuously fluorescing as it traverses the optical-excitation zone. In the absence of the patterned mask, the photodetector records a signal of essentially constant amplitude during the transit, that is, the signal spectrum has a narrow bandwidth. With the patterned mask the continuous fluorescence is detected as a time-dependent signal over a wider bandwidth (i.e., the signal spectrum has been spread). By using a pseudorandom transmission pattern, the recorded signal displays noiselike properties and the signal spectral power density is proportional to the ratio of the signal bandwidth and the spread bandwidth. Correlating the recorded signal with the known pattern recovers the fluorescence signal with high S/N discrimination.

A variety of predefined masks can be used, which include periodic, chirp, and pseudorandom patterns. The functional form of the mask influences the obtainable particle information as well as S/N discrimination. A periodic mask has the advantage that the particle speed can be readily determined (e.g., Fourier transform or electronic lock-in techniques); however, it is less satisfactory for accurately determining absolute position of the particle or handling multiple particles in the detection area. These issues are elegantly resolved by adopting a mask with a pseudorandomly defined pattern. Correlating the recorded time-varying signal with the mask pattern can detect multiple particles in the detection zone and precisely determine their absolute positions and separation, with spatial resolution related to the minimum feature size of the mask pattern. Also the combined advantages of periodic and pseudorandom masks can be obtained by integrating the two patterns in a single mask. In this case data analysis can accurately yield both speed and position of each particle in real time.

The experimental setup is illustrated in Fig. 2. Figure 2(a) schematically depicts the fluidic chip and the arrangement for fluidic handling, optical excitation, and collection of the spatially modulated fluorescence. The fluidic chip was formed with two closely spaced quartz slides to define a flow channel 200 μm wide and 25 μm deep. A pseudorandom mask pattern was photolithographically defined in a metal

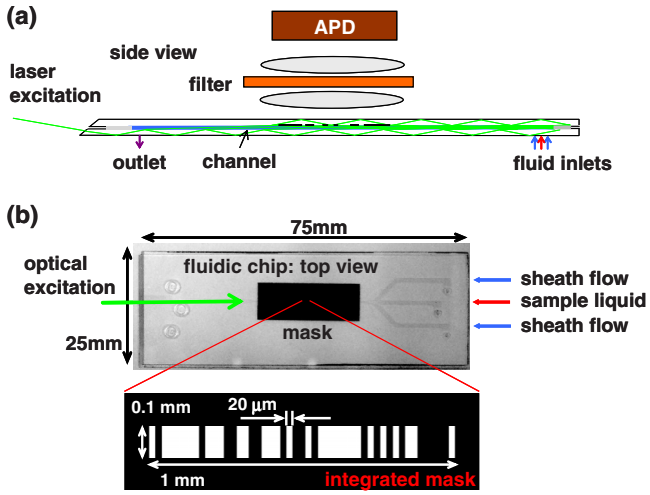

FIG. 2. (Color online) Experimental setup for spatially modulated collection of optically stimulated fluorescence emission from particles flowing through a fluidic chip: (a) schematic diagram and (b) fluidic chip with expanded image of integrated mask.

film deposited on the inside surface of the top slide. A syringe pump is used to control both the flow of the bead-containing (analyte) solution and the sheath flow; however, sheath fluid is not required but can be used to optimize the light-analyte interaction. Optical excitation can be provided by antiresonantly coupling laser light into the fluidic chip to achieve nearly uniform excitation along the path of the analyte flows.[6] In this configuration special flow schemes can be used to minimize background noise and allow high distributed excitation with reduced bleaching of the dyes. For example, the interaction between the guided excitation light and the analyte can be restricted to the detection zone by directing the analyte flow into the guided light beam just before the detection zone and directing the flow out of the beam right after the zone.

To illustrate the spatial modulation technique, experimental results are presented in Fig. 3 with real-time correlation analysis. The top graph shows the recorded signal (red) for a fluorescent particle (2 μm diameter) as it traverses the patterned zone. Cross correlation is used to characterize the degree of similarity between the recorded fluorescence signal $S(t)$ and the ideal signal $P(t)$ expected for a given mask pattern. The correlation signal $C(t)$ at time t is

$$C(t) = \int_{-T/2}^{T/2} P(\tau)S(\tau + t)d\tau, \tag{1}$$

where T is the transit time. The correlation signal consists of a broad triangularly shaped curve, of duration $2T$, topped by a sharp peak, as shown in the middle (black) graph of Fig. 3. The peak arises when the recorded signal perfectly aligns with the ideal signal; it is the signature for particle detection.

The derivative of the correlation signal (blue curve at the bottom in Fig. 3) provides quantization for the particle. The particle position is accurately obtained by identifying the zero-point crossing between the pair of adjacent peaks with maximum positive and negative amplitudes. This accurately locates the position of the sharp peak in the correlation signal and determines the position of the particle at any time during its transit. The peak-to-peak height of the adjacent peaks is proportional to the integrated fluorescence intensity of the particle as it traverses the patterned zone. An absolute cali-

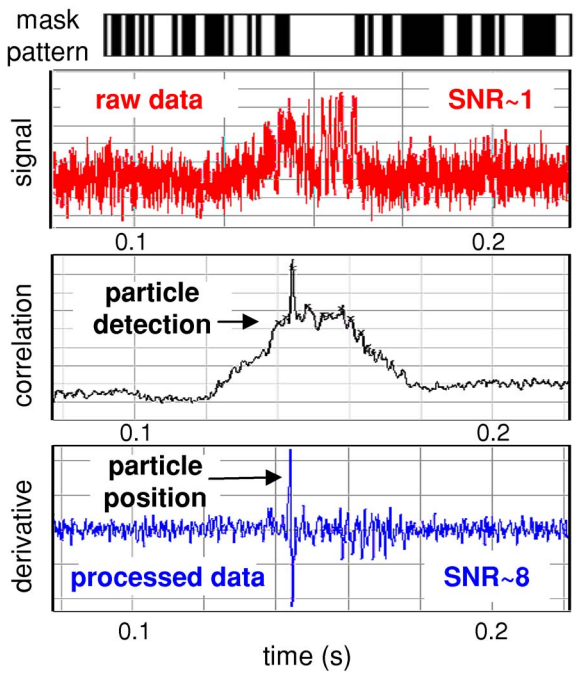

FIG. 3. (Color online) Experimental results and correlation analysis for modulated emission from weakly fluorescing beads traversing a patterned zone. The spatial mask at the top of the screenshot, with the white areas depicting the openings and the black areas the bars of the mask; the minimum feature size (4 μm) is the width of the smallest opening or bar.

bration of the signal can be obtained with calibration beads. To compare particles traveling at different speeds, the relative signals can be normalized to the transit times.

For the example in Fig. 3 the raw data display $S/N \approx 1$, while after the correlation analysis the exact particle position is reliably determined with $S/N \sim 8$. Alternative signal filtering techniques, for example, a low pass filter, could also improve S/N. However, the exact particle position within the excitation zone would be lost; this information is encoded in the high-frequency components of the spatial modulation signal. In addition, conventional filtering would not distinguish between particle signal and background. With the spatial modulation technique and fluorescent beads, we have demonstrated the following: (1) detected particles with $S/N < 1$, (2) distinguished multiple beads (diameter: 6 μm) simultaneously traversing the detection zone down to a separation distance of <10 μm, which is particularly important for high count rates at high particle concentrations, and (3) detected fluorescent beads (diameter: 0.6 μm) at an excitation power density <5 W/cm^2, which indicates the applicability of conventional light-emitting diodes.

The sensitivity and dynamic range of the spatial modulation technique are illustrated in Fig. 4 with a histogram of particle count as a function of fluorescence intensity for a mixture of fluorescent microbeads with three different diameters. For this experiment the excitation was provided by a 532 nm laser directly through the mask so that both the excitation and the fluorescence of the beads were spatially modulated by the mask. The excitation power density was estimated to be 10 W/cm^2. The fluorescence and scattered excitation light were collected and collimated with a 20× microscope objective (NA=0.4), filtered with a 585 nm band pass filter with a spectral width of 40 nm, refocused with a

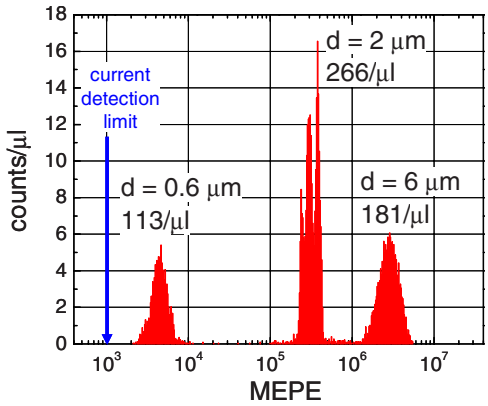

FIG. 4. (Color online) Histogram of particle count as a function of fluorescence intensity for a mixture of fluorescent beads with three diameters d. The absolute calibration in units of MEPE was estimated by comparing the 2 μm beads with calibration beads. The detection limit with the current experimental setup is $\sim 10^3$ MEPE.

20× objective, and detected with a pixelated avalanche photodiode. The fluidic chip utilized both sheath flow and analyte flow, as shown in Fig. 2(b), in the flow ratio (sheath:analyte:sheath) of 5:1:5, with an analyte flow of ~ 10 μl/min. In the vertical dimension of the fluid channel the flow speed distribution was parabolic, with the speed of the beads ranging from ~300 to 700 mm/s. All of the beads were tagged with the same dye with peak emission at 612 nm so that the emission intensity varied with bead diameter. The three clearly separated peaks in Fig. 4 correspond from left to right to bead diameters of 0.6, 2, and 6 μm, respectively, with an intensity variation of ±30%. The absolute calibration in units of molecules of equivalent phycoerythrin (MEPE) was estimated by using a commercial flow cytometer (BD FACS) to compare the fluorescence intensity of the 2 μm beads with calibration beads of known intensity (BD Quantibrite). The results demonstrate a dynamic range of over three orders of magnitude. From the S/N performance we estimate that the detection limit is approximately 1000 fluorescence molecules for the current setup. Finally, as a point of reference, the intensity from CD4+ lymphocytes stained with 1:1 conjugates of CD4-PE has been reported to correspond to $\sim 5 \times 10^4$ MEPE;[7] our preliminary measurements of fluorescence intensity from individual tagged CD4 + lymphocytes cells yielded $\sim 1 \times 10^5$ MEPE, in reasonable agreement with the reported value.

In conclusion, we have introduced a fundamental redesign for the optical detection system to characterize fluorescent particles in a flow cytometer. The new technique should enable the realization of practical instruments for resource-limited POC applications and thereby contribute to improving health care on the global scale.

[1]H. M. Shapiro and P. F. Cytometry (Wiley, Hoboken, NJ, 2005).
[2]R. A. Hoffman, *Flow Cytometry: Instrumentation, Applications, Future Trends and Limitations*, Springer Series on Fluorescence (Springer, Berlin, 2008), Vol. 6, pp. 307–342.
[3]W. R. Rodriguez, PLoS Med. **2**, e182 (2005).
[4]R. C. Dixon, *Spread Spectrum Systems*, 3rd ed. (Wiley New York, 1994).
[5]J. K. Holmes, *Spread Spectrum Systems for GNSS and Wireless Communications* (Artech House, Norwood, MA, 2007).
[6]O. Schmidt, M. Bassler, P. Kiesel, N. M. Johnson, and G. H. Döhler, Appl. Phys. Lett. **88**, 151109 (2006).
[7]K. A. Davis, B. Abrams, S. B. Iyer, R. A. Hoffman, and J. E. Bishop, Cytometry **33**, 197 (1998).